Confocal and other scanning microscopes
Confocal systems in the NIF
Lasers
Lasers

When laser is pumped, photons are spontaneously emitted as excited electrons return to lower energy levels.

Photons reflect off mirrors and start to stimulate other electrons to emit their photons.

A chain reaction of photons begins and the laser begins to "lase."

Full operation.
Remember the PSF?

Image of a Small Specimen

1.4NA objective
\( \lambda = 0.48 \, \mu m \)

Image size does not change

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Excitation volume: the min

The diffraction limit described by Ernst Abbe. The smallest diameter of your focal volume will be:

By underfilling the back aperture of the objective, you practically decrease the NA of your objective so the focal volume will be bigger (in xy and in z).
So for a wavelength of 488 nm, and a 100x objective with a NA of 1.4, \( d = \frac{488}{2 \times 1.4} = 174 \text{ nm} \) (impossible theoretical limit actually)
Lasers

- The medium or laser material can be a gas or it can be a solid like a crystal doped with specific ions to obtain different properties (wavelength, pulse width etc...)
- The laser beam comes out coherent: propagating in one direction, phase locked, same waveform and same frequency. Can be focused in a diffraction limited spot.
- The intensity profile follows a gaussian distribution.
Scanning microscopy
Detectors - PMT/HyD

• The PMT and the HyD both convert photons into electrons @ high efficiency.

• A PMT will multiply the converted electrons across many steps of dynodes before collection at the Anode and pre-amplification (this is where gain happens) at the Electron read-out step.

• A HyD is able to convert the photon at much higher efficiency over fewer steps which reduces the noise from multiple amplifications on the dynodes found in the PMT.
Image Construction - PMT/HyD Confocal

Hence the phrase “Laser Scanning”
Gain and Offset - The Big Picture

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High Voltage (HV) vs. Gain

• Some microscopes allow you to directly adjust the voltage on the dynodes of the PMT.

• The manufacturer will often specify normal range for voltage on the PMT dynodes. Downside of gain: additional noise can be introduced to the image.

• Gain is different in that it multiplies the final value of the collected charge from the PMT/HyD.
Good things to think about before taking a confocal image..

• Do I actually require the benefits of confocal?
  • Many researchers will use a 10-20x objective to capture a large field confocal image when you could capture nearly the same quality image with a widefield microscope and in a much shorter time.
  • Confocal can’t make up for a bad objective.
  • **Confocals are used to reject background coming from above or below your plane of interest.**

• When you look through the eyepiece you are not in confocal view.
  • The eyepiece is ”widefield mode”
  • This means that the optical section of the eyepiece is also much thicker so often you’ll need to readjust your focal plane to match confocal mode. Do this before you increase laser power and gain.
Confocal microscopy

Single photon excitation illuminates the entire hourglass. Addition of the pinhole aperture blocking photons not coming from the focal plane.
Out of focus fluorescence

Detector
Focusing lens
Pinhole
Objective
Focal plane
The Pinhole

- Constricting the pinhole further can see resolution gains BUT only when the signal is VERY STRONG

- Opening the pinhole will result in a brighter image but will drastically lower the resolution and increase the optical section. This is the same as wide-field when we do this.
Image Acquisition on a Confocal
How should I format my image?

• Optical vs. Digital resolution
  • Presuming you have maximized your optical resolution you should decide on a pixel size that is appropriate to capture the size of your specimen (remember Nyquist).
  • Many modern microscopes will calculate this value for you.
  • But what makes for a good pixel size?

Pixel format equates to a real world pixel size

Common Formats:
- 512x512
- 1024x1024
- 2048x2048
Choosing your dye configuration

• Most microscopy systems have a dye configuration assistant; here is where you will select your dyes.

• Ordering may matter here as some dyes are more likely to bleach than others.

• Your image is being built one pixel at a time. You can scan a single line then switch dyes or you can scan an entire frame then switch.

• Usually we stick to line sequential to avoid crosstalk and to build all channel images simultaneously to visualize our entire image at once.
Excitation and Emission

- **Excitation** – This is the wavelength of light that we will project onto the sample (represented by the dotted line on the spectrum in the image).

- **Emission** – This is the light that is emitted from the fluorophore and sent back through the objective (this is the white line in the image).

- In some microscopes we can control the emission collection...
Zoom – A way to decrease pixel size without adding pixels

Zoom = 1.5

Note: many systems will default to 1.5 zoom for certain obj. so as not to capture the uncorrected edges of the field

Zoom = 3
Setting up your image

• Laser power – We start with a very low percentage of laser power then work our way up as needed. Often <20% is required.
  • Keep in mind the % of the laser power is related to overall strength of the laser.

• Use Hi-Lo view – this is a LUT. Maximum intensity value pixels shown in blue here.

• If possible, before adding more laser power reduce scan speed to maximize light absorption at a point source.
Offset – Setting the baseline of your image

- In Hi-Lo view on a Leica system green represents a pixel with an intensity value of 0.

- Think of offset as the threshold energy value required to detect a photon.

- If the offset is too high then information will be completely removed from your image. This is worse than saturation because no information will be left behind.
Image Averaging and Accumulation

• Because we build an image a pixel at a time we have tremendous flexibility in how we create our image from consecutive acquisitions.

• We can average lines or frames of our images to reduce noise. **This will not get rid of background.**

• OR we can accumulate signal from consecutive lines or frames to add our signal together. This can often keep noise low while brightening the signal in our image.

• With accumulation however, you can end up saturating your image.
Optical Section –
The 3rd Dimension

- Notice while pixel size changes in the x-y plane, our optical section size does not change.
- When performing z-stacks use your knowledge of optical sections to think about step size
- Think in VOXELS! (volumetric pixels)
Confocal examples
Spinning disk examples
2-photon excitation

![Jablonski Diagram](from Brad Amos/Science Photo Library, London)
2P images

Chettih and Harvey, Nature 2019
Find the core online for more info on equipment and services: https://nif.hms.harvard.edu/

Follow us on Twitter for events and classes: https://twitter.com/hmsneuro

HMS wide microscopy cores and services, register for emailing list: https://microscopy.hms.harvard.edu/

Resources:
• microscopyU
• Olympus microscopy resource center
• Leica Science lab