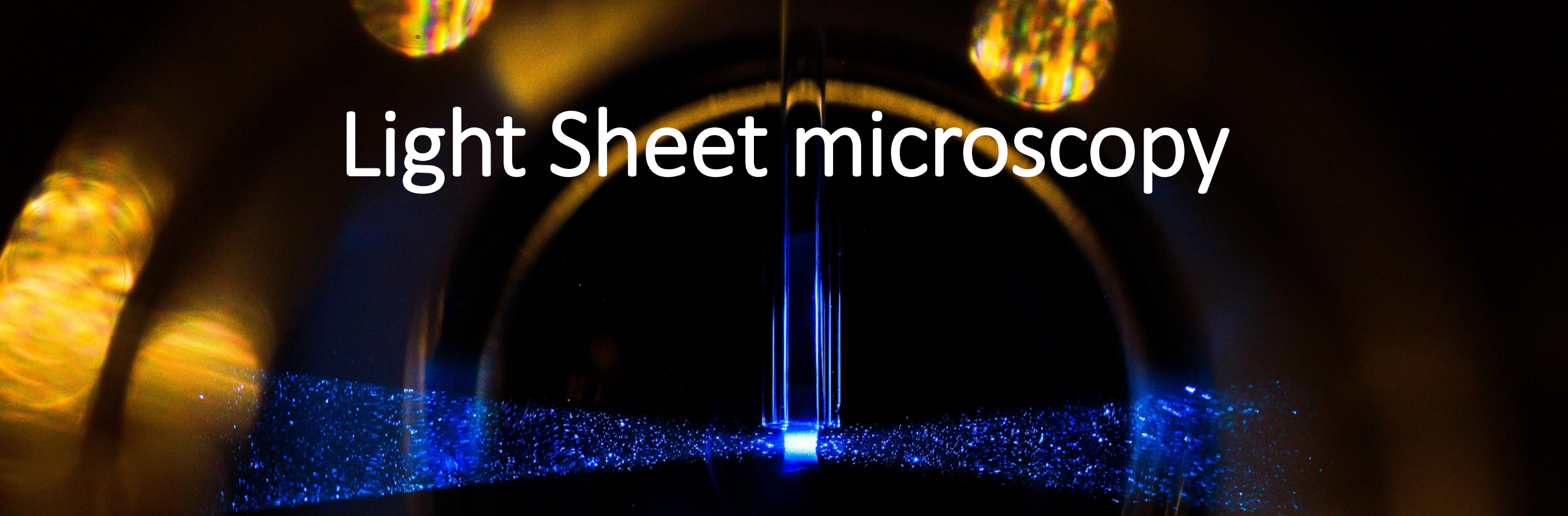
Light Sheet microscopy



Jiří Černý

j.cerny@img.cas.cz
Light Microscopy Core Facility

Speaker: Barbora Radochová

barbora.radochova@fgu.cas.cz
Microscopic Core Facility

IMG

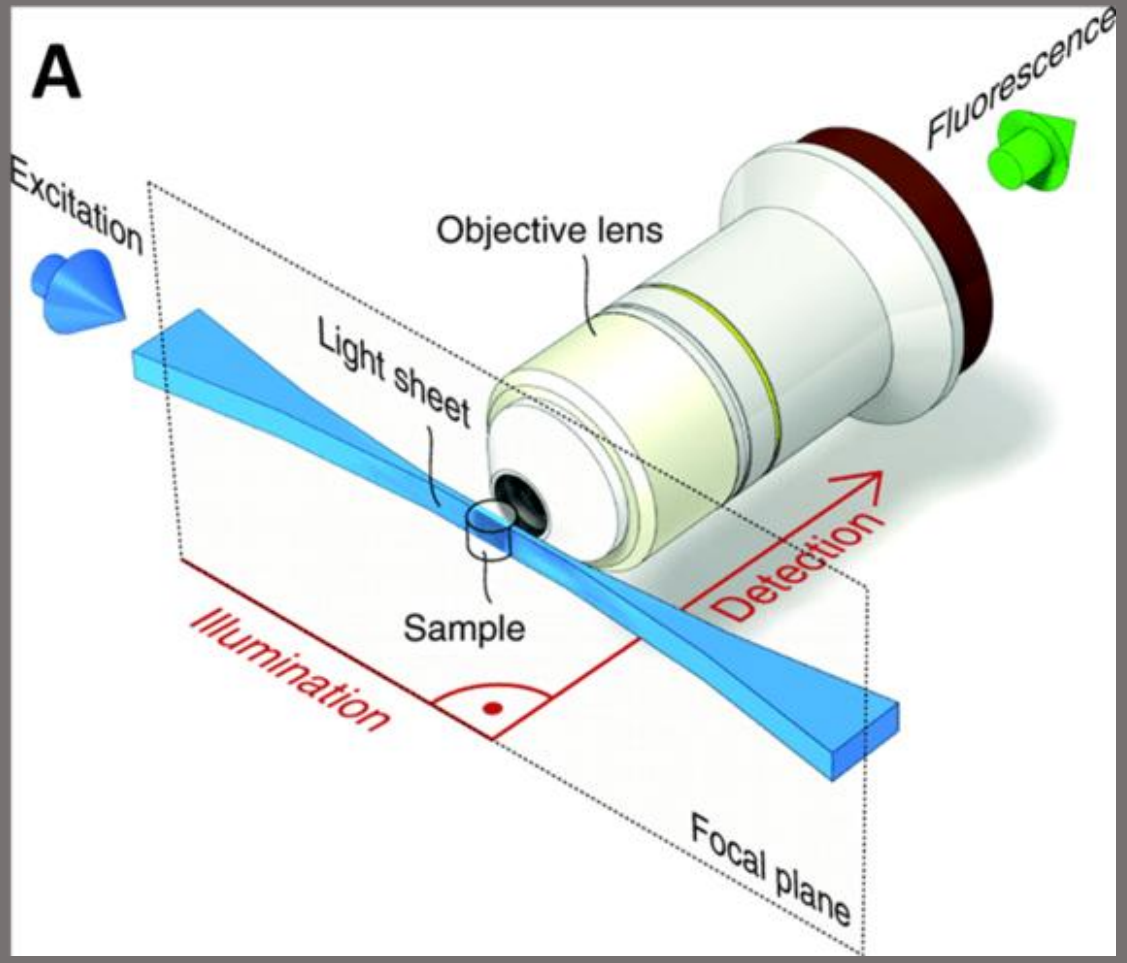
How to acquire a large biological object

Microscope Comparisons

Name	Signal	Resolution	Fluorescent	Size	Imaging Time	Cost ($)	Photobleaching
Magnetic resonance imaging	Magnetic	mm	No, contrast agent	M	hr	Millions	NA
Computed tomography	Radioactive	<mm	No, contrast agent	cm	min	Millions	NA
Confocal	Laser	<micron	Yes	micron	msec	200,000	Yes
2-Photon	Laser	<micron	Yes	mm	msec	500,000	Less
Light sheet fluorescence microscopy	Laser	micron	Yes	>cm	msec	30,000	Least

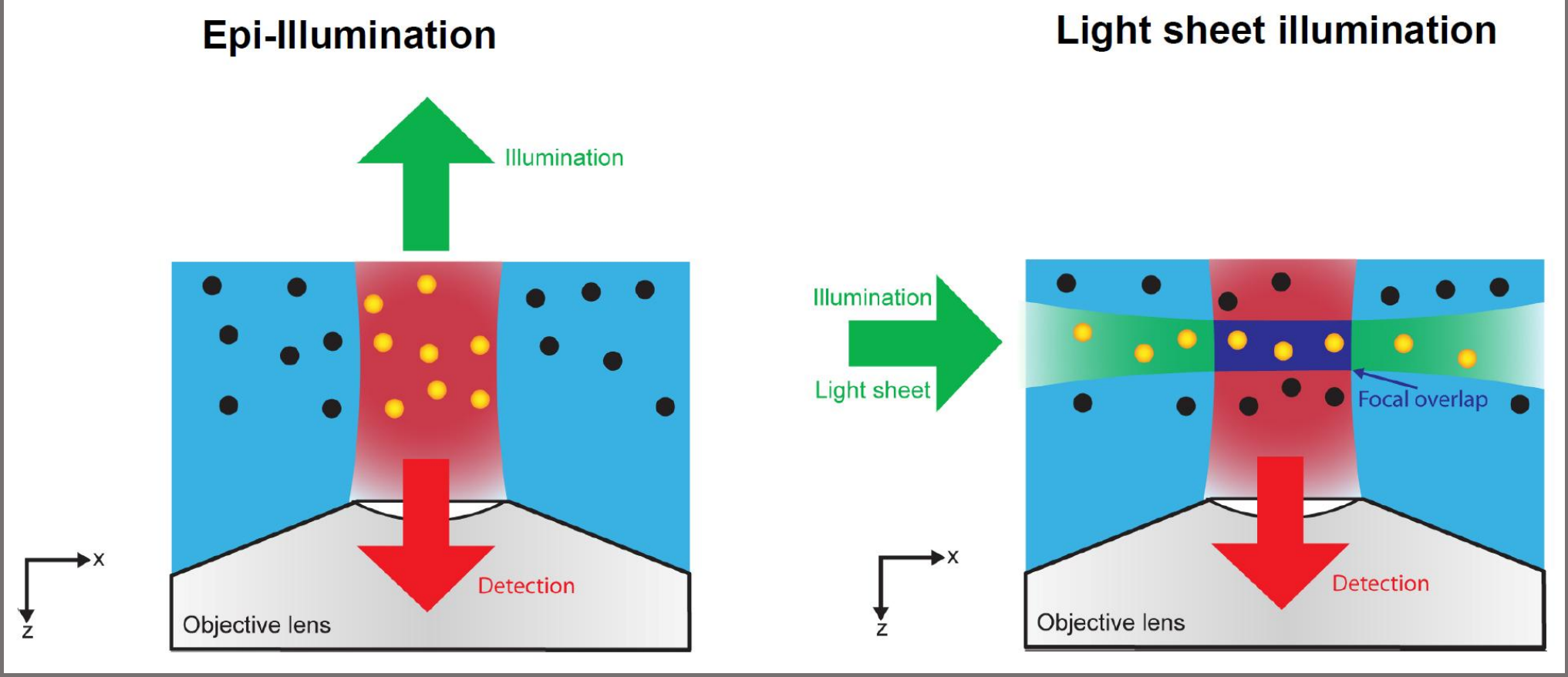
Santi, Light Sheet Fluorescence Microscopy, 2011

Light Sheet Microscopy - principle



- The axis of illumination is **perpendicular** to the detection axis
- The sample is illuminated by a thin sheet of light
- The lightsheet is aligned with the focal plane of the detection objective

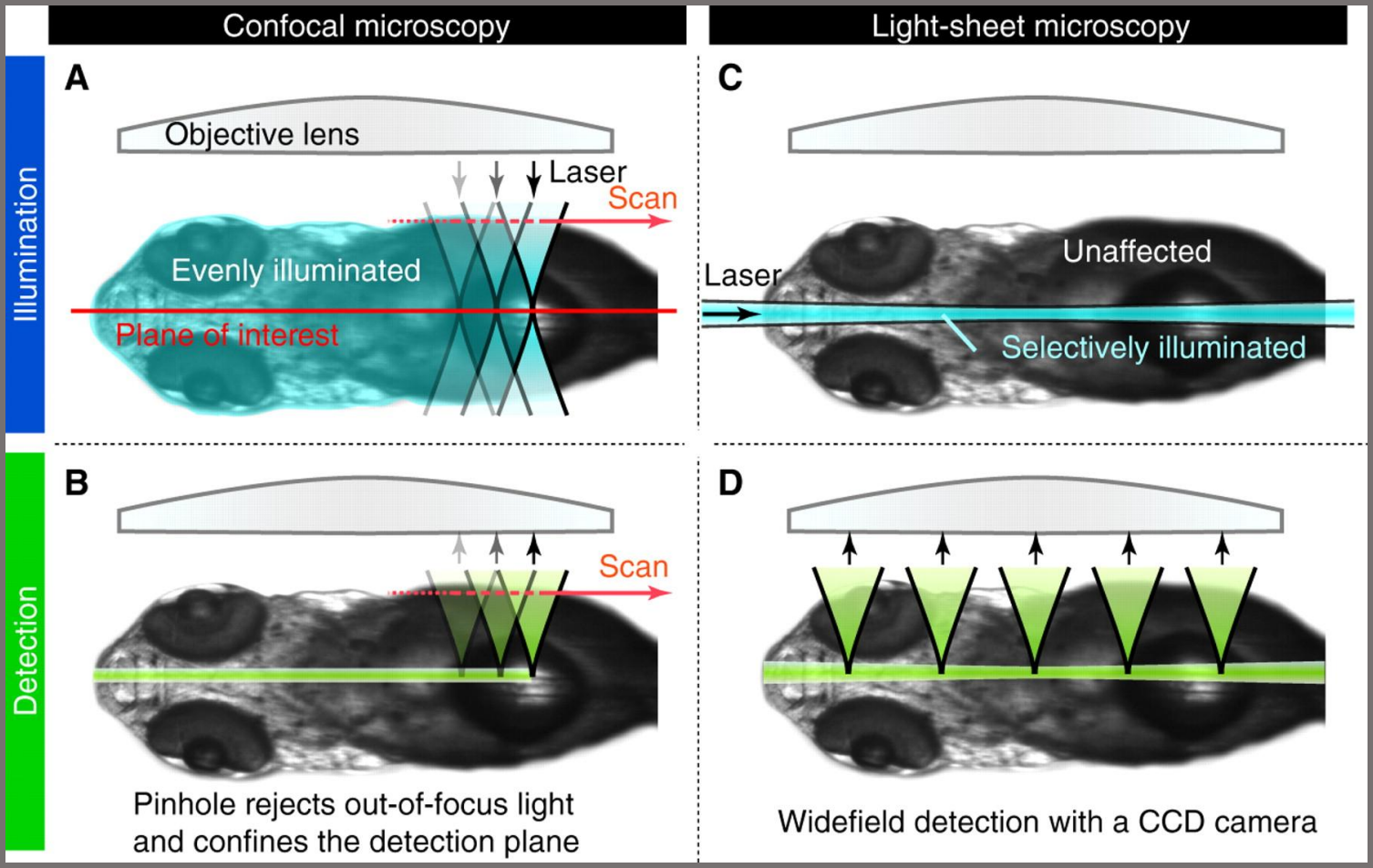
Light Sheet Microscopy - principle



PhD thesis of Jörg Ritter (2011), University of Bonn, Germany

- Inherent optical sectioning capability of the illumination method
- **No excitation of out-of-focus** fluorescence
- **Minimal photo toxicity** – reduced bleaching and photo-damage

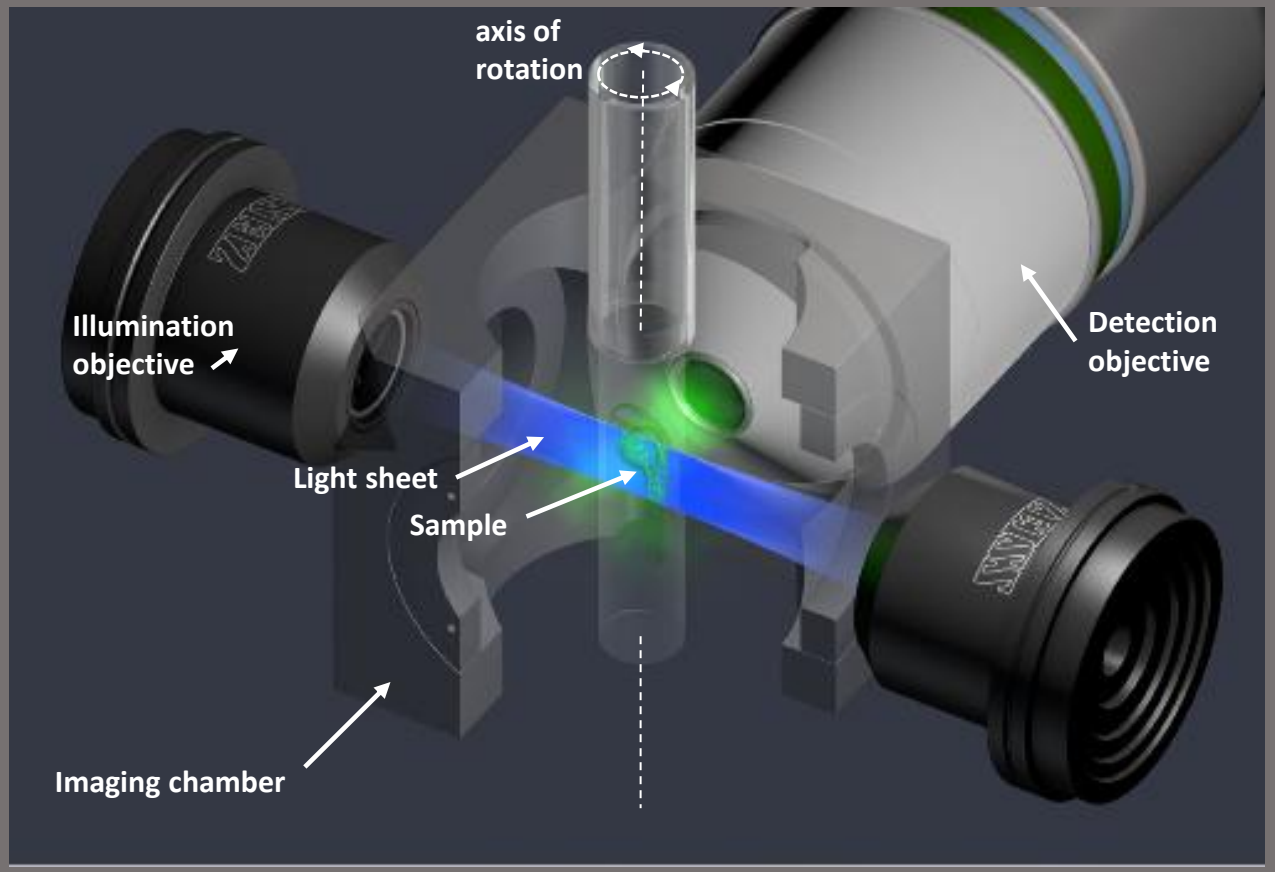
Light Sheet Microscopy - principle

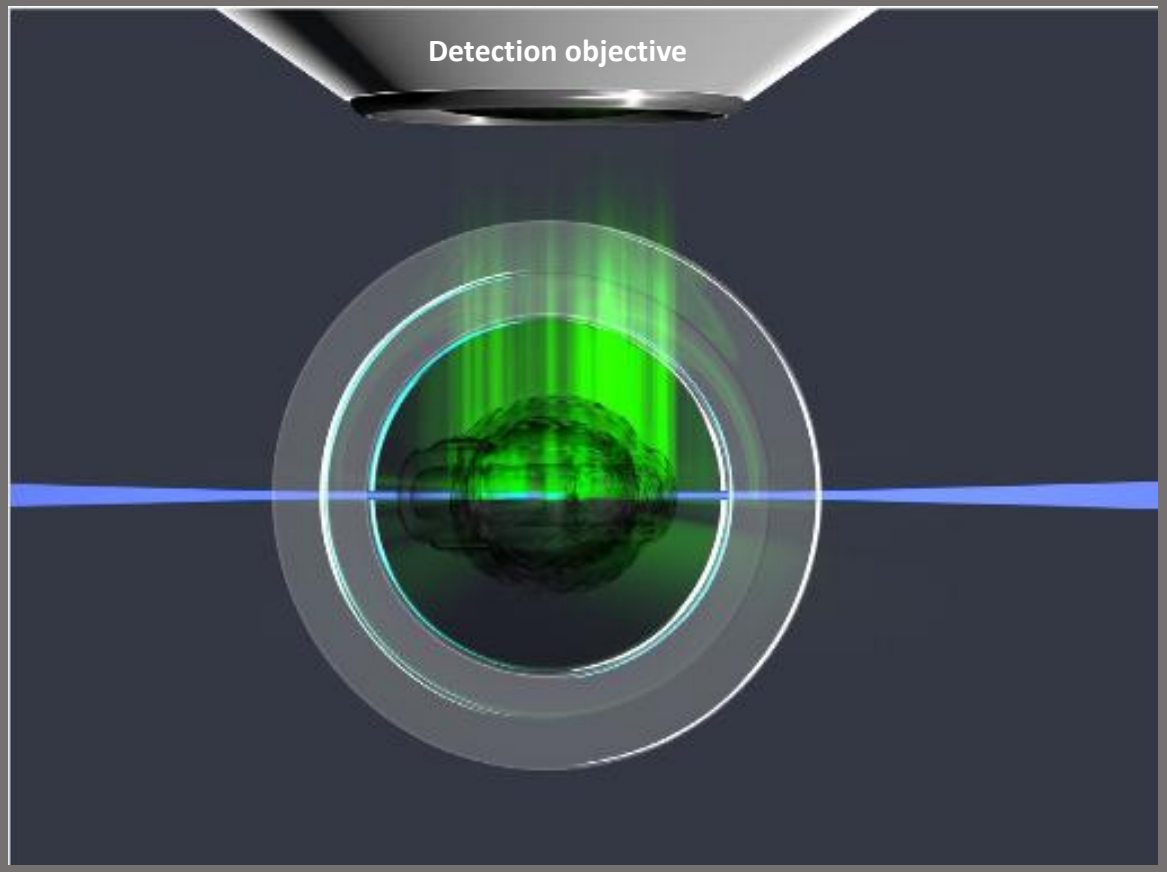


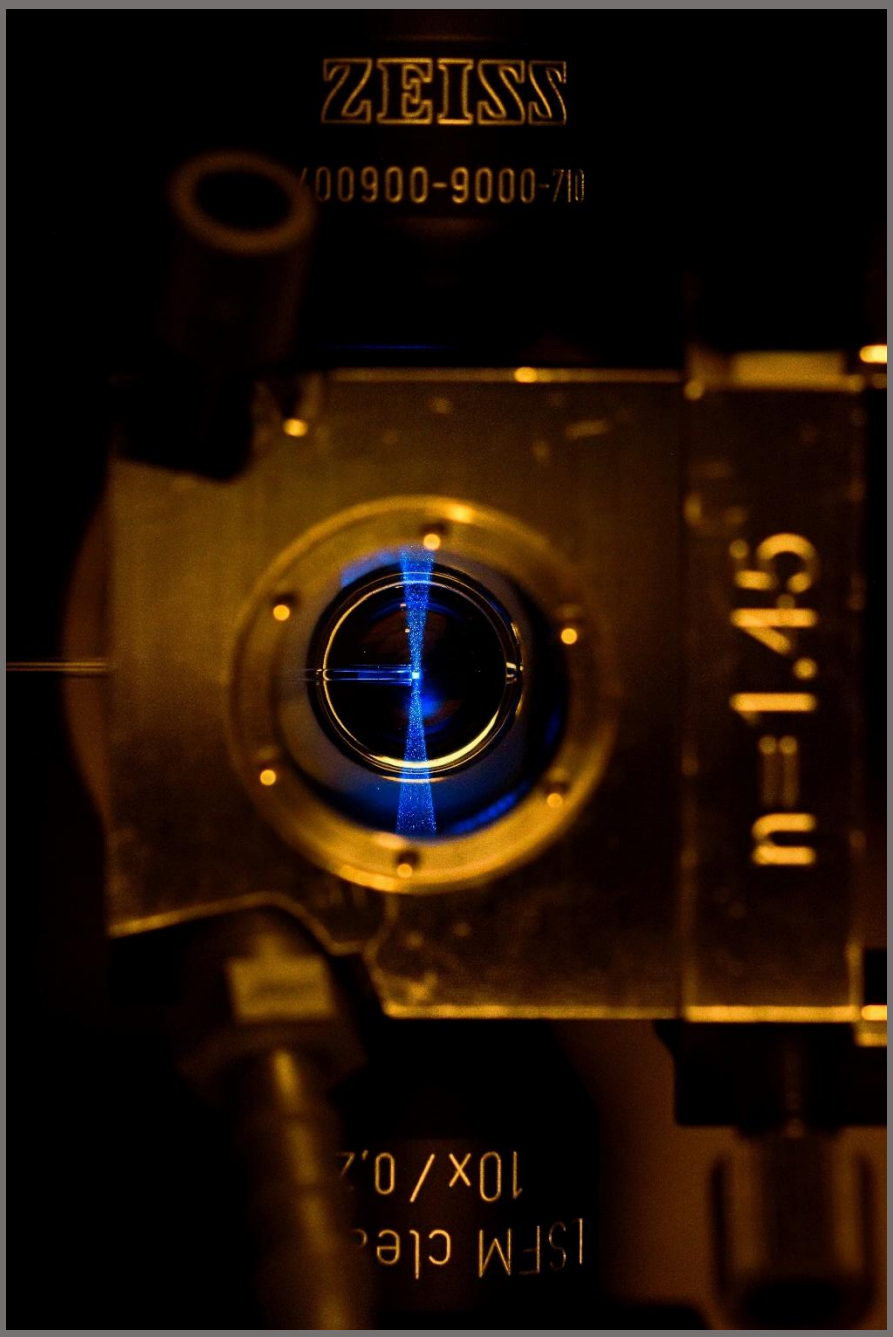
Huisken, Stainier,2009

- Fast and sensitive detection with camera (EMCCD, sCMOS)

Light Sheet Microscopy - setup

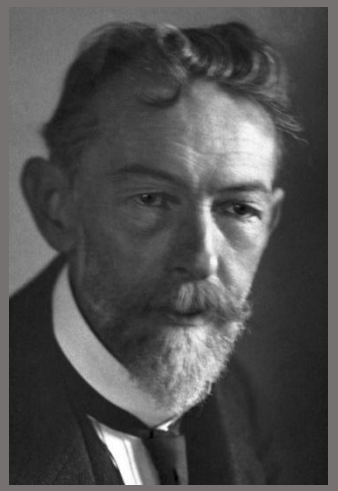




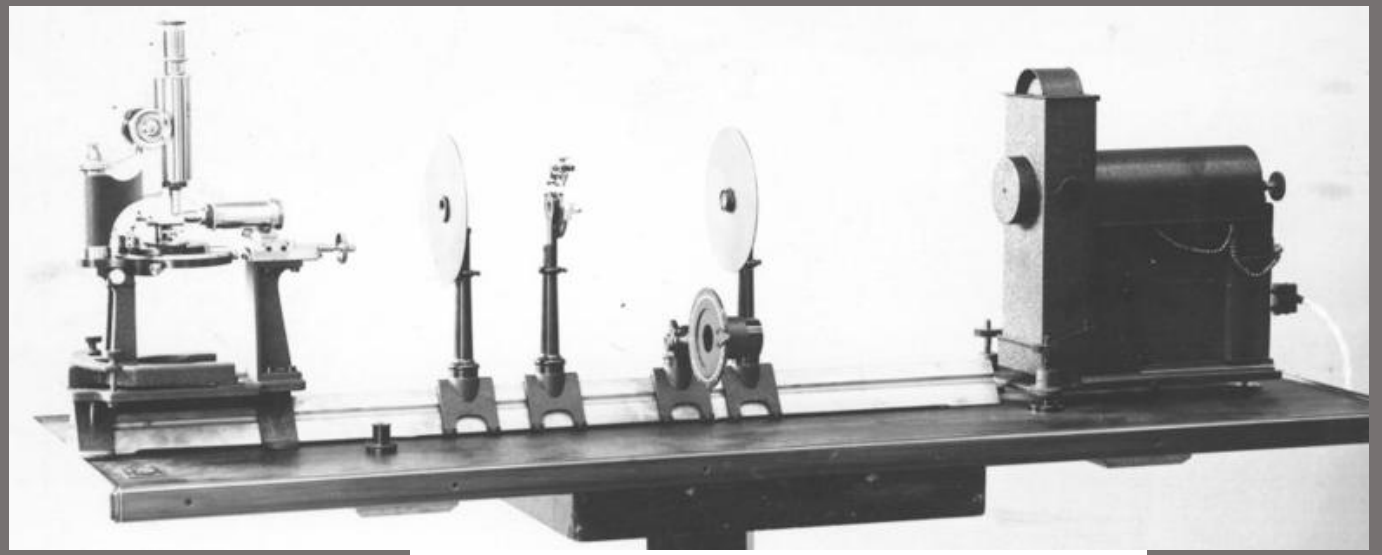

ZEISS
n=1.45


n 1.45

History



Richard Adolf Zsigmondy
The Nobel Prize in Chemistry 1925



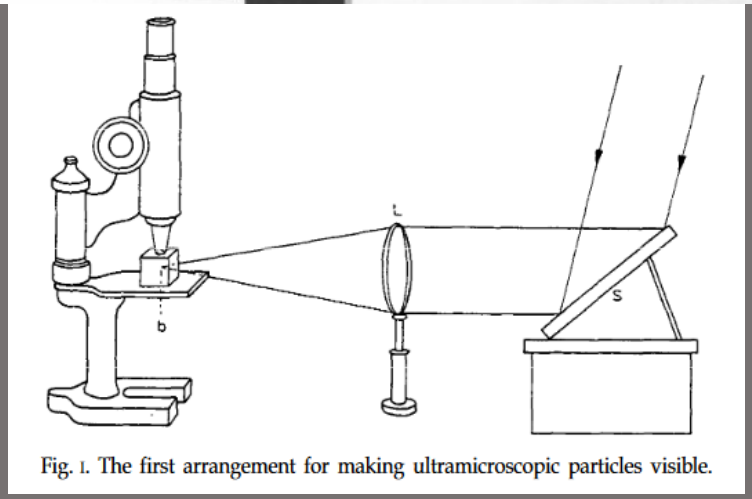


Fig. 1. The first arrangement for making ultramicroscopic particles visible.

- Zsigmondy and Siedentopf (1903), sunlight side-on illumination of colloidal gold solution, called „ultramicroscopy“

<https://www.nobelprize.org/prizes/chemistry/1925/zsigmondy/facts/>

- Jan Huisken, et. al, 2004 Science, *Optical sectioning deep inside live embryos by selective plane illumination microscopy*

Family of light-sheet microscopy techniques

- Acronyms

  LSFM: Light Sheet based Fluorescence Microscopy

  SPIM: Selective (or Single) Plane Illumination Microscopy

  LSBM: Light-Sheet based Microscopy

  LSIM: Light Sheet Illumination Microscopy

  UM: Ultramicrosopy (*Zsigmondy and Siedentopf*)

  OPFOS: Orthogonal Plane Fluorescence Optical Sectioning

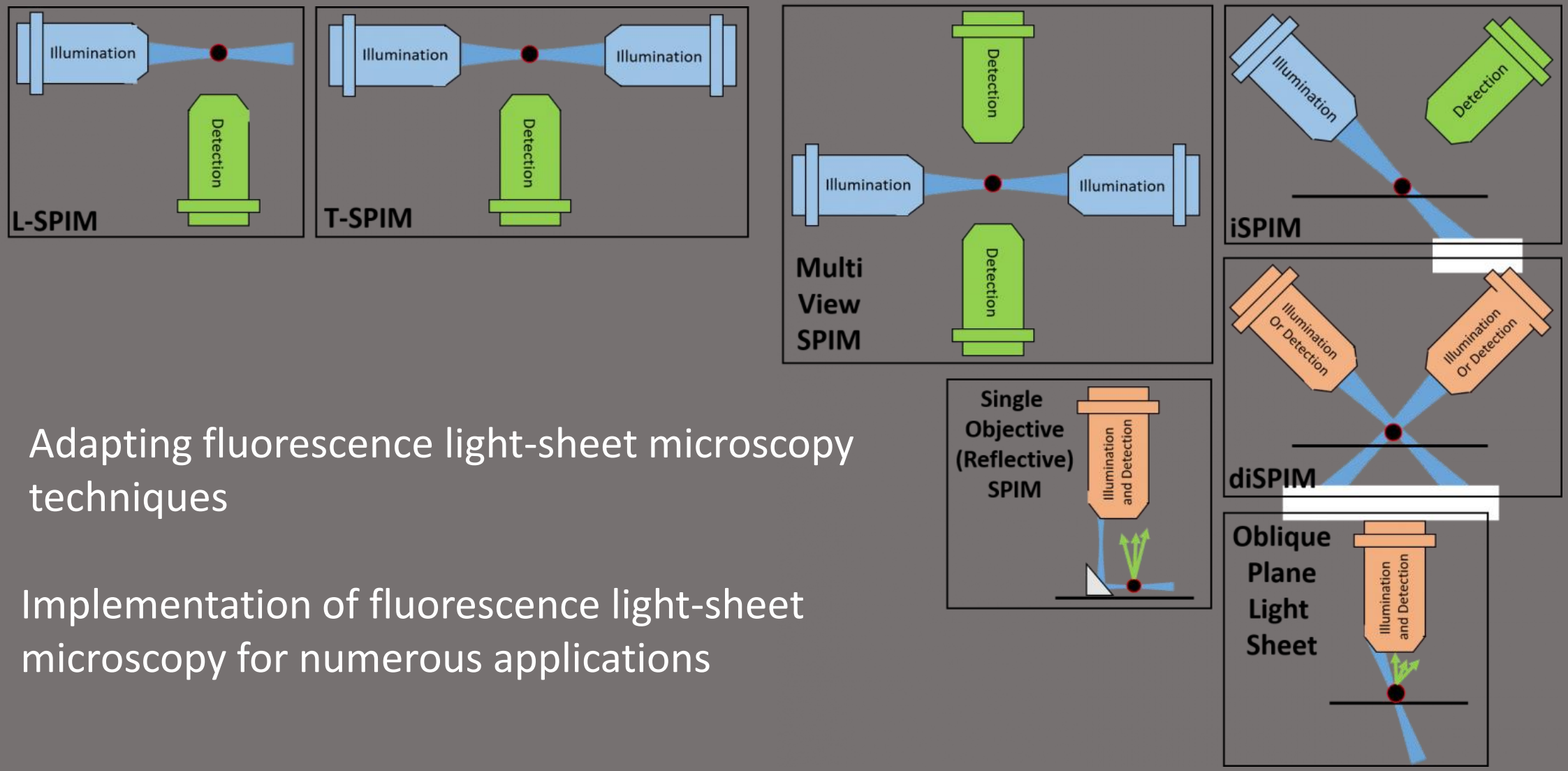
  TSLIM: Thin-Sheet Laser Imaging Microscope

  OCPI: Objective Coupled Planar Illumination microscopy

  OPM: Oblique Plane Microscopy

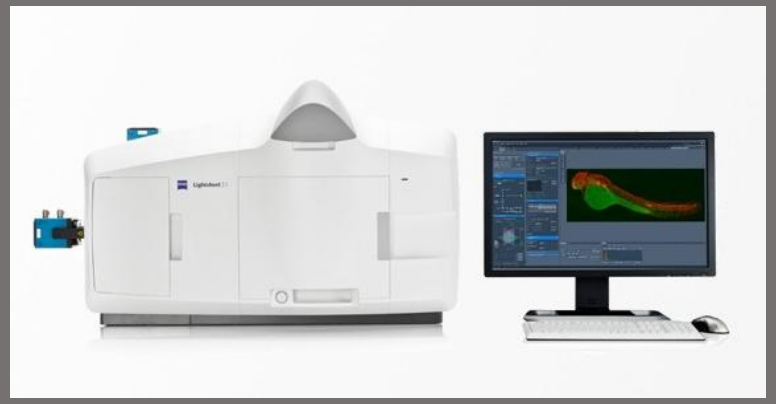
  ...

Family of light-sheet microscopy techniques



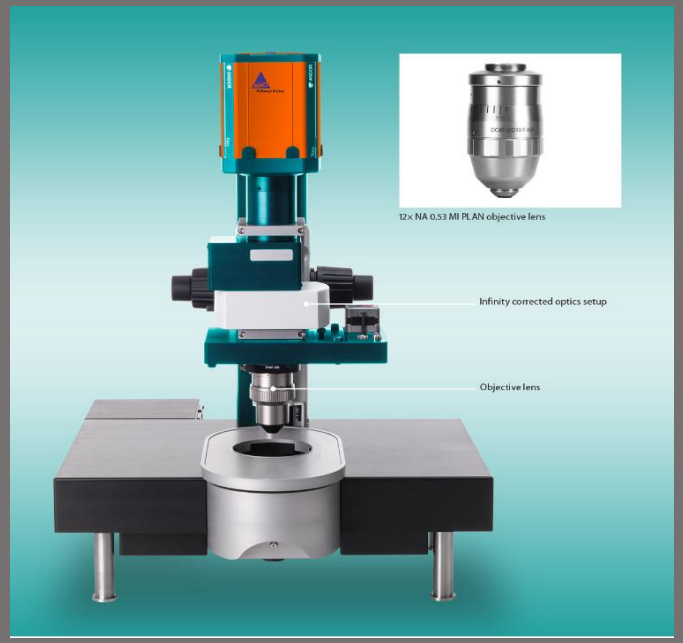
- Adapting fluorescence light-sheet microscopy techniques
- Implementation of fluorescence light-sheet microscopy for numerous applications

Commercial systems

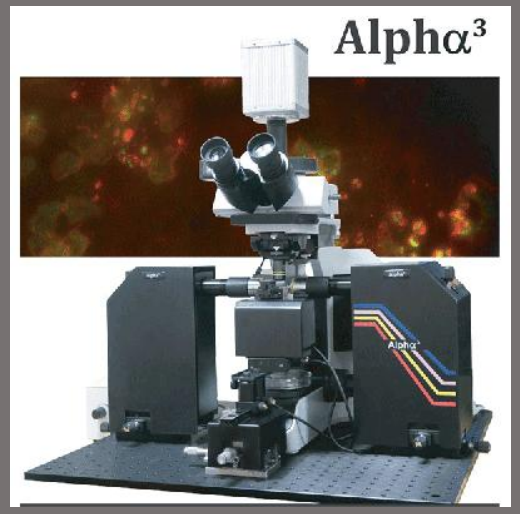


Lightsheet Z.1, Carl Zeiss

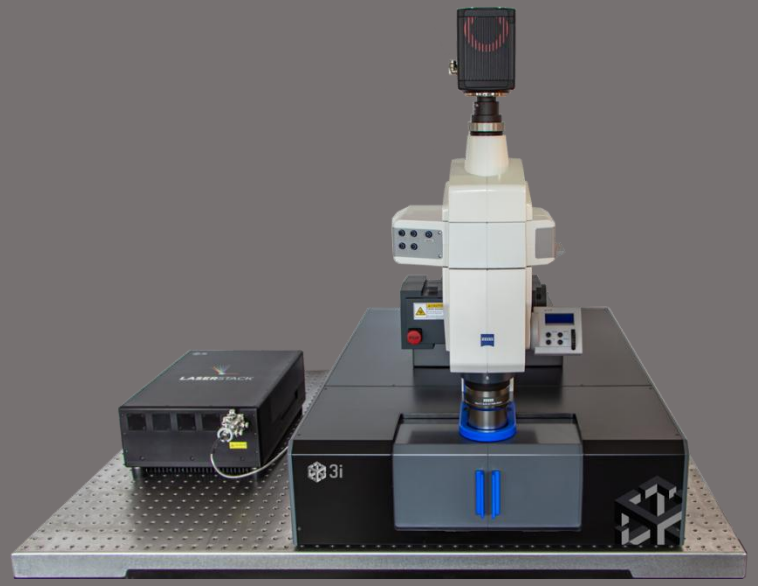
Viventis LS2 Live, Leica



UltraMicroscope II, LaVision, BioTec



Alpha3, PhaseView

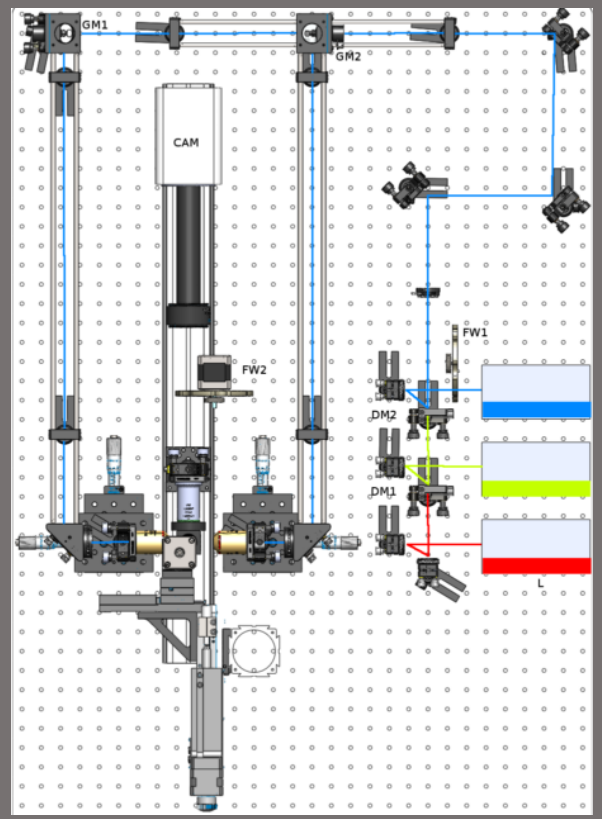


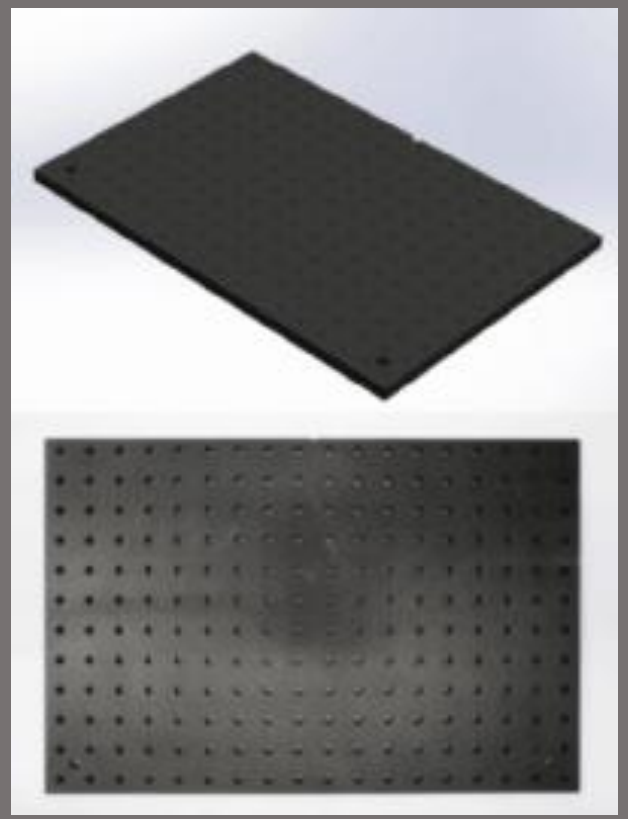
Cleared Tissue LightSheet, 3i

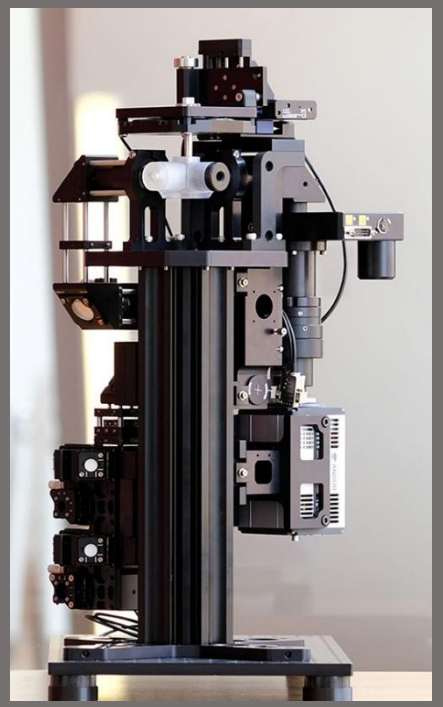
MuVi SPIM, Luxendo

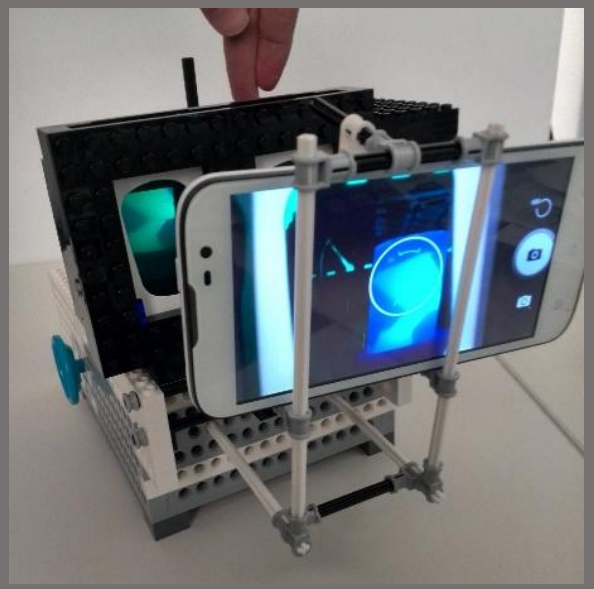
Home built systems

- „Built around the sample“
- OpenSPIM platforms
- Portable LS microscopes (compact “kits” sent to users)
- Creative projects for education purposes....



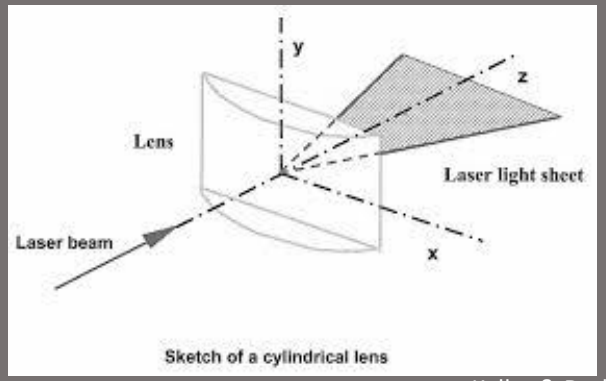






https://openspim.org/
https://www.physikinstrumente.com/en/applications/microscopy/flamingo-lightsheet-fluorescence-microscopy/
https://lemolish.mystrikingly.com/ (LEGOLish: Light Sheet Imaging for everybody)

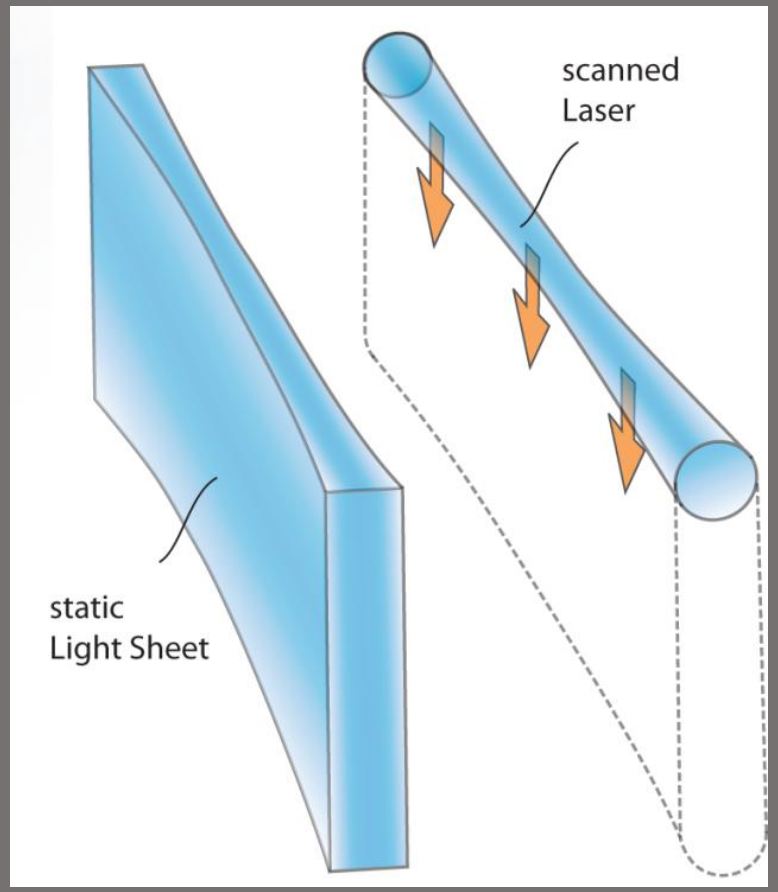
Shaping the lightsheet



Keller & Dodt, 2012

Static Light Sheet

- Laser beam expanded in one dimension with a cylindrical lens
- Easy to integrate (no moving parts)
- Entire field of view illuminated at once

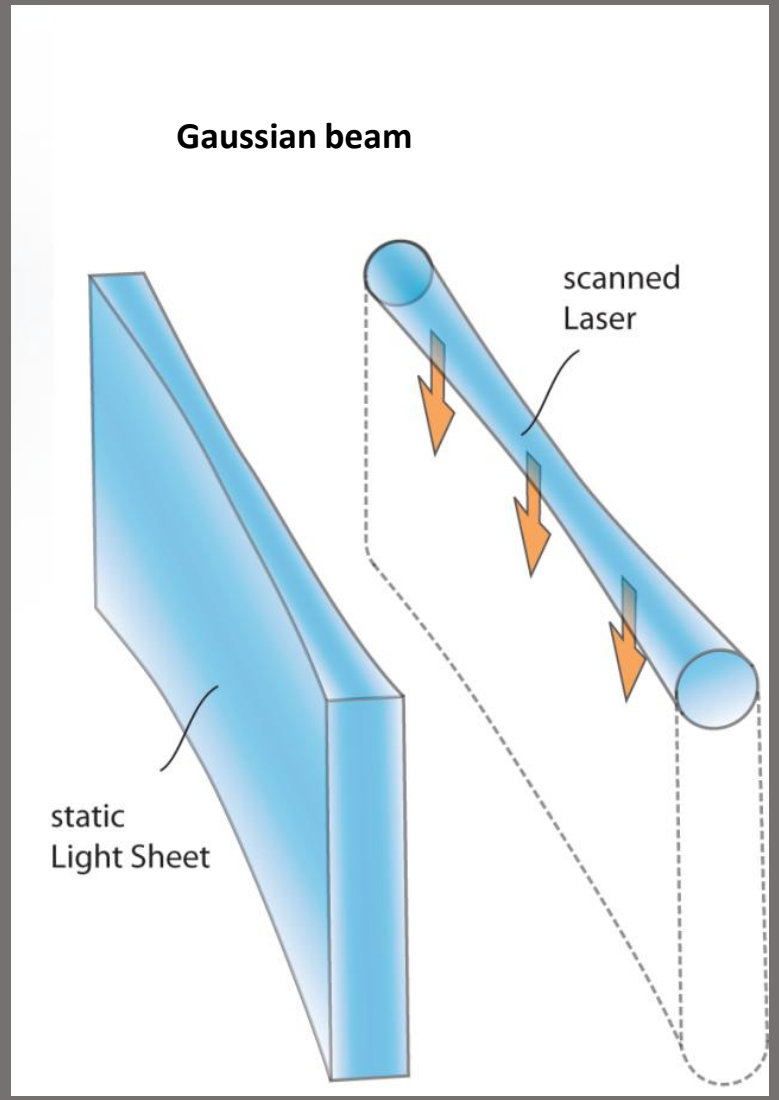


Weber and Huisken, 2011

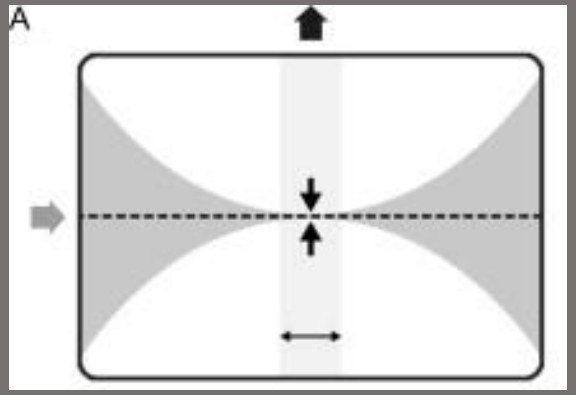
Scanned (virtual) Light Sheet

- Rapidly scanning a beam up and down
- Higher local intensities are generated
- Each line of the scanned light sheet is illuminated equally

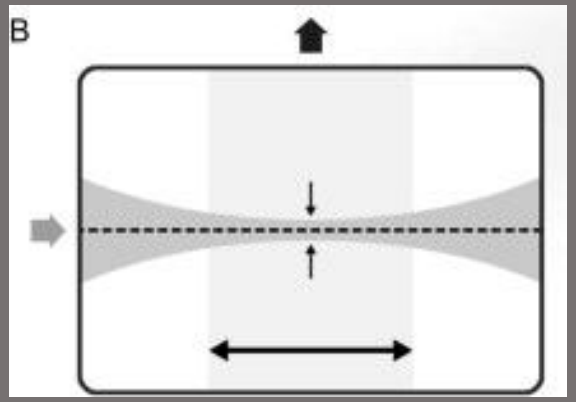
Shaping the lightsheet



Weber and Huisken, 2011



Small field of view
Thin light sheet

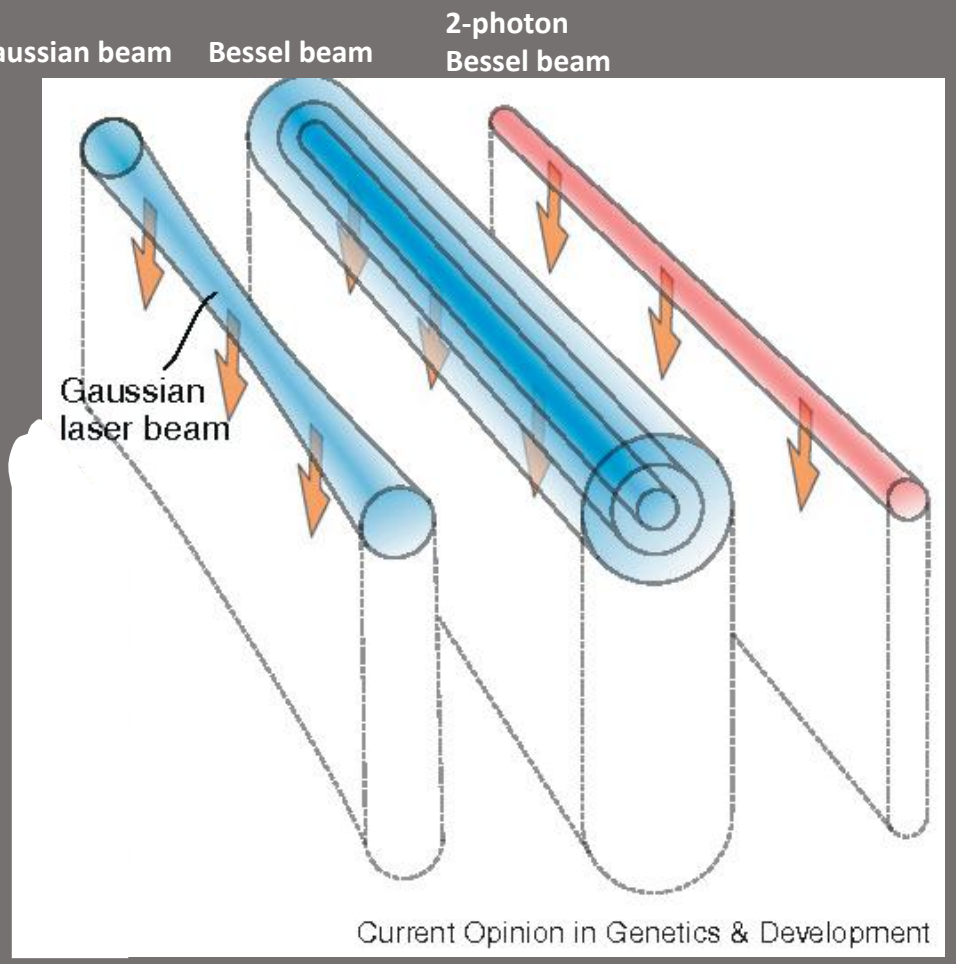


Large filed of view
Thick light sheet

- Light sheet has hyperbolic profile in xz plane

Weber and Huisken, 2014

Shaping the lightsheet



- Bessel beam – thin and long core surrounded by a ring system
- „Non-diffracting“ and „self-reconstructing“ ability
- Large portion of beam energy in side lobes
  - ✓ Vital for self-healing properties of the beam
  - X Contribute to out of focus background
- 2-photon Bessel beam – excitation only in the central core

Weber and Huisken, 2011

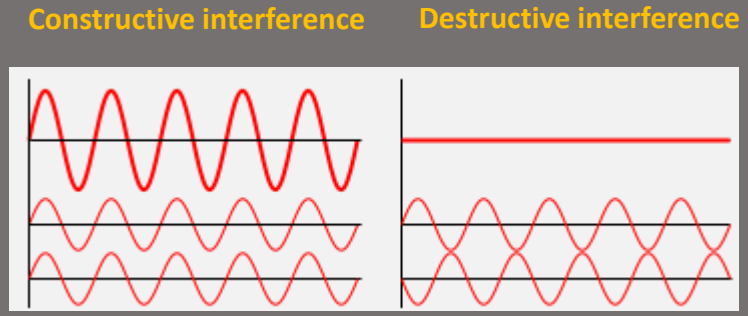
Lattice lightsheet

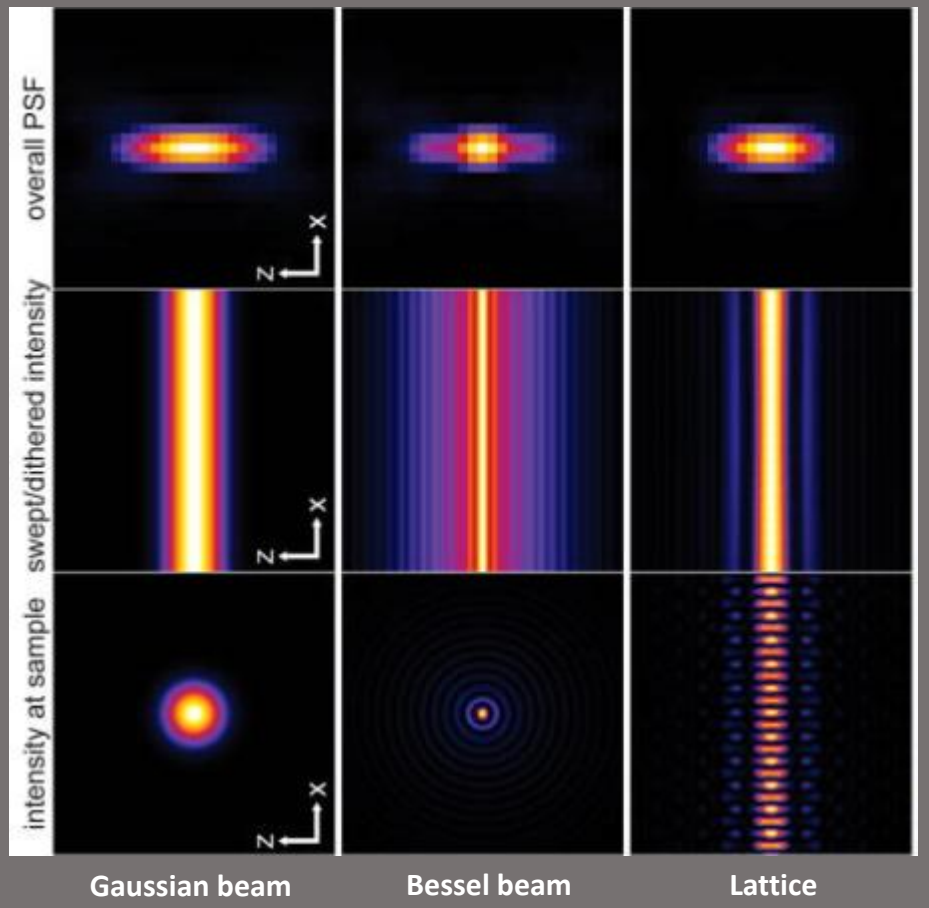
- Improved **Bessel beam** light sheet microscopes in axial (z) resolution
- Reducing the intensity of the outer lobes of the Bessel functions by **destructive interference**
- Two-dimensional lattice of regularly spaced Bessel beams is created
  - spacing between the beams triggers destructive interference

✓ improved resolution, low phototoxicity, fast acquisition

→ dynamic cellular interactions, subcellular processes *in vivo*

→ up to 100 µm in depth



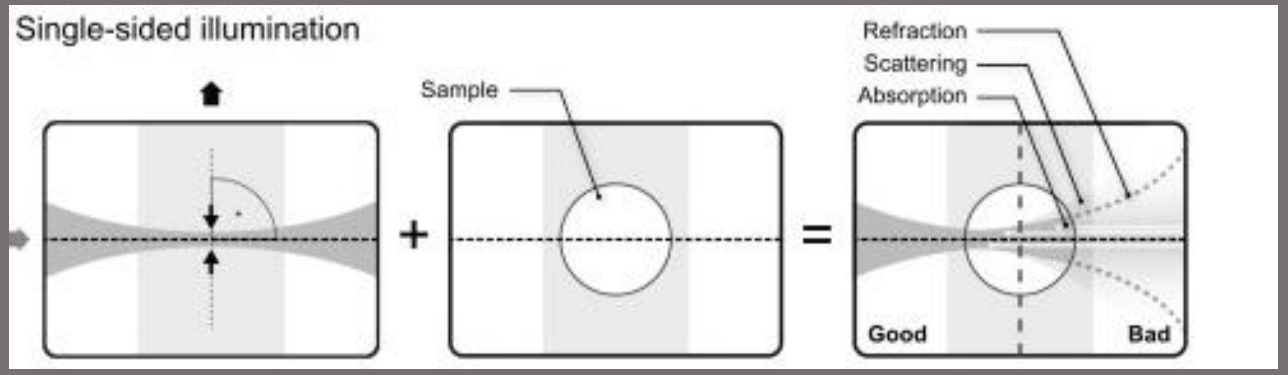


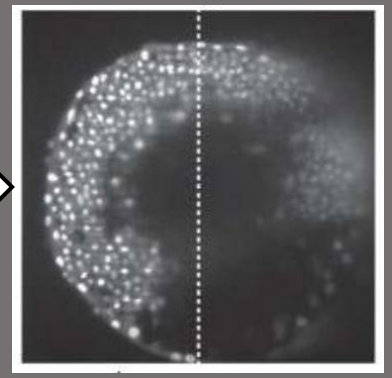
en.wikipedia.org
Chen,..Betzig, 2014

Artefacts

Optical properties of the sample → Absorption, Scattering, Refraction

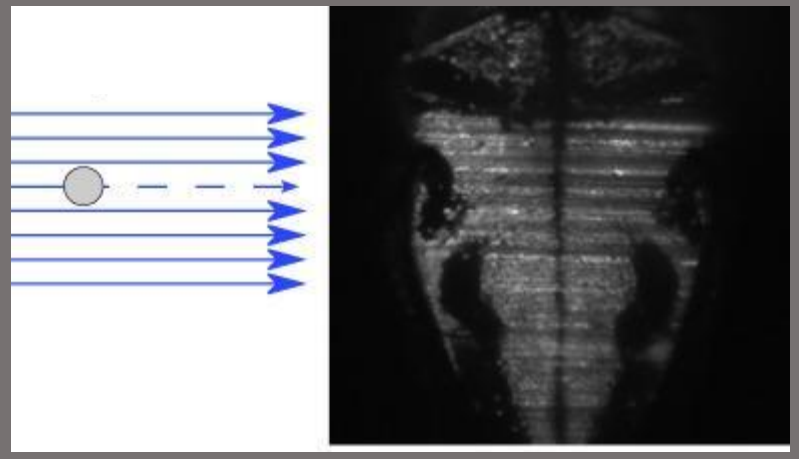
- Limited penetration depth of the light sheet





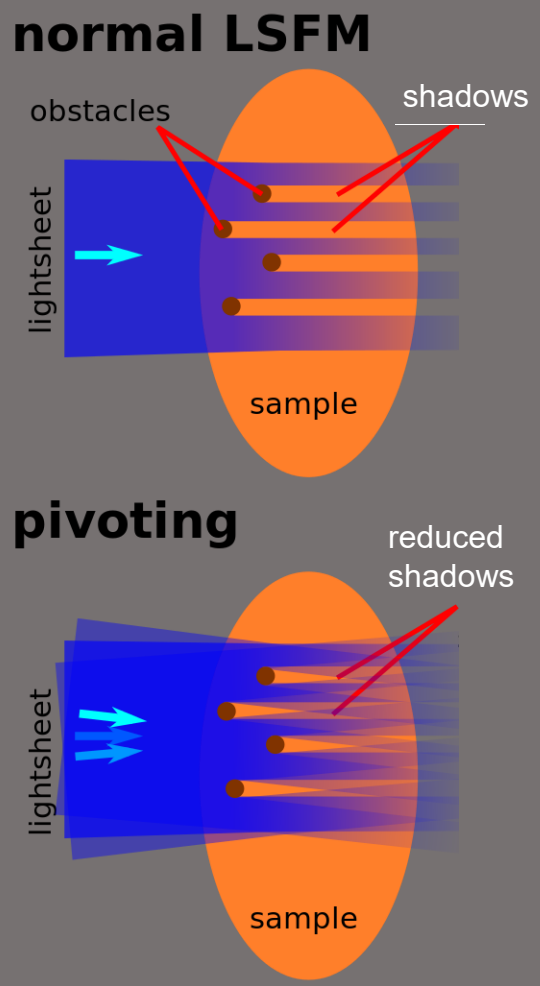
Weber et al., 2014

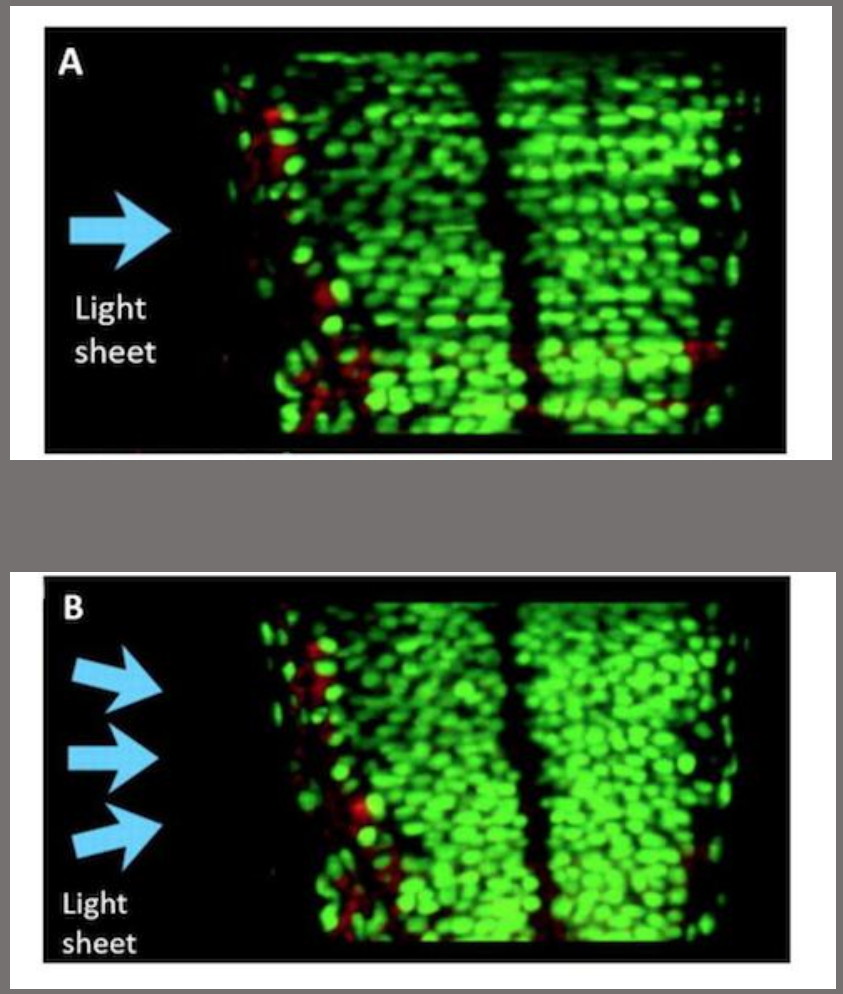
- Shadow Stripes



Taylor et al., 2018

Pivot scan

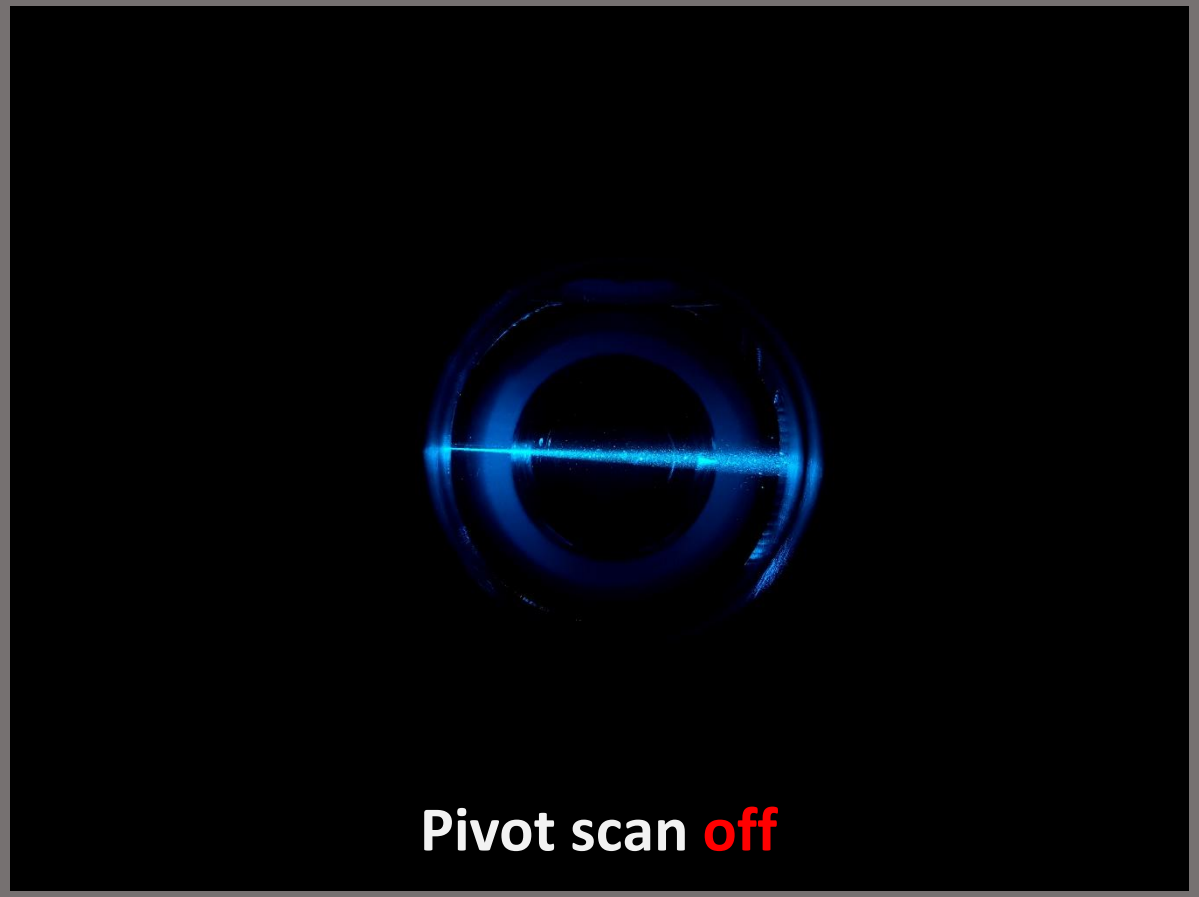




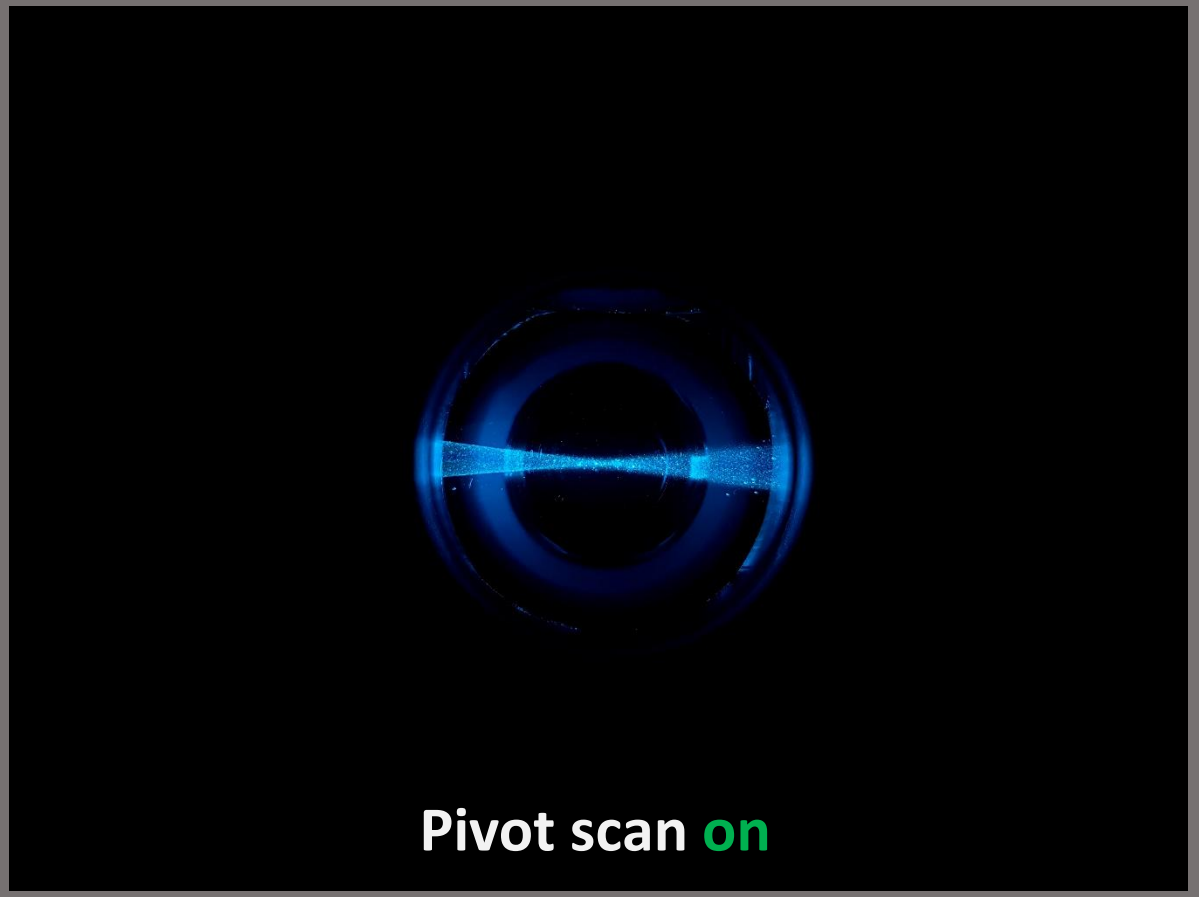
- Lightsheet's direction of incidence changed rapidly (~1 kHz rate) by a few degrees (~10°)
- Light can reach regions behind „obstacles“
- More even illumination
- Elimination of shadows

en.wikipedia.org

Weber et al., 2014



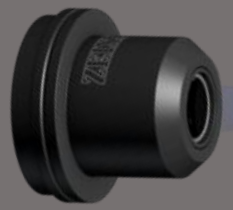
Pivot scan off

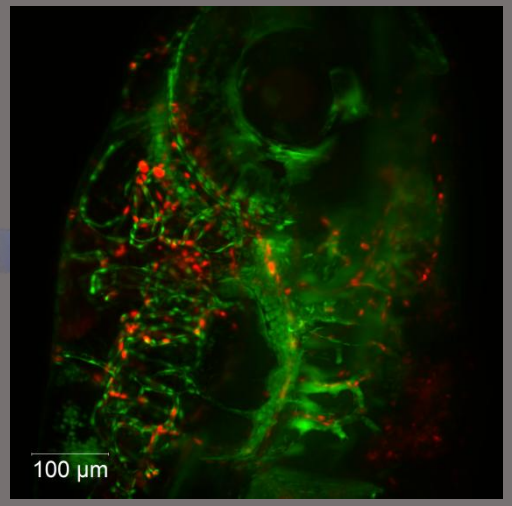


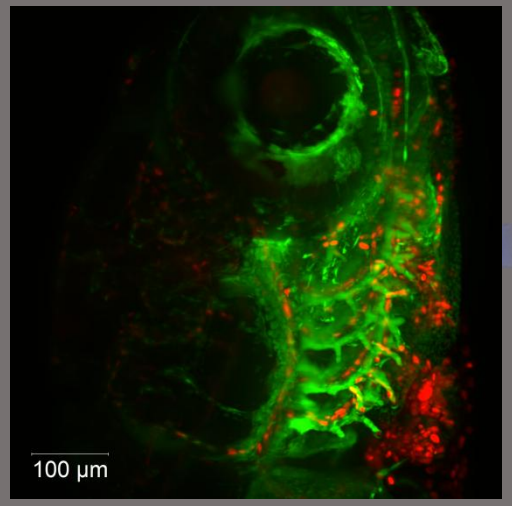
Pivot scan on

Dual side illumination

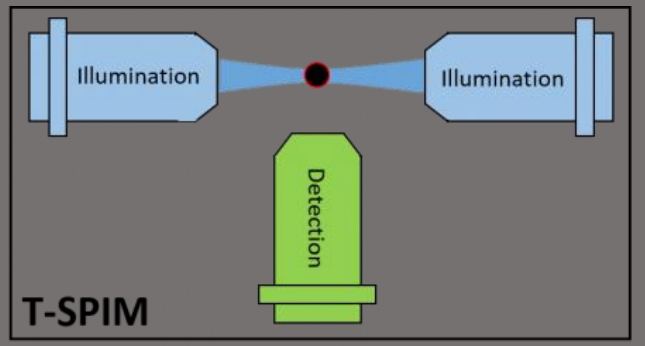
Left side illumination



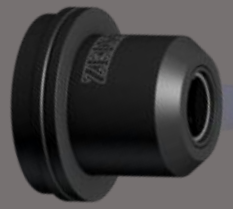


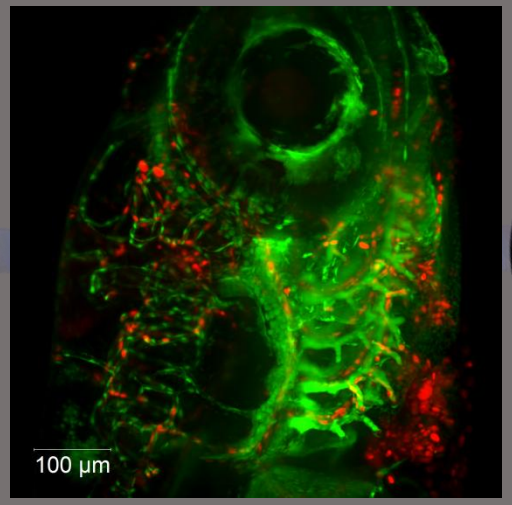


Right side illumination

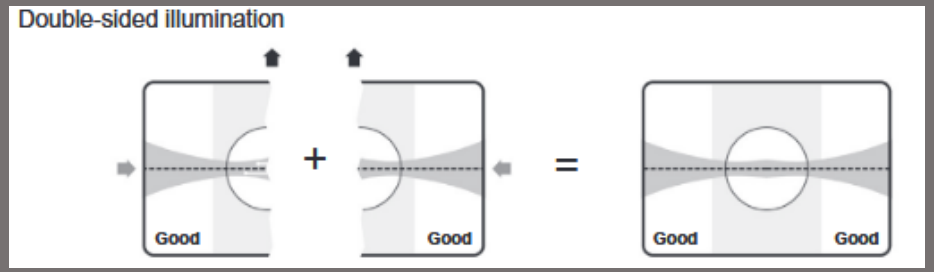


Dual side fusion





- Two illumination objectives (two light sheets)
- Alignment to the same plane
- More homogenous illumination
- Higher penetration depth



Dr. Cathleen Teh, IMCB, Singapore
Image provided by Zeiss

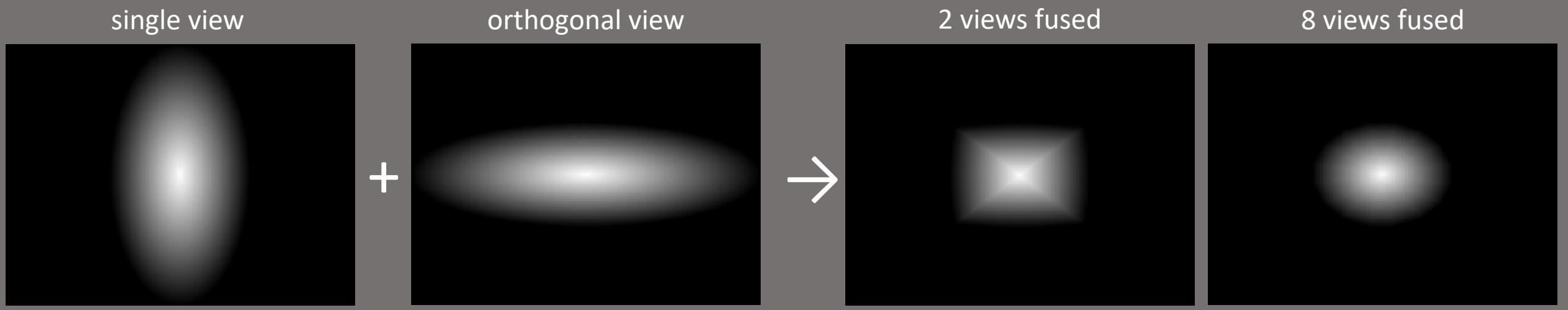
Weber et al., 2014

Multiview imaging

- Rotation of the sample and sequential acquisition of multiple stacks
- Complementary information from different viewing angles
- Registration and fusion of individual datasets

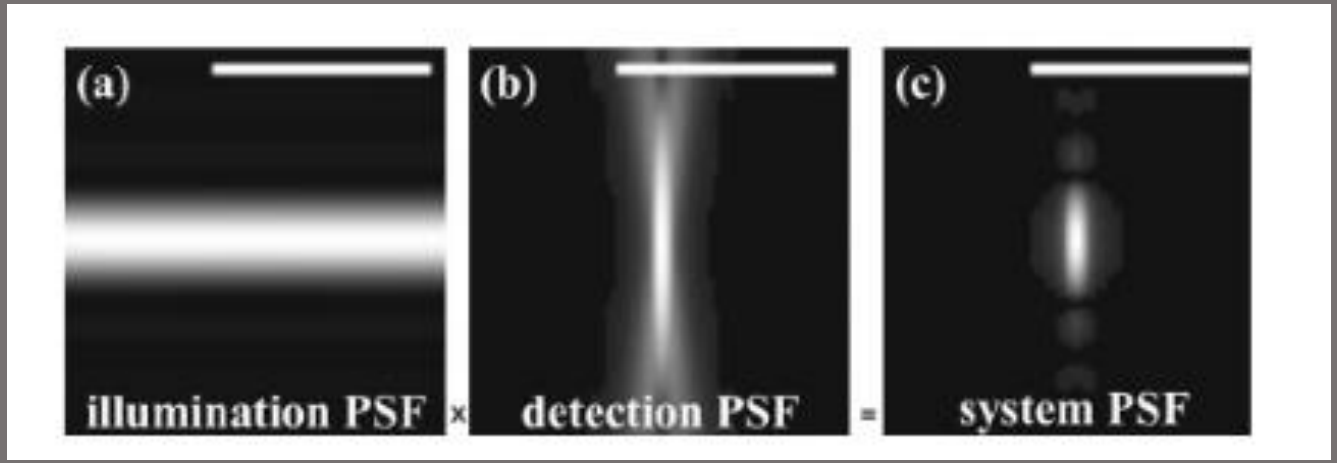
Dr. Cathleen Teh, IMCB, Singapore
Image provided by Zeiss

Multiview imaging



- Potential improvement of the resolution in z
- Fusion of the images from each view
- Combining complementary information from different views
- Achieving isotropic 3D resolution

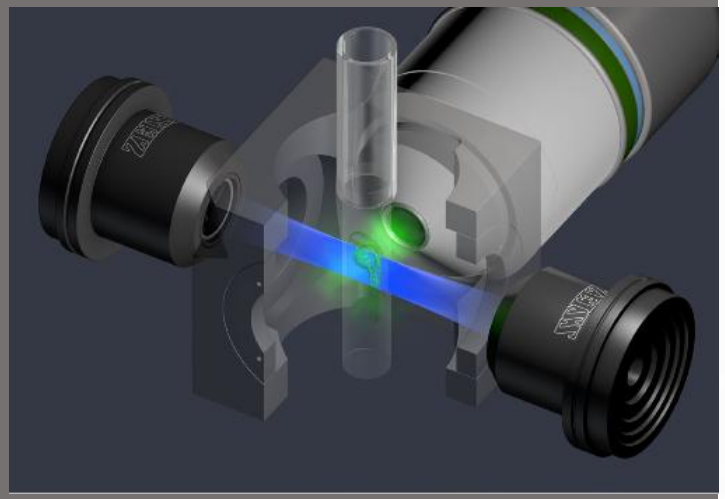
Resolution

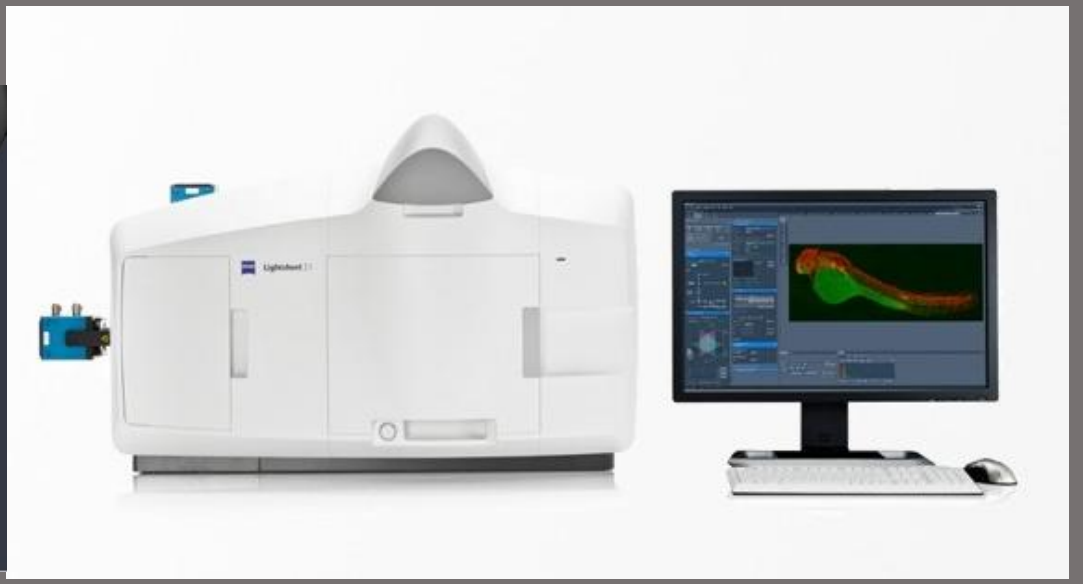


Christoph J. Engelbrecht and Ernst H. K. Stelzer, 2006

- **The effective PSF in Z is convolution of 2 PSFs, one from illumination objective and one from detection objective**
  - XY resolution: comparable to widefield
  - Z resolution: depending on NA of detection objective
    - lightsheet determines the optical section for LOW NA objective → improves Z resolution
    - objective itself determines the optical section for HIGH NA objective

ZEISS Lightsheet Z.1



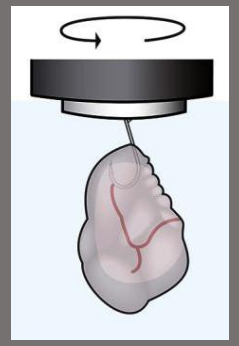


Lightsheet Z.1, Carl Zeiss

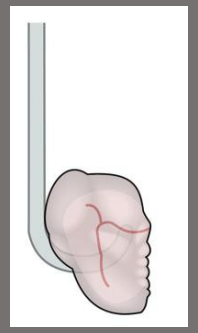
- Combines cylindrical optics and a beam scanning mechanism
- 2-side illumination
- Reducing stripes by pivoting the light sheet
- Precision sample/positioning system
  - z-stack, sample rotation, multiview imaging, tile scan
- 2 sCMOS monochromatic cameras for two-color simultaneous imaging

Sample preparation / mounting

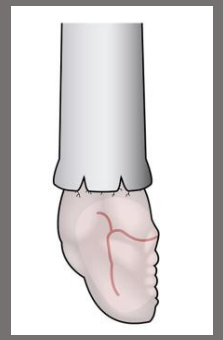
HANGING



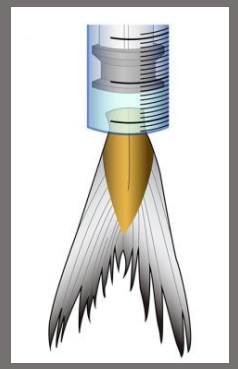
Hooked



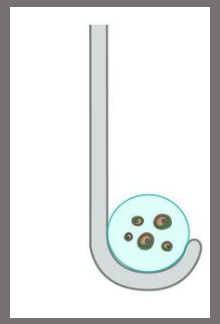
Glued



Clamped / Submerged



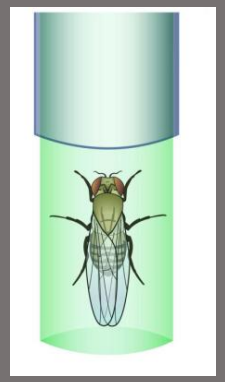
Suspended



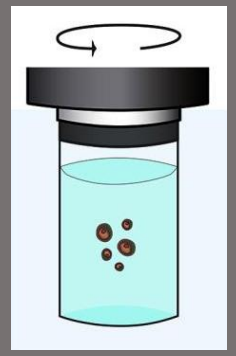
EMBEDDED

Syringe

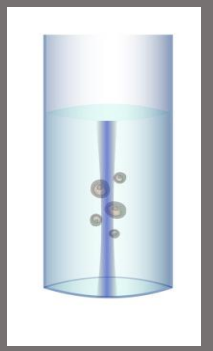
Glass Capillaries



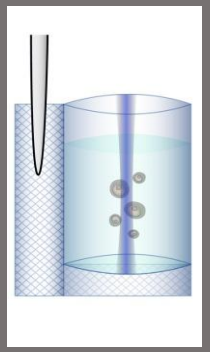
ENCLOSED

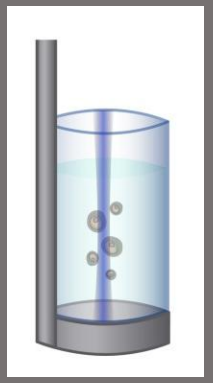


FEP Tubing



Polymer Foil





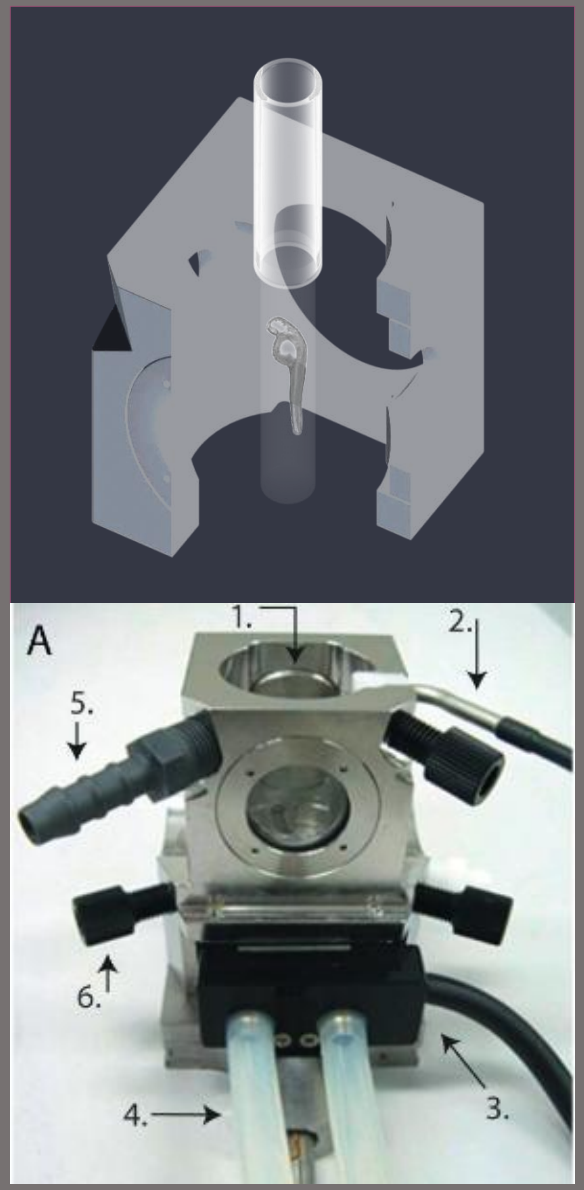
Agarose Beaker

Carl Zeiss Microscopy GmbH, Lightsheet Z.1 Sample preparation, 2013

Applications / Samples

- Living samples
  - Transparent
  - Slightly opaque samples → sample positioning , dual side illumination, multiview
  - Fully opaque sample → imaging limited to its surface
- Fixed, stained samples
- Fixed & optically cleared specimens

Imaging of living samples

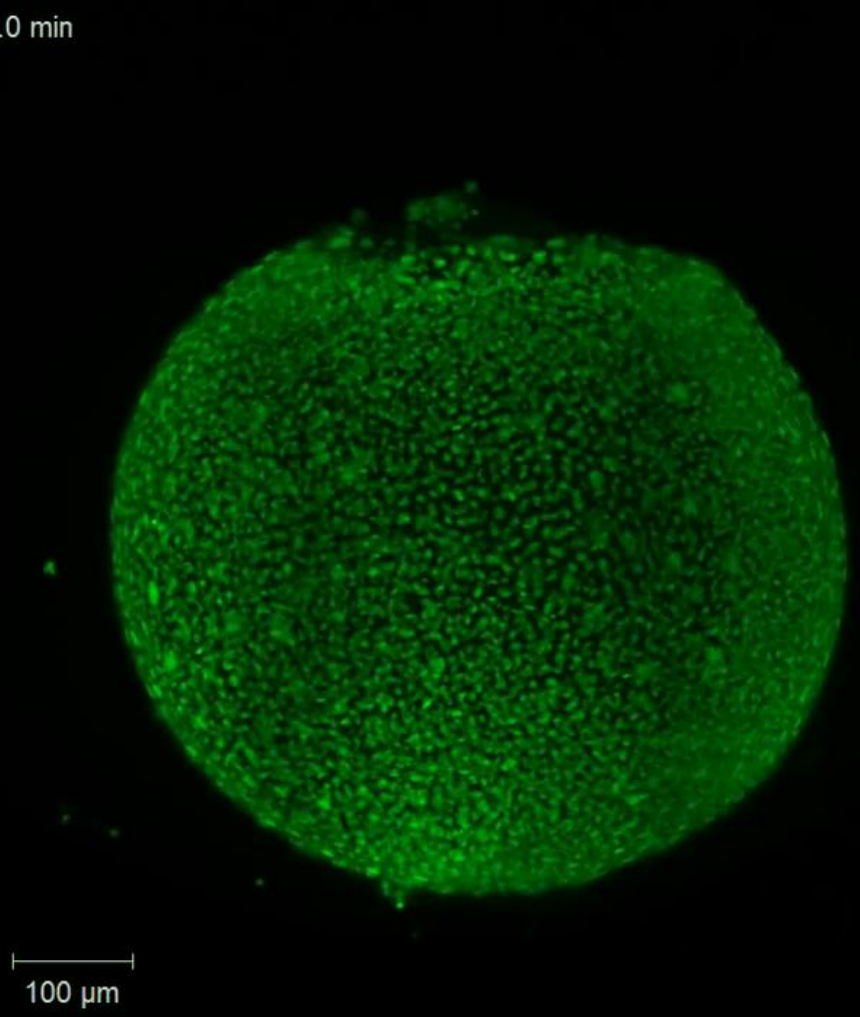


Chamber for aqueous sample environment

- Sample mounted vertically
- Physiological conditions maintained
- Temperature (10 °C - 42 °C) and CO_2 controlled incubation
- Possibility to exchange media, supply nutrients to the specimen
- Water immersion detection objectives, refractive index matched beam path

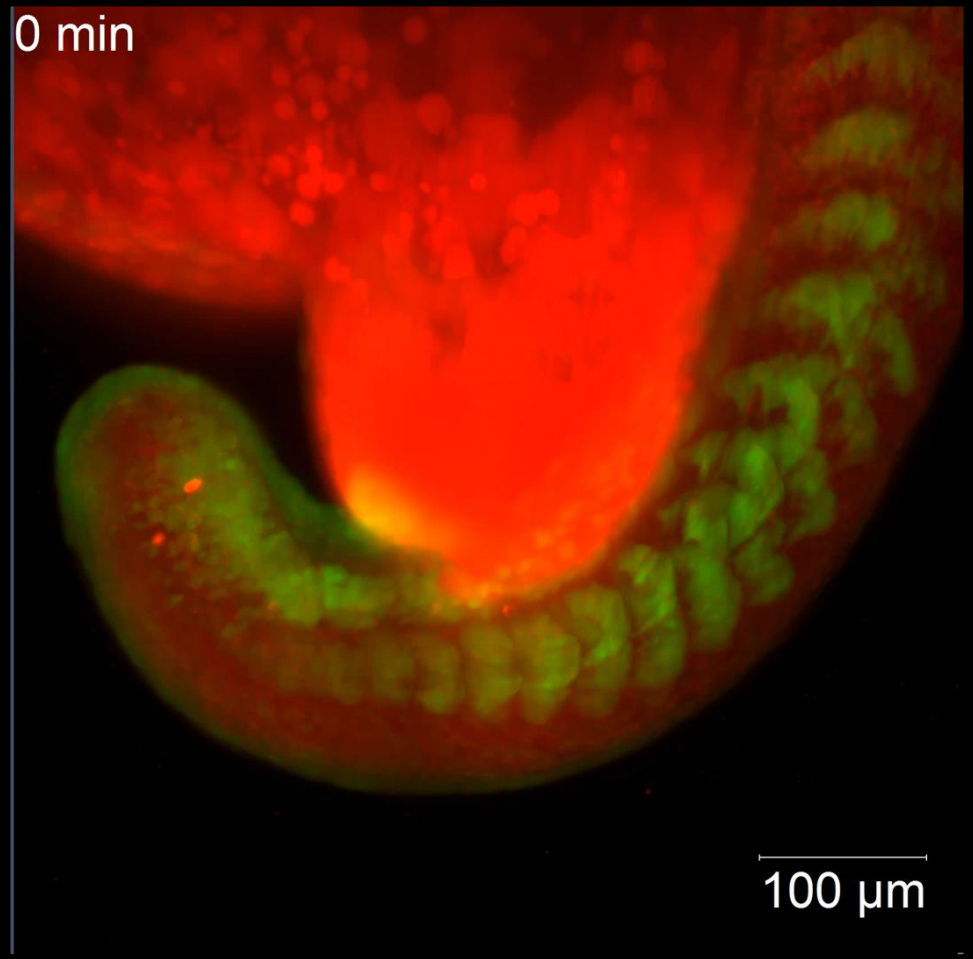
→Multiday experiments, long-term live imaging

- Fluorescent imaging of cellular dynamics in embryos and small organisms



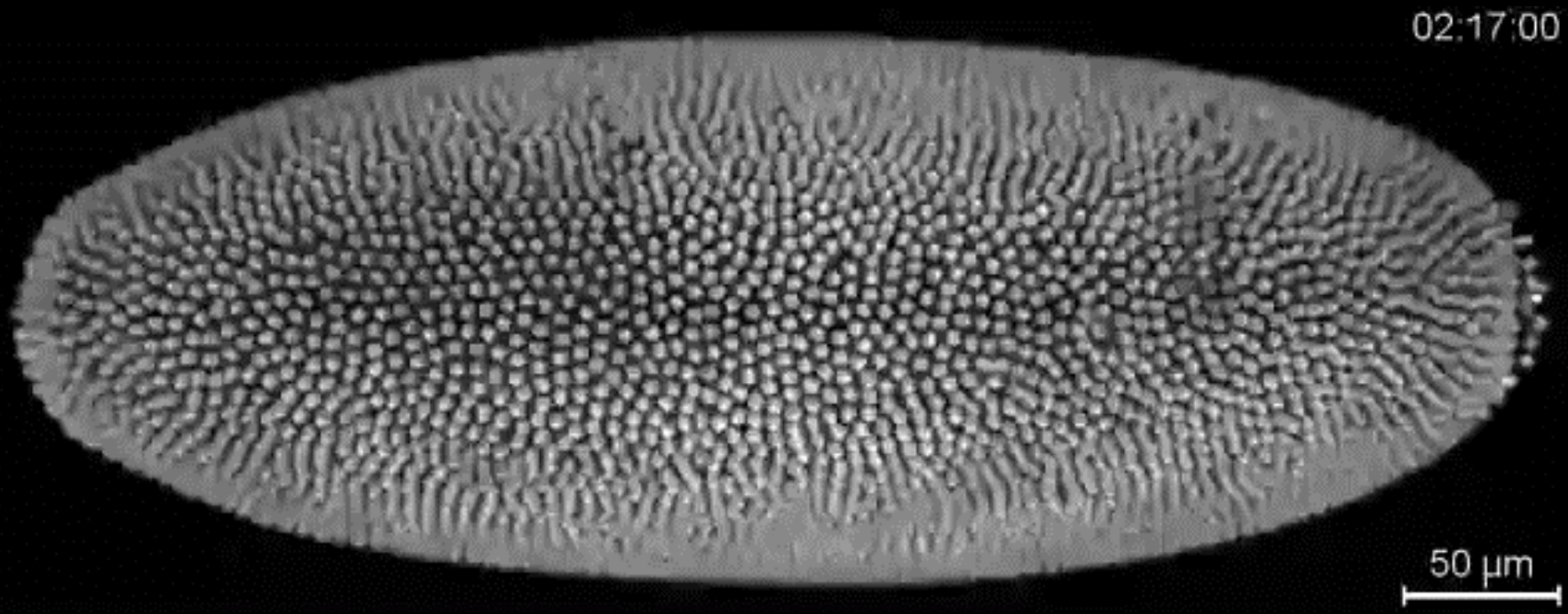
Zebrafish embryo ca. 2 somite stage at the start of the movie.
Stained with Transgenic H2B: Histone2B-egfp.
By Zeiss

- Fluorescent imaging of cellular dynamics in embryos and small organisms



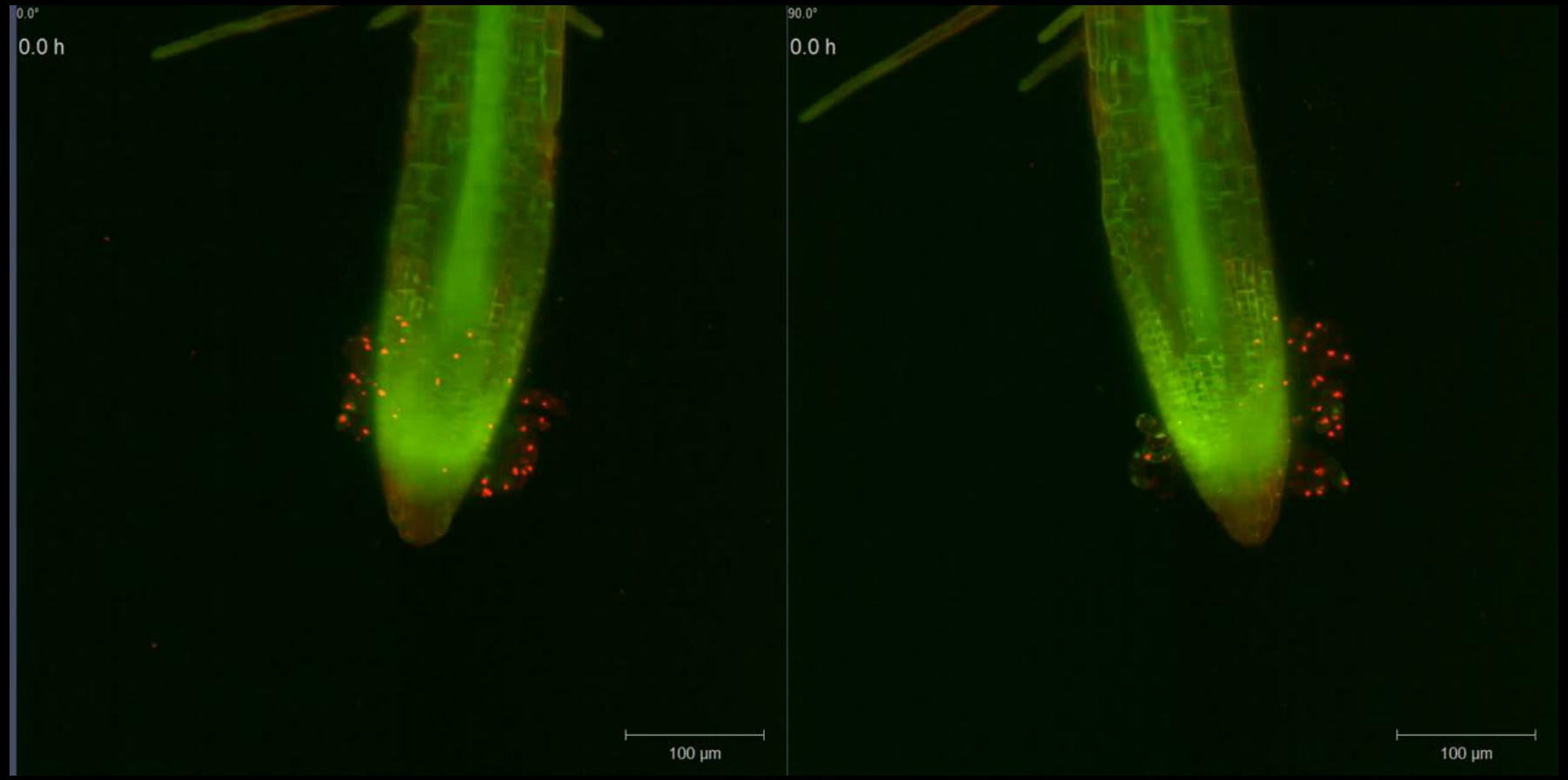
Zebrafish embryo, 12h, muscle development
Green: GFP, Red: Bodipy; Views: 1, Dual side fusion
Objective: W Plan Apochromat 20x/1.0.
*Courtesy of Dr. Ono Yosokue, Philip Ingham Lab, IMCB, A*STAR, Singapore*

- Fluorescent imaging of cellular dynamics in embryos and small organisms



Histone 2A -mRFP labelled Drosophila Embryos, 30 sec intervals over 11 hours.
Ventral half of the embryo.
Amat, F. et al, Nature Methods, July 20th 2014

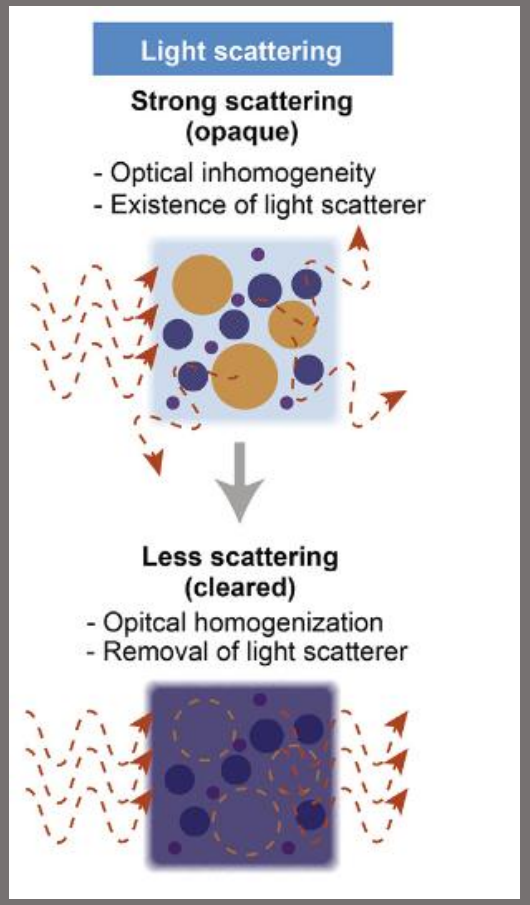
- Imaging of developmental processes and physiological measurements in plants

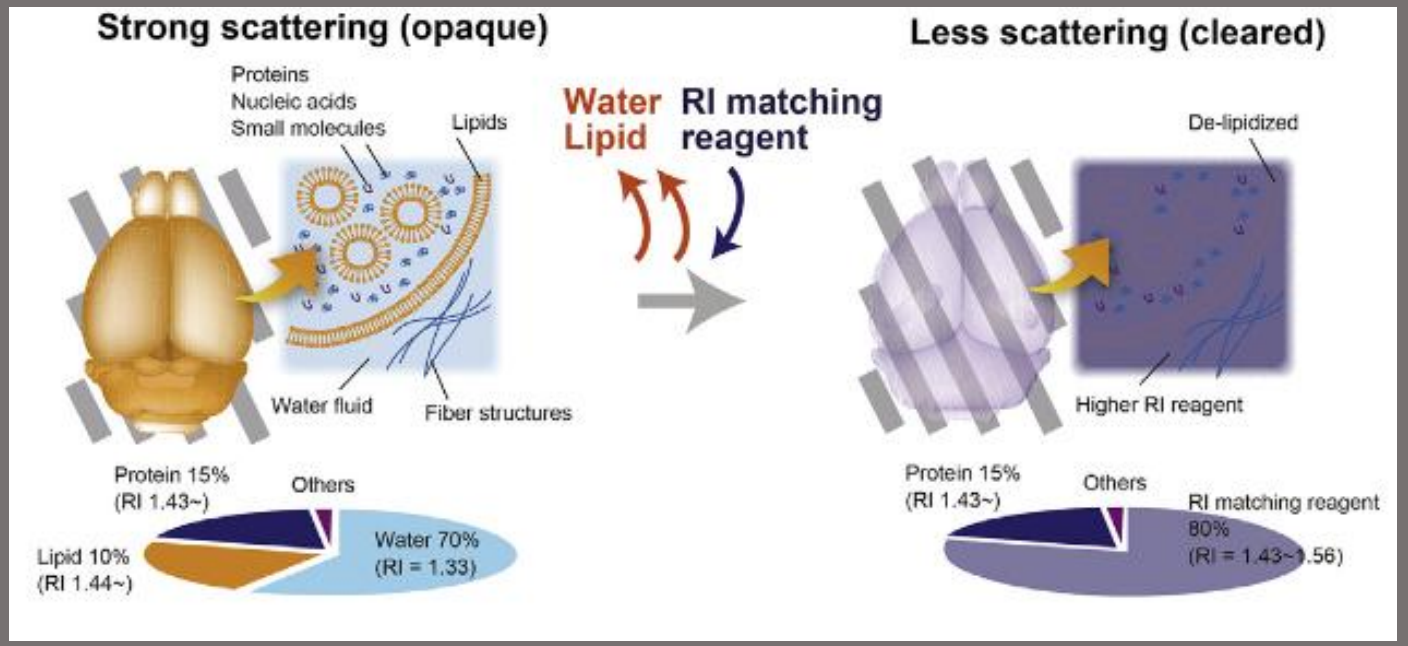


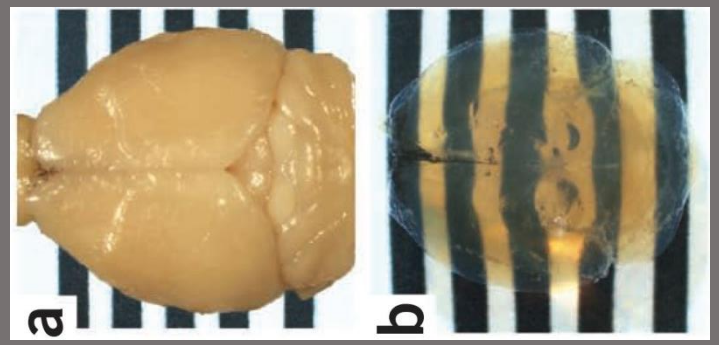
Arabidopsis root growth, 12h timelapse
GFP (green) & autofluorescence. Scaling = 0.581 µm.
Sample courtesy of Dr. Axel Mithoefer, Max-Planck-Institute fur chemische Oekologie, Jena, Germany.

(https://twitter.com/samaj_lab)

Tissue clearing

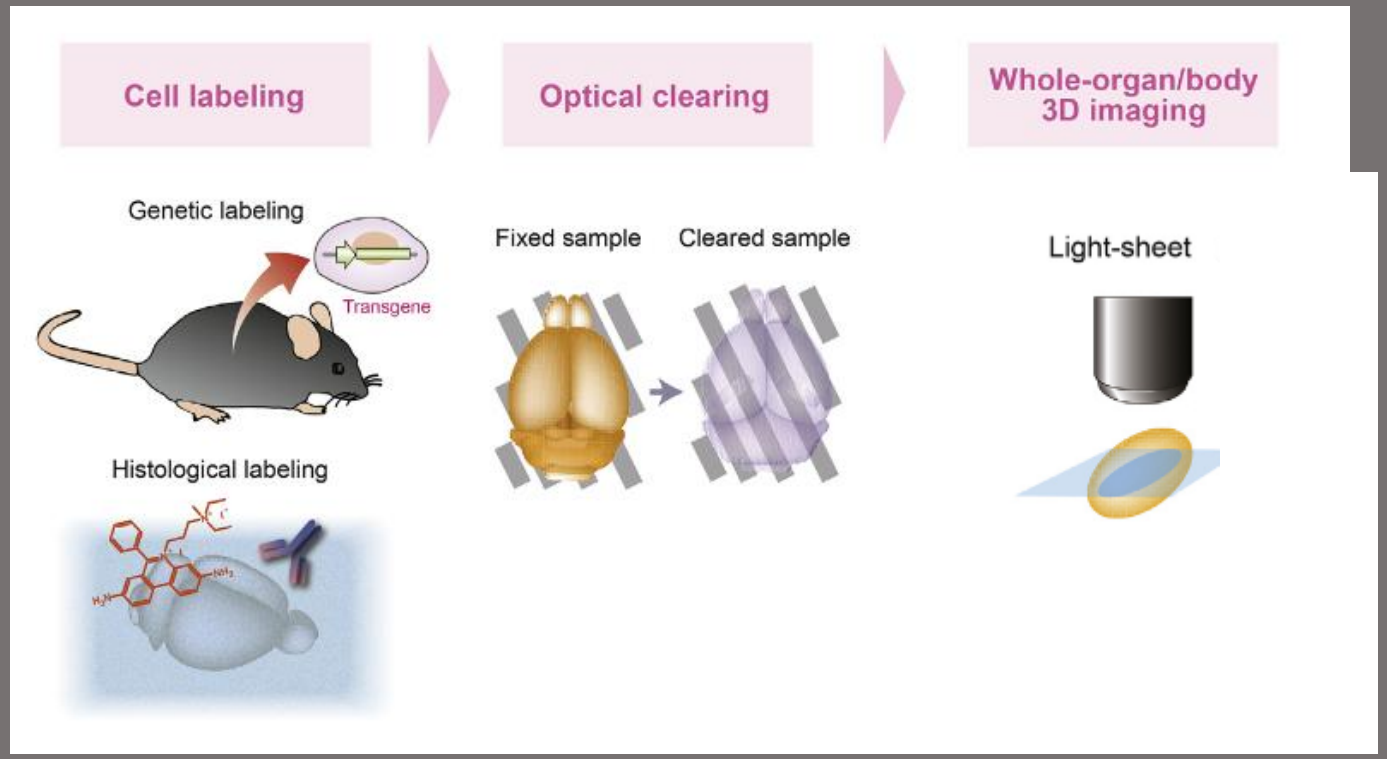


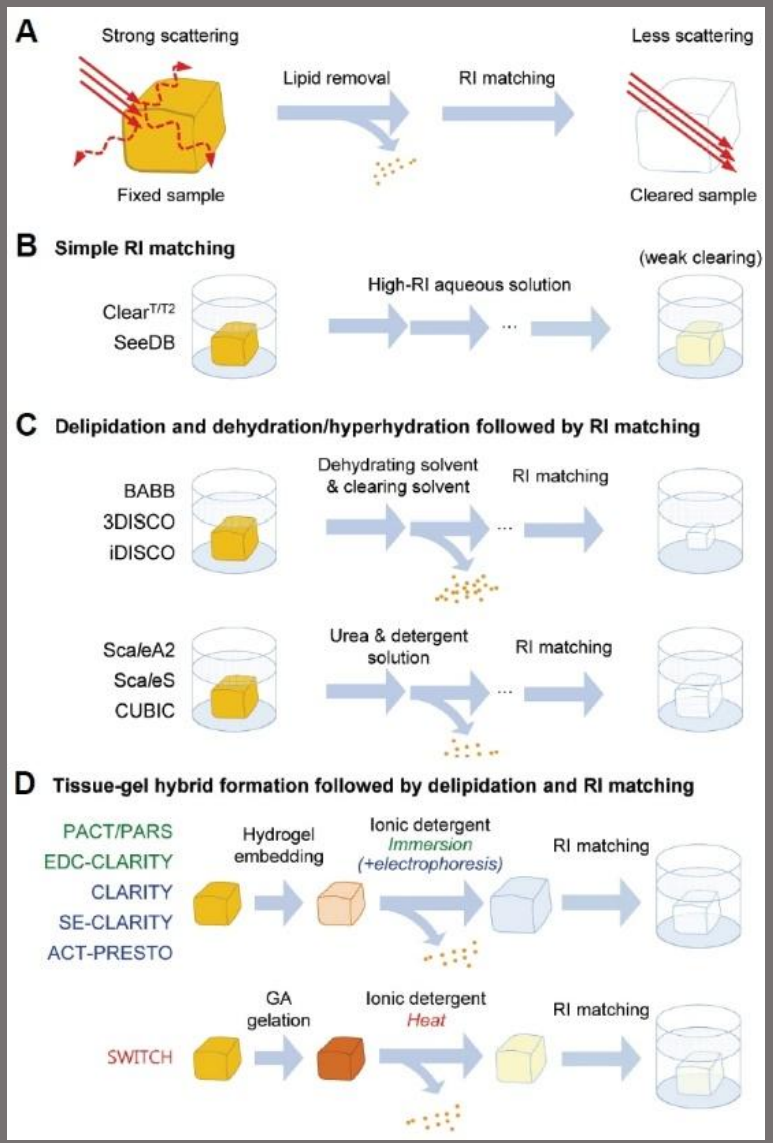




- The opacity of the sample is caused by light scattering.
- Light scattering in biological tissues can be reduced by **removal of lipid** and **RI matching**

Susaki & Ueda, Cell Chemical Biology, 2015





- Variety of clearing protocols – time to clear, fluorescence preservation, compatibility with tissue staining, toxicity, alterations in tissue morphology…

- Lightsheet Z.1 is compatible with aqueous based clearing protocols –

  ScaleS (RI=1,44), ScaleA2 (RI=1,38), CUBIC (RI=1,48), PACT (RI=1,38 – 1,48) …

Immersion	Magnif.	NA	WD [mm]
Clearing Corr n=1.38	20x	1.0	5.6
Clearing Corr n=1.45	20x	1.0	5.6

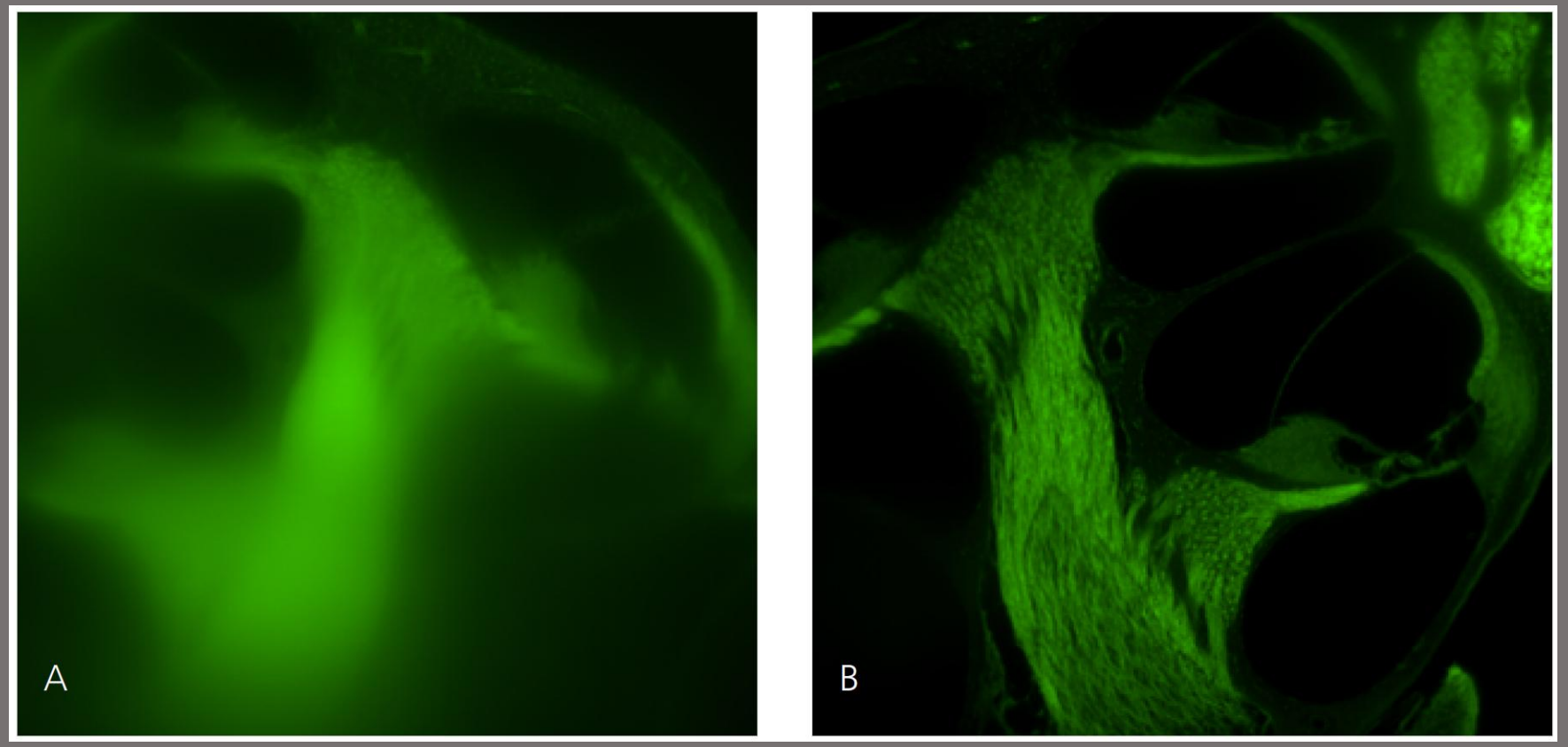
Comparison of clearing techniques

Solvent based	Final RI	Key components	Time to clear	Immunostaining demonstrated	Alterations in tissue morphology	FP emission	Detergent used	Lipid preserved	Electrophoresis	Hydrogel embedding	Clearing solution perfused	Toxic
Spatleholz	1.55	Benzylbenzoate/Methylsalicilate	Months	NO	Shrinkage	NO	NO	NO	NO	NO	NO	YES
BABB	1.55	Benzylalcohol/Benzylbenzoate	Days	YES	Shrinkage	Yes, but only ½ day	NO	NO	NO	NO	NO	YES
3DISCO	1.56	Dichloromethane/Dibenzylether	Hours-Days	Limited	Shrinkage	Yes, but only 1-2 days	NO	NO	NO	NO	NO	NO (DBE)
iDISCO	1.56	Dichloromethane/Dibenzylether	Hours-Days	YES	Shrinkage	Yes, but only 2-4 days	NO	NO	NO	NO	NO	NO (DBE)
Simple Immersion	**Final refractiveindex**	**Key components**	**Time to clear**	**Immunostaining demonstrated**	**Alterations in tissue morphology**	**FP emission**	**Detergent used**	**Lipid preserved**	**Electrophoresis**	**Hydrogel embedding**	**Clearing solution perfused**	**Toxic**
Sucrose	1.44	Sucrose	1 day	YES	Shrinkage	YES	Triton (2%)	NO	NO	NO	NO	NO
FocusClear	1.47	Diatrizoic acid	Hours-Days	YES	NO	YES	Tween 20	YES	NO	NO	NO	NO
ClearT[*]	1.44	Formamide	Hours-Days	YES	NO	NO	NO	YES	NO	NO	NO	NO
ClearT2[*]	1.44	Formamide/PEG	Hours-Days	YES	NO	YES	NO	YES	NO	NO	NO	NO
SeeDB	1.48	Fructose/Thioglycerol	Days	NO	NO	YES	NO	YES	NO	NO	NO	NO
FRUIT[*]	1.48	Fructose/Thioglycerol/Urea	Days	NO	Minimal expansion	YES	NO	YES	NO	NO	YES	NO
TDE[^]	1.42	2,2'-thiodiethanol	Days-Weeks	YES	NO	YES	8% SDS (optional)	NO	Optional	Optional	NO	NO
Hyperhydration	**Final refractive index**	**Key components**	**Time to clear**	**Immunostaining demonstrated**	**Alterations in tissue morphology**	**FP emission**	**Detergent used**	**Lipid preserved**	**Electrophoresis**	**Hydrogel embedding**	**Clearing solution perfused**	**Toxic**
Scale A2	1.38	4M Urea 10% Glycerol	Weeks	NO	Expansion	YES	Triton X100 (0.1%)	NO	NO	NO	NO	NO
Scale U2	1.38	4M Urea 30% Glycerol	Months	NO	NO	YES	Triton X100 (0.1%)	NO	NO	NO	NO	NO
CUBIC	CUBIC1-1.38CUBIC2-1.48	4M Urea/50% Sucrose	Days	YES	Expansion	YES	Triton X100 (50%)	NO	NO	NO	NO	NO
Whole-Body CUBIC	1.38	4M Urea	Days	YES	Expansion	YES	Triton X100 (10%)	NO	NO	NO	YES	NO
Hydrogel Embedding	**Final refractive index**	**Key components**	**Time to clear**	**Immunostaining demonstrated**	**Alterations in tissue morphology**	**FP emission**	**Detergent used**	**Lipid preserved**	**Electrophoresis**	**Hydrogel embedding**	**Clearing solution perfused**	**Toxic**
CLARITY	1.45	FocusClear/80% Glycerol	Days	YES	Slight Expansion	YES	SDS (8%)	NO	YES	YES	NO	NO
PACT	1.38-1.48	Histodenz	Days-Weeks	YES	Slight Expansion	YES	SDS (8%)	NO	NO	YES	NO	NO
PARS	1.38-1.48	Histodenz	Days	YES	NO	YES	SDS (8%)	NO	YES	YES	YES	NO

Refractive index mismatch

RI mismatch of 0.03

RI match



- The matching of RI between objective, medium, and specimen

Carraro et al., 2015

Advantages

- Multi-color fluorescence imaging
- Optical sectioning (no out of focus excitation)
- Minimal photo toxicity
- Fast and sensitive detection with camera
- Multi-view acquisition by rotation of the sample
- Good penetration in scattering tissues

Challenges – sample preparation

- Sample mounting
  - Specific mounting techniques, unique for different samples
  - Stability (gravity, rotational movement..)
  - Include fluorescent beads for multiview registration
- Optical properties of the sample
  - Transparent samples
  - Opaque samples – dual illumination, multiview…
  - Cleared samples – fixed only
- Staining/labeling protocols need to be adapted for thick samples, clearing protocols

Challenges – big data production

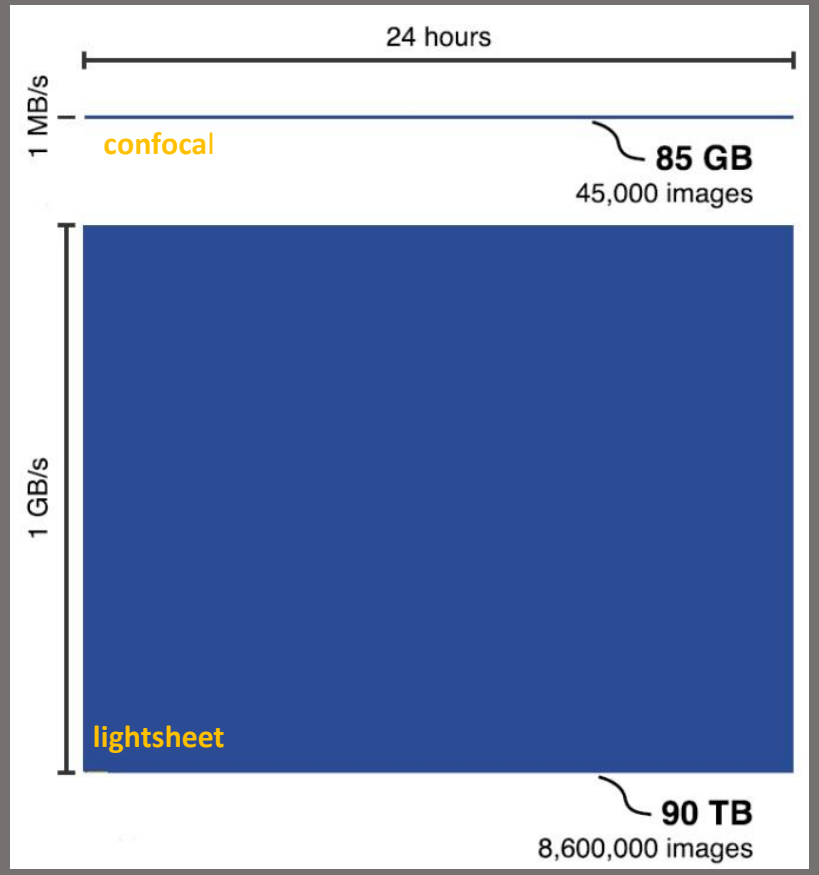
- Amounts of data (range of terabytes)
  - Dual side fusion = two dataset
  - Multiview imaging = multiple datasets
  - Time series …

  - Storage capacity - volume of raw data
  - Data transfer rates
  - Computing power for data processing & analysis (e.g. Multiview experiments + time series…)

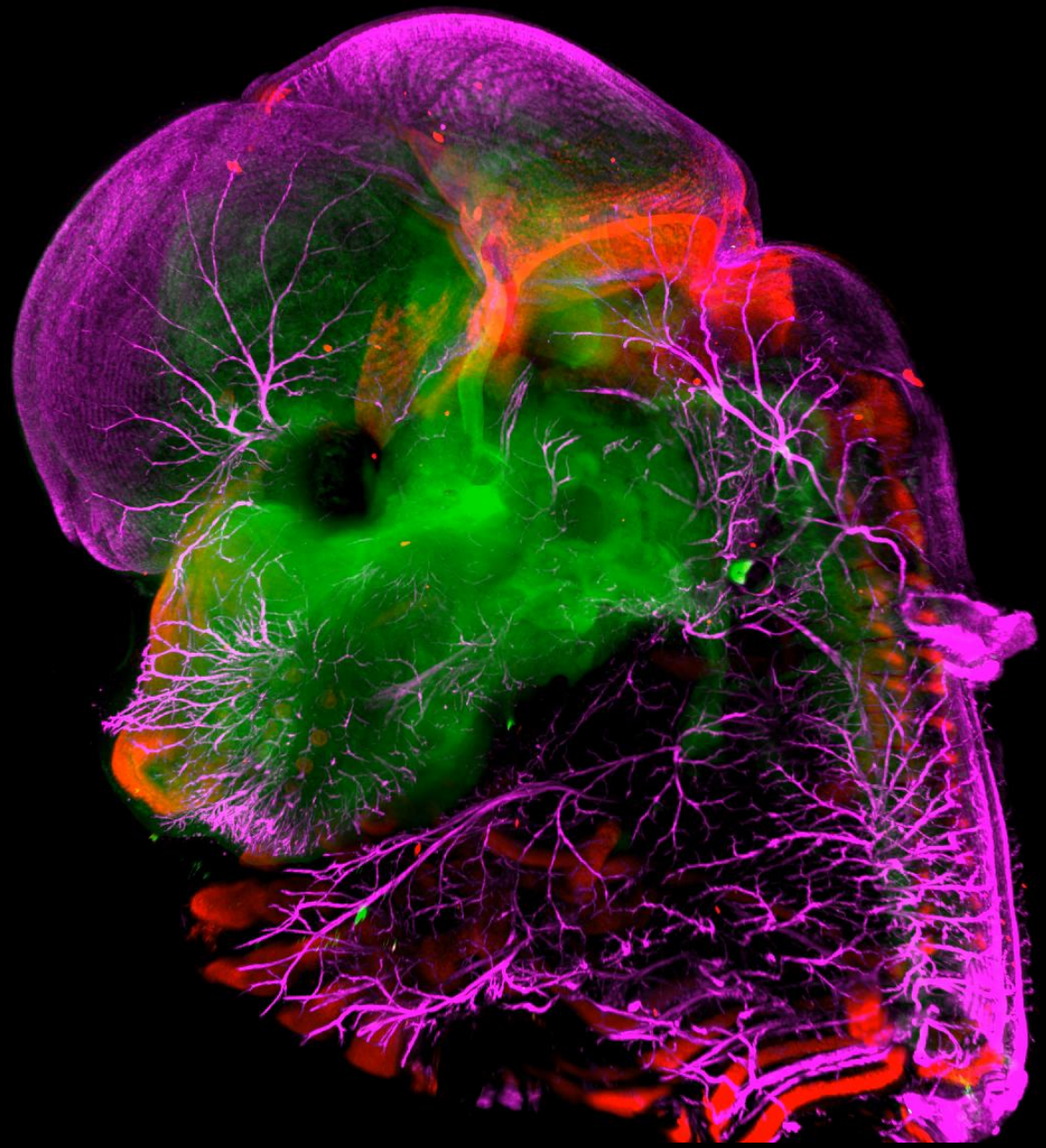
  - Choose the right imaging parameters – **balance between image quality and data size**
    - Lightsheet thickness, z-steps
    - Region of interest of the camera
    - Illumination, number of angles, movie frame rate, interval and duration

    ….
  - Plan your data processing steps and analysis before starting the experiment

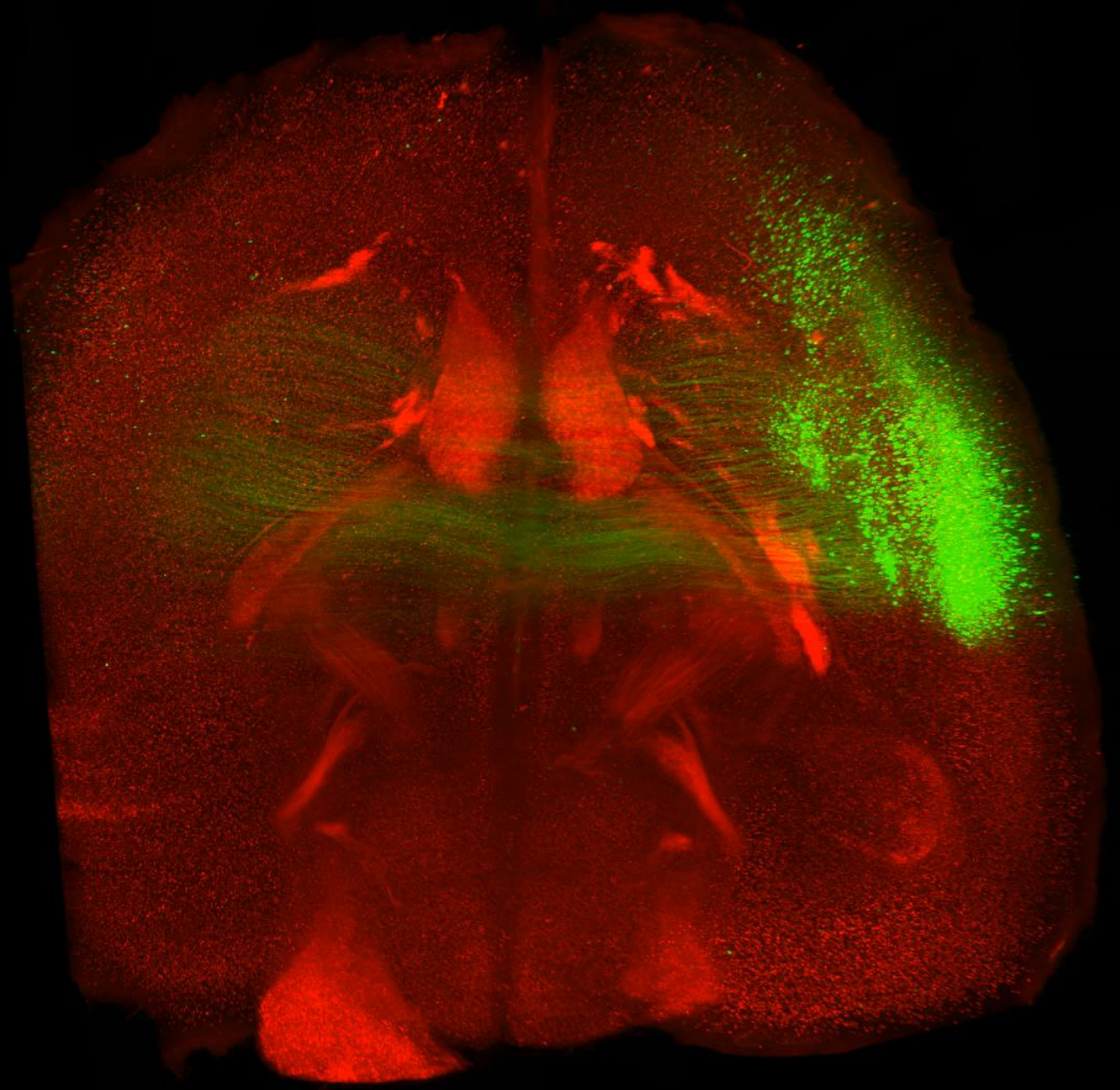
Comparison of the data rates and the total amount of data:



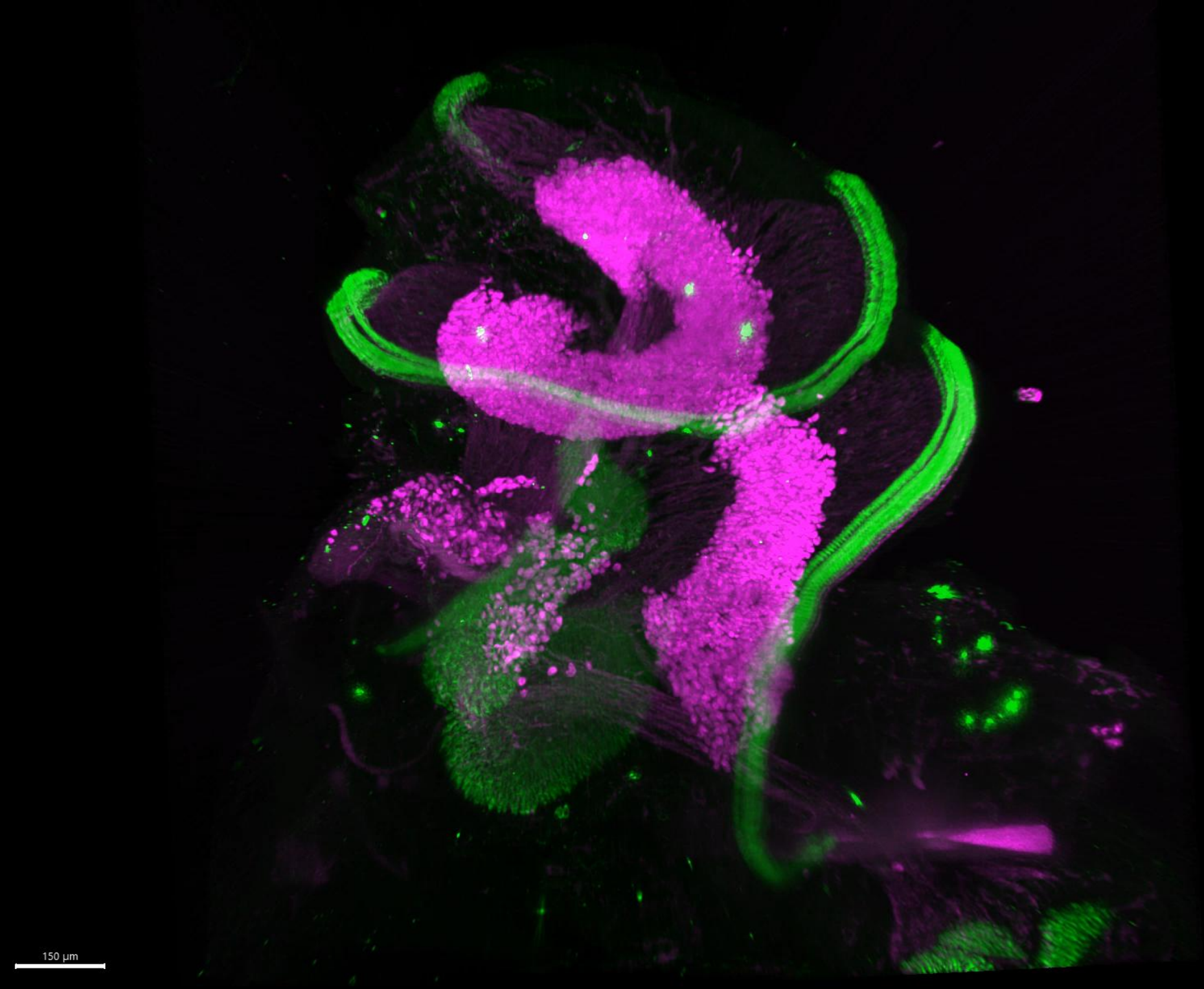
adapted from Schimd et al., 2015



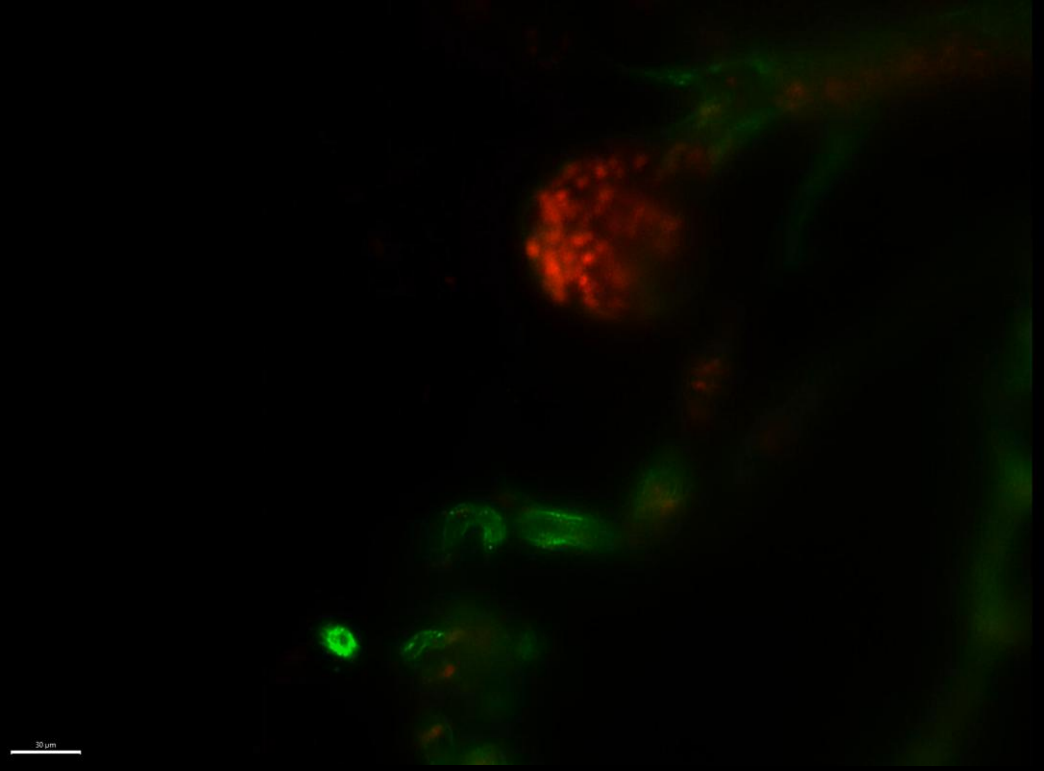
RNDr. Ondřej Machoň, Ph.D.
Department of developmental biology, IEM CAS

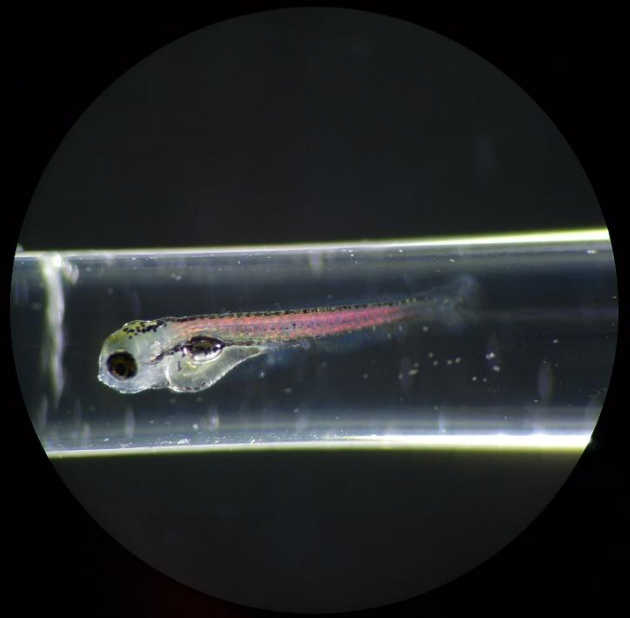


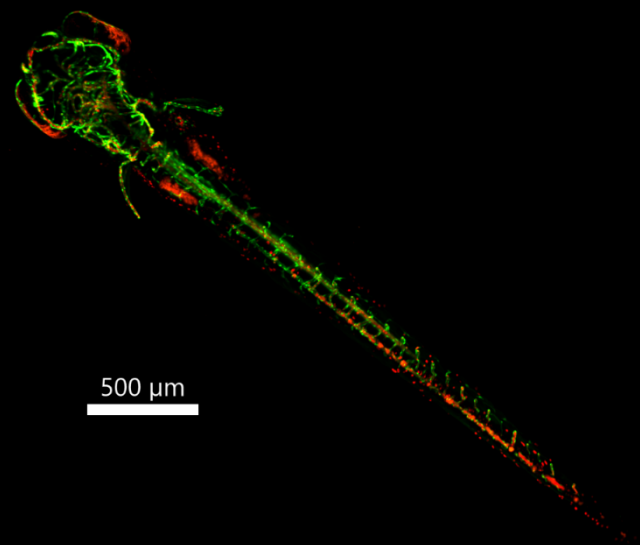
Mgr. Natálie Procházková
Optophysiology of neuronal circuits,
Department of Physiology, 2nd Medical School
of Charles University



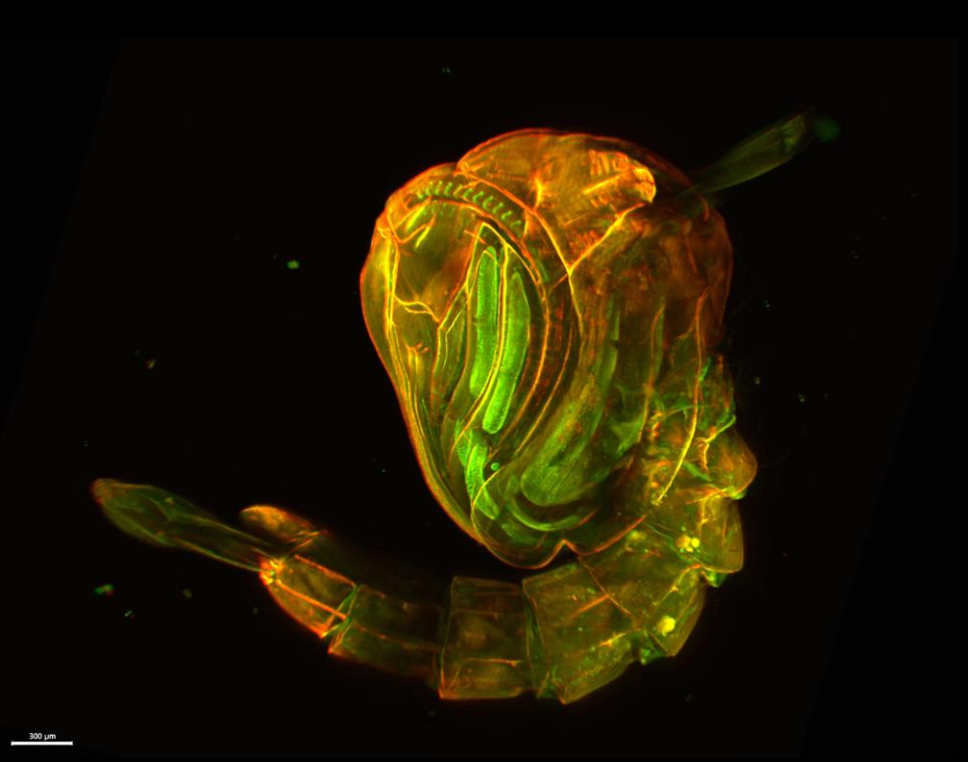
Mgr. Valéria Fabríciová
Ing. Romana Bohuslavová, Ph.D.
Mitra Tavakoli
Laboratory of Molecular Pathogenetics, IBT CAS







Mgr. Ondřej Svoboda, Ph.D.
Laboratory of Cell Differentiation, IMG CAS


300 µm


200 µm


500 µm


70 µm

Acknowledgement

<u>Light Microscopy Core Facility</u>

MUDr. Ondrej Horváth
Ing. Ivan Novotný, Ph.D.
Mgr. Helena Chmelová, Ph.D.
Ing. Martin Čapek, Ph.D.
RNDr. Michaela Blažíková, Ph.D.
Mgr. Jan Valečka

Thank you
for your attention