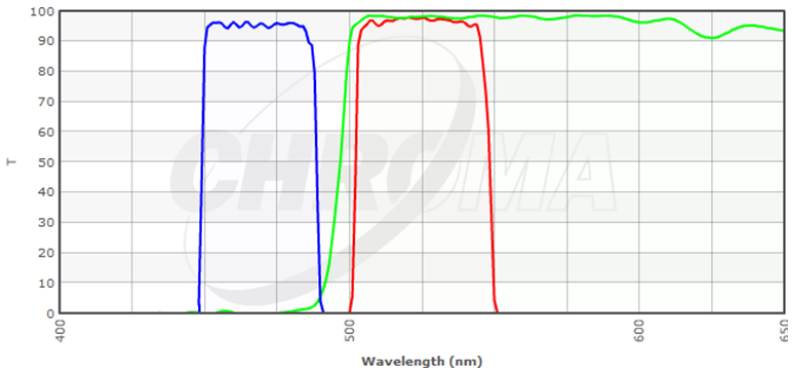
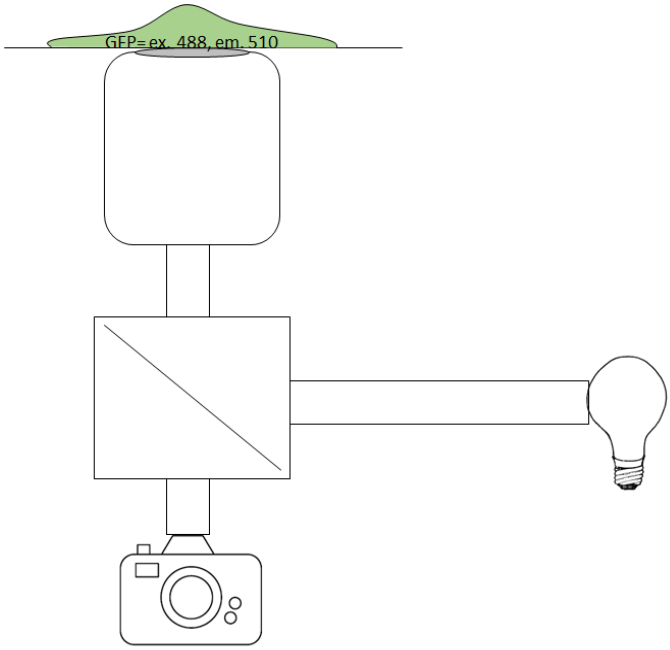
Biophysics 210
Discussion Section 5: Fluorescence Microscopy

1) Draw a standard infinity-conjugated brightfield setup as we discussed the first couple weeks, including camera; tube, objective, condenser, and collector lenses; sample; and light source. Where should the filter cubes for fluorescence microscopy be placed? Add one (containing an excitation filter, emission filter, and dichroic mirror) to your drawing and trace the light path for fluorescence imaging (hint: your light source will be located in a different place!). Which parts of your brightfield setup are now no longer in use?

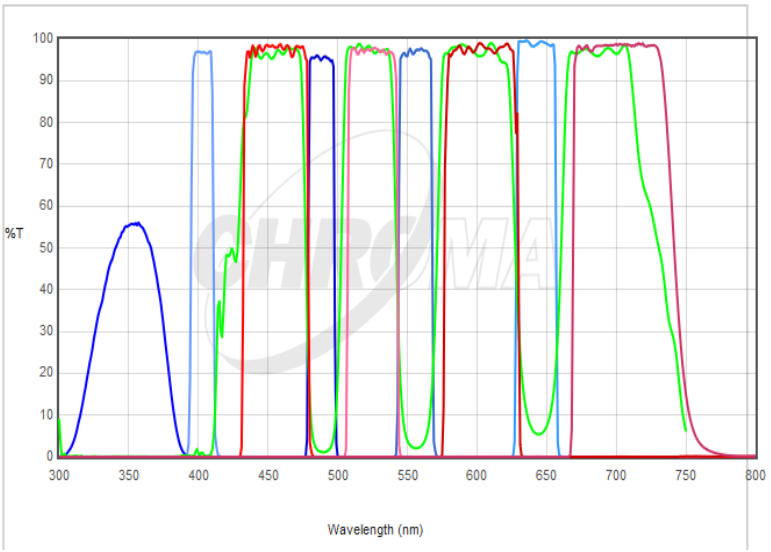
2) Below is the spectrum for a filter cube- label which lines depict the excitation filter, dichroic mirror, and emission filter.

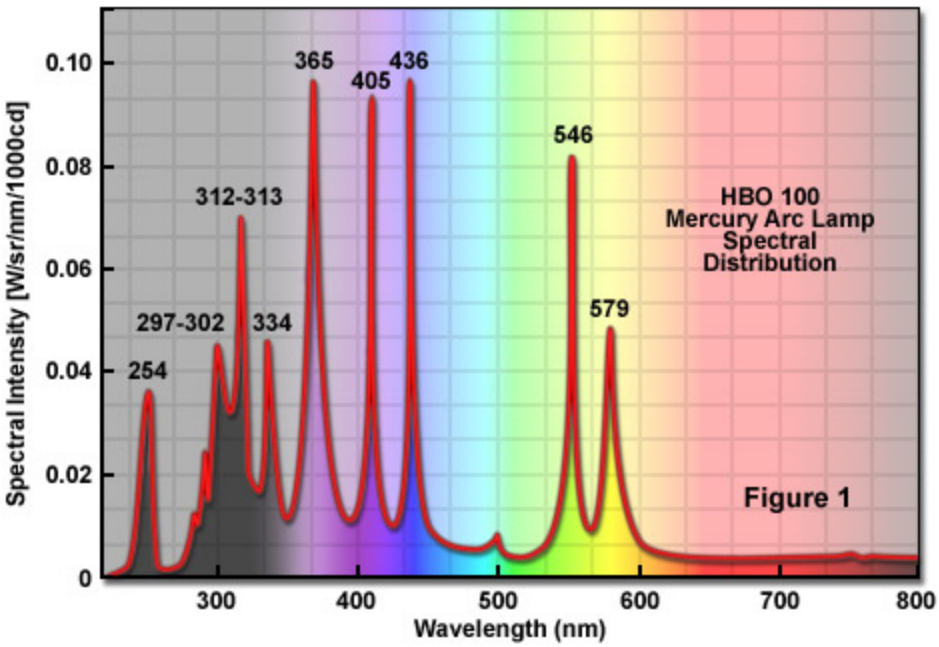


Given this spectrum, diagram on the setup below what would happen when you illuminate this cube with a white arc lamp (which we'll idealize as emitting 400nm, 450nm, 500nm, and 550nm light simultaneously). Draw in the position of the excitation and emission filters and what happens to each of those 4 starting beams after it leaves the lamp. If some of the light that hits the sample is reflected instead of being absorbed, what happens to it?



3) Your lab microscope is equipped with a mercury arc lamp and a set of separate emission and excitation wheels with a quad-bandpass dichroic (sometimes called a "Sedat quad"); the spectra are below.

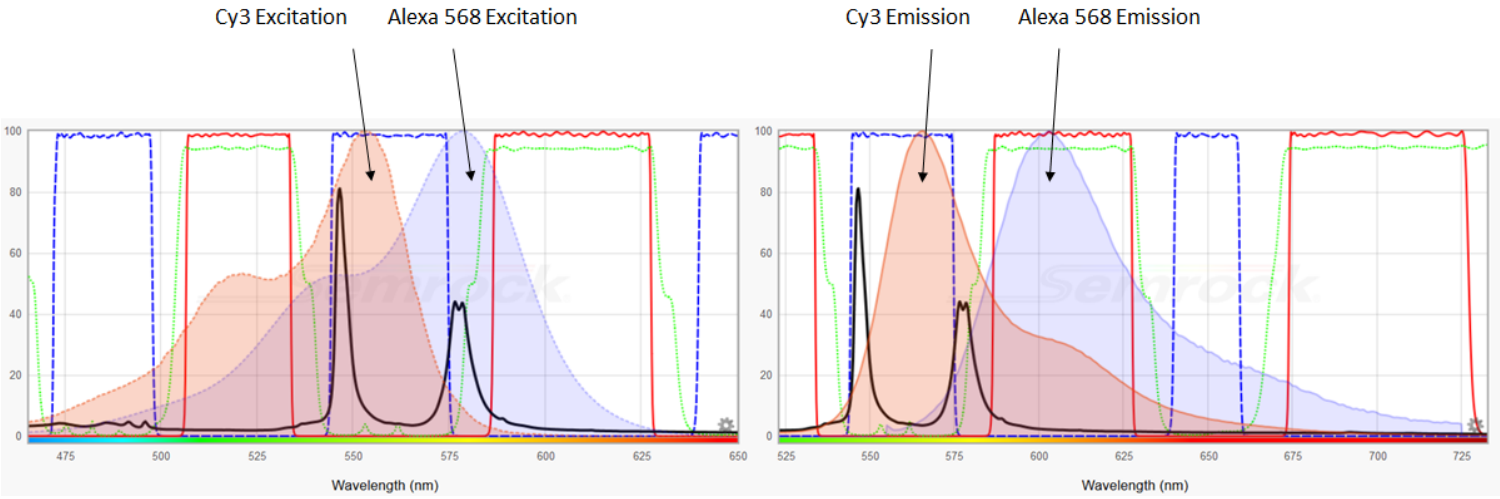




a. Below are the list of filters that make up this Sedat quad. What does each do? What do the first and second numbers refer to? What about the designations “x” and “m”? (You can ignore the D and ET designations for the moment).
D350/50x, ET402/15x, ET490/20x, ET555/25x, ET645/30x, ET455/50m, ET525/36m, ET605/52m, ET705/72m

b. Which of the bright lines in your arc lamp spectrum are going to be useful for microscopy based on this setup? Which are unusable?

c. If you had a very low-abundance protein you were attempting to label and image on this microscope, would you label it in the green, red, or far red channel? Why?
d. Consider the excitation and emission spectra of Alexa568 (blue shading) and Cy3 (orange shading) overlaid with your filters below (or at this link). Which would you choose to label a low-abundance epitope in your samples? Which would you choose if you had a high abundance epitope and were concerned about bleedthrough into your far red channel?



4) You are studying cell-cell interactions in a live animal (such as c. elegans, drosophila, or zebrafish) and need to select 3 different fluorescent proteins to label 3 kinds of cells (e.g. neurons, glia, and muscles).

a. You decide to use a confocal microscope, which has 4 lasers- 405, 488, 561, and 640 nm. Since you will be using live specimens, which channel will you exclude and why?
b. To get the best images you'll need to make sure to pick bright proteins so that you can achieve a reasonable signal to noise ratio. What two optical parameters do we use to determine brightness, and what does each one mean in a practical sense?
c. Select a trio of bright fluorescent proteins (one for each laser) from the Nikon center's list that will work well with the laser lines you chose.
d. Find and compare the emission spectra of the 3 proteins you chose on the Chroma spectra viewer , Semrock spectra viewer, or in the papers for this discussion. What should be the centers and widths of the emission filters to use to get maximal light from each protein with minimal crosstalk between the three? (You may certainly look up the properties of existing filters if you like, but it's not necessary; you can approximate what you'll need just from looking at the spectra).

5)You are studying Notch-Delta signaling during cell differentiation in live zebrafish, and need to fluorescently label 3 proteins (Delta, Centrin, and Mib) involved in this signaling for imaging on the same microscope.

a. In order to not perturb the structure, function, and interaction of these proteins with their partners, which parameter or parameters (in addition to those above) must you now consider when selecting which fluorescent proteins to fuse them to?
b. You may want to do time-lapse imaging of these fish. Which fluorescent protein property will be important for making this possible?
c. Choose a set of fluorescent proteins that will work well for this experiment.