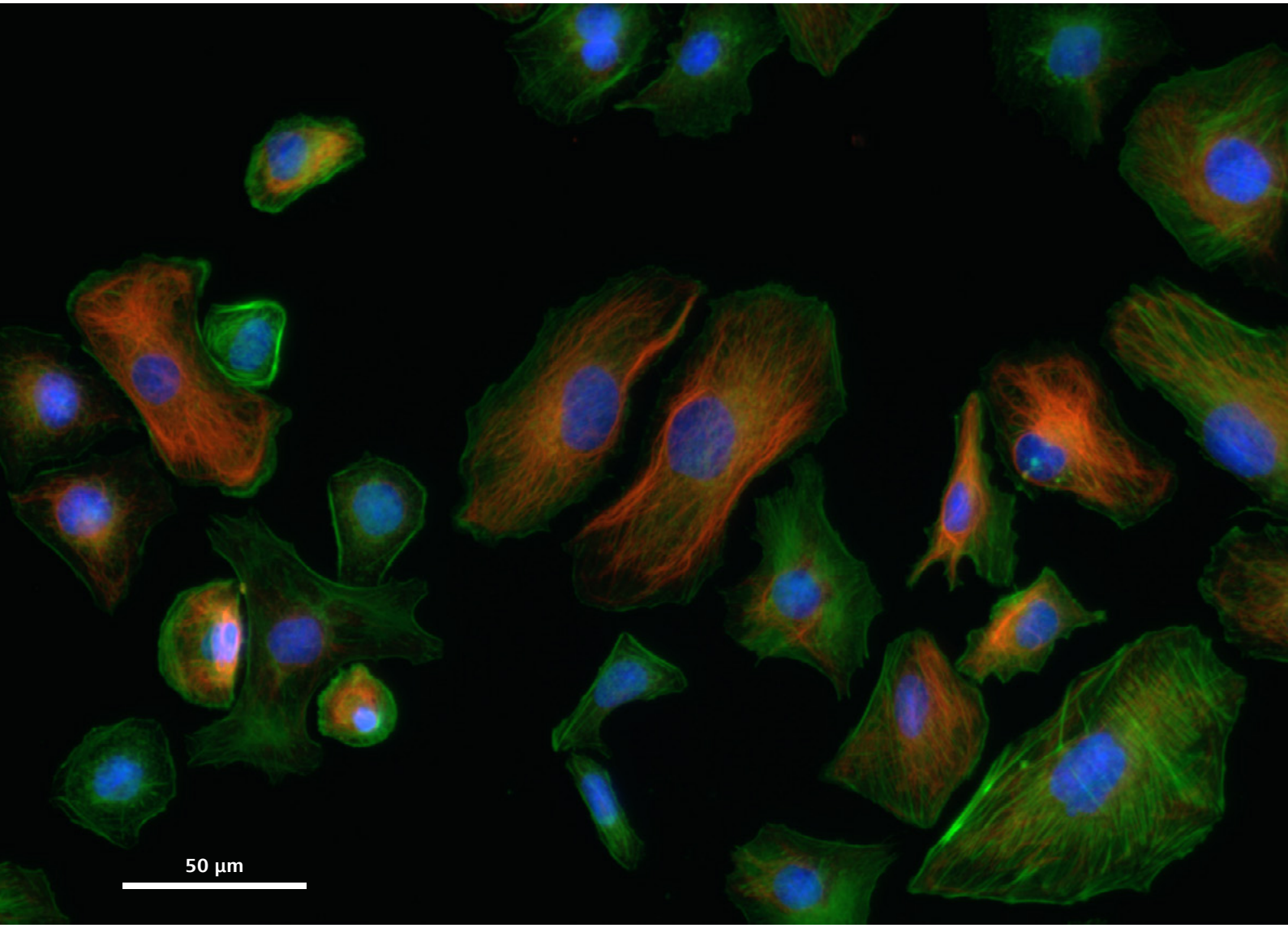

Principles of Fluorescence and Fluorescence Microscopy

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Fluorescence is the property of atoms and molecules, so called fluorophores, to absorb light at a particular wavelength and to subsequently emit light of longer wavelength. Fluorescence microscopy can be based on autofluorescence or the addition of fluorescent dyes. It is mainly used in biology and medicine to observe structures and processes within a specimen.

Introduction

In 1852, the Irish physicist and mathematician Sir George Gabriel Stokes first observed fluorescence when sunlight, filtered through a purple glass window, hit a flask of quinine water and blue light was emitted. Stokes observed this blue light because it travelled through a glass of white wine, which filtered out the purple light from the window, leaving only the blue light emitted by the quinine[1]. Stokes observation also exemplifies the principle of the fluorescence microscope — without the light-filtering abilities of the purple glass window and the glass of white wine, Stokes would not have been able to observe any fluorescence at all. Using Stokes' observation and the green fluorescent protein (GFP) as examples, this article will explain fluorescence and fluorescence microscopy.

The Principle of Fluorescence

A fluorophore is a molecule that can fluoresce. This means that the molecule can absorb and emit photons, or particles of light, of different wavelengths. For instance, the quinine in Stokes' flask was able to absorb purple light and emit blue light. Fluorescence can therefore be defined as the emission of light (photons) at one wavelength resulting from absorption of light (photons) at another, typically shorter, wavelength. On the atomic level, absorption of a photon by an electron in the fluorophore causes the electron to jump into an orbital further away from the atom nucleus (i.e., into a higher energy, or excited, state) (Figure 1).

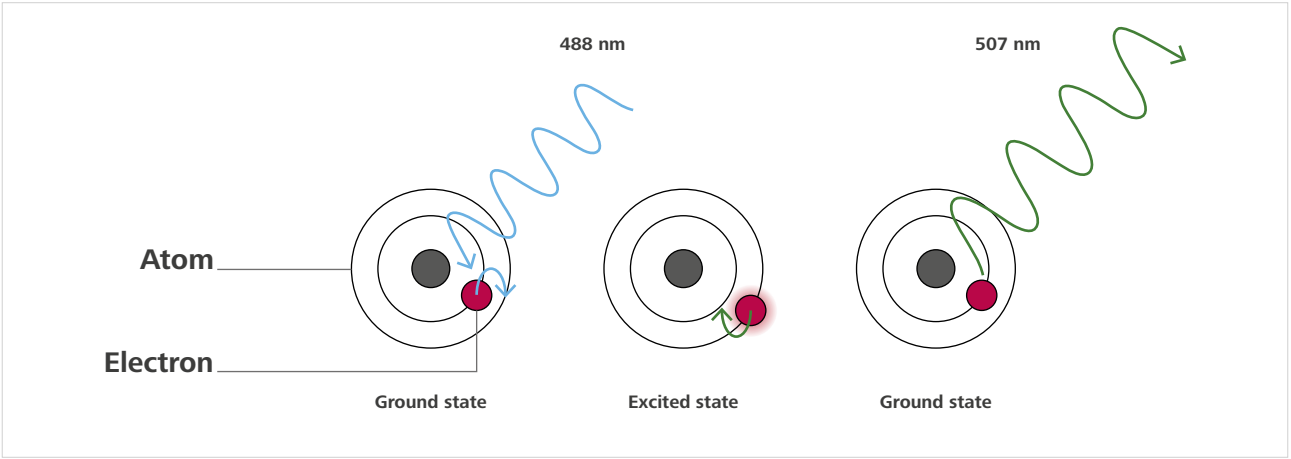

Figure 1 *The basic principle of fluorescence using GFP as an example. In GFP, fluorescence is generated when electrons in the atoms located in the modified Tyr66 residue of the mature fluorophore are excited by light with a wavelength of 488 nm and subsequently returns to the ground state emitting the surplus energy as light with a longer wavelength of 507 nm. Image of GFP structure courtesy of National Institute of General Medical Sciences, National Institutes of Health.*

However, an electron in the excited state is unstable and when it returns to its ground state, a longer wavelength photon is emitted to dissipate the excess energy – this is known as fluorescence.

The electron states and transitions in a fluorophore are more complex than described above but can be simply illustrated with a 'Jablonski diagram', conceived by the Polish physicist Professor Aleksander Jablonski in 1933[2]. In the Jablonski diagram (Figure 2, top), a blue vertical arrow indicates the absorption of a photon by a molecule, which causes an electron to jump from the ground state (S_0) to a higher energy singlet state (S_1 and S_2) within femtoseconds (10^{-15} s). Molecular vibrations, in which the inter-nuclear distances vary over time, further cause the electron to exist in discrete energy levels within each singlet state. These are known as vibrational levels. As a result of these discrete energy states, electrons can absorb photons with a range of wavelengths that can shift the electron into a higher energy level.
The range of wavelengths able to induce fluorescence in this manner is directly related to the excitation spectrum of a fluorophore (Figure 2, bottom).

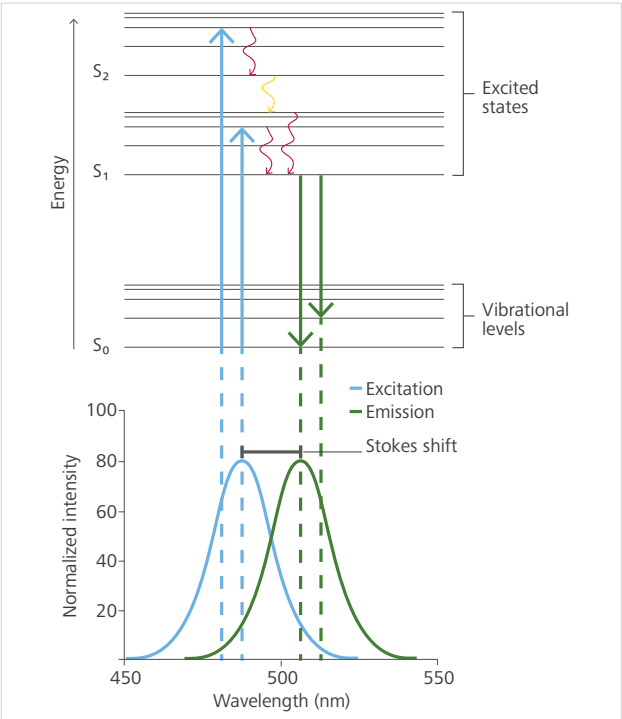

Figure 2 *The fundamental physics of fluorescence. Top. Jablonski diagram illustrating the processes underlying excitation and fluorescence emission at the level of the electron using GFP as an example. Absorption, blue arrow; fluorescence, green arrow; vibrational relaxation, red wavy arrow and internal conversion, orange wavy arrow. Bottom. Excitation and emission spectra of GFP. The graph shows the spectra as simple Gaussian distributions centered on the peak excitation (488 nm) and emission (507 nm) wavelengths of GFP.*

Once excited, there are different ways an electron can dissipate the excess energy and return to its ground state. Two such ways, indicated by wavy arrows in the Jablonski diagram, are internal conversion and vibrational relaxation (Figure 2, top). These are non-radiative energy dissipations that occur within picoseconds (10^{-12} s). In vibrational relaxation, the electron returns to the lowest energy level of its current singlet state by transferring vibrational energy to nearby molecules, whereas internal conversion is the transition between singlet states (e.g., S_2 to S_1). In efficient fluorophores, energy dissipation between S_1 and the ground state results in the emission of a photon and occurs within nanoseconds (10^{-9} s). As an electron can return to any of the vibrational levels of the ground state, photons covering a range of wavelengths can be emitted (known as the 'emission spectrum' of the fluorophore, Figure 2, bottom).

Finally, because some of the excitation energy of the electron is dissipated by non-radiative measures, the emitted photon will be of lower energy (longer wavelength) than the absorbed photon. This is termed the 'Stokes shift' (Figure 2, bottom). This is what Stokes observed when quinine was excited by purple light and emitted blue.

Fluorophores and Immunofluorescence

In 1962, the fluorescent property of the jelly fish Aequorea Victoria (Figure 3) was found to be attributable to green fluorescent protein (GFP)[3].

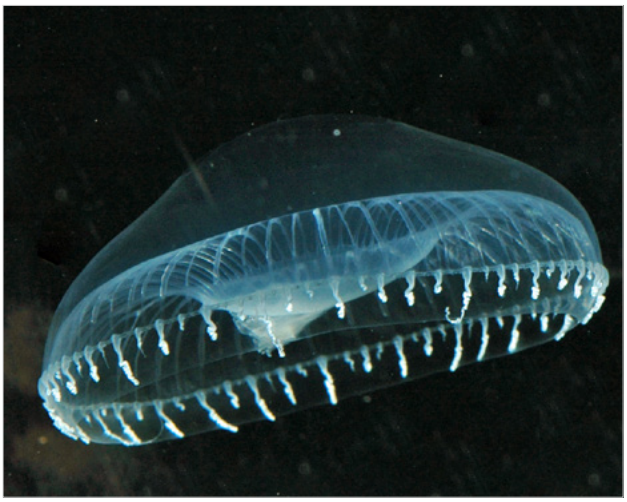

Figure 3 Aequorea victoria, *also called crystal jelly (https://en.wikipedia.org/wiki/Aequorea_victoria)*

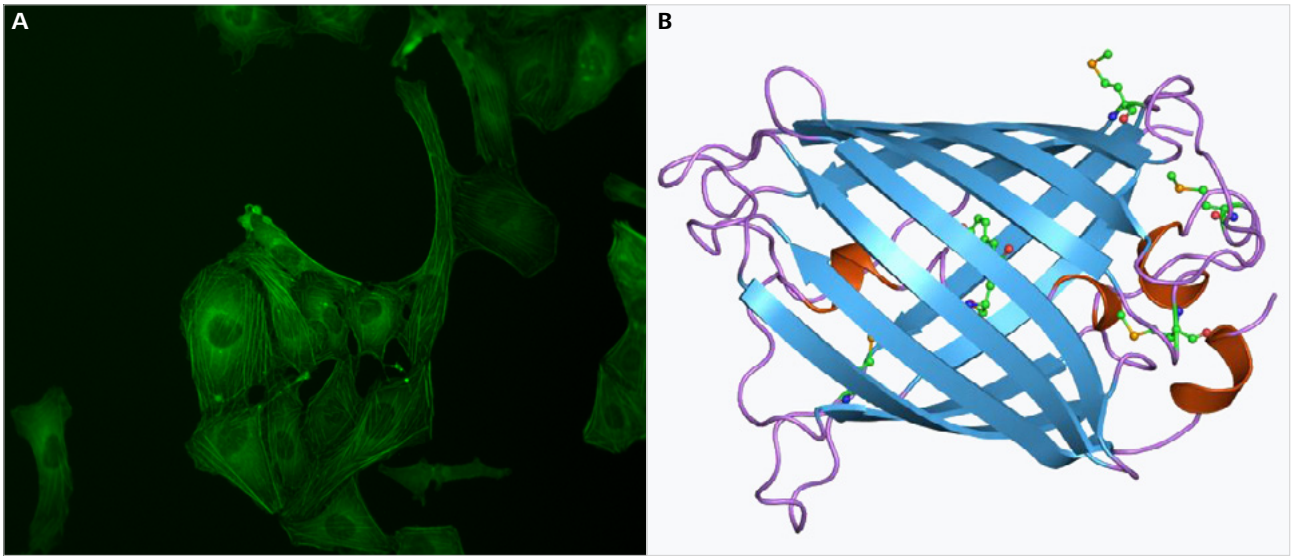

Figure 4 *A) U2OS cells, GFP Actin stained; B) Structure of the Aequorea victoria green fluorescent protein (https://en.wikipedia.org/wiki/Green_fluorescent_protein)*

GFP is a small 27 kDa protein folded into a beta-barrel structure (Figure 4).

The mature fluorophore protrudes into the cavity of this beta-barrel. After translation of the protein, the fluorophore is spontaneously formed by modifying the structure of a tripeptide centered on Tyrosine 66. From the 1990s onwards, the amino acid sequence and structure of wild type GFP has been mutated to create fluorescent proteins of greater stability and brightness as well as a suite of different colors[4]. Fluorescent proteins can be used in a variety of applications as either reporters, or fused to proteins of interest, and can track protein localization as well as examining a range of cellular processes within live cells.

Before the discovery of GFP, and still to this day, small organic molecules have been used as fluorescent labels. These are mostly based on the structure of xanthene and cyanine dyes but a plethora of labels exist. Some are sensitive to their environment, making them attractive sensors of pH, voltage, or ion concentration. Other small organic dyes are used as markers of organelles (e.g., Mito- and LysoTracker dyes) or DNA (e.g., the fluorescent DNA intercalators ethidium bromide, 4,6-diamidino-2-phenylindole (DAPI) and Hoechst). Importantly, a major breakthrough for the use of small organic dyes as fluorescent labels came in 1941, when they were first conjugated to antibodies, thereby inaugurating the field of immunofluorescence[5].

Immunofluorescence is one of the most widely used biological techniques for fluorescence microscopy. In order to visualize an antigen of interest, cells or tissue are incubated with an antibody conjugated to a fluorophore. The fluorophore can either be conjugated to the primary antibody, which recognizes the antigen, or to a secondary antibody, which recognizes the primary antibody. This is termed direct and indirect immunofluorescence, respectively (Figure 5).

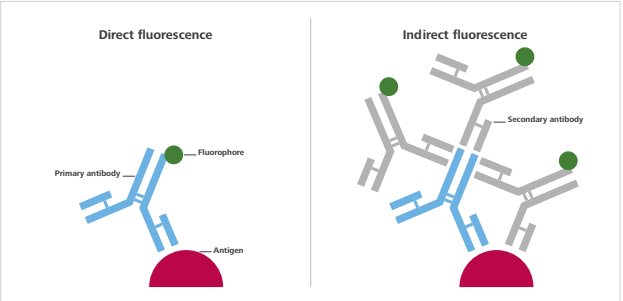

Figure 5 *Direct vs. indirect fluorescence*

Advantages of direct immunofluorescence include a shorter protocol and less chance of cross-reactivity, but the greatest disadvantage is the low signal intensity. With indirect immunofluorescence, multiple secondary antibodies may bind the same primary antibody to amplify the fluorescent signal. However, this two-step labeling protocol also increases complexity and may lead to cross-reactivity within the sample.

The Fluorescence Microscope

The main requirement of a fluorescence microscope is to illuminate a specimen with light of an excitatory wavelength whilst simultaneously collecting and separating the comparatively weaker light emitted by the sample. In the example of Stokes' observation, these tasks are performed by the purple-stained glass window and the glass of white wine, respectively. In the fluorescence microscope, the filter cube provides this separation of light.

Figure 6 illustrates the basic setup of an inverted widefield fluorescence microscope imaging a sample containing GFP.

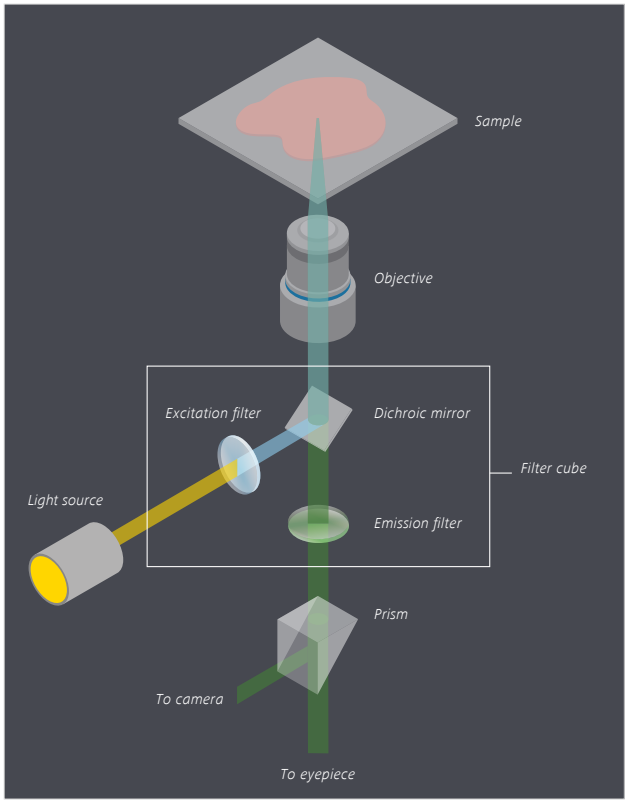

Figure 6 *Basic setup of an inverted widefield fluorescence microscope imaging a sample containing GFP.*

The first important component is the light source. To produce the optimal wavelength of excitatory light for a particular fluorophore, an excitation filter is inserted in the light path between the light source and the sample. This is the first of three components in the filter cube, which consist of an excitation filter, dichroic mirror, and an emission or barrier filter. The excitation filter is usually a bandpass filter, meaning that it transmits light of a narrow range of wavelengths while blocking others.

Once filtered, the excitatory light is reflected towards the sample by a dichroic mirror. This is another filter that sits at a 45° angle relative to both the light from the light source and the sample. While reflecting the excitatory light toward the sample, the spectral characteristics of the dichroic mirror are such that longer wavelength emitted light is transmitted through the filter to the detector. Therefore, the dichroic mirror also needs to match the excitation and emission spectra (i.e., the Stokes shift) of the chosen fluorophore for optimal imaging.

En route to the sample, the excitation light is focused by the objective. In the widefield fluorescence microscope, the objective functions as a condenser and also to magnify the sample. In addition, the objective captures the light emitted from the sample and transmits it back towards the dichroic mirror in the filter cube.

Although the dichroic mirror should prevent excitatory light from reaching the detector, an emission filter is often inserted between these components to block extraneous excitatory light or background fluorescence. When imaging a single fluorophore, the emission filter can be a longpass filter, which transmits light of longer wavelengths whilst blocking shorter wavelengths. However, both the excitation and emission filters are usually bandpass filters with a narrow range of transmitted wavelengths. This enables imaging of more than one fluorophore in a single specimen.

The setup of the filter cube becomes more complex when imaging more than one fluorophore. The fluorescence microscope can accommodate several filter cubes tailored to match the excitation and emission spectra of a number of fluorophores. Switching between filter cubes enables imaging of several fluorophores in a single sample. However, even a slight difference in alignment of the filter cubes can result in a misalignment of the images produced by the different fluorescence channels. To overcome this problem, the excitation filters can be mounted on filter wheels that use the same dichroic mirror for all fluorescence channels. This solves

the alignment issue and enables rapid switching between excitation and emission filters. However, it also means that the dichroic mirror, in this instance termed a polychromatic mirror, needs to specifically reflect the excitation wavelengths for several fluorophores while still transmitting the emission signal for each of these. The key to using multiple fluorophores for a single experiment is to choose those whose emission spectra overlap as little as possible.

Aided by its wide range of applications with relatively few requirements, fluorescence microscopy has long been an essential tool for investigating all aspects of cellular and molecular biology. Today, the fluorescence microscope (Figure 7) has been adapted and improved to make a suite of ever more specialized and sensitive microscopes available to researchers.

This, in connection with the on-going development of optimized and specialized fluorescent probes, firmly places fluorescence microscopy as a constant cornerstone of biological research.

Figure 7 *ZEISS Axioscope 5 fluorescence microscope with LED illumination Colibri 3*

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[5] Coons, A. H., Creech, H. J. & Jones, R. N. Immunological properties of an antibody containing a fluorescent group. Proceedings of the Society for Experimental Biology and Medicine 47, 200–202 (1941).

Suggested reviews for further reading

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- Lichtman, J. W. and J. A. Conchello. Fluorescence microscopy. Nat Methods 2(12), 910–919 (2005). Excellent short review of the basic principles of fluorescence and the fluorescence microscope.
- Sanderson, M. J., et al. Fluorescence microscopy. Cold Spring Harb Protoc 2014(10): pdb top071795 (2014). Review detailing the principles and characteristics of how different fluorescence microscopes form images.
- Shaner, N. C., et al. A guide to choosing fluorescent proteins. Nat Methods 2(12): 905–909 (2005). Short review with helpful tables for choosing fluorescent proteins and filter sets to match specific applications.
- Waggoner, A. Fluorescent labels for proteomics and genomics. Curr Opin Chem Biol 10(1): 62–66 (2006). Short review on the history and characteristics of small organic molecules used as fluorescent labels.
- Zhang, J., et al. Creating new fluorescent probes for cell biology. Nat Rev Mol Cell Biol 3(12): 906–918 (2002). Description of recent advances in the design and applications of fluorescent proteins and small organic dyes.

Suggested links for further learning

- Matching Filter Sets with Microscope Light Sources Interactive setup displaying wavelengths and intensity of light emitted by different microscope light sources with the possibility to choose excitation filter sets matching the requirements of ones fluorophore.
- Filter Wheel Wavelength Selection Interactive illustration of the principle of filter wheels.
- Introduction to Fluorescence Microscopy Excellent description of the principle of fluorescence, components and properties of the fluorescence microscope.
- Introduction to Fluorescent Proteins Comprehensive description of the history, structure, adaptations and characteristics of fluorescent proteins.

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