Imaging Beyond the Basics: Optimizing Settings on the Leica SP8 Confocal
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):**
- **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

Simultaneous scan

Sequential: 2 scans

Sequential: 3 scans

Apply the detector settings you want
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”
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
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

<table>
<thead>
<tr>
<th>By Frame</th>
<th>By Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min 46 sec</td>
<td>1 min 52 sec</td>
</tr>
</tbody>
</table>

“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

<table>
<thead>
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<th>By Line + Bidirectional</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min 46 sec</td>
<td>1 min 52 sec</td>
<td>56 sec</td>
<td></td>
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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
Scan speed is fixed at 8000 lps.
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

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<th>By Line + Bidirectional</th>
<th>By Line + Resonant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min 46 sec</td>
<td>1 min 52 sec</td>
<td>56 sec</td>
<td>17 sec</td>
</tr>
</tbody>
</table>

*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

By Frame
- 3 min 46 sec

By Line
- 1 min 52 sec

By Line + Bidirectional
- 56 sec

By Line + Resonant*
- 17 sec

By Line + Resonant* + Bidirectional
- 9 sec

25X Faster!

*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*
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

Pixel intensity values

Frequency
Fill, but do not exceed the dynamic range of the detector
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