BMB/Bi/Ch 173 – Winter 2017

Problem Set 7.2: Fluorescence Microscopy– Assigned 2-23-17. Due 2-28-17 by 10:30am

TA: Wen Zhou (201 Kerckoff, office hour: Fri 2/24 5-6pm, Mon 2/27 5-6pm or by appointment

110 points total

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

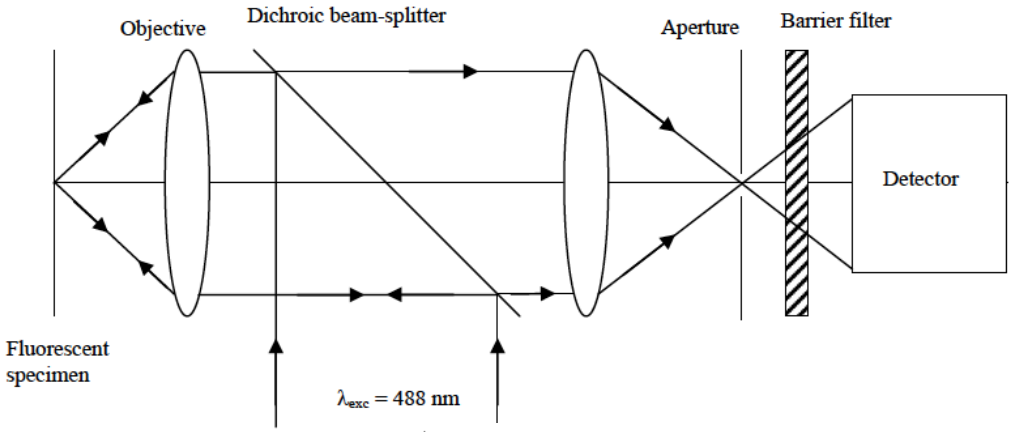
2. **(30 points) Fluorescent Labeling Methods:** For your project, you decide to examine the localization of "your Favorite Protein" (yFavP).
   a. (10 points) You are unsure of whether to study yFavP using immunofluorescence or a genetic fusion. Explain one advantage and one disadvantage for each of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

   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?

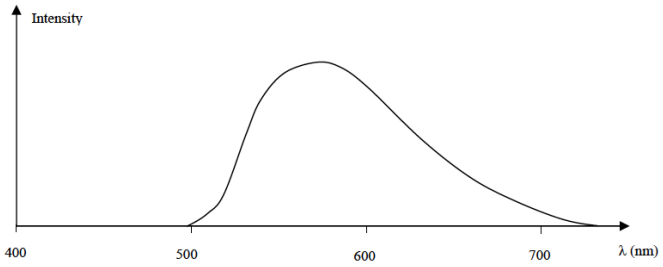
      ii.(10 points) Just like for light microscopy, electron microscopy can be done using genetic fusions (chemical reactions) or immunocytochemistry (immuno-EM, using gold-conjugated antibodies). Explain one advantage and one disadvantage for using a chemical reaction via a genetic fusion versus gold-labeled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

      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.

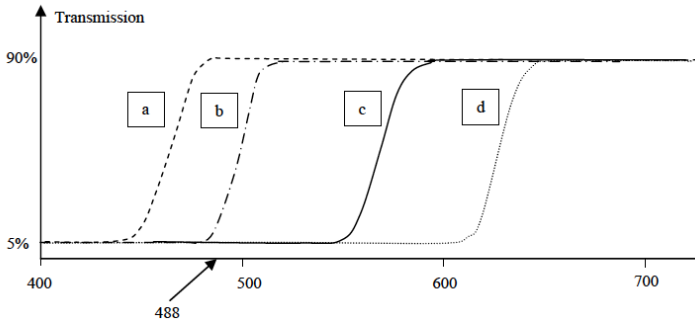
3. **(50 points) Microscope setup, fluorescent spectrums, and photobleaching:** Now you have your yFavP fused with a fluorescent protein:
   a. (10 points) You are asked to select a suitable dichroic beam-splitter and barrier filter for confocal fluorescence microscopy. A simplified schematic drawing of the set-up is shown in the figure below.

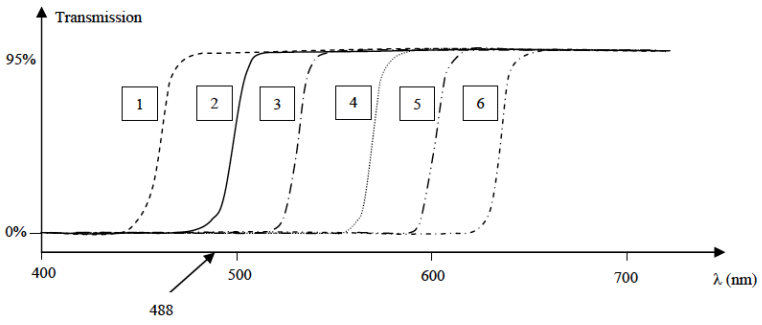


Your fluorescent protein has its emission spectrum shown here:



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.





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)

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

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

c. (6 points)Name another technique you could use instead of confocal microscopy and explain how it overcomes the issue of photobleaching.

d. (20 points) 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
- Rab5
- RCAS1
- Lamin B1
- LAMP1
- Calnexin
- COX IV
- PMP70
- MitoTracker Red
- Fibrillarin

ii. (10 points) What is fluorescent crosstalk/bleed-through, and how does it affect your choice of fluorophores in multicolor imaging?