

## Bi 1X, Spring 2011

### Week 1

- **Session 2: Microscopy and Powers of Ten**
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#### **Introduction: The Relative Sizes of Things in Biology**

IBM's short film "Powers of Ten" uses orders of magnitude to take a new look at physics, from the tiny subatomic particles in a proton to the huge, sweeping arms of distant galaxies. Many advances have been made that now take us well beyond the picture painted in 1977 and concern exotic states of matter at very small and very large scales.

Many other advances, in biology and medicine, fit comfortably below 1 meter. In a perfect world, we could use microscopes to resolve all of these scales in the ponds, but diffraction, the way light scatters and spreads when it meets small objects, prevents us from seeing it all. What is the resolution limit of the microscope you are using? You may find this site useful:

<http://www.microscopyu.com/articles/formulas/formulasresolution.html>

The scales of different organisms dictates in part how they interact with their environment: a frog swims just fine by kicking, but *E. coli* and other microbes would have trouble moving by a similar motion due to an increase in the influence of inertia. To get around that, they have found other ways of moving with flagella. For a great explanation of the importance of size in motion, see "Life at low Reynolds number" by EM Purcell (check out the "Readings" section on the Bi1x website).

#### **Goal:**

Your mission is to investigate, like the scientists in the Powers of Ten video, what is happening in the Caltech ponds at each power of 10 from  $10^{-1}$  meters to  $10^{-8}$  meters. Turn in a gallery of photos with descriptions, one for each order of magnitude, using the pictures you took with microscopes in class. For orders of magnitude outside the capabilities of your microscope, add pictures you can take or find on your own (remember to always cite sources). Be sure to discuss the biology of what's happening at each order of magnitude in the pond and how it relates to the world at large in a short paragraph with each picture. In addition, use Matlab to convert pixels to microns on the gratules for each magnification and add scale bars to each picture. Concatenate images taken at different fluorescent channels using Matlab with a caption on the false colors and scale bars.

## Protocol:

Throughout the course, you will acquaint yourself with modes of light microscopy: bright-field (BF) and phase contrast (PC). Additionally, certain samples are either naturally fluorescent or they have been modified chemically or genetically to have specific fluorescent markers. You will examine these samples using fluorescence microscopy – a mainstay of modern biological microscopy.

1. Go over the Micro-Manager Manual attached at the end of this handout with your TA.
2. Understand the basic mechanisms and light paths of light and fluorescent microscopy, know the location of: eyepieces, objectives, stage, focus adjustments, condenser aperture diaphragm, field stop, phase ring, lumen, fluorescent filter block turret (i.e. dichroic), and intensity filters (excitation and emission).
3. Be able to setup Köhler Illumination in bright-field.
4. Familiarize yourself with the basics of objectives: magnification, depth-of-field, working distance, numerical aperture (objective and condenser), and the effect of imaging medium (air vs. oil) on resolution.
5. Have a working understanding of how to capture images with a CCD camera, including how contrast, saturation, binning, exposure time, frame rate and image format are all related to the quality of video or still images.
6. Spatially calibrate your microscope at three different magnifications: use a “graticule” to directly correlate pixel size with spatial dimension, estimate the error in your calibration.
7. Take images on the following samples and save for future analysis. Make sure to write down the magnification, exposure time, and fluorescent channels used in your notebook!
  - a. Prefixed cells with organelles fluorescently labeled
  - b. Pond water. Your TA will show you how to mount the specimens.
  - c. Optional: Diatoms – unicellular organisms with a cell wall made of silica called frustule, which can be a way to test the resolving power of a microscope.

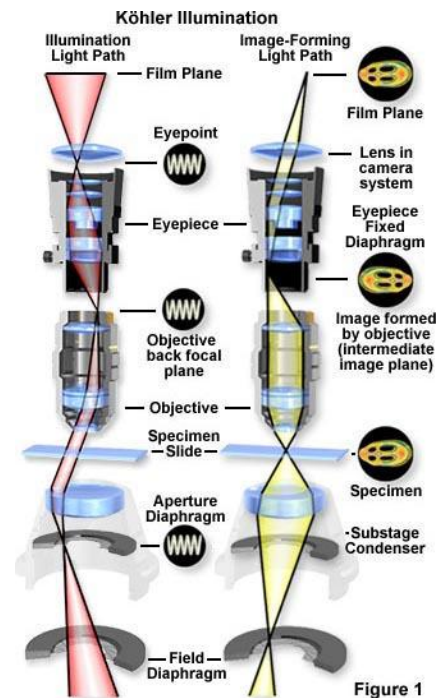


Figure 1

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## **Micro-Manager Manual**

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### **Introduction: Why Micro-Manager?**

Micro-Manager (MM) is a microscope control software developed in Ron Vale's Lab at UCSF with support from NIH. The idea was to provide a free, open-source platform based on Java using ImageJ (which is also funded by NIH). Basically, MM tries to imitate the function of packages such as Meta-Morph, ImagePro and SlideBook, but for free whereas these other packages go for up to \$10,000.

In this course, we will be using mostly the GUI user interface. However, MM has a good scripting language. Its libraries for microscope control can also be accessed from Matlab, giving the chance to integrate data acquisition and data analysis.

Because it's a program in constant development it will always have some bugs. Please report any of them to the TAs so we can pass that information along to the programmers and, also, *be patient!*

The next few paragraphs give guidelines to be followed every time you sit in front of the microscope.

### **Some safety words**

The Olympus scopes (Kratos, Nemesis, Wheels) use external filter wheels. This means that when using fluorescence if you look through the eyepiece you might get some of the UV excitation light. **DO NOT LOOK THROUGH THE EYEPIECE WHEN USING FLUORESCENCE.** This is not a concern in the Zeiss.

After turning on the fluorescence lamp it should be kept on for **at least 30 minutes**. When turned off it should be kept off for **at least 30 minutes**. Changes to the bulb before it's equilibrated can break it.

### **Turning everything on**

There's no particular order you should turn things on. However, make sure to turn on the fluorescence **only if** you need it and to turn everything off once you're done with your experiment.

*Special warning about the scopes with a Lumen (Nemesis and Wheels):* The Lumen combines a fluorescence light source with fluorescence filter wheel and an ND filter wheel. Both wheels are controlled from the Prior controller. The only role of the switch on the Lumen is to turn the

fluorescence lamp on and off. If, for any reason, you need to restart your system and turn all controllers off and back on make sure you don't do this with the Lumen! You'd only be hurting the fluorescence light bulb.

*Special warning about Wheels:* When turning on the Prior controller you might hear a weird “buzzing” sound coming from the brightfield shutter. If you hear that just turn the Prior controller off and back on after one minute.

### **Logging in**

Use the **Bi1** account. If you find the computer already logged on, make sure to log off and log on using the **Bi1** account. Do not install any software or change anything in the computer configuration.

*Make sure to log yourself on the microscope logbook and to write down the number on the fluorescence illumination source (if you're using it).*

### **Data storage**

You should take data and save it in a folder inside the “**Bi1X2011**” folder located on the desktop. Always save data directly to the local machine first! After that you can move it to a USB drive or to the course server (snowdome) in a folder inside the “**Bi1X2011**”. In order to mount snowdome on a Windows machine you need to connect to \\snowdome.caltech.edu\aph162. The username is “aph162” and the password is “162sharing”.

### **Loading Micro-Manager**

On the desktop there should be a link to “MicroManager1.3.XX”, with “XX” being some version. The software keeps getting updated constantly so load the latest one if there happen to be multiple links.

MicroManager will ask you for a configuration file. Make sure that it's loading the “.CFG” file located in the folder “C:\MMConfig”. The “.CFG” file should have the name of the scope you're using. The previous user might have used a different configuration file, that's why it's key you make sure you choose and load the right one.

*Special warning about Zeiss:* Unfortunately, the camera on Zeiss sometimes has issues when starting MicroManager. If you have any problems just turn off the scope and camera, turn them back on and restart the computer.

### **Setting the dichroic mirror**

Each scope has different dichroics. In this course we will use only one of them unless otherwise noted by your TA. Please consult with your TA to make sure that the dichroic is set to the right position.

## **Making sure everything's ready**

Make sure that all diaphragms are open and that you know what phase ring (if any) is set on the condenser before starting.

## **Live mode**

Each scope has a channel defined as “BrightField” and one defined “BrightField-Live”. The difference between the two is that the latter bins the image. This decreases the resolution of the image, but allows for a much faster update of the screen with exploring your sample in “Live” mode. When you actually take data you can use “BrightField”, but you should use “BrightField-Live” when moving through your sample.

Note that the “Auto shutter” option needs to be enabled. If you wanted to open the shutter manually in order to look at a sample in brightfield through the eyepiece, for example, you can unselect it and click on “Open” or “Close”.

## **Multi-D Acquisition**

This is one of the great strengths of Micro-Manager. It allows you to go to multiple XY-positions, take Z-slices, take pictures using different channels (i.e. different wavelengths of excitation), perform image-based autofocus, and do time lapse microscopy! Make sure you familiarize yourself with this window. Also, before clicking on “Acquire!” check which of these options are set. For example, if you are interested in taking a snapshot of the particular position you're on, be sure to deselect the “Use XY list” option.

## **Time-lapse microscopy**

Before taking a movie it is very important to make sure that the results are saved to disk choosing “Save files to acquisition directory”. Also, you should select “Display in Live window”. This will only show on the screen the very last frame that was taken. It ensures that there are no memory problems.

## **Autofocus**

This option is only available together with the multiple “Use XY list” option. If you want to do this for a single position, just choose a single position on the position list! You'll find a couple of parameters regarding the autofocus that can be adjusted.

- *Channel*: This is the channel that will be used to do the image-based autofocus. We usually use “BrightField”.
- *1st number of steps and 1st step size*: If the parameters are 5 and 1, respectively, it will take a total of 11 snapshots for the first search range. Each one will be spaced by 1 micron and will be centered on the starting position.

- *2nd number of steps and 2nd step size*: After doing the first search it will perform a finer focus. One set of parameters that works is 6 and 0.3.
- *Threshold*: Set this to 1.
- *Crop ratio*: Fraction of the image should be used for finding the focus. Often, you'll start with one cell in the middle of the field. You want most of the information to come from that area. In that case a crop ratio of 0.25 is good. If you're focusing on samples that span the whole image you can set that to 1.

### **Perfect Focus System (Nikon scopes only)**

The Nikon scopes (Hermes and Bolt) come with a unique Perfect Focus System (PFS) that automatically corrects focus drift in real time during a prolonged period of time-lapse imaging. Thus, be sure to deselect the "Autofocus" option while taking a time-lapse movie. Consult with your TAs for detailed manual.