
HOW TO USE THE CONFOCAL MICROSCOPES

1) UPRIGHT CONFOCAL MICROSCOPE

a) SETTING UP THE MICROSCOPE

- The system and PC should be left on at all times. It should only be necessary to turn on the two monitors using the switches on the screens. However, if the system has undergone a shutdown you will first need to turn on the two switches on the side of the laser box (PC ON and System ON)
- Turn on the mercury lamp (if you need it). The lamp controller unit is marked HBO 100 and is below the air table with the confocal on.
N.B Once the lamp is on it must be left on for at least 30 minutes before switching off. After switching off it cannot be switched on again for at least 30 minutes.
- Turn on the PC and open the "LSM 510Meta" software
- When the program has loaded choose "Scan New Images" and click "Start Expert Mode"
- The program will load and a main menu bar come up on the screen.
- Place the required objective into the microscope. BE VERY CAREFUL – THEY ARE VERY EXPENSIVE. Make sure you have recorded in the software which objectives you are using. To do this, on the main menu bar click the "Maintain" button and then "Objective". Click one of the objective positions and double click on the correct objective from the list. The information in the box should change, and then close the window.
- Go to main menu, click "Acquire" and select "laser". To turn on the Argon laser click the "Standby" button and then when possible then click "On", at the bottom of that box there is an output %. When the software opens, it starts at 25% and you should move this up to set the tube current to 6.1A. If this requires > 50% the software will warn you. You should not go beyond 50%. The other lasers can just be switched on by clicking "On".

N.B Only turn on the lasers you will need as this helps to extend the lifetime of the lasers.

Use the following:-

Argon 458, 477, 488, 514 nm - mainly for green staining e.g. FITC, SYTO 9

He/Ne 543 – for red staining – e.g. Texas Red, PI and rhodamine

He/Ne 633 – for far-red stains

N.B The laser lifetime is reduced by repeatedly switching on and off. If there is 2 hours or less between the end of your booking and the start of the next, please leave any lasers you have used on at the end of your session and allow the next user to switch them off.

- Place your sample under the microscope and move the correct objective over it. If you are using an oil dipping objective remember to add a drop of oil. Once the objective is in place it can be carefully lowered using the lever.

b) VIEWING WITH THE MERCURY LAMP

- To see the fluorescent labels using the mercury lamp first ensure that the LSM software is switched on as described above. Click the VIS button on the left side of the microscope and set the correct filter for your label. Under the eyepiece is a grey wheel which controls the filter. The current filter is shown by the number above the laser warning triangle. Use
 - 1 = for obtaining the confocal image
 - 2 = blue e.g. DAPI
 - 3 = green e.g. FITC/SYTO 9
 - 4 = red e.g. TRITC/RHOD/Texas Red

5 = to use as a 'normal' microscope with a halogen light source

- Pull out the black slide handle on the right of the microscope and lift the black shutter between the lamp and the microscope on the left hand side. Use the control pad to the right of the microscope to focus the microscope by moving the objectives up and down with the up and down arrows. Choose "speed" or "slow" buttons to switch between coarse and fine focus. Once you have selected an area you wish to image close the black slide handle and drop the shutter to block off the mercury lamp, as long exposure to the light can bleach your samples, and switch the filter back to 1

c) VIEWING WITH HALOGEN LIGHT

- If you prefer, you can view your sample using the halogen bulb. This will allow you to see a normal microscope image of your sample but will not show fluorescence.
- To do this, turn on the halogen bulb (control box on table marked SNT 12V 100W) and use the knob to increase the light intensity. You can view your sample using the VIS button and filter set 5. You then focus the microscope as described above. Once you have chosen an area of interest turn the lamp intensity back down and switch the filter back to 1.

2) INVERTED CONFOCAL MICROSCOPE

a) SETTING UP THE MICROSCOPE

- The system and PC should be left on at all times. It should only be necessary to turn on the two monitors using the switches on the screens. However, if the system has undergone a shutdown you will first need to turn on the single switch on the desk next to the inverted microscope. This will switch power on to the PC, lasers and confocal.
- Turn on the mercury lamp if you need it. The lamp controller is located behind the table with the PC and marked ebq 100.
N.B Once the lamp is on it must be left on for at least 30 minutes before switching off. After switching off it cannot be switched on again for at least 30 minutes.
- Turn on the computer and select the "FCS LSM 510Meta" program
- When the program has loaded choose "Scan New Images" and click "Start Expert Mode"
- The program will load and a main menu bar come up on the screen.
- Select the appropriate objective by pressing the left and right "objective" arrows on the right hand side of the microscope. This will rotate the turret and move through the objectives. The current objective is indicated on the display on the microscope.
- If you wish to change an objective for one not currently in the turret you can remove and replace one CAREFULLY. You then need to indicate this to the software. On the main menu bar click on "Maintain" and then "Objective". Select the objective you have removed and from the "potential objectives folder" double click on the new objective. Then close the window.
- Go to main menu, click "Acquire" and select "laser". To turn on the Argon laser click the "Standby" button and then when possible then click "On". N.B The argon laser then needs about 30 minutes to warm up on this microscope before you should attempt to use it. Once the laser is warmed up you will be able to increase the current output. At the bottom of that box there is an output %. When the software opens, it starts at 25% and you should move this up to set the tube current to 6.1A. If this requires > 50% the software will warn you. You should not go beyond 50%. The other lasers can just be switched on by clicking "On".

N.B Only turn on the lasers you will need as this helps to extend the lifetime of the lasers.

Use the following:-

Argon 458, 477, 488, 514 nm - mainly for green staining e.g. FITC, SYTO 9

He/Ne 543 – for red staining – e.g. Texas Red, PI and rhodamine

He/Ne 633 – for far-red stains

N.B The laser lifetime is reduced by repeatedly switching on and off. If there is 2 hours or less between the end of your booking and the start of the next, please leave any lasers you have used on at the end of your session and allow the next user to switch them off.

- Place your sample on the microscope (coverslip side down) and move the correct objective under it. If you are using an oil dipping objective remember to add a drop of oil.

a) VIEWING WITH THE MERCURY LAMP

- To view an area of fluorescence using the mercury lamp click the VIS button on the LSM510 software on the PC – the button on the side of this microscope does not work. Set the correct filter for your stain. This is done by using the left and right “reflector” arrows on the right of the microscope to change the filter set. The one in use currently is shown by the display at the top of the microscope. The settings are numbered from 1 to 5. Use

1 = for obtaining the confocal image


2 = blue e.g. DAPI

3 = green e.g. FITC/SYTO 9


4 = red e.g. TRITC/RHOD/Texas Red


5 = to use as a ‘normal’ microscope with a halogen light source

- Allow the mercury beam path onto your sample by pressing the “FL on/off” button on the right of the microscope. Use the control pad and joystick to the left of the microscope to move the slide and select the area of interest. Focus the microscope by turning the focus knob, on the right of the microscope.

N.B If you have followed the above steps but are still unable to see anything through the eyepiece, check that the knob below the eyepiece is pointing at . It should be left like this at all times.

b) VIEWING WITH HALOGEN LIGHT

- **N.B. Due to a problem with the system this option is NOT currently available**
- If you wish to view your sample with the halogen light, click the VIS button on the LSM510 software on the PC – the button on the side of the microscope does not work. Select filter set 5 by using the buttons marked “reflector” on the right of the microscope and reading the display at the top of the microscope. Switch the halogen bulb on by pressing HAL on the right hand side of the microscope and pressing the  button at the front of the microscope to increase bulb intensity. Again, use the control pad to the left of the microscope to move the slide and focus by turning the focus knob.

N.B If you have followed the above steps but are still unable to see anything through the eyepiece, check that the knob below the eyepiece is pointing at . It should be left like this at all times.

3) IMAGING A SAMPLE WITH A SINGLE LABEL

- Once you have the area you wish to image return the filter/reflectors to 1 (must be done manually on the upright microscope) and ensure that the microscope is set to LSM rather than VIS. (**N.B** This will happen automatically once the laser starts to scan).
- Return to the LSM software that you opened in the setting up stage. From the main “Acquire” menu, open the “Config” and “Scan” windows by clicking on the buttons.

Setting up the Configuration

- The confocal configuration is preset for many fluorophores. If your fluorophore is pre-loaded you simply choose the correct configuration.

- In the “Config” window choose the Channel mode button. Click on “Single Track” and then select the picture on the right showing a disk with “Config” underneath. A list of the preset configurations will come up, click on the down arrow and find the one you need. If there is a relevant “narrow band” option choose that one and click “apply”. If you accidentally press “Store” click NO when the computer asks if you are sure!

Initial scan settings

- Move to the scan window and select the “Mode” button and underneath that select “Frame”.
- Make sure the objective you are using to look at the sample is the one shown in the window (or your measurements and scale bar will be wrong later). If not click the down arrow and choose the correct objective. If you can’t see the correct objective in this list you have not set it up properly – go back and read the set up section above.
- Frame size is usually 512 x 512 and this is fine for the setting up process, but you need to select “optimal” when you are collecting images you want to save.
- The line step should be 1 and the speed at about 4 or 5 initially. You can reduce it further if your image is too noisy
- The scan direction should be single → and the data depth 8 bit
- Go to the top of the window and click on the “Channel” tab. This shows how much the pinhole is open, click the ‘1’ button at the right of the counter (next to the max button) – this changes the pinhole value to as close to 1 Airy unit it can – this is the OPTICALLY optimum unit. However it MAY NOT be the optimum for your sample depending on the staining intensity and you may need to increase this later. Don’t open it too much as you will blur your image.

Obtaining and optimising the image

- On the “Scan” menu box click “Find”.

You now have an image that needs optimising.

- With scan on “continuous” check the focus by moving the objective up and down slightly using the fine control.
- Once you have the image in focus click on the “Palette” button on the right side of the window containing your image. Click on the “Range Indicator” and your picture should now be black and white but may contain red and blue areas – these indicate a loss of information. Red tells you that that part of the image is above the limit of the detector while blue means that it is below.
- To get rid of the red from the image, go to “Scan” window, select “Channels” and reduce the “Detector Gain”. Click single – this retakes the image with your changed setting. Continue until there is only a slight speckle of red in your image. To get rid of the blue do the same with the value for the “amplifier offset”. N.B. You need to ensure that you DO have some red and blue pixels in your image to ensure that you are covering the full detector range.
- Click “Palette” again and choose “no palette” and colour should return to your picture.
N.B Each time you scan a spot you do bleach it so remember that if you have had to collect a number of images in the optimisation process the fluorescence will be greater when you move to a new area and so it may be worth dropping the gain a little further to compensate for this.
- You can also change the scan speed, line averaging, pinhole diameter (don’t open too much though) and % laser transmission to improve image. The effects of these will be discussed in the training
- Once you are happy with the settings, click “optimal” for the frame size and “single” to obtain an image and once complete save it using the “save” button on the right of the image.

4) IMAGING A SAMPLE WITH MORE THAN ONE LABEL

- Set up the microscope and focus the confocal upon the sample in the same way as described above.
- In the “config” window select the “Multi Track” tab

- Use the “add track” button to add the required number of tracks – make sure you have one for each separate fluorophore on your sample
- Highlight the first track in the list and go to the button marked “store/apply single track”
- This gives the list of available single track configurations – select one which is appropriate for your first fluorophore. N.B It is best to use configurations which have band pass filters i.e. avoid using configurations with an LP filter, particularly for the fluorophores one your sample with the lowest emission wavelengths.
- Select the second track on the list and follow the steps above to select a second single track which is suitable for your next fluorophore.
- Continue in this way until you have tracks for all the fluorophores on your sample.
- Each track has a check box next to it. If this box is ticked the track is on and will be used to obtain an image, if there is no tick the track is currently off. When the track is on, the channel is also listed under the channel tab of the “scan” window. To turn tracks on and off click the tick box with your mouse. You can also highlight one track from the list by clicking on its name. The configuration for this particular track is then shown in the “config” window.
- Firstly, with all the tracks ticked, go through each of the channels and check in the “scan” window and check that they are all imaging the same thickness of optical slice. If they are not it is necessary to change the pinhole for some channels to make them the same.
- Turn of all but one channel and optimise the detector settings using the same procedure described above for a single channel.
- When you are happy with one channel, switch this off and go onto the next. Do this until all the channels have been optimised. N.B. Some settings have to be the same for all channels – and the software will ensure that if you do change it for one channel it is changed for them all. These are those in the mode tab of the “scan” window such as scan speed, frame size, scan average etc. The settings under the channel tab can be different for each channel.
- When you are happy with the settings for all the fluorophores, turn all the channels on, click “optimal” for the frame size and “single” to obtain an image and once complete save it using the “save” button on the right of the image.

5) TURNING OFF THE CONFOCAL

- Remove the sample and, if using the upright microscope, remove objectives and return to their containers. If you have used oil, wipe them CAREFULLY with the lens tissues.
- Close all the windows on the computer except for the main LSM software window.
- Select “Laser” and click “Off” for all the lasers. Switch off the mercury lamp.
N.B It is not a good idea to repeatedly turn the lasers off and on, so if there is a user booked on in the 2 hours after you please leave all the confocal lasers on that you have used and let the next user turn them off if they do not require them. If you have used the two-photon laser, please check to see if they have booked it as well and if so leave it on for them, otherwise switch it off and turn the key to standby. Please also leave the mercury lamp on if you have used it and let them switch this off if they do not need it.
If there is a gap of more than two hours before the start of the next booking, it is OK to turn off everything.
- If no-one else is using the confocal after you go back to the main LSM menu and select “File” and “Exit”. This will take you back to the very first menu window.
- Switch off the monitors using the buttons on the screen.
- N.B. Leave the computer and system ON.