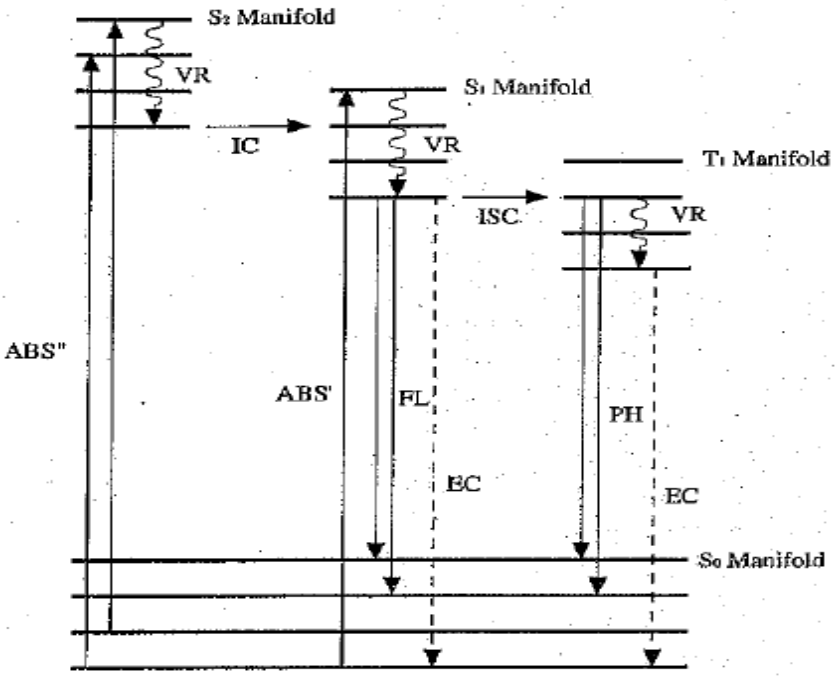
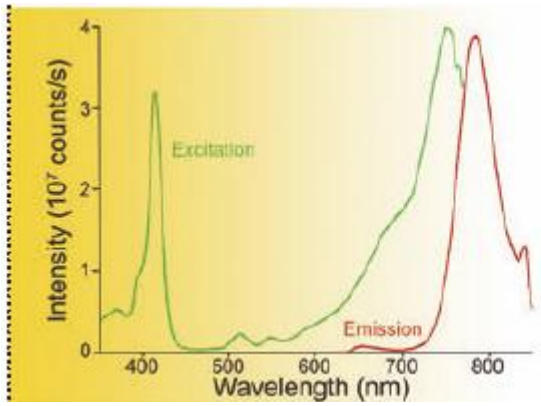
CHEM 524 -- Outline (Part 15) - Luminescence — 2013

XI. Molecular Luminescence Spectra (Chapter 15)

Kinetic process, competing pathways fluorescence, phosphorescence, non-radiative decay

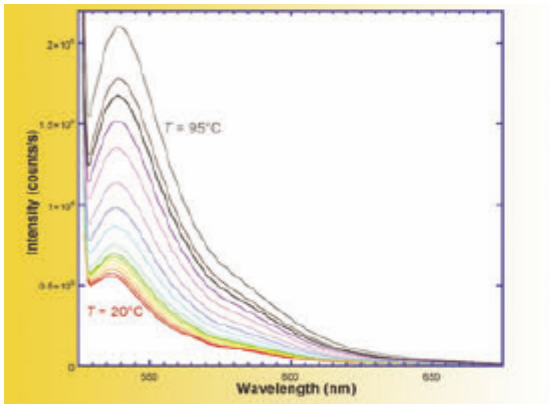


Jablonski diagram summarizes



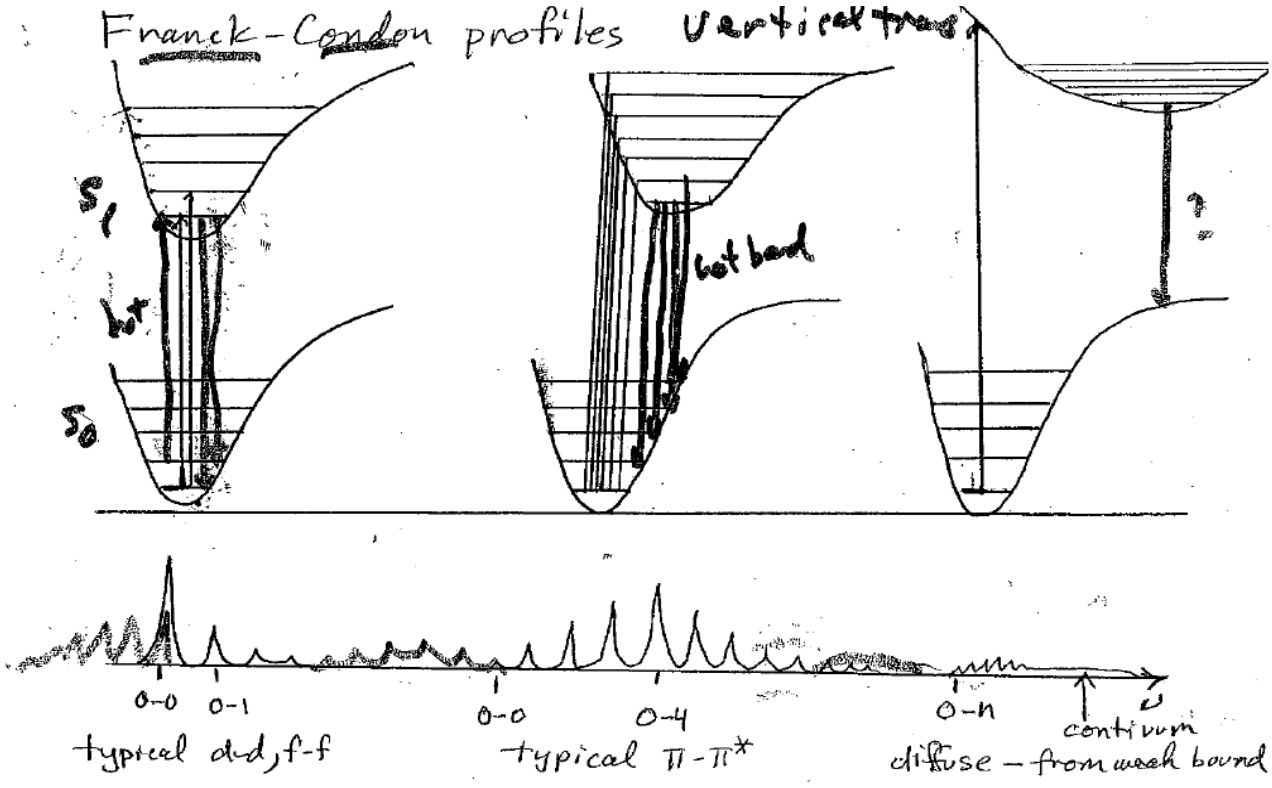
Measuring the ratio-corrected excitation of chlorophyll in the near-IR region supplies information on leaf senescence. The ratio-corrected excitation and emission spectra of chlorophyll from most green vegetation are shown.

Excitation vs. emission



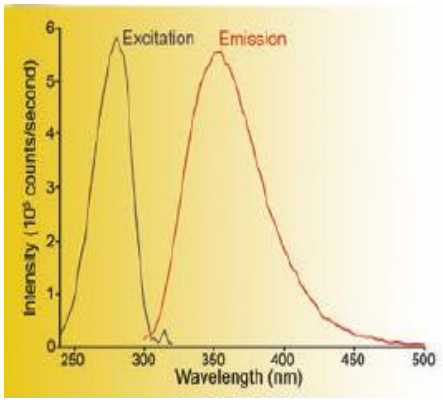
TET, a donor and fluorescent dye, was attached to the 5′ end of a DNA fragment, and QSY, an acceptor or quencher, was attached to the 3′ end. The DNA formed a loop, with a stem keeping the donor and acceptor together at low temperatures. When heated, the loop opened, removing the acceptor from the donor's proximity, and the fluorescence increased. Excitation was at 521 nm.

Energy transfer - distance

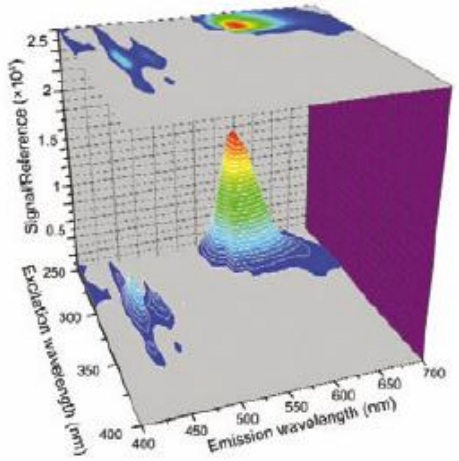


A. Two kinds of measurements (both depend on quantum yield: Q = ϕ_F/ϕ_A ϕ→flux photons)

1. Luminescence – scan spectra as function of λ_{lum}, excited at fixed $\lambda_{ex}\pm\Delta\lambda$
2. Excitation – scan spectra as function of λ_{ex}, detect at fixed λ_{lum} $\pm\Delta\lambda$
   [equivalent to absorption under conditions, const. Quantum yield]



Excitation and emissio
of 10^{-6} *M* tryptophan.
were automatically acq
blank-subtracted, and
in 20 s.



3-D matrix scar
excitation wave
sample of petro
is divided by th
detector to rem
spectral variatio
excitation light.
scans from san
can be used for
and analysis of

Spectra contain contributions from excitation, Raman and scatter and fluorescence/phosphor,
need to sort out: excitation and scatter (Raman) fixed energy relation – shift together
fluorescence and phosphorescence fixed by molecule energetics – don't shift, broader

Gratings still have orders to watch out for as well
Separate excitation from fluorescence is partial role of monochromator, also orthog.optics, filter

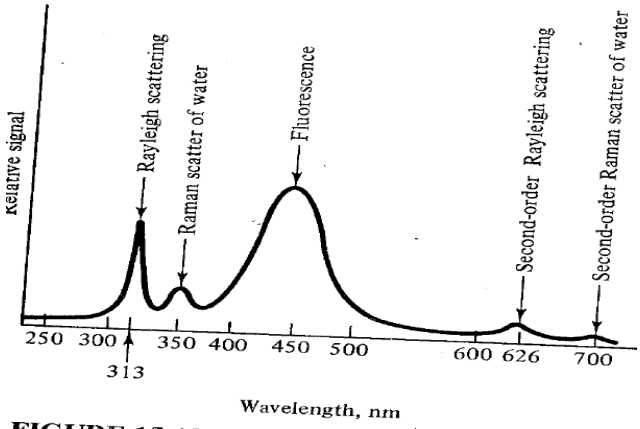
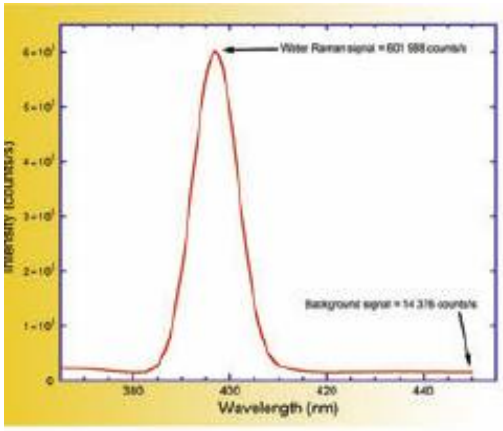


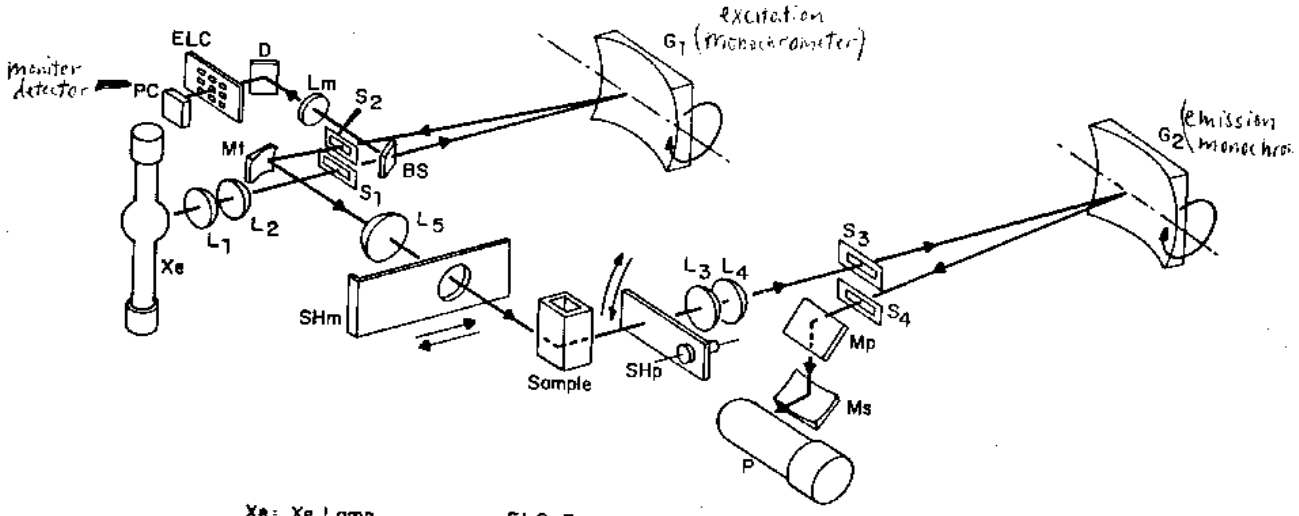
FIGURE 15-18 Hypothetical fluorescence spectrum of a species in water excited by the 313-nm mercury line. The hypothetical molecule is ...



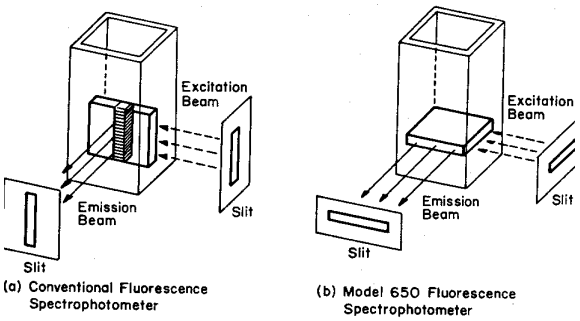
Water-Raman
typical Fluorol
Excitation was
integration tin
bandpass, wit
every 0.5 nm
Signal (at 397
background (a
are indicated i

B. Instrumentation -- typically can control both λ_{Lum}, λ_{ex},

1. Old model: PE Model 650 – typical optics - source Xe, monochromator -- fast, PMT detect minimal optics, shutters (for E_{dark}), slits (resolution, excit.-left, lumin. $\Delta\lambda$ -right), reference



Horizontal slit, allows larger excited volume imaged onto luminescence monochromator



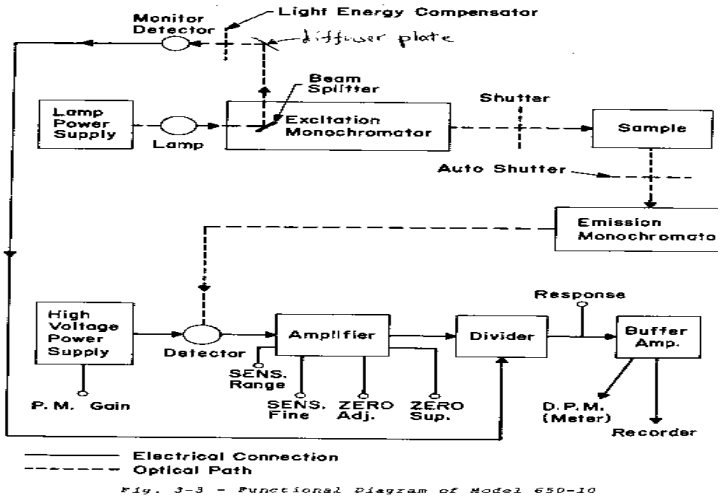


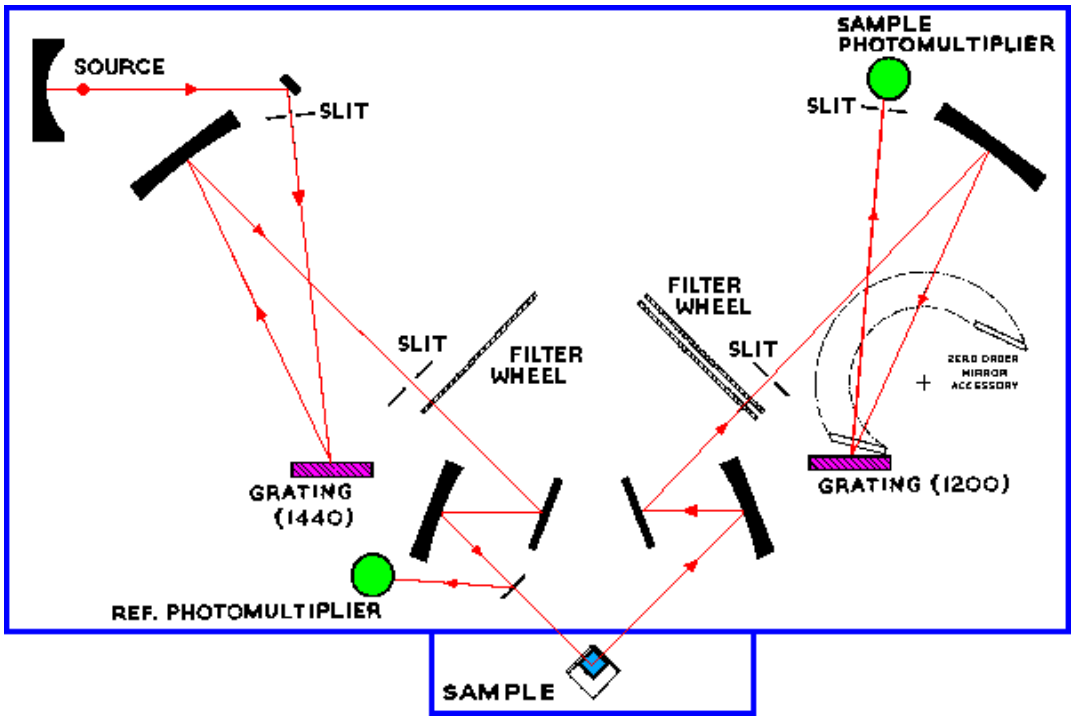
Fig. 3-3 - Functional Diagram of Model 650-10

Stabilization (source instability) - eg arc wander, fluctuate at slit – normalize →

Options: thermal (broad, slow) or quantum detector (spectral, fast)– two options (only a above)

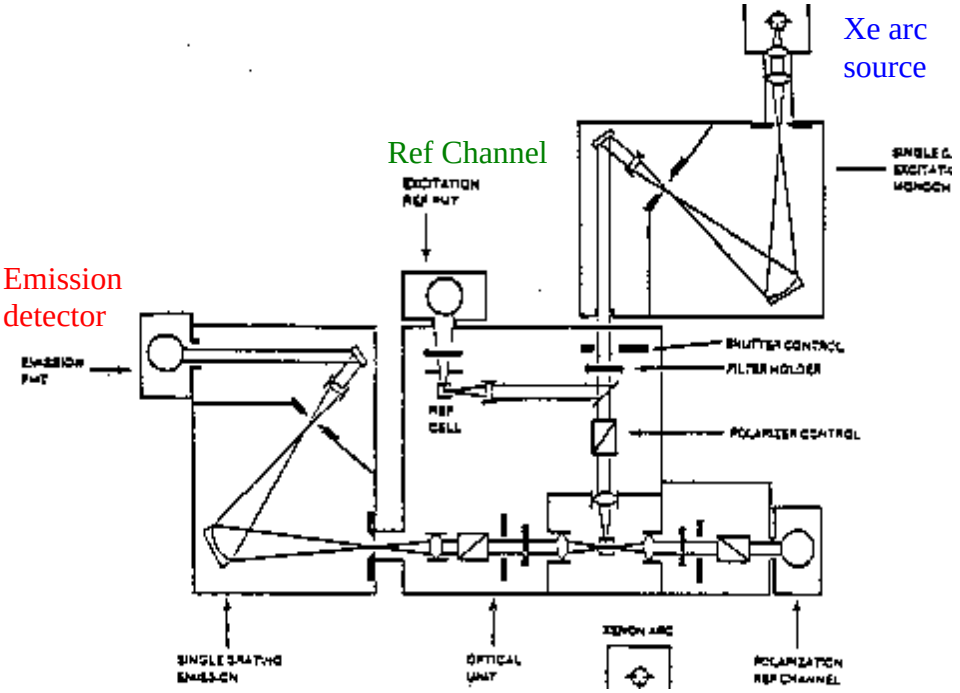
a. beam split off portion of excitation monochromator beam to broad band detector

b. split out part of excitation to broad band dye in separate cell and detect its fluorescence



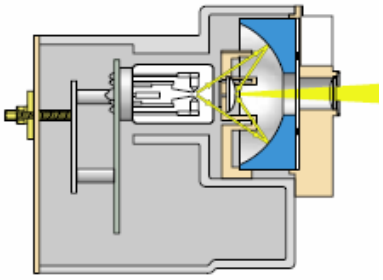
Underlying design for modern version, Perkin Elmer LS-50 and LS-55

2. Alternate models -- SLM - modular design, alternate: double mono excite (low scatter); stabilize with braod band dye fluorescence ("ref cell", after $\lambda_{ex} \pm \Delta\lambda$) -- modular

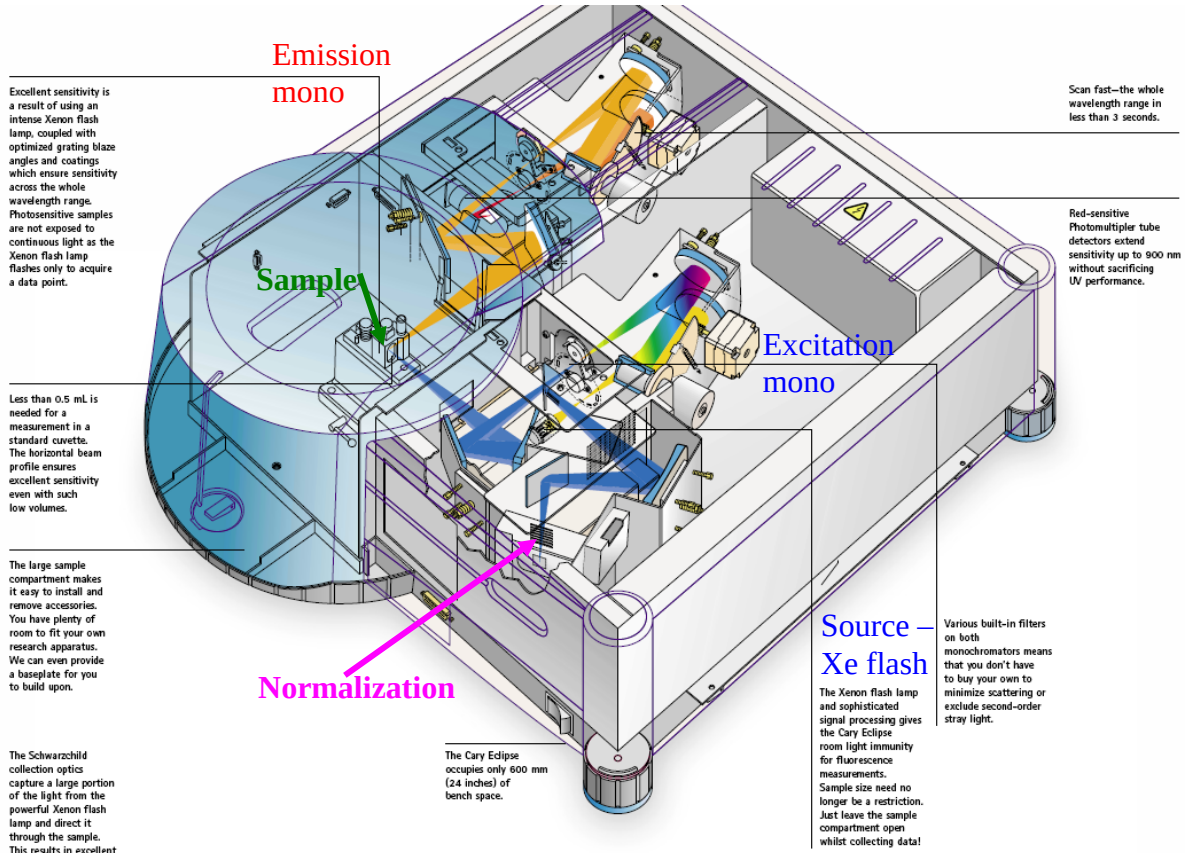


Varian Source optics (next page)

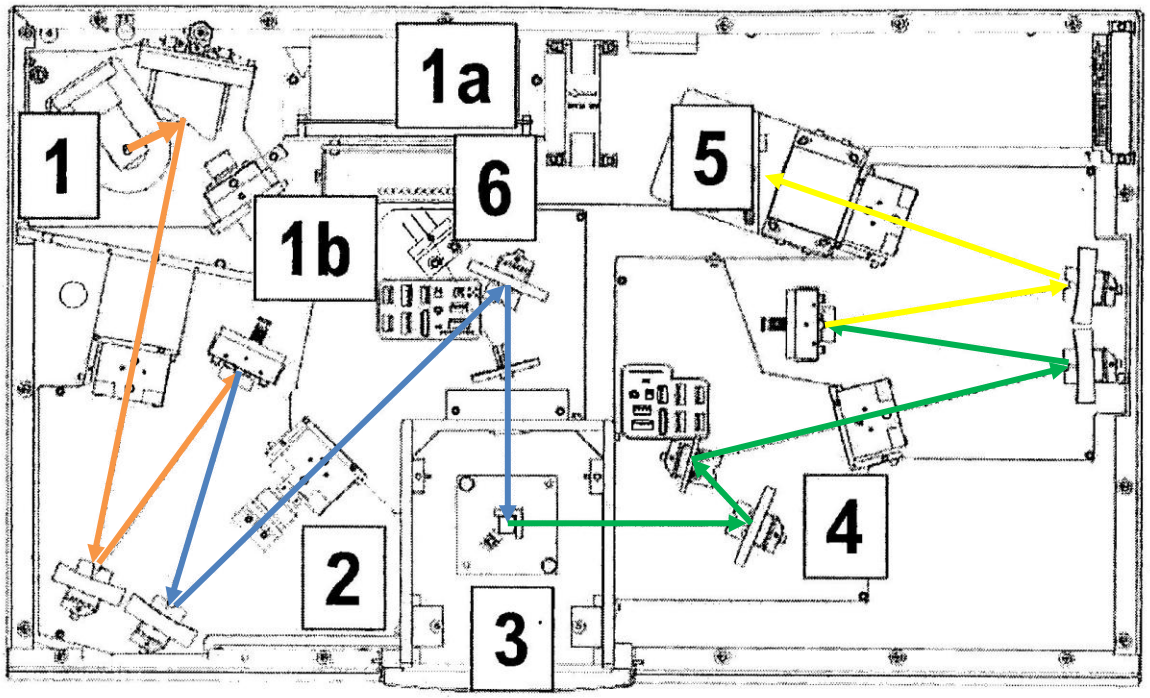
The Schwarzchild collection optics capture a large portion of the light from the powerful Xenon flash lamp and direct it through the sample. This results in excellent sensitivity and low signal noise.



Modern ones – Varian Cary Eclipse – uses Xe flash lamp, collect with fast optics (above)



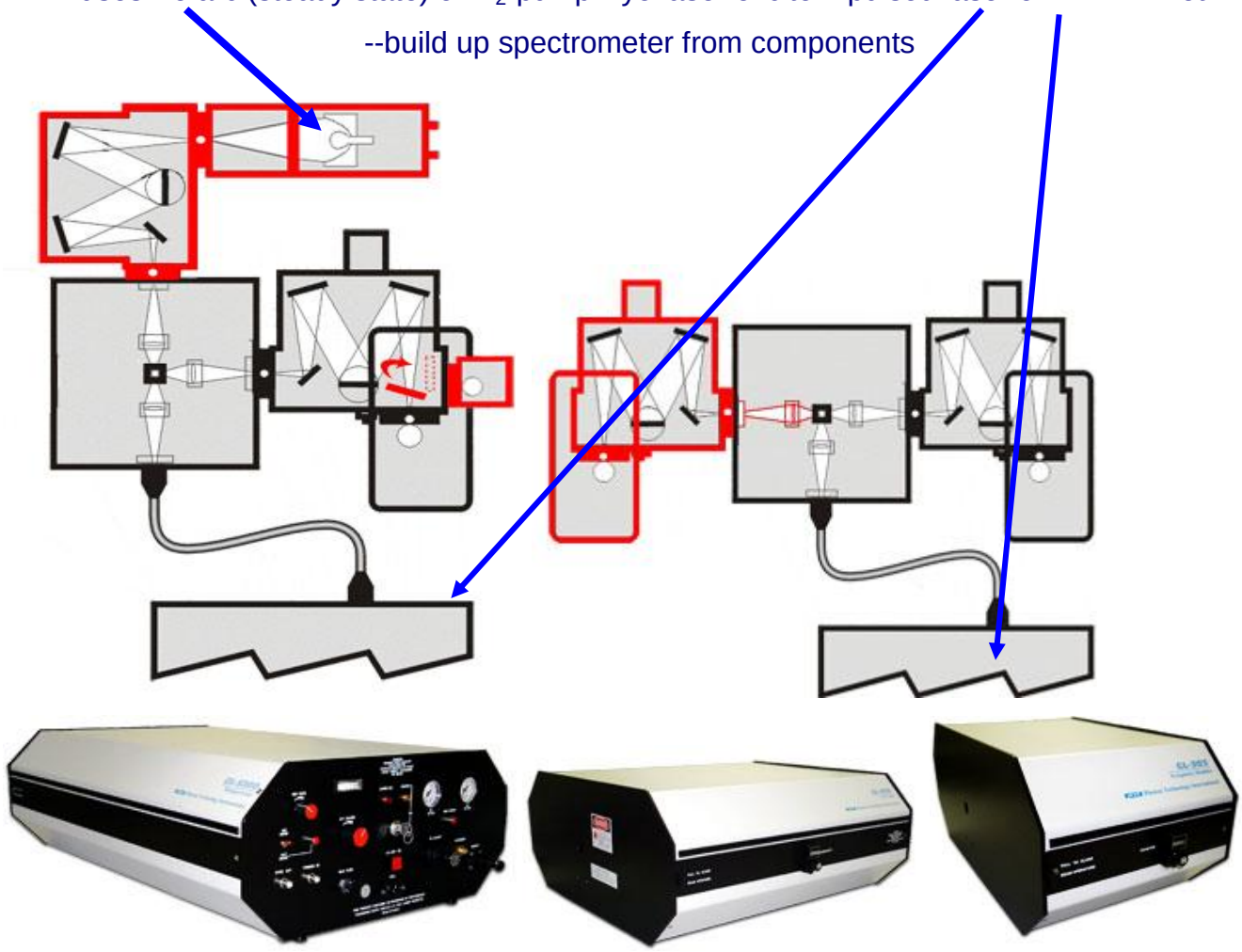
J-Y Horiba Fluoromax– in 4315 SES– conventional Xe source, high sensitivity, ms response



1 Xenon arc-lamp and lamp housing
1a Xenon-lamp power supply
1b Xenon flash lamp (FluoroMax®-4P only)
2 Excitation monochromator
3 Sample compartment
4 Emission monochromator
5 Signal detector (photomultiplier tube and housing)
6 Reference detector (photodiode and current-acquisition module)

PTI—uses Xe arc (steady state) or N_2-pump Dye laser excite – pulsed laser or LED → lifetime

--build up spectrometer from components

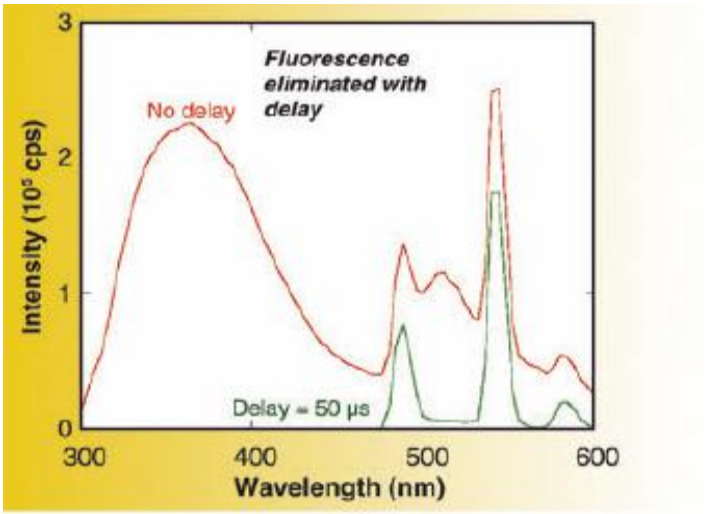


N_2 laser Dye laser (oscill.+ampl.) Frequency doubler (BBO)

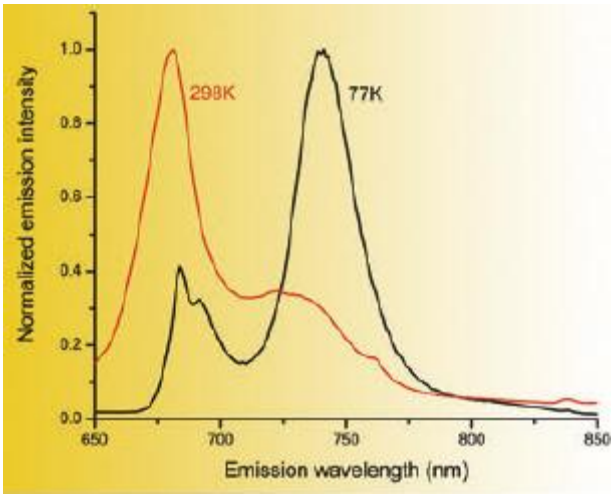
C. ***Measurements*** – Link to mini-review by Frank Bright, and example slides - UK bio-fluores.

1. Excitation -- increase sensitivity over absorption or
   selectivity for specific component (that has luminescence) or
   part of molecule (eg. Trp in proteins)
2. Fluorescence
   -- normally see $S_1 \rightarrow S_0$ since relax vibrations fast to lowest vibration in excited state,
   --if excite S_1 in absorption, then A and F overlap, if there are hot bands - more overlap
   --if excite S_n (n>1) then have a gap, $\Delta E > (E_{Sn} - E_{S1})$
   --intensity proportional to quantum yield: $Q = \phi_F/\phi_A$ $\phi \rightarrow$flux photons, and lifetime, τ_F
3. Phosphorescence – $T_1 \rightarrow S_0$ -- intersystem cross to triplet ($\Delta S \neq 0$)

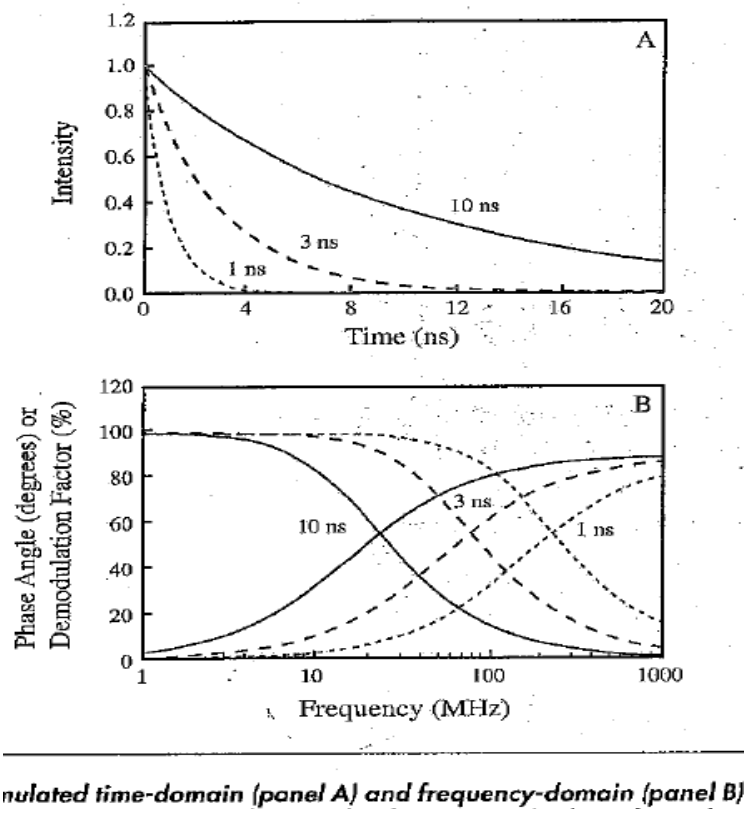
   slow time characteristic - discriminates process, temperature dependent (compete with
   radiationless decay or vibrational relax to ground state from S_1)
   sensitive to heavy atoms (promote ISC - spin-orbit couple)
   lowering temperature can enhance phosphorescence detection, reduce non-radiative



Luminescence detected for mixture of peptide, terbium, and fluoresc
curve) and without (red curve) a 50-µs phosphorimeter delay. Note h
removes unwanted fluorescence, leaving only long-lived phosphores



Cooling reduced relative fluorescence w/r/t phosphorescence, by reducing non-radiative decay, allowing more ISC



nulated time-domain (panel A) and frequency-domain (panel B)

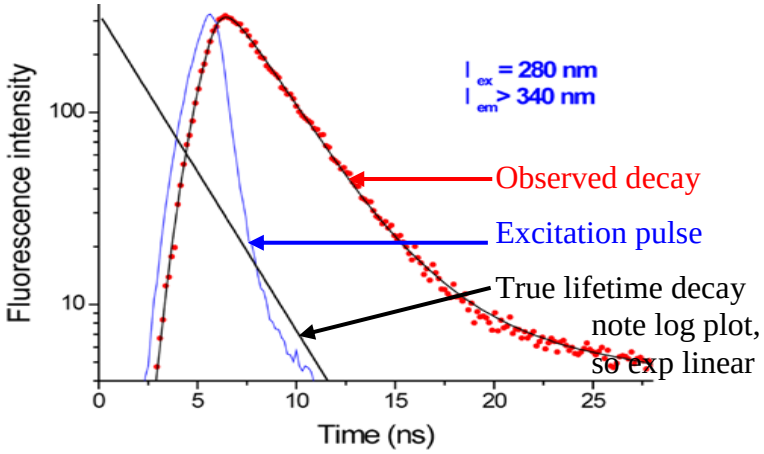
Examples from Bright review
Two ways of lifetime sensing

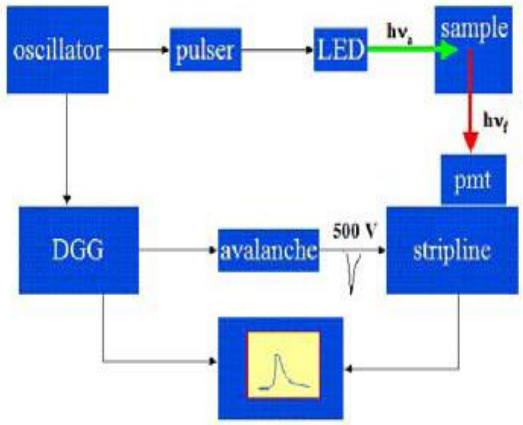
Time domain decay,
fit I s. t to exponential, e^{-kt}
like 1st order kinetics

Frequency domain phase shift
Faster decay has less phase shift,
i.e. apparent at higher frequencies

4. Time dependence -- lifetime: $\phi = \phi^o \exp(-t/\tau)$ where τ -lifetime, $(\tau)_{1/2} = 0.69\ \tau$

Time-domain lifetime measurements (excitation and fluores. time can be comparable):
phosphorescence -- measure decay directly $\phi(t)$, $\tau_P = (k_P + k_{nr}')^{-1} \sim 10^{-4}$-$10^{4}$ sec
fluorescence – must deconvolve exciting pulse, $E_F'(t) = \int E_{ex}(t')E_F(t-t')dt'$
Observe I_F variation: E'_F, real is E_F, decay time: $\tau_F = (k_F + k_{nr})^{-1} \sim 10^{-5}$-$10^{-8}$ sec



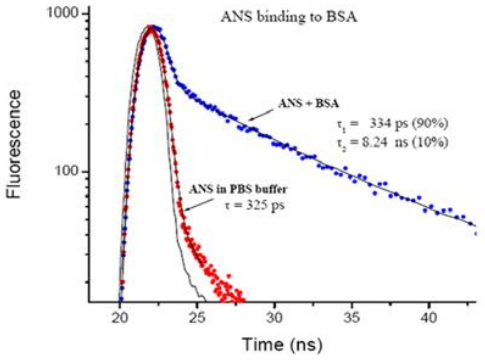


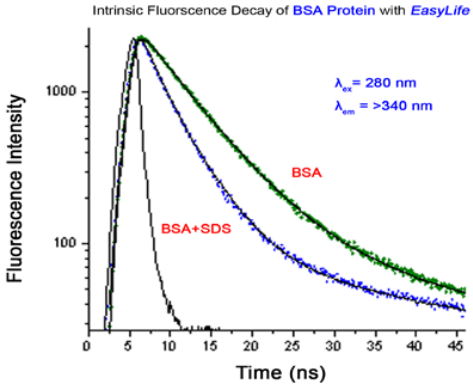
Method – laser/flash/diode – excite; detect fast PMT/avalanche and boxcar/transient digitize Stroboscopic→pulse the source and look at sequential time delays to plot decay of fluorescence with gated PMT and time resolved data storage (Trp fluor. w/Easy Life)

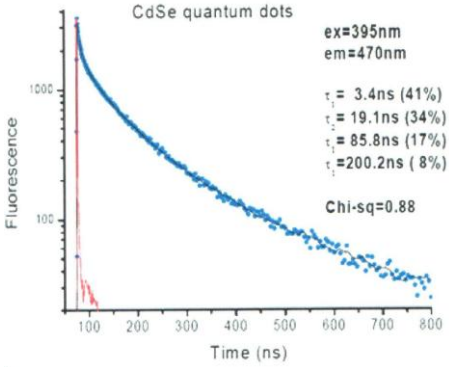
[REPEAT: from Wiki: When decay exponential is similar in time to the excitation pulse or detection response is wide, the measured fluorescence, M(t), will not be purely exponential. The instrumental response function, IRF(t) will be convolved or blended with the decay function, F(t).

$$M(t) = IRF(t) \otimes F(t)$$

The decay function (and corresponding lifetimes) cannot be recovered by direct deconvolution using Fourier transforms because division by zero will produce errors and noise will be amplified. The instrumental response of the source, detector, and electronics can be measured, usually from scattered excitation light. The IRF can then be convolved with a trial decay function to produce a calculated fluorescence, which can be compared to the measured fluorescence. The parameters for the trial decay function can be varied until the calculated and measured fluorescence curves fit well. This is known as reconvolution or reiterative convolution, and can be performed by software.]



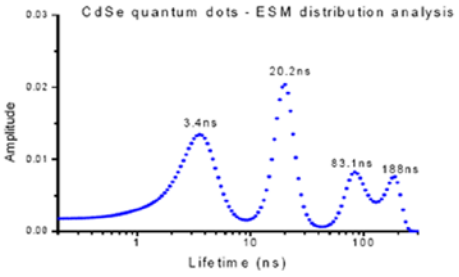


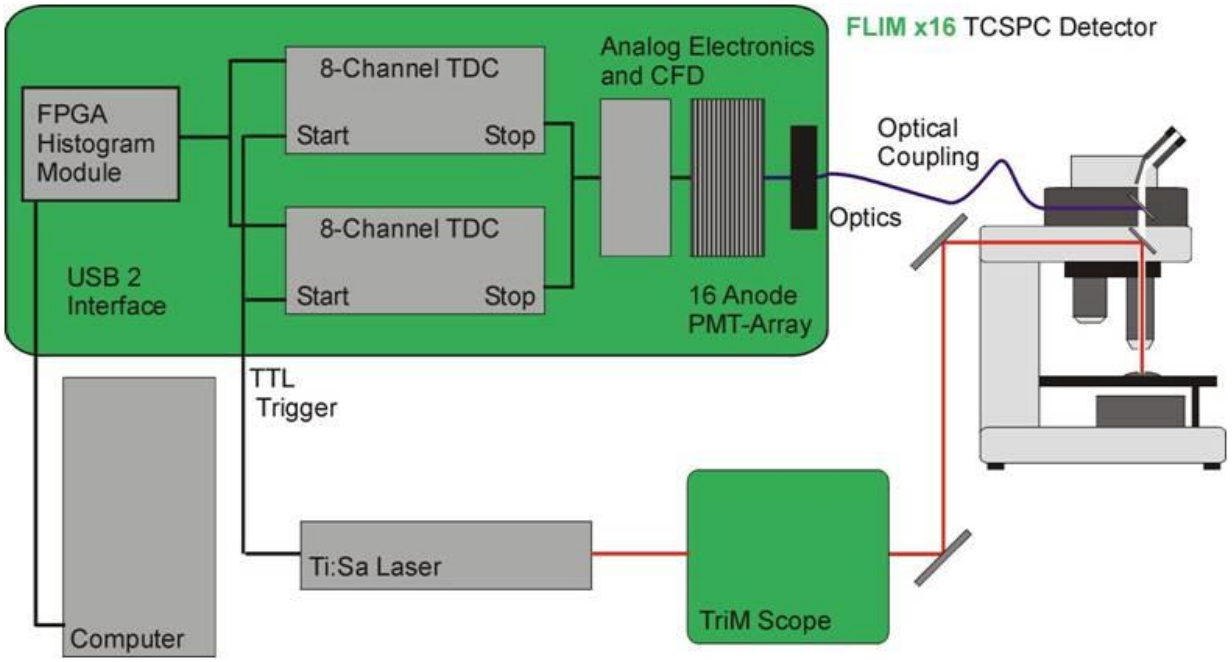


Double exponential, ANS free-fast fast fluorescence, see decay almost same as excitation, when bound to BSA-slows fluorescence, almost straight line in log plot (exp)

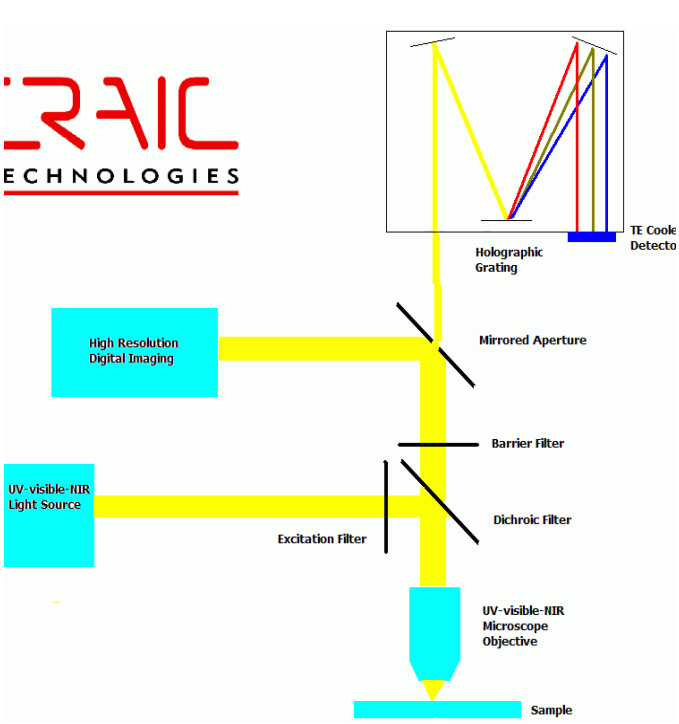
(middle) similarly BSA+SDS faster decay, double exponential decay, note, shifted t = 0

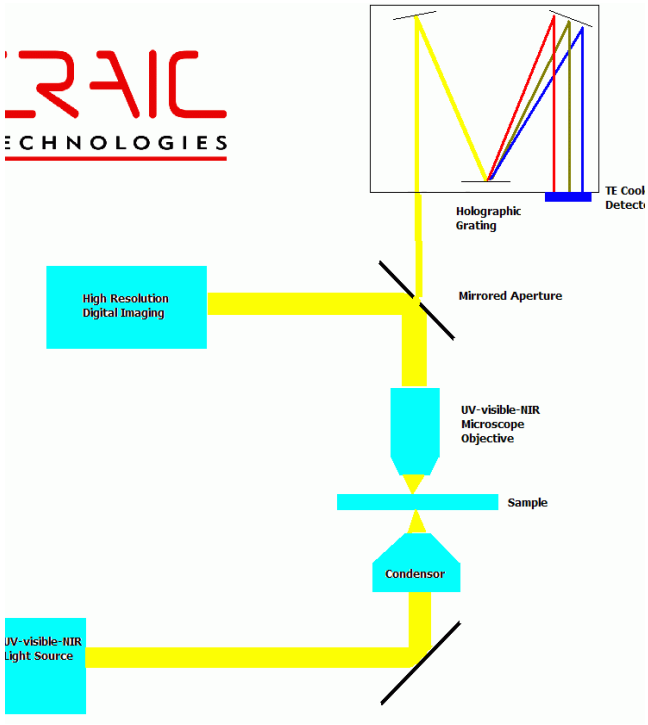
Works for quantum dots as well, CdSe slower fluorescence but multiexponential, can resolve component distribution





Couple to microscope, now popular for following spatial aspects, imaging, single molecule

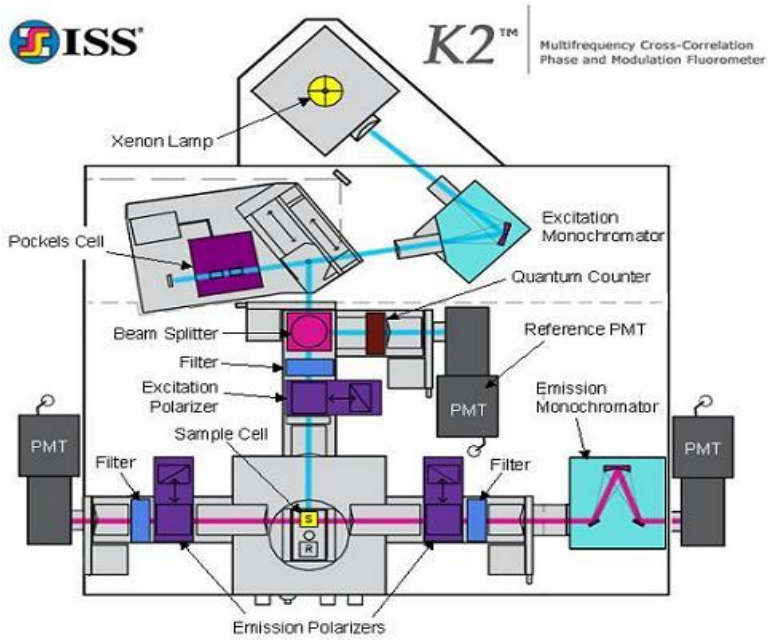


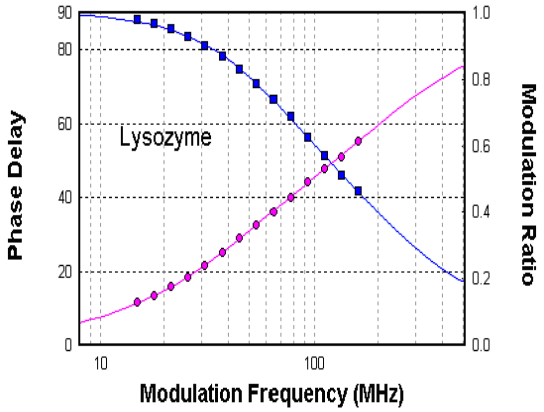


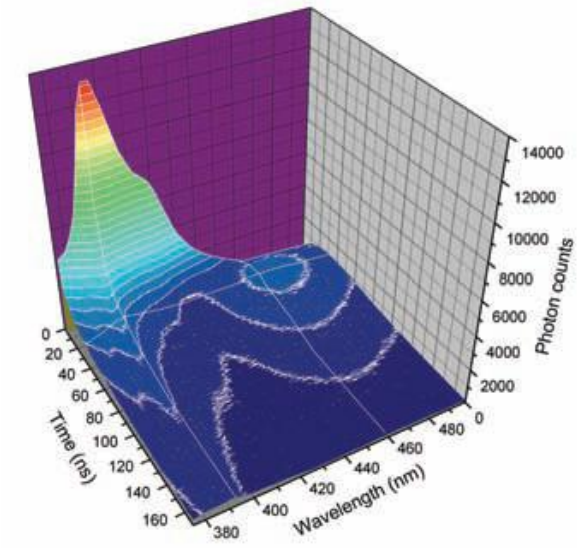
Fluorescence microspectrom. (also reflectance) Absorbance (transmission) microspectrom

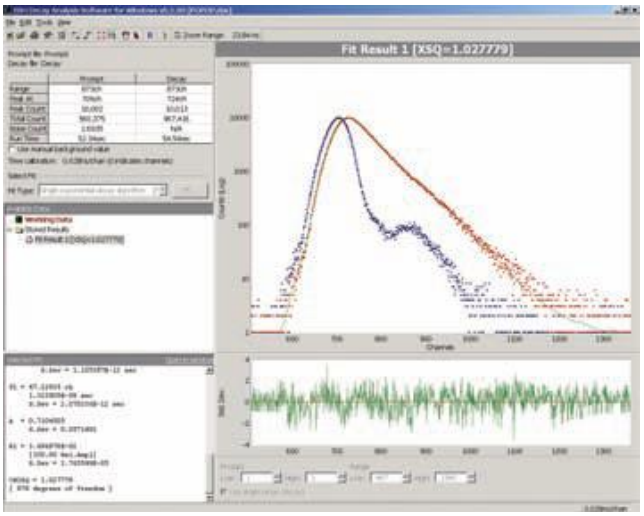
Phase resolved method -- modulate source (cyclic repeat), lifetime induces a phase shift

[From Wiki: Alternatively, fluorescence lifetimes can be determined in the frequency domain by a phase-modulated method. The intensity of a continuous wave source is modulated at high frequency, by an acousto-optic modulator for example, which will modulate the fluorescence. Since the excited state has a lifetime, the fluorescence will be delayed with respect to the excitation signal, and the lifetime can be determined from the phase shift. Also, y-components to the excitation and fluorescence sine waves will be modulated, and lifetime can be determined from the modulation ratio of these y-components. Hence, 2 values for the lifetime can be determined from the phase-modulation method.]





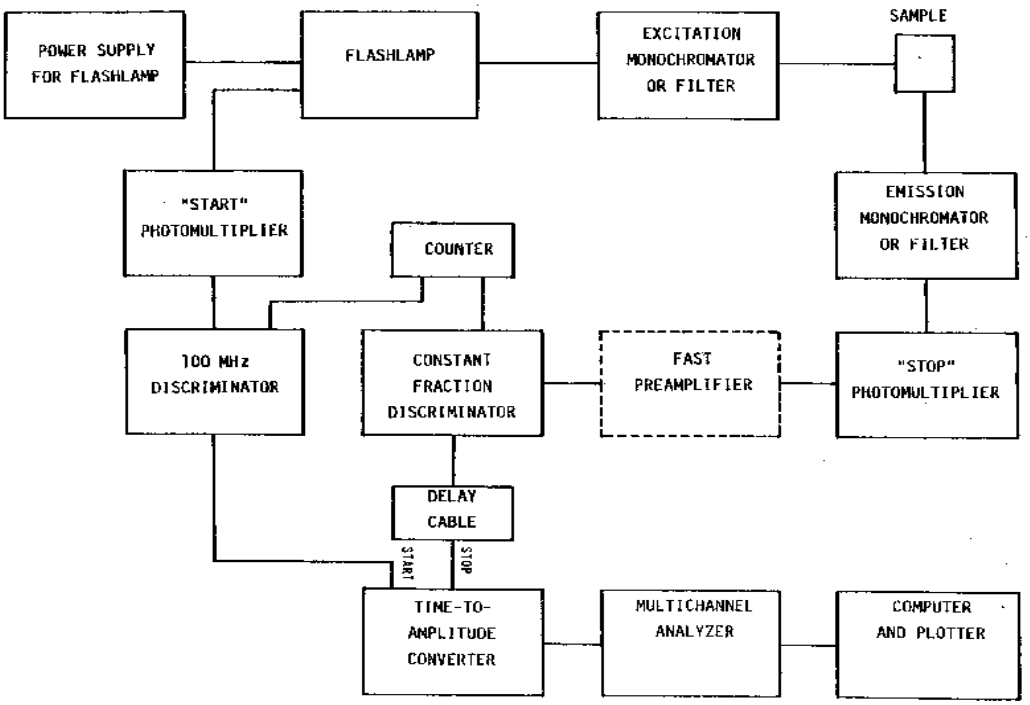


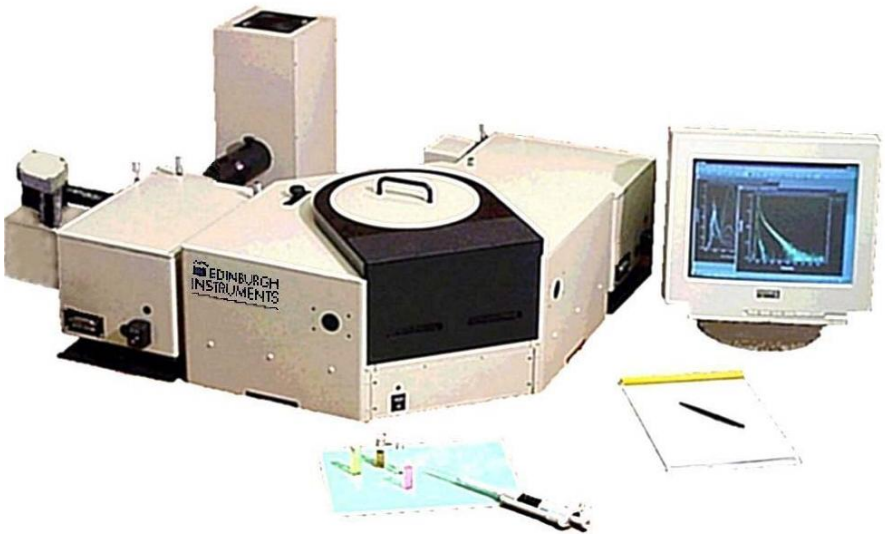


TCSPC (J-Y) 3-D plot: lifetime, freq., inten. POPOP decay in MeOH (red), lamp pulse (blue)

-- pulse correlation scheme

SCHEMATIC DIAGRAM OF FLUORESCENCE LIFETIME INSTRUMENT





Combined Fluorescence Lifetime & Steady State Spectrometer

Lifetime ranges 10 ps - 10 s, UV - Vis - NIR spectral range, Single Photon Counting sensitivity

Many accessories for variations on standard luminescence (e.g. Varian Cary Eclipse)

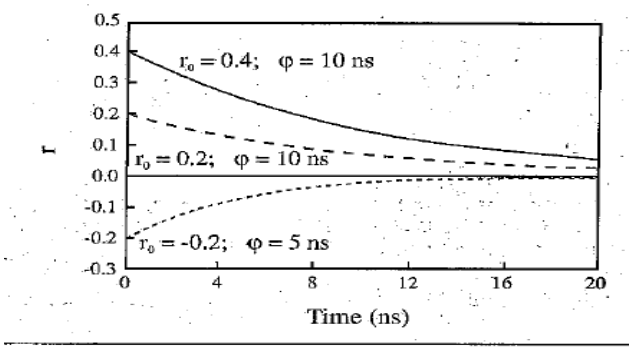
5. *Quenching*--external conversion--loss of excitation to another species

$k_{ec} = k_q[Q]$ Stern-Volmer relation: $(\phi_{FQ}^{o})^{-1} = (\phi_F^{o})^{-1} + K_Q[Q]/(\phi_F^{o})$

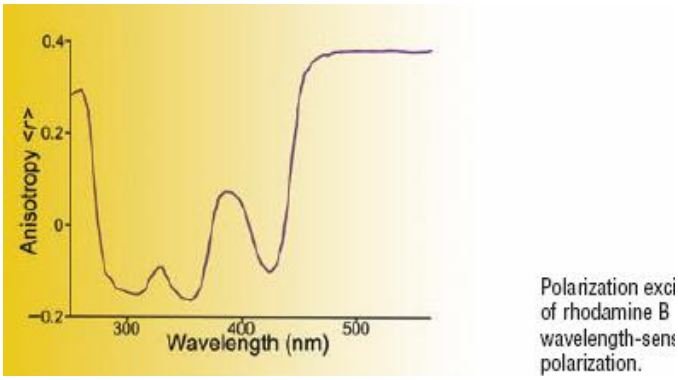
$\phi_F^{o} = k_q/(k_F + k_{IC} + k_{ISC})$ -- use dipole acceptor for S_1 and triplet for T_1

[Alternate formulation: The dependence of the emission intensity, *F* on quencher concentration *[Q]* is given by the Stern-Volmer equation: $F_0/F = T_0/T = 1 + k_q T_0[Q]$ where: T and T_o is the lifetime in the presence and absence of quencher, respectively, and k_q is the bimolecular rate constant for reaction of quencher with fluorophore. The product of $k_q T_o$ is referred to as the ***Stern-Volmer constant*** or K_{SV}]

6. *Polarization anisotropy* $r = (R - 1)/(R + 2)$ polarization ratio: $R = \phi_{||}/\phi_{\perp}$

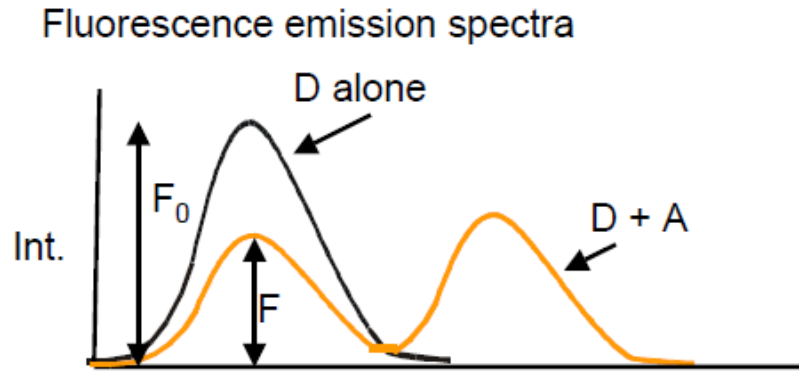


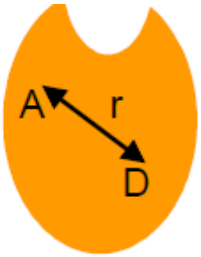
Simulated time-resolved anisotropy decay profiles for several



Polarization exci
of rhodamine B
wavelength-sens
polarization.

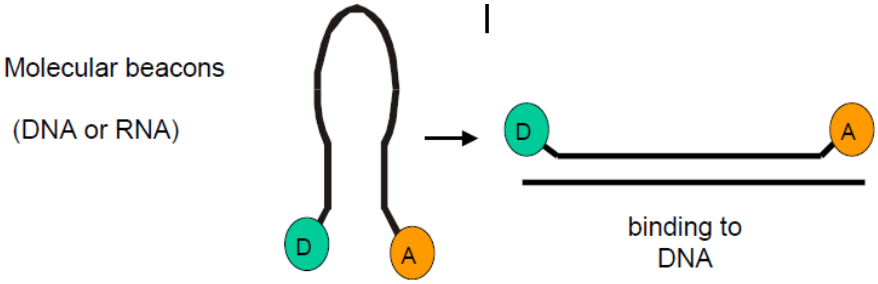
7. *Linearity* $E_L \sim c$ for $A < 0.005$
8. *Saturation* -- approach equilibrium with excited state
9. *Detection Limit* -- 0.1-10 pM -- strength of fluorescence
10. Distance measurements, FRET – measure efficiency (E) of fluorescence energy transfer
    Förster theory: $E = R_0^6/(r^6 + R_0^6)$ → R_0= distance of separation at which E = 50%





Measure decrease in F for donor caused by the presence of the acceptor

Efficiency of transfer $E = 1 - F/F_0$



Label ends of biopolymer
Measure distances between sites in protein (above) or sense binding in DNA or folding of a protein/peptide

Spec Sheet comparisons: J-Y Horiba Fluoromax 4 (analogous to Dept. instrum.)

The source
Starting with a xenon source that supplies prime UV performance, we mount the bulb vertically, because horizontal mounting leads to sagging, instability, and shorter arc-life. The xenon source is focused onto the entrance-slit of the excitation monochromator with an elliptical mirror. Besides ensuring efficient collection, the reflective surface keeps all wavelengths focused on the slit, unlike lenses (with chromatic aberrations that make them optimally efficient only at one wavelength).

The slits
The slits are bilateral, continuously adjustable by the software in units of bandpass (wavelength) or millimeters. This preserves maximum resolution and instant reproducibility.

The excitation monochromator
The excitation monochromator is an aspheric design which ensures that the image of the light diffracted by the grating fits through the slit. The gratings themselves are blazed and planar, avoiding the two major disadvantages of the more common concave holographic gratings: poor polarization performance and inadequate imaging during scans that throws away light. The unique wavelength-drive scans the grating at speeds as high as 80 nm/s. The grating's grooves are blazed to provide maximum light in the UV and visible region.

The reference detector
Before the excitation light reaches the sample, a photodiode reference detector monitors the intensity as a function of time and wavelength to correct for any change in output due age or wavelength. The photodiode detector is traceable to NIST standards out to 1000 nm, and requires no maintenance.

The sample chamber
A spacious sample chamber is provided to allow the use of a wide variety of accessories for special samples, and encourage the user to experiment with many sample schemes.

The emission monochromator
All the outstanding features of the excitation monochromator are also incorporated into the emission monochromator. Gratings are blazed to provide maximum efficiency in the visible. Correction-factor files traceable to NIST lamps remove optical artifacts from the optical path through the monochromator.

The detector
Emission-detector electronics employ photon-counting for the ultimate in low-light-level detection. Photon-counting concentrates on signals that originate from fluorescence photons, ignoring the smaller pulses originating in photomultiplier-tube electronics. Lower-performance fluorometers with analog detection—in contrast—simply add noise and signal together, hiding low signals within the noise. The emission-detector housing also contains an integral high-voltage supply which is factory-set to provide the maximum count-rate, while eliminating most of the dark noise.

Computer-control
The entire control of the FluoroMax®-4 originates in your PC, from our most powerful software, FluorEssence™. On start-up, the system automatically calibrates and presents itself for new experiments, or stored routines instantly called from memory. Professional, publication-ready plots and data-analysis are based on world-renowned Origin®.

Accessories

LAMP, xenon replacement, 150 W ozone-free	250 µL reduced volume cell	CELL-HOLDER, single-position thermostatted, with magnetic stirrer
CUVETTE, 4 mL, quartz, capped	Adapter for F-3012	POLARIZER, automated L-format
CUVETTE, 4 mL, quartz, stoppered	Janis Cryostat	PHOSPHORIMETER upgrade
LIQUID-NITROGEN DEWAR (spare)	Fiber optic adapter	WINDOWS for the FluoroMax®-4 sample compartme
HOLDER for solid samples	HOLDERS (2) for filters	Filter Holder
FILTERS, 1″× 2″ (2.5 cm × 5 cm), cut-on, set	CELL-HOLDER, automated four-position thermostatted, with magnetic stirrer	TCSPC upgrade
FILTERS, 2″ × 2″ (5 cm × 5 cm), cut-on, set	CELL-HOLDER, automated dual-position thermostatted, with magnetic stirrer	MICROSCOPE INTERFACE
CELL, HPLC flow	LIQUID-NITROGEN DEWAR assembly	PLATE-READER, Microwell
TEMPERATURE BATH	PORT, injector	CELL, reduced volume 500 µL, with adapter
PELTIER DRIVE, sample heater/cooler		STOPPED-FLOW accessory
INJECTOR, autotitration		TRIGGER accessory, external
QUANTUM-YIELD accessory		

Specifications:

Optics	All-reflective for focusing at all wavelengths and precise imaging for microsamples
Source	Ozone-free xenon lamp eliminates venting
Spectrometers	Plane-grating Czery-Turner design maintains focus at all wavelengths
Excitation	200–950 nm, optimized in the UV
Emission	200–950 nm, optimized in the visible
Bandpass	0–30 nm, continuously adjustable from computer
Wavelength Accuracy	± 0.5 nm
Scan Speed	80 nm/s
Integration Time	1 ms to 160 s
Emission Detector	Photomultiplier, range 200–850 nm
Reference Detector	Photodiode selected for stability
Water-Raman Signal	400,000 counts/second minimum at 350 nm excitation, 397 nm emission, 5 nm bandpass, 1 s integration time
Signal-to-Noise Ratio	3000:1 (steady-state mode)
Nanosecond Lifetime Option	Lifetime range 200 ps–0.1 ms (100 ps optional; may degrade CW performance slightly) Minimum resolution < 7 ps/channel Excitation with interchangeable NanoLEDs: 265–785 nm TCSPC detection
Phosphorescence Lifetime Option (in FluoroMax®-4P)	Lifetimes down to 10 µs Delay variable 50 µs–10 s Sampling time variable 50 µs–10 s Excitation with broadband pulsed UV xenon lamp Flash rate 0.05–25 Hz Flash duration 3 µs FWHM; low-intensity tail > 30 µs Flashes per data point 1–999
System Control	PC, with FluorEssence™ software
Dimensions (w × h × d)	32.5″ × 11″ × 19″; 82.6 cm × 28 cm × 48 cm
Sample Compartment (w × h × d)	5.5″ × 7″ × 7″; 14 cm × 18 cm × 18 cm
Power Requirements	5 A, 120 V; 2.5 A, 240 V; 50 or 60 Hz, single-phase
Weight	75 lbs; 34 kg

Perkin Elmer LS 45 and LS 55 – only sales pitch, e.g. like first part of J-Y above

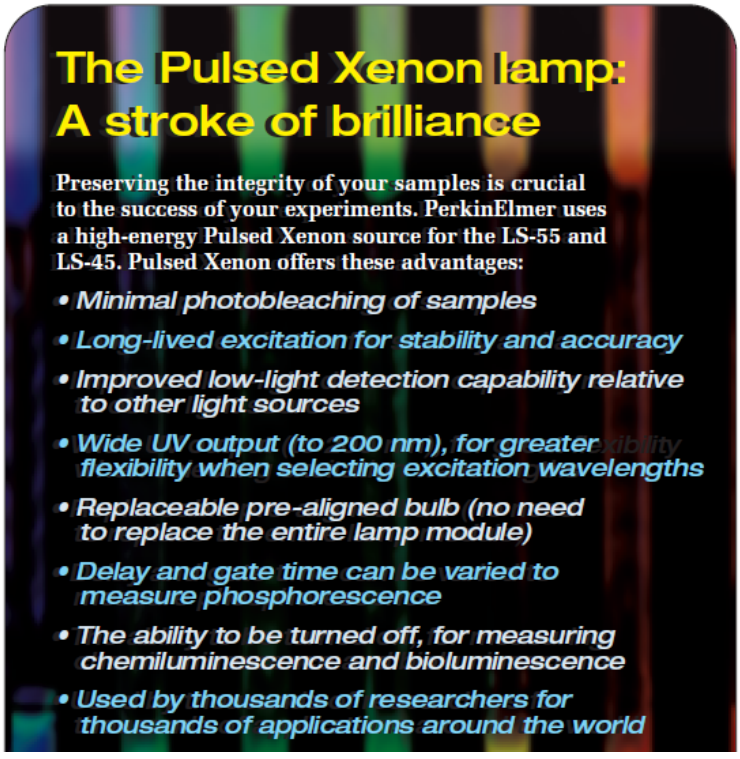
The PerkinElmer LS-55 and LS-45 Fluorescence Spectrometers provide your laboratory with the ultimate blend of high performance, reliability, ease-of-use, durability, and versatility. The LS-55 and LS-45 are the right choice to meet your laboratory's current and future applications needs. Indispensable features of these instruments include:

Ultimate flexibility. No instruments handle a wider range of bioscience applications than the LS-55 and LS-45. These instruments are ideally suited for bioresearch including cell-biology, immunology, enzymology, protein analysis, and microplate-based measurements.

Broad range of accessories. The wide range of accessories specifically designed for the LS-55 and LS-45 is versatile enough to handle virtually any type of sample.

Reliable optical performance. The LS-55 features continuously variable slits, in increments of 0.1 nm, for ultimate control of measurement conditions. The holographic gratings of the LS-55 minimize stray light for the highest sensitivity, accuracy and reproducibility. The LS-45 has fixed slits, for sturdy, dependable daily use.

Durable, long-lived light source. Unlike conventional light sources, the Pulsed Xenon lamp minimizes photobleaching of samples. This preserves the integrity of the sample and delivers accurate and uncompromised results.



User-friendly software. Unique to PerkinElmer, FL WinLab™ software is a comprehensive package that provides the most powerful and flexible system for data collection and analysis. Built-in validation protocols assure you that the instrument is working properly.

Versatility. Multiple accessory options allow you to configure exactly the system you need, whatever your lab's specific needs. Purchase only the accessories you need now and add more accessories as your applications expand.

LS-55:
Scanning and plate reading – *in one system*

The plate-reader accessory lets you switch from a fully functional fluorescence spectrometer to a well-plate reader in seconds. The plate reader is mounted on the front, providing easy access to standard multi-well plates.

Completely automated system
Read up to 96 well plates in either the X or Y direction.

Greatest range of applications
The excitation light is collected via a fiberoptic bundle that directs light onto the well, requires no alignment, and works over a range of wavelengths.

Results in "real time"
Data is saved as an ASCII file that is compatible with most word processing and spreadsheet software packages.

Varian Cary same type approach, brochure focus on capabilities, not numerical specifications

EasyLife V – OBB Corp, measures fluorescence lifetimes – excite with pulsed LED lamp, must select what region you wish to excite, purchase different LED for each,
measure total fluorescence (select with filter),

Specifications:

- **Lifetime range:** 100 ps to 3 µs
- **Sensitivity:** 400 picomolar fluorescein
- **Excitation:** OBB proprietary nanosecond LEDs
- **Optical pulse width:** 1.5 ns (typical)
- **Excitation range available:** 280-670 nm
- **Emission range:** 185-680 nm, Optional to 900 nm
- **Wavelength selection:** 2" square or 1" round filters
- **Detection:** Patented lifetime detector
- **Typical acquisition time:** 20 s (sample dependent)
- **Timescale (menu selectable):** Linear, arithmetic and logarithmic
- **Acquisition mode:** Sequential or random
- **Sample holder:** Single 1 x 1 cm cuvette
- **Software:** FeliX GX
- **Lifetime analysis:** Complete package: 1-4 exponential, global, non-exponential, micelle kinetics, lifetime distribution (ESM and MEM), anisotropy, FRET calculator
- **QuickStart DVD:** Included

* All specifications subject to change without notice