Inside the LSM 880 NLO + Airyscan

Overview of the Newest High-End Point Scanning Solution from Carl Zeiss Microscopy

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Outline of Discussion

ZEISS LSM 880 NLO + Airyscan @ WashU

1. Existing System Overview
2. **LSM 880** Design and Considerations
3. Principles of the **Airyscan**
4. Additional Enabling Components
5. And ... the ApoTome!
6. Summary / Questions

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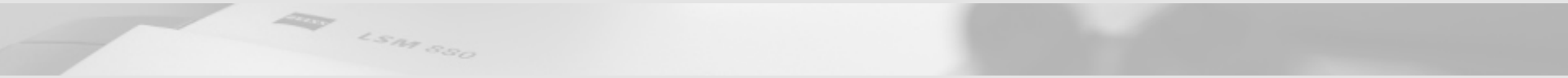

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System At-a-Glance
Hardware Specifications

LSM 880 scanhead; 34-channel (GaAsP)

Airyscan superresolution detector (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, CO_2)

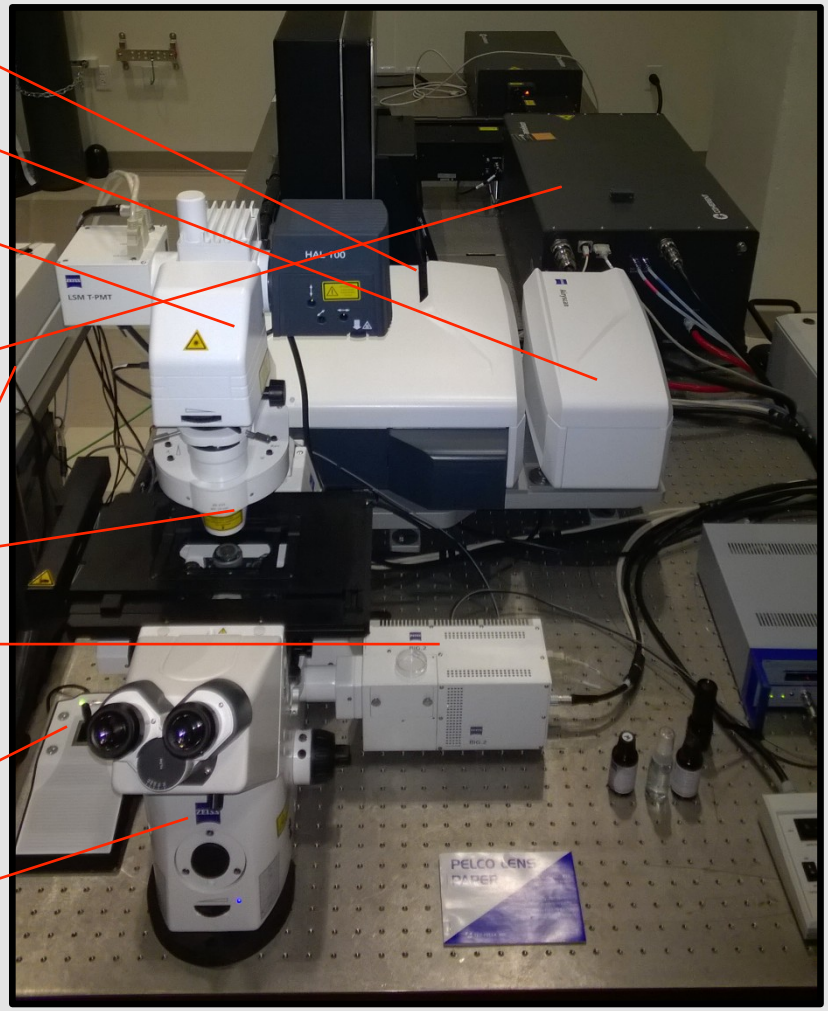
Objectives:
20x/0.8
40x/1.2 W
40x/1.3 oil

External NDD,
reflected light
(2-channel GaAsP)

Motorized XY stage + Z-piezo insert

Observer.Z1 inverted microscope
(with Definite Focus, ~~820-860 nm~~)

(Insanely long and low anti-vibration table)



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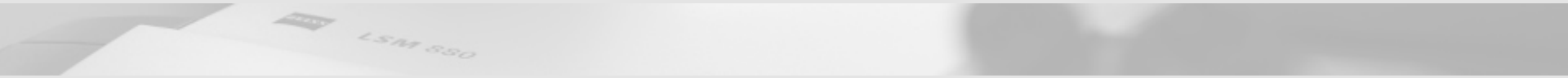

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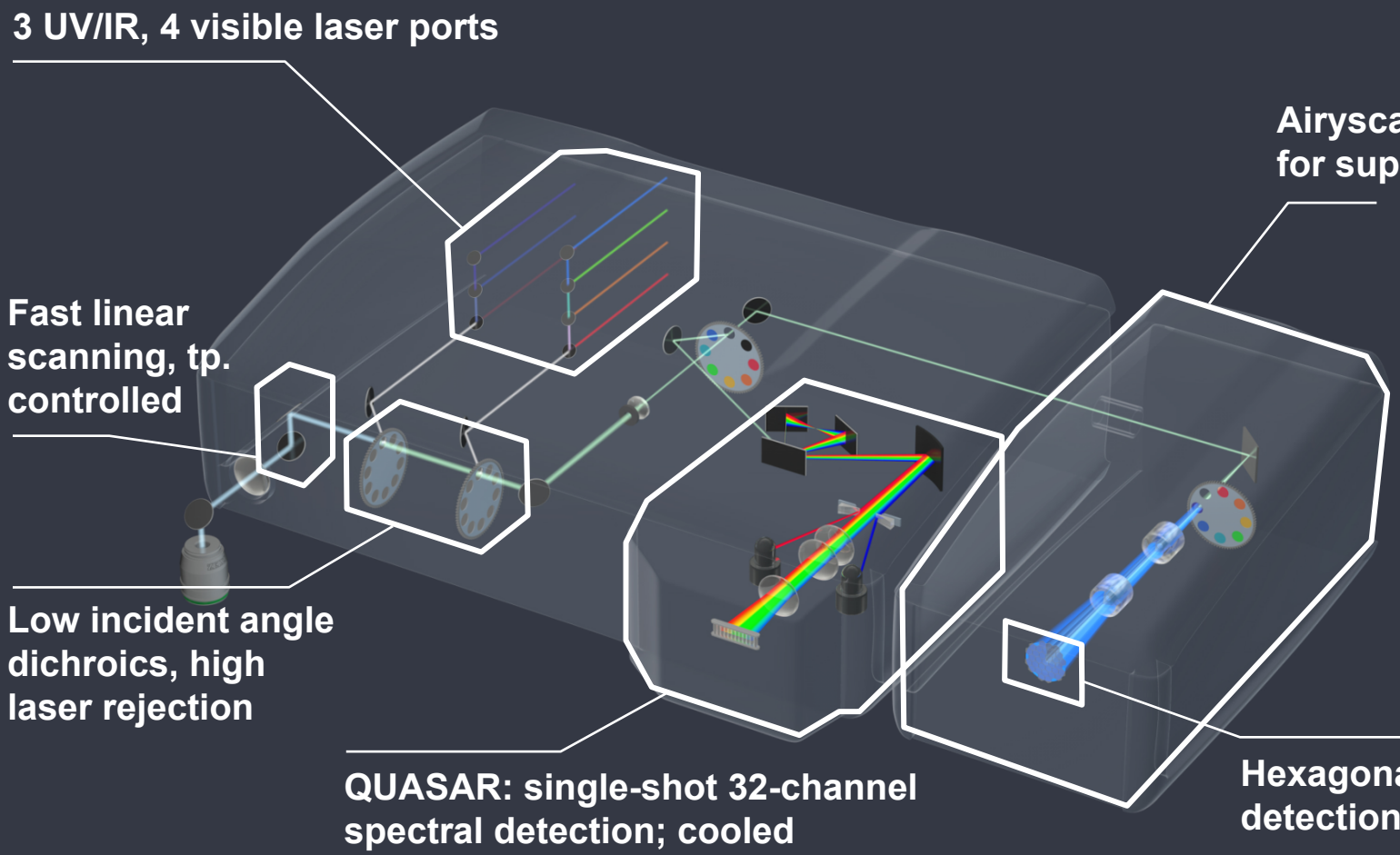
Inside the ZEISS LSM 880

System Footprint

Inside the ZEISS LSM 880

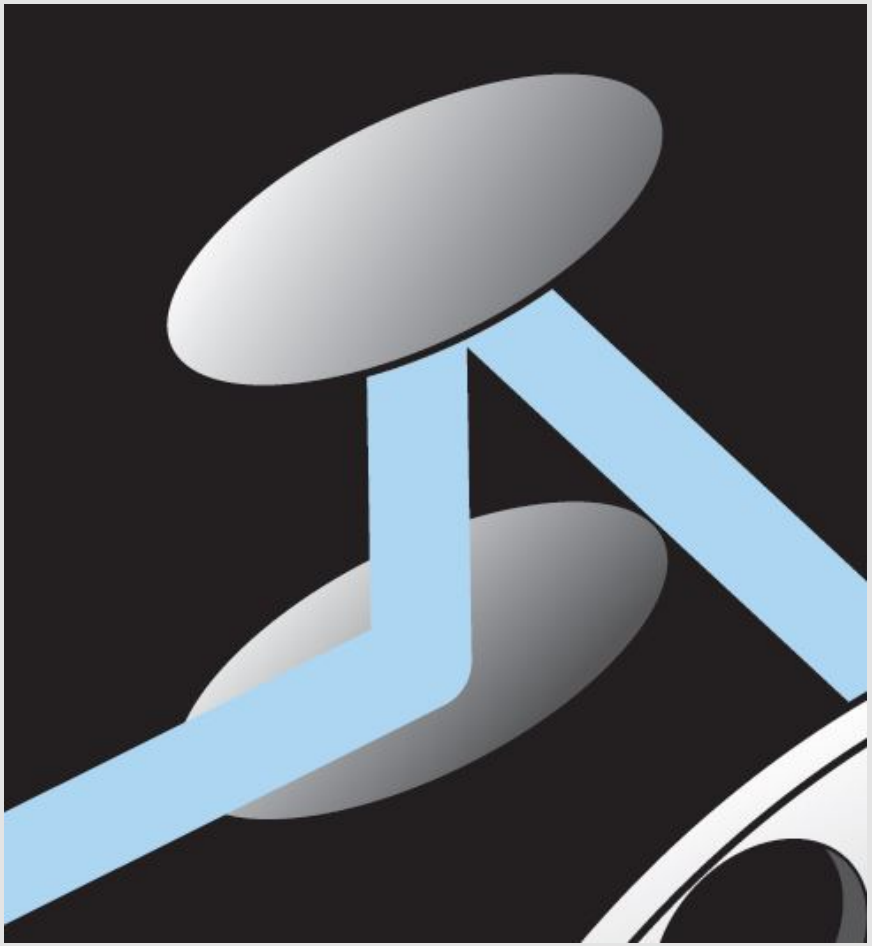
Added Speed, Sensitivity, and Resolution



Inside the ZEISS LSM 880

Linear Scanners

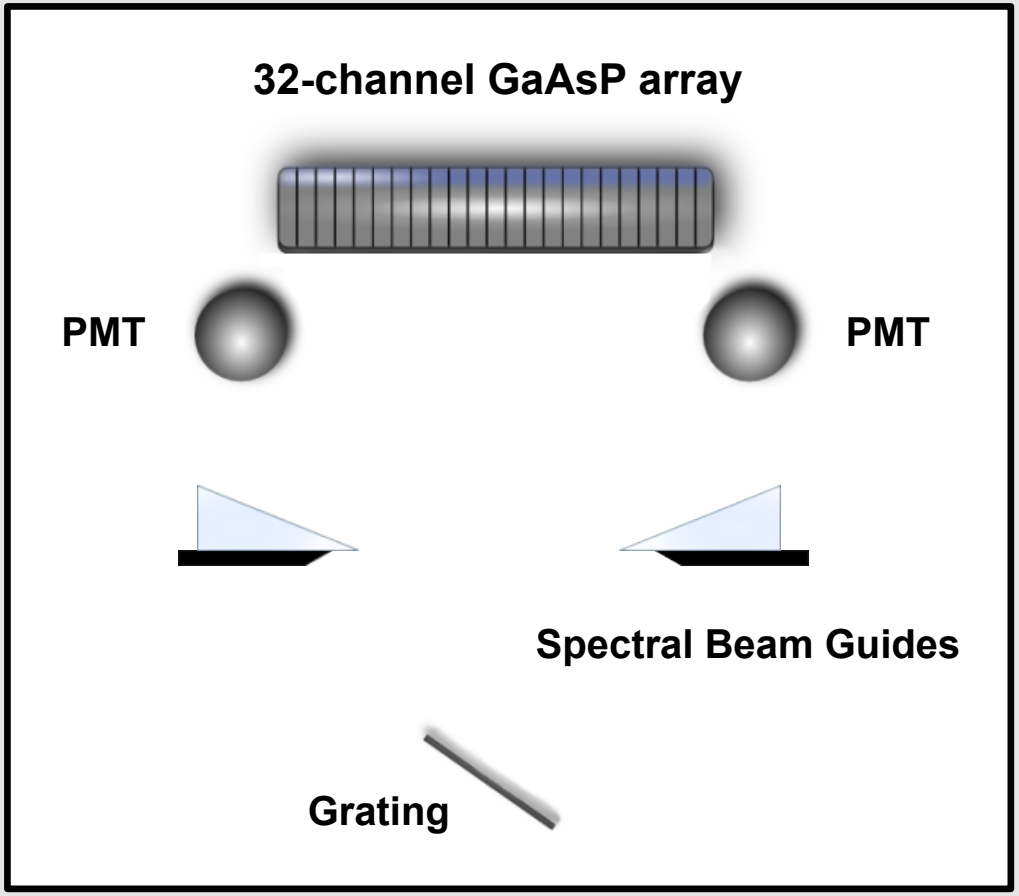
- Fastest **linear scanning** frequencies and amplitudes available
  - At 512 x 512 pixels → **13 fps**
  - At 512 x 16 pixels → **430 fps**
  - At max speeds → **4x larger field of view**
- Full 0.6 – 40x scanning zoom, freely rotatable in 360°
- Full liquid cooling of scanning components and surrounding electronics



Inside the ZEISS LSM 880

Detection Unit

- Signal is directed to detectors via prisms and beam guides
  - **Fully definable collection window**
  - No secondary dichroics or fixed emission filters



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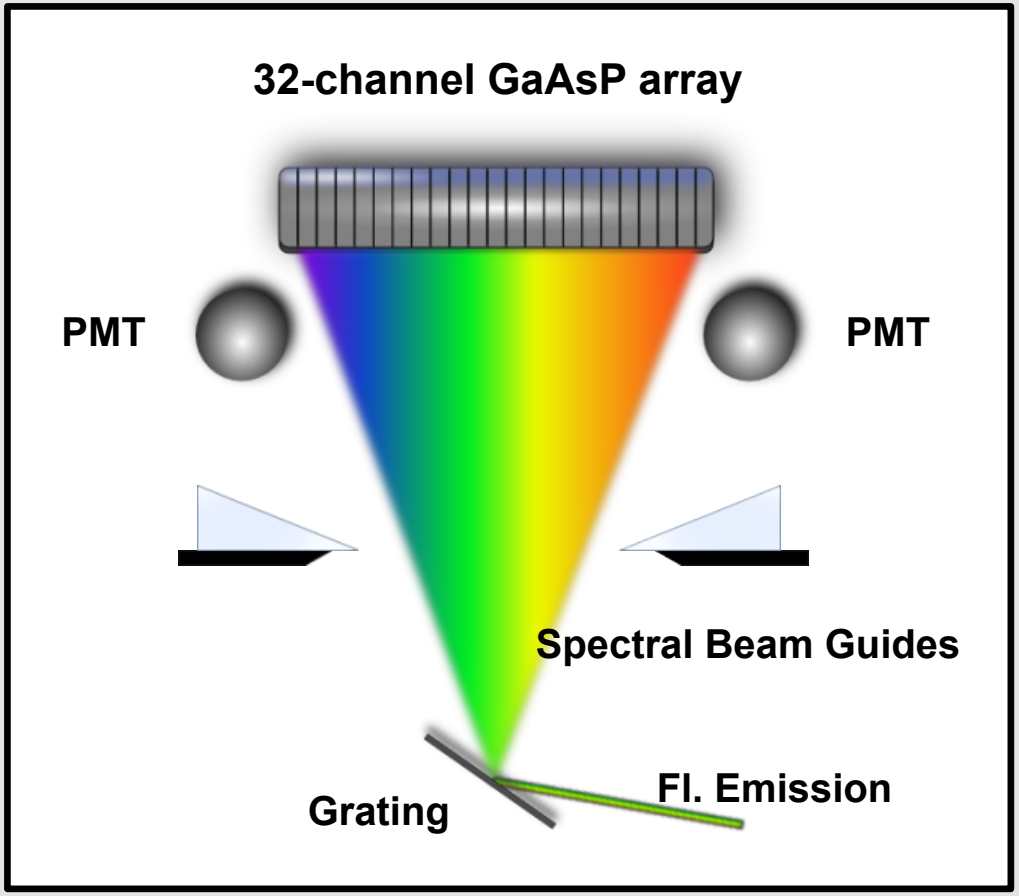

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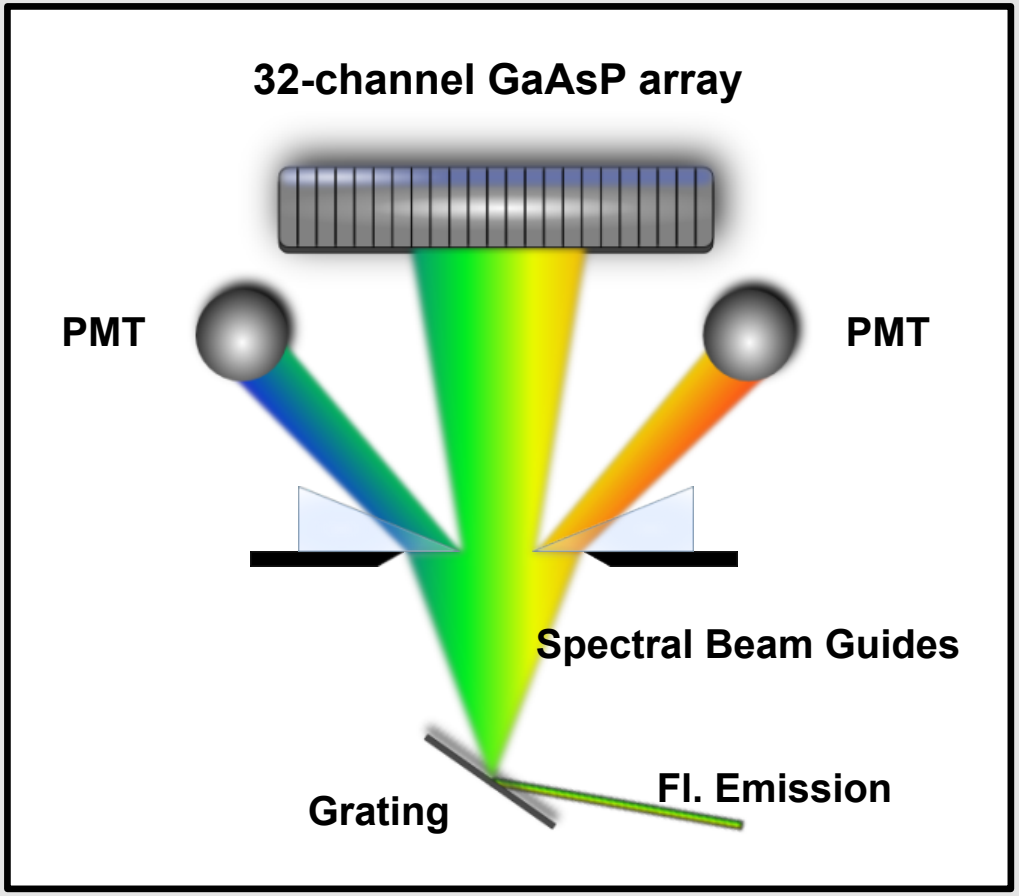
Detection Unit

- Signal is directed to detectors via prisms and beam guides
  - **Fully definable collection window**
  - No secondary dichroics or fixed emission filters
- Light reaching center detector array remains linearly dispersed
- Flanking PMTs can pick off spectrum as needed



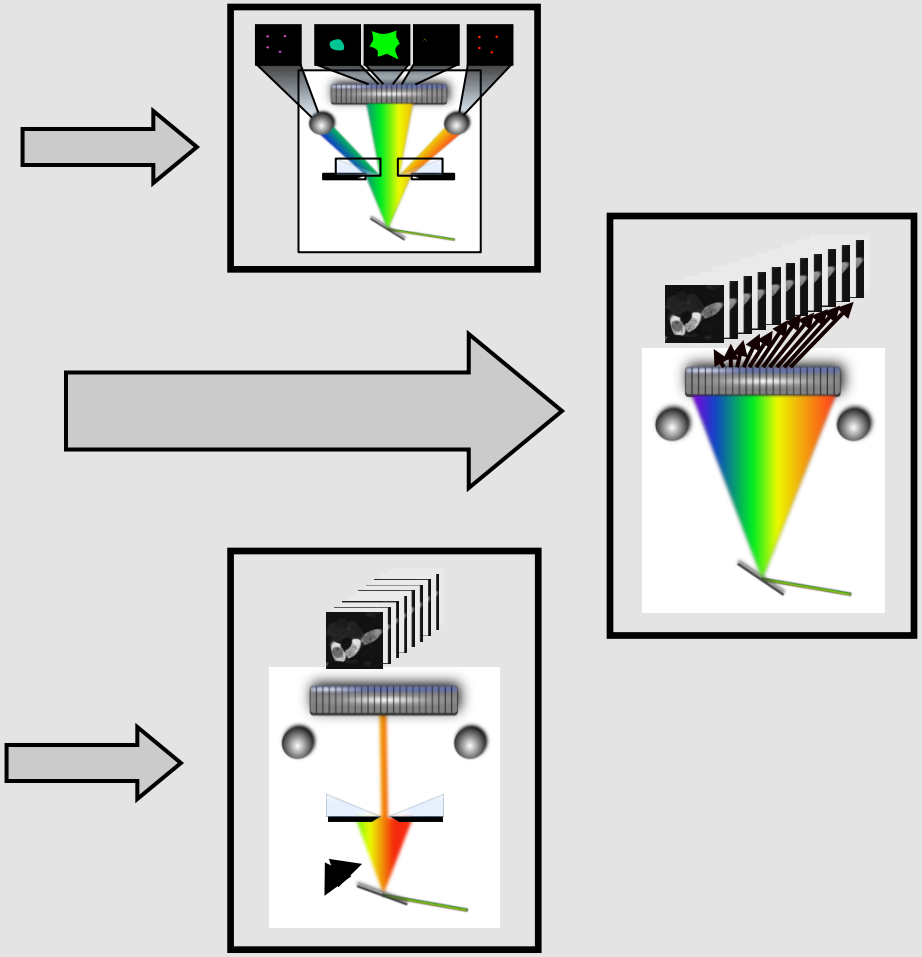
Inside the ZEISS LSM 880

Detection Modes

1. Variable **multi-channel** detection
   - Full freedom of detection windows, widths with 1 nm resolution
   - Range from 391 – 749 nm
   - Up to 10 channels simultaneously

2. Simultaneous **full spectrum** collection (“Lambda Mode”)
   - Up to 34 contiguous spectral segments in a single scan; full emission range
   - Collection of entire spectral signature
   - Subsequent unmixing of fluorophores into channels

3. High-res **“spectrometer”** mode
   - Sequential scanning across spectrum for demanding unmixing applications
   - Up to 3 nm resolution collection



Fast Spectral Imaging

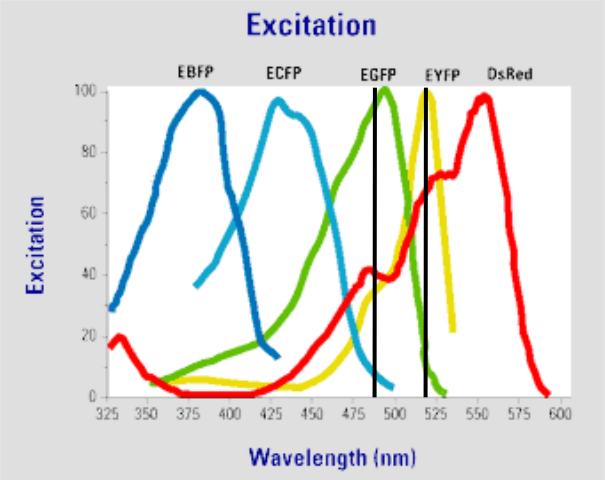
Unmixing of Overlapping Fluorophores

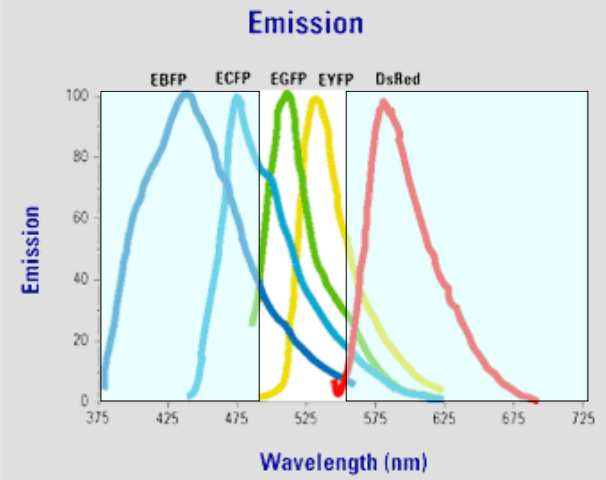
CHALLENGE:

- Conditions of excitation and emission cross-talk from spectrally-adjacent fluorophores

SOLUTION:

- Use of robust and sensitive spectral detection (via GaAsP array) to unmix overlapping signals





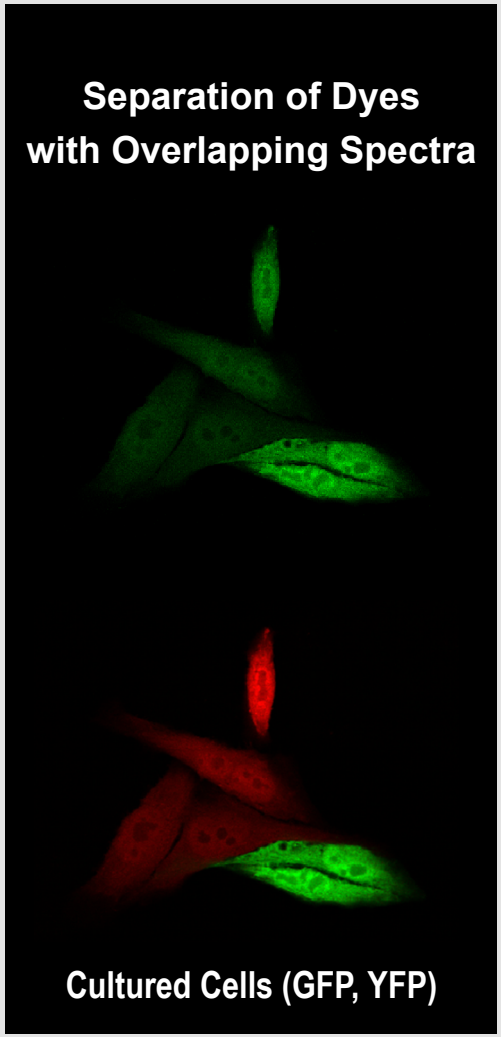
Fast Spectral Imaging

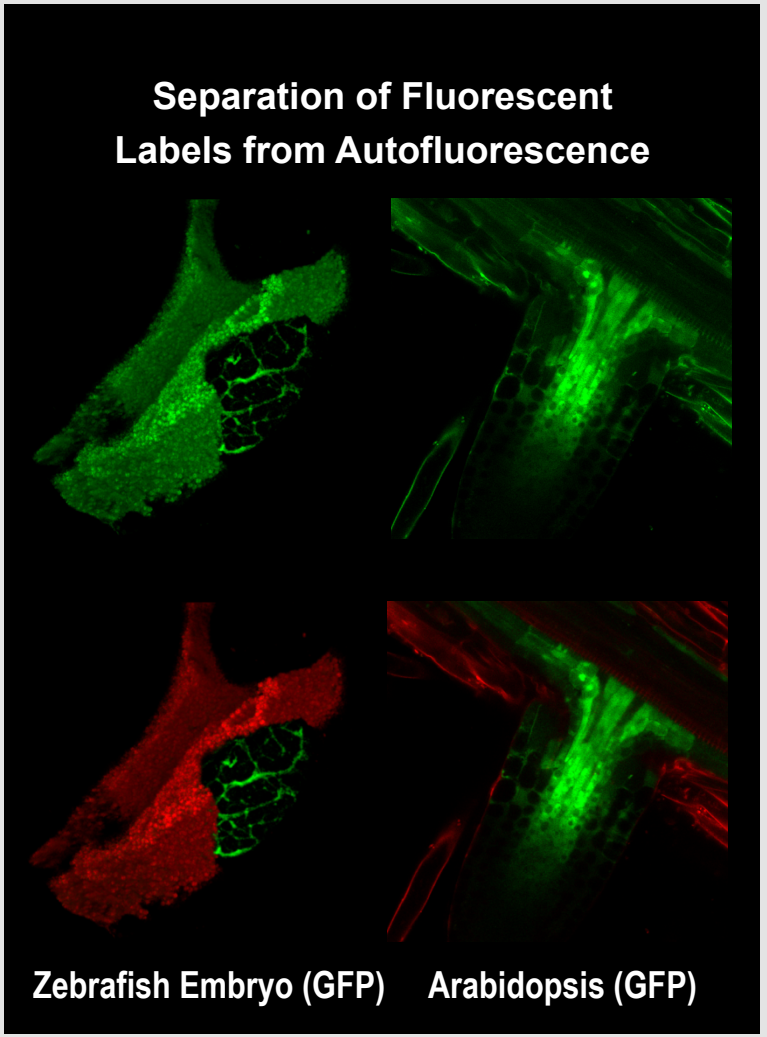
Unmixing of Overlapping Fluorophores

Imaging task:

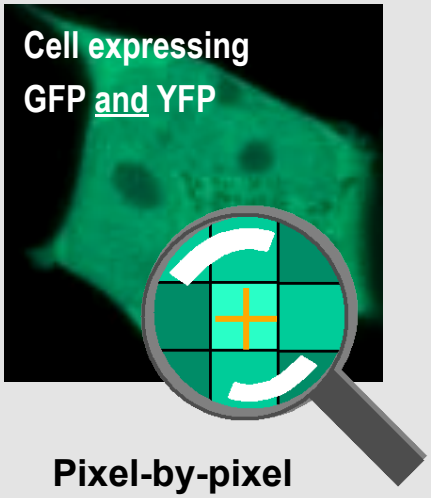
Without Linear Unmixing

With Linear Unmixing

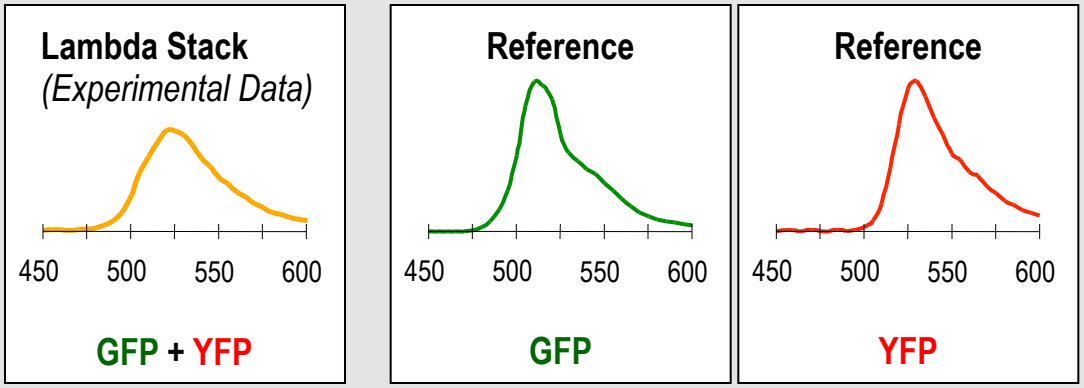




Linear Unmixing:
How Does it Work?



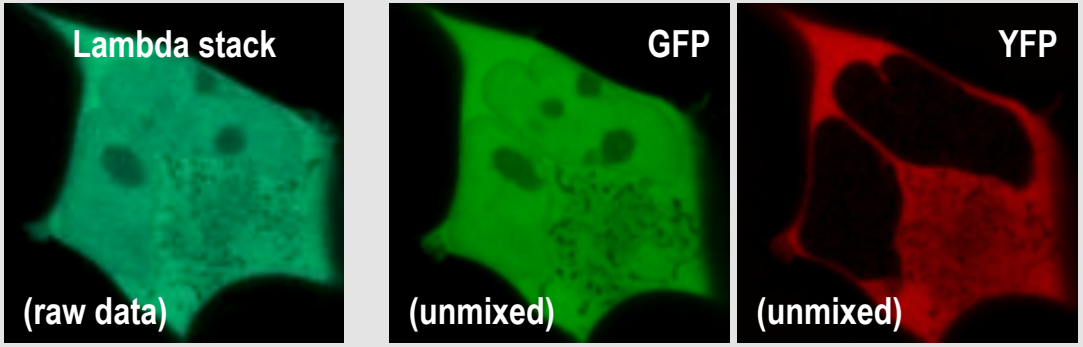
Pixel-by-pixel analysis



100 = 68 + 32

Relative contribution of GFP and YFP

Linear unmixing determines the relative contribution of each fluorophore in **every pixel** of an image

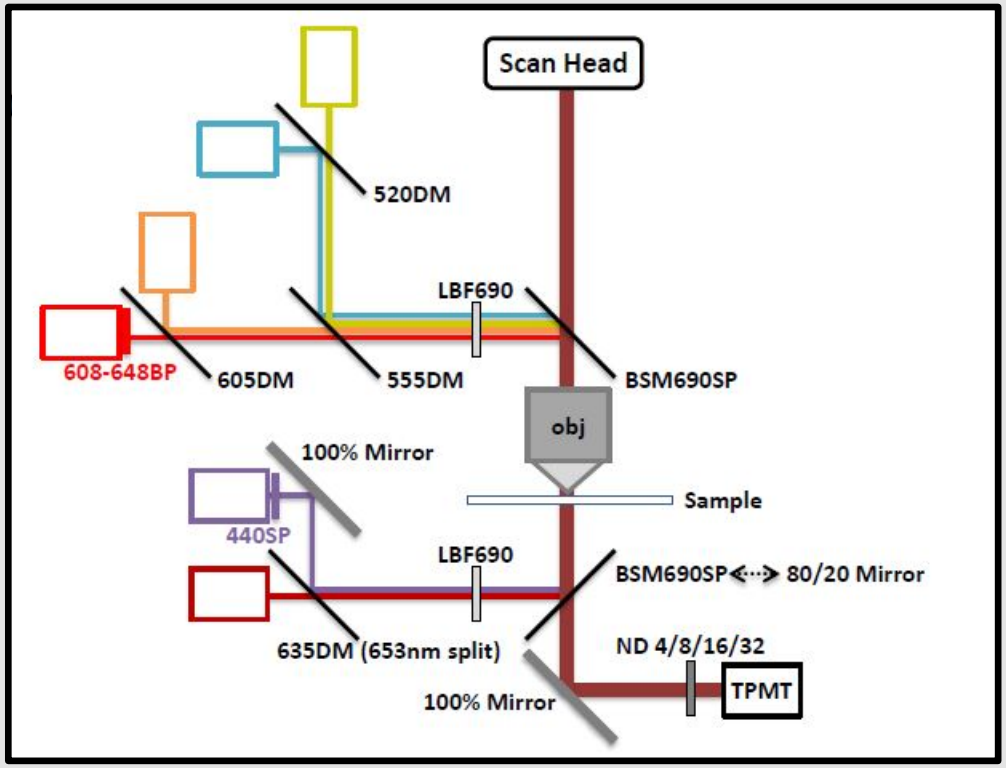


Spectral Imaging: Applications
Using Multiple Excitation Options

GOAL:
Detect 6 fluorophores in single scan with highest signal-to-noise possible

Strategy #1: Use 32-element internal detector (+ pinhole) with combination of visible lasers (5 wavelengths)

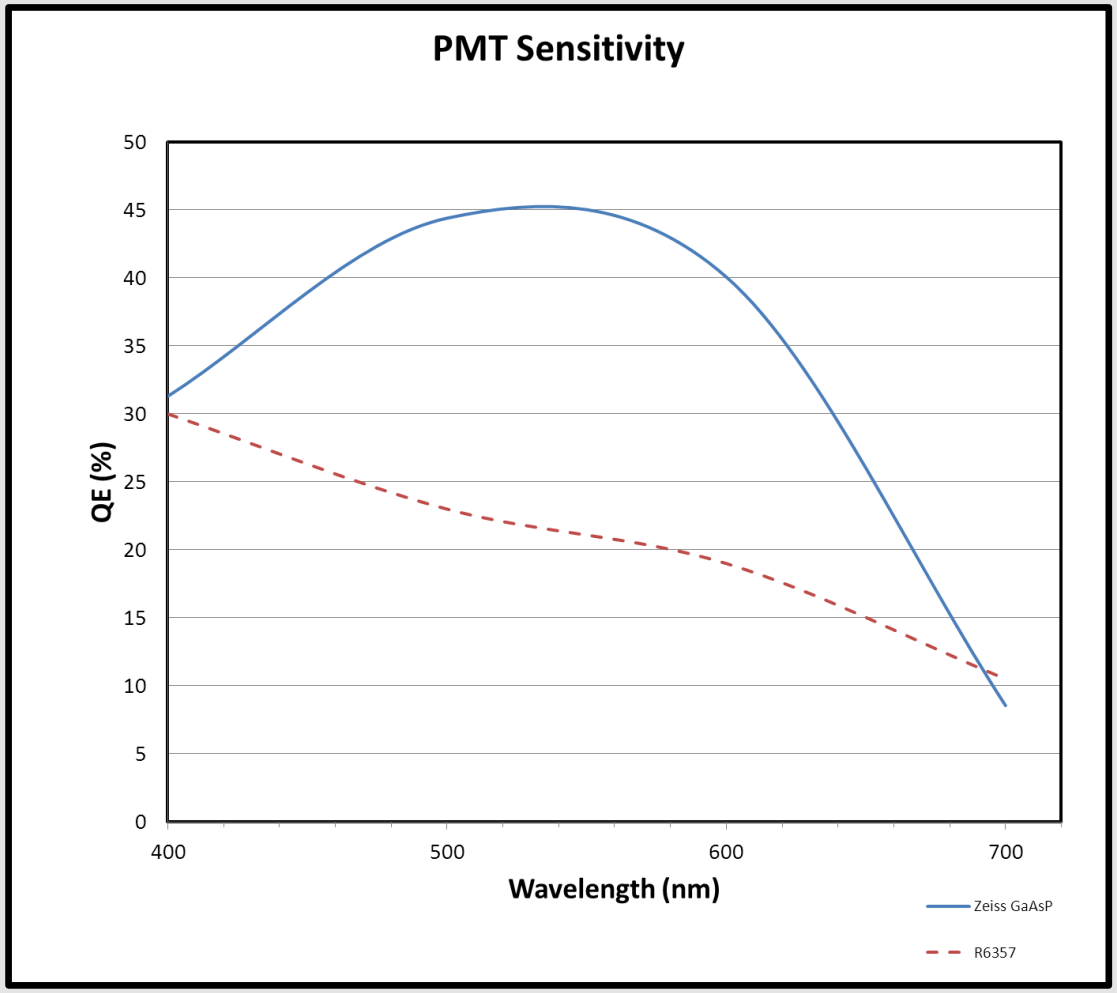
Strategy #2: Use 2 separate IR wavelengths (830, 1040 nm, exploiting specific cross-sections) with readout on 6 NDDs on TL and RL path



*(LSM + Examiner.Z1 detector schematic courtesy of **Dawen Cai**, University of Michigan)*

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

Using the GaAsP Detectors:
Integration Mode

- GaAsP detectors permit two methods of reading signals – **integration mode** and **photon counting mode**

- Under **integration mode** (conventional), signal read with constant frequency (40 MHz, oversampling)
  - Average photons over pixel dwell time is basis for pixel grey value
  - *Integration is reason why scan speed setting has no influence on image brightness – only signal/noise ratio*

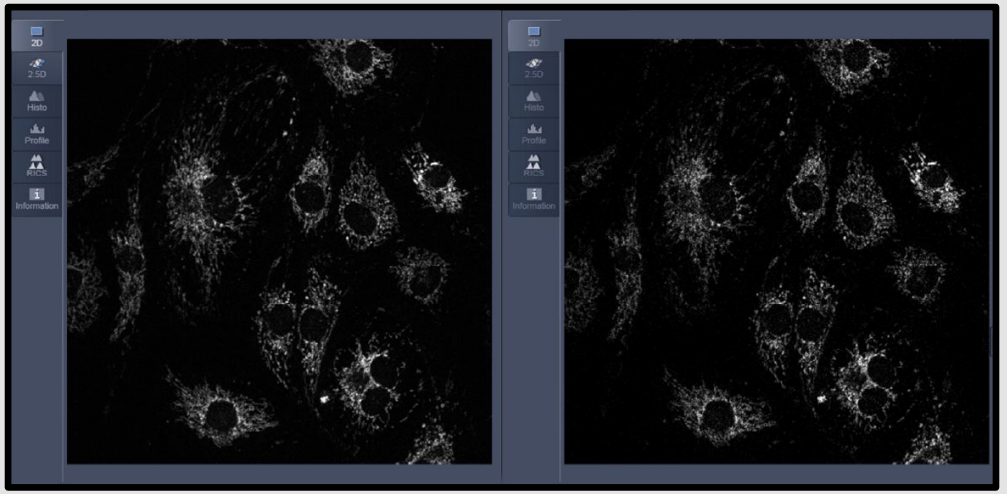
Using the GaAsP Detectors:
Photon Counting Mode

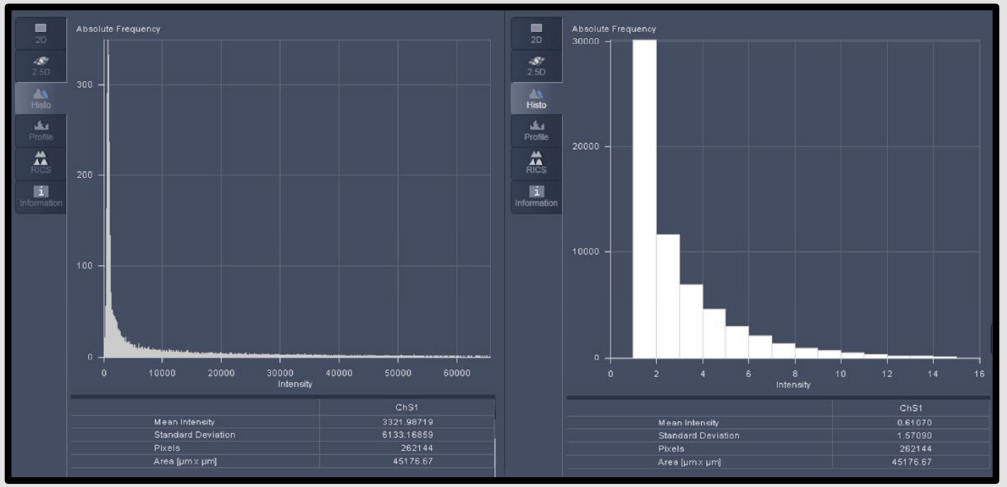
- GaAsP detectors permit two methods of reading signals – **integration mode** and **photon counting mode**

- With **photon counting mode**, master gain locked at maximum voltage (1250 V) to assess single photon events
  - Useful if image quality in integration mode with high gain is insufficient
  - *Operates at different count rate (15 MHz) and is cumulative; here dwell time and scanning speed directly affect signal intensity*
  - *For 1.5 µs dwell time, maximum detectable photons = 15 x 1.5 = 22*

Using the GaAsP Detectors:
Applications

- Image on left is 16-bit **integration** image taken with **0.1% laser power**
- Image on right is a **photon counting** image taken with **0.01% laser power**
- Added sensitivity thus allows for **more gentle** imaging approaches – or can be traded outright for **greater speed**





External GaAsP Detectors

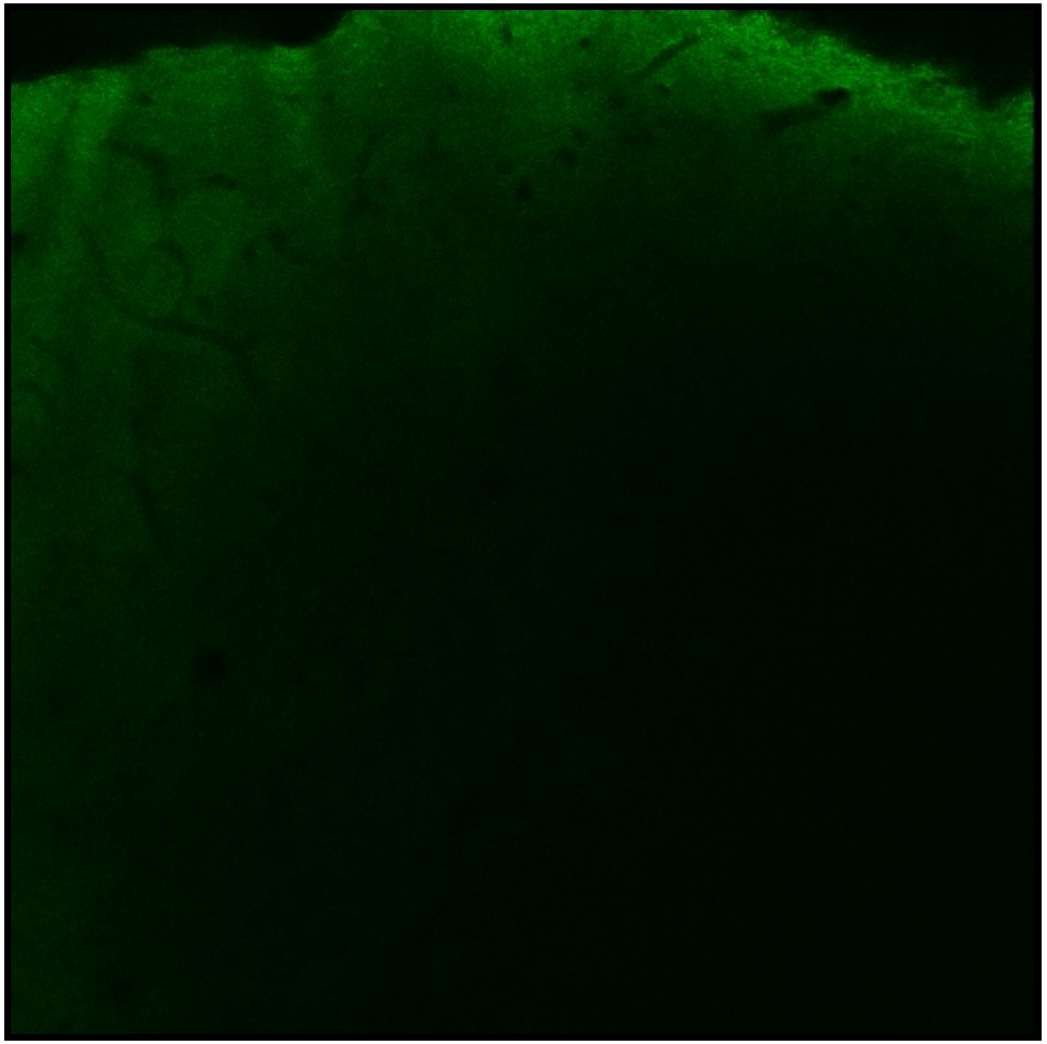
"BiG.2" 2-Channel GaAsP as NDD

- BiG.2 (GaAsP) detector can be used on any NDD port/mount on all NLO microscope stands
- Works with customizable filters, yielding 2-channel readouts (integration or photon counting modes)
- Works on transmitted or reflected light NDD path

Comparison of Detectors

514 nm Excitation, Internal MA-PMT

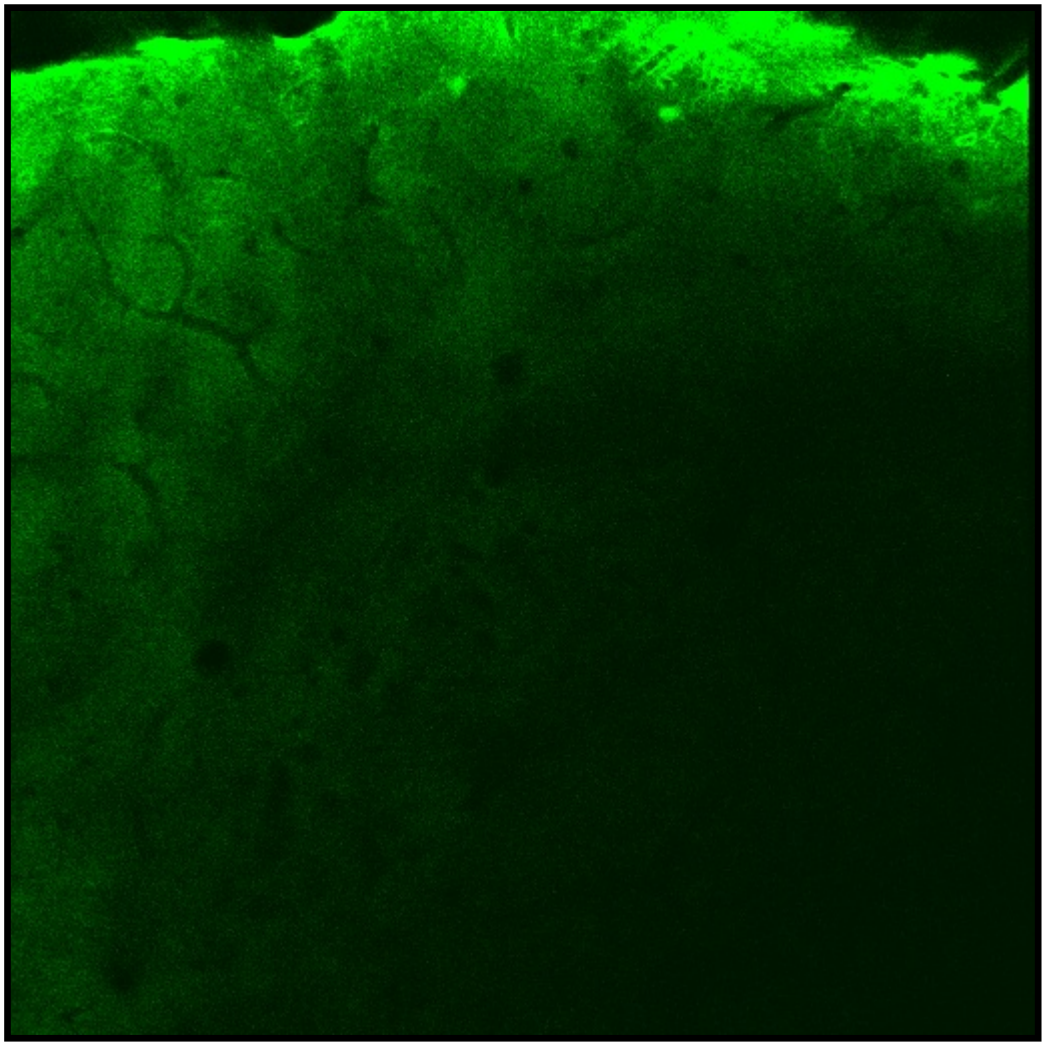
Mouse brain: YFP-labelled tissue; 80 µm deep



Comparison of Detectors

870 nm Excitation, Internal MA-PMT

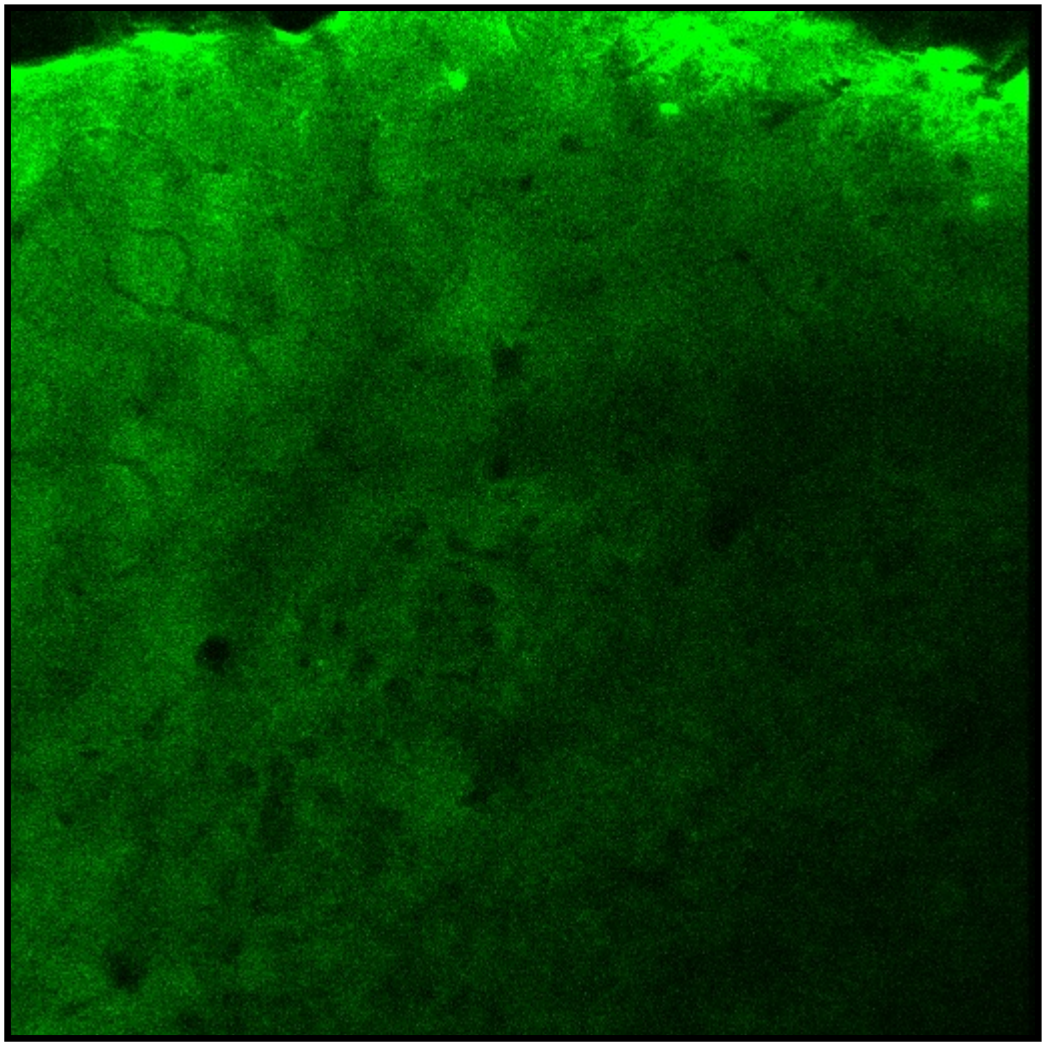
Mouse brain: YFP-labelled tissue; 80 µm deep



Comparison of Detectors

870 nm Excitation, NDD MA-PMT

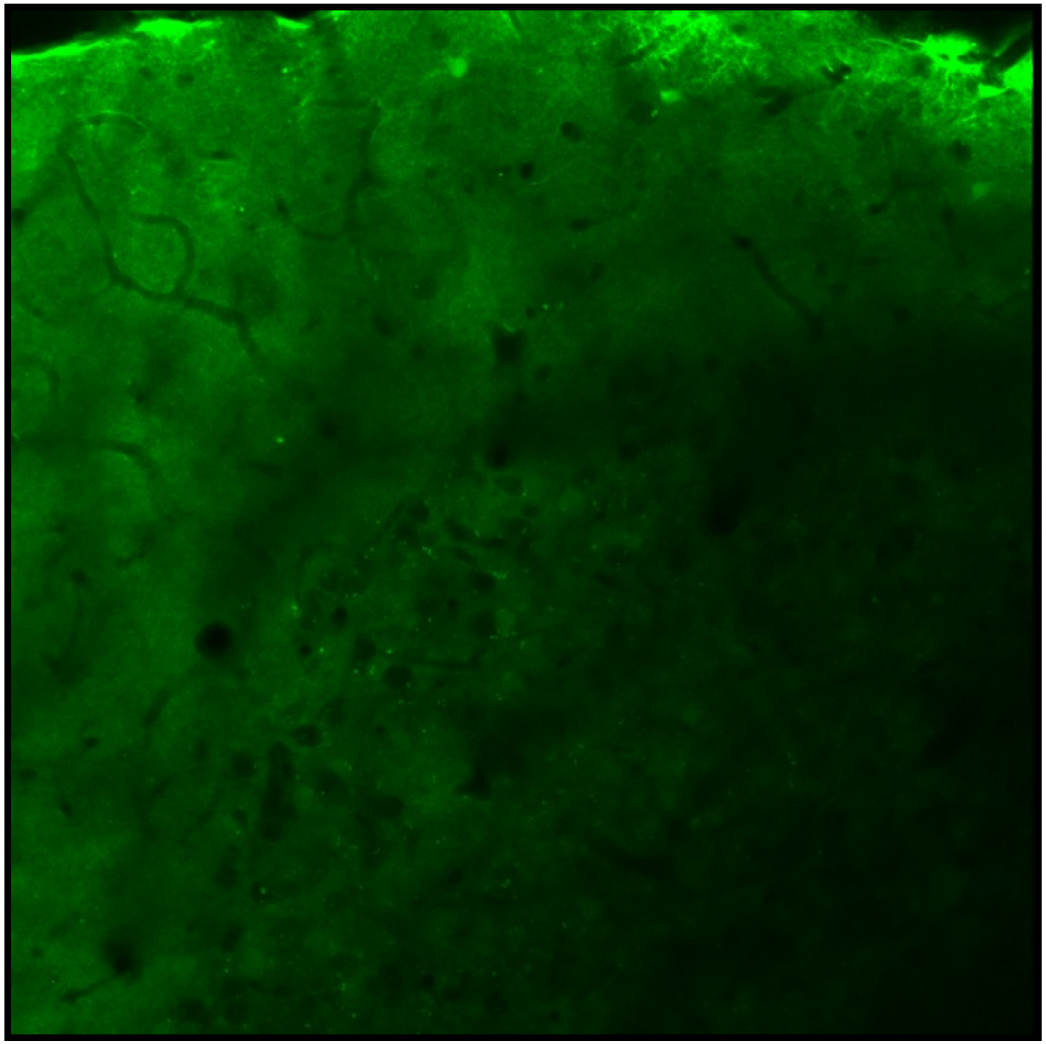
Mouse brain: YFP-labelled tissue; 80 µm deep



Comparison of Detectors

870 nm Excitation, NDD GaAsP (BiG.2)

Mouse brain: YFP-labelled tissue; 80 µm deep



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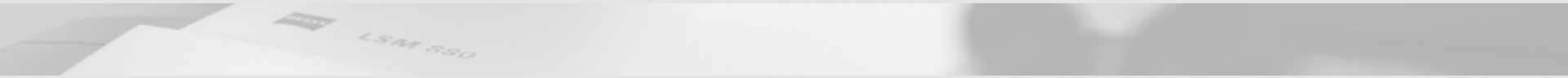

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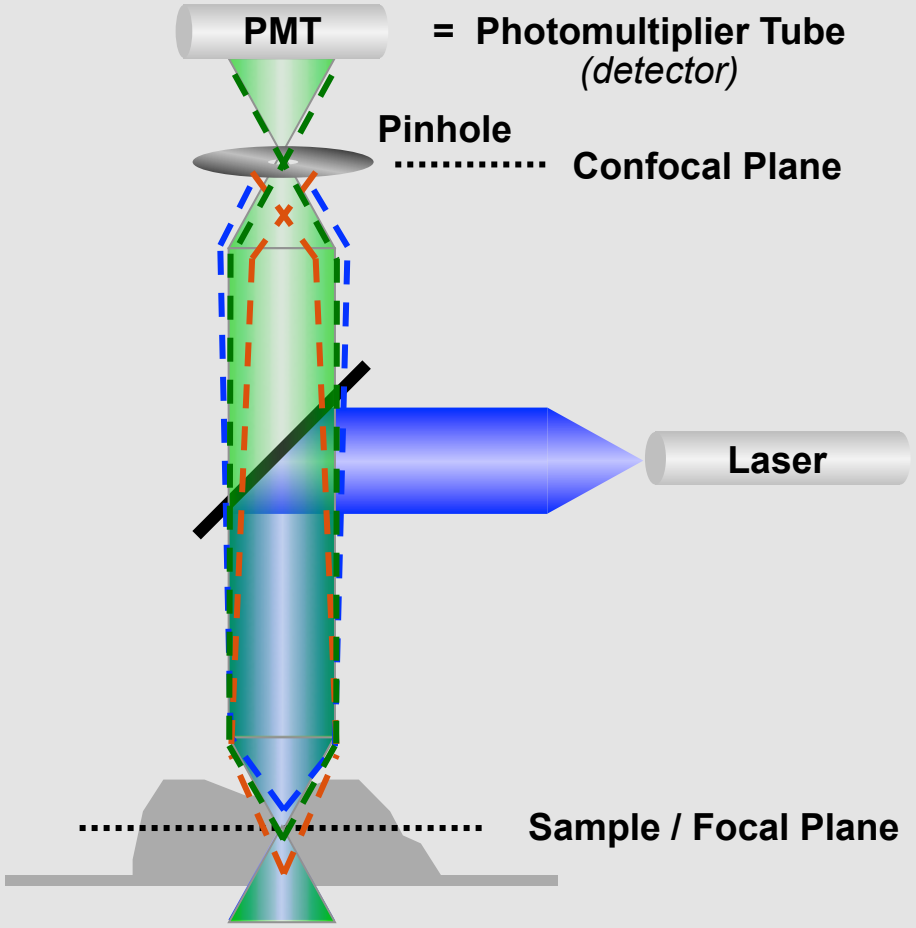
The Confocal Principle

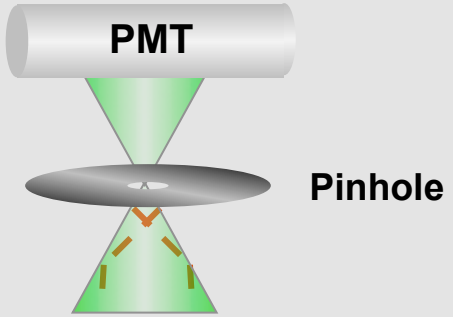
Sectioning via Rejection of Out-of-Focus Signal

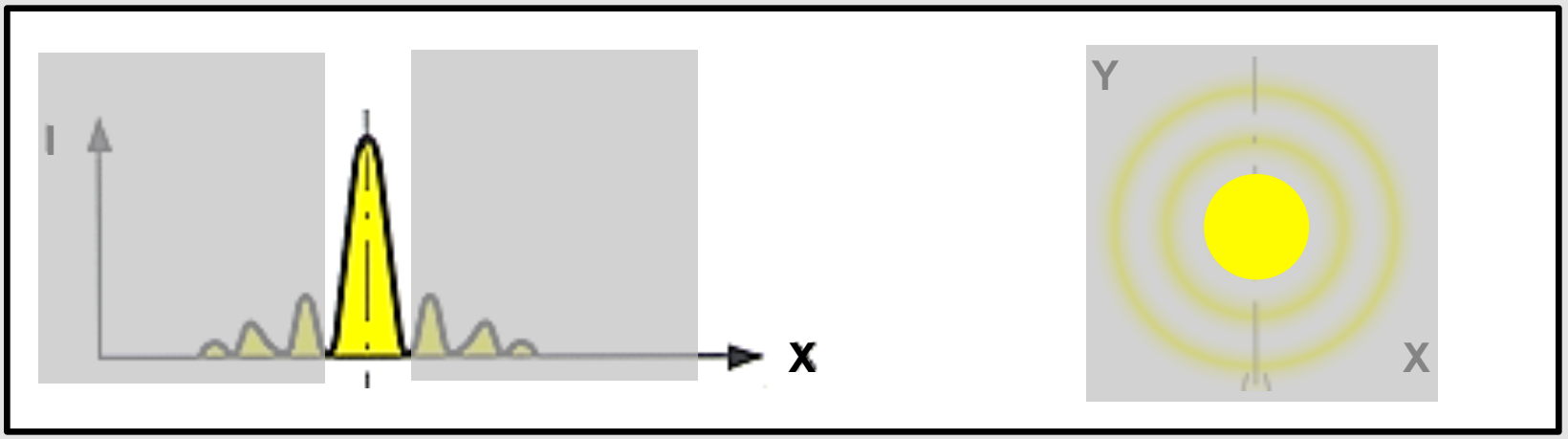
- Characteristic point-wise illumination via laser (filling the back focal plane of objective)
- Pinhole **prevents detection of out-of-focus signals**
  - Minute diaphragm situated in conjugate focal plane
- The thickness of resulting **optical section** influenced by:
  - Numerical aperture of lens
  - Wavelength of excitation light
  - **Pinhole diameter**



The Confocal Principle
Limits of the Pinhole Rejection

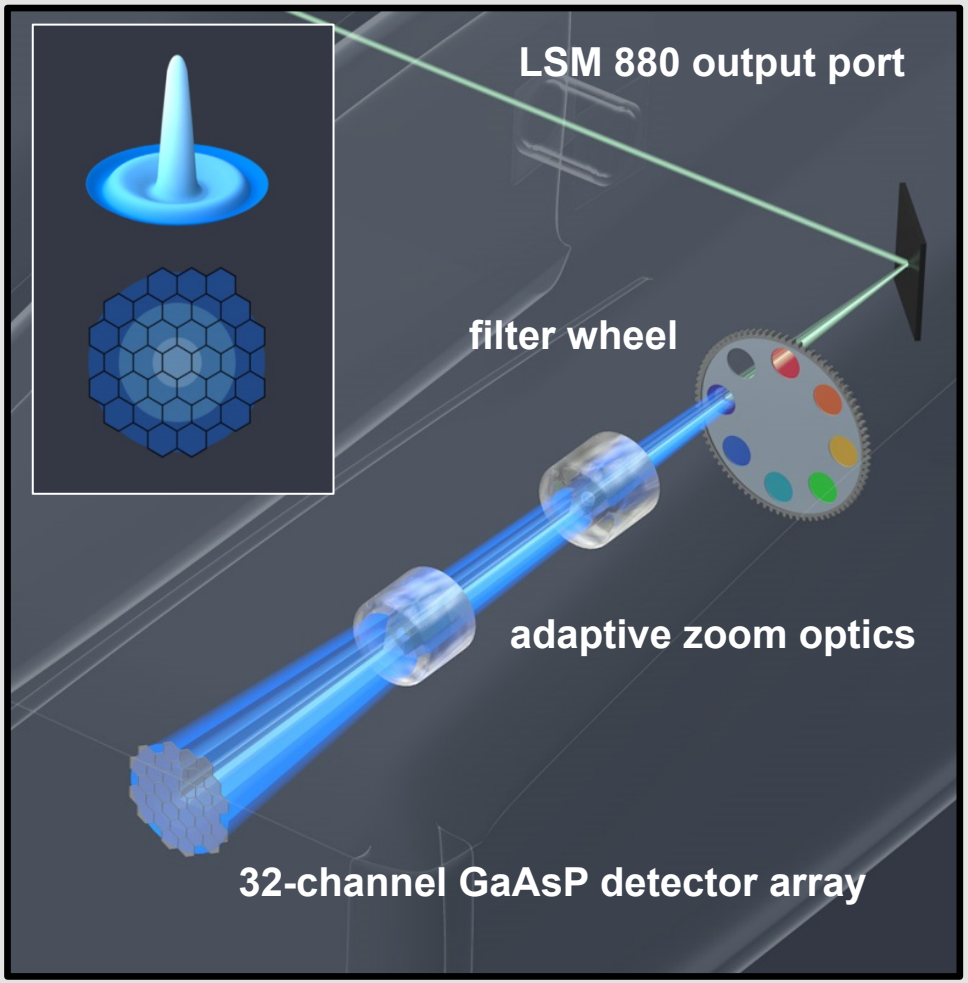
- Mechanical pinhole is rejecting emitted photons based on diameter
- 1 Airy Unit (“AU”) often acts as an ideal compromise between thin optical sections and reasonable signal levels

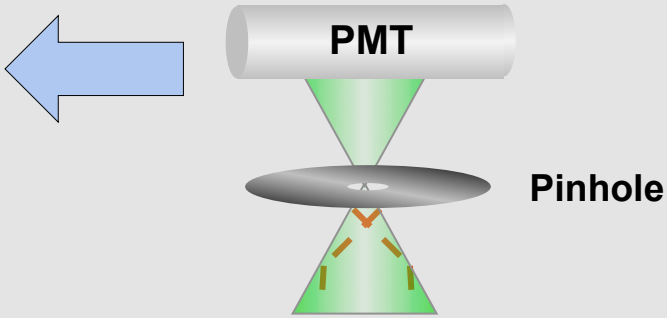


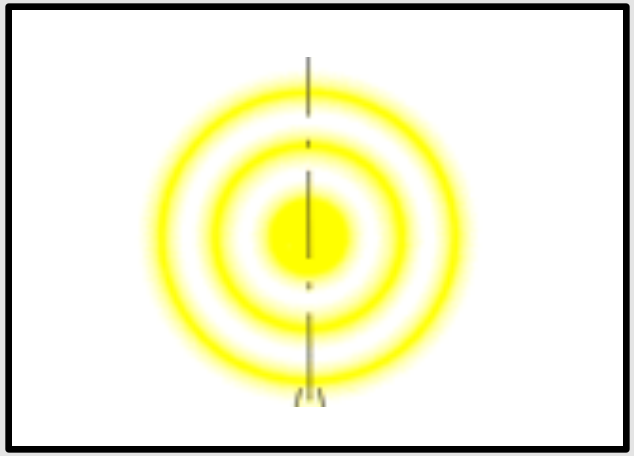


The Airyscan Principle

Unique 32-Channel GaAsP Design

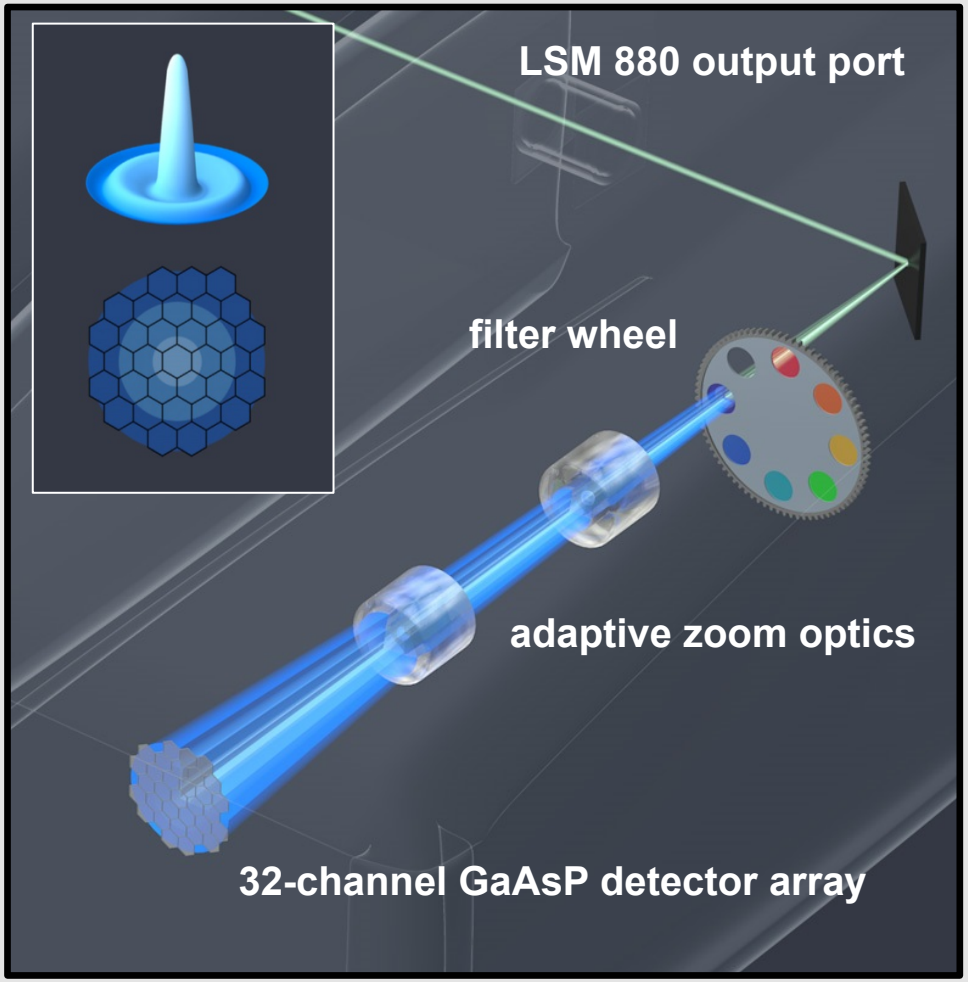






The Airyscan Principle

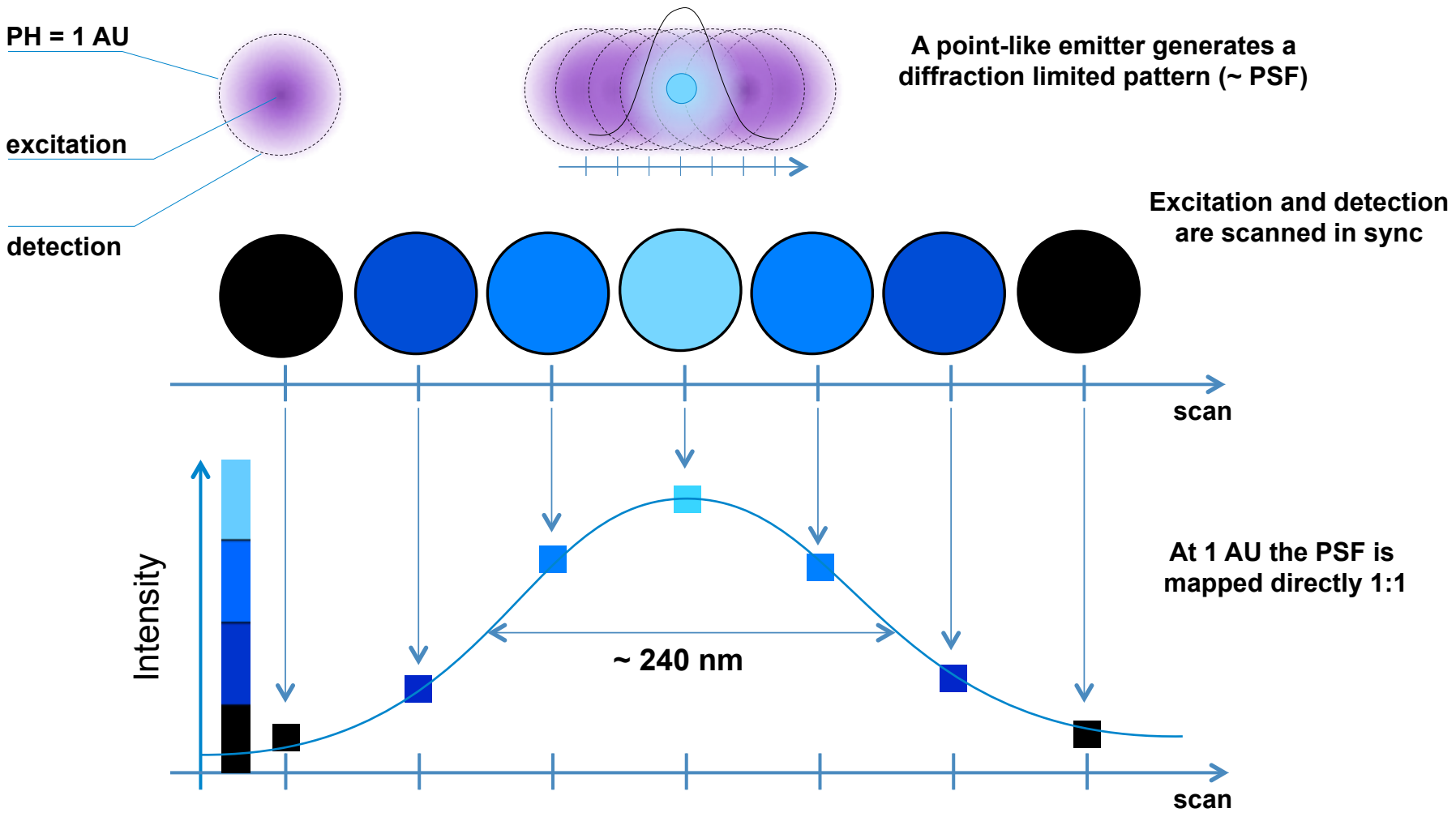
Unique 32-Channel GaAsP Design



- Instead of throwing light away at the pinhole, a 32-channel area detector collects all light of an Airy pattern simultaneously
  - Each pixel thus contains an image of 32 smaller subunits

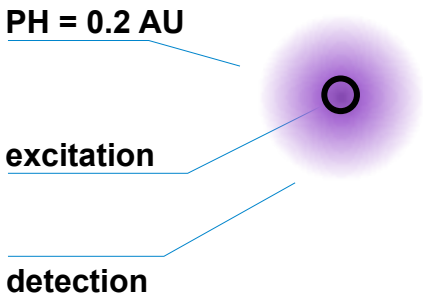
Conventional Scanning Confocal

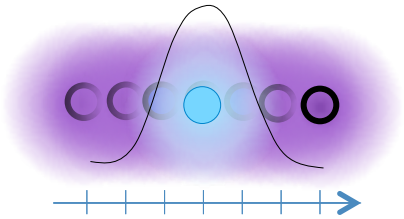
The 1 Airy Unit (AU) Pinhole Setting as a Standard



Conventional Scanning Confocal

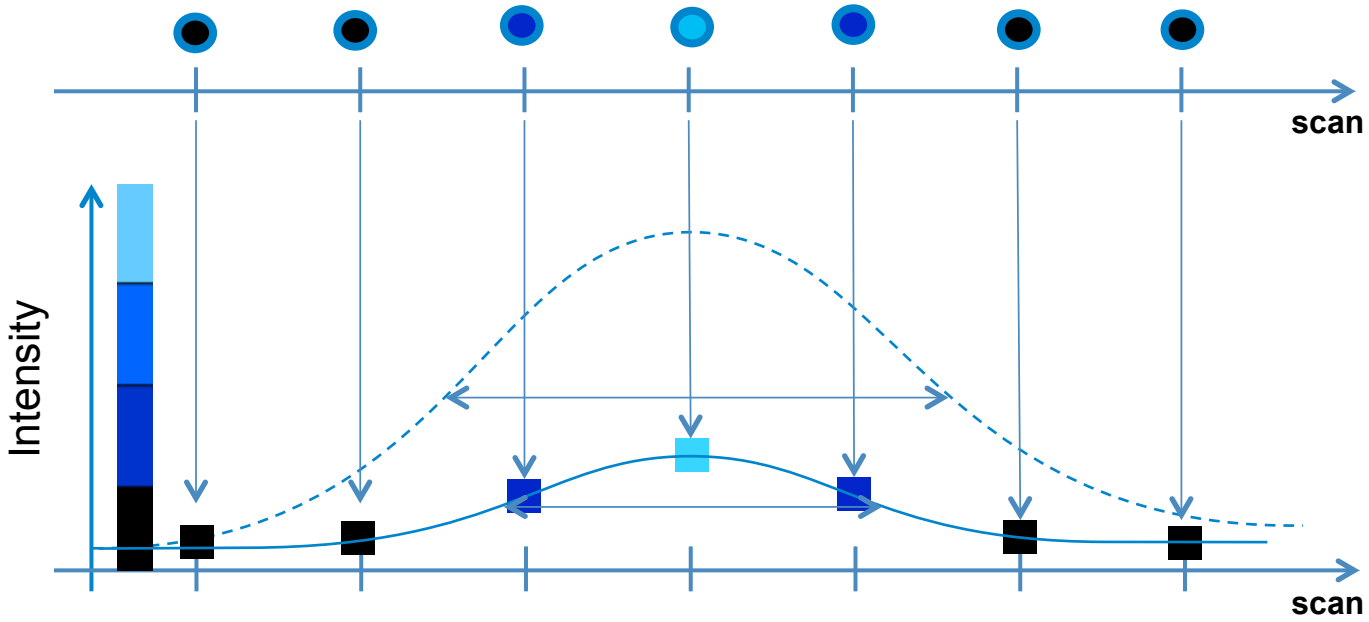
Smaller Pinholes Can Improve Resolution, But…





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

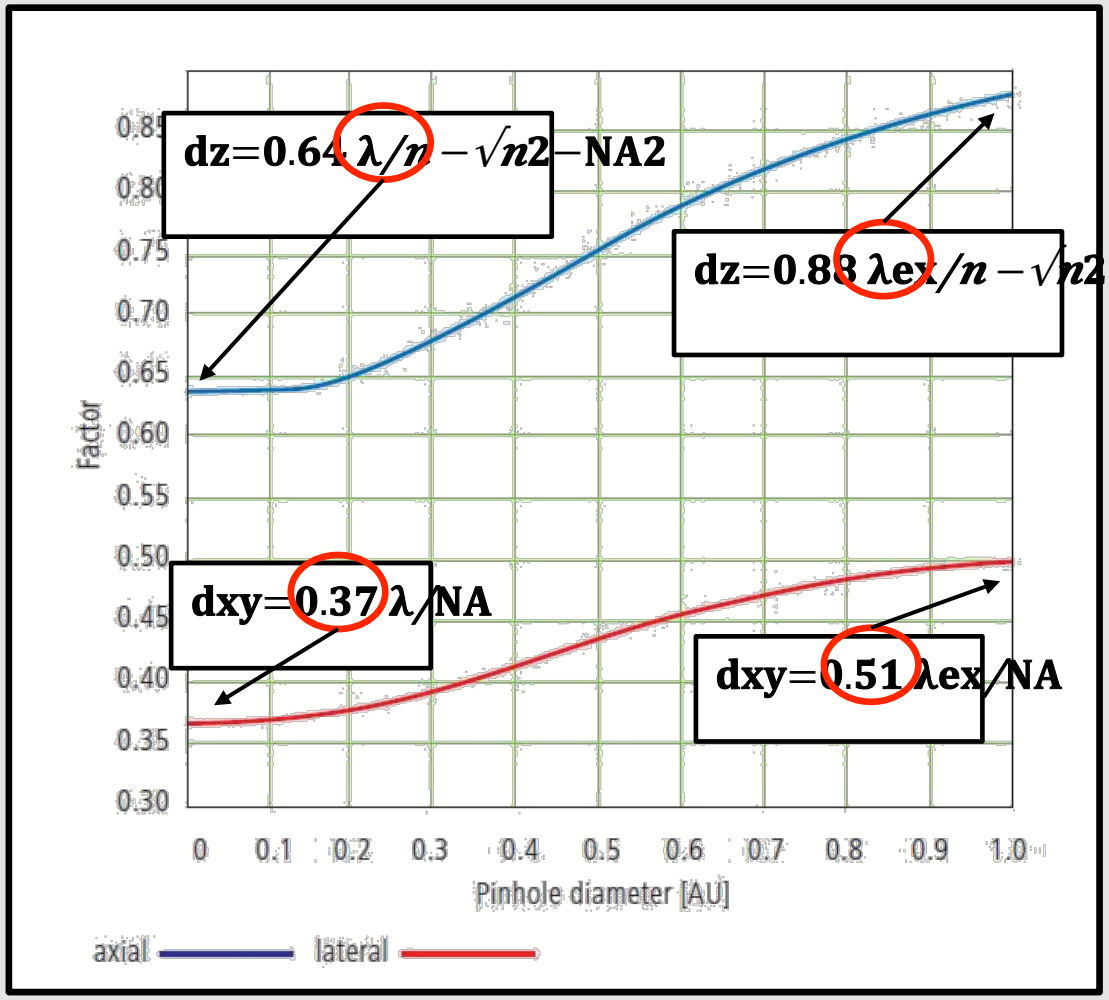
With a conventional detector, the PSF is weaker but *narrower*



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**



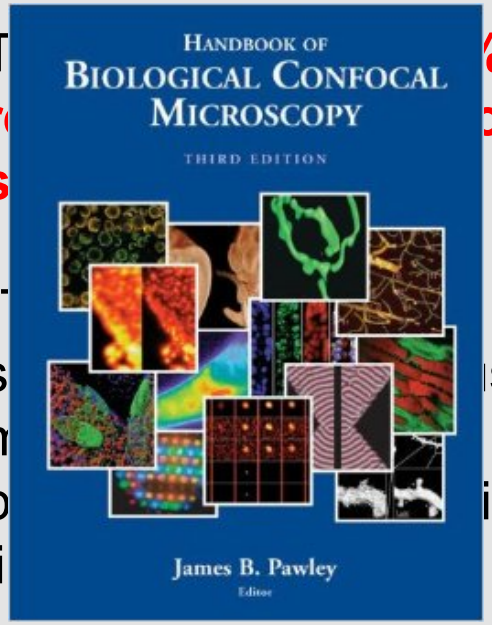
Conventional Scanning Confocal

At <<1 AU, Signal Loss Dominates Resolution Gain

However, there is a second constraint on the choice of pinhole size. Because almost all of the light originating from the plane of focus will pass through a properly-aligned pinhole, 1 Airy unit in size, one might expect that there could be no reason for ever wanting to use any other aperture size. This might be the case if the diameter of the pinhole did not also affect the spatial resolution of the microscope in both the *xy*-plane and, to a lesser extent, in *z*. If the pinhole is made very small (<0.1 Airy units), the *xy*-resolution of the instrument is improved by ~40% over that set by the Abbe limit, but only at the cost of reducing the signal level by 95%. As the pinhole is made larger, it begins to accept more light while the *xy*-resolution is reduced. When it equals 1 Airy unit, 80% of the light originating from the focus plane is accepted, while a 10% resolution gain is still being realized. On the other hand, when the pinhole is opened still more, any extra light that it accepts must be that originating from either above or below the focus plane, and this reduces the optical sectioning effect as well as providing more photons.

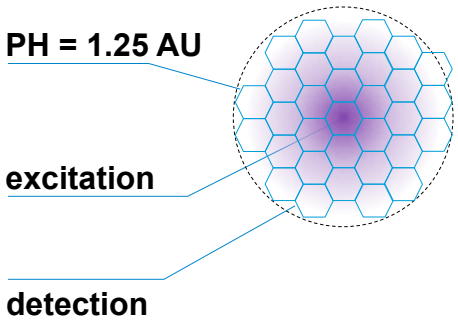
(J. Pawley, *Handbook of Biol. Confocal Microscopy*, 1995)

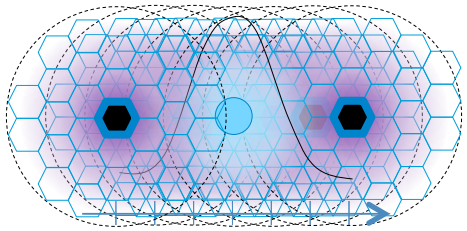
- The potential to increase resolution by simply closing the pinhole is **not** a new insight
- T… % r… on s…
- (T… s… s… m… b… ical li…



Airyscan: ~0.2 AU Scanning, No Loss

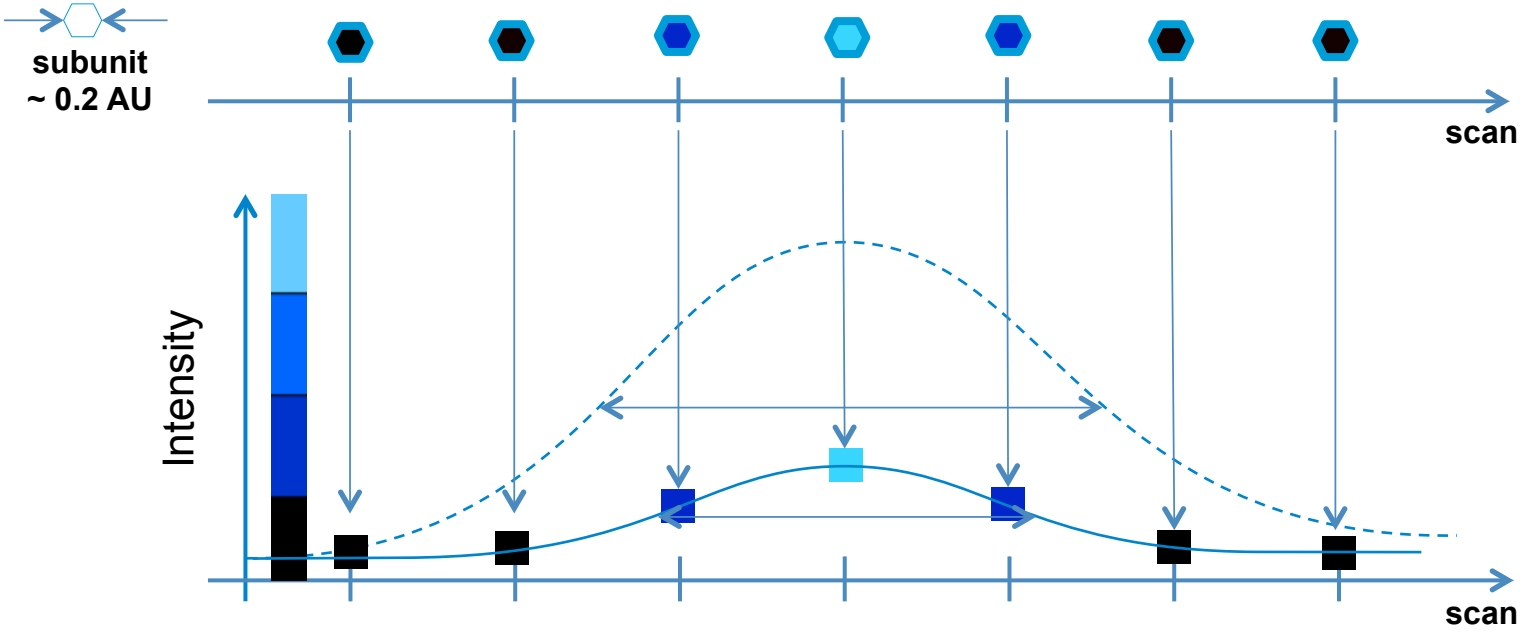
A Single Element Improves Resolution





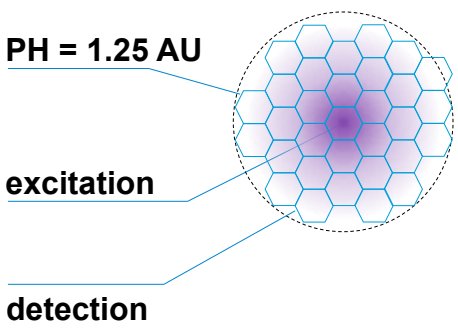
A point-like emitter generates a diffraction limited pattern (~ PSF)

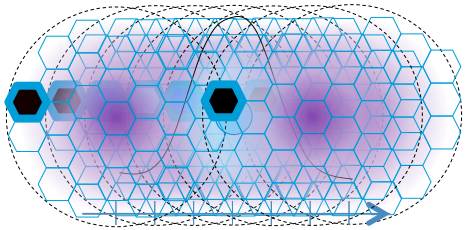
By collecting just the central element, the PSF is weaker but *narrower*



Airyscan: ~0.2 AU Scanning, No Loss

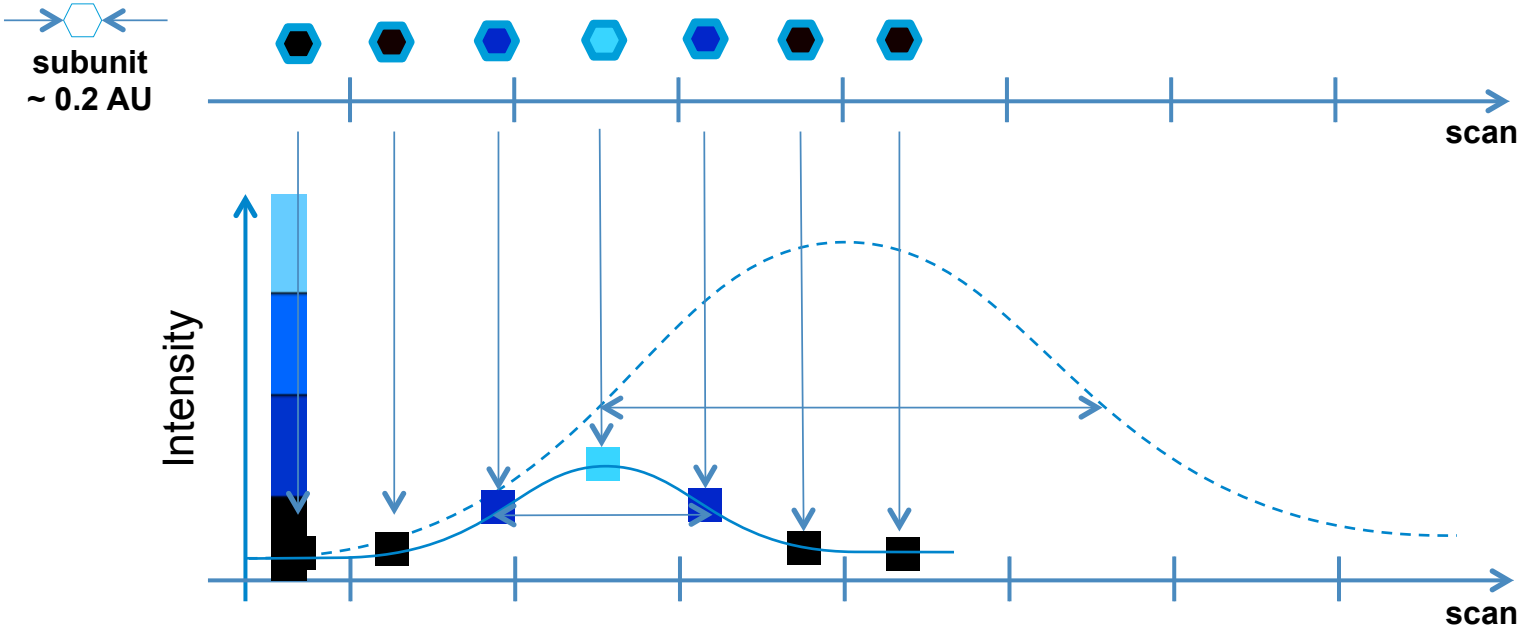
An Offset Element Further Improves Resolution





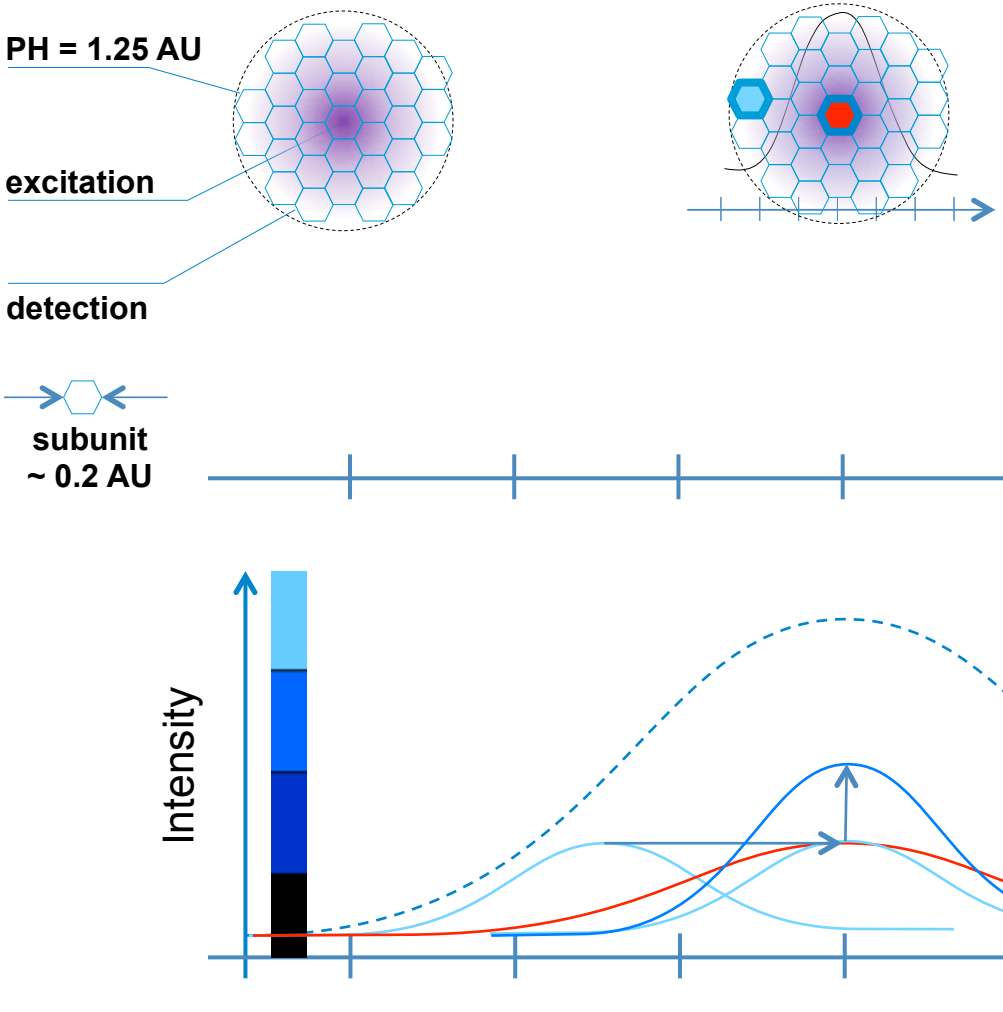
A point-like emitter generates a diffraction limited pattern (~ PSF)

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



Airyscan: ~0.2 AU Scanning, No Loss

Combining the Data

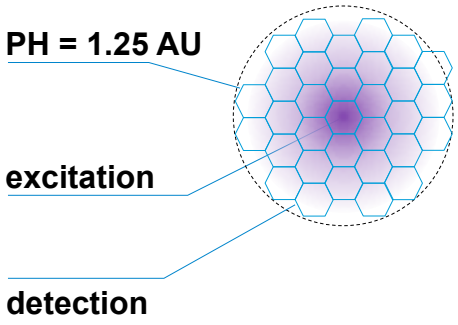


An Airyscan image is formed by:

1. **Reassigning the offset signal**
2. **Summing the contributions**

Airyscan: ~0.2 AU Scanning, No Loss

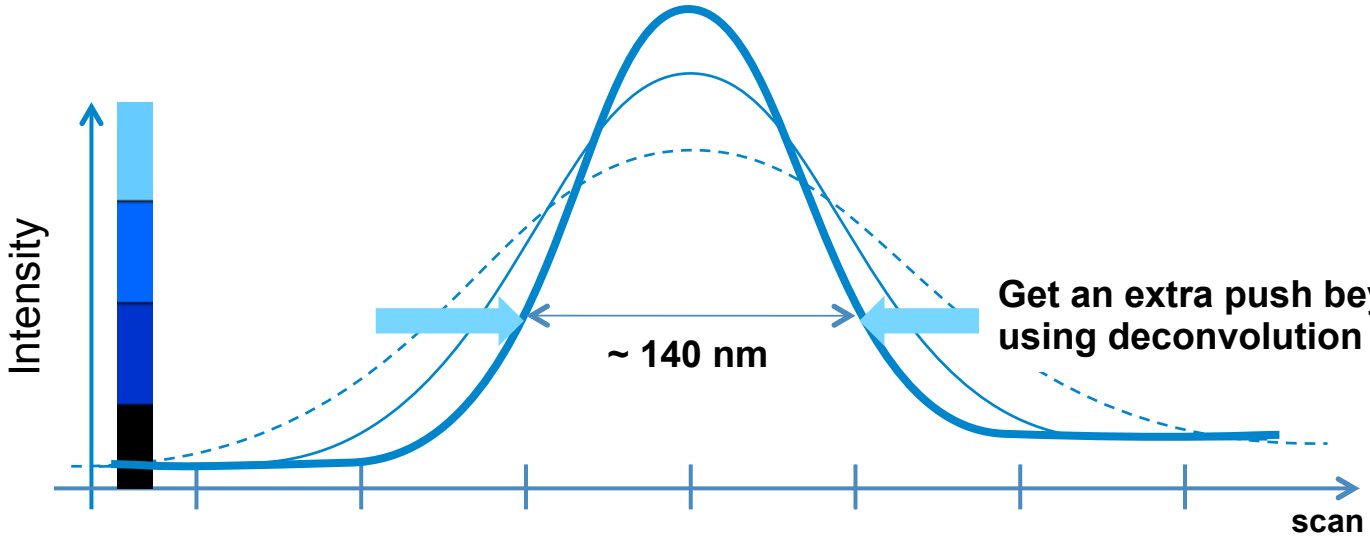
Simultaneous Mapping of 32 Elements



subunit
~ 0.2 AU

All elements are acquired simultaneously, and can be remapped for better resolution and sensitivity

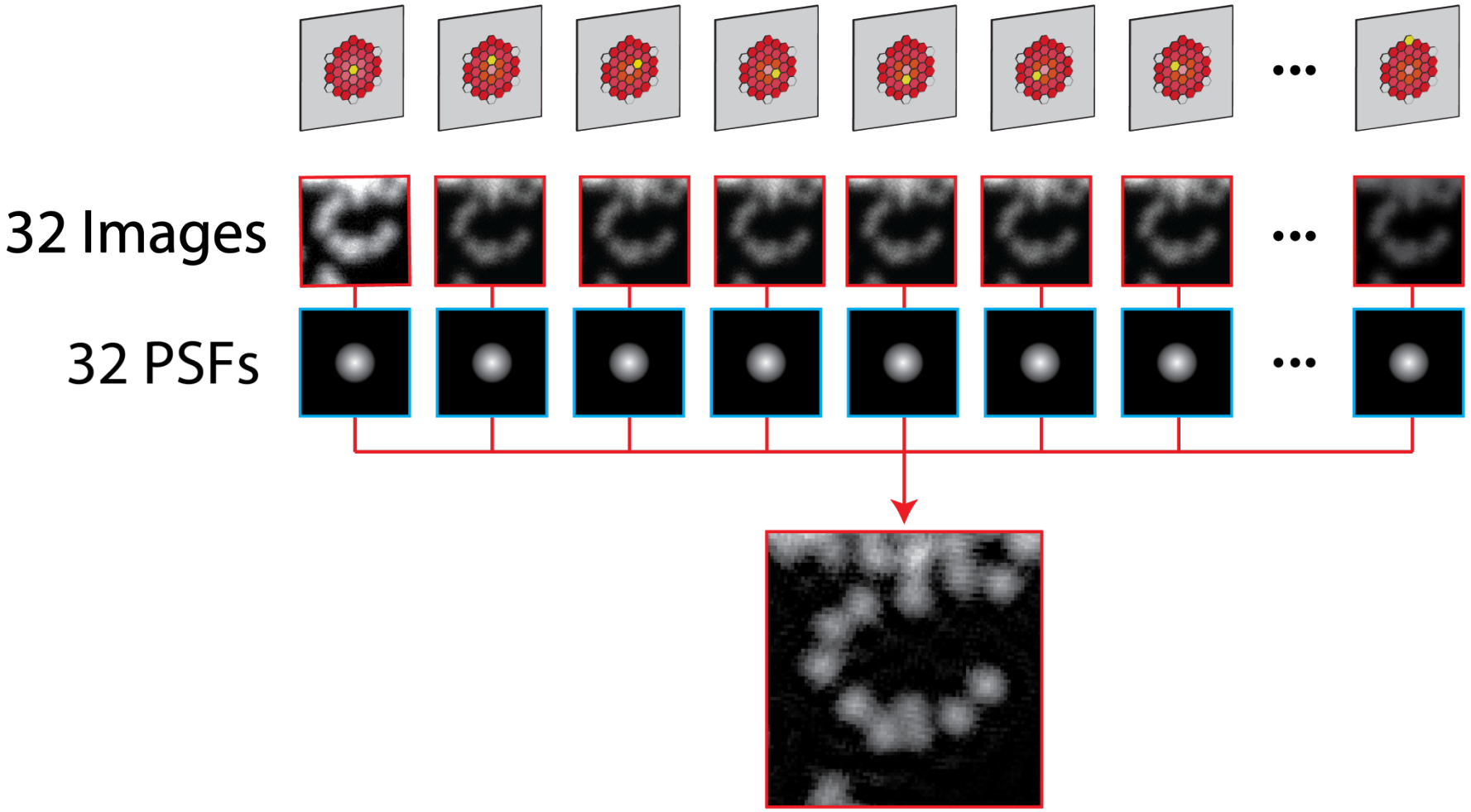
The PSF is mapped directly √2x



Get an extra push beyond the limit using deconvolution (down to 1.7x)

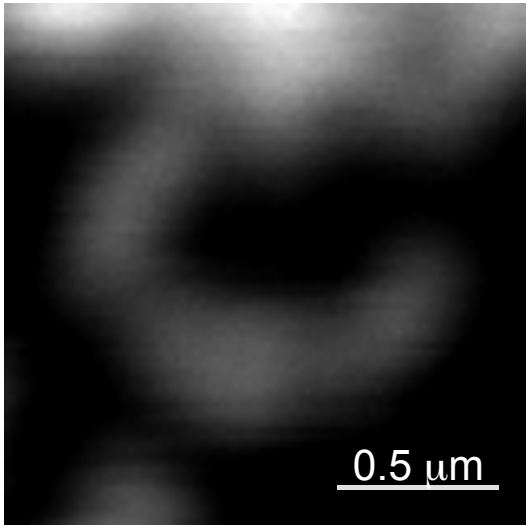
Airyscan Processing

Isotropic 1.7x Resolution Improvement



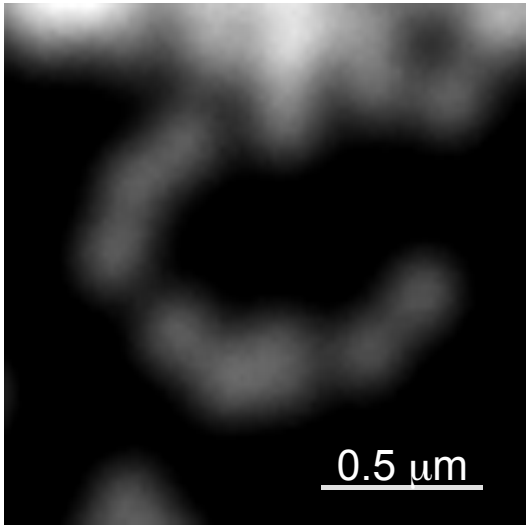
Airyscan Processing
Detector-Wise Deconvolution

170 nm fluorescent beads

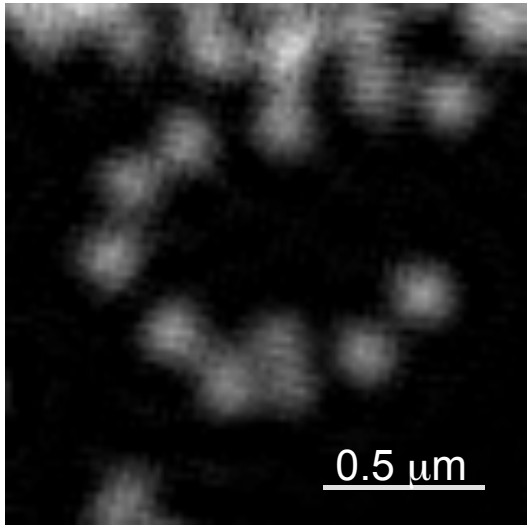


Confocal microscope
Plan-Apochromat 63x/1.4
633nm illumination

Approx. resolution: 260 nm



Pixel reassignment
1.4x improved resolution



Airyscan processing
1.7x improved resolution

Advanced Concepts of the Airyscan

Similar Principles and Innovations

Sheppard, C.J., Super-resolution in confocal imaging. Optik, 1988. 80(2): p. 53-54

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

Muller, C.B. and J. Enderlein, Image scanning microscopy. Phys Rev Lett, 2010. 104(19): p. 198101

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]

Sheppard, C.J., S.B. Mehta, and R. Heintzmann, Superresolution by image scanning microscopy using pixel reassignment. Opt Lett, 2013. 38(15): p. 2889-2892

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)

York, A.G., et al., Resolution doubling in live, multicellular organisms via multifocal structured illumination microscopy. Nat Methods, 2012. 9(7): p. 749-754

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)

Roth, S., Sheppard, C.J., Wicker, K., and R. Heintzmann, Optical photon reassignment microscopy (OPRA). Optical Nanoscopy, 2013. 2(5): p. 1-6

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

York, A.G., et al., Instant super-resolution imaging in live cells and embryos via analog image processing. Nat Methods, 2013. 10(11): p. 1122-1126

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

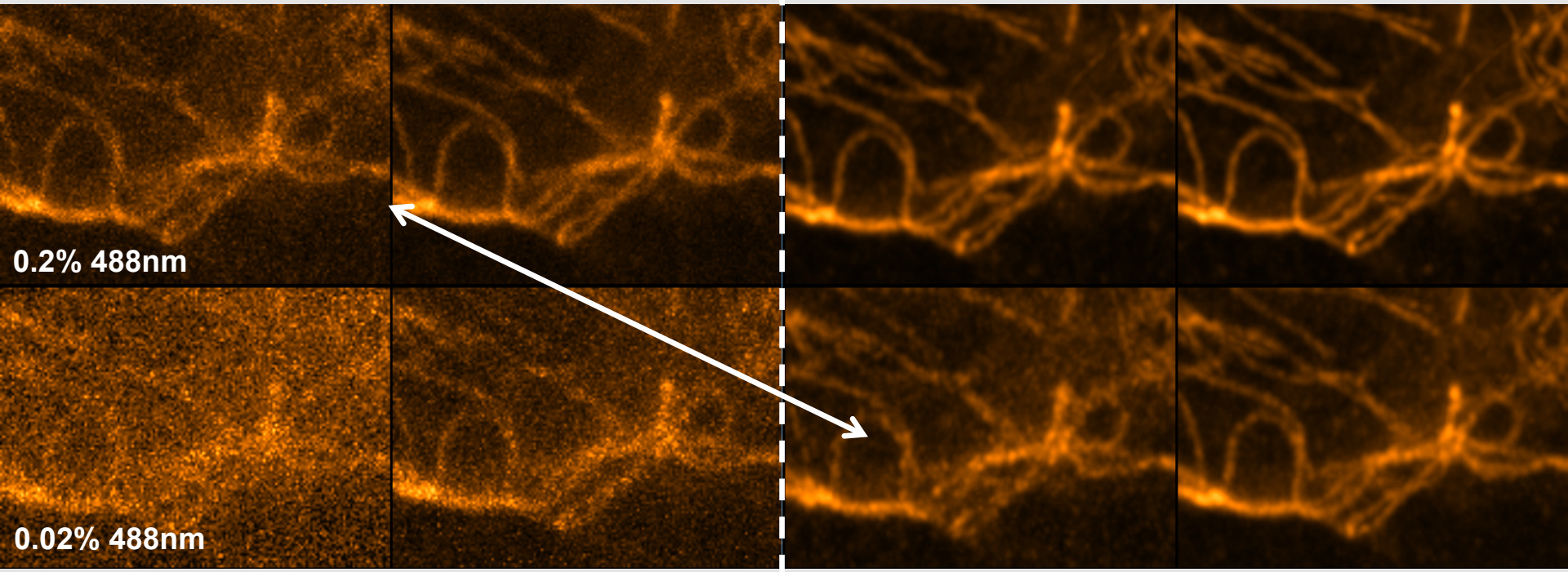
Comparing the Airyscan
Resolution with Other Techniques

Feature	Airyscan	SIM	PALM/STORM	STED
Resolution (X-Y, Z)[1]	140 nm, 400 nm	120 nm, 350 nm	20 nm, 50 nm	60 nm, 120 nm
Fluor choice[2]	●●●●●	●●●	●●	●●
Objective choice[3]	●●●●	●●	●●	●●
Sample thickness[4]	~100 microns	10-20 microns	5-10 microns	10-20 microns
Sample prep[5]	●●●●●	●●●●●	●●●	●●●
Live-cell imaging[6]	●●●●	●●	●	●●
2-Photon capable	●●●●	●	●	●

1. **Typical/reported values for GFP with 63x/1.4 NA objective**
2. **Works with all fluorophores between 400-700 nm**
3. **Compatible with a wide variety of objectives**
4. **Works on any sample that can be imaged with a confocal microscope**
5. **Standard sample preparation protocol (no special buffers or reagents required)**
6. **Supports gentle imaging of live-cells for extended periods of time**

LSM 880 + Airyscan

Signal-to-Noise Comparison

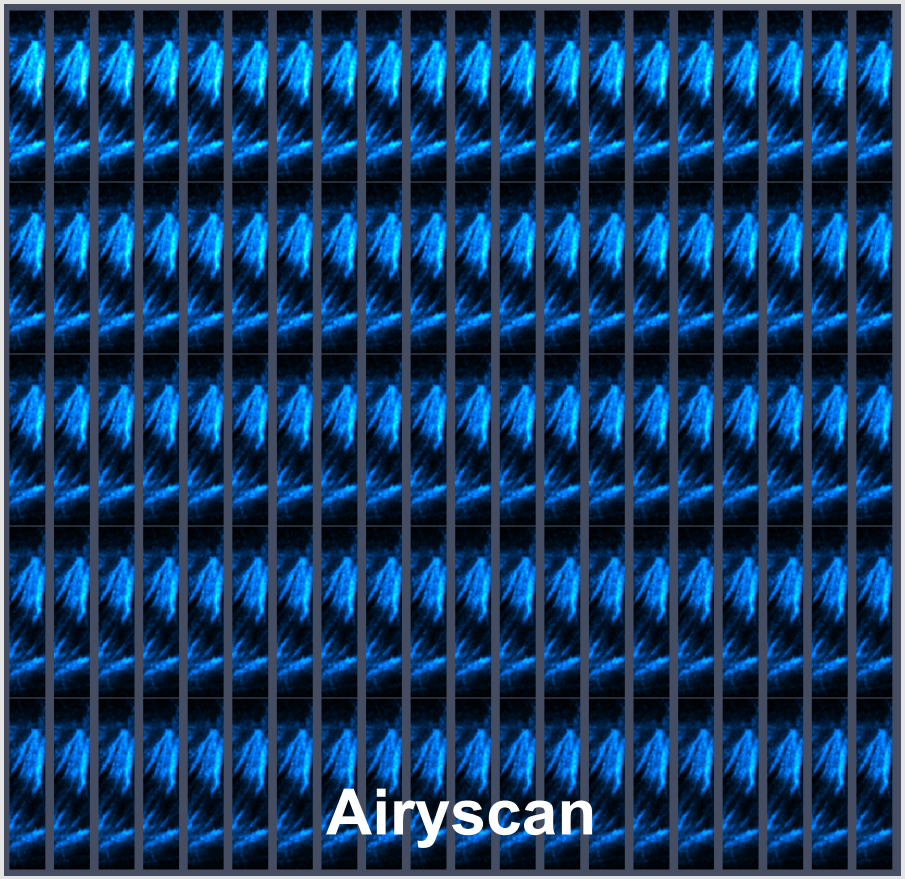


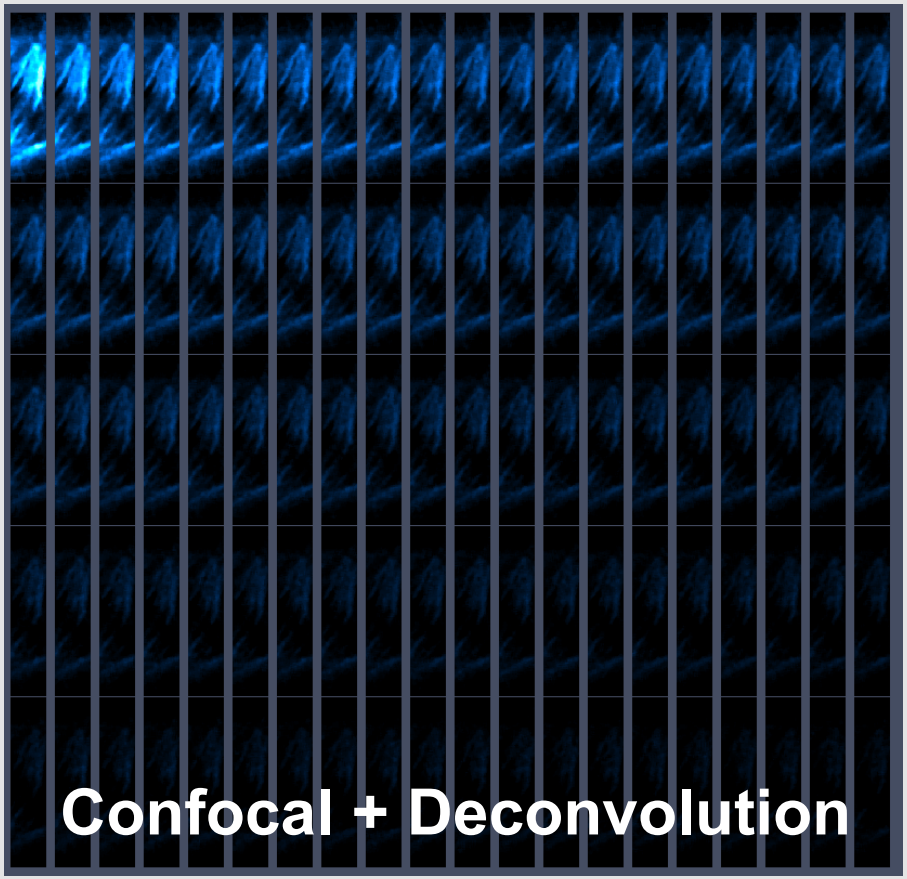
- Same sample: stable, hard to bleach
- Identical imaging parameters beyond than these stated above
- All images scaled with best fit display settings (0.4% top and bottom)

Efficiency of the Airyscan

Comparisons to Confocal + Deconvolution Only

In order to obtain the same result, the confocal imaging conditions are relatively harsh:



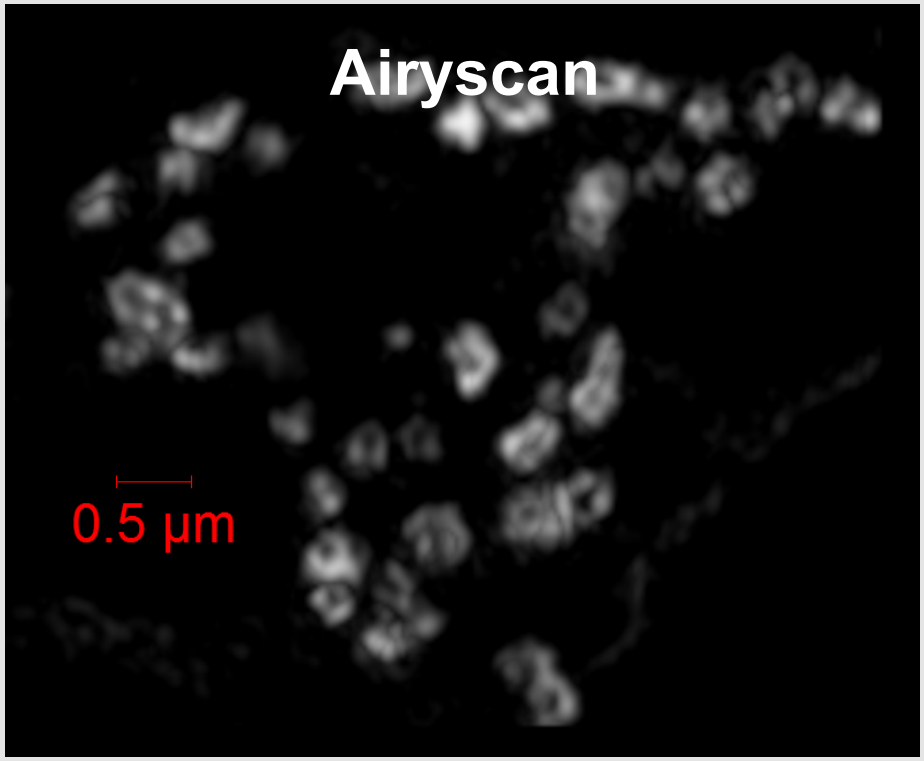


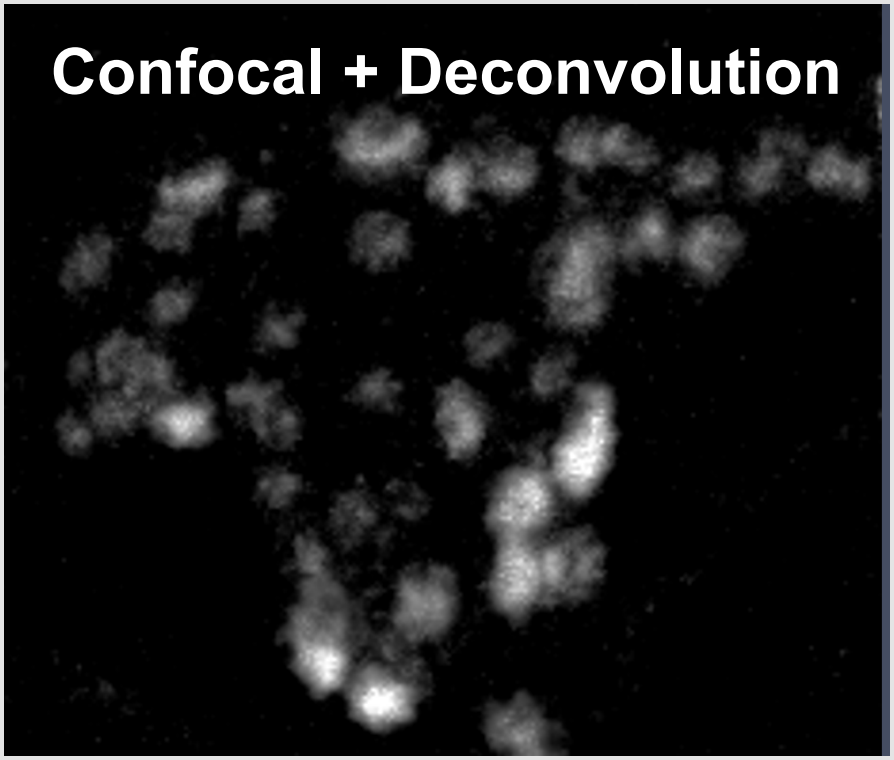
The lower efficiency of a confocal (0.3 AU) + DCV strategy yields **very apparent bleaching**

Efficiency of the Airyscan

Comparisons to Confocal + Deconvolution Only

If the source signal is dim, the light-limited aspects of deconvolution alone renders it highly prone to a number of **image processing artifacts**:

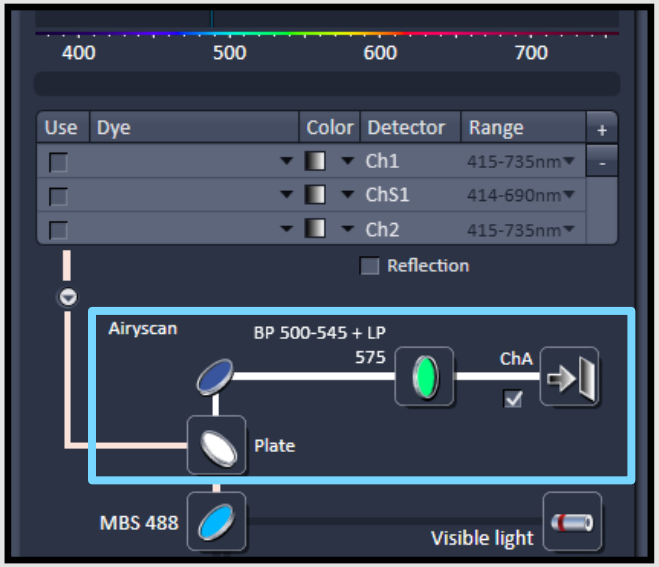


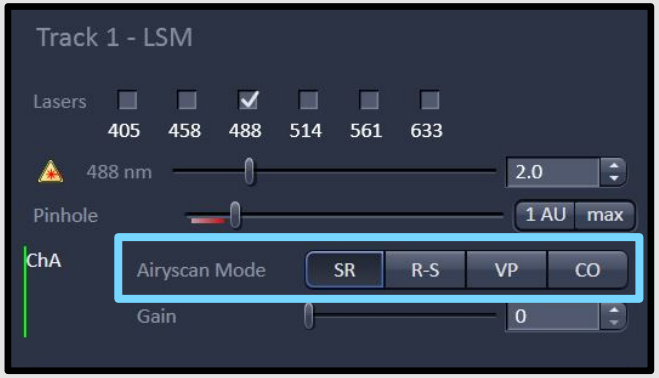


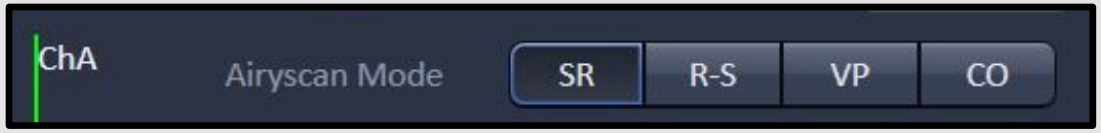
Neuromuscular junctions, Jan Pielage (FMI, Zürich)

Airyscan: Software Interface

Ease-of-Use, Multiple Collection Modes







SR (Superresolution Mode)

- Uses area detector to produce effectively small pinholes; 140 nm res. in XY, 400 nm in Z

R-S (Sensitivity Mode)

- Detector fits slightly larger Airy pattern (2 AU) to rapidly boost signal-to-noise over resolution

VP (Virtual Pinhole Mode)

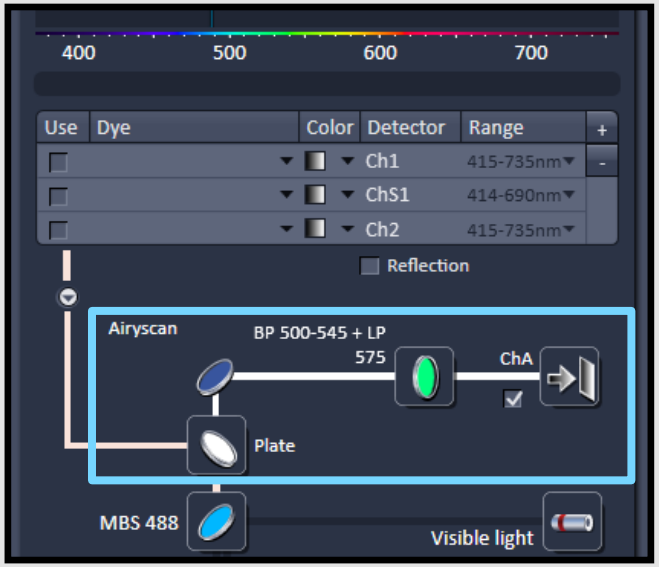
- Detector fits much larger Airy pattern (> 3 AU) to permit adjustment of pinhole post-hoc

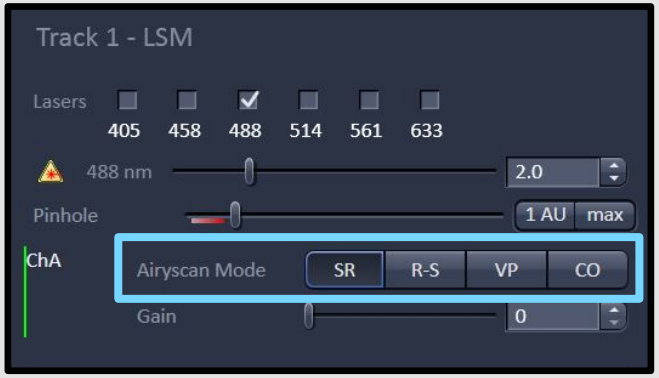
CO (Confocal Mode)

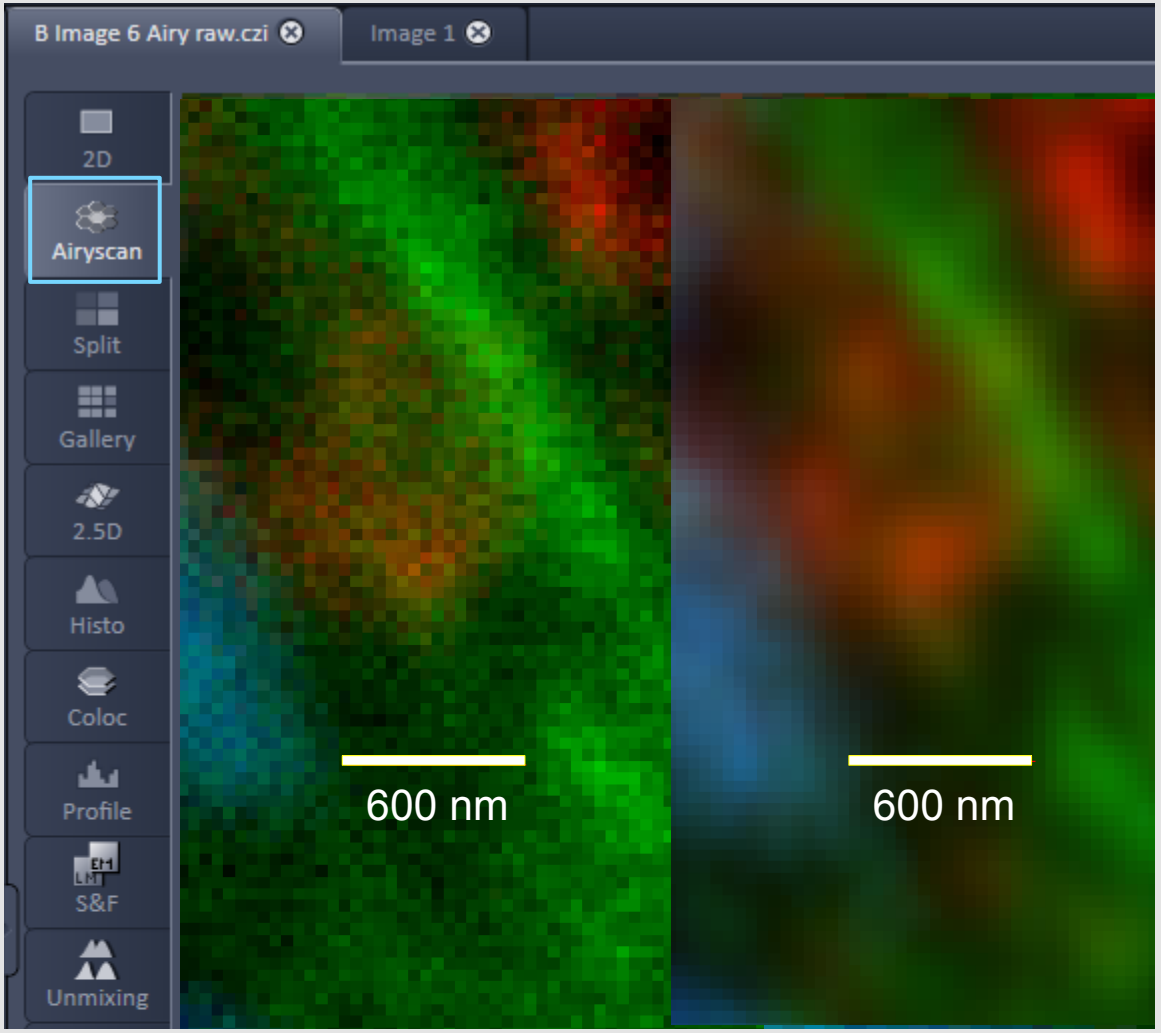
- Uses the sum total signal from the area detector; serves as an extra channel

Airyscan: Software Interface

Ease-of-Use, Multiple Collection Modes



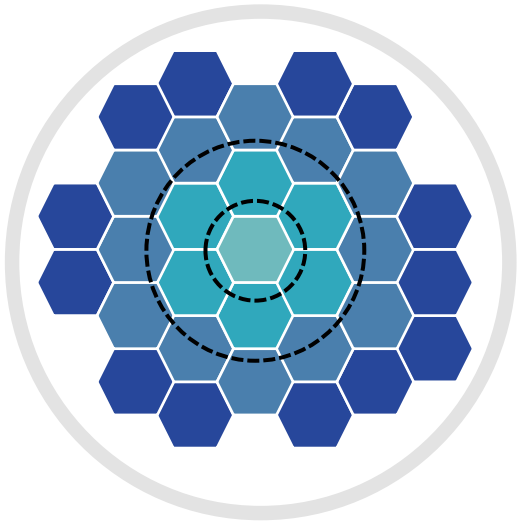




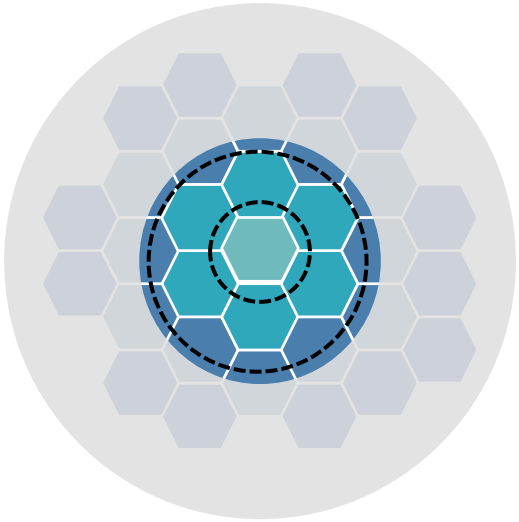
Airyscan: Virtual Pinhole Mode

Optimization of Slice Thickness

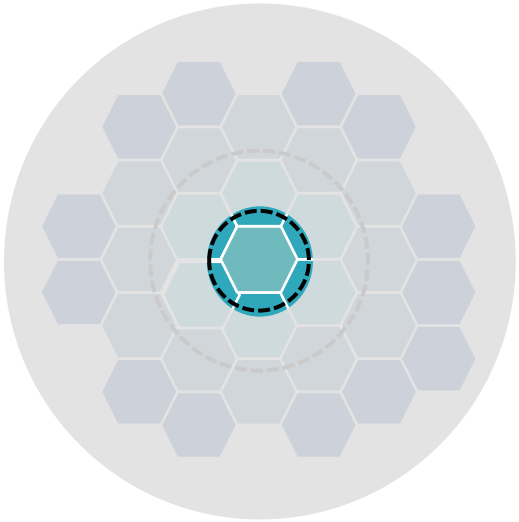
- A virtual pinhole can be applied after imaging to display more or less of the captured Airy pattern



Large Virtual Pinhole



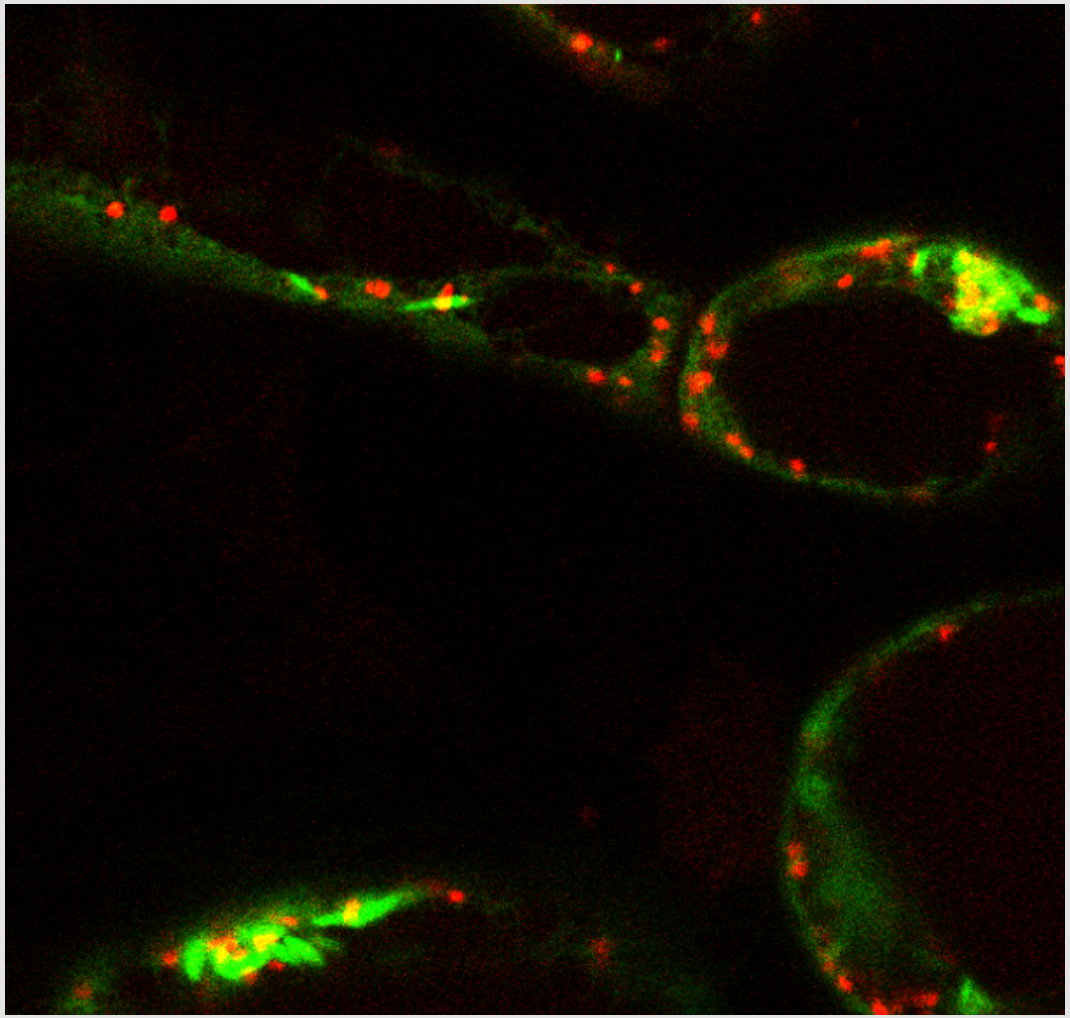
Medium Virtual Pinhole

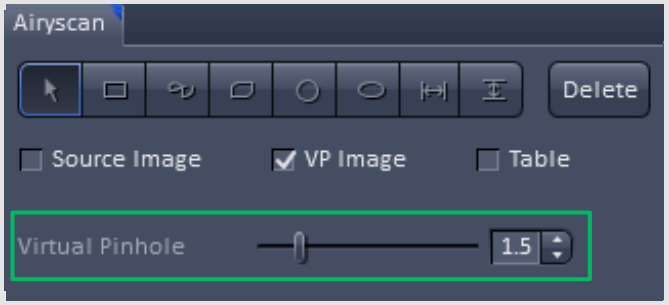


Small Virtual Pinhole

Airyscan: Virtual Pinhole Mode

Optimization of Slice Thickness

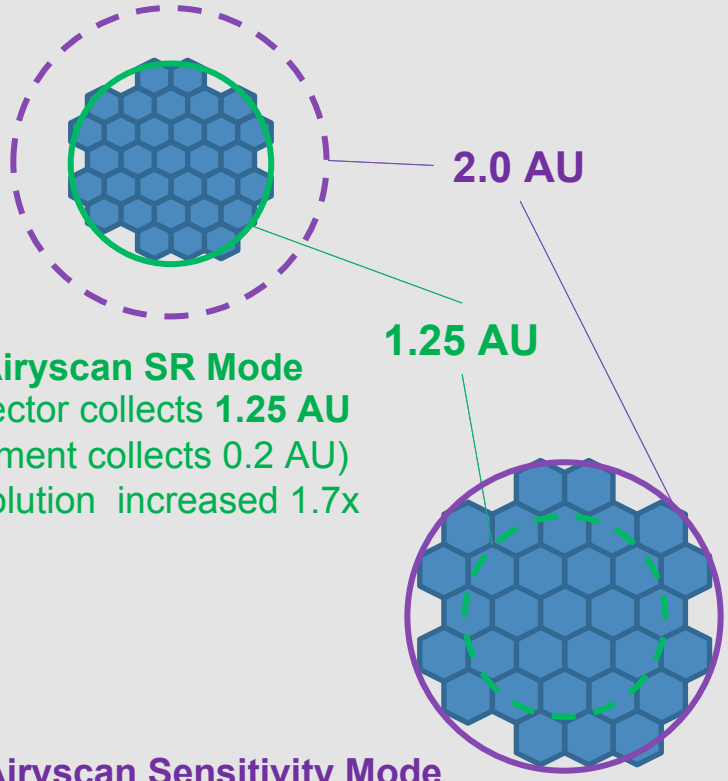




- Different virtual pinhole settings are selectable after imaging via simple software slider

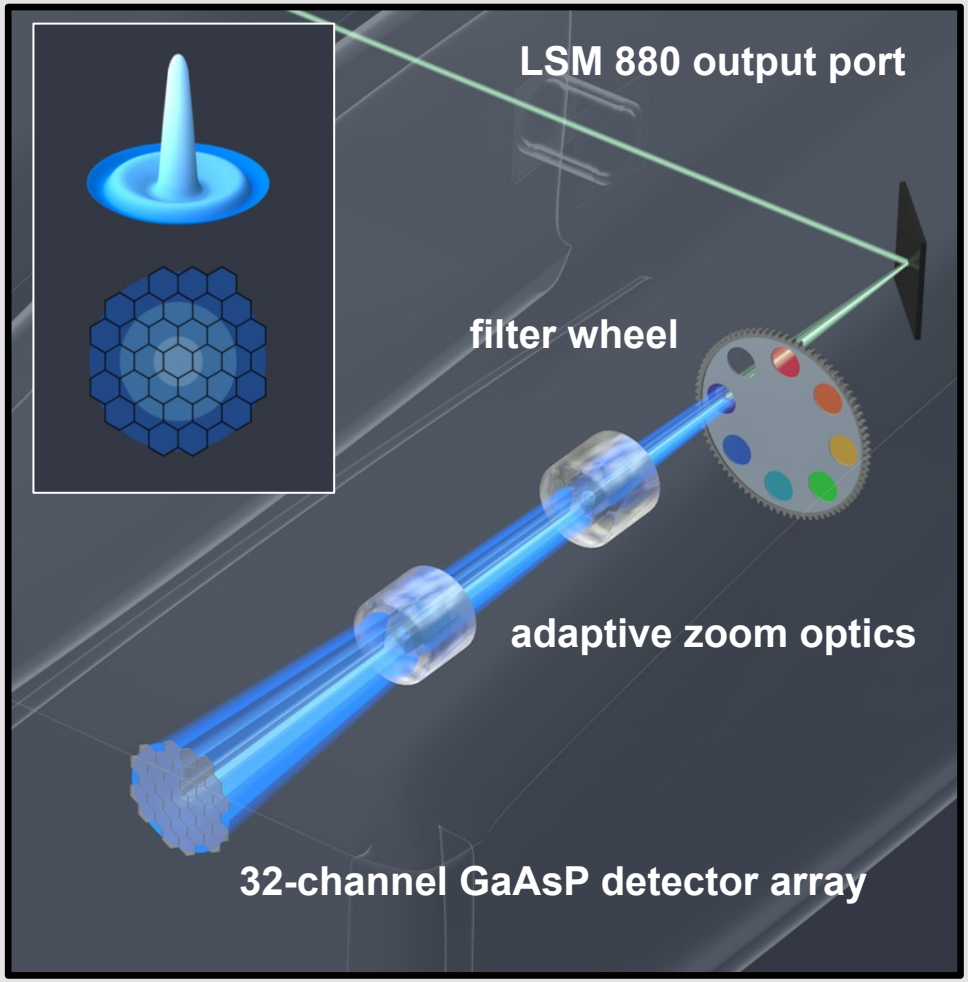
Airyscan: Sensitivity Mode

Finding Best Balance of Resolution / Sensitivity



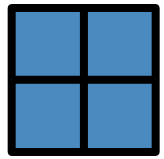
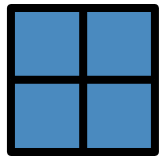
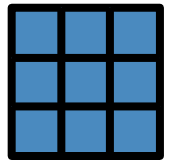
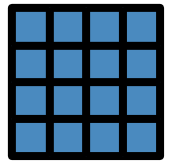
Airyscan SR Mode
Detector collects **1.25 AU**
(Element collects 0.2 AU)
Resolution increased 1.7x

Airyscan Sensitivity Mode
Detector collects **2.0 AU**
(Element collects 0.3 AU)
Resolution ~1.2-1.6x increased
depending on pixel sampling



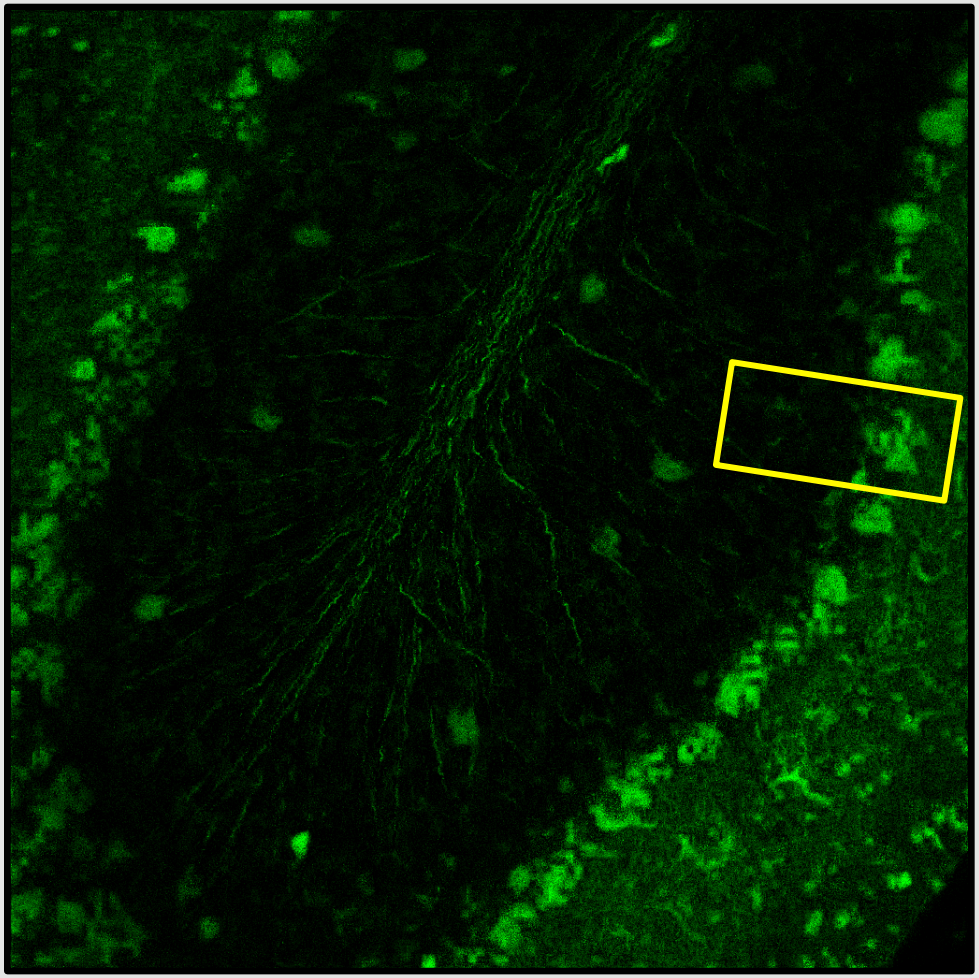
Airyscan: Sensitivity Mode

Finding Best Balance of Resolution / Sensitivity

Detector: Pixel Count	Resolution Improvement Factor	SNR Gain vs Confocal GaAsP @ 1 AU	Relative Acquisition Time Increase	
Confocal GaAsP: Nyquist	1x	1x	1x (2D) 1x (3D)	
Airyscan Sensitivity Mode: Nyquist	1x	4-8x	1x (2D) 1x (3D)	
Airyscan Sensitivity Mode: 1.5x Nyquist	1.45x	4-8x	2.27x (2D) 3.33x (3D)	
Airyscan Resolution Mode: 2x Nyquist	1.7x	4-8x	4x (2D) 8x (3D)	

Airyscan: Sensitivity Mode

Mode Comparisons with 2-Photon Excitation

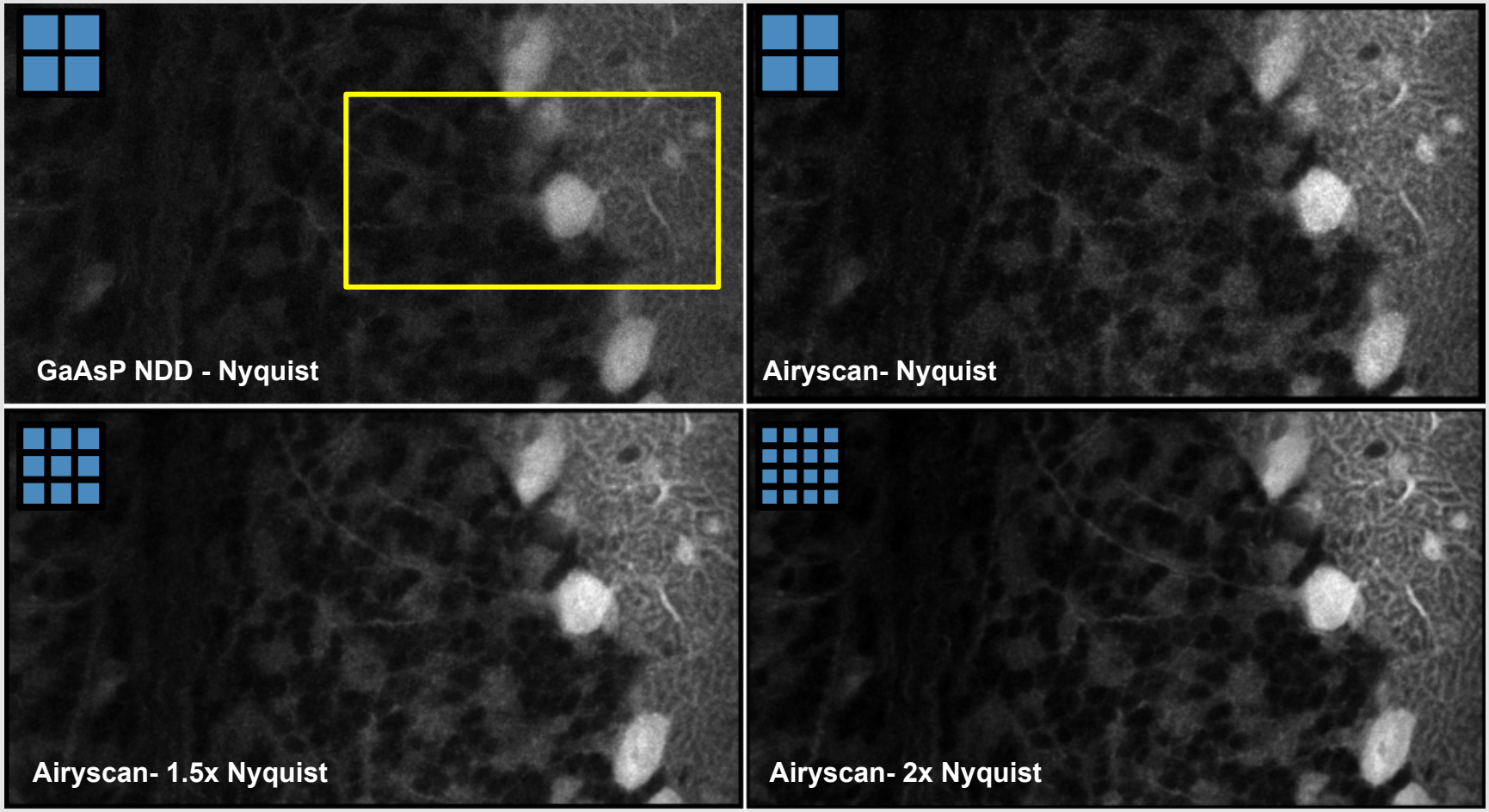


Drosophila brain section, eGFP in motor neurons, 25x/0.8 LD LCI Plan Apo

Airyscan: Sensitivity Mode

Mode Comparisons with 2-Photon Excitation

300 microns depth with 900 nm excitation



Drosophila brain section, eGFP in motor neurons, 25x/0.8 LD LCI Plan Apo

Airyscan: Sensitivity Mode

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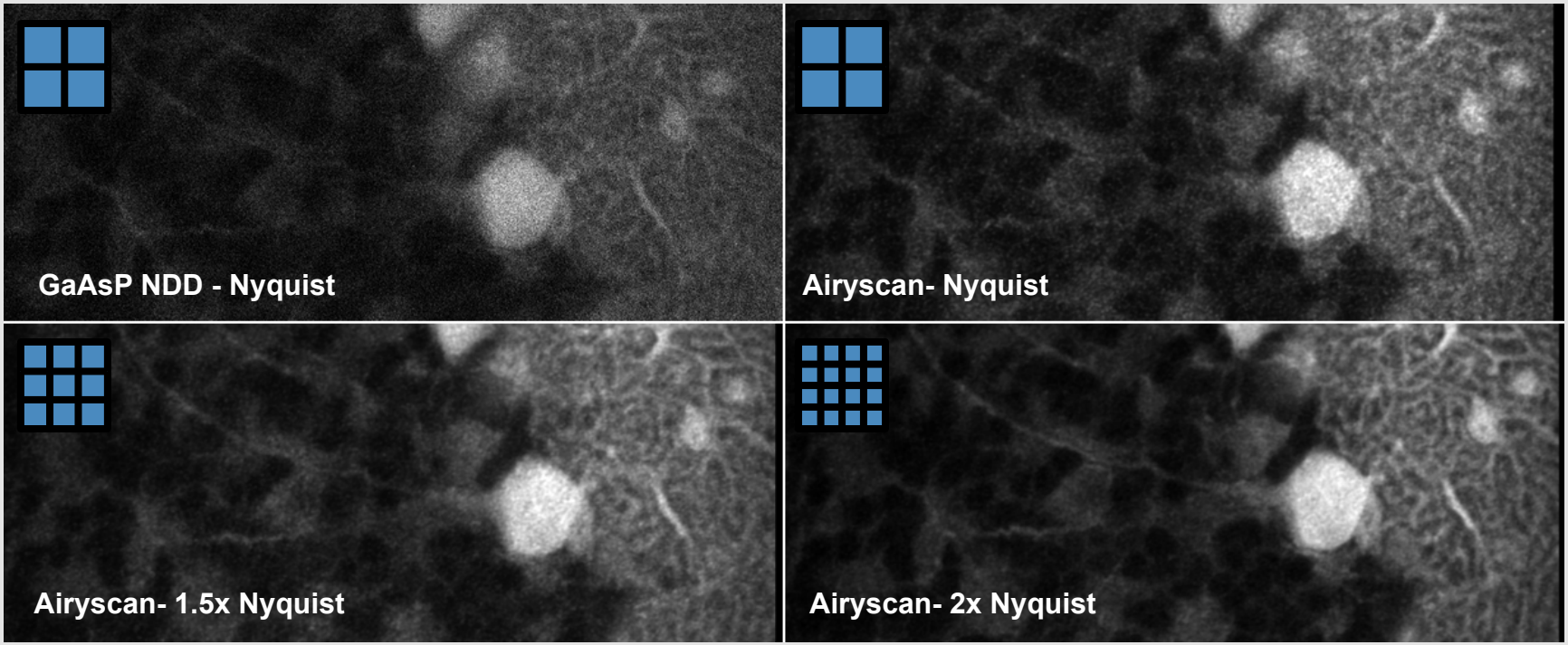

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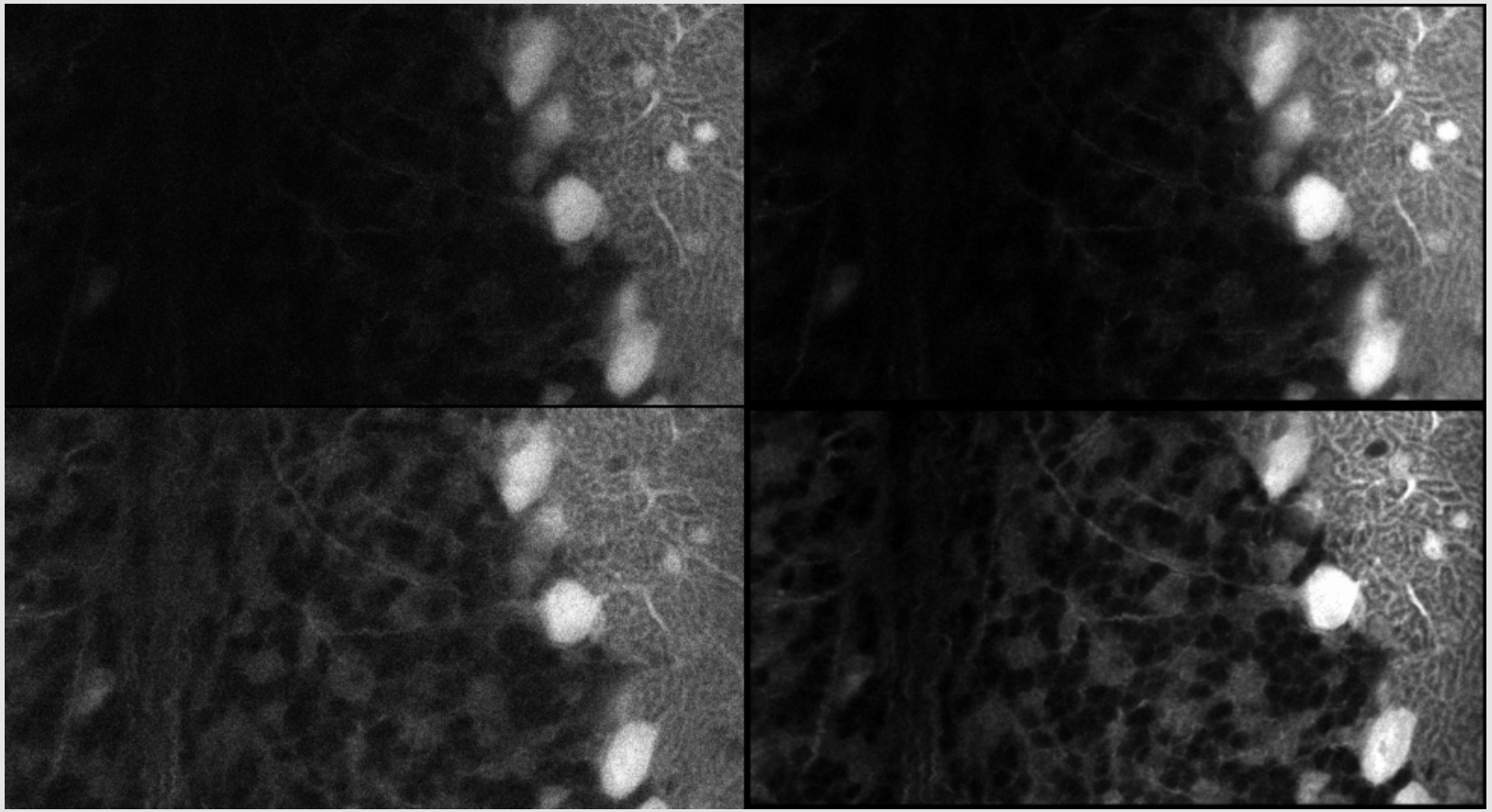
Airyscan: Sensitivity Mode

Comparing GaAsP NDD and Airyscan



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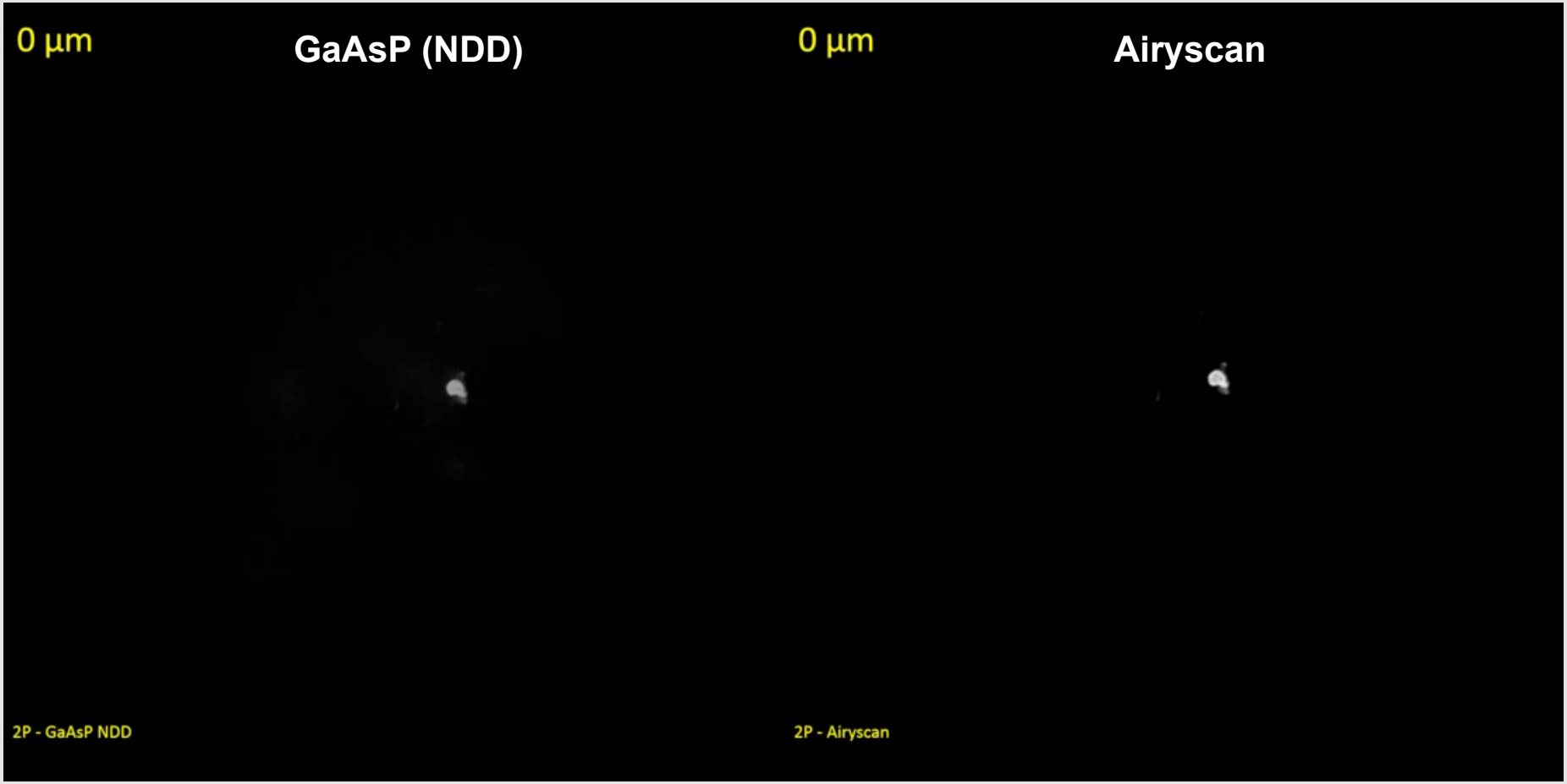
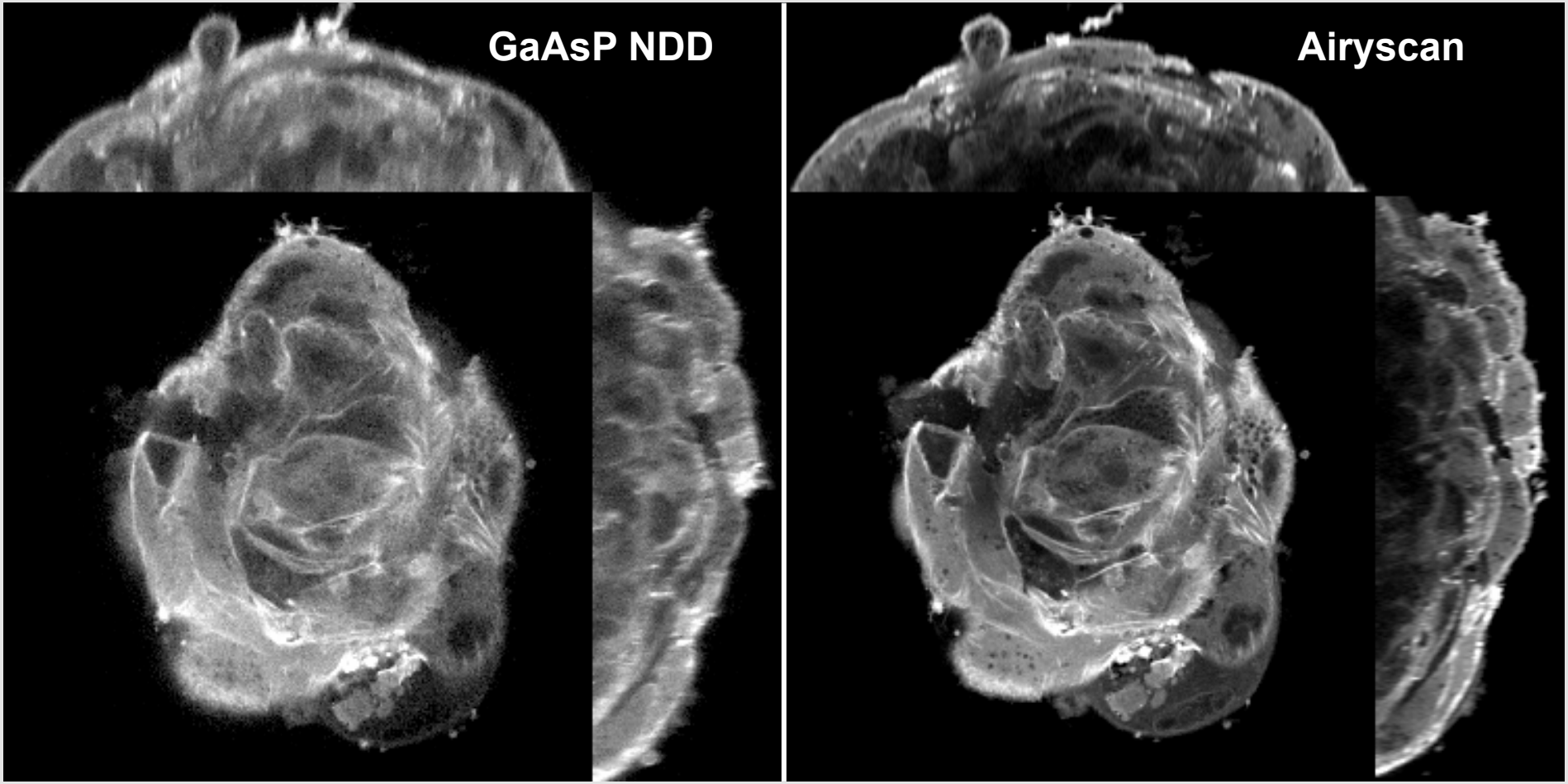


FoLu cell spheroid expressing GFP-actin, imaged with 40x/1.1 LD C-Apo, 40 um Z-stack with 900 nm excitation

Airyscan: Sensitivity Mode

Comparing GaAsP NDD and Airyscan



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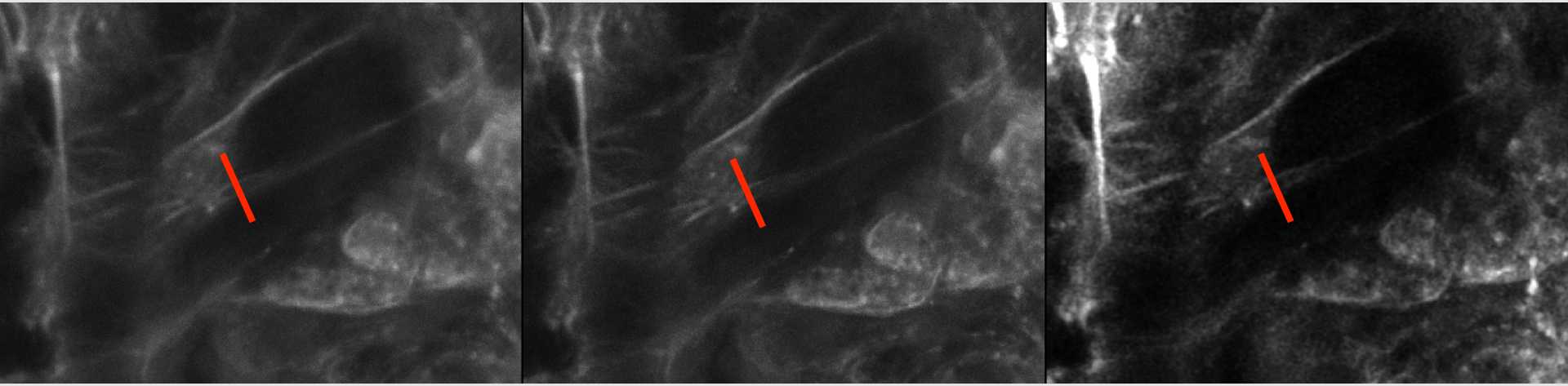
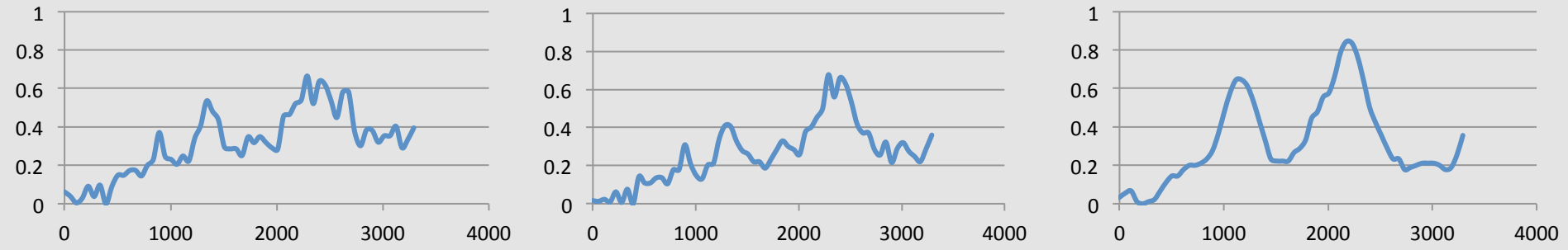
Airyscan: Sensitivity Mode

Comparing GaAsP NDD and Airyscan

GaAsP NDD | **GaAsP NDD - Decon** | **Airyscan**





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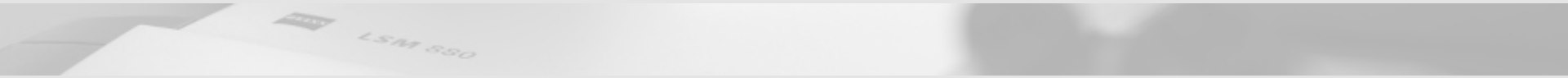
Outline of Discussion

ZEISS LSM 880 NLO + Airyscan @ WashU



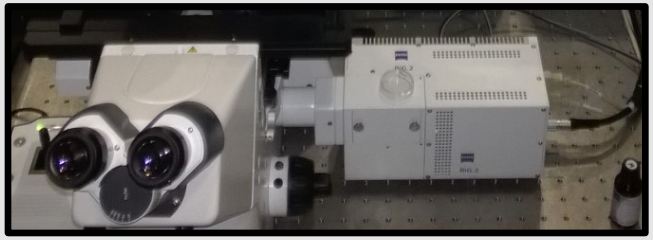
1. Existing System Overview
2. **LSM 880** Design and Considerations
3. Principles of the **Airyscan**
4. **Additional Enabling Components**
5. And ... the ApoTome!
6. Summary / Questions

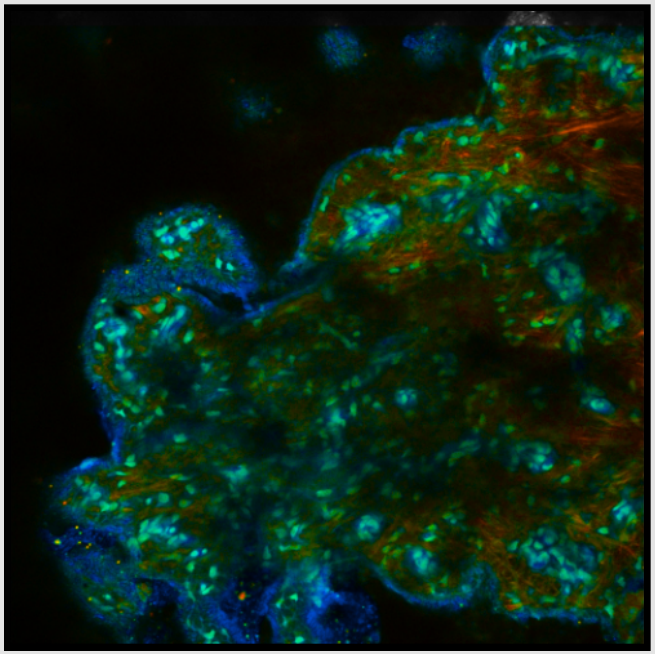
Potential System Upgrades

Fluorescence Lifetime Imaging Microscopy

- Existing NDD BiG.2 detector can be used as **IR FLIM** readout
  - Time-correlated single photon counting is used to plot **temporal** distribution of the excited state lifetime (~100s of ps)
  - Repeating counts at each scan pixel also permits **spatial** distribution of lifetimes
  - Resulting color maps can yield information about microenvironment (FRET, pH, ion concentrations, protein binding, etc)

(*Need only synchronizing electronics from PicoQuant or Becker & Hickl using SMA ports of BiG.2*)

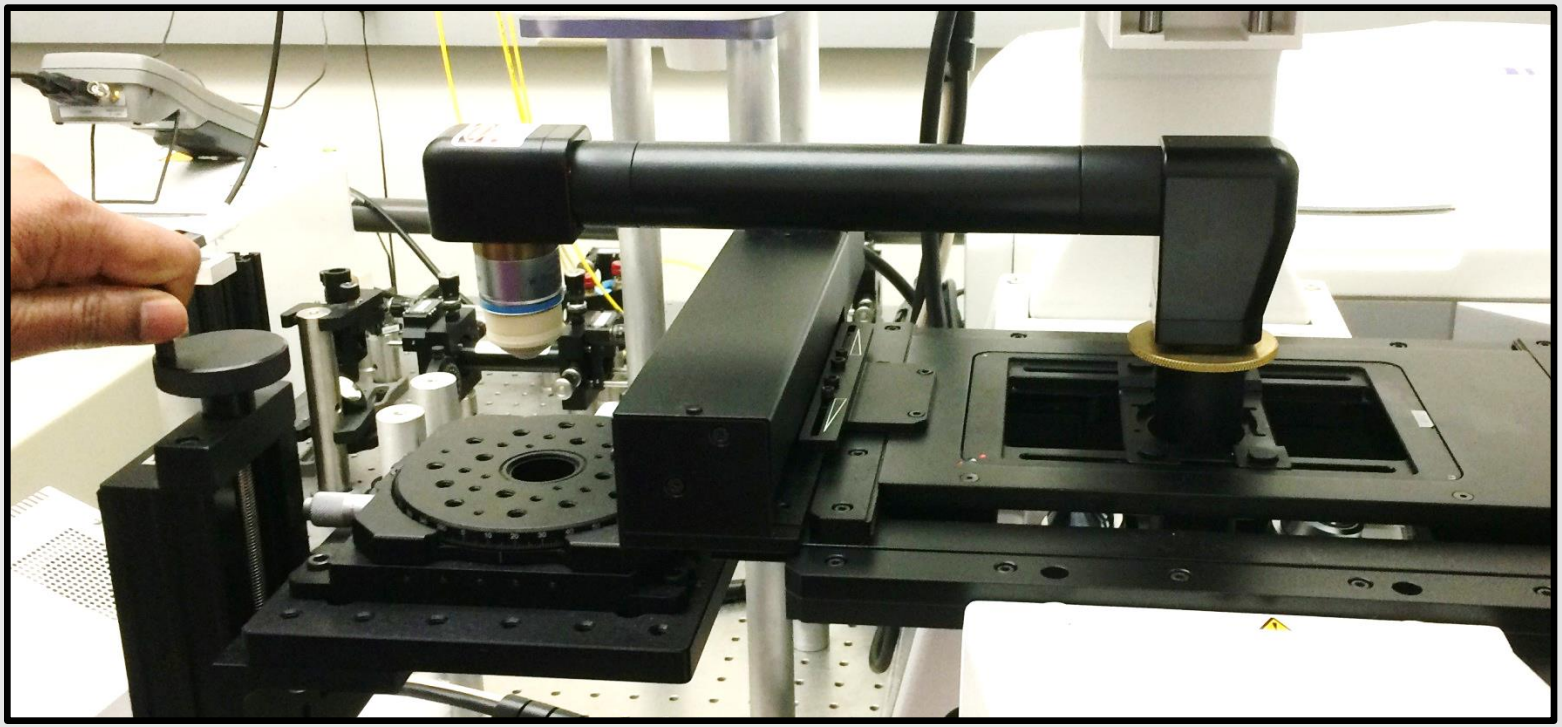




Skin tissue (pig) stained with ethylene blue; 1100 nm exciation (OPO); lifetime image

Potential System Upgrades
Objective Inverter, LD Objectives for Clearing

- Additional specialty dipping objectives with longer parfocal lengths can be utilized via an objective inverter (*LSM Tech*)
  - LD Plan-Apochromat 20x/1.0 (WD = 5.6 mm) for cleared tissues



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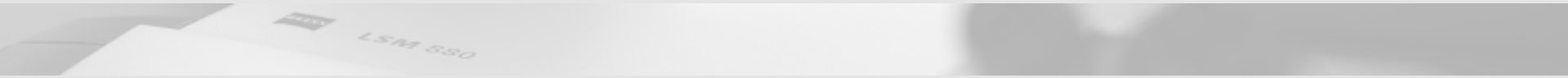

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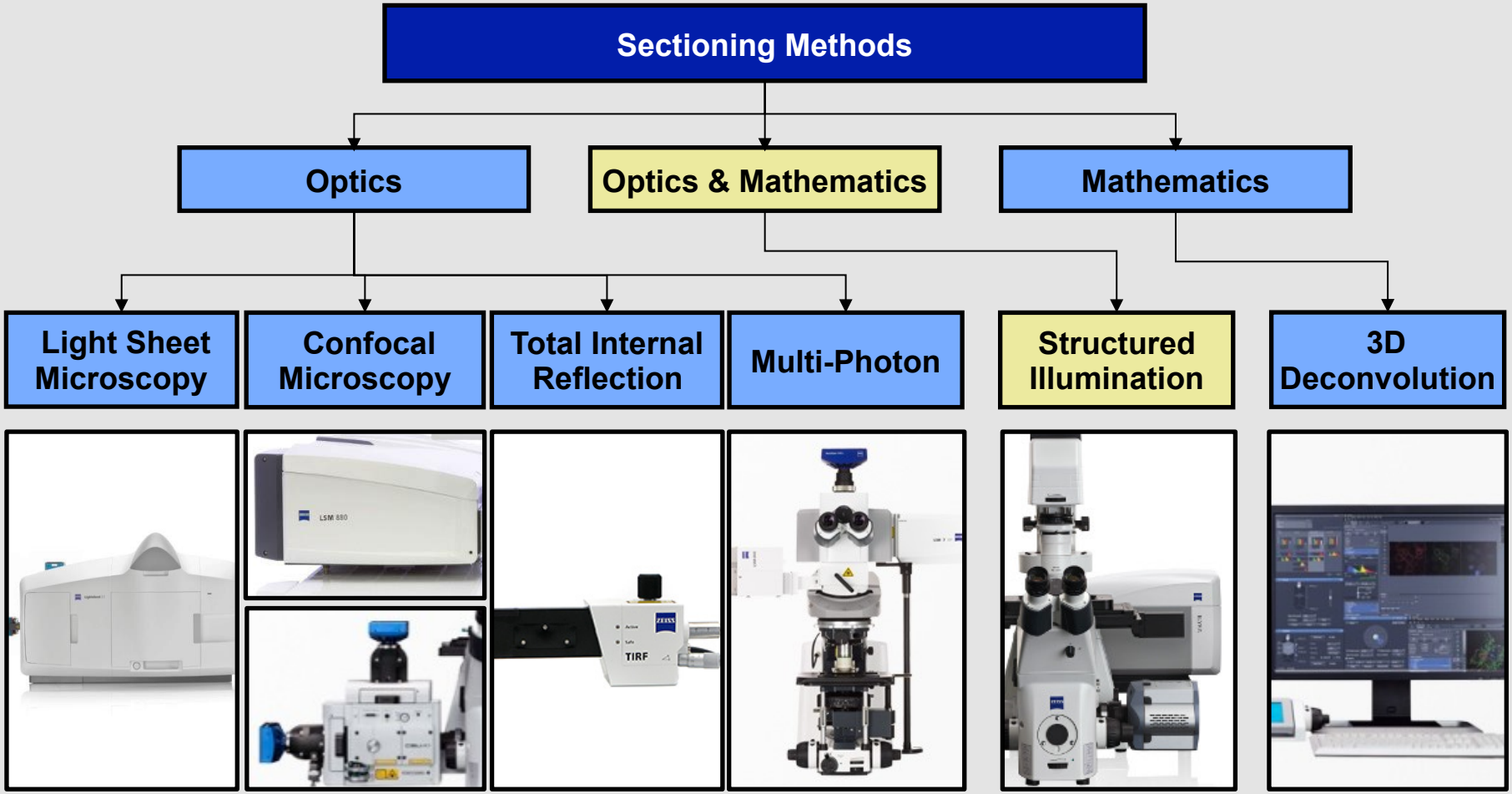
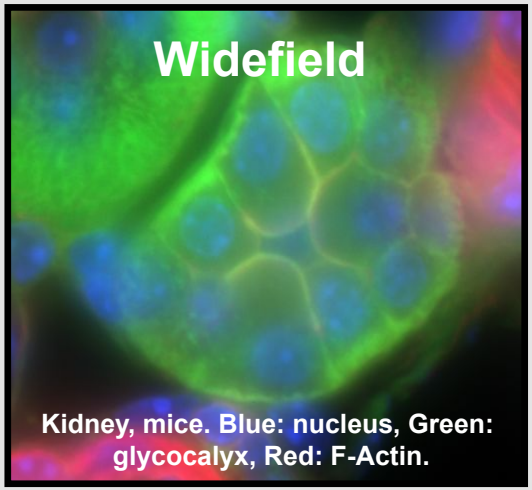
Optical Sectioning Techniques

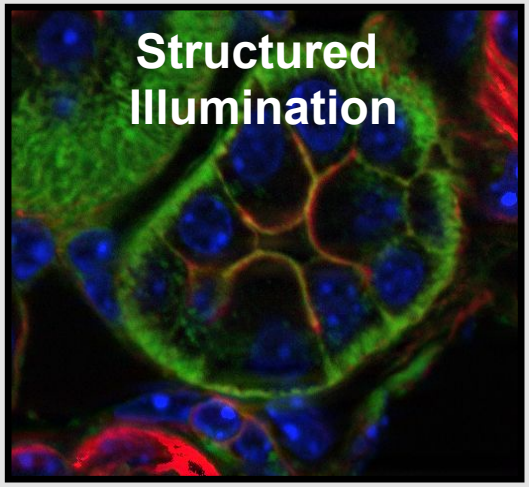
Hierarchy of Common Approaches

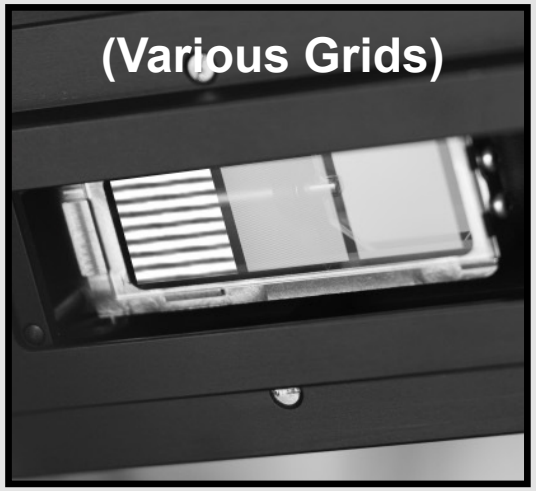


Structured Illumination

Principles of the Apotome







- Structured illumination imaging exploits a combination of **patterned excitation light** and **post-processing** to create an optical section

- Superimpose a **moving grid** over the image in light path; sharpness of the grid lines coincides with a given focal plane of specimen
  - When the sample is moved out-of-focus, the grids are also out-of-focus

Structured Illumination

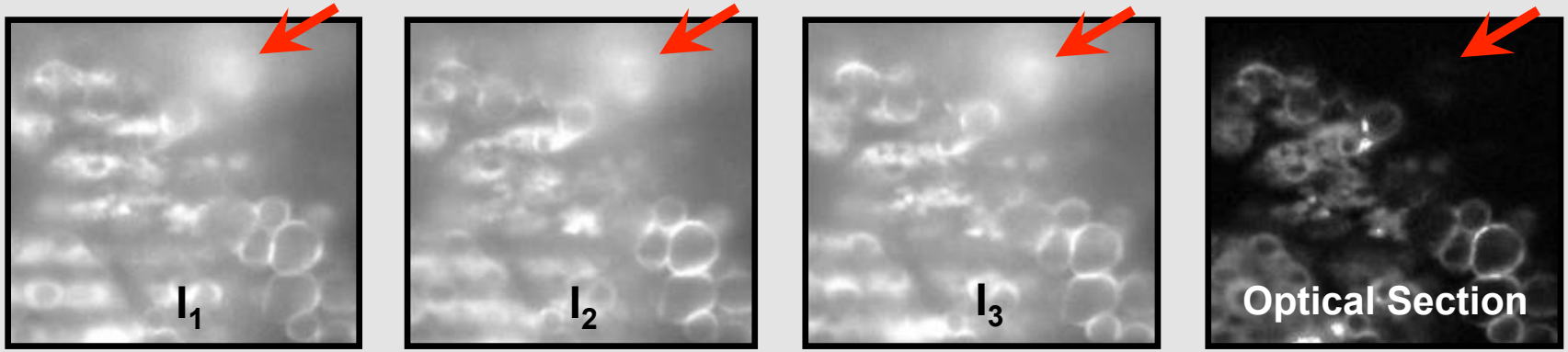
Principles of the Apotome

- Insert a grid structure into conjugate image plane of specimen
- Grid moved laterally over three positions; image is collected at each position
- Processed optical section is **dependent on wavelength, NA, and grid spacing**

Structured Illumination

Principles of the Apotome

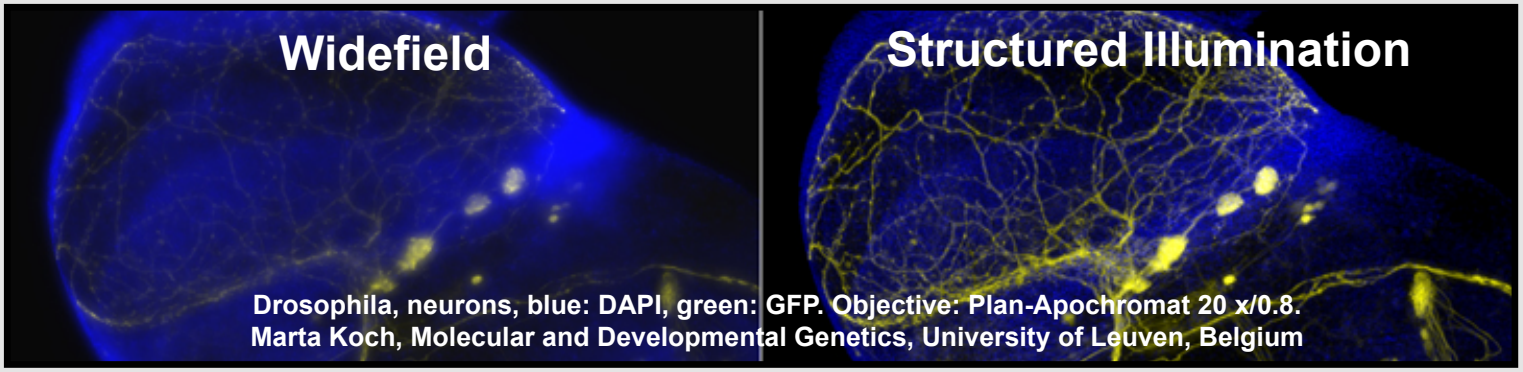
- Optical section generated by combining **three or more images** of equal phase shift (*e.g.* – I_1, I_2, I_3)
  - Result is calculated by simple least squares processing step
  - Blurred, out-of-focus regions aren't obscured by grid lines, so pixel values ("I") all cancel out during processing and become dark



$$\text{Intensity}_{\text{section}} = \sqrt{(I1-I2)2+(I1-I3)2+(I2-I3)2}$$

Structured Illumination

Resolution Improvements



- Resulting images **less prone to aberrations than deconvolution**
  - Grids removed; increased signal/noise
  - Enhanced axial resolution (**sectioning**) and lateral resolution (**contrast**)

Objective	M	NA	Section Thickness (µm)
EC Plan-Neofluar	20x	0.5	5.4
Plan-Apochromat	20x	0.8	1.5
Plan-Apochromat	40x	1.4	1.1
Plan-Apochromat	63x	1.4	0.7

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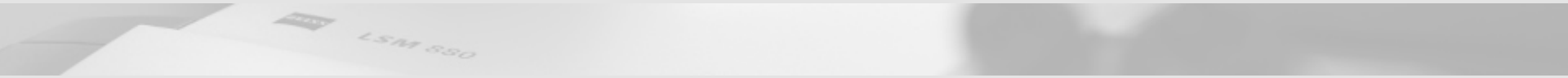

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We make it visible.