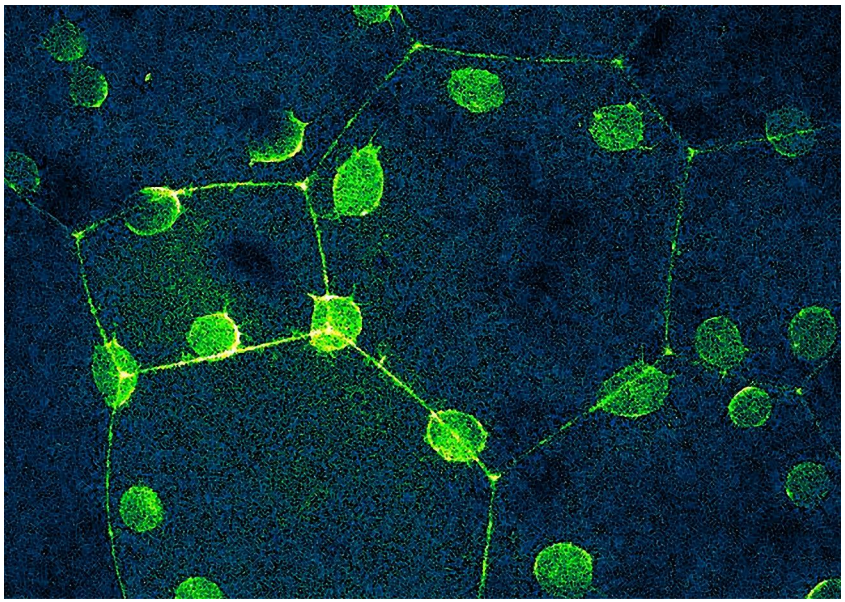
EMBO Practical Course

Optics, Forces & Microscopy 2026



# Light sheet, expansion and single molecule microscopy

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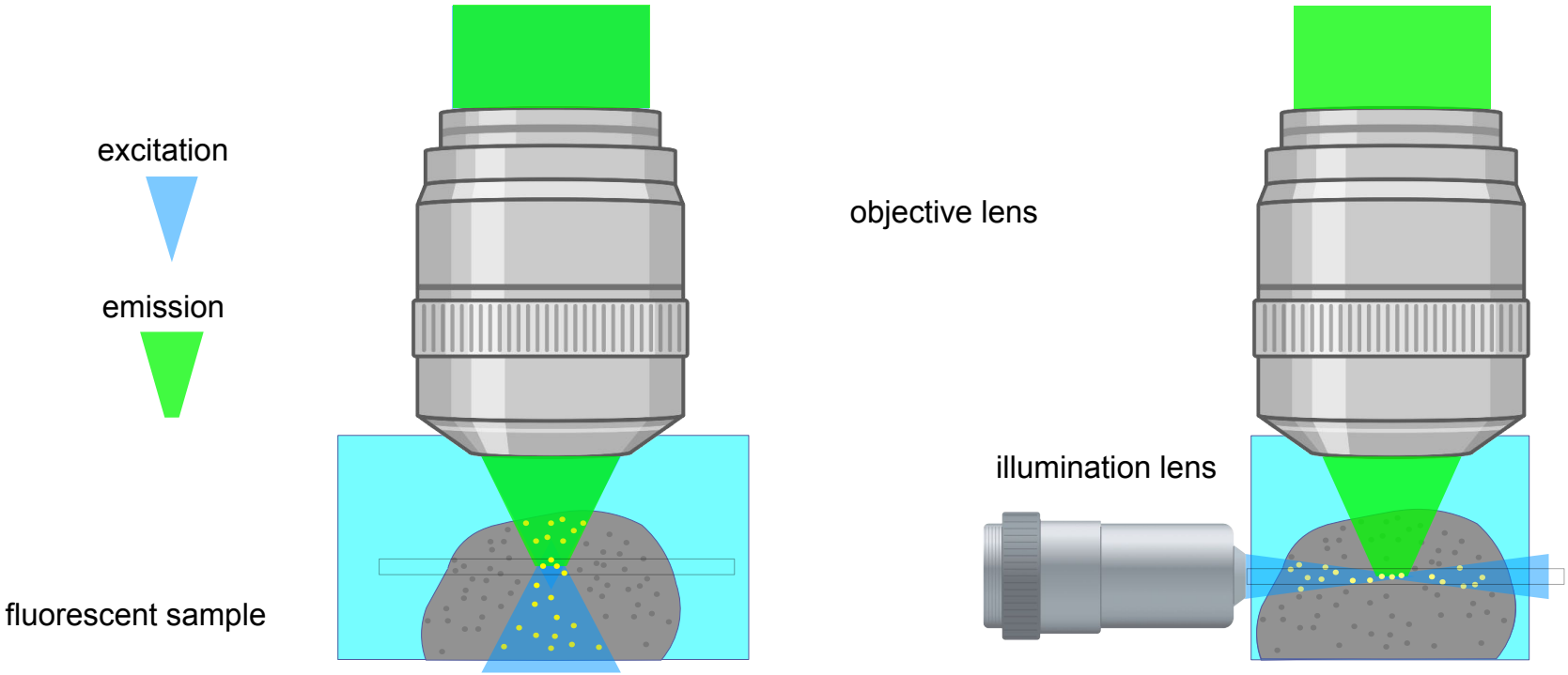
Clausius-Institute of Physical and Theoretical Chemistry
Rheinische Friedrich-Wilhelms-Universität Bonn

Light sheet fluorescence microscopy

# High contrast by light-sheet based  microscopy

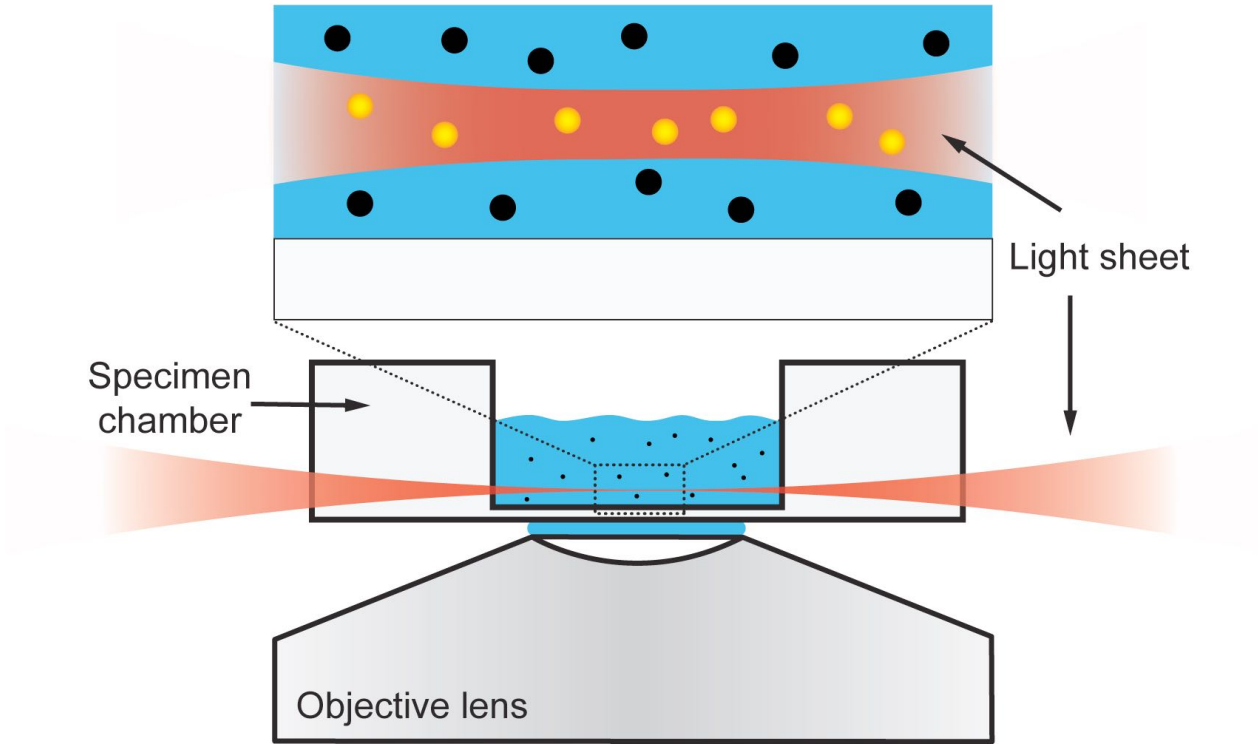



epi-fluorescence

focal plane illumination

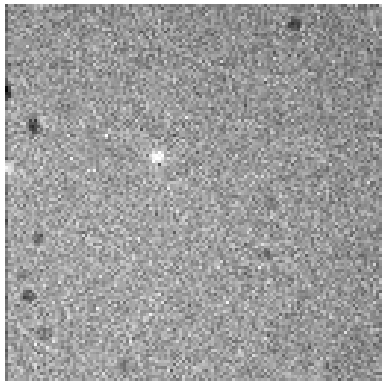
Zsigmondy, 1903
Voie et al, 1993
Huisken et al., 2004
Dodt et al., 2007
.....

# Single molecule imaging in solution

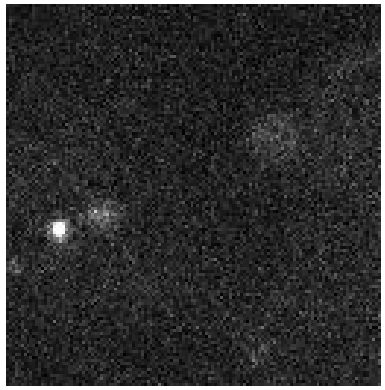


# Comparison between epi- and light sheet illumination

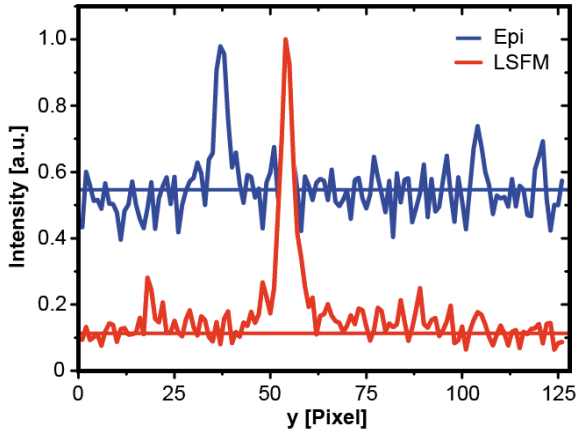
500 kDa dextran-Atto633 in buffer
40X, NA 1.2W objective lens
Image field 19.2 µm
Image acquisition 100 Hz, display 33 Hz



Epi-illumination
Contrast 0.37

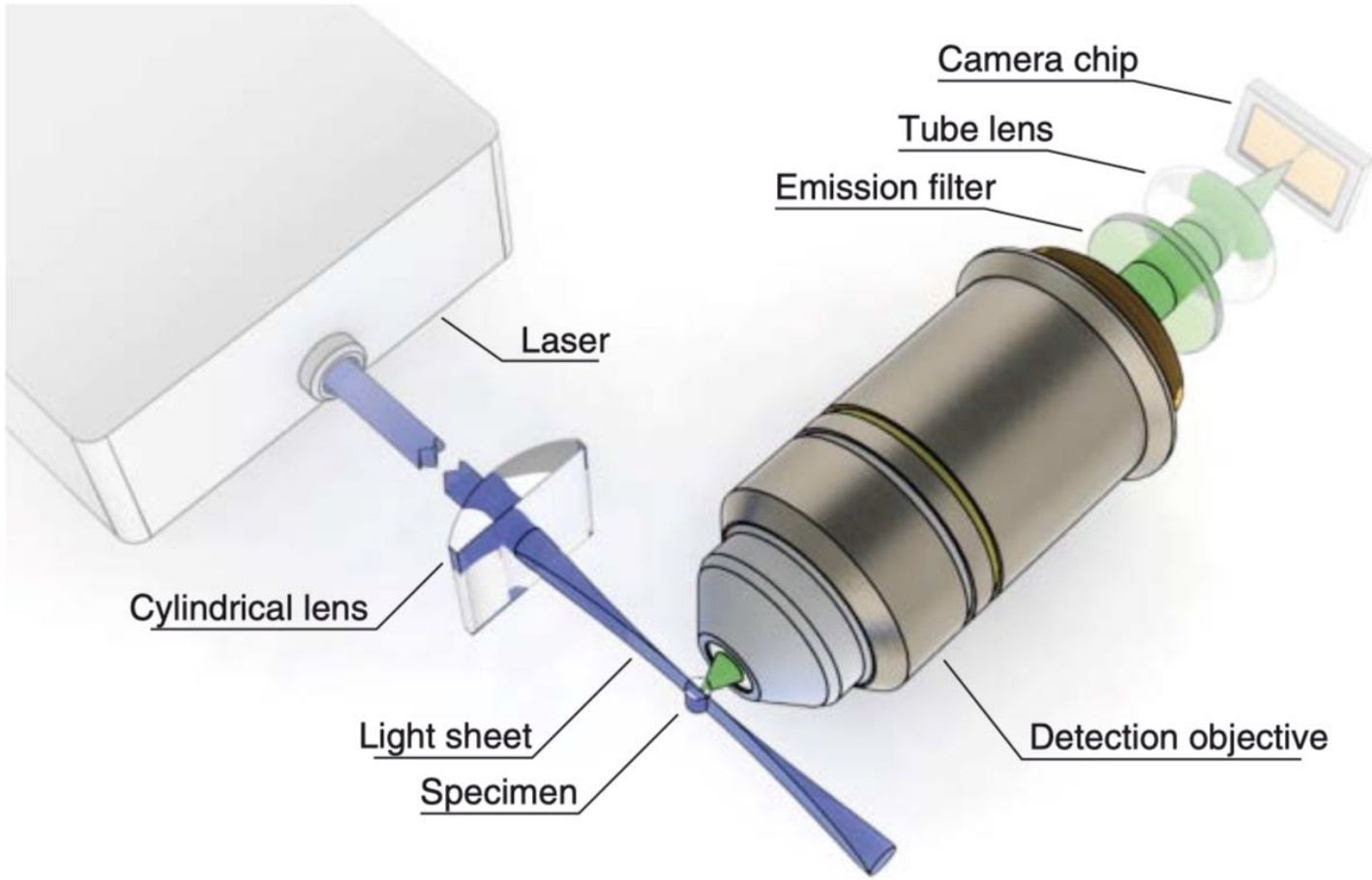


Sheet illumination
Contrast 0.97



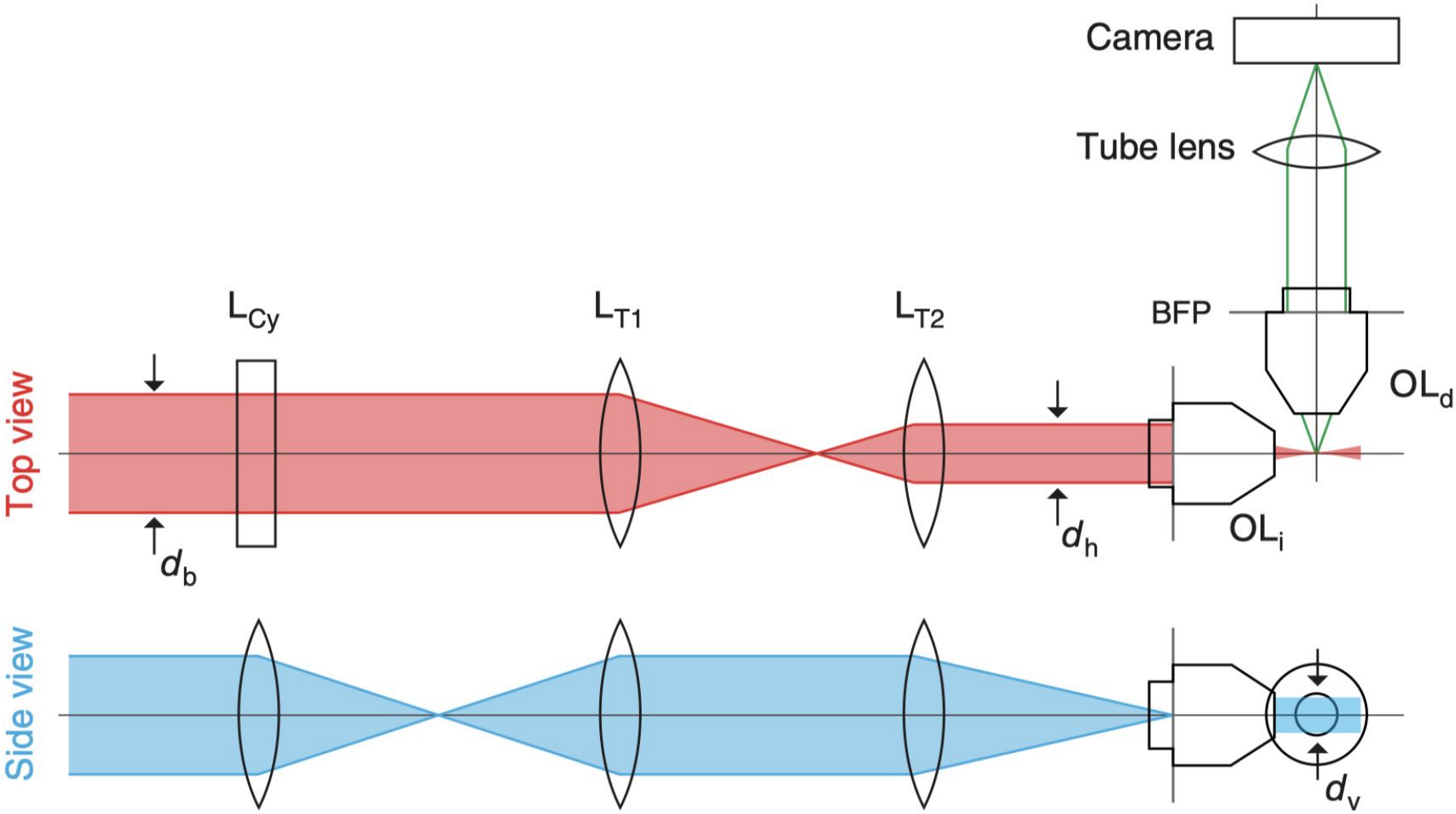

D= 7.1 ± 0.2 µm²/s
as theoretically expected

# Principle of light sheet microscopy



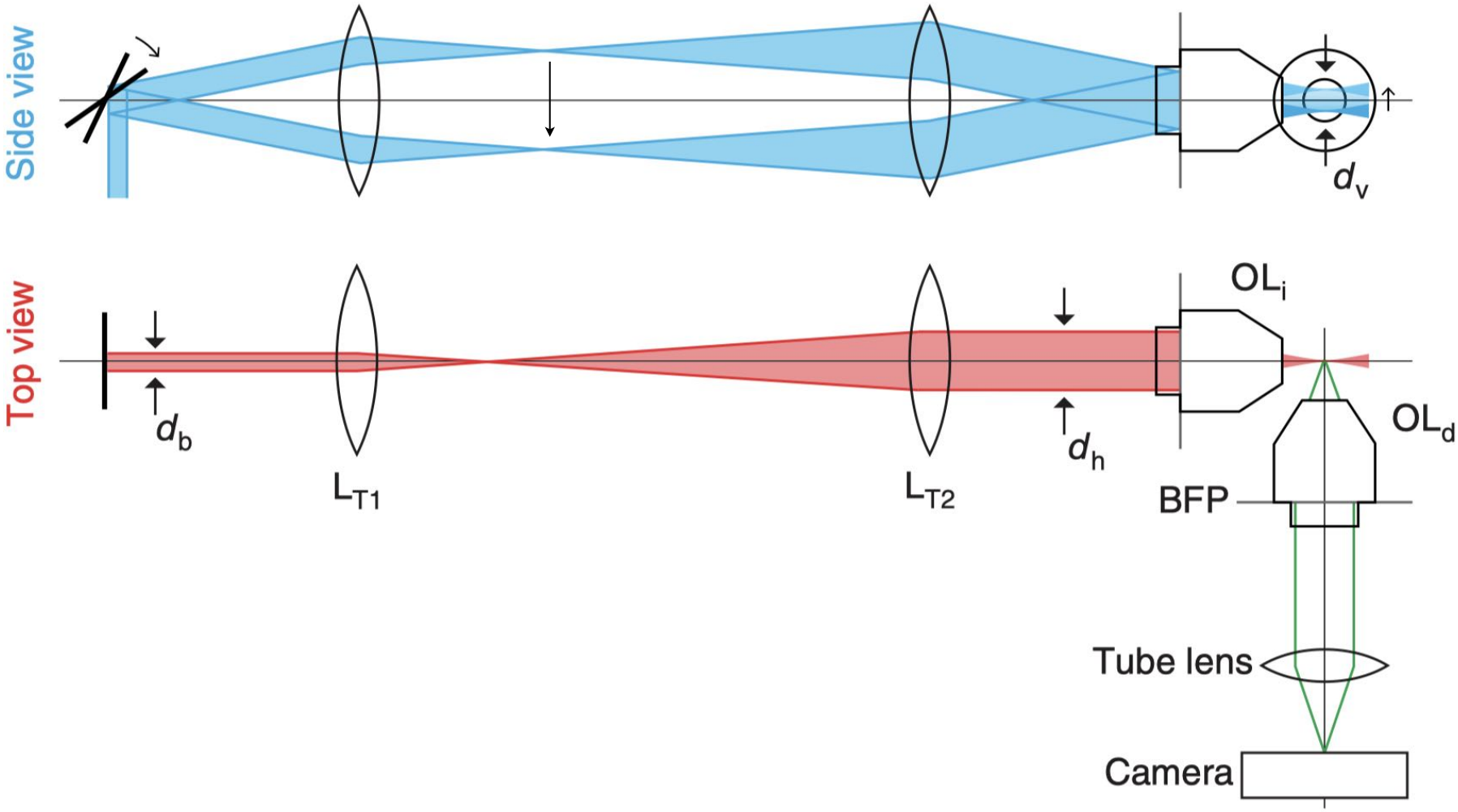

Shah, Weber and Huisken, Chapter 7 „Light Sheet Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

# Light Sheet Microscopy using a cylindrical lens

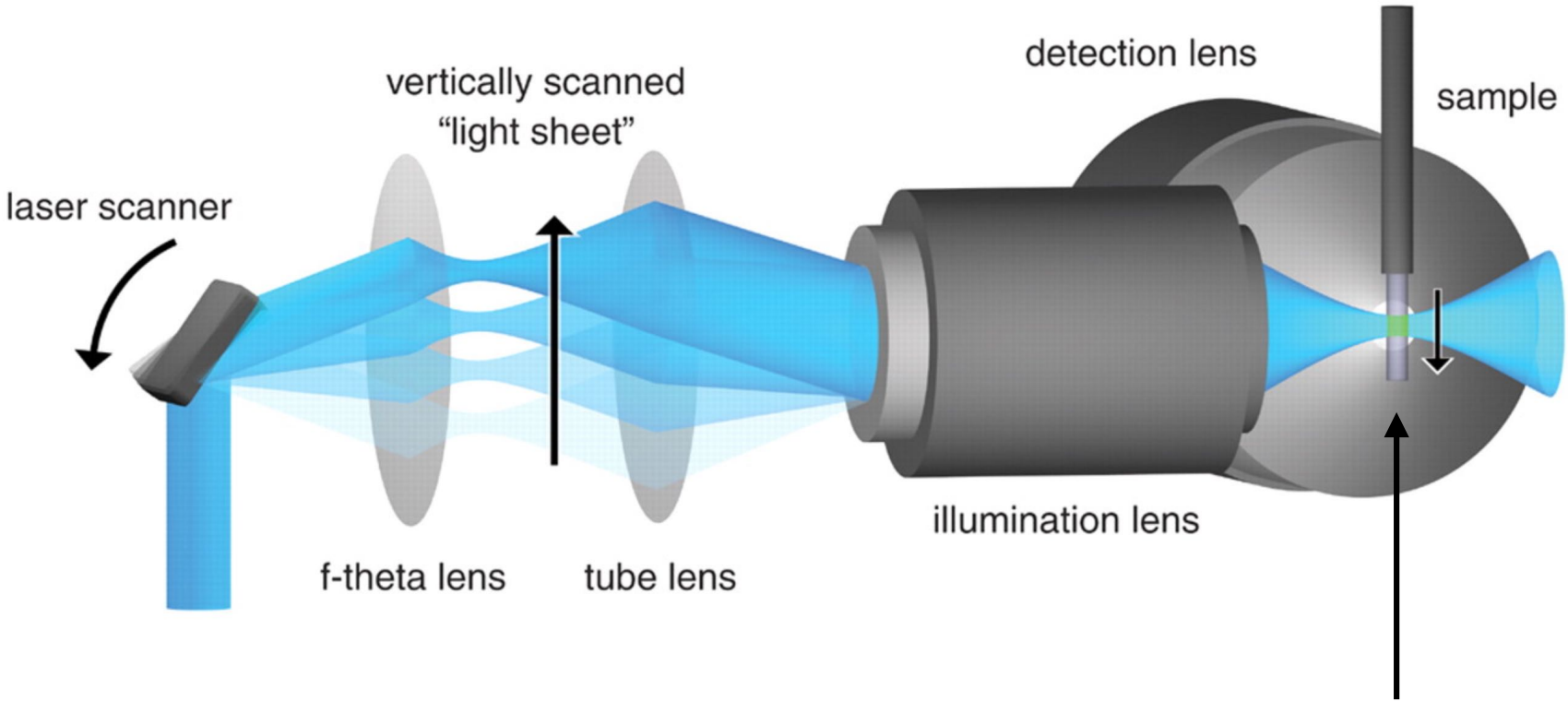



Shah, Weber and Huisken, Chapter 7 „Light Sheet Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

# Scanned Light Sheet Microscopy



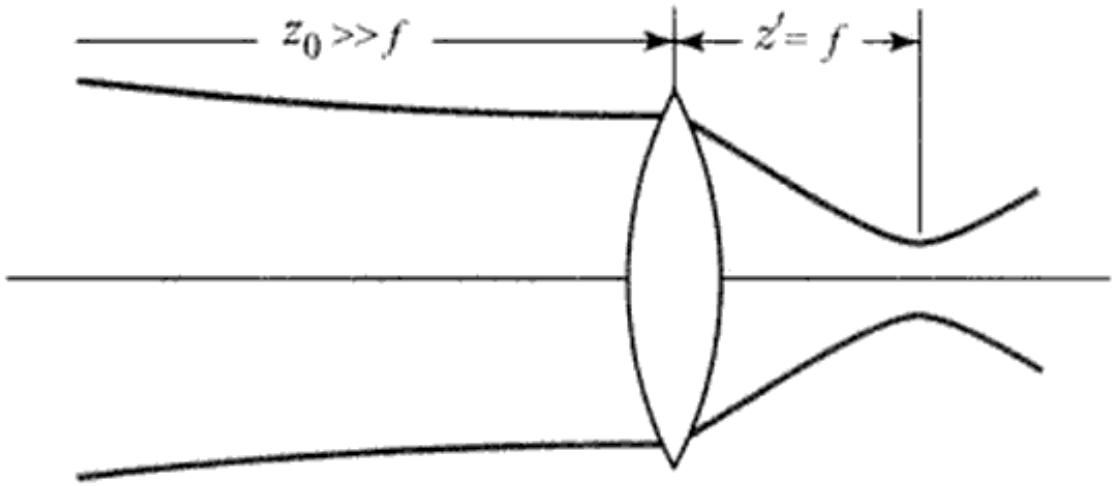
# Scanned Light Sheet Microscopy




Scan circular beam rapidly vertically across the object plane

Philipp J. Keller, et al., Science 322, 1065 (2008)

# Focus of a Gaussian beam



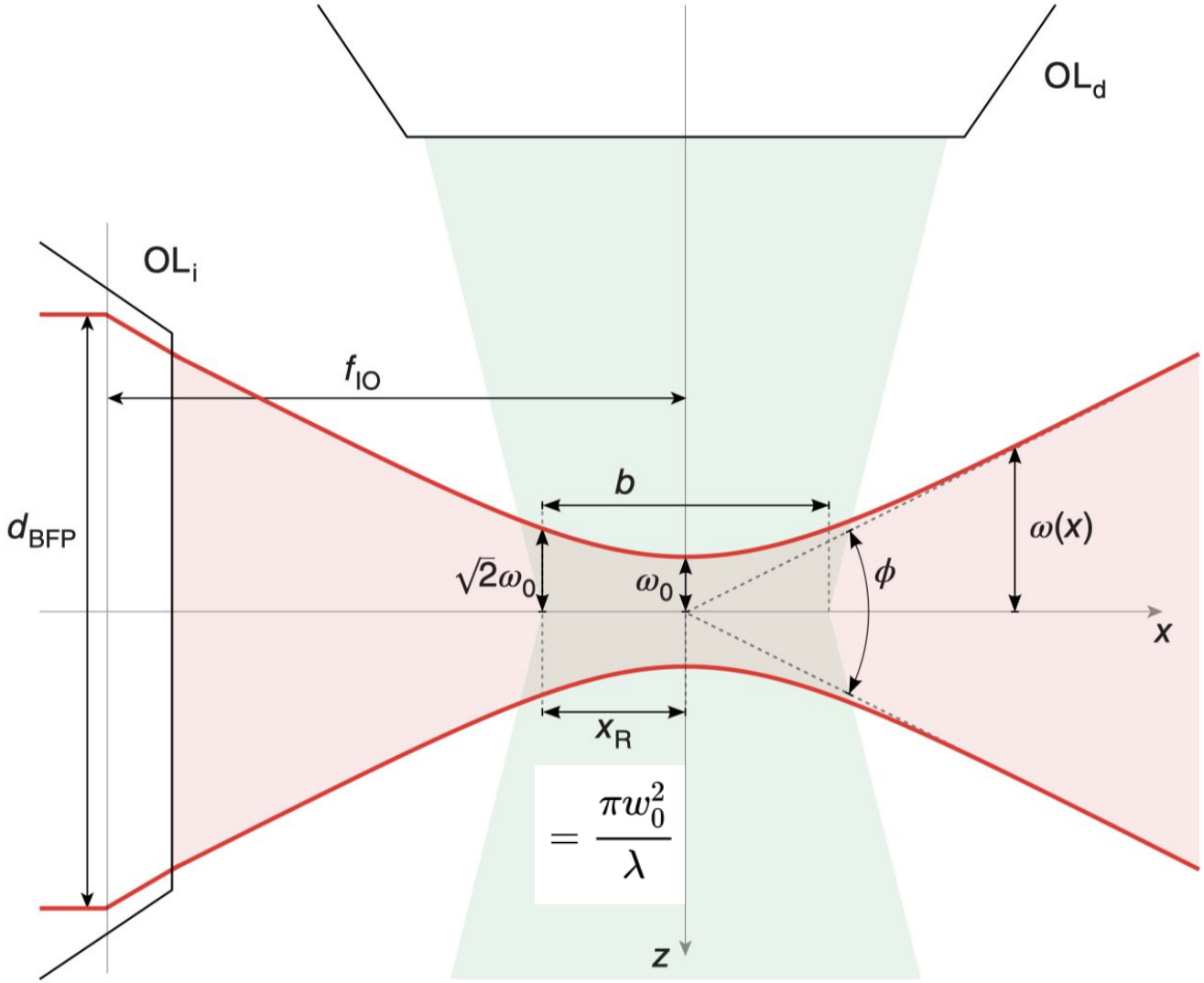

Lens at the waist of the Gaussian beam and focal length of the incident beam >> f

then $$w_0' = \frac{\lambda f}{\pi w_0} \propto \frac{1}{w_0}$$

and

$$z' = f$$

# Relationship between light sheet dimensions and field of view

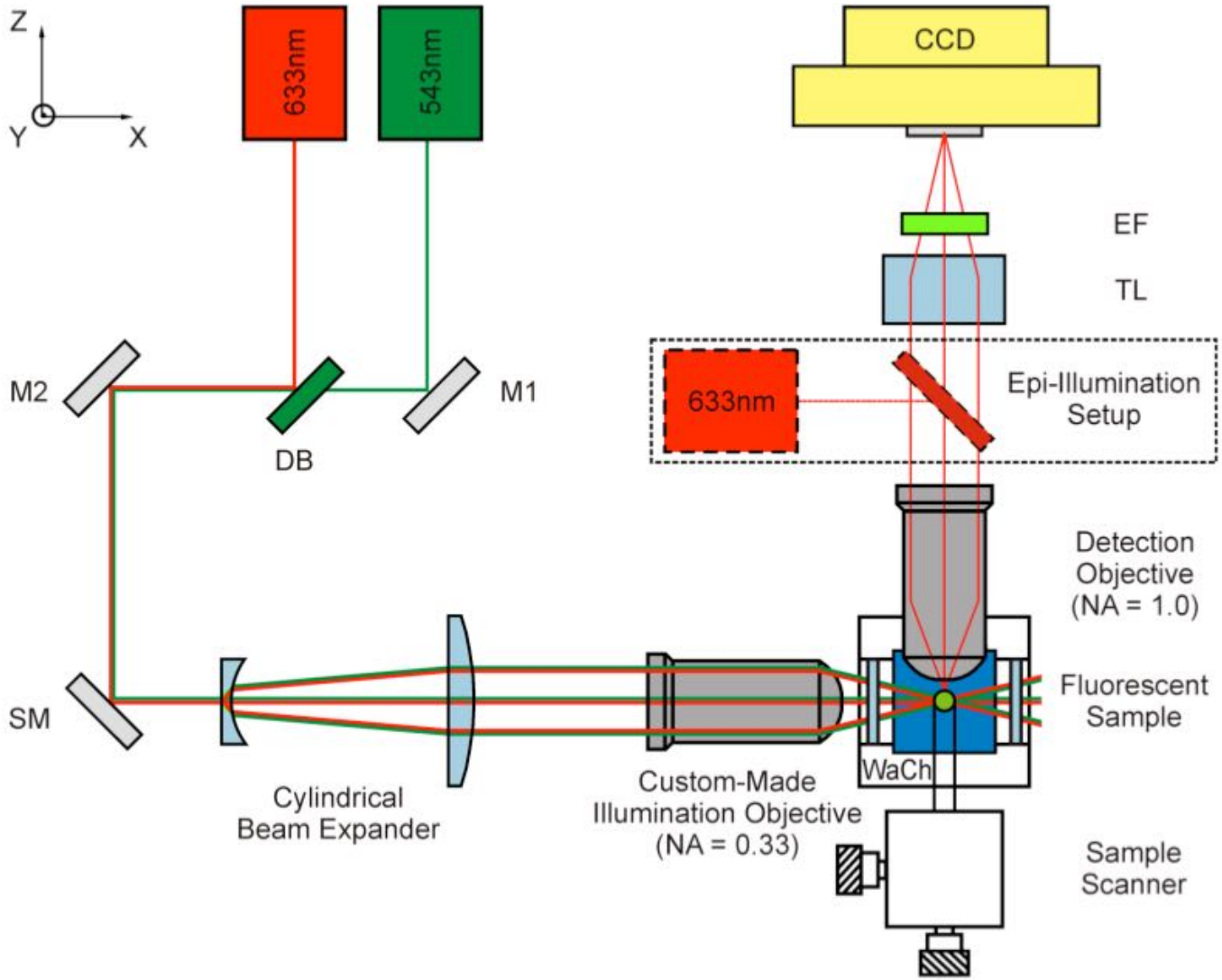



Gaussian beam illumination:
limited observation field size
Width and length are closely related to each other



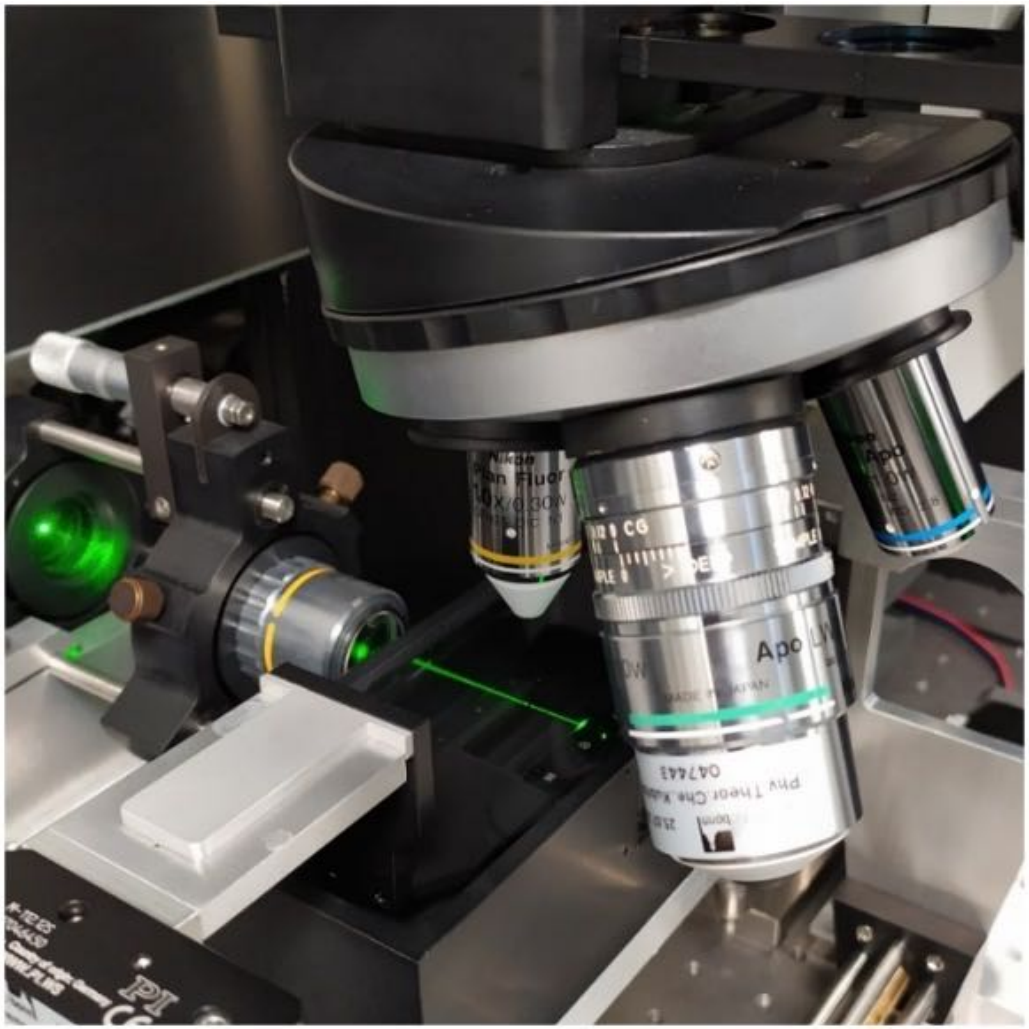
Shah, Weber and Huisken, Chapter 7 „Light Sheet Microscopy“ in „Fluorescence Microscopy“, ed. U. Kubitscheck, 2017 Wiley VCH

# Light sheet fluorescence microscopy

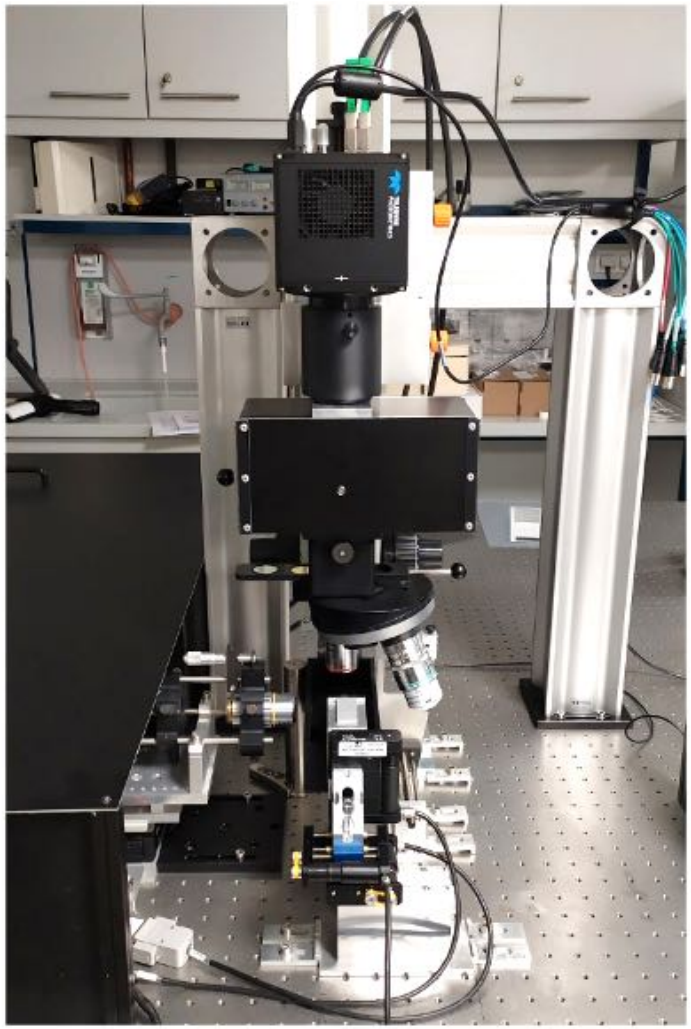


# Light sheet fluorescence microscopy: LSFM

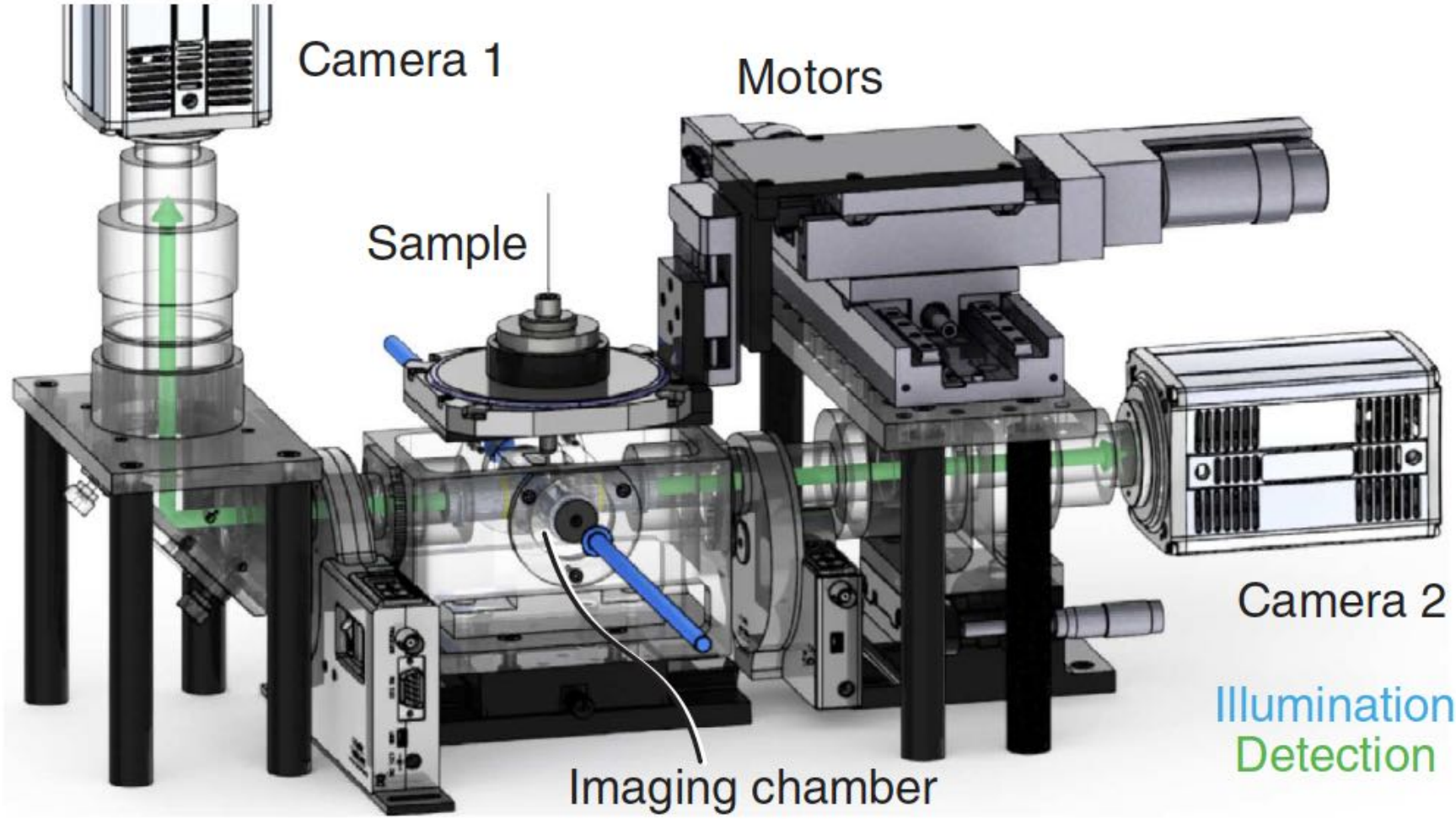
illumination from the side



imaging towards the top

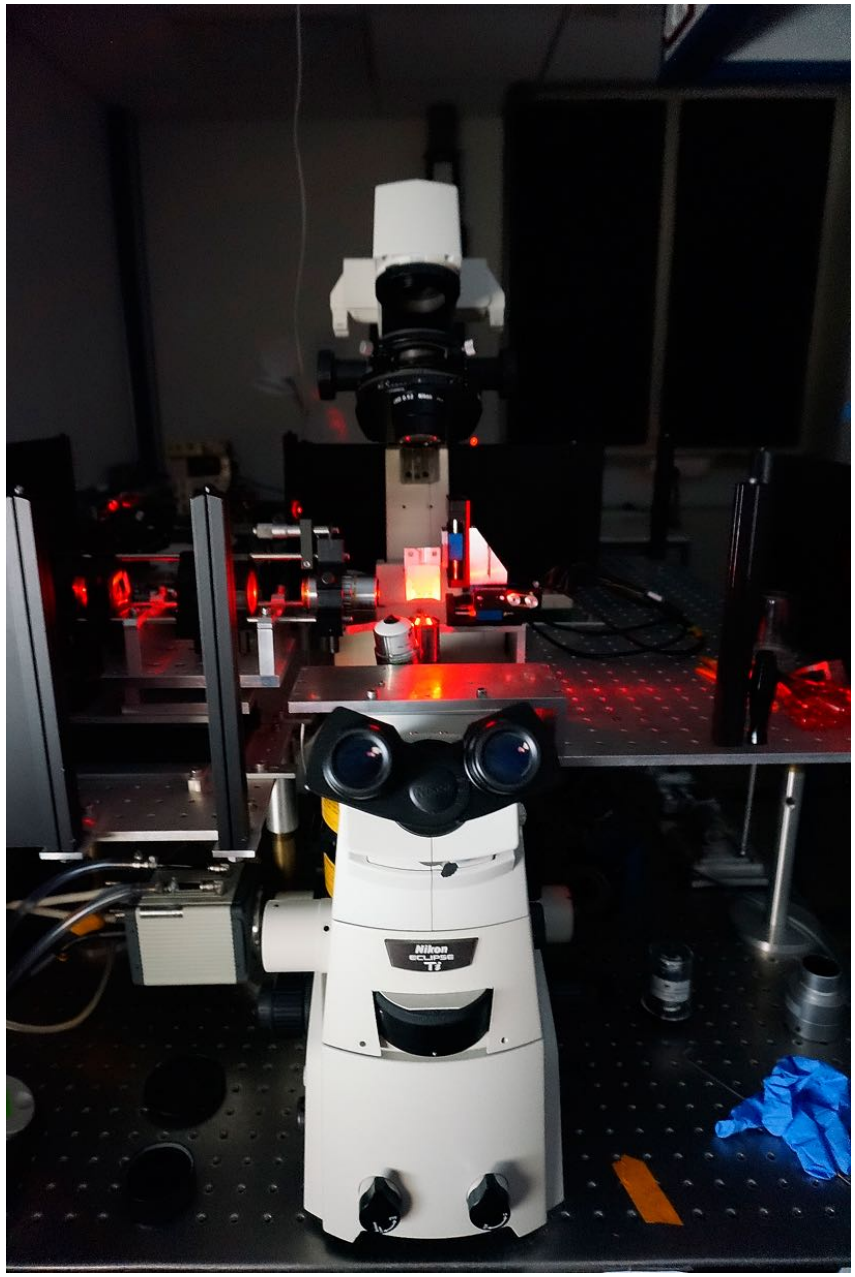


# Example of state-of-the-art setup (Jan Huisken)

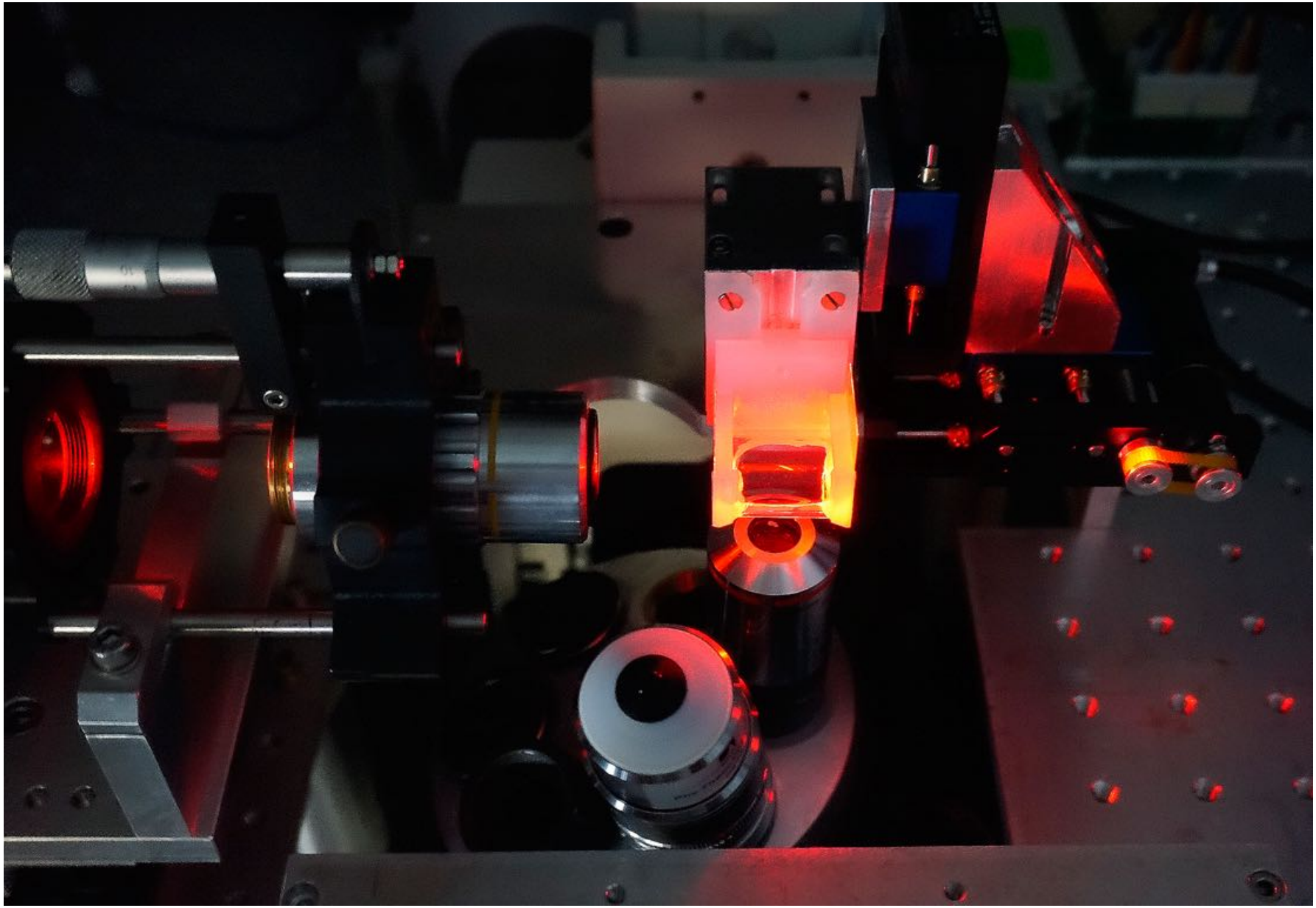



Schmid, ….. and Huisken, 2013, Nature Comm. 4:2207

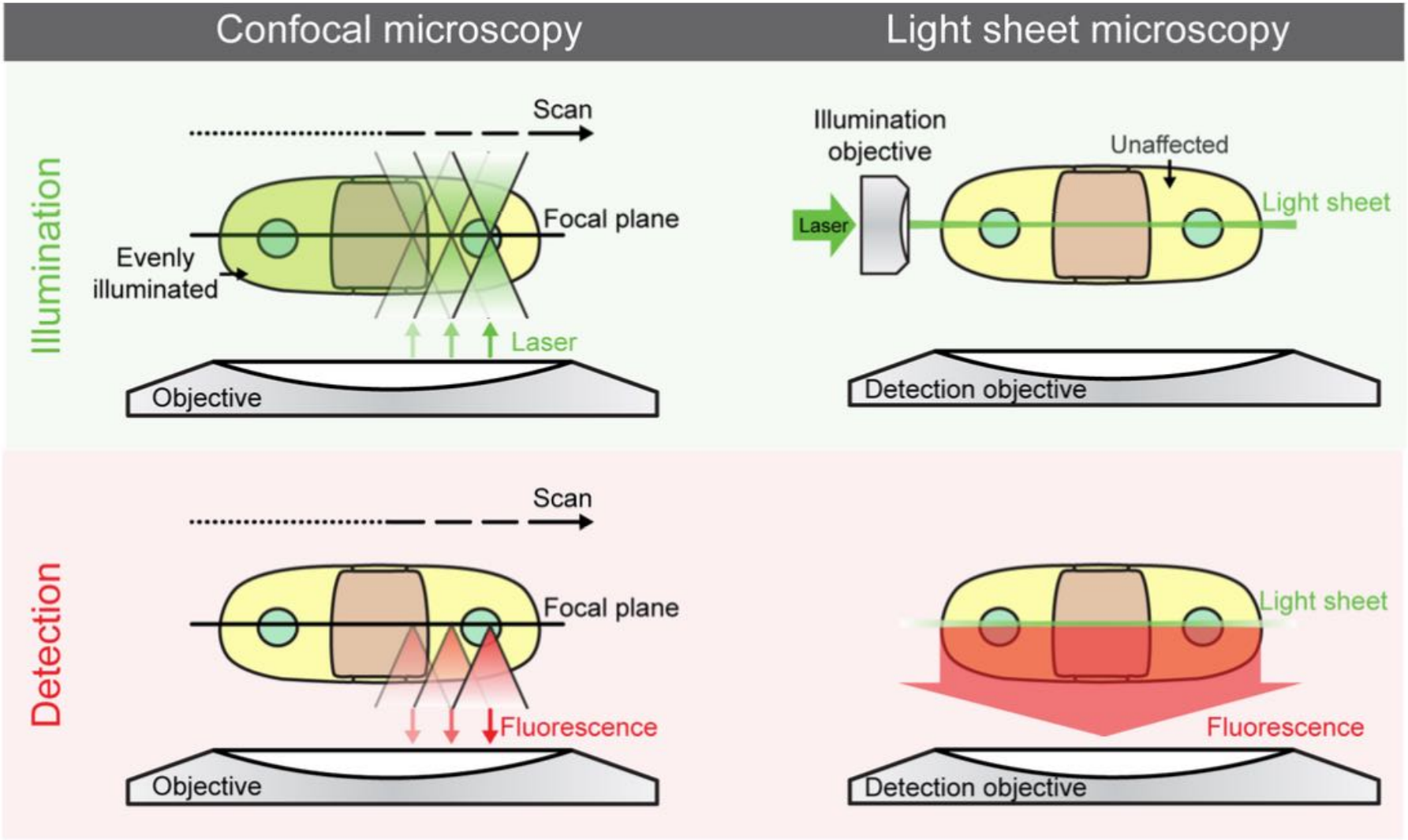
# Light sheet microscopy of expanded samples



# Light sheet microscopy of expanded samples



# Image generation in confocal and light sheet microscopy

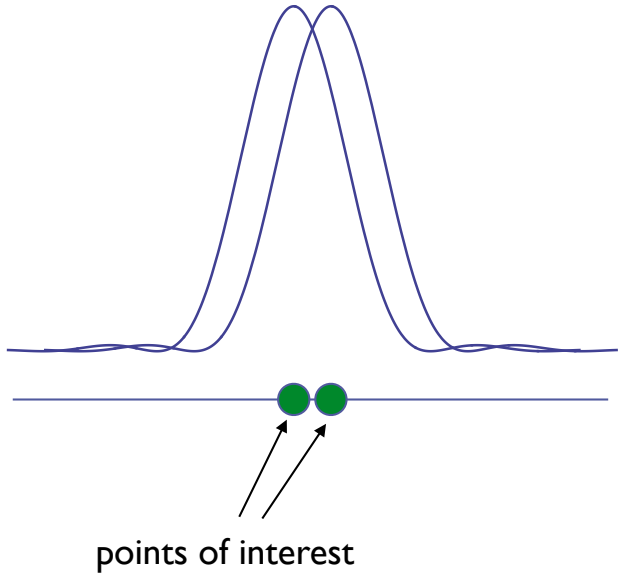



Jörg Ritter, Bonn 2011

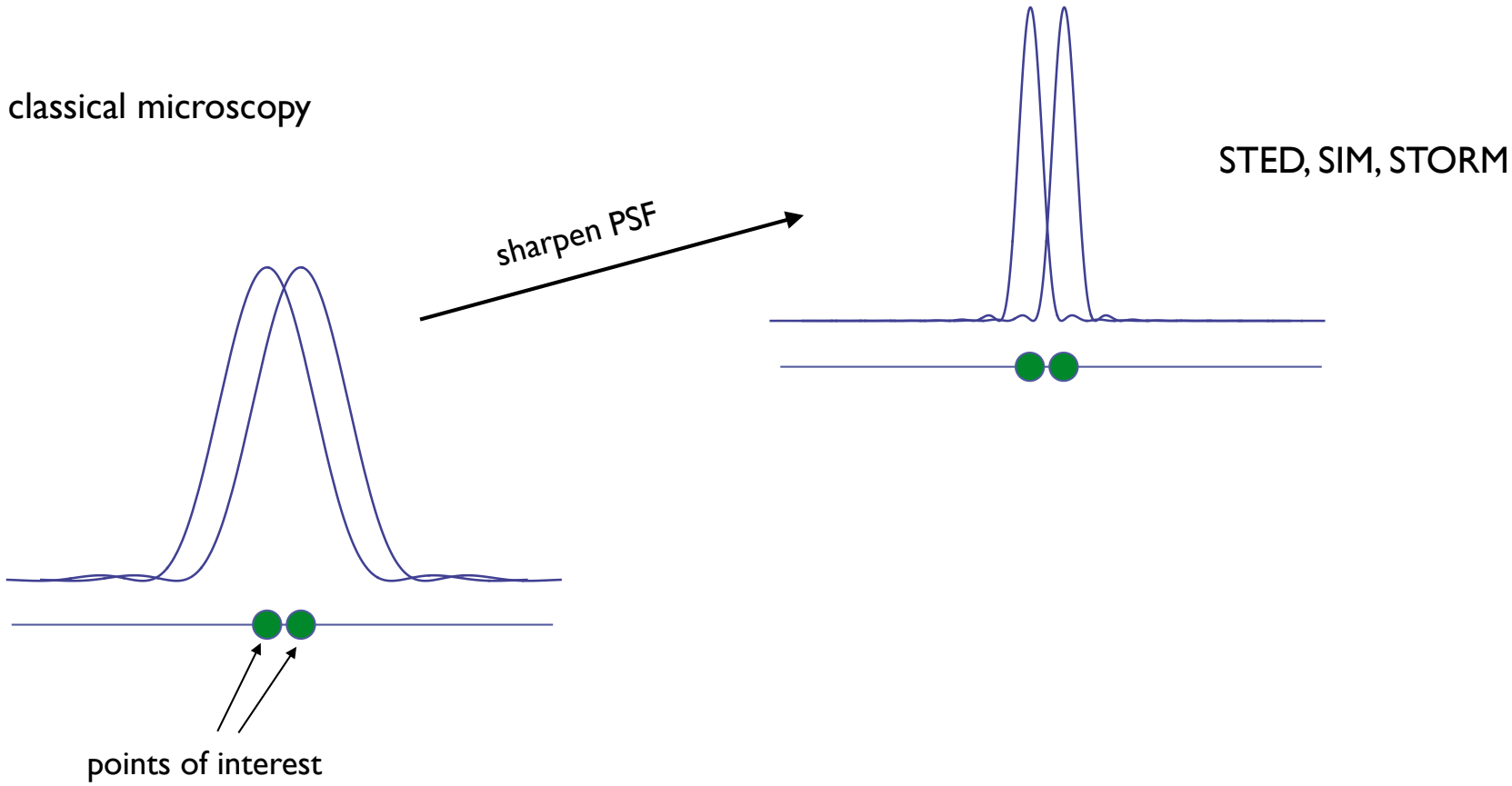
# Expansion microscopy

# How to image a large sample with sub-resolution features?

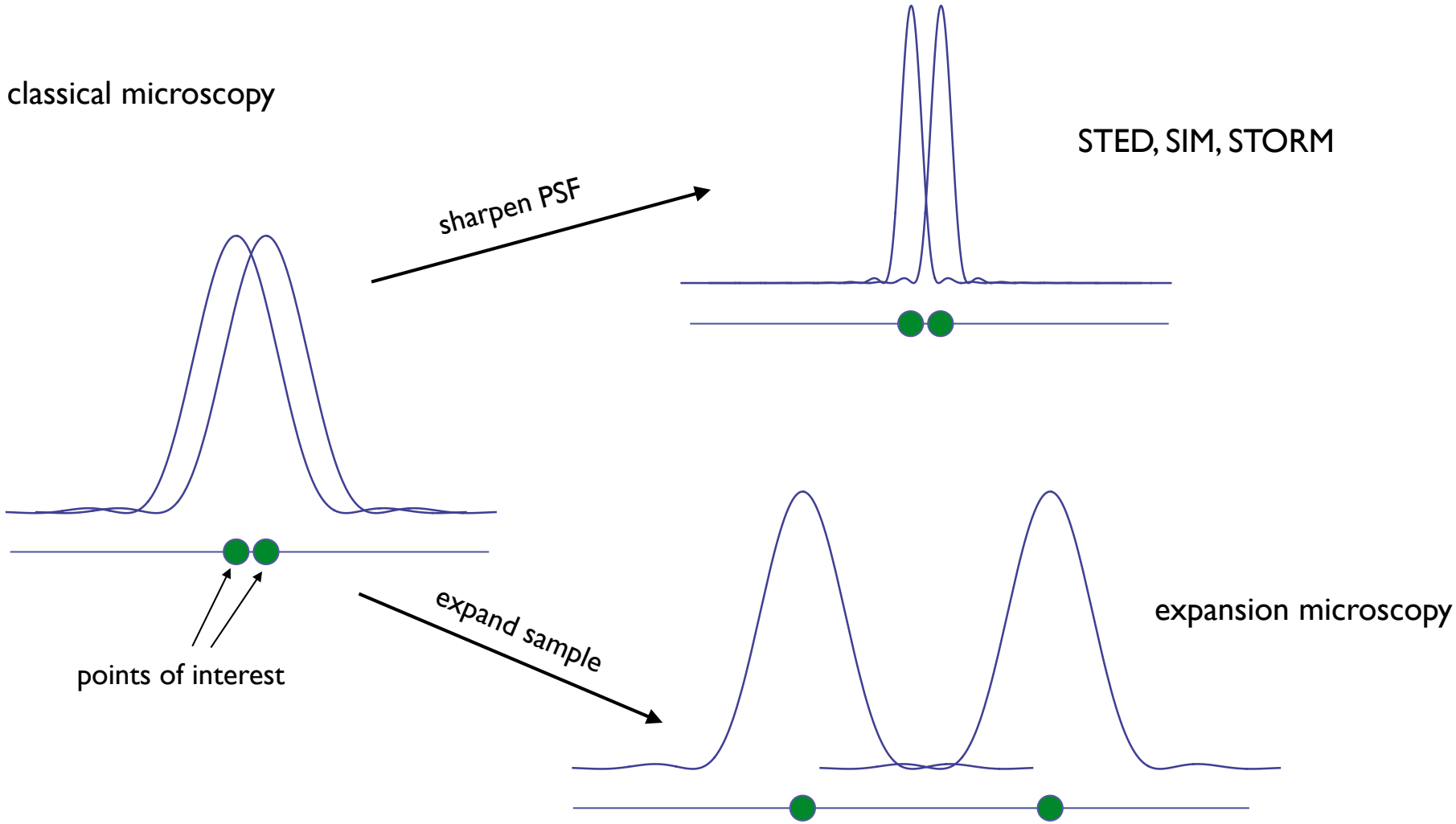
classical microscopy



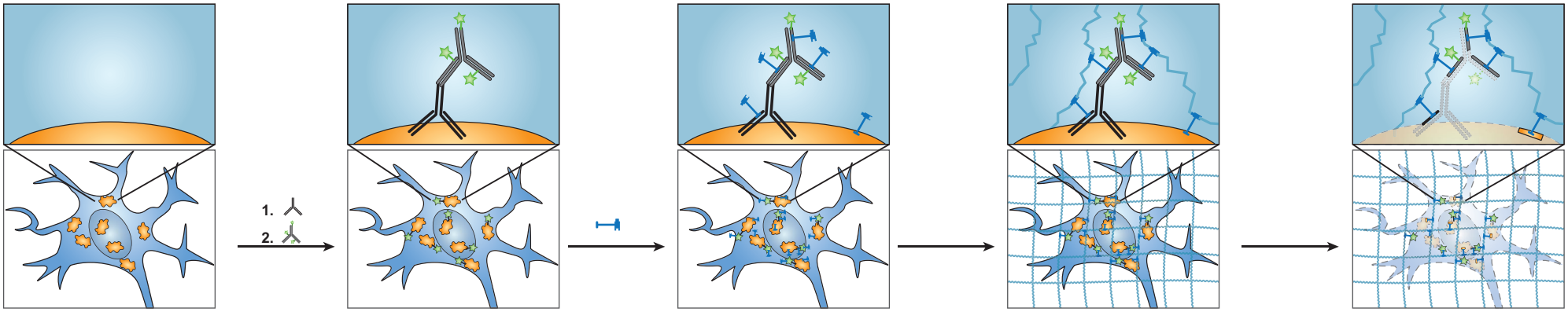
# Increase optical resolution by „sharpening“ the point spread function



# … or use expansion microscopy

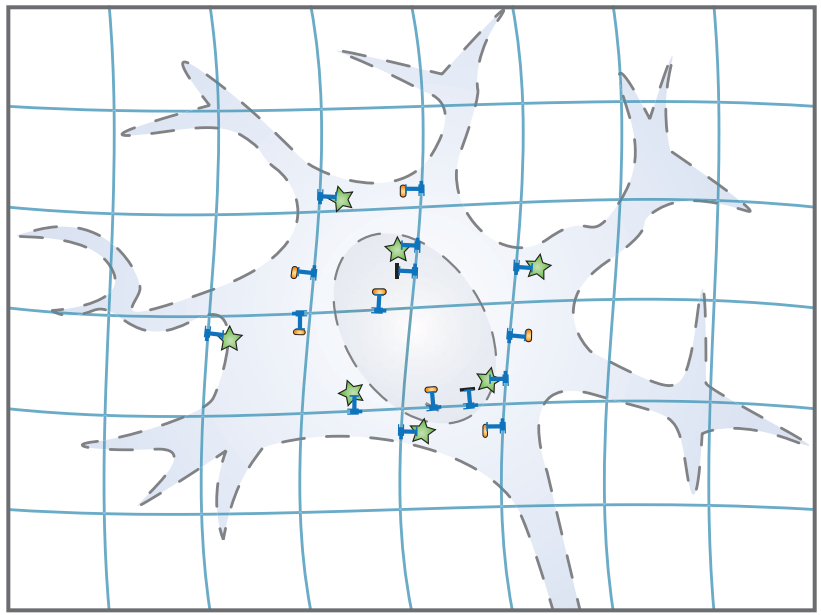


# Biochemical procedure of expansion




**Advantages**:
Transparent sample
Refractive index of water

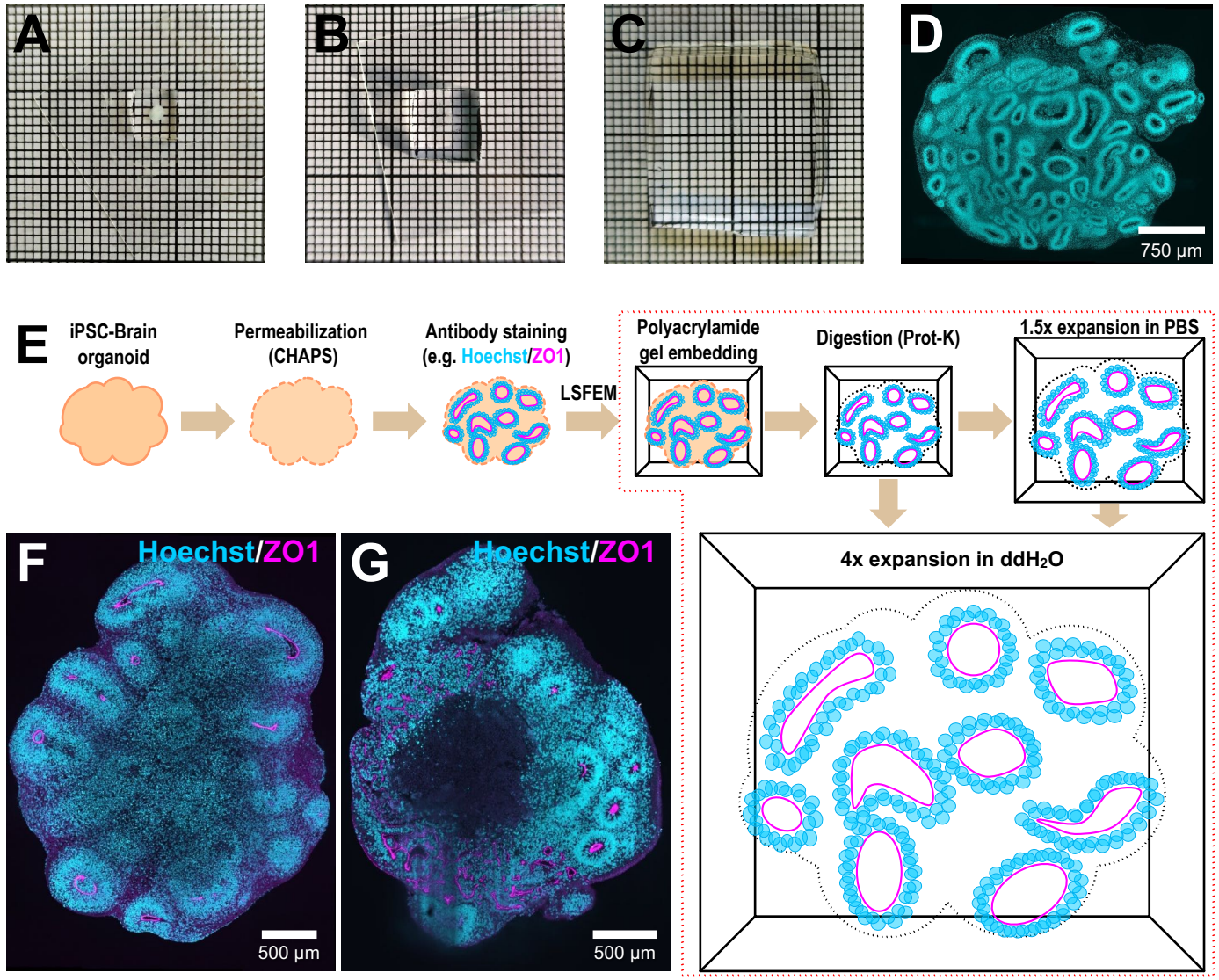
Expansions up to 20x



Expansion 4x

Expansion according to: Chozinski, ….. and Boyden, Nature Methods (2016)

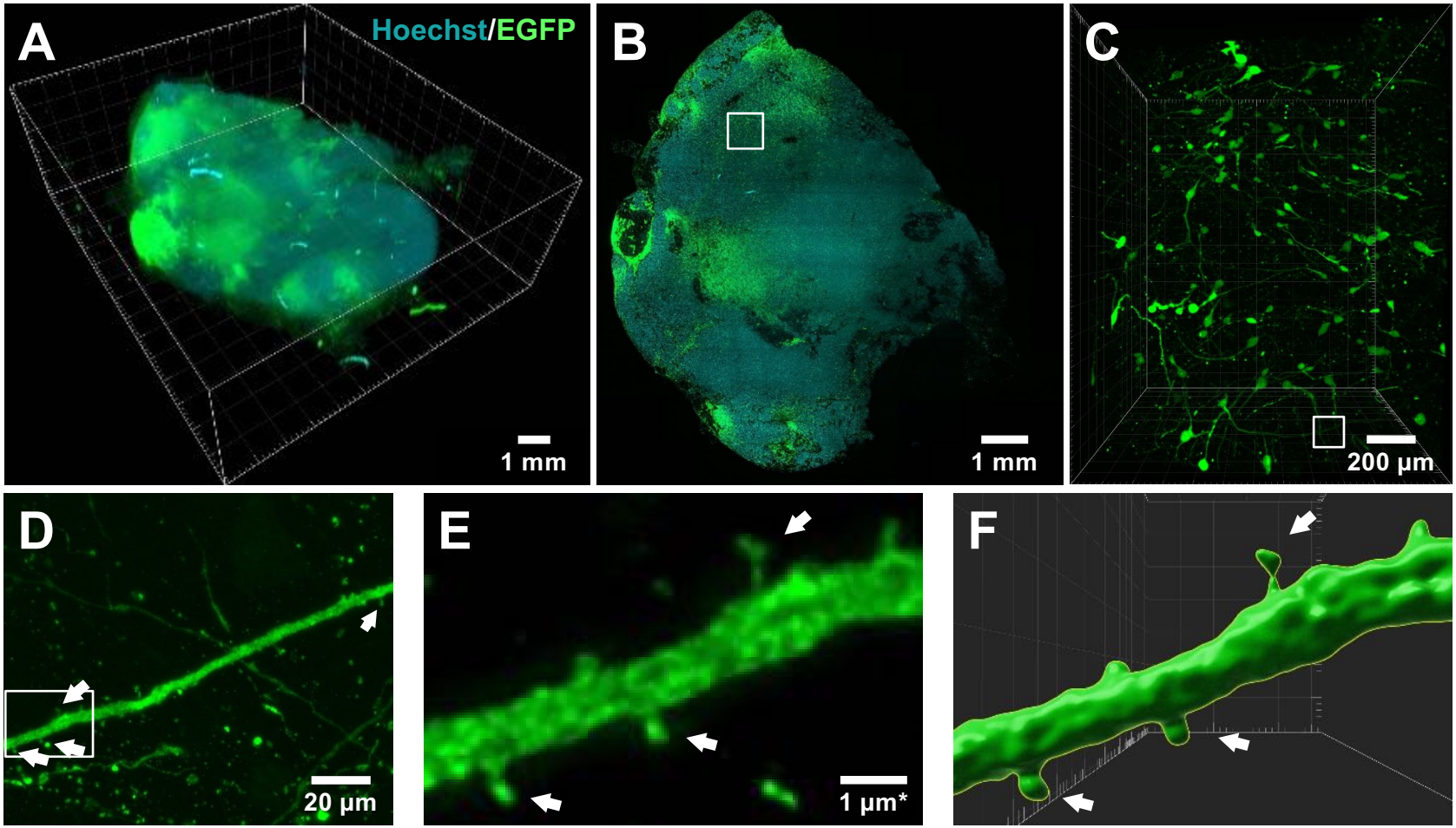
# Brain organoid sample preparation for LFSM




(A) Two months old brain organoid embedded in a polyachrylamide gel. (B) Two months old organoid after proteinase K digestion, which resulted in a clearing of the organoid and an approximately 1.5-fold expansion. (C) The same organoid after expansion in bidistilled water, which yielded an approximately 4-fold expansion. (D) Optical section of the cleared and 1.5-fold expanded organoid in a depth of 1.2 mm. (E) Pipeline for organoid sample preparation: fixation, permeabilization, immunostaining for identifying specific cell types or structures and nuclear staining. The tight junction marker ZO1 - magenta, and nuclear staining Hoechst – cyan, are used as example. Then the sample is embedded in and chemically linked to a polyacrylamide gel. Next, digestion using proteinase K (Prot-K) renders the sample transparent. Placing the sample in PBS leads to expansion of 1.5x, while placing the sample in bi-distilled water leads to a 4x expansion. Optical sections of (F) a three months old brain spheroid and (G) a two months old brain organoid.

Rodríguez-Gatica et al., Development 2022

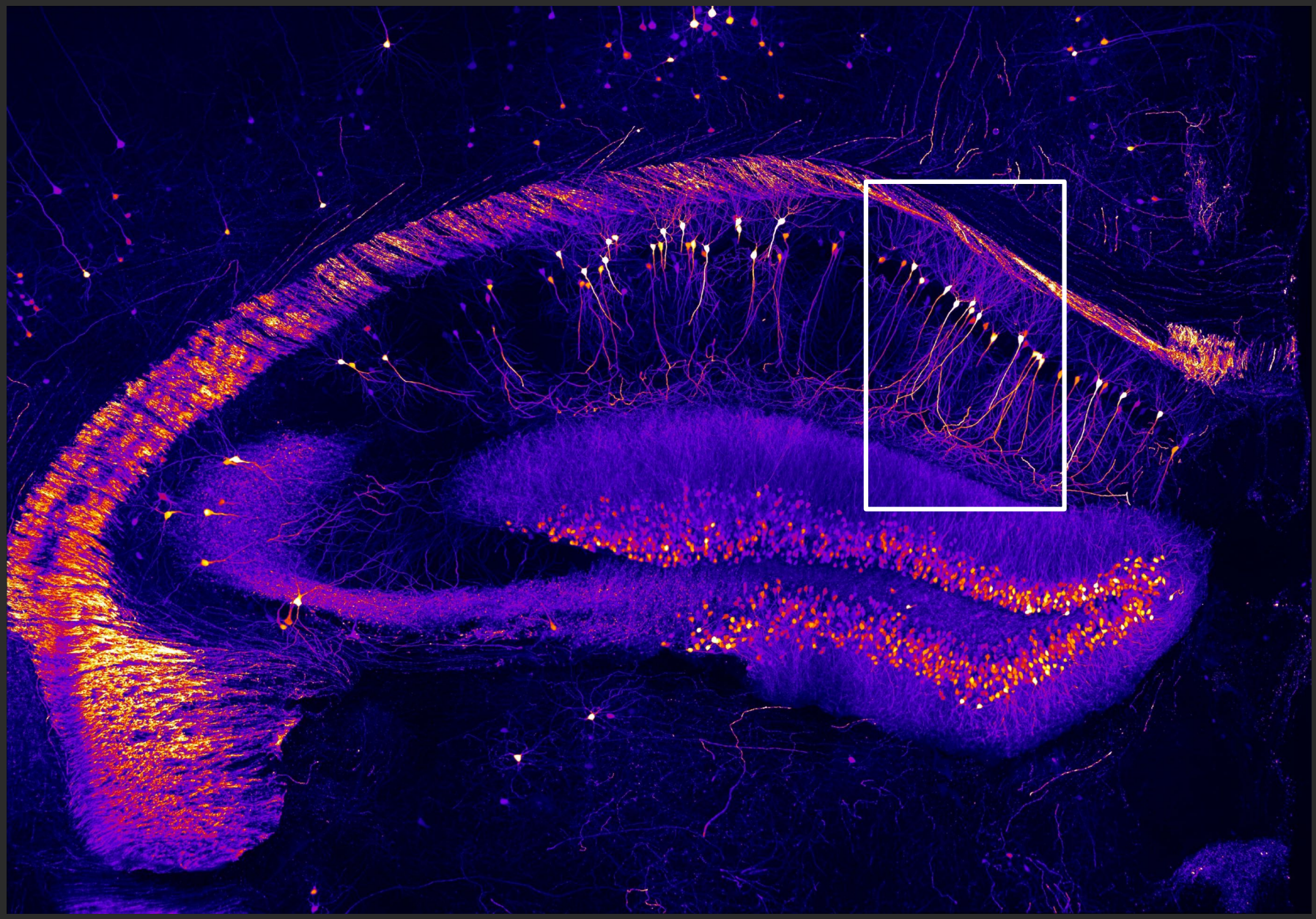
# Five months old brain organoid containing GFP-positive cells




(A) 3D view, volume 13.1 x 14.9 x 5.2 $mm^3$.
(B) Optical slice at a depth of 1.8 mm. The image was obtained using a 10x NA 0.3 objective lens. Size 13.1 x 14.9 $mm^2$
(C) Rendering of a 3D stack with a volume of 1.25 x 1.55 x 1.3 $mm^3$ as marked in (B). The image was obtained using a 25x NA 1.1 objective in the same sample after a 4-fold expansion.
(D) Magnification of the region marked in (C), 185 x 132 $\mu m^2$ revealing spine-like structures.
(E) Magnification of the region marked in (D). The adjusted scale bar 1 $\mu m$* considered the expansion and physically corresponded to 4 $\mu m$.
(F) Surface rendering of the neural projection revealed spine-like structures.
For (C) to (E), the shown image data were deconvolved.

Rodríguez-Gatica et al., Development 2022

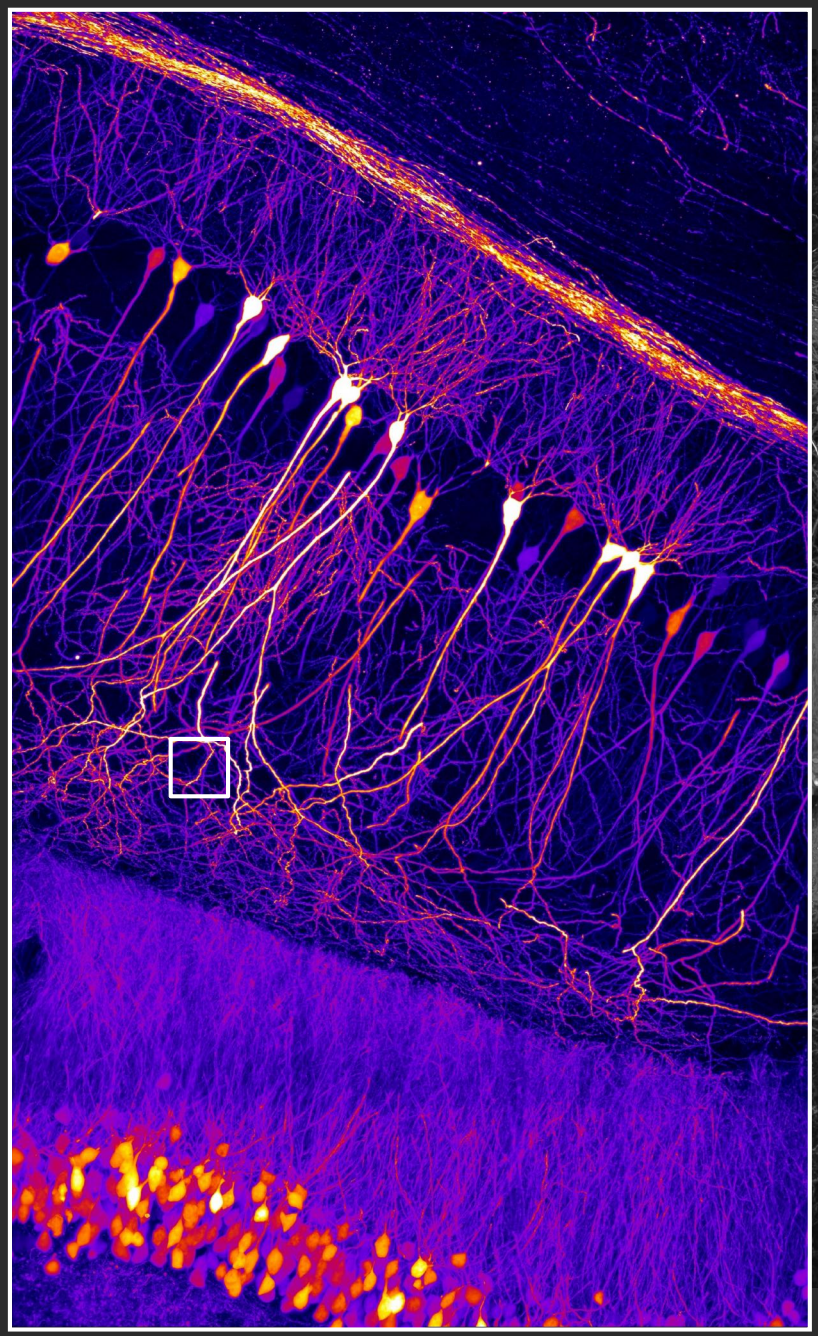
# Mouse brain: CA1-Region and *Gyrus dentatus* with granule cells

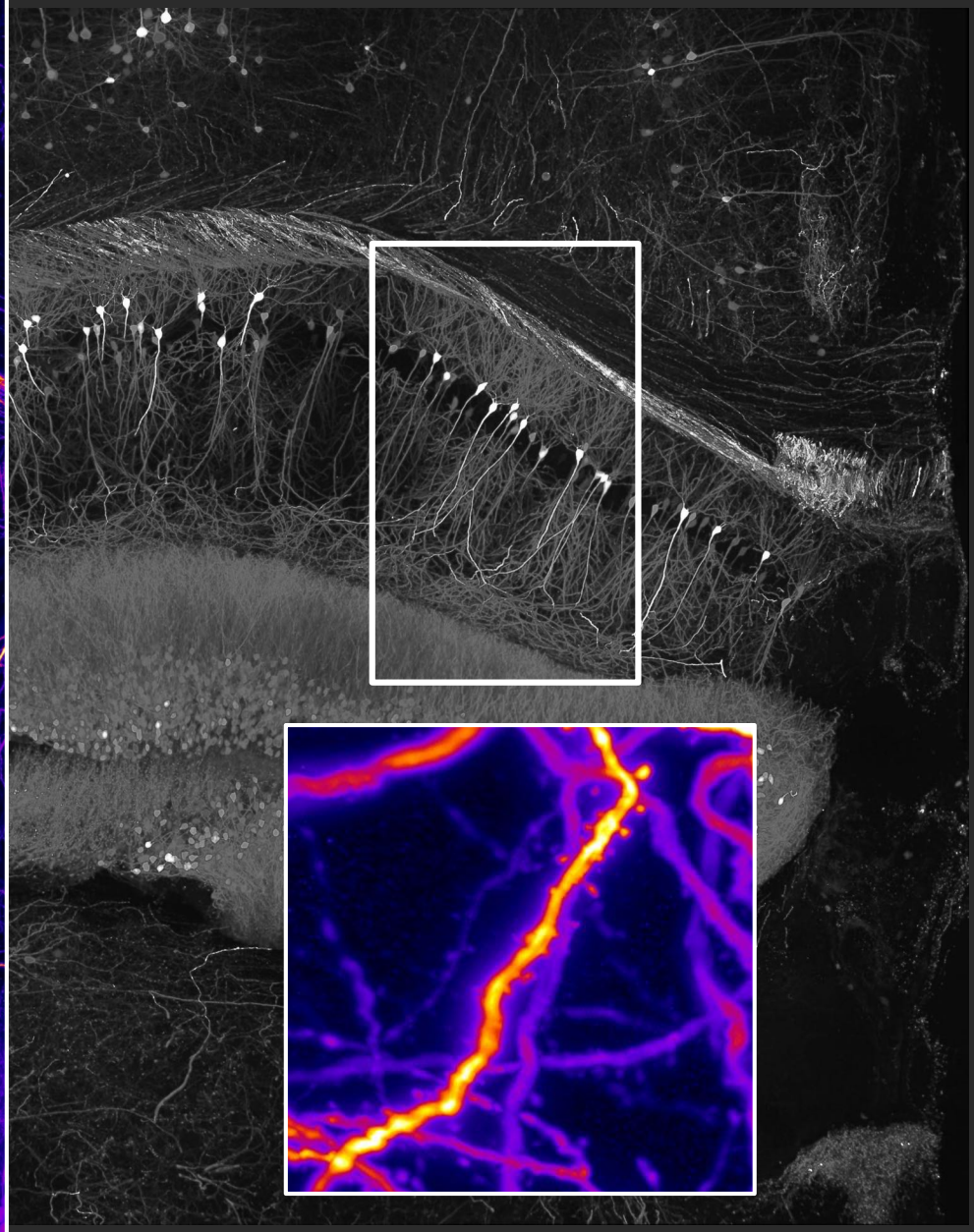


*Kelly et al., submitted*

Maximum-Intensity-Projection of 434 sections (10 tiles)
LSFM 4x/ NA 0.2 Air objective
Size 8.8 x 6.13 $mm^2$

# Mouse brain: CA1-Region and *Gyrus dentatus* with granule cells





*Kelly et al., submitted*

Maximum-Intensity-Projection of 434 sections (10 tiles)
LSFM 4x/ NA 0.2 Air objective
Size 8.8 x 6.13 $mm^2$

# Summary: light sheet fluorescence expansion microscopy

Imaging of extended brain slices

Super resolution (laterally $\lesssim$ 80 nm, axially $\lesssim$ 300 nm)

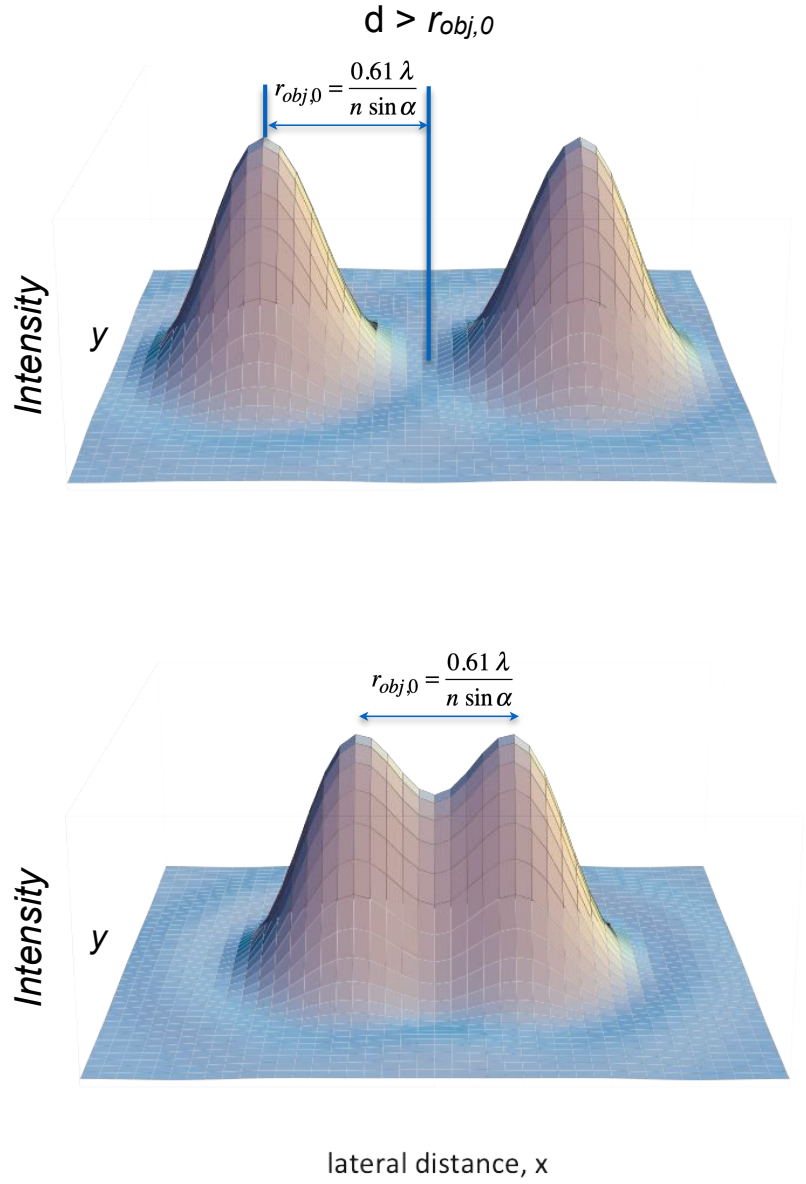
Neuronal connectivity details (spines, pre- and postsynaptic structures)

Imaging of organoids on the meso-, micro- and nanoscale

E.g., analysis of mouse brain connectomics

# Single molecule microscopy

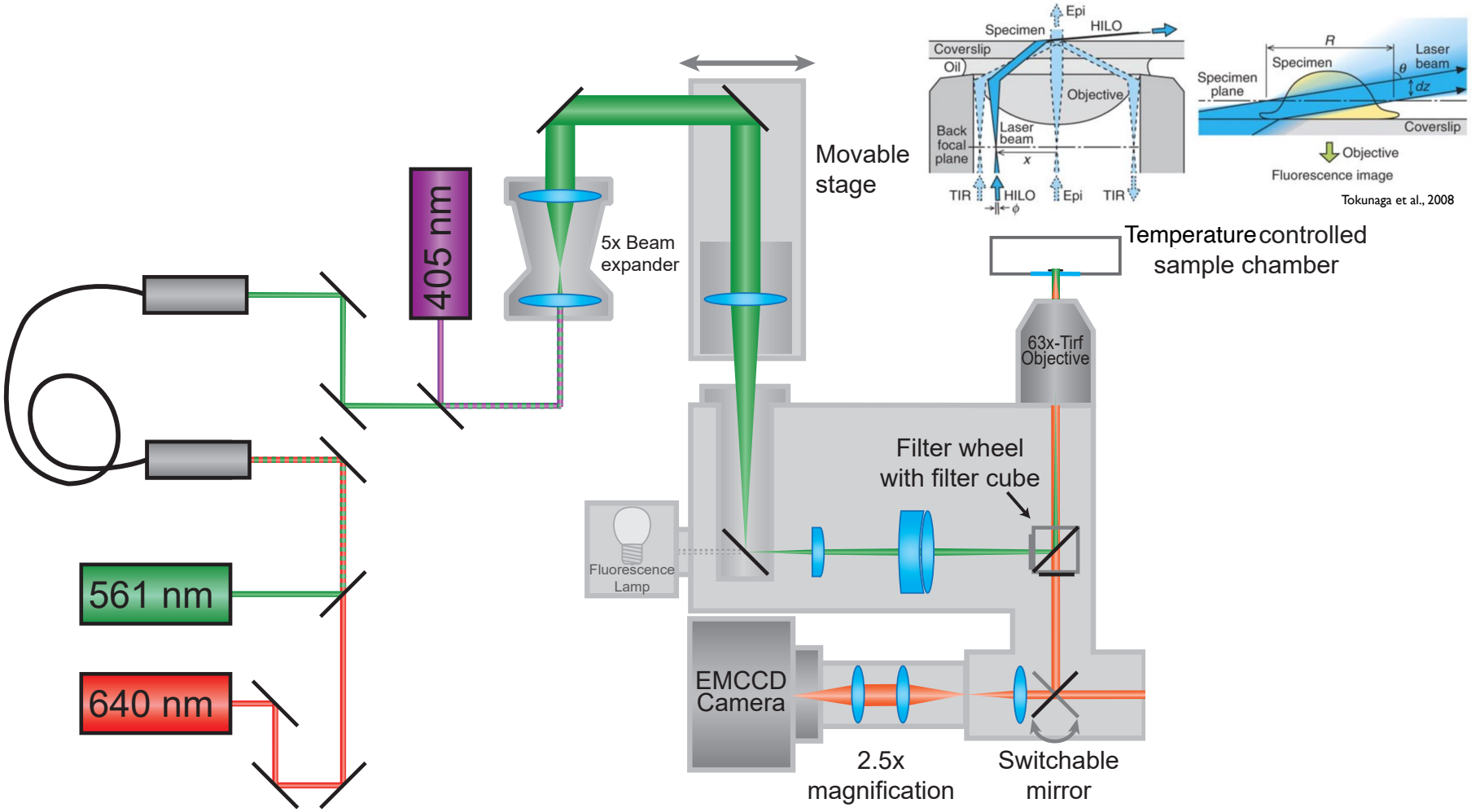
# Sum of Point Spread Functions for Incoherent Point Objects






From “Fluorescence Microscopy: From Principles to Biological Applications“, edited by Ulrich Kubitscheck. Wiley-VCH, Weinheim, 2nd edition

# Single molecule microscope

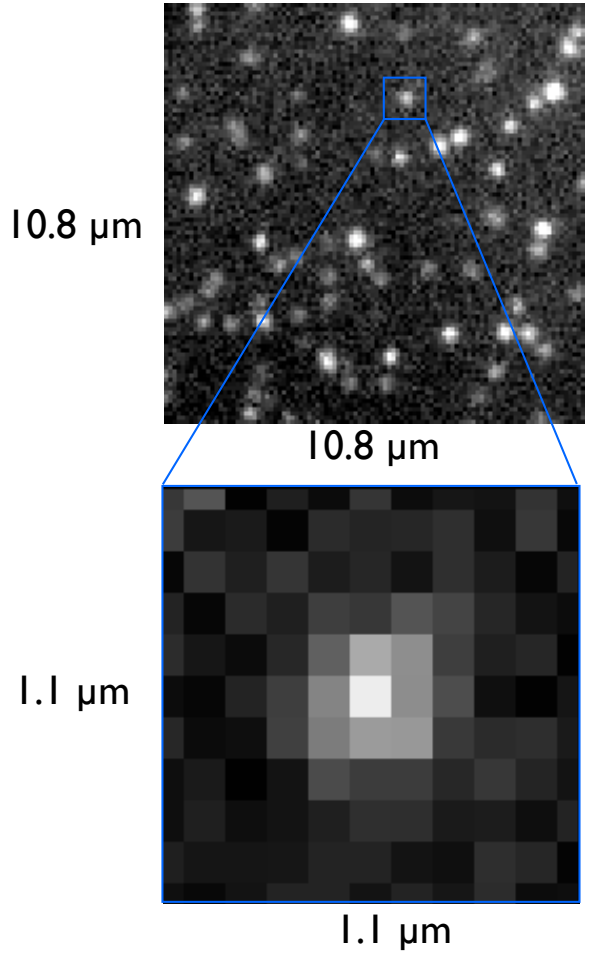


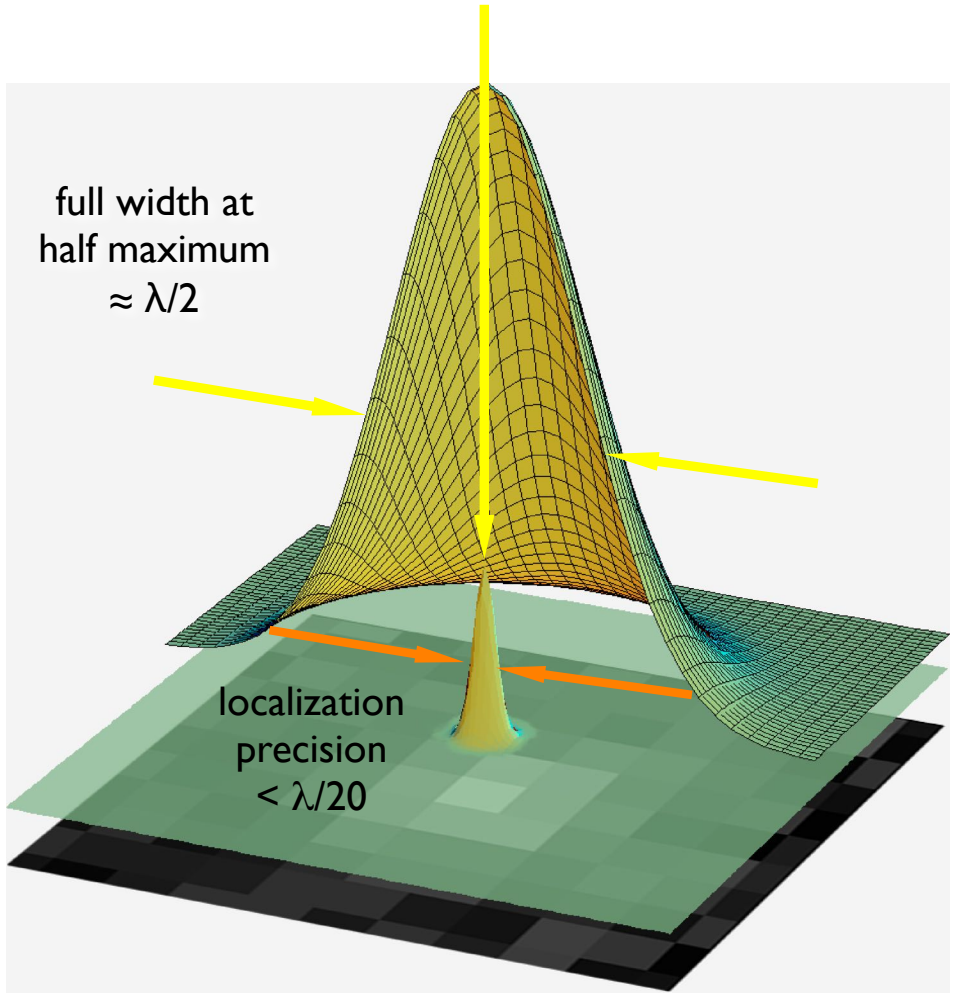

Highly inclined optical sheet microscope *HILO*

# Localisation of single molecules

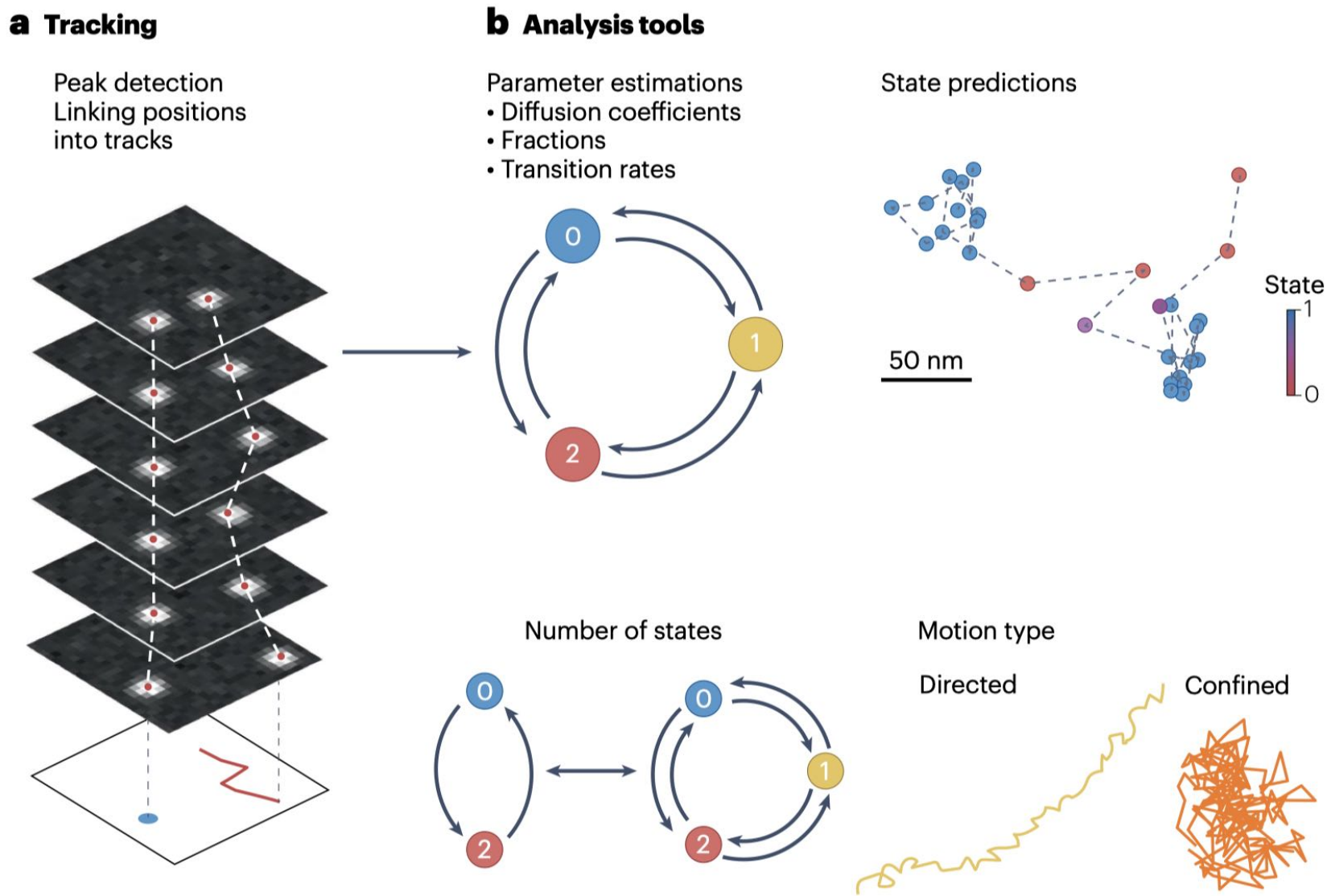
Single, surface-attached
Oyster565 molecules

ex 633 nm, em >650 nm, 2kW/cm-2, 100 ms



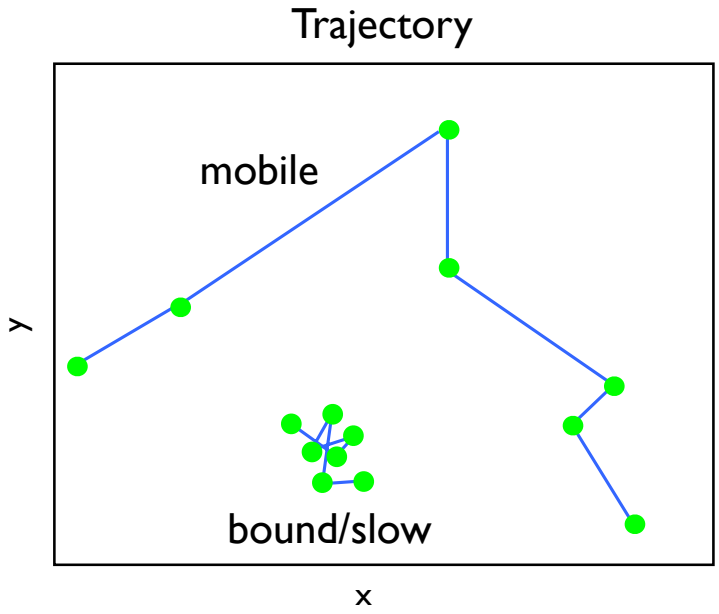
# Tracking and analysing motion of single molecules

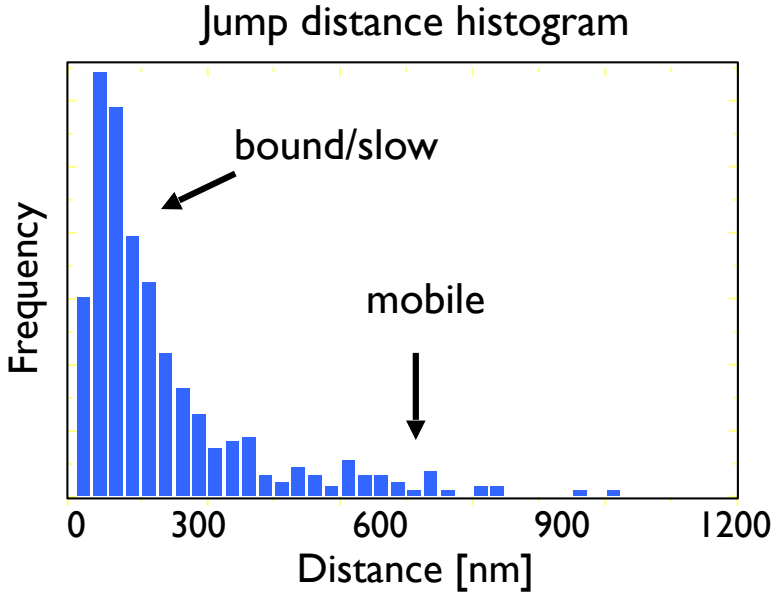



Framework for single-particle tracking. (a) movie acquisition, peak detection followed by a linking step to reconstruct tracks. (b) Different analysis tools estimate parameters such as diffusion coefficients, fractions and transition rates of different states, the number of states and the types of motion.
Tracks can also be labelled with time-dependent information.

Simon F, Weiss LE, Teeffelen S van. A guide to single-particle tracking. Nat Rev Methods Prim 2024; 4:66.

# Tracking and jump distance mobility analysis




mobile and immobile fractions
diffusion coefficients
velocities
dissociation constants

Probability distribution of jump distances for one diffusing species

$$p(r,t)\,dr = \frac{1}{4\pi D\,t} e^{-r^2/4Dt} 2\pi r\,dr$$

*r,* distance
*t*, time lag
*D*, diffusion coefficient

Probability distribution of jump distances for N diffusing species

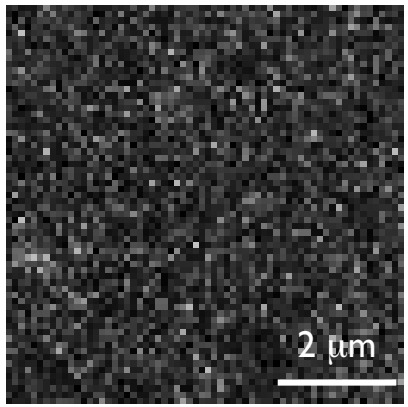
$$p'(r,\,t)dr = \sum_{j=1}^{N} \left( \frac{f_j}{2D_j t} \right) e^{-\frac{r^2}{4D_j t}} r dr$$

$f_j$, size of fraction j
$D_j$, diffusion coefficient of fraction j

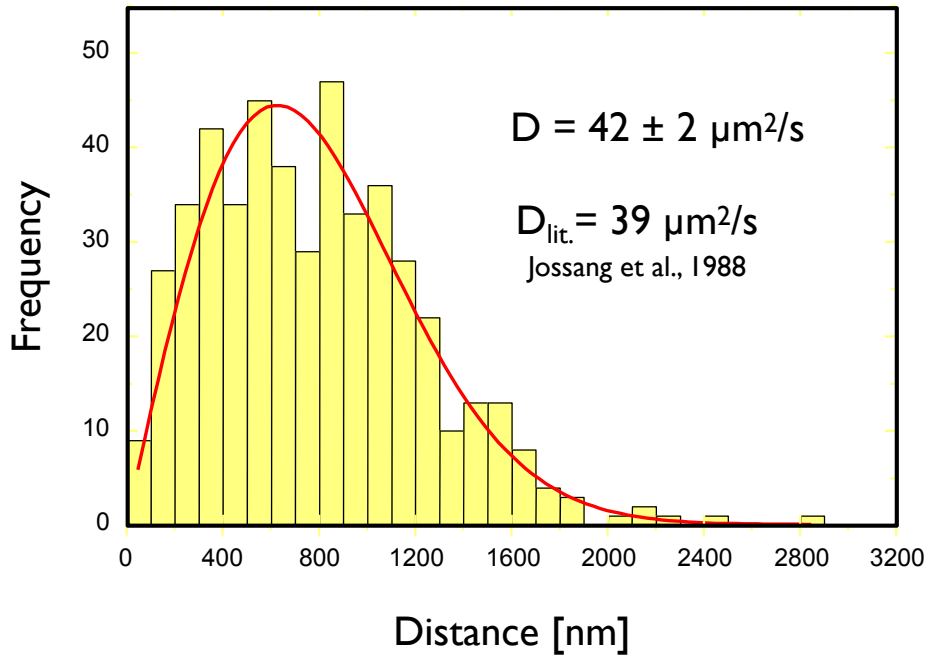
# Imaging single fluorescent proteins in solution

IgG-Alexa633 in buffer

Imaging rate 340 Hz
Display 15-fold slow motion

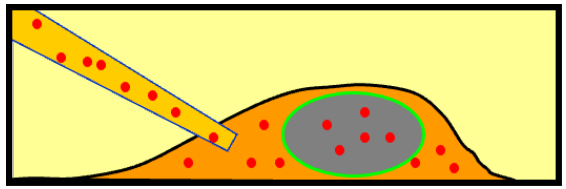



Histogram for time step 8.8 ms

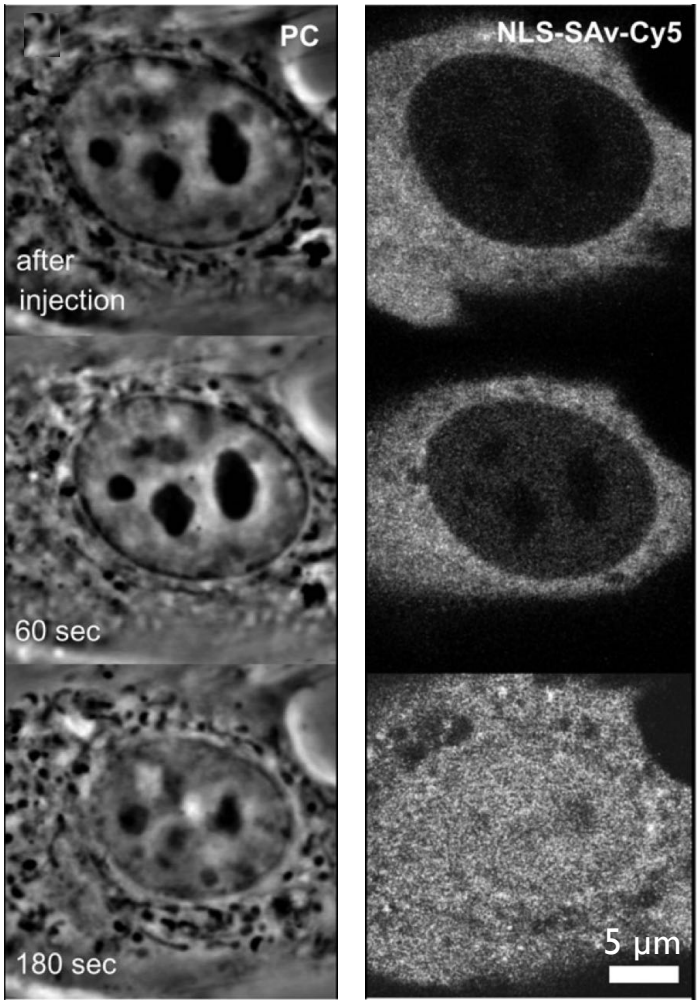



Grünwald et al., *ChemPhysChem* 2008

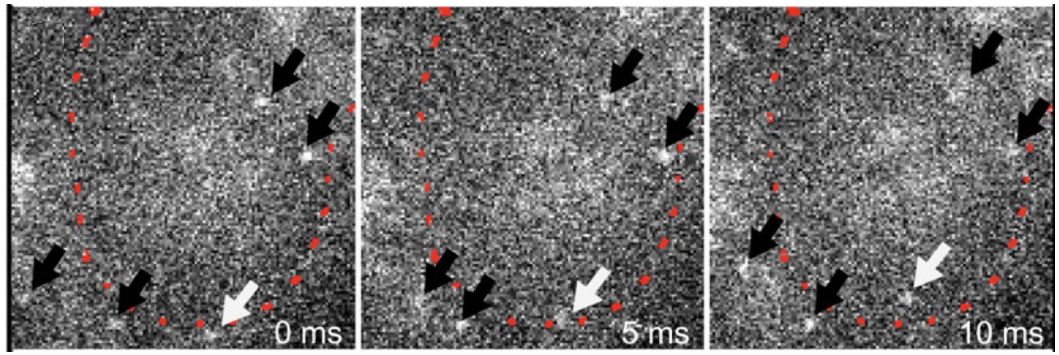
# Single streptavidin-Cy5 molecules in living cells



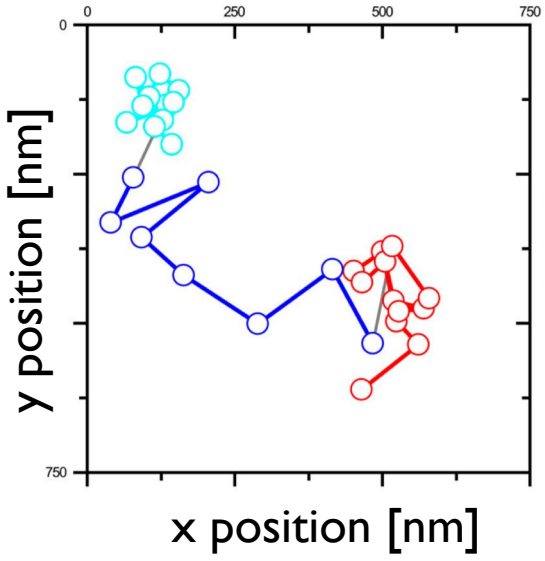
Microinjection of streptavidin-Cy5 molecules
with nuclear localization signal into cytoplasm

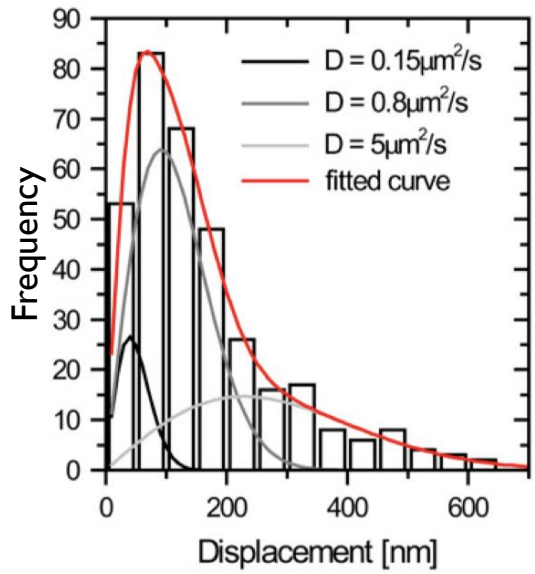



phase contrast SAv-Cy5 fluorescence




single streptavidin-Cy5 molecules




Grünwald et al., *Biophys J* 2008

# Mobility of inert protein molecules in the cell nucleus

nuclear structure affects mobility and local concentration of proteins

retardation and a range of mobility

no restricted access to nucleoli or other nuclear compartments

fastest motions are 7-fold less than in aqueous buffer

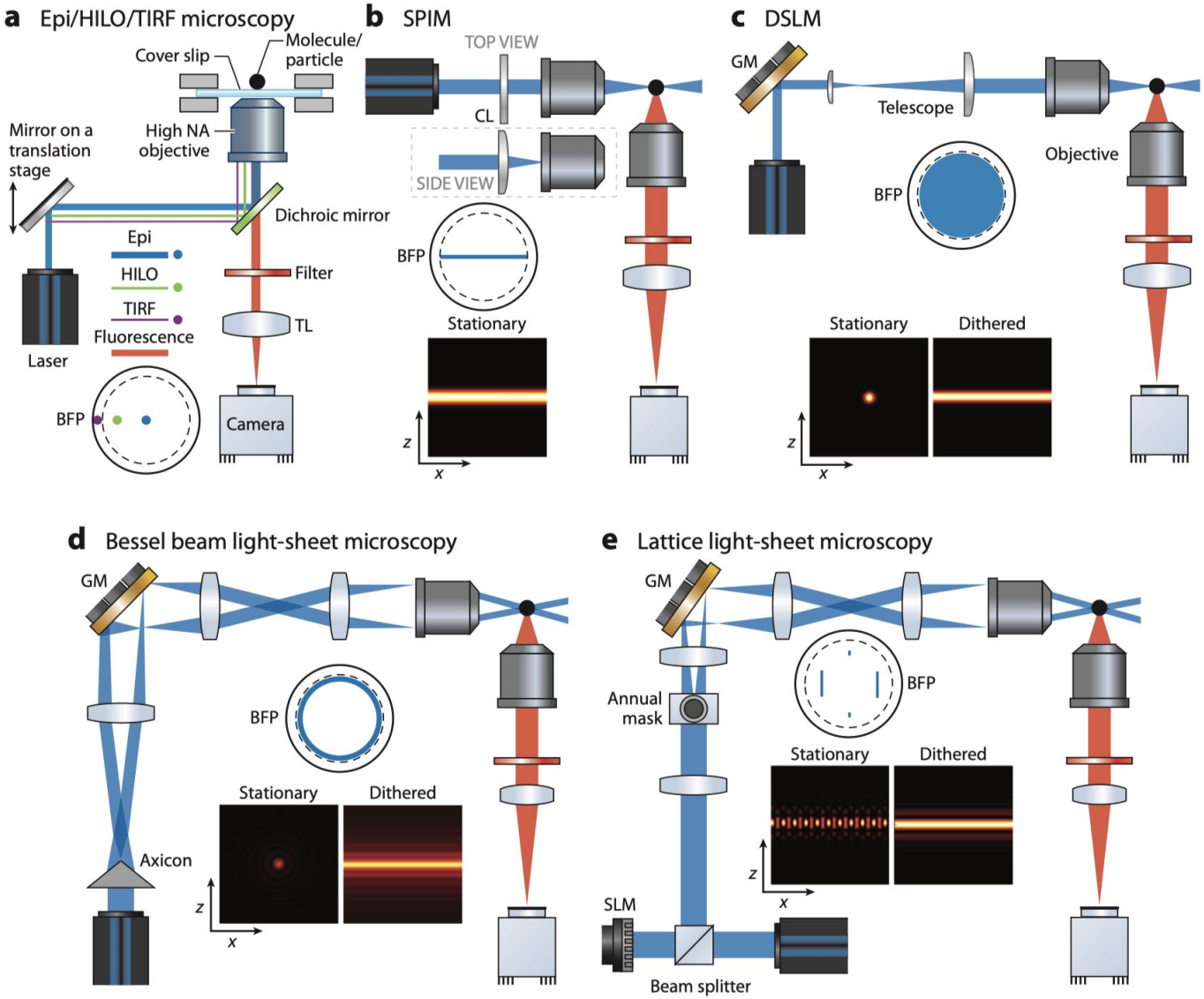
Diffusion time from center to nuclear envelope (nucleus ∅ 25 µm):

43 kDa ovalbumin 8.3 s

60 kDa streptavidin 16 s

Grünwald et al., *Biophys J* 2008, Speil & Kubitscheck, *BBA* 2010

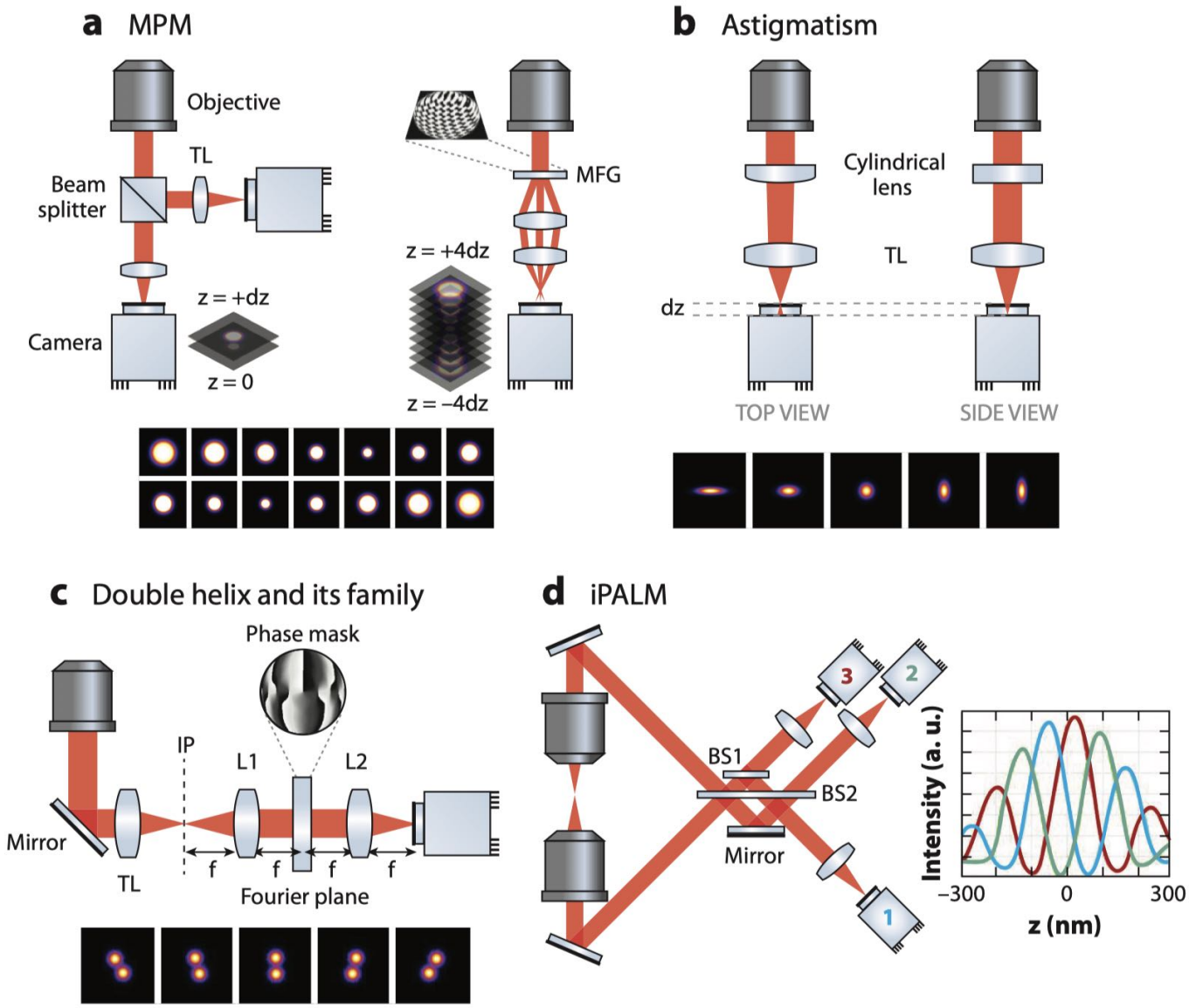
# Different 2D-SMT and imaging techniques




(a) Epi/HILO/TIRF microscopy (87), HILO (80), and TIRF (88). (b) SPIM (83). (c) DSLM (89). (d) Bessel beam light-sheet microscopy (90). (e) Lattice light-sheet microscopy (91) for generating an ultrathin illumination plane and a large field of view. The dashed circle at the BFP denotes the critical angle position (assuming a glass/water interface). Abbreviations: 2D, two-dimensional; BFP, back focal plane; CL, cylindrical lens; DSLM, digital scanned laser light-sheet fluorescence microscopy; Epi, epiluminescence; GM, galvo mirror; HILO, highly inclined and laminated optical sheet; NA, numerical aperture; SLM, spatial light modulator; SMT, single-molecule tracking; SPIM, selective plane illumination microscopy; TIRF, total internal reflection fluorescence; TL, tube lens.

Nguyen TD, Chen Y-I, Chen LH, Yeh H-C. Recent Advances in Single-Molecule Tracking and Imaging Techniques. Annu Rev Anal Chem 2023; 16:253–84.

# Different 3D-SMT and imaging techniques




(a) MPM: (left) biplane microscopy and (right) 9-plane MPM. (b) PSF engineering with astigmatism. (c) PSF engineering using a phase mask in the Fourier plane. L1 and L2: two lenses in the 4f system. (d) iPALM. Abbreviations: 3D, three-dimensional; BS1, 66:33 beam splitter; BS2, 50:50 beam splitter; dz, focus step between successive planes; f, lens focal length; IP, intermediate plane; iPALM, interferometric photoactivation and localization microscope; MFG, multifocus grating; MPM, multifocal plane microscopy; PSF, point-spread-function; TL, tube lens.

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