INTRODUCTION TO CONFOCAL MICROSCOPY

Matt Renshaw
October 2018
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<tr>
<th>Date</th>
<th>Topic</th>
<th>Presenter</th>
<th>Location</th>
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<tr>
<td>17th Oct</td>
<td>Intro to Light Microscopy</td>
<td>Kurt Anderson</td>
<td>Auditorium 1</td>
<td>10:00</td>
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<tr>
<td>24th Oct</td>
<td>Intro to Confocal Microscopy</td>
<td>Matt Renshaw</td>
<td>Auditorium 1</td>
<td>10:00</td>
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<tr>
<td>31st Oct</td>
<td>Intro to Live Cell Imaging</td>
<td>Deborah Aubyn</td>
<td>Seminar room 4</td>
<td>10:00</td>
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<td>7th Nov</td>
<td>Intro to Optical Sectioning</td>
<td>Donald Bell</td>
<td>Seminar room 3 &amp; 4</td>
<td>11:00</td>
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<td>14th Nov</td>
<td>Intro to Light Sheet Microscopy</td>
<td>Alessandro Ciccarelli</td>
<td>Seminar room 4 &amp; 5</td>
<td>10:00</td>
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<tr>
<td>28th Nov</td>
<td>Intro to Multiphoton Microscopy</td>
<td>Rocco D'Antuono</td>
<td>Seminar room 4 &amp; 5</td>
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Introduction to confocal microscopy

Widefield

Confocal

(yes, both are GFP z stacks)
widefield

confocal

normalised intensity vs. distance (microns)
Take home message

Confocal microscopy improves the optical resolution and contrast of your images by reducing out of focus light
Talk outline...

- Basics of fluorescence microscopy and the principle of confocal microscopy
- Multi-dimensional image acquisition with a point scanning confocal microscope
- Advanced applications
Epifluorescence light path

Sample and slide
Objective lens
Dichroic mirror
Emission filter
Detector

Jablonski diagram

Stokes shift

www.leica-microsystems.com/science-lab/fluorescence-in-microscopy/
svi.nl/FluorescenceMicroscope
Microscopy is:
- creating a magnified, blurred image of your object of interest.

Components of blur...

1. **Point Spread Function**

![Diagram showing how light waves interfere and converge on the focal point, creating a diffraction pattern called the Airy Disk.](image)

Light waves interfere and converge on the focal point, creating a diffraction pattern called the Airy Disk.
Separating objects - the Airy Disk

Rayleigh limit is when the central maxima of one Airy Disk is in line with the first minima of the second Airy Disk

Size of the Airy Disk determines minimum resolvable distance of 2 objects

http://olympus.magnet.fsu.edu/
Size of the Airy Disk determines minimum resolvable distance of 2 objects.
Sub-resolution bead in 3D

Graph showing data with a peak at a distance of 6 µm. Two sets of images labeled XY and YZ, with indications of focal planes.
Point Spread Function: the 3D diffraction pattern
Sources of blur

1. Point Spread Function
2. Light from out of focus areas
Pinhole blocks diffraction rings

Optical section at resolution limit
Pinhole increases contrast by excluding light from the diffraction rings

Does the pinhole increase resolution?
Laser scanning confocal

Widefield illumination  Laser scanning
Image is built up pixel by pixel

Emission light is descanned = focussed onto a static position
No pinhole
Pinhole excludes out of focus light
Numerical Aperture

- Depth of focus
- PSF
- size of the airy disk

are all related and defined by the angle of light collected by the objective.
• Numerical Aperture determines:
  • how much light from the sample is collected by the objective
  • resolution
  • the thickness of sample that appears in focus
- Numerical aperture \( NA = n \sin \alpha \)

- Resolution \( d = \frac{1.22 \cdot \lambda}{2 \cdot NA} \)

- Depth of focus \( D \propto \frac{n \cdot \lambda}{NA^2} \)
Optical Sectioning: Confocal Microscopy

Other optical sectioning techniques are available.

See: Intro to Optical Sectioning talk
Talk outline...

- Basics of fluorescence microscopy and the principle of confocal microscopy
- Multi-dimensional image acquisition with a point scanning confocal microscope
- Advanced applications
The Tetrahedron of Frustration

Sample health

Spatial resolution

Temporal resolution

Signal-to-noise ratio
Standard applications

- Although there are subtle differences, all of our confocals have these basic functions
- Software varies, but settings are there somewhere...
Excitation and Emission
Detection

- Adaptable wavelength selection
- Simultaneous or sequential detection
### Brief comparison of detectors

<table>
<thead>
<tr>
<th></th>
<th>PM T Alkali</th>
<th>PM T GaAsP</th>
<th>APD/SPAD</th>
<th>HyD</th>
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<tbody>
<tr>
<td>PDE (500 nm) %</td>
<td>25</td>
<td>37</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Pulse noise %</td>
<td>60 %</td>
<td>60 %</td>
<td>5 %</td>
<td>3 %</td>
</tr>
<tr>
<td>Pulse width/μs</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>0.1 (</td>
<td>1</td>
</tr>
<tr>
<td>Dark #/s</td>
<td>15,000</td>
<td>15,000</td>
<td>300 (</td>
<td>2,500</td>
</tr>
<tr>
<td>max M #/s</td>
<td>very low</td>
<td>very low</td>
<td>20</td>
<td>150</td>
</tr>
<tr>
<td>Area/mm²</td>
<td>50</td>
<td>50</td>
<td>0.05</td>
<td>8</td>
</tr>
<tr>
<td>Afterpulsing</td>
<td>high</td>
<td>high</td>
<td>medium</td>
<td>low</td>
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[www.leica-microsystems.com/science-lab/]
Optimising acquisition settings

- Laser power
- Gain
- Number of pixels
- Scanning speed
- Size of scanning area
- Averaging
Increase laser power = increase signal

Increase laser power = increase photo damage
Increase laser power = increase signal

Increase laser power = increase photo damage
Increase gain = increase signal

Increase gain = increase noise
Increase gain = increase signal

Increase gain = increase noise
Finding a compromise
Finding a compromise

Noise can be negative, addition of offset prevents false zeros
Finding a compromise

Noise can be negative, addition of offset prevents false zeros
Achieving resolution: number of pixels

512 x 512 ~500µm per pixel

1024 x 1024 ~250µm per pixel

More pixels, more time
Achieving resolution: number of pixels

512 x 512 ~500µm per pixel  
2048 x 2048 ~125µm per pixel

More pixels, more time
Achieving resolution: optical zoom

1024 x 1024 ~250µm per pixel

Zooming reduces size of scanned area

1024 x 1024 ~125µm per pixel
Line averaging reduces noise, but takes longer

Averaging 1
Line averaging reduces noise, but takes longer

Averaging 4
Line averaging reduces noise, but takes longer

Averaging 8
Reduce scanning speed, reduce noise
Reduce scanning speed, reduce noise
Imaging 3D volumes
Imaging deep, increases light scattering

Laser power and gain can be adjusted through z stack to compensate
Talk outline...

- Basics of fluorescence microscopy and the principle of confocal microscopy
- Multi-dimensional image acquisition with a point scanning confocal microscope
- Advanced applications
Nikon W1 spinning disk confocal
- Speed
- Reduced photo-toxicity
- Photo manipulation
Resonant scanning on the Olympus FV3000

- rapidly oscillating resonant mirror scanners
- Very fast frame speeds (~400 per second)
- Permits very low laser power
- Combined with rolling averaging (post processing) can detect high quality images of dynamic processes
Zeiss LSM 880 with Airyscan
Pinhole diameter, resolution and transmission

0.2 AU = 1.4 x improvement
Airyscan detector is 32x 0.2 AU mini GaAsP detectors
Spectral imaging and unmixing

- separating fluorophores with close emission spectra
  
e.g. Alexa fluor 555 and Mito Tracker orange
Whole spectrum of emitted light collected by spectral detector (ChS)
8.7 nm intensity bins

415 nm

686 nm
Alexa 555 labelled microtubules and Mito tracker orange mitochondria
Multiphoton

- Multiphoton laser allows very deep tissue imaging
- See Introduction to Multiphoton Imaging talk (Rocco, 28th Nov)
- MP lasers can also be used for cutting experiments
MP – ablation experiments

Sophie Herszterg, Vincent lab
Fluorescent Recovery After Photobleaching

- Bleach region of interest
- Measure recovery of fluorescence
Using FRAP to investigate cell-cell interactions
New Leica SP8 FALCON

Time-resolved, single-photon detection

Pulsed, white-light laser
Fluorescence Lifetime Imaging (FLIM)

- Quantify FRET
- Detect protein interactions

Roman Fedoryshchak, Treisman lab
Thank you for your attention!

Good sources of info... (Google is always useful too)

- https://www.leica-microsystems.com/science-lab/
- https://www.microscopyu.com/
- http://olympus.magnet.fsu.edu/index.html
- http://zeiss-campus.magnet.fsu.edu/
- http://micro.magnet.fsu.edu/
- https://svi.nl/
- Imaging Helpdesk (twice monthly in cafe)