BMB/Bi/Ch 173 2017
Problem Set 7.2: Fluorescence Microscopy

1. **(30 points) Fluorescence Imaging Techniques:** For each of the following, briefly describe how the technique works (distinguish principles), what information can be obtained with the technique and their limitations (if any):
   a. Deconvolution
   b. Confocal
   c. 2-photon
   d. TIRF
   e. FRET
   f. PALM

Technique	Key distinguishing principle	Limitations and Issues	Information
Deconvolution	Mathematical post-processing of images to improve resolution by estimating and correcting for the PSF.	Possible errors in estimation and correction.	Higher resolution spatial images.
Confocal	Confocal pinholes are used after the illumination source and before the detector to illuminate a point in focus and accept only emitted fluorescence from the in focus point.	Photobleaching from exposure of area outside of imaged point Signal is reduced from only accepting information at imaged point	Spatial information about a fluorescent object at an axial height.
2-photon	Excitation of a fluorophore with two photons of lower energy when they interact at the same time. The illumination source is focused in such a way as to excite flourophore at a spot in the focal plane.	More expensive than confocal	Same as confocal
TIRF	An evanescent wave propagating from 100 nm from a spot where illumination is totally reflected excites fluorophores in a narrow region near the surface of reflection.	The area that can be imaged is limited to the 100 nm area near the reflection surface.	High resolution spatial information near the surface of reflection.

FRET	A donor fluorophore is excited and transfers energy to a nearby acceptor fluorophore, stimulating emission.	Tagging molecules can be difficult. Spatial information can be limited.	Small distances (10-100 A) between fluorophores.
PALM	Fluorescent molecules are activated and photobleached in a distribution such that the center of fluorescence can be determined.	Resolution improved only in x-y plane.	High-resolution spatial information about fluorescent molecules beyond the diffraction limit.

2. **(30 points) Fluorescent Labeling Methods:** For your project, you decide to examine the localization of "your Favorite Protein" (yFavP).
   a. (10 points) You are unsure of whether to study yFavP using immunofluorescence or a genetic fusion. Explain one advantage and one disadvantage for each of these two techniques.

Immunofluorescence:
Advantages: you are examining the localization of the native protein, easy (requires no cloning and expression of fusion) Disadvantages: fixation and permeabilization required– prevents examination of living cells and is very damaging to the cell
Genetic fusion:
Advantages: can be used in living cells, no harsh conditions necessary so the cell is preserved
Disadvantages:fusions can cause mislocalization or loss of function for the protein

   b. (20 points) You decide you would like to do correlated light and electron microscopy to determine the localization of yFavP.

      i.(4 points) What additional information can electron microscopy provide as compared to fluorescence microscopy alone?

Electron microscopy puts the localization of the protein within the context of the cell as a whole. For example, you would be able to determine if the protein is found in a particular region or type of organelle.

      ii.(10 points) Just like for light microscopy, electron microscopy can be done using genetic fusions (chemical reactions) or immunocytochemistry (immuno-EM, using gold-conjugated antibodies). Explain one advantage and one disadvantage for using a chemical reaction via a genetic fusion versus gold-labeled antibodies for localization in correlated light and electron microscopy.

Chemical via genetic fusion:

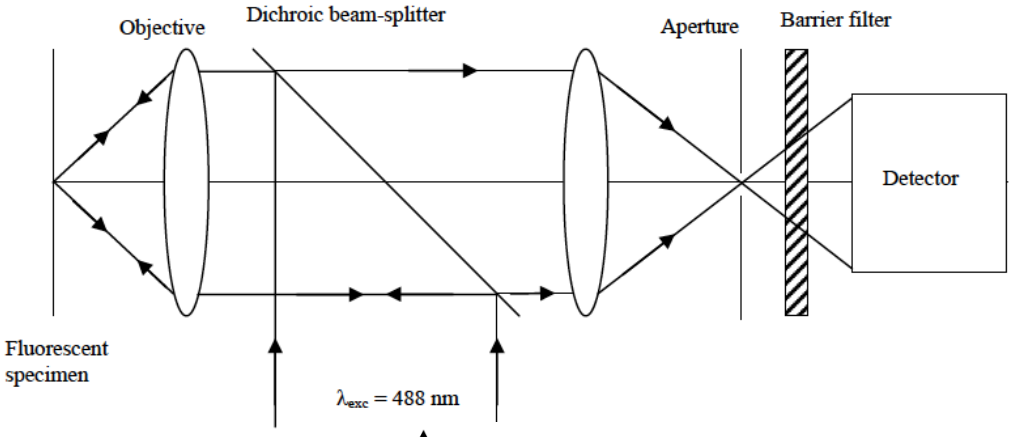
Advantages: no need for antibodies or expensive gold; the fluorescence microscopy can be done on live cells; no permeabilization step needed for either fluorescence or EM studies
Disadvantages: the DAB stain gives a large coating radius and may not provide very distinct spots for localization if you want a precise localization

Immuno-EM
Advantages: gold particles are distinct spots and provide very clear localization
Disadvantages: fixation and permeabilization needed for both light and electron microsopy, destroying ultrastructures

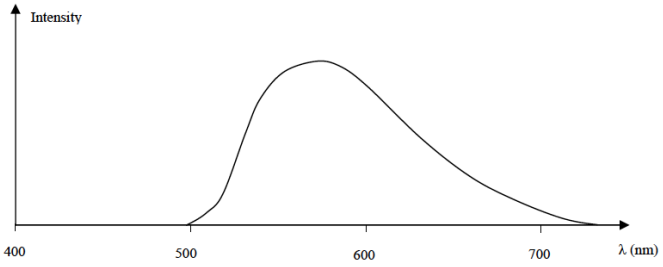
iii.(6 points) After considering your options, you decide you want to use a genetic fusion. Choose a genetic fusion for yFavP and explain how it can be used for both fluorescence and electron microscopy.

Answers may differ. Example: Add a tetracysteine motif on the N- or C-terminus of the protein, and label using FlAsH/ReAsH which are fluorescent when bound to protein. Additionally, these dyes catalyze the photoconversion of DAB into a precipitate that binds osmium. As osmium is visible in the electron microscope, FlAsH/ReAsH are also EM-compatible tags.

3. **(40 points) Microscope setup, fluorescent spectrums, and photobleaching:** Now you have your yFavP fused with a fluorescent protein:
   a. (10 points) You are asked to select a suitable dichroic beam-splitter and barrier filter for confocal fluorescence microscopy. A simplified schematic drawing of the set-up is shown in the figure below.

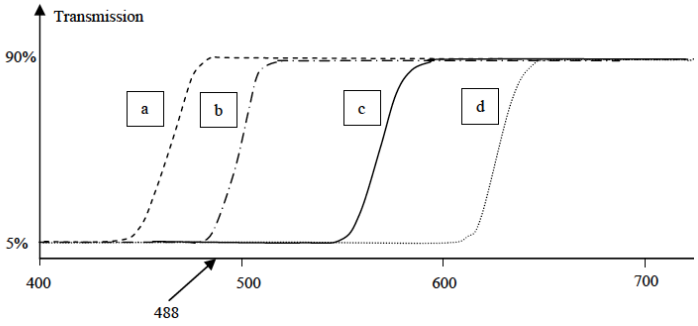


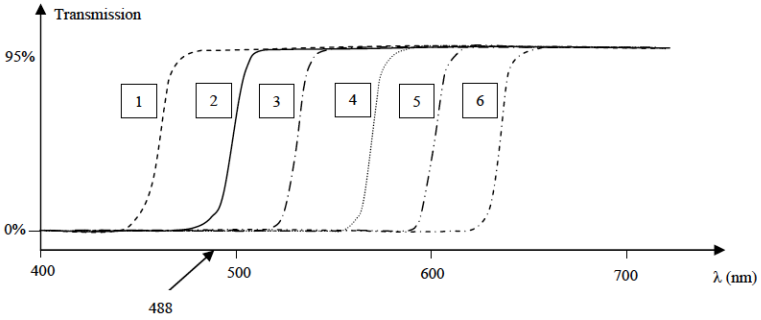
Your fluorescent protein has its emission spectrum shown here:



You have a choice of 4 different dichroic beam-splitters (a-d), and a selection

of different barrier filters (1-6), whose transmission curves are given in the figure below. Since the light intensity is very low, it is important that as many fluorescence photons as possible can reach the detector. However, it is also important that absolutely no laser light (which is many orders of magnitude stronger than the fluorescent light!) can reach the detector. Select a suitable dichroic beam-splitter (a-d) and barrier filter (1-6) to give the best results under the circumstances. Explain how you make your choice.





A suitable choice would be beam-splitter b and barrier filter 3. In this way most of the laser light will be reflected towards the specimen, and the fluorescent light will be efficiently transmitted by the beam-splitter (a would reflect very little laser light towards the specimen, whereas c and d would mean that we waste fluorescent light). Barrier filter 3 is the best choice, given the requirement that we must block all laser light (note that all beam-splitters transmit a few percent of the laser light reflected back from the specimen) and transmit as much as possible of the fluorescent light.

b. (14 points) Your cells are around 8um thick, and you have been imaging them on a spinning disc confocal at 0.1um steps in z for optical sectioning, therefore 80 optical sections to image through your sample. You have noticed that your fluorescent proteins appear to be dimmer in later z sections due to photobleaching. You realized due to the diffraction limitation, there is no point to do these fine steps. What is the resolution due to diffraction limit? (Your objective has a NA=1.4)

x,y resolution=0.6*wavelength of emission/NA
since it wasn't clarified in the lecture to use emission wavelength, no points deducted for using excitation wavelength

The axial (z) resolution has typically only half the resolution of the pixel in the x- and y-dimension. Factors affecting axial resolution are the objective numerical aperture (NA) and pinhole diameter. Increasing the NA and/or decreasing the diameter of the pinhole will increase the z-resolution. In any case, the elucidation of sample depth information is always less than the x- and y-resolution due to the blurring effect of the PSF. Given the equation below, what is a proper z step for your confocal imaging experiment?

$$dz \cong \frac{0.64 \cdot \lambda_{exc}}{n - \sqrt{n^2 - NA^2}}$$

This equation represents an ideal situation with infinite small pinhole
n is the refractive index of the immersion medium, typically n = 1.518

Z step larger than 335nm

Practically, why don't you want infinite small or very small pinholes?

The intensity of excitation light would be too small, resulting in the need of prolonged exposure and imaging time would be very long (since you are doing z-stack acquiring, imaging time is a very practical concern).

c. (6 points)Name another technique you could use instead of confocal microscopy and explain how it overcomes the issue of photobleaching.
You could use 2-photon microscopy, which relies on two photons of lower energy being absorbed to excite a fluorophore. The sample is scanned, and since two photons are required, the sample is able to fluoresce only at the focal point. This reduces photobleaching outside of focal point and phototoxicity compared to confocal. You could also use light sheet, which does not illuminate flurophores outside of the focal depth and therefore also decreases photobleaching/toxicity.

d. You want to determine the organelle in which yFavP is found using fluorescence microscopy.
i. (10 points) Dyes and marker proteins can be used to fluorescently label organelles or other cellular structures. To which cellular structures are each of the following specific?
- DAPI – Nucleus (DNA)
- Rab5 – early endosome
- RCAS1 – Golgi
- Lamin B1 – nuclear envelope
- LAMP1 – lysosome
- Calnexin – ER
- COX IV – mitochondria
- PMP70 – peroxisome
- MitoTracker Red – mitochondria
- Fibrillarin – nucleolus

ii. (8 points) What is fluorescent crosstalk/bleed-through, and how does it affect your choice of fluorophores in multicolor imaging?
Bleed-though/crosstalk is when your two fluorophores are both excited at the same time. This happens when the emission/excitation spectra of the two fluorophores are very close, as is the case for GFP and YFP shown below. Two

negatives can happen when crosstalk/bleed-through occurs. First, there can be significant photobleaching if your second fluorophore is also excited while imaging your first fluorophore, causing a decrease in image quality for the second fluorophore. Second, if the emission of both fluorophores is captured at the same time while imaging, artefacts will occur. Therefore, you must choose fluorophores with non-overlapping excitation/emission spectra when doing multicolor imaging.