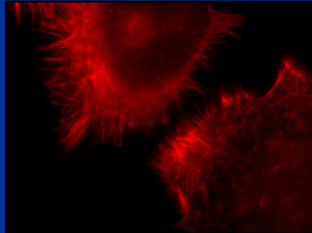


Cell imaging Fluorescence



Fluorescence

- Fluorescence is the molecular absorption of light energy at one wavelength and nearly instantaneous emission at another wavelength
- Emitted light has longer wavelength than that absorbed (Stoke's shift)

Fluorochromes

- Chemical components that fluoresce
- Have two characteristic spectra
 - Excitation (wavelength that is absorbed)
 - Emission (wavelength that is emitted)
- Can be a natural part of the system
- Or a probe attached to a specific part of the cell

Use of fluorochromes in microscopy

- Each fluorochrome requires special set of filters appropriate for the excitation and emission spectra
- Some of the spectra for different fluorochromes overlap (check before co-localization studies)
- Check your available filter sets before choosing the fluorochrome
- Some fluorochromes are visible in several filters – find the optimal one

Why do we use fluorescence in microscopy?

- Added contrast
- A probe for localization and quantification of specific molecules in cells
- Cells can be fixed or alive

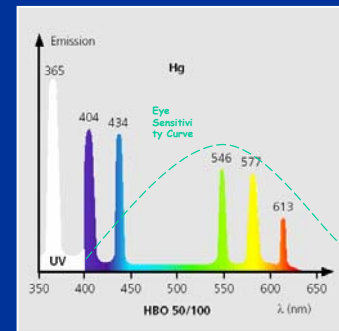
Fluorescence in microscopy

- Most fluorochromes do not damage cells
 - can be used in the microscopy of living specimens
- They can be bound to antibodies and other specific molecules
 - permitting the exact localization
 - observation and measurement of changes such as translocation, secretion
 - use of different fluorochromes allows for localization of several molecules in the same sample (cell)

What is needed for fluorescence microscopy?

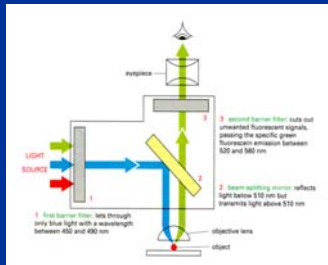
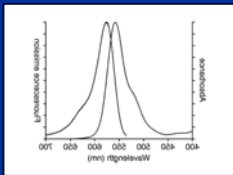
- Good light microscope modified for fluorescence
- High-intensity lamp (usually super-pressure mercury or xenon lamps)
- High numerical aperture of the objectives
 - allows for high contrast of the fluorescent areas against a dark background
 - doubling of the objective aperture allows four times more fluorescent light to be detected
- Fluorescence filter set – each fluorochrome requires a specific set of filters
- <http://www.zeiss.com/4125681F004CA025/Contents-Frame/C7B4C762D6F8A09A85256B4B0075CE56>

Mercury (Hg) light source

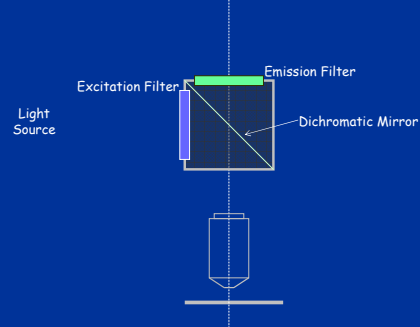


Fluorescence filter sets

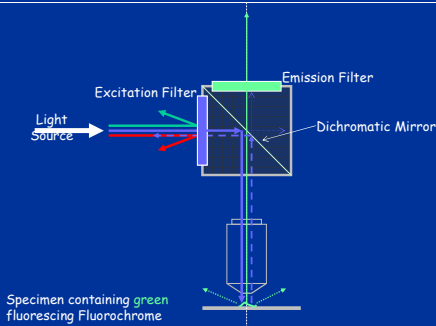
- First barrier filter – excitation (absorbance) filter
- Beam splitting mirror – dichromatic beam splitter
- Second barrier filter – emission filter



Epi - Fluorescence



Epi - Fluorescence



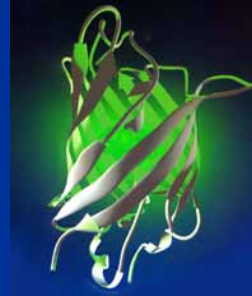
Problems with fluorescence

- Bleaching of the sample
 - Fluorescence is a short time phenomenon
 - Emitted light “bleaches” with longer exposures

Fluorescence microscopy in living cells

- Fluorochrome is a part of the system – autofluorescence
- A gene for fluorescent protein can be fused to the gene of interest and act as a reporter
- Most popular is green fluorescent protein - GFP

Structure of the GFP molecule



- Mutations of single amino acids change the wavelengths of the emitted light

Our class protocol

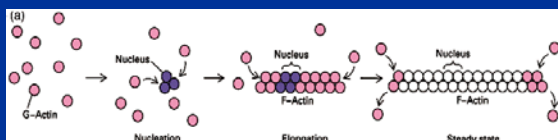
- We used a probe for actin (phalloidin) fused to rhodamine to visualize actin cytoskeleton in 3T3 cells

Cytoskeleton

- A network of fibers in the cytosol that supports cell shape, confers mechanical resilience and participates in cell movement
- 3 types of fibers
 - Actin – microfilaments
 - Intermediate filaments
 - Microtubules

Actin cytoskeleton

- Actin exists in two states
 - Monomeric – globular G-actin
 - Polymeric – filamentous F-actin
- Actin cytoskeleton is dynamic
 - filaments grow and shrink constantly and rapidly



Toxins can disrupt the monomer-polymer equilibrium of actin

- Cytochalasin D
- Latrunculin
- Phalloidin

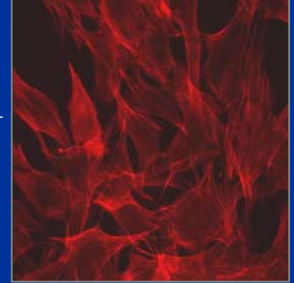
Phalloidin

- Fungal toxin from *Amanita phalloides* (death cap)
- Prevents depolymerization of F actin
- Binds at the interface between subunits in F actin
- Locks adjacent subunits together
- Fluorescently labeled phalloidin is used in microscopy to visualize F actin
- Any type of fluorochrome can be attached to phalloidin



Our experiment

- Phalloidin that locks actin in filamentous (F actin) state was coupled to rhodamine
- What we see is F actin that fluoresces red in rhodamine filter set but not in FITC filter set



Our experiment

- We want to fix the cells so they retain their shape throughout the procedure
- Rhodamine phalloidin has to enter the cell to bind to actin therefore we have to permeabilize the cells (punch holes in the membranes)
- Rhodamine is a fluorochrome and has to be protected from light to avoid photobleaching (we have to work in the dark) from the step when we add rhodamine phalloidin

Homework

- Find a fluorochrome, write its name, chemical composition and excitation and emission spectra