Biomedical Imaging Unit

SP8 Confocal System

Location: LB60A

Basic User Guide for Authorised / Experienced Users.

Any problems, please find Dave Johnston and let him know

Startup Procedure

(1) Switch on the 2 wall sockets labelled *SP8* and the wall socket labelled *HPX lamp.*

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(2) Make sure that there is nothing in the way on the microscope stage.

Switch on the 3 green switches on the main console (under RHS of desk) - the order of switch on isn't important but do them one at a time to prevent a power surge.
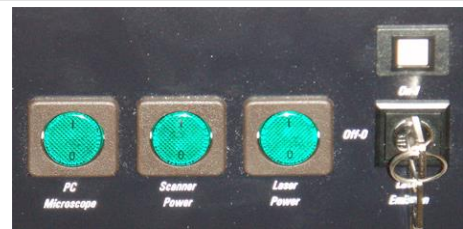
Turn the laser key 90 degrees clockwise to start the lasers (the square indicator above the key will light up yellow).

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(3) The microscope control box (under the microscope table) should permanently be left on (green status light above switch on box front is illuminated) and therefore it powers up when the PC-Microscope switch on the console is switched on.

The information panel on the microscope stand will illuminate and display a start-up message.

The microscope stage will initialise.

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(4) With the intensity dial on the HPX short arc mercury reflector lamp starter box set to **minimum** (fully anticlockwise), switch on its power switch (illuminates green).

The intensity dial should then be set 3 clicks clockwise to position 4 for normal use (dial positions = 0 / 12.5 / 25 / 50 / 100 % of maximum intensity).

The Shutter button (bottom left) should be in the out (remote) position.

The lamp is ready to use immediately the arc strikes but takes a few minutes to stabilise fully and
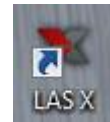
warm up to maximum intensity.

(5) Switch on the PC and log onto the local machine as user TCS-user with password "*SP8*" (without the quotes).

Turn on the keyboard illumination (LHS of middle 4 silver buttons on top of keyboard – (button cycles illumination; on-blue, on red, on violet, off)

(6) Start the LAS software using the icon at top right of the monitor screen.

(7) On the start up screen, check that the configuration is set to *machine.xlhw*, that the microscope is set to *DMI8* and that customised user settings is *off*.

> **note**: if you want to use the software to simply analyse previously captured data, rather than to acquire new data, you can use the *Simulator SP8* configuration mode as it will load up faster.
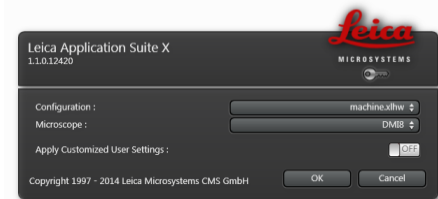
Click on *OK* to continue start up.

As the microscope and system components initialise, the text in the start up screen will update (it takes a couple of minutes to complete).

(8) Initialise the stage when prompted to do so (if you do not initialise the stage, tile scan and mark-re-find functions will be disabled for the session).
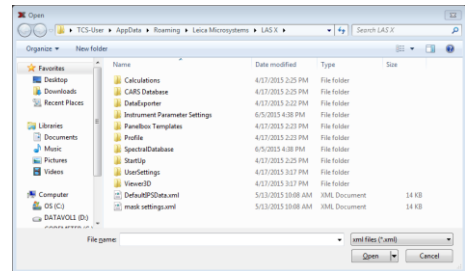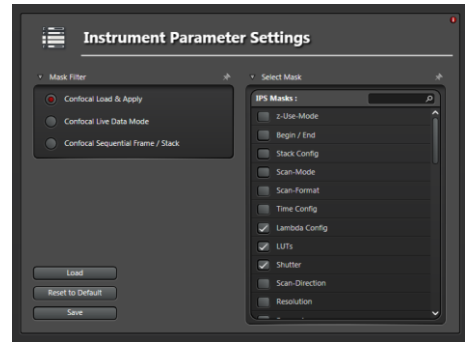

(9) Once initialisation is complete you will see the LAS user interface across the screen.

(10) Configuration tab, *IPS* icon:

Click on *Load* button, a *file open* window appears, pointing to the correct directory.
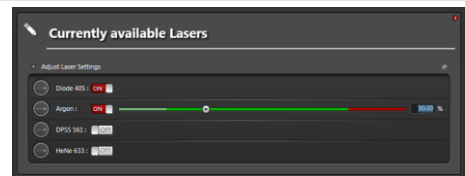
Select *mask settings.xml* and click on *Open*.

(11) Configuration tab, *USB Panel* icon:

In the Load/Save control panel setting dropdown, select *User Settings – BIU*.

(12) Configuration tab, *Laser Config* icon:

Turn on the lasers needed for your session – if you select the Argon laser, move the slider to 25-30% power setting.

(13) *Acquire* tab – *Acquisition* sub tab – *XY* section

Default Settings:
    Format: 1024 x 1024 *¹
    Speed: 600 *²
    Zoom:0.75 *³
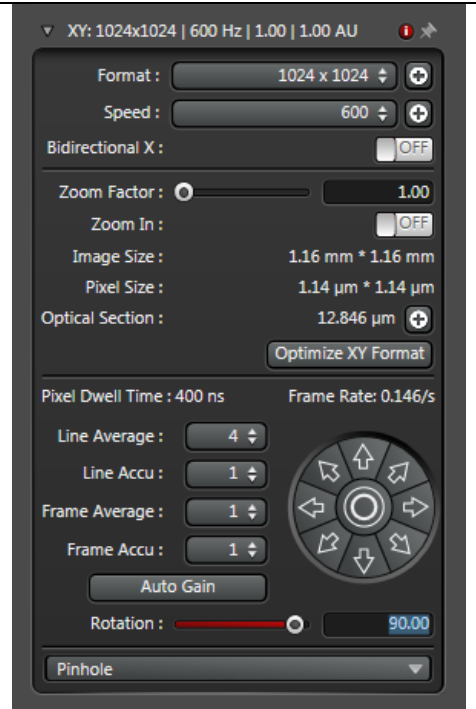   Line Average; 4
    Rotation 90
        (Recommended values for routine imaging)

*¹ For deep, system optimised, Z series, it may be worth using 800 x 800 as this is faster for not a lot of lost resolution in X and Y. To set this value click on the + button on RHS of *Format* line and type in the values.

*² Speeds faster than 600 force a zoom factor.

*³ Default zoom on startup is 1. Reset to 0.75 as zoom 0.75 on SP8 gives the same size field of view as zoom 1 on SP5 .

XY: 1024x1024 | 600 Hz | 1.00 | 1.00 AU	
Format :	1024 x 1024
Speed :	600
Bidirectional X :	OFF
Zoom Factor :	1.00
Zoom In :	OFF
Image Size :	1.16 mm * 1.16 mm
Pixel Size :	1.14 µm * 1.14 µm
Optical Section :	12.846 µm
	Optimize XY Format
Pixel Dwell Time : 400 ns	Frame Rate: 0.146/s
Line Average :	4
Line Accu :	1
Frame Average :	1
Frame Accu :	1
	Auto Gain
Rotation :	90.00
Pinhole	

You are now ready to configure the microscope for image acquisition.

Shutdown Procedure

note: once switched on for the day, the system is normally left running and is only shut down at the end of the day.

Remove your sample and use lens tissue to wipe away any immersion fluid from the objectives and that has dripped down onto the nosepiece.

Leave the nosepiece with the x10 objective in position.

Close your experiment file(s).

Copy your data to a portable hard drive or across the network. There is a shortcut to Research Temporary on the desktop, else you can double click on the BIU filestore icon under *Network Location* on explorer's *Computer* window
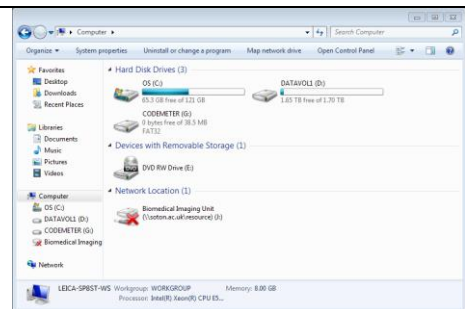
You will need to login as:
      User: *soton\your_UoS_username*
      Password: *your UoS password*
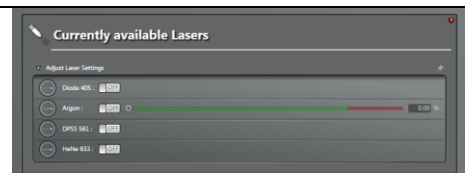
If you need to remap the drive, its full address is:

\\soton.ac.uk\resource\Biomedical Imaging Unit

If you are not the last user of the day.
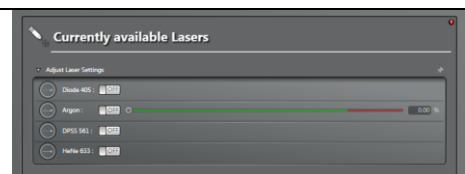
Go to the *Configuration > Laser Config* tab and turn off all of the lasers.

Leave the rest of the system running.

If you are the last user of the day and you have been asked to shut down the system.

Go to the *Configuration > Laser Config* tab and turn off all of the lasers.

Shut down the software – it will run a few tests on shutdown.

Shut down the PC

Turn the laser emission key on the console 90º to the left. (*Off* position)

Turn off the HPX lamp (power switch on lamp box).

Wait until you hear the laser cooling fan shut down (several minutes), then turn off the 3 console switches.

Turn off the wall switches labelled *SP8* (x2) and *HPX lamp*.

Turn off any desk lamps, the radio etc., both at the units and at the wall switches. Leave the router wall switch on if anyone is using the lightsheet system.

Turn off the room lights if you are the last to leave.

Basic Instructions for Imaging Setup

In LAS-X Software

(1) *Configuration* tab – *Laser Config* sub tab:
     Activate any lasers required for session, set argon laser to 25-30% for routine use

(2) *Configuration* tab – *IPS* sub tab:
     Load *mask settings.xml* file

(3) Configuration tab – *USB pane*l sub tab
     Load *BIU* setting

(4) Acquire tab – Acquisition, sub tab:
- Set Format = 1024 x 1024
- Set Speed = 600 Hz
- Set Line Average = 4
- Set Zoom = 0.75   **note**: default zoom setting at startup = 1.0 – but zoom 0.75 gives equivalent field of view to SP5 at zoom 1.0
- Set Rotation = 90º

(5) Acquire tab – Beam Path Settings:

Set up from Scratch
- Assign fluorophores to PMTs, make those PMTs active and assign false colour LUTs
- Activate UV and Visible laser AOBS as needed.
- Power up the individual laser lines required (25-30% starting value)
- Adjust PMT sliders on spectrum to detect peak fluorescence and avoid laser lines and spectral overlap
- Activate transmitted light detector if required and select desired image contrast mode

Or

Set up from a previous Experiment
- Open a previous experiment file, select a data set, right click on it and select *Properties* in the dropdown
- Click on *Apply Settings* in the *Properties* window, wait a few seconds for the system to respond, then close the *Properties* window.

Or

Set up using the *Dye Assistant*
- Open the *Dye Assistan*t window
- Add the fluorophores for your sample to the *Dye Assistant*
- Inspect the various options offered and select the one you wish to use.
- The system will set up automatically. **Note**: you will still need to set the laser powers in the AOTF (they default to 1%) and the gains and offsets.

(6) In LAS-AF Software: start *Live* image acquisition

For each PMT / channel in turn:
     Click on its image space on RHS of monitor
     Set smart offset to -1% using control panel
     Turn gain up until image appears

Adjust gain for maximum dynamic range using the over and under exposure LUT (a few pixels of blue where signal is brightest, background to green).

If you have a negative control / secondary antibody only control slide, image this first and set the gain to the highest value for which you still see nothing in the image – capture a blank image and rename it, including the PMT voltage and objective in the image name (e.g. FITC control x20 75v). Repeat for each controlled channel. For any subsequent images, do not set the gain to a higher value than this else you will record non-specific background. If you change objective or laser power, you will need to repeat the control. If you don't have a secondary only control – WHY NOT!

Turn each laser off in turn by setting its AOBS slider to 0% whilst watching the image channel s on the RHS of the monitor. If the channel for the fluorophore excited by that laser does not become blank, you have problems of spectral bleed through of other fluorophores into that channel. If the **pattern** seen in any other channel changes, you have problems of bleed through of that fluorophore into other channels (note: the **brightness** of images in other channels may reduce as the laser being switched off may contribute to excitation of those fluorophores). If you see spectral bleed through you will need to eliminate it by adjusting relative laser powers, gains and spectral detection windows or by using sequential imaging.

(7) Focus up and down through sample and set upper and lower limits of the required Z stack In *Z stack* window. Limits are set / re-set when the beginning and end boxes are red, unlocked by selecting the delete icon (trash can).

(8) Stop live imaging and set number of Z slices or Z slice spacing required.

(9) Start image series capture

(10) At end of image series capture, rename image stack in *Experiment* tab and save experiment file (save file in *D:\User Data\your folder*).

Key operational differences between SP8 and SP5

Apart from the obvious look / theme / skin of the software running on the 2 systems (LAS-AF on SP5, LAS-X on SP8), the vast majority of the functionality and location of features is common to them both. There are, however, a few exceptions which are detailed below

Hardware

No x63 oil objective on SP8 (can be moved from SP5 if really needed)
No 594 nm laser on SP8 – use 561 nm laser
No resonant scanner on SP8
X63 glycerol lens has separate correcting collar settings for room temperature and 37°C imaging
Y axis on smartmove controller moves in the opposite direction (very annoying!).

Software (1) Config tab:
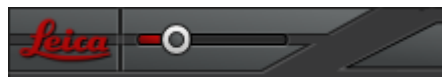The individual configuration icons have subtly different names, e.g.:

<p style="text-align:center">SP5 Lasers = SP8 Laser Config
SP5 *** = SP8 USB Panel
SP5 IPS Masks = SP8 IPS</p>

Software (2) Acquire Tab:

Settings are selected / on / locked when red.

You can increase or decrease the size of the LHS settings boxes using the small slider at very top left of the screen.

To select options that are not on the default dropdown menus, instead of selecting the *More* option in the dropdown, use the + button at the RHS of the dropdown.

Scan speed 600 Hz (fastest rate for full field of view scanning) is now on the default dropdown list on SP8.

Zoom 0.75 on SP8 gives the same field of view as zoom 1.0 on SP5 (**note**: the software defaults to zoom 1.0 on startup, so you need to remember to reset it to 0.75).

Z stack settings: you need to set the start point before the stop point and to delete both points to define a new stack. You don't have to unlock and relock values, just click on the *Begin* and *End* buttons to set / reset. The schematic for the Z stack isn't really of any use unless you drag its slider to the RHS before collecting each stack (it resets when you delete stack settings).

Brightfield detector mode (brightfield, phase, DIC, polarising etc.) is set using the *Fluo Turret* dropdown above the spectrum, rather than on a dropdown at RHS of the detector's on/off button.
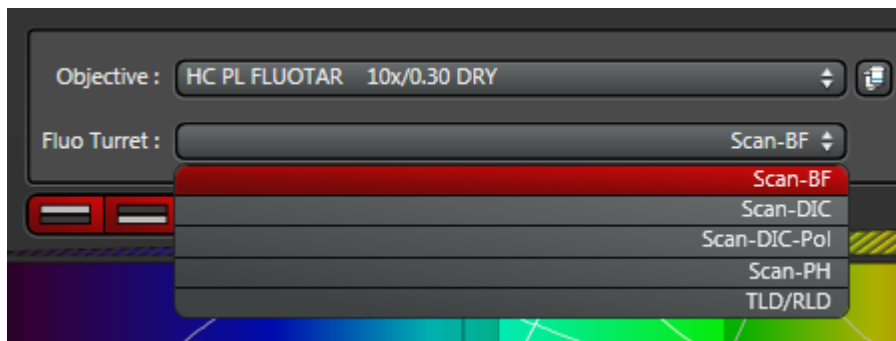
When you apply a set of properties from a previous image set, the dye curves / names are not restored unless you it is a sequential mode set (very annoying – and reported as a bug!). You also have to open the properties of a previous image in order to apply settings, there is no *Apply* icon at the bottom of the *Acquisition* sub tab.

If you use *Z-compensation*, the *Z-compensation* window looks different as it plots a graph of the compensation curve for each channel, rather than just listing the points.

Software – Dye Assistant

This is a very useful feature of the new software to help you set up imaging for a new experiment / dye combination from scratch.

Click on the *Dye Assistant* icon (RHS of the 3 icons above the spectrum) to open the *Dye Assistant* window.

Click on the … button to the RHS of the *Please select a dye* >>> command and select a dye from the dyes database.

Repeat for each dye in the experiment.

The Dye Assistant window will generate a series of different imaging modes for the dye combination, showing how much of the emission profile of each dye will be captured (*Yield*) and the potential for spectral bleed through due to overlapping spectra (Crosstalk).

Evaluate the different options and decide on the one which you want to use. Click on the *Apply* button to the RHS of the desired option and the system will set itself up for these default settings. **Note**: it will turn on the AOTFs and lasers but only set the laser power to 1%

so you will need to increase the power manually. You will also need to adjust gains and offsets as normal.

If you want to tweak the suggested setup and see the effects of the adjustments, click on the *Edit…* button beside the desired option. A new window will open in which you can adjust the slider positions and see how this affects the crosstalk. Then apply the edited settings using the *Apply* button on this window.