

# Operating Checklist for using the Laser Scanning Confocal Microscope, Leica DM IRBE.

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## Operating Checklist for using the Laser Scanning Confocal Microscope, Leica DM IRBE .

### Pre-Check

- Check logbook
- Check which light-sources you want to use:
  1. brightfield and/or DIC - tungsten light
  2. conventional fluorescence - mercury (HBO) bulb
  3. laser fluorescence
    - argon (Ar) laser, exciting at 488nm (blue)
    - krypton (Kr) laser, exciting at 568nm (green)
    - helium-neon (HeNe), laser, exciting at 633nm (red)

Find the laser you need with your fluorochrome, if your fluorochrome is not listed below, use the Bio-Rad chart on the wall to determine the excitation wavelength you need, and thus the laser you need.

FLUOROCHROME	LASER
ACRIDINE ORANGE	Ar
BODIPY 558/568 PHALLOIDIN	Kr
CY5	HeNe
GFP	Ar
DASPMI	Ar
Dil	Kr
FITC (Fluorescein)	Ar
PROPIDIUM IODIDE	Kr
RHODAMINE 123	Kr
TOTO-3	HeNe
TRITC (Rhodamine)	Kr
YOYO-1	Ar

### Startup

- Turn on the fan/filter above the door
- Remove DUST COVER from microscope and printer
- Follow steps in **THIS ORDER ONLY** (also see poster):
  1. If you will be using epifluorescence, turn on power supply for the mercury bulb (it should be left on for at least 15 minutes before it is turned off)
  2. Turn on microscope (black toggle switch on left side of microscope)
  3. Turn on the laser fan(s)(red toggle switches next to intensity LEVEL knob)
  4. If you want to use the Kr and/or Ar laser, check if intensity LEVEL of Kr and/or Ar laser are turned to minimum (round knob left of laser keys). Turn key to **start**, hold for two seconds and release, yellow light below the key should stay on

5. Turn on scan electronics “always if computer is used” (red toggle switch on box on top of Ar laser)
6. If you want to use the HeNe laser, turn key on scan electronics box to ‘ON’
7. Turn on the monitors (lower right of each screen)
8. Turn on the printer (toggle switch on left side towards the back)
9. Turn on the computer (big red button on upper right of computer)

## Computer Setup

### Log on

- Wait until screen shows log-on sign (in middle of the two screens)
- Press CONTROL + ALT + DEL (on keyboard)
- Use mouse to drag dialog box onto left screen (to move the box, put cursor on blue bar at the top of the box, and press the left mouse button)
- Enter USERNAME and PASSWORD (it is handy to use Science Network password)
- Click on OK with left mouse button and wait till hourglass cursor disappears
- Close ‘Welcome’ window
- Click on TCS NT icon or click on START (left lower corner) followed by PROGRAMS, Leica TCS NT and TCS NT
- **Wait** a few minutes till the hourglass cursor disappears

### TCS software

- Click on ACQUIRE IMAGES (left corner)
- Select initial filter setting and click OK

FLUOROCHROME	FILTER SETTINGS
ACRIDINE ORANGE	FITC
BODIPY 558/568 PHALLOIDIN	TRITC
CY5	CY5
GFP	FITC
DASPMI	FITC
Dil	TRITC
FITC (Fluorescein)	FITC
PROPIDIUM IODIDE	TRITC
RHODAMINE 123	TRITC
TOTO-3	CY5
Transmitted light	TRANS
TRITC (Rhodamine)	TRITC
YOYO-1	FITC
<b>Or relevant combination</b>	

- Wait for hourglass to go away
- Click on FILTERS icon (leave window open for reference)

## Microscope Setup

### Mounting specimen

- Tilt back tungsten light source and condenser gently until it stops
- **GENTLY** insert the slide on the galvano stage, **SPECIMEN SIDE DOWN**, and use clips to hold the specimen in place
- Return the light source and condenser to the forward, upright position

### Getting an image (brightfield)

- Turn the wheel on the lower left (behind the microscope power switch) to control the intensity of transmitted tungsten light (intensity displayed on L.E.D. on front panel of microscope)
- Follow steps to setup for brightfield imaging:
  1. Right top slider – pushed in (to view through oculars)
  2. Right bottom slider – pushed in (always)
  3. Right wheel – turn to SCAN or 1.6X (enlarges viewed image 1.6X)
  4. Left top slider – pushed in (blocks Hg light if Hg lamp is on)
  5. Left bottom flat slider – pulled out (Wollaston prism for DIC)
  6. Left top wheel – turn to BF (bright field)
  7. Left bottom wheel - turn to SCAN or 1 (blank)
  8. Condenser wheel – turn to BF (bright field)

### Viewing (focussing and magnification)

- Adjust the eyepieces (interpupillary distance and focus)
- Use traverse control on the right to move stage
- Check that 5X objective is in place (objective in use is also displayed in L.E.D.)
- If focus is way off, use the fast coarse drive behind the right focus knob. The upper button moves the objective up, the lower button moves it down .
- Focus with the focus knob (towards back moves objective up closer to specimen, towards you moves objective down away from the specimen)
- To change “focus” step size use STEP button on front panel of microscope. Selected step size S1 (fine), S2 (medium) and S3 (coarse) is visible in L.E.D.
- To change objectives use the black push buttons behind the left focus knob. Upper button moves to higher power, lower button moves to lower power (objective sequence: 5X, 10X, 20X, 40X oil, 100X oil). **DO NOT MOVE TURRET BY HAND**
- Adjust focus using focus knob. **DO NOT USE THE FAST COARSE DRIVE ABOVE 5X OBJECTIVE**
- If you use the 40X oil or 100X oil objective, apply oil as follows:
  1. Push the LEARN button on the L.E.D. display once
  2. Wait for the lens to move to the far right
  3. Put a **VERY SMALL** drop of oil over the lens using the syringe + tube gizmo
  4. Push LEARN again and the lens will go back to original position
- Set Köhler illumination to increase quality of viewed image

## **Brightfield**

- Go to 'Microscope setup' (page 3) and follow the instructions on 'Getting an image (brightfield)'
- Focus image

## **Differential Interference Contrast – DIC**

### **Short version**

- Follow steps **only** if microscope is already setup for brightfield:
  1. Left bottom slider – pushed in (Wollaston prism for DIC)
  2. Left top wheel - turn to 40 (for 40X DIC) or to D (for 100X DIC)
  3. Condenser wheel – turn to 40 (for 40X DIC) or to 100 (for 100X DIC)
  4. Put polarization filter on top of the condenser in place
  5. Focus image
  6. To get the desired shear plane, tweak the upper left wheel one way or the other. You also may need to adjust the polarizing filter on top of the condenser

### **Complete version**

- Turn the wheel on the lower right (behind the microscope power switch) to control the intensity of transmitted tungsten light (intensity displayed on L.E.D. on front panel of microscope)
- Follow steps to setup for DIC imaging:
  1. Right top slider – pushed in (to view through oculars)
  2. Right bottom slider – pushed in (always)
  3. Right wheel – turn to SCAN or 1.6X (enlarges viewed image 1.6X)
  4. Left top slider – pushed in (blocks Hg light if Hg lamp is on)
  5. Left bottom slider – pushed in (Wollaston prism for DIC)
  6. Left top wheel – turn to 40 (for 40X DIC) or to D (for 100X DIC)
  7. Left bottom wheel - turn to SCAN
  8. Condenser wheel – turn to 40 (for 40X DIC) or to 100 (for 100X DIC)
  9. Put polarization filter on top of the condenser in place
  10. Focus image
  11. To get the desired shear plane, tweak the upper left wheel one way or the other. You also may need to adjust the polarizing filter on top of the condenser

## Conventional Fluorescence

### Short version

- Follow steps **only** if microscope is already setup for brightfield:
- Check that mercury bulb is turned on (see instructions on 'startup' page 1)
  1. Left top slider - in middle position (for mercury bulb with heat filter)
    - pulled out (for mercury bulb with no heat filter)
  2. Left bottom wheel – turn to 2 for fluorescein (FITC)
    - turn to 3 for Rhodamine (TRITC)
  3. Turn the intensity of the tungsten light down with the wheel behind microscope switch (0 V(voltage) displayed on L.E.D.)
  4. Focus image

### Complete version

- Check or mercury bulb is turned on (see instructions on 'startup' page 1)
- Follow steps to setup for conventional fluorescence imaging:
  1. Right top slider – pushed in (to view through oculars)
  2. Right bottom slider – pushed in (always)
  3. Right wheel – turn to SCAN or 1.6X (enlarges viewed image 1.6X)
  4. Left top slider - in middle position (for mercury bulb with heat filter)
    - pulled out (for mercury bulb with no heat filter)
  5. Left bottom slider – pulled out (Wollaston prism for DIC)
  6. Left top wheel – turn to BF (bright field)
  7. Left bottom wheel – turn to 2 for fluorescein (FITC)
    - turn to 3 for Rhodamine (TRITC)
  8. Condenser wheel – any position (does not matter)
  9. Turn the intensity of the tungsten light down with the wheel behind the microscope switch (0 V(voltage) displayed on L.E.D.)
  10. Focus image

## Laser Fluorescence

- Check if laser(s) to use is/are turned on (see instructions on 'startup' page 1). An orange light beneath the key should be on
- Setup the microscope for either brightfield or DIC (see instructions on 'brightfield' or 'DIC')
- Focus image
- Turn the intensity of the tungsten light down with the wheel behind the microscope switch (0 V(voltage) displayed on L.E.D.)
- **Pull right top slider out (sent light to photo multiplier tubes)**
- Go to 'Imaging (TCS)' (page 6) and follow the instructions

## Digital Imaging for Brightfield and DIC

- Check if Ar laser is turned on (see instructions on ‘startup’ page 1). An orange light beneath the key should be on
- Setup the microscope for either brightfield or DIC. See instructions on ‘brightfield ’ or ‘DIC’ (page 4)
- Remember to set Köhler illumination to increase quality of viewed image
- Turn the knob at the very top of the condenser so that the white line is down (towards scan)
- **Pull out the right top slider (send light to PMT’s)**
- Go to ‘Imaging (TCS)’ (page 6) and follow the instructions (leave tungsten light on when scanning)

## Imaging (TCS)

- Check that the right top slider is out (directs light to PMT’s)

### TCS Tools – Acquisition

- If ‘settings’ window is not open yet, click FILTERS icon to show filter settings (leave open for reference)
- Check for or select proper FILTER SETTING in SETTINGS box
- Select color of image collected by active PMT in SETTINGS box
- Use the LENS icon to select the lens **which is actually used**
- Use MODE to select the plane of scanning (xy or xz section)
- Use FORMAT to specify the number of pixels for scanning (64x64, 128x128, 256x256, 512x512, 1024x1024)
- Click on SCAN icon to activate continuous scanning mode and click the same button again to STOP (sample is scanned according to the selected parameters)
- Turn off SCAN whenever possible to minimize bleaching (see also ‘Improving the Image’ in attachment I)
- If at this point no image appears on the right screen go to ‘Trouble shooting’ in text box

### Trouble shooting

- Check that all the sliders are properly set
- Check that the specimen is still in view and focus (look through oculars)
- Check that the top slider is pulled out (send light to photo multiplier tube)
- Check that the laser light is actually reaching the specimen during scanning. If not, restart ‘TCS NT’ software
- Check that the intensity level of the laser is high enough (LEVEL knob on laser box)
- Check that gain is high enough (PMT knob on panel box). See instructions ‘TCS Panel box’ (page 7)

- Go to 'TCS Panel Box' (page 7) for easy adjustment of zoom, pinhole, z-position, gain and offset
- Use ACCUM  $\Sigma$  icon to define # of scans of a single section to be averaged (1, 2, 4, 8, 16, 32 etc.)
- Use CONTR icon to change contrast while scanning. Keep mouse button pressed, movements will change pinhole while scanning
- Use PINHOLE icon to change the pinhole size. Keep mouse button pressed, movements will change pinhole while scanning. Keep size between 0.6 and 2.0 (see 'Pinhole' attachment I)
- Use ZOOM icon to magnify the image electronically. If zoom >1, the arrow buttons can be used to move the scan field in scanning mode
- Use the SPEED icon to change the scan speed (dwelling time of the laser on each point)
- Use ZOOMIN icon to zoom in on selected area in a defined white box

### **TCS Panel box**

- Use the panel box knobs for easy adjustment of zoom, pinhole, z-position, gain and offset
- Click on SETTINGS (upper left corner of left screen) followed by PREFERENCES. A dialog box on the right screen appears
- In dialog box click on PANEL BOX
- While scanning, use Z-POS knob to find the brightest plane in your sample
- While scanning, use gain (PMT) and OFFSET knobs to adjust respectively bright and dark areas in collected images

**NOTE:** To minimize cheating select GlowOvUr (glow over under) in SETTING box. Adjust gain (PMT) and OFFSET knobs to a level where a little bit of blue (gain) and green (OFFSET) can be seen in the image

- Use zoom to increase magnification electronically on the screen
- Use PINHOLE knob to change the size of the pinhole. Keep size between 0.6 and 2.0 (see 'Pinhole' attachment I)
- To assign different functions to the panel box knobs, click on the drop down menu underneath the knobs shown on the screen

### **TCS Image**

- Use icons in 'TCS Image' window to adjust layout of the image on the right screen
- If two PMT's are in use, use CHN1 icon to show image collected by PMT 1 and CHN2 icon to show image collected by PMT 2 (see setting box on left screen)
- If only one PMT is in use, use CHN1 icon to show the image
- Use OVERLAY icon to overlay the images in Chn1 (image shows green) and Chn2 (image shows red)
- Use SINGLE icon to show one (selected) image (Chn 1, Chn 2 or Overlay)
- Use TILED icon to show multiple (selected) images (Chn 1, Chn 2 and Overlay)
- Use 256, 512 or 1024 icon to change the format of the image

- Use CLUT (color lookup table) icon to select a color for the image in Chn1 and Chn2 as follows:
  1. Click CLUT icon and two color bars will show on the right side of the image
  2. Select an image and click on the corresponding color bar (Chn1 – left bar, Chn2 - right bar)
  3. Select a color in the CLUT box
- Use PRINT icon and SNAPS icon to print or save an image (go to ‘Image Storage’ page 12, and follow instructions on ‘Printing’ and ‘Saving’)
- To improve the image but **know** the trade off, see ‘Improving the Image’ in Attachment I

### **Imaging (TCS) – 3D**

- Continue to get a 3D image if a desired single image is already obtained using instructions under ‘Imaging TCS’ (page 6)

### **TCS Tools – Acquisition**

- Use BEGIN  $\uparrow$  icon and END  $\downarrow$  icon to define specific positions in sample (in between these two positions a selectable number of optical sections can be defined):
  - Click SCAN and use z-position knob (CCW) to define upper section
  - Hit BEGIN  $\uparrow$
  - Use z-position knob (CW) to define lower section and hit END  $\downarrow$
  - Click STOP
- Use SECT # icon to select number of sections in the z-plane. To define optimal number of sections for 3D imaging :
  - Click SECT # icon and select USER DEFINED
  - In ‘User Defined Section’ window enter step size ( $\mu\text{m}$ ). Use VOXEL SIZE X in ‘TCS Image’ window (on right screen)
  - Hit calculate
  - If ‘Stack Parameters Correction’ window shows up, select which parameter to change
  - Hit OK in ‘User Defined Section’ window
- Use ACCUM  $\Sigma$  icon to define # of scans of a single section to be averaged (1, 2, 4, 8, 16, 32 etc.)
- Click on SERIES icon to start scanning the series of optical sections (after position and # sections have been defined)

### **TCS Image**

- Use GALLERY to show individual optical sections of a series showing either one channel (single) or two channels (tiled) or an overlay (single)
- For other icon descriptions see ‘TCS Image’ (page 7)

## **TCS Tools – View**

- Use ORIGINAL icon to display original individual images of an image series:
  - Use FIRST icon to display the first image of a series
  - Use LAST icon to display the last image of a series
  - Use PREV icon to display the previous image of a series
  - Use NEXT icon to display the next image of a series
  - Use SELECT icon to select an image (number) of a series you want to display
- Use START icon to display the images as a movie
- Use EXT FOC (extended focus) icon to average gray scale values from a image series and displays the result as an ‘extended focus’ image (to get original images back hit ORIGINAL)
- Use PROJ (project) icon to display the maximum gray scale value along the z-axis as a ‘maximum projection’ (to get original images back hit ORIGINAL)
- Use CONTRAST icon to change contrast (linear) of series images in selected channel
- Use GAMMA icon to change contrast (nonlinear) of series images in selected channel. Displays dark areas in a lighter way without significantly changing the light areas
- Use FILTER icon to smoothen the image or to sharpen the contours of series images
- Use MODIFY icon to replace the original data with the image modified with ‘contrast’, ‘gamma’ and ‘filter’
- Use TOPO icon to get a topological image (higher areas represent brighter areas)
- Use STEREO icon together with SINGLE icon in ‘TCS image’ window, to generate a single red/green stereo image of one channel. Use special glasses to see 3D effect
- Use STEREO icon together with TILED icon in ‘TCS image’ window, to generate a stereo pair of one channel or overlay. Cross your eyes to see 3D effect

## **Quantification**

### **Define area**

- Obtain a desired image using instructions under ‘Imaging TCS’ (page 6) or ‘Imaging (TCS)- 3D’ (page 8)
- Several optical sections can be selected from the entire stack by utilizing GALLERY icon in ‘TCS-image’ window:
  - Click GALLERY button in ‘TCS-image’ window
  - Choose first optical section with the cursor
  - Hold the shift button to collect subsequent optical sections
  - In upper left corner on left screen, select FILE, SAVE SELECTED and select SCANNER FILE to save image in personal folder on data drive ‘Data (I:)’
  - In upper left corner on left screen, select FILE, OPEN, to open file
- Select QUANTIFY in ‘TCS-tools’ box

- Select an area for quantification:
  - Use POLY icon to select an area of irregularly shape for quantification
  - Use ELLIPSE icon to select an ellipse shaped area for quantification
  - Use RECT icon to select an rectangular shaped area for quantification
  - Use LINE icon to select an line shape for quantification
- Move cursor to image, a cross will appear
- Click left mouse button and define an area on the image by either holding the mouse button down or use it to connect points in small line segments
- Double click to close defined area
- Use CLEAR icon to clear all defined areas on image

NOTE: Multiple areas can be defined but only one at a time can be selected for analysis. To select area, highlight area using the cursor

### Analysis

- Use analysis tools to measure certain features (intensity, area, roughness etc.) in image after area or line is defined (see 'Define area' above)
- Select HISTO icon to create an intensity histogram on the intensity in a marked area of an image. In addition area measurements ( $\mu\text{m}_-$ ) are done
- Select PROFILE icon to create an intensity profile on the intensity along the edge of a marked area. In addition length measurements ( $\mu\text{m}$ ) are done
- Use STACK icon to produce a xz-intensity profile of a series along the edge of a marked area including a ratio of channel 1 and channel 2
- Use SURF icon to determine additional statistical data on the geometry of a marked area such as surface and various roughness parameters

NOTE: A topological image is required for this function and parameters of roughness are calculated only for rectangular areas:

- define a rectangle area on the image using RECT icon
- click VIEW in 'TCS-tools' box and select TOPO icon
- click QUANTIFY in 'TCS-tools' box and select SURF icon
- To save and/or print image go to 'Image Storage' (page 12)
- To save graph:
  - Click SNAP icon in lower right corner of 'analysis (surface, histogram, profile)' window
  - Click ANNOTATE in 'TCS Tools' box
  - Click FILE, SAVE AS and change the name from 'TCS-DOCUMENTATION' to something specific before saving the image in personal folder on data drive "Data (I)"
- To print graph:
  - Click PRINT icon in lower right corner of 'analysis (surface, histogram, profile)' window
  - To change the headers of the image click on SETUP on the left screen and click on HEADER/FOOTER in the 'Page Setup' window
  - Click COSTUM HEADER and make changes in 'Left Section' (i.e. your name), 'Middle Section' (i.e. file name) and 'Right Section' (if desired)
  - Click OK in 'Header/Footer' window and 'Setup' window
  - Click PRINT on left screen followed by OK in the 'Print' window

## Time lapse movie

- Use time lapse to store images over time and show them as a movie
- Check that you are in ACQUISITION mode (in 'TCS-Tools' window)
- Click MODE icon and select 'xyt' scanning mode
- Click on SETTINGS (upper left corner of left screen) followed by PREFERENCES. A dialog box on the right screen appears
- In dialog box change the number in the 'Simple Time-lapse Delay' box. The number represent # of seconds in between two scans
- Click SECT # icon in 'TCS-Tools' window and select the number of scans (sect #) you want to make
- Click SERIES icon in 'TCS-Tools' window to start scanning
- Wait till scanning is done
- To view images/movie:
  - Select VIEW in 'TCS-tools' window
  - Use ORIGINAL icon to display original individual images of an image series
  - Use FIRST, LAST, PREV, NEXT and SELECT icon to view one image out of an series
  - Use PLAY icon to display the images as a movie

## Bleed Over

- If working with double or triple stained specimen, the emitted  $\lambda$  from one stain (for example FITC) can appear in the wrong channel and increase the signal for the other stain (for example TRITC). This is called **bleeding over**. Two options are possible to avoid this:

### Option 1 – cleaner

- Selecting FITC filter setting in 'settings' window and collect an image
- Click FILE followed by SAVE AS and save image in **your** folder as a SCANNERFILE
- Select TRITC filters in 'settings' window. Reset gain (PMT), OFFSET and ACCUM  $\Sigma$  (if necessary) and collect an image. If 3D imaging is used, do not change BEGIN, END and SECT #
- Click FILE followed by SAVE AS and save image as a SCANNERFILE in **your** folder on data drive 'Data (I:)'
- Use MERGE application to put images together:
  - Click on START followed by PROGRAMS, Leica TCS NT and MERGE
  - A "Get input file(s)" window will open
  - Click ADD and select FITC scanner file in your folder. Click OPEN
  - Click ADD and select TRITC scanner file in your folder. Click OPEN
  - Enter OUTPUT FILE complete (for example: I:\ **your** name \ file name)
  - Click START MERGE button followed by EXIT
  - Go to FILE, OPEN and select 'output file name'. Chn1 and Chn2 are visible on right screen

## **Option 2 – simpler**

- Select FITC/TRITC filter setting in ‘settings’ window and collect an image
- In the ‘settings’ window click on the picture of the laser to reveal the ‘Acoushco Optical Timing Filter’ (AOTF) window
- In the ‘AOTF’ window, turn the TRITC to 0% by sliding the 568 slider all the way to the left
- If there is an image in chn2, lower, while scanning, the 488 until there is no image in chn2
- If needed, collect this image as proof that there is no bleed over. For saving options go to ‘Saving’ (page 13)
- In the ‘AOTF’ window, turn 568 back up to 100% and collect an image. For saving options go to ‘Saving’ (page 13)

## **Image Storage**

### **Legend Info**

- To change legend info in the ‘TCS Image’ window, click SETTINGS on left screen, followed by PREFERENCES
- In ‘preferences’ box click LEGEND INFO
- Fill in the empty spaces in the ‘Legend info’ box on the left screen (default legend will display USER, INSTITUTE, COMMENT 1 – 3)
- Click OK
- Leave ‘preferences’ box open for easy access to ‘Legend Info’ and ‘Panel Box’

### **Printing**

- Check that the printer is ON
- If printing an image without legend go to (1) and if printing an image with legend follow instructions under (2):
  1. If only printing the image, click PRINT icon in the ‘TCS Image’ window
    - To change the headers of the image click on SETUP on the left screen and click on HEADER/FOOTER in the ‘Page Setup’ window
    - Click COSTUM HEADER and make changes in ‘Left Section’ (i.e. your name), ‘Middle Section’ (i.e. file name) and ‘Right Section’ (if desired)
    - Click OK in ‘Header/Footer’ window and ‘Setup’ window
    - Click PRINT on left screen followed by OK in the ‘Print’ window
  2. - If printing an image with legend, check that you are in ‘Acquisition’
    - Click SNAP icon in the ‘TCS Image’ window
    - Select ‘ANNOTATE’ in ‘TCS Tools’ window on left screen
    - Click PRINT icon in ‘TCS Tools’ window
    - To change the headers of the image click on SETUP on the left screen and click on HEADER/FOOTER in the ‘Page Setup’ window
    - Click COSTUM HEADER and make changes in ‘Left Section’ (i.e. your name), ‘Middle Section’ (i.e. file name) and ‘Right Section’ (if desired)
    - Click OK in ‘Header/Footer’ window and ‘Setup’ window
    - Click PRINT on left screen followed by OK in the ‘Print’ window

## **Saving**

- If you want to save images on the computer, save images in **your personal** folder on data drive "Data (I:)".
  - **NOTE: If cumulative size of YOUR PERSONAL files approach 600 MB, go to 'Clean Up Data Drive (I:)' (page 13)**
  - To save **image and legend** info as shown on the screen go to (1)
  - To save one **selected image** (out of a series) without legend info, go to (2)
  - To save all **individual images** (without legend info) and be able to read the information on this (TCS) computer, follow instructions under (3)
  - To save all **individual images** including **legend** info and be able to pull up individual images on any computer, follow instructions under (4)
1.
    - One image including legend info will be saved as an Excel workbook file
    - Click SNAP icon in the 'TCS Image' box
    - Click ANNOTATE in 'TCS Tools' box
    - Click FILE, SAVE AS and change the name from 'TCS-DOCUMENTATION' to something specific before saving the image
  2.
    - One selected image in one channel can be saved as a single tif file
    - No legend info will be saved
    - Check that you are in ACQUISITION mode (in 'TCS-Tools' window)
    - Select SINGLE and one channel (CHN1 or CHN2) in 'TCS Image' window
    - Click FILE and SAVE SELECTED on left screen
    - Select 'SCANNER FILE' to save image
  3.
    - All individual images will be saved as files to be opened by this (TCS) computer (for example, you are able to run movies, make stereo pairs and merge images)
    - Legend info will not be saved and no other computer will be able to see anything except one section in chn1 (filename.tif)
    - Click FILE and SAVE AS on left screen
    - Select 'SCANNER FILE' to save image
  4.
    - All images are saved separately in a folder as individual tif files. Any computer can pull up any image (in one channel) at a time. The set of files will not be recognized as a series
    - Legend will be saved as a text file
    - Click FILE and SAVE AS on left screen
    - Select 'EXPORT FILE' to save image (folder and text file will get the same chosen name)

## **Clean Up Data Drive '(I:)'**

### **Copy files to a Zip Disc**

- Place Zip Disc in blue drive on top of the computer
- Close all programs
- To open 'window explorer' click on WINDOW NT EXP. icon
- Click on 'Removable disc (D:)' to see content on Zip Disc
- Select 'Data (I:)' in 'Exploring - (C:)' window and open **your** personal folder
- If you want to organize data by date, hit MODIFIED header

- To find out how big your files are highlight one or more (by holding shift key) files and select FILE followed by PROPERTIES
- Click OK in 'Properties' to close window
- Select one or more (by holding shift key) files to be copied
- Drag file(s) to the REMOVABLE DISC (D:) in the all folders column
- Wait until 'Copying' window disappears
- Select 'REMOVABLE DISC (D:)' to see or files are copied
- Press round grey button on blue drive to remove Zip Disc

### **Copy files to a CD**

- Place CD in drive 'E'
- Close all programs
- Click START, PROGRAMS, ADAPTEC EASY CD CREATOR, EASY CD CREATOR.....and wait (**hour glass will not appear**).....until program starts up
- Select DATA CD and hit NEXT
- Select files and folders to be copied and hit ADD NOW after each selection
- When done hit NEXT
- Select PERFORM TEST in ADAPTEC EASY CD CREATOR WIZARD window followed by NEXT
- Select CREATE CD NOW followed by FINISH

### **WRITING OF CD WILL START**

- Click OK in the CD CREATION PROCESS-UNTITLED window
- Go to FILE, EXIT
- Click NO in EASY CD CREATOR window

#### **NOTE:**

Some problems seem to arise when you try to add on files on a recordable CD (CD-R). It is suggested to copy at once as many MB (up to 600) on a CD-R.

### **Delete copied files**

- Close all programs
- To open 'window explorer' click on WINDOW NT EXP. icon
- Select 'Data (I:)' in 'Exploring - (C:)' window and open **your** personal folder
- To find out how big your files are highlight one or more (by holding shift key) files and select FILE followed by PROPERTIES
- To close 'Properties' window, click OK
- Select one or more (by holding shift key) files to be deleted
- Go to FILE, DELETE
- Click YES in the 'Confirm Multiple File Delete' window
- Wait till files are deleted
- Click FILE, CLOSE to close WINDOW EXPLORER
- Click on RECYCLE BIN icon
- Empty trash can by selecting FILE and EMPTY RECYCLE BIN
- Click YES in the 'Confirm Multiple File Delete' window
- Close 'Recycle Bin' window

## Remove specimen

- To move the lens away from your specimen, press black **BOTTOM** button behind the **RIGHT** focus knob
- Tilt back tungsten light source and condenser until it stops
- **GENTLY** remove the slide on the galvano stage and return the light source and condenser to the forward, upright position

## Shut Down

Remove slide with sample **first** (see 'Remove Specimen' above)

1. Mercury lamp
  - Turn off Hg bulb if used (it must have been **ON** for a minimum of 10 minutes)
2. Lasers
  - Turn intensity to minimum for Kr and Ar laser (LEVEL knob on laser box)
  - Turn key to off for Kr, Ar and HeNe laser
  - **WAIT** for 10 min before turning of the laser fan(s) on Ar and Kr laser box
3. Computer
  - Menu (upper left), click on FILE, then click on EXIT
  - Click on START (lower left corner of screen)
  - Click on SHUT DOWN, then click YES
  - Wait for the sign stating it is OK to SHUT OFF
  - Turn off the computer (red button on computer)
  - Turn off the monitors (lower right of each screen)
4. Microscope
  - If specimen isn't removed, go to 'Remove Specimen' (above)
  - Push right top slider in
  - If you used the 40X and/or 100X oil lenses, clean lenses of oil as follows:
    - Push the LEARN button on the L.E.D. display once
    - Wait for the lens to move to the far right
    - Clean lens of oil. **NEVER TOUCH A LENS WITH ANYTHING EXCEPT LENS PAPER!**
    - If oil has dripped down the sides of the lenses and/or onto the turret, be sure to clean it off
    - Push LEARN again and the lens will go back to original position
  - Cycle the objective turret back until the 5X objective is in place (use black push buttons behind the left focus knob)
  - Turn the intensity of the tungsten light down with the wheel behind the microscope switch (0 V(voltage) displayed on L.E.D.)
  - Turn the microscope OFF (black toggle switch on left side of microscope)
  - Wait 5 – 10 minutes (to cool Hg bulb) before putting the DUST COVER back on
5. Turn off Scan Electronics (red toggle switch on box on top of Ar laser)
6. Turn off the printer and put the cover back on
7. Sign out Logbook
8. **10 MINUTES LATER** turn off the laser fan and cover the microscope
9. Turn off the fan/filter above the door
10. Leave the room **CLEAN** and **TIDY!**

## ATTACHMENT 1:

### Improving the Image More Light

Procedure	Trade off
Turn up laser intensity	Faster bleaching of sample
Increase pinhole size	Faster bleaching of sample and decreasing resolution
Raise gain (PMT cw)	Amplifies light, doesn't gather more
Slower scan speed	Faster bleaching of sample

### Better Light

Procedure	Trade off
Decrease pinhole size (increasing resolution)	Less light
Accumulate (average out noise)	Faster bleaching of sample
Use Glow Over Under (minimize cheating)	None

### Minimize Bleaching

Turn off SCAN whenever possible!

Procedure	Trade off
Increase scan speed	Less light, 4X zoom at FAST
Fewer accumulations	More noise

## Pinhole

When you set the lens, the machine automatically sets the pinhole size to the diameter that optimizes the balance between resolution and light intensity. It calls that size 1.00. The working range of the pinhole size is 0.60 → 2.00: < 1.00 = better resolution > 1.00 = more light

