Confocal, Airyscan and Structured Illumination Superresolution microscopy – Mayandi Sivaguru
Theory, Light Path, Resolution Comparison

Performance comparison

Why and When Choose Airyscan OR SR-SIM Superresolution

Speed, data size, and processing

Specialized Applications and Live Cell Imaging

Summary
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Summary
Diffraction limit and PSF of an Optical System

Lateral
0.61λ/NA

Axial
2nλ/NA²

Excerpt from Point Spread Function by Jeff Lichtman iBiology.org
### Superresolution PSF Dimensions - Theoretical comparisons

<table>
<thead>
<tr>
<th>Method</th>
<th>CLSM</th>
<th>STED</th>
<th>CW-STED</th>
<th>3D-SIM</th>
<th>PALM/STORM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{em}$  [nm]</td>
<td>460-670</td>
<td>670</td>
<td>520</td>
<td>620</td>
<td>460</td>
</tr>
<tr>
<td>$D_{xy}$ [nm]</td>
<td>180-250</td>
<td>60</td>
<td>70</td>
<td>130</td>
<td>110</td>
</tr>
<tr>
<td>$D_z$ [nm]</td>
<td>500-700</td>
<td>700</td>
<td>560</td>
<td>340</td>
<td>280</td>
</tr>
<tr>
<td>$V_{x,y,z}$ [$10^{-3} \mu m^3$]</td>
<td>10-23</td>
<td>1.3</td>
<td>1.5</td>
<td>3.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

- Courtesy NYTHERS

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**Shermelleh et al., JCB 2010, a Review**

![Nobel Laureates Pushed Limits of Microscopes](image)

Airyscan: 352, 147
Confocal Microscopes - Light Path - Optical Sectioning, Resolution

Images are from Zeiss Campus
Structured Illumination Microscopy (SIM)
Concepts (Real Space)
SR-SIM: Structured Illumination Microscopy Concepts (Frequency Space)
SR-SIM: Structured Illumination Microscopy-Theory

Concepts (Frequency Space)

Matts Gustafsson 1960-2011
Photo Courtesy of Paul Fetters, Nature Methods

SR-SIM: Structured Illumination Microscopy-Theory

Concepts (Frequency Space)

Klaus Weisshart, 2014 Zeiss White Paper

S(f_x+2f_c')

S(f_x)

S(f_x−2f_c')

2f_c

Gustafsson et al., Biophys J 2008
System Components
ELYRA illumination module

Sample illumination in all camera-based detection modes in ELYRA is made possible by:

... a single illumination module
... the same laser module
... the same set of lasers

of fluorochromes:

405 nm (50 mW)
488 nm (100 mW)
561 nm (100 mW)
642 nm (100 mW)
LSM 880 and Airyscan Light path

The additional information is used to get better signal-to-noise, resolution or speed.
Inside the LSM 880
Coupling of the Airyscan Module

- Efficient handling at laser input
- Apochromatic pinhole optics
- Fastest linear scanning, tp. controlled
- Low incident angle dichroics, high laser rejection
- QUASAR: single-shot spectral detection; cooled and improved electronics, higher data throughput
- Airyscan detector for superresolution
- Hexagonal GaAsP detection array
Resolution Limits of a Confocal LSM
Effects of Smaller Pinhole Sizes

- As pinhole is reduced below 1 AU, wave optical properties begin to dominate
- An infinitely small pinhole yields identical illumination and detection PSFs
- Both lateral and axial resolution criteria can be reduced by a factor of 1.4

\[ d_z = \frac{0.64 \lambda}{n - \sqrt{n^2 - NA^2}} \]
\[ d_{xy} = \frac{0.37 \lambda}{NA} \]
\[ d_z = \frac{0.88 \lambda_{ex}}{n - \sqrt{n^2 - NA^2}} \]
\[ d_{xy} = \frac{0.51 \lambda_{ex}}{NA} \]
Resolution Limits of a Confocal LSM
At Small Pinholes, Signal Loss > Resolution Gains

Small pinhole diameters lead to improved resolution steadily until about 0.2 AU, results in deeper dips between two objects.

However, constricting the pinhole actually yields a drastic reduction in signal below 1 AU.
Airyscan Detection
Farewell to the Pinhole

- 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 Detection

The light-efficient detection concept in Airyscan overcomes a classical limitation of confocal LSMs

Emission of a point-like emitter

Confocal detection (pinhole: 1.0 AU)
- Popular setting in biomedical confocal imaging!
- Acceptable signal-to-noise ratio (SNR)
- LSM’s resolving power far below its potential maximum

Confocal detection (pinhole: 0.2 AU)
- Improved spatial resolution
- Very poor SNR (vast portion of signal rejected by the pinhole!)
- Pinhole setting not useful in biomedical imaging!

Airyscan detection (“sub Airy” sampling)
- Every element compares to a pinhole set to 0.2 AU.
- Array captures light normally rejected at small pinhole diameters
Advanced Concepts of the Airyscan
Conventional Scanning Confocal (1 AU)

A point-like emitter generates a diffraction limited pattern (~ PSF)

At 1 AU the PSF is mapped directly 1:1

Excitation and detection are scanned in sync

Intensity

~ 240 nm

Carl Zeiss Microscopy

11/3/2015
Advanced Concepts of the Airyscan
Airyscan – A Single Element Improves Resolution

PH = 1.25 AU
excitation
detection

A point-like emitter generates a diffraction limited pattern (~ PSF)

By collecting just the central element, the PSF is weaker but narrower
Advanced Concepts of the Airyscan

Airyscan – An Offset Element Improves Resolution

A point-like emitter generates a diffraction limited pattern (~ PSF)

PH = 1.25 AU

excitation
detection

By an element offset from the center, the resolution is still improved

Intensity

scan

scan
Advanced Concepts of the Airyscan

Airyscan – Combining the Data

An Airyscan image is formed by:
1. Reassigning the offset signal
2. Summing the contributions

PH = 1.25 AU
excitation
detection

subunit
~ 0.2 AU

scan

Intensity
scan
Advanced Concepts of the Airyscan
Reducing the Effective PSF
Airyscan Processing
Immediate Pixel Reassignment
Airyscan Processing

Isotropic 1.7x Resolution Improvement
detector wise deconvolution

32 Images

32 PSFs
Airyscan Processing
Detector-Wise Deconvolution

170 nm fluorescent beads

Confocal microscope
Plan-Apochromat 63x/1.4
633nm illumination

Approx. resolution: 260 nm

Pixel reassignment
1.4x improved resolution

Approx. resolution: 185 nm

Airyscan processing
1.7x improved resolution

Approx. resolution: 153 nm
Why Linear Scanners?
Ensuring Versatility and Quantifiable Results

Linear Scanning (Zeiss)
- natively linear
- no discarding of the signal; time-efficient
- 85% on
- 15% off
- Duty Cycle

Sine Scanning
- requires equalization
- 12% discarded
- usable pixel time
- 60% on
- 40% off
- Duty Cycle

- Linear scans feature:
  - 29% higher S/N*
  - 66% longer pixel time*
  - Lower light dosage**
  - Decreased photodamage**
  - Constant scan speed
  - Uniform excitation

- Sine (and resonant) scanners yield images that are inherently not quantitative

*Compared at same imaging speed and FOV
**Compared at same S/N level
What Defines Sensitivity?
Aspects of Signal and Noise

- Improved S/N ratio: Black background
- Improved signal recording: Crisp details, clear image data
Detectors-QE: Confocal, Airyscan and SR-SIM

![Graph showing quantum efficiency vs. wavelength at room temperature for different types of detectors: Airyscan, Confocal, and SR-SIM.](image)
What Defines Sensitivity?  
And What Does Increased Sensitivity Enable?

- **Better image quality**
  - Higher signal-to-noise with detection of faint signals; look deeper

- **Faster scanning**
  - Shorter pixel dwell times, reduced need for averaging

- **Longer imaging**
  - Lower laser power prevents phototoxicity

Cultured 2h8 cells labeled with extremely low expression of GFP and mCherry. *Courtesy A. Bruckbauer Cancer Research, London, UK*
LSM SNR Increases over 30 years

Ideal System =
100% QE
detector and 0%
light losses

Normalised SNR
Why and When Choose Airyscan OR SR-SIM Superresolution

Speed, data size, and processing

Specialized Applications and Live Cell Imaging

Summary

Theory, Light Path, Resolution Comparison

Performance comparison
Experimental PSFs-3 Channels

488 nm

561 nm

Confocal

130-170 nm Airyscan FWHM

SR-SIM

120-157 nm FWHM
**SENSITIVITY**

- No need for additional spatial sampling; uses photons discarded in conventional confocals with mechanical pinhole.

**SPEED**

- Higher inherent S/N ratio can be traded for faster imaging as needed.

**RESOLUTION**

- A $1.7\times$ higher resolution in all three dimensions, yielding 5 times smaller confocal volumes.

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**SNR Comparison on Microtubules**

<table>
<thead>
<tr>
<th>Single scan</th>
<th>GaAsP</th>
<th>Average 4</th>
<th>Airyscan</th>
<th>Average 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2% 488nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02% 488nm</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

For the same resolution, a standard confocal pinhole would have to be closed to 0.2 AU (with consequent loss in S/N).
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Thin Vs Thick samples

SR-SIM

Jennifer Mitchell, Martha Gillette Lab

Airyscan

Urban and Barclay, Surangi Punyasena Lab

Jingyi Fei, TJ Ha Lab

CARL R. WOESE INSTITUTE FOR GENOMIC BIOLOGY
Where Science Meets Society
### Comparison in 3D Performance

<table>
<thead>
<tr>
<th>Method</th>
<th>Maximum Intensity</th>
<th>Single Plane</th>
<th>Line Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conical</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Echocardiography</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Helixina sp.</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>Anyscan</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Helixina sp.</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
<tr>
<td>SR-SIM</td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Notes:**
- The tables and images above compare different methods in 3D performance, highlighting maximum intensity, single plane, and line profile graphs.
- The images show various samples with corresponding analysis metrics.
Confocal/AS/SR-SIM Comparison

<table>
<thead>
<tr>
<th></th>
<th>Croton hirtus</th>
<th>Dactylis glomerata</th>
<th>Helianthus sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Confocal</strong></td>
<td><img src="confocal.png" alt="Image" /></td>
<td><img src="confocal.png" alt="Image" /></td>
<td><img src="confocal.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>Intensity vs Relative distance (µm)</strong></td>
<td><img src="intensity_graph.png" alt="Graph" /></td>
<td><img src="intensity_graph.png" alt="Graph" /></td>
<td><img src="intensity_graph.png" alt="Graph" /></td>
</tr>
<tr>
<td><strong>Aryscan</strong></td>
<td><img src="aryscan.png" alt="Image" /></td>
<td><img src="aryscan.png" alt="Image" /></td>
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<td><img src="intensity_graph.png" alt="Graph" /></td>
</tr>
<tr>
<td><strong>SR-SIM</strong></td>
<td><img src="sr-sim.png" alt="Image" /></td>
<td><img src="sr-sim.png" alt="Image" /></td>
<td><img src="sr-sim.png" alt="Image" /></td>
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</tbody>
</table>
SR-SIM Resolving the Areolae and Granulae of grass pollen

SEM data Mander et al., 2013, Royal Society Proceeding
SR-SIM in Resolving *Areolae* and *Granulae* Contrast in Pollen

SEM data Mander et al., 2013
Royal Society Proceeding

Sivaguru et al., 2015, MRT (in preparation)
SR-SIM resolving the Granulae and Areolae of grass pollen

Unlabeled pollen
Why and When Choose Airyscan OR SR-SIM Superresolution

Speed, data size, and processing

Specialized Applications and Live Cell Imaging

Summary
SR-SIM

<table>
<thead>
<tr>
<th>Image Format</th>
<th>Single SR-SIM Frame$^{(2)}$</th>
<th>Time Series$^{(3)}$ (10 SR-SIM frames)</th>
<th>Z-stack$^{(4)}$ (2 μm, 16 SR-SIM frames)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004 x 1002 px (full frame)</td>
<td>1.7 sec</td>
<td>17.1 sec</td>
<td>14.6 sec</td>
</tr>
<tr>
<td>512 x 512 px (subarray)</td>
<td>1.4 sec</td>
<td>14.1 sec</td>
<td>9.6 sec</td>
</tr>
<tr>
<td>256 x 256 px (subarray)</td>
<td>1.4 sec</td>
<td>13.6 sec</td>
<td>9.2 sec</td>
</tr>
</tbody>
</table>

$^{(2)}$ 15 individual images recorded per SR-SIM frame (at three pattern rotations)

$^{(3)}$ 150 individual images recorded without pausing representing 10 SR-SIM frames (same Z-level)

$^{(4)}$ 240 individual images recorded corresponding to 16 SR-SIM frames at different Z-levels (spacing between Z-levels: 0.133)

Airyscan

At 512 x 512 pixels → 13 fps

At 512 x 16 pixels → 430 fps

At max speeds → 4x larger field of view

Image Size 16 times larger

1024x1024

Confocal Mode ()/Airyscan Mode/After Processing
Theory, Light Path, Resolution Comparison

Performance comparison

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Specialized Applications and Live Cell Imaging

Summary
Live Cell Imaging Mitochondria - Ratiometric ROS indicator - SR-SIM

Vladimir and Jessica, Rex Gaskin's Lab
FRET Acceptor Photobleaching in LSM 880 with GaAsP detector

Xinyu K, Debra Leckband Lab

Airyscan Live Cell Imaging

Ryan Lake, Chemistry Dept.,

CARL R. WOESE INSTITUTE FOR GENOMIC BIOLOGY
Where Science Meets Society
Mitosis in HeLa EB3 EGFP-Histone 2B mCherry, Airyscan Superresolution_Jan Ellenberg, EMBL
Theory, Light Path, Resolution Comparison

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Summary
Choosing the right optical system for your application*

<table>
<thead>
<tr>
<th>When and Where?</th>
<th>Airyscan-SR LSM 880</th>
<th>SR-SIM</th>
<th>Confocal 700, 710</th>
</tr>
</thead>
<tbody>
<tr>
<td>XY Resolution (Thin Samples-Cells-Colocalization)</td>
<td>XXXX</td>
<td>XXXXX</td>
<td>X</td>
</tr>
<tr>
<td>XY Resolution (Thick samples-Tissues)</td>
<td>XXXXX</td>
<td>XXX</td>
<td>X</td>
</tr>
<tr>
<td>Dual/Multiple Channels simultaneous</td>
<td>XXXXX</td>
<td>X</td>
<td>XXX</td>
</tr>
<tr>
<td>Speed</td>
<td>XXXX</td>
<td>XXX</td>
<td>X</td>
</tr>
<tr>
<td>Z-Depth Penetration and Resolution</td>
<td>XXXX</td>
<td>XXX</td>
<td>XXX</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>XXX</td>
<td>XXXXX</td>
<td>X</td>
</tr>
<tr>
<td>Specialized techniques-FRAP, FRET</td>
<td>XXXXX</td>
<td>X</td>
<td>XXX</td>
</tr>
</tbody>
</table>
Collaborators
Bruce W. Fouke, Geology, UIUC
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