An Introductory Guide to Light Microscopy

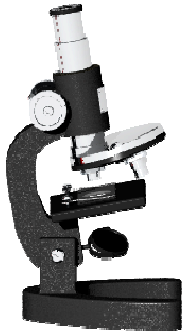
16 Apr to 14 May 2007

Fluorescence and Fluorescence Microscopy

April 23

$E = h\nu$

Michael Hooker
Microscopy Facility



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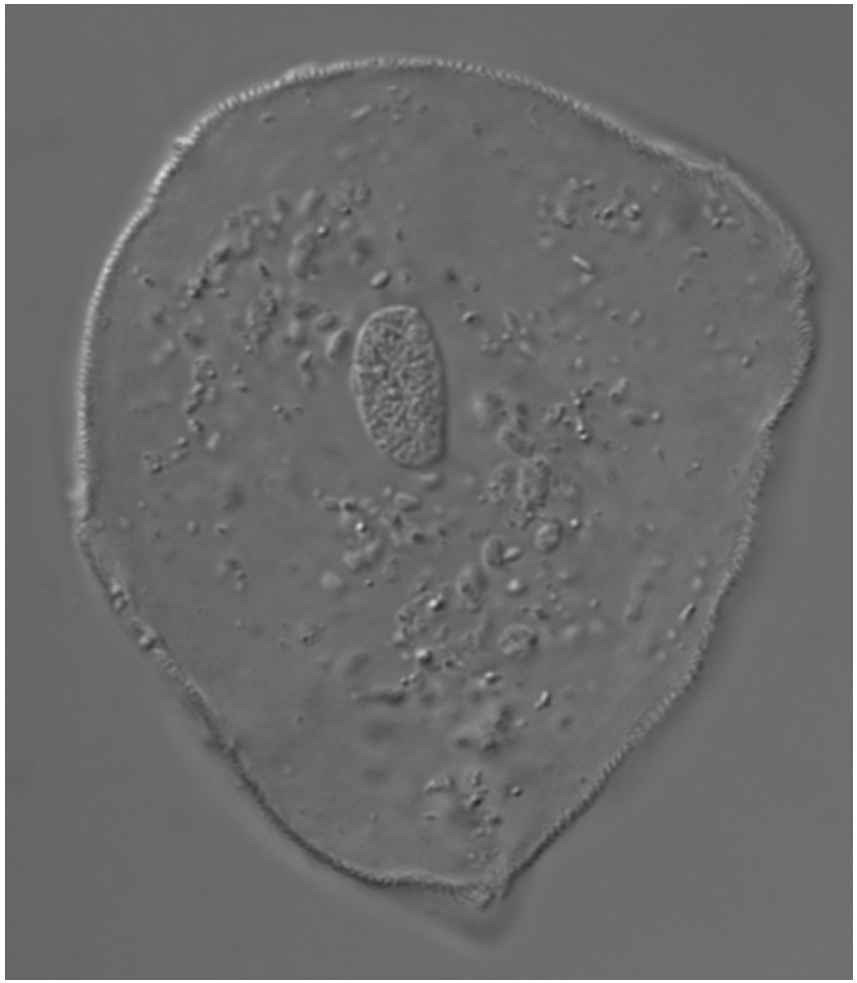
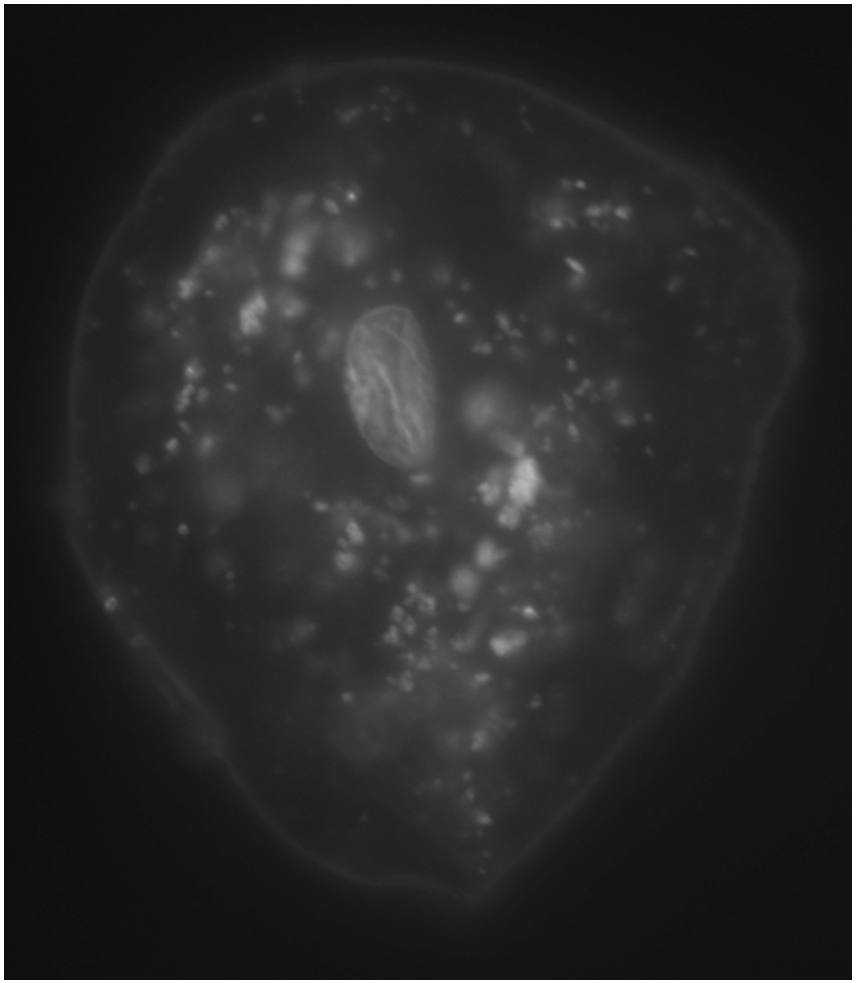
Live Buccal Epithelial cells



Transmitted light (DIC)



Fluorescence
FM 1-43 membrane dye

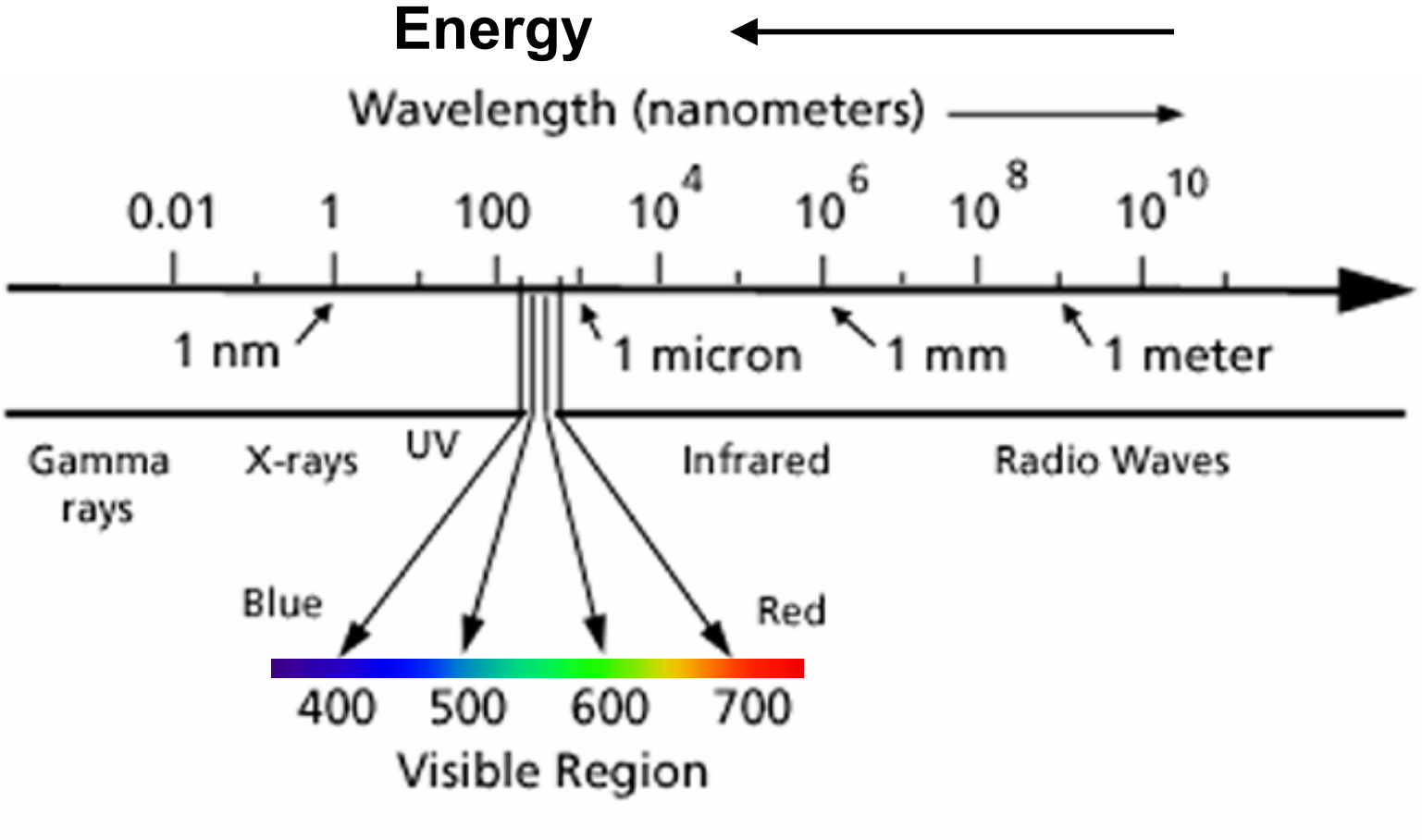
What is fluorescence?

The molecular absorption of a photon that triggers the emission of another photon with a longer wavelength

Why use Fluorescence?

1. **Improved contrast over transmitted light**
2. **Ability to detect low abundance structures**
3. **Ability to detect sub-resolution molecules**
4. **Specificity for detection of more than one biomarker per sample**

Electromagnetic Spectrum



Adapted from http://acept.asu.edu/PiN/rdg/color/color.shtml

Definition of fluorescence again

The molecular absorption of a photon that triggers the emission of another photon with a longer wavelength

Beer's Law

$$A = \varepsilon \ell C$$

A = absorption
ε = molar absorbtivity (L/mol/cm)
= α/2.303
(α = 4πk/**λ**, k = extinction coefficient, **λ = wavelength**)
ℓ = path length of the sample
C = concentration of the compound (mol/L)

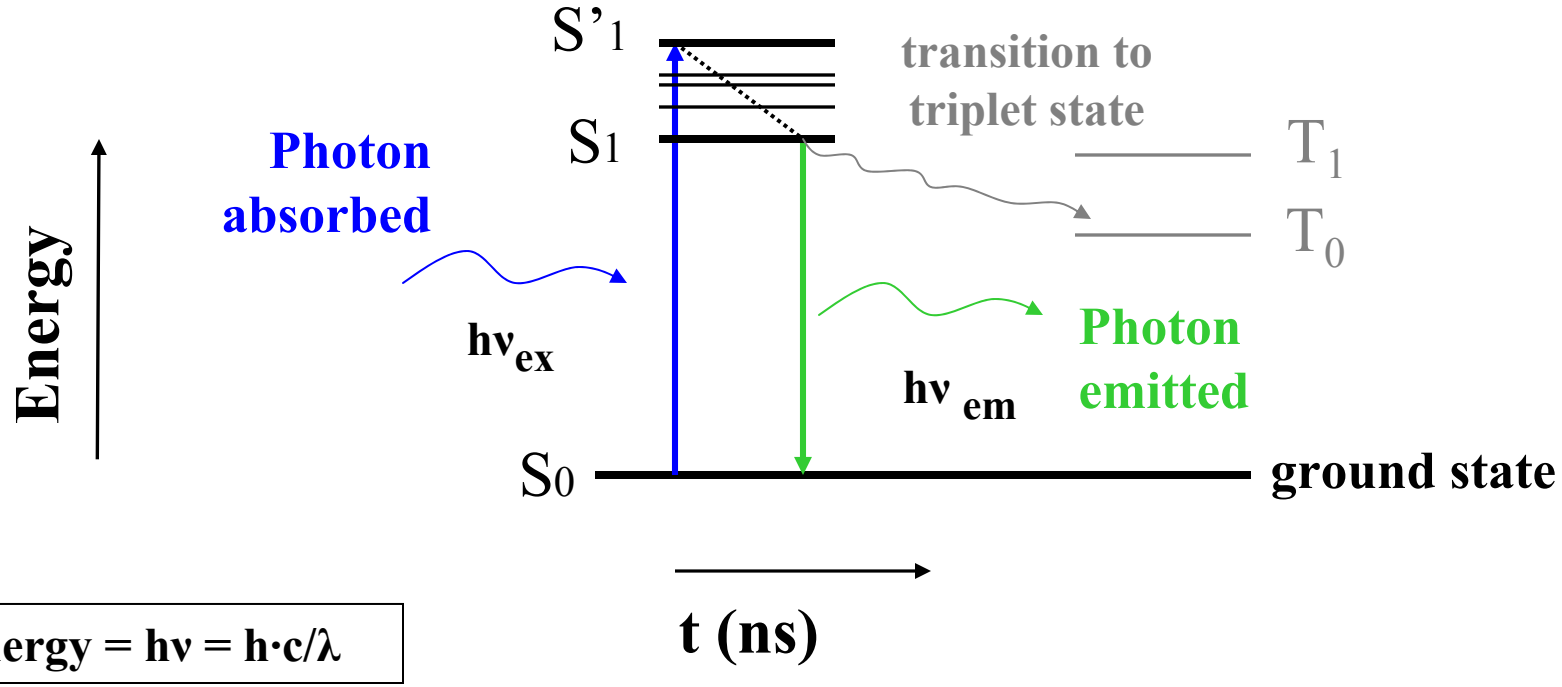
The absorbance of a fluorescent molecule in suspension is dependent on the:

- …concentration of the fluorophore (how much there is)
- …path length of the sample (how much of the suspension the light has to go through)
- …wavelength of light

Molecular Fluorescence

Jablonski diagram

(Simplified)



Energy = hν = h·c/λ

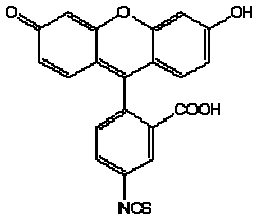
- **Absorbed photon's energy must be tuned to fluorophore electron structure**
- **Emission light has a longer wavelength than the excitation light**

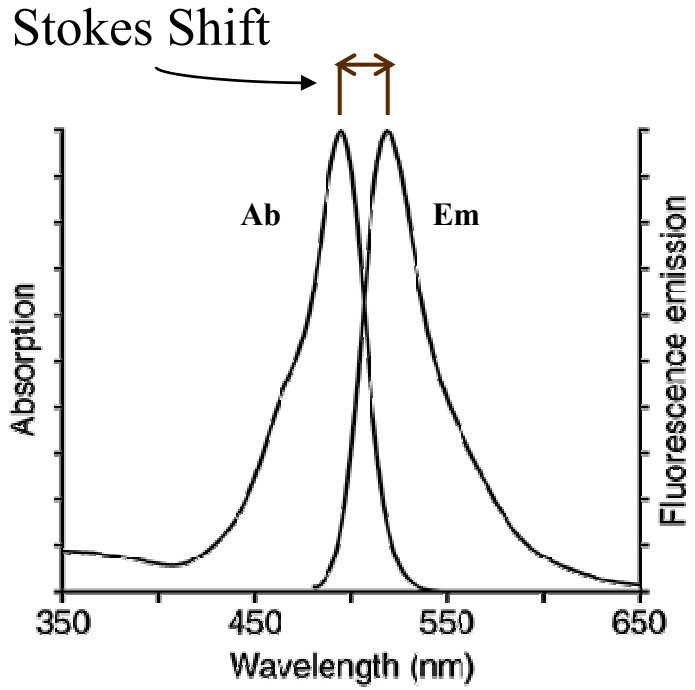
Fluorescence Spectra

- Fluorescent compounds each have a spectrum of excitation wavelengths and a spectrum of emission wavelengths (or λs).

- The **Stokes shift** is the difference (in wavelength) between the band maxima of the absorption and the emission spectra for a fluorophore

FITC





Quantum Yield

- A measure of the fluorescence efficiency of a molecule
- Is the proportion of absorbed photons that are released as emitted photons

$$\Phi = \frac{\text{\# photons emitted}}{\text{\# photons absorbed}}$$

- FITC (fluorescein) has a quantum yield of 0.6
- Using a fluorophore with a low quantum yield means a larger amount of illumination light is necessary to get a minimum number of emitted photons for collection by the detector.
- An important consideration for low concentration proteins or for live cell imaging, eg fluorescent proteins (eCFP =0.4, eYFP = 0.61).
- Most standard fluorophores used with antibodies have high quantum yield values (>0.6).

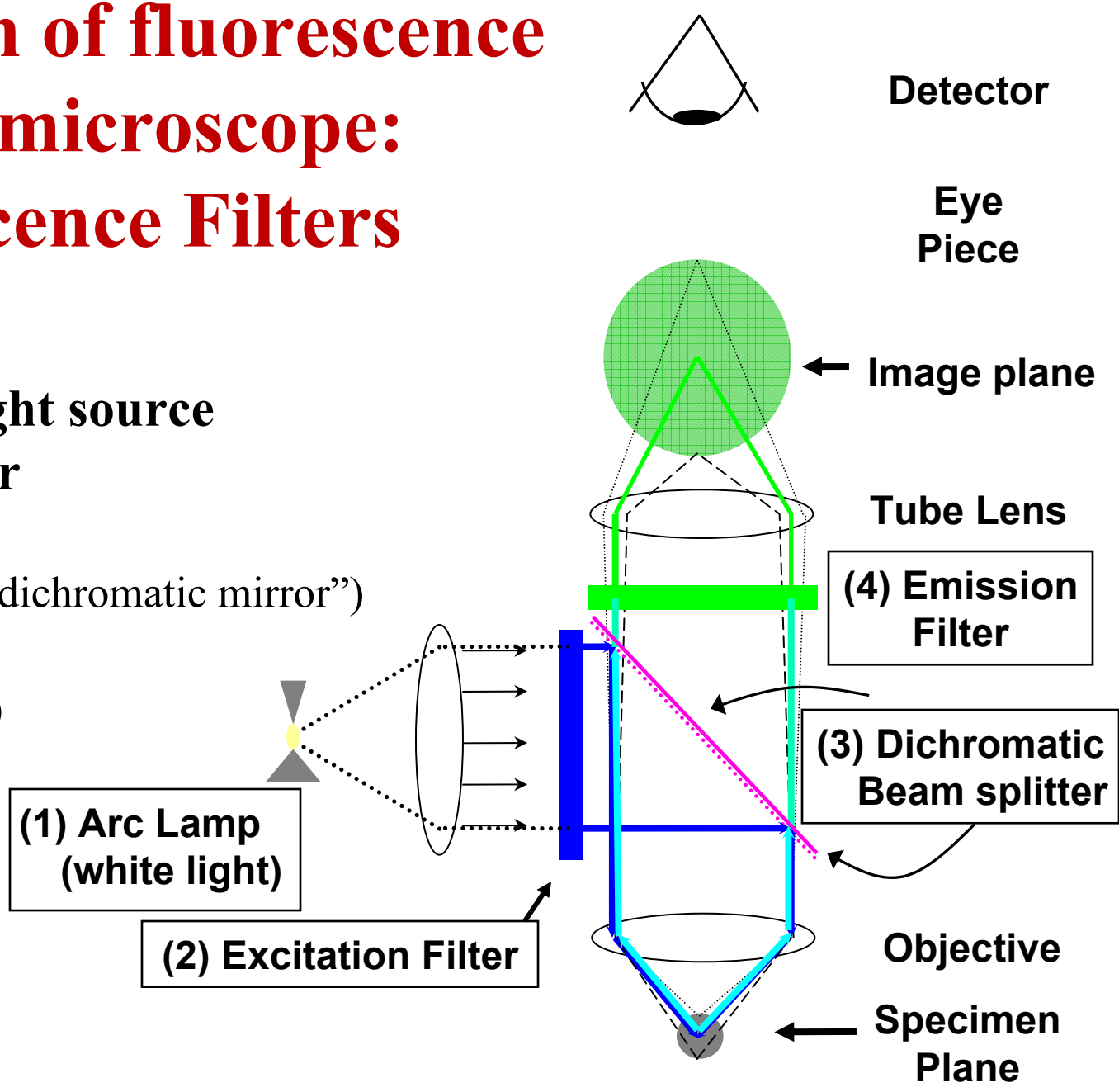
Fluorescence Spectra

- Typically, a fluorophore is referred to with it's peak excitation and emission wavelengths.
- Spectra can be influenced by the fluorophore's environment (e.g., water vs. EtOH, pH)
- Narrow spectral characteristics allow for easier separation of multiple fluorophores in the same sample.

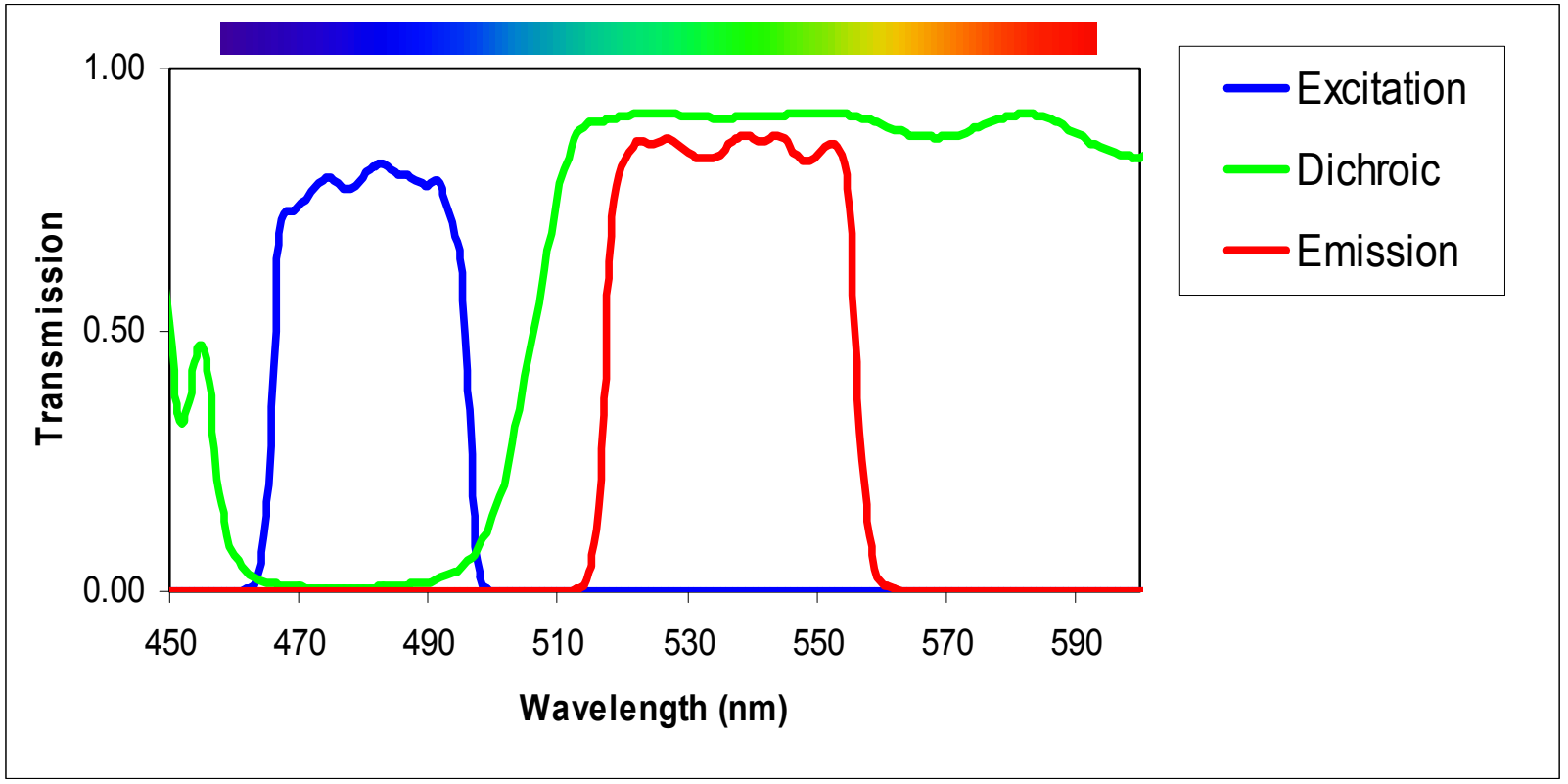
Visualization of fluorescence through the microscope: Epi-Fluorescence Filters

Components

1. **Strong white light source**
2. **Excitation Filter**
3. **Beam Splitter**
   (aka "dichoric" or "dichromatic mirror")
4. **Emission Filter**
   (aka "barrier filter")

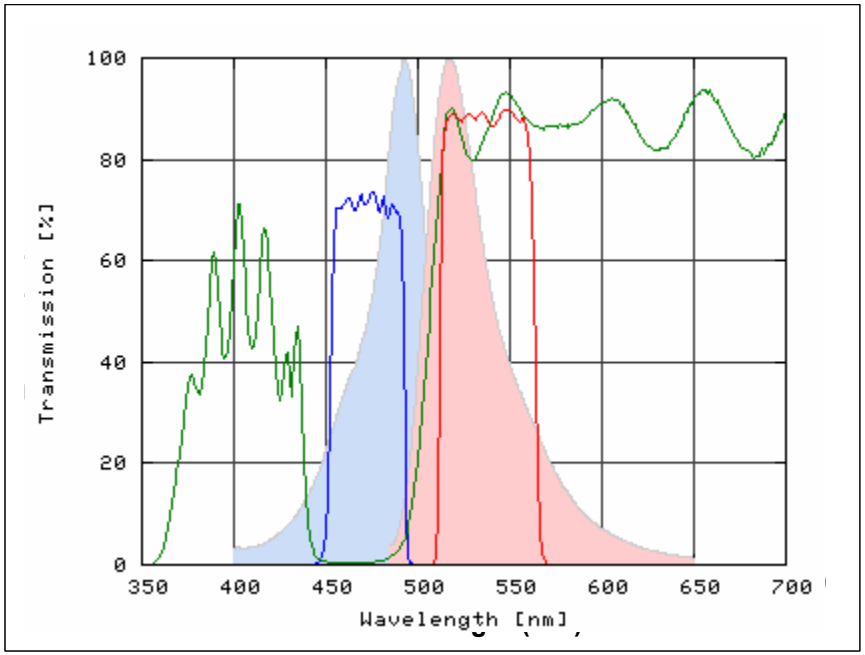


Fluorescent Filter Transmission Characteristics



Filter demonstration

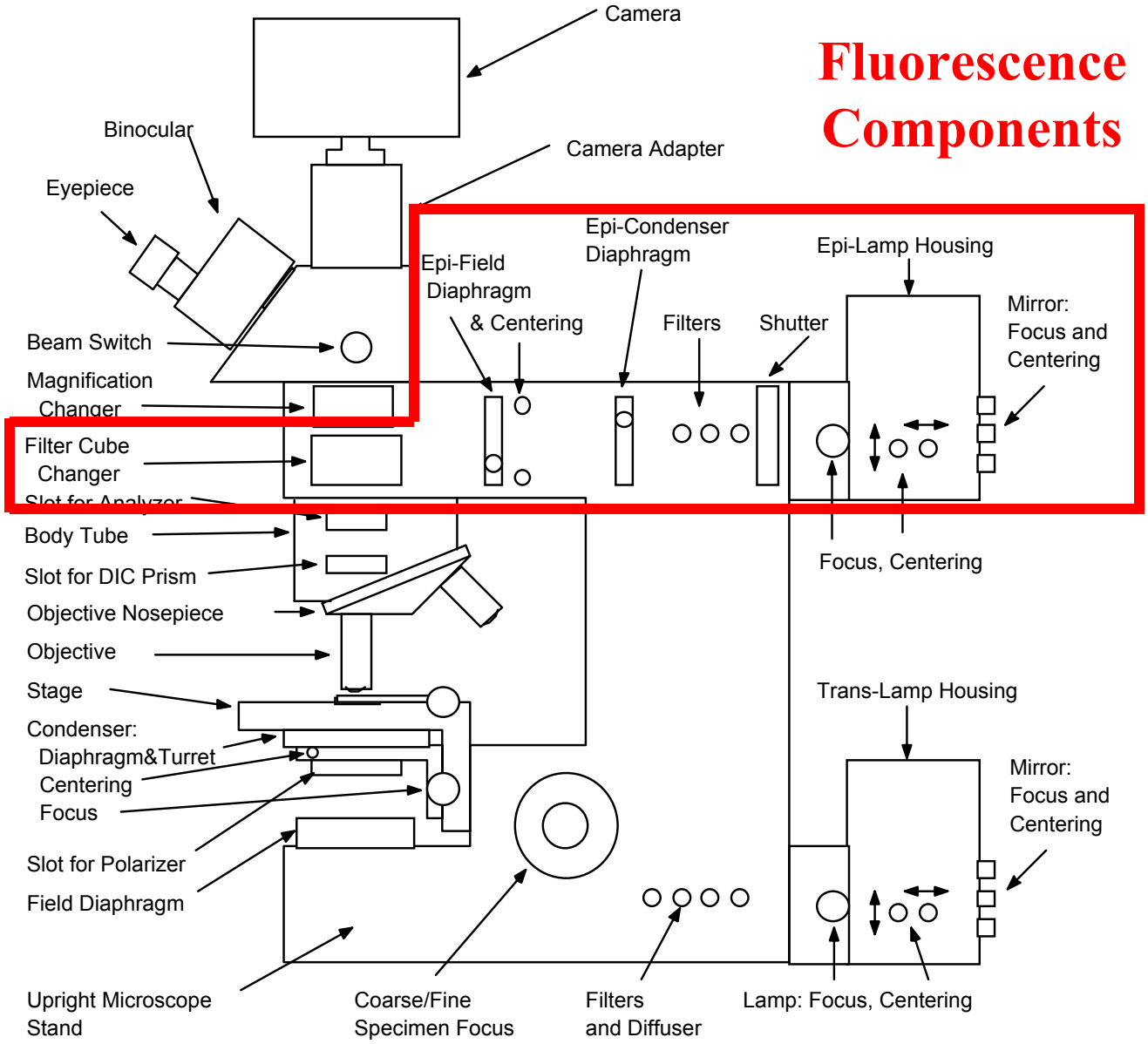
So, when you have a filter set that matches your fluorophore spectra, the graphs should look something like this….



Excitation (filled blue) and Emission (filled red) spectra for FITC overlaid with FITC filter set (blue line=ex, red line=em, green line = dichroic)

Taken from: Omega optical Curvo-O-Matic, https://www.omegafilters.com/front/curvomatic/spectra.php

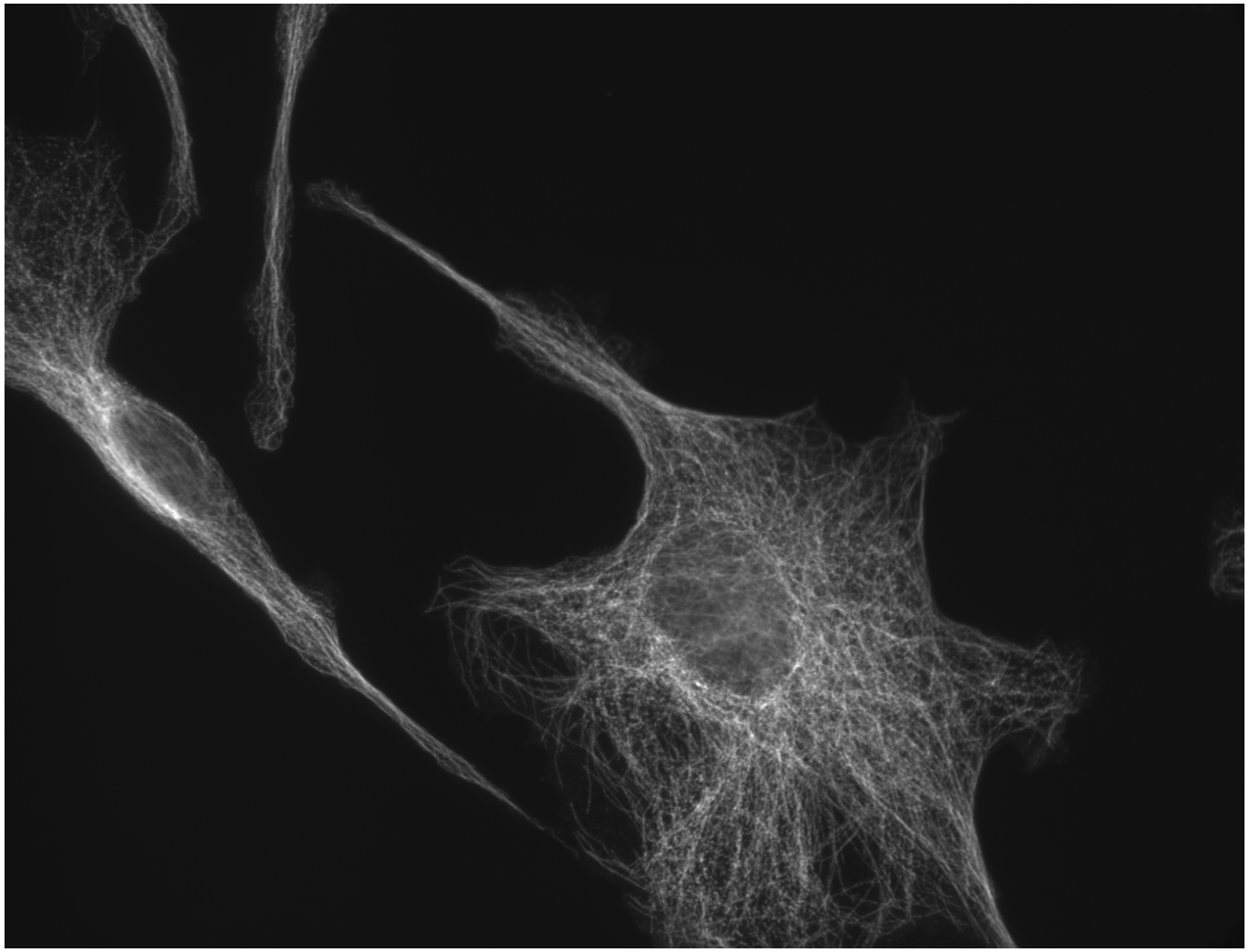
Parts of a typical microscope



Fluorescence Components

From E.D. Salmon

Single channel fluorescence image



Fluorescence filter lingo

Filter cube	The combination of excitation filter, beam splitter and emission filter contained in a cube. When ordering, it refers to the cube-shaped object mounted in the microscope that holds all or some of the filter set components.
Beam Splitter (BS)	The category term for dichromatic (splits light two ways) or polychromatic (splits light > 2 ways) mirrors.
Long pass (LP)	Transmits wavelengths longer than that specified wavelength. Ex: 565LP transmits most visible wavelengths longer than 565nm
Short pass (SP)	Transmits wavelengths shorter than specified wavelength. Ex: 565SP transmits most visible wavelengths shorter than 565nm
Band pass (BP)	Transmits a specific range of wavelengths. Designated by middle value and total range. Filters can have multiple bands. Ex: 565/40 transmits wavelengths 545nm-585nm

Excitation filter = exciter
Emission filter = emitter ≈ barrier filter
Filters absorb or deflect non-transmitted wavelengths
Mirrors reflect non-transmitted wavelengths

Images taken from:
www.molecularexpressions.com

Fluorescence filter lingo Illustrated Edition

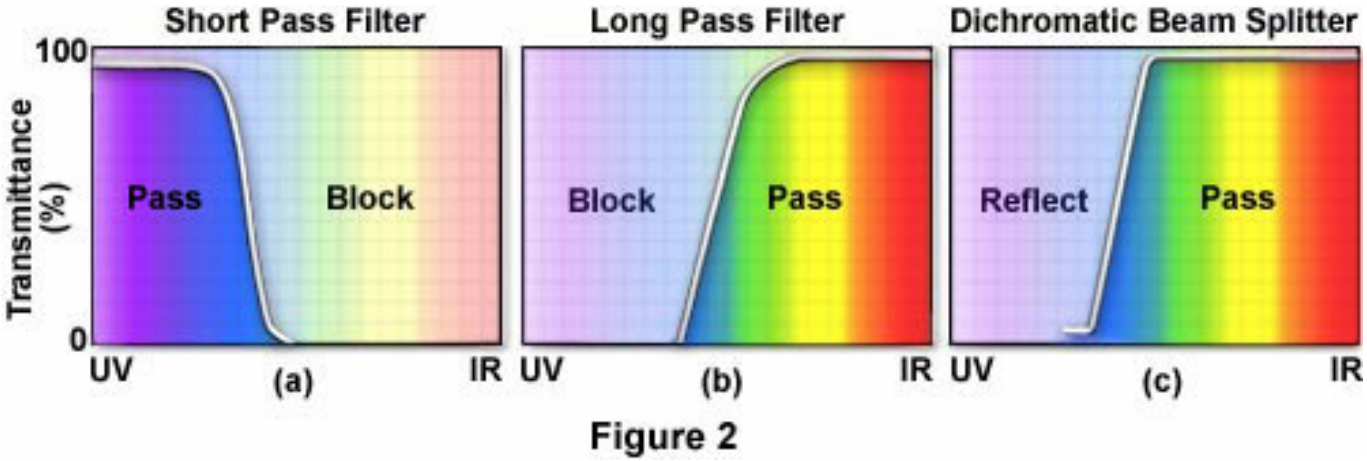


Figure 2

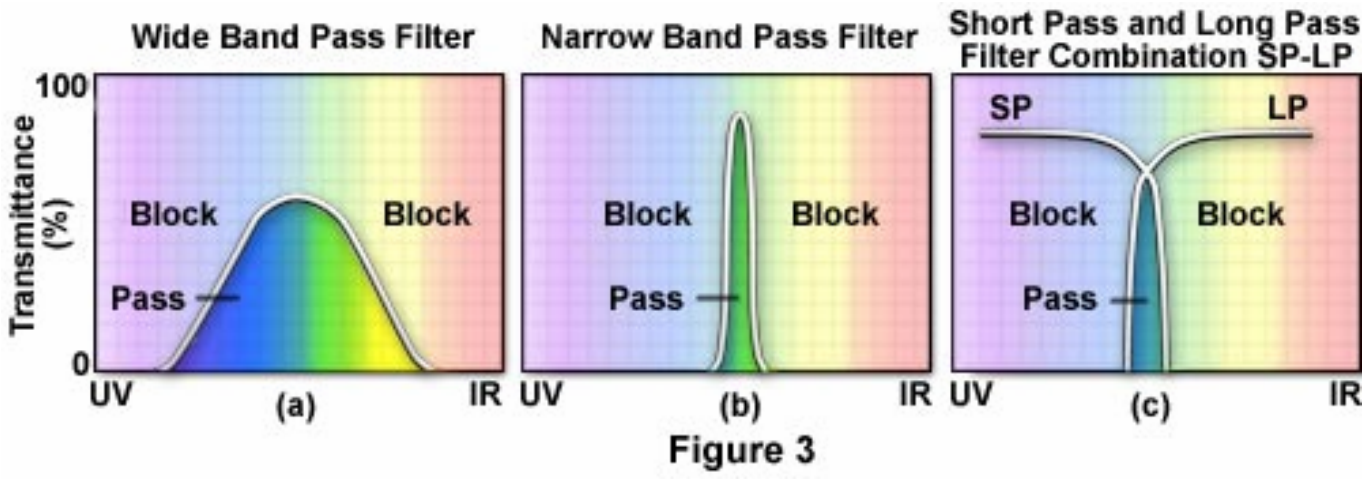
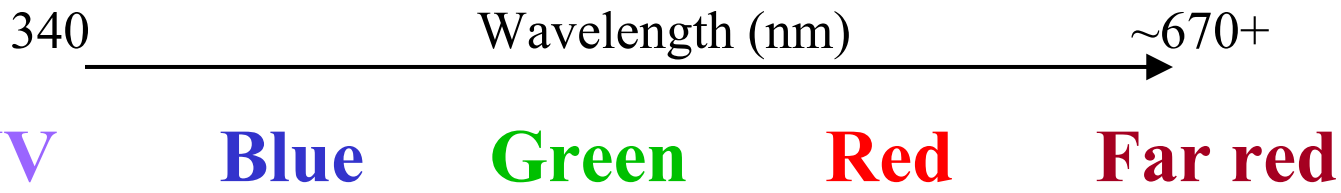


Figure 3

Images taken from:
www.molecularexpressions.com

Fluorescence Color "ladder"

Standard fluorescent probes tend to follow the following excitation and emission characteristics in relation to the visible color spectrum:



Excite → Emit
(DAPI like)

Excite → Emit
(FITC like)

Excite → Emit
(Rhodamine & TexRed like)

Excite → Emit
(CY5 like)

Table of Fluorochromes

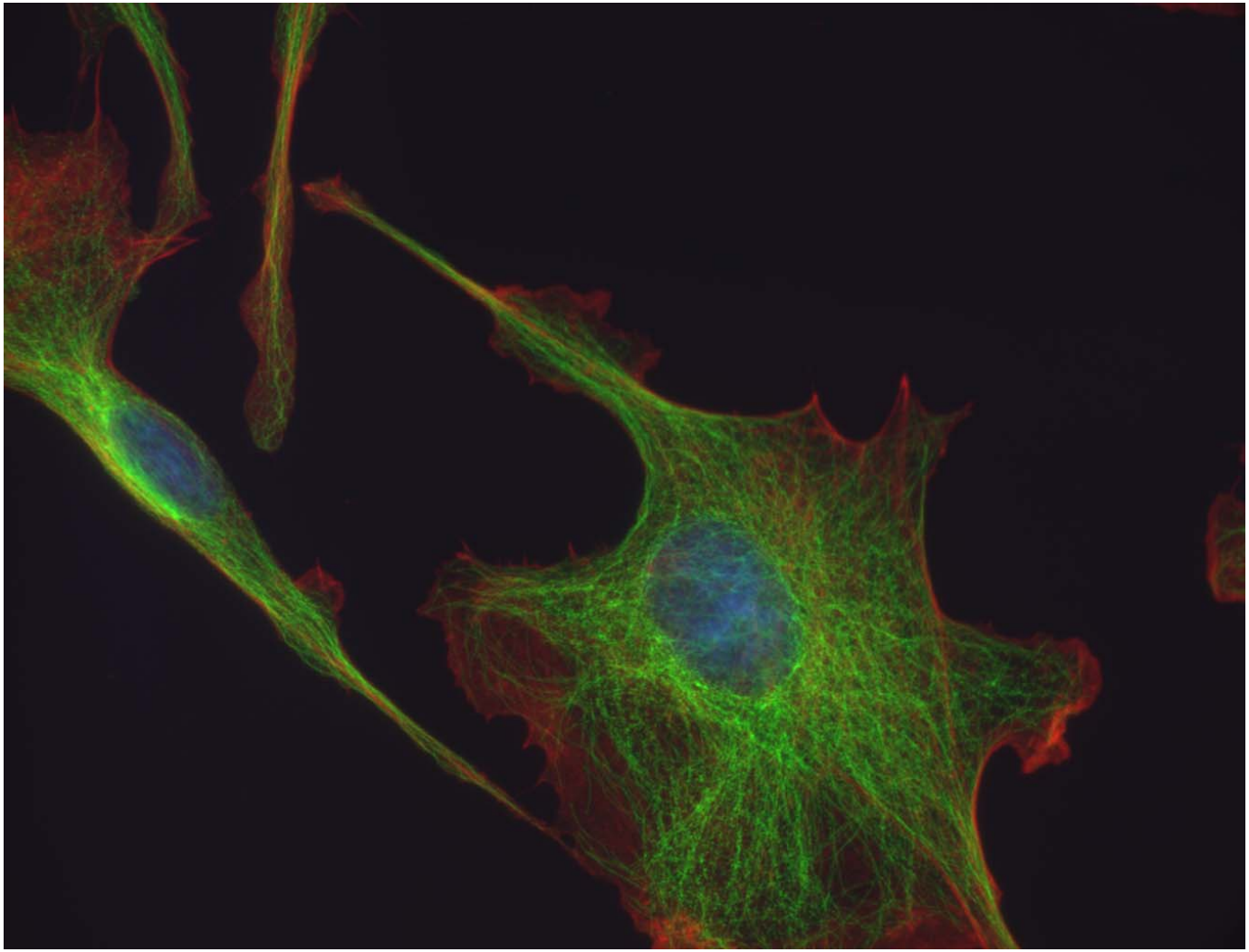
Probe	Ex (nm)	Em (nm)	
Reactive and conjugated probes			
Fluorescein	495	519	FITC; pH sensitive
TRITC	547	572	TRITC
Texas Red	589	615	Sulfonyl chloride
Alexa Fluor dyes *(Molecular Probes)*			
Alexa Fluor 350	346	445	
Alexa Fluor 488	494	517	
Alexa Fluor 568	578	603	
Alexa Fluor 594	590	617	
Alexa Fluor 647	650	668	
Cy Dyes *(AP Biotech)*			
Cy2	489	506	
Cy3	(512);550	570;(615)	
Cy5	(625);650	670	

Adapted from Salk Flow Cytometry home page
http://flowcyt.salk.edu/fluo.html

Probe	Ex (nm)	Em (nm)	
Nucleic acid probes			
DAPI	345	455	AT-selective
SYTOX Green	504	523	DNA
Propidium Iodide (PI)	536	617	
TOTO-3, TO-PRO-3	642	661	
Cell function probes			
Fura-2	340/380	510	AM ester. Low/High Ca^{++},
Fluo-3	506	526	AM ester. pH > 6, Ca^{++}
Fluorescent Proteins			
BCECF	490	535	pH
eBFP	380	440	(*Clontech*) Quantum yield 0.18
"GFP"	396,475	508	Quantum yield 0.77
eCFP	434	477	(*Clontech*) Quantum yield 0.40
eGFP	489	508	(*Clontech*) Quantum yield ~0.60
eYFP	514	527	(*Clontech*) Quantum yield 0.61
Other probes			
mRFP1	584	607	(*Tsien lab*) Quantum yield 0.25
Calcein	496	517	pH > 5

Adapted from Salk Flow Cytometry home page (http://flowcyt.salk.edu)

Multi-channel fluorescence image

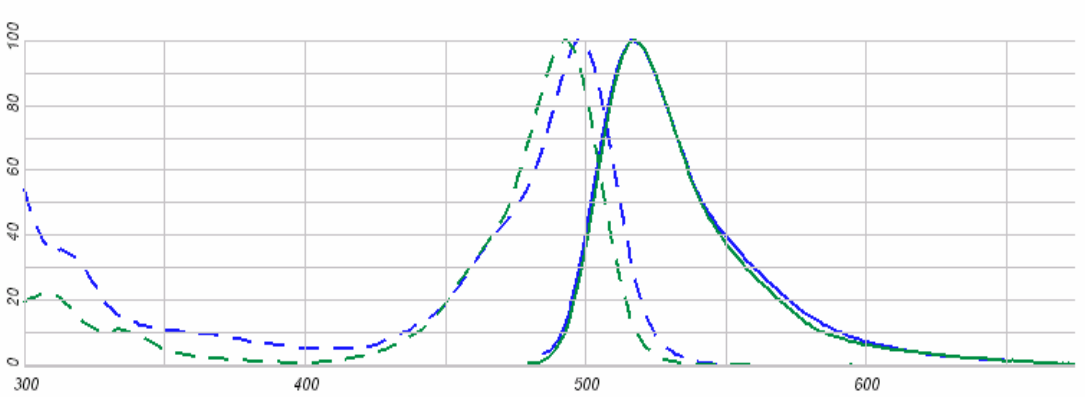


Labeling multiple structures in the same sample: Multiple fluorophores

To be able to distinguish different structures, you MUST be able to spectrally separate each fluorophore from the others

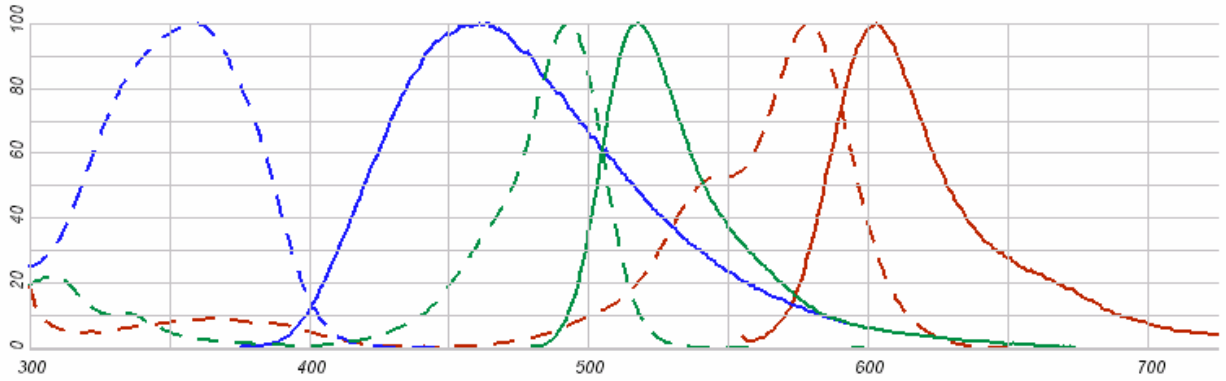
Spectral overlap

AlexaFluor488
FITC



Spectral separation

DAPI
AlexaFluor488
AlexaFluor568



http://probes.invitrogen.com/resources/spectraviewer

The Big Problem: Bleed-through and Crosstalk

Spectral overlap increases as more structures are stained simultaneously (limited λ range, limited chemistry of fluorophores).

This, in turn, increases the risk of detecting multiple fluorophores in the emission wavelength range for one fluorophore

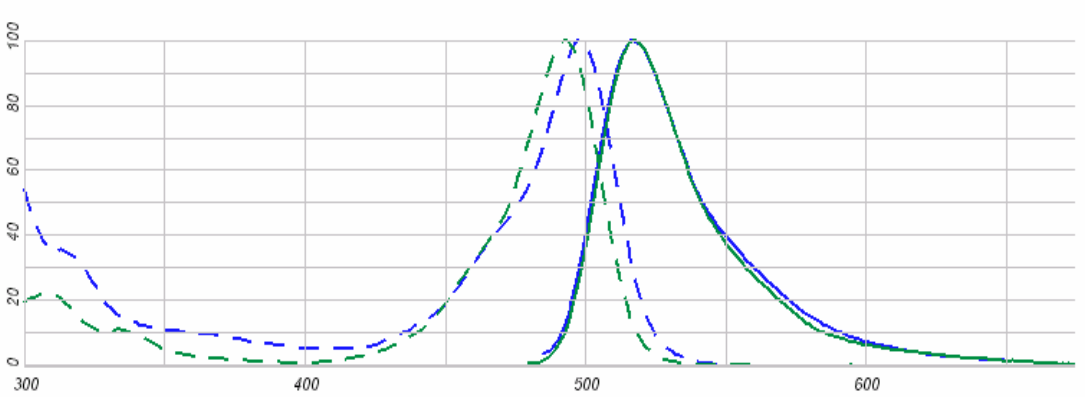
Always test for bleed-through/crosstalk with a new set of fluorophores by creating a single-stained sample for each structure included in the multi-stained sample(s).

Labeling multiple structures in the same sample: Multiple fluorophores

To be able to distinguish different structures, you MUST be able to spectrally separate each fluorophore from the others

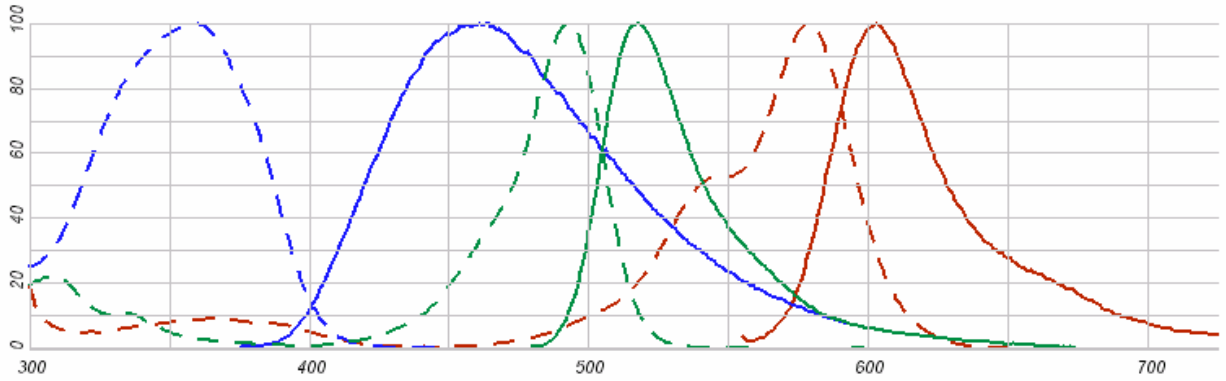
Spectral overlap

AlexaFluor488
FITC



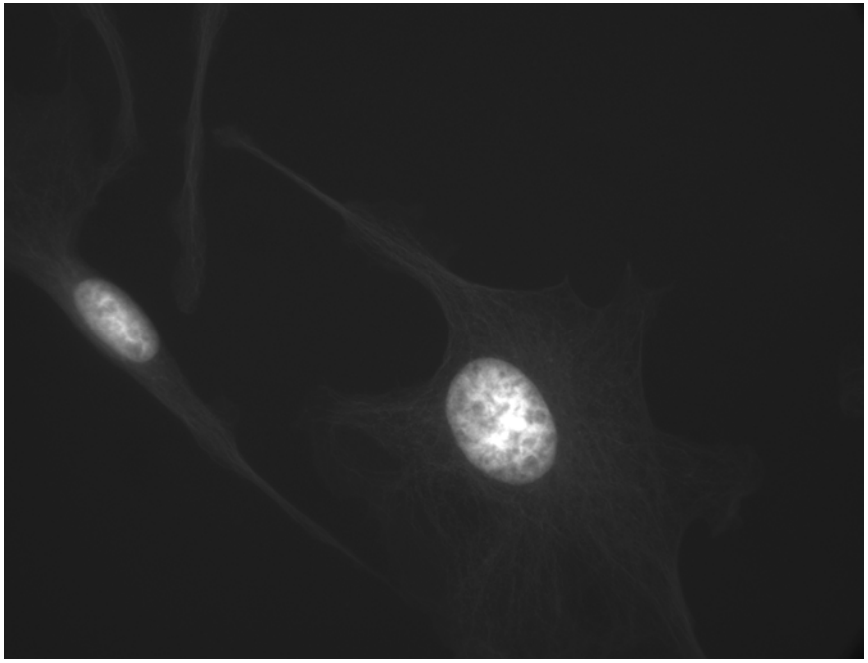
Spectral separation

DAPI
AlexaFluor488
AlexaFluor568

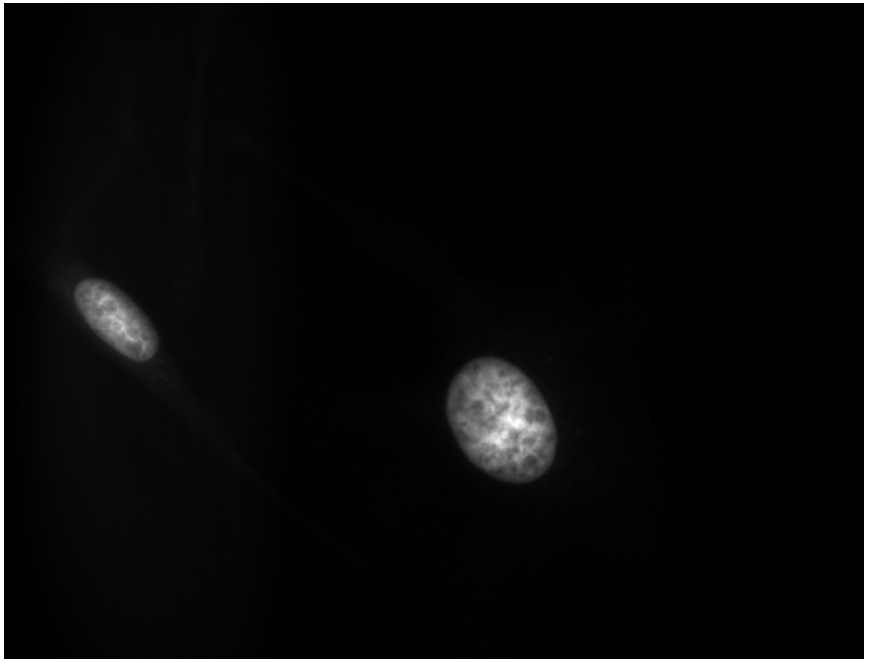


http://probes.invitrogen.com/resources/spectraviewer

Bleed-through



Improper emission filter (in this case, a long pass filter) allows fluorescence from the AlexaFluor488-labeled tubulin to appear with the DAPI



Use of a band-pass emission filter eliminates the AlexaFluor488 fluorescence from the DAPI image

Preventing/Overcoming Bleed-through

- Choose fluorophores with better spectral separation to work with the available fluorescence filters
- Adjust filter selection for more specific excitation and/or emission
- Adjust fluorophore intensities in sample (have lower-wavelength fluor(s) less bright)
- Image each fluorophore separately

Brightness

- For a population of fluorophores

  Brightness $\propto$ I C

  - I = intensity of exciting light
  - C = concentration of fluorophore

- The relationship is approximately linear for low to moderate levels of I and C

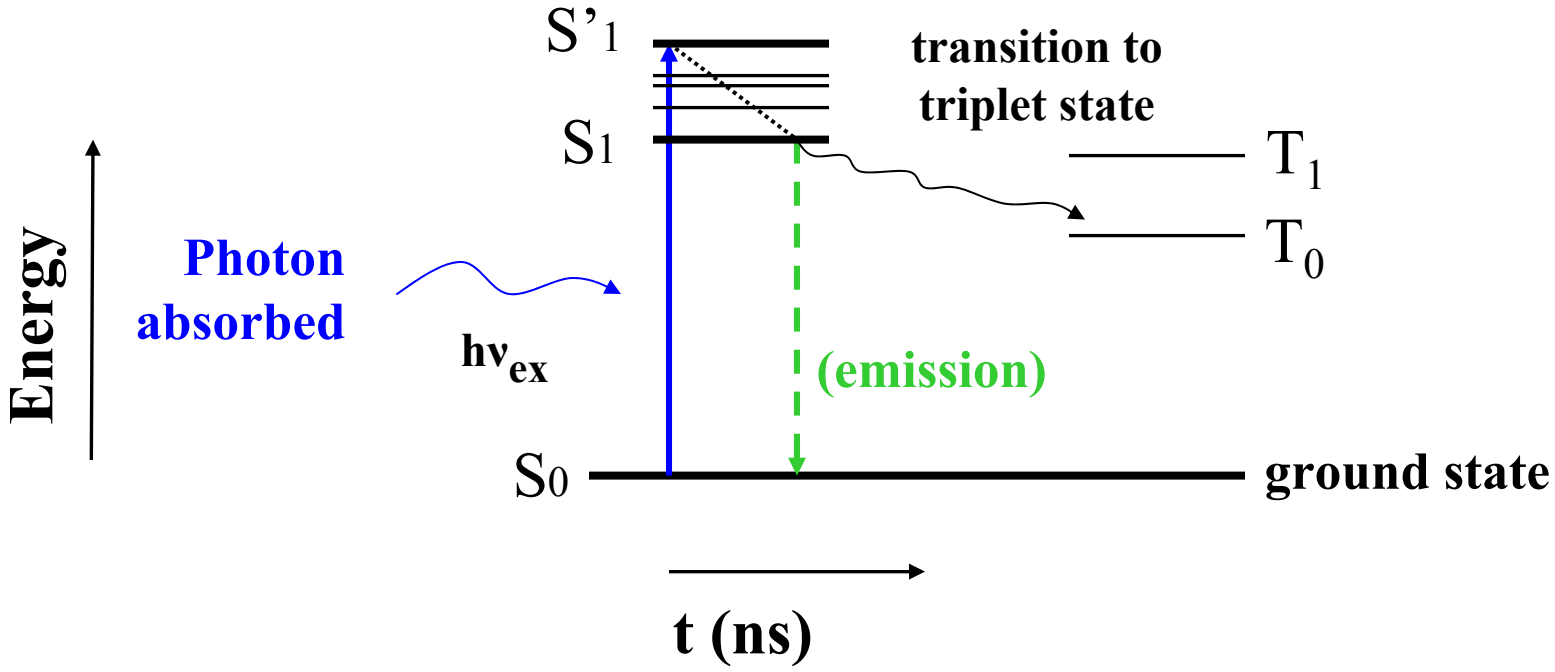
- The relationship becomes non-linear at the high end
  - **At very high C, you get self-quenching**
    - Application of any amount of light does not result in fluorescence because they are absorbing the emission energy of adjacent molecules
  - **At high I, you can get fluorescence saturation**
    - At very high I, you can have the situation where most of the excitable electrons are in the excited state, leaving fewer at the ground state for excitation by the light. Therefore, increasing the I will not result in increased fluorescence

Other Potential Problems

- **Auto-fluorescence**
  - … of your sample (different tissue types, dead cells, collagen, NADPH, chlorophyll, lipofusion, etc.)
  - … of the sample holder (plastic, animal serum, phenol red)
  - … fixation/staining protocol (e.g. glutaraldehyde)
  - **Test for auto-fluorescence by using a sample with no fluorescent modifications**
- **Photobleaching**
  - Fluorophores have a limited number of excitation-emission cycles.
  - Excessive intensity or longevity of fluorophore excitation can result in loss of fluorescence ability of the fluorophore.
  - More recently developed fluorescent compounds are less prone to photobleaching (therefore, more photostable)
  - Always shut the fluorescence illumination when not looking at or imaging your sample.
  - Addition of oxygen scavengers reduces the rate of photobleaching.
- **Fluorescence Saturation**
- **Quenching**

Photobleaching

Jablonski diagram



Electrons can lose energy in non-radiative ways

- Electrons that move to the triplet state are highly reactive.
- When they react with oxygen, the electron is no longer available for the fluorescence energy state transitions.

Like a light house in the fog…

Fluorescent specimens are their own light source.

Therefore, you can *detect* molecules/structures without *resolving* their structure, giving you the ability to see sub-resolution objects.

Example: You can detect the presence of a lighthouse in the fog by seeing the light even though you cannot resolve the structure of the lighthouse itself.

Ways to make your sample fluorescent

1. Dyes
   - "Structure" dyes
     - DAPI, Sytox Green (fluoresce when intercalate with DNA)
     - FM 1-43 (fluorescent when interact with lipid)
   - Indicator dyes
     - Fura-2 (free vs. bound Ca^{2+}), pH indicator dyes
2. Immunohistochemistry
   - Use of antibodies to label specific structures
     - Directly conjugated primary antibodies
     - Fluorescent Secondary antibody
     - Streptavidin/Biotin antibody detection
3. Fluorescent Proteins
   - Use of molecular biology to attach a fluorescent protein to your protein of interest.
4. Quantum Dots

And others…

Immunofluorescence

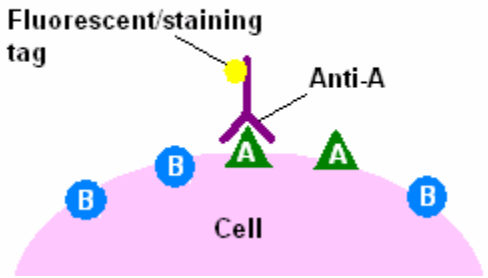
- Common term for fluorescence immunohistochemistry, but also includes use of fluorescent dyes for structure detection. Often referred to as IF.
- There is definitely an art form to the science of IF.
- Every combination of sample type + antibody + target structure or molecule can require adjustments, additions or subtractions to a “standard” protocol.
- Every step in the protocol has caveats that can dramatically effect your sample structure or staining efficiency (or not). And there are often many steps.
- Use minimum amount of reagents necessary

The basic idea of IF

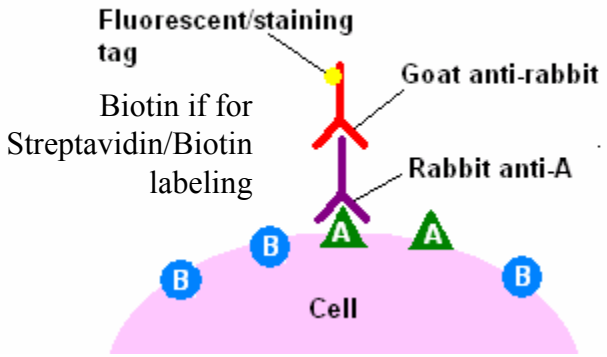
Specific detection of target molecules using antibodies (immunohistochemistry) or fluorescent dyes (chemicals that directly label a structure) to create fluorescent contrast between your structure of interest and the background

Two types of immunohistochemistry:

Direct



Indirect



Images taken from Wikipedia.com

What's the correct protocol?

Well, that depends on a lot of things…

- Sample type
- Structure(s) of interest
- Antibody characteristics
  - Affinity and binding properties
  - Species considerations
- Requirements for any dyes or non-antibody labeling substances used

If you are starting from scratch, start with several protocols from the literature or Current Protocols to determine what might work best for all of the structures you want to visualize in your sample

Generic Protocol for IF

1. Fix
2. Permeablize (for antibody penetration)
3. Wash 3x (incubate for some time each wash)
4. Block for non-specific binding
   - use serum from the species in which your secondary antibody/ies were raised, not only BSA
5. Primary antibody/ies (in blocking solution)
6. Wash 3x (incubate for some time each wash)
7. Secondary antibody/ies in blocking solution
   - also add structural stains (eg, fluorescent phalloidin)
8. Wash 3x (incubate for some time each wash)
9. DAPI, Sytox Green, other quick dyes; Streptavidin (steps 7-8)
10. Wash 1x (quickly)
11. Store in PBS or Mount with appropriate mounting media*

    ***Not just any mounting media will do!!**
12. Store in the dark in the cold

IF Generic Protocol breakdown

1. **Fixation**

Common fixatives

Paraformaldehyde, Formalin, Ethanol, Methanol, Acetone, Glutaraldehyde

Optimal fixative depends on:

- Sample type (cell type or tissue type)
- Structure(s) of interest (ex: actin does not fix well in methanol)
- Antibody affinity (some antibodies work with methanol fixation but not paraformaldehyde)

Fixation time:

- Dependent on sample type.
- Thicker samples require longer fixing time at low temperature for penetration.

IF Generic Protocol breakdown

2. Permeablize

Use of a mild detergent to poke holes in the cell membranes for access of any membrane impermeable labeling molecules, such as antibodies or dyes.

Common permeablization agents

- TritonX100 (0.1%)
- Saponin
- Tween20

3. Wash (3 times)

Each wash step should include a short incubation to allow excess to dissipate into washing liquid

IF Generic Protocol breakdown

4. ****Block****

- **Essential** step to reduce non-specific binding of the antibodies (primary and secondary) to your sample.
- Best to use serum (5% in buffer) from the species in which your secondary antibody/ies were raised, not just BSA
  - Unnecessary if no antibodies will be used

5. **Primary antibody/ies**

- Should be diluted in the blocking solution
- Incubation time will depend on sample type (thicker samples require longer incubation)
- Excess primary antibody concentration or incubation can result in non-specific staining and high background.
- Can combine multiple primary antibodies in the same step as long as there are no species problems

IF Generic Protocol breakdown

6. **Wash 3x** same as before

7. **Secondary antibody/ies in blocking solution**
   - Similar theory to primary antibodies
   - Diluted into blocking solution
   - Typically use very high dilution from stock (1:500-1:2000 dilution)
   - Can also add structural stains to the cocktail (e.g. fluorescent phalloidin)
   - Be careful of your species when combining multiple antibodies

8. **Wash 3x** same as before

IF Generic Protocol breakdown

9. **Quick dyes (optional)**
    - DAPI, Sytox Green, FM 1-43
    - Quick 1-5minute staining

10. **Wash 1x** short incubation to rinse the excess quick dye

11. **Store in PBS or Mount with proper mounting media**
    - **Not just any mounting media will do!!**
    - Different fluorophores react differently to different mounting media. More about this later

12. **Store in the dark in the cold**
    - This helps preserve the fluorescence of the fluorophores.

A few common adjustments

1. Streptavidin/Biotin
   - Used to amplify a weak antibody signal
   - Add an additional set of “antibody” + wash steps before step 9
2. Phalloidin and phallacidin (as examples)
   - Substances that label filamentous actin
   - Can be fluorescently labeled with standard fluorophores
   - Used in the staining protocol as you would use a secondary antibody, but not antibody is involved.
3. Fluorescent Proteins
   - No “staining” required if native fluorescence is preserved in the fixation step
   - Often loose their native fluorescence with dehydrating fixatives (ethanol, methanol, etc.), though not always. Antibodies are available for most xFPs if needed, but use native fluorescence is cleanest

Mounting Media (MM)

and you thought this was the easy part

- Watch out for incompatibilities between a specific MM and a fluorescent compound (e.g. Cy2 and Vectasheild)
- The closer your MM refractive index (RI) is to that of glass (1.5), the higher your transmission efficiency is from your fluorescent sample to the detector.
- However, as the MM's RI reaches 1.5, you can loose the diffraction necessary for transmitted light contrast (ie DIC)
- Use a staining step for DAPI, not MM containing DAPI
- Hardening MM can cause structures to change in thick samples

Careful of your antibody species

A few things to watch out for with antibodies:

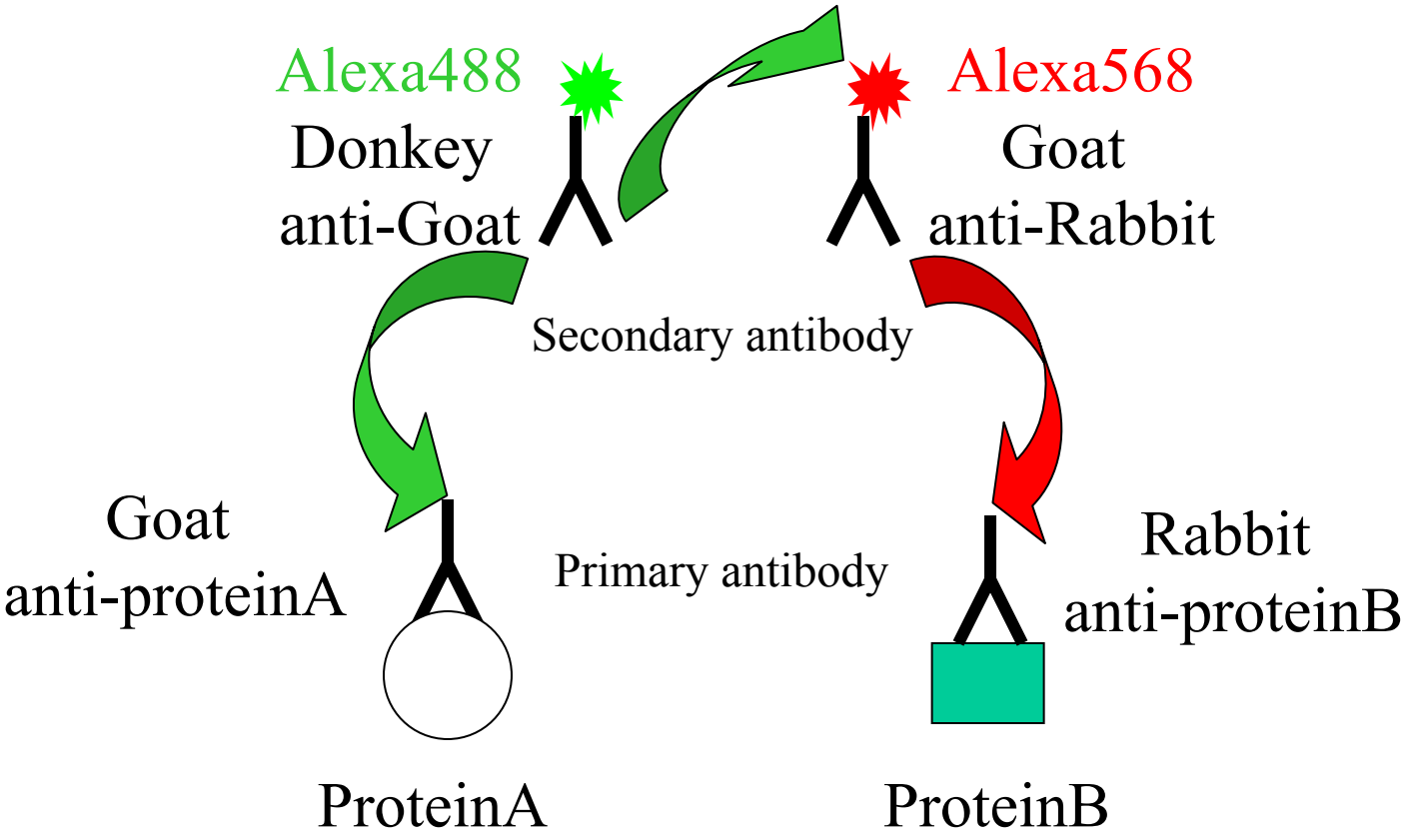
Secondary antibody cross-reactivity

- The host species of one secondary (2º) antibody is the same as the target species of the other 2º antibody
  - Ex: 1ºA = rabbit anti-proteinA 2ºA* = *goat anti-rabbit
    1ºB = goat anti-proteinB 2ºB* = *donkey anti-goat

    Since 2ºB recognizes 2ºA, both proteinA and proteinB would be labeled with 2ºB*. This can be avoided by sequential staining (label for proteinB, then go back and label for proteinA).

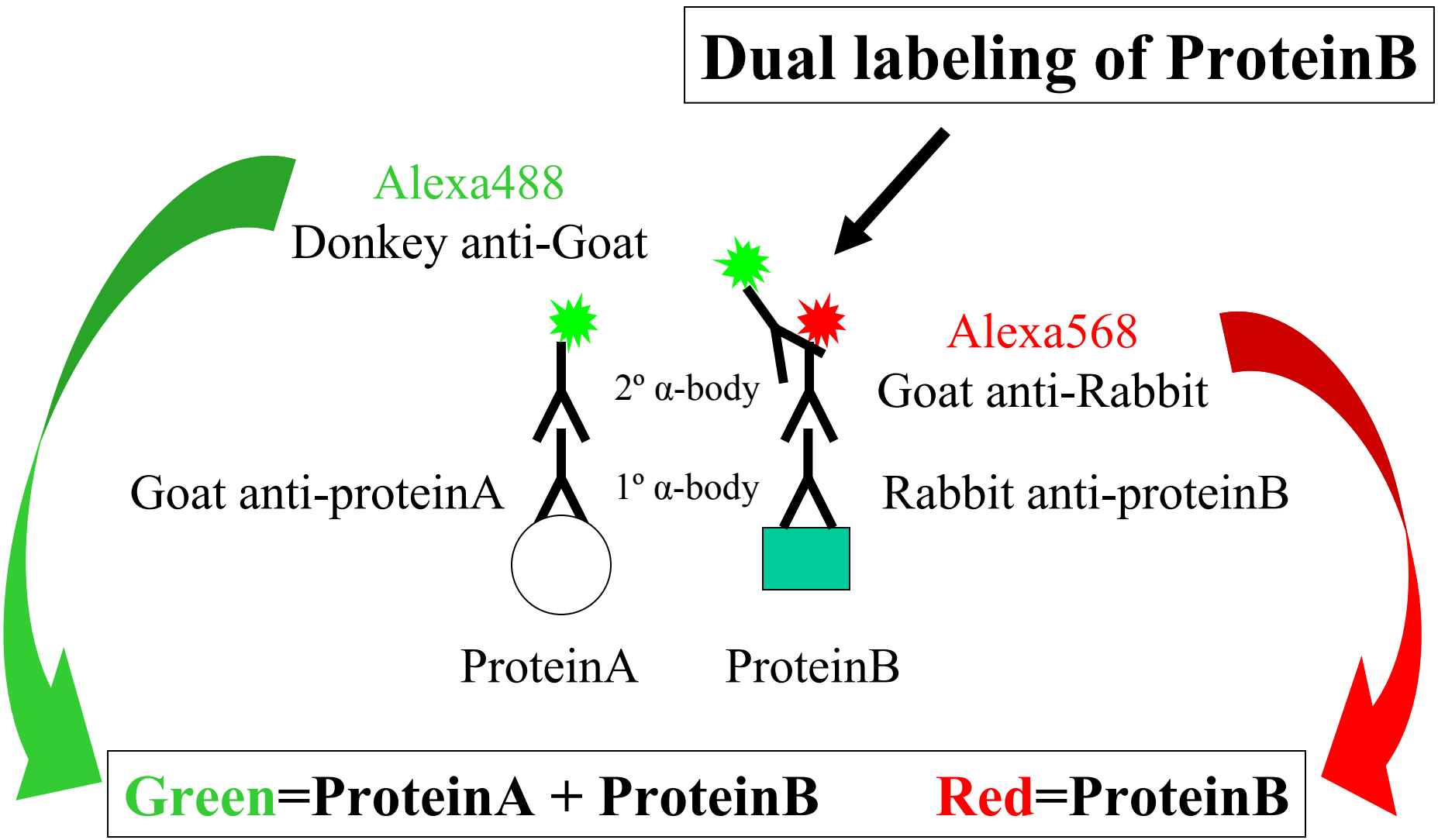
Your sample contains elements that will be detected as the target of one of the secondary antibodies (e.g., using an anti-mouse 2º antibody on mouse tissue)

Secondary Antibody Cross-reactivity



Secondary Antibody Cross-reactivity

Dual labeling of ProteinB



Green=ProteinA + ProteinB **Red=ProteinB**

If in doubt, ***test it out!!***

If you think that a new (or old) component of your staining protocol or imaging system is causing strange results:

- Minimize the number of variables for a test sample or two and try to pin-point where things may be going wrong.
- Run entire IF protocol without primary Ab(s)
- It may be in the sample preparation OR it may be in the imaging system!

Remember: IF is not a wholly predictable problem at the beginning—use empirical evaluation of a well thought out preparation protocol to determine its success.

Summary

- Fluorescence is the absorption of a photon that results in the release of a photon of longer wavelength
- Fluorescent compounds absorb and emit a spectrum of wavelengths
- Filters and dichromatic mirrors are used to separate the excitation and emission spectra
- Separation of multiple fluorescent compounds requires that they have distinct spectra
- Preparation of specimens with fluorescent compounds for imaging is often complex

The 5 Main Components of Sample Fluorescence Intensity

1. Intensity of Illumination light
2. Amount of target
3. Amount of probe
4. Spectral Characteristics of light path
5. Detector Sensitivity **Next Week**

Resources

http://probes.invitrogen.com/resources/education/

http://www.molecularexpressions.com

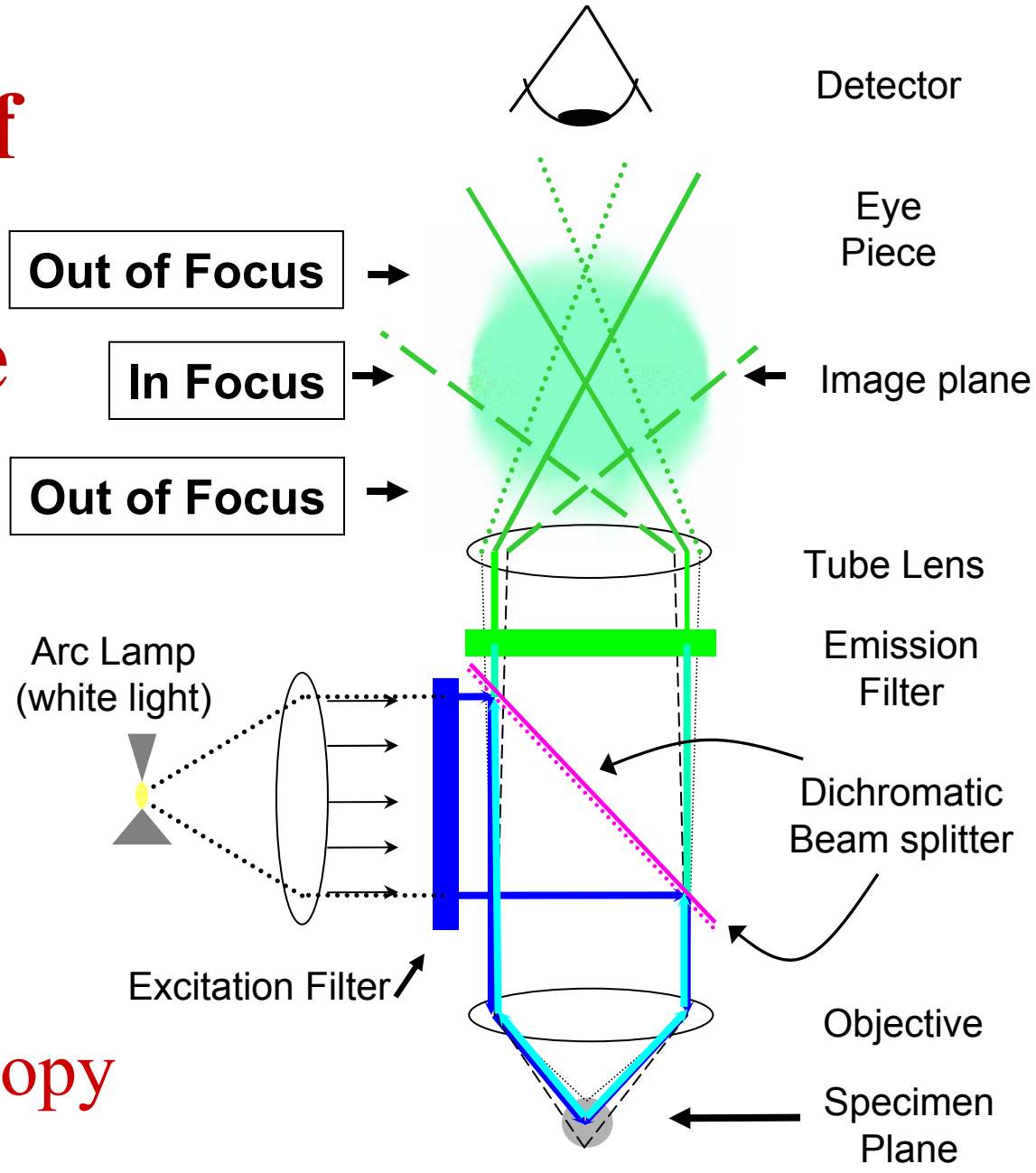
https://www.omegafilters.com/curvo2/index.php

Spector, DL and Goldman, RD (ed.s). Basic Methods in Microscopy. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2006.

Murphy, D. Fundamentals of Light Microscopy and Electronic Imaging. New York, NY: Wiley-Liss, Inc., 2001.

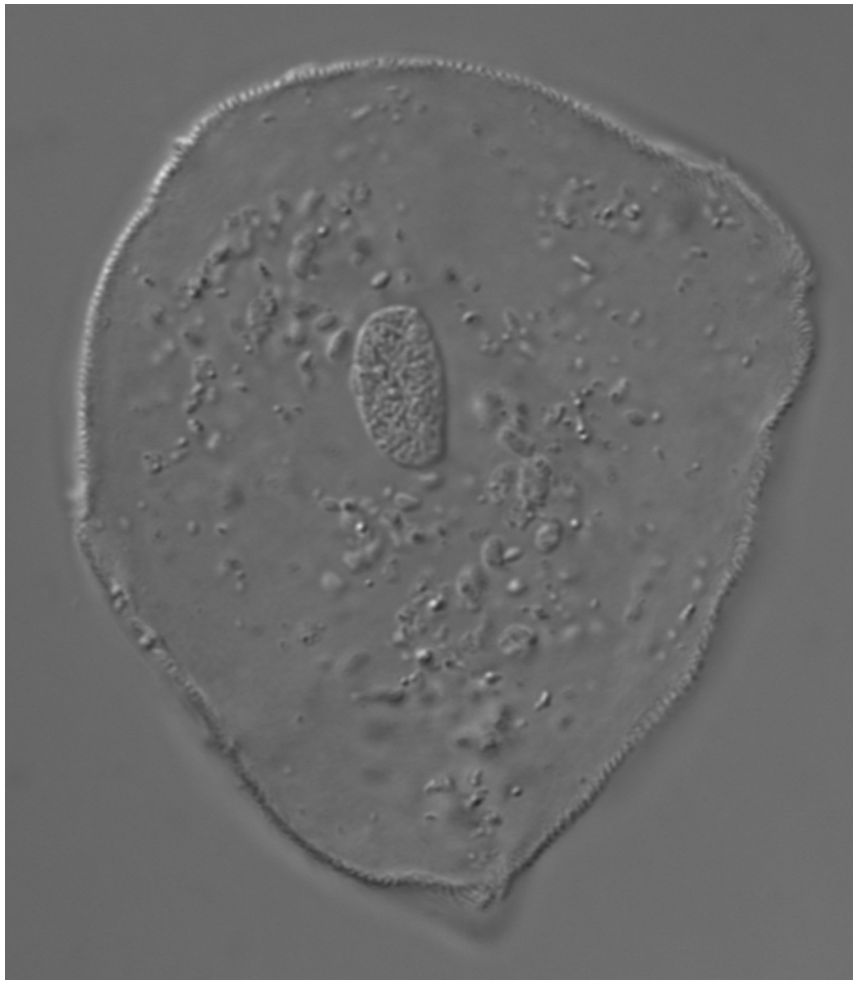
Current Protocols in Cell Biology (avail. online with UNC-CH subscription or through Invitrogen/Molecular Probes) http://www.mrw.interscience.wiley.com/cp/cpcb

Limitation of Widefield Fluorescence

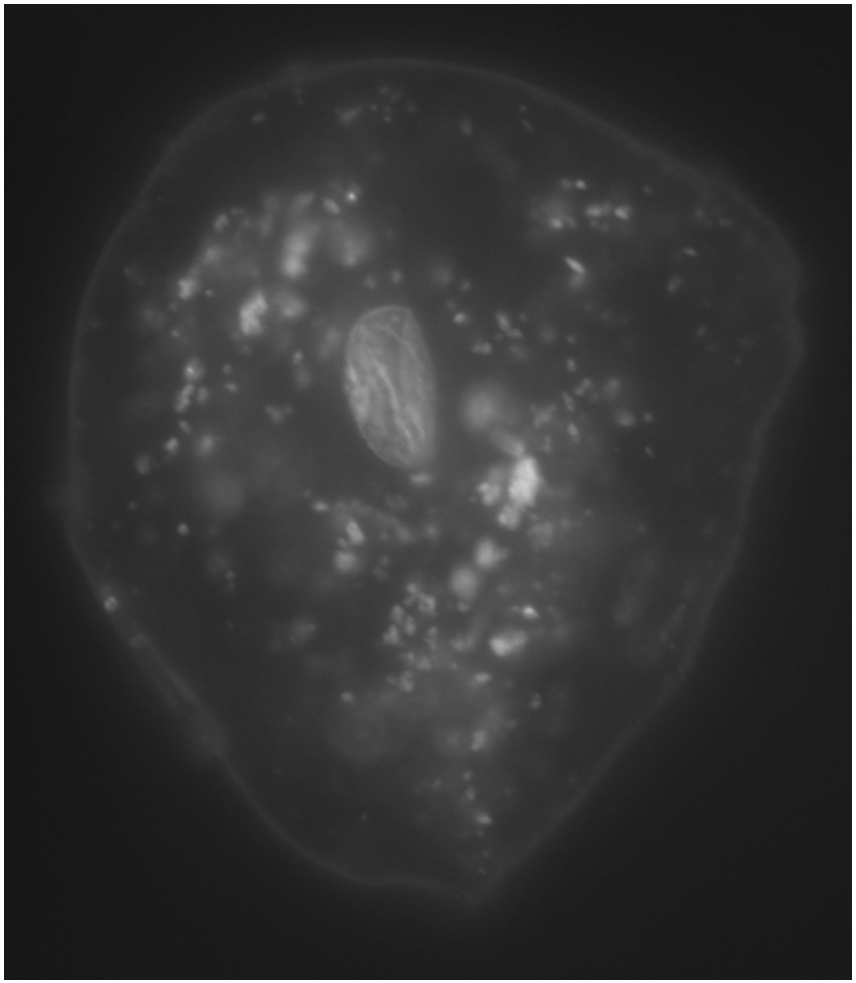


Solution:
Confocal Microscopy
In 2 weeks

Live Buccal Epithelial cells



Transmitted light (DIC)



Fluorescence
FM 1-43 membrane dye