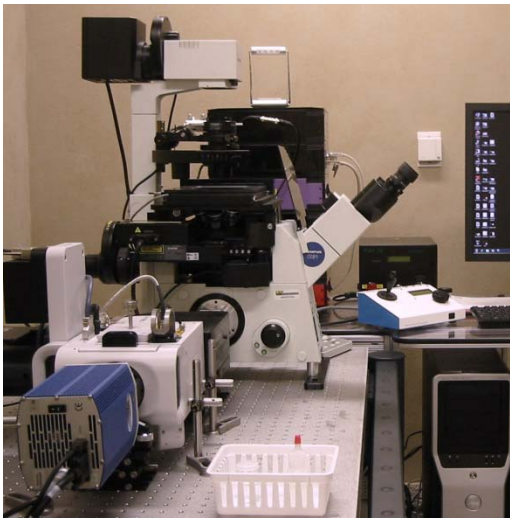
3i Olympus SPD Microscope User Guide

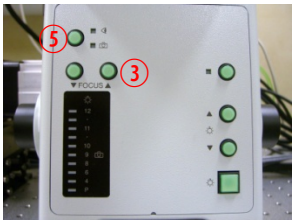


A. System Start-up.

1. Sign into the log book on the desk.
2. Turn on the entire microscopy system with the main switch ①.
3. Turn on (or wake up) the computer and select "USER" and enter the password on the log-in window.
4. Turn the laser switcher key ② to "I".
5. Start Slidebook 5.0 x64.

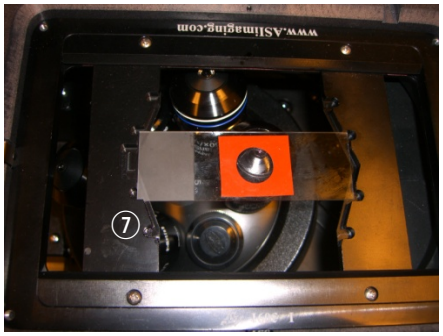
B. Hardware Control and Setting.

1. Microscope is controlled with buttons, knobs, and remote controller boxes. It also can be controlled by the Slidebook program.

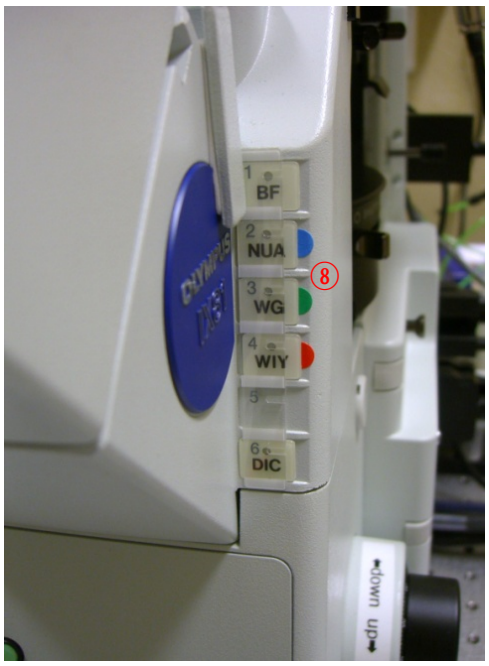


2. Focus is adjusted by moving objective lens coarsely with Focus Buttons ③ or finely with knobs ④ (note the direction of lens movement by the knobs; "up" lens goes up, "down" lens goes down). The extent of movement by knob can be adjusted to coarse (C) or fine (F) by pressing the F/C button. (Once Slidebook program is started, more precise focus can be adjusted with Galvo stage movement (see below).
3. Push the light path button ⑤ (eye or camera) to see and focus onto the sample through eyepieces.

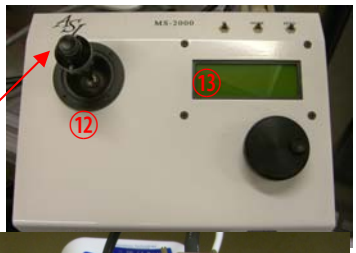
4. Select lower mag lens (10x) by pressing 10X button on the controller box ⑥. Put the sample slide on the adjustable holder ⑦.



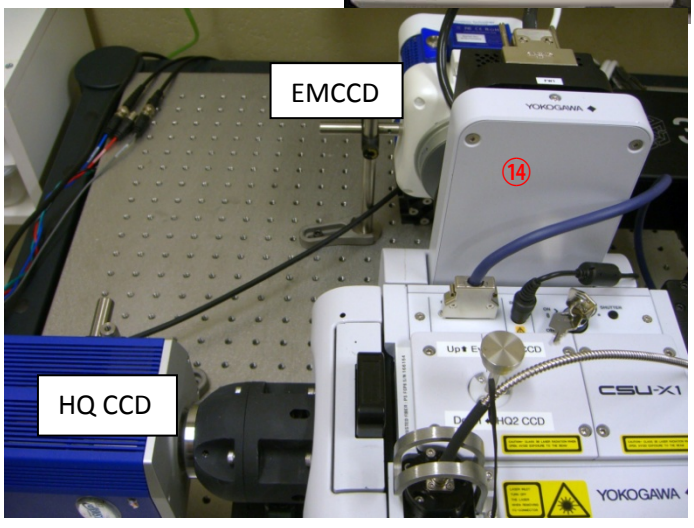
5. For bright field viewing, press "TSHT" (Transmission light Shutter) button on the controller box, which open or close alternatively by pressing. Choose DIC (differential interference contrast)(filter box ⑧ position 6) or normal bright-field (filter box position 5) by pressing the "filters" buttons ⑨ on the controller box.
   For DIC, match the condenser prism ⑩ with the objective lens by pressing "AP prism" button ⑪.



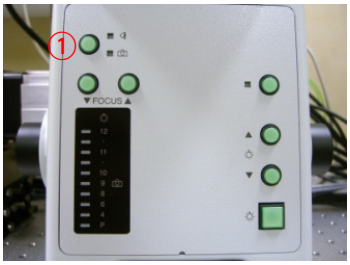
6. For immunofluorescence viewing, press "RSHT" (Reflective shutter) button. Select the appropriate filter set by pressing "filters" buttons ⑨ (pressing each button turns the filter wheel to opposite directions).
   "NUA" (position 2) for DAPI-kind staining
   "WG" (position 3) for green fluorescence
   "WIY" (position 4) for red fluorescence.



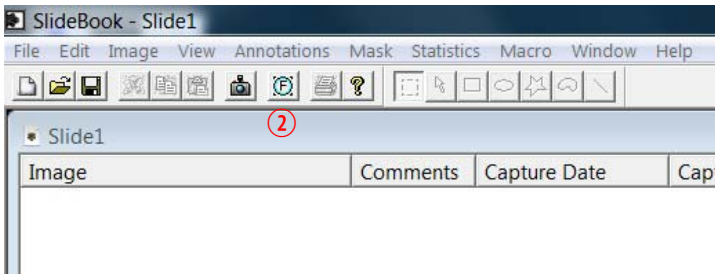
7. Use X-Y stage controller joystick ⑫ to move the stage. By pushing the joystick button, the speed of movement can be adjusted to fast (f) or slow (s) (the selection is displayed on the LCD panel ⑬).
8. **Camera selection**: the 3i SPD system has two cameras; **EMCCD for high-speed** imaging and **HQ2 CCD for high-resolution** imaging. Either one can be used by placing the filter wheel box ⑭ in the front of the camera and directing the light path.
9. Now you find a region of interest on the slide and are ready for imaging.



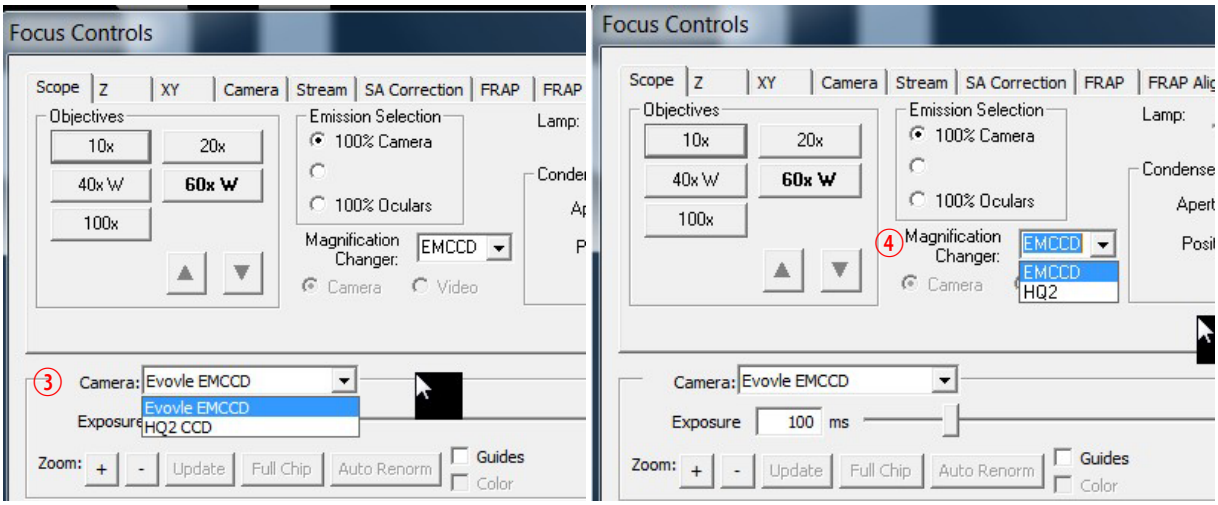
C. Overview of Focus Control window and Basic image acquisition.



1. To change the light path to camera, push the Eyepiece/Camera selection button ① on the microscope (Camera icon will be lightened.)
2. To set up image acquisition, click **Focus (F) button** ② to open the ***Focus Control*** and **live Camera view** window.

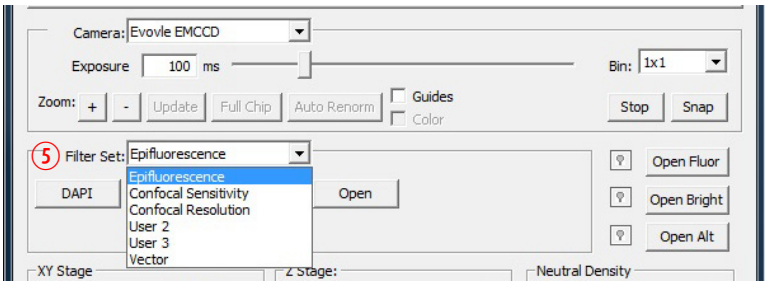


3. Select the **Camera** ③ you will use from the menu and match the **Magnification Changer** ④ with the selected camera.

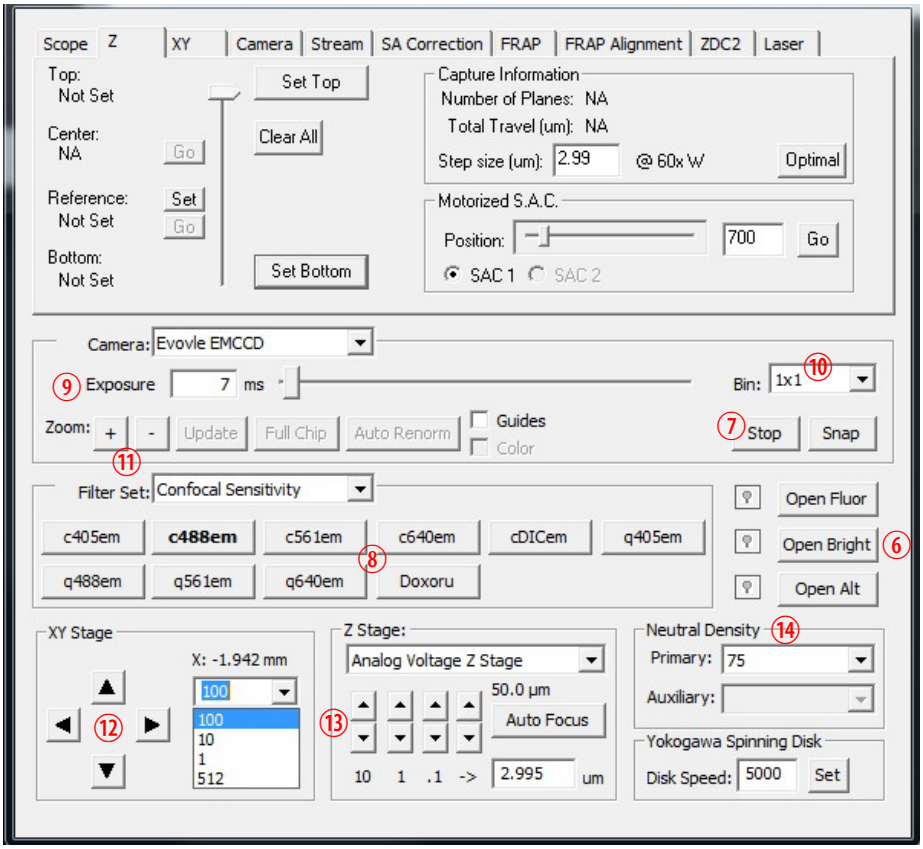


4. Select appropriate filter sets for the selected camera from the **Filter Set** menu ⑤.

Evolve EMCCD -> Confocal Sensitivity (laser excitation) or Epifluorescence (Xenon light excitation)
HQ2 CCD -> Confocal Resolution (laser excitation) or Epifluorescence.



5. Click on appropriate light source button ⑥. Click on the same button to close.
   For normal epifluorescence filter set, click on **Open Fluor** button to illuminate with Xenon light.
   For bright-field imaging (such as DIC), click on **Open Bright** button to turn on halogen lamp.
   For laser excitation for confocal imaging , click on **Open Alt** button.
6. Click on **Start** button ⑦, if not. (Camera begins to collect light.). Click any **filter button** ⑧ to visualize the image on the camera screen. (*Note: close the light source when you are not imaging, otherwise it will bleach the sample.*)
7. **Other Imaging Settings**.



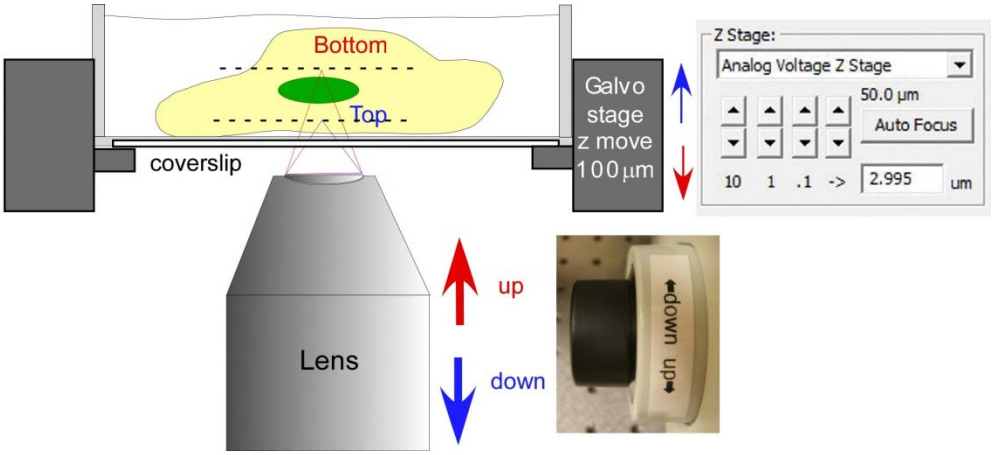
Exposure ⑨: Drag the button to control camera exposure time.

Bin ⑩: increase sensitivity and speed of image acquisition at the expense of resolution.

Zoom ⑪: digital zooming in or out.

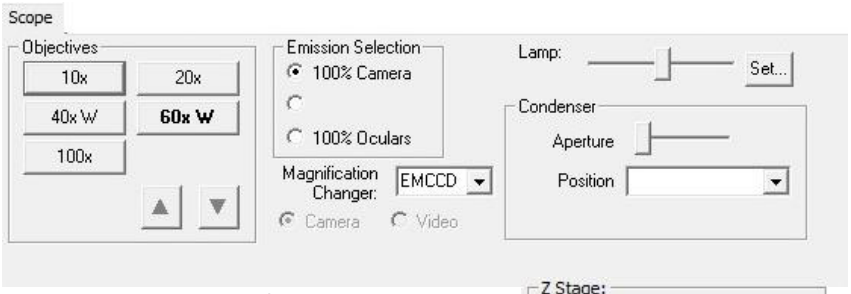
XY Stage ⑫: clicking on the arrowhead button move the stage. The extent of movement can be set by drop-down menu. (you can use the joystick on the XY-stage controller box.)

Z stage ⑬: Focus movement directions by arrowhead tabs are identical to those by the microscope knobs. However, arrowhead-clicking moves the Galvo stage (**Analog Voltage Z stage**), whereas the knob moves the lens to focus. Use these arrowhead tabs to set the top and bottom limits of z series.

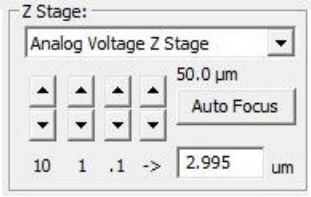


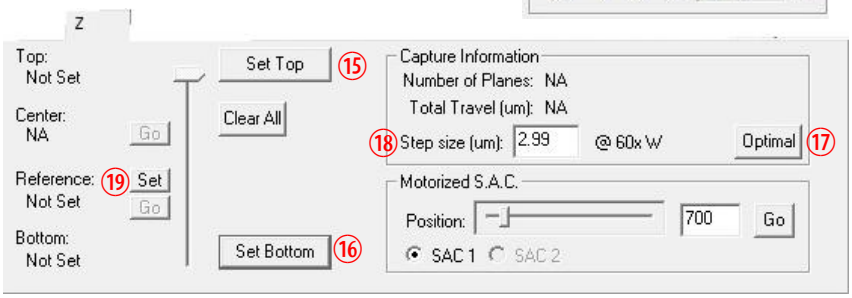
Neutral Density ⑭: It controls the intensity of laser light; the higher, the stronger.

8. **Scope** tab: controls the microscope objective, light path (emission selection to camera or objectives), halogen lamp for bright field viewing, and the condenser/aperture.



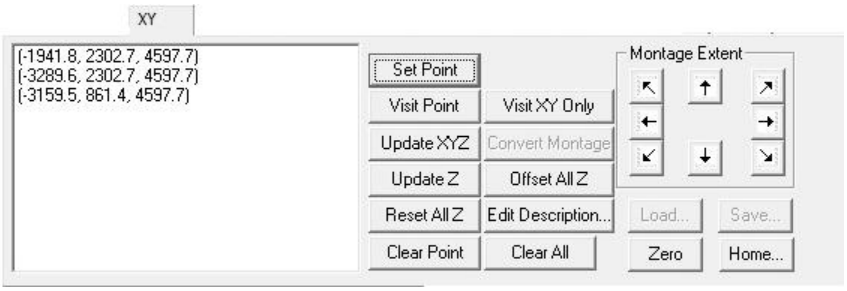
9. **Z** tab: defines the Z series. To set z-series, click the up-arrowhead to focus onto the region close to the coverslip. Then click **Set Top** button⑮. Focus deeper by clicking down-arrowhead, and click **Set Bottom**⑯. The capture information window shows the number of planes and total travel based on the step size. The **Optimal** button⑰ will calculate the optimal z-step for the used objective based on the theoretical resolution of the lens. You can adjust this step size by entering a value in the **Step size**⑱ field.





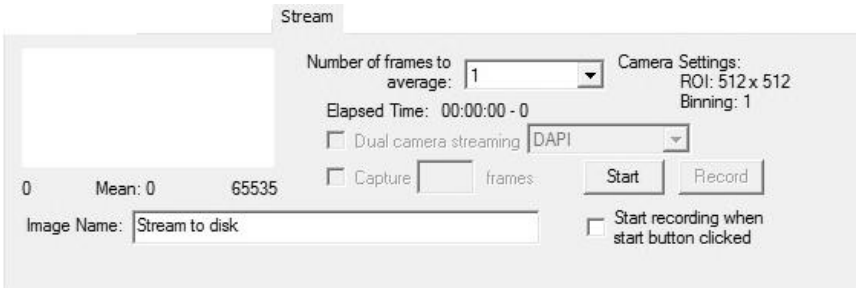
Reference Set button⑲ define the current z position as a reference point that can be used for z-series image capture in the **Capture** window.

10. **XY** tab: You can set multiple points for imaging different regions on a sample or making montage.
    * **Set Point** – add the current xyz location to the list.
    * **Clear Point** – removes the currently selected location from the list.
    * **Update Z or XYZ** – reset the z or xyz position of the selected location to the current z or xyz position.
    * **Reset All Z** – resets the z positions of all points to the current z position.
    * **Offset All Z** – update the z positions of all points to the same extent of change in the current z position.



Montage Extent: sets the boundaries that will be used to create a montage image. The boundary can be defined by selecting one of the following sets of coordinates: upper left/lower right, upper right/lower left, or top/bottom/left/right. Find the corners of the region and click appropriate arrow buttons to set the locations of the corners. It will automatically generate the coordinates for the montage image for Multiple XY location Capture in the capture window.

11. **Stream** tab: this function allows running the CCD camera at the fastest possible speed. When this streaming capture is used, the Capture window won't be working; only Focus window will function to control hardware. The best use for this will be rapid

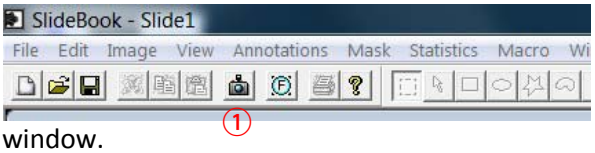


2D timelapse imaging.

To run camera in this stream mode, click **Start** button to see the quality of image at the exposure time you choose. You can average the image by selecting the Number of frames to average from the drop-down list. To record the image, name the file in the **Image name** field and press **Record**.

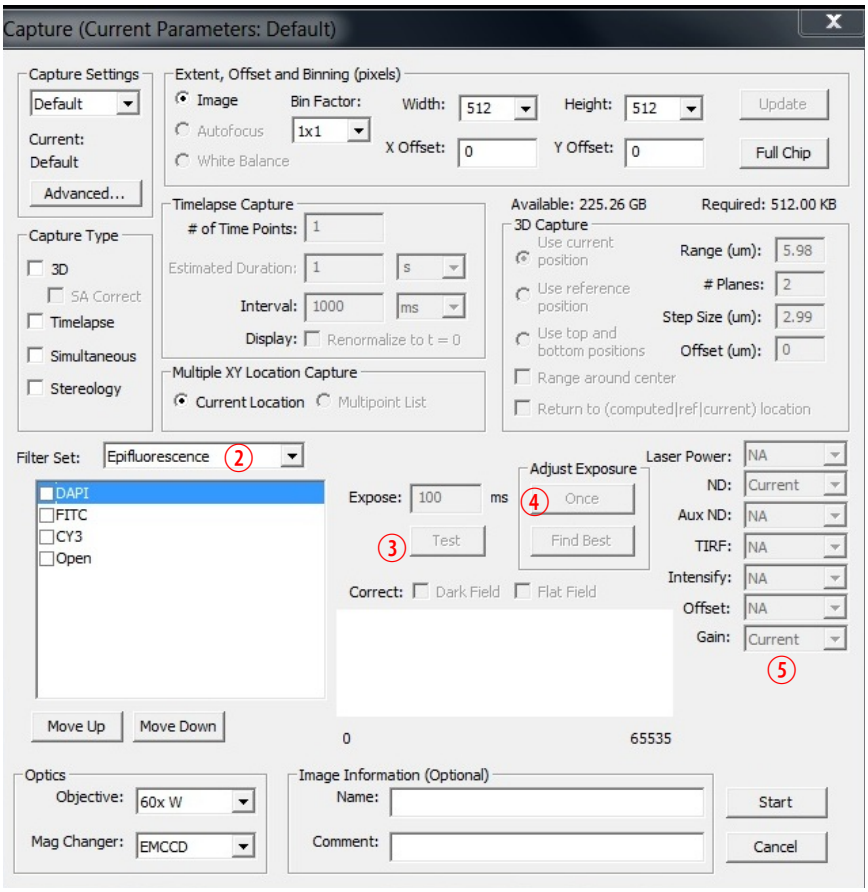
D. Capturing Image.

Capture window allows you to set up actual acquisition of images (2D, 3D, 3D timelapse, and 4D etc..). Click **Camera** ①icon to open the



window.

Select the same **Filter Set** ②as you selected in the **Focus** window. Check the boxes for filters you want to image. In order to obtain a proper camera exposure time, select a filter and click **Test** button③. If the image is not acceptable, click **Once** button④ in **Adjust Exposure** box, it will adjust the proper exposure time based on the current image histogram. Repeat this process for each filter. (*note: you can use the same **ND**, **Intensify**, and **Gain** setting for all different filters by selecting **Current** from the menu – it will use the settings of the Focus window.* ⑤*)*

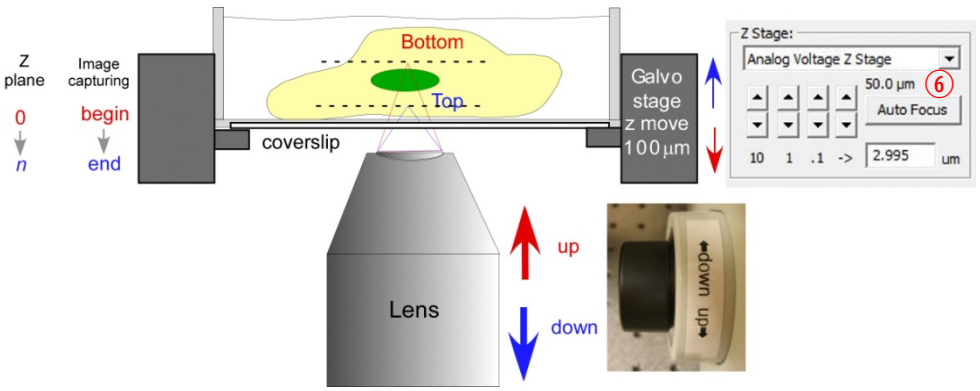


For a simple 2D image, click **Start** button. You can put a file name in the **Name** box and notes in the **Comment** box, which will be saved with the image.

<u>3D image capture.</u>

Although you can focus to different z plane by the focus knob (moving lens) or clicking arrowheads (moving Analog Voltage Z Galvo stage), setting "Top and Bottom" limit works only with the Galvo stage. By starting with *the Galvo stage at the middle (50 µm) position*⑥, you can have 50 µm above and 50 µm below the current focal plane (The total movement range of the stage is 100 µm). Move the lens up or down with the focus knob to focus onto the tentative middle of the sample's z axis range.

Click up-arrowhead, which moves the stage up,

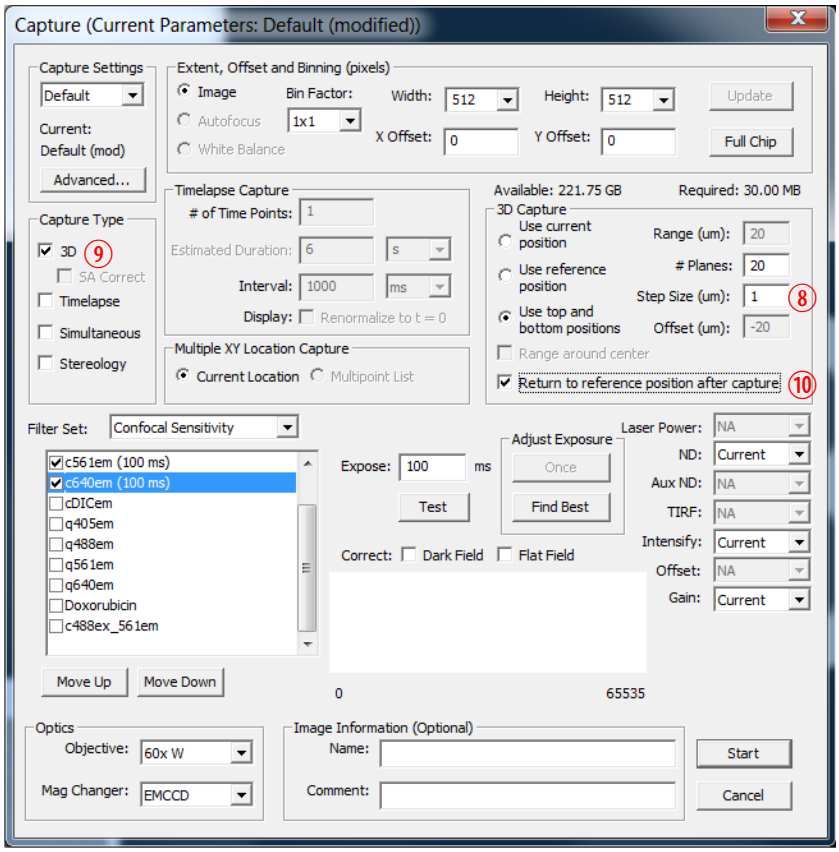


and find the top limit of your sample (*note the Top position is the region close to the coverslip*). Click **Set Top** button.

Click down-arrowhead until you find the bottom limit. Click **Set Bottom** button.

By sliding the bar, you can go to different z planes within the z series limit you just set.

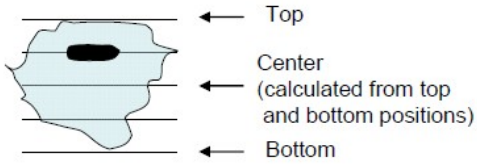
The step size (µm) box⑦ shows a number that is the distance between imaging scans. You can change this step size by typing a specific number or click Optimal to obtain an optimal size based on the objective used. (You can also set this later in the **Capture** control window ⑧).

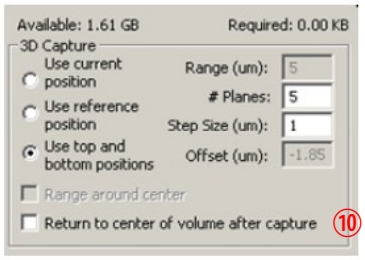


Open the Capture Window. Select **3D** as **Capture Type** ⑨.

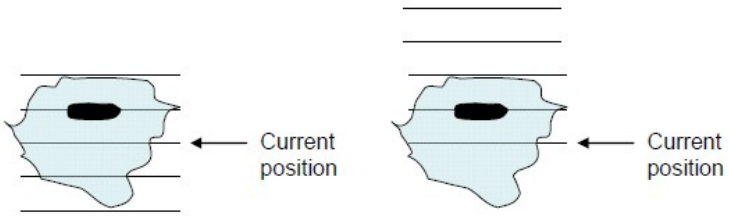
In the **3D Capture** option box, you can set the range of z series in various ways.

The basic method will be to use the Top and Bottom you set in the focus window.

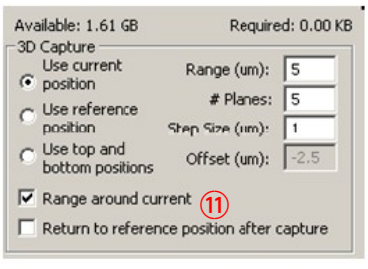


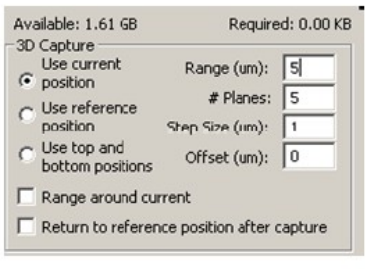


Click **Return to Center of volume after capture** box, so that the focus goes to the center of z series after capturing ⑩.

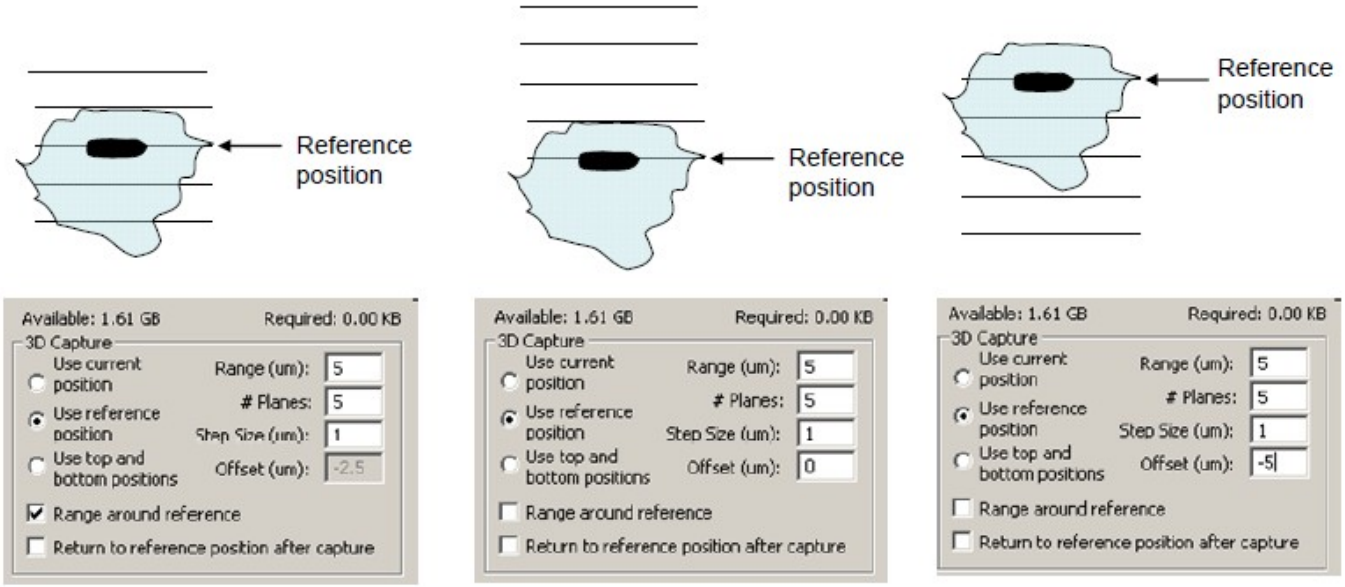


Alternatively, you can use the current position to capture z series. Note the difference in the capturing range by selecting **Range around current** option ⑪.





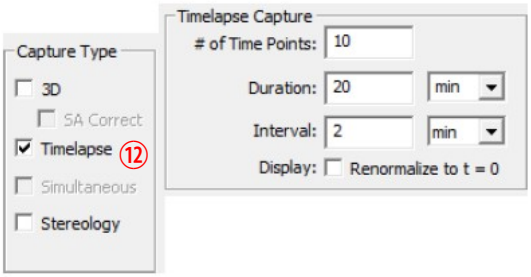
Finally, you can use the Reference position you may set in the Focus window. *Note the differences with selection of* ***Range around reference*** *option.*



Click on **Start** to capture 3D.

2D Time lapse capture

In the Capture window, check the **Timelapse** checkbox ⑫ in the **Capture Type** section. This allows you to set various timelapse parameters. Enter the desired values in the following fields:



- # of Time Points- the total number of timepoints to be captured.
- Duration- Total length of time for the capturing. Units of time (ms, second, min, or hours) can be selected from the drop down menu. (Due to the inability to predict time required for hardware movement, this option is only available when the interval for the capture is over 30 seconds.)
- Interval - Delay between the beginnings of one timepoint to the beginning of the next timepoint. The interval unit can be selected from the drop down menu.
- Display: Renormalize checkbox: When checked, the minimum and maximum pixel intensity values for the first timepoint in a timelapse series will be used to determine the renormalization values of all subsequent timepoints (only affects image display during capture and won't affect the actual captured image).

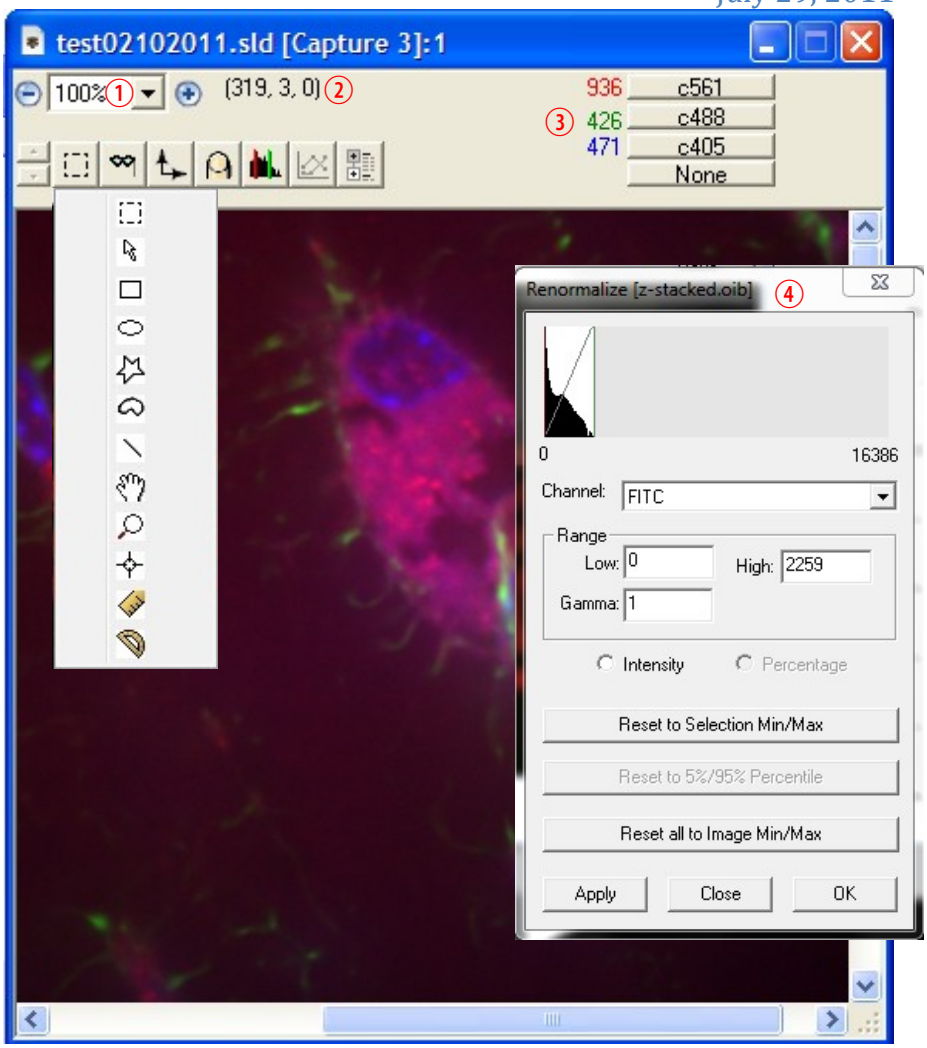
Saving Images

When image capturing is done, the Slidebook automatically stores the images in a Slide *n*. You need to SAVE this Slide that contains the captured images in to your user folder and it will be saved as filename.sld. Use the **File/Save** Slide command (Ctrl+S). It is advised to save the slide file often; especially you are collecting large timelapse images, in case of power outage or computer crash.

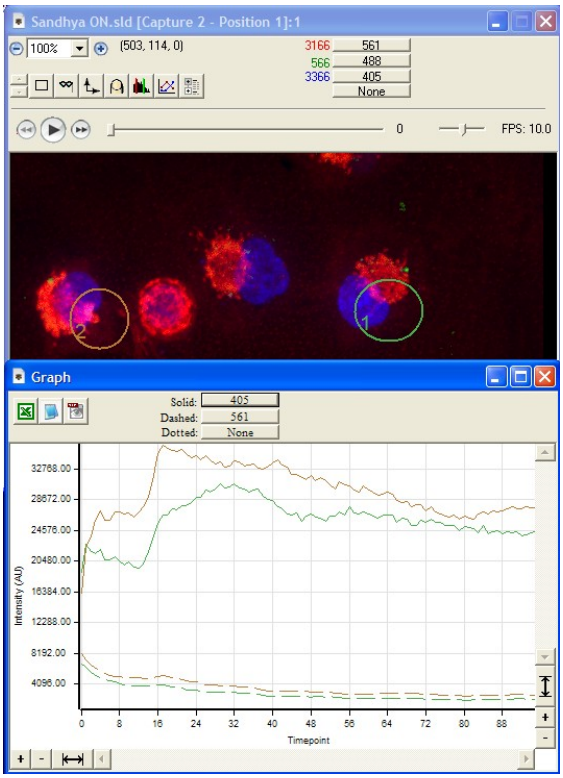
E. Viewing and Adjusting Images

View menu allows you to display images in many different ways including 3D reconstruction.

Main view (Ctrl+K): The Main View windows contains various tools to explore and adjust images.

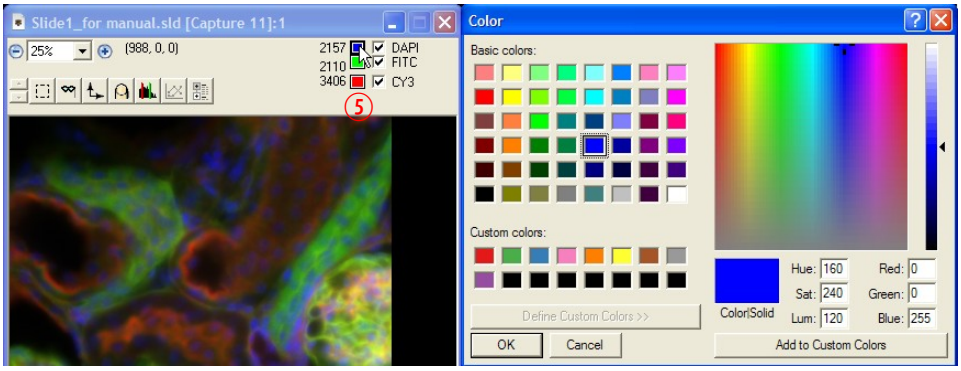


- **Zoom Control**①: zooming the image.
- **Plane selection** : scroll up and down to different z planes of 3D image.
- **X, Y, Z, (T) position**②: indicate the current x, y, z, t location of the cross-hair pointer (pixel).
- **Pixel intensity values**③: shows the signal intensity of the pixel located by the cross-hair pointer.
- **Tool Menu** : provides various tools to draw and set ROIs, measure and annotate.
- **Axis Menu** : select the view angle, x, y, or z.
- **Adjust Renormalization** : allows you to change the range of intensities that are displayed for any channel you choose. A histogram ④shows the relative number of pixels on the y axis and intensity values on the x axis for the specific channel.
  Click on the red and green bars in the histogram window and drag them to left or right, observing the change in the display. The red and green bars set the minimum and maximum intensities in the display respectively. You can enter specific numbers to the low and high range boxes. After entering numbers, click Apply button to see the change.
- **Set Default View** : this button let you set the default view settings for an image, such as color assignment of the channel, renormalization information, and the z position of an opened image. If you close the Main View without clicking this button, the changes that you just made will be lost.
- **Show Graphs** : when you draw ROIs on a timelpase image, this button shows the intensity change of those ROIs over time.
- **View Settings** : allows you to set various viewing options such as background color, display styles, tile view options, and the loop speed of timelapse images.



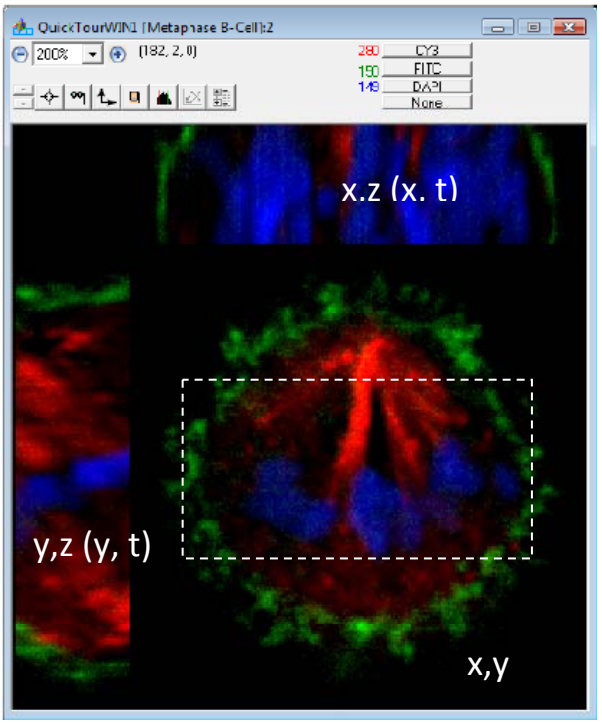
* **<u>Changing Displaying Color of Channels</u>**: View menu allows you to choose different way to colorize each channel in addition basic RGB (Red Green Blue) color scheme, such as monochrome, pseudocolor, and user-defined color. In the User-Defined Color

scheme⑤, you can assign any color to each channel by clicking the colored box and selecting any color in the pop-up window.

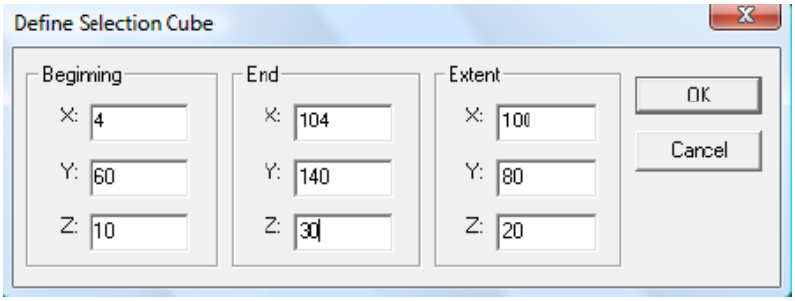


Three View (Ctrl+T): This method allows you to view all three axes simultaneously (works with 3D image). The window shows an ***xz*** pane on the top, a ***yz*** pane on the left, and an ***xy*** pane in the middle.

Choose the point selection tool icon  in the tool menu. Click on the ***xy*** pane, move the pointer, and notice the changes in the **xz** and **yz** panes, which represent the cross-sectional view of the region selected by the point selection tool. Hold the mouse button down and drag it around the image to see the cross-sectional view of different regions.

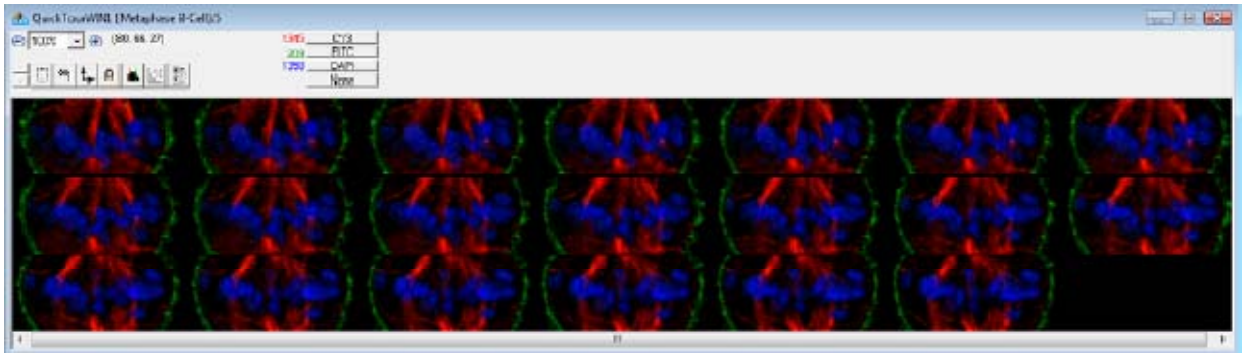


With the marquee tool  , you can make 3D selections. Draw a rectangle around the region of your interest in the *xy* pane. Open **View/Define Selection Cube** and the **x** and **y** extent will show the



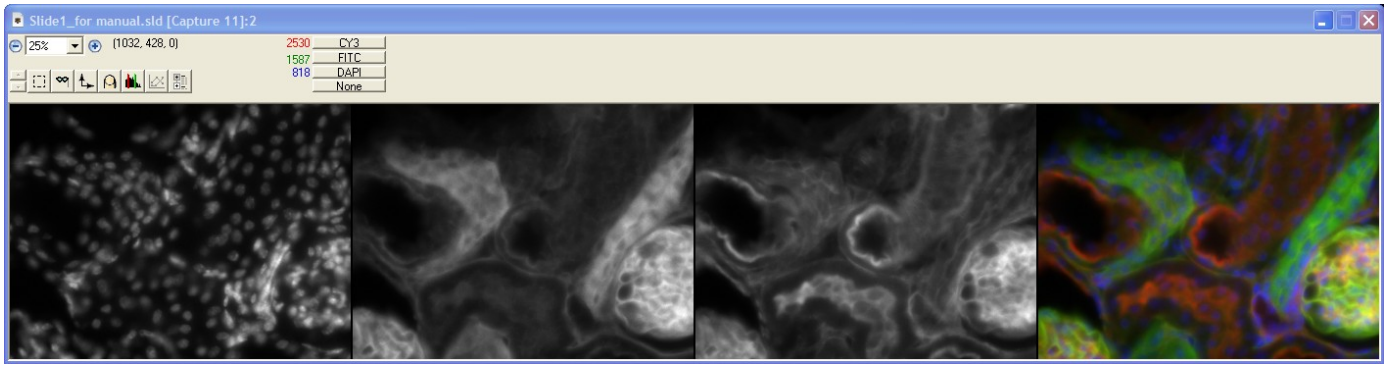
dimension of the rectangle you just draw with the marquee tool.
Enter numbers for Beginning and End for the **z** dimension to select the **z series range**. Press OK, it will show the dashed boxes for selected dimensions (you can crop the selected dimension out to a new image file).

Tile View (Ctrl+L): it shows a plane-by-plane mosaic from Main view or 3D selection with Define selection cube box.

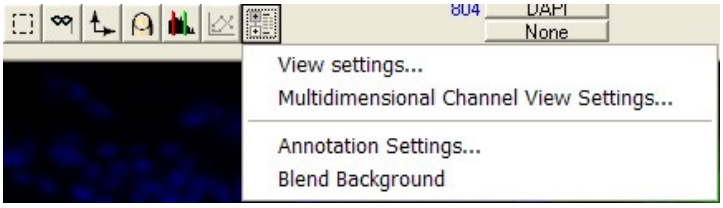


Select the hand tool  from the tool menu, click and drag around a pane of the mosaic. It will adjust the position of the display cube within the image (it will take time to see the effect of movement if the series has many images).

Multidimensional Channel View: It displays the individual channel side-by-side together with the over-laid image.

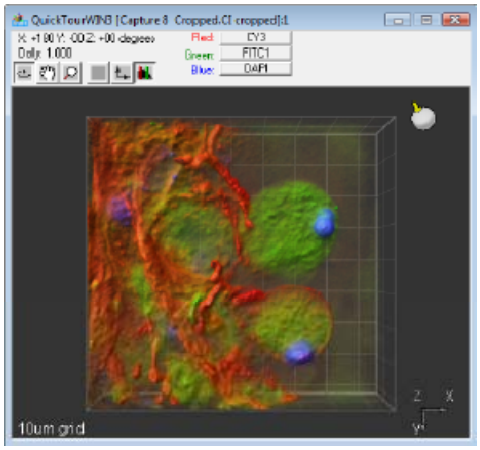


You can change the number of channels to show, their order, channel layout, and color option with **View Setting/Multidimensional Channel View Settings** option.

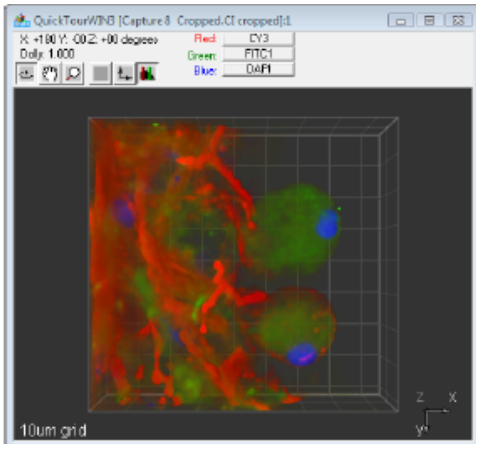


***3D Volume View (3D Volume Rendering)*:** This allows you to make interactive 3D reconstruction. Two options are available; **High Speed** will render the 3D image fast with less image quality, whereas **High Quality** will provide better quality 3D image that takes little longer to reconstruct.
3D image can be displayed in different rendering modes;

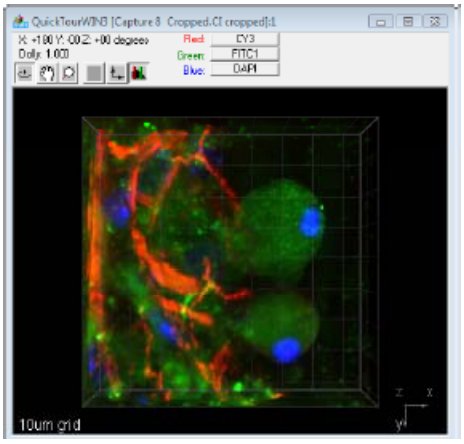
- **Dynamic Lighting** – illuminate your object with different shadow angle by changing the lighting angle. Useful for determining surface characteristics.
- **Fixed Lighting** – uses a fixed lighting for shading, approximated surface characteristics.
- **Maximum Intensity Projection (MIP)** –displays the brightest pixels through the axis perpendicular to the screen.
- **X Ray** – similar to MIP, but displays the sums of pixels through the axis perpendicular to the screen. Useful for visualizing density or concentration difference.



Dynamic Lighting

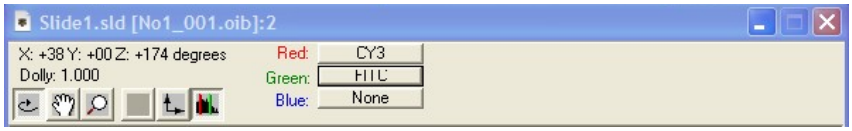


Fixed Lighting



MIP

Working with 3D Volume Views



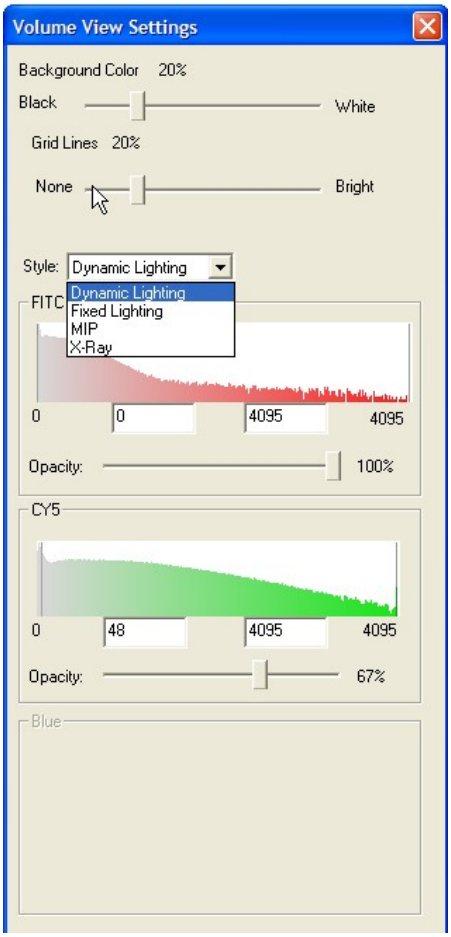
Rotation tool  : click on the icon and then click on the image and drag in the direction that you wish to rotate.

Zoom tool  : click on the icon and then click and drag on the image to zoom in and out.

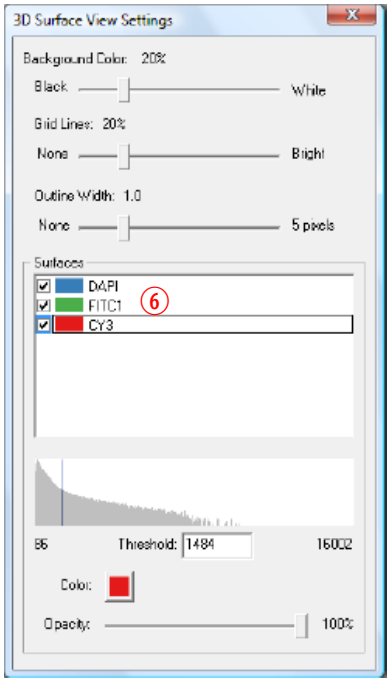
Hand tool : click on the icon and then click and drag on the image to move the image in the field of view.

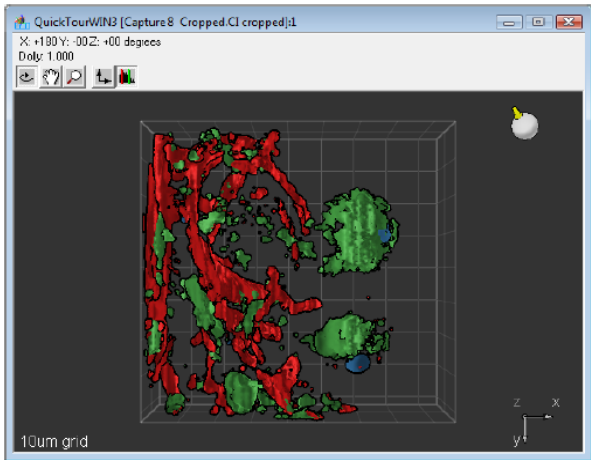
Axis Tool : this option allows you to instantly reorient the image to display a specific face of the 3D image.

Volume View Settings : This setting window automatically opens when you create a 3D volume view. In this window, you can change the rendering styles and display. Change the background color and the grid line visibility with the sliders. Each channel can be renormalized and its opacity can be adjusted with the **Opacity** control slider.



3D Surface View: displays graphical representation of intensity. Useful for visualizing spatial relationship among the objects in the image.





Similar to 3D Volume view settings, 3D Surface View settings window controls the background, grid line, extent of surface presentation (by histogram slider), opacity, and color of the surface (click on the **Color** box⑥, choose any color you want to use).

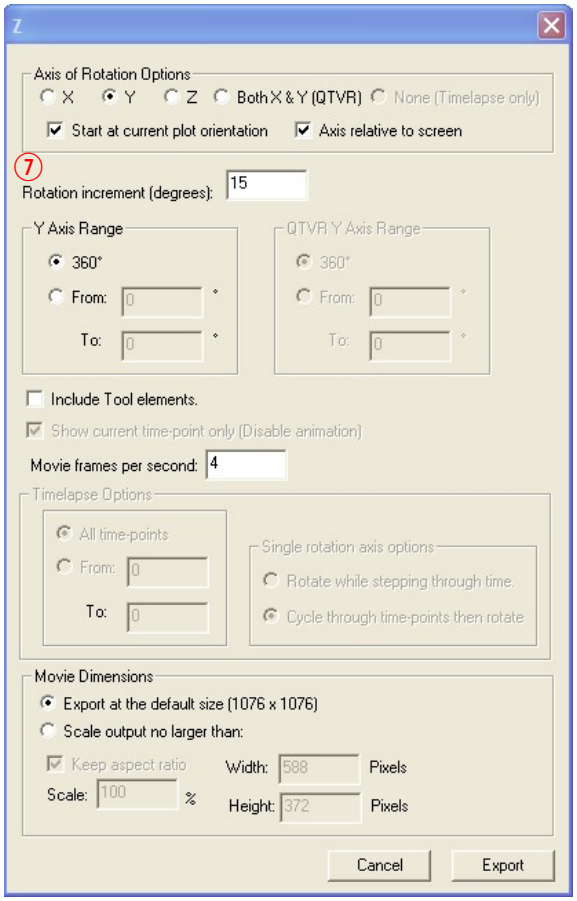
<u>Processing and Exporting Images</u>

If you want to open the images on your computer, the images need to be exported as Tiff image files or movie files. If the image will be used for intensity analysis or processing in other imaging program, it is recommended to export using **Image/Export** menu command as described below.

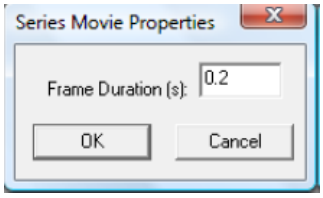
1. **View/Export ▸ :** This command allows you to export images as different file types (BMP, Tiff, or movies). The exported images will be 8-bit, which can be manipulated in Photoshop, Word, or Powerpoint etc. If you want to show annotations such as scale bars, time stamps, etc, you should export the image using this **View/Export** menu command. Open an image using Main View, Three View, Tile View or Channel View of the image.

- **TIFF…** will export only the current display shown on the monitor regardless of image types (i.e. 2D, 3D, 2D timelpase etc). It includes the annotation you put on the image.
- **TIFF Series…** will export the multiple images series such as 2D timelapse, 3D, or 4D. All timepoints or z-series planes will be exported as individual TIFF files (one image for each z or t plane). Thus, it is recommended putting these files into a separate folder. It includes the annotations.

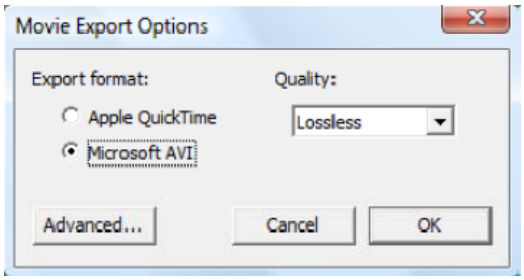
- **Default Views of All Images as TIFFs…** will export a series of TIFF files, one for each image in the slide. Each TIFF file will be a single plane similar to the thumbnail image (default view).
- **Movie…** it will open a window ⑦ for options for exporting 3D Volume/Surface view rendered images into movie files (AVI or Quicktime).



2. **View/Create Series Movie**… ▸ **:** Scanning through time lapse images or z-series can be converted into movies with this menu. It will open the



Series Movie Properties box. Enter the speed at which you want the movie to play in the **Frame Duration**(s) field. 0.2 second usually gives agood viewing speed. Click **OK**, then the **Movie Export Options** dialog box will appear.



Select the movie file format (AVI or Quicktime) and Quality (compression). Click **OK**.
***(Advanced..** button allows you to choose options in compression methods.)*

2**. Image/Export/Channel Intensities as a 16-bit TIFF file**...: This command will export a 16-bit file that can be used for intensity analysis or image processing in other image processing programs. This command exports each imaged channel of the image into separate files. For example, a 2D timelapse with 3 channels (ex; DAPI, Green, Red fluorescence) and 10 timepoints will be exported into 3 individual Tiff files containing single channel and 10 timepoints. If the image is 3D timelapse, it will be exported into 3 multiplane, multitimepoint Tiff files for the 3 channels.

G. System Turning-Off.

When you finish the imaging, remove your sample and clean the immersion media (water or oil) on the lens with LENS PAPER. Select 10x lens and exit the Slidebook program. Write down the log-off time on the Log book. Turn off the laser box key to O position and log off (or shut-down) the windows. Turn off the whole system with the main switch.