

Leica SP8 STED

WLL: white light laser

TL: transmitted light

RL: reflected light

CS: confocal scanning

PMT Trans: transmitted light detector

FLUO filter tube: DAPI (blue); FITC-LP (green); RHOD (red)

Lasers

Excitation Lasers:

405 Diode 50 mW (continuous): 405 nm

Ar laser 65 mW (continuous): 458, 476, 488, 496, 514 nm set 30% at configuration

SuperK WLL 1.5 mW (pulsed): 470-670 nm 78 Mhz; set 70% at configuration

Notes: I always suggest using WLL since it is not strong power and less bleaching.

At configuration page set 30% for Ar and 70% for WLL.

Start with < 10% excitation laser power and increase if needed.

Double excitation laser power when switch from confocal mode to STED mode.

STED 3X Lasers:

592nm (continuous), 660nm (continuous), 775nm (pulsed)

STED 3X CW 592 STED: Vortex donut (FWHM): $xy < 80$ nm; $z =$ confocal

592 STED: Z donut (FWHM): $xy < 150$ nm; $z < 175$ nm

660 STED: Vortex donut (FWHM): $xy < 80$ nm; $z =$ confocal

660 STED: Z donut (FWHM): $xy < 150$ nm; $z < 175$ nm

STED 3X gated

592 STED: Vortex donut (FWHM): $xy < 50$ nm; $z =$ confocal

592 STED: Z donut (FWHM): $xy < 130$ nm; $z < 130$ nm

660 STED: Vortex donut (FWHM): $xy < 50$ nm; $z =$ confocal

660 STED: Z donut (FWHM): $xy < 130$ nm; $z < 130$ nm

STED 3X pulsed

775 STED: Vortex donut (FWHM): $xy < 50$ nm; $z =$ confocal

775 STED: Z donut (FWHM): $xy < 130$ nm; $z < 130$ nm

Notes: CW-Continuous Wavelength

Only SuperK WLL and 775 STED are suitable for STED pulsed.

775 STED can only be used in STED pulsed.

STED gated means gated detection and is compatible with all STED lasers.

STED CW and STED gated are compatible with all excitation lasers.

At Laser configure page set 100% for STED laser 775nm, 80% for STED lasers 592nm and 660nm.

Best resolution is achieved by STED pulsed: $xy < 50$ nm; $z < 130$ nm

Suggested laser power: 5% (775) for STED pulsed; >50% (592/660) for STED CW

Objective Lens

10x: air-immersion

20x: multi-immersion (air and oil) retractable, suggested for confocal imaging

40x (not installed): oil-immersion, suggested for high magnification confocal imaging

100x: oil-immersion, the only STED-compatible lens for STED imaging

Notes: 20x lens has lock/VS retractable switch, lock mode is not good for imaging

STED imaging must use 100x lens.

Microscope Mode

BF transmitted:	bright field imaging
DIC transmitted:	contrast enhanced imaging
POL transmitted:	polarized light imaging
Fluo incident:	conventional fluorescence imaging
Fluo DIC:	contrast enhanced fluorescence imaging with detectors
CS:	confocal scanning with detectors

Notes: Use transmitted mode or Fluo incident mode with eyepiece to find sample at low mag (first 20x lens), switch to 100x lens to locate samples and switch to CS mode to live image and collect data with detectors.

Detectors

HyD: good for low fluorescence intensity; smart gain sweet spot 0-200%

PMT: good for high fluorescence intensity; smart gain sweet spot 300-850V

Notes: PMT-PhotoMultiplier Tube, much faster than CCD/CMOS.

HyD is more sensitive than PMT and less noise.

HyD has three modes: Standard, Counting, and BrightR.

BrightR is just to get good-looking images and not accurate on pixel intensity.

BrightR should not be used for quantitative imaging.

Sample preparation

- Must use #1.5 cover slip (0.17mm thickness)
- Please refer to manufacture sample preparation guide for more details on sample preparation, e.g. dye selection.

System starts up

- Log in Badger and enable the Leica STED.
- Turn on **Fluorescence source (#1)**. It is white light excitation source (not laser) for conventional light/inverted fluorescence microscopy and epi-fluorescence.
- Turn on **PC/microscope (#2)**, let the computer and microscope power up.
- Turn on **Scanner power (#3)**.
- Turn on **Laser power (#4)**.
- Turn **Laser Emission key (#5)** to switch on laser.
- The white LED light source above the sample stage is for transmitted microscopy (e.g. eyepiece).
- Log in computer: ASRC-user, no password.

Software starts up

- Double click **LAS X** icon on desktop.
- In configuration pop up menu:
 - Confocal mode only:
 - Machine.xlhw
 - DMI8
 - STED off

- STED mode:
 - Machine.xlhw
 - DMI8
 - STED on
- Imaging Processing only:
 - Simulator SP8
 - DMI8
- It takes a few minutes for LAS X to start. Choose 100x objective lens on microscope.
- Select **Configuration** tab and select **Laser Config** to turn on laser needed in experiment:
 - Turn on 405nm diode if needed (this is the shortest excitation wavelength).
 - Turn on Argon if needed, adjust power to 15-30%.
 - Turn white light laser (WLL), adjust power to 70%.
 - Turn on three STED lasers if needed: adjust 592nm and 660nm STED power to 80%, 775 STED to 100%.

Note: in case STED 592nm or 660nm laser cannot be turned on due to interlock issue, you can turn off and turn on emission key for that laser.

Note: in case STED 775nm laser cannot be turn on due to WLL frequency issue, you WLL must be set at **80 MHz** in order to turn on STED 775nm laser: **Acquire** > SwitchToWLL > right above wavelength selector bar.
- Make sure 100x objective lens is selected and at least STED 592nm laser is on and warmed up (turn bright green to grey-green). STED 592nm laser is the main laser; the other two STED lasers are controlled by this one.
- Select **STED** in **Configuration** tab, click **Beam Align** to start automatic laser alignment between excitation and STED lasers. Alignment is done in a few minutes and return “success” message.
- Select **Acquire** tab, choose Acquisition Mode, e.g. xyz.
- Standard settings:
 - XY: 1024 x 1024 or 512 x 512
 - Speed: 400 Hz
 - Bidirectional X: on
 - Zoom factor: 1
 - Set appropriate averaging and accumulation: start with 1 for both.

Samples loading

- Switch to 20x lens and apply a small drop of oil to the cover glass side, load glass slide to sample stage.
- Use transmitted mode or Fluo incident mode with white excitation light source (not laser) eyepiece to locate sample at low mag fist (20x lens) by moving stage with XY control and to focus on samples with the course/fine Z wheel on microscope.
- Switch to 100x lens to locate and focus samples
- Switch to CS mode to live image and collect data with detectors.

Optics & detectors configuration

- **Project** tab: give project name and data saving directory at bottom of the tab. Then create a new project (autosave is recommended) from the top functions buttons, you can also load saved project if needed.
- **Acquire** tab: switch to WLL/conventional laser panel, select laser line, emission collection, channels for display, etc. This is starting from scratch.

- **Acquire** tab: standard optics&detector configurations can be load from **Load/Save: single setting**, right above laser selection panel. Alternative way to load optics&detector configurations is to load a previous save project in **Project** tab, choose the save image that you plan to repeat the experiment with the same optical and detector settings, right click the image and load the setting.
- Standard configuration:
 - TLD detector should be used for bright field imaging.
 - PMT detectors should be used for bright/strong signals
 - HyD detectors should be used for dim/weak signals and STED
 - Emission collection must be at least 10nm away from excitation and STED lines
 - Start with <5% laser power and 10% depletion (STED) laser
- Set **Begin**, **End**, and **Step** for Z-stack if you plan to do Z-stack collection.

Standard STED configuration

- Align lasers in **STED** of **Configuration** tab with 100x lens. Align lasers in the beginning, 20mins later and every 3 hours.
- Always use the HyD detectors.
- Start with 5% laser in confocal mode (STED laser is off) to obtain decent images, you may also need to select parameters for image format, speed, average, accumulation, etc.
- Once you are satisfied with confocal image, you can double excitation laser power and start with 10% STED laser.
Note: good results with standard samples: 10-30% 592nm, 10-40% 660nm, ~50% 775nm. These settings can be a good start points for user specific samples. Other settings/parameters that must be tuned to achieve good STED imaging are described in the following steps, for example, average, accumulation, gating.
- Pinhole should be ~0.75 AU for maximum resolution.
- For STED data acquisition, 4-6 line average and 2-3 frame accumulation are good choice to increase SNR.
- A good pair of dyes for co-localization: Atto647 and Alexa594 with STED 775nm.
 - If using more than one STED lasers in sequential mode, the sequential order must be from long wavelength to shorter one and scanning must be between frames.
 - If using only one STED with multi-dyes in sequential mode, scanning could be between lines or frames.
 - It is always a good practice to do frame/stack sequential, not line seq.
- For 775 nm STED, increase from 40%-60%-100%. For Z donut, increase from 20%.
- Gated imaging: 0.3-6 ns for confocal; 1-6 ns for STED (e.g. 1.5-6ns with HyD2). Gating is to remove excitation laser reflection.
- Balance depletion laser power and gating
- Test for depletion laser-sample interaction:
 - Configure and adjust channels in confocal mode
 - Turn off excitation line(s)
 - Start scan and turn up depletion laser while looking for signal, checking for any signal/artifact in image
 - Reset to confocal mode and check for any depletion laser-induced damage

Suggested STED acquisition setting (starting points):

Format: 1024 x 512

Speed: 400

WLL: 5-15%

STED 592: 10-30%

Pix size: 25-50 nm
Line Avg: 4-6
Frame Accu: 2-3

STED 660: 10-40%
STED 775: ~50%
HyD gating: 1.5-6 ns

Shutting down

- Turn off all lasers in LAS-X: first in **Acquire** tab followed by turning off laser in **Configuration**.
- Save data/project, exit program and shut down computer.
- Turn off **laser emission key #5** (if using the Argon laser wait 2-3 minutes before proceeding to next step)
- Turn off **laser power #4, scanner power #3** and **PC/Microscope switches #2**
- Turn off **Fluorescence source #1**
- Disable Leica STED in badger.
- Clean objective lens and working area, take all materials that you bring to the facility with you

LAS X offline program is available for download here (<https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/>). You can manipulate data/images with offline version.

Huygen Suites (deconvolution)

Huygen essential is for confocal images
Huygen professional is for STED