CNS STANDARD OPERATING PROCEDURE

Two Photon Fluorescence Imaging

CNS instrument ID: LZR001

CNS document ID: SOP107

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CHANGE LOG

Revision	Date	Author	Changes
0.1	02/23/2011	A McClelland	Initial draft for two photon fluorescence SOP; portions taken from Multiphoton SOP030.
0.2	03/11/2011	E. Martin	Formatting. Assigned #107.
0.3	03/14/2011	A McClelland	Updated Fig 9
0.4	04/13/2011	A McClelland	Changed which spectrometer to use to measure wavelength. Changed which PMT detector to use

1. Purpose

1.1. This document specifies the work instructions for the CNS Two Photon Fluorescence Imaging System (instrument ID #LZR001) located in room G04 of the LISE building. If you see an area where more clarification is needed, if additional information is needed, or if you have suggestions on how to make this guide more useful in the lab, please contact CNS.

1.2. Note that this document is not a detailed instrument manual and does not intend to be one. For detailed questions, please refer to the manuals present in the lab, or ask CNS personal for help.

2. Scope

2.1. These work instructions are applicable to using the Coherent Mira 900D femtosecond laser with the Olympus Fluorview 300 for two photon fluorescence imaging

2.2. CNS Two Photon Fluorescence Imaging System consists of the following devices:

2.2.1. Coherent Mira 900D Ti:Sapphire laser (~1.5 W @ 720-950 nm, tunable wavelength, cw or pulsed mode fs or ps)

2.2.2. Beam routing optics (mirrors, lenses, wave plates, polarizers, …) and mounts used to manipulate, and direct the laser beam to the confocal laser scanning microscopes.

2.2.3. Instrument Control PC

3. Applicable Documents

PLEASE LEAVE ALL HARDCOPIES IN THE LAB

3.1. CNS Safety Guidelines and Policies as described at http://www.cns.fas.harvard.edu/safety/

3.2. Harvard University Radiation Safety Manual (hardcopy in the lab)

3.3. American National Standard for Safe Use of Lasers (hardcopy in the lab)

3.4. Coherent Verdi V-10 manual (hardcopy in the lab)

3.5. Coherent Mira 900D(P/F) manual (hardcopy in the lab)

3.6. CNS Standard Operating Procedure #SOP071 "Olympus FV300 Confocal Fluorescence Microscope"

3.7. Before starting, please read the following carefully:

3.7.1. This manual was developed to assist in the training process of users. Be aware that only the basic operation details will be presented. Please contact the CNS staff for more assistance if required.

3.7.2. Changes may occur when a new software version or patch is installed. Please contact the CNS staff if you are not sure about new features and functions.

NOTE:

This instrument is subject to laser safety regulations.

Prior any usage, every instrument user has to complete EHS laser safety training. The schedule of training is here:
http://www.uos.harvard.edu/ehs/radiation/rad_training_laser.shtml

CNS has created a form (#FM007 Pre-Training Requirements for Laser Use) that describes all necessary steps in detail.

All requirements of this form must be completed and approve before the user can work with the instrument.

4. What is two photon fluorescence and why is it a useful technique?

4.1. Two photon fluorescence uses two photons from a femtosecond infrared laser pulse to excite a chromophore to an excited electronic state. The chromophore then typically relaxes to a lower vibrational energy level in the excited state and finally emits a photon as fluorescence. The absorption of two photons is an unlikely process and thus needs a high density of photons to occur. Thus the two photon fluorescence only occurs at the focal point of the beam as can be seen in the photo below. This localization of signal to the focal spot allows for Z scans of the sample. Another advantage of two photon fluorescence is that near infrared light has a deeper penetration depth in biological samples than visible light. Second Harmonic Generation (SHG) is similar to two photon fluorescence, but has much stricter selection rules, the discussion of which are beyond the scope of this document. Two photon fluorescence can often be performed with the autofluorescence of a biological sample avoiding the need for toxic fluorescent dyes.

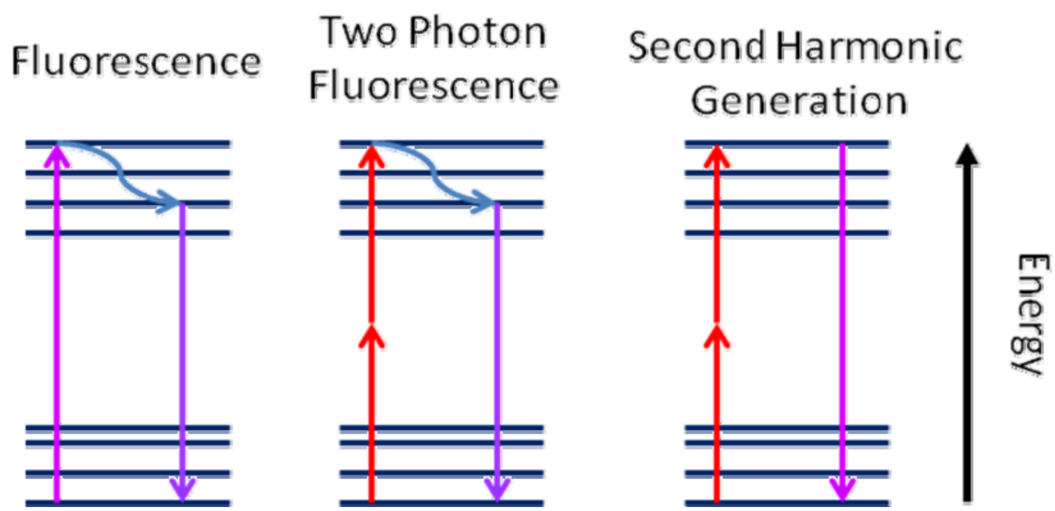

Figure 1 Energy diagrams comparing single photon fluorescence, two photon fluorescence, and second harmonic generation.

Figure 2 Demonstration of the localization of the two photon fluorescence signal to the focal spot of the near infrared laser beam. Image borrowed from

http://belfield.cos.ucf.edu/one%20vs%20two-photon%20excitation.html

5. Coherent Mira 900D Femtosecond/Picosecond Ti:Sa laser

5.1. System overview

5.1.1. Identify the parts of the laser system with the help of Figure 3.

5.1.2. Contact CNS personnel immediately if there are parts missing or if the laser system seems to be running (and it should not) or if you read any error messages on the screens.

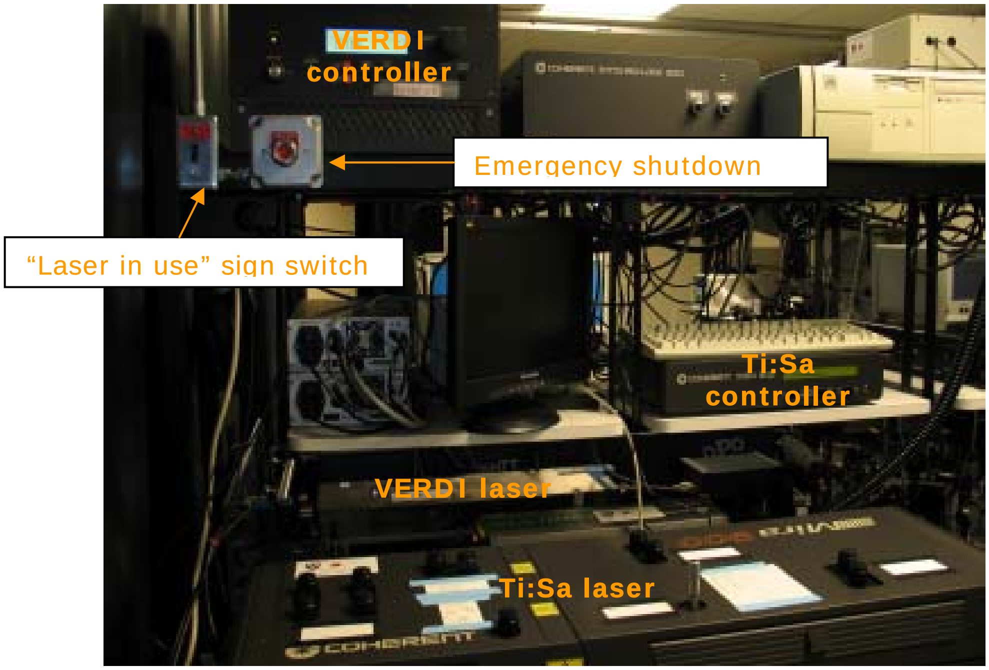

Figure 3: Ti:Sa laser system overview. NOTE the position of the “Laser in use” sign switch and the emergency power shutdown button.

5.2. Safety preparations

5.2.1. Remove any reflective objects from your body (rings, watches, bracelets, etc.). It is easy to get an accidental stray reflection from such items. Reflected laser light can be very dangerous.

5.2.2. Search and find an appropriate laser safety goggle.

5.2.3. Check the blocking specification written on the goggle. The safety glass must block with **more than 5 OD** in the wavelength range where you want to operate the laser.

5.2.4. Wear the goggles from now on until you shut down the laser.

5.2.5. Locate the emergency shutdown button (Figure 3). *Push it in case of emergency only* – this will trigger an immediate and complete power down of the whole instrument.

5.2.6. Close the laser safety curtain.

5.2.7. Switch on the “Laser in use” sign; the switch is located near the emergency shutdown button (Figure 3).

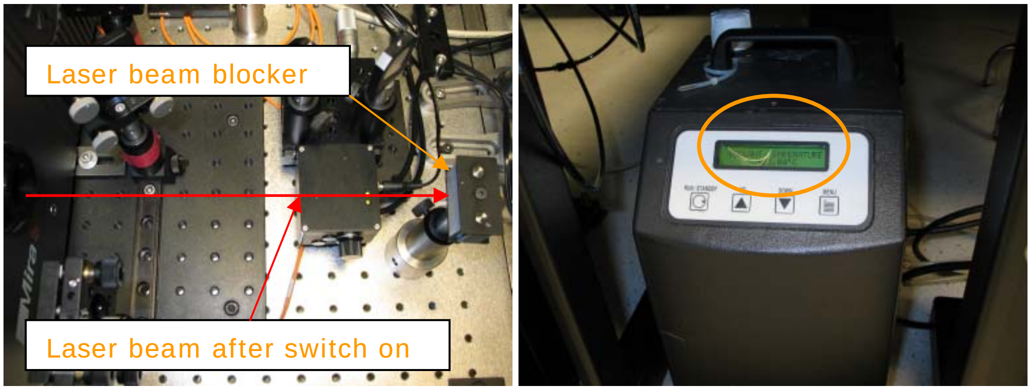

Figure 4: Laser output beam blocker in default position (left); water chiller (right) in normal operation mode (22-23 degC)

5.3. Switching on

5.3.1. Make sure that the output of the Ti:Sa laser is blocked with a beam blocker (Figure 4, left).

5.3.2. Check the status of the water chiller (Figure 4, right): The chiller should be in normal mode (NOT in STANDBY), showing NO ERRORS, and the coolant temperature should be 18 C.

5.3.3. Check the status of the VERDI and Ti:Sa controllers. You should find them as shown in Figure 3: Both in standby mode, the LCD screens on. The key on the VERDI controller should be in the "Standby" position.

5.3.4. CAUTION: NEVER switch any of the controllers off. If any one of them is switched off, do not proceed and contact CNS immediately.

5.3.5. Check the pump power setting on the VERDI laser controller: Default value is 10W (see Figure 5). If this is not the case, not proceed and contact CNS staff.

Figure 5: VERDI controller with VERDI laser switched on: Shutter closed (left), shutter open (right)

5.3.6. Switch the VERDI laser on by turning the key to the “on” position (see Figure 5).

5.3.7. The diode pump current on the display will increase. Wait till it reaches a stable value around 21 A.

5.3.8. Make sure that “cw” mode is selected on the Ti:Sa controller (Figure 6 left).

5.3.9. Also make sure that the relative humidity in the cavity is < ~ 15% (Figure 6). Contact CNS personnel, if the value is larger.

5.3.10. Press the “Shutter open” button on the Verdi laser controller (Figure 5 right) and wait ~15 minutes for the laser to stabilize. The current will jump to 25 A.

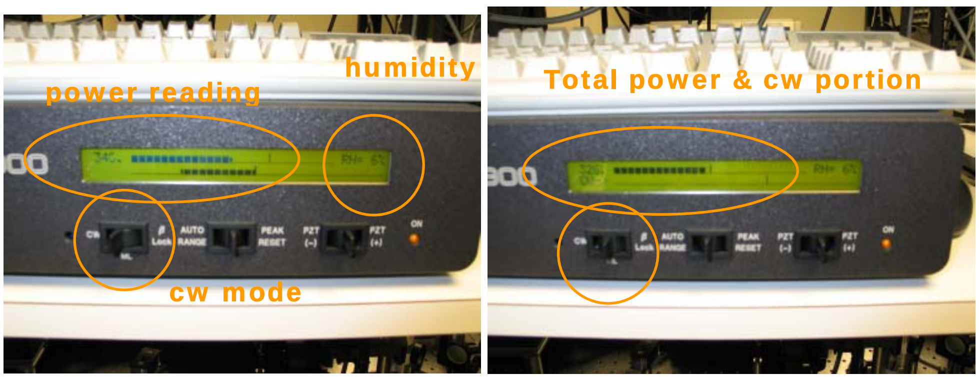

Figure 6: Ti:Sa controller: cw mode (left), modelocked (pulsing) mode (right)

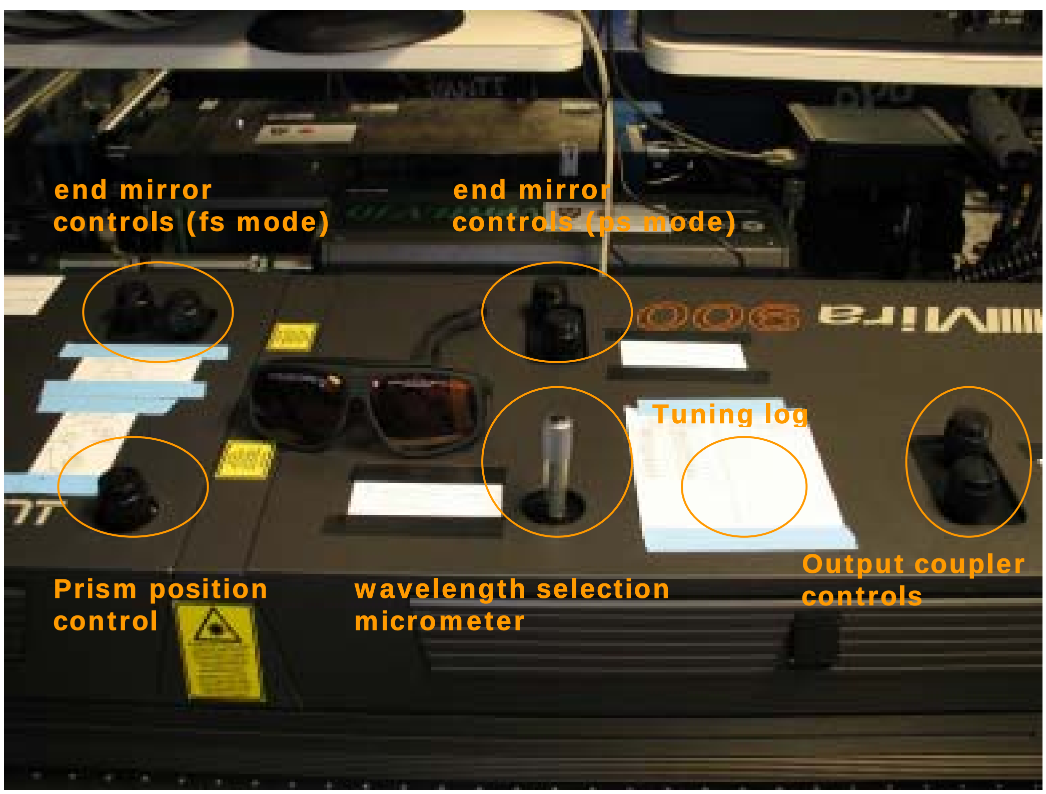

Figure 7: Ti:Sa laser cavity controls.

5.3.11. Identify the Ti:Sa laser cavity controls (Figure 7): fs end mirror, prism position, wavelength selection and output coupler controls.

5.3.12. Observe the power reading on the Ti:Sa controller (Figure 6) and maximize the value with the end mirror and output coupler controls.

5.3.13. Use the IR viewer or an IR viewing card to check laser output directly at the beam blocker.

5.4. Modelocking

5.4.1. To enable laser pulsing, switch to “ML” on the Ti:Sa controller (Figure 6 right).

5.4.2. Observe the lower row on the power display; residual cw laser power is shown here. In addition, the total power can fluctuate, indicating unsuccessful attempts to start modelocking (pulsing) mode.

5.4.3. To stabilize modelocking and to remove residual cw laser light, adjust the output coupler width first. It might be necessary to compromise some of the total power.

5.4.4. In addition, you could apply SLIGHT adjustments to output coupler position (indicated with an “H”), wavelength selection and prism position.

5.4.5. If these attempts to start modelocking are not successful, contact CNS personnel as the laser cavity might need realignment.

5.5. Wavelength tuning

5.5.1. Flip down the mirror that is directly after the Faraday isolator. (See fig 9 for reference.)

5.5.2. The output can be read on the screen of the APE controller that is on the shelf to the left.

5.5.3. To tune the wavelength:

5.5.3.1. SLOWLY turn the wavelength selection micrometer (Figure 7) to change the wavelength in steps of 10-20 nm.

5.5.3.2. While suppressing cw light, maximize power by adjusting the prism position (in the direction indicated on the cavity), as well as end mirror and output coupler controls. Only slight adjustments should be applied to the end mirror and output coupler controls.

5.5.3.3. Repeat these steps until you reach the desired wavelength.

5.5.3.4. If modelocking cannot be restored, you might be out of the specified tuning range. NOTE that the wavelength range available for cw mode is larger than the one available for ML mode!

5.5.3.5. Flip the mirror back up.

6. Beam routing and alignment optics

6.1. Ti:Sa beam routing is sketched on the table in black marker. Flip up the mirror to direct the beam towards the microscope. Refer to Fig 9.

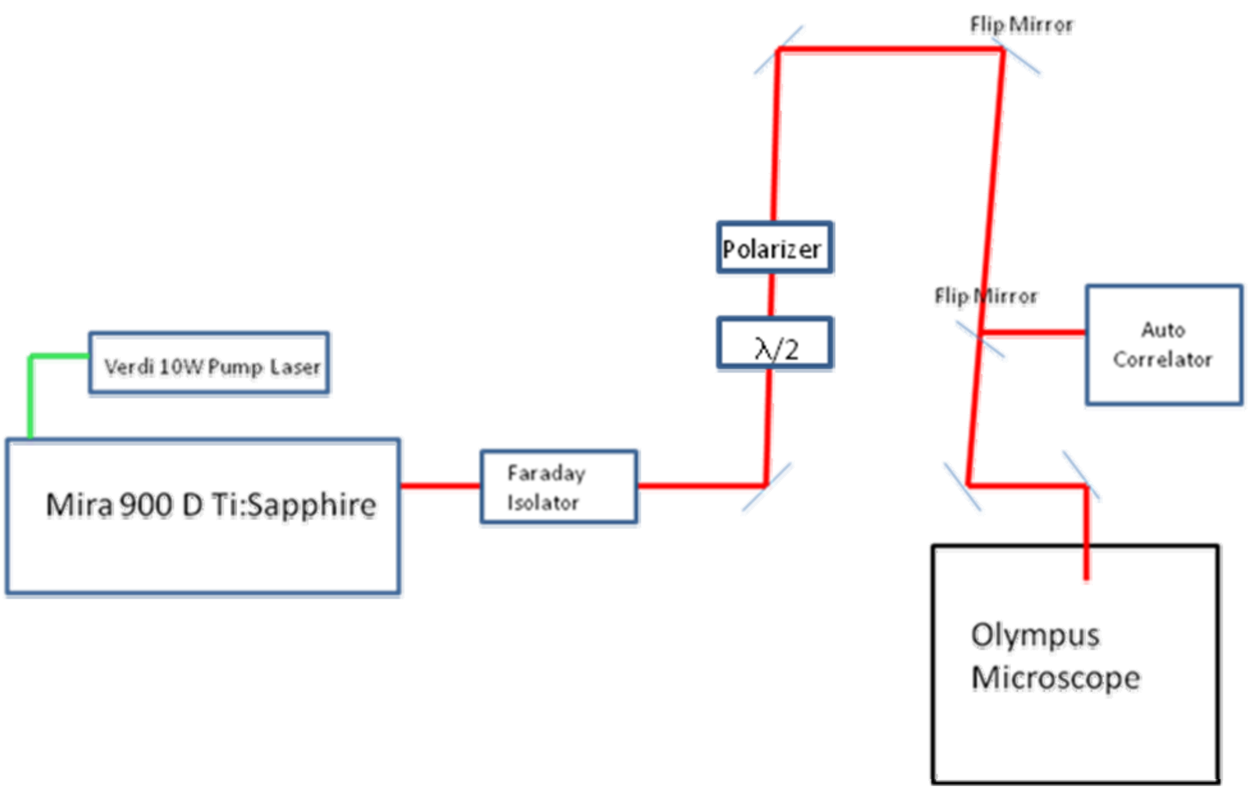

Figure 8: Beam routing of Ti:Sa on the optical table.

Note that the laser beams are usually well aligned. Do not attempt to realign unless you are certain that the beams are off!

6.2. Beam fine adjustment

6.2.1. Make small adjustments to the steering mirrors to route the beam through the irises installed in the beam path. The first mirror is the most likely mirror to have been bumped by another user.

6.3. Pulse length autocorrelation measurements

6.3.1. Move the flip mirror into the beam path to send the laser to the autocorrelator

6.3.2. Autocorrelation or pulse length measurement

6.3.2.1. Direct the beam into the small hole to the left of the window

6.3.2.2. Make sure the beam reflections are centered on the crosses on the autocorrelator alignment window.

6.3.2.3. Use the autocorrelator/spectrometer controller to read the autocorrelation/pulse length result

6.3.2.3.1. Check the setting of alpha (the crystal angle in the autocorrelator). The angle needs to be adjusted for different wavelengths. For example alpha should be set to 340 for 1064nm and to 365 for 816nm using the black buttons labeled "tuning".

6.3.2.3.2. Change the gain with the large black knob to adjust the height of the peak on the screen.

6.3.2.3.3. The "scan range" may need to be adjusted with the black buttons also to get femtosecond resolution.

6.3.2.4. The autocorrelator reading should be in the few hundred femtosecond range

6.3.2.5. Refer to the autocorrelator manual for further details on theory and operation.

7. Replace the dichroic mirror in the scan head

7.1. Put on gloves to prevent accidental fingerprints on the optics

7.2. CAREFULLY, remove the cover to the scan head box

Figure 9 Cover removed from scan head box.

7.3. Locate the dichroic mirror the separates the excitation beam from the signal beam

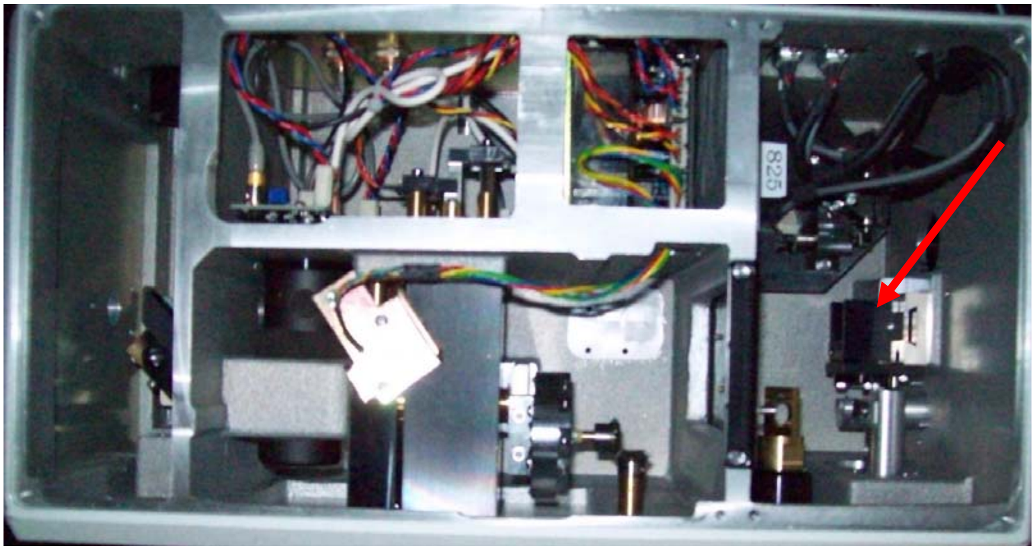

Figure 10 Inside the scan head box. The dichroic mirror that separates the excitation beam from the signal beam is on the right.

7.4. Insert an allen wrench into the hole on the front right of the scan head and loosen the screw holding the dichroic mirror

Figure 11. Gently insert Allen wrench into hole on right side of scan head to loosen the screw that holds the dichroic mirror in place.

7.5. When the screw is loosened, pull the mirror holder straight up.

7.6. Put the visible dichroic mirror in a protective plastic box and put it on the rack of optics. Do NOT leave it on the computer work station as there is a high probability of it accidentally being knocked off the computer stand.

Figure 12 Dichroic mirror for the two photon fluorescence which is kept on the shelf in the rack of optics. The visible dichroic filter should be stored in a protective plastic box on this shelf when it is not in use.

7.7. Install the Semrock FF670 filter. Tighten the screw with the allen wrench

7.8. Replace the cover to the scanhead box.

8. 8 Switch Cables to Multiphoton Detector

8.1. IMPORTANT! Make sure that the power supply to the microscope is OFF!
8.2. Unplug the BNC connectors from the PMTs in the back of the laser scan head
8.3. Plug Channel 2 into the BNC connector for the CARS/Multiphoton signal collection PMT
8.4. Unplug the parallel port connector from the back of the laser scan head and connect it to the control board for the CARS/Multiphoton signal PMT

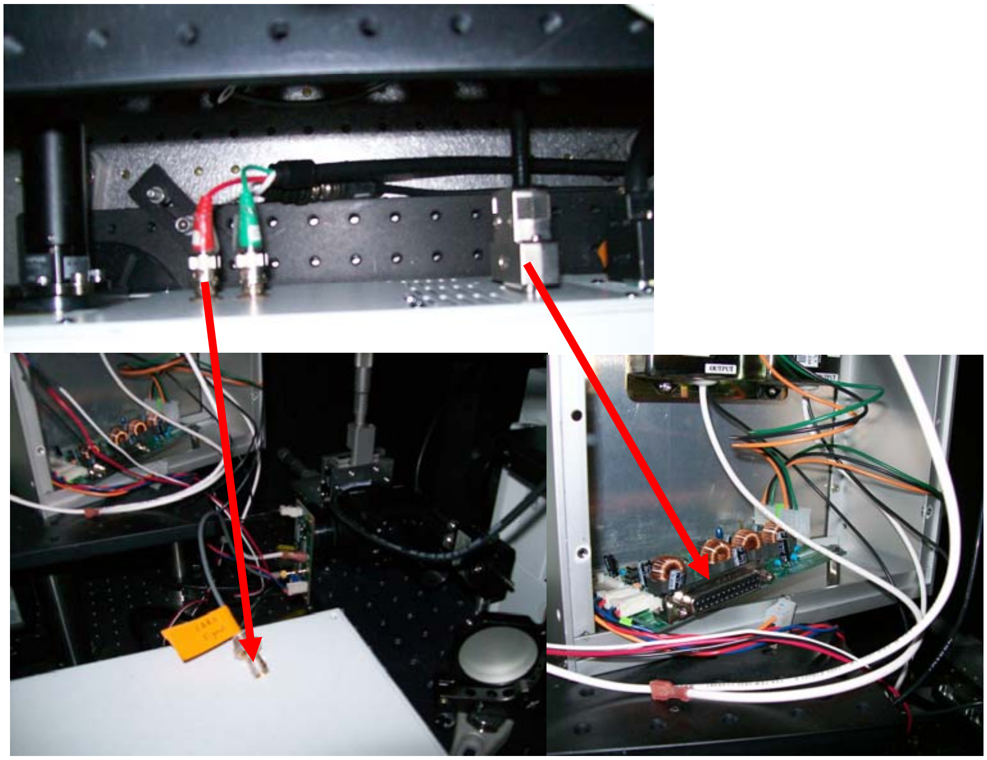

Figure 13 Changing PMT connector connections

8.5. Set the microscope filter wheel to setting 1

8.6. Change filters in the tube in front of the CARS/Multiphoton PMT to appropriate filters for your sample

9. Beam coupling Olympus FV300 Confocal Microscope

9.1. Flip down the mirror that sends the beam to the auto correlator

9.2. With the help of an IR viewing card or an IR viewer, check the presence of the IR laser spot at the sample position.

9.3. Slightly adjust silver steering mirrors just outside the black box to center the spot.

10. Two photon fluorescence imaging

10.1. Load your sample on the microscope stage

10.2. Proceed as you normally would for confocal fluorescence imaging

10.3. Refer to SOP071 Olympus FV300 Confocal Fluorescence Microscope for help in operation of the microscope.

11. Shut Down Procedure

11.1. Press the “Shutter open” button (Figure 5 right) to close the shutter.

11.2. Place the laser beam blocker back in its default position in front of the Ti:Saphire (Figure 4, left).

11.3. On the VERDI controller, turn the key to the “Standby” position (Figure 5 left).

11.4. Select “cw” mode on the Ti:Sa controller (Figure 6 left).

11.5. Switch off the “Laser in use” sign (Figure 3).

11.6. Replace the visible dichroic mirror in the scan head. Reversing the steps in section 7 of this SOP.

11.7. Email your data to yourself. Please use an online file storage service (such as dropbox.com) if your files are too big to e-mail. CNS has had several issues with viruses on USB storage devices taking down instruments.

11.8. Log out of the interlock system

11.9. DO NOT SWITCH OFF the controllers!! Leave them in standby

APPENDIX A - CERTIFICATION: DEMONSTRATION OF SKILLS

Name:	Operation:
Date:	Tested by:

SAFETY

	Pass	Fail
Which laser safety goggles have to be used?	☐	☐
Where is the emergency shutdown button?	☐	☐
Where is the switch for the “laser in use” sign?	☐	☐
How and when must the laser safety curtain be closed?	☐	☐

DEMONSTRATION of SKILLS

Ti:Sa system

	Pass	Fail
Demonstrate correct order of steps for startup	☐	☐
Demonstrate optimizing laser output	☐	☐
Demonstrate tuning the laser wavelength	☐	☐
Demonstrate shutdown	☐	☐
Demonstrate changing the dichroic mirror	☐	☐

Beam routing

	Pass	Fail
Demonstrate ability to check and correct beam routing to microscope	☐	☐
Demonstrate pulse length/autocorrelation measurement and optimization	☐	☐
Demonstrate spectrum measurement	☐	☐
Demonstrate ability to check and correct beam routing to Olympus confocal	☐	☐

VALIDATION

Certification: Pass ☐ Fail ☐

Trainee signature: ______________________________
(Required if pass or fail)

Certified by: ______________________________