# Resolution Roll Call:
## Optical Sectioning System Overviews

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

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January 2019

# 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

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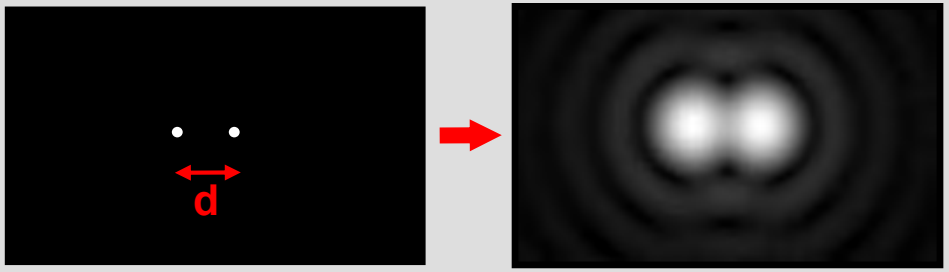
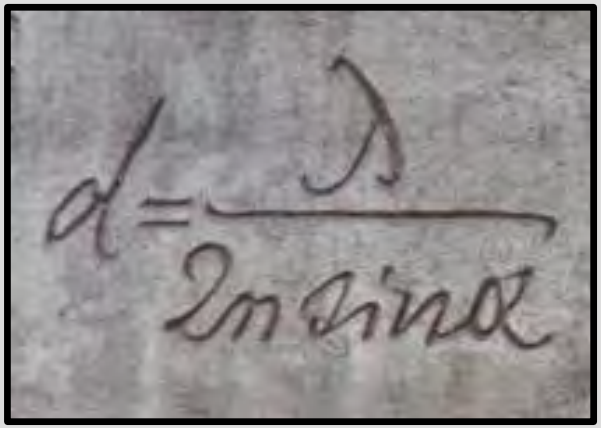
# Aspects of Resolution

## Defining the Limits

- 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



# Aspects of Resolution

## Historical Context

- Thomas A. Edison's electric lamp (1880)

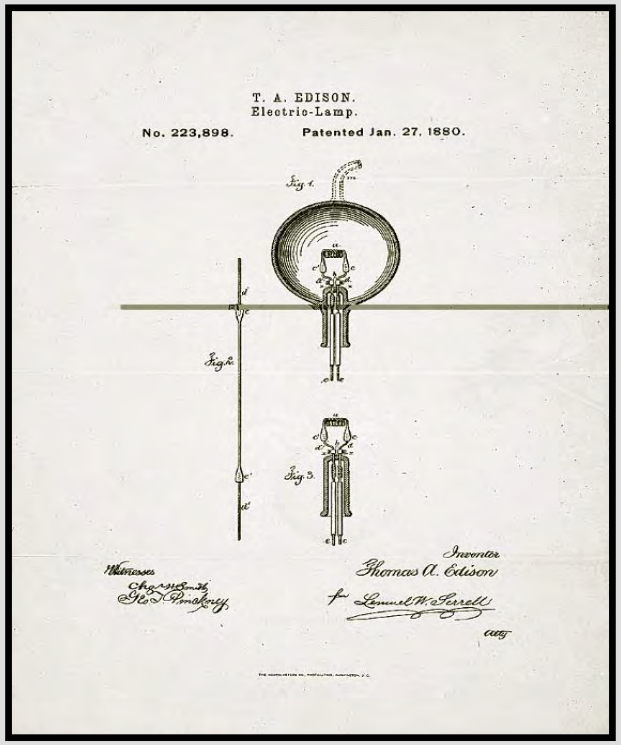

T. A. EDISON.
Electric-Lamp.
No. 223,898. Patented Jan. 27, 1880.

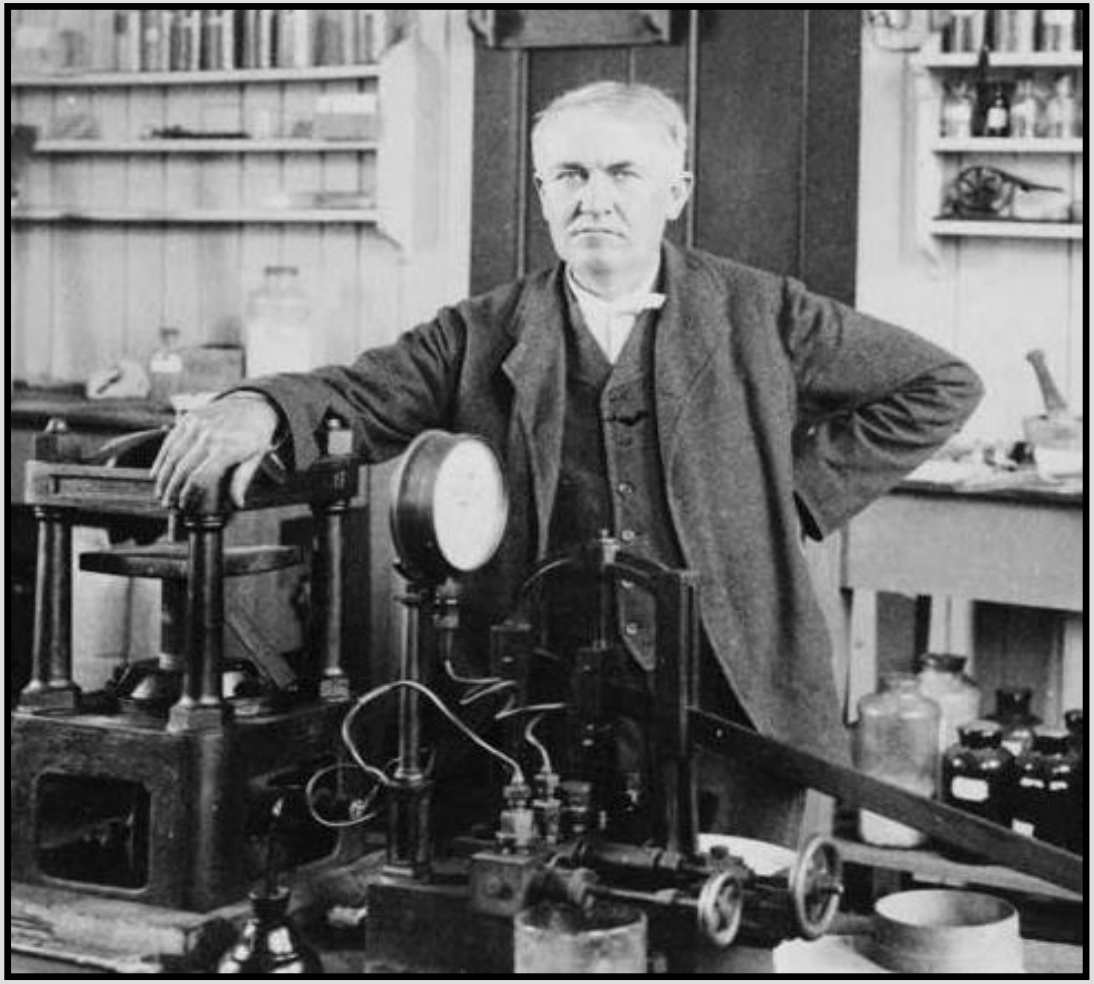
Fig 1.

Fig 2.

Fig 3.

Witnesses
Chas. H. Smith
Geo. D. Pomeroy.

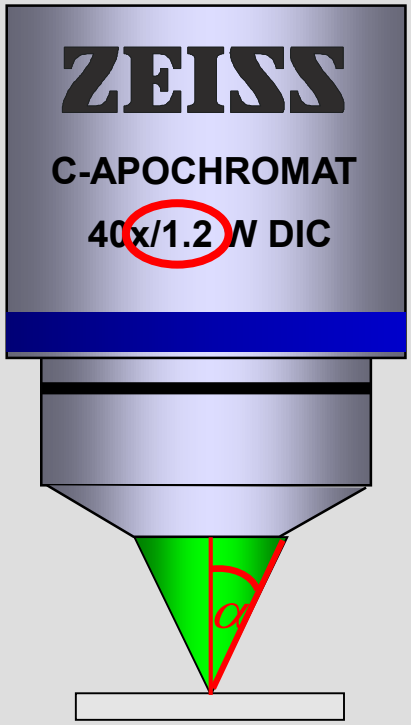
Inventor
Thomas A. Edison
by Samuel McFarell
atty



# Aspects of Resolution

## Defining the Limits

- Numerical aperture **(NA)** is the defining characteristic for resolution




$$\mathbf{NA} = n \sin \alpha$$

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

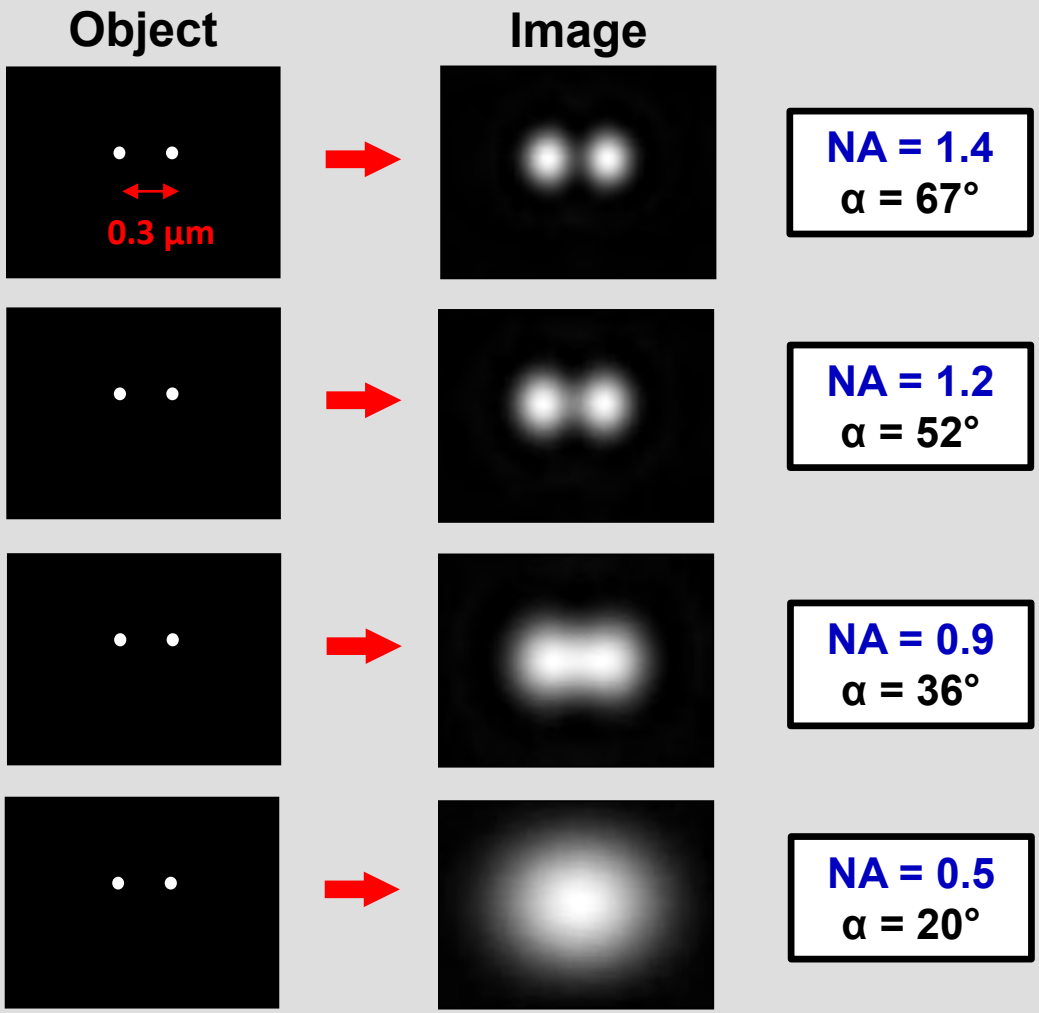
- **Resolution criteria is dependent on both wavelength and NA** (**not magnification**)

$$\mathbf{d_{xy}} = \frac{\lambda}{2(n \sin \alpha)} = \frac{0.5\,\lambda}{\mathbf{NA}}$$

# 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?*



# Aspects of Resolution

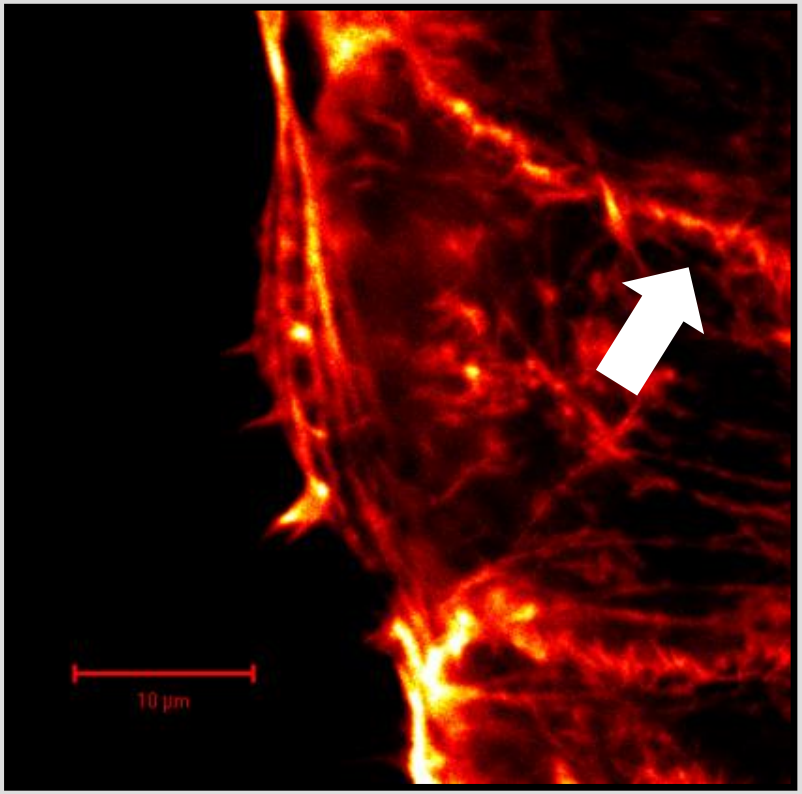
## Comparison of Example Objectives

| Magnification | NA | Resolution (XY) |
|---|---|---|
| 4x | 0.10 | 2.75 µm |
| 4x | 0.20 | 1.375 µm |
| 10x | 0.25 | 1.10 µm |
| 10x | 0.45 | 0.61 µm |
| 20x | 0.40 | 0.69 µm |
| 20x | 0.75 | 0.37 µm |
| 40x | 0.65 | 0.42 µm |
| 40x | 1.40 | 0.20 µm |
| 63x | 0.75 | 0.37 µm |
| 63x | 1.30 | 0.25 µm |
| 100x | 1.25 | 0.22 µm |
| 100x | 1.40 | 0.20 µm |

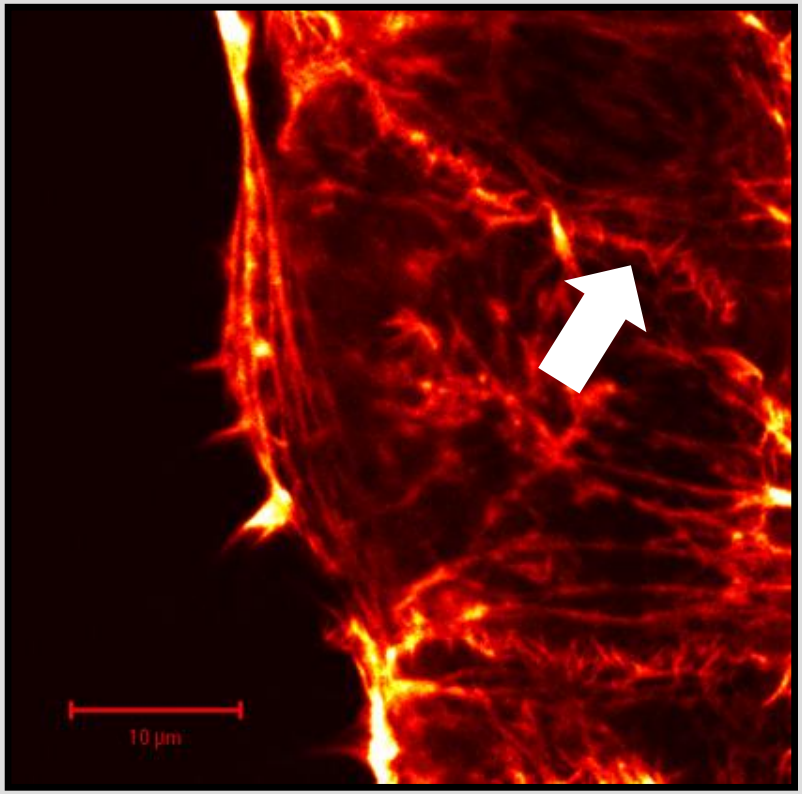
# Aspects of Resolution

## Comparison of Example Objectives

**Plan-Apochromat 63x/1.4 Oil**



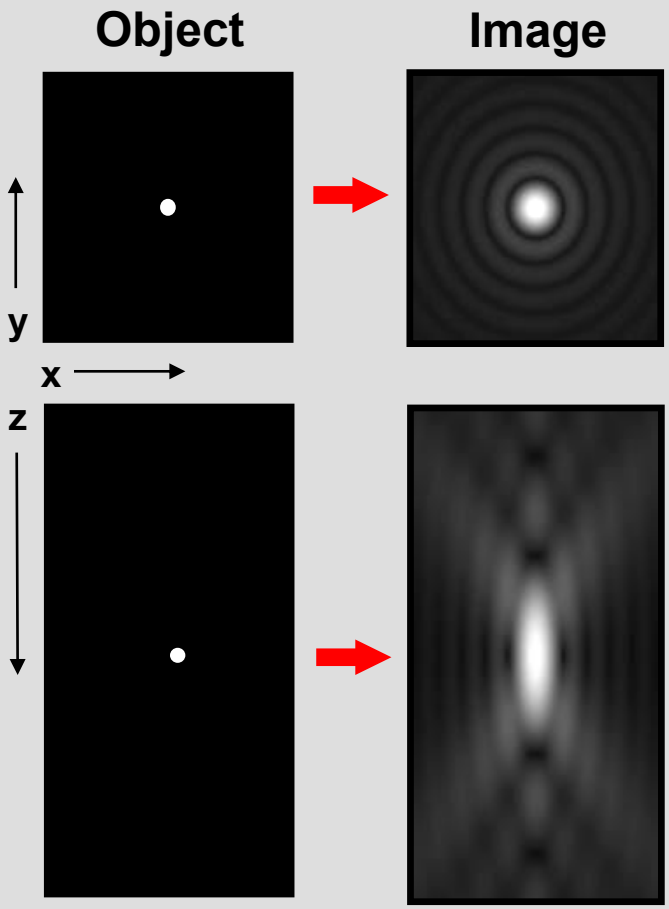

**Alpha-Fluar 100x/1.45 Oil**




- Even small increases in NA can yield key improvements in imaging

# Aspects of Resolution

## Limits in the Z Dimension




$$d_{xy} = \frac{0.5\,\lambda}{NA}$$

**lateral resolution (XY) limit ~ 200 nm**

$$d_z = \frac{2\lambda}{NA^2}$$

**axial resolution (Z) limit ~ 550 nm**

- 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

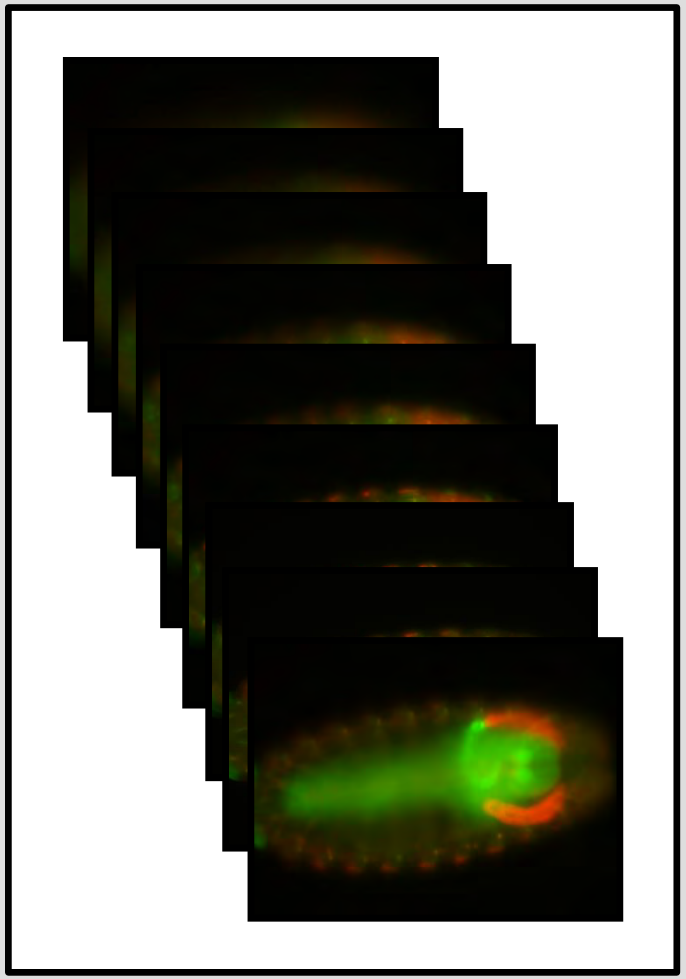
# Aspects of Resolution

## Comparison of Example Objectives

| Magnification | NA | Resolution (XY) | Resolution (Z) |
|---|---|---|---|
| 4x | 0.10 | 2.75 µm | 110 µm |
| 4x | 0.20 | 1.375 µm | 27.5 µm |
| 10x | 0.25 | 1.10 µm | 17.6 µm |
| 10x | 0.45 | 0.61 µm | 5.43 µm |
| 20x | 0.40 | 0.69 µm | 6.87 µm |
| 20x | 0.75 | 0.37 µm | 1.95 µm |
| 40x | 0.65 | 0.42 µm | 2.60 µm |
| 40x | 1.40 | 0.20 µm | 0.56 µm |
| 63x | 0.75 | 0.37 µm | 1.95 µm |
| 63x | 1.30 | 0.25 µm | 0.65 µm |
| 100x | 1.25 | 0.22 µm | 0.70 µm |
| 100x | 1.40 | 0.20 µm | 0.56 µm |

# Aspects of Resolution

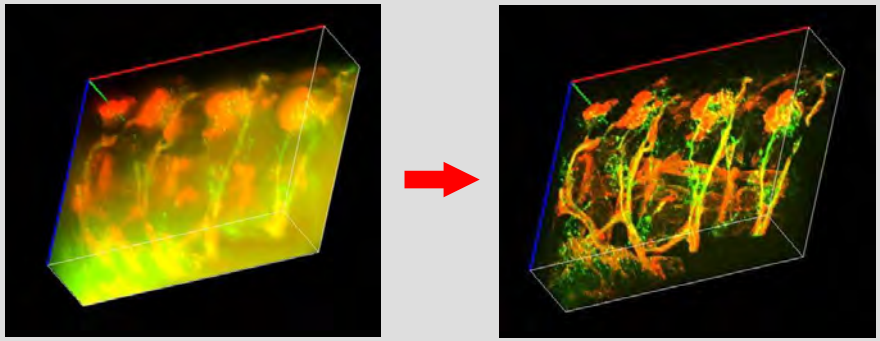
## Enhancing Axial Resolution



**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**



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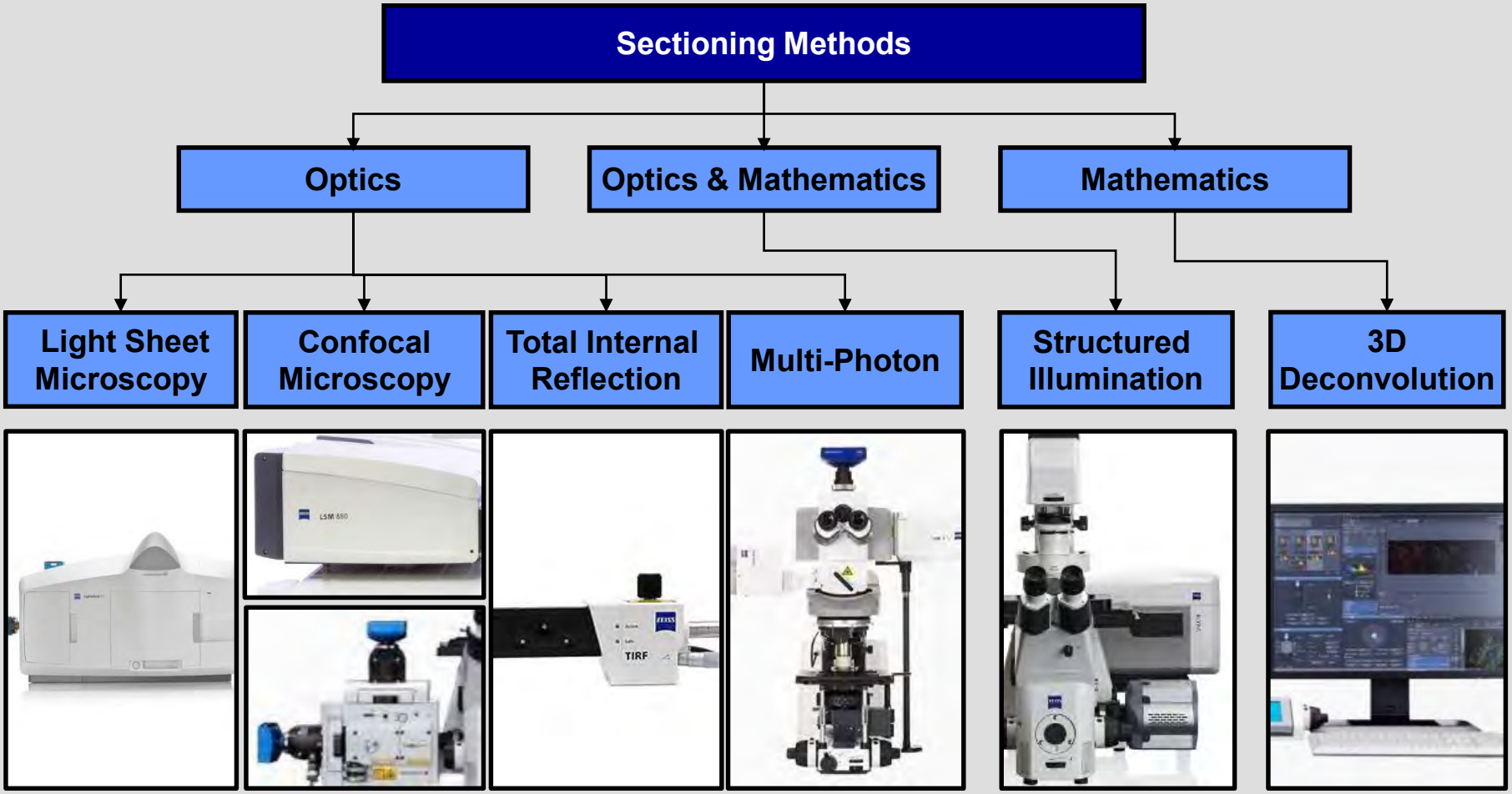
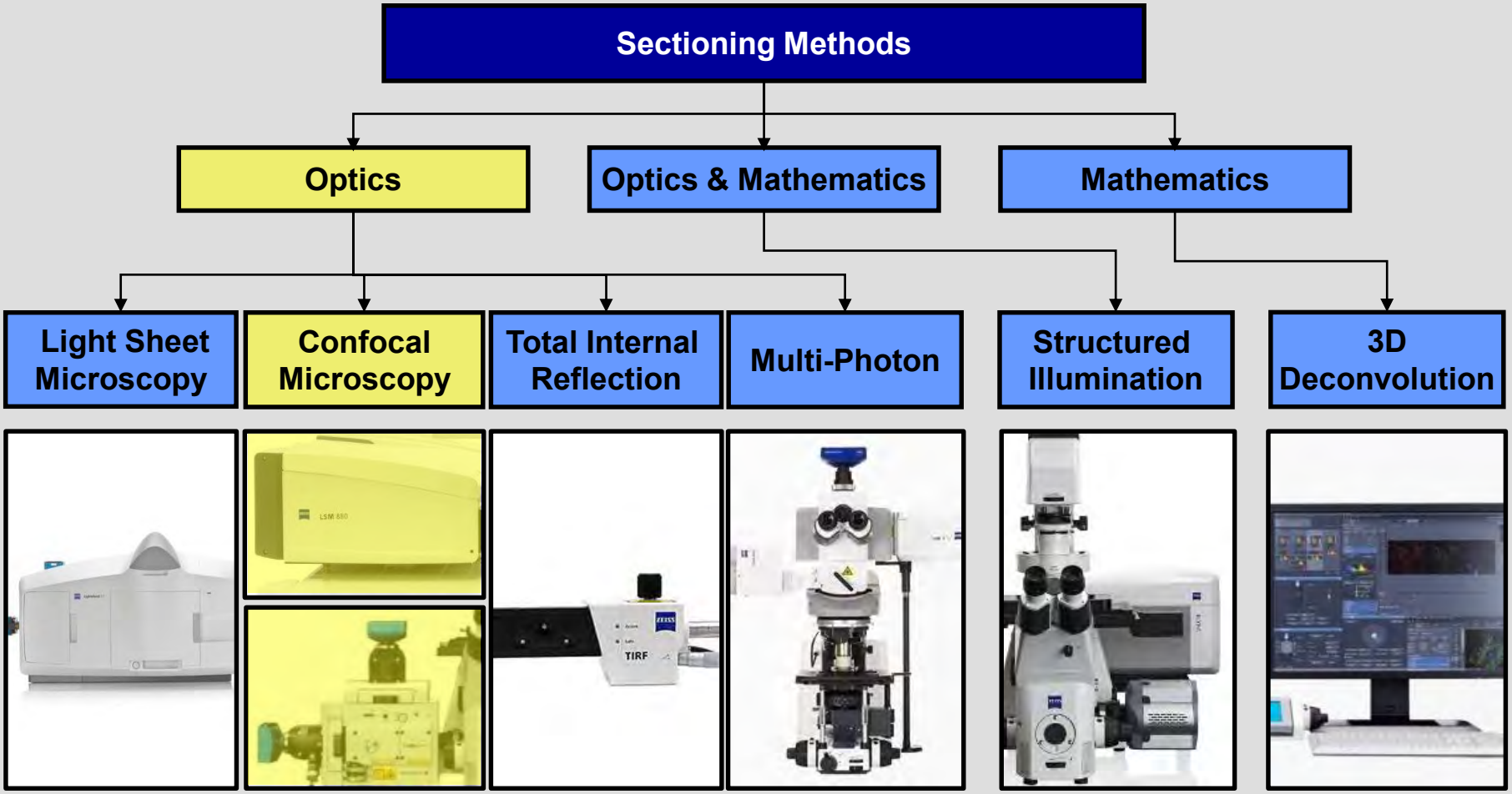
# Optical Sectioning Techniques

## Hierarchy of Common Approaches



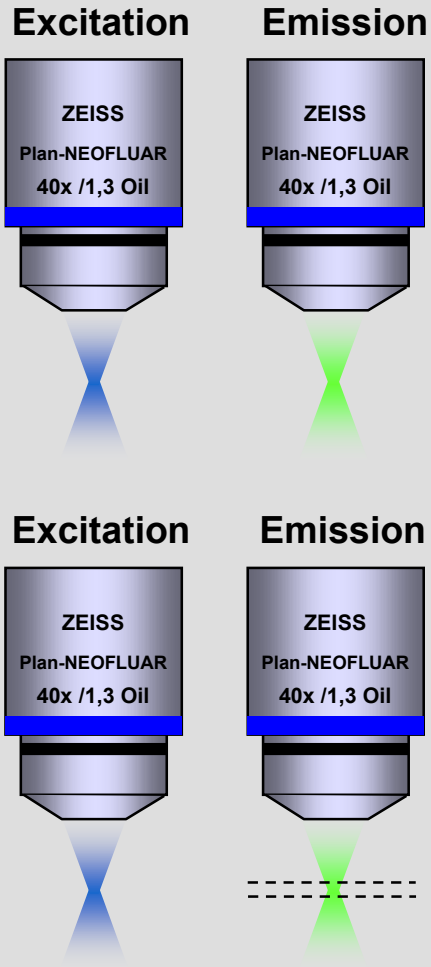
# Optical Sectioning

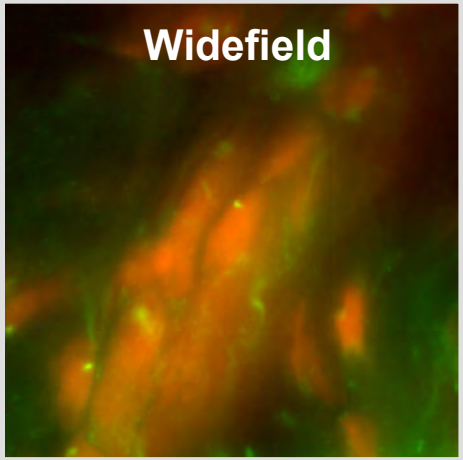
## Confocal Microscopy

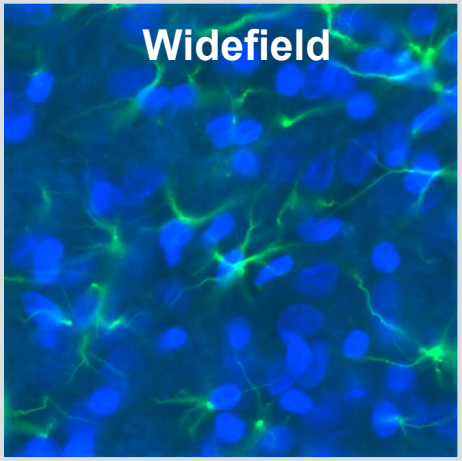


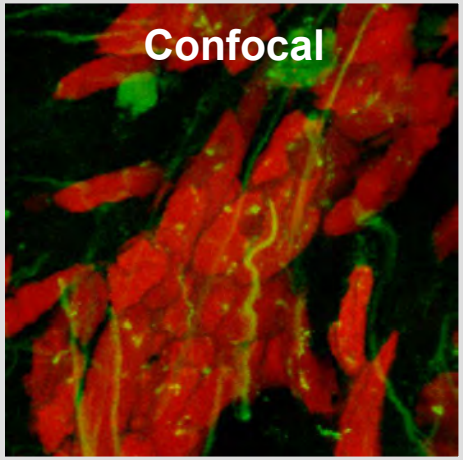
# Confocal Microscopy

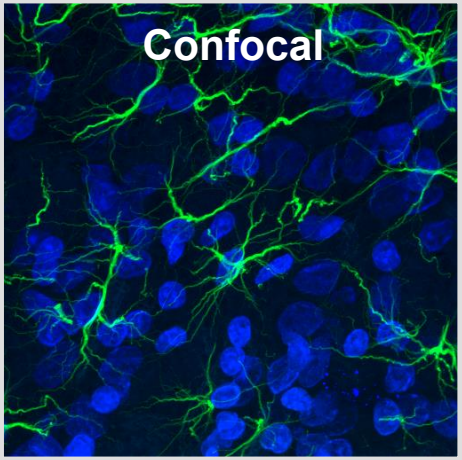
## Rejection of Out-of-Focus Signal



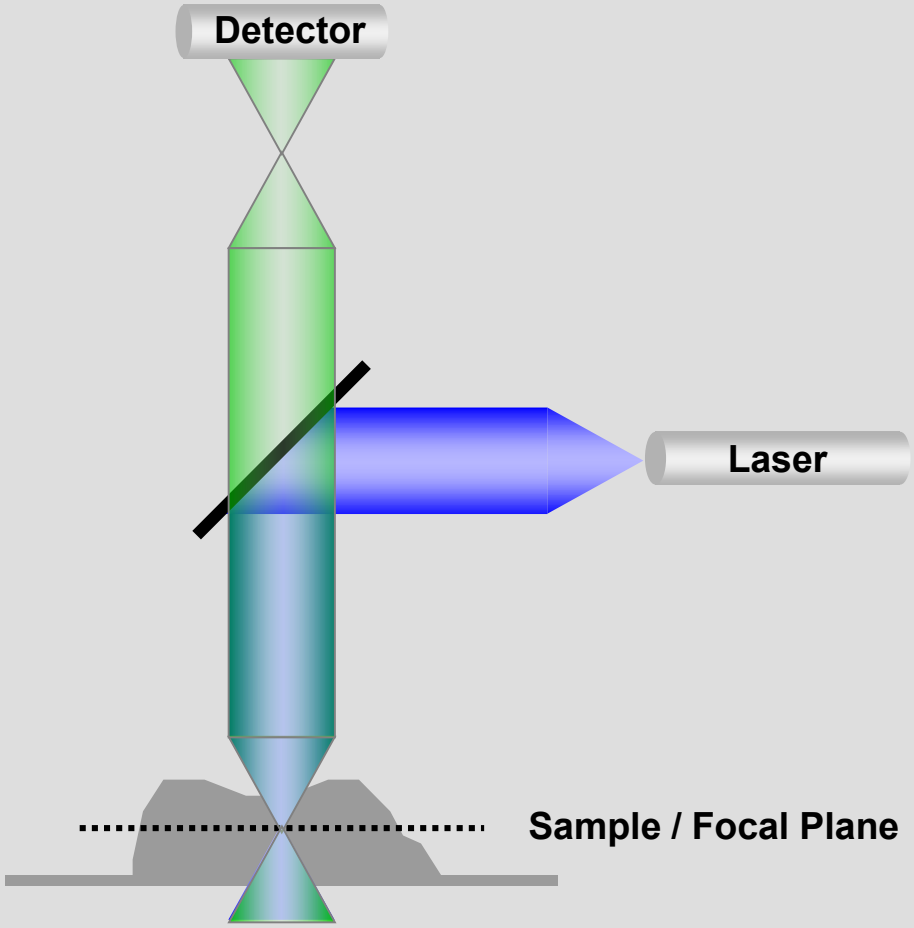



# Confocal Microscopy

## Rejection of Out-of-Focus Signal

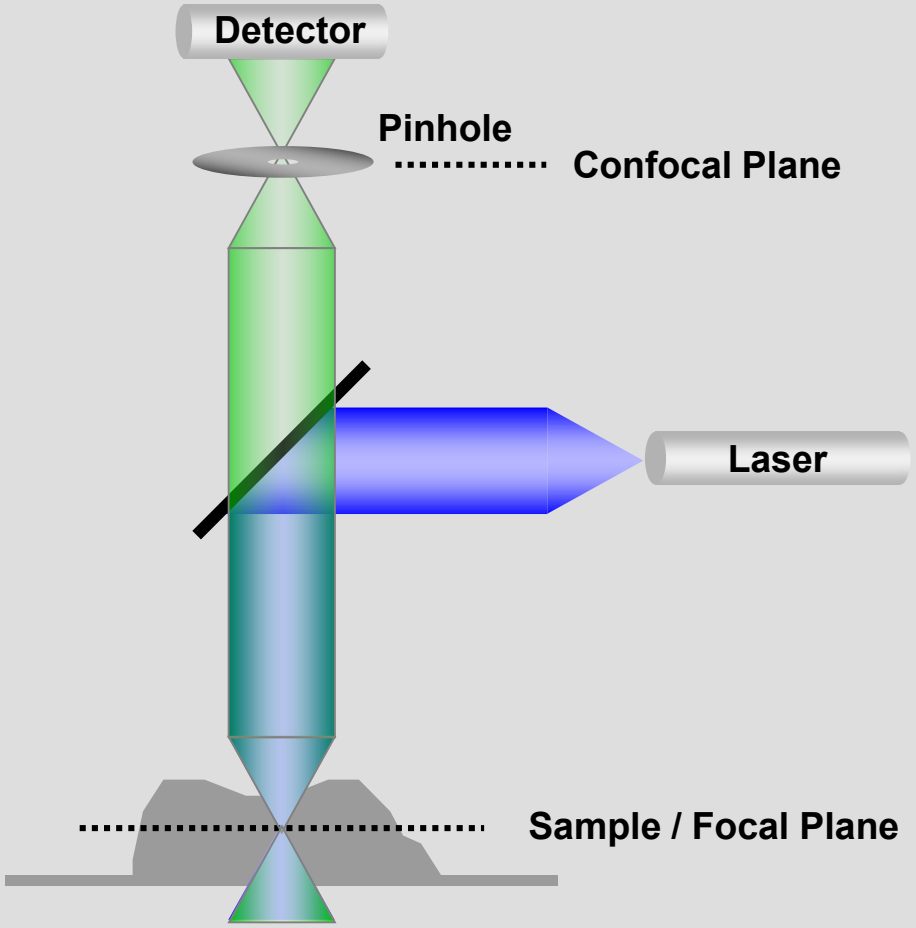
- Laser excitation and sample emission separated by **dichroic**



# Confocal Microscopy

## Rejection of Out-of-Focus Signal

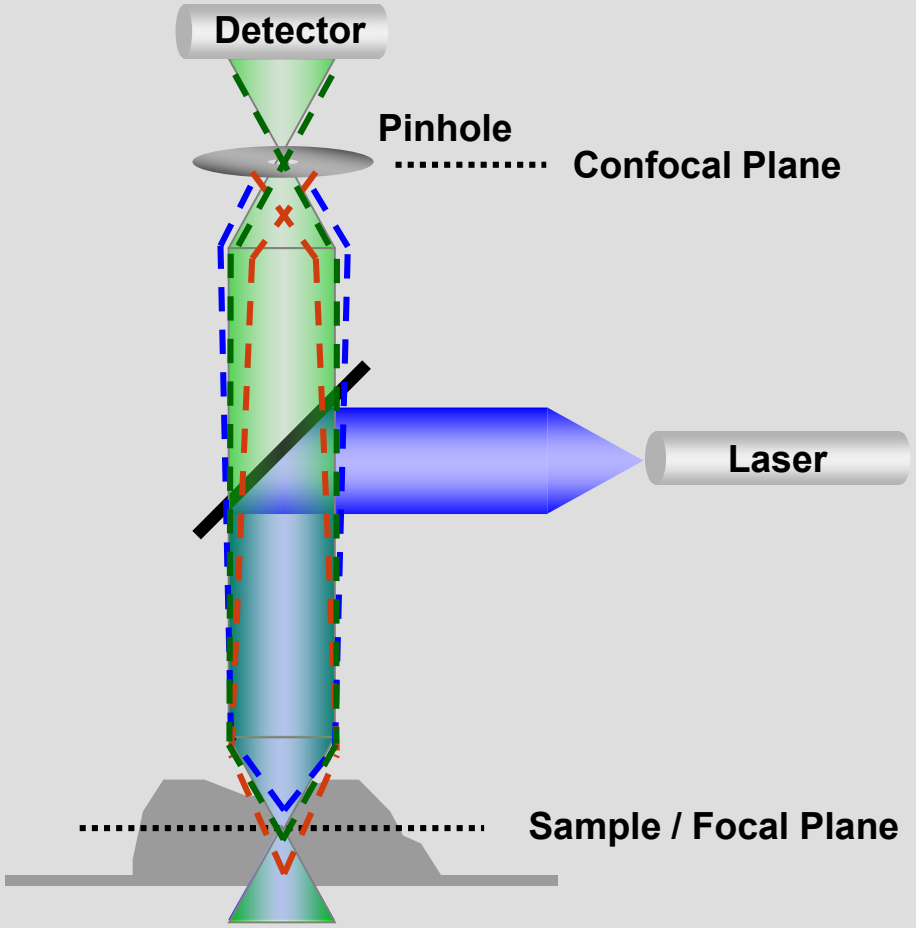
- Laser excitation and sample emission separated by **dichroic**
- The **pinhole(s)** prevent detection of out-of-focus signal
  - Diaphragm situated in conjugate focal plane



# Confocal Microscopy

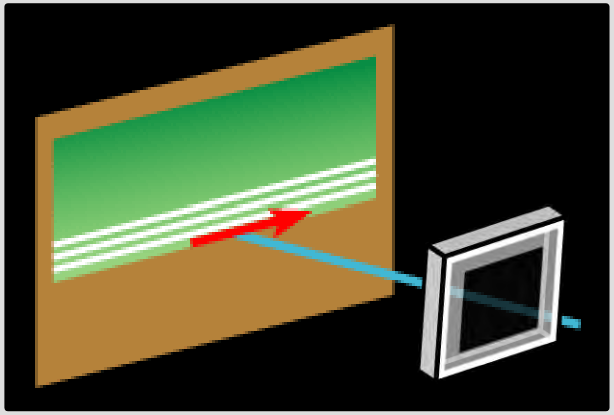
## Rejection of Out-of-Focus Signal

- 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**

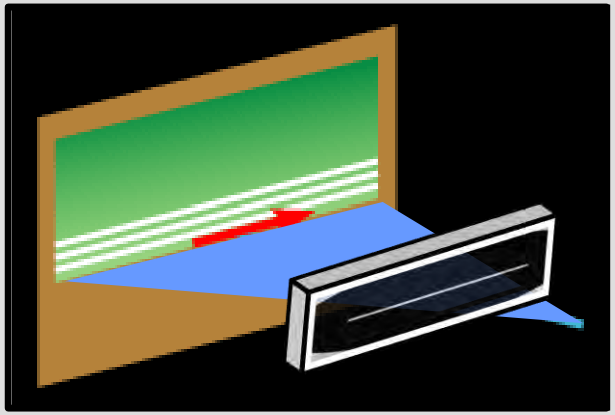


# Confocal Microscopy
## Methods for Scanning

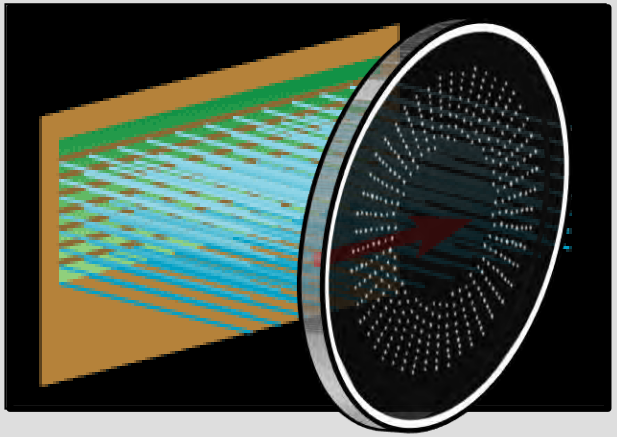
- Increased scanning speeds are possible by **multipoint approaches**
  - Such approaches sacrifice **pinhole versatility** at emission side and thus have less control over the optical section thickness



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



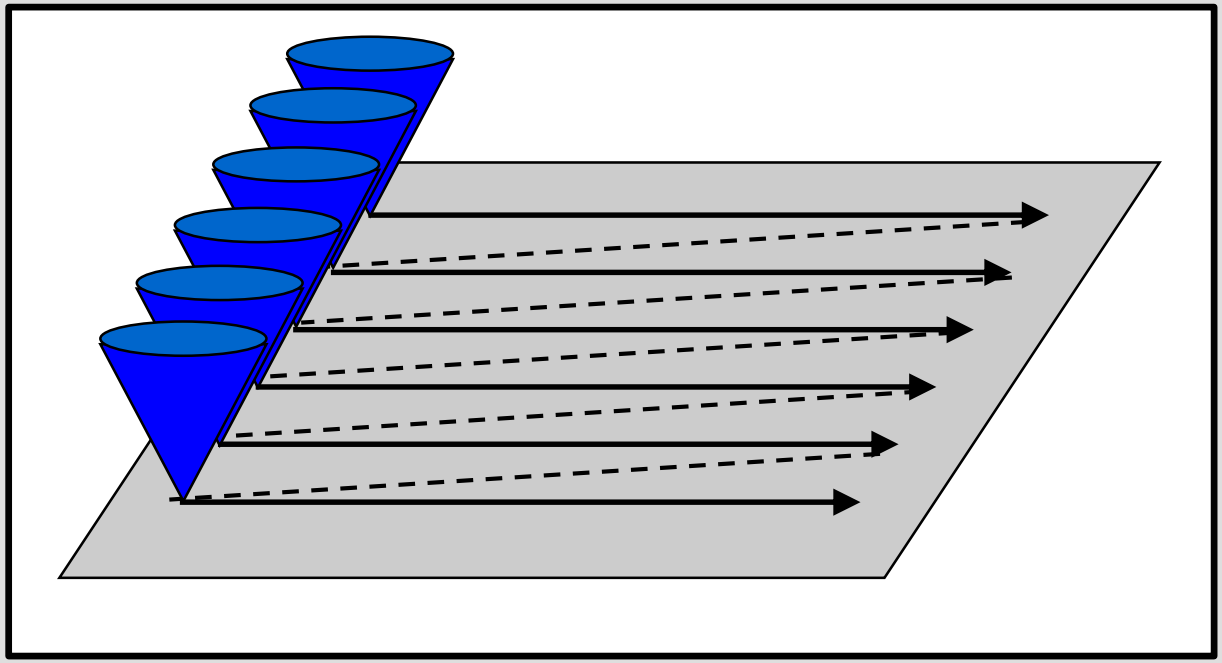
**Line Scanning Confocal**
*(single line or slit at a time)*



**Spinning Disk Confocal**
*(~1000 points at a time)*

# Point-Scanning Confocal

## Basic Principles



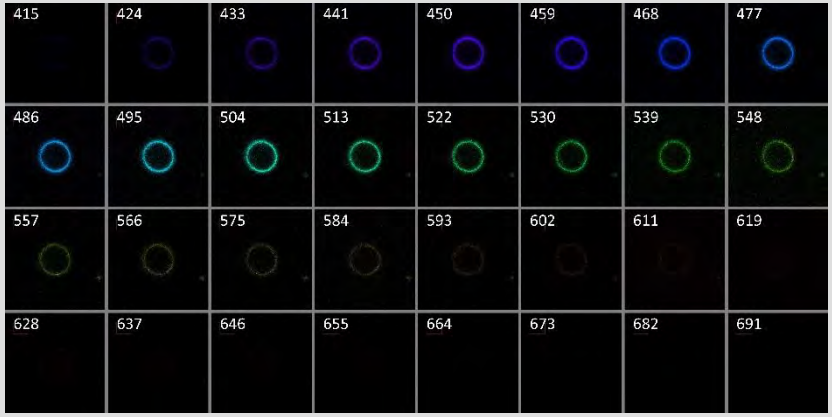
- A **diffraction-limited laser spot** is moved across the sample via two independent scanning mirrors
  - The resulting image is generated a **single point at a time**

# Point-Scanning Confocal

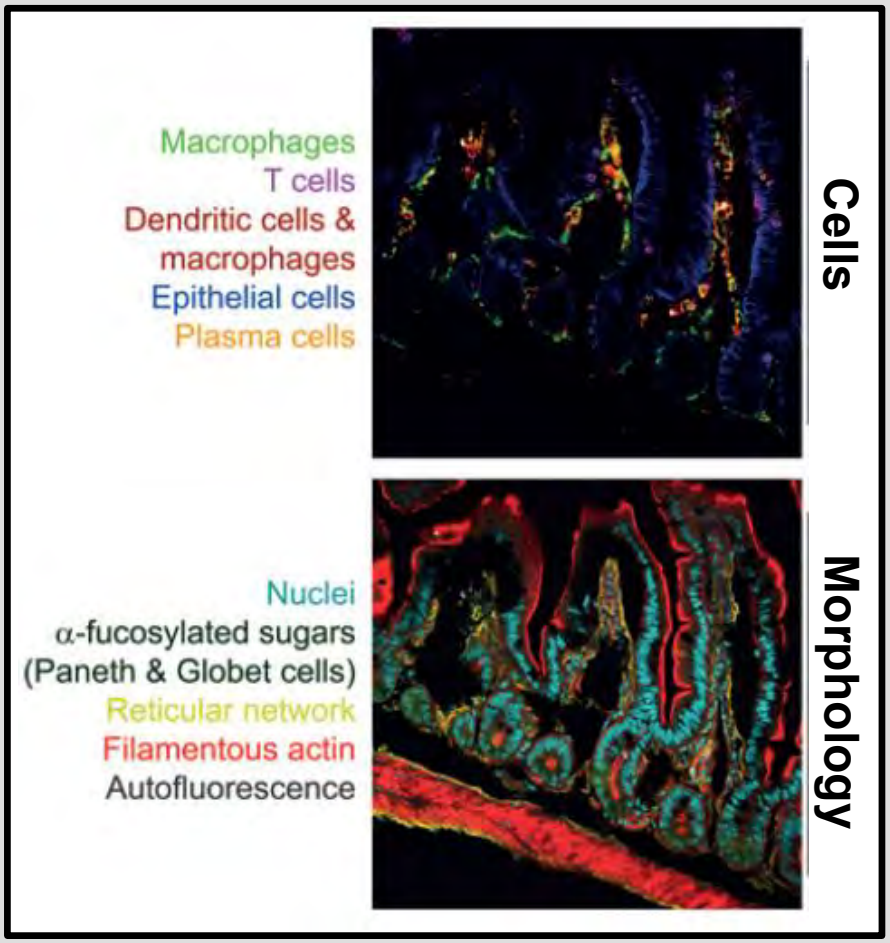
## Common/Unique Applications

## 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)**

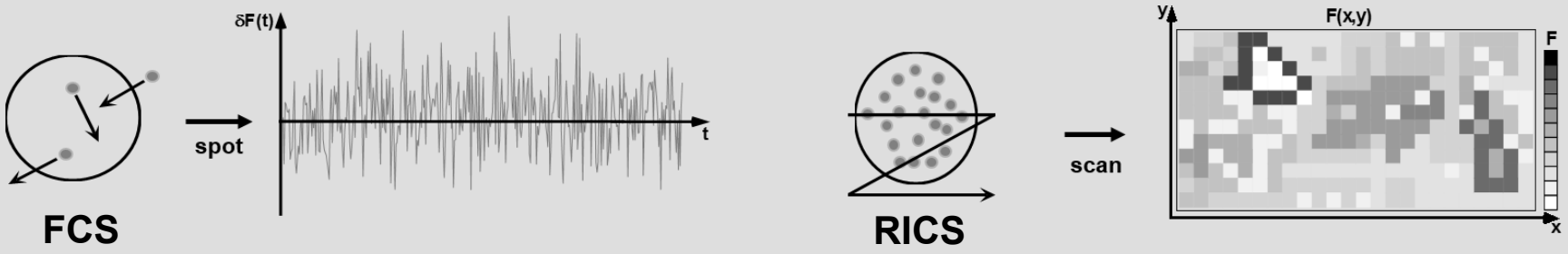
# Point-Scanning Confocal

## Common/Unique Applications

## 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”)

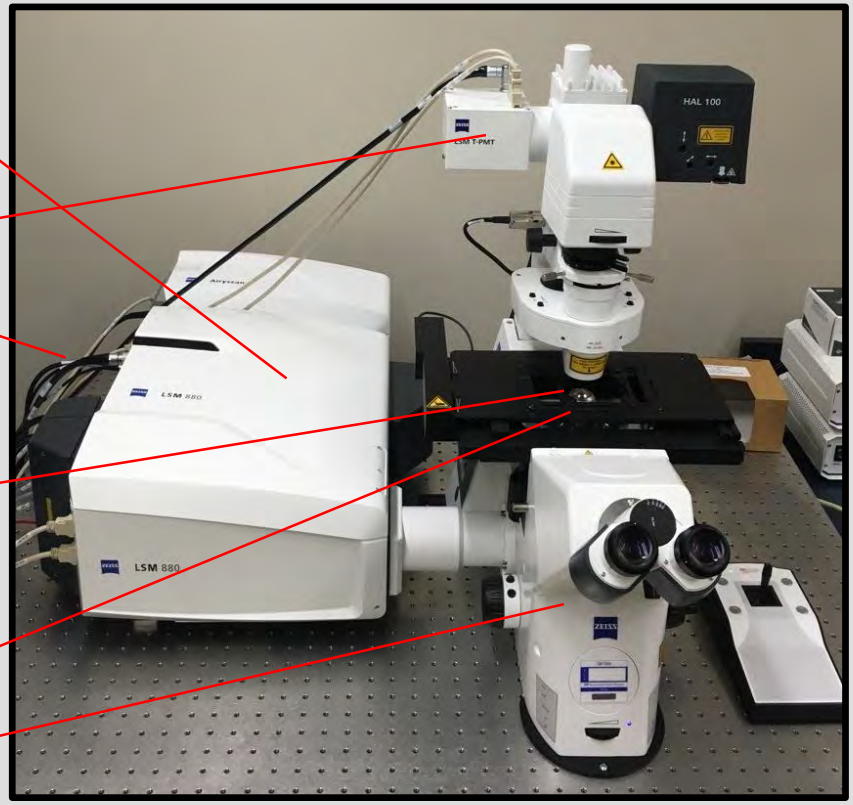
Laser lines: 405, 458, 488, 514, 561, 633 nm

Objectives:

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

Motorized XY stage + Z-piezo insert

Observer inverted microscope

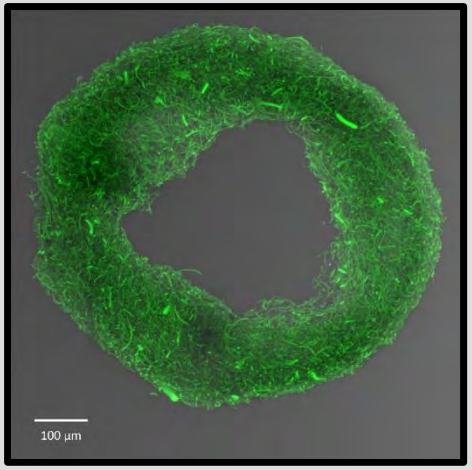


# Point-Scanning Confocal

## Practical Perspective

### ADVANTAGES:

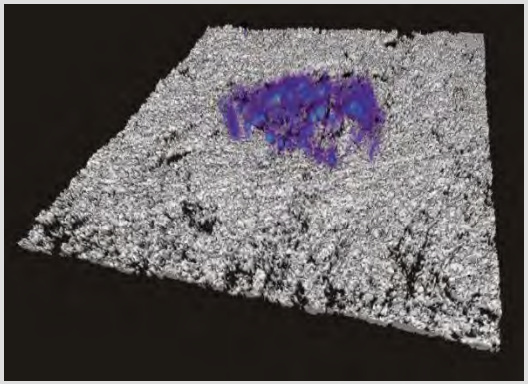
- Nearly **ubiquitous** 3D modality
- Easy to combine multiple **channels in parallel** (spectral imaging)
- Precise **pixel photomanipulation possible**
- Works with **reflected mode imaging** (non-fluorescent structures)




**FITC electrospun fiber scaffold (Xie Lab, Univ. Nebraska Med.)**

### DISADVANTAGES:

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



**Fluorescent flake on paper (reflection)**

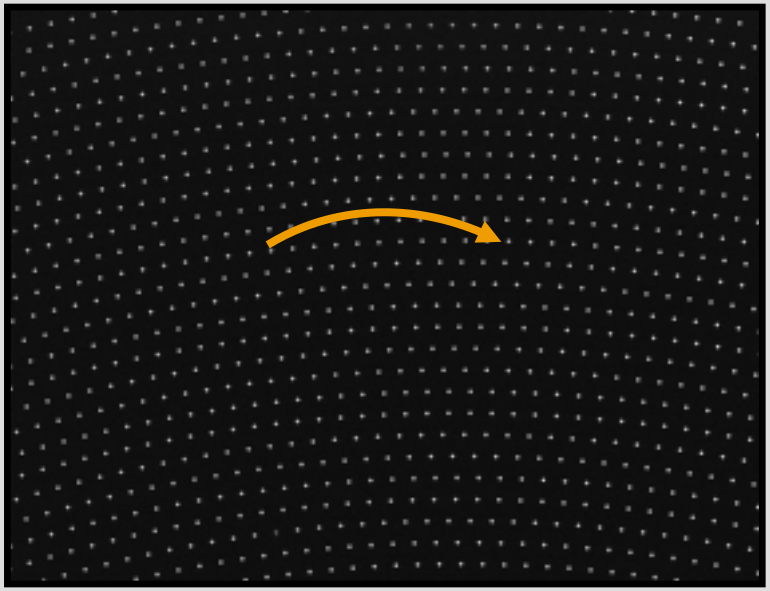
# Spinning Disk Confocal

## Basic Principles

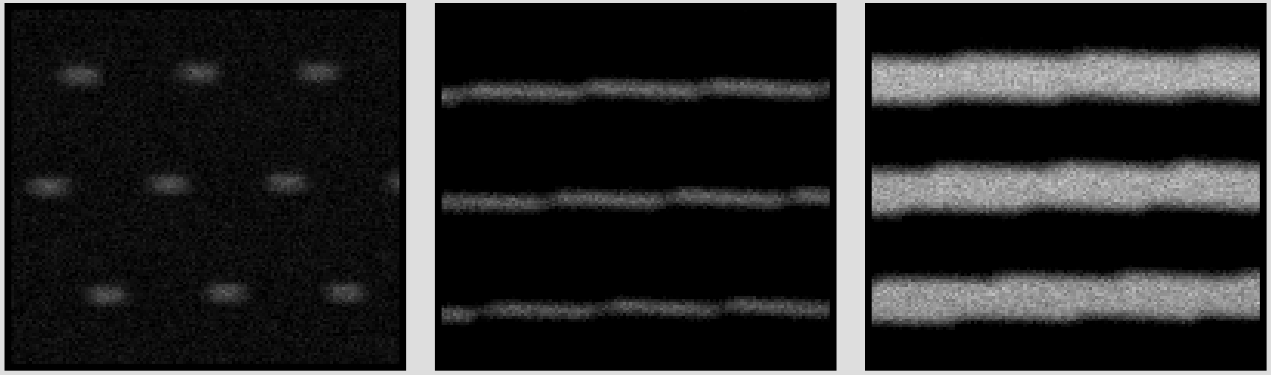
- A single **rotating disk** containing **hundreds of pinholes** scans laser light across the sample
  - Rotation **speed is adjustable** (~1500 – 5000 RPM)
- Paul Nipkow patented concept in 1884, later became basis for mechanical television in 1920s

# 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

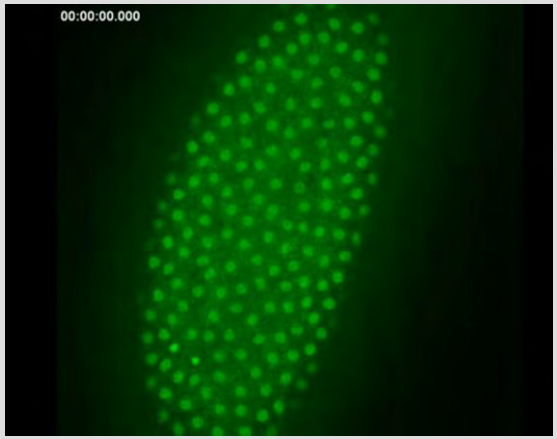
# Spinning Disk Confocal

## Basic Principles

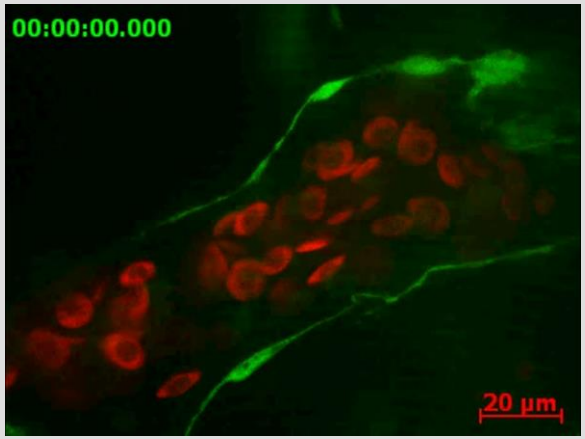
- Pinholes **illuminated by laser** (via focusing of microlens array)
  - Projected onto sample
- Emission collected through objective; **passes pinhole array**
  - Only in-focus signals
- Beamsplitter passes **fluorescence to area detector** (*e.g.* – CCD)
  - High-speed confocal

# Spinning Disk Confocal

## Common/Unique Applications



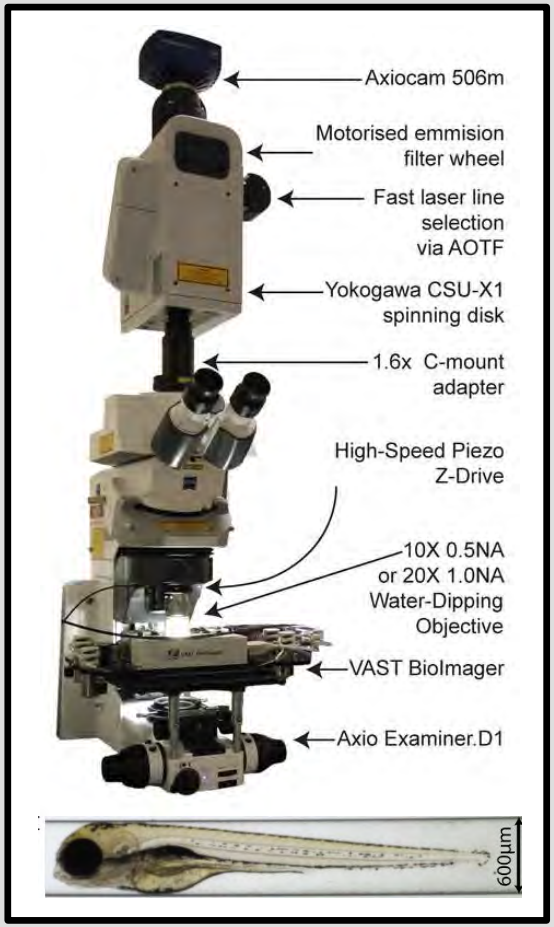

Drosophila embryo (projection)




Zebrafish red blood cells

## 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)

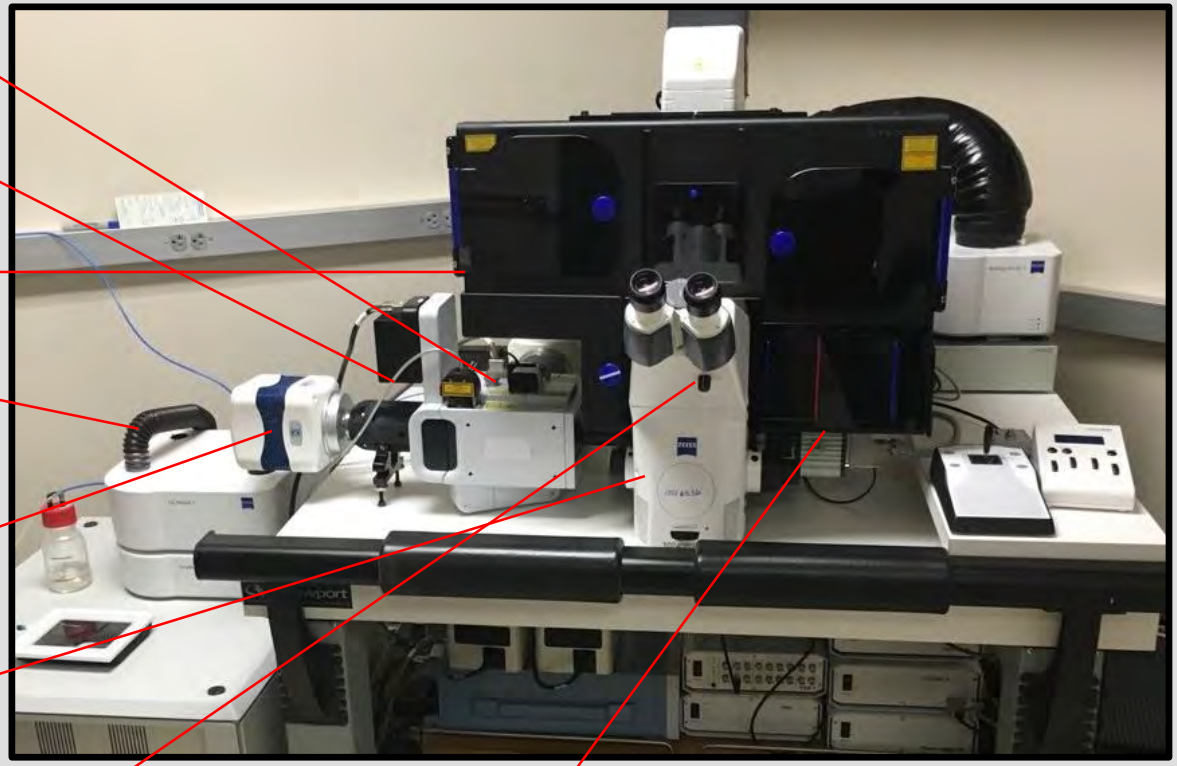
Laser-safety incubator chamber

Temperature, $CO_2$ 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:

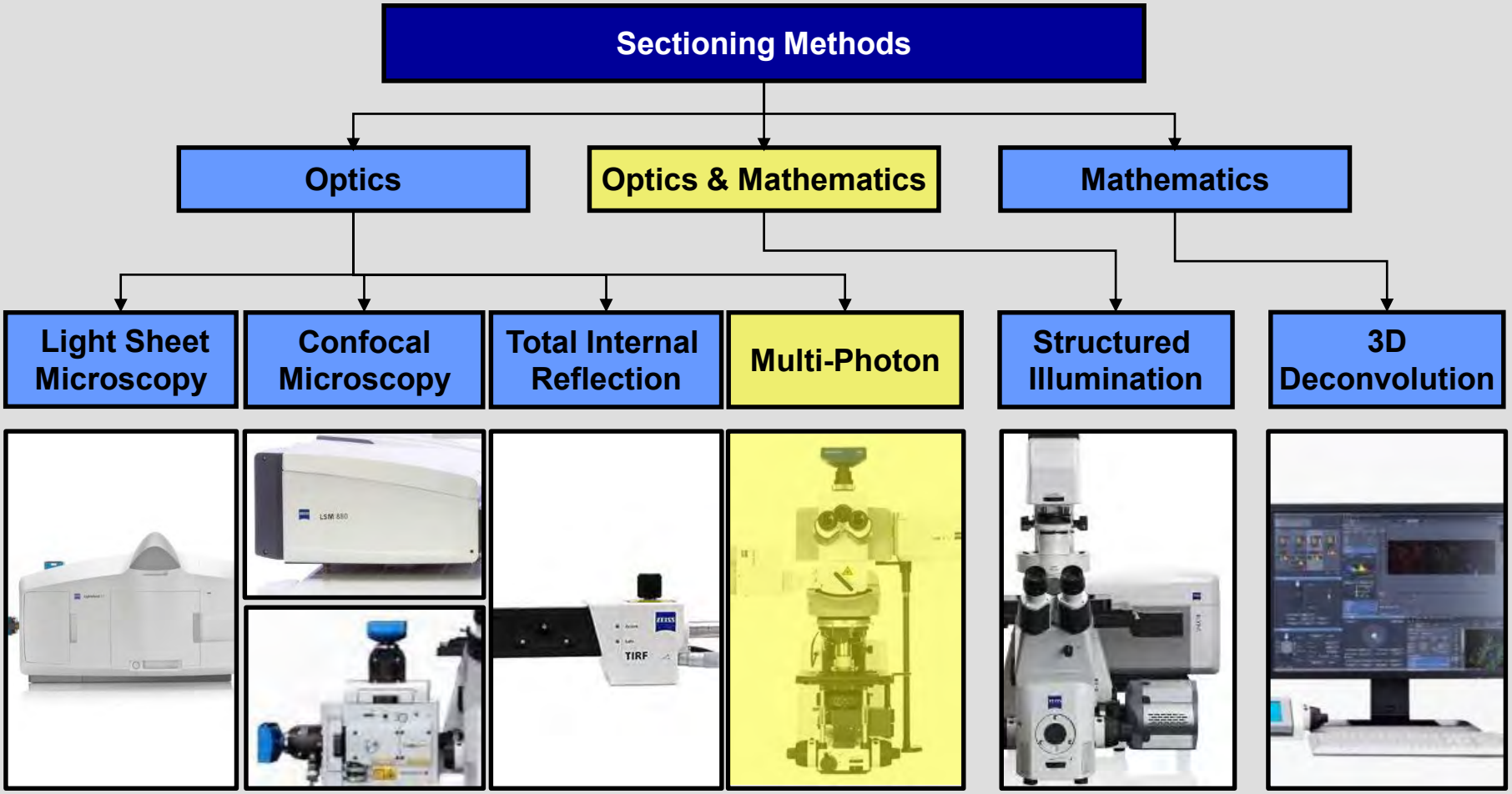
- High speed acquisition **ideal for live cell/tissue dynamics**; capture rate is typically limited only by camera(s)
- Excitation spots are constantly moving; generally accepted as one of the **most gentle confocal imaging strategies**; low photobleaching
- Robust, relatively inexpensive setup that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

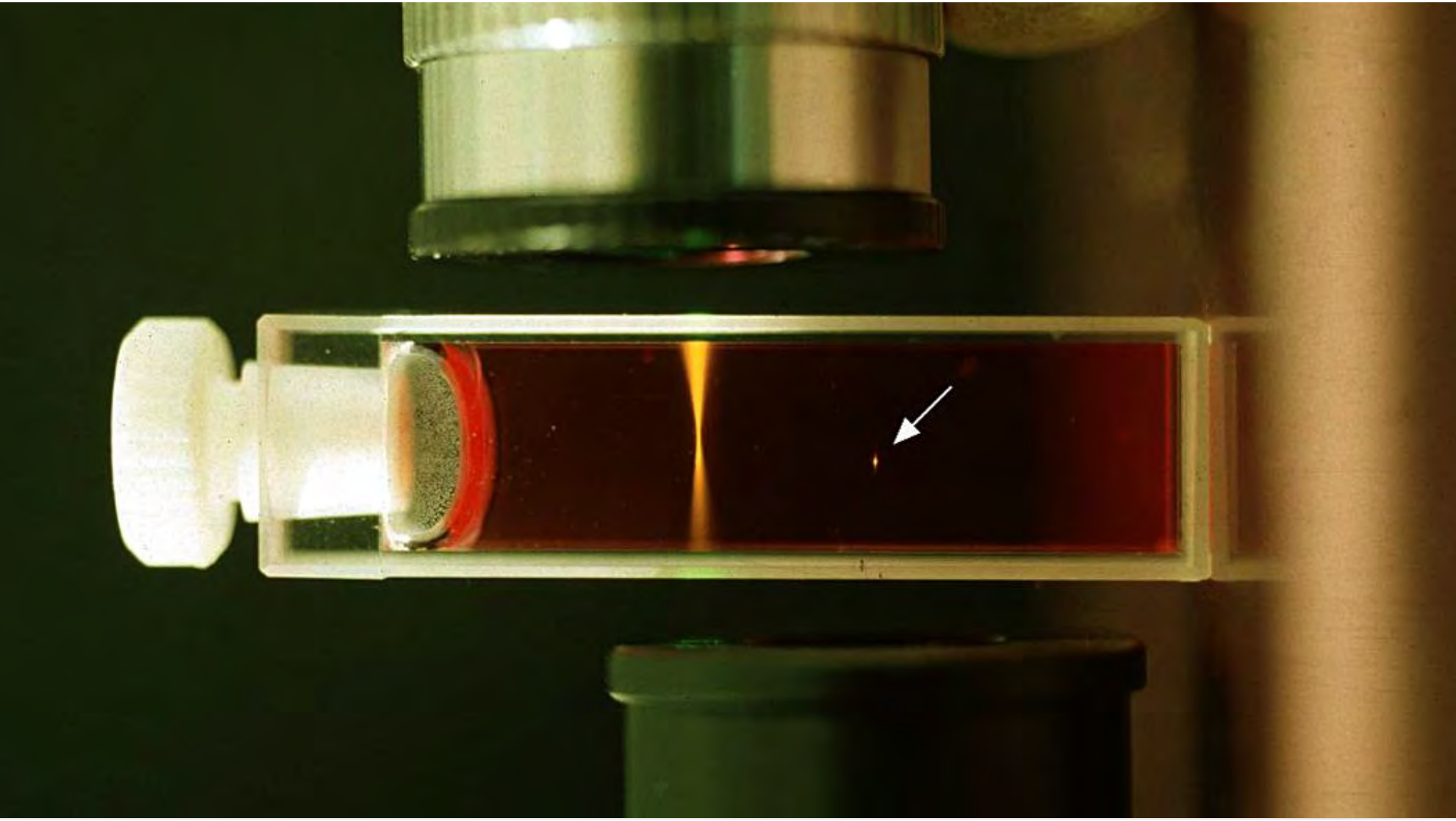
# Optical Sectioning Techniques

## Multi-Photon Microscopy



# Multi-Photon Microscopy

## Principles of the Two-Photon Effect



# 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)

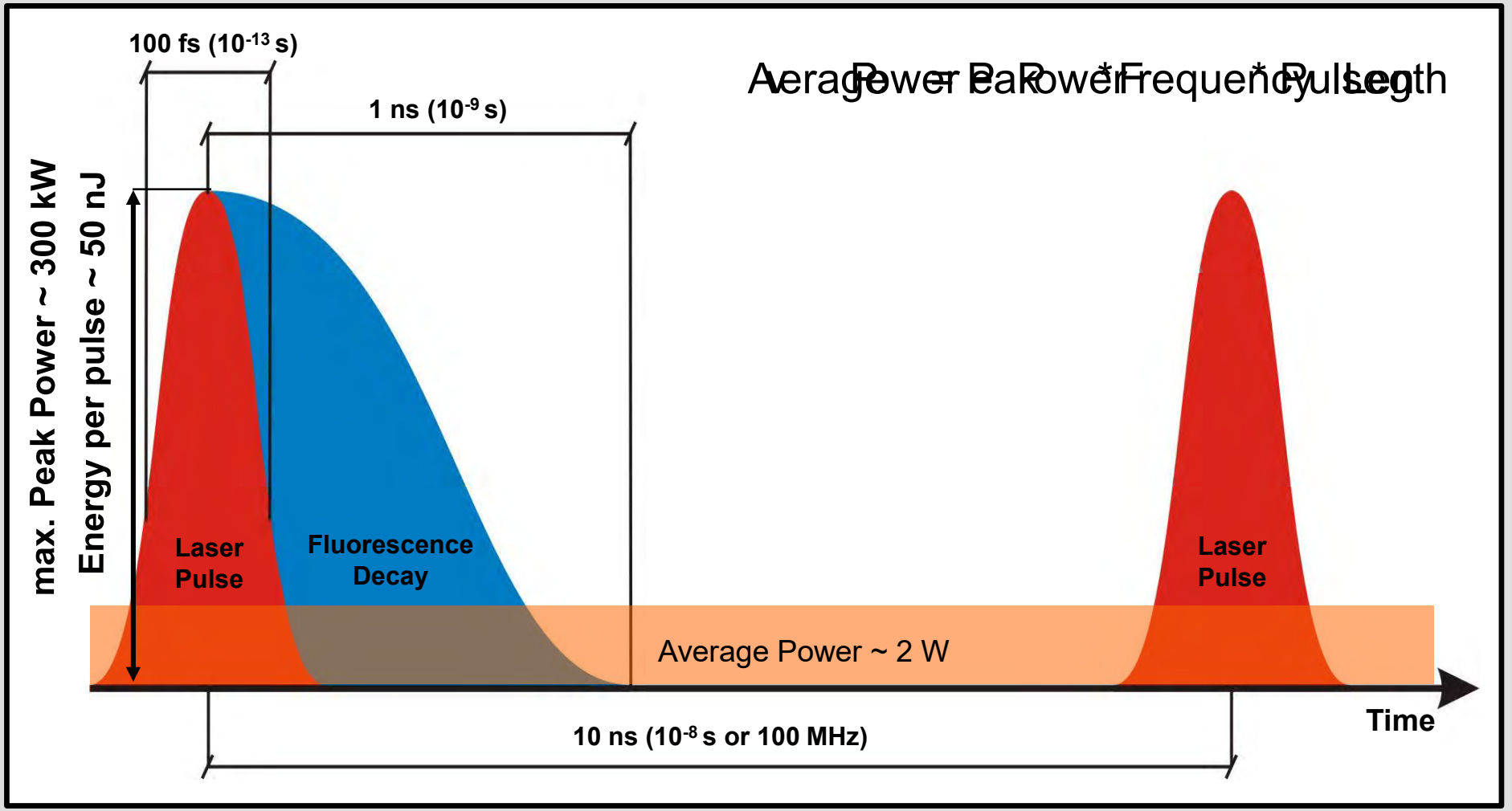
- A **single-photon** instrument is known as a “linear” imaging technique
  - Excitation of fluorophore is **directly proportional to the laser intensity**

- 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

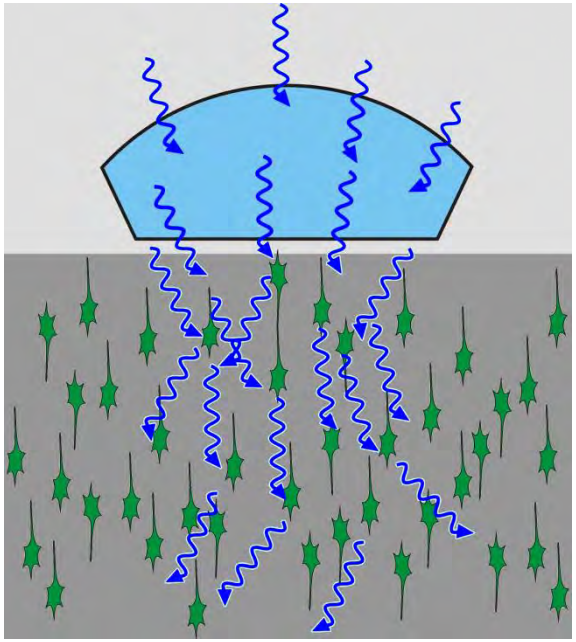
## Excitation by a Pulsed IR Laser



# Multi-Photon Microscopy

## IR Laser Yields Increased Penetration

**Short Wavelength Light**
(visible light)

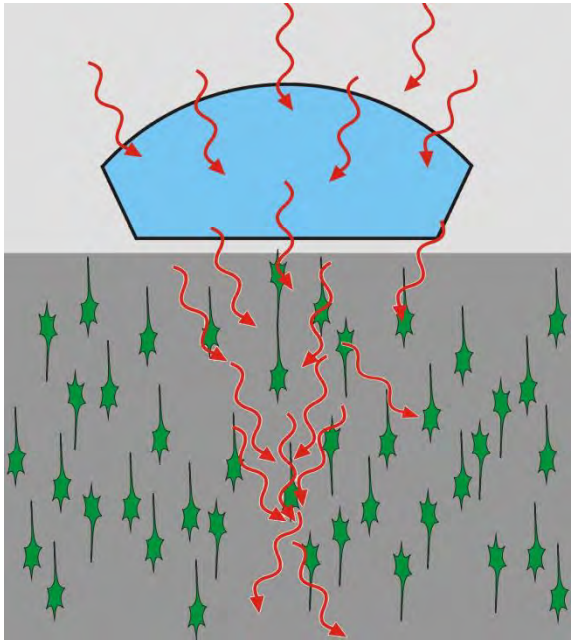


**Low penetration depth:**
Scattering of photons compromises the focusing of light

**Objective Lens**

**Thick Tissue**
(*e.g.* - brain)

**Long Wavelength Light**
(near infrared)

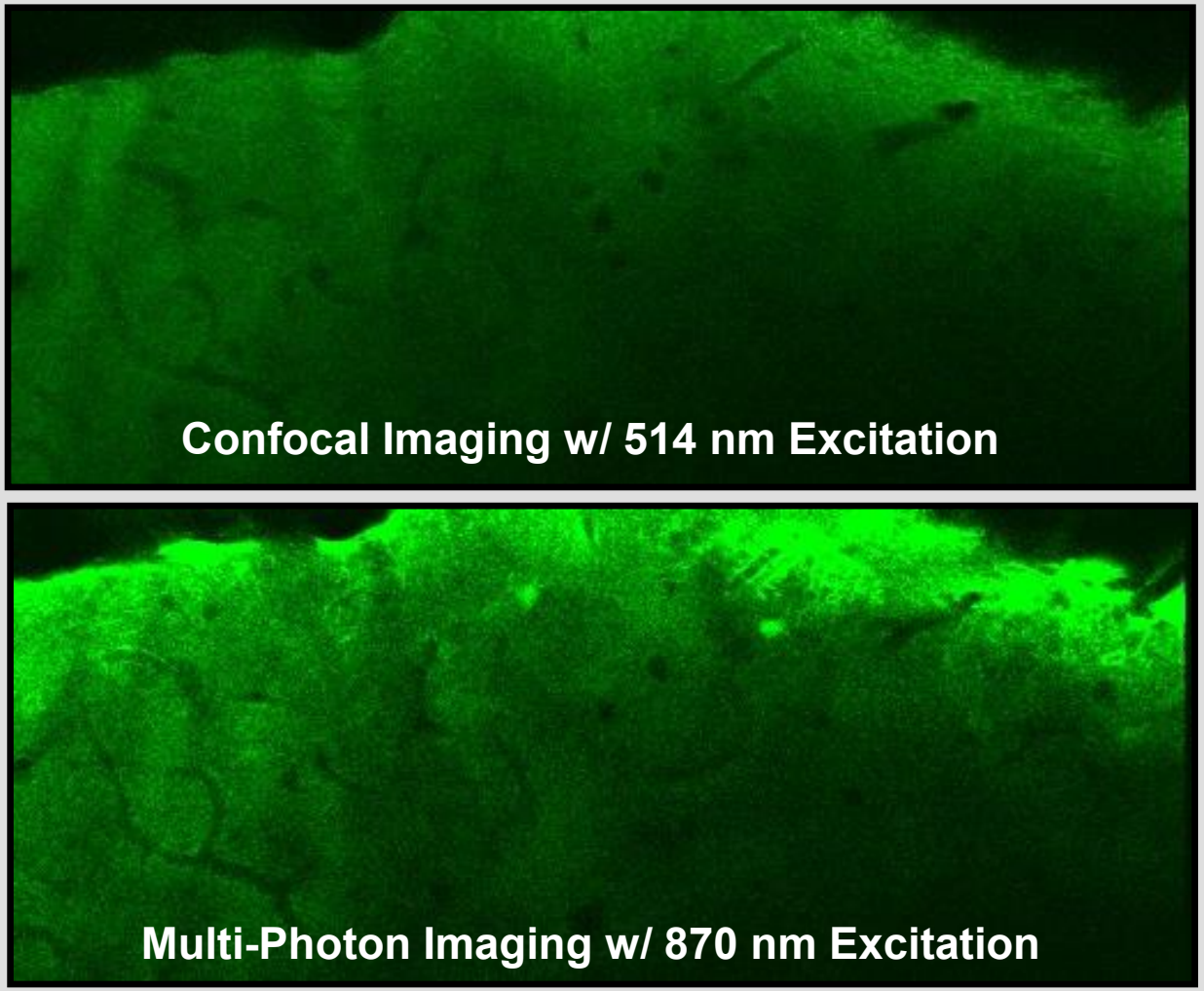


**Increased penetration depth:**
More efficient focusing due to reduced scattering of photons

# Multi-Photon Microscopy

## Depth Considerations

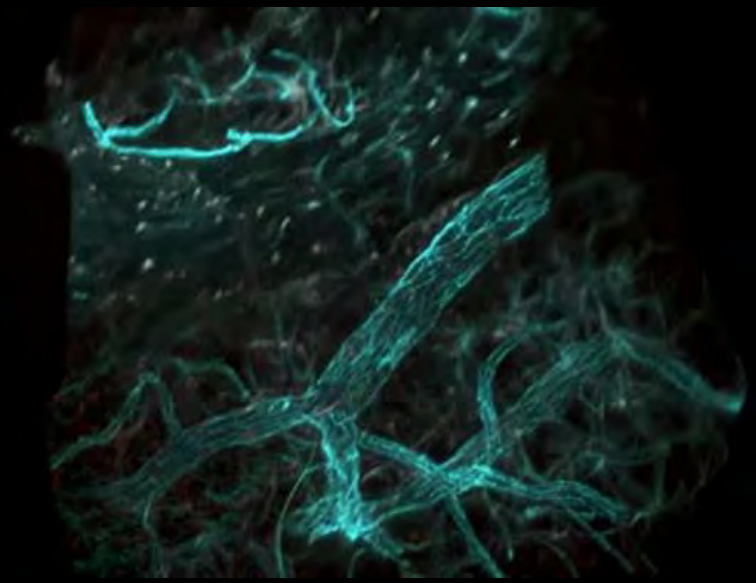
**Mouse brain: YFP-labelled tissue; 80 µm deep**



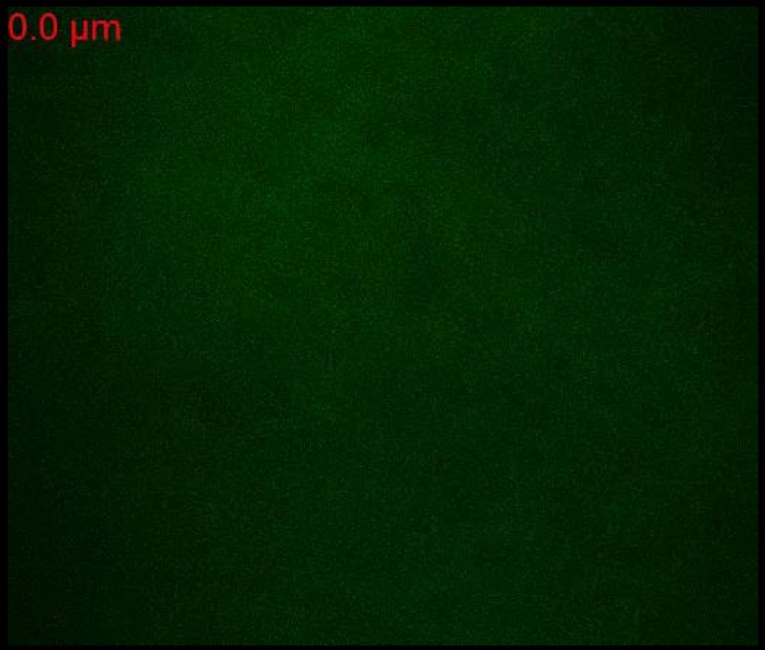
# Multi-Photon Microscopy
## Common/Unique Applications

### WHAT ARE THE USES?

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



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




**Mouse brain cleared with Sca*l*e**
**Hiroshi Hama et al *(RIKEN BSI, Wako, Japan)***

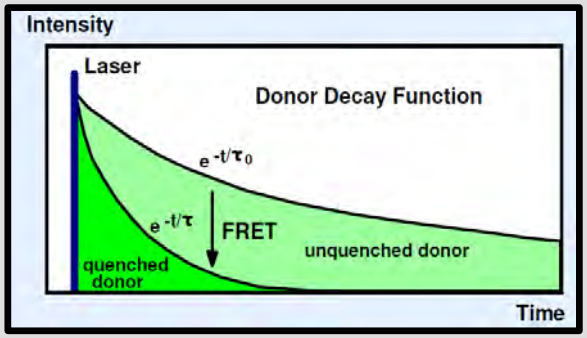
- High depth (>5 mm) imaging of **optically-cleared** tissues and organs

# Multi-Photon Microscopy

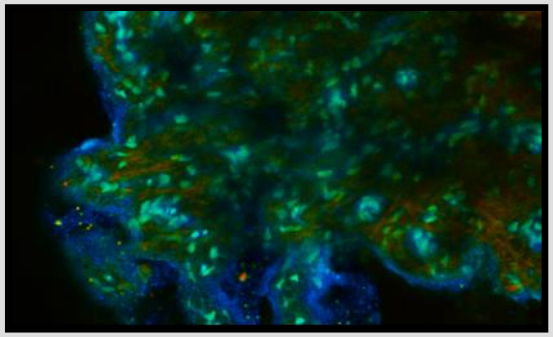
## Common/Unique Applications

## WHAT ARE THE USES?

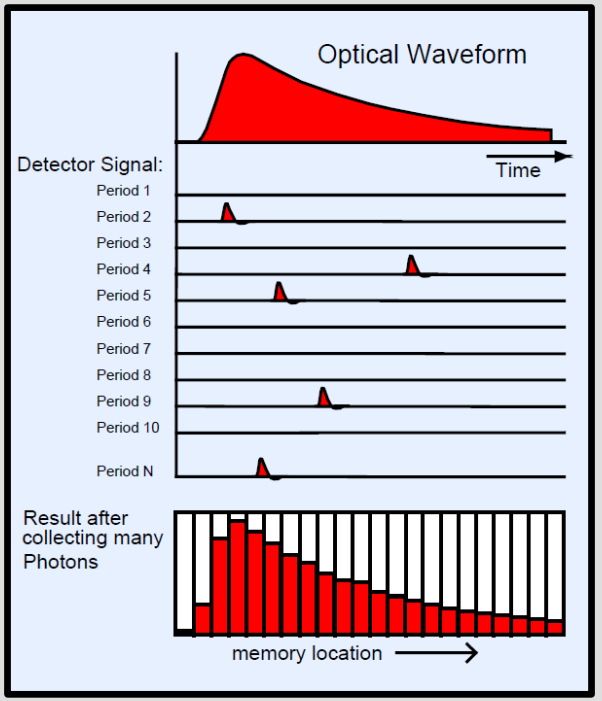
- Pulsed lasers can enable measurement of fluorescence lifetime (**FLIM**)
  - Photon counting is used to plot **temporal** distribution of the excited state (~100s of ps)
  - Repeating counts at each scan pixel reveals **spatial** distribution of lifetimes
  - Lifetimes are **sensitive to microenvironment**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

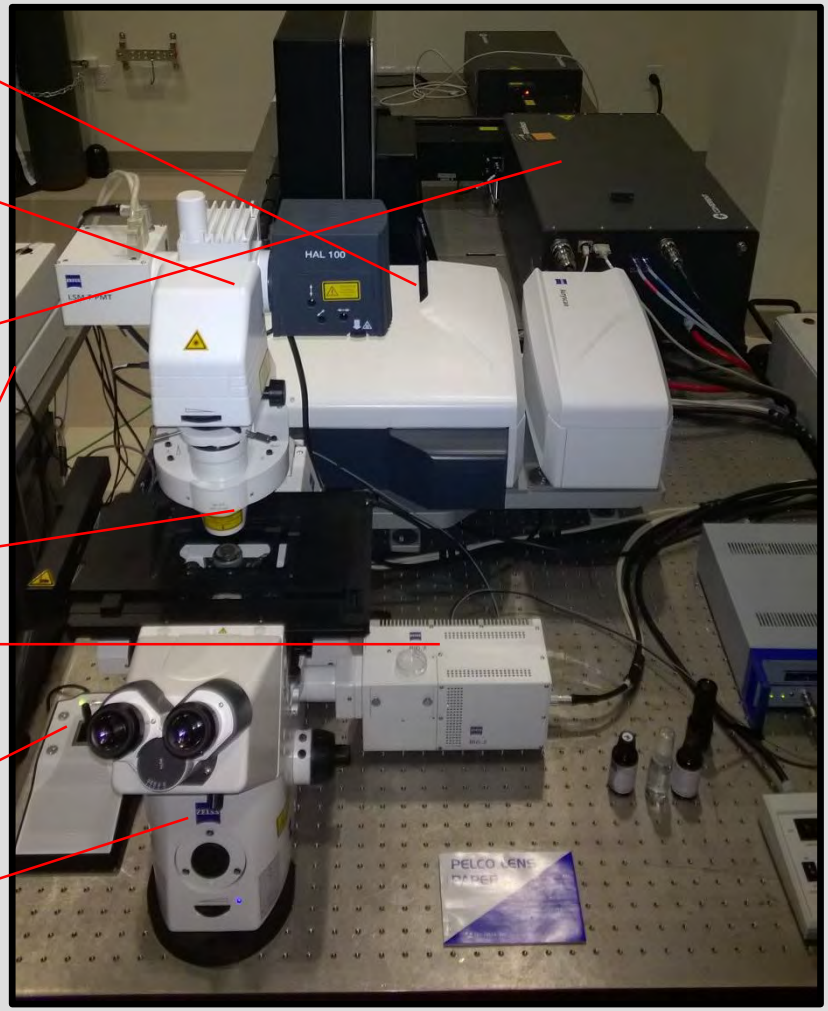
Incubation accessories (temperature, $CO_2$)

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 inverted microscope
(with Definite Focus)



# Multi-Photon Microscopy

## Practical Perspective

### ADVANTAGES:

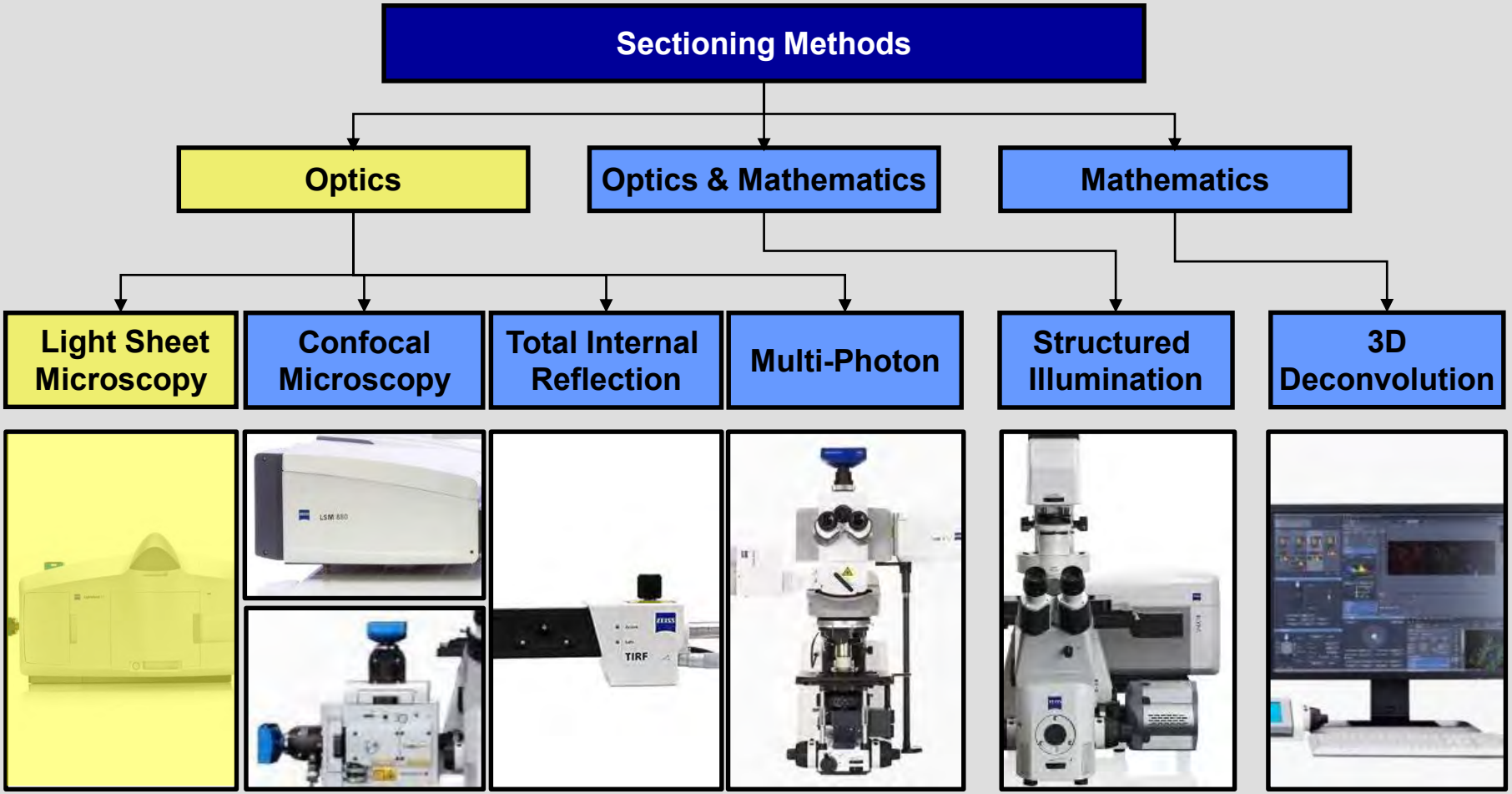
- Permits **deep imaging of tissue or whole animal** due to low scattering of IR light and non-descanned detection (no pinhole)
- IR **light less toxic to live** cells/organisms
- Optical sectioning occurs entirely through excitation; **limits bleaching to the focal plane only**

### 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

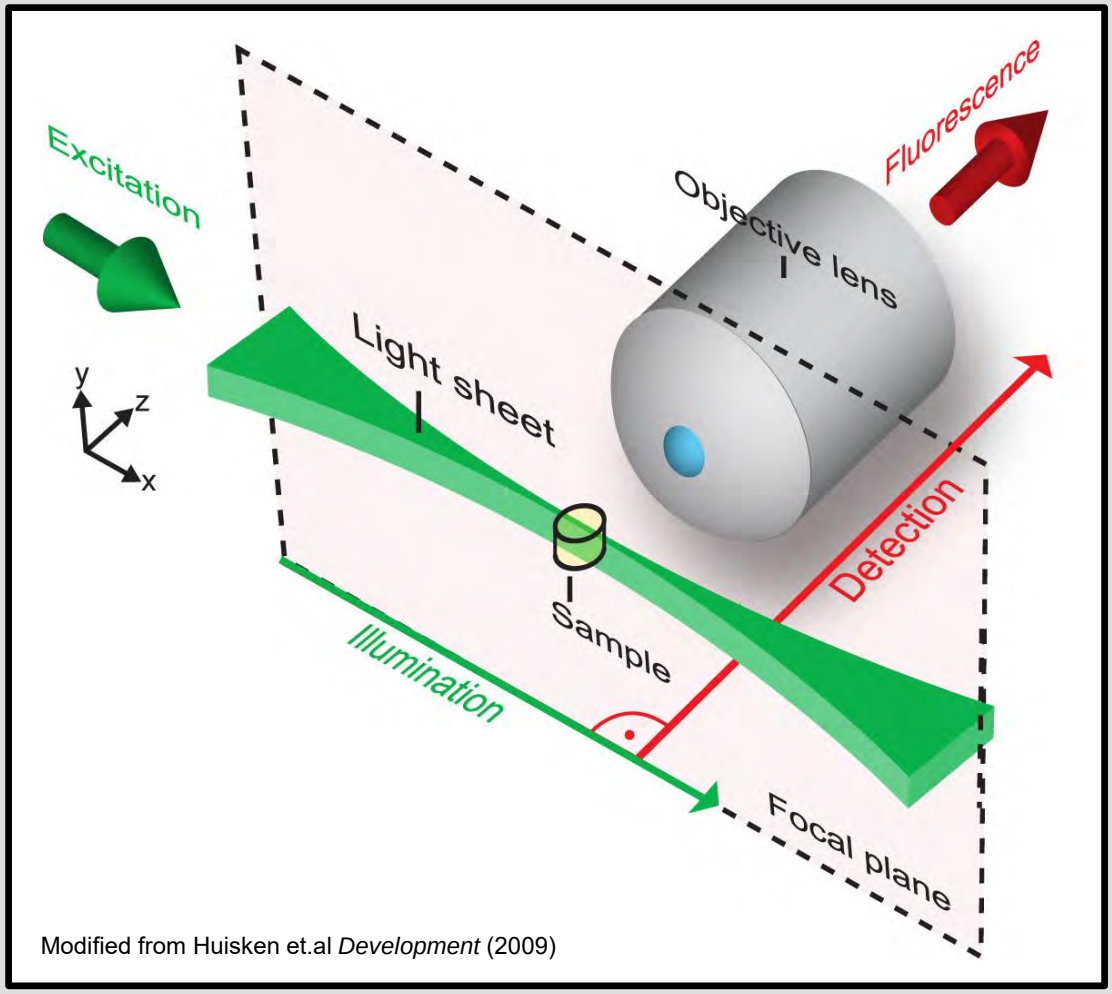


# Light Sheet Fluorescence Microscopy

## Fundamental Characteristics of LSFM

## PRINCIPLES

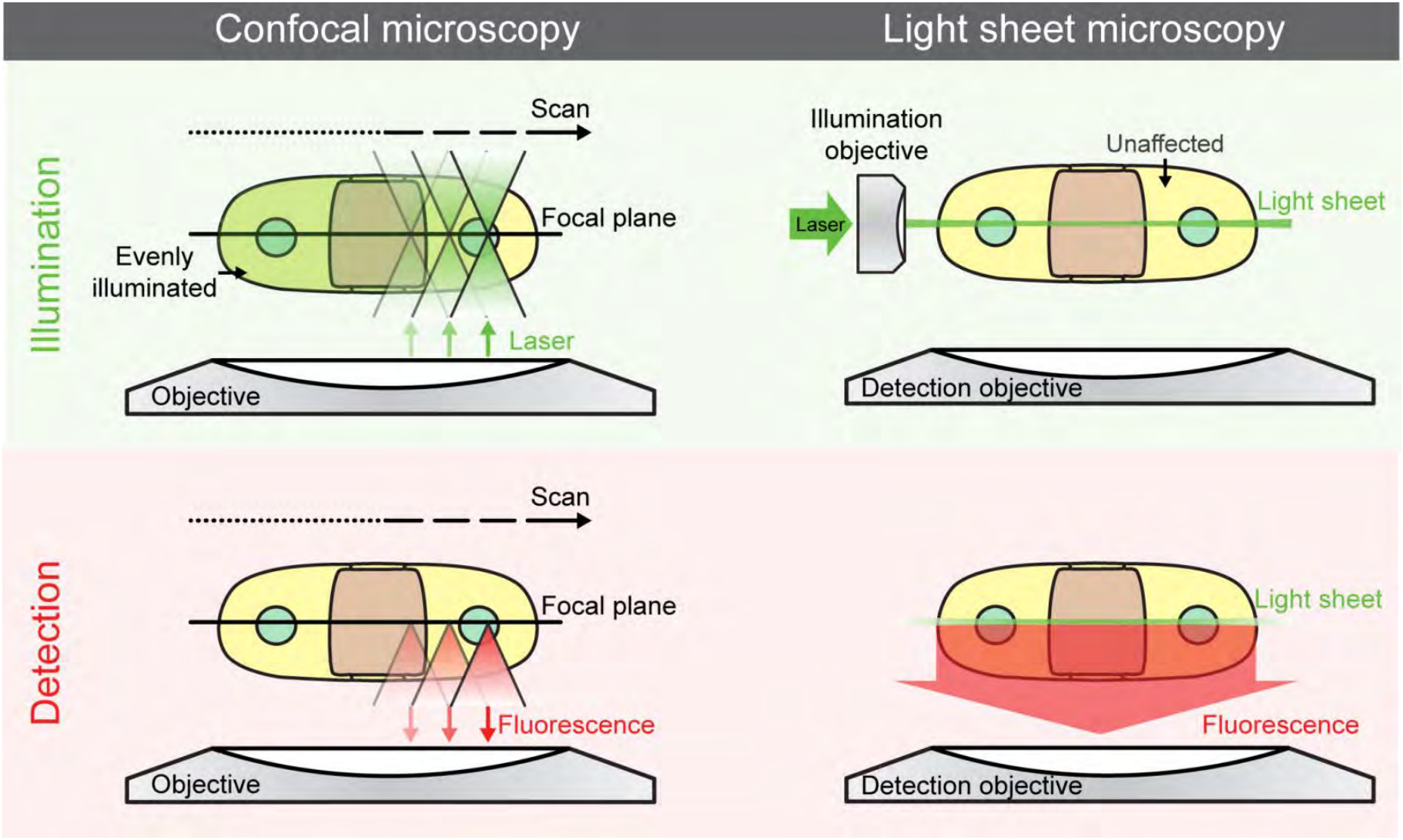
- Orthogonal light paths for illumination and detection
- Horizontal microscope configuration
- Whole field of view illuminated; camera-based collection




Modified from Huisken et.al *Development* (2009)

# Light Sheet Fluorescence Microscopy

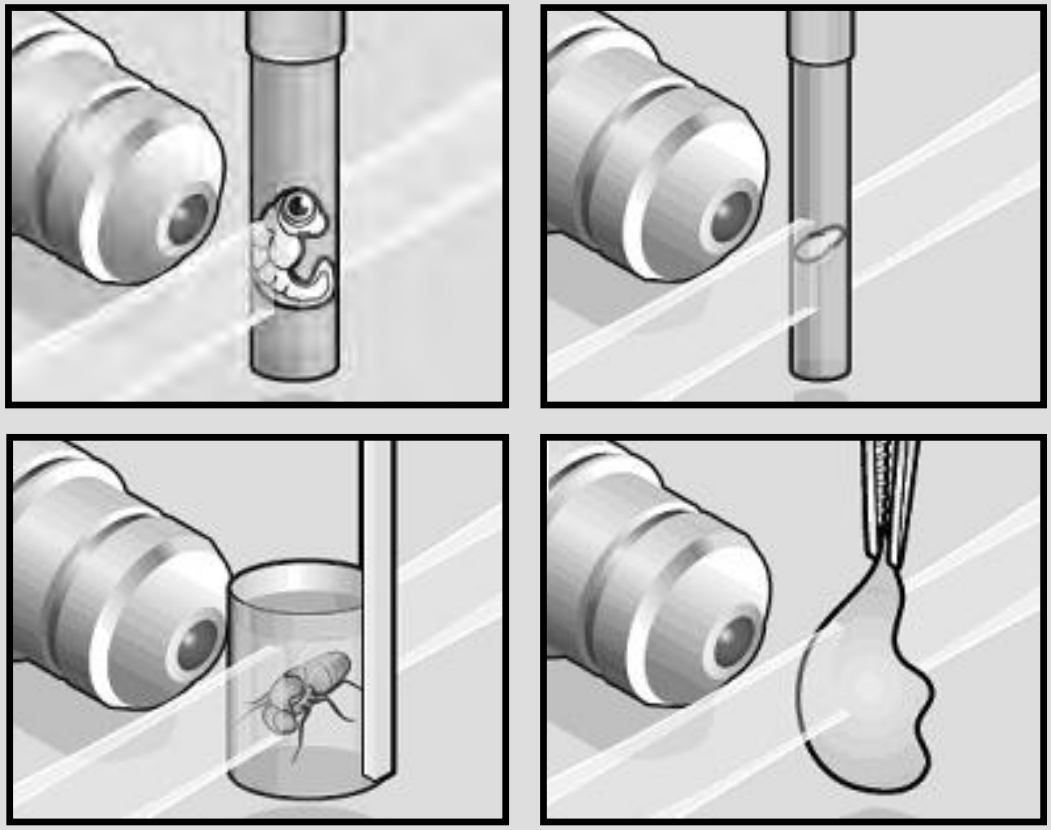
## Comparison to Confocal Microscopy



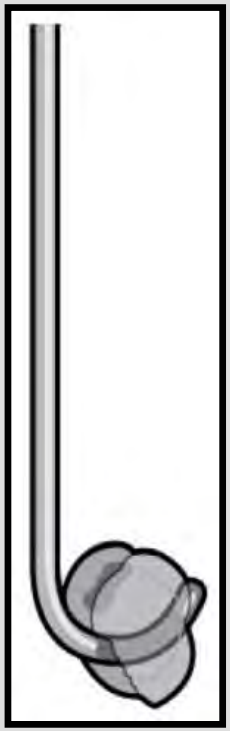
# Light Sheet Fluorescence Microscopy

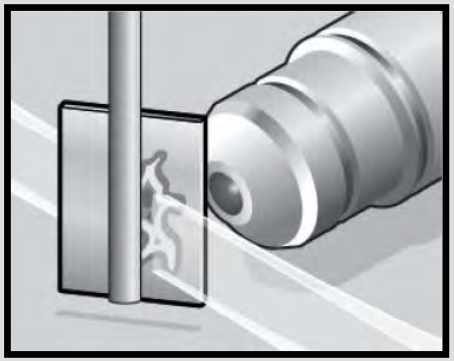
## Samples Types and Preparation

Reynaud et al., HFSP J. (5) 2008



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



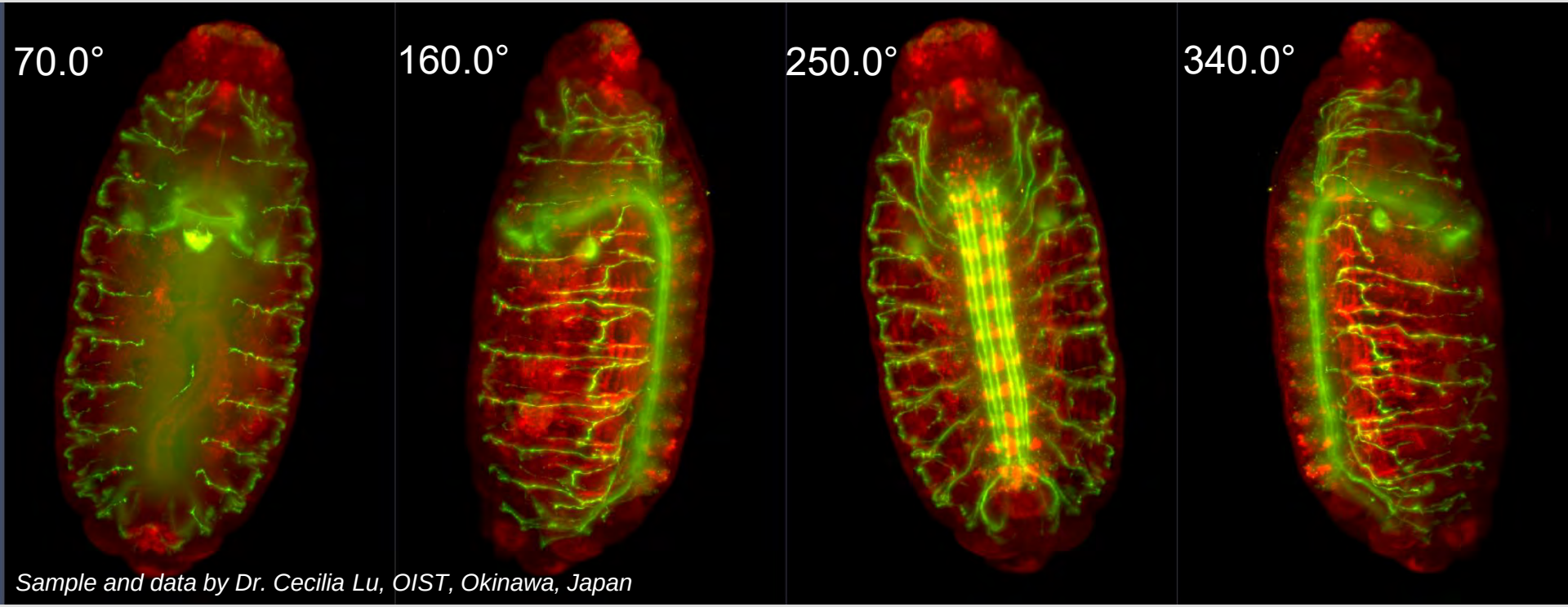


*(Compromised solution for coverslipped samples)*

Adapted agarose capillaries, cups, hooks, clamps, and adhesives permit a variety of sample formats

# Light Sheet Fluorescence Microscopy

## Multiview Imaging

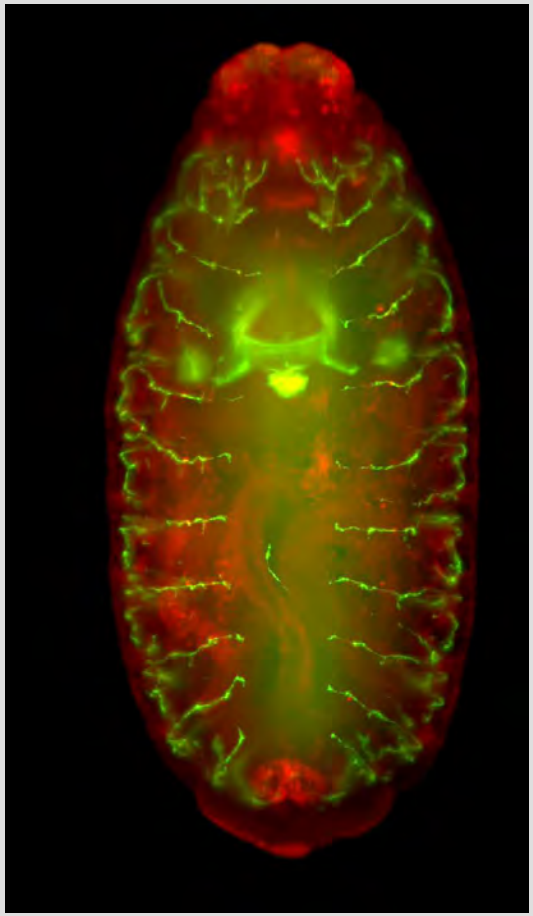



- Larger samples benefit from applying illumination features (*e.g.* – two-sided illumination, pivot scanning) to different rotational views
  - Complementary information from **unique viewing angles**; **resolution gain**

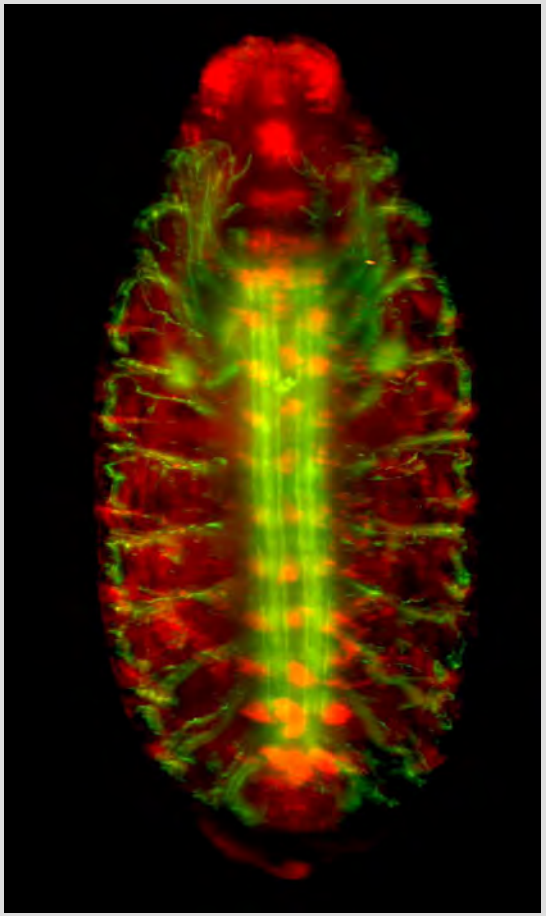
# Light Sheet Fluorescence Microscopy

## Multiview Imaging – Fusion

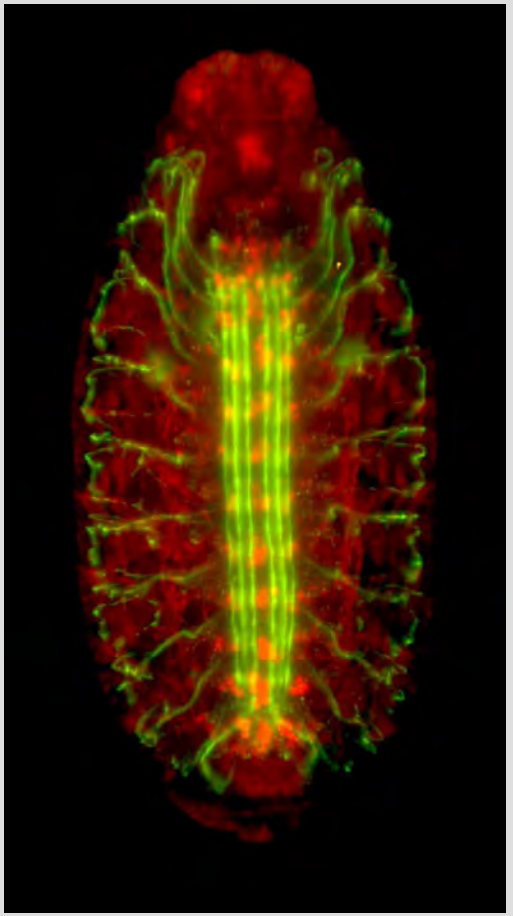
**Z-Stack View 1**



**Registration + Fusion**



**Z-Stack View 2**

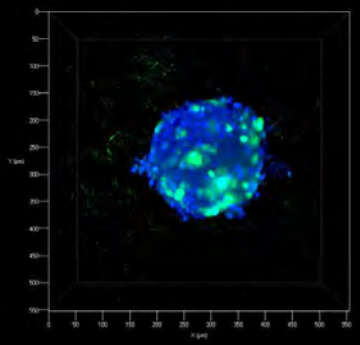


# Light Sheet Fluorescence Microscopy

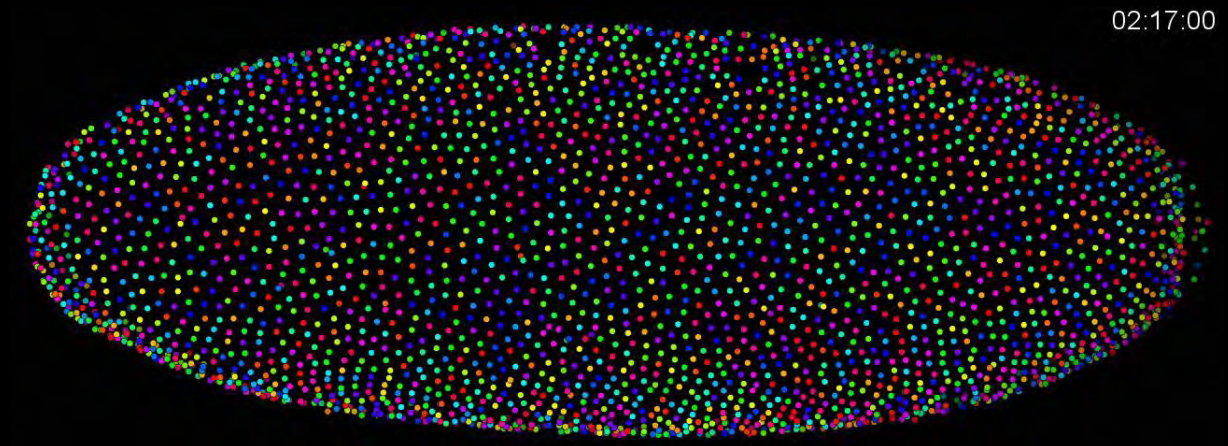
## Common/Unique Applications

## WHAT ARE THE USES?

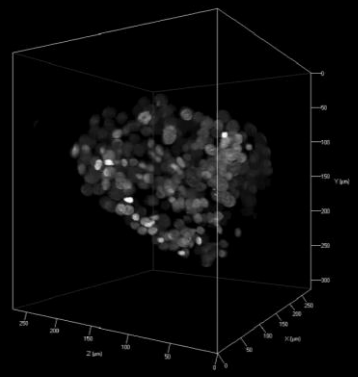
- Tracking and invasion of cells in unperturbed **3D culture** models (spheroids, seeded scaffolds, invasion assays) and intact organisms



**Spheroid cultures**

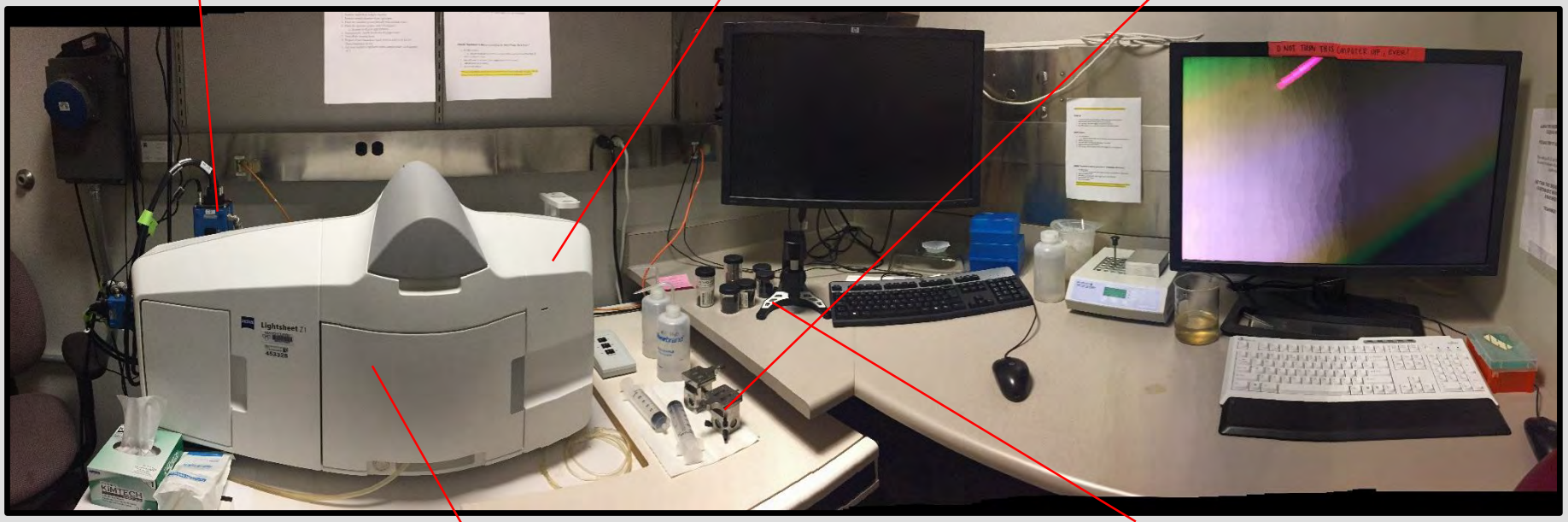



**Drosophila embryo lineage reconstruction (H2A), 30 s interval over 11 hr, 10 frame avg**
**Amat et al. (*Nature Methods 2014)***



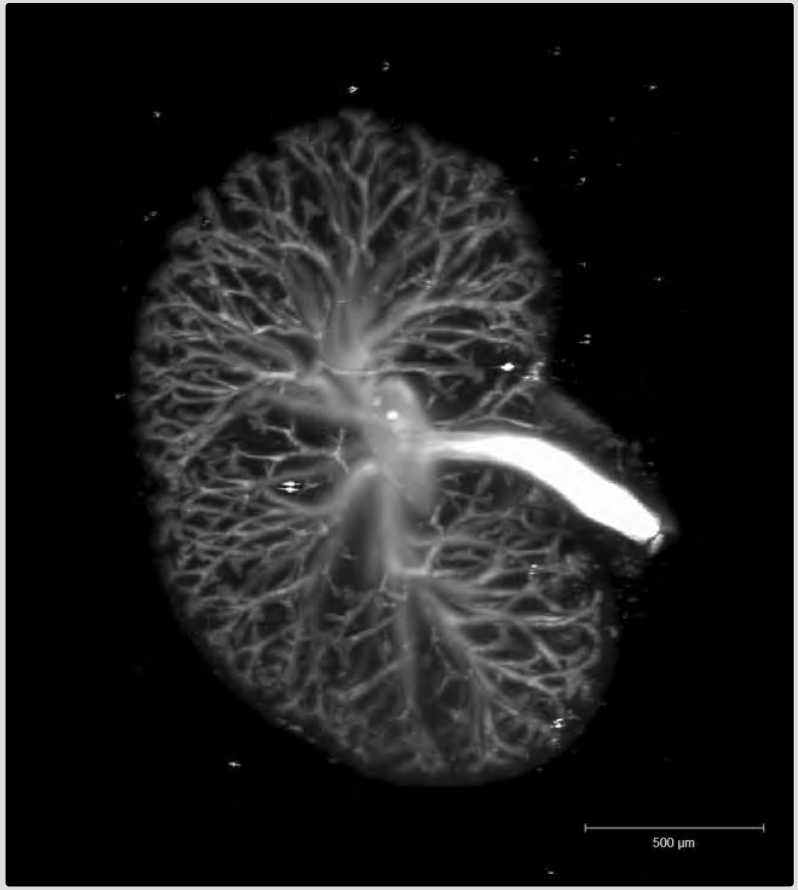
# Light Sheet Fluorescence Microscopy

## Typical System Footprint – ZEISS Lightsheet Z.1



# Light Sheet Fluorescence Microscopy

## Practical Perspective




**E15.5 mouse kidney cleared with glycerol (blood vessels, eGFP)**
**Renata Prunskaite-Hyyryläinen *(Univ. Oulu, Finland)***

## 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**

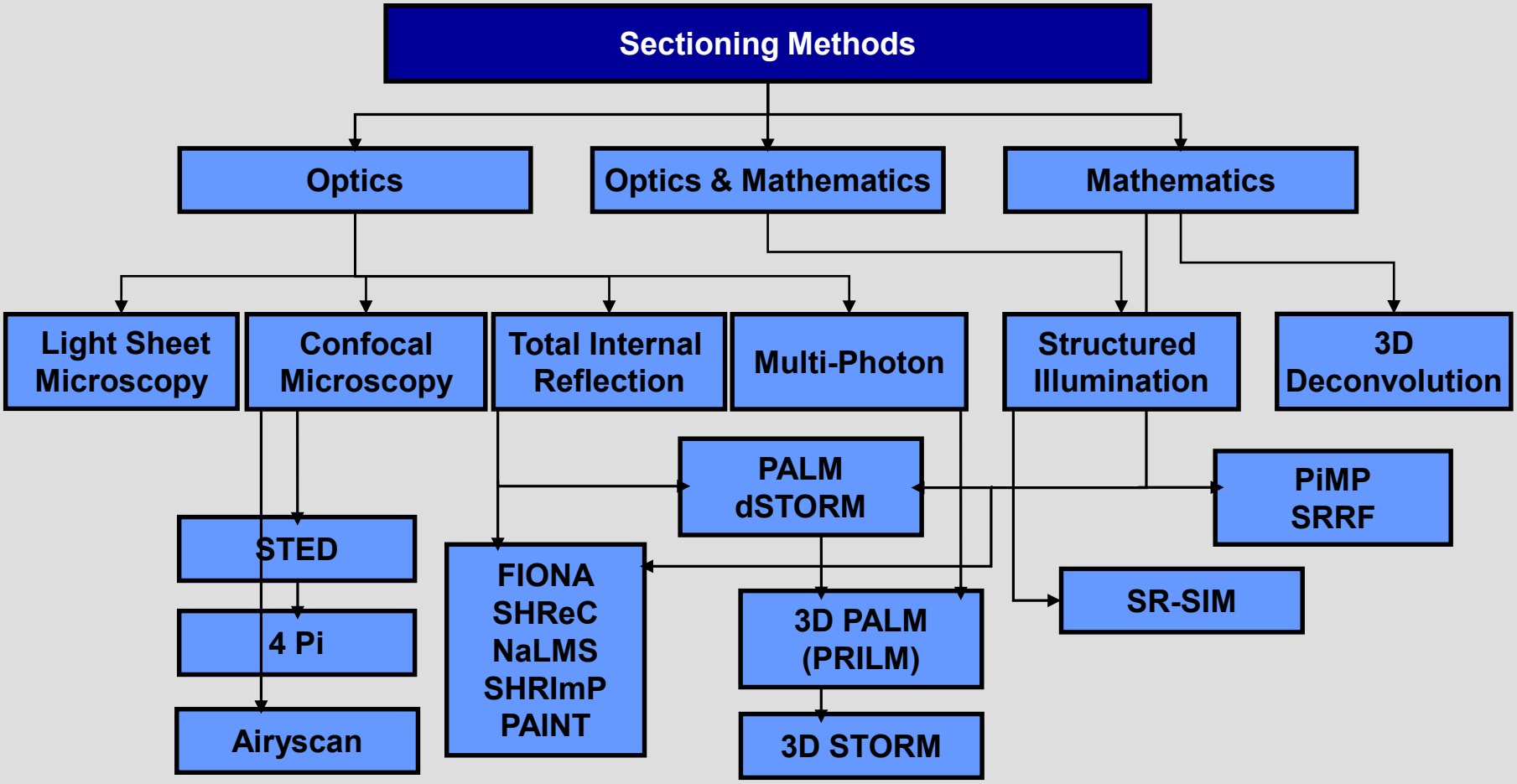
# Outline of Discussion

## 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

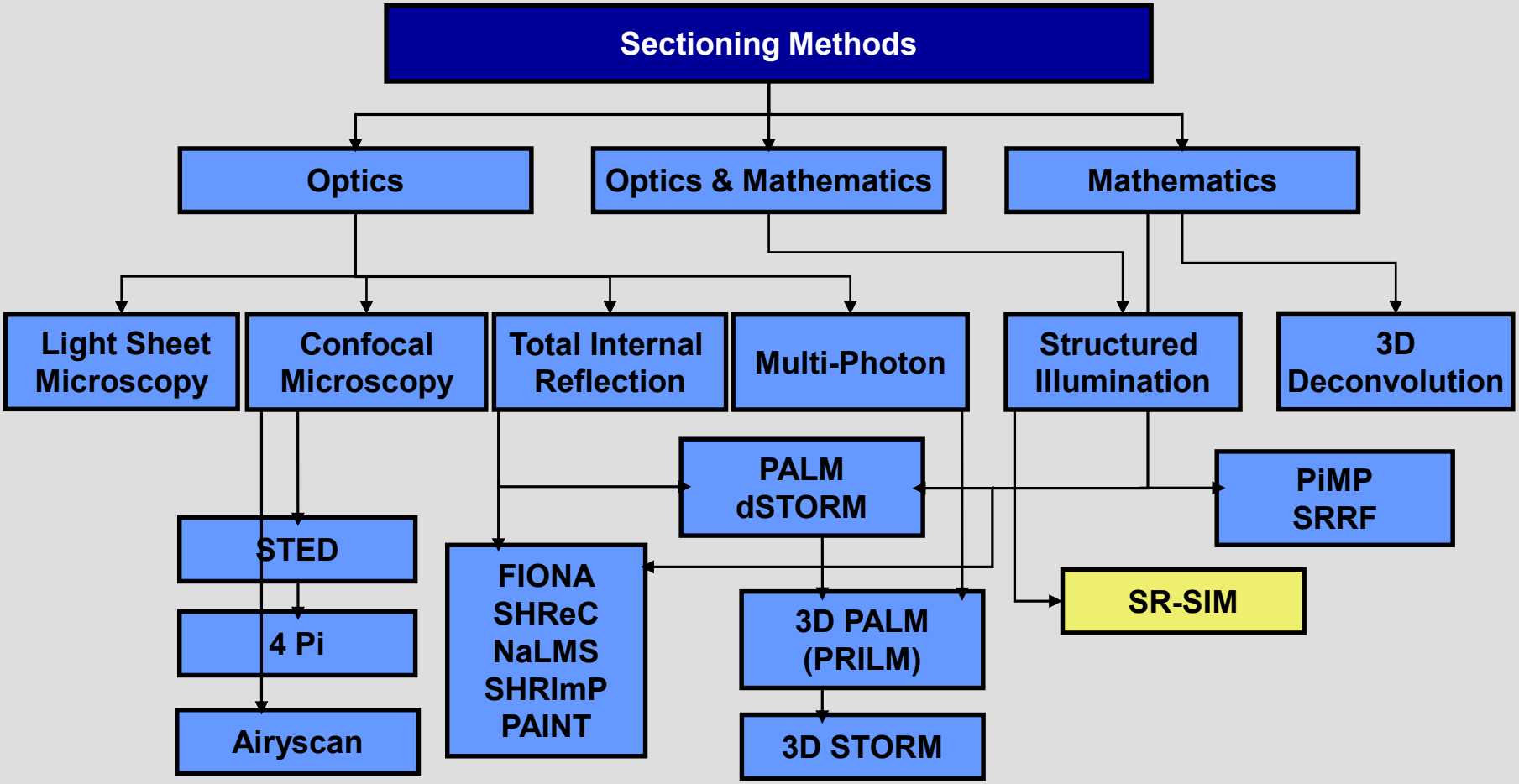
# Optical Sectioning Techniques

## Common Superresolution Approaches



# Optical Sectioning Techniques

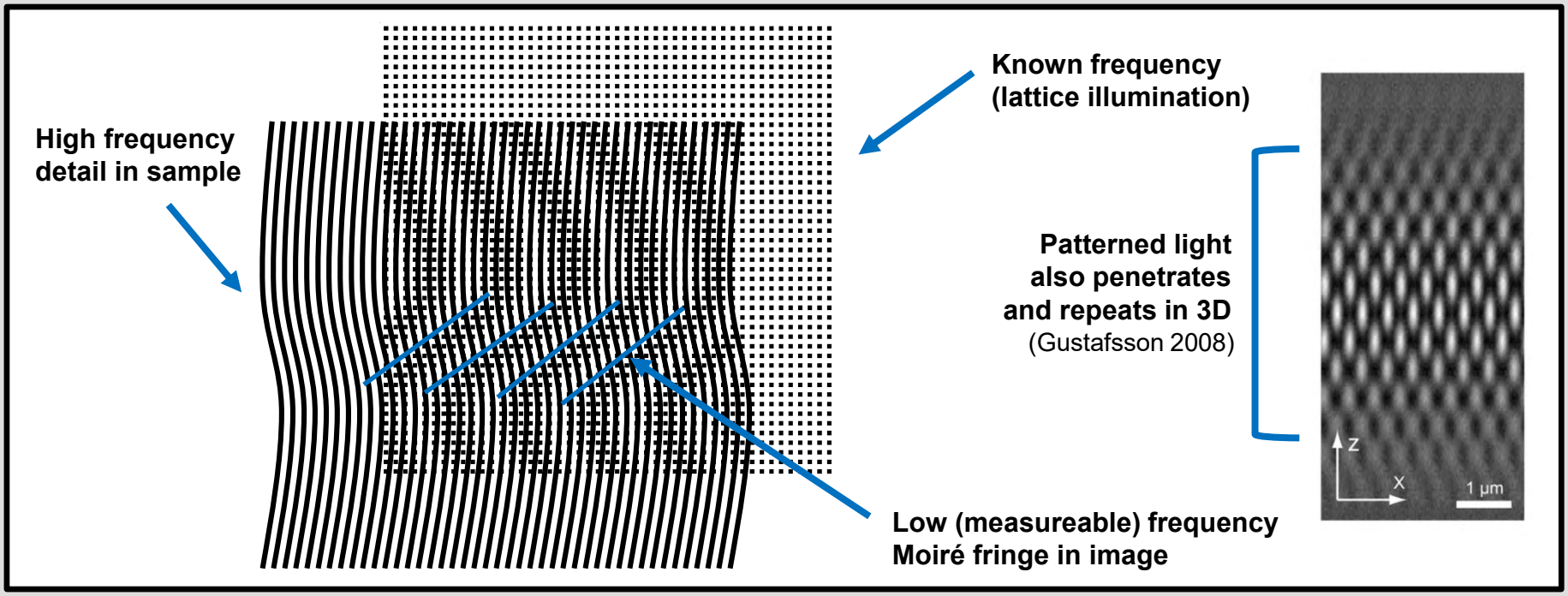
## Common Superresolution Approaches



# Structured Illumination Microscopy

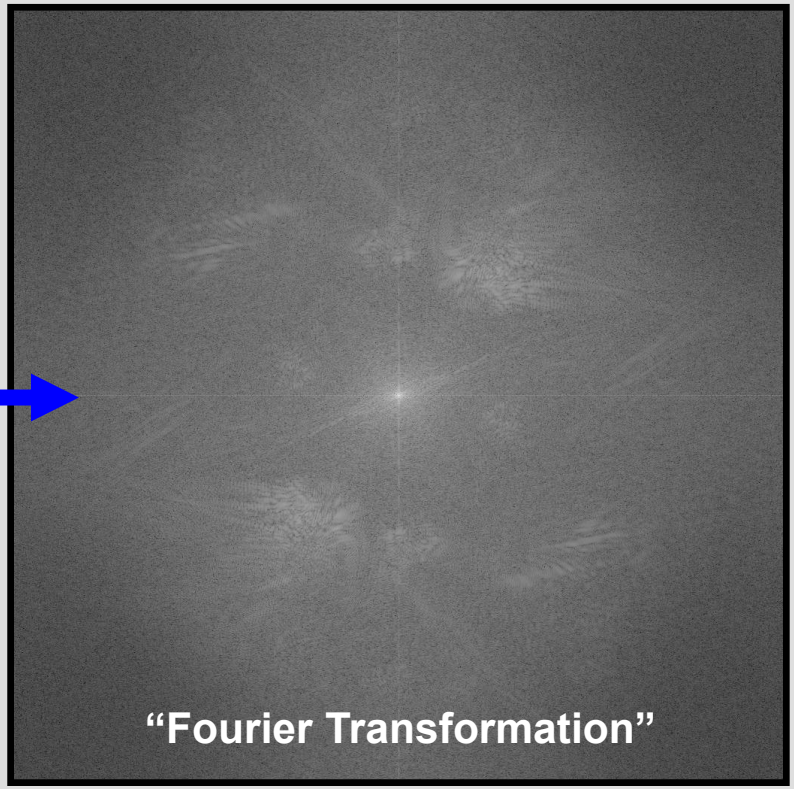
## Basic Principles

- 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

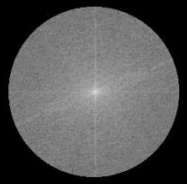
## Understanding Frequency Space

# Structured Illumination Microscopy

## Fourier Spectrum – Objective Limit

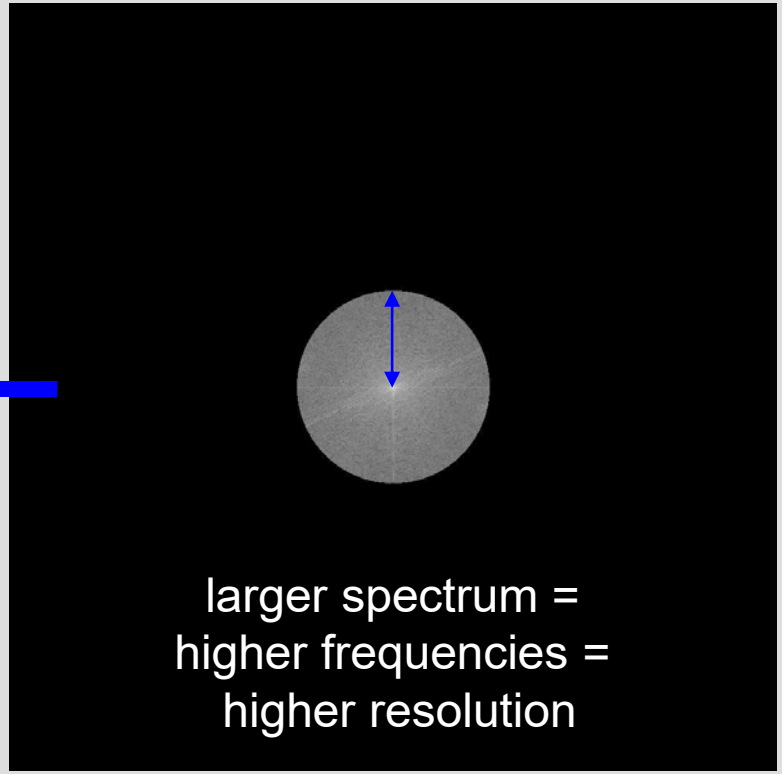
Optical system always performs its own ***inverse*** Fourier transformation from back focal plane to image plane
(Note limited radius of objective)



smaller spectrum =
lower frequencies =
lower resolution

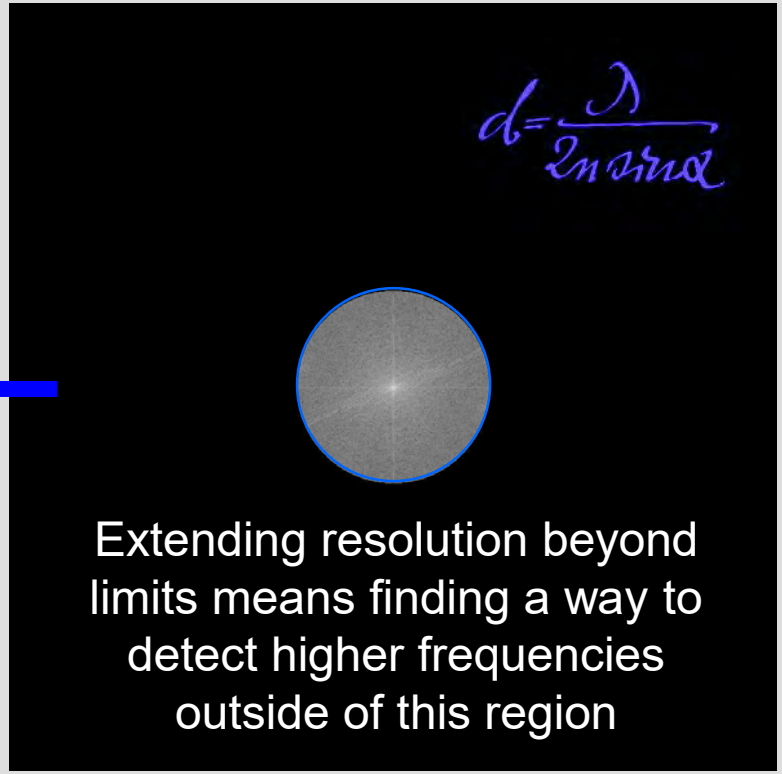
# Structured Illumination Microscopy

## Fourier Spectrum – Objective Limit



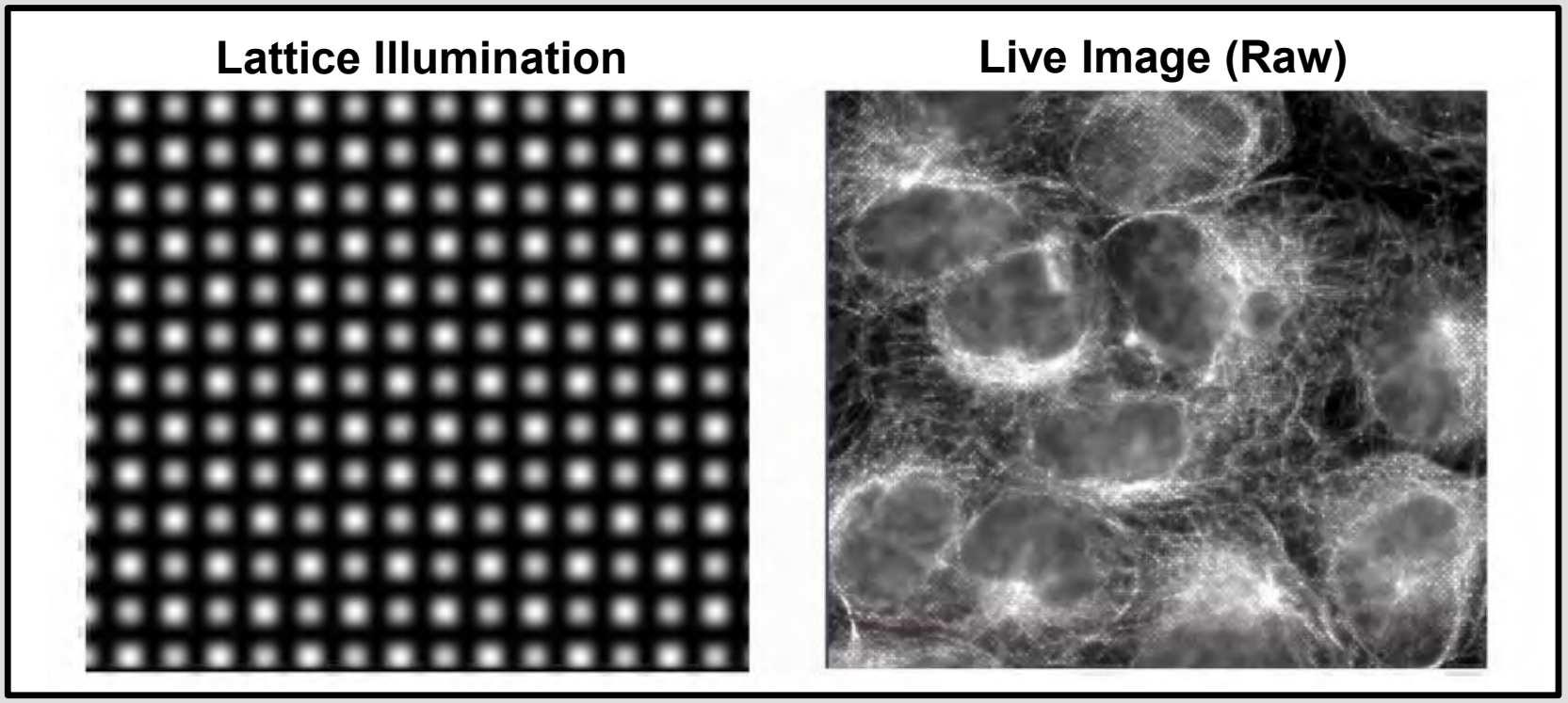
# Structured Illumination Microscopy

## Fourier Spectrum – Objective Limit



# Structured Illumination Microscopy

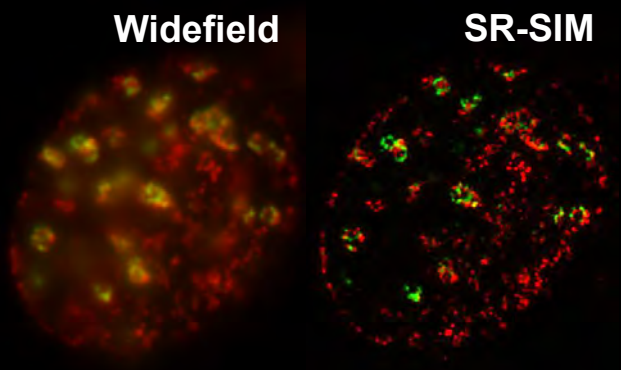
## Acquisition Process




- 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

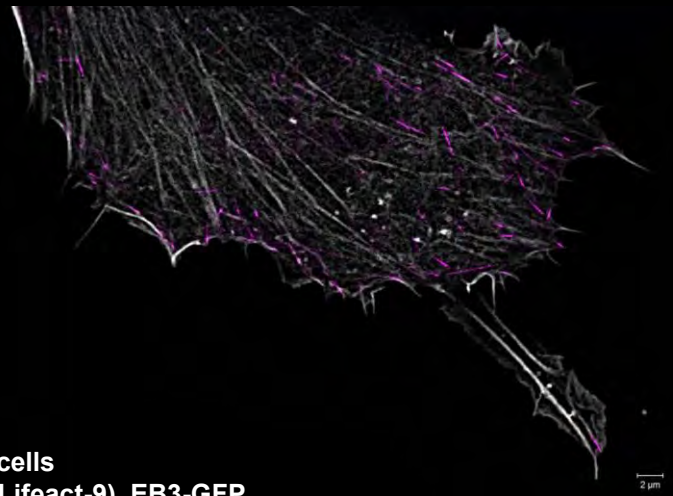
## Common/Unique Applications



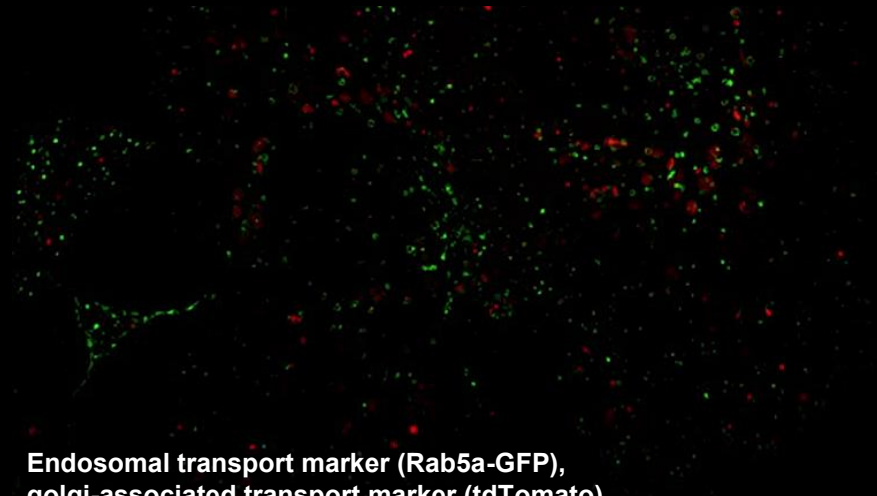

**Myoblast C2C12, BrdU + Dnmt1**
**Schermelleh lab *(LMU Munich)***

## 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



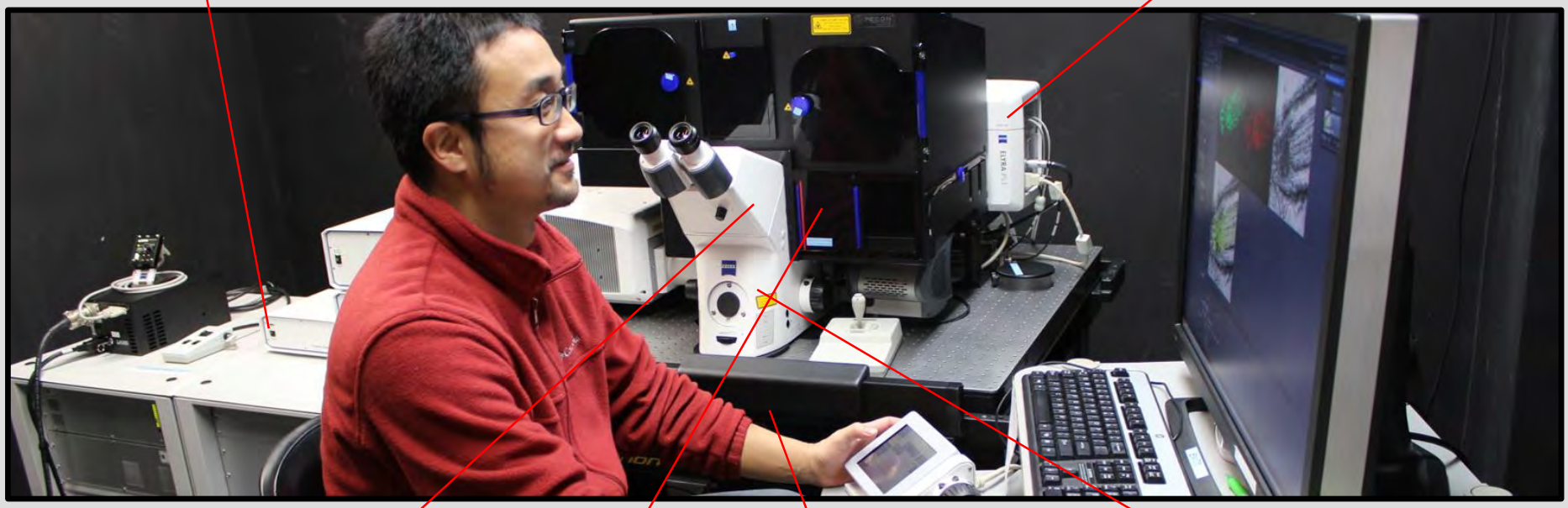
**U2Os cells**
**actin (Lifeact-9), EB3-GFP**



**Endosomal transport marker (Rab5a-GFP),**
**golgi-associated transport marker (tdTomato)**

# Structured Illumination Microscopy

## Typical System Footprint – ZEISS Elyra 7 (SIM)



# Structured Illumination Microscopy

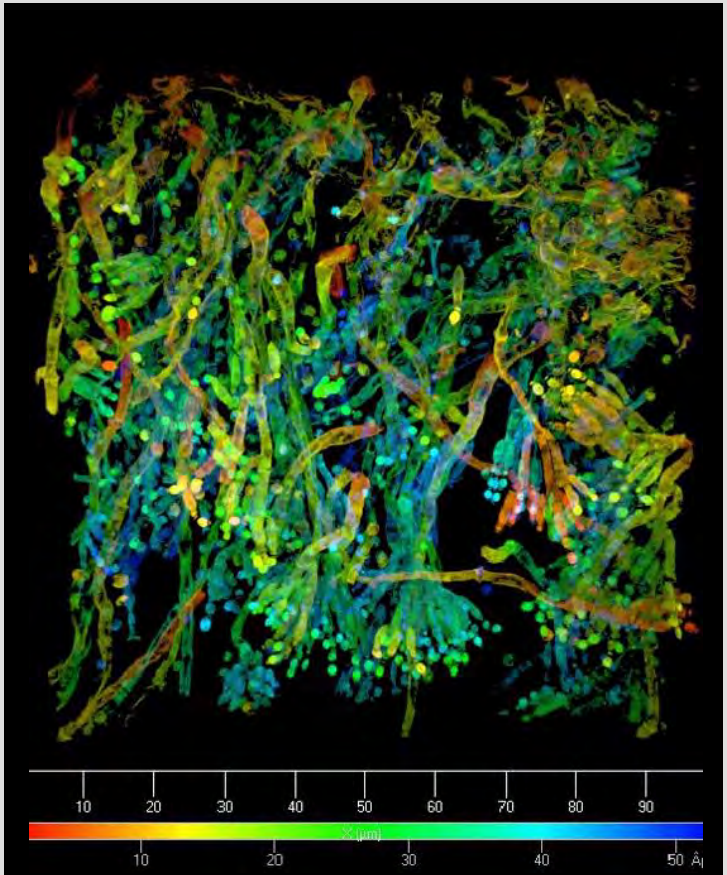
## Practical Perspective

### 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:

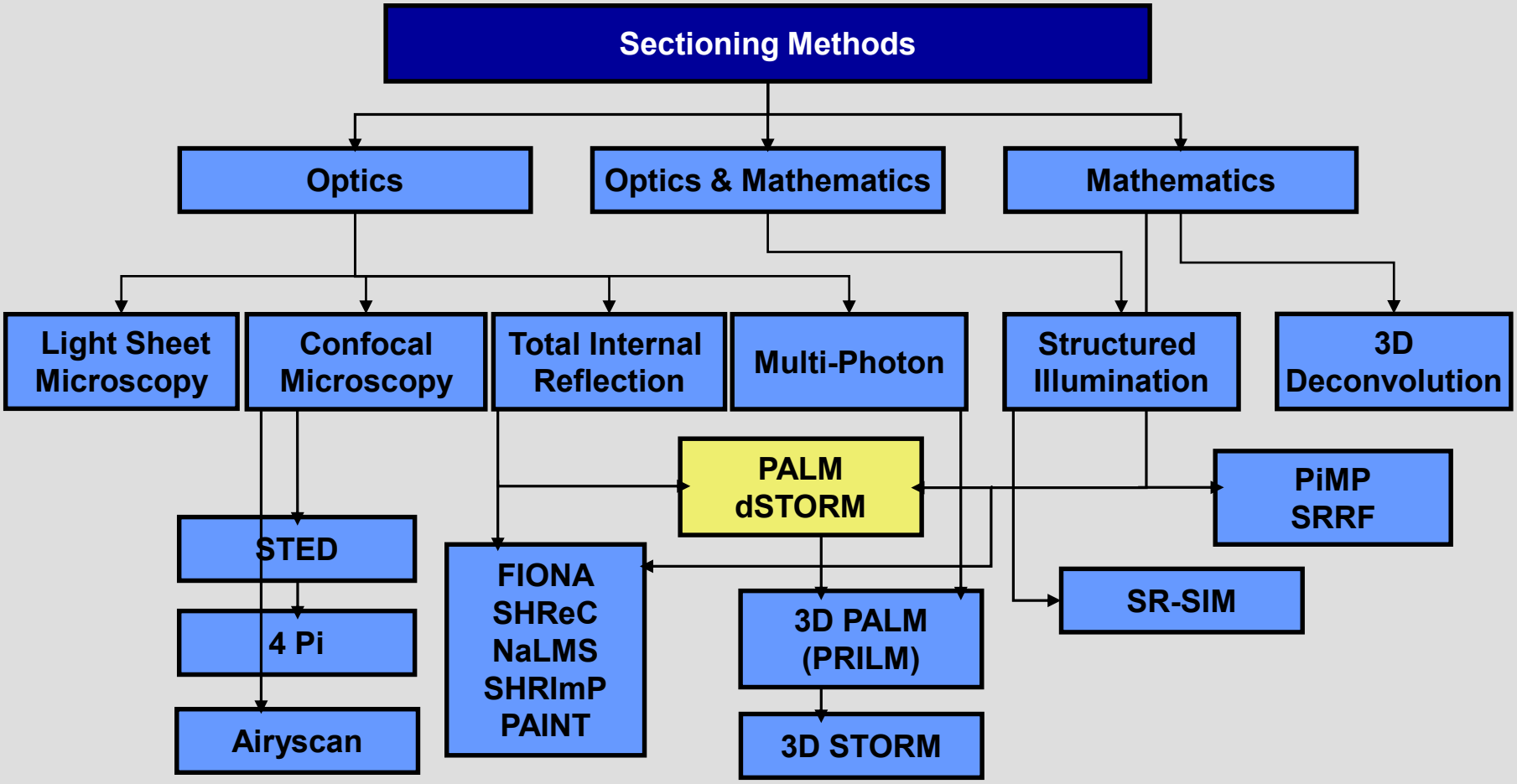
- Largely **incompatible with thicker (>50 micron) samples** due to scattering and loss of patterned illumination with depth




**Penicillium autofluorescence, depth-coded**
**90 x 90 x 50 um, 422 Z planes**

# Optical Sectioning Techniques

## Common Superresolution Approaches



# Localization Microscopy

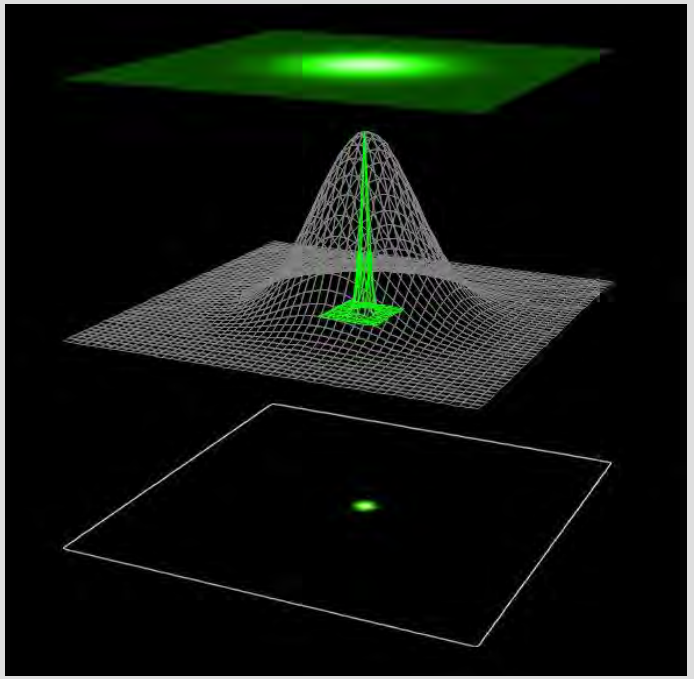
## Basic Principles

- 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

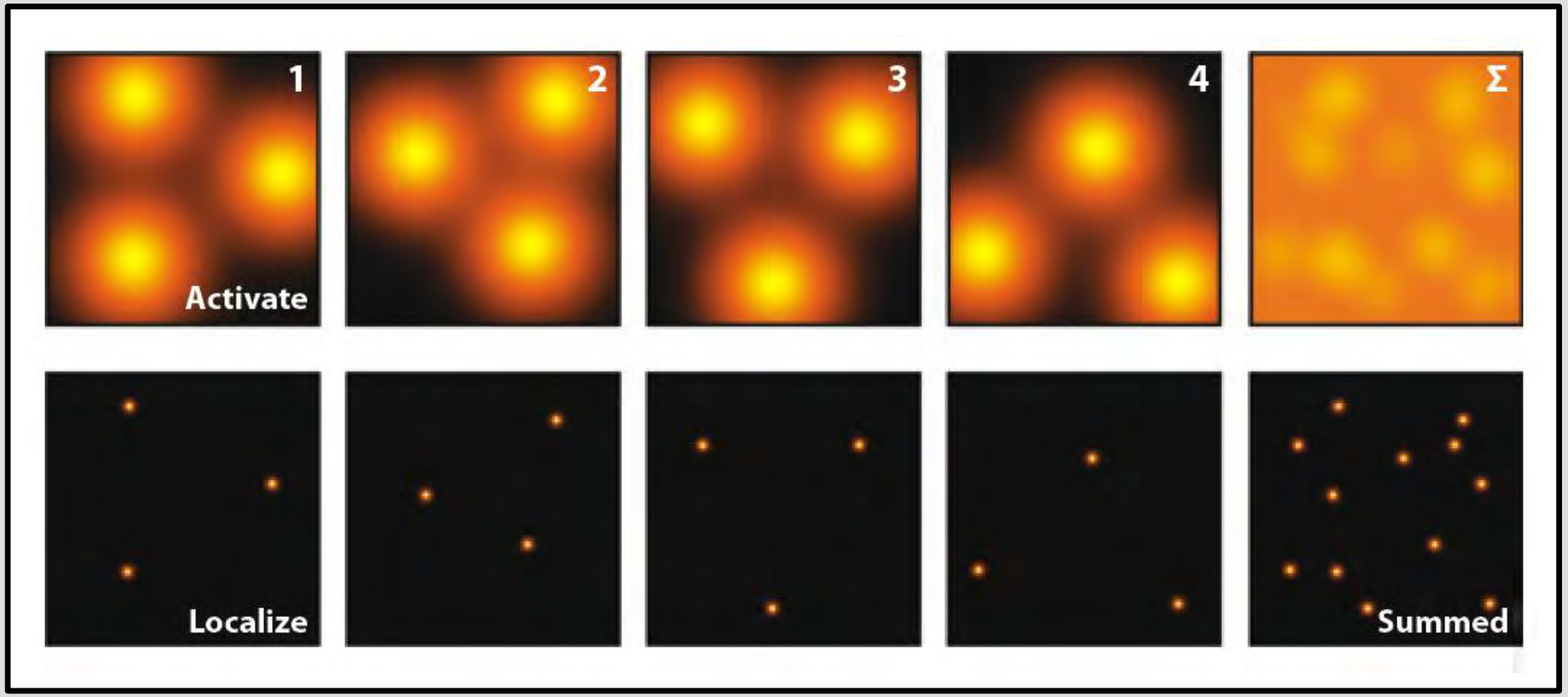
- Lateral resolution up to 10 times greater than widefield imaging

- TIRF-based illumination yields excellent Z-resolution (~100 nm)



# Localization Microscopy

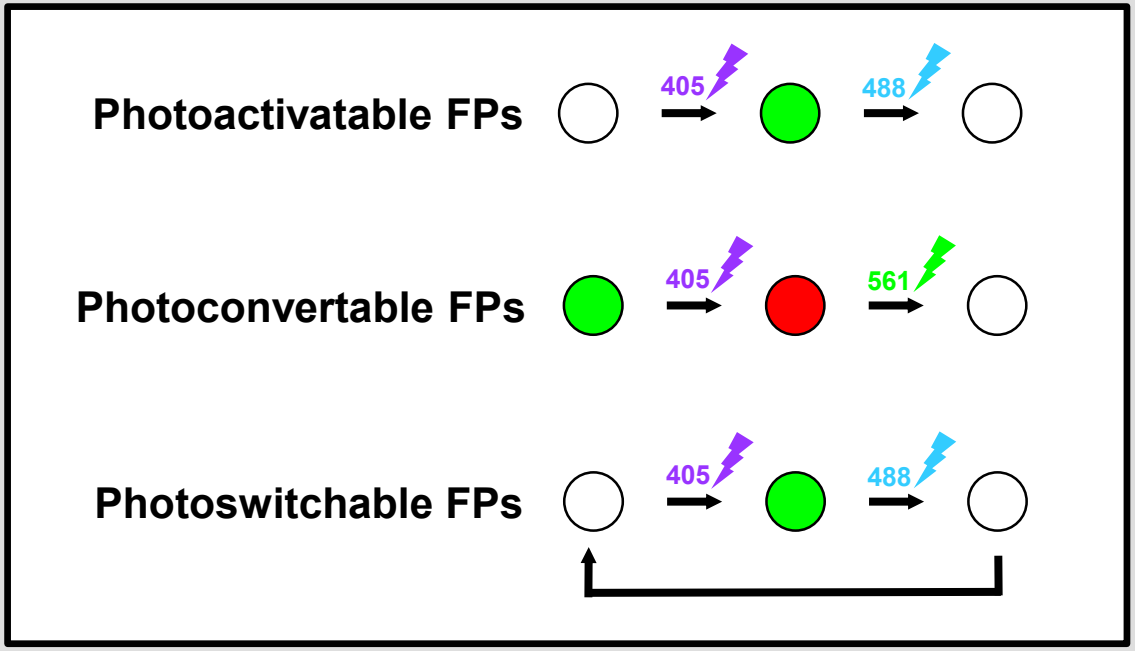
## Single Molecule Localization



# Localization Microscopy

## PALM Experiment Design

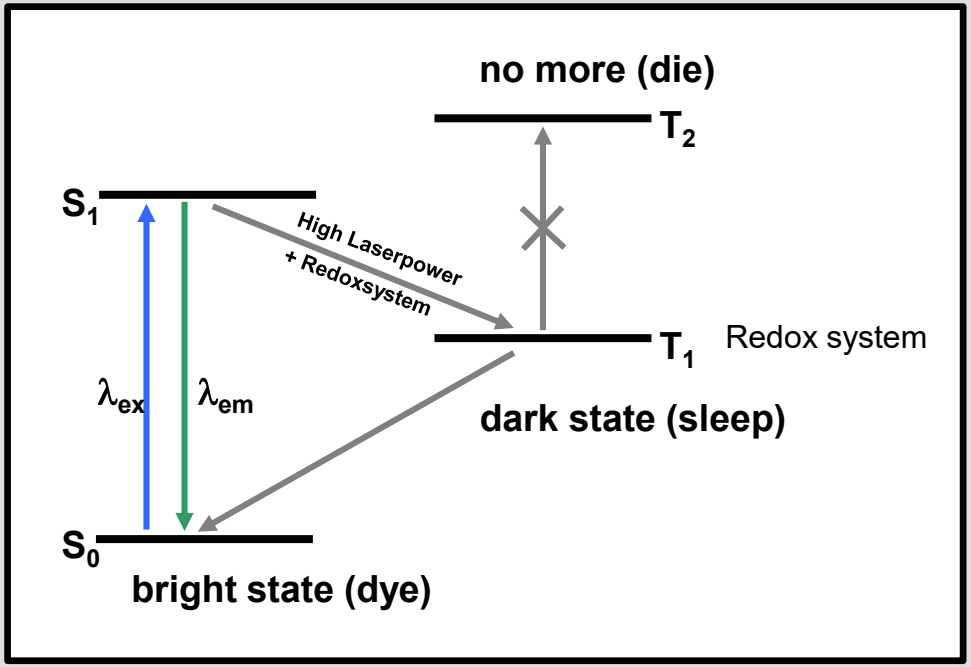
- PALM relies on fluorochromes that can be either **photoactivated**, **photoconverted**, or reversibly **photoswitched** to control emission over time

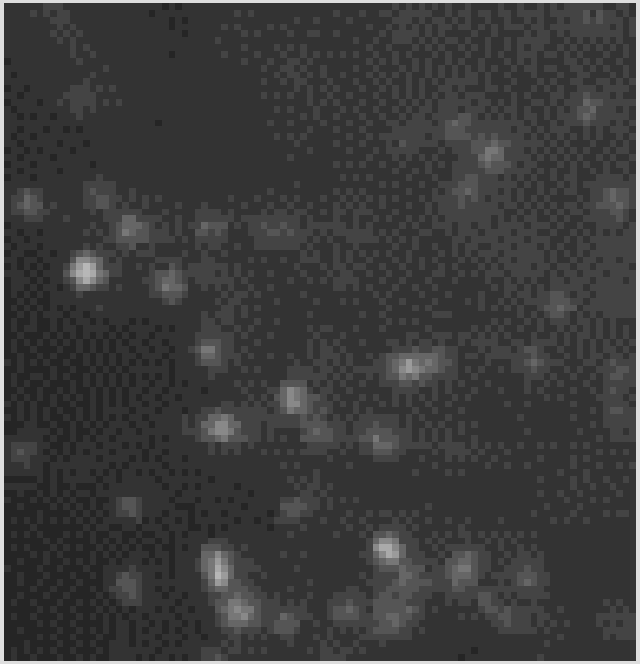



| | | |
|---|---|---|
| Dendra | 405/488 | 1 (2006) |
| Dronpa | 400 ⇄ 490 | 2 (2004) |
| EosFP | 390 | 3 (2004) |
| Kaede | 400 | 4 (2002) |
| KFP1 | 580 ⇄ (490) | 5 (2003) |
| KikGR | 400 | 6 (2005) |
| Padron | 460 ⇄ 405 | 7 (2008) |
| PA-GFP | 400 | 8 (2002) |
| PA-mCherry | 400 | 9 (2009) |
| PA-mRFP1-1 | 400 | 10 (2005) |
| PS-CFP2 | 400 | 11 (2004) |
| rsCherry | 550 ⇄ 450 | 12 (2008) |
| rsCherryRev | 450 ⇄ 550 | 12 (2008) |
| rsFastLime | 400 ⇄ 490 | 13 (2007) |

# Localization Microscopy

## dSTORM: Dye, to Die, to Sleep?



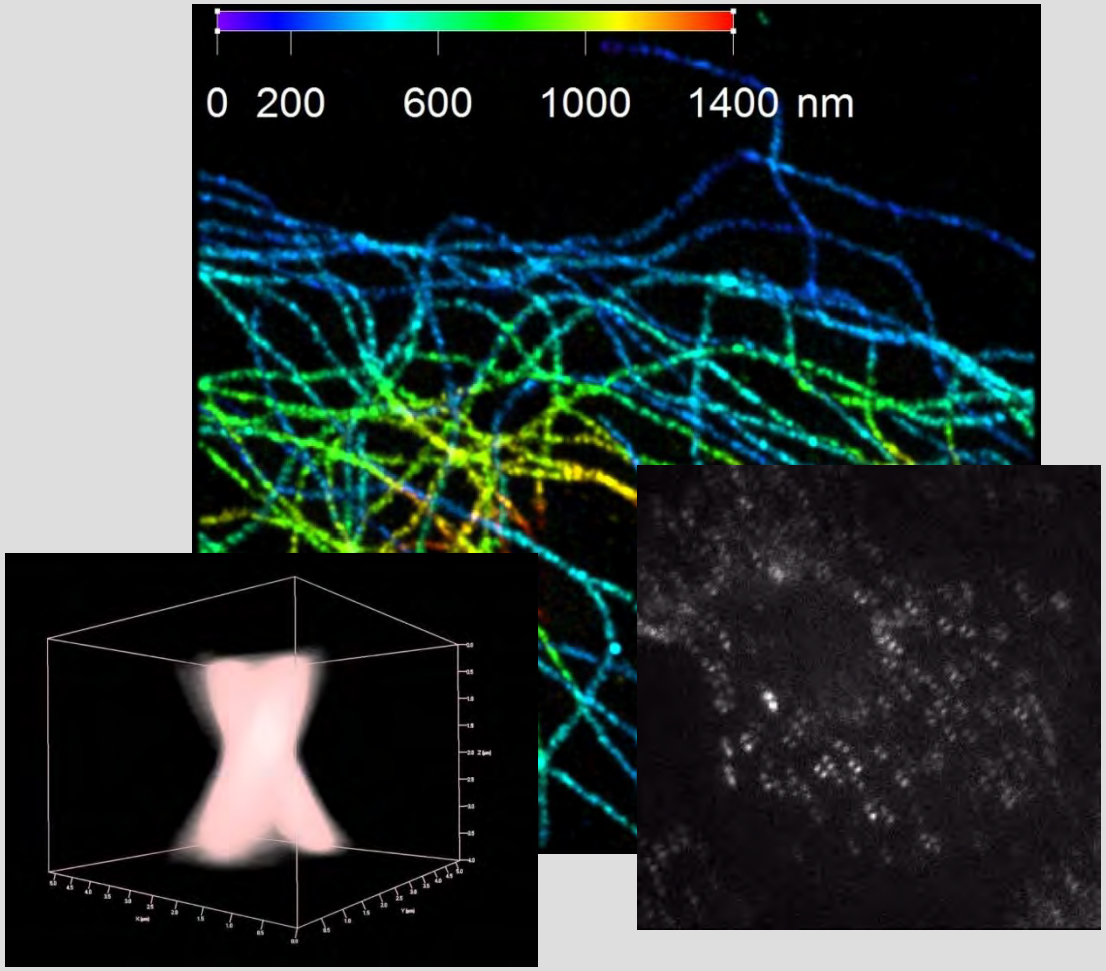
- Instead of using fluorescent proteins, high laser powers (with a specialized reducing cocktail to prevent bleaching) can be used to keep conventional fluorescent dyes in a **dark state**, from which they wake up stochastically

# 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

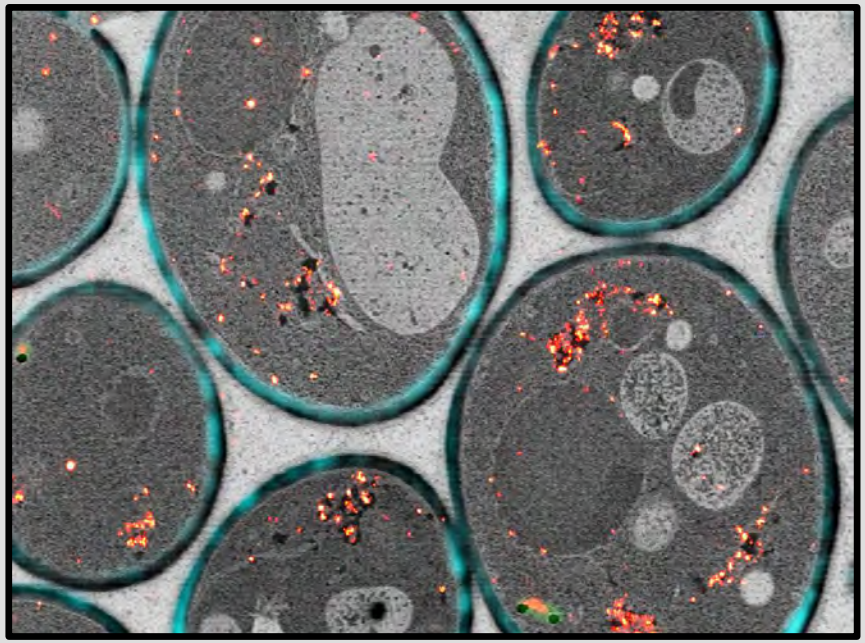


# Localization Microscopy

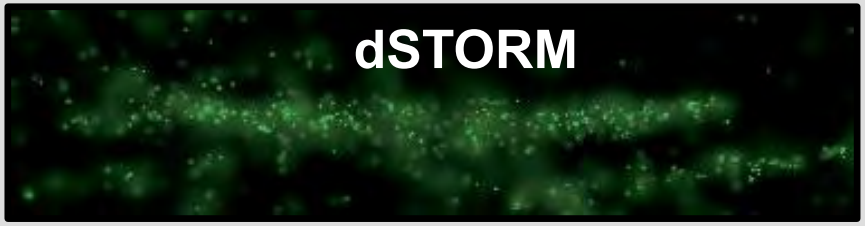
## Common/Unique Applications

### 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**)



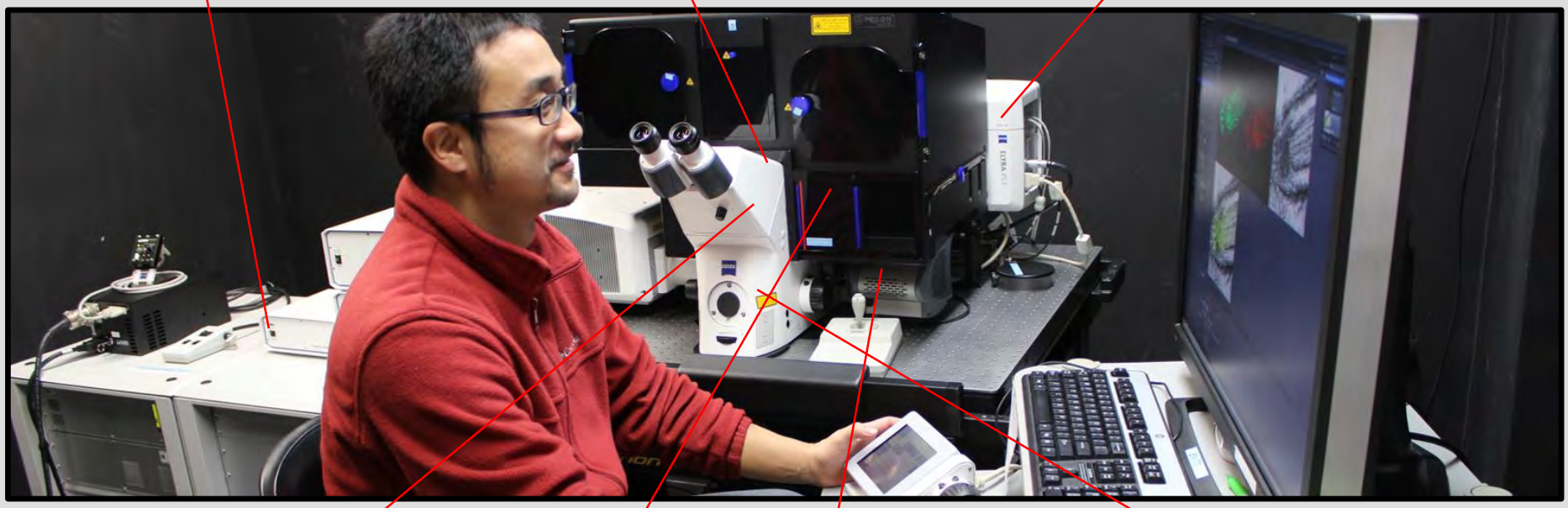
**Ultrathin *S. cerevisiae* overlay SIM, dSTORM (hA1ar), FE-SEM**
**J. Caplan, K. Czymmek (*Delaware Biotech. Instit.*)**


**Focal adhesion complexes (Alexa 488), Martin Bastmeyer (*Univ. Karlsruhe, Germany*)**

# Localization Microscopy

## Typical System Footprint – ZEISS Elyra 7 (SMLM)



# Localization Microscopy

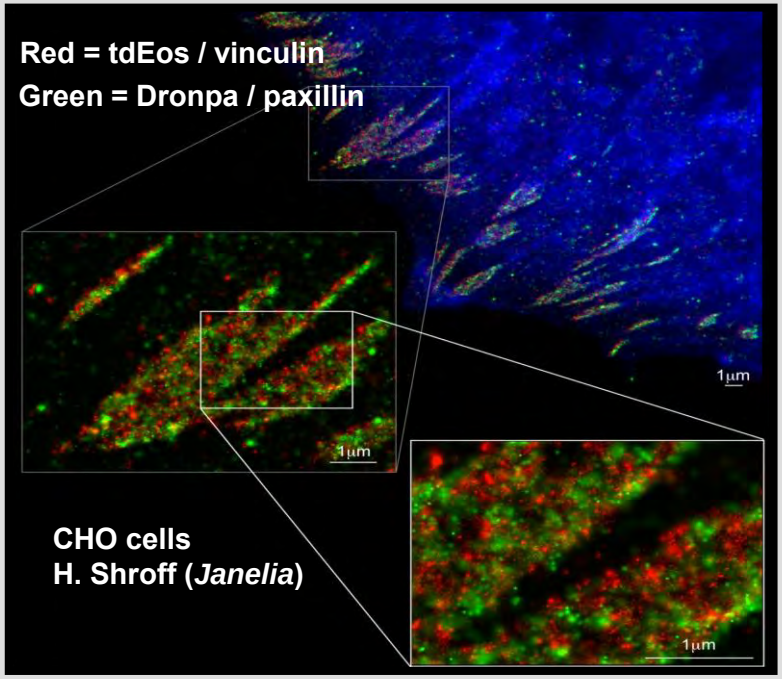
## Practical Perspective

### ADVANTAGES:

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

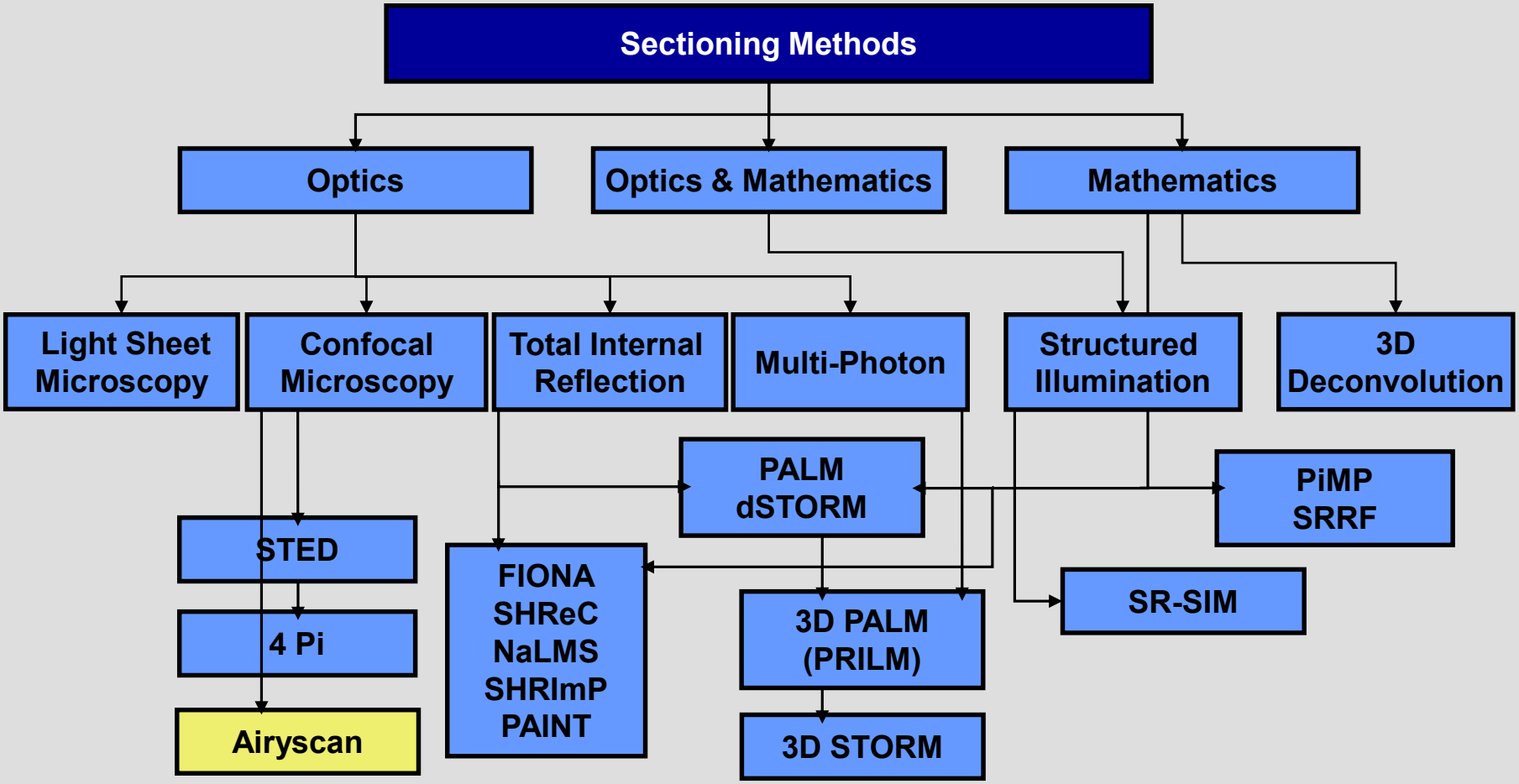
### DISADVANTAGES:

- 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**

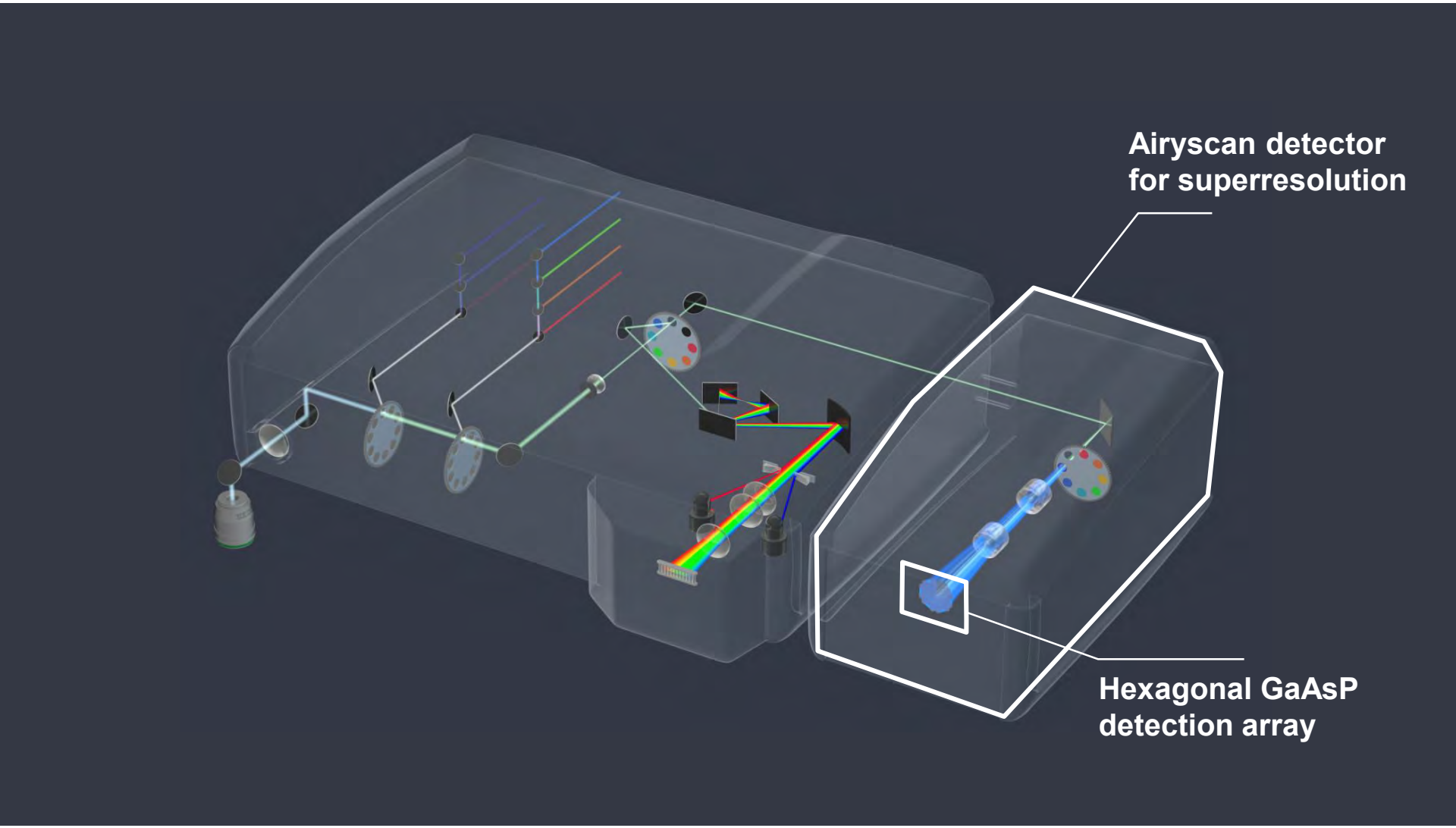


# Optical Sectioning Techniques

## Common Superresolution Approaches

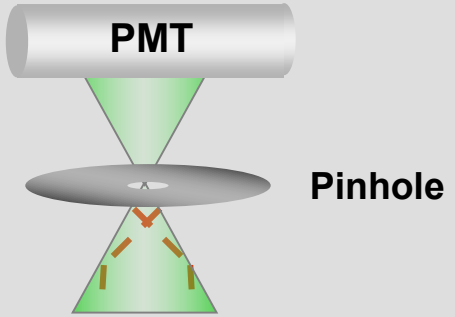


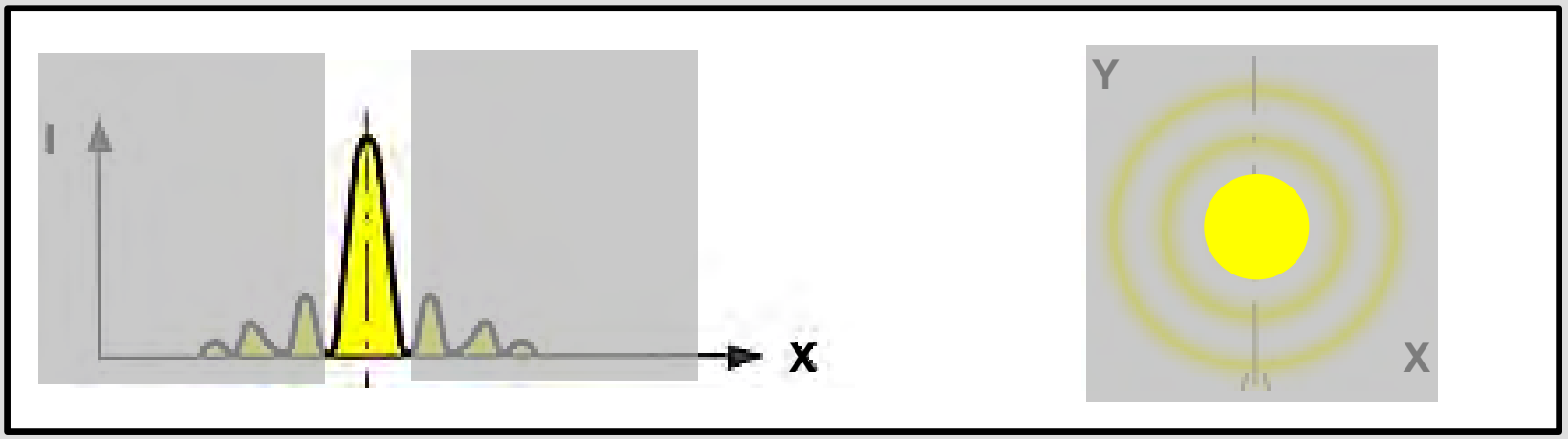
# Airyscan
## Dedicated Superresolution Detector



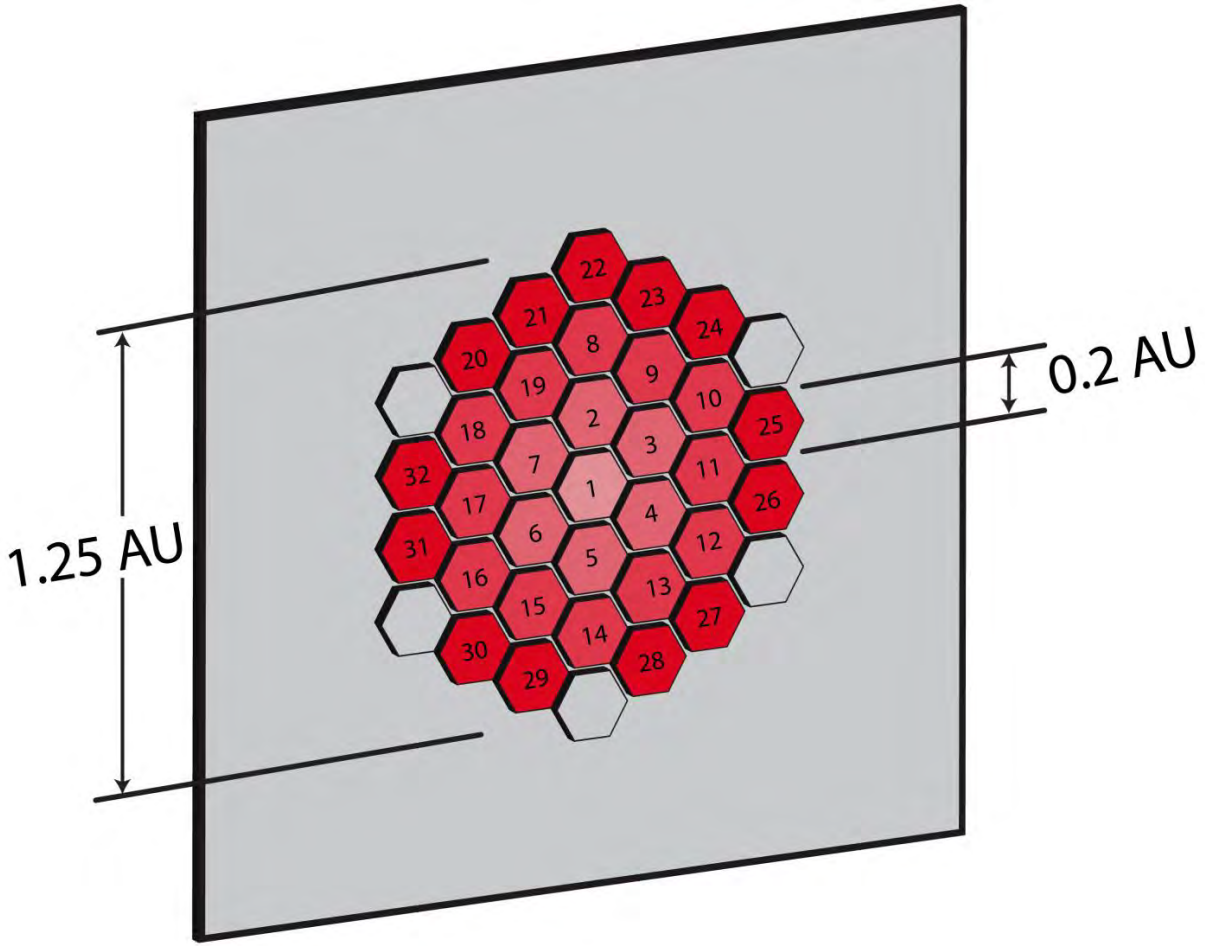
# Airyscan
## Basic Principles

- 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

**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)

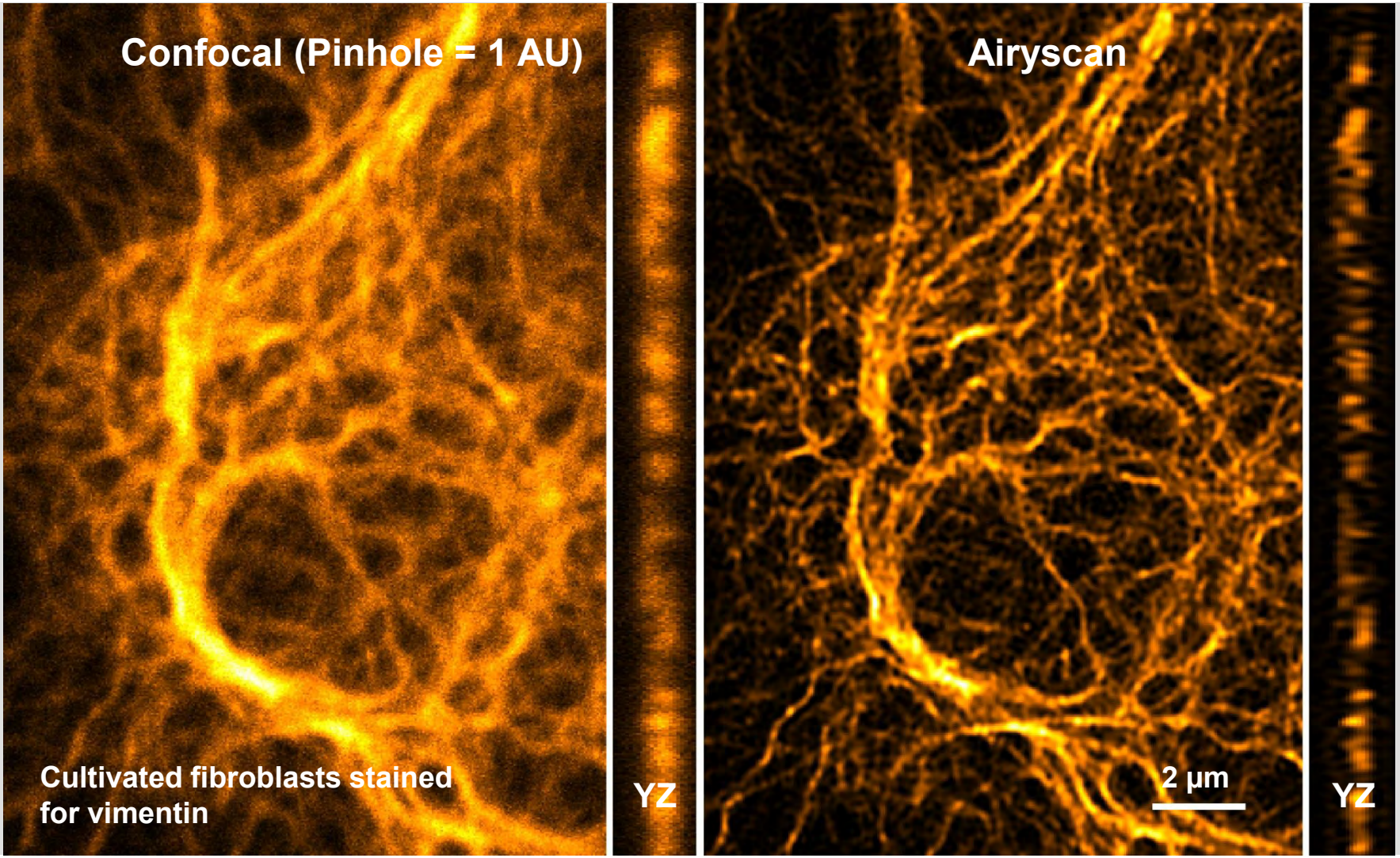
**York, A.G., et al., Resolution doubling in live, multicellular organisms via multifocal structured illumination microscopy. Nat Methods, 2012. 9(7): p. 749-754**
- Parallelized the image scanning microscopy procedure using illumination patterns via a digital micromirror device
- Multifocal pattern (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

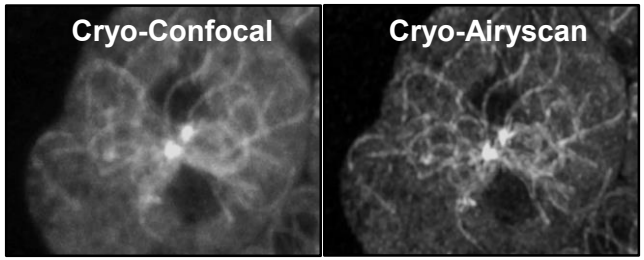
**York, A.G., et al., Instant super-resolution imaging in live cells and embryos via analog image processing. Nat Methods, 2013. 10(11): p. 1122-1126**
- Parallelized the re-scan approach using microlens and pinhole array, coupled with second microlens array
- Second microlens array used to locally contract 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

# Airyscan

## Isotropic Resolution Improvements (2-fold)



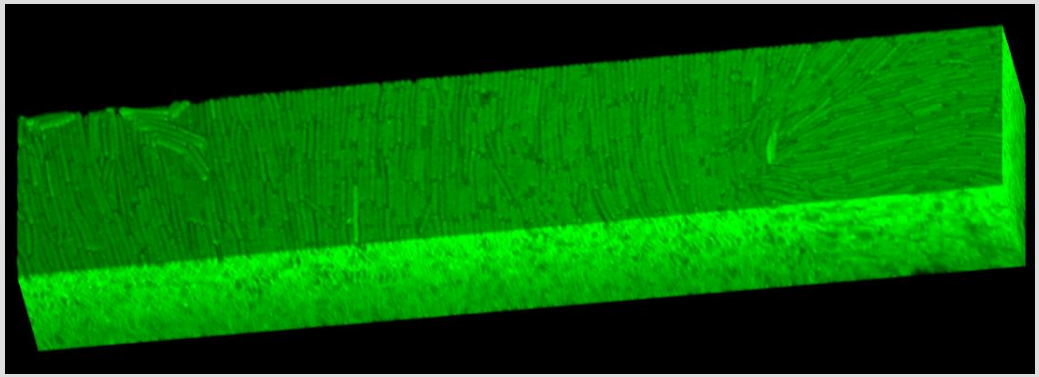
# Airyscan
## Common/Unique Applications




**Dictyostelium discoideum (slime mold)**
*Plunge-freezing on EM support; tubulin-mRFP*

## WHAT ARE THE USES?

- New benchmark in point-scanning resolution for **thicker specimens**



**Biofilm (3D render), collected with Airyscan, 63x/1.4 oil**

- 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”)

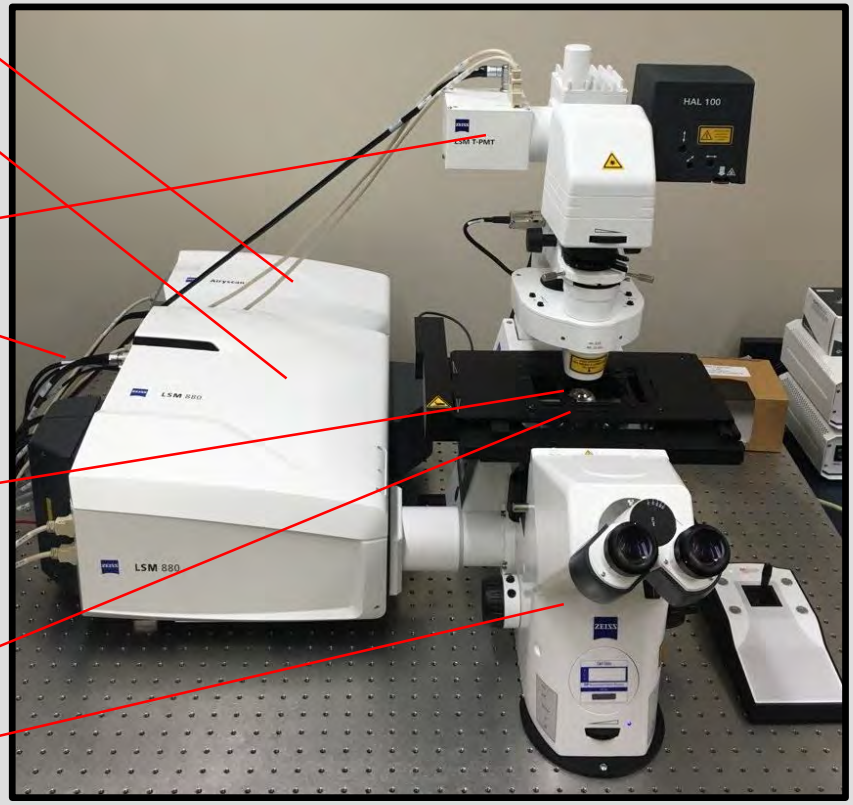
Laser lines: 405, 458, 488, 514, 561, 633 nm

Objectives:

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

Motorized XY stage + Z-piezo insert

Observer inverted microscope



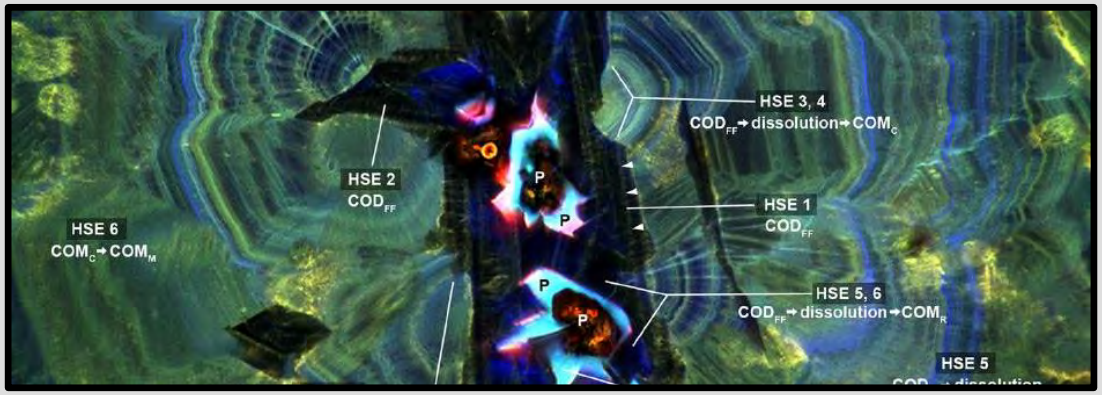
# Airyscan
## Practical Perspective

## ADVANTAGES:

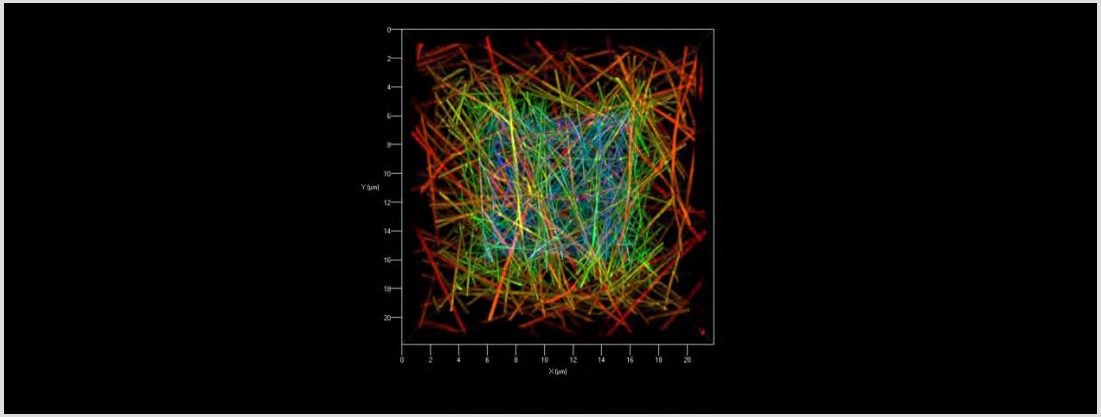
- Relatively versatile **superresolution tool at high depths**
- No specific sample preparations required

## 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



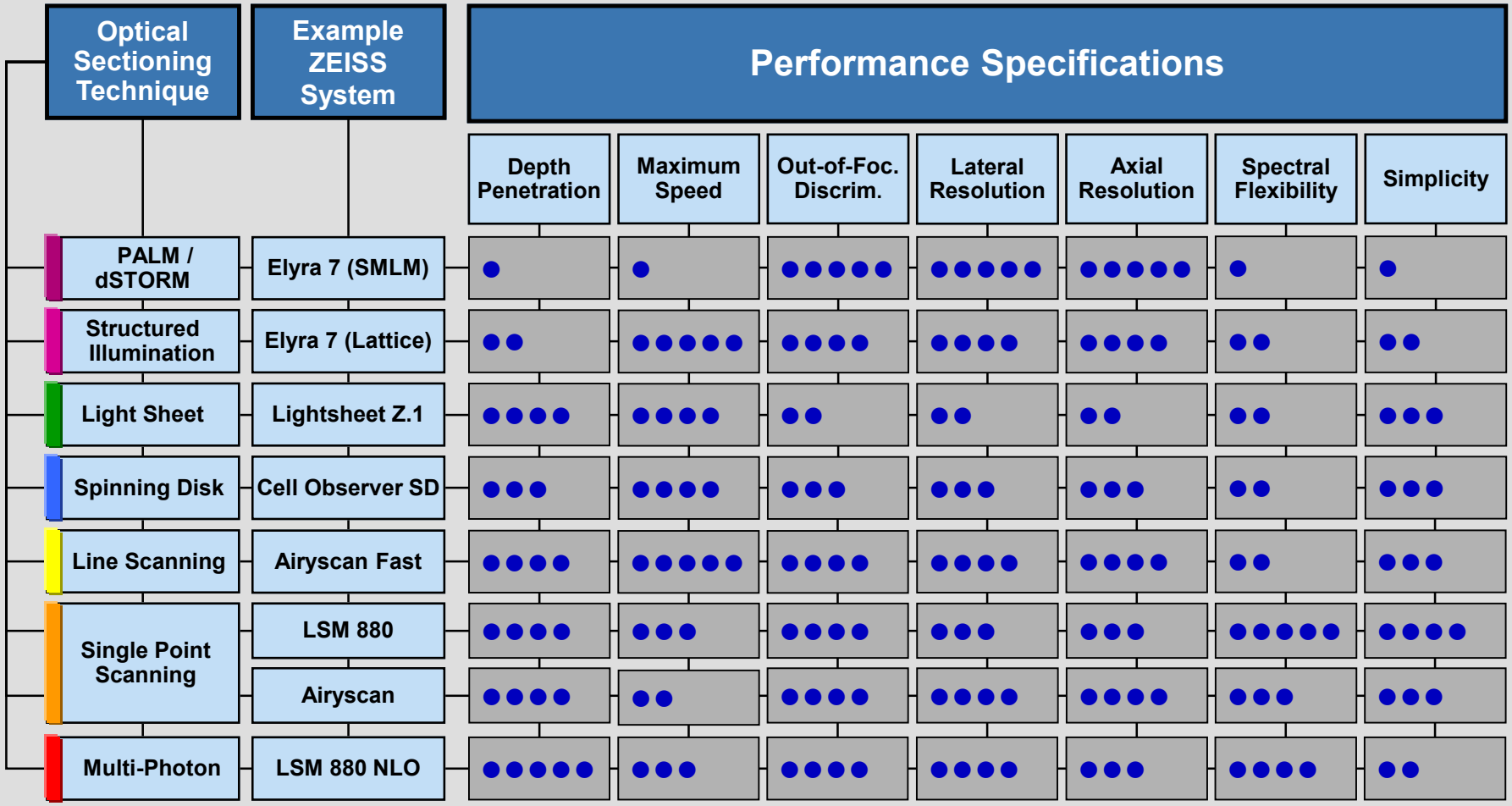
# Summary
## The Right Tool for the Job?

- 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



# 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.