Microscopy Optical Sectioning

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Of Light Microscopy
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The Eyes of Science
• Confocal Microscopy In Principle
• Point Scanning Confocal
• Spinning Disk Confocal
• Swept Field Confocal
• Total Internal Reflection Fluorescence (TIRF)
1. Define optical section in terms of the mathematics:

- Lateral resolution: for diffraction limited techniques: where $d$ is the smallest resolvable unit, $\lambda$ is the wavelength of light, and $NA$ is the numerical Aperture of the objective lens.

$$d_{xy} = \frac{1.22\lambda}{2NA}$$

Example: For a 1.4NA objective at 550nm, the resolution limit is 240nm.
1. Define optical section in terms of the mathematics:

- Axial resolution (focal volume): Again, for diffraction limited techniques, the axial resolution is given as: where $\eta$ is the refractive index of the media.

$$d_z = \frac{2\lambda \cdot \eta}{(NA_{obj})^2}$$

Example: For a 1.4NA objective in oil/oil, at 550nm, the resolution limit is 850nm.
Point Spread Function

- PSF defines the propagation of electromagnetic radiation or other imaging light rays from a point source or point object.
- The degree of spreading of the point object is a measure of the imaging system.
- The effect of the imaging system on a point object is termed the convolution of the optical system.
- Displayed by imaging a sub micron point in 3D (Z stack) and displaying as a volumetric airy disc. (X-Z projection)
Conventional light microscopy is limited by multiple focal planes in the same section, therefore:

- Physically cut thinner sections
  - Technically difficult
  - Highest resolution
  - Highest sensitivity
- Optical sectioning using confocal microscopy
  - Technically simple
  - Multiple section planes available
  - Complex computing
Principal of Confocal microscopy

Move pinhole or Specimen in Z Axis to get multiple focal planes
Pinhole size and resolution

- Pinhole size should be related to width of point spread function
- Width of point spread function = resolution of lens \( \times \) magnification of lens = 1 Airy unit
  
  - 100x / 1.4 NA: resolution = 220nm, so 1 Airy unit = 22 \( \mu \text{m} \)
  - 40x / 1.3 NA: resolution = 235nm, so 1 Airy unit = 9.4 \( \mu \text{m} \)
  - 20x / 0.75 NA: resolution = 407nm, so 1 Airy unit = 8.1 \( \mu \text{m} \)
  - 10x / 0.45 NA: resolution = 678nm, so 1 Airy unit = 6.8 \( \mu \text{m} \)
Confocal and Widefield Fluorescence Microscopy

Figure 1
Diffraction limited volume:

$NA = 1.4$

$\eta$ (Refractive Index) = 1.515

$\lambda$ (Wavelength) = 550 nm

$$d_{xy} = \frac{1.22 \lambda}{(2NA)} = \frac{1.22 \cdot 0.55 \ \mu m}{(2 \cdot 1.4)} = 0.24 \ \mu m = 240 \ nm$$

$$d_z = \frac{2\lambda \cdot \eta}{(NA_{obj})^2}$$

$$= \frac{2 \cdot (0.55 \ \mu m) \cdot 1.515}{(1.4)^2} = 0.85 \ \mu m = 850 \ nm$$
Components of a confocal

Light source
Generally laser based

Controller and image acquisition computer

Detector
PMT or CCD

Microscope

Scan Head w/pinholes
Once the “Z-stack” has been collected and sent to the computer, Sections of the stack can be reconstructed into a 3D volume.
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How a Point Scanner Works

The pinhole will only provide information about a single point in space. Therefore move specimen or move the exciting light.

- Note that the mirrors also descan the returning light
- Many systems have been developed to control the scan mirrors
- Also motorize Z-axis to sample points in vertical axis

X and Y Galvo Mirrors scan the whole field in a raster pattern.
Commonly used Lasers

- Argon (Ar) 457, 488, 514 nm
- Krypton-Ar (Kr-Ar) 488, 568, 647 nm
- Helium-Neon (He-Ne) lots single line sources 543, 594, 633 nm
- He-Cadmium (He-Cd) 325 - 441 nm
- Diodes 350, 405, 442, 473, 488, 491,514, 532, 561,593, 638, more coming!
Photomultipliers (PMTs)

- Maximize the light budget returning from the scan head by intensification.
- Subject to electrical noise; too much gain will degrade image and demand excessive averaging.

PMTs must be matched with detection wavelength.
Computer builds digital image from scanned data
Due to PMT Noise, Averaging is commonly used to enhance S/N.
Multi-photon excitation does not excite out-of-focus light, so you can get rid of pinhole.
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Spinning Disc Confocal

Figure 5

Nikon

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SFC Design Overview

- Supravideo rate, high frequency slit scanner.
- Variable pinhole, linear array field scanner.
- CCD camera-based detector.
- Linear scanning at high frequency minimizes phototoxicity.
SFC Design Overview, cont’d.

- Variable pinhole sizes match various objective lenses.
- Linear pinhole array (1-D) and separate excitation and emission light path minimize emission crosstalk.
SFC Aperture Positions

Slit scanning positions
- 35 μm slit
- 70 μm slit

Field scanning positions
- 30 μm pinholes (x32)
- 45 μm pinholes (x32)
- 60 μm pinholes (x32)
- 90 μm pinholes (x32)
Excitation slits and pinholes

Emission slits and pinholes
Laser beam is shaped into a line, then scanned
Slit scanner lightpath (simplified)
Low frequency galvo sweep

High frequency galvo sweep
Field-scanner lightpath
Galvo “Sweep”
Piezo movement of Pinhole image

CCD Frame #1 (or odd #s)

CCD Frame #2 (or even #s)
Piezos move a fixed distance

Time to move depends on exposure time

It takes 2 frames for one complete “piezo sweep”

Galvo moves a fixed distance

Time to move depends on exposure time

Galvo sweeps multiple cycles/frame, and piezos sweep only once per frame

Galvo cycles multiple times / frame
Field-scanning combines multiple galvo sweeps with bidirectional piezo movement.
Galvo "Sweep"

Piezo step
Galvo, Piezos stationary

Sweeping galvo only

Sweeping galvo & piezos

Piezo sweep synch with frame rate
Performance

- Slit scan imaging to 1000Hz with appropriate detector.
- Pinhole scan imaging to 100Hz.
- Axial resolution from $\sim 0.4\mu m$ to $1.2\mu m$ over 6 scanning modes.
10 minute timelapse (35um slit) Beta cell with eGFP labeled insulin.
Calcium sparks at 80 FPS in smooth muscle cells (Fluo4)
Drosophila Embryo Heart Beating 50 FPS, GFP expressed in smooth muscle.
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What is Evanescent Wave or Total Internal Reflection Fluorescence (TIRF) Microscopy?

- An optical phenomenon that can occur when light strikes the interface between two media of different refractive indices.

- When light is totally internally reflected it does not propagate through the sample, but causes an electromagnetic field (termed the evanescent wave) that extends in the z-direction.

- The evanescent wave intensity decreases exponentially, and extends only a few hundred nanometers into the second media.
Criterion for TIR

- $N_1 > N_2$
- The angle of incidence must be $\geq$ the critical angle ($\theta_C$)

Glass ($n=1.515$)

Live cells ($n=1.33-1.38$)
The energy of the evanescent wave at any point in z is a function of location in the field and the penetration depth.

The penetration depth of the evanescent wave is a function of the illumination wavelength.

only 50nm to 100nm is typically useful illumination.
Why is this useful for fluorescence microscopy?

- The evanescent wave will typically only excite fluorophores within 50nm-80nm of the cover slip.

- Excitation of this small proportion of fluorophores in the thin optical section results in an extremely large increase in the S/N.

- As a result of the increase in S/N very small signals, such as single molecule fluorescence, can be resolved above the noise floor.
TIRF is all about

SIGNAL to NOISE!
Epi-Fl Image
5mm deep solution of 200nm fluorophor coated polystyrene micro-spheres.

3D Intensity Histogram of image on the left

SIGNAL to NOISE
Epi-fluorescence = 1.3
For this specimen

Low Intensity (black) → High Intensity (white)
TIRF Image
5mm deep solution of 200nm fluorophor coated polystyrene micro-spheres.

SIGNAL to NOISE
TIRF = 35
For the same specimen

3D Intensity Histogram of image on the left

Low Intensity (black)  High Intensity (white)
What are some of the applications of TIRF?

- Endocytosis and Exocytosis
- Dynamics of membrane associated proteins
- Protein arrangement at membranes
- Focal adhesions
- Growth cone migration
- Receptor/Ligand interactions
- Biophysical studies of single molecule behavior

- ??? For you to figure out
When a laser is focused at the back aperture plane of the objective in the low NA regions (left) it causes epi-illumination, however when the laser focus is moved to the high NA regions, beyond the n of the specimen, the light is totally internally reflected (right). Consequently, the aperture plane darkens and reveals an “Eclipse” of light at the perimeter.
High NA objective lenses are necessary for Total Internal Reflection, utilizing the lens method, so that light can come out of the lens at angles at or beyond the critical angle for TIR.

\[ NA = n \sin \theta \]

- Numerical Aperture
- Mounting Media Refractive Index
- Angle of the Cone of illumination

\[ \theta \]
Special objective lenses provide the NA Necessary to achieve Total Internal Reflection Through the objective lens.
COS Cell Expressing GFP Clathrin
(Taken with “white light” TIRF)

Images by: Prof. Dan Axelrod, Univ. of Michigan
Aplysia Growth Cone, primary culture
Alexa 488 Actin
Camera: Roper Coolsnap HQ, 0.1Hz

Imaged by Andy Schaefer, Paul Forscher Lab., Yale University
Aplysia Growth Cone, primary culture
Alexa 488 Actin
Alexa 568 Tubulin
Camera: Roper Coolsnap HQ, 0.1Hz

Imaged by Andy Schaefer, Paul Forscher Lab., Yale University
Aplysia Growth Cone, primary culture
Alexa 488 Actin (speckled concentration)
Alexa 568 Tubulin
Camera: Roper Coolsnap HQ, 0.1Hz

Imaged by Andy Schaefer, Paul Forscher Lab., Yale University
Thank You