Biophysics 210: Biological Light Microscopy
Discussion Section 5: Confocal, Two-photon, and TIRF microscopy
Tuesdays 1-2:30pm
Location: Genentech Hall Rm N114

Helpful Equations

Airy disk diameter in object space = 1.22 λ/NA

Resolution of the objective = 0.61λ/NA

1. What is the role of the pinhole in confocal microscopy? What is the optimal size for a pinhole if you are using a Plan Apo 100x/1.4 NA objective? What is the optimal size for a pinhole if you are using a Plan Apo 40x/0.95 NA objective? What happens when the size of the pinhole is increased? What happens when the size of the pinhole is decreased?

2. When using a laser scanning confocal you have control over many parameters that directly impact the quality of the image you will achieve. One of these parameters is how many points you sample in the scan field which determines the number of pixels your final image is composed of. How many pixels will you need in your image to reach Nyquist sampling for a 100 um x 100 um area using a Plan Apo 100x/1.4 NA objective? Can you have too many pixels when you scan?

3. You have a sample where you have labeled a low abundant protein and the signal is very weak. When you check your staining on a widefield microscope you confirm that your experiment worked and then take the sample to image on a confocal microscope. On the laser scanning confocal the signal seems weaker than you thought from what it looked like on the other microscope. Why is this? What are the ways that you can increase the signal on the microscope for laser scanning confocal and for spinning disk confocal?

4. Why is spinning disk confocal a good choice for live cell imaging? What advantage would a laser scanning confocal have over the spinning disk? What limits the temporal resolution for your experiment when using a spinning disk confocal?

5. What are the similarities and differences between laser scanning confocal and 2-photon microscopy? Why are you able to image deeper into tissue when using a 2-photon microscope versus a standard laser scanning confocal?

6. In TIRF microscopy what is the critical angle and what are the sample requirements in order to reach it? How can you determine if you have reached the critical angle and are in TIRF? What happens if the incident angle of the laser is greater than the critical angle? What happens if the incident angle of the laser is less than the critical angle?

7. In many cases you have to determine which technique is most appropriate to use for your experiment. Below a few examples are listed. Discuss the pros and cons for each of the techniques you learned about this week and determine which technique would be the best to use.

    Case 1: Your experiment requires you to determine what cell types have been infected after a virus expressing eGFP is injected in a specific brain region. You have stained a 30 micron thick brain section for the GFP expressed by the virus and 2 cell specific markers.

    Case 2: Your experiment requires you to follow your protein of interest fused to GFP to determine how the protein is trafficked through the cell after a treatment. You need to image the cells for at least 30 minutes to follow the entire process.

    Case 3: Your experiment requires you to determine if your gene of interest plays a role in endocytosis. You have tissue culture cells expressing your protein of interest fused to GFP and clathrin-mRuby2.

    Case 4: Your experiment requires you to follow the dendritic spine dynamics of a subset of neurons labeled with GFP in a 400 micron thick live brain slice under different stimulus patterns over time. You need to make sure to only image neurons at the center of the slice to maintain as many of the neuronal processes as possible.

    Case 5: You want to monitor the recovery of patients with hypertrophic scars, which are raised, dense collagen scars that are an abnormal response to wound healing. To do this you decide to compare the structure of isotropic collagen I and IV fibers, and non-isotropic elastin fibers in hypertrophic scar tissues with those of normal skin in ex-vivo skin samples. Skin tissue is known to strongly scatter light due to melanin and hemoglobin. What method(s) would be best to image? Do you need more than one?