Quick Reference Guide for DMC Marianas Users

This guide is for using the Marianas with Slidebook 6.0 software. Instructions on advanced imaging methods can be found in the Slidebook software by pressing 'F1'. The "SlideBook 5.5 Manual.pdf" from Intelligent-Imaging Innovations, Inc. is on the Core C Website. However, Slidebook 6 provides new features and a redesigned interface.

SlideBook is organized using a graphical menu and icon system. There is a customizable *Quick Access toolbar* across the top of the application with the rest of the program functionality being found in a tabbed system. The tabs are designed to group functionalities and improve workflow while minimizing the number of clicks required to accomplish tasks.

The exact arrangement of the command groups in each tab may vary from the example below or as seen in the Slidebook Help (F1).

The Slidebook Tutorial is an excellent place to become familiar with the software. Press 'F1' to open the help menu, search for 'Tutorial' or scroll in the index. Several topics are available.

Marianas Start Up and Shut Down.

Starting Slidebook with microscope.

1. Reserve your usage period on the web-based calendar: cajal.oto.washington.edu/corec;
2. Turn on the fluorescent lamp "**A**" (black box on shelf to right of microscope);
3. Turn on the power strip "**B**" under the shelf, back left of the enclosure, (microscope, filters and main camera);
4. [Optional] Evolve camera, power on;
    a. push button switch under its near side, below the label "On/Off"
5. Press the button labeled "**FL**" on the right side of the microscope (Figure 3)
    a. the condenser tower displays "FL" in its upper right corner
6. Inspect the positions of the fluorescence apertures and ND filters (Figure 5);
    a. both levers should be fully up
    b. the ND slider should be fully inserted to position 3
7. Turn on the computer and log in as the Marianas user;
8. Start the Slidebook 6.0 software (yellow icon in the taskbar);
    a. ignore error messages that "Camera 01 cannot be found" by pressing 'OK'. They only indicate that the Evolve is not turned on.
    b. you might see a message to try again to initialize the microscope - press 'OK'
9. Mount your specimen in the stage, coverslip facing down (it is an inverted microscope);
10. Open Slidebook's Focus Window by Home>Focus icon.

Software only, without microscope.

1. Reserve your usage period on the web-based calendar: cajal.oto.washington.edu/corec;
2. Turn on the computer and log in as the Marianas user;
3. Hold down the shift key while starting Slidebook to ignore the microscope;
    a. there is a Slidebook software workstation located in the inner room, same login

Shut down

1. Remove specimen from stage;
2. If working with oil or water immersion objectives, clean objectives and any spills;
3. Inspect the microscope, air table and bench for spills and debris;
    a. rinse ANY saline or culture media splatter with distilled water to remove the salt
4. Select the 10X objective, then return microscope to focus position;
5. Transfer files to personal media or servers;
6. Delete files from Marianas computer hard drives (D: or F:);
7. Quit Slidebook;
    a. if someone is signed up within the next 3 hours, leave everything on
8. Turn off equipment (computer, xenon lamp, power strip) in any order (see #6 and #7);
9. Turn off Evolve camera, if used;
10. Clean up any other messes you might have made;
11. Close the microscope enclosure, it doubles as a dust cover;
12. If the amount of time taken for the imaging session differs from the reserved time, please edit your reservation in the on-line calendar.
13. Remove any trash, pipet tips, waste liquids, broken glass, styrofoam containers, etc.

Reserving the Marianas.

1. ALL USE MUST BE RESERVED IN THE CALENDAR: http://cajal.oto.washington.edu/corec/.
2. User names do not appear in the list until the user has received training from DMC staff or other designated individuals.
   a. Secondhand training from other users is not allowed
3. A valid budget number may be added in the 'Notes' section when making a reservation;
4. Instruction on reserving time is given at the time of training;
5. A link to the on-line instructions is at the top of the calendar, "**Using this Calendar**".

View brightfield/DIC images

(for single plane, single channel capture)

See 'Optimizing the Image' for details.

Press 'F1' at any time for a comprehensive Help resource.

Note that Slidebook is configured to switch the beamsplitter for either camera or eyepieces when any filter is chosen from the selected filter set.

1. Open the Focus Window from Home>Focus icon;
2. Turn on the halogen lamp by clicking "Open Bright" button on the right of the window
   a. adjust intensity with 'Lamp' slider, upper right of Focus Window
      i. [manual control] turn on lamp by pressing the button labeled "HAL on/off" on the front, right side of the scope (Figures 2,3)
      ii. [manual control] intensity control is a rocker switch on the front of the scope (Figure 1)
3. Change magnification by clicking on an objective button in the Focus Window;
4. Select "Eyepieces" filters from the Focus Window (default);
   a. Select "BFvis" filter for brightfield
   b. Select "DICvis" for DIC
      i. rotate the polarizer to 0 degrees, see Figure 5
5. Route the image to the eyepieces by cliitter (in the 'Scope' tab)
   a. select "100% Eyepieces" in the Focus Window
   b. [option] click eyepieces icon next to the camera selector
6. Focus on your sample and position it with the stage controller joystick;
   a. joystick powers up with 'Fast' motion, press button on joystick tip for 'Slow'
   b. note the "f" and "s" in the joystick display, right end of X and Y lines
7. Set Koehler Illumination (see chapter in this binder).;
8. Review specimen in eyepieces.

Capture Brightfield/DIC images
(for single plane, single channel capture)
A brief explanation on capturing a single channel, single plane image. Underlined words refer to command tabs. Refer to the Slidebook manual or press 'F1' for more advanced capture options such as multi-channel capture, 3D, 4D, montage, etc. Also refer to 'Optimizing Images' in this guide. Note that Slidebook is configured to switch the beamsplitter for either camera or eyepieces when any filter is chosen from the selected filter set.

1. Open the Focus Window from <u>Home</u>>Focus icon;
2. Select the "HQ2" filters from 'Filter Set:' in the Focus Window
    a. select "BF" filter or "DIC" filter
3. The beamsplitter switches to the camera when a filter is selected
    a. [option] select "CoolSnap HQ" in the Focus Window
4. Click on the "Live" button to begin camera capture
    a. camera is active by default when Focus Window is opened
5. Turn on the halogen lamp with "Open Bright"
    a. software waits 2 seconds for the halogen lamp to reach full intensity
6. Adjust lamp intensity with the slider in upper right of focus window or switch on microscope;
7. Adjust exposure time with the slider in the focus window;
8. Focus your specimen while viewing the live camera display;
9. Click on <u>Home</u>>Capture icon in the toolbar;
10. Select the "HQ2" filters in the Capture Window
    a. select "BF" filter for bright field or "DIC" for DIC
11. Use the 'Test'or 'Live', and 'Stop' buttons to assess exposure times and fine focus
    a. if using 'Live', click it again after changing exposure time
    b. default exposure time is 100 ms, enter a new value to change
12. Adjust image intensities by changing exposure time and lamp voltage
    a. intensities don't need to be more than 2/3 of the histogram
    b. 'Test Once' calculates an exposure time to change the intensity by 50%
    c. 'Best Exposure' calculates an exposure that fills the histogram
13. Capture only a portion of the field:
    a. drag a box over the area in the focus window that you want to capture
    b. click the button labeled "Update" near the right of the Capture window
14. Enter a name for for the image at the bottom of the window, and click "OK"
15. Slide book captures the image and adds it to the open slide file;

Exposure times and lamp intensity for transmitted light images should be sufficient to avoid saturation and yet allow visualization of dark (opaque) objects.

View Fluorescent Images (eyepieces).

Note that Slidebook is configured to switch the beamsplitter for either camera or eyepieces when any filter is chosen from the selected filter set.

1. Open the Focus Window from <u>Home</u>>Focus icon;
2. Select 'Eyepieces' filter list (default);
3. Select the desired fluorescent filter:
    a. select 'Triple' to excite DAPI, FITC, Cy3 simultaneously
    b. the other buttons select for individual fluorophores
4. Click on 'Open Fluor' in the Focus Window
    a. *Prevent photobleaching: open the fluorescence shutter <u>only</u> while actually visualizing the specimen, not while taking notes or answering your phone!*
5. Adjust focus and frame the field of view;
6. If fluorescence is too bright with short excitation time, then reduce excitation intensity:
    a. [recommended] use the ND filter slider at the back of the microscope
        i. position 3 is 100% transmission
        ii. filters in positions 1 and 2 may be changed to reduce the amount of light
    b. the aperture iris ('A') also reduces fluorescent excitation (Figure 4) by some non-reproducible amount
7. Click on 'Close Fluor' in the Focus Window when finished

Capture Fluorescence Images

(single plane, single channel capture)

Advanced capture options such as multi-channel capture, 3D, 4D, montage, etc. are described in the Slidebook manual. See 'Optimizing the Exposure' in this document.

1. Select the "HQ2" filters from 'Filter Set:' in the Focus Window
    a. selecting any filter will switch the beamsplitter to the camera
2. The Focus Window opens with the camera active, controlled by the 'Live/Stop' button;
3. Open the lamp shutter with 'Open Fluor';
4. Re-focus your sample while observing the live camera window;
    a. if there is no image to the focus window:
        i. check "100% HQ2" in the Focus Window
        ii. check that the 'Camera:' selector is set to "PM1394Camera00"
        iii. is there excitation light to the sample
5. Control intensity with the camera exposure time slider in the Focus window;
6. Close the shutter;
7. Click on <u>Home</u>>Capture" to open Capture window;
8. Select the "HQ2" filter set
    a. select the desired fluorescent channel
9. Click on the "Test" button to get an initial image;
10. Adjust the exposure time
    a. click the "Test Once" button or enter a new value for 'Test'
    b. the 'Live' button allows viewing exposure on a live image
    c. press the 'Live' button each time the exposure time is changed
11. Capture the image

       a. enter an image name at the bottom of the window, comments are optional

       b. click 'OK'

12. Slidebook will capture the image and display it in the active slide window;

       a. see 'Optimizing the Exposure', below

13. Capturing only a portion of the field:

       a. drag a box over the area you want to capture

       b. click the button labeled 'Update'

       c. return to full chip by clicking on 'Full Chip'

Control Fluorescent Excitation

Figure 4 shows the position of the 3 controls for excitation intensity:

1. The Field Stop (F) controls the diameter of the excitation light in the field of view;
2. The Fluorescence Aperture (A) controls the intensity of the excitation light
   a. provides poor control and reproducibility
3. The Neutral Density slider has 3 positions:
   a. Position 3 transmits 100% of incoming light
   b. Position 2 transmits 10%
   c. Position 1 is currently empty but other ND filters are available

ND slider positions 1 and 2 can be easily changed to different filters.

Optimize the Captured Image

Remember, <u>any</u> light exposure causes photobleaching. Observe the histogram in the Capture window as you test the exposure. Exposure times that display the brightest intensities at 1/3 to 1/2 of the maximum histogram is usually sufficient for fluorescent imaging. Attempting to fully fill the histogram usually causes high levels of photobleaching with little gain in signal to noise ratio. Sometimes, the histogram must be filled, even saturating the brightest structures, in order to image weakly fluorescing regions.

Monitoring Image Appearance

1. Slidebook defaults to display the focus window with histogram stretch to make the dimmest images visible.
2. The best way to monitor image intensity is to observe the histogram while testing exposure time. The histogram is more objective than the images's appearance.
3. A grainy image indicates little light is reaching the camera and noise is very significant.
4. A red mask indicates saturated pixels. No information regarding spatial or intensity variation is possible from saturated pixels.
5. White regions lacking visible detail are over-exposed and close to saturation.
6. A solid black image indicates too much light is going to the camera and protective circuitry is blanking the chip to prevent damage.
7. Apply image corrections for dark field and flat field.
8. Make sure your coverslip is 170 μm thick (#1.5) and clean.

<u>Image Corrections.</u>

Dark field and flat field corrections remove image artifacts originating in the microscope and electronics. The HQ2 camera and electronics add about 100 intensity units to every image pixel. This elevates background and alters measurements of low intensities. Illumination sources rarely

illuminate the field of view evenly, and there may be aberrations from the imaging optics. Such non-uniformities across the field of view affect image quality and may alter measured intensities.

Slidebook can correct these aberrations at acquisition. Read the section on Image Corrections for full details and how to apply correction to images that have already been captured and saved.

1. Perform dark field correction to subtract camera and electronic artifacts
    a. Click the 'Dark Field' check box in Capture window (just above the image name box)
2. Perform Flatfield correction to normalize the field of view
    a. Click the 'Flatfield' check box in Capture window (just above the image name box)

Which Camera to Use?

The Marianas is equipped with 2 cooled, monochrome CCD cameras from Photometrics, designed for distinctly different imaging capabilities.
1. **CoolSnap HQ2** - recommended for "routine" imaging, high resolution, , brightfield and relatively bright fluorescence.
2. **Evolve 512** - designed for weak fluorescence, short exposure times, low excitation levels for live cells, provides much less resolution than the HQ2.

Switching Between Cameras.

Note that Slidebook is configured to switch the beamsplitter for either camera or eyepieces when any filter is chosen from the selected filter set.
1. Open the Slidebook Focus window;
2. Select the filter set for the desired camera;
3. Select a filter by clicking on its button.
4. Manually setting the camera:
    a. find the "Camera:" menu, between the objective buttons and the Exposure time slider
    b. HQ2 - select "PM1394Camera00"
    c. Evolve - select "PM1394Camera01" (sorry, naming is limited by the camera driver)
        i. the Evolve has a separate Gain slider which must be kept below 900
5. The beamsplitter ("Emission Selection") will switch automatically when changing between cameras, or click on the desired camera name in the beamsplitter window;
6. Select the filter set for the selected camera - each camera must use its own filter set;
7. Choose the desired filter;
8. Set exposure time
    a. 100 ms is a good starting point for the HQ2
    b. ~25 ms for the Evolve
9. Note: When switching from the Evolve to the HQ2, click on 'Full Chip' in the Capture Window, or else Slidebook will read only a 512x512 region from the HQ2;

The Slidebook Cook Book.

Basic operations that make microscopy easier.

I. Display Image Channels.

• The image view window will display up to 4 channels by default.
• A channel will be displayed in color if it is defined with a color, some are not.
• "None" (no channel) is selected to display channels collected with filters that do not have a default color selection;
• The number of displayed channels will change according to image content;

A. Channel Appearance.

1. Select a channel by clicking on its name, e.g. "DAPI, "FITC", "DIC", "None", etc.;
2. The selected image will appear in that display channel.

B. Assign Channel Color.

1. Click on Home>'Mode'>'User Defined Color';
2. Click on a small square appearing to the left of each channel;
3. Select the desired color;
4. Click on 'OK'.

II. XY Stage Control.

Use the motorized stage to speed your research.

A. Stage Speed.

The motorized XY stage has 2 speeds: "Slow" and "Fast" (default). The push button on the top of the joystick toggles between speeds each time it is pressed. The current speed setting is indicated by "f" or "s" at the right hand end of the X and Y coordinate display on the controller.

B. Centering the Camera Field.

Automatically center the specimen in a live camera display.
1. Open the Home command tab;
2. Open the 'Tools' menu (White Arrow icon);
3. Click on 'Select' (arrow icon);
4. Double click on any point in a live camera window;
5. The stage moves that point to the center of the camera field.
Note: You must be 'Live' to see the result, although the stage moves even if the camera is stopped.

Works with both Focus and Capture windows.

C. Maintaining Field when Switching Lenses.

This trick reduces time spent hunting for the specimen after switching from dry lens to an immersion objective lens.
1. Find the desired field of view with a low magnification objective;
2. Center the field or item of interest, and bring into focus;
3. Close shutter;

4. Open the "XY' tab in the Focus Window;
5. Click on 'Set Point';
6. Return to the 'Scope' tab;
7. Select the immersion objective;
8. Lower the immersion objective (manual de-focus or press the down focus button on microscope, Figure 3);
9. Drive the specimen out of the way with the joystick;
10. Apply immersion oil or water to the lens, as appropriate;
11. Go to XY tab again;
12. Click on 'Visit Point' (or double click on that point in the list);
13. The stage will position itself over the lens and the lens will raise;
14. Open the shutter;
15. Start camera, if necessary, or view by eyepieces;
16. Adjust fine focus.

https://www.intelligent-imaging.com/techanswers.php
How can I read SlideBook SLD files in ImageJ FIJI?

III. File Export.

There are 2 options for opening the *.sld files outside of Slidebook.
1. Bio-Formats plugin for Fiji opens *.sld files on computers properly configured, section A.
2. Export as TIFF, Matlab or video, as explained in the remaining sections B - J.

The Slidebook file format (*.sld) cannot be opened by most other software. However, Slidebook can export images in formats suitable for quantitative use or for qualitative purposes (journal figures and projection images). Time-lapse and 3D images can exported as animations (movies).

Image intensities are digitized at 14-bits/pixel (0-16,383) for the CoolSnap HQ2 at at16-bits/pixel (0-32,767) for the Evolve 512. Bit-depths above 8-bits must be stored in a 16-bit file to preserve the recorded intensities. This is essential for most image processing, including deconvolution and intensity-based quantitation. Saving as 8-bits per pixel (or 24-bits: 3 channels of 8-bit pixels) will scale the original intensity range into only 256 intensity values, adequate for simple morphometric analysis (how many, how big) and for presentation use. Exporting as 8-bits is not acceptable for image processing for feature extraction or for intensity based measurements.

Press 'F1' in Slidebook for Help, search for "export" and select 'Topic: Export'.

A.   Opening *.sld files in Fiji;

As of August, 2016, the Bio-Formats plugin for Fiji can open Slidebook files. This requires that you have a current version of Fiji and Java version 8 installed on your computer. (Java 8 is also called "Java 1.8"). It is very convenient since you only need to work with 1 copy of your *.sld file instead of multiple copies of TIFFs. Also, Fiji reads metadata, so you will always have pixel size, z-step and time interval available. These are usually lost by the TIFF format.

Fiji is a Java based, public domain software application that will run on MacOSX, Windows and various flavors of Linux. The Bio-Formats plugin for Fiji can open ~170 file formats and save to about 2 dozen formats, and is bundled with Fiji. You will need the following on your computer:

1. Current version of Fiji;
2. Java 8 (also known as version 1.8);
3. The Slidebook file library.

1. You don't have Fiji installed?

This website will guide you through installation of Fiji:
http://fiji.sc/#download

You may need to change Fiji's memory allocation to no more than 75% of your computer's RAM. Open Fiji, Edit>Options>Memory& Threads> enter the RAM. Don't worry, it will only take what it needs when it has to open a large file. You can also open files with Bio-Formats as "Virtual Stacks", in which only the currently viewed image is actually held in RAM.

2. Install or update to Java 8.

Java installation instructions:
https://java.com/en/download/help/mac_install.xml

Download the latest Java SE Development Kit for your platform

http://www.oracle.com/technetwork/java/javase/downloads/jdk8-downloads-2133151.html

Click the 'Accept License Agreement' then click on the link in the right hand column for your operating system. Install by opening the downloaded file and following the instructions. Note: the runtime version of Java is only for web browsers and will not support Fiji.

3. Install the Slidebook library.

Details for setting up Fiji from the Slidebook website:
https://www.intelligent-imaging.com/techanswers.php - SLDinFiji

B. Exporting Images from Slidebook.

Export images in 16-bit file formats to preserve full intensity range for deconvolution, intensity-based quantitation and most image processing. It sounds more complicated that it really is.

1. 16-bit TIFF (OME).

Slidebook offers 2 options for 16-bit TIFF export: TIFF and OME-XML TIFF. Both export each channel as a single file. Both formats provide the option to write a text file containing image information, named as filename.log, where "filename" is the name of the first channel of the image (shortest wavelength), e.g. "Pretty Cell 17_C0.log", in addition to "Pretty Cell 17_C0.tif".

TIFF: does not retain metadata such as pixel size, z-step or channel and collection information. The metadata is solely in the log file.

OME TIFF: stores metadata in a separate XML file that can be read by Bio-formats, Huygens and other software recognizing the OME TIFF format. Other software will open an OME TIFF as a standard TIFF. Slidebook implements the XML format as a separate metadata file "Pretty Cell 17_C0.xml". Usually, software that reads the XML TIFF can be directed to open the first channel of the multi-channel images, "Pretty Cell 17_C0.tif", and will automatically locate by matching the filename and read "Pretty Cell 17_C0.xml" to obtain metadata and presence of additional channels. Usually, the additional channels are opened and merged, but there is variation in implementation.

2. MATLAB.
   Exports images in the format used by Matlab for data analysis.

C. Exporting Quantitative images;

1. Open a Slide file and highlight an image to export (it does not have to opened);
2. <u>Home</u>>Export menu (yellow arrow icon)>Quantitative Export>16-bit TIFF (OME);
3. Browse to the destination directory;
4. Select 'Write Log File;
5. Under 'Advanced'
   a. Select 'Include OME XML'
   b. Edit name template from:
      %N_XY%P_Z%Z_T%T_C%C
   c. Set to:
      %N_%c - names files by the entered name and channel name, e.g. "16-cmyc_DAPI.tiff"
         the other %variables designate XY coordinates, z-planes, time points
6. Image Range: [optionally] choose to export only the selected image or a set of images in the Slide file;

Note: "Quantitative" images can be converted to "qualitative" images at a later date. The reverse is not true, once exported as an 8-bit or 24-bit image, or jpeg, the original intensities can never be recovered.

D. Naming Quantitative Images:

Each channel is saved in its own file.
'%N' selects the image name, as it appears in the .sld.
'%c' appends the filter name used for the channel, e.g. "Image0001_DAPI.tiff".
'%C' appends the channel number for the image, e.g. "Image0001_C0.tiff".
The log file will be named "Image0001_DAPI.log" or "Image0001_C0.log", depending on the naming scheme.
The Y, Z and T options are used when saving substacks as XY locations, z-steps or timepoints and can be deleted from the naming string.

E. Home>Export> Qualitative Export

1. Generates 8-bits per channel and 24-bit (RGB) TIFF and .AVI movies suitable only for presentations, such as journal figures, Photoshop, PowerPoint and movies.
2. Scaling intensities to 8-bits is unsuitable for deconvolution, intensity-based measurements and most image processing.

3. Z-series and time-lapse images exported under 'Tiff series' will save as 1 image file per z-step or time point. No, you still can't save an RGB z-series or time-lapse.
4. "What You See Is What You Get" (WYSIWYG). Slidebook exports 8-bit and 24-bit images exactly as they appear on the screen, including effects of histogram adjustment and gamma.
5. Channel histograms are automatically stretched by default, the image minimum intensity is set to zero and the maximum intensity is set to 255, regardless of the original values.
6. Histogram stretch may mean each channel appears equally bright, regardless of the original recorded intensity range.
7. The relative brightness between multiple channels may be retained by opening the Renormalize window and selecting 'Reset All to Selection Minimum/Maximum'. This stretches the histogram using the darkest intensity and brightest intensity from all channels.
8. 24-bit TIFF (RGB) can have no more than 3 separate channels. Additional channels will be blended into the red, green and blue RGB channels. They cannot be separated after blending.
9. If you have 4 or more channels in an image, you may want to use Quantitative Export (XML TIFF) and open the image in Fiji so that you can edit the channels before merging.
10. Each image channel of an RGB image is still 8-bits per pixel.
11. TIFF and AVI formats lose all metadata for pixel size, z-step, time interval, channel name, etc.
12. Most research software stores multi-channel images in order of shortest wavelength to longest wavelength. But, computers display the first image in an RGB as red and the last image as blue. Photoshop often opens exported RGB images with the first channel displayed as red and the red channel displayed as blue.

F. Scaling intensities - Qualitative Export.

1. Open an image view window in Slidebook;
2. Open the Renormalization window (icons at image top);
3. Select a channel;
4. Click on 'Reset to Selection Minimum/Maximum';
5. [Optional] Further adjust by dragging from the right or left edges of histogram
6. [Optional] Adjust Gamma by dragging the diagonal line in the histogram;
7. [Optional] Preserve relative channel brightness: 'Reset All to Selection Minimum/ Maximum';
8. Click on 'Apply'.

G. Export as 8-bit TIFF - Qualitative Export.

1. Click on Home>Export>'Export all channels as 8-bit TIFFs'
Each channel in an image is exported as 8-bits monochrome.
Exporting images with more than 3 channels allows merging as layers in Photoshop to control final appearance.

H. Export images as 24-bit RGB - Qualitative Export.

1. Click on Home>Export>'RGB TIFF (24-bit)'
   a. the displayed image is saved as color RGB
   b. A z-series or time-lapse will only save the displayed frame as a single image

I. Tiff Series - Qualitative Export.

1. All frames from a z-series or time-lapse as individual 24-bit RGB images.
If this is what you want, create a new directory to receive the pile of files about to be created.

J. Movie Export - Qualitative Export.

These are strictly reserved for qualitative presentations of time and spatial relationships.
1. Adjust the histogram to achieve desired contrast;
2. Add optional Annotations such as time stamps or scale bar;
3. Select Home>Export>'Create series movie'
4. Enter Frame duration - this is the inverse of frame rate (fps)
    4.1. "1" means a framerate of 1 fps, 0.2 = 5 fps, etc.
5. Enter 'OK' ;
6. Select movie file format
    6.1. ".wmv" - Windows format, play on Mac by VLC and Fiji, but not QuickTime X
    6.2. "h.264" - Play with Windows Media Player, or on Mac with QuickTime X, VLC, Fiji

These movies are qualitative because they use compression to reduce file size. Compression removes fine details and alters intensities. Movie formats are not acceptable for analysis based on intensity levels or morphometry. Quantitation of time-lapse imaging or 3D optical volumes should be performed within Slidebook or on images exported as 16-bit OME-XML TIFF.

IV. Slide Tilt Correction for Montages.

A specimen seldom lies parallel to the plane of focus. The slope or tilt of a specimen causes it to move up or down through the objective's plane of focus as the stage moves.

A montage region of a specimen may be defined by 3 corners of the rectangle and the axial position of those 3 points can define a plane with known angle of tilt. Slidebook can calculate the specimen's axial shift as a function of stage position and adjust the objective focus to compensate. The catch is that you need to identify the same axial position in the specimen for each of the 3 corners. For example, you may look for a reflection at the interface between coverslip and mounting medium, or for bright objects on the surface of the specimen nearest the coverslip. If the 3 corners are defined at different focal levels within the specimen and do not correlate with the specimen's physical orientation, the focus will be adjusted incorrectly.

This is compatible with standard montages as well as with collecting stacks for stereology. Press 'F1' and search for 'XY tab of focus controls' or search for 'stereology' for details.

A. Setting Tilt Correction for montages

1. Open the XY tab in the Focus window and clear all XY points;
2. Open the 'Advanced…' capture settings in the Capture window;
3. Open the 'Focus' tab of the Advanced Capture window;
4. Select 'Enable 3 point Z interpolation for montage capture';
    4.1. this is grayed out if there are any XY positions in the XY tab
5. Click on 'OK' to close the Advanced window;

6. Return to the XY tab and select 'Set Extent';
7. Identify 3 corners that encompass your montage region;
8. Move the stage to the 3 corners of the region consecutively
    8.1. bring each field into focus
    8.2. click on their corresponding buttons, such as UR, LR, LL;
9. The single line in XY point list for this montage will end with "3D", i.e. "3X5 3D", indicating that the tilt correction has been set for a montage of 3X5 fields;
10. Continue to the 'Z' tab to set focus limits, if collecting a 3D montage;
11. Go to the Capture window and set the channels;
12. Acquire montage;

B. Refining Tilt Compensation.

If you need to refine the specimen tilt definition, such as switching to higher NA objective.
1. Select the higher NA objective;
2. Go to the XY tab;
3. Click on 'Visit Extent' button;
4. Click on one of the corners of the Montage Extent, the stage will move to the position;
5. Bring the reference axial level into focus, such as the "top" of the section;
6. Click on 'Set Extent';
7. Click on the corner button used to select the corner;
8. Refine the next 2 corners of the montage by repeating steps 3-7.
Just be sure to select the 3 corners in sequence, whether clockwise or counter-clockwise.

C. Using Tilt Correction for Stereology.

In stereology, we typically collect a low magnification montage containing the area to be sub-sampled. Then, 3D volumes are collected with a high magnification objective that is more affected by slide tilt.

Before capturing a low magnification montage:
1. Open the 'Advanced…' capture settings in the Capture window;
2. Open the 'Focus' tab of the Advanced Capture;
3. Select 'Enable 3 point Z interpolation for montage capture';
    3.1. this is grayed out if there are any XY positions in the XY tab
4. Click on 'OK' to close the Advanced window;
5. Collect the low magnification montage;
6. Switch to the high magnification objective lens;
7. Go through the steps for "Refining the tilt compensation", above
8. Return to creating the stereology ROI and setup.
Note, the Z interpolation option only needs to be selected 1 time after Slidebook is started.

The Slidebook 5.5 Manual Index

The Slidebook 6.0 manual is somewhat different and available from F1 in Slidebook.
Chapter 2 provides a tour of the Slidebook software using images found in the /Quick Tour
directory located in the User Files directory.

Slidebook 6.0
Press 'F1' for help.

Slide tilt correction overview:
Select "montage" from list of key words in the index for an overview of setting the extent of a montage and slide tilt correction. Also view the Stereology section in this guide.

Detailed directions for focus compensation:
Search for "Collecting a Stereology Montage" and "Optional: Refine "Plane" of Montage
Note that Focus interpolation cannot be turned on in the Advanced Capture tab if there are already XY points in the XY tab.

Slidebook Supplements.

Available in this manual and by PDF.

1) Stereology
   a. instructions updated to version 6
   b. the manual for stereology on Slidebook 4
2) How to Perform DIC Microscopy - includes setting Koehler illumination
3) How to Exchange objective lenses on the turret, and setting the objective's coverslip correction collars.
4) Using Autofocus
5) Using the electric timer for the Marianas heated enclosure
6) Mid-volume Capture of a Single Image
7) Dark Frame and Flat Field Correction in Slidebook

Stage Up

Stage Down

"FL" Fluorescence
Shutter Open/Close

"HAL" Halogen Lamp
On/Off
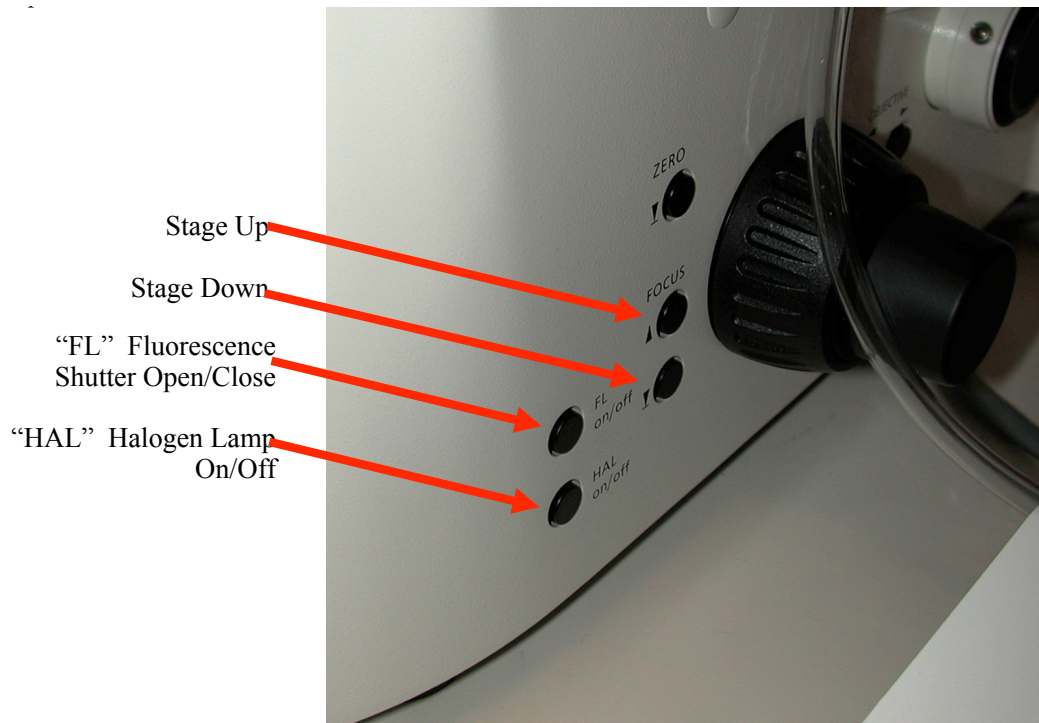
Reflector Indicator

Reflector Labels

Reflector Selection
(filters cubes)
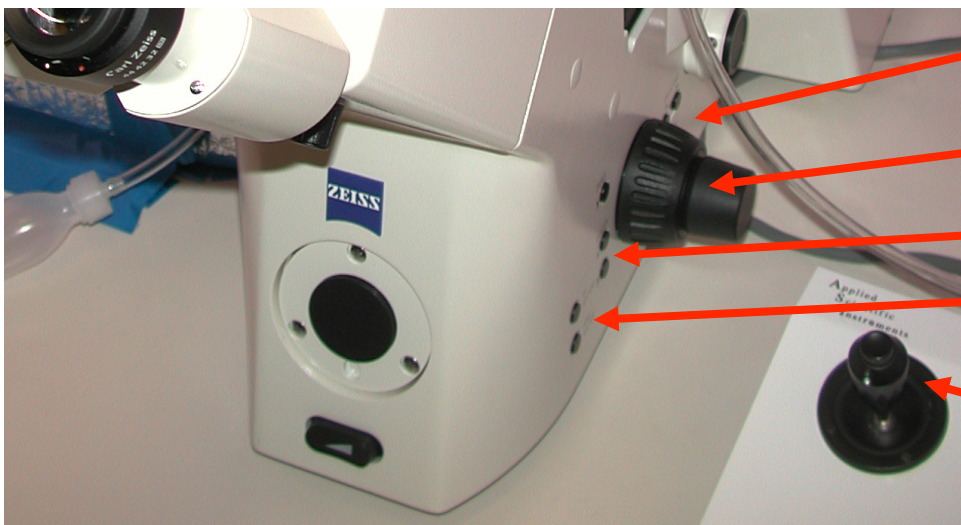
Objective Selection

Fluorescence Field
Stop (field size)

Fluorescence
Aperture (intensity)

Neutral Density
Filters (intensity)

witch-
S ON!!

More Buttons
see Figure 4

Coarse/Fine
Focus

Stage UP/
DOWN

Illumination
Controls

Stage Joystick

Figure 3. Zeiss Axioplan 200M, Right Side, Front Controls

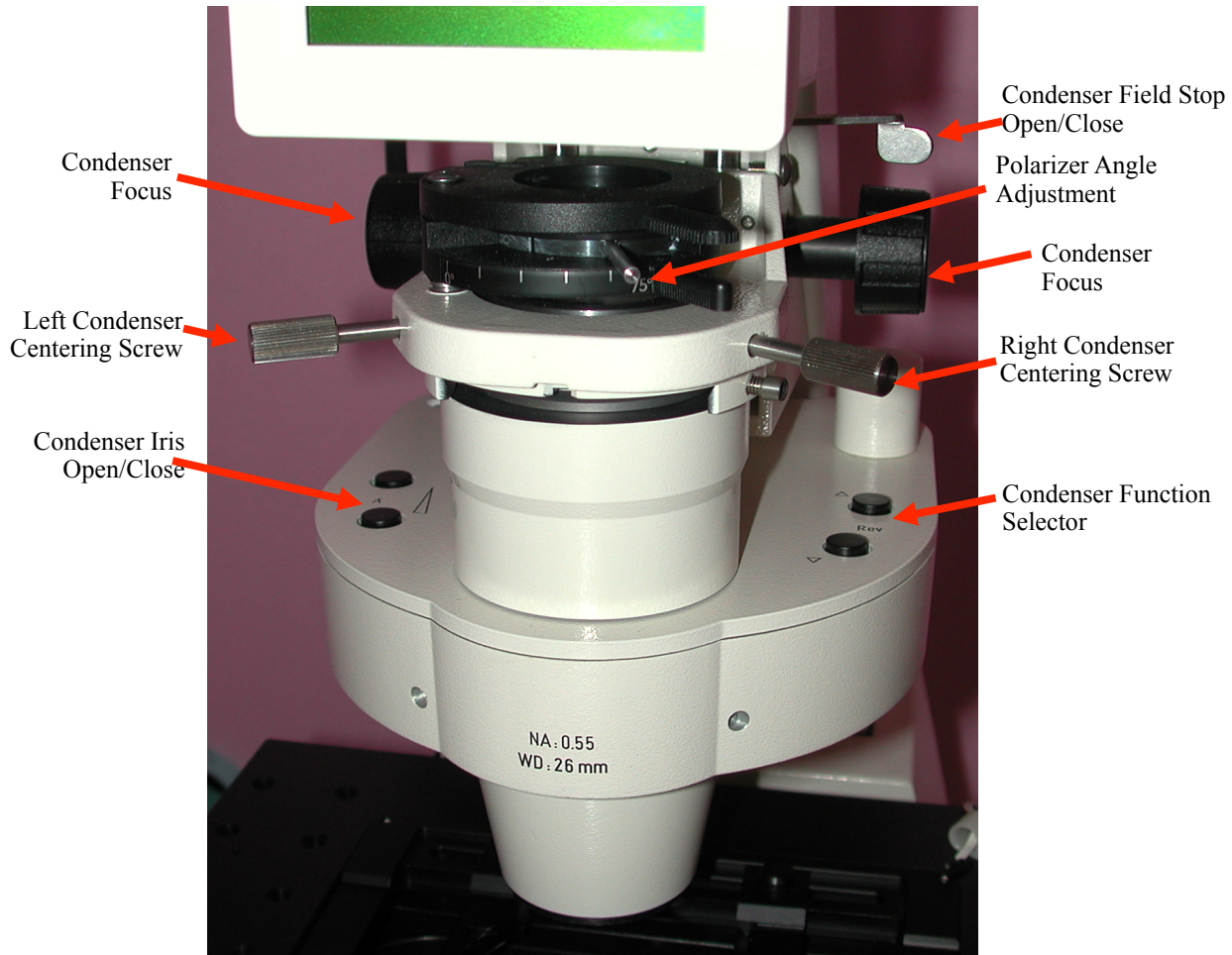**Figure 4. Zeiss Axioplan 200M, Right Side, Side and Back Controls**

Figure 5. Zeiss Axioplan 200M, Condenser

Marianas Live Cell Imaging System Overview

The Marianas imaging system is composed of hardware components integrated by Intelligent Imaging Innovations, Inc. (3i), Denver, CO. and controlled by their Slidebook software. It provides a highly automated platform for critical imaging applications such as timelapse, deconvolution, FRET and ratiometric imaging, as well as routine brightfield and fluorescence image capture.

Major Components

- Axiovert 200M inverted microscope (Zeiss)
- Motorized X,Y stage (ASI, LS-2000)
- Shuttered 175 W xenon lamp coupled with a liquid light guide (Sutter, LD)
- Lambda 10-3 filterwheel controller (Sutter) with filter wheels for excitation and emission
- CoolSnap HQ cooled monochrome camera, 6.74 µm pixels, 1392 x1030 (Photometrics)
- Evolve 512 EMCCD camera, 16.0 µm pixels, 512 x 512 (Photometrics)
- Heated enclosure.

Table 1. Objective lenses.

Magnification	NA	Type	Pixel size°	Working Distance
5X	0.15	Plan NeoFluor	1.249 μm/p	13.6 mm
10X	0.3	Plan NeoFluor	0.624 μm/p	5.6 mm
20X	0.75	Plan Apochromat	0.314 μm/p	610 μm
25X***	0.8	LCI-PlanNeofluor	0.244 μm/p	600 μm
40X	0.75	Plan NeoFluor	0.156 μm/p	500 μm
63XW*	1.2	C-Apochromat	0.099 μm/p	240 μm
63X**	1.4	Plan Apochromat	0.099 μm/p	180 μm
100X	1.4	Plan Apochromat	0.062 μm/p	180 μm

Scales as of June 9, 2016
*water immersion with coverslip correction collar, for live cell imaging
**Removed to make room for the 63XW lens, but available upon request.
***Multi-immersion with media immersion correction collar, installed as needed
°Pixel size may change slightly with modifications to the system, check image info.

Description of Fluorescence System:

Fluorescence excitation is provided by a 175 W xenon lamp coupled to the microscope by a liquid light guide. The light guide scrambles light to create a more uniform illumination across the field of view. The illuminator incorporates a filter wheel and a shutter to select the excitation wavelengths and control light exposure.

None of the filter cubes contain excitation filters because excitation is controlled at the illuminator. The filter cubes for camera-specific filter sets contain only a dichroic mirror. A filter wheel at each camera selects the appropriate barrier filters for each fluorescent channel. Avoid looking through the eyepieces while using a camera-specific cube. You will give a painful dose of excitation light.

The Triple cube, used for viewing through the eyepieces, is the only filter cube that contains an emission filter. This emission filter prevents out-of-band excitation light from reaching the eyes. Although the Triple cube may be used with each camera, the camera-specific filter sets will deliver better performance.

This design has several advantages over relying on a single filter cube for each wavelength.
1. The filter cube does not move when collecting multiple channels, reducing the chance that different channel images will be mis-aligned.
2. Different combinations of excitation and emission filters may be created for non-standard fluorescent probes, to the degree allowed by the spectral characteristics of the dichroic mirrors.
3. It is much faster to rotate a filter wheel than a filter turret, roughly 30 ms per filter position.

Fluorescence Filter Sets

The tables list filter combinations as *currently configured*. The filter combinations were named for the most commonly used fluorophore <u>at the time the filter was created</u>. Other combinations of excitation and emission filters may be possible, depending upon availability of dichroic mirrors. Please note that the filter combination used for your particular fluorophore may not be named as such. For example, the 'Cy3' filter set works with rhodamine, Alexa568, DSRed, etc.

Filter naming: Bandpass filters are identified by a central wavelength and bandwidth. The Cy3 excitation filter "555/28" has a central wavelength of 555 nm and and a bandwidth of 28 nm. The 555/28 transmits an excitation band from 541 nm to 569 nm.

Table 2. Visible Filter Set

Name	Excitation	Emission	Internal Turret
DAPIvis	403/12	505/25	3 (Triple)
FITCvis	490/20	520/30	3 (Triple)
Cy3is	555/28	600/50	3 (Triple)
Triple	Open	RGB	3 (Triple)
BFvis	Halogen	NA	4 (Bright)
DICvis	Halogen	NA	1 - DIC

Eyepieces filter sets. These use the "Triple" cube turret position.
Filter names end with "vis".

Table 3. Camera Filter Sets

Name	Excitation	Emission	Internal Turret
DAPI	350/50	457/50	2 (Sedat)
FITC	490/20	528/38	2 (Sedat)
Cy3	555/28	617/73	2 (Sedat)
Cy5	635/20	685/40	2 (Sedat)
FM1-43	490/20	617/73	2 (Sedat)
Fluorogold	403/12	528/38	2 (Sedat)
Far Cy5	645/20	685/40	5 (FRET)
CFP	427/10	472/30	5 (FRET)
YFP	504/12	542/27	5 (FRET)
CFPExYFP*	427/10	542/27	5 (FRET)
LysoBluepH3.0*	360/40	457/50	2 (Sedat)
LysoYellowpH9.0*	403/12	542/27	2 (Sedat)
Flash**	Open	None	2 (Sedat)

Cannot be used with eyepieces
*FRET setting
**Used for caged compound release
Each fluorophore name ends in "HQ" or "Evolve" to indicate the camera for which the filter set was created.

The best way to match filters and fluorescent labels is to match the bandwidth of wavelengths for both excitation and emission filters with the excitation and emission spectra of the fluorescent label. This may be done by "eyeballing" the spectra or using on-line tools listed on the DMC website.

Marianas Quick Reference Guide.

Additional documents in the Quick Reference Guide and on the DMC website.
Dark Field and Flat Field Corrections in Slidebook
Stereology (with slide tilt correction)
Autofocus
DIC (Differential Interference Contrast)
Exchanging Objectives (useful when changing between 63X water and 63X oil)
Mid-Volume Capture
Timer

Current capabilities with the Slidebook software:

Image capture:
Multiple channel fluorescence
Mixed fluorescence and transmitted light image capture
Monochrome and color brightfield and DIC image capture
Timelapse imaging up to 10 fps
Z-stack capture for 3D imaging and deconvolution
Timelapse with Z-stack capture (4D)
Montage and Multi-point sampling
Capture from selected regions of the camera chip
Stereology - optical disector
FRET
Autofocus

Image processing and analysis:
Particle analysis
Co-localization, in 2D and 3D
Densitometry in 2D and 3D
Deconvolution
Stereology
3D feature extraction
Rotation projections
QuicktimeVR interactive renderings