Imperial College London

Facility for Imaging by Light Microscopy

Observing Life As It Happens

QUICKSTARTGUIDE

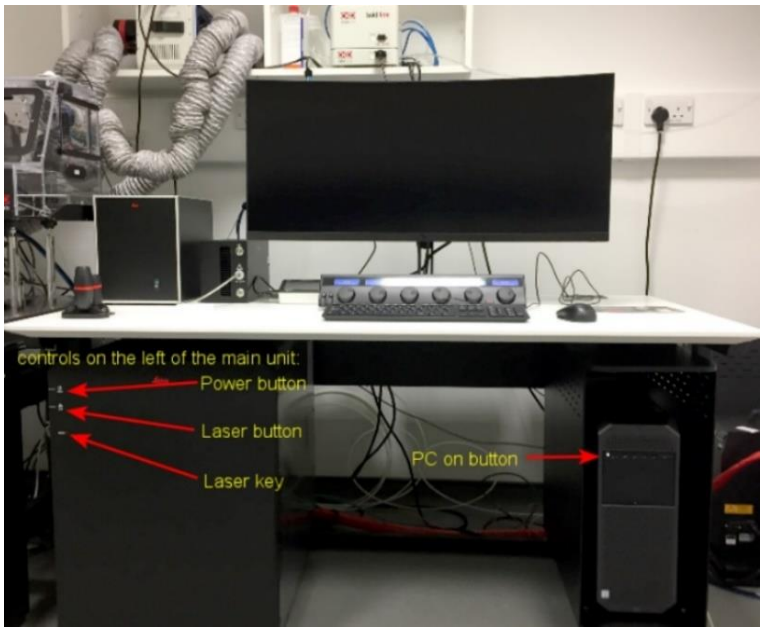
HCF3 - STED
LEICA Stellaris 8
inverted

(ICTEM 312)

NB All users must have completed the on-line “Introduction to Laser Safety” from Imperial College safety department and read the “Laser Safety HCF3 STED” document on PPMS

Contents

Startup Procedure



- Start PC
- On the central power unit - Switch on:
    - Power
    - Laser
    - turn on Laser Emission Key

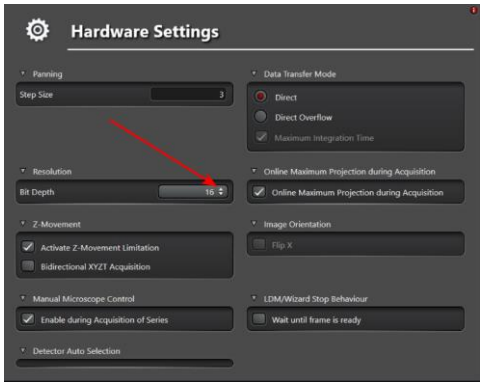
- !!! IMPORTANT !!! The microscope stage will auto calibrate (move) – to avoid trapped fingers
  DO NOT LOAD A SAMPLE UNTIL THE MICROSCOPE HAS FINISHD STARTING UP
- Login (IC network account)
- Wait until the TFT screen on the front of the microscope has finished booting
- On the STED Laser power unit, insert the laser keys and switch on the laser power buttons, (do not turn on the keys yet)
- Place the safety cover over condensor
- Start LEICA Application Software “LAS X” on desktop

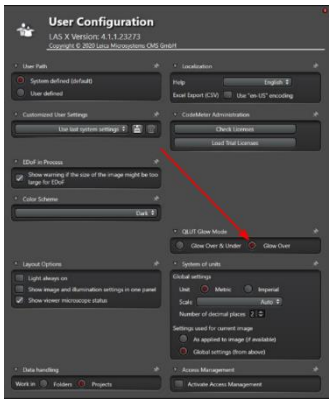
Software Setup

- In the start-up window from the drop-down menu select machine.xlhw
- Select the STED option

“Configuration” tab

- Select "Hardware" and set the required Bit Depth – 16 Bit

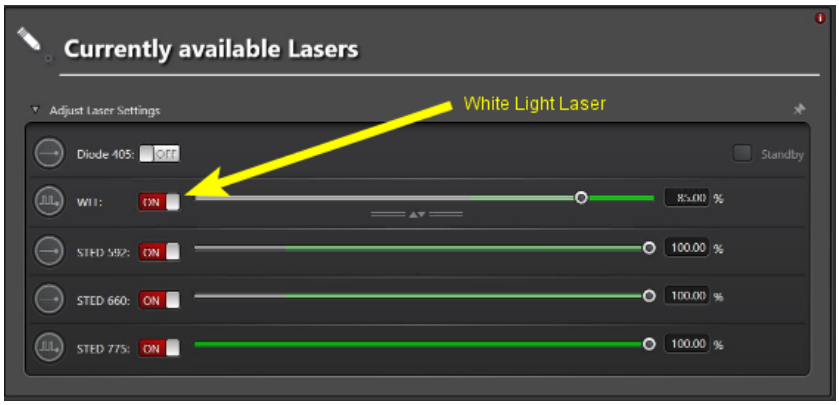




- Change the range indicator lookup table to Glow Over in the “User Configuration”

STED Lasers

- On the STED Laser power unit turn on the laser keys
- In the configuration tab, select "Laser Config" and turn on required lasers starting with the WLL (white light) laser

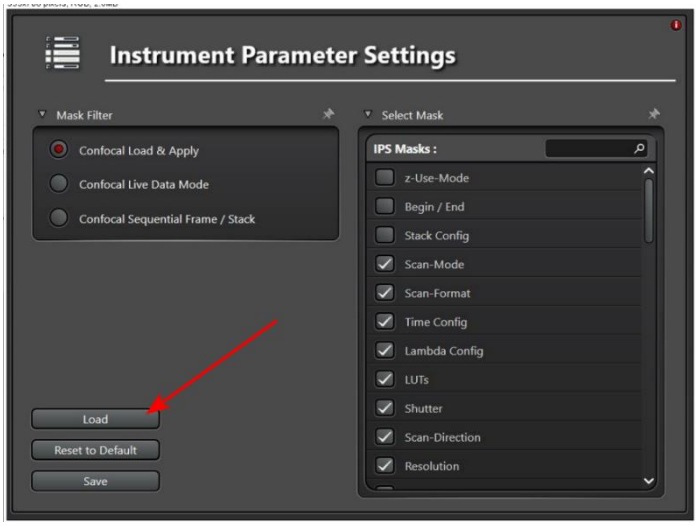


NB if the STED laser will not turn on try turning off the STED laser key and then turning it back on again. NB. The STED 775 laser will not start unless the WLL laser is already on

Re-Using settings

In the **Configuration** tab:

- Select IPS
- Select Load
- Select All_Users.xml from the E:\ drive
- Then applying or loading experimental settings will load all image settings



Software / Image Acquisition

Select “Acquire” tab and choose the desired acquisition mode eg XYZ

Channel Setup – Loading an existing setup

Load settings (Acquire” tab):

- Either, from a previously saved Image. Open the saved image/library in the “projects” tab and select the image. Then click on the APPLY icon on the menu above (NB. The objective, pixel number, bit depth and zoom and averaging are not re-loaded and may need re-setting).
- Or use the Load channel setup option from the main setup window to open a previously created and saved channel setup

Channel Setup - Automatic using the “Dye Assistant”

- select the Dye Assistant setup button (1)
- a new window will open allowing channel selection by fluorophore.
- clicking on the "..." button will load a search window allowing dye to be selected
- change the detector to STED by clicking on the detector type (2)
- repeat adding channels as necessary (a new line will automatically appear for each new channel)
- select the sequential scan method of choice ((3) - use frame sequential) by pressing apply. This will setup the channels and detectors automatically

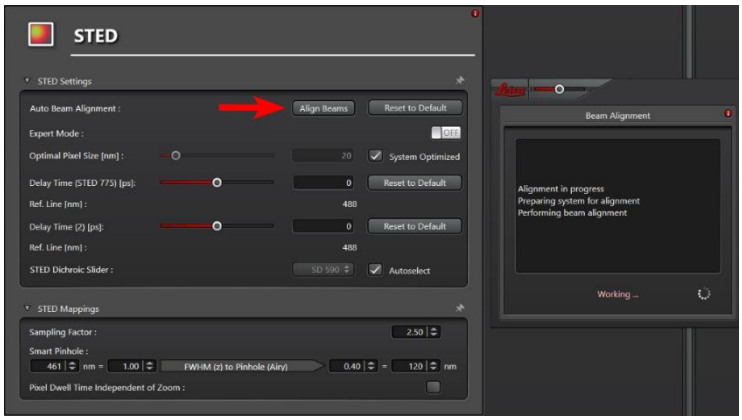
Channel Setup – New manual setup

Searching for fluorophores, and dragging/dropping into channels

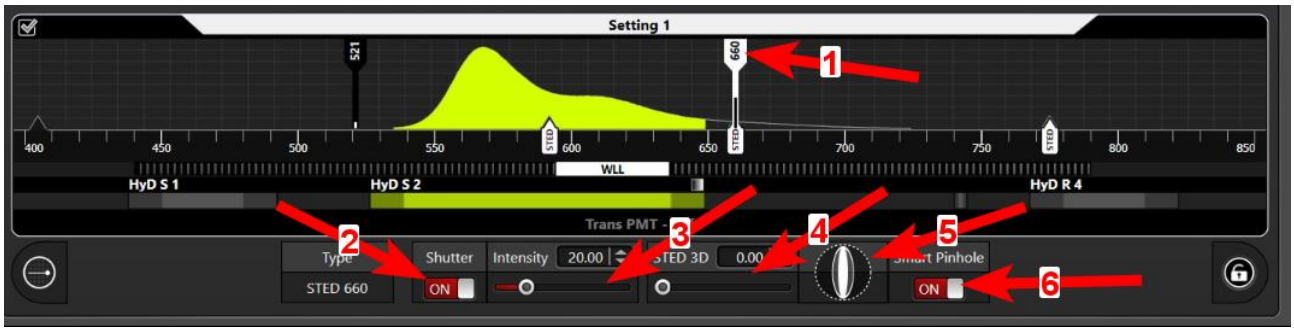
1. type in the dye – STED dyes have a C (confocal mode) and S (STED mode) above them
2. select sequential method (Stack - between stacks, Frame - between frames, Lines - between lines). Between lines is not recommended for STED.
3. select the S mode and drag the dye onto the detector region (it will auto select the detectors)
4. adjust detector range if required and add further tracks/dyes as required using the (+) symbol

Aligning STED Lasers

- Needs to be done at startup after the lasers have warmed up for 30 minutes
- Under the configuration select STED
- Run the Align beams (takes about 3 minutes)

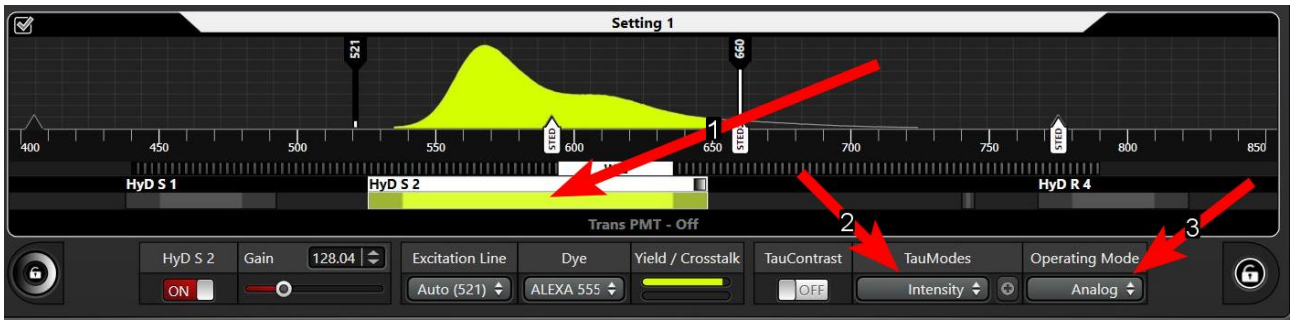


The Detector Settings



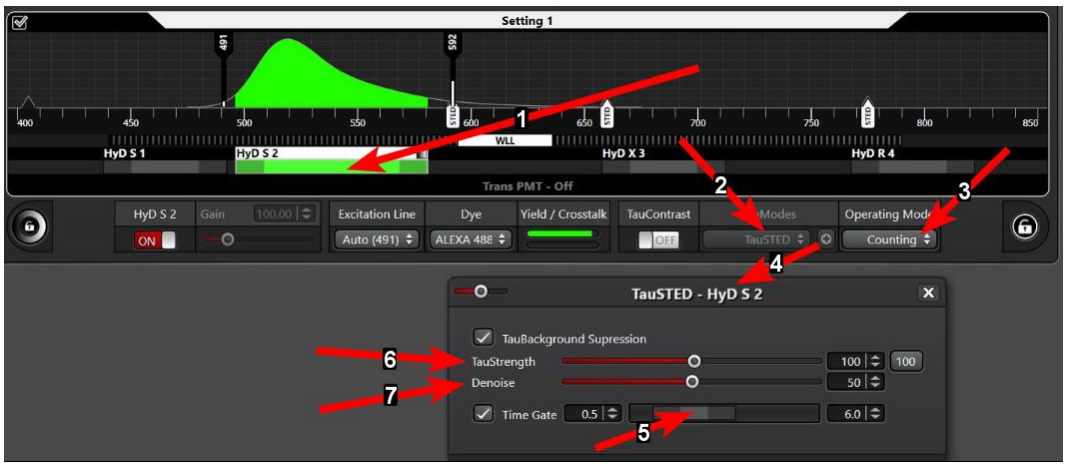
1. STED Laser – Select this to display laser settings below
2. STED Laser Shutter – switch this off to select just confocal mode
3. STED Laser Intensity – this controls the depletion efficiency. If this is set too high then it can result in high photobleaching of the sample. Try to keep the excitation laser intensity low so that you don't need higher depletion laser intensities.
4. STED 3D – changes the depletion beam to reduce the Z volume emission by adjusting the pinhole
5. Graphic displaying the theoretical illumination shape
6. Smart Pinhole – when ticked the pinhole is altered when the STED 3D slider is moved

Analog Detection Mode



1. Select detector – this will display the detector settings
2. Select Intensity
3. Select Analog

TauSTED Mode

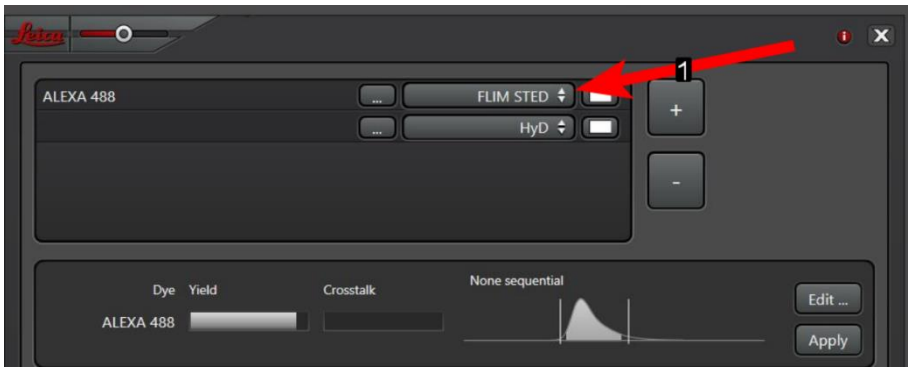


1. Select detector – this will display the detector settings
2. Select TauSTED
3. Counting mode will be automatically selected
4. Click the “+” symbol to display the fluorescent lifetime gate
5. Adjust to select a lifetime range
6. Adjust Tau strength
7. Adjust denoising filter

TauGating Mode

1. Select detector – this will display the detector settings
2. Select TauGating
3. Counting mode will be automatically selected
4. Click the “+” symbol to display the fluorescent lifetime gate
5. Adjust to select a lifetime range

FLIM STED, using the FLIM module



- Select FLIM STED in the Dye assistant tool will setup the channels correctly for FLIM STED
- Select FLIM under acquisition Mode tab
- See FLIM guide

Setting up STED

- Find the sample using high na objective (x86 water immersion for live samples or 100x oil immersion for fixed and mounted samples
- Set-up the channels as above
- Switch off the STED shutter and use “Fast Live” to find and focus the sample
- In the X_Y window setup the acquisition parameters: number of pixels, zoom, averaging/accumulation/speed. For best results set pixel size to be better than Nyquist (eg 1024, zoom 5)
- Try to keep excitation laser intensity low (use slower speed and or accumulation if possible)
- Open STED shutter and adjust STED parameters
- Capture image

Image Format

Use the XY panel to Set

- Image format (1) 1024 x 1024 is typical but depends on image requirements
- zoom factor (2) - (If required, move the Zoom Area using the arrow icons, NB Zooms above 4 results in empty magnification at 1024 x 1024 pixels)
- Averaging (3) - required to give you sufficient image quality:
  - line averaging for live imaging
  - line or frame averaging for fixed cells
- Pinhole (4) - is preset at 1AU but may be adjusted to change Z volume

Z-Stacks

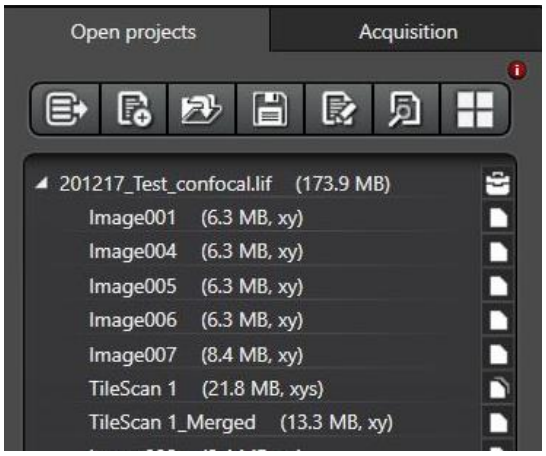
- Adjust focus in "live" mode to start of Z- stacck and select Begin (1)
- Adjust focus in "live" mode to end of Z- stacck and select End (2)
- Set step size - the "optical section" size (z) can be read from the X-Y panel or the "+" button will allow Nyquist settings to be applied

When finished with Z stack mode there is a "Trash" button to remove the stack settings

Capturing Images

Once the setup is compplete the image is recoreded using either “Capture Image” for a single image or use “Start” for an image sequence (Z-stack, tiling or time series)

Saving Data



- Images are stored in a library (.lif file format)
- Every time Capture Image or Start is pressed the image is added (but NOT SAVED) to the library in the “Open projects” tab.
- Right clicking on the individual image names allows renaming or deleting.
- To save the images click the “Save” icon above.
- The first time it will prompt for a location and the library name.
- Then every time you capture an image press the “Save” icon to update the library or you could lose your data.

Shutdown Procedure

- In the Configuration tab and laser config, **switch off lasers**
- Close LASX
- Update booking if necessary.
- Remove your samples & clean objective lenses with fresh lens tissue
- Remove safety cover from condensor
- Clear up the desk
- Save files onto the server
- On the STED Laser power unit - Switch off:
    - Laser Keys
    - Power buttons
- On the central power unit - Switch off:
    - Laser Key
    - Laser
    - Power
- Shut down PC or sign out for next user