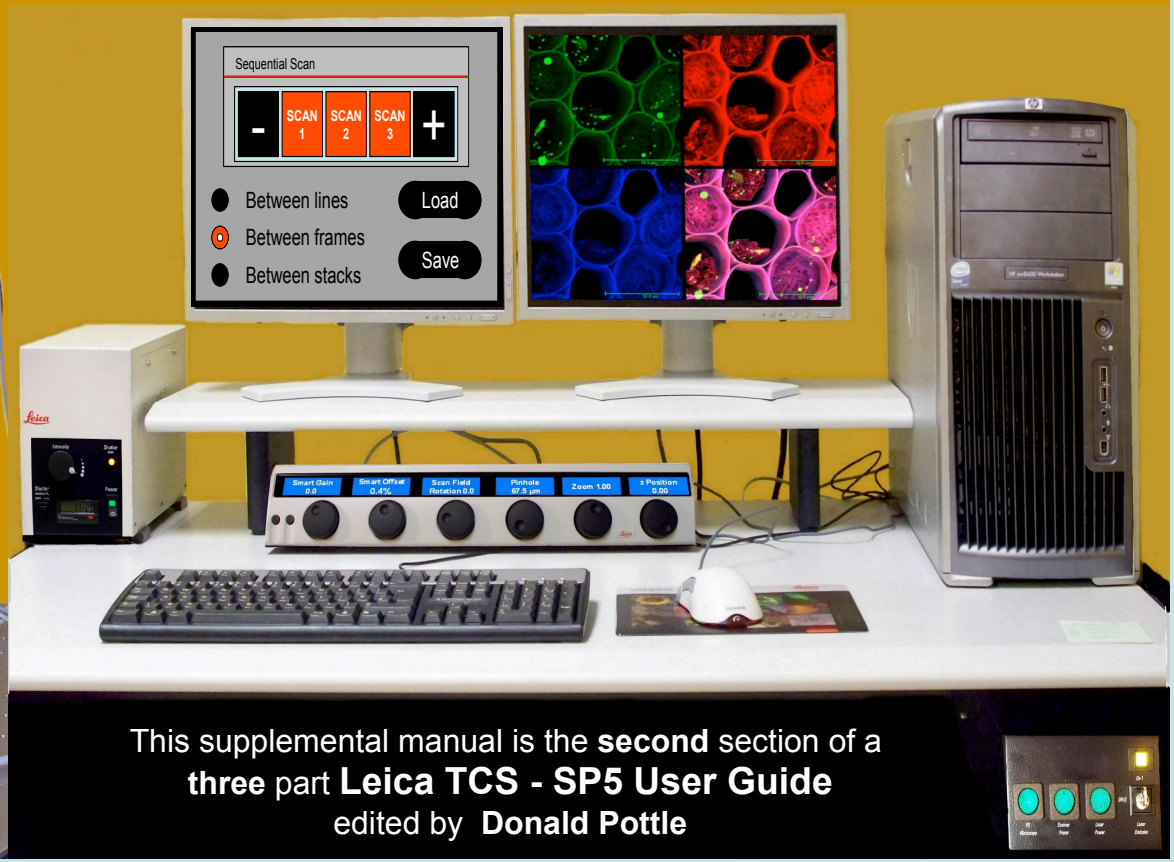
TCS – SP5 User Guide 2. SEQUENTIAL SCANNING

DWP -
Mar. 12, 2009

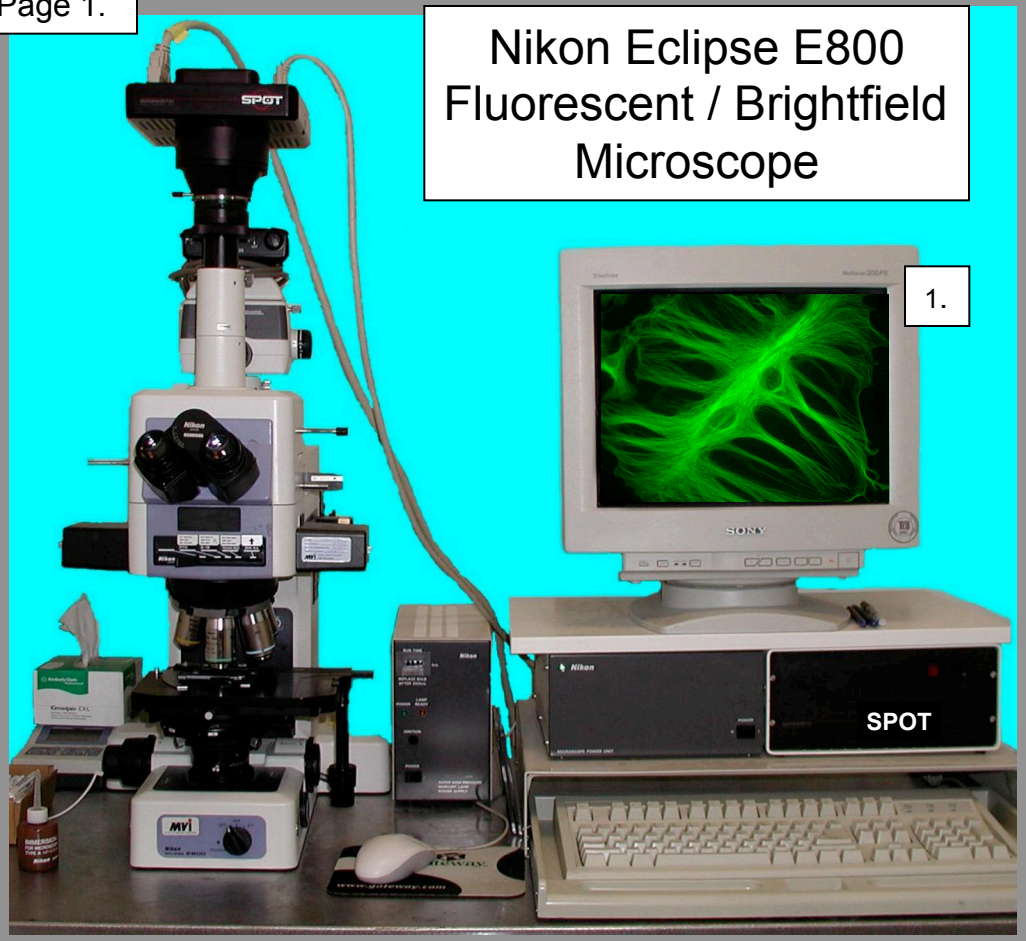
Schepens
149 W

Using the Leica TCS - SP5 Confocal Laser Scanning Microscope

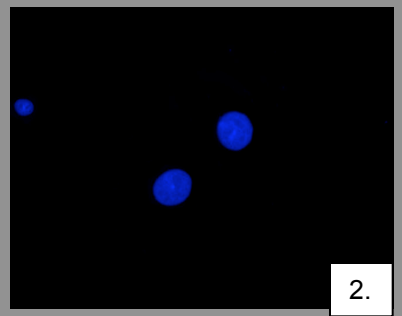


This supplemental manual is the **second** section of a **three** part **Leica TCS - SP5 User Guide**
edited by **Donald Pottle**

Nikon Eclipse E800 Fluorescent / Brightfield Microscope



1.



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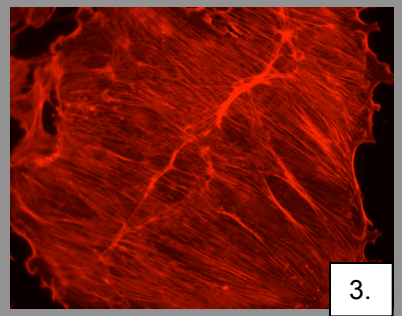
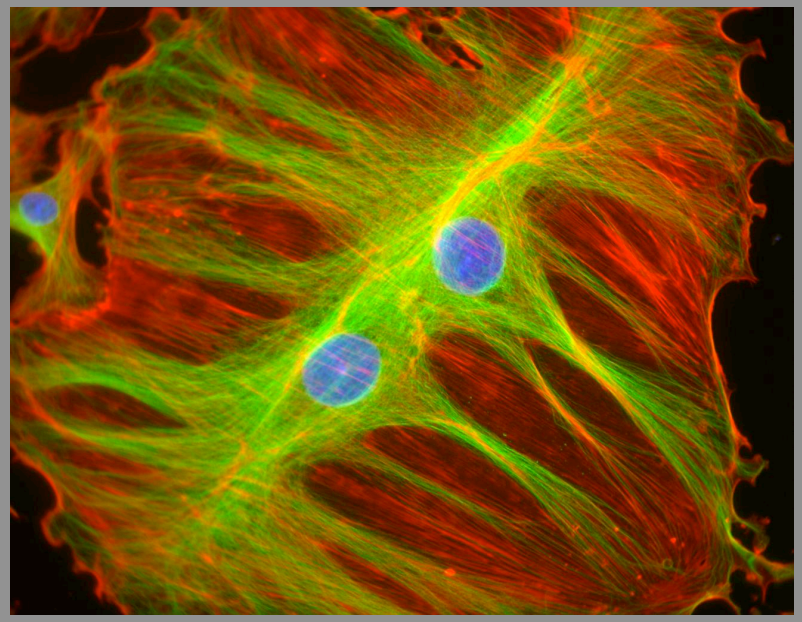


3.

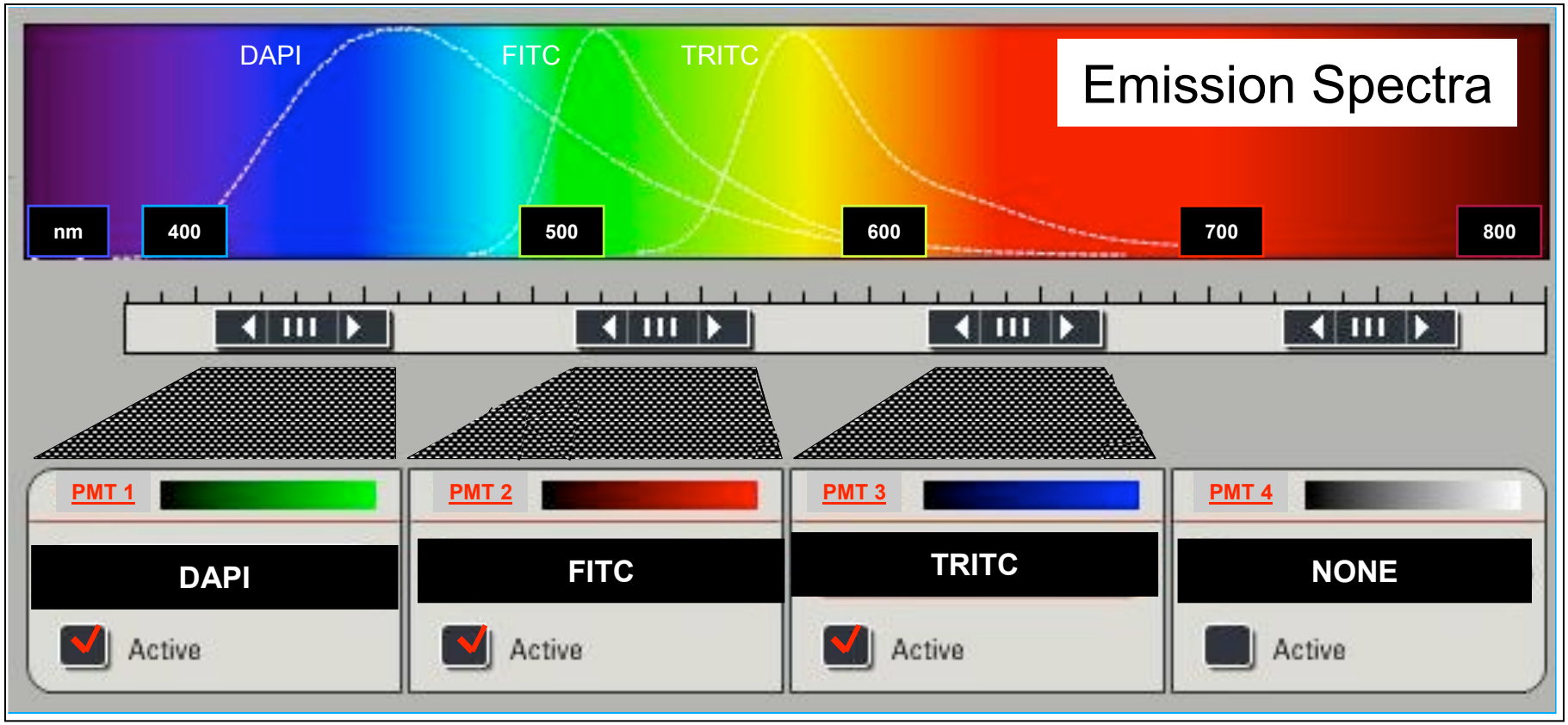


Bovine Pulmonary Arterial Epithelial cells – Molecular Probes® Fluo Cells #2 – 400x Triple labeled showing 1. Microtubules, 2.Nuclei (DAPI) and 3.Filamentous Actin and a merged image – DWP -2005

A standard fluorescent microscope may sometimes be a better choice than a confocal microscope if your specimen is less than ~ 6m thick. The filter cubes are used sequentially to reduce crosstalk. Generating and processing images takes seconds compared to several minutes using confocal microscopy.

For your consideration

SEQUENTIAL SCANNING – why we need to do it

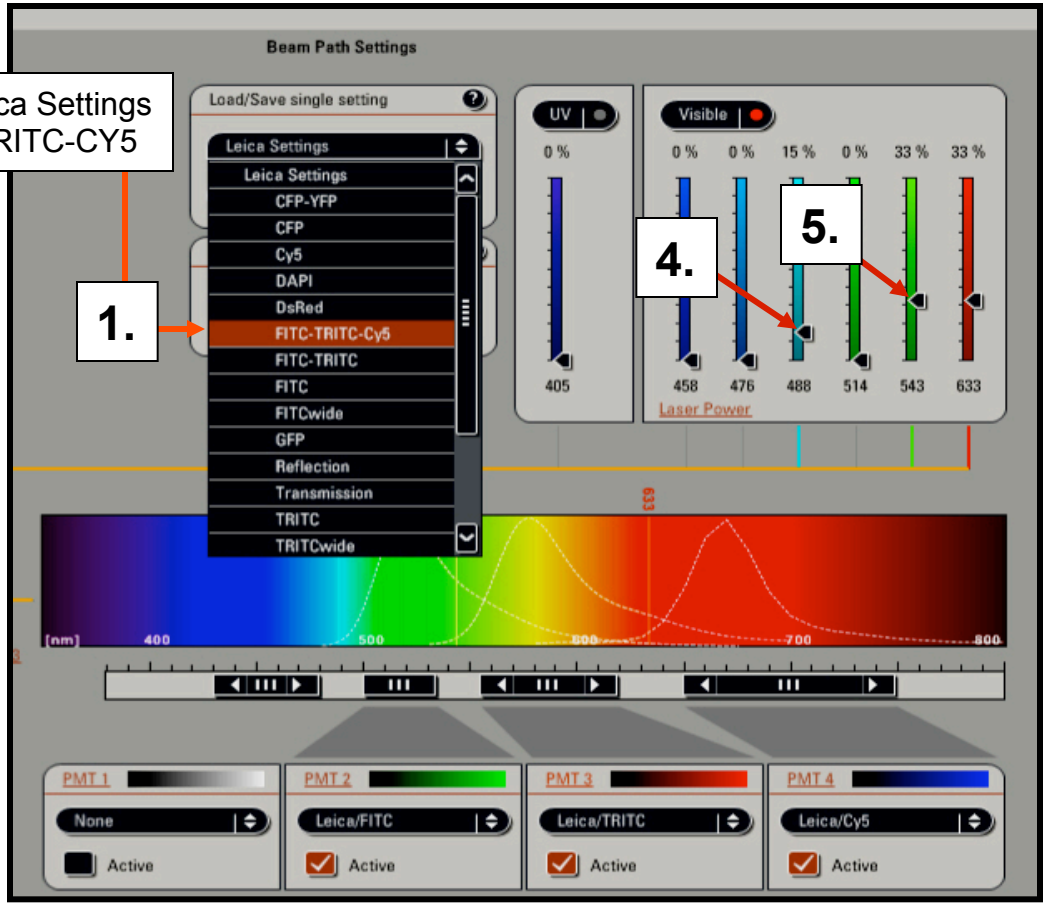


Fluorophores used in fluorescent / confocal microscopy often have emission spectra that overlap, like those shown in the spectral graph above. This causes confusion while interpreting image data. Sometimes crosstalk among simultaneously scanned channels can be controlled by adjusting the detection sliders so that they rest beneath the peaks of their corresponding emission curves in the beam path window. When this is insufficient to control crosstalk (esp. DAPI), scanning can be done using only one channel at a time. This is called sequential scanning.

SEQUENTIAL SCANNING

Testing your specimen for Cross-Talk

e.g. Leica Settings
FITC-TRITC-CY5



1. Load the parameters of at least two fluorophores that you are interested in simultaneously scanning.

2. Click **<Live>** to start scanning.

3. Adjust the gain, offset, and detection sliders for each image in the image template window as you normally would.

4. In the beam path setting box, pull down the slider for the first fluorophore to 0%. The image corresponding to that slider should fade to black. *If an image still appears, even a faint one, then there is crosstalk between at least two channels.*
   Raise the slider for the first fluorophore to its previous position until its corresponding image is restored in the image template window.

5. Lower the next slider to 0%. The image corresponding to that slider should fade to black. If it does not, crosstalk is present and sequential scanning is advised.

SEQUENTIAL SCANNING

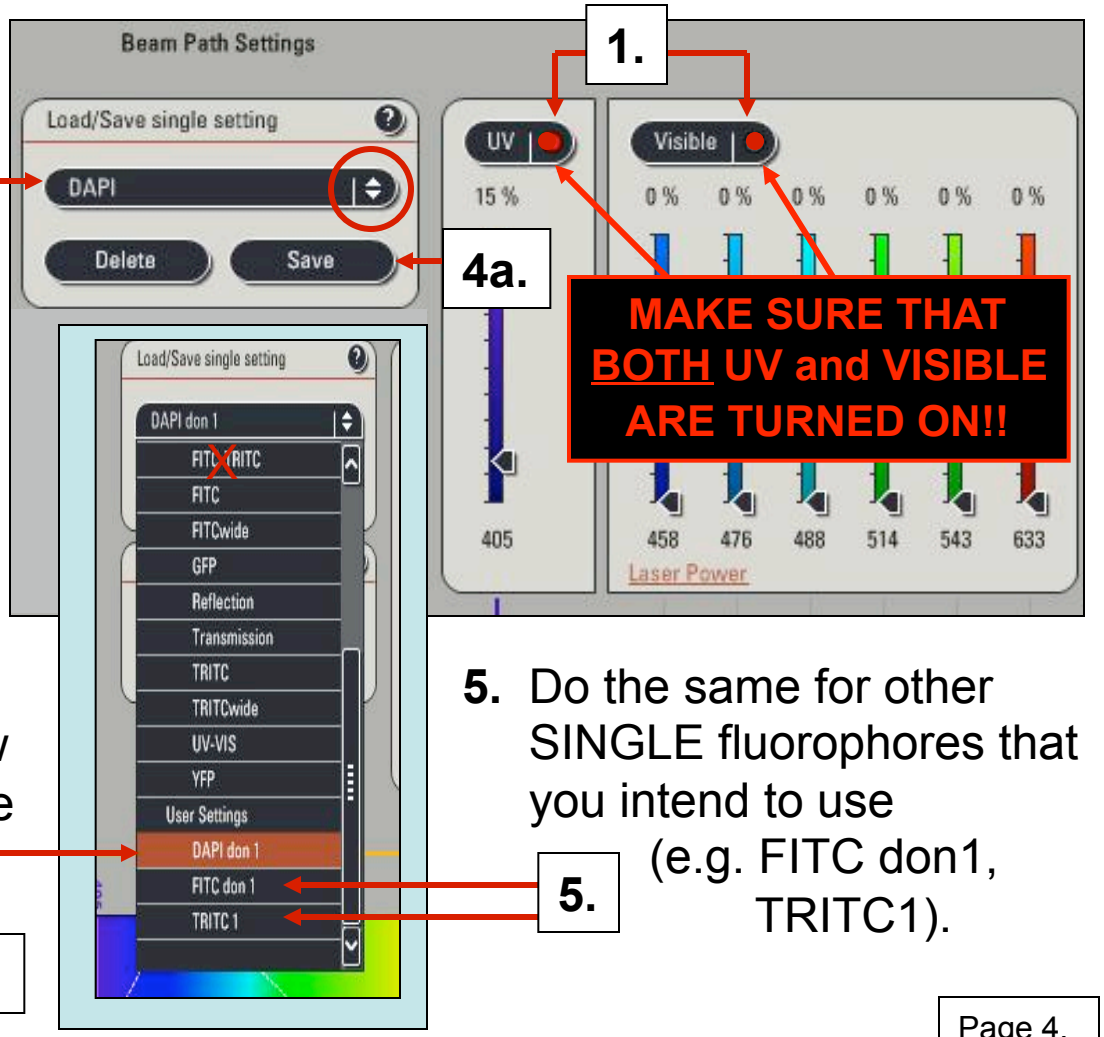
Setting up Sequential Scanning for Multiple Fluorophores

1. Click **both** the **UV** and **Visible** lasers in the beam path window if **DAPI** is to be used with other fluorophores.

2. Double-click a SINGLE preset fluorophore parameter in the Leica Settings drop-down menu (e.g. DAPI).

3. Click Live and optimize the Gain, Offset and detector slider for a DAPI image as you normally would.

4a. Click <Save>, name your new setting and **4b. <**save> it in the User Settings section of the drop-down menu (e.g. DAPI don1).

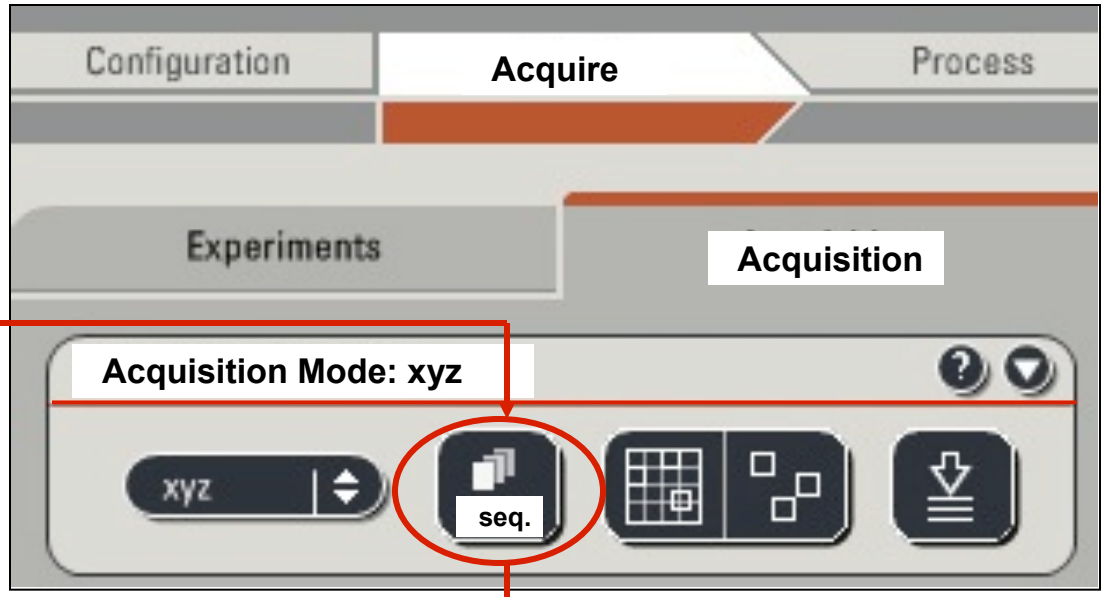


5. Do the same for other SINGLE fluorophores that you intend to use (e.g. FITC don1, TRITC1).

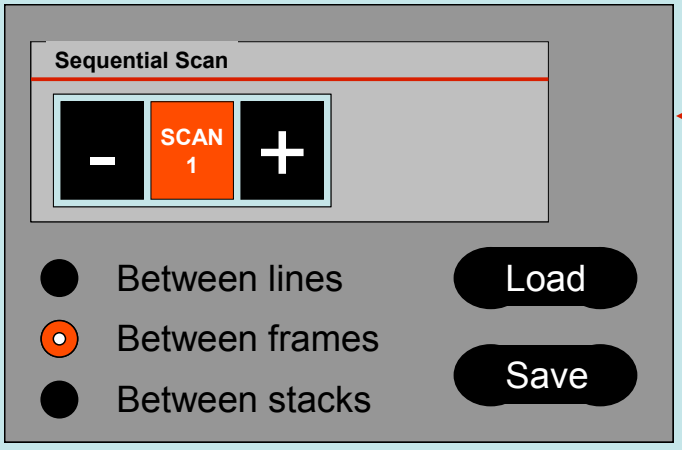
SEQUENTIAL SCANNING

Setting up the Sequential Scan window

6. Click on the **Sequential Scan** icon under the <**Acquire**>, <**Acquisition**>, and <**Acquisition Mode: xyz**> window.



The Sequential Scan window appears.



7. Select (Click) Between lines [live specimens], Between frames, or Between stacks (not a common choice). “Between frames” is only used as an example. “Between lines” may be a better choice for your sample.

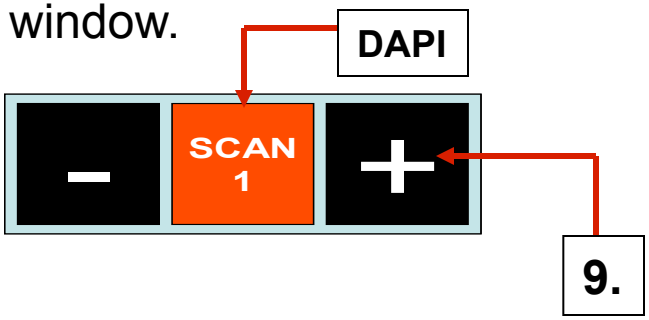
SEQUENTIAL SCANNING

Setting up the Sequential Scan window

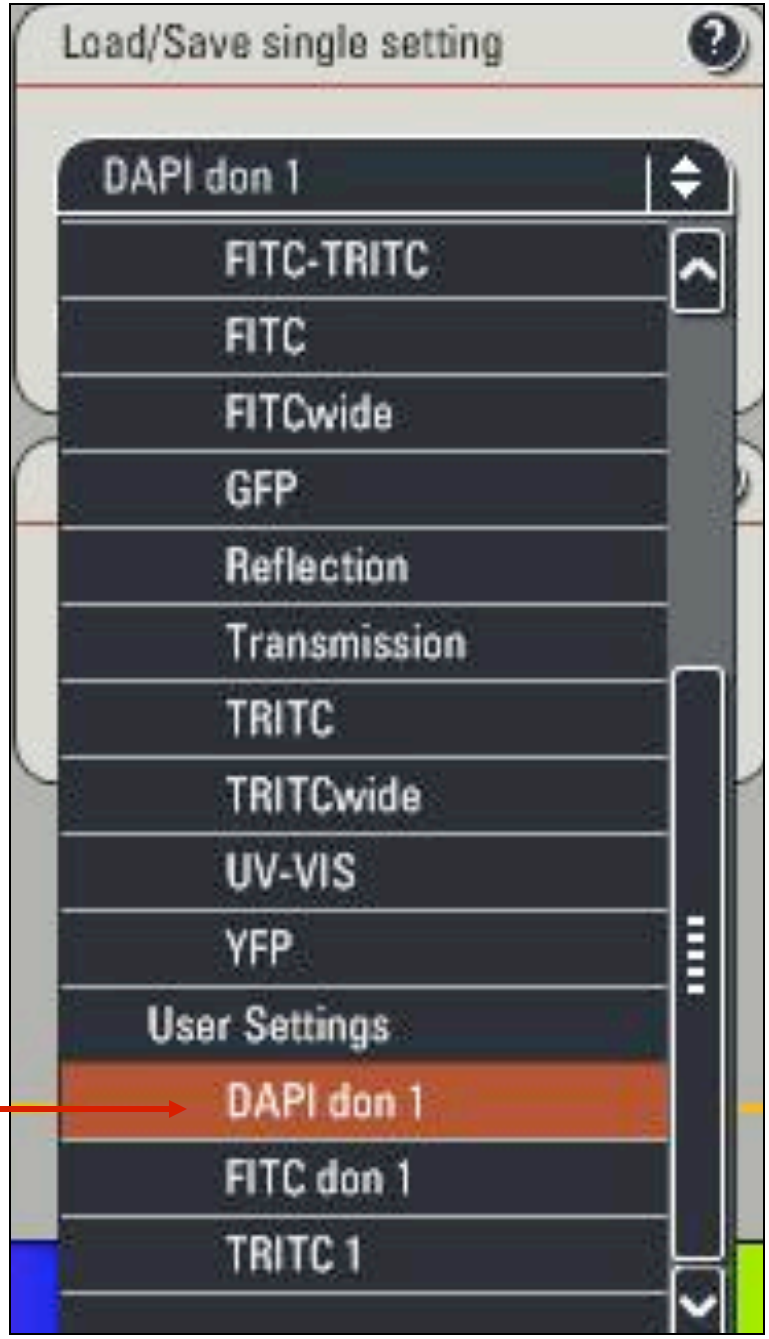
To set up the order of the sequenced scans,

8. **Click on** the UV fluorophore (**DAPI don1** in this example) in the User Settings drop-down menu that you created earlier.

   Always make **your UV** selection first. Doing so will automatically link your UV fluorophore settings (DAPI) for the first setting (SCAN 1) in the Sequential scan window.

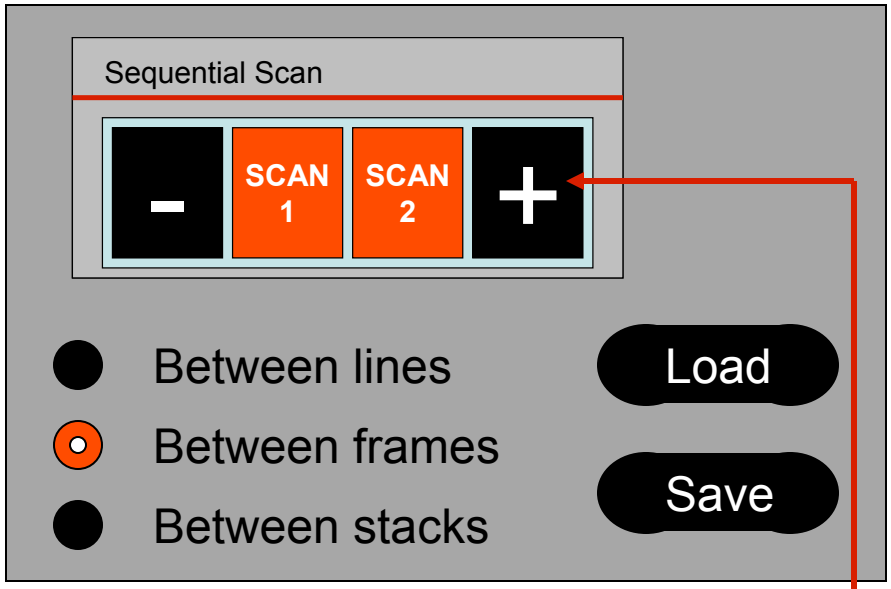


9. Click on the + in the sequential scan window (**one time only**).



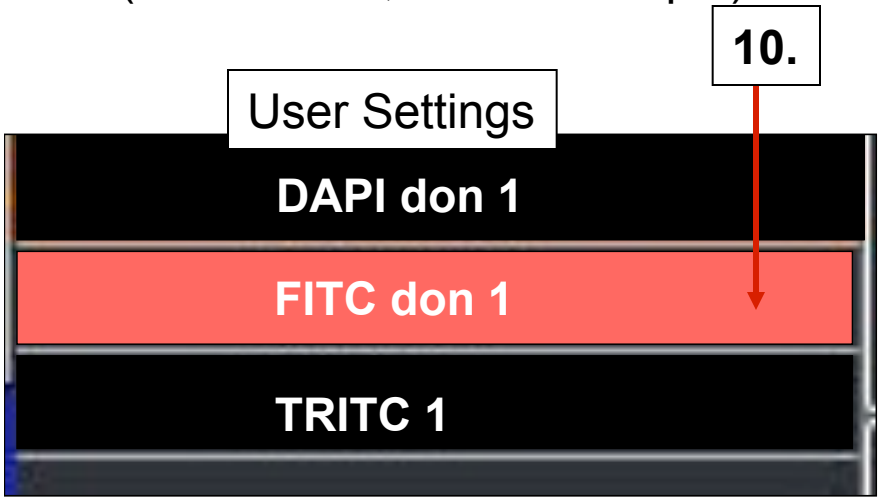
SEQUENTIAL SCANNING

Setting up the Sequential Scan window



Scan 2 appears in the Sequential Scan window.

10. Double-Click on your second fluorophore in the User Settings drop-down menu.
(**FITC don 1**, in this example)

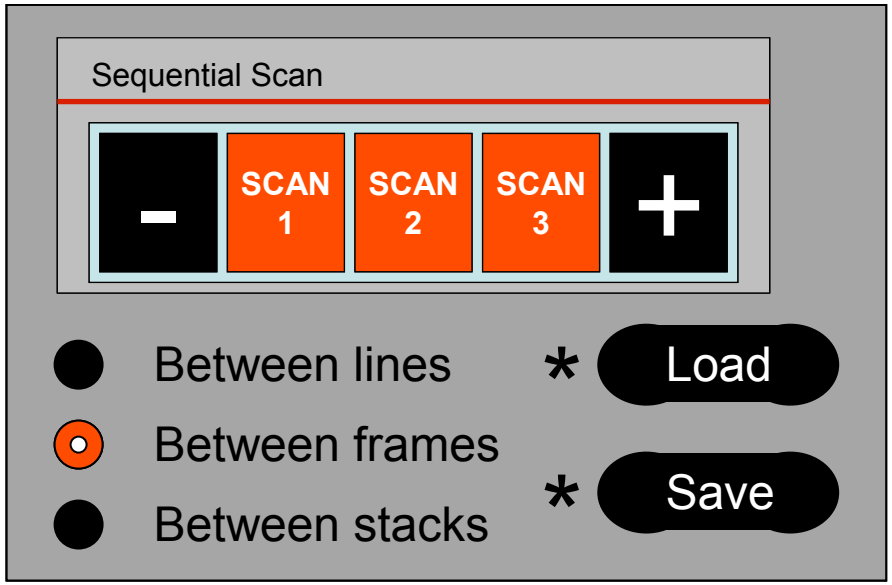


The parameters for **FITC don 1** now become linked to the second scan in the sequence (SCAN 2).

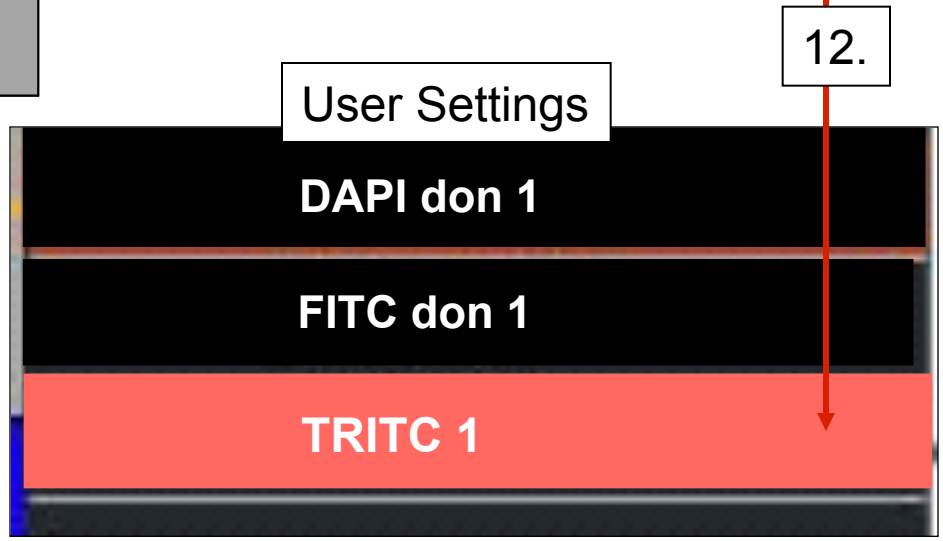
11. Click, once again, on the + in the sequential scan window (**one time only**).

Setting up the Sequential Scan window

The next scan in the sequential scanning order appears (SCAN 3).



12. Double-Click on your third fluorophore in the User Settings drop-down menu. (**TRITC 1**, in this case)



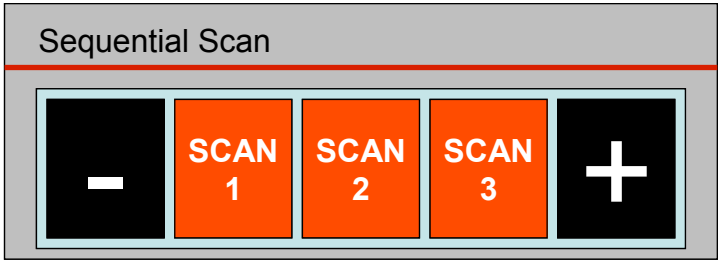
The parameters for **TRITC 1** now become linked to the third scan in the sequence (SCAN 3).

* Your Sequential scan fluorophore parameters and sequencing order can be saved and later recalled through <Load> and <Save>.

SEQUENTIAL SCANNING

Setting up the Sequential Scan window

13. Check that each of the three scan icons are correctly registered with the fluorophores that you selected. Clicking on a scan icon should cause each single corresponding fluorophore to appear in the beam path window in their correct position and order. Clicking on − will delete scan icons.



When you are certain that you have successfully made all of the adjustments leave the **Sequential Scan** box expanded. If you need to go back to a previous scan, click on the corresponding number.

14. Click Live and adjust the **Z POS** knob to find the brightest level of your sample on the monitor. Find the best image plane for making Gain and Offset adjustments.

15. Adjust the Gain, Offset, Field Rotation and Zoom functions as you might normally do.

16. Select Format and the desired number of line or frame averages in the XY window (again, as you normally would for any scanning).

17. Determine the **<Begin>** and **<End>** of your region of interest in the Z window - Click **<STOP>** and decide on the step size and/or number of sections that you wish to scan.

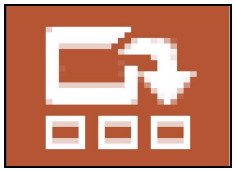
(#s 14 through 17 were explained in detail earlier)

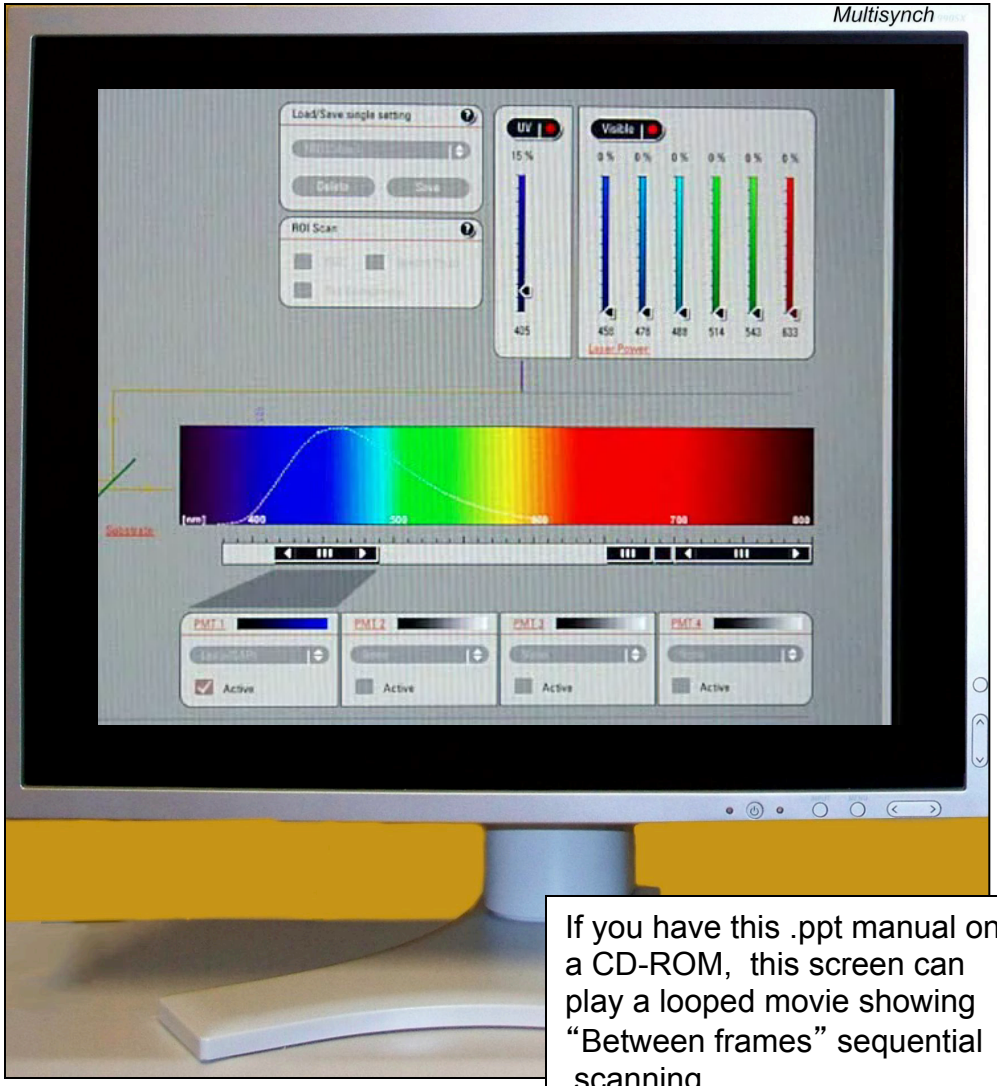
Reminder

Sequential settings can be saved via the "**Save**" button in this window (to *your own* image data folder on Drive D or your flash drive). In the future you can recall the settings by clicking on "**Load**". Recalling a saved sequential setting also recalls all of the other other parameters under "Acquisition" (zoom, format, etc). To exit the sequential scan, click again on the "Seq" icon. Do not save anything within the popup message. It is recommended that you recheck the parameters of any recalled / presaved sequential settings.

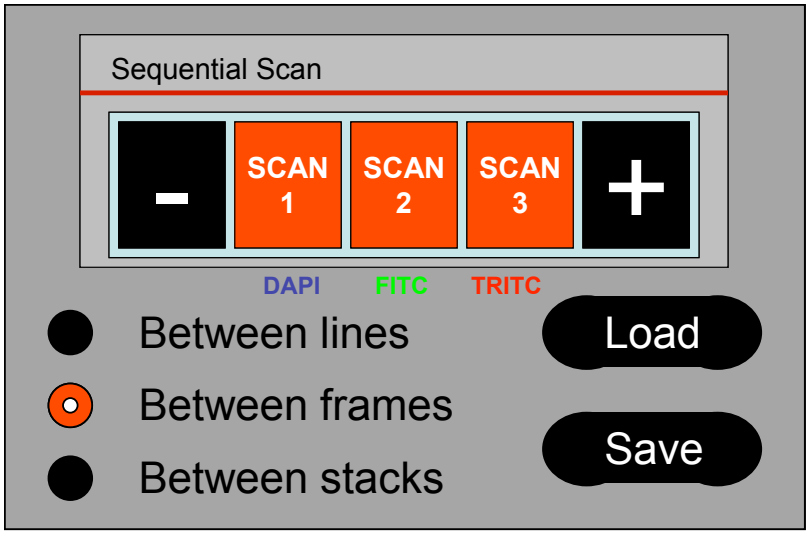
18. Click 

to begin sequential scanning.

19. Click on the **<**  **>** button to view the images as they are scanned.



If you have this .ppt manual on a CD-ROM, this screen can play a looped movie showing “Between frames” sequential scanning.



In this example, <Between frames> is selected and when <Start> is clicked, each fluorophore, its power slider %, corresponding assigned color and emission spectrum appear and switch sequentially in real time. This effect will not appear during “Between lines” scanning.

SEQUENTIAL SCANNING

Reminder

As a practical matter, selecting <Between lines> and selecting line averaging may be among the best selections for your work.

SEQUENTIAL SCANNING

Processing Sequentially Scanned Images

Sequentially scanned image data can be processed in the same ways as simultaneously scanned series and projections. For merging images adding measurement scale bars, orthogonal sectioning, saving and transferring image data to the D drive or to your USB flash drives the Microscope Work Station can be used. For creating movies, 3D reconstructions and rotations, please use the off-line confocal workstation facilities.

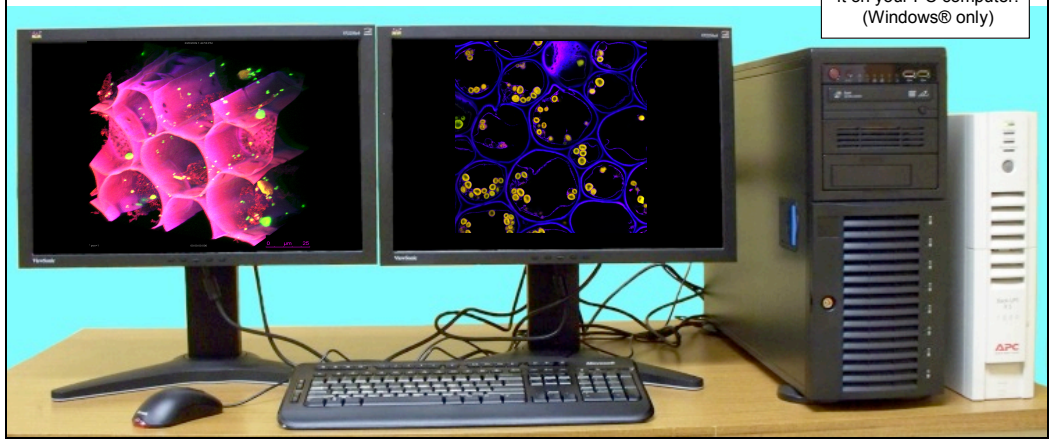
TCS – SP5 Off-Line Confocal Simulator

Processing image data for 3D rotations and movies takes substantial time on our confocal microscope work station. Clients are asked to transfer data to our off-line work station (Confocal Simulator) so that the microscope can be available for other users. Two LAS software versions are available:

LAS AF — Room 249 West
Full processing version
Hardware key required

LAS AF Lite — Modified home version
No rotation capability
single monitor

Please ask for the LAS AF Lite application if you would like to install it on your PC computer. (Windows® only)



If you have this .ppt manual on a CD-ROM, click on the images in the monitors and the movies will start.