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An Introduction to the Theory and Practice of Light Microscopy

$E = h\nu$

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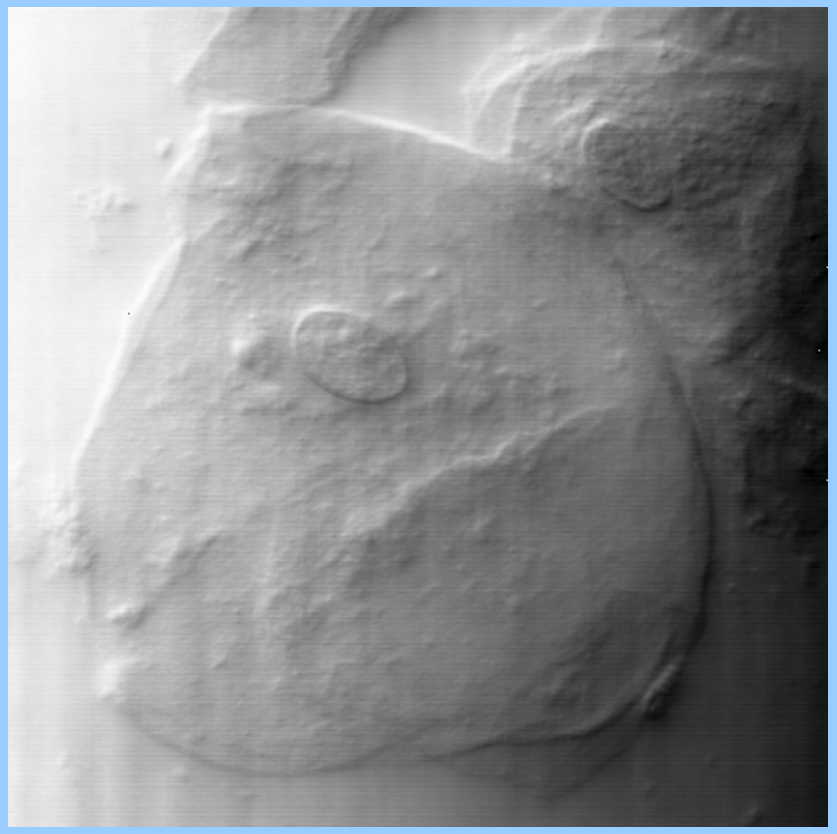
Part 2 - Fluorescence

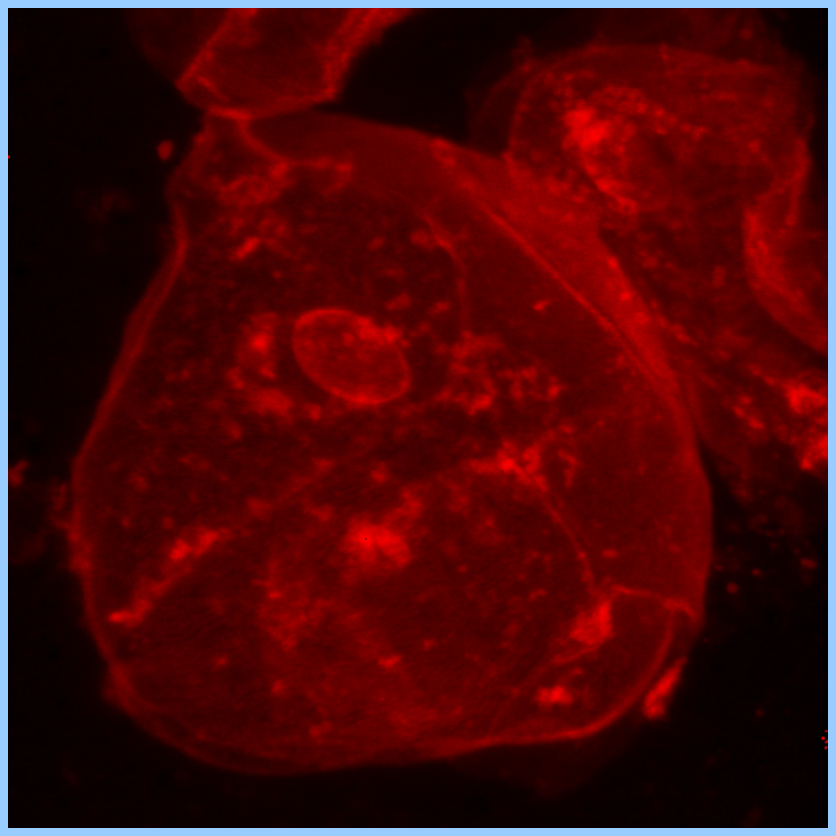
Why use Fluorescence?

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

Live Buccal Epithelial cells

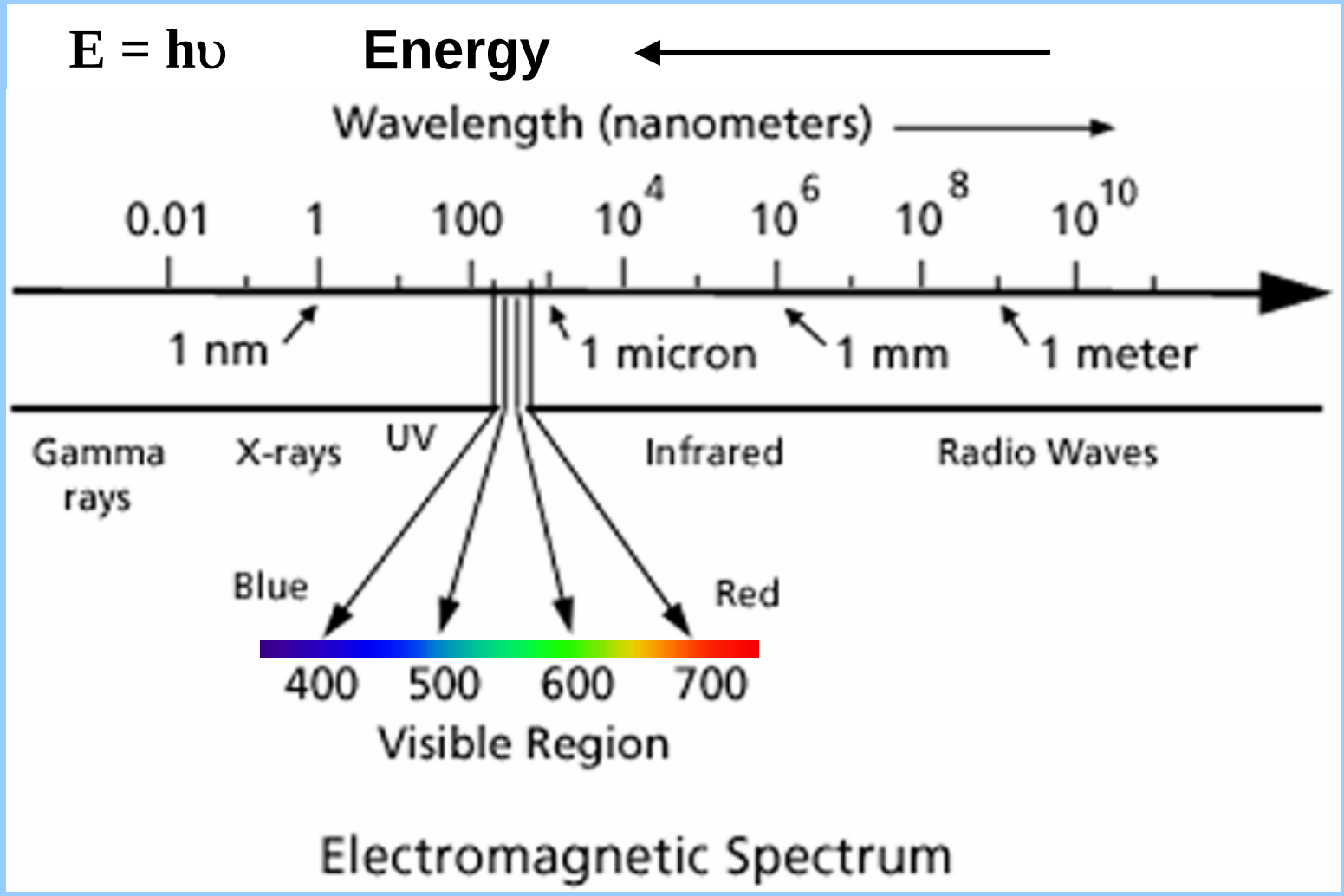


Transmitted



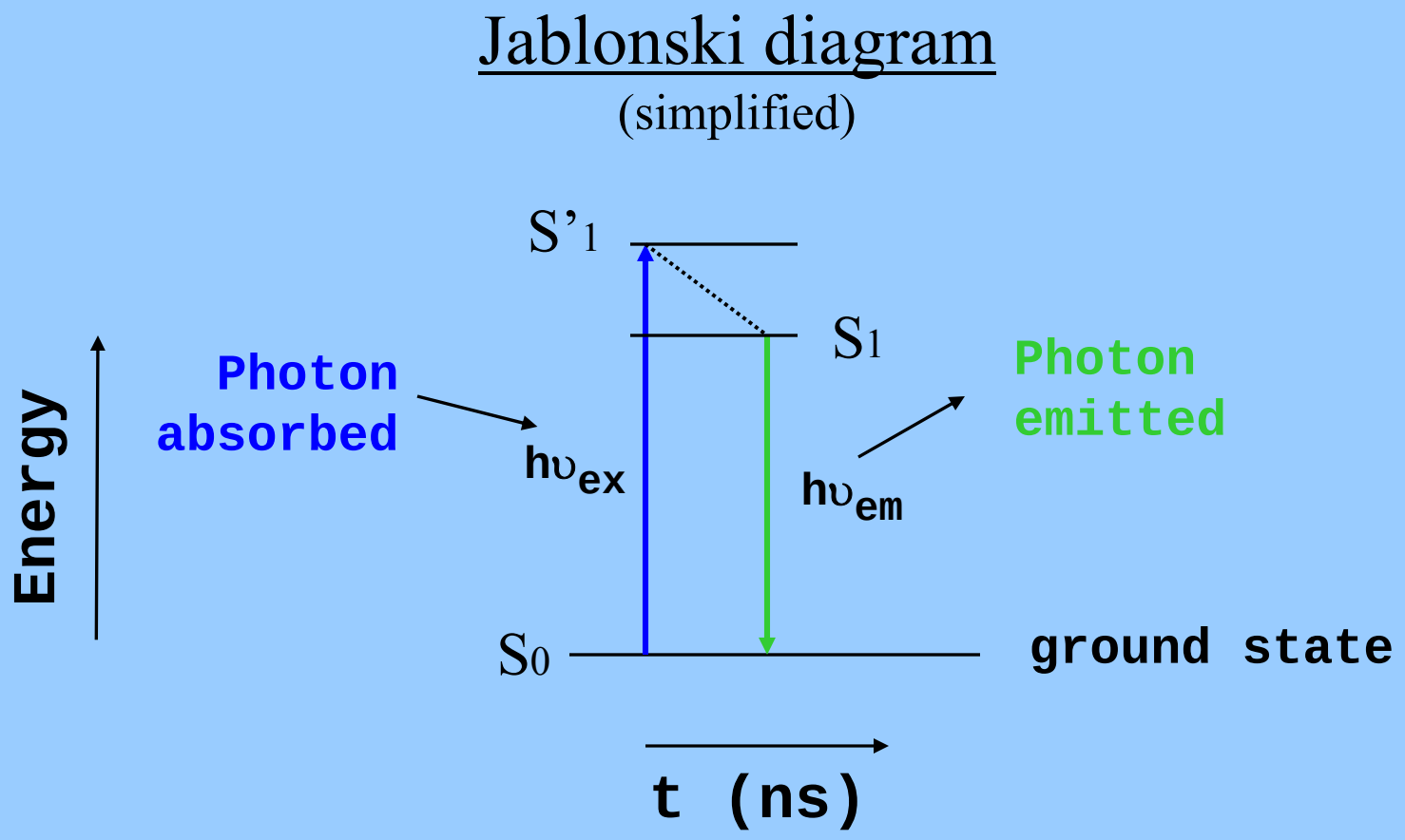
Fluorescence
FM 1-43 membrane dye

A little physics reminder…



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

Molecular Fluorescence



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

Like a light house in the fog…

Important conclusion:
Fluorescent specimens are their own light source.

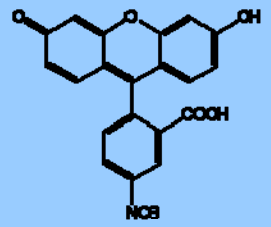
Therefore, you can *detect* molecules/structures without *resolving* their structure, giving you the ability to see sub-resolution objects.

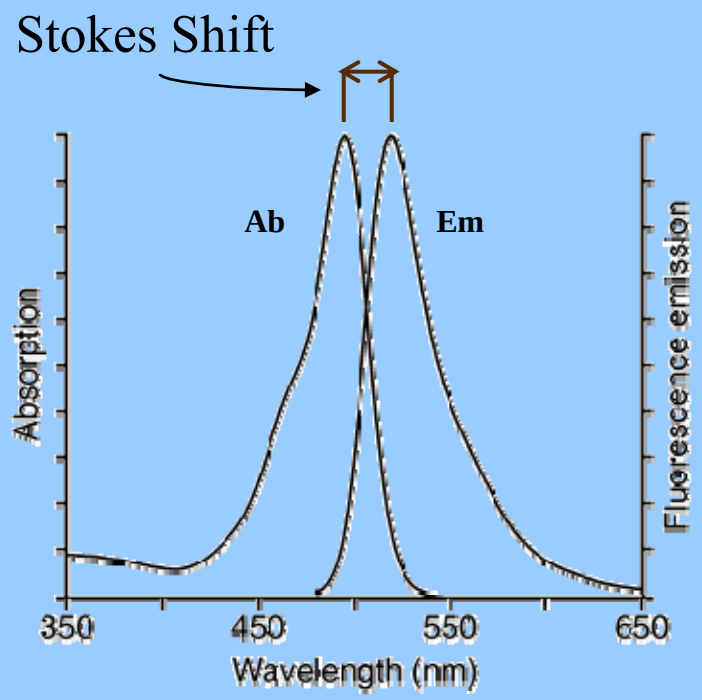
Example: You can detect the presence of a lighthouse in the fog by seeing the light even though you cannot resolve the structure of the lighthouse itself.

Fluorescence Spectra

- Fluorescent compounds each have a spectrum of excitation wavelengths and a spectrum of emission wavelengths (or λs).
- Typically, the peak excitation and emission wavelengths are used to describe a fluorophore's characteristic.
- Spectra can be influenced by the environment (e.g., water vs. EtOH, pH)
- The **Stokes shift** is the difference (in wavelength) between the band maxima of the absorption and emission spectra for a fluorophore

FITC

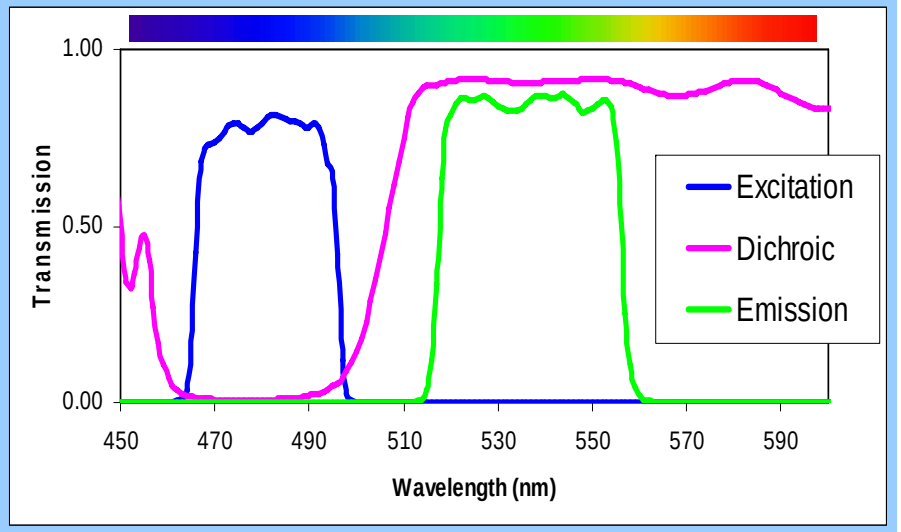


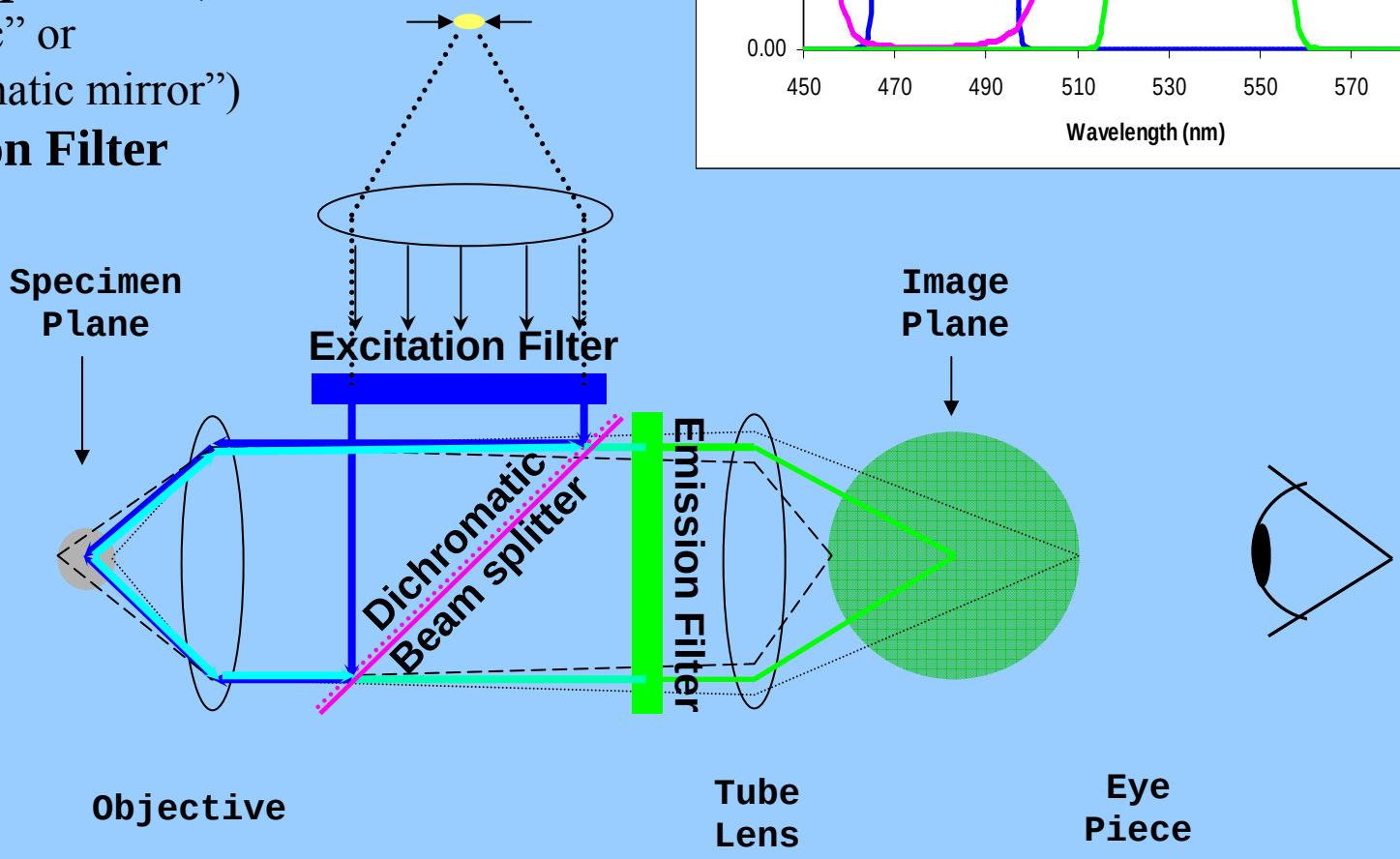


Epi-Fluorescence Filters

Components

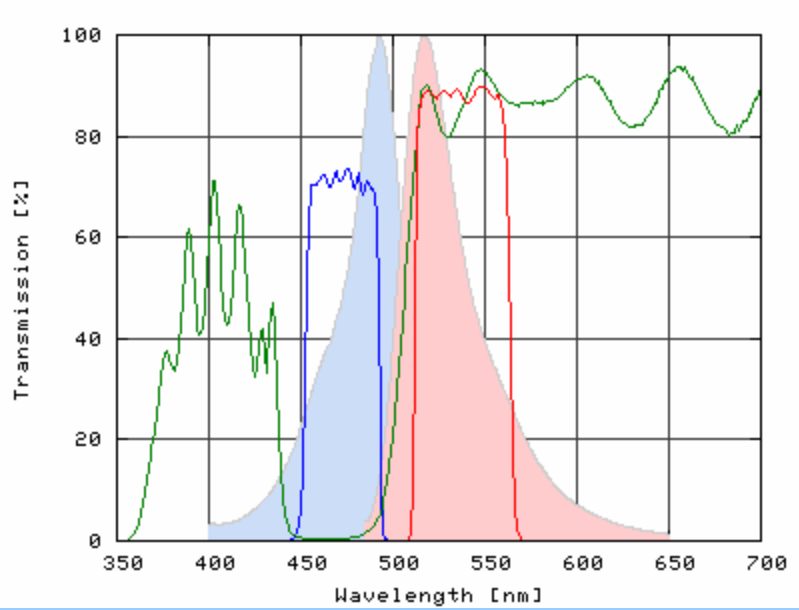
1. **Strong white light source**
2. **Excitation Filter**
3. **Beam Splitter** (aka "dichoric" or "dichromatic mirror")
4. **Emission Filter**





Filter demonstration

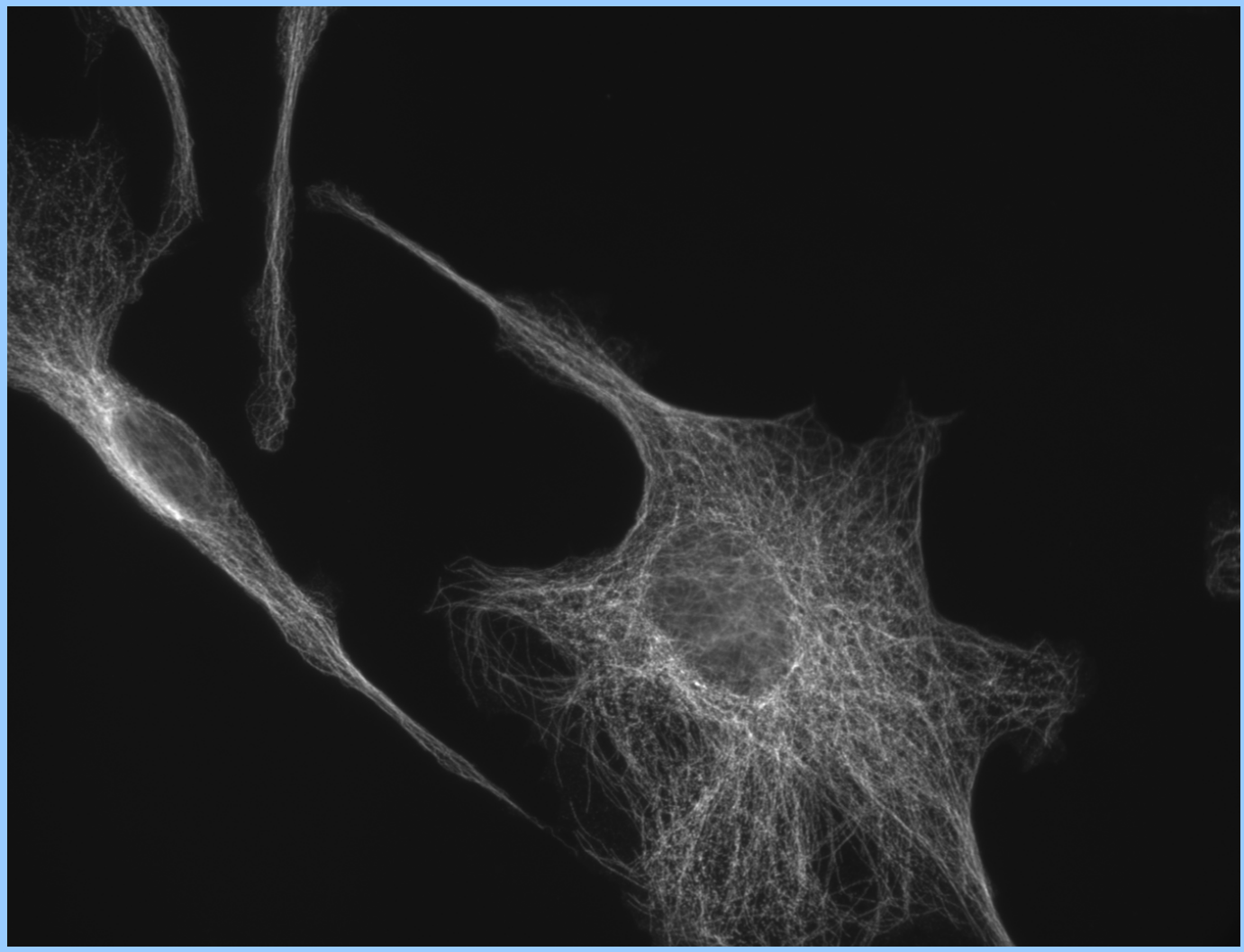
So, when you have a filter set that matches your fluorophore spectra, the graphs should look something like this….



FITC excitation (filled blue) and emission (filled red) spectra 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

Single channel fluorescence image



Fluorescence filter lingo

Filter cube	The combination of excitation filter, beam splitter and emission filter contained in a cube holder. 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 p
Long pass (LP)	T velength. E than 565nm
Short pass (SP)	T ngth. E r than 565nm
Band pass (BP)	Transmits a e value and total ran Ex: 565/40 t

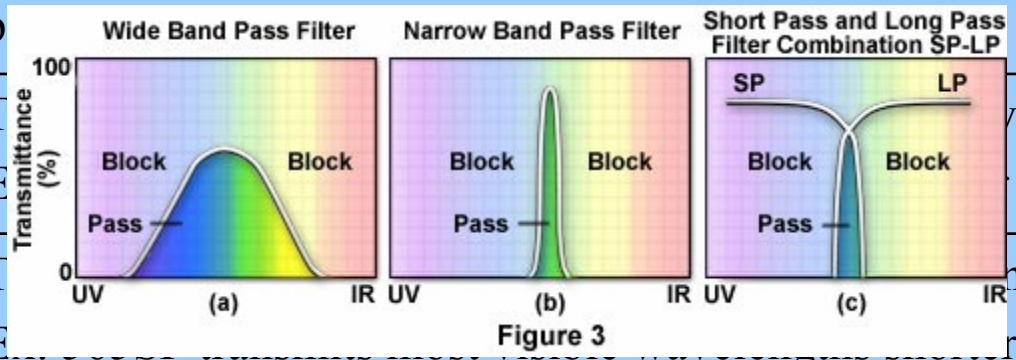


Figure 3

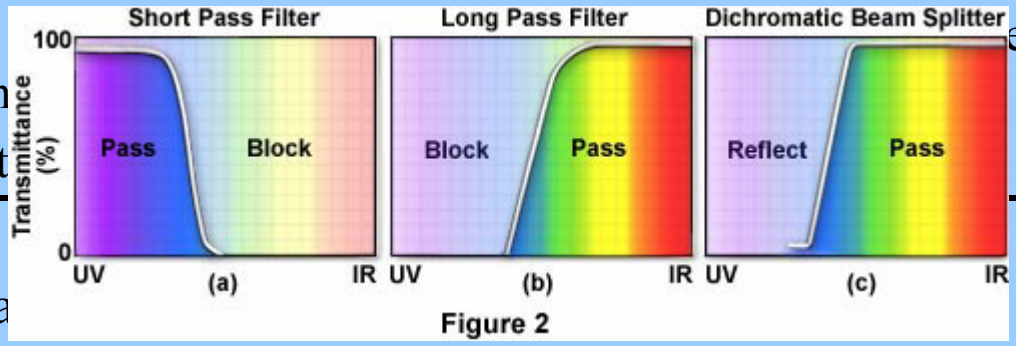


Figure 2

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

Images taken from:
www.molecularexpressions.com

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

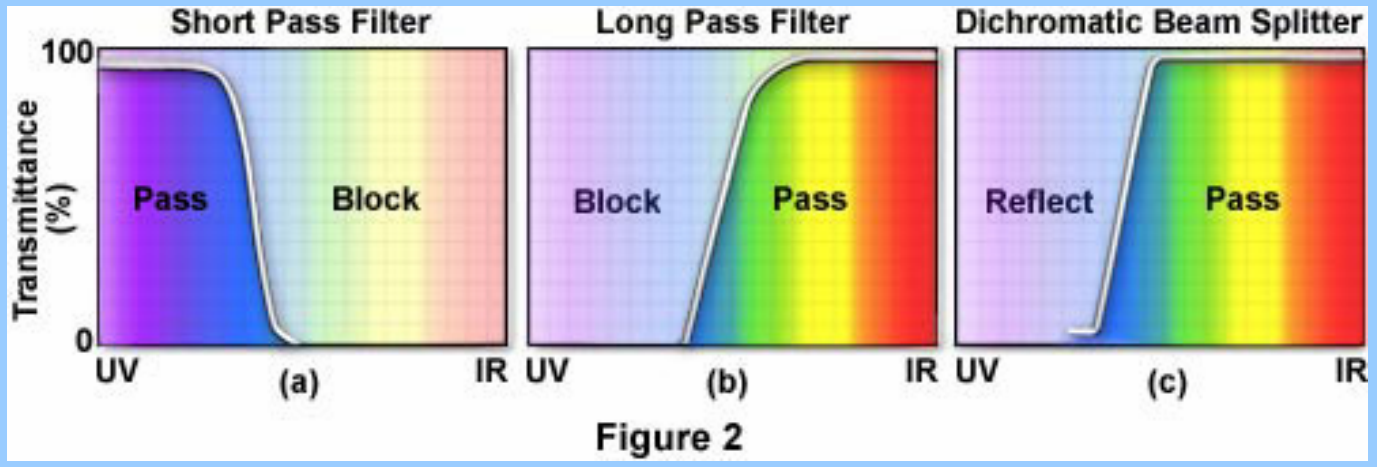


Figure 2

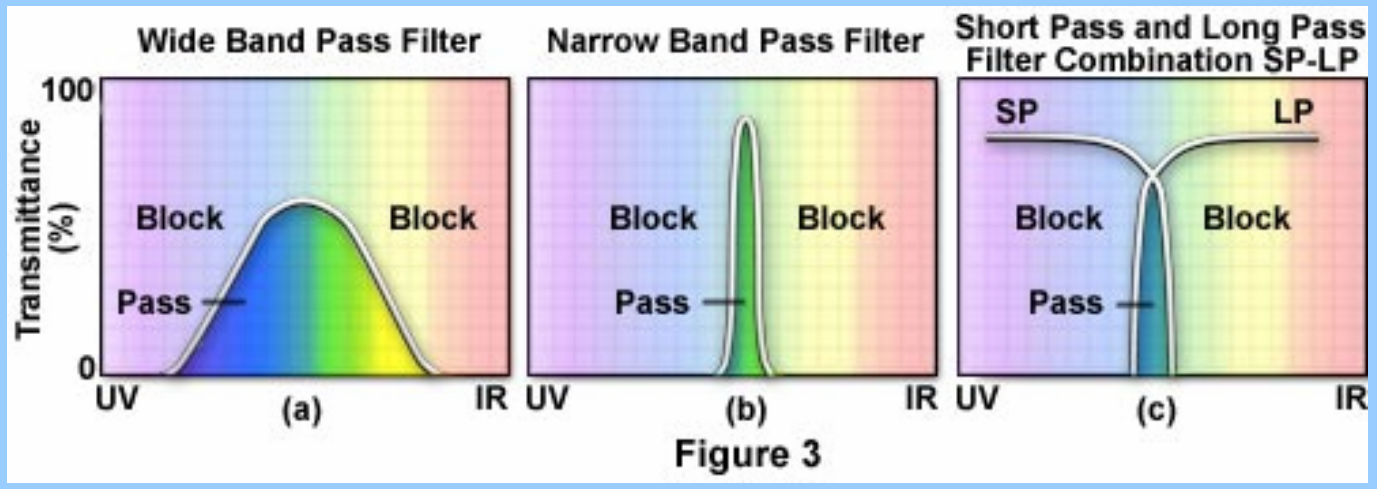


Figure 3

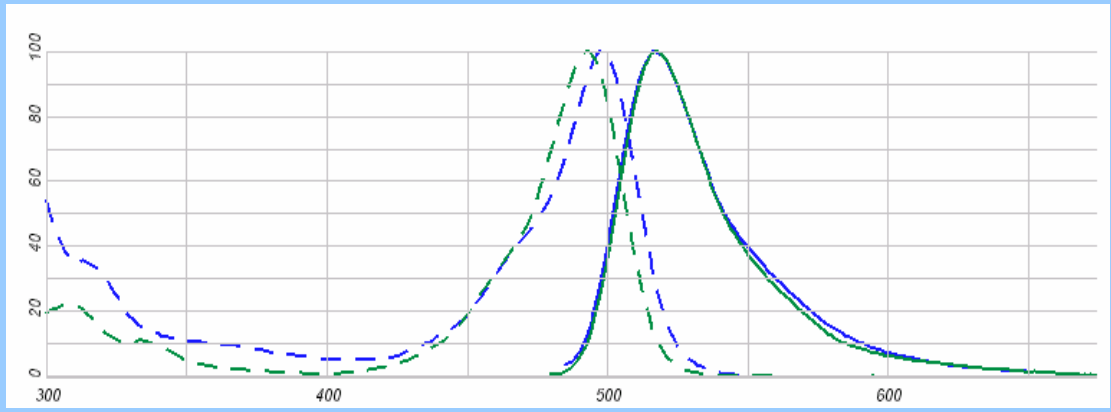
Images taken from:
www.molecularexpressions.com

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

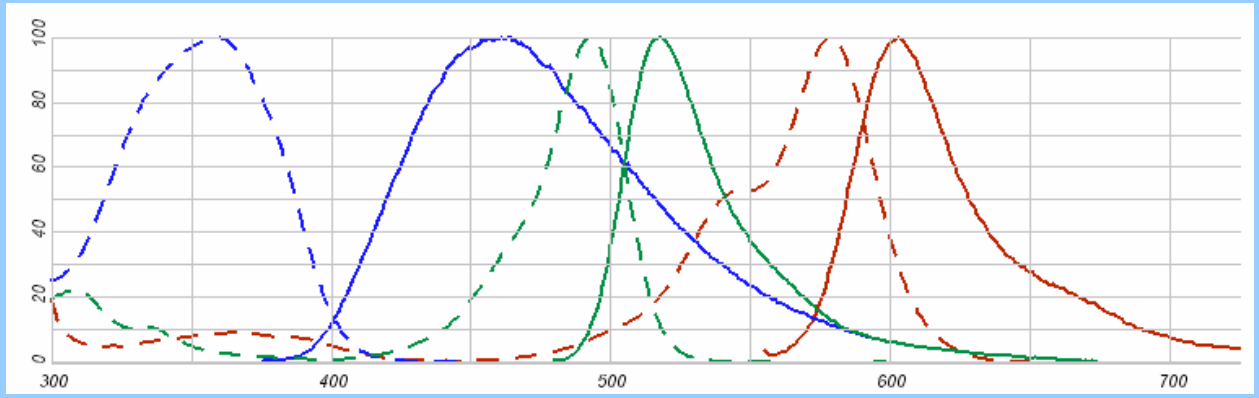
Spectral overlap

AlexaFluor488
FITC



Spectral separation

DAPI
AlexaFluor488
AlexaFluor568



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

Fluorescence Color "ladder"

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

UV **Blue** **Green** **Red** **Far red**

Excite ⟶ Emit

(DAPI like)

Excite ⟶ Emit

(FITC like)

Excite ⟶ Emit

(Rhodamine &TexRed like)

Excite ⟶ Emit

(CY5 like)

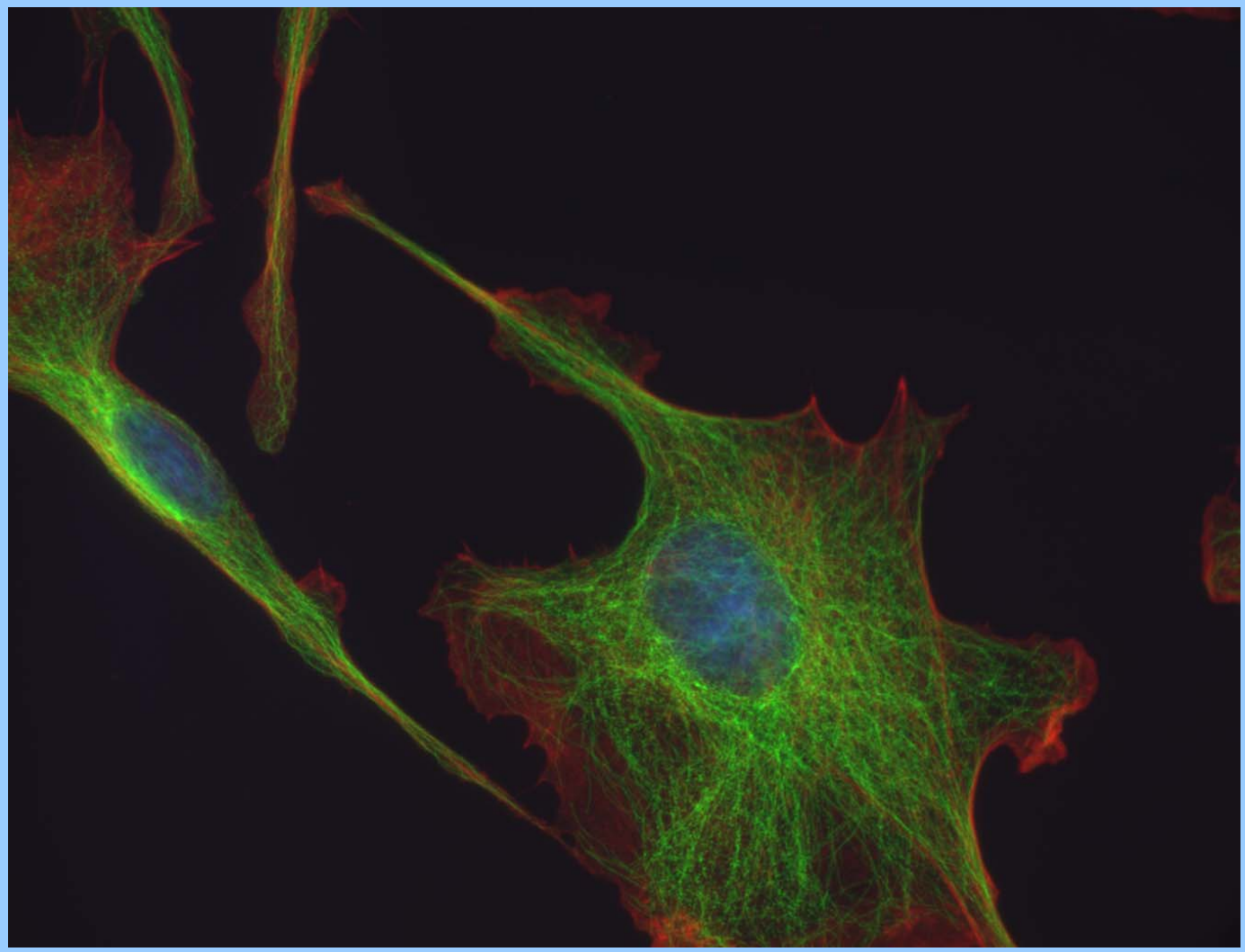
Table of Fluorochromes

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	

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



The Big Problem: Bleedthrough and Crosstalk

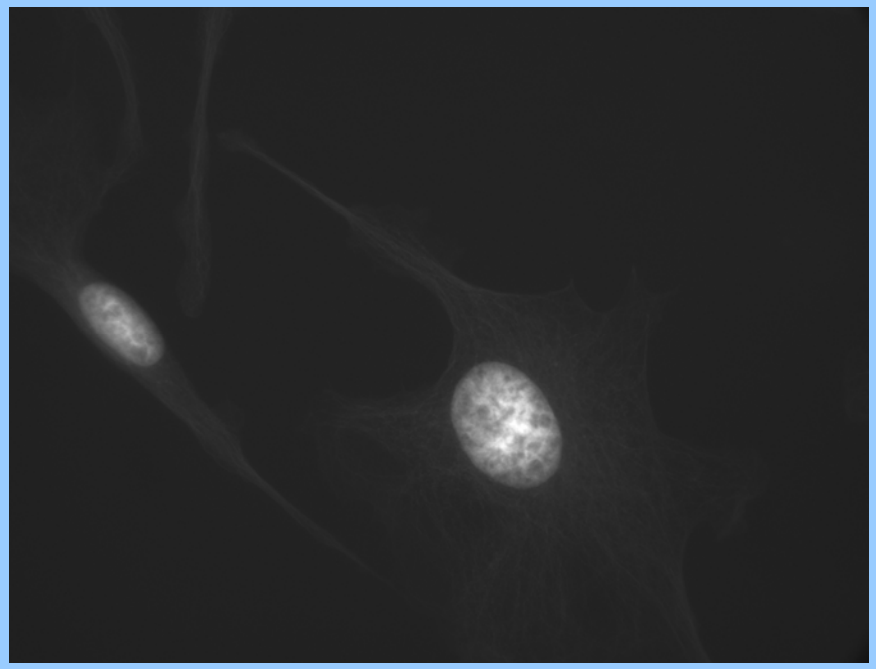
Spectral overlap increases as more structures are stained simultaneously (limited λ range, limited chemistry of fluorophores). This, in turn, increases the risk of detecting multiple fluorophores in the emission wavelength range for one fluorophore

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

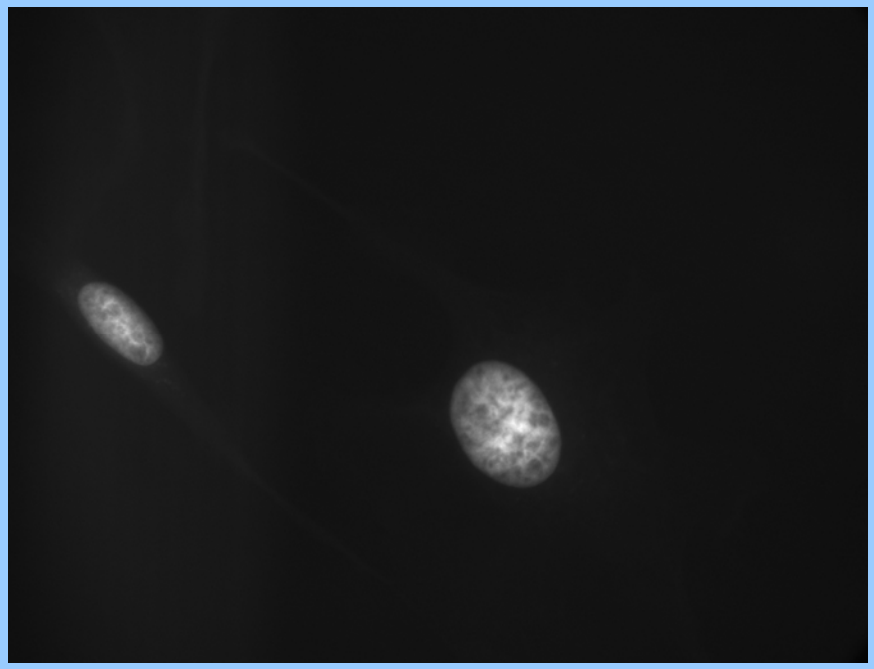
A few methods to overcome bleedthrough:

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

Bleedthrough



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

Other Potential Problems

- Auto-fluorescence
  - … of your sample (different tissue types, dead cells, collagen, NADPH, chlorophyll, lipofusion, etc.)
  - … of the sample holder (plastic, Phenol-red)
  - … fixation/staining protocol (e.g. glutaraldehyde)
  - Test for auto-fluorescence 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.
  - Newer fluorophores are less prone to photobleaching
  - 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

Ways to make your sample glow

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

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.

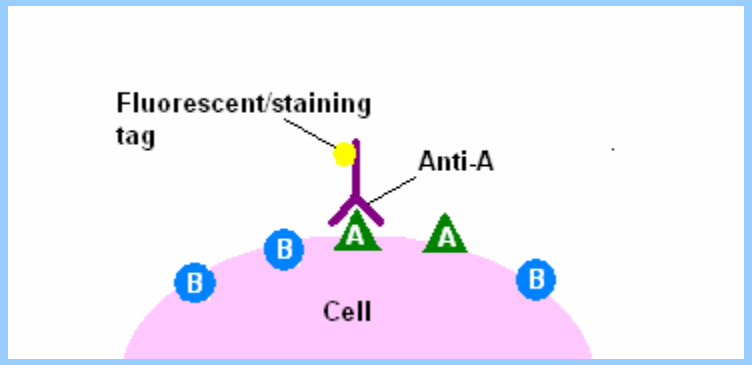
Every step in the protocol has caveats that can dramatically effect your sample structure or staining efficiency (or not). And there are often many steps.

The basic idea

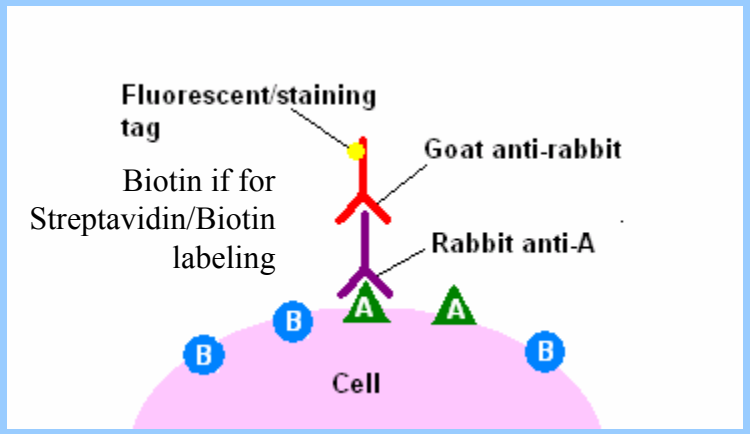
Specific detection of target molecules using antibodies (immunohistochemistry) or fluorescent dyes (chemicals that directly label a structure) to create contrast between the structure and the background

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 see which works best for all of the structures you want to visualize in your sample type

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 just 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
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

Careful of your antibody species

A few things to watch out for with antibodies:

- 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 anti-mouse 2º antibody on mouse tissue)

Mounting Media (MM)

and you thought this was the easy part

- Watch out for incompatabilities between a specific MM and label (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
- Preferable to do separate DAPI staining step than to use MM containing DAPI
- Hardening MM can cause structures change in thick samples

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, minimizing the number of variables for a test sample or two and try to pin-point where things may be going wrong.

It may be in the sample preparation OR it may be in the imaging system!

Run entire protocol without primary Ab(s)

Resources

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

http://www.molecularexpressions.com

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

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

Limitation of wide field