**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 **1**
* Turn on **PC computer** switch **2** on table panel, let computer boot up
* Turn on **scanner power** **3** and **laser power** **4** buttons
* Turn on **laser emission** key **5**
* Log onto computer using your Hawk ID and Hawk password
* Turn on the **STED laser power 6 and key 7**
* **Software start up**
* Double click on **LAS-X** icon
* In Configuration pop up menu:
  + For confocal only mode select:
    - Machine-no STED.xlhw
    - microscope: DMI6000
    - STED **OFF**
  + For STED mode select:
    - Machine STED.xlhw
    - microscope: DMI6000
    - 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 configuration**
  + If performing STED imaging turn on **STED 660 nm** depletion laser, adjust power to 100% (laser will beep) **FIRST**
  + 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%
* 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 under Laser configuration in the configuration tab
* Save data, exit program and shut down computer
* Turn off the **STED laser key 7 and box 6**
* 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 mercury lamp **1**
* Record usage in log book.