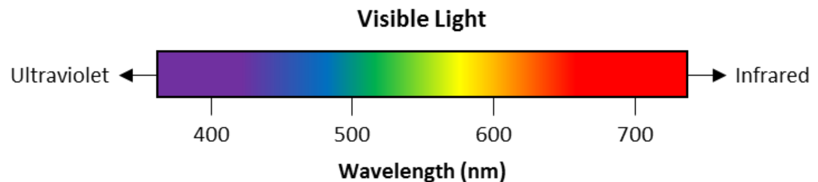
Fluorescence Microscopy

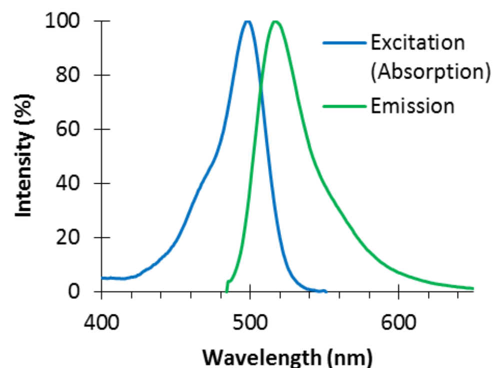
Fluorescence microscopy is a very powerful way to visualize difficult-to-see objects, in particular because fluorescent probes can be designed to recognize objects with high specificity. The design of fluorescent probes is beyond the scope of this short reading. Suffice it to say that, in fluorescent microscopy, specimens are labeled (stained) with fluorescent dyes (**fluorophores**) that emit light that can be seen using a microscope.



Physical Basis of Fluorescence

First, it is important to remember the correspondence between the wavelength of light and color (see diagram, above) and that wavelength and energy are inversely related. For example, light with wavelengths < 350 nm is ultraviolet and is higher energy (think: sunburn, tanning beds) than violet light (wavelength ~400 nm).

Fluorescence is a property of some atoms and molecules to absorb light and, after a brief interval, to re-emit light. Each type of fluorescent molecule can absorb light only of specific wavelengths, and also only emits light of specific wavelengths. These are represented graphically as **excitation** (or absorption) and **emission spectra**, where the peaks indicate the wavelengths that are best absorbed and most frequently emitted, respectively. The fluorophore with the spectra shown in the graph on the right is excited best by ~490 nm light, but can also be excited (although not as well) by other wavelengths from 400-530 nm. When excited, the fluorophore will most often emit ~510 nm light, but might also emit light with wavelengths from 490-640 nm, although not as often. The wavelength of the emitted light is always greater than the wavelength of the excitation light because there is energy loss in this process and wavelength and energy are inversely related.



Different fluorescent molecules have different characteristic excitation and emission spectra and are colloquially referred to by the color of their peak emission light (not their excitation light). The spectra show above are for a “green” dye because it emits green-ish light. Fluorophores are often grouped together by their emission wavelengths. For example, Alexa 488, Green Fluorescent Protein, and Fluorescein are all “green” dyes: although their spectra are different, they all emit green light.

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Fluorescence microscopes contain glass **filters** that only transmit specific wavelengths of light. These filters are used to select the wavelengths necessary to excite a fluorophore and capture its emission. The light path of a fluorescence microscope is different than that of a transmitted light microscope:

1. White light (containing a broad range of wavelengths) enters the microscope and encounters the **excitation filter**. Only wavelengths necessary to excite the fluorophore pass through the filter; all others are blocked. For example, the excitation filter for a green dye might pass 450-490 nm light (refer to spectra, above).

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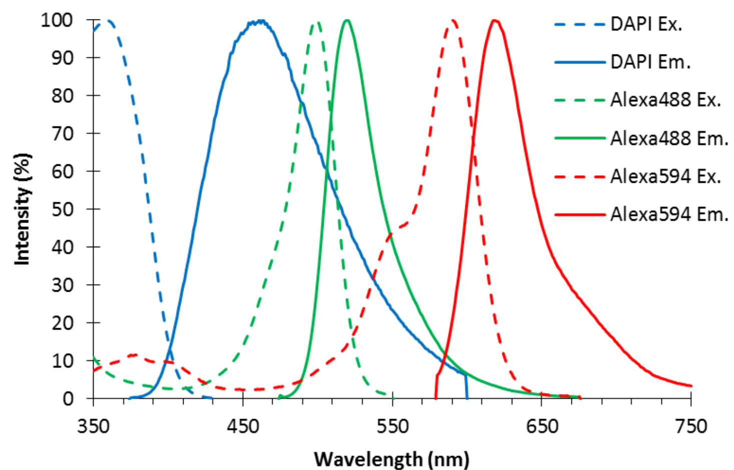
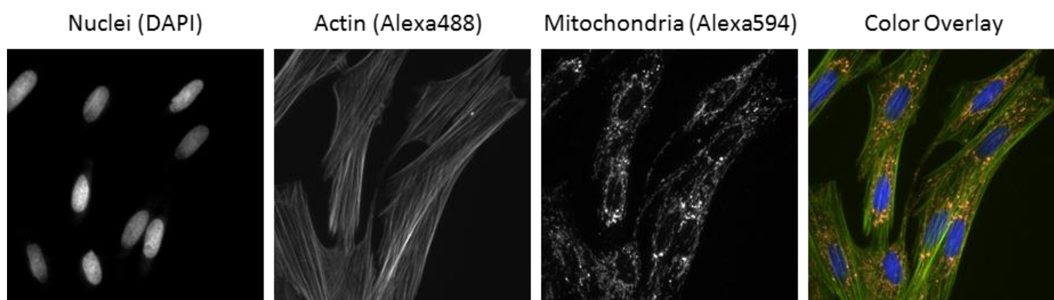
2. The excitation light then exits the objective and illuminates the specimen.
3. The fluorophores in the specimen are excited and emit light at a longer wavelength.
4. The emitted light is collected by the objective and goes to the **emission filter**. Only wavelengths emitted by the fluorophore are allowed to pass; all others are blocked. For example, the emission filter for a green dye might pass 500-550 nm light. The emission filter might seem unnecessary, but consider that the objective is not only "seeing" the specimen's fluorescence, but also the excitation light. The emission filter blocks the excitation light and ensures that the specimen is completely dark except for the areas labeled by the fluorophore.
5. The light then travels to the oculars and/or camera.

Excitation and emission filters are paired in "sets" (or "cubes"), each designed to image one family of fluorophores (one set for green, one for red, etc.). It is therefore possible to label different features of a specimen simultaneously using different "colors" of fluorophores. For example, you could label nuclei with DAPI (a blue dye), actin with Alexa 488 (a green dye), and mitochondria with Alexa 594 (a red dye). You can visualize each of these fluorophores independently by changing filter sets.



Since you are only ever looking at one "color" of fluorophore at a time, there is no need to use a color camera. Most fluorescence microscopes are equipped with monochrome ("black and white") cameras because they are more sensitive than color cameras. In the earlier example, you would acquire a monochrome image using each of the three filter sets and these three images would then be colored and combined using a computer program.



One major problem with fluorescence microscopy is **photobleaching**. Fluorescent molecules can only be excited a finite number of times before they become damaged and no longer fluoresce. The phenomenon of fluorophores "dying" or "going dark" is called photobleaching. The practical implication of this is that the longer you shine excitation light on your specimen, the more fluorophores you will "kill" and the dimmer your specimen will become until you can no longer see any fluorescence emission. To minimize photobleaching, only illuminate your sample when you are actively viewing or imaging your specimen.

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