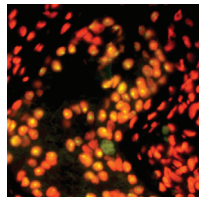


Fluorochromes

W. Roy Overton, PhD

Attaching fluorescent compounds to antibodies, cells, microorganisms and microscopic beads enables scientists and clinicians to gain tremendous amounts of information about the biology and pathology of cells from humans, animals, plants and microbes using fluorescence microscopy or flow cytometry. Following is a discussion of these fluorescent compounds, which are known as fluorochromes.

Physics of Light

Among the properties of many compounds is the ability to absorb one color of light and then emit a different color of light. This causes the compound to appear to be glowing, or fluorescing, and the compound is referred to as a fluorochrome. To better understand how a fluorochrome works, it is necessary to examine this process at the subatomic level. As can be seen in Figure 1, electrons normally spin around the nucleus of an atom at a distance that is referred to as the electron's ground state. If the atom is hit by photons of light that can excite the electron, then the electron will move up to a higher energy state that is farther from the nucleus. The electron is unable to maintain that distance from the nucleus due to the electron's magnetic attraction to the protons in the nucleus and so it quickly drops to a slightly closer distance, called the lowest singlet excited state. Moving to the lowest singlet excited state causes the electron to release a little of the energy that it got from the light, but it releases the energy as heat. The electron then returns to its original ground state, releasing the rest of the energy that it absorbed from the light. This energy is released as light, but since some energy was released as heat, there is less energy in that light. Thus, the emitted light appears to be a different color than the light that excited the atom.

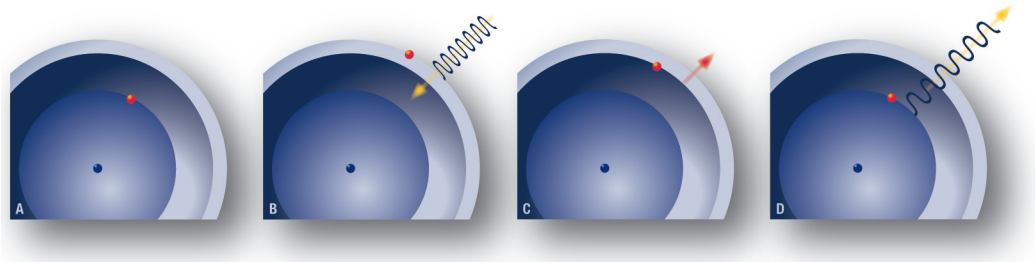


Figure 1. A) Electrons in an atom (red) are normally found at their ground state. B) When an electron is excited by high-energy light, such as ultraviolet light, the electron moves from the ground state to a higher energy state. C) The electron is unable to sustain the higher energy state and quickly drops to the lowest singlet excited state. The energy lost is given off as heat. The amount of energy lost determines the Stokes shift. D) The electron then falls back to its ground state, and energy is emitted as light with a longer wavelength and less energy than the light that excited the electron.

Light travels in waves that determine the color of the light. If the light has a lot of energy, its waves are shorter than a wave of light with less energy (Figure 2). The wavelength of the light determines the color of the light. Very high-energy light, such as ultraviolet (UV) light, has very short wavelengths. The wavelength of UV light is less than 400 nm. Because the wavelength is so short, the human eye is unable to see UV light, but electronic photodetectors are able to detect and measure this invisible light. Visible light has wavelengths from 400 nm to 700 nm, which produce the colors violet, blue, green, yellow, orange and red. Above 700 nm is the infrared range, which is also invisible to the human eye but can be felt as heat or detected by electronic photodetectors.

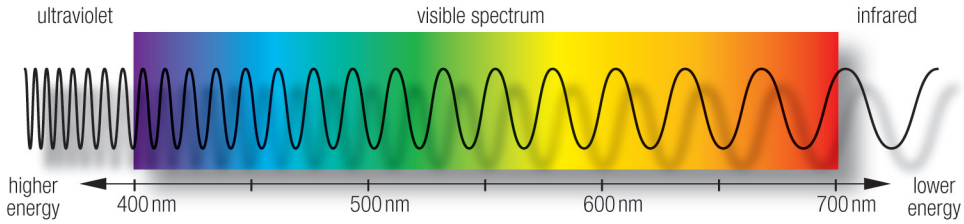
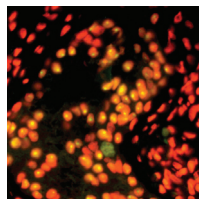


Figure 2. High-energy light has short wavelengths, and low-energy light has long wavelengths.

Excitation and Emission

In flow cytometry, laser light is usually used to excite the fluorochromes. These lasers produce light in the UV and/or visible range. Fluorochromes are selected based on their abilities to fluoresce with the wavelengths of light produced by the lasers. Therefore, if a flow cytometer has only one laser that produces only 488 nm light, then only fluorochromes that are excited by 488 nm light can be used. The chemical properties of the fluorochrome determine whether its electrons can be excited to the higher energy state by a specific wavelength of laser light. If the electrons can be excited to the higher energy state, the chemical properties of the fluorochrome will also determine the amount of energy lost as heat when the electrons drop back down to the lowest singlet excited state and the wavelength of light produced when the electrons return to their ground state.

The difference in the wavelength of the light that excites the electrons and the light that is emitted is called the Stokes shift and is determined by the amount of energy lost as heat. Some fluorochromes have a small Stokes shift, and the excitation and emission wavelengths have almost the same wavelengths, but other fluorescent compounds have large Stokes shifts. For example, the fluorochrome, fluorescein, can be excited by blue-green light and its Stokes shift is only about 25 nm, which means that the light emitted is green. This contrasts with another fluorochrome, phycoerythrin, which can also be excited by blue-green light but has a large Stokes shift. Thus, the light emitted is yellow-orange. In flow cytometry, a laser beam of a single wavelength can be used to excite several fluorochromes with different Stokes shifts and, thereby, produce a variety of fluorescent colors. This is the basis of multicolor flow cytometry. If additional



colors are needed, a second or even third laser with different wavelengths can be used to excite additional fluorochromes.

The electrons of a fluorochrome can be excited by a range of wavelengths of light. For example, the fluorochrome, fluorescein, will fluoresce when hit by light with a wavelength between 430 nm and 520 nm (Figure 3A). However, the closer the excitation wavelength is to 495 nm, the more fluorescence will be produced. This optimal wavelength is called the excitation peak. Similarly, the light produced by fluorochromes has a range of wavelengths. The emission of light from fluorescein, as shown in Figure 3B, ranges from 490 nm to 630 nm, and the emission peak is approximately 520 nm.

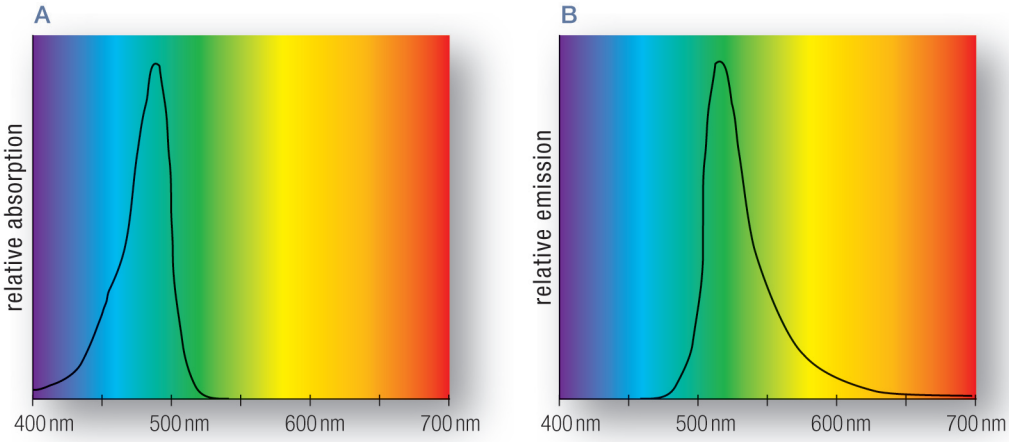


Figure 3. A) Fluorescein excitation. B) Fluorescein emission.

Fluorochrome Selection

Knowing the excitation and emission properties of fluorescent compounds makes it possible to select combinations of fluorochromes that will work together optimally on a specific flow cytometer with specific lasers. However, for a fluorochrome to be useful in a biological application, it must attach to or be contained within a particle of biological significance. Some fluorochromes are useful because they bind to specific chemical structures, such as antibodies (See Chapter 5) or the nucleic acids in DNA or RNA. Fluorochromes that are used most often in flow cytometry are ones that attach in some way to biologically significant molecules and are excitable by the lasers that are commonly found on commercial flow cytometers.

Many fluorochromes can be attached to antibodies, which will then bind to specific chemical structures on or inside of cells. If these chemical structures are unique to a specific type of cell, then the fluorochrome will identify that cell type. This technique of identifying cells using fluorescent antibodies is called immunophenotyping. A list of the fluorochromes used most often in immunophenotyping is shown in Table 1 with their peak excitation and emission wavelengths and the laser wavelengths most often used to excite them on a flow cytometer. Table 2 shows the lasers that can generate the required wavelengths of light to excite the various fluorochromes. Some other common

applications of fluorochromes in flow cytometry include the detection of intracellular calcium, measurement of the relative amount of cellular DNA or RNA, and measurement of transcription levels using a fluorescent protein as a reporter gene. Fluorochromes used for these applications are shown in Table 3.

Table 1. Fluorochromes for Immunophenotyping

Fluorochrome	Excitation Peak (nm)	Emission Peak (nm)	Laser Wavelengths (nm)
AMCA	345	440	334-364, 351-356
Alexa 350	350	445	334-364, 351-356
Marina Blue	365	460	334-364, 351-356, 405, 407
Cascade Blue	395	420	405, 407
Cascade Yellow	400	550	405, 407
Pacific Blue	405	455	405, 407
Alexa 430	435	540	458
Per-CP	490	670	488
FITC	495	520	488
Alexa 488	500	520	488
Alexa 532	532	555	514
TRITC	545	580	568
Alexa 546	560	570	568
Phycoerythrin (PE)	565	575	488, 514, 568
PE-Texas Red	565	615	488, 514
PE-Cy5	565	670	488, 514
PE-Cy5.5	565	695	488, 514
PE-Cy7	565	770	488, 514
Alexa 568	568	605	568
Alexa 594	594	620	568
Texas Red	595	615	568
Alexa 633	630	650	633, 635, 647
Alexa 647	647	670	633, 635, 647
Allophycocyanin (APC)	650	660	633, 635, 647
Cy5	650	665	633, 635, 647
APC-Cy7	650	770	633, 635, 647
Alexa 660	660	690	633, 635, 647
Cy5.5	675	695	633, 635, 647
Alexa 680	680	700	633, 635, 647
Alexa 700	700	720	633, 635, 647

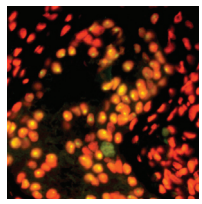


Table 2. Common Laser Wavelengths in Flow Cytometry

Laser	UV	Violet	Blue	Blue-Green	Green	Yellow	Red
Argon	334-364		458	488	514		
Solid-State Violet Laser		405					
Krypton	351-356	407				568	647
Helium-Neon							633
Red Diode							635

Table 3. Fluorochromes for Other Flow Cytometry Applications

Application	Fluorochrome	Excitation Peak (nm)	Emission Peak (nm)	Laser Wavelengths (nm)
Calcium	Indo-1 (calcium)	325	400	334-364, 351-356
Calcium	Indo-1 (no calcium)	345	485	334-364, 351-356
Calcium	Fura Red	485	675	458, 488
Calcium	Fluo-3	500	540	488
DNA Content	Hoechst 33342	355	455	334-364, 351-356
DNA Content	DAPI	360	460	334-364, 351-356, 405, 407
DNA Content	Acridine Orange	495	535	488
DNA Content	Propidium Iodide	305	620	334-364, 351-356
		535	620	488, 514, 568, 633, 647
DNA Content	7-AAD	545	650	488, 514, 568
DNA Content	To-Pro-3	640	655	633, 635, 647
Reporter Gene	eCFP	430	475	458
Reporter Gene	eGFP	495	510	488
Reporter Gene	eYFP	520	535	514
Reporter Gene	Ds-Red	555	585	514, 568
Reporter Gene	HcRed	590	620	568

There are many other chemical and physical properties of fluorochromes that determine where and when these dyes are useful in various biological assays. For example, some of the fluorochromes that bind to DNA, such as Hoechst 33342, can get into living cells, but most DNA-binding fluorochromes cannot get past the cell membrane. The fluorescent dyes that cannot get past a viable cell membrane, such as propidium iodide, are often used to distinguish live from dead or dying cells.

In order to select the best fluorochromes for use in a specific application on a particular flow cytometer, it is necessary to know the laser configuration of the flow cytometer and the physical and chemical characteristics of the fluorochromes that are available for that application.

References

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