Fluorescence Microscopy

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Ruston, Louisiana
Microscopy Workshop

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Terms and concepts to know:

- Signal to Noise
- Excitation (Absorption)
- Emission
- Wavelength
- Photon
- Spectrum
- Barrier filter
- Beam-splitting mirror

Visible spectrum (of light) and energy

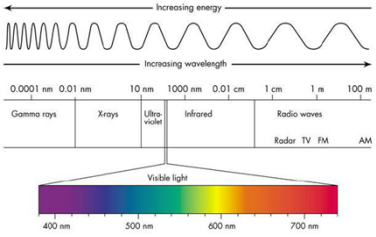
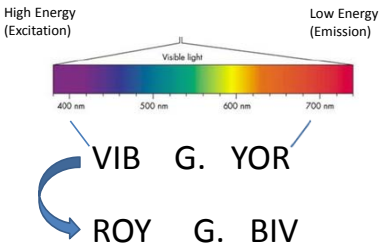


Image modification from: http://www.kollewin.com/blog/electromagnetic-spectrum/

Visible spectrum (of light): the colors



Fluorescence: Excitation and Emission

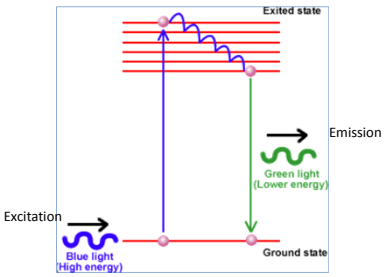


Image modification from: http://www.bristol.ac.uk/synaptic/research/techniques/

Specific molecules can be located in cells by fluorescence microscopy

- Fluorescent molecules *absorb* light at one wavelength and *emit* it at another, longer wavelength.
- If such a compound is illuminated at its absorbing wavelength and then viewed through a filter that allows only light of the emitted wavelength to pass, it is seen to glow against a dark background. Because the background is dark, the signal to noise ratio is increased over most ordinary, white-light stains.
- The same number of molecules of an ordinary stain viewed conventionally would be practically invisible because they would give only the faintest tinge of color to the light transmitted through this stained portion of the cell.

Specific molecules can be located in cells by fluorescence microscopy-2

- The fluorescent dyes used for staining cells are detected by a fluorescence microscope.
- The microscope is similar to a conventional scope, except that the light source is passed through two filters, one to filter the light before it hits the sample, and one to filter the light obtained from the sample.
- The first filter is chosen so that passes only wavelengths that excite the particular fluorescent probe, while the second blocks out this light and passes only wavelengths emitted when the dye fluoresces. (Figure 9-12).

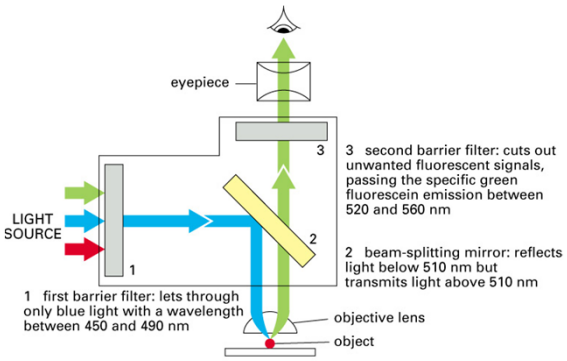


Figure 9–12. Molecular Biology of the Cell, 4th Edition.

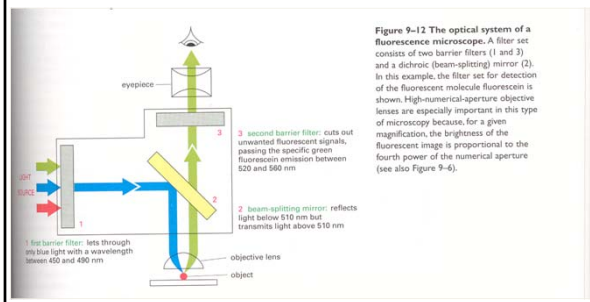


Figure 9–12 The optical system of a fluorescence microscope. A filter set consists of two barrier filters (1 and 3) and a dichroic (beam-splitting) mirror (2). In this example, the filter set for detection of the fluorescent molecule fluorescein is shown. High-numerical-aperture objective lenses are especially important in this type of microscopy because, for a given magnification, the brightness of the fluorescent image is proportional to the fourth power of the numerical aperture (see also Figure 9–6).

Specific molecules can be located in cells by fluorescence microscopy-3

- Fluorescence microscopy is most often used to detect specific proteins of other molecules in cells and tissues.
- By coupling fluorescent dyes to antibodies, highly specific staining reagents can be obtained.
- Two commonly used fluorescent dyes used for cell staining are **fluorescein**, which emits green light when excited with blue light, and **rhodamine**, which emits red light when excited with green-yellow light.
- By coupling one type of antibody to the fluorescein and one to rhodamine, the distribution of different molecules can be compared in the same cell, because of the distinct color differences– the two molecules are visualized separately in the microscope by switching back and forth between the appropriate filter sets which excite and collect light from fluorescein or rhodamine.
- As more fluorescent dyes are synthesized, the excitation and emission and spectrum (figure 9-13) can be utilized to visualize 3 or more dyes in the same sample (Figure 9-14).

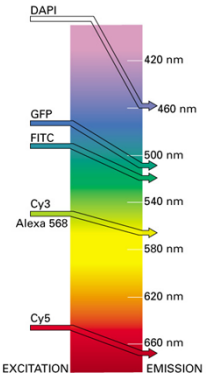


Figure 9–13. Molecular Biology of the Cell, 4th Edition.

The maximum excitation and emission wavelengths of several commonly used fluorescent dyes are shown in relation to the corresponding colors of the spectrum.

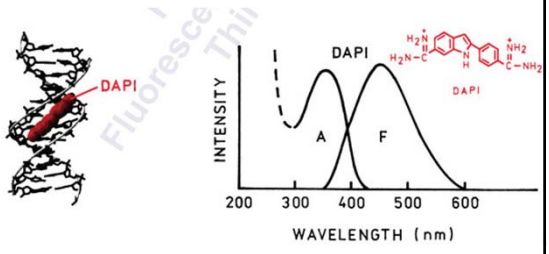
The photon emitted by a dye molecule is necessarily of lower energy (higher wavelength) than the photon absorbed and this accounts for the difference between the excitation and emission peaks.

GFP is a green fluorescent protein, not a dye.

DAPI is widely used as a general fluorescent DNA dye that absorbs UV light and fluoresces bright blue.

The other dyes shown are all commonly used to fluorescently label antibodies and other proteins.

Example Flurophore (Dye): DAPI



Modified Image from: J.R. Lakowicz: "Principles of Fluorescence Spectroscopy", 3rd Edition.

Dapi staining: improved signal to noise

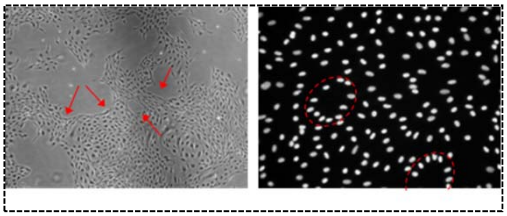
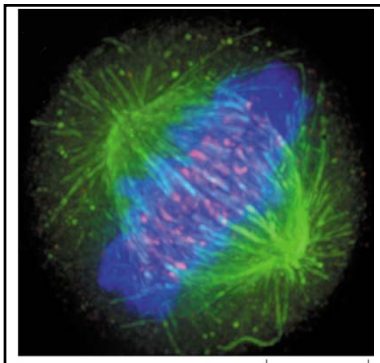


Figure 3. ***Rat brain microvascular endothelial cells (RBMVECs) remodeling into tubular orientations in culture. Endothelial cells shown using phase microscopy (Panel A), and fluorescent staining of nuclei using DAPI (Panel B). Note curved growth patterns, as indicated by arrows in 3A and circled by ovals in 3B, demonstrating potential tube formation in culture.***

*****Note: MONOCHROME CAMERA******



Multiple-fluorescent-probe microscopy. In this composite micrograph of a cell in mitosis, three different fluorescent probes have been used to stain three different cellular components. The spindle microtubules are revealed with a green fluorescent antibody, centromeres with a red fluorescent antibody and the DNA of the condensed chromosomes with the blue fluorescent dye DAPI.

Figure 9–14. Molecular Biology of the Cell, 4th Edition.

Fluorescence Microscopy and Antibodies: Revealing structure of the cell

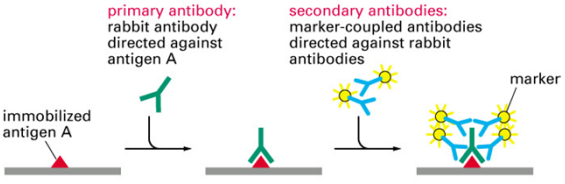
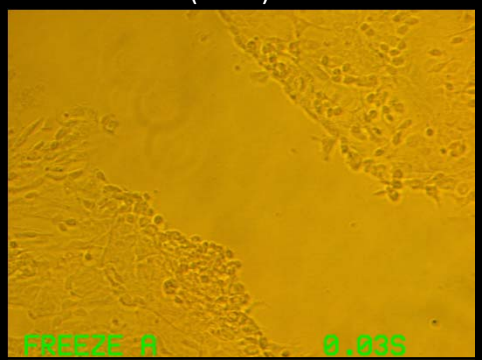


Figure 9–16. Molecular Biology of the Cell, 4th Edition.

Astrocytes: Glial Fibrillary Acidic Protein (GFAP)



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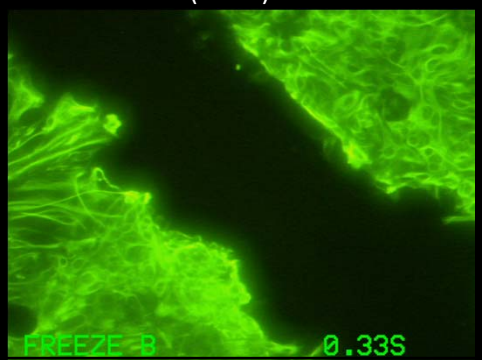
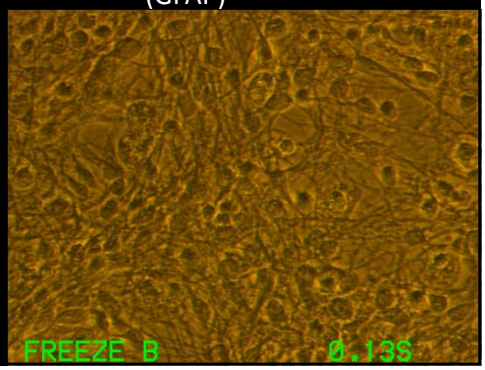


Astrocytes: Glial Fibrillary Acidic Protein (GFAP)

Culture= Neurons and Astrocytes



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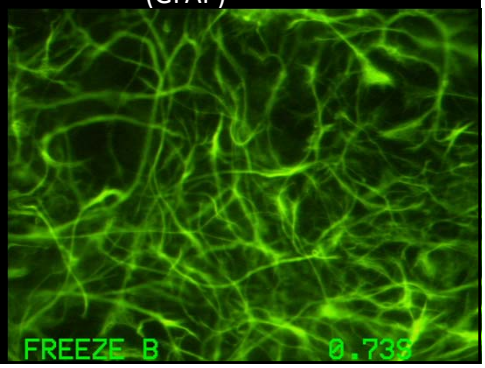
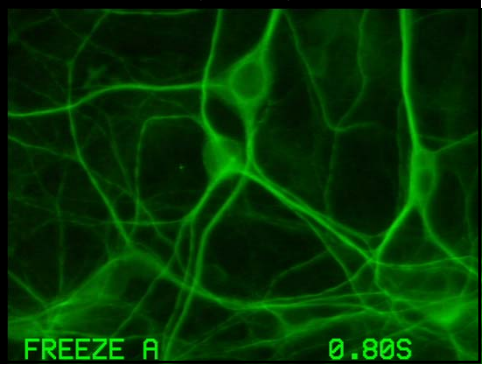


Neuronal Markers: Microtubule Associated Protein-2 (MAP-2)

Culture= Retinal Neurons



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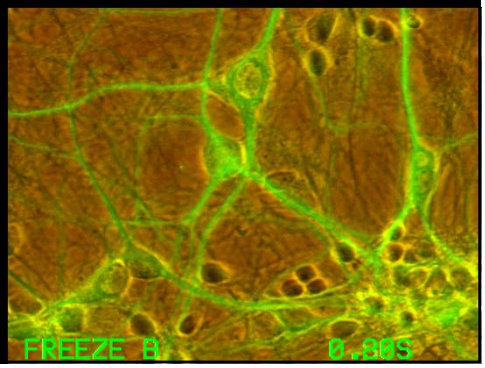


New Fluorescent Microscopy Tools: Green Fluorescent Protein and Quantum Dots

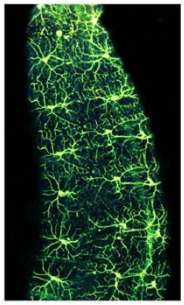
Nobel Prize in Chemistry- 2008:
"for the discovery and development of the green fluorescent protein, GFP"

Osamu Shimomura
1/3 of the prize
USA
Marine Biological Laboratory (MBL) Woods Hole, MA, USA; Boston University Medical School Massachusetts, MA, USA
b. 1928
(in Kyoto, Japan)

Martin Chalfie
1/3 of the prize
USA
Columbia University New York, NY, USA
b. 1947

Roger Y. Tsien
1/3 of the prize
USA
University of California San Diego, CA, USA; Howard Hughes Medical Institute
b. 1952

New Fluorescent Microscopy Tools: GFP-2



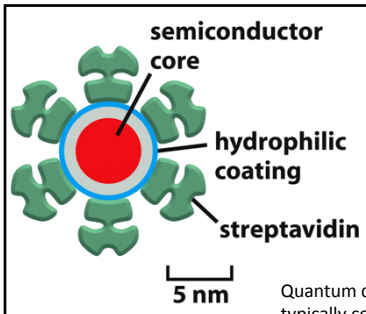
Green fluorescent protein (GFP) as a reporter. For this experiment, carried out in the fruit fly, the GFP was joined (using recombinant DNA techniques) to a fly promoter that is active only in a specialized set of neurons. This image of a live fly embryo was captured by a fluorescence microscope and shows approximately 20 neurons, each with long projections (axons and dendrites) that communicate with other (nonfluorescent) cells. The neurons shown here are located just under the surface of the animal and allow it to sense its immediate environment. (From W.B. Grueber et al., *Curr. Biol.* 13:618-626, 2003. With permission from Elsevier).

****Note: Other colors are available now***

Figure 9-25 *Molecular Biology of the Cell* (© Garland Science 2008)

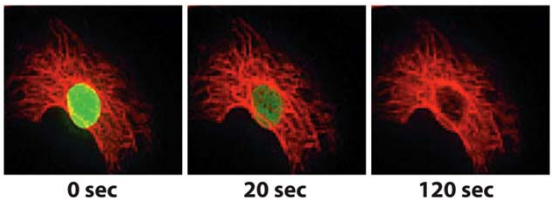
Nanotech imaging-quantum dots

- Organic dyes have the disadvantage of sometimes fading quickly when continuously illuminated.
- More stable inorganic fluorochromes have recently been developed composed of tiny crystals of semiconductor material, called quantum dots.
- These nanoparticles, when coupled to other probes such as antibodies, are ideal for tracking molecules over time.



Quantum dots: quantum dots are typically composed of semiconductor cores such as cadmium selenide, with a coating to make them water-soluble

Figure 9-16a *Molecular Biology of the Cell* (© Garland Science 2008)



Quantum dots can be coupled to protein probes such as antibodies or streptavidin and, when introduced into a cell, will bind to a protein of interest. Different-sized quantum dots ***emit*** light of different colors (larger dots emit at longer wavelength), but they all absorb across a broad spectrum.

Quantum dots can keep shining for weeks, unlike most fluorescent organic dyes. In this cell, a nuclear protein is labelled green, with the organic dye alexa 488, while microtubules are stained red with quantum dots bound to streptavidin. On continuous exposure to blue light the fluorescent dye fades quickly while the quantum dots continue to fluoresce.

Figure 9-16b *Molecular Biology of the Cell* (© Garland Science 2008)

Videos!

http://www.viddler.com/explore/benchfly/videos/273/