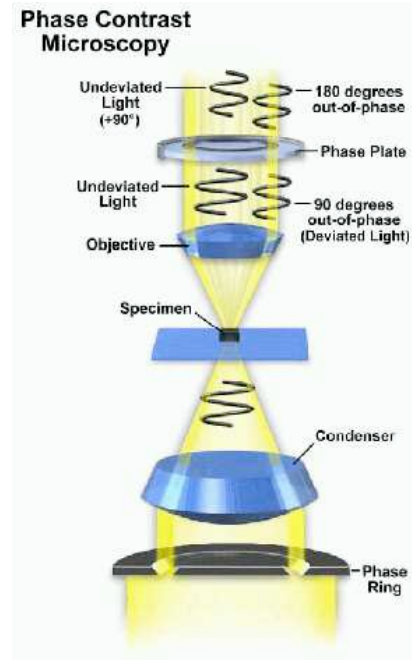


Objectives:

1. DNA Flow experiment
2. Photobleaching curve
3. Movie of E. coli growth

Phase microscopy:

The problem is that these biological samples don't create enough amplitude differences in light for our cameras to detect, nor do they create any sort of color that our eyes would detect (the cameras still wouldn't, since there are no colour filters on the ccd chip). Thus, we need a method of creating contrast in the specimens. One approach is to add dye molecules to the samples so that they can be seen under fluorescence, another is to encode fluorescent proteins, such as GFP (green fluorescent protein) in the target of interest. Yet another way is externally modulate the light so the heretofore transparent specimens become opaque (see diagram on the right). In this laboratory, the type of brightfield microscopy used to create contrast is called "phase contrast". In brief, a $\pi/2$ phase shift is introduced into the zero-order components of the Fourier transform of the object through the use of a spatial filter at the back focal plane of the objective (Fourier plane). What we first notice from the diagram is that the *phase ring*, since it is a new source of light, is conjugate to the *phase plate*. Light that passes through the specimen that does not diffract is therefore focused onto an *annular ring* in the phase plate, and hence encounters a $\pi/2$ phase shift. The annular ring also absorbs some light. Light that the specimen does diffract misses the ring and is spread over the entire back focal plane. Thus the zeroth order light is reduced in intensity, and the diffracted light and zero-order light are out-of-phase by half a wave. These two combine to yield an image of higher contrast.



Photobleaching of fluorescent E. coli (required):

1. Follow the growth movies protocol, except using PBS instead of LB to avoid autofluorescence.
2. When imaging, add a fluorescence channel. You might want to leave the fluorescence (lumen) shutter open, depending on your individual preferences

Growth movies protocol:

Preparing the agar pads:

1. Stretch a piece of parafilm on a glass surface (or the bench). Place a 24x50mm or 24x60mm coverslip on the parafilm.
2. Pipette 2-3ml of LB with 1.5% low melting temperature agarose.
3. Drop another coverslip on top in order to "sandwich" the agarose. This step might be easier to do with your gloves off.
4. Let it dry for 30-60 minutes.

Setting up the pads:

1. Cut two squares 3-4mm squares of agarose and put them on slide. Cover the pads while they're drying (without touching them!). The idea is to reduce pad drying before you put in any cells. We're making two pads in case something goes wrong with one!
2. Spot 2 μ l of cells on each one of the pads. Remember that you shouldn't touch the pad with the pipette tip!
3. Close the petri dish again and let dry for a couple of minutes. Depending on how dry the pad is we might put it at 37°C to speed up the drying.
4. Place the pads on a microscopy dish. This will be either one of the Wilco dishes (the big ones) or the Matek dished (the smaller ones).
5. Place the dish on the stage corresponding to your scope. Make sure that the environmental chambers are properly closed. The idea behind this step is to let the pad equilibrate with the temperature of the scope.
6. After 5 minutes seal the dish using parafilm. Make sure the parafilm doesn't interfere with the placement of the dish on the scope. This step is meant to reduce evaporation from the pad which would result in drying.
7. Put a drop of oil on the objective and place the dish on the corresponding holder.

Setting up the movie:

1. Load "Micro-Manager".
2. Go to the "Multi-D Acquisition" window and open the XY list (next to the "Use XY list" option).
3. Find the cells and move around the pad. Find 5-10 different positions where you see interesting things. For example, you might want to include some areas with only one or two cells in the middle and some other areas with a lot more cells. Mark the positions on the XY list.
4. Set up the channels to be used. You should use the brightfield setting without binning. Make sure that you have a reasonable exposure!
5. Set up the autofocus by selecting "Autofocus" and open its option dialogue. Let's choose the following options:
 - 1st number of steps: 6
 - 1st step size: 1
 - 2nd number of steps: 6
 - 2nd step size: 0.3
 - Threshold: 1
 - Crop ratio: 0.25

What do these options mean? Check out the Micro-Manager manual on the course website. Explain how the search for the optimal focus will go in your homework.

Note for the Nikon: The Nikon scope does not need any software autofocus. You'll have to set up the Perfect Focus. Also, make sure that the "Hardware Autofocus" options "Switch off for XY move" and "Switch off for Z move" are selected. Ask your TA how to work with Perfect Focus.

6. Choose "Save files to acquisition directory" and select a folder where you want to save your images. Also choose "Single Window" in the "Display" option.
7. Before you start taking your movie, make sure that everything is working. Take only one frame at each position. If one of the frames fails to focus go back to the position and make sure it hasn't drifted out of the range of the autofocus search. If you're having issues with this ask you TA!
8. Now you're ready to take your movie. Think carefully about how often you want to take frames. What's the maximum time resolution you could get? What's the limiting factor (the bottleneck) in the acquisition at each position? You'll probably want to take a frame every 5 minutes.

Take a movie, making sure all the exposure times, number of frames, etc., makes sense.