

CMI STANDARD OPERATING PROCEDURE

Fluoview 300 laser scanning confocal microscope

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REQUIREMENTS FOR EQUIPMENT USAGE:

1. CMI user
2. Completion and signing of Microscope Safety Checklist
3. Certification by Peter Owens or Kerry Thompson

REVISION LOG

Revision	Date	Author	Changes
1.0	5/3/13	P.Owens	Initial draft

1. Purpose

1.1. This document specifies the work instructions for the CMI Olympus Fluoview 300 Fluorescence Confocal Microscope located in room 105 of Block F in Anatomy. 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 the CMI.

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 CMI personnel for help.

2. Scope

2.1. These work instructions are applicable to **all** work that is carried out using the Olympus FV 300 Fluorescence Confocal Microscope.

3. Applicable Documents

PLEASE LEAVE ALL HARDCOPIES IN THE LAB

3.1. CMI Safety Manual

3.2. MSDS Sheets

3.2.1. Immersion Oil

3.2.2. Isopropanol

3.3. Olympus IX71 manual (hardcopy in the lab, soft copy on the instrument PC)

3.4. Olympus FV300 manual (hardcopy in the lab, soft copy on the instrument PC)

3.5 The CMI access policy , available online at

http://imaging.nuigalway.ie/access%20policy/cmi_access_policy_1.5.pdf

3.6. Before starting, please read the following carefully:

3.6.1. 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 CMI staff for more assistance if required.

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

4. Confocal Microscopy Primer

Although the basic idea behind confocal microscopy was initially proposed by Marvin Minsky at Harvard back in 1957, the technique did not become widespread until the 1990s because of many technological limitations especially with regards to computer power. Over the last 20 years confocal microscopy has exploded from a cutting-edge technique to an important tool in biological research, especially in cell biology and genetics.

From the Olympus Fluoview Resource Center: *“Confocal microscopy offers several advantages over conventional widefield optical microscopy, including the ability to control depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the capability to collect serial optical sections from thick specimens. The basic key to the confocal approach is the use of spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus.”*

The Olympus website has a number of java tutorials that can be quite helpful in understanding the operation of the instrument: <http://www.olympusfluoview.com/java/index.html>.

Optical bright field microscopy has (almost) no optical sectioning capability along the optical z (axial) axis: Considering a 3D volume with constant signal source density, it can be readily seen that every plane in z direction contributes equally to the final signal.

To filter out these light signals from out-of-focus planes, an aperture (‘pinhole’) is introduced in a spot that is confocal with the focus. The open diameter of the pinhole defines the effective thickness of the optical section that contributes now to the signal.

The pinhole is also filtering light from other spots in the xy object plane, so that effectively only one pixel can be imaged at a time. Two and three dimensional images are obtained by either scanning the sample or scanning the focus spot in x, y and z direction.

The CMI Olympus Confocal Microscope is a spot scanning system where the sample remains at a fixed location. Spot scanning is achieved by imposing an angle on the illumination beam before the objective lens focuses the beam down to a spot on the sample (using a series of movable mirrors). Spot scanning, in which the laser is scanned point-by-point over the sample at various speeds from slightly below 1 frame/second (for 512 x 512 pixels) to several seconds per frame (at slower rates over larger areas).

As the pinhole has to stay confocal with the spot at all times, it is either necessary to scan the pinhole synchronously with the spot, or to descanned the back-reflected light before it hits the pinhole. The latter is the scheme used within the Olympus confocal (and most other systems on the market).

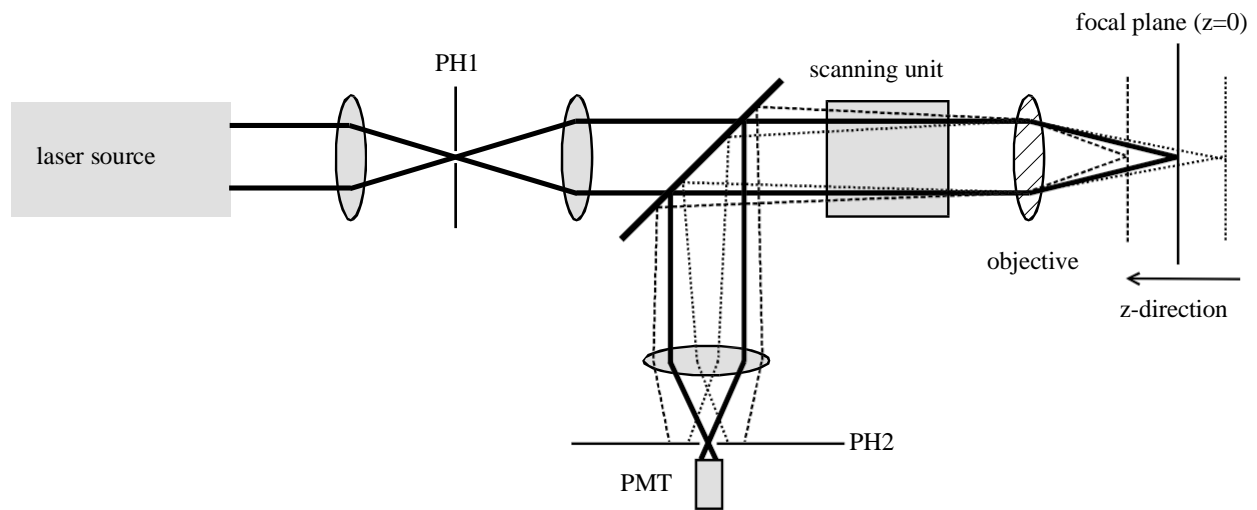


Figure 1: Confocal laser scanning microscope working principle: The pinhole PH2 is confocal with the light spot in the focal plane. Pinhole PH1 is used to generate a truly point-like light source.

For a more detailed description of the principle of confocal microscopy, please refer to one of the many textbooks or articles available either in print or online <http://www.olympusfluoview.com/java/index.html>.

5. Responsibilities

- 5.1. Operators of this equipment are responsible for the following:
 - 5.1.1 Complying with all safety regulations.
 - 5.1.2 Compliance with procedures and specifications contained in this document.
 - 5.1.3 Reporting misuse of the instruments, or in a manner inconsistent with this specification, by any personnel, to the supervising CMI staff.
 - 5.1.4 Maintaining a clean workspace. **Food and drink are NOT allowed!**
 - 5.1.5 Last-minute bioprep work should be done in the cell culture room XXX (observe sign-up rules for that room) and not in the Fluoview room.
 - 5.1.6 Reporting any and all maintenance issues/concerns to the supervising CMI staff member immediately.

6. System Overview

- 6.1 The Olympus FV300 Confocal Microscope consists of the following devices:
 - 6.1.1 Instrument Control PC
 - 6.1.2 Olympus Control Electronics (FV5-PSU)
 - 6.1.3 Epifluorescence illumination lamp (Mercury Lamp, Filter turret used for this)
 - 6.1.4 Olympus IX71 Inverted Microscope with 0.55 NA condenser
 - 6.1.5 FV 300 confocal scanhead and PMT detection units
 - 6.1.6 Several excellent Olympus microscope objectives:
 - 1. UPlanSAPO 10x/0.4 N.A.
 - 2. UPlanSAPO 20x/0.75 N.A.
 - 3. UPlanSAPO 40x/0.90 N.A.
 - 4. UPlanFLN 100x 1.30 NA oil
 - 6.1.7 RGB laser combiner, Argon (488 nm), green He:Ne (543 nm), red HeNe (633 nm).

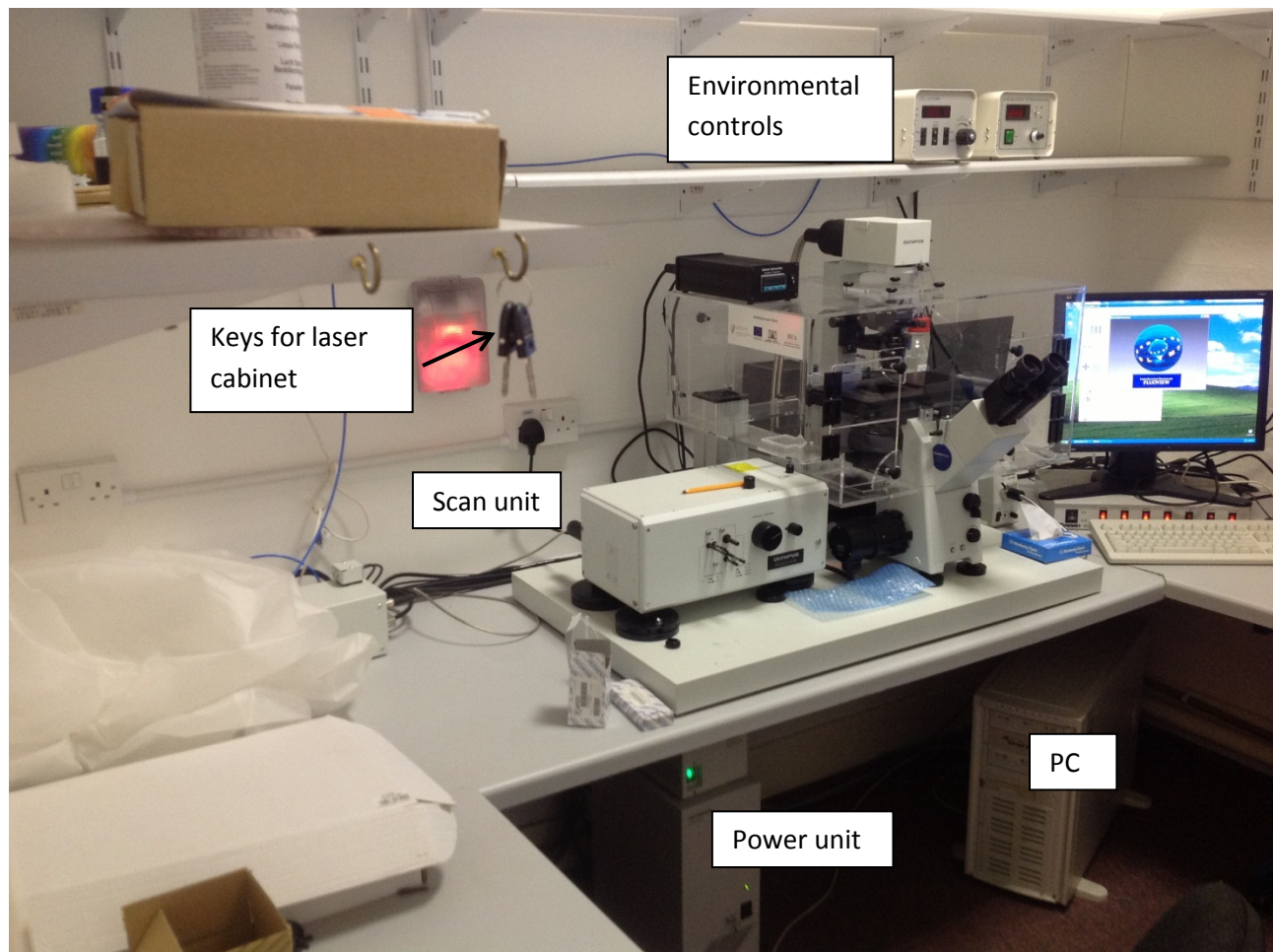


Figure 2 Overview of System Layout

- 6.2. Two confocal imaging and one transmitted light non-descanned detectorS are available. Several confocal pinholes and barrier filters are also available in addition to optics that route both the incident laser light and the confocal fluorescence. Here is a diagram from the Olympus FV300 manual available in lab both in hardcopy and softcopy on the computer.

1-3 Light Path Diagram

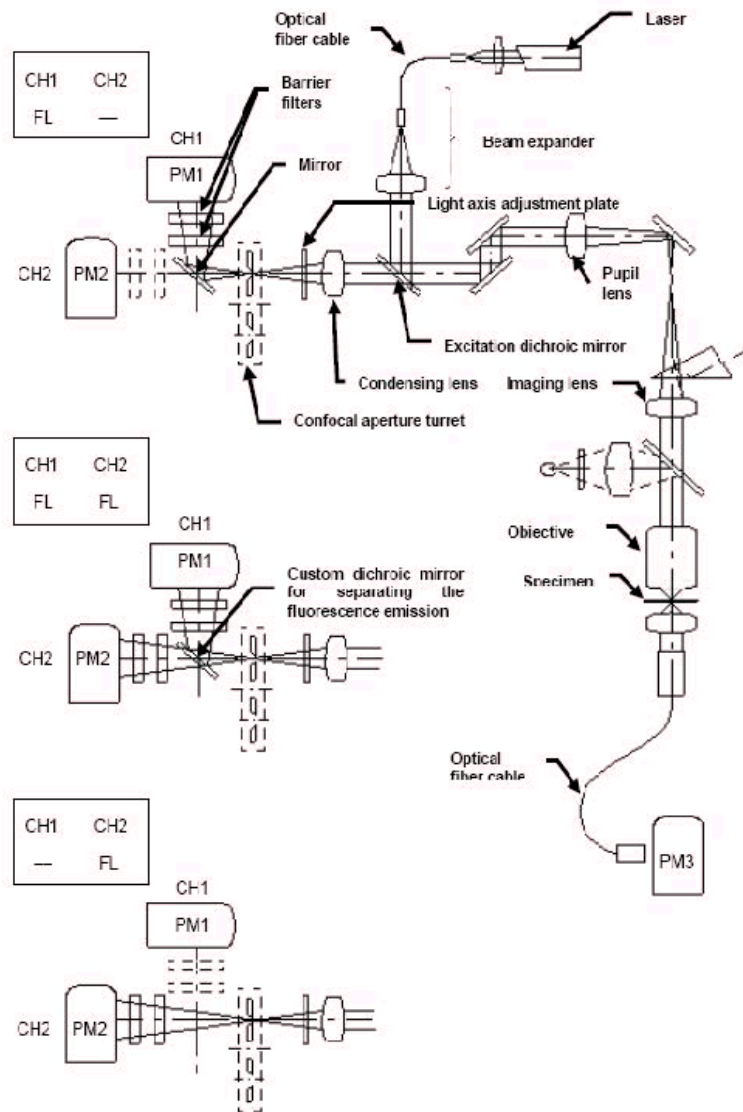


Figure 3: Olympus FV300 Light Path Diagram

6.3. Familiarise yourself with the Layout of the Inverted Microscope:



Figure 4: IX71 Inverted Microscope for use with FV300

- 6.4. Make sure that you use the “camera” position of the light path selector dial when switching from visible inspection using the eyepieces to the laser scanning mode otherwise no image will be formed. The “eye” position prevents laser light from traveling up into the binocular and should be in place whenever you look into the microscope eyepieces.

6.5. Familiarize yourself with the Confocal Scan Unit and its features:

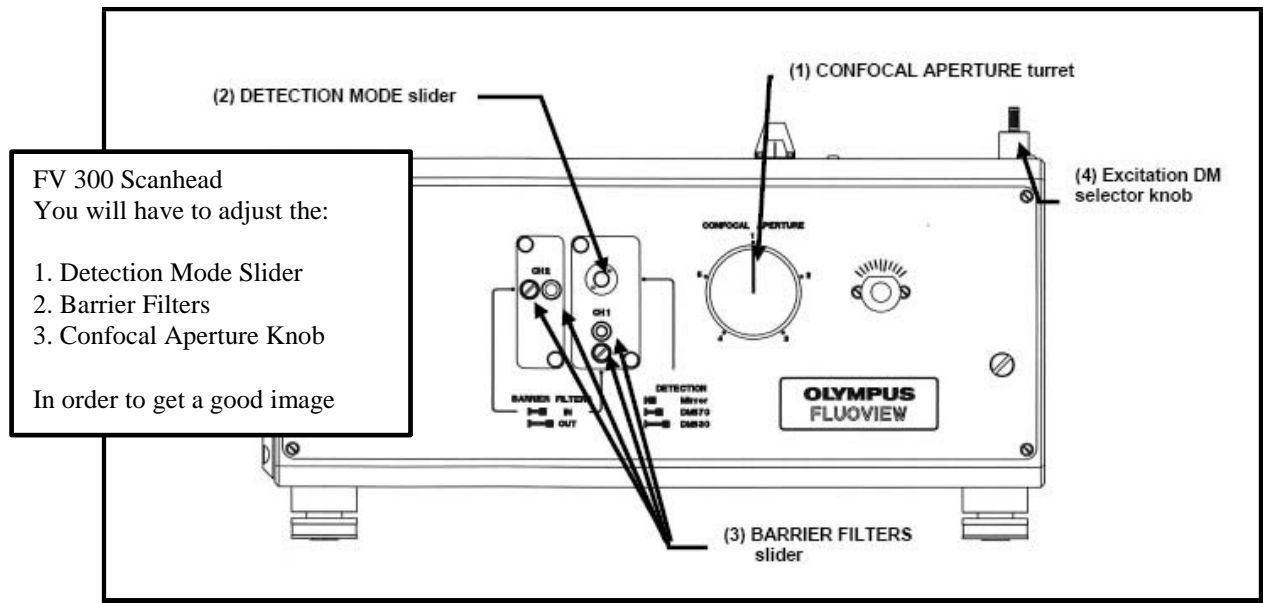
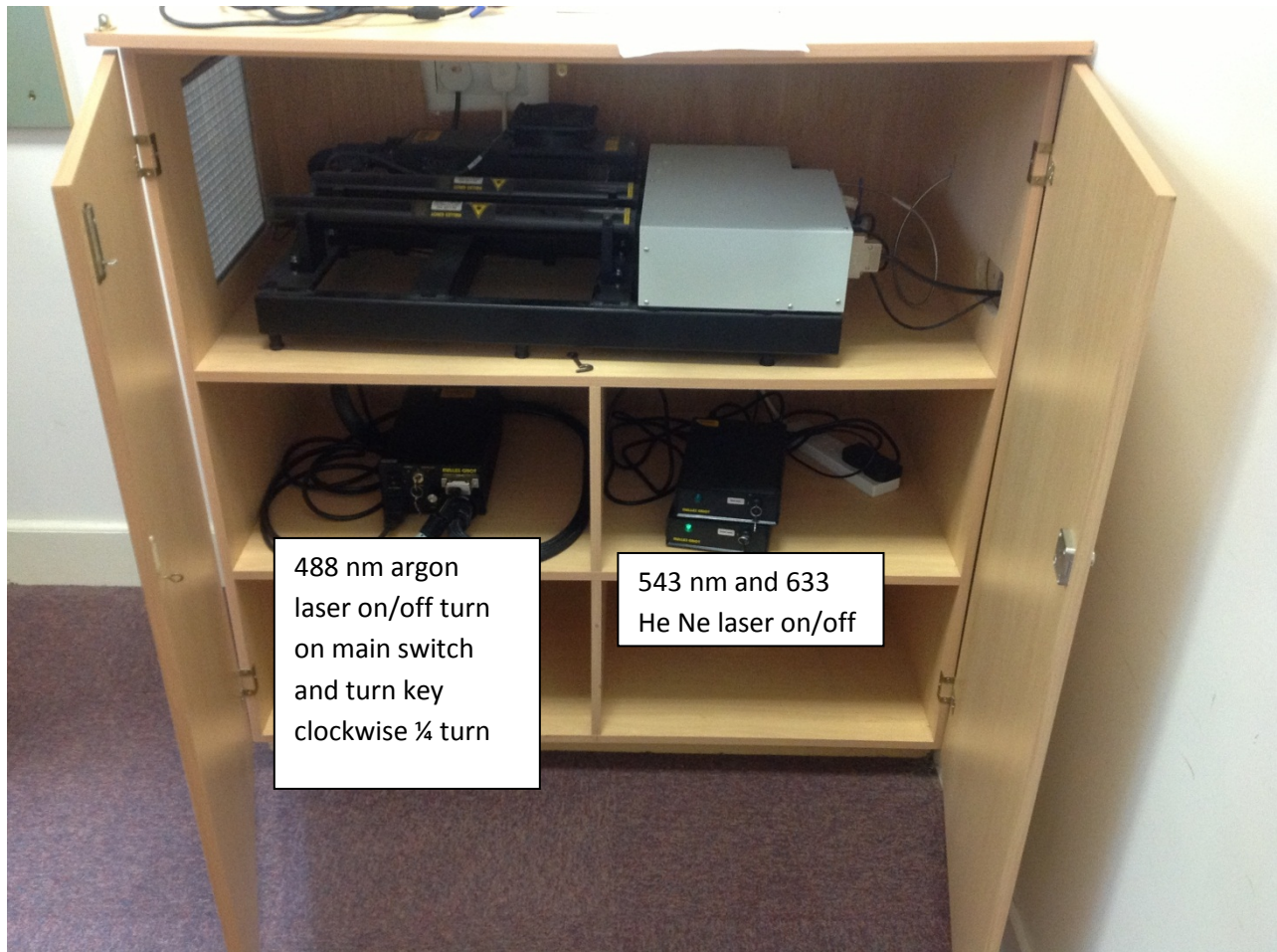


Figure 5: Olympus Fluoview Scanhead Exterior Description

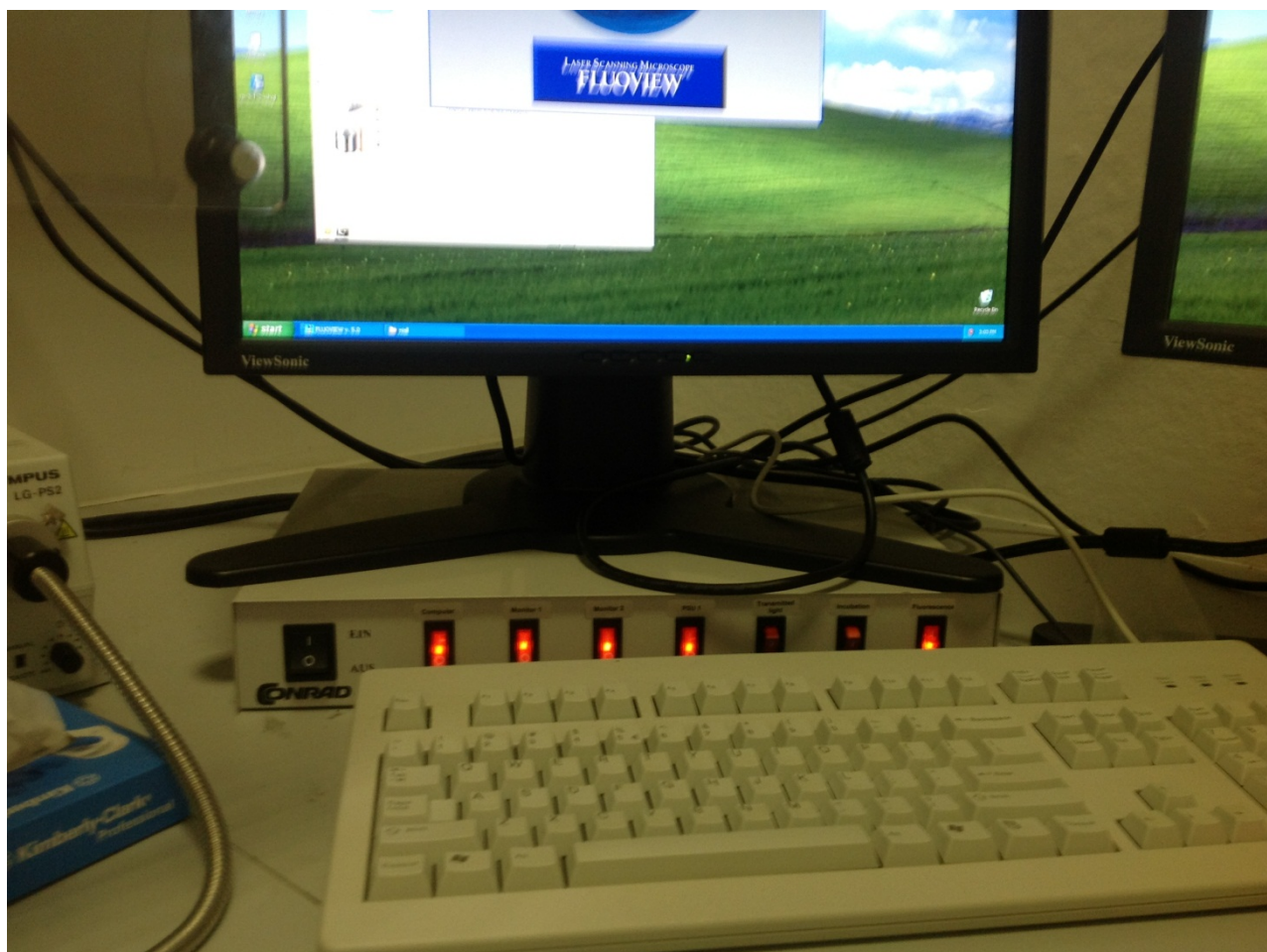
6.6. Initially the CONFOCAL APERTURE Wheel should be set to position “5”, which is the largest confocal pin. After a good image has been obtained, the confocal pinhole can be adjusted to position “1” or “2” to improve the contrast and resolution of your image. Further instruction on the bandpass filter and PMT detector selection rods will be provided.

7.0 Start Up Procedure

- 7.1 Book equipment on CMI web booking system.
- 7.2 Turn on laser light source used for your experiment, get key for laser cabinet from inside room 105. Turn on the lasers by turning the keys on the power supplies located on the shelf above the microscope to start them warming up. Allow ~20 minutes for everything to stabilize.



- 7.3. Turn the microscope unit on using switches on the power control unit. Only turn on the equipment that is needed. Ie if not doing transmitted light imaging such as dic , do not turn on the transmitted light switch.



Wait 2 minutes for the microscope to initialize.

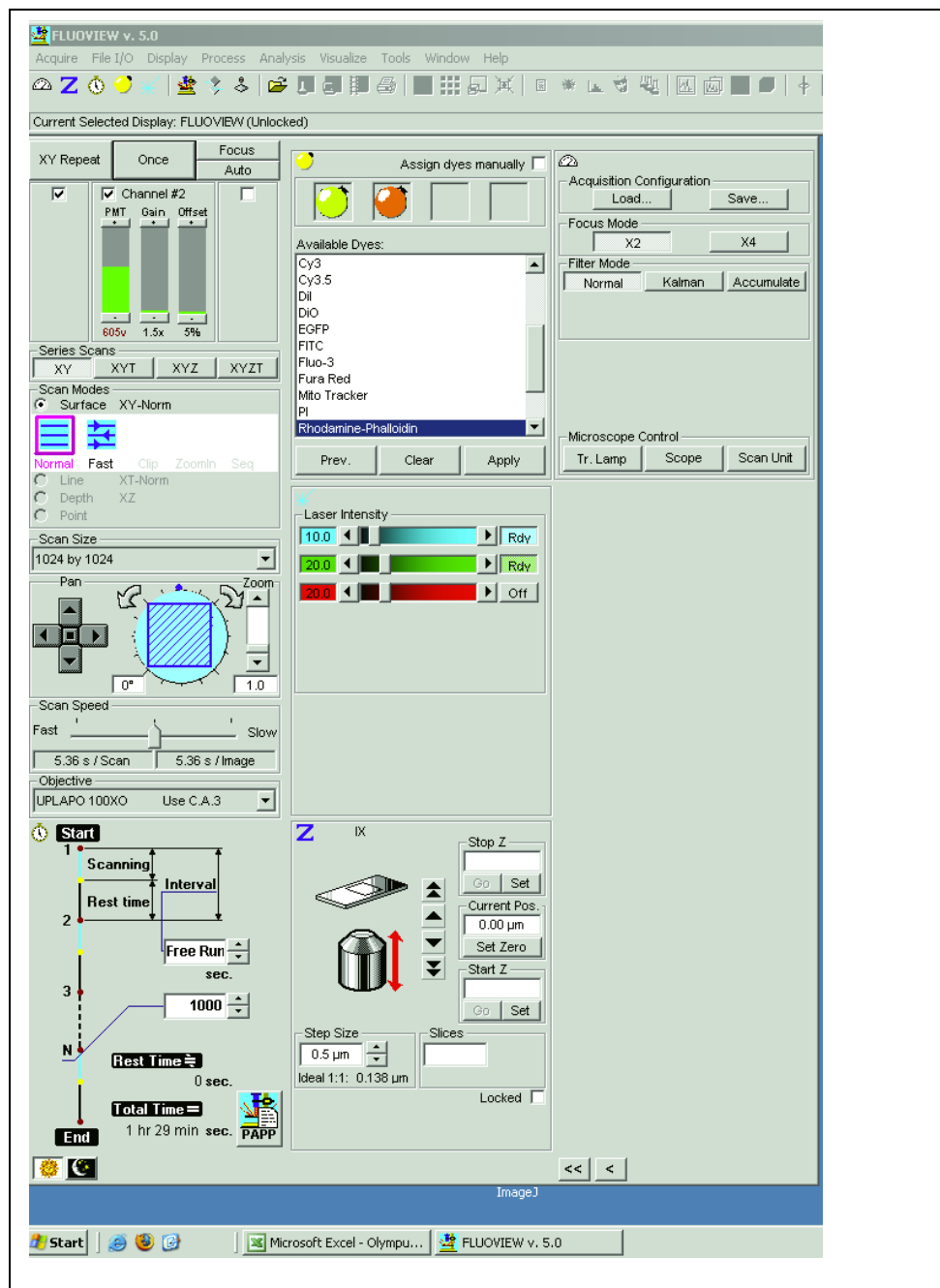
- 7.4. Turn on PC, Start the Fluoview software with a double click on the desktop shortcut titled "Fluoview" on the right side of the computer screen



- 7.5. Load your sample on the microscope. CAUTION: Avoid any hard contact of the objective lens with your sample at any time.
- 7.6. Check that the light path selector dial on the front of the microscope body is in the eye position. Check that the filter wheel below the nose piece is in position 2 or 3 (for FITC , TRITC epi fluorescence observation. Read the number from the right side of the microscope body.
- 7.7. Start with a low power objective and find the region of interest on your sample. The manual XY stage control is to the right of the microscope body.
- 7.8. Use the focus knob which is also on the right side of the microscope body to bring your sample into focus being careful not to crash the objective into the sample.
- 7.9. Rotate the objective turret to switch to a higher power objective if desired. If applicable, put ONE SMALL drop of immersion medium on the microscope objective lens. Remember: Water ONLY on water immersion lenses, oil ONLY on oil immersion lenses. Immersion oil is provided close to the microscope.
- 7.10 When the image is in focus, and correct location is observed, turn the light path selector dial to the camera mode from the eye mode for laser scanning confocal work (position 1 on filter turret).

CAUTION: Avoid wrong immersion contact (oil on water lens, water on oil lens). Do NOT REMOVE objective lenses from the microscope unless approved by CMI personnel. If you accidentally place water on an oil objective lens or vice versa , wipe it off with lens tissue / isopropanol (no kimwipes, THEY ARE TOO HARSH ON THE GLASS!) and notify CMI staff.

- 7.11. Familiarise yourself with the picture on this page. All tools necessary to obtain a confocal image are available on one screen with the Olympus Fluoview software.



7.12. Selecting Channels for Imaging, the acquire menu

- 7.12.1 Detection Channel Window. XY Repeat/ Focus is provided for you above the detection channels in order for you to optimize your focus and other parameters. There are three check boxes for Channel 1, Channel 2, and Channel 3, the transmitted light PMT. The transmitted light PMT is very useful for overlaying a confocal fluorescence image on top of a laser confocal image and can serve as a brightfield/DIC/non-descanned detector channel.

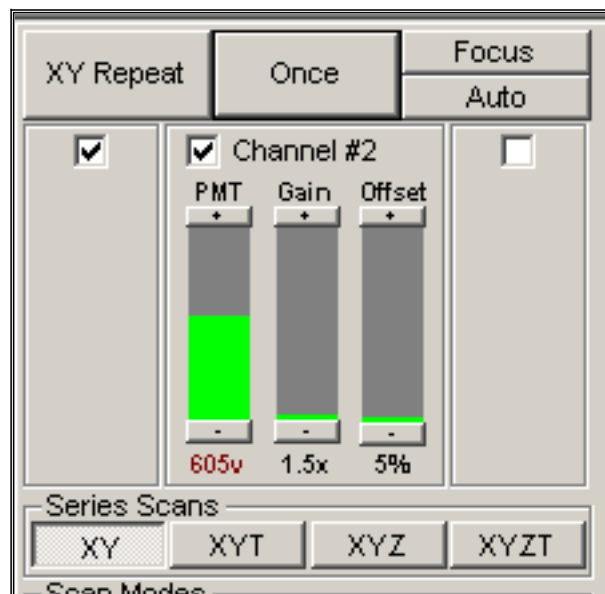


Figure 7: Channel Selection Window

- 7.12.2 Make sure that the PMT settings are always set to low levels initially, ~200 V, then gradually increase the voltage in order to see your image. The PMTs should not be run above 800 V. If you need to run the PMT above 800 V that is indicative of poor sample preparation or improper laser/filter selection. Fluoview will warn you with a yellow and red color change in the volt bar that you are approaching unsafe limits.

7.13. Laser Line Excitation and Attenuation

- 7.13.1 Switch to the Laser Intensity Menu now. Make sure you enable the “Rdy” dial for the laser line you intend to use. Any combination of 488 nm, 543 nm, and 633 nm is available. With the right combination of barrier filters you can excite a wide variety of conventional dyes with these combinations. One word of advice: the 488 nm Ar:Ion is much more powerful than the 543 or 633 nm lines and shouldn’t be run at full power. The slider bar adjusts a neutral density (ND) filter wheel in the laser rack that controls the power throughput through the scanhead.

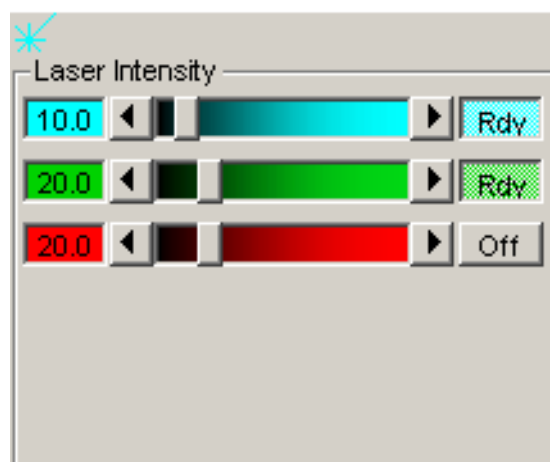


Figure 8: Laser Excitation Control Window

7.14. Confocal acquisition modes

7.14.1. Confocal Scanning Modes in 2 and 3 Dimensions

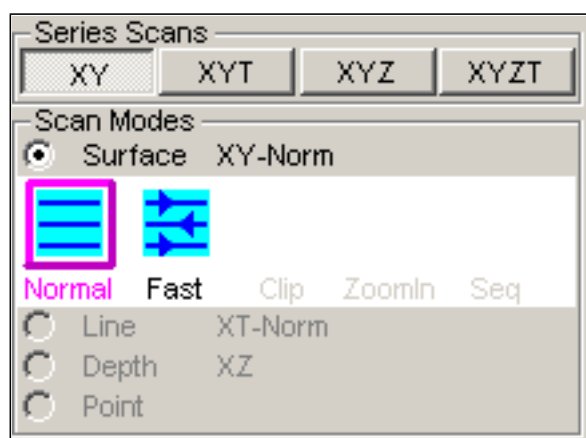


Figure 9: Confocal Configuration Control Window

7.14.2. Standard XY, XYT, XYZ (3-D), and XYZT (3D time) are located all in one place on the Olympus Fluoview software along with the options to do a standard serial point-scanning under Normal (recommended) or a FAST scan (recommended for fast events with strong signal). Line scans and XZ depth scans along with point scans (for bleaching experiments) are also available

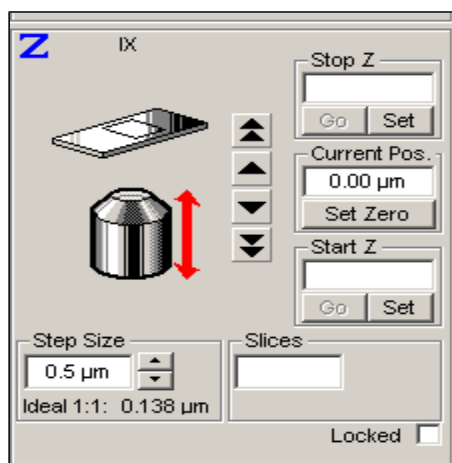


Figure 10: 3D Image Acquisition Mode

7.14.3. The current position you are imaging (with XY) is defined as “Current Pos.” the motorized objective will move down by the amount you set (generally ranges from -200 microns to 200 microns are reasonable). The motorized objective will then move up through the focal plane to a top position defined as “Stop Z.” For example, to image one micron beads over 20 microns when we are currently at the focal plane. Type -10 at “Start Z” and 10 at Stop Z and the objective will move down and record individual XY frames at various z positions, going through the focus and then stopping 10 microns above the focal plane. Thus, find the focal plane and use this feature to take 3D stacks. However, you should observe Nyquist sampling, with a 60x 1.2NA water or 100x 1.4 Oil, the z-resolution is not much better than 1 micron, so a 0.5 micron step size (no smaller than 0.3 micron) is reasonable to prevent oversampling. *Remember to check the “locked” box in the lower right to give Z control to the computer. Never change the focus manually while the “locked” box is checked. Also remember to change to confocal pinhole to an appropriate size to reject light that is out of the focal plane.* A table of appropriate pinhole settings for different objectives is in the Fluoview manual and also suggested by the software in the acquisition window.

7.14.4. Setting up XYT

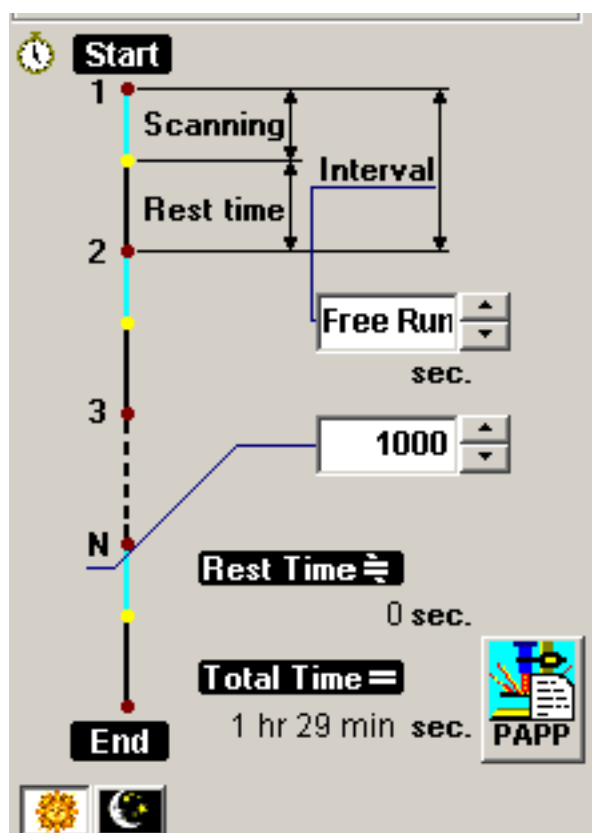


Figure 11: Configuring a time series

7.14.5. The Fluoview software will engage the lasers and then rest and then re-engage the lasers depending on the Interval you set between scans. You could also set Free-Running which allows a continual scan (but increases photobleaching). Fluoview has a maximum memory, as a result, the Free Running mode or short intervals won't permit as many repetitions. At slower intervals you can image for up to a day or longer.

7.15. Filter Selection

7.15.1. In addition to appropriate laser lines, good sample focus, correct confocal pinhole settings, and confocal alignment (done by CMI staff), the last and most important part of an experiment is knowing a) where you sample fluoresces b) picking and choosing the appropriate filters (bandpass and longpass) to image your sample.

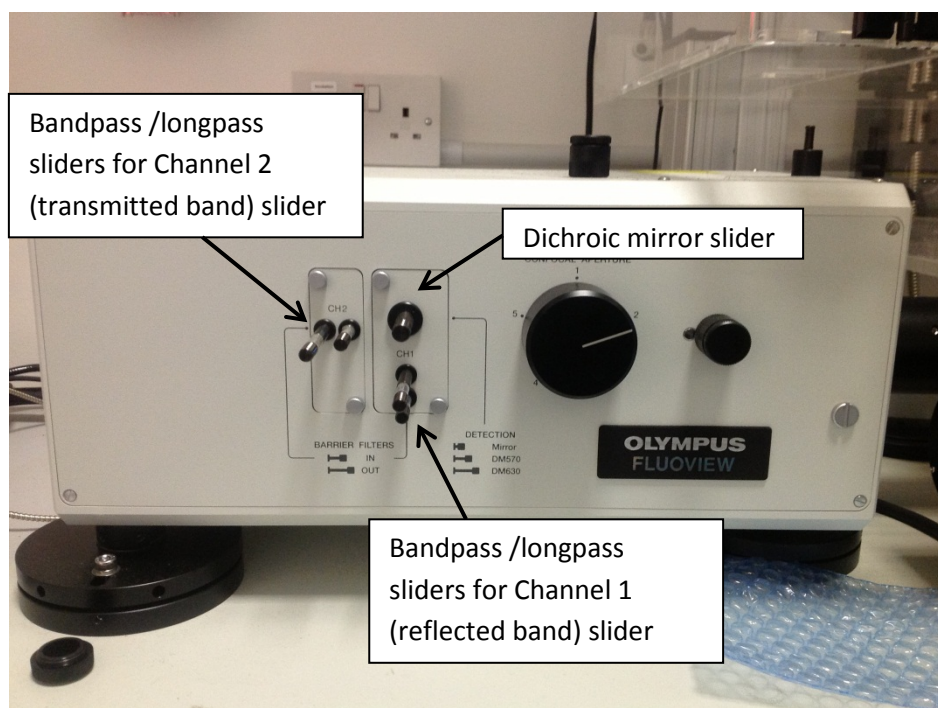


Figure 12: Filter Combinations

The Olympus FV300 provides a simple set of push/pull rods that supply bandpass and long pass filters to modify the emission wavelength range that is going to the detectors. There is also three positions that allow one to steer the beam to the detectors:

- 1: Pushed all the way in directs emission light to Channel 1 detector
- 2: Pushed half way ; splits light at 570 nm, emission below goes to Channel 1, emission above 570 nm goes to channel 2 detector.
- 3: Out all the way ; splits light at 630 nm, emission below goes to Channel 1, emission above 630 nm goes to channel 2 detector.

7.15.3. Typical Filter Configurations:

Alexafluor 488: 488 nm laser, 510 LP, BA 530 nm
 FITC: 488 nm laser, 510 nm LP, BA 530 nm
 TRITC: 543 nm laser, 565 nm LP
 Propidium Iodide: 543 nm laser, 565 IF
 Rhodamine B: 543 nm laser, 565 IF
 TO-PRO3: 633 nm laser, 650 IF filter.

8. Shut Down Procedure

- 8.1. Discard cover slips and glass slides in the red sharps container. This includes BioHazard material.
- 8.2. CLEAN UP the workplace, and leave it better than you found it.
- 8.3 Turn off the 488 nm Argon laser first by turning the key, ¼ turn anticlockwise. The cooling fans will still be on but after a period of approximately 10 minutes the fans will quieten down to low power mode. The system is now sufficiently cooled so you can now turn off the main power switch.
- 8.4. Turn off the He-Ne lasers by turning the keys on the power supplies to off.
- 8.5. Turn off the power switches below the pc monitor (except pc power)
- 8.6. Close the Fluoview software
- 8.7. Shut down the PC.
- 8.8. Sign out on the log-book.

9. APPENDIX A - CERTIFICATION: DEMONSTRATION OF SKILLS

Name:	Operation:
Date:	Tested by:

DEMONSTRATION of SKILLS

	Pass	Fail
Demonstrate software start up and configuration	<input type="checkbox"/>	<input type="checkbox"/>
Demonstrate loading a sample on the tool	<input type="checkbox"/>	<input type="checkbox"/>
Demonstrate finding the sample and taking images	<input type="checkbox"/>	<input type="checkbox"/>
Demonstrate changing between VIS and LSM mode	<input type="checkbox"/>	<input type="checkbox"/>
Demonstrate changing the lens during the measurement session	<input type="checkbox"/>	<input type="checkbox"/>
Demonstrate finishing and cleaning up	<input type="checkbox"/>	<input type="checkbox"/>

SAFETY

Where are cover slips, glass slides discarded?	<input type="checkbox"/>	<input type="checkbox"/>
Where are biological samples discarded?	<input type="checkbox"/>	<input type="checkbox"/>
Which chemicals used require careful handling?	<input type="checkbox"/>	<input type="checkbox"/>

VALIDATION

Certification: Pass ☐ Fail ☐

Trainee signature:

Certified by :

Date: