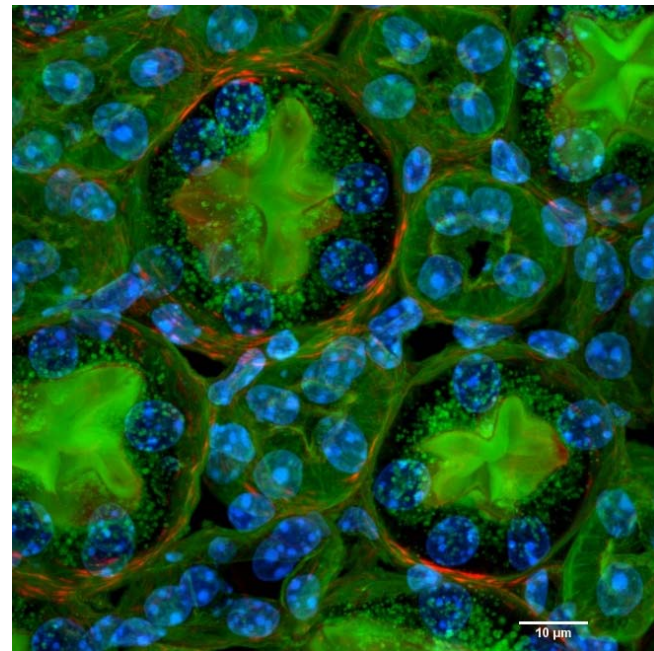


# Imaging Beyond the Basics: Optimizing Settings on the Leica SP8 Confocal



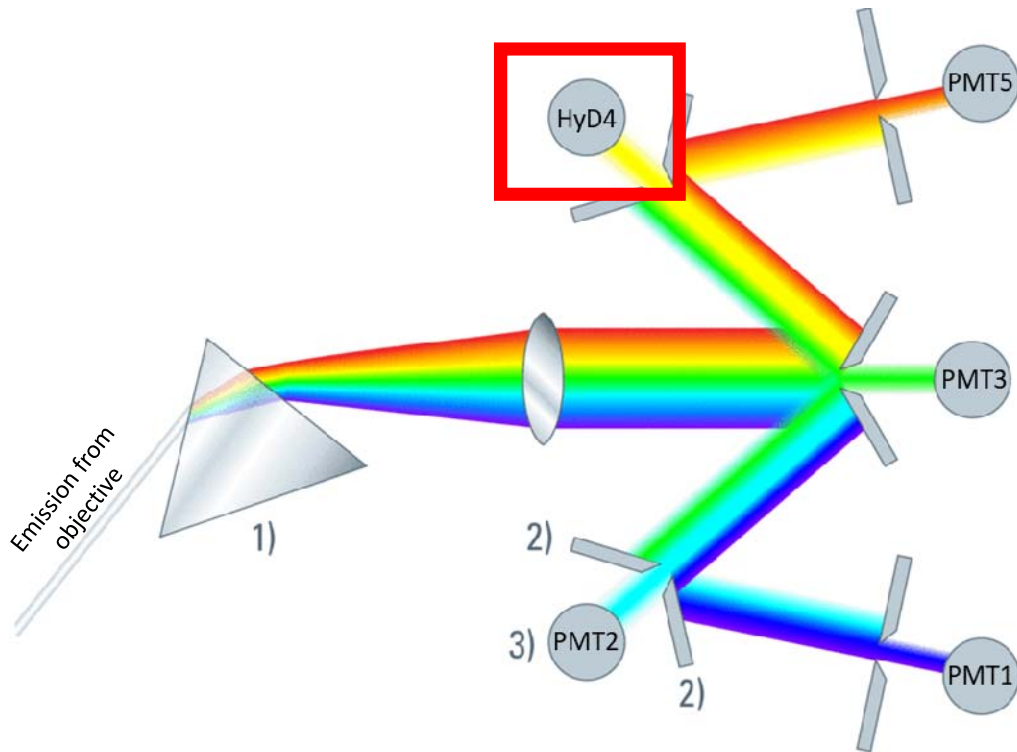
# Today's Goal:

- Introduce some additional functionalities of the Leica SP8 confocal
  - HyD vs. PMT detectors
  - Dye Assistant
  - Scanning “By Frame” vs. “By Line”
  - Bi-directional and resonant scanning
  - Optimizing resolution and pixel size
  - Using the Histogram and QuickLUT
  - Linear Z compensation



# Spectral Detection with the Leica SP8

- Light emitted from the sample passes through a prism
- There are 5 detectors in the scan head
- Movable slits and mirrors in front of the detectors determine what wavelengths are captured



# PMT vs. HyD Detectors

## Photomultiplier tubes (PMT): Detectors 1,2,3,5



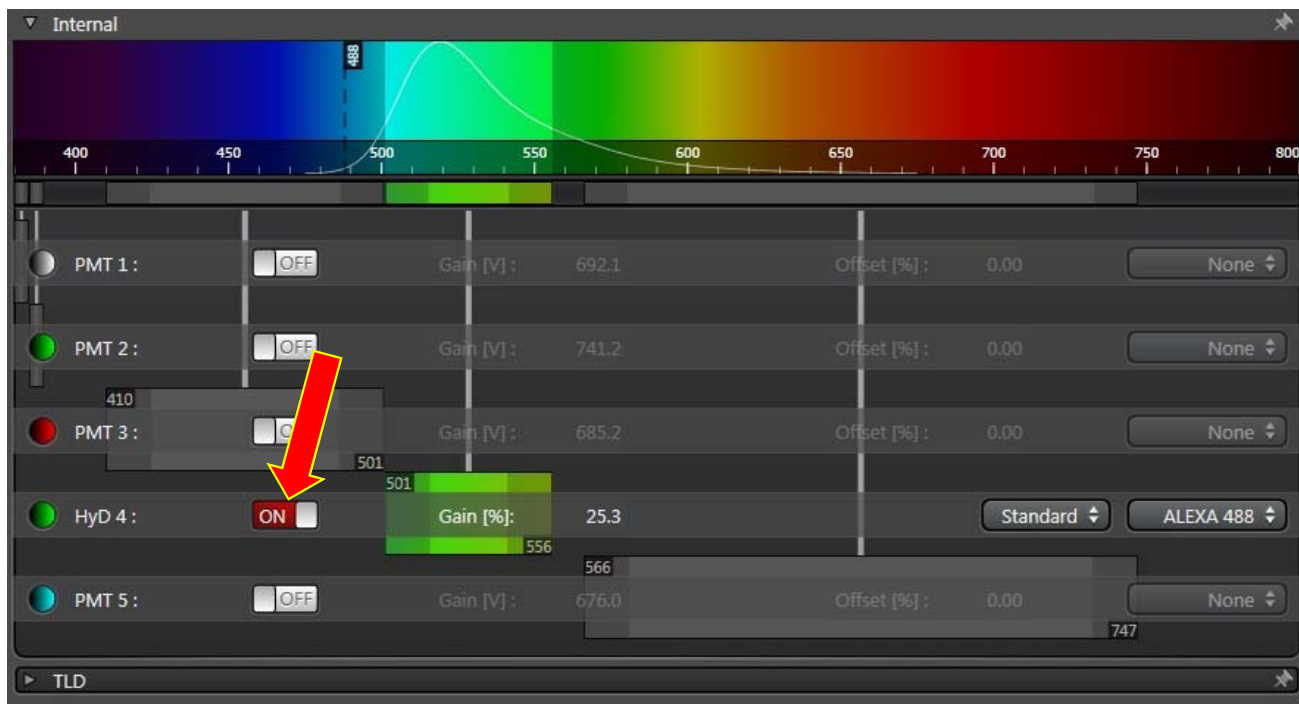
- Convert photons to photoelectrons
- Low sensitivity (30% QE)
- Inexpensive

## Hybrid Detector (HyD) Detector 4:



- Cross between PMT and APD
- More sensitive (45% QE) – Use for low light applications
- Lower Noise
- Expensive
- Can be damaged

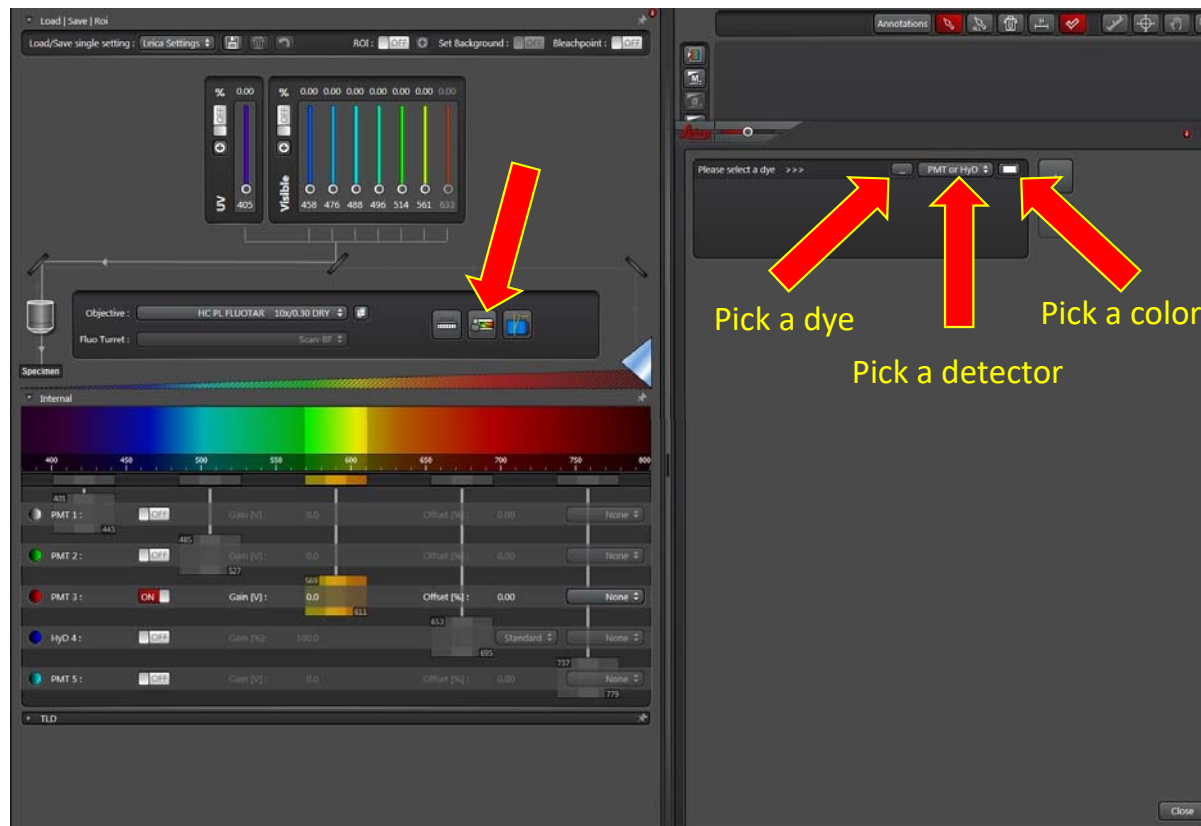
# Using the HyD Detector on the SP8



- Auto shutoff will engage if HyD is exposed to too much light
- Start with low laser power and gain
- Gain is in % (not V)

# Dye Assistant

- A wizard to help you configure the excitation and detection settings quickly



# Dye Assistant

- A wizard to help you configure the detector quickly

The screenshot displays the Dye Assistant software interface, which is used for configuring a detector. The interface is divided into several sections:

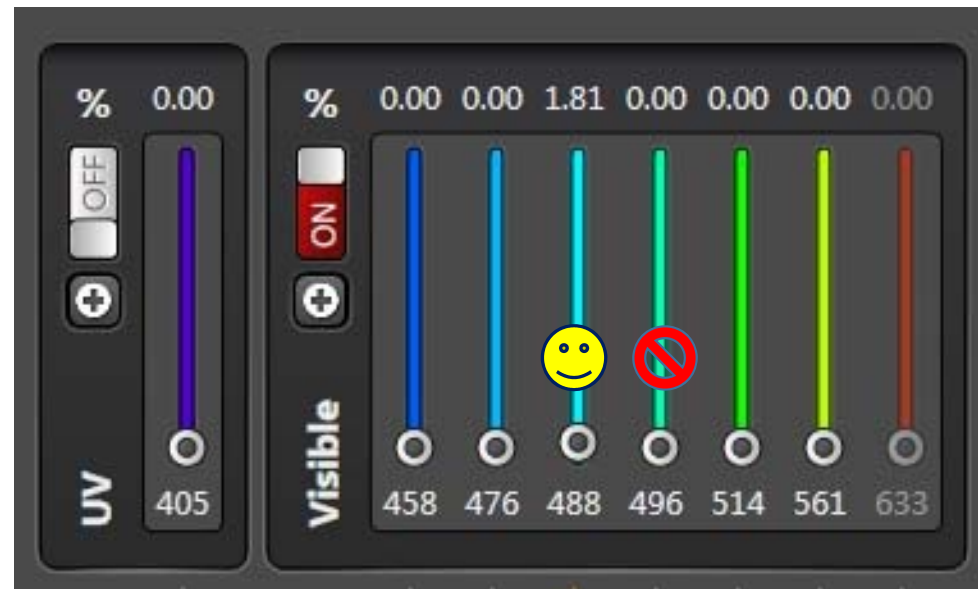
- Top Left:** A control panel with sliders for 'UV' and 'Variable' settings, and a 'Specimen' section with a color calibration bar.
- Bottom Left:** A detailed configuration panel for the detector, showing parameters for five PMTs (PMT 1 to PMT 5) and a TLD (Tandem Laser Diode) section. Each PMT has a 'Gain [V]' and 'Offset [mV]' setting.
- Right Panel:** A configuration wizard for the detector, showing settings for ALEXA 568, ALEXA 488, and DAPI. It includes a 'Dye Yield' section with color-coded bars and a 'Crosstalk' section with line graphs showing the relationship between different dyes. The wizard offers five scan configurations: 'None sequential', 'Line sequential: 2 sequences', 'Frame or stack sequential: 2 sequences', 'Line sequential: 3 sequences', and 'Frame or stack sequential: 3 sequences'.

Annotations on the right side of the image highlight the differences between scan methods:

- A blue bracket groups the 'None sequential' and 'Line sequential: 2 sequences' options, labeled 'Simultaneous scan'.
- A blue bracket groups the 'Frame or stack sequential: 2 sequences' and 'Line sequential: 3 sequences' options, labeled 'Sequential: 2 scans'.
- A red arrow points to the 'Line sequential: 3 sequences' option, labeled 'Apply the detector settings you want'.
- Two blue triangles on the far right are labeled 'crosstalk' and 'speed', indicating that simultaneous scans have lower crosstalk and higher speed compared to sequential scans.

# Dye Assistant Note

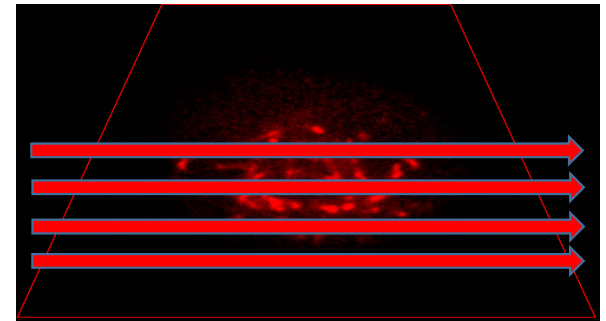
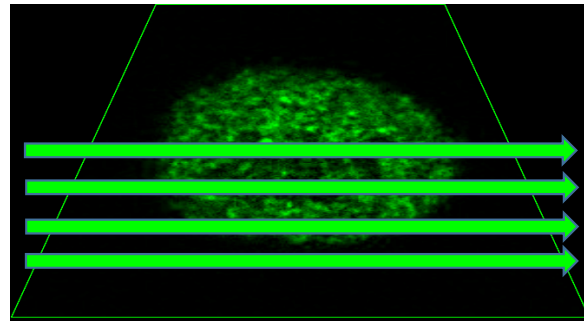
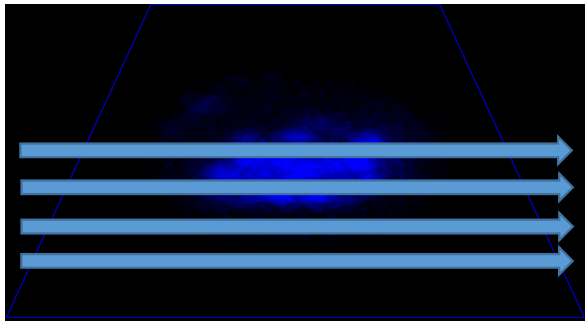
- The Wizard will choose the 496 nm laser for Alexa 488
- While 496 nm is closer to the actual excitation peak of Alexa 488...
- ....The 488 nm laser is much stronger
- You will have to manually choose 488 nm excitation for this channel





# Scanning Sequentially “By Line”

- Scans 1 line of each channel, one after the other
- All channels will appear to be captured simultaneously
- Wavelength sliders cannot move between channels during this type of scan – NO MOVING PARTS
- Fastest method of sequential scanning
- Slightly less photon efficient than “By Frame”



The screenshot shows a microscopy software interface with several key components:

- Left Panel (Acquisition Mode):** Contains settings for XY (512x512), Speed (400), Bidirectional X, Zoom Factor (2.54), Image Size (114.26 μm x 114.26 μm), Pixel Size (223.61 nm x 223.61 nm), Optical Section (1.038 μm), Pixel Dwell Time (1.2 μs), Frame Rate (0.258/s), Line Average, Line Accu, Frame Average, Frame Accu, Rotation (0.00), Pinhole, Z-Stack (Begin, End, Z Position, Z Size, Re-Center, z-Galvo), and Sequential Scan (Seq 1, Seq 2, Seq 3).
- Top Panel (Configuration):** Includes Load/Save/ROI, Load/Save single setting, Lencia Settings, ROI, Set Background, and Bleachpoint.
- Center Panel (Spectral):** Features a spectral graph with a color scale from 400 to 800 nm. Below the graph is a table of detector settings:

Detector	Gain (%)	Offset (%)
PMT 1	734.8	0.00
PMT 2	64.4	0.00
PMT 3	73.0	0.00
HyD 4	100.0	Standard
PMT 5	8.0	0.00

**Right Panel (Image View):** Displays a 2x2 grid of images: top-left (blue channel), top-right (green channel), bottom-left (red channel), and bottom-right (merged image). A vertical color bar on the left of this panel shows a gradient from blue to red.

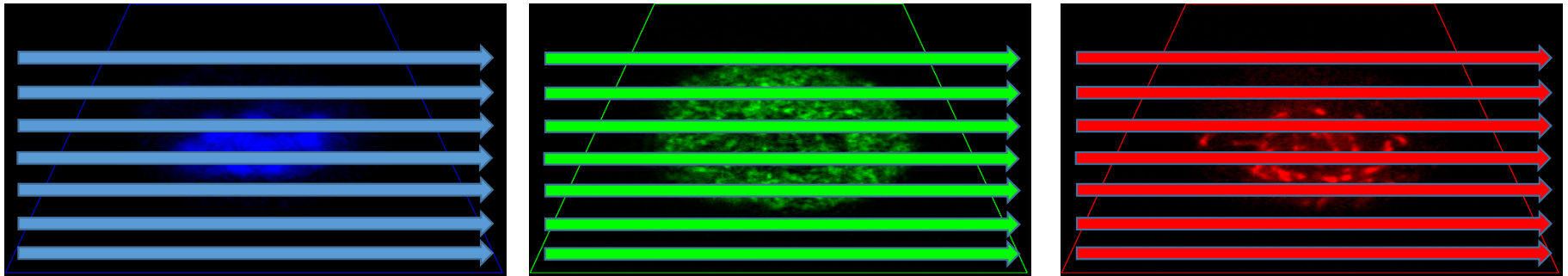
**Annotations:** Three yellow arrows point from the text below to the wavelength sliders for PMT 1, PMT 2, and PMT 3 in the spectral graph. A yellow box highlights the 'Sequential Scan' section, with an arrow pointing to the 'Seq 1' button.

**Text:** "All wavelength slider positions must not change between sequences"

**Bottom Panel:** Includes Autofocus, Stop, Capture Image, Start, and a status bar showing "Preview x=512 y=512 (786 KB) Size 114.26 μm x 114.26 μm".

# Scanning Sequentially “By Frame”

- Scans entire image of one channel before moving to the next channel
- All channels will be captured one by one
- Wavelength sliders can move between frames during this type of scan –MOVING PARTS
- Slowest method of sequential scanning
- More range/flexibility in setting emission bandwidth, more photon efficient



- One application would be to use the HyD detector for multiple channels

The screenshot displays a complex software interface for fluorescence microscopy. On the left, the 'Acquisition' panel includes settings for 'Acquisition Mode', 'Format' (512 x 512), 'Speed' (900), 'Zoom Factor' (2.54), 'Image Size' (114.26 μm x 114.26 μm), 'Pixel Size' (223.61 nm x 223.61 nm), 'Optical Section' (1.038 μm), 'Frame Rate' (0.124/s), and 'Z-Stack' parameters. The central panel shows 'Load | Save | ROI' options, a 'Specimen' diagram, and a spectral graph with a 'Wavelength' slider. Below the graph is a table of PMT settings:

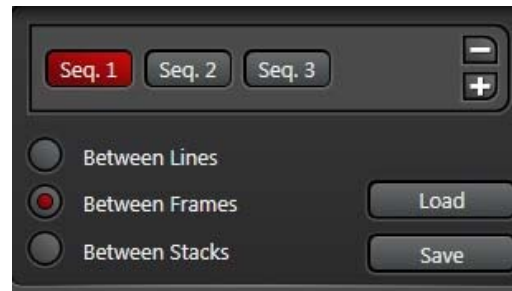
Channel	Gain [V]	Offset [mV]
PMT 1	644.8	0.00
PMT 2	639.2	0.00
PMT 3	527.8	0.00
HyD 4	100.0	Standard
PMT 5	0.0	0.00

At the bottom left, a yellow box highlights 'Seq 1', 'Seq 2', and 'Seq 3' buttons, with an arrow pointing to the text: 'Wavelength slider positions can be changed between sequences'. The right side of the interface features a 2x2 grid of live images: top-left shows blue fluorescence, top-right shows green fluorescence, bottom-left shows a merged blue and green image, and bottom-right shows a zoomed-in view of the green channel. The bottom status bar shows 'Series005 x=512 y=512 z=1 (786 KB)' and 'Size: 114.26 μm x 114.26 μm'.

# Acquisition Speed Comparison

- 400 lps scan speed
- 512 x 512 pixels
- 3 channels
- 10 um z range, 30 planes

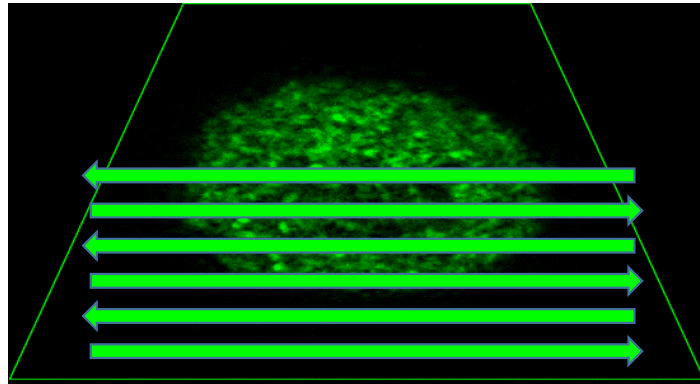
By Frame	By Line			
3 min 46 sec	1 min 52 sec			



“Between Stacks” not recommended

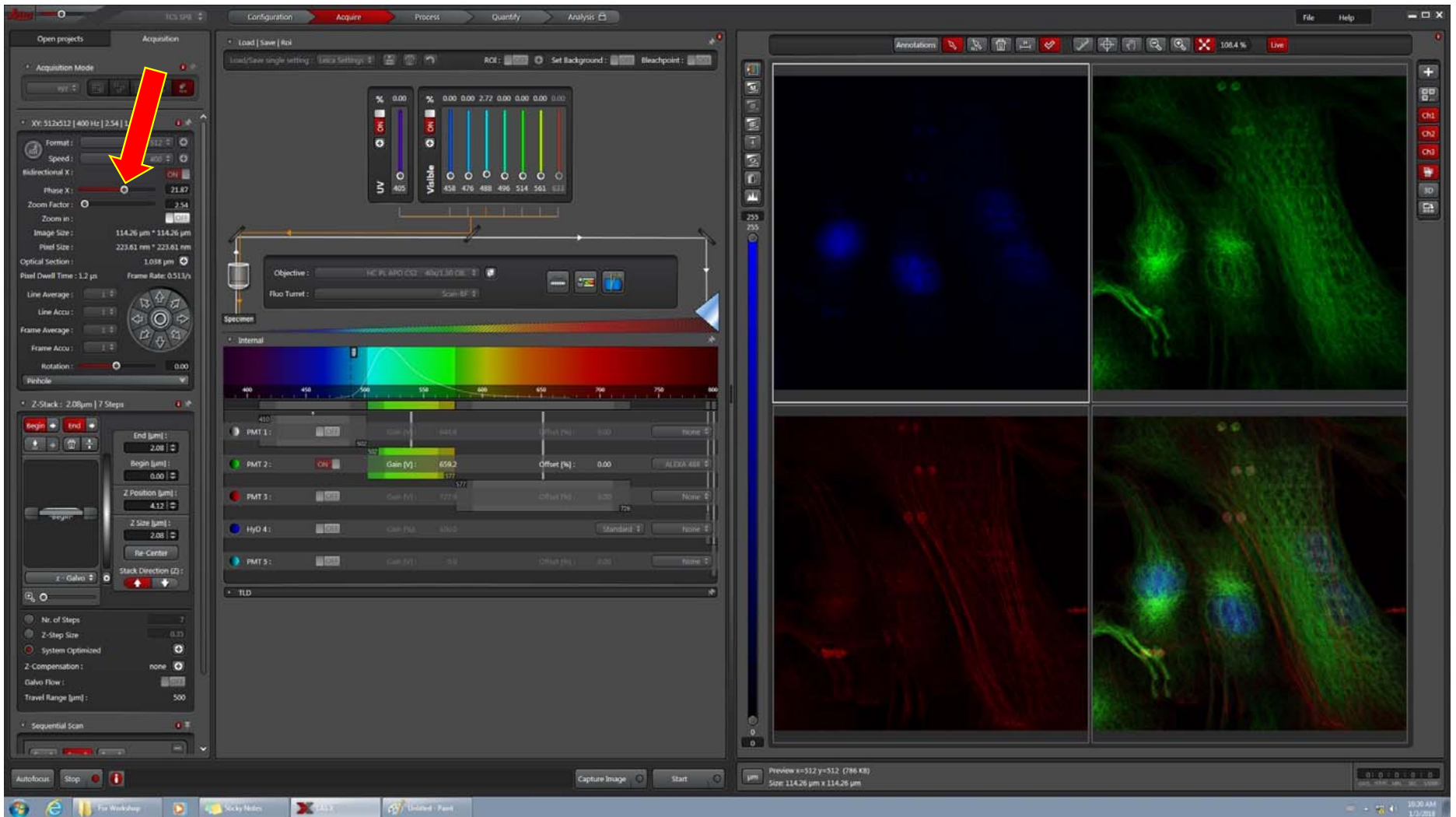
# Bi-Directional Scanning

- Capture is usually done in only one direction of the beam scan
- Imaging can also be done on the return pass of the beam
- 2X as fast
- Reverses the direction in which pixels are recorded
- Alignment of the scan phase is needed

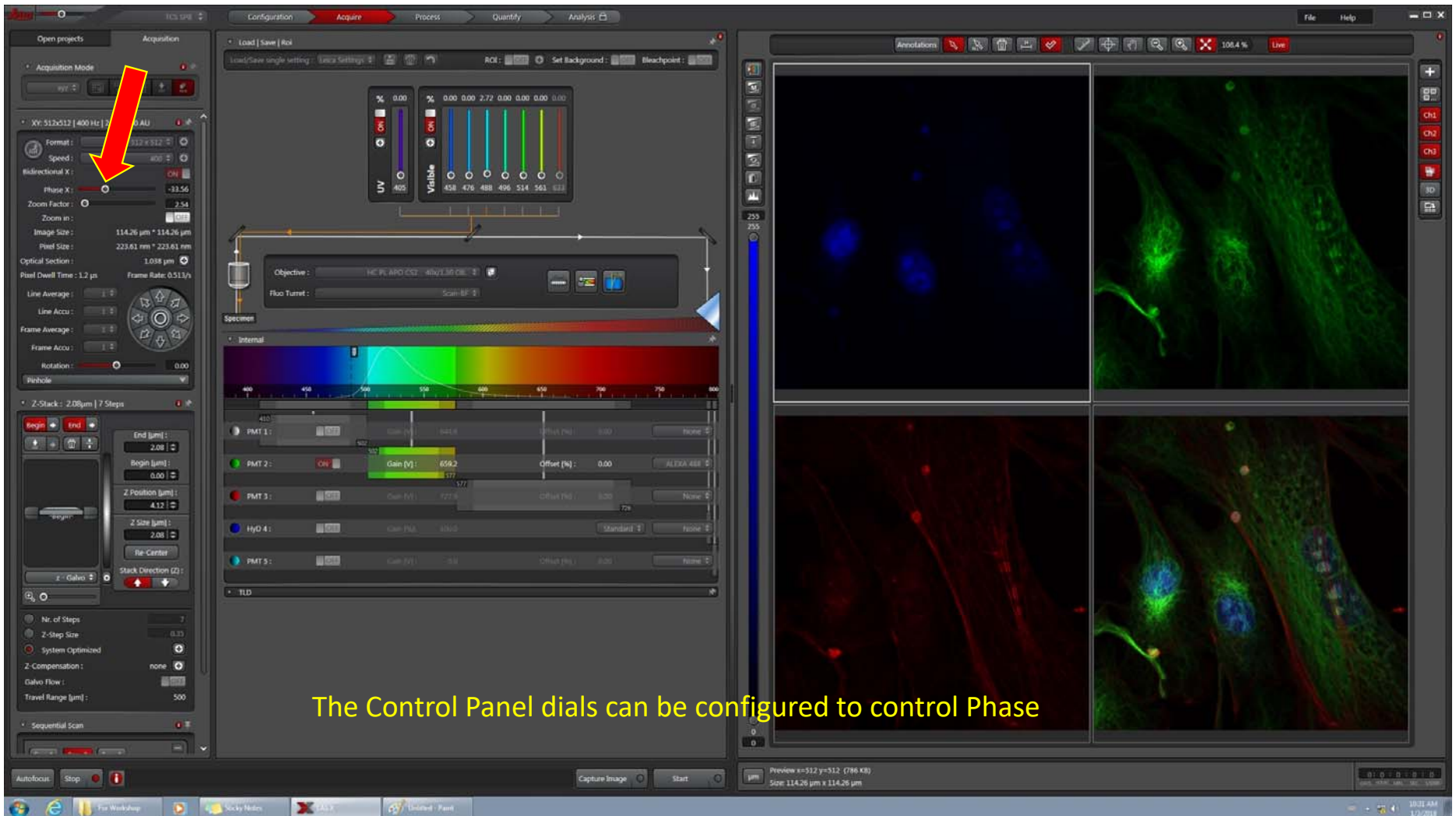












The Control Panel dials can be configured to control Phase

# Acquisition Speed Comparison

- 400 lps scan speed
- 512 x 512 pixels
- 3 channels
- 10 um z range, 30 planes

<b>By Frame</b>	<b>By Line</b>	<b>By Line + Bidirectional</b>		
3 min 46 sec	1 min 52 sec	56 sec		

# Resonant Scanning for large samples

- The excitation beam is usually raster scanned by the movement of galvanometer driven mirrors – *flexible scan speeds but slow*
- These can be replaced by faster “resonant” scanning mirrors which oscillate more rapidly, - *fast but fixed scan speed*
- Select Resonant “On” at Startup



Resonant Mirror





# Acquisition Speed Comparison

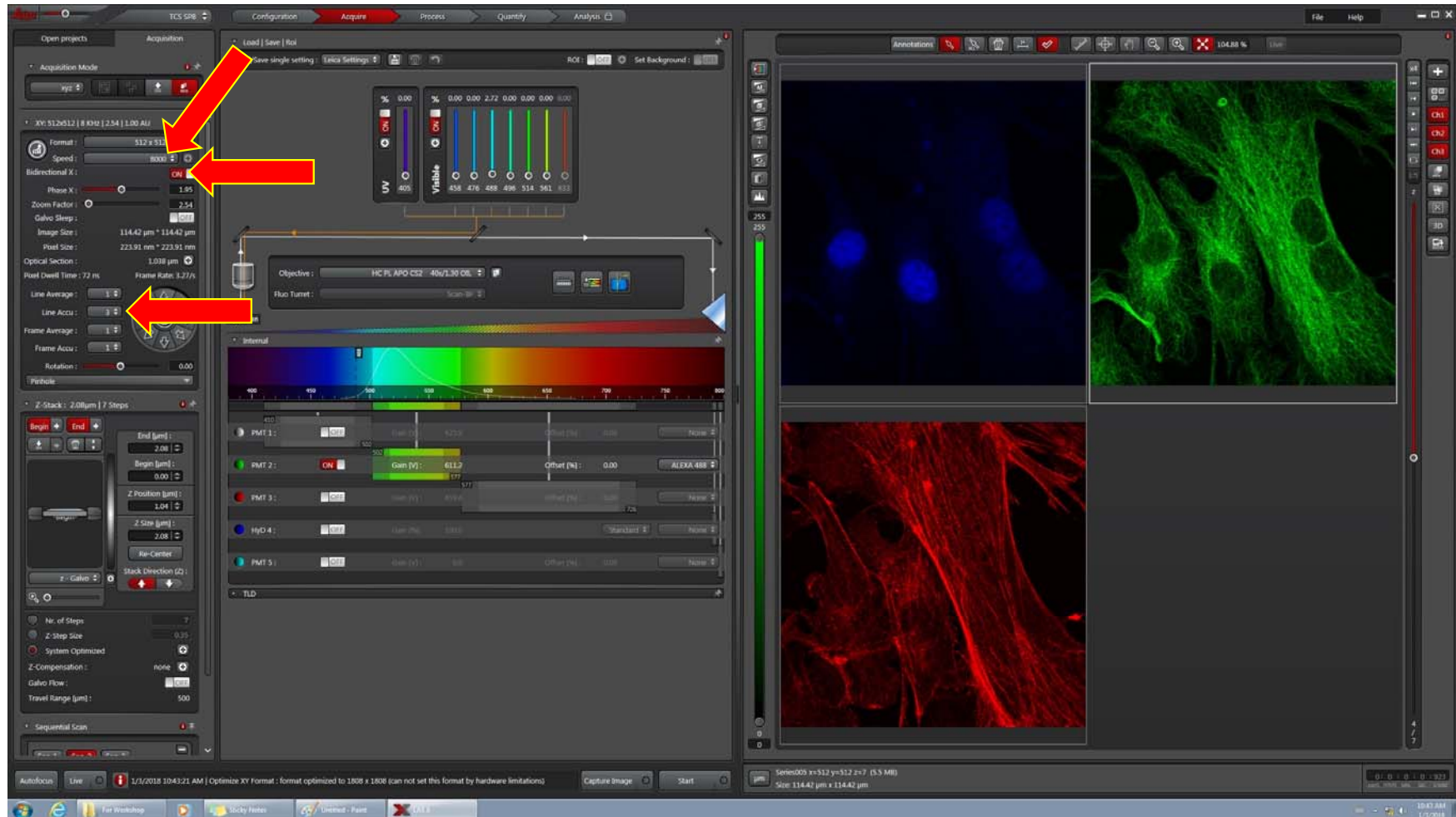
- 400 or 8000 lps scan speed
- 512 x 512 pixels
- 3 channels
- 10 um z range, 30 planes

<b>By Frame</b>	<b>By Line</b>	<b>By Line + Bidirectional</b>	<b>By Line + Resonant*</b>	
3 min 46 sec	1 min 52 sec	56 sec	17 sec	

\*w/3 line accumulations



# Combining “By Line” + Resonant + Bi-directional



# Acquisition Speed Comparison

- 400 or 8000 lps scan speed
- 512 x 512 pixels
- 3 channels
- 10 um z range, 30 planes

**25X Faster!**

<b>By Frame</b>	<b>By Line</b>	<b>By Line + Bidirectional</b>	<b>By Line + Resonant*</b>	<b>By Line + Resonant* + Bidirectional</b>
3 min 46 sec	1 min 52 sec	56 sec	17 sec	9 sec

\*w/3 line accumulations



# Optimizing Resolution and Pixel Size

- Each objective lens is capable of achieving only so much resolution
- The pixel size of the image must be set properly to achieve the max resolution (lens resolution / 2.3)
- There are two ways to do this:

- 1. Increase zoom factor



- 2. Increase the number of pixels



- The software has a button that will increase the number of pixels to maximize resolution for a given lens
- However, more pixels take longer to scan
- *Pixels smaller than theoretical best size have no additional benefit*



YCS SPM Configuration Acquire Process Quantify Analysis

Open projects Acquisition

Acquisition Mode xyz

Speed: 912 x 912  
Bidirectional X: 400  
Zoom Factor: 1.00  
Zoom In:  
Image Size: 290.62 µm x 290.62 µm  
Pixel Size: 568.74 nm x 568.74 nm  
Optical Section: 1.038 µm  
Pixel Dwell Time: 1.2 µs Frame Rate: 0.258/s  
Line Average: Line Accu: Frame Average: Frame Accu: Rotation: 0.00  
Pinhole

Z-Stack: Z Position [µm]: 2.23 Z-Size [µm]: 0.00 z-Galvo

Nr. of Steps: 1 Z-Step Size: 0.00 System Optimized: Z-Compensation: none Galvo Flow: Travel Range [µm]: 500

Sequential Scan: Seq. 1 Seq. 2 Seq. 3

Load | Save | Roll

Load/Save single setting: [Lisca Settings] RCI: Set Background: Blackpoint

UV: 405 Variable: 458 476 488 496 514 561 612

Objective: HC PL APO CS2 40x/1.30 Obj. Fluo Turret: Scan-BF 9

Specimen

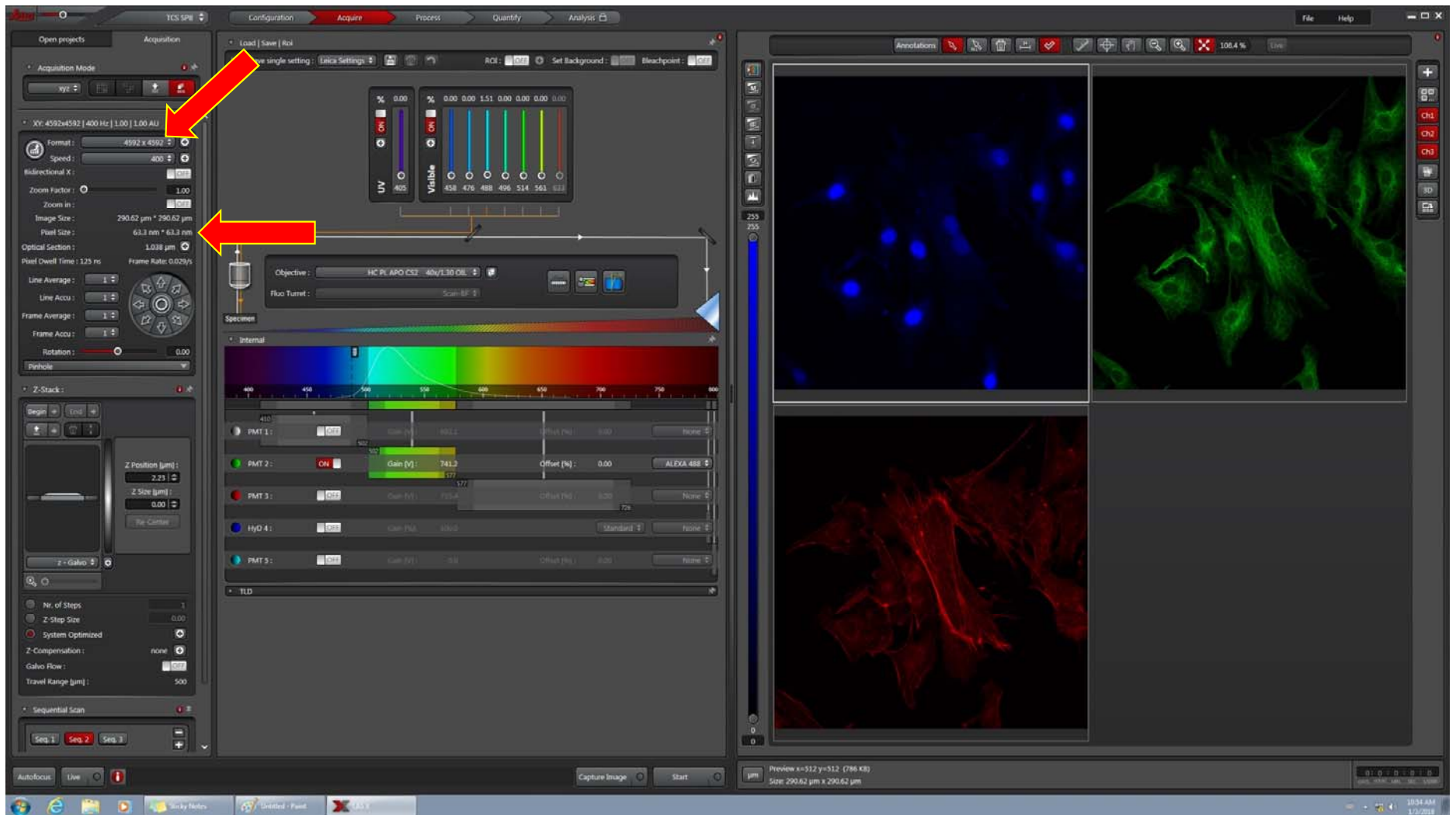
Internal

PMT 1: Gain [V]: 892.2 Offset [V]: 0.00  
PMT 2: Gain [V]: 741.3 Offset [V]: 0.00 ALEXA 488  
PMT 3: Gain [V]: 725.4 Offset [V]: 0.00  
HYD 4: Gain [V]: 0.00 Offset [V]: 0.00 Standard  
PMT 5: Gain [V]: 0.00 Offset [V]: 0.00  
TLD

Annotations 108.4% Live

Preview x=512 y=512 (786 KB) Size: 290.62 µm x 290.62 µm

10:35 AM 1/2/2018



Configuration Acquire Process Quantify Analysis

Open projects Acquisition

Acquisition Mode: xyz

XY: 512x512 | 400 Hz | 9.02 | 1.00 AU

Format: 512 x 512

Speed: 400

Bidirectional X:

Zoom Factor: 9.02

Zoom in:

Image Size: 32.23  $\mu$ m x 32.23  $\mu$ m

Pixel Size: 61.07 nm x 61.07 nm

Optical Section: 1.038  $\mu$ m

Pixel Dwell Time: 1.2 ps

Frame Rate: 0.258/s

Line Average: 1

Line Accu: 1

Frame Average: 1

Frame Accu: 1

Rotation: 0.00

Pinhole:

Z-Stack:

Begin End

Z Position [ $\mu$ m]: 1.15

Z Step [ $\mu$ m]: 0.00

z - Galvo

Nr. of Steps: 1

Z-Step Size: 0.00

System Optimized:

Z-Compensation: none

Galvo Flow:

Travel Range [ $\mu$ m]: 500

Sequential Scan:

Seq 1 Seq 2 Seq 3

Load | Save | ROI

Line: single setting: ERICA Settings

ROI:  Set Background:  Bleachpoint:

UV:  ON

Visible:  ON

Objective: HC PL APO CS2 40x/1.30 Oil

Fluo Turret: Scan BF

Specimen

Internal

Channel	Gain [V]	Offset [mV]	Filter
PMT 1	741.2	0.00	ALEXA 488
PMT 2	177	0.00	None
PMT 3	177	0.00	None
HyD 4	177	0.00	Standard
PMT 5	177	0.00	None

TLD

Annotations

100.4 %

255 255

0 0

Preview x=512 y=512 (796 KB)

Size: 32.23  $\mu$ m x 32.23  $\mu$ m

10:55 AM 1/3/2018

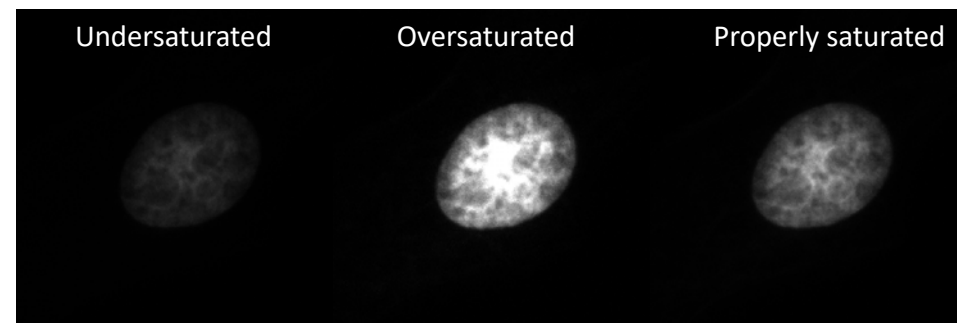
# Optimizing Images with the Histogram or Quick LUT

“Your eyes can deceive you. Don’t trust them.”

-Obi-Wan Kenobi

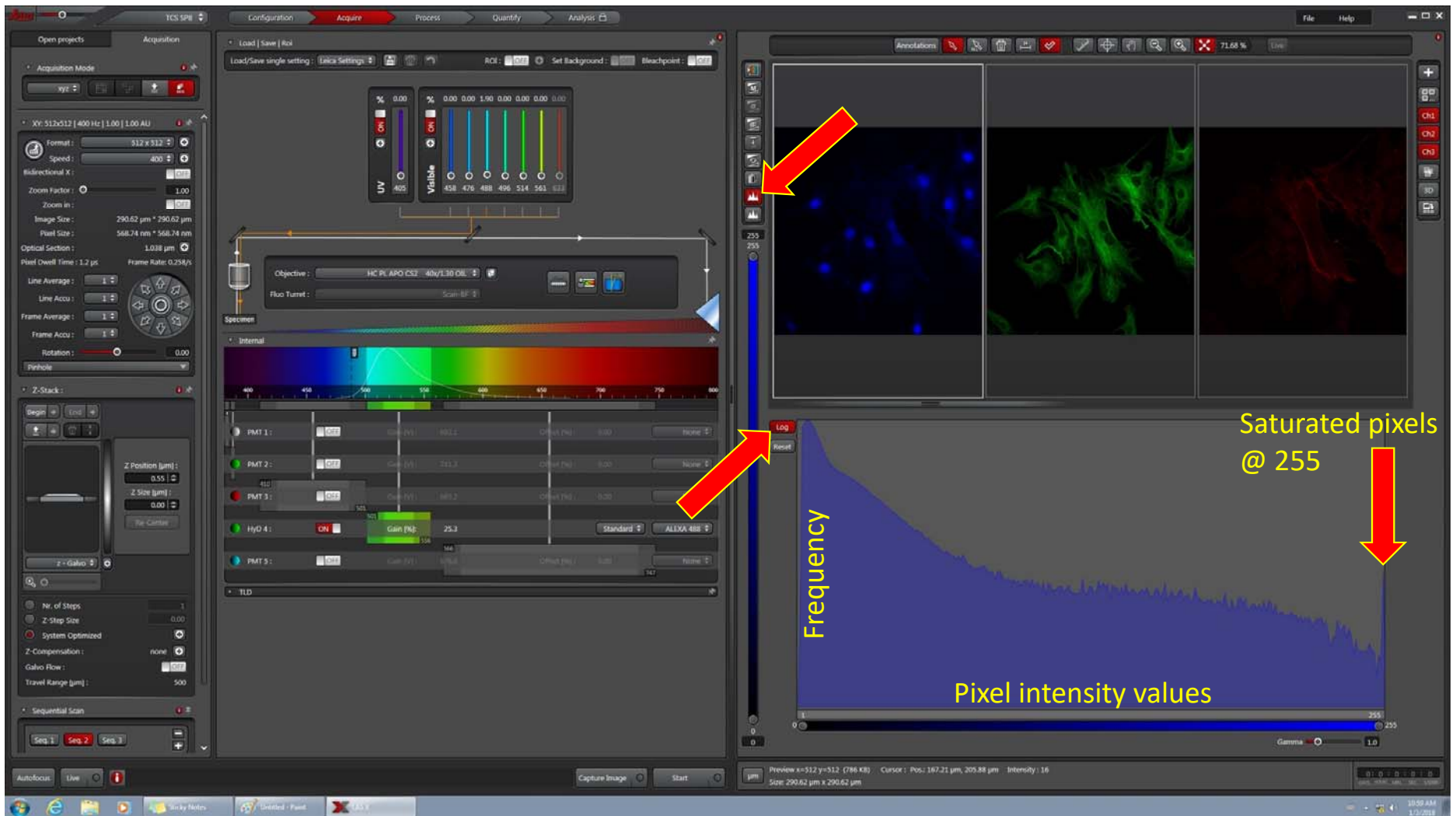


- Images which are under or oversaturated are not using the dynamic range of the detector



- These images are missing information
- There are quantitative tools to help you choose the best laser power and gain





The screenshot displays a comprehensive fluorescence microscopy software interface, likely from a Zeiss system, organized into several functional panels:

- Acquisition Panel (Left):** Contains settings for the microscope's operation, including XY coordinates (512x512), speed (400), zoom factor (6.02), image size (48.31 μm x 48.31 μm), pixel size (94.52 nm x 94.52 nm), and optical section parameters like pixel dwell time (1.2 μs) and frame rate (0.258/s). It also includes controls for line/frame averaging and rotation.
- Configuration Panel (Top Center):** Shows the current objective lens (HC PL APO CS2 40x/1.30 Oil) and the scan field (Scan 8F 5). It features a 'Load/Save single setting' section and a 'Variable' control with a color-coded bar.
- Internal Panel (Middle):** Displays a spectral power distribution graph and a table of detector settings for five PMTs and HyD detectors. The table includes columns for PMT/Channel name, Gain, Offset, and other parameters.
- Live Image Panel (Right Top):** Shows a real-time fluorescence image of a cell, with a zoom level of 114.26% and a 'Live' indicator.
- Histogram Panel (Right Bottom):** A graph showing the intensity distribution of the image data. A yellow text overlay reads: "Fill, but do not exceed the dynamic range of the detector". The histogram shows a peak at the low end of the intensity scale, indicating that the signal is saturated.

The bottom of the interface shows a Windows taskbar with various application icons and a system clock indicating 11:05 AM on 1/7/2018.

The screenshot displays a microscopy software interface with several key sections:

- Acquisition Settings (Left):** Includes parameters for Xr: 512x512 [400 Hz] [6.02] 1.00 AU, Format: 512 x 512, Speed: 400, Image Size: 48.31 μm x 48.31 μm, and Z-Stack settings.
- Configuration (Top):** Shows 'Acquire' mode, ROI, and various control buttons.
- Internal Spectral Plot (Middle):** A graph showing intensity across a wavelength range from 400 to 800 nm. A red arrow points to the 'Internal' section of the plot.
- Fluorescence Image (Right):** A large image showing a cell with a bright blue nucleus. A red arrow points to the top-left corner of the image area. The text 'Blue pixels are saturated (intensity = 255)' is overlaid on the image.
- System Information (Bottom):** Shows 'Preview: ex512.yr512 (786 KB)' and 'Size: 48.31 μm x 48.31 μm'.



Configuration Acquire Process Quantity Analysis

Open projects Acquisition

Acquisition Mode

Format: 512 x 512  
Speed: 400  
Bidirectional X:  
Zoom Factor: 6.02  
Zoom in:  
Image Size: 48.31  $\mu\text{m}$  x 48.31  $\mu\text{m}$   
Pixel Size: 94.53 nm x 94.53 nm  
Optical Section:  
Pixel dwell Time: 1.2  $\mu\text{s}$  Frame Rate: 0.258/s  
Line Average: 1  
Line Accu: 1  
Frame Average: 1  
Frame Accu: 1  
Rotation: 0.00  
Pinhole

Z-Stack:  
Begin End  
Z Position ( $\mu\text{m}$ ): 2.64  
Z Size ( $\mu\text{m}$ ): 0.00  
z-Galvo  
Nr. of Steps: 1  
Z-Step Size: 0.00  
System Optimized  
Z-Compensation: none  
Galvo Flow:  
Travel Range ( $\mu\text{m}$ ): 500  
Sequential Scan  
Seq 1 Seq 2 Seq 3

Load/Save single setting: Leica Settings  
ROI: Set Background: Bleedpoint:

UV: 405  
Visible: 458 476 488 496 514 563 633

Objective: HC PL APO CS2 40x/1.30 Obj.  
Fluo Turret: Scan EF 9

Specimen

Internal

PMT 1: Gain [V]: 652.1 Offset [V]: 0.00  
PMT 2: Gain [V]: 743.3 Offset [V]: 0.00  
PMT 3: Gain [V]: 611.8 Offset [V]: 0.00  
HyD 4: Gain [V]: 25.0 Standard  
PMT 5: Gain [V]: 554 Offset [V]: 0.00  
TL0

Annotations

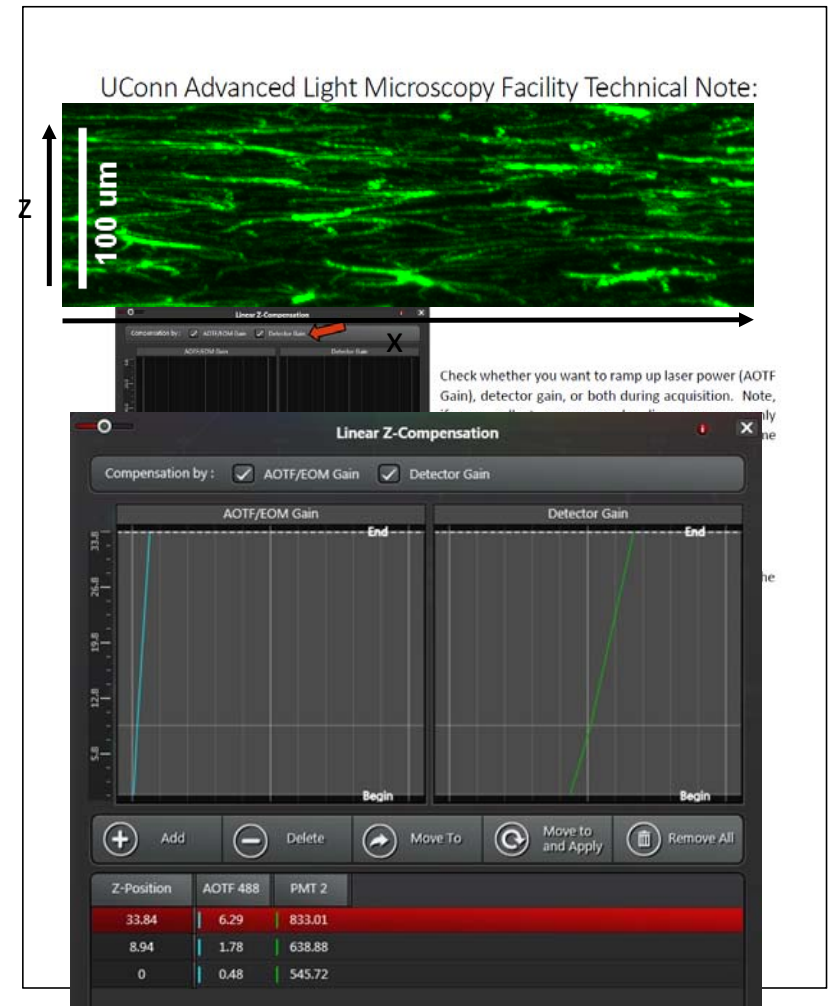
Decrease laser power and/or gain until blue saturation indicator just disappears

Preview x=512 y=512 (786 KB)  
Size: 48.31  $\mu\text{m}$  x 48.31  $\mu\text{m}$

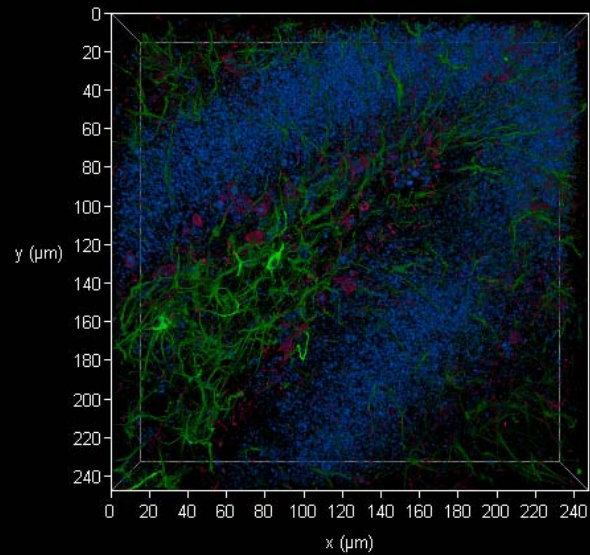
11:03 AM  
1/12/2018

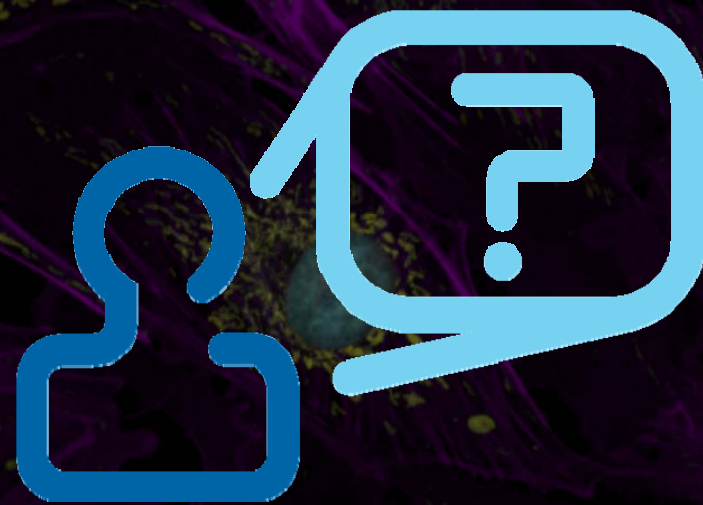
# Linear Z Compensation

- Optical aberrations get worse the deeper you image into a specimen
- One result is decreasing signal during z stacks (usually noticeable > 20  $\mu\text{m}$ )
- Laser power and gain can be automatically increased as a function of depth to help keep intensity constant through the sample
- [confocal.uconn.edu/resources/](http://confocal.uconn.edu/resources/)



# 120 $\mu\text{m}$ z stack through mouse hippocampus





[confocal.uconn.edu](http://confocal.uconn.edu)