Biophysics 210: Biological Light Microscopy
Discussion Section 2: Microscope Objectives, Numerical Aperture, and Resolution

In this discussion section, we will examine the resolution achievable by different microscope systems. The aim is to gain a deeper understanding of the factors that control the achievable resolution in the microscope.

Some useful equations:

- Snell's law (refraction): $n_1 \sin \theta_1 = n_2 \sin \theta_2$
- Rayleigh resolution limit (x-y): $d_{min} = 0.61 \lambda/NA$
- Axial resolution limit $z_{min} = 2 \lambda n/NA^2$
- Where λ is the wavelength of light, NA is the objective numerical aperture, and n is the refractive index between the sample and the objective

1. You have a microscope with the following objectives: 10x / 0.45 NA , 20x / 0.75 NA, 40x / 1.3 NA, and 100x / 1.4 NA. For each objective, calculate the resolution in both the x-y and z directions. What is the likely immersion medium for each lens?

2. Below are a few examples of experiments where imaging is used. Discuss which objectives (magnification and NA) would be able to distinguish the features described and which one would be the best choice.
   Example 1: You are examining yeast cells and want to determine if your protein of interest is located in the mitochondria. Mitochondria are normally 4 x 0.8 microns in size.
   Example 2: You are examining HEK293 cells and want to measure the percentage of your protein in interest that is located in the nucleus of the cell. The cells are approximately 13 microns across and 5 microns thick and the nucleus is approximately 8 microns across.
   Example 3: You are examining stem cells and want to count the number of cells that are induced to express a cell-specific marker with various treatments. The cells are between 30-40 microns in diameter.
   Example 4: You have stained a tissue section to identify tumor cells and are trying to measure how large of an area the tumor is covering. The section you are working with is approximately 2 x 3 mm and the cells are 20 microns in diameters.

3. What problems are you likely to encounter when using an 100x Achromat objective to image a 4-color stained tissue section (stained with DAPI, FITC, Cy3, and Cy5)? What kind of lens could you use to circumvent this problem?

4. **a.** Sketch the ray diagram for an infinity corrected oil-immersion lens imaging a sample mounted in an index-matched mounting medium (i.e. the refractive index of the top lens of

the objective, the oil, coverslip, and sample are all the same). Include all of these elements in your drawing.

b. Repeat the same sketch for an air objective, where now there is air between the top lens of the objective and the coverslip. What happens to the light rays as they exit the coverslip? Why is the resolution of this lens lower than that of the oil-immersion objective?

5. **a.** Sketch the ray diagram for an oil immersion lens (1.4 NA) imaging a mammalian cell in culture media. Assume that refractive index of the objective top lens, oil, and coverslip is 1.515 and that the refractive index of the culture medium is 1.38. Assume that you're imaging a point inside the cell, several microns above the coverslip. Pay careful attention to how the rays propagate as they move from one refractive index to the next.
   b. Based on your result from part a, would there be any advantage in replacing the 1.4 NA objective lens with a 1.49 NA objective lens? Please explain.

6. Olympus makes a 1.65 NA objective lens for TIRF microscopy. It uses sapphire coverslips (n = 1.76) and diiodomethane (n = 1.74) for the immersion oil. We'll discuss TIRF microscopy in a later session, but based on what you have learned so far, would this lens have much utility for imaging live cells not in TIRF?

7. A paper by Siyushev et al. (Applied Physics Letters **97**, 241902 (2010)) discusses imaging nitrogen-vacancy centers in diamond. These are defects in the diamond crystal lattice that fluoresce with very narrow linewidths and essentially no photobleaching. However, diamond has a very high refractive index (n=2.4) that complicates imaging it. In this paper they use an air lens with a numerical aperture of 0.85 to image these nitrogen vacancy centers. They also machine the diamond sample they are imaging into a hemisphere. Why? Hint: sketch a ray diagram of this lens imaging into a planar sheet of diamond or into the curved surface of the hemisphere.