Digital microscope software manual

Quick Start for Metamorph

(edited by Xuejun Sun, April 15, 1999, revised Jan 26, 2008)

Content

I) Safety /regulations

1) Fluorescent lamp:

a. In the very unlikely event that a **lamp explodes** (sounds like a gun shot), clear the room immediately and close the door. Post a sign that no one should enter the room. Inform Sun or Gerry or Building system.

b. Fluorescence uses high intensity light sources, do not attempt to change, remove or modify any optics. Direct exposure of fluorescence lamp light may cause **blindness**.

c. **Fluorescent light sources** should not be turned on and off frequently. Check if the lamp was just turned off (touch the lamp housing, if it is hot, come back in 15mins). Hot lamp may not ignite or it may explode.

d. Before turning off the lamp, make sure no one has signed up for the instrument after you within 2hr (check signup site for the scope, if so, leave the lamp on and close the shut).

e. If you have gone for a short coffee/lunch break, leave the lamp on.

2) Microscope

a. No wet **Neil polish** on any scope! It needs to be dry for at least for 30mins before viewing.

b. If a **lens crashes into a slide**, do not attempt to clean by yourself. Inform Sun or Gerry.

c. If you are not sure about anything, ask for help. Do not force or change any microscope accessories.

d. Take extreme care with lenses, **nothing but lens paper** should touch lenses. When you wipe oil off, do not press directly on the lens surface.

e. If you are responsible for any **damage** to any instrument in the Facility, your lab will be responsible for the repair cost.

f. **BE GENTLE** with the scope! Focusing mechanism has a precision in the order of 10-50nm, cracking it too fast will damage it. Prisms are precisely aligned, excessive force will cause misalignments.

g. If you need perfusion setup on any scope, be extremely careful to check for leaks. Use minimum amount of perfusion solution and ensure no excessive amount of solution is close to scope or electronics. Talk to Facility staff first!

3) Computer Data storage and backup

a. Users are responsible for their own data. ***The Facility is not responsible for any data loss*** *due to whatever reason (either accidental deletion or hardware/Operating system failure).* Therefore, back up your data!

b. Any data left on computers in the Facility **older than 6 months** will subject to **deletion** without notice (we do send a notice out before reinforce the rule though).

c. **Data left on Desktop and/or we could not identify the owner** will be deleted from the systems. Always save data in your own folders.

4) Instrument access:

a. All instruments in the Facility can be booked on first come first serve bases, up to 2 weeks ahead of usage. This can be done online at the following URL:

   i. **http://www.cellnucleus.com/facilityweb/login.aspx**

b. A **user name and password** is required to access the site. Once a user is trained, s/he will be given the access. For detailed booking rules, please see the usage rules on the web site.

c. All usage must be logged! No-shows will be charged. All uses without login will be treated as no-shows.

II) Start the system

1) **Important:** ***Always turn on the Fluorescent lamp with all other hardware off!***
2) If the computer is running, but the fluorescent lamp is off and you need the fluorescent light, shut down the whole system and start from the next step.
3) If all system is down and you need fluorescent light, switch on the Fluorescence power supplier.
4) **Wait ~20-60 seconds** to let the Fluorescent lamp to ignite (the display stops flashing).
5) Switch on the power bar and press the computer power button.
6) Logon the computer with **Confocal** as user name and "**confocal2**" as password.
7) Turn on Uniblitz shutter, Stage power supplier and microscope if it is equipped with.
8) Switch on the Camera.
9) Start Metamorph by double click the **Metamorph_hardware** shortcut on desktop (there are 2, one with hardware control, other for offline use only).
10) Login as with your lab profile. If you switched on all the hardware, it should start without any warning messages. Otherwise, you need to quit the software and restart it.

III) Shut off the system.

1) Turn off Fluorescence Lamp.
2) Wipe oil off scope objective if you used oil lens. Use lens paper and never press directly on lens surface. ***Make sure Sun or Gerry show you at least once how to do this!***
3) Quit software (Metamorph).
4) Login your usage.
5) Turn off Camera.
6) Shut down computer.
7) **Turn off power bar** which will turn off all other devices.
8) COVER up microscope (**make sure again that the camera is off**)

IV) Use of the Zeiss Axioplan IIM microscope

You need at least one training session to work independently!

Most of the moving part of the microscope is motorized. You can drive the microscope through the Metamorph software or by the control buttons on the microscope. This manual guides you through some of the most often used operations.

Usage of Microscope independent of Metamorph control.

1) Change filter cubes and/or objectives:

a) Change **filter** by the 2 buttons on the left side of the microscope right below the focusing knob. The color dots in the little window on the filter wheel indicate which one is on.

b) Change **objective:** Drop the stage first is a good practice to protect the lenses from being scratched. Be careful with dry or oil lenses, familiarize with direction of the turret movement and the buttons so that dry lenses would not swing into immersion oil. Lenses can be changed by using the 2 buttons on the right side of the microscope below the focusing knob.

2) Switch between fine and course focus.

A button upper to the focus knob is the switch which toggles between fine and coarse focus. It is obvious which mode the scope is in when you look through the eye pieces.

3) Move stage

If **motorized stage** is on, use the joystick on the right side of the microscope. Press the top button will make it move REALLY fast.

To lower the stage: The stage can be lowered by the 2 buttons on the front base of the scope. This is useful for adding immersion oil, changing lenses/slides and cleaning of lens.

Remember to return the stage to upper position before power off the scope!

4) For DIC images, (using bright field microscope)

There are 3 things you need to do:

a) **Turn on** the bright field light by pressing the button next to the 3200K button on the right side of the microscope. The illumination intensity is controlled by rotating the black knob right above the power switch of the scope.

b) Setup Kholler illumination (If you do not know how, ask).

c) Set fluorescent filter wheel to DIC position (use filter wheel control button).

V) Acquire a fluorescence image (single wavelength) with Metamorph

Basically, the program acquires an image through a CCD camera. Main issue with the camera is that you have to find the best exposure time. This depends on stain intensity and the lens used.

1) Start the system as in section I.
2) Select the lens you want to use. But I recommend starting with lowest magnification (10X).
3) Place your sample on the scope. Add immersion oil if needed.
4) Direct light to the sample. This includes:

   a) Opening of the **Uniblitz shutter** (use shutter manual switch or Metamorph shutter control button);

   b) Opening of the **microscope shutter (**use microscope light source selector, located on right side of the scope (black button);

   c) Selecting the correct **filter cube** (filter wheel buttons on the left side of the microscope) and

   d) Directing the lights toward eye pieces (beam select slider on upper part of the scope).
5) Focus on you sample and choose an area you need to acquire a picture.

6) Close the shutter.

7) Direct the light toward the camera by pulling the sliding rod to the out most position (100% light goes to camera).

8) In Metamorph program, go to main **Acquire menu** and select **Acquire…**

9) An window shows up (figure 1):

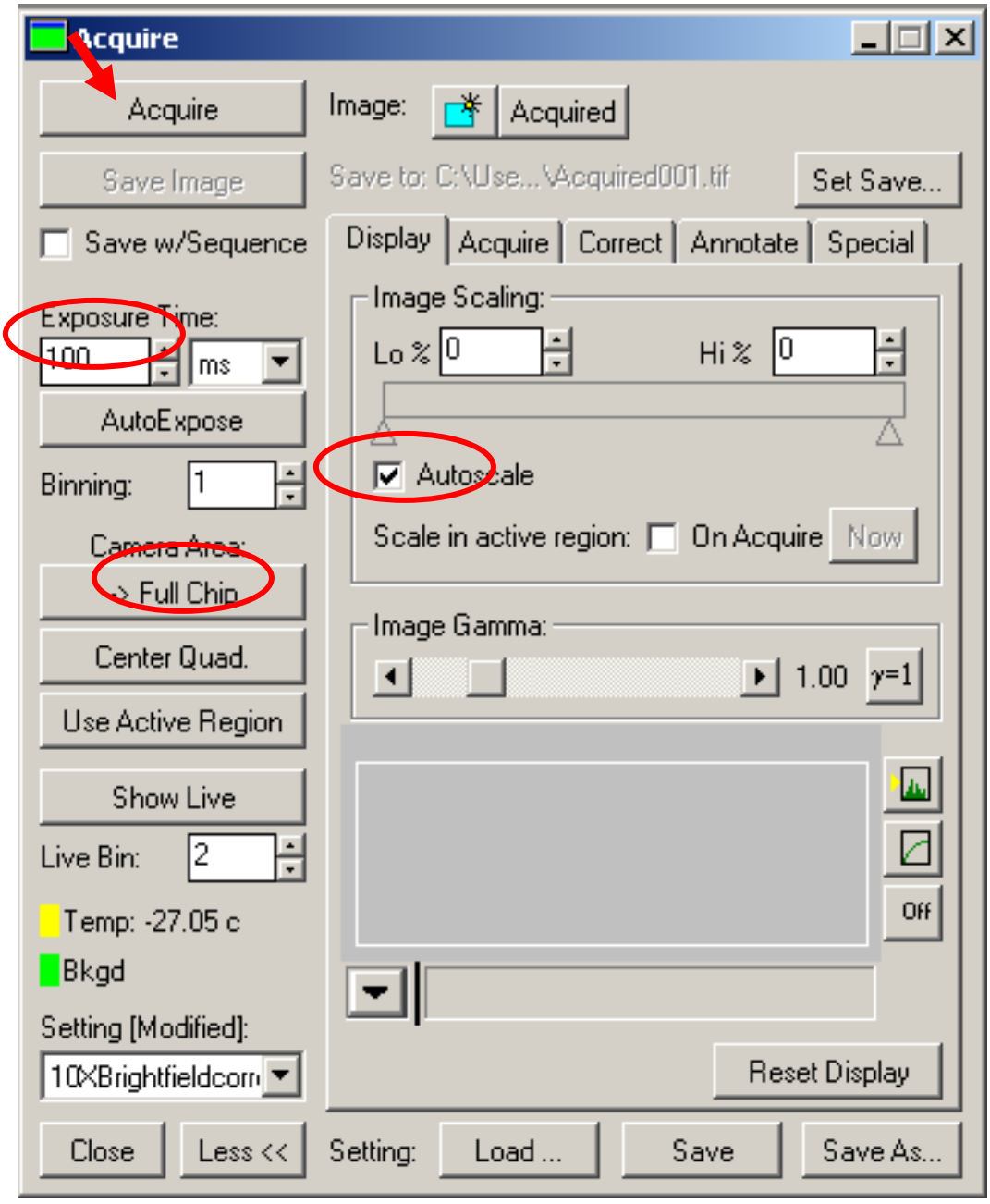


Figure 1: Acquire window. All important fields are highlighted with oval.

10) Try an **Exposure time** for the dye you are using – say 100ms

11) Make sure **Autoscale** is checked and **Camera area** is set at **full Chip**.

12) Click on **Acquire** (arrow in figure 1) button in the window.

13) An Image window will show up.

14) Uncheck **Autoscale**.

15) Look at the **high%** text box.

16) If the value indicates **4095**, it means some pixels in the image are saturated. Reduce the exposure time.

17) If the value is **below 1000**, it means the exposure time is too low. Increase it.

18) The target value should be around 3500-4000 for a bright sample with proper exposure time. However, anywhere between 1500-4000 is acceptable. If exposure is too long or the sample bleaches fast, the high pixel value should be around 1500.

19) If small area of interest is needed, use the region tool, draw an area of interest. Then click on **use active region**.

20) Click **Acquire** to acquire an image.

21) Save the picture and move to next field of view.

VI) Acquire multiple wavelength images

This is best done in **Multidimensional Acquisition** window under **Apps**.

1) Set up microscope and slide as in previous sections.

2) Go to **Apps** select **Multidimensional Acquisition …**

3) Following window shows up (Fig. 2).

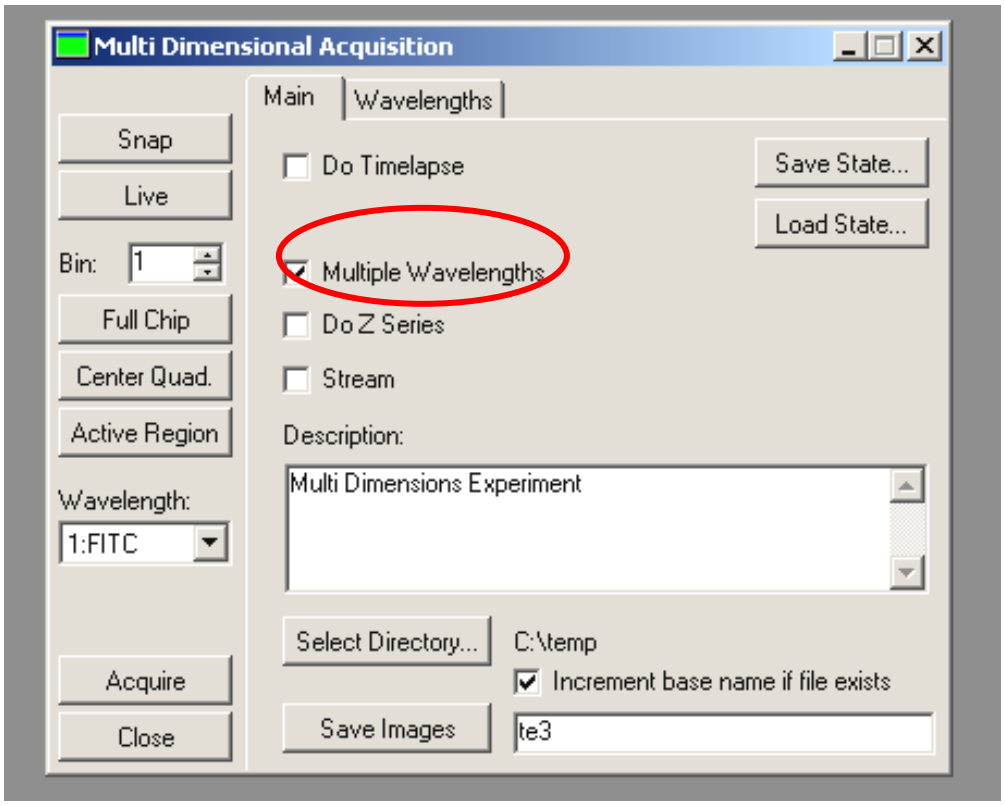


Figure 2, Multi dimensional acquisition window

4) Under the main tab, check type of experiment you want to do (e.g. **Multiple Wavelengths**).
5) Click on **Wavelengths** lab (figure 3).

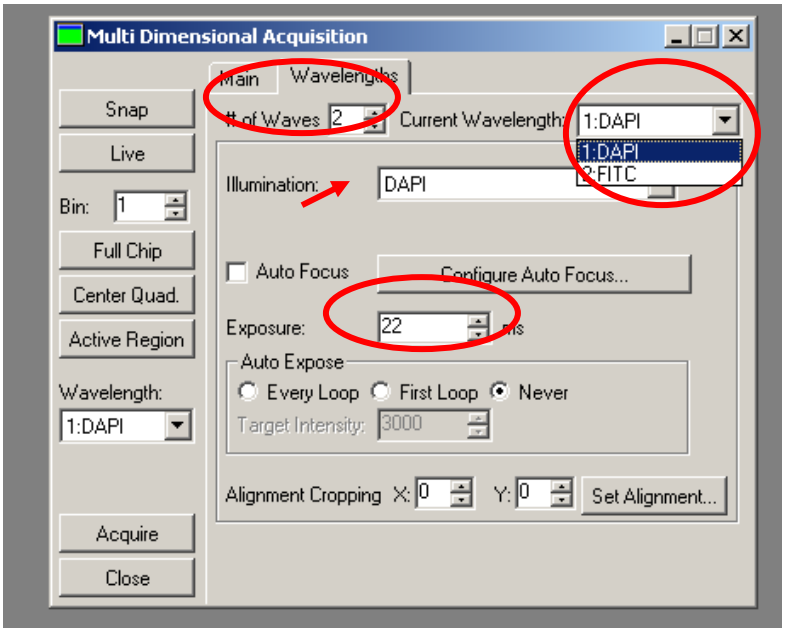


Figure 3, Multiwavength control window.

6) Enter **# wavelengths** you want to acquire (e.g. 2).
7) Select **current wavelength**. And specify the wavelength you want to acquire under **illumination** (arrow). You need to specify each wavelength for each stain you have with your sample.
8) Make sure **Autofocus** is unchecked
9) I normally determine exposure time manually (check **never** for **Auto exposure**). If you want **auto-exposure**, you can use. But it will be slow and the sample will be bleached more. For manual exposure set, enter an **exposure** value (200ms is a good start in most of the cases).
10) Click on **Full chip** to make sure the program is set to take picture at maxi. size. It will take a picture with current wavelength (DAPI).
11) Go to main Menu, select **Display** and select, **Scale…**
12) In the new **Scale Image window (fig 4)**, make sure the **Range** is set 12 bits.
13) Check **AutoScale**.
14) If you see the image, click on **live**, manually focus on the microscope for best focus. If you do not see an image, exposure is not set right. Continue on next step.
15) To determine if exposure time is appropriate, go to **scale image Window,**
16) **Check** and then **uncheck Autoscale** and read the value in **high scale.** An appropriate exposure should yield a value in the range or 3500-4000.

17) If **High scale** indicates 4095, some pixels are saturated, reduce exposure, if it is below 1000, increase exposure time. Acceptable range is 1500-4000. If exposure is too long or dye bleaches, set exposure so the high scale range is in the 1500 range. Otherwise, set it to 3500-4000.

18) Set exposure time for another channel the same way by selecting the second wavelength under **current wavelengths** in Multiple dimensional acquisition window. And go through step 10 –15 for the other channel(s).

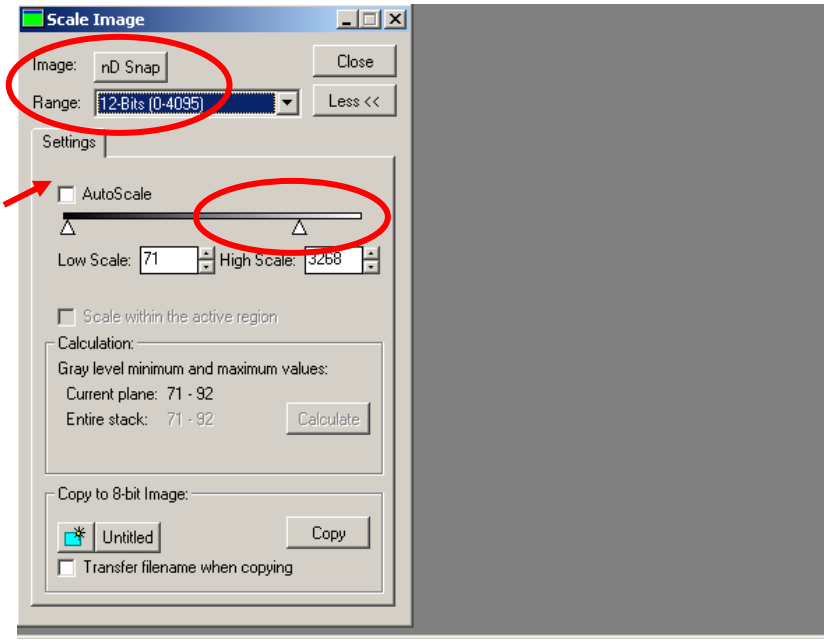


Figure 4: Scale Image window

19) Once appropriate exposure times are made, use region tool, draw rectangle region around the cells or area you want to take pictures in the snapped image window.

20) Click on use active region.

21) Click on **main** tab (**Fig 5**) . Enter experiment information if you wish in the description window. This will be saved with your image files (you can view them from Image info window under Edit in main menu). This information will only be saved if it is entered BEFORE images were taken.

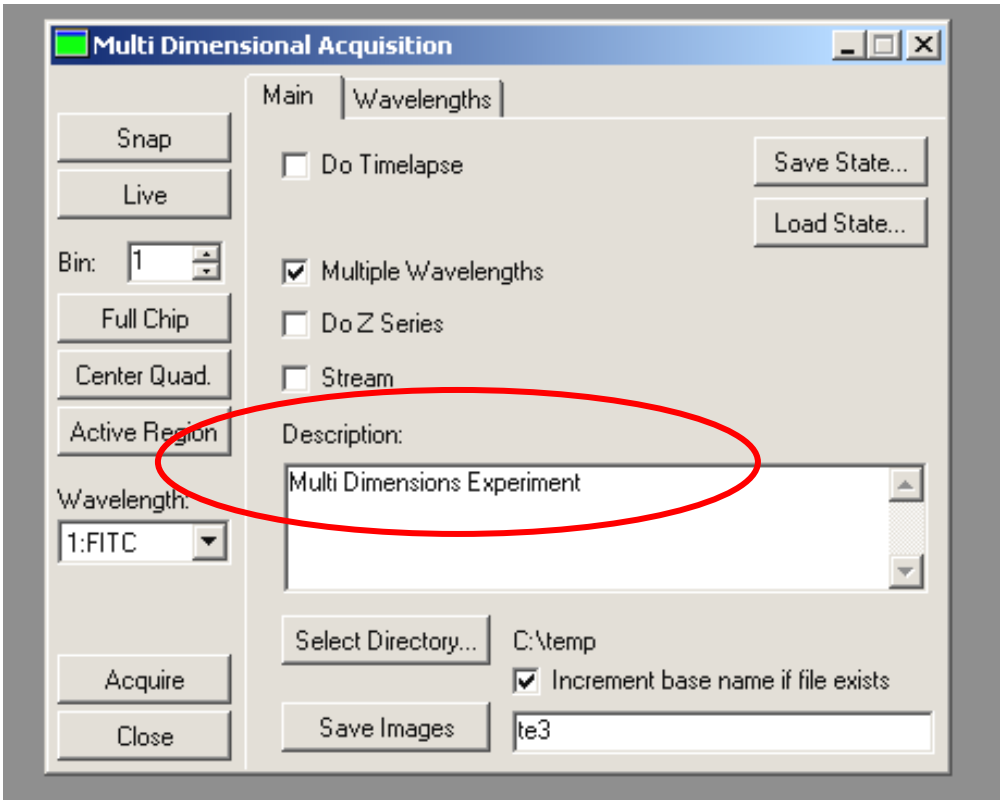


Fig. 5 Exp. Information can be entered in the description box.

22) Select the folder you want to save by clicking on **Select Directory** to specify where you want to save the images.

23) Enter base file name in **Save Images** text box. Check **Increment Base nam**e if file exists. This will automatically add a digit to last file name when you save the file.

24) Base file name needs not to be too long (especially if you want to deconvolve the images, UNIX computer does not like special characters or spaces in the files name). All information such as date, wavelengths etc. will be saved together with each image. So there is no need to be integrated into file name.

25) Save image by clicking on **Save images** button. This will save all the wavelengths.

VII) Acquire Z series

This is best done in **Multidimensional Acquisition** window under **Apps**.

1) Set up microscope and slide as in previous sections.

2) Go to **Apps** select **Multidimensional Acquisition …**

3) Following window shows up (Fig. 6).

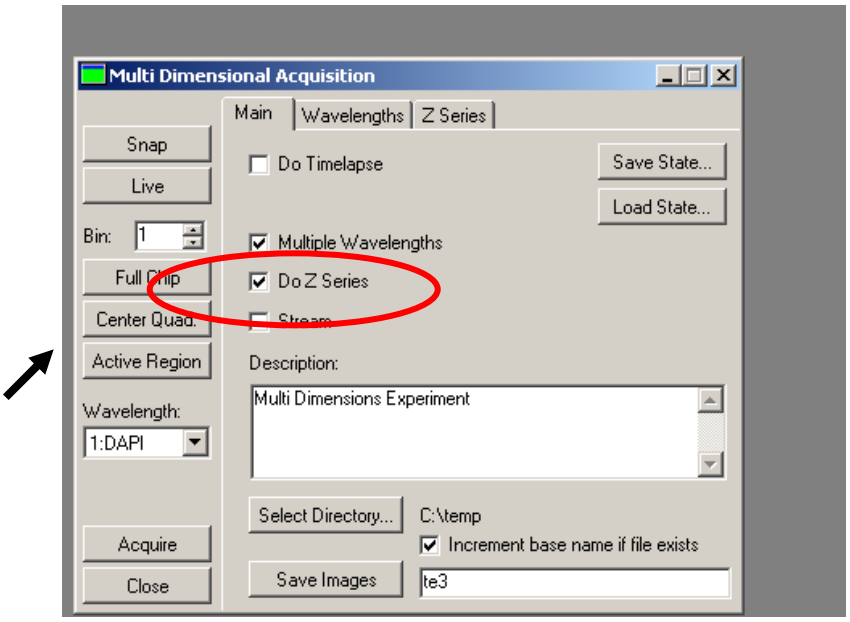


Figure 6, Multi dimensional acquisition window. For z-series, check **Do Z series** box

4) Check type of experiment you need (e.g. Multiwavelength, z-series)

5) Set up **exposure time** and **wavelengths** as in previous section.

6) Under the main tab, check **Do Z series** box.

7) Click on **Z-series** lab (**Figure** 7).

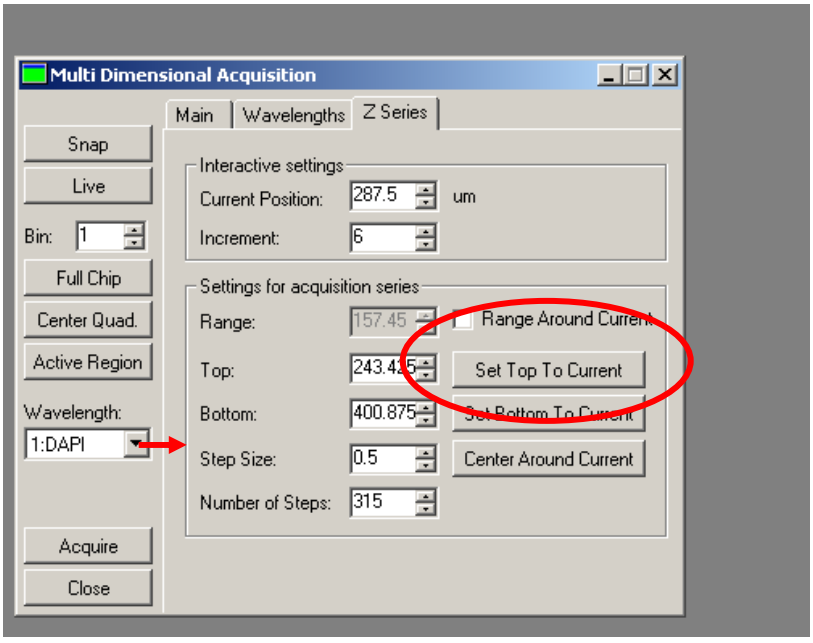


Figure 7, Z series control window.

8) For Z series, you need to define 3 parameters: a) where the top of your specimen is and b) where the bottom of the specimen is and c) what is the z step size. To set these parameters,

9) Select a wavelength which is more stable and do not have too long exposure time.

10) Click on live

11) Manually focus to the top of the cell and click **Set Top to Current** (oval in **Fig 7**).

12) Then manually focus to the bottom of the cell and clickc **Set bottom to current**.

13) Then stop live image (F2). Enter a z step size (arrow in Fig 7). This value can be 0.2-0.5um for 40X above oil lenses we have. Generally, for stable dyes and/or deconvolution, use 0.2 for less stable ones, use 0.5.

14) The top/bottom setting is not critical so long as you have all the structure included. But if you want to perform deconvolution (a mathematical way to remove out of focus light), you need to collect at least 50% more on top and 50% more on bottom.

15) Collect the Z series by click on **Acquire** button.

16) Save the stack image.

Note: Alternatively, if you do not have to set the top and bottom precisely (specially for deconvolution) or if your specimen bleaches quickly, you can use the range around current function to do 3-D stack. This is specially useful for mono layer of cells where thickness of cells does not change greatly. To use this function:

1) Focus in the middle of the cell
2) Check **Range around current**.
3) Enter a range to thickness you want to collect (e.g. for adherent cells, its typically **10-15um**).
4) Enter a step size (**0.2-0.5)**.
5) Click **Acquire**.

This often over shoot a bit which is required for deconvolution anyway.

VIII) Overlay multiple wavelength images (obtain a color image from multiple staining)

1) In MM main menu, go to **Display**, select **Overlay Images….**
2) In new window (**Fig 8**), select the number of images to overlay (there is always a B/W image. So it should be the number of channels (3) you have plus one).
3) If multiple wavelengths are individual image windows, **uncheck Images from Stack**.
4) Check **Auto balance**, which works well in most of the time.
5) Uncheck boost colocalization (often, this will yield an overly contrasted image).
6) Assign color for each channel. This is done in hue selection window. You can create whatever color you want. But pure RGB is best (in the pull down menu (oval)).
7) Select source images for each channel (e.g. DAPI for blue channel, FITC for green, etc).
8) Check **Show preview** box. This will give a preview of a small area. You can drag the box (arrow) in the preview image area to see how new image look like.
9) If image is too dark, you can
    a) Boost its brightness by increasing the brightness of the original window or

b) Uncheck **auto balance** and change the **balance** (**Bal)** value for each channel or increase overlay brightness.

10) Click **Apply**, a new overlay image appears.

11) Save the image if you like.

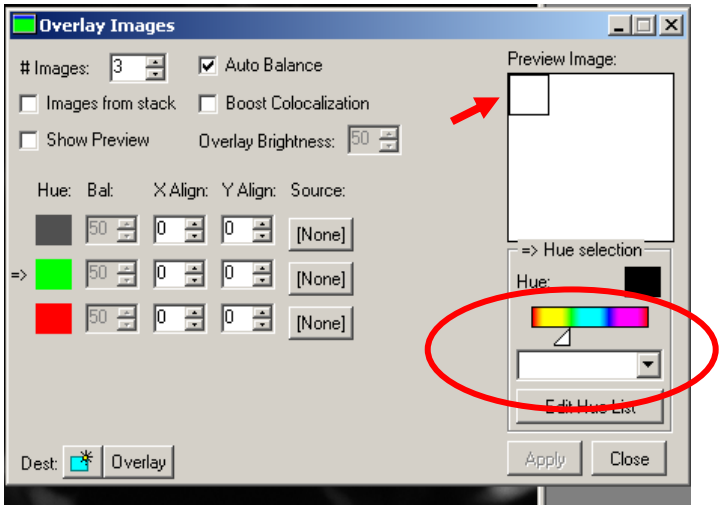


Figure 8: Overlay image window.

IX) Common Task/questions:

1) How to collect images of fixed sizes.

a. You can use fixed region tool to always collect images of the same sizes. To do this.

b. Go to main Menu tool bar, click on Region property (arrow in Fig. 9).

c. Check **Lock region size** (oval in fig 9) and enter the size you want (circle in Fig 9, e.g. 500X500).

d. If you cannot draw a region freely, it is because someone checked this option. Uncheck it and it will disable it.

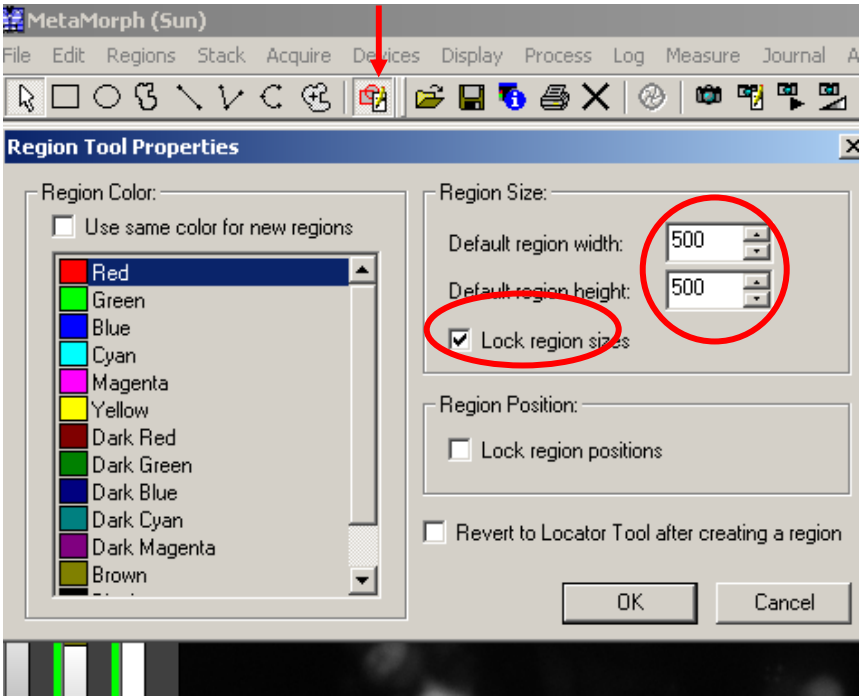


Figure 9, Region tool property

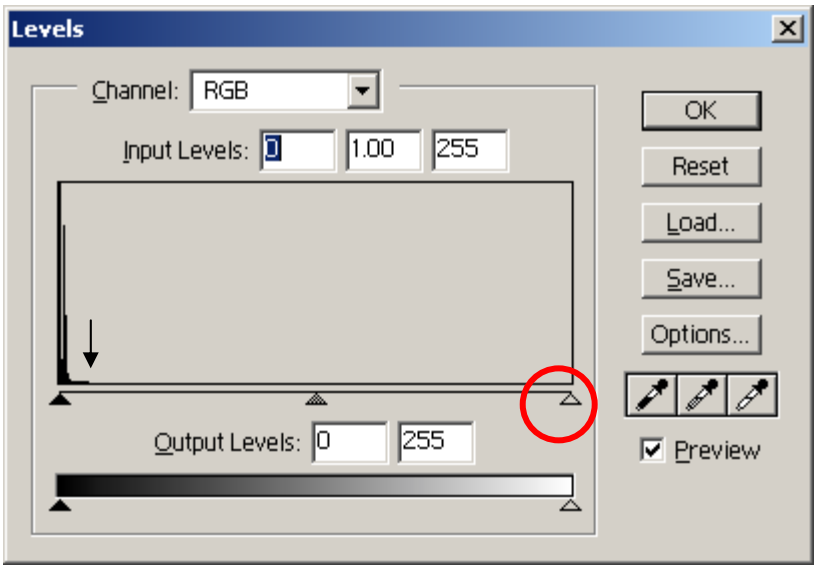
2) Open 16 bit tif images of Metamorph in Photoshop

a. All image files in Metamorph are saved either as 16 bit tiff or stacks. You cannot open stacks in Photoshop (you need to export it as series of tiffs). This section deals with individual Metamorph files.

b. In photoshop, open the file as usual. If the file does not open, it means it is either a stack file or you saved the file as pseudo-color. Then you have to either export the stack as series of tiffs or resave the file as monochrome tiff in Metamorph.

c. The window will be totally black because Photoshop displays only 8 bit image.

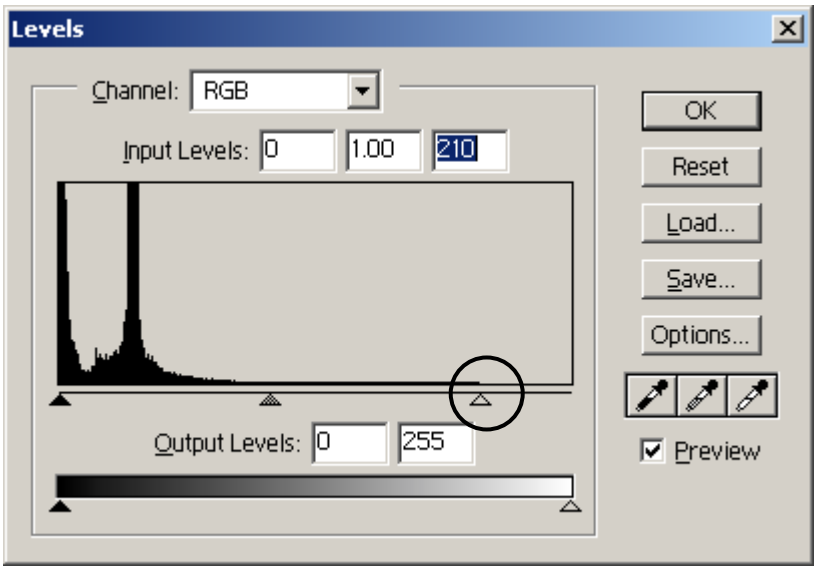
d. Press **ctrl_L** together, you will have a new window:



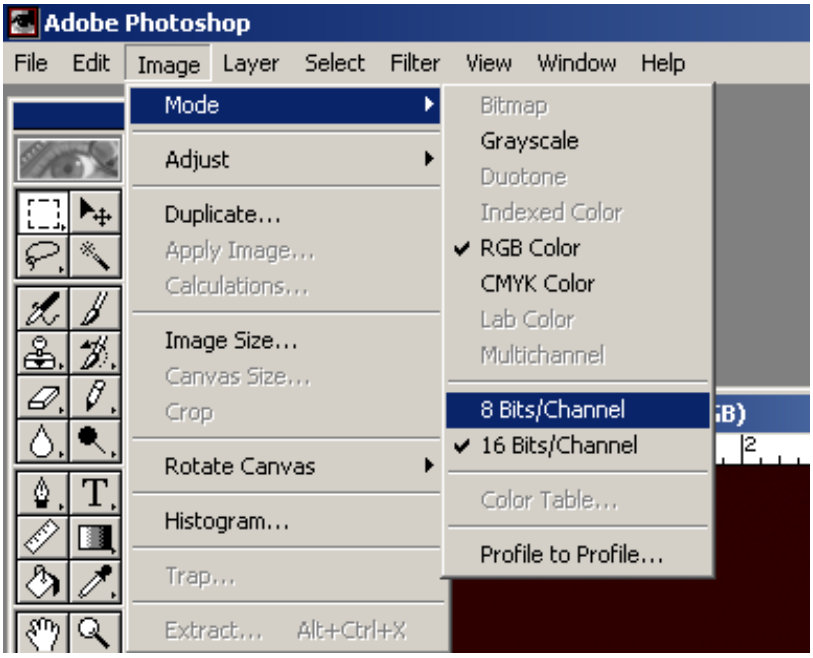
e. Drag the open triangle (Circle) to the first column in the right (arrow) in the image histogram. Be careful not drag the triangle too far.

f. Click OK.

g. Repeat step d-f. This time, the histogram is easier to be seen. Drag the open triangle to the first column at the right end of the histogram.



h. Click ok and go to main menu, Image, select mode and choose 8 bit.



i. Save the image. This process converts a 16 bit file into an 8 bit one. Do not overwrite the original file as some information is lost during this process.

3) Making 3-d reconstruction in Metamorph.

Three-d reconstruction for fluorescence images is best made in Imaris which is true 3-d software. However Metamorph allows you to reconstruct with min or average pixel value. This is sometimes useful for bright field images.

a) Open 3-D stack file

b) Go to Main Menu select **Stack**, select **3-d reconstruction…**.

c) In the new widow (Figure 10), check **Custom** in **predefined angles**.

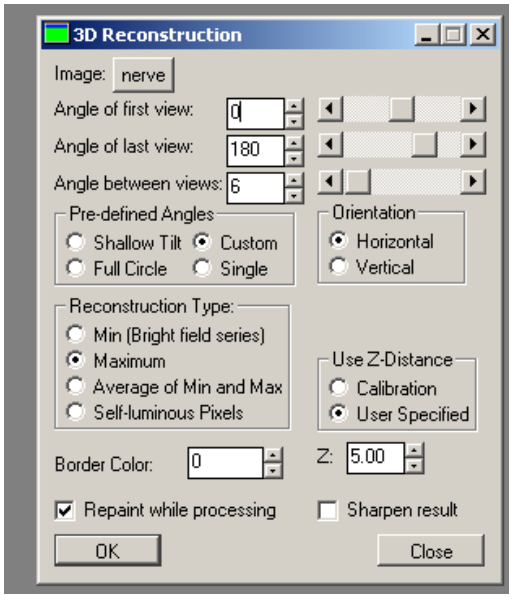


Figure 10. 3-D reconstruction window.

d) Enter the following values:

   i) Angle of first view: 0;

   ii) Angle of last view: 180;

   iii) Angle between view: 6.

   iv) Check the following options: **Maximum** for **reconstruction** type (for fluorescence); **User specified** for Use Z-distance.

   v) Enter the proper z value. This is the ratio between z/x or y dimension of the pixels. Here are pixel dimensions of lenses we have:

   100X, 0.064um, 63X: 0.102um, 40X, 0.16, 20X: 0.31; You need to calculate the ratio according to the z step of the image you have. (e.g. for 63X with z step of 0.3um, the value should be 0.3/0.102 =~3).

e) Click Ok

f) Reconstruction will start and then you can make a movie to view it.

4) Making Movies in Metamorph.

There are 2 types of movies: Movie to view on screen or movie to be saved no hard disk for presentation.

For quick view on screen, first Zoom the image to appropriate size. Go to main Menu, select Stack and choose Movie. Check F for forward playing. And check S to make window black other than the movie.

To make a movie to save on HD.

a) Process the image as you wish. (such as:

   i) Brightness/contrast, Gamma adjustment,

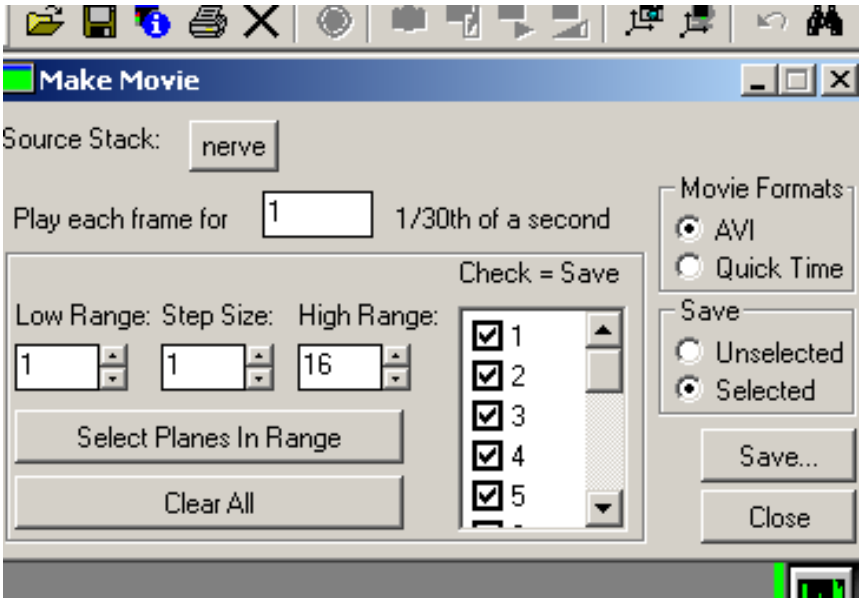
ii) Pseudo-coloring;

iii) Crop out all unnecessary information as a movie file will become too big to insert into powerpoint.

iv) Remove unnecessary frames (see following section).

v) Preview the movie with above instruction.

b) Go to Main menu, select **Stack** and choose **Make Movie.**



c) In the new Window, check the type of movie you want to make, in the **movie formats** field. For PCs, make AVI type. For Mac, choose Quicktime.

d) Set length of time for playing each frame. Typical value is 3 (100ms/frame).

e) Select the frames you want to make the movie. This will affect file size. If your movie file is too big, try to select every other frame (choose a step size of 2 and then clear all and select planes in the range).