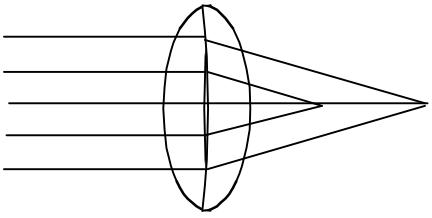
IGB Fluorescence Microscope Quick Reference Guide

Turn everything on:
1) Turn on the X-cite source and turn the dial to full intensity.
2) Turn on the fluorescent microscope on the right side of its base.
3) Turn on the Lambda 10-3
4) Turn on the Uniblix shutter (black box)
5) Turn on the MCU-28
6) Turn on the computer-Follow this sequence.

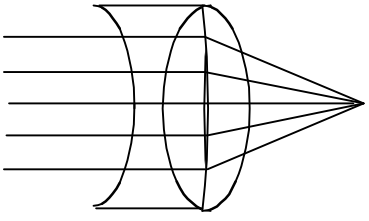
Preparing to image a sample
The optics (objectives) is optimized to image microscope slides with a mounted coverslip (#1.5 or 0.17mm). Coverslips that are 22 x 60 mm are available from Fisher. If you do not use a coverslip your sample will be bleached more readily and chromatic and spherical aberration is more likely.[1]

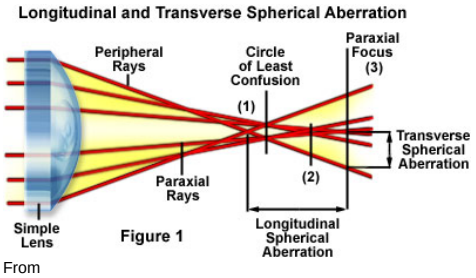
[1]Chromatic aberration is the inability for light made up of different wavelengths to focus to a single point. Spherical aberration occurs when light rays passing through different matter which has different refractive indices and the light gets bent when there is a mismatch.

a lens system with chromatic aberration

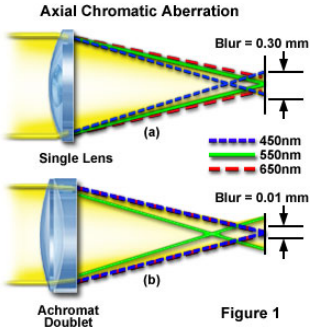


a lens system without chromatic aberration





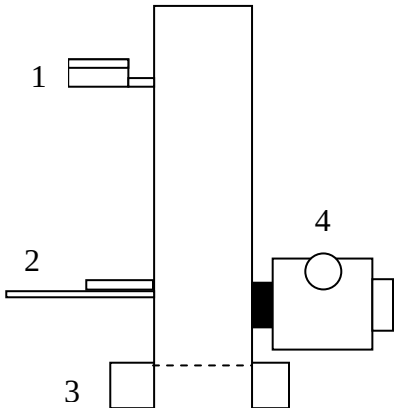
From
http://micro.magnet.fsu.edu/primer/java/aberrations/spherical/index.html



From
http://micro.magnet.fsu.edu/primer/java/aberrations/chromatic/index.html

Prolong Gold is a mounting medium available from Invitrogen used for fluorescent samples (could be obtained from invitrogen supply center, ask Shiv). Put the product down on top of the sample on the microscope slide, pipetting the volume directly onto the sample where the areas of interest are located. (The exact amount to pipet down must be worked out by trial and error). Next, place one edge of the coverslip into the Prolong Gold and hold the rest of the slide at a 45˚ angle. Then, carefully lower the coverslip into the Prolong Gold, trying to avoid air bubbles. To cure the mounted sample, keep it at room temperature for 24 hours in the dark. Then store it at 4˚C until you are ready to analyze it. The Prolong Gold has a refractive index of 1.45 (when cured), which is close to the refractive index of oil.

Preparing the Instrument
1) Make sure the polarizer is out
2) Make sure the analyzer is unengaged.
3) Make sure the filter turret contains the FITC, Cy3, DAPI, etc. filters
4) Make sure the apotome is out, so that you can see the black bar.

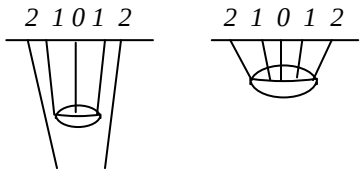


The microscope has two turrets. One contains objectives, and one contains filters. Choose an appropriate objective. Start with the 5 or 10X objective and move to more powerful magnifications, if necessary. The 10X and 20X objectives work in air and should not be immersed in oil. More powerful lenses should be immersed in oil. The oil is used to match the refractive index of the sample.

The numerical aperture of the 40X or 63X oil objectives are around 1.4, but the numerical aperture of the 20X objective is only 0.8. The accuracy of counting increases with increasing numerical aperture, because more photons are being collected.

20X
2 mm working distance 0.8 NA

40X
0.2 mm working 1.4 NA



The 40X objective has a larger aperture than the 20X objective. The 40X aperture is closer to the sample, and has a greater NA (numerical aperture), since more of the higher angle/order (for example 2˚) light rays are collected. While these are excellent features, the 40X lens has a reduced depth of field. When choosing an objective, you must choose between a greater working distance or greater accuracy. (Accuracy/resolution increases as the number of photons being collected increases. In other words, this results in higher resolution images).

lens	NA
10X	0.45
20X	0.8
40X	1.4
63X	1.4

Mount your sample
1) The coarse and fine focus adjusts are located on the sides of the microscope. Bring the objective down until you hear the microscope beep.
2) Bring the stage close to you with the joystick.

Starting the software
1) Login to the computer by entering the domain "IGB," and your username and password.
2) Double click on MTB2004 Configuration on the desktop.
3) Make sure "IGB" is the active configuration by right-clicking on "IGB." Click "Apply," then click "OK."
4) Double-click on "AxioVision Rel. 4.6." This is the acquisition software.

There are three cameras that can be used with the fluorescence microscope. In this case, use the AxioCam MRm. It is located on the side port left. It is recognized in the software as "AxioCam MR3," where the number "3" is the driver release upgrade. It has {1388 x 1040} pixel resolution, with 6.45 x 6.35 µm (2/3") CCD. This camera is high resolution monochrome. The IGB website lists more specific information on the microscope, such as the quantum efficiency curves and wavelengths of light the microscope is most sensitive to.

High speed efficiency cameras are installed on this microscope, but we will not be using them right now.

The software controls 95% of the instrument. Here are the three things the software controls, and the window through which each aspect is controlled:

aspect	Window
microscope	Microscope
channel (Cy3, Cy5, FITC)	Multidimensional Acquisition
camera	AxioCam MR3

Whichever windows you leave open at the end of a session will appear when the software is restarted. If a window is accidentally closed, here is how they can be reopened: "Microscope" is a blue icon on the menu across the top of the software window. "Multidimensional Acquisition" is on the 6D acquisition menu. "AxioCam MR3" can be accessed by going to Acquisition > Select Camera > AxioCam MR3. (This sequence will activate the AxioCam MR3, but no window will appear). Then go to Acquisition > Camera > AxioCam MR3. (This sequence will bring the AxioCam MR3 window). All camera specifications, chip size and frame rates are available at the http://core.igb.uiuc.edu website.

Multidimensional Acquisition (MA)
On the MA window, click on "Load." (Not the "V" drop down menu on the Load button).

Select an experiment, usually stored under your name.

Put a name on “Image name” to save pictures.

Use a new channel for each unique fluorescent dye you are using. If you have a single dye, use a single channel. To turn on/off channels, right click on the channel number tabs.

Left click on a channel number tab to make it active.

Under exposure, click on “Fixed.”

Adjust the “Hardware Settings”:

On the drop-down list for “During acquisition,” select “User FITC” (usually selected and stored in your experiment). Then click “Go.”

On the drop-down list for “After acquisition,” select “Turn off FL2” (also usually selected or custom made for your sample).

“User FITC” and “Turn off FL2” are macros which configure the microscopes filters and shutters.

Under “Focus,” select “Current” (also usually selected for your method).

Microscope

Under”Common Tab” “Lightpath,” select Sideport L (to send the light to the AxiocamMrm) or to “Eye” to see the sample through the oculars (using your eyes) and focus the sample and change the light path to camera once you find your sample and focus.

Under “Objective,” select “20.”

Under “Micrsocope Manager,” **deselect/uncheck** “Enable light manager.” (This setting is used for bright field).

Focusing on the sample

Multidimensional Acquisition

Under “Hardware Settings,” under the listing “During Acqisition,” click the “Go” next to “User FITC.”

The light on the microscope will be turned on. You will want to limit the amount of time your sample is exposed to light, in order to avoid photobleaching. Thus, when you are exploring your slide, you will use the macros under Multidimensional Acquisition to turn on and off the light when you start and finish exploring, respectively.

When you are exploring the slide thorugh the objectives on the microscope, you will have the lightpath directed to the “Eyes” listed on the lightpath diagram. If you want a live image to appear on the computer screen, or if you want to take a picture of your sample, you will need to redirect the lightpath to the Sideport left.

Look in the microscope and bring the objective up by turning the fine focus knob counter clockwise (CCW). Focus on your sample. After this first time, when you manually focus the microscope onto your sample, the software will remember the position of the objectives, and be able to replicate this.

After focusing, turn off the lamp by pressing FL on/off on the base of the microscope.

The 10X and 20X objectives should be used in air. If someone has mistakenly used oil on one of these objectives inform the core staff immediately.

Obtaining a live image

Click on the “Live” icon on the menu.

Under the Hardware Settings in “Multidimensional Acquisition,” click “Go.”

An image will appear in the live window screen.

Click on “Linear” and “over exposure” icons in the menu on the live window. When these settings are enabled, the image will turn red where it is being overexposed.

Optimizing the exposure level of the preview

The goal is to adjust the exposure so that the histogram on AxioCam MR3 to be in the middle, i.e., one should have few dark pixels (0 intensity) and few saturated pixels (4095 intensity). So the user uses the entire dynamic range of the available grey level depths in the camera (that is what the Linear display does). Note changing the linear or min max grey levels does not change the raw data but only the display mapping.

Never change the exposure weight (left at 100%)– adjust only the exposure time.

After optimizing the preview image, click the “Go” button next to Hardware Settings - After acquisition – User FL Light Off 2 to avoid photobleaching.

Obtaining an actual image

In the Multidimensional Acquisition window, key in the exposure time you found in the previous step. It may be necessary to change the units from seconds to milliseconds. That means you can enter the same number in the exposure camera window from 1-999 which directly stands as milliseconds, in case if your exposure requires a second or more 1.2s for example, then you enter 1200 milliseconds in the Multidimensional Acquisition window for that

respective channel. Do the same for all the channels you wish/have selected.

Click "Start," to take a real image.

Once the image is taken, click on the various buttons under the image to change between the linear or other settings (display mappings), so you can see if the image is to your satisfaction. (again regardless of the setting/mapping you select to display the image the raw data grey scale intensities available in the image will remain the same).

If you want the image to be in false color, click on the button showing the color wheel so that it reads "On" instead of "Off."

Under the File menu, select "Save As" > Storage > E: > Users > (your name). Save this initial file as a .zvi file. (This can only be opened by the fluorescence microscope software) or a lower end version could be downloaded from
http://www.zeiss.com/C12571950028B51A/AxioVisionLEEN?OpenForm

To add annotations, go to Annotations > Scale Bar > Measure > Profile. Then draw a box. To save your image with the profile shown, export the file and save it as a .tif file. Select the following boxes:

__ convert to 8-bit (if you wish to change the bit depth otherwise uncheck this box to retain original grey scale-for quantification and image analysis, it is strongly advised to keep the original bit depth).

Check

__ burn in annotations

0% compression

(The relative intensities depend on how many bits are used. 16 bit ranges from 0 to 4096 while 8 bit only ranges from 0 to 256. When a file is compressed or changed bit depth, you lose information.)

To view the profile, click on the blue icon at the bottom of the window > profile. This will display actual values.

The default y-axis for the profile is pixels. If you wish to change the units to micrometers, go to Measure > Scalings > 10X MRM > Apply selection to image > Activate selection. (On the "10X MRM" level, choose the correct objective and camera). Usually, this is done automatically, you need to do this only if the x axis is already not in micrometers.

To save the profile, export it in the same way that you exported the annotated image.

You can also create a table. To export the table, select the table window, and go to File > Save as and save a csv or excel file to be directly opened in Excel.

If for any reason you need to print what you actually see on the screen and to remind yourself about the controls you are currently using, use the print screen on the key board and export the file to photoshop and edit and save it for future use. The print screen option will produce images up to 600 x 1200.

Typically, you will have about four files for each image you acquire:

1) The initial .zvi image
2) The .tiff of the image with the profile bar or scale bar.
3) The .tiff of the intensity profile
4) The data table showing the values in the intensity profile.
5) Or a gallery showing all different channels and scale made from gallery mode under the image window. Select capture image after you activate the color and channel names.

Exiting the software

After you have saved all of the files that you want, exit the software by clicking on the red X at the corner of the software main window.

The menus in the software that are open when you exit the software will be kept when you log in the next time.

Shutting everything down:

First log off on the system and walk away if your appointment is between 8-5 Mon-Fri, if not, shut down the computer after logging off.

Turn off everything that was turned on at the beginning. The order in which things are turned off is less crucial now than at the beginning. Do not forget to turn off the fluorescent light (Excite 100).

A basic microscope system information is available on the <http://core.igb.uiuc.edu> website under instruments. Go to "Resources" > Molecular Expressions > Virtual Microscopy, this will provide all the basic and advanced information about the different microscopic techniques.
http://micro.magnet.fsu.edu/primer/index.html
The resources page also has information on immunofluorescence protocols, how to handle digital images and ethics in digital imaging. First time microscope users handling scientific digital images for posters, presentations and publications are strongly advised to go through with these articles. Prepared by Hannah Ihms (PI: Ryan Bailey) and edited by Shiv (questions: 333-9896, 417-9593)