IMPRS NeuroCom

Microscopical Imaging in Neuroscience

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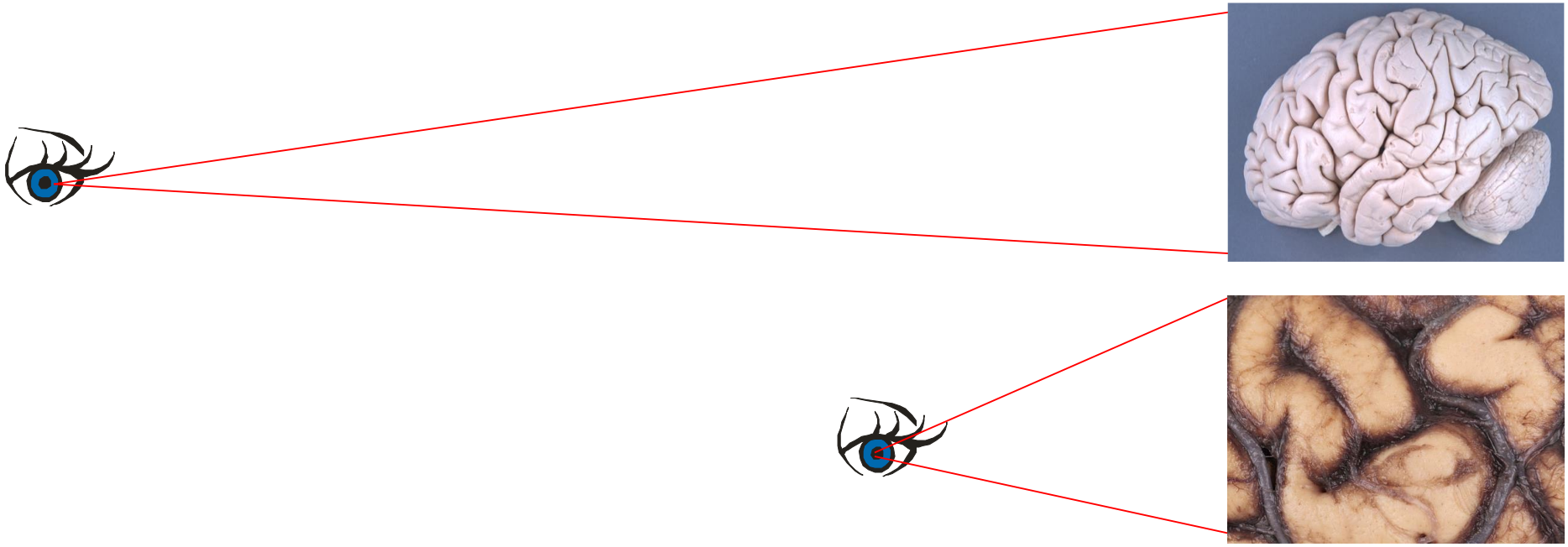
Microscopy

The basics:

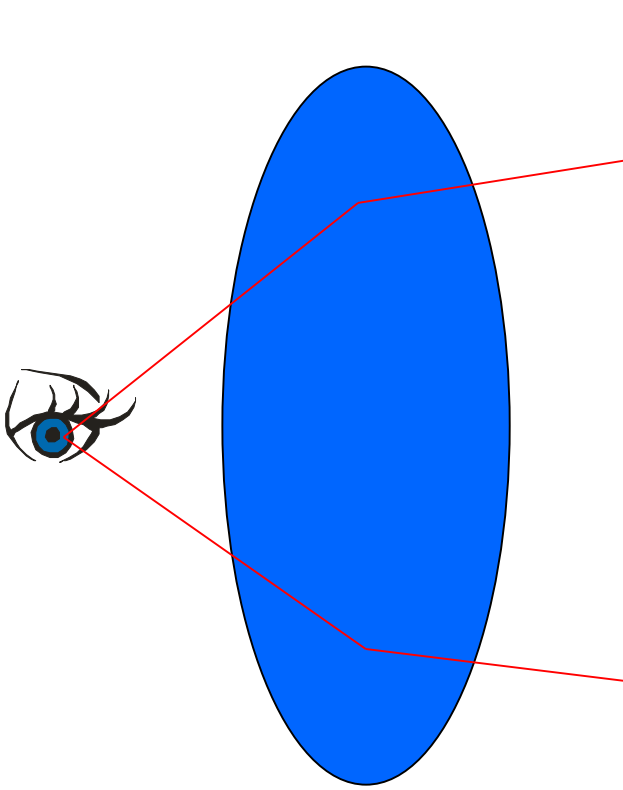
Seeing! Determined by the "resolving power", the smallest distance between neighboring pixels that can be perceived separately. The resolution is limited by the angle that two rays form in the eye that hit adjacent photoreceptors.
The resolving power can be increased by changing the angle, under which an object appears.

This can be achieved on the one hand by approaching the eye

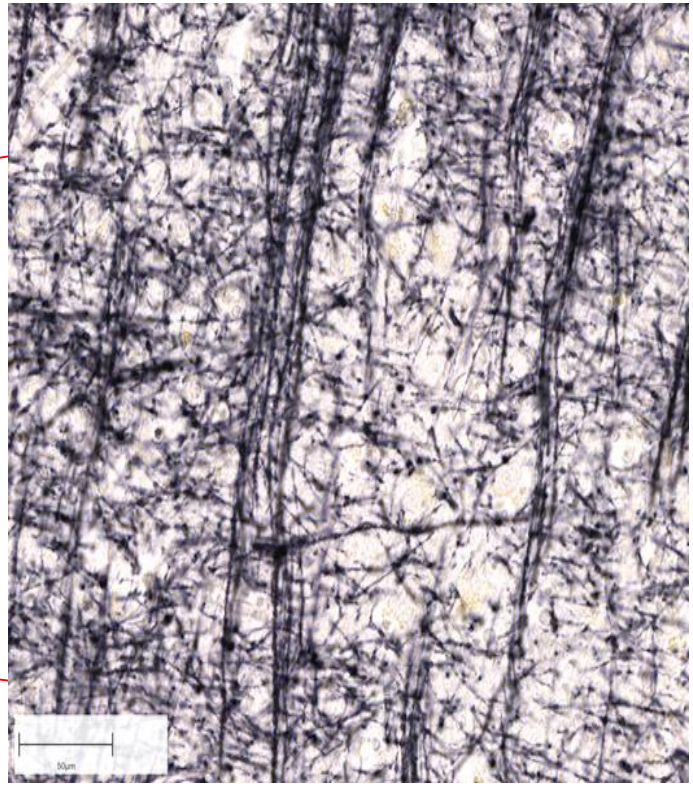


Microscopy

or by optical aids, e.g. through "microscopes / lenses" of all kinds.



Lens



Myelinated fibres human cortex

MICROSCOPY

Greek

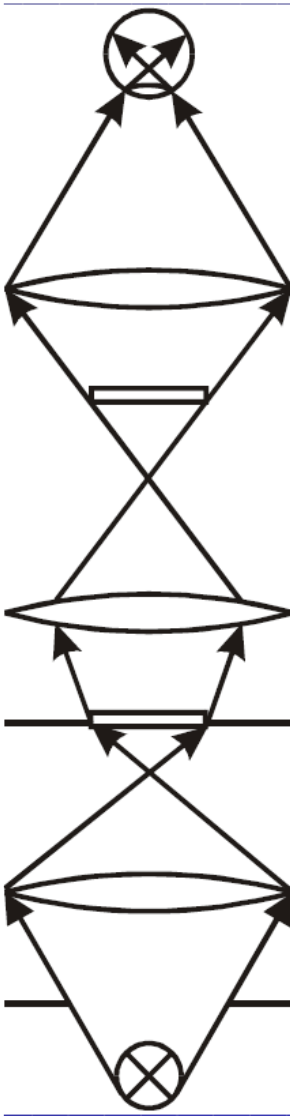
mikro = small

skopien = to observe

“To observe small things“

Microscopy

Principle of the light microscope:



Eye or camera

virtual, right-sided, magnified image

Eyepiece as a magnifying glass (2. magnification)

Intermediate image

real, inverted, magnified image

Lens (1. magnification, variable)

Specimen

Condenser

Illumination with aperture

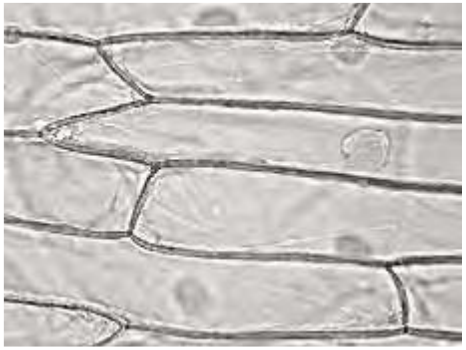
Microscopy

Light microscopic techniques:

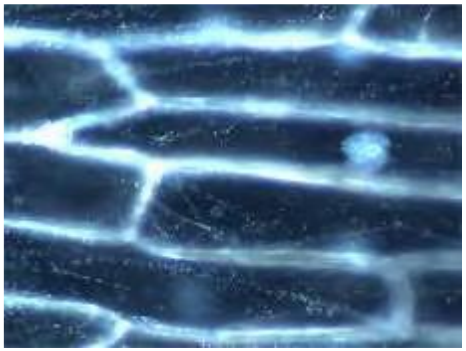
Brightfield microscopy:
Different absorption of light

Without tissue

With tissue

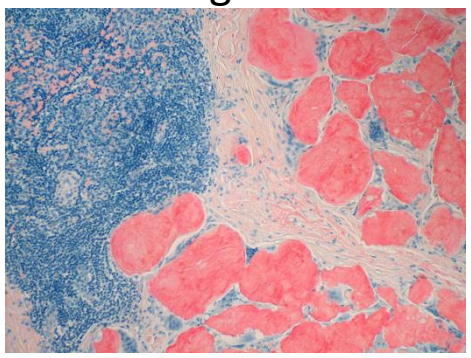


Dark field microscopy:
No direct light in the lens-sample, stray light, Tyndall effect

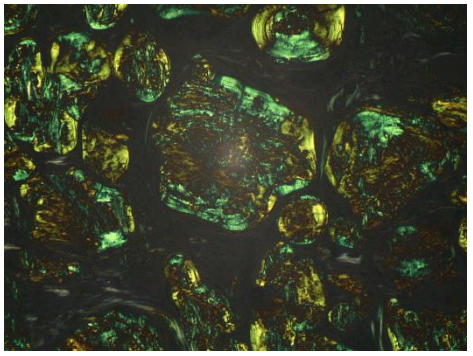


Polarizing microscopy:
Different indices of refraction of light.
It is used to examine optically anisotropic (birefringent) objects.

Brightfield



Polarisation

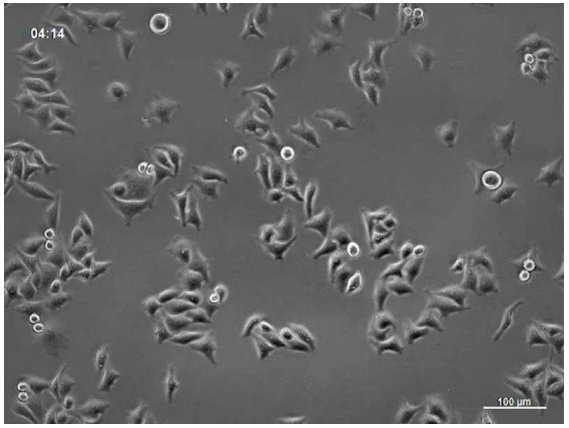


Microscopy

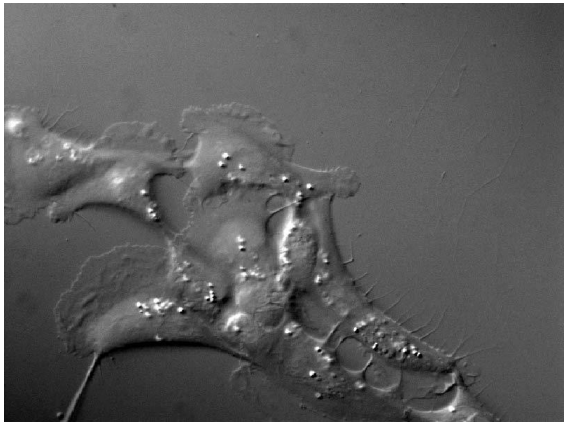
Light microscopic techniques:

Contrast microscopy:
Contrast-enhancing procedures that deliver high-contrast images of transparent, unstained specimens

Phase contrast microscopy (Ph1-3):
Differences in the density of structures are shown.



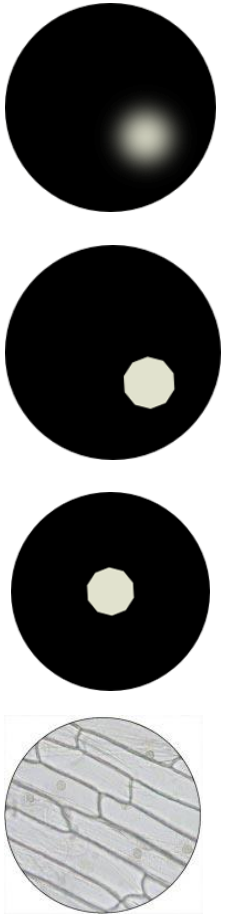
Differential interference contrast (DIC):
Similar to phase contrast, differences in the density of structures are made visible. Advantages compared to the phase contrast: offers sufficient contrast even with high resolution.



Microscopy

Köhler Illumination

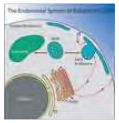
Through Koehler illumination arrangement
a uniformly illuminated image field is achieved
without the image of the light source in the specimen plane.
In addition, by centering the light source one achieves a maximum
light output, which is of great importance especially for the contrast method.



Microscopy

<u>Resolution and magnification:</u>

A higher magnification does not automatically go hand in hand with a higher resolution.



The **resolving power** is mainly represented by the **numerical aperture** of the objective.

Higher magnification with the same resolution, the picture appears blurry and details are not resolved.

Microscopy

numerical aperture(NA)

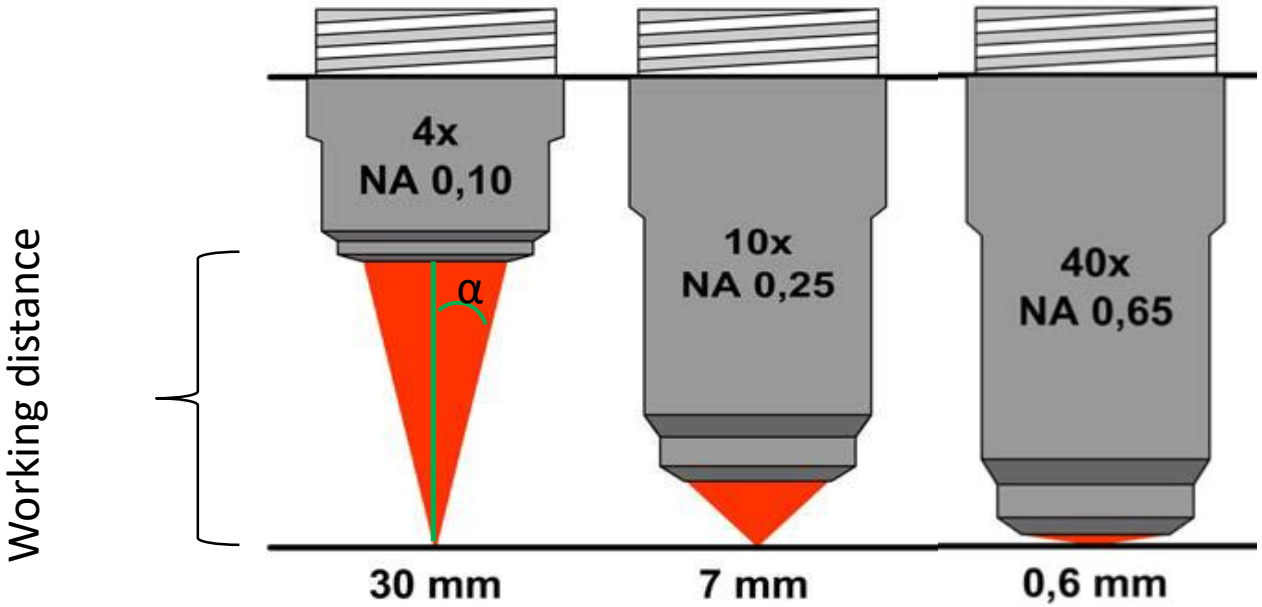
NA = n sin α

n = Refractive index of the medium (between specimen and objective)

α = half the opening angle of the lens

The numerical aperture controls the resolution and the contrast!

high aperture	**low contrast, high resolution**
low aperture	**high contrast, low resolution**



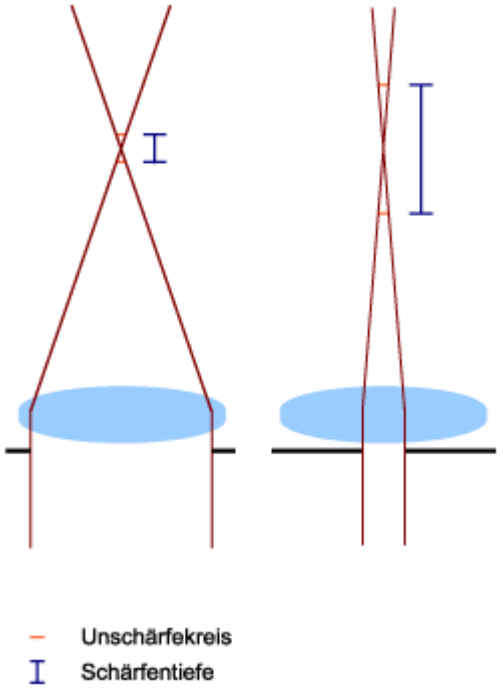
Microscopy

Depth of field (imaging depth / depth of field) (DOF):

$$DOF = n \times \lambda \ / \ 2x \ (NA)^2$$

n = refractive index of the medium (between specimen and objective)

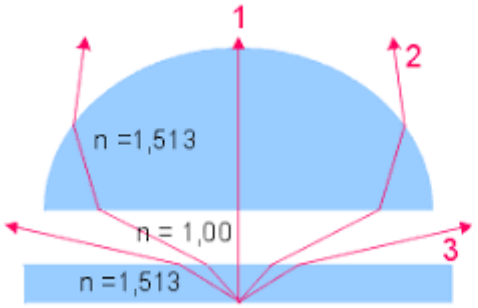
The imaging depth describes the layer thickness in the object, in which this appears sharp (depth of focus / depth of field).
The **higher the NA** of the lens, the
the **smaller the depth of field** / imaging depth in the object



Microscopy

Important indices of refraction

Vacuum:	1
Air:	1,0003
Water:	1,33
Glass:	1,4 - 1,8
Cover slip:	1,513

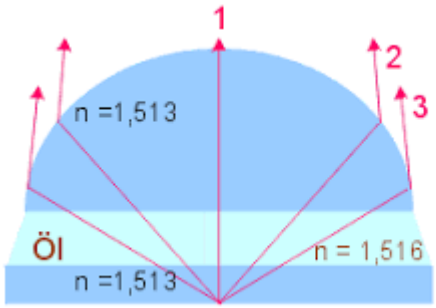


Objective lens

Cover lip

Immersion media:

Immersion oil:	1,516
Canada balm:	~1,52
Epoxyd resin:	~1,56



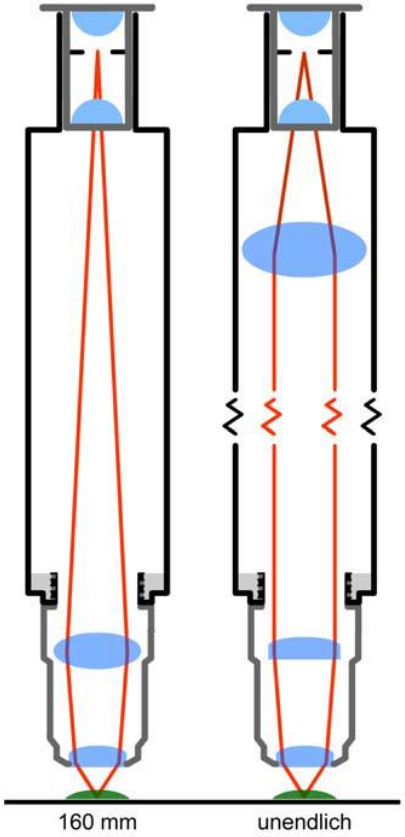
Objective lens

Cover lip

Microscopy

Lenses (labeling) and tube length:

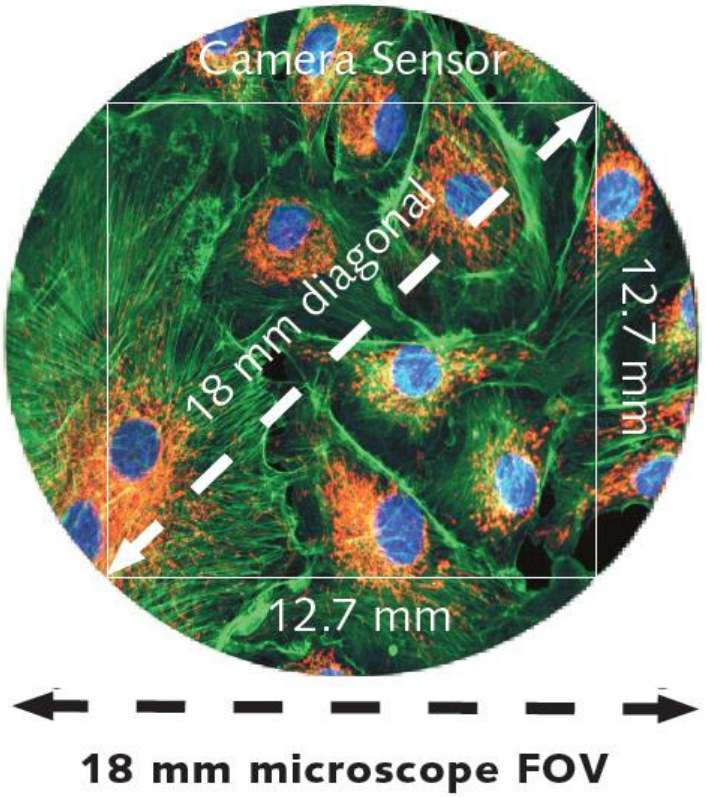
PlanC N (Objektivserie, Objektivklasse(Planachromat))
20x / 0.40 (Magnification/ numerical aperture)
∞ / 0.17 (Tube length / cover glass thickness)
/ FN 22 (Field of view (Field Number))

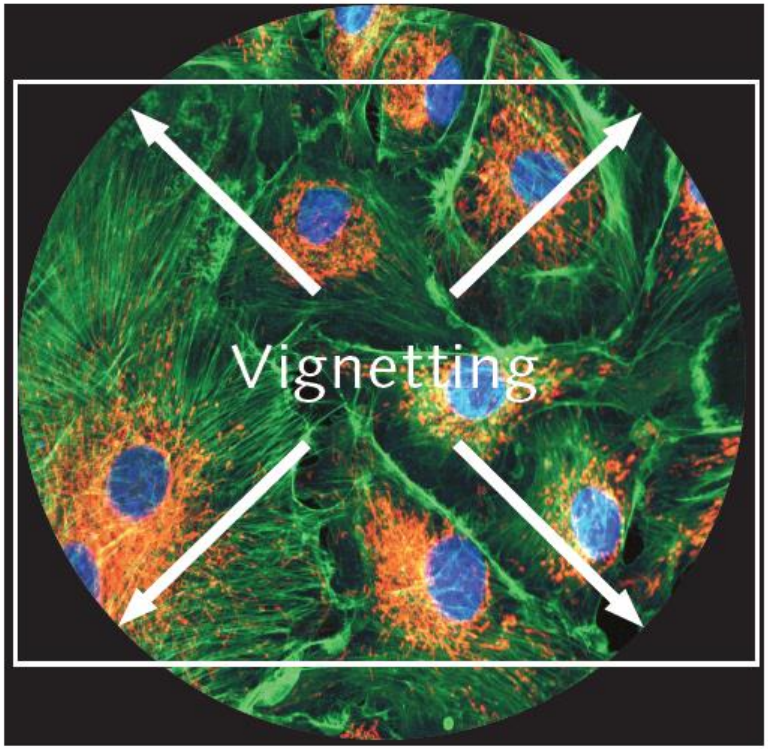


Microscopy

Field of View (FOV) and Field Number (FN):

FOV = Field Number (FN) / Objective Magnification = 22 / 20 = 1,1 mm





Microscopy

Resolution (d) and Abbe diffraction limit:

λ = wavelength of the light used
NA = numerical aperture of the objective

$$d = \lambda / 2xNA$$

So e.g. light with 500nm with a 20x, 40x or 63x objective with an NA of 1.0 results in a resolution of 250nm (~roughly the diffraction limit, means I can distinguish between two points that are 250nm apart), it doesn't matter whether I use 20x, 40x, 63x objective !!!

Resolution of 250nm only applies "in plane" x-y direction.

The z-direction has a poorer resolution by a factor of ~2-4 (500-1000nm)

Why???

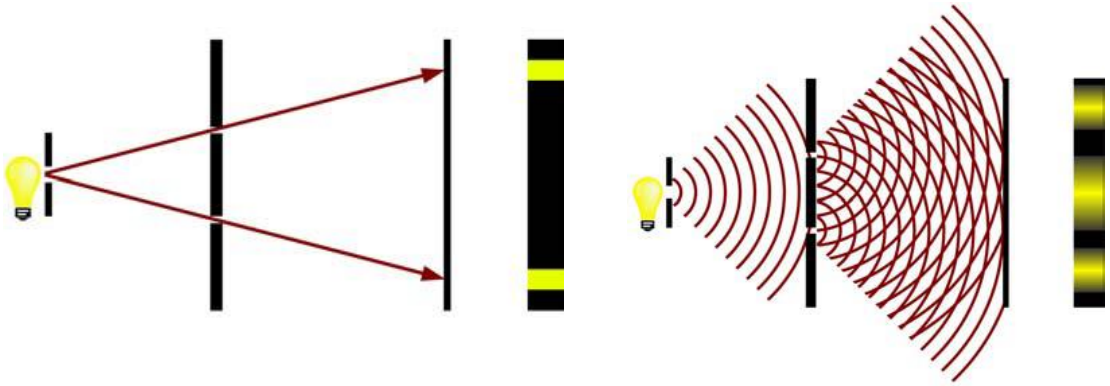
Microscopy

The resolution (d) is disturbed by various imaging errors

Chromatic aberration
Spherical aberration

and by diffraction of light

Due to the wave properties of light, the real behavior deviates strongly from what one would expect

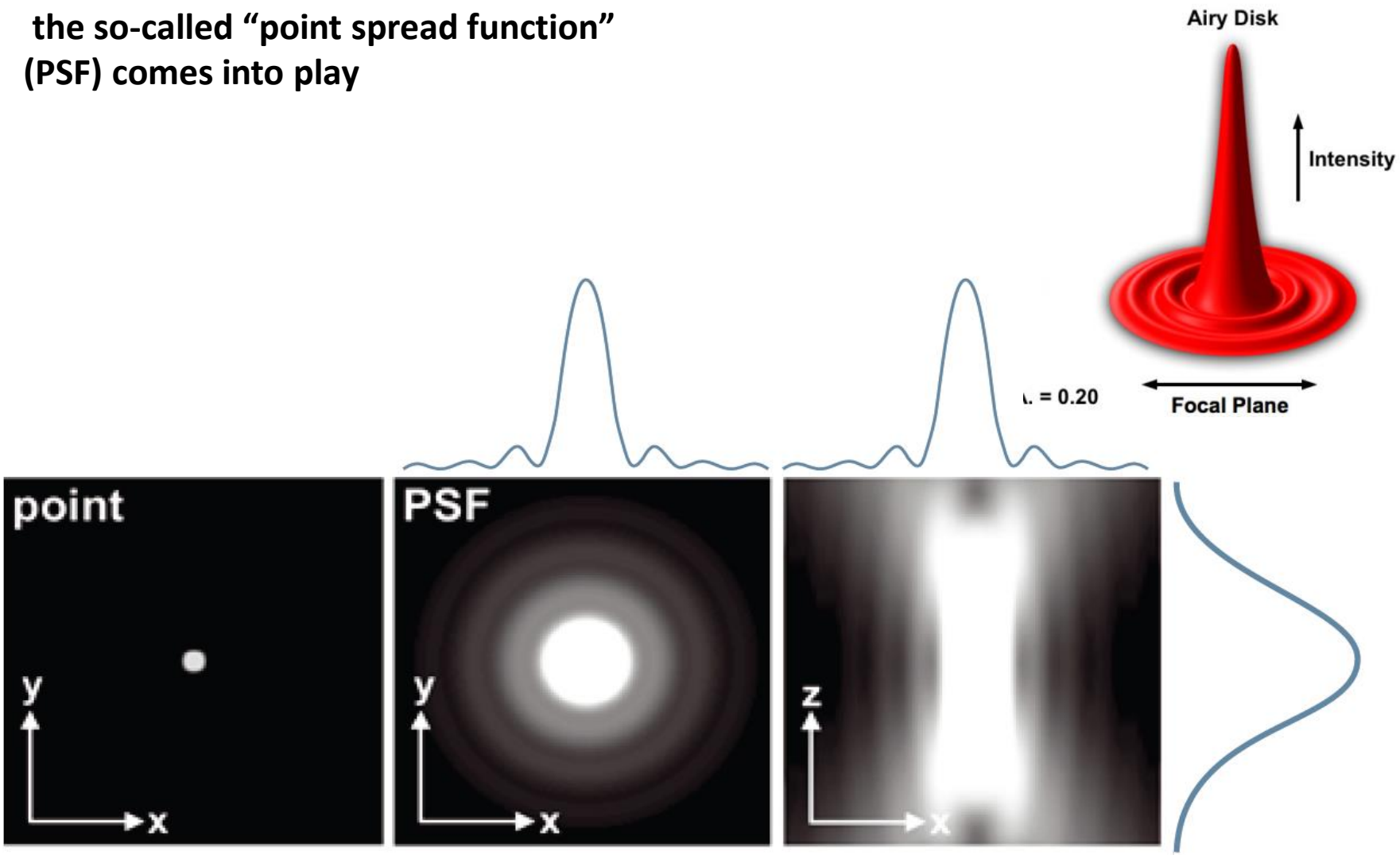


Expected Image

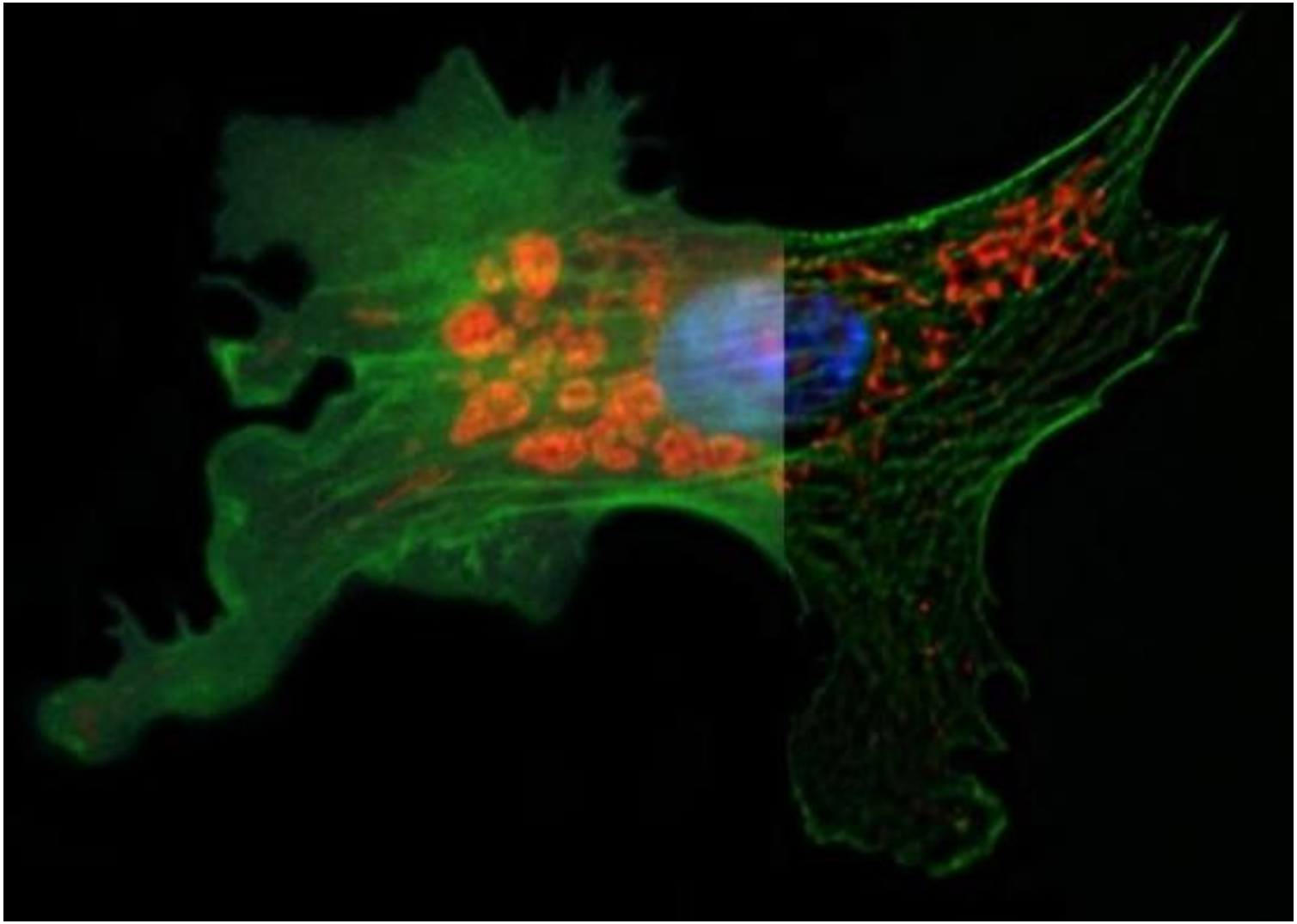
Real Image

Point spread function (PSF)

the so-called “point spread function” (PSF) comes into play



Deconvolution

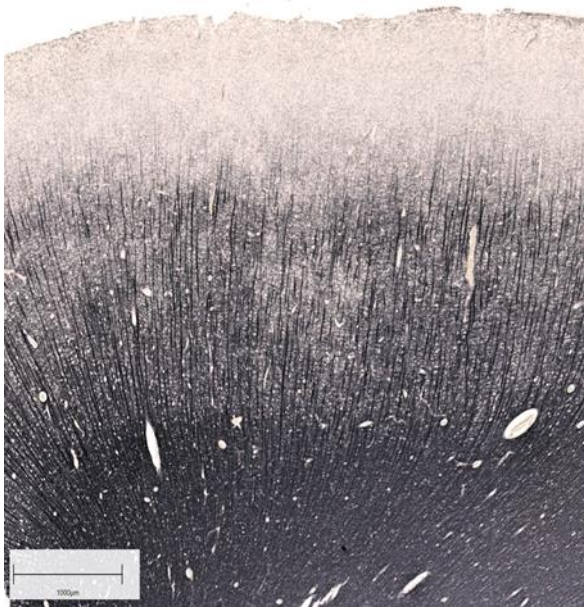


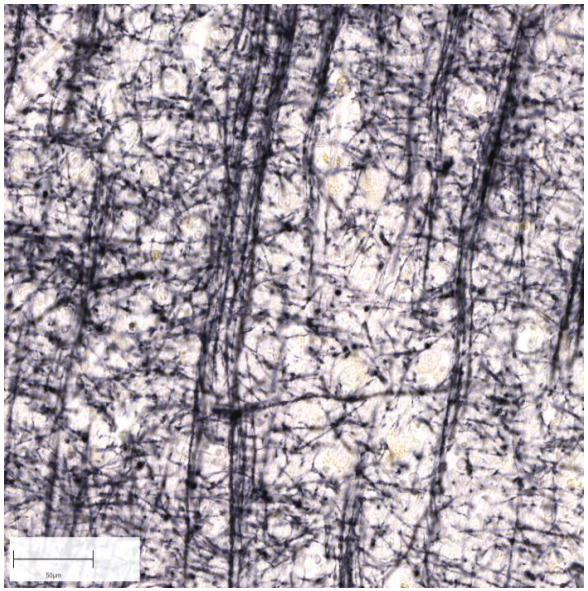
https://svi.nl/Huygens-Deconvolution

Brightfield Microscopy

Standard light microscopic systems

Zeiss Axiophot with RGB AxioCam HRC Camera



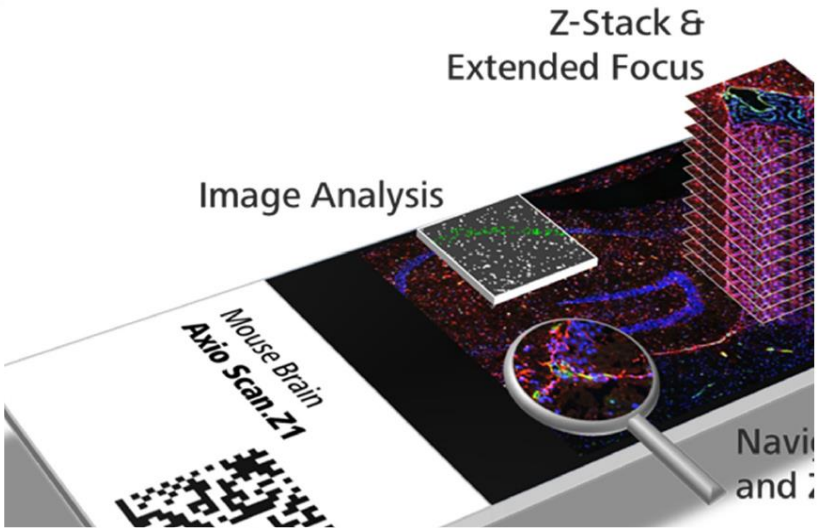


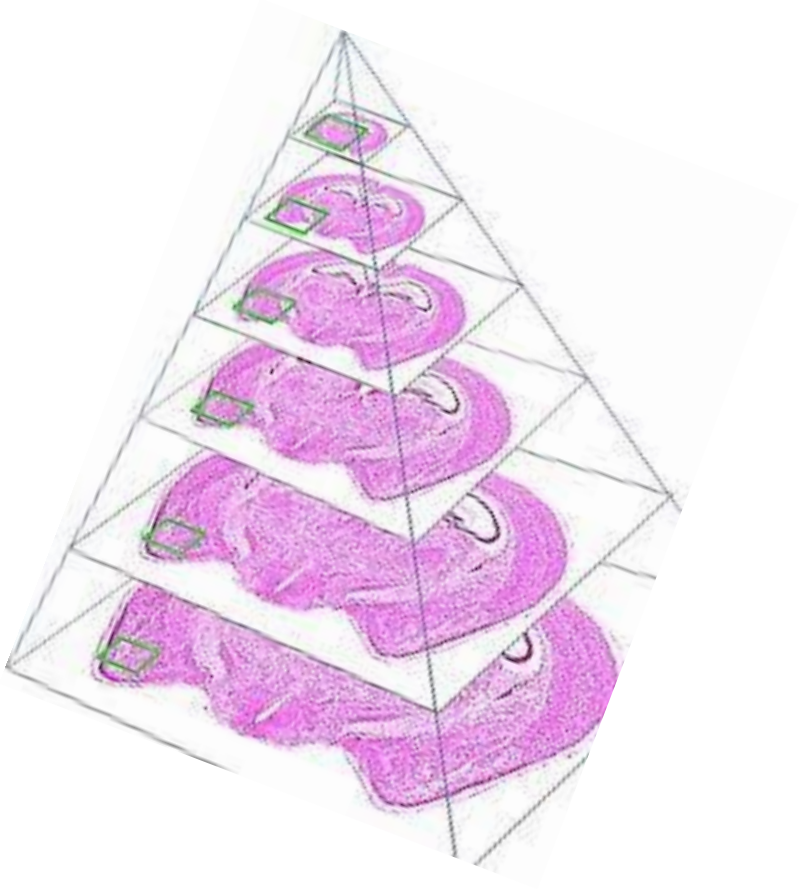
Files size around **4-8 MB**

FOV, even with the most expensive 2,5x objective with **0.1 NA**, is just around **20mm** and you have a resolution of ~**2-3mm**.

Digital Automated Microscopy

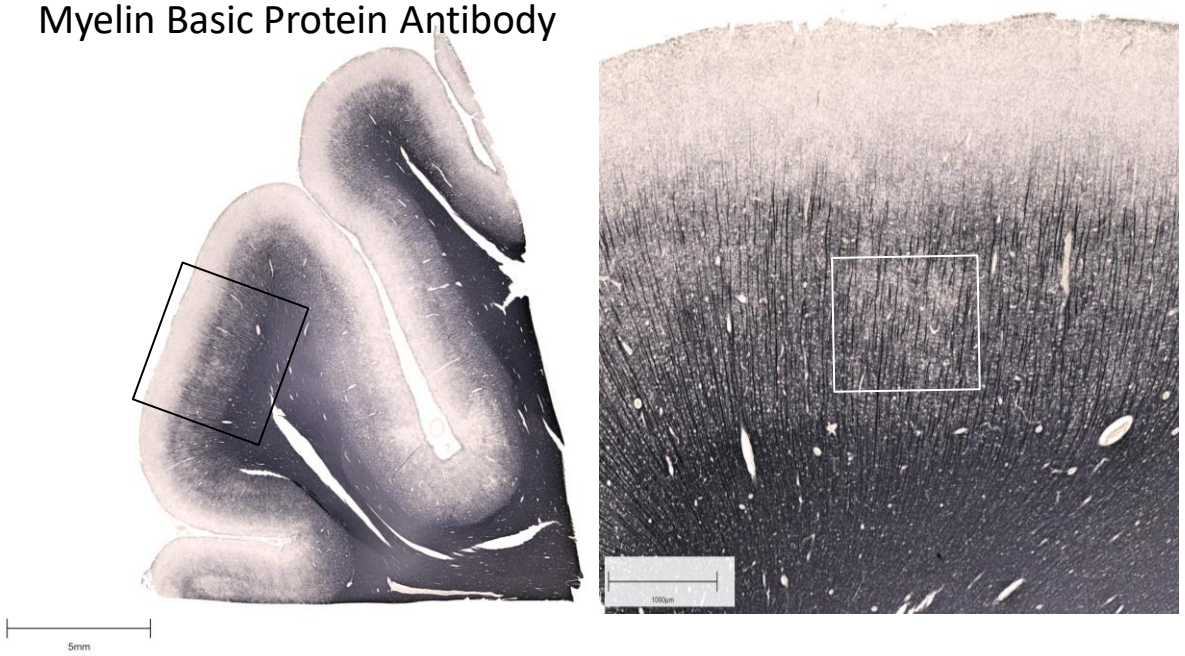
Zeiss AxioScan.Z1 Automated Microscope available since 2017/18

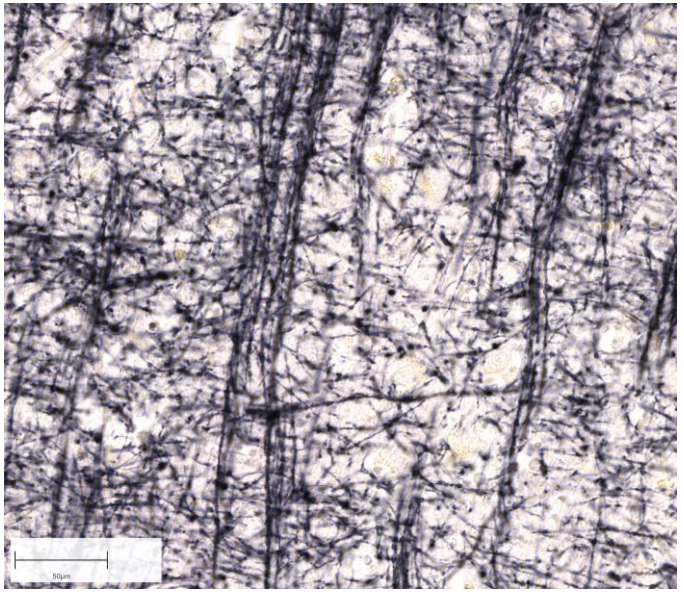


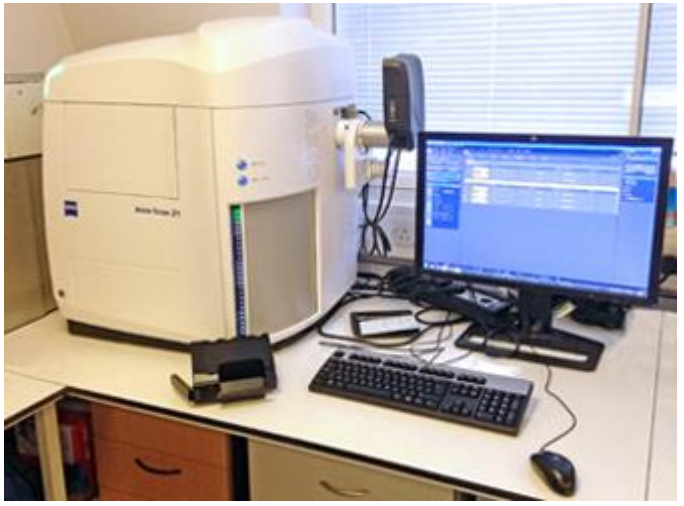


Digital Automated Microscopy

Myelin Basic Protein Antibody







Serial cut recording with the Zeiss AxioScan.Z1 microscope
100 slides (76x26mm)
50 slides (76x52mm)
25 slides (102x76mm)
Resolution 250-1000nm x-y approx. 1 µm in the z direction
Z-stacks with EDF (Extended Depth of Focus) and
MIP (Multi Image Projection)
Light microscopy
Fluorescence microscopy DAPI, Cy2, Cy3, Cy5 and Cy7
Objectives 5x (NA 0.25), 10x (NA 0.45), 20x (NA 0.5),
20x (NA 0.8), and 40x (NA 0.95)
Recording speed ~ 2cm^2 per minute with 20x objective
Price approx. 200,000 Euros

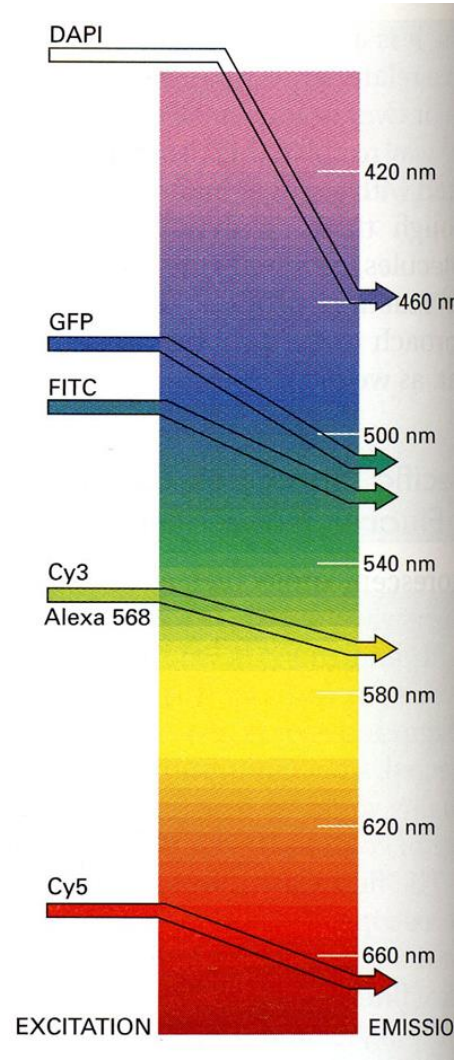
Switch to Zeiss ZEN

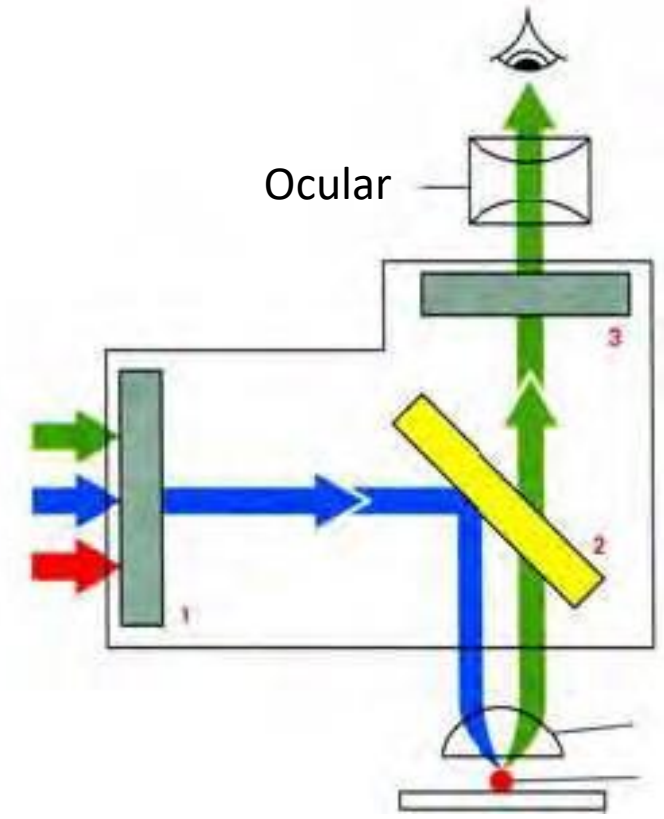
Epi-Fluorescence Microscopy

Principle of fluorescence microscopy:

Excitation = short wave length

Emission = long wave length





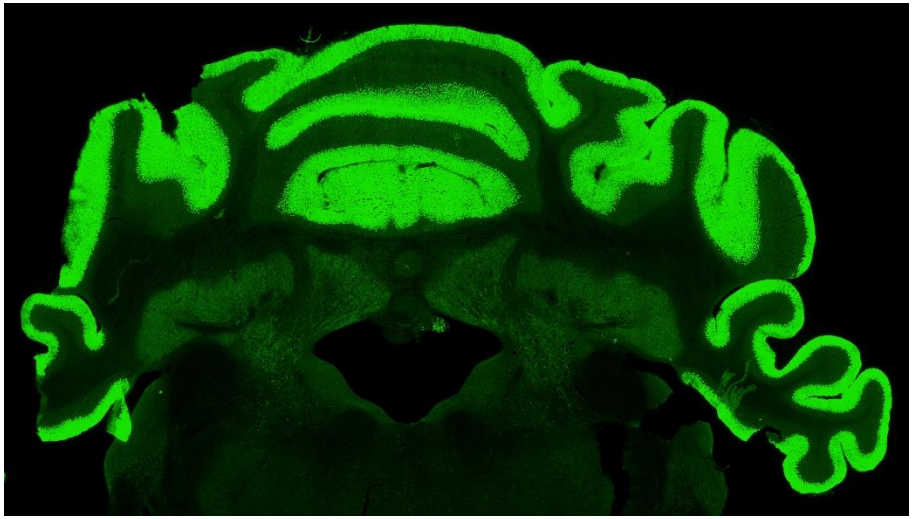
Light source:
HBO, HPX
Colibri LED

1. Excitation filter
(allows light of the excitation wavelength (s) to pass)
2. Dichroic mirror
(breaks excitation light, lets emission light pass)
3. Emission filter
(specifically lets the emission light pass)

Epi-Fluorescence Microscopy

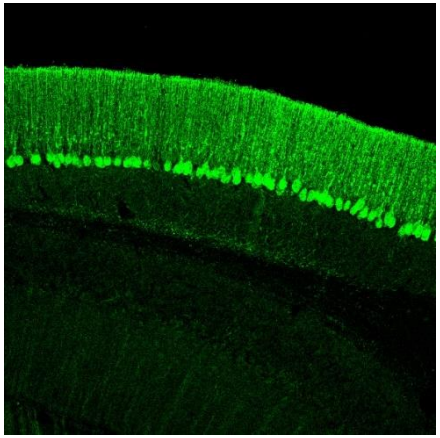
Epi-Fluorescence microscopic system

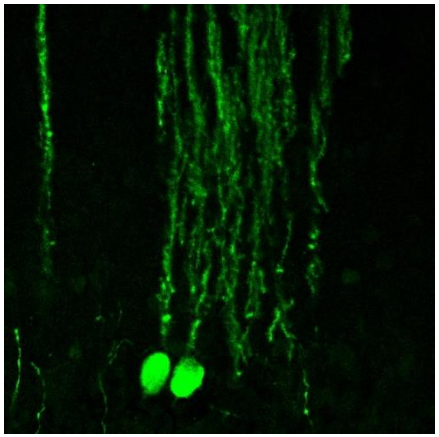
Zeiss Axioscope with monochrome AxioCam ICm



Images taken at 2,5x (0.05 NA), 10x (0.3 NA) and 40x (1.2NA)

Files size around **2-6 MB**





Widefield Illumination

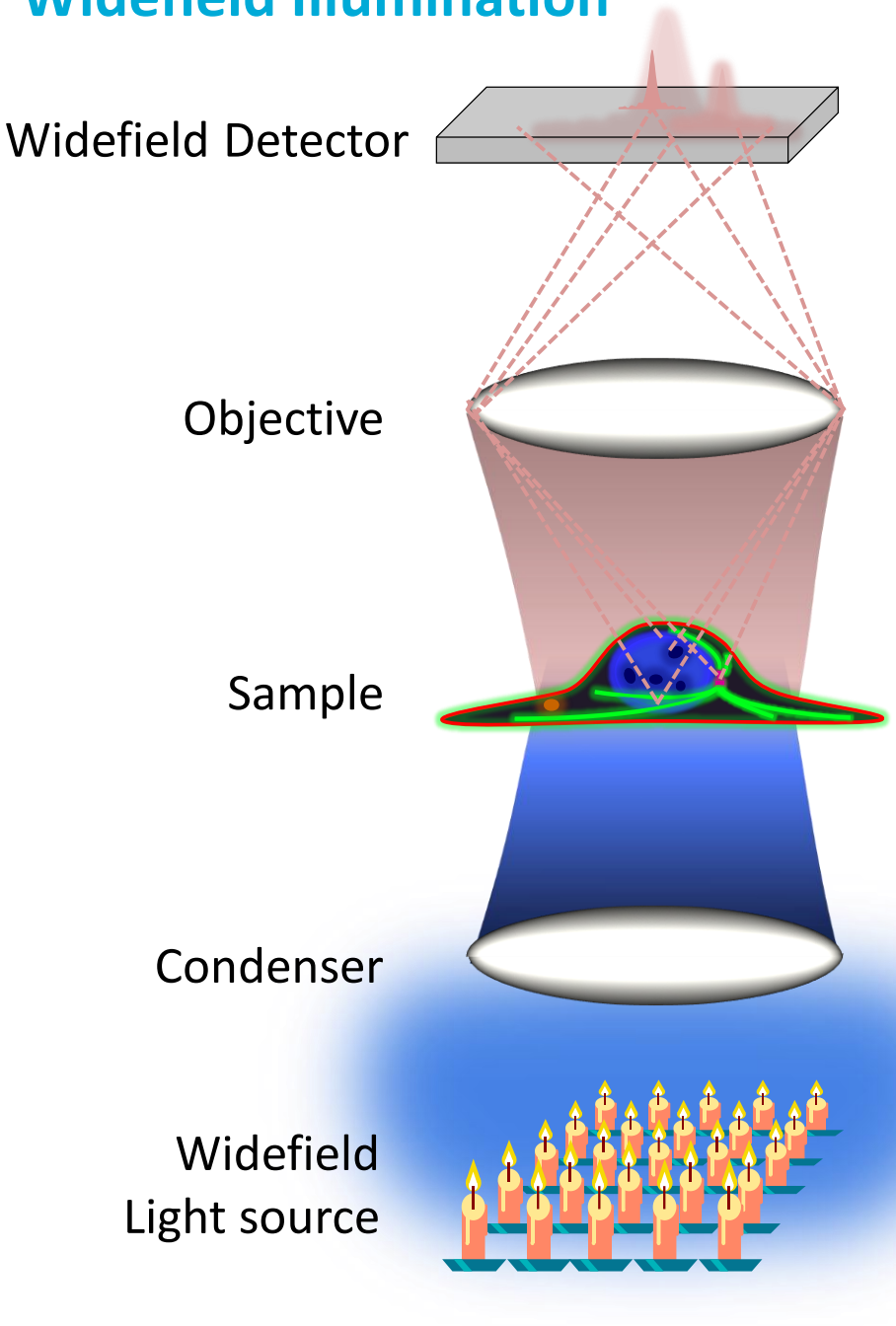


Image = In-focus + Defocus PSF's

Pro's

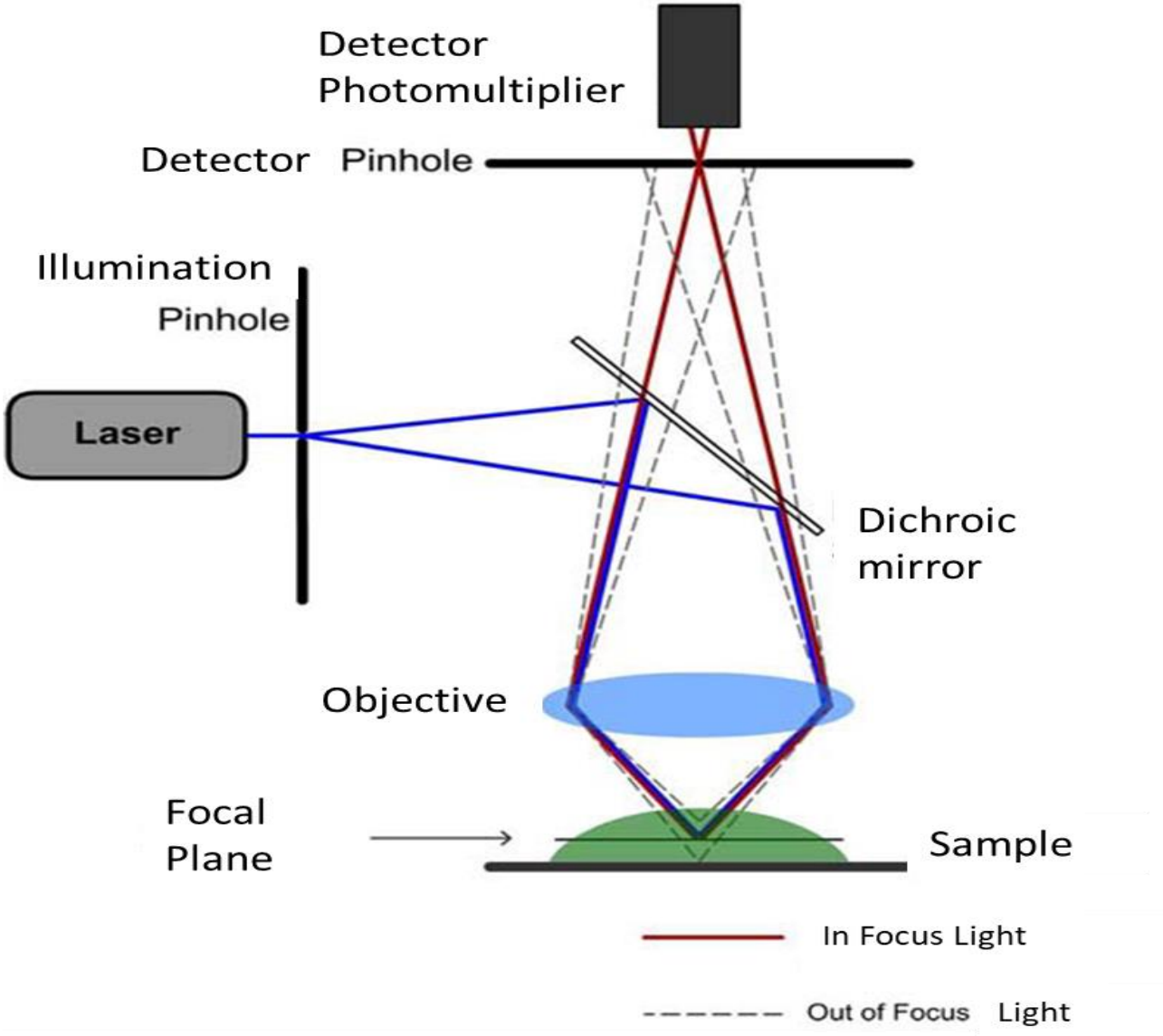
- Many points are imaged simultaneously, increasing the image capture speed

Con's

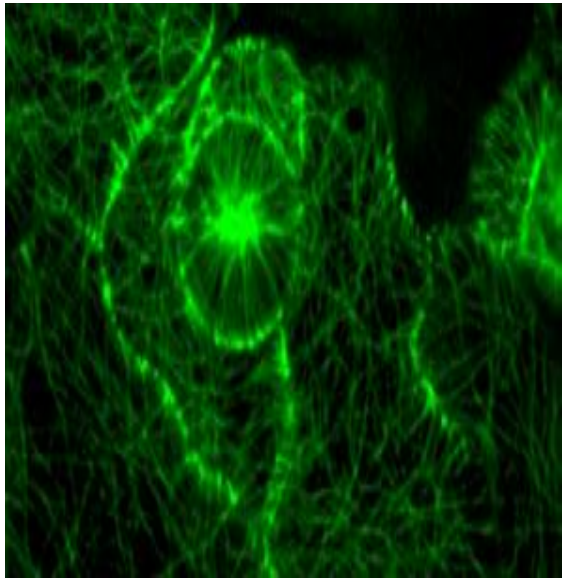
- Out of focus light reduces the contrast of the image
- Out of focus excitation leads to excessive photo damage

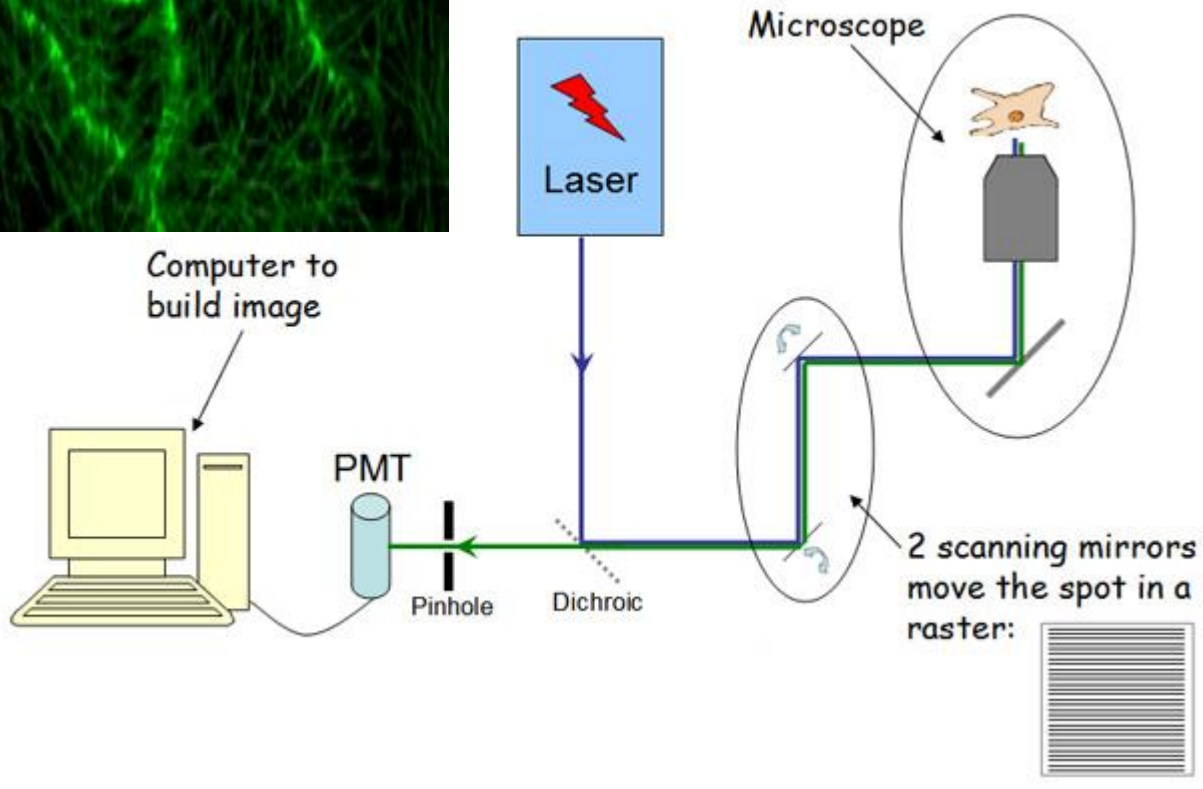
Confocal Laser Scanning Microscopy

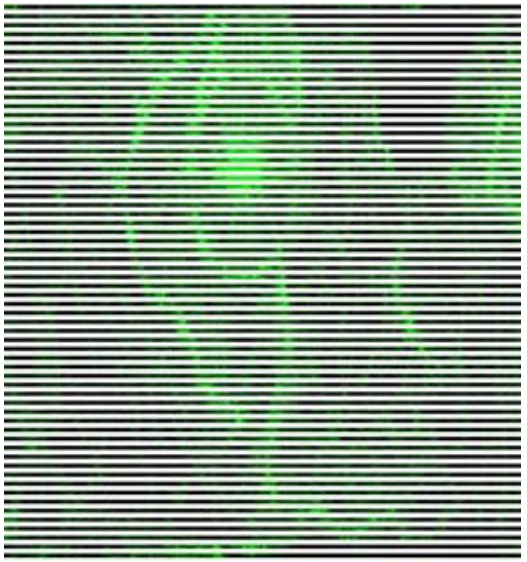
Principle of CLSM (Confocal Laser Scanning Microscope):



Confocal Laser Scanning Microscopy







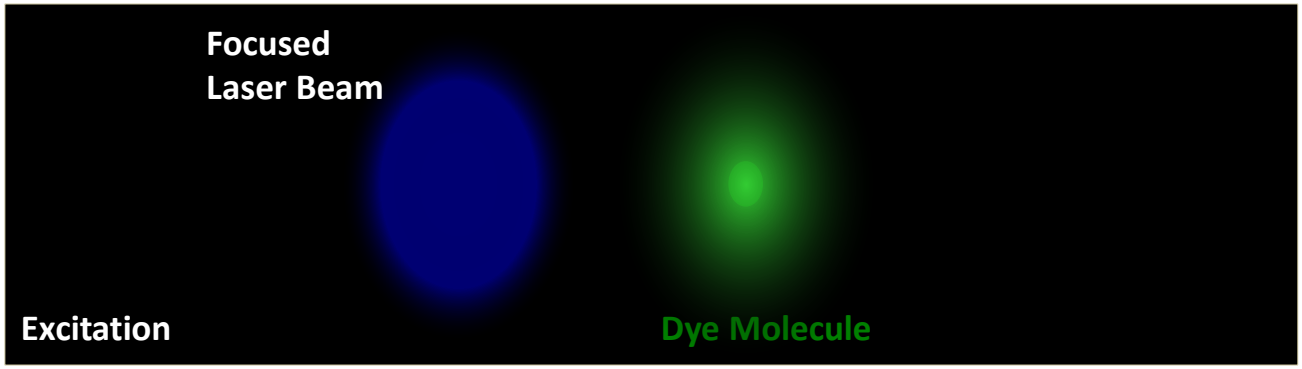
Confocal Laser Scanning Microscopy

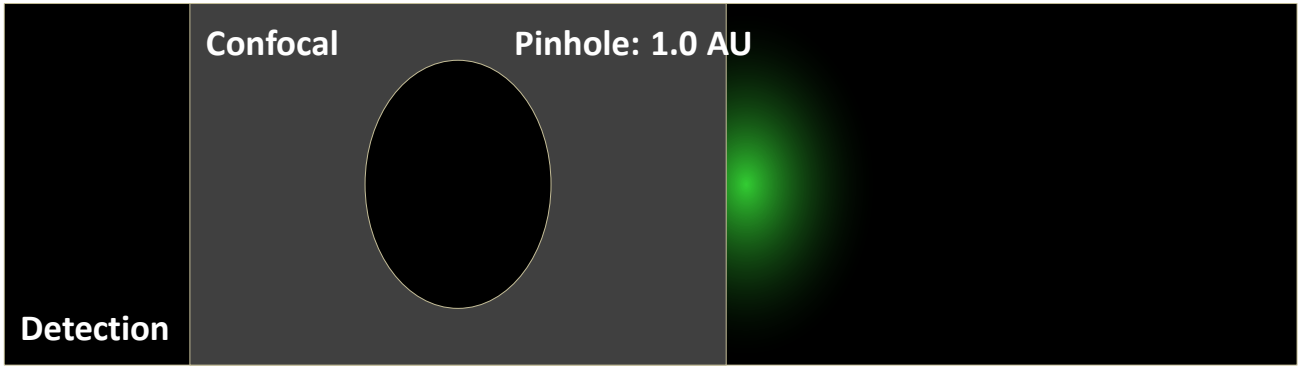
LSM 880-NLO Fast Airyscan

Confocal Laser Scanning Microscopy

Pinhole vs Airyscan

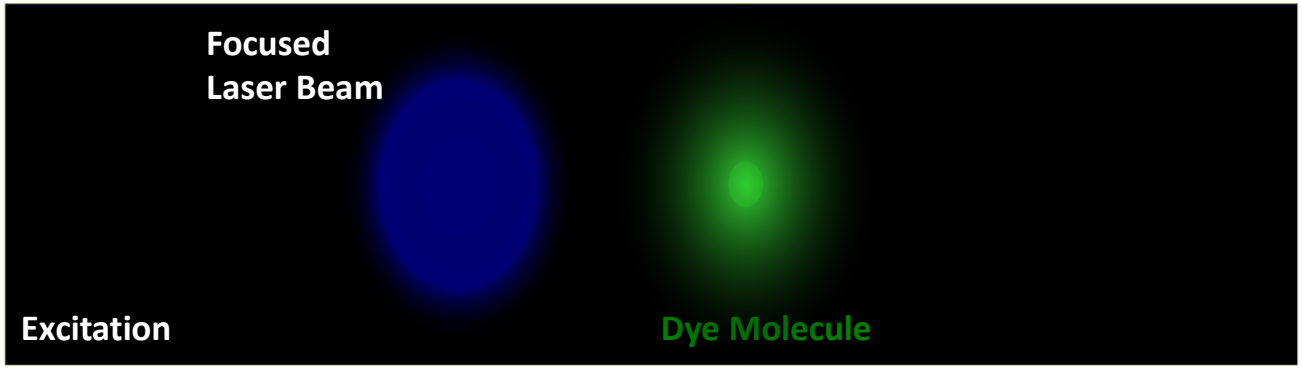
Confocal imaging with pinhole at 1 AU

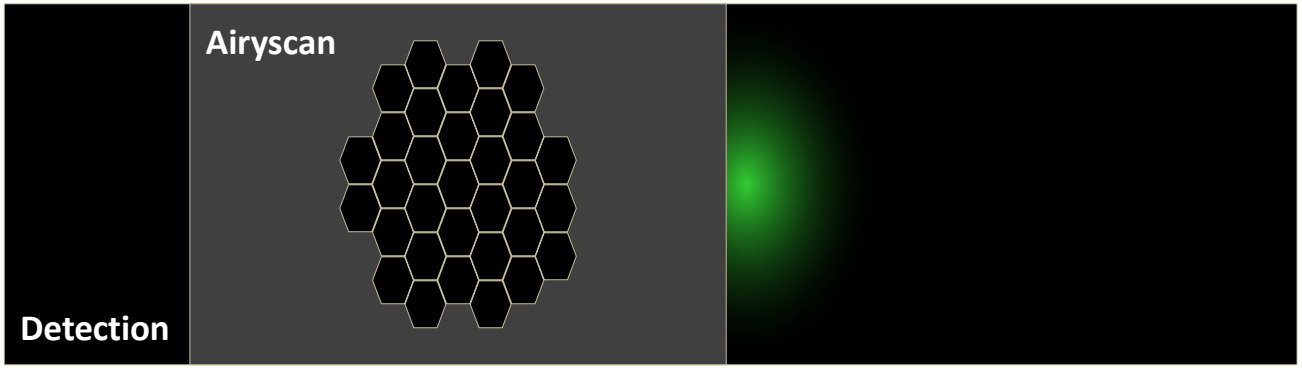




Airyscan

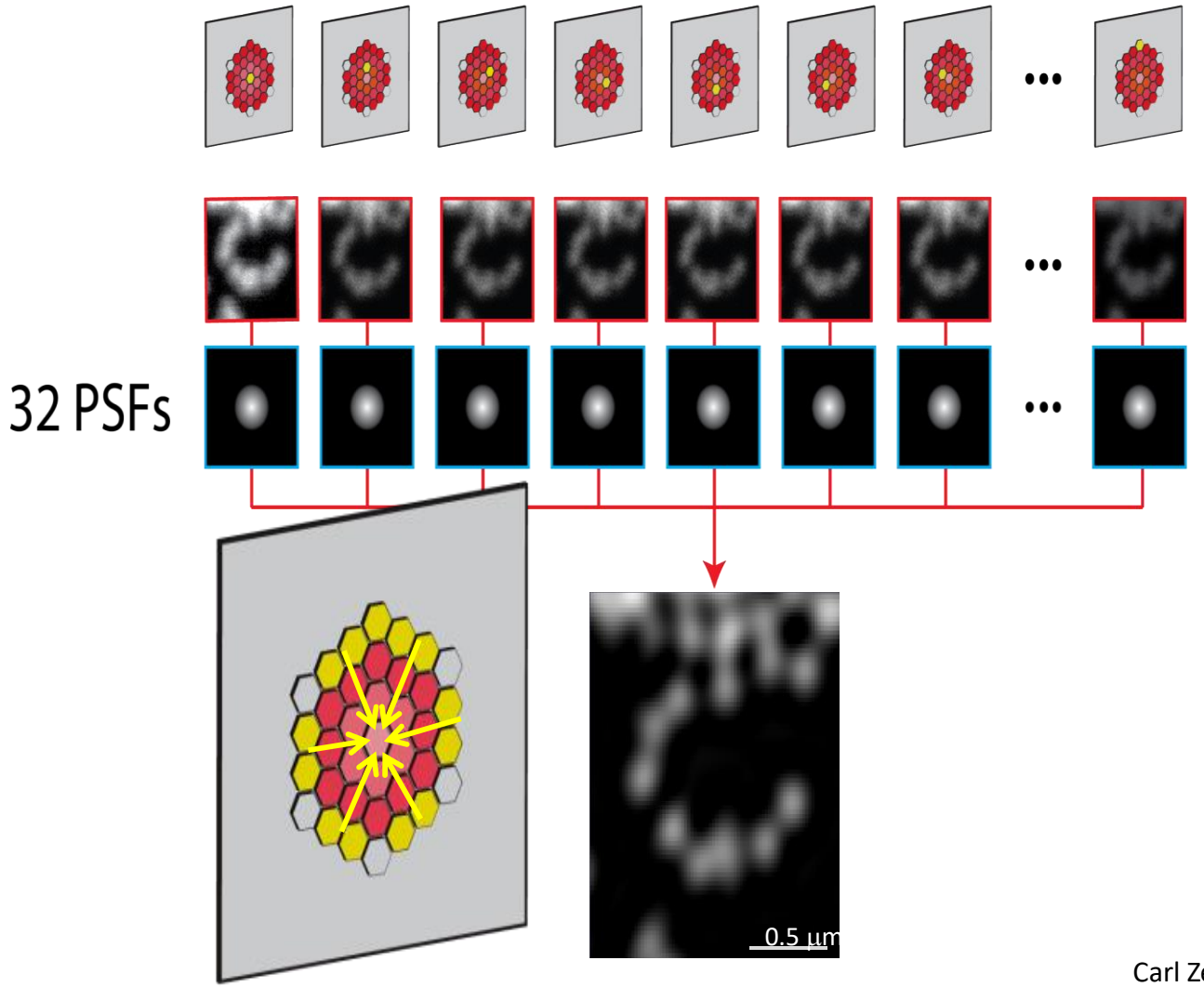
Confocal imaging with Airyscan





Confocal Laser Scanning Microscopy

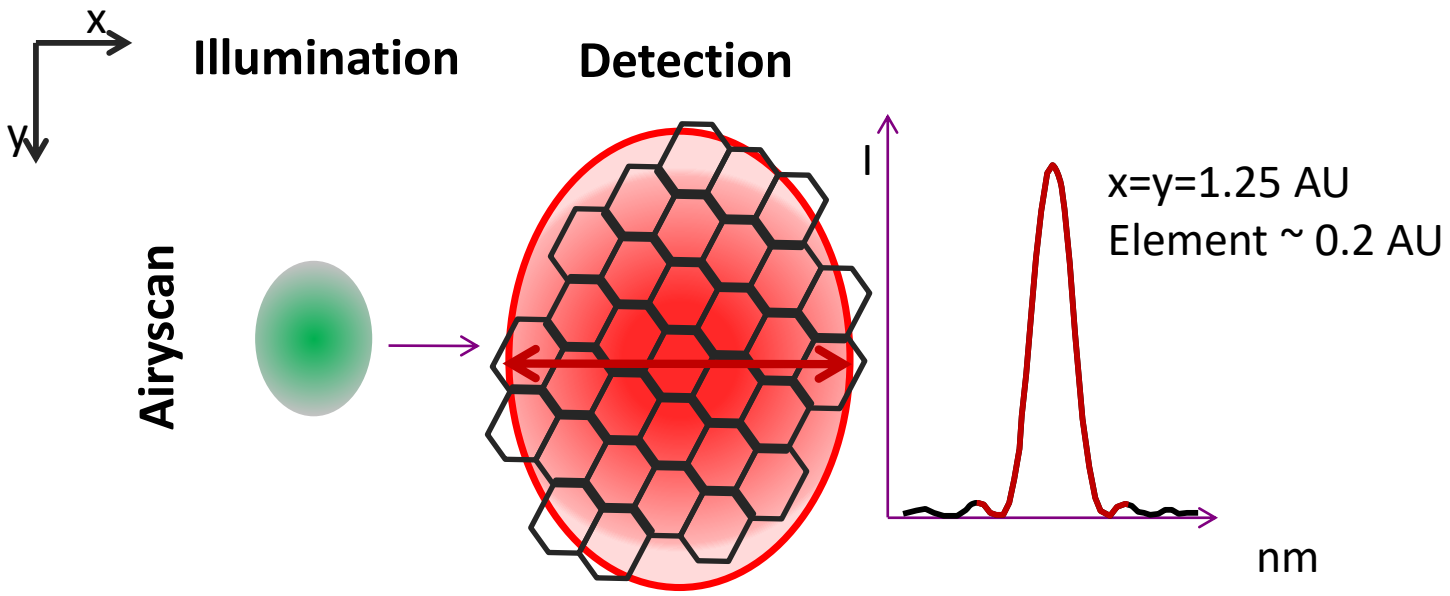
Airyscan processing / detector deconvolution and spatial assignment



Carl Zeiss Microscopy

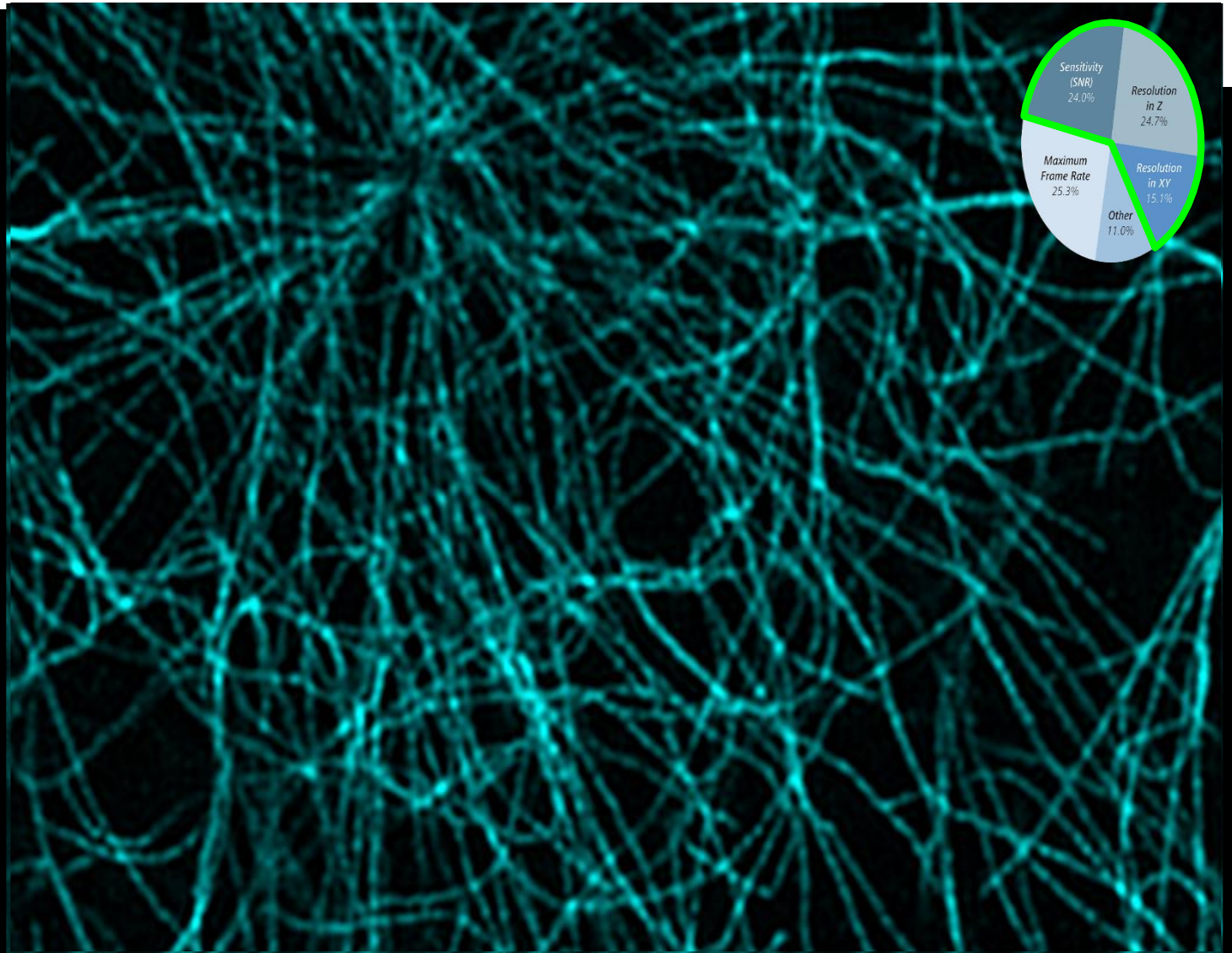
Airyscan

Superresolution and Sensitivity at Speed



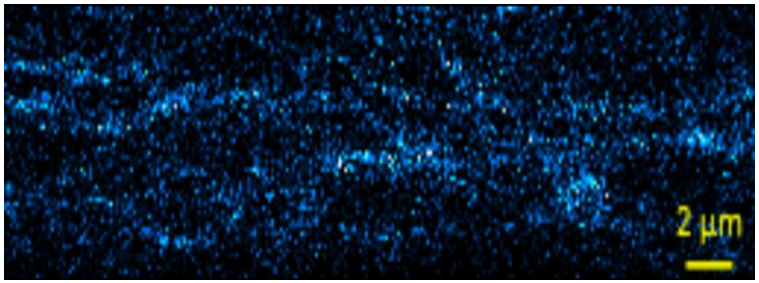
Airyscan / new confocal imaging

~80-120 nm lateral resolution with a single image

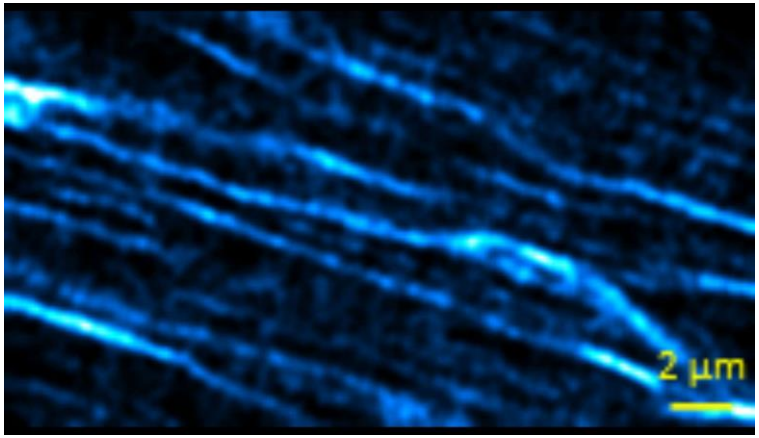


Confocal Laser Scanning Microscopy

Airyscan / highSNR and high Speed



Standard Scanner
– **40-80 FPS**



Airyscan Mode
– **96 FPS**

- Resolution: ~ 2-3x
- Speed: 2-3x
- Signal to noise: 4x

Cardiomyocyte Cells with tubulin-EMTB to measure microtubule buckling
Images and Samples Courtesy of Ben Prosser, UPENN – "Detyrosinated microtubules buckle and bear load in contracting cardiomyocytes", Science April 2016

Point Scanning Confocal Illumination

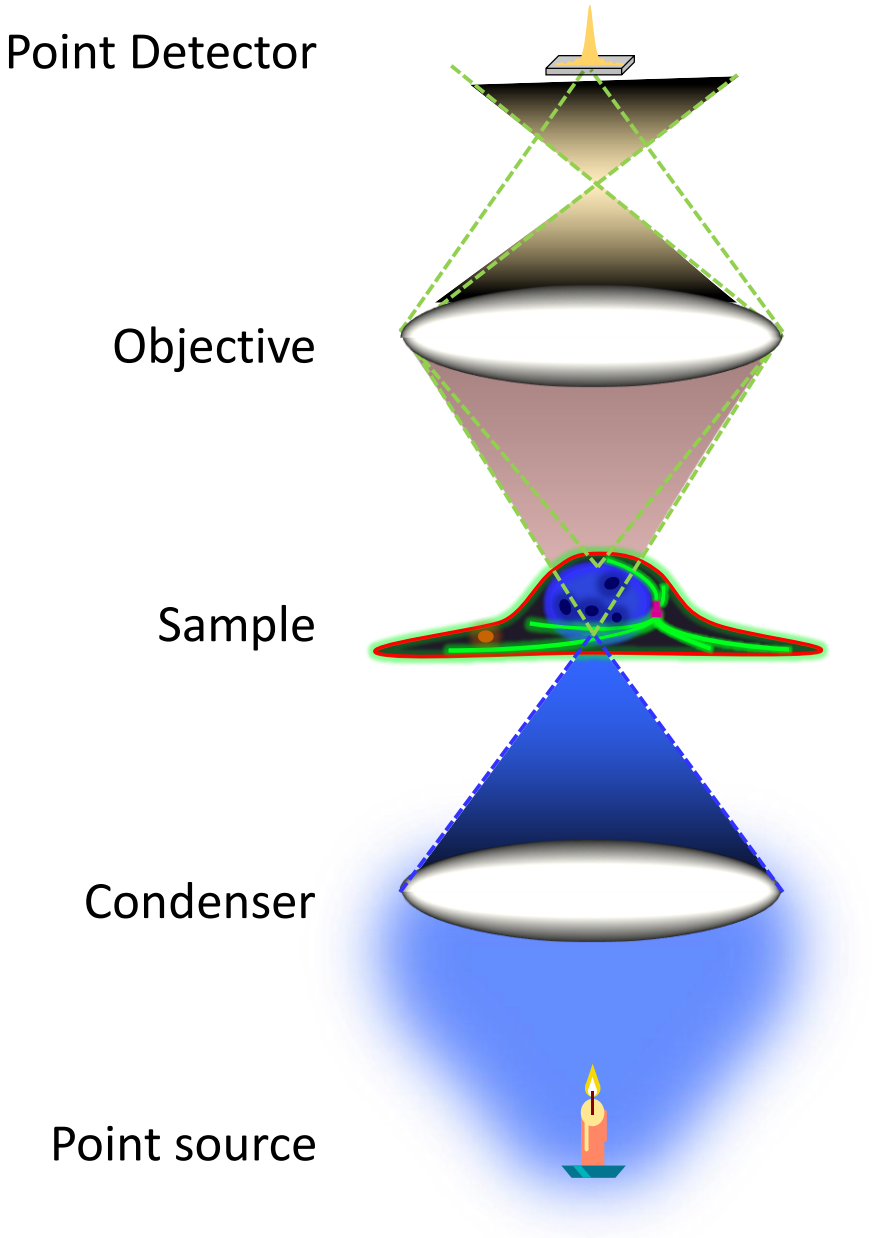


Image = In-focus PSF's only

Pro's

- Out of focus light does not obscure the contrast of the image

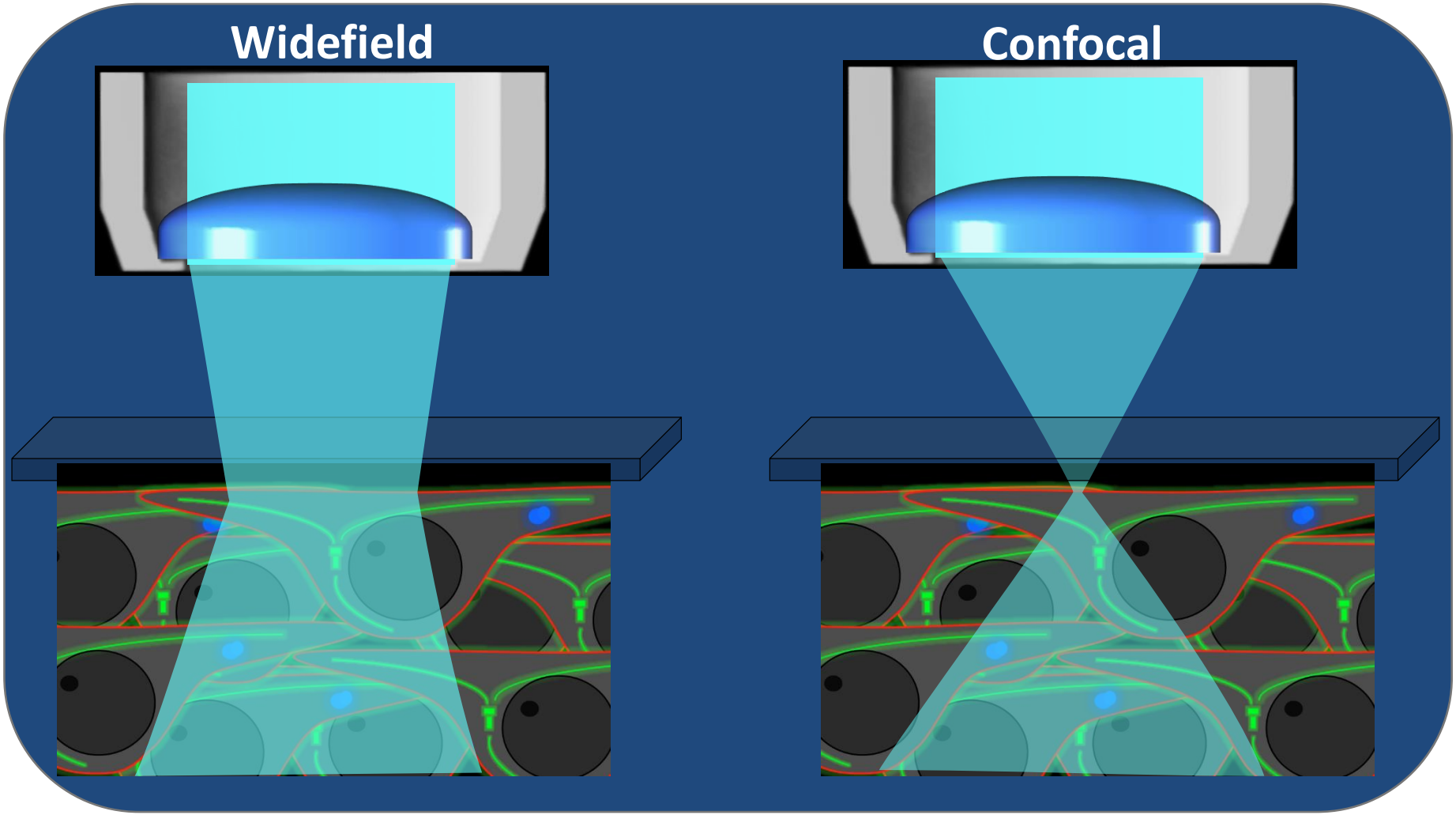
Con's

- One point is imaged at a time, meaning image collection is slow.
- Out of focus excitation leads to excessive photodamage
- Photon efficiency is reduced by the need for a pinhole

Switch to Zeiss ZEN

Conventional Illumination

Both widefield and confocal microscopy induce significant photobleaching/photo-toxicity during 3D image acquisition



Orthogonal plane imaging | Selective Plane illumination Microscopy

- First described by Siedentopf and Zsigmondy in 1903
- First used for fluorescence imaging by Voie et al., in 1994
- The term SPIM was coined by Huisken et al., in 2004

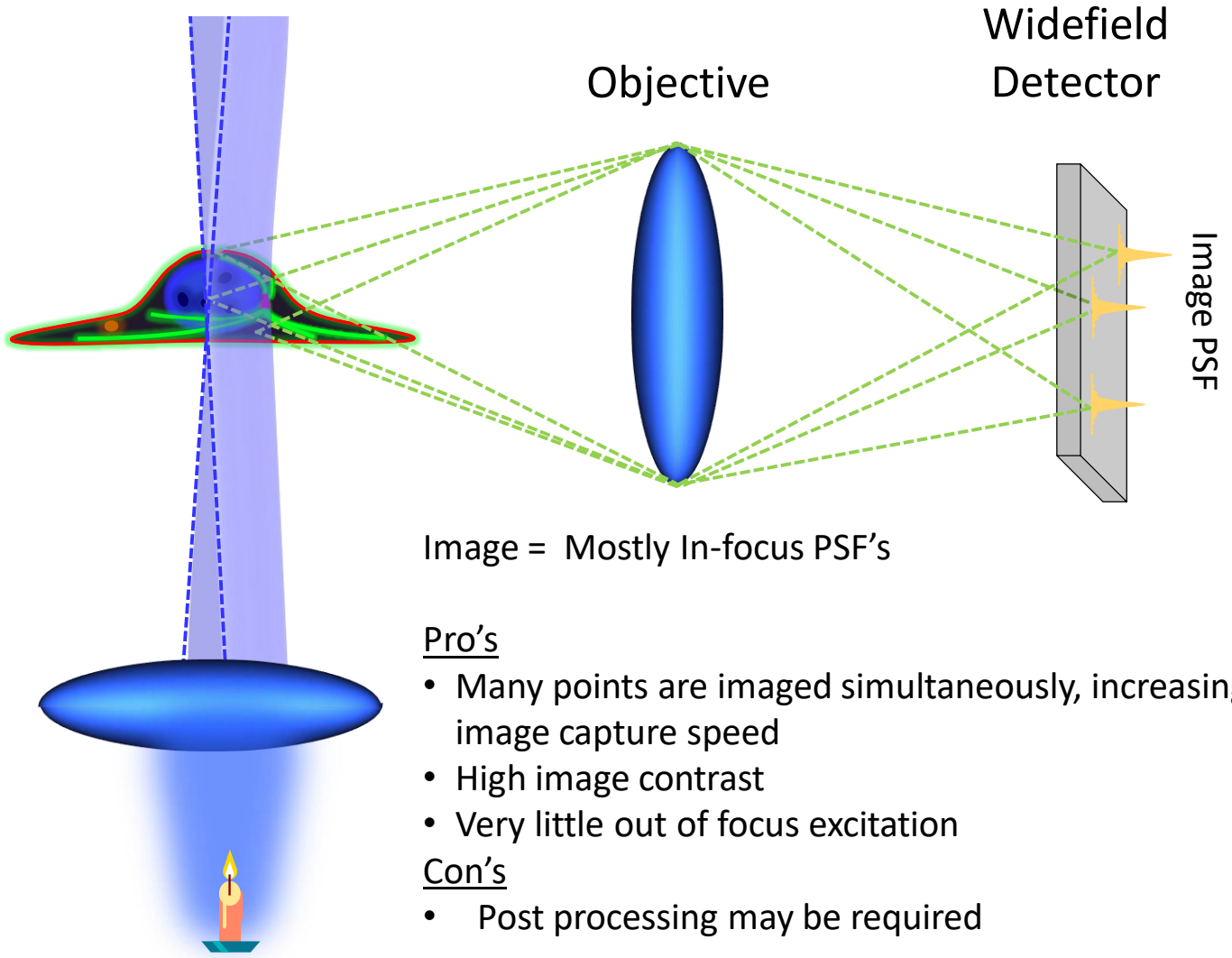


Image = Mostly In-focus PSF's

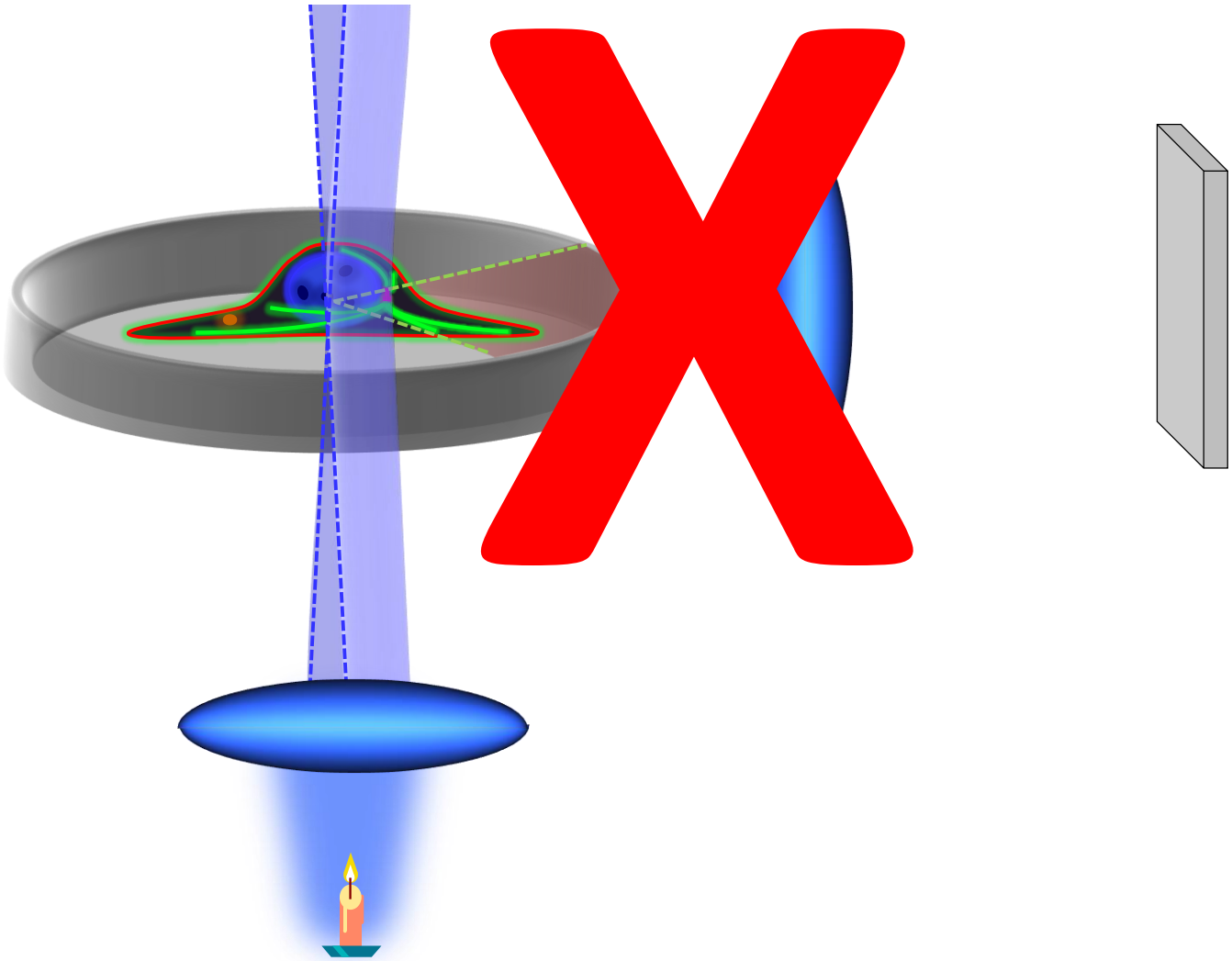
Pro's

- Many points are imaged simultaneously, increasing the image capture speed
- High image contrast
- Very little out of focus excitation

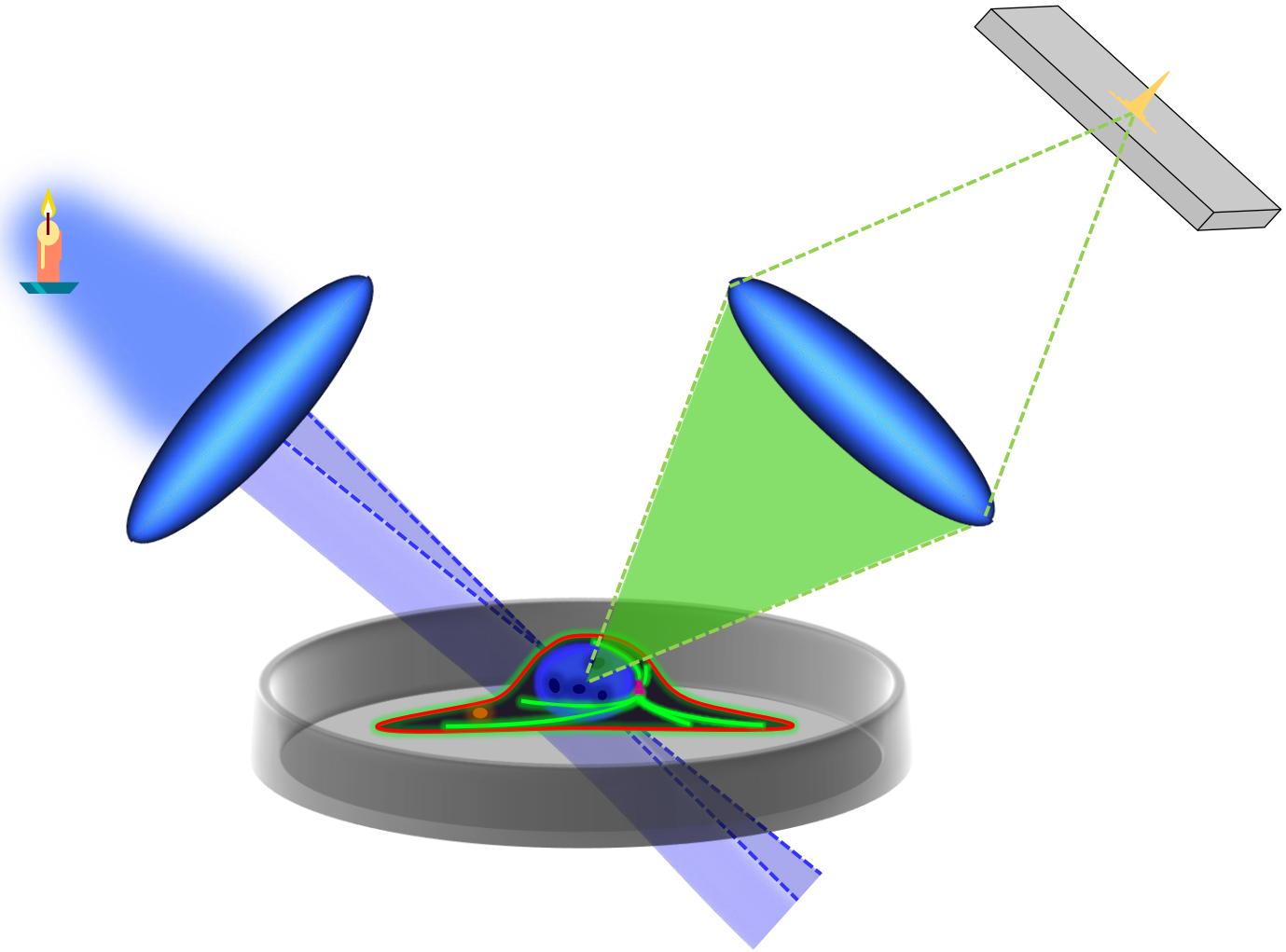
Con's

- Post processing may be required

SPIM| Sample Viewing



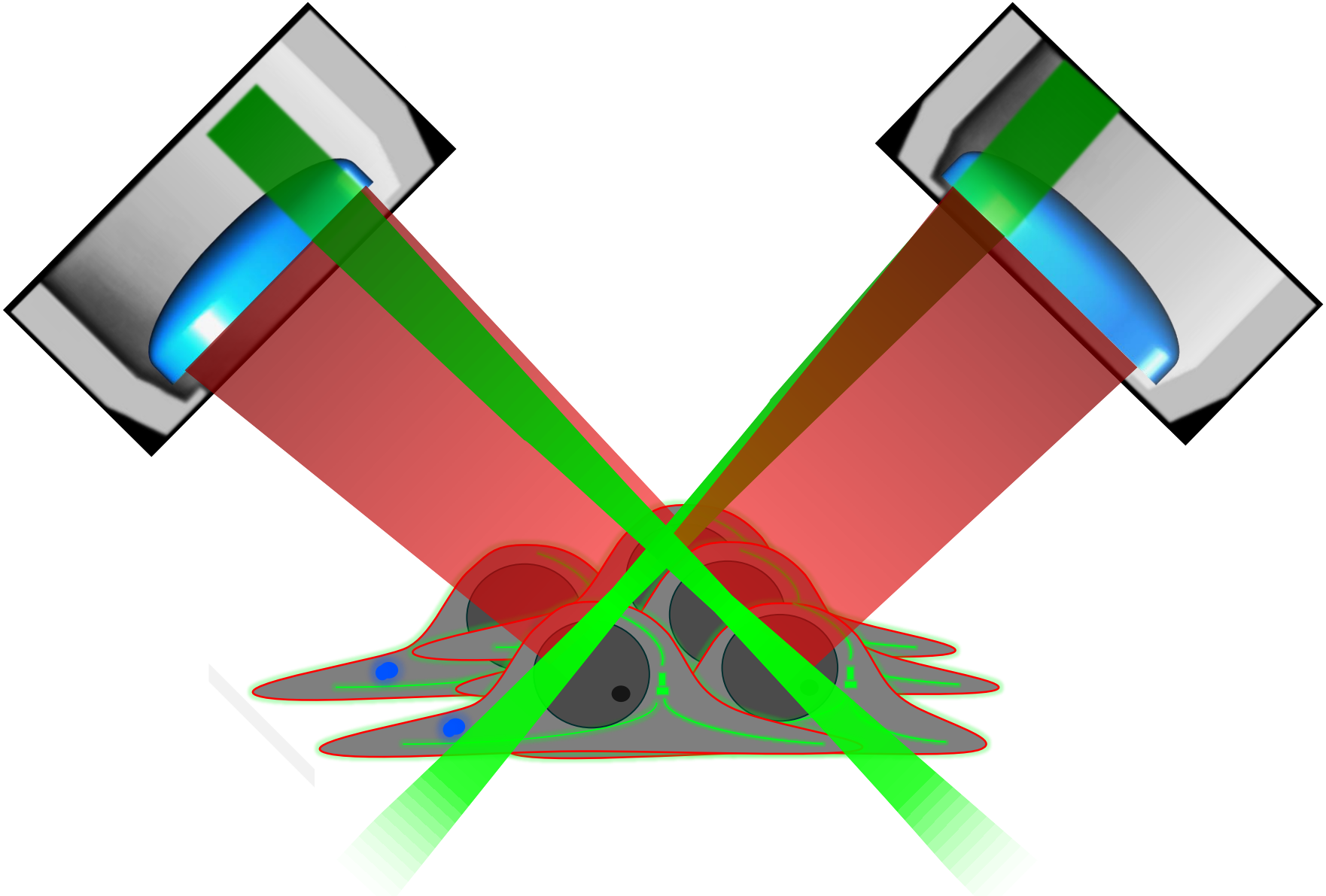
SPIM| Inverted Selective Plane Illumination



Marianas Lightsheet | University of Leipzig, PFI-System

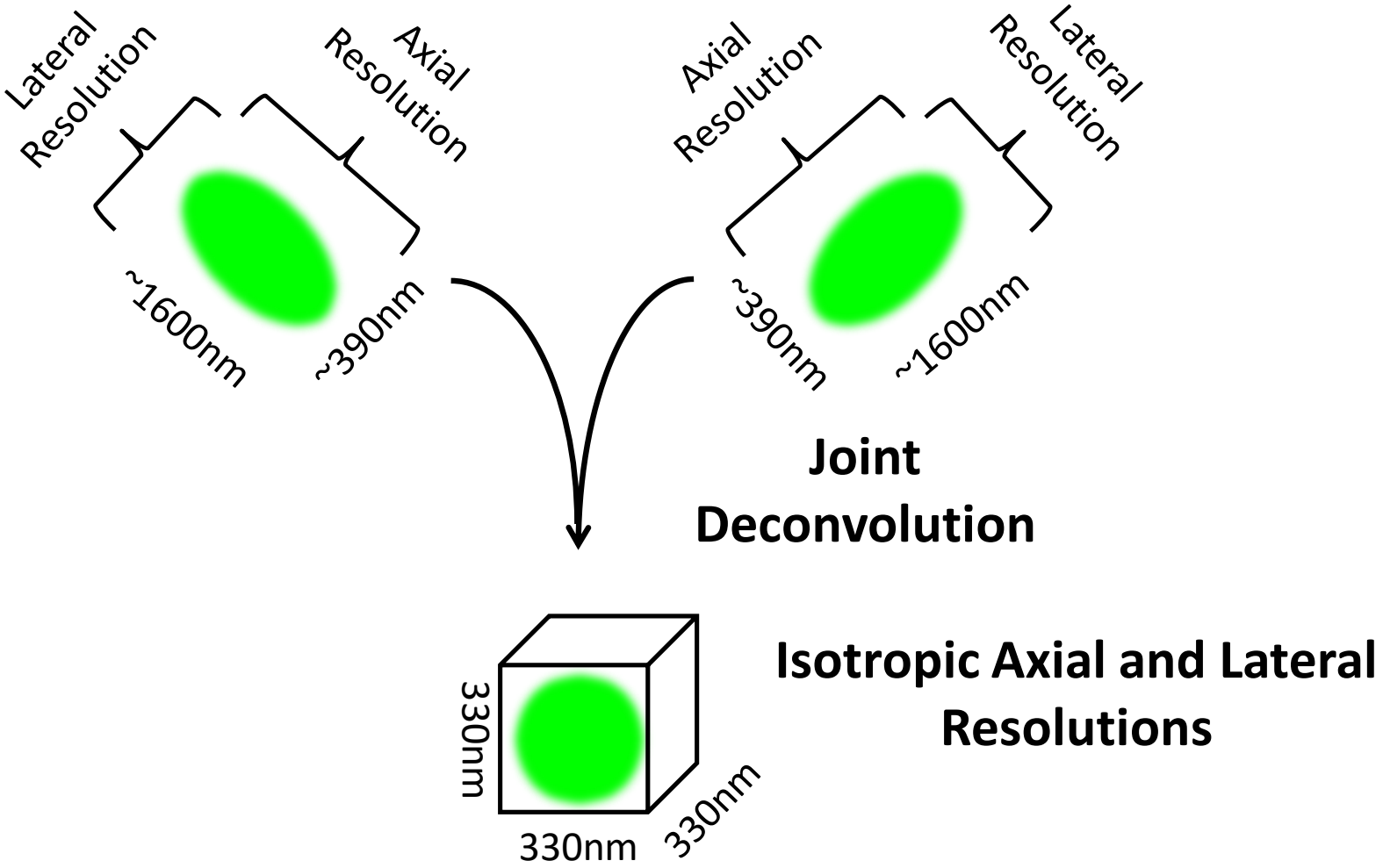
Marianas Lightsheet | Imaging Modes - diSPIM

Piezo/Slice Scanning: Dual acquisition of image stacks using a coordinated movement of the slice position and the piezo. One volume from each side.



Marianas Lightsheet |

Resolution of 40x 0.8NA objectives, with a sheet thickness of 3µm



Scale of Sample