**Leica SP8 STED/Confocal Operation Guide**

* **Notes:**
	+ **The 100x oil lens is the only STED-compatible lens and should be used for STED imaging only!! Use the 40x oil for high magnification confocal imaging.**
	+ **The galvo z-stage is delicate; be gentle when loading and removing samples**
	+ **The focus controls on the microscope and joystick are for initial focus only. The z-galvo control on the panel must be used for imaging, e.g. setting up z-stack positions.**
	+ **This is only a guide. STED imaging is more complicated and sample-dependent than confocal imaging. Please contact CMRF staff for assistance.**
* **System start up**
* Enter name and start time in log book
* Turn on **FL6000** epi fluorescence lamp
* Turn on **PC computer** switch on table panel, let computer boot up
* Turn on **scanner power** and **laser power** buttons
* Turn on **laser emission** key
* Log onto computer: TCS user, password: leicasted
* **Software start up**
* Double click on **LAS-AF** icon
* In Configuration pop up menu:
	+ For confocal only mode select:
		- machine.xlhw
		- microscope: DMI 6000
		- STED **off**
	+ For STED mode select:
		- machine.xlhw
		- microscope: DMI 6000
		- STED **on**
* If tiling or multi-point imaging function are needed, when prompted **Initialize stage**. **Tilt back the condenser column before initializing!**
	+ Note: Tiling, multi-point and image rotation are not compatible with STED imaging
* Select **Configuration** tab at top of screen
* Select **Laser config**
	+ Turn on **405 diode** laser if needed
	+ Turn on **Argon laser** if needed (for CFP, YFP or photobleaching), adjust power to 15%
	+ Turn on **white light laser (WLL)** if needed, adjust power to 70%
	+ If performing STED imaging turn on **STED 660 nm** depletion laser, adjust power to 100% (laser will beep)
* Select **Acquire** tab
* If desired, in the menu to the right select a color scheme (**dark 3** works well) and change LUT to **glow over** (eliminates the green background when using HyD detectors)
* Select **Experiments** tab and configure save to location
* Select **Acquisition** tab
* For standard imaging select **xyz** in acquisition mode drop down menu
* General Settings:
	+ Set scan speed to **600** lps
	+ Turn bidirectional scanning **ON**
	+ Set averaging and accumulation as appropriate
* **Configure optical path**
	+ Select a general, simultaneous configuration from **Load/Save: single setting**
	+ Turn on lasers, set/adjust laser lines, emission bands, channels, etc. as desired
	+ **General configuration notes:**
		- Channels with bright signals (e.g. DAPI, reflected light) should be configured with **PMT** detectors
		- Channels with dim signals, and STED, should be configured with **HyD** detectors
		- Detection bands should be at least 5nm (shorter wavelengths) to 10nm (longer wavelengths) away from excitation lines
		- Start with ~5% laser power
* If sequential scanning is desired select **SEQ** button in upper left
	+ In the sequential menu select **+** to duplicate the current configuration to equal the total number of detection channels (i.e. if you have a 3 channels click **+** twice)
	+ For each channel, adjust the laser lines for the other channels to zero (do not turn off lasers) and turn off the detectors for the other channels
	+ Save configuration
	+ **Note: This is the best method for storing and recalling sequential configurations.**
	+ Configurations can also be reused from previously saved images:
		- Right click on image, select **properties** then **apply settings**
	+ **Note: Please see a CMRF staff member if you have any questions regarding configurations.**
* Choose **LIVE** button in bottom corner to start scan
* Adjust gain (and offset if using PMTs) on smart panel
	+ Ideally stay below 130% on HyD channels, and 800V on PMT channels.
	+ **Note: The HyD detectors are very sensitive to bright signals. Do not use with bright fluorescent channels. Overexposures will result in detector shutdown.**
* If performing a z-stack set **Begin**, **End** and **Step**.
* Select Capture to **Acquire** single channel or **Start** to capture all channels
* **General STED Considerations**
	+ Align lasers at the beginning, after 20 minutes and every 3-4 hours (or more often) thereafter
		- Select 100x lens (sample not needed)
		- In the **Config**, **STED** menu select **Align Beams**
	+ Use HyD detectors
	+ Start with ~10% depletion laser
	+ Pinhole should be ~0.75 AU for maximum resolution
	+ Use accumulate to allow lower detector voltages
	+ Use gating, start with 0.3 – 6 ns
	+ 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
	+ Always use Frame or Stack sequential, not line seq.
* **Shutting down**
* Turn off all lasers
* Save data, exit program and shut down computer
* Turn off **laser emission key** (if using the Argon laser wait 2-3 minutes before proceeding to next step)
* Turn off **laser power**, **scanner power** and **PC/Microscope switches**
* Turn off mercury lamp
* Record usage in log book.