Resolution Roll Call: Optical Sectioning System Overviews

Principles and Comparisons of Fluorescence-Based High-Resolution Imaging Modalities

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Outline of Discussion
High Resolution Optical Sectioning

1. Basic Aspects of Resolution in X, Y, and Z
2. Modalities for Enhancing Axial Resolution
3. Advanced Approaches for Exceeding the Resolution Limit
4. Summary of Techniques
5. Questions
Outline of Discussion
High Resolution Optical Sectioning

1. Basic Aspects of Resolution in X, Y, and Z
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Ernst Abbe deduced the “resolution limit” of lenses at Carl Zeiss’ Jena workshop in 1873
- Expanded concepts of optical imaging using wave properties of light

Resolution defined as the minimum distance (d) necessary to distinguish two point-like objects in lateral space

$$d = \frac{\lambda}{2\sin \alpha}$$
Aspects of Resolution
Historical Context

- Thomas A. Edison’s electric lamp (1880)
Numerical aperture (\textit{NA}) is the defining characteristic for resolution.

\[ \text{NA} = n \sin \alpha \]

- \( n \) = refractive index of immersion media (describes how light propagates relative to vacuum)
- \( \alpha \) = half angle; acceptance cone of light

Resolution criteria is dependent on both wavelength and NA (\textit{not magnification})

\[ d_{xy} = \frac{\lambda}{2(n \sin \alpha)} = \frac{0.5 \lambda}{\text{NA}} \]
Aspects of Resolution
Comparing Numerical Aperture

- All other things constant, changes in NA influence the image resolution.
- In practical terms, this has significance in all applications involving structural or molecular localization.
- One object/puncta or actually several?

<table>
<thead>
<tr>
<th>Object</th>
<th>Image</th>
<th>NA</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Object Image](0.3 µm)</td>
<td>![Image](NA = 1.4, α = 67°)</td>
<td>NA = 1.4</td>
<td>α = 67°</td>
</tr>
<tr>
<td><img src=".." alt="Object Image" /></td>
<td>![Image](NA = 1.2, α = 52°)</td>
<td>NA = 1.2</td>
<td>α = 52°</td>
</tr>
<tr>
<td><img src=".." alt="Object Image" /></td>
<td>![Image](NA = 0.9, α = 36°)</td>
<td>NA = 0.9</td>
<td>α = 36°</td>
</tr>
<tr>
<td><img src=".." alt="Object Image" /></td>
<td>![Image](NA = 0.5, α = 20°)</td>
<td>NA = 0.5</td>
<td>α = 20°</td>
</tr>
</tbody>
</table>
# Aspects of Resolution

## Comparison of Example Objectives

<table>
<thead>
<tr>
<th>Magnification</th>
<th>NA</th>
<th>Resolution (XY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x</td>
<td>0.10</td>
<td>2.75 μm</td>
</tr>
<tr>
<td>4x</td>
<td>0.20</td>
<td>1.375 μm</td>
</tr>
<tr>
<td>10x</td>
<td>0.25</td>
<td>1.10 μm</td>
</tr>
<tr>
<td>10x</td>
<td>0.45</td>
<td>0.61 μm</td>
</tr>
<tr>
<td>20x</td>
<td>0.40</td>
<td>0.69 μm</td>
</tr>
<tr>
<td>20x</td>
<td>0.75</td>
<td>0.37 μm</td>
</tr>
<tr>
<td>40x</td>
<td>0.65</td>
<td>0.42 μm</td>
</tr>
<tr>
<td>40x</td>
<td>1.40</td>
<td>0.20 μm</td>
</tr>
<tr>
<td>63x</td>
<td>0.75</td>
<td>0.37 μm</td>
</tr>
<tr>
<td>63x</td>
<td>1.30</td>
<td>0.25 μm</td>
</tr>
<tr>
<td>100x</td>
<td>1.25</td>
<td>0.22 μm</td>
</tr>
<tr>
<td>100x</td>
<td>1.40</td>
<td>0.20 μm</td>
</tr>
</tbody>
</table>
Aspects of Resolution
Comparison of Example Objectives

- Even small increases in NA can yield key improvements in imaging
Aspects of Resolution
Limits in the Z Dimension

- The same principles of resolution extend into the **axial (Z) dimension**
  - For widefield imaging system, **minimum resolved distance in Z** is larger than in XY

**WHY WORSE IN Z?**
- Out-of-focus light interference in sample
- Non-symmetrical wavefront from lens along the optical axis

\[
d_{xy} = \frac{0.5 \lambda}{NA}
\]

lateral resolution (XY) limit ~ 200 nm

\[
d_{z} = \frac{2\lambda}{NA^2}
\]

axial resolution (Z) limit ~ 550 nm
# Aspects of Resolution

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<tr>
<td>4x</td>
<td>0.10</td>
<td>2.75 µm</td>
<td>110 µm</td>
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<tr>
<td>4x</td>
<td>0.20</td>
<td>1.375 µm</td>
<td>27.5 µm</td>
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<td>10x</td>
<td>0.25</td>
<td>1.10 µm</td>
<td>17.6 µm</td>
</tr>
<tr>
<td>10x</td>
<td>0.45</td>
<td>0.61 µm</td>
<td>5.43 µm</td>
</tr>
<tr>
<td>20x</td>
<td>0.40</td>
<td>0.69 µm</td>
<td>6.87 µm</td>
</tr>
<tr>
<td>20x</td>
<td>0.75</td>
<td>0.37 µm</td>
<td>1.95 µm</td>
</tr>
<tr>
<td>40x</td>
<td>0.65</td>
<td>0.42 µm</td>
<td>2.60 µm</td>
</tr>
<tr>
<td>40x</td>
<td>1.40</td>
<td>0.20 µm</td>
<td>0.56 µm</td>
</tr>
<tr>
<td>63x</td>
<td>0.75</td>
<td>0.37 µm</td>
<td>1.95 µm</td>
</tr>
<tr>
<td>63x</td>
<td>1.30</td>
<td>0.25 µm</td>
<td>0.65 µm</td>
</tr>
<tr>
<td>100x</td>
<td>1.25</td>
<td>0.22 µm</td>
<td>0.70 µm</td>
</tr>
<tr>
<td>100x</td>
<td>1.40</td>
<td>0.20 µm</td>
<td>0.56 µm</td>
</tr>
</tbody>
</table>
Aspects of Resolution
Enhancing Axial Resolution

**REQUIREMENTS:**
- Collect images with Z step sizes smaller than the target resolution
  - Motorized focus drive
- Imaging method that results in a clearer “slice” of the source signal
  - Optical sectioning technique
Outline of Discussion

High Resolution Optical Sectioning

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Optical Sectioning Techniques
Hierarchy of Common Approaches

Sectioning Methods

Optics
- Light Sheet Microscopy
- Confocal Microscopy

Optics & Mathematics
- Total Internal Reflection
- Multi-Photon

Mathematics
- Structured Illumination
- 3D Deconvolution
Optical Sectioning
Confocal Microscopy

Sectioning Methods

- Optics
- Optics & Mathematics
- Mathematics

- Light Sheet Microscopy
- Confocal Microscopy
- Total Internal Reflection
- Multi-Photon
- Structured Illumination
- 3D Deconvolution
Confocal Microscopy
Rejection of Out-of-Focus Signal

Excitation
ZEISS Plan-NEOFLUAR 40x /1.3 Oil

Emission
ZEISS Plan-NEOFLUAR 40x /1.3 Oil

Widefield

Excitation
ZEISS Plan-NEOFLUAR 40x /1.3 Oil

Emission
ZEISS Plan-NEOFLUAR 40x /1.3 Oil

Confocal
Confocal Microscopy
Rejection of Out-of-Focus Signal

• Laser excitation and sample emission separated by dichroic
Confocal Microscopy

Rejection of Out-of-Focus Signal

- Laser excitation and sample emission separated by **dichroic**
- The **pinhole(s)** prevent detection of out-of-focus signal
  - Diaphragm situated in conjugate focal plane

![Confocal Microscopy Diagram]

[Diagram showing laser excitation, dichroic separation, pinhole(s) preventing out-of-focus signal detection, and diaphragm situated in conjugate focal plane.]
Confocal Microscopy
Rejection of Out-of-Focus Signal

• Laser excitation and sample emission separated by dichroic

• The pinhole(s) prevent detection of out-of-focus signal
  - Diaphragm situated in conjugate focal plane

• Thickness of resulting optical section is influenced by:
  - Numerical aperture of lens
  - Wavelength of excitation light
  - The shape, spacing, and diameter of the pinhole
Increased scanning speeds are possible by multipoint approaches. Such approaches sacrifice pinhole versatility at emission side and thus have less control over the optical section thickness.

**Point Scanning Confocal**  
*(single point at a time)*

**Line Scanning Confocal**  
*(single line or slit at a time)*

**Spinning Disk Confocal**  
*(~1000 points at a time)*
A \textbf{diffraction-limited laser spot} is moved across the sample via two independent scanning mirrors.
- The resulting image is generated a \textit{single point at a time}.
Point-Scanning Confocal
Common/Unique Applications

WHAT ARE THE USES?
• Easy-to-implement simultaneous detector layout enables true **spectral imaging**
  - Separation of closely-overlapping fluorophores, identification of autofluorescent populations

32-channel spectral readout of giant unilamellar vesicle (GUV) labelled with laurdan

Visualization of gut immune cell populations
Hugues Lelouard et al (Aix Marseille Univ, France)
WHAT ARE THE USES?

- Control of pointwise illumination with nanosecond-scale photon readout permits measurement/modeling of molecular dynamics
  - FRAP → mobility (parameters of diffusion, viscosity, size), transport kinetics
  - FCS → diffusivity, concentration, transport and binding kinetics
  - RICS → spatial maps of diffusivity, concentration(s)
  - N&B → concentration, oligomerization

Detailed characterization of any aqueous compartment or environment via fluorescence!
# Point-Scanning Confocal

## Typical System Footprint – ZEISS LSM 880

- **LSM 880 scanhead; 34-channel (GaAsP)**
- **Transmitted light detector ("T-PMT")**
- **Laser lines:** 405, 458, 488, 514, 561, 633 nm
- **Objectives:**
  - 10x/0.3
  - 25x/0.8 oil/W
  - 40x/1.2 W
  - 40x/1.3 oil
  - 63x/1.4 oil
  - 63x/1.4 oil
  - 100x/1.46 oil
- **Motorized XY stage + Z-piezo insert**
- **Observer inverted microscope**
Point-Scanning Confocal
Practical Perspective

ADVANTAGES:
• Nearly **ubiquitous** 3D modality
• Easy to combine multiple **channels in parallel** (spectral imaging)
• Precise **pixel photomanipulation possible**
• Works with **reflected mode imaging** (non-fluorescent structures)

DISADVANTAGES:
• Relatively **slow point-by-point acquisition**
• High **laser powers may cause photobleaching** or photodamage
• Photocathode-based **detectors are less sensitive than cameras**

FITC electrospun fiber scaffold (Xie Lab, Univ. Nebraska Med.)
Fluorescent flake on paper (reflection)
Spinning Disk Confocal
Basic Principles

• A single **rotating disk** containing **hundreds of pinholes** scans laser light across the sample
  - Rotation **speed is adjustable** (~1500 – 5000 RPM)

• Paul Nipkow patented concept in 1884, later became basis for mechanical television in 1920s
Spinning Disk Confocal
Basic Principles

- Pinholes are arranged along slightly curved line (Archimedean spiral)

- Over a set exposure time, these lines assemble into one full scan of the field of view
  - 12 scans per complete rotation, 50 μm pinhole diameter, 250 μm apart

- To evenly scan, rotation speed and exposure time are synchronized
Spinning Disk Confocal
Basic Principles

- Pinholes *illuminated by laser* (via focusing of microlens array)
  - Projected onto sample

- Emission collected through objective; *passes pinhole array*
  - Only in-focus signals

- Beamsplitter passes *fluorescence to area detector* (*e.g.* – CCD)
  - High-speed confocal
WHAT ARE THE USES?

- Projects requiring **high-throughput 3D imaging**, up to 2 simultaneous channels
- Increasingly central to **screening studies** (e.g. – Zebrafish sorting VAST BioImager; automated iPS cell screening)

Automated high-resolution screen (Zebrafish)
Jason Early et al *(Univ. Edinburgh, UK)*
Spinning Disk Confocal
Typical System Footprint – ZEISS Cell Observer SD

Yokogawa CSU-X1A 5000 spinning disk scanhead

Laser lines (50 mW)

Laser-safety incubator chamber

Temperature, CO₂ and humidity controllers

2x Evolve 512 EMCCD cameras (dual configuration)

Observer inverted stand (with motorized XY stage)

Objectives:
- 40x/1.2 W
- 63x/1.4 oil
- 100x/1.46 oil

DirectFRAP photomanipulation unit
Spinning Disk Confocal
Practical Perspective

ADVANTAGES:
• High speed acquisition ideal for live cell/tissue dynamics; capture rate is typically limited only by camera(s)
• Excitation spots are constantly moving; generally accepted as one of the most gentle confocal imaging strategies; low photobleaching
• Robust, relatively inexpensive setup that offers scalability

DISADVANTAGES:
• Confocal pinhole diameter is fixed and optimized only for a subset of magnifications; lower resolution than point-scanning confocals
• Thick, scattering samples lead to pinhole crosstalk, results in increased background
Optical Sectioning Techniques
Multi-Photon Microscopy

Sectioning Methods

Optics
- Light Sheet Microscopy
- Confocal Microscopy

Optics & Mathematics
- Total Internal Reflection
- Multi-Photon

Mathematics
- Structured Illumination
- 3D Deconvolution
Multi-Photon Microscopy

Principles of the Two-Photon Effect
Multi-photon excitation requires that the fluorophore simultaneously absorbs two (or more) photons. This process requires an extremely high photon density (i.e., the focal volume of the objective lens).

A single-photon instrument is known as a “linear” imaging technique. Excitation of fluorophore is directly proportional to the laser intensity.

A multi-photon system is known as a “non-linear” technique (NLO). Excitation of fluorophore is dependent on the square of the laser intensity. No confocal pinhole required; excitation only probable near focal plane.

How is the high photon density obtained?
Multi-Photon Microscopy
Excitation by a Pulsed IR Laser

Average Power = Peak Power * Frequency * Pulse Length

Energy per pulse ~ 50 nJ

max. Peak Power ~ 300 kW

100 fs (10^-13 s)
1 ns (10^-9 s)
10 ns (10^-8 s or 100 MHz)

Average Power ~ 2 W

Laser Pulse
Fluorescence Decay

Carl Zeiss Microscopy
1/28/2019
Multi-Photon Microscopy
IR Laser Yields Increased Penetration

Short Wavelength Light
(Visible light)

Long Wavelength Light
(Near infrared)

Low penetration depth:
Scattering of photons compromises the focusing of light

Objective Lens

Thick Tissue
(e.g. - brain)

Increased penetration depth:
More efficient focusing due to reduced scattering of photons
Multi-Photon Microscopy
Depth Considerations

Mouse brain: YFP-labelled tissue; 80 µm deep

Confocal Imaging w/ 514 nm Excitation

Multi-Photon Imaging w/ 870 nm Excitation
WHAT ARE THE USES?

- Label-free imaging of collagen, myosin, starch via second harmonic generation (SHG)

- High depth (>5 mm) imaging of optically-cleared tissues and organs

Mesenterium, SHG collagen (cyan, 800 nm), endothelial cells (white), F. Kiefer (MPI Munster)

Mouse brain cleared with Scale Hiroshi Hama et al (RIKEN BSI, Wako, Japan)
WHAT ARE THE USES?

- Pulsed lasers can enable measurement of fluorescence lifetime (FLIM)
  - Photon counting is used to plot **temporal** distribution of the excited state (~100s of ps)
  - Repeating counts at each scan pixel reveals **spatial** distribution of lifetimes
  - Lifetimes are **sensitive to microenvironment** (FRET, pH, ion concentration, binding, etc)

Presence of FRET (protein-protein interaction) reduces donor lifetime

Skin (pig) stained with ethylene blue; 1100 nm ex. lifetime image

Principle of time-correlated single photon counting (TCSPC), via B&H
Multi-Photon Microscopy
Typical System Footprint – ZEISS LSM 880 NLO

- LSM 880 scanhead; 34-channel (GaAsP)
- VIS laser lines: 458, 488, 514, 561, 633 nm
- IR laser: Coherent Discovery dual beam
  Output A: 690-1010 nm + 1070-1300 nm
  Output B: 1040 nm
- Incubation accessories (temperature, CO₂)
- Objectives:
  - 20x/0.8
  - 40x/1.2 W
  - 40x/1.3 oil
- Motorized XY stage + Z-piezo insert
- External NDD, reflected light
  (2-channel GaAsP)
- Observer inverted microscope
  (with Definite Focus)
Multi-Photon Microscopy
Practical Perspective

ADVANTAGES:
• Permits **deep imaging of tissue or whole animal** due to low scattering of IR light and non-descanned detection (no pinhole)
• IR **light less toxic to live** cells/organisms
• Optical sectioning occurs entirely through excitation; **limits bleaching to the focal plane only**

DISADVANTAGES:
• Not useful or recommended for thin samples; IR wavelength and power **can boil sample** (where water dominant absorber)
• Broad fluorophore absorption cross-section; **multi-labeling tricky**
• No ability to adjust optical slice thickness (**e.g.** – for weak samples)
• IR pulsed lasers render the system **expensive, large footprint**
Optical Sectioning Techniques
Light Sheet Microscopy

Sectioning Methods

Optics
- Light Sheet Microscopy

Optics & Mathematics
- Confocal Microscopy
- Total Internal Reflection

Mathematics
- Multi-Photon
- Structured Illumination
- 3D Deconvolution
Light Sheet Fluorescence Microscopy
Fundamental Characteristics of LSFM

PRINCIPLES

• Orthogonal light paths for illumination and detection

• Horizontal microscope configuration

• Whole field of view illuminated; camera-based collection

Modified from Huisken et al. Development (2009)
Light Sheet Fluorescence Microscopy
Comparison to Confocal Microscopy

<table>
<thead>
<tr>
<th>Confocal microscopy</th>
<th>Light sheet microscopy</th>
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</thead>
<tbody>
<tr>
<td><strong>Illumination</strong></td>
<td><strong>Illumination</strong></td>
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<tr>
<td>Evenly illuminated</td>
<td>Objective</td>
</tr>
<tr>
<td>Scan</td>
<td>Laser</td>
</tr>
<tr>
<td><strong>Focal plane</strong></td>
<td>Unaffected</td>
</tr>
<tr>
<td>Laser</td>
<td>Light sheet</td>
</tr>
<tr>
<td><strong>Objective</strong></td>
<td>Detection objective</td>
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<table>
<thead>
<tr>
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<tr>
<td><strong>Focal plane</strong></td>
<td>Light sheet</td>
</tr>
<tr>
<td>Detection</td>
<td><strong>Fluorescence</strong></td>
</tr>
<tr>
<td><strong>Objective</strong></td>
<td>Detection objective</td>
</tr>
<tr>
<td><strong>Fluorescence</strong></td>
<td></td>
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</table>
Reynaud et al., HFSP J. (5) 2008

Multiple configurations ideal for medium-sized to large living specimens in an aqueous solution

(Compromised solution for coverslipped samples)

Adapted agarose capillaries, cups, hooks, clamps, and adhesives permit a variety of sample formats
Larger samples benefit from applying illumination features (e.g. two-sided illumination, pivot scanning) to different rotational views.

- Complementary information from unique viewing angles; resolution gain

Sample and data by Dr. Cecilia Lu, OIST, Okinawa, Japan
Light Sheet Fluorescence Microscopy
Multiview Imaging – Fusion

Z-Stack View 1
Registration + Fusion
Z-Stack View 2
WHAT ARE THE USES?

• Tracking and invasion of cells in unperturbed **3D culture** models (spheroids, seeded scaffolds, invasion assays) and intact organisms

Drosophila embryo lineage reconstruction (H2A), 30 s interval over 11 hr, 10 frame avg
Amat et al. (*Nature Methods* 2014)
Light Sheet Fluorescence Microscopy

Typical System Footprint – ZEISS Lightsheet Z.1

Objectives:
- 5x/0.16
- 20x/1.0 water
- 40x/1.0 water

Laser lines (50 mW)

2x PCO.edge CMOS cameras (dual configuration)

Additional sample chambers

Lightsheet Z.1 unit (with temperature controllers)
ADVANTAGES:
• Fast and gentle optical sectioning
• Multiview positioning allows for more efficient light delivery at high depths
• Enables large format cleared tissue imaging

DISADVANTAGES:
• Light sheet thickness ranges from 1 to 10 microns thick; subcellular resolution possible but lower than confocal imaging
• High throughput requires considerations for large datasets

E15.5 mouse kidney cleared with glycerol (blood vessels, eGFP)
Renata Prunskaitė-Hyyryläinen (Univ. Oulu, Finland)
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Optical Sectioning Techniques
Common Superresolution Approaches

Sectioning Methods

- Optics
  - Light Sheet Microscopy
    - STED
    - 4 Pi
    - Airyscan
  - Confocal Microscopy
    - 3 Pi
  - Total Internal Reflection
  - Multi-Photon
    - PALM (dSTORM)
    - 3D PALM (PRILM)
    - 3D STORM

- Optics & Mathematics
  - FIONA
  - SHReC
  - NaLMS
  - SHRImP
  - PAINT

- Mathematics
  - Structured Illumination
    - SR-SIM
  - 3D Deconvolution
    - PiMP SRRF
Optical Sectioning Techniques
Common Superresolution Approaches

- Optics
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    - PiMP
    - SRRF
    - SR-SIM

- Mathematics
  - 3D Deconvolution
Structured Illumination Microscopy

Basic Principles

- Method of casting patterned light in highly-controlled manner to yield composite images with **double resolution in X, Y, and Z**
  - Use of **grid or lattice** illumination reveals Moiré patterns with lower frequencies that are not hindered by the resolution limit of an objective.
Structured Illumination Microscopy
Understanding Frequency Space

“Fourier Transformation”
Structured Illumination Microscopy
Fourier Spectrum – Objective Limit

Optical system always performs its own *inverse* Fourier transformation from back focal plane to image plane (Note limited radius of objective)

smaller spectrum = lower frequencies = lower resolution
Structured Illumination Microscopy
Fourier Spectrum – Objective Limit

larger spectrum = higher frequencies = higher resolution
Structured Illumination Microscopy
Fourier Spectrum – Objective Limit

Extending resolution beyond limits means finding a way to detect higher frequencies outside of this region.
Structured Illumination Microscopy
Acquisition Process

- Final image is reconstructed from ~15 images, each with different phase (position) of the lattice light pattern
  - High speeds are possible via rapid sweeping of lattice + camera readout
Structured Illumination Microscopy
Common/Unique Applications

WHAT ARE THE USES?
- Resolved capture of vesicle trafficking, membrane ruffling, and dynamic processes
  - 110-120 nm XY, 300 nm Z
- Minimal sample prep often triggers renewed assessments of colocalization

Myoblast C2C12, BrdU + Dnmt1
Schermelleh lab (LMU Munich)

U2Os cells
actin (Lifeact-9), EB3-GFP

Endosomal transport marker (Rab5a-GFP),
golgi-associated transport marker (tdTomato)
Structured Illumination Microscopy
Typical System Footprint – ZEISS Elyra 7 (SIM)

Laser lines (100 mW)

Objectives:
- 63x/1.2 W
- 63x/1.4 oil
- 100x/1.46 oil

Observer inverted stand (with motorized XY stage)

Elyra 7 beam path module

Darkening chamber

PCO.edge CMOS camera (SIM)
ADVANTAGES:
• Relatively easy entry to superresolution; low learning curve
• Maintains a large field of view
• Demodulation processing retains quantitative information
• Concepts can be easily tailored for live cell experiments (up to 255 frames/sec with lattice SIM)

DISADVANTAGES:
• Largely incompatible with thicker (>50 micron) samples due to scattering and loss of patterned illumination with depth
Optical Sectioning Techniques
Common Superresolution Approaches

Sectioning Methods

Optics
- Light Sheet Microscopy
  - STED
  - 4 Pi
  - Airyscan

Confocal Microscopy
- 3D STORM

Total Internal Reflection
- PALM
dSTORM
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  - PAINT

Multi-Photon
- 3D PALM (PRILM)
- 3D STORM

Structured Illumination

Mathematics
- SR
  - PiMP
  - SRRF

3D Deconvolution
- SR-SIM
Localization Microscopy

Basic Principles

- Refers to a number of conceptually similar time-sensitive techniques
  - Photoactivated Localization Microscopy (Palm)
  - direct Stochastic Optical Reconstruction Microscopy (dSTORM)

- Use of emission-limiting conditions, weighting algorithms to pinpoint (or “localize”) individual fluorescent molecules

- Lateral resolution up to 10 times greater than widefield imaging

- TIRF-based illumination yields excellent Z-resolution (~100 nm)
Localization Microscopy
Single Molecule Localization

1. Activate
2. Localize
3. Summed
PALM relies on fluorochromes that can be either **photoactivated**, **photoconverted**, or reversibly **photoswitched** to control emission over time.
Instead of using fluorescent proteins, high laser powers (with a specialized reducing cocktail to prevent bleaching) can be used to keep conventional fluorescent dyes in a **dark state**, from which they wake up stochastically.
Localization Microscopy
Extending High Resolution into 3D

• Extended Z capture range (up to 1.4 micron) is possible using approaches of **PSF engineering**

• Double phase ramp within back aperture of objective creates a double helix pattern for each emitter
  - Allows for **pinpointing of Z position** for single molecules
WHAT ARE THE USES?

- Understanding clustering, aggregation at the nanoscale
- Precise correlation of function (fluorescent labelling) with ultrastructure (electron microscopy)
  - Correlative Light and Electron Microscopy (CLEM)

Ultrathin *S. cerevisiae* overlay SIM, dSTORM (hA1ar), FE-SEM
J. Caplan, K. Czymmek (*Delaware Biotech. Instit.*)

Focal adhesion complexes (Alexa 488), Martin Bastmeyer (*Univ. Karlsruhe, Germany*)
Localization Microscopy
Typical System Footprint – ZEISS Elyra 7 (SMLM)

- Laser lines (200 mW)
- 3D PALM beam path mechanism
- Elyra 7 module
- Objectives: 100x/1.46 oil
- Darkening chamber
- Observer inverted stand (with motorized XY stage)
- Andor iXon EMCCD camera (PALM/dSTORM)
Localization Microscopy
Practical Perspective

ADVANTAGES:
• Provides **highest resolution available** for fluorescence approaches
• Light path is **always optimized for TIRF**, providing additional out-of-the-box versatility

DISADVANTAGES:
• Optimizing blinking, fluorophore concentration, and imaging buffers requires **expertise, trial-and-error**
• Extending to **multi-color imaging doubles complexity** of acquisition and experiment design
• Even with 3D-based tools, **depths are generally limited to <10 microns**

Red = tdEos / vinculin
Green = Dronpa / paxillin

CHO cells
H. Shroff (*Janelia*)
Optical Sectioning Techniques
Common Superresolution Approaches

Sectioning Methods

Optics
- Light Sheet Microscopy
  - STED
  - 4 Pi
  - Airyscan

Confocal Microscopy
- Confocal Microscopy
  - STED
  - 4 Pi
  - Airyscan

Total Internal Reflection
- Total Internal Reflection
  - FIONA
  - SHReC
  - NaLMS
  - SHRImP
  - PAINT

Optics & Mathematics
- PALM dSTORM

Multi-Photon
- PALM dSTORM
  - 3D PALM (PRILM)
  - 3D STORM

Mathematics
- Structured Illumination
  - PiMP SRRF
- 3D Deconvolution
  - SR-SIM
Airyscan
Dedicated Superresolution Detector

Airyscan detector for superresolution

Hexagonal GaAsP detection array
Airyscan
Basic Principles

• Mechanical pinhole is rejecting emitted photons based on diameter

• 1 Airy Unit (“AU”) often acts as an ideal compromise between thin optical sections and reasonable signal levels
Airyscan
Basic Principles

- 32 GaAsP detectors in hexagonal lattice
- Each detector approximately 0.2 AU in diameter
- Total detection area approximately 1.25 AU in diameter
- Simultaneous improvement in resolution and signal
Airyscan
Innovations in the Literature

First theorized about pinhole plane image detection and reassignment
Proposed reassignment to position halfway between excitation/detection positions for improving resolution
With identical PSFs, this reassigned position corresponds to the most probable position of an emitter

First to implement Sheppard’s concept using a camera as an area detector
A full camera image was captured for each laser spot position moving across an object
Pixels with a greater displacement from the given optical axis yield narrower effective PSFs [at those pixels]

Argued that an off-axis detector can improve resolution up to 1.53-fold (assuming no Stokes shift)
(Normalized transverse coordinate vd = 0 yields 1.39-fold resolution for zero pinhole; vd = 2.75 yields 1.45-fold)

Parallelized the image scanning microscopy procedure using illumination patterns via a digital micromirror device
Multifocal pattern (e.g. – spinning disk) is shifted after each image, followed by postprocessing (2x scaling, summing)
Resulting resolution reached ~145 nm laterally and 400 nm axially (at 480 x 480 pixels, ~1 final 2D per second)

First to implement hardware-based pixel reassignment by introducing a re-scanning unit in the detection path
Expanded the beam in pupil plane by a certain factor, which shrinks the corresponding image on the detector
Confocal sectioning possible by combining a pinhole in the detection path prior to rescanning

Parallelized the re-scan approach using microlens and pinhole array, coupled with second microlens array
Second microlens array used to locally contract each pinholed emission; galvo scan to sum over camera exposure
Claim lateral resolution of ~145 nm and axial resolution of ~350 nm, albeit with fixed pinholes
Airyscan
Isotropic Resolution Improvements (2-fold)

Confocal (Pinhole = 1 AU)

Airyscan

Cultivated fibroblasts stained for vimentin

2 µm

YZ
WHAT ARE THE USES?

- New benchmark in point-scanning resolution for thicker specimens
- High(er) resolution cryo imaging of vitrified specimens (plunge freezing)
  - Compatibility with cryo-EM
Airyscan

Typical System Footprint – (Confocal Upgrade)

Airyscan superresolution detector (GaAsP)

- LSM 880 scanhead; 34-channel (GaAsP)
- Transmitted light detector ("T-PMT")
- Laser lines: 405, 458, 488, 514, 561, 633 nm

Objectives:
- 10x/0.3
- 25x/0.8 oil/W
- 40x/1.3 oil
- 63x/1.4 oil
- 40x/1.2 W
- 100x/1.46 oil

Motorized XY stage + Z-piezo insert

Observer inverted microscope
ADVANTAGES:
• Relatively versatile superresolution tool at high depths
• No specific sample preparations required

DISADVANTAGES:
• Oversampling initially yields larger file sizes
• Airyscan concept requires a complete point-scanning confocal system

Autofluorescence, calcium oxalate crystals in kidney stone (Sivaguru et al, Nature Scientific Reports 2018)

Collagen network, depth color-coded (100 microns thick, 633 nm ex.)
# Outline of Discussion

**High Resolution Optical Sectioning**

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Summary
The Right Tool for the Job?

- No aforementioned tool/technique is inherently better than another

- Selecting the **wrong imaging modality** is likely to have a **far greater consequence** than any performance differences between competitor systems with the same class
  - The “best” point-scanning confocal is about 3x more sensitive than “worst”
  - For study of development in 3D, even the “worst” light sheet system will be 50-100x more gentle than the “best” point-scanning confocal
## Summary

### Comparison of Techniques

<table>
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<th>Optical Sectioning Technique</th>
<th>Example ZEISS System</th>
<th>Performance Specifications</th>
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<tr>
<td>PALM / dSTORM</td>
<td>Elyra 7 (SMLM)</td>
<td>Depth Penetration: ● ● ● ● ●</td>
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<td>Structured Illumination</td>
<td>Elyra 7 (Lattice)</td>
<td>Maximum Speed: ● ● ● ● ● ●</td>
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<td>Light Sheet</td>
<td>Lightsheet Z.1</td>
<td>Out-of-Foc. Discrim.: ● ● ● ●</td>
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<td>Spinning Disk</td>
<td>Cell Observer SD</td>
<td>Lateral Resolution: ● ● ● ●</td>
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<td>Line Scanning</td>
<td>Airyscan Fast</td>
<td>Axial Resolution: ● ● ● ● ●</td>
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<td>Single Point Scanning</td>
<td>LSM 880</td>
<td>Spectral Flexibility: ● ● ● ●</td>
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<td>Multi-Photon</td>
<td>Airyscan</td>
<td>Simplicity: ● ● ● ● ● ● ●</td>
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<td>LSM 880 NLO</td>
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*Legend: ● indicates better performance.*

- Elyra 7 (SMLM)
- Elyra 7 (Lattice)
- Lightsheet Z.1
- Cell Observer SD
- Airyscan Fast
- LSM 880
- LSM 880 NLO

*Note: The table summarizes the performance specifications of various optical sectioning techniques.*
## Outline of Discussion
High Resolution Optical Sectioning

1. Basic Aspects of Resolution in X, Y, and Z
2. Modalities for Enhancing Axial Resolution
3. Advanced Approaches for Exceeding the Resolution Limit
4. Summary of Techniques
5. Questions
We make it visible.