

BMB/Bi/Ch 173 – Winter 2017

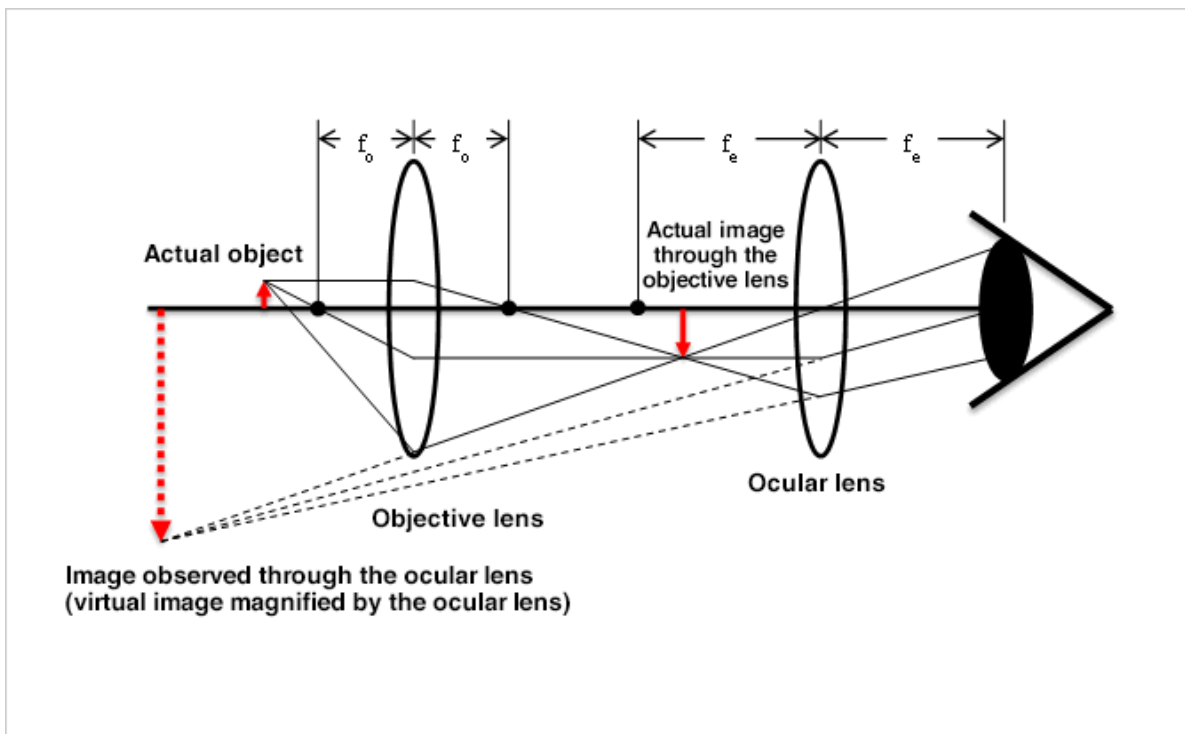
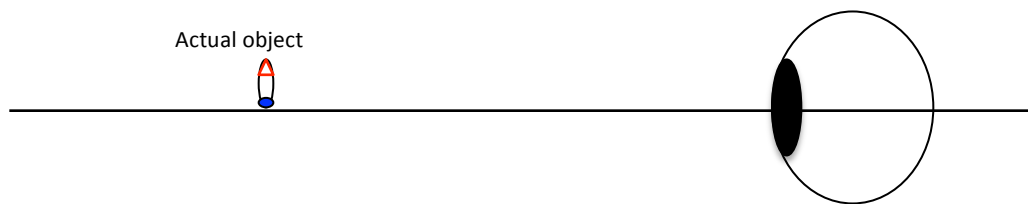
Problem Set 7.1: Light Microscopy Basics – Assigned 2-21-17. Due 2-28-17 by 10:30am

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

90 points total

1. (34 points) Microscopy and Lens Basics

- a. (10 points) You have a cell with two stains at the two poles. Draw the components of a compound microscope between the object and the eyeball, and the ray diagram. Be sure to label all the lenses, and their corresponding focal points. Draw the real image projected through objective lens and on retina, and the virtual image observed through the eyepiece. Be sure to label the locations of the dyes.



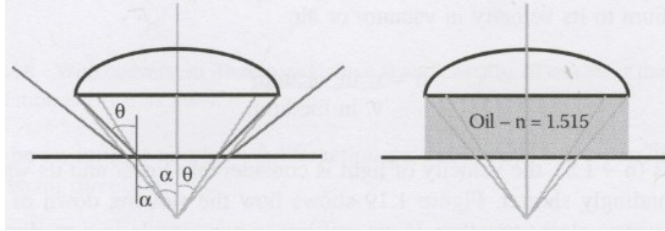
- b. (6 points) What are the fundamental resolution limits for wide-field optical light microscopy?

The fundamental limitations for traditional wide-field optical light microscopy are the wavelength of light used and the numerical aperture of the lens.

- c. (10 points) How does oil immersion increase the resolving power of a lens? Please draw a simple diagram to illustrate your answer.

The first reason is the refractive index of oil (~1.5) is higher than air (~1), which increases the numerical aperture. Since the resolution is dependent on the numerical aperture (resolution =  $0.6\lambda / NA$ ), there is an increase in resolution.

The path of light from an aqueous biological sample through the oil to the glass of the lens passes through very similar refractive indices so more of the information is collected. Any diagram showing this gets credit.



- d. (8 points) You are using a lens without oil immersion (index of refraction in air  $n = 1$ , half-angle  $\theta = 48^\circ$ ). What is the numerical aperture of the lens? If you are using light with a wavelength of 532 nm, what is the resolution of the lens?

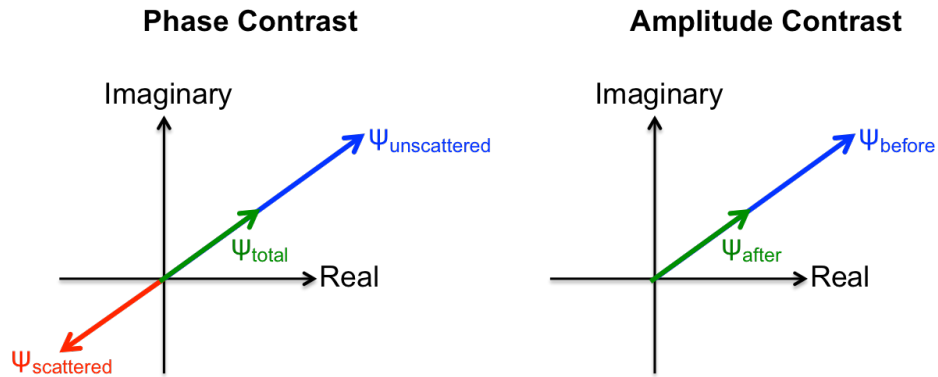
$$NA = n \sin \theta = 1 * \sin(48) = 0.743$$

$$\text{Resolution} = 0.6\lambda / NA = 0.6 * (532 \text{ nm}) / NA = 437 \text{ nm}$$

**2. (26 points) Contrast in Light Microscopy**

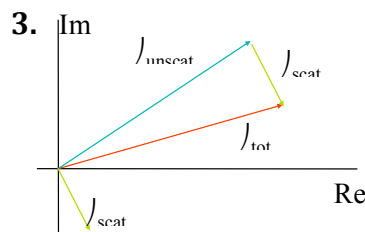
- a. (10 points) What is the difference between amplitude and phase contrast? Please draw Argand diagrams to illustrate.

Amplitude contrast is formed by absorption of light and scattered light not collected by optical system. This leads to a direct subtraction from the intensity of the recorded wave. Phase contrast is formed by a phase shift in the scattered wave interfering with the unscattered wave. The interference leads to a reduction in the intensity of the recorded wave.



- b. (8 points) How is contrast of cells created in bright field microscopy? Why may this be inadequate for imaging? (you can use Argand diagram in your answer)

First, brightfield microscopy transmits amplitude contrast; however, if the sample is very transparent then this contrast is inadequate. Additionally, brightfield microscopy does introduce *some* phase contrast, with scattered light emerging from the sample with a  $90^\circ$  phase delay with respect to unscattered radiation. However, this scattered light has little effect on the amplitude of the total wave, making the contrast from phases very minimal.



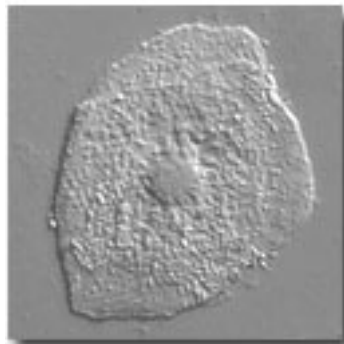
- c. (8 points) How can you increase the amplitude contrast? Give at least 2 examples and briefly describe their limitations.

Stains (eosin, hematoxylin) can help increase the amplitude contrast. In order to use stains, the samples must be fixed, embedded, and sectioned. Therefore, stains such as H&E cannot be used to image living cells/tissues. Enzymatic amplification (peroxidase, alkaline phosphatase, gold/silver enhancement). However, these often a) requires antibody being available, and b) in order to visualize intracellular compartments, the cells still need to be killed and permeabilized.

- d. (30 points) Below shown are 3 different biological samples imaged with phase contrast and DIC.

human buccal mucosa epithelial (cheek) cell

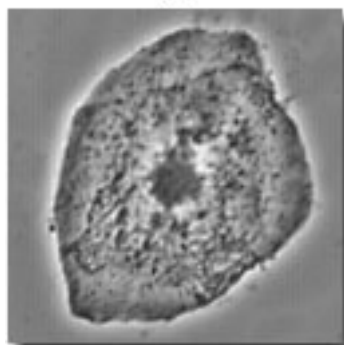
A thick section of mouse kidney tissue



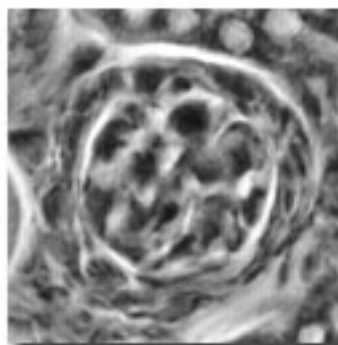
(a)



(c)



(b)



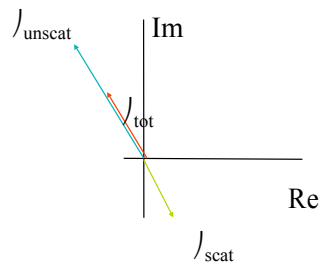
(d)

- Using diagrams, briefly describe how contrasts were generated with the phase contrasts and DIC.
- Among images (a)-(d), which are images acquired with Phase Contrast mode, which are images acquired with DIC. How can you tell?
- There are certain biological content that are more clearly visible in one mode than the other. Fill out the table below with "V" or "X" or "Maybe", to mark what you can see in each mode

	Phase contrast	DIC
Nucleus of human cheek cell	V	V
Numerous bacteria on the surface of the human cheek cell	X	V
Bundles of cells enclosed within a tubule in kidney section	V	Maybe
Some nuclei in the kidney section	V	X

In a few sentences (3 max), briefly compare the information observed in the two modes. What biological contents are more visible in each mode, and why?

(b)(d) were taken in phase contrast mode. The light halo around the cells is indicative of phase contrast mode. Phase contrast mode creates contrast by introducing an additional  $90^\circ$  phase shift between unscattered and scattered beams. This is done with a "Zernike" phase plate, which accelerates the unscattered beam by  $90^\circ$  and reduces its intensity by  $\sim 90\%$ . With the scattered and unscattered beams now  $180^\circ$  out of phase and more similar in amplitude, they destructively interfere to create contrast in the image.



(a)(c) were taken in Differential Interference (DIC) mode. The appearance of a shadow on one edge and a highlight on the opposite edge is indicative of DIC. In DIC, Wollaston prisms are used to split plane---polarized light into two waves ("O" and "E"). When the two waves interact with the sample in different ways (ex. one goes through the sample while one does not), a phase shift is introduced. When the waves are recombined, elliptically polarized light rather than plane---polarized light results. While the analyzer blocks the unaffected plane---polarized light, a component of the elliptically polarized light remains as signal.

Phase contrast yields image intensity values as a function of specimen optical path length magnitude, with very dense regions (those having large path lengths) appearing darker than the background. Alternatively, specimen features that have relatively low thickness, or a refractive index less than the surrounding medium, are rendered much lighter when superimposed on the medium gray background.

The situation is quite distinct for differential interference contrast, where optical path length gradients (in effect, the rate of change in the direction of wave front shear) are primarily responsible for introducing contrast into specimen images. Steep gradients in path length generate excellent contrast, and images display a pseudo three-dimensional relief shading that is characteristic of the DIC technique. Regions having very shallow optical path slopes, such as those observed in

extended, flat specimens, produce insignificant contrast and often appear in the image at the same intensity level as the background.

A human buccal mucosa epithelial (cheek) cell, revealing the nucleus, cytoplasmic inclusions, and numerous bacteria on the upper surface, is presented in Figure (a), imaged with differential interference contrast. The same viewfield with phase contrast illumination is illustrated in Figure (b). The phase contrast image features pronounced halos around the cellular periphery and nucleus, which are absent in the differential interference contrast image. Optical sectioning DIC microscopy investigations (not illustrated) reveal that the bacteria are present (almost exclusively) on the membrane surface that is bathed in surrounding media as opposed to lying on the underside of the cell. This fact cannot be unambiguously determined with phase contrast.

In Figure (c), a thick section of murine kidney tissue imaged with differential interference contrast reveals a bundle of cells enclosed within a tubule. A phase contrast image (Figure (d)) of the same area is confusing and disturbed by the presence of phase halos outside the plane of focus. However, several of the cellular nuclei appear visible in the phase contrast image, which are not distinguishable in DIC.