**Quick start guide to the Zeiss LSM700.**

**If you have any questions about using this equipment please contact Louise Howell on Ext: 4182 or email:** **lhowell@icr.ac.uk** **Thank you.**

**Switching on the system.**

* Switch on the mains switches at the wall (labelled 1-3).

 Only switch on #4, (the definite focus one) if, you require CO2, heat and the definite focus for live cell imaging/time series.

* Switch on the lasers by turning the key from centre position to the right, the yellow LED should then come on.
* Switch on the computer. Click on Zen user, password is: **zeiss12**. Leave 5 mins.

2 messages will come on screen – 1) Adding info can unintentionally change or delete values and cause components to stop working correctly etc – **Click – YES**

2) The keys and values contained in C:\Program Data\Microsoft Windows\Start etc have been successfully added to the registry – **Click - OK.**

* Double click on Zen 2009 then click on Start system.
* If you just want to open up existing images for processing and don’t need the hardware just click on **image processing.**

**Viewing your sample.**

* Carefully lift up the microscope by the top being careful not to push back too hard.
* Using the microscope touchscreen click: **Home, microscope, control, objectives.** Select relevant objective – 10x dry, 20x dry, 40x oil and 63x oil. Place your slide coverslip facing down. N.B. Take care when switching objectives to ensure you don’t go from an oil objective to a dry objective! Once you have finished with an oil objective always remove the oil by **gently** wiping with **lens** tissue.
* Then click on **Reflector** to choose the relevant filter cube e.g. DAPI or GFP, click on filter of choice. Click on **Lightpath** tab then eye to view. For fluorescence click **RL** (reflected light) illumination on (white when on) – you will see light going to the slide. Carefully focus your sample.
* For brightfield click **TL** (transmitted light) illumination on. If you are having problems visualising the specimen, click on Home and the **‘Make it visible’** button – this will set the microscope to a standard state in which the specimen is visible. For **DIC** click on **POL TL** under reflector tab, this is only available on 40x and 63x. For normal **brightfield select Pos 5 or 6.**
* In order to see brightfield must have the silver switch on top of the microscope pointing towards the right, towards HAL100 (to go to the screen it should point the other way).

**Confocal imaging: Configurations – Using Smart Set-Up.**

* On the computer click on **Acquisition tab** then tick the **show manual tools** box (next to smart set-up) this will open the Laser, Imaging Setup and Light Path Windows under the Setup manager.
* The best and easiest way to get started is to click on **‘Smart Setup’** then choose your dyes from the drop down menu. The software will automatically pseudo colour your dyes, you can change the suggested colour with the right down arrow.
* The software will offer 3 different configurations for imaging your sample: **Fastest, Best Signal and Best Compromise**. Make your selection and click on **Apply** to implement the configuration.
* For multi-labelling experiments it is generally best to select **Best Signal** as this will capture each channel sequentially and therefore reduce any bleedthrough problems which may occur.
* Open the **Imaging set-up menu** to show the configuration of the light path. To view the details of a particular track in the Light Path window highlight the track in the Imaging set up window. In the light path tool the part of the emission spectrum detected by the system is displayed by a filled curve, the parts of the spectra which are actually detected by the system are filled with colour.
* Once the smart set up parameters have been chosen and the tracks checked to look out for the possibility of bleedthrough, then you need to click on: **‘Acquisition mode’** and click **‘Show all’.**

**Scanning Parameters: Acquisition mode.**

* **Scan mode –** **select frame. Line step – select 1.**
* **Frame size** – generally a frame size of 1024 x 1024 or above at zoom 1 will produce good resolution images. A larger frame size means you will collect more pixels per unit area scanned, effectively increasing the resolution of the image. However, larger frame sizes take longer to scan and therefore increase photobleaching. Click on **Optimal** for an optimal image resolution according to Nyquist theorem.
* **Scan speed –** a speed of 7-8 should produce good results, a speed of 6-7 excellent results. Slower scan speeds will result in a less noisy image but increases the risk of photobleaching effects.
* **Averaging –** improves the image by increasing the signal to noise ratio. Select **mean** averaging, **select the number** of times you want the image to be averaged, generally an average of 2 – 4 is enough to produce a good resolution image. Under **Mod**e choose **line** **averaging.**
* **Bit depth –** 8, 12 or 16 bit. Generally 8 bit images are fine but 12 or 16 bit images are recommended for intensity measurements and publications (but will obviously result in larger file sizes).
* **Scan direction –** default setting – unidirectional.
* **Scan area –** you can zoom in using this window or use the **crop tool** below the image window.

**Scanning Parameters: Channels.**

* In the Channels window you can set the **laser power, detector gain, detector offset and pinhole for each channel.** Click on a channel to select it and the parameters for that channel will be displayed below. If working with multiple channels uncheck all except one and highlight that one to optimise the settings for that channel.
* Set the **pinhole size to 1AU** (Airy Unit) for the best compromise between depth discrimination and detection efficiency, based on the excitation wavelength and numerical aperture.
* **Gain** – the voltage on the PMT detector which detects the emitted light from your sample. Initially set the gain quite high, around 800 to detect any signal and then adjust accordingly.
* **Digital offset** – sets the background/minimum intensity level of the image.
* Laser power – generally set quite low around 2-5% for samples with a reasonable fluorescent signal.

**Scanning.**

* Start a live scan by clicking on the **Live** button, this will scan the sample continously at a high rate of speed until you click on the **stop** button.
* While scanning click on the **range indicator button** in the dimensions tab below the image.

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* The image will now appear in monochrome in the gray scale mode, with the pixels that are at mimimum level in blue and the pixels which are too bright/saturated in red (0 and 255 for an 8 bit image).
* In order to get the full ‘dynamic range’ reduce the gain until there are no or only a few red pixels and increase the offset until all the blue pixels disappear and then make it slightly positive – usually around 1-2.
* If the image is very saturated at a gain of 600-700 then it may be best to reduce the laser power as this will result in less photobleaching. However, a high gain will increase the noise levels. **N.B.** For quantification avoid all red and blue pixels – saturated pixels cannot be quantified.
* Repeat these steps for all the image channels and when you are ready to take the image check all the channels to activate them and click **Snap** to take the final image.

**Transmitted light image.**

* To take a transmitted light image (brightfield, DIC) highlight one of your channels (generally have the T-PMT coming off track 1), ensure the **show all** button is clicked on the **Light Path** tool. Check the box next to the T-PMT to activate the transmitted light PMT, de-activate the dye by unticking on the light path and hightlight the T-PMT in the channels window.
* Ensure that the silver switch on top of the microscope is pointed to the left to put the image to the screen and start a live scan. Adjust the gain of the T-PMT channel until you get an image and decrease the offset to increase contrast if necessary.
* When the image is at the correct intensity and contrast take a snap. The fluorescent image can be collected at the same time by re-selecting the dye in the light path and clicking on Snap. Alternatively you can overlay the separate images under the processing tab – this can be useful if your brightfield image is more focused on a slightly different z plane to your fluorescent signal.

**Collecting a Z stack.**

* Start a **Live scan**. Focus up and down through your sample to find the brightest focal plane and adjust the gain and offset until there are no or very few red pixels and no blue pixels seen using the range indicator.
* Check the box next to **Z stack** and then click on Live scan.
* Adjust the focus in one direction until you identify where you want the Z stack to start. If you need to take a Z stack of the whole sample in Z you need to adjust the focal plane until you don’t see any fluorescence. Click on **Set first.**
* Focus in the other direction until you identify where you want the Z stack to end, click **Set Last.**
* Click the arrow next to **Optimize Sectioning and Step** to open up the window. Click on **Optimal**. The computer will calculate the optimal number of slices and ensure correct Nyquist sampling (slices will overlap by half their thickness).
* Also check here to make sure that the **optical slice thickness** is the same for all channnels. If it isn’t then you need to adjust the pinhole in the channels window so that all the channels match. This is very important especially for co-localisation studies. **N.B.** If you have to alter the pinhole size you will need to alter the gain intensity, check using the range indicator.
* Once you have all the settings sorted out then click on **Start experiment** to start the Z stack.
* You can view the Z-stack in gallery mode by clicking **Gallery** next to the image window.
* To view the stack as a maximum intensity projection, click on **3D** and **Maximum.** To create image click on **create image**, you can also do this under the **image processing** tab.
* To render the series click on the **Series tab.** Choose your turning axis e.g. can turn around X and Y, number of frames and difference angle.

**Saving the images.**

* Double click on image to be saved, then in top left hand corner of the screen click on file, save as and scroll down to Data D – User – your folder name and then name and save your image.
* Always save as an LSM file as this will store all the hardware settings used i.e. the laser settings, gain, zoom factor etc. These files can only be opened using Zen software such as Zen Lite which you can download for free. If you would like to open your files in Photoshop or Powerpoint you will need to **export** them by clicking on **file, export** and scrolling down to an appropriate format.
* In the Export window select the file format: **Select TIF** file if you need to preserve the format of your image (if you need to analyse the data). JPEG files will be more compressed but cannot be analysed. In the export window select the file format, generally select **contents of image window** or **full resolution image** window for single images with overlays such as scale bars.
* Choose video for windows to export an animation such as time series, z series or 3D render series.

**Saving your settings/configuration.**

* You can save your configuration in the window below the Acquisition tab by naming it e.g. dapi-488-555 sequential and clicking on the save symbol. This means the next time you can simply reload these settings by clicking on the open file symbol and selecting your configuration.
* **Re-use button** - you can re-use the settings from any image by opening the image (the lsm file) and clicking on the re-use button found next to the crop button in the Dimensions tab below the image window.

**Switching off the system.**

* Save your images and back up onto an external hard-drive. It is your responsibility to save your files so it is recommended that you keep at least 2 copies of important images.
* Clean the oil objectives you have used by **gently** wiping with **lens** tissue.
* Switch objectives to an empty position.
* Turn off laser by turning key back to the centre.
* Shut down the computer.
* Turn off the main switches at the wall.
* Ensure that everything is completely switched off.
* Cover microscope with dust cover.