BMB/Bi/Ch 173 – Winter 2018 - Homework Set 7.2

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Problem 5 (30 points) Fluorescent Labeling Methods

For your thesis, you decide to examine the localization of "your Favorite Protein" (yFavP).

5.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),*
- *could use small molecule dyes with better fluorescent properties than fluorescent proteins,*
- *antibodies can target specific states (like phosphorylation)*

Disadvantages:

- *fixation and permeabilization required – prevents examination of living cells and is very damaging to the cell,*
- *antibody binding is not 100% efficient,*
- *off-target binding,*
- *artifacts can be introduced by fixation process,*
- *good antibodies are required and can be time-consuming to develop*

Genetic fusion:

Advantages:

- *can be used in living cells,*
- *no harsh conditions necessary so the cell is preserved*

Disadvantages:

- *fusions can cause mislocalization, aggregation, or loss of function for the protein*

5.b. (5 points) You decide you would like to do correlated light and electron microscopy to determine the localization of yFavP. What additional information can electron microscopy provide as compared to fluorescence microscopy alone?

Live cell fluorescence allows one to observe dynamic processes. However, fluorescence experiments only give you information about what you've illuminated with the fluorophores unless you correlate it with the phase or DIC image.

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 without also having to tag that organelle with a fluorescent probe.

5.c. (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*

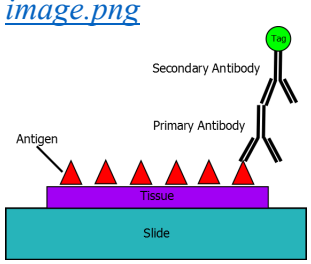
Disadvantages:

- *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*
- *Many choices of antibodies (as opposed to few choices for genetic fusion)*

Disadvantages:

- *Fixation and permeabilization are needed for both light and electron microsopy, destroying ultrastructures*
- *There is a relatively large distance between the gold bead and the target, so localization can be difficult to interpret in micrographs if the target of interest is in a crowded location.*
    - *https://upload.wikimedia.org/wikipedia/commons/6/67/Immunolabeling_process_image.png*
    - 

5.d. (5 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 vary.

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.

Example: MiniSOG (mini Singlet oxygen generator)
http://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.1001041

Example: APEX fingerprinting https://www.sciencedirect.com/science/article/pii/S2211124716305083

Problem 6 (20 points) – Microscopy Techniques and Troubleshooting:

Having chosen a labeling method, you begin to examine the localization of yFavP using confocal microscopy.

6.a. (10 points) You are using a fluorophore that photobleaches quite easily, and you find it hard to get quality images over time. 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.

6.b. (10 points) Your images suggest that yFavP is forming interesting structures in the cell, but these structures are too blurry in your images for you to come to any conclusions. Explain how deconvolution could be used to improve the images you have taken.

Deconvolution convolves the image with the inverse point spread function to remove some blurring and produce a sharper image. The PSF of the microscope must be known.

Problem 7 (30 points) –Subcellular Localization:

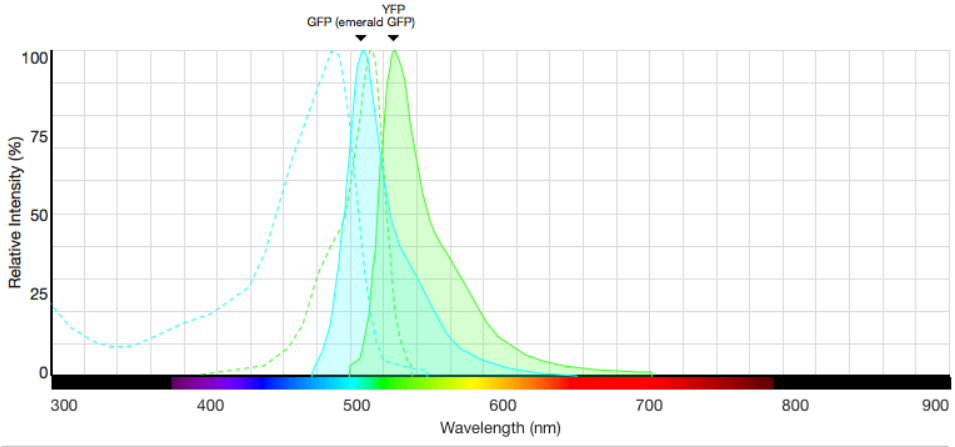
You want to determine the organelle in which yFavP is found using fluorescence microscopy.

7.a. (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?

i. DAPI – Nucleus (DNA)
ii. Rab5 – early endosome
iii. RCAS1 – Golgi
iv. Lamin B1 – nuclear envelope
v. LAMP1 – lysosome
vi. Calnexin – ER
vii. COX IV – mitochondria
viii. PMP70 – peroxisome
ix. MitoTracker Red – mitochondria
x. Fibrillarin – nucleolus

7.b. (10 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, artifacts will occur. Therefore, you must choose fluorophores with non-overlapping excitation/emission spectra when doing multicolor imaging



7.c. (10 points) Based on your initial localization assays, you suspect yFavP interacts with a protein on the surface of the inner surface of the cell. Explain how intermolecular FRET could be used to study this investigation. How close do the fluorophores need to be for a successful FRET interaction? (suggested source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5038762/)

Fluorescence resonance energy transfer (FRET) happens when a donor fluorophore gets excited and its emission excites an acceptor fluorophore to also emit. FRET is sensitive to local interactions, on the 1 to 10 nm distance scale.

Problem 8 (20 points) –Fluorescence Imaging on Cell Surfaces

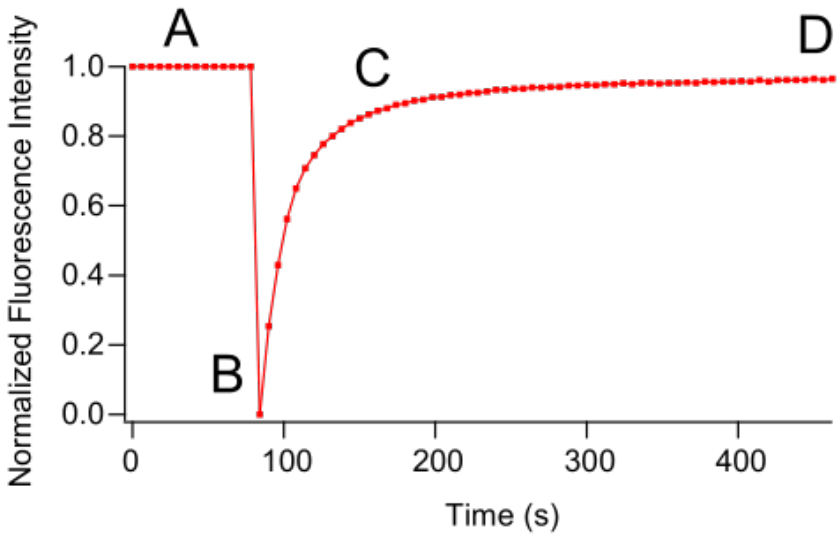
Your investigation of yFavP led you to another protein, "your second Favorite Protein" (yFavP2), which is found on the cell surface.

8.a. (10 points) Your labmate suggests using TIRF microscopy to study yFavP2. Describe how TIRF works and why it is well suited to your studies of yFavP2. What are the advantages of TIRF over other methods?

In TIRF microscopy, the sample is illuminated from below. The incoming light is shone at the sample past its critical angle, which causes total internal reflection (all light is reflected rather than passing through the medium). Under the conditions of total internal reflection, an evanescent field propagates ~100 nm into the sample, allowing for the excitation of fluorophores near the boundary. TIRF microscopy is well suited to the study of yFavP2 because it is a cell-surface protein, meaning it will be found on the boundary that is accessible via TIRF. An advantage to TIRF is that it illuminates a small sample area on the boundary, reducing background and phototoxicity.

8.b. (10 points) You are particularly interested in the mobility of yFavP2 on the cell surface, so you choose to carry out a fluorescence recovery after photobleaching (FRAP) experiment. Explain how FRAP works, and draw an example trace of fluorescence over time during a FRAP experiment.

In FRAP, a section of the cell membrane is exposed to an intense light so as to photobleach the fluorophores in that section. When the light is turned off, 2D diffusion within the membrane allows for unbleached fluorescent molecules to move into that section, eventually recovering to the same level of fluorescence as the rest of the membrane. The mobility of the fluorescent molecule is related to how quickly the fluorescence is recovered.



A: before bleaching
B: immediately after bleaching
C: 2D diffusion is restoring intensity
D: uniform intensity is restored

(from https://upload.wikimedia.org/wikipedia/commons/thumb/a/a7/Frap_diagram.svg/604px-Frap_diagram.svg.png)