PCB 4023 – Cell Biology

Lab 1: Light Microscopy

Name: ____________________ SSN: _______________
Name: ____________________ SSN: _______________

N.B. Since this document is in “pdf” format, the URLs (web addresses) cannot be linked. To use them, simply highlight and copy the address and paste it into the address box of your browser.

N.B. You may wish to bring a calculator.

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Preparation assignment (to be completed before lab):

1) Visit the “The Powers of 10” web site to begin your appreciation of the concept of scale; URL: http://micro.magnet.fsu.edu/primer/java/scienceopticsu/powersof10/index.html

2) For the basic principals of light microscopy visit Dr. Stephen Wolniak’s site from the University of Maryland. Read carefully the section on Brightfield Microscopy and then skim the following sections on phase, fluorescence, polarized light and confocal microscopy; URL: http://www.life.umd.edu/CBMG/faculty/wolniak/wolniakmicro.html

3) For an introduction to microscopy, use the on-line tutorial provided by the University of Chicago Medical School for its histology course; URL: http://www.finchcms.edu/anatomy/histohome/lectures/tutorial/scope.html

4) Optional: If your computer has Quick-Time capability, you may wish to examine the “One-Minute Microscope Alignment” from the University of California at Davis Medical School. Be forewarned, the download time is a bit lengthy: URL: http://medocs.ucdavis.edu/CHA/400/faculty/microscope_how-to.htm

5) Once you have worked your way through each of the above sites, answer the questions below pertaining to each subject. These will be due when you turn in your handout (Lab 2; Epithelium).

Additional Web resources:

N.B. The great-grandmother of all on-line microscopy primers can be found at Florida State University (URL: http://micro.magnet.fsu.edu/primer/index.html). If you follow all the links, this primer runs to several hundred pages and its depth is well beyond the scope (Pun! Get it!) of this course. However, if you have a question about microscopy, this is a good place to research it.

A good history of microscopy up through the 19th century (and aside from the source of illumination used in your Leica ATC 2000, it’s technology is basically 19th century) can be found at the University of Tennessee at Memphis (URL: http://www.utmem.edu/%7Ethjones/hist/hist_mic.htm). Additional historical links can be found at the FSU Microscopy Primer (URL: http://micro.magnet.fsu.edu/primer/resources/history.html).

Microscopy and histology are full of eponyms (words derived from persons names). A good but incomplete reference for medical, and to a lesser degree, biological eponyms is the following site; URL: http://www.whonamedit.com.

Know of any other microscopy links you have found helpful or interesting? E-mail me the link at condon@fiu.edu, and let me know what and why you found it informative and/or interesting.
Questions pertaining to Web Assignment (Due Week 2):

1) Powers of 10
   a. Over how many orders of magnitude (powers of ten) can light microscopy extend human vision?

2) Principles of Light Microscopy
   a. Define resolution.

   b. What three, non-constant factors determine the resolution of a microscope?

   c. What is the equation for computing the resolution of a compound microscope?

   d. What is numerical aperture? What is the typical range of numerical apertures on an objective lens? How would you determine the numerical aperture of a microscope’s objective lens?

   e. What is the significance of two objectives with equal magnification but different numerical apertures. Would you choose the lens with the higher or lower numerical aperture?

   f. Often microscopists will close down the [substage] condenser aperture using a diaphragm to increase the contrast of an image. What is the trade-off and why?

   e. Under what conditions is it possible to have an objective with a numerical aperture greater than 1.0?

   f. What is the theoretical limit of resolution in light microscopy?

   g. Why will blue light provide higher resolution than red light? [Hint: Find a spectrum and examine the corresponding wavelengths of blue and red light.]

   h. How is the total magnification of an image produced by a compound microscope computed?

   i. What is spatial aberration? Give an example. What might chromatic aberration be?

   j. What two apertures are regulated by diaphragms to control illumination? Which is used to increase contrast in the image? Which is used to adjust for optimal illumination?

3) Microscopy Tutorial
a. What is the difference between a simple and compound microscope? [Hint: Think about the number of lenses situated between the object and its image.]

b. Define magnification

c. Define field of view. What is its relationship to magnification?

Lab Assignment:

Work through the following sections. Make sure to answer all questions marked by “?” These will be evaluated when you turn in your handout next week. When you finish with the assignment, and if there is time remaining, you can examine slides from your histology slide set to get used to operating your microscope.

1. Microscopy etiquette
2. Components of a compound light microscope
3. Setting up and operating the microscope
4. Establishing Köehler illumination
5. Using an oil immersion lens
6. Question Time
7. Inversion
8. Depth of field
9. Additional terms / concepts

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1. Microscopy etiquette

Always carry the microscope using two hands; one under the base and the other under the arm.

Always put the x4 lens in position before inserting or removing a slide.

Always make sure the coverslip is facing up.

Always focus the image at a lower magnification before moving to a higher magnification.

Always change objectives by grasping the nose piece, not the objective.

When using the high-dry (x40) lens, go back the way you came, i.e. x40 > x10 > x4 (NOT x40 > x100 > x4).

Always clean the oil immersion objective and slide immediately after use.

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2. Components of a compound light microscope

Identify the following components on the Leica ATC 2000 microscope (see handout mcrscp.ds4)

1) ocular lens (eyepiece)
2) head (or body tube)
3. Setting up and operating the microscope

Retrieve the microscope from the cabinet using both hands
Unwind the electrical cord and plug the microscope into outlet
Loosen the knurled screw clamping the head to the arm and swivel the head 180° so it is facing the same way as the stage; retighten the screw
Turn on light source switch; the brightness of illumination can be adjusted using the lamp / brightness control knob.
Making sure the x4 objective is in the viewing position; if not, use the nose piece to swivel it into position
Mount a slide out of your slide box onto the stage: Retract the stage clip and place the slide (COVERSLIP-SIDE UP) on the stage and guide it into position; gently release the stage clip, locking the slide into position
Using a plastic ruler, measure and record the interpupillary distance (the distance in mm between the middle of your pupils in parallel gaze) of yourself and your lab-mate

Student A: i.p. distance = ______ mm
Student B: i.p. distance = ______ mm

Adjust the i.p. distance of your oculars using the i.p. scale; if your microscope lacks this scale, simply adjust the oculars empirically so that a single field of view is obtained when looking through both objectives.

Adjust the focusing ocular to each student’s vision and record the approximate position:
First, look through the ocular that does not have a separate focusing ring; focus the image using the main focusing knobs. Hint: If you are having trouble focusing on the specimen, focus on the edge of the coverslip first, then move the specimen into view using the stage controls.
Second, look through the ocular that does have a focusing ring; turn the ring to bring the image into sharp focus (do not use the main focusing knob here)
Now the images in both oculars should be focus at all objective magnifications; record the position of the focusing ring for each observer

Student A: ocular position = ______
Student B: ocular position = ______

To change objectives, grasp the knurled ring of the nosepiece and turn it in the appropriate direction. Do not use the objectives to rotate the nosepiece; this damages the lens and is poor microscopy practice.
Always focus the image before changing objectives; if you don’t focus before moving to a higher power (and longer lens), you may drive the lens into the glass slide, damaging both.
When increasing magnification, always rotate from x4 to x10 to x40 to x100; when decreasing magnification, reverse this order. Do not rotate x40 to x100 to x4 if you are not planning to use the x100 lens; the latter comes very close to the slide and swiveling it across the slide risk damaging both the lens and slide.

Always put the x4 lens in position before inserting or removing a slide.

When you are finished with the microscope, unplug the electrical cord and wrap it around the base; reverse the nose piece and return the instrument to the cabinet.

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4. Establishing Köhler illumination

N.B. Have an instructor check and initialize your adjustment when you are finished

1. Make sure the condenser diaphragm is fully open; lower the condenser; place a specimen on the stage and focus on it using your x10 objective
2. Turn the ring of the field aperture diaphragm so that its edges obscure the periphery of the field of view
3. Raise the condenser until the edges of the field aperture are clearly focused
   a. it may be necessary to center the field aperture diaphragm using the condenser centering screws
4. Re-check illumination by re-opening the field aperture diaphragm and adjusting the focus, if necessary; then reset the field aperture diaphragm as in (2) and make sure the edges are still focused.
5. Adjust the substage condenser. Each lens has its own optimal setting which is obtained by looking through the ocular, opening the diaphragm (brightest image) and then closing it down until the image just starts to dim. This level of illumination will provide the sharpest image.

? Why is it best to have optimal illumination (i.e., what property of microscopy will be maximized under this condition)?

? Theoretically, Köhler illumination should be re-established when changing objectives. Why is it a best practice to use the highest (or near-highest) magnification if only one such adjustment is to be made?

? Optional: Who the dickens was Köehler?

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5. Using an oil immersion lens

N.B. Have an instructor check and initialize your observation after step 3

For good image quality with the x100 lens, you must place a drop of oil so that it fills the space between the lens and the slide. To use such an oil-immersion lens:

1. Use the x40 lens to select the area to be viewed; focus the image.
2. Rotate the nosepiece (lens turret) halfway towards the 100X lens, then place one [small] drop of immersion oil over the area to be viewed.
3. Rotate the 100X lens into position, so that the tip of the lens lies immersed in the oil and focus the image
4. When done, ROTATE THE TURRET TO BRING THE x4 LENS INTO POSITION, NOT THE x40 LENS. The 40X lens lies close enough to the slide that it will pick up oil (which you will have to clean off the lens) if you are not careful. Clean the x100 lens with a piece of lens paper.

5. Clean the slide with a Kim wipe (saliva works well) before viewing the slide at lower magnifications and/or before putting it back in the slide box.

6. **Question Time:** Answer the following questions:

? What is the magnification of the ocular lens?

? For each objective lens on your microscope record the following:

<table>
<thead>
<tr>
<th>lens 1</th>
<th>lens 2</th>
<th>lens 3</th>
<th>lens 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>color code:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>magnification:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*numerical aperture:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>**field of view:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>***resolution:</td>
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</tbody>
</table>

* The numerical aperture is the number following the magnification

** You can use a clear plastic ruler at the two lowest magnifications; ask your instructor for a stage micrometer for the two highest magnifications.

*** Show your work! The wavelength of green light is 530 nanometers (0.530 µm); assume the numerical aperture of your condenser is equal to the numerical aperture of the objective; it isn’t but this will provide a good approximation. [This assumption is why the formula for resolution is sometimes given as d = 0.61 / N.A.]

? What is the relationship between magnification and the field of view?

? Which light source will provide greater resolution than green light, blue or red light? Why?

? With which objectives might you be able to resolve mitochondria (approximate diameter = 1 µm). A red blood cell (approx. diameter = 7 µm)?

FYI The infinity symbol on the objective lens indicates it is an infinity-corrected objective (as opposed to an older design finite tube length objective) indicating that no set distance is required between the nosepiece (where the objective lens screws in) and the top of the observation tube. In [older] finite tube length objectives, the infinity symbol is replaced by a number indicating the optimal distance between the opening of the nosepiece to the top of the observation tube; insertion of any accessories in this light path will result in spherical aberration.

FYI The number following the infinity symbol (typically 0.17) refers to the thickness, in millimeters, of the cover glass that was designed for use with this objective. This becomes in issue only with
7. Inversion

1. Using a microscope, slide, cover slip, water, scissors, and newspaper make a wet-mount slide of a small-case letter "e". Your instructors can assist or "e" slides might already be available.
   a. Cut a small-case letter "e" from the sheet provided
   b. Put the letter "e" on a microscope slide, right side up.
   c. Using a dropper bottle, put a small drop of water on the letter "e".
   d. Cover the letter "e" with a cover slip.
   e. Make a sketch of the slide showing the slide, coverslip and object
2. Look at the letter "e" using the low power objective lens.
   ? What is the total magnification when using low power?
   ? Draw what you see.
   ? How does the image differ in terms of orientation from the unmagnified image:
   ? Move the slide (mechanical stage) to the right. Which way does the image appear to move? Explain.
   ? Move the slide (mechanical stage) away from you. Which way does the image appear to move? Explain.
3. Rotate the nose piece to the medium power objective lens and observe the letter "e".
   ?. What is the total magnification when using medium power?
   ?. Draw what you see.
4. Rotate the nose piece to the high-dry power objective lens and observe the letter "e".
   ?. What is the total magnification when using high power?
   ?. Draw what you see.

8. Depth of field

Although histological slides look thin and flat, at the level of microns they are considerably thick and irregular with a varying amount of topology. How much (i.e., the vertical distance) of this landscape
which remains in focus at any given focal plane is the depth of field (see below for a more formal
definition). In general, the greater the depth of field of an objective, the fewer number of focal planes
(focusing steps) are necessary to image the entire specimen. In this excursive you will qualitatively
compare the depth of field of the three dry (i.e., the non-oil immersion) objectives by focusing through an
extremely thick specimen. [Depth of field can actually be quantified, but what the hay, we’re not physics
geeks.]

Obtain or prepare a slide with three colored threads that overlap one another at a single locus.
Starting with the x4 objective, count the number of focal planes necessary to focus through the entire
specimen. In doing so, note that at any one focal plane only a portion of any of the threads is in focus at
that level. Repeat the exercise using the x10 and x40 objectives.

What is the relationship between the magnification of an objective and its depth of field?

9. Additional terms / concepts

parfocal – operating condition in which objects in focus at low magnification, remain in focus at
higher magnification

working distance – the vertical distance in millimeters, or a decimal fraction of a millimeter, from
the front of the objective to the cover glass or uncovered specimen, when the specimen is in focus

What is the relationship between the magnification of an objective and its working
distance?

depth of field – or object space; the distance from the nearest object plane in focus to that of the
farthest plane also simultaneously in focus (same as in photography); in microscopy the depth of field is
very short (literally microns).

depth of focus – or image space; a concept beyond the kithe and ken of most mortals; often
incorrectly equated with depth of field.