Confocal and Light Sheet Microscopy

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Most Modern Microscopy is Based on Fluorescence

Fluorescent dyes are useful because the intensity of emissions can be quantified (unlike colorimetric stains) and sensitivity is increases since ‘background’ is dark (unlike for transmitted light). **Photon Budget** is how many photons a dye can produce before it goes dark.

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**Fluorescence**
- Occurs in 1-10 nanoseconds
- Random in time
- Longer wavelength emissions
- Highly Inefficient (~10^-6)
- Brightness = QY x EC
Conceptual Fluorescence Microscope Design

**Bright-Field Microscope**
- Condenser
- **Absorbing** Sample
- Objective
- Camera

**Fluorescence Microscope**
- Condenser
- **Fluorescing** Sample
- Objective
- Color Filter
- Camera
Conceptual Epifluorescence Microscope Design

**Bright-Field Microscope**

- Condenser
- Objective
- Sample (Absorbing)
- Camera

**Fluorescence Microscope**

- Objective & Condenser
- Sample (Fluorescing)
- Dichroic Mirror
- ‘Illuminator’
- Camera
Practical Epifluorescence Microscope Design

1. Excitation light is shined through (not into) the objective.

2. The objective functions also as the condenser lens.

3. Color-selective dichroic distinguishes the excitation from emissions.
Resolution is Often Limited by Magnification and Camera Pixel Size
Out of Focus Emissions are a Problem

Fluorescence generated outside the focal plane still also reaches the camera, which adds ‘background’ or ‘blur’ that can greatly reduce image contrast.

Lots of out-of-focus light reaches the camera.
Typical Epifluorescence Image
Confocal Microscopy
The Goal of Confocal Microscopy

Instead of exciting the entire sample at once with collimated light, confocal microscopy scans a focused point of light back-and-forth across the sample to create an image sequentially. This design allows out-of-focus light to be blocked with a small (10’s um) aperture called a pinhole.

Excitation concentration highest in focal plane

Remaining out-of-plane emissions blocked by pin-hole

Optical section thickness is distinct from depth of field
Optical Sectioning from a Wave Perspective

Recall that due to diffraction, light is focused to an intensity profile called an Airy disk. The width of the Airy disk is measured in ‘Airy Units’. The pinhole should be ‘just big enough’ to let most of the Airy disk pass.

Note that the Airy Units in the image plane are wavelength and magnification dependent.
A modular set of optics called the ‘scan head’ handle the point scanning, filters the excitation / emissions, and contains the pinhole and detectors.
How the Scanning Works

Mirrors attached to electric motors scan the excitation light. Feed back from the mirrors tells a computer where in the sample the point is located.
Scan Spacing Often Determines Resolution

Because the image is collected via scanning, sampling rate usually limits resolution in practice. Higher resolution can be obtained by A) decreasing the scan area or B) scanning more pixels.... as long as the point-to-point displacement remains greater than the width of the excitation PSF.

Small Scan Area; Higher Resolution

Large Scan Area; Low Resolution

The spot size is constant and determined by the objective’s numerical aperture.
Z-Stacks

By successively moving the axial (z) location of the focal plane within the sample, a series, or ‘stack’, of (2D) optical sections can be collected that together constitute a sampled 3D image.
A Confocal Optical Section vs Epifluorescence
Many points can be scanned in parallel with the use of a spinning disk of aligned lenses and pinholes. The holes are imaged onto the sample. 100s fps for one color.

Scan Pattern: Rotation of spiral hole pattern sweeps points across the scan area.
Spinning-disk confocal
## When is confocal preferred over wide-field?

<table>
<thead>
<tr>
<th></th>
<th>Epifluorescence</th>
<th>Confocal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Contrast:</strong></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td><strong>3D images?:</strong></td>
<td>No (Sort of, with deconv.)</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Sensitivity:</strong></td>
<td>High (Single molecule)</td>
<td>Low (100s molecules)</td>
</tr>
<tr>
<td><strong>Speed:</strong></td>
<td>High (20 msec)</td>
<td>Slow (+1 sec)</td>
</tr>
<tr>
<td><strong>Photodamage:</strong></td>
<td>‘1x’ per image</td>
<td>‘10-100x’ per image</td>
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</table>
Light Sheet Microscopy
Why Not Confocal?

Biologists often want to image large 3D volumes, but:...

1. **Point scanning techniques are really slow.** E.g. 10 us/px x 1M px/slice x 100 slices = 17 min per 100 slices.

2. **Point scanning techniques are harsh.** High laser powers traveling through the entire sample for each image kills the sample and bleaches fluorophores.
What is Light Sheet Microscopy?

Light sheet microscopes use one light path for excitation and a separate (typically) light path for detection. The excitation path creates a sheet of light in the sample that is perpendicular to the detection axis:

Sample is moved or sheet is scanned to collect stack.
Many Different Kinds of Light Sheets!

<table>
<thead>
<tr>
<th>Type</th>
<th>BFP</th>
<th>Lens</th>
<th>Longitudinal Cross-Section</th>
<th>Thickness</th>
</tr>
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<tbody>
<tr>
<td>All Optical LS</td>
<td></td>
<td>Cylindrical Lens</td>
<td></td>
<td>&gt;2 um</td>
</tr>
<tr>
<td>(Scanning not required)</td>
<td></td>
<td>Low NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scanned LS</td>
<td></td>
<td>Low NA</td>
<td>long</td>
<td>&gt;2 um</td>
</tr>
<tr>
<td>Scanned LS</td>
<td></td>
<td>High NA</td>
<td>short</td>
<td>1 um</td>
</tr>
<tr>
<td>Bessel Beam LS</td>
<td>C</td>
<td>High NA</td>
<td></td>
<td>0.5 um</td>
</tr>
<tr>
<td>Lattice LS</td>
<td>O</td>
<td>High NA</td>
<td></td>
<td>0.4 um</td>
</tr>
</tbody>
</table>

Different kinds of light sheets are used for different kinds of samples!
Lattice Light Sheet Microscopy

Enables very high contrast, fast, gentle 3D live cell imaging

(Chen, Science, 2014)
Overview of Lattice Light Sheet Design
Dual Inverted Light Sheet Microscopy

DiSPIM collects two views in rapid succession to achieve nearly isotropic xyz resolution, but contrast is not as high as for lattice.

https://lakdawalalab.com/dispim-movie-gallery/
Overview of DiSPIM Light Sheet Design

Kumar, Nat Meth, 2014
Light Sheet: Summary

1) Optical sectioning is provided by planar illumination, typically from ‘the side’.

2) Camera is used for acquisition, so is very sensitive and high speed.

3) Requires an optically ‘clear’ sample to maintain the planar illumination.

4) Huge datasets (>100 GB) are difficult to analyze.