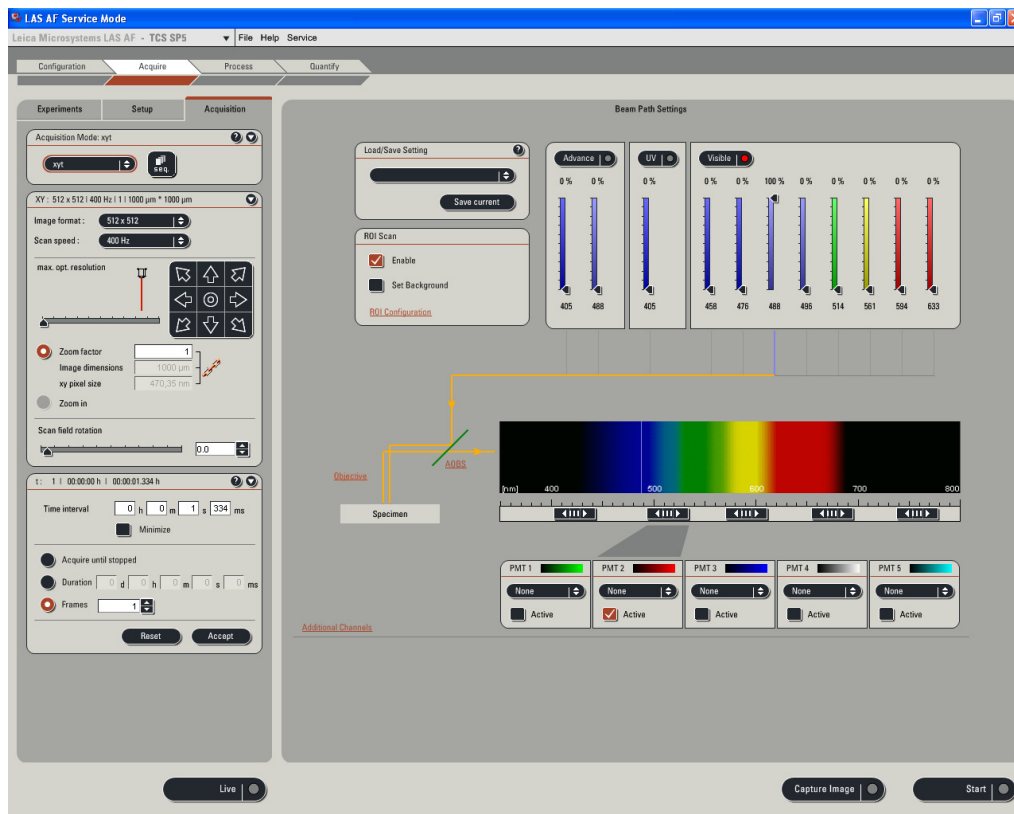


Leica TCS SP5 LASAF New User Guide



This Application Note was created by:

Myriam Gastard, Ph.D.
Leica Application and Technology Support Group, Life Science Division
Exton, PA 19341, USA

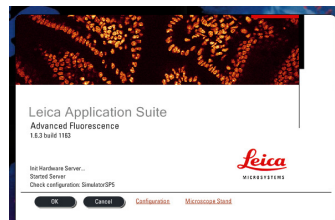
QUICK-START FOR SP5

STARTING YOUR SP5 system:

- 1- Turn on the laser, scan, and computer/Mic buttons on your console (push green buttons, turn your laser's key).

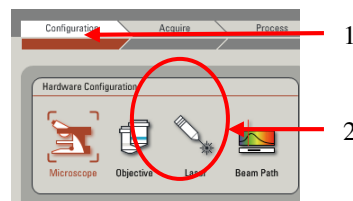


- 2- Logon to Windows.
- 3- Double Click on LAS AF icon on your computer.
- 4- Click on Start in the LAS AF window.

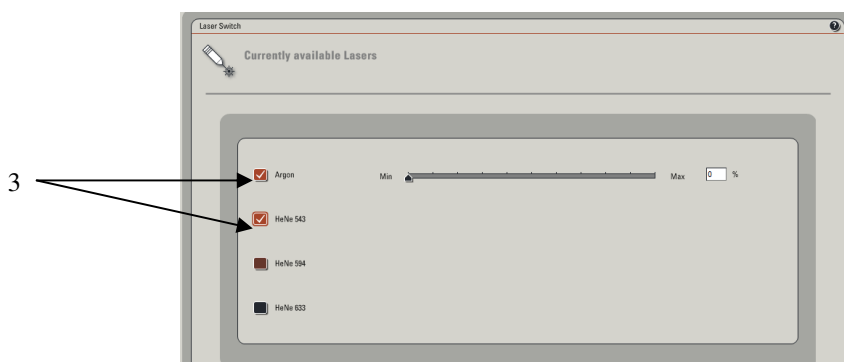


ACTIVATE YOUR LASERS:

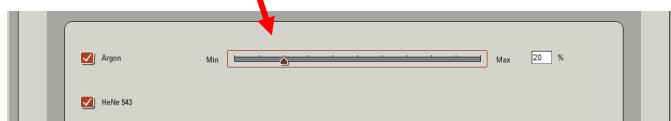
- 1- Click on the **Configuration** tab.
- 2- Click on **laser**.



- 3- Activate the laser(s) needed for your experiment by checking the box(es). *If you do not know which laser(s) to activate, then check every boxes to be sure that the needed laser(s) will be turned on.*



If you are using the Argon laser, do NOT forget to put the digital power slider at 20-30%.




SETUP for ACQUISITION OF IMAGES:

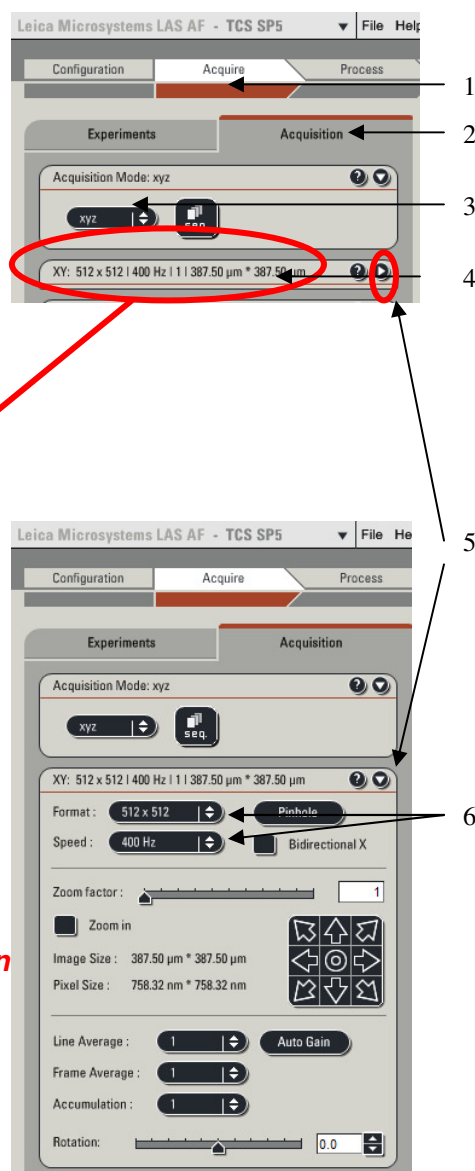
- 1- Click on **Acquire**.
- 2- The window will automatically open on the Acquisition mode.
- 3- The acquisition will be automatically be on **xyz** scanning mode.
- 4- The format of your image is automatically displayed in **512x512** pixels. The speed is automatically chosen at **400 Hz** and the image size as well as the pixel size is automatically calculated and displayed.



- 5- Imaging parameters (XY Window) can be changed by opening the drop-down window.

⇒ Click on the arrowhead. 

- 6- In the opened XY window, image format and scanning speed can be changed. **We encourage new users not to change these parameters at first. A better understanding of your confocal system will allow you to modify later on scan format and speed when appropriate.**



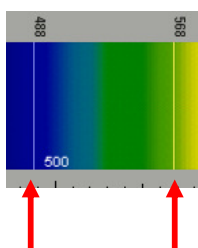
BEAM PATH SETTINGS:

- 1- Click on **Visible** to activate the laser(s).
- 2- Select the laser and their intensity by moving the sliders up or down (AOTF%, between **20-30%** to begin with).

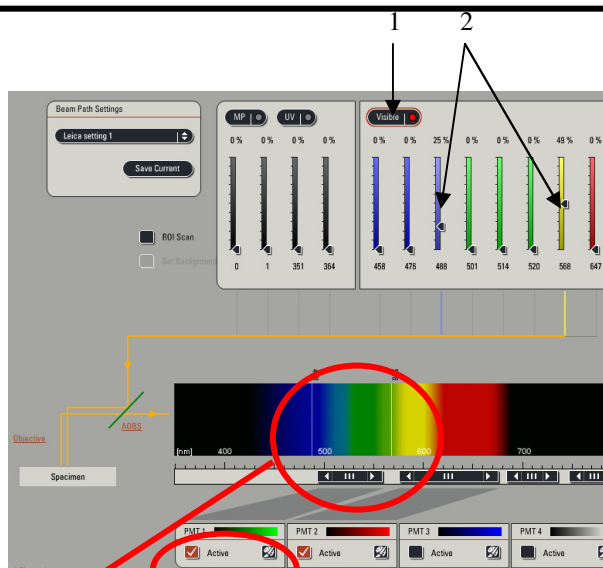
Choice of the laser line(s) is depending on the fluorophore(s) your sample is labeled with.

For instance:

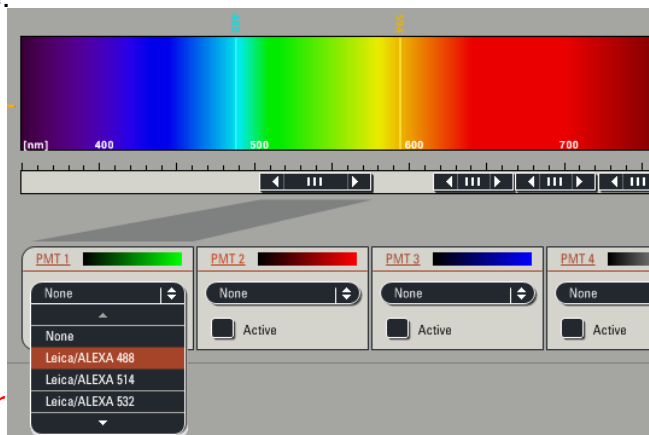
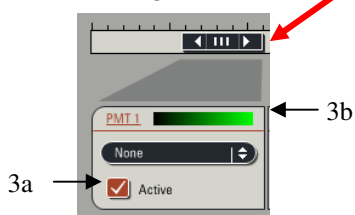
- *Alexa 488, FITC or GFP will be excited using the 488 Argon laser line.*
- *Alexa 568 is excited using the 568 laser line or the 543 laser line if your system is not equipped with a 568 laser line.*



An active laser line will be expressed as a line on the spectrum.

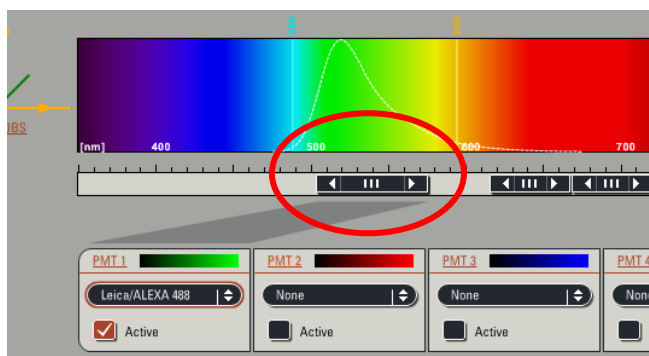


- 3- **Activate** the PMTs (3a) by clicking on the **Active** button and chose the color for your fluorophore emission (3b). A gray shadow will then appear underneath the PMT bar confirming that the PMT is active.

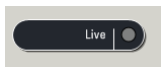


- 4- Click on **None** to open the drop-down window, and choose the fluorophore emission wavelength. This step will help you in setting your PMT
In our example: Alexa 488 was chosen for the PMT1.

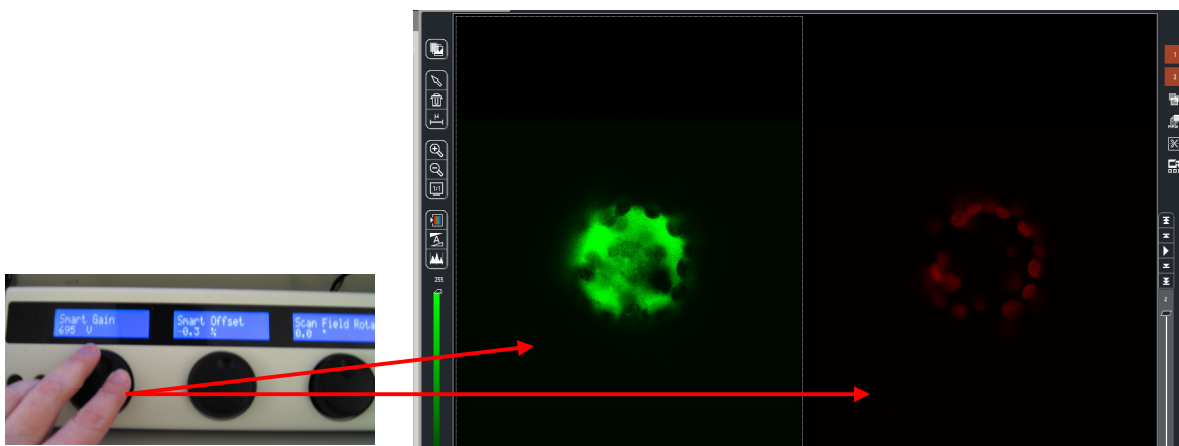
- 5- Place the PMT bars in correspondence with the fluorophore wavelength by sliding it left and right. The slider can also be resized by clicking on the right or left side of it. *Also, double clicking on the slider will open a window where you can enter the begin and end position of the slider*



- 7- Click on the **Live** button (lower left corner of your setup screen) to check a live image of your sample.





- 8- Turn the Smart Gain knob until you can visualize your signal. If you have more than 1 fluorescence, and then more than 1 PMT activated, your viewer screen (right monitor) will be separated in 2 halves.
- Click on one half of the viewing screen to select the channel, and adjust your gain using the **Smart Gain** knob.
 - Then, click on the other half of the screen to select the other channel, and adjust the gain for this channel using the **Smart Gain** knob again.

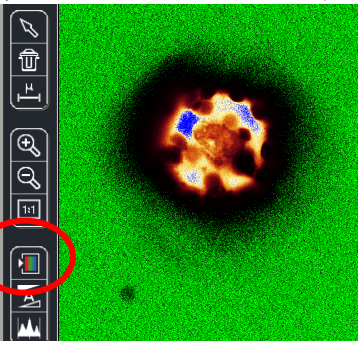


- 9- Adjust your gain and offset, using the **QLUT** button (Quick Look Up Table) to change your image color as intensity values. Set up your intensity as shown below with few blue (saturated) pixels, most orange and white pixels, and your background as mostly green pixels (using your Smart Offset button).

Gain adjustment

Offset adjustment



Blue = saturated signal = 255

Black to Orange to white = signal between 1 to 254

Green = no signal = 0

Gain adjustment

Offset adjustment

Click twice on the **QLUT** button to go back to your original colors.

- 10- Adjust if necessary the **laser intensities** (as described in 2) and **PMTs** bars position (as described in 5).

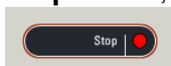
If the image is still too dim or not visible at all:

- Enhance the laser power using the vertical slider (AOTF %) until you can see an image on the screen.
- Adjust the PMT Smart Gain.

PS: A smart gain value lower than 400 V would mean that you can lower the laser power and go up the smart gain until about 900-1000 V). A smart gain between 1100-1250 would suggest going up on the laser power (AOTF %).

REMEMBER: By enhancing the AOTF% you will expose your sample to more laser exposition, hence your sample **will bleach faster**. On the other hand, enhancing the gain won't expose your sample to more laser exposition, and it will protect your sample from too much laser exposition. Thus, in order to protect your sample signal, it is better to first adjust your gain, and then if not enough signal is found to enhance your AOTF %.

Click on the **Stop** button, and then click on the **Capture Image** button to acquire an image.

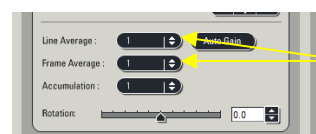


CHANGING THE QUALITY OF YOUR IMAGE ACQUISITION:

- 1- Averaging the **Line** number and/or the **Frame** number can dramatically enhance the quality of the acquired image.

⇒ **Acquisition** ⇒ **XY window**

Under **Acquire** and **Setup**, click on the arrows of either **Line** and/or **Frame** buttons and choose the averaging number (for instance 1-4).

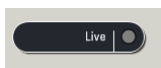


1

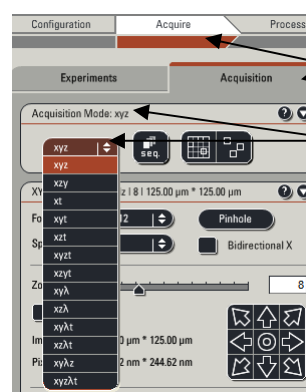
ACQUISITION OF A Z-STACK:

- 1- Under the **Acquire** Tab, go to **Acquisition**.
- 2- Click on **Scan Modes** in the **Acquisition Mode** and select **xyz**.

- 3- Go to **Live Mode**.



- 4- Move at the top of your sample (on the Z plan, using the z-position knob), and set the position of your Z-Stack by clicking on the **Begin** arrowhead.

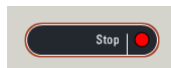


1

2

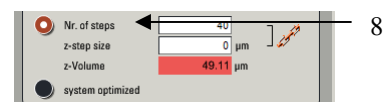
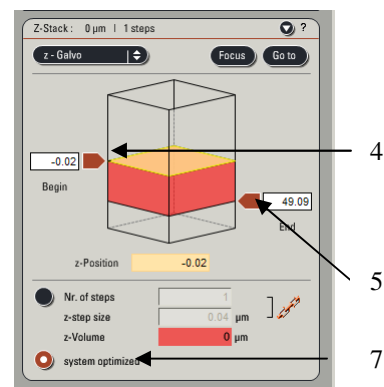
- 5- Move at the bottom of your sample (or region of interest, using the z-position knob), And set the bottom position of your Z-Stack by clicking on the **End** arrowhead.

- 6- Click on **Stop**.

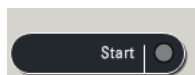


- 7- To set the number of z-steps, you can choose **system optimized** if you desire to obtain the optimal number of image calculated for your Z-Stack size (depending on your objective, zoom and image format).

- 8- If you choose to enter the number of z-steps or the z-step size then click on **Nr. of steps**.



- 9- Click on **Start**, and your Z-Stack will begin and end automatically when finished.



- 10- Your z-stack will be automatically saved under **Experiment**, and under a name as: **Serie001 (56.4 MB, xyz)**.

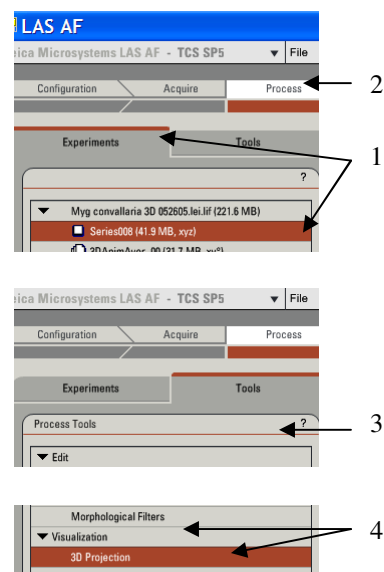
PS: You can rename your experiment by Right clicking on the name and click on “Rename” and then type a new name.

3-DIMENSIONAL PROJECTION:

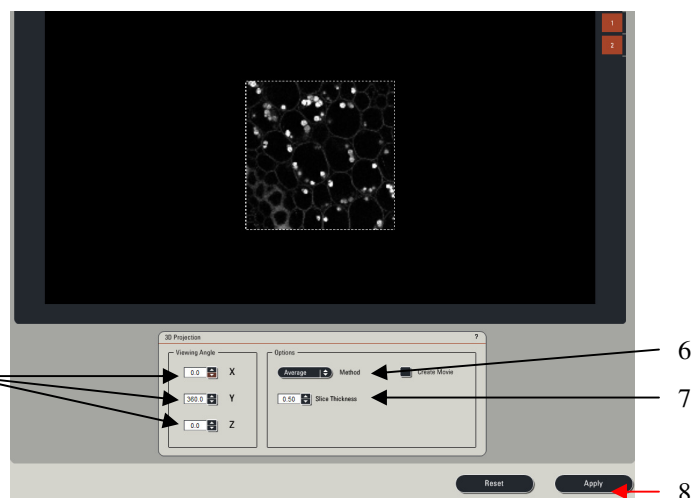
- **3D projection without animation:**

After acquiring a z-stack (or series), you can process your data to a 3-D projection.

- 1- Under **Experiment**, click on your **Series** name.
- 2- Go to **Process**.
- 3- Click on **Tool**.
- 4- In the **Process Tools**, click under **Visualization** and **3D Projection**, located at the bottom of the list.

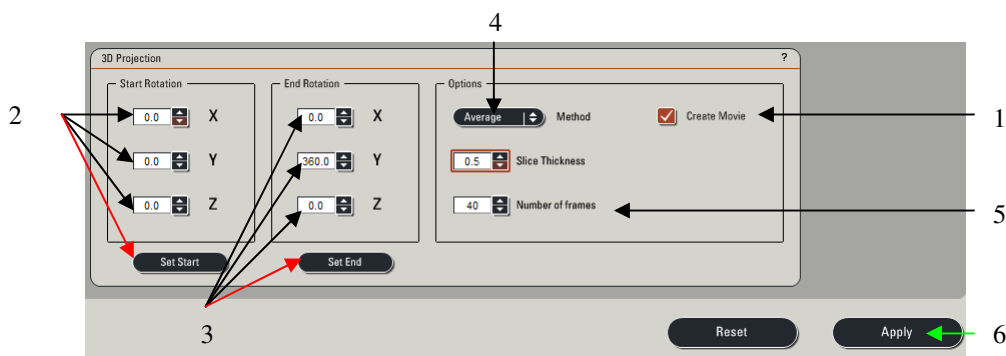


- 5- Do NOT change the **X**, **Y**, and **Z** plans if you just need to see a simple projection .
- 6- Enter **Maximum** in the Method drop down list (**Average** can be used if your fluorescence intensity is very strong and a max projection saturates completely the signal).
- 7- Enter **1** in the **Slice Thickness**.
- 8- Click on **Apply**.



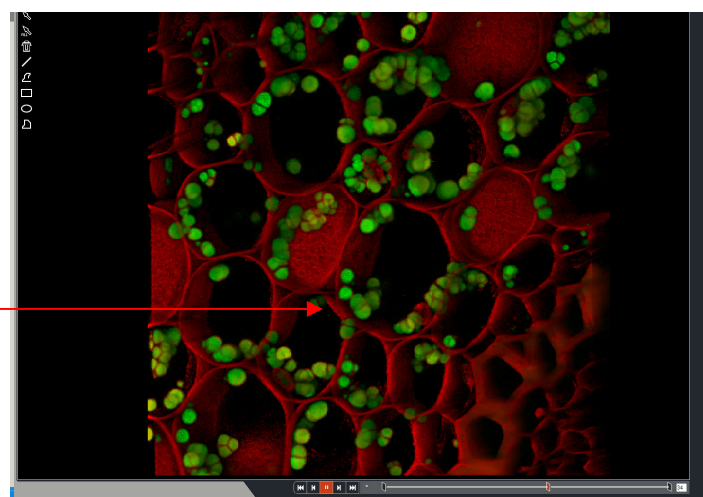
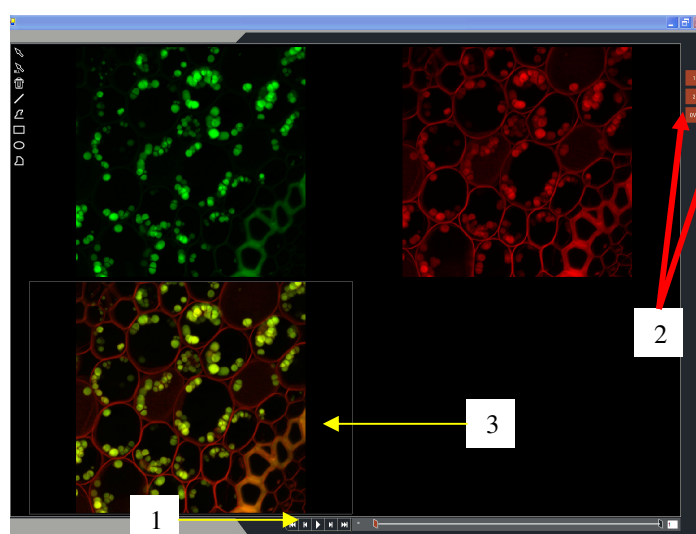
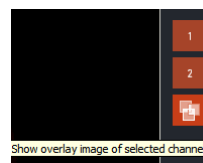
• **3D projection with Animation:**

- 1- Click on **Create a Movie**.
- 2- Enter the **Start Rotation** angle (in degree) corresponding to the start view of the movie (example: -190, Start Rotation), and click on **Set Start**.
- 3- Enter the **End Rotation** angle (in degree) corresponding to the end view of the movie (example: 190, End Rotation), and click on **Set End**.
- 4- Under **Options**, enter the **Method** in the drop down list (ex: Maximum).
- 5- Enter the **Number of Frames** (= number of frame needed to do the rotation. Higher the number and slower the speed of rotation; example: 70 for a complete rotation for a 512x512 z-stack series).
- 6- Click on **Apply**.



The 3D movie can be visualized on your right screen.

- 1- Click the Play (▶) button, to begin the movie.
- 2- Click the Overlay (📄) button to visualize both colors.
- 3- Double click on the overlay image to have the movie full screen.
- 3- Click on the **Stop** button to end the movie.



Shutdown Procedure

If no one else is signed up to use the system for 2 hours, shut the system down in the following order:

- Save your images and exit the software.
- Shut down the computer via the Start menu of Windows
- Turn off the mercury lamp power supply and write the elapsed time on sign-in sheet.
- Turn the laser key counterclockwise. **Leave the laser power button on (button #3).**
- Switch off the Scanner power button (button #2) .
- When the computer is completely shut down, switch off the PC/Microscope button (button #1).
- Wait 5 minutes, then switch off the laser power button (button #3). This button controls the fan that cools the lasers, and laser life will be significantly shortened if this rule is not followed. You can be doing other housekeeping things while you wait – cleaning off the objective and your slides, etc.