# A GUIDE TO LIGHT MICROSCOPY & CONFOCAL IMAGING


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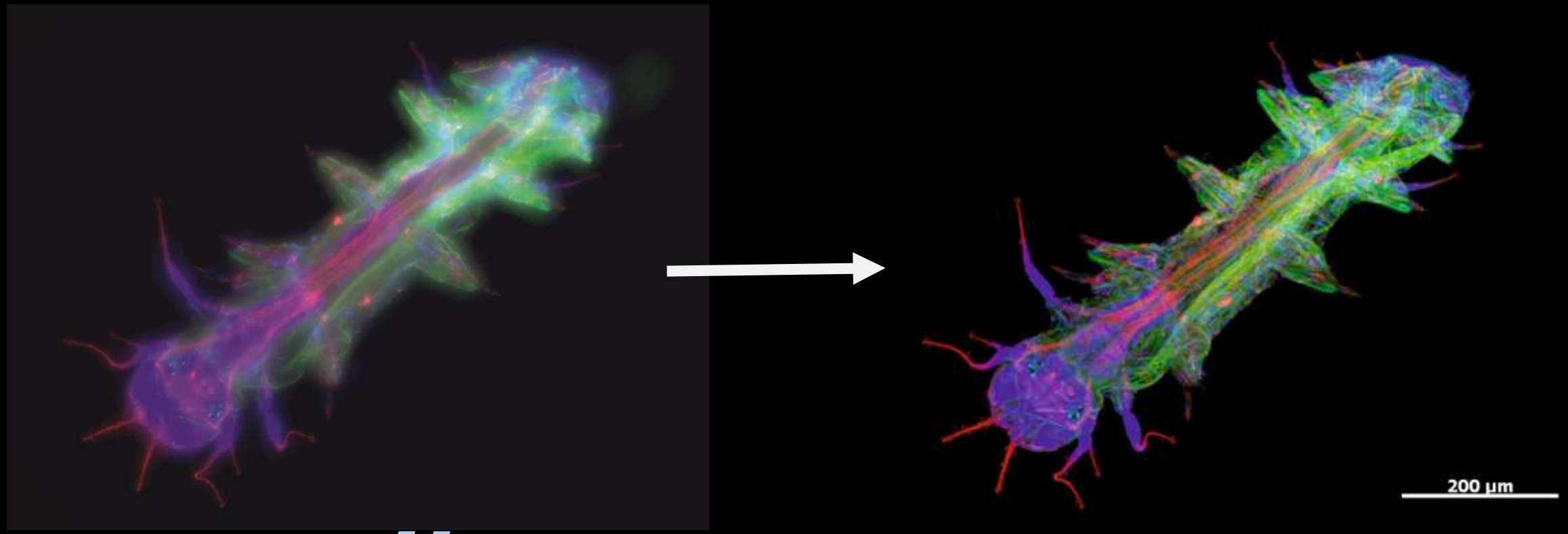
Application Specialist, Research Microscopy Solutions

# ZEISS LSM 880 IN NTNU

## Highest-End Confocal Microscope For Life Science Research

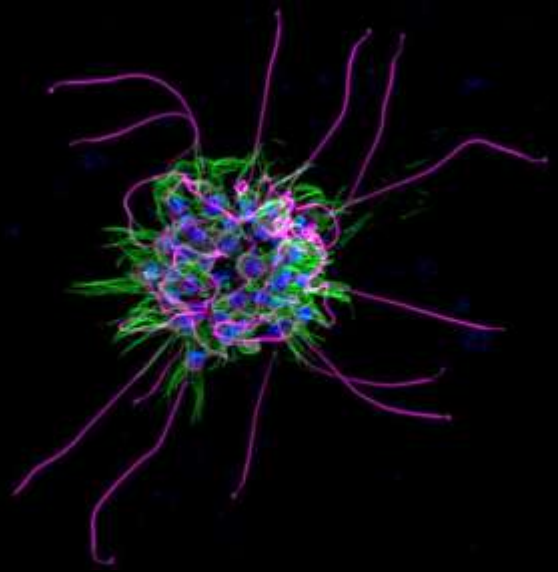
# Optical Sectioning | Extract the Layer of the Image

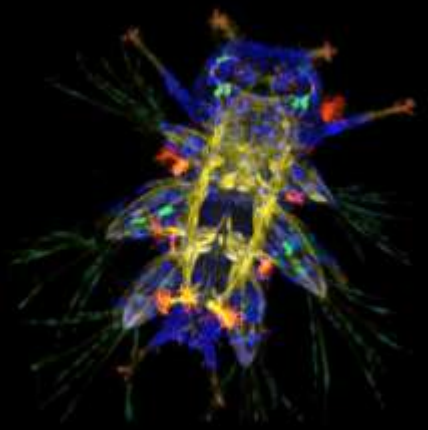


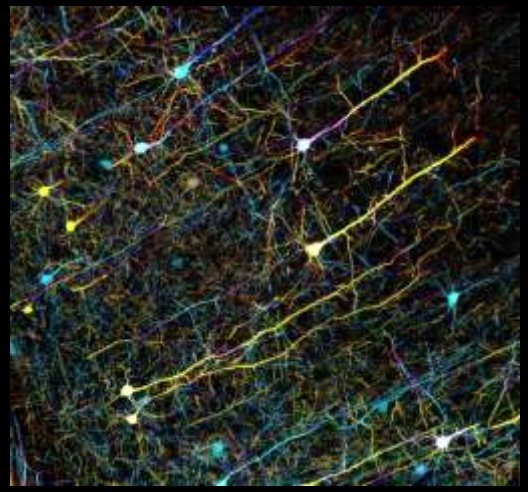

"We want clear image!"

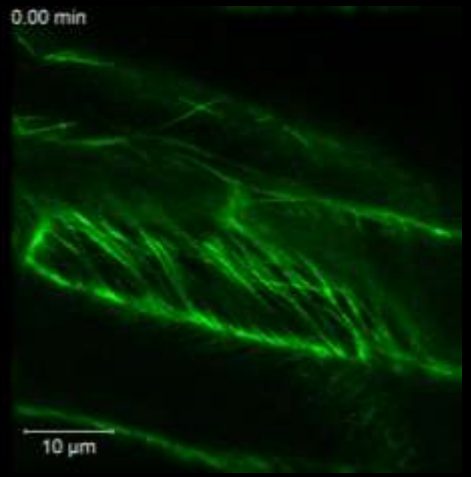
*V. Wilkens, University of Osnabrueck, Germany., Optical Sectioning*

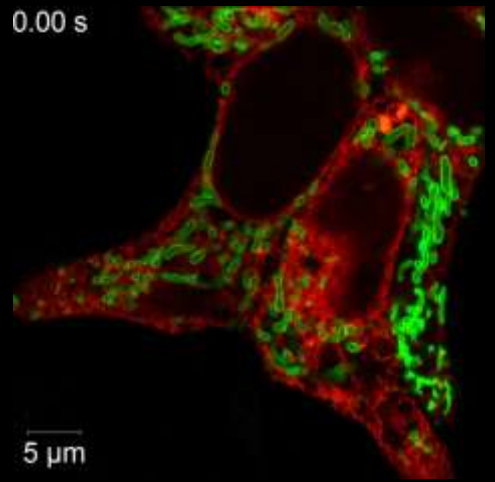
# Confocal microscopy allows you to optically section thick samples

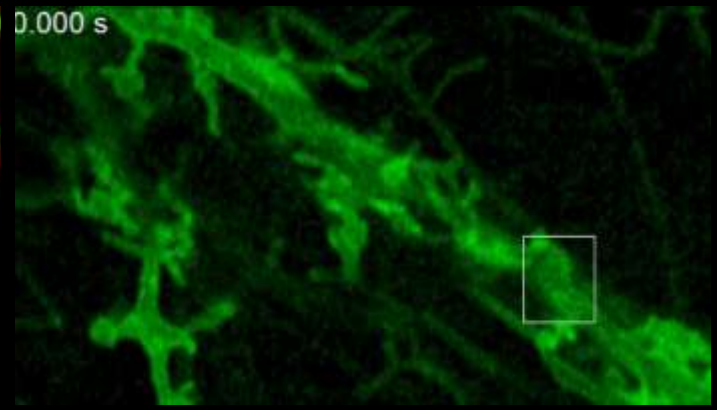






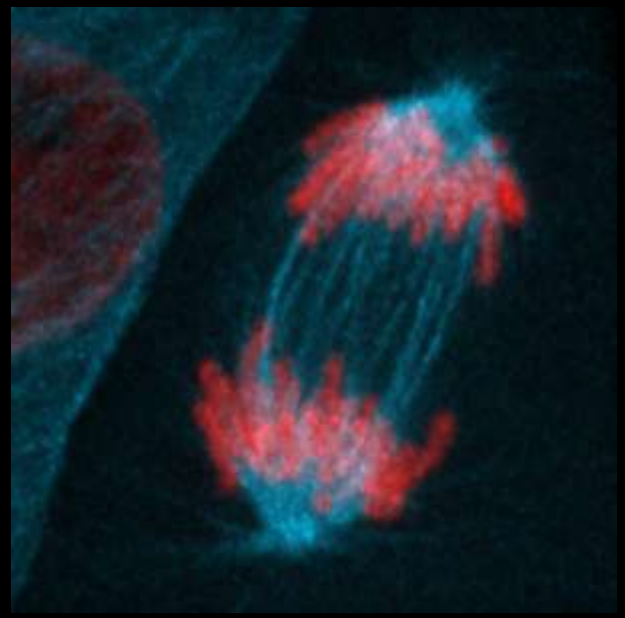


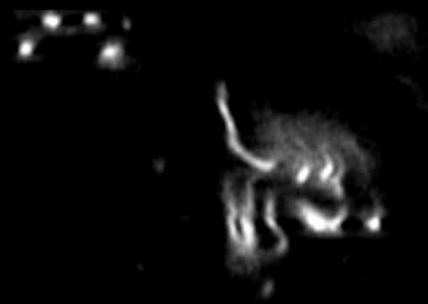




Holly Aaron (UC Berkeley); N. Kenny, K. McClelland, S. Miller (U of Oxford, U of Queensland, U of Cambridge), D. Reiff (U of Freiburg); Y. Zuo, A. Aharon, A. Schnulz (U of California Santa Cruz); Courtesy of Balazs Erdi, Max F. Perutz (Vienna Biocenter, Austria); Jason D Vevea (University of Wisconsin-Madison, USA); O. Samajova (Faculty of Science, Palacky University Olomouc, Poland)

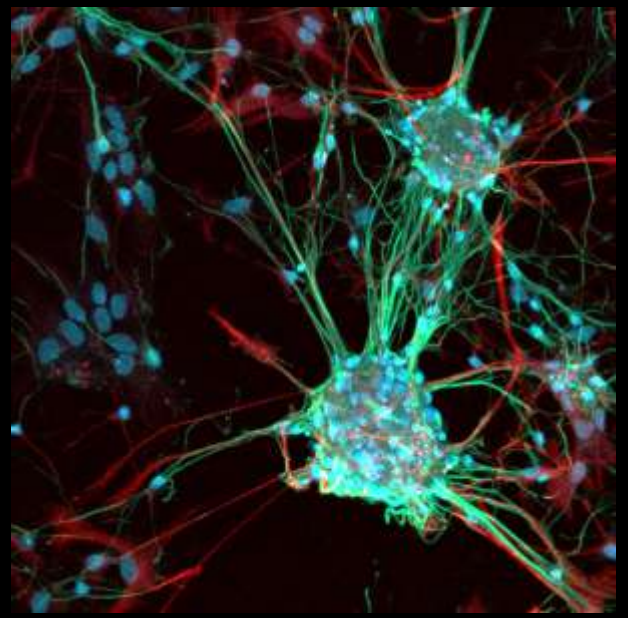
# **LSM** | Fast and Gentle Multiplex Imaging



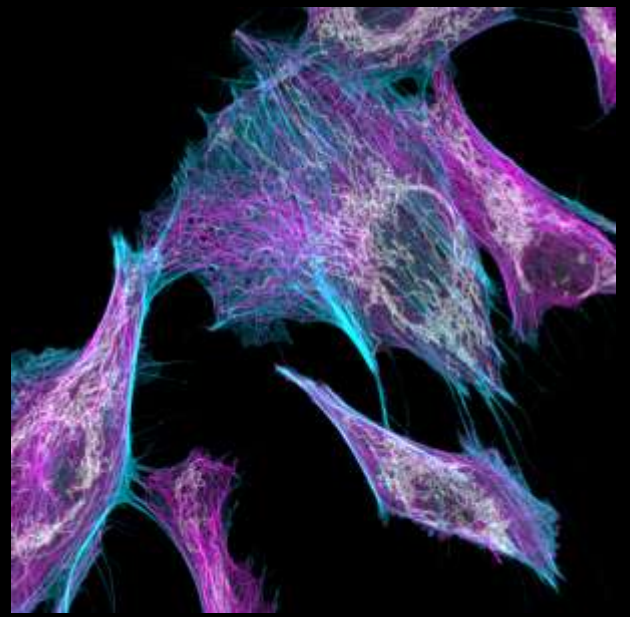
Highest sensitivity



Fast &
High throughput

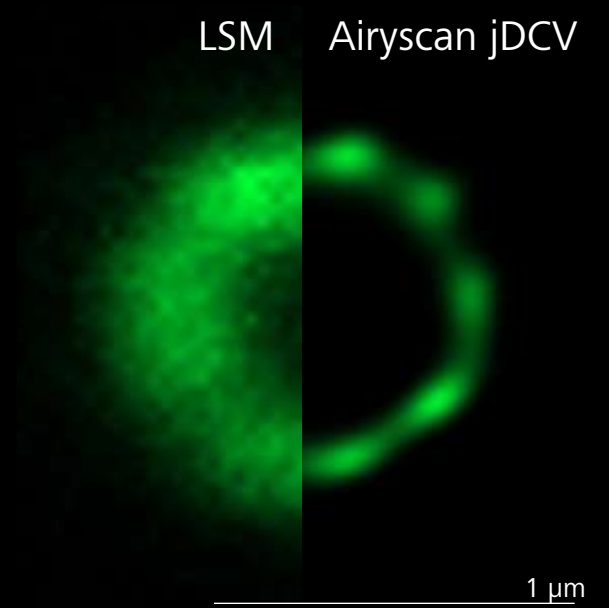


High resolution



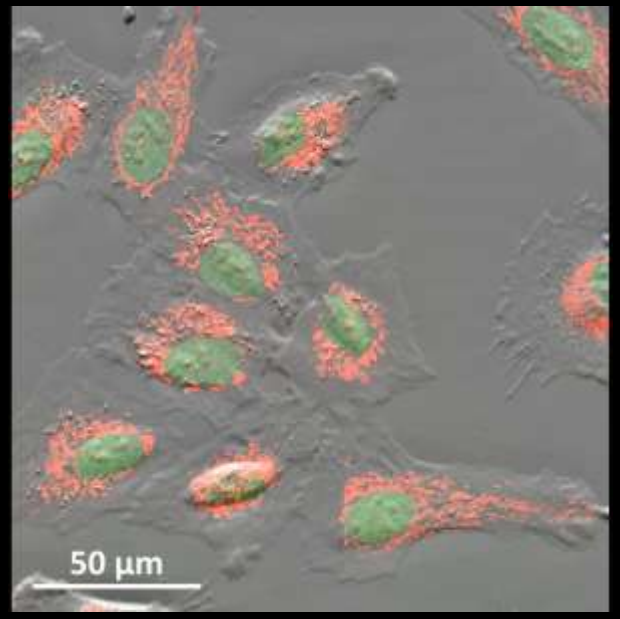
Spectral
multiplexing

# **LSM 880** | Versatile Confocal Platform




## Airyscan 2

Superresolution

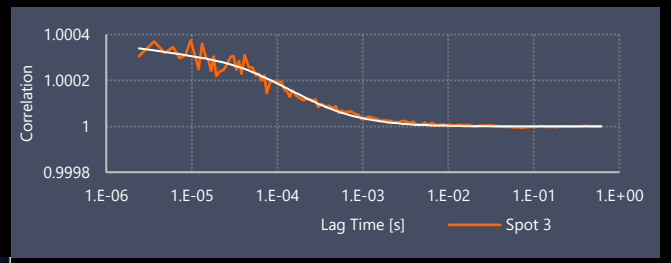


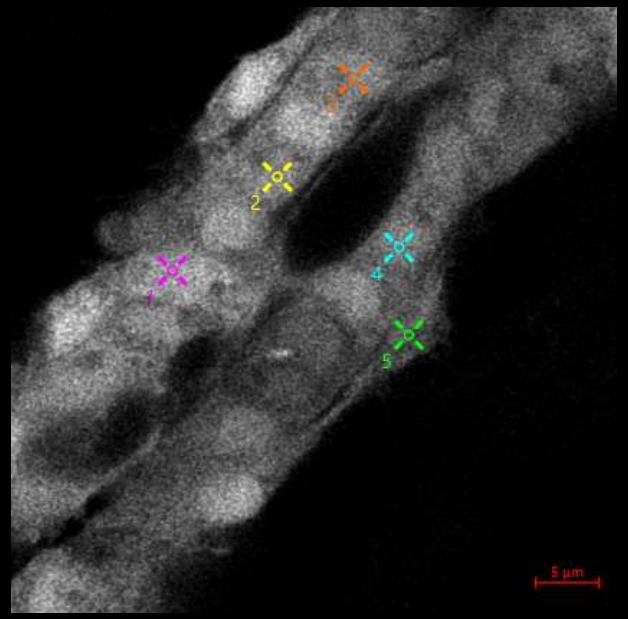

## Incubation Module

Live cell imaging

## AI Sample Finder

Automated imaging startup



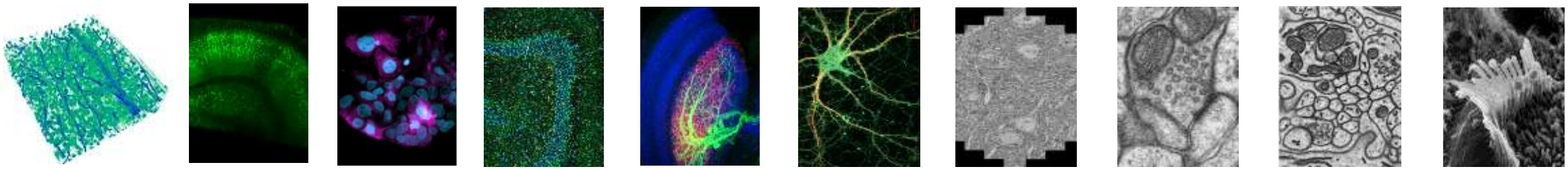



## Dynamic Profiler

Gain molecular info

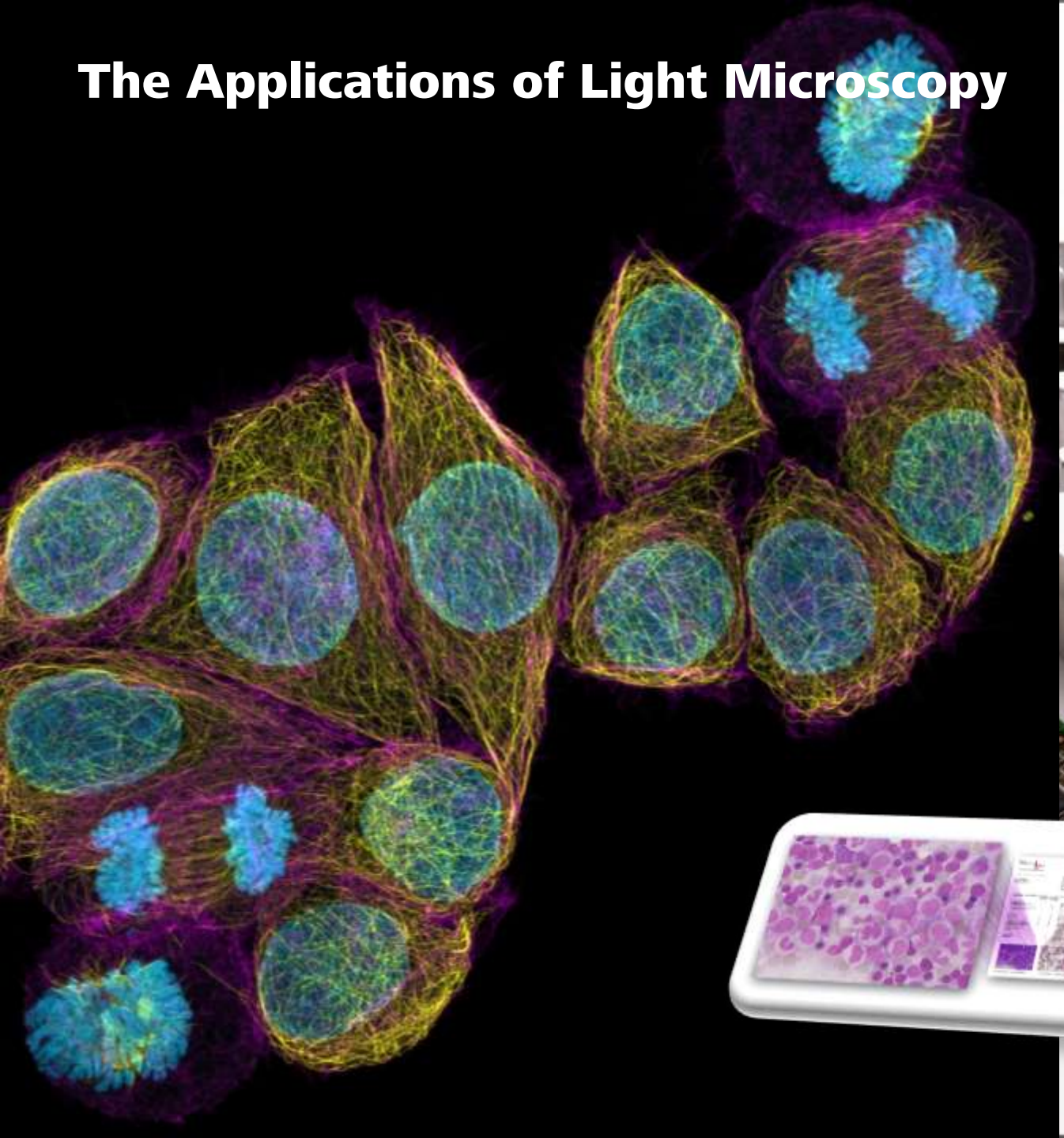
# Keep the Context of Your Experiments

| X-ray | Light Microscopy | | | | | Electron Microscopy | | | Ion |
|---|---|---|---|---|---|---|---|---|---|



| X-ray | Lightsheet | Lattice Lightsheet | Widefield | LSM Airyscan | Super-resolution | MultiSEM | Field Emission Scanning Electron | Focused Ion Beam | Helium Ion |
|---|---|---|---|---|---|---|---|---|---|
| 500 nm | 500 nm | 290 nm | 250 nm | 120 nm | 20 nm | 5 nm | < 2 nm | < 1 nm | < 0.5 nm |

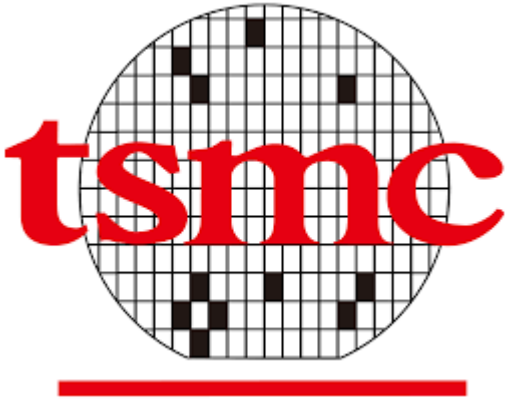
# The Applications of Light Microscopy



# The Applications of Light Microscopy



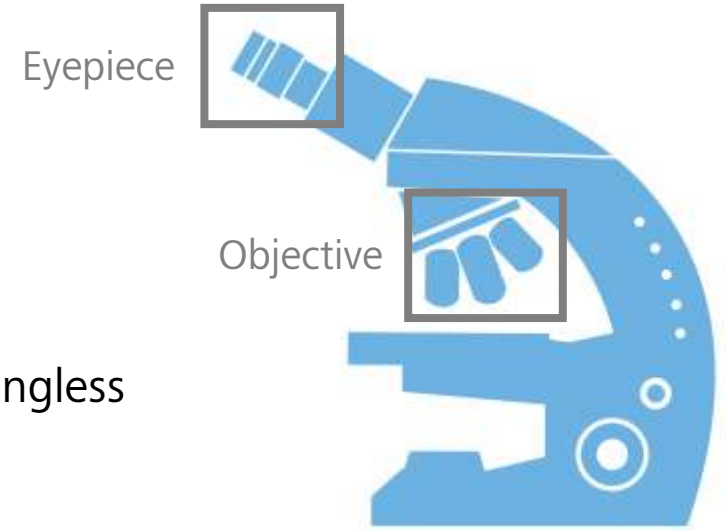






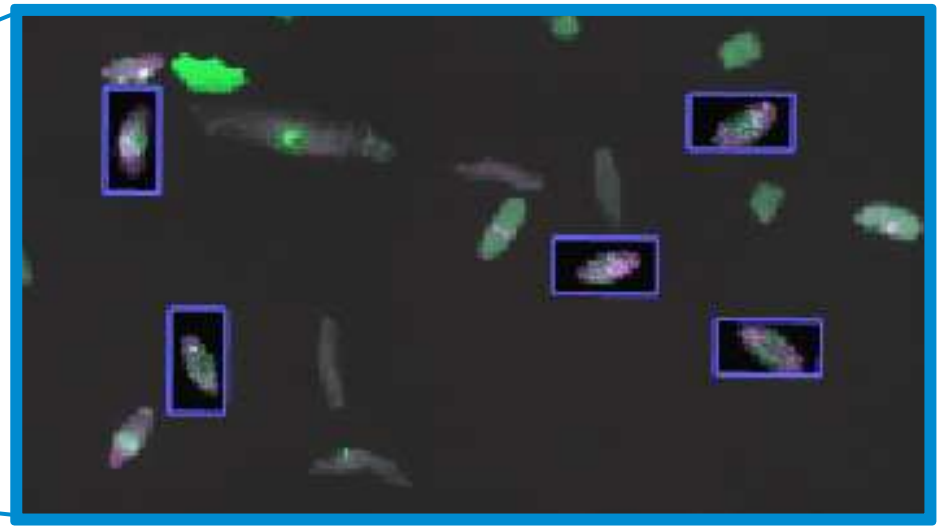



# Magnification and Resolution




- Magnification? 100x? 1000x? 999999999x?
- Total magnification = **Objective** magnification x **Eyepiece** magnification

- **~1500x** is the limit of Light Microscopes, magnification above 1500x is meaningless
- Why?.

# Magnification and Resolution

# Magnification and Resolution

Magnification alone is not enough:

Resolution determines what we see.


# Resolution of Your Eyes

Definition:

The resolution limit is reached, when two point-like objects can not be imaged as two distinct structures anymore.

The **distance** between the objects is called the resolution limit.

d = 10 cm

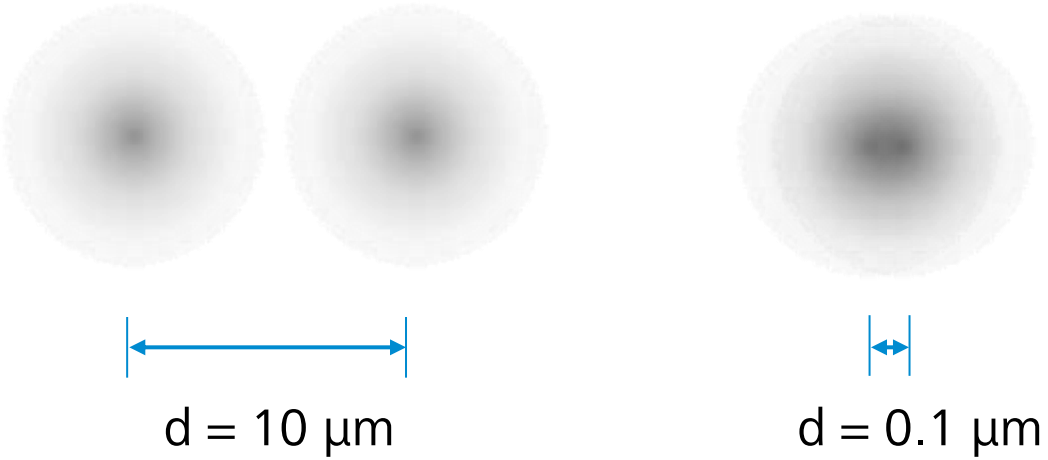
d = 0.1 cm

# Resolution of Microscopes

Definition:

The resolution limit is reached, when two point-like objects can not be imaged as two distinct structures anymore.

The **distance** between the objects is called the resolution limit.



# Resolution

**Rayleigh criterion**

$$d_0 = \frac{1.22\lambda}{N.A_{obj.} + N.A._{Cond}}$$

or more simply

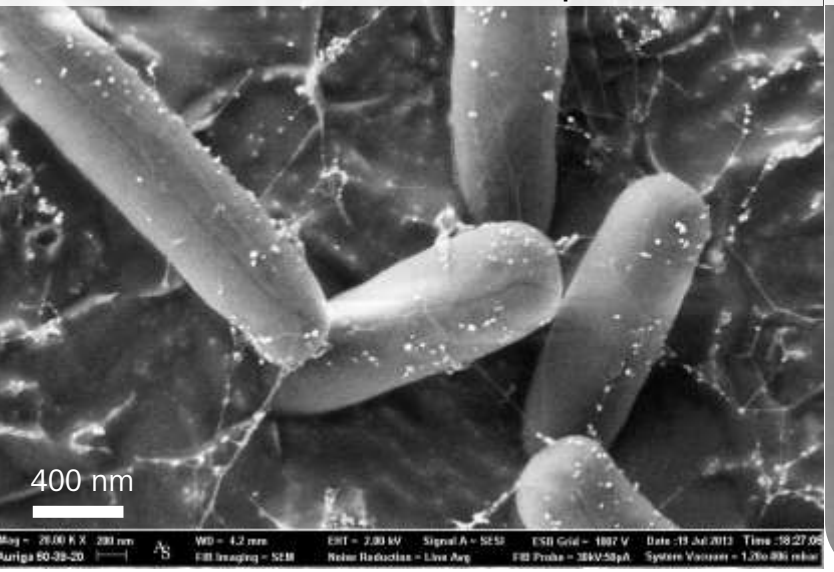
**Abbe limit**

$$d_0 = \frac{\lambda}{2N.A}$$

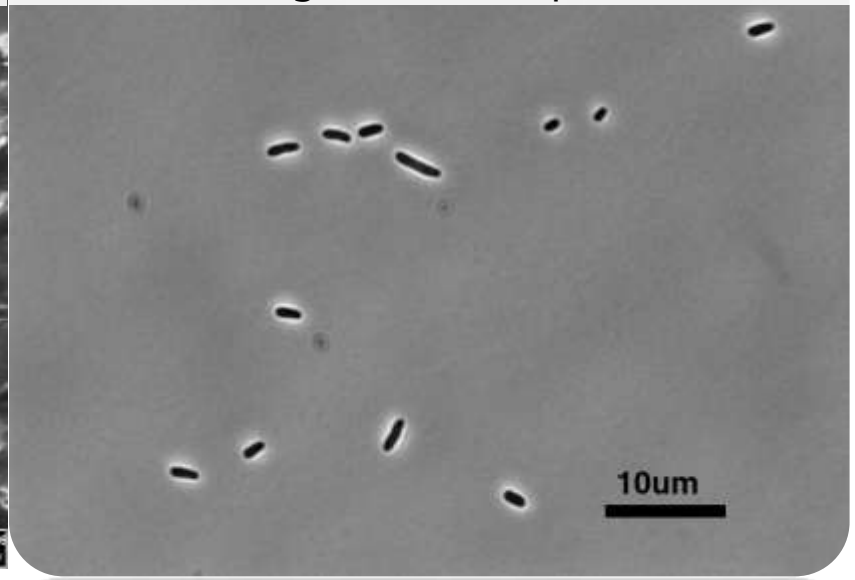
$\lambda$ = wavelength of light, e.g. 550 nm (green)

The resolution of light microscope **$d_0$= 200 ~ 300 nm**

Electron microscope



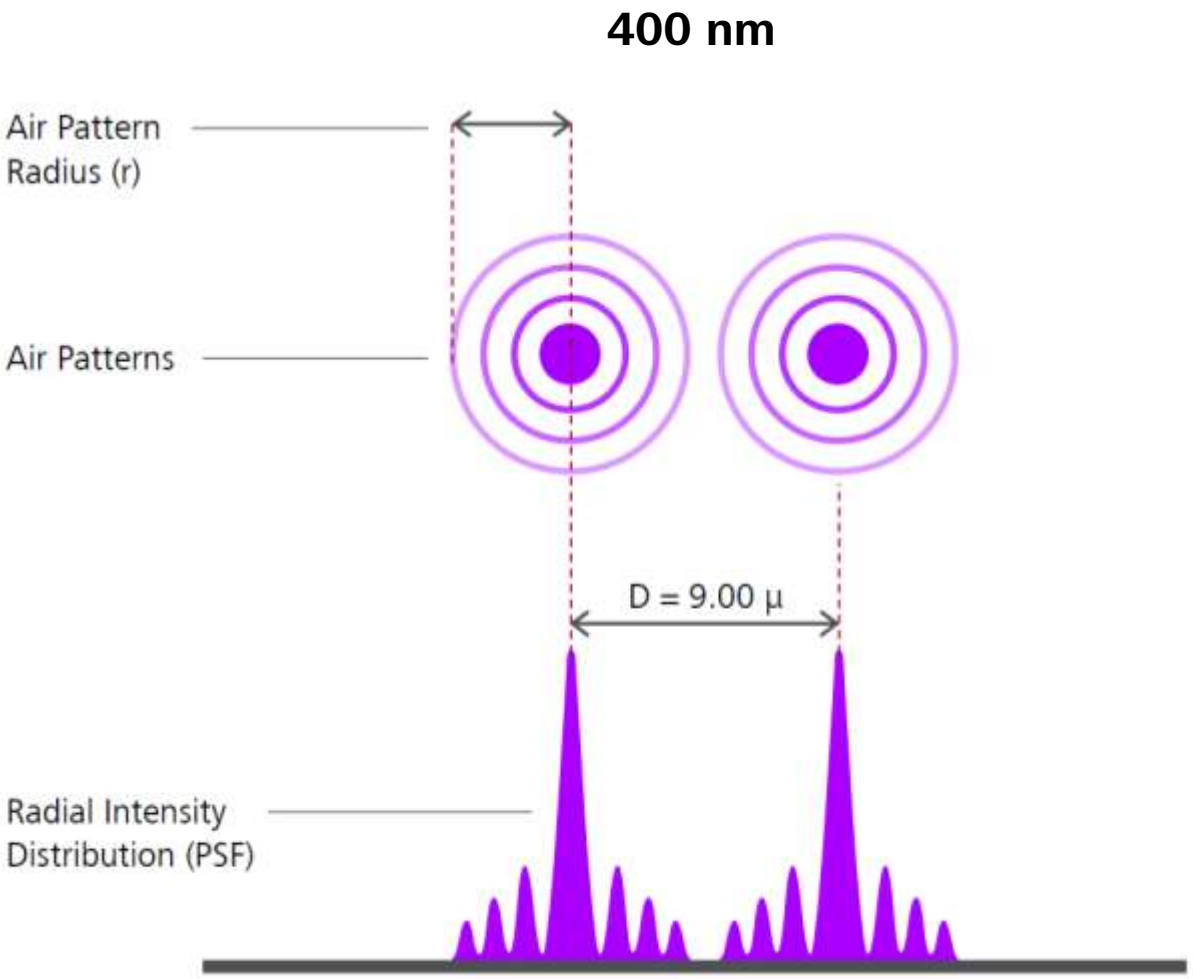

Light microscope

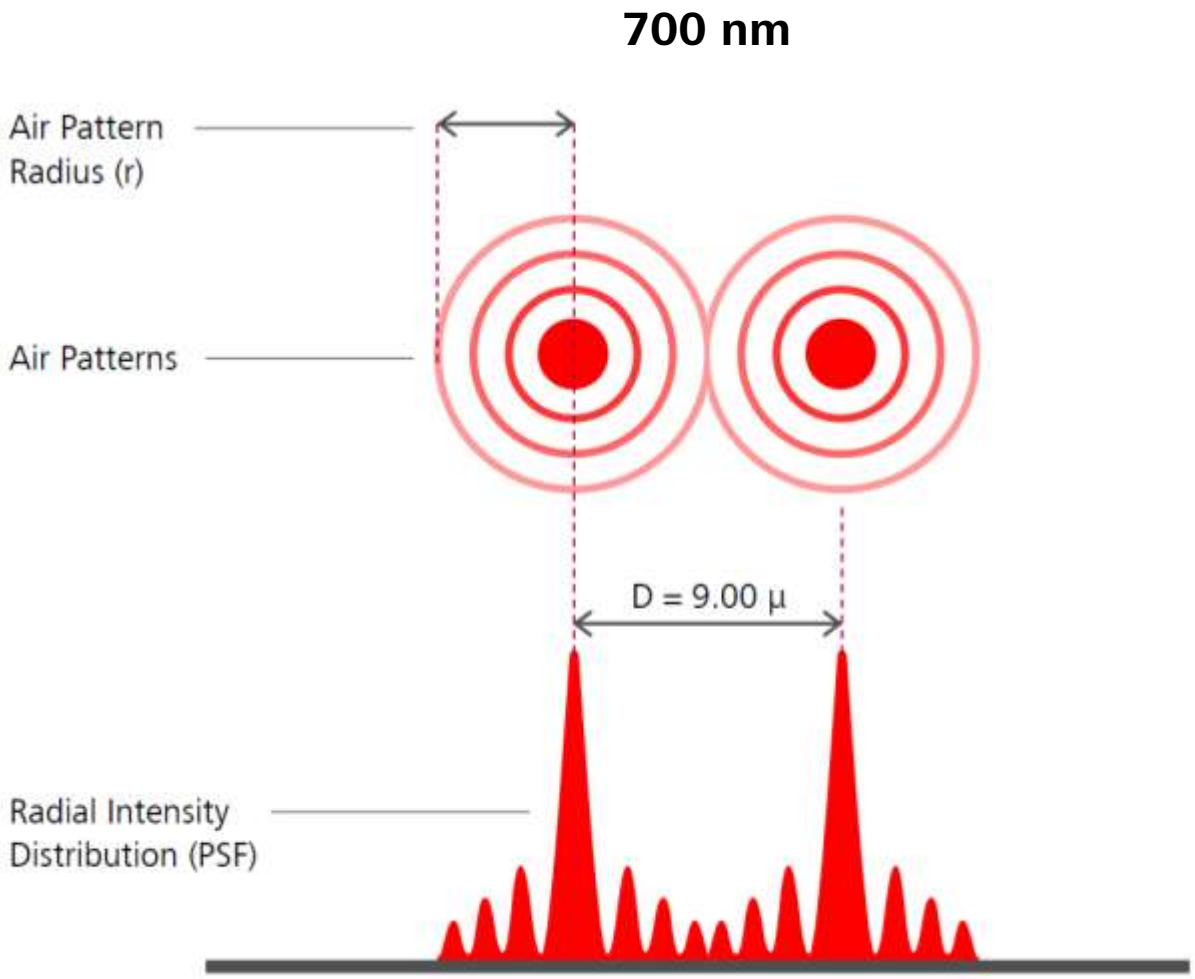



*E. coli*
0.5 x 2 µm

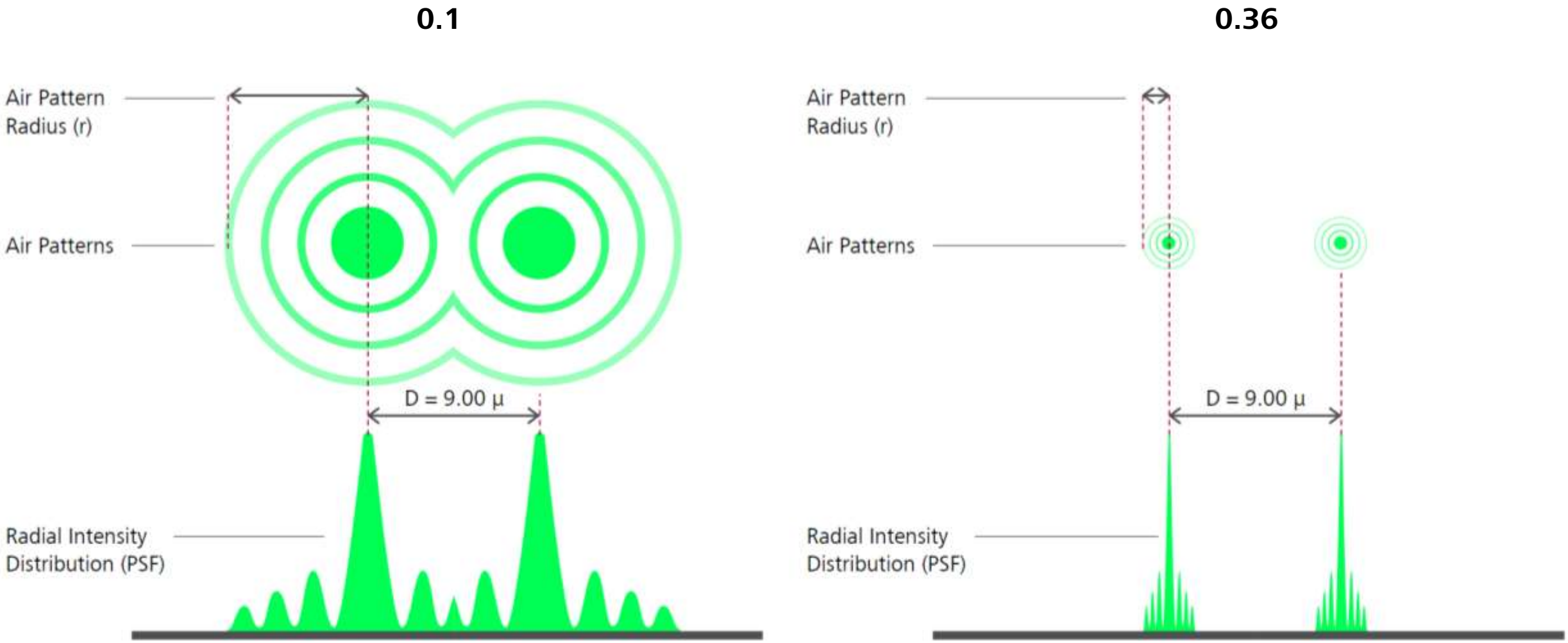
**Ernst Abbe**

# Resolution – Wavelength

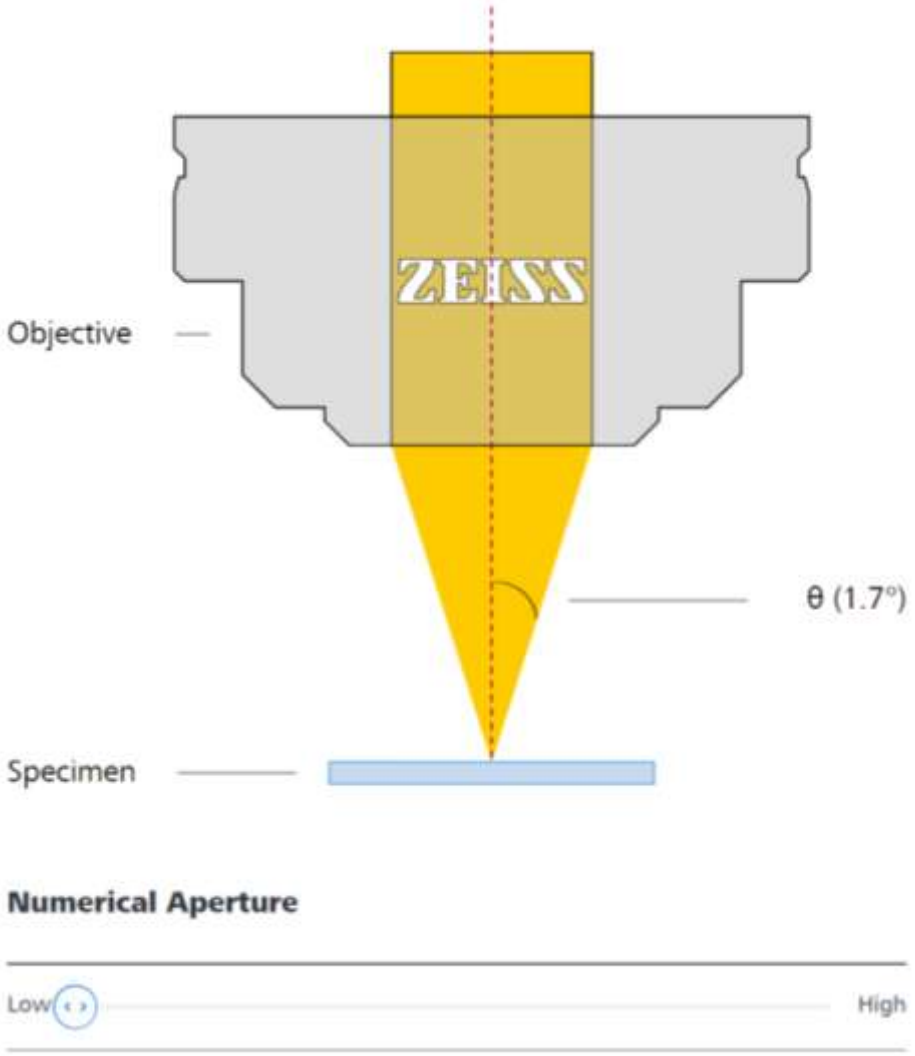


# Resolution – N.A.



# Resolution – N.A.



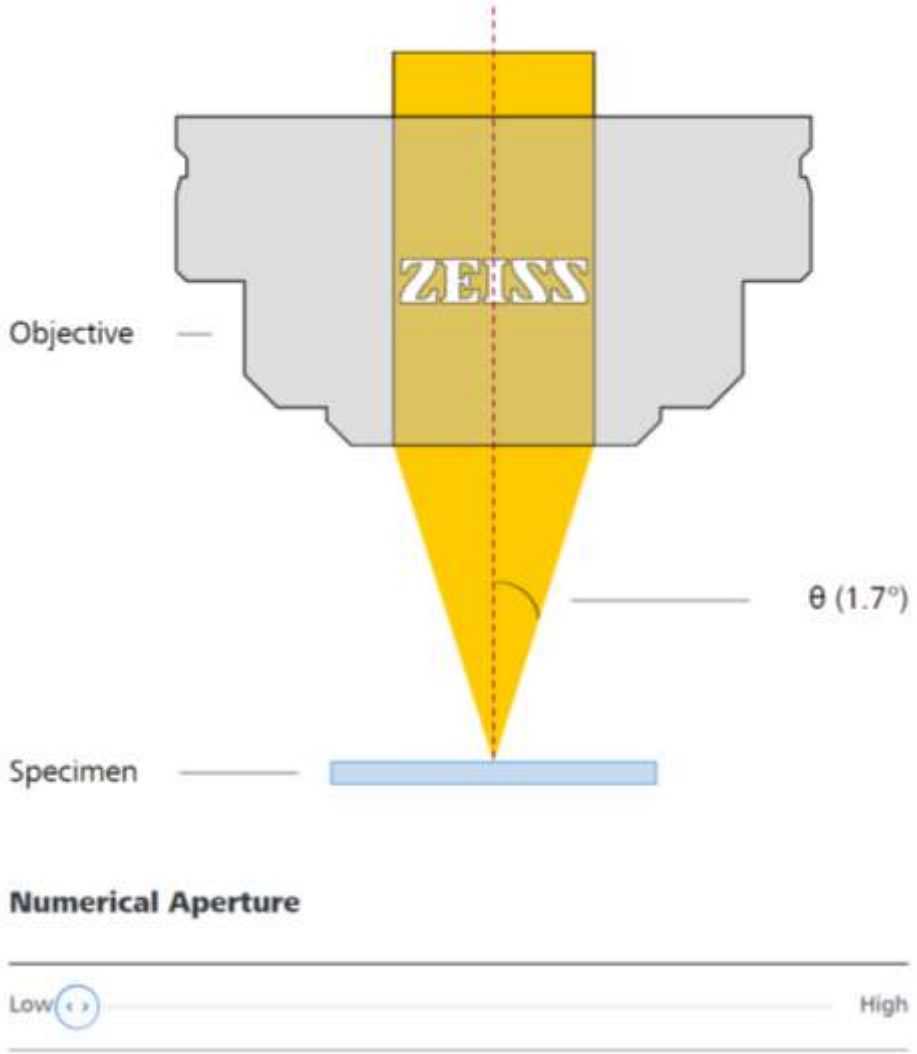

Numerical Aperture (NA) = n • sin θ

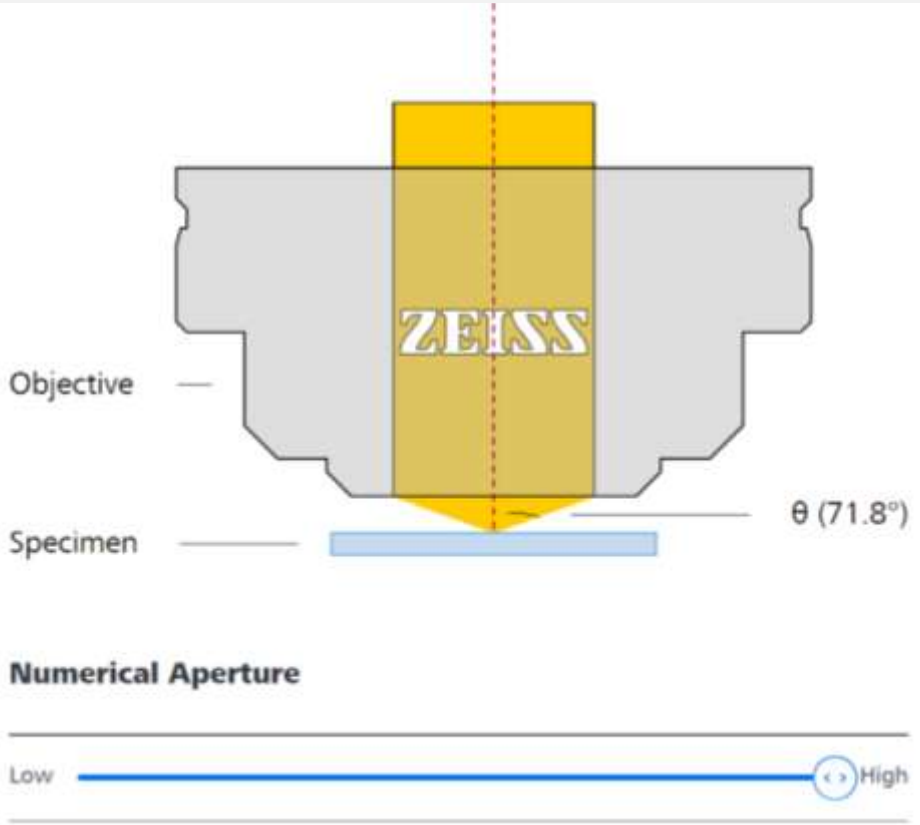
N.A. determines the brightness and resolution of an image formed by an objective

# Resolution – N.A.

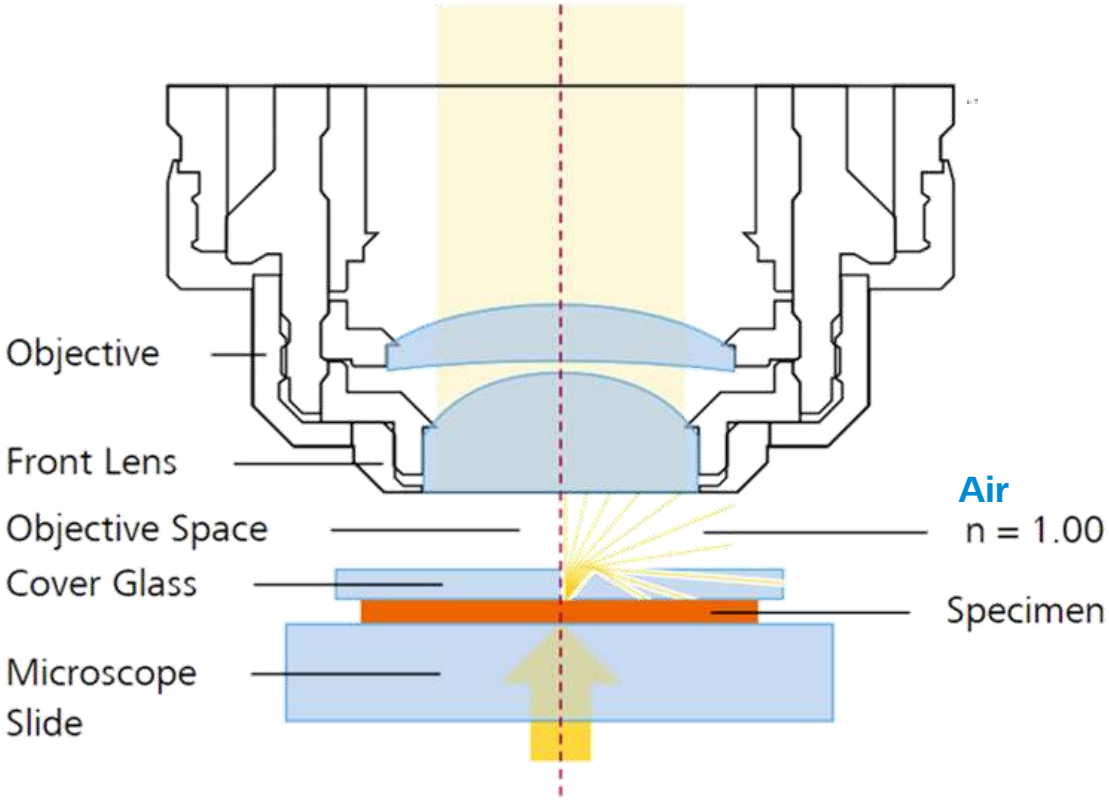
Higher NA offers

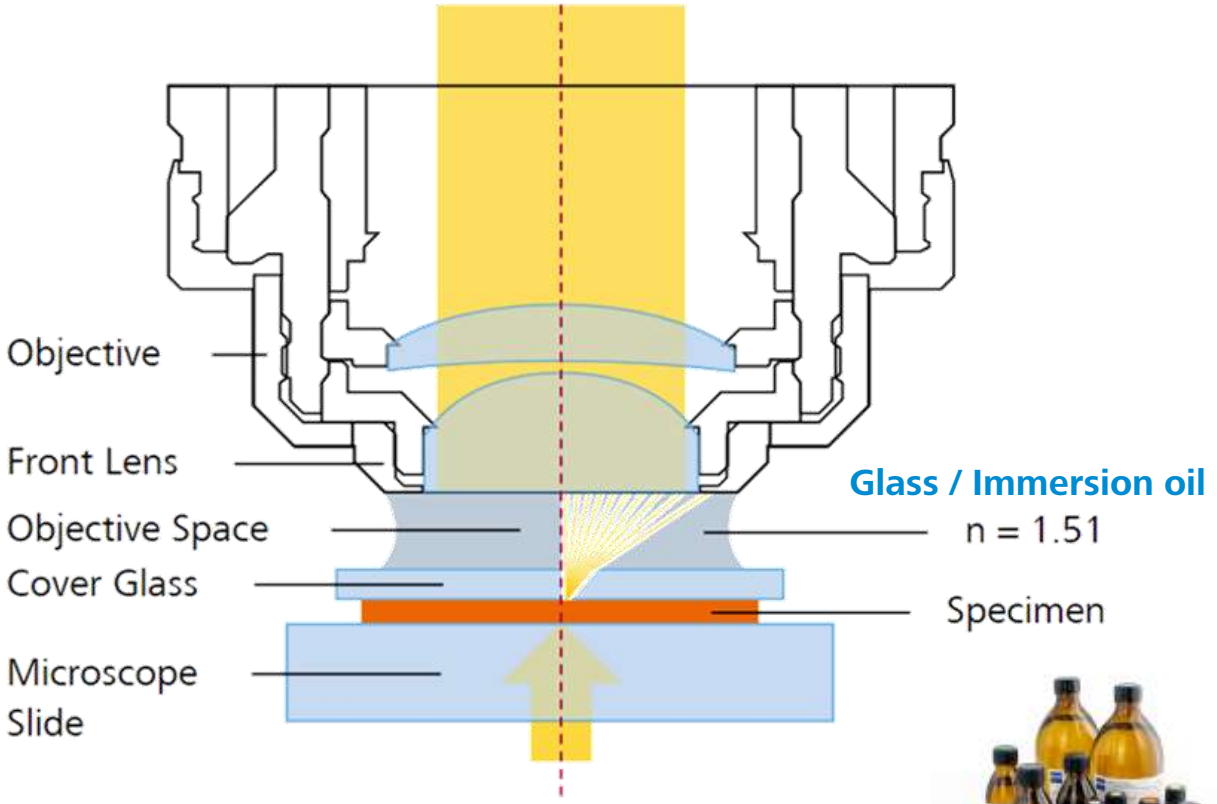
- Better resolution & brighter image
- Reduced working distance & sensitive to spherical aberration



# Immersion & Refractive Index



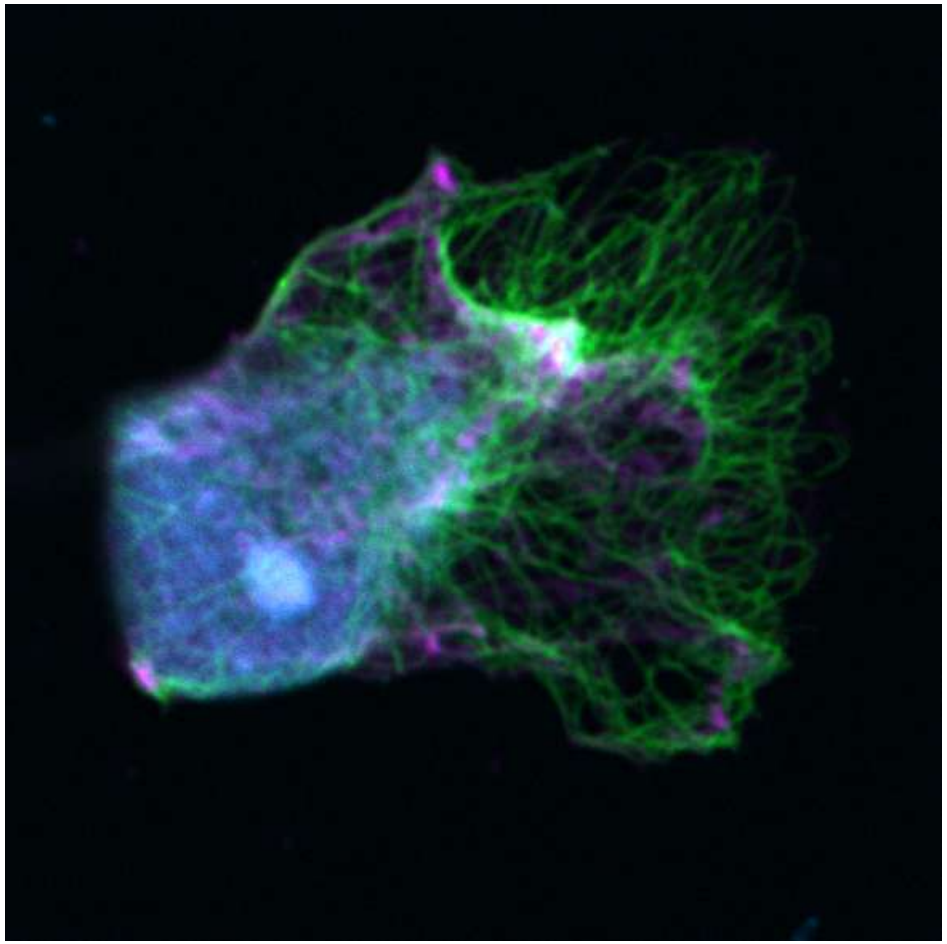

**Refractive Index (n)**

Low High

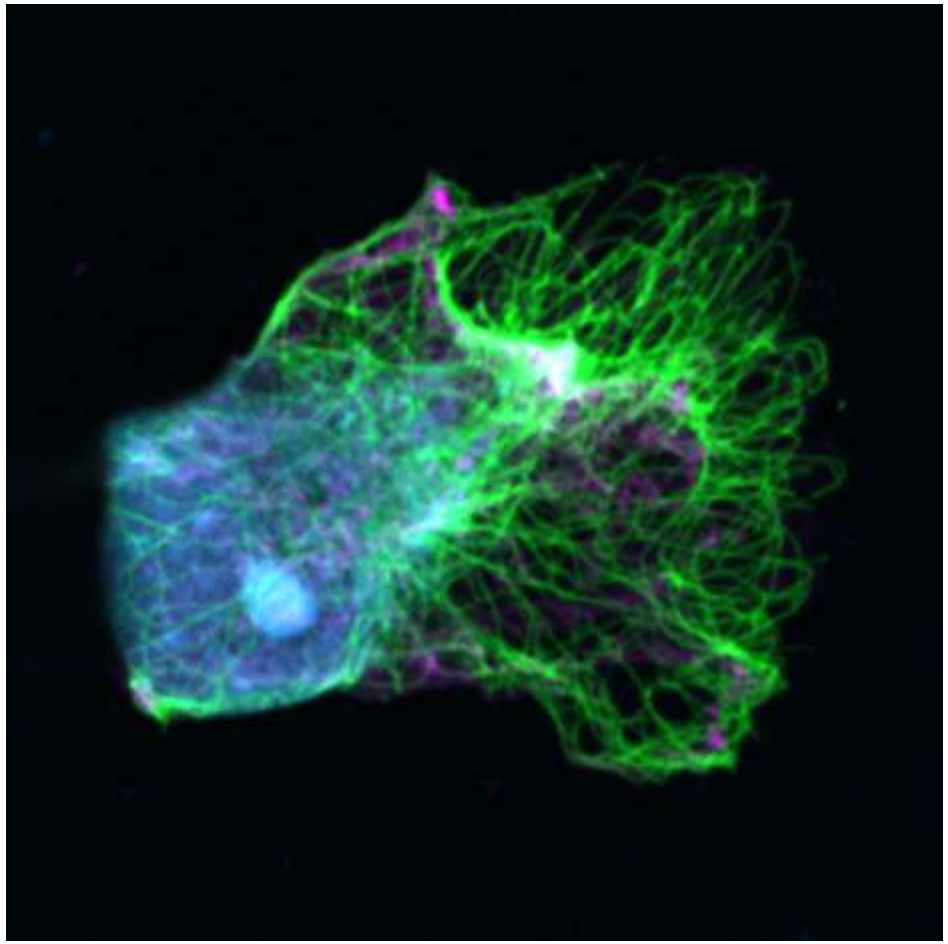
**Refractive Index (n)**

Low High

# Higher NA + Immersion = Higher Resolution

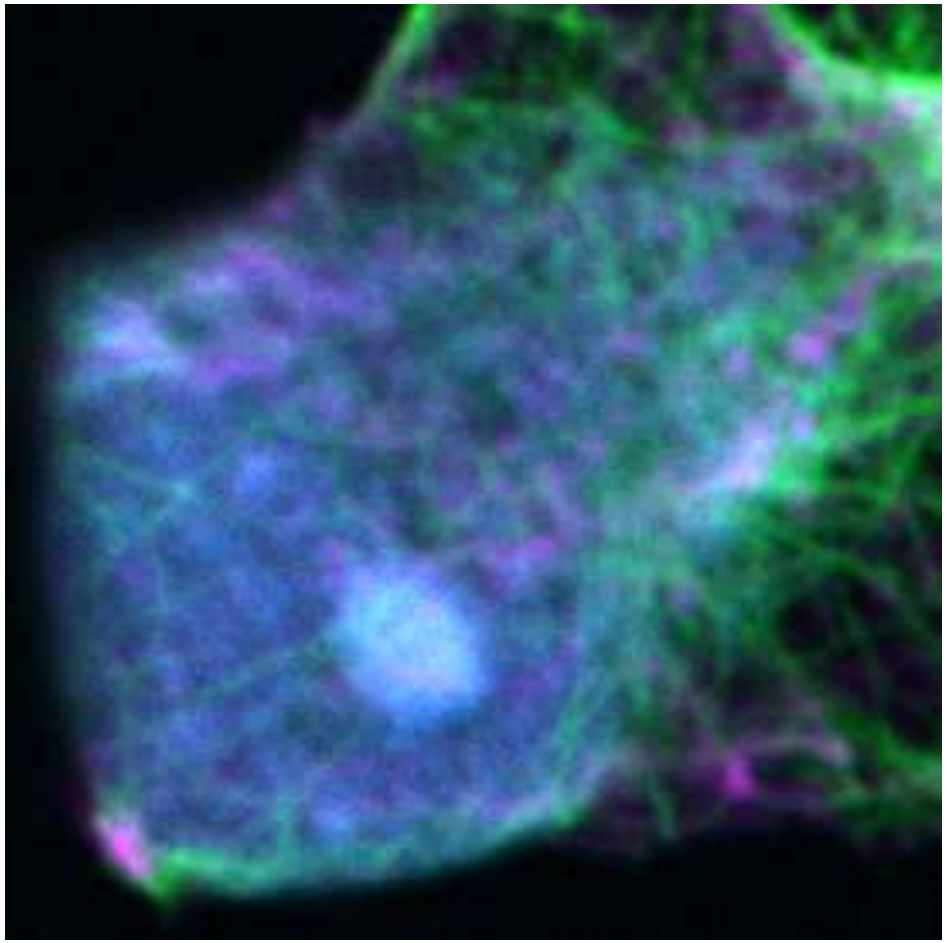


40x / 0.95 air

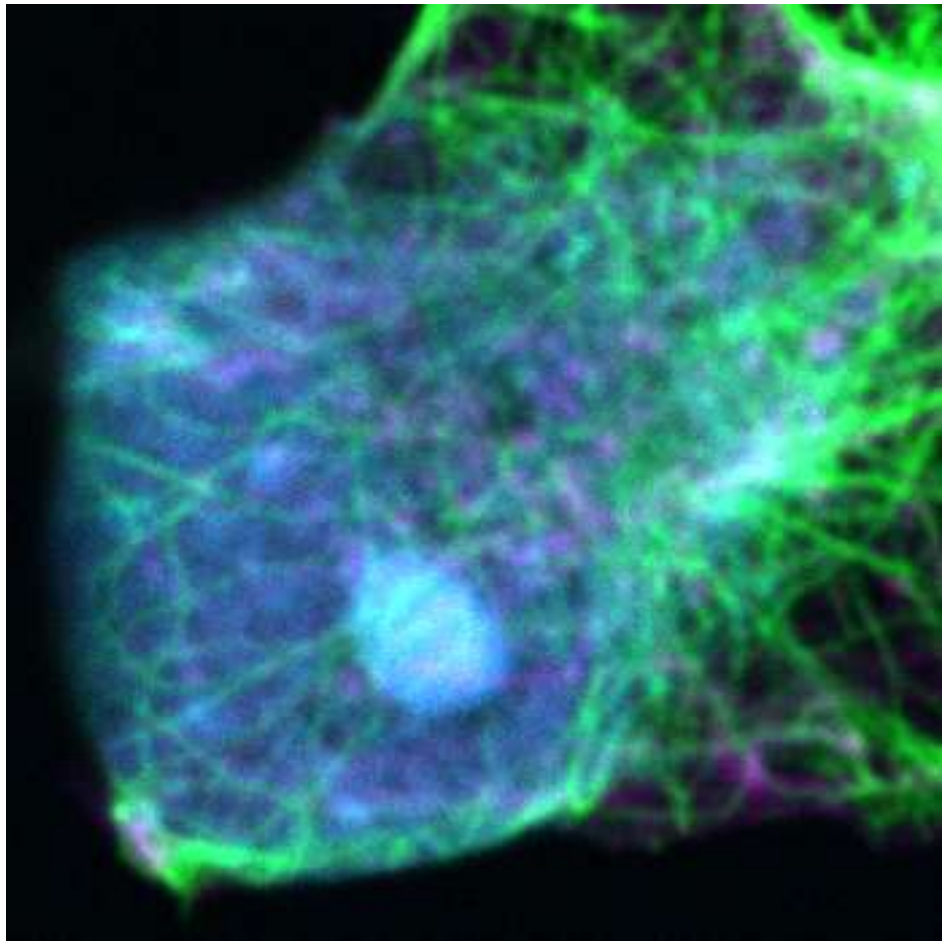


40x / 1.2 water

# Higher NA + Immersion = Higher Resolution



40x / 0.95 air

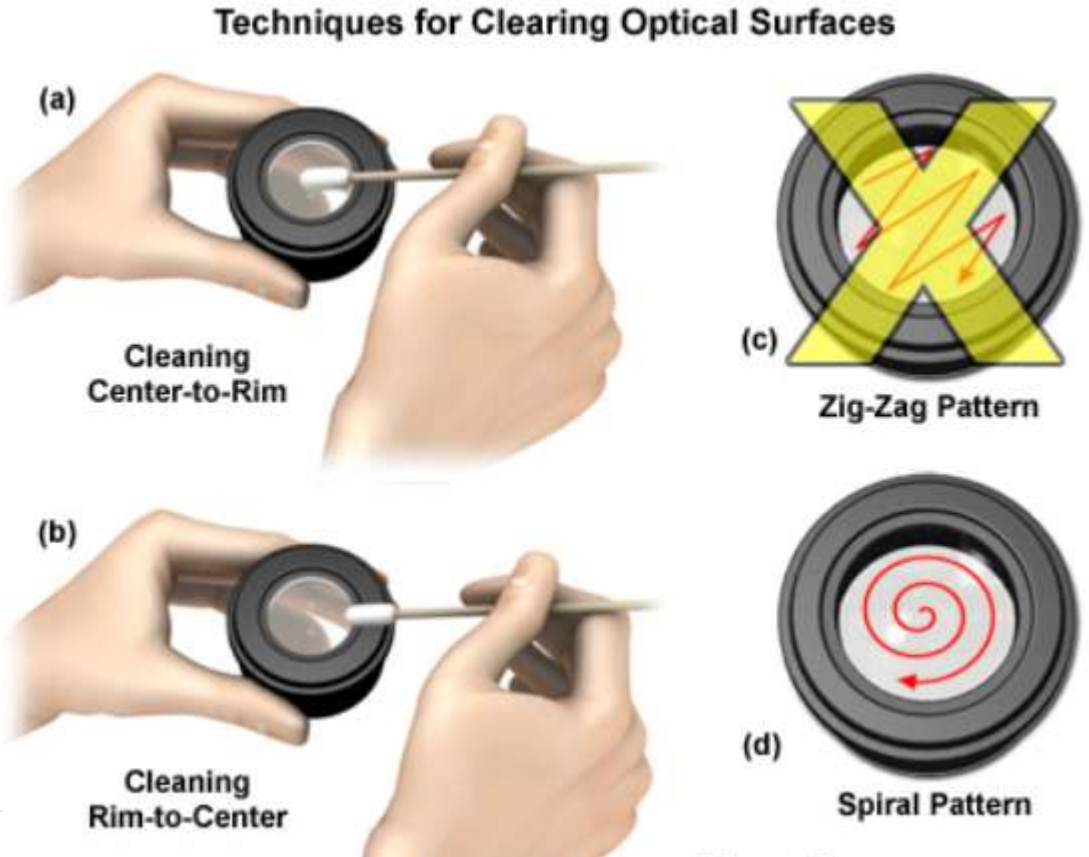


40x / 1.2 water

# Immersion Objectives



Figure 4

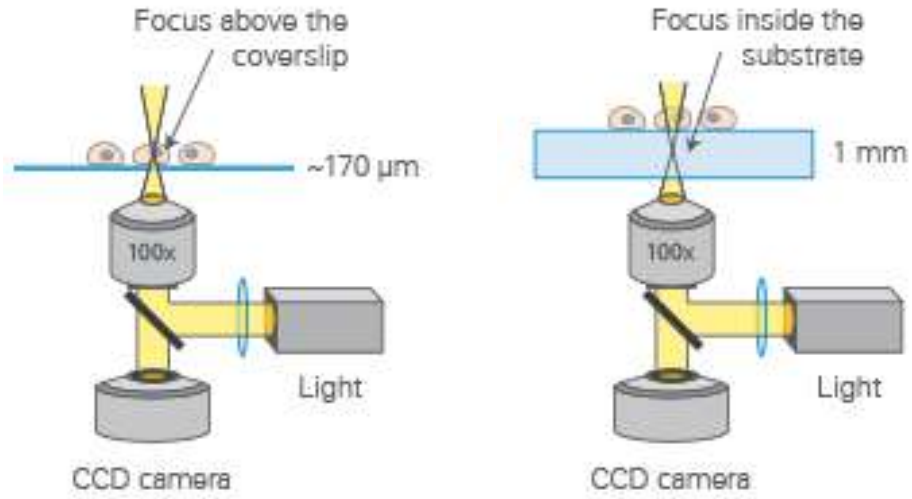


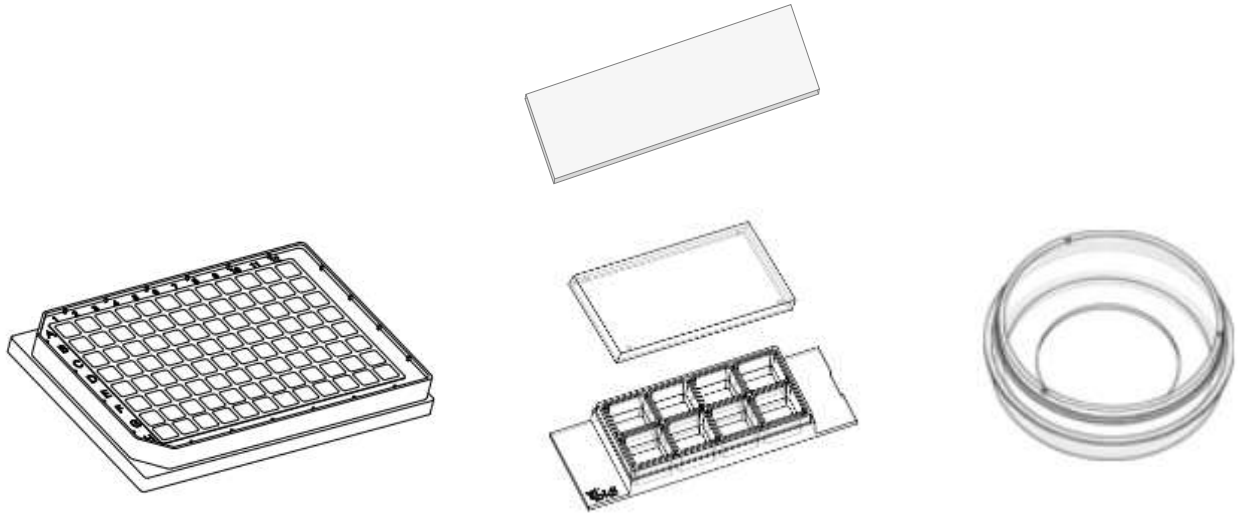
# Immersion & Refractive Index



Multi-Immersion objectives (**L**ive **C**ell **I**maging-objectives) can be used when working with different immersion media (oil, glycerol, water)

# Sample Carrier Thickness





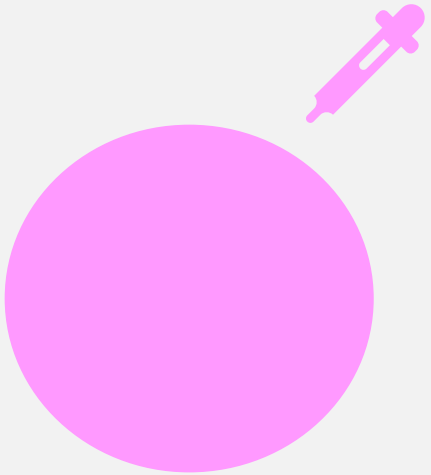
Thickness no. 1 (0.13-0.16 mm)
Thickness no. 1.5 (0.16-0.19 mm)
Thickness no. 1.5H (0.165-0.175 mm)

# Contrast Methods

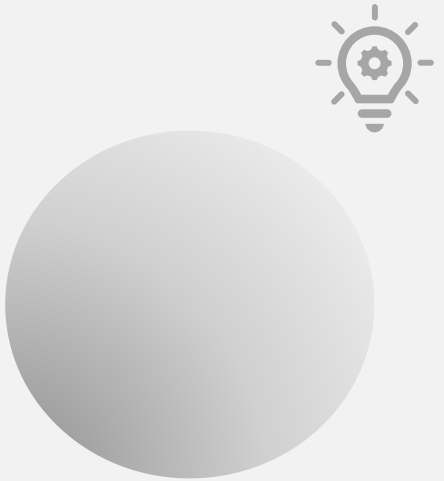
# Contrast Methods



Low contrast object

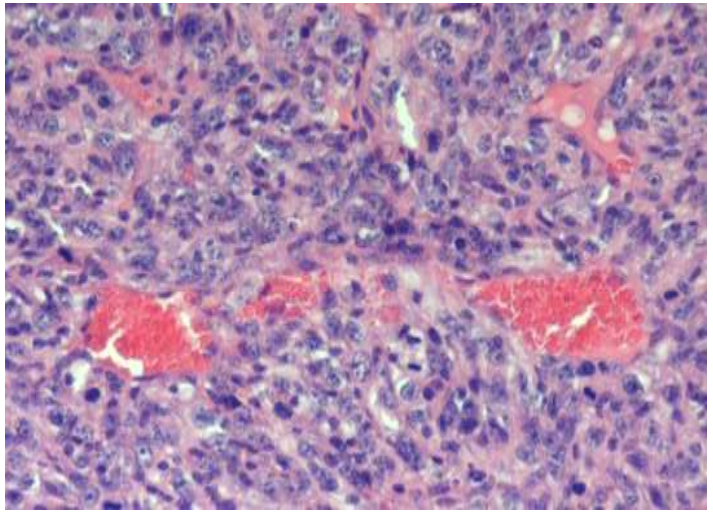


Stain



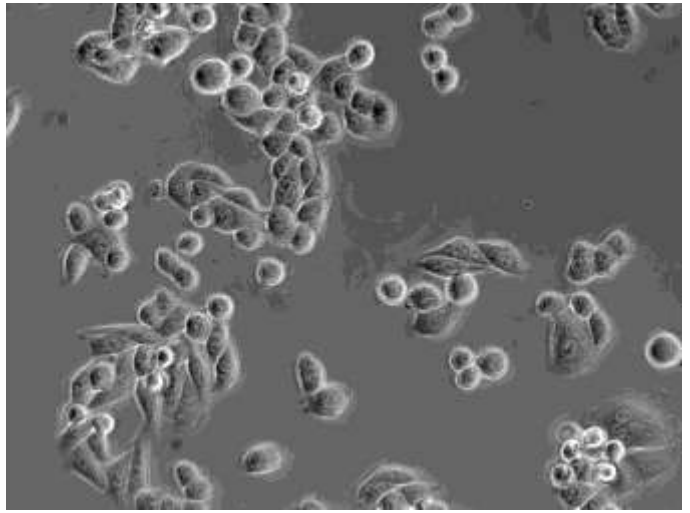
Shadow

# Contrast Methods



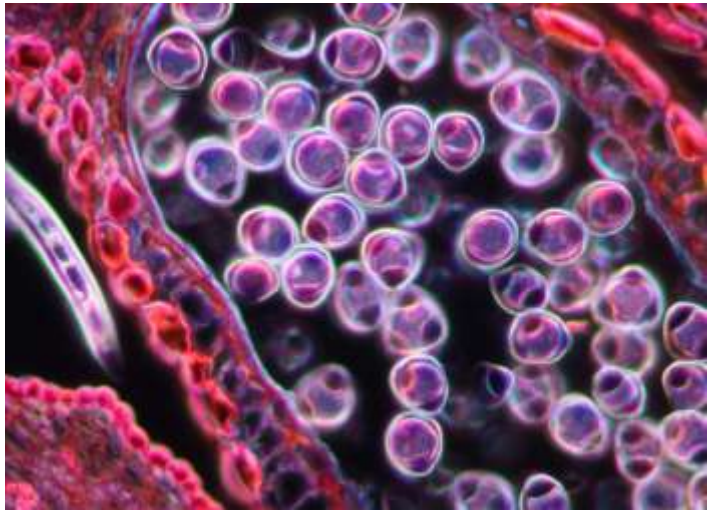
**Brightfield**

Colorful samples



**Phase contrast**

Colorless samples



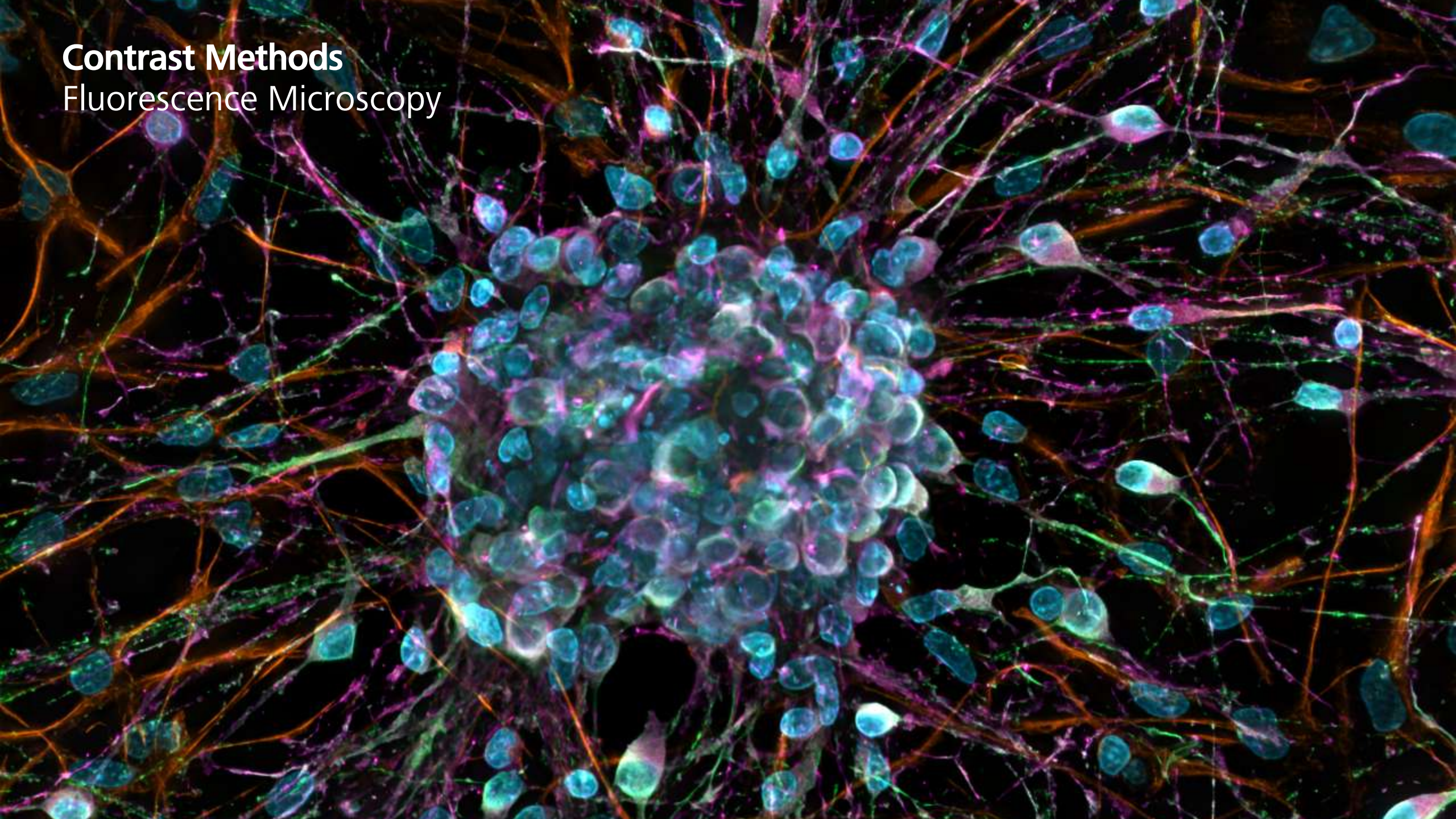
**Dark field**

Translucent samples

**DIC** (Differential Interference Contrast)

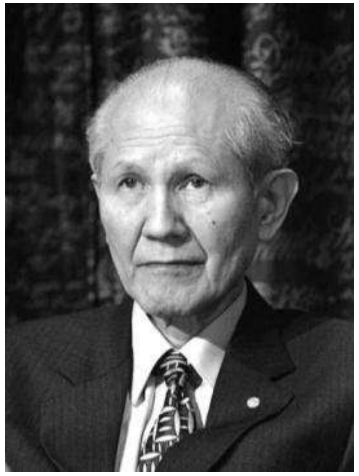
Colorless samples

# Contrast Methods
## Fluorescence Microscopy



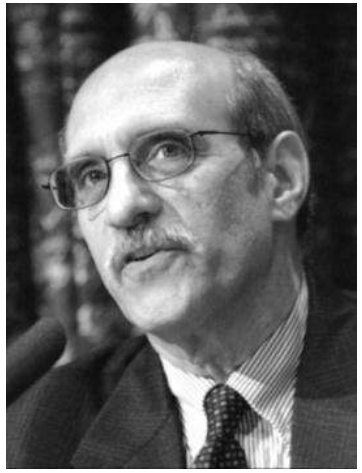
# The Moment Proteins Became Visible In Living Organisms

**1994** Fluorescence proteins make it possible to study organisms and cells in vivo

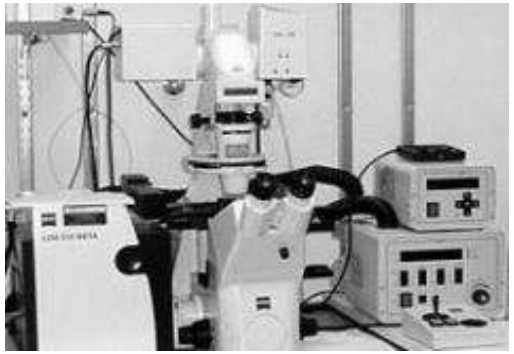


Osamu Shimomura
(Nobel Laureate, 2008)

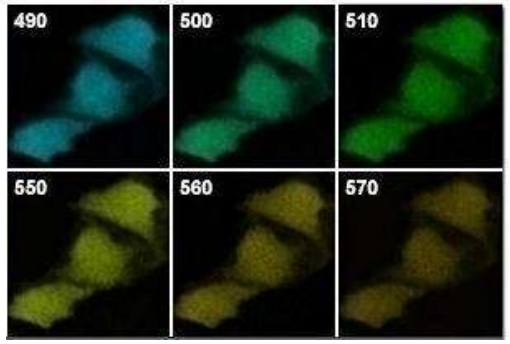
Roger Y. Tsien
(Nobel Laureate, 2008)



Martin Chalfie
(Nobel Laureate, 2008)

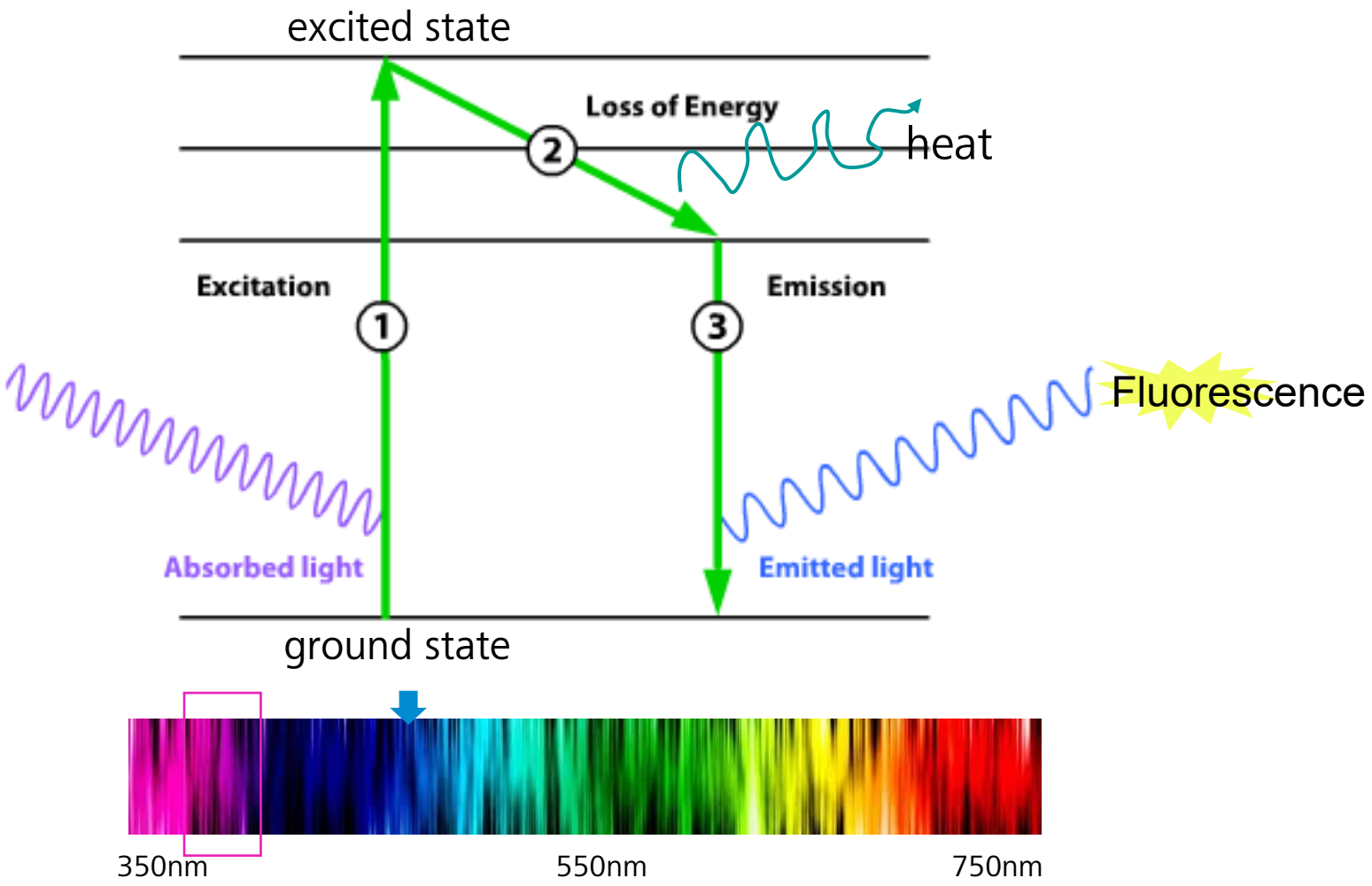


LSM 510 Meta from ZEISS

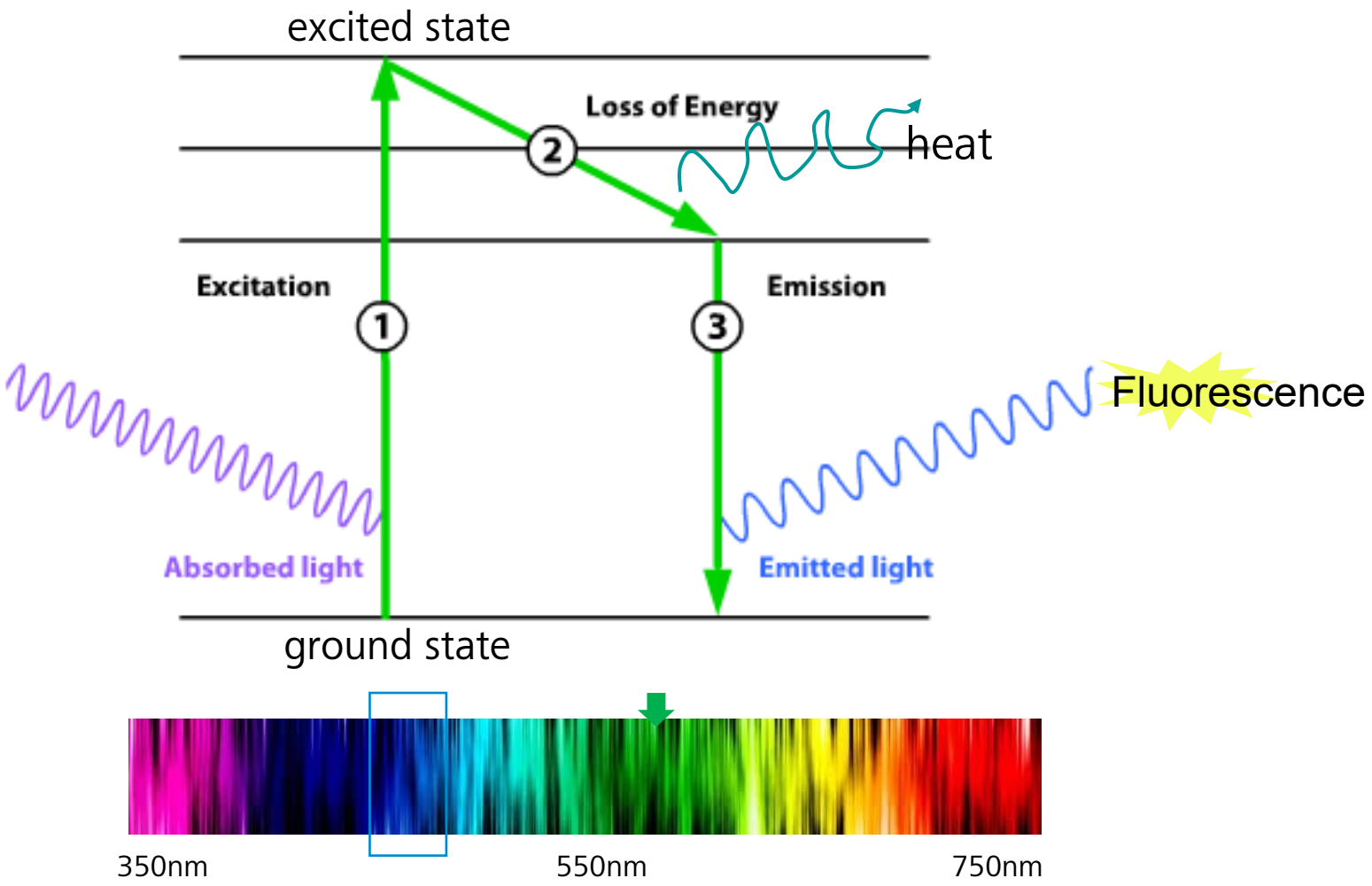



Spectral image of GFP labelled cells

# Contrast Methods
## Fluorescence Contrast (FL)



# Contrast Methods
## Fluorescence Contrast (FL)



# Why Do We Need Optical Sectioning
## The Fundamental Problem


- We want focused image
- We don’t like unfocused image

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# Why Do We Need Optical Sectioning
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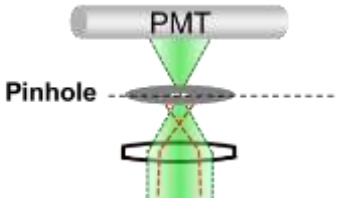
- We want focused image
- We don’t like unfocused image
- Unfocused images are annoying

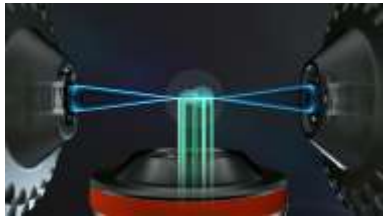
# Optical Sectioning

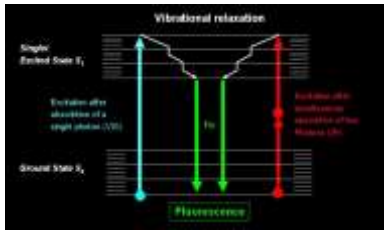
## Extracting The Layer Of The Image

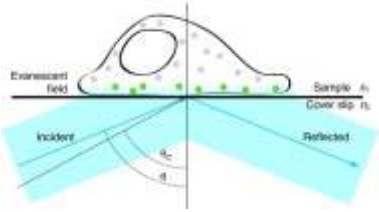
# General Optical Sectioning Methods

- Optical Sectioning Methods
  - Removing out-of focus light (downstream strategy)
    - Deconvolution
    - Structured Illumination
  - Blocking out-of focus light (detection strategy)
    - Confocal Methods
  - Avoiding out-of focus light (excitation strategy)
    - Light sheet
    - Multi-Photon
    - Total Internal Reflection



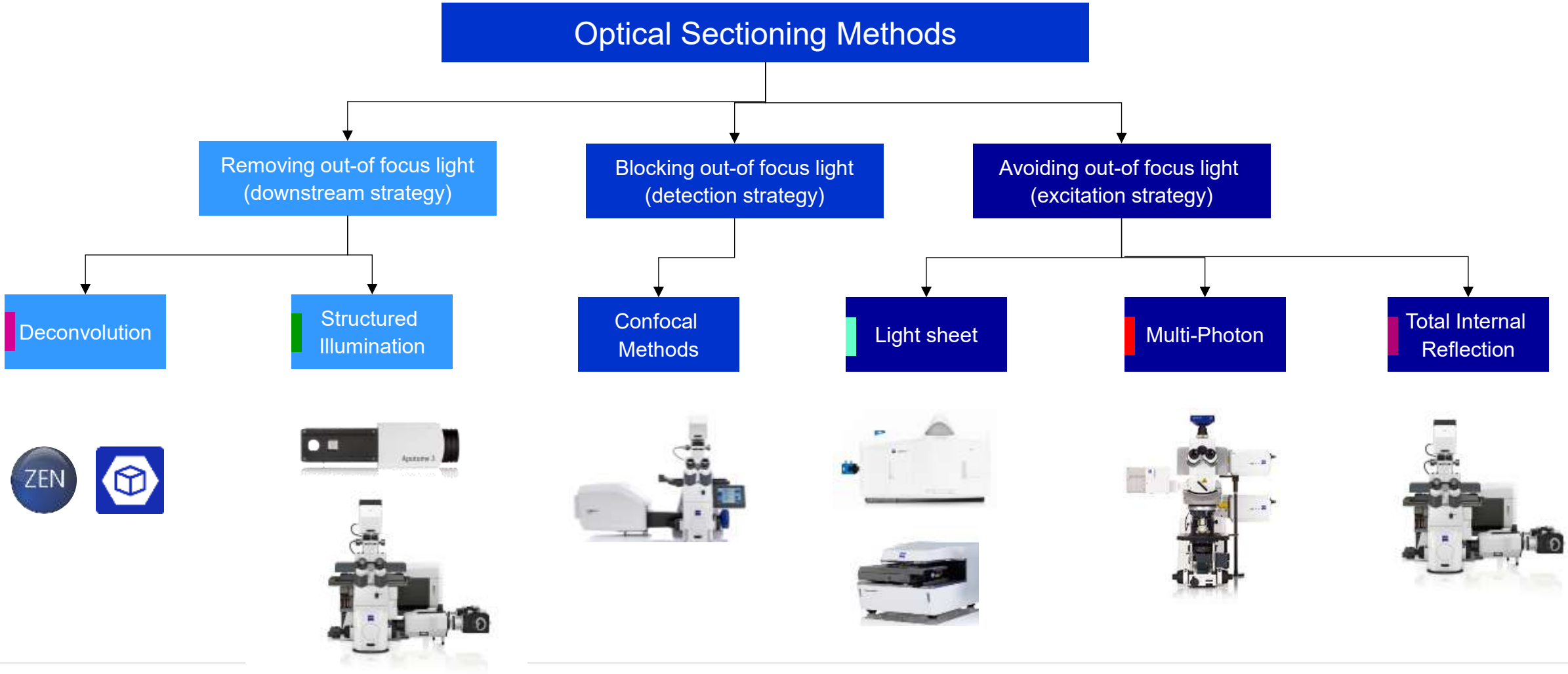






[Total Internal Reflection Microscopy (TIRF) Service - Creative Biostructure (creative-biostructure.com)]

# General Optical Sectioning Methods



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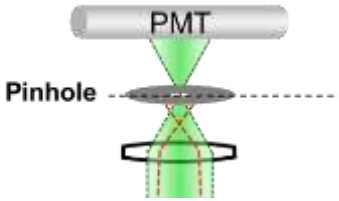
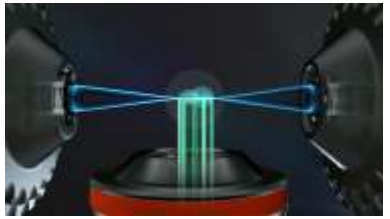
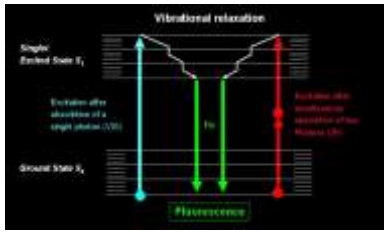
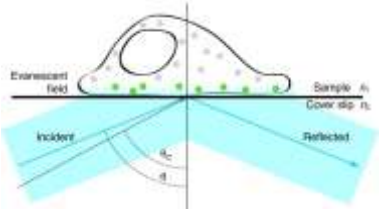


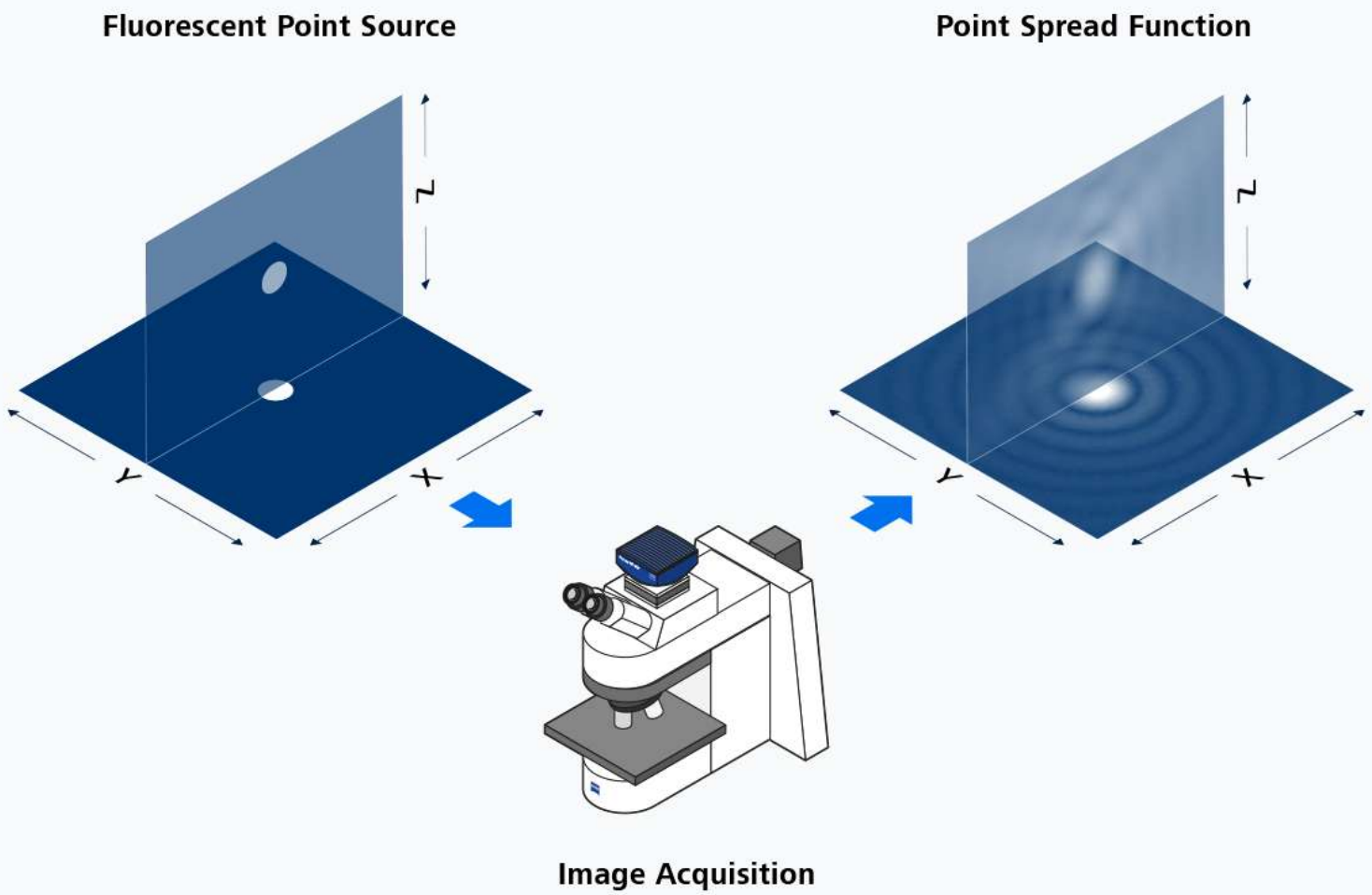





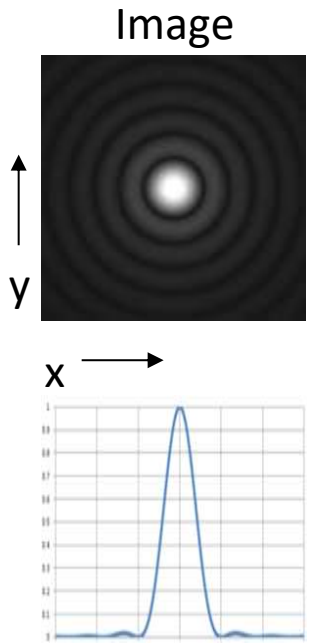

[Total Internal Reflection Microscopy (TIRF) Service - Creative Biostructure (creative-biostructure.com)]

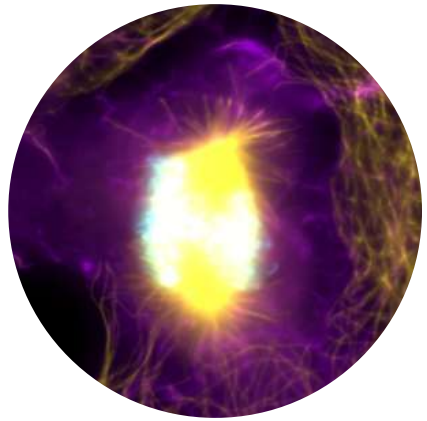
# Lights Were Convolutioned Before You See Them




**Point-Spread-Function**

The image of a point is not a point. It's a complex 3-dimensional diffraction pattern.

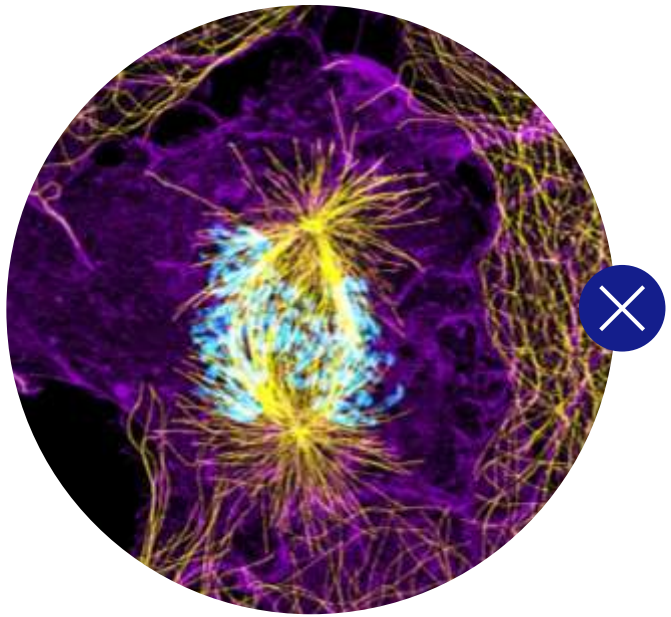


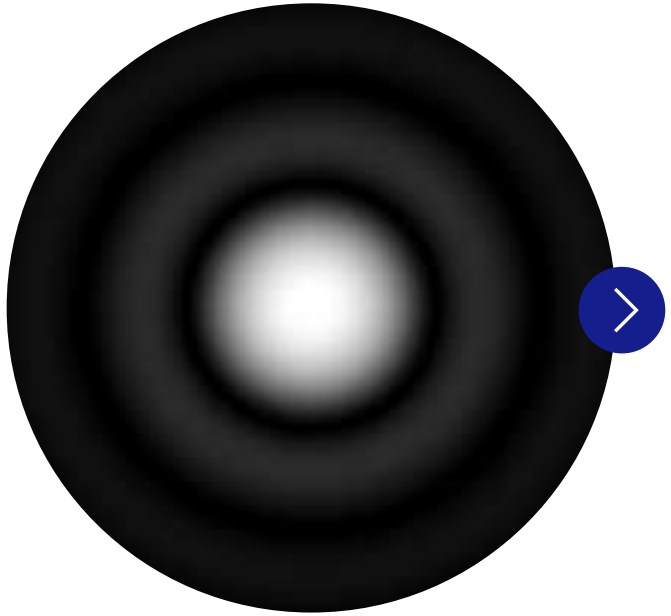
# Imaging in Mathematical Terms

"Convolution" of the Object with the PSF

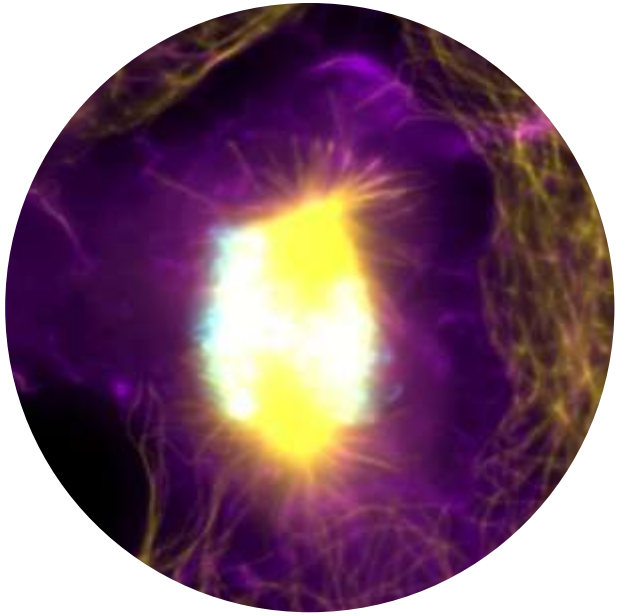
Sample

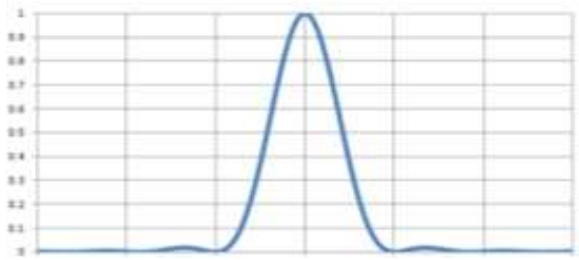


PSF



Image





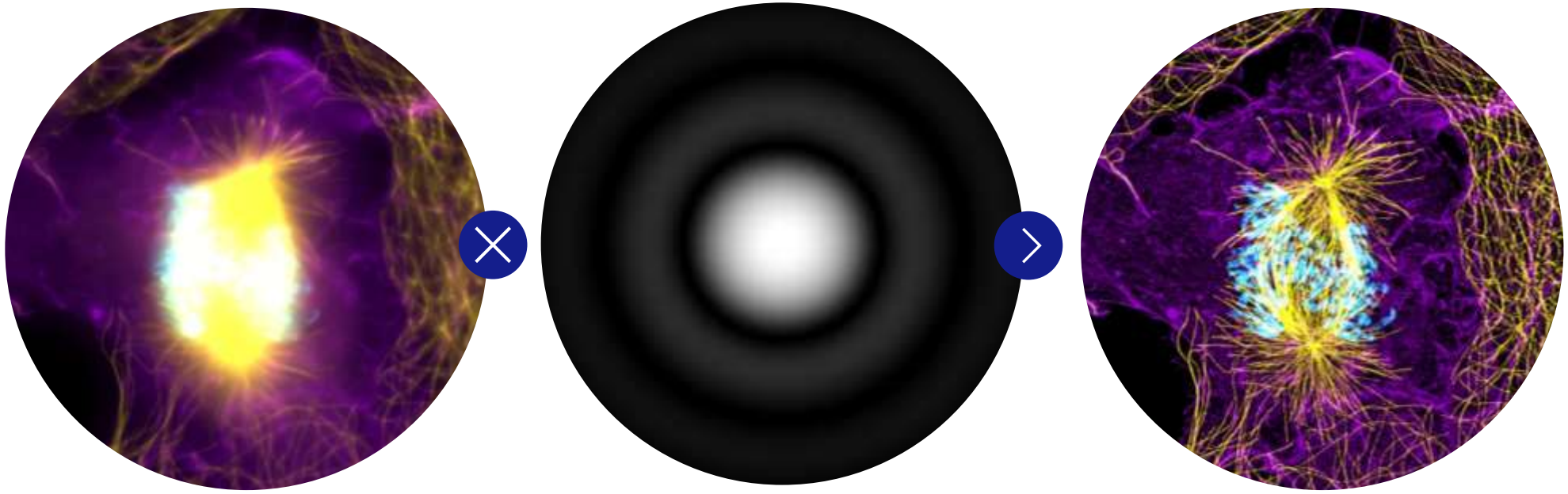
# Inverting the Imaging-Process with Mathematics

## A Deconvolution of the Image

Sample

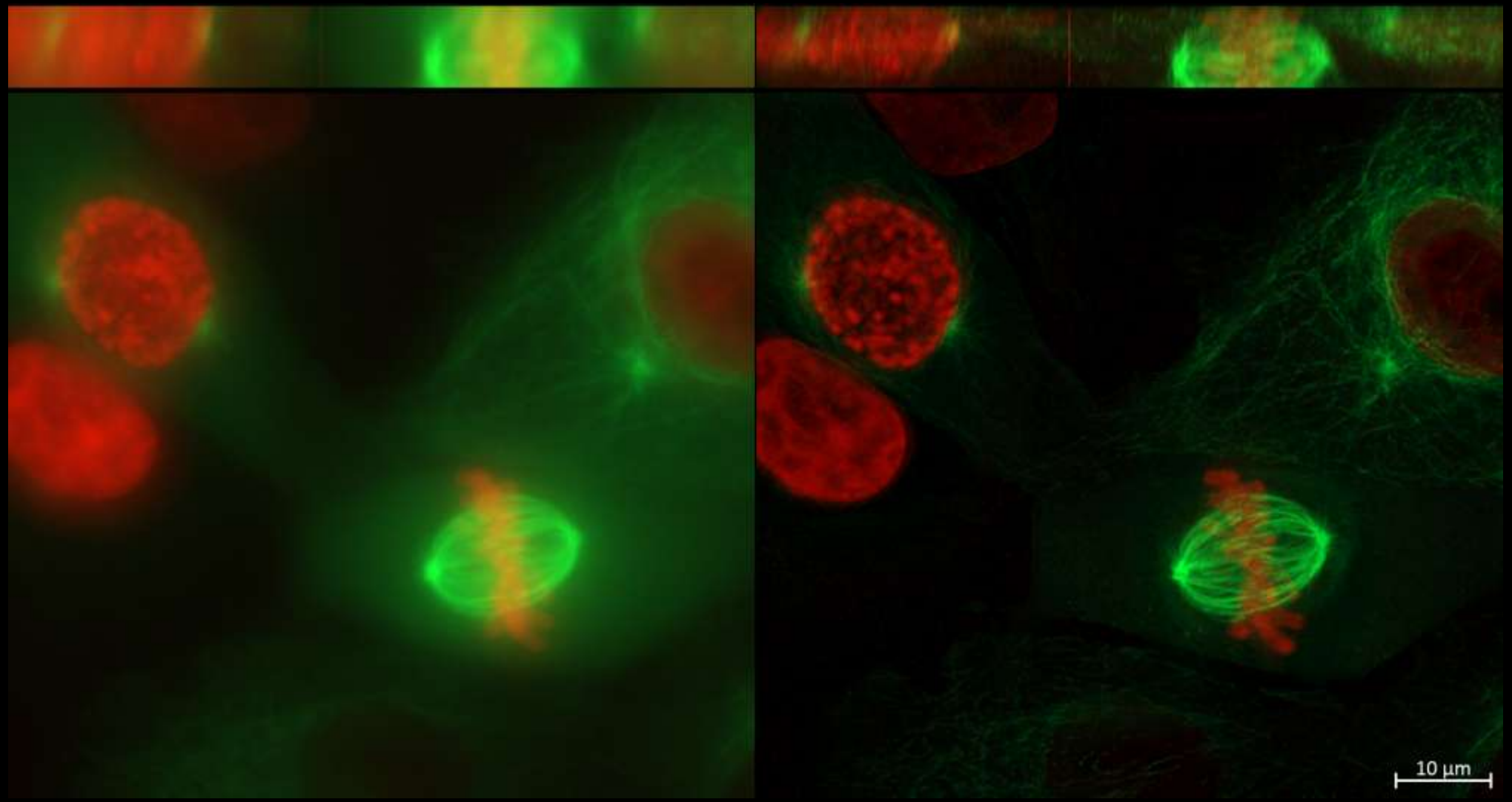
Deconvolution with PSF

Image



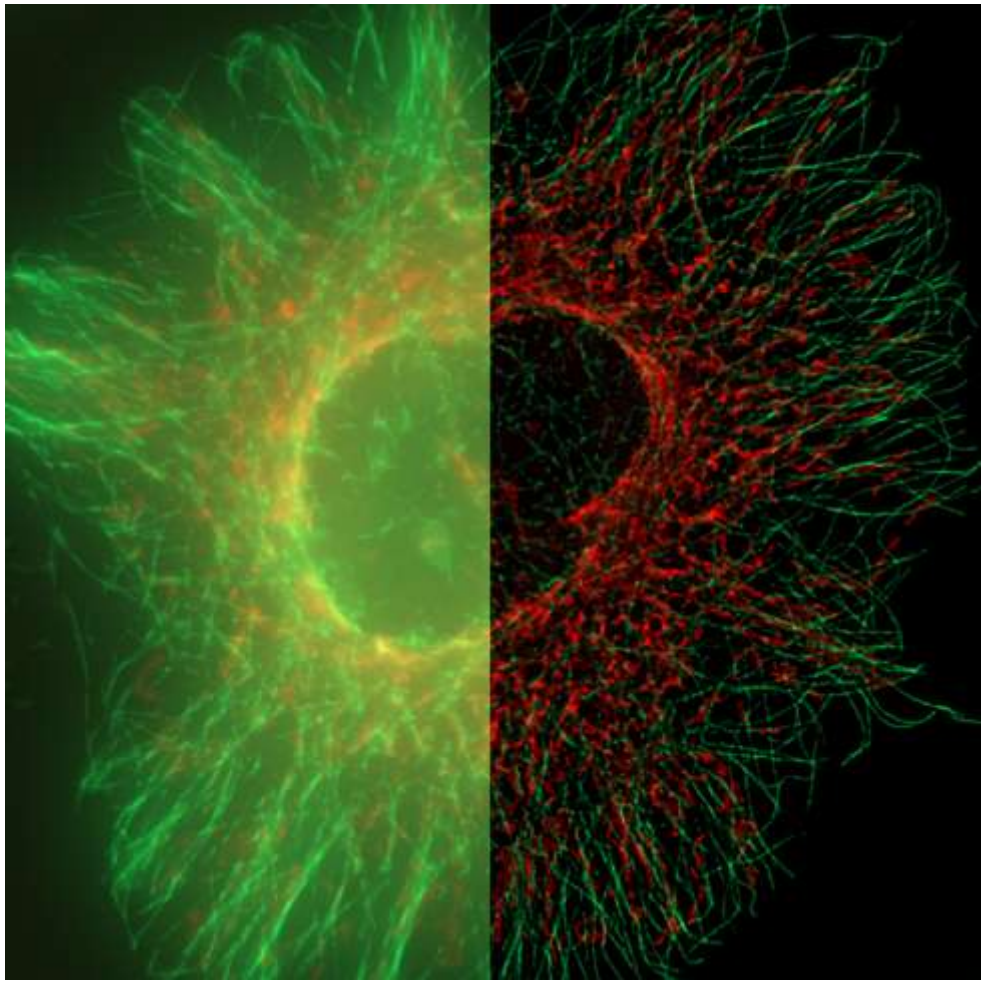
**“Re-assignment” of “photons“**

# Widefield Imaging with Deconvolution



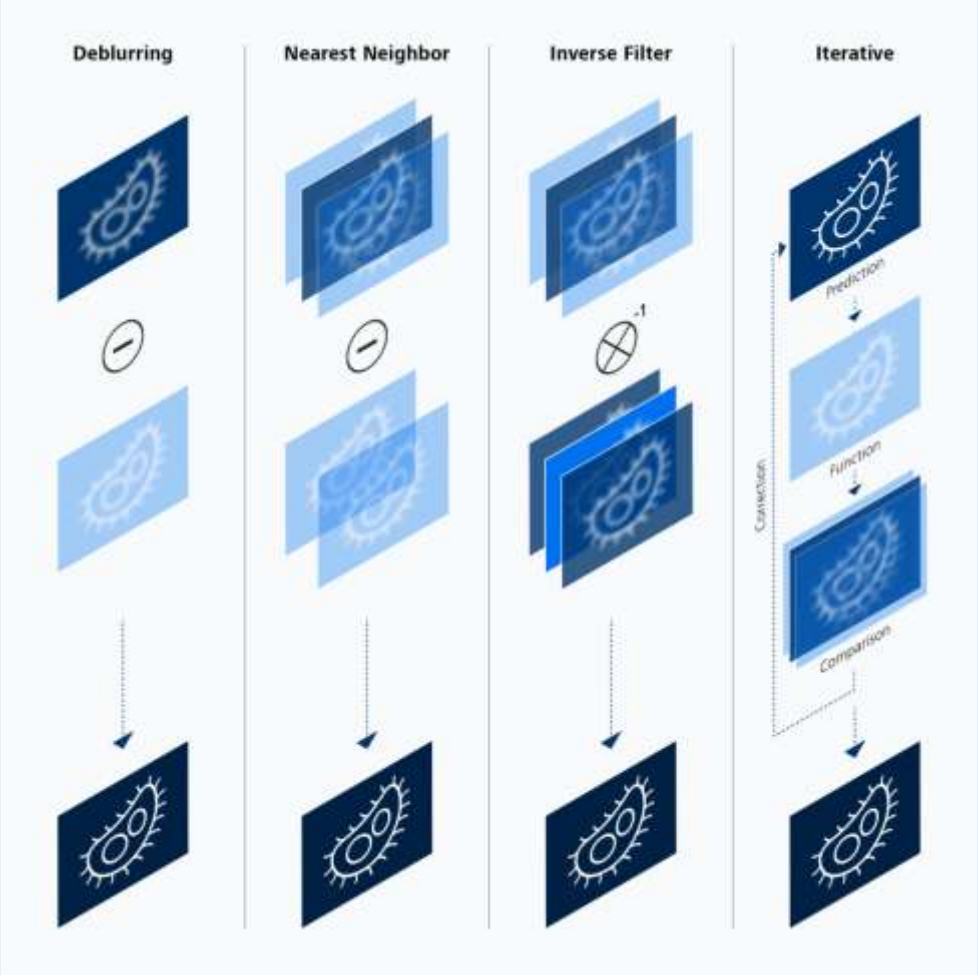
# Deconvolution Algorithms

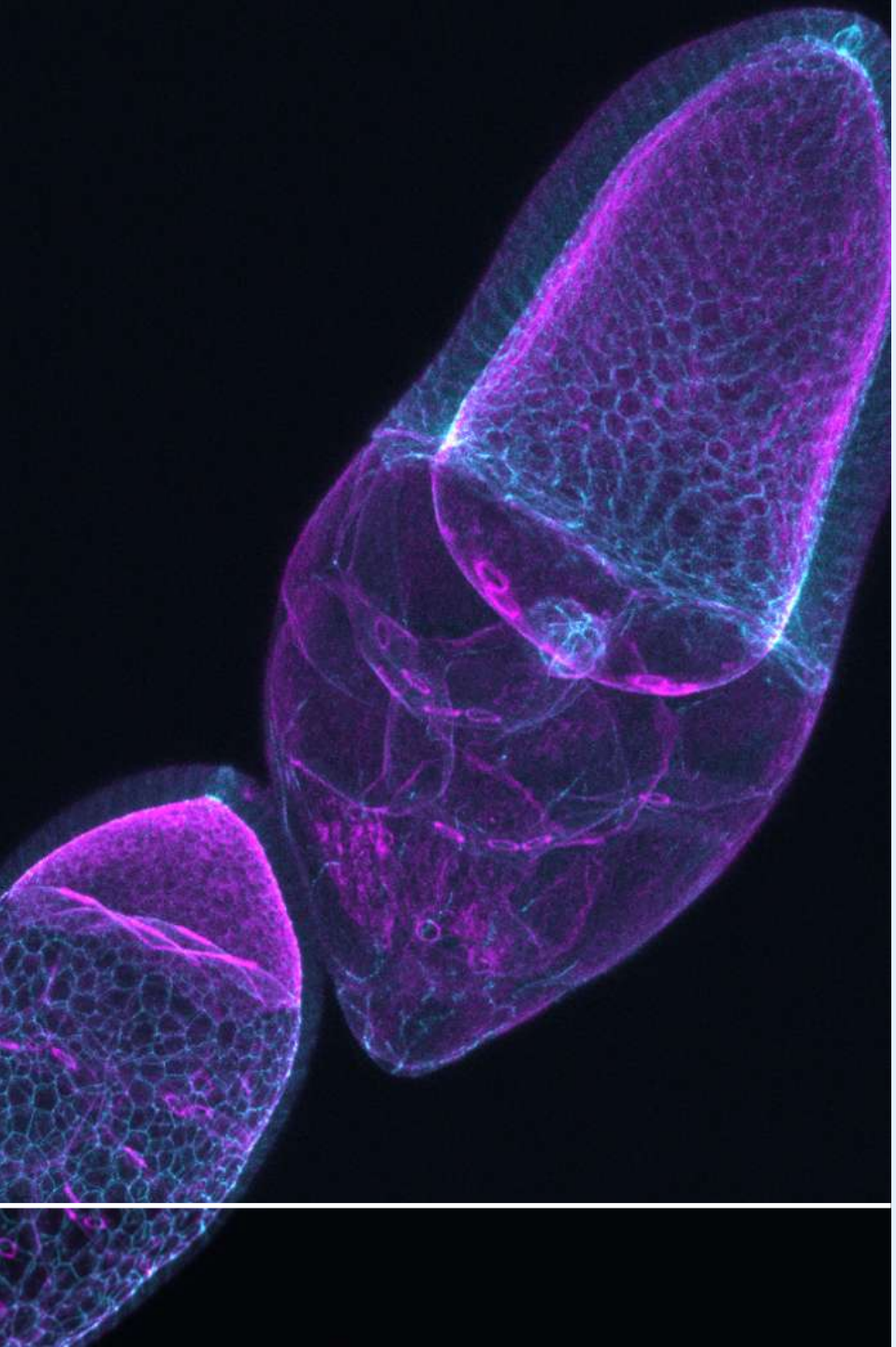
Raw Deconvolution



U2OS cells labeled for mitochondria (TOM20-mCherry) and microtubules (Tubulin-GFP) structures before and after Constrained Iterative Deconvolution.

Fast Slow



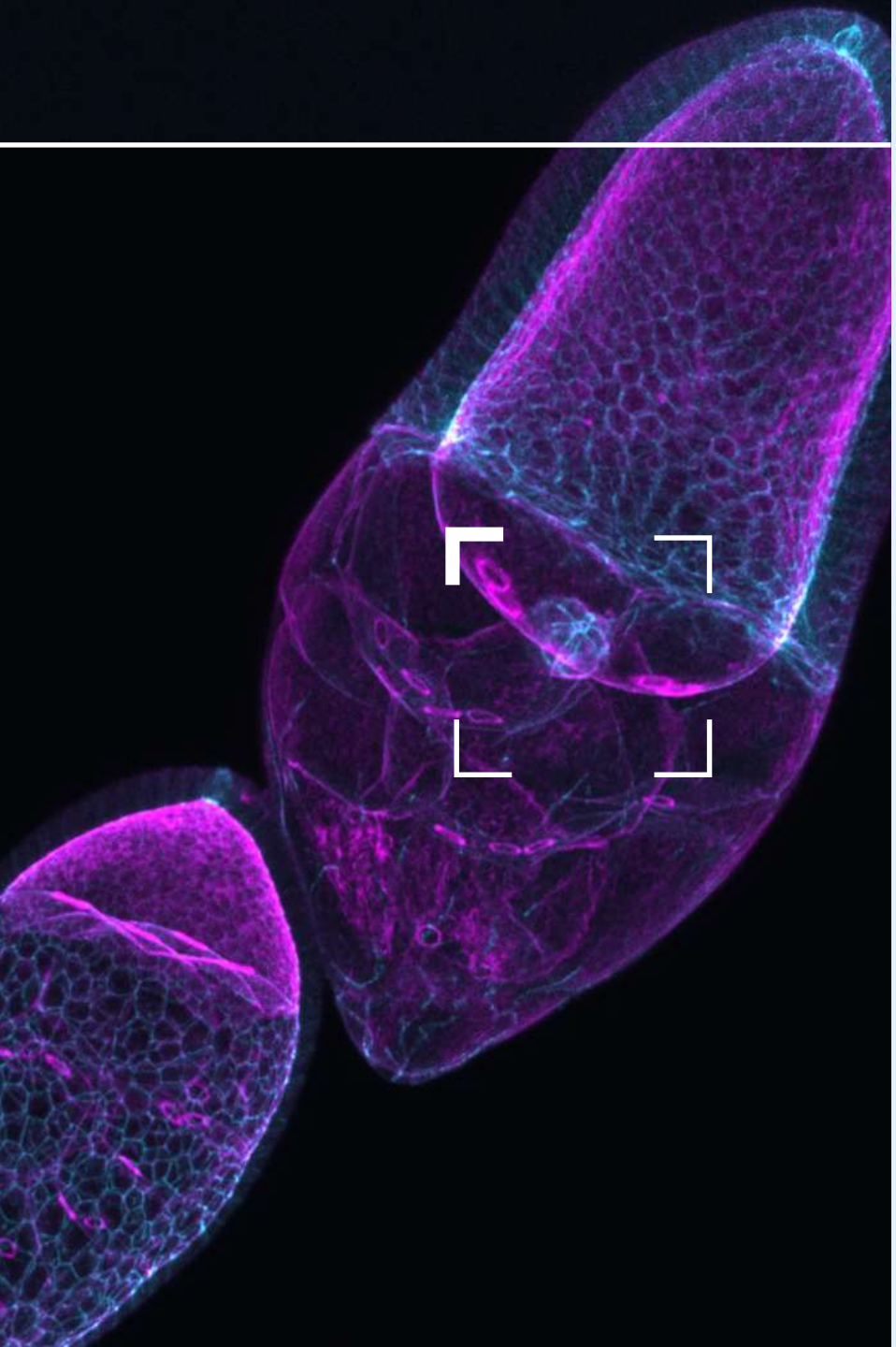


# A unique confocal experience

## LSM Plus

Drosophila egg chambers stained for F-actin (Phalloidin, magenta) and DE-Cadherin (cyan)

Sample courtesy of Thea Jacobs, AG Luschnig, WWU Münster, together with T. Zobel, Münster Imaging Network, Germany



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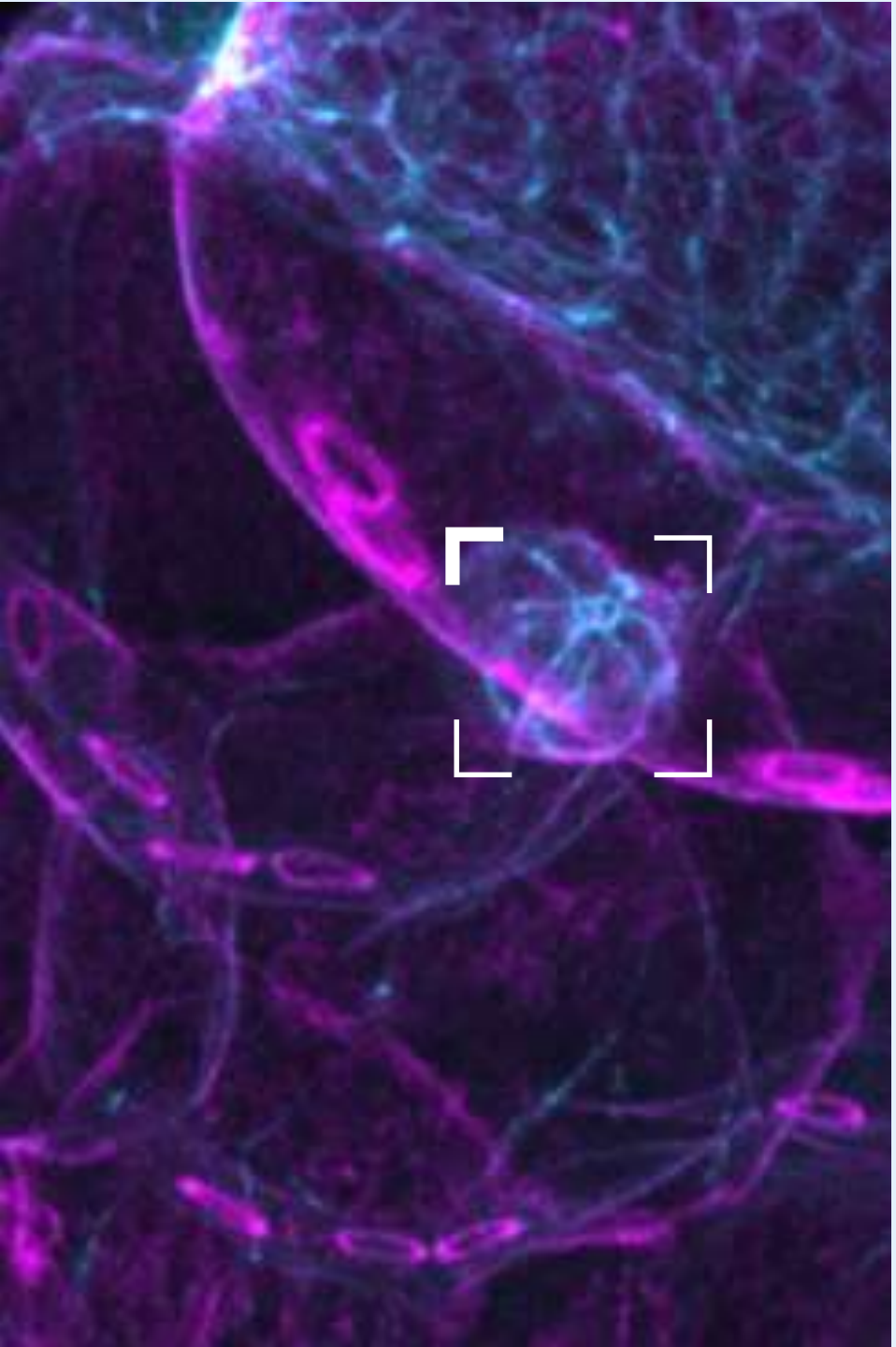

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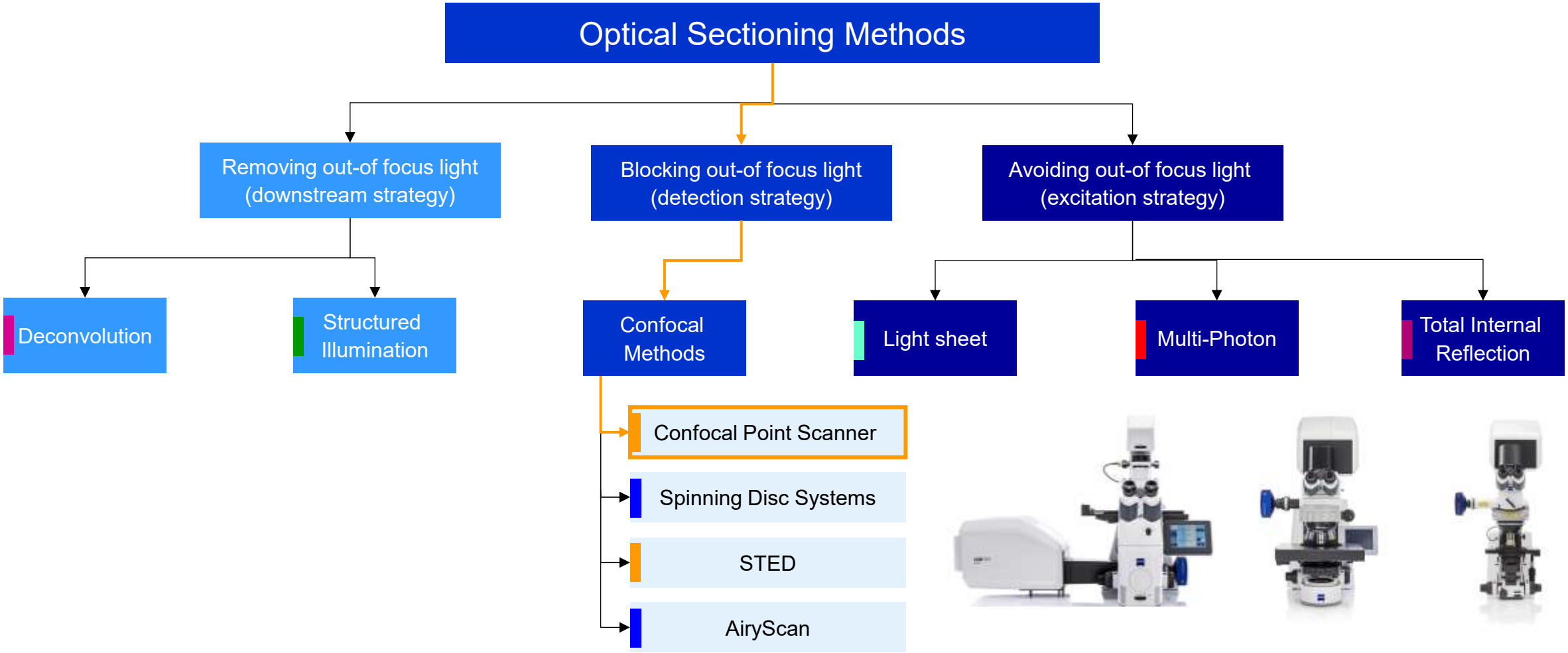
# General Optical Sectioning Methods



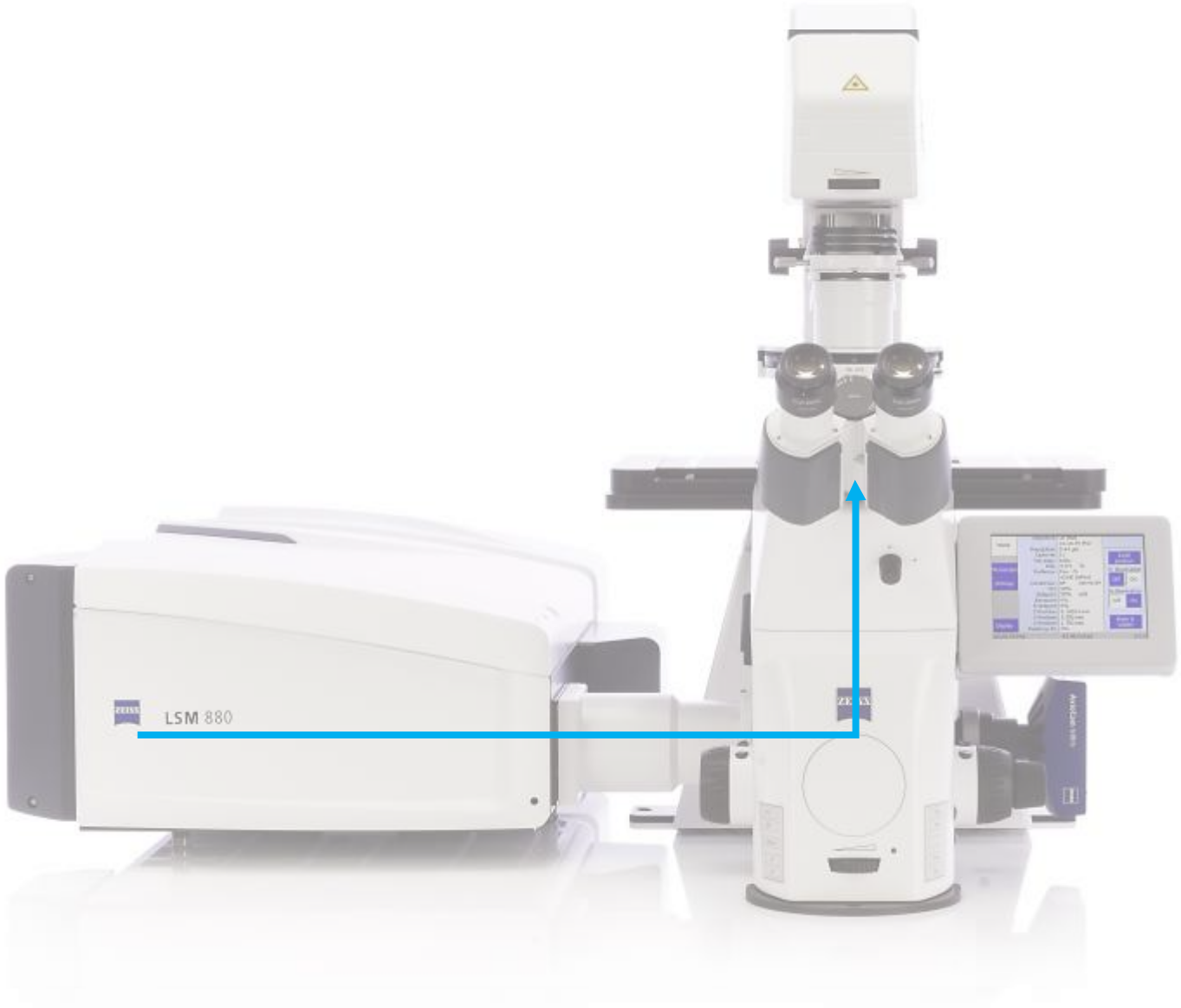
# ZEISS LSM Confocal

## Inverted microscope

## Software

## Scanning module

# ZEISS LSM Confocal



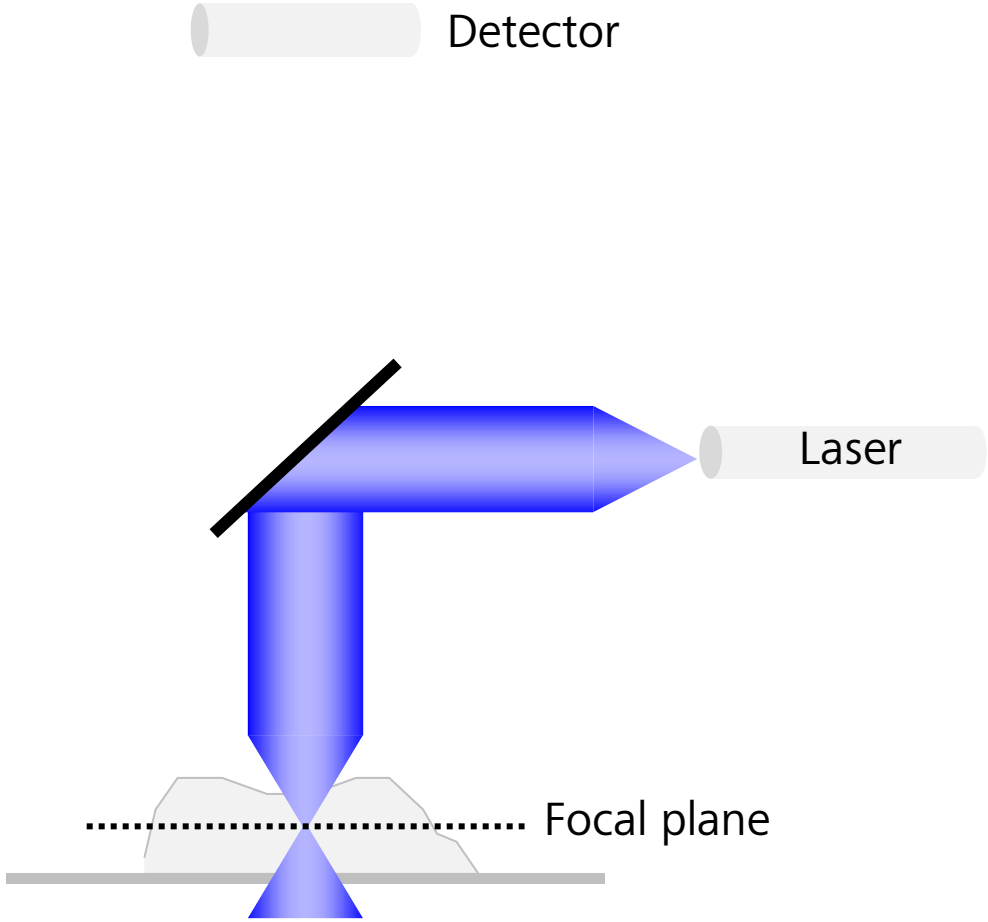

# ZEISS LSM Confocal


# Point Scanning Confocal Microscopes

## Confocal principle

**Spot Illumination**

A laser beam which is focussed to a diffraction limited spot illuminates the sample and is used for fluorescence excitation.

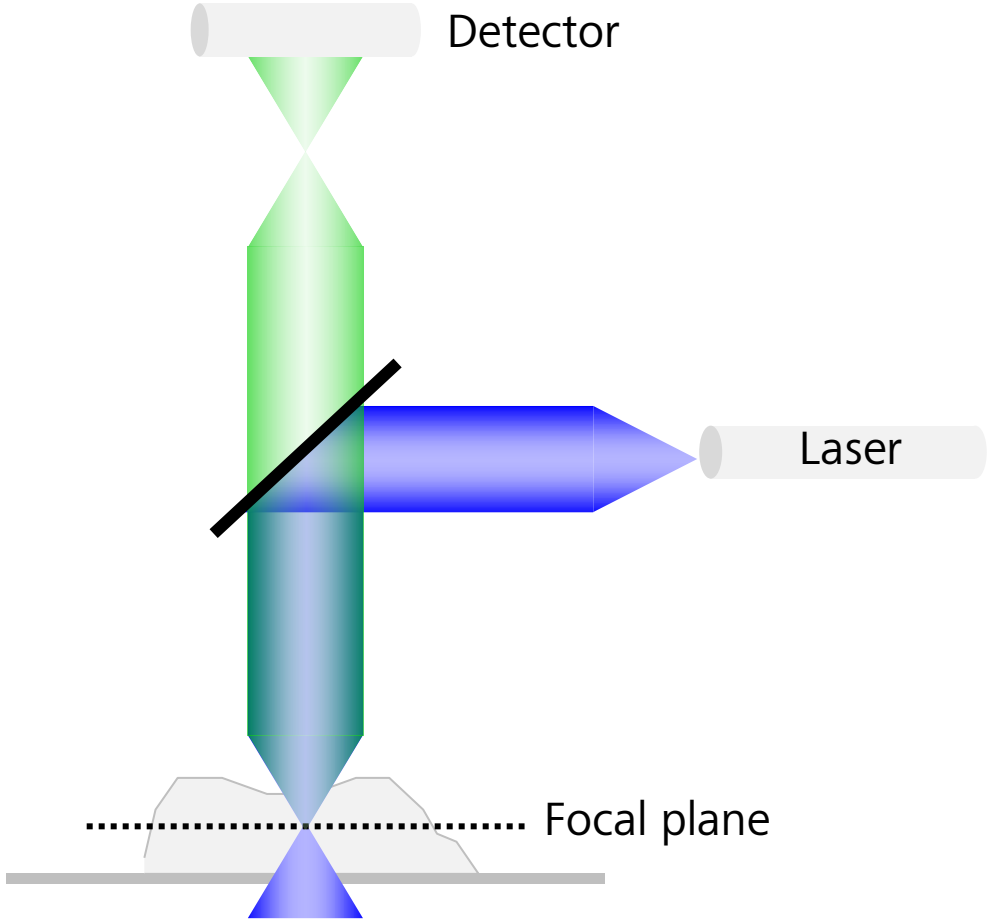


# Point Scanning Confocal Microscopes

## Confocal principle

**Spot detection**

The emitted fluorescence light is separated from the excitation light by appropriate beamsplitters and is usually detected by a photomultiplier.



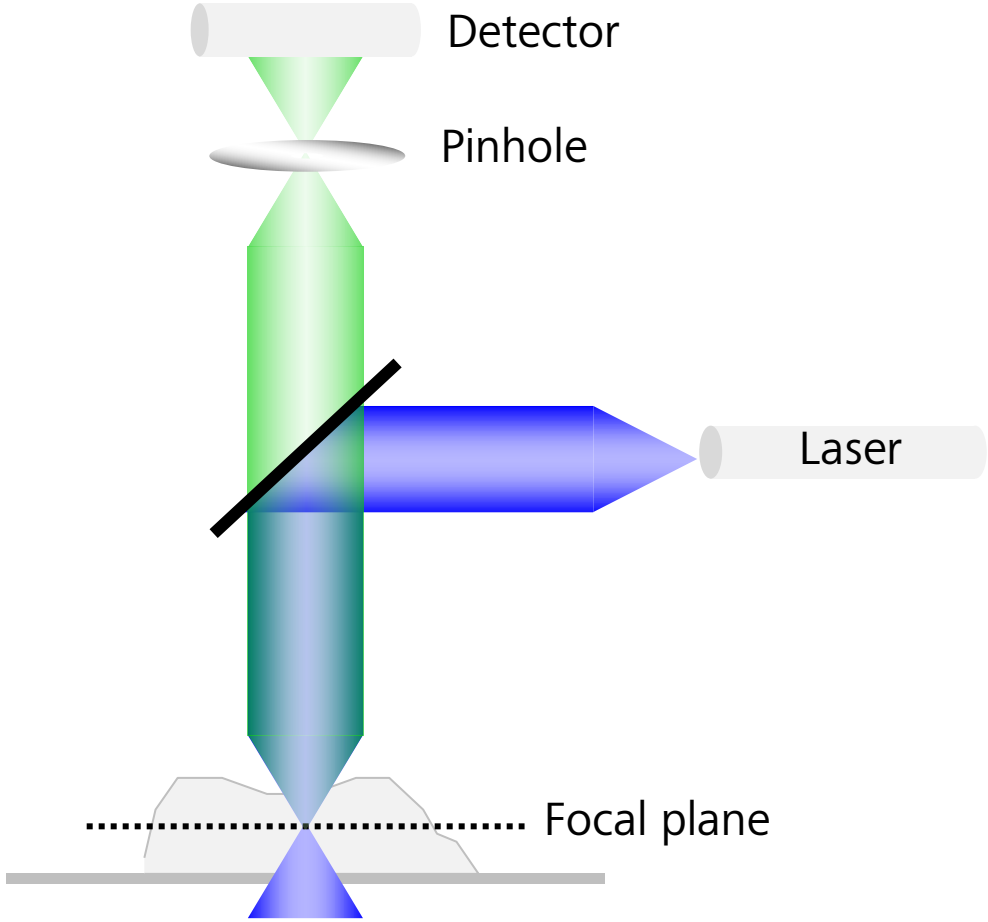
# Point Scanning Confocal Microscopes

## Confocal principle

**Spot detection**

The emitted fluorescence light is separated from the excitation light by appropriate beamsplitters and is usually detected by a photomultiplier.

The crucial part is the pinhole, which is placed in front of the detector – in a conjugated plane to the focal plane of the objective.

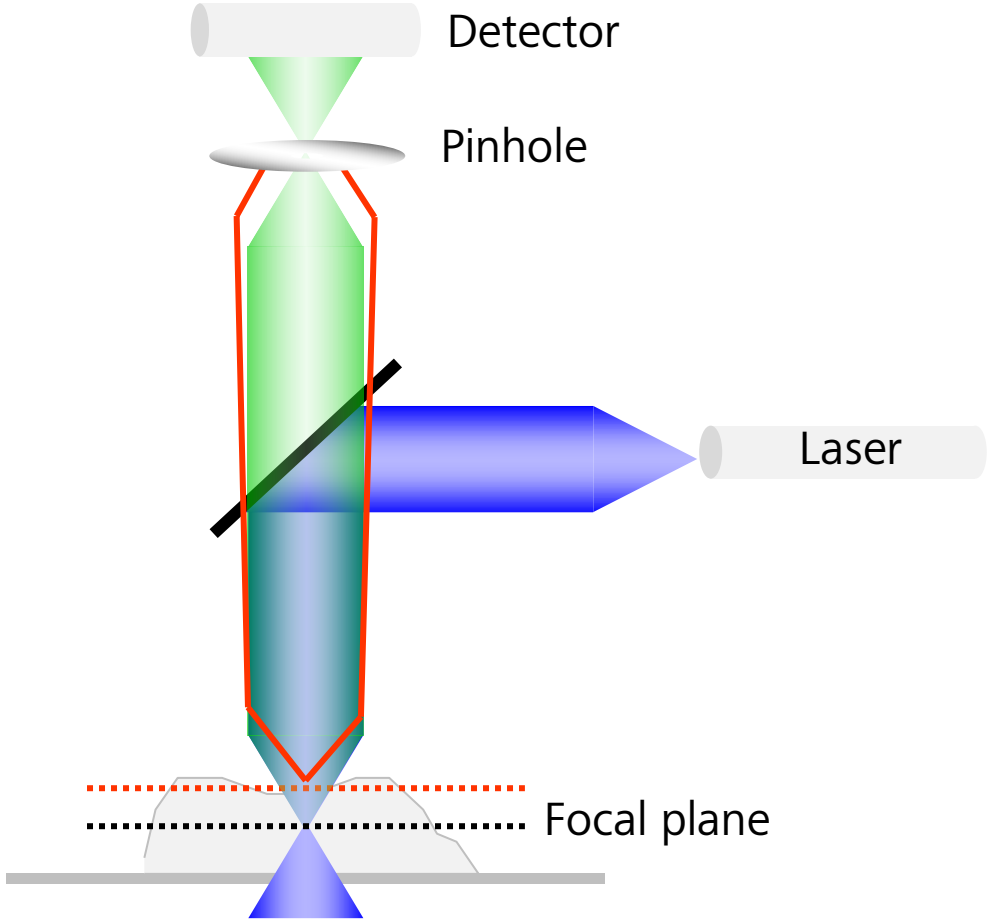


# Point Scanning Confocal Microscopes

## Confocal principle

**Spot detection**

This pinhole simply blocks fluorescence light, which originates from above or below the focal plane of the objective.



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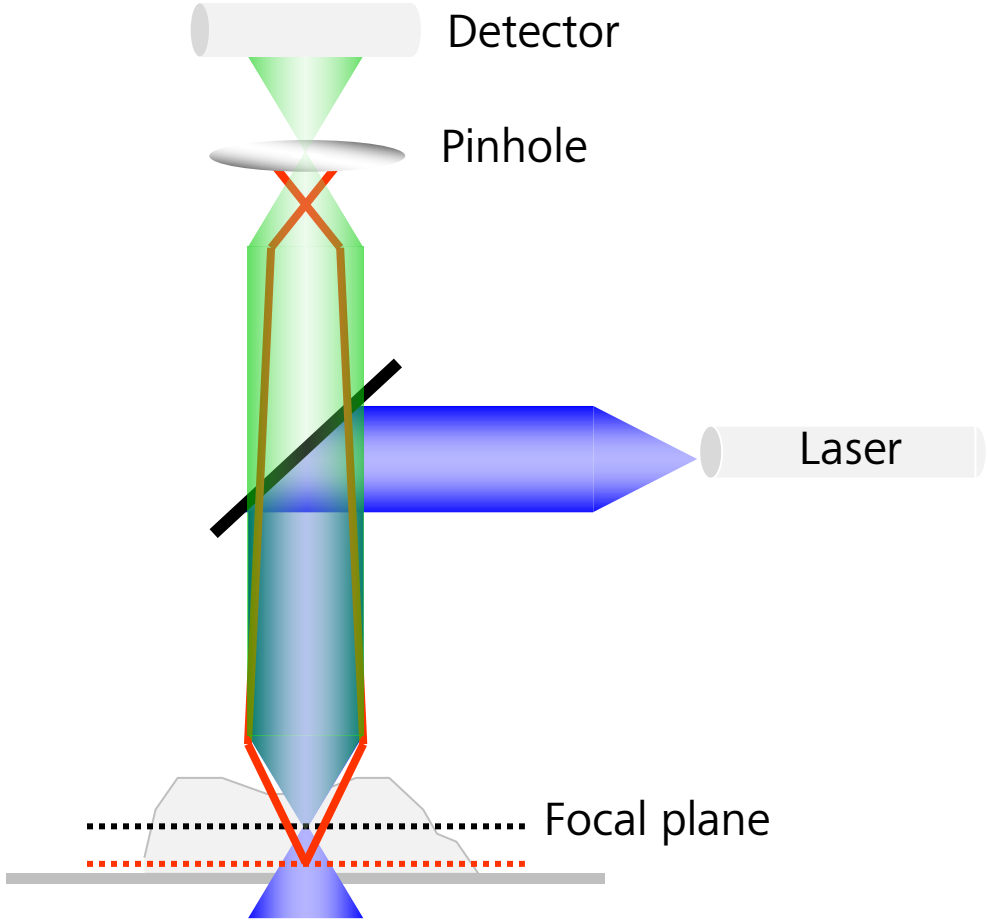

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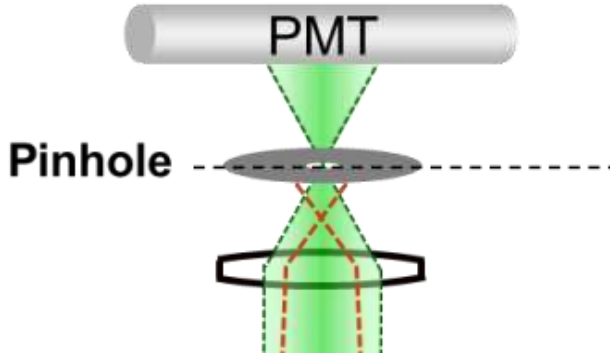
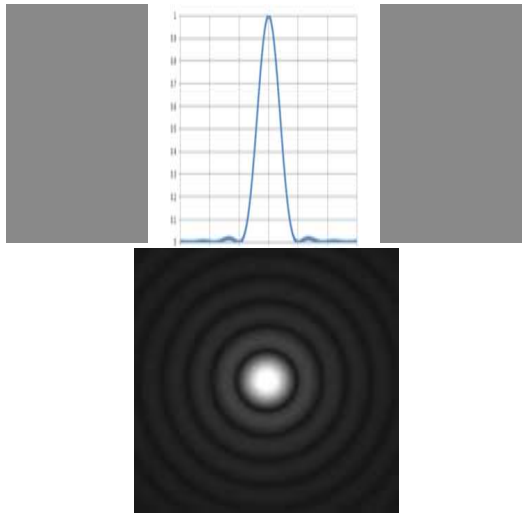
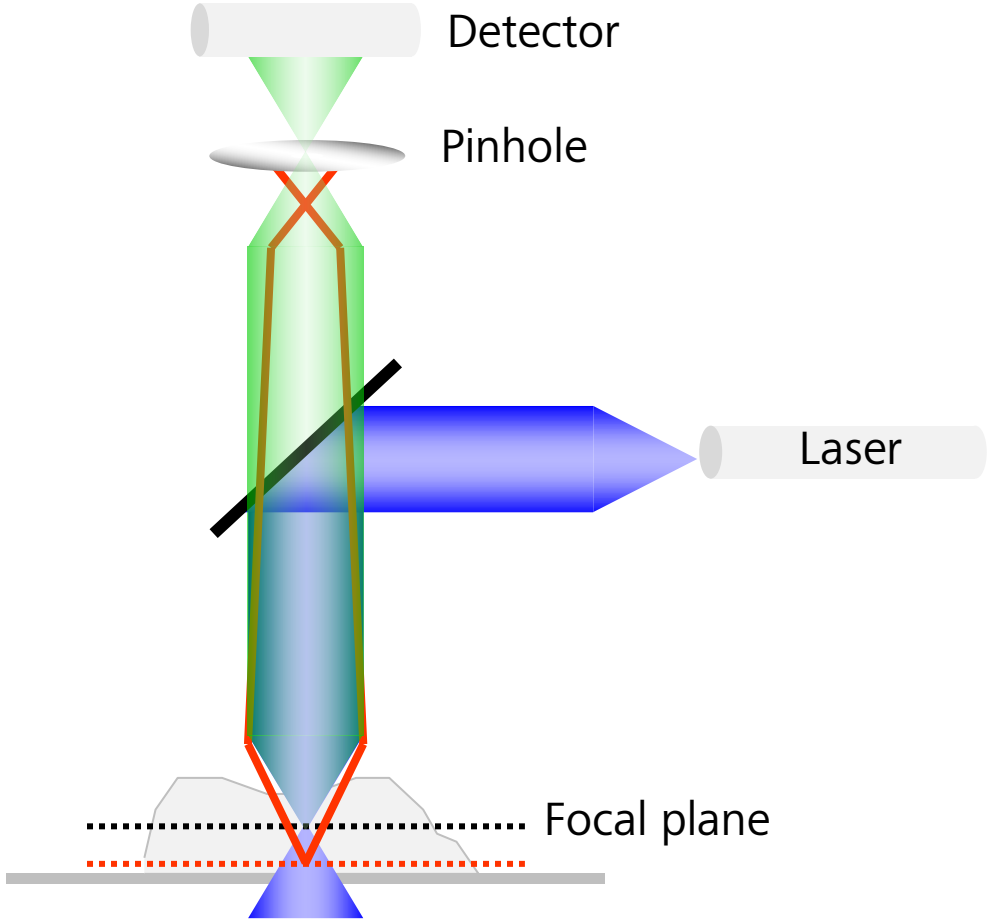
## Confocal principle

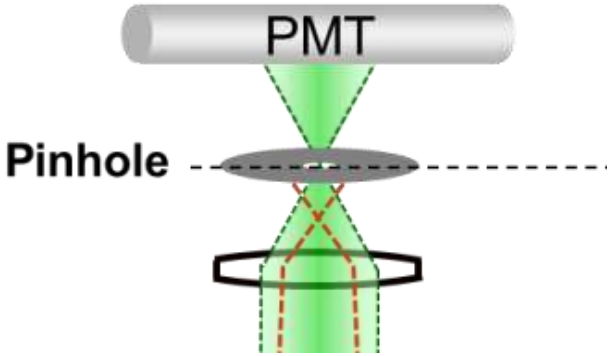


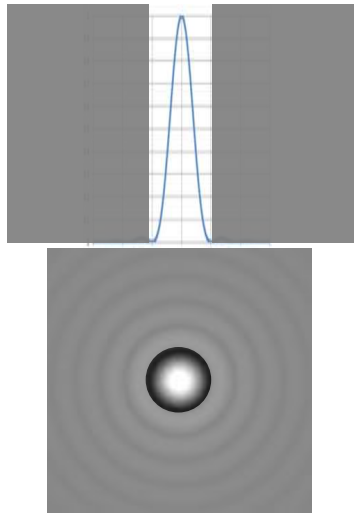



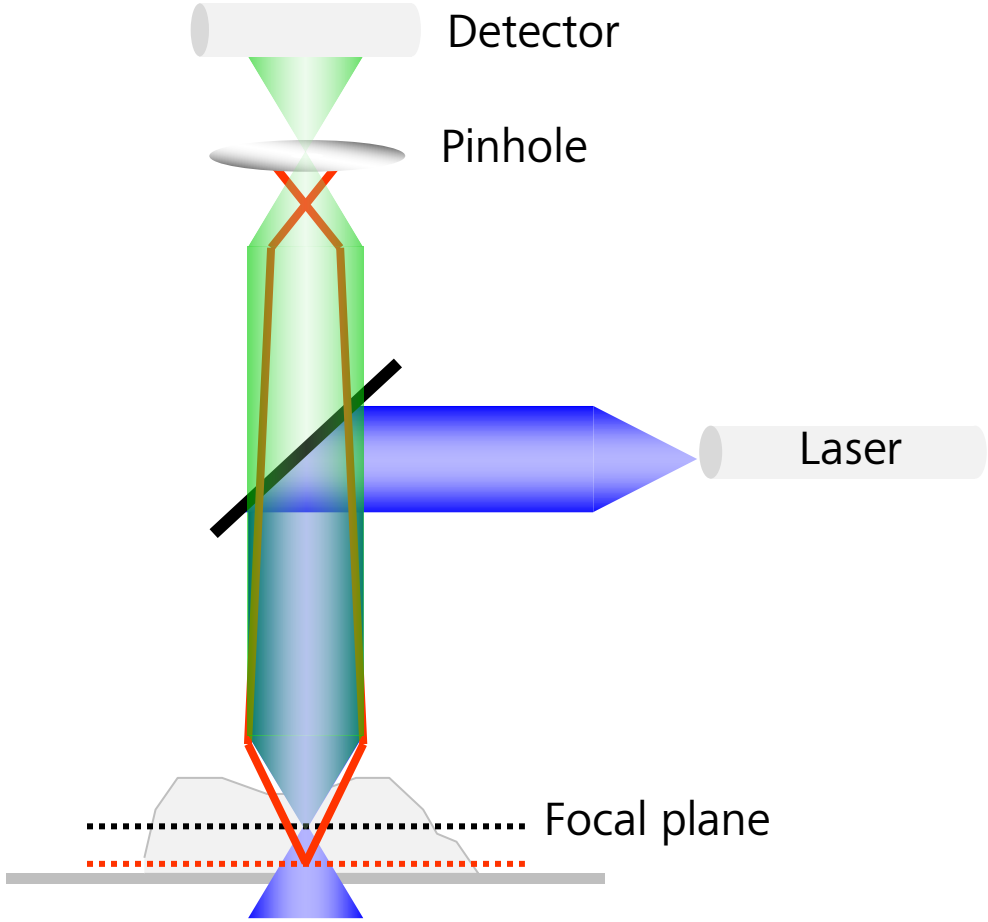


# Point Scanning Confocal Microscopes

## Confocal principle





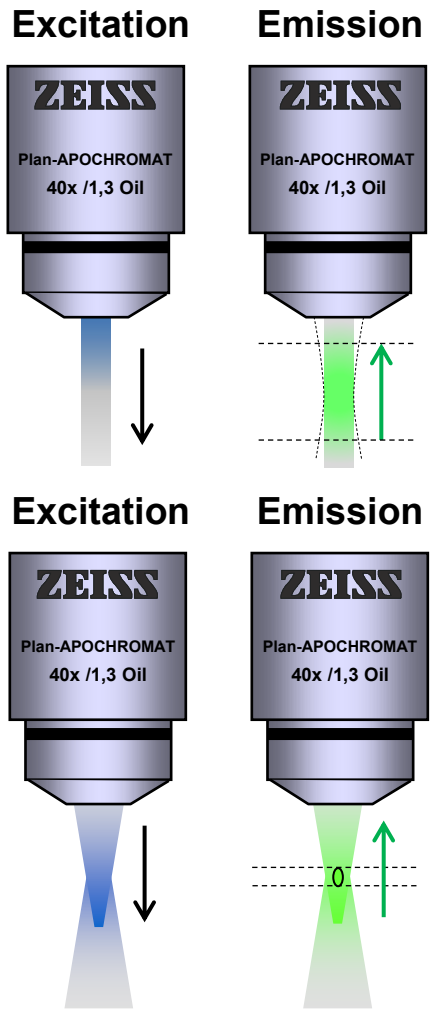
# From a Single Spot to a Complete Image

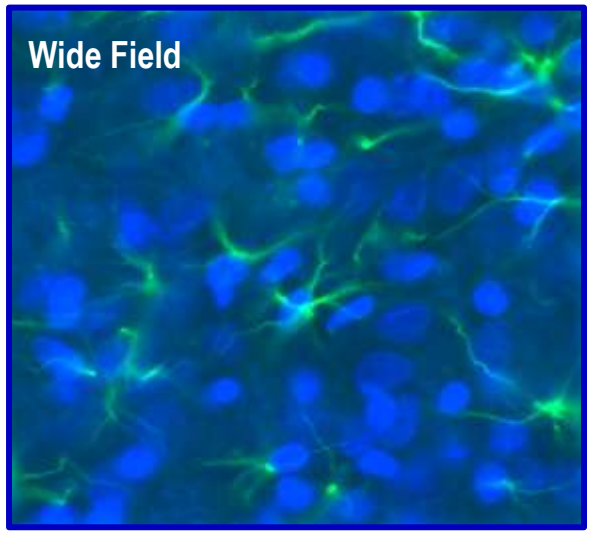
## Spot Illumination Requires Two-dimensional Scanning

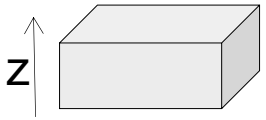
**X-Y scanning**

To generate a two-dimensional image, the laser spot is scanned in x and y direction to illuminate the whole field of view.

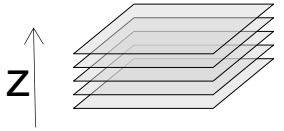
This is usually done by scanning mirrors.




limited z-resolution
thick sections




high z-resolution
3D via sectioning

**The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany**

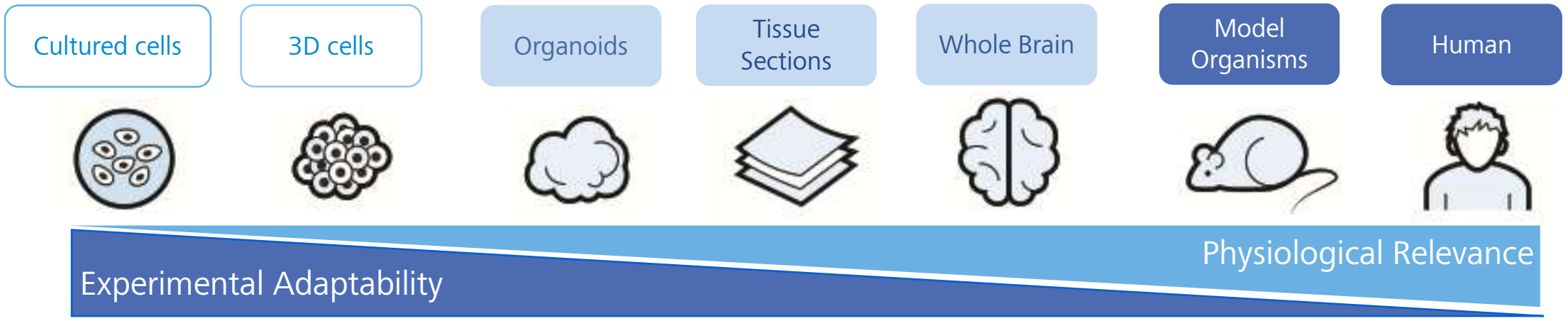
# Breeding Research on the Way to a Plant-Based Bioeconomy


"Microscopy is an important link between the different research groups."

**Michael Melzer | IPK Gatersleben**

**Your needs our motivation**

# Scaling from 2D Cell Cultures to New 3D Model Systems




Adapted from https://academic.oup.com/ib/article-abstract/8/6/672/5115178

## Your needs our motivation

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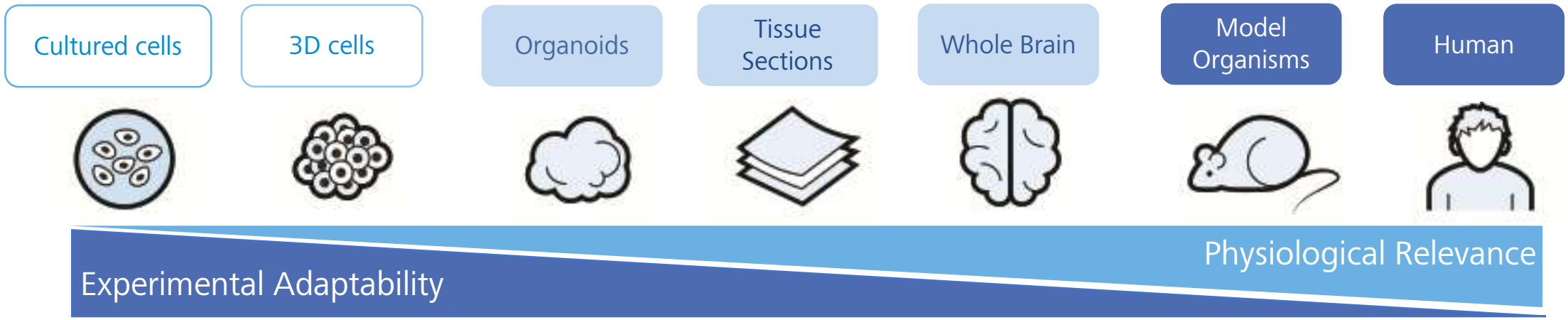
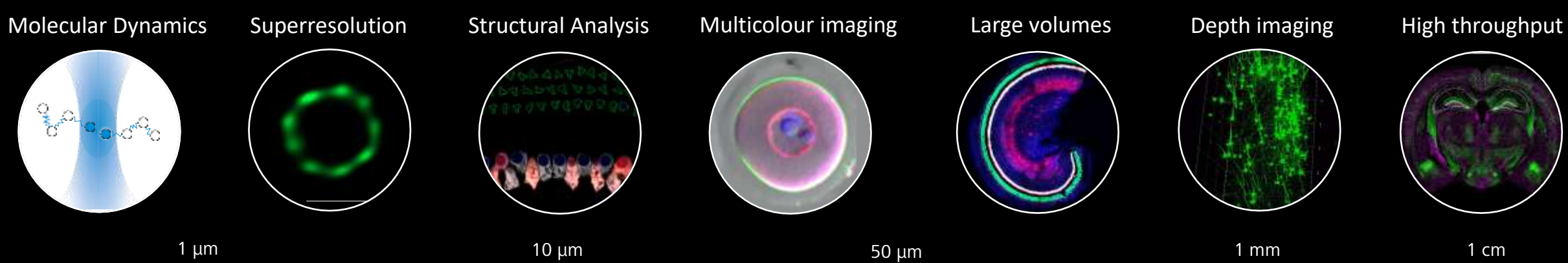


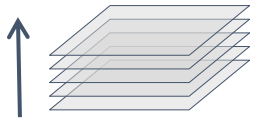

Adapted from https://academic.oup.com/ib/article-abstract/8/6/672/5115178



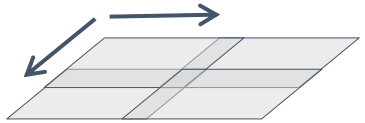
# Integrated Imaging Platform

**Confocal imaging**

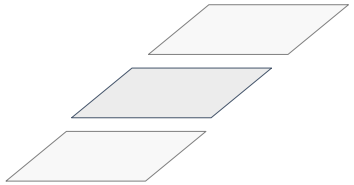
Snap



Z-Stack



Tiles



Time Series

**Bleaching**

Photomanipulation

**Software**

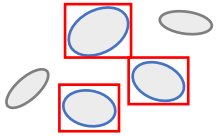
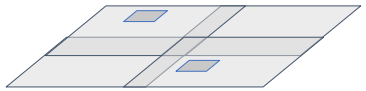


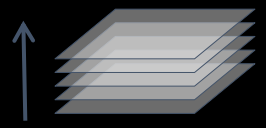
Image Analysis

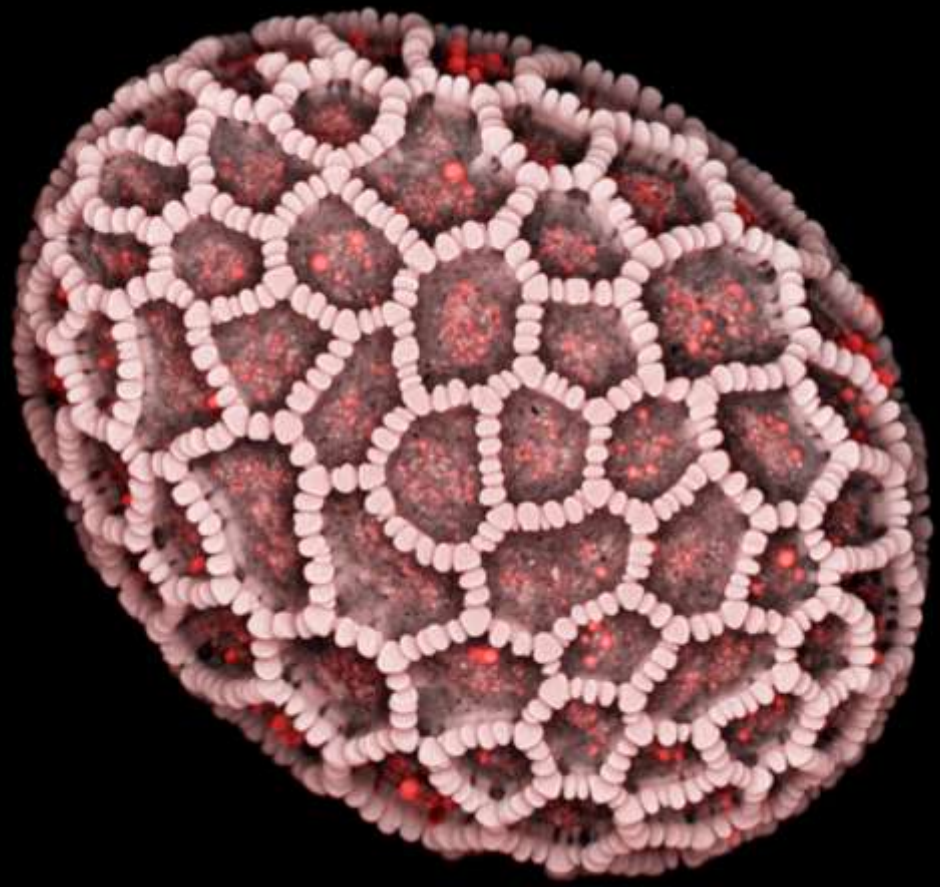


ZEN connect

Correlative Microscopy

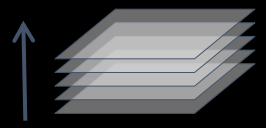
# High Resolution Optical Sectioning



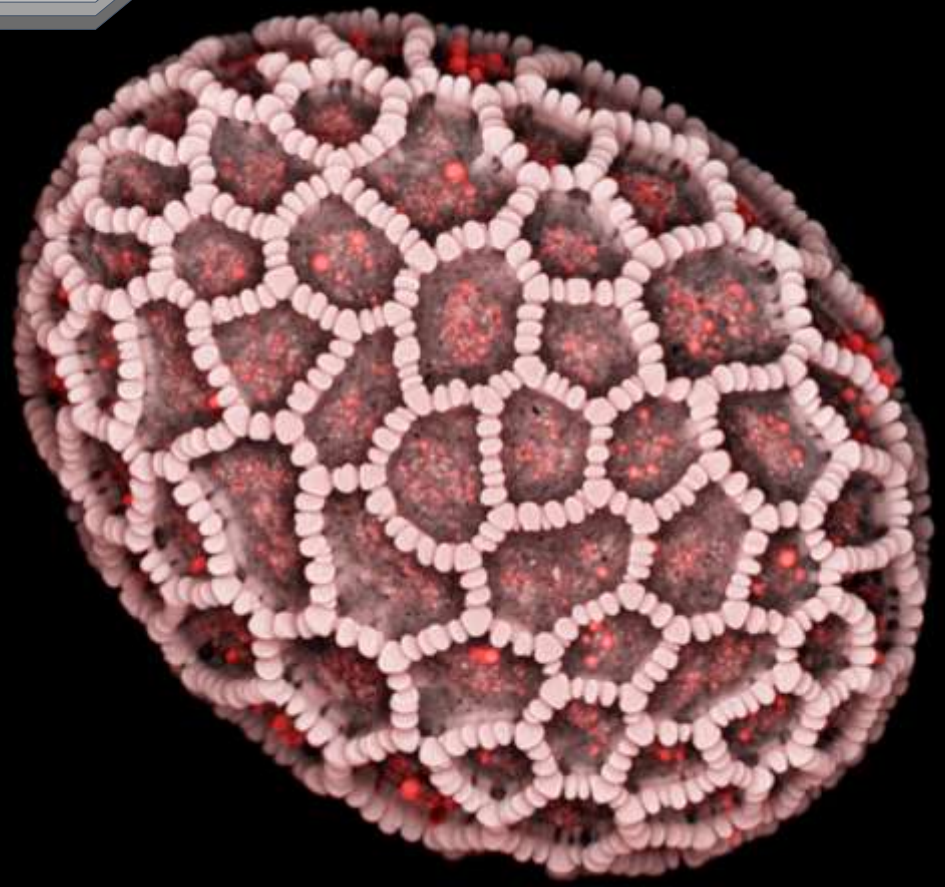


*Lilium auratum* pollen grain. Airyscan Multiplex mode. Courtesy of Jan Michels, Zoological Institute, Kiel University

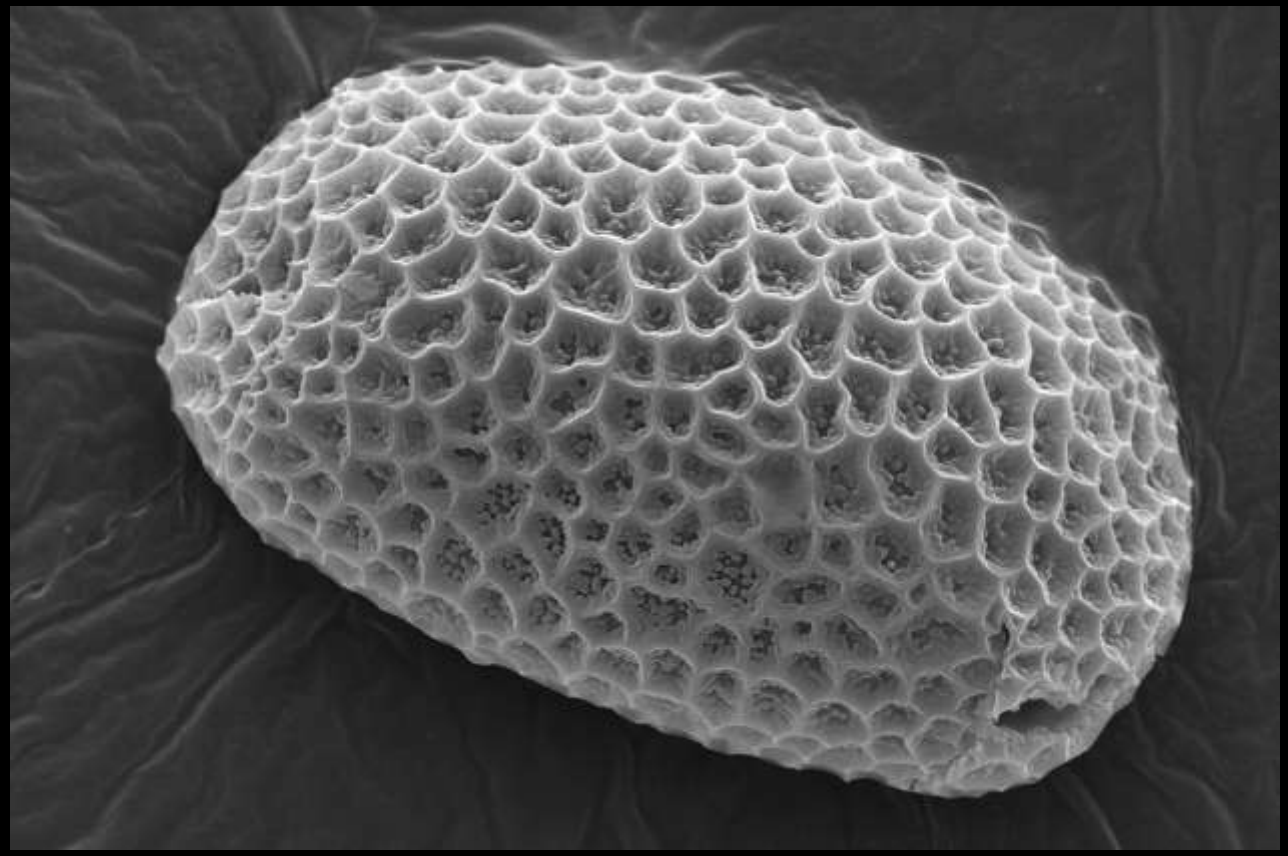
# High Resolution Optical Sectioning



LSM

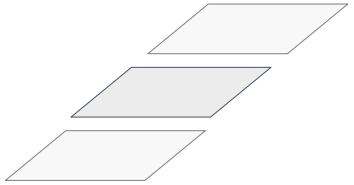


SEM

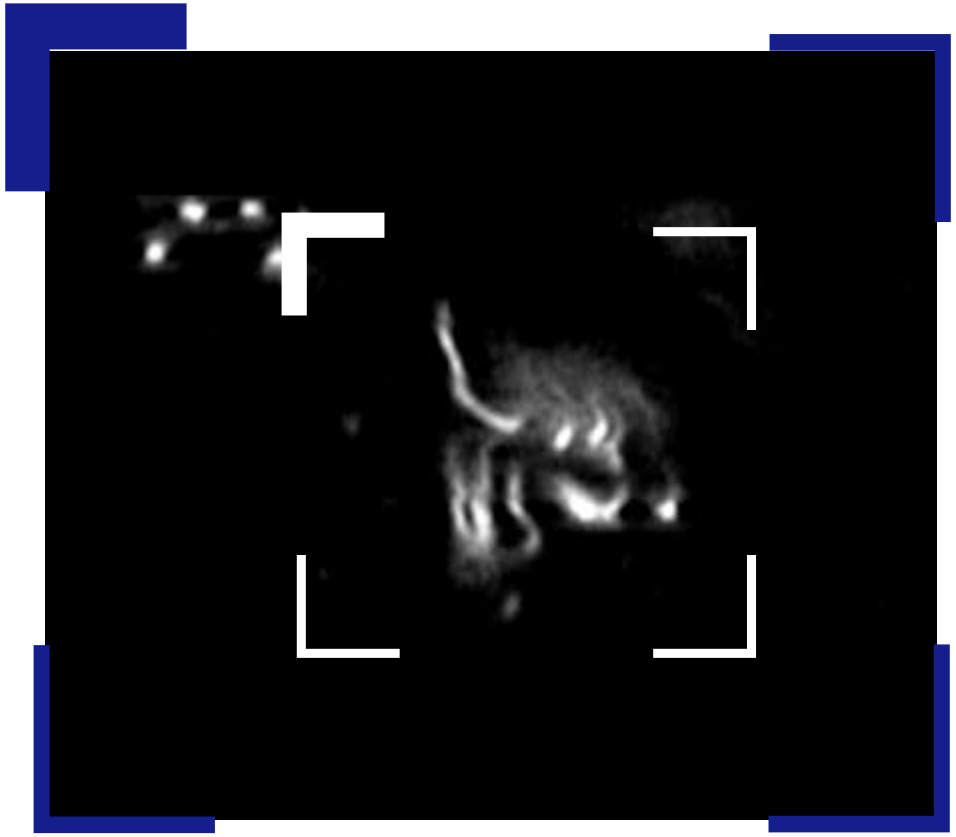


*Lilium auratum* pollen grain. Airyscan Multiplex mode. Courtesy of Jan Michels, Zoological Institute, Kiel University

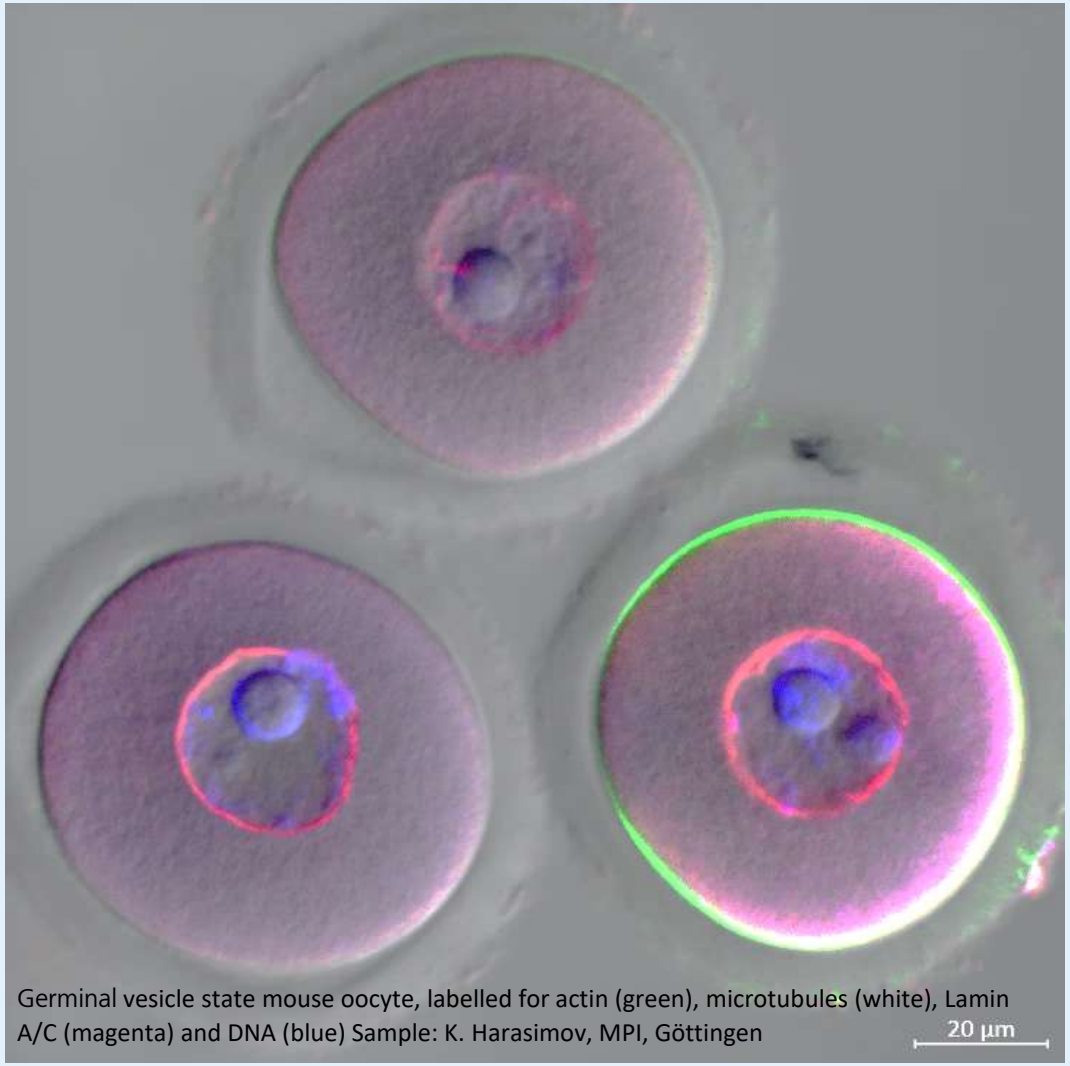
# Sensitive & Speedy Imaging



Time series

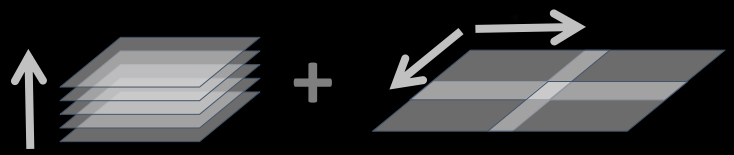


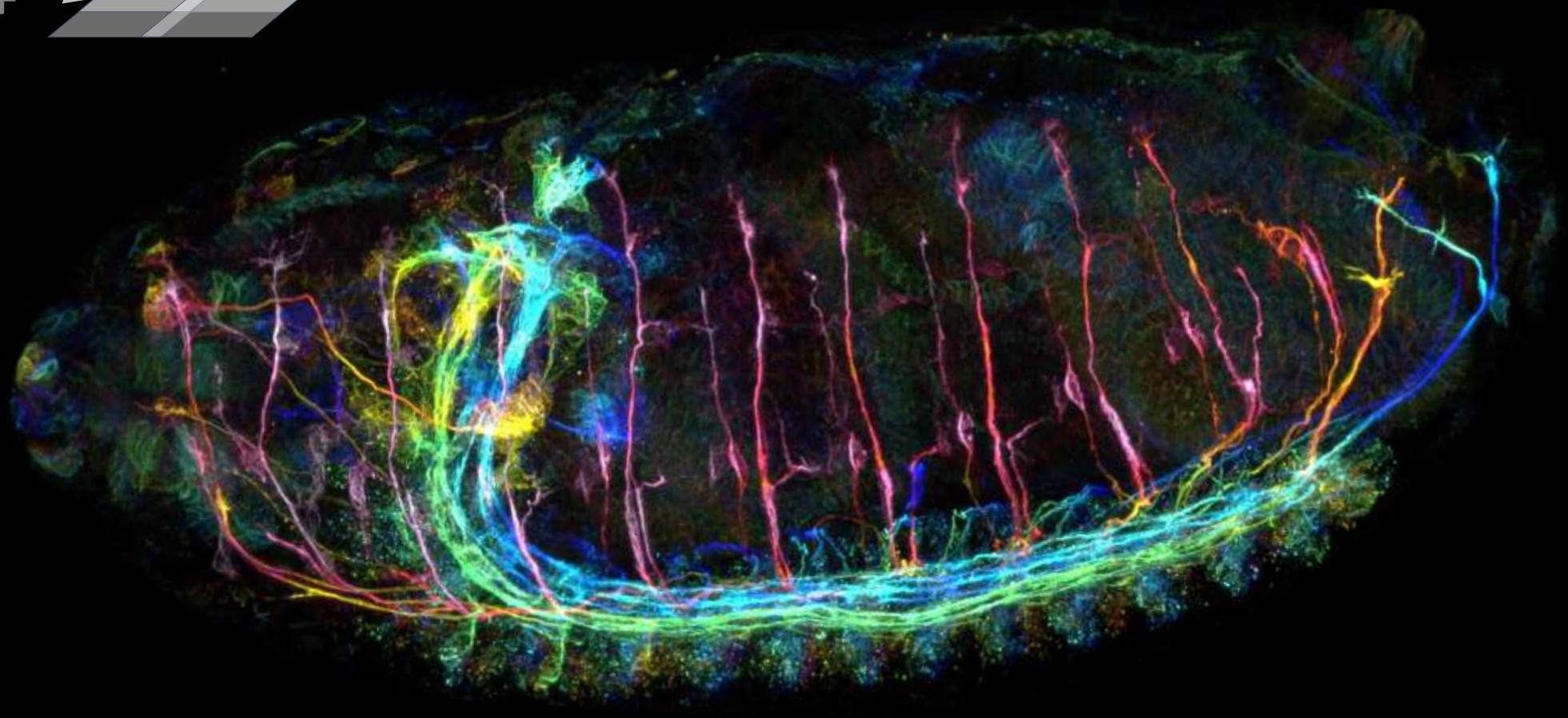
Data courtesy of Ann-Kathrin Günther & Dr. Gregor Eichele, MPI for Biophysical Chemistry, Göttingen, Germany




Germinal vesicle state mouse oocyte, labelled for actin (green), microtubules (white), Lamin A/C (magenta) and DNA (blue) Sample: K. Harasimov, MPI, Göttingen

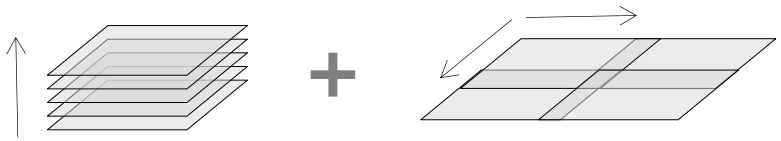
# Acquire Large Volumes at Best Quality





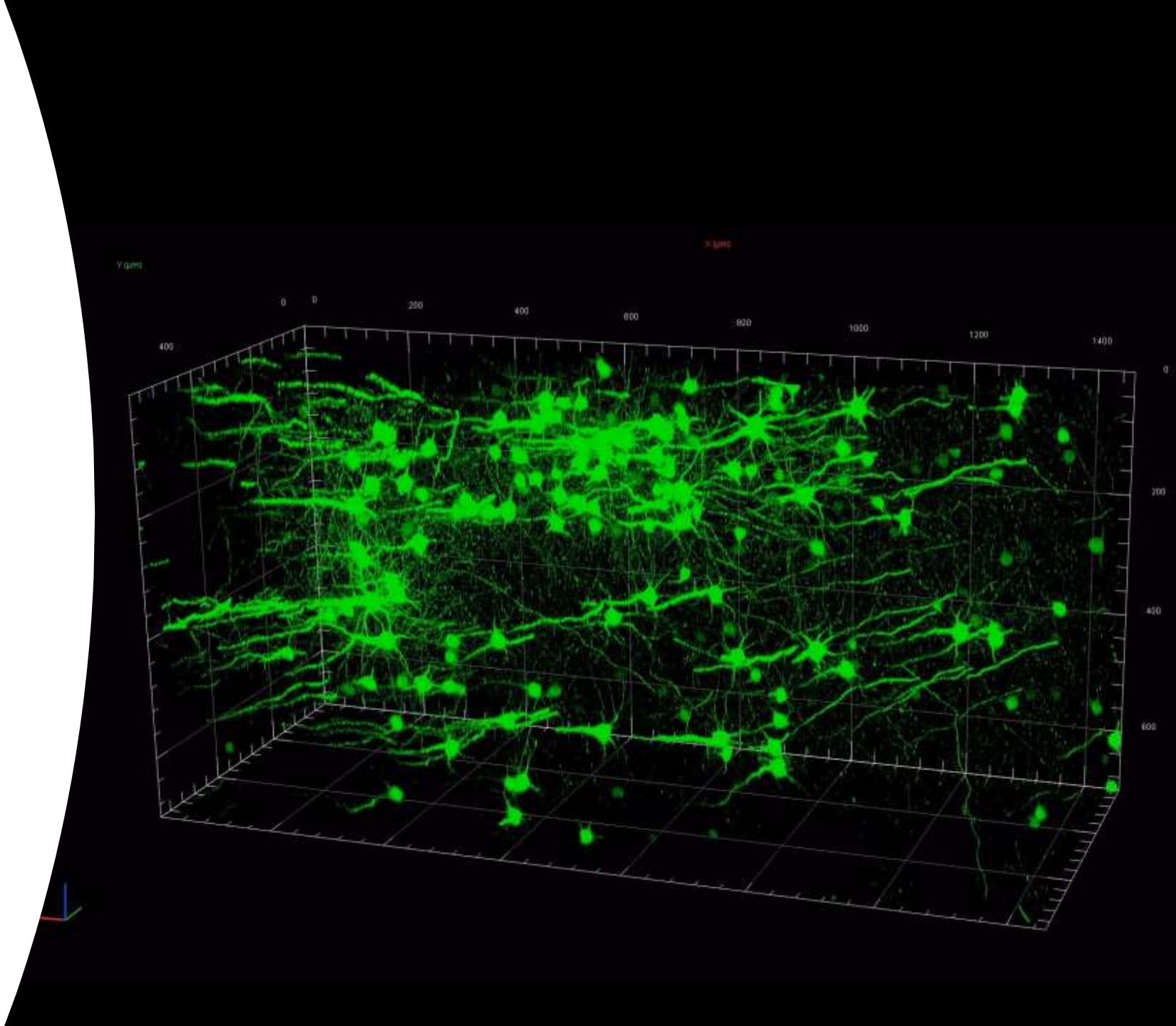
*Drosophila melanogaster, CNS and PNS depth coded*, Airyscan Multiplex mode. Courtesy of Julia Sellin, LIMES, Bonn, Germany

# Large Volume Imaging



**Adult mouse brain**
Thy1-GFP (Neurons)
CLARITY

12 tiles and 800µm z-stack
Total sample depth 1.4mm

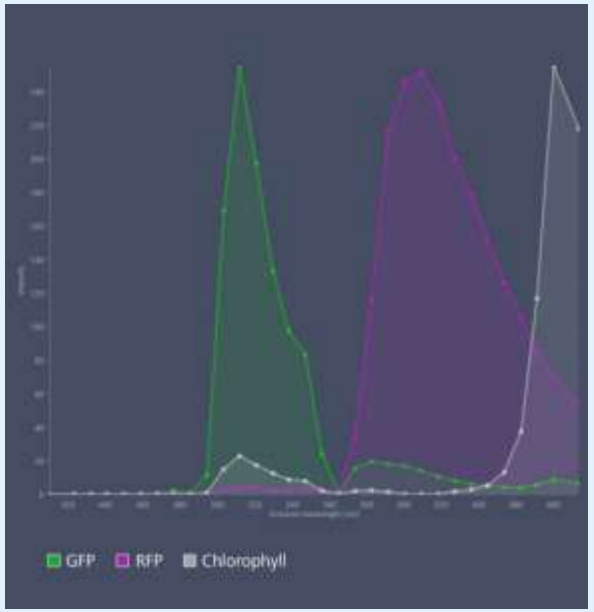



Tobias Ruff, Max Planck Institute of Neurobiology,
Martinsried (Munich), Germany.

# User-friendly experiment design

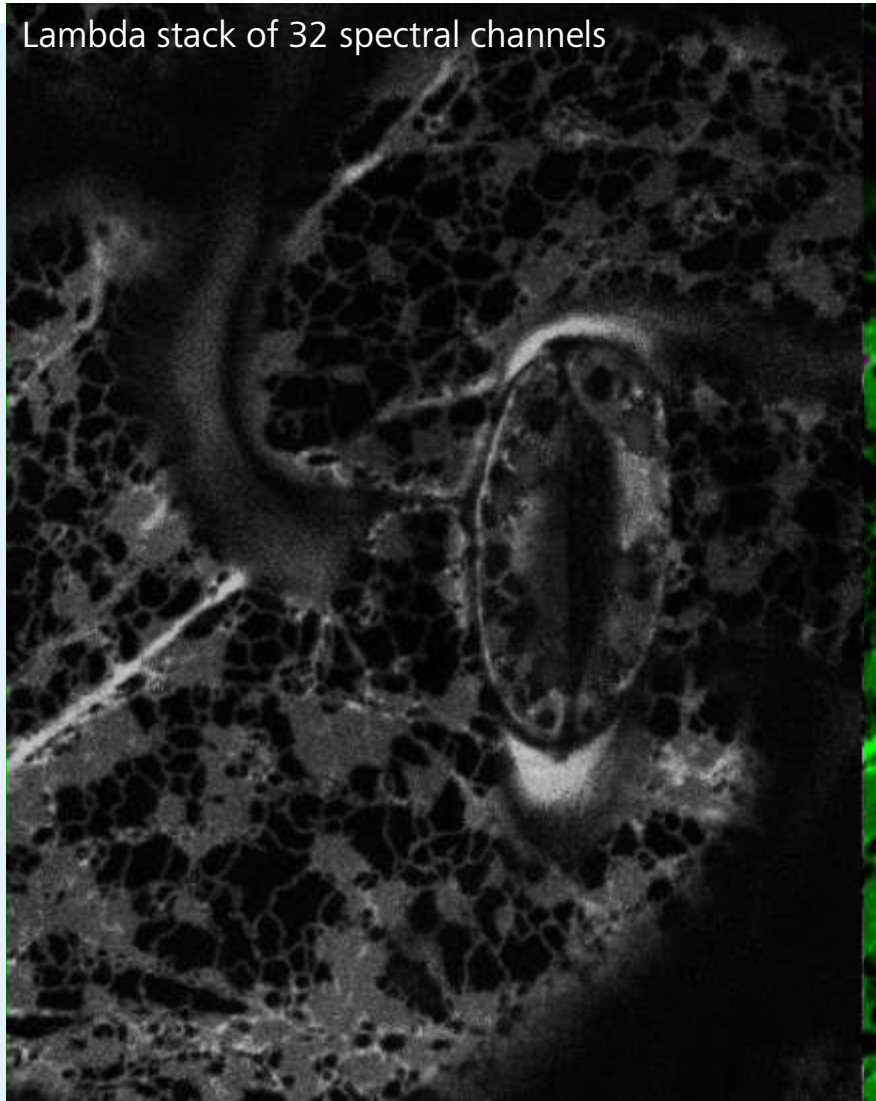
## Customized spectral imaging mastered with ease

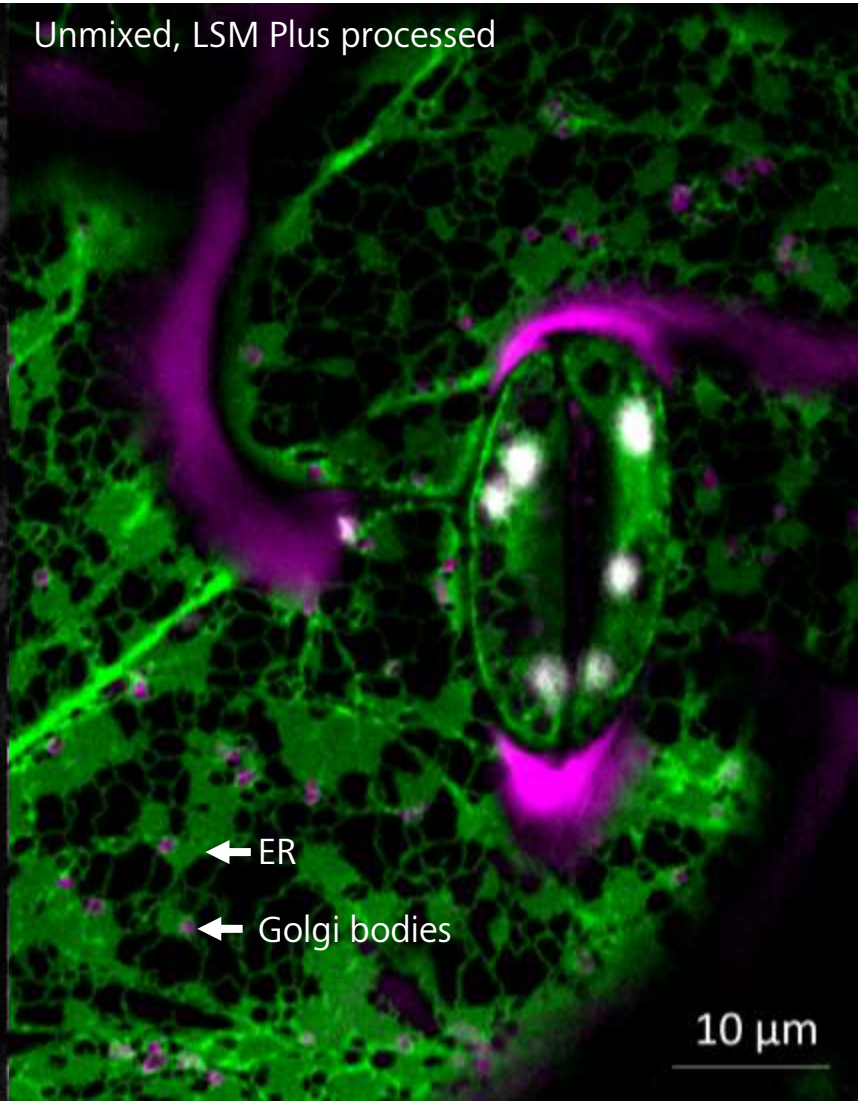
### Separating autofluorescence




Arabidopsis leaf expressing GFP-HDEL (labeling the endoplasmatic reticulum) and ST-mRFP (labeling Golgi bodies)

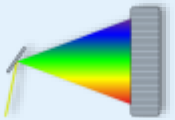
Sample courtesy of Verena Kriechbaumer, Oxford Brookes University, UK



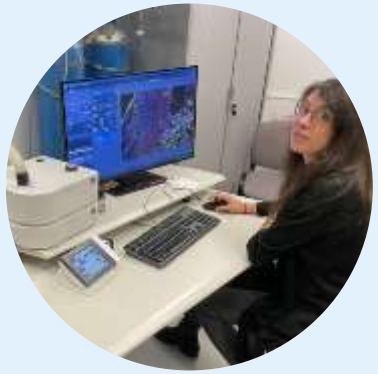



# Spatial Biology Studies in Lung Tissue using Spectral Microscopy

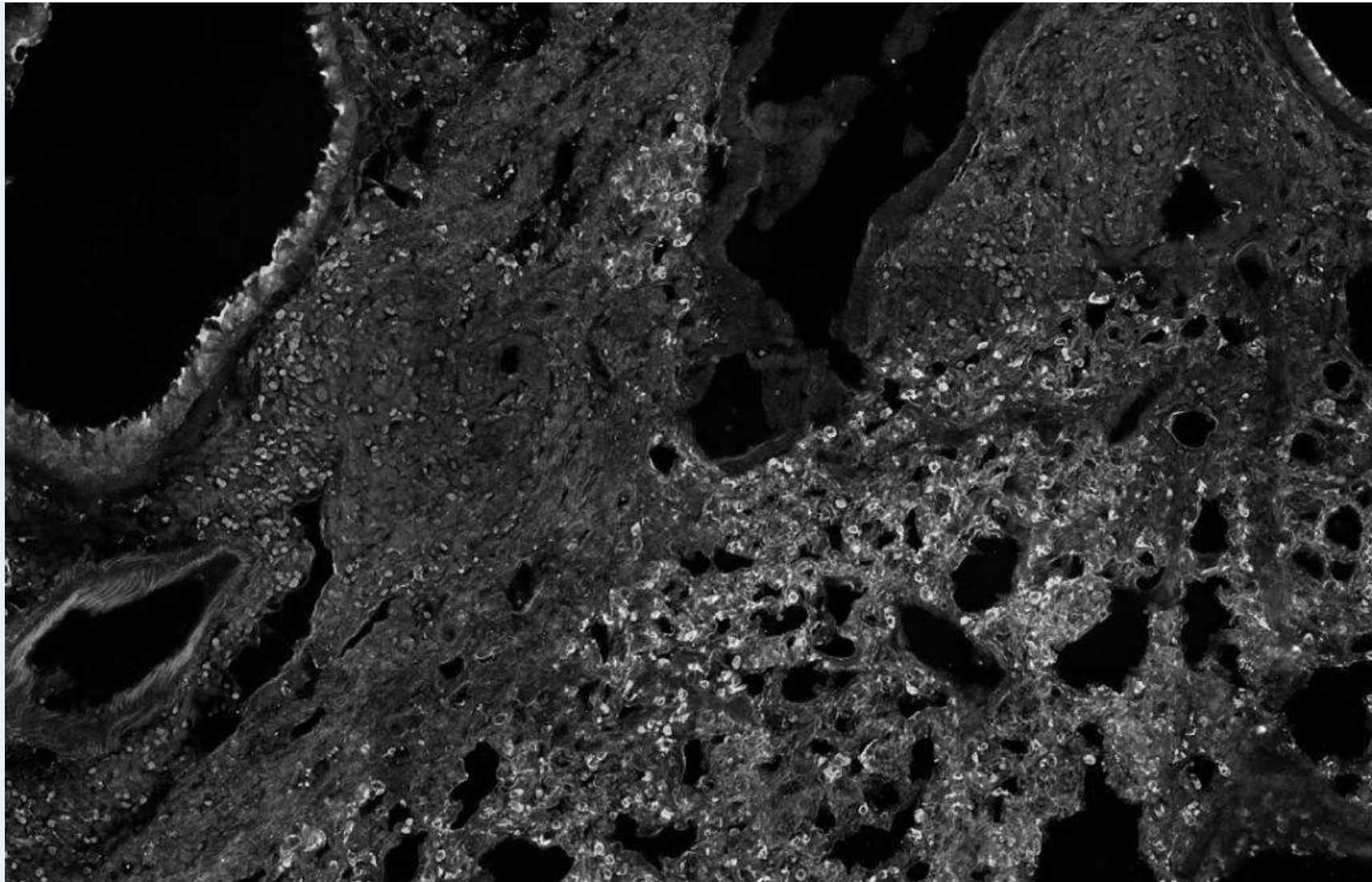
## Spectral Unmixing



Identification of macrophage niches in wounded lungs

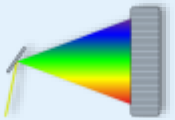


**Cecilia Ruscitti**
PhD Student at the Laboratory of Immunophysiology
Supervised by Dr. Thomas Marichal, University of Liège, Belgium



# Spatial Biology Studies in Lung Tissue using Spectral Microscopy

## Spectral Unmixing



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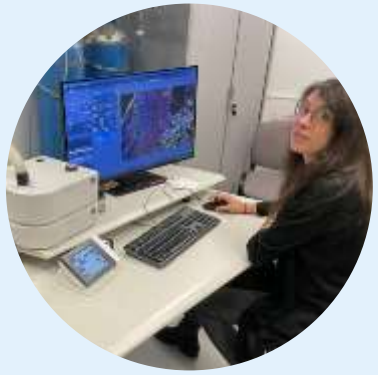

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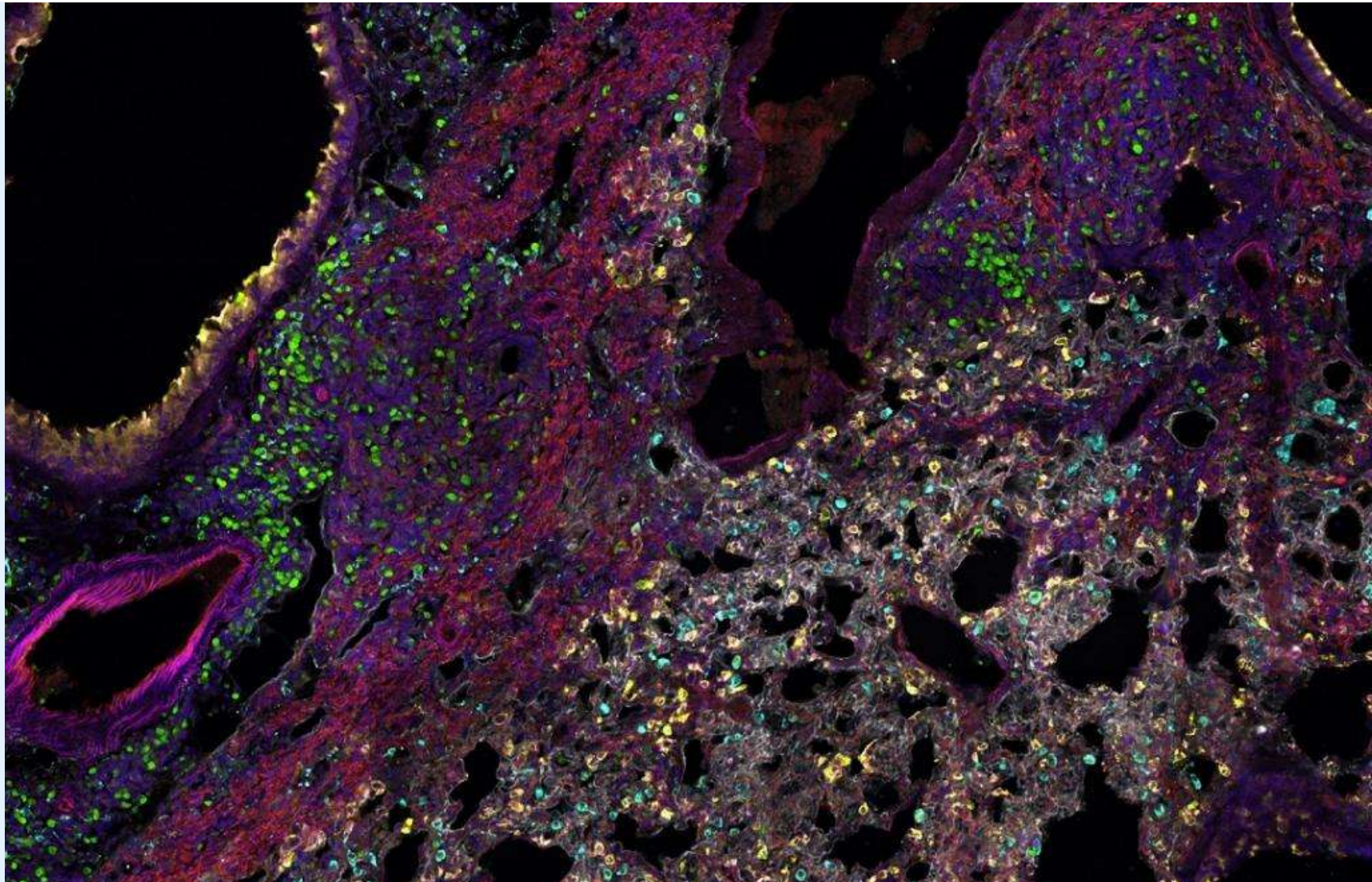
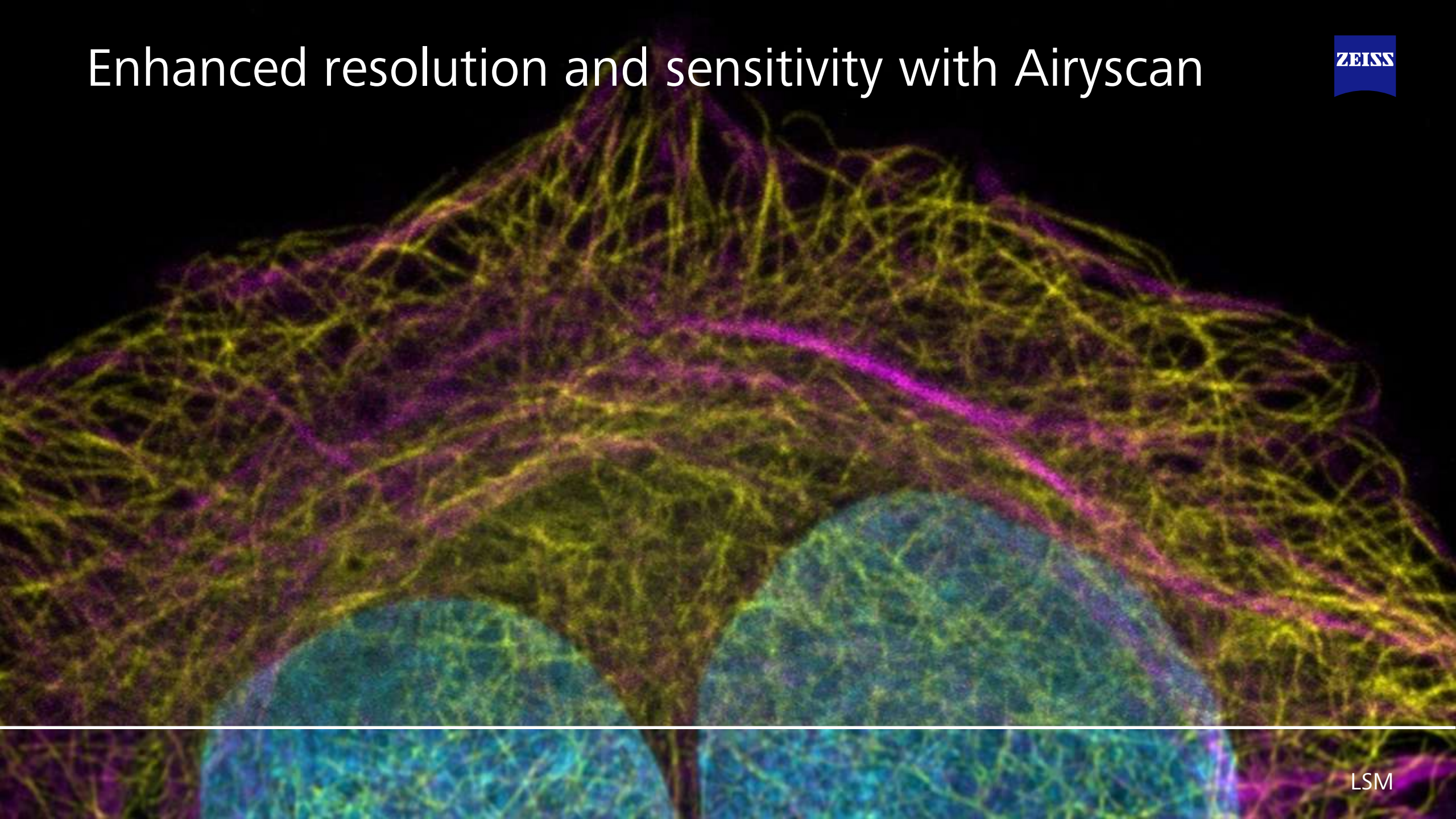
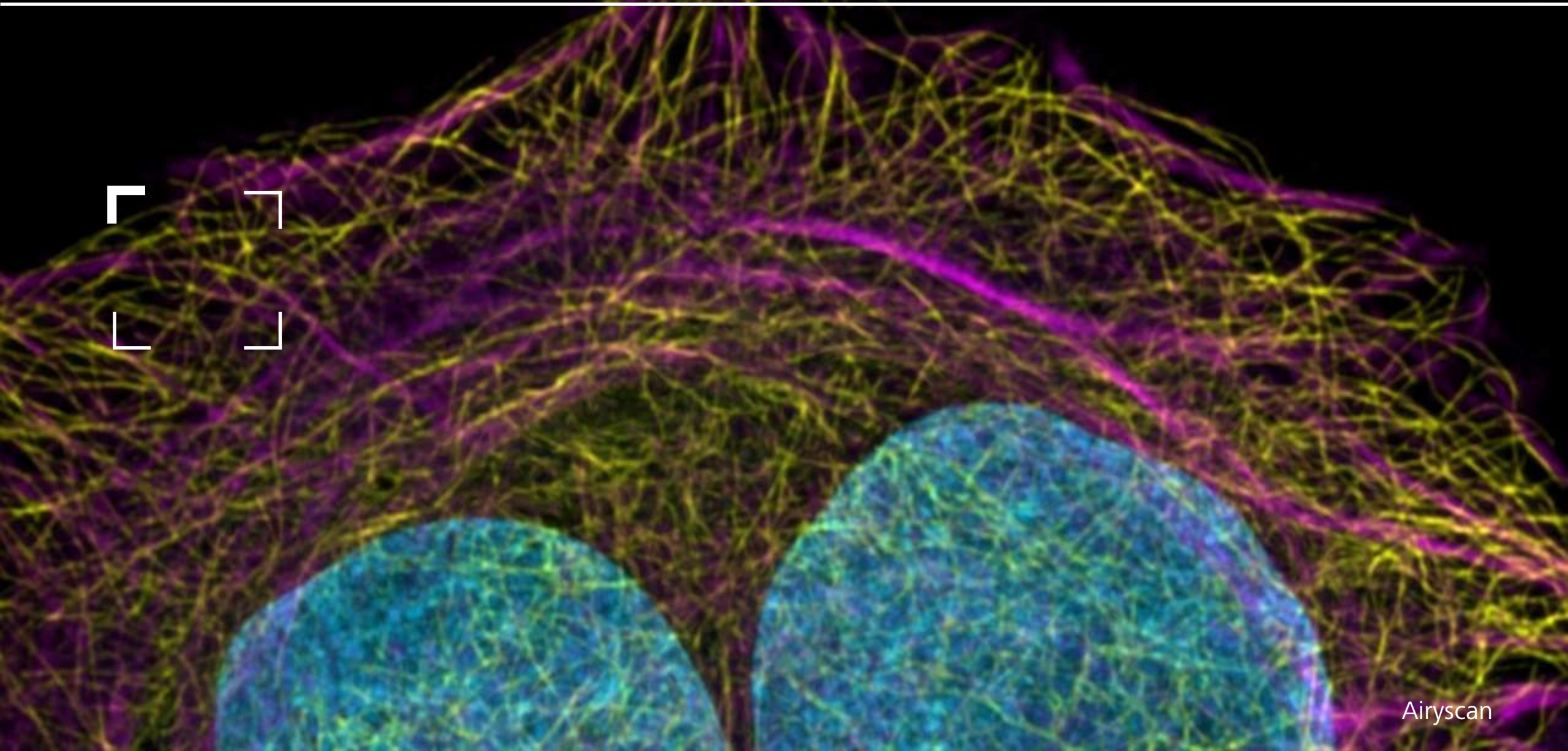


# Enhanced resolution and sensitivity with Airyscan

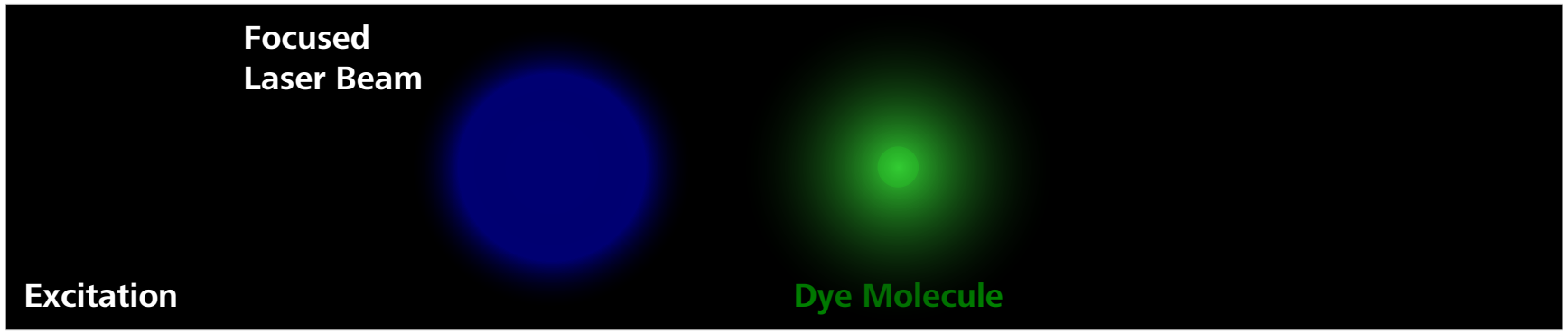


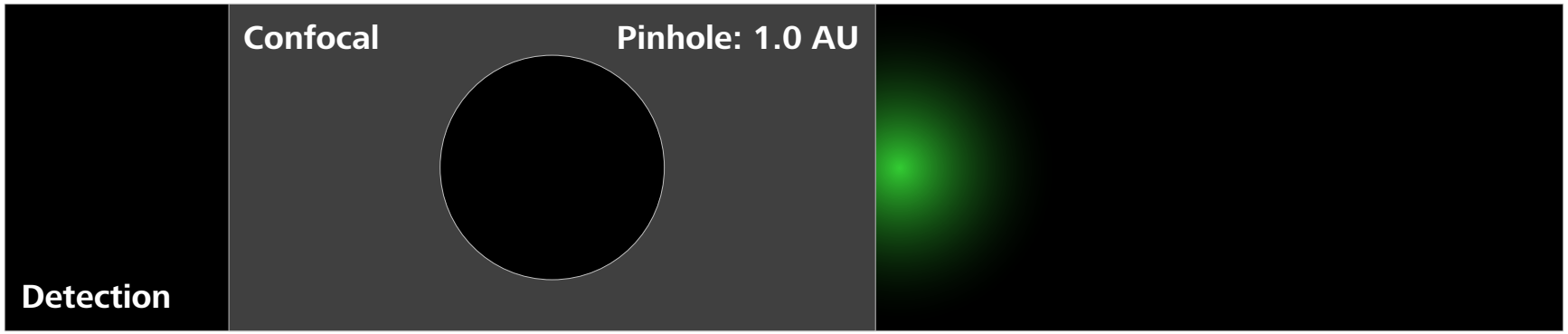
LSM

# Enhanced resolution and sensitivity with Airyscan

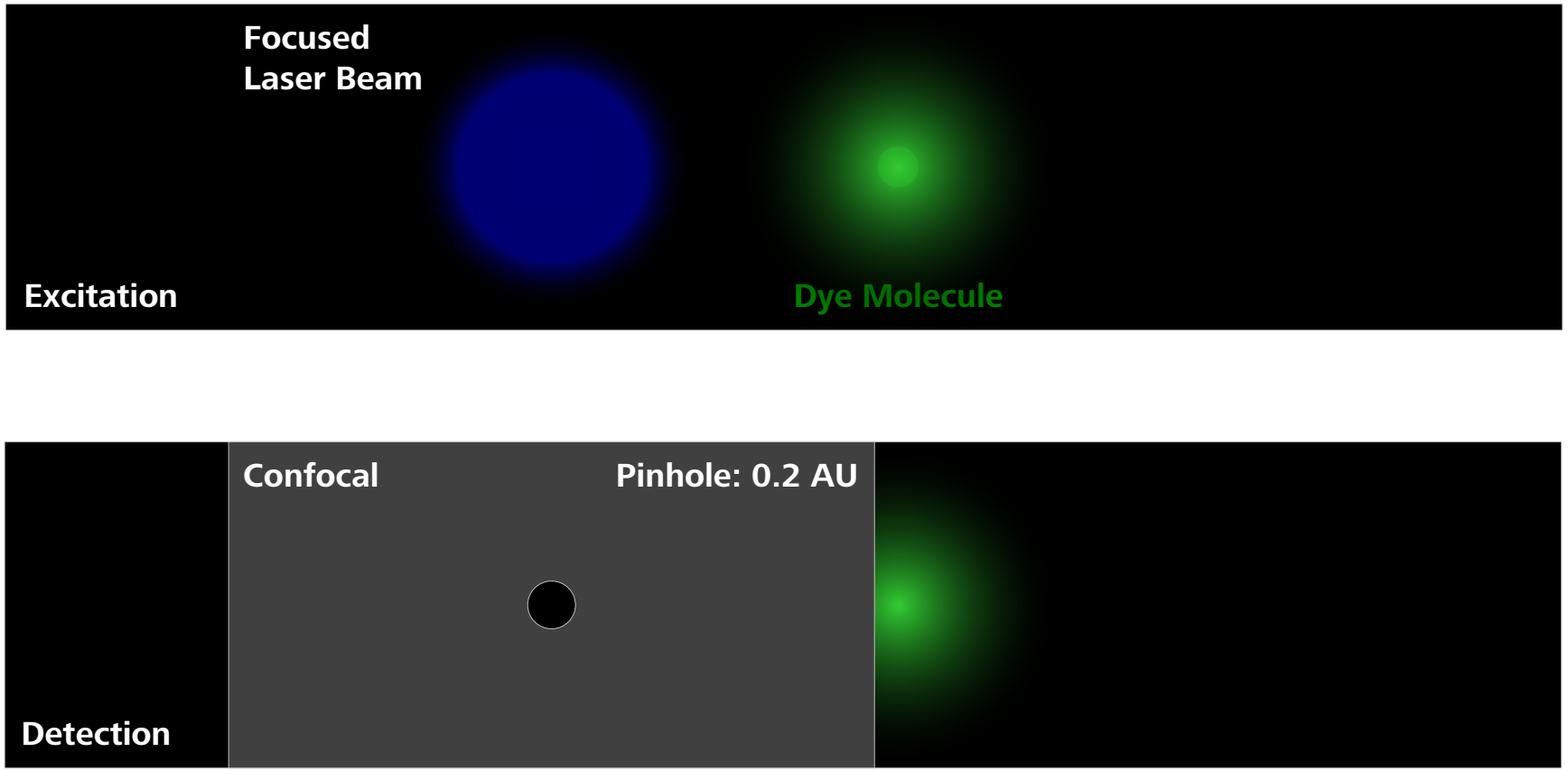


# Confocal Imaging with Pinhole at 1 AU

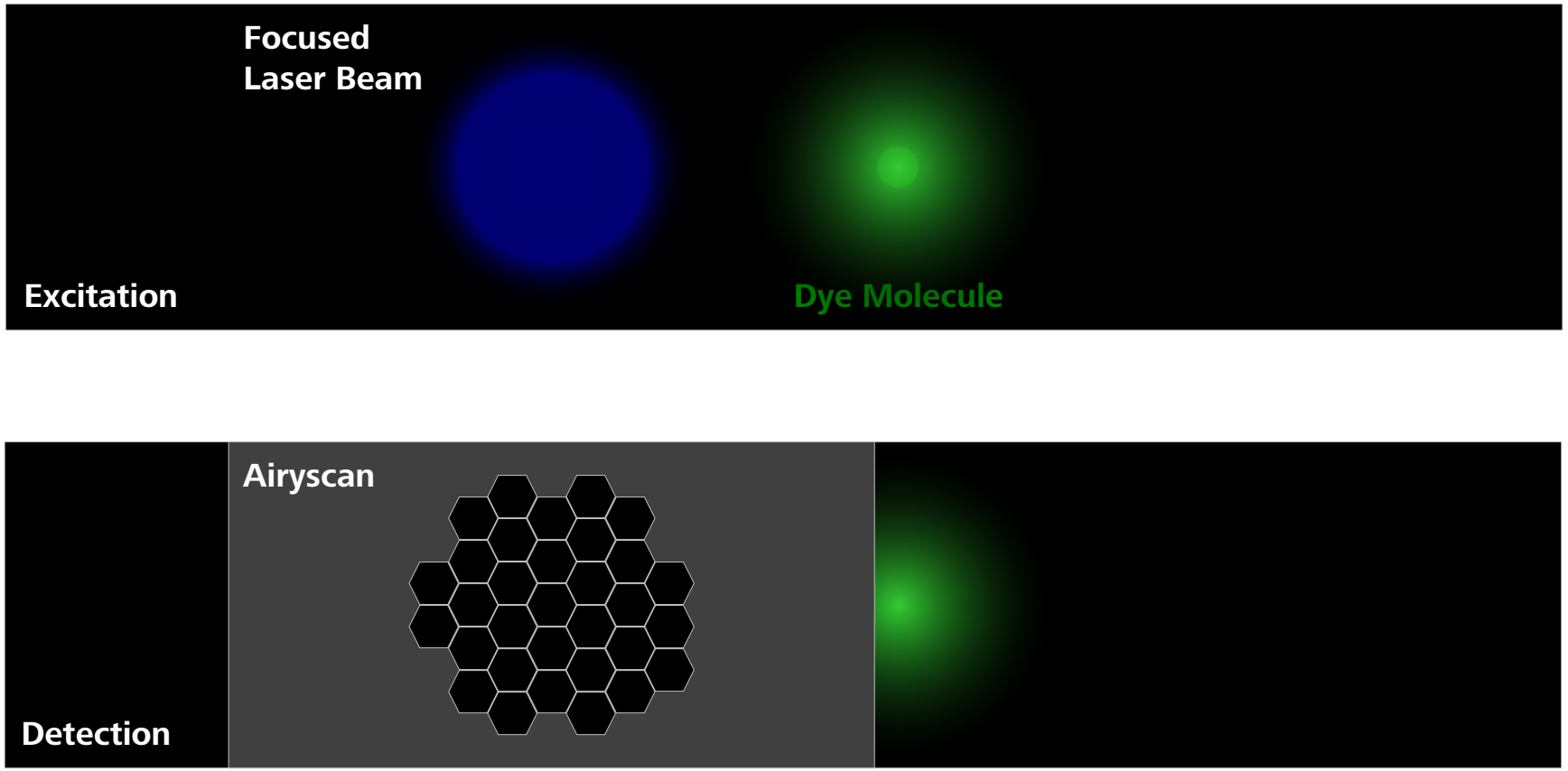


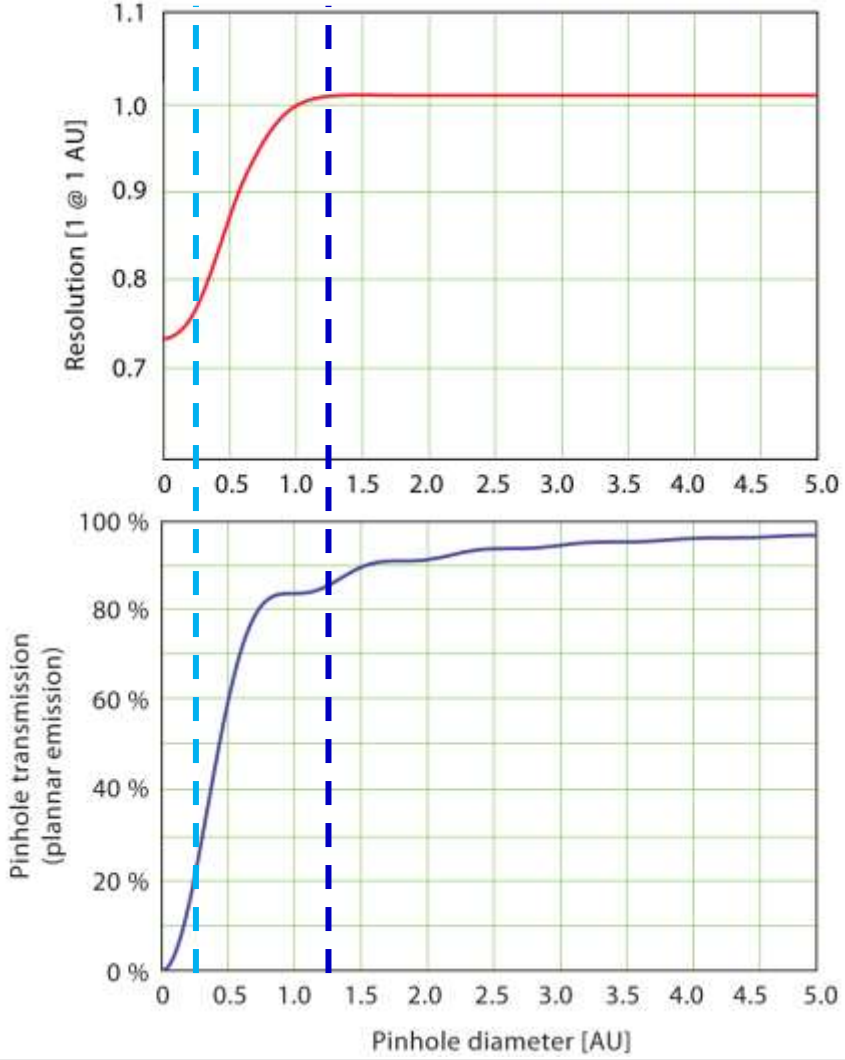
# Confocal Imaging with Pinhole at 0.2 AU

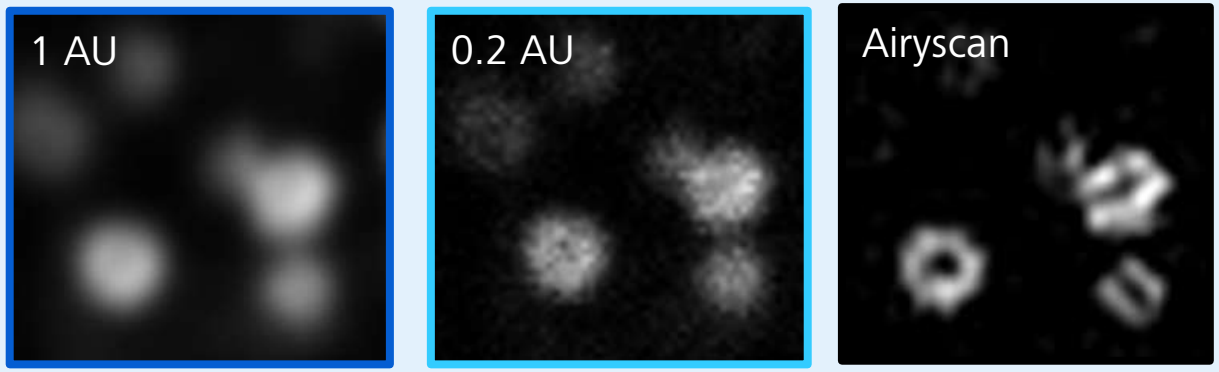


# Confocal Imaging with Airyscan

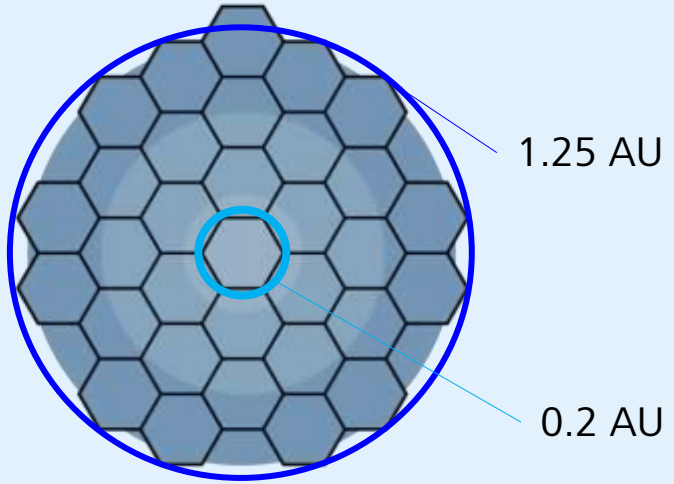


# Enhanced resolution and sensitivity with Airyscan



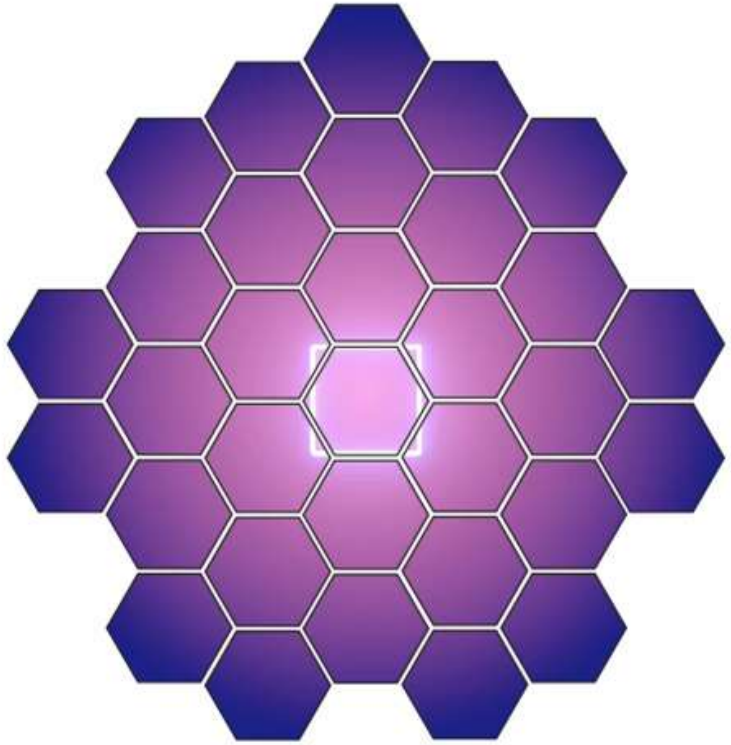

**120 nm lateral**
**350 nm axial**

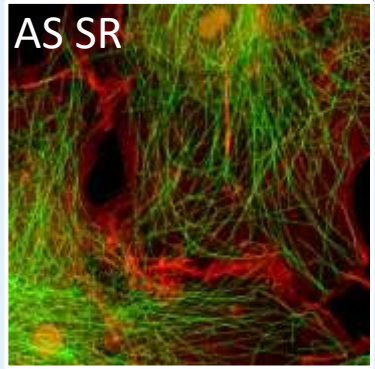



Neuromuscular junction, bruchpilot, *Drosophila melanogaster* Sample courtesy of J. Pielage, Basel, Switzerland

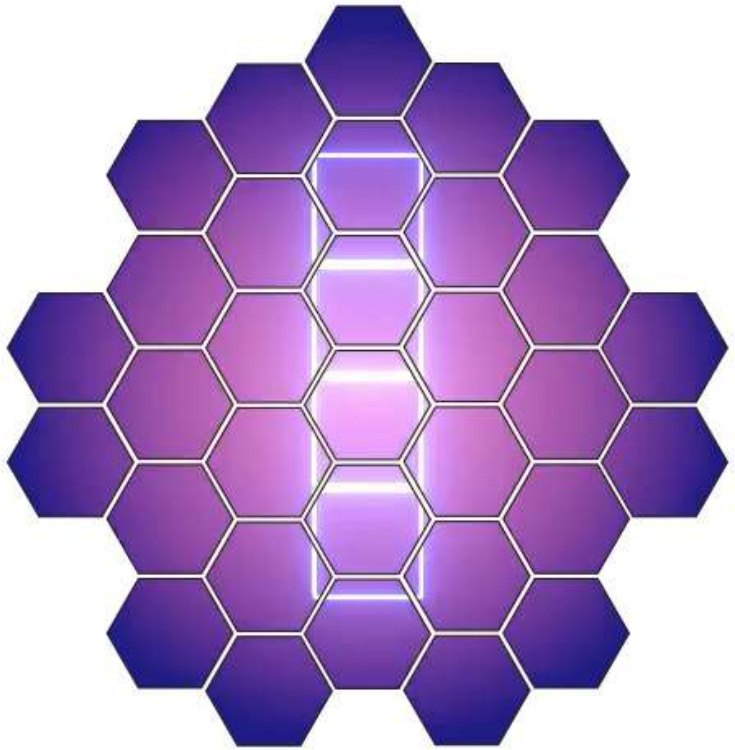
# Efficient super-resolution imaging through parallelization
Airyscan

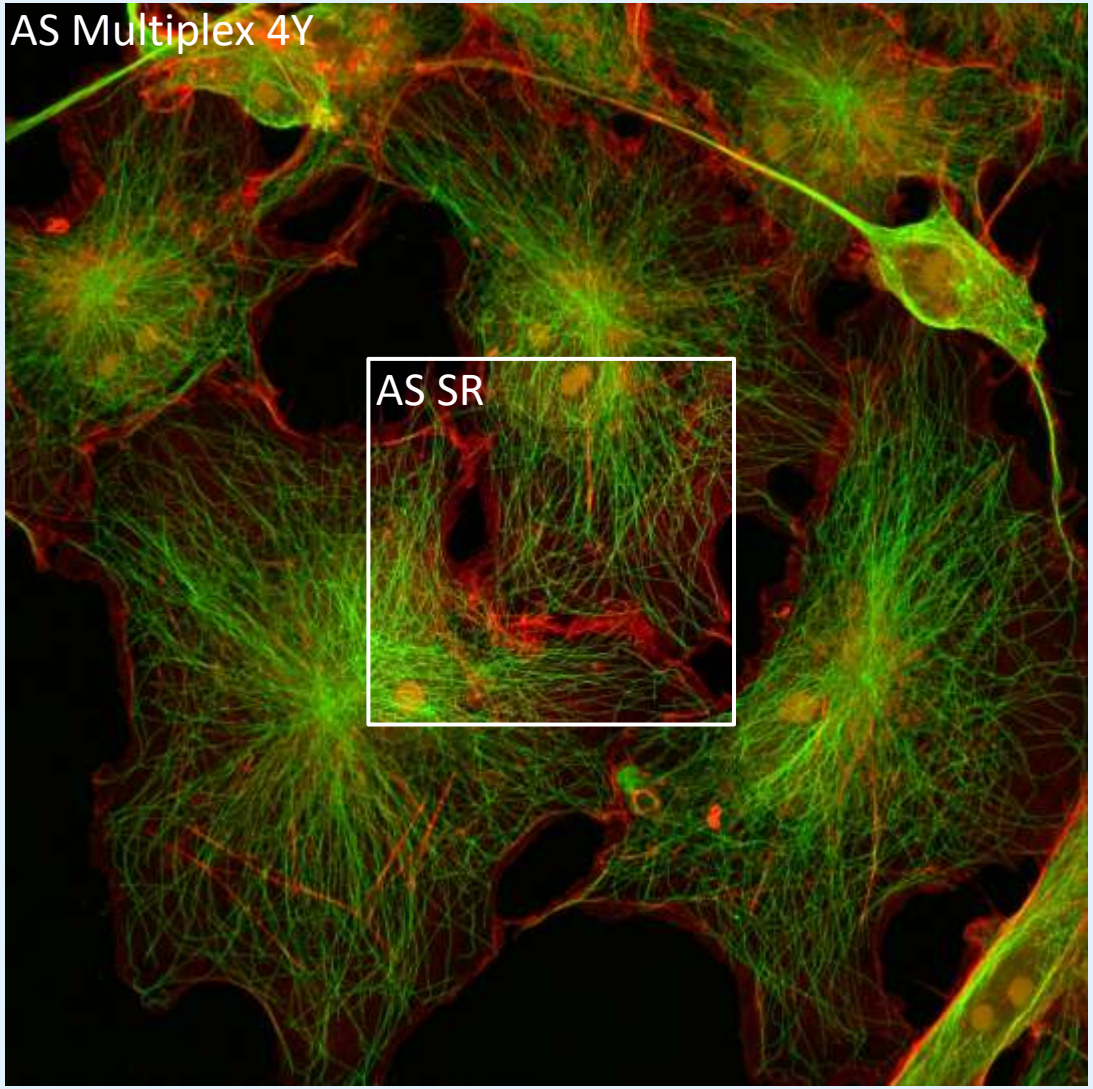
AS Multiplex 4Y



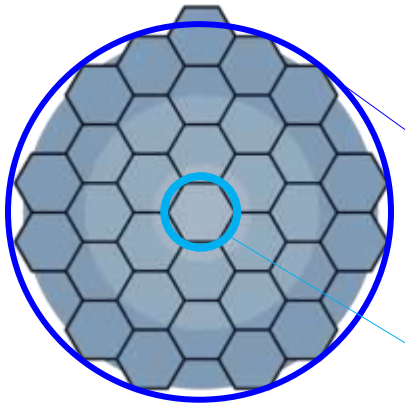


# Efficient super-resolution imaging through parallelization
Airyscan





# Gentle super-resolution imaging
## Airyscan

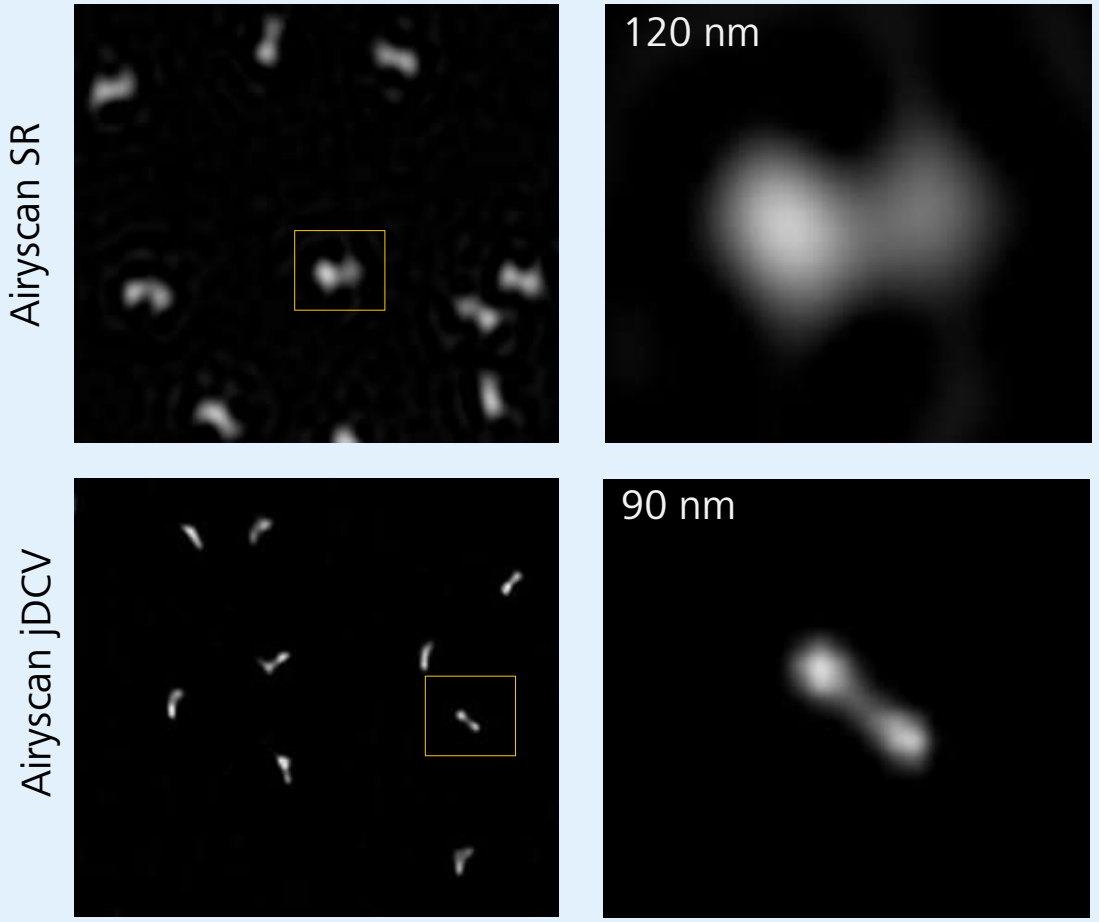


1.25 AU

0.2 AU

linear
Wiener DCV

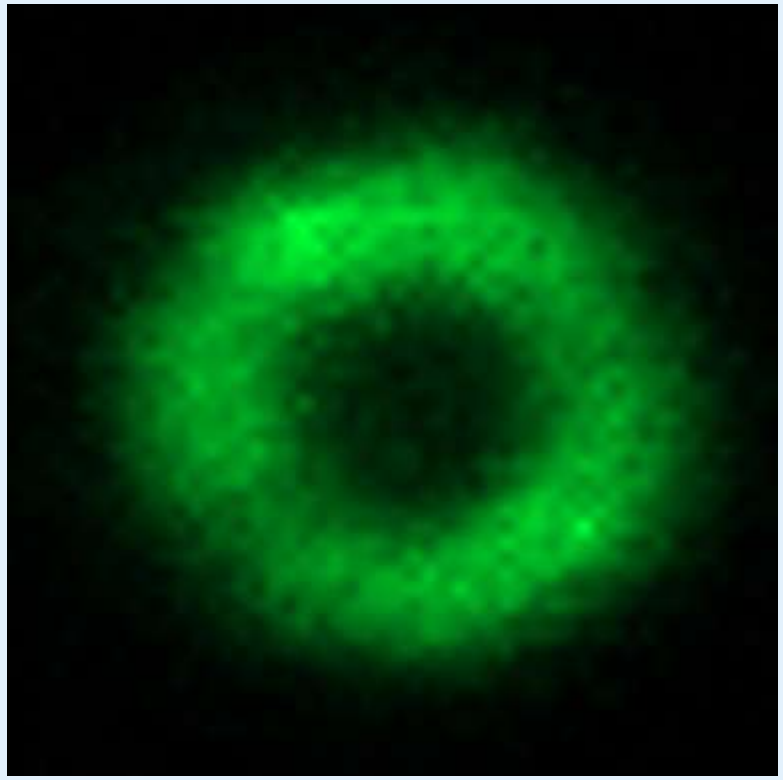
iterative
joint DCV



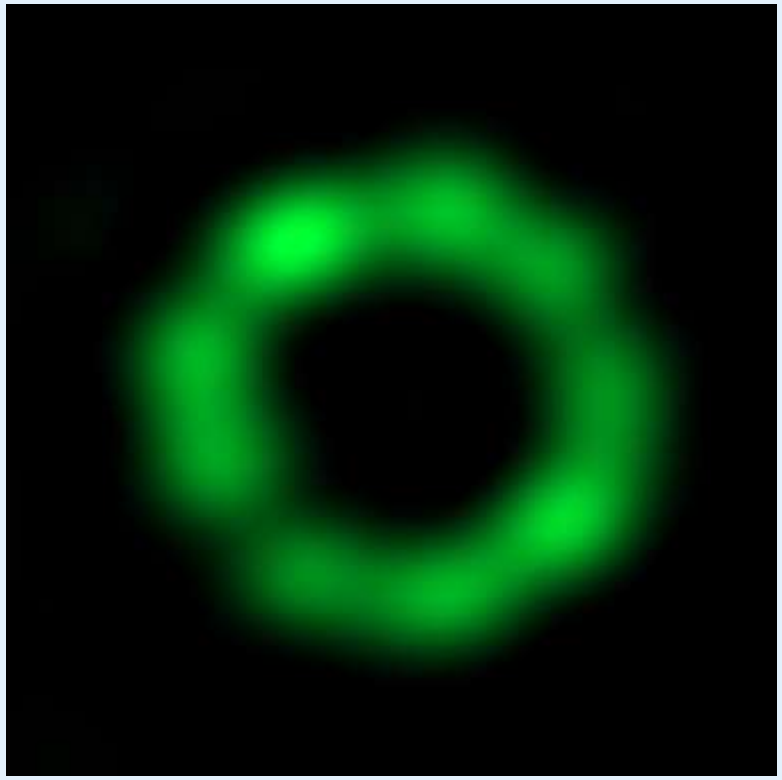

GATTA-SIM nanoruler

# Airyscan Joint Deconvolution

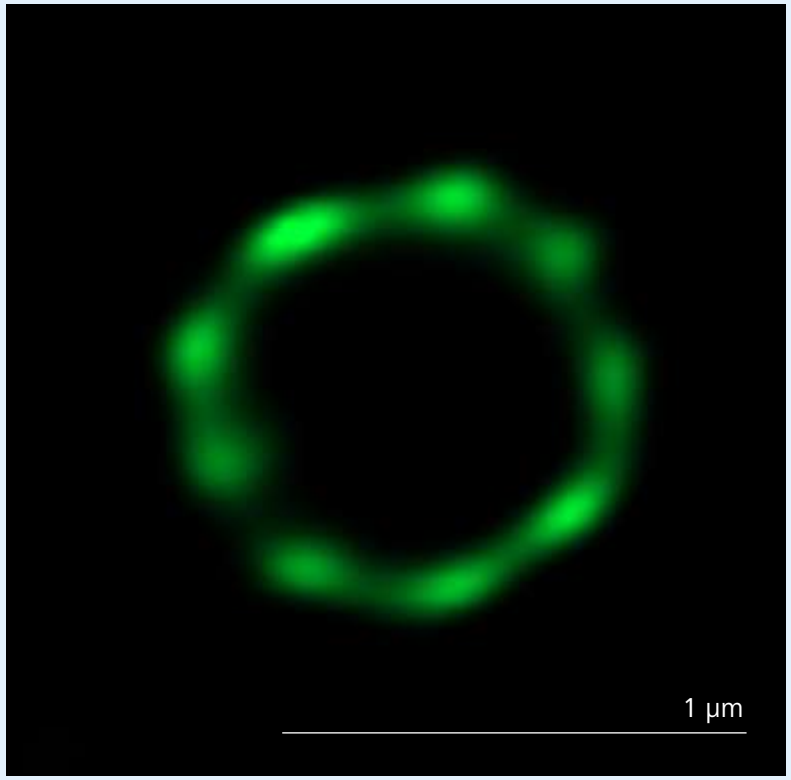
LSM



Airyscan SR



Airyscan jDCV



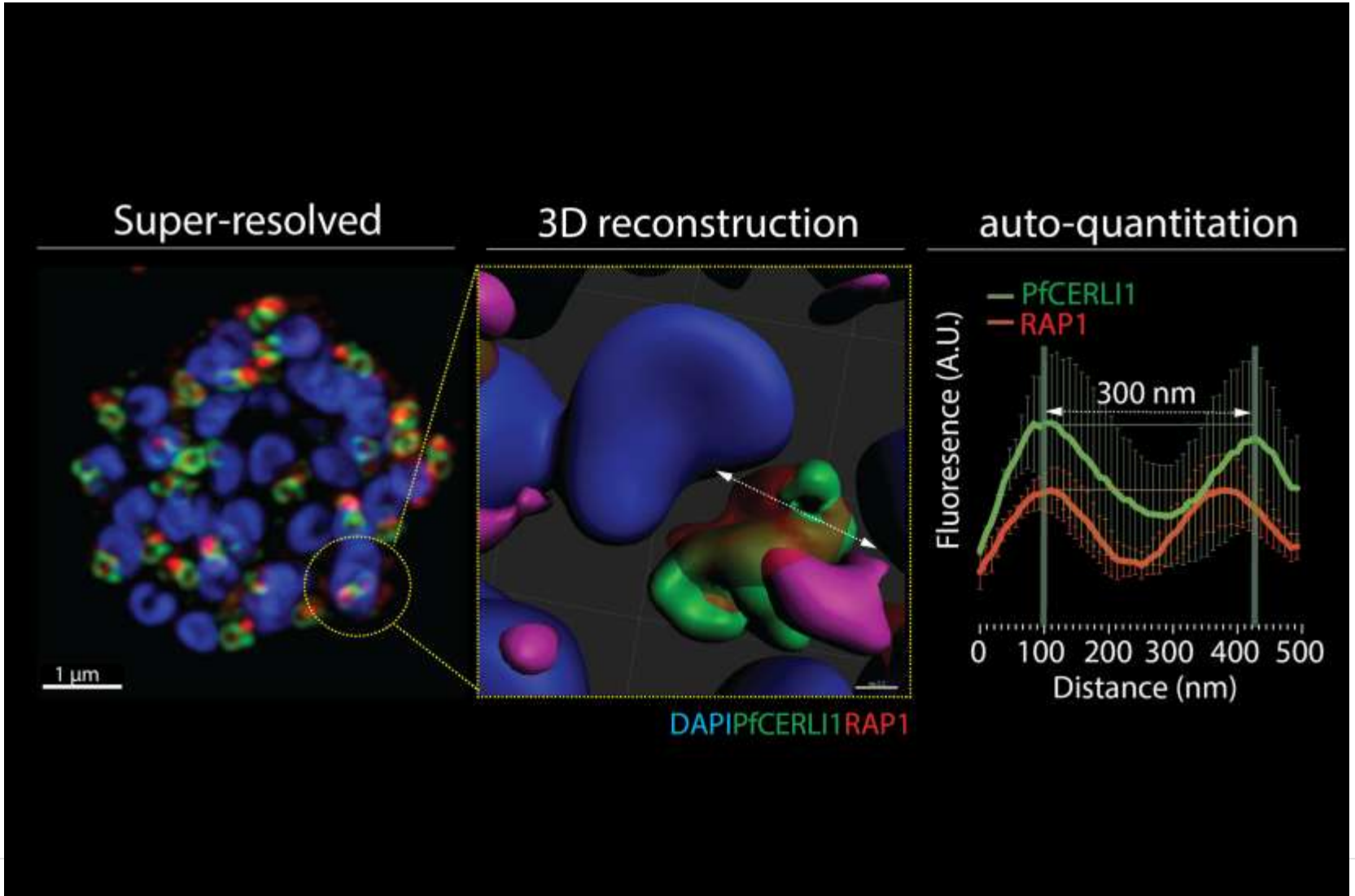

HeLa cell, 4x expanded and labelled with acetylated alpha tubulin. Courtesy of S. Zhang, Prof. Liou Yih-Cherng's lab, Singapore

# Exploring Malaria Parasite Entry into Red Blood Cells

## Airyscan 2 Superresolution

Quantitative analysis of malaria invasion organelles

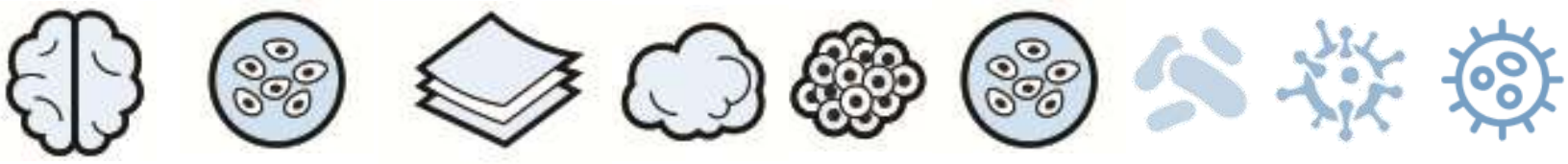
**Dr. Sonja Frölich**
Wilson Laboratory, Research Centre of Infectious Diseases, University of Adelaide, Australia



# Keep the Context of Your Experiments

| X-ray | Light Microscopy | | | | | Electron Microscopy | | | Ion |
|---|---|---|---|---|---|---|---|---|---|

| X-ray | Lightsheet | Lattice Lightsheet | Widefield | LSM Airyscan | Super-resolution | MultiSEM | Field Emission Scanning Electron | Focused Ion Beam | Helium Ion |
|---|---|---|---|---|---|---|---|---|---|
| 700 nm | 500 nm | 290 nm | 250 nm | 120 nm | 20 nm | 5 nm | < 2 nm | < 1 nm | < 0.5 nm |



# Developing the Future

**Versa Series**
**Global No.1**

Non-destructive & 3D imaging for sample quality measurement

# Developing the Future

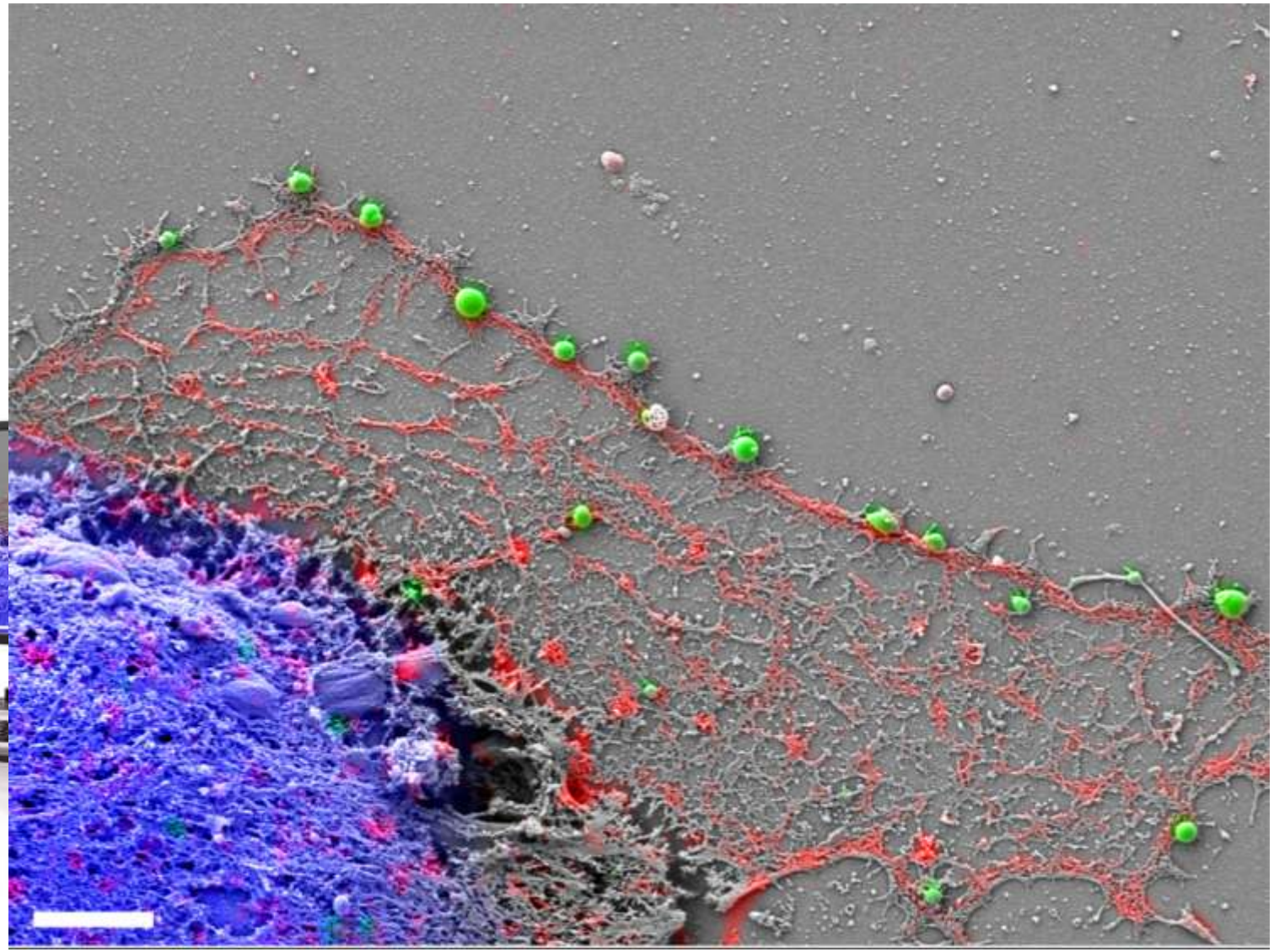
Zebrafish vertebrae

**Versa Series**
**Global No.1**

Non-destructive & 3D imaging for sample quality measurement

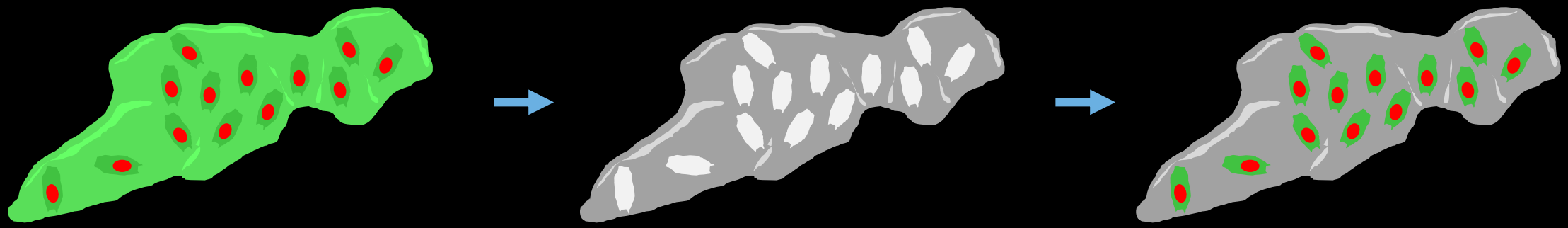
# Not Only But Also
## Superresolution-XRM-SEM Correlative Microscopy


GeminiSEM 360



# Verify bone cells on coral bone

Coral bone  Bone cell



**LSM 980 NLO**
Localize bone cells

**Cryo Gemini 360**
Visualize cell morphology

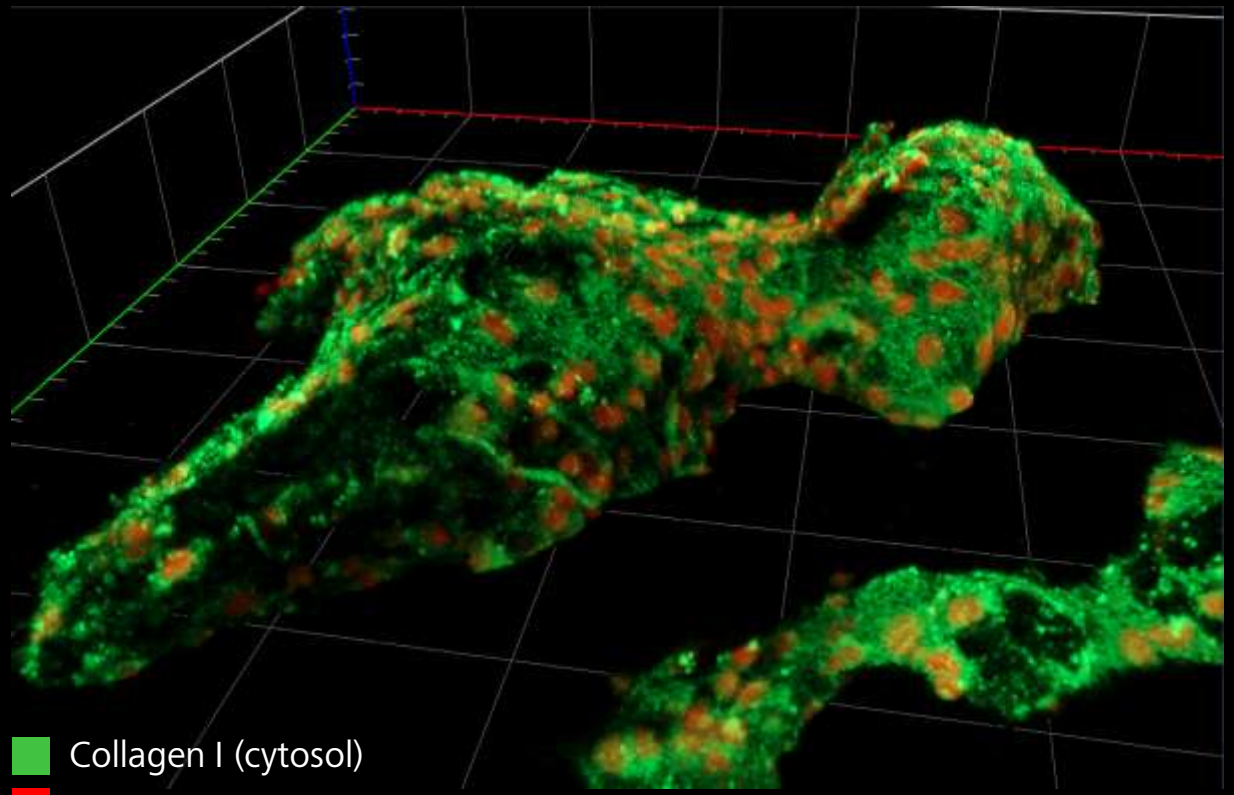
**ZEN connect**
Verify bone cells on coral bone


**Challenges**

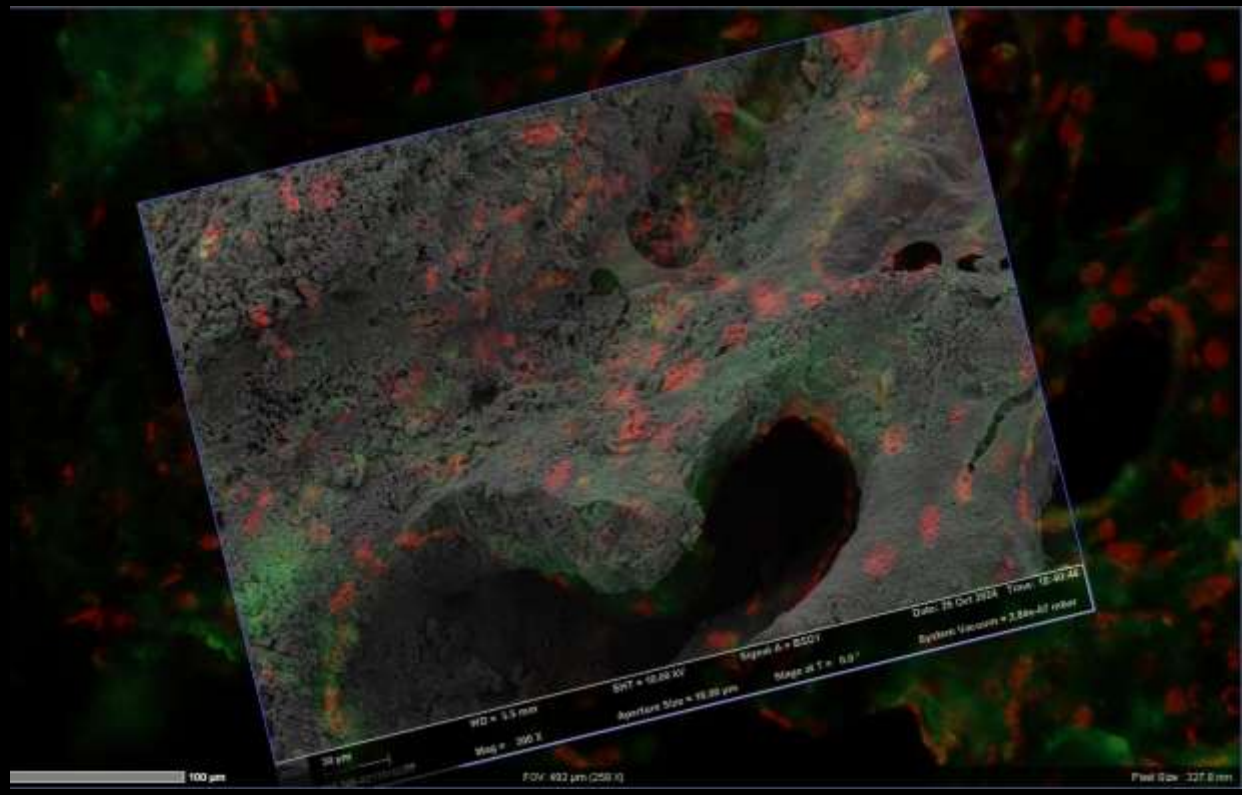
- Autofluorescence from coral bone merged with immunolabeled bone cells.
- Bone cells showed little contrast against coral bone (SE, BSE)
- How to alleviate drifting during sample transfer

# Verify bone cells on coral bone

LSM 980 NLO



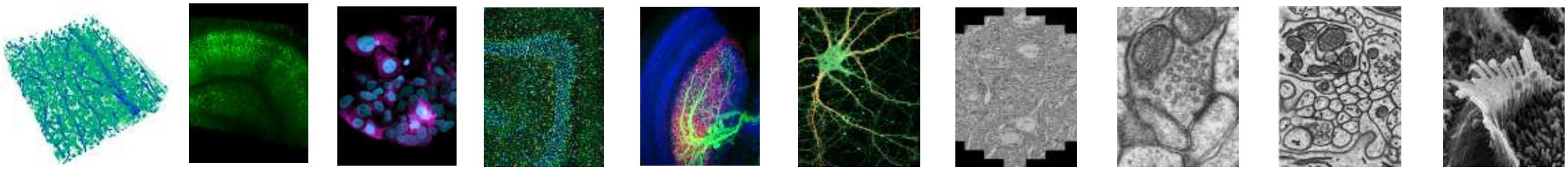

LSM 980 NLO + Cryo Gemini 360



CLEM enabled visualization of transparent targets on opaque substrate.

# Keep the Context of Your Experiments

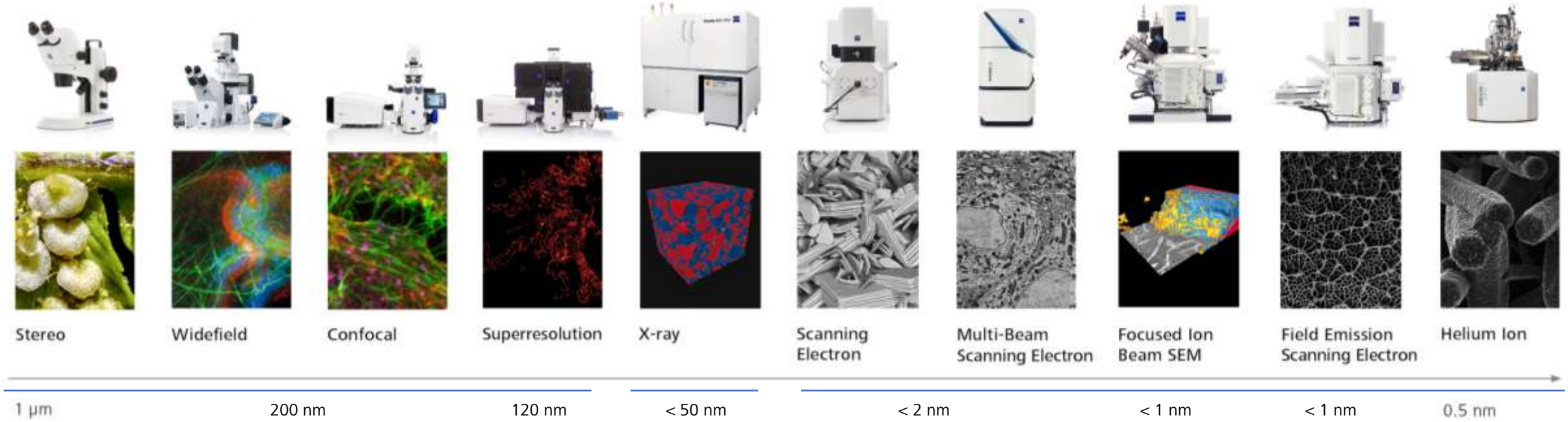
| X-ray | Light Microscopy | | | | | Electron Microscopy | | | Ion |
|---|---|---|---|---|---|---|---|---|---|



| X-ray | Lightsheet | Lattice Lightsheet | Widefield | LSM Airyscan | Super-resolution | MultiSEM | Field Emission Scanning Electron | Focused Ion Beam | Helium Ion |
|---|---|---|---|---|---|---|---|---|---|
| 700 nm | 500 nm | 290 nm | 250 nm | 120 nm | 20 nm | 5 nm | < 2 nm | < 1 nm | < 0.5 nm |

**CORRELATIVE MICROSCOPY SOLUTION**

# Insights For Science Discoveries




LM
Light Microscopy

XRM
X-Ray Microscopy

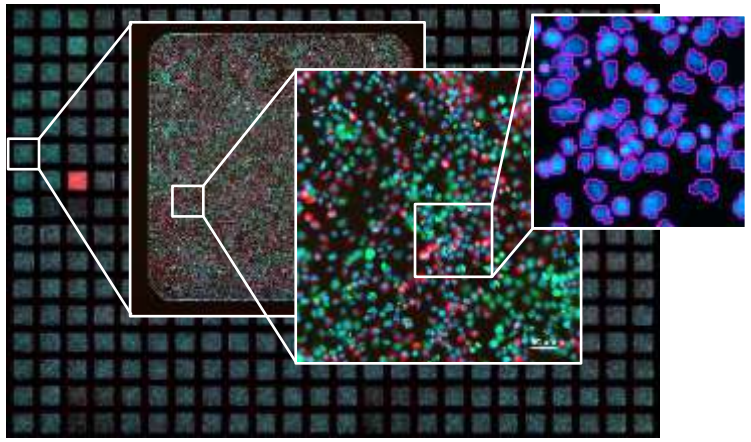
EM
Electron Microscopy

## CORRELATIVE MICROSCOPY SOLUTION

# Integrated Solution for Analytic Imaging

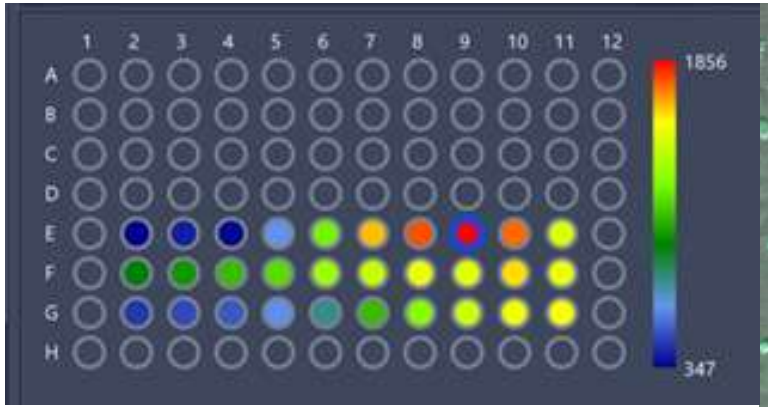
## Smart Acquisition


**Direct Processing** | Parallel acquisition and data processing

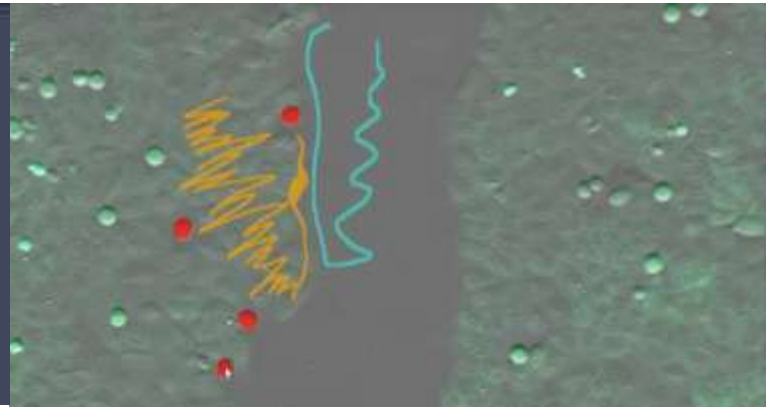


**Guided Acquisition**

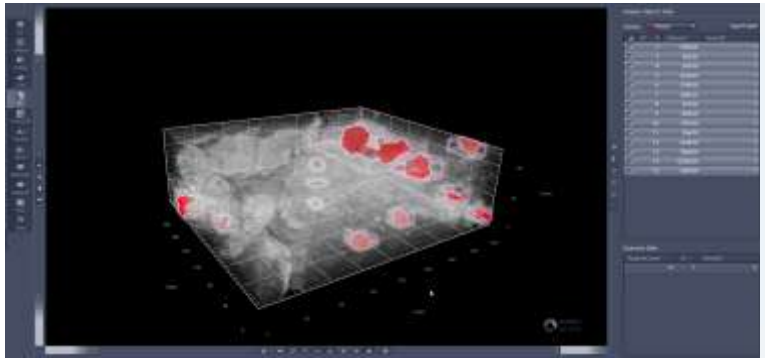
## Image Analysis



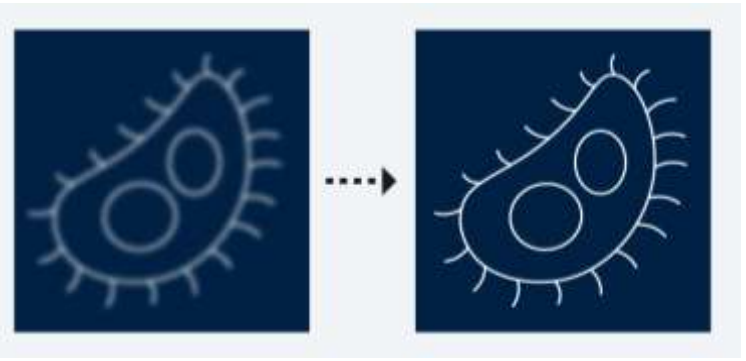

**Bio Applications**



**Intellesis** | AI machine learning



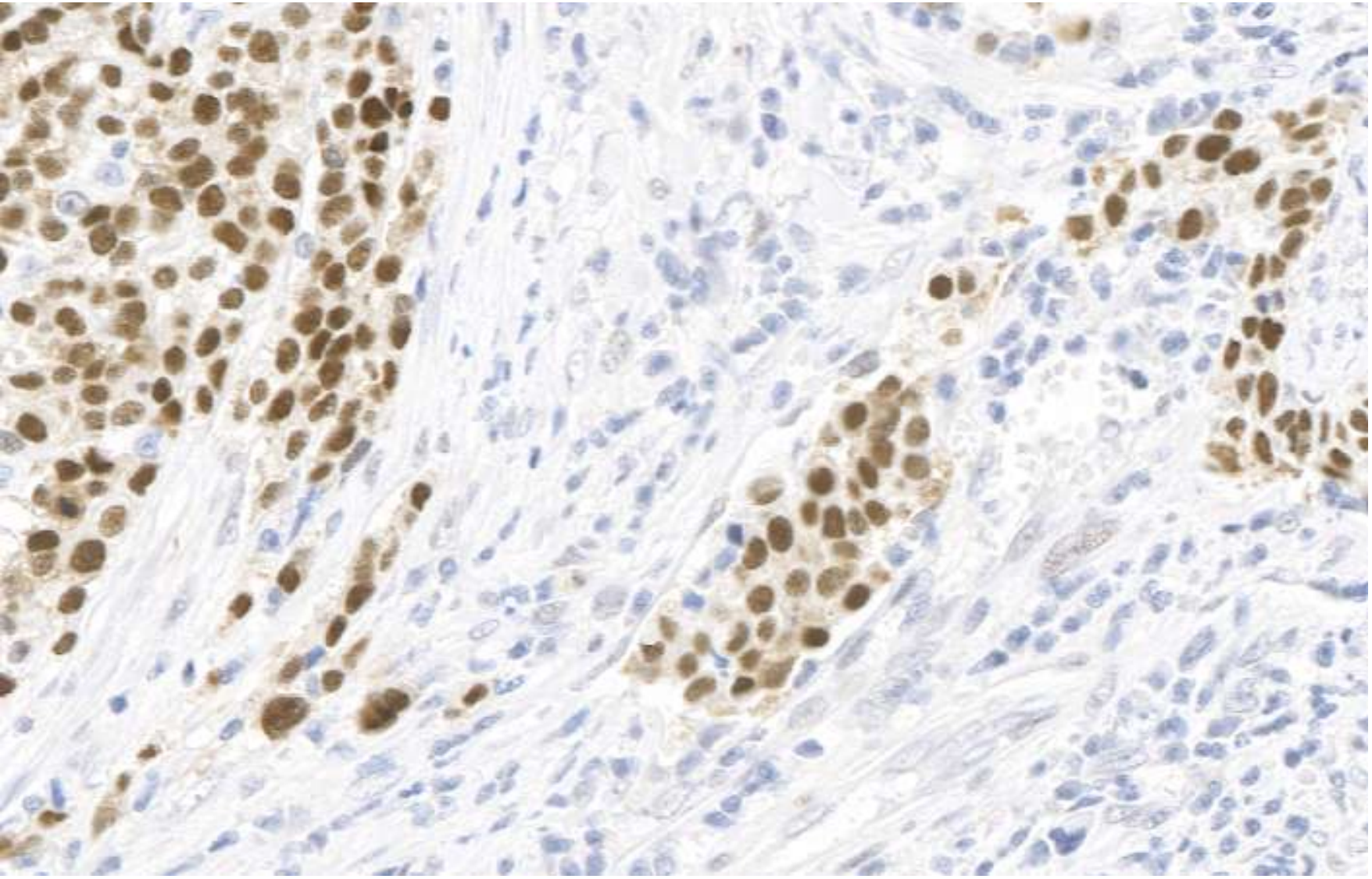
**3D segmentation**



**Deconvolution Toolkit**

# Effortless Image Acquisition and Analysis

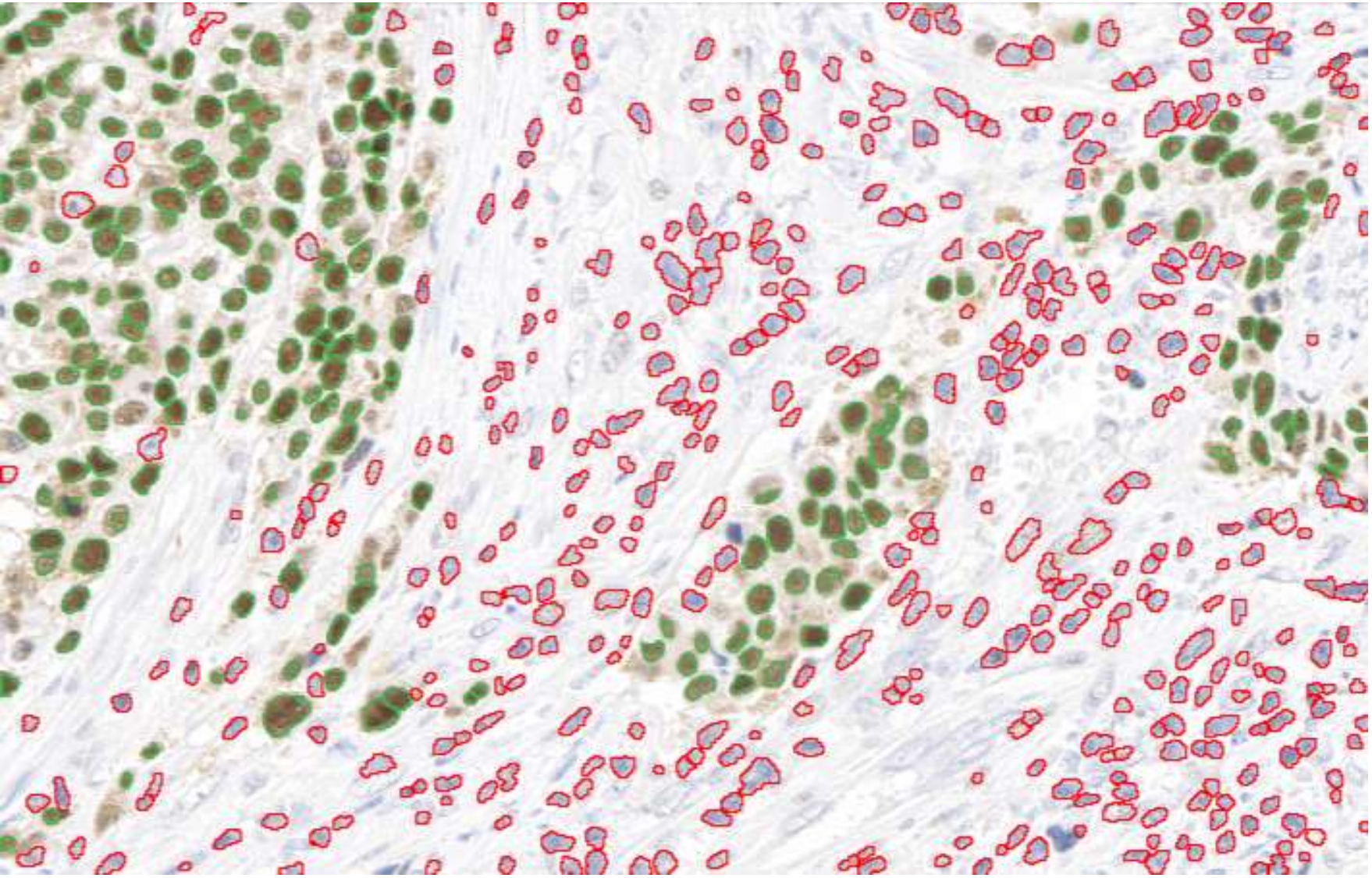
# Microscopy Images: A Picture is worth a thousand words!



How Many Cells are DAB positive?

A: 1-10%
B: 10-20%
C: 20-30%
D: 30-40%

# Microscopy Images: A Picture is worth a thousand words!



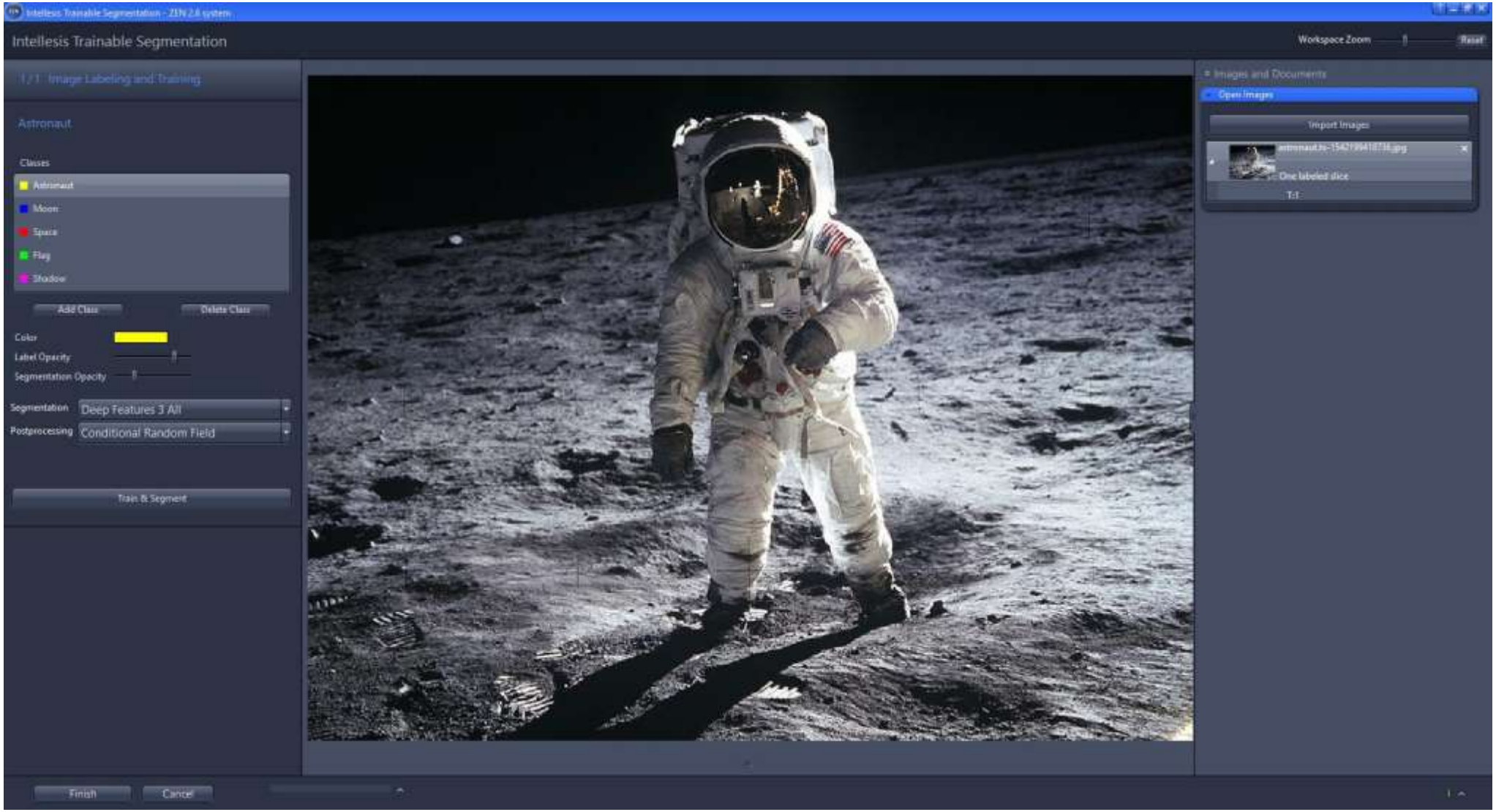
How Many Cells are DAB positive?

A: 1-10%
B: 10-20%
C: 20-30%
D: 30-40%

$$DAB = \frac{234}{234 + 418}\ \% = 35.9\%$$

# AI Image Segmentation | Zen Intellesis

## Machine Learning & Training Offline Analysis



# AI Image Segmentation | Zen Intellesis

## Machine Learning & Training Offline Analysis

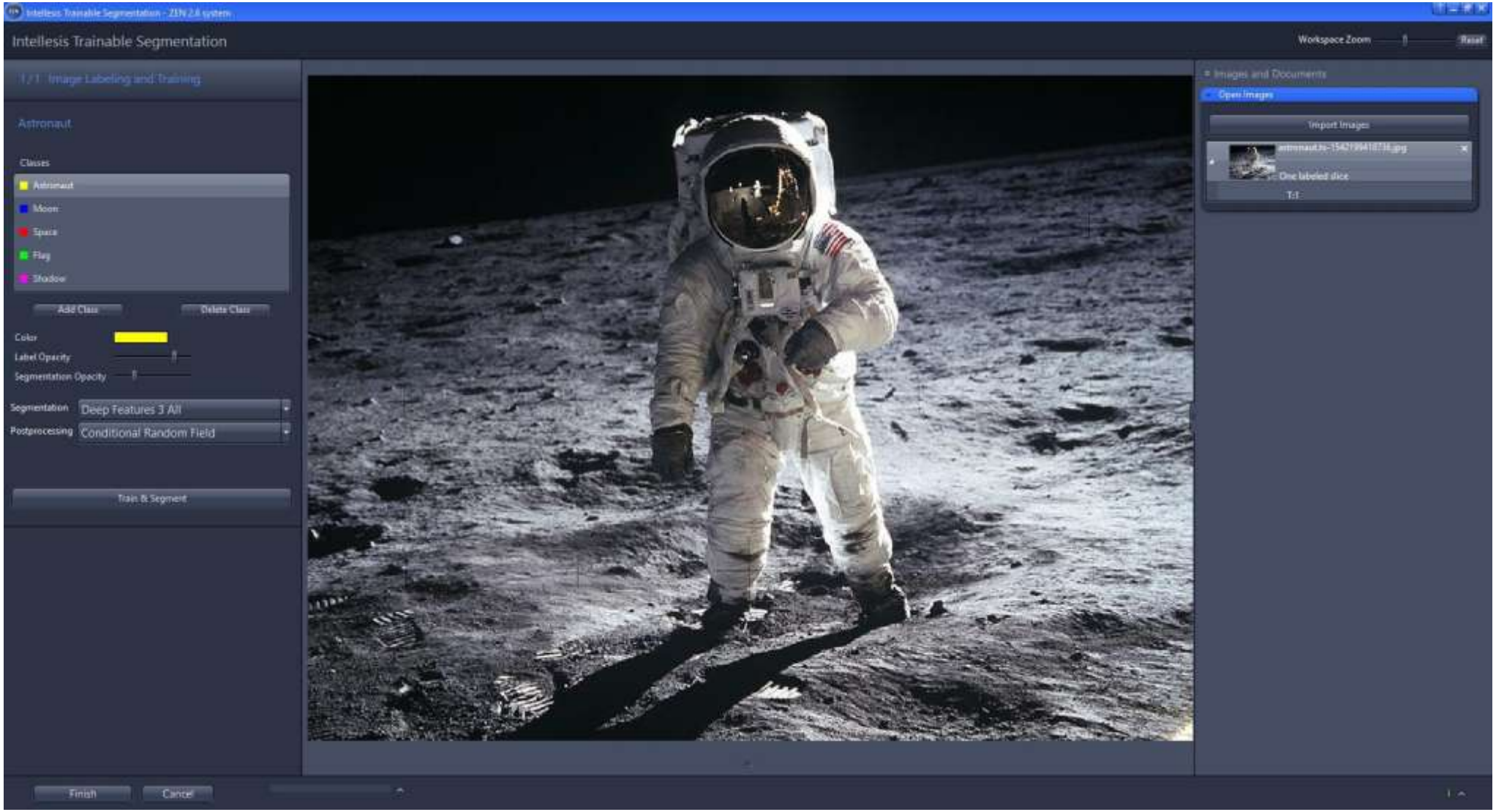
Astronaut

Moon

Space

Flag

Shadow



# AI Image Segmentation | Zen Intellesis

## Machine Learning & Training Offline Analysis

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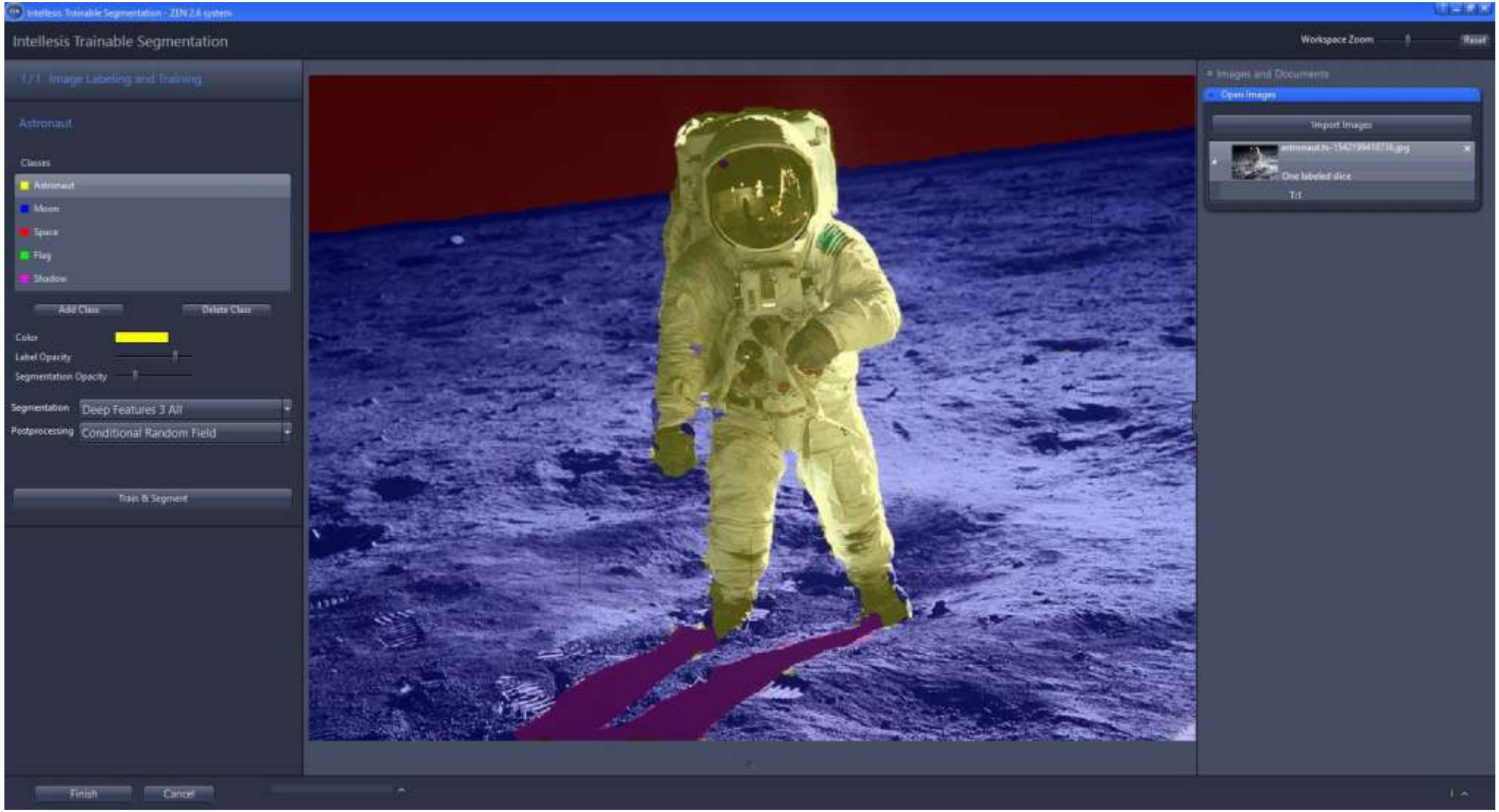


# ZEISS Image Analysis Software

ZEN

## Image Analysis

Flexible analysis pipeline

## BioApps

AI-powered image analysis for specific application

arivis

## arivis Pro

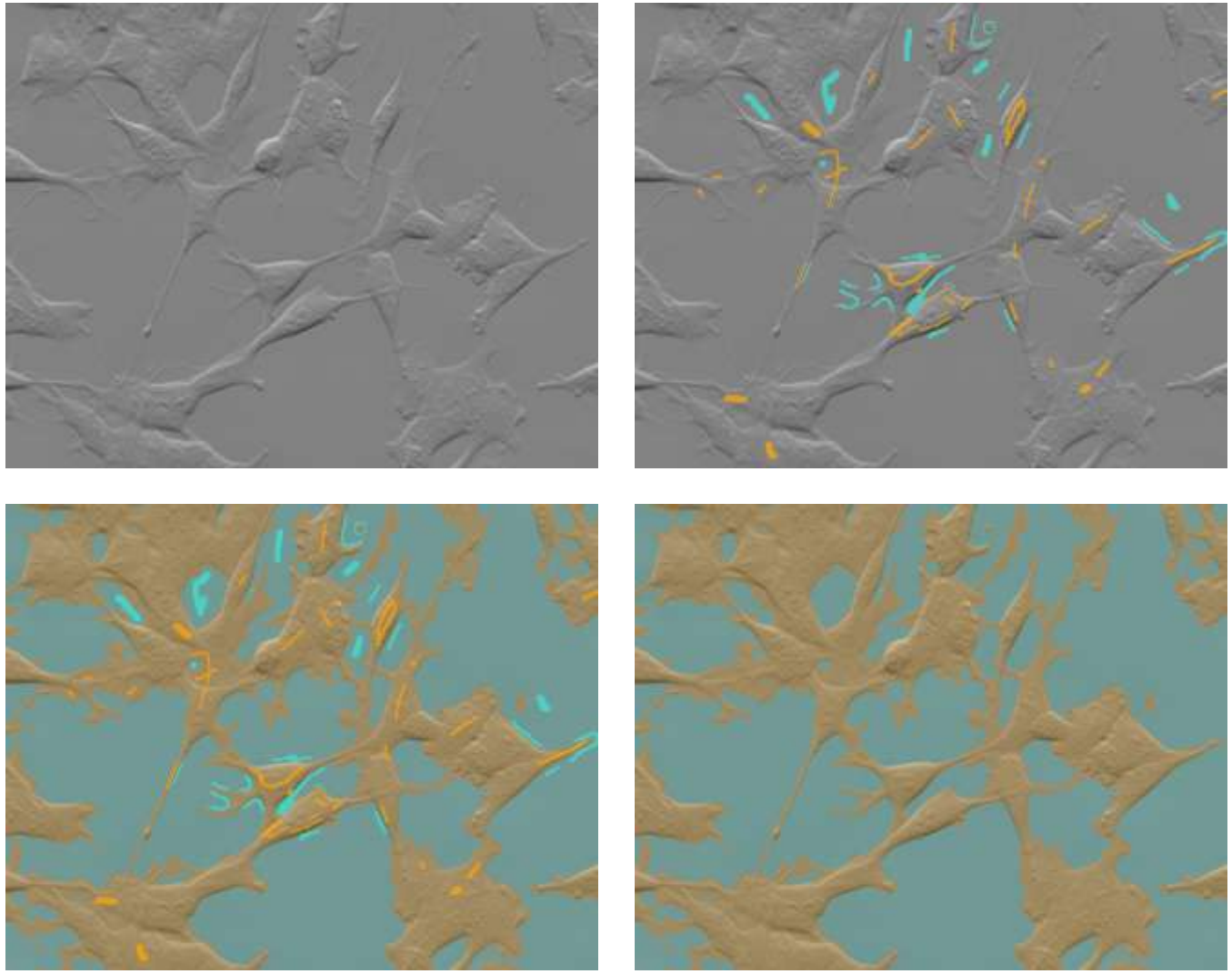
3D image analysis and visualization

Local AI image analysis

## arivis Cloud

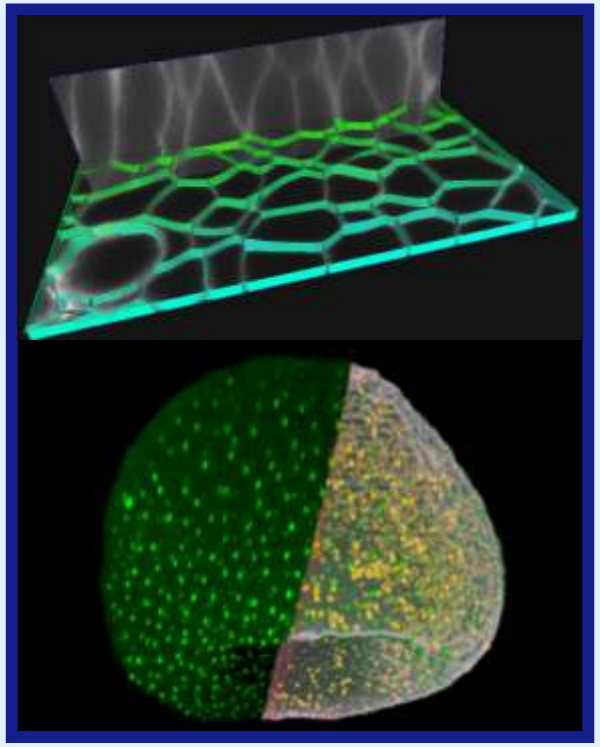
Cloud-based AI image analysis

# Intellesis – Simple User Interface



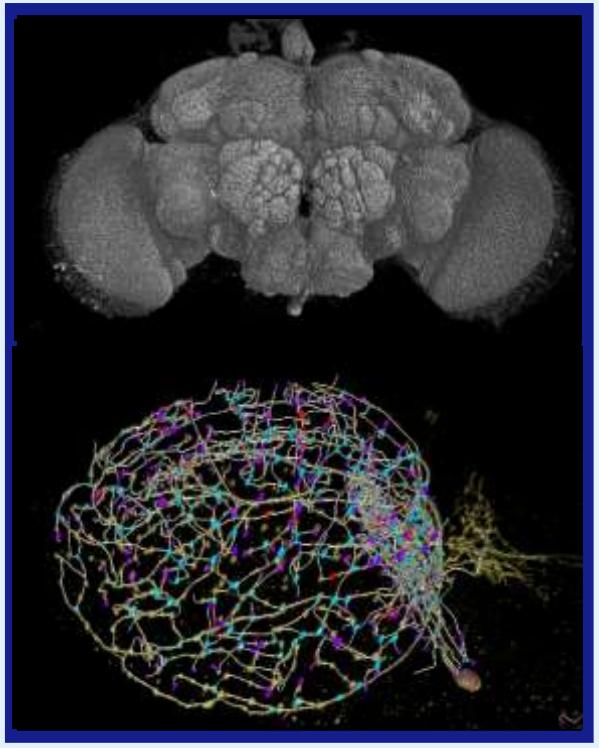
- Cells image using Phase-Gradient Contrast on a CD7
- Labeled with 2 classes inside Intellesis Training UI
- Feature Extractor: DeepFeatures256 + CRF Postprocessing

# Out of the Box Solutions For All Research Topics in **arivis Pro**



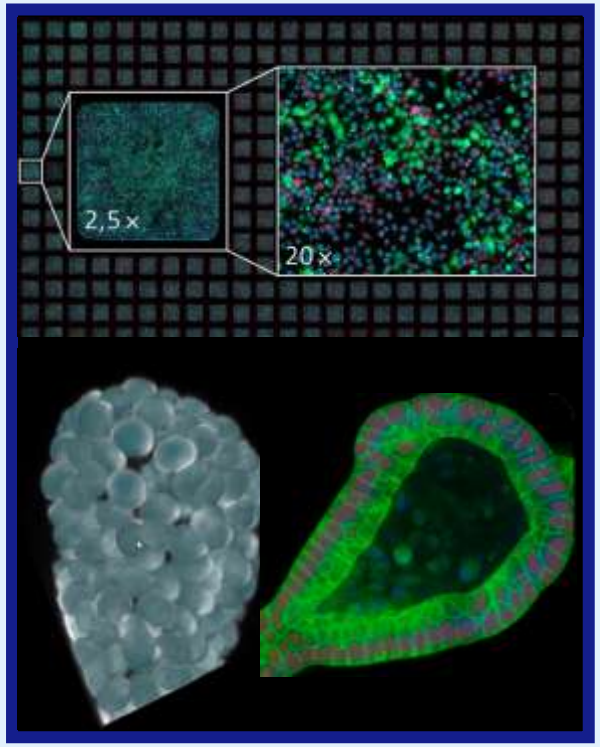
## Developmental Biology

Cell and Organelle Tracking
3D and 4D Analysis
Membrane Segmentation



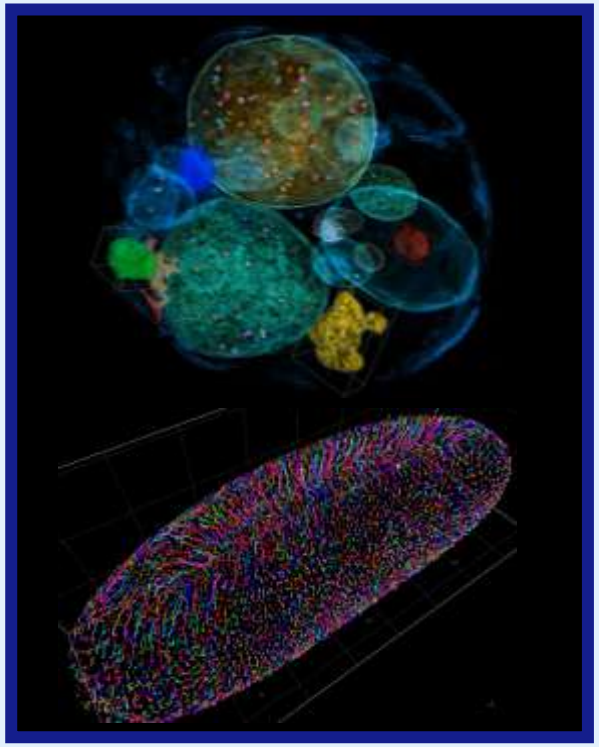
## Neuroscience

Compartment Analysis
Distribution Analysis
Stitching / Multi-view image reconstruction




## High Content

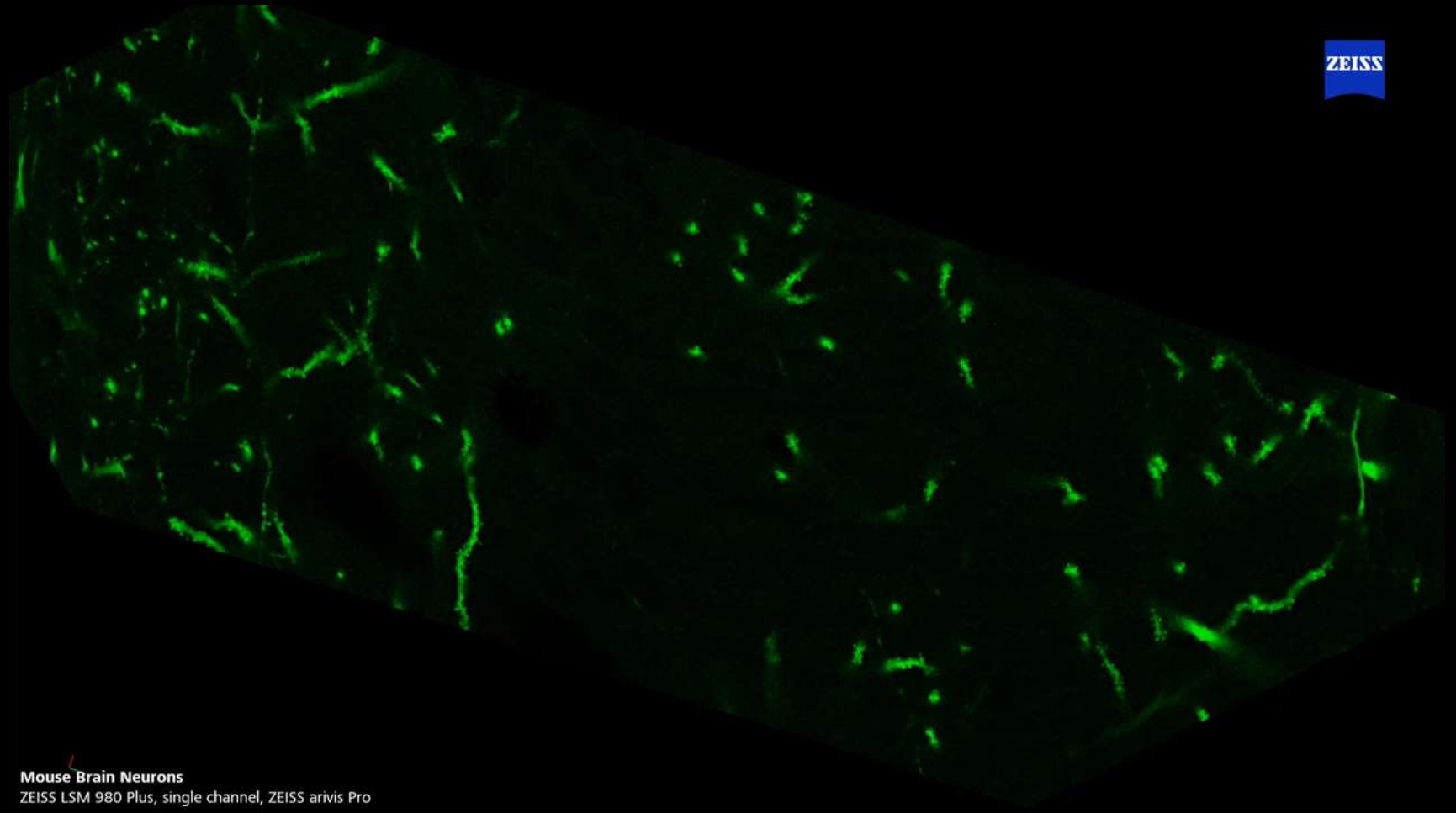
Well-by-well analysis
Cell counting
Organoids and Spheroids



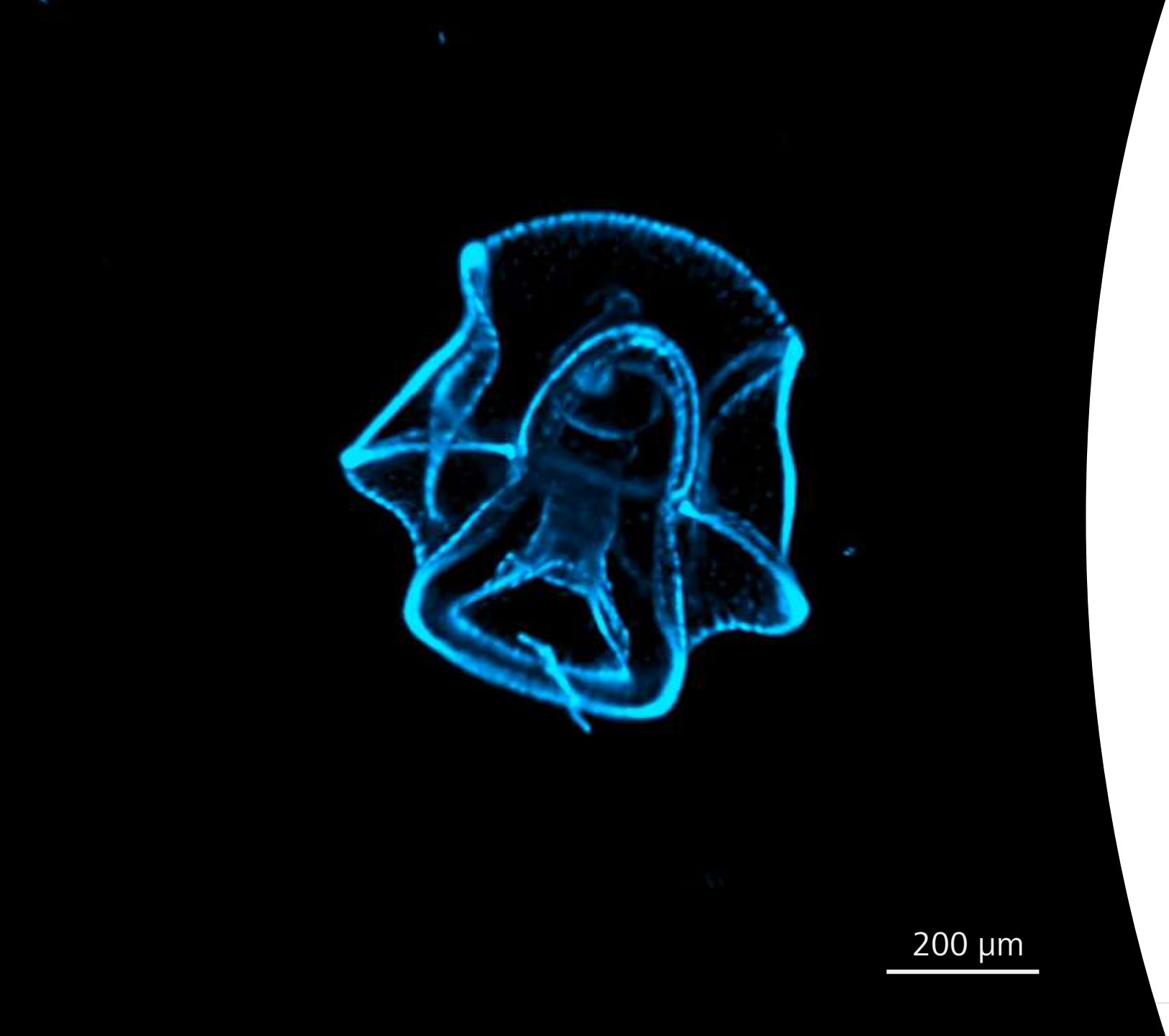
## Cell Biology

Organelle Analysis
Distance Measurements
Compartment Analysis

# Gain Spatial Information using 3D Reconstruction



**Mouse Brain Neurons**
ZEISS LSM 980 Plus, single channel, ZEISS arivis Pro




# Lightfield 4D

## One Snap. One volume

Free swimming larvae of the bat star (Patiria miniata) stained with Hoechst 33342 (cyan, nuclei).

Sample courtesy of Peter Lenart and Jasmin Jakobi, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany

# One snap. One volume.

Acquired @
80 volumes/second

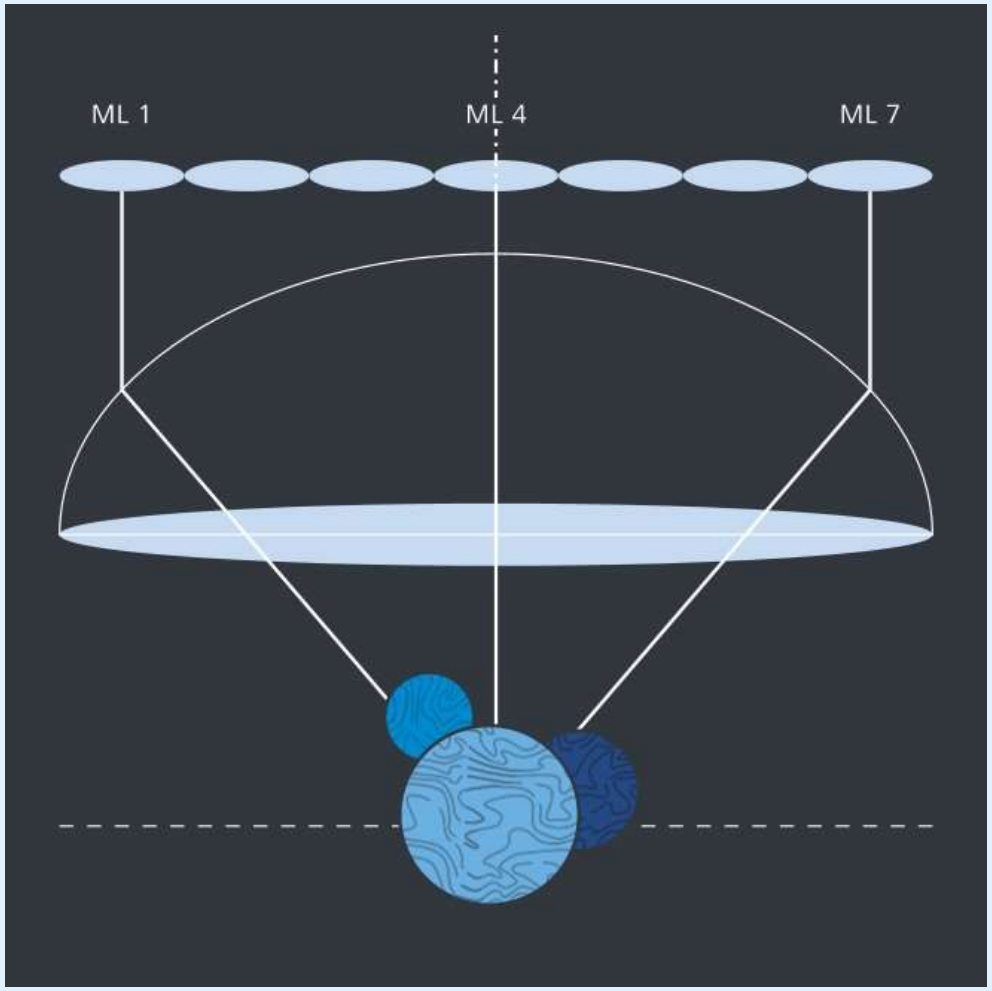
723 x 723 x 430 um $^{3}$

Investigating the morphology and cardiac wall movement of the developing zebrafish heart.

Sample courtesy of Stone Elworthy and Emily Noël, School of Biosciences, University of Sheffield, UK

# Lightfield 4D
Insights into the technology behind it

# Lightfield 4D
## Insights into the technology behind it

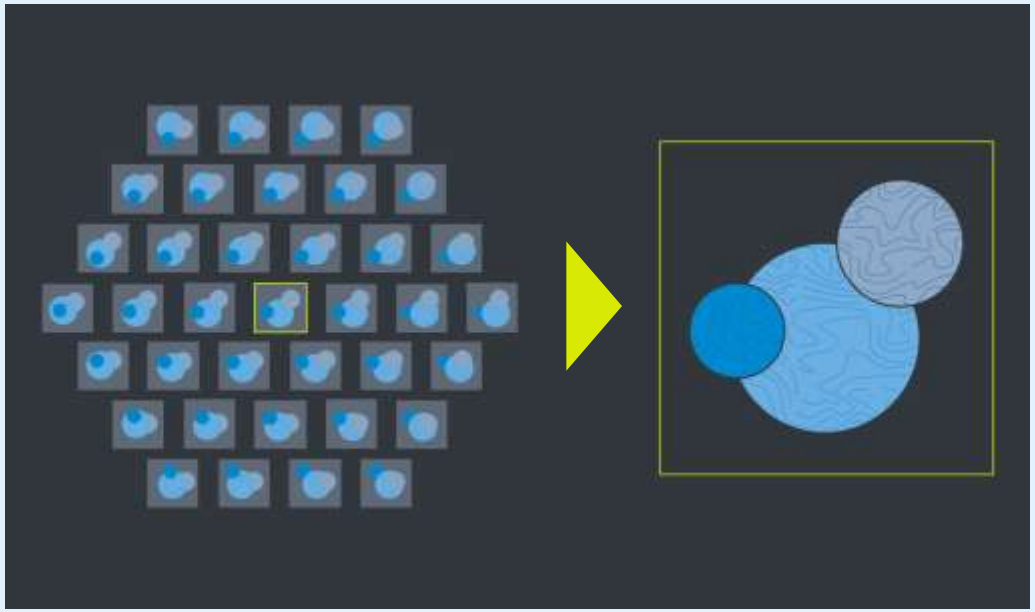



Multi lens array

Objective lens

Focal plane, sample

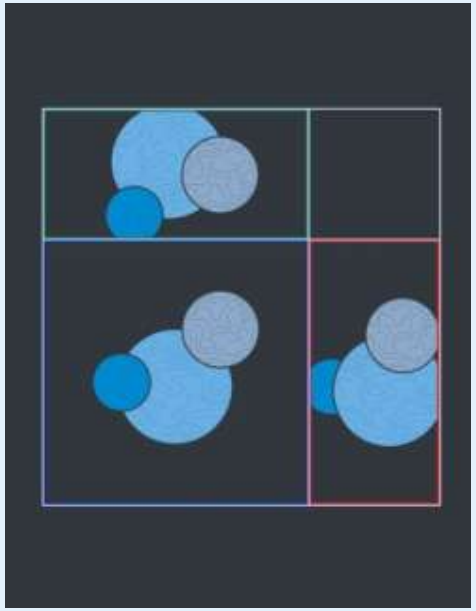
# Lightfield 4D
## Insights into the technology behind it



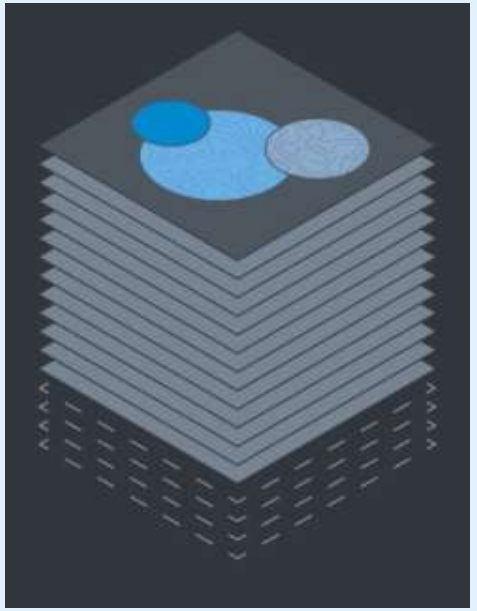
Readout of all 37 views



Parallax view

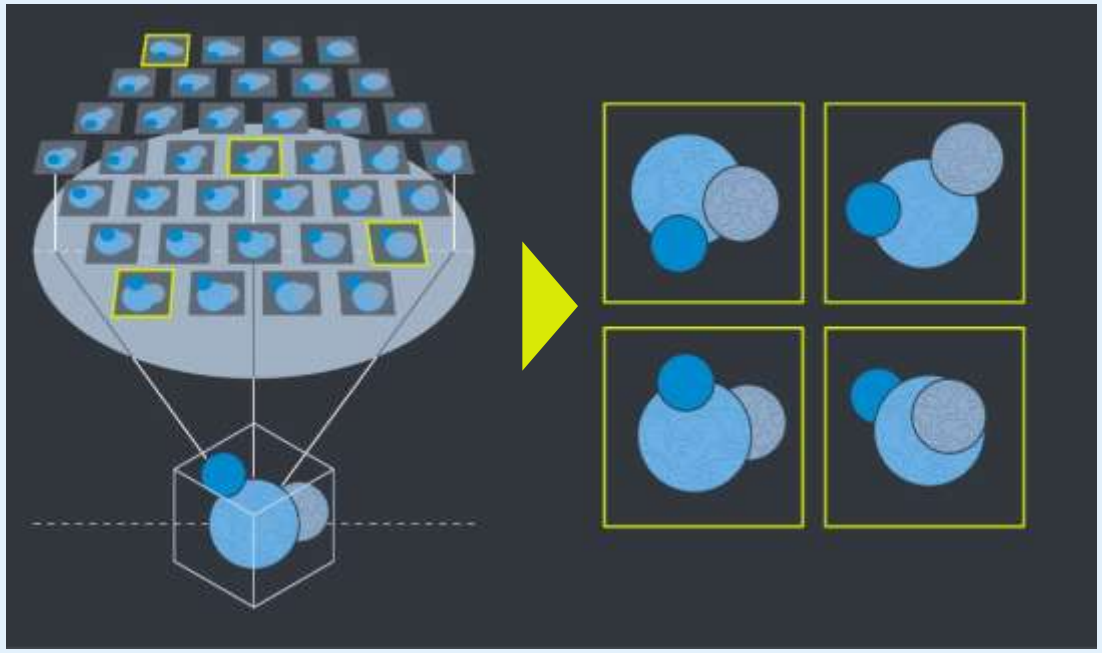


3D result (ortho view)



Final output: reconstructed z-stack for subsequent analysis with ZEN and arivis

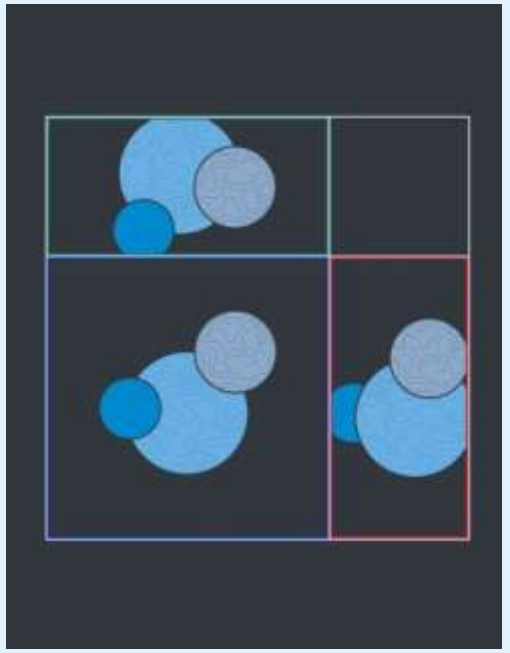
# Lightfield 4D
## Insights into the technology behind it



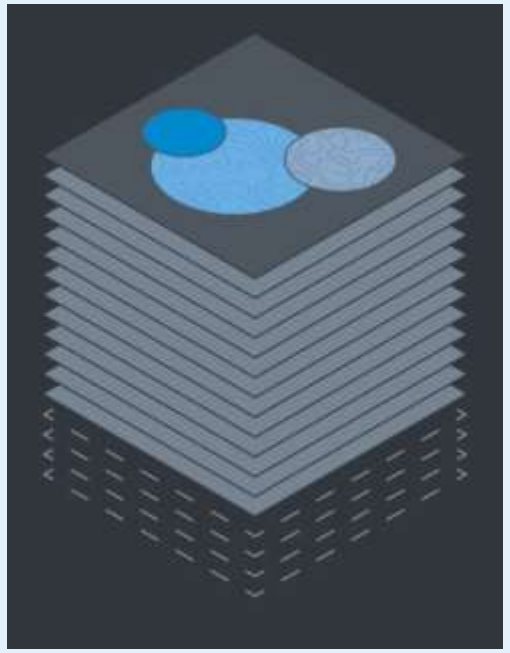
Readout of all 37 views



Views from different angles



3D result (ortho view)



Final output: reconstructed z-stack for subsequent analysis with ZEN and arivis

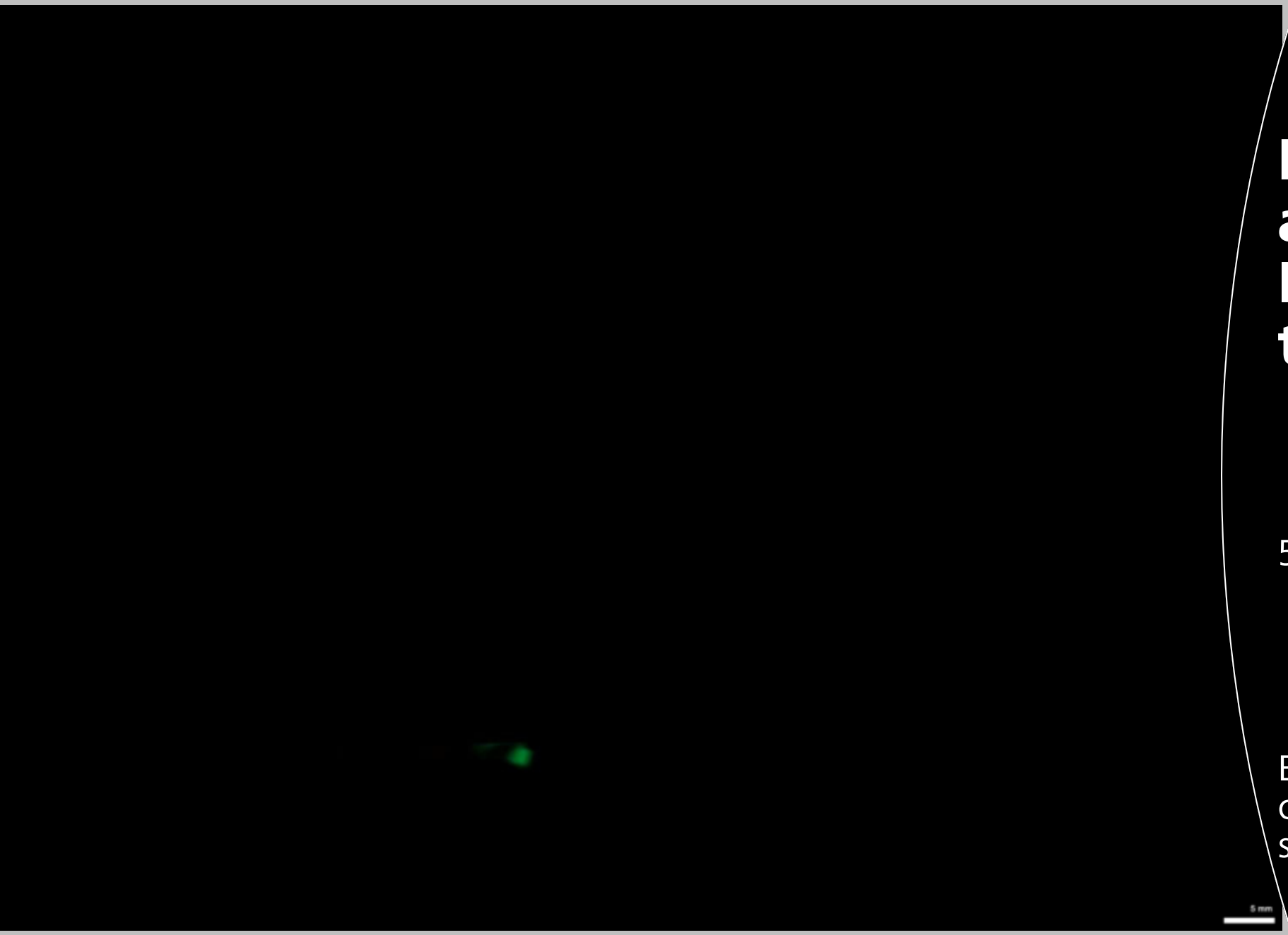

Courtesy of Toby Andrews and Rashmi Priya, The Francis Crick Institute

# One snap. One volume.

Acquired @
80 volumes/second
for 2.4 seconds

361 x 361 x 109 um $^3$

Red blood cells flowing through the tail vasculature of the developing zebrafish embryo

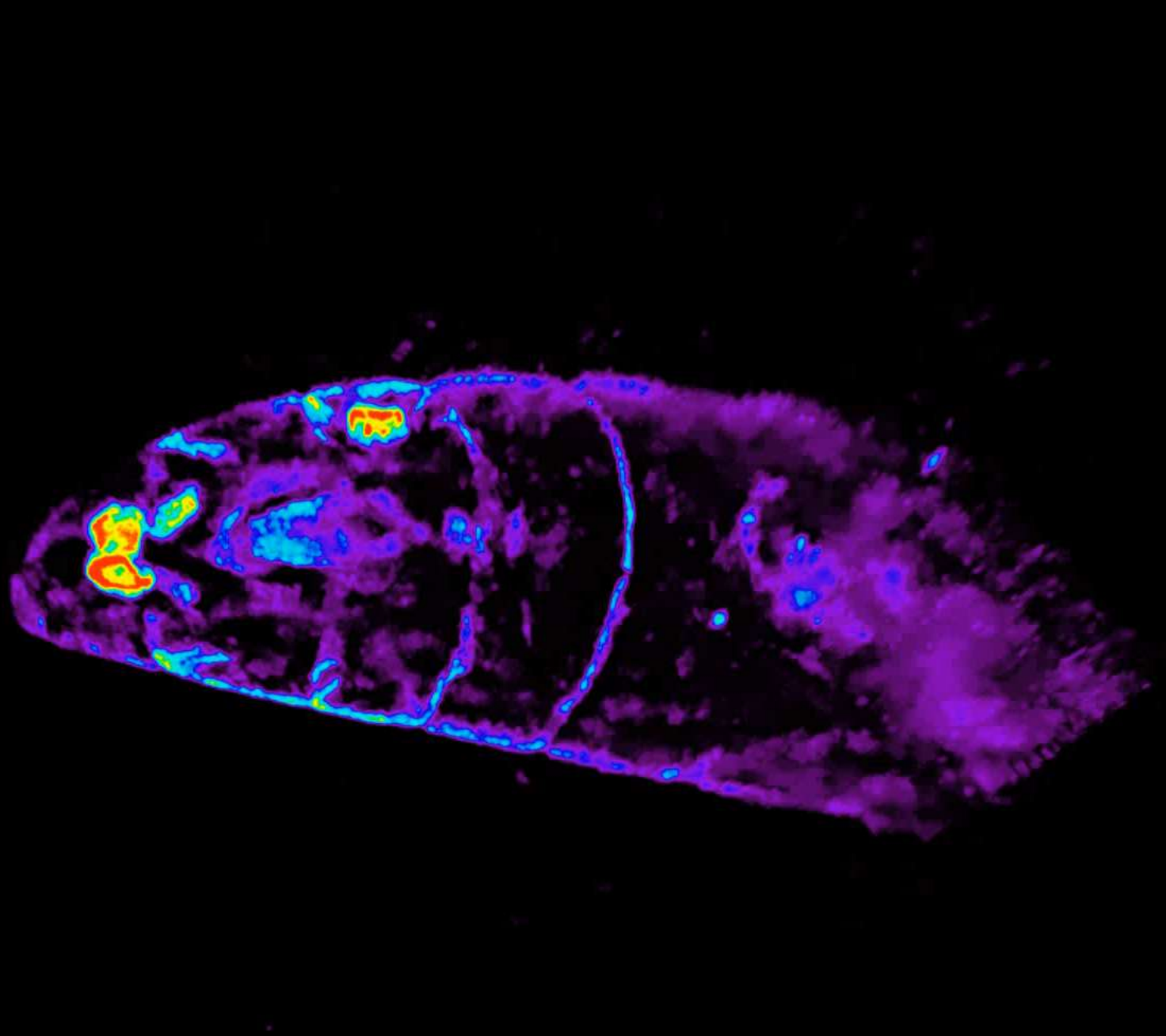



# Fast acquisition. Increased throughput

585 x 585 x 278 um $^{3}$

Efficient volume imaging of cleared spheroids with subsequent cell counting.

Sample courtesy of InSphero AG. Schlieren, Switzerland



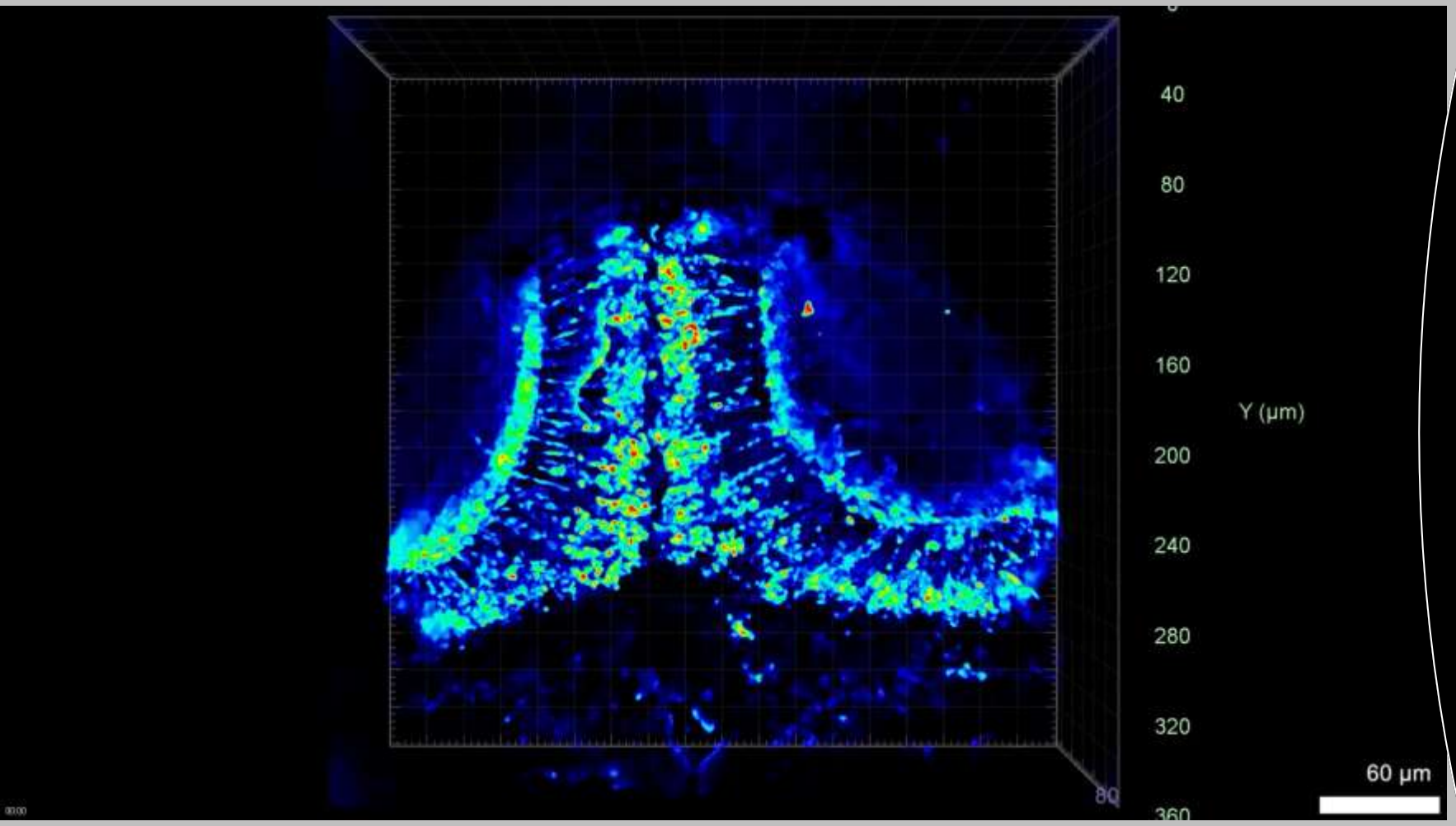
# One snap. One volume.

34 Volumes/second for roughly 9 seconds

585 x 585 x 278 um $^{3}$

Sensitive 3-dimensional imaging of muscle activation during lightly constrained crawling behavior of *Drosophila melanogaster* 1$^{st}$ instar larvae.

Sean Sweeney, Department of Biology, University of York, United Kingdom.




# One imaging platform. Endless possibilities

1. Lightfield 4D: acquire the neuronal activity @ 10 volumes per second
   361 x 361 x 109 um
   3

2. Airyscan MPLX: structural information without moving the sample

The thinking zebrafish: Analyzing neuronal activity in developing organisms

Sample courtesy of Anton Nikolaev, University of Sheffield, UK . Data acquired at Wolfson Light Microscopy Facility in the School of Biosciences at the University of Sheffield.


Seeing beyond