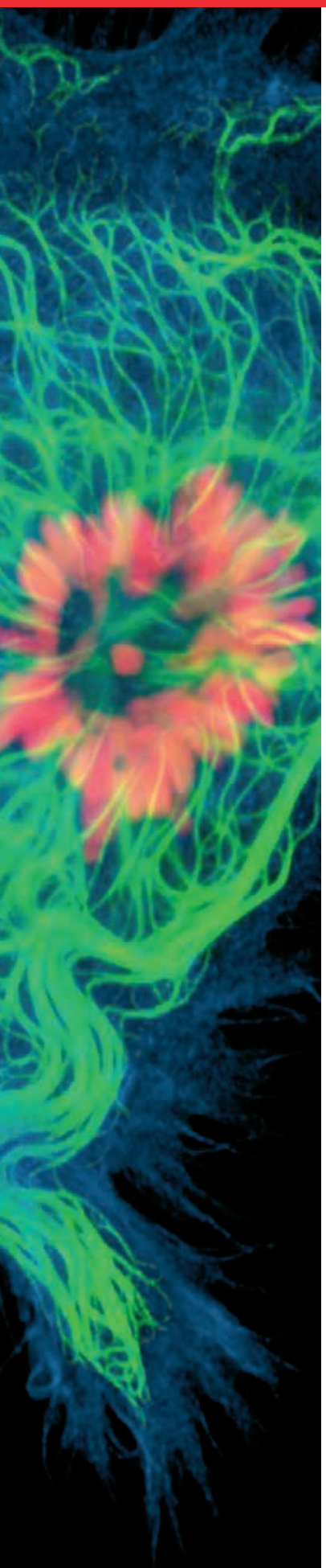
invitrogen



Molecular Probes™ Handbook

A Guide to Fluorescent Probes and Labeling Technologies

11th Edition (2010)

CHAPTER 15

Assays for Cell Viability, Proliferation and Function

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CHAPTER 15

Assays for Cell Viability, Proliferation and Function

15.1 Overview of Probes for Cell Viability, Cell Proliferation and Live-Cell Function

Cell viability, cell proliferation and many important live-cell functions—including apoptosis, cell adhesion, chemotaxis, multidrug resistance, endocytosis, secretion and signal transduction—can be stimulated or monitored with various chemical and biological reagents. Many of these processes lead to changes in intracellular radicals (Chapter 18), free-ion concentrations (Chapter 19, Chapter 20, Chapter 21) or membrane potential (Chapter 22) that can be followed with appropriately responsive fluorescent indicators. This chapter discusses Molecular Probes® reagents and assays for detecting these diverse cell processes in live cells. Many of the assays in this chapter can be analyzed on a cell-by-cell basis and some are equally suitable for detection with a fluorescence microscope, flow cytometer or microplate reader. Most of the assays have the capacity for high-throughput analysis.

Our viability and cytotoxicity assay reagents (Section 15.2) and kits (Section 15.3) are principally used to enumerate the proportion of live and dead cells in a population.[1] In contrast, proliferation assays such as the Click-iT® EdU cell proliferation assay (Section 15.4) are primarily designed to monitor the growth rate of a cell population or to detect daughter cells in a growing population. Fluorescence-based cell viability and proliferation assays are generally less hazardous and less expensive than radioisotopic techniques, more sensitive than colorimetric methods and more convenient than animal testing methods. Unlike ^{51}Cr-release assays, fluorescence-based assays of cell-mediated cytotoxicity do not require large samples, which can be difficult to obtain.[2] Furthermore, fluorescence-based protocols are more convenient than the trypan blue–based exclusion assay. This common colorimetric method for determining cell viability must be completed within 3–5 minutes because the number of blue-staining cells increases with time after addition of the dye.[3]

Fluorescent dye–based assays for cell viability and cytotoxicity are reliable and easy to perform. Our stand-alone reagents for these assays are described in Section 15.2, whereas our kits for viability and cytotoxicity analysis are discussed in Section 15.3. Molecular Probes® LIVE/DEAD® Viability Assay Kits (Section 15.3) give researchers a choice of viability/cytotoxicity assays suitable for bacteria, fungi or higher eukaryotic cells. Our LIVE/DEAD® Reduced Biohazard Cell Viability Kit and LIVE/DEAD® Fixable Dead Cell Stain Kits (Section 15.3) permit the original viability status of a mixed-cell population to be determined following aldehyde fixation of the sample in order to kill pathogens or in preparation for antibody staining. In each case, these viability assay kits provide the reagents and a simple protocol for simultaneous quantitation of live and dead cells. We also offer several proliferation assay kits that enable researchers to rapidly monitor numbers of adherent or nonadherent cells based on the presence of newly replicated DNA, total nucleic acid content or total protein content (Section 15.4). Section 15.5 focuses on our probes for monitoring apoptosis, including reagents for selectively detecting apoptotic cells based on their cell-permeability properties, as well as conjugates of annexin V phosphatidylserine-binding protein for detecting phosphatidylserine externalization. Additionally, our Premo™ Autophagy Sensors and other fluorescent probes are useful for examining the role of autophagy in normal and diseased cells.

In addition to our probes for cell viability, cell proliferation, apoptosis and autophagy, several of the reagents for live-cell function described in Section 15.6 can be used to develop assays that measure a particular biochemical parameter of interest. There is a significant overlap between probes for cell viability and probes for live-cell functions. For example, fluorogenic esterase substrates are commonly used to detect viability and proliferation, as well as to monitor cell adhesion, apoptosis and multidrug resistance. Likewise, cell-permeant and cell-impermeant nucleic acid stains are widely applicable to many live-cell function assays. We have organized discussions in this chapter according to several commonly studied cell processes in order to highlight the many published applications for these probes and foster the development of new applications.

The diversity of live cells and their environments (Figure 15.1.1) makes it impossible to devise a single viability or enumeration assay applicable to all cell types. Because viability is not easily defined in terms of a single physiological or morphological parameter,[1] it is often desirable to combine several different measures, such as enzymatic activity, membrane permeability and oxidation–reduction (redox) potential. Each assay method has inherent advantages and limitations and may introduce specific biases into the experiment; thus, different applications often call for different approaches.

REFERENCES

1. Cell Death Differ (2009) 16:3; **2**. Hum Immunol (1993) 37:264; **3**. J Histochem Cytochem (1985) 33:77.

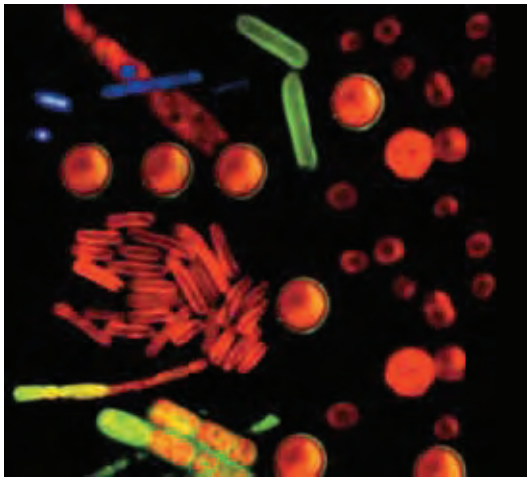

Figure 15.1.1 Collage of images of cyanobacteria stained with various blue- or green-fluorescent probes to complement the natural red autofluorescence from chlorophyll and phycobilisomes. The round cells are *Synechocystis* sp. (strain PCC 6803), and their membranes were labeled with green-fluorescent BODIPY® FL propionic acid (D2183). The cylindrical cells are *Synechococcus* sp. (strain PCC 7942), stained with blue-fluorescent DAPI (D1306, D3571, D21490). The filamentous cyanobacteria, *Anabaena cylindrica*, were labeled with either the green-fluorescent cytosolic stain, CellTracker™ Green BODIPY® (C2102), or with the lipophilic membrane stain BODIPY® FL proprionic acid (D2183). The image was contributed by Mary Sarcina, University College, London.

15.2 Viability and Cytotoxicity Assay Reagents

Fluorometric assays of cell viability and cytotoxicity are easy to perform with the use of a fluorescence microscope, fluorometer, fluorescence microplate reader or flow cytometer,[1] and they offer many advantages over traditional colorimetric and radioactivity-based assays. This section describes our numerous reagents for conducting viability and cytotoxicity assays in a wide variety of cells, including those of animal origin as well as bacteria and yeast. Following this discussion of individual reagents is Section 15.3, which contains a thorough description of each of our viability and cytotoxicity kits, including the:

- LIVE/DEAD® Viability/Cytotoxicity Kit (L3224)
- LIVE/DEAD® Reduced Biohazard Cell Viability Kit (L7013)
- LIVE/DEAD® Fixable Dead Cell Stain Kits (8 different fluorescent stain kits and the Sampler Kit L34960)
- LIVE/DEAD® Cell-Mediated Cytotoxicity Kit (L7010)
- LIVE/DEAD® Sperm Viability Kit (L7011)
- LIVE/DEAD® Cell Vitality Assay Kit (L34951)
- LIVE/DEAD® Violet Viability/Vitality Kit (L34958)
- Vybrant® Cell Metabolic Assay Kit, with C_{12}-resazurin (V23110)
- Vybrant® Cytotoxicity Assay Kit, G6PD release assay (V23111)
- LIVE/DEAD® Yeast Viability Kit (L7009)
- LIVE/DEAD® *Funga*Light™ Yeast Viability Kit (L34952)
- *Funga*Light™ CFDA AM/Propidium Iodide Yeast Vitality Kit (F34953)
- LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits (L7007, L7012, L13152, L34856)
- *Bac*Light™ RedoxSensor™ Vitality Kits (B34954, B34956)
- *Bac*Light™ Bacterial Membrane Potential Kit (B34950)
- ViaGram™ Red$^+$ Bacterial Gram Stain and Viability Kit (V7023)

Also discussed both in this section and Section 15.3 are our unique single-step reagents and kits for assessing gram sign and for simultaneously determining gram sign and viability of bacteria, as well as our novel fluorescent antibiotics. Section 15.4 describes our important probes for quantitating cell proliferation, analyzing the cell cycle and detecting the presence of bacteria and mycoplasma.

Table 15.1 Esterase substrates for cell viability studies.

Esterase Substrate (Cat. No.)	Properties in Cells	pK_a of Product *
BCECF AM (B1150, B1170, B3051)	• Quite well retained • Released during cytolysis • pH-sensitive fluorescence	7.0
Calcein AM (C1430, C3099, C3100MP)	• Quite well retained • Released during cytolysis • Not as pH-sensitive as BCECF	5
Carboxyeosin diacetate, succinimidyl ester (CEDA, SE; C22803; Section 15.4)	• Well retained by reaction with amines • Useful for DAB photoconversion • Phosphorescent	<5 †
Carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA, C369)	• Moderately well retained • Not as pH-sensitive as CFDA	4.8
Carboxyfluorescein diacetate (5(6)-CFDA, C195)	• Moderately well retained • pH-sensitive fluorescence	6.4
Carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA, AM; C1354)	• Easier to load than CFDA yet yields the same product upon hydrolysis • pH-sensitive fluorescence	6.4
Carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE; C1157; Section 15.4)	• Well retained by reaction with amines • Not completely released during cytolysis • pH-sensitive fluorescence	6.4 †
CellTracker™ Green CMFDA (CMFDA; C2925, C7025)	• Well retained by reaction with thiols • Not completely released during cytolysis • pH-sensitive fluorescence	6.4 †
Chloromethyl SNARF®-1, acetate (C6826)	• Well retained by reaction with thiols • Not completely released during cytolysis • Long-wavelength, pH-sensitive fluorescence	7.5 †
Fluorescein diacetate (F1303)	• Poorly retained • pH-sensitive fluorescence • Inexpensive	6.4
Oregon Green® 488 carboxylic acid diacetate (carboxy-F_2FDA, O6151)	• Moderately well retained • Not as pH-sensitive as CFDA	4.7

* Approximate pK_a values in aqueous solvents. The actual pK_a of the indicator will vary somewhat depending upon experimental conditions. † pK_a of the unconjugated hydrolysis product; after conjugation to an intracellular amine or thiol, the actual pK_a value may be different.

Viability/Cytotoxicity Assays Using Esterase Substrates

We prepare a wide variety of fluorogenic esterase substrates—including calcein AM, BCECF AM and various fluorescein diacetate derivatives—that can be passively loaded into adherent and nonadherent cells. These cell-permeant esterase substrates serve as viability probes that measure both enzymatic activity, which is required to activate their fluorescence, and cell-membrane integrity, which is required for intracellular retention of their fluorescent products.

As electrically neutral or near-neutral molecules, the esterase substrates freely diffuse into most cells. In general, cell loading of acetate or acetoxymethyl ester derivatives is accomplished by initially preparing a 1–10 mM stock solution of the dye in dimethylsulfoxide (DMSO) and then diluting the stock solution into the cell medium to a final concentration of 1–25 µM (Loading and Calibration of Intracellular Ion Indicators—Note 19.1). Once inside the cell, these nonfluorescent substrates are converted by nonspecific intracellular esterases into fluorescent products that are retained by cells with intact plasma membranes. In contrast, both the unhydrolyzed substrates and their products rapidly leak from dead or damaged cells with compromised membranes, even when the cells retain some residual esterase activity. Low incubation temperatures and highly charged esterase products usually favor retention, although the rate of dye loss from viable cells also depends to a large extent on cell type (see "Multidrug Resistance" in Section 15.6). For example, mast cells and epithelial cells actively secrete many polar products.[2,3] Table 15.1 lists Molecular Probes® esterase substrates that have been used for cell viability studies and compares their cell loading, retention and pH sensitivity. Many of the applications of these esterase substrates—for example, viability, cytotoxicity and adhesion assays—closely parallel those of ^{51}Cr-release assays, except that the fluorescent probes do not carry the risks or the disposal costs associated with the use of radioactive materials.

CellTrace™ Calceins: Calcein AM, Calcein Blue AM, Calcein Violet AM and Calcein Red-Orange AM

Of the dyes listed in Table 15.1, calcein AM (C1430, C3099, C3100MP; Figure 15.2.1) stands out as the premier indicator of cell viability due to its superior cell retention and the relative insensitivity of its fluorescence to pH in the physiological range.[4–7] Calcein AM, also called CellTrace™ calcein green AM (C34852), is a widely used probe for assays of cell adhesion, chemotaxis and multidrug resistance (Section 15.6). Calcein (C481, Section 14.3), which is the hydrolysis product of calcein AM, is a polyanionic fluorescein derivative (Figure 15.2.2) that has about six negative charges and two positive charges at pH 7.[8] Calcein is better retained in viable cells than are fluorescein, carboxyfluorescein and BCECF (Figure 15.2.3) and tends to have brighter fluorescence in a number of mammalian cell types. Calcein AM has the ability to penetrate intact cornea, revealing cell viability, morphology and organization of living cornea.[9,10] Furthermore, unlike some other dyes—including BCECF AM—calcein AM does not interfere with leukocyte chemotaxis or superoxide production, nor does it affect lymphocyte–target cell conjugation.[6,11–14] Leakage of calcein from calcein AM–loaded cells has been used to measure the increase in membrane permeability that occurs above physiological temperatures,[15] as well as to assay for cytotoxic T lymphocyte activity.[5] Fluorescence of extracellular calcein that has leaked from cells or that has been lost during secretion, lysis or ATP-dependent anion transport can be quenched by 5 µM Co^{2+} ion or by Mn^{2+} ion. Heavy-atom quenching of calcein provides a means of detecting dye leakage and quantitating only the intracellular fluorescence.[16]

Dihydrocalcein AM (Figure 15.2.4) is a reasonably stable, chemically reduced form of calcein AM that requires *both* hydrolysis by intracellular esterases and oxidation within the cell to produce the green-fluorescent calcein dye. Dihydrocalcein AM resembles 2′,7′-dichlorodihydrofluorescein diacetate (D399), the important indicator for oxidative activity in cells (see below), except that its oxidation product (calcein, Figure 15.2.2) should be better retained in cells than is the oxidation product of 2′,7′-dichlorodihydrofluorescein diacetate. Dihydrocalcein AM (D23805) is available as a set of 20 vials, each containing 50 µg of the product.

Calcein blue AM, also called CellTrace™ calcein blue AM (C1429, C34853; Figure 15.2.5), is a viability indicator for use with instruments optimized for the detection of blue fluorescence.[17,18] This tracer possesses AM esters that allow its passive diffusion across cell membranes. Before deesterification, CellTrace™ calcein blue AM is only weakly fluorescent (excitation/emission maxima ~322/435 nm). Upon cleavage of the AM esters by intracellular esterases, however, this tracer becomes relatively polar and is retained by cells for several hours. In addition, its fluorescence intensity increases and its fluorescence spectra shifts to longer wavelengths, with excitation/emission

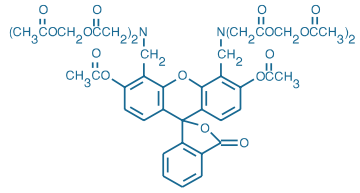

Figure 15.2.1 Calcein, AM (C1430).

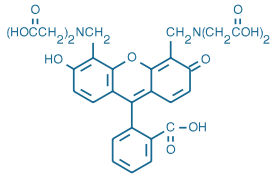

Figure 15.2.2 Calcein (C481).

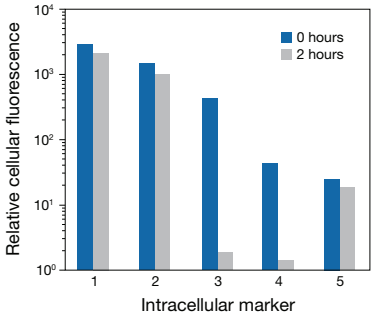

Figure 15.2.3 Loading and retention characteristics of intracellular marker dyes. Cells of a human lymphoid line (GePa) were loaded with the following cell-permeant acetoxymethyl ester (AM) or acetate derivatives of fluorescein: **1**) calcein AM (C1430, C3099, C3100MP), **2**) BCECF AM (B1150), **3**) fluorescein diacetate (FDA, F1303), **4**) carboxyfluorescein diacetate (CFDA, C1354) and **5**) CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, C2925, C7025). Cells were incubated in 4 µM staining solutions in Dulbecco's modified eagle medium containing 10% fetal bovine serum (DMEM+) at 37°C. After incubation for 30 minutes, cell samples were immediately analyzed by flow cytometry to determine the average fluorescence per cell at time zero (0 hours). Retained cell samples were subsequently washed twice by centrifugation, resuspended in DMEM+, maintained at 37°C for 2 hours and then analyzed by flow cytometry. The decrease in the average fluorescence intensity per cell in these samples relative to the time zero samples indicates the extent of intracellular dye leakage during the 2-hour incubation period.

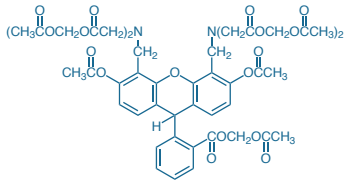

Figure 15.2.4 Dihydrocalcein, AM (D23805).

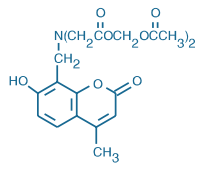

Figure 15.2.5 Calcein blue, AM (C1429).

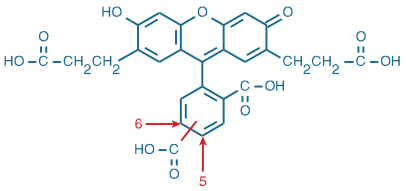

Figure 15.2.6 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF acid, B1151).

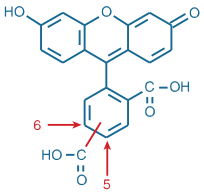

Figure 15.2.7 5-(and-6)-Carboxyfluorescein (C194).

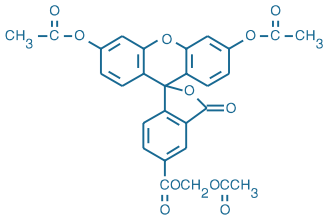

Figure 15.2.8 5-CFDA, AM (5-carboxyfluorescein diacetate, acetoxymethyl ester; C1354).

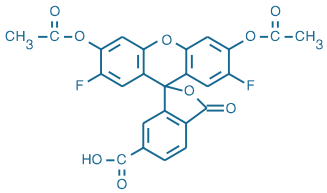

Figure 15.2.9 Oregon Green® 488 carboxylic acid diacetate (carboxy-DFFDA, O6151).

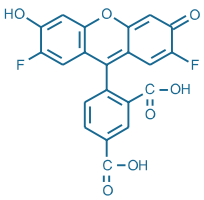

Figure 15.2.10 Oregon Green® 488 carboxylic acid (O6146).

maxima of ~360/449 nm. Calcein blue AM is useful for viability measurements in combination with our SYTOX® Green nucleic acid stain (see below) and other green- or red-fluorescent probes.

Calcein Violet AM (C34858) is optimized for use in flow cytometry. The enzymatic conversion of the virtually nonfluorescent cell-permeant calcein violet AM to the intensely fluorescent calcein violet (excitation/emission maxima ~400/452 nm) is efficiently excited by the 405 nm violet diode laser.

We also offer CellTrace™ calcein red-orange AM (C34851). Upon cleavage by intracellular esterases, CellTrace™ calcein red-orange (excitation/emission maxima ~576/589 nm) is well retained by live cells that possess intact plasma membranes. Unlike calcein AM, CellTrace™ calcein red-orange AM is intrinsically fluorescent; thus, an additional wash step may be necessary to minimize background fluorescence from dye that is not taken up by cells. CellTrace™ calcein red-orange AM is a useful cell tracer and indicator of cell viability and can be used in combination with green-fluorescent probes such as the Fluo-4 Ca^{2+} indicator (Section 19.3). [19]

BCECF AM

BCECF AM (B1150, B1170, B3051) is extensively used for detecting cytotoxicity and for determining the ability of surviving cells to proliferate.[20,21] The intracellular hydrolysis product of BCECF AM, BCECF (B1151, Section 20.2), has 4–5 negative charges (Figure 15.2.6), a property that considerably improves its cell retention in viable cells over that of fluorescein or carboxyfluorescein (Figure 15.2.3). However, because the emission intensity of BCECF is only half-maximal at pH 7.0 (pK_a = 6.98)—and is even further reduced in a cell's acidic compartments—the signal intensity of BCECF may be less than optimal in some cell viability and cell adhesion assays.

Using monoclonal antibodies known to either enhance or inhibit natural killer (NK) cell function, researchers found that BCECF AM was at least as effective as ^{51}Cr for measuring NK activity. Furthermore, the fluorescence-based assay could be performed with smaller samples.[22] BCECF AM has also been used to screen for trypanocidal activity [23] and viability of islets.

Fluorescein Diacetate

Fluorescein diacetate (FDA, F1303) was one of the first probes to be used as a fluorescent indicator of cell viability.[24–26] FDA is still occasionally used to detect cell adhesion [27] or, in combination with propidium iodide (P1304MP, P3566, P21493), to determine cell viability.[28,29] However, fluorescein (F1300, Section 20.2), which is formed by intracellular hydrolysis of FDA, rapidly leaks from cells (Figure 15.2.3). Thus, other cell-permeant dyes such calcein AM and BCECF AM are now preferred for cell viability assays.

Carboxyfluorescein Diacetate and Its Derivatives

The high leakage rate of fluorescein from cells [26,30] prompted the development of carboxyfluorescein diacetate (CFDA), which was originally used to measure intracellular pH [31] but was soon adapted for use as a cell viability indicator.[32,33] Upon hydrolysis by intracellular nonspecific esterases, CFDA forms carboxyfluorescein (C194, C1904; Section 14.3). As compared with fluorescein, carboxyfluorescein contains extra negative charges (Figure 15.2.7) and is therefore better retained in cells [6] (Figure 15.2.3). CFDA is moderately permeant to most cell membranes, with uptake greater at pH 6.2 than at pH 7.4.[31] The mixed-isomer preparation of CFDA (5(6)-CFDA, C195) is usually adequate for cell viability measurements; however, we also prepare high-purity single isomers of CFDA (C1361, C1362). In addition, we offer the electrically neutral AM ester of CFDA (5-CFDA, AM; C1354; Figure 15.2.8), which can be loaded into cells at lower concentrations than CFDA. Upon hydrolysis by intracellular esterases, this AM ester also yields carboxyfluorescein.[34–36] CFDA, CFDA AM and sulfofluorescein diacetate (see below) have been proposed for detection of living organisms on Mars.[37] Hemoglobin can be used to quench extracellular fluorescence due to leakage of probes or leakage of products, such as fluorescein or carboxyfluorescein.[38] Alternatively, antibodies directed against the fluorescein hapten (Section 7.4, Table 7.8) or the membrane-impermeant reagent trypan blue can be used to quench low levels of extracellular fluorescence of some fluorescein-based dyes.

CFDA has been used as a viability probe with a variety of cells, including bacteria,[39] fungi (e.g., *Saccharomyces cerevisiae*),[40] spermatozoa,[41] natural killer (NK) cells [19,42] and tumor cells.[43] Cytotoxicity assays using either CFDA or 5-(and 6-)carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA, C369) show good correlation with results obtained using the radioisotopic ^{51}Cr-release method.[19,44] With its low pK_a, carboxy-DCFDA is frequently used as a selective

probe for the relatively acidic yeast vacuole.[45–47] Oregon Green® 488 carboxylic acid diacetate (carboxy-DFFDA, O6151, Figure 15.2.9) also exhibits a low pK_a (~4.7) and may be similarly useful as a vital stain. Its intracellular hydrolysis product—Oregon Green® 488 carboxylic acid (O6146, Section 1.5, Figure 15.2.10)—is more photostable than carboxyfluorescein.

Sulfofluorescein Diacetate

Sulfofluorescein diacetate (SFDA, S1129), which is converted by intracellular esterases to fluorescein sulfonic acid (F1130, Section 14.3), is more polar than CFDA and consequently may be more difficult to load into some viable cells. However, SFDA's polar hydrolysis product, fluorescein sulfonic acid, is better retained in viable cells than is carboxyfluorescein.[48–51] SFDA was used to stain live bacteria and fungi in soil; little interference from autofluorescence of soil minerals or detritus was observed.[17,52]

CellTracker™ Green CMFDA

The CellTracker™ dyes are thiol-reactive fluorescent dyes that are retained in many live cells through several generations (Figure 15.2.3) and are not transferred to adjacent cells in a population (Figure 15.2.11, Figure 15.2.12, Figure 15.2.13), except possibly through gap junctions. These dyes represent a significant breakthrough in the cellular retention of fluorescent probes and are ideal long-term tracers for transplanted cells or tissues (Section 14.2).

CellTracker™ Green CMFDA (C2925, C7025) freely diffuses into the cell, where its weakly thiol-reactive chloromethyl moieties react with intracellular thiols and their acetate groups are cleaved by cytoplasmic esterases (Figure 15.2.14), generating the fluorescent product (Figure 15.2.15). The other CellTracker™ probes (coumarin, BODIPY® and tetramethylrhodamine derivatives; Section 14.2) do not require enzymatic cleavage to activate their fluorescence. Because the CellTracker™ dyes may react with both glutathione and proteins, cells with membranes that become compromised after staining may retain some residual fluorescent conjugates. However, use of a membrane-impermeant probe such as propidium iodide (P1304MP, P3566, P21493), SYTOX® Blue (S11348, S34857), SYTOX® Orange (S11368), SYTOX® Red (S34859), SYTOX® AADvanced™ (S10274, S10349) or one of our "dimeric" or "monomeric" nucleic acid stains (see below) in combination with CellTracker™ Green CMFDA should permit relatively long-term cytotoxicity assays. CellTracker™ Green CMFDA and ethidium homodimer-1 (EthD-1, E1169) have been used to detect viable and nonviable cells in rat and human coronary and internal thoracic arteries sampled at autopsy[53] and in connective tissue explants.[54]

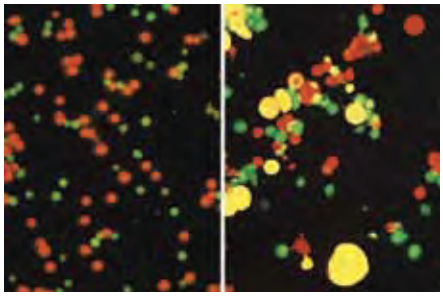

Figure 15.2.11 HL60 cells that have been stained with CellTracker™ Orange CMTMR (C2927) and then mixed with WEHI 7.1 cells stained with CellTracker™ Green CMFDA (C2925, C7025) (left). Several minutes after initiating cell–cell electrofusion, a CMTMR-stained HL60 cell is observed fusing with a CMFDA-stained WEHI 7.1 cell; cytoplasmic mixing is evident by the appearance of yellow fluorescence. After electrofusion is complete, dual-fluorescing (yellow) hybrids can be easily distinguished (right). Images contributed by Mark J. Jaroszeski, University of South Florida.

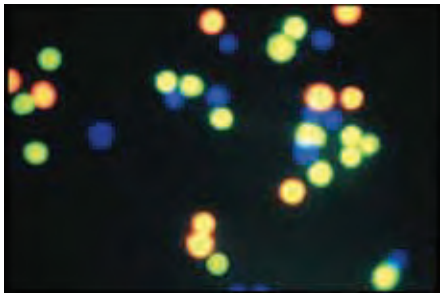

Figure 15.2.12 Individual populations of mouse myeloma (P3X) cells stained with our reactive tracers—CellTracker™ Orange CMTMR (C2927), CellTracker™ Green CMFDA (C2925) and CellTracker™ Blue CMAC (C2110). Each of three cell populations was stained with a different tracer and then the populations were mixed, demonstrating that these tracers allow simultaneous long-term monitoring of different groups of cells in transplantation and other assays.

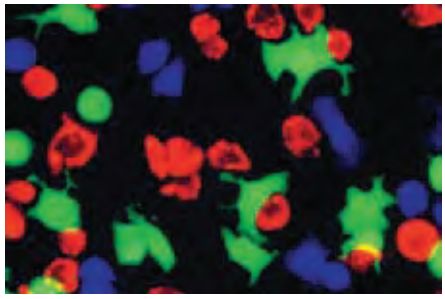

Figure 15.2.13 Rat basophilic leukemia (RBL) cells labeled in suspension with CellTracker™ Blue CMAC (C2110), CellTracker™ Green CMFDA (C2925, C7025) or CellTracker™ Orange CMTMR (C2927) prior to plating. The image was acquired using optical filter sets appropriate for DAPI, fluorescein and the Texas Red® dye.

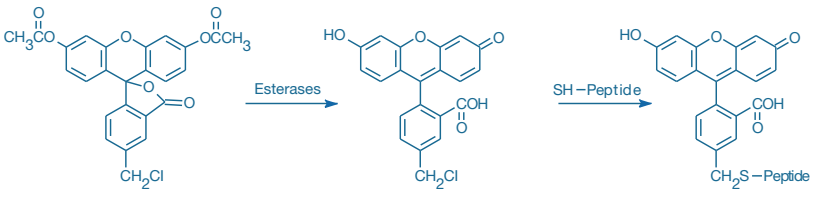

Figure 15.2.14 Intracellular reactions of our fixable CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate; C2925, C7025). Once this membrane-permeant probe enters a cell, esterase hydrolysis converts nonfluorescent CMFDA to fluorescent 5-chloromethylfluorescein, which can then react with thiols on proteins and peptides to form aldehyde-fixable conjugates. This probe may also react with intracellular thiol-containing biomolecules first, but the conjugate is nonfluorescent until its acetates are removed.

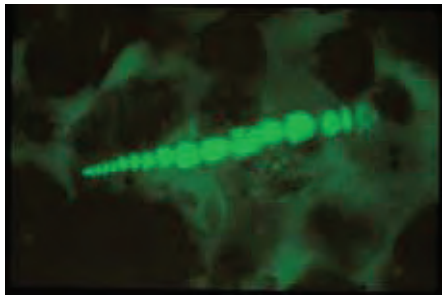

Figure 15.2.15 Detection of organisms in marine sediments by incubating an intact sediment core sample with the fixable, cell-permeant CellTracker™ Green CMFDA (C2925, C7025). The core sample was subsequently embedded, sectioned and examined for fluorescently labeled organisms. The micrograph reveals the microorganism *Leptohalysis scotti*, a marine benthic foraminifera. Image contributed by Joan M. Bernhard, Wadsworth Center, New York State Department of Health.

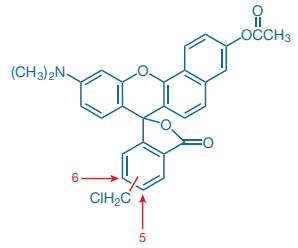

Figure 15.2.16 5-(and-6)-Chloromethyl SNARF®-1, acetate (C6826).

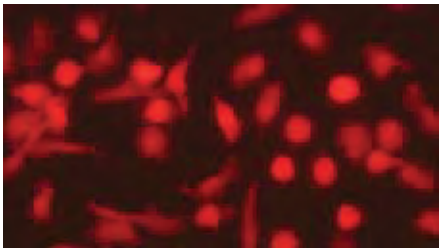

Figure 15.2.17 Human neutrophils loaded with 5-(and-6)-chloromethyl SNARF®-1 acetate (C6826).

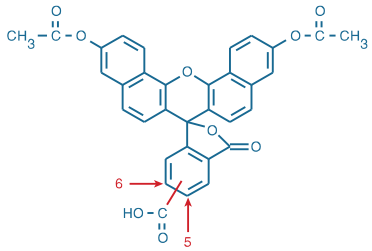

Figure 15.2.18 5-(and-6)-Carboxynaphthofluorescein diacetate (C13196).

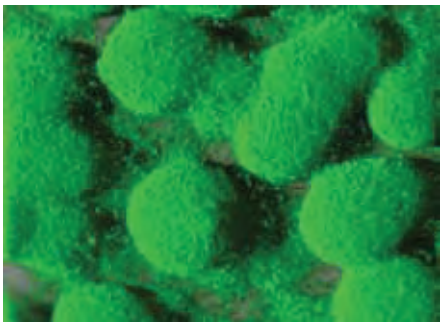

Figure 15.2.19 FilmTracer™ FM® 1-43 green biofilm cell stain (F10317) applied to a *Pseudomonas aeruginosa* biofilm. FilmTracer™ FM® 1-43 green biofilm cell stain appears to bind to the cell membrane. This stain has been shown to work equally well on *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli*, exhibiting exceptional cell specificity in each case. The image was obtained using a Leica TCS-SP2 AOBS confocal microscope and a 63×/0.9 NA water immersion objective. Image contributed by Betsey Pitts and Ellen Swogger, Center for Biofilm Engineerig, Montana State University.

Chloromethyl SNARF®-1 Acetate

Chloromethyl SNARF®-1 acetate (C6826, Figure 15.2.16) is the only cell-tracking dye (and pH indicator) that exhibits bright red cytoplasmic fluorescence (Figure 15.2.17) when excited at the same wavelengths used to excite the green-fluorescent hydrolysis product of CMFDA. The spectral characteristics of these two dyes permit simultaneous tracking of two cell populations by either fluorescence microscopy or flow cytometry. The large Stokes shift of the SNARF® fluorophore also makes chloromethyl SNARF®-1 acetate useful as a viability indicator in cells that exhibit green autofluorescence when excited by the 488 nm spectral line of the argon-ion laser.

Carboxynaphthofluorescein Diacetate

Carboxynaphthofluorescein diacetate (C13196, Figure 15.2.18), which is cleaved by intracellular esterases to yield red-fluorescent carboxynaphthofluorescein (excitation/emission maxima ~598/668 nm), is the only long-wavelength tracer of this type that can be passively loaded into live cells.[55] Like chloromethyl SNARF®-1 acetate, carboxynaphthofluorescein diacetate is usually used in combination with a green-fluorescent tracer for detecting cell–cell interactions.

FilmTracer™ Biofilm Stains

Bacterial biofilms present a unique set of challenges for fluorescent staining and subsequent imaging. A typical biofilm not only exhibits heterogeneous thickness throughout the surface, placing stringent restrictions on stain penetration, but also contains regions of widely varying environmental conditions. Evidence suggests that bacterial cells exist in various physiological states within these biofilm microenvironments. Furthermore, biofilms contain many poorly defined components (e.g., the extracellular polymeric matrix) that differ with species and conditions.

FilmTracer™ calcein violet, FilmTracer™ calcein green and FilmTracer™ calcein red-orange biofilm stains (F10320, F10322, F10319) are acetoxymethyl (AM) ester derivatives of fluorescent indicators that provide reliable indication of esterase activity in live cells and are of particular use in biofilm applications. FilmTracer™ calcein violet and FilmTracer™ calcein green biofilm stains are colorless and nonfluorescent until the AM ester is hydrolyzed. FilmTracer™ calcein red-orange biofilm stain is fluorescent prior to cleavage; however, the intracellular fluorescence is much brighter than background fluorescence after rinsing.

FM® dyes are lipophilic styryl compounds useful as general-purpose probes for investigating endocytosis and for identifying cell membrane boundaries (Section 14.4). These styryl dyes are easily applied to cells, where they bind rapidly and reversibly to the plasma membrane with strong fluorescence enhancement.[56–58] FilmTracer™ FM® 1-43 green biofilm cell stain (F10317) has been used successfully to stain the cell bodies in a complex biofilm mileu, including *Pseudomonas aeruginosa* (Figure 15.2.19), *Escherichia coli*, *Staphylococcus* sp., *Acidothiobacillus caldus* and *Vibrio cholerae*.[59]

Our SYPRO® Ruby stain labels most classes of proteins, including glycoproteins, phosphoproteins, lipoproteins, calcium binding proteins, fibrillar proteins and other proteins that are difficult to stain[60] (Section 9.3). FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain (F10318) is a specially formulated version of the SYPRO® Ruby stain and has been found to stain the matrix of *Pseudomonas aeruginosa* (ATCC 15442) and some strains of *Escherichia coli*; it does not stain *E. coli* K-12, which does not produce cellulose. As with all the biofilm stains, staining patterns may vary depending upon the organism and the matrix composition.

Viability/Cytotoxicity and Gram Stain Assays Using Nucleic Acid Stains

Viability assessments of animal cells, bacteria and yeast frequently employ polar and therefore cell-impermeant nucleic acid stains to detect the dead-cell population. Nucleic acid stains are most often used in combination with intracellular esterase substrates (see above), membrane-permeant nucleic acid stains (see below), membrane potential–sensitive probes (Chapter 22), organelle probes (Chapter 12) or cell-permeant indicators (Chapter 19, Chapter 20 and Chapter 21) to simultaneously detect the live-cell population. Although many other cell-impermeant dyes can be used to detect dead cells, the high concentrations of nucleic acids in cells, coupled with the large fluorescence enhancement exhibited by most of our nucleic acid stains upon binding, make cell-impermeant nucleic acid stains the logical candidates for viability probes. See Table 8.2 for

a list of several cell-impermeant nucleic acid stains and Section 8.1 for a general discussion of dye binding to nucleic acids.

SYTOX® Nucleic Acid Stains

Many polar nucleic acid stains will enter eukaryotic cells with damaged plasma membranes yet will not stain dead bacteria with damaged plasma membranes. SYTOX® Green nucleic acid stain (S7020) is a high-affinity probe that easily penetrates eukaryotic cells and both gram-positive and gram-negative bacteria with compromised plasma membranes, yet is completely excluded from live cells.[61] After brief incubation with the SYTOX® Green nucleic acid stain, dead bacteria fluoresce bright green when excited with the 488 nm spectral line of the argon-ion laser or any other 470–490 nm source (Figure 15.2.20). These properties, combined with its ~1000-fold fluorescence enhancement upon nucleic acid binding, make our SYTOX® Green stain a simple and quantitative dead-cell indicator for use with fluorescence microscopes, fluorometers, fluorescence microplate readers or flow cytometers (Figure 15.2.21). We have taken advantage of the sensitivity of the SYTOX® Green nucleic acid stain in our ViaGram™ Red+ Bacterial Gram Stain and Viability Kit (V7023) and in our Single Channel Annexin V/Dead Cell Apoptosis Kit (V13240, Section 15.5). An important application of the SYTOX® Green nucleic acid stain is the high-throughput screening of bacteria for antibiotic susceptibility by fluorescence microscopy, by flow cytometry or in a fluorescence microplate reader.[62]

The SYTOX® Green nucleic acid stain as a tool for viability assessment is not restricted to bacteria; it is also a very effective cell-impermeant counterstain in eukaryotic systems (Section 12.5). It can be used in conjunction with blue- and red-fluorescent labels for multiparameter analyses in fixed cells and tissue sections (Figure 15.2.22, Figure 15.2.23, Figure 15.2.24). Furthermore, it should be possible to combine the SYTOX® Green nucleic acid stain with one of the membrane-permeant nucleic acid stains in our SYTO® Red-, SYTO® Blue- or SYTO® Orange-Fluorescent Nucleic Acid Stain Sampler Kits (S11340, S11350, S11360) for two-color visualization of dead and live cells.

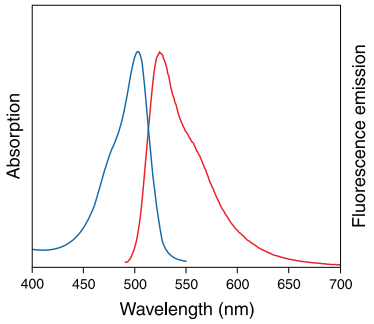

Figure 15.2.20 Absorption and fluorescence emission spectra of the SYTOX® Green nucleic acid stain bound to DNA.

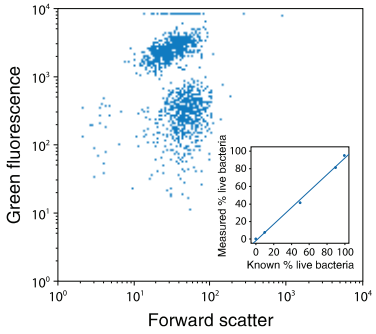

Figure 15.2.21 Quantitative flow cytometric analysis of *Escherichia coli* viability using the SYTOX® Green nucleic acid stain (S7020). A bacterial suspension containing an equal number of live and isopropyl alcohol–killed *E. coli* was stained with SYTOX® Green and analyzed using excitation at 488 nm. A bivariate frequency distribution for forward light scatter versus log fluorescence intensity (collected with a 510 nm longpass optical filter) shows two clearly distinct populations. When live and dead bacteria were mixed in varying proportions, a linear relationship between the population numbers and the actual percentage of live cells in the sample was obtained (see inset).

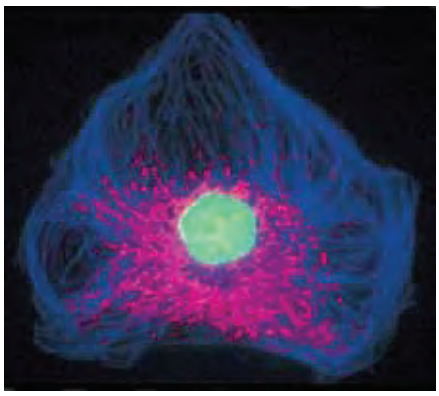

Figure 15.2.22 Bovine pulmonary artery endothelial cells (BPAEC) incubated with the fixable, mitochondrion-selective MitoTracker® Red CMXRos (M7512). After staining, the cells were formaldehyde-fixed, acetone-permeabilized, treated with DNase-free RNase and counterstained using SYTOX® Green nucleic acid stain (S7020). Microtubules were labeled with a mouse monoclonal anti–ß-tubulin antibody, biotin-XX goat anti–mouse IgG antibody (B2763) and Cascade Blue® NeutrAvidin™ biotin-binding protein (A2663). This photograph was taken using multiple exposures through bandpass optical filters appropriate for Texas Red® dye, fluorescein and DAPI using a Nikon® Labophot 2 microscope equipped with a Quadfluor epi-illumination system.

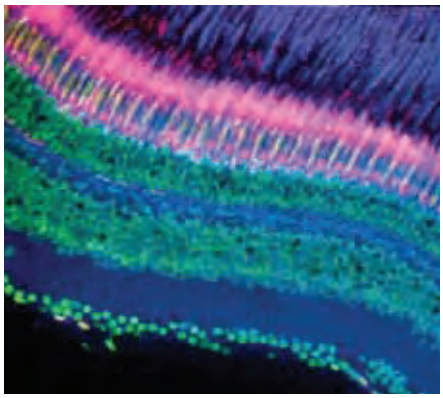

Figure 15.2.23 A frozen section of zebrafish retina stained with mouse monoclonal antibody FRet 43 in conjunction with Texas Red®-X goat anti–mouse IgG (T6390), Alexa Fluor® 350 wheat germ agglutinin (W11263) and SYTOX® Green nucleic acid stain (S7020).

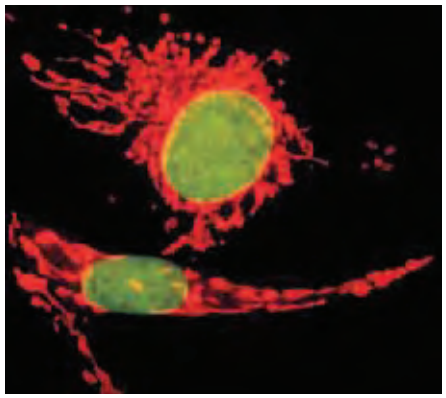

Figure 15.2.24 The mitochondria of bovine pulmonary artery endothelial cells stained with MitoTracker® Red CM-H_2XRos (M7513). The cells were subsequently fixed, permeabilized and treated with RNase. Then the nuclei were stained with SYTOX® Green nucleic acid stain (S7020). The multiple-exposure photomicrograph was acquired using a fluorescence microscope equipped with bandpass filter sets appropriate for fluorescein and Texas Red® dyes.

Like the SYTOX® Green reagent, our SYTOX® Orange (S11368) and SYTOX® Blue nucleic acid stains (5 mM solution in dimethylsulfoxide (DMSO), S11348; 1 mM solution in DMSO, S34857) are high-affinity nucleic acid stains that only penetrate cells with compromised plasma membranes. The SYTOX® Orange nucleic acid stain (S11368, Figure 15.2.25) has absorption/emission maxima of 547/570 nm when bound to DNA and is optimally detected using filters appropriate for rhodamine dyes. As with the other SYTOX® dyes, the SYTOX® Orange stain is virtually nonfluorescent except when bound to nucleic acids and can be used to detect cells that have compromised membranes without a wash step.

Our SYTOX® Blue nucleic acid stain labels both DNA and RNA with extremely bright fluorescence centered near 480 nm (Figure 15.2.26), making it an excellent fluorescent indicator of cell viability (Figure 15.2.27). Unlike many blue-fluorescent dyes, the SYTOX® Blue stain is efficiently excited by the 405 nm violet diode laser. The brightness of the SYTOX® Blue complex with nucleic acids allows sensitive detection of stained cells with fluorometers, fluorescence microplate readers, flow cytometers and epifluorescence microscopes. Quantitation of membrane-compromised bacterial cells carried out with the SYTOX® Blue stain yields results identical to those obtained in parallel assays using the SYTOX® Green stain. Like the SYTOX® Green stain, the SYTOX® Blue stain does not interfere with bacterial cell growth. Because their emission spectra overlap somewhat, we have found that it is not ideal to use SYTOX® Blue stain and green-fluorescent dyes together in the same application, except when the green-fluorescent dye is excited beyond the absorption of the SYTOX® Blue dye (e.g., at >480 nm). However, emission of the SYTOX® Blue complex with nucleic acids permits clear discrimination from red- and orange-fluorescent probes, facilitating development of multicolor assays with minimal spectral overlap between signals.

SYTOX® Red dead cell stain (S34859) is a simple and quantitative single-step dead-cell indicator for use with red laser–equipped flow cytometers. SYTOX® Red dead cell stain is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes but will not cross uncompromised cell membranes. After brief incubation with SYTOX® Red stain, the nucleic acids of dead cells fluoresce bright red when excited with 633 or 635 nm red laser light. SYTOX® Red nucleic acid stain has absorption/emission maxima of 633/660 nm when bound to DNA and is optimally detected using filters appropriate for Alexa Fluor® 647 dye. SYTOX® Red dead cell stain is distinct from other dead cell probes like 7-AAD and PI, which require 488 nm excitation.

Unlike the other SYTOX® stains, SYTOX® AADvanced™ Dead Cell Stain Kits (S10274, S10349) provide separate vials of dried dye and anhydrous DMSO to help ensure a stable shelf life. SYTOX® AADvanced™ dead cell stain is a high-affinity nucleic acid stain for the detection of dead cells and analysis of cell cycle using the common 488 nm spectral line of the argon-ion laser in flow cytometry. The dye is spectrally similar to 7-AAD but exhibits rapid uptake kinetics and relatively low cellular staining variability. SYTOX® AADvanced™ dead cell stain penetrates cells more efficiently than does 7-AAD, providing better separation of live and dead cell fluorescence signals. SYTOX® AADvanced™ dead cell stain can also be used with fixed cells for DNA content analysis when paired with RNAse treatment.

Dimeric and Monomeric Cyanine Dyes

The dimeric and monomeric cyanine dyes in the TOTO® and TO-PRO® series (Table 8.2) are essentially nonfluorescent unless bound to nucleic acids and have extinction coefficients 10–20 times greater than that of DNA-bound propidium iodide. Spectra of the nucleic acid–bound dyes cover the entire visible spectrum and into the infrared region (Figure 15.2.28). These dyes are typically impermeant to the membranes of live cells[63] but brightly stain dead cells that have compromised membranes. However, YO-PRO®-1 and other polar stains are taken up by some live cells via the $P2X_{1-7}$ receptors[64] as well as by apoptotic cells (Section 15.5), so care needs to be taken when assaying cell viability with nucleic acid stains. The POPO™-1 and BOBO™-1 dyes may be useful blue-fluorescent dead-cell stains, and the YOYO®-3 and TOTO®-3 dyes and the corresponding YO-PRO®-3 and TO-PRO®-3 dyes have excitation maxima beyond 600 nm when bound to DNA. Our JOJO™-1 and JO-PRO™-1 dyes exhibit orange fluorescence (~545 nm) upon binding to nucleic acids and can be excited with a 532 nm Nd:YAG laser. The LOLO™-1 nucleic acid stain has longer-wavelength fluorescence (~580 nm). Our Nucleic Acid Stains Dimer Sampler Kit (N7565)

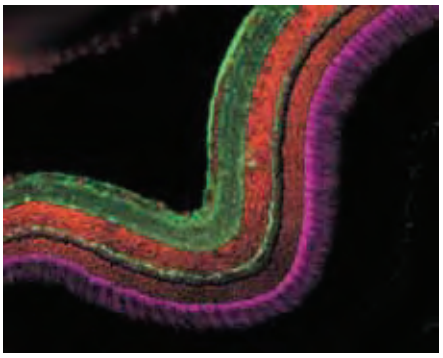

Figure 15.2.25 A zebrafish cryosection incubated with the biotin-XX conjugate of mouse monoclonal anti–α-tubulin antibody (A21371). The signal was amplified with TSA™ Kit #22, which includes HRP–streptavidin and Alexa Fluor® 488 tyramide (T20932). The sample was then incubated with the mouse monoclonal FRet 6 antibody and was visualized with Alexa Fluor® 647 goat anti–mouse IgG (A21235), which is pseudocolored magenta. Finally, the nuclei were counterstained with SYTOX® Orange nucleic acid stain (S11368).

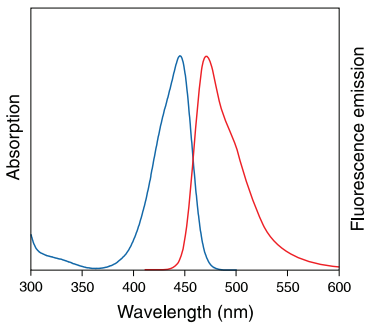

Figure 15.2.26 Absorption and fluorescence emission spectra of SYTOX® Blue nucleic acid stain bound to DNA.

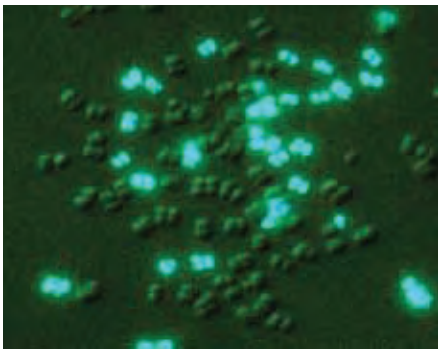

Figure 15.2.27 A mixed population of live and isopropyl alcohol–killed *Micrococcus luteus* stained with SYTOX® Blue nucleic acid stain (S11348), which does not penetrate intact plasma membranes. Dead cells exhibit bright blue-fluorescent staining. The image was acquired using a longpass optical filter set appropriate for the Cascade Blue® dye.

provides a total of eight cyanine dyes that span the visible spectrum. This kit should be useful when screening dyes for their utility in viability and cytotoxicity assays.

One cell viability assay utilizes YOYO®-1 fluorescence before and after treatment with digitonin as a measure of the dead cells and total cells, respectively, in the sample.[65] The YOYO®-3 dye was used as a stain for dead cells in an assay designed to correlate cell cycle with metabolism in single cells.[66] In addition to their use as dead-cell stains, both the dimeric and monomeric cyanine dyes are also proving useful for staining viruses. TOTO®-1 dye–staining and flow cytometric analysis gave better discrimination of live and dead lactic acid bacteria in several species than did propidium iodide.[67] Viruses stained with the YOYO®-1 and POPO™-1 dyes have been employed to identify and quantitate bacteria and cyanobacteria in marine microbial communities.[68] The YO-PRO®-1 dye has been used to count viruses in marine and freshwater environments by epifluorescence microscopy[69] and is also selectively permeant to apoptotic cells[70] (Section 15.5). TO-PRO®-3 (T3605, Section 15.4) has been utilized to demonstrate transient permeabilization of bacterial cells by sublethal doses of antibiotics.[71]

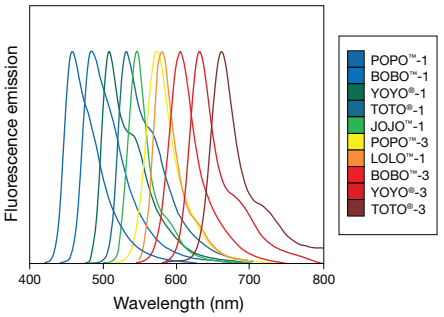

Figure 15.2.28 Normalized fluorescence emission spectra of DNA-bound cyanine dimers, identified by the color key on the sidebar.

Ethidium and Propidium Dyes

The red-fluorescent, cell-impermeant ethidium and propidium dyes—ethidium bromide (15585-011), ethidium homodimer-1 (EthD-1, E1169; Figure 15.2.29) and propidium iodide (P1304MP, P3566, P21493)—can all be excited by the argon-ion laser and are therefore useful for detecting and sorting dead cells by flow cytometry.[72,73] Moreover, these dyes have large Stokes shifts and may be used in combination with fluorescein-based probes (such as calcein, CellTracker™ Green CMFDA or BCECF) or green-fluorescent SYTO® dyes (Table 8.3) for two-color applications (Figure 15.2.30). Both propidium iodide and ethidium bromide have been extensively used to detect dead or dying cells,[74–78] although ethidium bromide may be somewhat less reliable because it is not as highly charged. EthD-1 and propidium iodide are superior to ethidium bromide for two-color flow cytometric viability assays in which either BCECF AM or calcein AM is used as the live-cell stain because their spectra do not overlap as much with those of the green-fluorescent esterase probes.[21]

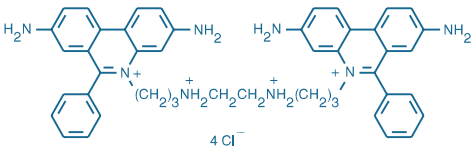

Figure 15.2.29 Ethidium homodimer-1 (EthD-1, E1169).

With its high affinity for DNA and low membrane permeability,[79–82] EthD-1 is often the preferred red-fluorescent dead-cell indicator. EthD-1 binds to nucleic acids 1000 times more tightly than does ethidium bromide and undergoes about a 40-fold enhancement of fluorescence upon binding.[81,83] When used as a viability indicator, EthD-1 typically does not require a wash step. Also, the high affinity of EthD-1 permits the use of very low concentrations to stain dead cells, thus avoiding the use of large quantities of the potentially hazardous ethidium bromide or propidium iodide. EthD-1, the dead-cell indicator in our LIVE/DEAD® Viability/Cytotoxicity Kit (L3224, Section 15.3), has been used alone[84] or in combination with calcein AM[85] to detect tumor necrosis factor activity and to assay neuronal cell death.[75,86,87] Ethidium homodimer-2 (E3599, Figure 15.2.31), which we use as the DEAD Red™ necrotic-cell indicator in our LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1 (L7013, Section 15.3), has a particularly low dissociation rate from cellular nucleic acids, permitting its use for selective marking of dead-cell populations that need to be observed over several hours. Our DEAD Red™ nucleic acid stain has proven useful for determining brain stem lesion size *in vivo* in rats following a neurotoxin injection.[88] Live and dead cells of the yeast-like fungus *Aureobasidium pullulans* have been identified on microscope slides as well as leaf surfaces using CellTracker™ Blue CMAC (C2110, Section 14.2) in conjunction with the DEAD Red™ nucleic acid stain.[89]

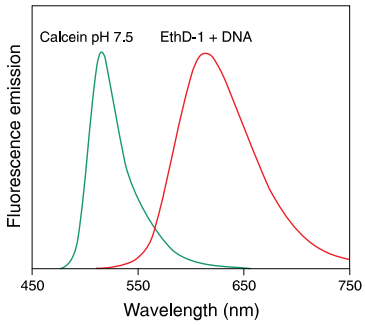

Figure 15.2.30 Normalized fluorescence emission spectra of calcein (C481) and DNA-bound ethidium homodimer-1 (EthD-1, E1169), illustrating the clear spectral separation that allows simultaneous visualization of live and dead eukaryotic cells with Molecular Probes® LIVE/DEAD® Viability/Cytotoxicity Kit (L3224).

Ethidium Monoazide

Ethidium monoazide (E1374) is a fluorescent photoaffinity label that, after photolysis, binds covalently to nucleic acids in solution and in cells with compromised membranes.[90,91] A mixed population of live and dead cells incubated with this membrane-impermeant dye can be illuminated with a visible-light source, washed, fixed and then analyzed in order to determine the viability of the cells at the time of photolysis. Thus, ethidium monoazide reduces some of the hazards inherent in working with pathogenic samples because, once stained, samples can be treated with fixatives before analysis by fluorescence microscopy or flow cytometry.[91] Immunocytochemical analyses requiring fixation are also compatible with this ethidium monoazide–based viability assay. We have developed an alternative two-color fluorescence-based assay for determining the original viability of fixed samples that employs our cell-permeant, green-fluorescent SYTO® 10 and cell-impermeant, red-fluorescent DEAD Red™ nucleic acid stains; the LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1 (L7013) is described in Section 15.3.

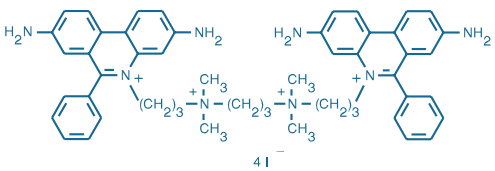

Figure 15.2.31 Ethidium homodimer-2 (EthD-2, E3599).

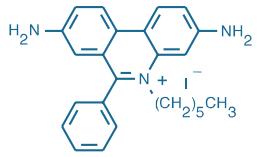

Figure 15.2.32 Hexidium iodide (H7593).

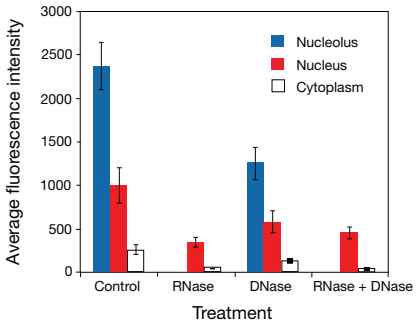

Figure 15.2.33 Methanol-fixed bovine pulmonary artery endothelial cells treated with RNase, DNase or both, and then labeled with SYTO® RNASelect™ Green cell stain (S32703). Removal of RNA with RNase prevented nucleolar labeling and greatly decreased nuclear and cytoplasmic labeling. Use of DNase resulted in less of a loss of label intensity in these cell compartments, reflecting the RNA-selective nature of this dye.

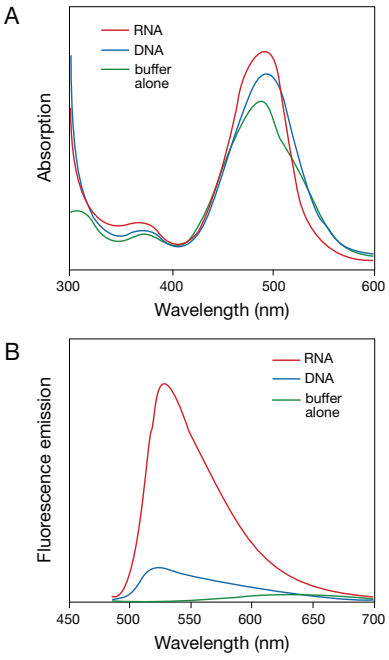

Figure 15.2.34 Absorption (**A**) and fluorescence emission (**B**) spectra of SYTO® RNASelect™ green-fluorescent cell stain (S32703) in the presence of *Escherichia coli* DNA or in buffer alone.

Hexidium Iodide: A Fluorescent Gram Stain

Ethidium bromide is only marginally permeant to cell membranes or bacteria; however, we found that our hexidium iodide stain (H7593) has the right combination of polarity and permeability (Figure 15.2.32) to allow it to rapidly stain most gram-positive bacteria while being excluded by the less-permeant membranes of most gram-negative bacteria.[92] Combining the red-orange–fluorescent hexidium iodide reagent with a green-fluorescent, membrane-permeant nucleic acid stain—as in our LIVE *Bac*Light™ Bacterial Gram Stain Kit (L7005, Section 15.3)—enables taxonomic classification of most bacteria in minutes, using a single staining solution, with no fixatives or wash steps. This rapid gram stain assay should be useful in both clinical and research settings. The validity of using hexidium iodide in combination with the SYTO® 13 green-fluorescent nucleic acid stain to correctly predict the gram sign of 45 clinically relevant organisms, including several known to be gram variable, has been demonstrated.[92] The method of use of hexidium iodide as a gram stain is described further in Section 15.3.

SYTO® Nucleic Acid Stains

Our SYTO® family of dyes, all of which are listed in Table 8.3, are essentially nonfluorescent until they bind to nucleic acids, whereupon their fluorescence quantum yield may increase by 1000-fold or more. These dyes are freely permeant to most cells, although their rate of uptake and ultimate staining pattern may be cell dependent. Their affinity for nucleic acids is moderate and they can be displaced by higher-affinity nucleic acid stains such as SYTOX® Green, propidium iodide, the ethidium dimers and all of the monomeric and dimeric nucleic acid stains described above. Because the membrane of intact cells offers a barrier to entry of these higher-affinity nucleic acid stains, it is common to combine, for instance, a green-fluorescent SYTO® dye with a red-fluorescent, high-affinity nucleic acid stain such as propidium iodide, one of the ethidium homodimers, or TOTO®-3 for simultaneous staining of the live- and dead-cell populations. Although the green-fluorescent SYTO® dye will still bind to nucleic acids in dead cells, it will be displaced or its fluorescence quenched by the red-fluorescent dye, resulting in a yellow-, orange- or red-fluorescent dead-cell population. This principle is the basis of our LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits (L7007, L7012, L13152), our LIVE/DEAD® Sperm Viability Kit (L7011) and our LIVE *Bac*Light™ Bacterial Gram Stain Kit (L7005), which are all discussed in Section 15.3. Four sampler kits of the SYTO® dyes (S7572, S11340, S11350, S11360) provide a total of 27 SYTO® dyes with emission maxima that range from 441 nm to 678 nm. All of the SYTO® dyes in the sampler kits are also available individually (Section 8.1), as well as several other SYTO® green-fluorescent (S32704, S34854, S34855) nucleic acid stains. The SYTO® 13 green-fluorescent nucleic acid stain (S7575) has been used in combination with:

- Ethidium bromide for studies of tissue cryopreservation [93]
- Hexidium iodide for simultaneous viability and gram sign of clinically relevant bacteria [92]
- Ethidium homodimer-1 for quantitation of neurotoxicity [75,94]
- Propidium iodide to detect the effects of surfactants on *Escherichia coli* viability [95]

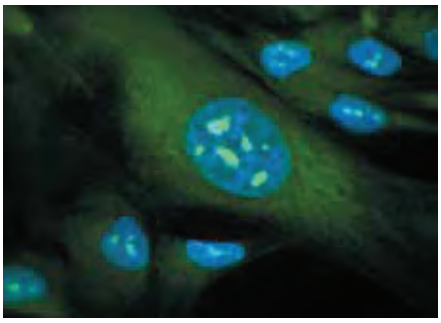

Figure 15.2.35 Methanol-fixed MRC-5 cells stained with SYTO® RNASelect™ green-fluorescent cell stain (S32703). Nuclei were stained with DAPI (D1306, D3571, D21490); the densely stained areas are nucleoli.

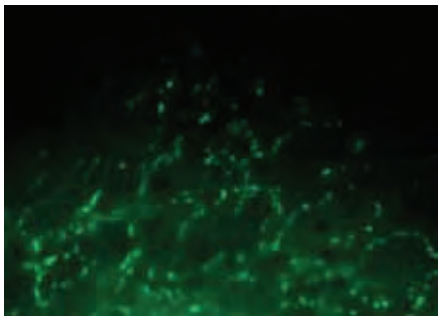

Figure 15.2.36 Methanol-fixed MRC-5 cells stained with SYTO® RNASelect™ green-fluorescent cell stain (S32703). Nuclei were stained with DAPI (D1306, D3571, D21490); the densely stained areas are nucleoli.

SYTO® RNASelect™ Green-Fluorescent Cell Stain

SYTO® RNASelect™ green-fluorescent cell stain (S32703) is a cell-permeant nucleic acid stain that selectively stains RNA (Figure 15.2.33). Although virtually nonfluorescent in the absence of nucleic acids, SYTO® RNASelect™ stain exhibits bright green fluorescence when bound to RNA (absorption/emission maxima ~490/530 nm), but only weak fluorescence when bound to DNA (Figure 15.2.34). Filter sets that are suitable for imaging cells labeled with fluorescein (FITC) will work well for imaging cells stained with SYTO® RNASelect™ stain (Figure 15.2.35, Figure 15.2.36).

Eukaryotic cells stained with the SYTO® RNASelect™ dye show a staining pattern consistent with that of an RNA-selective probe. Maximal fluorescence is observed in the nucleoli, with faint fluorescence throughout the nucleus. Weak fluorescence is also seen throughout the cytoplasm, predominantly associated with mitochondria. The RNA localization of the SYTO® RNASelect™ stain is further supported by RNase and DNase treatments: 1) upon treatment with RNase, the nucleolar and cytoplasmic intensities are significantly reduced, as compared with control cells; 2) upon treatment with DNase, there is no significant loss of fluorescence; and 3) upon treatment with both RNase and DNase, the staining pattern is the same as that observed with RNase treatment alone.

Because the SYTO® RNASelect™ green-fluorescent cell stain is cell permeant, it is suitable for use in live cells. After the cells have been stained, they may be fixed in methanol with minimal loss of the staining pattern. If desired, cells can also be fixed in methanol before staining RNA with the SYTO® RNASelect™ stain. Fixation with formaldehyde alters the staining pattern and is not recommended.

FUN® 1 Dye: A Unique Stain for Assessing Viability of Yeast and Fungi

While structurally related to the SYTO® dyes, FUN® 1 dye (F7030) contains substituents (Figure 15.2.37) that apparently make them chemically reactive with intracellular components of yeast, provided that the yeast are metabolically active. FUN® 1 dye is freely taken up by several species of yeast and fungi and converted from a diffusely distributed pool of yellow-green–fluorescent intracellular stain into compact red-orange– or yellow-orange–fluorescent intravacuolar structures, respectively (Figure 15.2.38). Conversion of FUN® 1 dye to products with longer-wavelength emission (Figure 15.2.39) requires both plasma membrane integrity and metabolic capability. Only metabolically active cells are marked clearly with fluorescent intravacuolar structures, while dead cells exhibit extremely bright, diffuse, yellow-green fluorescence.[61,96] The FUN® 1 cell stain is also available as a component in our LIVE/DEAD® Yeast Viability Kit (L7009, Section 15.3).

7-Aminoactinomycin D

7-AAD (7-aminoactinomycin D, A1310; Section 15.4) is a fluorescent intercalator that undergoes a spectral shift upon association with DNA. 7-AAD/DNA complexes can be excited by the argon-ion laser and emit beyond 610 nm (Figure 15.2.40), making this nucleic acid stain useful for multicolor fluorescence microscopy, confocal laser-scanning microscopy and flow cytometry. 7-AAD appears to be generally excluded from live cells, although it has been reported to label the nuclear region of live cultured mouse L cells and salivary gland polytene chromosomes of *Chironomus thummi thummi* larvae.[97] 7-AAD is also a useful marker for apoptotic cell populations (Section 15.5) and has been utilized to discriminate dead cells from apoptotic and live cells.[98] In addition, 7-AAD can be used in combination with conjugates of R-phycoerythrin (Section 6.4) in three-color flow cytometry protocols.

Viability/Cytotoxicity Assays That Measure Oxidation or Reduction

The generation of reactive oxygen species (ROS) is inevitable for aerobic organisms and, in healthy cells, occurs at a controlled rate. Under conditions of oxidative stress, ROS production is dramatically increased, resulting in subsequent alteration of membrane lipids, proteins and nucleic acids. Oxidative damage of these biomolecules is associated with aging[99,100] and with a variety of pathological events, including atherosclerosis, carcinogenesis, ischemic reperfusion injury and neuorodegenerative disorders.[101,102]

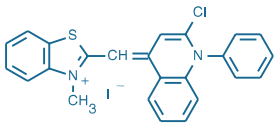

Figure 15.2.37 FUN® 1 cell stain (F7030).

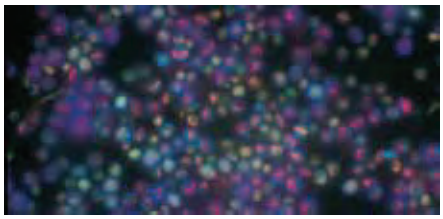

Figure 15.2.38 A culture of *Saccharomyces cerevisiae* incubated in medium containing the FUN® 1 viability indicator (F7030) and the counterstain Calcofluor White M2R, both of which are provided in our LIVE/DEAD® Yeast Viability Kit (L7009). Metabolically active yeast process the FUN® 1 dye, forming numerous red-fluorescent cylindrical structures within their vacuoles. Calcofluor stains the cell walls fluorescent blue, regardless of the yeast's metabolic state. The yeast were photographed in a single exposure through an Omega® Optical triple bandpass filter set.

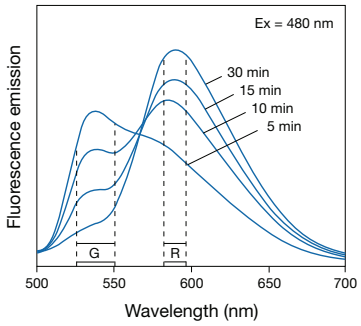

Figure 15.2.39 Fluorescence emission spectra of a *Saccharomyces cerevisiae* suspension that has been stained with the FUN® 1 cell stain, which is available separately (F7030) or in our LIVE/DEAD® Yeast Viability Kit (L7009). After the FUN® 1 reagent was added to the medium, the fluorescence emission spectrum (excited at 480 nm) was recorded in a spectrofluorometer at the indicated times during a 30-minute incubation period. The shift from green (G) to red (R) fluorescence reflects the processing of FUN® 1 by metabolically active yeast cells.

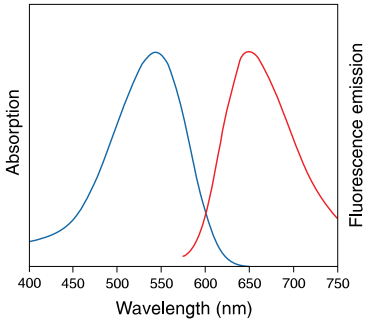

Figure 15.2.40 Absorption and fluorescence emission spectra of 7-aminoactinomycin D (7-AAD) bound to DNA.

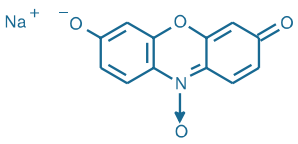

Figure 15.2.41 Resazurin, sodium salt (R12204).

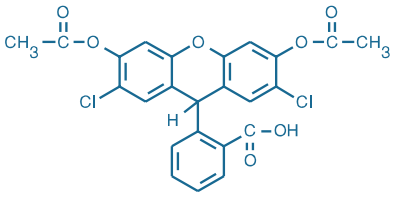

Figure 15.2.42 2′,7′-Dichlorodihydrofluorescein diacetate (2′,7′-dichlorofluorescin diacetate; H_2DCFDA; D399).

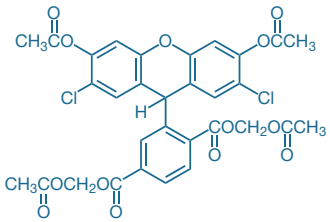

Figure 15.2.43 6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester), (C2938).

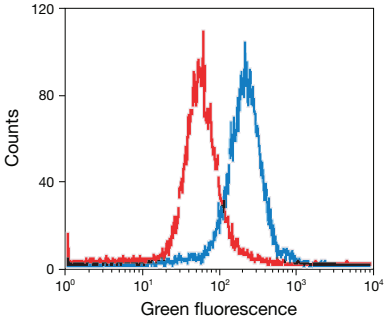

Figure 15.2.44 An oxidative burst was detected by flow cytometry of cells labeled with 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, C6827). Jurkat cells were incubated with 100 nM CM-H_2DCFDA. The cells were washed and resuspended in either phosphate-buffered saline (PBS, red) or PBS with 0.03% H_2O_2 (blue). The samples were analyzed on a flow cytometer equipped with a 488 nm argon-ion laser and a 525 ± 10 nm band-pass emission filter.

Metabolically active cells can oxidize or reduce a variety of probes, providing a measure of cell viability and overall cell health. This measure of viability is distinct from that provided by probes designed to detect esterase activity or cell permeability. Detecting oxidative activity and ROS in cells is also discussed in Section 18.2.

High-Purity Resazurin

Resazurin (R12204, Figure 15.2.41) has been extensively used as an oxidation–reduction indicator to detect bacteria and yeast in broth cultures and milk,[103,104] to assess the activity of sperm [105,106] and to assay bile acids [107,108] and triglycerides.[109] Resazurin reduction also occurs with other mammalian cells, including neurons,[110] corneal endothelial cells,[111] lymphocytes, lymphoid tumor cells and hybridoma cells.[112] Furthermore, resazurin has been used in high-throughput screening assays for compounds that act against *Mycobacterium tuberculosis*.[113] However, correlation of the results obtained with resazurin and bioluminescent assays for ATP has been reported to be poor.[114] Resazurin has been reported to be useful for quantitatively measuring cell-mediated cytotoxicity,[115] cell proliferation [116,117] and mitochondrial metabolic activity in isolated neural tissue.[118]

Dodecylresazurin: A Superior Probe for Cell Metabolic Studies

Dodecylresazurin (C_{12}-resazurin), which is available only as a component of our Vybrant® Cell Metabolic Assay Kit (V23110, Section 15.3), LIVE/DEAD® Cell Vitality Assay Kit (L34951, Section 15.3) and Metabolic Activity/Annexin V/Dead Cell Apoptosis Kit (V35114, Section 15.5), has several properties that make it superior to resazurin (and alamarBlue®) for detecting metabolic activity in cells:

- C_{12}-resazurin is freely permeant to the membranes of most cells.
- Less C_{12}-resazurin is required for equivalent sensitivity relative to resazurin.
- Unlike resazurin, which yields a product (resorufin) that rapidly leaks from viable cells, the product of reduction of C_{12}-resazurin—C_{12}-resorufin—is relatively well retained by single cells, permitting flow cytometric assay of cell metabolism and viability on a single-cell basis.
- The fluorescence developed by reduction of C_{12}-resazurin is directly proportional to cell number, and the assay is capable of detecting very low numbers of cells, even in a high-throughput microplate assay.

Dihydrorhodamines and Dihydrofluoresceins

Fluorescein, rhodamine and various other dyes can be chemically reduced to colorless, non-fluorescent leuco dyes. These "dihydro" derivatives are readily oxidized back to the parent dye by some reactive oxygen species (Section 18.2) and thus can serve as fluorogenic probes for detecting oxidative activity in cells and tissues.[119–121] Because reactive oxygen species are produced by live but not dead cells, fluorescent oxidation products that are retained in cells can be used as viability indicators for single cells or cell suspensions. Some probes that are useful for detecting oxidative activity in metabolically active cells include:

- H_2DCFDA [122] (2′,7′-dichlorodihydrofluorescein diacetate, D399, Figure 15.2.42), carboxy-H_2DCFDA (5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate, C400) and the acetoxymethyl ester of H_2DCFDA (C2938, Figure 15.2.43), all of which require both intracellular deacetylation and oxidation to yield green-fluorescent products [123–125]
- CM-H_2DCFDA (chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester, C6827), which is analogous to H_2DCFDA, except that it forms a mildly thiol-reactive fluorescent product after oxidation by metabolically active cells (Figure 15.2.44), permitting significantly longer-term measurements [126,127]
- Dihydrocalcein AM (D23805), our newest dihydrofluorescein derivative, which is converted intracellularly to calcein, a green-fluorescent dye with superior cell retention (Figure 15.2.3)
- Dihydrorhodamine 123 (D632, D23806; Figure 15.2.45) and dihydrorhodamine 6G (D633), which are oxidized in viable cells to the mitochondrial stains rhodamine 123 [128–131] and rhodamine 6G,[132,133] respectively
- Dihydroethidium (also known as hydroethidine; D1168, D11347, D23107; Figure 15.2.46), which forms the nucleic acid stain ethidium following oxidation [134,135] and has proven useful for detecting the viability of intracellular parasites [136]

- Luminol (L8455), which is useful for chemiluminescence-based detection of oxidative events in cells rich in peroxidases, including granulocytes [137–140] and spermatozoa [141]

These probes are all described in more detail in Section 18.2, which includes products for assaying oxidative activity in live cells and tissues.

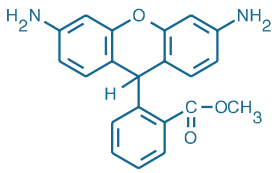

Figure 15.2.45 Dihydrorhodamine 123 (D632).

Image-iT® LIVE Green Reactive Oxygen Species Detection Kit

The Image-iT® LIVE Green Reactive Oxygen Species Detection Kit (I36007) provides the key reagents for detecting reactive oxygen species (ROS) in live cells, including:

- Carboxy-H_2DCFDA (5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate)
- Hoechst 33342
- *tert*-Butyl hydroperoxide (TBHP)
- Dimethylsulfoxide (DMSO)
- Detailed protocols for fluorescence microscopy assays

This assay is based on carboxy-H_2DCFDA (5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate), a reliable fluorogenic marker for ROS in live cells.[142,143] In addition to carboxy-H_2DCFDA, this kit provides the common inducer of ROS production *tert*-butyl hydroperoxide (TBHP) as a positive control [144–147] and the blue-fluorescent, cell-permeant nucleic acid stain Hoechst 33342. Oxidatively stressed and nonstressed cells can be reliably distinguished by fluorescence microscopy using this combination of dyes and the protocol provided (Figure 15.2.47).

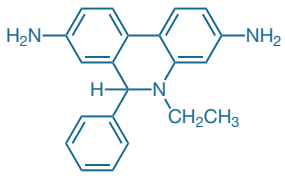

Figure 15.2.46 Dihydroethidium (hydroethidine, D1168).

RedoxSensor™ Red CC-1 Stain

RedoxSensor™ Red CC-1 stain (2,3,4,5,6-pentafluorotetramethyldihydrorosamine, R14060; Figure 15.2.48) passively enters live cells and is subsequently oxidized in the cytosol to a red-fluorescent product (excitation/emission maxima ~540/600 nm), which then accumulates in the mitochondria. Alternatively, this nonfluorescent probe may be transported to the lysosomes where it is oxidized. The differential distribution of the oxidized product between mitochondria and lysosomes appears to depend on the redox potential of the cytosol.[148–150] In proliferating cells, mitochondrial staining predominates; whereas in contact-inhibited cells, the staining is primarily lysosomal (Figure 15.2.49).

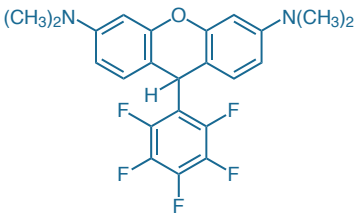

Figure 15.2.48 RedoxSensor™ Red CC-1 (R14060).

Tetrazolium Salts

Tetrazolium salts are widely used for detecting redox potential of cells for viability, cytotoxicity and proliferation assays.[151–156] Following reduction, these water-soluble, colorless compounds form uncharged, brightly colored but nonfluorescent formazans. Several of the formazans precipitate out of solution and are useful for histochemical localization of the site of reduction or, after solubilization in organic solvent, for quantitation by standard spectrophotometric techniques.

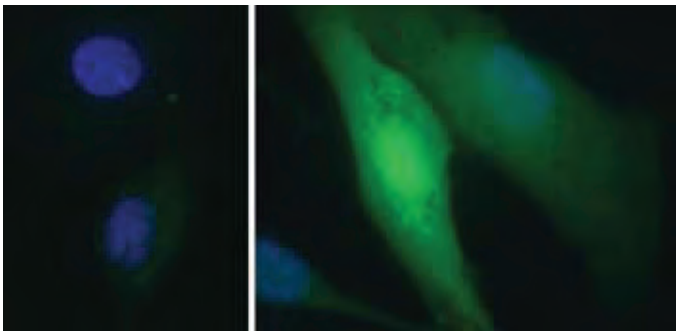

Figure 15.2.47 Detection of oxidative stress in live cells using the Image-iT® LIVE Reactive Oxygen Species (ROS) Kit (I36007). Live bovine pulmonary artery endothelial cells were treated with *tert*-butyl hydroperoxide to induce oxidative stress (right) or were left untreated (left). Cells were then labeled with carboxy-H_2DCFDA, which fluoresces when oxidized by ROS, and nuclei were stained with blue-fluorescent Hoechst 33342. The stressed cells exhibited green fluorescence, signaling an increase in ROS, whereas the untreated cells showed minimal fluorescence.

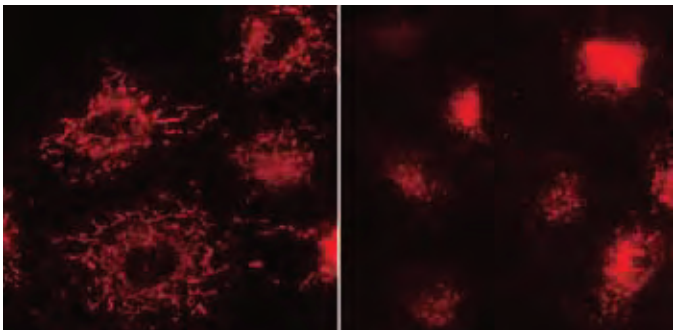

Figure 15.2.49 Cellular proliferation state determines the distribution of the oxidized product of RedoxSensor™ Red CC-1 (R14060). Normal rat kidney (NRK) cells in different growth states were stained with RedoxSensor™ Red CC-1. In proliferating cells (left), the oxidized dye accumulates in mitochondria. In quiescent cells (right), the oxidized product localizes in the lysosomes.

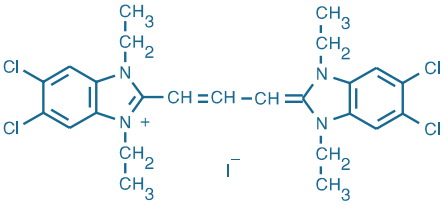

Figure 15.2.50 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, T3168)

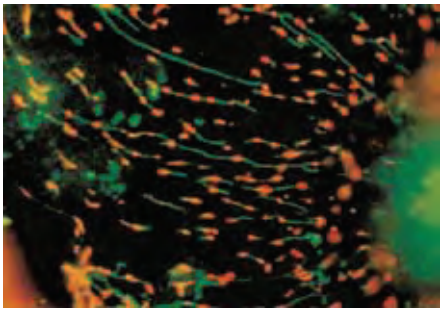

Figure 15.2.51 Potential-dependent staining of mitochondria in CCL64 fibroblasts by JC-1 (T3168). The mitochondria were visualized by epifluorescence microscopy using a 520 nm longpass optical filter. Regions of high mitochondrial polarization are indicated by red fluorescence due to J-aggregate formation by the concentrated dye. Depolarized regions are indicated by the green fluorescence of the JC-1 monomers. The image was contributed by Lan Bo Chen, Dana Farber Cancer Institute, Harvard Medical School.

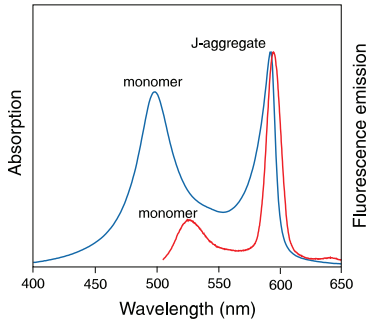

Figure 15.2.52 Absorption and fluorescence emission (excited at 488 nm) spectra of JC-1 in pH 8.2 buffer containing 1% (v/v) DMSO.

Reduction of MTT (M6494) remains the most common assay for tetrazolium salt–based viability testing.[157–159] The Vybrant® MTT Cell Proliferation Assay Kit (V13154, Section 15.4) provides a simple method for determining cell number using standard microplate absorbance readers. MTT has also been used to measure adhesion of HL60 leukemia cells onto endothelial cells.[160] In addition to dehydrogenases, MTT is reduced by glutathione *S*-transferase[161] (GST). Therefore, MTT may not always be a reliable cell viability probe in cells treated with compounds that affect GST activity.

Unlike MTT's purple-colored formazan product, the extremely water-soluble, orange-colored formazan product of XTT (X6493) does not require solubilization prior to quantitation, thereby reducing the assay time in many viability assay protocols. Moreover, the sensitivity of the XTT reduction assay is reported to be similar to or better than that of the MTT reduction assay.[152] The XTT reduction assay is particularly useful for high-throughput screening of antiviral and antitumor agents and for assessing the effect of cytokines on cell proliferation.[156,162–165] NBT (N6495) forms a deep blue–colored precipitate that is commonly used to indicate oxidative metabolism.[166,167]

Other Viability/Cytotoxicity Assay Methods

A viable cell contains an ensemble of ion pumps and channels that maintain both intracellular ion concentrations and transmembrane potentials. Active maintenance of ion gradients ceases when the cell dies, and this loss of activity can be assessed using potentiometric dyes, acidotropic probes, Ca^{2+} indicators[122] (Chapter 19) and pH indicators[122] (Chapter 20).

Potentiometric Dyes

We offer a variety of dyes for detecting transmembrane potential gradients (Chapter 22), including several cationic probes that accumulate in the mitochondria of metabolically active cells (Section 12.2). The mitochondrion-selective rhodamine 123[122] (R302, R22420) has been used to assess the viability of lymphocytes,[168] human fibroblasts,[169] Simian virus–transformed human cells[170] and bacteria;[171] however, rhodamine 123 is not taken up well by gram-negative bacteria.[171] Rhodamine 123 has also been used in combination with propidium iodide (P1304MP, P3566, P21493) for two-color flow cytometric viability assessment.[172]

The methyl and ethyl esters of tetramethylrhodamine (T668, T669) accumulate in the mitochondria of healthy cells in an amount related to the membrane potential. The dyes are nontoxic and highly fluorescent and do not form aggregates or display binding-dependent changes in their fluorescence efficiency, permitting continuous monitoring of cell heath.[173]

Other potential-sensitive dyes that have proven useful in viability studies include several fast-response styryl dyes and slow-response oxonol and carbocyanine dyes. The fast-response styryl dyes such as di-4-ANEPPS (D1199, Section 22.2) give relatively large fluorescence response to potential changes. Di-4-ANEPPS was used for rapid measurement of toxicity in frog embryos.[174] The symmetrical bis-oxonol dyes[122] (B413, B438; Section 22.3) have been used for viability assessment by flow cytometry[175–177] and imaging. These slow-response dyes have also been employed to determine antibiotic susceptibility of bacteria by flow cytometry,[178,179] and our *Bac*Light™ Bacterial Membrane Potential Kit (B34950, Section 15.3) provides the carbocyanine dye $DiOC_2(3)$ along with the proton ionophore CCCP for detecting membrane potential in both gram-positive and gram-negative bacteria.

The green-fluorescent cyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, T3168, M34152; Figure 15.2.50) exists as a monomer at low concentrations or at low membrane potential; however, at higher concentrations (aqueous solutions above 0.1 µM) or higher potentials, JC-1 forms red-fluorescent "J-aggregates" (Figure 15.2.51) that exhibit a broad excitation spectrum and an emission maximum at ~590 nm (Figure 15.2.52). JC-1 has been used to investigate apoptosis,[180,181] as well as mitochondrial poisoning, uncoupling and anoxia.[182] The ability to make ratiometric emission measurements with JC-1 makes this probe particularly useful for monitoring changes in cell health. We have discovered another carbocyanine dye, JC-9 (3,3′-dimethyl-β-naphthoxazolium iodide, D22421; Figure 15.2.53), with potential-dependent spectroscopic properties.

Acidotropic Stains

Membrane-bound proton pumps are used to maintain low pH within the cell's acidic organelles. Our complete selection of stains for lysosomes and other acidic organelles, including LysoTracker® and LysoSensor™ probes, is described in Section 12.3.

The lysosomal stain neutral red (N3246), which was first used for viability measurements by Ehrlich in 1894, has been employed in numerous cytotoxicity, cell proliferation and adhesion assays.[183–188] Although usually used as a chromophoric probe, neutral red also fluoresces at ~640 nm in viable cells and has been detected using a fluorescence microplate reader.[189] Furthermore, the fluorescence of neutral red and BCECF AM (or SYTOX® Green nucleic acid stain) can be measured simultaneously using a single excitation wavelength of 488 nm,[189] suggesting that neutral red may be an effective probe for multicolor flow cytometric determination of cell viability. Our neutral red is highly purified to reduce contaminants that might interfere with these observations.

Acridine orange (A1301, A3568) concentrates in acidic organelles in a pH-dependent manner. The metachromatic green or red fluorescence of acridine orange has been used to assess islet viability [190] and bacterial spore viability [191] and to monitor physiological activity in *Escherichia coli*.[192]

LysoTracker® Green DND-26 (L7526, Section 12.3) was used in a fluorometric assay of cryopreserved sperm to demonstrate both acrosomal integrity and sperm viability.[193] Of several methods used, LysoTracker® Green DND-26 staining was the quickest and easiest to use and gave excellent correlation with the SYBR® 14 staining method used in our LIVE/DEAD® Sperm Viability Kit (L7011, Section 15.3). When observed with a fluorescence microscope, sperm appeared to lose their green-fluorescent LysoTracker® Green DND-26 staining and instead exhibited red-fluorescent propidium iodide staining within about 30 seconds after motility ceased.[194,195]

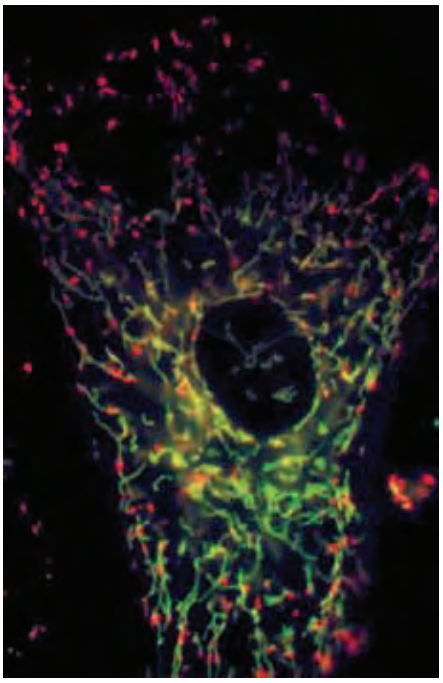

Figure 15.2.53 A viable bovine pulmonary artery endothelial cell incubated with the ratiometric mitochondrial potential indicator, JC-9 (D22421). In live cells, JC-9 exists either as a green-fluorescent monomer at depolarized membrane potentials, or as a red-fluorescent J-aggregate at hyperpolarized membrane potentials.

Fluorescent Glucose Analogs

Measurements of glucose uptake can be used to assess viability in a variety of organisms. 2-NBD-deoxyglucose (2-NBDG, N13195) has been used to monitor glucose uptake in living pancreatic β-cells,[196] the yeast *Candida albicans*[197] and the bacteria *Escherichia coli*.[198–200] We also offer the fluorescent nonhydrolyzable glucose analog 6-NBD-deoxyglucose (6-NBDG, N23106). Using this probe, researchers have studied glucose uptake and transport in isolated cells [201–203] and intact tissues.[204] Although sensitive to its environment, NBD fluorescence typically displays excitation/emission maxima of ~465/540 nm and can be visualized using optical filters designed for fluorescein.

Fluorescent Antibiotics and Related Probes

Fluorescent Polymyxins

Polymyxin B is a cyclic cationic peptide antibiotic that binds to the lipopolysaccharide (LPS) component of the outer membrane of gram-negative bacteria and increases its permeability to lysozyme and hydrophobic compounds. Molecular Probes® fluorescent BODIPY® FL (P13235), Oregon Green® 514 (P13236) and dansyl (P13238) derivatives of polymyxin B are available. Dansyl polymyxin B, which fluoresces weakly when free in solution, becomes highly fluorescent (excitation/emission ~340/485 nm) upon binding to intact cells or LPS.[205] The binding of dansyl polymyxin B to LPS can be displaced by a variety of polycationic antibiotics, as well as by Mg^{2+}. Consequently, dansyl polymyxin B displacement experiments can be used to assess the binding of various compounds, such as antibiotics and macrophage cationic proteins, to LPS and intact bacterial cells.[206–208] Dansyl polymyxin has also been used to localize regions of high anionic lipid content in both sperm [209] and aggregated human platelets,[210] and to analyze the morphology of lipid bilayers in preparations of acetylcholine receptor clusters from rat myotubules.[211] Green-fluorescent BODIPY® FL and Oregon Green® 514 polymyxin B provide additional fluorescent color options for these experiments. Our fluorescent LPS is described in Section 13.3 and Section 16.1.

Fluorescent Penicillin Analogs

BOCILLIN™ FL penicillin and BOCILLIN™ 650/665 penicillin (B13233, B13234) are green- and infrared-fluorescent penicillin analogs, respectively, that bind selectively and with high affinity to penicillin-binding proteins present on the cytoplasmic membranes of

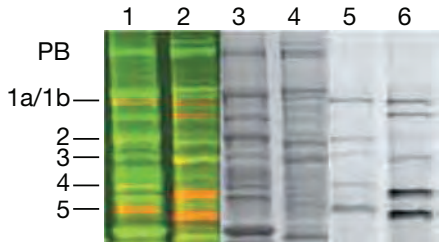

Figure 15.2.54 Detection of penicillin-binding proteins (PBPs) from *Escherichia coli* and *Pseudomonas aeruginosa*. The membrane fractions from *E. coli* and *P. aeruginosa* were prepared as previously described and labeled with BOCILLIN™ 650/665 penicillin (B13234). The labeled membranes were separated on an SDS-polyacrylamide gel, stained with SYPRO® Ruby and visualized using a Typhoon imager. The location of PBPs from *E. coli* are labeled to the left of the gels. Lanes 1, 3 and 5 are *E. coli* membrane preparations; lanes 2, 4 and 6 are *P. aeruginosa* membrane preparations; lanes 1 and 2 are overlays of images obtained from total protein (green) and PBP (red) scans; lanes 3 and 4 are total protein visualized with the SYPRO® Ruby protein gel stain; lanes 5 and 6 are PBPs as detected by BOCILLIN™ 650/665. Image used with permission from Wiley VCH publishers.

eubacteria.[212,213] When electrophoresed under nonreducing conditions, the dye-labeled penicillin-binding proteins are easily visible in the gel with sensitivity in the low nanograms[214] (Figure 15.2.54). BOCILLIN™ FL penicillin, synthesized from penicillin V and the BODIPY® FL dye (spectrally similar to fluorescein), has been used to determine the penicillin-binding protein profiles of *Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus pneumoniae*, and these binding profiles are found to be similar to those reported by researchers using radioactively labeled penicillin V.[212] Fluorescently labeled penicillin has also been used for direct labeling and rapid detection of whole *E. coli* and *Bacillus licheniformis*[215] and of *Enterobacter pneumoniae*.[216] The β-lactam sensor-transducer (BlaR), an integral membrane protein from *Staphylococcus aureus*, covalently and stoichiometrically reacts with β-lactam antibiotics, including BOCILLIN™ FL penicillin, by acylation of its active-site serine residue.[217]

BODIPY® FL Vancomycin

BODIPY® FL vancomycin (V34850), which contains a single BODIPY® FL dye per vancomycin molecule, is a green-fluorescent analog of this important antibiotic, which is active against gram-positive bacteria, including enterrococci. BODIPY® FL vancomycin is useful for detecting vancomycin binding sites[218,219] and for the study and detection of vancomycin-resistant enterococci[220,221] (VRE, Section 15.6).

Antimalarial Agent

The phosphonate antibiotic FR-31564 (fosmidomycin, F23103) is an effective antimalarial agent that functions by blocking a mevalonate-independent methylerythritol phosphate (MEP) pathway of isoprene synthesis.[222–225] The antibiotic activity of fosmidomycin is potentiated by glucose 1-phosphate.[226,227] This antibiotic is also active against several gram-negative bacteria.[228,229]

REFERENCES

1. J Immunol Methods (2000) 243:155; **2**. Biochem Biophys Res Commun (1990) 172:262; **3**. EMBO J (1986) 5:51; **4**. J Immunol Methods (1994) 177:101; **5**. J Immunol Methods (1994) 172:227; **6**. J Immunol Methods (1994) 172:115; **7**. Hum Immunol (1993) 37:264; **8**. Biophys J (1977) 18:3; **9**. J Cell Biol (1994) 125:1077; **10**. Cornea (1996) 15:599; **11**. Biophys J (1995) 68:1207; **12**. Cytometry (1995) 19:366; **13**. Cytometry (1991) 12:666; **14**. J Immunol Methods (1991) 139:281; **15**. Biophys J (1995) 68:2608; **16**. Biochemistry (1998) 37:2243; **17**. Appl Environ Microbiol (2000) 66:4486; **18**. Science (1991) 251:81; **19**. J Neurosci (2008) 28:143; **20**. J Immunol Methods (1994) 172:255; **21**. Cytometry (1990) 11:244; **22**. J Immunol Methods (1989) 122:15; **23**. Trop Med Parasitol (1995) 46:45; **24**. Cryobiology (1977) 14:322; **25**. Vox Sang (1974) 27:13; **26**. Proc Natl Acad Sci U S A (1966) 55:134; **27**. J Immunol Methods (1993) 157:117; **28**. Cancer Res (1989) 49:3776; **29**. J Histochem Cytochem (1985) 33:77; **30**. Cytometry (1986) 7:70; **31**. Biochemistry (1979) 18:2210; **32**. Immunol Lett (1981) 2:187; **33**. J Immunol Methods (1980) 33:33; **34**. Biochemistry (1995) 34:1606; **35**. Cytometry (1992) 13:739; **36**. J Immunol Methods (1990) 130:251; **37**. Proc SPIE-Int Soc Opt Eng (1999) 3755:24; **38**. J Immunol Methods (1987) 100:261; **39**. Cytometry (1994) 15:213; **40**. Appl Environ Microbiol (1994) 60:1467; **41**. Biol Reprod (1986) 34:127; **42**. J Immunol Methods (1986) 86:7; **43**. Anticancer Res (1994) 14:927; **44**. J Immunol Methods (1992) 155:19; **45**. J Cell Biol (1995) 128:779; **46**. Methods Enzymol (1991) 194:644; **47**. Methods Cell Biol (1989) 31:357; **48**. J Cell Biol (1990) 111:3129; **49**. J Immunol Methods (1990) 133:87; **50**. FEBS Lett (1986) 200:203; **51**. Biotechniques (1985) 3:270; **52**. Appl Environ Microbiol (1995) 61:3415; **53**. J Vasc Res (1995) 32:371; **54**. Connect Tissue Res (1996) 33:233; **55**. J Cell Biol (1996) 135:1593; **56**. EMBO J (1993) 12:5209; **57**. J Neurosci (1993) 13:834; **58**. Science (1992) 255:200; **59**. J Bacteriol (2008) 190:311; **60**. Electrophoresis (2000) 21:2509; **61**. Plant Methods (2008) 4:15; **62**. Appl Environ Microbiol (1997) 63:2421; **63**. Appl Environ Microbiol (1994) 60:3284; **64**. Physiol Rev (2002) 82:1013; **65**. Anal Biochem (1994) 221:78; **66**. Cytometry (1999) 37:14; **67**. Appl Environ Microbiol (2001) 67:2326; **68**. Appl Environ Microbiol (1995) 61:3623; **69**. Limnol Oceanogr (1995) 40:1050; **70**. J Immunol Methods (1995) 185:249; **71**. J Microbiol Methods (2000) 42:3; **72**. J Immunol Methods (2010) 358:81; **73**. J Immunol Methods (1982) 52:91; **74**. Appl Environ Microbiol (1995) 61:2521; **75**. Neuron (1995) 15:961; **76**. Anal Biochem (1994) 220:149; **77**. J Immunol Methods (1992) 149:133; **78**. J Immunol Methods (1990) 134:201; **79**. Nucleic Acids Res (1995) 23:1215; **80**. Nucleic Acids Res (1992) 20:2803; **81**. Biochemistry (1978) 17:5078; **82**. Biochemistry (1978) 17:5071; **83**. Proc Natl Acad Sci U S A (1990) 87:3851; **84**. J Immunol Methods (1995) 178:71; **85**. Cytometry (1995) 20:181; **86**. J Neurosci (1995) 15:6239; **87**. J Neurosci (1994) 14:2260; **88**. J Appl Physiol (1998) 85:2370; **89**. Biotechniques (2000) 29:874, 882; **90**. Appl Environ Microbiol (2006) 72:1997; **91**. Methods Mol Biol (2009) 510:415; **92**. Appl Environ Microbiol (1998) 64:2681; **93**. J Physiol (1997) 502:105; **94**. Cytometry (1998) 32:66; **95**. Cytometry (1997) 29:58; **96**. J Cell Biol (1994) 126:1375; **97**. Histochem J (1985) 17:131; **98**. Cytometry (1992) 13:204; **99**. Mitochondrion (2003) 2:361; **100**. Ann N Y Acad Sci (2000) 908:219; **101**. J Cell Mol Med (2002) 6:175; **102**. Free Radic Biol Med (2001) 31:164; **103**. Appl Environ Microbiol (1990) 56:3785; **104**. J Dairy Res (1990) 57:239; **105**. Hum Reprod (1994) 9:1688; **106**. Fertil Steril (1991) 56:743; **107**. Steroids (1981) 38:281; **108**. Clin Chim Acta (1976) 70:79; **109**. Clin Chem (1983) 29:171; **110**. J Neurosci Methods (1996) 70:195; **111**. Invest Ophthalmol Vis Sci (1997) 38:1929; **112**. J Immunol Methods (1994) 170:211; **113**. Antimicrob Agents Chemother (1997) 41:1004; **114**. Gynecol Oncol (1995) 58:101; **115**. J Immunol Methods (1998) 213:157; **116**. J Clin Lab Anal (1995) 9:89; **117**. J Immunol Methods (1994) 175:181; **118**. J Neurosci Res (1996) 45:216; **119**. Arch Toxicol (1994) 68:582; **120**. Brain Res (1994) 635:113; **121**. Chem Res Toxicol (1992) 5:227; **122**. Methods Mol Biol (2010) 594:57; **123**. Biochemistry (1995) 34:7194; **124**. Cell (1993) 75:241; **125**. J Immunol (1983) 130:1910; **126**. J Pharmacol Exp Ther (2002) 300:9; **127**. J Biol Chem (1999) 274:19323; **128**. Cytometry (1994) 18:147; **129**. Methods Enzymol (1994) 233:539; **130**. Naturwissenschaften (1988) 75:354; **131**. J Med Chem (1987) 30:1757; **132**. J Cell Physiol (1993) 156:428; **133**. Biochem Biophys Res Commun (1991) 175:387; **134**. FEMS Microbiol Lett (1994) 122:187; **135**. J Immunol Methods (1994) 170:117; **136**. J Immunol Methods (1991) 140:23; **137**. J Appl Physiol (1994) 76:539; **138**. J Leukoc Biol (1993) 54:300; **139**. J Biochem (Tokyo) (1989) 106:355; **140**. Biochem Biophys Res Commun (1988) 155:106; **141**. J Cell

REFERENCES—*continued*

Physiol (1992) 151:466; **142**. Am J Physiol Heart Circ Physiol (2000) 279:H2424; **143**. J Natl Cancer Inst (1999) 91:1138; **144**. Lipids (2001) 36:57; **145**. Cancer Res (2001) 61:1392; **146**. Histochem Cell Biol (2003) 120:319; **147**. Am J Physiol (1997) 272:C1286; **148**. Am J Pathol (2009) 174:101; **149**. Am J Physiol Renal Physiol (2007) 292:F523; **150**. Free Radic Biol Med (2000) 28:1266; **151**. J Immunol Methods (1995) 179:95; **152**. J Infect Dis (1995) 172:1153; **153**. J Appl Bacteriol (1993) 74:433; **154**. J Immunol Methods (1993) 160:89; **155**. J Immunol Methods (1993) 157:203; **156**. J Immunol Methods (1991) 142:257; **157**. J Cell Biol (1995) 128:201; **158**. Cancer Res (1994) 54:3620; **159**. Cell (1994) 77:817; **160**. J Immunol Methods (1993) 164:255; **161**. Biotechniques (1998) 25:622; **162**. J Immunol Methods (1993) 159:81; **163**. J Immunol Methods (1992) 147:153; **164**. J Natl Cancer Inst (1989) 81:577; **165**. Cancer Res (1988) 48:4827; **166**. Clin Chim Acta (1993) 221:197; **167**. J Leukoc Biol (1993) 53:404; **168**. Clin Bull (1981) 11:47; **169**. J Cell Biol (1981) 91:392; **170**. Somatic Cell Genet (1983) 9:375; **171**. J Appl Bacteriol (1992) 72:410; **172**. Cancer Res (1982) 42:799; **173**. Biophys J (1988) 53:785; **174**. Bull Environ Contam Toxicol (1993) 51:557; **175**. Cytometry (1994) 15:343; **176**. J Immunol Methods (1993) 161:119; **177**. Jpn J Pharmacol (1991) 57:419; **178**. J Appl Bacteriol (1995) 78:309; **179**. J Microsc (1994) 176:8; **180**. J Biol Chem (2008) 283:5188; **181**. Nat Protoc (2007) 2:2719; **182**. Cardiovasc Res (1993) 27:1790; **183**. Clin Chem (1995) 41:1906; **184**. Cell Biol Toxicol (1994) 10:329; **185**. Anal Biochem (1993) 213:426; **186**. Biotech Histochem (1993) 68:29; **187**. Nat Protoc (2008) 3:1125; **188**. Biotechnology (N Y) (1990) 8:1248; **189**. In Vitro Toxicol (1990) 3:219; **190**. In Vitro Cell Dev Biol (1988) 24:266; **191**. Biotech Histochem (1992) 67:27; **192**. J Microbiol Methods (1991) 13:87; **193**. Biol Reprod (1997) 56:991; **194**. Development (1995) 121:825; **195**. J Immunol Methods (1989) 119:45; **196**. J Biol Chem (2000) 275:22278; **197**. Appl Microbiol Biotechnol (1996) 46:400; **198**. J Microbiol Methods (2000) 42:87; **199**. Biochim Biophys Acta (1996) 1289:5; **200**. Biosci Biotechnol Biochem (1996) 60:1899; **201**. Cytometry (1997) 27:262; **202**. Biochemistry (1995) 34:15395; **203**. Biochim Biophys Acta (1992) 1111:231; **204**. Histochem J (1994) 26:207; **205**. Antimicrob Agents Chemother (1986) 29:496; **206**. Antimicrob Agents Chemother (1993) 37:453; **207**. Antimicrob Agents Chemother (1991) 35:1309; **208**. Infect Immun (1988) 56:693; **209**. Proc Natl Acad Sci U S A (1980) 77:6601; **210**. Thromb Res (1988) 50:605; **211**. Exp Cell Res (1991) 195:79; **212**. Antimicrob Agents Chemother (1999) 43:1124; **213**. J Biol Chem (2000) 275:17693; **214**. Electrophoresis (2001) 22:960; **215**. Biochem J (1993) 291:19; **216**. Biochem J (1994) 300:141; **217**. J Biol Chem (2003) 278:18419; **218**. PLos Patholog (2009) 5:e1000285; **219**. Antimicrob Agents Chemother (2007) 51:3267; **220**. Clin Microbiol Infect (2008) 14:766; **221**. N Engl J Med (2001) 344:1427; **222**. J Infect Dis (2004) 189:901; **223**. Proc Natl Acad Sci U S A (2004) 101:7451; **224**. Science (1999) 285:1502; **225**. Science (1999) 285:1573; **226**. Eur J Clin Microbiol (1987) 6:386; **227**. Infection (1987) 15:465; **228**. J Antibiot (Tokyo) (1980) 33:44; **229**. J Antibiot (Tokyo) (1980) 33:24.

DATA TABLE 15.2 VIABILITY AND CYTOTOXICITY ASSAY REAGENTS

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A1301	301.82	L	H_2O, EtOH	500	53,000	526	H_2O/DNA	1, 2
A3568	301.82	RR,L	H_2O	500	53,000	526	H_2O/DNA	1, 2, 3
B1150	~615	F,D	DMSO	<300		none		4, 5
B1170	~615	F,D	DMSO	<300		none		4, 5
B3051	~615	F,D	DMSO	<300		none		3, 4, 5
B13233	661.46	F,D,L	H_2O, DMSO	504	68,000	511	MeOH	
B13234	653.44	F,D,L	DMSO	646	78,000	659	MeOH	
C195	460.40	F,D	DMSO	<300		none		6
C369	529.29	F,D	DMSO	<300		none		7
C400	531.30	F,D	DMSO, EtOH	290	5600	none	MeCN	8
C1354	532.46	F,D	DMSO	<300		none		9
C1361	460.40	F,D	DMSO	<300		none		6
C1362	460.40	F,D	DMSO	<300		none		6
C1429	465.41	F,D,L	DMSO	322	13,000	437	DMSO	10
C1430	994.87	F,D	DMSO	<300		none		11
C2925	464.86	F,D	DMSO	<300		none		6
C2938	675.43	F,D,AA	DMSO	291	5700	none	MeOH	8
C3099	994.87	F,D	DMSO	<300		none		3, 11
C3100MP	994.87	F,D	DMSO	<300		none		11
C6826	499.95	F,D	DMSO	<350		none		12
C6827	577.80	F,D,AA	DMSO	287	9100	none	MeOH	8
C7025	464.86	F,D	DMSO	<300		none		6
C13196	560.52	F,D	DMSO	<300		none		13
C34851	789.55	F,D,L	DMSO	576	90,000	589	DMSO	14
C34852	994.87	F,D	DMSO	<300		none		11
C34853	465.41	F,D	DMSO	322	13,000	437	DMSO	10
D399	487.29	F,D	DMSO, EtOH	258	11,000	none	MeOH	8
D632	346.38	F,D,L,AA	DMF, DMSO	289	7100	none	MeOH	15, 16
D633	444.57	F,D,L,AA	DMF, DMSO	296	11,000	none	MeOH	15, 16
D1168	315.42	FF,L,AA	DMF, DMSO	355	14,000	see Notes	MeCN	15, 17
D11347	315.42	FF,L,AA	DMF, DMSO	355	14,000	see Notes	MeCN	15, 17
D22421	532.38	D,L	DMSO, DMF	522	143,000	535	$CHCl_3$	18
D23107	315.42	FF,D,L,AA	DMSO	355	14,000	see Notes	MeCN	17, 19
D23805	1068.95	F,D	DMSO	285	5800	none	MeCN	20
D23806	346.38	F,D,L,AA	DMSO	289	7100	none	MeOH	16, 19
E1169	856.77	F,D,L	DMSO	528	7000	617	H_2O/DNA	1, 21, 22
E1374	420.31	F,LL	DMF, EtOH	462	5400	625	pH 7	23
E3599	1292.71	F,D,L	DMSO	535	8000	624	H_2O/DNA	1, 3, 21, 22
F1303	416.39	F,D	DMSO	<300		none		6
F7030	528.84	F,D,L	DMSO	508	71,000	none	pH 7	3, 24
F23103	205.08	F,D,L	H_2O	<300		none		
H7593	497.42	L	DMSO	518	3900	600	H_2O/DNA	1, 25
L8455	177.16	D,L	DMF	355	7500	411	MeOH	26
M6494	414.32	D,L	H_2O, DMSO	375	8300	none	MeOH	27, 28
N3246	288.78	D,L	H_2O, EtOH	541	39,000	640	see Notes	29
N6495	817.65	D,L	H_2O, DMSO	256	64,000	none	MeOH	27
N13195	342.26	F,L	H_2O	466	20,000	540	MeOH	30

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DATA TABLE 15.2 VIABILITY AND CYTOTOXICITY ASSAY REAGENTS—*continued*

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
N23106	342.26	F,L	DMSO	475	25,000	552	H_2O	30
O6151	496.38	F,D	DMSO	<300		none		31
P1304MP	668.40	L	H_2O, DMSO	535	5400	617	H_2O/DNA	1, 32
P3566	668.40	RR,L	H_2O	535	5400	617	H_2O/DNA	1, 3, 32
P21493	668.40	L	H_2O, DMSO	535	5400	617	H_2O/DNA	33
R302	380.83	F,D,L	MeOH, DMF	507	101,000	529	MeOH	
R12204	251.17	L	H_2O, MeOH	604	60,000	none	MeOH	34
R14060	434.41	F,D,L,AA	DMSO	239	52,000	none	MeOH	15, 35
R22420	380.83	F,D,L	MeOH, DMF	507	101,000	529	MeOH	33
S1129	518.43	F,D	DMSO	<300		none		36
S7020	~600	F,D,L	DMSO	504	67,000	523	H_2O/DNA	1, 3, 37, 38
S7575	~400	F,D,L	DMSO	488	74,000	509	H_2O/DNA	1, 3, 37, 38, 39
S11348	~400	F,D,L	DMSO	445	38,000	470	H_2O/DNA	1, 3, 37, 38
S11368	~500	F,D,L	DMSO	547	79,000	570	H_2O/DNA	1, 3, 37, 38
S32703	~800	F,D,L	DMSO	491	107,000	532	H_2O/RNA	1, 3, 37, 38
S32704	~350	F,D,L	DMSO	484	67,000	505	H_2O/DNA	1, 3, 37, 38
S34854	~400	F,D,L	DMSO	483	65,000	503	H_2O/DNA	1, 3, 37, 38
S34855	~400	F,D,L	DMSO	480	66,000	502	H_2O/DNA	1, 3, 37, 38
S34859	~450	F,D,L	DMSO	640	92,000	658	H_2O/DNA	1, 3, 37, 38
T668	500.93	F,D,L	DMSO, MeOH	549	115,000	573	MeOH	
T669	514.96	F,D,L	DMSO, EtOH	549	109,000	574	MeOH	
T3168	652.23	D,L	DMSO, DMF	514	195,000	529	MeOH	40
V34850	1723.35	F,D,L	H_2O, DMSO	504	68,000	511	MeOH	
X6493	674.53	F,D	H_2O, DMSO	286	15,000	none	MeOH	41
15585-011(EtBr)	394.31	RR,L	H_2O	518	5200	605	H_2O/DNA	1,3,42

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. Spectra represent aqueous solutions of nucleic acid–bound dye. EC values are derived by comparing the absorbance of the nucleic acid–bound dye with that of free dye in a reference solvent (H_2O or MeOH).
2. Acridine orange bound to RNA has Abs ~460 nm, Em ~650 nm. (Methods Cell Biol (1994) 41:401, Cytometry (1982) 2:201)
3. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
4. MW value is approximate. BCECF AM is a mixture of molecular species. Lot-specific average MW values are printed on product labels.
5. BCECF AM is colorless and nonfluorescent until converted to BCECF (B1151) by acetoxymethyl ester hydrolysis.
6. Acetate hydrolysis of this compound yields a fluorescent product with similar pH-dependent spectral characteristics to C1904.
7. C369 is converted to a fluorescent product (C368) after acetate hydrolysis.
8. Dihydrofluorescein diacetates are colorless and nonfluorescent until both of the acetate groups are hydrolyzed and the products are subsequently oxidized to fluorescein derivatives. The materials contain less than 0.1% of oxidized derivative when initially prepared. The oxidation products of C400, C2938, C6827, D399 and D2935 are 2′,7′-dichlorofluorescein derivatives with spectra similar to C368.
9. Hydrolysis of the acetate and acetoxymethyl ester groups of C1354 yields C1359.
10. Acetoxymethyl ester hydrolysis of calcein blue, AM yields the corresponding iminodiacetic acid. The iminodiacetic acid product is water soluble and has similar spectroscopic properties to 7-hydroxy-4-methylcoumarin (H189).
11. Calcein AM is converted to fluorescent calcein (C481) after acetoxymethyl ester hydrolysis.
12. C6826 is converted to a fluorescent product with spectra similar to C1270 after acetate hydrolysis.
13. C13196 is converted to a fluorescent product (C652) after acetate hydrolysis.
14. Calcein red-orange, AM is converted to the corresponding nitrilotriacetic acid after acetoxymethyl ester hydrolysis. The nitrilotriacetic acid product is water-soluble and has similar spectroscopic properties to the AM ester form.
15. This compound is susceptible to oxidation, especially in solution. Store solutions under argon or nitrogen. Oxidation may be induced by illumination.
16. D632, D23806 and D633 are essentially colorless and nonfluorescent until oxidized. Oxidation products are R302 (from D632 and D23806) and R634 (from D633).
17. Dihydroethidium has blue fluorescence (Em ~420 nm) until oxidized to ethidium (Ex/Em = 518/605 nm in H_2O/DNA). The reduced dye does not bind to nucleic acids. (FEBS Lett (1972) 26:169)
18. JC-9 exhibits long-wavelength J-aggregate emission at ~635 nm in aqueous solutions and polarized mitochondria.
19. This product is supplied as a ready-made solution in DMSO with sodium borohydride added to inhibit oxidation.
20. D23805 is colorless and nonfluorescent until the AM ester groups are hydrolyzed and the resulting leuco dye is subsequently oxidized. The final product is calcein (C481).
21. Although this compound is soluble in water, preparation of stock solutions in water is not recommended because of possible adsorption onto glass or plastic.
22. E1169 in H_2O: Abs = 493 nm (EC = 9100 $cm^{-1}M^{-1}$). E3599 in H_2O: Abs = 498 nm (EC = 10,800 $cm^{-1}M^{-1}$). Both compounds are very weakly fluorescent in H_2O. QY increases >40-fold on binding to dsDNA.
23. E1374 spectral data are for the free dye. Fluorescence is weak, but intensity increases ~15-fold on binding to DNA. After photocrosslinking to DNA, Abs = 504 nm (EC ~4000 $cm^{-1}M^{-1}$), Em = 600 nm. (Nucleic Acids Res (1978) 5:4891, Biochemistry (1980) 19:3221)
24. F7030 is fluorescent when bound to DNA (Em = 538 nm). Uptake and processing of the dye by live yeast results in red-shifted fluorescence (Em ~590 nm).
25. H7593 in H_2O: Abs = 482 nm (EC = 5500 $cm^{-1}M^{-1}$), Em = 625 nm (weakly fluorescent).
26. This compound emits chemiluminescence upon oxidation in basic aqueous solutions. Emission peaks are at 425 nm (L8455) and 470 nm (L6868).
27. Enzymatic reduction products are water-insoluble formazans with Abs = 505 nm (M6494) and 605 nm (N6495) after solubilization in DMSO or DMF. See literature sources for further information. (Histochemistry (1982) 76:381, Prog Histochem Cytochem (1976) 9:1)
28. M6494 also has Abs = 242 nm (EC = 21,000 $cm^{-1}M^{-1}$) in MeOH.
29. Spectra of N3246 are pH-dependent (pK_a ~6.7). Data reported are for 1:1 (v/v) EtOH/1% acetic acid.
30. Fluorescence of NBD and its derivatives in water is relatively weak. QY and τ increase and Em decreases in aprotic solvents and other nonpolar environments relative to water. (Biochemistry (1977) 16:5150, Photochem Photobiol (1991) 54:361)
31. Acetate hydrolysis of this compound yields a fluorescent product with similar spectral characteristics to O6146.
32. Propidium iodide in H_2O: Abs = 493 nm (EC = 5900 $cm^{-1}M^{-1}$), Em = 636 nm (weakly fluorescent). Fluorescence is enhanced >10-fold on binding to dsDNA.
33. This product is specified to equal or exceed 98% analytical purity by HPLC.
34. Enzymatic reduction of resazurin yields resorufin (R363).
35. R14060 is colorless and nonfluorescent until oxidized. The spectral characteristics of the oxidation product (2,3,4,5,6-pentafluorotetramethylrosamine) are similar to those of T639.
36. S1129 is converted to a fluorescent product (F1130) after acetate hydrolysis.
37. This product is essentially nonfluorescent except when bound to DNA or RNA.
38. MW: The preceding ~ symbol indicates an approximate value, not including counterions.
39. The fluorescence quantum yield (QY) of SYTO® 13 dye bound to dsDNA is 0.56 (measured at 22°C).
40. JC-1 forms J-aggregates with Abs/Em = 585/590 nm at concentrations above 0.1 µM in aqueous solutions (pH 8.0). (Biochemistry (1991) 30:4480)
41. Enzymatic reduction product is a water-soluble formazan, Abs = 475 nm.
42. Ethidium bromide in H_2O: Abs = 480 nm (EC = 5600 $cm^{-1}M^{-1}$), Em = 620 nm (weakly fluorescent). Fluorescence is enhanced >10-fold on binding to dsDNA.

PRODUCT LIST 15.2 VIABILITY AND CYTOTOXICITY ASSAY REAGENTS

Cat. No.	Product	Quantity
A1301	acridine orange	1 g
A3568	acridine orange *10 mg/mL solution in water*	10 mL
B1150	2′,7′-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM)	1 mg
B3051	2′,7′-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM) *1 mg/mL solution in anhydrous DMSO*	1 mL
B1170	2′,7′-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM) *special packaging*	20 x 50 µg
B13234	BOCILLIN™ 650/665 penicillin, sodium salt	1 mg
B13233	BOCILLIN™ FL penicillin, sodium salt	1 mg
C1429	calcein blue, AM	1 mg
C34853	calcein blue, AM *for flow cytometry* *for UV excitation* *special packaging*	20 x 50 µg
C34852	calcein green, AM *for flow cytometry* *for 488 nm excitation* *special packaging*	20 x 50 µg
C34851	calcein red-orange, AM *special packaging*	20 x 50 µg
C34858	calcein violet, AM *for flow cytometry* *for 405 nm excitation* *special packaging*	20 x 25 µg
C1430	calcein, AM	1 mg
C3099	calcein, AM *1 mg/mL solution in anhydrous DMSO*	1 mL
C3100MP	calcein, AM *special packaging*	20 x 50 µg
C2938	6-carboxy-2′,7′-dichlorodihydrofluoresce in diacetate, di(acetoxymethyl ester)	5 mg
C400	5-(and-6)-carboxy-2′,7′ -dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) *mixed isomers*	25 mg
C369	5-(and-6)-carboxy-2′,7′ -dichlorofluorescein diacetate (carboxy-DCFDA) *mixed isomers*	100 mg
C13196	5-(and-6)-carboxynaphthofluorescein diacetate	10 mg
C2925	CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate)	1 mg
C7025	CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) *special packaging*	20 x 50 µg
C1361	5-CFDA (5-carboxyfluorescein diacetate) *single isomer*	100 mg
C1362	6-CFDA (6-carboxyfluorescein diacetate) *single isomer*	100 mg
C195	5(6)-CFDA (5-(and-6)-carboxyfluorescein diacetate) *mixed isomers*	100 mg
C1354	5-CFDA, AM (5-carboxyfluorescein diacetate, acetoxymethyl ester)	5 mg
C6827	5-(and-6)-chloromethyl- 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) *mixed isomers* *special packaging*	20 x 50 µg
C6826	5-(and-6)-chloromethyl SNARF®-1, acetate *mixed isomers* *special packaging*	20 x 50 µg
D399	2′,7′-dichlorodihydrofluorescein diacetate (2′,7′-dichlorofluorescin diacetate; H_2DCFDA)	100 mg
D23805	dihydrocalcein, AM *special packaging*	20 x 50 µg
D1168	dihydroethidium (hydroethidine)	25 mg
D23107	dihydroethidium (hydroethidine) *5 mM stabilized solution in DMSO*	1 mL
D11347	dihydroethidium (hydroethidine) *special packaging*	10 x 1 mg
D632	dihydrorhodamine 123	10 mg
D23806	dihydrorhodamine 123 *5 mM stabilized solution in DMSO*	1 mL
D633	dihydrorhodamine 6G	25 mg
D22421	3,3′-dimethyl-α-naphthoxacarbocyanine iodide (JC-9; $DiNOC_1(3)$)	5 mg
E1169	ethidium homodimer-1 (EthD-1)	1 mg
E3599	ethidium homodimer-2 (EthD-2) *1 mM solution in DMSO*	200 µL
E1374	ethidium monoazide bromide (EMA)	5 mg
F1303	FDA (fluorescein diacetate)	1 g
F10322	FilmTracer™ calcein green biofilm stain	20 x 50 µg
F10319	FilmTracer™ calcein red-orange biofilm stain	20 x 50 µg
F10320	FilmTracer™ calcein violet biofilm stain	20 x 25 µg
F10317	FilmTracer™ FM® 1-43 green biofilm matrix stain	1 mg
F10318	FilmTracer™ SYPRO® Ruby biofilm matrix stain	200 mL
F23103	fosmidomycin, sodium salt (FR-31564)	25 mg
F7030	FUN® 1 cell stain *10 mM solution in DMSO*	100 µL
H7593	hexidium iodide	5 mg
L8455	luminol (3-aminophthalhydrazide)	25 g
M6494	MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide)	1 g
N13195	2-NBDG (2-(*N*-(7-nitrobenz-2-oxa- 1,3-diazol-4-yl)amino)-2-deoxyglucose)	5 mg
N3246	neutral red *high purity*	25 mg
N23106	6-(*N*-(7-nitrobenz-2-oxa- 1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG)	5 mg
N6495	nitro blue tetrazolium chloride (NBT)	1 g
N7565	Nucleic Acid Stains Dimer Sampler Kit	1 kit
O6151	Oregon Green® 488 carboxylic acid diacetate (carboxy-DFFDA) *6-isomer*	5 mg
P13235	polymyxin B, BODIPY® FL conjugate, trifluoroacetic acid salt *mixed species*	100 µg
P13238	polymyxin B, dansyl conjugate, trifluoroacetic acid salt *mixed species*	100 µg
P13236	polymyxin B, Oregon Green® 514 conjugate, trifluoroacetic acid salt *mixed species*	100 µg
P1304MP	propidium iodide	100 mg
P3566	propidium iodide *1.0 mg/mL solution in water*	10 mL

continued on next page

PRODUCT LIST 15.2 VIABILITY AND CYTOTOXICITY ASSAY REAGENTS—*continued*

Cat. No.	Product	Quantity
P21493	propidium iodide *FluoroPure™ grade*	100 mg
R14060	RedoxSensor™ Red CC-1 *special packaging*	10 x 50 µg
R12204	resazurin, sodium salt	10 mg
R302	rhodamine 123	25 mg
R22420	rhodamine 123 *FluoroPure™ grade*	25 mg
S1129	SFDA (5-sulfofluorescein diacetate, sodium salt)	25 mg
S32704	SYTO® 10 green fluorescent nucleic acid stain *5 mM solution in DMSO*	100 µL
S7575	SYTO® 13 green fluorescent nucleic acid stain *5 mM solution in DMSO*	250 µL
S34854	SYTO® 9 green fluorescent nucleic acid stain *5 mM solution in DMSO*	100 µL
S34855	SYTO® BC green fluorescent nucleic acid stain *5 mM solution in DMSO*	100 µL
S11350	SYTO® Blue Fluorescent Nucleic Acid Stain Sampler Kit *SYTO® dyes 40–45* *50 µL each*	1 kit
S7572	SYTO® Green Fluorescent Nucleic Acid Stain Sampler Kit *SYTO® dyes 11–14, 16, 21, 24, and 25* *50 µL each*	1 kit
S11360	SYTO® Orange Fluorescent Nucleic Acid Stain Sampler Kit *SYTO® dyes 80–85* *50 µL each*	1 kit
S11340	SYTO® Red Fluorescent Nucleic Acid Stain Sampler Kit *SYTO® dyes 17 and 59–64* *50 µL each*	1 kit
S32703	SYTO® RNASelect™ green fluorescent cell stain *5 mM solution in DMSO*	100 µL
S10349	SYTOX® AADvanced™ Dead Cell Stain Kit *for flow cytometry* *for 488 nm excitation* *100 tests*	1 kit
S10274	SYTOX® AADvanced™ Dead Cell Stain Kit *for flow cytometry* *for 488 nm excitation* *500 tests*	1 kit
S34857	SYTOX® Blue dead cell stain *for flow cytometry* *1000 assays* *1 mM solution in DMSO*	1 mL
S11348	SYTOX® Blue nucleic acid stain *5 mM solution in DMSO*	250 µL
S7020	SYTOX® Green nucleic acid stain *5 mM solution in DMSO*	250 µL
S11368	SYTOX® Orange nucleic acid stain *5 mM solution in DMSO*	250 µL
S34859	SYTOX® Red dead cell stain *for 633 or 635 nm excitation* *5 µM solution in DMSO*	1 mL
T3168	5,5′,6,6′-tetrachloro- 1,1′,3,3′-tetraethylbenzimidazolylcarboc yanine iodide (JC-1; $CBIC_2(3)$)	5 mg
T669	tetramethylrhodamine, ethyl ester, perchlorate (TMRE)	25 mg
T668	tetramethylrhodamine, methyl ester, perchlorate (TMRM)	25 mg
15585-011	UltraPure™ ethidium bromide *10 mg/mL*	10 mL
V34850	vancomycin, BODIPY® FL conjugate (BODIPY® FL vancomycin)	100 µg
X6493	XTT (2,3-bis-(2-methoxy-4- nitro-5-sulfophenyl)-2H- tetrazolium-5-carboxanilide)	100 mg

15.3 Viability and Cytotoxicity Assay Kits for Diverse Cell Types

This section contains a thorough description of Molecular Probes® viability and cytotoxicity kits. Fluorometric assays of cell viability and cytotoxicity are easy to perform with the use of a fluorescence microscope, fluorometer, fluorescence microplate reader or flow cytometer,[1,2] and they offer many advantages over traditional colorimetric and radioactivity-based assays. Also discussed in this section are our unique single-step kits for assessing gram sign and for simultaneously determining gram sign and viability of bacteria, as well as the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit designed specifically for bacterial biofilms.

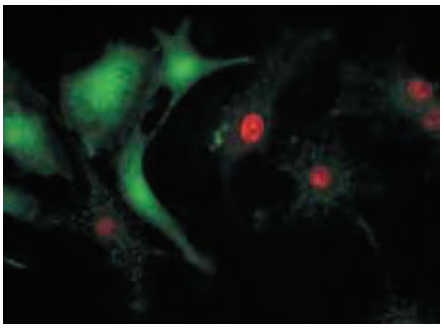

Figure 15.3.2 A mixture of live and ethanol-killed bovine pulmonary artery epithelial cells stained with the reagents in our LIVE/DEAD® Cell Viability/Cytotoxicity Assay Kit (L3224). Live cells fluoresce bright green, whereas dead cells with compromised membranes fluoresce red-orange.

Viability Assay Kits for Animal Cells

To facilitate use of our unique cell viability and cytotoxicity assay technology, we have developed several important products (Table 15.2) that combine fluorescent reagents to yield, in most cases, two-color discrimination of the population of live cells from the dead-cell population by simply adding the reagents, incubating for a brief period and observing the results without any wash steps required. These facile assays are ideal for high-throughput screening applications and, in most cases, for imaging, fluorometry and flow cytometry.

LIVE/DEAD® Viability/Cytotoxicity Kit for Animal Cells

Our LIVE/DEAD® Viability/Cytotoxicity Kit (L3224) for animal cells provides an exceptionally easy fluorescence-based method for determining viability of adherent or nonadherent cells and for assaying cytotoxicity. The kit comprises two probes: calcein AM and ethidium homodimer-1. Calcein AM is a fluorogenic esterase substrate that is hydrolyzed to a green-fluorescent product (calcein); thus, green fluorescence is an indicator of cells that have esterase activity as well as an intact membrane to retain the esterase products. Ethidium homodimer-1 is a high-affinity, red-fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells. The LIVE/DEAD® viability/cytotoxicity assay offers several advantages:

- **Simplicity**. The reagents are simultaneously added to the cell suspension, which is then incubated for 30–45 minutes. No wash steps are required before analysis.
- **Specificity and reliability**. Green-fluorescent cells are live; red-fluorescent cells are dead (Figure 15.3.1, Figure 15.3.2).
- **Versatility**. The LIVE/DEAD® viability/cytotoxicity assay is compatible with adherent cells such as astrocytes,[3] nonadherent cells and certain tissues.[4–6] Results can be analyzed by fluorescence microscopy using standard fluorescein longpass filter sets, as well as by flow cytometry (Figure 15.3.3) or fluorometry. The fluorescence emissions of the two probes are easily resolved (Figure 15.3.4).
- **Simple quantitation**. Flow cytometric measurements yield only two populations; there are rarely any doubly stained cells (Figure 15.3.3). Quantitative assays of bulk cells can be made using a fluorescence microplate reader or fluorometer.
- **Suitability for high-throughput screening**. The ease, reliability and low cost of the LIVE/DEAD® Viability/Cytotoxicity Kit make it an economical assay for high-throughput screening of cytotoxic agents.

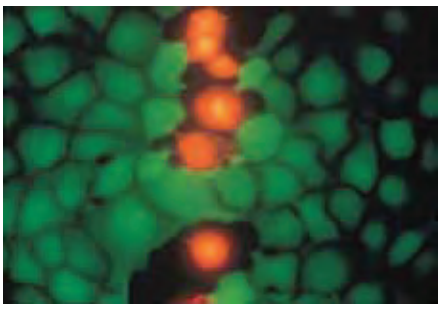

Figure 15.3.1 Live and dead kangaroo rat (PtK2) cells stained with ethidium homodimer-1 and the esterase substrate calcein AM, both of which are provided in our LIVE/DEAD® Viability/Cytotoxicity Kit (L3224). Live cells fluoresce a bright green, whereas dead cells with compromised membranes fluoresce red-orange.

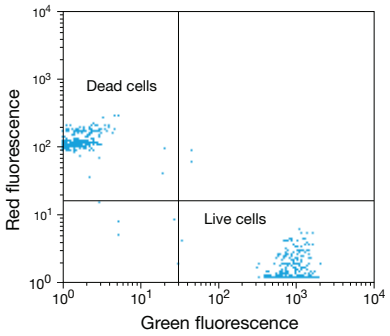

Figure 15.3.3 Flow cytometric viability assay using the LIVE/DEAD® Viability/Cytotoxicity Kit (L3224). A 1:1 mixture of live and ethanol-fixed human B cells was stained with calcein AM and ethidium homodimer-1 according to the kit protocol. After 5 minutes, flow cytometric analysis was carried out with excitation at 488 nm. The resulting bivariate frequency distribution shows the clear separation of the green-fluorescent (530 nm) live-cell population from the red-fluorescent (585 nm) dead-cell population.

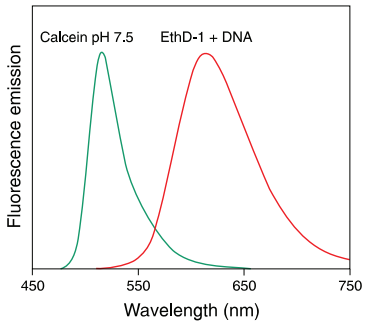

Figure 15.3.4 Normalized fluorescence emission spectra of calcein (C481) and DNA-bound ethidium homodimer-1 (EthD-1, E1169), illustrating the clear spectral separation that allows simultaneous visualization of live and dead eukaryotic cells with the LIVE/DEAD® Viability/Cytotoxicity Kit (L3224).

Table 15.2 Molecular Probes® assay kits for cell viability, cell counting and bacterial gram staining.

Cat. No.	Kit Name	Kit Components	# Assays	Assay Principle
Assay Kits for Mammalian Cells				
L3224	LIVE/DEAD® Viability/ Cytotoxicity Kit	• Calcein AM • Ethidium homodimer-1 • Detailed protocols	1000 microscopy assays, 1000 microplate assays or 300 flow cytometry assays	Membrane-permeant calcein AM is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, and membrane-impermeant ethidium homodimer-1 labels nucleic acids of membrane-compromised cells with red fluorescence.
L7013	LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1	• SYTO® 10 nucleic acid stain • DEAD Red™ nucleic acid stain • Detailed protocols	100 microscopy assays or 100 flow cytometry assays	Membrane-permeant SYTO® 10 dye labels the nucleic acids of live cells with green fluorescence, and membrane-impermeant DEAD Red™ dye labels nucleic acids of membrane-compromised cells with red fluorescence. Subsequent fixation inactivates pathogens without distorting the staining pattern.
L23105 L34957 L34959 L34955 L23101 L23102 L10120 L10119 L34960 †	LIVE/DEAD® Fixable Dead Cell Stain Kits *	• Fluorescent reactive dye • DMSO • Detailed protocols	200 flow cytometry assays	Live cells react with the fluorescent reactive dye only on their surface to yield weakly fluorescent cells. Cells with compromised membranes react with the dye throughout their volume, yielding brightly stained cells. Subsequent fixation inactivates pathogens without distorting the staining pattern.
L7010	LIVE/DEAD® Cell-Mediated Cytotoxicity Kit	• $DiOC_{18}(3)$ • Propidium iodide • Detailed protocols	2000 microscopy assays or 200 flow cytometry assays	Target cells are preincubated with the green-fluorescent membrane stain $DiOC_{18}(3)$ and then mixed with effector cells in the presence of the red-fluorescent, membrane-impermeant propidium iodide. Live and dead target cells retain their green-fluorescent membrane stain; target and effector cells with compromised membranes exhibit red-fluorescent nucleic acid staining; live effector cells are nonfluorescent.
L7011	LIVE/DEAD® Sperm Viability Kit	• SYBR® 14 nucleic acid stain • Propidium iodide • Detailed protocols	1000 microscopy assays or 200 flow cytometry assays	Membrane-permeant SYBR® 14 nucleic acid stain labels live sperm with green fluorescence, and membrane-impermeant propidium iodide labels the nucleic acids of membrane-compromised sperm with red fluorescence.
L34951	LIVE/DEAD® Cell Vitality Assay Kit	• Dodecylresazurin (C_{12}-resazurin) • SYTOX® Green nucleic acid stain • DMSO • Phosphate-buffered saline • Detailed protocols	1000 flow cytometry assays	Metabolically active cells reduce C_{12}-resazurin to red-fluorescent C_{12}-resorufin, and cells with compromised membranes (usually late-apoptotic and necrotic cells) are labeled with the green-fluorescent SYTOX® Green nucleic acid stain.
L34958	LIVE/DEAD® Cell Vitality Assay Kit	• CellTrace™ calcein violet AM • Aqua-fluorescent reactive dye • DMSO • Detailed protocols	200 flow cytometry assays	Live cells are labeled with CellTrace™ calcein violet AM, and dead cells with the aqua-fluorescent reactive dye. Both stains are compatible with the 405 nm violet laser diode.
V23110	Vybrant® Cell Metabolic Assay Kit	• Dodecylresazurin (C_{12}-resazurin) • DMSO • Resorufin • Detailed protocols	200 flow cytometry assays or 1000 microplate assays	Nonfluorescent C_{12}-resazurin is reduced to fluorescent C_{12}-resorufin by viable cells; the resulting signal is proportional to the number of cells present.
V23111	Vybrant® Cytotoxicity Assay Kit	• Resazurin • DMSO • Reaction mixture • Reaction buffer • Lysis buffer • Detailed protocols	1000 microplate assays	Damaged and dying cells release glucose 6-phosphate into the surrounding medium, which is detected by an enzymatic process that leads to the reduction of resazurin into red-fluorescent resorufin.
Assay Kits for Yeast				
L7009	LIVE/DEAD® Yeast Viability Kit	• FUN® 1 cell stain • Calcofluor White M2R • Detailed protocols	>1000 microscopy assays or 1000 microplate assays	Plasma membrane integrity and metabolic function of fungi are required to convert the yellow-green–fluorescent intracellular staining of FUN® 1 into red-orange–fluorescent intravacuolar structures; Calcofluor White M2R labels cell-wall chitin with blue fluorescence regardless of metabolic state.
L34952	LIVE/DEAD® *Funga*Light™ Yeast Viability Kit *for flow cytometry*	• SYTO® 9 nucleic acid stain • Propidium iodide • Detailed protocols	~200 flow cytometry assays	Membrane-permeant SYTO® 9 dye generally labels all yeast in a population—those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only yeast with damaged membranes, causing displacement of the SYTO® 9 stain.
F34953	*Funga*Light™ CFDA AM/ Propidium Iodide Yeast Vitality Kit *for flow cytometry*	• 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA AM) • Propidium iodide • Dimethylsulfoxide (DMSO), anhydrous • Detailed protocols	~200 flow cytometry assays	The cell-permeant CFDA AM is combined with the membrane integrity indicator propidium iodide to evaluate the vitality of yeast cells by flow cytometry or microscopy. With an appropriate stain mixture, esterase-active yeast with intact cell membranes stain fluorescent green, whereas yeast with damaged membranes stain fluorescent red.

***** The LIVE/DEAD® Fixable Dead Cell Stain Kits are available in eight fluorescent colors (excitation/emission maxima): blue (350/450 nm, L23105), aqua (367/526 nm, L34957), yellow (400/575 nm, L34959), violet (416/451 nm, L34955), green (495/520 nm, L23101), red (595/615 nm, L23102), far red (650/665 nm, L10120) and near infrared (750/775 nm, L10119), as well as in the LIVE/DEAD® Fixable Dead Cell Stain Sampler Kit (L34960), which contains all eight fluorescent fixable dead cell stains. **†** 320 flow cytometry assays.

Table 15.2 Molecular Probes® assay kits for cell viability, cell counting and bacterial gram staining—*continued*.

Cat. No.	Kit Name	Kit Components	# Assays	Assay Principle
Assay Kits for Bacteria				
L7012 L7007 L13152	LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit	• SYTO® 9 nucleic acid stain • Propidium iodide • *Bac*Light™ mounting oil • Detailed protocols	For Kit L7007 and L7012: >1000 microscopy assays, 1000 microplate assays or 200 flow cytometry assays. For Kit L13152: each applicator set allows ~1000 microscopy assays, 50 microplate assays or 10 flow cytometry assays	Membrane-permeant SYTO® 9 dye labels live bacteria with green fluorescence; membrane-impermeant propidium iodide labels membrane-compromised bacteria with red fluorescence. In Kit L7007, the stains are supplied in a mixed, two-component formulation, whereas in Kit L7012 the stains are provided as separate solutions. In Kit L13152, the separate dyes are dry and premeasured into pairs of polyethylene transfer pipettes.
L34856	LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit	• SYTO® 9 nucleic acid stain • Propidium iodide • Microsphere standard • Detailed protocols	100 flow cytometry assays	Membrane-permeant SYTO® 9 dye labels live bacteria with green fluorescence; membrane-impermeant propidium iodide labels membrane-compromised bacteria with red fluorescence. The calibrated suspension of polystyrene microspheres serves as a standard for the volume of suspension analyzed and is clearly distinguishable from stained bacteria in a fluorescence versus side scatter cytogram.
B34954	*Bac*Light™ RedoxSensor™ Green Vitality Kit	• RedoxSensor™ Green reagent • Propidium iodide, an indicator of membrane integrity • Sodium azide • Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) • Detailed protocols	200 flow cytometry assays	The RedoxSensor™ Green reagent reports reductase activity in both gram-positive and gram-negative bacteria (although differences in signal intensity may be observed based upon cell wall characteristics), providing an accurate view of this important aspect of bacterial vitality. Following reduction, the RedoxSensor™ Green reagent will produce a stable green-fluorescent signal (excitation/emission maxima ~490/520 nm) in 10 minutes that is compatible with formaldehyde fixation techniques.
B34956	*Bac*Light™ RedoxSensor™ CTC Vitality Kit	• 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) • SYTO® 24 green-fluorescent nucleic acid stain • 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) • Detailed protocols	50 tests by flow cytometry or microscopy	This kit contains 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), for detection of respiration-linked dehydrogenase activity. SYTO® 24 and DAPI counterstains facilitate the differentiation of cells from debris and the calculation of total cell numbers.
B34950	*Bac*Light™ Bacterial Membrane Potential Kit	• $DiOC_2(3)$ in DMSO • CCCP in DMSO • Phosphate-buffered saline (PBS) • Detailed protocols	100 flow cytometry assays	At low concentrations, $DiOC_2(3)$ exhibits green fluorescence in all bacterial cells, but it becomes more concentrated in healthy cells that are maintaining a membrane potential, causing the dye to self-associate and the fluorescence emission to shift to red. CCCP is included in the kit for use as a control because it eradicates the proton gradient, eliminating bacterial membrane potential.
V7023	ViaGram™ Red$^+$ Bacterial Gram Stain and Viability Kit	• DAPI • SYTOX® Green nucleic acid stain • Texas Red®-X conjugate of wheat germ agglutinin (WGA) • Sodium bicarbonate • *Bac*Light™ mounting oil • Detailed protocols	200 microscopy assays	Membrane-permeant DAPI labels live bacteria with blue fluorescence; membrane-impermeant SYTOX® Green nucleic acid stain labels bacteria with compromised membranes with green fluorescence. Simultaneous Texas Red®-X WGA staining produces red-fluorescent surface labeling of gram-positive bacteria.
L7005	LIVE *Bac*Light™ Bacterial Gram Stain Kit	• SYTO® 9 nucleic acid stain • Hexidium iodide • *Bac*Light™ mounting oil • Detailed protocols	>1000 microscopy assays, 1000 microplate assays or 200 flow cytometry assays	When gram-negative and gram-positive bacteria are simultaneously stained with the membrane-permeant SYTO® 9 dye and hexidium iodide, gram-negative bacteria fluoresce green and gram-positive bacteria fluoresce red.
B7277	Bacteria Counting Kit (Section 15.4)	• SYTO® BC bacteria stain • Suspended microsphere standard • Detailed protocols	100 flow cytometry assays	The membrane-permeant SYTO® BC stain labels both gram-positive and gram-negative bacteria with green fluorescence; the calibrated suspension of polystyrene microspheres serves as a standard for the volume of suspension analyzed.
ATP Assay Kit for All Cell Types				
A22066	ATP Determination Kit	• Luciferin • Luciferase • ATP • Dithiothreitol (DTT) • Reaction buffer • Detailed protocols	1000 microplate assays	Luciferase catalyzes the chemiluminescent reaction of luciferin, ATP and oxygen, producing a photon and other chemical by-products. Stores of ATP are higher in viable cells; therefore, the signal strength correlates with population viability.

* The LIVE/DEAD® Fixable Dead Cell Stain Kits are available in eight fluorescent colors (excitation/emission maxima): blue (350/450 nm, L23105), aqua (367/526 nm, L34957), yellow (400/575 nm, L34959), violet (416/451 nm, L34955), green (495/520 nm, L23101), red (595/615 nm, L23102), far red (650/665 nm, L10120) and near infrared (750/775 nm, L10119), as well as in the LIVE/DEAD® Fixable Dead Cell Stain Sampler Kit (L34960), which contains all eight fluorescent fixable dead cell stains. † 320 flow cytometry assays.

Several laboratories have established the validity of the LIVE/DEAD® viability/cytotoxicity assay for use with animal cells and tissues. Published applications have included measuring the toxic effects of tumor necrosis factor[7] (TNF), β-amyloid protein,[8] adenovirus E1A proteins,[9] tetrodotoxin (TTX) binding to Na^+ channels,[10] methamphetamines,[11] mitogenic sphingolipids[12] and photodynamic therapy.[13] This assay has also been adapted to quantitate lymphocyte-mediated cytotoxicity by flow cytometry,[14] cell-mediated cytotoxicity by fluorescence microscopy[15] and the viability of boar sperm by fluorescence microscopy.[16]

The LIVE/DEAD® Viability/Cytotoxicity Kit is intended for use with animal cells that can be analyzed within about an hour of adding the dyes to the cells. The kit components, number of assays and assay principles are summarized in Table 15.2. This kit's two viability probes—calcein AM (C1430, C3099, C3100MP) and ethidium homodimer-1 (E1169)—are also available separately (see Section 15.2) and may be used in combination with other probes for discrimination of live and dead cells. When assays need to be conducted over longer periods or when hazardous samples are being analyzed, we recommend our LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1 (L7013, see below).

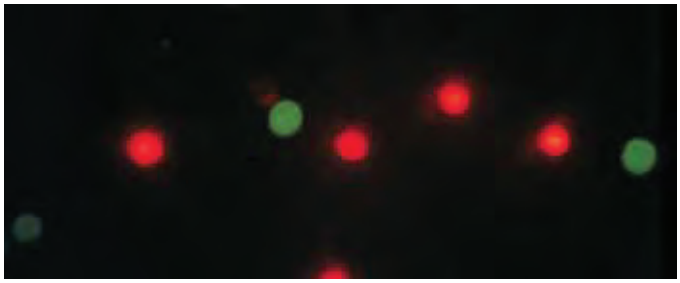

Figure 15.3.5 A mixture of live and dead goat lymphocytes stained with the LIVE/DEAD® Reduced Biohazard Viability/Cytotoxicity Kit (L7013) and subsequently fixed with 4% glutaraldehyde. This image was photographed in a single exposure through an Omega® Optical triple bandpass filter set.

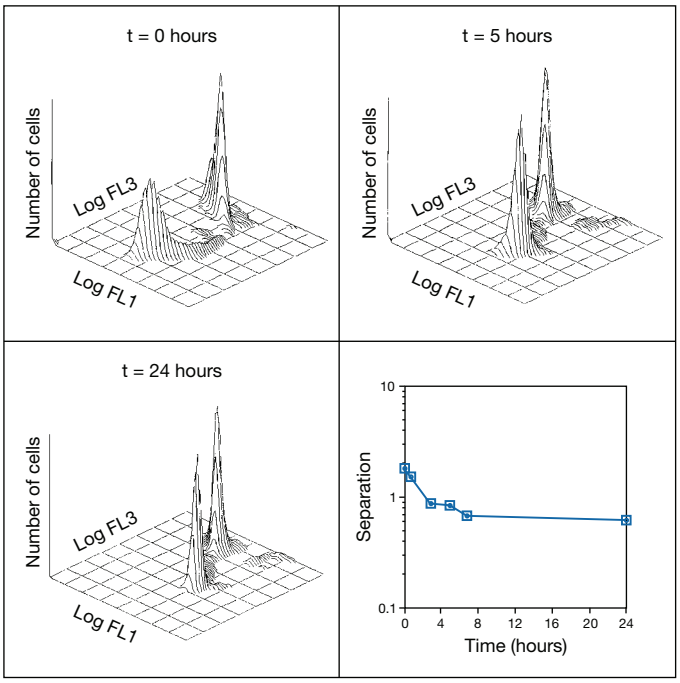

Figure 15.3.6 Flow cytometric analysis of a mixed population of live and complement-treated goat lymphocytes stained using the reagents and protocols provided in our LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1 (L7013) and monitored over a 24-hour period. The panels (left to right, top to bottom) represent the distribution of SYTO® 10 green fluorescence and DEAD Red™ red fluorescence in lymphocytes at 0, 5 and 24 hours after fixation. Data are on a logarithmic scale. The lower right panel is a plot of the separation between the live- and dead-population peaks as a function of time.

LIVE/DEAD® Reduced Biohazard Cell Viability Kit

Rigorous precautions are necessary during analysis of biohazardous specimens.[17,18] Therefore, fixation procedures that inactivate cells yet produce minimal distortion of their characteristics are highly advantageous.[19] The LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1 (L7013) provides a two-color fluorescence assay for animal cell viability that is designed to reduce the risk associated with handling potential biohazards such as viral, bacterial or protozoan pathogens.

Viability analysis with the LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1 is provided by the cell-permeant, green-fluorescent SYTO® 10 and cell-impermeant, red-fluorescent DEAD Red™ (ethidium homodimer-2) nucleic acid stains. The dye concentrations and their relative affinities are balanced so that a cell population exposed simultaneously to both dyes becomes differentially stained—live cells fluoresce green and dead cells fluoresce red (Figure 15.3.5). This assay is simple, fast and can be carried out using a fluorescence microscope, flow cytometer or fluorescence microplate reader. Moreover, the staining pattern of a cell population is retained for several hours after fixation (Figure 15.3.6).

Our LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1 has several unique features:

- **Reduced handling risks**. This kit allows viability staining to take place while the potentially pathogenic sample is well contained. Subsequent treatment with 4% glutaraldehyde (or less effectively with formaldehyde) permits safer handling during analysis, without disrupting the distinctive staining pattern. Glutaraldehyde is known to inactivate cells and viruses, while preserving their overall morphology.[20] In addition, the high sensitivity and specificity of the assay mean that sample sizes can be very small, further reducing potential biohazards.
- **Specificity and reliability**. Live cells initially fluoresce green, and dead cells fluoresce red. With time, this discrimination is reduced but can still be detected, even after 24 hours (Figure 15.3.6).
- **Independence from enzymatic activity**. Because it relies on two nucleic acid stains that differ in their membrane permeability, this assay equates loss of cell viability with loss of membrane integrity. Consequently, the assay is totally independent of variations in enzymatic activity or electrical potential of the cell.
- **Versatility**. The analysis is readily quantitated with a fluorescence microscope or flow cytometer (Figure 15.3.6). This kit's protocol includes methods for analyzing the viability of nonadherent cells, as well as adherent cells on coverslips.
- **Convenience**. Cells can be stained and fixed at various times during the experiment, and the results can be analyzed several hours later, without loss of the discrimination pattern.

The kit components, number of assays and assay principles are summarized in Table 15.2.

LIVE/DEAD® Fixable Dead Cell Stain Kits

The LIVE/DEAD® Fixable Dead Cell Stain Kits (three of which were formerly named LIVE/DEAD® Reduced Biohazard Cell Viability Kits #2, #3 and #4) employ an amine-reactive fluorescent dye to evaluate mammalian cell viability by flow cytometry (Figure 15.3.7).[20] The LIVE/DEAD® Fixable Dead Cell Stain Kits are identical except for the fluorescent color of the amine-reactive dye:

- LIVE/DEAD® Fixable Blue Dead Cell Stain Kit, for UV excitation (L23105, excitation/emission maxima ~350/450 nm)
- LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation (L34957, excitation/emission maxima ~367/526 nm)
- LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit, for 405 nm excitation (L34959, excitation/emission maxima ~400/575 nm)
- LIVE/DEAD® Fixable Violet Dead Cell Stain Kit, for 405 nm excitation (L34955, excitation/emission maxima ~416/451 nm)
- LIVE/DEAD® Fixable Green Dead Cell Stain Kit, for 488 nm excitation (L23101, excitation/emission maxima ~495/520 nm)
- LIVE/DEAD® Fixable Red Dead Cell Stain Kit, for 488 nm excitation (L23102, excitation/emission maxima ~595/615 nm)
- LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit, for 633 or 635 nm excitation (L10120, excitation/emission maxima ~650/665 nm)
- LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation (L10119, excitation/emission maxima ~750/775 nm)
- LIVE/DEAD® Fixable Dead Cell Stain Sampler Kit, for flow cytometry (L34960), which contains one vial of each of the eight different fluorescent reactive dyes

In cells with compromised membranes, the dye reacts with free amines both in the cell interior and on the cell surface, yielding intense fluorescent staining. In viable cells, the dye's reactivity is restricted to the cell-surface amines, resulting in less intense fluorescence. The difference in intensity between the live and dead cell populations is typically greater than 50 fold (Figure 15.3.8), and this fluorescence intensity discrimination is preserved following formaldehyde fixation, using conditions that inactivate pathogens. These single-color assays use only one channel of a flow cytometer, making the reactive dyes in the LIVE/DEAD® Fixable Dead Cell Stain Kits compatible with multiparameter staining experiments; appropriate flow cytometer channels may vary depending on the instrument. The assays can also be used to detect dead cells by fluorescence microscopy; however, the difference in fluorescence intensity of the live and dead cells can be appreciable, making it relatively difficult to simultaneously photograph the two populations. The kit components, number of assays and assay principles are summarized in Table 15.2.

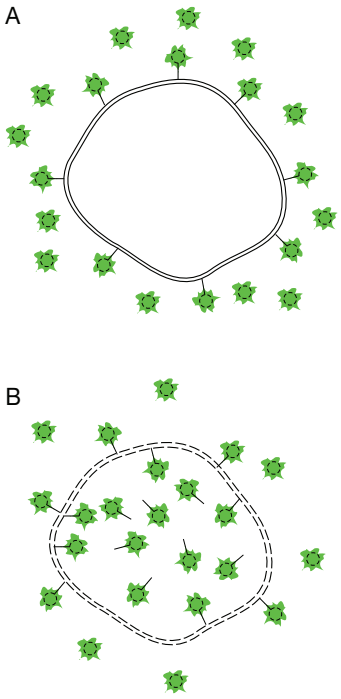

Figure 15.3.7 Principle of our LIVE/DEAD® Fixable Green Dead Cell Stain Kit (L23101). Live cells (**A**) react with the kit's green-fluorescent, amine-reactive dye only on their surface to yield weakly fluorescent cells. Cells with compromised membranes (**B**) react with the dye throughout their volume, yielding brightly stained cells. In both cases, the excess reactive dye is subsequently washed away.

ArC™ Amine-Reactive Compensation Bead Kit

Optimized for use with the LIVE/DEAD® Fixable Dead Cell Stain Kits, the ArC™ Amine Reactive Compensation Bead Kit (A10346) is a tool designed to remove spectral overlap of the fixable dead-cell stains with other standard fluorophores. This kit provides two polystyrene microsphere samples: ArC™ Reactive Beads, which are reactive to all dyes in the Live/Dead Fixable Dead Cell Stain Kits, and Negative Control Beads, which have no reactivity. The two components provide negative and positive populations that can be used to help accurately set compensation when using the Live/Dead Fixable Dead Cell Stains.

LIVE/DEAD® Cell-Mediated Cytotoxicity Kit

Cytotoxicity triggered by a natural defense mechanism may be much slower than cell lysis triggered by a cytotoxic reagent. Our LIVE/DEAD® Cell-Mediated Cytotoxicity Kit (L7010) is intended for cytotoxicity assessments extending over time periods that are too long for effective use of cytoplasmic markers, such as calcein, which may leak out or become sequestered. This kit is based directly on procedures developed by Kroesen and colleagues for measuring natural killer (NK) cell–mediated, lymphokine-activated killer (LAK) cell–mediated and T cell–mediated cytotoxicity by fluorescence microscopy.[21] The assay has also been adapted for rapid flow cytometric analysis of NK cell activity.[22–24]

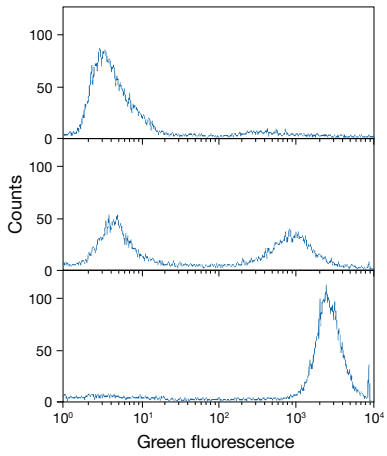

Figure 15.3.8 Live and dead cells distinguished by flow cytometry using the LIVE/DEAD® Fixable Green Dead Cell Stain Kit (L23101). The LIVE/DEAD® Fixable Green Dead Cell Stain Kit was used to differentially stain live and dead Jurkat cells taken from a healthy culture (top panel), an aged culture (middle panel) and a heat-killed culture (bottom panel). Following the staining reaction, the cells were fixed in 3.7% formaldehyde and analyzed by flow cytometry. Nearly identical results were obtained using unfixed cells (data not shown).

Figure 15.3.10 Flow cytometric analysis of Jurkat cells using the LIVE/DEAD® Cell Vitality Assay Kit (L34951). Jurkat human T-cell leukemia cells were first exposed to 10 µM camptothecin for 4 hours at 37°C, 5% CO_2. The cells were then treated with the reagents in the LIVE/DEAD® Cell Vitality Assay Kit as specified in the kit protocol and analyzed by flow cytometry. This dot plot of SYTOX® Green fluorescence versus resorufin fluorescence shows resolution of live-, injured- and dead-cell populations.

Analysis of cell-mediated cytotoxicity using this kit is easy. In order to distinguish target cells, cultures are labeled overnight with $DiOC_{18}(3)$, a green-fluorescent membrane stain (Section 14.4). Target cells are then washed free of excess $DiOC_{18}(3)$ and combined in various proportions with effector cells. After a suitable incubation period, propidium iodide, a red-fluorescent, membrane-impermeant nucleic acid stain, is added. Propidium iodide labels dead effector cells, as well as dead target cells once their plasma membranes are compromised. Because the target cells retain the green-fluorescent membrane stain, both live and dead effector cells and live and dead target cells can readily be discriminated with a fluorescence microscope. Dead target cells exhibit both green-fluorescent membrane staining and red-fluorescent nuclear staining, whereas dead effector cells show only red-fluorescent nuclear staining. Live target cells have only green-fluorescent membrane staining, and live effector cells are unstained. The kit components, number of assays and assay principles are summarized in Table 15.2.

LIVE/DEAD® Sperm Viability Kit

The LIVE/DEAD® Sperm Viability Kit (L7011), developed in collaboration with Duane L. Garner, provides a novel fluorescence-based method for analyzing the viability of sperm in different species.[25–28] The LIVE/DEAD® Sperm Viability Kit contains the membrane-permeant SYBR® 14 nucleic acid stain, along with the conventional dead-cell stain, propidium iodide. Using this combination of dyes, researchers can rapidly distinguish live and dead cells with visible-light excitation (Figure 15.3.9), thus avoiding the harmful effects of UV exposure and allowing flow cytometric analysis of sperm viability to be performed using an argon-ion laser excitation source. When semen is incubated briefly with these two stains, live sperm with intact membranes fluoresce bright green, whereas sperm cells with damaged membranes fluoresce red. Garner and colleagues assessed bovine sperm viability with flow cytometry and with fluorescence microscopy; both techniques allowed live and dead cells to be visualized simultaneously.[28,29] Furthermore, it was reported that neither the ability to fertilize oocytes nor the development of the embryos was affected by SYBR® 14 staining of porcine sperm.[25] The effect of two-photon illumination on the viability of human sperm stained with these reagents has also been analyzed.[30] This assay is particularly useful for evaluating the viability of cryopreserved sperm.[31–35]

The dyes provided in the LIVE/DEAD® Sperm Viability Kit stain cells more rapidly than conventional stains (within 5–10 minutes), and both label DNA, thereby avoiding the ambiguity that may arise from targeting separate cellular components. The membrane-permeant SYBR® 14 stain provided in the LIVE/DEAD® Sperm Viability Kit should also serve as a valuable tool for labeling and tracking live sperm, thus facilitating analysis of their motility and abundance in

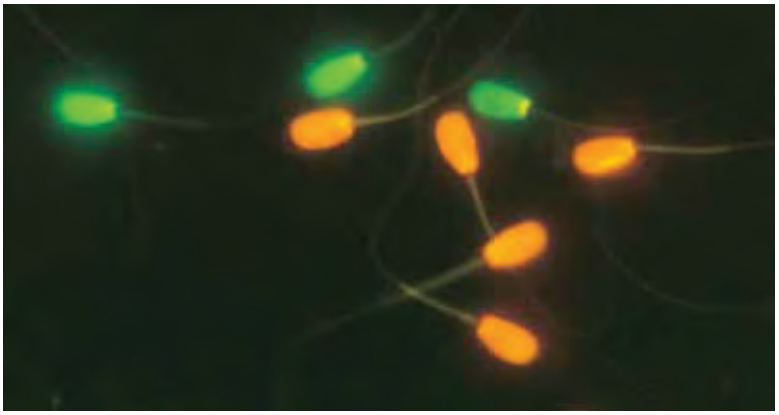

Figure 15.3.9 A mixture of live and dead bovine sperm cells stained with the dyes provided in our LIVE/DEAD® Sperm Viability Kit (L7011). Live sperm with intact membranes are labeled with our proprietary cell-permeant nucleic acid stain, SYBR® 14, and fluoresce green. Dead sperm, which have been killed by unprotected freeze-thawing, are labeled with propidium iodide (P1304MP, P3566, P21493) and fluoresce red-orange. The image was contributed by Duane L. Garner, School of Veterinary Medicine, University of Nevada, Reno, and Lawrence A. Johnson, USDA Agricultural Research Service.

semen samples. Reliable viability measurements with bovine,[28] porcine, ovine, murine,[26] goat, turkey[27] and human sperm[26,30] have been published. The kit components, number of assays and assay principles are summarized in Table 15.2.

Conventional sperm viability assays have employed mixtures of two or three dyes, including fluorescein diacetate derivatives, rhodamine 123 and reduced nucleic acid stains such as dihydroethidium[27,36–39] (hydroethidine, D1168, D11347, D23107; Section 15.2). Acridine orange (A1301, A3568), which fluoresces at different wavelengths when bound to DNA and RNA,[40,41] and the UV light–excitable nucleic acid stains Hoechst 33258 (H1398, H3569, H21491) and Hoechst 33342[42–44] (H1399, H3570, H21492) are frequently used to determine sperm viability and DNA content and to trace sperm–oocyte fusion.[45,46] These nucleic acid stains are described in Section 8.1.

LIVE/DEAD® Violet Viability/Vitality Kit

The LIVE/DEAD® Violet Viability/Vitality Kit (L34958) provides a two-color cell viability and vitality assay that enables the simultaneous identification of live and dead cells. The assay employs two fluorescent probes—calcein violet AM and aqua-fluorescent reactive dye—to indicate recognized parameters of cell health (intracellular esterase activity and plasma membrane integrity, respectively). Both dyes utilize 405 nm violet diode laser excitation, allowing other laser lines to be used for conventional fluorophores. The kit components, number of assays and assay principles are summarized in Table 15.2.

LIVE/DEAD® Cell Vitality Assay Kit

The LIVE/DEAD® Cell Vitality Assay Kit (L34951) provides a simple two-color fluorescence assay that distinguishes metabolically active cells from injured cells and dead cells. The assay is based on the reduction of C_{12}-resazurin to red-fluorescent C_{12}-resorufin in metabolically active cells and the uptake of the cell-impermeant SYTOX® Green nucleic acid stain in cells with compromised plasma membranes (usually late apoptotic and necrotic cells). Dead cells emit mostly green fluorescence, whereas the healthy, metabolically active cells emit mostly red fluorescence (Figure 15.3.10). The injured cells have lower metabolic activity and, consequently, reduced red-fluorescent emission. Because they possess intact membranes, however, injured cells accumulate little SYTOX® Green dye and, therefore, emit very little green fluorescence.

Nonfluorescent resazurin, which can be reduced by viable cells to red-fluorescent resorufin, has been extensively used to detect the metabolic activity of many different cell types, from bacteria to higher eukaryotes.[47–49] Resazurin is nontoxic and stable in culture media, allowing researchers to continuously monitor proliferating cells[50] and to investigate cytotoxicity in both conventional[51] and high-throughput applications.[52] The LIVE/DEAD® Cell Vitality Assay Kit includes a lipophilic version of resazurin, C_{12}-resazurin, which is more permeable to live cells and, after reduction to C_{12}-resorufin, is far better retained than the nonlipophilic resorufin. These characteristics result in brighter signals and better detection limits. The kit components, number of assays and assay principles are summarized in Table 15.2.

Vybrant® Cell Metabolic Assay Kit

One potential drawback in the use of resazurin as a substrate that can detect metabolic activity in live cells (R12204, Section 15.2) is the relatively poor uptake of the substrate and poor cellular retention of the reduced by-product, resorufin. We have found that the lipophilic dodecylresazurin (C_{12}-resazurin), included as a component in the Vybrant® Cell Metabolic Assay Kit (V23110), surpasses resazurin alone in cell permeability and its reduction product (C_{12}-resorufin) is very well retained, permitting single-cell analysis of the substrate's turnover by flow cytometry (Figure 15.3.11). This enhanced uptake, turnover and retention of C_{12}-resazurin by metabolically active cells translates into much brighter signals and far better detection limits when compared with assays using resazurin alone (Figure 15.3.12).

C_{12}-resazurin can be used in any viability/cytotoxicity assay that employs resazurin for both conventional and high-throughput applications. C_{12}-resorufin, which is the reduction product of C_{12}-resazurin, has the same absorption/emission maxima as unmodified resorufin (~571/ 585 nm, Figure 15.3.13); therefore, no changes in instrumentation are required in

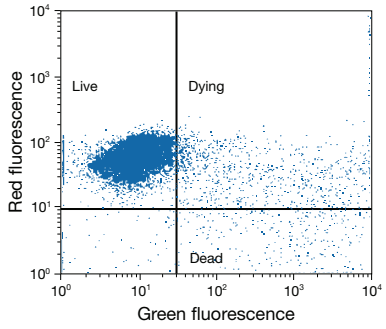

Figure 15.3.11 Flow cytometric analysis of Jurkat cells stained with C_{12}-resazurin. Cells were loaded with 0.1 µM C_{12}-resazurin, a component of the Vybrant® Cell Metabolic Assay Kit (V23110), and 1 mM SYTOX® Green (S7020). After a 15-minute incubation, the cells were analyzed. Healthy (live) cells reduce C_{12}-resazurin into red-fluorescent C_{12}-resorufin and exclude the cell impermeant green-fluorescent SYTOX® Green. Dead cells show little reduction of the C_{12}-resazurin, but strong staining by SYTOX® Green. Cells indicated in the figure as dying are of indeterminate viability, showing both reduction of C_{12}-resazurin and compromised membrane integrity.

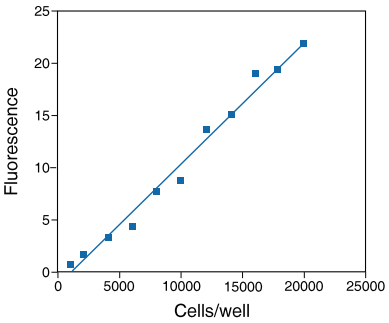

Figure 15.3.12 Detection limit of C_{12}-resazurin and linear response with increasing cell number using our Vybrant® Cell Metabolic Assay Kit (V23110). Jurkat cells were loaded with 5 µM C_{12}-resazurin for 15 minutes. The resulting signal was measured in a fluorescence microplate reader with excitation/emission at 530/590 nm. For comparison, the detection limit for resazurin in a similar experiment was ~8000 cells/well (data not shown).

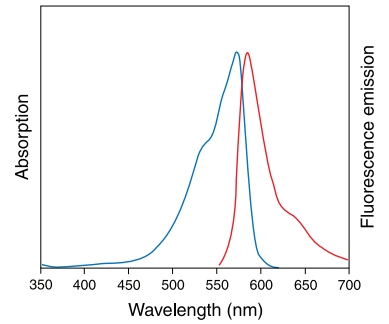

Figure 15.3.13 Absorption and fluorescence emission spectra of resorufin in pH 9.0 buffer.

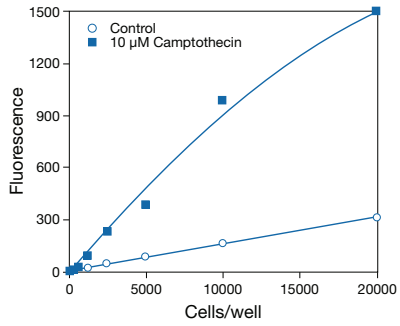

Figure 15.3.15 Detection of dead and dying cells using the Vybrant® Cytotoxicity Assay Kit (V23111). Jurkat cells were treated with 10 µM camptothecin for 6 hours, then assayed for glucose 6-phosphate dehydrogenase release. An untreated control sample is shown for comparison. The fluorescence was measured in a microplate reader (excitation/emission ~530/590 nm). A background of 55 fluorescence units was subtracted from each value.

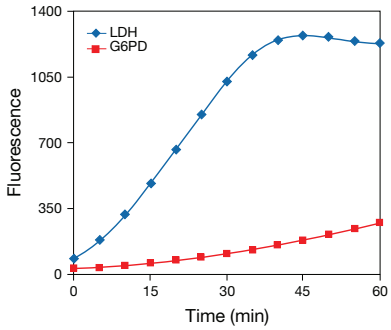

Figure 15.3.16 10% bovine serum was assayed for the presence of lactate dehydrogenase (LDH, blue) and glucose 6-phosphate dehydrogenase (G6PD, red). G6PD was assayed using the Vybrant® Cytotoxicity Assay Kit (V23111); LDH was detected using a similar method, in which LDH reduces lactate to generate NADH. The result clearly shows that, over the time course of the experiment, the serum generates a much lower signal in the G6PD assay than in the LDH assay.

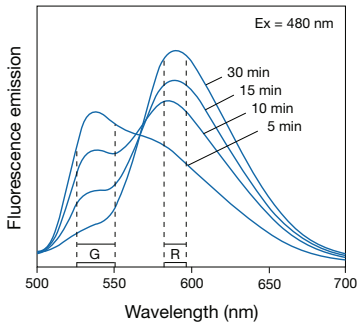

Figure 15.3.17 Fluorescence emission spectra of a *Saccharomyces cerevisiae* suspension that has been stained with the FUN® 1 cell stain, which is available separately (F7030) or in our LIVE/DEAD® Yeast Viability Kit (L7009). After the FUN® 1 reagent was added to the medium, the fluorescence emission spectrum (excited at 480 nm) was recorded in a spectrofluorometer at the indicated times during a 30-minute incubation period. The shift from green (G) to red (R) fluorescence reflects the processing of FUN® 1 by metabolically active yeast cells.

order to use the kit in place of a resazurin-based assay. The Vybrant® Cell Metabolic Assay Kit (V23110) contains:

- Dodecylresazurin (C_{12}-resazurin)
- DMSO
- Resorufin
- Detailed protocols for the assay

Each kit contains sufficient reagents for 500–1000 assays in a fluorescence microplate or about 10,000 flow cytometry assays.

Vybrant® Cytotoxicity Assay Kit

The LIVE/DEAD® kits generally assay cell death via probes that gain entry to the interior of the cell as a result of plasma membrane damage. In contrast, the Vybrant® Cytotoxicity Assay Kit (V23111) monitors the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6PD) from damaged cells into the surrounding medium. G6PD is a ubiquitous enzyme that is part of the pentose phosphate pathway, and is crucial for cellular antioxidant defenses via its production of NADPH.[53,54] Detection of G6PD is by a two-step enzymatic process that leads to the reduction of resazurin into the red-fluorescent resorufin (Figure 15.3.14). The resulting signal is proportional to the amount of G6PD released into the cell medium, which correlates with the number of dead cells in the sample (Figure 15.3.15).

The Vybrant® Cytotoxicity Assay Kit contains all enzymes and substrates needed to detect the release of G6PD from damaged and dying cells. The assay can be completed in less than an hour and is effective with as few as 500 cells per sample. Resorufin, the end product of the G6PD cytotoxicity assay, has absorption and emission maxima at ~571 nm and 585 nm, respectively (Figure 15.3.13), placing the fluorescent signal beyond the autofluorescence of most biological samples. In addition, the levels of G6PD in serum commonly used for cell culture are lower than those of lactate dehydrogenase (LDH), an enzyme often used in similar assays, thus resulting in lower background signals (Figure 15.3.16). The Vybrant® Cytotoxicity Assay Kit (V23111) contains:

- Resazurin (5 vials)
- DMSO
- Reaction mixture (diaphorase, glucose 6-phosphate and $NADP^+$)
- Reaction buffer
- Cell-lysis buffer
- Detailed protocols for the assay

Sufficient reagents are provided for about 1000 assays in a fluorescence microplate reader.

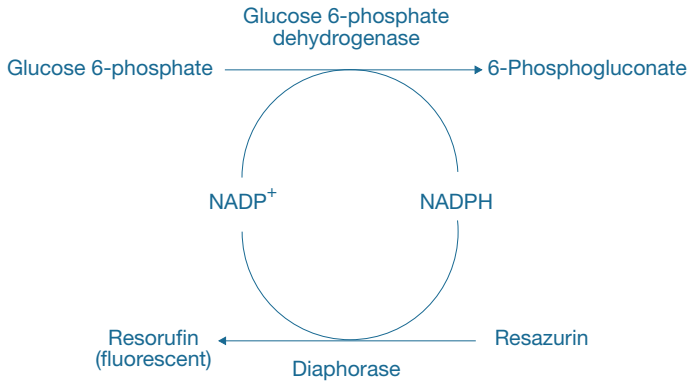

Figure 15.3.14 Principle of the coupled enzymatic assay for detection of glucose 6-phosphate dehydrogenase activity. Oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase results in the generation of NADPH, which in turn leads to the reduction of resazurin by diaphorase to yield fluorescent resorufin.

Viability Assay Kits for Yeast

LIVE/DEAD® Yeast Viability Kit

Our LIVE/DEAD® Yeast Viability Kit (L7009) provides an extremely simple and sensitive assay for discriminating viable yeast and fungi in complex mixtures or in pure cultures.[55–57] This kit contains our unique two-color fluorescent viability probe, the FUN® 1 dye, which has low intrinsic fluorescence, moderate affinity for nucleic acids and exceptional membrane permeability. Also included is the UV light–excitable counterstain Calcofluor White M2R, which labels the cell walls of yeast and fungi fluorescent blue, regardless of the cell's metabolic state.[58–60]

The FUN® 1 viability probe displays some extraordinary spectral properties when used to stain metabolically active yeast and fungal cells, exploiting normal endogenous biochemical processing mechanisms that appear to be well conserved among different fungal species. The FUN® 1 stain passively diffuses into a variety of cell types and initially stains the cytoplasm with a diffusely distributed green fluorescence. However, in several common species of yeast and fungi, subsequent processing of the dye by live cells results in the formation of distinct vacuolar structures with compact form that exhibit a striking red fluorescence, accompanied by a reduction in the green cytoplasmic fluorescence[57,61] (Figure 15.3.17). Formation of the red-fluorescent intravacuolar structures requires both plasma membrane integrity and metabolic capability. Dead cells fluoresce bright yellow-green, with no discernable red structures.

FUN® 1 stain can be used alone or together with Calcofluor White M2R to determine the metabolic activity of single fungal cells by manual or automated fluorescence microscopy (Figure 15.3.18, Figure 15.3.19). Both live and dead cells may be viewed simultaneously by fluorescence microscopy using a standard fluorescein longpass optical filter set. FUN® 1 dye staining can also be used to assay the viability of suspensions of fungal cells using a fluorescence microplate reader or a fluorometer. The FUN® 1 reagent has been extensively used for flow cytometric analysis of yeast, including of their susceptibility to antifungal agents.[62–66]

The LIVE/DEAD® Yeast Viability Kit has been tested on several fungal species, including *Candida albicans*, *Candida pseudotropicalis* and several strains of *Saccharomyces cerevisiae*, under a variety of experimental conditions. Formation of the red-fluorescent structures was observed not only in logarithmically growing cells but also in nonculturable cells with residual metabolic activity. The LIVE/DEAD® Yeast Viability Kit should be particularly useful for detecting very low numbers of live or dead fungal cells, even in complex mixtures such as blood. The kit components, number of assays and assay principles are summarized in Table 15.2. The FUN® 1 cell stain is also available separately (F7030, Section 15.2).

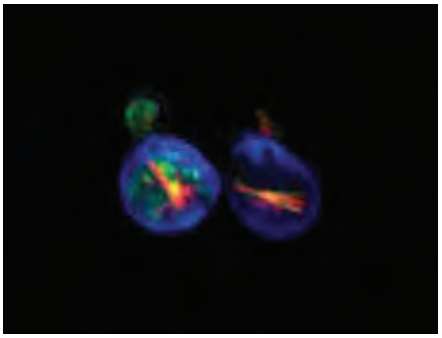

Figure 15.3.18 *Saccharomyces cerevisiae* stained with FUN® 1 cell stain, which generates red-fluorescent intravacuolar structures, and with Calcofluor White M2R, a blue-fluorescent cell wall stain. Both probes are provided in our LIVE/DEAD® Yeast Viability Kit (L7009); FUN® 1 cell stain is also available separately (F7030). A series of z-section images was acquired with a Deltavision wide-field optical sectioning microscope (Applied Precision, Inc.). A three-dimensional projection movie was generated from a deconvolved z-image stack.

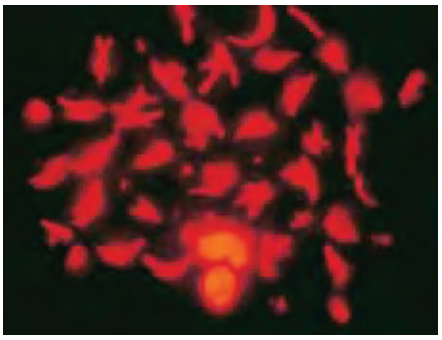

Figure 15.3.19 *Saccharomyces cerevisiae* stained with the FUN® 1 dye, available separately (F7030) or in our LIVE/DEAD® Yeast Viability Kit (L7009). Metabolically active yeast process the FUN® 1 dye, forming numerous red fluorescent cylindrical structures within their vacuoles.

LIVE/DEAD® *Funga*Light™ Yeast Viability Kit

The LIVE/DEAD® *Funga*Light™ Yeast Viability Kit (L34952) allows researchers to easily, reliably and quantitatively distinguish live and dead yeast in minutes. The kit contains solutions of SYT0® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy yeast cells. When used alone, the SYTO® 9 stain generally labels all yeast in a population—those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates yeast with damaged membranes, displacing SYTO® 9 stain. Thus, with an appropriate mixture of the SYTO® 9 and propidium iodide stains, yeast with intact cell membranes stain fluorescent green, whereas yeast with damaged membranes stain fluorescent red. The background remains virtually nonfluorescent. Furthermore, this kit also accommodates fine-tuning of the dye combinations so that optimal staining of yeast can be achieved under a variety of experimental conditions. The kit components, number of assays and assay principles are summarized in Table 15.2.

*Funga*Light™ CFDA AM/Propidium Iodide Yeast Vitality Kit

The *Funga*Light™ CFDA AM/Propidium Iodide Yeast Vitality Kit (F34953) combines a cell-permeant esterase substrate with a membrane integrity indicator to evaluate the vitality of yeast cells by flow cytometry or microscopy. The acetoxymethyl ester (AM) of the esterase substrate 5-carboxy-fluorescein diacetate (CFDA) allows this reagent to permeate cell membranes. Once inside the cell, the lipophilic blocking and diacetate groups are cleaved by nonspecific esterases, resulting in a fluorescent, charged form that leaks out of cells very slowly.

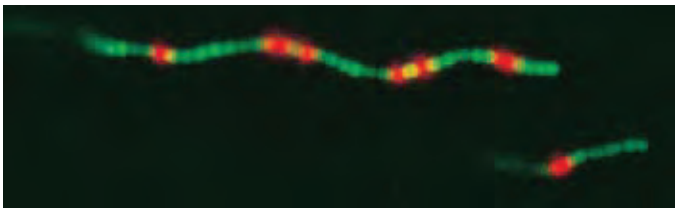

Figure 15.3.20 Use of our LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7007, L7012, L13152) to identify individual live and dead bacteria along a chain of *Streptococcus pyogenes*. This image was photographed in a single exposure through an Omega® Optical triple bandpass filter set.

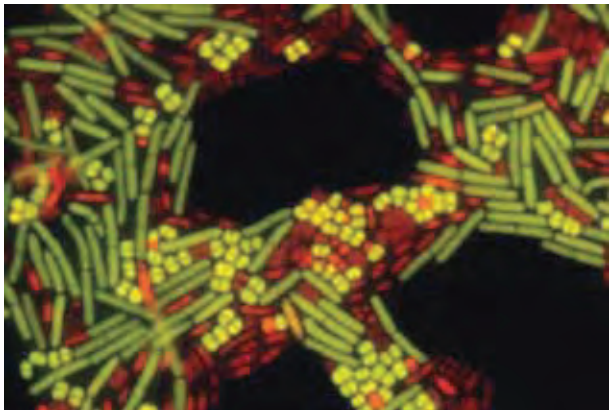

Figure 15.3.21 A mixed population of live and isopropyl alcohol–killed *Micrococcus luteus* and *Bacillus cereus* stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7007, L7012). Bacteria with intact cell membranes exhibit green fluorescence, whereas bacteria with damaged membranes exhibit red fluorescence. Prior to imaging, the bacteria were placed onto a polycarbonate filter and immersed in *Bac*Light™ mounting oil. This multiple exposure image was acquired with a triple-bandpass optical filter set appropriate for simultaneous imaging of DAPI, fluorescein and Texas Red® dyes.

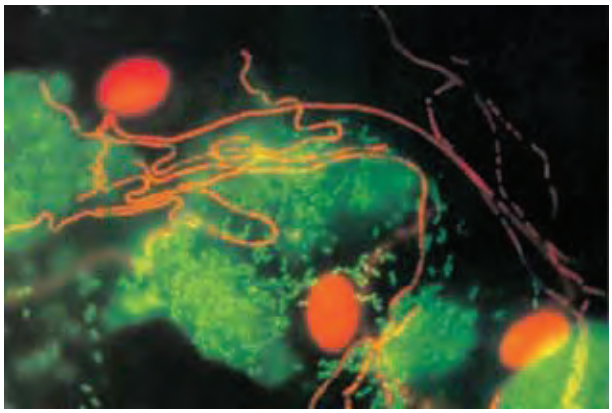

Figure 15.3.22 Live and dead bacteria visualized on freshly isolated human cheek epithelial cells using our LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7007, L7012, L13152). When incubated with the SYTO® 9 and propidium iodide nucleic acid stains provided in this kit, live bacteria with intact cell membranes fluoresce green and dead bacteria with compromised membranes fluoresce red. This image was photographed in a single exposure through an Omega® Optical triple bandpass filter set.

In contrast, the membrane integrity indicator, propidium iodide, penetrates yeast with damaged membranes. With an appropriate mixture of the CFDA AM and propidium iodide stains, esterase-active yeast with intact cell membranes stain fluorescent green, whereas yeast with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are 492/517 nm for CFDA AM and 530/635 nm for propidium iodide. The kit components, number of assays and assay principles are summarized in Table 15.2.

Viability Assay and Gram Stain Kits for Bacteria

LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits

Molecular Probes® original LIVE/DEAD® Viability/Cytotoxicity Kit (L3224, see above) is a proven tool for assessing viability of animal cells but is generally not suitable for use with bacterial and yeast cells.[67] Consequently, we have developed the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits (L7007, L7012, L13152), which provide two different nucleic acid stains—the SYTO® 9 dye and propidium iodide—to rapidly distinguish live bacteria with intact plasma membranes from dead bacteria with compromised membranes[55,57,68] (Figure 15.3.20, Figure 15.3.21).

This assay has several significant features:

- **Ease of use**. The reagents are simultaneously added to the bacterial suspension, which is then incubated for 5–10 minutes. No wash steps are required before analysis.
- **Specificity**. Live bacteria fluoresce green and dead bacteria fluoresce red. Live and dead bacteria can be distinguished and quantitated in minutes, even in a mixed population of bacterial species (Figure 15.3.22).
- **Reliability**. The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits yield consistent results in tests on a variety of eubacterial genera (Table 15.3). It can also be used to assess the viability of *Eurioplasma eurilytica* and *Mycoplasma hominus* mycoplasma as well as cysts of the protozoan parasite *Giardia muris*[69] (Figure 15.3.23).
- **Validity**. Viability measurements in fresh cultures of bacteria typically correlate well with enumeration techniques involving growth in liquid or solid media. However, variable results have been found using the LIVE/DEAD® *Bac*Light™ reagents to assess viability in some marine bacteria from environmental samples.
- **Versatility**. Bacteria can be stained in suspension or immobilized on microscope slides or filter membranes. Protocols are provided for bacterial viability analysis using a fluorescence microscope, fluorometer (Figure 15.3.24) or fluorescence microplate reader.

The intensities of the fluorescence signals produced by the SYTO® 9 and propidium iodide nucleic acid stains can be adjusted by mixing different proportions of the dye solutions provided in the LIVE/DEAD® *Bac*Light™ Kits. We have balanced the dye concentrations so that, for most bacteria, equal volumes of the two solutions provided give balanced staining of most species. The background remains virtually nonfluorescent, allowing live and dead cells to be easily differentiated in any fluorescence microscope equipped with

a longpass fluorescein or comparable optical filter set. Under certain conditions, bacteria with compromised membranes may recover and reproduce, even though such bacteria may be scored as dead in this assay. Conversely, some bacteria with intact membranes may be unable to reproduce in nutrient medium, yet be scored as live.[70] Combining several different measures of viability, such as membrane permeability, enzyme activity and redox potential, offers a more thorough assessment of bacterial viability and eliminates the inherent limitations of any single viability assay.

The LIVE/DEAD® *Bac*Light™ viability assay has been used to estimate total and viable bacteria in drinking water,[71] to quantitate total and viable concentrations of aerosolized *Pseudomonas fluorescens* by fluorescence microscopy [72] and to measure the reliability of disinfection agents on reducing the viability of *Cryptosporidium parvum* and *Giardia muris* cysts.[69,73,74] The number of live natural planktonic bacteria, as determined with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit, reportedly correlated well with the number of bacteria with high DNA content (HDNA), as determined with SYTO® 13 green-fluorescent nucleic acid stain (S7575), leading to the recommendation that %HDNA be used as an index of the percentage of actively growing bacterial cells in marine plankton samples.[75] The reagents in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit have been utilized in a high-throughput fluorescence-based screen for bacterial mechanosensitive ion-channel (MscL) activity that replaces otherwise tedious and difficult assays.[76]

Our original packaging of the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7007), in which the dyes were mixed at different proportions in two solutions, is still available for customers who have already developed protocols using that formulation. However, we recommend use of the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7012), which is more flexible because it provides separate solutions of the SYTO® 9 and propidium iodide nucleic acid stains, thus facilitating calibration of bacterial fluorescence at each of the two emission wavelengths in quantitative assays. Kit L7007 was designed primarily for use in fluorescence microscopy; Kit L7012 is equally well suited for use in fluorescence microscopy and is better suited than Kit L7007 for use in quantitative analysis with a fluorometer (Figure 15.3.24), fluorescence microplate reader, flow cytometer or other instrumentation.

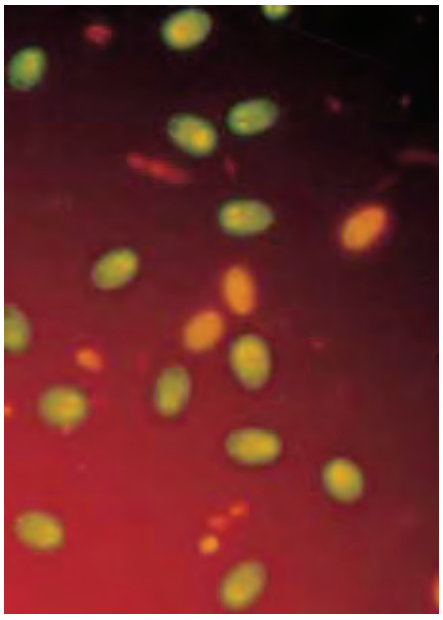

Figure 15.3.23 *Giardia muris* cysts stained with the reagents in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7007, L7012). When incubated with the SYTO® 9 and propidium iodide nucleic acid stains provided in this kit, live bacteria with intact cell membranes fluoresce green and dead bacteria with compromised membranes fluoresce red.

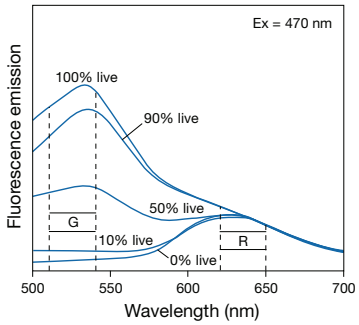

Figure 15.3.24 Viability analysis of bacterial suspensions comprising various proportions of live and isopropyl alcohol–killed *Escherichia coli* using the reagents in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7007, L7012, L13152). Live and dead bacteria are stained fluorescent green (G) by SYTO® 9 and fluorescent red (R) by propidium iodide, respectively. Bacterial suspensions that have been incubated in the two stains simultaneously and then excited at 470 nm exhibit a fluorescence spectral shift from red to green as the percentage of live bacteria in the sample is increased.

Table 15.3 Some organisms that have been successfully stained with our LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits.

Bacteria	
Gram-Positive	**Gram-Negative**
• *Bacillus cereus*	• *Agrobacterium tumefaciens*
• *Bacillus subtilis*	• *Edwardsiella ictaluri*
• *Clostridium paerfringens*	• *Escherichia coli*
• *Lactobacillus* sp.	• *Deleya aquamarina*
• *Micrococcus luteus*	• *Helicobacter pylori*
• *Mycobacterium phlei*	• *Klebsiella pneumoniae*
• *Propionibacterium* sp.	• *Legionella pneumophila*
• *Staphylococcus aureus*	• *Pseudomonas aeruginosa*
• *Streptococcus pyogenes*	• *Pseudomonas syringae*
	• *Salmonella oranienburg*
	• *Serratia marcescens*
	• *Shigella sonnei*
	• *Zymomonas* sp.
Mycoplasma	
• *Eurioplasma eurilytica*	
• *Mycoplasma hominus*	
Protozoa	
• *Giardia muris* cysts	

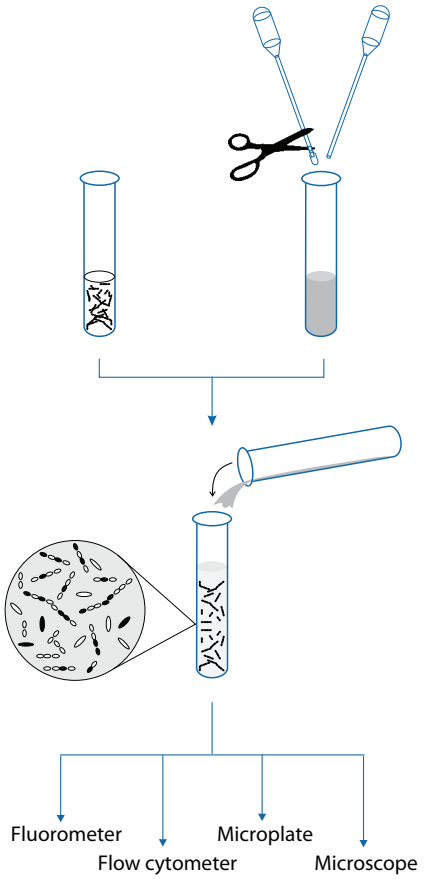

Figure 15.3.25 The convenient and versatile procedure for using the specially packaged LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L13152). Simply dissolve the premeasured dyes in buffer, mix with the bacterial sample and observe the fluorescence.

For added convenience, our LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L13152) provides the separate stains dry and premeasured into pairs of polyethylene transfer pipettes (Figure 15.3.25). Kit L13152 has several advantages:

- The stains are dry, without DMSO or other potentially harmful solvents, allowing viability determination of solvent-sensitive microorganisms—just dissolve the dyes in virtually any aqueous medium and then add them to the cells.
- The stains are premeasured and supplied in sealed polyethylene transfer pipettes, eliminating the need for pipetting microliter volumes—perfect for educational settings, where there is a need to simplify handling and minimize equipment expenditures.
- This stain formulation does not require refrigeration and is chemically stable, even in poor conditions—storage at 37°C for more than six months produces no detectable changes, making the assay well suited to field testing and other situations where storage or use conditions are less than optimal.

Each of our LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits includes a procedure for mounting bacteria stained with the reagents in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit on filter membranes and a proprietary mounting oil that we have found to be useful for the direct epifluorescence filter technique[77] (DEFT). The kit components, number of assays and assay principles are summarized in Table 15.2. The SYTO® 9 nucleic acid stain is also available separately (S34854, Section 15.2).

LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit

Accurate detection and enumeration of the live and dead bacteria in a sample is an important aspect of many experimental procedures in biotechnology. Because of the marked differences in morphology, cytology and physiology among the many bacterial genera, a universally applicable direct-count viability assay has been very difficult to attain. Conventional direct-count assays of bacterial viability are based on metabolic characteristics or membrane integrity. However, methods relying on metabolic characteristics often only work for a limited subset of bacterial groups,[78] and methods for assessing bacterial membrane integrity commonly have high levels of background fluorescence.[79] Both types of determinations suffer from being very sensitive to growth and staining conditions.[80,81] The LIVE/DEAD® *Bac*Light™ Bacterial Counting and Viability Kit (L34856) allows researchers to reliably distinguish and quantitate live and dead bacteria with the aid of a flow cytometer, even in a mixed population containing a range of bacterial types.

This kit utilizes a mixture of two nucleic acid stains—the green-fluorescent SYTO® 9 dye and red-fluorescent propidium iodide—for viability determinations, as well as a calibrated suspension of beads for accurate sample volume measurements. The SYTO® 9 and propidium iodide stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO® 9 stain generally labels all bacteria in a population—those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present. With an appropriate mixture of the SYTO® 9 and propidium iodide stains, bacteria with intact cell membranes fluoresce bright green, whereas bacteria with damaged membranes exhibit significantly less green fluorescence and they often also fluoresce red. The cell type and the gram character influence the amount of red-fluorescent staining exhibited by dead bacteria. Both the SYTO® 9 and propidium iodide stains are efficiently excited by the 488 nm spectral line of the argon-ion laser found in most flow cytometers, and their nucleic acid complexes can be detected in the green and red channels, respectively; the background remains virtually nonfluorescent.

The calibrated suspension of microspheres serves as a reference standard for sample volume. The size and fluorescence of the beads in this microsphere standard have been carefully chosen to ensure that they will be clearly distinguishable from any stained bacteria population in a fluorescence versus side scatter cytogram. A bacterial culture is simply stained with the optimal mixture of SYTO® 9 dye and propidium iodide, and then a fixed number of microspheres are added before analyzing the sample on a flow cytometer. Live and dead bacteria and the microspheres are all easily distinguished in a plot of fluorescence versus side scatter (Figure 15.3.26). The concentration of both the live bacteria and the dead bacteria can then be determined from the ratio of bacteria events to microsphere events in the cytogram (Figure 15.3.27).

The kit components, number of assays and assay principles are summarized in Table 15.2. The SYTO® 9 nucleic acid stain is also available separately (S34854, Section 15.2).

*Bac*Light™ RedoxSensor™ Green Vitality Kit

Bacterial oxidation–reduction activity is an informative parameter for measuring cell vitality. Bacterial oxidases and reductases engage in important functions involving the electron transport chain, catabolic and anabolic pathways and xenobiotic compound metabolism.[82] The RedoxSensor™ Green reagent, available in the *Bac*Light™ RedoxSensor™ Green Vitality Kit (B34954), is an indicator of bacterial reductase activity; this reductase activity is, in turn, a reliable marker for changes in electron transport chain function and for changes in vitality that occur following antibiotic treatment. The RedoxSensor™ Green reagent penetrates both gram-positive and gram-negative bacteria, although differences in signal intensity may be observed based upon cell wall characteristics. Following reduction, the RedoxSensor™ Green reagent will produce a stable green-fluorescent signal (excitation/emission maxima ~490/520 nm) in about 10 minutes that is compatible with formaldehyde fixation techniques. The *Bac*Light™ RedoxSensor™ Green Vitality Kit is useful for measuring the effects of antimicrobial agents and for monitoring cultures in fermenters.

The fluorescence intensity of cells stained with the RedoxSensor™ Green reagent is altered when cells are treated with reagents that disrupt electron transport, such as sodium azide or carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which are both included in this kit. Species differences in responses may be observed. For example, sodium azide disrupts the fluorescence response in *Escherichia coli*, and CCCP disrupts fluorescence response in *Staphylococcus aureus*. The *Bac*Light™ RedoxSensor™ Green Vitality Kit has been tested on logarithmically growing cultures of the following bacterial species: *Micrococcus luteus*, *Staphylococcus aureus*,

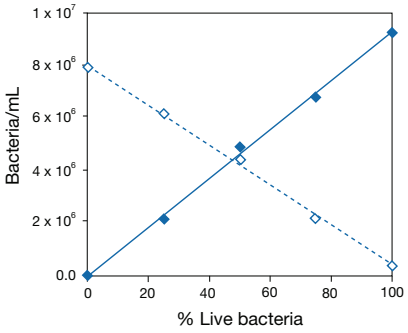

Figure 15.3.27 Best-fit linear regression analysis generated using the LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit (L34856). Suspensions of live (untreated, (—) (r^2=0.9982)) and dead (alcohol-treated, (– – –),(r^2=0.9974)) *Staphylococcus aureus* were mixed at various live:dead ratios. Mixtures were stained according to the kit protocol and analyzed in triplicate by flow cytometry. Values of bacteria/mL were calculated according to the equation provided in the kit protocol; the mean values are shown above. This experiment may be performed to determine optimal dye concentrations, to practice the cell-staining procedure or to generate a standard curve for unknown samples.

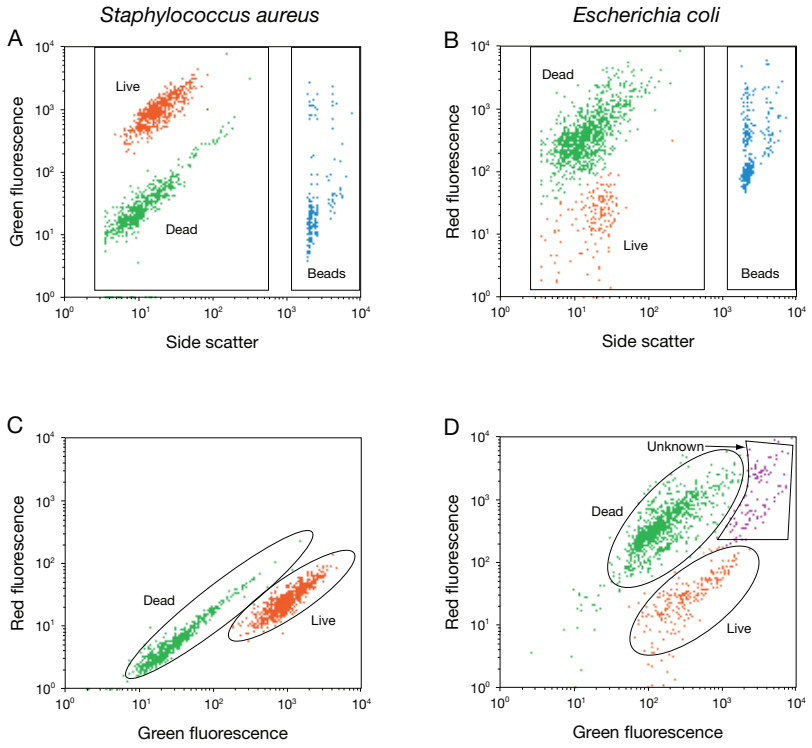

Figure 15.3.26 Analysis of bacterial cultures using the LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit (L34856). Suspensions of live (untreated) and dead (alcohol-treated) *Staphylococcus aureus* (**A** and **C**) and *Escherichia coli* (**B** and **D**) were stained with the SYTO® 9 nucleic acid stain and propidium iodide and then analyzed by flow cytometry according to the kit protocol. The green or red fluorescence versus side scatter cytogram (**A** or **B**) was used to gate the bacterial population and the bead population (left and right boxes, respectively). Events in the bacteria region of each cytogram are also displayed in red fluorescence versus green fluorescence cytograms (**C** and **D**). Live and dead bacteria/mL can be calculated from either the fluorescence versus side scatter cytogram or the green fluorescence versus red fluorescence cytogram, depending on which one shows the best separation of the live and dead populations. The position of the live and dead populations in these cytograms may be dependent on cell type and gram character. Some samples may exhibit events that fall outside the defined regions and should be evaluated appropriately (e.g., see panel **D**).

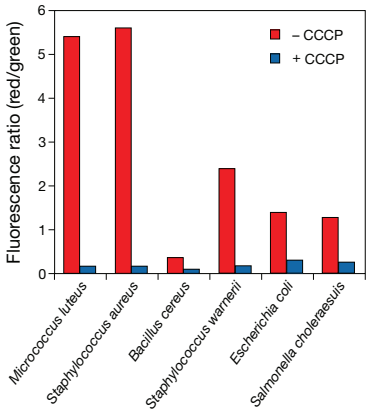

Figure 15.3.28 Detection of membrane potential in various bacteria with the *Bac*Light™ Bacterial Membrane Potential Kit (B34950). Red/green fluorescence ratios were calculated using population mean fluorescence intensities for gram-positive (*Micrococcus luteus, Staphylococcus aureus, Bacillus cereus* and *Staphylococcus warnerii*) and gram-negative (*Escherichia coli* and *Salmonella choleraesuis*) bacteria incubated with 30 µM $DiOC_2(3)$ for 30 minutes in either the presence or absence of 5 µM CCCP, according to the kit protocol.

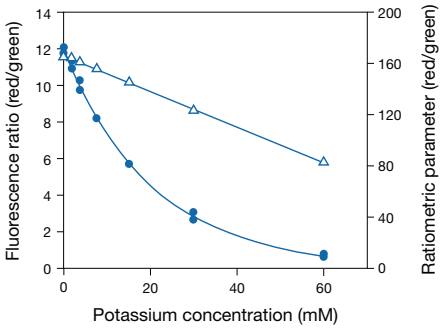

Figure 15.3.29 Response of *Staphylococcus aureus* to valinomycin and external potassium ions, as measured by flow cytometry using the *Bac*Light™ Bacterial Membrane Potential Kit (B34950). Samples containing *S. aureus* were treated with 5 µM valinomycin in different concentrations of potassium buffer, and then stained using 30 µM $DiOC_2(3)$ for 30 minutes, according to the kit protocol. Data are expressed either using a ratiometric parameter based on the formula provided in the kit protocol (Δ, right axis) or as the ratio of population mean red-fluorescence intensity/mean green-fluorescence intensity (●, left axis).

Bacillus cereus, *B. subtilis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella choleraesuis*. Most gram-positive bacteria stain more efficiently than many gram-negative bacteria; and the response of each bacterial system should be investigated and optimized.

Each *Bac*Light™ RedoxSensor™ Green Vitality Kit contains:

- RedoxSensor™ Green reagent
- Propidium iodide, an indicator of membrane integrity
- Sodium azide
- Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)
- Detailed protocols

At the recommended reagent dilutions and volumes, this kit contains sufficient material to perform ~200 tests by flow cytometry. Although the *Bac*Light™ RedoxSensor™ Green Vitality Kit has been developed for flow cytometric analysis, it may be appropriate for other analysis platforms.

*Bac*Light™ RedoxSensor™ CTC Vitality Kit

The *Bac*Light™ RedoxSensor™ CTC Vitality Kit (B34956) provides effective reagents for evaluating bacterial health and vitality, and these staining reagents can withstand fixation procedures. This kit contains 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which has been used by researchers to evaluate the respiratory activity of many bacterial populations derived from environmental sources including food,[83,84] soil,[85] stone[86] and marine and fresh water,[87–90] as well as populations undergoing drug efficacy evaluations. Briefly, healthy cells respiring via the electron transport chain will absorb and reduce CTC into an insoluble, red-fluorescent formazan product. Cells not respiring or respiring at slower rates will reduce less CTC, and consequently produce less fluorescent product, giving a semiquantitative estimate of healthy versus unhealthy bacteria. SYTO® 24 green-fluorescent nucleic acid stain and DAPI are provided as counterstains to assist the researcher in differentiating cells from debris and in calculating total cell numbers. Bacteria labeled with CTC and either counterstain may be evaluated immediately or after storage, with or without fixation, using a flow cytometer equipped with appropriate excitation sources (CTC in combination with SYTO® 24 green-fluorescent nucleic acid stain: single 488 nm argon-ion laser; CTC in combination with DAPI: dual UV and 488 nm lasers). Bacteria stained using the *Bac*Light™ RedoxSensor™ CTC Vitality Kit may also be viewed using most standard epifluorescence microscopes equipped with the appropriate filters; for visualizing CTC, SYTO® 24 and DAPI staining, we recommend optical filters optimized for the Texas Red® dye, FITC and DAPI, respectively.

Each *Bac*Light™ RedoxSensor™ CTC Vitality Kit contains:

- 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)
- SYTO® 24 green-fluorescent nucleic acid stain
- 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI)
- Detailed protocols

At the recommended reagent dilutions and volumes, this kit contains sufficient material to perform at least 50 tests by flow cytometry or microscopy.

*Bac*Light™ Bacterial Membrane Potential Kit

The *Bac*Light™ Bacterial Membrane Potential Kit (B34950) provides a fluorescent membrane-potential indicator dye, $DiOC_2(3)$, along with a proton ionophore (CCCP) and premixed buffer. At low concentrations, $DiOC_2(3)$ exhibits green fluorescence in all bacterial cells, but it becomes more concentrated in healthy cells that are maintaining a membrane potential, causing the dye to self-associate and the fluorescence emission to shift to red. The red- and green-fluorescent bacterial populations are easily distinguished using a flow cytometer. CCCP is included in the kit for use as a control because it eradicates the proton gradient, eliminating bacterial membrane potential.[91,92]

Using the *Bac*Light™ Bacterial Membrane Potential Kit, we have detected membrane potentials in all bacteria tested (including logarithmically growing cultures of *Micrococcus luteus,*

Staphylococcus aureus, Bacillus cereus, *Staphylococcus warnerii*, *Escherichia coli* and *Salmonella choleraesuis*), although the magnitude varies with species (Figure 15.3.28). For many gram-positive species, such as *M. luteus* and *S. aureus*, the $DiOC_2(3)$ red:green ratio has been shown to vary with the intensity of the proton gradient (Figure 15.3.29). In gram-negative bacteria, such as *E. coli* and *S. choleraesuis*, a $DiOC_2(3)$ response is observed in the presence of a membrane potential but the response does not appear to be proportional to proton gradient intensity.

Each *Bac*Light™ Bacterial Membrane Potential Kit contains:

- $DiOC_2(3)$ in dimethylsulfoxide (DMSO)
- CCCP in DMSO
- Phosphate-buffered saline (PBS)
- Detailed protocols

Using the recommended reagent dilutions and volumes, this kit provides sufficient $DiOC_2(3)$ to perform approximately 100 individual assays by flow cytometry; sufficient CCCP is provided for 30 depolarized control samples. The *Bac*Light™ Bacterial Membrane Potential Kit is designed to assay bacterial concentrations between 10^5 and 10^7 organisms per mL. Note that $DiOC_2(3)$ and CCCP are inhibitors of respiration, rendering the cells nonculturable beyond the brief time window required for staining and analysis.

LIVE *Bac*Light™ Bacterial Gram Stain Kit

The LIVE *Bac*Light™ Bacterial Gram Stain Kit (L7005) is based on differential nucleic acid staining of *live* gram-negative and gram-positive bacteria. The gram stain is one of the most important and widely used differential stains for the taxonomic classification of bacteria in both clinical and research settings. The original method involves several steps, including heat fixation of the bacteria, a two-step staining protocol, alcohol extraction and counterstaining. Over the years, several improved gram-staining techniques have been developed, but most still involve cell-fixation or cell-permeabilization steps that kill the bacteria being tested. Our single-step LIVE *Bac*Light™ Bacterial Gram Stain Kits can overcome many of the problems inherent in these labor-intensive, fixation-dependent procedures.

Unlike conventional gram stain procedures, the LIVE *Bac*Light™ Bacterial Gram Stain Kit allows researchers to rapidly classify bacteria as either gram-negative or gram-positive in minutes using a single staining solution, no fixatives and no wash steps. The LIVE *Bac*Light™ Bacterial Gram Stain Kit contains our green-fluorescent SYTO® 9 and red-fluorescent hexidium iodide nucleic acid stains. These two dyes differ in both their spectral characteristics and in their ability to label live gram-negative and gram-positive bacteria. When a mixed population of live gram-negative and gram-positive bacteria is simultaneously stained with the membrane-permeant SYTO® 9 dye in combination with hexidium iodide, gram-negative bacteria fluoresce green, and the gram-positive bacteria fluoresce red-orange (Figure 15.3.30, Figure 15.3.31). Dead bacteria do not exhibit predictable staining patterns. Gram-negative and gram-positive organisms can be easily differentiated in any fluorescence microscope equipped with a standard fluorescein longpass optical filter set or by flow cytometry.[93] The assay provides a sensitive indicator system for analyzing low numbers of bacteria in the presence of background material because the unbound reagents exhibit low fluorescence when not bound to nucleic acids. The LIVE *Bac*Light™ Bacterial Gram Stain Kit should be a useful tool for measuring dynamic changes in the composition of bacterial populations.

Our LIVE *Bac*Light™ Bacterial Gram Stain Kit provides separate solutions of the SYTO® 9 and hexidium iodide nucleic acid stains, thus facilitating calibration of bacterial fluorescence at each of the two emission wavelengths in quantitative assays. The kit is well suited for use in fluorescence microscopy, as well as for use in quantitative analysis with a fluorometer (Figure 15.3.32), fluorescence microplate reader, flow cytometer[94] or other instrumentation. The kit includes a procedure for mounting bacteria stained with the LIVE *Bac*Light™ Bacterial Gram Stain Kit on filter membranes, as well as the proprietary *Bac*Light™ mounting oil that we have found to be useful for the direct epifluorescence filter technique[77] (DEFT). The kit components, number of assays and assay principles are summarized in Table 15.2. Hexidium iodide (H7593), the gram-positive bacteria–selective nucleic acid stain, and the SYTO® 9 nucleic acid stain (S34854) are both available separately (Section 15.2). The validity of using hexidium iodide in combination with the SYTO® 13 green-fluorescent nucleic acid stain to correctly predict the

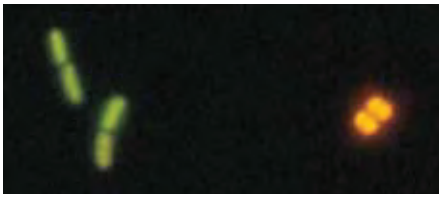

Figure 15.3.30 Live *Micrococcus luteus* and *Salmonella oranienburg* bacteria stained with the LIVE *Bac*Light™ Bacterial Gram Stain Kit (L7005). Gram-positive *M. luteus* cells fluoresce orange, whereas gram-negative *S. oranienburg* cells fluoresce green. This image was photographed in a single exposure through an Omega® Optical triple bandpass filter set.

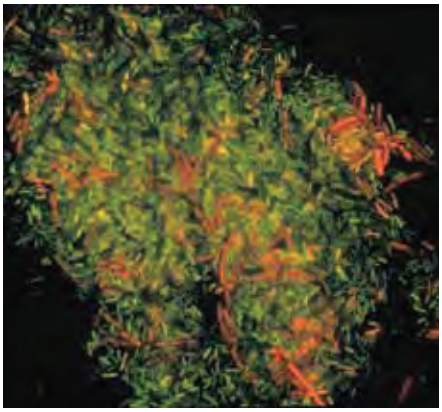

Figure 15.3.31 A mixed population of *Bacillus cereus* and *Pseudomonas aeruginosa* stained with the dye mixture provided in our LIVE *Bac*Light™ Gram Stain Kit (L7005). When live bacteria are incubated with this kit's cell-permeant nucleic acid stains, gram-positive organisms fluoresce orange and gram-negative organisms fluoresce green. The bacteria were photographed through an Omega® Optical triple bandpass filter set.

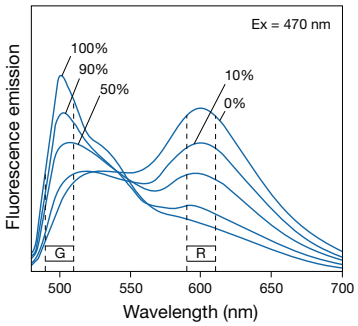

Figure 15.3.32 Analysis of the percentage of gram-negative *Escherichia coli* in mixed suspensions containing gram-positive *Staphylococcus aureus* using the reagents in the LIVE *Bac*Light™ Bacterial Gram Stain Kit (L7005). Live gram-negative and gram-positive bacteria are stained fluorescent green (G) by SYTO® 9 and fluorescent red (R) by hexidium iodide, respectively. Bacterial suspensions that have been incubated in the two stains simultaneously and then excited at 470 nm exhibit a fluorescence spectral shift from red to green as the percentage of gram-negative bacteria in the sample is increased.

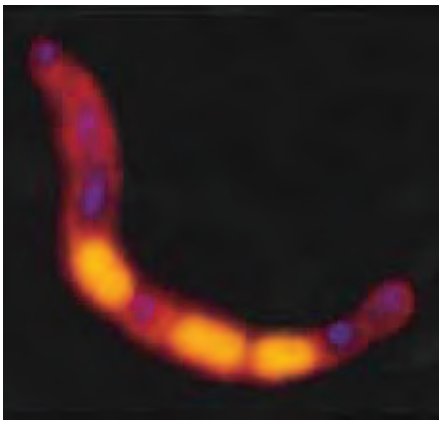

Figure 15.3.33 *Bifidobacterium* sp. bacteria stained with the ViaGram™ Red+ Bacterial Gram Stain and Viability Kit (V7023). While all cells exhibit a red-fluorescent surface stain (gram-positive), live cells exhibit blue-fluorescent internal staining and dead cells with compromised membranes exhibit yellow-green–fluorescent internal staining. This image was obtained by taking multiple exposures through bandpass optical filter sets appropriate for DAPI, fluorescein and Texas Red® dye.

gram sign of 45 clinically relevant organisms, including several known to be gram variable, has been demonstrated.[93]

ViaGram™ Red+ Bacterial Gram Stain and Viability Kit

The ViaGram™ Red+ Bacterial Gram Stain and Viability Kit (V7023) provides an easy, three-color fluorescent staining protocol that differentially stains many gram-negative and gram-positive bacterial species and, at the same time, discriminates live from dead cells based on plasma membrane integrity. This kit contains three reagents: two nucleic acid stains for viability determination—the blue-fluorescent cell-permeant DAPI and the green-fluorescent cell-impermeant SYTOX® Green nucleic acid stain—as well as the red-fluorescent Texas Red®-X wheat germ agglutinin (WGA) for gram sign determination. Bacteria with intact cell membranes stain fluorescent blue with DAPI, whereas bacteria with damaged membranes stain fluorescent green with the SYTOX® Green nucleic acid stain. The background remains virtually nonfluorescent. The Texas Red®-X WGA component selectively binds to the surface of gram-positive bacteria, providing a red-fluorescent cell-surface stain that effectively distinguishes them from gram-negative bacteria, even in the presence of the viability stains. Thus, with three fluorescent colors, the four possible combinations of live or dead, gram-negative and gram-positive cells are discriminated with a fluorescence microscope (Figure 15.3.33, Figure 15.3.34). This kit also includes a procedure for mounting bacteria on filter membranes and the *Bac*Light™ mounting oil, which we have found to be useful for the direct epifluorescence filter technique [77] (DEFT). The kit components, number of assays and assay principles are summarized in Table 15.2.

FilmTracer™ LIVE/DEAD® Biofilm Viability Kit

The FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (L10316) provides a two-color fluorescence assay of bacterial viability based on membrane integrity, and has proven useful for a diverse array of bacterial genera, including those growing in biofilm communities. The kit utilizes a mixture of SYTO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide, which differ both in their spectral characteristics and in their ability to penetrate healthy bacteria. SYTO® 9 stain generally labels all bacteria in a population—those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes; SYTO® 9 stain fluorescence is thereby reduced in cells where both dyes are present. When treated with an appropriate mixture of these stains, bacteria with intact cell membranes display green fluorescence while those with damaged membranes display red fluorescence.

We offer several individual fluorescent FilmTracer™ biofilm stains, which are described in Section 15.2):

- FilmTracer™ calcein red-orange biofilm stain (F10319)
- FilmTracer™ calcein violet biofilm stain (F10320)

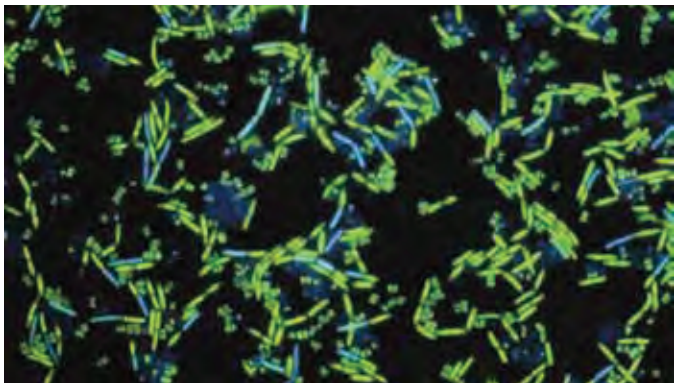

Figure 15.3.34 A mixed population of live and isopropyl alcohol–killed *Micrococcus luteus* and *Bacillus cereus* simultaneously stained with DAPI (D1306, D3571, D21490) and SYTOX® Green nucleic acid stain (S7020), the viability determination components of the ViaGram™ Red+ Bacterial Gram Stain and Viability Kit (V7023). Bacteria with intact cell membranes are stained exclusively with the cell-permeant DAPI nuclear stain and exhibit blue fluorescence, whereas cells with damaged membranes are stained with both fluorophores and exhibit green fluorescence. This image was acquired with a UV longpass optical filter set.

- FilmTracer™ calcein green biofilm stain (F10322)
- FilmTracer™ FM® 1-43 green biofilm cell stain (F10317)
- FilmTracer™ SYPRO® Ruby biofilm matrix stain (F10318)

Wheat Germ Agglutinin Sampler Kit

Fluorescent lectins have proven useful in microbiology applications. Fluorescent wheat germ agglutinin (WGA) conjugates selectively stain chitin in fungal cell walls,[95] as well as the surface of gram-positive but not of gram-negative bacteria. Fluorescent WGA has also been shown to bind to sheathed microfilariae and has been used to detect filarial infection in blood smears.[96] Our Wheat Germ Agglutinin Sampler Kit (W7024) provides 1 mg samples of four of our brightest fluorescent WGA conjugates, spanning the spectrum from blue to red. Included in this kit are conjugates of the blue-fluorescent Alexa Fluor® 350, green-fluorescent Oregon Green® 488, orange-fluorescent tetramethylrhodamine and red-fluorescent Texas Red®-X dyes (Table 7.10). See Section 7.7 for more information on lectins, including additional WGA and concanavalin A (Con A) conjugates.

Reverse Transcriptase Assay for Retrovirus Titering

The EnzChek® Reverse Transcriptase Assay Kit (E22064) is designed to provide a convenient and efficient method for measuring reverse transcriptase activity. This kit has been used to determine HIV-1 reverse transciptase heterodimer activity,[97] to measure SARS-CoV viral titers in 293T host cultures [98] and to quantitate reverse transciptase activity in Friend virus (FV) culture supernatants.[99]

The key to this assay is our PicoGreen® dsDNA quantitation reagent, which preferentially detects dsDNA or RNA–DNA heteroduplexes over single-stranded nucleic acids or free nucleotides. In the assay, the sample to be measured is added to a mixture of a long poly(A) template, an oligo(dT) primer and dTTP. Reverse transcriptase activity in the sample results in the formation of long RNA–DNA heteroduplexes, which are detected by the PicoGreen® reagent at the end of the assay. In less than an hour, samples can be read in a fluorometer or microplate reader with filter sets appropriate for fluorescein (FITC). The assay is sensitive, detecting as little as 0.02 units of HIV reverse transcriptase and has about a 50-fold linear range.

The EnzChek® Reverse Transcriptase Assay Kit contains sufficient reagents for approximately 1000 assays using a fluorescence microplate reader:

- PicoGreen® dsDNA quantitation reagent
- Bacteriophage lambda DNA standard
- Poly(A) ribonucleotide template
- Oligo dT16 primer
- TE buffer, polymerization buffer and an EDTA solution
- Detailed protocols

ATP Determination

The luciferin–luciferase bioluminescence assay is extremely sensitive; most luminometers can detect as little as 1 picomole of pre-existing ATP or ATP as it is generated in kinetic systems. This sensitivity has led to its widespread use for detecting ATP in various enzymatic reactions, as well as for measuring viable cell number,[100] for monitoring ATP release from cells [101–104] and for detecting low-level bacterial contamination in samples such as blood, milk, urine, soil and sludge.[105–109] The luciferin–luciferase bioluminescence assay has also been used to determine cell proliferation and cytotoxicity in both bacterial [110,111] and mammalian cells,[112,113] and to distinguish cytostatic versus cytocidal potential of anticancer drugs on malignant cell growth.[114]

We offer a convenient ATP Determination Kit (A22066) for the sensitive bioluminescence-based detection of ATP with recombinant firefly luciferase and its substrate luciferin. This assay is based on luciferase's absolute requirement for ATP to produce light. In the presence of Mg^{2+}, luciferase catalyzes the reaction of luciferin, ATP and O_2 to form oxyluciferin, AMP, CO_2, pyrophosphate and ~560 nm light (Figure 15.3.35).

The ATP Determination Kit (A22066) contains:

- Luciferin (5 × 3 mg)
- Luciferase
- ATP
- Dithiothreitol (DTT)
- Concentrated reaction buffer
- Detailed protocols for ATP quantitation

Unlike most other commercially available ATP detection kits, our ATP Determination Kit provides the luciferase and luciferin packaged separately, which enables researchers to optimize the reaction conditions for their particular instruments and samples. The ATP Determination Kit provides sufficient reagents to perform 200 ATP assays using 0.5 mL sample volumes or 500 ATP assays using 0.2 mL sample volumes.

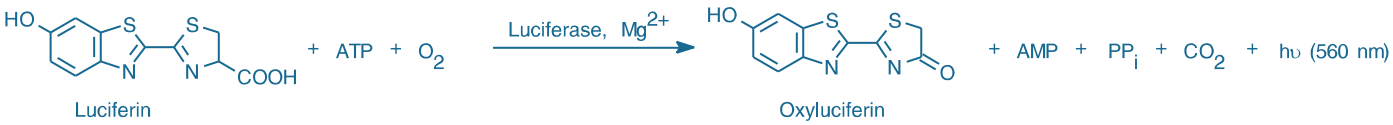

Figure 15.3.35 Reaction scheme for bioluminescence generation via luciferase-catalyzed conversion of luciferin to oxyluciferin.

REFERENCES

1. J Immunol Methods (2000) 243:155; **2**. Prog Neurobiol (2009) 88:221; **3**. J Neurosci (1995) 15:5389; **4**. Biol Bull (1995) 189:218; **5**. Ophthalmologe (1995) 92:452; **6**. J Cell Sci (1993) 106:685; **7**. Cytometry (1995) 20:181; **8**. J Biol Chem (1995) 270:23895; **9**. J Biol Chem (1995) 270:7791; **10**. J Neurosci (1994) 14:2464; **11**. J Neurosci (1994) 14:2260; **12**. J Biol Chem (1994) 269:6803; **13**. Photochem Photobiol (1996) 63:111; **14**. J Immunol Methods (1994) 177:101; **15**. Hum Immunol (1993) 37:264; **16**. Theriogenology (1995) 43:595; **17**. Practical Flow Cytometry, 3rd Ed., Shapiro HM, Ed. (1995) p. 250; **18**. Methods Cell Biol (1994) 42 Pt B:359; **19**. Methods Cell Biol (1994) 42 Pt B:295; **20**. J Immunol Methods (2006) 313:199; **21**. J Immunol Methods (1992) 156:47; **22**. J Immunol Methods (1995) 185:209; **23**. J Immunol Methods (1993) 166:45; **24**. J Immunol Methods (1997) 204:135; **25**. Theriogenology (1996) 45:1103; **26**. Biol Reprod (1995) 53:276; **27**. Poult Sci (1995) 74:1191; **28**. J Androl (1994) 15:620; **29**. Theriogenology (1996) 45:923; **30**. Nature (1995) 377:20; **31**. Biol Reprod (1997) 56:143; **32**. Biol Reprod (1997) 57:1401; **33**. Reprod Dom Anim (1997) 32:279; **34**. Biol Reprod (1998) 58:786; **35**. Biol Reprod (1997) 56:991; **36**. Theriogenology (1993) 39:1009; **37**. Gamete Res (1989) 22:355; **38**. Biol Reprod (1986) 34:127; **39**. J Histochem Cytochem (1982) 30:279; **40**. Methods Cell Biol (1990) 33:401; **41**. J Histochem Cytochem (1977) 25:46; **42**. J Androl (1991) 12:112; **43**. Cytometry (1987) 8:642; **44**. Cytometry (1980) 1:132; **45**. Biol Reprod (1994) 50:987; **46**. J Reprod Fertil (1992) 96:581; **47**. J Neurosci Methods (1996) 70:195; **48**. Appl Environ Microbiol (1990) 56:3785; **49**. J Dairy Res (1990) 57:239; **50**. J Immunol Methods (1997) 210:25; **51**. J Immunol Methods (1998) 213:157; **52**. Antimicrob Agents Chemother (1997) 41:1004; **53**. J Biol Chem (2000) 275:40042; **54**. Nucleic Acids Res (1986) 14:2511; **55**. FEMS Microbiol Lett (1995) 133:1; **56**. J Cell Biol (1994) 126:1375; **57**. Biotechnol Intl (1997) 1:291; **58**. J Cell Biol (1993) 123:1821; **59**. J Cell Biol (1991) 114:111; **60**. J Cell Biol (1991) 114:101; **61**. Appl Environ Microbiol (1997) 63:2897; **62**. J Clin Microbiol (2001) 39:2458; **63**. J Clin Microbiol (1997) 35:5; **64**. Cytometry (1998) 31:307; **65**. Mycoses (1996) 39:45; **66**. Yeast (1998) 14:147; **67**. J Microbiol Methods (1993) 17:1; **68**. Cytometry (1997) 29:58; **69**. Int J Parasitol (1996) 26:637; **70**. Microbiol Rev (1987) 51:365; **71**. J Microbiol Methods (1999) 37:77; **72**. Appl Environ Microbiol (1996) 62:2264; **73**. J Appl Microbiol (2004) 96:684; **74**. Int J Parasitol (1997) 27:787; **75**. Appl Environ Microbiol (1999) 65:4475; **76**. Biochim Biophys Acta (2001) 1514:165; **77**. Appl Environ Microbiol (1980) 39:423; **78**. J Appl Bacteriol (1992) 72:410; **79**. Lett Appl Microbiol (1991) 13:58; **80**. J Microbiol Methods (1991) 13:87; **81**. Current Microbiol (1980) 4:321; **82**. J Microbiol Methods (1997) 29:161; **83**. Cytometry (2003) 54A:27; **84**. Appl Environ Microbiol (2003) 69:2857; **85**. Biodegradation (2002) 13:285; **86**. J Microbiol Methods (2003) 52:75; **87**. Int J Food Microbiol (2004) 92:327; **88**. Antimicrob Agents Chemother (2008) 52:1446; **89**. Appl Environ Microbiol (2003) 69:7462; **90**. J Microbiol Methods (2003) 52:47; **91**. Antimicrob Agents Chemother (2000) 44:827; **92**. Cytometry (1999) 35:55; **93**. Appl Environ Microbiol (1998) 64:2681; **94**. Appl Environ Microbiol (1998) 64:515; **95**. Invest Ophthalmol Vis Sci (1986) 27:500; **96**. Int J Parasitol (1990) 20:1099; **97**. Biochemistry (2009) 48:7646; **98**. Biochem Biophys Res Commun (2008) 371:356; **99**. Science (2008) 321:1343; **100**. J Biolumin Chemilumin (1995) 10:29; **101**. Am J Physiol (1998) 275:C1391; **102**. J Biolumin Chemilumin (1996) 11:149; **103**. Neuroscience (1995) 66:915; **104**. FEBS Lett (1985) 185:323; **105**. Anal Biochem (1988) 175:14; **106**. Bio/Technology (1988) 6:634; **107**. J Clin Microbiol (1984) 20:644; **108**. J Clin Microbiol (1983) 18:521; **109**. Methods Enzymol (1978) 57:3; **110**. Biotechnol Bioeng (1993) 42:30; **111**. J Biolumin Chemilumin (1991) 6:193; **112**. Biochem J (1993) 295:165; **113**. J Immunol Methods (1993) 160:81; **114**. J Natl Cancer Inst (1986) 77:1039.

PRODUCT LIST 15.3 VIABILITY AND CYTOTOXICITY ASSAY KITS FOR DIVERSE CELL TYPES

Cat. No.	Product	Quantity
A10346	ArC™ Amine Reactive Compensation Bead Kit *for use with amine reactive dyes* *for flow cytometry compensation* *100 tests*	1 kit
A22066	ATP Determination Kit *special packaging* *200–1000 assays*	1 kit
B34950	*Bac*Light™ Bacterial Membrane Potential Kit *for flow cytometry* *100 assays*	1 kit
B34956	*Bac*Light™ RedoxSensor™ CTC Vitality Kit *for flow cytometry and microscopy*	1 kit
B34954	*Bac*Light™ RedoxSensor™ Green Vitality Kit *for flow cytometry*	1 kit
E22064	EnzChek® Reverse Transcriptase Assay Kit *1000 assays*	1 kit
L10316	FilmTracer™ LIVE/DEAD® Biofilm Viability Kit	1 kit
F34953	*Funga*Light™ CFDA, AM/propidium iodide Yeast Vitality Kit *for flow cytometry*	1 kit
L7005	LIVE *Bac*Light™ Bacterial Gram Stain Kit *for microscopy and quantitative assays* *1000 assays*	1 kit
L13152	LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit *10 applicator sets* *500 assays*	1 kit
L7007	LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit *for microscopy* *1000 assays*	1 kit
L7012	LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit *for microscopy and quantitative assays* *1000 assays*	1 kit
L34856	LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit *for flow cytometry* *100 assays*	1 kit
L34951	LIVE/DEAD® Cell Vitality Assay Kit *C_{12}-resazurin/SYTOX® Green* *1000 assays*	1 kit
L7010	LIVE/DEAD® Cell-Mediated Cytotoxicity Kit *for animal cells* *2000 assays*	1 kit
L34957	LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit *for 405 nm excitation* *200 assays*	1 kit
L23105	LIVE/DEAD® Fixable Blue Dead Cell Stain Kit *for UV excitation* *200 assays*	1 kit
L34960	LIVE/DEAD® Fixable Dead Cell Stain Sampler Kit *for flow cytometry* *320 assays*	1 kit
L10120	LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit *for 633 or 635 nm excitation* *200 assays*	1 kit
L23101	LIVE/DEAD® Fixable Green Dead Cell Stain Kit *for 488 nm excitation* *200 assays*	1 kit
L10119	LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit *for 633 or 635 nm excitation* *200 assays*	1 kit
L23102	LIVE/DEAD® Fixable Red Dead Cell Stain Kit *for 488 nm excitation* *200 assays*	1 kit
L34955	LIVE/DEAD® Fixable Violet Dead Cell Stain Kit *for 405 nm excitation* *200 assays*	1 kit
L34959	LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit *for 405 nm excitation* *200 assays*	1 kit
L34952	LIVE/DEAD® *Funga*Light™ Yeast Viability Kit *for flow cytometry*	1 kit
L7013	LIVE/DEAD® Reduced Biohazard Cell Viability Kit #1 *green and red fluorescence* *100 assays*	1 kit
L7011	LIVE/DEAD® Sperm Viability Kit *200–1000 assays*	1 kit
L3224	LIVE/DEAD® Viability/Cytotoxicity Kit *for mammalian cells*	1 kit
L34958	LIVE/DEAD® Violet Viability/Vitality Kit *for 405 nm excitation* *200 assays*	1 kit
L7009	LIVE/DEAD® Yeast Viability Kit *1000 assays*	1 kit
V7023	ViaGram™ Red+ Bacterial Gram Stain and Viability Kit *200 assays*	1 kit
V23110	Vybrant® Cell Metabolic Assay Kit *with C_{12}-resazurin* *500–1000 assays*	1 kit
V23111	Vybrant® Cytotoxicity Assay Kit *G6PD release assay* *1000 assays*	1 kit
W7024	Wheat Germ Agglutinin Sampler Kit *four fluorescent conjugates, 1 mg each*	1 kit

15.4 Assays for Cell Enumeration, Cell Proliferation and Cell Cycle

Cell proliferation and the characterization of agents that either promote or retard cell proliferation are extremely important areas of cell biology and drug-discovery research. We offer both traditional reagents for assessing cell proliferation and cell cycle—in particular the Hoechst nucleic acid stains and probes for 5-bromo-2'-deoxyuridine (BrdU) incorporation during cell division—as well as some exceptional tools developed in our laboratories, including the Click-iT® EdU cell proliferation assay. For simply detecting the presence or counting the number of cells, fluorescent stains that identify cells by their characteristic morphology or light-scattering properties may be sufficient.

Cell Enumeration and Cell Proliferation Assays for Animal Cells

Reagents for counting cells and quantitating cell proliferation are valuable research tools. Most cell proliferation assays estimate the number of cells either by incorporating a modified nucleotide into cells during proliferation or by measuring total nucleic acid or protein content of lysed cells.[1,2] Several of our nucleic acid stains (Section 8.1) and nucleotides (Section 8.2) have proven useful in nucleic acid labeling protocols. Here we describe our Click-iT® EdU cell proliferation assay, which provides a superior alternative to bromodeoxyuridine (BrdU) or ^{3}H-thymidine incorporation methods for measuring new DNA synthesis. Alternatively, our CyQUANT® Cell Proliferation Assay Kits use the CyQUANT® GR, CyQUANT® NF or CyQUANT® Direct nucleic acid stains to measure increases in nucleic acid content that accompany cell proliferation

Click-iT® Tools for Cell Proliferation Analysis

Click-iT® labeling technology employs a bioorthogonal reactive chemistry for the *in situ* labeling of specific molecular populations, such as newly synthesized nucleic acids, in an experimental time window of interest. The Click-iT® labeling reaction is based on a copper-catalyzed azide–alkyne cycloaddition[3,4] and derives its high degree of specificity from the fact that the azide and alkyne reaction partners have no endogenous representation in biological molecules, cells, tissues or model organisms.[5–7] Click-iT® labeling technology and the details of the click reaction are discussed in Section 3.1. For a complete list of azide and alkyne derivatives compatible with Click-iT® labeling technology, see Table 3.1. Here we highlight the Click-iT® EdU cell proliferation assay.

The Click-iT® EdU cell proliferation assay provides a superior alternative to bromodeoxyuridine (BrdU) or ^{3}H-thymidine incorporation methods for measuring new DNA synthesis.[8,9] The alkynyl nucleoside analog EdU (5-ethynyl-2'-deoxyuridine; A10044, E10187, E10415) is incorporated into DNA during the synthesis phase (S phase) of the cell cycle and is subsequently detected by copper (I)–catalyzed click coupling to an azide-derivatized fluorophore[10] (Figure 15.4.1). The small size of the click-coupled fluorophore compared to that of antibodies required for immunodetection of BrdU enables efficient penetration of complex samples without the need for harsh cell treatment, simplifying the assay considerably. The Click-iT® EdU assay protocol is compatible with both adherent cells and cell suspensions. From start to finish, the EdU detection assay is complete in as little as 90 minutes, as compared with the antibody-based BrdU method, which takes 6–24 hours to complete. In addition, the Click-iT® EdU cell proliferation assay can be multiplexed with surface and intracellular marker detection using Alexa Fluor® dye–labeled secondary antibodies[11–14] (Section 7.2) (Figure 15.4.2).

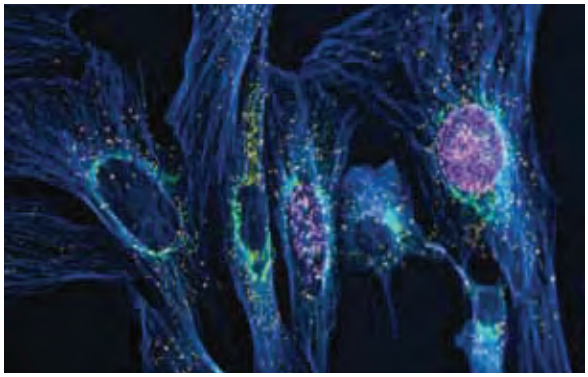

Figure 15.4.2 Multicolor imaging with the Click-iT® EdU Imaging Kits. Muntjac cells were treated with 10 µM EdU for 45 minutes. Cells were then fixed and permeabilized, and EdU that had been incorporated into newly synthesized DNA was detected by the far-red–fluorescent Click-iT® EdU Alexa Fluor® 647 HCS Assay (C10356, C10357). Tubulin was labeled with an anti-tubulin antibody and visualized with an Alexa Fluor® 350 goat anti–mouse IgG antibody (A21049). The Golgi complex was stained with the green-fluorescent Alexa Fluor® 488 conjugate of lectin HPA from *Helix pomatia* (edible snail) (L11271), and peroxisomes were labeled with an anti-peroxisome antibody and visualized with an orange-fluorescent Alexa Fluor® 555 donkey anti–rabbit IgG antibody (A31572).

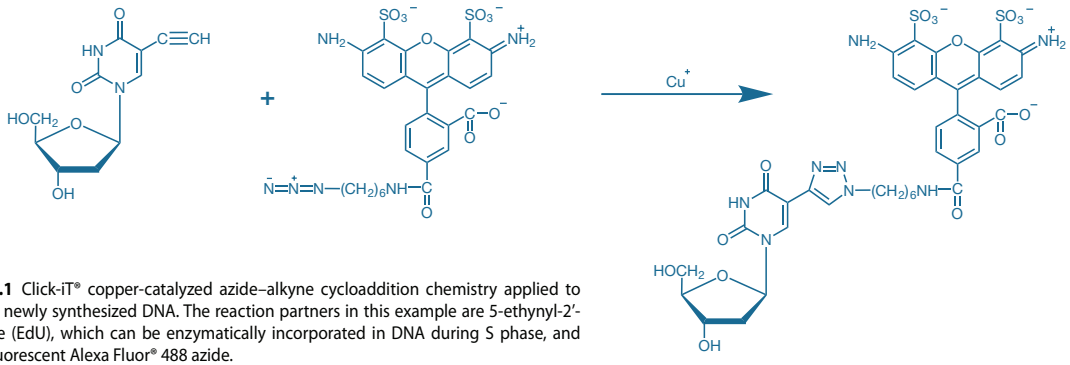

Figure 15.4.1 Click-iT® copper-catalyzed azide–alkyne cycloaddition chemistry applied to detection of newly synthesized DNA. The reaction partners in this example are 5-ethynyl-2'-deoxyuridine (EdU), which can be enzymatically incorporated in DNA during S phase, and the green-fluorescent Alexa Fluor® 488 azide.

Although the majority of applications are in cultured mammalian cells, Click-iT® EdU reagents and methods have also been successfully applied to a wide range of model organisms including:

- *Escherichia coli* [15]
- *Caenorhabditis elegans* [12]
- *Drosophila* [16]
- Zebrafish [17]
- Mouse [13]
- Plants [18,19] (alfalfa, *Arabidopsis*, grape, maize, rice and tobacco)

The Click-iT® EdU Flow Cytometry Assay Kits provide all the reagents needed to perform 50 assays using 0.5 mL reaction buffer per assay, including the nucleoside analog EdU and all components for fixation, permeabilization and labeling whole blood samples, adherent cells or suspension cells.[9,20] Additionally, two cell-cycle stains compatible with the fluorescence excitation and emission characteristics of the fluorescent azide detection reagents are included. We offer three Click-iT® EdU Flow Cytometry Assay Kits:

- Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (C35002)
- Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (A10202)
- Click-iT® EdU Pacific Blue™ Flow Cytometry Assay Kit (A10034)

The Click-iT® EdU Imaging Kits contain all of the components needed to label and detect incorporated EdU on 50 coverslips using 0.5 mL reaction buffer per test, as well as the blue-fluorescent Hoechst 33342 nuclear stain for performing cell-cycle analysis on adherent cell samples. We offer four Click-iT® EdU Imaging Kits:

- Click-iT® EdU Alexa Fluor® 488 Imaging Kit (C10337)
- Click-iT® EdU Alexa Fluor® 555 Imaging Kit (C10338)
- Click-iT® EdU Alexa Fluor® 594 Imaging Kit (C10339)
- Click-iT® EdU Alexa Fluor® 647 Imaging Kit (C10340)

The Click-iT® EdU HCS Assay Kits contain all of the materials needed to label and detect incorporated EdU in adherent cells in 96-well microplates and 100 µL reaction buffer per assay. For cell registration or DNA profiling, these kits also include the blue-fluorescent HCS NuclearMask™ Blue stain (H10325, Section 12.5). We offer four Click-iT® EdU HCS Assay Kits:

- Click-iT® EdU Alexa Fluor® 488 HCS Assay Kit (2-plate size, C10350; 10-plate size, C10351)
- Click-iT® EdU Alexa Fluor® 555 HCS Assay Kit (2-plate size, C10352; 10-plate size, C10353)
- Click-iT® EdU Alexa Fluor® 594 HCS Assay Kit (2-plate size, C10354; 10-plate size, C10355)
- Click-iT® EdU Alexa Fluor® 647 HCS Assay Kit (2-plate size, C10356; 10-plate size, C10357; Figure 15.4.2)

In addition to these kits, our Click-iT® EdU Microplate Assay Kit (C10214) provides a simple and rapid workflow with fewer wash steps resulting in a substantial time-savings advantage over traditional BrdU colorimetric or fluorescent cell proliferation assays. This assay uses Oregon Green® 488 azide for click coupling to synthetically incorporated EdU. The signal is amplified using immunodetection of the Oregon Green® 488 fluorophore by a rabbit anti–Oregon Green® horseradish peroxidase (HRP) conjugate followed by fluorogenic or chromogenic detection with our Amplex® UltraRed HRP substrate. The Click-iT® EdU microplate assay has been successfully tested in HeLa, A549, U2OS and A541 cells with a variety of reagents that modulate DNA synthesis, including the DNA synthesis inhibitor aphidicolin and the mitotic inhibitor paclitaxel. The Click-iT® EdU Microplate Assay Kit contains sufficient reagents for performing 400 individual assays in a 96-well plate format.

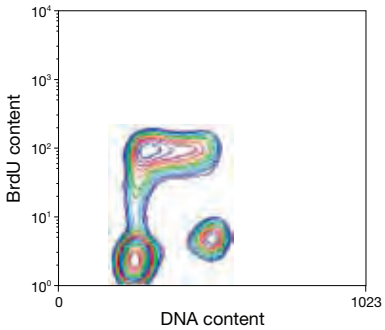

Figure 15.4.3 Detection of proliferation in Wil2S Lymphoma B cells. Cells were treated with 10 µM 5-bromo-2′-deoxyuridine (BrdU, B23151) in culture medium for 1 hour, then pelleted and fixed with cold 70% ethanol. After treatment with RNase and 4 M HCl (to denature the DNA), the cells were labeled with anti-BrdU antibody and detected using green-fluorescent Alexa Fluor® 488 goat anti–mouse IgG antibody (A11001). In addition, the cells were labeled with red-fluorescent propidium iodide (P1304MP, P3566, P21493) to assess the total cellular DNA content. The cells were analyzed by flow cytometry using 488 nm excitation; the fluorescent signals were collected at ~525 nm for the Alexa Fluor® 488 dye and at ~675 nm for propidium iodide. Increased BrdU incorporation is indicative of actively proliferating cells.

Proliferation Assays Using Bromodeoxyuridine Incorporation

Incorporation of 5-bromo-2′-deoxyuridine (BrdU, B23151) into newly synthesized DNA permits indirect detection of rapidly proliferating cells with fluorescently labeled anti-BrdU antibodies or certain nucleic acid stains, thereby facilitating the identification of cells that have progressed through the S phase of the cell cycle during the BrdU labeling period.[2,21,22] We offer fluorescent conjugates of the mouse monoclonal anti-BrdU antibody clone MoBU-1 labeled with our brightest and most photostable dyes:

- Alexa Fluor® 488 anti-BrdU antibody (B35130, B35139)
- Alexa Fluor® 555 anti-BrdU antibody (B35131)
- Alexa Fluor® 594 anti-BrdU antibody (B35132)
- Alexa Fluor® 647 anti-BrdU antibody (B35133, B35140)
- Pacific Blue™ anti-BrdU antibody (B35129)

This anti-BrdU antibody is also available biotinylated (B35138), as well as unlabeled (B35128, B35141). The unlabeled mouse anti-BrdU can be detected with our anti-mouse secondary antibodies (Table 7.1) using either flow cytometry (Figure 15.4.3) or imaging.

Because fluorescence of the Hoechst 33258 (H1398, H3569, H21491) and Hoechst 33342 (H1399, H3570, H21492) dyes bound to DNA is *quenched* at sites where BrdU is incorporated, Hoechst dye fluorescence can also be used to detect BrdU incorporation in single cells.[1,23–26] This technique has been employed to quantitate the noncycling cell fraction, as well as the fraction of cells that are in G_1 and G_2 of two subsequent cycles.[27] The addition of ethidium bromide as a counterstain that is *insensitive* to BrdU incorporation allows the resolution of G_1, S and G_2 compartments of up to three consecutive cell cycles.[28,29]

Unlike the fluorescence of Hoechst dyes, the fluorescence of TO-PRO®-3 (T3605) and LDS 751 (L7595) is considerably *enhanced* by the presence of bromodeoxyuridine in DNA. In conjunction with propidium iodide (P1304MP, P3566, P21493; Section 8.1), these nucleic acid stains have been used to discriminate BrdU-labeled cells from nonproliferating cells by flow cytometry [30,31] and with an imaging system for automated cell proliferation.[32]

Proliferation Assays Using ChromaTide® Nucleotides

In the strand break induction by photolysis (SBIP) technique, proliferating cells that have incorporated BrdU into newly synthesized DNA are subjected to Hoechst 33258 staining, followed by UV photolysis to induce DNA strand breaks [33] (Figure 15.4.4). Once the cells are fixed, strand breaks can be detected *in situ* using mammalian terminal deoxynucleotidyl transferase (TdT), which covalently adds labeled nucleotides to the 3'-hydroxyl ends of these DNA fragments.[34–37] Break sites have traditionally been labeled with biotinylated or haptenylated dUTP conjugates (Section 8.2) in conjunction with antibodies to the hapten (Section 7.4) or conjugates of streptavidin [37,38] (Section 7.6). However, a single-step procedure has been described that uses our ChromaTide® BODIPY® FL-14-dUTP (C7614, Figure 15.4.5) as a TdT substrate for directly detecting DNA strand breaks both in BrdU-labeled cells following SBIP and in apoptotic cells [33,39–42] (Section 15.5; Figure 15.4.6, Figure 15.4.7).

The single-step BODIPY® FL dye–based assay has several advantages over indirect detection of biotinylated or haptenylated nucleotides. With direct detection procedures, no secondary detection reagents are required; fewer protocol steps translate into less chance for error and more immediate results. Moreover, the yield of cells with direct detection procedures is reported to be about three times greater than that of multistep procedures employing biotin- or digoxigenin-conjugated dUTP. Although both BODIPY® FL dye– and fluorescein-labeled nucleotides can be detected with fluorescence microscopy or flow cytometry, the BODIPY® FL dye–labeled nucleotides provide ~40% stronger signal than fluorescein-labeled nucleotides when assaying strand breaks in apoptotic versus nonapoptotic cells. In addition, fading of the fluorescence of the incorporated BODIPY® FL dUTP is less than that of the corresponding fluorescein dUTP analog.[39] Unlike traditional proliferation assays based on BrdU incorporation, no DNA

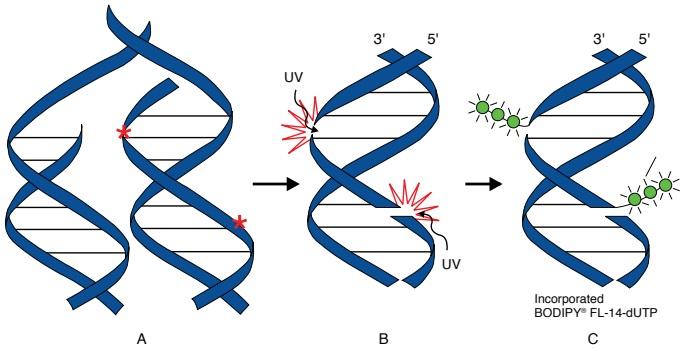

Figure 15.4.4 Schematic diagram showing the sequence of events in the strand break induction by photolysis (SBIP) technique. **A**) Proliferating cells that have incorporated BrdU (*) into newly synthesized DNA are **B**) exposed to UV light in order to induce DNA strand breaks. If the cells are stained with Hoechst 33258 prior to UV illumination, the photolysis efficiency is increased. **C**) Once the cells are fixed, the 3'-hydroxyl ends exposed at these strand breaks can be directly labeled *in situ* using mammalian terminal deoxynucleotidyl transferase (TdT) and our ChromaTide® BODIPY® FL-14-dUTP (C7614).

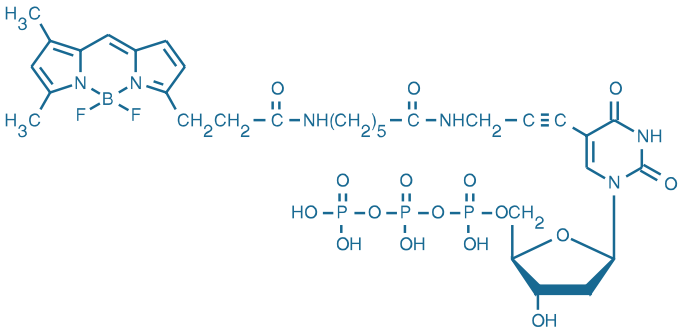

Figure 15.4.5 ChromaTide® BODIPY® FL-14-dUTP (C7614).

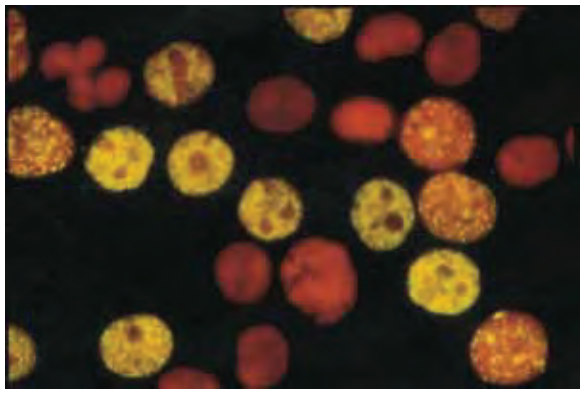

Figure 15.4.6 Detection of BrdU-incorporating cells using the SBIP (Strand Breaks Induced by Photolysis) technique. Exponentially growing human promyelocytic leukemia (HL60) cells were incubated with 20 µM BrdU (B23151) for 40 minutes and then with 20 µg/mL Hoechst 33258 (H1398, H3569, H21491) in the presence of 2% DMSO for an additional 20 minutes. After this incubation, the cells were exposed to 300 nm UV light for 5 minutes to selectively photolyze DNA that contained the incorporated BrdU, and then fixed in 70% ethanol. Subsequent incubation of the permeabilized cells with the ChromaTide® BODIPY® FL-14-dUTP (C7614) in the presence of exogenous terminal deoxynucleotidyl transferase resulted in incorporation of the fluorophore into DNA strand breaks, thereby labeling the S-phase cells. The DNA was counterstained with the red-fluorescent nucleic acid stain propidium iodide (P1304MP, P3566, P21493); therefore, the BODIPY® FL dye–labeled DNA appears yellow. The image was contributed by Zbigniew Darzynkiewicz, Cancer Research Institute, New York Medical College.

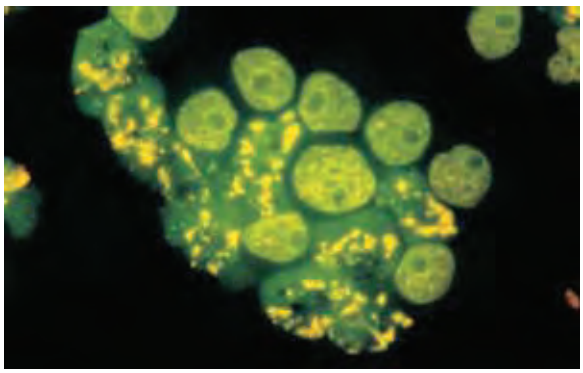

Figure 15.4.7 HL-60 cells treated with camptothecin for 3 hours. The DNA strand nicks characteristic of apoptosis were detected with the TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling) assay using the fluorescently labeled nucleotide ChromaTide® BODIPY® FL-14-dUTP (C7614). Image contributed by Zbigniew Darzynkiewicz, Cancer Research Institute, New York Medical College.

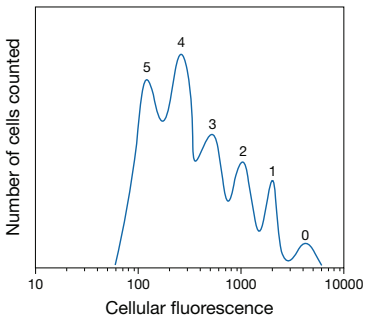

Figure 15.4.8 Tracking of asynchronous cell division using 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA SE or CFSE; C1157; V12883) labeling and flow cytometry. Cell division results in sequential halving of the initial fluorescence, resulting in a cellular fluorescence histogram. The peaks labeled 0, 1, 2, 3, 4 and 5 represent successive generations.

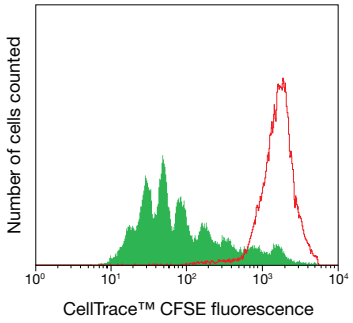

Figure 15.4.9 Following cell proliferation in human peripheral blood lymphocytes using the CellTrace™ CFSE Cell Proliferation Kit (C34554). Human peripheral blood lymphocytes were harvested and stained with CellTrace™ CFSE (carboxyfluorescein diacetate, succinimidyl ester; 5(6)-CFDA, SE) on day 0. A portion of the population was arrested at the parent generation using mitomycin C (red peak). The remainder of the sample was stimulated with phytohemagglutinin and allowed to proliferate for 5 days. Solid green peaks represent successive generations.

heat- or acid-denaturation steps are required with SBIP in order to visualize the labeled strand breaks, allowing simultaneous detection of the morphology of nuclear proteins and other cellular constituents by immunocytochemical analysis. The narrow emission spectrum of the BODIPY® FL dye–labeled nucleotides is especially useful for multicolor labeling experiments.

An elegant technique has been described that permits tracking of labeled chromosomes through mitosis by metabolic incorporation of microinjected fluorescent nucleotides, including our fluorescein-12-dUTP (C7604, Section 8.2), into DNA using endogenous cellular enzymes.[43–45] The procedure does not interfere with subsequent progress through the cell cycle, and fluorescent strands of DNA can be followed as they assemble into chromosomes and segregate into daughters and granddaughters.[43–45] Presumably, injection of 5'-bromo-2'-deoxyuridine triphosphate (BrdUTP, B21550), followed by detection of the incorporated BrdU with one of our Alexa Fluor® conjugates of anti-BrdU would also be suitable for studying mitosis. The corresponding ribonucleotide (BrUTP, B21551) that has been microinjected into cells is incorporated into RNA of a nucleolar compartment,[46,47] a process that should also be detectable with fluorescent anti-BrdU conjugates.

Proliferation Assay Using the Succinimidyl Ester of Carboxyfluorescein Diacetate and Related Probes

The succinimidyl ester of carboxyfluorescein diacetate (5(6)-CFDA, SE or CFSE, C1157) is currently the most widely used probe for generation analysis of cells, although our succinimidyl ester of Oregon Green® 488 carboxylic acid diacetate (O34550, C34555; see below) offers several important advantages over this fluorescein derivative. CFDA SE spontaneously and irreversibly couples to both intracellular and cell-surface proteins by reaction with lysine side chains and other available amine groups. When cells divide, CFDA SE labeling is distributed equally between the daughter cells, which are therefore half as fluorescent as the parents. As a result, each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity (excitation/emission maxima ~495/525 nm) that is readily detected by a flow cytometer (Figure 15.4.8), fluorescence microscope or fluorescence microplate reader. CFDA SE is available as a single vial containing 25 mg (C1157). CFDA SE is also available conveniently packaged for cell tracing applications in our Vybrant® CFDA SE Cell Tracer Kit (V12883, Figure 15.4.8) and for cell proliferation studies in our CellTrace™ CFSE Cell Proliferation Kit (C34554, Figure 15.4.9). The fluorescent CFDA SE product has excitation/emission maxima of ~492/517 nm and can be detected using a fluorescence microscope, flow cytometer or fluorescence microplate reader. Each kit includes 10 single-use vials of CFDA SE (500 µg each in Kit V12883, 50 µg each in Kit C34554), as well as high-quality anhydrous DMSO and a complete protocol.

CFDA SE produces more homogeneous cellular labeling and, consequently, much better intergenerational resolution than other cell-tracking dyes, such as the membrane marker PKH26. Using flow cytometric analysis of CFDA SE labeling, researchers can resolve 8 to 10 successive generations of lymphocytes.[48,49] In transplanted cells the signal of CFDA SE can be traced *in vivo* for weeks.[50,51] The feasibility of using cell-permeant fluorescent tracers to follow cell division of natural killer (NK) cells, B cells, T cells, thymocytes, lymphocytes, fibroblasts and hematopoietic cells has been demonstrated with CFDA SE.[49,52–62] For instance, researchers have used CFDA SE labeling to show that transplantable hematopoietic cells proliferate *in vitro* in response to stimulation by a growth factor cocktail.[63] These studies helped provide direct evidence that the hematopoietic potential of cultured stem cells is limited by homing activity and not by proliferative capacity. Because the first division results in the largest change in fluorescence intensity, this method is particularly useful for detecting subsets of cells within a population that are resistant to cell division. The method is not limited to mammalian cells; it has also been applied to determine the number of cell divisions in stained *Lactobacillus plantarum*.[64]

Like CFDA SE, the succinimidyl ester of Oregon Green® 488 carboxylic acid diacetate (carboxy-DFFDA SE) should be a useful tool for following proliferating cells. This Oregon Green® 488 probe passively diffuses into cells, where it is colorless and nonfluorescent until its acetate groups are removed by intracellular esterases to yield a highly green-fluorescent, amine-reactive dye. Upon reaction with intracellular amines, the probe forms Oregon Green® 488 conjugates that are well-retained by cells. Unlike fluorescein derivatives, however, Oregon Green® 488

derivatives exhibit bright green fluorescence that is not pH dependent at typical cellular pH values. Moreover, Oregon Green® 488 probes are usually brighter and more photostable than fluorescein probes. We offer carboxy-DFFDA SE in a 1 mg unit size (O34550) and specially packaged in a set of 20 vials, each containing 50 µg (CellTrace™ Oregon Green® 488 carboxylic acid diacetate succinimidyl ester, C34555).

The intracellular conjugates of 5-(and 6-)carboxyeosin diacetate succinimidyl ester (C22803) have absorption and emission spectra at longer wavelengths than CFDA SE, which may make this probe useful in combination with CFDA SE for studies of proliferation of mixed-cell populations. Eosin conjugates are more effective singlet-oxygen generators than are simple fluorescein derivatives, potentially resulting in their utility for photoablation of cells.

The succinimidyl ester of SNARF®-1 carboxylic acid, acetate (S22801) is also designed to serve as a cell tracer and indicator of cell division. However, unlike the green-fluorescent CFDA SE–labeled cells, cells labeled with the succinimidyl ester of SNARF®-1 carboxylic acid, acetate exhibit red fluorescence when excited near 488 nm. Although the fluorescence intensity of this SNARF® derivative in cells may be weaker than that of cells labeled with CFDA SE, its red fluorescence is easily distinguished from the green fluorescence of CFDA SE–labeled cells. The SNARF® dyes have been predominantly used as indicators of intracellular pH (Chapter 20).

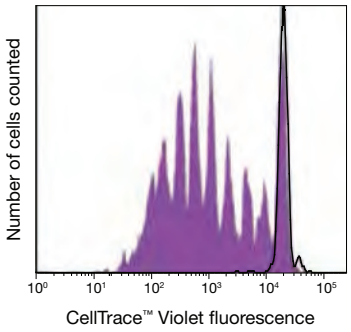

Figure 15.4.10 Human peripheral blood lymphocytes were harvested and stained with CellTrace™ Violet stain (C34557). The violet peaks represent successive generations of cells stimulated with mouse anti–human CD3 and Interleukin-2 and grown in culture for 7 days. The peak outlined in black represents cells that were grown in culture for 7 days with no stimulus.

CellTrace™ Violet Cell Proliferation Kit

CellTrace™ Violet stain is an esterase-activated phenolic fluorophore with a succinimidyl ester substituent for coupling to cell surface and intracellular amines. It is functionally analogous to CFSE, equally partitioning between daughter cells during division resulting in successive 2-fold reductions in cell-associated fluorescence intensity. When analyzed by flow cytometry, this progressive label partitioning provides a direct indication of cell proliferation status (Figure 15.4.10). In contrast to CFSE, CellTrace™ Violet stain is optimally excited by 405 nm violet diode lasers and generates blue fluorescence (emission peak ~455 nm). Consequently, it can be used in combination with CFSE to track cells from different origins after mixing or to analyze proliferation of GFP-expressing cells. The CellTrace™ Violet Cell Proliferation Kit (C34557) includes the CellTrace™ Violet stain together with dimethylsulfoxide (DMSO) for preparation of a stock solution.

CyQUANT® Cell Proliferation Assay Kit

Because cellular DNA content is highly regulated, it is closely proportional to cell number. Therefore, changes in nucleic acid content can serve as a sensitive indicator of overall cell proliferation, as well as of cytotoxic events or pathological abnormalities that affect cell proliferation. Our CyQUANT® Cell Proliferation Assay Kit (C7026) provides an excellent method both for enumerating cells in a population and for measuring their proliferative activity. This assay is an important development for the rapid and quantitative screening of agents that affect cell proliferation. The CyQUANT® assay is based on the use of our green-fluorescent CyQUANT® GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids.[65] The assay protocol is simple: the culture medium is removed (nonadherent cells require brief centrifugation); the cells are frozen, thawed and lysed by addition of the CyQUANT® cell buffer containing detergent and the CyQUANT® GR dye; and fluorescence is then measured directly in a fluorometer or fluorescence microplate reader (Figure 15.4.11). No washing steps, growth medium changes or long incubations are required.

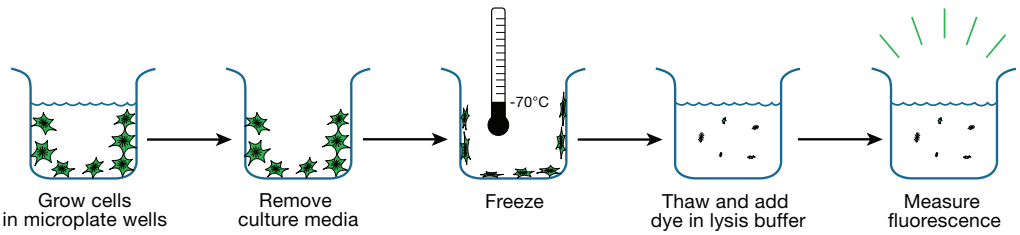

Figure 15.4.11 The simple procedure for using the CyQUANT® Cell Proliferation Assay Kit (C7026).

The CyQUANT® cell proliferation assay has a number of significant advantages over other proliferation assays:

- **Sensitivity and linearity.** The CyQUANT® assay is linear from 50 or fewer cells to at least 50,000 cells in 200 µL volumes (Figure 15.4.12); increasing the dye concentration extends the linear range to at least 250,000 cells. Methods that employ Hoechst 33258 [66] (H1398, H3569, H21491) or Hoechst 33342 [67] (H1399, H3570, H21492) to measure cell number and proliferation are much less sensitive—detection limits of 500 cells for Hoechst 33258 [66] or 2500 cells for Hoechst 33342 [67]—and have much smaller effective ranges.
- **No radioactivity.** Unlike assays that measure ^{3}H-thymidine incorporation, the CyQUANT® assay does not require radioisotopes and thus does not have the hazards or the expense associated with use, storage and disposal of radioisotopes.
- **Quick and easy protocol.** The CyQUANT® assay is a single-step procedure that requires no lengthy incubation steps and can be completed within an hour (Figure 15.4.11).
- **Specificity and reliability.** The assay is specific for total nucleic acids, with essentially no interference from other cell components. No wash steps are required because cellular growth media do not significantly interfere with CyQUANT® GR fluorescence. The CyQUANT® assay is reliable for cell quantitation, even without treatment to eliminate cellular RNA. However, addition of RNase or DNase permits the easy quantitation of DNA or RNA, respectively, in the sample.
- **Convenience.** Unlike assays that use tetrazolium salts, ^{3}H-thymidine, BrdU, neutral red or methylene blue, [67–70] the CyQUANT® procedure is not dependent on cellular metabolism. Thus, cells can be frozen and stored prior to assaying, with no reduction in signal, or they can be assayed immediately after collection. Time-course assays are simplified because data obtained from stored samples taken at widely different time intervals can be assayed together with a single standard curve determination.

We have found the CyQUANT® Cell Proliferation Assay Kit to be useful for assaying widely disparate cell types, including:

- Human neonatal fibroblasts, keratinocytes, melanocytes, umbilical vein endothelial cells (HUVEC) and dermal microvascular endothelial cells (DMVEC)

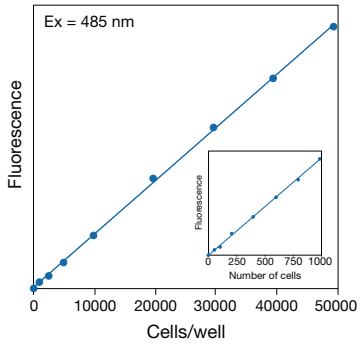

Figure 15.4.12 Quantitation of NIH 3T3 fibroblasts using the CyQUANT® Cell Proliferation Assay Kit (C7026). Fluorescence measurements were made using a microplate reader with excitation at 485 nm and emission detection at 530 nm. The linear range of the assay under these conditions is from 50 to 50,000 cells per 200 µL sample. The inset shows the linearity that can be obtained at very low numbers of cells.

- Murine fibroblasts (NIH 3T3 and CRE BAG 2 cells) and myeloma (P3X63A68) cells
- Madin–Darby canine kidney (MDCK) cells
- Chinook salmon embryo (CHSE) cells
- Rat basophilic leukemia (RBL) and glioma (C6) cells

Determination of total cell number using the CyQUANT® GR reagent is potentially useful for quantitating cell adhesion (see "Cell Adhesion" in Section 15.6) and for determining the total number of cells in a tissue. Each CyQUANT® Cell Proliferation Assay Kit (C7026) includes:

- CyQUANT® GR reagent
- Cell-lysis buffer
- DNA standard for calibration
- Detailed protocols

The kit supplies sufficient materials for performing 1000 assays based on a 200 µL sample volume or a proportionately lower number of assays with a larger sample volume. The CyQUANT® cell-lysis buffer (a 20X concentrate, C7027) is also available separately and has been formulated to produce efficient lysis, to protect nucleic acids from nuclease activity and to dissociate proteins that may interfere with dye binding to nucleic acids. It may prove generally useful in the development of other assays that require cell lysis.

CyQUANT® NF Cell Proliferation Assay Kit

The CyQUANT® NF Cell Proliferation Assay Kit provides a fast and sensitive method for counting cells in a population and measuring proliferation in microplate format. [71] This assay can be completed in 1 hour, with no washes, cell lysis, long incubations or radioactivity required, and it is not dependent on physiological activities that may exhibit cell number–independent variability. The CyQUANT® NF assay eliminates the freeze-thaw cell lysis step of the original CyQUANT® cell proliferation assay by using a cell-permeant DNA-binding dye in combination with a plasma membrane–permeabilization reagent. The CyQUANT® NF assay protocol requires only aspiration of growth medium (for adherent cells), replacement with dye binding solution, incubation for 30–60 minutes and then measurement of fluorescence in a microplate reader. The CyQUANT® NF assay has a linear detection range from at least 100 to 20,000 cells per well in most cell lines using a 96-well microplate format and a 100 µL assay volume.

The CyQUANT® NF Cell Proliferation Assay Kit can be used with either a 96-well or 384-well microplate format and is available in two configurations: a 200-assay kit (C35007) and a 1000-assay kit (C35006) for high-throughput applications.

Each kit contains:

- CyQUANT® NF dye reagent
- Dye delivery reagent
- Concentrated Hank's balanced salt solution (HBSS)
- Detailed protocols

CyQUANT® Direct Cell Proliferation Assay Kit

CyQUANT® Direct Cell Proliferation Assay is a fluorescence-based proliferation and cytotoxicity assay for microplate readers. The no-wash, homogeneous format and fast add-mix-read protocol makes the CyQUANT® Direct assay ideal for high-throughput screening (HTS) applications. The assay can be completed in 1 hour, with no washes, cell

lysis, temperature equilibrations or radioactivity required, and the signal is stable for several hours to provide work-flow convenience. With a dynamic range from less than 50 to more than 20,000 cells of most adherent and suspension cell types, the CyQUANT® Direct assay can be used in 96-, 384- or 1,536-well microplate formats, and is compatible with most HTS and high-content screening (HCS) readers. Because the experimental protocol does not include a lysis step, the assay can conveniently be multiplexed using a spectrally distinct fluorescent or a luminescent readout.

The CyQUANT® Direct Cell Proliferation Assay Kit is available in two configurations: a 10-plate assay kit (C35011) and a 100-plate assay kit (C35012) for high-throughput applications. Each kit contains:

- CyQUANT® Direct nucleic acid stain
- CyQUANT® Direct background suppressor
- Detailed protocols

FluoReporter® Blue Fluorometric Nucleic Acid Assay Kit

The FluoReporter® Blue Fluorometric dsDNA Quantitation Kit (F2962) provides the protocols developed by Rago and colleagues [72] for analyzing cellular DNA with the blue-fluorescent Hoechst 33258 nucleic acid stain. The kit enables researchers to detect ~10 ng of isolated calf thymus DNA or ~1000 mouse NIH 3T3 cells in a 200 µL sample (substantially lower levels are detectable using our CyQUANT® Cell Proliferation Assay Kit described above).

With this kit, quantitation of cellular DNA is rapid, and all manipulations can be carried out in microplate wells. The cells are lysed by freezing them in distilled water, which circumvents the requirement for extraction procedures used in other Hoechst 33258 dye–based protocols.[69,73–76] The diluted dye solution is then added to the lysed cells, and fluorescence is measured. Kit components include:

- Hoechst 33258 in DMSO/H_2O
- TNE buffer
- Detailed protocol

Each kit provides sufficient reagents for assaying approximately 2000 samples using a fluorescence microplate reader.

Vybrant® MTT Cell Proliferation Assay Kit

The convenient Molecular Probes® Vybrant® MTT Cell Proliferation Assay Kit (V13154) simplifies the task of counting cells with a microplate absorbance reader. The colorimetric MTT assay, developed by Mossman, is based on the conversion of the water-soluble MTT to an insoluble purple formazan.[77,78] This formazan is then solubilized, and its concentration determined by optical density at 570 nm. The Vybrant® MTT Cell Proliferation Assay Kit provides a sensitive assay with excellent linearity up to approximately 10^6 cells per well. Each kit includes:

- MTT
- Sodium dodecyl sulfate (SDS)
- Detailed protocol

This kit provides sufficient materials for ~1000 assays using standard 96-well microplates. Numerous variations and modifications of the MTT assay have been published.[79–81] In addition to dehydrogenases, MTT is reduced by glutathione *S*-transferase [82] (GST). Therefore, MTT may not always be a reliable cell viability probe in cells treated with compounds that affect GST activity.

Vybrant® DiI Cell-Labeling Solution

Analysis by mass spectrometry and HPLC indicates that the dye we use in our Vybrant® DiI cell-labeling solution (V22885) is structurally identical to the dye called PKH 26. DiI is a red-fluorescent lipophilic tracer that, in addition to being extensively used for cell tracing (Section 14.4), has been utilized for generational analysis of cells undergoing division.[52,83–86] Unlike the PKH 26 dye, which requires a special cell-labeling medium and low ionic strength for successful cell loading, our Vybrant® DiI cell-labeling solution is simply added to cells in normal growth medium. Dividing cells distribute the lipophilic tracer approximately equally between daughter cells. It is usually possibly to follow at least three or four generations of cells by flow cytometry, although asynchronous division times can quickly complicate the measurements. The dyes in our Vybrant® DiO, Vybrant® DiD and Vybrant® CM-DiI Labeling solutions (V22886, V22887, V22888) may have similar utility for tracing cells through cell division. CM-DiI contains a thiol-reactive chloromethyl that allows the dye to covalently bind to cellular thiols. Thus, unlike other membrane stains, this label is well retained in some cells throughout fixation and permeabilization steps; see Section 14.4 for more information.

CountBright™ Absolute Counting Beads

Flow cytometry provides a rapid method for quantitating cell characteristics, however most flow cytometers cannot directly provide the cell concentration or absolute count of cells in a sample. Absolute cell counts have been widely used in quantitating cell populations and disease progression [87–89] and are generally obtained either by combining a separate cell concentration determination from a hematology analyzer with flow cytometry population data (multiple-platform testing) or by adding an internal microsphere counting standard to the flow cytometry sample (single-platform testing). The single-platform method is preferred as it is technically less complicated and more accurate than multiple-platform testing.[90]

CountBright™ absolute counting beads (C36950) are a calibrated suspension of microspheres that are brightly fluorescent across a wide range of excitation and emission wavelengths and contain a known concentration of microspheres. For absolute counts, a specific volume of the microsphere suspension is added to a specific volume of sample, such that the ratio of sample volume to microsphere volume is known. The volume of sample analyzed can be calculated from the number of microsphere events and then used with cell events to determine cell concentration. In general, at least 1000 bead events should be acquired to assure a statistically significant determination of sample volume. Sufficient reagents are provided for 100 flow cytometry assays, each using 50 µL of counting beads per test.

CountBright™ absolute counting beads are broadly fluorescent and can be used with either a fluorescence or scatter threshold. Fluorescence can be excited by wavelengths from UV to 635 nm; fluorescence emission can be read between 385 nm and 800 nm. The fluorescence intensity of the microspheres has been adjusted to be about 5–50 times brighter than the anticipated intensities of typically stained cells. When using a scatter threshold, the microsphere signal should be above the threshold. The microspheres can be gated by a single parameter, but a combination of parameters can be used to resolve microspheres from cells and other events.

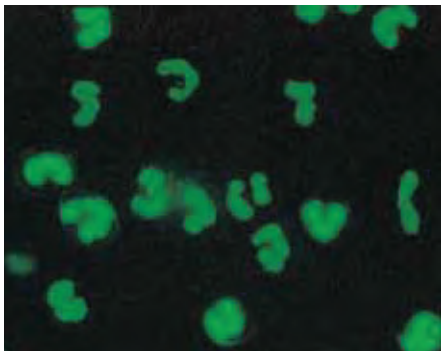

Figure 15.4.13 Adherent cells from human peripheral blood stained with the SYTO® 13 dye (S7575), one of the six visible light–excitable cell-permeant nucleic acid stains in our SYTO® Green Fluorescent Nucleic Acid Stain Sampler Kit #1 (S7572). The multilobed nuclei of these polymorphonuclear leukocytes are particularly striking in this field of view.

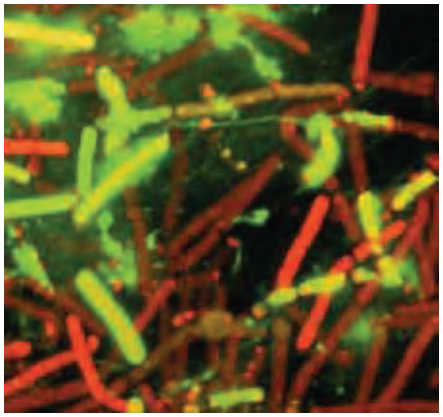

Figure 15.4.14 A mixture of live and heat-killed *Bacillus cereus* cells simultaneously stained with the cell-permeant SYTO® 59 red-fluorescent nucleic acid stain (S11341) and the cell-impermeant SYTOX® Green nucleic acid stain (S7020), each at a concentration of 5 µM. Bacteria with intact cell membranes stain fluorescent red, whereas bacteria with damaged membranes stain fluorescent green. This maximum-projection image was generated from a series of 10 images taken at 0.2 µm increments through the specimen with a Leica confocal laser-scanning microscope.

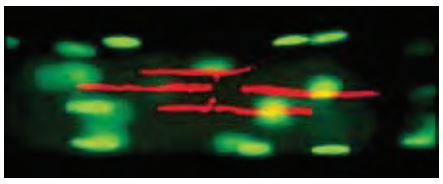

Figure 15.4.15 Pseudocolored photomicrograph of the synaptic region of fluorescently labeled living muscle fibers from the lumbricalis muscle of the adult frog *Rana pipiens*. Six hours after isolation of the muscle fibers, acetylcholine receptors were stained with red-fluorescent tetramethylrhodamine α-bungarotoxin (T1175) and myonuclei were stained with the green-fluorescent SYTO® 13 live-cell nucleic acid stain (S7575). Photo contributed by Christian Brösamle, Brain Research Institute, University of Zurich, and Damien Kuffler, Institute of Neurobiology, University of Puerto Rico.

CountBright™ absolute counting beads can be used with any sample type, including no-wash/lysed whole blood. The microspheres in the reagents are approximately 7 µm in diameter and have settling properties similar to lymphocytes. The accuracy of cell counts based on CountBright™ absolute counting beads depends on sample handling and the precise delivery of the volume of beads. The CountBright™ absolute counting beads must be mixed well to assure a uniform suspension of microspheres. After vortexing for 30 seconds, the microsphere suspension can be pipetted by standard techniques; however, more viscous solutions such as blood require reverse pipetting to facilitate accurate volume delivery. Cell suspensions may be diluted but should be assayed without wash steps. Other sample preparation steps that can lead to cell or microsphere loss should also be avoided. For antibody protocols, CountBright™ absolute counting beads should be used with reagents titered for no-wash staining.

Detection and Enumeration Assays for Microorganisms and Viruses

Detecting Bacteria, Yeast and Plankton Using Nucleic Acid Stains

We recommend our SYTO® nucleic acid stains (see the complete list in Table 8.3) for simple detection of the presence of bacteria, yeast and other microbial cells [91–95] (Figure 15.4.13, Figure 15.4.14, Figure 15.4.15). The SYTO® dyes are essentially nonfluorescent except when bound to nucleic acids, where they become highly fluorescent, often with quantum yields exceeding 0.5. Consequently, it is usually not necessary to remove unbound stains before analysis. SYTO® dyes are available with blue, green, orange or red fluorescence. The SYTO® dyes rapidly penetrate the membranes of almost all cells, including bacteria and yeast. The various cell types can often be identified by their characteristic morphology or, in the case of flow cytometric applications, by their light-scattering properties.

The SYTO® 11 and SYTO® 13 green-fluorescent nucleic acid stains show exceptional ability to penetrate tissues for at least 100 µm, including untreated, unfixed human brain tissue, where they were used to enumerate cells by confocal laser-scanning microscopy.[96] Simultaneous labeling with a green-fluorescent SYTO® dye and a red-fluorescent nucleic acid stain—most often propidium iodide, ethidium homodimer-1 or -2, TOTO®-3 or TO-PRO®-3 (Table 8.2, Table 8.4)—is frequently used to assess cell viability (Section 15.2). Although some of the SYTO® dyes show higher quantum yields on DNA or RNA, they should not be considered specific stains for either of these nucleic acids. We offer four different SYTO® Nucleic Acid Stain Sampler Kits (S11350, S7572, S11360, S11340; Section 8.1).

In addition to its use for cell-cycle analysis (see below) and nuclear staining, DAPI (D1306, D3571, D21490) is frequently employed for DNA content–based counting of bacterial cells [97–99] and for detecting malarial infections by fluorescence microscopy.[100]

*Bac*Light™ Bacterial Stains

The *Bac*Light™ Green and *Bac*Light™ Red bacterial stains (B35000, B35001) are fluorescent, non–nucleic acid labeling reagents for detecting and monitoring bacteria. Bacteria stained with the *Bac*Light™ Green and *Bac*Light™ Red bacterial stains exhibit bright green (excitation/emission maxima ~480/516 nm) and red (excitation/emission maxima ~480/516 nm) fluorescence, respectively, and can be resolved simultaneously using the appropriate flow cytometry channels. Although these dyes were specifically chosen for flow cytometry applications, bacteria stained with these *Bac*Light™ reagents can also be visualized by fluorescence microscopy with only minor, if any, adjustments in the staining concentrations. Furthermore, the *Bac*Light™ bacterial staining patterns are compatible with formaldehyde or alcohol fixation methods.

These *Bac*Light™ bacterial stains efficiently label a variety of different bacteria species. The intensity of the staining appears to depend on several factors, including gram character, outer membrane composition and overall membrane integrity. In the species we tested, gram-positive bacteria generally exhibited brighter fluorescence than gram-negative bacteria, and cells with compromised membranes accumulated more dye than intact cells (Figure 15.4.16).

Bacteria Counting Kit

Accurate enumeration of low numbers of bacteria in samples must be performed daily in many quality-control laboratories.

To facilitate this determination by flow cytometry (Figure 15.4.17), we have developed the Bacteria Counting Kit (B7277), which provides:

- Cell-permeant, green-fluorescent SYTO® BC nucleic acid stain to label bacteria
- Fluorescent polystyrene microspheres to calibrate the volume of bacterial suspension analyzed
- Detailed protocols

SYTO® BC dye, which is also available separately (S34855, Section 8.1), is a high-affinity nucleic acid stain that easily penetrates both gram-negative and gram-positive bacteria, producing an exceptionally bright green-fluorescent signal. The calibrated suspension of polystyrene microspheres contains beads that exhibit a uniform density, low-level fluorescence and optimal size to clearly separate the light scattering of the microspheres from that of most bacteria.

The Bacteria Counting Kit is particularly valuable for monitoring antibiotic sensitivity because it provides a convenient and accurate means for assessing changes in a bacterial population over time. A sample of the population is simply diluted, stained briefly with the SYTO® BC dye, mixed with a fixed number of microspheres and analyzed on a flow cytometer. Signals from both the stained bacteria and the beads are easily detected in the green-fluorescence channel of most

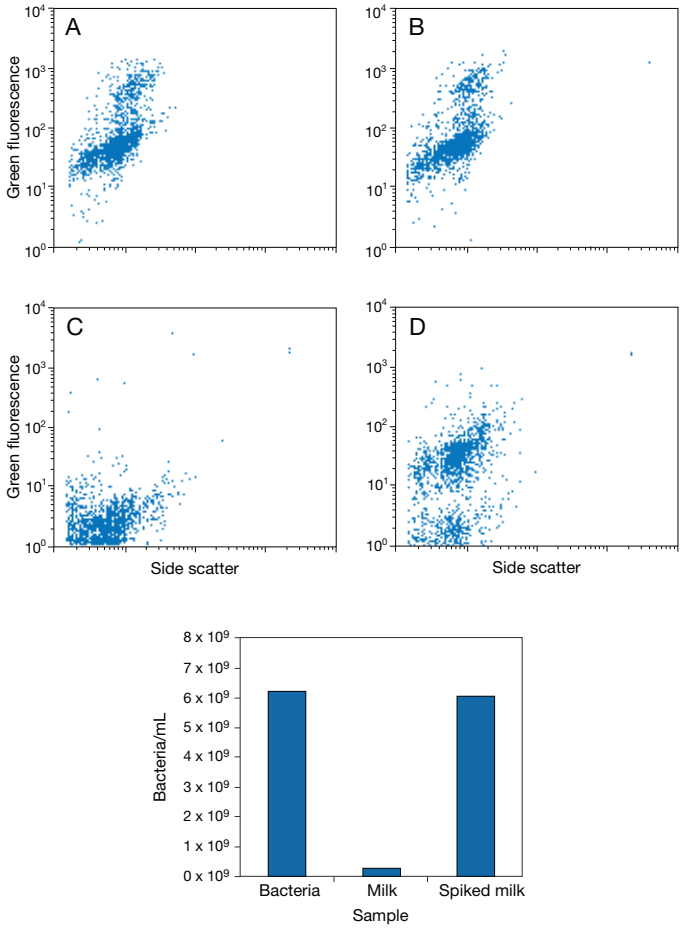

Figure 15.4.17 Detection and counting of bacteria in milk using the Bacteria Counting Kit (B7277). Equal numbers of bacteria were suspended in 150 mM NaCl or a mixture of milk and 150 mM NaCl and assayed using the protocol provided with the kit. As shown in the lower bar chart, the presence of milk does not affect the outcome of the assay. The upper panels plot green fluorescence versus side scatter for: **A**) bacteria alone, **B**) bacteria alone, **C**) milk alone and **D**) bacteria mixed with milk (spiked milk). Panels B–D also contain reference beads, which appear in the upper right corner of the respective plots.

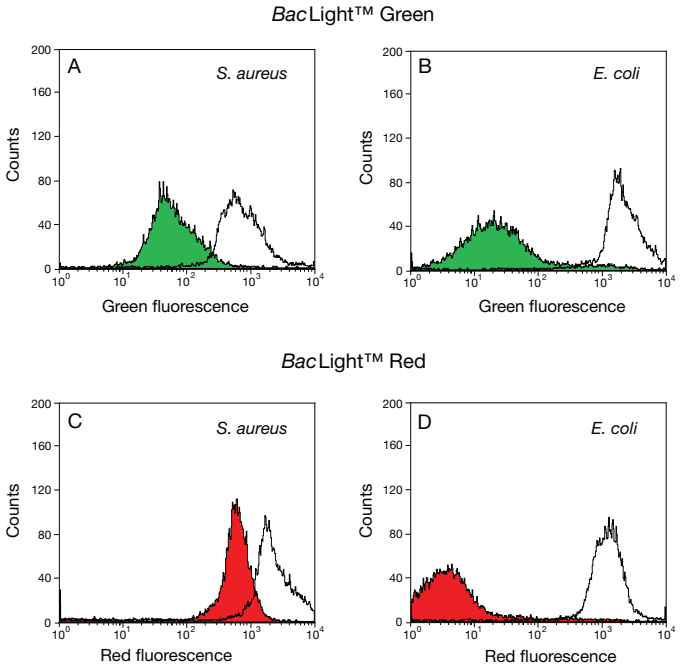

Figure 15.4.16 Flow cytometry histograms showing fluorescence of live and dead gram-positive and gram-negative bacteria stained with the *Bac*Light™ bacterial stains. Untreated and alcohol-treated gram-positive (*Staphylococcus aureus*, (**A** and **C**)) and gram-negative (*Escherichia coli*, (**B** and **D**)) bacteria were each stained separately with 100 nM of either the *Bac*Light™ Green (**A** and **B**) or the *Bac*Light™ Red (**C** and **D**) bacterial stains (B35000, B35001) in 0.85% NaCl buffer and then analyzed by flow cytometry. The histograms for the untreated (colored histogram curve) and alcohol-treated (uncolored histogram curve) bacteria samples were overlaid for each species and *Bac*Light™ bacterial stain.

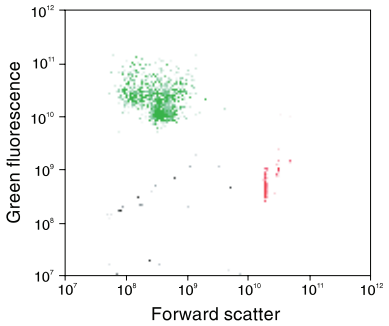

Figure 15.4.18 Flow cytometric enumeration of *Bacillus cereus* using the Bacteria Counting Kit (B7277). In this plot of forward scatter versus fluorescence, signals in the upper lefthand frame represent bacteria stained with SYTO® BC bacteria stain; signals in the lower right-hand frame represent microsphere particles, which serve as a standard used to indicate sample volume.

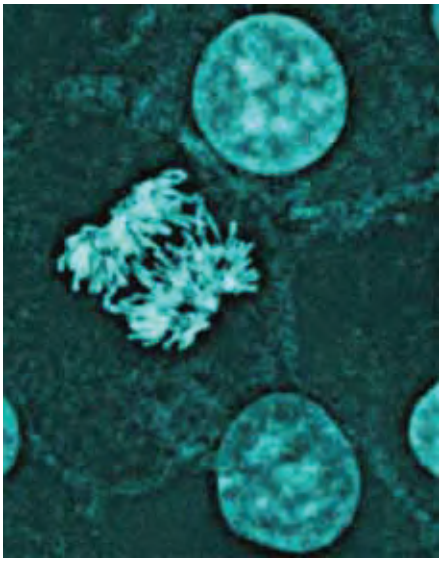

Figure 15.4.19 A mycoplasma-contaminated African green monkey fibroblast cell line was stained with cell-permeant, blue-fluorescent Hoechst 33342 nucleic acid stain (H1399, H3570, H21492), simultaneously illustrating both cell nuclei and mycoplasma. The image was acquired using filters appropriate for DAPI. Image contributed by Heiti Paves, Laboratory of Molecular Genetics, National Institute of Chemical Physics and Biophysics, Estonia.

flow cytometers and can be distinguished on a plot of forward scatter versus fluorescence (Figure 15.4.18). The density of the bacteria in the sample can be determined from the ratio of bacterial signals to microsphere signals in the cytogram. The Bacteria Counting Kit can be used with a variety of gram-negative and gram-positive species of bacteria and provides sufficient reagents for approximately 100 flow cytometry assays.

The fluorescent microspheres in our Bacteria Counting Kit have also been recommended for the enumeration of yeast.[101] We offer a wide selection of labeled beads (Section 6.5, Section 23.1, Section 23.2) that may also prove useful for yeast quantitation and viability assays by flow cytometry.[102]

Cell Culture Contamination Detection Kit

Molecular Probes® Cell Culture Contamination Detection Kit (C7028) uses a simple and effective procedure for visually screening cell cultures for contamination by yeast (and other fungi) or by gram-negative or gram-positive bacteria. This kit not only serves to detect the contaminants, but also identifies the contaminant type, enabling the researcher to choose an appropriate course of action.

A sample of the suspected culture is subjected to two slide-staining protocols. One sample slide is stained with Calcofluor White M2R, a UV light–excitable, blue-fluorescent stain specific for fungal cell walls. A second slide is stained with SYTO® 9 nucleic acid stain to identify all bacteria irrespective of gram signature, and also with the Texas Red®-X conjugate of wheat germ agglutinin (WGA), which selectively stains gram-positive bacteria.[103,104] Gram-positive bacteria on the second slide typically exhibit a green-fluorescent interior with red-fluorescent cell-surface staining, whereas gram-negative bacteria show only green-fluorescent interior staining. Staining and examination of the slides under a fluorescence microscope can be typically performed in less than one hour. Each Cell Culture Contamination Detection Kit contains:

- Green-fluorescent SYTO® 9 nucleic acid stain
- Blue-fluorescent Calcofluor White M2R fungal cell wall stain
- Red-fluorescent Texas Red®-X WGA, for positive identification of gram-positive bacteria
- Buffer for reconstituting Texas Red®-X WGA
- Detailed protocols

This kit provides sufficient material for approximately 200 contamination detection assays. The SYTO® 9 nucleic acid stain, which is also available separately (S34854, Section 15.2), has been used to detect lactic acid–producing bacteria in wine.[105]

MycoFluor™ Mycoplasma Detection Kit

Mycoplasma infections are generally difficult or impossible to detect during routine work with cultured cells because these intracellular pathogens cannot be observed by standard light microscopy. However, mycoplasma infections can be detected with the Hoechst dyes[106,107] (Figure 15.4.19) or with DAPI[108,109] (Section 8.1). Hoechst 33258, either alone[110] or in combination with merocyanine 540[111] (M24571, Section 18.2), has been utilized to eradicate mycoplasma infections from cell cultures. Mycoplasma infections are relatively common. Estimates of contaminated cultures in the United States range from 5% to 35%, whereas the contamination rate is postulated to be much higher in those countries where systematic detection and elimination are not practiced.[112] Not only do mycoplasma cause physiological and morphological distortions that can affect experimental results, but contamination can quickly spread to other cell lines.

The MycoFluor™ Mycoplasma Detection Kit (M7006) provides an extremely rapid and sensitive fluorescence microscopy–based assay for the visual identification of mycoplasma infection in laboratory cell cultures and media. In order to detect mycoplasma, the fluorescent MycoFluor™ reagent is added directly to the culture medium, with or without cells present, and the stained sample is then examined under a fluorescence microscope. The test for the presence of mycoplasma in live or fixed cell cultures takes about 15 minutes from when the reagent is added until when the sample is viewed with a fluorescence microscope equipped with DAPI optical filters. The detection of mycoplasma in cell media requires about 30 minutes, depending on the amount of centrifugation required to concentrate potential contaminants.

Also provided with this kit are mycoplasma MORFS (Microscopic Optical Replicas for Fluorescence assays), which serve as inert positive controls that mimic the size, shape and fluorescence intensity of mycoplasma stained with the blue-fluorescent MycoFluor™ reagent and viewed by fluorescence microscopy. The optical properties of the mycoplasma MORFS enable the researcher to discriminate between stained mycoplasma and other fluorescent material without introducing infectious biological agents into the laboratory environment. No previous experience with mycoplasma testing is required.

Each MycoFluor™ Mycoplasma Detection Kit supplies sufficient materials for at least 100 tests of live cells, fixed cells or culture media. Kit contents include:

- Concentrated MycoFluor™ reagent
- Suspension of mycoplasma MORFS
- Coverslip sealant
- Cotton swab
- Reference micrographs
- Detailed protocols

Alternatively, the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits (L7007, L7012, L13152; Section 15.3) are useful for detecting mycoplasma infections.[113] Researchers have determined that the reagents in our LIVE/DEAD® *Bac*Light™ Kits can be used for viability determinations in *Eurioplasma eurilytica* and *Mycoplasma hominus* mycoplasma and in the cysts of the protozoan parasite *Giardia muris* (Figure 15.4.20).

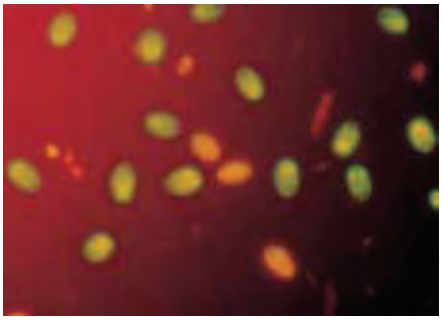

Figure 15.4.20 *Giardia muris* cysts stained with the reagents in the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (L7007, L7012). When incubated with the SYTO® 9 and propidium iodide nucleic acid stains provided in this kit, live bacteria with intact cell membranes fluoresce green and dead bacteria with compromised membranes fluoresce red.

Detecting Viruses Using Nucleic Acid Stains

Because of their small size, direct detection of viruses by fluorescence requires highly sensitive reagents or, much more commonly, an amplification scheme. However, direct enumeration of marine viral abundance in seawater using SYBR® Green I nucleic acid gel stain [114–116] (S7563, S7567, S7585; Section 8.3; Figure 15.4.21), YO-PRO®-1 (Y3603, Section 8.1) and DAPI [117] (D1306, D3571, D21490) has been reported.

The slow off-rate of our dimeric nucleic acid stains, such as TOTO®-1 and YOYO®-1 (T3600, Y3601), has permitted their use for labeling nucleic acids, including viral RNA,[118] prior to microinjection into live cells to follow their trafficking. Similar staining techniques may permit tracing of viral uptake and transport by live cells.

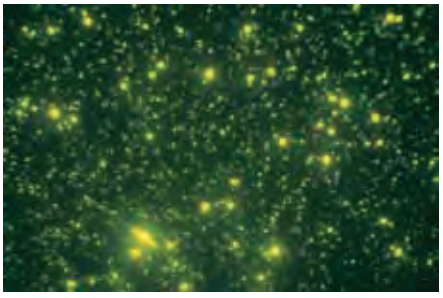

Figure 15.4.21 An environmental sample containing marine viruses (smallest dots), bacteria (larger, brighter dots), and a diatom (long thin cell with prominent nucleus) stained with SYBR® Green I nucleic acid stain (S7563, S7567, S7585). Image contributed by Jed Fuhrman, University of Southern California.

Countess® Automated Cell Counter

The Countess® automated cell counter (C10227, C10310, C10311; Figure 15.4.22) uses Trypan Blue staining combined with a sophisticated image analysis algorithm to count all cells in a sample—even mildly clumpy samples—as well as assess viability and measure average cell size. The Countess® instrument allows for accurate cell and viability counts and completes all calculations in about 30 seconds, using as little as 5 µL of your sample; a cell viability percentage value is calculated automatically.

The measurement range of the Countess® automated cell counter extends from 1×10^4 to 1×10^7 cells/mL, with an optimal range from 1×10^5 to 4×10^6 cells/mL, which is broader than that of a hemocytometer. Furthermore, the Countess® instrument counts cell size from 5 to 60 µm and counts beads from 4.5 to 60 µm. Countess® Test Beads (C10284) are designed to provide a reliable and rapid calibration standard for users who choose to operate the instrument outside its default parameters. The Countess® instrument has been validated for use with more than 20 common cell types, including primary cells, blood cells, yeast cells (without viability assessment), insect cells and fish cells.

The Countess® automated cell counter does not require a computer. Disposable chamber slides (C10228, C10312, C10313, C10314, C10315) provide rapid setup, with no cleaning, buffers or maintenance required other than battery replacement after approximately 5 years of use. Trypan Blue stain is also available as a stand-alone reagent (T10282). More information is available at www.invitrogen.com/handbook/countess.

Figure 15.4.22 The Countess® automated cell counter provides fast, easy and accurate cell counting without using a hemocytometer.

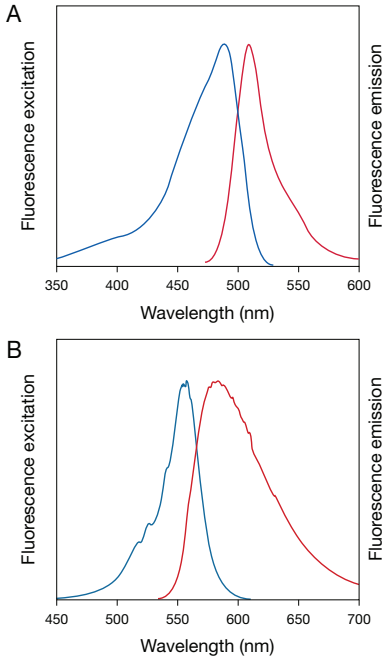

Figure 15.4.24 Fluorescence excitation and emission spectra for **A**) Premo™ geminin–GFP and **B**) Premo™ Cdt1–RFP.

Premo™ FUCCI Cell Cycle Sensor

In 2008, Miyawaki and colleagues developed the Fluorescence Ubiquitination Cell Cycle Indicator (FUCCI), a fluorescent protein (FP)–based sensor that employs a Red Fluorescent Protein (RFP) and a Green Fluorescent Protein (GFP), each of which is fused to one of two different regulators of the cell cycle: Cdt1 and geminin.[119] Ubiquitin E3 ligases add ubiquitin to the Cdt1–GFP and geminin–RFP fusions, thereby targeting these proteins for proteasomal degradation. Temporal regulation of E3 ligase activity results in the biphasic cycling of the geminin and Cdt1 fusions through the cell cycle.

In the G_1 phase, the geminin–GFP fusion is degraded, leaving only the Cdt1–RFP fusion and resulting in red-fluorescent nuclei. During the G_1/S transition, Cdt1 levels decrease and geminin levels increase. Because both proteins are present in the cells, both GFP and RFP fluorescence is visible and the cell nuclei appear yellow when the green and red images are overlaid. In the S, G_2 and M phases, the Cdt1–RFP fusion is degraded, leaving only the geminin–GFP fusion and resulting in green-fluorescent nuclei. This dynamic color change from red to yellow to green serves as an indicator of the progression through cell cycle and division (Figure 15.4.23).

The Premo™ FUCCI Cell Cycle Sensor (P36232) combines the Cdt1 and geminin FP constructs with the powerful BacMam gene delivery and expression system (BacMam Gene Delivery and Expression Technology—Note 11.1). The genetically encoded and pre-packaged reagents are ready for immediate use, eliminating the need to purify plasmid. The BacMam reagent is simply added to cells for 1–2 hours, after which the cells are treated with an enhancer reagent for another 1–2 hours, washed, incubated overnight and then visualized using fluorescence microscopy or high-throughput imaging platforms (Figure 15.4.23). BacMam transduction is efficient and reproducible in most cell types, including primary and stem cells, without apparent cytotoxic effects.

The Premo™ FUCCI Cell Cycle Sensor is designed for live-cell imaging of cell cycle progression and can be used to assess the effect of drugs, siRNA or other factors on the transition of cells through the cell cycle. The fluorescence signals from geminin-GFP and Cdt1-RFP (Figure 15.4.24) have been demonstrated to be resistant to fixation with 4% formaldehyde and permeabilization with 0.1% Triton X-100, thereby enabling processing of labeled cells with antibodies to other cellular targets. Each kit contains all of the components needed to label cells with the Premo™ FUCCI Cell Cycle Sensor using a transduction volume of 2 mL; however, the protocol can easily be adjusted for larger or smaller volumes.

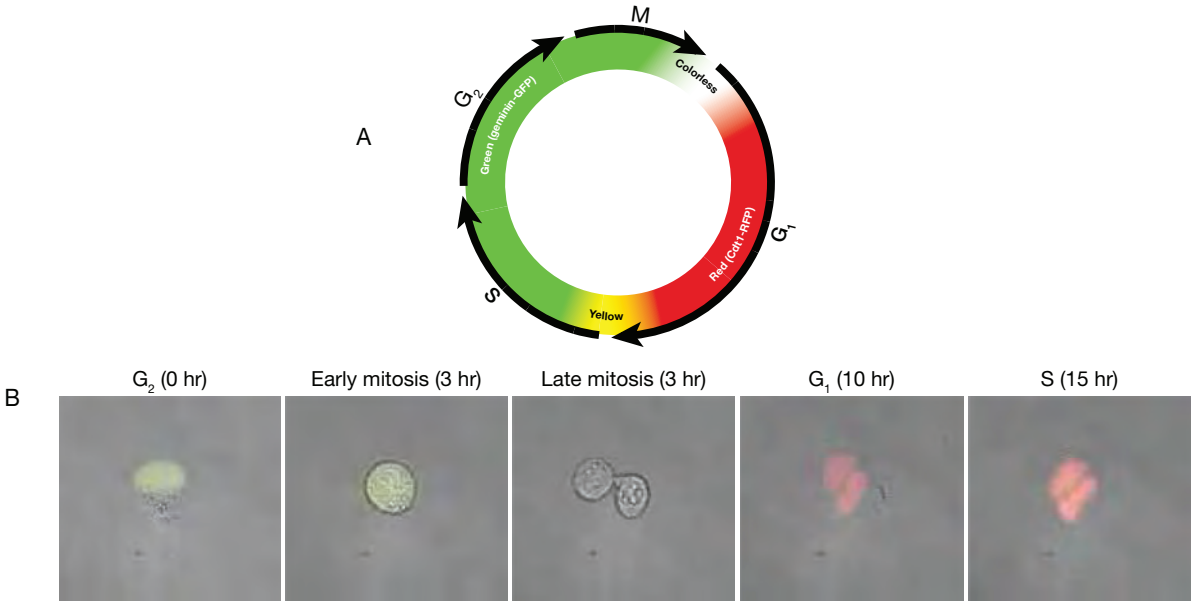

Figure 15.4.23 Imaging cell-cycle progression in live cells with Premo™ FUCCI Cell Cycle Sensor. **A**) Schematic of cell-cycle progression with nuclear fluorescence changes. **B**) U2OS cells were transduced with Premo™ FUCCI Cell Cycle Sensor, and images were collected over 15 hours.

Nucleic Acid Probes for Cell-Cycle Analysis

Live-cell studies of cellular DNA content and cell-cycle distribution are useful for investigating tumor behavior and suppressor gene mechanisms, for monitoring apoptosis and for detecting variations in growth patterns arising from a variety of physical, chemical and biological means. In a given population, cells will be distributed among three major phases of the cell cycle: G_0/G_1 phase (one set of paired chromosomes per cell), S phase (DNA synthesis with variable amount of DNA) and G_2/M phase (two sets of paired chromosomes per cell, prior to cell division).[120–123] DNA content can be measured using fluorescent, DNA-selective stains that exhibit emission signals proportional to DNA mass. Flow cytometric analysis of these stained populations is then used to produce a frequency histogram that reveals the various phases of the cell cycle. This analysis is typically performed on permeabilized or fixed cells using a cell-impermeant nucleic acid stain,[23,124–126] but is also possible using live cells and a cell-permeant nucleic acid stain.[127,128] While the choices for fixed cell staining are varied, there are only a few examples of useful cell-permeant nucleic acid stains.

Vybrant® DyeCycle™ Stains

All Vybrant® DyeCycle™ stains are DNA-selective, cell membrane–permeant and nonfluorescent until bound to double-stranded DNA. Once bound to DNA, these dyes emit a fluorescence signal that is proportional to DNA mass. Staining cells with the Vybrant® DyeCycle™ stains is simple: suspended cells are incubated in the presence of a Vybrant® DyeCycle™ stain, and fluorescence is measured directly—no additional treatment or centrifugation is required. Fluorescence data can then be used to generate a frequency histogram that reveals the various phases of the cell cycle.

We offer four Vybrant® DyeCycle™ stains for flow cytometry analysis of DNA content in live cells:

- Vybrant® DyeCycle™ Violet stain [129] (V35003)
- Vybrant® DyeCycle™ Green stain (V35004)
- Vybrant® DyeCycle™ Orange stain (V35005)
- Vybrant® DyeCycle™ Ruby stain (V10273, V10309)

The Vybrant® DyeCycle™ stains spectrally match commonly available excitation sources, placing cell-cycle studies within reach of all flow cytometrists and allowing simultaneous staining of the cell population for other parameters. Vybrant® DyeCycle™ Violet stain (excitation/emission maxima ~396/437 nm) is well suited for the 405 nm laser line (Figure 15.4.25) but can also be used with UV excitation. Vybrant® DyeCycle™ Green stain (excitation/emission maxima ~506/534 nm) is efficiently excited with the 488 nm spectral line of the argon-ion laser (Figure 15.4.26). Vybrant® DyeCycle™ Orange stain (excitation/emission maxima ~519/563 nm) can be excited using either the 488 nm or 532 nm laser lines (Figure 15.4.27). Vybrant® DyeCycle™ Ruby stain (excitation/emission maxima ~638/686 nm) can be excited using the 635 nm red diode laser (Figure 15.4.28).

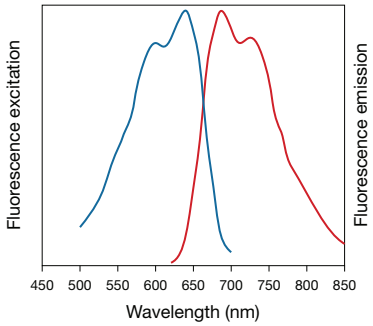

Figure 15.4.28 Fluorescence excitation and emission spectra of Vybrant® DyeCycle™ Ruby stain bound to DNA.

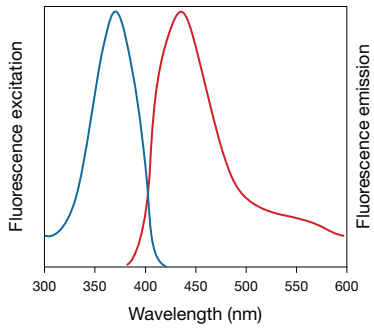

Figure 15.4.25 Fluorescence excitation and emission spectra of Vybrant® DyeCycle™ Violet stain bound to DNA.

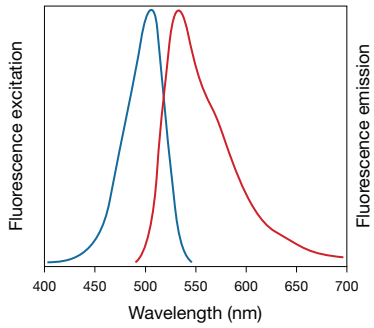

Figure 15.4.26 Fluorescence excitation and emission spectra of Vybrant® DyeCycle™ Green stain bound to DNA.

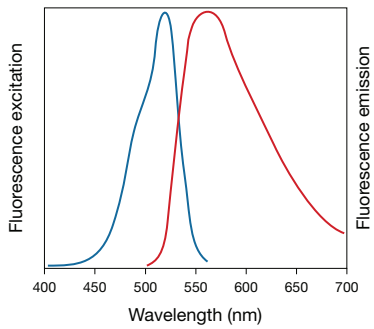

Figure 15.4.27 Fluorescence excitation and emission spectra of Vybrant® DyeCycle™ Orange stain bound to DNA.

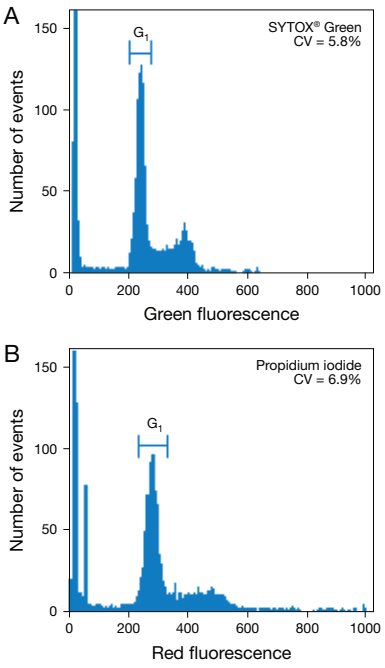

Figure 15.4.31 Comparison of DNA content histograms obtained with **A**) SYTOX® Green nucleic acid stain (S7020) and **B**) propidium iodide (P1304MP, P3566, P21493). Human B cells were suspended in permeabilizing buffer (100 mM Tris, pH 7.4, 154 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% Nonidet™ P-40) and then stained for 15 minutes with 0.5 µM SYTOX® Green or 5 µM propidium iodide. Flow cytometric analysis of the stained cells was carried out with excitation at 488 nm. SYTOX® Green staining produces a significantly narrower G_1 phase peak, indicated by the smaller coefficient of variation (CV).

FxCycle™ Stains for Cell-Cycle Analysis

FxCycle™ Violet stain (F10347) is a violet laser–excited dye used for cell-cycle analysis in fixed cells. FxCycle™ Violet stain (4',6-diamidino-2-phenylindole, dihydorchloride or DAPI) preferentially stains dsDNA, exhibiting a ~20-fold fluorescence enhancement upon binding. Using FxCycle™ Violet for cell cycle analysis increases the ability to multiplex by freeing up the 488 nm and 633 nm lasers for other cellular analyses, such as immunophenotyping, apoptosis analysis and dead cell discrimination. As compared with several commonly used dyes (propidium iodide and 7-AAD), FxCycle™ Violet stain overlaps less with other fluorescence detection channels, resulting in minimal compensation requirements and more accurate data (Figure 15.4.29).

FxCycle™ Far Red stain (F10348) is useful for flow cytometric analysis of DNA content in fixed cells. Because FxCycle™ Far Red stain binds to both RNA as well as DNA, the addition of RNase A is required for DNA content analysis. This dye takes advantage of the commonly available 633/635 nm excitation sources, with emission around 660 nm (Figure 15.4.30), and is a good choice for DNA content analysis in multicolor cell-cycle studies.

SYTOX® Green Nucleic Acid Stain

SYTOX® Green nucleic acid stain (S7020) is particularly useful for cell-cycle analysis on RNase-treated fixed cells (Figure 15.4.31). In particular, the SYTOX® Green dye produces lower coefficients of variation than propidium iodide (P1304MP, P3566, P21493; Section 8.1), leading to improved resolution of cell phase. Figure 15.4.31 shows a comparison of DNA content histograms obtained with SYTOX® Green nucleic acid stain and propidium iodide after flow cytometric analysis.

Hoechst 33258, Hoechst 33342 and DAPI

The nucleic acid stains most frequently used for cell-cycle analysis—Hoechst 33258 (H1398, H3569, H21491), Hoechst 33342 (H1399, H3570, H21492) and DAPI (D1306, D3571, D21490)—bind to the minor groove of DNA at AT-rich sequences. Hoechst 33342, which more rapidly permeates cells than Hoechst 33258, is commonly used for determining the DNA content of viable cells without detergent treatment or fixation [127,128] (Figure 15.4.32). The Hoechst dyes and DAPI can be excited with a mercury-arc lamp, the UV spectral lines of the argon-ion laser or the 325 nm spectral line of the He-Cd laser. These blue-fluorescent nucleic acid stains preferentially bind to AT-rich sequences and also exhibit higher quantum yields when bound to AT-rich nucleic acids, thus introducing a strong bias into the measurements of nuclear DNA content.[130,131] As a consequence, data obtained with Hoechst 33342 and DAPI correlate very well with each other but less well with data obtained with propidium iodide, a red-fluorescent, cell-impermeant nucleic acid stain [132] (P1304MP, P3566, P21493; Section 8.1). Hoechst 33342 is used in the high-speed sorting of X chromosome– and Y chromosome–bearing sperm based on their DNA content.[133,134]

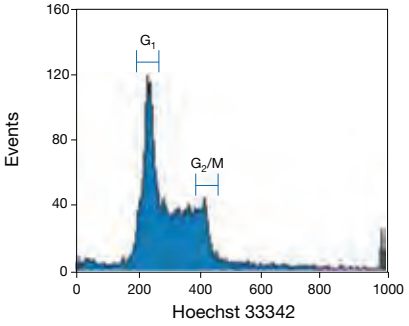

Figure 15.4.32 DNA content histogram for WIL2S cells. The cells were fixed in ethanol, treated with RNase and stained with 2 µg/mL Hoechst 33342 (H1399, H3570, H21492). Flow cytometric analysis of the stained cells was carried out using excitation at 350 nm.

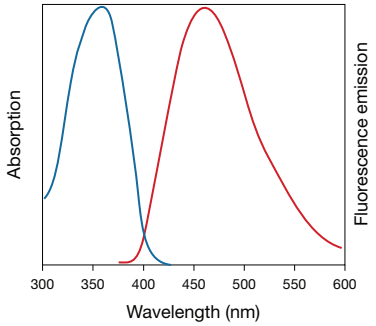

Figure 15.4.29 Absorption and fluorescence and emission spectra of FxCycle™ Violet stain bound to DNA.

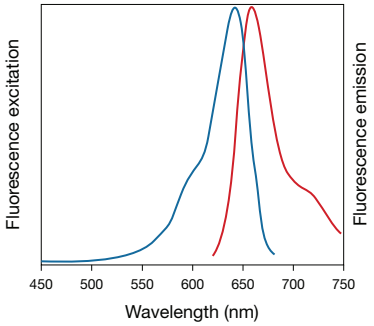

Figure 15.4.30 Fluorescence excitation and emission spectra of FxCycle™ Far Red stain bound to DNA.

HCS Mitotic Index Kit

Histones are core proteins of DNA in eukaryotic cells. This histones are organized as octamers, around which the DNA is wrapped. The phosphorylation of histone 3 (H3) is involved in condensation of chromatin during mitosis and reaches a peak during mitosis. Mitotic H3 phosphorylation occurs at Ser10 of the amino terminus, and there is a tight correlation between H3 (Ser10) phosphorylation, chromosome condensation and segregation during mitosis. This event can serve as an indication of mitotic progression or inhibition within the context of drug profiling.

The HCS Mitotic Index Kit (H10293) was developed to measure mitotic cells using automated imaging and anlysis and can be combined with other measurements such as DNA profiling, general cytotoxicity or immunocytochemical detection of choice targets. This kit includes sufficient reagents for two 96-well plate assays, including:

- Polyclonal rabbit anti–phospho-H3 antibody
- Alexa Fluor® 488 goat anti–rabbit IgG antibody
- DAPI
- HCS NuclearMask™ Deep Red stain
- Detailed protocols

The primary antibody against phosphorylated histone H3 (Ser10) serves as a sensitive index of mitosis and is detected using the green-fluorescent Alexa Fluor® 488 secondary antibody. The blue-fluorescent DAPI and near-infrared–fluorescent HCS NuclearMask™ Deep Red stain provide two choices for DNA profiling and cell demarcation during image analysis. The HCS Mitotic Index Kit represents a powerful image-based assay for the identification of compounds that affect mitotic progression (Figure 15.4.33).

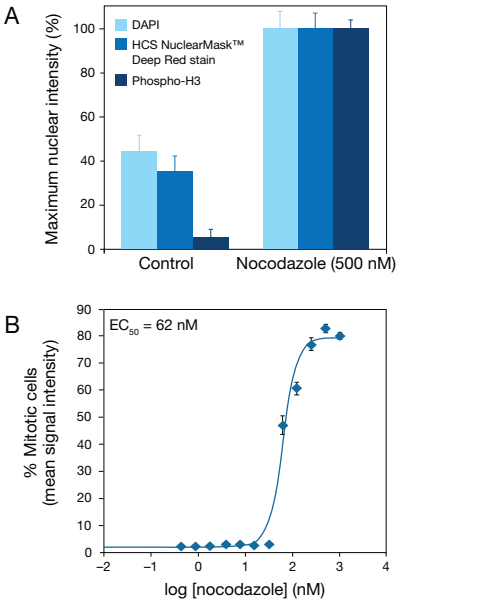

Figure 15.4.33 Imaging of nocodazole-induced mitotic arrest in A549 cells using the HCS Mitotic Index Kit (H10293). **A**) Quantitation of cells treated with 500 nM nocodazole for 24 hr. Nuclear segmentation and DNA content were measured using DAPI or HCS NuclearMask™ Deep Red stain. The strong increase in both phosphohistone 3 and DNA staining is indicative of mitotic cells. **B**) Dose response to 0–1,000 nM nocodazole after 24 hr incubation. The curve generated by nonlinear regression was used to determine the EC_{50} value for nocodazole.

REFERENCES

1. Exp Cell Res (1987) 173:256; **2**. Science (1982) 218:474; **3**. Chem Rev (2009) 109:4207; **4**. Chem Rev (2008) 108:2952; **5**. Angew Chem Int Ed Engl (2009) 48:6974; **6**. Biochemistry (2009) 48:6571; **7**. ACS Chem Biol (2006) 1:644; **8**. J Immunol Methods (2009) 350:29; **9**. Biotechniques (2008) 44:927; **10**. Proc Natl Acad Sci U S A (2008) 105:2415; **11**. J Biol Chem (2010) 285:1544; **12**. Genetics (2009) 183:233; **13**. J Neurosci Methods (2009) 177:122; **14**. Nucleic Acids Res (2009) 37:e31; **15**. Methods (2009) 48:8; **16**. Science (2009) 325:340; **17**. Proc Natl Acad Sci U S A (2009) 106:14896; **18**. Plant Methods (2010) 6:5; **19**. Proc Natl Acad Sci U S A (2009) 106:11806; **20**. Cytometry A (2009) 75:862; **21**. Methods Cell Biol (1994) 41:297; **22**. Proc Natl Acad Sci U S A (1983) 80:5573; **23**. Methods Cell Biol (1994) 41:195; **24**. J Histochem Cytochem (1977) 25:913; **25**. Proc Natl Acad Sci U S A (1973) 70:3395; **26**. Cytometry (2000) 41:89; **27**. Proc Natl Acad Sci U S A (1983) 80:2951; **28**. Methods Cell Biol (1994) 41:327; **29**. Exp Cell Res (1988) 174:319; **30**. Cytometry (1999) 37:221; **31**. Cytometry (1994) 17:310; **32**. Cytometry (2001) 45:13; **33**. Cytometry (1995) 20:172; **34**. Cytometry (1995) 20:257; **35**. Cytometry (1995) 20:245; **36**. J Histochem Cytochem (1993) 41:7; **37**. J Cell Biol (1992) 119:493; **38**. Cancer Res (1994) 54:4289; **39**. Exp Cell Res (1996) 222:28; **40**. Cell Prolif (1995) 28:571; **41**. Cell Biology: A Laboratory Handbook, 2nd Ed., Vol. 1, Celis JE, Ed. (1998) p. 341; **42**. Cytometry (1997) 27:1; **43**. J Biol Chem (2000) 275:16788; **44**. Dev Biol (1999) 206:232; **45**. J Cell Biol (1999) 144:813; **46**. Histochem Cell Biol (2000) 113:181; **47**. Mol Biol Cell (1999) 10:211; **48**. Immunol Cell Biol (1999) 77:509; **49**. J Immunol Methods (1994) 171:131; **50**. J Immunol Methods (1990) 133:87; **51**. Transplant Proc (1992) 24:2820; **52**. Nat Protoc (2007) 2:2049; **53**. J Exp Med (1999) 189:265; **54**. J Immunol (1999) 162:735; **55**. Cell Immunol (1998) 188:1; **56**. Cytometry (1998) 34:143; **57**. Eur J Immunol (1998) 28:1040; **58**. J Immunol (1998) 161:5260; **59**. J Immunol (1998) 160:3666; **60**. Br J Haematol (1997) 98:528; **61**. J Biol Chem (1999) 274:3541; **62**. J Exp Med (1996) 184:277; **63**. Blood (1999) 94:2161; **64**. Lett Appl Microbiol (1997) 25:295; **65**. J Immunol Methods (2001) 254:85; **66**. Anal Biochem (1992) 207:186; **67**. J Immunol Methods (1991) 142:199; **68**. Anal Biochem (1993) 213:426; **69**. In Vitro Toxicol (1990) 3:219; **70**. Exp Cell Res (1979) 124:329; **71**. J Biomol Screen (2008) 13:527; **72**. Anal Biochem (1990) 191:31; **73**. J Immunol Methods (1993) 162:41; **74**. Cancer Res (1989) 49:565; **75**. Anal Biochem (1983) 131:538; **76**. Anal Biochem (1980) 102:344; **77**. J Neurochem (1997) 69:581; **78**. J Immunol Methods (1983) 65:55; **79**. J Immunol Methods (1994) 168:253; **80**. Cancer Res (1987) 47:943; **81**. J Immunol Methods (1986) 93:157; **82**. Biotechniques (1998) 25:622, 626; **83**. J Immunol Methods (1999) 230:99; **84**. Blood (1999) 94:2595; **85**. Leuk Res (1993) 17:873; **86**. Anticancer Res (1998) 18:4243; **87**. J Acquir Immune Defic Syndr (2005) 39:32; **88**. Br J Haematol (2001) 115:953; **89**. Br J Haematol (1999) 106:1059; **90**. MMWR Recomm Rep (2003) 52:1; **91**. J Microbiol Methods (2008) 72:157; **92**. Anal Chem (2007) 79:7510; **93**. J Med Microbiol (2005) 54:843; **94**. J Biol Chem (2007) 282:12112; **95**. J Med Microbiol (2005) 54:77; **96**. Cytometry (1998) 32:66; **97**. J Microbiol Methods (1994) 20:255; **98**. J Microbiol Methods (1994) 19:89; **99**. Microbiol Rev (1994) 58:603; **100**. J Parasitol (1979) 65:421; **101**. Yeast (1998) 14:147; **102**. J Immunol Methods (1998) 211:51; **103**. Appl Environ Microbiol (1990) 56:2245; **104**. J Clin Microbiol (1979) 10:669; **105**. Lett Appl Microbiol (1999) 28:23; **106**. J Immunol Methods (1980) 38:315; **107**. Exp Cell Res (1977) 104:255; **108**. Leukemia (1992) 6:335; **109**. J Immunol Methods (1992) 149:43; **110**. Cytogenet Cell Genet (1983) 36:584; **111**. J Immunol Methods (1994) 168:245; **112**. Nature (1989) 339:487; **113**. Microbiology (2006) 152:913; **114**. Appl Environ Microbiol (1999) 65:45; **115**. Aquat Microbial Ecol (1998) 14:113; **116**. Nature (1999) 399:541; **117**. Aquat Microbial Ecol (1997) 13:225; **118**. Proc Natl Acad Sci U S A (1996) 93:12643; **119**. Cell (2008) 132:487; **120**. Methods Mol Biol (2004) 281:301; **121**. Current Protocols in Cytometry, Robinson JP, Ed. (2004) 7.0.1; **122**. Cytometry A (2004) 58:21; **123**. Lab Chip (2008) 8:68; **124**. Methods Cell Biol (1994) 41:231; **125**. Methods Cell Biol (1994) 41:219; **126**. Methods Cell Biol (1994) 41:211; **127**. Arch Pathol Lab Med (1989) 113:591; **128**. J Histochem Cytochem (1977) 25:585; **129**. Nat Protoc (2008) 3:1187; **130**. Biochemistry (1990) 29:9029; **131**. J Histochem Cytochem (1976) 24:24; **132**. Cytometry (1992) 13:389; **133**. Cytometry (1998) 33:476; **134**. Cytometry (1996) 25:191.

DATA TABLE 15.4 ASSAYS FOR CELL ENUMERATION, CELL PROLIFERATION AND CELL CYCLE

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
B21550	612.99	FF,L	H_2O	<300		none		1
B21551	628.98	FF,L	H_2O	<300		none		1
B23151	307.10	F,L	DMSO	<300		none		
B35000	671.88	F,D,L	DMSO	490	119,000	516	MeOH	
B35001	724.00	F,D,L	DMSO	588	81,000	645	MeOH	
C1157	557.47	F,D	DMF, DMSO	<300		none		2
C7614	~908	FF,L	H_2O	504	68,000	513	pH 8	1, 3, 13
C22803	873.05	F,D	DMSO	<300		none		4
C34555	593.45	F,D,L	DMSO	<300		none		5
D1306	350.25	L	H_2O, DMF	358	24,000	461	H_2O/DNA	6, 7
D3571	457.49	L	H_2O, MeOH	358	24,000	461	H_2O/DNA	
D21490	350.25	L	H_2O, DMF	358	24,000	461	H_2O/DNA	6, 7, 8
H1398	623.96	L	H_2O, DMF	352	40,000	461	H_2O/DNA	6, 9, 10
H1399	615.99	L	H_2O, DMF	350	45,000	461	H_2O/DNA	6, 9, 11
H3569	623.96	RR,L	H_2O	352	40,000	461	H_2O/DNA	1, 6, 9, 10
H3570	615.99	RR,L	H_2O	350	45,000	461	H_2O/DNA	1, 6, 9, 11
H21491	623.96	L	H_2O, DMF	352	40,000	461	H_2O/DNA	6, 8, 9, 10
H21492	615.99	L	H_2O, DMF	350	45,000	461	H_2O/DNA	6, 8, 9, 11
L7595	471.98	L	DMSO, EtOH	543	46,000	712	H_2O/DNA	6
O34550	593.45	F,D,L	DMSO	<300		none		5
S7020	~600	F,D,L	DMSO	504	67,000	523	H_2O/DNA	1, 6, 12, 13
S22801	592.56	F,D	DMSO	<350		none		14
T3600	1302.78	F,D,L	DMSO	514	117,000	533	H_2O/DNA	1, 6, 12, 15
T3605	671.42	F,D,L	DMSO	642	102,000	661	H_2O/DNA	1, 6, 12, 15
V22885	933.88	L	see Notes	549	148,000	565	MeOH	16
V22886	881.72	L	see Notes	484	154,000	501	MeOH	16
V22887	1052.08	L	see Notes	644	193,000	663	MeOH	16
V22888	1051.50	F,L	see Notes	553	134,000	570	MeOH	16
Y3601	1270.65	F,D,L	DMSO	491	99,000	509	H_2O/DNA	1, 6, 12, 15

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
2. Acetate hydrolysis of this compound yields a fluorescent product with similar pH-dependent spectral characteristics to C1904.
3. The molecular weight (MW) of this product is approximate because the degree of hydration and/or salt form has not been conclusively established.
4. Acetate hydrolysis of C22803 yields a fluorescent product with spectra similar to 5-carboxyeosin, Abs = 519 nm (EC = 100,000 $cm^{-1}M^{-1}$), Em = 542 nm in pH 9 buffer.
5. Acetate hydrolysis of this compound yields a fluorescent product with similar spectral characteristics to O6146.
6. Spectra represent aqueous solutions of nucleic acid-bound dye. EC values are derived by comparing the absorbance of the nucleic acid-bound dye with that of free dye in a reference solvent (H_2O or MeOH).
7. DAPI in H_2O: Abs = 342 nm (EC = 28,000 $cm^{-1}M^{-1}$), Em = 450 nm. The fluorescence quantum yield of DAPI bound to dsDNA is 0.34, representing an ~20-fold increase relative to the free dye in H_2O. (Photochem Photobiol (2001) 73:585)
8. This product is specified to equal or exceed 98% analytical purity by HPLC.
9. MW is for the hydrated form of this product.
10. The fluorescence quantum yield of Hoechst 33258 bound to dsDNA is 0.42, representing an ~30-fold increase relative to the free dye in H_2O. (Photochem Photobiol (2001) 73:585)
11. The fluorescence quantum yield of Hoechst 33342 bound to dsDNA is 0.38, representing an ~10-fold increase relative to the free dye in H_2O. (Photochem Photobiol (2001) 73:585)
12. This product is essentially nonfluorescent except when bound to DNA or RNA.
13. MW: The preceding ~ symbol indicates an approximate value, not including counterions.
14. S22801 is converted to a fluorescent product with spectra similar to C1270 after acetate hydrolysis.
15. Although this compound is soluble in water, preparation of stock solutions in water is not recommended because of possible adsorption onto glass or plastic.
16. This product is supplied as a ready-made staining solution.

PRODUCT LIST 15.4 ASSAYS FOR CELL ENUMERATION, CELL PROLIFERATION AND CELL CYCLE

Cat. No.	Product	Quantity
B35000	*Bac*Light™ Green bacterial stain *special packaging*	20 x 50 µg
B35001	*Bac*Light™ Red bacterial stain *special packaging*	20 x 50 µg
B7277	Bacteria Counting Kit *for flow cytometry*	1 kit
B35130	BrdU, mouse monoclonal antibody (Clone MoBU-1), Alexa Fluor® 488 conjugate *0.2 mg/mL*	350 µL
B35139	BrdU, mouse monoclonal antibody (Clone MoBU-1), Alexa Fluor® 488 conjugate *for flow cytometry* *100 tests*	1 each
B35131	BrdU, mouse monoclonal antibody (Clone MoBU-1), Alexa Fluor® 555 conjugate *0.2 mg/mL*	350 µL
B35132	BrdU, mouse monoclonal antibody (Clone MoBU-1), Alexa Fluor® 594 conjugate *0.2 mg/mL*	350 µL
B35133	BrdU, mouse monoclonal antibody (Clone MoBU-1), Alexa Fluor® 647 conjugate *0.2 mg/mL*	350 µL
B35140	BrdU, mouse monoclonal antibody (Clone MoBU-1), Alexa Fluor® 647 conjugate *for flow cytometry* *100 tests*	1 each
B35138	BrdU, mouse monoclonal antibody (Clone MoBU-1), biotin conjugate *0.2 mg/mL*	350 µL
B35129	BrdU, mouse monoclonal antibody (Clone MoBU-1), Pacific Blue™ conjugate *for flow cytometry* *100 tests*	1 each
B35128	BrdU, mouse monoclonal antibody (Clone MoBU-1), unconjugated *0.1 mg/mL*	350 µL
B35141	BrdU, mouse monoclonal antibody (Clone MoBU-1), unconjugated *for flow cytometry* *100 tests*	1 each
B23151	5-bromo-2′-deoxyuridine (BrdU)	100 mg
B21550	5-bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP) *10 mM in TE buffer*	25 µL
B21551	5-bromouridine 5′-triphosphate (BrUTP) *10 mM in TE buffer*	25 µL
C22803	CEDA, SE (5-(and-6)-carboxyeosin diacetate, succinimidyl ester) *mixed isomers*	5 mg

PRODUCT LIST 15.4 ASSAYS FOR CELL ENUMERATION, CELL PROLIFERATION AND CELL CYCLE—*continued*

Cat. No.	Product	Quantity
C7028	Cell Culture Contamination Detection Kit *200 assays*	1 kit
C34554	CellTrace™ CFSE Cell Proliferation Kit *for flow cytometry*	1 kit
C34555	CellTrace™ Oregon Green® 488 carboxylic acid diacetate, succinimidyl ester (carboxy-DFFDA, SE) *cell permeant* *mixed isomers*	20 x 50 µg
C34557	CellTrace™ Violet Cell Proliferation Kit *for flow cytometry*	1 kit
C1157	5(6)-CFDA, SE; CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester) *mixed isomers*	25 mg
C7614	ChromaTide® BODIPY® FL-14-dUTP *1 mM in TE buffer*	25 µL
C35002	Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit *50 assays*	1 kit
A10202	Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit *50 assays*	1 kit
A10034	Click-iT® EdU Pacific Blue™ Flow Cytometry Assay Kit *50 assays*	1 kit
C10350	Click-iT® EdU Alexa Fluor® 488 HCS Assay *2-plate size*	1 kit
C10351	Click-iT® EdU Alexa Fluor® 488 HCS Assay *10-plate size*	1 kit
C10352	Click-iT® EdU Alexa Fluor® 555 HCS Assay *2-plate size*	1 kit
C10353	Click-iT® EdU Alexa Fluor® 555 HCS Assay *10-plate size*	1 kit
C10354	Click-iT® EdU Alexa Fluor® 594 HCS Assay *2-plate size*	1 kit
C10355	Click-iT® EdU Alexa Fluor® 594 HCS Assay *10-plate size*	1 kit
C10356	Click-iT® EdU Alexa Fluor® 647 HCS Assay *2-plate size*	1 kit
C10357	Click-iT® EdU Alexa Fluor® 647 HCS Assay *10-plate size*	1 kit
C10337	Click-iT® EdU Alexa Fluor® 488 Imaging Kit *for 50 coverslips*	1 kit
C10338	Click-iT® EdU Alexa Fluor® 555 Imaging Kit *for 50 coverslips*	1 kit
C10339	Click-iT® EdU Alexa Fluor® 594 Imaging Kit *for 50 coverslips*	1 kit
C10340	Click-iT® EdU Alexa Fluor® 647 Imaging Kit *for 50 coverslips*	1 kit
C10214	Click-iT® EdU Microplate Assay *400 assays*	1 kit
C36950	CountBright™ absolute counting beads *for flow cytometry* *100 tests*	5 mL
C10227	Countess® automated cell counter *with box of 50 cell counting chambers and Trypan Blue*	1 kit
C10311	Countess® Automated Cell Counter Lab Starter Kit *with 101 boxes of 50 cell counting chamber slides and Trypan Blue*	1 kit
C10310	Countess® Automated Cell Counter Starter Kit *with 11 boxes of 50 cell counting chamber slides and Trypan Blue*	1 kit
C10228	Countess® cell counting chamber slides *for use with Countess® automated cell counter* *box of 50 with Trypan Blue*	1 kit
C10312	Countess® Cell Counting Chamber Slides, 500 Slides (1000 Counts) *for use with Countess® automated cell counter* *10 boxes of 50 slides* *with Trypan Blue*	1 kit
C10313	Countess® Cell Counting Chamber Slides, 1250 Slides (2500 Counts) *for use with Countess® automated cell counter* *25 boxes of 50 slides* *with Trypan Blue*	1 kit
C10314	Countess® Cell Counting Chamber Slides, 2500 Slides (5000 Counts) *for use with Countess® automated cell counter* *50 boxes of 50 slides* *with Trypan Blue*	1 kit
C10315	Countess® Cell Counting Chamber Slides, 5000 Slides (10,000 Counts) *for use with Countess® automated cell counter* *100 boxes of 50 slides* *with Trypan Blue*	1 kit
C10285	Countess® power cord with four adapter cords *for use with Countess® automated cell counter*	1 set
C10284	Countess® test beads *for use with Countess® automated cell counter* *1 x 10^6 beads/mL ±10%*	1 mL
C10286	Countess® USB drive *for use with Countess® automated cell counter* *1 Gbyte*	each
C7027	CyQUANT® cell lysis buffer *20X concentrate*	50 mL
C7026	CyQUANT® Cell Proliferation Assay Kit *for cells in culture* *1000 assays*	1 kit
C35011	CyQUANT® Direct Cell Proliferation Assay *for 10 microplates*	1 kit
C35012	CyQUANT® Direct Cell Proliferation Assay *for 100 microplates*	1 kit
C35006	CyQUANT® NF Cell Proliferation Assay Kit *1000 assays*	1 kit
C35007	CyQUANT® NF Cell Proliferation Assay Kit *200 assays*	1 kit
D1306	4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI)	10 mg
D21490	4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) *FluoroPure™ grade*	10 mg
D3571	4′,6-diamidino-2-phenylindole, dilactate (DAPI, dilactate)	10 mg
A10044	EdU (5-ethynyl-2′-deoxyuridine)	50 mg
E10187	EdU (5-ethynyl-2′-deoxyuridine)	500 mg
E10415	EdU (5-ethynyl-2′-deoxyuridine)	5 g
F2962	FluoReporter® Blue Fluorometric dsDNA Quantitation Kit *200–2000 assays*	1 kit
F10348	FxCycle™ Far Red stain *for flow cytometry* *500 assays*	1 set
F10347	FxCycle™ Violet stain *for flow cytometry* *500 assays* *DAPI*	1 set
H10293	HCS Mitotic Index Kit *2-plate size*	1 kit
H1398	Hoechst 33258, pentahydrate (bis-benzimide)	100 mg
H3569	Hoechst 33258, pentahydrate (bis-benzimide) *10 mg/mL solution in water*	10 mL
H21491	Hoechst 33258, pentahydrate (bis-benzimide) *FluoroPure™ grade*	100 mg
H1399	Hoechst 33342, trihydrochloride, trihydrate	100 mg
H3570	Hoechst 33342, trihydrochloride, trihydrate *10 mg/mL solution in water*	10 mL
H21492	Hoechst 33342, trihydrochloride, trihydrate *FluoroPure™ grade*	100 mg
L7595	LDS 751	10 mg
M7006	MycoFluor™ Mycoplasma Detection Kit	1 kit
O34550	Oregon Green® 488 carboxylic acid diacetate, succinimidyl ester (carboxy-DFFDA, SE) *mixed isomers*	1 mg

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PRODUCT LIST 15.4 ASSAYS FOR CELL ENUMERATION, CELL PROLIFERATION AND CELL CYCLE—*continued*

Cat. No.	Product	Quantity
P36232	Premo™ FUCCI Cell Cycle Sensor	1 kit
S22801	SNARF®-1 carboxylic acid, acetate, succinimidyl ester *special packaging*	10 x 50 µg
S7020	SYTOX® Green nucleic acid stain *5 mM solution in DMSO*	250 µL
T3605	TO-PRO®-3 iodide (642/661) *1 mM solution in DMSO*	1 mL
T3600	TOTO®-1 iodide (514/533) *1 mM solution in DMSO*	200 µL
T10282	Trypan Blue stain 0.4% *for use with Countess® automated cell counter*	2 x 1 mL
V12883	Vybrant® CFDA SE Cell Tracer Kit	1 kit
V22888	Vybrant® CM-DiI cell-labeling solution	1 mL
V22887	Vybrant® DiD cell-labeling solution	1 mL
V22885	Vybrant® DiI cell-labeling solution	1 mL
V22886	Vybrant® DiO cell-labeling solution	1 mL
V35004	Vybrant® DyeCycle™ Green stain *5 mM solution in DMSO* *200 assays*	400 µL
V35005	Vybrant® DyeCycle™ Orange stain *5 mM solution in DMSO* *200 assays*	400 µL
V10309	Vybrant® DyeCycle™ Ruby stain *2.5 mM solution in DMSO* *100 assays*	100 µL
V10273	Vybrant® DyeCycle™ Ruby stain *2.5 mM solution in DMSO* *400 assays*	400 µL
V35003	Vybrant® DyeCycle™ Violet stain *5 mM in water* *200 assays*	200 µL
V13154	Vybrant® MTT Cell Proliferation Assay Kit *1000 assays*	1 kit
Y3601	YOYO®-1 iodide (491/509) *1 mM solution in DMSO*	200 µL

15.5 Assays for Apoptosis and Autophagy

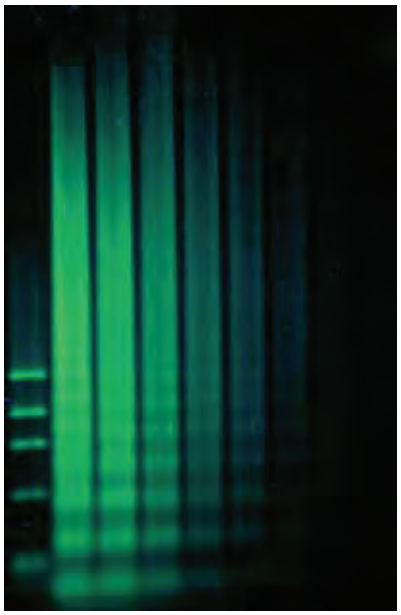

Figure 15.5.1 DNA extracts from camptothecin-treated HL-60 cells separated on an agarose gel and stained with SYBR® Green I nucleic acid gel stain (S7563, S7567, S7585). The 200 to 5000 bp DNA fragments characteristic of apoptotic cells (which appear as "ladders") are clearly visualized with this sensitive nucleic acid stain. Cell preparations were gifts of Zbigniew Darzynkiewicz, Cancer Research Institute, New York Medical College.

Apoptosis (programmed cell death) is the genetically controlled ablation of cells during normal development. Inappropriately regulated apoptosis is implicated in disease states such as Alzheimer disease, stroke and cancer.[1,2] Apoptosis is distinct from necrosis in both the biochemical and the morphological changes that occur. In contrast to necrotic cells, apoptotic cells are characterized morphologically by compaction of the nuclear chromatin, shrinkage of the cytoplasm and production of membrane-bound apoptotic bodies. Biochemically, apoptosis is distinguished by fragmentation of the genome and cleavage or degradation of several cellular proteins.

As with cell viability, no single parameter fully defines cell death in all systems; therefore, it is often advantageous to use several different approaches when studying apoptosis. Several methods have been developed to distinguish live cells from early and late apoptotic cells and from necrotic cells; these are described below and in a number of review articles.[3–5] Anti-cancer drug candidates failing to induce apoptosis are likely to have decreased clinical efficacy, making apoptosis assays important tools for high-throughput drug screening.[6–9]

Apoptosis Assays Using Nucleic Acid Stains

DNA Stains for Detecting Apoptotic Cells

The characteristic breakdown of the nucleus during apoptosis comprises collapse and fragmentation of the chromatin, degradation of the nuclear envelope and nuclear blebbing, resulting in the formation of micronuclei. Therefore, nucleic acid stains can be useful tools for identifying even low numbers of apoptotic cells in cell populations. Several nucleic acid stains, all of which are listed in Section 8.1, have been used to detect apoptotic cells by fluorescence imaging or flow cytometry.[10–13]

DNA fragmentation can also be detected *in vitro* using electrophoresis. DNA extracted from apoptotic cells and then separated by gel electrophoresis reveals a characteristic ladder pattern of low molecular weight DNA fragments.[14] Our ultrasensitive SYBR® Green I nucleic acid stain (S7567, Section 8.4) allows the detection of even fewer apoptotic cells in these applications (Figure 15.5.1).

Membrane Permeability/Dead Cell Apoptosis Kit

Our Membrane Permeability/Dead Cell Apoptosis Kit (V13243) detects apoptosis based on changes that occur in the permeability of cell membranes. This kit contains ready-to-use solutions of both the YO-PRO®-1 and propidium iodide nucleic acid stains. Our YO-PRO®-1 nucleic acid stain (also available as a stand-alone reagent, Y3603) selectively passes through the plasma membranes of apoptotic cells and labels them with moderate green fluorescence.[15–19]

The dyes included in this kit are effectively excited by the 488 nm spectral line of the argon-ion laser and are useful for both flow cytometry (Figure 15.5.2) and fluorescence microscopy (Figure 15.5.3). The kit components, number of assays and assay principles are summarized in Table 15.4.

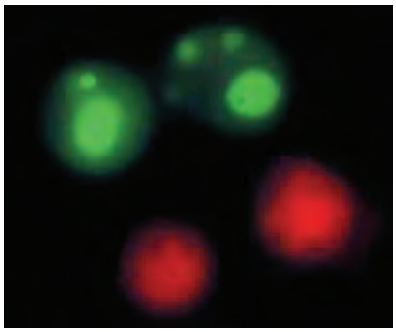

Figure 15.5.3 Apoptosis induced in Jurkat cells with 10 µM camptothecin. The cells were then treated with the reagents in the Membrane Permeability/Dead Cell Apoptosis Kit (V13243). Apoptotic cell nuclei were labeled with YO-PRO®-1 dye (green) (Y3603). Necrotic cells were detected with propidium iodide (red) (P1304MP, P3566, P21493).

Chromatin Condensation/Dead Cell Apoptosis Kit

The Chromatin Condensation/Dead Cell Apoptosis Kit (V13244) provides a rapid and convenient assay for apoptosis based upon fluorescence detection of the compacted state of the chromatin in apoptotic cells. This kit contains ready-to-use solutions of the blue-fluorescent Hoechst 33342 dye (excitation/emission maxima ~350/461 nm when bound to DNA), which stains the condensed chromatin of apoptotic cells more brightly than the chromatin of nonapoptotic cells, and the red-fluorescent propidium iodide (excitation/emission maxima ~535/617 nm when bound to DNA), which is permeant only to dead cells with compromised membranes. The staining pattern resulting from the simultaneous use of these dyes makes it possible to distinguish normal, apoptotic and dead cell populations by flow cytometry or fluorescence microscopy.[20] The 351 nm spectral line of an argon-ion laser or other suitable UV source is required for excitation of the Hoechst 33342 dye, whereas propidium iodide can be excited with the 488 nm spectral line of an argon-ion laser. The kit components, number of assays and assay principles are summarized in Table 15.4.

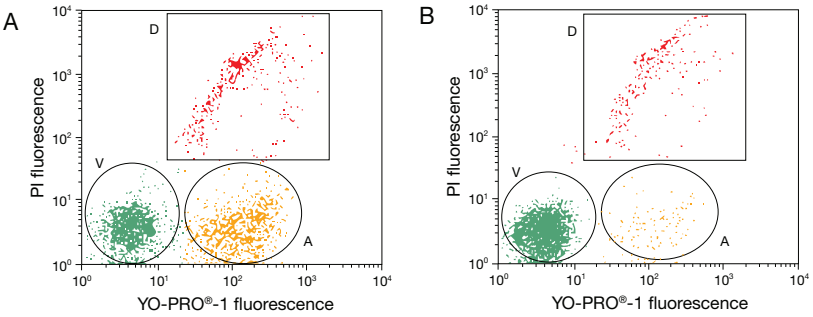

Figure 15.5.2 Flow cytometric analysis of Jurkat cells using the Membrane Permeability/Dead Cell Apoptosis Kit (V13243). Jurkat human T-cell leukemia cells were first exposed to 10 µM camptothecin for 4 hours (**A**) or left untreated (as control, **B**). Cells were then treated with the reagents in the kit and analyzed by flow cytometry. Note that the camptothecin-treated cells (**A**) have a significantly higher percentage of apoptotic cells (indicated by an "A") than the basal level of apoptosis seen in the control cells (**B**). V = viable cells, D = dead cells.

Table 15.4 Molecular Probes® apoptosis assay kits.

Cat. No.	Kit Name	Probe(s) for Apoptotic Cells (Abs/Em) *	Probe for Necrotic or Live Cells (Abs/Em) *	Number of Assays	Kit Features
V13240	Single Channel Annexin V/Dead Cell Apoptosis Kit	Alexa Fluor® 488 annexin V (495/519)	SYTOX® Green nucleic acid stain (504/523)	50 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	Because this Alexa Fluor® 488 annexin V–based assay uses only the green fluorescence channel on the flow cytometer, other parameters can be measured simultaneously using fluorescent probes that have different emission spectra.
V13241 V13245	Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit	Alexa Fluor® 488 annexin V (495/519)	Propidium iodide (535/617)	50 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	Apoptotic cells are labeled with annexin V conjugated to our exceptionally bright and photostable green-fluorescent Alexa Fluor® 488 dye. Necrotic cells are labeled with red-fluorescent propidium iodide.
V13242	FITC Annexin V/ Dead Cell Apoptosis Kit	FITC annexin V (494/519)	Propidium iodide (535/617)	50 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	Similar to Kit V13241 except that it contains the fluorescein conjugate of annexin V.
V13243	Membrane Permeability/Dead Cell Apoptosis Kit	YO-PRO®-1 dye (491/509)	Propidium iodide (535/617)	200 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	This assay detects changes in cell membrane permeability with YO-PRO®-1 dye, a green-fluorescent nucleic acid stain that is permeant to apoptotic cells but not to live cells. Necrotic cells are labeled with red-fluorescent propidium iodide.

* Approximate absorption and emission maxima, in nm.

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Table 15.4 Molecular Probes® apoptosis assay kits—*continued*.

Cat. No.	Kit Name	Probe(s) for Apoptotic Cells (Abs/Em) *	Probe for Necrotic or Live Cells (Abs/Em) *	Number of Assays	Kit Features
V13244	Chromatin Condensation/Dead Cell Apoptosis Kit	Hoechst 33342 (346/460)	Propidium iodide (535/617)	200 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	This assay uses Hoechst 33342 in combination with propidium iodide to distinguish between the condensed chromatin of apoptotic cells and the looser chromatin structure in live cells.
V23200	Vybrant® Apoptosis Assay Kit #6	Biotin-X annexin V and Alexa Fluor® 350 streptavidin (345/442)	Propidium iodide (535/617)	50 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	Apoptotic cells are labeled with the biotin-X conjugate of annexin V in conjunction with blue-fluorescent Alexa Fluor® 350 streptavidin. Necrotic cells are labeled with red-fluorescent propidium iodide.
V23201	Chromatin Condensation/ Membrane Permeability/Dead Cell Apoptosis Kit	Hoechst 33342 (346/460) and YO-PRO®-1 dye (491/509)	Propidium iodide (535/617)	200 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	This kit is a combination of Kits V13243 and V13244. All three dyes can be excited by a UV laser, or by a combination of UV and 488 nm excitation.
V35112	PE Annexin V/Dead Cell Apoptosis Kit	R-phycoerythrin (R-PE) annexin V (496/578)	SYTOX® Green nucleic acid stain (504/523)	50 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	Apoptotic cells are labeled with annexin V conjugated to the intensely orange-fluorescent R-phycoerythrin (R-PE). Necrotic cells are labeled with the green-fluorescent SYTOX® Green nucleic acid stain. Both probes can be excited by the 488 nm spectral line of the argon-ion laser.
V35113	APC Annexin V/Dead Cell Apoptosis Kit	Allophycocyanin annexin V (650/660)	SYTOX® Green nucleic acid stain (504/523)	50 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	This kit is similar to Kit V35112 except that it contains the allophycocyanin conjugate of annexin V. Apoptotic cells are labeled with the intensely far-red–fluorescent allophycocyanin annexin V. Necrotic cells are labeled with green-fluorescent SYTOX® Green nucleic acid stain. These populations can easily be distinguished using a flow cytometer equipped with both the 488 nm spectral line of the argon-ion laser and the 633 nm spectral line of the He-Ne laser for excitation.
V35114	Metabolic Activity/ Annexin V/Dead Cell Apoptosis Kit	Allophycocyanin annexin V (650/660)	SYTOX® Green nucleic acid stain (504/523)	50 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	This kit is identical to Kit V35113 except that it also contains C_{12}-resazurin, which is reduced by viable cells to the orange-fluorescent C_{12}-resorufin (Abs/Em = 571/585 nm). Apoptotic, necrotic and live cell populations can easily be distinguished using a flow cytometer equipped with both the 488 nm spectral line of the argon-ion laser and the 633 nm spectral line of the He-Ne laser for excitation.
V35116	Mitochondrial Membrane Potential/Annexin V Apoptosis Kit	Alexa Fluor® 488 annexin V (495/519)	MitoTracker® Red CMXRos (578/599)	50 flow cytometry assays, each containing 2×10^5 to 1×10^6 cells in a 1 mL volume	Apoptotic cells are labeled with annexin V conjugated to our exceptionally bright and photostable green-fluorescent Alexa Fluor® 488 dye. Live cells are labeled with MitoTracker® Red CMXRos, which exhibits bright red fluorescence in the presence of a mitochondrial transmembrane potential. Apoptotic and live cell populations can easily be distinguished using a flow cytometer and the 488 nm spectral line of the argon-ion laser.
V35123	Violet Membrane Permeability/Dead Cell Apoptosis Kit	PO-PRO™-1 dye (435/455)	7-Aminoactinomycin D (7-AAD) (546/650)	200 flow cytometry assays, each containing 1×10^6 cells in a 1 mL volume	This assay detects changes in cell membrane permeability with PO-PRO™-1 dye, a violet-fluorescent nucleic acid stain that is permeant to apoptotic cells but not to live cells. Necrotic cells are labeled with red-fluorescent 7-AAD. Apoptotic and necrotic cell populations can easily be distinguished using a flow cytometer equipped with a violet laser and an argon-ion laser.
A35135	Violet Chromatin Condensation/Dead Cell Apoptosis Kit	Vybrant® DyeCycle™ Violet dye (370/440)	SYTOX® AADvanced™ dead cell stain (546/647)	200 flow cytometry assays, each containing 1×10^6 cells in a 1 mL volume	This assay uses Vybrant® DyeCycle™ Violet stain to stain the condensed chromatin of apoptotic cells more brightly than the chromatin of normal cells. The SYTOX® AADvanced™ stain labels only necrotic cells, based on membrane integrity. The staining pattern resulting from the simultaneous use of these stains makes it possible to distinguish normal, apoptotic and necrotic cell populations by flow cytometry.
A35136	Violet Annexin V/Dead Cell Apoptosis Kit	Pacific Blue™ annexin V conjugate (415/455)	SYTOX® AADvanced™ dead cell stain (546/647)	50 flow cytometry assays, each containing 1×10^6 cells in a 1 mL volume	The violet-excitable Pacific Blue™ annexin V conjugate stains phosphatidylserine in the exposed cell membrane surface. The SYTOX® AADvanced™ stain labels only necrotic cells, based on membrane integrity. With this assay, apoptotic cells show bright violet fluorescence, dead cells show red fluorescence, and live cells show dim violet fluorescence, allowing users to rapidly and reliably distinguish these populations.
A35137	Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit	F2N12S (405/530 for apoptotic and dead cells, 405/585 for live cells)	SYTOX® AADvanced™ dead cell stain (546/647)	100 flow cytometry assays, each containing 1×10^6 cells in a 1 mL volume	This assay detects changes in cell membrane asymmetry using the ratiometric 405 nm–excitable F2N12S dye and SYTOX® AADvanced™ stain for dead cell detection. Apoptotic and necrotic cell populations can easily be distinguished using a flow cytometer equipped with a violet laser and an argon-ion laser.

* Approximate absorption and emission maxima, in nm.

Chromatin Condensation/Membrane Permeability/Dead Cell Apoptosis Kit

The Chromatin Condensation/Membrane Permeability/Dead Cell Apoptosis Kit (V23201) combines the detection principles used in the two related kits described above. Three nucleic acid stains—Hoechst 33342, YO-PRO®-1 and propidium iodide—are utilized to identify the blue-fluorescent live-cell population, the green-fluorescent apoptotic population and the red-fluorescent dead-cell population by flow cytometry. The stains are provided as separate solutions to facilitate optimization of the assay for the cell line under study and the equipment available. Once optimized, however, the assay can be completed using simultaneous staining with a mixture of the three nucleic acid stains and either UV excitation of all three dyes or with a combination of UV excitation for the Hoechst 33342 dye and excitation by the 488 nm spectral line of the argon-ion laser. The kit components, number of assays and assay principles are summarized in Table 15.4.

Violet Membrane Permeability/Dead Cell Apoptosis Kit

Like the Membrane Permeability/Dead Cell Apoptosis Kit, our Violet Membrane Permeability/Dead Cell Apoptosis Kit (V35123) detects apoptosis based on changes that occur in the permeability of cell membranes (Table 15.4). This kit contains ready-to-use solutions of both PO-PRO™-1 and 7-aminoactinomycin (7-AAD) nucleic acid stains. Our PO-PRO™-1 nucleic acid stain (also available as a stand-alone reagent, P3581) selectively passes through the plasma membranes of apoptotic cells and labels them with violet fluorescence. Furthermore, annexin V labeling of apoptosis yields poor results with trypsinized cells, whereas PO-PRO™-1 dye provides the same efficiency for detecting apoptosis with trypsinized cells as it does with suspension cells. Necrotic cells are stained with the red-fluorescent 7-AAD, a DNA-selective dye that is membrane impermeant but that easily passes through the compromised plasma membranes of necrotic cells. Live cells are not appreciably stained by either PO-PRO™-1 or 7-AAD. The dyes included in this kit are effectively excited by a flow cytometer that uses both the 405 nm spectral line of the violet laser and the 488 nm spectral line of the argon-ion laser for excitation. The kit components, number of assays and assay principles are summarized in Table 15.4.

Comet (Single-Cell Gel Electrophoresis) Assay to Detect Damaged DNA

The Comet assay, or single-cell gel electrophoresis assay, is used for rapid detection and quantitation of DNA damage from single cells.[21,22] The Comet assay is based on the alkaline lysis of labile DNA at sites of damage. Cells are immobilized in a thin agarose matrix on slides and gently lysed. When subjected to electrophoresis, the unwound, relaxed DNA migrates out of the cells. After staining with a nucleic acid stain, cells that have accumulated DNA damage appear as fluorescent comets, with tails of DNA fragmentation or unwinding (Figure 15.5.4). In contrast, cells with normal, undamaged DNA appear as round dots, because their intact DNA does not migrate out of the cell. The ease and sensitivity of the Comet assay has provided a fast and convenient way to measure damage to human sperm DNA,[23] evaluate DNA replicative integrity,[24] monitor the sensitivity of tumor cells to radiation damage [25] and assess the sensitivity of molluscan cells to toxins in the environment.[26] The Comet assay can also be used in combination with FISH to identify specific sequences with damaged DNA.[25] Comet assays have traditionally been performed using ethidium bromide to stain the DNA; [21] however, use of the SYBR® Gold and SYBR® Green I stains [27,28] improves the sensitivity of this assay (Figure 15.5.4).

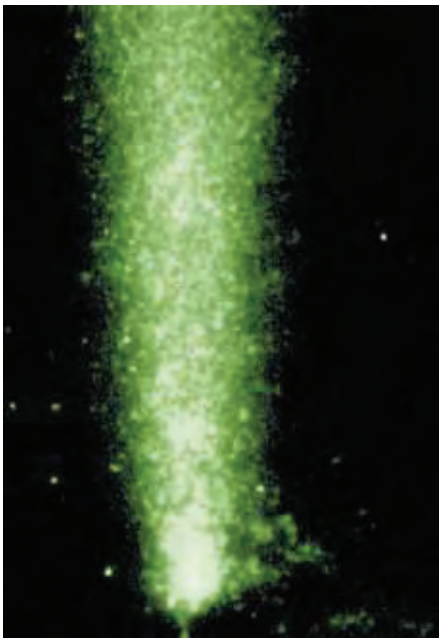

Figure 15.5.4 Comet assay with SYBR® Green I nucleic acid gel stain (S7563, S7567, S7585). DNA fragmentation associated with oxidative DNA damage was visualized using Trevigen's CometAssay® kit. HL-60 cells were treated with H_2O_2 and immobilized onto a Trevigen CometSlide™ for analysis. The cells were gently lysed, washed and treated with endonuclease. Slides were subjected to electrophoresis in alkaline electrophoresis buffer and stained with SYBR® Green I stain.

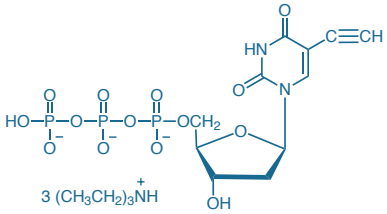

Figure 15.5.5 The EdUTP nucleotide, provided in the Click-iT® TUNEL Imaging Assay Kits.

Apoptosis Assays that Detect DNA Strand Breaks

Click-iT® TUNEL Alexa Fluor® Imaging Assays

The terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay—based on the incorporation of modified dUTPs by terminal deoxynucleotidyl transferase (TdT) at the 3′-OH ends of fragmented DNA—is probably the most widely used *in situ* test for studying apoptotic DNA fragmentation.[29,30] For a sensitive and reliable TUNEL imaging assay, it is vital that the modified nucleotide is an efficient substrate for TdT. We have developed a Click-iT® TUNEL imaging assay that incorporates an alkyne-modified dUTP (Figure 15.5.5) at the 3′-OH ends of fragmented DNA using TdT and then detects the enzymatically

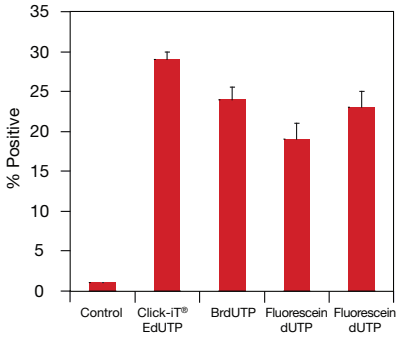

Figure 15.5.7 TUNEL assay comparison—percentage positives detected. HeLa cells were treated with 0.5 µM staurosporine for 4 hours. Following fixation and permeabilization, TUNEL imaging assays were performed according to the manufacturer's instructions. The percent positives were calculated based upon the corresponding negative control. Imaging and analysis was performed using a Thermo Fisher Scientific Cellomics® ArrayScan® II.

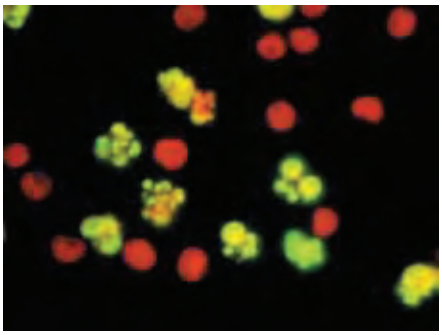

Figure 15.5.8 Human lymphoma cells treated with camptothecin for 4 hours and stained using the APO-BrdU™ TUNEL Assay Kit (A23210). Cells containing DNA strand nicks characteristic of apoptosis are detected by TUNEL and fluoresce green, while necrotic cells are stained with red-fluorescent propidium iodide.

incorporated nucleotide using a copper (I)–catalyzed click reaction with an azide-derivatized fluorophore (Figure 15.5.6).

The Click-iT® labeling reaction is based on a copper-catalyzed azide–alkyne cyloaddition [31,32] and derives its high degree of specificity from the fact that the azide and alkyne reaction partners have no endogenous representation in biological molecules, cells, tissues or model organisms.[33–35] The minimally modified EdUTP nucleotide (Figure 15.5.5) used in the Click-iT® TUNEL imaging assay is rapidly incorporated by TdT, allowing samples to be rapidly fixed in order to preserve late-stage apoptotic cells, thereby lessening the possibility of false-negative results due to cell detachment and subsequent loss. Compared with assays that use one-step incorporation of dye-modified nucleotides, the fast and reliable Click-iT® TUNEL imaging assay can detect a higher percentage of apoptotic cells under identical conditions in two hours or less (Figure 15.5.7). The Click-iT® TUNEL Imaging Assay Kits are available with a choice of azide-derivatized Alexa Fluor® dyes, providing flexibility for combination with other apoptosis detection reagents. They include:

- Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay (C10245)
- Click-iT® TUNEL Alexa Fluor® 594 Imaging Assay (C10246)
- Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay (C10247)

The Click-iT® TUNEL assays have been tested in HeLa, A549 and CHO K1 cells with a variety of reagents that induce apoptosis, including staurosporine, and multiplexed with antibody-based detection of other apoptosis biomarkers such as cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3 and phosphohistone 2B. It has also proven effective for detection of apoptosis induced by siRNA knockdown of the DEC2 transcription factor in human MCF-7 breast cancer cells.[36] Click-iT® labeling technology and the details of the click reaction are discussed in Section 3.1. For a complete list azide and alkyne derivatives compatible with Click-iT® labeling technology, see Table 3.1.

APO-BrdU™ TUNEL Assay Kit

Because DNA fragmentation is one of the most reliable methods for detecting apoptosis, we have collaborated with Phoenix Flow Systems to offer the APO-BrdU™ TUNEL Assay Kit (A23210), which provides all the materials necessary to label and detect the DNA strand breaks of apoptotic cells.[30] When DNA strands are cleaved or nicked by nucleases, a large number of 3′-hydroxyl ends are exposed. In the APO-BrdU™ assay, these ends are labeled with BrdUTP and terminal deoxynucleotidyl transferase (TdT) using the TUNEL technique described above. Once incorporated into the DNA, BrdU is detected using an Alexa Fluor® 488 dye–labeled anti-BrdU monoclonal antibody (Figure 15.5.8). This kit also provides propidium iodide for determining total cellular DNA content, as well as fixed control cells for assessing assay performance.

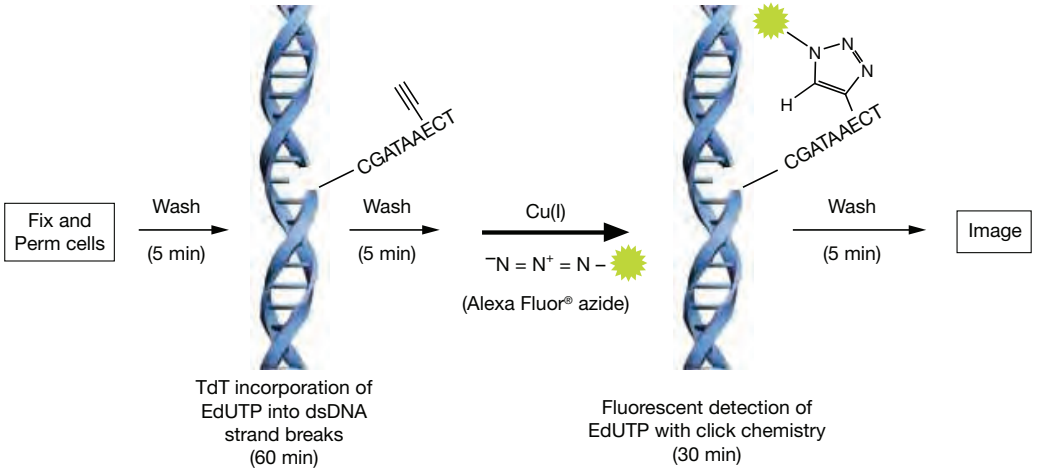

Figure 15.5.6 Detection of apoptosis with the Click-iT® TUNEL imaging assay.

The APO-BrdU™ TUNEL Assay Kit includes complete protocols for use in flow cytometry applications, though it may also be adapted for use with fluorescence microscopy. Each kit contains:

- Terminal deoxynucleotidyl transferase (TdT), for catalyzing the addition of BrdUTP at the break sites
- 5-Bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP)
- Alexa Fluor® 488 dye–labeled anti-BrdU mouse monoclonal antibody PRB-1, for detecting BrdU labels
- Propidium iodide/RNase staining buffer, for quantitating total cellular DNA
- Reaction, wash and rinse buffers
- Positive control cells (a fixed human lymphoma cell line)
- Negative control cells (a fixed human lymphoma cell line)
- Detailed protocols

Sufficient reagents are provided for approximately 60 assays of 1 mL samples, each containing approximately 1 x 10^6 cells/mL.

Detecting DNA Strand Breaks with ChromaTide® Nucleotides

Break sites have traditionally been labeled with biotinylated dUTP, followed by subsequent detection with an avidin or streptavidin conjugate [37–40] (Section 7.6, Table 7.9). However, a more direct approach for detecting DNA strand breaks in apoptotic cells is possible via the use of our ChromaTide® BODIPY® FL-14-dUTP (C7614) as a TdT substrate [41,42] (Figure 15.5.9). The single-step BODIPY® FL dye–based assay has several advantages over indirect detection of biotinylated or haptenylated nucleotides, including fewer protocol steps and increased cell yields. BODIPY® FL dye–labeled nucleotides have also proven superior to fluorescein-labeled nucleotides for detection of DNA strand breaks in apoptotic cells because they provide stronger signals, a narrower emission spectrum and less photobleaching [41] (Figure 15.5.9).

In situ DNA modifications by labeled nucleotides have been used to detect DNA fragmentation in what may be apoptotic cells in autopsy brains of Huntingtons and Alzheimer disease patients.[43–46] DNA fragmentation is also associated with amyotrophic lateral sclerosis.[47]

Analogous to TdT's ability to label double-strand breaks, the *E. coli* repair enzyme DNA polymerase I can be used to detect single-strand nicks,[48,49] which appear as a relatively early step in some apoptotic processes.[50–52] Because our ChromaTide® BODIPY® FL-14-dUTP (C7614) and ChromaTide® fluorescein-12-dUTP [53,54] (C7604) are incorporated into DNA by *E. coli* DNA polymerase I, they are also effective for *in situ* labeling with the nick translation method.[55]

High-Content Analysis of Genotoxicity and Cytotoxicity

In mammalian cells, a double-strand break (DSB) in genomic DNA is a potentially lethal lesion. One of the earliest known responses to DSB formation is phosphorylation of H2A histones. Specifically, DNA damaging agents induce phosphorylation of histone variant H2AX at Ser139, leading to the formation of DNA foci at the site of DNA DSBs.[56–58]

The HCS DNA Damage Kit (H10292) was developed to enable simultaneous quantitation of two cell health parameters, genotoxicity and cytotoxicity, by high-content analysis in the same cell (Figure 15.5.10). DNA damage is measured as an indication of genotoxicity and accomplished by specific antibody-based detection of phosphorylated H2AX (Ser139) in the nucleus. Cytotoxicity is measured with the Image-iT® DEAD Green™ viability stain (also available as a stand-alone reagent, I10291),

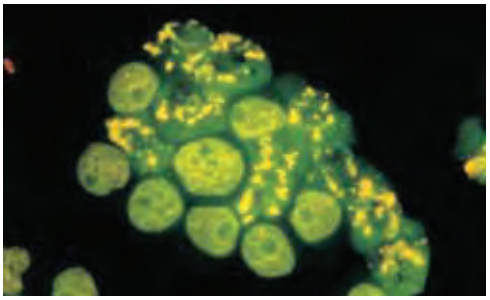

Figure 15.5.9 HL-60 cells treated with camptothecin for 3 hours. The DNA strand nicks characteristic of apoptosis were detected with the TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling) assay using the fluorescently labeled nucleotide ChromaTide® BODIPY® FL-14-dUTP (C7614). Image contributed by Zbigniew Darzynkiewicz, Cancer Research Institute, New York Medical College.

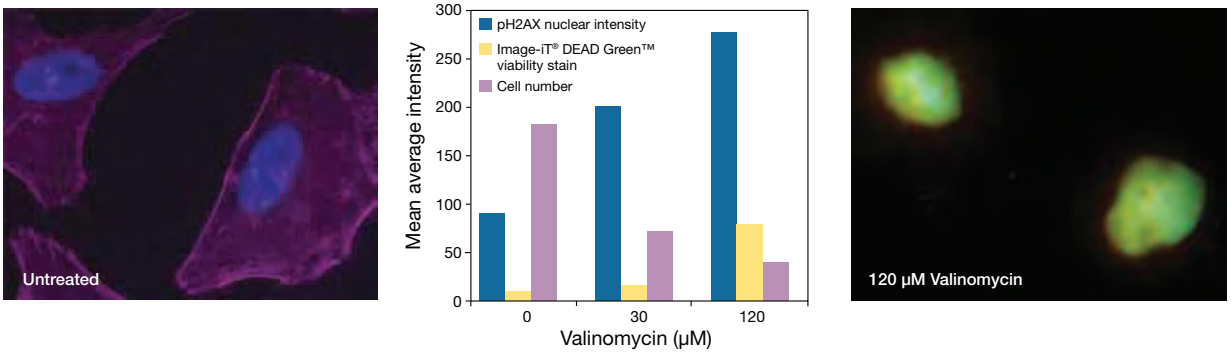

Figure 15.5.10 Detection of genotoxicity and cytotoxicity in valinomycin-treated A549 cells using the HCS DNA Damage Kit (H10292). A549 cells were treated with 30 μM or 120 μM valinomycin for 24 hr before performing the assay. With increasing concentrations of valinomycin, cells showed genotoxic effects as indicated by detection with a pH2AX antibody in conjunction with Alexa Fluor® 555 goat anti–mouse IgG antibody (orange fluorescence), and cytotoxic effects as indicated by staining with the Image-iT® DEAD Green™ viability stain (green fluorescence). Blue-fluorescent Hoechst 33342 was used as a nuclear segmentation tool, and Alexa Fluor® 647 phalloidin was used to visualize F-actin (pseudocolored magenta). The image on the left shows untreated cells with intact F-actin cytoskeletons and no evidence of cytotoxicity or genotoxicity. The image on the right shows cells treated with 120 μM valinomycin, which completely disrupted the actin cytoskeletons, increased levels of DNA damage and compromised plasma membrane integrity.

a cell-impermeant, nonfluorescent, high-affinity DNA stain that forms highly fluorescent and stable dye–nucleic acid complexes when bound to DNA. Thus, staining of nuclear DNA by the Image-iT® DEAD Green™ viability stain cannot occur in live cells due to the impermeability of the plasma membrane to the stain. Drugs and test compounds that lead to serious cell injuries, including plasma membrane permeability, allow entry of the Image-iT® DEAD Green™ viability stain, enabling discrimination of dead cells. Hoechst 33342, which stains nuclear DNA in live and dead cells, is included in the kit as a segmentation tool for automated image analysis.

The HCS DNA Damage Kit contains sufficient material to perform the DNA damage assay on two 96-well plates when used as described in the protocol provided:

- Image-iT® Dead Green viability stain
- pH2AX mouse monoclonal antibody
- Alexa Fluor® 555 goat anti–mouse IgG antibody
- Hoechst 33342 nucleic acid stain
- Detailed protocols

Because no single parameter fully defines cell viability in all systems, it is often useful to use multiple approaches to study cytotoxicity. In addition to the HCS DNA Damage Kit, we offer the HCS LIVE/DEAD® Green Kit (H10290) and the HCS Mitochondrial Health Kit (H10295), both of which employ the Image-iT® DEAD™ Green viability stain. Additionally, the HCS LIVE/DEAD® Kit provides a choice of two nucleic acid stains—the far-red–fluorescent HCS NuclearMask™ Deep Red stain (H10294, Section 12.5) and the blue-fluorescent Hoechst 33342 (H1399, H3570, H21492; Section 12.5)—for use as cell-permeant nuclear segmentation tools. The HCS Mitochondrial Health Kit provides Image-iT® DEAD Green viability stain and Hoechst 33342 stain, as well as the MitoHealth stain, which accumulates in the mitochondria of live cells in proportion to the mitochondrial membrane potential. Both of these kits provide sufficient reagents to perform the assays on two 96-well plates, using the protocol provided.

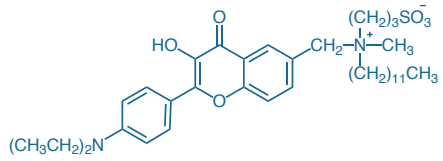

Figure 15.5.11 F2N12S, a component of the Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit (A35137).

Apoptosis Assays that Detect Membrane Asymmetry

Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit

The Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit (A35137) provides a simple and fast method for detecting apoptosis with dead-cell discrimination by flow cytometry. The violet ratiometric membrane asymmetry probe, F2N12S (4′-*N*,*N*-diethylamino-6-(*N*-dodecyl-*N*-methyl-*N*-(3-sulfopropyl))ammoniomethyl-3-hydroxyflavone, Figure 15.5.11), is a novel violet diode–excitable dye for the detection of membrane phospholipid asymmetry changes during apoptosis. This dye exhibits an excited-state intramolecular proton transfer (ESIPT) reaction, resulting in a dual fluorescence with two emission bands corresponding to 530 nm and 585 nm and producing a two-color ratiometric response to variations in surface charge.[59] This ratiometric probe is therefore a self-calibrating indicator of apoptotic transformation, which is independent of probe concentration, cell size and instrument variation, such as fluctuations of laser intensity or sensitivity of the detectors.

Given that apoptosis modifies the surface charge of the outer leaflet of the plasma membrane, F2N12S can be used to monitor changes in membrane asymmetry that occur during apoptosis through a change in the relative intensity of the two emission bands of the dye[59] (Figure 15.5.12). The F2N12S-based apoptosis assay allows samples to be analyzed after a 5-minute incubation at room temperature and does not require special buffers or wash steps. This kit can be paired with other reagents such as MitoProbe™ $DiIC_1(5)$ or annexin V for multiparametric analysis of apoptosis and viability. The kit components, number of assays and assay principles are summarized in Table 15.4.

Annexin V Conjugates

The human vascular anticoagulant annexin V is a 35–36 kilodalton, Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for the anionic phospholipid phosphatidylserine (PS). In normal

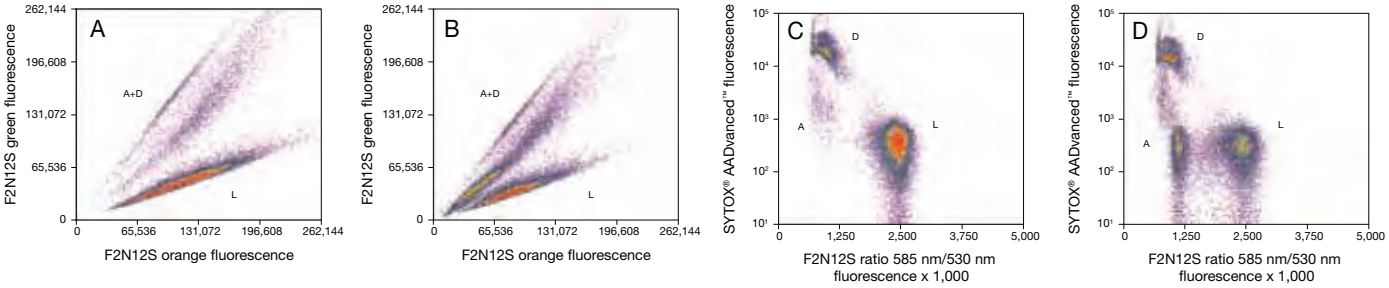

Figure 15.5.12 Jurkat cells (T-cell leukemia, human) treated with 10 µM camptothecin for 4 hours (**B** and **D**) or untreated (**A** and **C**), control. Cells were stained according to the protocol. Samples were analyzed on a flow cytometer with 405 nm excitation using 585 nm and 530 nm bandpass filters for F2N12S and 488 nm excitation for SYTOX® AADvanced™ dead cell stain using a 695 nm bandpass filter. In panels A and B, live cells can be discriminated from apoptotic and dead cells by the relative intensities of the two emission bands from F2N12S. In C and D, SYTOX® AADvanced™ dead cell stain fluorescence is plotted against a derived ratio parameter from the two emission bands (585/530 nm) of F2N12S. A = apoptotic cells, L = live cells, D = dead cells.

viable cells, PS is located on the cytoplasmic surface of the cell membrane. In apoptotic cells, however, PS is translocated from the inner to the outer leaflet of the plasma membrane, exposing PS to the external cellular environment where it can be detected by annexin V conjugates.[60] In leukocyte apoptosis, PS on the outer surface of the cell marks the cell for recognition and phagocytosis by macrophages.[61,62]

Highly fluorescent annexin V conjugates provide quick and reliable detection methods for studying the externalization of phosphatidylserine,[63–65] an indicator of intermediate stages of apoptosis. Nuclear fragmentation, mitochondrial membrane potential flux and caspase-3 activation apparently precede phosphatidylserine "flipping" during apoptosis, whereas permeability to propidium iodide and cytoskeletal collapse occur later. The difference in fluorescence intensity between apoptotic and nonapoptotic cells stained by our fluorescent annexin V conjugates, as measured by flow cytometry, is typically about 100-fold (Figure 15.5.13). Our annexin V conjugates are available as stand-alone reagents, each suitable for 50–100 flow cytometry assays or many more imaging assays, or in several variations of our apoptosis assay kits (Table 15.4). Our annexin V conjugates include:

- Alexa Fluor® 488 annexin V [66] (A13201, Figure 15.5.14)
- Fluorescein (FITC) annexin V (A13199)
- Oregon Green® 488 annexin V (A13200)
- Alexa Fluor® 555 annexin V (A35108)
- R-phycoerythrin (R-PE) annexin V (A35111)
- Alexa Fluor® 568 annexin V (A13202)
- Alexa Fluor® 594 annexin V [67,68] (A13203)
- Alexa Fluor® 647 annexin V [64] (A23204)
- Allophycocyanin (APC) annexin V (A35110)
- Alexa Fluor® 680 annexin V [69] (A35109)
- Alexa Fluor® 350 annexin V (A23202)
- Pacific Blue™ annexin V (A35122)
- Biotin-X annexin V [65] (A13204)

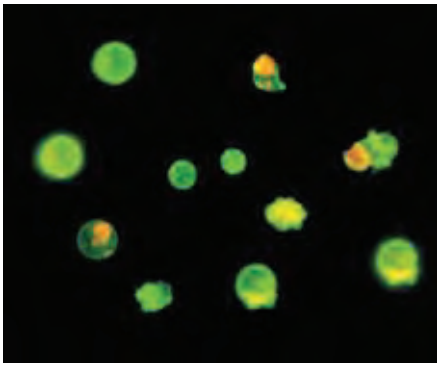

Figure 15.5.14 Jurkat human T-cell leukemia cells treated with 1 µM camptothecin. The externalized phosphatidylserine, a characteristic of early-stage apoptotic cells, was detected with Alexa Fluor® 488 annexin V (A13201). The late-stage apoptotic and necrotic cells were stained with propidium iodide (P1304MP, P3566, P21493). The image was acquired using bandpass filters appropriate for fluorescein.

Single Channel Annexin V/Dead Cell Apoptosis Kit

With the Single Channel Annexin V/Dead Cell Apoptosis Kit (V13240), apoptotic cells are detected based on the externalization of phosphatidylserine. This kit contains recombinant annexin V conjugated to the Alexa Fluor® 488 dye, our brightest and most photostable green fluorophore, to permit maximum sensitivity. In addition, the kit includes a ready-to-use solution of the SYTOX® Green nucleic acid stain. The SYTOX® Green dye is impermeant to live cells and apoptotic cells but stains necrotic cells with intense green fluorescence by binding to cellular nucleic acids. After staining a cell population with Alexa Fluor® 488 annexin V and SYTOX® Green dye in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show a higher level of green fluorescence and live cells show little or no fluorescence (Figure 15.5.15). These populations can easily be distinguished using a flow cytometer with the 488 nm spectral line of

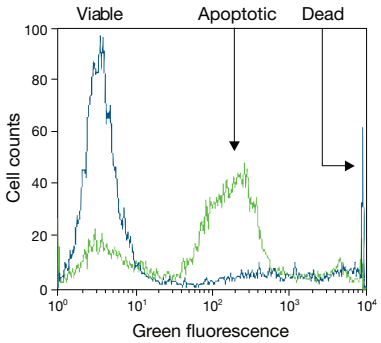

Figure 15.5.15 Flow cytometric analysis of Jurkat cells using the Single Channel Annexin V/Dead Cell Apoptosis Kit (V13240). Jurkat human T-cell leukemia cells were first exposed to 10 µM camptothecin for four hours green line) or left untreated (as control, blue line). Cells were then treated with the reagents in the kit and analyzed by flow cytometry. Note that the camptothecin-treated cells (green line) have a significantly higher percentage of apoptotic cells (intermediate green fluorescence) than the basal level of apoptosis seen in the control cells (blue line).

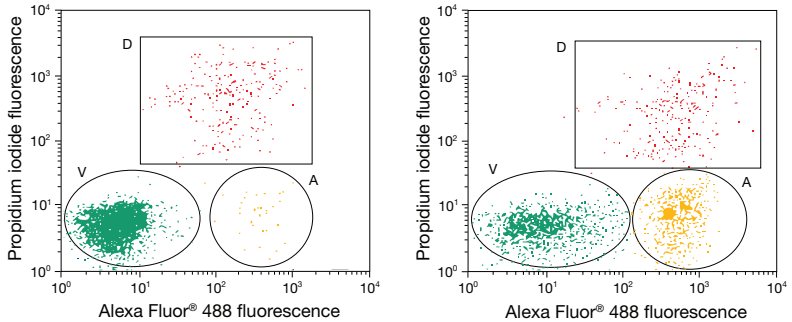

Figure 15.5.13 Flow cytometric analysis of Jurkat cells using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (V13241). Jurkat human T-cell leukemia cells were first exposed to 10 µM camptothecin for 4 hours (right) or left untreated (as control, left). Cells were then treated with the reagents in the kit and analyzed by flow cytometry. Note that the camptothecin-treated cells have a significantly higher percentage of apoptotic cells (indicated by an "A") than the basal level of apoptosis seen in the control cells. V = viable cells, D = dead cells.

an argon-ion laser for excitation. Both Alexa Fluor® 488 annexin and the SYTOX® Green dye emit a green fluorescence that can be detected in the green channel, freeing the other channels for the detection of additional probes in multicolor labeling experiments. The kit components, number of assays and assay principles are summarized in Table 15.4.

Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit

Like the Single Channel Annexin V/Dead Cell Apoptosis Kit, our Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (V13241) detects the externalization of phosphatidylserine in apoptotic cells.[70] This kit provides a sensitive two-color assay that employs our green-fluorescent Alexa Fluor® 488 annexin and a ready-to-use solution of the red-fluorescent propidium iodide nucleic acid stain. Propidium iodide is impermeant to live cells and apoptotic cells but stains necrotic cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with Alexa Fluor® 488 annexin V and propidium iodide in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence (Figure 15.5.13). These populations can easily be distinguished using a flow cytometer with the 488 nm spectral line of an argon-ion laser for excitation. The kit components, number of assays and assay principles are summarized in Table 15.4.

FITC Annexin V/Dead Cell Apoptosis Kit

The FITC Annexin V/Dead Cell Apoptosis Kit (V13242) is similar to the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, except that it contains fluorescein (FITC) annexin V in place of the Alexa Fluor® 488 conjugate. The kit components, number of assays and assay principles are summarized in Table 15.4.

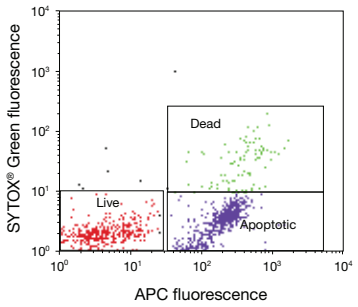

Figure 15.5.16 Flow cytometric analysis of Jurkat cells using the APC Annexin V/Dead Cell Apoptosis Kit (V35113). Jurkat human T-cell leukemia cells were first exposed to 10 µM camptothecin at 37°C, 5% CO_2. The cells were then treated with the reagents in the kit and analyzed by flow cytometry. The SYTOX® Green fluorescence versus allophycocyanin (APC) annexin fluorescence dot plot shows resolution of live, apoptotic and dead cell populations.

Vybrant Apoptosis Assay Kit

The Vybrant Apoptosis Assay Kit #6 (V23200) is similar to the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, except that it contains biotin-X annexin V and Alexa Fluor® 350 streptavidin in place of the Alexa Fluor® 488 conjugate. After staining a cell population with biotin-X annexin V in the provided binding buffer, Alexa Fluor® 350 streptavidin is added to fluorescently label the bound annexin V. Finally, propidium iodide is added to detect necrotic cells. Apoptotic cells show blue fluorescence, dead cells show red and blue fluorescence and live cells show little or no fluorescence. These populations can easily be distinguished using a flow cytometer with UV excitation for the Alexa Fluor® 350 fluorophore and 488 nm excitation for the propidium iodide. With this kit, fluorescence in the green channel is minimal. In the same experiment for apoptosis detection, the researcher can apply a green-fluorescent probe, for example an antibody labeled with the Alexa Fluor® 488 dye or with fluorescein. The kit components, number of assays and assay principles are summarized in Table 15.4.

PE Annexin V/ Dead Cell Apoptosis Kit and APC Annexin V/Dead Cell Apoptosis Kit

The PE Annexin V/ Dead Cell Apoptosis Kit and APC Annexin V/Dead Cell Apoptosis Kit (V35112, V35113) are similar to the Single Channel Annexin V/Dead Cell Apoptosis Kit, except that they contain either R-phycoerythrin (R-PE) annexin V or allophycocyanin (APC) annexin V instead of Alexa Fluor® 488 annexin V. In addition to the phycobiliprotein-conjugated annexin V, these kits include the SYTOX® Green nucleic acid stain, which is impermeant to live cells and apoptotic cells but stains necrotic cells with intense green fluorescence. After staining a cell population with R-PE annexin V and SYTOX® Green stain, apoptotic cells show orange fluorescence with very little green fluorescence, late apoptotic cells show a higher level of green and orange fluorescence and live cells show little or no fluorescence; these populations can easily be distinguished using a flow cytometer with the 488 nm spectral line of an argon-ion laser for excitation. After staining a cell population with APC annexin V and the SYTOX® Green stain, apoptotic cells show far-red fluorescence with very little green fluorescence, late apoptotic cells show a higher level of green and far-red fluorescence and live cells show little or no fluorescence (Figure 15.5.16); these populations can easily be distinguished using a flow cytometer

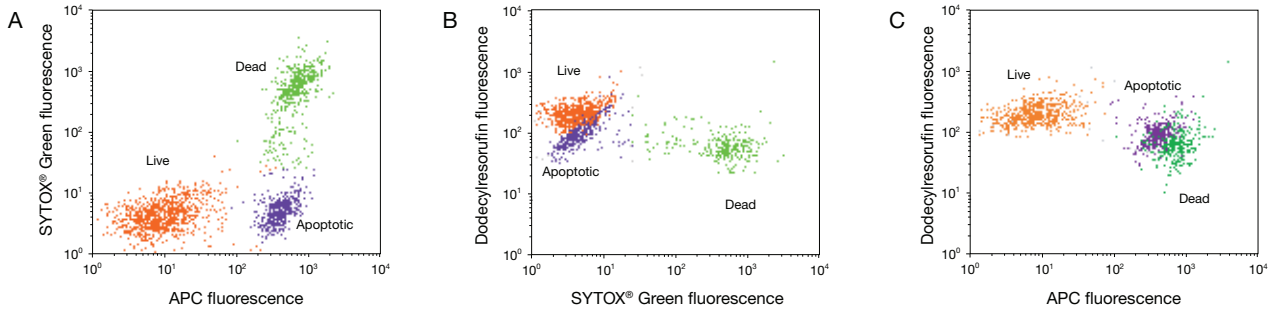

Figure 15.5.17 Flow cytometric analysis of Jurkat cells using the Metabolic Activity/Annexin V/Dead Cell Apoptosis Kit (V35114). Jurkat human T-cell leukemia cells were first exposed to either 10 µM camptothecin or 2 mM hydrogen peroxide for 4 hours at 37°C, 5% CO_2. The cells were then combined, treated with the reagents in the kit and analyzed by flow cytometry. **A**) The SYTOX® Green fluorescence versus allophycocyanin (APC) annexin fluorescence dot plot shows resolution of live, apoptotic and dead cell populations. The cell populations can be evaluated for metabolic activity using **B**) the dodecylresorufin fluorescence versus SYTOX® Green fluorescence dot plot and **C**) the dodecylresorufin fluorescence versus allophycocyanin fluorescence dot plot.

with both the 488 nm spectral line of an argon-ion laser and the 633 nm spectral line of a He-Ne laser for excitation. The kit components, number of assays and assay principles are summarized in Table 15.4.

Metabolic Activity/Annexin V/Dead Cell Apoptosis Kit

The Metabolic Activity/Annexin V/Dead Cell Apoptosis Kit (V35114) is an enhanced version of the APC Annexin V/Dead Cell Apoptosis Kit. Nonfluorescent C_{12}-resazurin is reduced by viable cells to orange-fluorescent C_{12}-resorufin.[71] After staining a cell population with allophycocyanin annexin V, C_{12}-resazurin and the SYTOX® Green stain, apoptotic cells show far-red fluorescence, intermediate orange fluorescence and no green fluorescence; late apoptotic cells show intense far-red and green fluorescence and little orange fluorescence; live cells show little or no green or far-red fluorescence but significant orange fluorescence (Figure 15.5.17). The kit components, number of assays and assay principles are summarized in Table 15.4.

Mitochondrial Membrane Potential/Annexin V Apoptosis Kit

The Mitochondrial Membrane Potential/Annexin V Apoptosis Kit (V35116) provides a rapid and convenient assay for two hallmarks of apoptosis—phosphatidylserine externalization and changes in mitochondrial membrane potential. Recombinant annexin V conjugated to the Alexa Fluor® 488 dye, our brightest and most photostable green fluorophore, provides maximum sensitivity for detecting phosphatidylserine externalization in apoptotic cells. Live cells are labeled with MitoTracker® Red CMXRos, which exhibits bright red fluorescence in the presence of a mitochondrial transmembrane potential. After staining a cell population with Alexa Fluor® 488 annexin V and MitoTracker® Red CMXRos dye in the provided binding buffer, live cells exhibit very little green fluorescence and bright red fluorescence, whereas apoptotic cells exhibit bright green fluorescence and decreased red fluorescence (Figure 15.5.18). These populations can easily be distinguished using a flow cytometer, and the 488 nm line of an argon-ion laser can be used to excite both dyes. The kit components, number of assays and assay principles are summarized in Table 15.4.

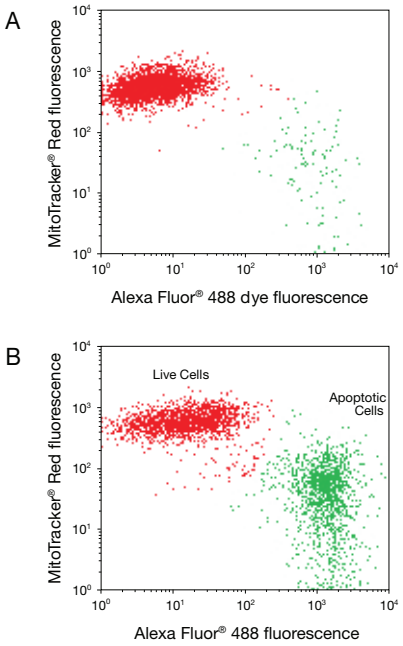

Figure 15.5.18 Flow cytometric analysis of Jurkat cells using the Mitochondrial Membrane Potential/Annexin V Apoptosis Kit (V35116). Jurkat human T-cell leukemia cells in complete medium were **B**) first exposed to 10 µM camptothecin for 4 hours or **A**) left untreated. Both cell populations were then treated with the reagents in the kit and analyzed by flow cytometry. Note that the apoptotic cells show higher reactivity for annexin V and lower MitoTracker® Red dye fluorescence than do live cells.

Violet Chromatin Condensation/Dead Cell Apoptosis Kit

The Violet Chromatin Condensation/Dead Cell Apoptosis Kit (A35135) provides a rapid and convenient assay for apoptosis based upon fluorescence analysis of the compacted state of the chromatin in apoptotic cells. The kit contains the cell-permeant Vybrant® DyeCycle™ Violet stain and the impermeant red-fluorescent SYTOX® AADvanced™ dead cell stain. The condensed chromatin of apoptotic cells are stained more brightly by Vybrant® DyeCycle™ Violet stain than the chromatin of normal cells. The SYTOX® AADvanced™ stain labels only necrotic cells, based on membrane integrity. The staining pattern resulting from the simultaneous use of these stains makes it possible to distinguish normal, apoptotic and necrotic cell populations by flow cytometry (Figure 15.5.19). The Vybrant® DyeCycle™ Violet and SYTOX® AADvanced™ stains are excited with the 405 nm violet diode laser and the 488 nm argon-ion laser, respectively.

Violet Annexin V/Dead Cell Apoptosis Kit

Like the Single Channel Annexin V/Dead Cell Apoptosis Kit, the Violet Annexin V/Dead Cell Apoptosis Kit (A35136) detects the externalization of phosphatidylserine in apoptotic cells.[70] This kit provides a sensitive two-color assay that employs our violet laser–excitable Pacific Blue™ annexin V (excitation/emission maxima ~415/460 nm) and the impermeant red-fluorescent SYTOX® AADvanced™ dead cell stain. The SYTOX® AADvanced™ stain labels only necrotic cells, based on membrane integrity. After staining a cell population with Pacific Blue™ annexin V and SYTOX® AADvanced™ stain in the provided binding buffer, apoptotic cells show blue fluorescence, dead cells show red fluorescence, and live cells show little or no fluorescence. These populations can easily be distinguished with a flow cytometer equipped with both a 405 nm violet diode laser and an argon-ion laser for excitation. The kit components, number of assays and assay principles are summarized in Table 15.4.

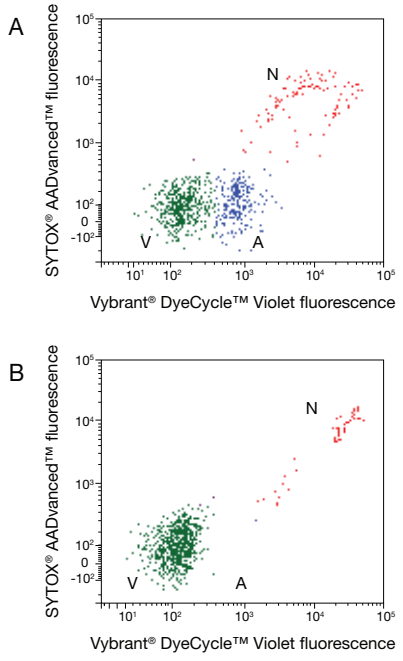

Figure 15.5.19 Jurkat cells (human T-cell leukemia) treated with 10 µM camptothecin for 6 hours (**B**) or untreated (as control, **A**). Cells were then mixed with the reagents in the kit and analyzed by flow cytometry using 405/488 nm dual excitation. Note that the campthothecin-treated cells (**B**) have a higher percentage of apoptotic cells than the basal level of apoptosis seen in the control cells (**A**). A=apoptotic cells, V = viable cells, N = necrotic cells.

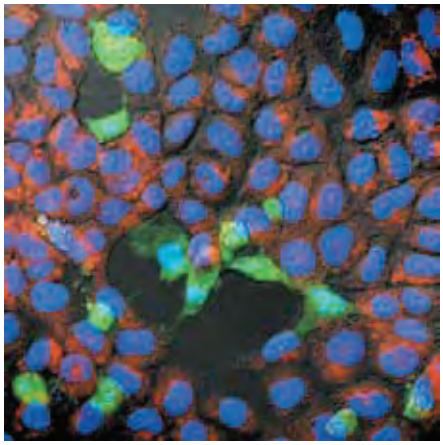

Figure 15.5.20 Detection of apoptosis in SK-N-MC neuroblastoma cells. Following a 6-hour exposure to hydrogen peroxide, cells were labeled with Hoechst 33342 (H1399, H3570, H21492), tetramethylrhodamine ethyl ester (TMRE, T669) and rhodamine 110, bis-L-aspartic acid amide (R22122) for 15 minutes. Apoptotic cells show green cytosolic fluorescence resulting from cleavage of the rhodamine 110, bis-L-aspartic acid amide substrate by active caspase-3. The staining pattern of the Hoechst 33342 dye reveals that the majority of the rhodamine 110–positive cells also contain condensed or fragmented nuclei characteristic of apoptosis. Furthermore, the rhodamine 110–positive cells are also characterized by an absence of polarized mitochondria, as indicated by their failure to load the positively charged mitochondrial indicator TMRE. The image was contributed by A.K. Stout and J.T. Greenamyre, Emory University.

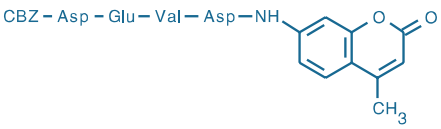

Figure 15.5.21 Z-DEVD-AMC substrate, a component of the EnzChek® Caspase-3 Assay Kit #1 (E13183).

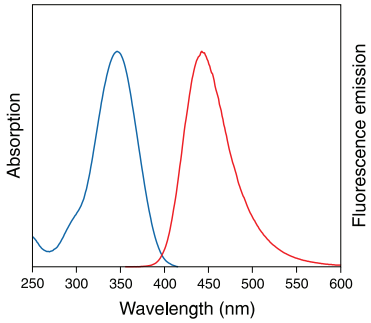

Figure 15.5.22 Absorption and fluorescence emission spectra of 7-amino-4-methylcoumarin in pH 7.0 buffer.

Apoptosis Assays Based on Protease Activity

Caspase-3 Substrates

A distinctive feature of the early stages of apoptosis is the activation of caspase enzymes. Members of the caspase (CED-3/ICE) family of *c*ysteine–aspartic acid *s*pecific proteases have been identified as crucial mediators of the complex biochemical events associated with apoptosis.[72–75] The recognition site for caspases is marked by three to four amino acids followed by an aspartic acid residue, with the cleavage occurring after the aspartate.[76] The caspase proteases are typically synthesized as inactive precursors. Inhibitor release or cofactor binding activates the caspase through cleavage at internal aspartates, either by autocatalysis or by the action of another protease.[73]

Caspase-3 (CPP32/apopain) is a key effector in the apoptosis pathway, amplifying the signal from initiator caspases (such as caspase-8) and signifying full commitment to cellular disassembly. In addition to cleaving other caspases in the enzyme cascade, caspase-3 has been shown to cleave poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase Cδ and actin.[74,77] We offer a selection of fluorogenic caspase substrates (Table 15.5). The Z-DEVD-R110 substrate[78,79]—a component of our EnzChek® Caspase-3 Assay Kit #2 (E13184) and RediPlate™ 96 EnzChek® Caspase-3 Assay Kit (R35100)—is available separately in a 20 mg unit size for high-throughput screening applications (R22120). This nonfluorescent bisamide is first converted by caspase-3 (or a closely related protease) to the fluorescent monoamide and then to the even more fluorescent rhodamine 110 (excitation/emission maxima ~496/520 nm). In addition, the bis-L-aspartic acid amide of R110 (D_2-R110, R22122), which contains rhodamine 110 (R110) flanked by aspartic acid residues, may serve as a substrate for a variety of apoptosis-related proteases, including caspase-3 and caspase-7,[78] and does not appear to require any invasive techniques such as osmotic shock to gain entrance into the cytoplasm (Figure 15.5.20).

Caspase-8 Substrates

Caspase-8 plays a critical role in the early cascade of apoptosis, acting as an initiator of the caspase activation cascade. Activation of the enzyme itself is accomplished through direct interaction with the death domains of cell-surface receptors for apoptosis-inducing ligands.[80,81] The activated protease has been shown to be involved in a pathway that mediates the release of cytochrome *c* from the mitochondria[82] and is also known to activate downstream caspases, such as caspase-3.[83] A R110-based fluorogenic substrate containing the caspase-8 recognition sequence Ile-Glu-Thr-Asp (IETD) is available (Z-IETD-R110, R22125, A22126; Table 15.5).

Other Caspase and Granzyme B Substrates

In addition to our R110-derived caspase-3 and -8 substrates, we offer R110-based substrates for caspase-1, -2, -6, -9 and -13, as well as for granzyme B (Table 15.5). Granzyme B, a serine protease contained within cytotoxic T lymphocytes and natural killer cells, is thought to induce apoptosis in target cells by activating caspases and causing mitochondrial cytochrome *c* release.[84]

Table 15.5 Fluorogenic substrates for caspase activity.

		Fluorophore †	
Peptide Sequence	**Target for Caspases ***	**R110**	**AMC**
YVAD	1, 4	R33750	
VDVAD	2	R33755	
DEVD	3, 6, 7, 8, 10	E13184 ‡ R22120 § R35100 **	E13183 ‡
VEID	6	R33754	
IETD	8, granzyme B	R22125 R22126 §	
LEED	13	R33753	
AAD	granzyme B	R33752	
Aspartic acid	generic	R22122	

* Caspase substrates can often be cleaved by multiple enzymes. The caspase most often associated with the given peptide sequence is listed first. † R110 = rhodamine 110; AMC = 7-amino-4-methylcoumarin. The absorption and emission maxima for the cleaved fluorophores at pH 7 are: 496/520 nm for R110 and 342/441 nm for AMC. ‡ EnzChek® Caspase-3 Assay Kit. § Bulk packaging. ** RediPlate™ 96 EnzChek® Caspase-3 Assay Kit.

EnzChek® Caspase-3 Assay Kits

Molecular Probes® EnzChek® Caspase-3 Assay Kits permit the detection of apoptosis by assaying for increases in caspase-3 and caspase-3–like protease activities. Our EnzChek® Caspase-3 Assay Kit #1 (E13183) contains the 7-amino-4-methylcoumarin (AMC)–derived substrate Z-DEVD-AMC (Figure 15.5.21) (where Z represents a benzyloxycarbonyl group). This substrate, which is weakly fluorescent in the UV spectral range (excitation/emission maxima ~330/390 nm), yields the blue-fluorescent product AMC (A191, Section 10.1, Figure 15.5.22), which has excitation/emission maxima of 342/441 nm upon proteolytic cleavage.

The EnzChek® Caspase-3 Assay Kit #2 (E13184) contains the R110-derived substrate, Z-DEVD-R110 [78,79] (Figure 15.5.23). This substrate is a bisamide derivative of R110, containing DEVD peptides covalently linked to each of R110's amino groups, thereby suppressing both the dye's visible absorption and fluorescence. Upon enzymatic cleavage by caspase-3 (or a closely related protease), the nonfluorescent bisamide substrate is converted in a two-step process first to the fluorescent monoamide and then to the even more fluorescent R110 (R6479, Section 10.1, Figure 15.5.24). Both of these hydrolysis products exhibit spectral properties similar to those of fluorescein, with excitation/emission maxima of 496/520 nm. The Z-DEVD-R110 substrate (R22120) is also available separately in a 20 mg unit size for high-throughput screening applications.

Either kit can be used to continuously measure the activity of caspase-3 and closely related proteases in cell extracts and purified enzyme preparations using a fluorescence microplate reader or fluorometer. AMC-based DEVD substrates, which yield blue fluorescence upon proteolytic cleavage, are widely used to monitor caspase-3 activity.[77,85] The longer-wavelength spectra and higher extinction coefficient of the green-fluorescent products of the R110-based substrate in Kit #2 (E13184) should provide even greater sensitivity.[78,79] The reversible aldehyde-based inhibitor Ac-DEVD-CHO can be used to confirm that the observed fluorescence signal in both induced and control cell populations is due to the activity of caspase-3–like proteases.[77] The EnzChek® Caspase-3 Assay Kits contain:

- Z-DEVD-AMC [86,87] (in Kit #1, E13183) or Z-DEVD-R110 [78,88] (in Kit #2, E13184)
- Dimethylsulfoxide (DMSO)
- Concentrated cell-lysis buffer
- Concentrated reaction buffer
- Dithiothreitol (DTT)
- Ac-DEVD-CHO, a reversible aldehyde-based inhibitor
- 7-Amino-4-methylcoumarin (AMC) (in Kit E13183) or rhodamine 110 (in Kit E13184) reference standard to quantitate the amount of fluorophore released in the reaction
- Detailed protocols

Each kit provides sufficient reagents for performing ~500 assays using a volume of 100 µL per assay.

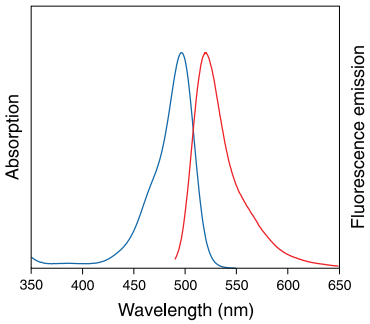

Figure 15.5.24 Absorption and fluorescence emission spectra of rhodamine 110 in pH 7.0 buffer.

RediPlate™ 96 EnzChek® Caspase-3 Assay Kit

Our EnzChek® Caspase-3 Assay Kit #2 is also available as a convenient RediPlate™ 96 EnzChek® Caspase-3 Assay Kit (R35100), which includes one 96-well microplate, contained in a resealable foil packet to help ensure the integrity of the fluorogenic components, plus all necessary buffers and reagents for performing the assay. The enzyme sample to be assayed is added to the microplate in a suitable buffer, along with any compounds to be tested. Then, after incubation, the resultant fluorescence is quantitated on a fluorescence microplate reader equipped with filters appropriate for the green-fluorescent R110, with excitation/emission maxima of 496/520 nm. The microplate consists of twelve removable strips, each with eight wells, allowing researchers to

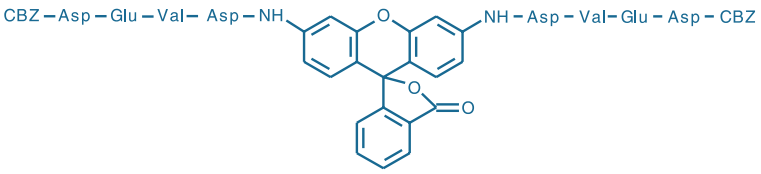

Figure 15.5.23 Z-DEVD-R110 substrate, a component of the EnzChek® Caspase-3 Assay Kit #2 (E13184).

perform only as many assay as required for the experiment. Eleven of the strips (88 wells) are preloaded with the Z-DEVD-R110 substrate. The remaining strip, marked with blackened tabs, contains a dilution series of free R110 that may be used as a fluorescence reference standard. The reversible aldehyde-based inhibitor Ac-DEVD-CHO, which is supplied in a separate vial, can be used to confirm that the observed fluorescence signal in both induced and control cell populations is due to the activity of caspase-3–like proteases.[77] Table 10.3 summarizes our other RediPlate™ 96 and RediPlate™ 384 Assay Kits for protease activity (Section 10.4), phosphatase activity (Section 10.3) and RNA quantitation (Section 8.3).

Image-iT® LIVE Green Caspase Detection Kits for Fluorescence Microscopy

The Image-iT® LIVE Green Caspase-3 and -7 Detection Kit, Image-iT® LIVE Green Caspase-8 Detection Kit and Image-iT® LIVE Green Poly Caspases Detection Kit (I35106, I35105, I35104) employ a novel approach to detect active caspases that is based on a fluorescent inhibitor of caspases (FLICA® methodology). The FLICA® inhibitor comprises a fluoromethyl ketone (FMK) moiety, which can react covalently with a cysteine, a caspase-selective amino acid sequence and a fluorescent carboxyfluorescein (FAM) reporter group. Essentially an affinity label, the FLICA® inhibitor is thought to interact with the enzymatic reactive center of an activated caspase via the recognition sequence, and then to attach covalently to a cysteine through the reactive FMK moiety.[89] The FLICA® inhibitor's recognition sequence is aspartic acid–glutamic acid–valine–aspartic acid (DEVD) for caspase-3 and-7 detection, leucine–glutamic acid–threonine–aspartic acid (LETD) for caspase-8 detection and valine–alanine–aspartic acid (VAD) for detection of most caspases (including caspase-1, -3, -4, -5, -6, -7, -8 and -9). Importantly, the FLICA® inhibitor is cell permeant and not cytotoxic; unbound FLICA® molecules diffuse out of the cell and are washed away. The remaining green-fluorescent signal (excitation/emission maxima ~488/530 nm) can be used as a direct measure of the amount of active caspase that was present at the time the inhibitor was added. FLICA® reagents have been used widely to study apoptosis with flow cytometry and microscopy.[90–94] Recent work indicates that cellular fluorescence from the bound FLICA® reagent is strongly linked to caspase activity in apoptotic cells; however, the interaction of the FLICA® reagent with other cellular sites may contribute to signal intensity in nonapoptotic cells.[95] The Image-iT® LIVE Green Caspase Detection Kit includes:

- FAM-DEVD-FMK caspase-3 and -7 reagent (in Kit I35106), FAM-LETD-FMK caspase-8 reagent (in Kit I35105) or FAM-VAD-FMK poly caspases reagent (in Kit I35104)
- Hoechst 33342
- Propidium iodide
- Dimethylsulfoxide (DMSO)
- Apoptosis fixative solution
- Concentrated apoptosis wash buffer
- Detailed protocols for fluorescence microcscopy assays

In addition to a specific FLICA® reagent, each kit provides Hoechst 33342 and propidium iodide stains, which allow the simultaneous evaluation of caspase activation, nuclear morphology and plasma membrane integrity. Sufficient reagents are provided for 25 assays, based on labeling volumes of 300 µL. These Image-iT® LIVE Green Caspase Detection Kits can also be used in combination with other reagents for multiparametric study of apoptosis.

Image-iT® LIVE Red Caspase Detection Kits for Fluorescence Microscopy

The Image-iT® LIVE Red Caspase-3 and -7 Detection Kit and Image-iT® LIVE Red Poly Caspases Detection Kit (I35102, I35101) are analogous to the Image-iT® LIVE Green Caspase Detection Kits except that the FLICA® reagent contains a red-fluorescent sulforhodamine (SR) reporter group instead of a green-fluorescent carboxyfluorescein (FAM) reporter group. This assay's red-fluorescent signal (excitation/emission maxima ~550/595 nm) can be used as a direct measure of the amount of active caspase that was present at the time the inhibitor was added. The Image-iT® LIVE Red Caspase Detection Kit includes:

- SR-DEVD-FMK caspase-3 and -7 reagent (in Kit I35102) or SR-VAD-FMK poly caspases reagent (in Kit I35101)
- Hoechst 33342
- SYTOX® Green nucleic acid stain
- Dimethylsulfoxide (DMSO)
- Apoptosis fixative solution
- Concentrated apoptosis wash buffer
- Detailed protocols for fluorescence microcscopy assays

In addition to a specific FLICA® reagent, each kit provides Hoechst 33342 and SYTOX® Green nucleic acid stains, which allow the simultaneous evaluation of caspase activation, nuclear morphology and plasma membrane integrity. Sufficient reagents are provided for 25 assays, based on labeling volumes of 300 µL.

Vybrant® FAM Caspase Assay Kits for Flow Cytometry

Like the Image-iT® Kits described above, the Vybrant® FAM Caspase Assay Kits for flow cytometry are based on a fluorescent caspase inhibitor (FLICA® methodology). We offer three different Vybrant® FAM Caspase Assay Kits designed to target different caspases. The Vybrant® FAM Caspase-3 and -7 Assay Kit (V35118) provides a FLICA® inhibitor containing the caspase-3 and -7 recognition sequence DEVD; the Vybrant® FAM Caspase-8 Assay Kit (V35119) provides a FLICA® inhibitor containing the caspase-8 recognition sequence Leu-Glu-Thr-Asp (LETD); and the Vybrant® FAM Poly Caspases Assay Kit (V35117) provides a FLICA® inhibitor containing the caspase recognition sequence Val-Ala-Asp (VAD), which is recognized by caspase-1, -3, -4, -5, -6, -7, -8 and -9. In addition to the selective FLICA® reagent, these kits contain the Hoechst 33342 and propidium iodide nucleic acid stains to permit simultaneous evaluation of caspase activation, membrane permeability and cell cycle. The Vybrant® FAM Caspase Assay Kits include:

- FAM-DEVD-FMK caspase-3 and -7 reagent (in Kit V35118), FAM-LETD-FMK caspase-8 reagent (in Kit V35119) or FAM-VAD-FMK poly caspases reagent (in Kit V35117)
- Hoechst 33342
- Propidium iodide
- Dimethylsulfoxide (DMSO)
- Apoptosis fixative solution
- Concentrated apoptosis wash buffer
- Detailed protocols for flow cytometry assays

Sufficient reagents are provided for 25 assays, based on labeling volumes of 300 µL. These Vybrant® FAM Caspase Assay Kits can be used in combination with other fluorescent probes, such as the

far-red–fluorescent allophycocyanin annexin V (A35110), for a multiparameter study of apoptosis.

Cathepsins and Calpains

The role of intracellular cathepsins and calpains in apoptosis is unclear, although an upstream role of cathepsin B in activation of some caspases[96,97] and cathepsins during apoptosis has been established.[98] Pepstatin A, which is a selective inhibitor of carboxyl (acid) proteases such as cathepsin D, has been reported to inhibit apoptosis in microglia, lymphoid cells and HeLa cells.[99–101] Consequently, our cell-permeant BODIPY® FL pepstatin derivative (P12271), which we have shown to inhibit cathepsin D *in vitro* (IC_{50} ~10 nM) and to target cathepsin D within lysosomes of live and fixed cells, has demonstrable utility for following the intracellular translocation of cathepsin D.[102,103]

Calpains are a family of ubiquitous calcium-activated thiol proteases that are implicated in a variety of cellular functions including exocytosis, cell fusion, apoptosis and cell proliferation.[101,104] Caspase-dependent downstream processing of calpain has been reported, suggesting that calpain may play a role in the degradation phase of apoptosis that is distinct from that of caspases.[105–107] One mechanism of caspase dependence appears to be processing of the endogenous calpain inhibitor calpastin by caspases.[108] However, calpain activation has also been reported to be upstream of caspases in radiation-induced apoptosis.[109] Our *t*-BOC-Leu-Met-CMAC fluorogenic substrate (A6520) has been used to measure calpain activity in hepatocytes following the addition of extracellular ATP [110] and may be of utility in detecting caspase-activated processing of procalpain in live single cells. Peptidase substrates based on our CMAC fluorophore (7-amino-4-chloromethylcoumarin, C2110; Section 10.1) passively diffuse into several types of cells, where the thiol-reactive chloromethyl group is enzymatically conjugated to glutathione by intracellular glutathione *S*-transferase or reacts with protein thiols, thus transforming the substrate into a membrane-impermeant probe. Subsequent peptidase cleavage results in a bright blue-fluorescent glutathione conjugate; see Section 10.4 for more information on AMC- and CMAC-based peptidase substrates.

Apoptosis Assays Using Mitochondrial Stains

A distinctive feature of the early stages of programmed cell death is the disruption of active mitochondria.[111–113] This mitochondrial disruption includes changes in the membrane potential and alterations to the oxidation–reduction potential of the mitochondria. Changes in the membrane potential are presumed to be due to the opening of the mitochondrial permeability transition pore, allowing passage of ions and small molecules. The resulting equilibration of ions leads in turn to the decoupling of the respiratory chain and then the release of cytochrome *c* into the cytosol.[114,115] These changes can be monitored using our extensive selection of potential-sensitive mitochondrial stains [3,5,64] (Section 12.2). We also offer several kits providing ready-to-use formulations of these reagents in flow cytometry or imaging protocols.

Image-iT® LIVE Mitochondrial Transition Pore Assay Kit for Fluorescence Microscopy

The Image-iT® LIVE Mitochondrial Transition Pore Assay Kit (I35103), based on published experimentation for mitochondrial transition pore opening,[116,117] provides a more direct method of measuring mitochondrial permeability transition pore opening than assays relying on mitochondrial membrane potential alone. This assay employs the acetoxymethyl (AM) ester of calcein, a colorless and nonfluorescent esterase substrate, and $CoCl_2$, a quencher of calcein fluorescence, to selectively label mitochondria. Cells are loaded with calcein AM, which passively diffuses into the cells and accumulates in cytosolic compartments, including the mitochondria. Once inside cells, calcein AM is cleaved by intracellular esterases to liberate the very polar fluorescent dye calcein, which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time. The fluorescence from cytosolic calcein is quenched by the addition of $CoCl_2$, while the fluorescence from the mitochondrial calcein is maintained. As a control, cells that have been loaded with calcein AM and $CoCl_2$ can also be treated with a Ca^{2+} ionophore such as ionomycin (I24222, Section 19.8) to allow entry of excess Ca^{2+} into the cells, which triggers mitochondrial pore activation and subsequent loss of mitochondrial calcein fluorescence. This ionomycin response can be blocked with cyclosporine A, a compound reported to prevent mitochondrial transition pore formation by binding cyclophilin D.

The Image-iT® LIVE Mitochondrial Transition Pore Assay Kit has been tested with HeLa cells and bovine pulmonary artery endothelial cells (BPAEC). Each Image-iT® LIVE Mitochondrial Transition Pore Assay Kit provides:

- Calcein AM
- MitoTracker® Red CMXRos, a red-fluorescent mitochondrial stain (excitation/emission maxima ~579/599 nm)
- Hoechst 33342, a blue-fluorescent nuclear stain (excitation/emission maxima ~350/461 nm)
- Ionomycin
- $CoCl_2$
- Dimethylsulfoxide (DMSO)
- Detailed protocols

Sufficient reagents are provided for 100 assays, based on labeling volumes of 1 mL.

MitoProbe™ Transition Pore Assay Kit for Flow Cytometry

The MitoProbe™ Transition Pore Assay Kit (M34153), based on published experimentation for mitochondrial transition pore opening,[116,117] provides a more direct method of measuring mitochondrial permeability transition pore opening than assays relying on mitochondrial membrane potential alone. As with the Image-iT® LIVE mitochondrial transition pore assay described above, this assay employs the acetoxymethyl (AM) ester of calcein, a colorless and nonfluorescent esterase substrate, and $CoCl_2$, a quencher of calcein fluorescence, to selectively label mitochondria. Cells are loaded with calcein AM, which passively diffuses into the cells and accumulates in cytosolic compartments, including the mitochondria. Once inside cells, calcein AM is cleaved by intracellular esterases to liberate the very polar fluorescent dye calcein, which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time. The fluorescence from cytosolic calcein is quenched by the addition of $CoCl_2$, while the fluorescence from the mitochondrial calcein is maintained. As a control, cells that have been loaded with calcein AM and $CoCl_2$ can also be treated with a Ca^{2+} ionophore such as ionomycin (I24222, Section 19.8) to allow entry of excess Ca^{2+} into the cells, which triggers mitochondrial pore activation and subsequent loss of mitochondrial calcein fluorescence. This ionomycin response can be blocked with cyclosporine A, a compound reported to prevent mitochondrial transition pore formation by binding cyclophilin D.

The MitoProbe™ Transition Pore Assay Kit has been tested with Jurkat cells, MH1C1 cells and bovine pulmonary artery endothelial cells (BPAEC). Each MitoProbe™ Transition Pore Assay Kit provides:

- Calcein AM
- $CoCl_2$
- Ionomycin
- Dimethylsulfoxide (DMSO)
- Detailed protocols

Sufficient reagents are provided for 100 assays, based on labeling volumes of 1 mL.

MitoProbe™ JC-1 Assay Kit for Flow Cytometry

The MitoProbe™ JC-1 Assay Kit (M34152) provides the cationic dye JC-1 and a mitochondrial membrane potential uncoupler, CCCP (carbonyl cyanide 3-chlorophenylhydrazone), for the study of mitochondrial membrane potential. JC-1 (Figure 15.5.25) exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm), due to concentration-dependent formation of red-fluorescent J-aggregates.[118–120] Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio, which is dependent only on the membrane potential and not on other factors such as mitochondrial size, shape and density, which may influence single-component fluorescence measurements. Use of fluorescence ratio detection therefore allows researchers to make comparative measurements of membrane potential and to determine the percentage of mitochondria within a population that respond to an applied stimulus. Subtle heterogeneity in cellular responses can be discerned in this way.[119,121] For example, four distinct patterns of mitochondrial membrane potential change in response to glutamate receptor activation in neurons have been identified using confocal ratio imaging of JC-1 fluorescence.[122]

JC-1 can be used as an indicator of mitochondrial potential in a variety of cell types, including myocytes [118] and neurons,[122] as well as in intact tissues [123] and isolated mitochondria.[121] JC-1 is more specific for mitochondrial versus plasma membrane potential and more consistent in its response to depolarization than some other cationic dyes such as $DiOC_6(3)$ and rhodamine 123.[124] The most widely implemented application for JC-1 is the detection of mitochondrial depolarization occurring in apoptosis.[124–127] Each MitoProbe™ JC-1 Assay Kit provides:

- JC-1
- Dimethylsulfoxide (DMSO)
- CCCP
- Concentrated phosphate-buffered saline (PBS)
- Detailed protocols

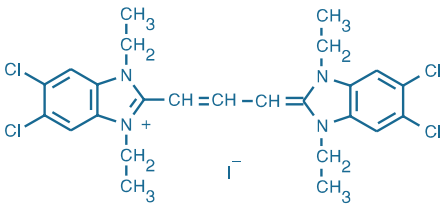

Figure 15.5.25 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; T3168).

Sufficient reagents are provided for 100 assays, based on a labeling volume of 1 mL.

MitoProbe™ $DiIC_1(5)$ and MitoProbe™ $DiOC_2(3)$ Assay Kits for Flow Cytometry

Cationic carbocyanine dyes have been shown to accumulate in cells in response to membrane potential,[128] and membrane potential changes have been studied in association with apoptosis.[129,130] The MitoProbe™ $DiIC_1(5)$ and MitoProbe™ $DiOC_2(3)$ Assay Kits (M34151, M34150) provide solutions of the far-red–fluorescent $DiIC_1(5)$ (1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide) and green-fluorescent $DiOC_2(3)$ (3,3′-diethyloxacarbocyanine iodide) carbocyanine dyes, respectively, along with a mitochondrial membrane potential disrupter, CCCP, for the study of mitochondrial membrane potential. These $DiIC_1(5)$ and $DiOC_2(3)$ carbocyanine dyes penetrate the cytosol of eukaryotic cells and, at concentrations below 100 nM, accumulate primarily in mitochondria with active membrane potentials. In the case of $DiOC_2(3)$, this accumulation is accompanied by a shift from green to red emission due to dye stacking, allowing the use of a ratiometric parameter (red/green fluorescence ratio) that corrects for size differences when measuring membrane potential in bacteria.[131,132] $DiIC_1(5)$ and $DiOC_2(3)$ stain intensities decrease when cells are treated with reagents that disrupt mitochondrial membrane potential, such as CCCP. Each MitoProbe™ $DiIC_1(5)$ and MitoProbe™ $DiOC_2(3)$ Assay Kit provides:

- $DiIC_1(5)$ (in Kit M34151) or $DiOC_2(3)$ (in Kit M34150)
- CCCP
- Detailed protocols for labeling cells with the short-chain carbocyanine dye, as well as with annexin V conjugates (not included)

Cells stained with $DiIC_1(5)$ can be visualized by flow cytometry with red excitation and far-red emission filters; cells stained with $DiOC_2(3)$ can be visualized by flow cytometry with blue excitation and green and red emission filters. $DiIC_1(5)$ can be paired with other reagents, such as propidium iodide and the green-fluorescent Alexa

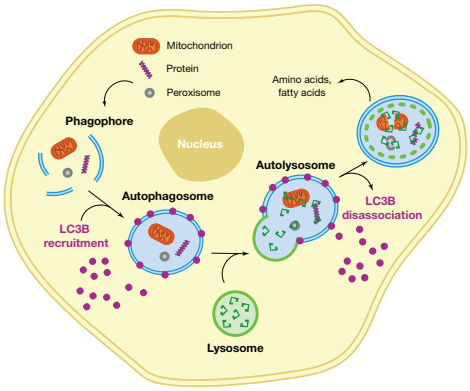

Figure 15.5.26. Schematic depiction of the multistep autophagy pathway in a eukaryotic cell. The first step involves the formation and elongation of isolation membranes, or phagophores. In the second step, which involves the LC3B protein, the cytoplasmic cargo is sequestered, and the double-membrane autophagosome is formed. Fusion of a lysosome with the autophagosome to generate the autolysosome is the penultimate step. In the fourth and final phase, the cargo is degraded.

Fluor® 488 annexin V (both provided in the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, V13241), for multiparameter study of vitality and apoptosis. $DiOC_2(3)$ can be paired with other reagents, such as the far-red–fluorescent allophycocyanin annexin V (A35110), for multiparameter study of vitality and apoptosis. Combining these short-chain carbocyanine dyes with annexin V conjugates results in superior resolution of subpopulations when compared with results obtained with other commonly used dyes.

Assays for Autophagy

Autophagy describes the segregation and delivery of cytoplasmic cargo, including proteins and organelles for degradation by hydrolytic enzymes. The process of autophagy begins with the formation and elongation of isolation membranes, or phagophores (Figure 15.5.26). The cytoplasmic cargo is then sequestered, and the double-membrane autophagosome fuses with a lysosome to generate the autolysosome. Finally, degradation is achieved through the action of hydrolytic enzymes within the autolysosome.

Autophagy was first described in 1963; however, only in the past decade has this pathway become the subject of intense study. Researchers have sought to gain further insight into the role basal autophagy plays in cell homeostasis and development, and to further elucidate the role of induced autophagy in the cell's response to stress, microbial infection, and disease.[1,133,134]

Autophagy Analysis Through Observation of LC3B

The LC3 protein plays a critical role in autophagy. Normally this protein resides in the cytosol, but following cleavage and lipidation with phosphatidylethanolamine, LC3 associates with the phagophore and can be used as a general marker for autophagic membranes (Figure 15.5.26). The new Premo™ Autophagy Sensor Kits (P36235, P36236) combine the selectivity of an LC3B–fluorescent protein chimera[6,135,136] with the transduction efficiency of BacMam technology (BacMam Gene Delivery and Expression Technology—Note 11.1), enabling unambiguous visualization of this protein in live cells (Figure 15.5.27). Recent improvements made to the BacMam system enable efficient transduction in a wider variety of cells, including neurons and neural stem cells (NSCs) with an easy, one-step protocol. To image autophagy, BacMam LC3B-FP is simply added to cells and allowed to incubate overnight for protein expression. Each Premo™ Autophagy Sensor Kit includes:

- BacMam LC3B-FP (GFP fusion, P36235; RFP fusion, P36236)
- Control BacMam LC3B (G120A)-FP
- Chloroquine diphosphate, to artificially induce phagosome formation
- Detailed protocols

Following treatment with chloroquine diphosphate, normal autophagic flux is disrupted, and autophagosomes accumulate as a result of the increase in lysosomal pH. The mutation in the control BacMam LC3B (G120A)-FP prevents cleavage and subsequent lipidation during normal autophagy, and thus protein localization should remain cytosolic and diffuse.

In addition to the Premo™ Autophagy Sensor Kits, we offer the LC3B Antibody Kit for Autophagy (L10382), which includes a rabbit polyclonal anti-LC3B antibody and chloroquine diphosphate for imaging autophagy via LC3B localization in fixed samples. The LC3B Antibody Kit for Autophagy has been validated for use with fluorescence microscopy and high-content imaging and analysis.

Imaging Autophagic Organelles

Two organelles that play a crucial role in auophagy are the mitochondria and lysosomes. Old, damaged and surplus mitochondria are major targets for autophagy, which in this case is sometimes referred to as "mitophagy." Degradation of mitochondria through this process can be used to recover their amino acids and other nutrients, as well as to remove damaged mitochondria from the cell. Fusion of a lysosome with the phagophore to form the autolysosome is the penultimate step of the autophagic pathway (Figure 15.5.26). A variety of reagents including fluorescent

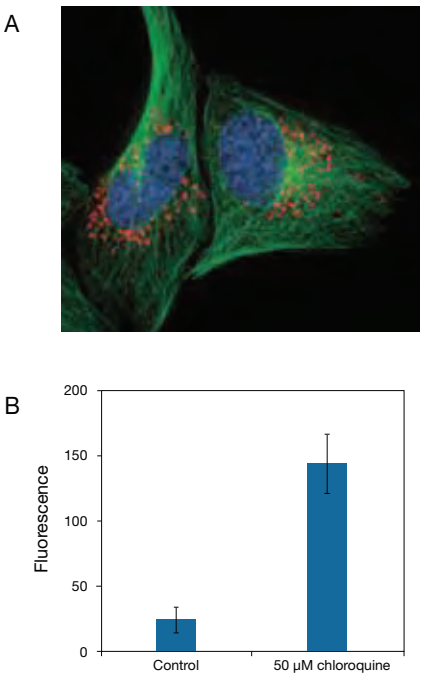

Figure 15.5.27 Detecting autophagy with the Premo™ Autophagy Sensor and fluorescence microscopy (**A**) or high-content imaging and analysis (**B**). (**A**) U2OS cells were co-transduced with the Premo™ Autophagy Sensor LC3B-RFP (P36236) and CellLight® MAP4-GFP (C10598). The following day, cells were treated with 50 µM chloroquine. The following day, cells were incubated with 1 µg/mL Hoechst 33342 before imaging. (**B**) HeLa cells were plated at 5000 cells per well and left to adhere overnight. Cells were then transduced with the Premo™ Autophagy Sensor LC3B-GFP. The following day, cells were incubated with 50 µM chloroquine or left untreated (control) for 16 hr. Quantitative analysis was performed by quantifying fluorescence from vesicular structures in the perinuclear region using the Thermo Scientific Cellomics® ArrayScan® VTI platform.

dyes, fluorescent protein (FP) chimeras and antibodies can be used to image mitochondria and lysosomes during basal and induced autophagy [133,137–139] (Table 15.6). For a description of our mitochondrial and lysosomal-selective organelle probes, see Section 12.2 and Section 12.3.

Visualizing Autolysosome Formation

In conjunction with the Premo™ autophagy sensors LC3B-GFP and LC3B-RFP, the fluorogenic protease substrates DQ™ Green BSA and DQ™ Red BSA can be used to accurately image the formation of the autolysosome in live cells.[140] DQ™ Green BSA and DQ™ Red BSA (D12050, D12051; Section 10.4) are bovine serum albumin (BSA) conjugates that have been labeled to such a high degree that the fluorescence is self-quenched. To visualize autolysosome formation, cells that express a GFP- or RFP-LC3 are incubated with the contrasting color of DQ™ BSA. The convergence of the lysosome with the autophagosome results in dequenching and release of brightly fluorescent fragments. The autolysosomes can then be identified by co-localization of green and red fluorescence.

Table 15.6 Fluorescent detection reagents for imaging mitochondria and lysosomes.

	Organic Dyes (e.g., MitoTracker® and LysoTracker® dyes)	BacMam-Based Fluorescent Proteins (e.g., CellLight® reagents)	Antibodies
How they work	Positively charged MitoTracker® dyes localize to actively respiring mitochondria; weakly basic LysoTracker® dyes accumulate in compartments with low pH.	Combine targeting sequence–fluorescent protein fusion with the transduction efficiency of BacMam to label organelles independently of function (i.e., pH, mitochondrial membrane potential).	Recognize specific target of interest (e.g., LAMP1, a lysosomal protein).
Applications	Live-cell imaging applications; fixable, thus compatible with antibody-based imaging applications.*	Live-cell imaging applications; fixable, thus compatible with antibody-based imaging applications. *†	Imaging fixed cells or tissue. Compatible with labeling with MitoTracker®, LysoTracker® and CellLight® reagents.
Typical workflow	Incubate cells with the MitoTracker® or LysoTracker® reagent for approximately 5–30 min.	The ready-to-use CellLight® reagent is added to live cells, followed by an overnight incubation to allow for protein expression.	Cells are fixed and permeabilized, then incubated with the antibody for labeling, and visualized with a fluorescently labeled secondary antibody. ‡

* Please consult the product manual or contact technical service for additional information on the fixability of these reagents. † With fluorescent protein constructs, anti-GFP or anti-RFP antibodies can be used to amplify fluorescence signals. ‡ Secondary antibody is required if the primary antibody is not directly labeled.

REFERENCES

1. Cell Death Differ (2009) 16:966; **2**. Biotechniques (2002) 32:648; **3**. Methods (2008) 44:222; **4**. Methods Mol Biol (2009) 559:19; **5**. Apoptosis (2007) 12:803; **6**. PLoS One (2009) 4:e7124; **7**. J Biomol Screen (2007) 12:510; **8**. J Biomol Screen (2006) 11:369; **9**. FEBS Lett (2006) 580:885; **10**. J Immunol Methods (2010) 358:81; **11**. Biochemistry (2008) 47:7539; **12**. Cytometry A (2008) 73:563; **13**. Cytometry A (2008) 73:496; **14**. Nat Protoc (2009) 4:125; **15**. J Cell Biol (2000) 150:145; **16**. Cancer Res (1997) 57:3804; **17**. Blood (1996) 87:4959; **18**. J Exp Med (1995) 182:1759; **19**. J Immunol Methods (1995) 185:249; **20**. Cytometry (1994) 17:59; **21**. Exp Cell Res (1988) 175:184; **22**. Int J Radiat Biol (1994) 66:23; **23**. Mol Hum Reprod (1996) 2:613; **24**. Irish J Med Sci (1997) 166:177; **25**. Mutagenesis (1998) 13:1; **26**. Mutat Res (1998) 399:87; **27**. J Histochem Cytochem (2003) 51:873; **28**. Biotechniques (1999) 27:846; **29**. Nucleic Acids Res (2009) 37:3452; **30**. J Histochem Cytochem (1999) 47:1101; **31**. Chem Rev (2009) 109:4207; **32**. Chem Rev (2008) 108:2952; **33**. Angew Chem Int Ed Engl (2009) 48:6974; **34**. Biochemistry (2009) 48:6571; **35**. ACS Chem Biol (2006) 1:644; **36**. Genes Cells (2010) 15:315; **37**. J Histochem Cytochem (1996) 44:959; **38**. Biotechniques (1995) 19:800; **39**. Exp Neurol (1995) 133:225; **40**. Jpn J Cancer Res (1994) 85:939; **41**. Exp Cell Res (1996) 222:28; **42**. Cytometry (1995) 20:172; **43**. Science (1998) 281:1303; **44**. Neuroreport (1995) 6:1053; **45**. Exp Neurol (1995) 133:265; **46**. J Neurosci (1995) 15:3775; **47**. Acta Neuropathol (Berl) (1994) 88:207; **48**. Cancer Res (1993) 53:1945; **49**. Int J Oncol (1992) 1:639; **50**. Trends Cell Biol (1995) 5:21; **51**. Jpn J Cancer Res (1993) 84:566; **52**. Nucleic Acids Res (1993) 21:4206; **53**. Cytometry (1998) 32:95; **54**. Toxicol Appl Pharmacol (1998) 148:176; **55**. J Histochem Cytochem (2001) 49:821; **56**. Nat Protoc (2008) 3:1187; **57**. Cell Prolif (2007) 40:1; **58**. Proc Natl Acad Sci U S A (2006) 103:9891; **59**. J Am Chem Soc (2007) 129:2187; **60**. Cytometry (1998) 31:1; **61**. J Immunol (1992) 148:2207; **62**. J Immunol (1993) 151:4274; **63**. Nat Protoc (2009) 4:1383; **64**. Nat Protoc (2007) 2:2719; **65**. Nano Lett (2006) 6:1863; **66**. J Biol Chem (2000) 275:23807; **67**. J Biol Chem (2001) 276:1127; **68**. J Biol Chem (2000) 275:25336; **69**. BMC Biochem (2009) 10:5; **70**. Proc Natl Acad Sci U S A (2002) 99:10706; **71**. Cancer Res (2008) 68:8384; **72**. Immunol Cell Biol (1998) 76:1; **73**. Science (1998) 281:1312; **74**. Trends Biochem Sci (1997) 22:388; **75**. Cell Death Differ (1999) 6:1067; **76**. J Biol Chem (1997) 272:17907; **77**. Nature (1995) 376:37; **78**. Biochemistry (1999) 38:13906; **79**. Bioorg Med Chem Lett (1999) 9:3231; **80**. Cell Death Differ (1999) 6:821; **81**. Science (1998) 281:1305; **82**. J Biol Chem (1999) 274:17484; **83**. Exp Cell Res (1999) 250:203; **84**. J Biol Chem (2001) 276:6974; **85**. J Neurosci Res (1998) 52:334; **86**. Biochemistry (2000) 39:16056; **87**. Oncogene (1998) 17:1295; **88**. J Biol Chem (2000) 275:288; **89**. Cell Death Differ (1999) 6:1081; **90**. Exp Cell Res (2000) 259:308; **91**. Biotechniques (2001) 31:608; **92**. Leukemia (2002) 16:1589; **93**. Cytometry (2002) 47:143; **94**. J Immunol Methods (2002) 265:111; **95**. Cytometry A (2003) 55:50; **96**. Kidney Int (1987) 31:112; **97**. FEBS Lett (1998) 438:150; **98**. J Biomed Mater Res (1988) 22:837; **99**. Mol Cells (1997) 7:742; **100**. Brain Res (1997) 746:220; **101**. Infect Immun (1994) 62:5126; **102**. J Biochem Biophys Methods (2000) 42:137; **103**. J Neurosci (2008) 28:6926; **104**. Biochem Biophys Res Commun (1997) 236:549; **105**. Cancer Res (1998) 58:1901; **106**. Proc Natl Acad Sci U S A (1999) 96:3200; **107**. J Cell Physiol (1994) 159:229; **108**. Methods Enzymol (1999) 294:117; **109**. Cell Death Differ (1998) 5:1051; **110**. J Biol Chem (1993) 268:23593; **111**. Science (2001) 292:624; **112**. Science (2000) 289:1150; **113**. Trends Cell Biol (2000) 10:369; **114**. Biochim Biophys Acta (1998) 1366:151; **115**. Science (1998) 281:1309; **116**. Biofactors (1998) 8:263; **117**. Biophys J (1999) 76:725; **118**. J Physiol (1995) 486:1; **119**. Proc Natl Acad Sci U S A (1991) 88:3671; **120**. Biochemistry (1991) 30:4480; **121**. Exp Cell Res (1996) 222:84; **122**. J Neurosci (1996) 16:5688; **123**. Methods (1999) 18:104; **124**. FEBS Lett (1997) 411:77; **125**. Exp Cell Res (1998) 245:170; **126**. J Neurosci (1998) 18:932; **127**. J Cell Biol (1997) 138:449; **128**. Proc Natl Acad Sci U S A (1979) 76:5728; **129**. Methods Cell Biol (2001) 63:467; **130**. Exp Cell Res (1994) 214:323; **131**. Methods (2000) 21:271; **132**. Cytometry (1999) 35:55; **133**. Methods Enzymol (2009) 453:417; **134**. J Biol Chem (2008) 283:10892; **135**. Autophagy (2008) 4:770; **136**. J Biol Chem (2009) 284:2383; **137**. Methods Enzymol (2009) 453:111; **138**. Methods Enzymol (2009) 453:217; **139**. Methods Mol Biol (2008) 445:29; **140**. Methods Enzymol (2009) 452:85.

DATA TABLE 15.5 ASSAYS FOR APOPTOSIS AND AUTOPHAGY

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A6520	554.10	F,D	DMSO	330	13,000	403	MeOH	1, 2
C7604	~993	FF,L	H_2O	496	68,000	523	pH 8	3, 4
C7614	~908	FF,L	H_2O	504	68,000	513	pH 8	3, 4
E13183	767.74	F,D,L	DMSO	325	16,000	395	pH 7	1, 2, 5
E13184	1515.46	F,D,L	DMSO	232	52,000	none	MeOH	5, 6
P3581	579.26	F,D,L	DMSO	435	50,000	455	H_2O/DNA	4, 7, 8, 9
P12271	1044.14	F,D,L	DMSO	504	86,000	511	MeOH	
R22120	1515.46	F,D	DMSO, DMF	232	52,000	none	MeOH	6
R22122	788.57	F,D	DMSO, DMF	232	55,000	none	MeOH	6
R22125	1515.55	F,D	DMSO, DMF	232	52,000	none	MeOH	6
R22126	1515.55	F,D	DMSO, DMF	232	52,000	none	MeOH	6
R33750	1495.56	F,D	DMSO, DMF	230	76,000	none	MeOH	6
R33752	1113.10	F,D	DMSO, DMF	232	57,000	none	MeOH	6
R33753	1571.57	F,D	DMSO, DMF	232	57,000	none	MeOH	6
R33754	1511.60	F,D	DMSO, DMF	232	57,000	none	MeOH	6
R33755	1597.65	F,D	DMSO, DMF	232	57,000	none	MeOH	6
Y3603	629.32	F,D,L	DMSO	491	52,000	509	H_2O/DNA	4, 7, 8, 9

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. Peptidase cleavage of this substrate yields 7-amino-4-methylcoumarin (A191).
2. Fluorescence of the unhydrolyzed substrate is very weak.
3. The molecular weight (MW) of this product is approximate because the degree of hydration and/or salt form has not been conclusively established.
4. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
5. Data represent the substrate component of this kit.
6. Peptidase cleavage of this substrate yields rhodamine 110 (R6479).
7. Spectra represent aqueous solutions of nucleic acid-bound dye. EC values are derived by comparing the absorbance of the nucleic acid–bound dye with that of free dye in a reference solvent (H_2O or MeOH).
8. This product is essentially nonfluorescent except when bound to DNA or RNA.
9. Although this compound is soluble in water, preparation of stock solutions in water is not recommended because of possible adsorption onto glass or plastic.

PRODUCT LIST 15.5 ASSAYS FOR APOPTOSIS AND AUTOPHAGY

Cat. No.	Product	Quantity
V13241	Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit *Alexa Fluor® 488 annexin V/propidium iodide* *50 assays* *for flow cytometry*	1 kit
A6520	7-amino-4-chloromethylcoumarin, *t*-BOC-L-leucyl-L-methionine amide (CMAC, *A*-BOC-Leu-Met)	5 mg
A23202	annexin V, Alexa Fluor® 350 conjugate *100 assays*	500 µL
A13201	annexin V, Alexa Fluor® 488 conjugate *100 assays*	500 µL
A35108	annexin V, Alexa Fluor® 555 conjugate *100 assays*	500 µL
A13202	annexin V, Alexa Fluor® 568 conjugate *100 assays*	500 µL
A13203	annexin V, Alexa Fluor® 594 conjugate *100 assays*	500 µL
A23204	annexin V, Alexa Fluor® 647 conjugate *100 assays*	500 µL
A35109	annexin V, Alexa Fluor® 680 conjugate *100 assays*	500 µL
A35110	annexin V, allophycocyanin conjugate (APC annexin V) *50 assays*	250 µL
A13204	annexin V, biotin-X conjugate *100 assays*	500 µL
A13199	annexin V, fluorescein conjugate (FITC annexin V) *100 assays*	500 µL
A13200	annexin V, Oregon Green® 488 conjugate *100 assays*	500 µL
A35122	annexin V, Pacific Blue™ conjugate *for flow cytometry* *100 assays*	500 µL
A35111	annexin V, R-phycoerythrin conjugate (R-PE annexin V) *50 assays*	250 µL
V35113	APC Annexin V/Dead Cell Apoptosis Kit *with APC annexin V and SYTOX® Green* *50 assays* *for flow cytometry*	1 kit
A23210	APO-BrdU™ TUNEL Assay Kit *with Alexa Fluor® 488 anti-BrdU* *60 assays*	1 kit
C7614	ChromaTide® BODIPY® FL-14-dUTP *1 mM in TE buffer*	25 µL
C7604	ChromaTide® fluorescein-12-dUTP *1 mM in TE buffer*	25 µL
V13244	Chromatin Condensation/Dead Cell Apoptosis Kit *Hoechst 33342/propidium iodide* *200 assays* *for flow cytometry*	1 kit
V23201	Chromatin Condesation/Membrane Permeability/Dead Cell Apoptosis Kit. *Hoechst 33342/YO-PRO®-1/propidium iodide* *200 assays* *for flow cytometry*	1 kit
C10245	Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay *for microscopy and HCS* *50–100 assays*	1 kit
C10246	Click-iT® TUNEL Alexa Fluor® 594 Imaging Assay *for microscopy and HCS* *50–100 assays*	1 kit
C10247	Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay *for microscopy and HCS* *50–100 assays*	1 kit
E13183	EnzChek® Caspase-3 Assay Kit #1 *Z-DEVD-AMC substrate* *500 assays*	1 kit
E13184	EnzChek® Caspase-3 Assay Kit #2 *Z-DEVD-R110 substrate* *500 assays*	1 kit
V13242	FITC Annexin V/ Dead Cell Apoptosis Kit *FITC annexin V/propidium iodide* *50 assays* *for flow cytometry*	1 kit
H10292	HCS DNA Damage Kit *2-plate size*	1 kit
H10290	HCS LIVE/DEAD® Green Kit *2-plate size*	1 kit
H10295	HCS Mitochondrial Health Kit *2-plate size*	1 kit
I10291	Image-iT® DEAD Green™ viability stain *1 mM solution in DMSO*	25 µL
I35106	Image-iT® LIVE Green Caspase-3 and -7 Detection Kit *for microscopy* *25 tests*	1 kit

continued on next page

PRODUCT LIST 15.5 ASSAYS FOR APOPTOSIS AND AUTOPHAGY—*continued*

Cat. No.	Product	Quantity
I35105	Image-iT® LIVE Green Caspase-8 Detection Kit *for microscopy* *25 tests*	1 kit
I35104	Image-iT® LIVE Green Poly Caspases Detection Kit *for microscopy* *25 tests*	1 kit
I35103	Image-iT® LIVE Mitochondrial Transition Pore Assay Kit *for microscopy*	1 kit
I35102	Image-iT® LIVE Red Caspase-3 and -7 Detection Kit *for microscopy*	1 kit
I35101	Image-iT® LIVE Red Poly Caspases Detection Kit *for microscopy*	1 kit
L10382	LC3B Antibody Kit for Autophagy *rabbit polyclonal LC3B* *includes autophagosome inducer*	1 kit
V13243	Membrane Permeability/ Dead Cell Apoptosis Kit *YO-PRO®-1/propidium iodide* *200 assays* *for flow cytometry*	1 kit
V35114	Metabolic Activity/Annexin V/Dead Cell Apoptosis Kit *with C_{12} resazurin, APC annexin V, and SYTOX® Green* *50 assays* *for flow cytometry*	1 kit
V35116	Mitochondrial Membrane Potential/Annexin V Apoptosis Kit *Alexa Fluor® 488 annexin V/MitoTracker® Red CMXRos* *50 assays* *for flow cytometry*	1 kit
M34151	MitoProbe™ $DiIC_1(5)$ Assay Kit *for flow cytometry* *100 assays*	1 kit
M34150	MitoProbe™ $DiOC_2(3)$ Assay Kit *for flow cytometry* *100 assays*	1 kit
M34152	MitoProbe™ JC-1 Assay Kit *for flow cytometry* *100 assays*	1 kit
M34153	MitoProbe™ Transition Pore Assay Kit *for flow cytometry* *100 assays*	1 kit
V35112	PE Annexin V/ Dead Cell Apoptosis Kit *with SYTOX® Green* *50 assays* *for flow cytometry*	1 kit
P12271	pepstatin A, BODIPY® FL conjugate	25 µg
P3581	PO-PRO™-1 iodide (435/455) *1 mM solution in DMSO*	1 mL
P36235	Premo™ Autophagy Sensor LC3B-GFP	1 kit
P36236	Premo™ Autophagy Sensor LC3B-RFP	1 kit
R35100	RediPlate™ 96 EnzChek® Caspase-3 Assay Kit *Z-DEVD-R110 substrate* *one 96-well microplate*	1 kit
R22122	rhodamine 110, bis-(L-aspartic acid amide), trifluoroacetic acid salt	1 mg
R33752	rhodamine 110, bis-(*N*-CBZ-L-alanyl-L- alanyl-L-aspartic acid amide) (Z-AAD-R110)	2 mg
R22120	rhodamine 110, bis-(*N*-CBZ-L-aspartyl- L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110) *bulk packaging*	20 mg
R22125	rhodamine 110, bis-(*N*-CBZ-L-isoleucyl- L-glutamyl-L-threonyl- L-aspartic acid amide) (Z-IETD-R110)	2 mg
R22126	rhodamine 110, bis-(*N*-CBZ-L-isoleucyl- L-glutamyl-L-threonyl- L-aspartic acid amide) (Z-IETD-R110) *bulk packaging*	20 mg
R33753	rhodamine 110, bis-(*N*-CBZ-L-leucyl-L- glutamyl-L-glutamyl-L- aspartic acid amide) (Z-LEED-R110)	2 mg
R33750	rhodamine 110, bis-(*N*-CBZ-L-tyrosinyl- L-valyl-L-alanyl-L-aspartic acid amide) (Z-YVAD-R110)	2 mg
R33755	rhodamine 110, bis-(*N*-CBZ-L-valyl-L-aspartyl- L-valyl-L-alanyl-L-aspartic acid amide) (Z-VDVAD-R110)	2 mg
R33754	rhodamine 110, bis-(*N*-CBZ-L-valyl-L-glutamyl- L-isoleucyl-L-aspartic acid amide) (Z-VEID-R110)	2 mg
V13240	Single Channel Annexin V/ Dead Cell Apoptosis Kit *Alexa Fluor® 488 annexin V/SYTOX® Green* *50 assays* *for flow cytometry*	1 kit
A35136	Violet Annexin V/Dead Cell Apoptosis Kit *Pacific Blue™ annexin V/SYTOX® AADvanced™* *for flow cytometry* *50 assays*	1 kit
A35135	Violet Chromatin Condensation/Dead Cell Apoptosis Kit *Vybrant® DyeCycle™ Violet and SYTOX® AADvanced™* *for flow cytometry* *200 assays*	1 kit
V35123	Violet Membrane Permeability/Dead Cell Apoptosis Kit *with PO-PRO™-1 and 7- aminoactinomycin D* *200 assays* *for flow cytometry*	1 kit
A35137	Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit *for flow cytometry* *100 assays*	1 kit
V23200	Vybrant® Apoptosis Assay Kit #6 *biotin-X annexin V/Alexa Fluor® 350 streptavidin/propidium iodide* *50 assays*	1 kit
V35118	Vybrant® FAM Caspase-3 and -7 Assay Kit *for flow cytometry* *25 assays*	1 kit
V35119	Vybrant® FAM Caspase-8 Assay Kit *for flow cytometry* *25 assays*	1 kit
V35117	Vybrant® FAM Poly Caspases Assay Kit *for flow cytometry* *25 assays*	1 kit
Y3603	YO-PRO®-1 iodide (491/509) *1 mM solution in DMSO*	1 mL

15.6 Probes for Cell Adhesion, Chemotaxis, Multidrug Resistance and Glutathione

Cell Adhesion Assays

The fundamental role of cell–cell and cell–matrix adhesion in the morphology and development of organisms, organs and tissues has made identification of molecular mediators of cell adhesion an important research focus in cell biology and immunology.[1–3] The useful review by Löster and Hortstkorte describes a number of different assays that can detect cell adhesion.[4] In a typical fluorescence-based cell adhesion assay, unlabeled cell monolayers in multiwell plates are incubated with fluorescently labeled cells and then washed to separate the adherent and nonadherent populations. Cell adhesion can then be determined simply by correlating the retained fluorescence with cell number. An ideal fluorescent marker will retain proportionality between fluorescence and cell number and introduce minimal interference with the cell adhesion process. Because adhesion is a cell-surface phenomenon, cytoplasmic markers that can be passively loaded are preferable to compounds that label cell-surface molecules, provided they are retained in the cell for the duration of the experiment or their leakage rate can be independently measured. Adhesion of fluorescent dye–labeled cells to matrices such as bone[5] can be directly observed by fluorescence microscopy using cells loaded with the permeant live-cell tracers described in Section 14.2 and Section 15.2 or the lipophilic dyes in Section 14.4. Alternatively, high molecular weight, cell-impermeant, fluorescent dextrans (Section 14.5) have been used to define the area *outside* of adherent cells, with the adherent cells themselves remaining unstained.[6] This same "negative-staining" method can also be used to assess cell spreading and progress toward confluency.

Cell Adhesion Assays Using Enzyme Substrates

Essentially all of the esterase substrates in Table 15.1 useful for monitoring cell viability can also be used for studying cell adhesion. As with cell viability studies, calcein AM (C1430, C3099, C3100MP) appears to best satisfy the criteria for assaying cell adhesion[7–9] and to have the least effect on cell viability and other cell functions.[7] The results obtained with leukocyte adhesion assays using calcein AM correlate well with those obtained with ^{51}Cr assays,[10,11] but the calcein AM protocols take less time and avoid the special handling required when using radioactive material. Calcein AM has been used in numerous cell adhesion assays, including those designed to measure:

- Binding of labeled Jurkat cells to vascular cell adhesion molecule-1 (VCAM1) in cell membrane preparations[12]
- Effects of E-selectin–binding peptides[13] and integrins[14] on neutrophil adhesion
- Integrin-mediated cell adhesion in transfected K562 cells[15,16] and BSC-1 cells[17]
- Leukocyte[7,11,18] and neutrophil[18–20] adhesion to endothelial cells
- Monocyte adhesion in HIV-infected cells[21]

Other fluorogenic esterase substrates that have been used to assess cell adhesion include BCECF AM[5,22–29] (B1150, B1170, B3051), carboxyfluorescein diacetate[7,30–32] (5(6)-CFDA, C195), fluorescein diacetate[33] (F1303), the succinimidyl ester of carboxyfluorescein diacetate[14] (5(6)-CFDA, SE; CFSE; C1157) and CellTracker™ Green CMFDA[23] (C2925, C7025), all of which are discussed in Section 15.2. Because of the possibility of slow calcein leakage from calcein AM–labeled cells, CellTracker™ Green CMFDA and 5(6)-CFDA SE are recommended for quantitative adhesion or aggregation assays that require incubation for more than about an hour.[14,34,35] Use of a combination of a green-fluorescent dye (usually calcein AM, BCECF AM or CMFDA) and a red-fluorescent dye—especially chloromethyl SNARF®-1 acetate (C6826), SNARF®-1 carboxylic acid, acetate, succinimidyl ester (S22801, Section 15.4) or carboxynaphthofluorescein diacetate (C13196, Section 15.2)—enables two-color measurements of adhesion or cell–cell aggregation in mixed cell cultures.[36] Calcein AM has also been used in a quantitative microplate assay of spreading of adherent cells on artificial or biological surfaces.[18]

In their review,[4] Löster and Hortstkorte describe the use of a number of other fluorogenic substrates for quantitating cell adhesion; among these are fluorescein diphosphate (F2999, Section 10.3) and ELF® 97 phosphate (Section 6.3) for lysosomal and membrane phosphatases, as well as various substrates for glycosidases (Section 10.2) and oxidases (Section 10.5).

Vybrant® Cell Adhesion Assay Kit

The Vybrant® Cell Adhesion Assay Kit (V13181) utilizes calcein AM to provide a fast and sensitive method for measuring cell–cell or cell–substratum adhesion.[7,13,14] Calcein AM is nonfluorescent but, once loaded into cells, is cleaved by endogenous esterases to produce calcein, a highly fluorescent and well-retained dye. Calcein provides a bright green-fluorescent, pH-independent, cytoplasmic cell marker that does not appear to affect the cell adhesion process.[9]

To perform this assay, samples of calcein AM–labeled cells are added to monolayers of unlabeled cells in a microplate. Following incubation to allow the labeled cells to adhere to the unlabeled cells, the samples are washed to remove any nonadhering labeled cells (Figure 15.6.1). The calcein fluorescence in this sample, as compared with that of an unlabeled control sample, can then be used to calculate the number of adherent cells. The absorption/emission maxima of calcein (~494/517 nm) are ideally suited for detection by a fluorescence microplate reader equipped with standard fluorescein optical filters.

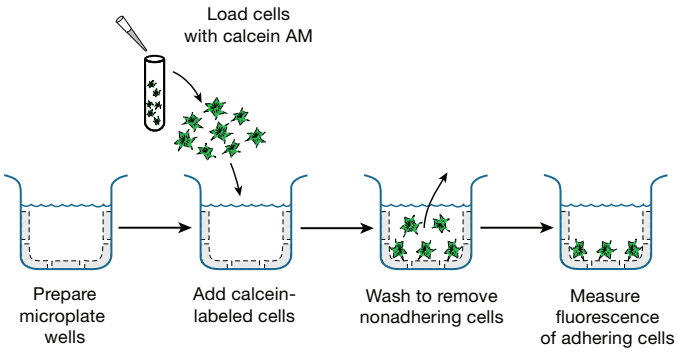

Figure 15.6.1 Principle of the Vybrant® Cell Adhesion Assay Kit (V13181). Microplate wells may be left untreated or precoated with extracellular matrix proteins, antibodies or other reagents.

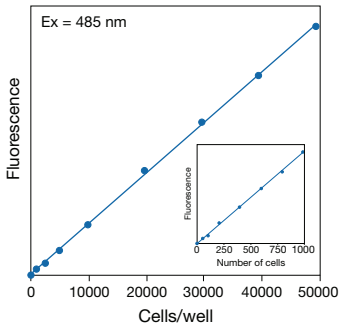

Figure 15.6.2 Quantitation of NIH 3T3 fibroblasts using the CyQUANT® Cell Proliferation Assay Kit (C7026). Fluorescence measurements were made using a microplate reader with excitation at 485 nm and emission detection at 530 nm. The linear range of the assay under these conditions is from 50 to 50,000 cells per 200 µL sample. The inset shows the linearity that can be obtained at very low numbers of cells.

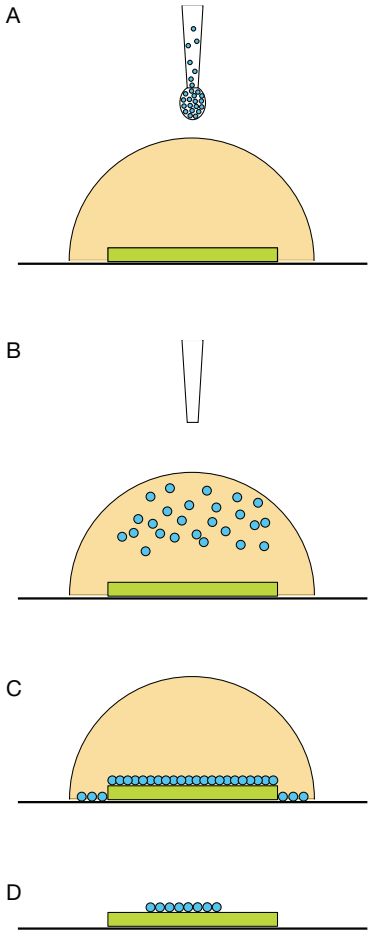

Figure 15.6.3 Schematic view of the microsphere adhesion assay. Membrane-coated microspheres are added to a living tissue slice in a drop of medium (**A**). The microspheres disperse through the medium and eventually cover the tissue slice (**B** and **C**). After incubation, nonadherent microspheres are removed by extensive washing, and the sample is ready for analysis by fluorescence microscopy (**D**).

In addition to calcein AM, the Vybrant® Cell Adhesion Assay Kit includes SYTOX® Green nucleic acid stain, an easy-to-use dead-cell indicator for assessing overall health of cells prior to performing the cell adhesion assay. As a fluorescent substitute for trypan blue, this high-affinity nucleic acid stain easily penetrates cells that have compromised membranes but will not cross the membranes of live cells. Upon binding to nucleic acids, the SYTOX® Green dye exhibits a >500-fold fluorescence enhancement and can be observed with standard fluorescein optical filters (excitation/emission ~504/523 nm).

The Vybrant® Cell Adhesion Assay Kit contains sufficient reagents to perform about 1000 assays using a fluorescence microplate reader, including:

- Calcein AM
- SYTOX® Green nucleic acid stain, an easy-to-use green-fluorescent indicator of the overall health of cells prior to performing the cell adhesion assay
- Detailed protocols

Cell Adhesion Assays Using the CyQUANT® Cell Proliferation Assay Kits

Our CyQUANT® Cell Proliferation Assay Kits (C7026, C35006, C35007, C35011, C35012; Section 15.4) can also serve as important tools for quantitating both cell–cell and cell–surface adhesion. The CyQUANT® GR, CyQUANT® NF and CyQUANT® Direct assays detect total nucleic acids in cells, with a linear detection range from 100 to at least 20,000 cells per well (Figure 15.6.2). To quantitate cell–surface adhesion, cells are simply permitted to adhere to the surface, gently washed to remove nonadherent cells and then analyzed to determine total nucleic acids in the adherent cells using the CyQUANT® assay protocol. As a control, the fluorescence of the total number of cells added to the well before the wash step can be determined by the same assay method to yield the percentage of adherent cells. It should also be possible to extend the CyQUANT® assay to studies of cell–cell adhesion by quantitating both the number of surface-adhering cells originally plated and the number of total cells after a second cell line has been introduced and allowed to adhere. A similar assay for cell adhesion based on DAPI (D1306, D3571, D21490; Section 8.1) has been reported.[37]

Microsphere Adhesion Assays

In a novel method for studying adhesion in live neural tissue slices, including hippocampal slices, fluorescent 4 µm microspheres (F8858, F8859; Section 6.5) are coated with isolated cell membranes from dissociated live cells. The membrane-coated microspheres are then seeded on live tissue slices, and after a short incubation time, the nonadherent microspheres are eliminated by washing (Figure 15.6.3). The pattern of tissue labeling of adherent microspheres can then be visualized by epifluorescence microscopy.[38] A flow cytometry–based microsphere adhesion assay has also been developed to determine integrin-specific adhesion, as well as to analyze integrin-mediated adhesion defects in B-lineage acute lymphoblastic leukemia.[39,40] FluoSpheres® microspheres of different colors (or sizes or intensities) (Section 6.5) can be used for simultaneous labeling of different adhesion factors.

Fluorescent Fibrinogen

Fibrinogen is a key component in the blood clotting process and can support both platelet–platelet and platelet–surface interactions by binding to the glycoprotein IIb-IIIa (GPIIb-IIIa) receptor [41–44] (also called integrin $\alpha_{IIb}\beta_3$). Although the mechanism is not well understood, activation of GPIIb-IIIa is required for fibrinogen binding, which leads to platelet activation, adhesion, spreading and microfilament reorganization of human endothelial cells *in vitro*.[41,44–46] Soluble fibrinogen binds to its receptor with a Ca^{2+}-dependent apparent K_d of 0.18 µM.[47] This binding is apparently mediated by the tripeptide sequence Arg–Gly–Asp (RGD), found both in fibrinogen and fibronectin, as well as some other proteins.[44,48–50]

Fluorescently labeled fibrinogen has proven to be a valuable tool for investigating platelet activation and subsequent fibrinogen binding. Fluorescein fibrinogen has been used to identify activated platelets by flow cytometry.[45,51–53] The binding of fluorescein fibrinogen to activated platelets has been shown to be saturable and can be inhibited completely by underivatized fibrinogen.[52] The preferential binding and accumulation of fluorescein fibrinogen at

the endothelial border of venular blood vessels has been studied by quantitative fluorescence microscopy.[54] We offer five fluorescent conjugates of human fibrinogen, which are useful for investigating platelet activation and subsequent fibrinogen binding using fluorescence microscopy or flow cytometry (Figure 15.6.4):

- Alexa Fluor® 488 human fibrinogen (F13191)
- Oregon Green® 488 human fibrinogen (F7496)
- Alexa Fluor® 546 human fibrinogen (F13192)
- Alexa Fluor® 594 human fibrinogen conjugate (F13193)
- Alexa Fluor® 647 human fibrinogen conjugate (F35200)

Fluorescent Gelatin and Collagen

Collagen is a major component of the extracellular matrix and, in vertebrates, constitutes approximately 25% of total cellular protein. This important protein not only serves a structural role, but also is important in cell adhesion and migration. Specific collagen receptors, fibronectin and a number of other proteins involved in cell–cell and cell–surface adhesion have been demonstrated to bind collagen and gelatin [55,56] (denatured collagen). We offer fluorescent conjugates of gelatin and collagen for use in studying collagen-binding proteins and collagen metabolism, as well as gelatinases and collagenases (Section 10.4), which are metalloproteins that digest gelatin and collagen.

We offer two green-fluorescent gelatin conjugates—fluorescein gelatin (G13187) and Oregon Green® 488 gelatin (G13186). When compared with the fluorescein conjugate, the Oregon Green® 488 conjugate exhibits almost identical fluorescence spectra but its fluorescence is much more photostable and less pH dependent. We also offer collagen-coated FluoSpheres® yellow-green–fluorescent microspheres (F20892, F20893; Section 16.1). By analogy to results obtained with fluorescein conjugates of these proteins, these highly fluorescent gelatin conjugates and collagen-coated microspheres are potentially useful for:

- Following integrin-mediated phagocytosis [57]
- Localizing surface fibronectin on cultured cells [58]
- Studying fibronectin–gelatin interactions in solution using fluorescence polarization [56,59]
- Visualizing gelatinase activity using *in situ* gel zymography [60]

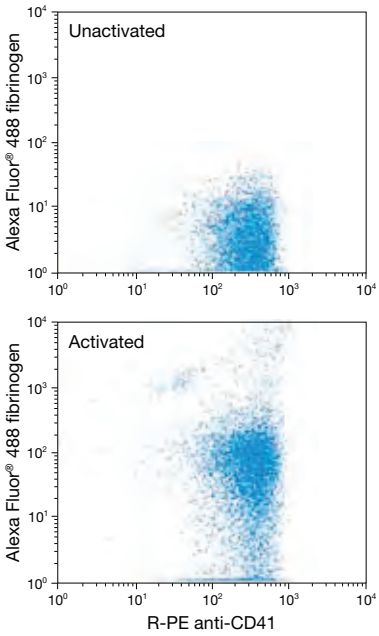

Figure 15.6.4 Interaction of fluorescently labeled fibrinogen with activated platelets. Whole blood was first incubated with an R-phycoerythrin (R-PE)–labeled anti-CD41 antibody to label the platelets. 20 µM adenosine 5′-diphosphate (ADP) was added in order to activate the platelets, then 2 µg/mL Alexa Fluor® 488 fibrinogen (F13191) was added and incubated with the sample for 5 minutes. Cells were analyzed by flow cytometry using excitation at 488 nm. Both activated and unactivated platelets show binding of the anti-CD41 antibody; however, only the activated platelets show strong binding by fibrinogen. A total of 5000 platelets are shown in each experiment.

Chemotaxis Assays

Direct Detection of Chemotaxis Using Cell Counting

Chemotaxis, defined as directed cell motion toward an extracellular gradient, plays an important role during fertilization, inflammation, wound healing and hematopoiesis.[61] Chemotaxis is typically assayed by determining the number of viable cells that have migrated through a special "chemotaxis chamber." A 96-well disposable chemotaxis chamber is reported to be suitable for fluorescence-based assays that are faster, less labor intensive and more sensitive than visually detected migration assays.[62] The probes used to follow chemotaxis in live cells are often the same esterase substrates that are used for assaying cell viability and cell adhesion (Table 15.1), including calcein AM [63–67] (C1430, C3099, C3100MP), BCECF AM [7,24] (B1150, B1170, B3051) and CellTracker™ Green CMFDA [68] (C2925, C7025). Calcein AM does not interfere with lymphocyte proliferation or with granulocyte or neutrophil chemotaxis or superoxide production,[7,64] and, unlike BCECF AM, calcein AM does not affect chemotaxis in leukocytes.[65]

Because chemotaxis involves translocation of whole cells, assays that simply count cell numbers—such as our CyQUANT® Cell Proliferation Assay Kit (C7026, Section 15.4)—are also quite reliable for following chemotaxis.[69] In addition, the green-fluorescent SYTO® 13 dye (S7575, Section 8.1) has been used to track the co-migration of separately stained populations of neutrophils using opposing gradients of leukotriene B_4 and interleukin 8 as the chemoattractants.[70]

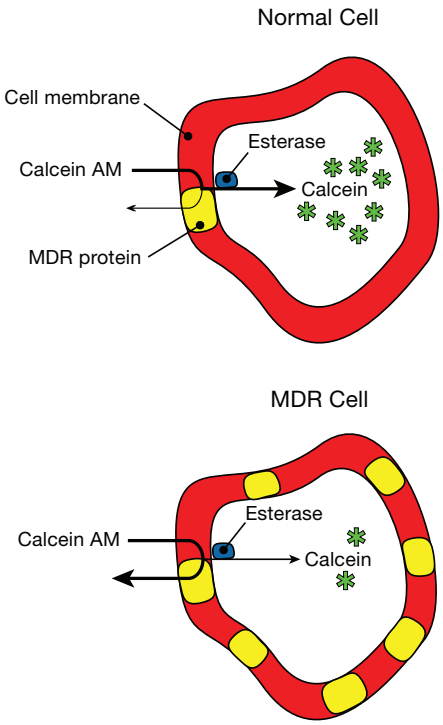

Figure 15.6.5 Principle of the Vybrant® Multidrug Resistance Assay Kit (V13180). In normal cells, nonfluorescent calcein AM readily diffuses across the cell membrane. Fluorescent calcein accumulates in the cytoplasm after cleavage of calcein AM by endogenous esterases. In MDR cells, overexpression of MDR transporter proteins increases expulsion of calcein AM from the cell membrane before enzymatic hydrolysis of its AM esters, thus reducing accumulation of intracellular calcein.

Probes for Chemotaxis Receptors

We prepare the fluorescein conjugate of the chemotactic hexapeptide *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (F1314), which binds to the fMLF receptor [71,72] (Section 16.1). We also offer fluorescein-labeled casein (C2990), which has been used to demonstrate casein-specific chemotaxis receptors in human neutrophils and monocytes with flow cytometry.[73,74] Neutrophils activated with phorbol myristate acetate have been shown to undergo a dose-dependent increase in binding of fluorescein-labeled casein.[75]

Multidrug Resistance Assays

Multidrug resistance (MDR) is a phenomenon representing a complex group of biological processes that are of growing interest in both clinical and experimental oncology.[76–78] The MDR phenotype is characterized by the acquired resistance of tumor cells to structurally and functionally dissimilar anticancer drugs. Among the many mechanisms contributing to this multidrug resistance are the following:

- Amplification of genes encoding drug-metabolizing enzymes
- Elevated levels of glutathione and glutathione-conjugating enzymes
- Mutated DNA topoisomerases
- Overexpression of plasma membrane ATP-dependent drug efflux pumps

Based on substrate and inhibitor profiles, at least four different plasma membrane ATP-dependent drug efflux pumps have been identified.[79,80] The activity of the verapamil-sensitive P-glycoprotein (Pgp) encoded by the *MDR1* gene leads to extrusion of anthracyclins, epipodophyllotoxins, *Vinca* alkaloids, coelenterazine and other cytostatic drugs.[81,82] BODIPY® FL paclitaxel (P7500; Section 11.2) is a substrate for Pgp-mediated transport that has been used as a probe for tumor spheroids.[83]

Many tumor cells do not express Pgp but export daunorubicin via a second, energy-dependent drug export mechanism.[84] A third, energy-dependent drug exporting mechanism is associated with a MDR-associated protein [85] (MRP), which shows high selectivity toward glutathione *S*-conjugates and is inhibited by *Vinca* alkaloids and probenecid.[86,87] A fourth vanadate- and verapamil-resistant but probenecid-sensitive glutathione *S*-conjugate–exporting system in mouse and rat fibroblasts has also been reported.[88] The probes commonly used to follow the transport of glutathione adducts are the same probes used to measure intracellular levels of glutathione (see below): monochlorobimane [89–92] (M1381MP) and CellTracker™ Green CMFDA [91,93,94] (C2925, C7025).

Obviously, the mechanisms of MDR are complex and, in some cases, have overlapping selectivity for the substrates.[80] We offer the Vybrant® Multidrug Resistance Assay Kit, along with a variety of useful fluorescent probes for monitoring various aspects of the MDR phenotype.

MDR Assays Using Acetoxymethyl Esters

The discovery that fluorescent calcium indicators such as indo-1 AM and fluo-3 AM (I1203, F1241, F23915; Section 19.3) and other dyes such as calcein AM (C1430, C3099, C3100MP), are rapidly extruded from cells expressing Pgp [95] presented a new class of highly sensitive probes for functional assays of the *MDR1*-encoded Pgp. Calcein AM—but not calcein—is an activator of Pgp in isolated membranes with a $K_d \leq 1$ µM.[96] Cells expressing the *MDR1*-encoded Pgp rapidly remove the nonfluorescent probe calcein AM, resulting in decreased accumulation of the highly fluorescent calcein in the cytoplasmic compartment.[87,96–98] Calcein AM is also a substrate for the MDR-associated protein (MRP), although in this case the hydrophilic free calcein anion is also exported.[99–101] Because calcein itself is not a substrate for the *MDR1*-encoded Pgp, MDR can be quantitatively assessed by measuring the net accumulation of intracellular fluorescence.[102] Cellular depletion of glutathione does not affect the extrusion of calcein AM by the *MDR1*-encoded Pgp.[99] Fluorescence of intracellular calcein can be distinguished from that of calcein that has leaked into the medium by adding Co^{2+} to totally quench the fluorescence of the extracellular dye.[101] The patented calcein AM assay for Pgp-related MDR is suitable for either flow cytometry [103] or fluorometry and is more rapid and significantly more sensitive than conventional assays based on doxorubicin accumulation.[104] Reduced accumulation of calcein in MDR cells can also be observed in single cells by fluorescence microscopy.[97,100]

Vybrant® Multidrug Resistance Assay Kit

The Vybrant® Multidrug Resistance Assay Kit (V13180), which is based on the fluorescence microplate–based method developed by Tiberghien and Loor,[98] provides a rapid and simple method for large-scale screening of MDR inhibitors. This assay utilizes the fluorogenic dye calcein AM as a substrate for efflux activity of Pgp. Upon hydrolysis by intracellular esterases, calcein is well retained in the cytosol and, unlike the hydrolysis product of other fluorescent Pgp substrates such as BCECF AM or fura-2 AM, its fluorescence is neither pH nor calcium dependent. MDR cells expressing high levels of Pgp rapidly extrude nonfluorescent calcein AM from the plasma membrane, reducing accumulation of fluorescent calcein in the cytosol[87,96,102] (Figure 15.6.5). The amount of Pgp activity is inversely proportional to the accumulation of intracellular calcein fluorescence. This assay is designed for use with fluorescence microplate readers and is particularly useful for rapid and sensitive screening of candidate Pgp inhibitors in MDR cell lines. The absorption/emission maxima of calcein (494/517 nm) are ideally suited for detection by instruments equipped with standard fluorescein filters. The Vybrant® Multidrug Resistance Assay Kit (V13180) contains sufficient reagents to perform approximately 10,000 assays using a fluorescence microplate reader, including:

- Calcein AM
- Cyclosporin A, a competitive inhibitor of drug binding to Pgp
- Verapamil, a calcium channel blocker that noncompetitively inhibits Pgp activity
- Detailed protocols

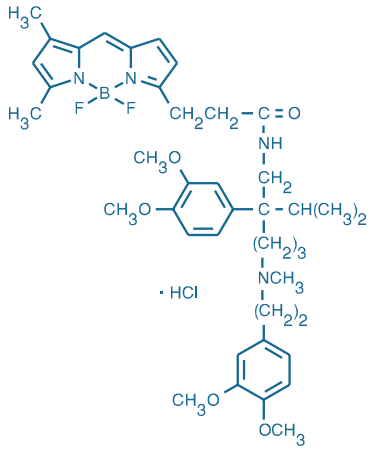

Figure 15.6.6 BODIPY® FL verapamil, hydrochloride (B7431).

MDR Assays Using Mitochondrial Probes

In the classical functional assay for MDR, doxorubicin efflux is measured.[105–107] The principal weakness of this assay is its low sensitivity, which has stimulated the search for other fluorochromes to monitor drug efflux. MDR cells overexpressing the Pgp have been identified using various mitochondrial probes (Section 12.2), including rhodamine 123 (R302, R22420), acridine orange 10-nonyl bromide (nonyl acridine orange, A1372) and rhodamine 6G[79,108–115] (R634). Furthermore, it has been reported that the fluorescence excitation spectrum of rhodamine 123 is different in drug-resistant and drug-sensitive cells.[116,117]

The potential-sensitive carbocyanine dyes, including dipentyl-, dihexyl- and diheptyloxacarbocyanines (D272, D273, D378; Section 22.3), also have advantages over the common doxorubicin assay for MDR.[105,118] Not only are these carbocyanine dyes more fluorescent, permitting use of lower dye concentrations, but their fluorescence increases upon binding to cell membranes,[119] unlike the fluorescence of doxorubicin, which is substantially quenched inside cells.[120] The ratiometric, potential-sensitive di-4-ANEPPS probe (D1199, Section 22.2) has been used to demonstrate that MDR cells have decreased electrical potentials.[121]

MDR Assays Using Nucleic Acid Stains

MDR cells have also been identified by their decreased accumulation of nucleic acid–binding dyes such as Hoechst 33258 (H1398, H3569, H21491; Section 8.1), Hoechst 33342 (H1399, H3570, H21492; Section 8.1) and ethidium bromide[115,122-124] (E1305, 15585-011; Section 8.1). SYTO® 16 green-fluorescent nucleic acid stain (S7578; Section 8.1) was shown to be a useful substrate for detecting Pgp-mediated resistance by flow cytometry and in single cells by confocal laser-scanning microscopy.[125] Our four SYTO® Fluorescent Nucleic Acid Stain Sampler Kits (S7572, S11340, S11350, S11360; Section 8.1) provide samples of SYTO® dyes with fluorescence covering the entire visible spectrum; these dyes may be screened for their utility in detecting MDR cells.

BODIPY® FL Verapamil and Dihydropyridine

The Ca^{2+}-channel blocker verapamil is one of several molecules known to inhibit Pgp-mediated drug efflux.[126] We offer the green-fluorescent BODIPY® FL verapamil probe (B7431, Figure 15.6.6) for the study of Pgp function and localization. Verapamil appears to inhibit drug efflux by acting as a substrate for Pgp, thereby overwhelming the transporter's capacity to expel other drugs. Our BODIPY® FL conjugate of verapamil, with spectral properties similar to fluorescein, also serves as a substrate for Pgp. This fluorescent verapamil derivative preferentially accumulates in the lysosomes of normal, drug-sensitive NIH 3T3 cells but is rapidly transported out of MDR cells, as revealed by fluorescence microscopy.[127–131] The outward transport of BODIPY® FL verapamil from MDR cells is inhibited by underivatized verapamil, as well as by excess vinblastine.[129]

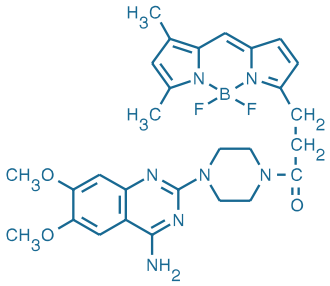

Figure 15.6.7 BODIPY® FL prazosin (B7433).

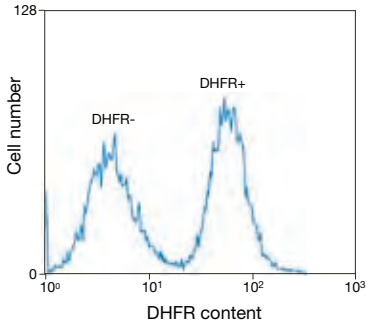

Figure 15.6.9 Analysis of dihydrofolate reductase (DHFR) content in CHO cells. A mixture of DHFR+ and DHFR- cells was stained with 1 µM fluorescein methotrexate (M1198MP) for 2 hours at 37°C. Following incubation with fluorescein methotrexate, cells were trypsinized, washed in phosphate-buffered saline and analyzed by flow cytometry using 488 nm excitation. Emission was collected at 525 nm.

Like verapamil, dihydropyridines are known to inhibit drug efflux. Consequently, our fluorescent dihydropyridines [132,133] labeled with either the green-fluorescent DM-BODIPY® (D7443, Section 16.3) or the orange-fluorescent ST-BODIPY® (S7445, Section 16.3) fluorophores may be useful MDR probes.

BODIPY® FL Vinblastine

The antimitotic agent vinblastine inhibits the Pgp-mediated efflux of a number of drugs and other probes from multidrug-resistant cells. Our BODIPY® FL conjugate of vinblastine (V12390) is useful as a highly fluorescent vinblastine analog.[134] A biologically active coumarin dye–labeled vinblastine has previously been described.[135]

BODIPY® FL Prazosin and BODIPY® FL Forskolin

Photoaffinity analogs of prazosin, an α_1-adrenergic receptor antagonist,[136,137] and forskolin, an adenylate cyclase activator, have been shown to selectively photolabel isolated Pgp.[138–141] Our green-fluorescent BODIPY® FL prazosin (B7433, Figure 15.6.7) and BODIPY® FL forskolin (B7469, Figure 15.6.8) are useful tools for probing MDR mechanisms.[82]

Methotrexate Resistance and Gene Amplification

Tumor cells often undergo gene amplification that leads to overexpression of dihydrofolate reductase (DHFR). This increased DHFR expression (Figure 15.6.9) confers enhanced tolerance to the cytotoxic effects of methotrexate.[76,115] For the study of antimetabolite resistance and spontaneous gene amplification, we offer fluorescent methotrexate conjugates. In addition to green-fluorescent fluorescein methotrexate (M1198MP), which has been used to visualize biochemical networks in living cells,[142] we provide Alexa Fluor® 488 methotrexate (M23271), which exhibits fluorescein-like spectral characteristics. The quantitative binding of fluorescein methotrexate (M1198MP) to dihydrofolate reductase (DHFR) enables researchers to isolate cells based on DHFR expression.[143–146] Our fluorescein cadaverine adduct, which was originally described by Gapski and colleagues,[147] appears to be equivalent to fluorescein lysine methotrexate in its applications.[143]

Glutathione Determination

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is the most abundant nonprotein thiol in mammalian cells. Glutathione plays a central role in protecting cells of all organs, including the brain,[149] against damage produced by free radicals, oxidants and electrophiles. A distinct mechanism of MDR involves the overexpression of energy-dependent membrane pumps dedicated to removal of glutathione *S*-conjugates from the cytoplasm by a multidrug resistance–associated protein [85,150] (MRP). An increased rate of efflux of glutathione from Jurkat T lymphocytes during anti-FAS/APO-1 antibody–induced apoptosis has been reported.[151]

Several fluorescent reagents have been proposed for determining cellular levels of glutathione and glutathione *S*-transferase (GST), which catalyzes the formation of glutathione *S*-conjugates, but no probe is without drawbacks in quantitative studies of live cells. The high but variable levels of intracellular glutathione and the multitude of GST isozymes make kinetic measurements under saturating substrate conditions difficult or impossible.[152,153] Isozymes of GST vary both in abundance and activity, further complicating the analysis.[154] Moreover, the fluorescent reagents designed to measure glutathione may react with intracellular thiols other than glutathione, including proteins in glutathione-depleted cells.[155] Therefore, precautions must be

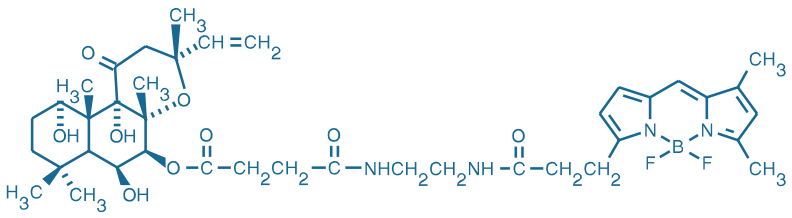

Figure 15.6.8 BODIPY® FL forskolin (B7469).

taken in applying the reagents mentioned here to quantitate either glutathione or GST in cells. A useful strategy is to test a variety of glutathione-sensitive dyes—those requiring glutathione *S*-transferase activity, as well as GST-independent fluorophores—under controlled experimental conditions in which glutathione is depleted.[156]

ThiolTracker™ Violet Glutathione Detection Reagent

The ThiolTracker™ Violet reagent (T10095, T10096) reacts with reduced thiols in intact cells and is up to 10-fold brighter than the bimanes traditionally used for gluthathione (GSH) detection. Staining is achieved by applying ThiolTracker™ Violet dye to live cells in thiol-free buffer and then directly imaging labeled cells using excitation with either a 405 nm violet diode laser or conventional xenon or mercury arc lamps (excitation/emission maxima ~404/526 nm, Figure 15.6.10). Alternatively, because this cell-permeant stain survives formaldehyde fixation and detergent extraction, labeled cells can be subjected to immunochemical analysis prior to imaging.

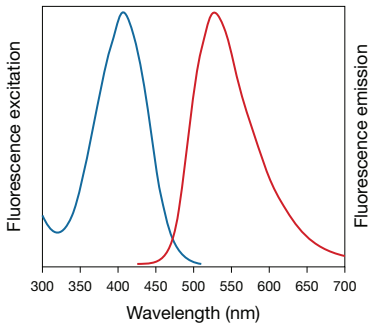

Figure 15.6.10 Fluorescence excitation and emission spectra of ThiolTracker™ Violet dye conjugated to glutathione (GSH) in pH 7.2 buffer.

Glutathione Determination with Monochlorobimane

Cell-permeant monochlorobimane (mBCl, M1381MP), which is essentially nonfluorescent until conjugated to thiols, has long been the preferred thiol-reactive probe for quantitating glutathione levels in cells and for measuring GST activity.[157,158] Because the blue-fluorescent glutathione adduct of monochlorobimane eventually accumulates in the nucleus, it is not a reliable indicator of the nuclear and cytoplasmic distribution of cellular glutathione.[159] Tissue glutathione levels can also be measured fluorometrically by adding both monochlorobimane and glutathione *S*-transferase to tissue homogenates.[160]

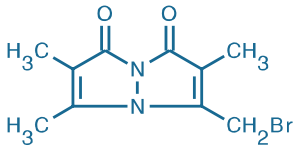

Figure 15.6.11 Monobromobimane (mBBr, M1378).

Monochlorobimane is reported to react more selectively with glutathione in whole cells than does monobromobimane (M1378, M20381; Section 2.3; Figure 15.6.11) and has proven useful for assaying drug resistance by flow cytometry [153,158,161] and by fluorescence microscopy.[162,163] Moreover, HPLC analysis has shown that glutathione is the only low molecular weight thiol in hepatocytes that reacts with monochlorobimane.[164] Results from glutathione determination with monochlorobimane have been shown to match those from an independent glutathione-specific assay using glutathione reductase.[165,166] However, although monochlorobimane was shown to be highly selective for glutathione in rodent cells, it was reported to inadequately label glutathione in human cells because of its low affinity for human glutathione *S*-transferases.[153] The reducing agent tris-(2-carboxyethyl)phosphine (TCEP, T2556; Section 2.1) has been used in place of dithiothreitol (DTT, D1532; Section 2.1) in a simplified monobromobimane-based assay for glutathione [167] and thus may also prove useful for monochlorobimane-based assays. In this monobromobimane-based glutathione assay, an extraction step is reportedly necessary to remove a fluorescent, reductive-dehalogenation side product of TCEP and monobromobimane.[168] Probenecid (P36400; Section 19.8) inhibits the ATP-dependent organic anion pump and blocks the loss of the fluorescent bimane–glutathione adduct from rat fibroblasts.[169] Monochlorobimane has also been employed to sort cells based on their expression of recombinant GST.[170]

Glutathione Determination with Visible Light–Excitable Thiol-Reactive Probes

CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, C2925, C7025) is a useful alternative to the UV light–excitable monochlorobimane for determining levels of intracellular glutathione.[171,172] CellTracker™ Green CMFDA can be excited by the argon-ion laser, and is compatible with flow cytometry and confocal laser-scanning microscopy applications. CellTracker™ Green CMFDA's enzymatic product has a much higher absorbance and fluorescence quantum yield than that of monochlorobimane. In conjunction with Hoechst 33342 (H1399, H3570, H21492; Section 8.1), CellTracker™ Green CMFDA has also been shown to be effective for analyzing intracellular thiol levels as a function of cell cycle using flow cytometry,[171,173] for following transport of the glutathione adduct to secretory vesicles in multidrug-resistant cells [91] and for detecting apoptotic cells, which have reduced levels of intracellular reduced glutathione.[174] Selectivity of CellTracker™ Green CMFDA for glutathione (versus thiolated proteins) was shown by the isolation of >95% of the intracellular fluorescent products as a mixture of the glutathione adduct and the unconjugated hydrolysis product, chloromethylfluorescein.[153] However, in these experiments, the high fluorescence of unconjugated chloromethylfluorescein resulted in significantly increased background levels. Because glutathione-depleting chemicals may also cause cell death, it has been recommended that

calcein AM be used to independently assess cell viability in assays that use CellTracker™ Green CMFDA to measure changes in the level of intracellular glutathione.[172]

Like CMFDA, chloromethyl SNARF®-1 acetate (C6826, Figure 15.6.12) forms adducts with intracellular thiols that are well retained by viable cells. The glutathione adduct of chloromethyl SNARF®-1 can be excited by the 488 nm spectral line of the argon-ion laser yet emits beyond 630 nm, which may prove advantageous in multicolor applications or when assaying autofluorescent samples. A number of our other CellTracker™ (Section 14.2) and MitoTracker® (Section 12.2) probes have thiol-reactive chloromethyl moieties and may be similarly useful for glutathione determination. All of these probes form glutathione *S*-conjugates that are likely to be transported from the cytoplasm by an MDR-associated protein [85,150] (MRP).

Glutathiolation Detection with BioGEE

Biotinylated glutathione ethyl ester (BioGEE, G36000; Figure 15.6.13) is a cell-permeant, biotinylated glutathione analog for detecting glutathiolation. Under conditions of oxidative stress, cells may transiently incorporate glutathione into proteins. Stressed cells incubated with BioGEE will also incorporate this biotinylated glutathione derivative into proteins, facilitating the identification of oxidation-sensitive proteins.[175] Once these cells are fixed and permeabilized, glutathiolation levels can be detected with a fluorescent streptavidin conjugate (Section 7.6, Table 7.9) using either flow cytometry or fluorescence microscopy. Proteins glutathiolated with BioGEE can also be extracted and analyzed by mass spectrometry or by western blotting methods in conjunction with fluorophore- or enzyme-labeled streptavidin conjugates.

Glutathione Determination With *o*-Phthaldialdehyde and Naphthalene-2,3-Dicarboxaldehyde

The reagent *o*-phthaldialdehyde (OPA, P2331MP) reacts with both the thiol and the amine functions of glutathione, yielding a cyclic derivative that is fluorescent. The spectra of the glutathione adduct of OPA (excitation/emission maxima of 350/420 nm) are shifted from those of its protein adducts.[176] This effect has occasionally been used to estimate glutathione levels in cells.[153,177] OPA has also been used as a derivatization reagent for the chromatographic determination of glutathione in cells, blood and tissues.[178,179]

The membrane-permeant naphthalene-2,3-dicarboxaldehyde (NDA, N1138) has been used to determine glutathione levels in single cells. Cells were treated with the NDA reagent and then analyzed by capillary electrophoresis;[180] glutathione labeling was reported to be complete within two minutes. The glutathione adduct of NDA can be excited by the 458 nm spectral line of the argon-ion laser.

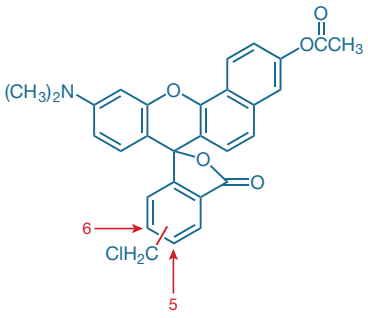

Figure 15.6.12 5-(and-6)-Chloromethyl SNARF®-1, acetate (C6826).

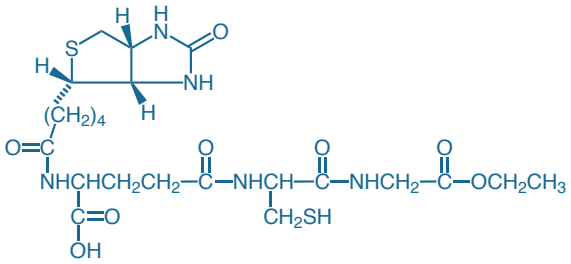

Figure 15.6.13 Glutathione ethyl ester, biotin amide (BioGEE, G36000).

REFERENCES

1. Pharmacol Rev (1998) 50:197; **2**. Cell (1996) 84:345; **3**. Annu Rev Biochem (1991) 60:155; **4**. Micron (2000) 31:41; **5**. Anal Biochem (1999) 267:37; **6**. Cytometry (1998) 33:41; **7**. J Immunol Methods (1994) 172:115; **8**. Cell Biol Toxicol (1994) 10:329; **9**. J Immunol Methods (1995) 178:41; **10**. Biotechniques (1997) 23:1056; **11**. J Immunol Methods (1993) 163:181; **12**. J Immunol Methods (1994) 175:59; **13**. J Biol Chem (1995) 270:21129; **14**. J Immunol Methods (1994) 172:25; **15**. J Cell Biol (1995) 130:745; **16**. J Cell Biol (1994) 127:1129; **17**. Proc Natl Acad Sci U S A (1993) 90:5700; **18**. Anal Biochem (1994) 219:288; **19**. J Immunol (1996) 157:3617; **20**. J Biol Chem (1994) 269:10008; **21**. J Immunol (1996) 156:1638; **22**. J Biol Chem (2000) 275:7052; **23**. J Cell Biol (1996) 133:445; **24**. Mol Biol Cell (1995) 6:661; **25**. J Biol Chem (1994) 269:1033; **26**. J Cell Biol (1994) 125:1395; **27**. J Biol Chem (1993) 268:8835; **28**. J Cell Biol (1993) 123:245; **29**. Biochem Biophys Res Commun (1990) 172:262; **30**. J Cell Biol (1994) 124:609; **31**. Dev Biol (1989) 135:133; **32**. J Cell Biol (1989) 109:3465; **33**. J Immunol Methods (1993) 157:117; **34**. Neuron (1996) 17:1089; **35**. Neuron (1996) 17:353; **36**. J Cell Biol (1996) 135:1593; **37**. J Immunol Methods (1993) 165:93; **38**. Biotechniques (1999) 26:466, 470, 472; **39**. J Immunol (2000) 165:442; **40**. Blood (1999) 94:754; **41**. J Biol Chem (1995) 270:28812; **42**. Thromb Res (1995) 77:543; **43**. Biochem J (1990) 270:149; **44**. Biochem Pharmacol (1987) 36:4035; **45**. J Biol Chem (1995) 270:11358; **46**. J Cell Biol (1987) 104:1403; **47**. J Biol Chem (1983) 258:12582; **48**. J Biol Chem (1998) 273:6821; **49**. Science (1986) 231:1559; **50**. Proc Natl Acad Sci U S A (1985) 82:8057; **51**. J Biol Chem (2000) 275:7795; **52**. Cytometry (1994) 17:287; **53**. J Lab Clin Med (1994) 123:728; **54**. Ann N Y Acad Sci (1983) 416:426; **55**. J Cell Sci (1992) 101:873; **56**. Arch Biochem Biophys (1983) 227:358; **57**. Biophys J (1996) 71:2319; **58**. J Cell Biol (1980) 87:14; **59**. Biochemistry (1993) 32:8168; **60**. FASEB J (1995) 9:974; **61**. Proc Natl Acad Sci U S A (2006) 103:1353; **62**. J Immunol Methods (1998) 213:41; **63**. Anal Biochem (2000) 280:11; **64**. Biophys J (1995) 68:1207; **65**. Cytometry (1995) 19:366; **66**. J Immunol (1996) 156:679; **67**. Immunol Invest (1996) 25:49; **68**. Am J Physiol (1998) 274:C182; **69**. Biotechniques (2000) 29:81; **70**. J Cell Biol (1999) 147:577; **71**. Biochemistry (1990) 29:313; **72**. J Biol Chem (1990) 265:16725; **73**. Inflammation (1983) 7:363; **74**. Inflammation (1977) 2:115; **75**. J Immunol (1987) 139:3028; **76**. Assay Drug Dev Technol (2007) 5:541; **77**. Proc Natl Acad Sci U S A (1994) 91:3497; **78**. Annu Rev Biochem (1993) 62:385; **79**. Biochemistry (1995) 34:32; **80**. Cytometry (1997) 29:279; **81**. Proc Natl Acad Sci U S A (2004) 101:1702; **82**. J Pharmacol Exp Ther (2009) 331:1118; **83**. Br J Cancer (2003) 89:1581; **84**. Cancer Res (1992) 52:17; **85**. Proc Natl

Acad Sci U S A (1994) 91:13033; **86**. Biochem Pharmacol (1996) 52:967; **87**. FEBS Lett (1995) 368:385; **88**. Int J Biochem Cell Biol (2004) 36:247; **89**. J Pharm Biomed Anal (1998) 18:585; **90**. Int J Radiat Biol (1998) 74:647; **91**. Int J Cancer (1998) 76:55; **92**. Int J Radiat Oncol Biol Phys (1989) 16:1315; **93**. J Biol Chem (1996) 271:30587; **94**. J Cell Sci (1998) 111:1137; **95**. Cytometry (1994) 17:343; **96**. J Biol Chem (1993) 268:21493; **97**. J Biol Chem (1996) 271:13668; **98**. Anticancer Drugs (1996) 7:568; **99**. FEBS Lett (1996) 383:99; **100**. Anticancer Res (1998) 18:2981; **101**. Biochemistry (1998) 37:2243; **102**. Biochim Biophys Acta (1994) 1191:384; **103**. Br J Cancer (1996) 73:849; **104**. Eur J Cancer (1993) 29A:1024; **105**. Cytometry (1987) 8:306; **106**. Cancer Res (1983) 43:5126; **107**. Cancer Res (1980) 40:3895; **108**. Anticancer Res (1995) 15:121; **109**. Cytometry (1994) 17:50; **110**. J Biol Chem (1994) 269:7145; **111**. Mol Pharmacol (1994) 45:1145; **112**. Proc Natl Acad Sci U S A (1994) 91:4654; **113**. FEBS Lett (1993) 329:63; **114**. Blood (1991) 78:1385; **115**. Cancer Res (1991) 51:4665; **116**. Exp Cell Res (1991) 196:323; **117**. Cancer Commun (1989) 1:145; **118**. J Cell Physiol (1986) 126:266; **119**. Biochemistry (1995) 34:3858; **120**. Biochemistry (1992) 31:12555; **121**. Biochemistry (1993) 32:11042; **122**. Anticancer Res (1993) 13:1557; **123**. Cell Biochem Funct (1992) 10:9; **124**. Cytometry A (2009) 75:14; **125**. Br J Cancer (1997) 76:1029; **126**. Cancer Res (1987) 47:1421; **127**. Biochem Biophys Res Commun (1995) 212:494; **128**. Biochim Biophys Acta (1995) 1237:31; **129**. Mol Pharmacol (1991) 40:490; **130**. Blood (1998) 91:4106; **131**. Biochemistry (1999) 38:6630; **132**. Biochemistry (1994) 33:11875; **133**. Proc Natl Acad Sci U S A (1992) 89:3586; **134**. Anticancer Res (1997) 17:3321; **135**. Biochemistry (1994) 33:12665; **136**. Cardiology (1986) 73:164; **137**. Life Sci (1980) 27:1525; **138**. J Biol Chem (1993) 268:11417; **139**. Biochem Pharmacol (1992) 43:89; **140**. J Biol Chem (1991) 266:20744; **141**. J Biol Chem (1989) 264:15483; **142**. Proc Natl Acad Sci U S A (2001) 98:7678; **143**. J Biol Chem (1982) 257:14162; **144**. Cancer Res (1990) 50:4946; **145**. Proc Natl Acad Sci U S A (1983) 80:3711; **146**. J Biol Chem (1978) 253:5852; **147**. J Med Chem (1975) 18:526; **148**. Clin Chem (1996) 42:64; **149**. Prog Neurobiol (2000) 62:649; **150**. J Biol Chem (1994) 269:29085; **151**. J Biol Chem (1996) 271:15420; **152**. Anal Biochem (1994) 217:41; **153**. Cytometry (1994) 15:349; **154**. Cancer Res (1991) 51:1783; **155**. Cytometry (1991) 12:366; **156**. Glia (2000) 30:329; **157**. Ann N Y Acad Sci (1993) 677:341; **158**. Cancer Res (1986) 46:6105; **159**. Biochem J (1993) 294:631; **160**. Anal Biochem (2000) 286:35; **161**. J Biol Chem (1988) 263:14107; **162**. J Biol Chem (2006) 281:17420; **163**. Cytometry (1995) 19:226; **164**. Anal Biochem (1990) 190:212; **165**. Cancer Res (1991) 51:4287; **166**. J Clin Lab Anal (1990) 4:324; **167**. Anal Biochem (1999) 272:107; **168**. Anal Biochem (2003) 318:325; **169**. Cytometry (1996) 23:78; **170**. Cytometry (1991) 12:651; **171**. Cytometry (1991) 12:184; **172**. Toxicol in Vitro (1996) 10:341; **173**. Cytometry (1993) 14:747; **174**. J Immunol (1997) 158:4612; **175**. Biochemistry (2000) 39:11121; **176**. Anal Biochem (1966) 14:434; **177**. Exp Cell Res (1986) 163:518; **178**. Clin Chem (1995) 41:448; **179**. Chromatographia (1993) 36:130; **180**. Anal Chem (1995) 67:4261.

DATA TABLE 15.6 PROBES FOR CELL ADHESION, CHEMOTAXIS, MULTIDRUG RESISTANCE AND GLUTATHIONE

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
B1150	~615	F,D	DMSO	<300		none		1, 2
B1170	~615	F,D	DMSO	<300		none		1, 2
B3051	~615	F,D	DMSO	<300		none		1, 2, 3
B7431	769.18	F,D,L	DMSO, EtOH	504	74,000	511	MeOH	
B7433	563.41	F,D,L	DMSO, EtOH	504	77,000	511	MeOH	
B7469	784.70	F,D,L	DMSO	504	79,000	511	MeOH	
C1430	994.87	F,D	DMSO	<300		none		4
C2925	464.86	F,D	DMSO	<300		none		5
C3099	994.87	F,D	DMSO	<300		none		3, 4
C3100MP	994.87	F,D	DMSO	<300		none		4
C6826	499.95	F,D	DMSO	<350		none		6
C7025	464.86	F,D	DMSO	<300		none		5
F1314	1213.41	F,L	pH >6, DMF	494	72,000	517	pH 9	
G36000	561.67	F,D	DMSO	<300		none		
M1198MP	979.08	F,L	pH >6, DMF	496	67,000	516	pH 9	
M1381MP	226.66	F,L	DMSO	380	6000	see Notes	MeOH	7
M23271	1055.06	F,D,L	DMSO	494	78,000	518	pH 7	
N1138	184.19	L	DMF, MeCN	419	9400	493	see Notes	8
P2331MP	134.13	L	EtOH	334	5700	455	pH 9	9
V12390	1043.02	F,D,L	DMSO, DMF	503	83,000	510	MeOH	

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. MW value is approximate. BCECF AM is a mixture of molecular species.
2. BCECF AM is colorless and nonfluorescent until converted to BCECF (B1151) by acetoxymethyl ester hydrolysis.
3. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
4. Calcein AM is converted to fluorescent calcein (C481) after acetoxymethyl ester hydrolysis.
5. Acetate hydrolysis of this compound yields a fluorescent product with similar pH-dependent spectral characteristics to C1904.
6. C6826 is converted to a fluorescent product with spectra similar to C1270 after acetate hydrolysis.
7. Bimanes are almost nonfluorescent until reacted with thiols. For monobromobimane conjugated to glutathione, Abs = 394 nm, Em = 490 nm (QY ~0.1–0.3) in pH 8 buffer. (Methods Enzymol (1987) 143:76, Methods Enzymol (1995) 251:133)
8. Spectral data are for the reaction product with glycine in the presence of cyanide, measured in pH 7.0 buffer/MeCN (40:60). (Anal Chem (1987) 59:1102) Unreacted reagent in MeOH: Abs = 279 nm (EC = 5500 $cm^{-1}M^{-1}$), Em = 330 nm.
9. Spectral data are for the reaction product of P2331MP with alanine and 2-mercaptoethanol. The spectra and stability of the adduct depend on the amine and thiol reactants. (Biochim Biophys Acta (1979) 576:440) Unreacted reagent in H_2O: Abs = 257 nm (EC = 1000 $cm^{-1}M^{-1}$).

PRODUCT LIST 15.6 PROBES FOR CELL ADHESION, CHEMOTAXIS, MULTIDRUG RESISTANCE AND GLUTATHIONE

Cat. No.	Product	Quantity
B1150	2′,7′-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM)	1 mg
B3051	2′,7′-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM) *1 mg/mL solution in anhydrous DMSO*	1 mL
B1170	2′,7′-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM) *special packaging*	20 x 50 µg
B7469	BODIPY® FL forskolin	100 µg
B7433	BODIPY® FL prazosin	100 µg
B7431	BODIPY® FL verapamil, hydrochloride	1 mg
C1430	calcein, AM	1 mg
C3099	calcein, AM *1 mg/mL solution in anhydrous DMSO*	1 mL
C3100MP	calcein, AM *special packaging*	20 x 50 µg
C2990	casein, fluorescein conjugate	25 mg
C2925	CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate)	1 mg
C7025	CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) *special packaging*	20 x 50 µg
C6826	5-(and-6)-chloromethyl SNARF®-1, acetate *mixed isomers* *special packaging*	20 x 50 µg
C7026	CyQUANT® Cell Proliferation Assay Kit *for cells in culture* *1000 assays*	1 kit
C35011	CyQUANT® Direct Cell Proliferation Assay *for 10 microplates*	1 kit
C35012	CyQUANT® Direct Cell Proliferation Assay *for 100 microplates*	1 kit
C35007	CyQUANT® NF Cell Proliferation Assay Kit *200 assays*	1 kit
C35006	CyQUANT® NF Cell Proliferation Assay Kit *1000 assays*	1 kit
F13191	fibrinogen from human plasma, Alexa Fluor® 488 conjugate	5 mg
F13192	fibrinogen from human plasma, Alexa Fluor® 546 conjugate	5 mg
F13193	fibrinogen from human plasma, Alexa Fluor® 594 conjugate	5 mg
F35200	fibrinogen from human plasma, Alexa Fluor® 647 conjugate	5 mg
F7496	fibrinogen from human plasma, Oregon Green® 488 conjugate	5 mg
F1314	formyl-Nle-Leu-Phe-Nle-Tyr-Lys, fluorescein derivative	1 mg
G13187	gelatin from pig skin, fluorescein conjugate	5 mg
G13186	gelatin from pig skin, Oregon Green® 488 conjugate	5 mg
G36000	glutathione ethyl ester, biotin amide (BioGEE) *glutathiolation detection reagent* *special packaging*	10 x 100 µg
M23271	methotrexate, Alexa Fluor® 488, inner salt (Alexa Fluor® 488 methotrexate) *mixed isomers*	500 µg
M1198MP	methotrexate, fluorescein, triammonium salt (fluorescein methotrexate)	1 mg
M1381MP	monochlorobimane (mBCl)	25 mg
N1138	naphthalene-2,3-dicarboxaldehyde (NDA)	100 mg
P2331MP	*o*-phthaldialdehyde (OPA) *high purity*	1 g
T10095	ThiolTracker™ Violet (Glutathione Detection Reagent) *180 assays* *set of 3 vials*	1 set
T10096	ThiolTracker™ Violet (Glutathione Detection Reagent) *for 5 microplates*	each
V12390	vinblastine, BODIPY® FL conjugate (BODIPY® FL vinblastine)	100 µg
V13181	Vybrant® Cell Adhesion Assay Kit	1 kit
V13180	Vybrant® Multidrug Resistance Assay Kit	1 kit