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

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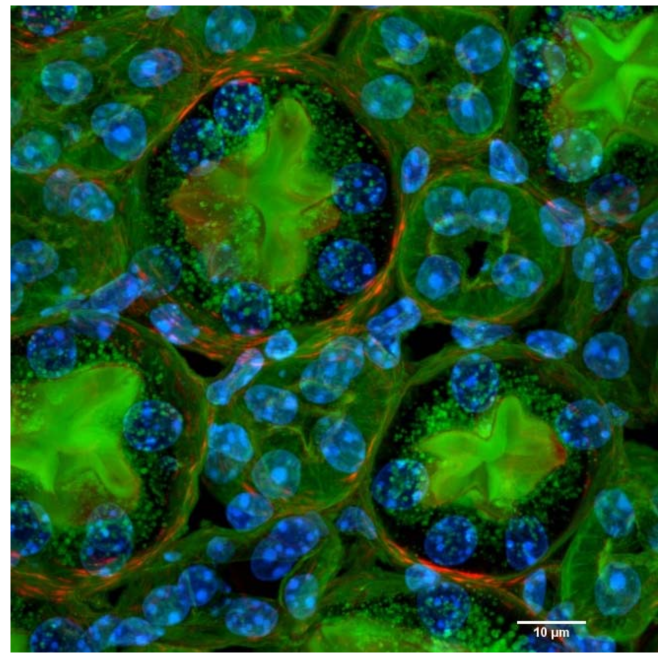
ADVANCED LIGHT MICROSCOPY FACILITY

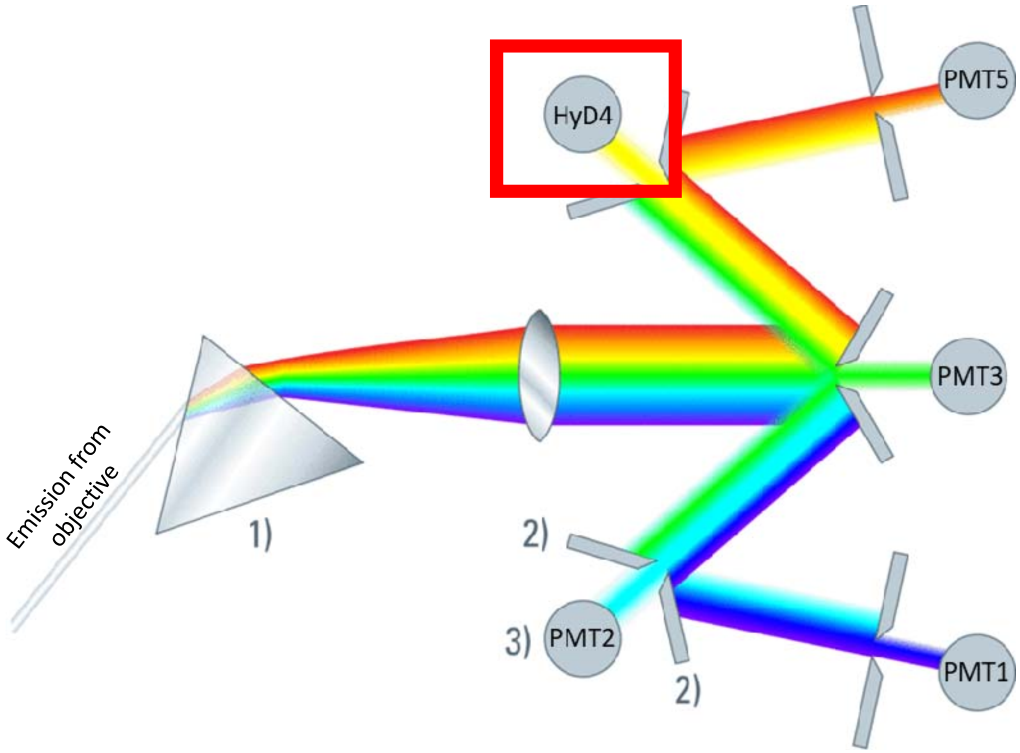
Todays Goal:

- 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

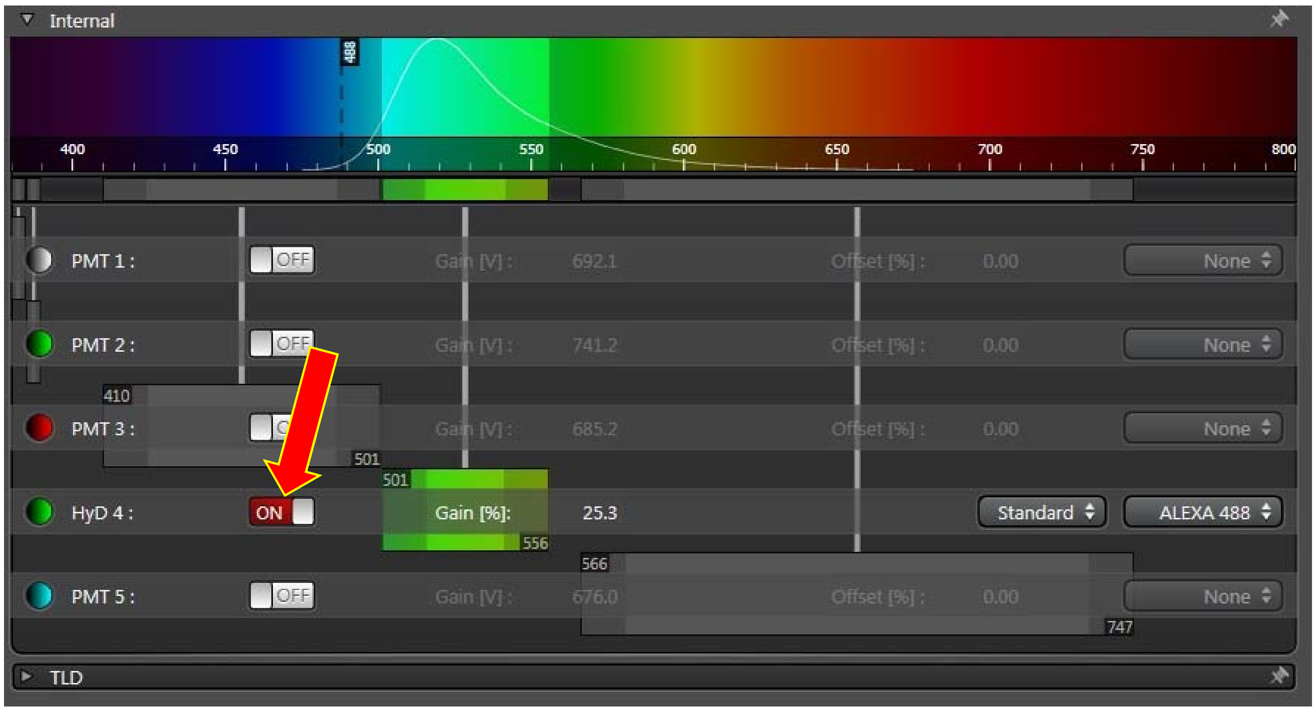
Photomultiplier tubes (PMT):
<u>Detectors 1,2,3,5</u>

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

Hybrid Detector (HyD)
<u>Detector 4:</u>

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

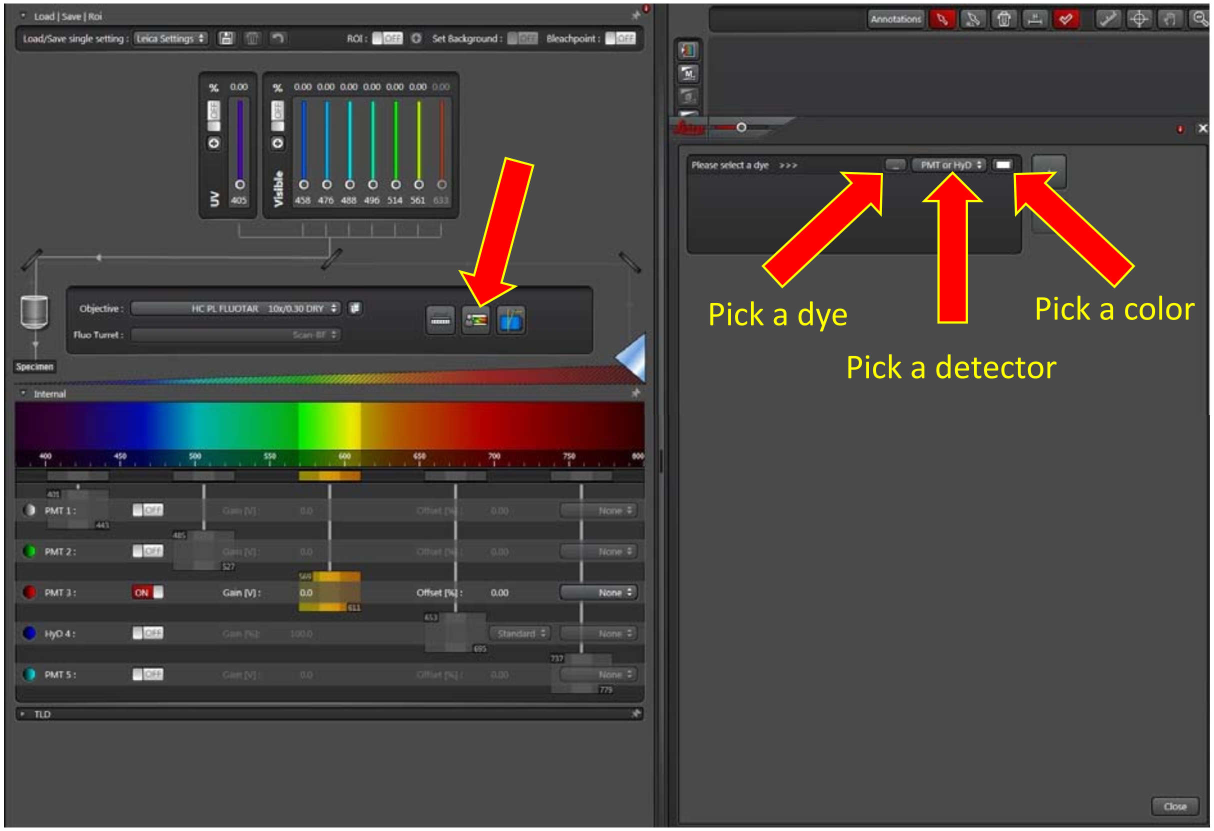
Using the HyD Detector on the SP8



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

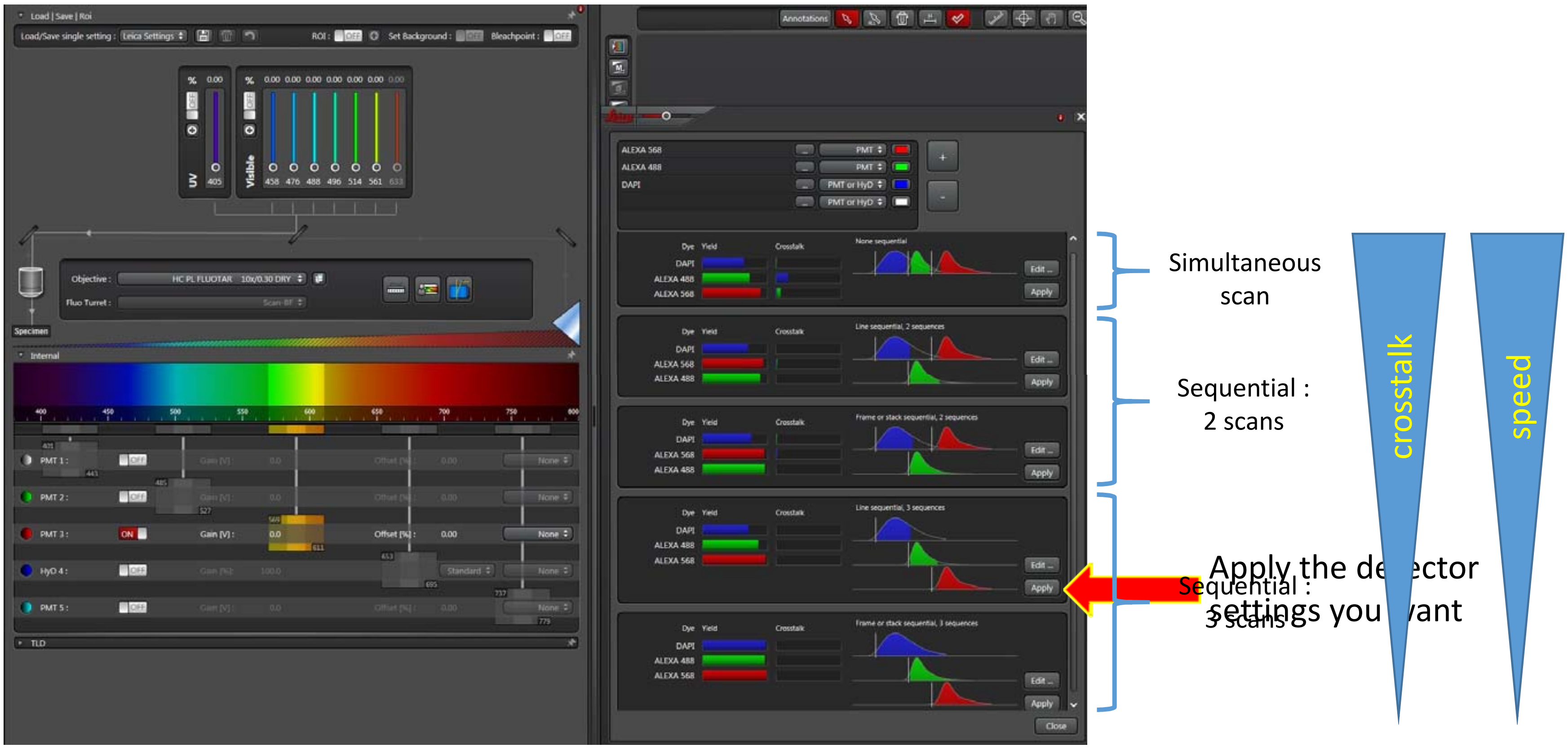
Dye Assistant

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



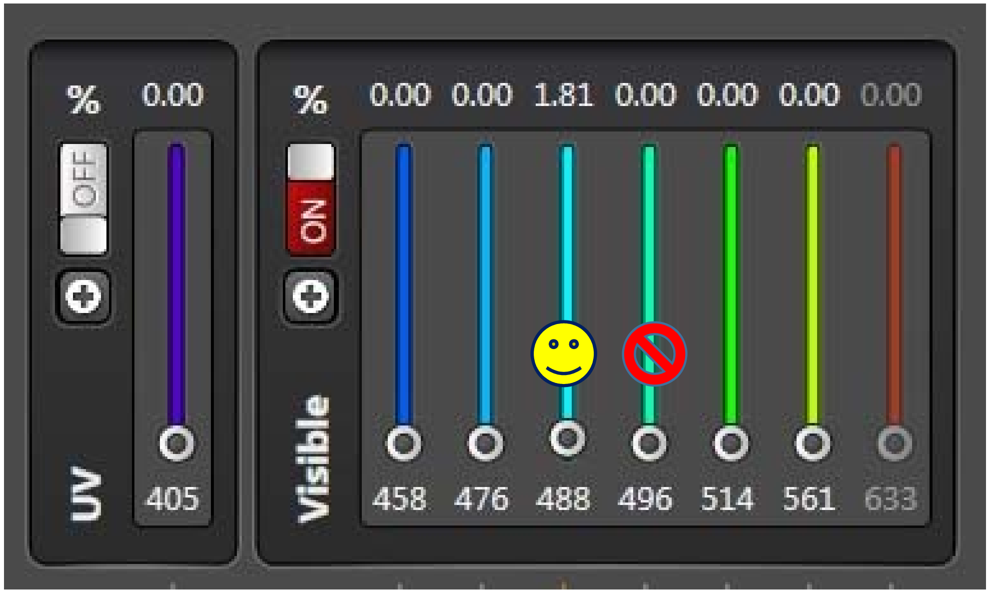
Dye Assistant

- A wizard to help you configure the detector quickly



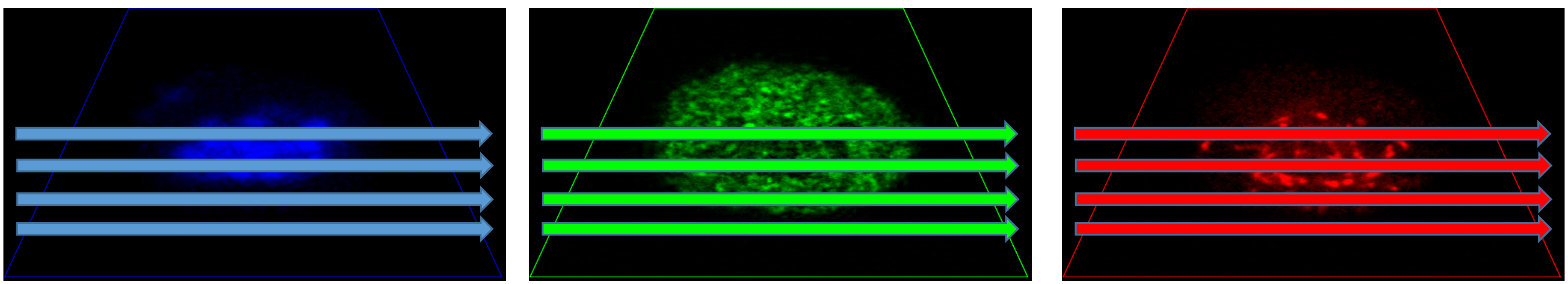
Dye Assistant Note

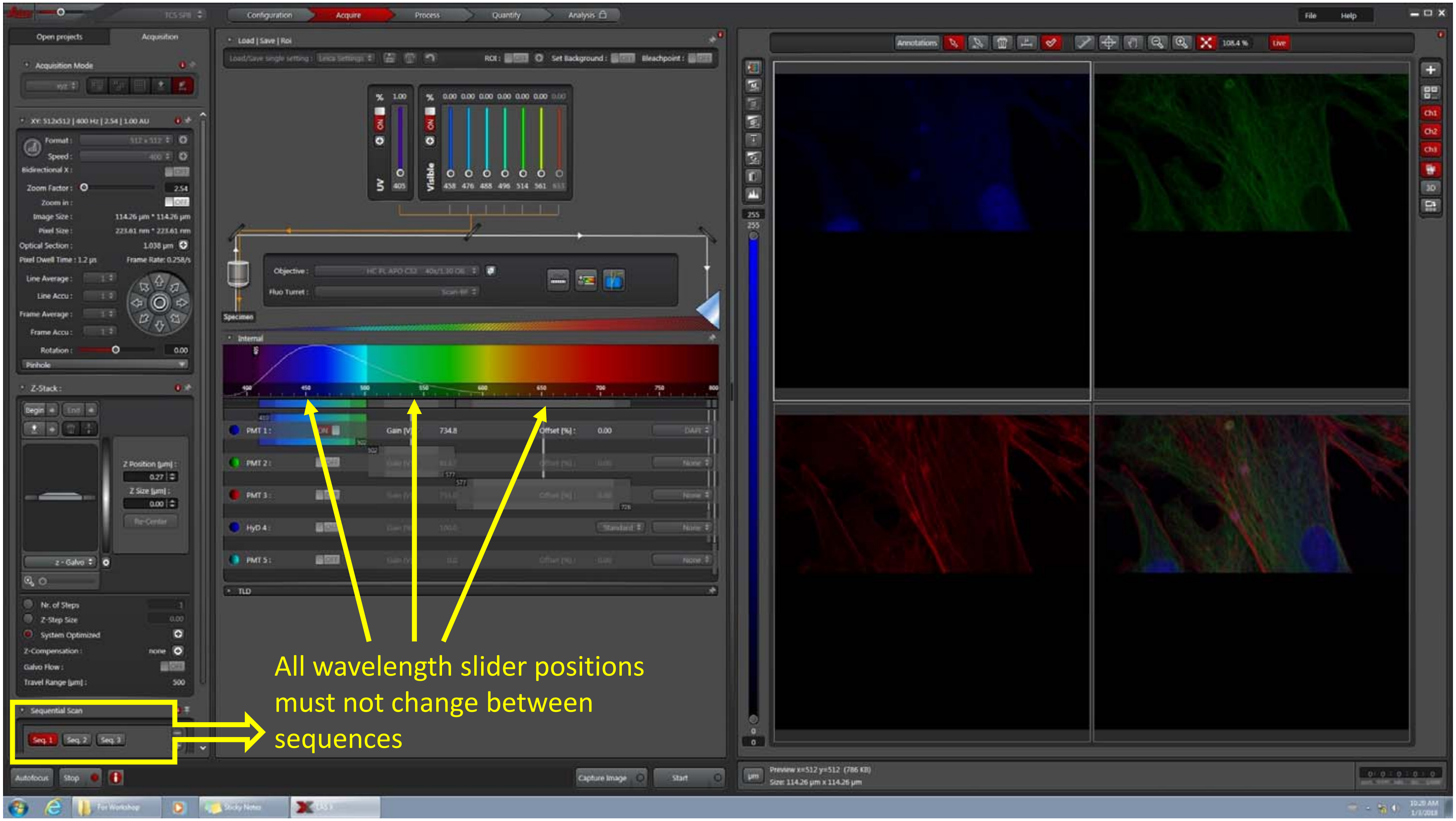
- The Wizard will choose the 496 nm laser for Alexa 488
- While 496 nm is closer to the actual excitation peak of Alexa 488…
- ….The 488 nm laser is much stronger
- You will have to manually choose 488 nm excitation for this channel



Scanning Sequentially "By Line"

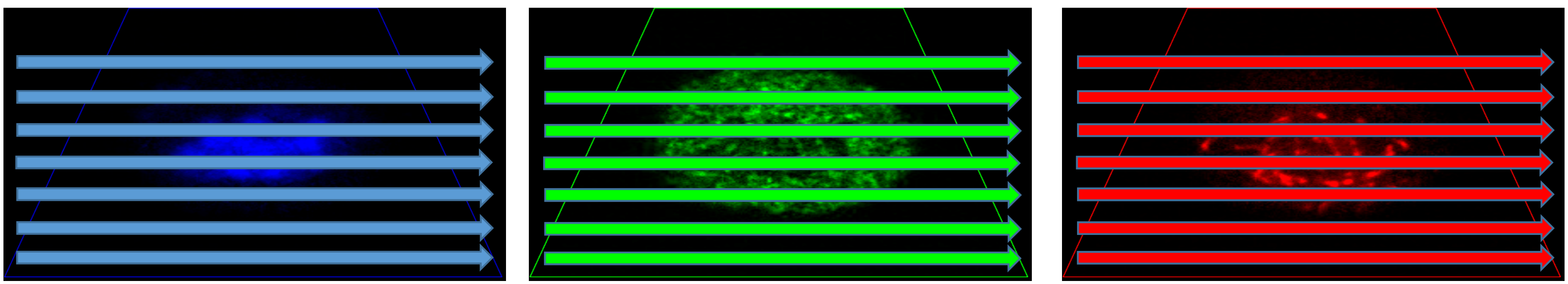
- Scans 1 line of each channel, one after the other
- All channels will appear to be captured simultaneously
- Wavelength sliders <u>cannot move</u> between channels during this type of scan – NO MOVING PARTS
- Fastest method of sequential scanning
- Slightly less photon efficient than "By Frame"



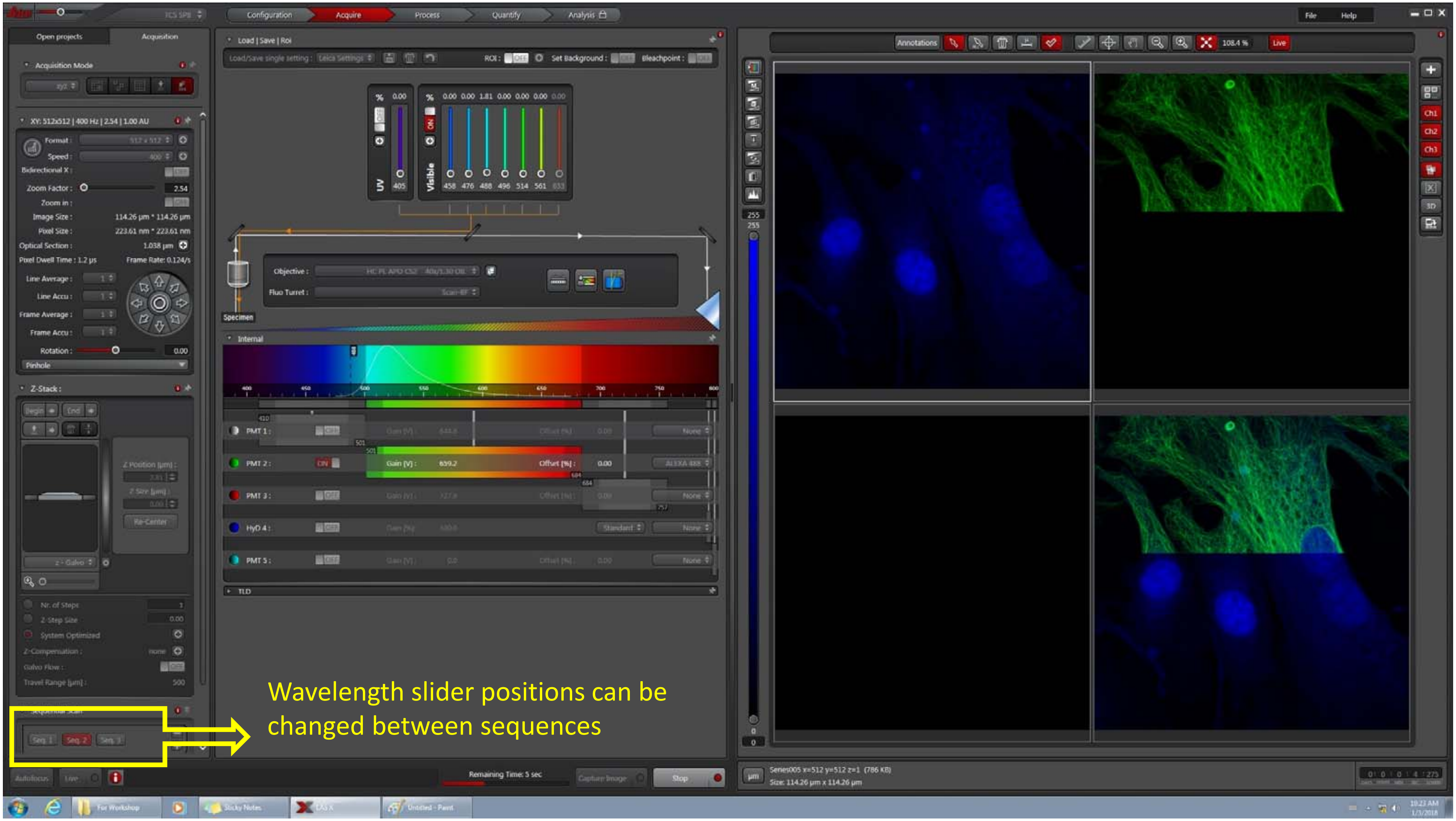

All wavelength slider positions must not change between sequences

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


Configuration
Acquire
Process
Quantify
Analysis
Open projects
Acquisition
Acquisition Mode
XY: 512x512 | 400 Hz | 2.54 | 1.00 AU
Format :
Speed :
Bidirectional X :
Zoom Factor :
Zoom in :
Image Size :
114.26 µm * 114.26 µm
Pixel Size :
223.61 nm * 223.61 nm
Optical Section :
1.038 µm
Pixel Dwell Time : 1.2 µs
Frame Rate: 0.124/s
Line Average :
Line Accu :
Frame Average :
Frame Accu :
Rotation :
Pinhole
Z-Stack :
Load | Save | Roi
ROI :
Set Background :
Bleachpoint :
UV
Visible
405
458
476
488
496
514
561
633
Objective :
Fluo Turret :
Specimen
Internal
PMT 1 :
PMT 2 :
Gain [V] :
639.2
Offset [%] :
0.00
PMT 3 :
HyD 4 :
PMT 5 :
TLD
Annotations
108.4 %
Live
Ch1
Ch2
Ch3
File
Help
Remaining Time: 5 sec
Stop
Series005 x=512 y=512 z=1 (786 KB)
Size: 114.26 µm x 114.26 µm
Seq. 1
Seq. 2
Seq. 3
Wavelength slider positions can be changed between sequences

Acquisition Speed Comparison

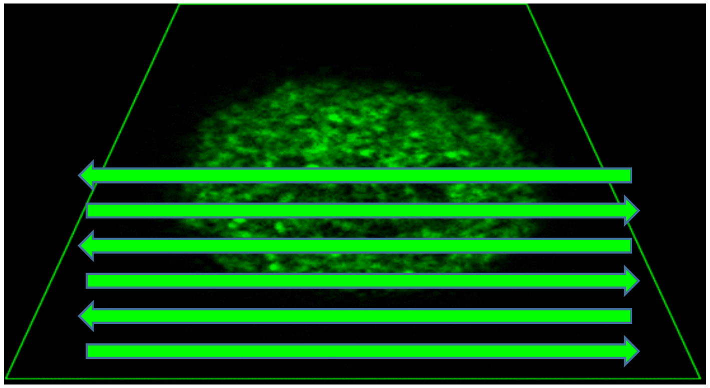
- 400 lps scan speed
- 512 x 512 pixels
- 3 channels
- 10 um z range, 30 planes

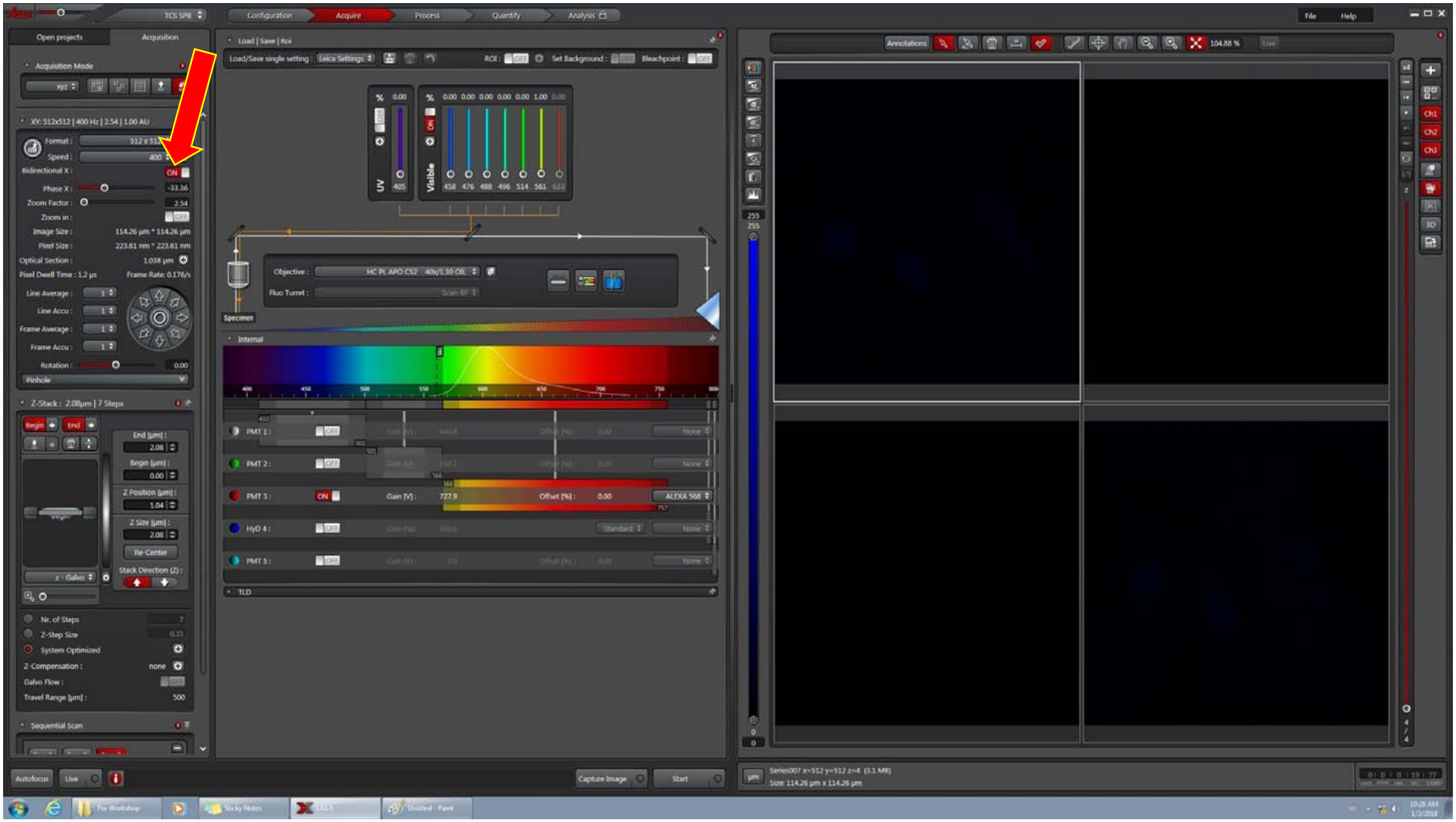
By Frame	By Line			
3 min 46 sec	1 min 52 sec			

“Between Stacks” not recommended

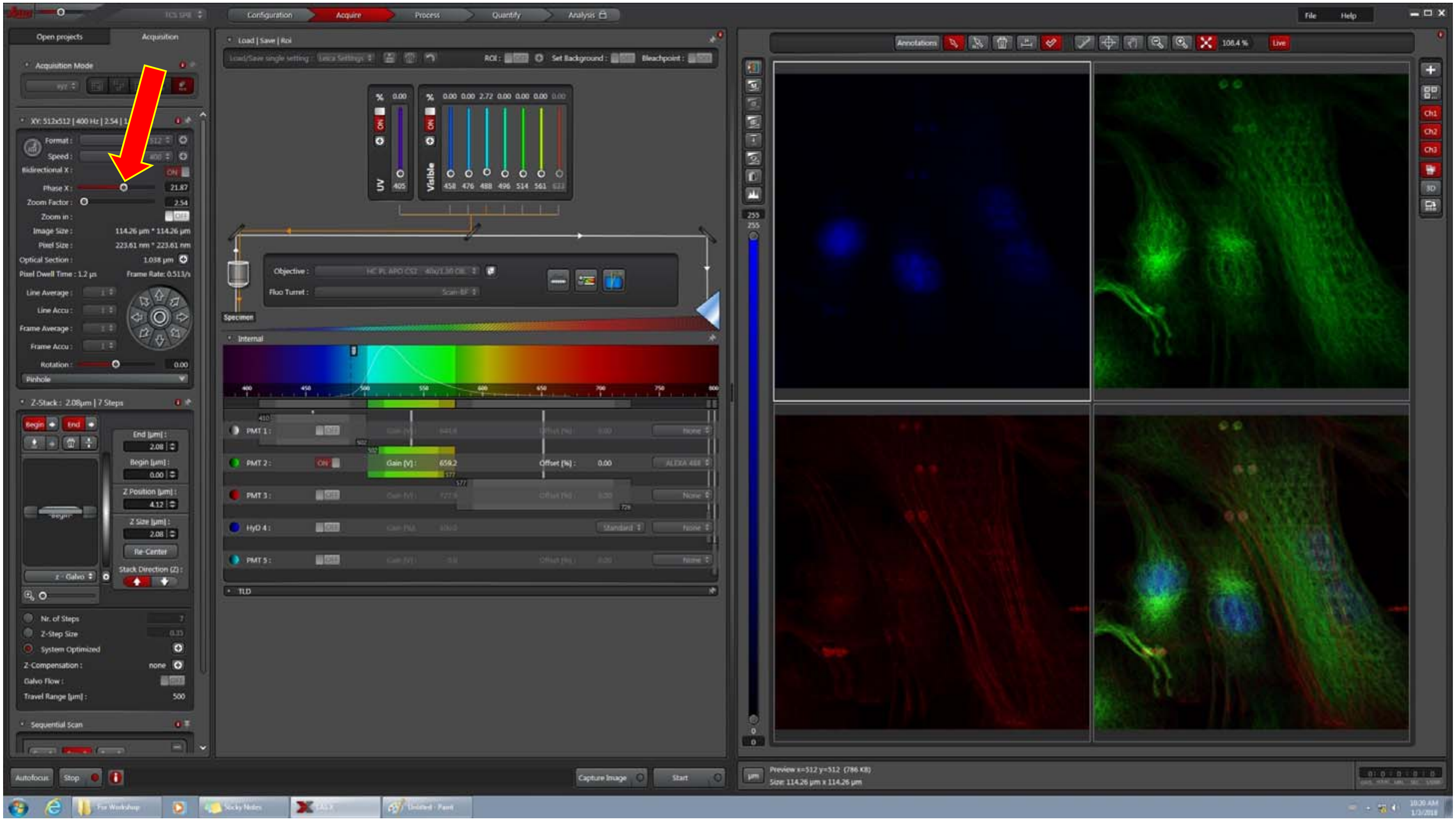
Bi-Directional Scanning

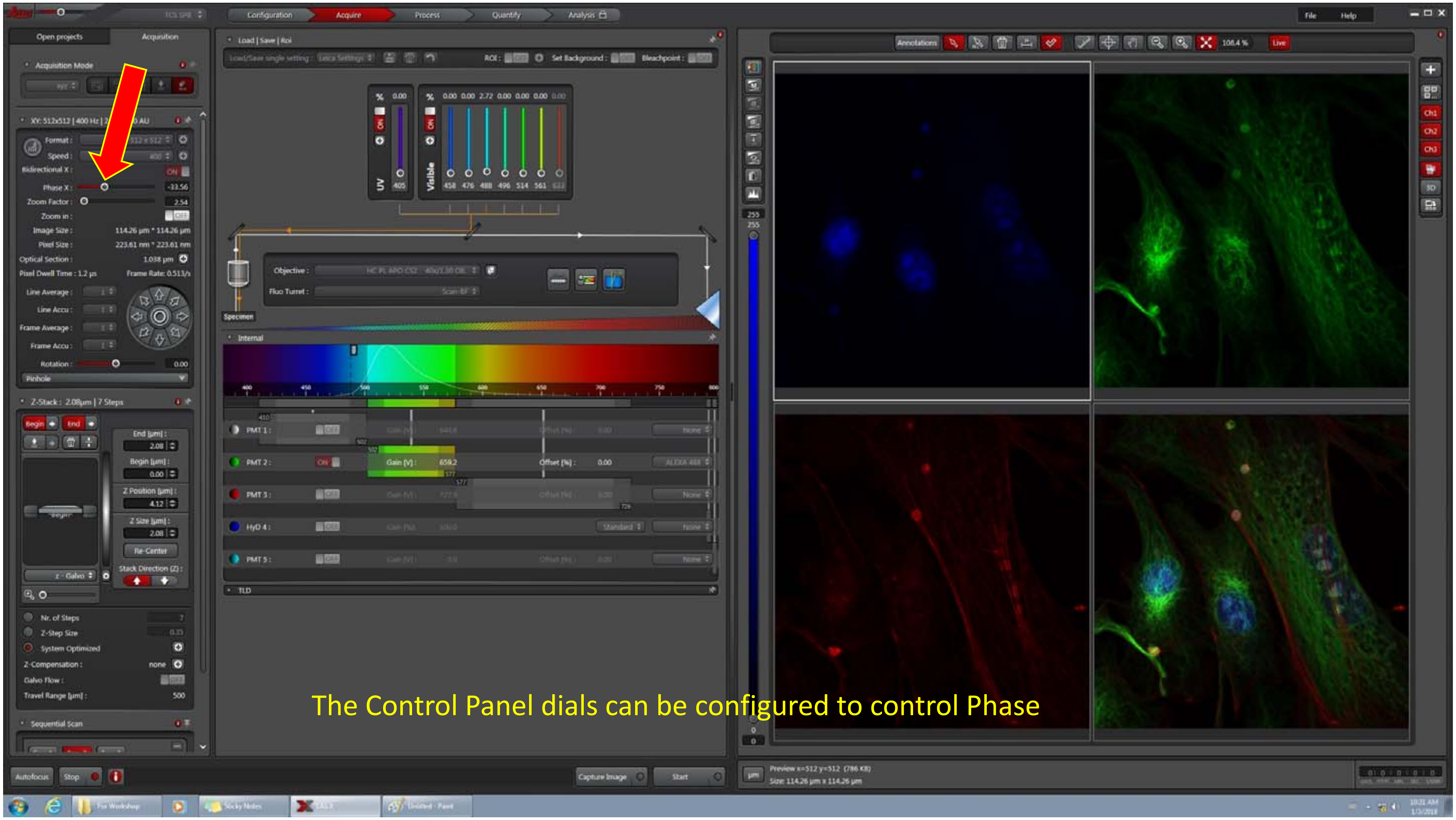
- Capture is usually done in only one direction of the beam scan
- Imaging can also be done on the return pass of the beam
- 2X as fast
- Reverses the direction in which pixels are recorded
- Alignment of the scan phase is needed





Configuration
Acquire
Process
Quantify
Analysis
Open projects
Acquisition
Acquisition Mode
Format :
Speed :
Bidirectional X :
ON
Phase X :
Zoom Factor :
Image Size :
Pixel Size :
Objective :
Fluo Turret :
Specimen
PMT 1 :
PMT 2 :
PMT 3 :
HyD 4 :
PMT 5 :
Gain [V] :
Offset [%] :
Z-Stack
Sequential Scan
Capture Image
Start


Configuration
Acquire
Process
Quantify
Analysis
Open projects
Acquisition
Acquisition Mode
Format :
Speed :
Bidirectional X :
Phase X :
21.87
Zoom Factor :
2.54
Zoom in :
Image Size :
114.26 µm * 114.26 µm
Pixel Size :
223.61 nm * 223.61 nm
Optical Section :
1.038 µm
Line Average :
Line Accu :
Frame Average :
Frame Accu :
Rotation :
Pinhole
Z-Stack : 2.08µm | 7 Steps
Begin
End
End [µm] :
2.08
Begin [µm] :
0.00
Z Position [µm] :
4.12
Z Size [µm] :
2.08
Re-Center
Stack Direction (Z) :
Nr. of Steps
Z-Step Size
System Optimized
Z-Compensation :
none
Galvo Flow :
Travel Range [µm] :
500
Sequential Scan
Load | Save | Roi
ROI :
Set Background :
Bleachpoint :
UV
Visible
Objective :
Fluo Turret :
Specimen
Internal
PMT 1 :
PMT 2 :
Gain [V] :
659.2
Offset [%] :
0.00
PMT 3 :
HyD 4 :
PMT 5 :
TLD
Annotations
Live
Autofocus
Stop
Capture Image
Start
File
Help



The Control Panel dials can be configured to control Phase

Acquisition Speed Comparison

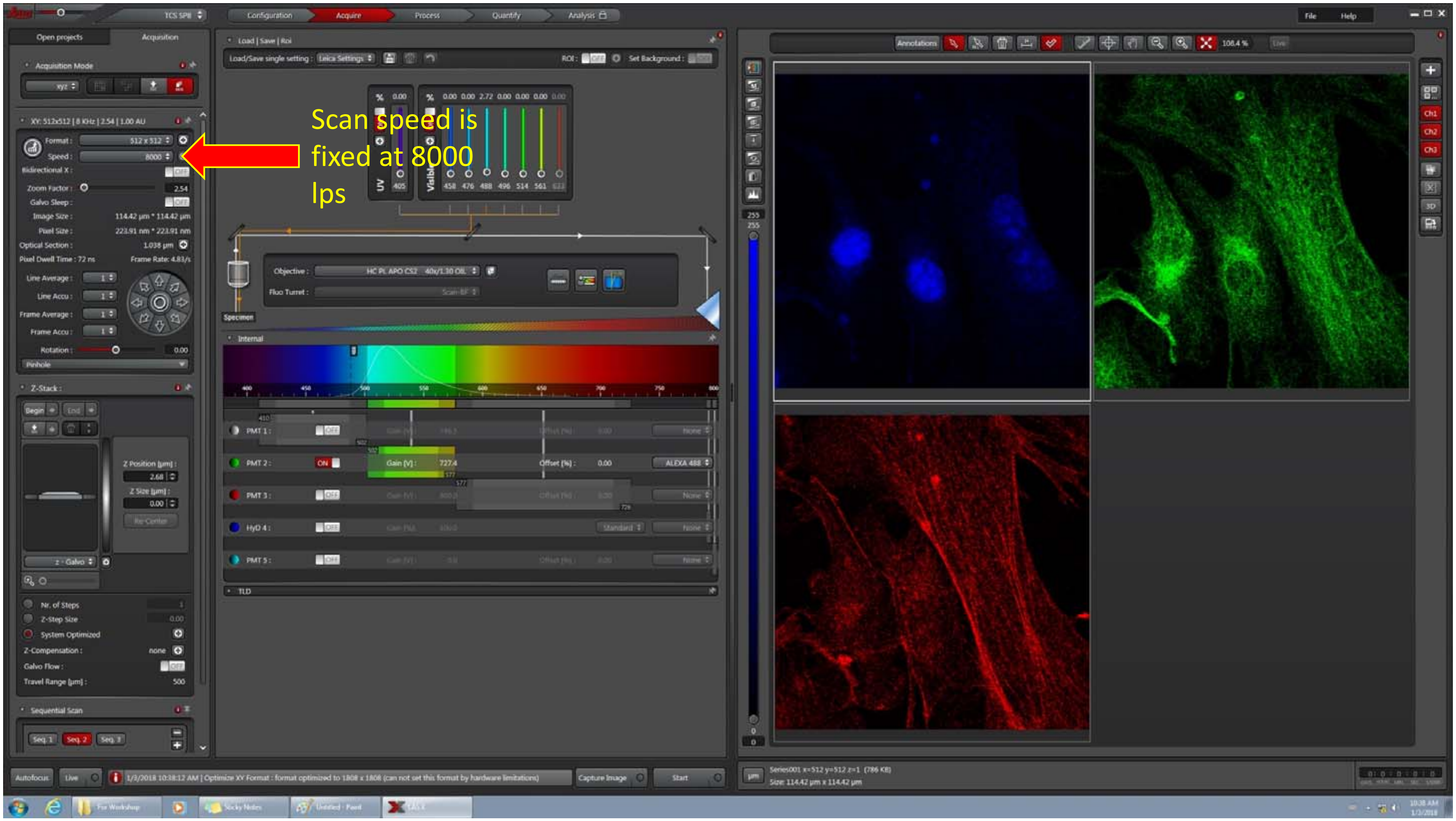
- 400 lps scan speed
- 512 x 512 pixels
- 3 channels
- 10 um z range, 30 planes

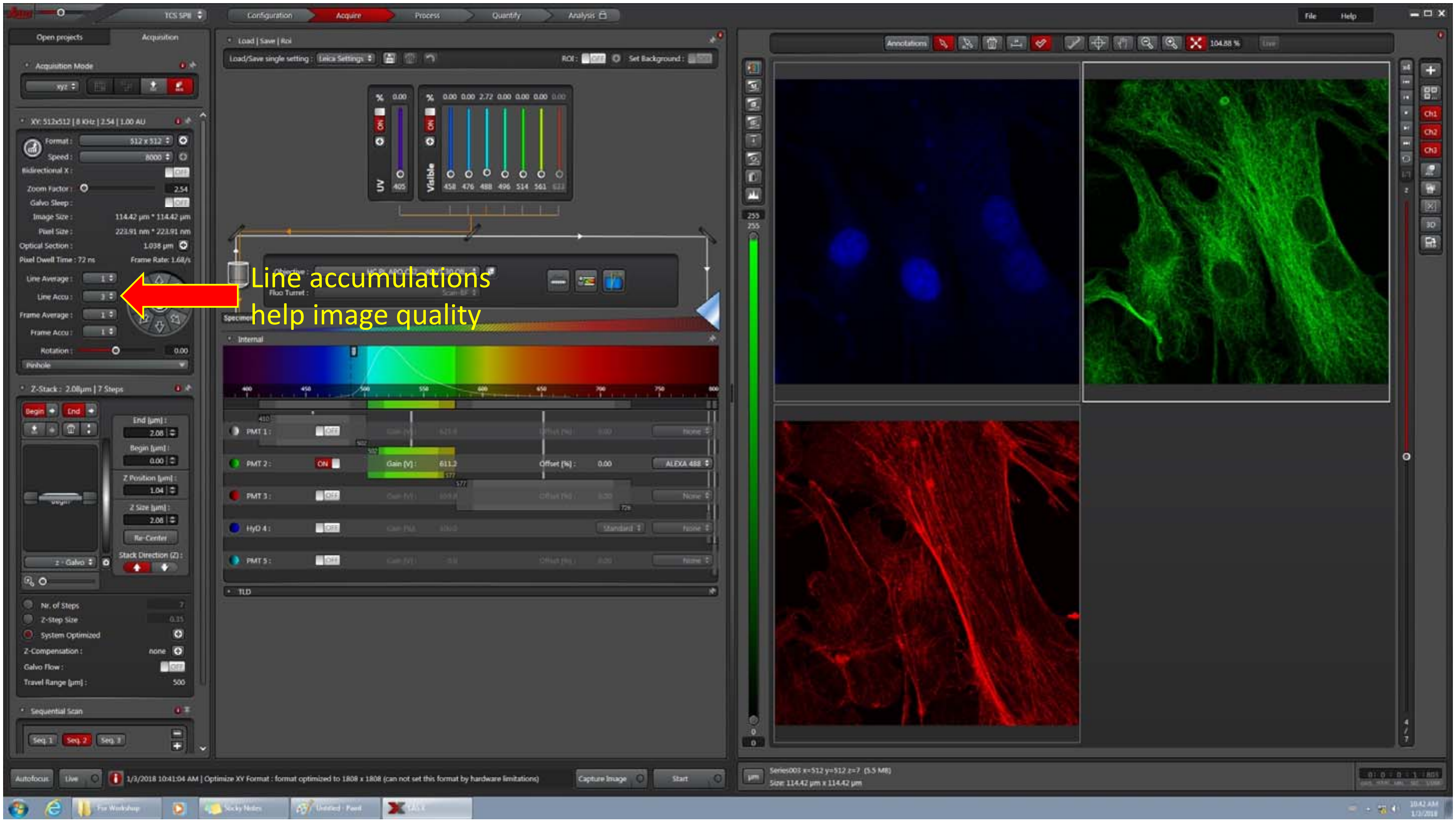
By Frame	By Line	By Line + Bidirectional		
3 min 46 sec	1 min 52 sec	56 sec		

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


Scan speed is fixed at 8000 lps
Configuration
Acquire
Process
Quantify
Analysis
Open projects
Acquisition
Acquisition Mode
Format :
Speed :
Z-Stack :
Sequential Scan
Objective :
Internal
PMT 1 :
PMT 2 :
PMT 3 :
HyD 4 :
PMT 5 :
Annotations
Capture Image
Start


Line accumulations
help image quality

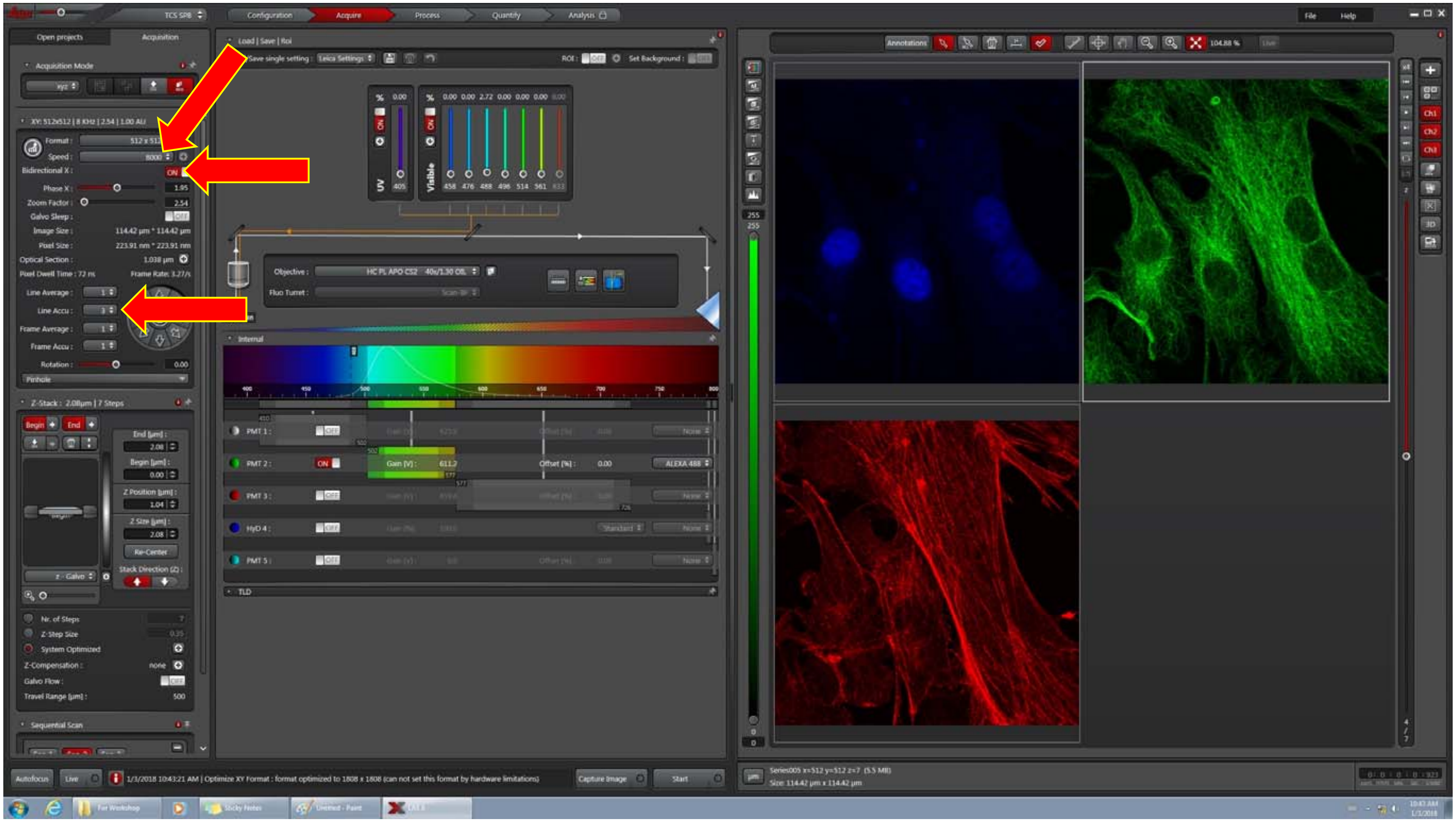
Acquisition Speed Comparison

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

By Frame	By Line	By Line + Bidirectional	By Line + Resonant*	
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!

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

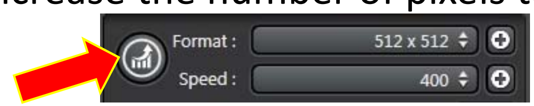
*w/3 line accumulations

Optimizing Resolution and Pixel Size

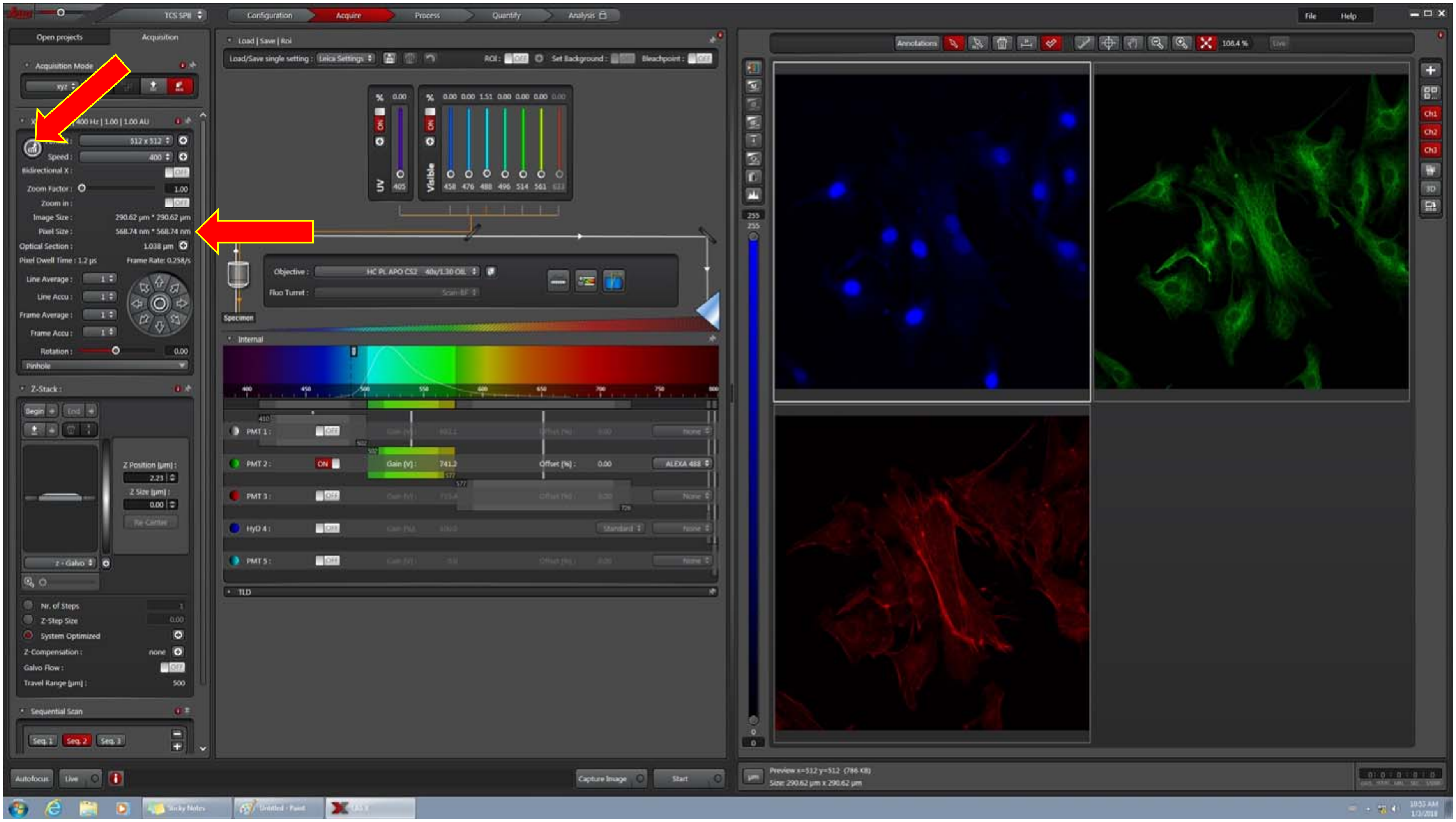
- Each objective lens is capable of achieving only so much resolution
- The pixel size of the image must be set properly to achieve the max resolution (lens resolution / 2.3)
- There are two ways to do this:
    - 1. Increase zoom factor

    - 2. Increase the number of pixels

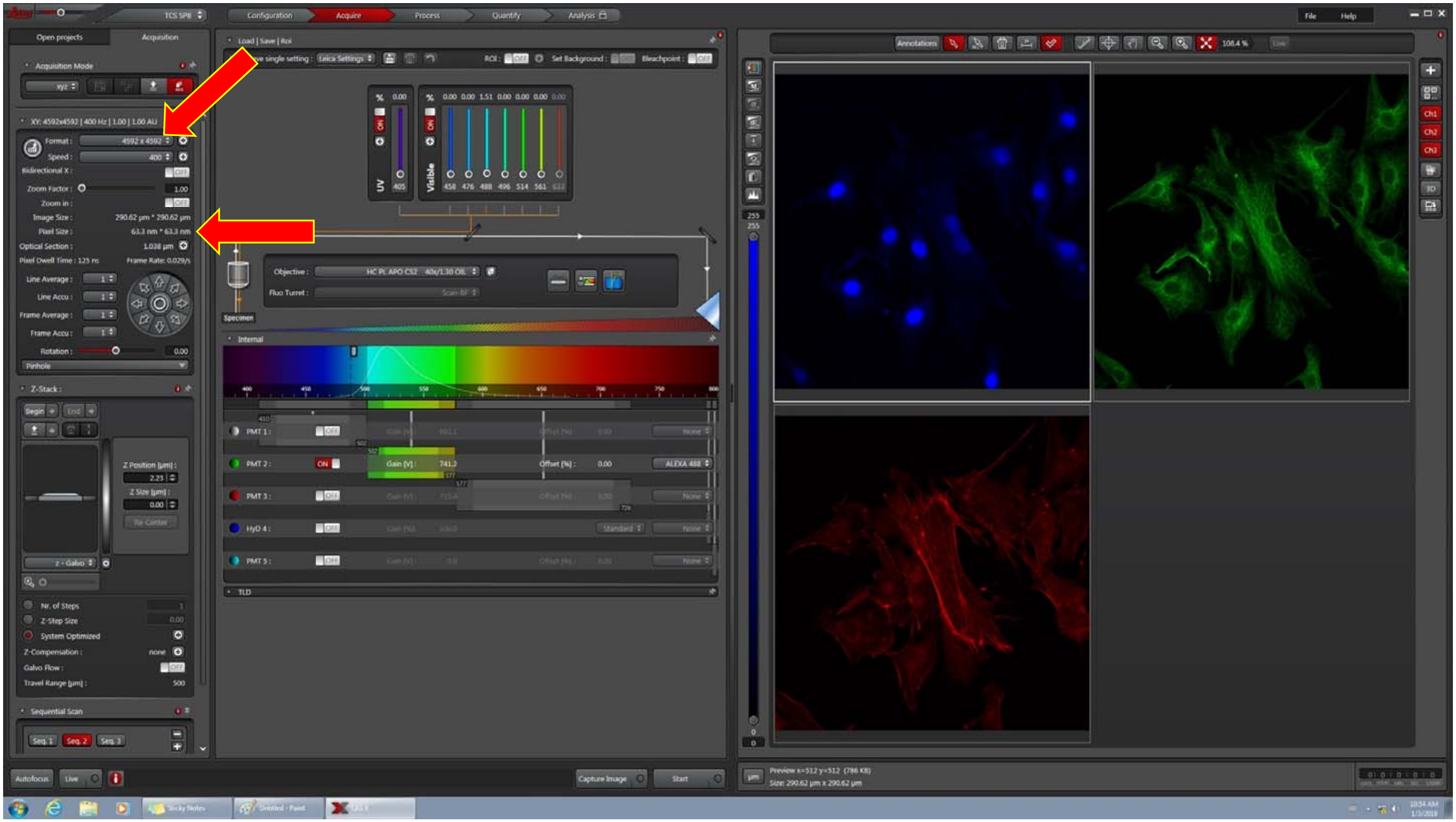
- The software has a button that will increase the number of pixels to maximize resolution for a given lens



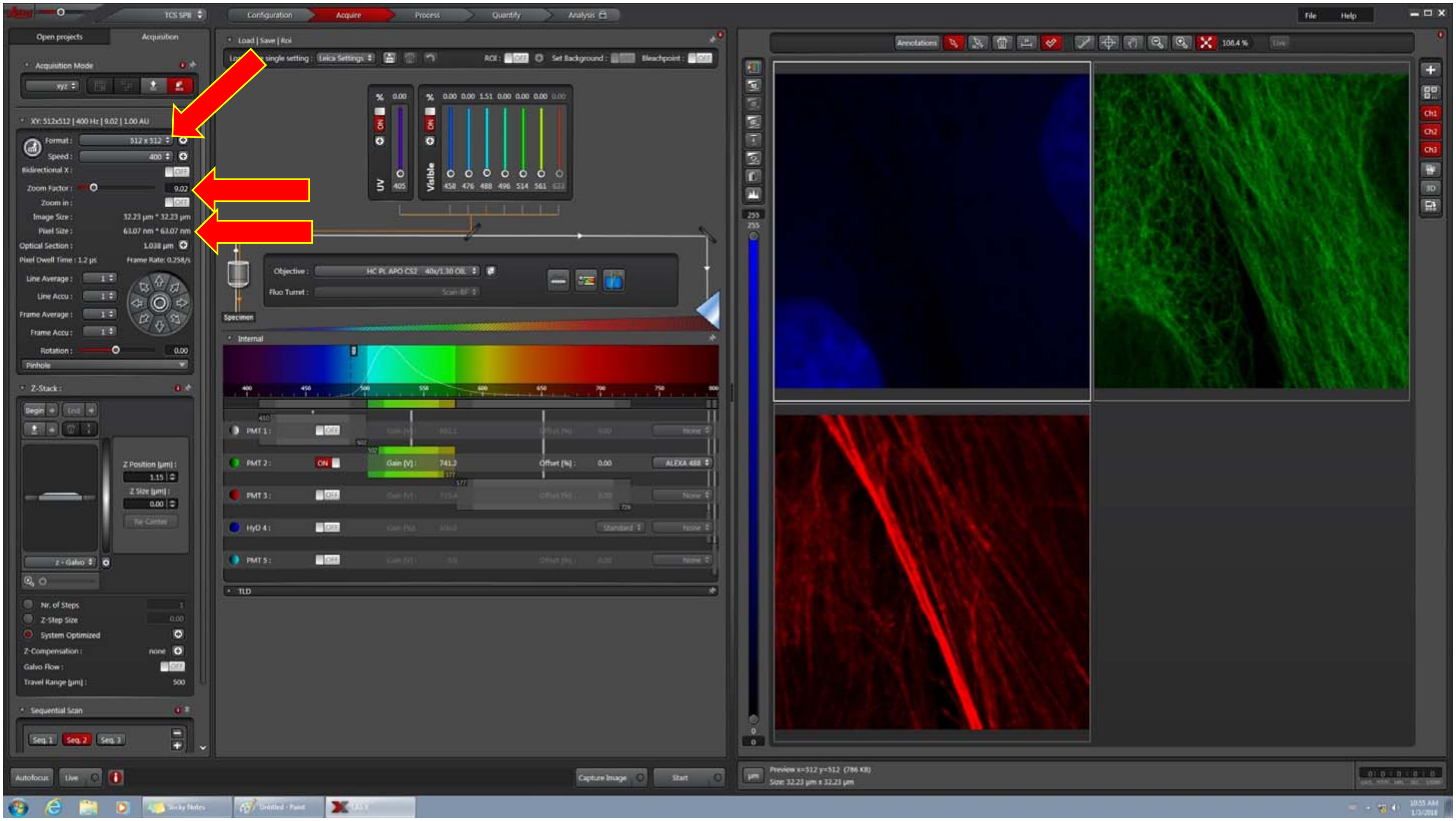
- However, more pixels take longer to scan
- *Pixels smaller than theoretical best size have no additional benefit*



TCS SP8
Configuration
Acquire
Process
Quantify
Analysis
File
Help
Open projects
Acquisition
Acquisition Mode
Load | Save | Roi
Speed :
Bidirectional X :
Zoom Factor :
Zoom in :
Image Size :
Pixel Size :
Optical Section :
Line Average :
Line Accu :
Frame Average :
Frame Accu :
Rotation :
Pinhole
Z-Stack :
Z Position [µm] :
Z Size [µm] :
Nr. of Steps
Z-Step Size
System Optimized
Z-Compensation :
Galvo Flow :
Travel Range [µm] :
Sequential Scan
Seq. 1
Seq. 2
Seq. 3
UV
Visible
Objective :
Fluo Turret :
Specimen
Internal
PMT 1 :
PMT 2 :
PMT 3 :
HyD 4 :
PMT 5 :
Gain [V] :
Offset [%] :
ALEXA 488
TLD
Annotations
Ch1
Ch2
Ch3
Autofocus
Live
Capture Image
Start



TCS SP8
Configuration
Acquire
Process
Quantify
Analysis
File
Help
Open projects
Acquisition
Acquisition Mode
XY: 4592x4592 | 400 Hz | 1.00 | 1.00 AU
Format :
4592 x 4592
Speed :
400
Bidirectional X :
Zoom Factor :
1.00
Zoom in :
Image Size :
290.62 µm * 290.62 µm
Pixel Size :
63.3 nm * 63.3 nm
Optical Section :
1.038 µm
Pixel Dwell Time : 125 ns
Frame Rate: 0.029/s
Line Average :
Line Accu :
Frame Average :
Frame Accu :
Rotation :
0.00
Pinhole
Z-Stack :
Begin
End
Z Position [µm] :
2.23
Z Size [µm] :
0.00
Re-Center
z - Galvo
Nr. of Steps
Z-Step Size
System Optimized
Z-Compensation :
none
Galvo Flow :
Travel Range [µm] :
500
Sequential Scan
Seq. 1
Seq. 2
Seq. 3
Autofocus
Live
Load | Save | Roi
Leica Settings
ROI :
Set Background :
Bleachpoint :
UV
Visible
405
458
476
488
496
514
561
Objective :
HC PL APO CS2 40x/1.30 OIL
Fluo Turret :
Specimen
Internal
PMT 1 :
PMT 2 :
ON
Gain [V] :
741.2
Offset [%] :
0.00
ALEXA 488
PMT 3 :
HyD 4 :
Standard
PMT 5 :
TLD
Capture Image
Start
Annotations
108.4 %
Ch1
Ch2
Ch3
3D
Preview x=512 y=512 (786 KB)
Size: 290.62 µm x 290.62 µm



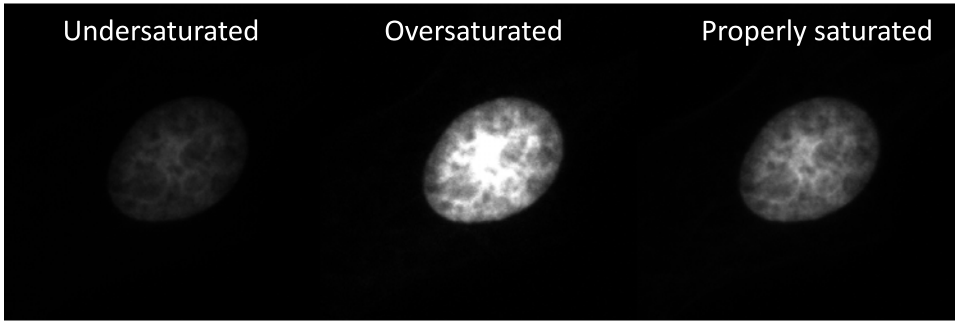
TCS SP8
Configuration
Acquire
Process
Quantify
Analysis
File
Help
Open projects
Acquisition
Acquisition Mode
xyz
XY: 512x512 | 400 Hz | 9.02 | 1.00 AU
Format :
512 x 512
Speed :
400
Bidirectional X :
Zoom Factor :
9.02
Zoom in :
Image Size :
32.23 µm * 32.23 µm
Pixel Size :
63.07 nm * 63.07 nm
Optical 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 [µm] :
1.15
Z Size [µm] :
0.00
z-Galvo
Nr. of Steps
Z-Step Size
System Optimized
Z-Compensation :
none
Galvo Flow :
Travel Range [µm] :
500
Sequential Scan
Seq. 1
Seq. 2
Seq. 3
Load | Save | Roi
Leica Settings
ROI :
Set Background :
Bleachpoint :
UV
Visible
405
458
476
488
496
514
561
Objective :
HC PL APO CS2 40x/1.30 OIL
Fluo Turret :
Specimen
Internal
PMT 1 :
PMT 2 :
ON
Gain [V] :
741.2
Offset [%] :
0.00
ALEXA 488
PMT 3 :
HyD 4 :
Standard
PMT 5 :
TLD
Annotations
108.4 %
Ch1
Ch2
Ch3
3D
Autofocus
Live
Capture Image
Start
Preview x=512 y=512 (786 KB)
Size: 32.23 µm x 32.23 µm

Optimizing Images with the Histogram or Quick LUT

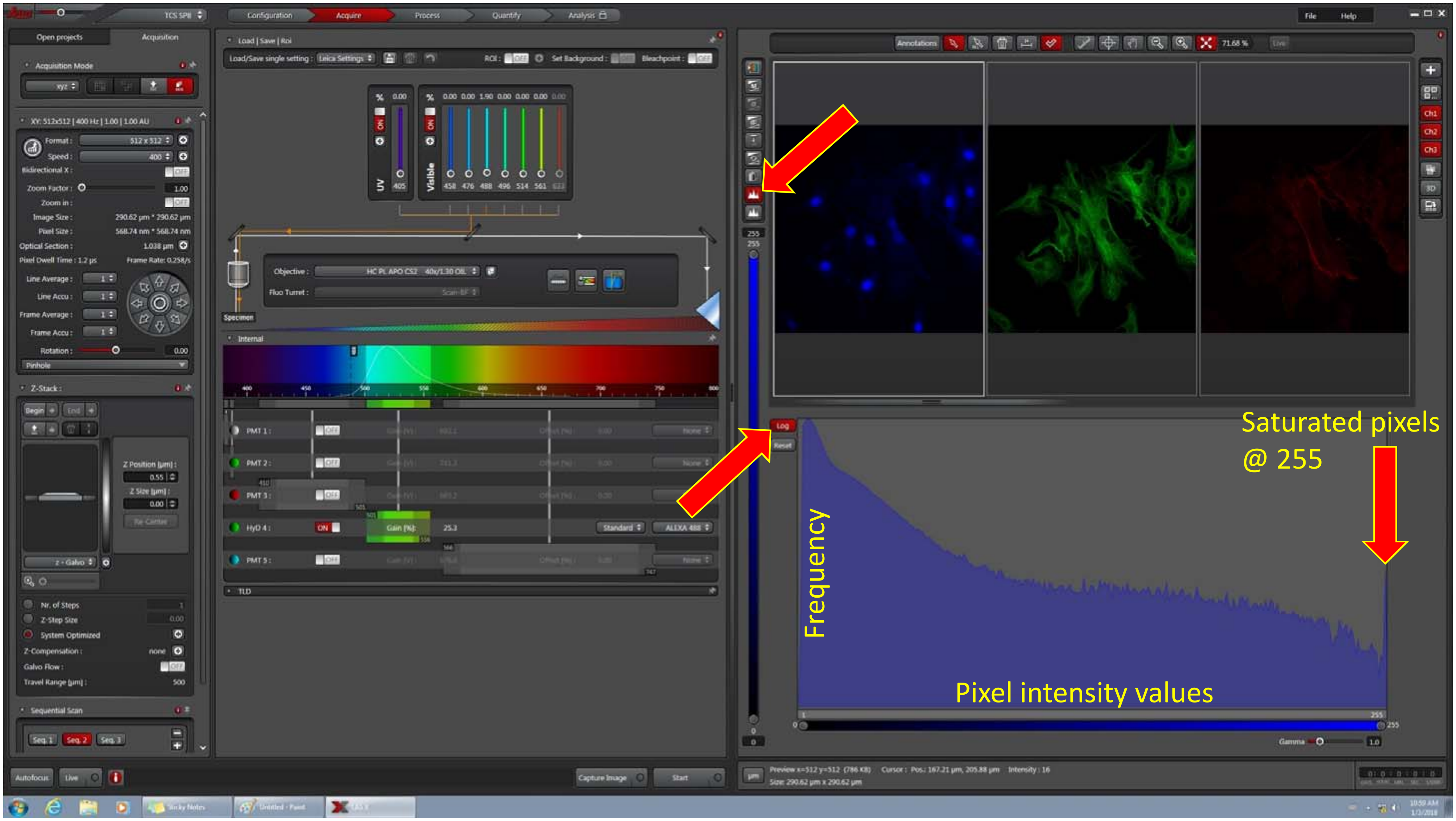
“Your eyes can deceive you. Don’t trust them.”

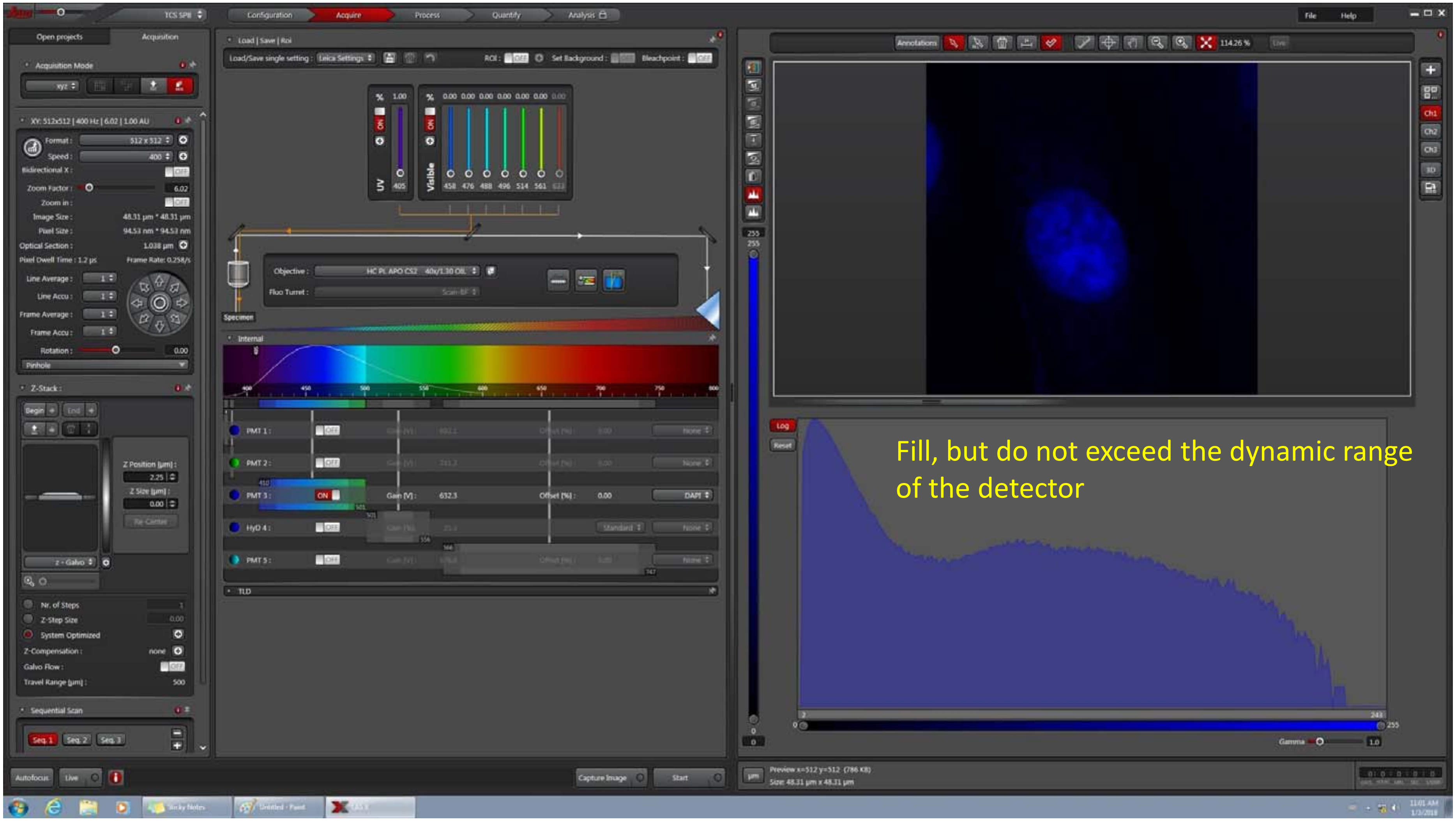
-Obi-Wan Kenobi

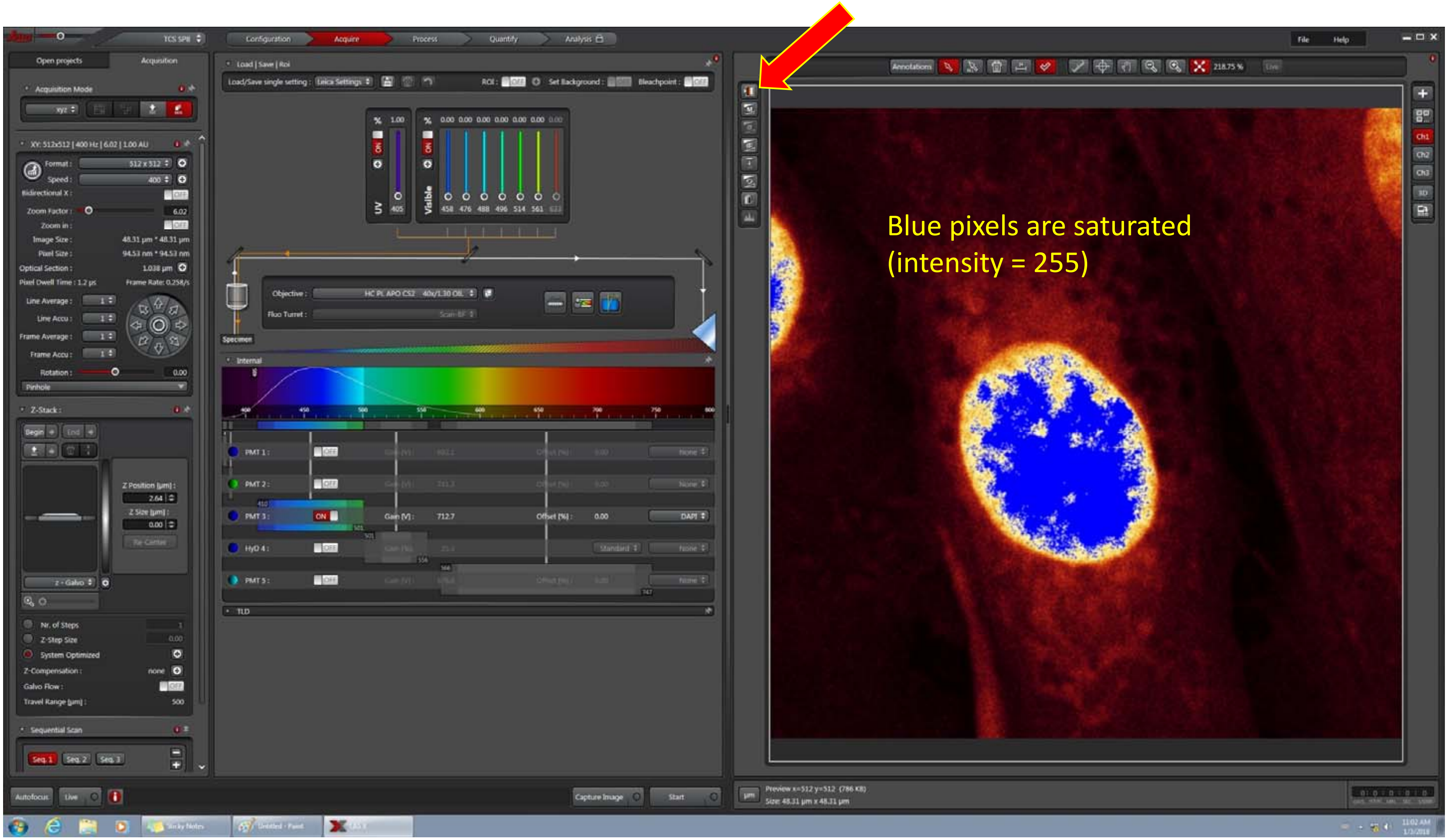
- Images which are under or oversaturated are not using the dynamic range of the detector

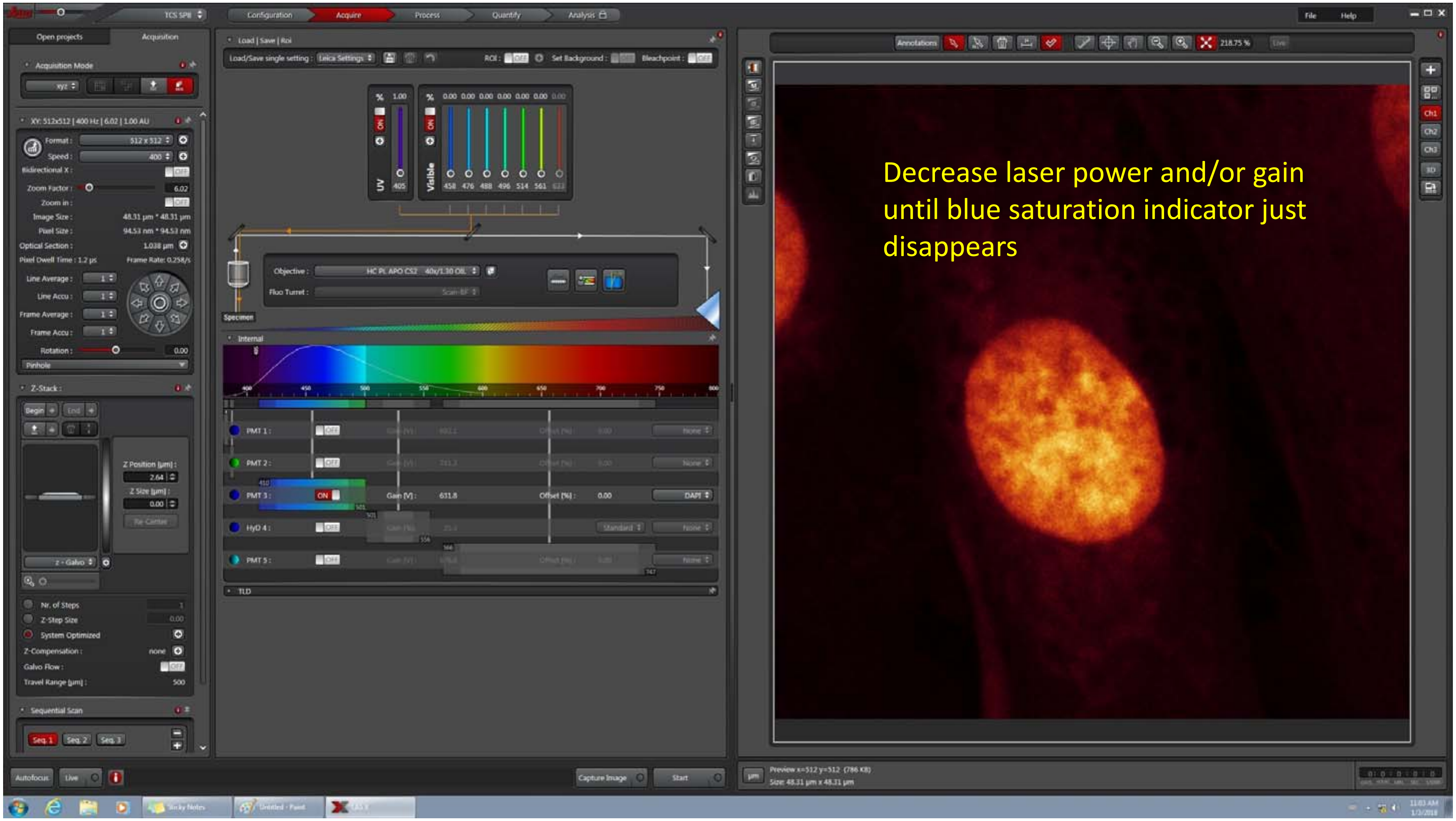


- These images are missing information
- There are quantitative tools to help you choose the best laser power and gain


Saturated pixels
@ 255
Frequency
Pixel intensity values

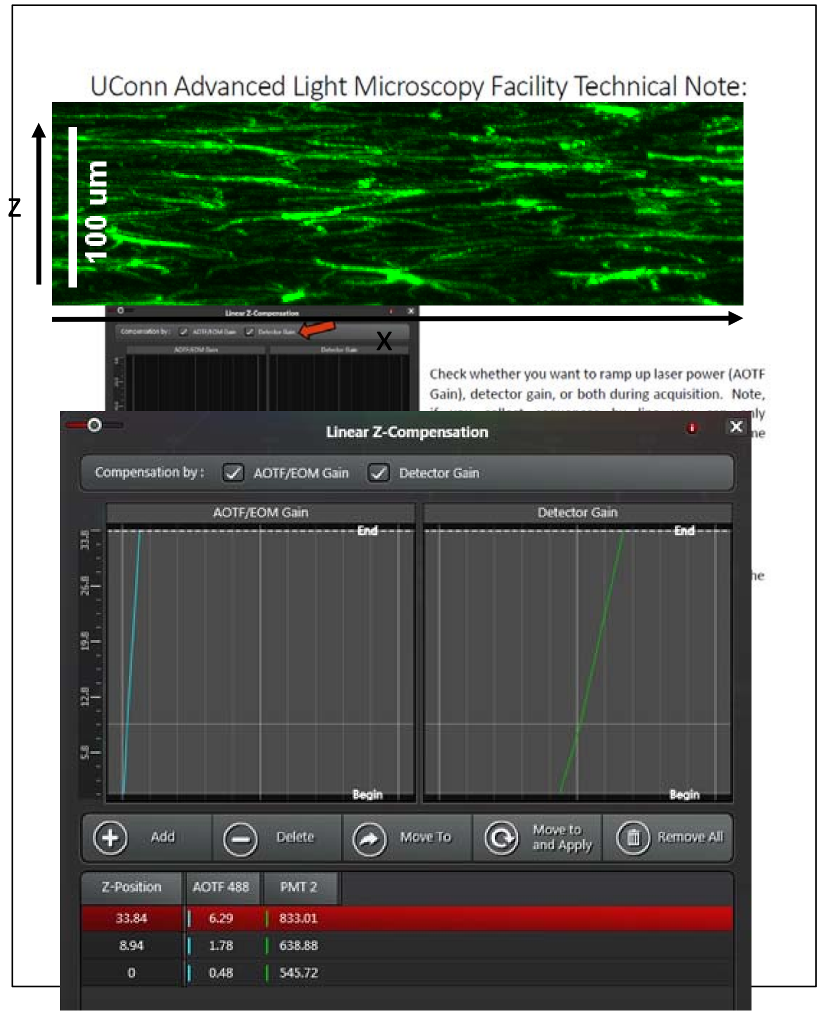

Configuration
Acquire
Process
Quantify
Analysis
Fill, but do not exceed the dynamic range of the detector
Capture Image
Start


Blue pixels are saturated
(intensity = 255)

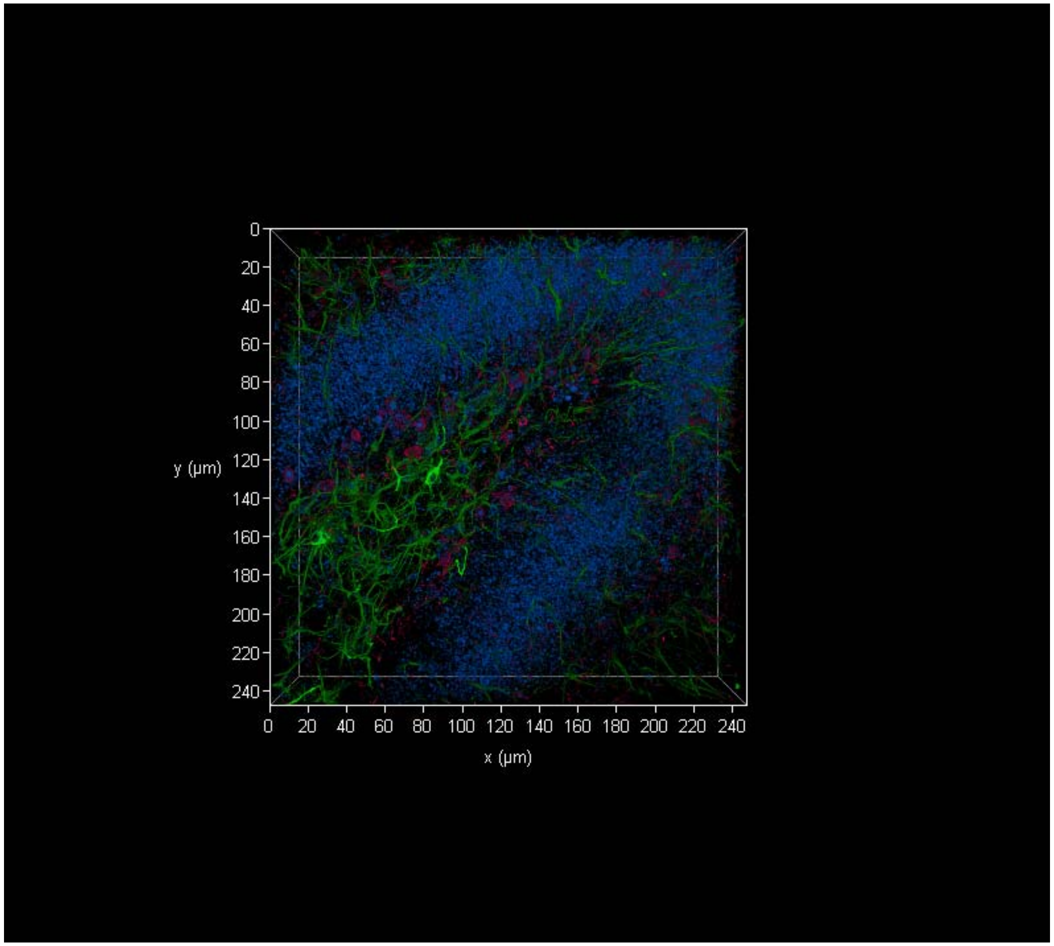

Decrease laser power and/or gain
until blue saturation indicator just
disappears

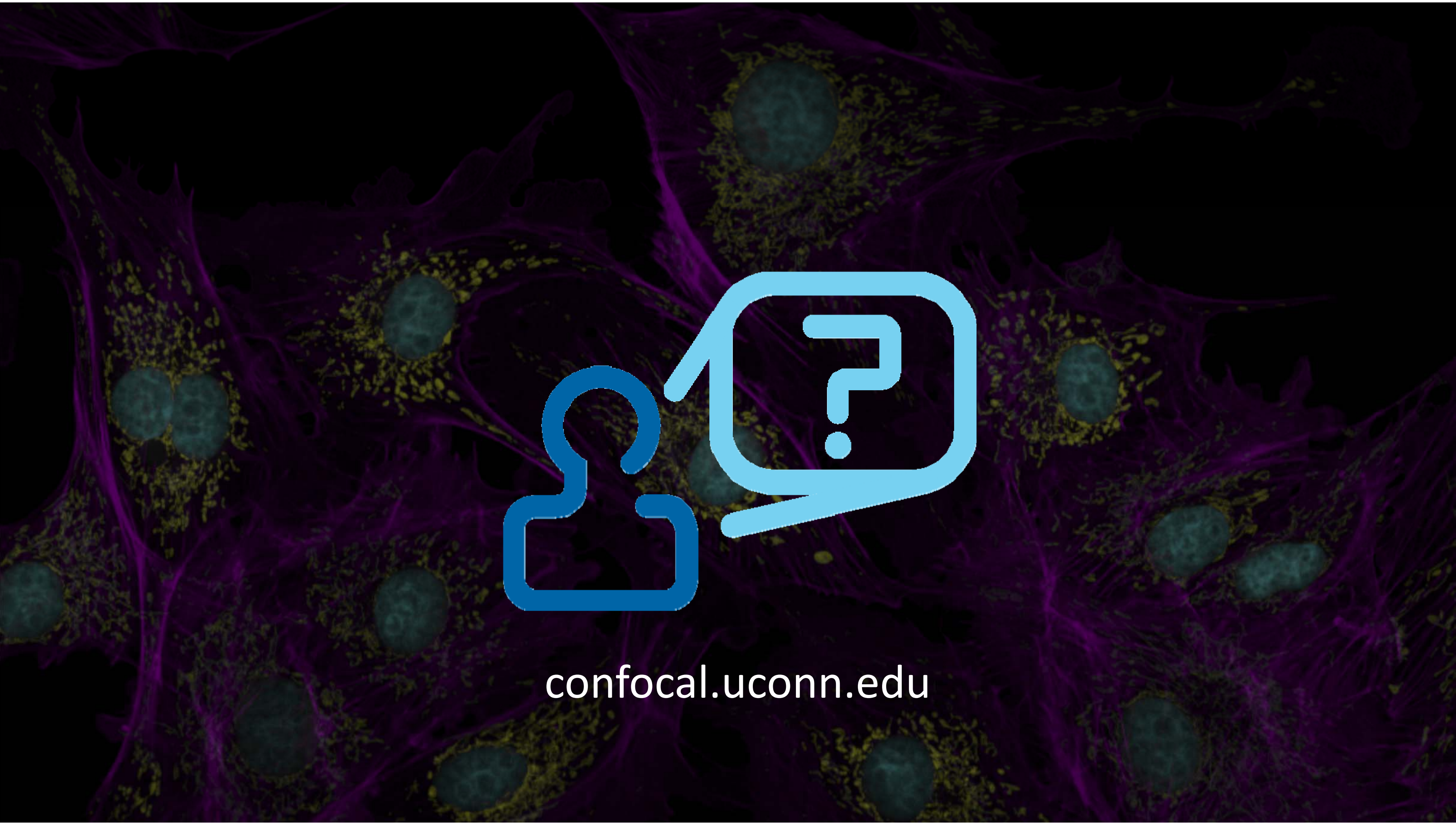
Linear Z Compensation

- Optical aberrations get worse the deeper you image into a specimen
- One result is decreasing signal during z stacks (usually noticeable > 20 um)
- 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/



120 um z stack through mouse hippocampus




confocal.uconn.edu