

Bi1x: Making movies of dividing cells

As a way to complement the bulk measurements you did last week we'll be measuring cell division at the single cell level under the microscope today. The idea is to not only compute the cell doubling time based on the movies you'll obtain. Today's experiment is also an introduction to the kinds of measurements that can be done on single cells and to the full use of an automated microscope.

Note: There is nomenclature here involving petri dishes, microscope slides and microscopy dishes. Make sure you know which one is which before starting the protocol! If in doubt, ask a TA!

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. Note that in the interest of time we'll be giving you some premade pads.

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 2ul 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 37C 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 dishes (the smaller ones).
5. Place the dish on the stage corresponding to your scope leaving the lid open. 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. If you close the lid and see condensation forming then open it again and wait some more.
6. After around 15 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 1.3.39". **NOTE:** It's very important to load this version of Micro-Manager. If not your movie might not work.
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
 - Channel: Brightfield

What do these options mean? Check out the Micro-Manager manual you were given at the beginning of the course. Explain how the search for the optimal focus will go.

Note for the Nikon scopes: 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 you 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 let's make sure that everything is working. Let's take only one frame. 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.

Assignment:

1. Answer the questions in the protocol marked in *italics* related to the autofocus and the time resolution.
2. Show the initial and final frames of all the positions you took data from. Did all cells divide?
3. Show a couple of frames of one or two of your favorite movies. You might want to look into the Matlab command *montage* in order to plot many frames right next to each other.
4. Estimate the cell doubling time by looking at your movies. An easy and fast way to look at the movies is with ImageJ by using the command "File → Import → Image Sequence".
5. Make sure to explain your reasoning carefully by showing a couple of division events with their respective time stamps. Time stamps are easy to add using "Plugins → Stacks → Time Stamper". Also add a scale bar by doing "Analyze → Tools → Scale Bar". How do you decide that a cell has divided already? Do all cells divide at the same rate? Do you get the same division rate in all positions on the pad?
6. Look at the movies corresponding to one or two positions and manually track the different cell division times. Draw a histogram of the division times you found and find the mean division time and the standard deviation. How does the division time compare to the bulk results you got last week? Is there any difference, why do you think this could be?