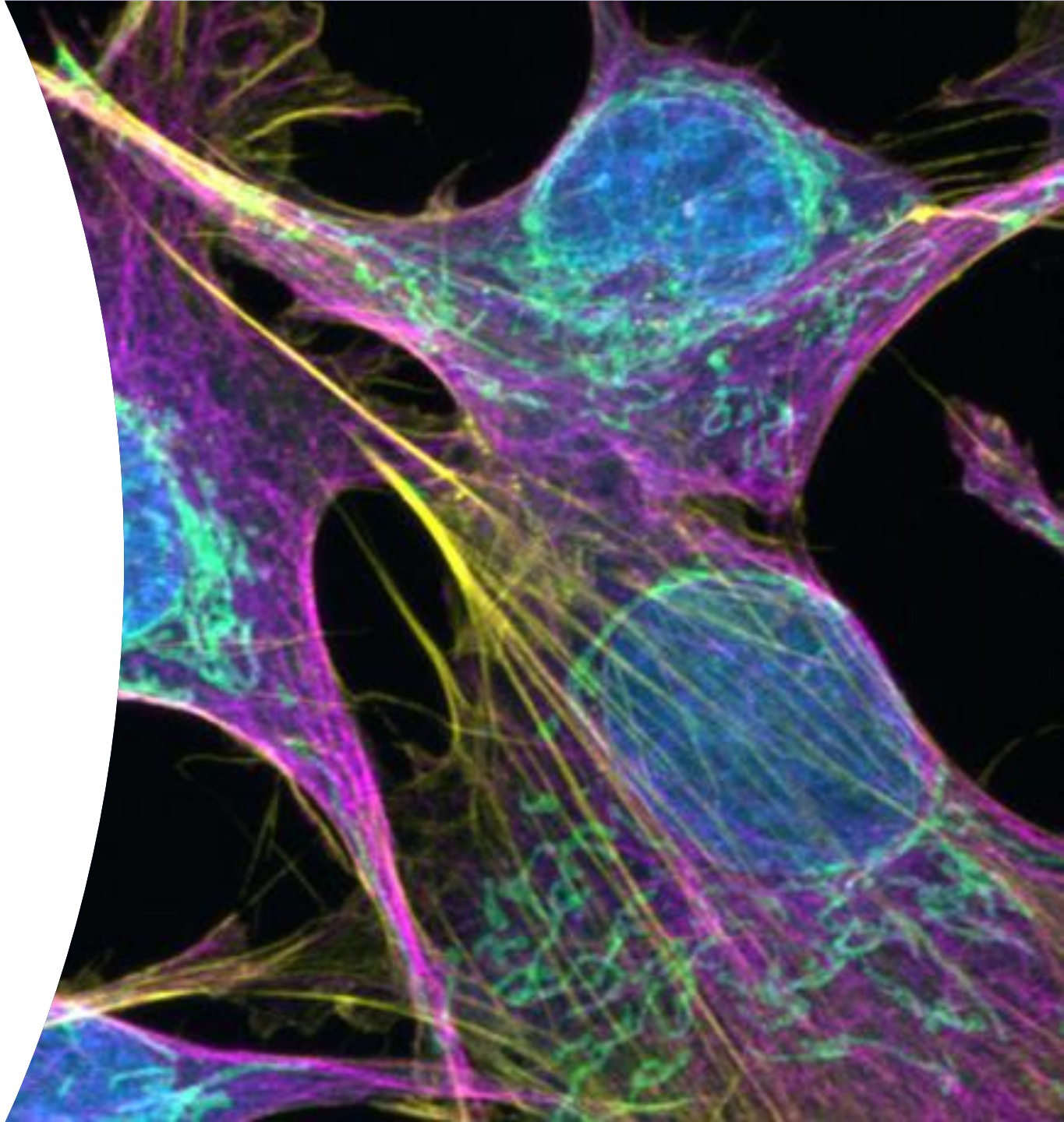
Confocal Principle



Jerry Fan
Application Engineer
ZEISS Research Microscopy Solutions Taiwan

Internet connection

10 years old laptop

Bioinformatics Research

Molecular Biology Research

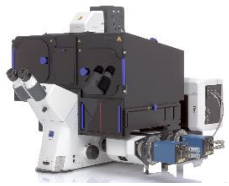
- DNA/RNA sequences
- Mass Spectrometer
- qPCR
- Fluorescence Microscopes
- Confocal Microscopes
- Electron Microscopes
- High-Speed Centrifuges
- Gel Electrophoresis
- Microplate Readers
- Incubators
- Pipettes & Pipettors
- Vortex Mixers
- Hot Plates
- Petri Dishes
- Well Plates
- Laboratory Freezers
- Autoclaves
- HPLC Systems
- Live-Cell Imaging
- Single-Cell Analysis

Keep the Context of Your Experiments

X-ray	Light Microscopy					Electron Microscopy			Ion



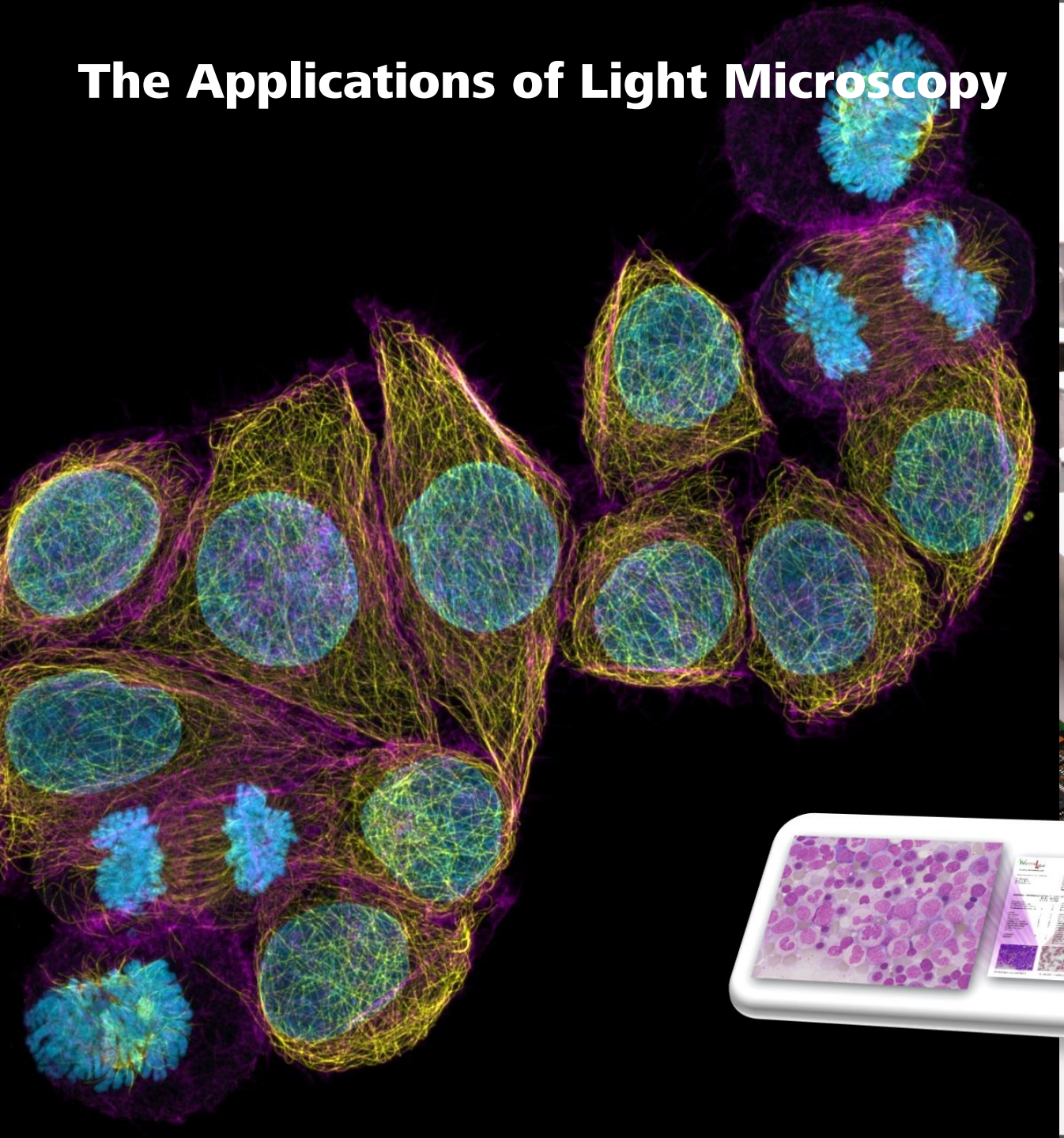


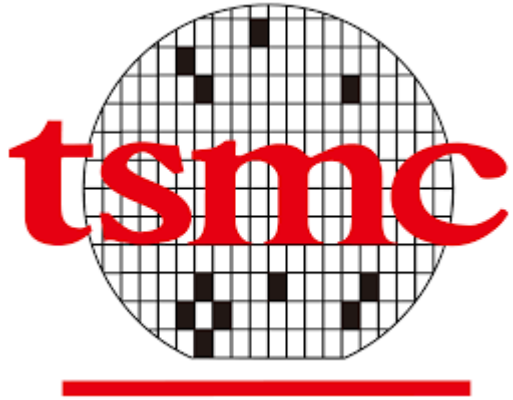


X-ray	Lightsheet	Lattice Lightsheet	Widefield	LSM Airyscan	Super-resolution	MultiSEM	Field Emission Scanning Electron	Focused Ion Beam	Helium Ion
700 nm	500 nm	290 nm	250 nm	120 nm	20 nm	5 nm	< 2 nm	< 1 nm	< 0.5 nm

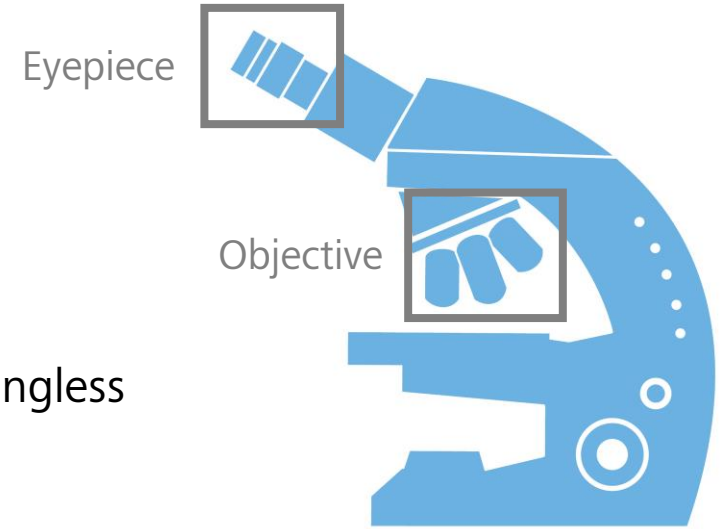
The Applications of Light Microscopy



The Applications of Light Microscopy

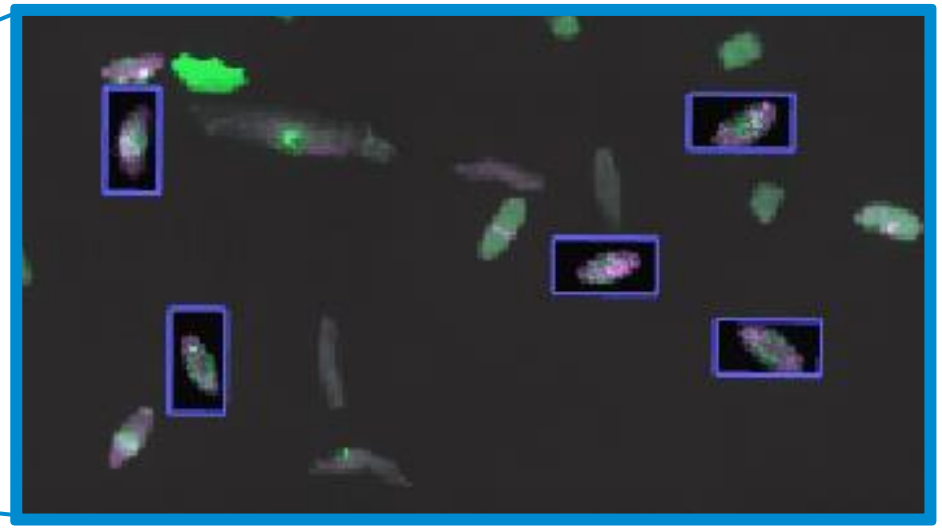


Magnification and Resolution



- 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

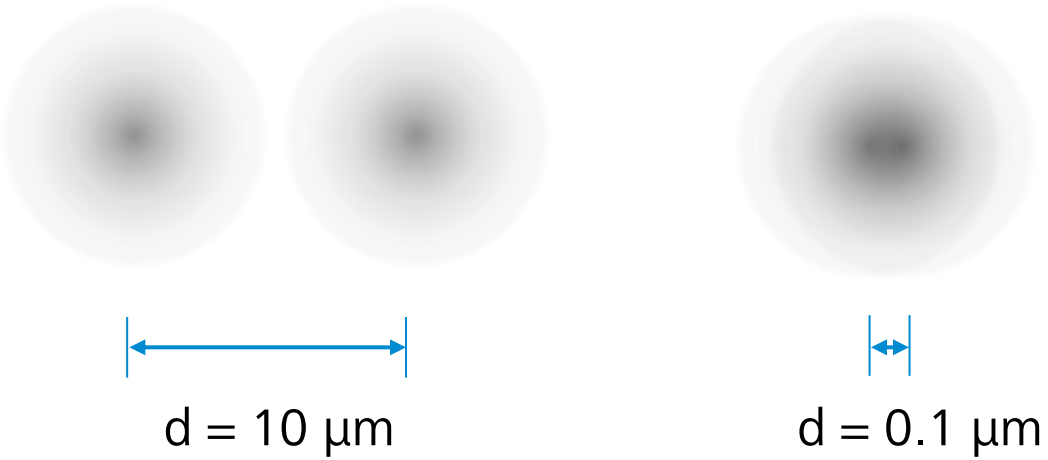
d = 0.1 cm

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.



Diffraction Limited Resolution

Rayleigh criterion

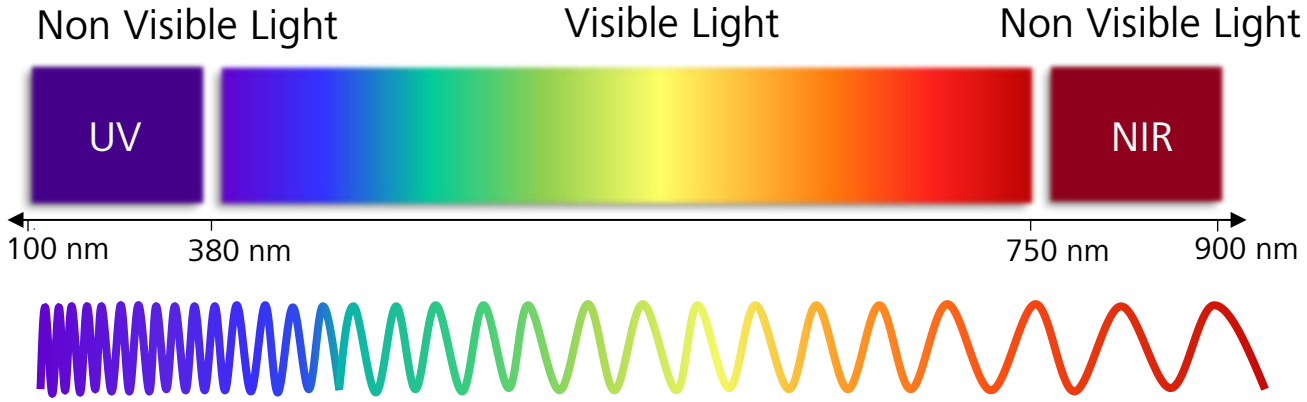
$$d_0 = \frac{1.22\lambda}{N.A_{obj.} + N.A._{Cond}}$$

or more simply

Abbe limit

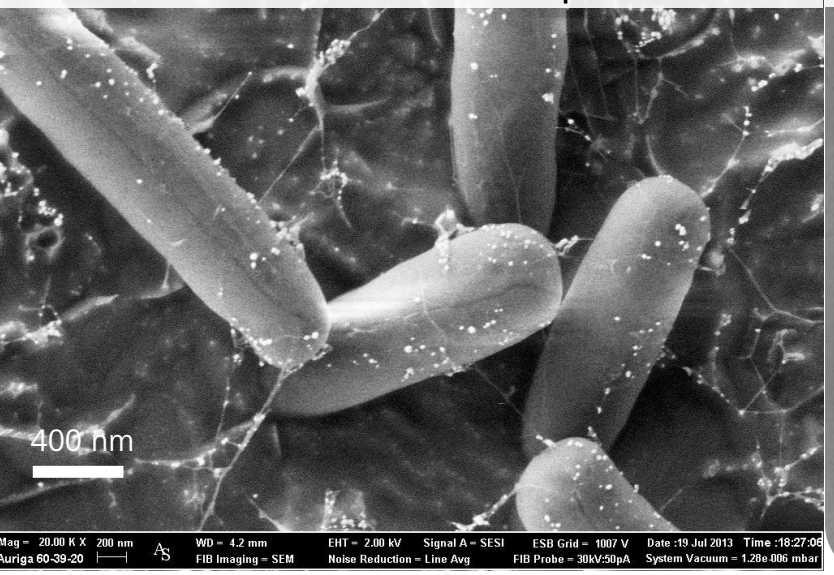
$$d_0 = \frac{\lambda}{2N.A}$$

λ = wavelength of light, e.g. 550 nm (green)

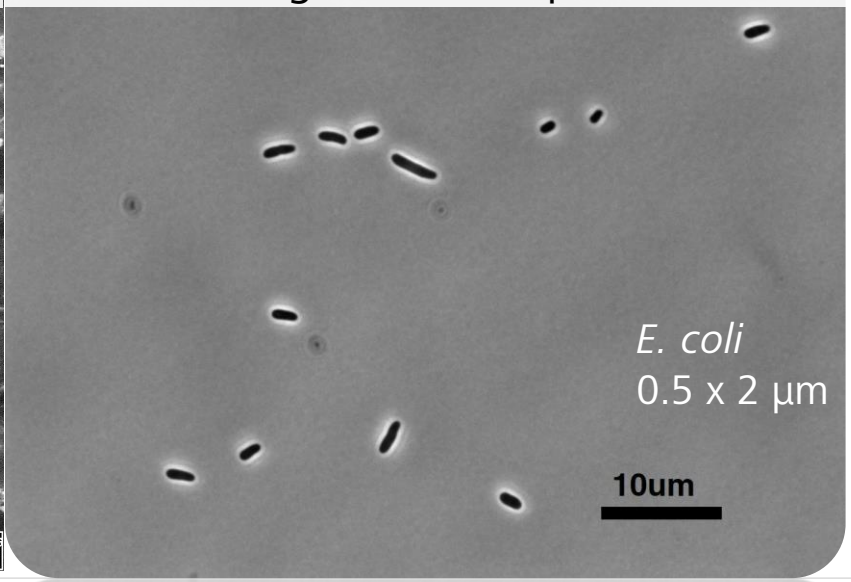


The resolution of light microscope **d_0= 200 ~ 300 nm**

Electron microscope



Light microscope



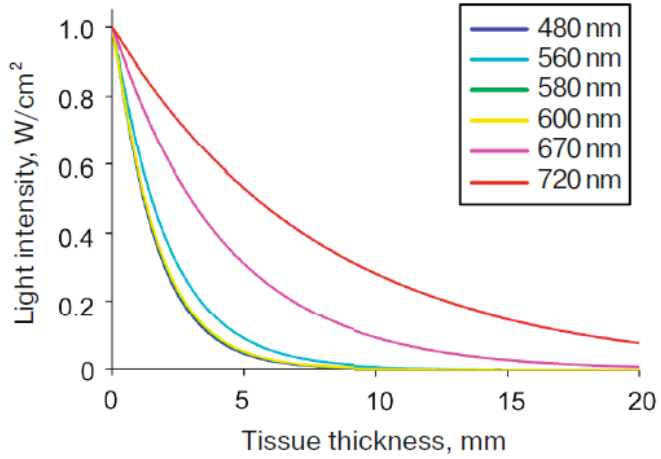
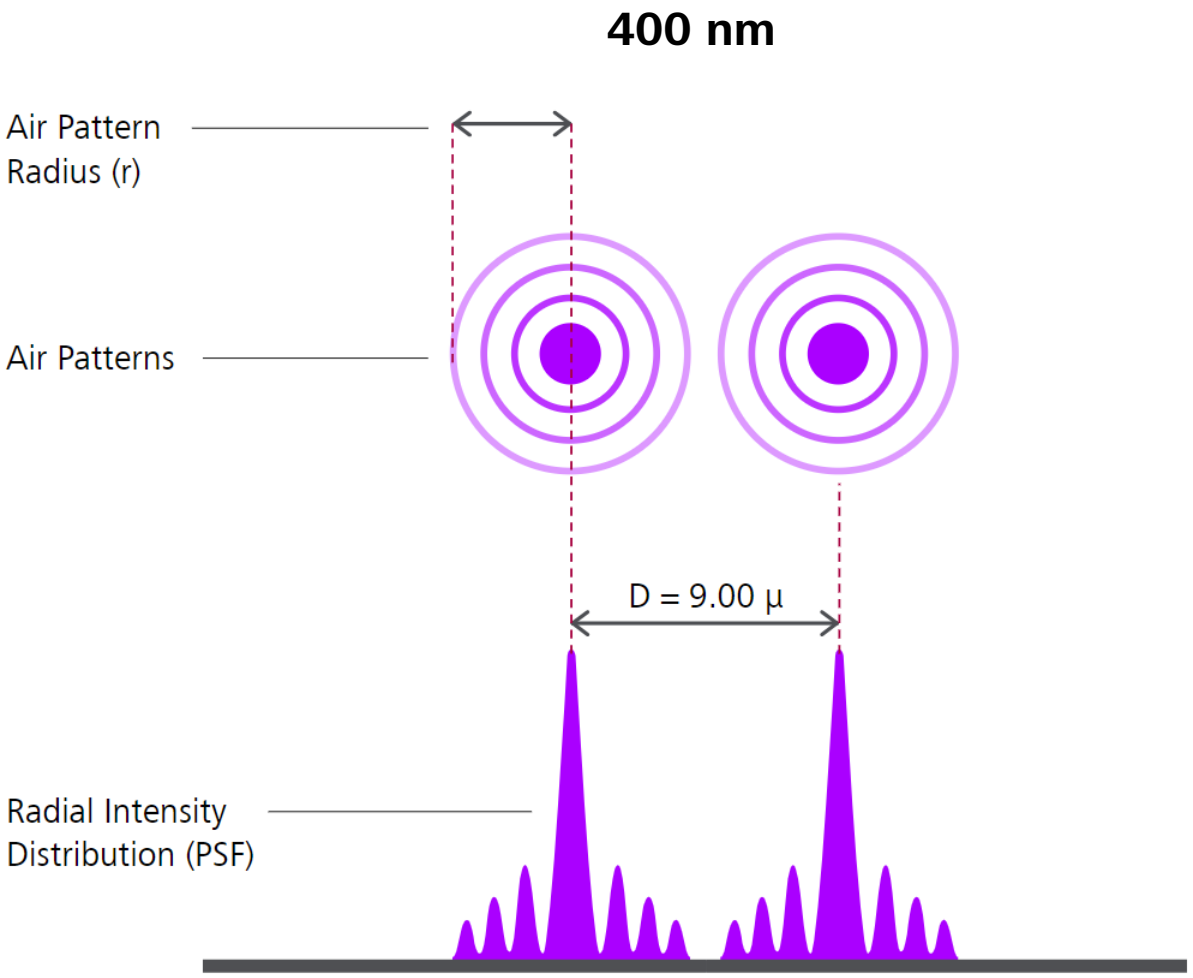
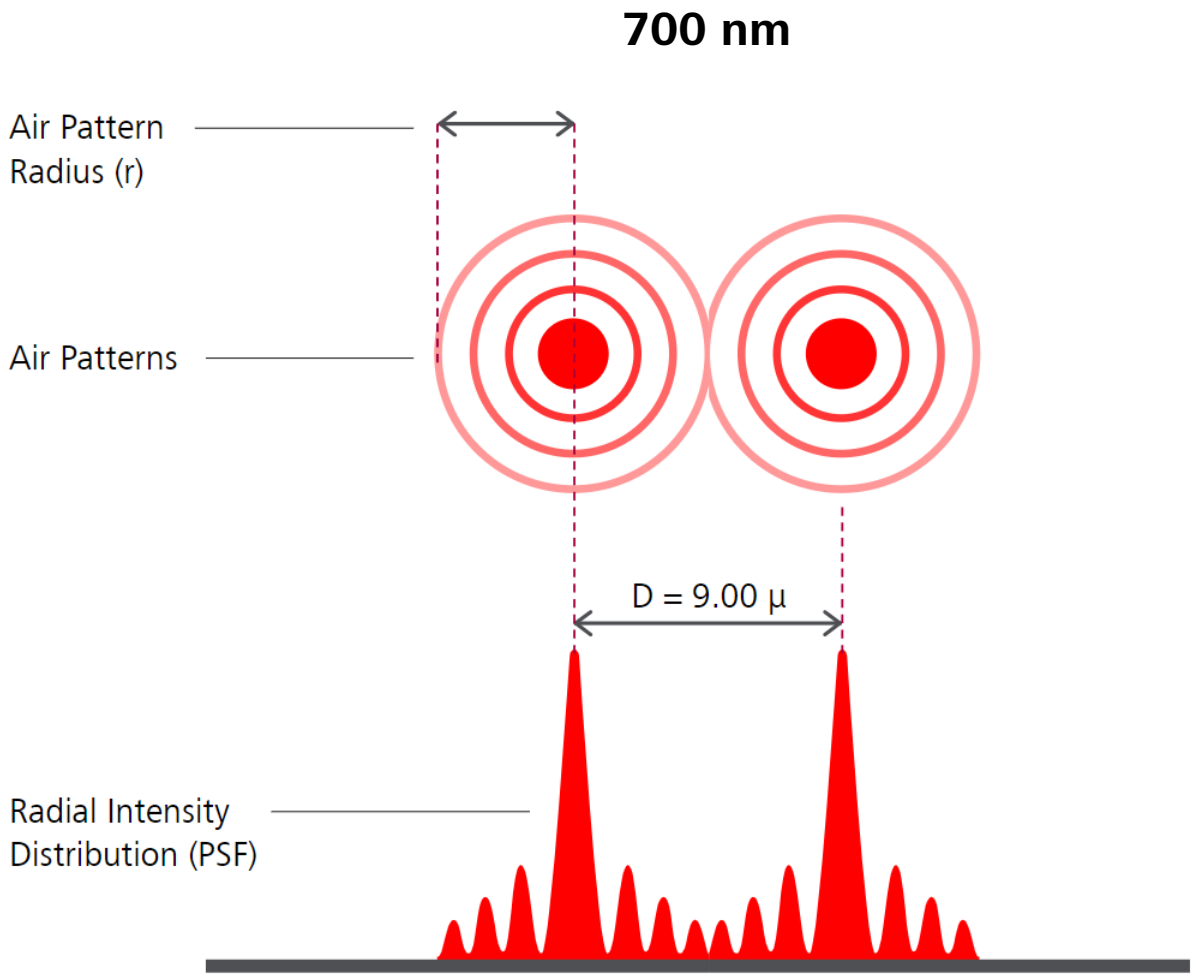


Image adapted from Karasev *et al.*, 2019

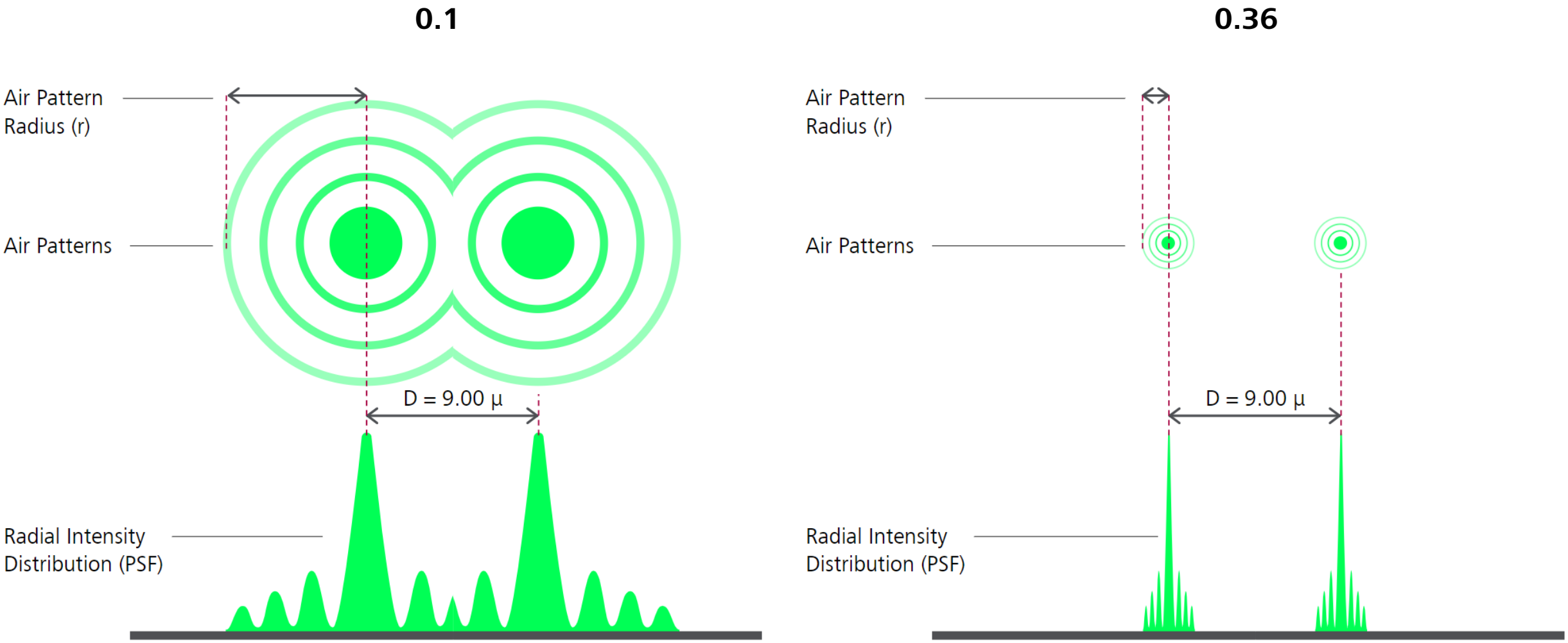
Longer wavelength being able to travel deeper into tissue

Resolution – Wavelength

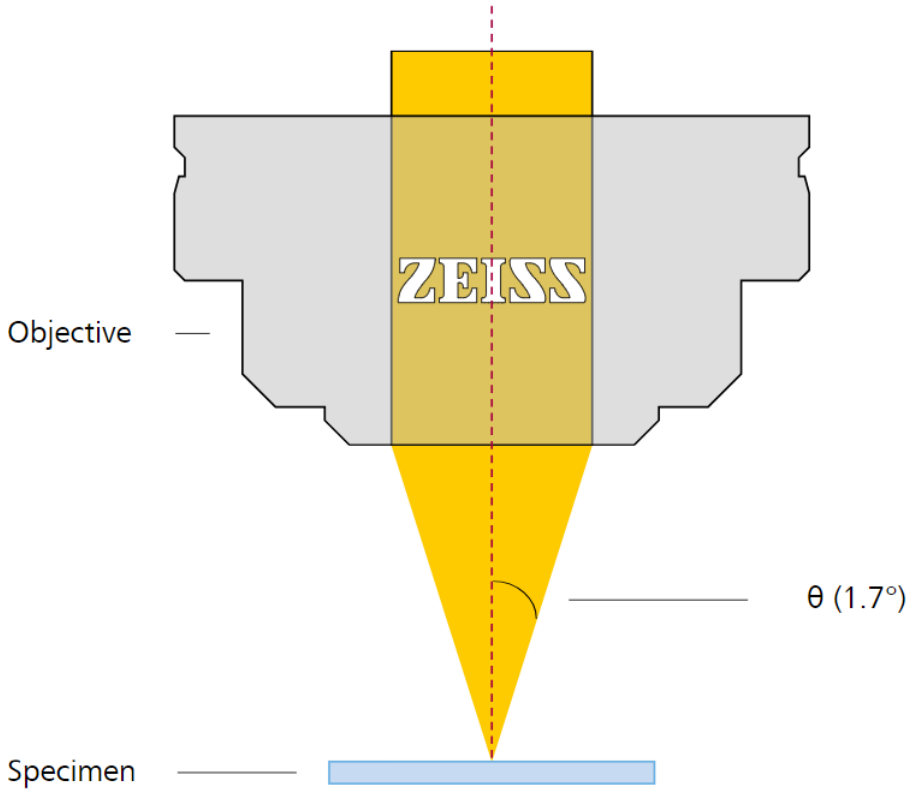




Resolution – N.A.



Resolution – N.A.



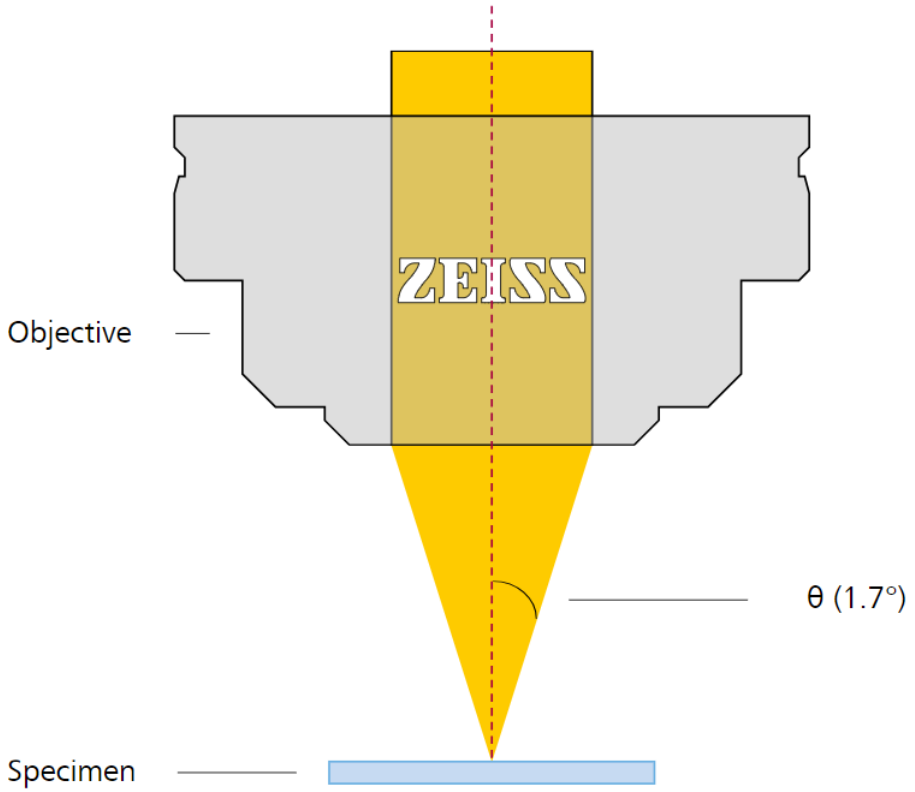
Numerical Aperture

Low ‹ › High

Numerical Aperture (NA) = n • sin θ

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

Resolution – N.A.

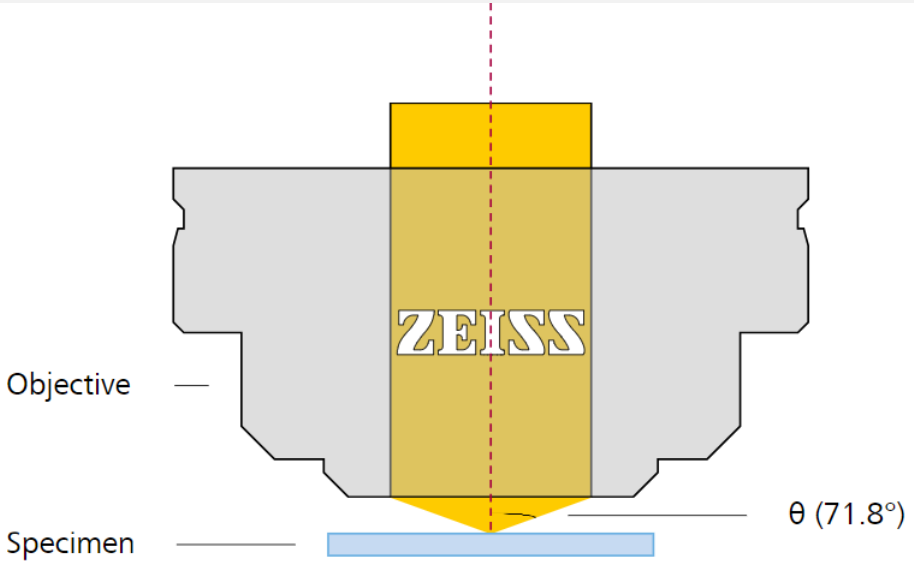


Numerical Aperture

Low ‹ › High

Higher NA offers

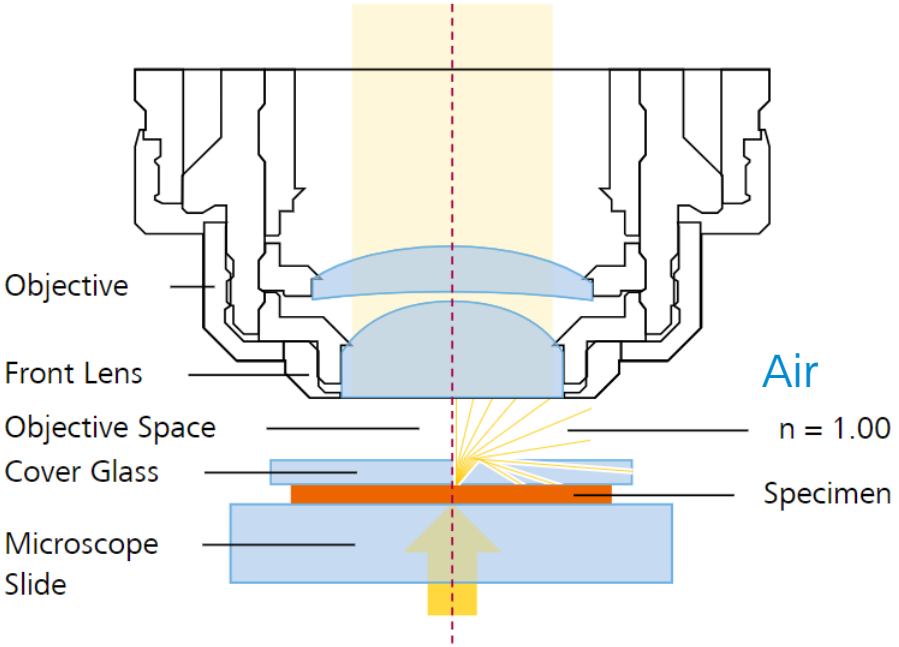
- 🙂 Better resolution & brighter image
- 🙁 Reduced working distance & sensitive to spherical aberration

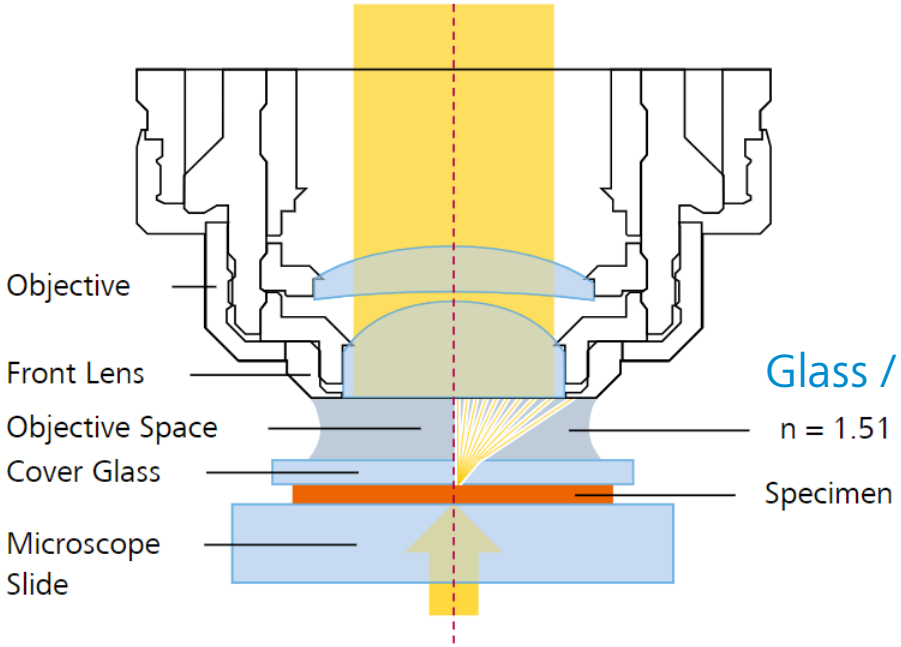


Numerical Aperture

Low ‹ › High

Immersion & Refractive Index





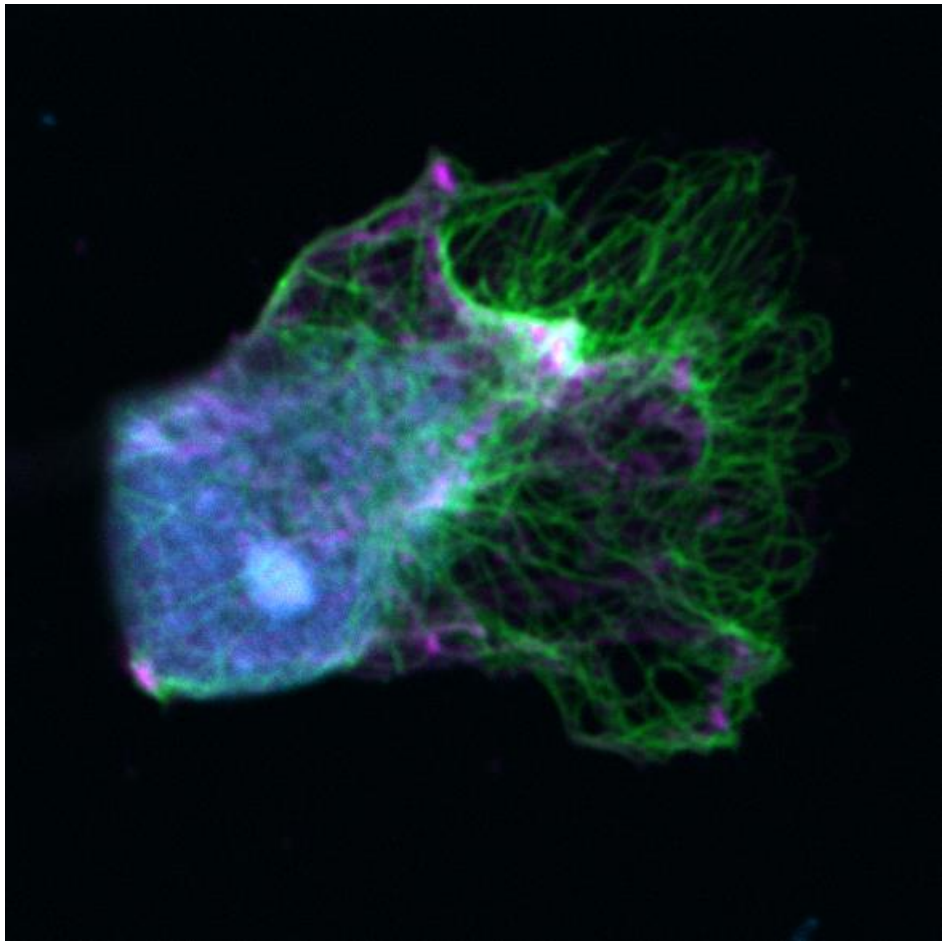
Refractive Index (n)

Low High

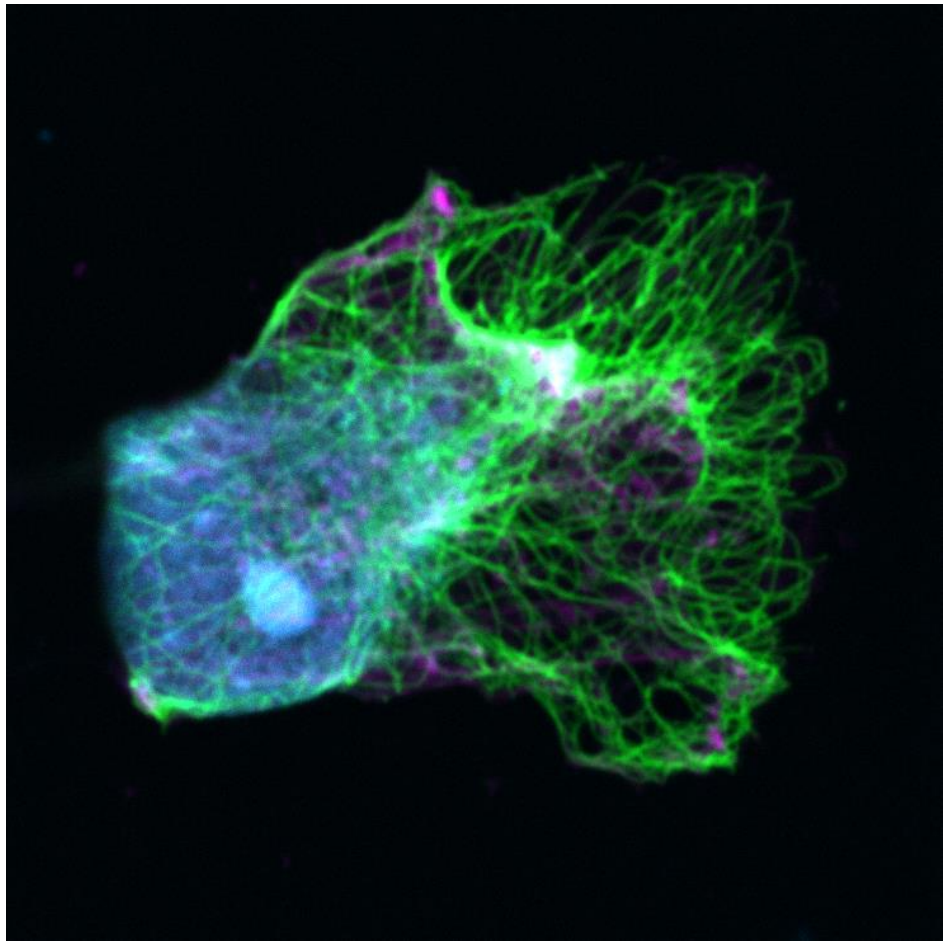
Refractive Index (n)

Low High

Higher NA + Immersion = Higher Resolution

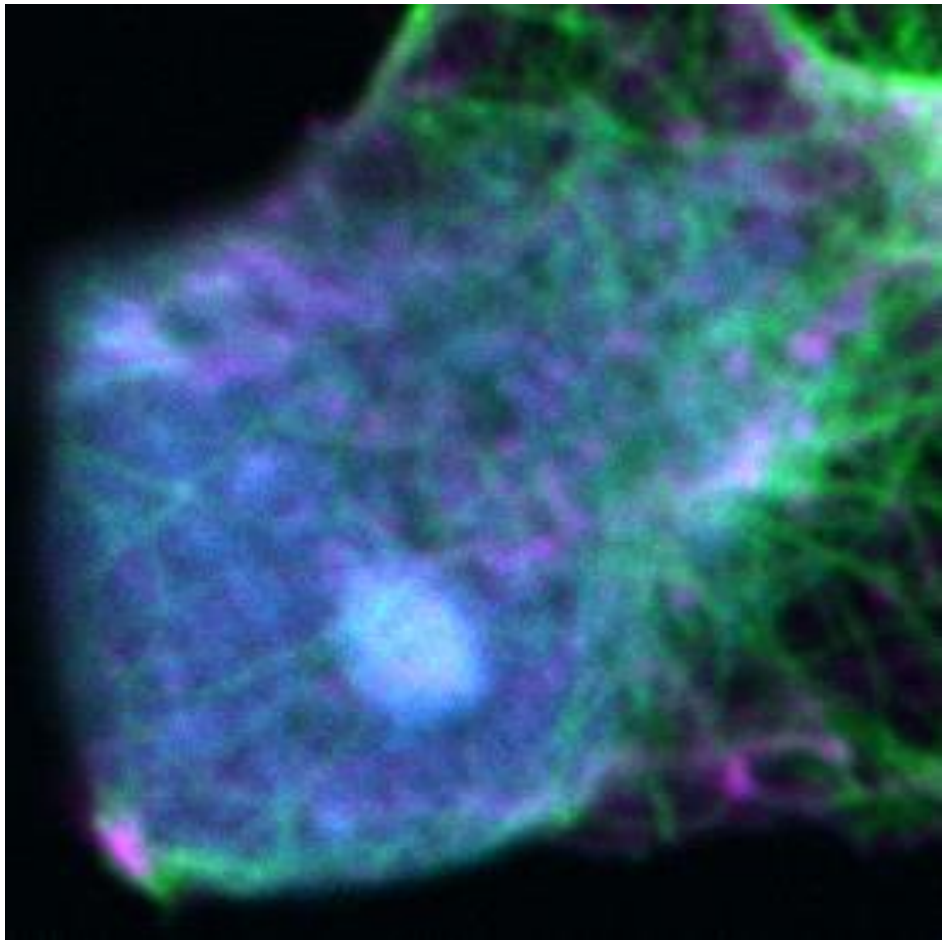


40x / 0.95 air

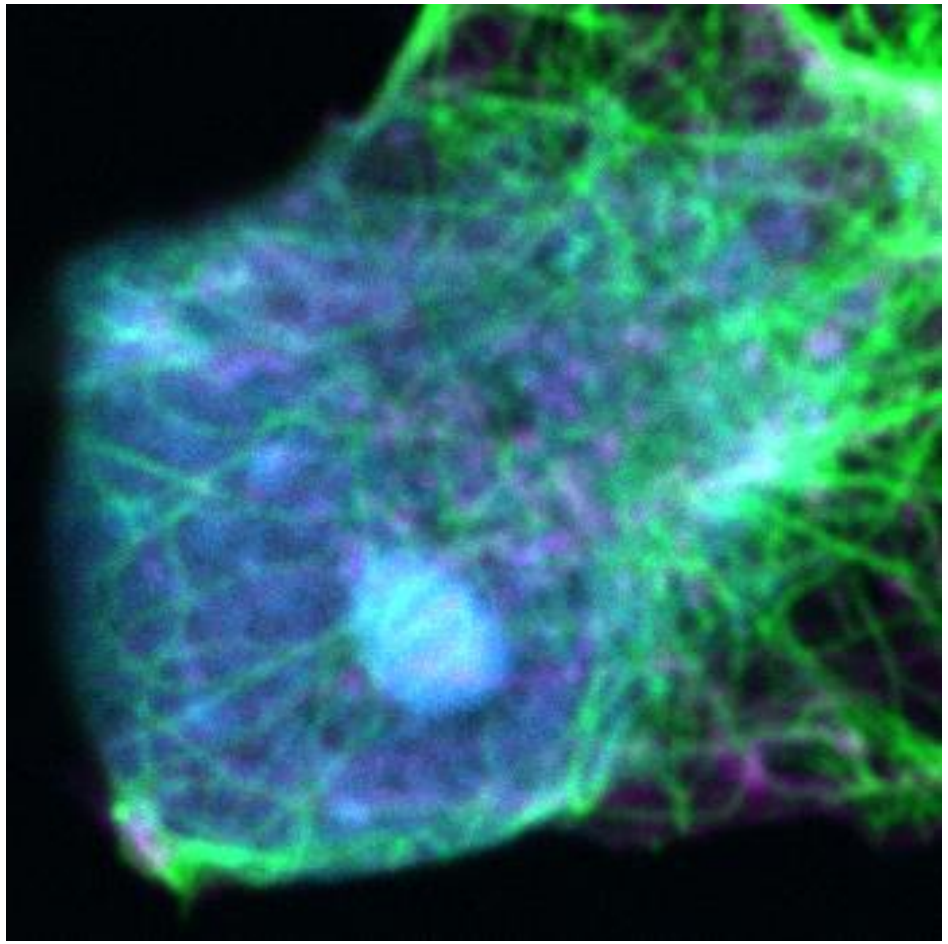


40x / 1.2 water

Higher NA + Immersion = Higher Resolution



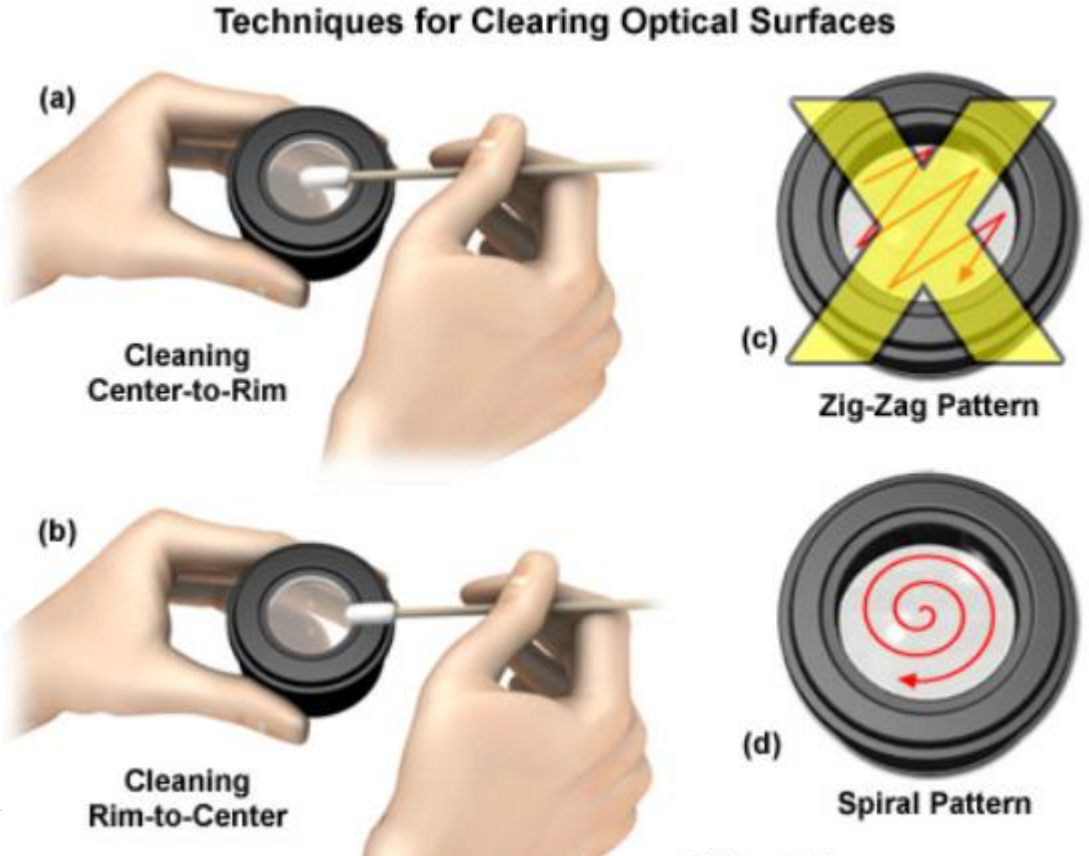
40x / 0.95 air



40x / 1.2 water

Immersion Objectives

Figure 4



Immersion & Refractive Index

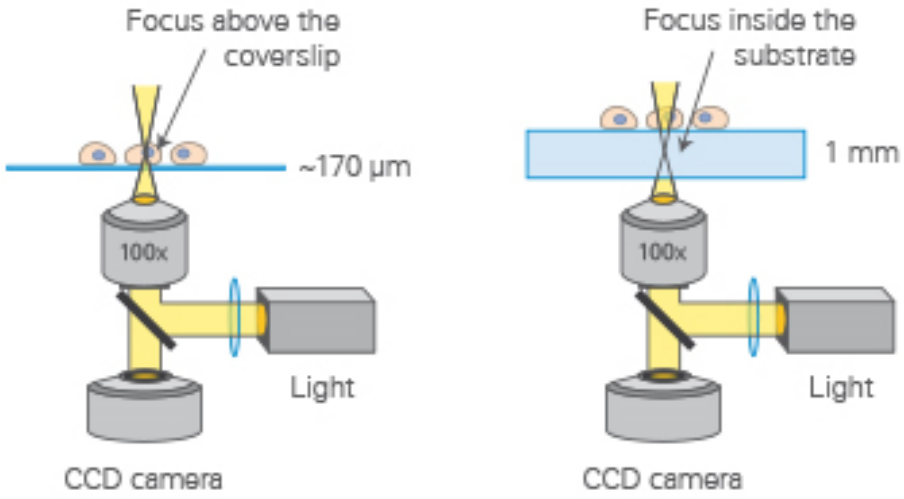
Sample objective only / might differ according to settings

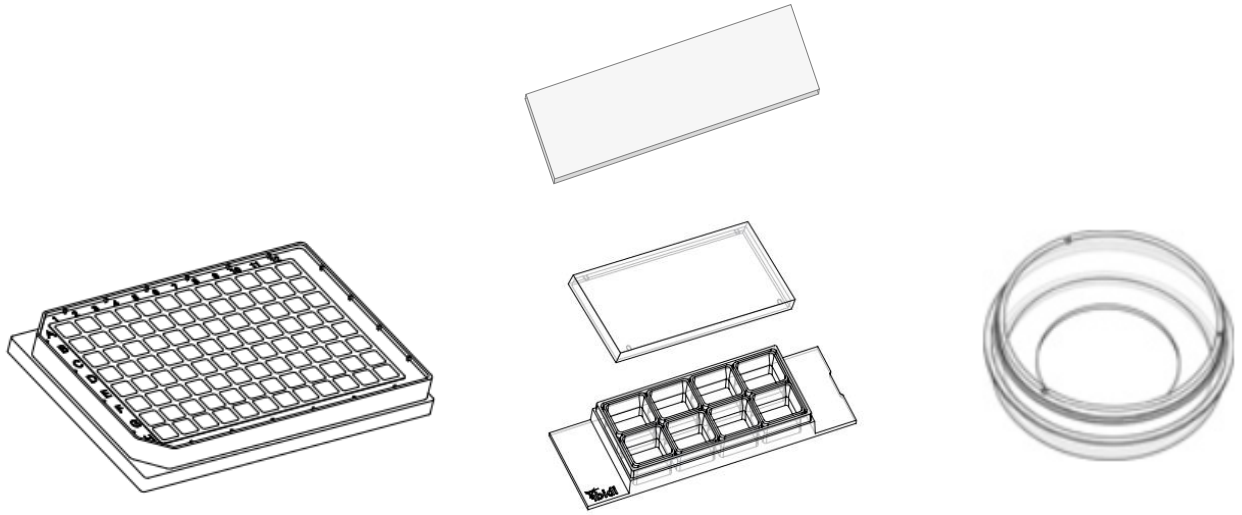
Mechanical Correction Collar

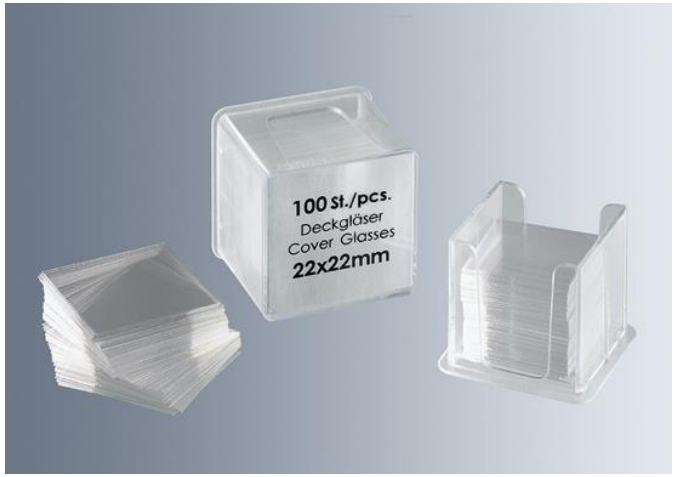
Cover glass thickness correction
Different Immersion (Oil, Glyc, Water)
Different Temperature
Adjusting an Iris Diaphragm

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







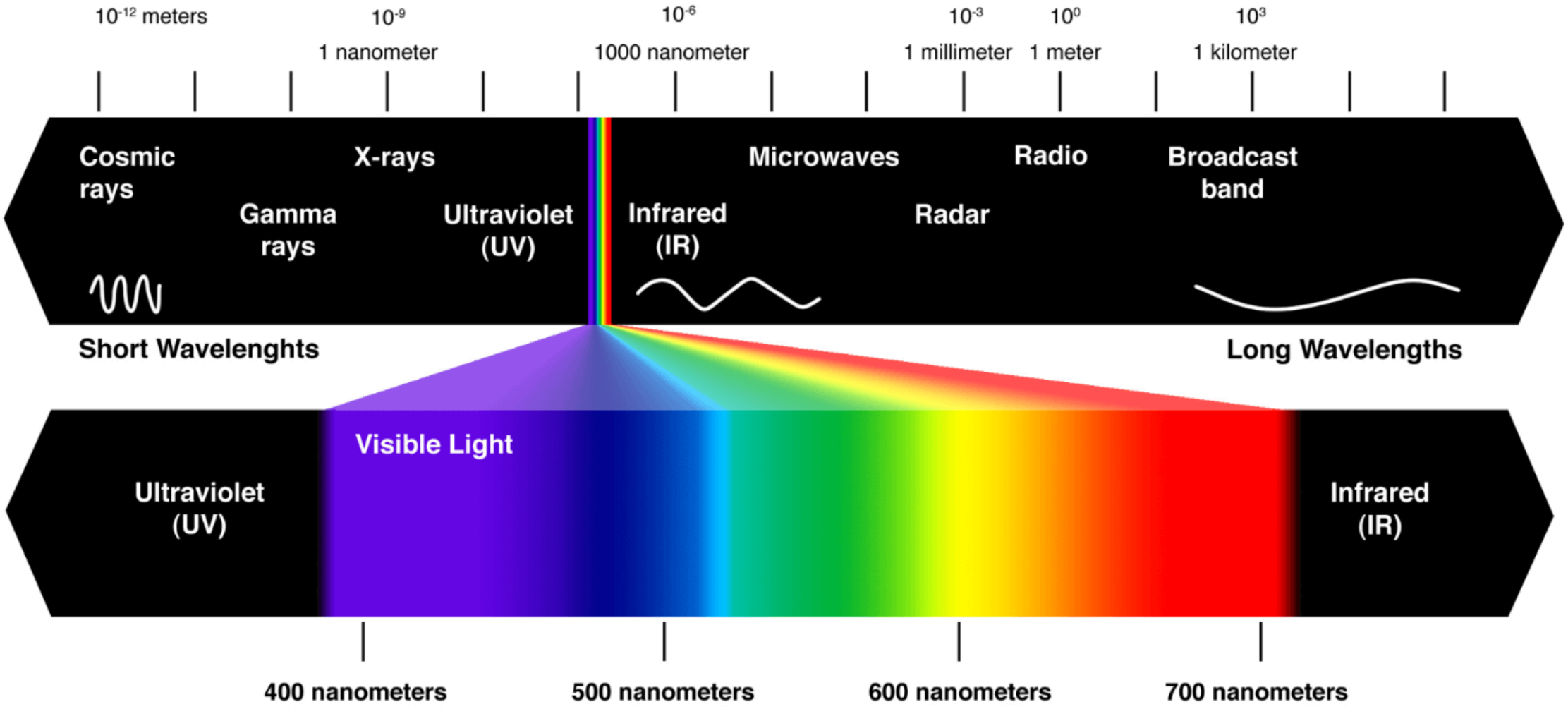
Thickness no. 1 (0.13-0.16 mm)

Thickness no. 1.5 (0.16-0.19 mm)

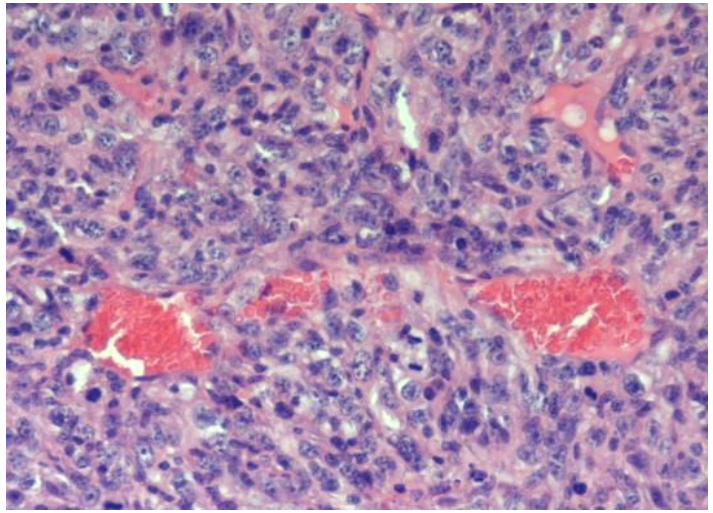
Thickness no. 1.5H (0.165-0.175 mm)

Contrast Methods

Sample Carrier Thickness



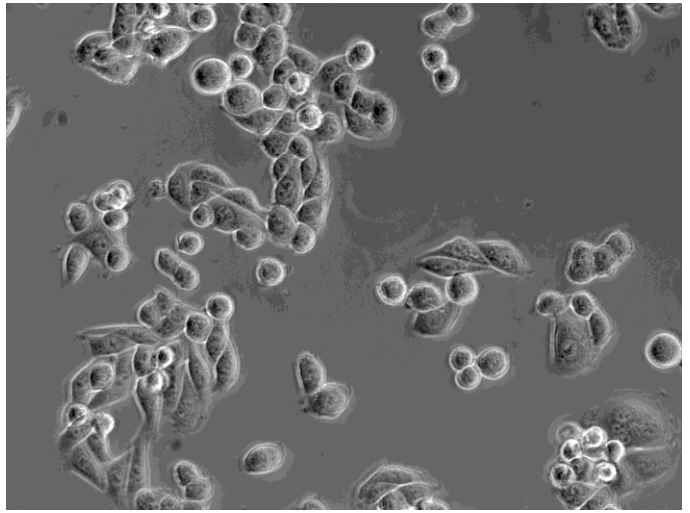
Contrast Methods of Transmitted Light



Brightfield

Colorful samples

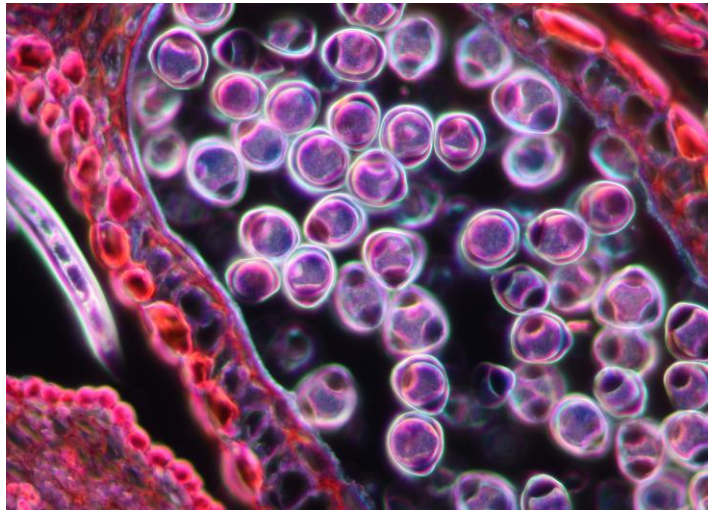
Widefield microscopes



Phase contrast

Colorless samples

Widefield microscopes



Dark field

Translucent samples

Widefield microscopes

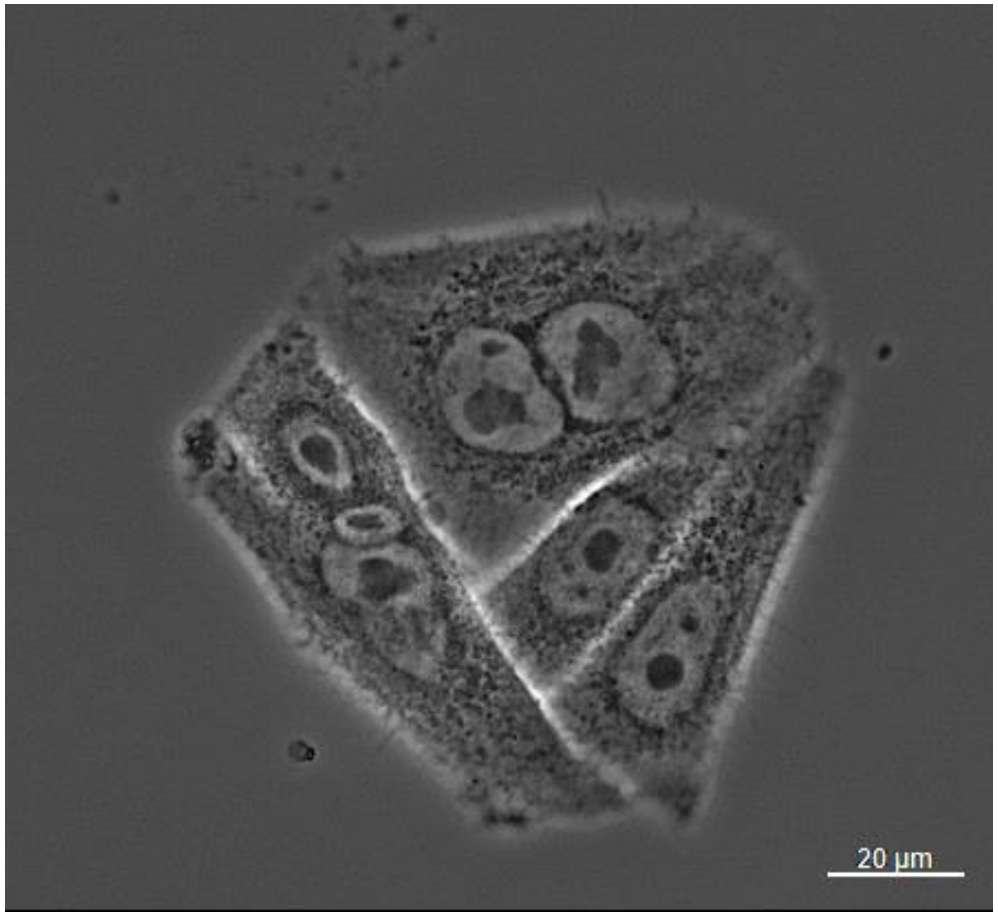
DIC (Differential Interference Contrast)

Colorless samples

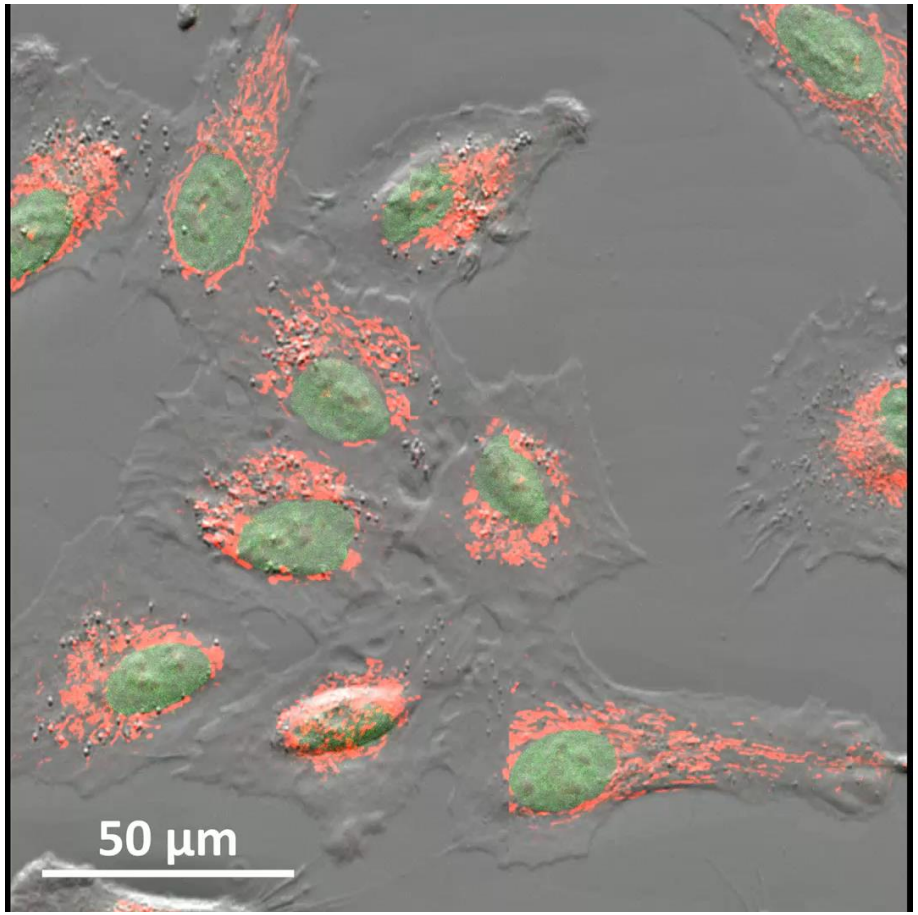
Widefield / confocal microscopes

Phase Contrast vs DIC

Ph (Phase Contrast)



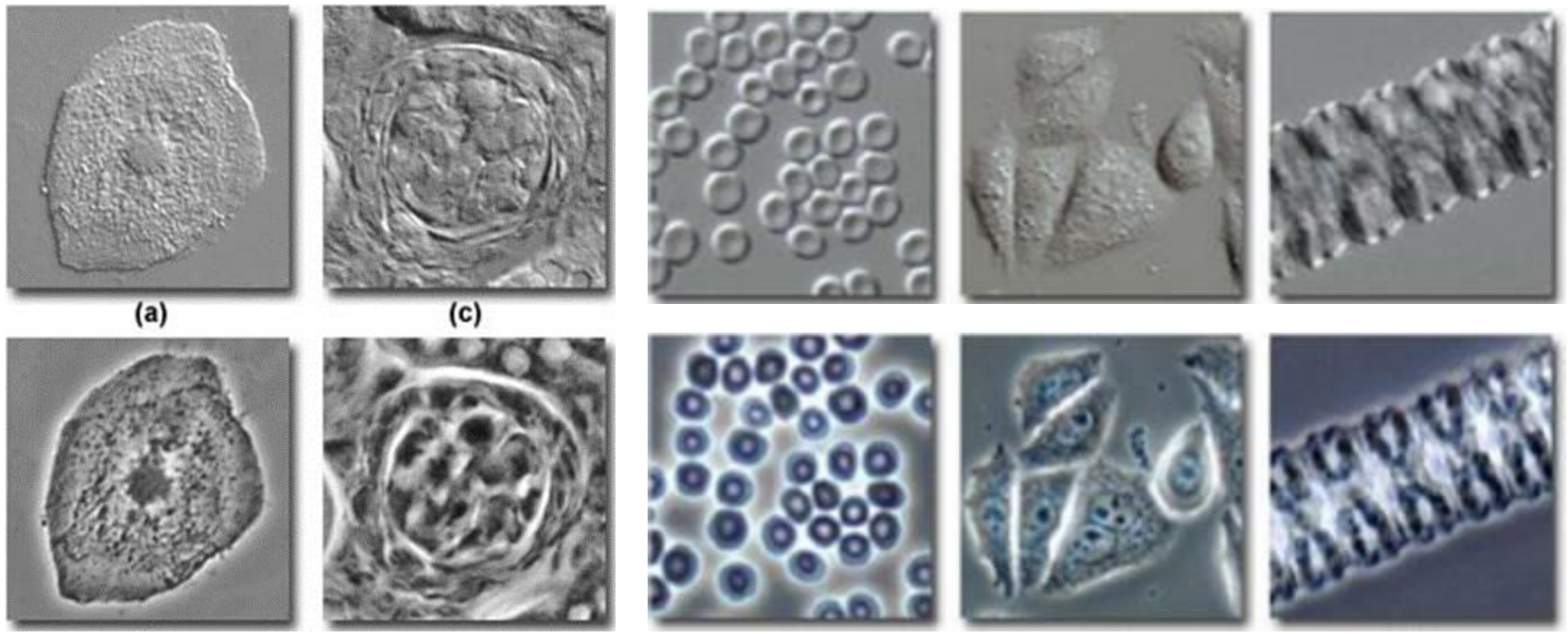
DIC (Differential Interference Contrast)



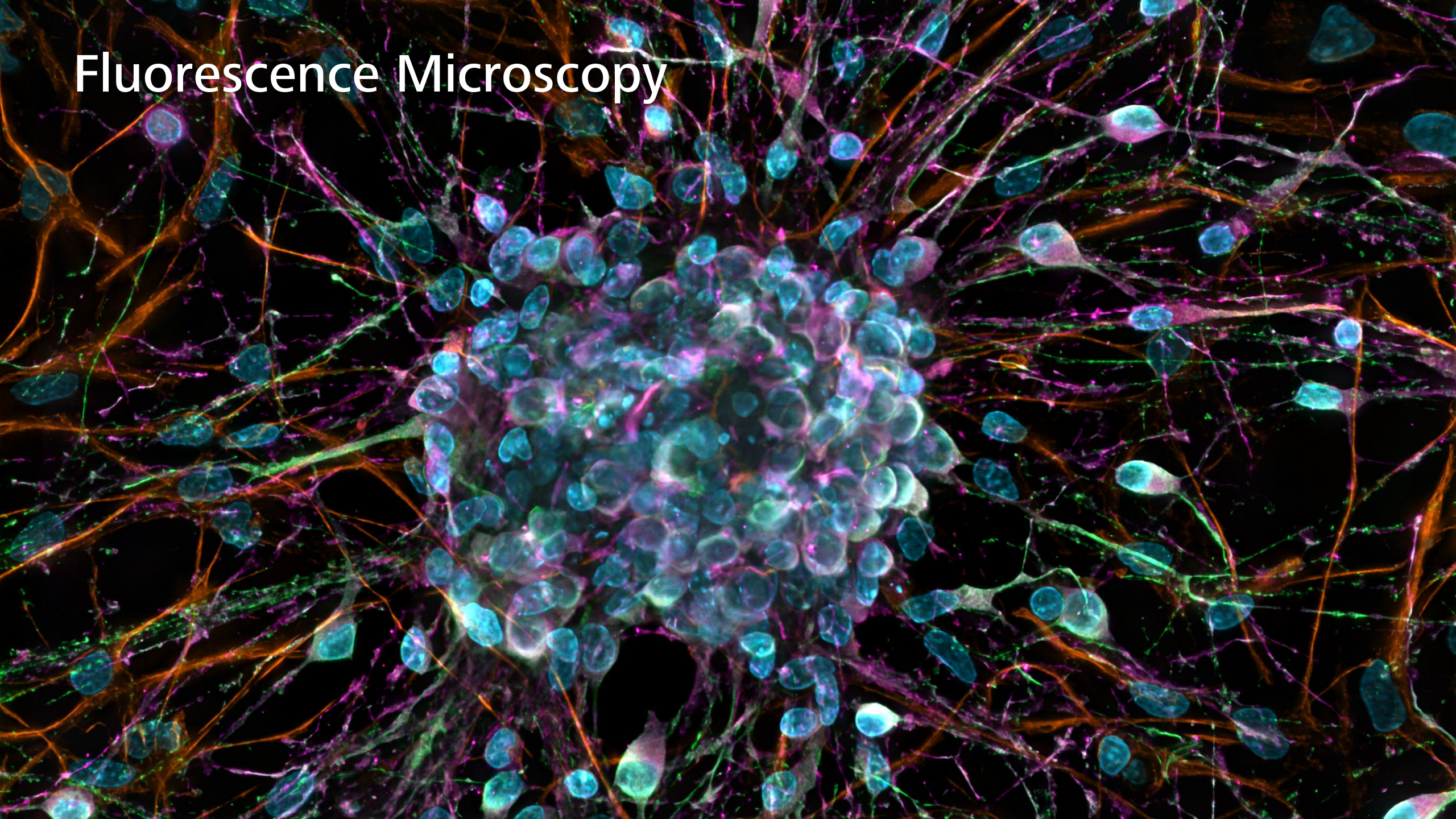
Phase Contrast vs DIC

DIC

Phase



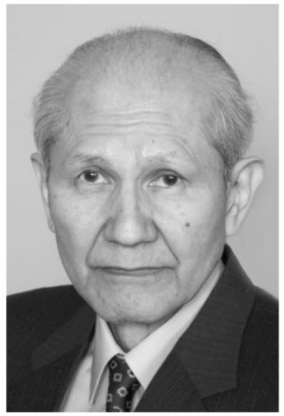
Fluorescence Microscopy



Fluorescence Contrast (FL)

- Specific, precision to molecule level
- Multiple staining
- High resolution
- 4D imaging
- Fluorescence bleaching ☹
- Gene transfection, fluorescent dyes
- Fluorescence filters
- Fluorescent light sources

The Nobel Prize in Chemistry 2008



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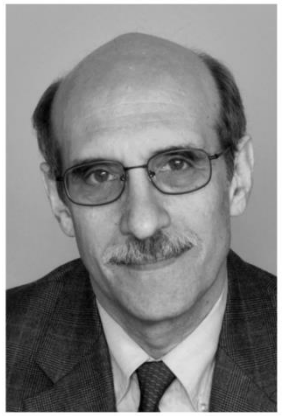
Osamu Shimomura



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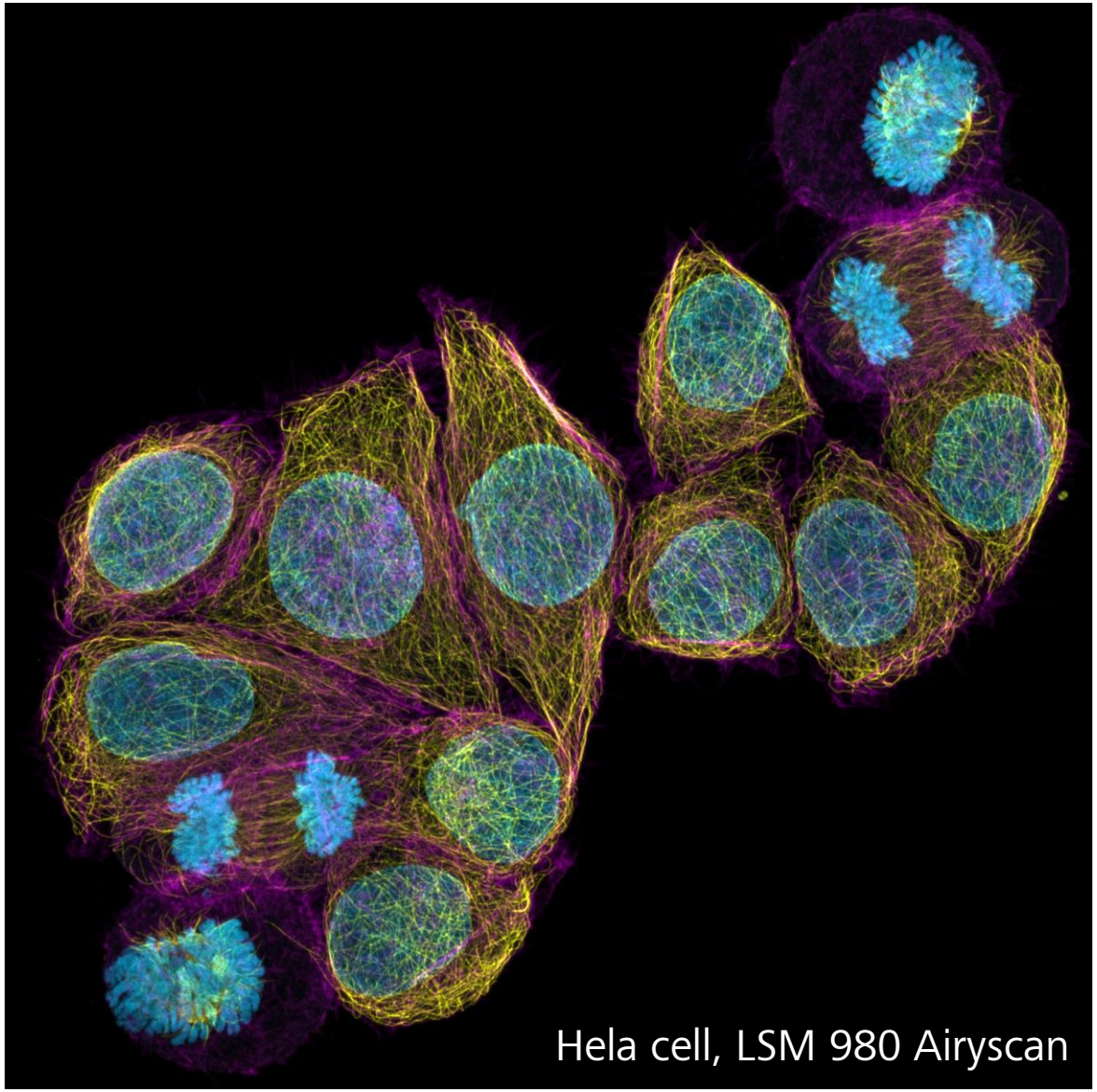
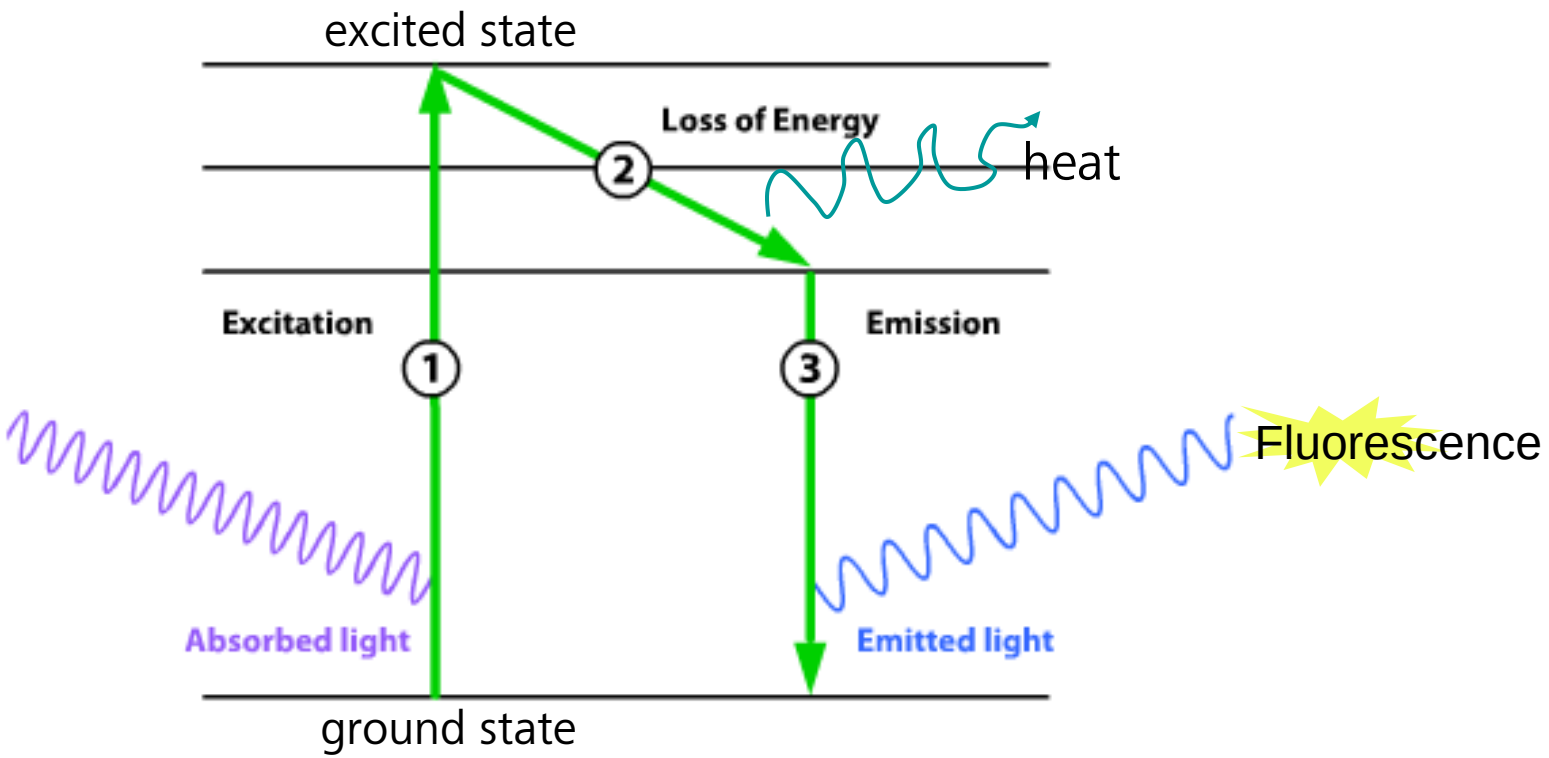
Martin Chalfie

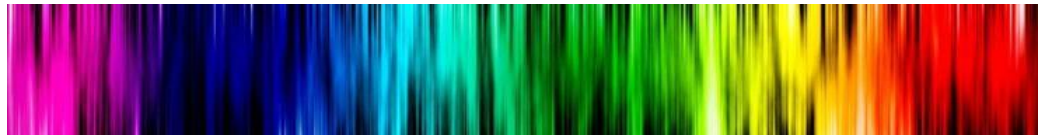
© The Nobel Foundation. Photo: U. Montan
Roger Y. Tsien



Hela cell, LSM 980 Airyscan

Fluorescence Contrast (FL)

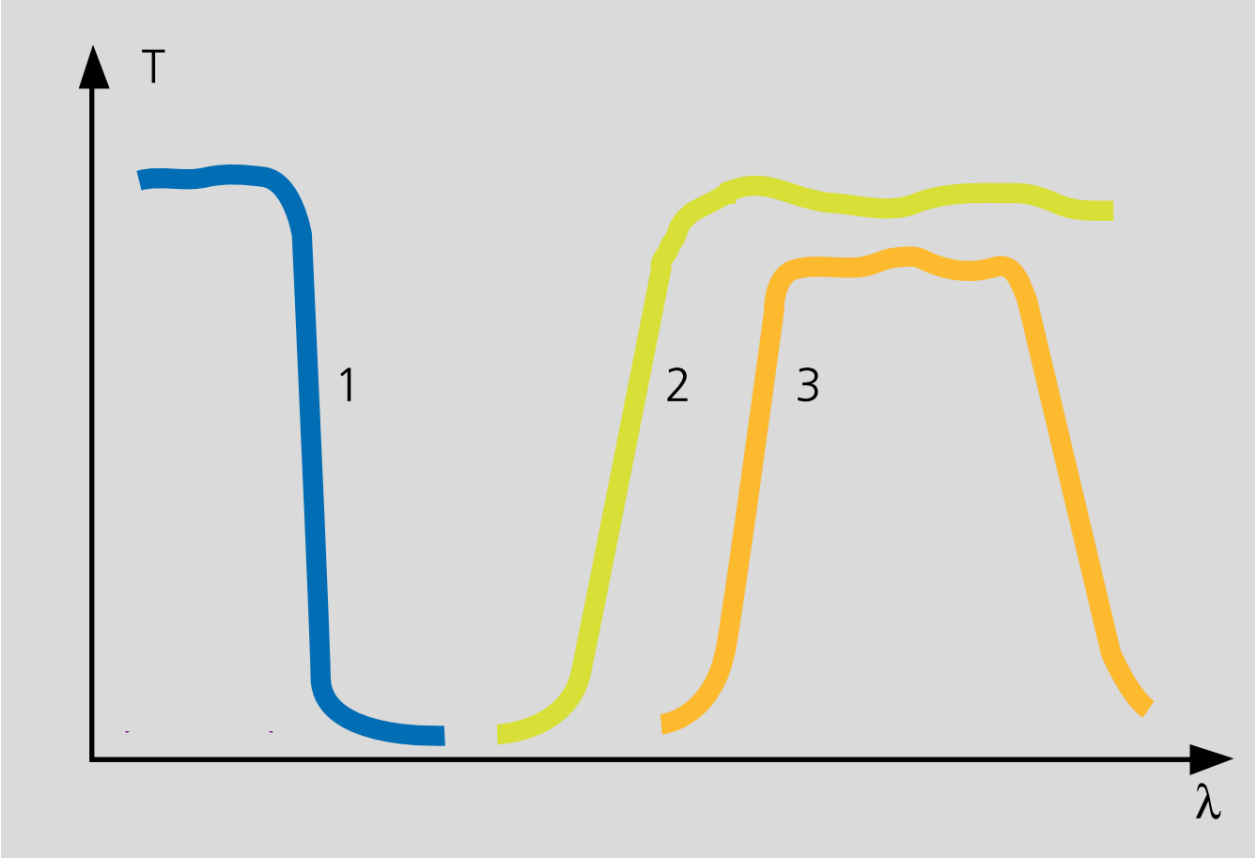




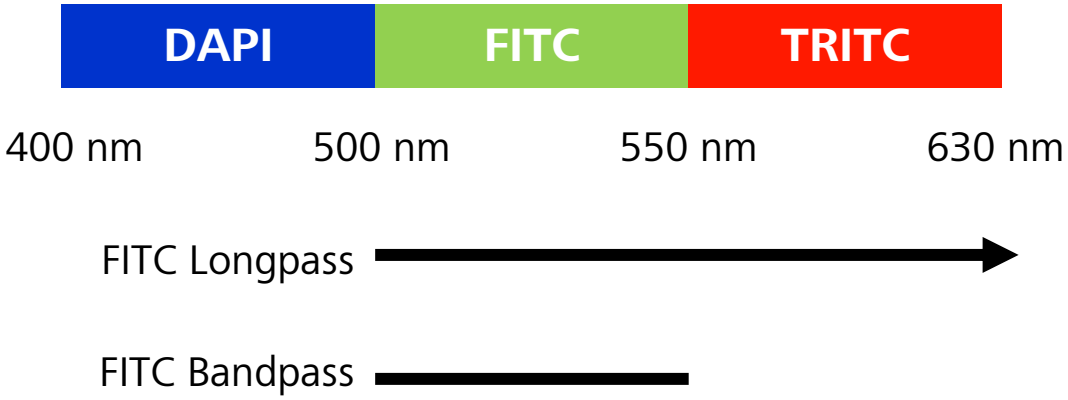
The Filter Sets for Fluorescence Microscopy

Light Path of Fluorescence Microscopy

Fluorescence Filters

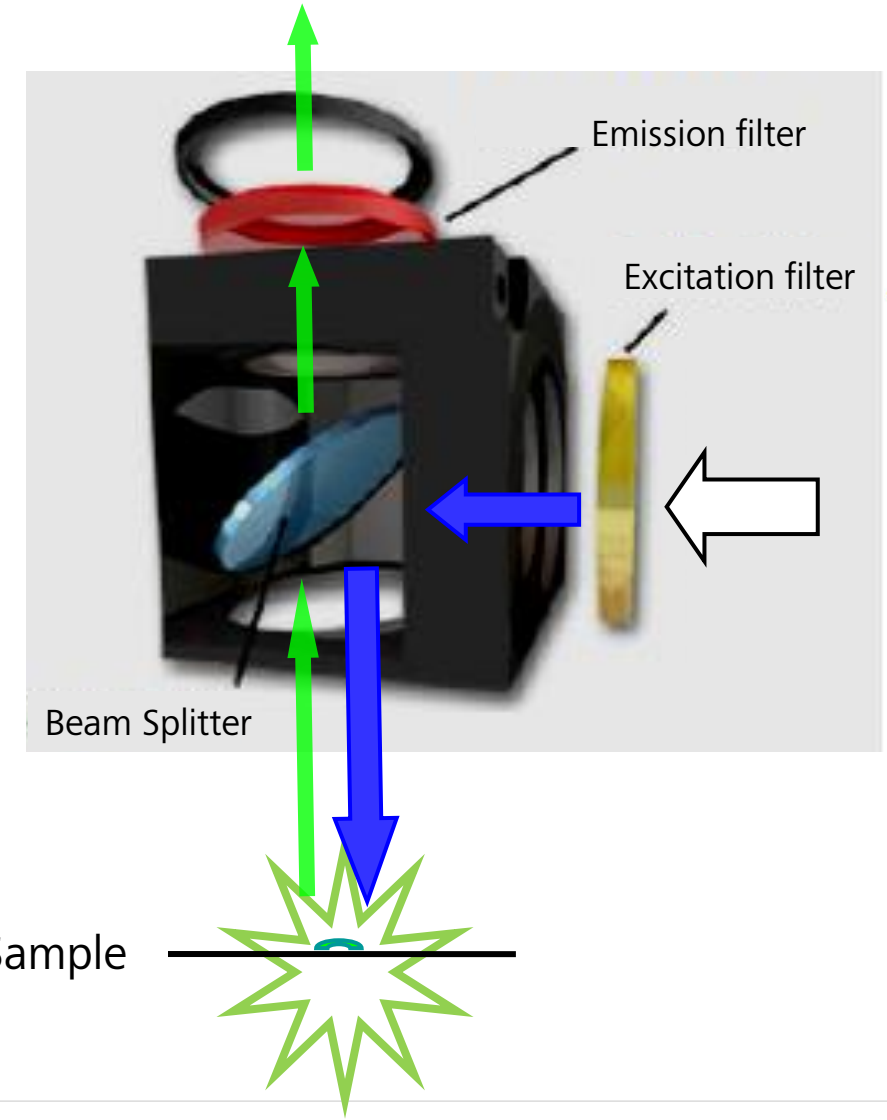


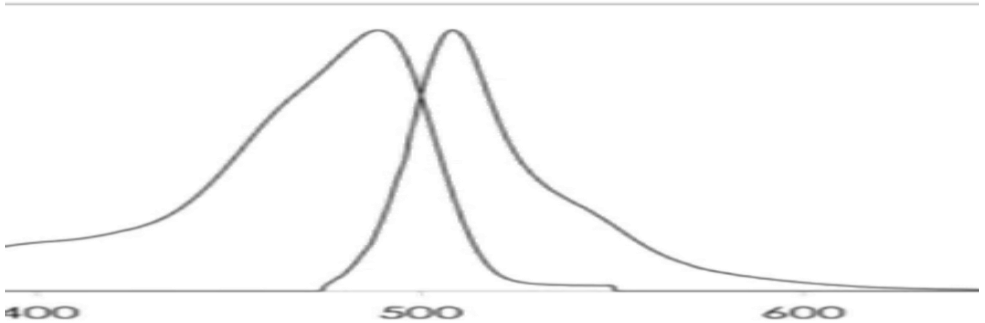
1. Shortpass filter
2. Longpass filter
3. Bandpass filter



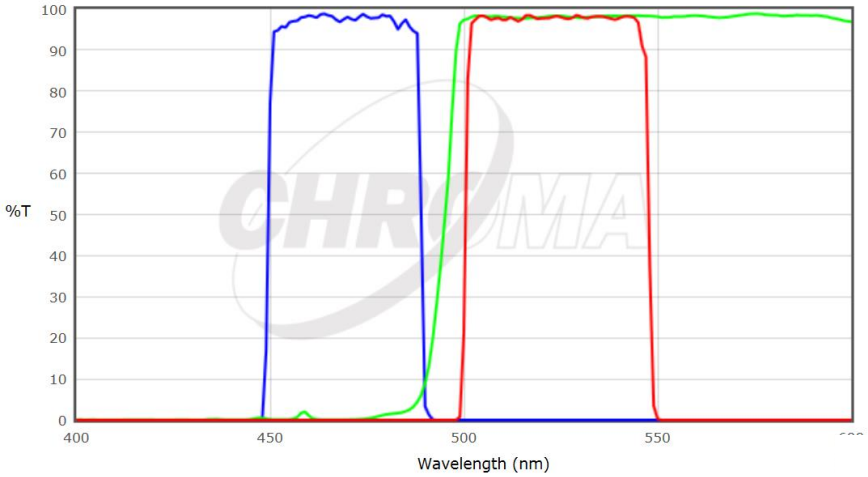
Fluorescence Filter

Fluorescence filter cube





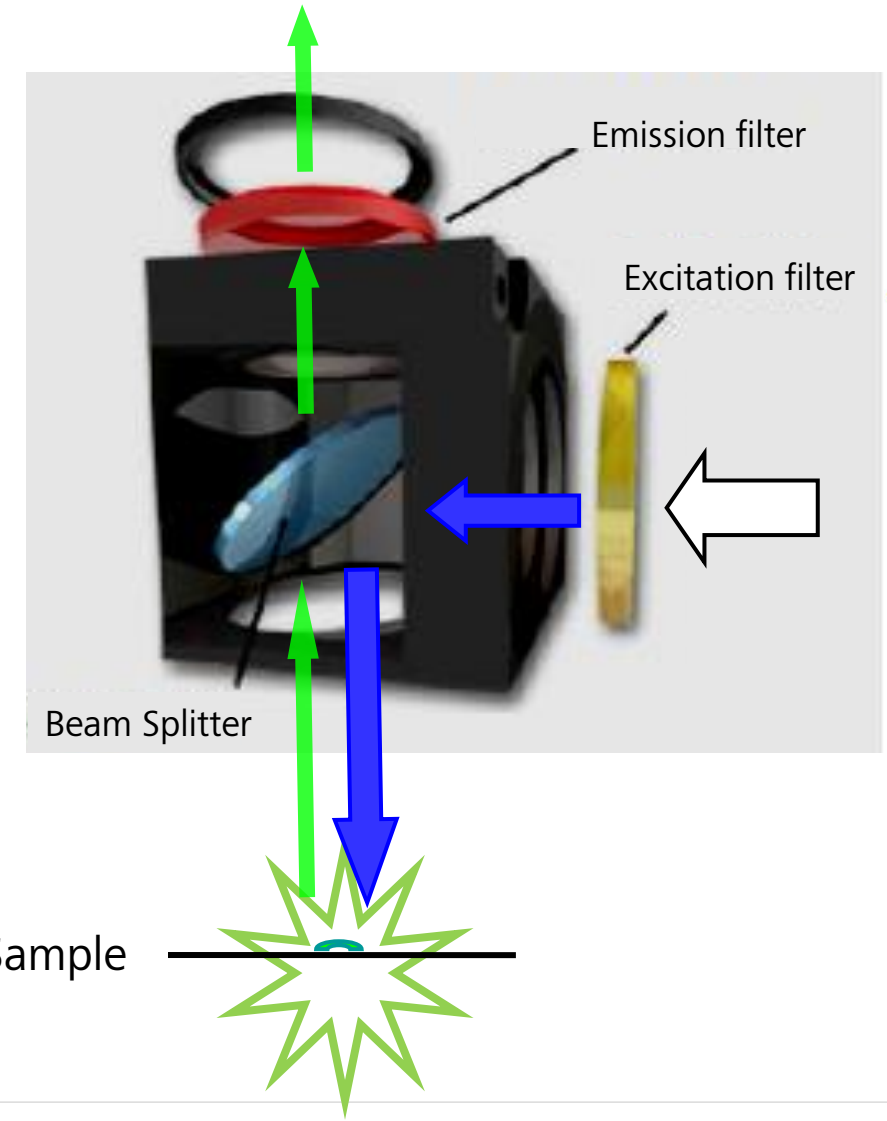
EGFP (Ex. 488 nm, Em. 507 nm)



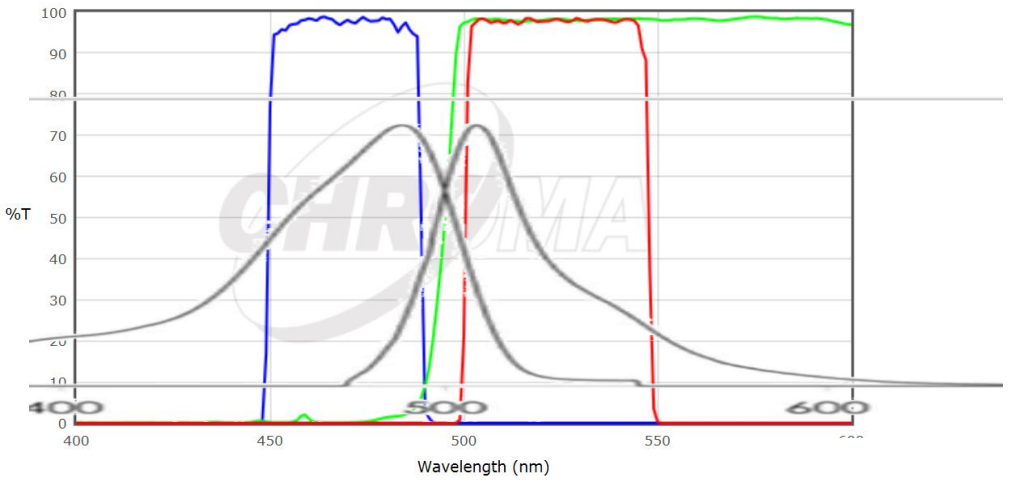
FILTERS	TYPE
ET470/40x	EX
T495lpxr	BS
ET525/50m	EM

Fluorescence Filter

Fluorescence filter cube



EGFP (Ex. 488 nm, Em. 507 nm)



FILTERS	TYPE
ET470/40x	EX
T495lpxr	BS
ET525/50m	EM

Spectra Viewer

Fluorochromes | Sets | Parts — Remove All

Current selection (0 of 5)

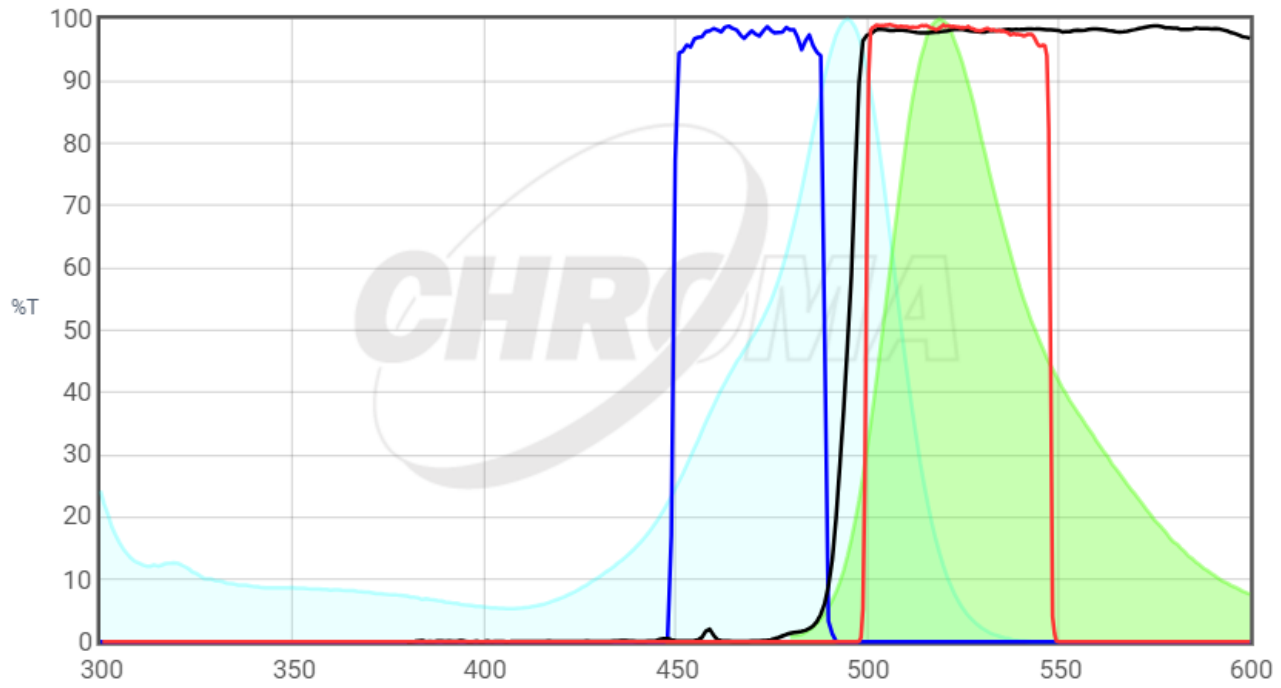
Search

NUMBER - NAME

- 11014v2 Narrow Blue
- 19000 AT – UV/DAPI Longpass
- 19001 AT - Aqua Longpass
- 19002 AT - GFP/FITC Longpass

Reload Plot

%T OD 300 to 600 update reset



Fluorochromes

FITC *(collapse)* — Remove | Add Compatible Sets

PLOT	FILTERS	TYPE	%T-OD	AOI	DATA
☑	FITC Excitation Spectra	EX	12.32 T/317nm	0°	ASCII
☑	FITC Emission Spectra	EM	0.00 T/317nm	0°	ASCII

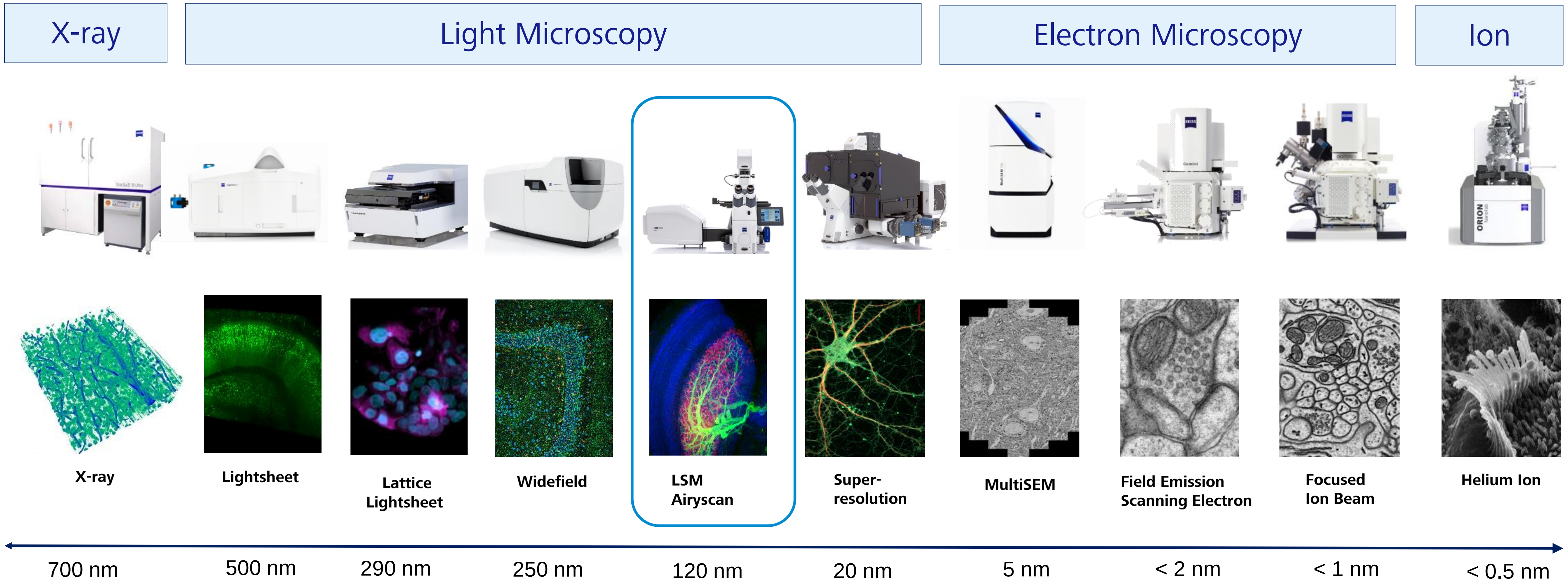
Catalog Sets

49002 *(collapse)* — Remove | Add to cart

PLOT	FILTERS	TYPE	%T-OD	AOI	DATA
☑	ET470/40x	EX	0.00 T/317nm	0°	ASCII
☑	T495lpxr	BS	0.00 T/317nm	45°	ASCII
☑	ET525/50m	EM	0.00 T/317nm	0°	ASCII

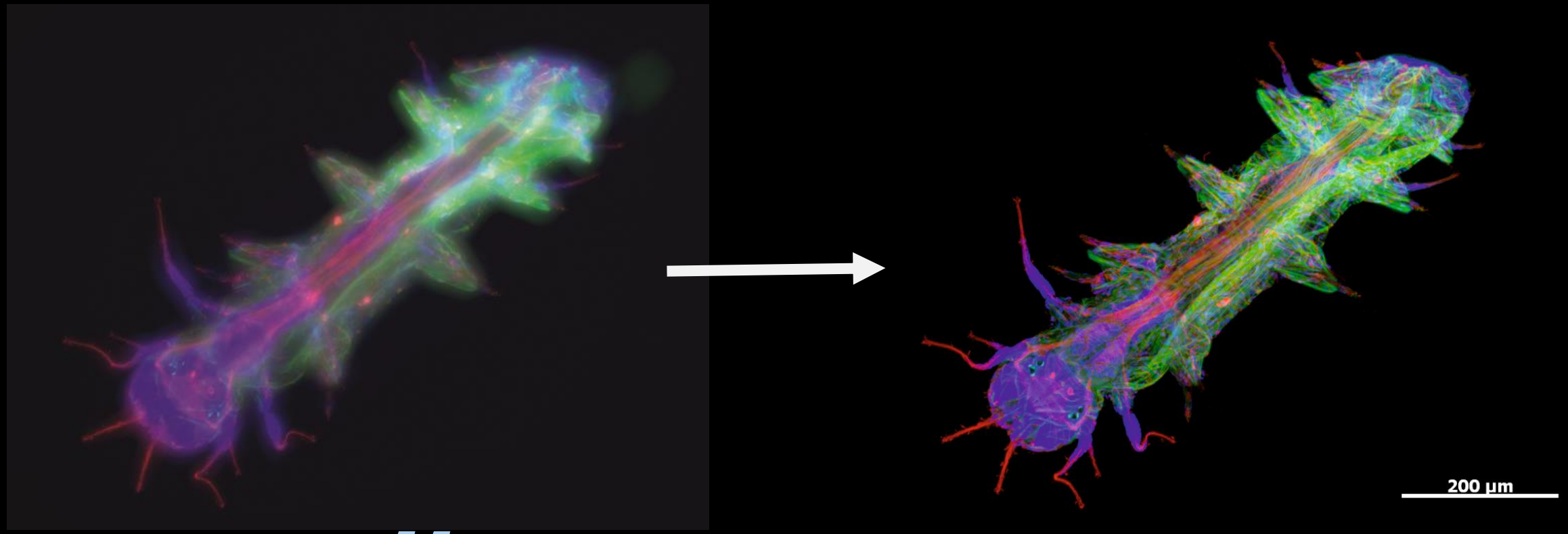
All transmission and blocking (OD) data are actual, measured spectra of representative production lots. Spectra varies slightly from lot to lot. Optical density values in excess of 6 may appear noisy because such evaluations push the resolution limit of low light level measurements.

Keep the Context of Your Experiments



ZEISS LSM Confocal

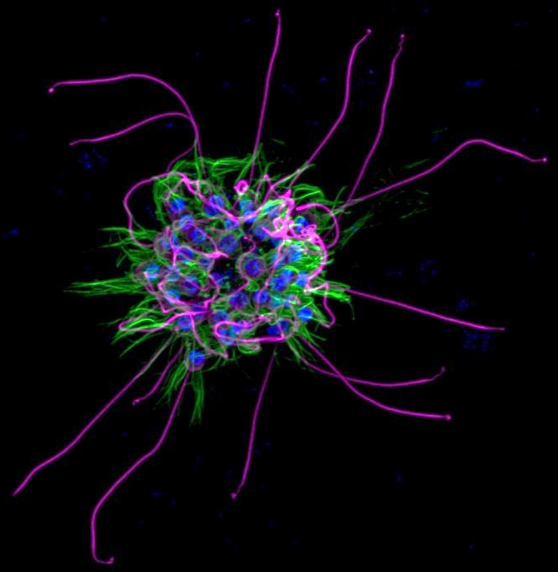
Optical Sectioning | Extract the Layer of the Image

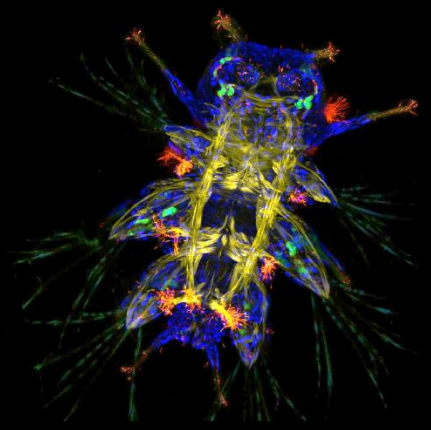


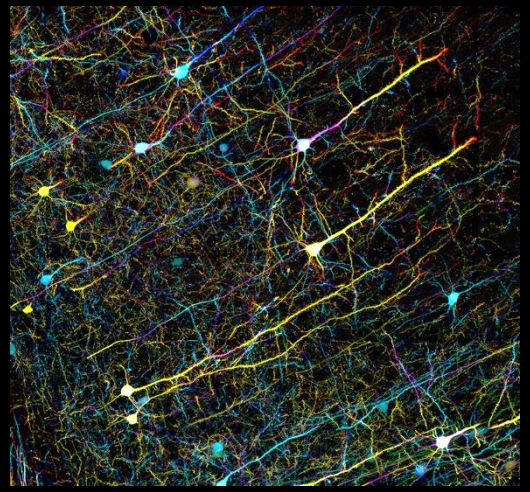
"We want focused image!"

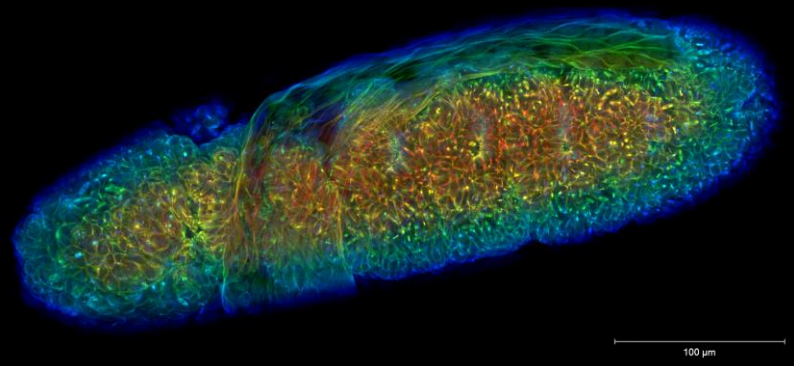
V. Wilkens, University of Osnabrueck, Germany., Optical Sectioning

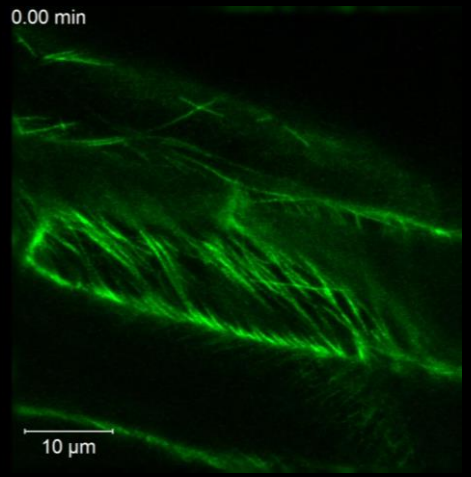
Confocal microscopy allows you to optically section thick samples

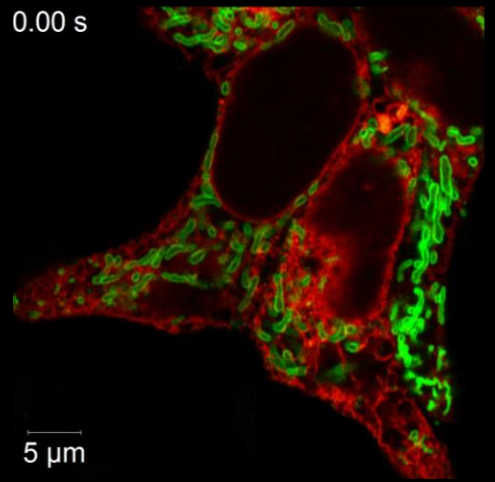


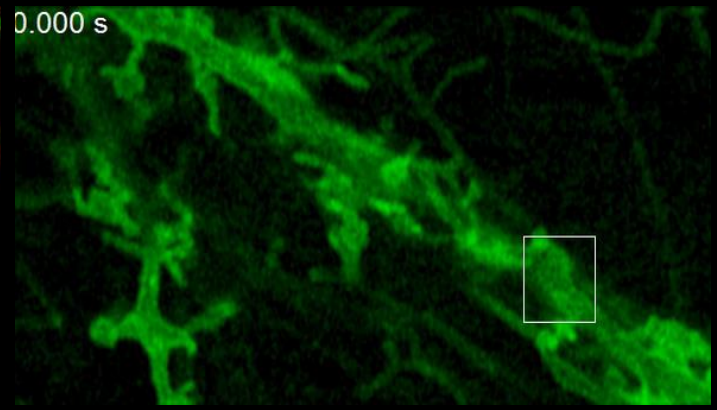






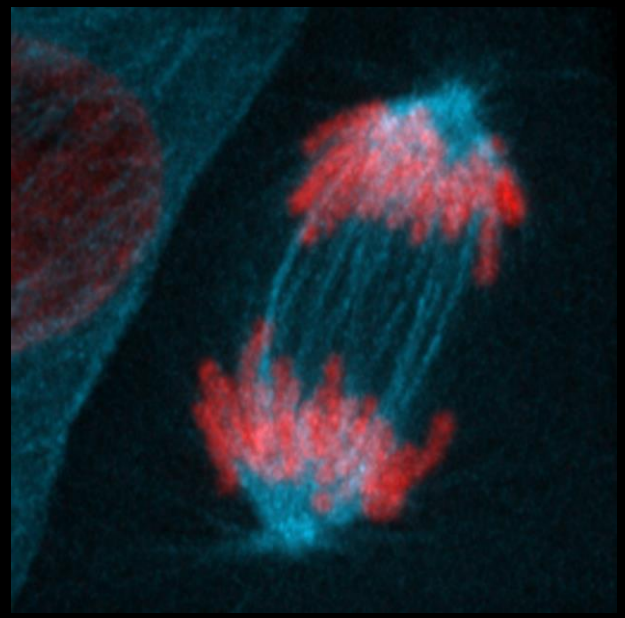




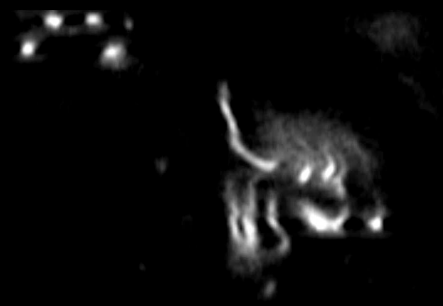


Holly Aaron (UC Berkeley); N. Kenny, K. McClelland, S. Miller (U of Oxford, U of Queensland, U of Cambridge), D. Reiff (U of Freiburg); Y. Zuo, A. Aharon, A. Schnulz (U of California Santa Cruz); Courtesy of Balazs Erdi, Max F. Perutz (Vienna Biocenter, Austria); Jason D Vevea (University of Wisconsin-Madison,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

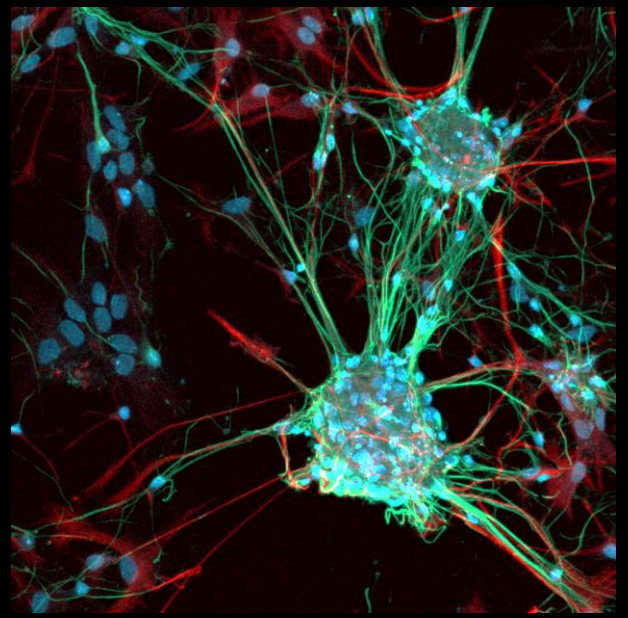
LSM | Fast and Gentle Multiplex Imaging



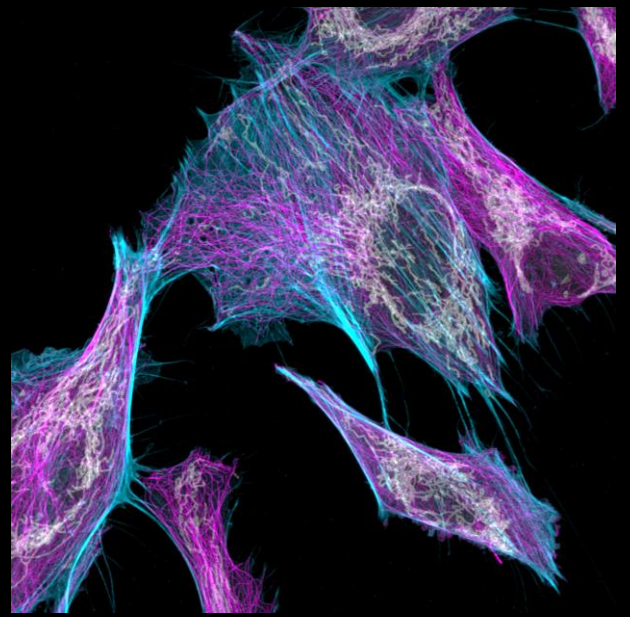
Highest sensitivity



Fast &
High throughput

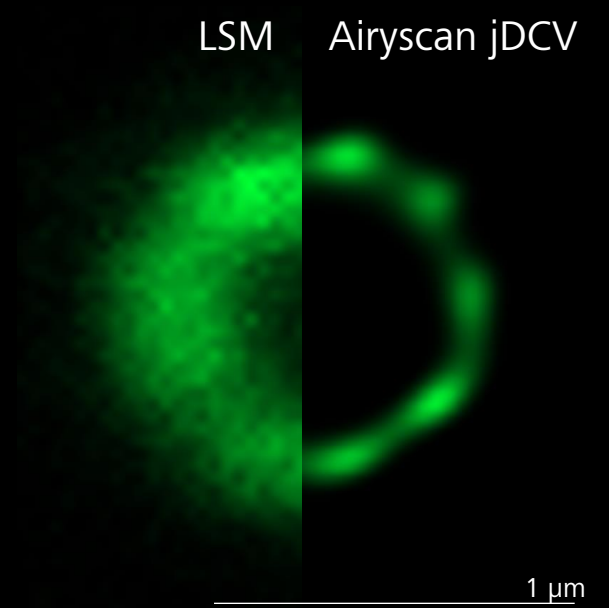


High resolution



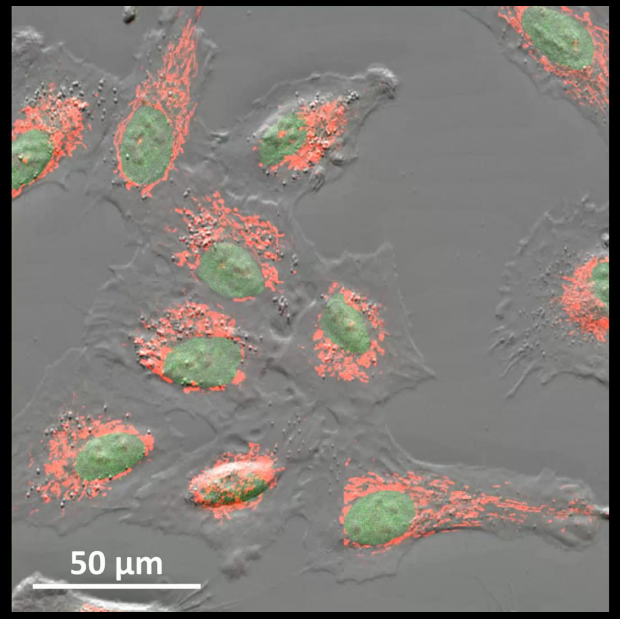
Spectral
multiplexing

LSM 9 Series | Versatile Confocal Platform



Airyscan 2

Superresolution

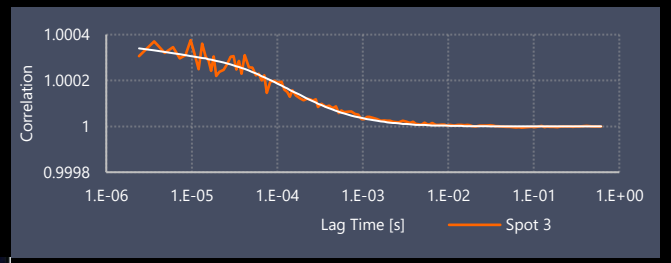


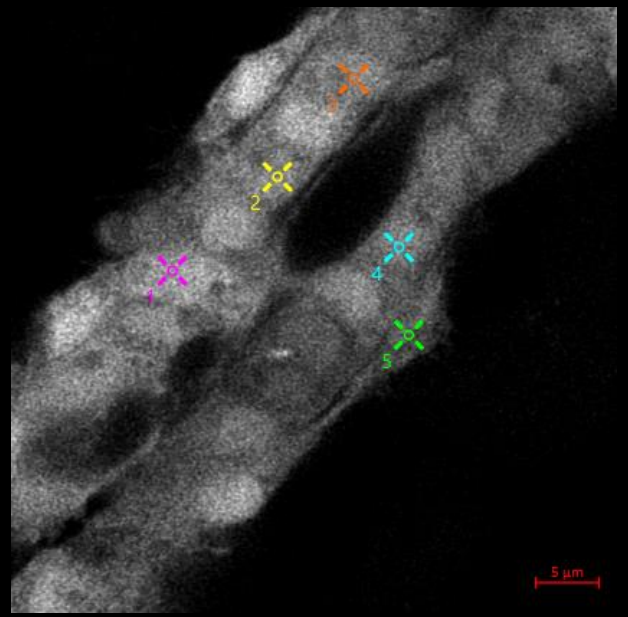
Incubation Module

Live cell imaging

AI Sample Finder

Automated imaging startup



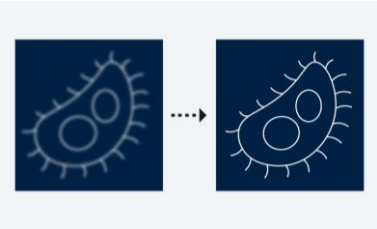


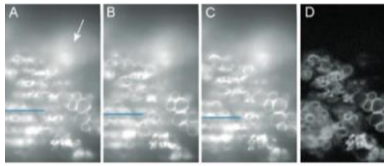
Dynamic Profiler

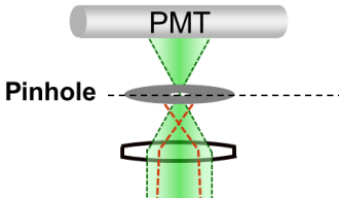
Gain molecular info

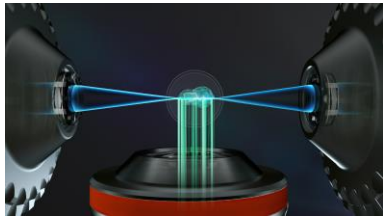
General Optical Sectioning Methods

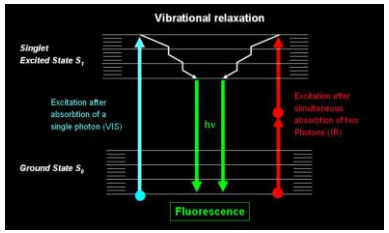
- Optical Sectioning Methods
  - Removing out-of focus light (downstream strategy)
    - Deconvolution
    - Structured Illumination
  - Blocking out-of focus light (detection strategy)
    - Confocal Methods
  - Avoiding out-of focus light (excitation strategy)
    - Light sheet
    - Multi-Photon
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

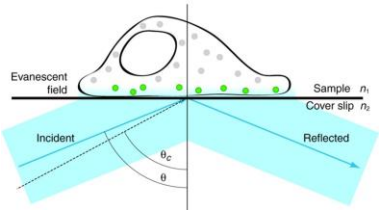






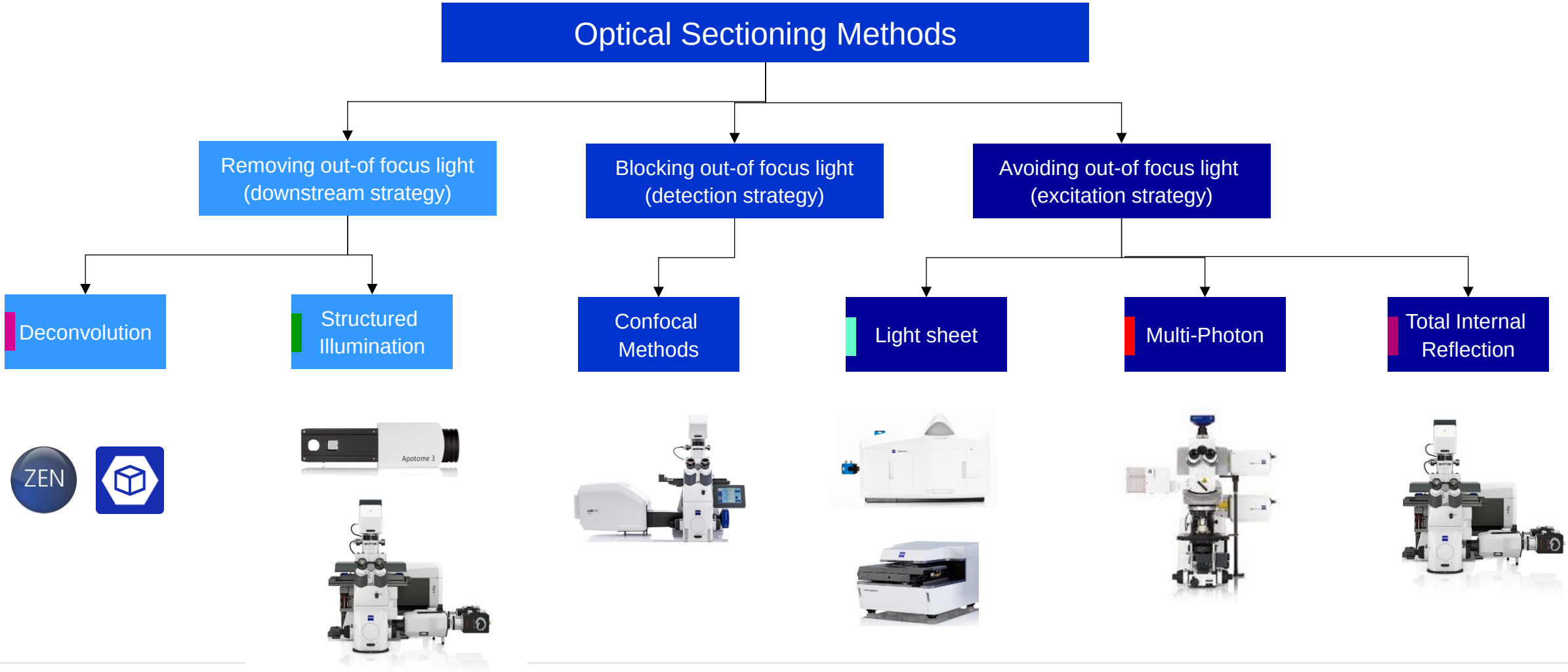






[Total Internal Reflection Microscopy (TIRF) Service - Creative Biostructure (creative-biostructure.com)]

General Optical Sectioning Methods



General Optical Sectioning Methods

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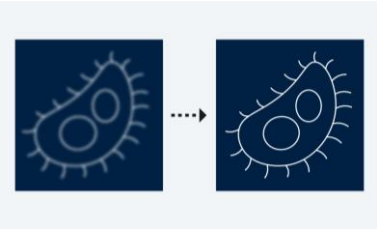
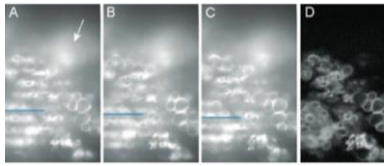
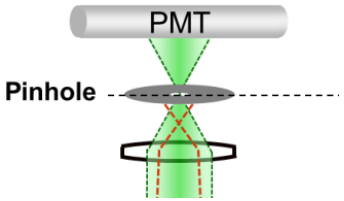
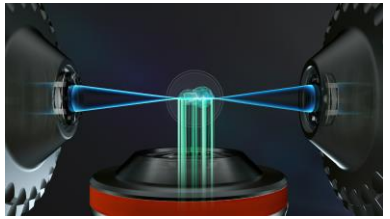
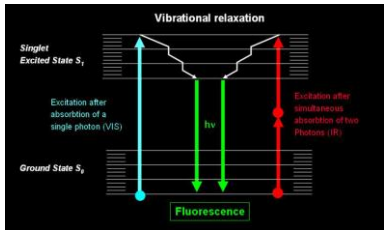
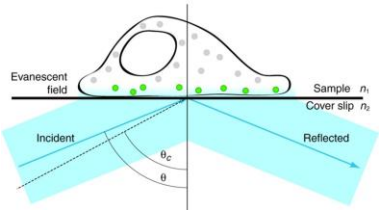






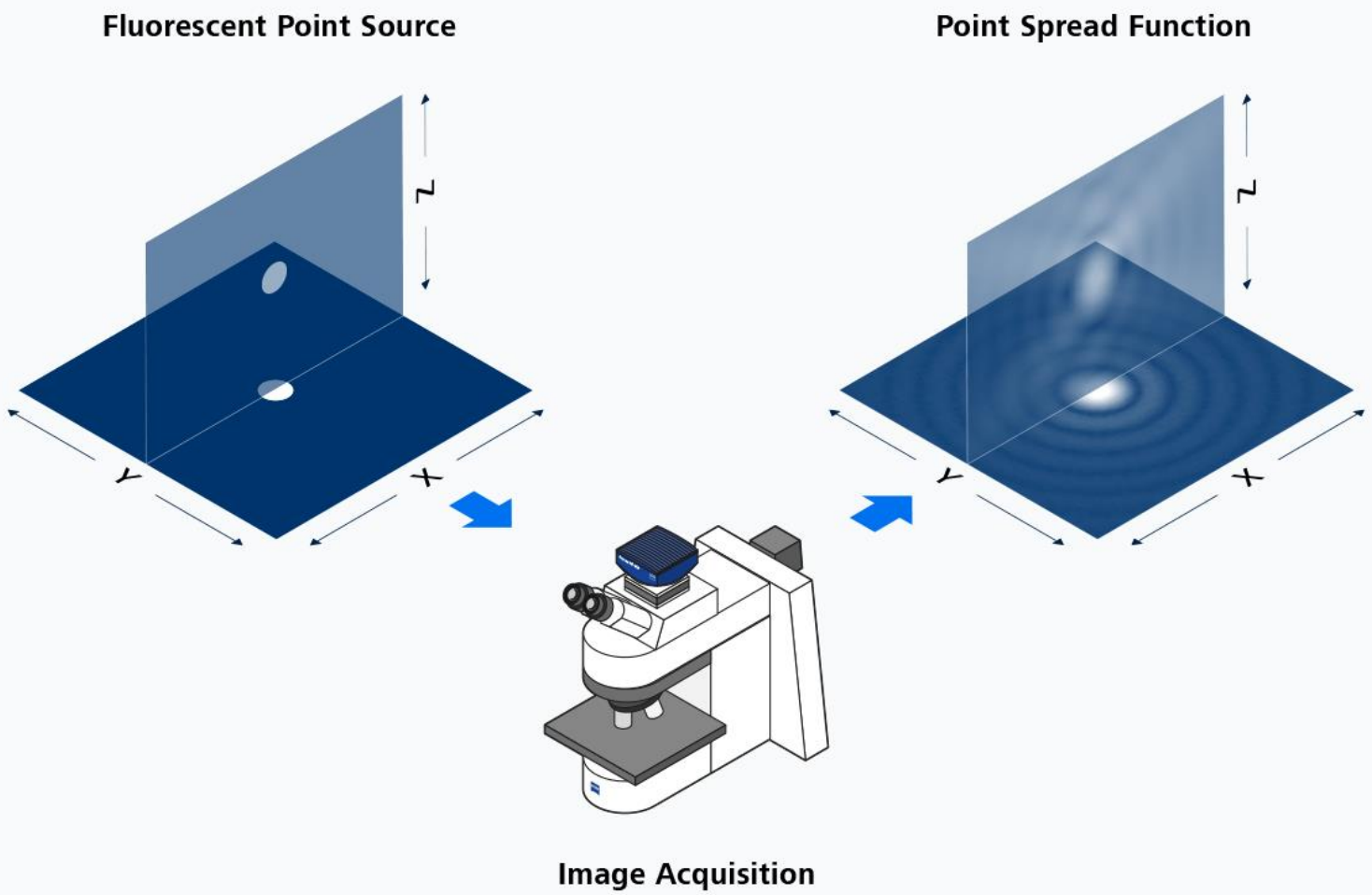






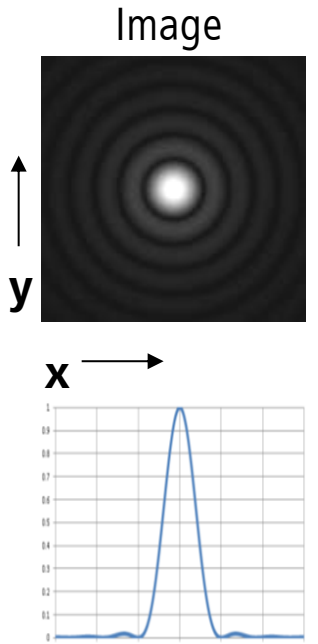
[Total Internal Reflection Microscopy (TIRF) Service - Creative Biostructure (creative-biostructure.com)]

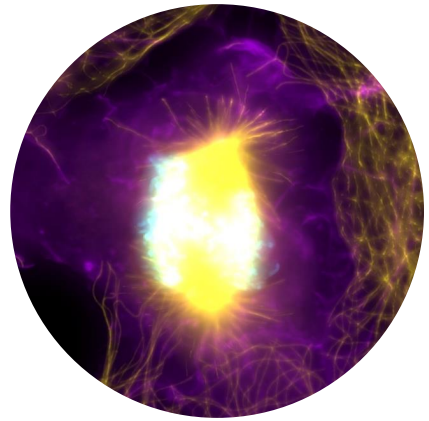
Noise-free Images are Physically Impossible



Point-Spread-Function

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

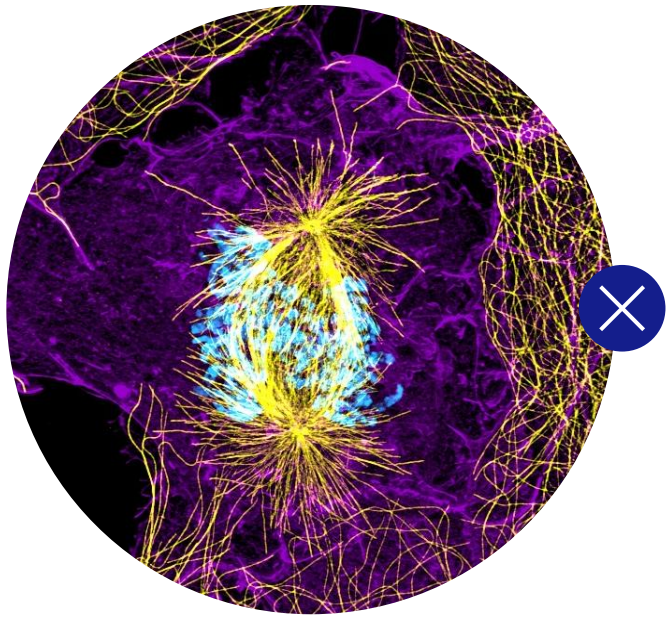




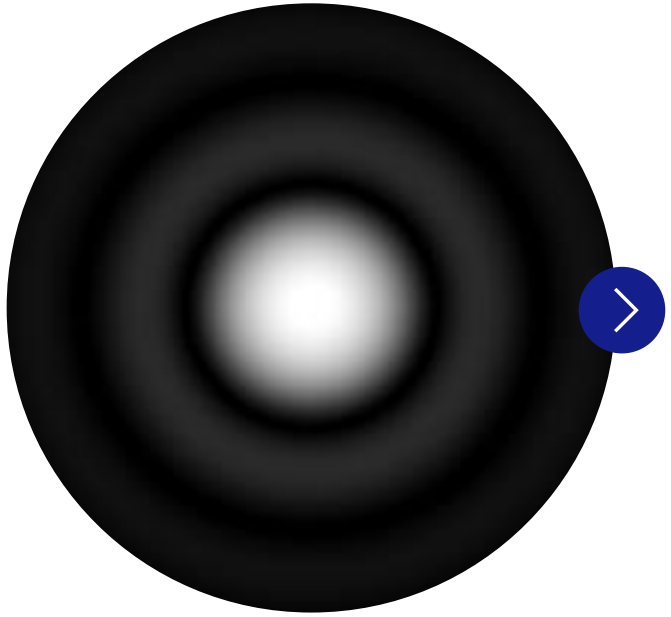
Imaging in Mathematical Terms

"Convolution" of the Object with the PSF

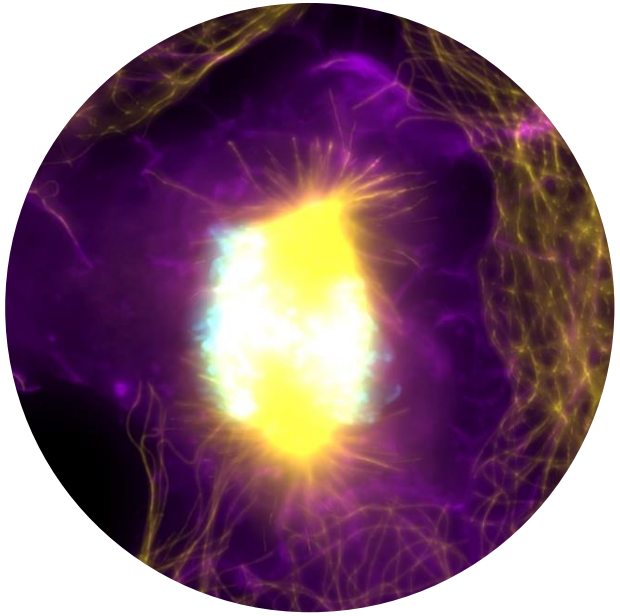
Sample

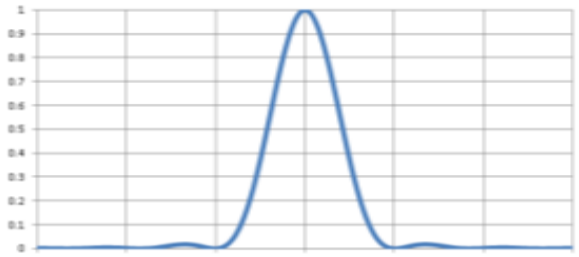


PSF



Image





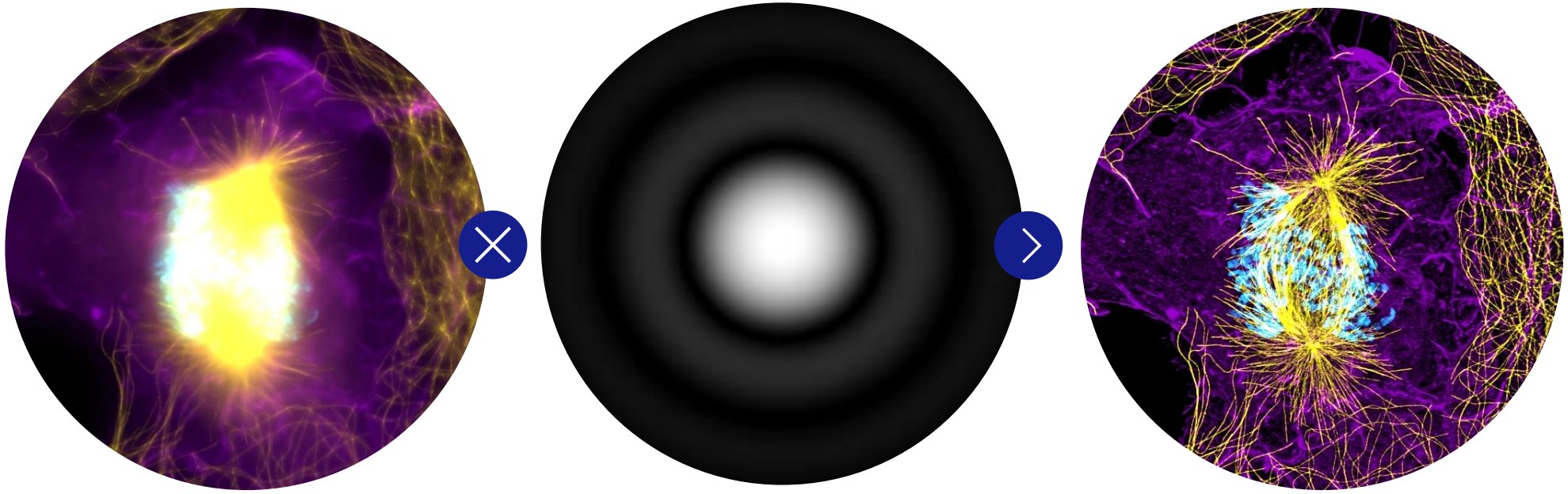
Inverting the Imaging-Process with Mathematics

A Deconvolution of the Image

Sample

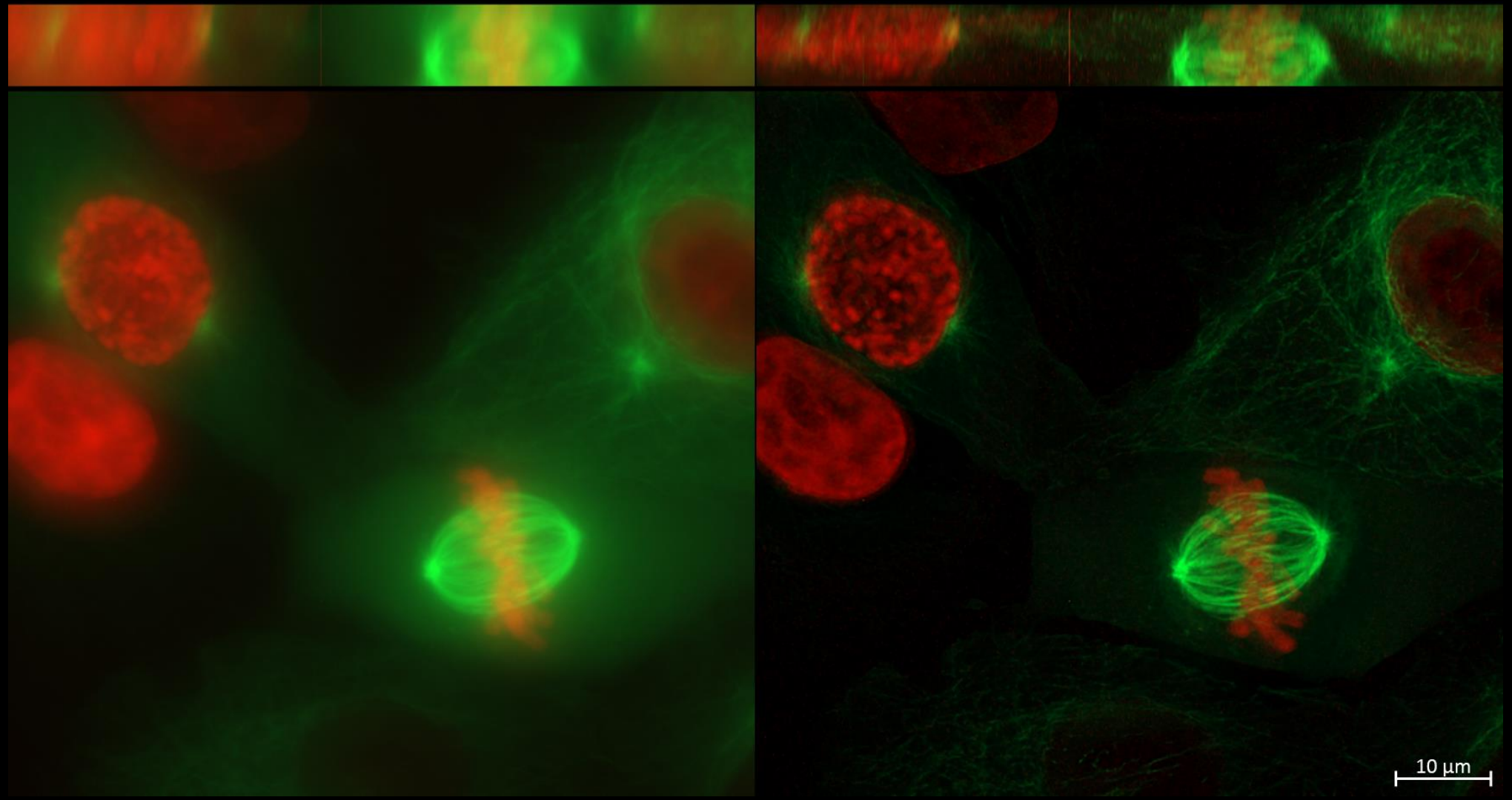
Deconvolution with PSF

Image



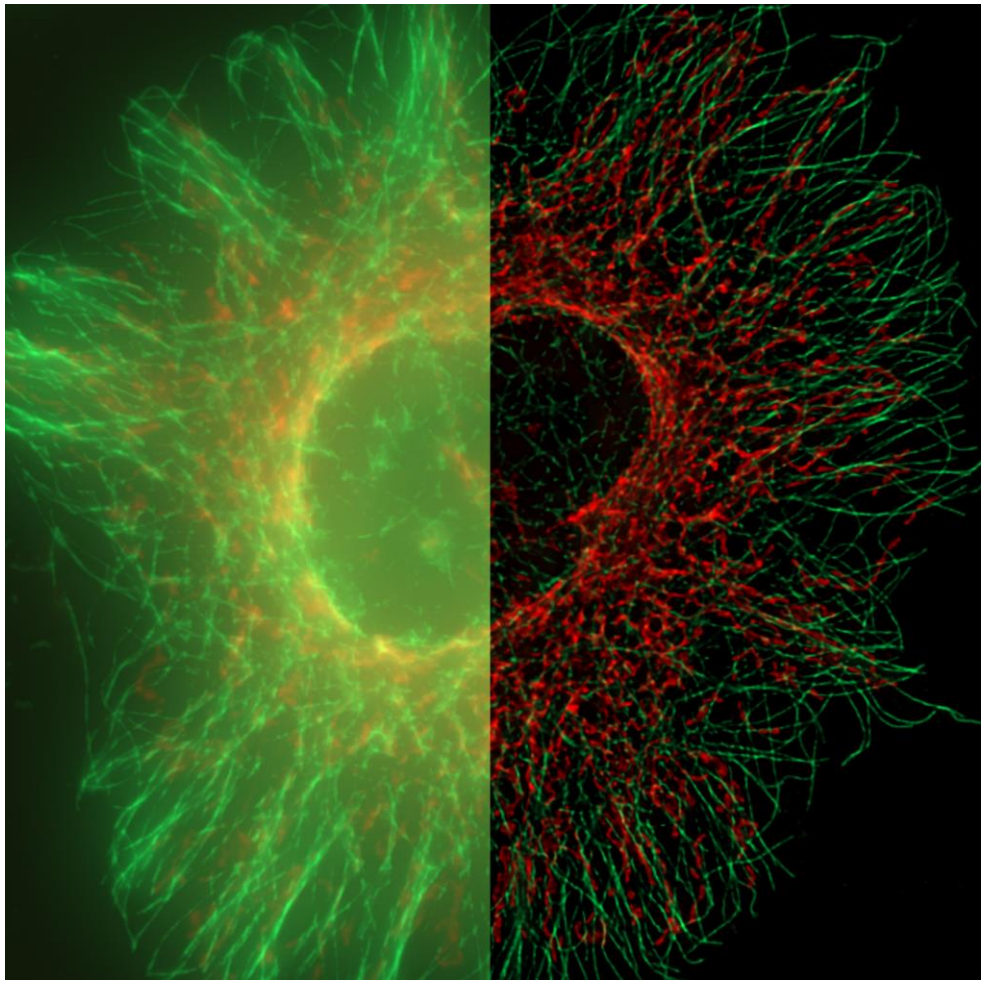
“Re-assignment” of “photons“

Widefield Imaging with Deconvolution



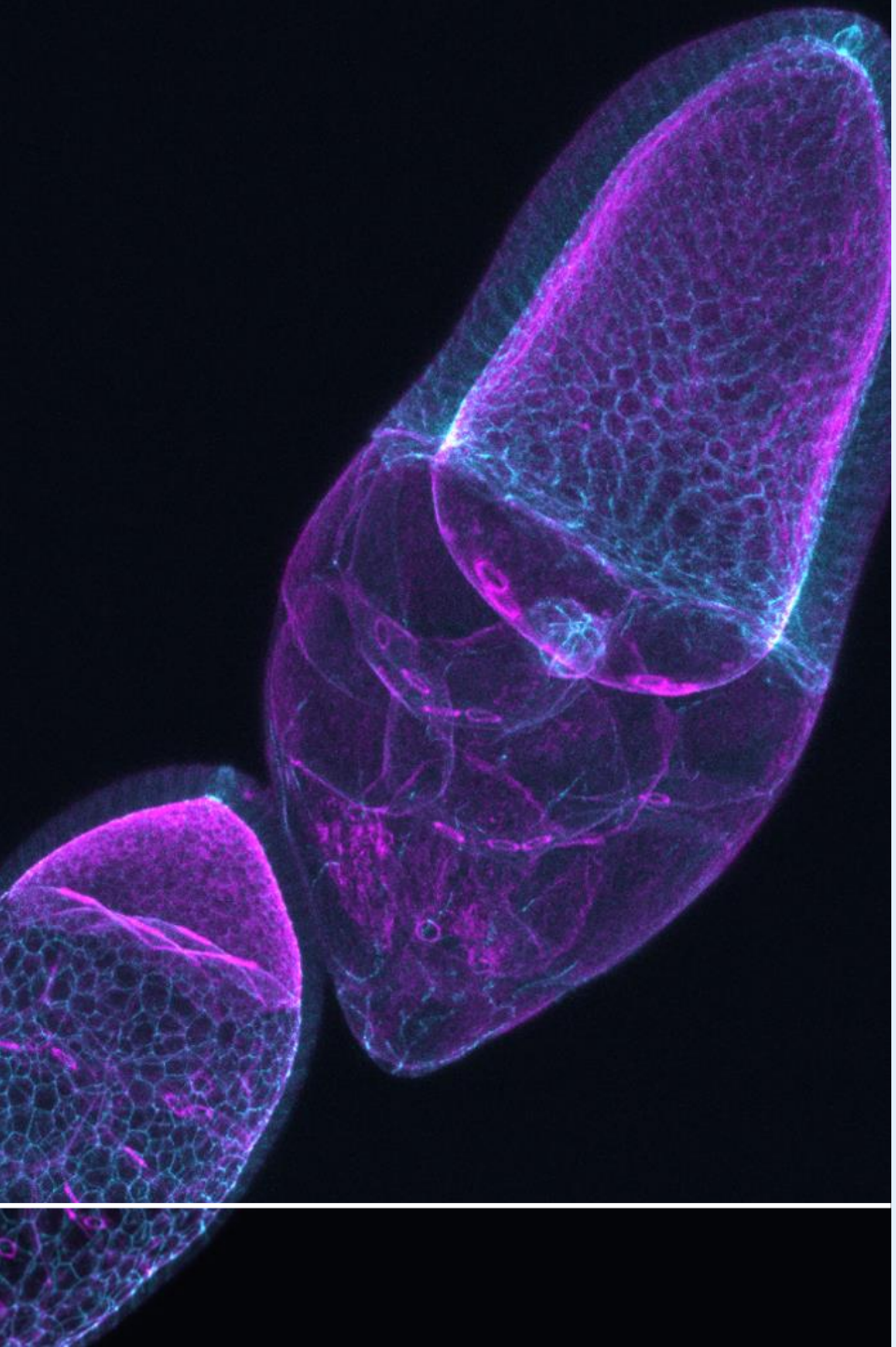
Deconvolution Algorithms

Raw Deconvolution



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

Fast Slow

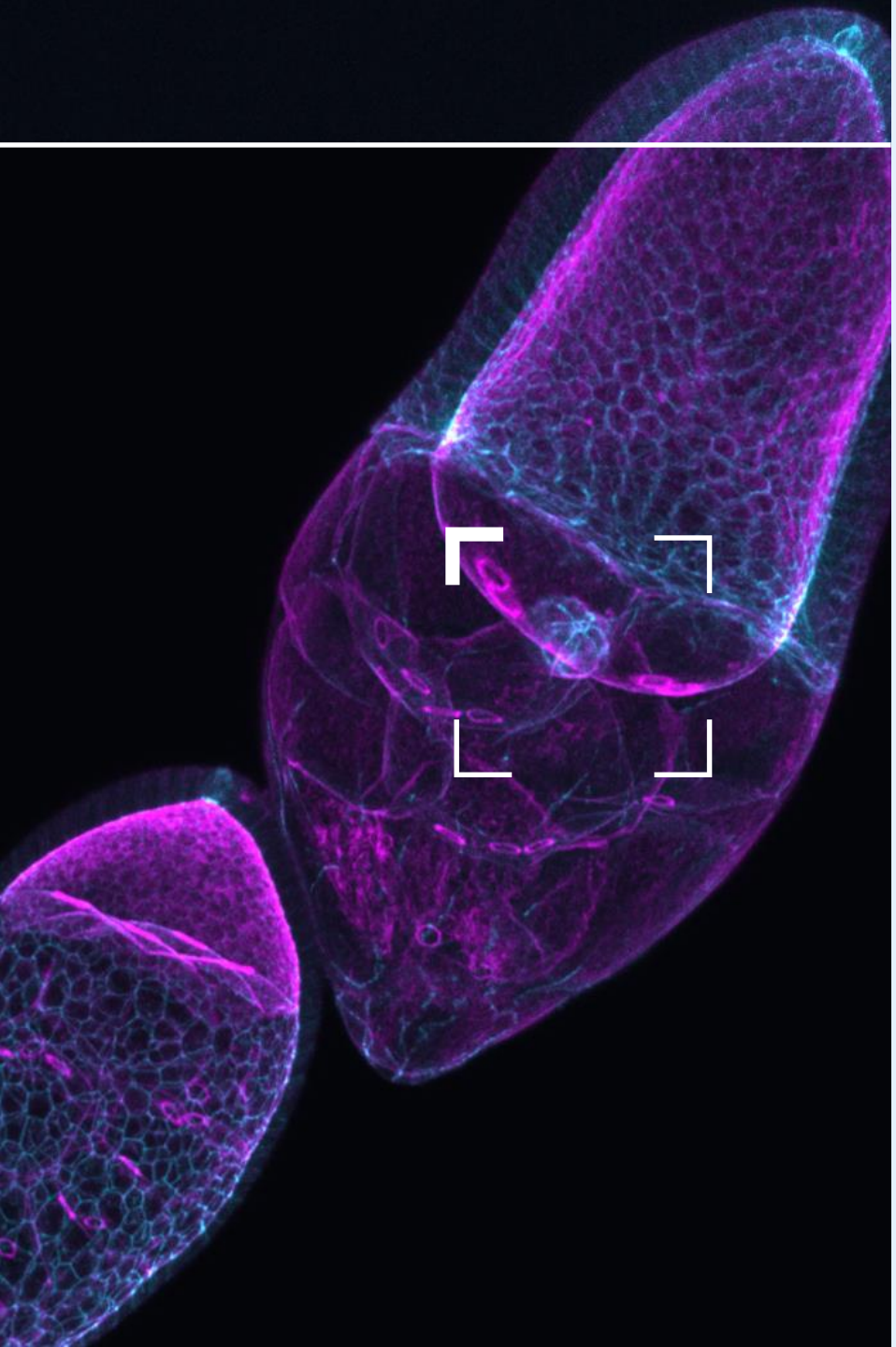


A unique confocal experience

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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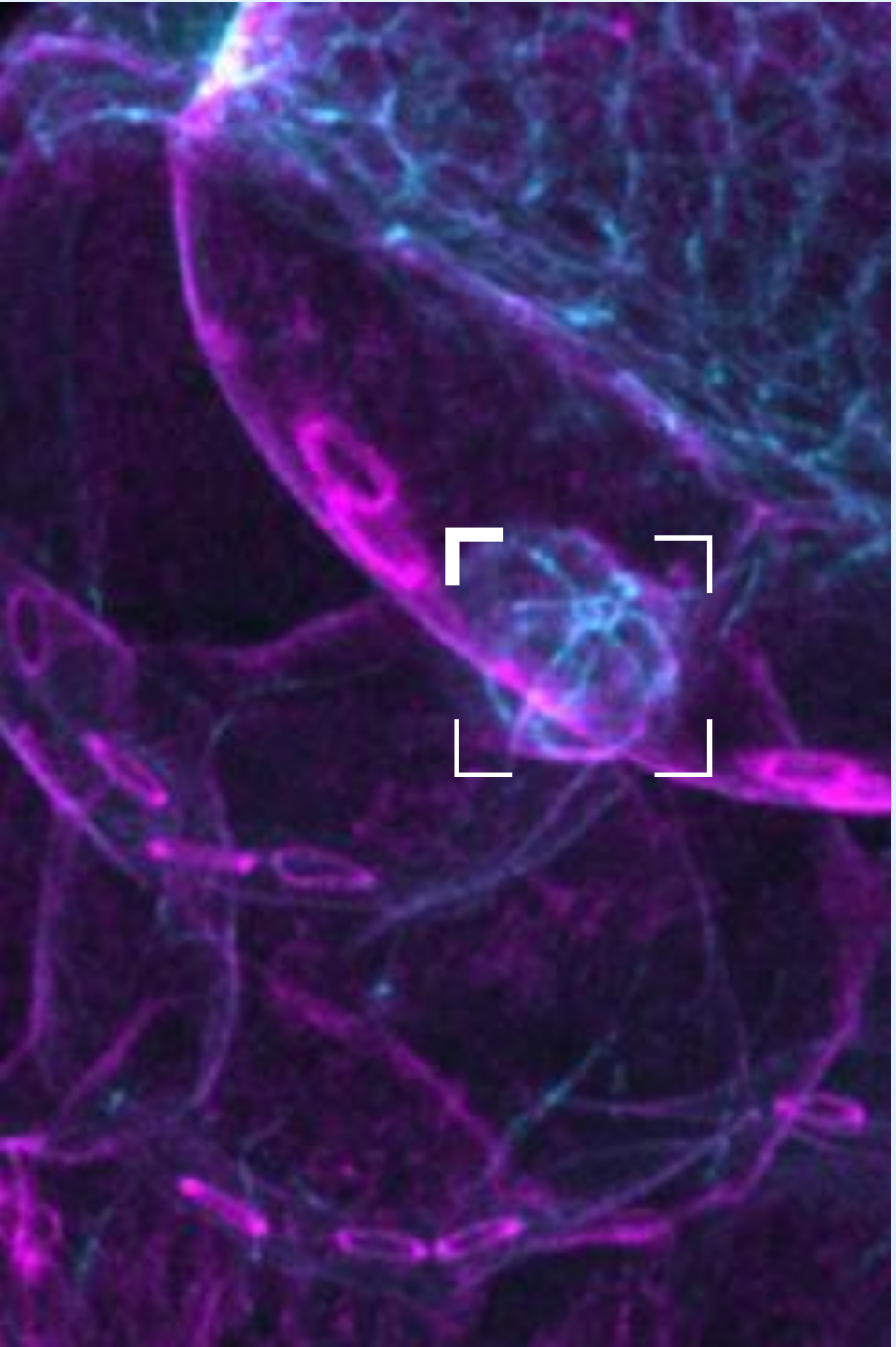

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Sample courtesy of Thea Jacobs, AG Luschnig, WWU Münster, together with T. Zobel, Münster Imaging Network, Germany

LSM Plus: Better data, faster

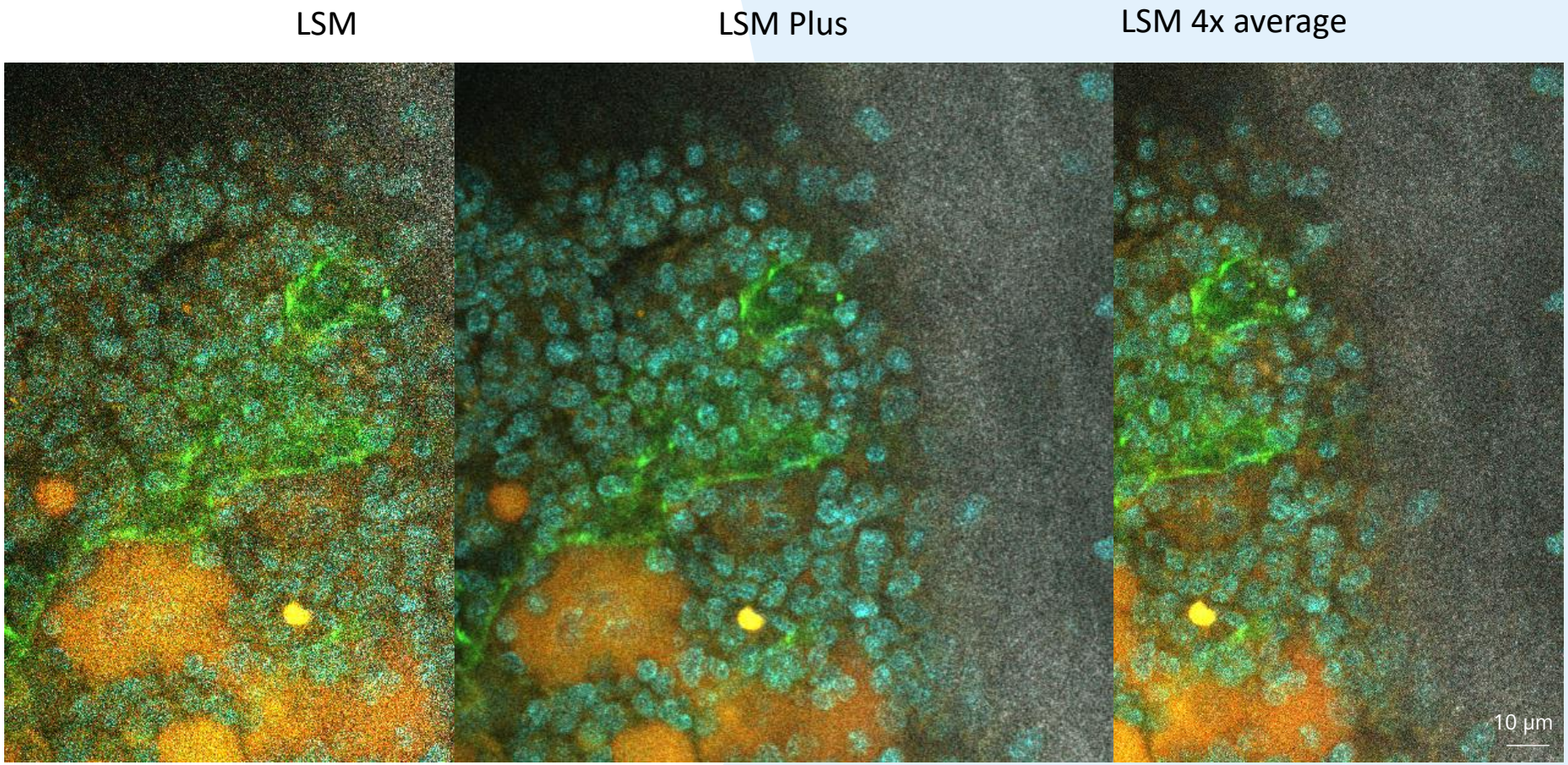
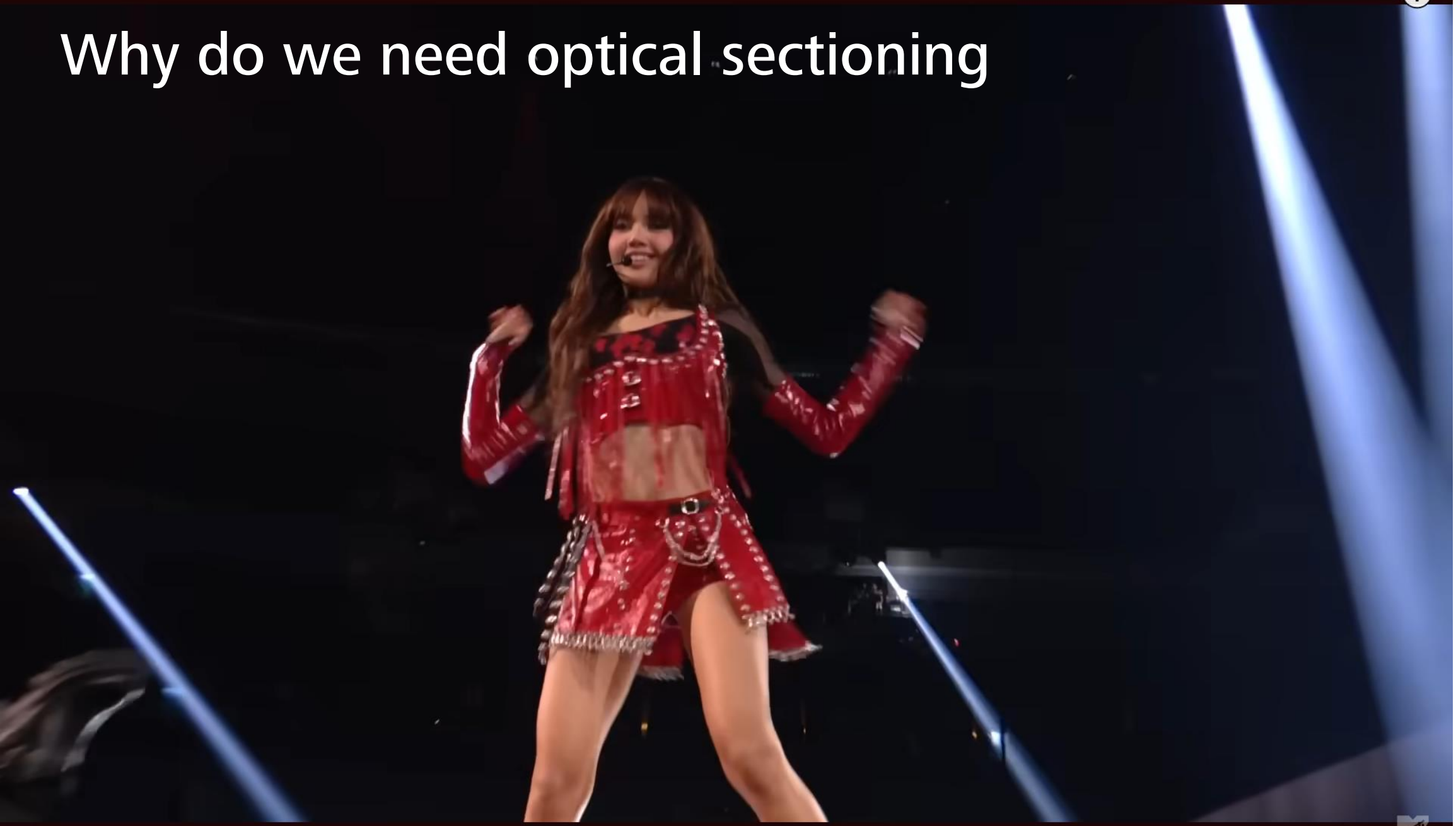


Image of bone marrow section showing bone (grey), endomucin vessels (green), dapi (blue) and megakaryocytes (red). Courtesy of George Adams (Imperial College London).

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
      - Confocal Point Scanner
      - Spinning Disc Systems
      - STED
      - AiryScan
  - Avoiding out-of focus light (excitation strategy)
    - Light sheet
    - Multi-Photon
    - Total Internal Reflection

Why do we need optical sectioning



We want focused image

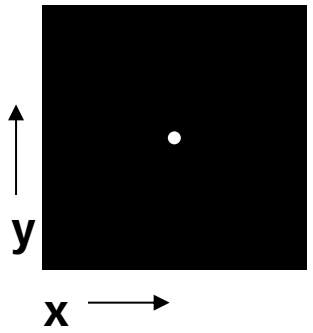
Unfocused images are annoying

The Point-spread-function of a Microscope

Point-Spread-Function

The image of a point is not a point.

fluorescent point source



The Point-spread-function of a Microscope

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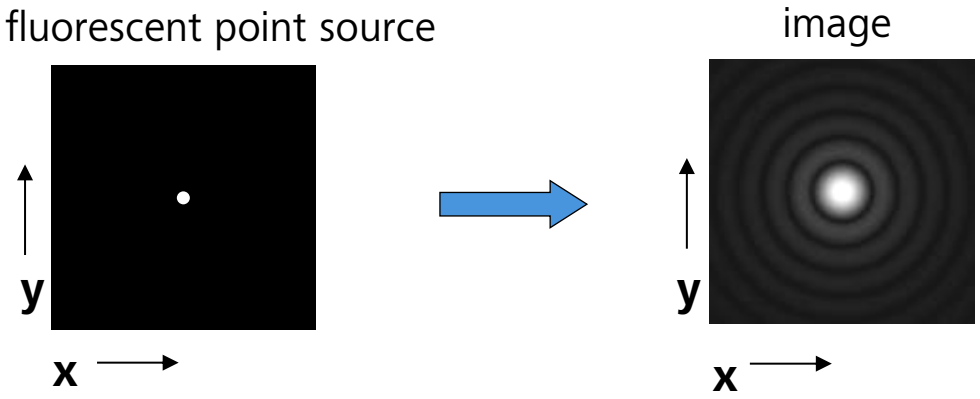
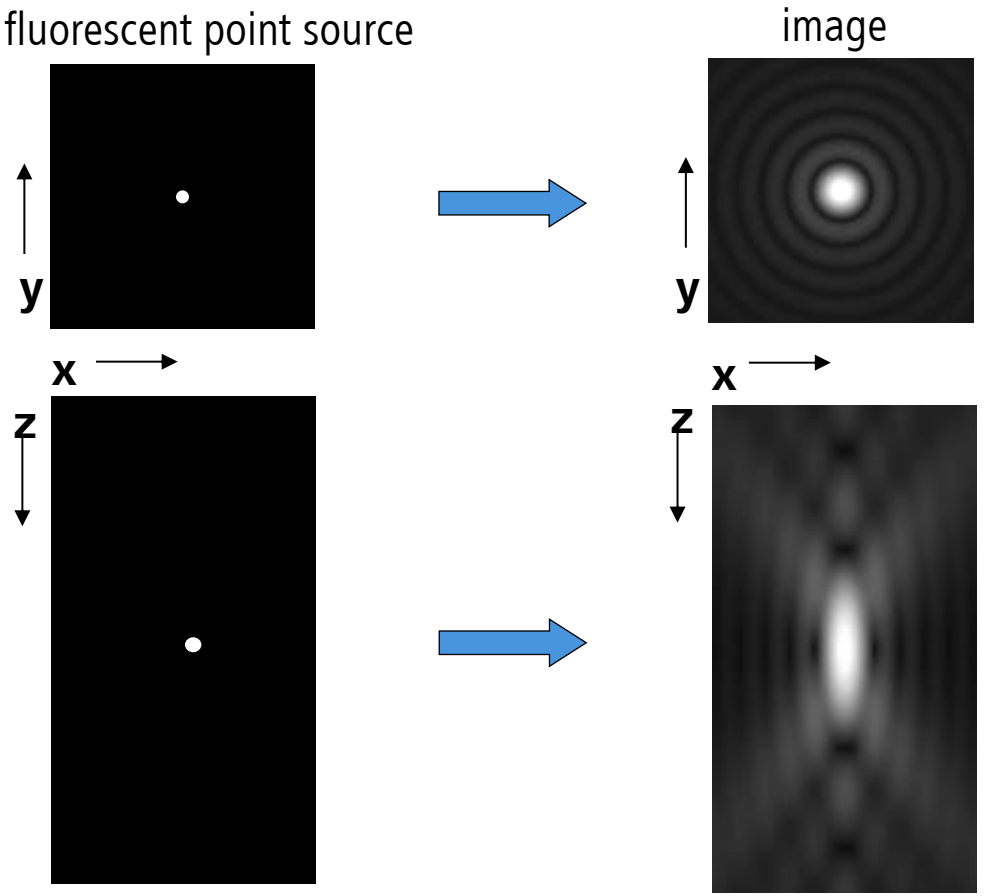


General Optical Sectioning Methods

Point-Spread-Function

The image of a point is not a point.

It's a complex 3D diffraction pattern.



General Optical Sectioning Methods

Point-Spread-Function

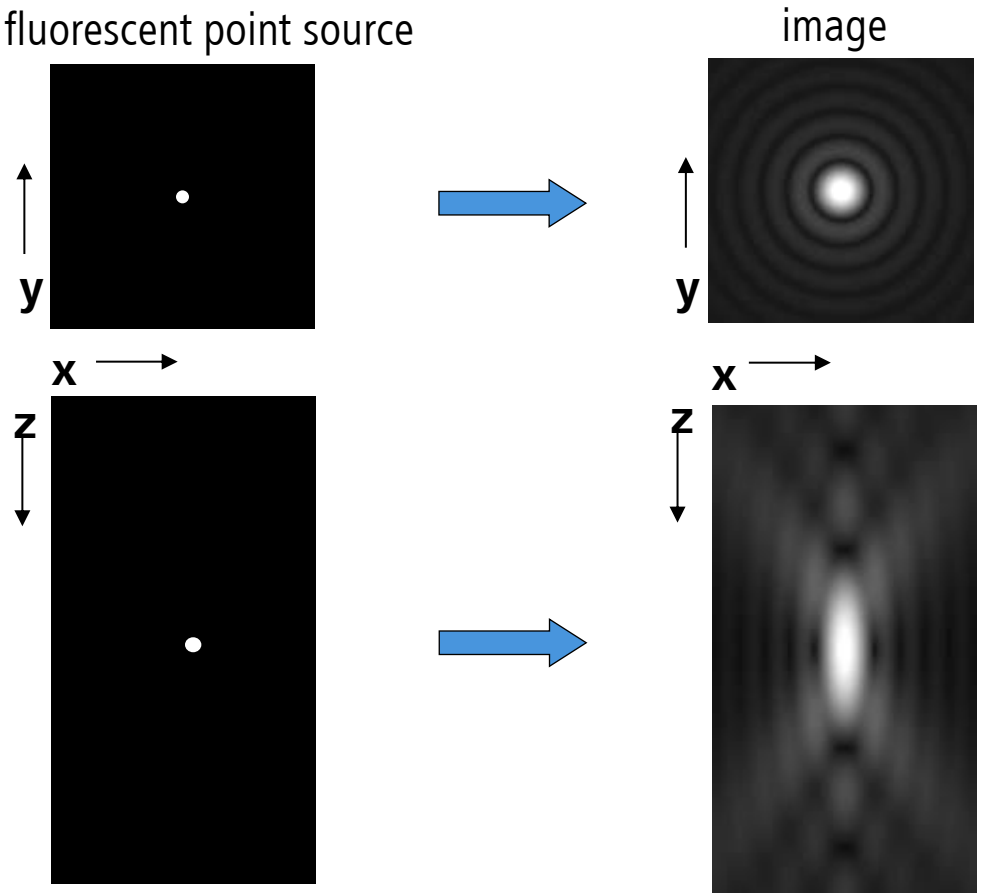
The image of a point is not a point.

It's a complex 3D diffraction pattern.

Dimensions of the central peak:

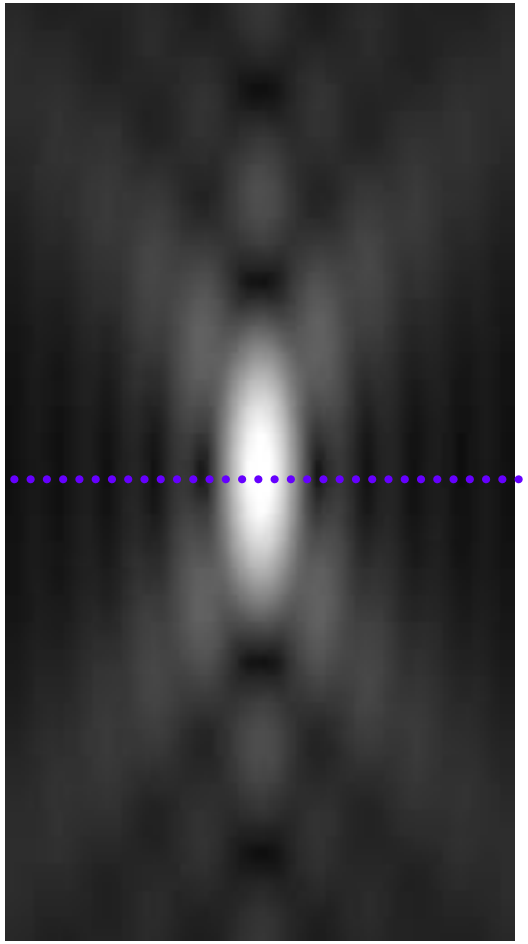
$$r_{lateral} \approx 0{,}6\frac{\lambda}{NA}$$

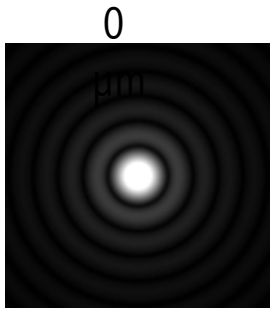
$$r_{axial} \approx 2\frac{n \cdot \lambda}{NA^2}$$



Defocusing of an Object

Conventional microscope





Defocusing of an Object

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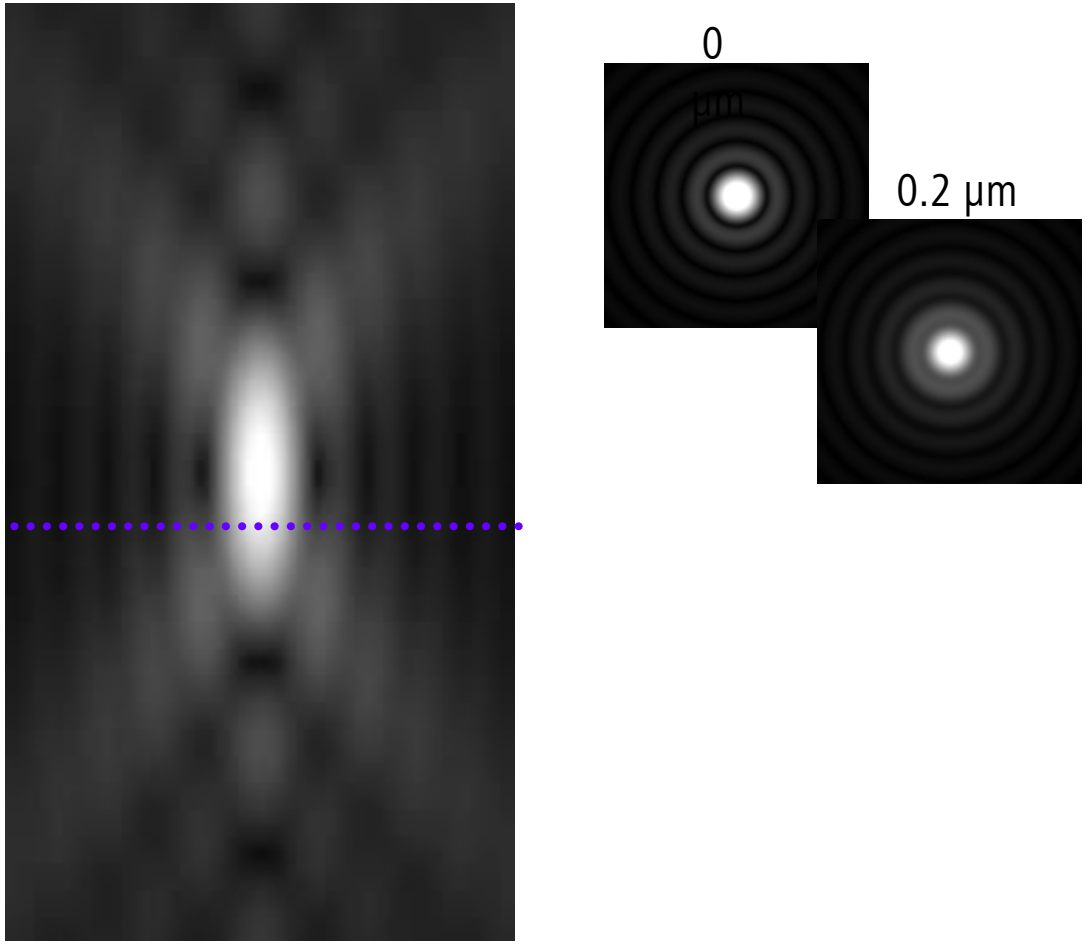
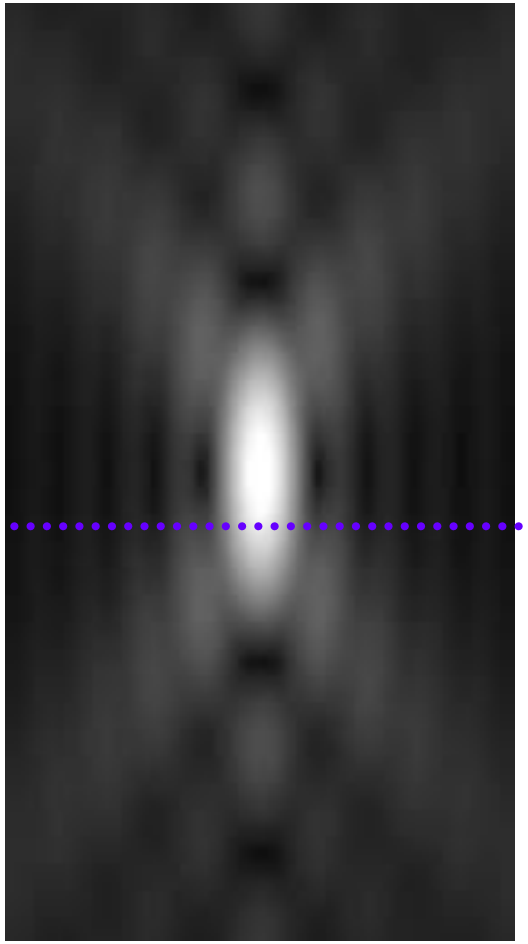
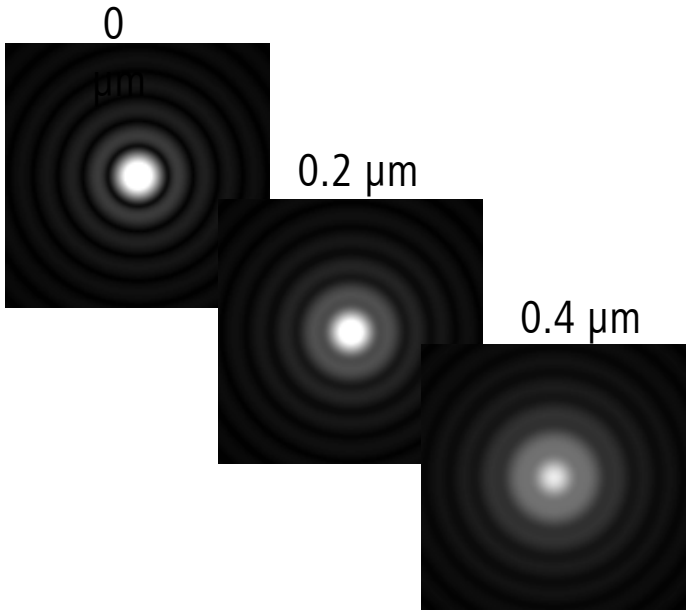


Defocusing of an Object

Conventional microscope





Defocusing of an Object

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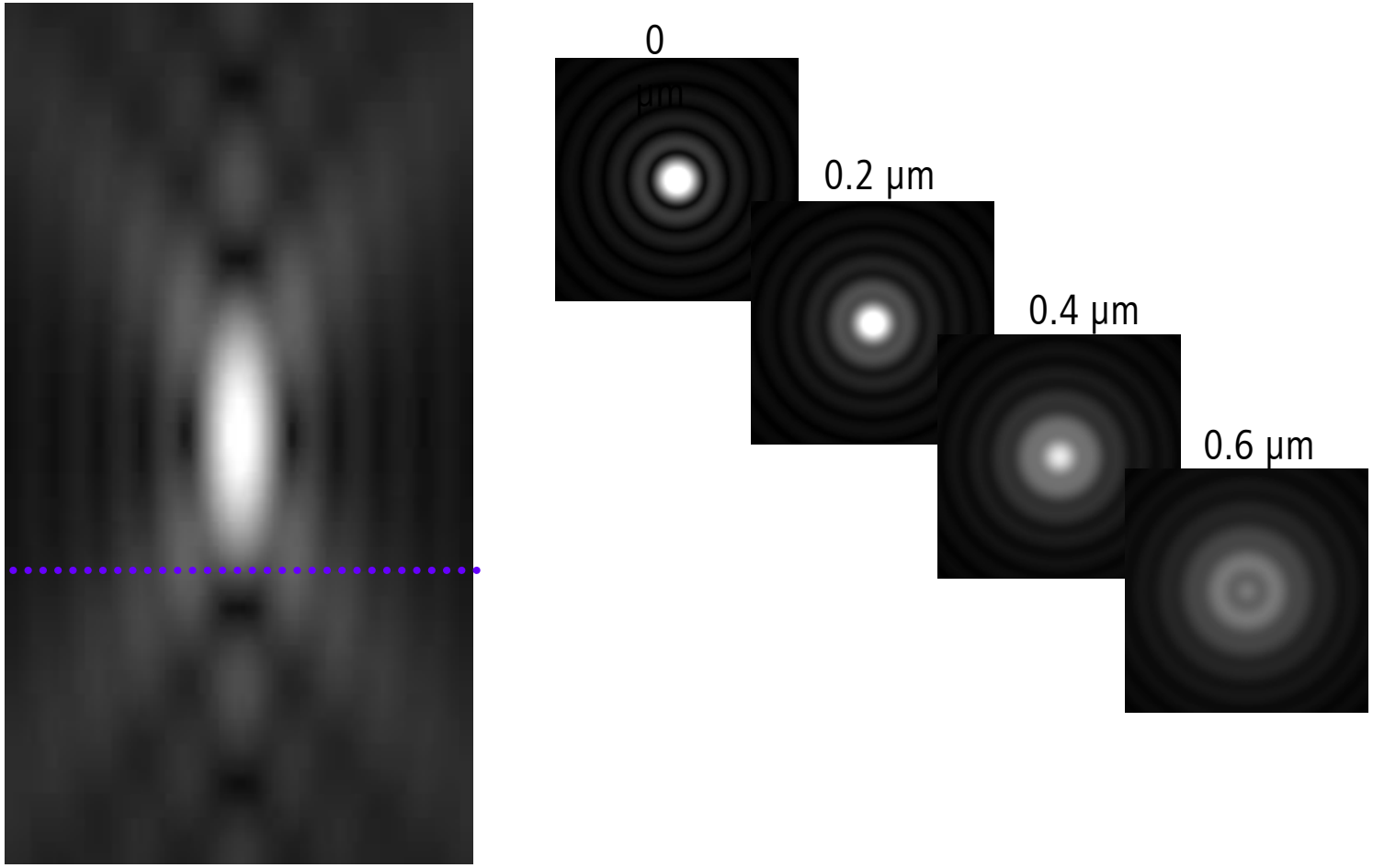
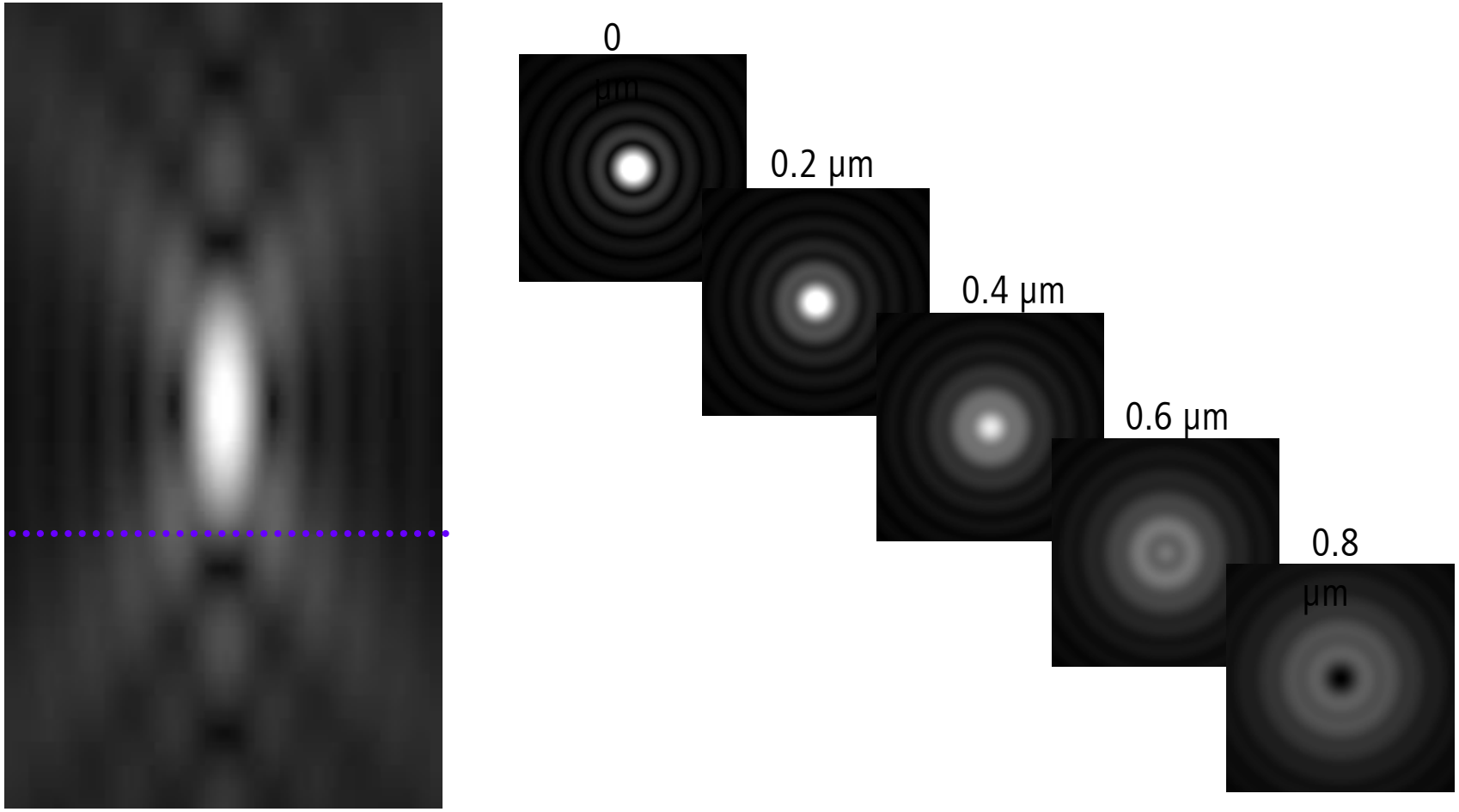


Defocusing of an Object

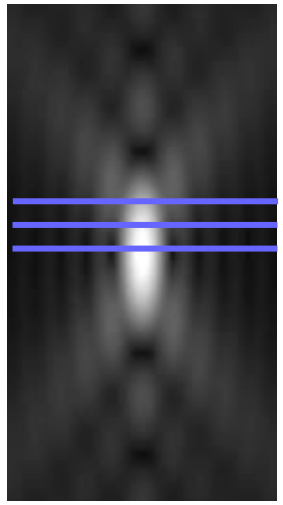
Conventional microscope

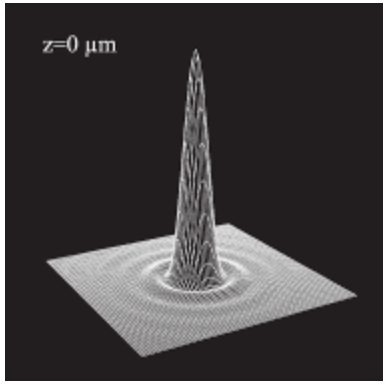


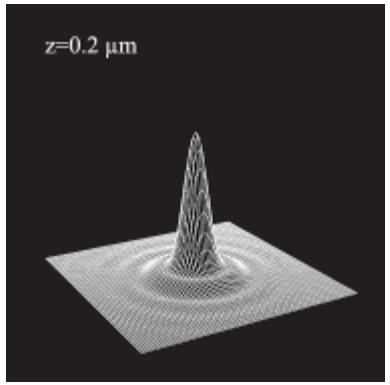
Defocusing of an object

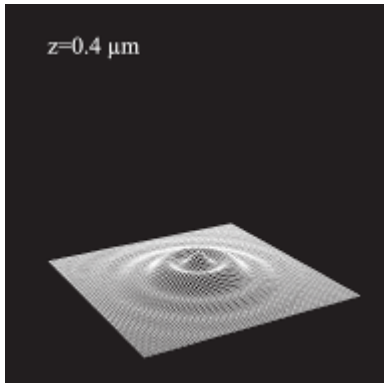
Conventional microscope

The integrated intensity in each image is <u>independent</u> of the distance from the focal plane!

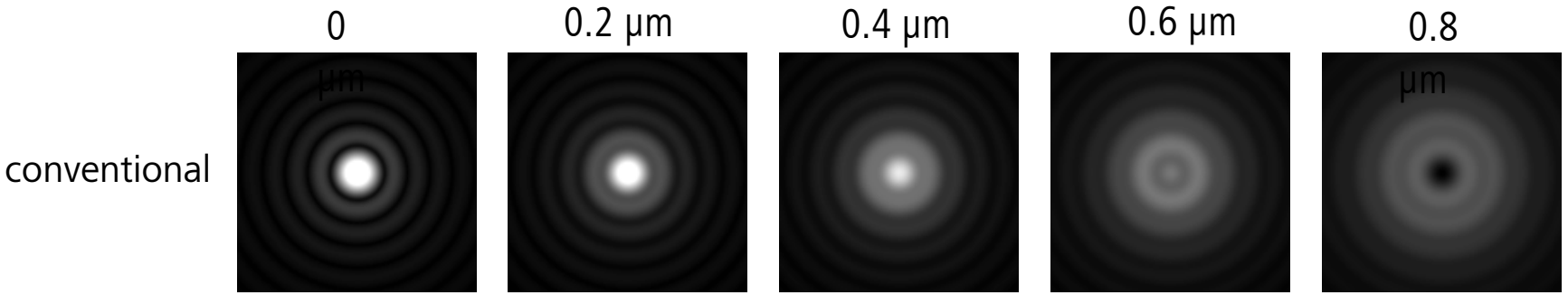






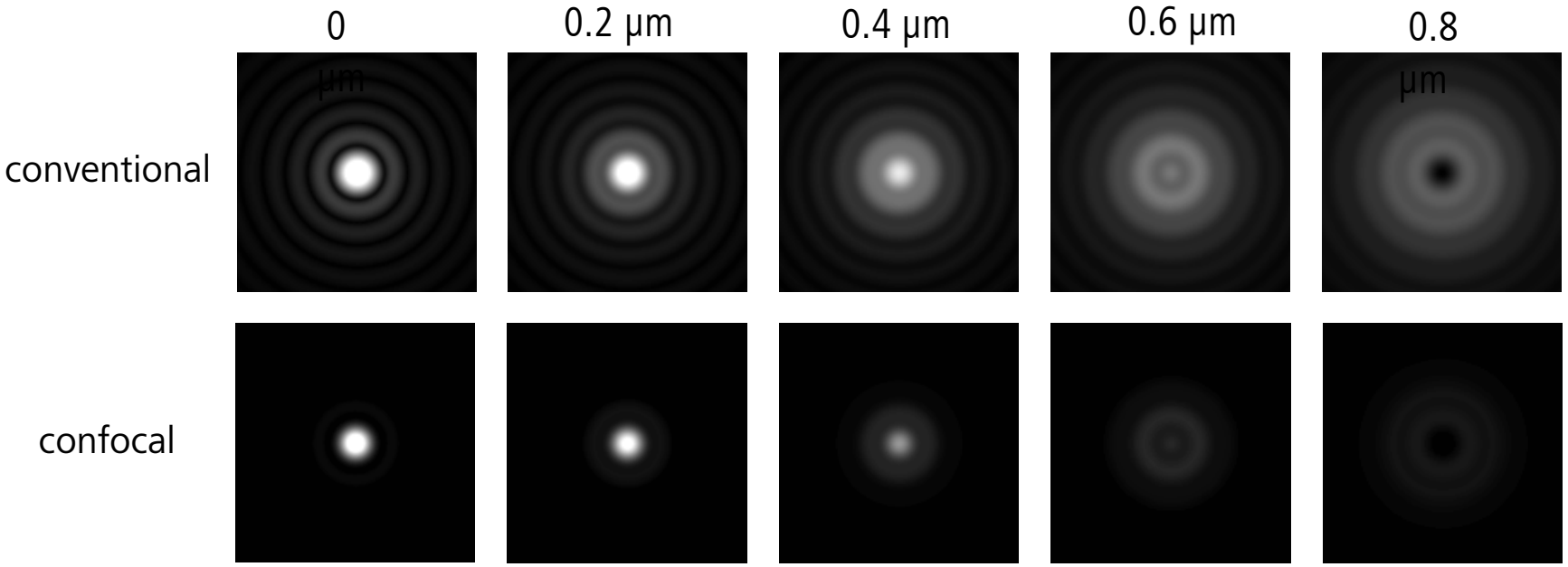


What is an optical section?



→ The integrated intensity in each image plane is **independent** of the axial position!

What is an optical section?



The integrated intensity in each image plane is **independent** of the axial position!

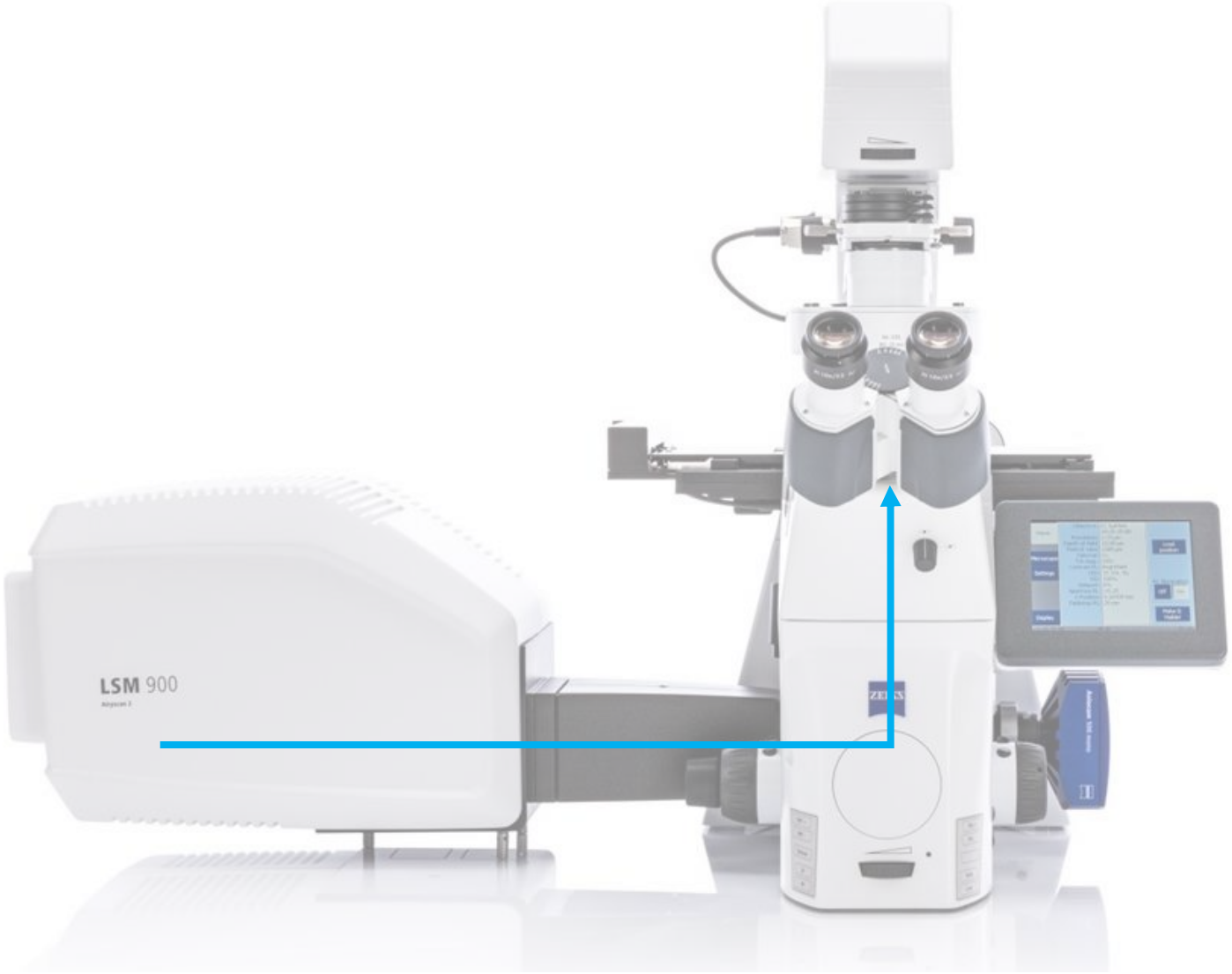
ZEISS LSM Confocal

Inverted microscope

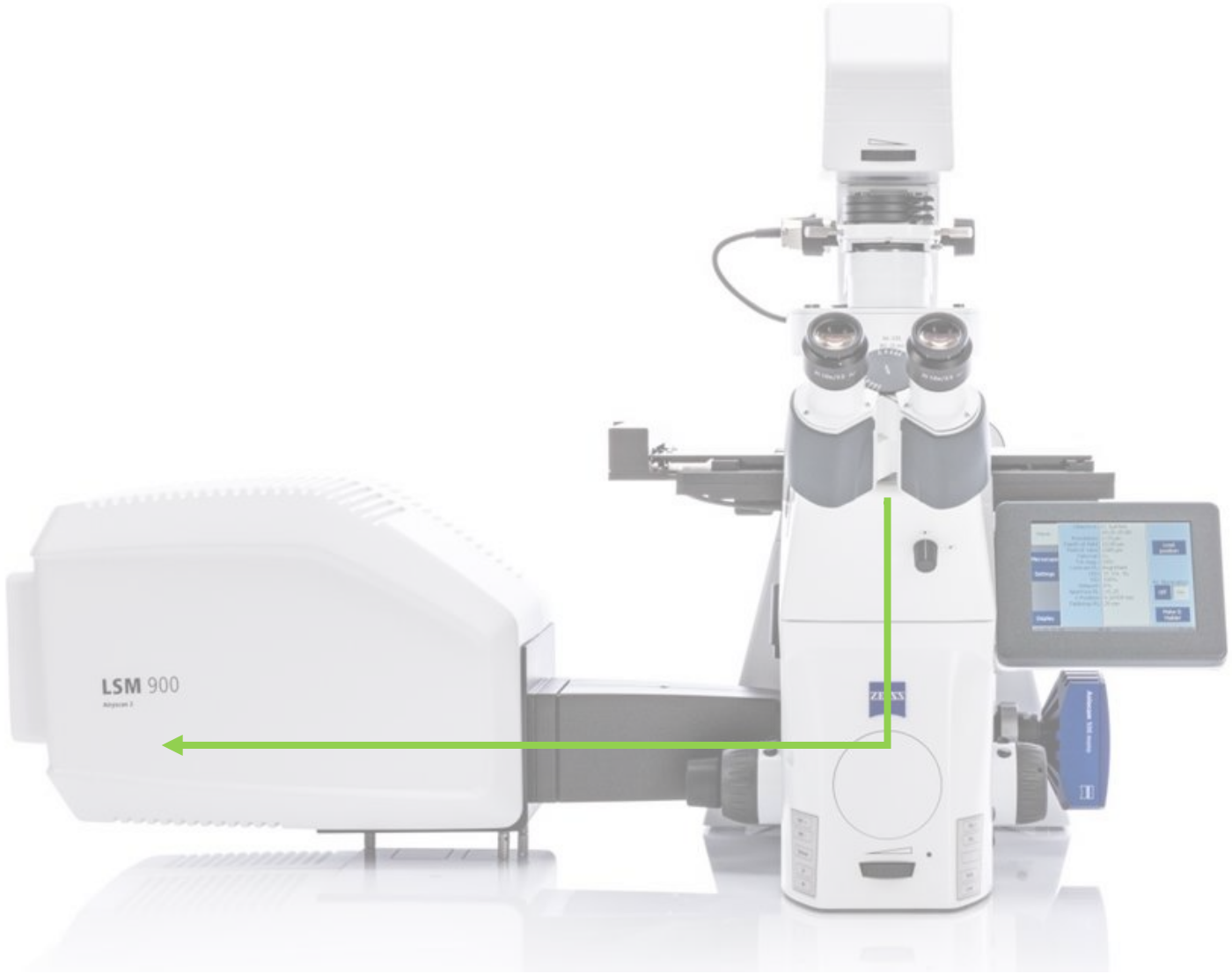
Software

Scanning module

ZEISS LSM Confocal



ZEISS LSM Confocal

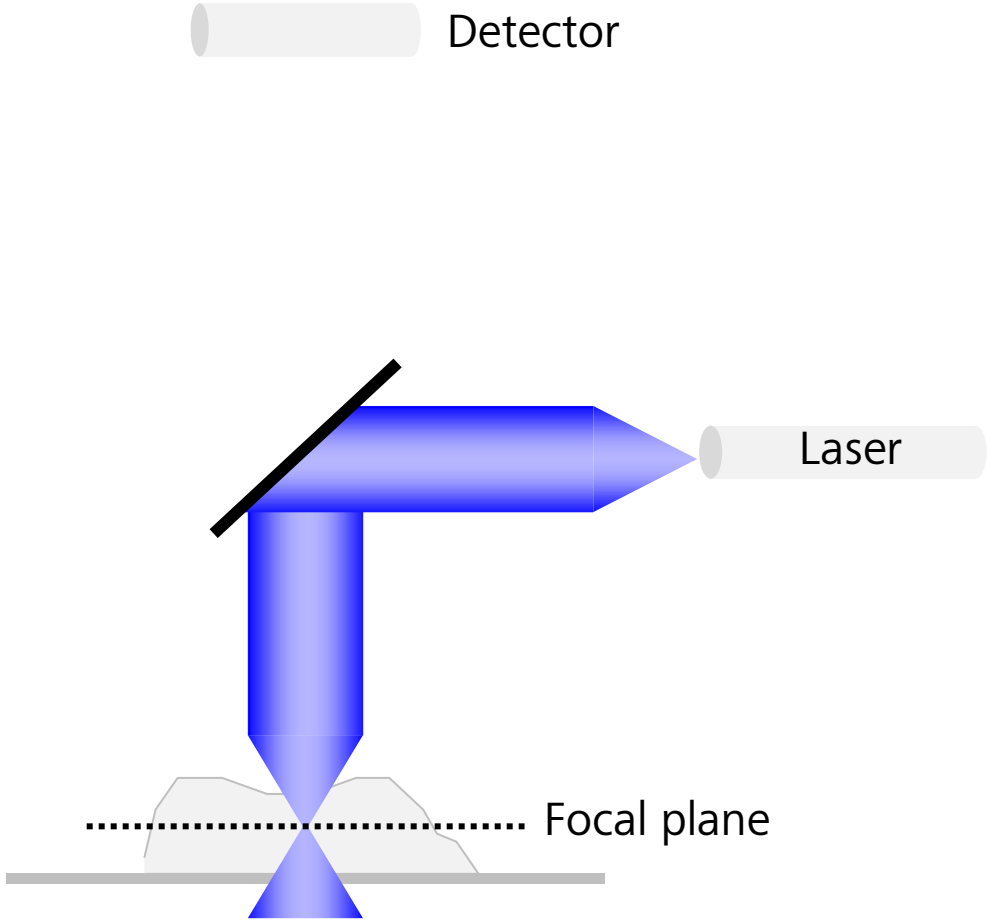


Point Scanning Confocal Microscopes

Confocal principle

Spot Illumination

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

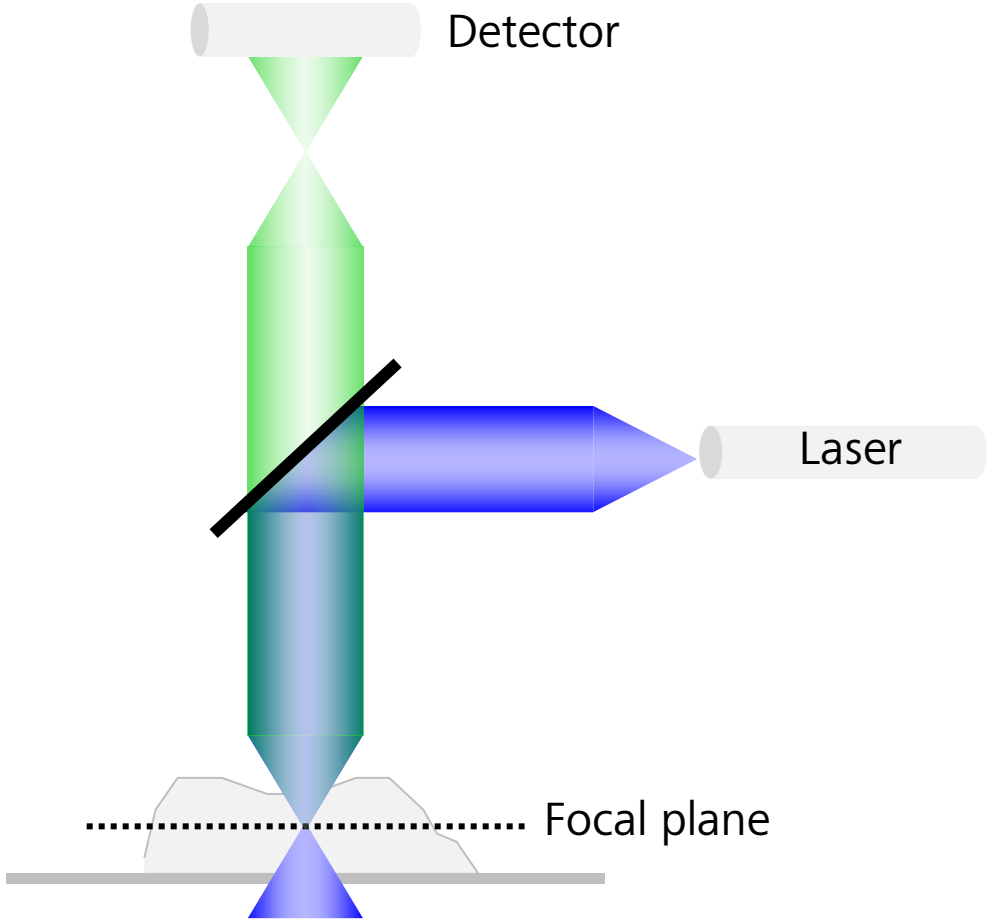


Point Scanning Confocal Microscopes

Confocal principle

Spot detection

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



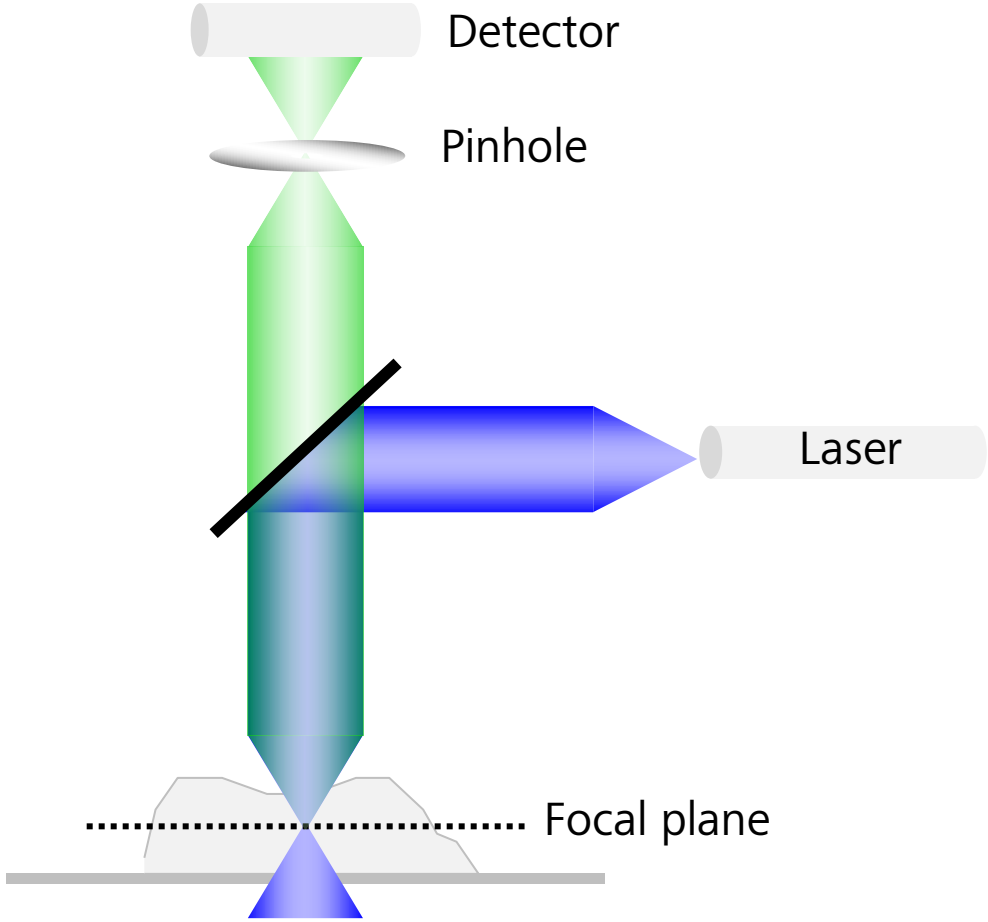
Point Scanning Confocal Microscopes

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.

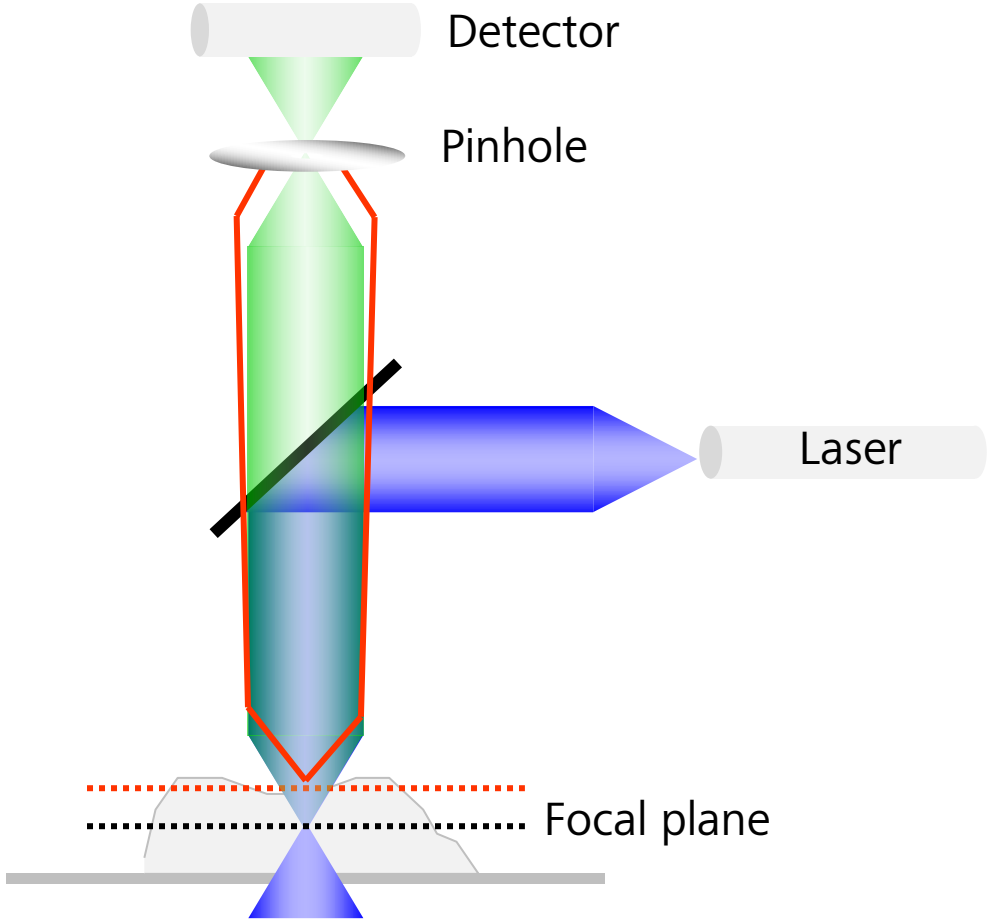


Point Scanning Confocal Microscopes

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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Confocal principle

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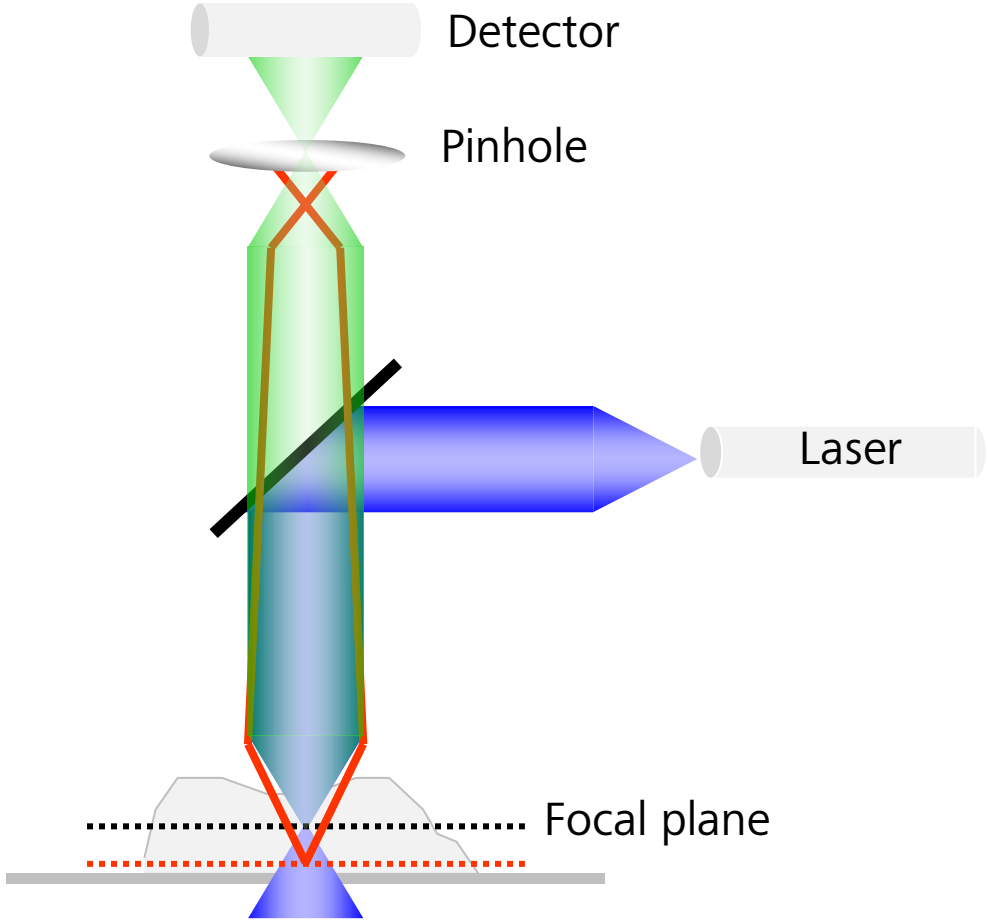
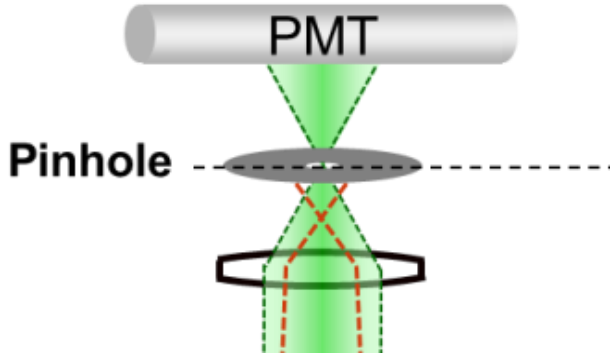
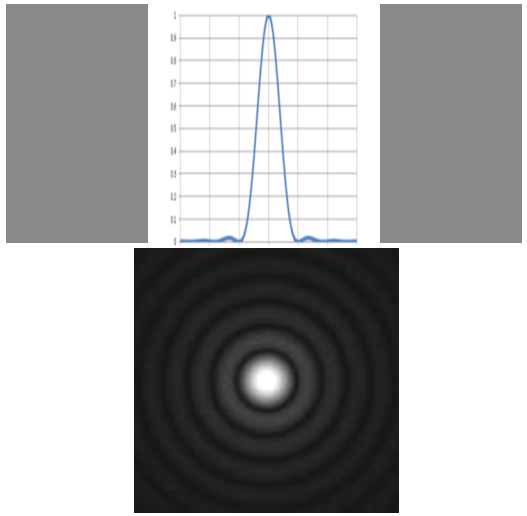
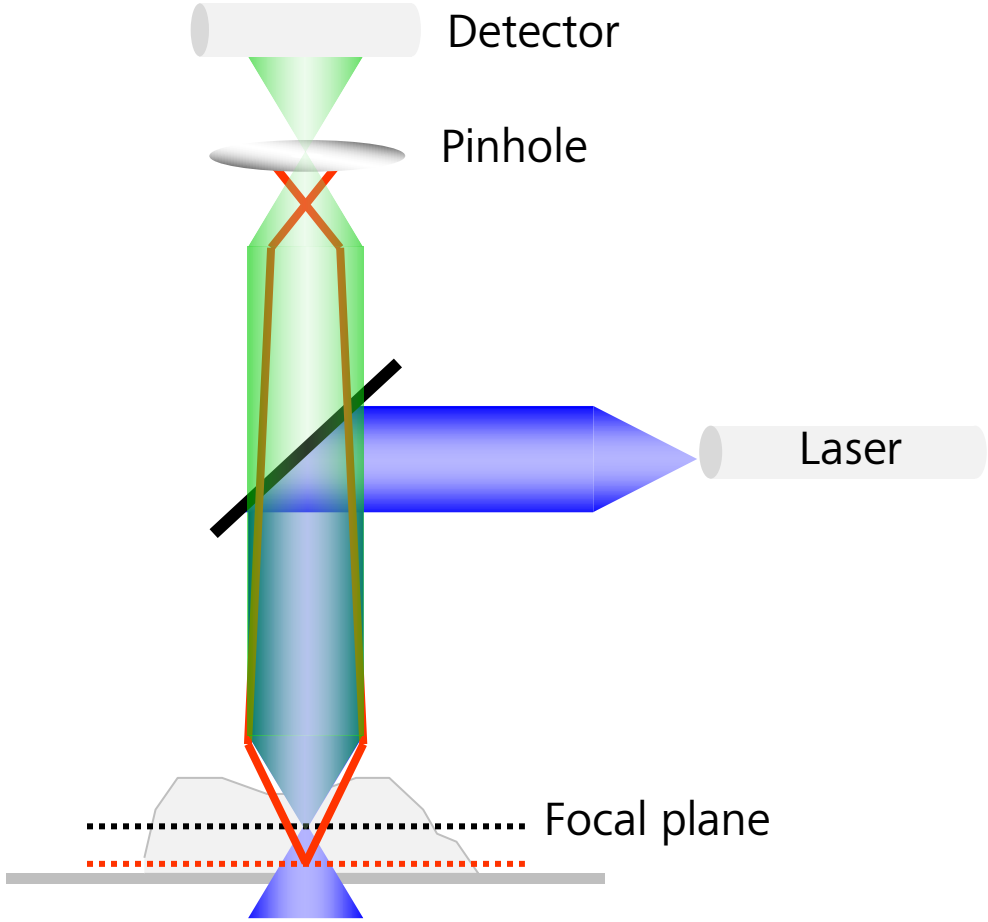


Point Scanning Confocal Microscopes

Confocal principle

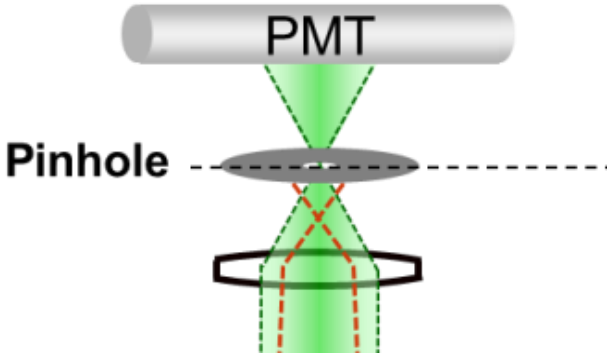


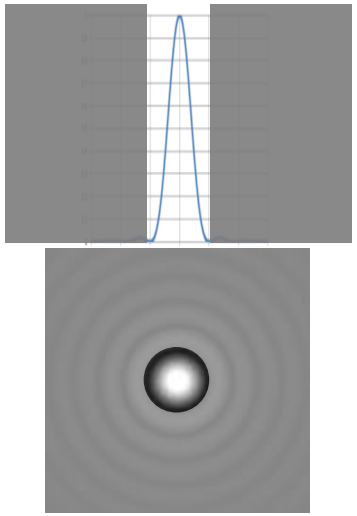


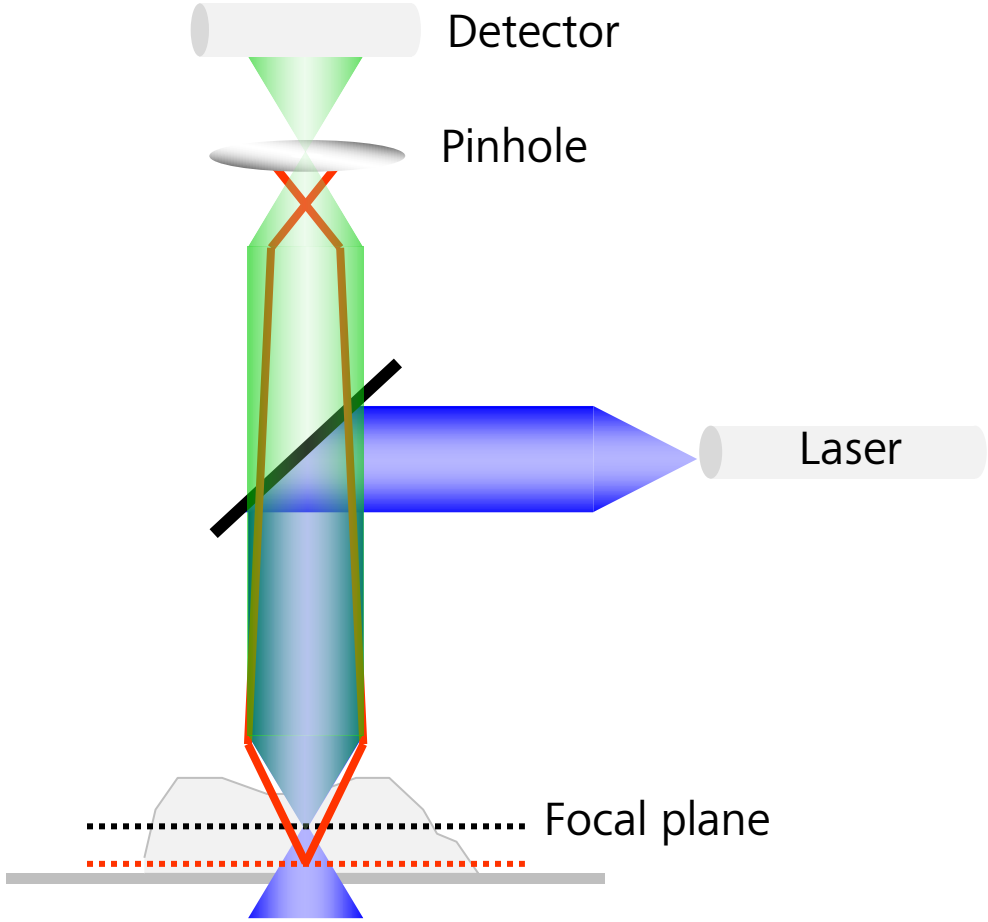


Point Scanning Confocal Microscopes

Confocal principle







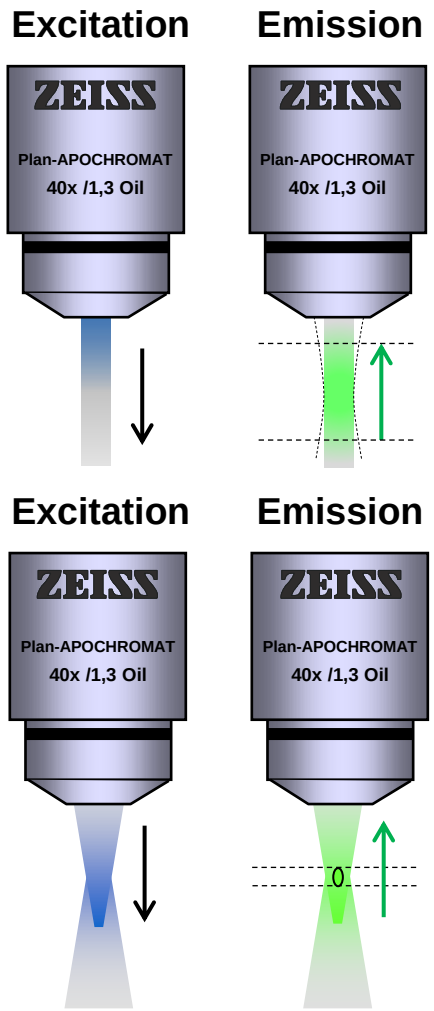
From a Single Spot to a Complete Image

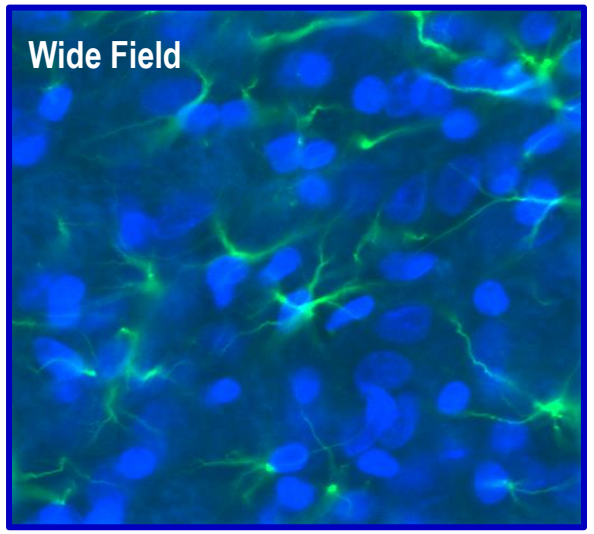
Spot Illumination Requires Two-dimensional Scanning

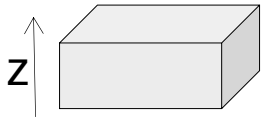
X-Y scanning

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

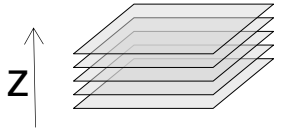
This is usually done by scanning mirrors.







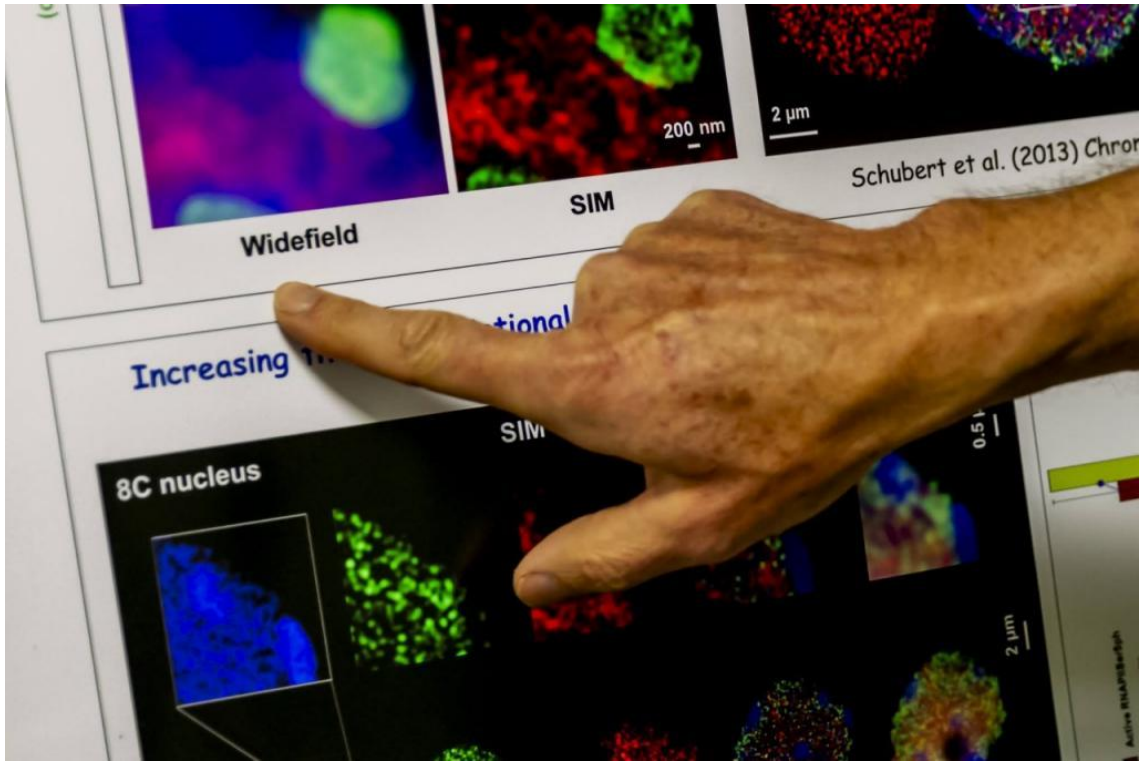
limited z-resolution
thick sections



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

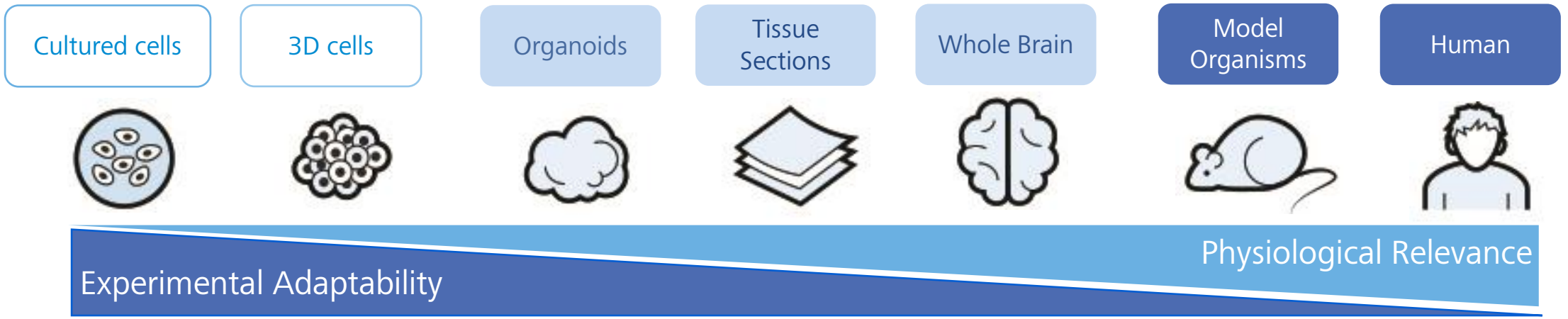


> Microscopy is an important link between the different research groups.

Michael Melzer | IPK Gatersleben

Your needs our motivation

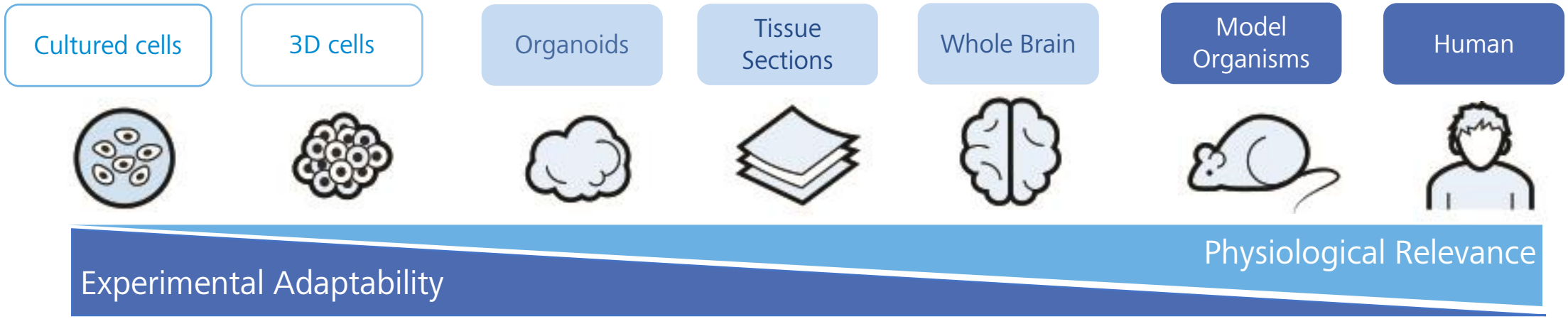
Scaling from 2D Cell Cultures to New 3D Model Systems



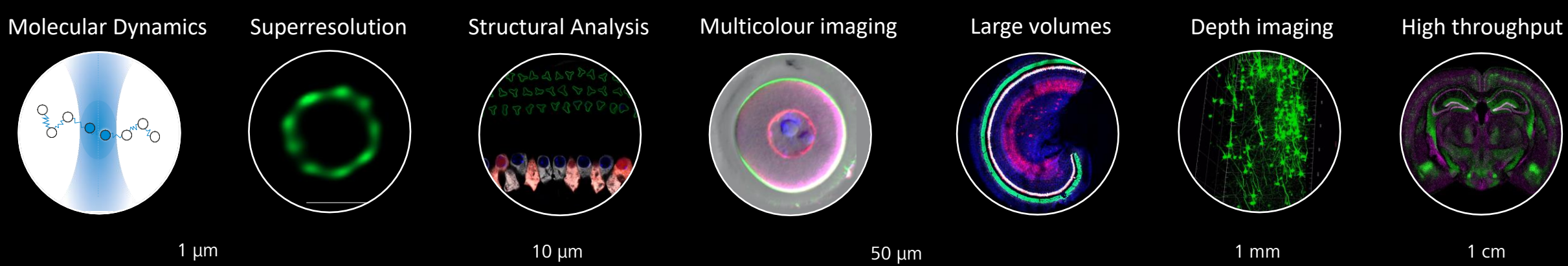
Adapted from https://academic.oup.com/ib/article-abstract/8/6/672/5115178

Your needs our motivation

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



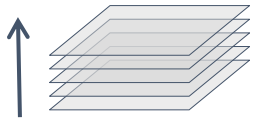
Adapted from https://academic.oup.com/ib/article-abstract/8/6/672/5115178



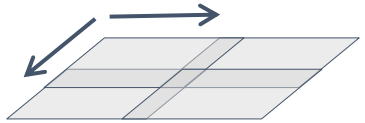
Integrated Imaging Platform

Confocal imaging

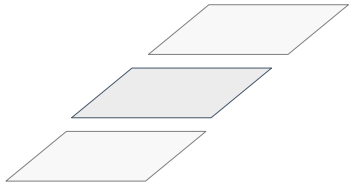
Snap



Z-Stack



Tiles



Time Series

Software

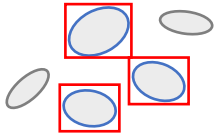
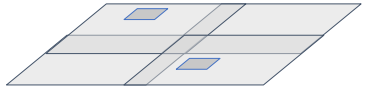
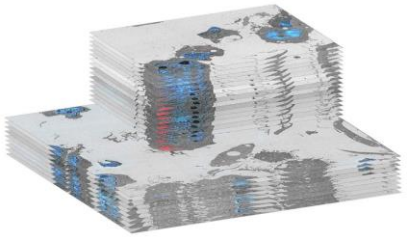


Image Analysis

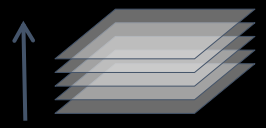


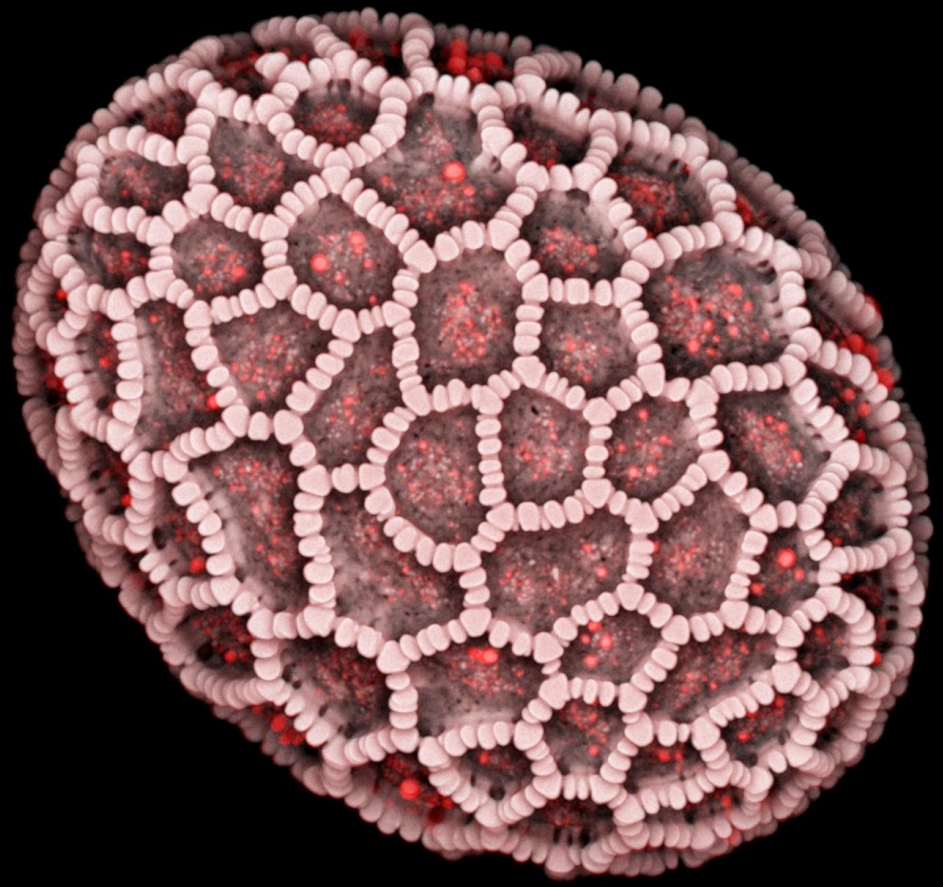
ZEN connect



Correlative Microscopy

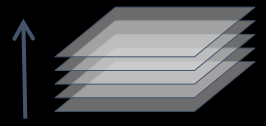
High Resolution Optical Sectioning



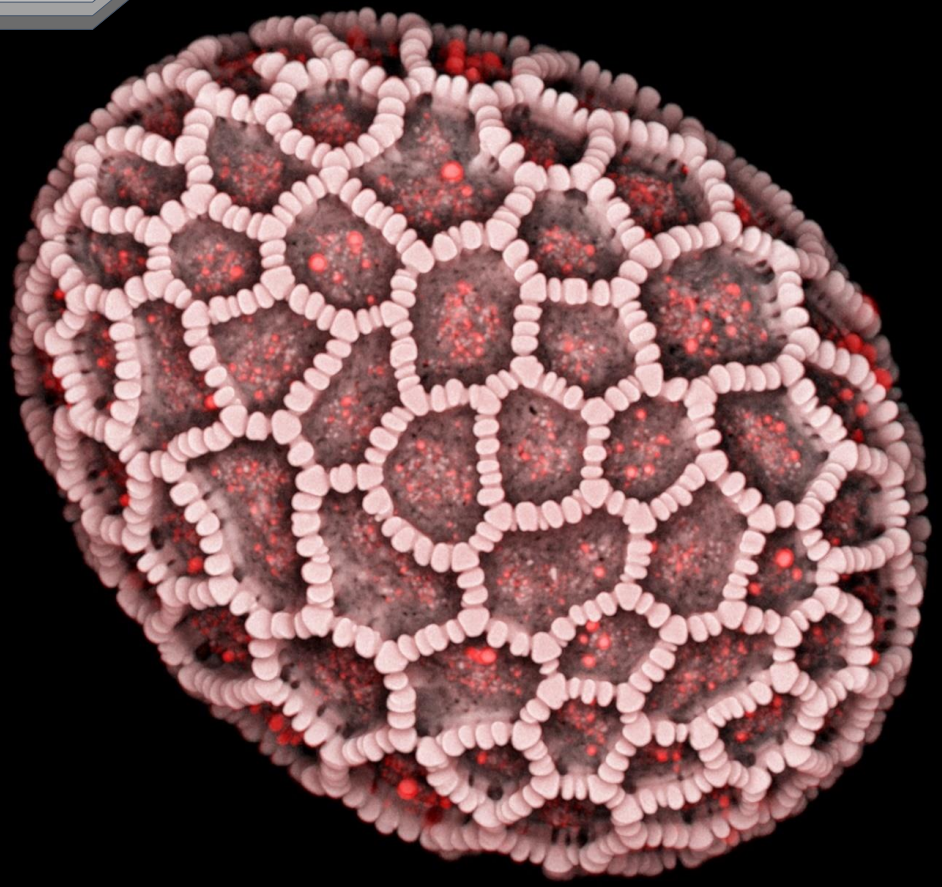


Lilium auratum pollen grain. Airyscan Multiplex mode. Courtesy of Jan Michels, Zoological Institute, Kiel University

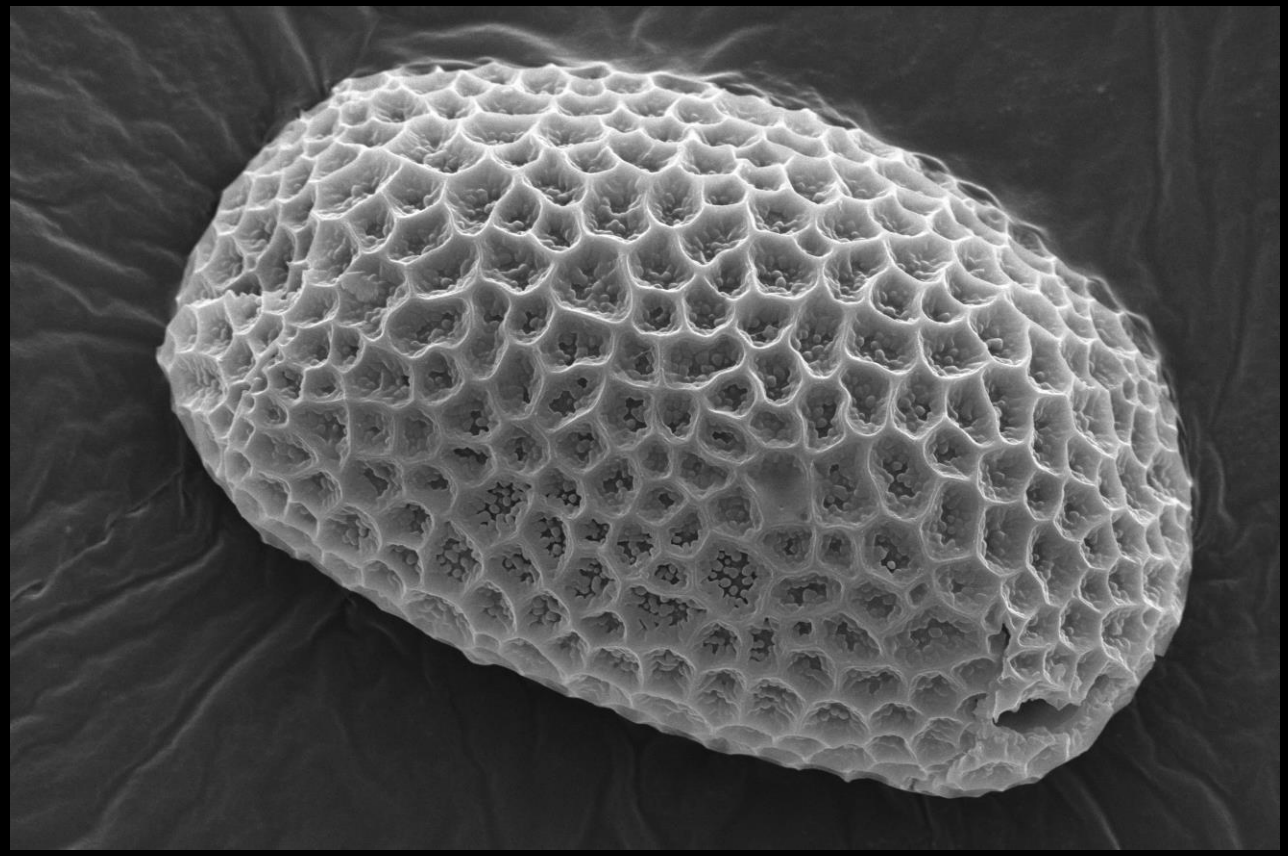
High Resolution Optical Sectioning



LSM

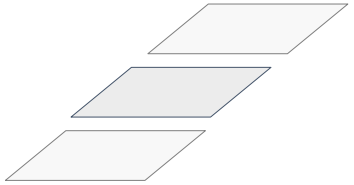


SEM

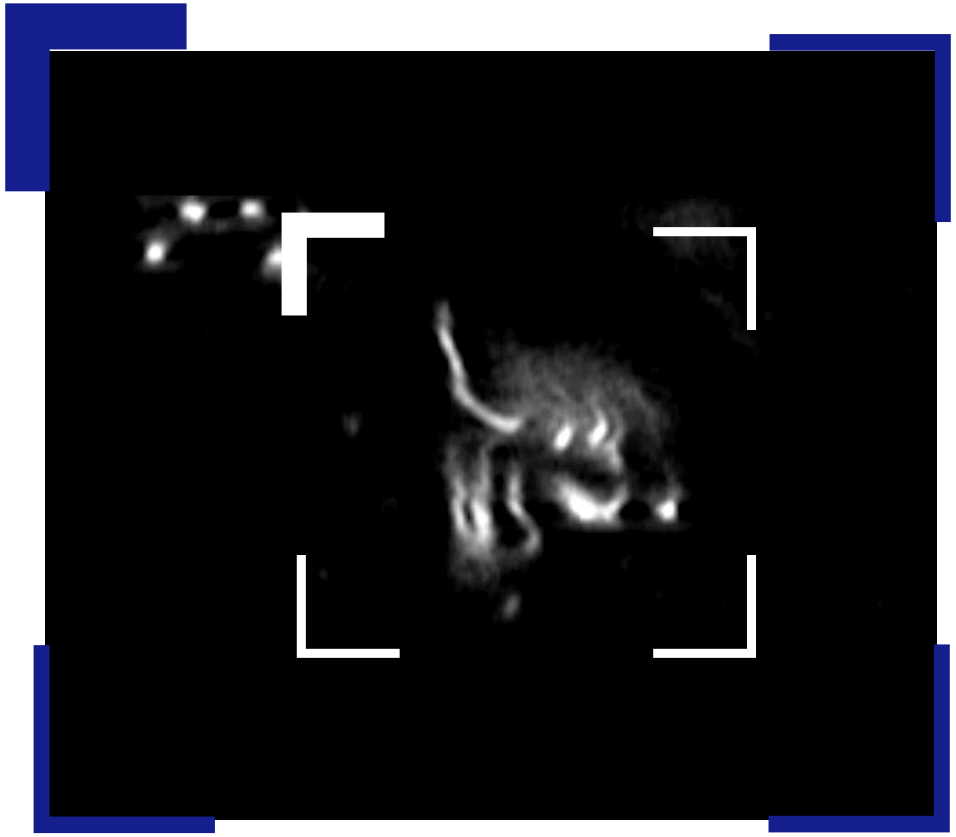


Lilium auratum pollen grain. Airyscan Multiplex mode. Courtesy of Jan Michels, Zoological Institute, Kiel University

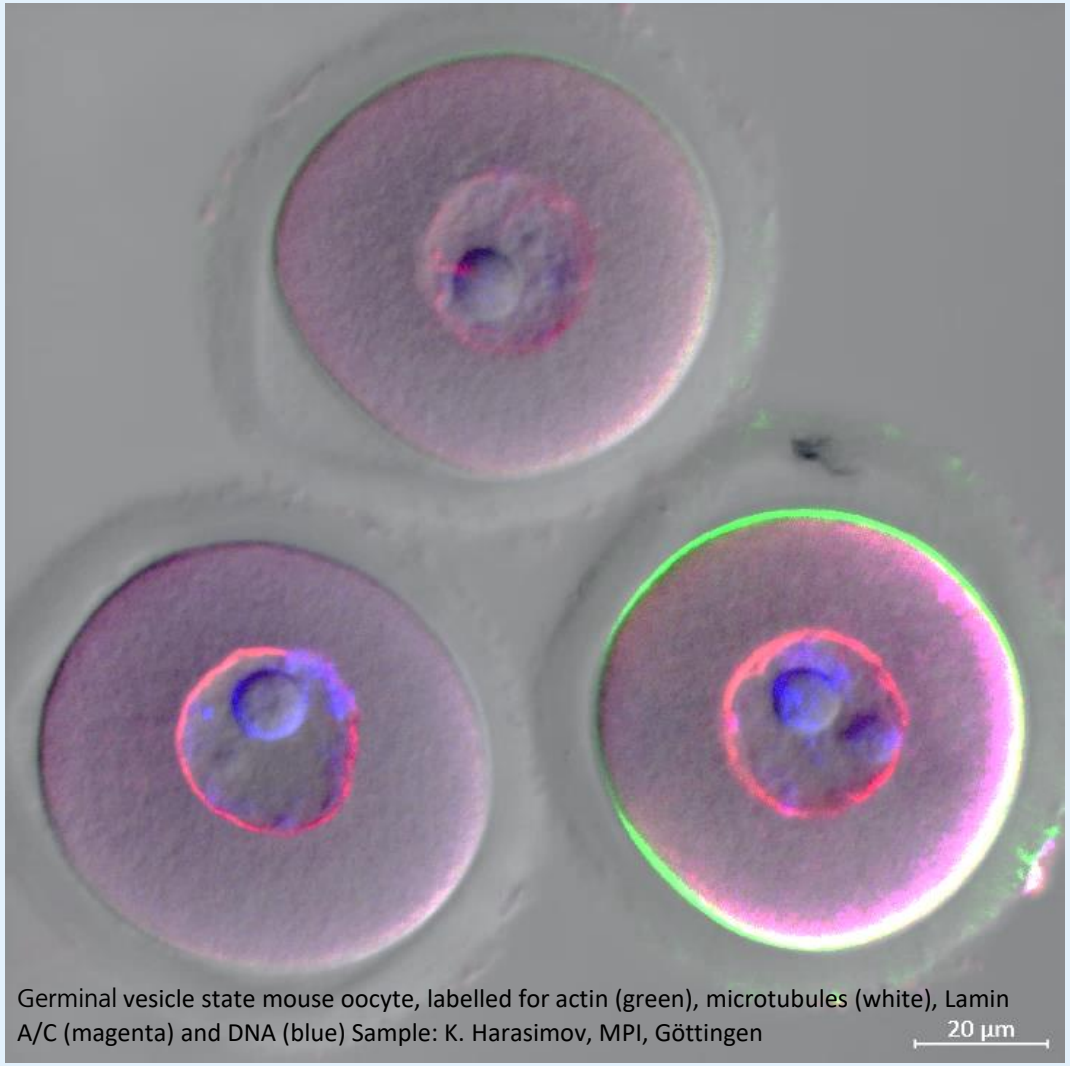
Sensitive & Speedy Imaging



Time series

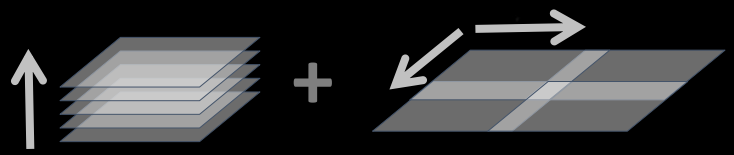


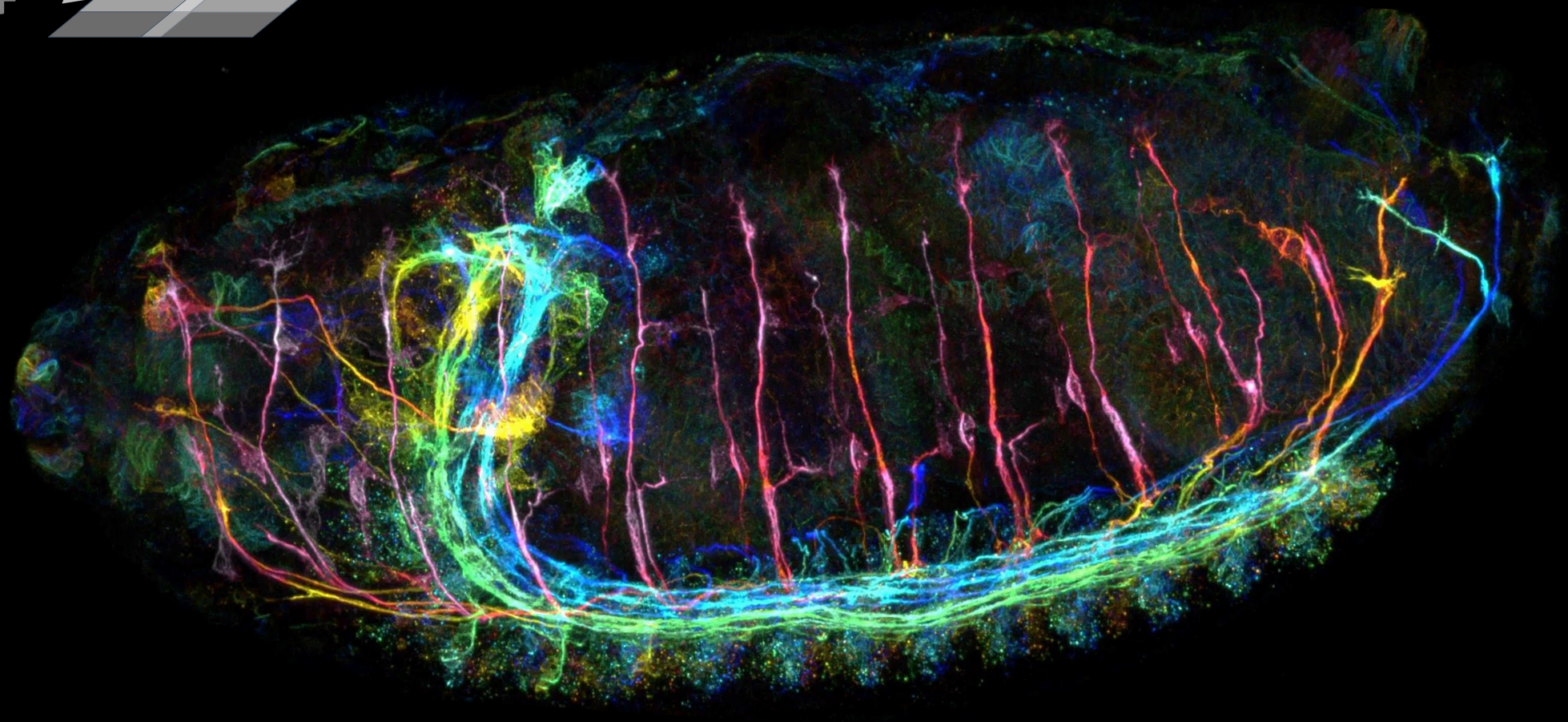
Data courtesy of Ann-Kathrin Günther & Dr. Gregor Eichele, MPI for Biophysical Chemistry, Göttingen, Germany



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

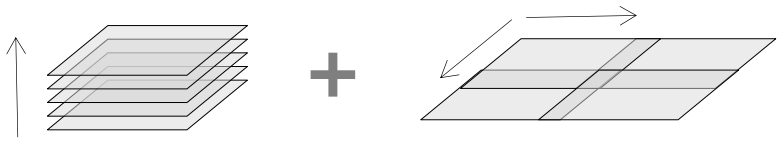
Acquire Large Volumes at Best Quality





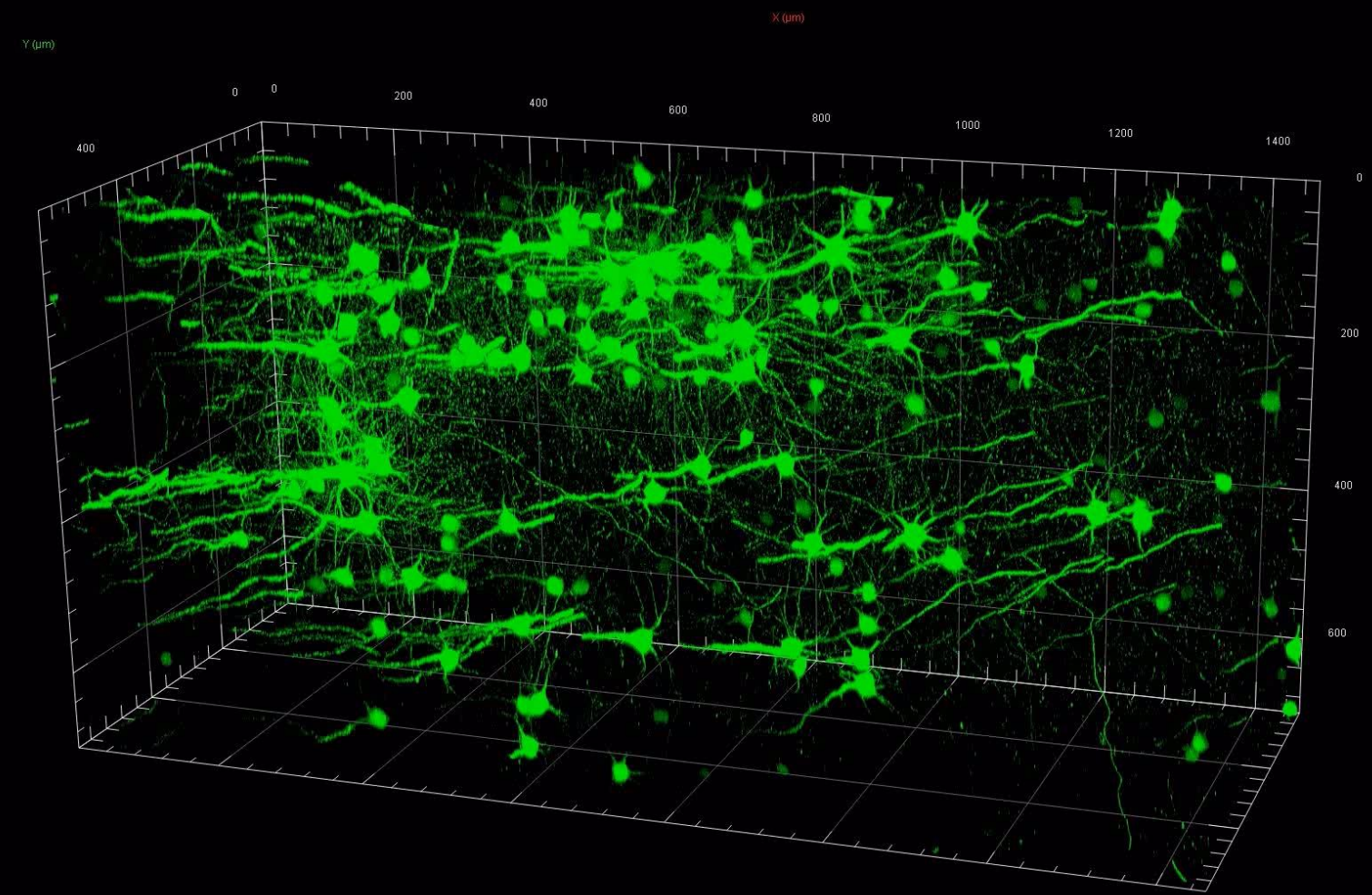
Drosophila melanogaster, CNS and PNS depth coded, Airyscan Multiplex mode. Courtesy of Julia Sellin, LIMES, Bonn, Germany

Large Volume Imaging



Adult mouse brain
Thy1-GFP (Neurons)
CLARITY

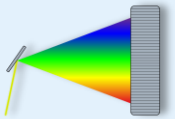
12 tiles and 800µm z-stack
Total sample depth 1.4mm



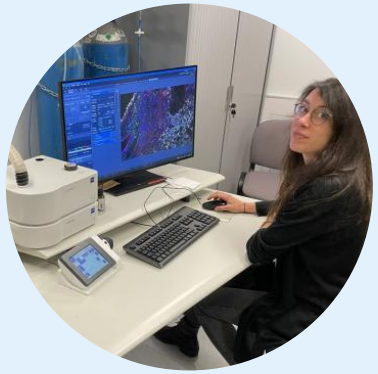
Tobias Ruff, Max Planck Institute of Neurobiology,
Martinsried (Munich), Germany.

Spatial Biology Studies in Lung Tissue using Spectral Microscopy

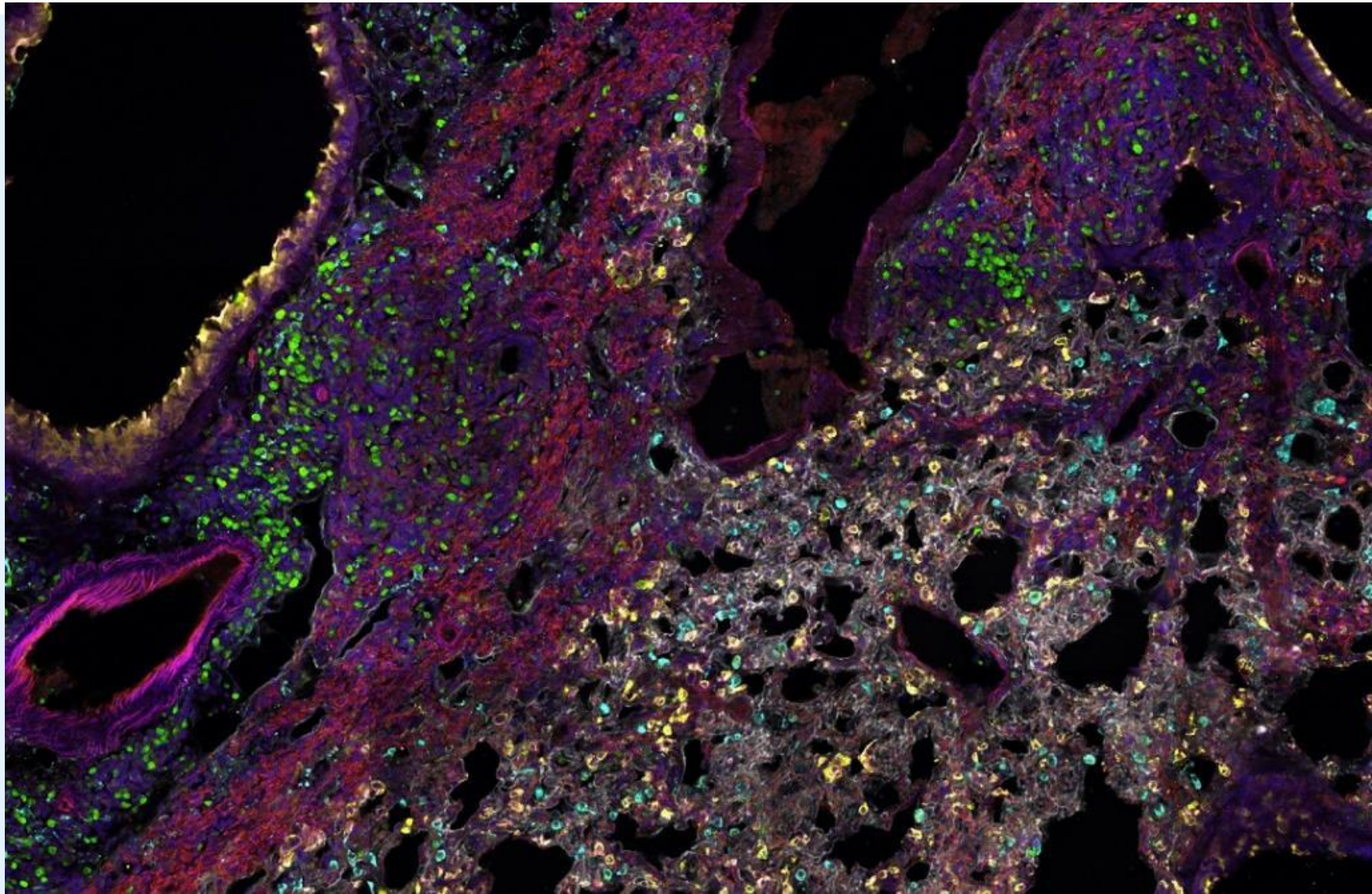
Spectral Unmixing



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

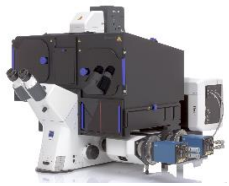


Keep the Context of Your Experiments

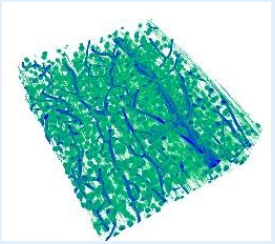
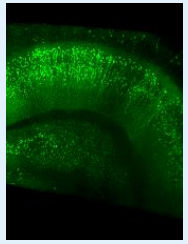
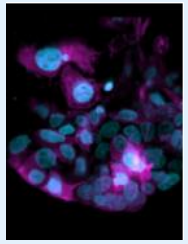
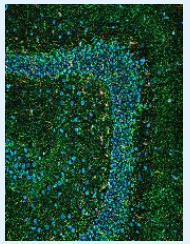
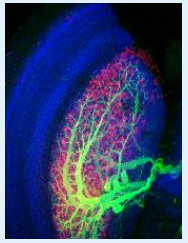
X-ray	Light Microscopy					Electron Microscopy			Ion

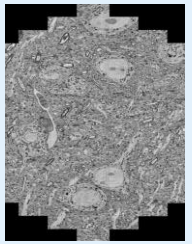
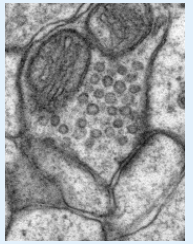
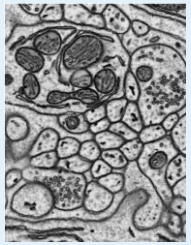







X-ray	Lightsheet	Lattice Lightsheet	Widefield	LSM Airyscan	Super-resolution	MultiSEM	Field Emission Scanning Electron	Focused Ion Beam	Helium Ion
700 nm	500 nm	290 nm	250 nm	120 nm	20 nm	5 nm	< 2 nm	< 1 nm	< 0.5 nm







Why Correlative Microscopy ?

X-ray	Light Microscopy	Electron Microscopy	Ion

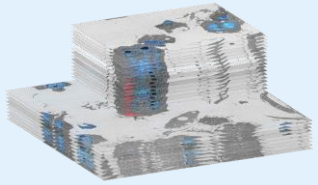
Dive into ultrastructure

Multi-dimensional research

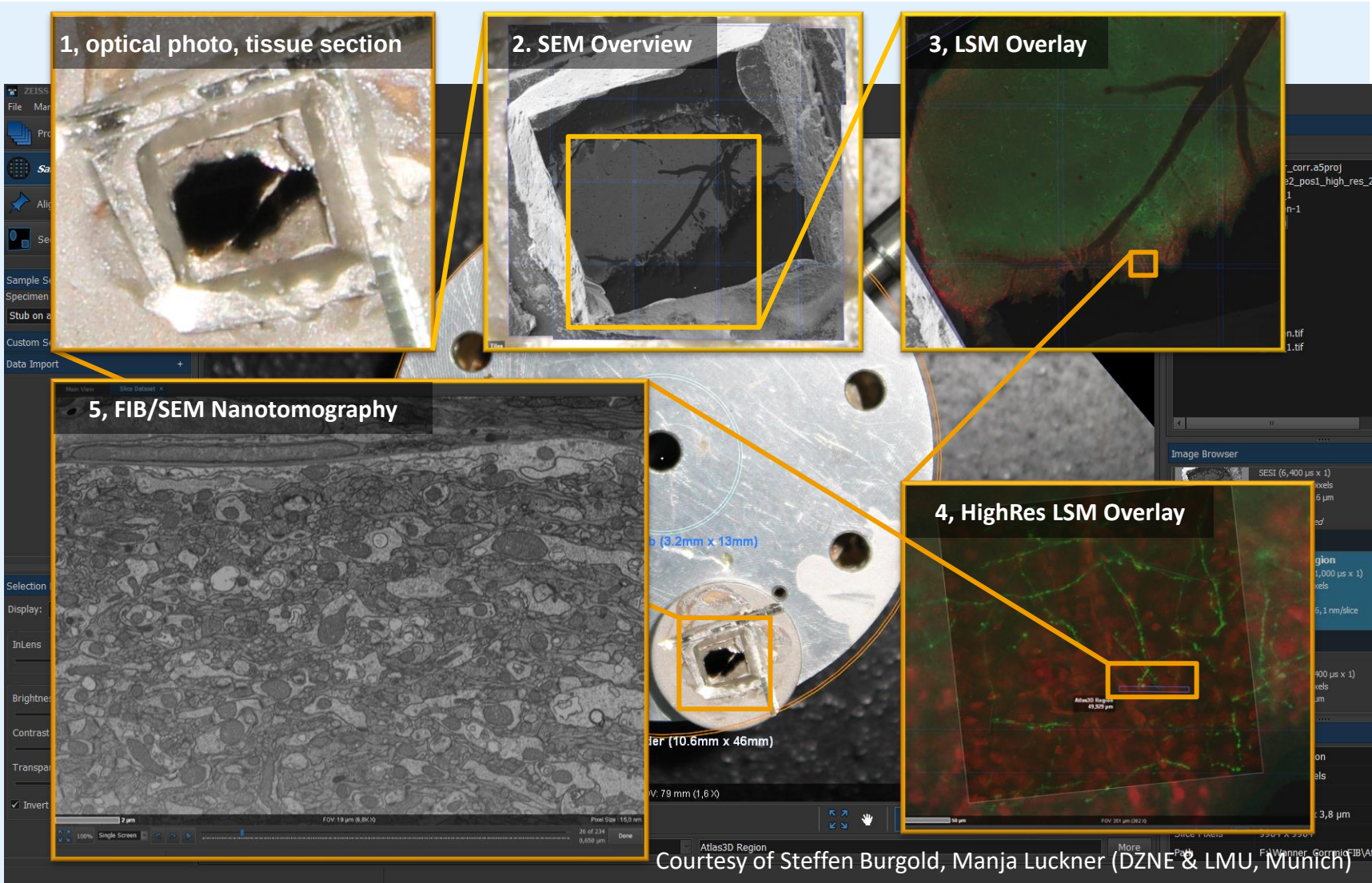
700 nm | 500 nm | 290 nm | 250 nm | 120 nm | 20 nm | 5 nm | < 2 nm | < 1 nm | < 0.5 nm

Confocal Microscope to FIB/SEM for Targeted Milling

Correlative Microscopy



- Connect various microscopy system (i.e., LM, EM, XRM)
- Combine analytical solution (i.e., Raman, EDS)

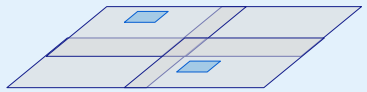


Courtesy of Steffen Burgold, Manja Luckner (DZNE & LMU, Munich)

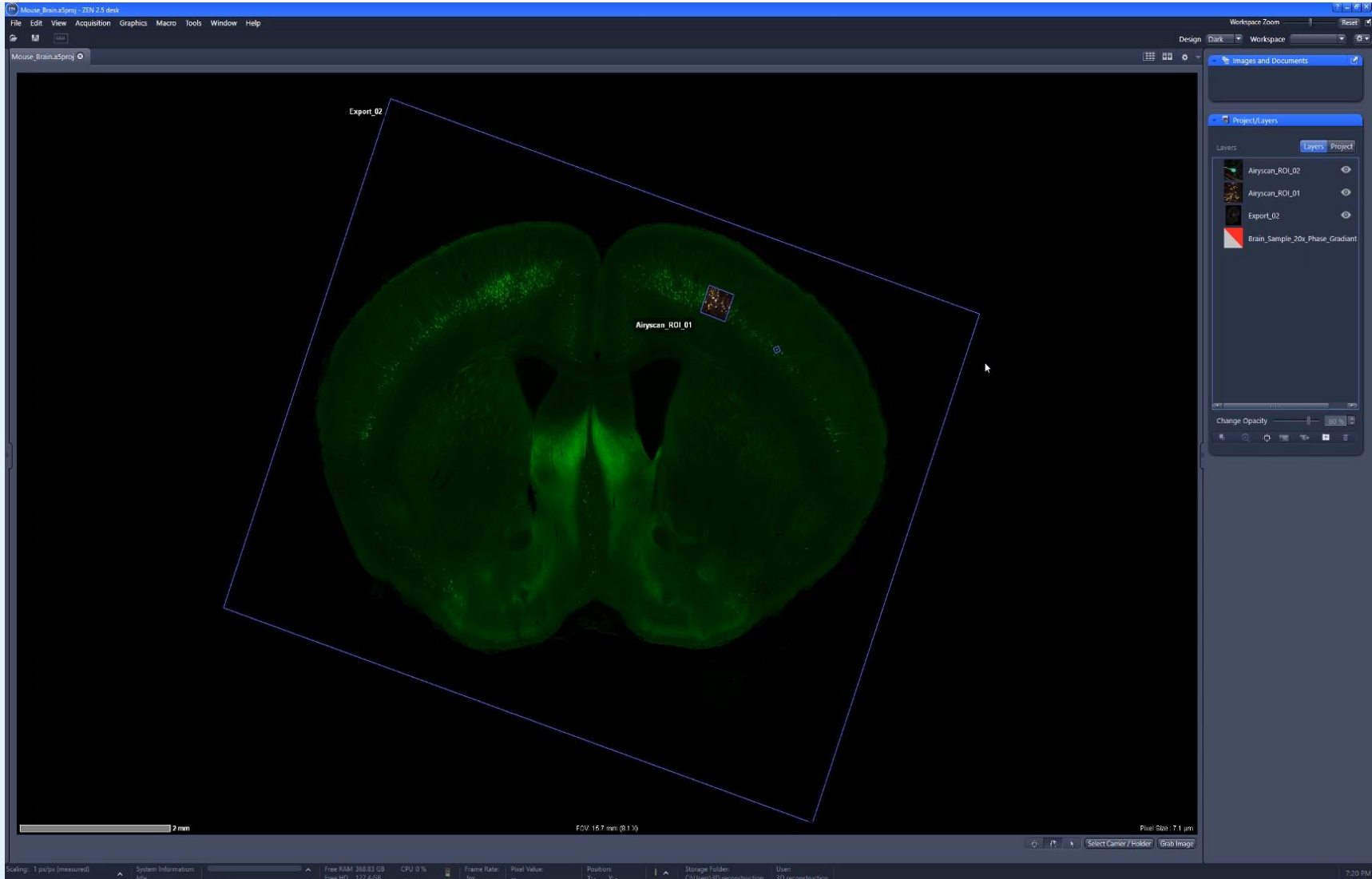
Effortless Image Acquisition and Analysis

Data Integration between Different Imaging Modalities

ZEN Connect

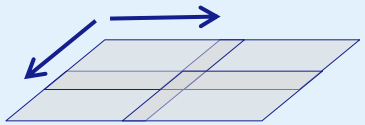


- Overlay and alignment of all your images
- Intelligent data management



Convenient Overview & Navigation

Navigated Imaging



Acquire general view of zebra fish at low magnification

Sample courtesy of J. Hartmann and D. Gilmour, EMBL,Heidelberg, Germany

Convenient Overview & Navigation

Navigated Imaging

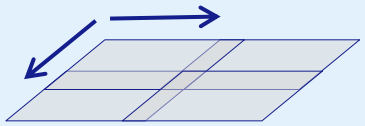
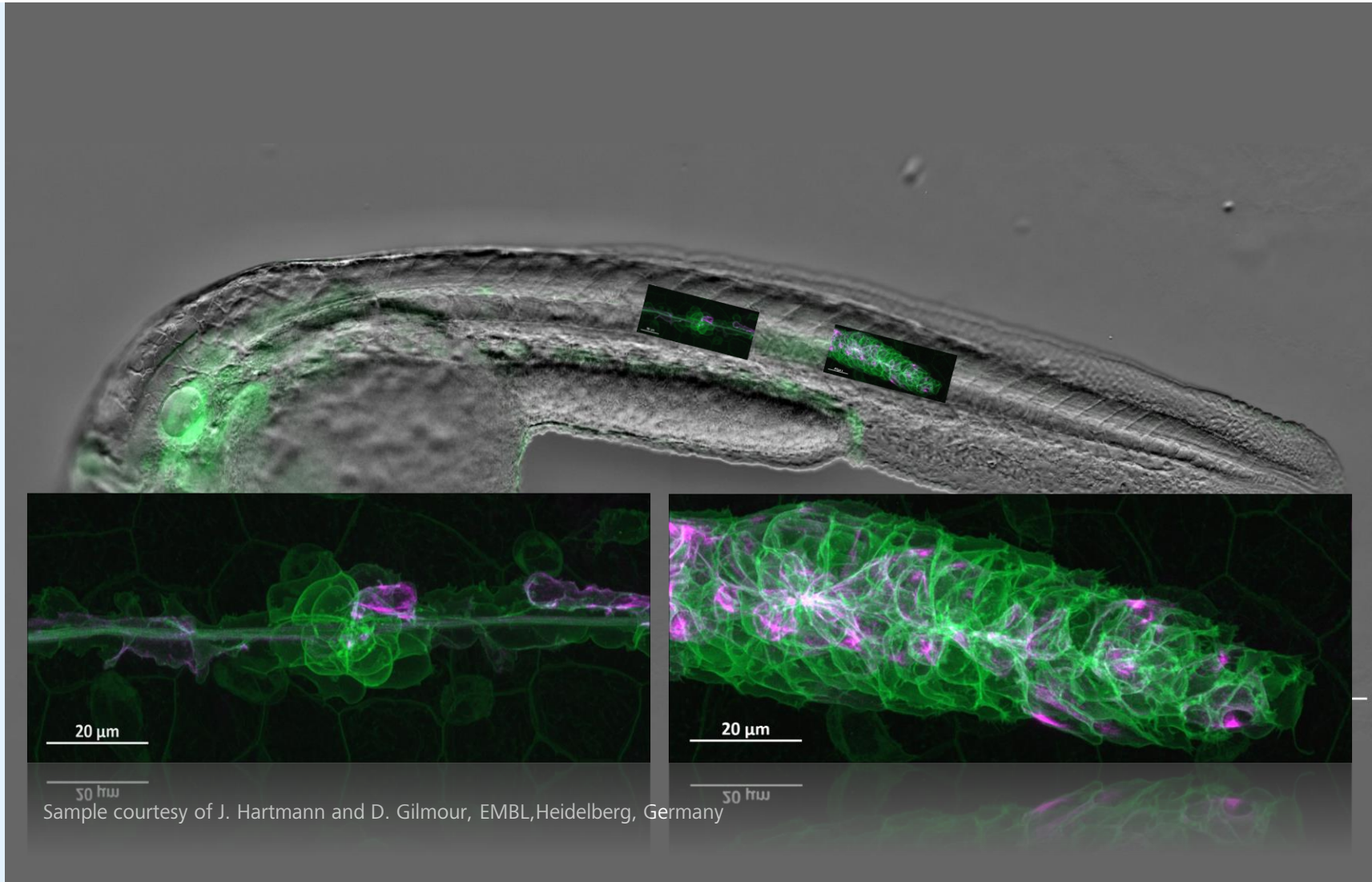
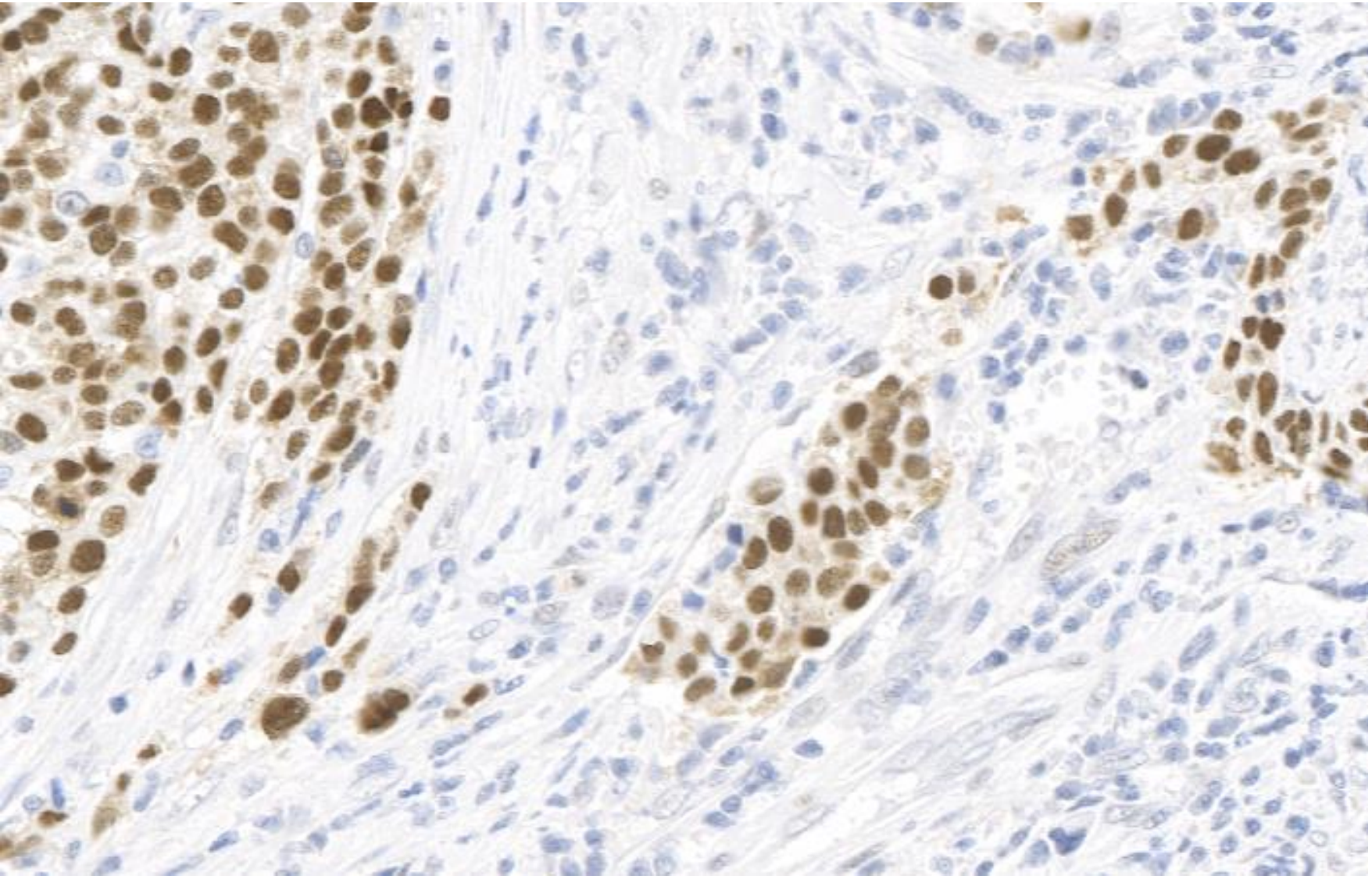


Image neuromast and lateral line with high resolution



Sample courtesy of J. Hartmann and D. Gilmour, EMBL,Heidelberg, Germany

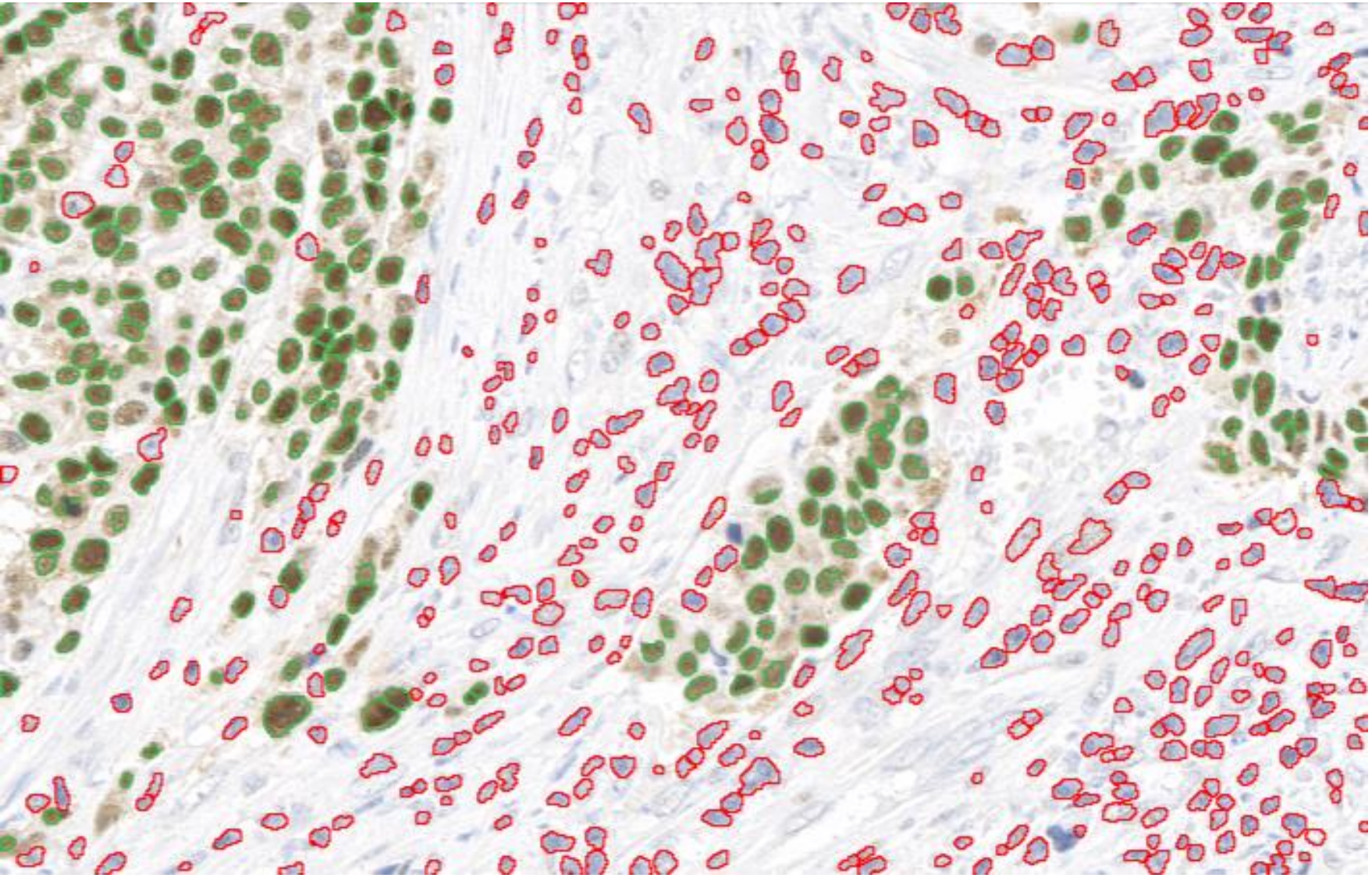
Microscopy Images: A Picture is worth a thousand words!



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%

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

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

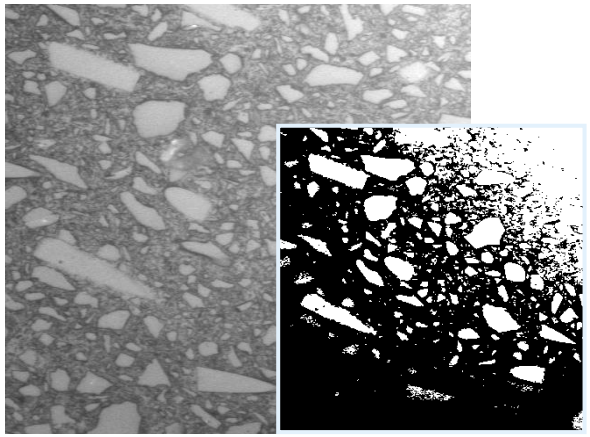
arivis Cloud

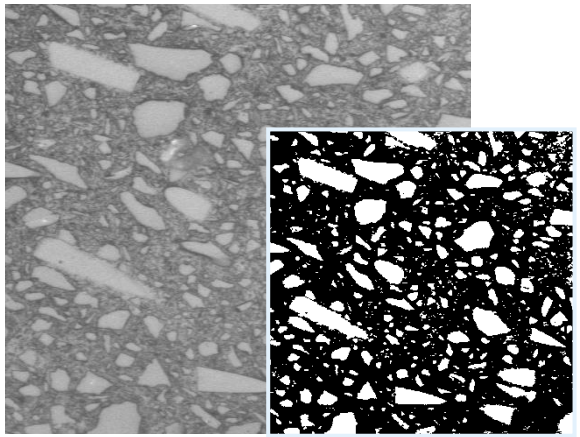
Cloud-based AI image analysis

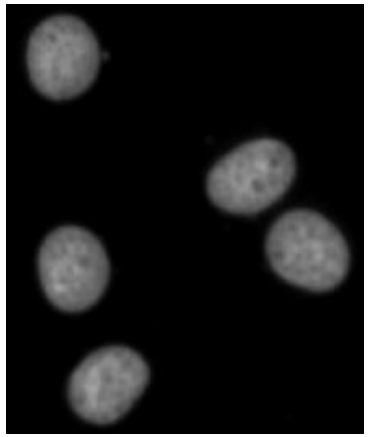
New Feature

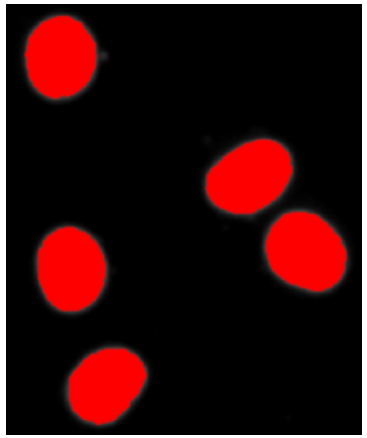
Image Analysis Workflow

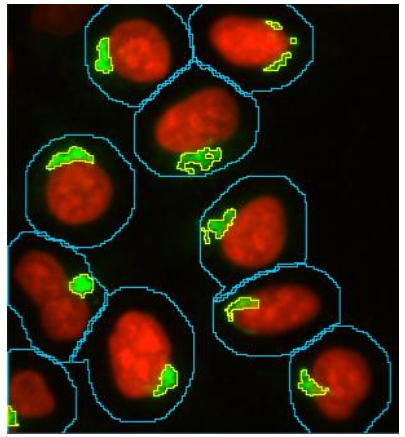
Pre-Processing → Segmentation → Feature Detection → Data Presentation











Object association

Heatmap for HCS

Scatter plot for relationship analysis

	ID	Intensity...	Area [µm²]	Roundness
1	2	3,828.036	156.287	0.710
2	3	3,557.861	169.015	0.569
3	4	4,241.335	146.652	0.802
4	5	3,663.464	160.925	0.762
5	6	3,336.314	155.927	0.718
6	7	4,302.819	153.608	0.736
7	8	3,443.283	173.962	0.697
8	9	3,737.238	166.439	0.826
9	10	4,315.105	158.297	0.688

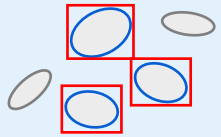
Feature measurements

Scatter plot for relationship analysis

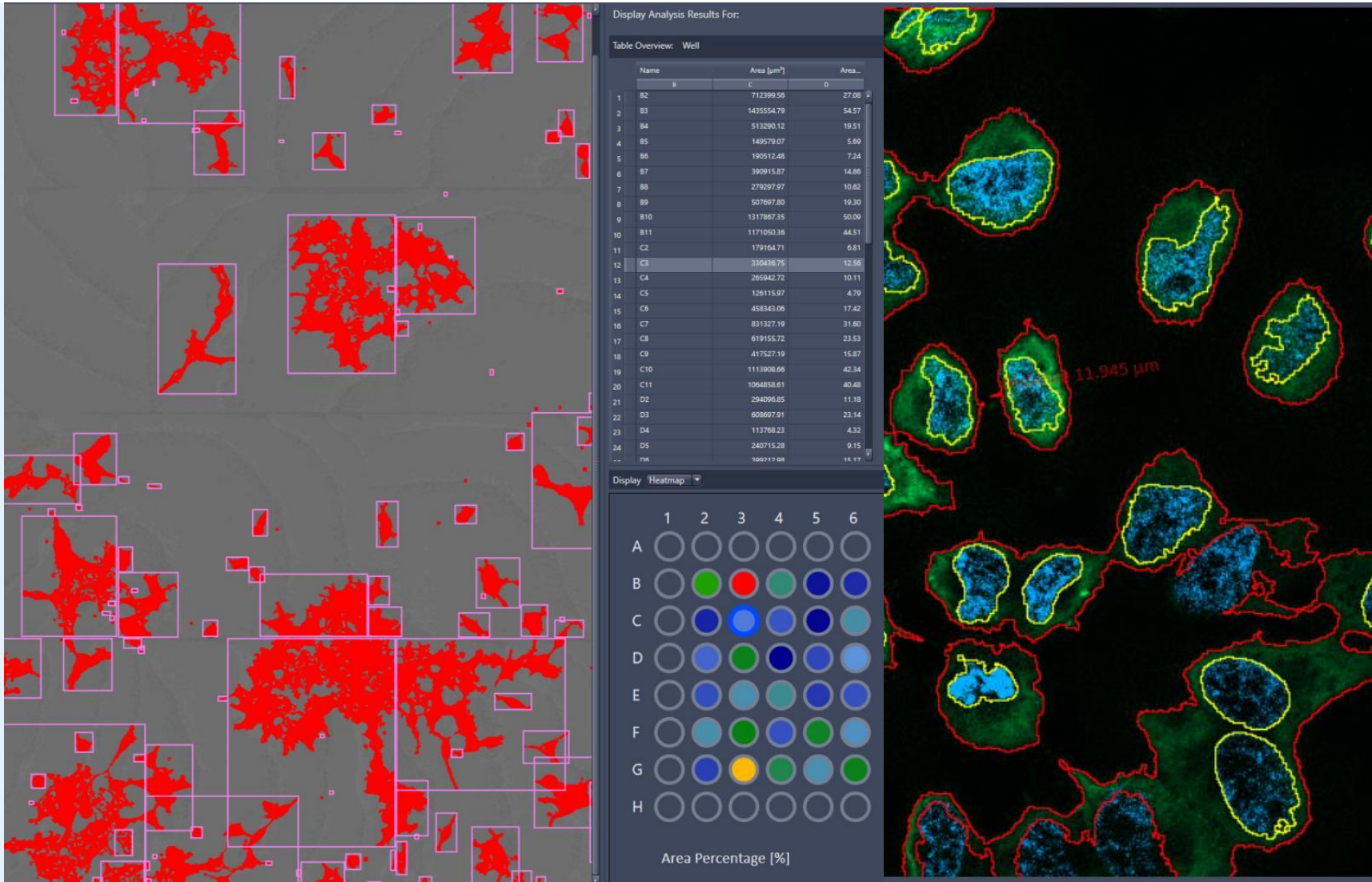
New Feature

Intuitive Analysis Workflow

ZEN Image Analysis

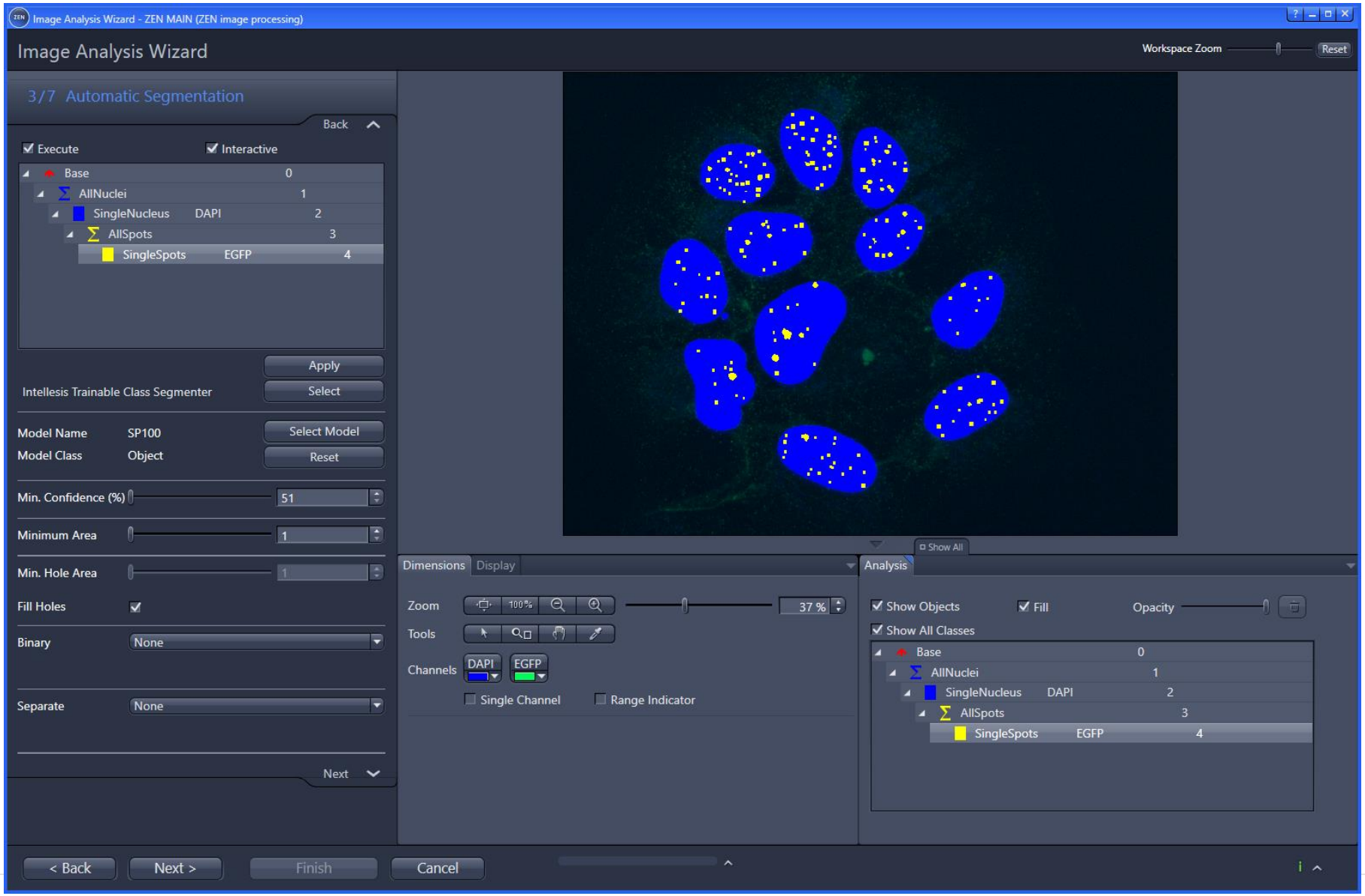


- Step-by-step analysis workflow
- Customize analysis
- Acquire statistical results



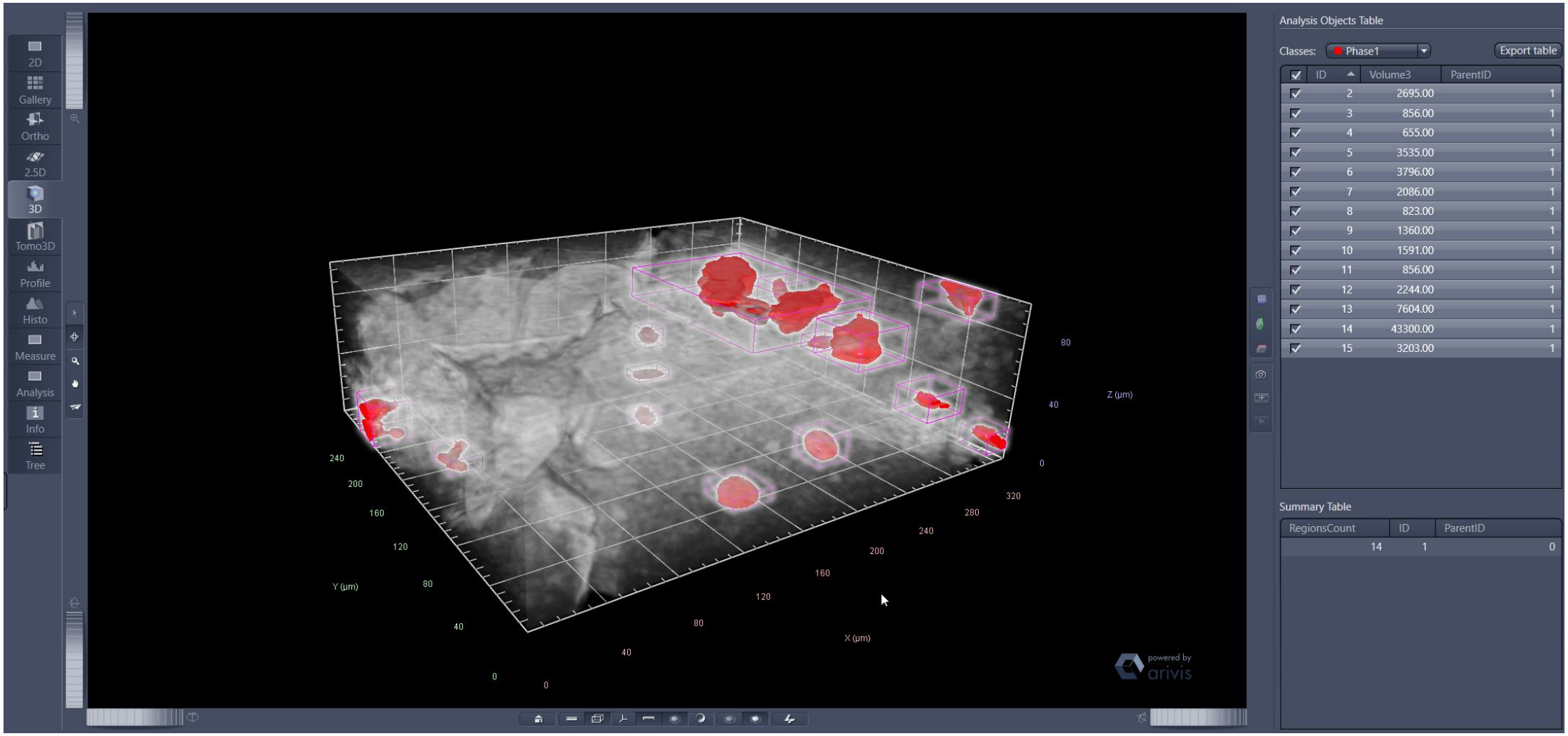
New Feature

2D Segmentation



New Feature

3D Segmentation using a RF-based model in ZEN blue

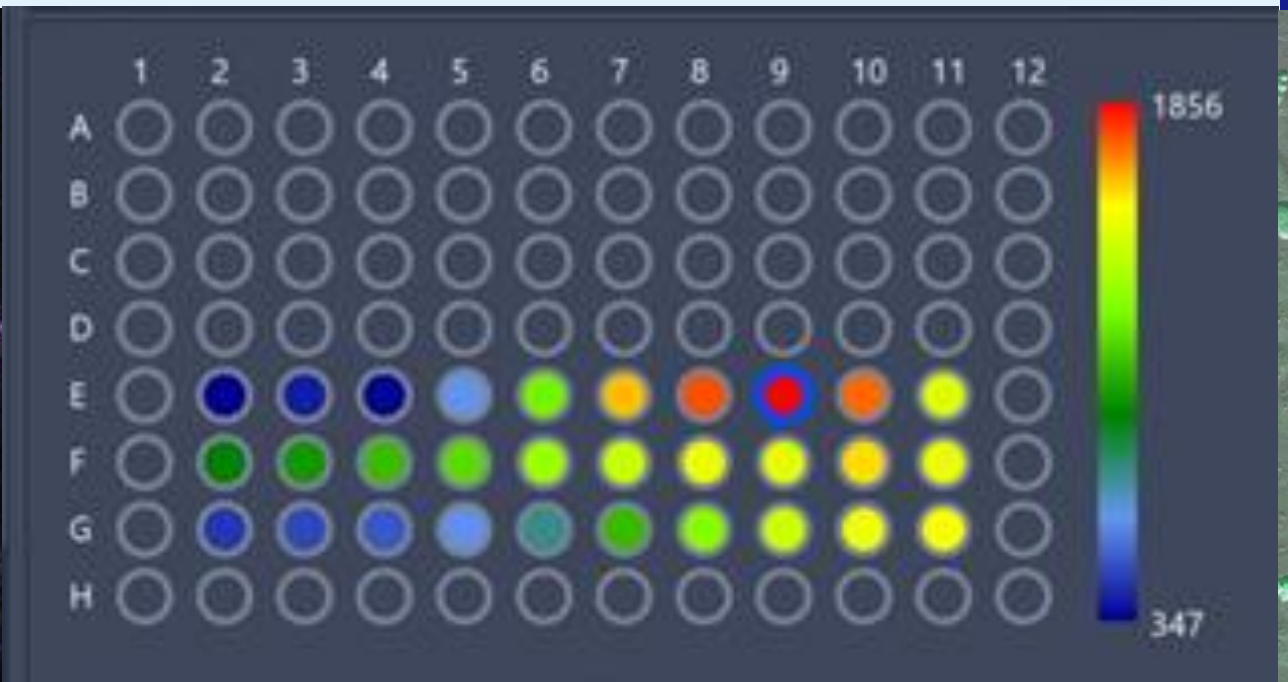


New Feature

Simple, Modular, AI-powered Image Analysis

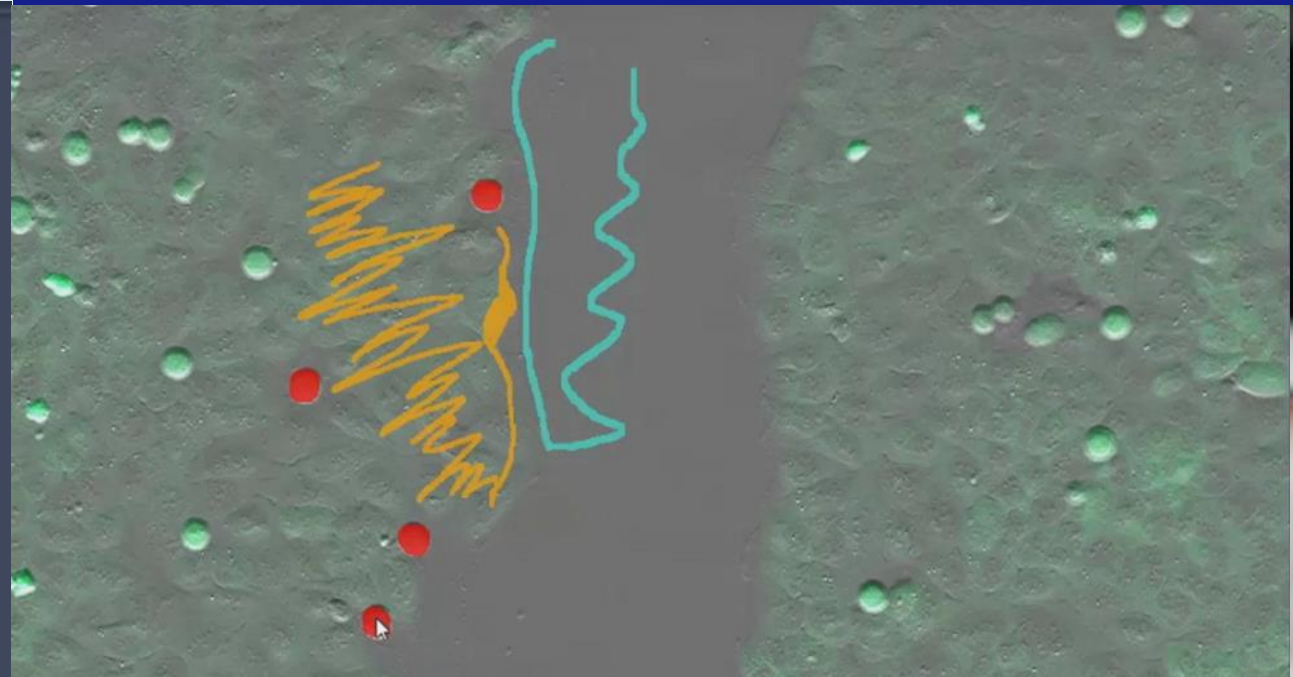
BioApps

- Application-specific tools for cell-based imaging laboratories
- Easy-to-use with an intuitive user interface
- Concise results in easy-to-read formats



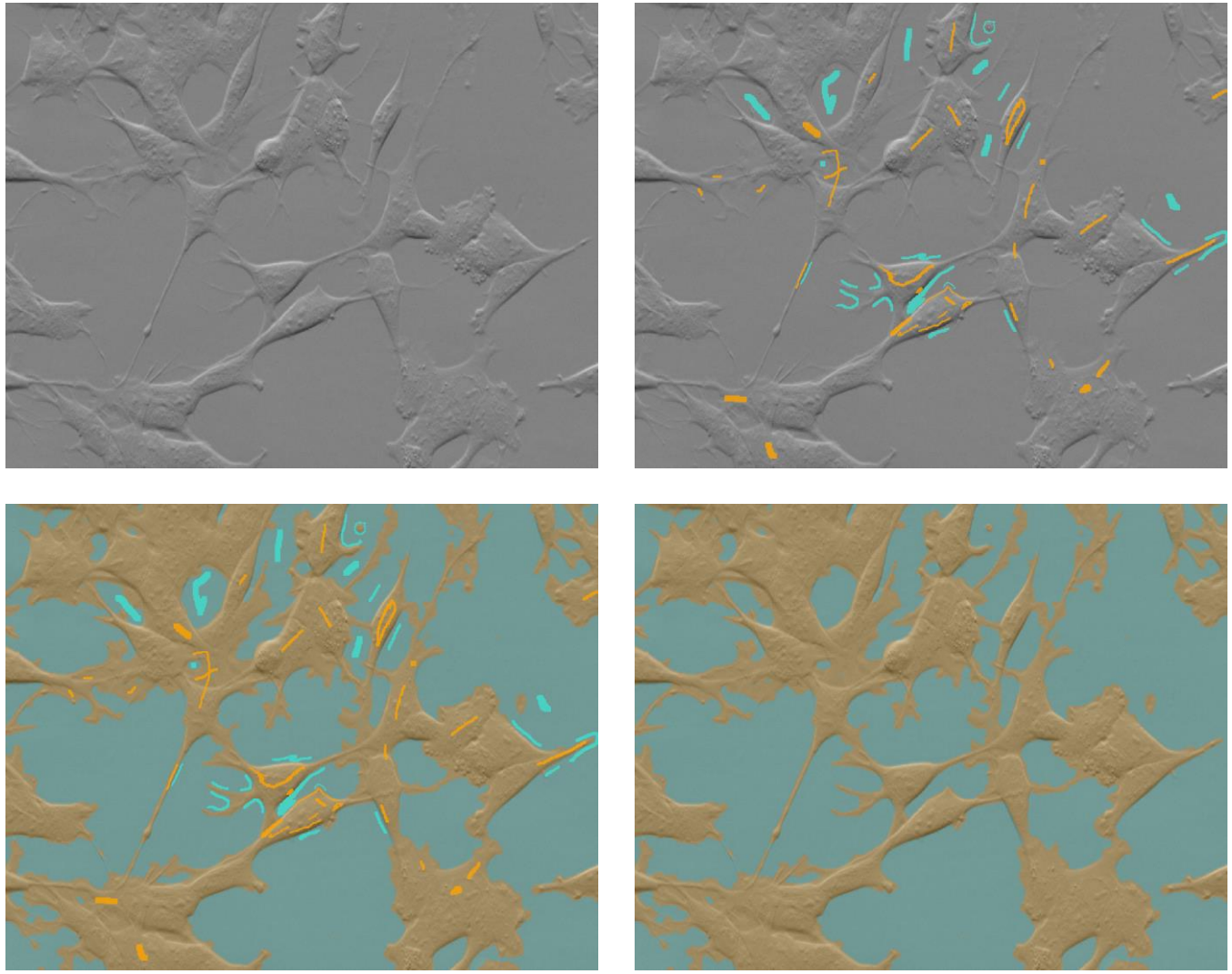
Intellesis

- Automated image segmentation powered by machine learning
- Use your expertise to train the software on your own images
- Analyze multimodal images from different sources



New Feature

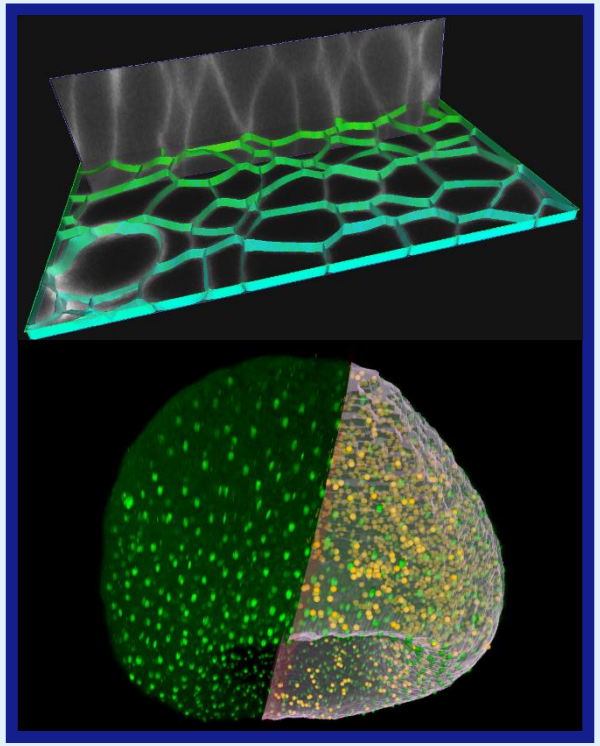
Intellesis – Simple User Interface



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

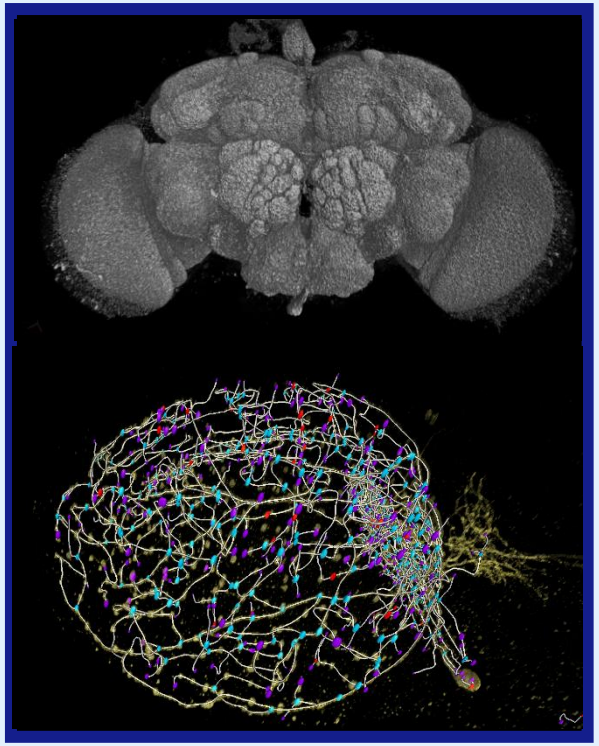
New Feature

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



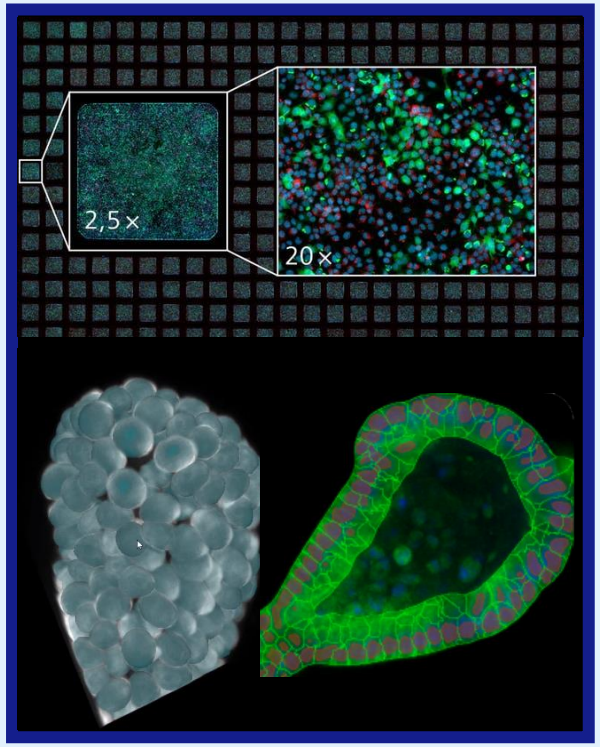
Developmental Biology

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



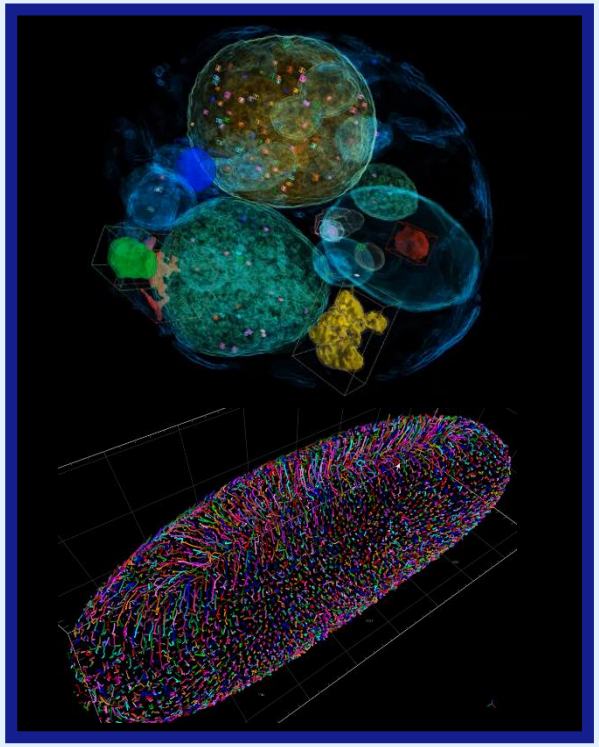
Neuroscience

Compartment Analysis
Distribution Analysis
Stitching / Multi-view image reconstruction



High Content

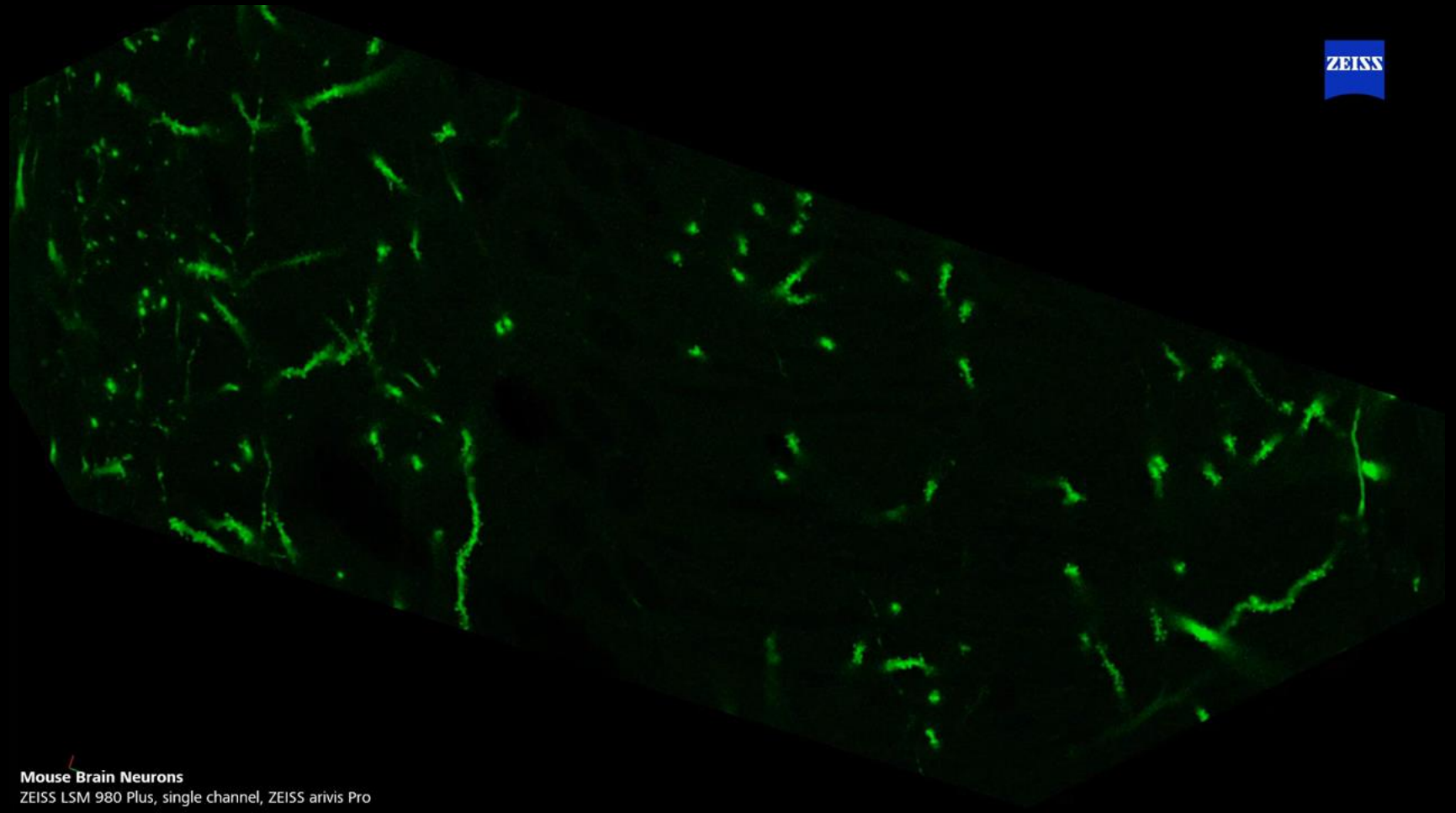
Well-by-well analysis
Cell counting
Organoids and Spheroids



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

Seeing beyond