LABORATORY 1: THE LIGHT MICROSCOPE

Prior to lab:
1) Read these instructions (p 1-6)
2) Go through the online tutorial, the “microscopy pre-lab online tutorial” link is on the course web site, on the “laboratory” page.

HAND IN: Fill in and print out the quiz from the online website, write your name on it and hand it in at the beginning of your lab session.

I. Various microscopic techniques are central to the study of cell biology. A thorough familiarity with light microscopy is essential not only to lab work but also to understanding and interpretation of images encountered in the literature.

Goals:

1. To become competent with use of the phase contrast microscope and adjustment for proper illumination and contrast (through online tutorials, hands-on work, quiz and finally a practical evaluation).

2. To understand magnification and resolution and how these parameters are used in light microscopy.

Important reminders:

1. Always check and adjust microscope alignment before using.

2. Do not drag the microscope across the bench. Lift it from the bench, and set it down it carefully.

3. Raise the stage slowly, to avoid hitting the objective with the slide. Watch the stage as you bring the slide toward the objectives.

4. Always use a cover glass over your sample.

5. Never allow anything to touch an objective lens. Even if you cannot dirt, the overall quality of the image will be seriously degraded. If you suspect your objective is not clean, tell the instructors. Never attempt to clean an objective yourself; more specifically, never use regular paper or tissue (they scratch the glass surface, damage which cannot be repaired).
II. Goal One: Proper use of the microscope

Diagram of a compound phase microscope:

A. parts of the microscope

1. *On/Off switch* – Powers the light source for the microscope (once it is plugged in).
2. *Brightness control* - Varies the intensity of the light shining up toward the specimen.
3. *Light source and mirror* – A tungsten lamp built into the base of the microscope directs light up toward the specimen via an angled mirror.
4. *Field diaphragm* – Adjusts the opening through which light from the source travels.
5. *Ocular* - This is the eyepiece through which you look to observe the specimen of interest. It contains a lens that acts to further magnify the image you see by a factor of 10.
6. *Objectives* – This series of lenses directly above the specimen serve to magnify and focus the image. The degree of magnification is written on each lens (4, 10, 20, 40).
7. **Stage** – A movable platform on which the slide with the specimen is placed.

8. **Stage clips** – Hold the slide in place.

9. **Position controls** – The larger knob moves the stage forward and backward; the smaller knob positions the stage clips to the left or right. Together, these can be used to move the slide into a good position for viewing the specimen.

10. **Condensers** – This is a series of lenses tucked below the stage that focus the illumination onto the specimen.

11. **Phase turret** – This allows the appropriate condenser lens to be put into place. The “A” setting is used for brightfield observations. The “Ph1” setting is for phase contrast for the 10X and 20X objectives, and “Ph2” setting is for 40X (important if the specimen is transparent). Note: there is no setting for the 4X objective, which cannot be used for phase contrast.

12. **Focus** – The larger knob is coarse focus, to be used only at low power settings (4X, 10X). The smaller knob is fine focus, and should be used to hone in on the image.

*Take a look at one of the objectives on your microscope:*

- **The level of magnification** is given (e.g. 10X, 40X), followed by the numerical aperture (e.g. 0.25) which gives an indication of the resolving power of the lens. **Underneath is the phase ring size** (e.g. Ph1) that tells what phase turret setting to use with that objective.

- **Note that some objectives are longer than others, be careful not to rotate objectives if there is not enough clearance on the stage.**

B. Theory of Brightfield and Phase Contrast microscopy

**Brightfield** microscopy is the most basic technique, in which the sample is visualized simply by illumination of white light. However this method does not provide enough contrast for samples that are transparent, such as unstained cells or non-pigmented tissue. For these specimens, it is necessary to use optical adjustments to increase contrast.

**Phase contrast** is a technique that exploits the slight differences in refractive index parts of the specimen and the microscope slide. Light that has has traveled through the sample has a wavelength that is slightly out of phase with light that has not. This technique depends on (1) a disc with a ring-shaped opening called the **phase annulus**. This disc, placed, beneath the condenser lens serves to produce a hollow cone of light to illuminate the sample. The second part is (2) another disc called the **phase plate** on the back of the objective lens. This disc selectively alters the phase and amplitude of the light that passed through the specimen, providing greater contrast.
C. Kohler illumination for brightfield viewing

For optimum illumination, the light is adjusted according to the method Köhler (J. Royal Micr. Soc. 2:261 (1894)). By using a lens called a condenser, the illuminating light is focused on the precise area that is imaged in the microscope eyepiece, thus eliminating stray light (which would reduce contrast) and subjecting the specimen to a minimum amount of light for visualization. Contrast and depth of focus are controlled by the condenser aperture. Irregularities in light intensity caused by bulb filament irregularities are minimized since the incident light is collected and focused by the condenser.

Procedure for achieving Kohler illumination:

1. Turn on the lamp, and adjust its intensity with the built-in transformer to an comfortable viewing level.

2. Adjust the oculars to a comfortable width for your eyes.

3. Insert a specimen slide under the 10X objective and bring the specimen into focus. Begin by setting the height of the stage relative to the objective while watching the stage from the side (not looking through the eyepieces), to prevent putting the objective through the slide! Be careful to use coarse focus only in the direction which carries the objective away from the slide, for the same reason.

4. Raise the condenser lens all the way, and set the phase turret at “A” for brightfield.

5. Close the field diaphragm almost completely.

6. Bring the edge of the field diaphragm image into focus by adjusting the condenser focusing knob on the left side of the condenser. The edges of the image should be sharp.

7. Open the field diaphragm image to almost fill the field of view but so that the edges are still visible. Now center the image using the two centering knobs on the field diaphragm.

8. Now open the field diaphragm image until the edges go just outside the field of view.
D. Proper alignment for phase contrast viewing

For phase optics to work properly, the rings of the phase annulus and phase plate must be aligned.

**Procedure for aligning phase rings:**

1. Rotate the phase turret to the stop corresponding to the objective being used (note that the 4X objective cannot be used for phase microscopy). This inserts the correct size phase annulus for the objective being used.

2. Carefully remove the ocular and insert a telescopic eyepiece (take care not to knock over the oculars that have been removed). Look into the microscope and observe the back of the objective lens, you will see two rings, a bright ring corresponding to the phase annulus (in the condenser), and a dark ring corresponding to the phase plate (behind the objective).

3. Make the two rings perfectly concentric using the phase centering knobs under the phase turret, this should require only minor adjustment, so not turn the knobs too far.

4. Repeat the procedure for the other objectives.

5. Replace the ocular.

C. Observation of specimens.

A convenient source of cells are the epithelial cells lining your mouth. Scrape a bit of tissue from your mouth with a toothpick and smear it on a slide. Add a *small* drop of saline buffer and a cover slip. Examine under bright field illumination. Change the condenser aperture or illumination settings to see what effects each of these has on the image. Examine with the 10X, 20X and 40X objectives. What structures can you see (look for organelles and bacteria)? Stain some cells with a drop of fast green instead of saline. What effect does this have on contrast (again adjust the condenser aperture)? Observe prepared stained slides (e.g. striated muscle). Compare the depth of field at various magnifications.

Compare the phase contrast images of both unstained and stained cells with the bright field images. Misalign the phase rings to see what effect this has. Mount some cells in glycerol (n = 1.47) or immersion oil (n = 1.52) instead of saline (n=1.3) and see what happens to the contrast. Why?
III. Goal Two: Magnification and resolution

*Magnification* is the number of times the image is enlarged over the actual size of the specimen,  \[ \text{Magnification} = \frac{\text{image size}}{\text{Object size}} \]

Note that the total magnification is the product of the magnification of the objective times that of the ocular. Magnification is only useful if the image can still be resolved, that is if the resolution is poor, further magnification will not help.

Resolution is the minimum distance by which two points must be separated in order to be distinguishable as two separate points. Because samples in microscopes are illuminated by light, the resolution of objects is limited by the properties of light.

The resolution distance is given by Abbe’s equation:  \[ D = \frac{0.612 \lambda}{n \sin \alpha} \]

Where:  
- $D$ = the resolution, or minimum distance to resolve 2 points
- $\lambda$ is the wavelength of illuminating light
- $n$ is the refractive index of the sample
- $\alpha$ is the angle of light captured by the objective

$n \sin \alpha$ is the numerical aperture (N.A.) of a lens, and is an indicator of the resolving power of that lens (The larger the N.A., the better the resolution).

Notes:
1. A smaller $D$ constitutes a higher resolution.
2. The smaller the wavelength $\lambda$, the better the resolution.
3. The smallest resolution by visible light is about 0.2µm (200nm)

**DUE Prior to next week’s fourth hour discussion:**
1. Watch short videos on (1) brightfield microscopy, (2)phase contrast and (3)Kohler illumination on the course website, Laboratory page
2. Read pp. 580-584 in your textbook
3. Review p.6 (part III) from this handout
4. Discussion will begin with a brief quiz on microscope adjustment

For more information on microsopy, see the website links on the course laboratory page: