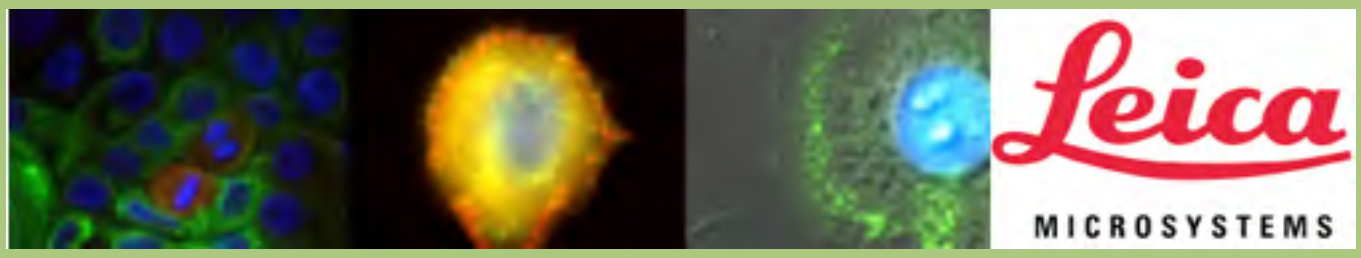
Leica SP5 Laser Scanning Confocal Microscope Training Session



Overview

1. Fluorescence - What is Fluorescence? How does a fluorescence microscope work? Principle of confocal and spinning disk confocal. Fluorescent proteins and dyes.
2. Hardware - What can this particular microscope image?
3. Quick Guide - How to get started: What to do and what not to do!

Web Help

Table of Fluorescent dyes:	http://flowcyt.salk.edu/fluo.html
Microscopy Resources Centre:	http://www.olympusmicro.com/primer/index.html
Fluorescence Spectra Viewer:	http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html

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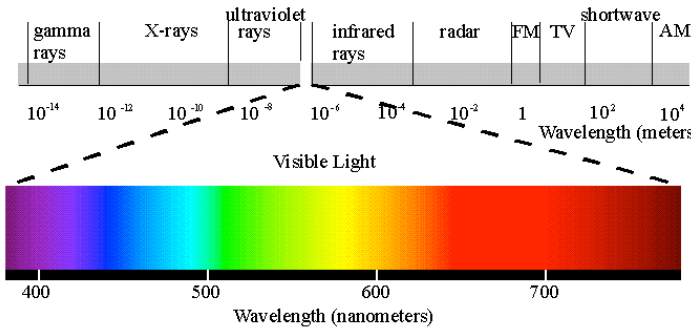
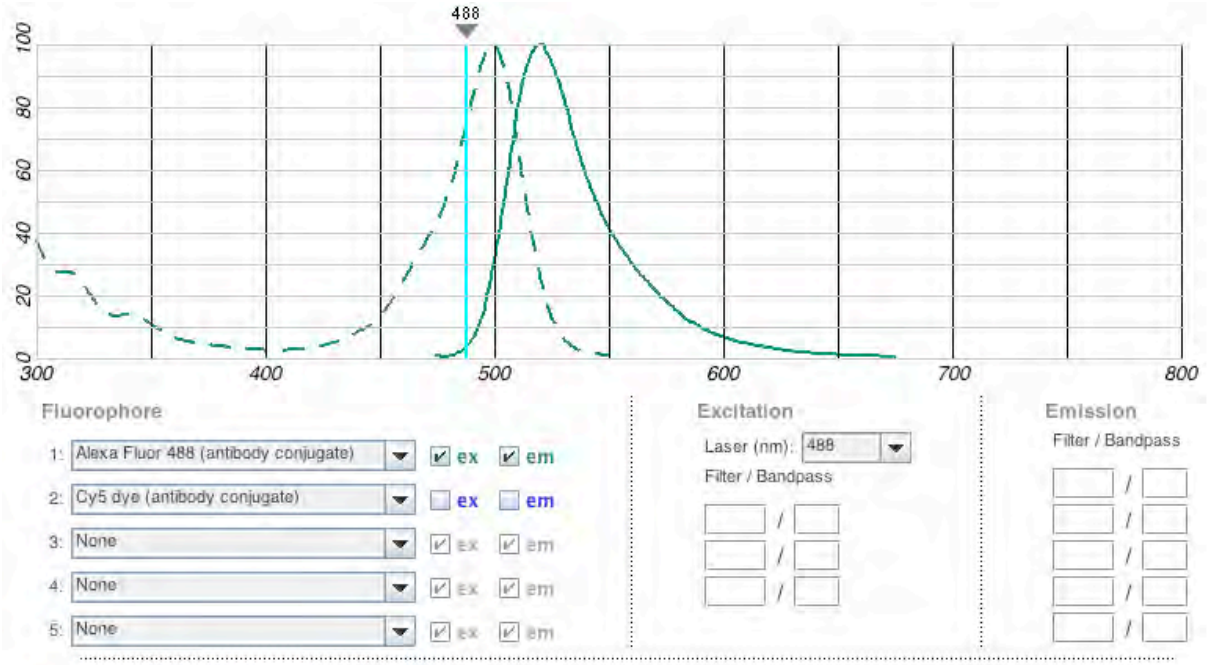
The wavelength of light

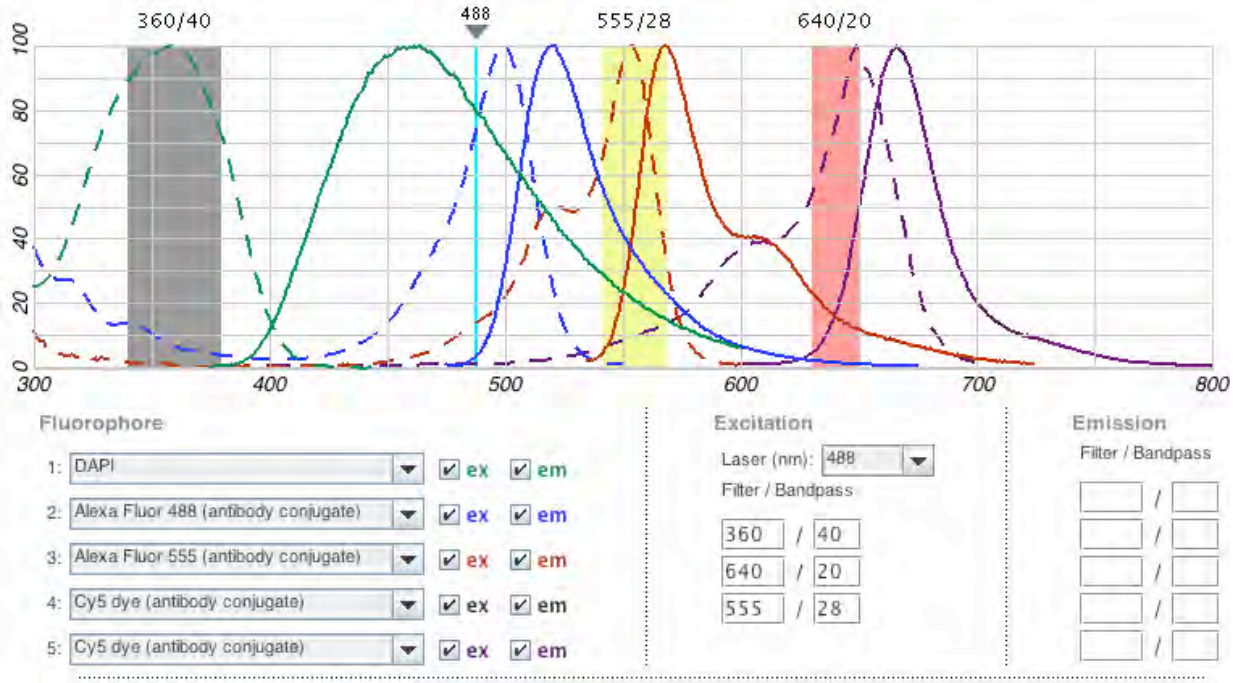
In light microscopy we use light to form an image in the microscope. Each photon in the visible light range has a quanta of energy that depends on its wavelength and can be calculated from the following equation:

$$E = hc/\lambda$$

Therefore longer wavelengths equate to lower energy waves, and shorter wavelengths equate to higher energy waves.

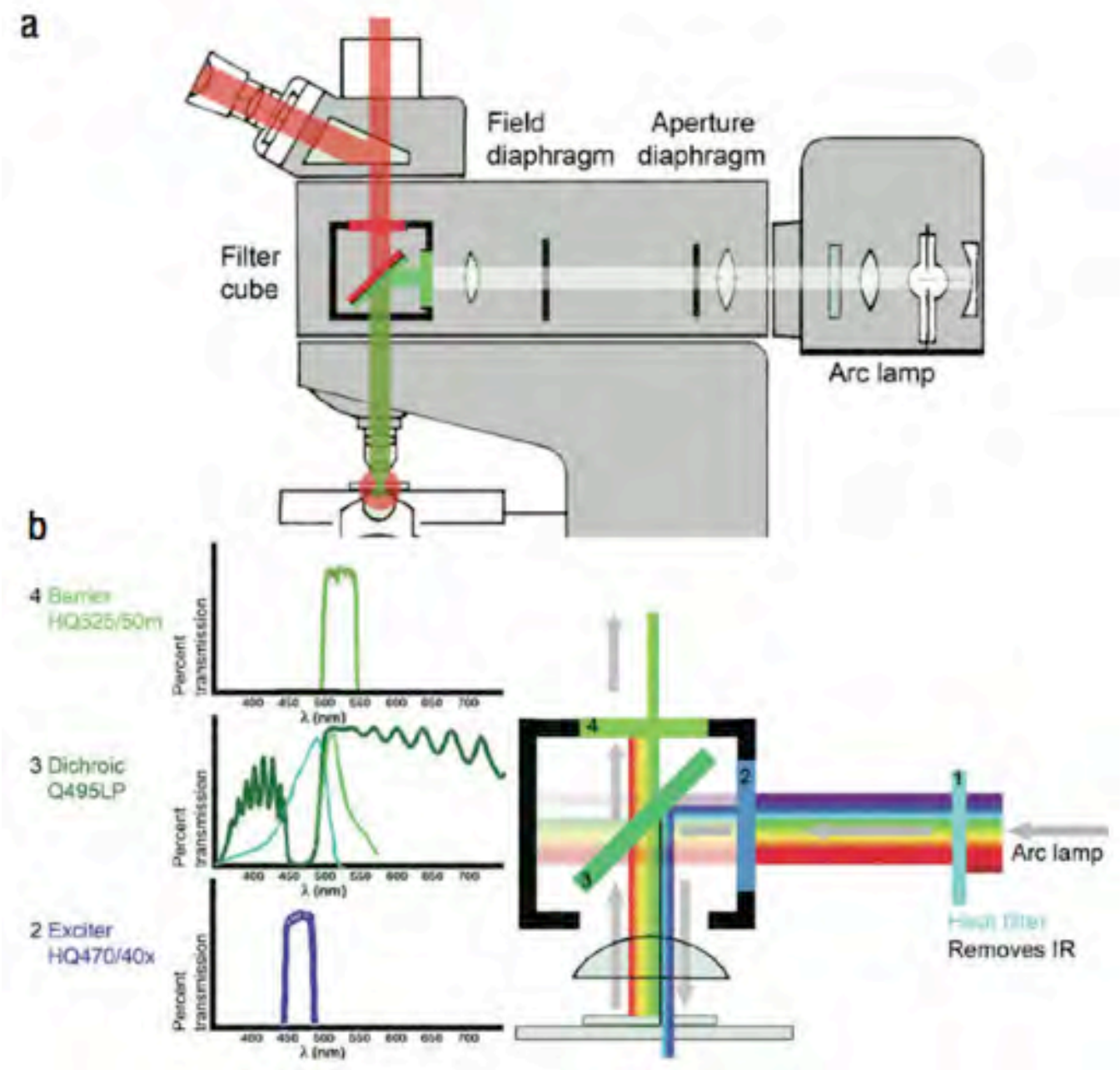






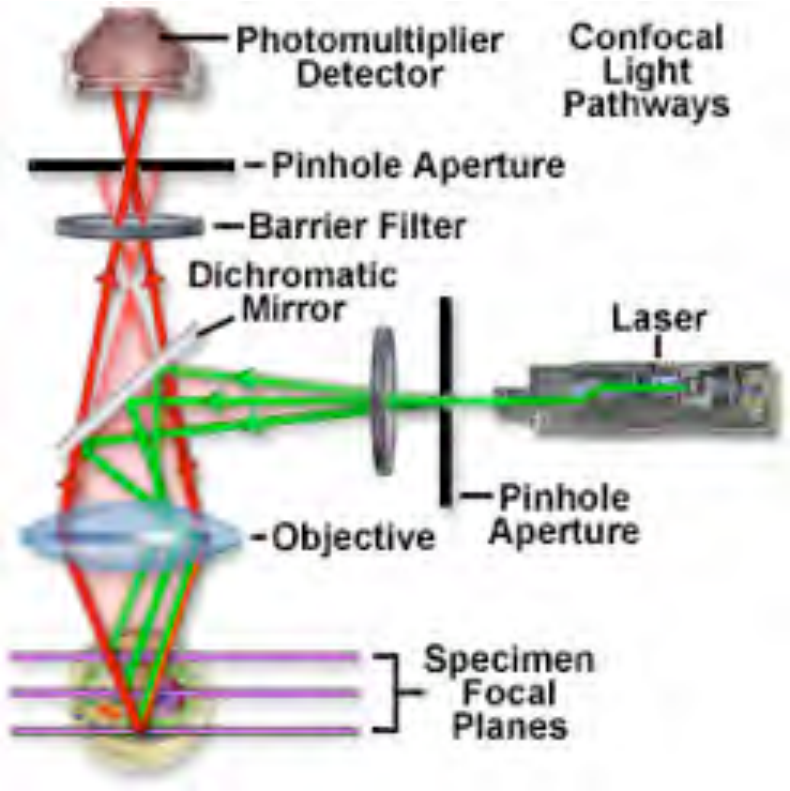
Fluorescence Spectra

In fluorescence microscopy, it's important to determine whether **your** fluorophore(s) will be visible in a **particular instrument** - in particular, the one you're using, with it's unique set of excitation sources, emission filters and beamsplitters. The images above are taken from the Fluorescence Spectra viewer available on invitrogen's website (url on page 1).



How does a fluorescence microscope work?

The fluorescence microscope works because it shows the spatial relationships among specific molecules (which you have labelled with a fluorochrome(s)). It achieves this high specificity by blocking the excitation light from reaching your eyes, only allowing the light emitted from the fluorochrome to be detected. The essence of the fluorescence microscope is the filter cube, composed of three glass filters (emission, excitation and dichroic) that are designed with special optical properties. They are coated with thin dielectric films and interference results in transmission of some wavelengths and reflection of others.



Confocal Microscopes

A laser scanning confocal microscope (LSM, LSCM, confocal) works by raster scanning a point of laser excitation across the sample (using two mirrors to translate the laser beam in x & y), and recording the number of emitted photons at each point by means of a photomultiplier tube. At any one position (the pixel dwell time is on the order of microseconds), only a single point is being sampled. As the laser traverses the sample and the data are recorded by the PMT, an image is constructed on the screen.

To reduce out of focus blur, "pinholes" are placed in front of the detector and the laser. These act to restrict the light that reaches the detector. Only emitted photons from the plane of focus will reach the detector and contribute to the formation of the image. A series of focal planes are imaged sequentially, generating 3D information from the sample.

A spinning disk confocal microscope differs slightly from a LSCM in how it forms the image. Instead of using mirrors to raster a single laser beam across the sample, the laser is directed through "nipkow disk" to generate the raster scan across the specimen and provide the out of focus light rejection. The disk rotates at several thousand rpm, providing an image of the entire field at once. As the entire image is present at one time, CCD cameras are used to detect the emitted photons, rather than the PMTs used in the LSCM.

Fluorescent Proteins/Dyes

The use of GFP as a protein tag has led to a revolution on fluorescent microscopy. Many fluorescent protein variants as well as organic dyes are available that you can use to stain your sample. Many can be imaged in live cells.

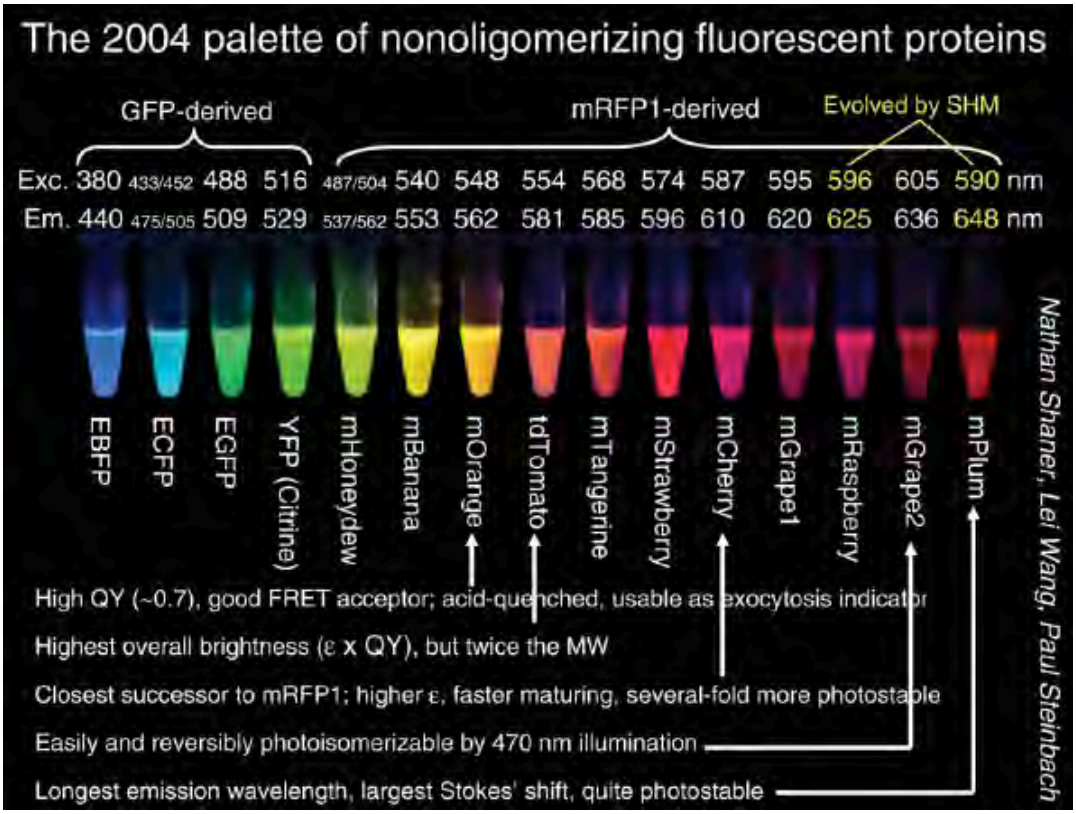


Table 1 | Properties of the best FP variants[a,b]

Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]	Tsien (4)	574	596	26	15	<4.5	Monomer
	J-Red[h]	Evrogen	584	610	8.8*	13	5.0	Dimer
	DsRed-monomer[h]	Clontech	556	586	3.5	16	4.5	Monomer
Orange	mOrange[g]	Tsien (4)	548	562	49	9.0	6.5	Monomer
	mKO	MBL Intl. (10)	548	559	31*	122	5.0	Monomer
Yellow-green	mCitrine[i]	Tsien (16,23)	516	529	59	49	5.7	Monomer
	Venus	Miyawaki (1)	515	528	53*	15	6.0	Weak dimer[j]
	YPet[g]	Daugherty (2)	517	530	80*	49	5.6	Weak dimer[j]
	EYFP	Invitrogen (18)	514	527	51	60	6.9	Weak dimer[j]
Green	Emerald[g]	Invitrogen (18)	487	509	39	0.69[k]	6.0	Weak dimer[j]
	EGFP	Clontech[l]	488	507	34	174	6.0	Weak dimer[j]
Cyan	CyPet	Daugherty (2)	435	477	18*	59	5.0	Weak dimer[j]
	mCFPm[m]	Tsien (23)	433	475	13	64	4.7	Monomer
	Cerulean[g]	Piston (3)	433	475	27*	36	4.7	Weak dimer[j]
UV-excitable green	T-Sapphire[g]	Griesbeck (6)	399	511	26*	25	4.9	Weak dimer[j]

[a]An expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. [b]The mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. [c]Major excitation peak. [d]Major emission peak. [e]Product of extinction coefficient and quantum yield at pH 7.4 measured or confirmed (indicated by *) in our laboratory under ideal maturation conditions, in (mM • cm)–1 (for comparison, free fluorescein at pH 7.4 has a brightness of about 69 (mM • cm)–1). [f]Time for bleaching from an initial emission rate of 1,000 photons/s down to 500 photons/s ($t_{1/2}$; for comparison, fluorescein at pH 8.4 has $t_{1/2}$ of 5.2 s); data are not indicative of photostability under focused laser illumination. [g]Brightest in spectral class. [h]Not recommended (dim with poor folding at 37 °C). [i]Citrine YFP with A206K mutation; spectroscopic properties equivalent to Citrine. [j]Can be made monomeric with A206K mutation. [k]Emerald has a pronounced fast bleaching component that leads to a very short time to 50% bleach. Its photostability after the initial few seconds, however, is comparable to that of EGFP. [l]Formerly sold by Clontech, no longer commercially available. mECFP with A206K mutation; spectroscopic properties equivalent to ECFP.

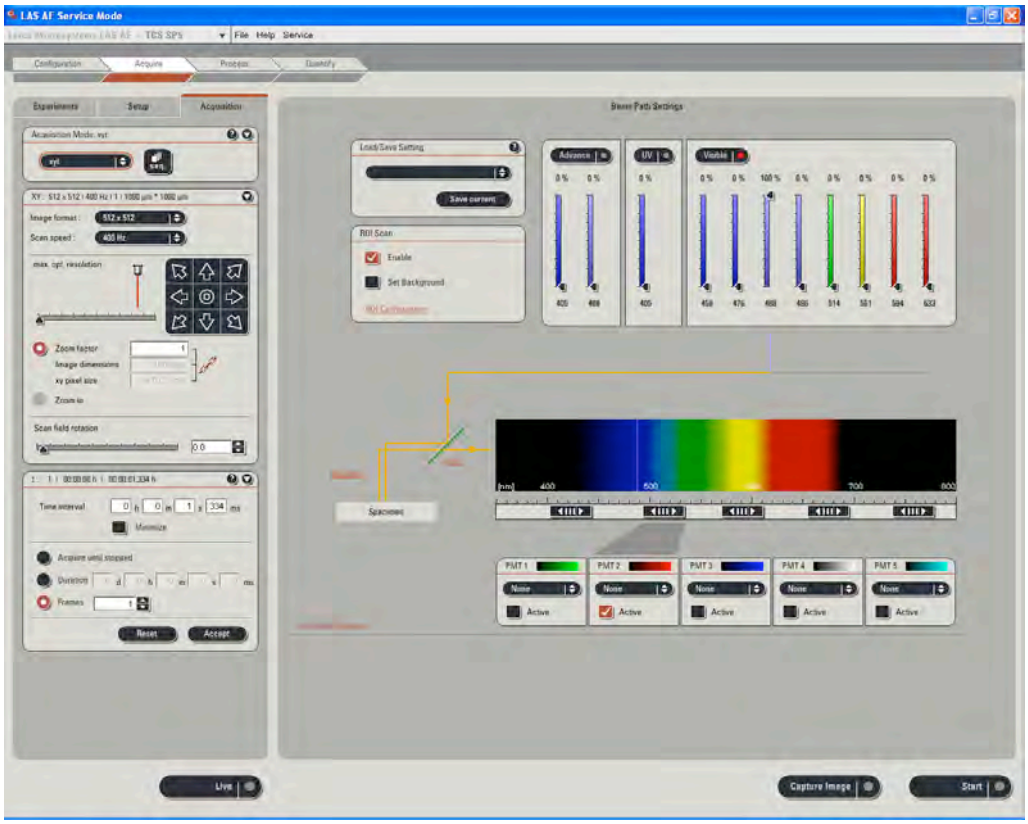
Table 1. Properties of Alexa Fluor® Dyes

Alexa Fluor® dye	Absorption max. (nm)	Emission max (nm)	Emission color*	Extinction coefficient**
Alexa Fluor® 350	346	442	Blue	19,000
Alexa Fluor® 405	401	421	Blue	34,000
Alexa Fluor® 430	433	541	Green/Yellow	16,000
Alexa Fluor® 488	496	519	Green	71,000
Alexa Fluor® 532	532	553	Yellow	81,000
Alexa Fluor® 546	556	573	Orange	104,000
Alexa Fluor® 555	555	565	Orange	150,000
Alexa Fluor® 568	578	603	Orange/Red	91,000
Alexa Fluor® 594	590	617	Red	73,000
Alexa Fluor® 610	612	628	Red	138,000
Alexa Fluor® 633	632	647	Far Red	239,000
Alexa Fluor® 635	633	647	Far Red	140,000
Alexa Fluor® 647	650	665	Near-IR***	239,000
Alexa Fluor® 660	663	690	Near-IR***	132,000
Alexa Fluor® 680	679	702	Near-IR***	184,000
Alexa Fluor® 700	702	723	Near-IR***	192,000
Alexa Fluor® 750	749	775	Near-IR***	240,000
Alexa Fluor® 790	784	814	Near-IR***	270,000

* Typical emission color seen through the eyepiece of a conventional fluorescence microscope with appropriate filters.
** Extinction coefficient at lmax in cm-1M-1.
*** Human vision is insensitive to light beyond ~650 nm; it is not possible to view near-IR fluorescent dyes.

Alexa Fluor antibody conjugates - see http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/alexa-fluor/Alexa-Fluor-Dyes-Across-the-Spectrum.html

Leica TCS SP5
LAS AF Guide for New Users



For Confocal Application Support and Technical questions:
Please contact our *One*Call™ Toll Free Number: 866-830-0735, Option 3
or EMAIL: Confocal@Leica-Microsystems.com

First, try to Call Steve @ 780.492.1613 or 780.566.2185 or stephen.ogg@ualberta.ca

QUICK-START FOR SP5

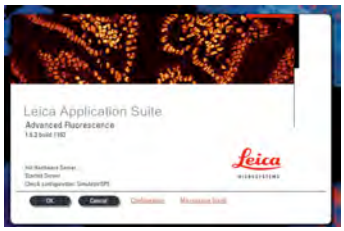
STARTING YOUR SP5 system:

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.

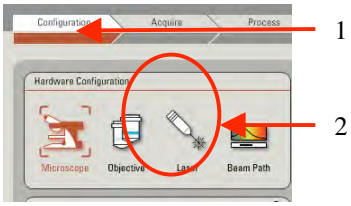
3- Double Click on LAS AF icon on your computer.

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.

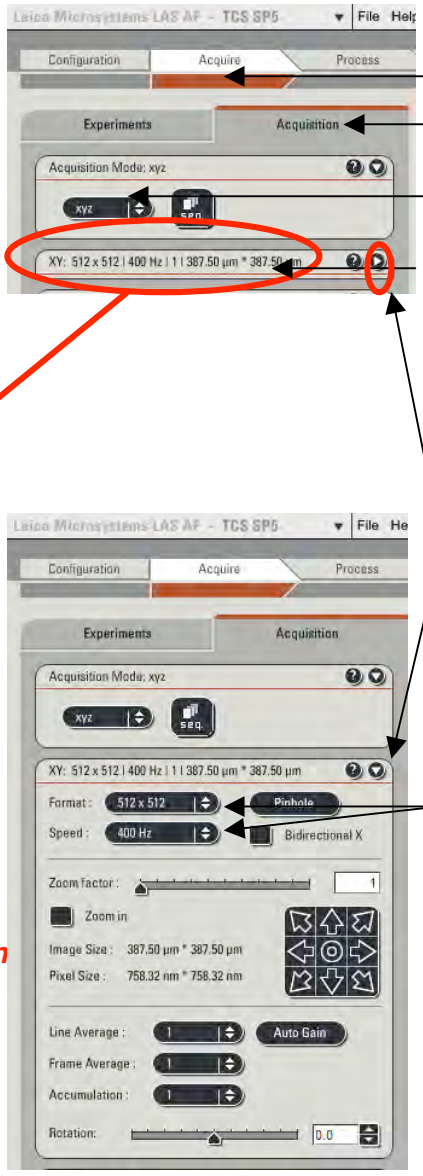
3- The acquisition will be automatically be on **xyz** scanning mode.

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.

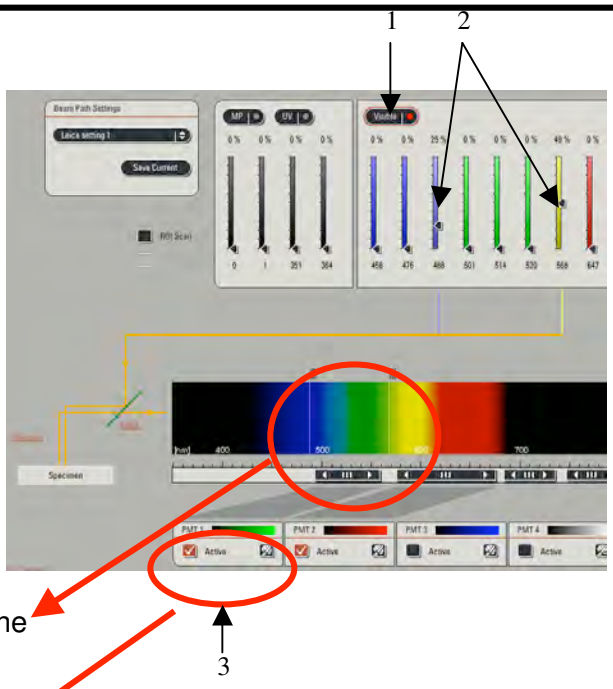
5- Imaging parameters (XY Window) can be changed by opening the drop-down window.

⇨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.***



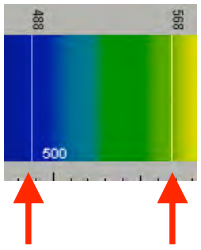
BEAM PATH SETTINGS:



1- Click on **Visible** to activate the laser(s).

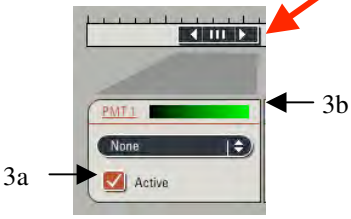
2- Select the laser and their intensity by moving the sliders up or down (AOTF%, between **<u>20-30%</u>** to begin with).
Choice of the laser line(s) is depending on the fluorophore(s) your sample is labeled with.
For instance:

- *Alexa 488, FITC or GFP will be excited using the 488 Argon laser line.*
- *Alexa 568 is excited using the568 laser line or the 543 laser line if your system is not equipped with a 568 laser line.*

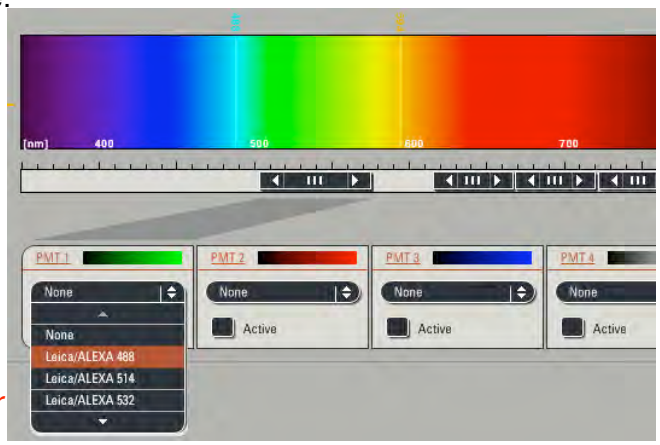


An active laser line will be expressed as a line on the spectrum.

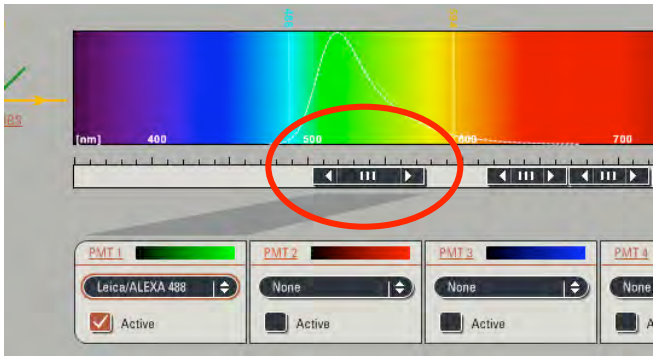
3- **Activate** the PMTs (3a) by clicking on the **Active** button and chose the color for your fluorophore emission (3b). A gray shadow will then appear underneath the PMT bar confirming that the PMT is active.



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.



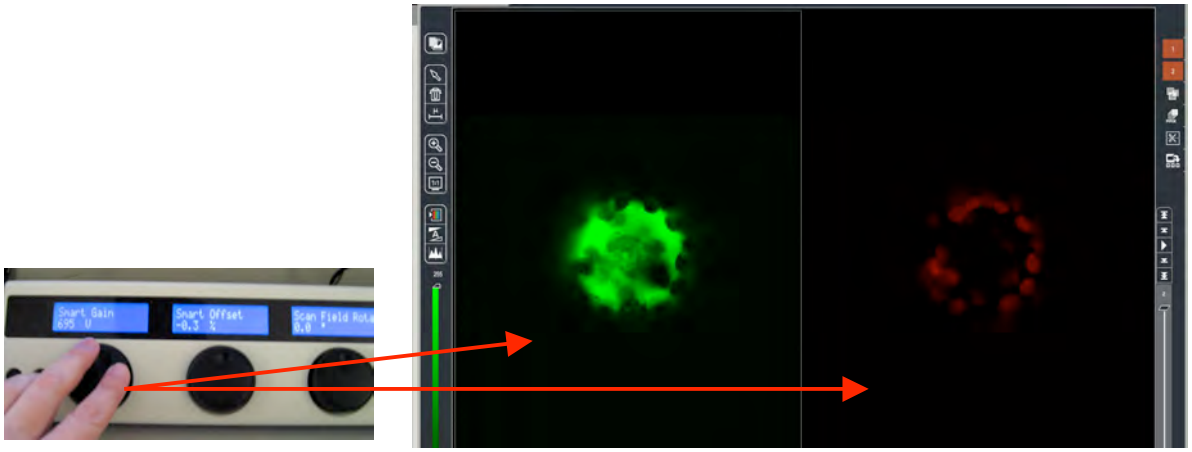
5- Place the PMT bars in correspondence with the fluorophore wavelength by sliding it left and right. The slider can also be resized by clicking on the right or left side of it. *Also, double clicking on the slider will open a window where you can enter the begin and end position of the slider*



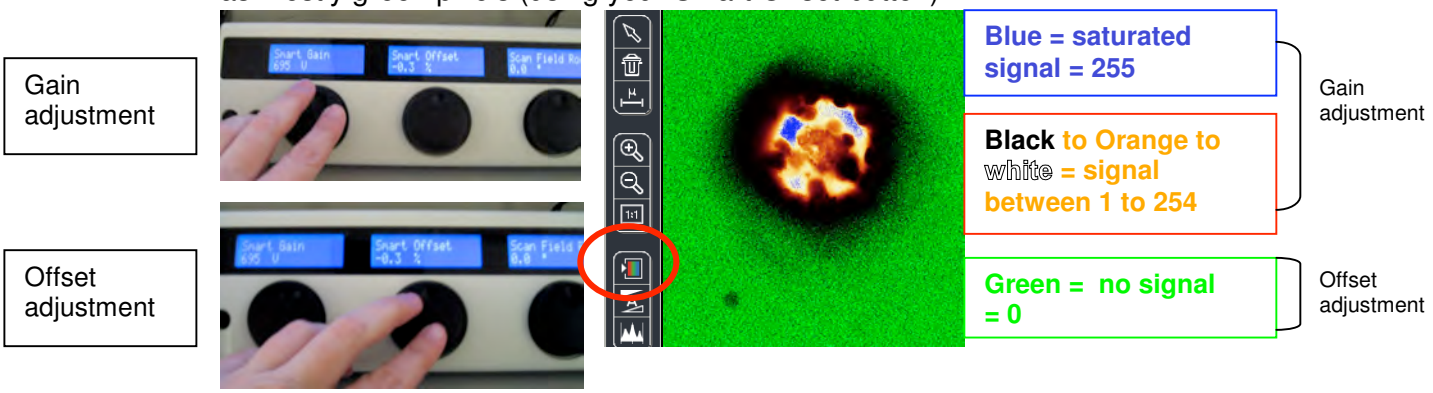
7- Click on the **Live** button (lower left corner of your setup screen) to check a live image of your sample.

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).



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).

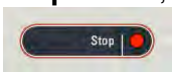
If the image is still too dim or not visible at all:

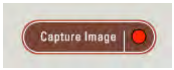
- Enhance the laser power using the vertical slider (AOTF %) until you can see an image on the screen.
- Adjust the PMT Smart Gain.

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.



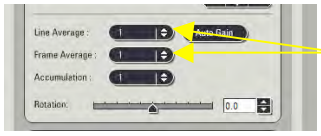


CHANGING THE QUALITY OF YOUR IMAGE ACQUISITION:

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

⇨ Acquisition ⇨ XY window

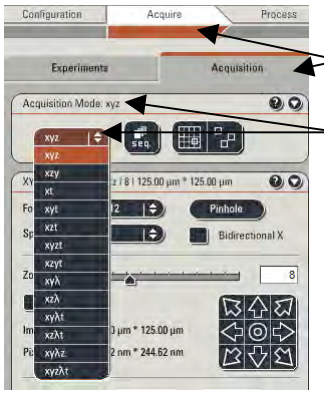
Under **Acquire** and **Setup**, click on the arrows of either **Line** and/or **Frame** buttons and choose the averaging number (for instance 1-4).



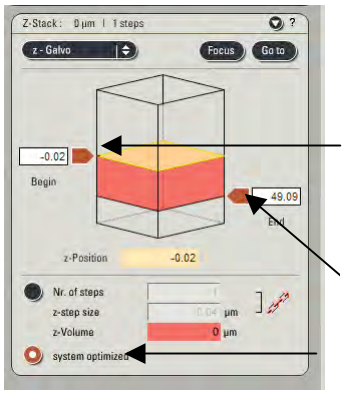
ACQUISITION OF A Z-STACK:

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.

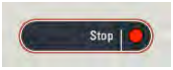
4- Move at the top of your sample (on the Z plan, using the z-position knob), and set the position of your Z-Stack by clicking on the **Begin** arrowhead.



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.

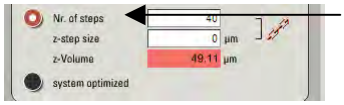


6- Click on **Stop**.

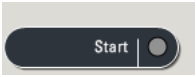


7- To set the number of z-steps, you can choose**system optimized** if you desire to obtain the optimal number of image calculated for your Z-Stack size (depending on your objective, zoom and image format).

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.



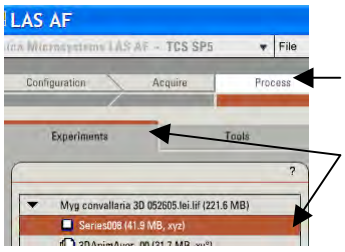
10- Your z-stack will be automatically saved under **Experiment**, and under a name as: **Serie001 (56.4 MB, xyz)**.

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:

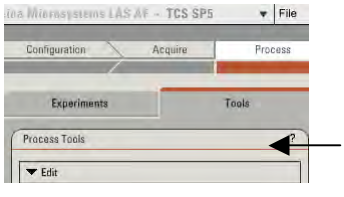
- **3D projection without animation:**

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



1- Under **Experiment**, click on your **Serie**s name.

2- Go to **Process**.



3- Click on **Tool**.

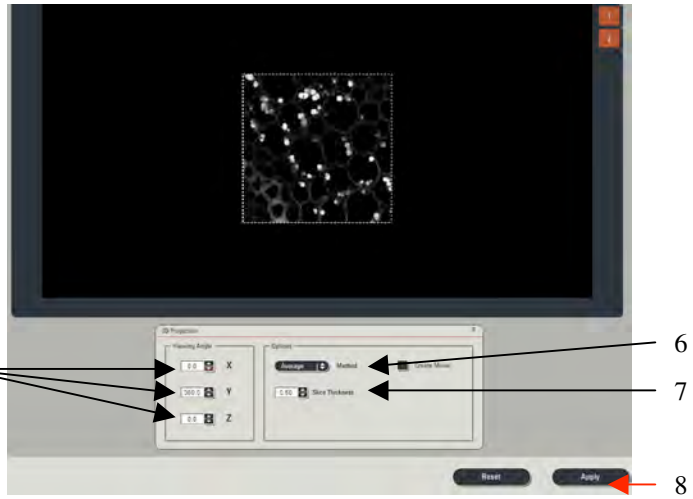
4- In the **Process Tools,** click under **Visualization** and **3D Projection**, located at the bottom of the list.

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).

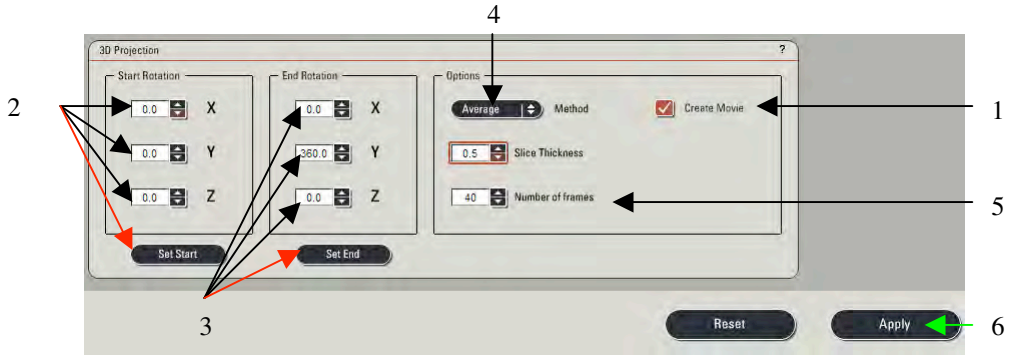
7- Enter **1** in the **Slice Thickness**.

8- Click on **Apply**.



- **<u>3D projection with Animation:</u>**

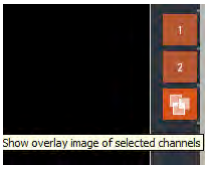
1- Click on **Create a Movie**.
2- Enter the **Start Rotation** angle (in degree) corresponding to the start view of the movie (example: –190, Start Rotation), and click on **Set Start**.
3- Enter the **End Rotation** angle (in degree) corresponding the end view of the movie (example: 190, End Rotation), 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.