

LEICA SP8 τ STED

Basic Operation

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STED Acquisition

System Basics

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Lasers

- 405nm
- Argon: 457nm, 488nm, 514nm
- WLL: 470nm-670nm
- STED: 592nm, 660nm, 775nm

Microscope

- The SP8 is mounted on an inverted stand, DMi8 from Leica
- Motorized XY stage with encoders
 - Motorized Z focus with encoders
 - Motorized turret of widefield cubes
 - Full incubation enclosure

Optics

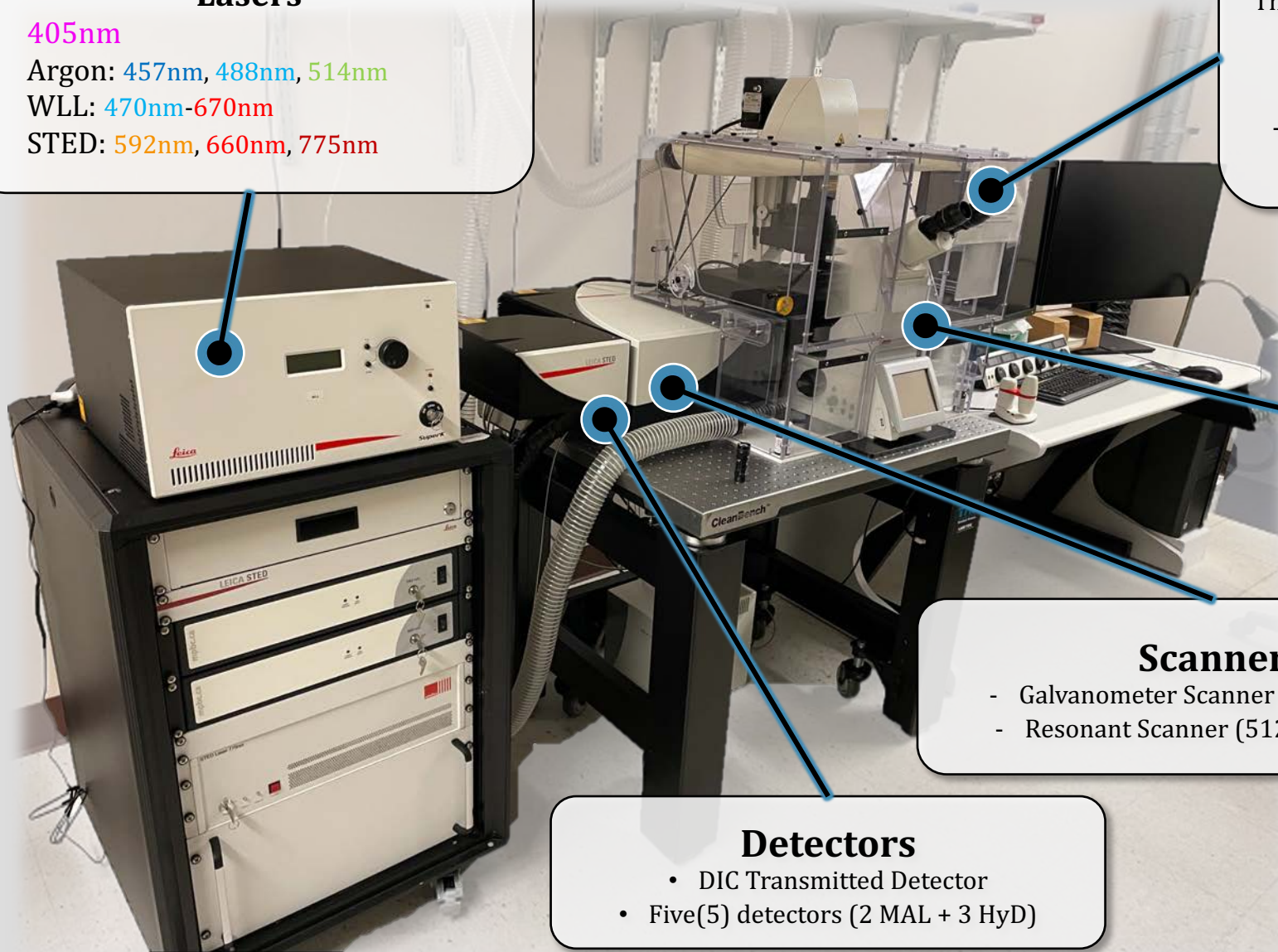
- HC PL Apo 10x/0.4
- HC PL Apo 40x/1.3 (oil)
- HC PL Apo 63x/1.4 (oil)
- HC PL Apo 93x/1.3 (glycerol)
- HC PL Apo 100x/1.4 (oil)

Scanner

- Galvanometer Scanner (max 8k x 8k)
- Resonant Scanner (512x512, 30fps)

Detectors

- DIC Transmitted Detector
- Five(5) detectors (2 MAL + 3 HyD)



Software Start

A. Overview

The Leica SP8 STED runs in *LAS-X software*. The system starts in a standard configuration, but requires a few steps **each time** the system starts.

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Software Navigation

Scanning Parameters

Multi-dimensional Parameters

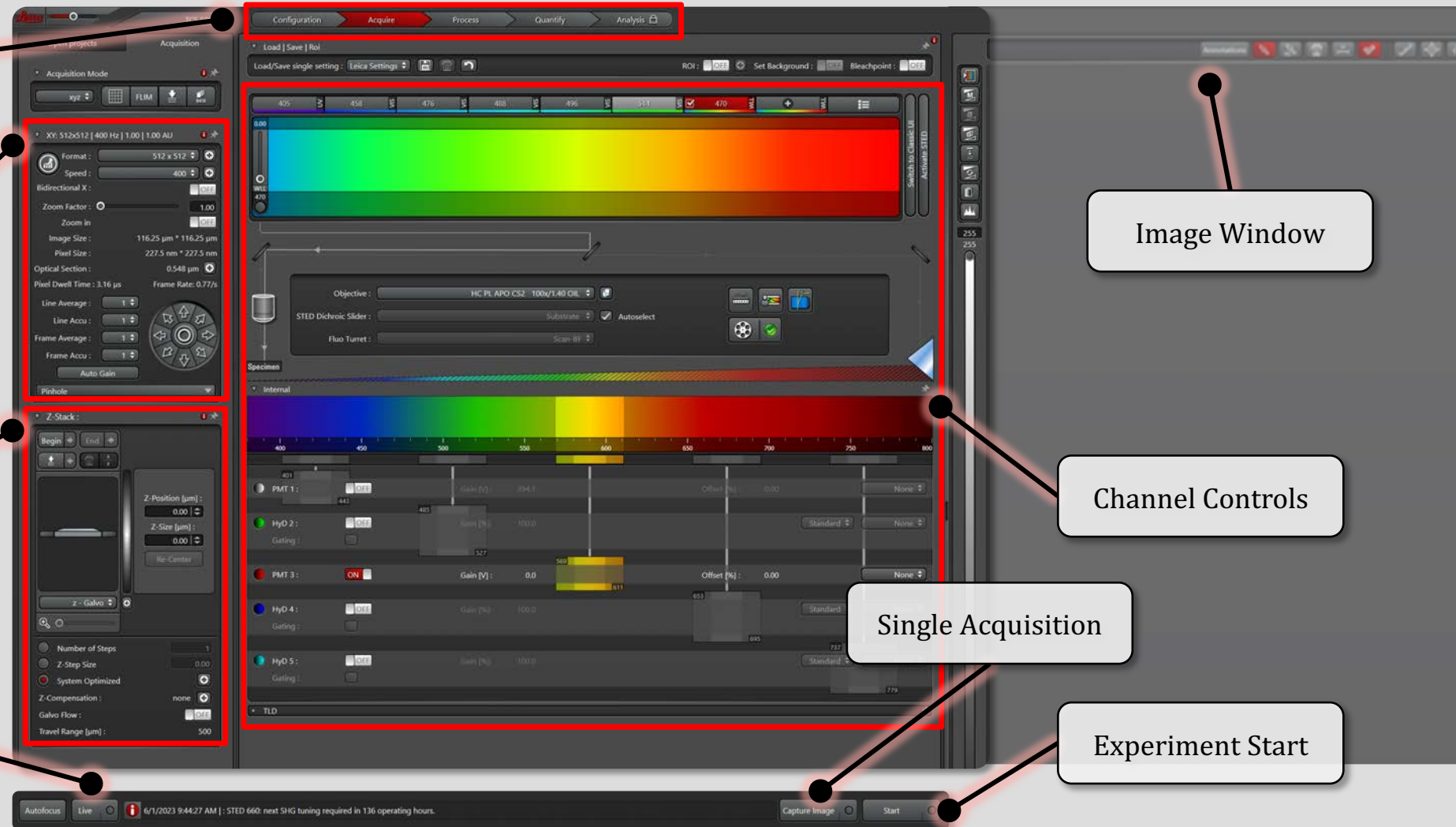
Live Scan

Image Window

Channel Controls

Single Acquisition

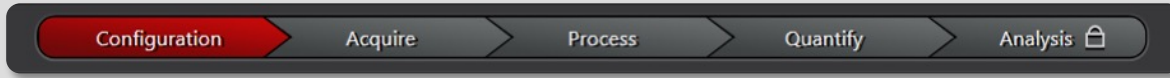
Experiment Start



Software Start

B. Initial Steps

- 1
- 2
- 3
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1 Turn on Lasers

WLL: Turn ON and set to 70%
STED Lasers: Turn on required lines and match WLL %

2 Align Beams

Click "ALIGN BEAMS" to calibrate the system for STED

Sample Finding and Focus

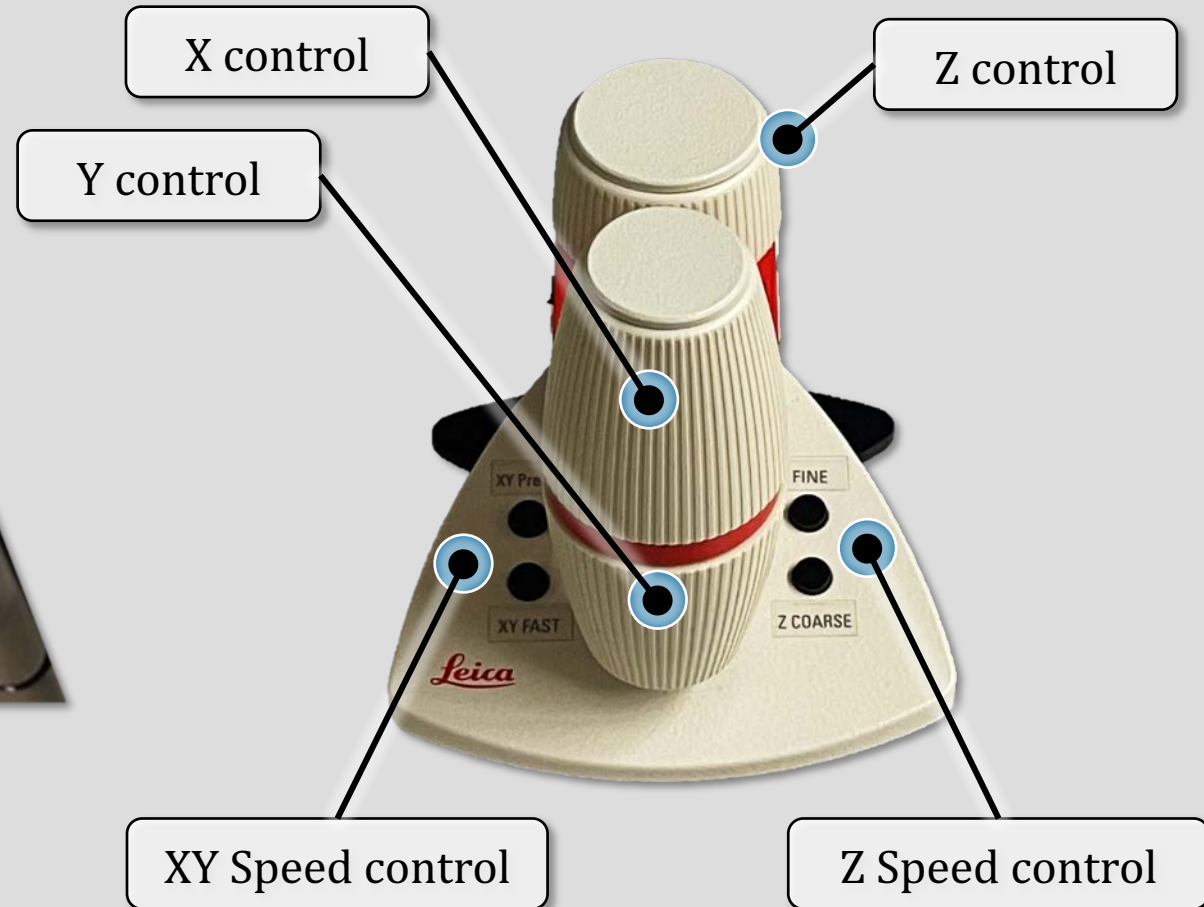
A. Stage Placement

The Stage Adapter can accommodate

- Slides
- Multi-well chambers



Joystick can control Stage (XY) and Focus (Z)



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Sample Finding and Focus

B. Eyepoint Viewing

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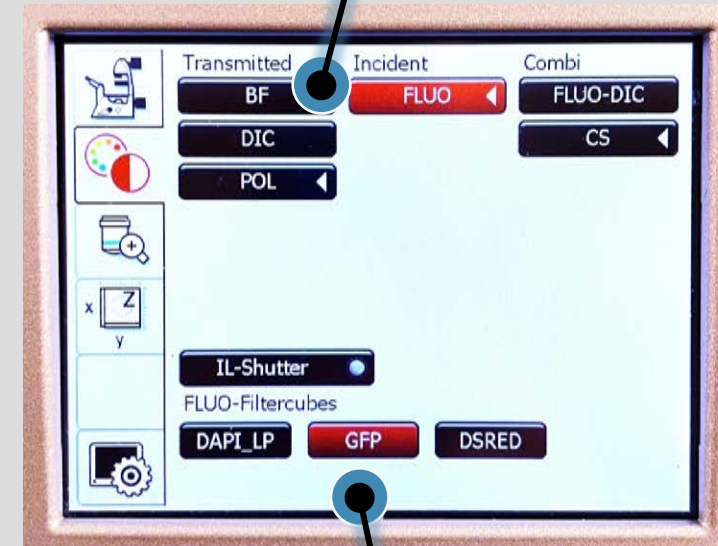
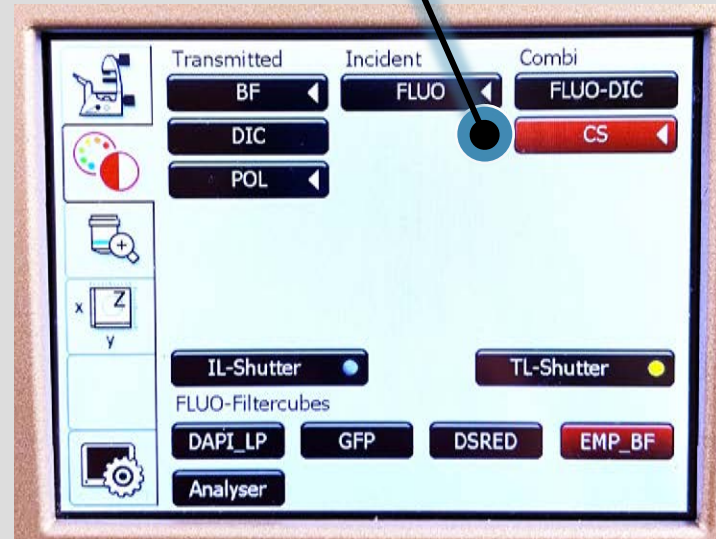
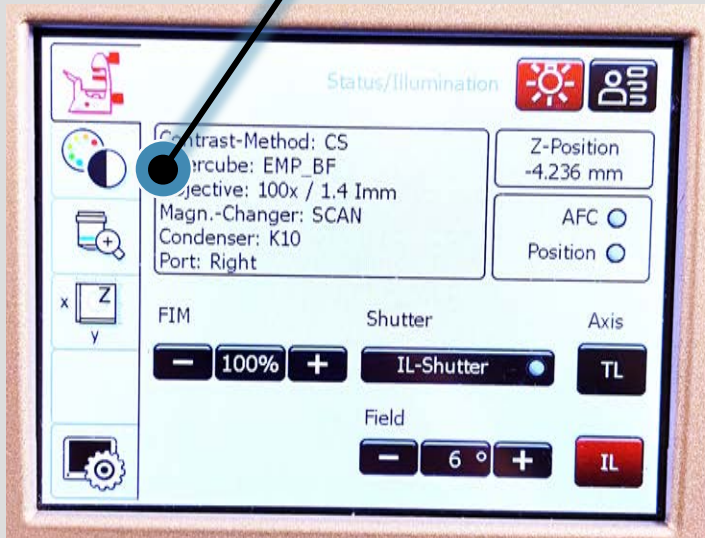
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Confocal Scanning Mode

Eyepiece Widefield Mode

Filters and Lightpath



Current Filter Set

Scanning and Capture

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Scan Array Size

Image Data

Average/Accumulate

Collect image multiple time to improve signal to noise

Pinhole Size

Controls optical section thickness and removes out of focus light

Live/Capture/Start

LIVE: constant scanning for optimization and setup
 CAPTURE: collects a single image with the current settings
 START: Begin Experiment

XY: 512x512 | 400 Hz | 1.00 | 1.00 AU

Format: 512 x 512

Speed: 400

Bidirectional X: OFF

Zoom Factor: 1.00

Zoom in: OFF

Image Size: 116.25 μ m * 116.25 μ m

Pixel Size: 227.5 nm * 227.5 nm

Optical Section: 0.548 μ m

Pixel Dwell Time: 3.16 μ s Frame Rate: 0.77/s

Line Average: 1

Line Accu: 1

Frame Average: 1

Frame Accu: 1

Auto Gain

Pinhole

Live Capture Image Start

Channel Settings

A. Setup

Configure Channels

Add Channel from Dye List

CHOOSE PMT or HYD

Select Multi-channel Method

More information about methods on next slide

The screenshot displays the 'Channel Settings' interface with the following components:

- Channel List:** A table with columns for dye name, PMT/HyD selection, and a plus/minus button.

ALEXA 647	PMT	+
ALEXA 568	HyD	+
ALEXA 488	HyD	+
	PMT or HyD	-
- Method Selection Panels:** Four panels showing different multi-channel methods:
 - Line sequential, 2 sequences:** Shows ALEXA 488 (green), ALEXA 647 (magenta), and ALEXA 568 (red) with their respective fluorescence intensity histograms.
 - Frame or stack sequential, 2 sequences:** Shows the same three dyes with histograms.
 - Line sequential, 3 sequences:** Shows ALEXA 488 (green), ALEXA 568 (red), and ALEXA 647 (magenta) with histograms.
 - Frame or stack sequential, 3 sequences:** Shows the same three dyes with histograms.
- Controls:** Each method panel includes 'Yield' and 'Crosstalk' sliders, and 'Edit ...' and 'Apply' buttons.

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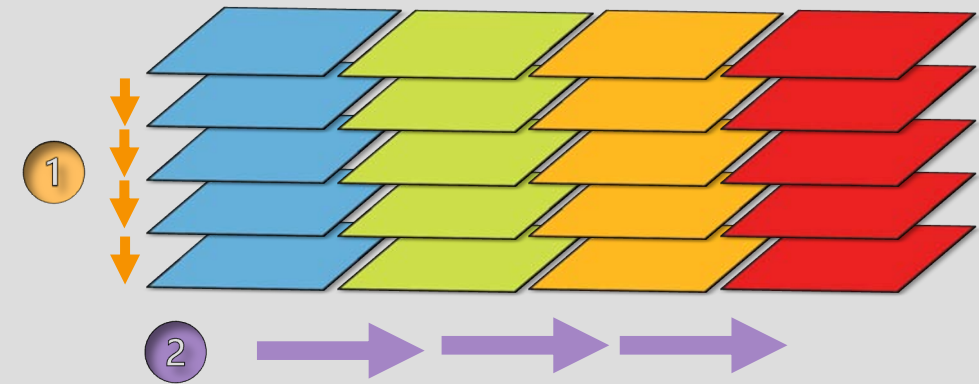
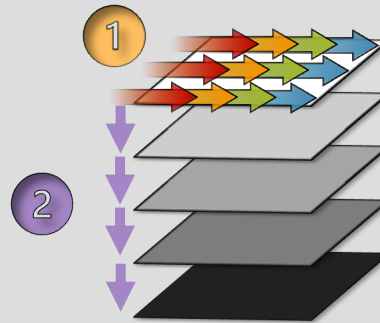
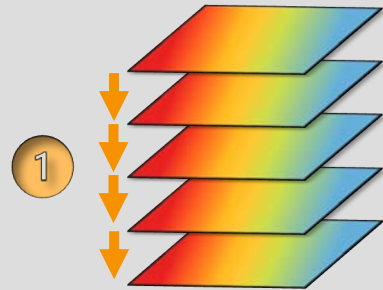
Channel Settings

B. Method

Simultaneous

Line Sequential

Frame/Stack Sequential



All channels collected at the same time

- FASTEST METHOD
- HIGH BLEEDTHROUGH
- Incompatible with multi-color STED

Each channel collected separately, one line of the image at a time.

- NO BLEEDTHROUGH
- SLOWER SCANNING
- Incompatible with multi-color STED

Each channel collected separately, one frame/stack of the image at a time.

- NO BLEEDTHROUGH
- MULTI-COLOR STED
- SLOWEST SCANNING

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Channel Settings

C. Controls

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Active Laser(s) and Power

Active Detector(s) and Gain

Seq. 1 Seq. 2 Seq. 3

Between Lines

Between Frames

Between Stacks

Load

Save

Multi-Channel Mode and Channel Selection*

- If a sequential mode is chosen, Select the specific channel for live viewing (e.g. SEQ 1)



Internal

Channel	Gain [V]	Gain [%]	Offset [%]	Ref. Line [nm]
PMT 1	0.0	0.00	0.00	None
HyD 2	100.0	100.0	0.30	ATTO 488
PMT 3	603.6	100.0	0.00	ALEXA 568
HyD 4	100.0	100.0	0.00	ALEXA 647
HyD 5	100.0	100.0	0.00	None

*The control windows above show three simultaneous channels being active

Channel Look Up Table Adjustment



Z-Stack Acquisition

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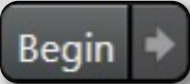
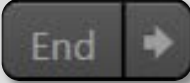
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To set stack parameters:

1. Focus to top; click 
2. Focus to bottom; click 
3. Confirm step size and adjust if needed

Visual Navigation
Slide  to see full stack

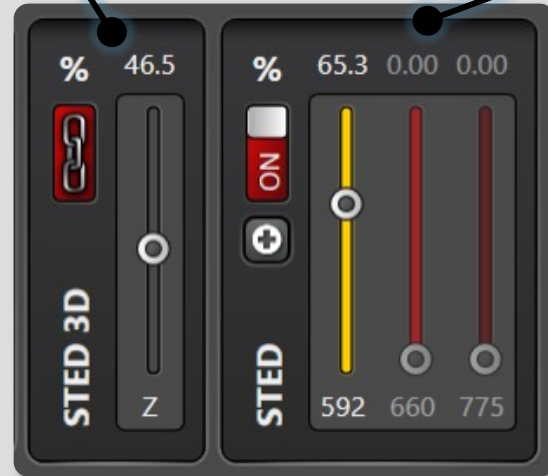
Stack Details

Step
Size/Number


STED

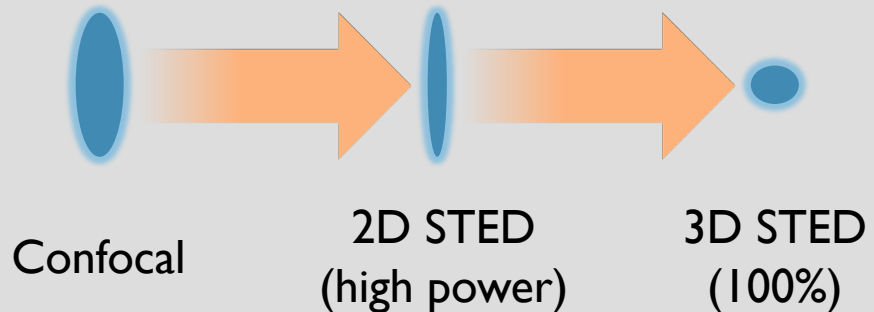
% of Depletion Line diverted
to 3D beam shaping

Depletion Line and Power



STED Multicolor Tips

1. STED Depletion Lines will photobleach any dyes that emit near that wavelength
Example: STED 592nm will bleach dyes like ATTO568, and mCherry
2. Optimize depletion power settings in a field of the sample you **DO NOT** plan to image with other wavelengths
3. Collect multiple colors using  **Between Stacks**
4. Configure your collection order from furthest RED to furthest BLUE
Example: Collect AlexaFluor 647 before ATTO568 before GFP



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