Leica TCS SPE Confocal Microscope Standard Operation Procedure Guide

Location: 5-085 Katz Centre, University of Alberta

Warnings:
- This microscope is only to be used by trained personnel. You MUST arrange for training through SMART Labs prior to using the instrument. **This guide is not a replacement for in-person training**. It is meant to be a quick reference for important features – **all trained personnel are still expected to generally know how to operate the microscope with little to no difficulty**.
- Abuse of the microscope will result in access privileges being revoked.
- If you do not understand how to do something, **ask**. The last thing we want happening is equipment breaking down.
- DO NOT USE LASERS AT OVER 30% POWER. THIS SHORTENS THEIR LIFESPAN AND BURNS YOUR SAMPLE.
- Do not overwrite others' data or settings. Save all of your own data on a separate storage device.
- You **must** sign into the microscope room, record your use of the confocal microscope on the hard copy usage log, and report your hours on the SMART Labs website (https://smart-network.nanofab.ualberta.ca/)

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Section 1: Setting Up

1. Ensure all objective lenses are rotated out of position. **This is so when the microscope stage initializes on startup it does not potentially hit an objective lens!**

2. Turn on the control unit (shown on the bottom right of the picture).

3. Turn on the lamp (shown on the top right). Record the bulb runtime at start and finish on the sign-in sheet by the computer.

4. Press the power switch on the laser module (shown on the left) and turn the key to the 'on' position.

5. Open the LAS X software. The microscope will initialize.

Ensure that the following configuration is selected for startup (ie. do not select Simulator mode):
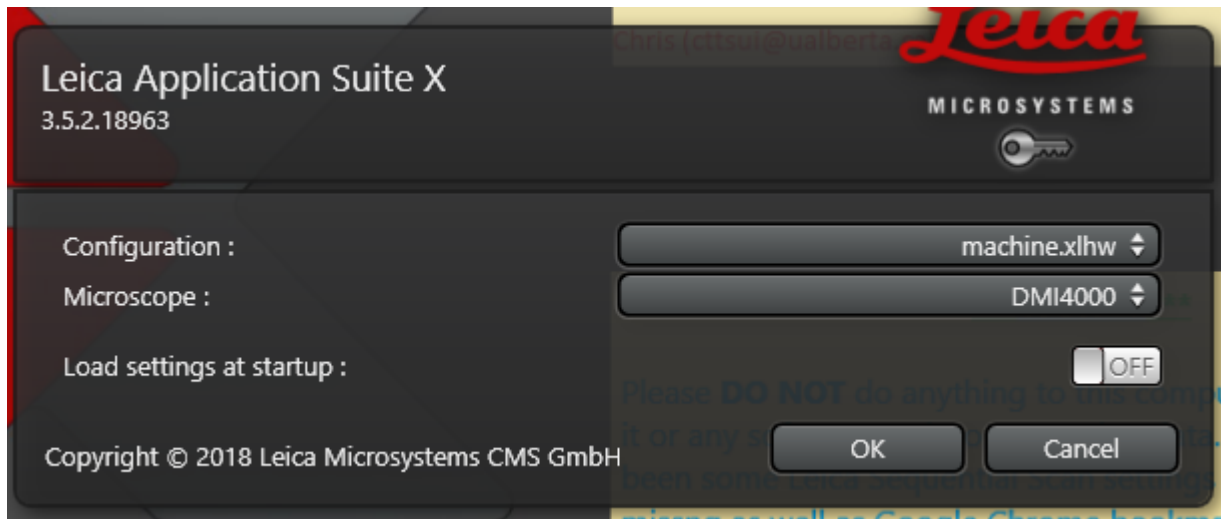
Troubleshooting: If during startup an error occurs, 99% of the time it can be resolved with a restart of either the software, the computer, or the microscope. If restarting the microscope: shut down all three boxes shown above and wait 15 minutes before restarting. This is so the lamp does not overheat and explode.

6. After initialization you can load your sample. Choose the appropriate stage insert for a slide or cell culture plate. Ensure that you use the insert screws to attach the insert onto the stage – this prevents z-axis drift during imaging.

7. Rotate your desired objective lens into place. This must be done by hand – be gentle!
    a. This microscope has three lenses:
        i. 10x Air
        ii. 40x Oil
        iii. 63x Oil
    b. If you do not know how to properly apply oil on your sample for the 40x and 63x lenses, **ASK FOR HELP**.
    c. Please ensure that after using oil immersion that the objective lens, stage and stage insert are cleaned to avoid buildup of old, sticky oil.
        i. Use lens paper to clean the objective lens. Wipe gently across the top of the lens in one direction only. **Do not apply pressure!**

Section 2: Loading Settings

1. To maximize the workspace in the application, stretch the window out with the mouse to use both monitors.

2. If you have previously used the microscope, load your settings in the Load/Save/ROI panel. You can adjust your settings profile or make new settings by following the steps below.

3. To save acquired images or load a previous project file click on the "Open Projects" tab on the top left of the interface.

4. Select your acquisition mode. The 'default' setting is XYZ (which allows you to take regular images on the XY plane with possible z-stacks).

5. For imaging in multiple channels click on the Sequential Scan Panel button (shown at the arrow above). This will open the Sequential Scan dialogue box on the left hand side of the software interface. To add or subtract channels simply click on the plus or minus sign. Whatever settings you end up establishing for your project can also be saved and loaded at a later time.

6. You can then adjust what channels you are imaging in the centre panel. To image in fluorescence mode toggle on PMT 1 (PMT = photomultiplier tube). Otherwise, to turn on brightfield toggle on PMT Trans.
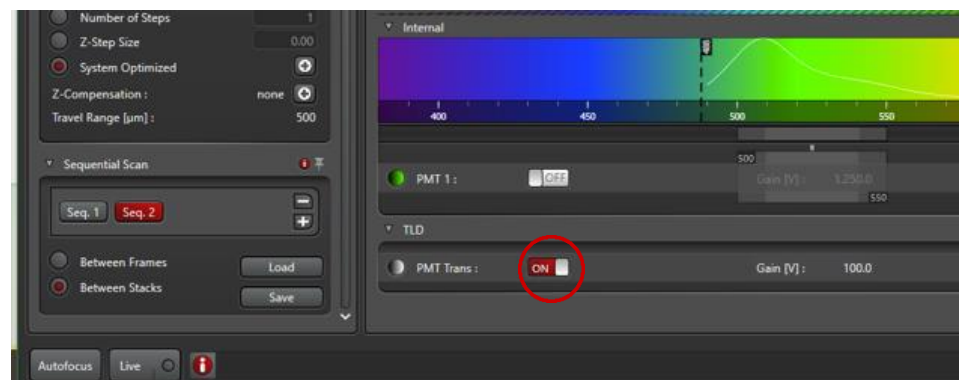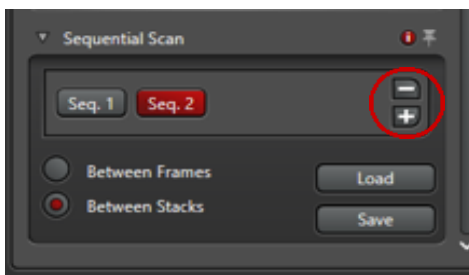
Section 3.1: Taking an Image

1. The image quality panel provides many options to change the type of image you will receive. These will depend on your preferences and sample.
   a. **Format**: The quality of image. For some images 256x256 will be fine, for others you may need 1024x1024 or higher.
   b. **Frame average**: This allows the microscope to take the same image multiple times, reducing background noise. This is done at the expense of acquisition time.
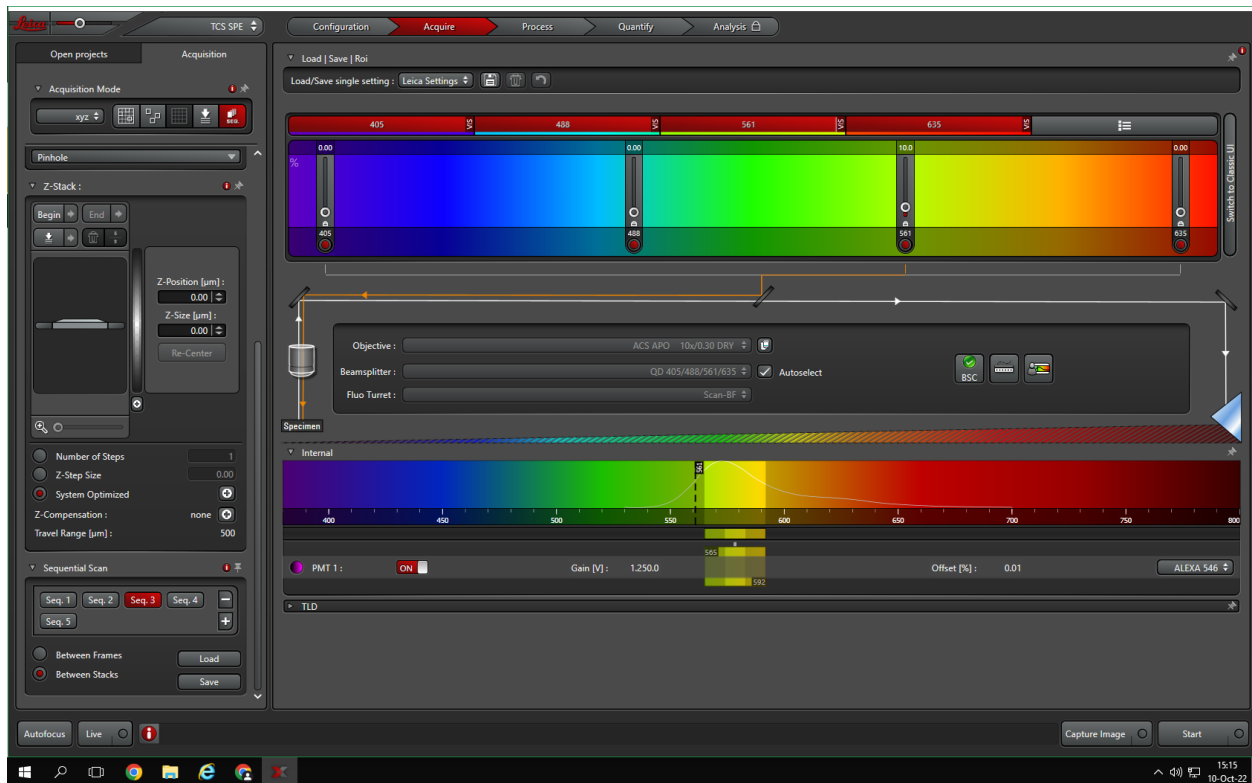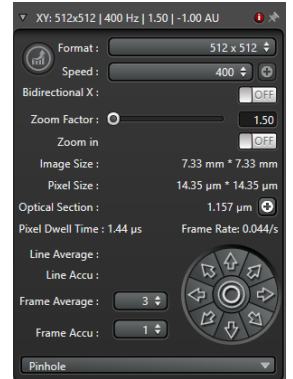
2. **Lasers**: There are four laser lines available (405 nm, 488 nm, 561 nm, 635 nm). You can adjust the laser intensity by dragging the slider or double-clicking the number above the slider, but **do not use the lasers beyond 30% power**. This should provide plenty of light for your sample. Try starting around 10-15% and adjust as needed.

3. **Detection**: This microscope utilizes a photomultiplier tube (PMT) for detecting emitted sample light. This section of the interface allows for adjustment of detection settings.
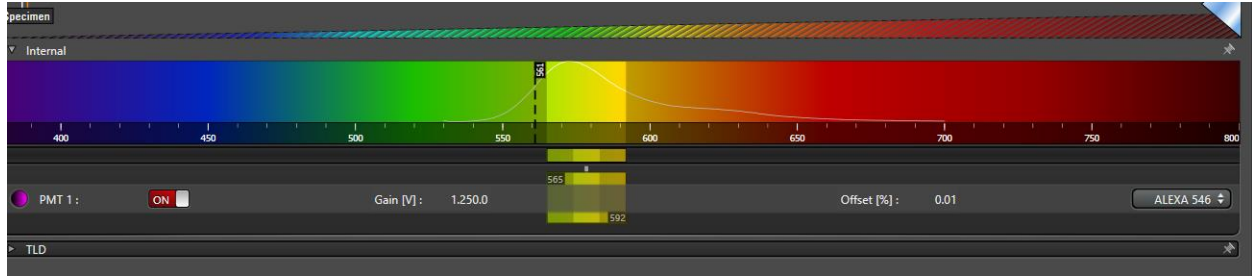
a. Gain [V] refers to **Smart Gain**. Refer to the section below for an explanation on what it does.
b. Offset [%] refers to **Smart Offset**. Refer to the section below for an explanation on what it does.
c. To the left of "PMT 1" is a coloured circle. Click it to change the pseudocolour of the imaging channel. There are many predefined selections available to you (or you can enable your own custom colour if you know its RGB combination!).
d. To the right of the interface is a drop-down list of many commonly-used fluorophores. Clicking a fluorophore of interest here will generate an emission curve on the rainbow-coloured band shown above. Adjust your detection sliders to cover the peak around the emission curve while taking care not to go further left of the excitation laser wavelength (shown as a vertical black line on the rainbow-coloured band.

4. Our setup features turn wheels for easy access to different settings. To change what these adjust as well as turn sensitivity, enter the knob control menu.

a. **Smart Gain**: This improves image brightness (up to a maximum of 1250 V) without further fluorescently exciting the sample. This is done by increasing the sensitivity of the photomultiplier tube (PMT) detector. There is typically more background signal when image brightness is increased this way.

    b. **Smart Offset**: This setting reduces the amount of background signal in the generated image. Typically this is best used in the green range of the light spectrum where many things naturally autofluoresce. When paired with the 488 nm laser it is recommended to set Smart Offset to **-0.2%**. Too high of an offset runs the risk of taking away meaningful features in your image.

    c. **Pinhole**: Confocal microscopy works by restricting out-of-focus light from reaching the detector through the use of a pinhole. Typically, imaging is done at 1.00 Airy Units (AU) – the larger the pinhole size the more light is allowed to pass through to the detector as the optical section becomes thicker.

    d. **Zoom**: Is digital zoom. Allows you focus on a subset of your current field of view – however, resolution will stay the same.

    e. **Z-Position**: Allows you to move up and down the z-axis of the sample. The range of movement is between -250.00 μm and 250.00 μm (ie. total range = 500.00 μm). This is *not* the same as using the black coarse adjust knob on the side of the microscope! More on that below.

5. At this point it is a good idea to have a test run. Turn on the livefeed ("Live" button) in the bottom left corner. You should start with a low resolution of 256x256 so that the resulting image will refresh frequently while you tune your settings to your requirements.

    a. To find the focal plane of your sample, either use the z-position knob on the control bar or the black coarse adjust knob found on the microscope.

6. Check for good image signal using the heatmap on the viewing screen (cycle through the pseudocolour/heatmap/greyscale viewing modes via the icon circled below). Heatmap is better for viewing livefeed images than the other two modes as it offers the best visual contrast between pixels in an image and helps determine the extent of pixel intensity and saturation.

    a. Saturated pixels appear when the intensity of pixels exceeds what is quantifiable by the detector (max intensity 255) – this will hinder your ability to quantitatively compare differences in signal between areas. On the heatmap saturated pixels appear in **blue**.

    b. Conversely, pixels that have an intensity value of 0 will show up as **green** on the heatmap. This may indicate empty space in the field of view or insufficient signal from a sample.

Section 3.2: Z-Stack Imaging

Z-stack imaging is beneficial for generating a more wholistic impression of your sample from top to bottom with improved resolution along the z-axis. Even monolayers of cells have a bit of thickness to them – generating a z-stack will also allow for a 3D reconstruction of the sample in post-acquisition software such as ImageJ.

Z-stack imaging is possible through LAS X's 'default' XYZ acquisition mode. It will take a 'deck' of images, slice-by-slice, through a distance along the z-axis that is predefined by the user. The Z-Stack dialogue window is as follows:
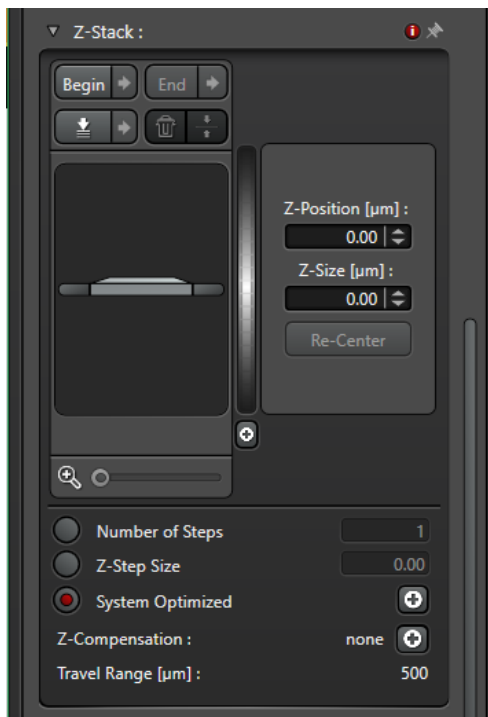
To enable Z-Stack, click on both 'Begin' and 'End' – both buttons will then turn red. To then set the beginning and end positions of the stack, either manually enter in the z-position in their respective fields or click on the 'Begin' and 'End' buttons again. The maximum possible size of a stack is 500 µm.

It is important to move up and down the z-axis using the z-position knob on the far right of the control bar **and not the black coarse adjust knob on the microscope itself**. This is because the black coarse adjust knob will set the absolute position of the sample while the z-position control knob sets its value relative to the position of the sample as determined by the black coarse adjust knob.

(*Example of what I mean by this*: You're looking at a sample at a z-position of 0.00 µm and then use the black coarse adjust knob to move to another plane. The z-position value of 0.00 µm on the control bar does not change – what happened is that that you've just defined a new '0.00 µm' for your sample on the microscope. Don't do this if you're setting up a z-stack!)

Once the beginning and endpoints have been defined the software will determine how many slices to image throughout the z-stack. This is done if the 'System Optimized' option is selected – otherwise, the user can manually enter in how many slices they would like in the stack, or the spacing in between slices. For best results in 3D reconstruction in post-acquisition rendering, leave it on 'System Optimized.'

Section 3.3: Mark and Find Imaging

You may wish to take multiple images at different prescribed points in your sample. You can simplify this by saving the positions of different images in the "Mark and Find" menu which can be opened in the acquisition mode menu mentioned above. To access Mark and Find click on the icon indicated by the arrow below.
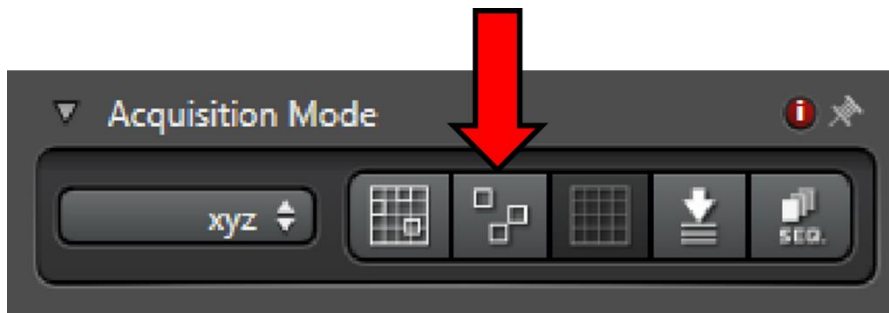

Select "Mark Position" from the buttons below the coordinate map. You can then return to this position at any time during the same imaging session by selecting from the 'Positions' drop-down menu. A position can also be redefined by clicking on the 'Redefine Position' icon. When you press "Start" the microscope will take images in sequence starting at position one. A map of positions can also be saved and re-loaded at another time.

Section 3.4: Timelapse Imaging

Timelapse imaging allows the user to image a sample at regularly scheduled intervals over a period. To enable this, you must select XYT as the acquisition mode.

The following dialogue box will appear allowing you to modify several parameters:
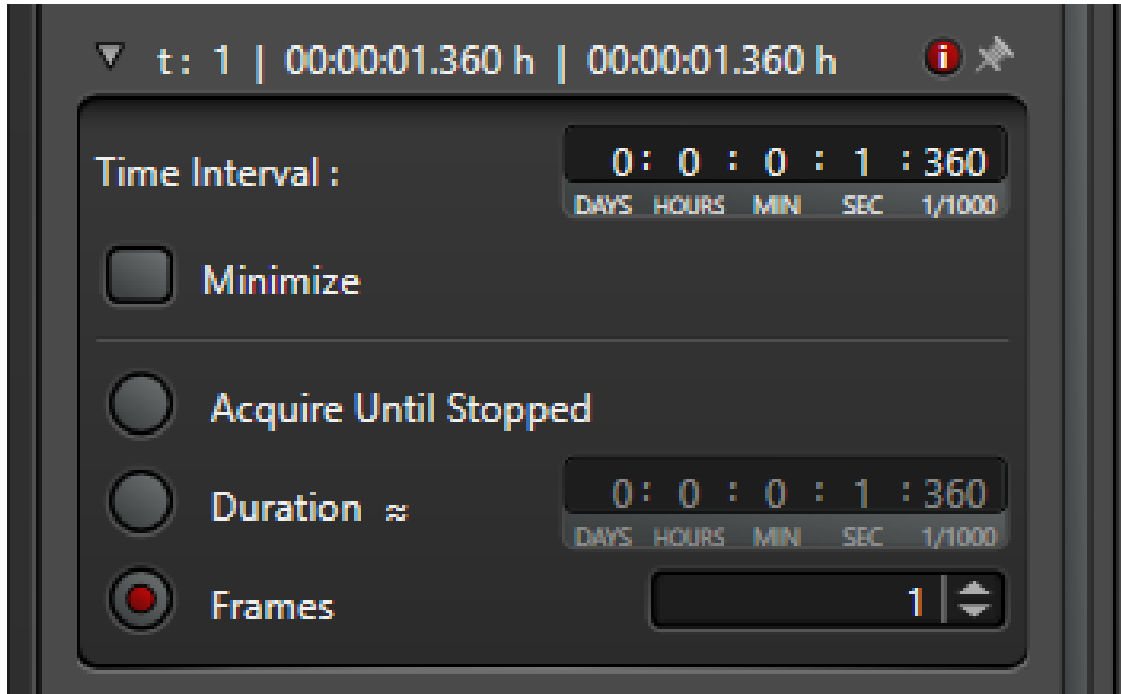
You can choose to have the software acquire images indefinitely until stopped, for a set amount of time, or for a set number of frames. The time interval between frames is also displayed – this will be dependent on several factors such as image resolution, number of positions being imaged (ie. you can pair timelapse imaging with Mark and Find), number of channels being imaged, frame averaging, etc.

Section 4: Other Things

- When looking for ways to improve image brightness, consider adjusting Smart Gain first before adjusting laser power. Smart Gain only modifies the sensitivity of the PMT detector whereas laser power will directly affect the excitability of the sample itself. You can also increase pinhole size to allow more emitted light to pass through to the detector at the cost of increased optical section thickness.
- To access images acquired during the current session and past sessions, click the "Open Projects" tab at the top left of the interface. Ensure all work is saved prior to shutting down the LAS X software.
- For viewing samples through the eyepiece:
  - Ensure the livefeed is off
  - Press the buttons below in the indicated order:

  - **Button 1**: Opens the eyepieces
  - **Button 2**: Selects a filter cube. On this microscope there are two filter cubes to choose from (GFP, Y5). Stop here if you want to just view your samples in epifluorescence mode – don't forget to also press the **Shutter button**.
  - **Button 3**: Can be found on the left-hand side of the microscope. This button allows regular light to be transmitted through the sample (ie. enable brightfield mode)
  - Press **Buttons 1, 2, and 3** in that order. Sometimes **Button 1** may have to be pressed a second time after, along with the **Shutter button**.
- For quick access to sequential scan settings used before, two options are available:
  - Sequential scan settings can be saved and re-loaded at the Sequential Scan settings dialogue.
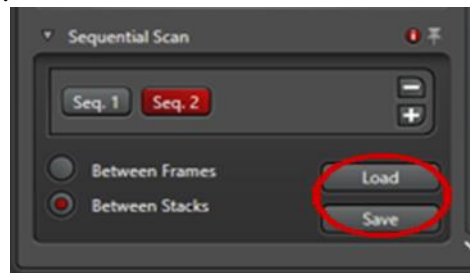
- o Alternatively, a previous image file can be loaded in the "Open Projects" tab. Select an individual image in a lif file and Right click > Properties to access its metadata. In the metadata box, click on "Apply Settings" at the bottom left to re-load the imaging settings used to acquire the image.
- Shutting down the microscope:
  - o Ensure all work is saved before closing LAS X
  - o Rotate the objective lens out of position!
  - o Shut down all 3 boxes, in any order. Before turning off the laser box ensure the key is in the "off" position.
  - o Put the dust cover back on the microscope
  - o Record shut down time and lamp usage on paper logsheet
  - o Log out of SMART Labs
  - o Turn lights off in room if you are the last one out