

NIKON PCM2000 LASER SCANNING CONFOCAL SYSTEM

September 1999 - Version 3.0

SECTION 1. PCM2000 HARDWARE

1.1 START UP

1. Argon laser

- Black box on floor under scope:
 - top button, toggle on (up) ✓ fan should come on
 - then key on (clockwise)
- Black box on tabletop ("Spectra Physics"):
 - ✓ make sure it's on stand-by, power (not current) and 50mW: key **ON** (clockwise)
 - ✓ green regulation and interlock lights should come on...after a few minutes, emission light will come on and gauge should read approximately 13 mW

2. HeNe laser

- Beige box on tabletop ("Uniphase"): key on (clockwise) ✓ orange light should come on

3. Remote fine focus (Z drive)

- Toggle on (left side of box) ✓ LED 0.0 should come on

4. Controller, Computer, and Monitor

If computer is on, but PCM Controller is off:

- Turn On PCM Controller
- Press <Ctrl><Alt> and **SHUT DOWN AND RESTART** Computer
- At **Windows NT, Windows NT (VGA), DOS/Win 3.11 - Confocal**
Select "**DOS/Win3.11**"
- Program Manager screen: double click PCM-2000 icon

If computer system is off:

✓ **To SEE IF THE MONITOR IS OFF FIRST**

- Turn On PCM Controller
- SMARTPRO power supply under computer:
black button on left---push down
- At **Windows NT, Windows NT (VGA), DOS/Win 3.11 - Confocal**
Select "**DOS/Win3.11**"
- Program Manager screen: double click PCM-2000 icon

1.2 SHUTTING DOWN

1. Remote fine focus (Z drive)

- Toggle OFF (left side of box) ✓ LED should go off

2. HeNe laser

- Beige box on tabletop ("Uniphase"): key off (counter-clockwise) ✓ orange light should go off

3. Argon laser

- Black box on tabletop ("Spectra Physics"):
 - ✓ make sure it's on stand-by, power (not current) and 50mW: Turn key **OFF** (counter-clockwise)

Allow the fan on the laser box to run for
10 minutes
to cool the laser

- Black box on floor under scope:
 - then turn key **OFF** (Counter-clockwise)
 - top button, toggle **OFF** (down) ✓ fan should go off

4. Controller, Computer, and Monitor

Exit Windows 3.11 but leave computer on, and at Windows NT Login Screen - This allows network access to the hard disk.

- Turn PCM Controller **OFF**
- Exit PCM 2000 Program [**FILE >> EXIT**]
- Exit Windows 3.11 Program manager [**FILE >> EXIT**]
- At C:> , press **<Ctrl><Alt>** To restart computer
- Computer will automatically start **Windows NT**
- Turn Off Computer Monitor!!

1.3 FLUORESCENCE PATHWAYS

FILTERS AND DICHOIC MIRRORS

NOTE: For all the 1.5cm wide filter holders:

- ☞ Position #1: All the way in selects Filter position ☞
- Position #2: One click out blocks laser
- Position #3: two clicks out no-filter

LASER BOX (On the floor)

— **NeHe Laser** (543.5nm):

Excitation filter:

- ☞ (1) All the way in selects (empty) Filter position ☞

— **Ar ion Laser** (457nm, 488nm, & 514nm)

Excitation filter:

- ☞ (1) All the way in selects 488nm bandpass Filter position ☞
- (2) One click out blocks laser
- (3) two clicks out no-filter (all lines)

Neutral Density Filter:

- ☞ (1) All the way out - 100% transmission ☞
- (2) One click in - 10% transmission
- (3) two clicks in - 3 % transmission
- (4) All the way in - 1% transmission

SCANNER MODULE (On top of scope)

— Excitation Filter: selection of excitation wavelengths

- ☞ All the way in selects (empty) filter position ☞

— Neutral Density Filter: reduces intensity of *all* excitation lines

- ☞ (1) All the way out - 100% transmission ☞
- (2) One click in - 10% transmission
- (3) two clicks in - 3 % transmission
- (4) All the way in - 1% transmission

— First Dichroic mirror - divides laser path from fluorescent path

RGB - reflects 488, 543.5 & 633nm laser lines
- transmits remaining wavelengths

505LP - reflects laser wavelengths below 505nm (i.e. 457nm & 488nm)
- transmits wavelengths above 505nm

475LP - reflects laser wavelengths below 475nm (i.e. 457nm)
- transmits wavelengths above 475nm

- Confocaling Pinholes: Selects confocaling depth
 - (1) In position - $20\mu\text{m}$ hole
 - (2) Middle position - open, no pinhole
 - (3) Out position - $50\mu\text{m}$ hole

- Second Dichroic Mirror selector: Splits fluorescent signal between the two detectors
 - RF** - (*Reflective Mirror*) reflects 100% of signal to Channel #1

 - 565LP** - reflects wavelengths below 565nm to Channel #1
 - transmits wavelengths above 565nm to Channel #2

 - HM** - (*Half-Mirror*) splits entire signal 50% to each detector

- Barrier Filter Holder #1:
 - ☞ (1) All the way in selects 500-530nm bandpass Filter position ☞
 - (2) One click out blocks all wavelengths
 - (3) two clicks out no-filter (all wavelengths transmitted)

- Barrier Filter Holder #2:
 - ☞ (1) All the way in selects 565nm longpass Filter position ☞
 - (2) One click out blocks all wavelengths
 - (3) two clicks out no-filter (all wavelengths transmitted)

Example Settings

1) FITC / Fluorescein Isothiocyanate - Excitation: 490nm, Emission: 525nm

Laser box:

- Argon ion Laser only (488nm)
- Argon emission filter (1.5cm blue handle): all the way in
- Neutral density filter (small blue handle): all the way out (100%T)

Laser Scanning Head:

- Excitation filter: all the way in
- Barrier filters 1 and 2: all the way in
- Neutral density filter: all the way out (100%T)
- First Dichroic Mirror: 505 nm
- Second Dichroic Mirror: RF mirror (for maximum signal or 565nm LP mirror for better wavelength selection)
- Channel #1 will have FITC Signal (Channel #2 may have an auto-fluorescent signal)

2) FITC & Propidium Iodide - Ext: 490nm/Em: 525nm & Ext: 536/Em:617nm

Or FITC & Texas Red - Ext: 490nm/Em: 525nm & Ext: 596/Em:615nm

Laser box:

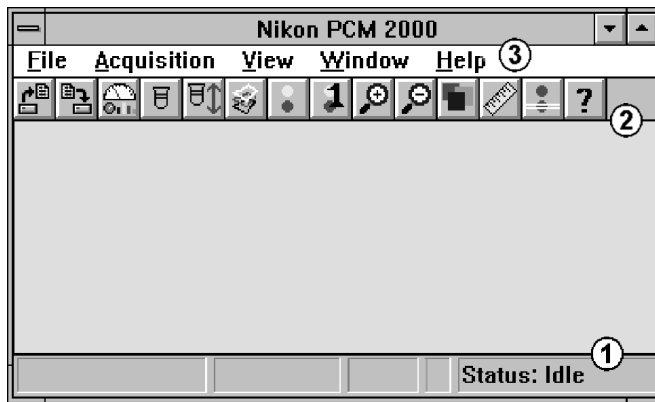
- He/Ne Laser ON (543.5nm ← Propidium Iodide)
- He/Ne emission filter (1.5cm blue handle): all the way in
- Argon ion Laser ON (488nm ← FITC)
- Argon emission filter (1.5cm blue handle): all the way in
- neutral density filter (small blue handle): all the way out (100%T)

Laser Scanning Head:

- excitation filter: all the way in
- barrier filters 1 and 2: all the way in
- neutral density filter: all the way out (100%T)
- First Dichroic Mirror: RGB
- Second Dichroic Mirror: 565nm LP
- Channel #1 will have FITC Signal
- Channel #2 will have Propidium Iodide signal


2. Capturing Images

MAIN PCM2000 SOFTWARE WINDOW



1 **SCANNER STATUS:** Reports current status of the laser scanning head.

SCANNING - Laser is actively scanning the specimen and collecting data

 *Specimen is being subjected to photobleaching*

IDLE - Laser is currently parked off the specimen and no live data is being collected

2 **TOOLBAR ICONS:**



OPEN FILE



ZOOM IN



SAVE FILE



ZOOM OUT



ACQUISITION MENU



VIEW MENU



DESIGNATE CURRENT OBJECTIVE



QUICK RULER TOOL



Z-FOCUS MENU



START/STOP X-T SCAN



Z-SECTIONING MENU



HELP




START/STOP CONTINUOUS SCAN



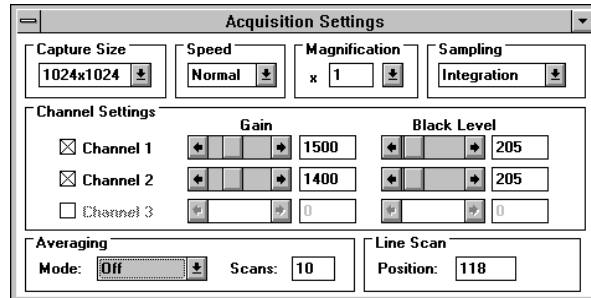
START/STOP SINGLE SCAN

2.1 GETTING STARTED / INITIAL SETTINGS

1. Follow all **START-UP** and **FLUORESCENCE PATHWAYS SETUP** Instructions, and in the Program Manager screen: double click PCM-2000 icon to start the PCM2000 Software

2. On computer system open the **ACQUISITION MENU** by double clicking on 

3. **ACQUISITION SETTINGS:**



3.1. Set **CAPTURE SIZE** ➤ **640x480**

3.2. Set **SPEED** ➤ **Normal**

3.3 Set **MAGNIFICATION** ➤ **x 1**

3.4 Set **SAMPLING** ➤ **Integration**

3.5 Set *both* **CHANNEL 1 & CHANNEL 2** ➤ to active


3.6 Set *both* **GAINS** ➤ **1400-1500**

3.7 Set **BLACK LEVELS** ➤ **205**

3.8 Set **AVERAGING MODE** ➤ **Off**

4. Select the 50 μ m pinhole to start with, and check that the trinocular microscope head is in the left position


5. Perform a single frame capture by clicking on the  icon

 **Watch** to See If You Are Saturating Either Detector! Red Led's on Pcm2000 Controller Box (Left of Computer System).

6. If neither detector is saturated, start **CONTINUOUS SCAN** mode by clicking on  icon

7. Adjust **GAIN** settings until image appears on screen.


8. Adjust **Z-FOCUS** position with the **REMOTE FINE FOCUS CONTROL** [Usually to obtain brightest image or optical plan of interest]

9. Click on  icon *ONCE* to stop scanning.

2.2 SEARCHING AROUND THE SLIDE


1. Setup acquisition for faster scanning:

- 1.1. Reduce **CAPTURE SIZE** ► **320x240** or **160x120**
- 1.2. Set **SPEED** ► **Normal**
- 1.3. Set **MAGNIFICATION** ► **x 1**
- 1.4. Set **SAMPLING** ► **Integration**
- 1.5. Set *both* **CHANNEL 1 & CHANNEL 2** ► to active
- 1.6. Leave current **GAIN** and **BLACK LEVEL** settings
- 1.7. Set **AVERAGING MODE** ► **Off**

2. Start **CONTINUOUS SCAN** mode by clicking on  icon

3. Reduce **GAINS** if needed, but don't be concerned about overly bright images

4. While laser is scanning, move microscope stage and/or fine focus to find and center specimen.

5. Click on  icon *ONCE* to stop scanning when desired area located.

6. Change **CAPTURE SIZE** ► **640x480** or **1024x1024** and adjust **ACQUISITION SETTINGS** as desired.

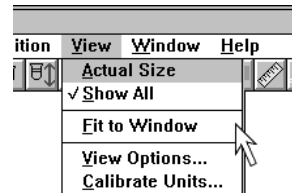
NOTE: TRY TO MINIMIZE LASER EXPOSURE OF THE SPECIMEN DURING THIS SEARCH STAGE, AS PHOTOBLEACHING OF THE FLUOROCHROME CAN OCCUR.

2.3 CAPTURING FINAL IMAGE

1. Set **CAPTURE SIZE** ► **1024x1024** and adjust **ACQUISITION SETTINGS** as desired.

NOTE: A 1024x1024 image size is larger than the computer's display area, go to:

VIEW > FIT TO WINDOW
to see the entire capture area.



2. In general frame averaging will significantly improve the final captured image.


2.2 Set **AVERAGING MODE** ► **Sum**

2.3 Set number of **SCANS** between 3-10 [Note: beyond ~ 24 scans shows little effect].

3. Perform a single frame capture by clicking on the  icon

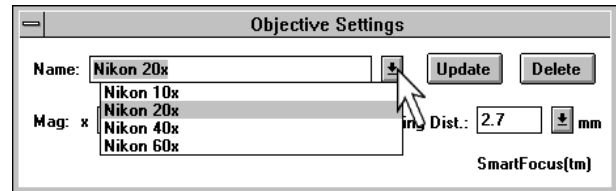
2.4 SAVING IMAGES

1. Set current objective lens being used.

1.1 Click on 

1.2 In **OBJECTIVE SETTINGS** window
NAME:, use pull-down menu and
select the current objective.

1.3 “Close” **OBJECTIVE SETTINGS** window

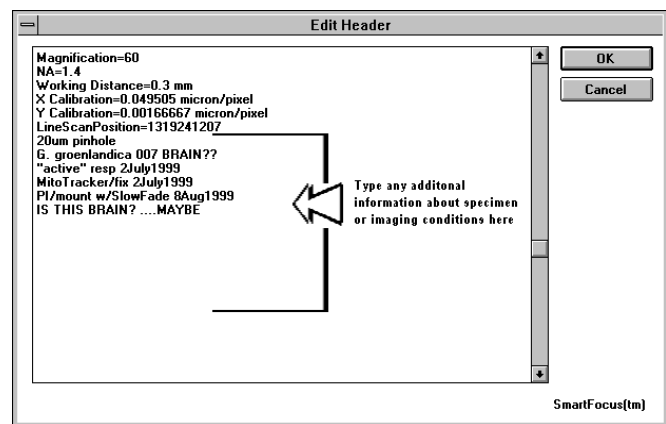


2. Edit image **HEADER**:

2.1 Open **EDIT HEADER** window:

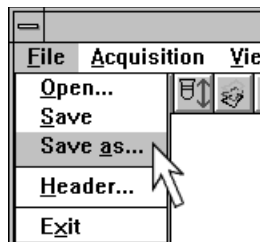


2.2 In **EDIT HEADER** window,
- Deselect the text by clicking
with the mouse pointer any
where in the field.
- Scroll to bottom of text and
type in “Pinhole Size” used to
capture the image.
- Enter any additional
information you would like
including, specimen type,
preparation, filters, etc. You
may add up to 60 additional
lines of text.



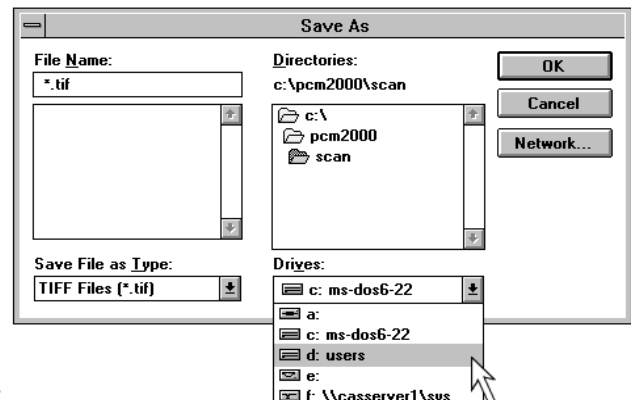
2.3 Click on “**OK**” to close the **EDIT HEADER** window.

3. Go to “**SAVE AS . . .**”

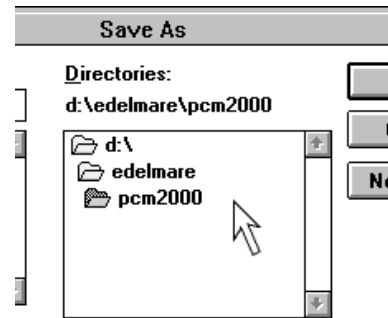


4. In the **SAVE AS** window:

4.1 Change the **Drives:** designation to “D: users”

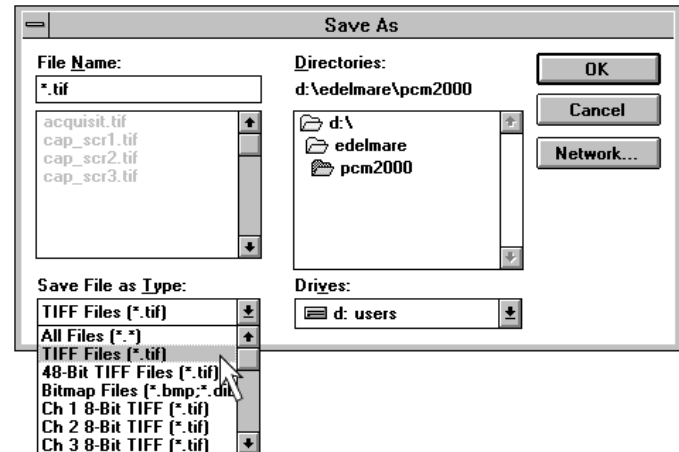


- 4.2 In the “**Directories:**” section change to your personal directory [use the mouse pointer to click through the directories]



- 4.3 In the “**Save File as Type:**” section make sure file type “**TIFF Files (*.tiff)**” is selected.

[This is the 24-bit RGB image type]



- 4.4 In the “**File Name:**” enter a file name.

NOTE: You are limited to only **EIGHT** characters plus the **.tif** ending. The following characters are **NOT** allowed: 'Spaces', /, \, ?, *, %

- 4.5 Click on **OK** to save the file.

2.5 NETWORK FILE TRANSFER

CONNECTING TO NETWORKED CASSERVER3

1. In main PCM2000 Windows Desktop double click on **NETWARE LOGIN** icon
2. **LOGIN** - tab
Name: [*Your User ID*]
Password: [*Your Password*]
3. Select **CONNECTIONS** - tab
✓ Select **SERVER**: Pull down and select **Casserver3**
CONTEXT: Pull down and select your context or type in from following table:

<u>Graduate Student in</u>	<u>Your Context is:</u>
Botany	bot.cas.std.mu
Chemistry	chm.cas.std.mu
Microbiology	mbi.cas.std.mu
Zoology	zoo.cas.std.mu
<u>Faculty in</u>	<u>Your Context is:</u>
Botany	bot.cas.mu
Chemistry	chm.cas.mu
Microbiology	mbi.cas.mu
Zoology	zoo.cas.mu
<u>All Undergraduates</u>	<u>Your Context is:</u> student.std.mu

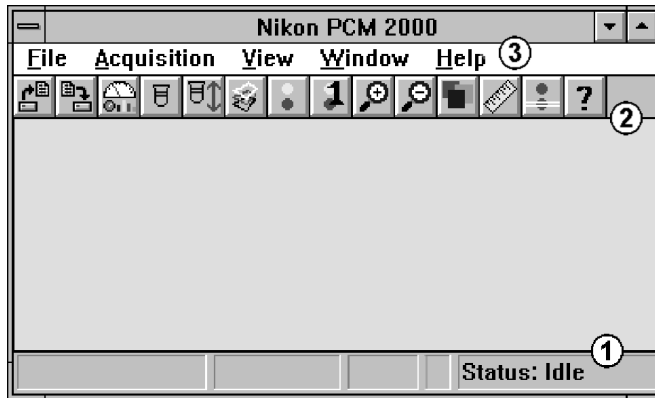
4. Click on **O.K.** button.

MANAGING FILES

1. In main PCM2000 Windows Desktop double click on **FILE MANAGER** icon
2. Select **WINDOW → NEW**
3. Select **DISK → NETWORK CONNECTIONS**
4. On right side of **NETWORK CONNECTIONS** window double-click through the following:
MU → (Maybe a second lower down MU →) CAS → . . .
(Scroll down to bottom) → CASSERVER3_USERS →
→ EMF → Users → [*Your User ID*]
5. On left side of **Network Connections** window select an empty driver letter (i.e. I) and click on the big MAP button in lower right of window.
6. Close **NETWORK CONNECTIONS** Window.
7. Now click on the new drive icon you just mapped (i.e. **K** up on menu bar).
8. Click and drag files from one drive window (i.e. **D:[YOUR FOLDER]**) to the networked drive **K:**)


3. PCM 2000 WINBRITE SOFTWARE

3.1 MAIN PCM2000 SOFTWARE WINDOW



1 **SCANNER STATUS:** Reports current status of the laser scanning head.

SCANNING - Laser is actively scanning the specimen and collecting data

 *Specimen is being subjected to photobleaching*

IDLE - Laser is currently parked off the specimen and no live data is being collected

2 **TOOLBAR ICONS:**



OPEN FILE



ZOOM IN



SAVE FILE



ZOOM OUT



ACQUISITION MENU



VIEW MENU



DESIGNATE CURRENT OBJECTIVE



QUICK RULER TOOL



Z-FOCUS MENU



START/STOP X-T SCAN



Z-SECTIONING MENU




HELP



START/STOP CONTINUOUS SCAN



START/STOP SINGLE SCAN

 Specific commands are covered in the instructional sections that follow.

3 **PULL-DOWN MENU ITEMS**

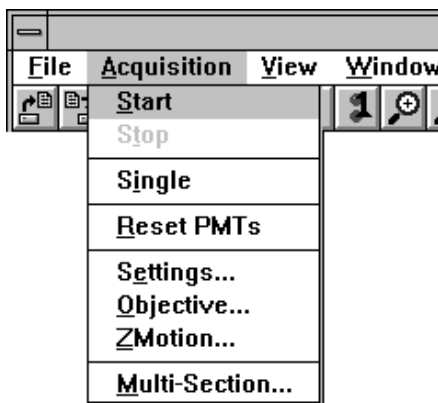
FILE:

- File management commands and editing of **HEADER** information



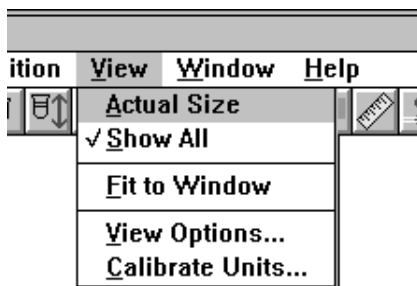
ACQUISITION:

- Controlling Capturing parameters



VIEW:

- Controls for changing the display conditions (i.e. Size of display, colors, and viewing channels separately)



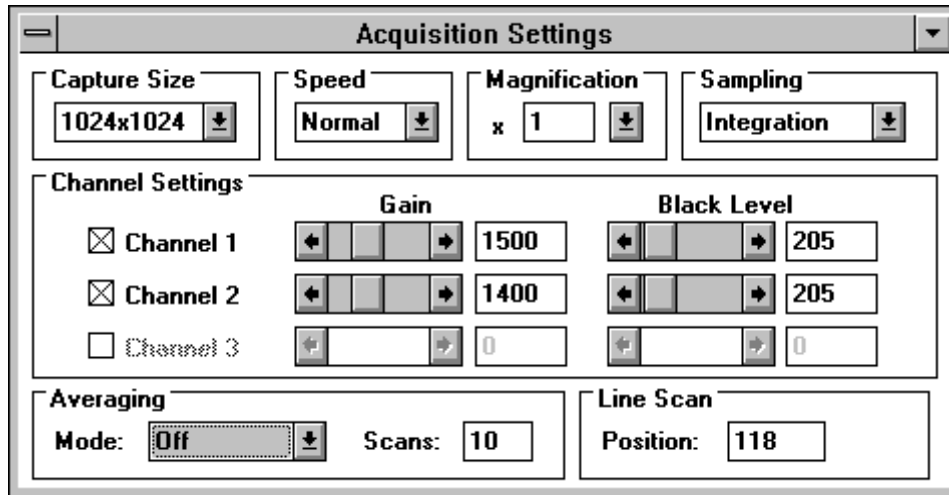
ACTUAL SIZE: displays data at 1:1 ratio with screen resolution. Default setting for live imaging display

SHOW ALL: Displays all three channels as a single RGB image.

FIT TO WINDOW: Shrinks the 1024x1024 image size to fit in the main window.

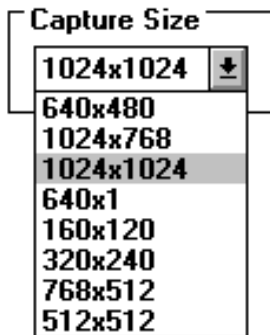
VIEW OPTIONS: Allows user to change colors associated with each channel and select four-panel view

3.2 THE ACQUISITION MENU



CAPTURE SIZES:

- Changes the size (resolution) of the image



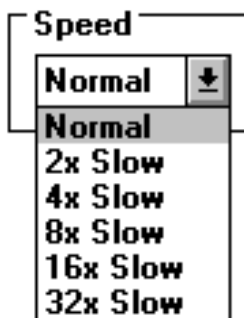
640x480 - quicker screen refresh, used for routine live imaging and fine adjustments to gains and focus.

1024x1024 - Slower screen refresh, used for publication quality captures !!!

160x120 - *Very fast*, nearly live video, used for moving specimens on microscope - very bright image due to pixel binning

320x240 - Fast scanning, used for specimen movement and location focus plains (i.e. top & bottom of specimen for Z-sectioning)

SCANNING SPEED:



- Changes the scanning speed or *dwell* time; Longer laser looks at any one point the higher the signal generated and detected.

MAGNIFICATION:

- Increases magnification of imaged area by decreasing the area scanned by lasers, resulting signal is still captured at same image resolution. Magnification can be selected via pull-down menu or by entering values into box; decimal values are acceptable.

SAMPLING: Leave at **INTEGRATION**.

CHANNEL SETTINGS: Controls for each photomultiplier tube (PMT) detector.

- By default Channel 1 is Green, Channel 2 is Red, and Channel 3 is Blue (Presently only two PMT's are available, and the Blue channel is inactive and assigned as 0's in the RGB image formats)
- Each PMT can be turned On or Off by selecting or de-selecting the check box at the left of the channel designation; regardless of any signals that may or may not be being sent to the detector.

GAIN values range from 0-4096 for each PMT, and are selectable via the slider bars or by entering whole numbers in the text boxes.

 **WARNING** 

You can seriously damage or destroy the PMT's by setting too high of a **GAIN** value! If saturated the PMT will shut off and the Red saturation LED will light on the PCM2000 controller box. **STOP** scanning immediately to reset any saturated PMT. In order to avoid saturation set both gains to 1400-1500 and capture a single scan when first viewing a sample.

The **GAIN** value determines the charge placed upon the vanes within the PMT's, and serves to amplify the incoming photon signal (similar to the volume control on a radio). In general the approximate ranges for signals are:

Gains of ~800-1600 are strong signals

Gains of 1600-2400 are good signals

Gains of 2400-3200 are poor signals

Gains over 3200 are questionable signals

- It is suggested that you modify the **SPEED**, Dichroic Mirror, Barrier Filters, and or laser intensity if possible to obtain gain settings below 2200.

BLACK LEVEL values range from 0-4096 for each PMT, and are selectable via the slider bars or by entering whole numbers in the text boxes. Black levels set a minimum cut off signal value, and are used to remove electronic noise. Generally a **BLACK LEVEL** setting of ~205 is desirable.

AVERAGING: Can significantly reduce the amount electronic noise in an image, by averaging a number of weaker signal frames (i.e. higher gains).

MODE: OFF; no frame averaging. **SUM;** frame averaging active.

SCANS: Number of frames to average.

 Leave averaging **OFF** until you are ready to capture your final image.

LINE SCAN - POSITION: Used to set the desire Y-value for capturing an X-T image.

STACKING IMAGES WITH IMAGE PRO PLUS (IN CONFOCAL ROOM)

1. Turn on computer (SMARTPRO power supply): black button on left---push down
 2. Select operating system NT version 4.00 (not VGA Mode).
 3. Log into Windows NT.
 4. Log into CASSERVER3:
 - Network Neighborhood
 - Entire Network
 - Netware Services
 - Netware Servers
 - To access CAS3 via CHM server, select CHMSERVER 1, login, close CHMSERVER, then select CASSERVER 3 from Netware Services window:
- Users
EMF
EMFShare
Your folder
5. Stack images.
 - Open all files in the stack using Image Pro Plus (IPWin 32)
 - Select icon with math symbols: Arithmetic logical operations
 - Source image: first image in the stack
 - Operation: maximum
 - Operand/Image: second image in the stack
 - Place result into: New image (it will be "untitled #whatever")
 - APPLY
 - To add remaining images to the stack:
 - Source image: untitled #... (same as above)
 - Operation: maximum
 - Operand/Image: next image in the stack
 - Place result into: Source image
 - APPLY
 - Repeat until all images are stacked
 - Save stacked image as new file (I store stacks on D drive. When I want to print them, I copy them to CHMSERVER or CASSERVER3)
 - Move individual images from "Incoming" to "Already Stacked"