**Lab 6 – Microscope Modalities**

**Fall 2017**

*Objective* The objective of this lab is to become familiar with setup of frequently used microscope modalities: Kohler Illumination, Brightfield, Darkfield, Phase Contrast (PC), Differential Interference Contrast (DIC)/Nomarksi, and epi-fluorescence.

*Resources* MicroscopyU (<https://www.microscopyu.com/>) and ZEISS campus (<http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/index.html> ), AxioObserve A1 user manual (<http://www.torontomicrofluidics.ca/cms/manuals/AxioObserverManual.pdf> , p.58-59)

prelab

1) Describe in your own words the difference between an amplitude object and a phase object.

**(2 points)**

2) What is the advantage of using Kohler illumination? Support your conclusion with a sketch.

**(2 points)**

3) In phase contrast microscopy the AC and DC components are made to interfere by introducing a π/2 phase offset. A typical phase contrast objective introduces an additional attenuation. Describe in your own words why this is done, and support your conclusion with a sketch.

**(2 points)**

4) Please sketch the system schematic of a DIC microscope and explain the principle

**(2 points)**

\*use resources online to answer these questions, if needed.

Part I Brightfield

*Discussion* The microscope image under brightfield illumination is, mostly, due to amplitude modulation. This is the preferred style of transmitted light microscopy for stained specimen such as those used in histopathology.

*Experiment*

1. For this lab, you will use a Zeiss AxioObserver A1 microscope. On the condenser, turn the wheel and set it to the “H” position (brightfield), then setup Kohler illumination. With the help of your TA verify that the microscope is aligned and producing an ideal image.
2. Locate a stained tissue sample, and mount it under the microscope. Adjust the condenser and focus until you achieve optimal contrast. For most samples, the optimal condenser position is near the top. The microscope used in this exercise is not parfocal and the system will need to be adjusted for each objective.
3. Mount the camera and acquire an image. Verify that the images are being saved as per-pixel linear intensity counts (8 or 16 bit). Instead of JPEGs or PNGs.

1. Acquire an image of a stained tissue or some other sample that fills the spatial frequency space. You will use this image in the write-up.

Part II Phase Contrast

*Discussion* In phase contrast, the AC and DC components of the field are brought to interfere. In a typical illumination geometry, the DC components lie on a ring similar to the ring in darkfield. Phase contrast is the most popular contrast enhancement technique used in live cell imaging.

*Experiment*

1. Set the wheel of condenser to a “Ph” position.
2. Mount or find a section of unstained material. Acquire two images, one under brightfield and the other under phase contrast illumination. You should see a big difference!

Part III Dic/Nomarski

*Discussion* Differential Interference Contrast (DIC) microscopy uses polarizing optics to represent the phase difference across the shear direction. Although the “relief” style image in DIC has weaker contrast compared to phase contrast, it benefits from marginally higher resolution, and doesn’t have the “halo” artifact typical of phase contrast. Additionally, the design can be used in a reflection geometry for tasks such as wafer defect inspection.

*Experiment*

1. Set the microscope condenser to the DIC position (DIC II with 40x), switch the beam splitter to the cube with a polarizer (labeled as the DIC position), and verify that you see the contrast change when you rotate the Nomarski/de Sénarmont compensator.
2. Now that you have a good DIC image, replace the sample with a piece of plastic or any other material that breaks the polarization. Now rotate the de Sénarmont compensator, record what you see through the eye piece. What can you say is a limitation of DIC?

**(2 points)**

1. Now set the compensator to half between excitation and full brightness. Acquire an image with the condenser open and closed. You will use this image in your write up.

Part IV Fluorescence Imaging

*Discussion* Cellular structures can be made to emit light in response to illumination. Here, the sample is excited in transmission with a high frequency wave (~400nm), and fluorescent emission is collected in reflection through a narrow band filter at a lower frequency (~500nm).

*Experiment*

1. Turn on the fluorescence source, and rotate the reflector cube to match the stain used in your sample, typically FITC or DAPI. The source may take a few minutes to “warm up”. You should see a blue, purple or other vivid colored excitation beam. Turn off the white light halogen illumination and open the reflected light shutter, verify that you can see the stained structures through the eyepiece.
2. Acquire an image and save it for your write up.

**(2 points)**

1. The fluorescence source has a limited life span. Have your TA verify that the source is off before leaving. You will lose points if the TA finds that the source has been left on.

**(-100 points)**

Part V Spatial coherence and image formation

*Discussion* The role of spatial coherence in image formation is an active area of research. Here you will note that the qualitative trends when the spatial coherence properties of light are modified by opening and closing the condenser on the microscope.

*Experiment*

1. For this experiment you will use a microscope with Köhler illumination and tunable condenser aperture.
2. The bright field position of the condenser contains an aperture. In our model, each point in the aperture produces a random wave that contributes a plain wave at the sample plane. Observe that opening and closing the condenser results in a qualitative change in the image. Describe what you see.

**(1 points)**

1. To quantify this change you will acquire images of two samples at three condenser positions – “open”, “closed”, and “half-open”.
2. Switch the light path to the camera port and focus a test sample. Verify that the images are raw recordings of the pixel sensor (instead of JPEG) and are the expected bit-depth of the camera (typically 12-bits). If unsure ask your TA.
3. For the first sample we will attempt to uniformly fill the spatial bandwidth of the imaging system. Such samples include a fine diffuser or a slide with histopathological prepared tissue. Now acquire the same field of view at the three condenser positions. Verify your images before continuing.
4. Next find a sample with an identifiable structure. For your write up you will draw a line profile through this structure and note the qualitative changes as the aperture is closed. A red blood cells or a scratch on the glass will work well. Record three images.
5. Have a group member send out the acquired images to your partners. You will need them to answer the lab questions!

QUESTIONS FOR WRITEUP

|  |  |
| --- | --- |
| **Image and FT Pair** | **Written Analysis and Discussion** (3 sentence limit) |
| Brightfield | **(2 points each)** |
| Phase Contrast compared to Brightfield (2 images) |  |
| DIC (2 images) |  |
| Fluorescence (2 images) |  |

1. The numeric aperture of your objective gives the maximal acquired angles, where NA=1.0 means all the angles are acquired. What does an NA>1.0 mean, and how does one use such an objective?

**(2 points)**

1. Because the image formed in fluorescence microscopy is due emission it is possible to improve the quality by modifying the excitation in ways that would otherwise be impossible for scattering modalities. In three or fewer sentences explain how TIRF excitation works, and why this may improve the image quality. Does this improve the lateral (XY) resolution of a conventional microscope? Why?

**(3 points)**

1. Show the images you acquired for the first sample in Part III side-by-side with log magnitude of the Fourier transform. What conclusions can you draw?

**(3 points)**

1. Using a tool such as ImageJ show the line profile of your chose structure as the condenser aperture is closed down, side-by-side with the acquired images. What conclusion can you draw?

**(3 points)**