

Molecular Dynamics MultiProbe 2010

The Molecular Dynamics MultiProbe 2010 laser scanning confocal microscope offers an Argon / Krypton laser (488, 568 and 647 nm excitation lines), reflectance mode, and a transmitted light detector and 2 PMT's for dual simultaneous collection.

ImageSpace runs on a Silicon Graphics Indigo2 machine, running IRIX 6.2 operating system. This manual will cover only necessary UNIX commands, as all of the controls for running the microscope are done using ImageSpace software. This manual is designed to highlight the functions used by most CSIF users, but cannot cover any of the subjects in depth. For a more complete description of functions, consult one of the Molecular Dynamics manuals located beside the computer.

ImageSpace uses 5 modules to control various data collection or management tasks. **ScanControl** is used to set confocal microscope parameters and to acquire the output images. **ImageDisplay** is the window where scanned images will be displayed, as well as where temporary files will be stored before they are saved. **DataManager** (is used for retrieving archived data sets. **ToolBox** contains all the measurement tools. **VolumeWorkbench** is used for making projections from section series.

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Powering on equipment:

In order to use the MD confocal, you must be trained by one of the CSIF staff, who will open a user account for you. When starting a session:

- 1) sign in name and starting time on log sheet
- 2) turn on the mercury arc lamp (only if you will use it), spark the lamp
- 3) turn on MD control box to right of table on the floor (red rocker switch)
- 4) turn on Laser (red button on front panel of confocal)
- 5) log onto SGI (double click icon above your name, enter your password)
All ImageSpace modules are accessed through grey menu in top left corner.
- 6) Open ScanControl module (hold down left mouse button, highlight ScanControl, release mouse button)

revised 12/5/95

Viewing specimen through eyepieces:

Step-by-Step for brightfield through eyepieces

Turn on transmitted light source, adjust level to ~4
block mercury arc (silver slider to CLOSED position - pull out)
Swing transmitted light detector out of light path
Filter block to OPEN
Eyepiece beamsplitter to back

Step-by-Step for epi-fluorescence through eyepieces

Turn on mercury arc lamp before starting session
Open light path to mercury arc (silver slider to OPEN position - push in)
Turn off transmitted light source
Filter block to B-2A for FITC, G-2A for Rhodamine
Eyepiece beamsplitter to back

Scanning an image:

At the microscope -

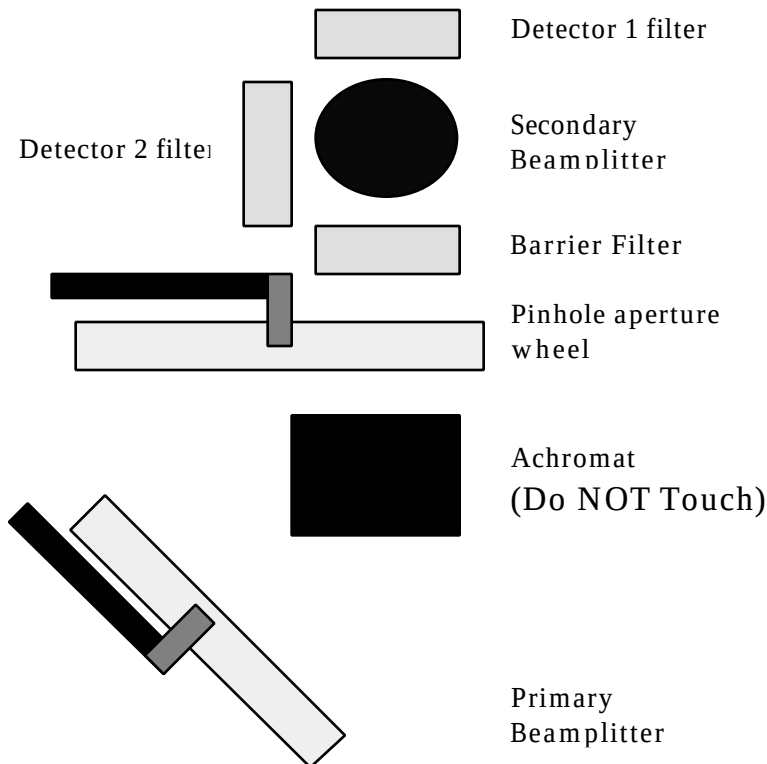
- 1) Select the desired excitation wavelength on the excitation wheel.
- 2) Press laser button on (red), the laser power is ~ 5 mW in standby mode and ~35 mW when actively scanning (do not change power level).
- 3) turn on PMT's that you will use
 - PMT 1 for single fluorescence channel,
 - PMT 1 & 2 for two fluorescence channels
- 4) Set voltage to about 600 (working range 400-700).
- 5) Set attenuation wheel to 10%, this is the percentage of the laser actually hitting the sample.
- 6) Arrange internal dichroics / beamsplitters according to fluorescent marker used and the chart on the microscope.

Inside confocal optical compartment -

The PRIMARY BEAMSPLITTER is the first of two optical components that prevent reflected excitation laser light from reaching the detector and contaminating the image. Turn wheel by pushing down the position lever, then spin **rim** of wheel. Make sure that lever rests in **small** groove, *not* in one of the large slots between filters.

BARRIER FILTERS transmit emission wavelength light. Long-pass filters (EFLP) collect all wavelengths equal to or longer than specified number. Band-pass filters (DFLP) transmit wavelengths within specified range (example 530DF30 would collect

30 nm centered around 530 nm = 515-545 nm). For reflected light, a polarizing filter is used. There are three barrier filter slots: one in front of the secondary beamsplitter and one in front of each detector. In general, for single fluorophore experiments, only the barrier filter in front of the secondary beamsplitter will have a filter, the other two will be filled with blanks. For double fluorescence experiments, the two filters in front of the detectors will specify the emission wavelengths collected for each channel and the filter in front of the secondary beamsplitter will be blank.



The SECONDARY BEAMSPLITTER divides the light into two paths. Emitted light with wavelengths longer than the beamsplitter setting are passed to detector 1. Emitted light with wavelengths shorter than the secondary beamsplitter setting is reflected to detector 2. For single label experiments, the hole is capped with a blank.

pinhole wheel -

- 50 μm - greatest depth resolution, image not as bright
- 100 μm - brighter, but with loss of z-axis resolution (larger depth of field)
- 200 μm - not confocal, close to standard fluorescent microscope

NOTE: If you have problems understanding any of the internal microscope settings - see Susan rather than experimenting with the microscope. The internal

parts can be damaged by dust and /or fingerprints, so take extreme care in handling all components.

ScanControl:

Box types -

Continuous value - click inside box (which turns pink) and type in value or, if slider appears, move the bar on the slider.

Choice parameter - display shows small circle at left of box. Place mouse on the box and hold down the mouse button. A pop-up menu will appear - move mouse to highlight desired selection, then release mouse.

Display only - cannot be changed (e.g. Current Z box)

Sliders -

Z position - these are alternatives to adjusting the focal plane with the manual fine focus control knob on the microscope. The left slide is the Coarse adjustment, the right slider is the Fine adjustment. After adjusting sliders, click on Set button, then stage will move.

Variable slider - a third slider will appear when the mouse pointer is clicked inside some continuous value boxes. Slide the bar up and down to adjust parameter within allowable limits.

To perform xy scan -

Set Objective to either 20X or 60X.

Choose **Image size** - 512 is a good starting point

Choose **Pixel size** - use largest pixel size to scan the entire field of view in the microscope.

Choose correct detector (1+2 are fluorescent channels, 3 is transmission)

Choose **TempScan** - **Scan Continuous** (to set parameters) or

- **Scan Section** (when parameters have been set already)

To Zoom in on a region, first do full field scan. Change **Pixel size** to smaller value, then place pointer in **Subregion box**, hold down mouse button and red square will appear over image - this is the area which will be zoomed in on. Move mouse to outline region of interest, release mouse button. *Note* that for *smallest pixel size* for each image size, you will be restricted to the center of original (largest pixel size) image - even if you zoomed to a middle pixel size first.

To perform xz scan -

Do full field xy scan as described above

Place mouse pointer in **Z-scan position box**, hold down mouse and red line will appear over xy image. Move line up / down to set line to where xz scan will be performed and release mouse button.

Choose **TempScan - Scan Vertical**

To perform section series (multiple xy planes along z axis) -

Do xz scan as described above

Click in the **Step size box**. Adjust the general (leftmost) slider to set section interval. In general set Step size larger than, but close to the Pixel size you are using.

Set start point with **Z-position fine adjustment slider** (rightmost) - hold down mouse button on green slider, red line / dot will appear in image area, adjust to that red line is at the TOP of the area of interest. (hint: go slightly above where you want to start collection). Click **SET** button at top of z-position sliders.

Set end point by clicking in the **# Sections box**. Adjust the general (leftmost) slider so that red line appears at the BOTTOM of area of interest (or so that # Sections X interval is sufficient to collect amount of data you want).

Choose **TempScan - Scan Series**

If your last scan is not through the sample, choose **TempScan - Continue Scan** and more sections will be added to current series. Click on **Cancel** to stop collection.

Step-by-Step for fluorescence - Single Channel Confocal

Turn off transmitted light, block mercury arc lamp

Filter block to OPEN

Eyepiece beamsplitter to front

Quarter waveplate out (right edge ~ level with edge of filter block)

Set excitation wheel and internal dichroics according to chart

turn on PMT 1 (Starting points: 500V with 10% attenuation)

Select **Detector** 1 in ScanControl

Step-by-Step for Reflection - Single Channel Confocal

Turn off transmitted light, block mercury arc lamp

Filter block to OPEN

Eyepiece beamsplitter to front

Quarter waveplate IN (right edge pushed in under filter block)

Set excitation wheel and internal dichroics according to chart

turn on PMT 1 (Starting points: 200V with 30% attenuation)

Select **Detector** 1 in ScanControl

Step-by-Step for Laser Transmittance - Single Channel

Turn off transmitted light, block mercury arc lamp
Filter block to OPEN
Eyepiece beamsplitter to front
Quarter waveplate OUT (right edge ~ level with edge of filter block)
Swing transmitted detector into light path
Starting points: 10% attenuation, gain set to 1, offset to zero
Set excitation wheel and internal dichroics according to chart
Select **Detector 3** in ScanControl

Collecting dual images:

Remember that when you collect two images simultaneously, the excitation wavelength selector must be set to excite both necessary wavelengths and the % of the laser light reaching the sample (attenuation) must be identical for both channels. It is suggested that you adjust the attenuation for the dimmer sample, and then turn down the voltage for the second channel to compensate for the higher energy input. (For a transmitted / fluor double label, usually the attenuation is higher for the transmitted channel.)

Dual fluorescence

Longer wavelength light will pass through secondary beamsplitter and be collected by detector 1. The shorter wavelength light will be reflected by the secondary beamsplitter to detector 2. For example, for a FITC / Texas Red double labelled sample, the red emission (TR) is collected in PMT 1 and the green emission (FITC) is collected in PMT 2. [for Smith Mark III users - this is backwards to the channel orientation you are used to.]

Fluorescence + Reflected

Reflectance mode takes advantage of either natural reflectance of the sample, silver grains (autoradiography) or immunogold particles. The emitted fluorescent light will pass through secondary beamsplitter and be collected by detector 1. The reflected light, which is shorter wavelength, will be reflected by the secondary beamsplitter to detector 2.

Scan speed -

The duration of an xy scan depends on the size of the image (number of pixels) and the number of detectors scanned, and is independent of objective magnification and pixel size. Approximate scan times (in seconds) are as follows:

	<u>1 PMT</u>	<u>2PMTs</u>
64 x 64	1	3
128 x 128	2	5
256 x 256	4	6
512 x 512	6	12
1024 x 1024	15	29

Optimization of image:

To increase signal

- use higher laser attenuation filter (1 - 100%)
- in ScanControl, increase **Count** (number of times section is scanned), and set **Accumulation** mode to **Add**.
- increase aperture size
- increase laser power
- increase **Photometric gain** in the ScanControl window **
this will amplify *both* the signal *and* the noise by the same factor.

Enhance separation

In the case where there is crosstalk between samples, you can use **Enhance separation (ToolBox)** to subtract a scaled version of one image from another. For examples, for a FITC / Rhod double labelled specimen, the green fluorochrome has a long tail that overlaps the red emission spectrum. To subtract this tail, first stain a sample with only FITC. Scan the sample in both FITC / Rhod channels (as if it were truly double labelled), and determine the Rhod signal / FITC signal. This ratio is the signal fraction you will subtract using **Enhance separation**.

To store confocal parameters used in scanning with the image file

Because many of the barrier filters and beamsplitters are manually adjusted, the software cannot keep track of which specific filters were used to collect the image. Other information (z-position, objective, etc.) are entered automatically by the software. To enter the filter set information, choose **Options: Annotation window** in the **ScanControl** window. A series of Choice Parameter input boxes will be shown. Each box contains the possible filters that could be used in each slot based on the filters CSIF owns. Choose the correct filter choices to reflect your scanning mode set-up.

Remember to change the parameters every time you change filters, otherwise the subsequent files will be listed with incorrect barrier filters / beamsplitters. After the file has been saved, you can, however, go back and correct the confocal parameters using the **ToolBox** module, **Utilities: Edit Parameters**.

ImageDisplay

Image area - center region

Stack area - vertically along left side

Menu buttons - along top

Info area - between Menu and Image areas

Stack Area - displays an icon for data item - single section, section series, etc. Icons rimmed in red are stored in temporary memory. The storage capacity of the program dictates that no more than 10 images can be maintained in the stack at any one time.

Therefore, the collection of the 11th data set will cause the 10th icon to “fall below” the red line and be lost from memory. To avoid the loss of precious data, periodically go through stack and save items to disk.

Save/Delete items from the stack - Position cursor over stack item, hold down right mouse button, menu of options will appear, move cursor to desired option and release mouse button.

Note: When you select an image (click on icon with left mouse button), the stack REARRANGES so that the chosen image is displayed AND its icon moves to the top of the stack. Therefore, the displayed image ALWAYS corresponds to the top icon.

Animate Stack - when a series is collected or displayed from disk, a series scrollbar will appear between stack and image area. Click on any number to display that section of the series. To animate the stack, choose **Utilities: Movie** and click start. You can change the interval between sections and whether the movie runs forward, backward or rocks back and forth.

Menu buttons

Window - lets you change the size of the window. Full screen will result in the Image Display taking up the full screen. To access the menu bar again, hold down the right mouse button and turn off the Full Screen (alternatively, F2 toggles between full screen and regular screen sizes).

Zoom -

Factor - allows choice of zoom factor (use F5 to cancel magnifying glass)

To resize back to “normal” window:

- 1) **Zoom: Reset**
- 2) **Image: Fit Image**

Image -

Image off - deletes confocal image, leaving only drawings. Be careful with this selection - if you hit it accidentally, you won't see any future images that are collected. *Check this setting if you unexpectedly don't see an image and you've checked everything else!*

Move - moves image within main image area (pointer will change to hand).

Dual - options for displaying simultaneously collected images side-by-side or as anaglyph (=overlay)

Series - controls display of series

Sequence - shows images one at a time (control with numbered scroll buttons)

Mosaic - shows all images in thumbnail view.

Utilities -

LUT - choose between grey, pseudo-colored or single color look-up tables

To make upper/lower limits display in color on grey LUT, position pointer at top of grey scale wedge, click middle mouse button, drag pointer to set lower limit of saturation color. Move pointer to bottom of wedge, click left mouse button, drag up to set upper limit of background color.

To change limit colors, choose **Options: Limit Colors**. Change sliders to set which colors display upper/lower values.

DataManager

Drag & Drop Icons - located in row on top panel

Info icon - displays collection information if you Annotated your confocal settings

Remove icon - similar to trash can on Macs

Backup/Restore icons - used to store to external devices, however there are no external devices attached to the SGI Indigo2 at present

ToolBox - will open ToolBox module

VolWorks - will open VolumeWorkbench module and use the dropped dataset as the input data for the projection

FileCabinets - will show your directory of files. Directory path is

“/usr/img/yourname”. Double clicking on the FileCabinet will result in the contents of the directory being displayed in lower portion of window. Each icon is a dataset (folder), which may contain individual sections, section series, projections or a combination of the above. Double-clicking on an icon will display the contents of the DataSet in a new window.

Small datatype icons are attached to a data set icon and provide information about the types of data stored in the dataset folder.

To move/copy a DataSet, drag DataSet over FileCabinet and a menu of options will be displayed.

Window button - found at top left of screen

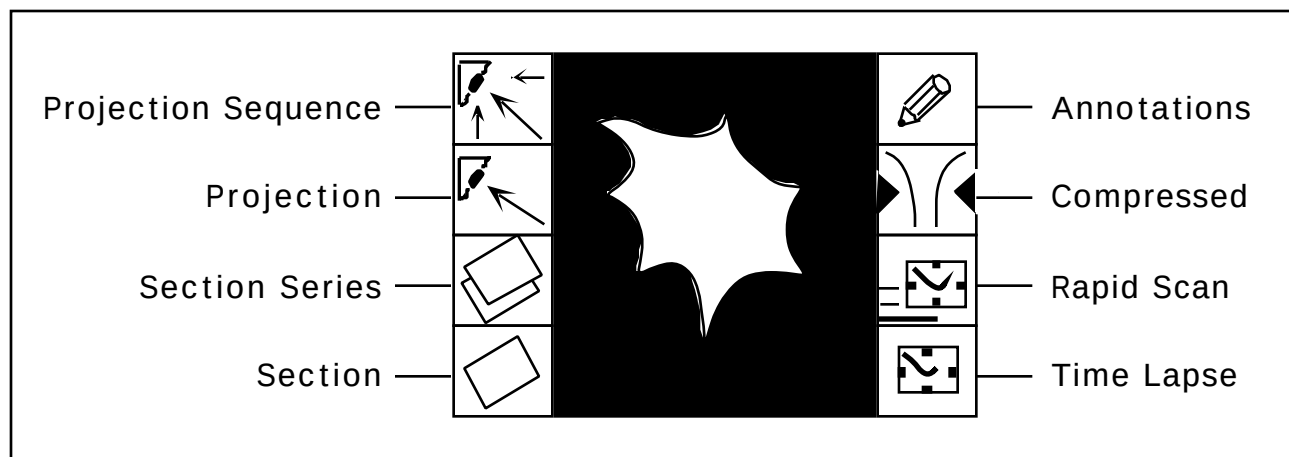
Options - enables choice of preferences for format of

Screen - Set to Multiple

DataSet order - controls whether alphabetized, displayed by date, etc.

Remove Data - Set to Delete

Data Set Icons



ToolBox

Basic Analysis

Area - measure intensity values for different drawn areas. Choose which of the menu of result parameters you want calculated by clicking on small buttons to the left of text items. Then select a shape for the area with one of the tool icons, draw shape on image, release mouse button and a result window will appear. All subsequent results will be added to this window.

To erase a drawn shape, select the + tool, click on shape (red dashed lines will appear), hold down right mouse button to access menu, choose **Edit: Cut**. To erase all shapes on the image, hold down right mouse button and choose **Edit: Select All**, followed by **Edit: Cut**.

Colocalization - makes 2D scatterplot of how dual labels are colocalized.

Dual Out - shows axes in red and green with yellow as overlay color.

Image inspection - draw shape on image, displays pixels on scatterplot

Diagram inspection - outline pixels on scatterplot, displays chosen pixels on image.

Histogram - outline an area with drawing tools, and graph window will appear.

Measurement log - can save results window to file.

Measure 2D - determine distance between points, pixel intensities of points or objects.

Object Count - will tabulate number of objects within a certain range of intensity values having a minimum specified number of pixels. Results window will display each object with size, intensity, position info.

Thresholded Area - will let you determine number of pixels above and below the upper and lower pixel intensity threshold limits that you set.

3D Analysis - measure pixel intensities along z-axis within series at a single point projected through the entire series or along a cylinder within the series.

Object Count - equivalent to ObjectCount 2D except that operation is performed on section series, not on single section.

Filters - listed from most to least smoothing performed:

Mean - smooths the most of all the filters, will obscure fine details

Alpha - smooths less than Mean

Median - suppresses spikes, medium smoothing, best for discrete noisy data

Gaussian - low pass filter, smooths data, may obscure edge definition, use for noisy data which is continuous

Gradient - emphasizes edges in images. Use for section series before generating projections. Enhances noise, smooth data first with Gaussian filter.

VolumeWorkbench

Projections are 2D renderings of a 3D data set. There are 7 types of projections available in ImageSpace, each computed with a different algorithm and displaying a slightly different aspect of the data set.

Look Through - voxel intensities along projection rays are summed. This is equivalent to “extended focus” because resulting images appear to have been collected by lens with a large depth of focus. The image shows both surface and internal structures.

- no prefiltering suggested
- surface extraction option - removes interior points and results in soap-bubble like image

Depth Coding - closer portions of specimen appear brighter and portions that are further away appear dimmer, with brightness inversely proportional to distance from viewer.

- no prefiltering suggested

Look Depth - combination of Look through and Depth coding algorithms. Layers are added, with deeper layers attenuated proportionally to their distance from the viewer before the addition.

- no prefiltering suggested
- surface extraction - removes interior points, soap-bubble like image

Maximum Intensity - displays maximum intensity value along each line of sight. Similar to Look through, but shows more detail and edges are cleaner.

- no prefiltering suggested
- invert intensities - will make dark portions of image light and vice versa.

Closest Intensity - displays first pixel value above the threshold value along the line of sight. Two effects can be generated - 1) opaque cube with intensities painted on sides or 2) a stack of opaque images.

- no prefiltering suggested
- use all intensities while rendering

Ray Model - Most complicated method, allowing you to control reflection, absorption and radiosity. The specimen is luminated from the direction of view. As light “hits” each voxel, a percentage of the light is reflected, a percentage is absorbed, and a percentage is used to light up the voxel (radiosity). Useful for fine-tuning the transparency of the output image.

- when reflection setting is greater than zero, try a 3x3x3 Gaussian filter to prefilter the data before rendering.
- use small percentages, as the light is diminished by these percentages for each section. The suggested defaults are: reflected 3%, absorbed 0%, radiosity 1%.

Surface Shading - displays the specimen as if it had an opaque, reflective surface. The orientation of the surface is calculated and shaded so that surfaces with a large tilt are dark and surfaces perpendicular to the viewer are bright.

- this render method is sensitive to noise, so prefilter the data with a 3x3x3 Gaussian filter. It may be necessary to filter the series more than once.
- this render method is also sensitive to intensity control. Intensity should be set so that pixel values representing background noise are not included in the projection calculation (see vol3, pg 7-26)

For dual channel projections - make each projection independently, making sure they are from the same angles and the same cut planes. To select channel, place mouse pointer over the **No. input** box and press down. A pop-up menu will show the available channels. Select first channel, do projection, save in folder where series is stored, then repeat for second channel and save again into same folder.

Projection type can be different for each channel. To view the projections together, choose **Utilities:Attach Band** (files MUST be in same folder for this to work). Enter one projection as **Input 1**, then the other as **Input 2**, select **Datatype** as projection. The two projections will now be stored as one file using the Input 1 filename. Use **Image: Dual: Anaglyph** to display in colors. Change colors with **Utilities:Grey/Color Adjust**.

APPENDIX A. Setup of laser, beamsplitters and filters

Fluorochrome Applied	Ar/Kr laser Excitation	Primary Beamsplitter	Barrier Filter *	Secondary BeamSpl *	Detector 1 Filter *	Detector 2 Filter *	sugg Attn
Single Scanning mode							
Transmission	488	no other filters required - make sure that detector is in light path					
Reflection ***	488	510 DRLP	POLAR	blank cap	empty	empty	
Lucifer Yellow	488	510 DRLP	510	blank	empty	empty	10%
Fluorescein (FITC)	488	510 DRLP	510	blank	empty	empty	10%
Phycoerythrin (PE)	488	510 DRLP	510	blank	empty	empty	10%
Rhodamine	568	488/568/647	590	blank	empty	empty	10%
Texas Red	568	488/568/647	590	blank	empty	empty	10%
Propidium Iodide	488	488/568/647	590	blank	empty	empty	10%
Dual Scanning Mode							
FITC & Rhod **	488/568	488/568/647	empty	565	590	530DF30	30%
FITC & TexasRed	488/568	488/568/647	empty	565	590	530DF30	30%
FITC & PI	488/568	488/568/647	empty	565	590	530DF30	30%
Fluo 3 & FuraRed	488/568	488/568/647	empty	565	590	530DF30	30%
FITC & Cy5	488/647	488/647 B/S	empty	650	660	530DF30	30%
Rhod & Cy5	ALL	488/568/647	empty	650	660	590	30%
Transmitted + FITC	488	510 DRLP	510	blank	empty	empty	
Trans + TexasRed	488/568	488/568/647	590	blank	empty	empty	
Reflected + Trans	488	510 DRLP	POLAR	blank	empty	empty	
Reflected + TexasRed	488/568	488/568/647	empty	565	590	POLAR	10%
* Extra filters and beamsplitters are in wood box to the left of the microscope							
** Note that with this combination of fluorochromes, the red signal is always contaminated by fluorescein emissions							
*** Quarter waveplate (below filter block on right of microscope) must be IN							
sugg Attn = suggested Attenuation starting point							
Blank = blank cap							
Empty = empty holder in place							