# *Challenges in Light Sheet Microscopy?*

**Emmanuel G. Reynaud**

**University College Dublin, Ireland**

# Summary

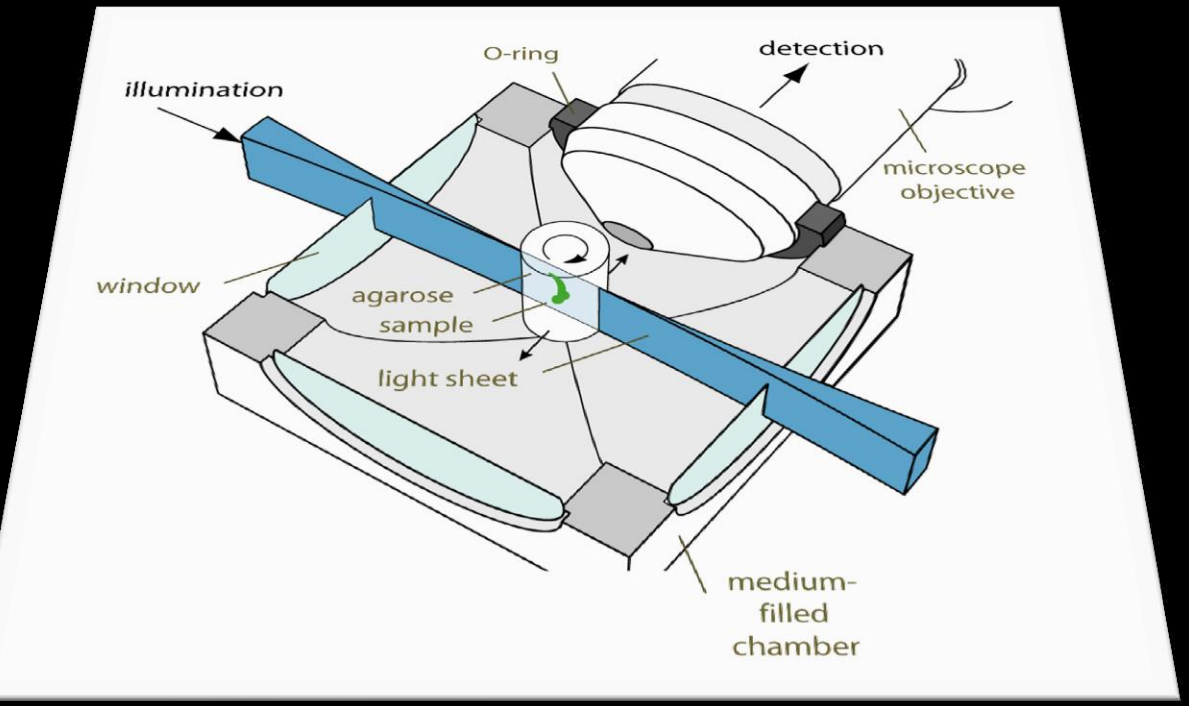
- Young and confusing
- Energy, time and space
- Sample preparation
- Image processing
- Data Deluge
- Conclusion

# The acronym war…

LS: Light Sheet or **LISH**
UM: Ultramicrosope
OPFOS: Orthogonal Plane Fluorescence Optical Sectioning
LSP: Light Scanning Photomicrography
SPIM: Selective (or Single) Plane Illumination Microscopy
TLSM: Thin laser light sheet microscope
DSLM: Digital Scanned Laser Light Sheet Microscope
LSFM: Light Sheet based Fluorescence Microscopy (very close to LSM???)
OCPI: Objective Coupled Planar Illumination microscopy
OPM: Oblique Plane Microscopy
TSLIM: Thin-Sheet Laser Imaging Microscope
LSBM: Light-Sheet based Microscopy
LISM: LIght Sheet Microscopy
**Light Sheet Illumination Microscopy (LSIM)**
…
**Planar Illumination Microscopy (PIM)**, Azimuthal microscopy

**But Light sheet microscopy is more often used…**

# Single Plane Illumination Microscopy




**Light sheet illumination:**
- optical sectioning
- no damage outside light sheet
- very low laser power

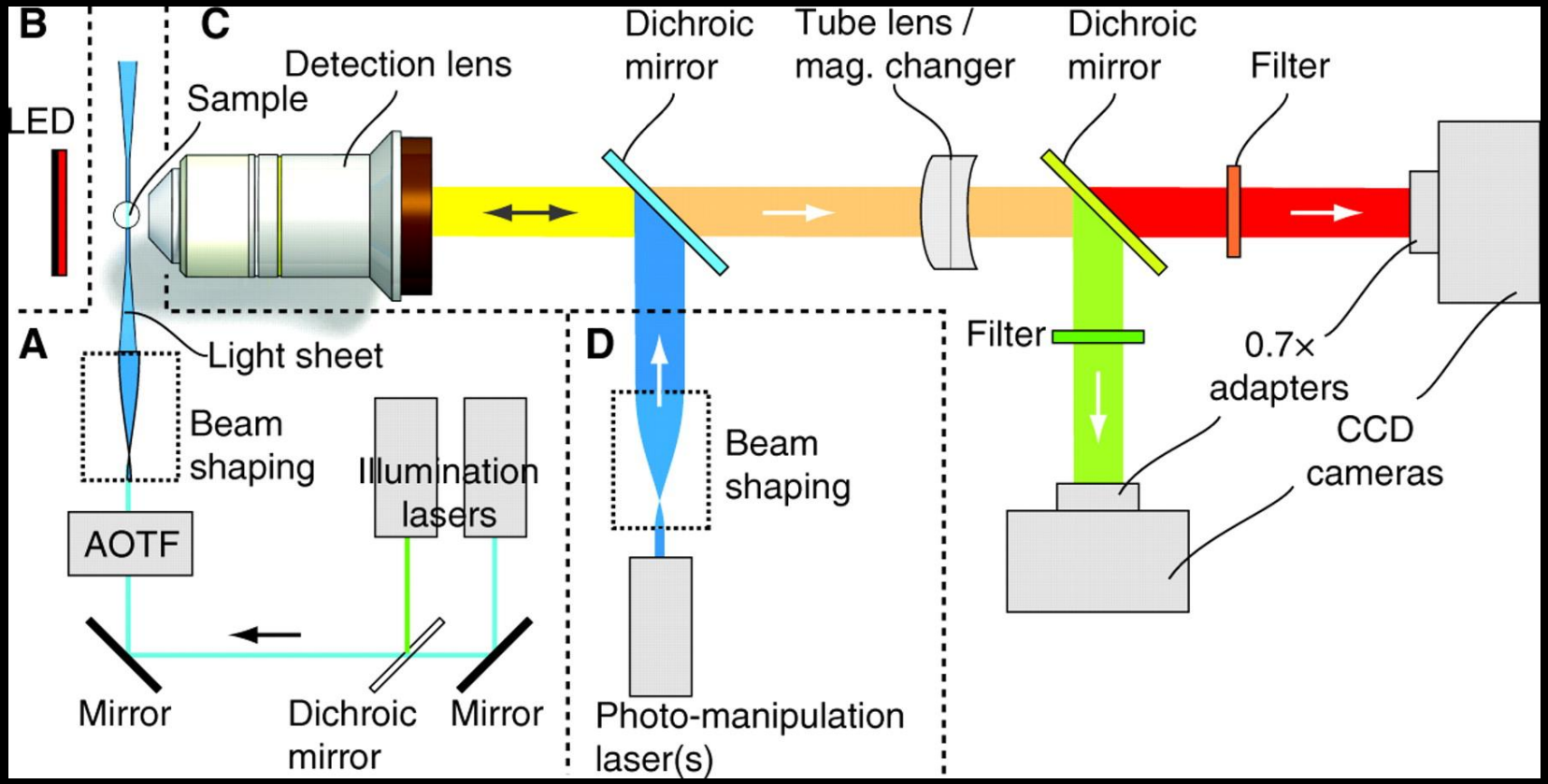
**Detection with regular lens:**
- focal plane overlaps light sheet
- water immersion or air lens
- variety of NA & magnification

**Sample mounted e.g. in agarose:**
- translation & rotation
- in medium / buffer
- physiological conditions

**Chamber:**
- aqueous medium
- minimized aberrations
- temperature controllable
- perfusion (environment)

Huisken, Swoger, Del Bene, Wittbrodt, Stelzer, Science 2004

# Single Plane Illumination Microscopy

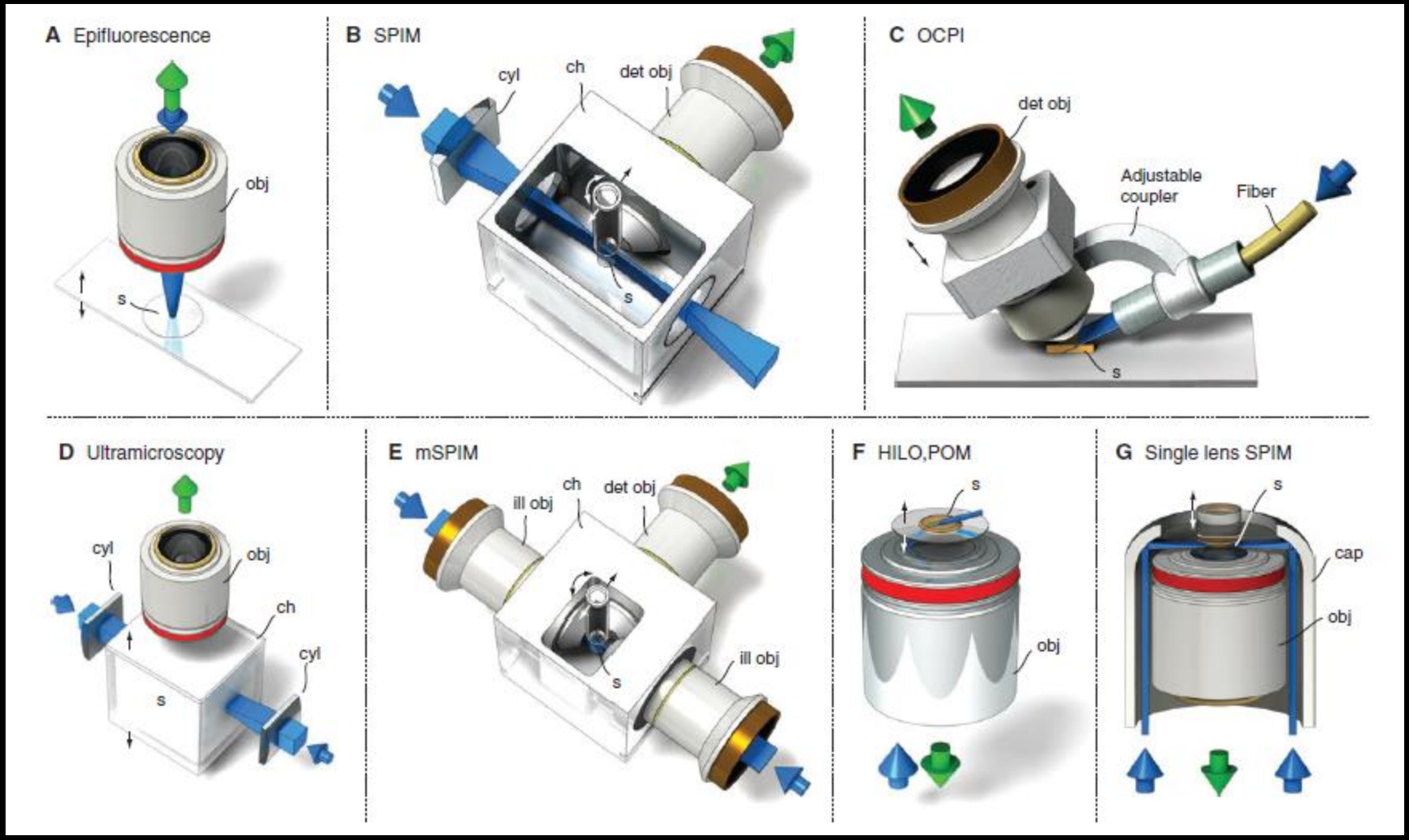



Huisken J , Stainier D Y R Development 2009;136:1963-1975

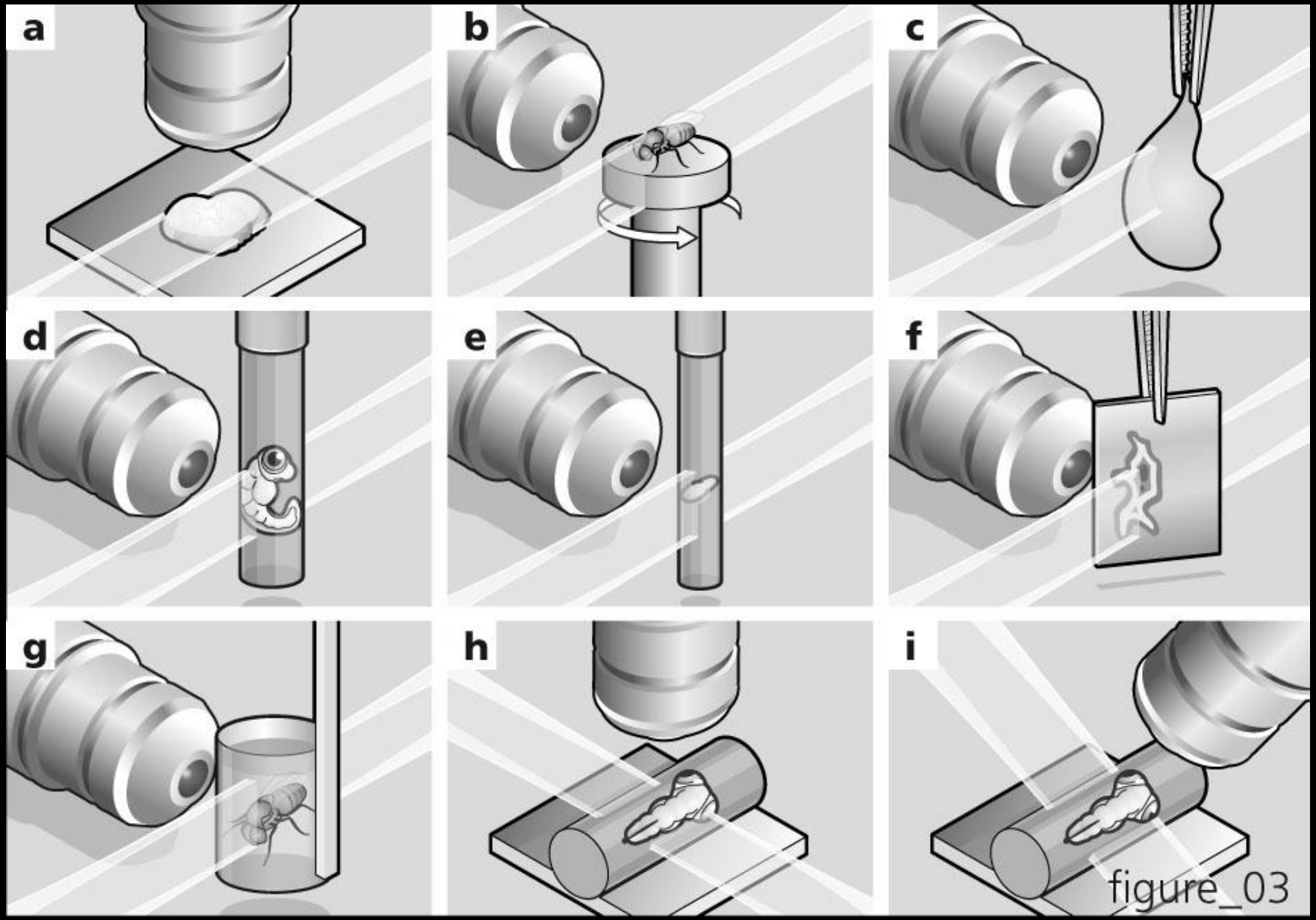
# Light Sheet Microscopy advantages…

| | | |
|---|---|---|
| Shorter time intervals | record images more often | 5 fps instead of 1 fps |
| Longer observation periods | record images for very long periods of time | days instead of hours |
| More images per stack | improve z-sampling | |
| Higher signal to noise ratio | cameras provide excellent dynamic range | 12 - 14 bits instead of 3 - 5 bits |
| Improved resolution | resolution determined by NA not statistics | isotropic resolution 200 nm |
| Excellent deconvolution | | isotropic resolution 150 nm dynamic range 8 - 10 bits |
| Multiple views | observe specimen not only from top | depending on specimen properties 2 – 18 stacks |
| Life time imaging | take advantage of high dynamic range | record more frequencies distinguish more lifetimes |

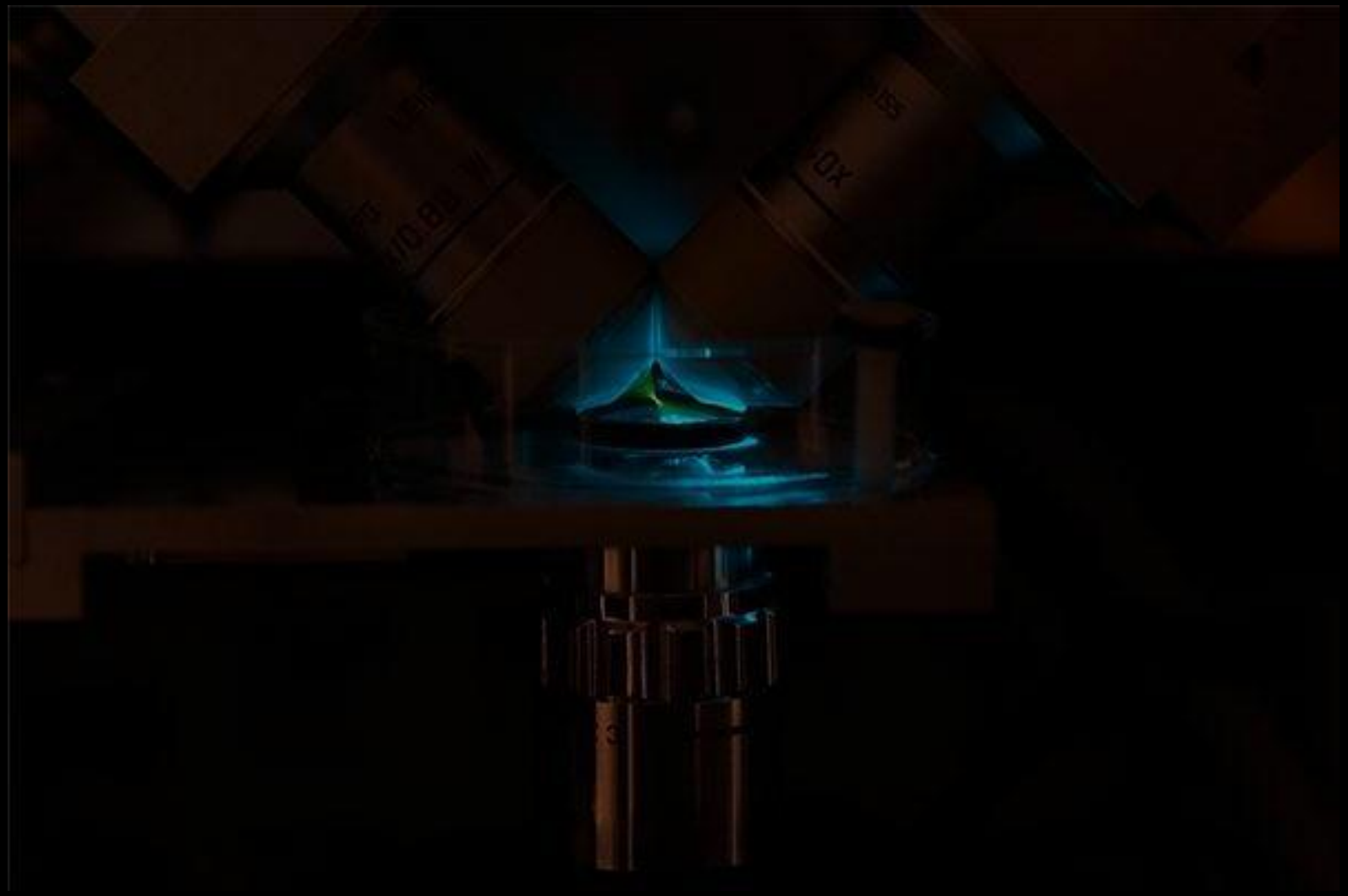
# SPIM and friends



# LSFM possibilities

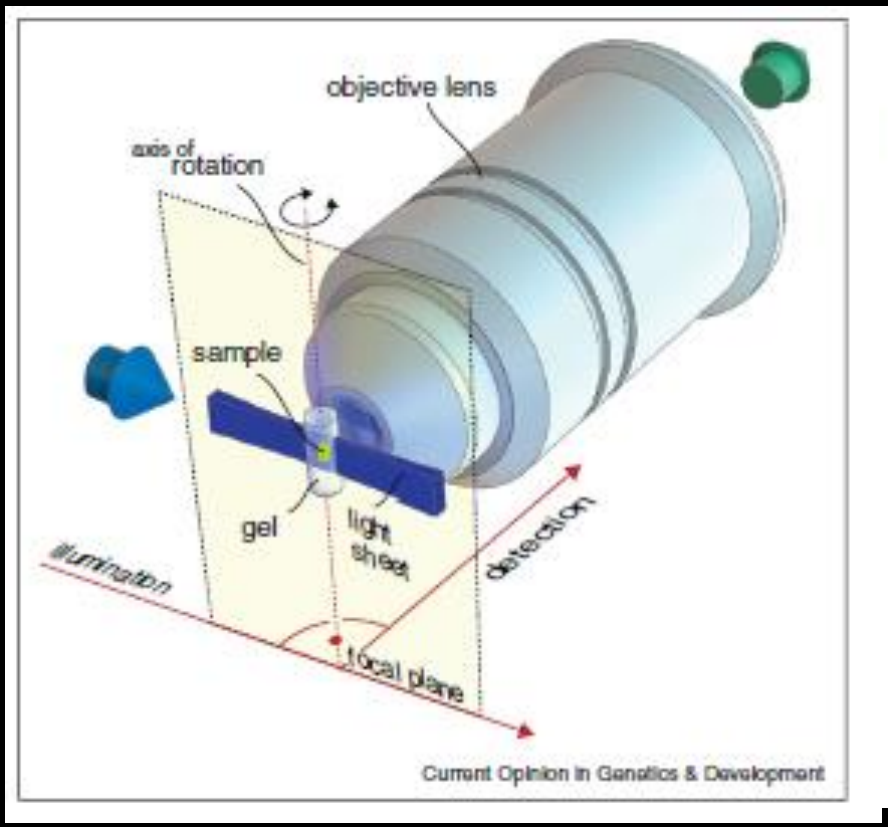


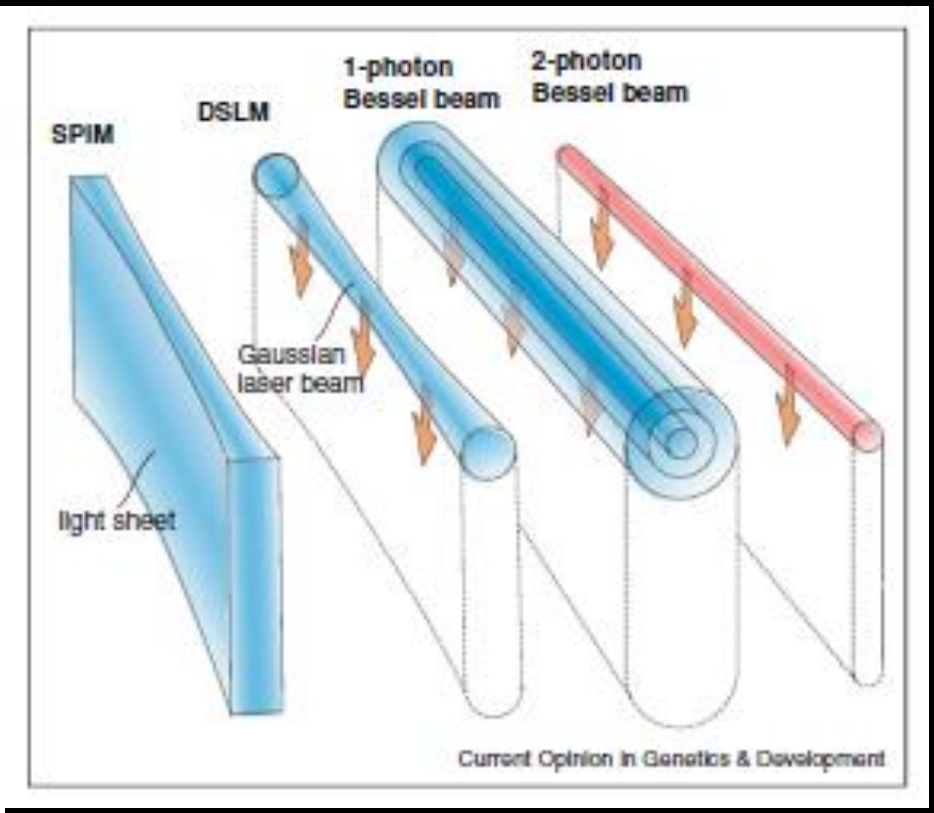
# SPIM and friends



Capoulade, J., Wachsmuth, M., Hufnagel, L. & Knop, M. Quantitative fluorescence imaging of protein diffusion and interaction in living cells. Nature Biotechnology

# SPIM and friends



# Light Sheet Illumination F…techniques

- **FRAP** (Fluorescence Recovery After Photobleaching)

- **Laser Nanosurgery**

- **FLIM (Fluorescence Life-Time Imaging)**

-**Structured illumination**

- **STED** (Stimulated Emission Depletion microscopy)

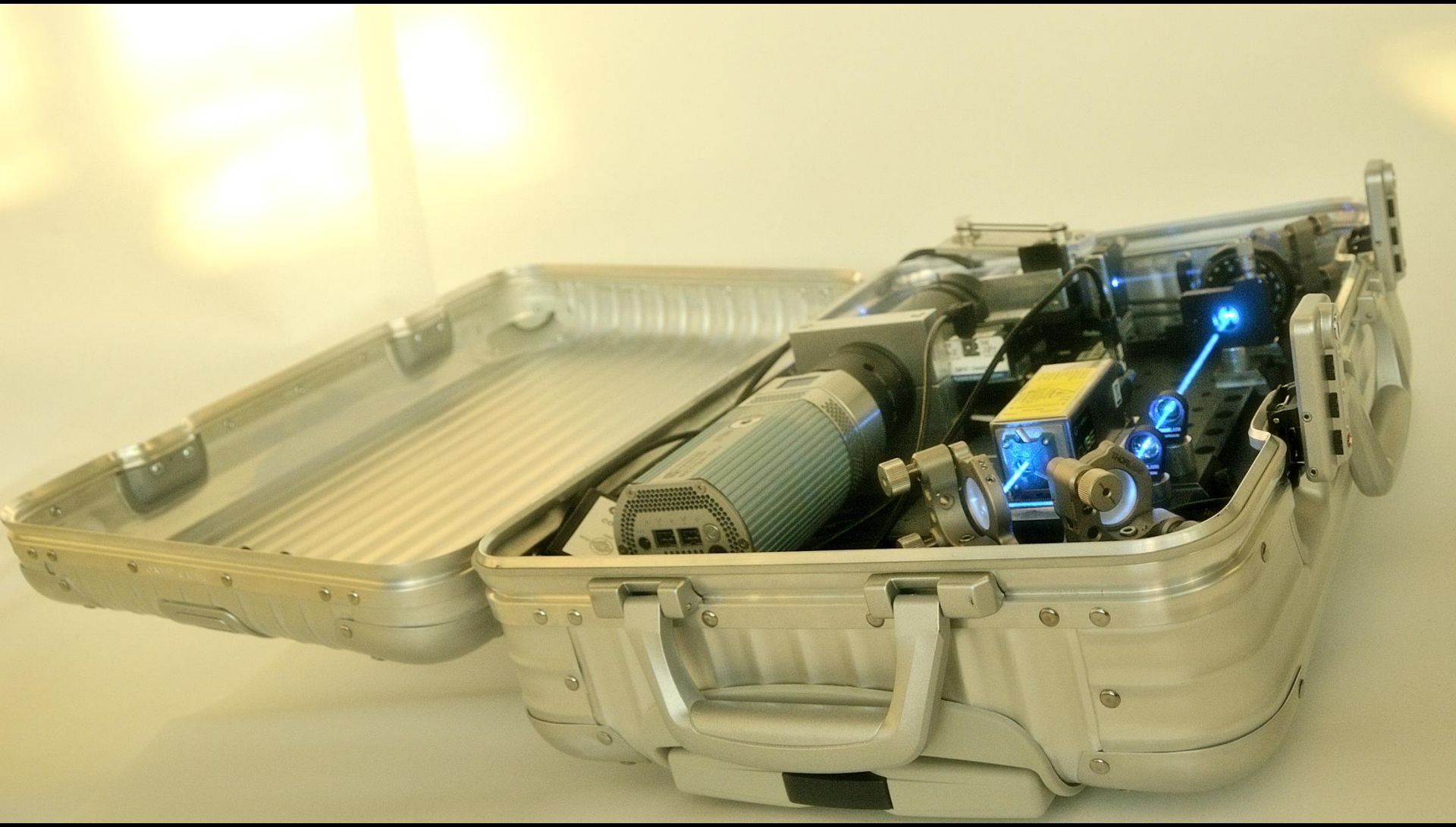
- **FCS** (Fluorescence correlation spectroscopy) and **FCCS**

- **STORM, PALM…**

- **Adaptive optics**

- **Cell biology applications up to weeks**

# The Open SPIM to travel with..



| Topic | Subtopic | Sample/Model Organism | Technique/ LSFM implementation | Reference |
|---|---|---|---|---|
| | | | | |
| Physics | Technical set up of MISERB | Fluorescent beads | MISERB | Fahrback et al, 2010 |
| | Structured illumination | Mouse cochlea | sTSLIM | Schroter et al, 2011 |
| | Light Sheet Characteristics | Fluorescent beads | SPIM | Ritter et al, 2008 |
| | Image formation | C. elegans | DSLM | Olarte et al, 2012 |
| | Image View fusion | live sea urchin embryo, live Danio rerioembryo | LSFM | Rubio-Guivernau et al, 2012 |
| | | | | |
| Biochemistry | Laser Microsurgery | In vitro microtubules | SPIM | Engelbrecht et al, 2007 |
| | Microtubule dynamic instability | In vitro microtubules | SPIM/DSLM? | Keller et al, 2008 |
| | mRNA nuclear export | Chironomus tentans Salivary Glands | SPIM | Siebrasse et al, 2012 |
| | Heterochromatin dynamics | MCDK cells, Drosophila melanogaster | LSFM (FCS) | Capoulade, 2011 |
| | Imaging of engineered gene expression | Drosophila melanogaster | SPIM | Ejsmont et al, 2009 |
| | | | | |
| Microbiology | Marine microbiology | Various bacteria, protozoa etc. | LSFM | Fuchs et al, 2002 |
| | | | | |
| Cell biology | Adaptive optics to improve imaging performance | Tumour spheroids | waoSPIM | Jorand et al, 2012 |
| | Intracellular imaging | Mammalian cell organelles | Bessel beam plane illumination | Planchon et al, 2011 |
| | Nuclear protein localisation | Cellular spheroids | SPIM | Zanacchi et al, 2011 |
| | Imaging large living samples | MCDK cell cysts | SPIM | Verveer et al, 2007 |
| | | | | |
| Plant Biology | Live imaging of root growth | Arabidopsis thaliana | DSLM | Maizel et al, 2011 |
| | Consecutive imaging of vertically growing root | Arabidopsis thaliana | SPIM | Sena et al, 2011 |
| | | | | |
| Developmental Biology | Imaging of developing organs | Danio rerio heart valve | SPIM | Scherz et al, 2008 |
| | Embryogenesis visualisation | Drosophila embryo | SPIM | Huisken et al, 2004 |
| | Zebrafish development | Danio rerio | mSPIM | Kaufmann et al, 2012 |
| | Cell identity lineaging and neurodevelopmental imaging | Caenorhabditis elegans | iSPIM | Wu et al, 2011 |
| | Gene Expression: hour glass model verification | Drosophila melanogaster | SPIM | Kalinka et al, 2010 |
| | | | | |
| Physiology | 3D reconstruction of inner ear | Cavia porcellus | OPFOS; LSFM | Hofman et al, 2009 |
| | Brain in vivo imaging | Microspheres | miniSPIM | Engelbrecht et al, 2010 |
| | 3D reconstruction for morphological analysis | Bast's valve | OPFOS | Hofman et al, 2007 |
| | Scan of whole brain | Mouse brain | LSFM | Mertz and Kim, 2010 |
| | Neural network imaging | Mouse brain | Ultramicroscope | Dodt et al, 2007 |
| | Sectioning of thick tissues | Mouse cochlea/zebrafish inner ear, brain/ rat brain | TSLIM | Santi et al, 2009 |
| | Imaging neuronal activity | Mouse vomeronasal cells | OCPI | Holekamp et al, 2008 |
| | Imaging of immunolabelled receptors | Mouse | SPIM | Klohs et al, 2008 |
| | Optical sectioning | Meriones unguiculatus cochlea, Hippocampus reidi head, Xenopus laevis | OPFOS | Buytaert et al, 2012 |
| | | | | |
| Large organism general biology | Whole organism 3D reconstruction | Ormia ochracea; Emblemasoma auditrix | LSP | Huber et al, 2001 |
| | Whole organism 3D reconstruction | Drosophila melanogaster | Ultramicroscope | Jahrling et al, 2010 |
| | Imaging copepod gut contents | Calanus pacificus | PLIF | Jaffe et al, 2009 |

# Light Sheet Illumination

- Architecture (upright, azimuthal...)
- Illumination (lens, scanner, Bessel beams…)
- LISH angle (90 degrees…)
- Add-ons (nanosurgery, adaptive optics…)
- Camera numbers
- Drivers and computers

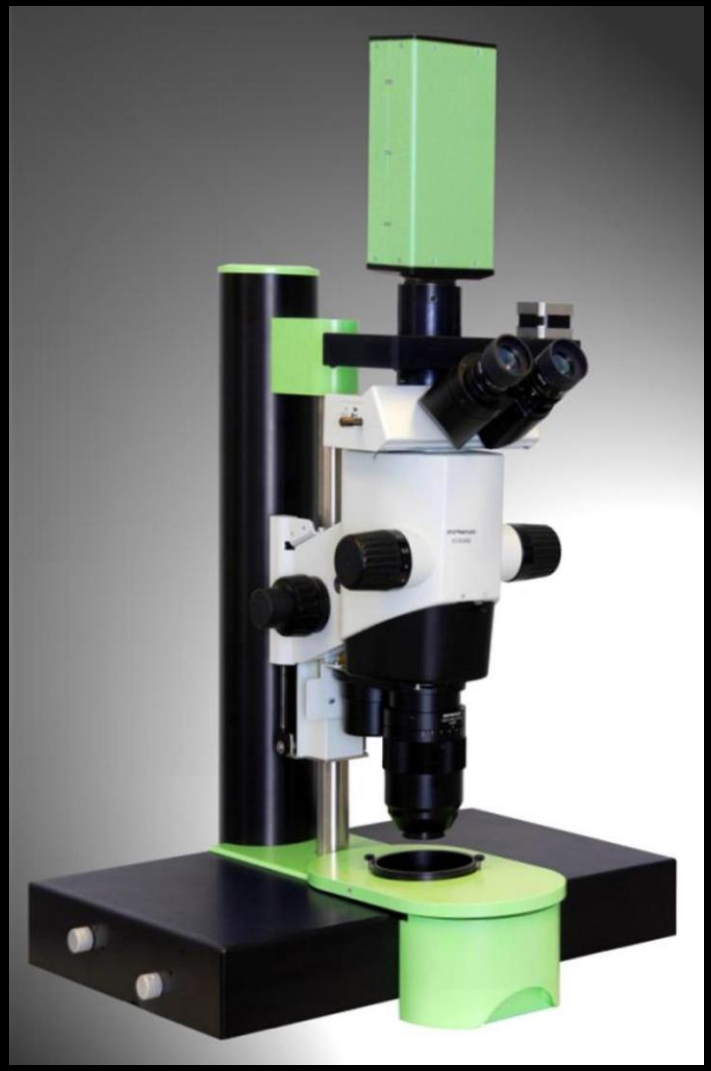
# Light Sheet Use

- Single plane (FCS...)
- Single stack
- Multiview stacks
- + Time

# Ultramicroscope

**Whole brain imaging at cellular resolution**
**3D high speed imaging without bleaching**
**High throughput phenotype screening**

**LAVISION BIOTECH**



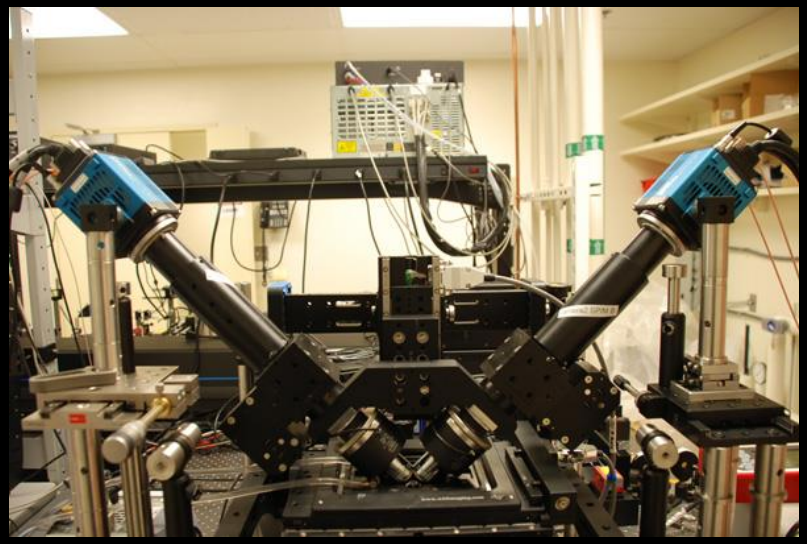
H. Siedentopf and R. Zsigmondy,
Ann. Phys. 10, 1-39 (1903)

# Light Sheet-based Fluorescence Microscope for long-term live imaging

- **Turn-key**
- **Compact**
- **Laser Safe**

Carl Zeiss Microscopy GmbH,
Olaf Selchow, BioSciences

Applied Scientific Instrumentation

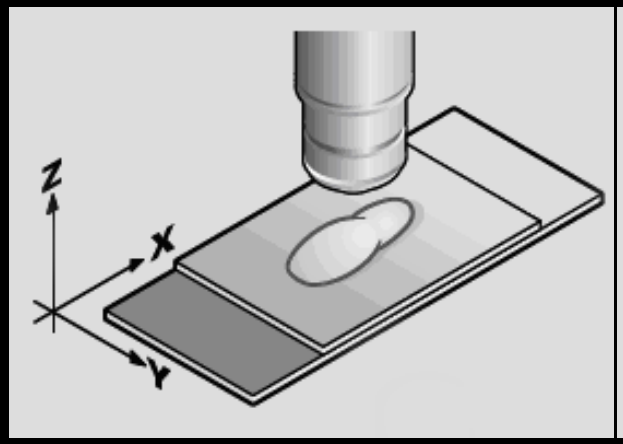


# Sample preparation

- The sample is a 3D object
- Different sizes and types of samples
- Mounting techniques
  - Embedded samples
  - Hanging samples
  - Flat samples
  - Enclosed samples
- Cell biology applications

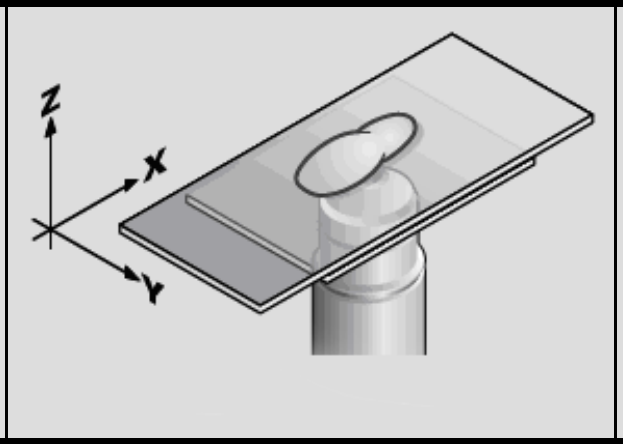
# The sample is a 3D object

Upright



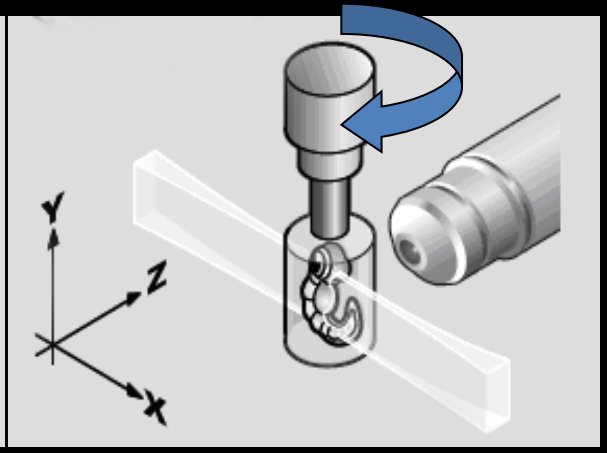

. Glass support
. Fixed orientation
. Fixative
. Mounting media
. Pressure…

Inverted



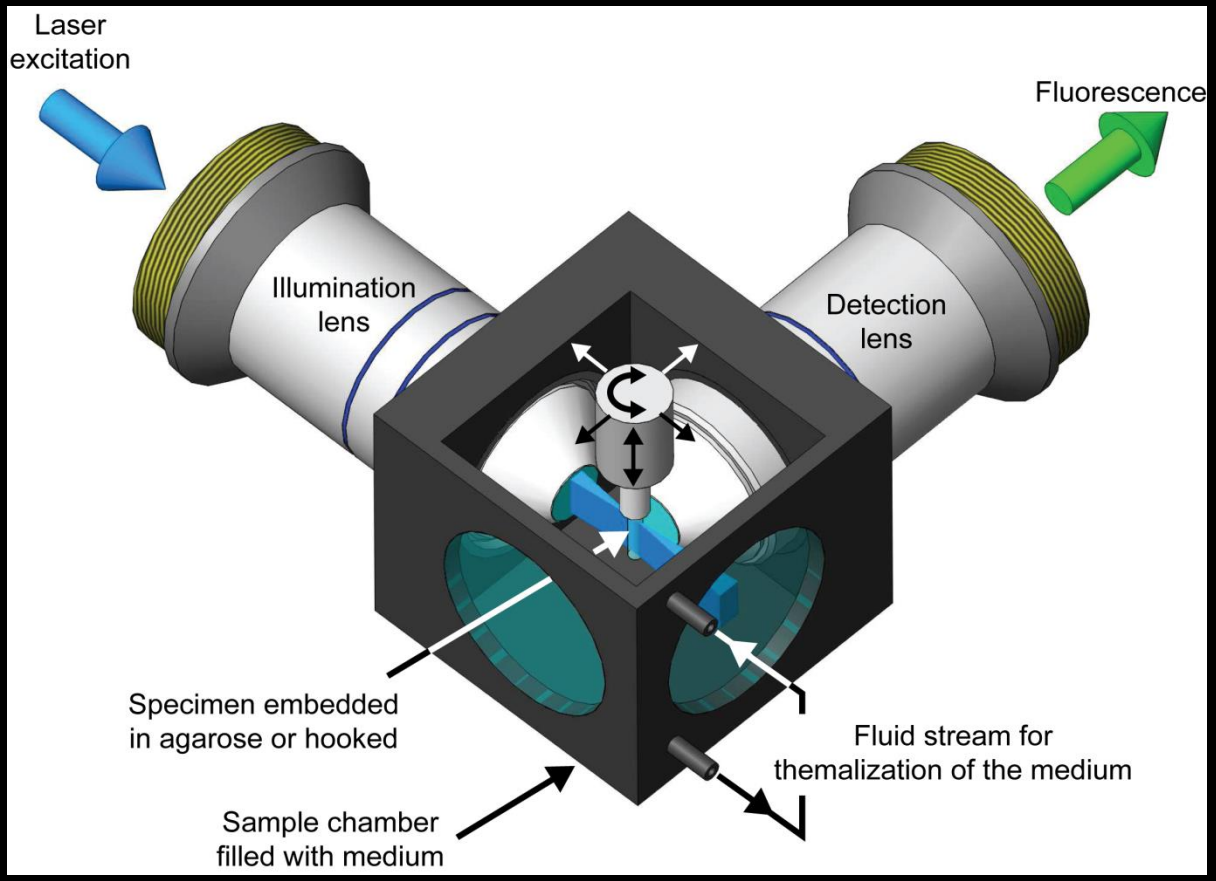


LSFM

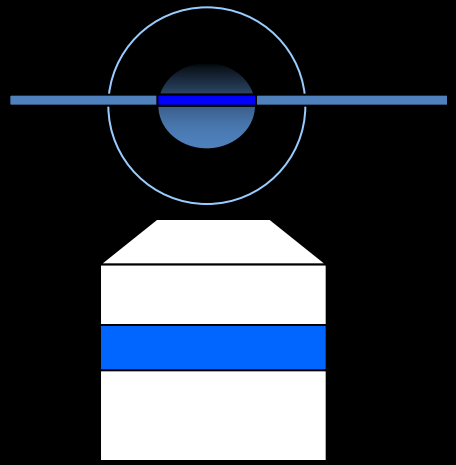



. Support from above
. Rotation
. Fixative
. Objective

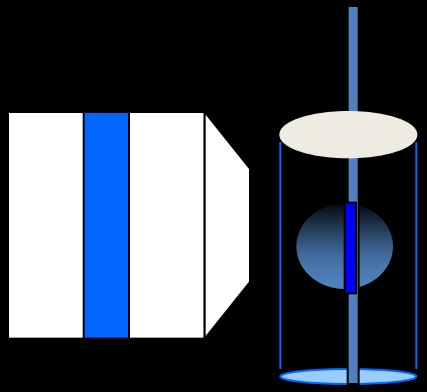
# LSFM specificities




TOP VIEW

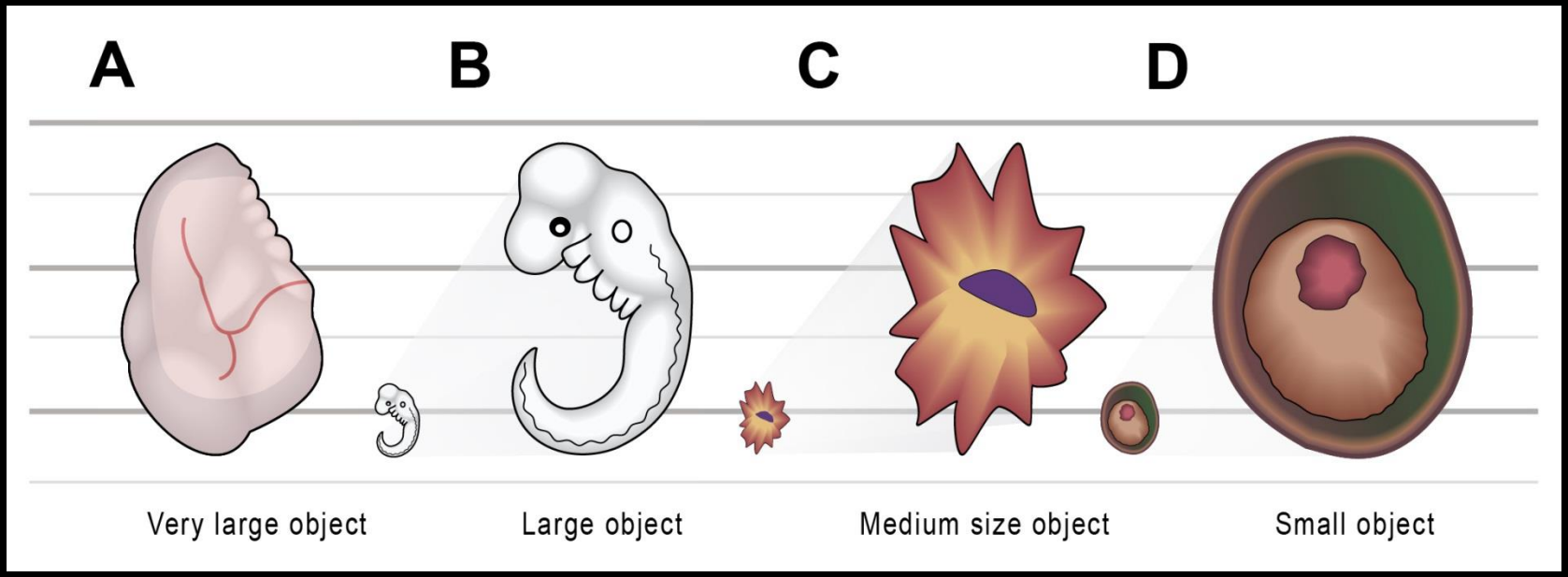


SIDE VIEW



-Wet environment (stability…)
-Light sheet (penetration)
-Objective (obstacle…)
- Hanging mounting (gravity…)

# Different sizes



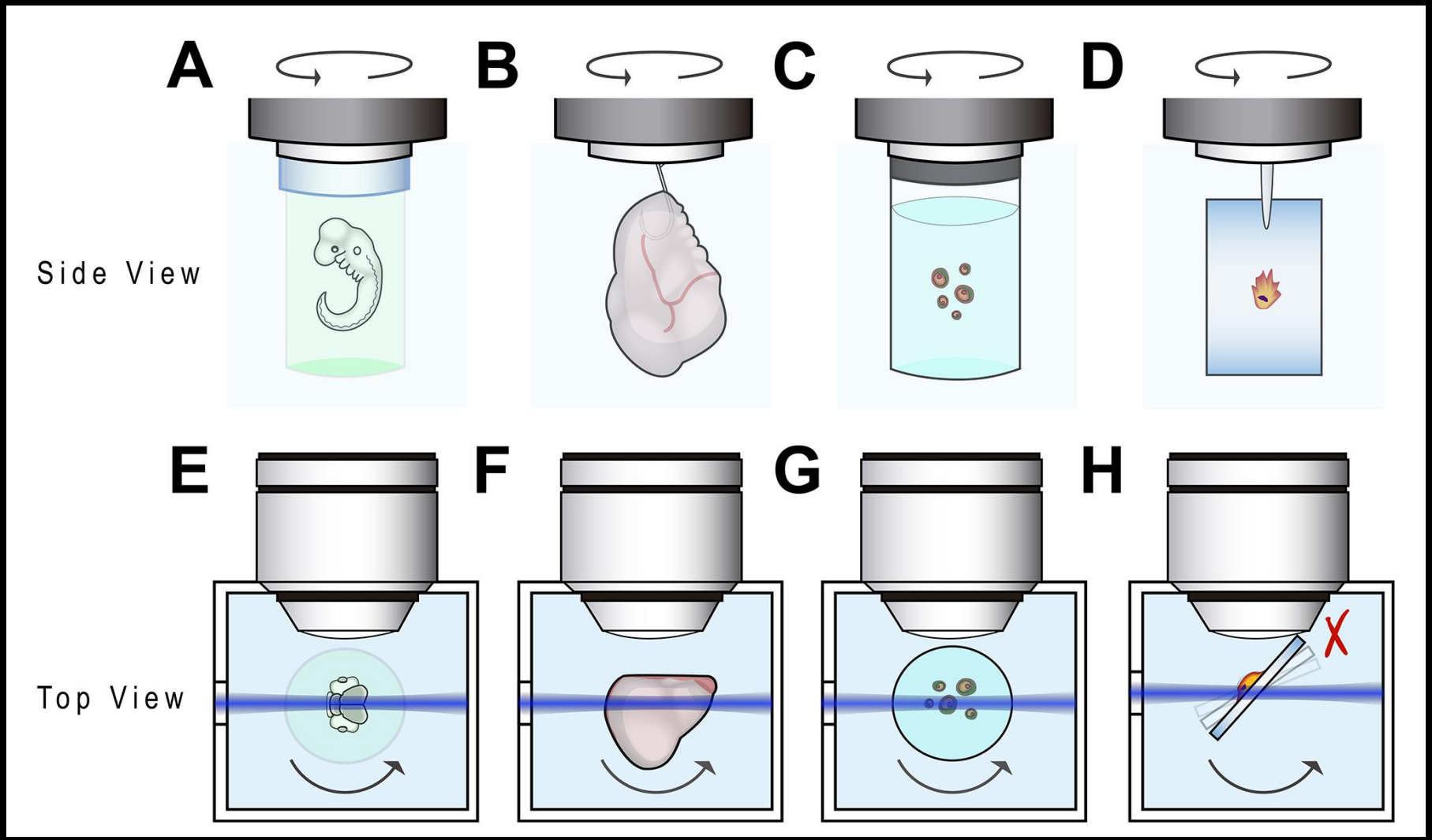
# Different sizes and types of samples

- 2.5X, 5X, 10X, 20X, 40X, 63X, 100X
- Large samples (mm)
    - *Mus musculus* (brain)
    - *Anopheles Gambiae…*
- Mid range samples (μm)
    - *Drosophila melanogaster* ovaries
    - Cell clusters, cysts…
- Small samples (μm to nm…)
    - *Saccharomyces cerevisae*
    - Single microtubules

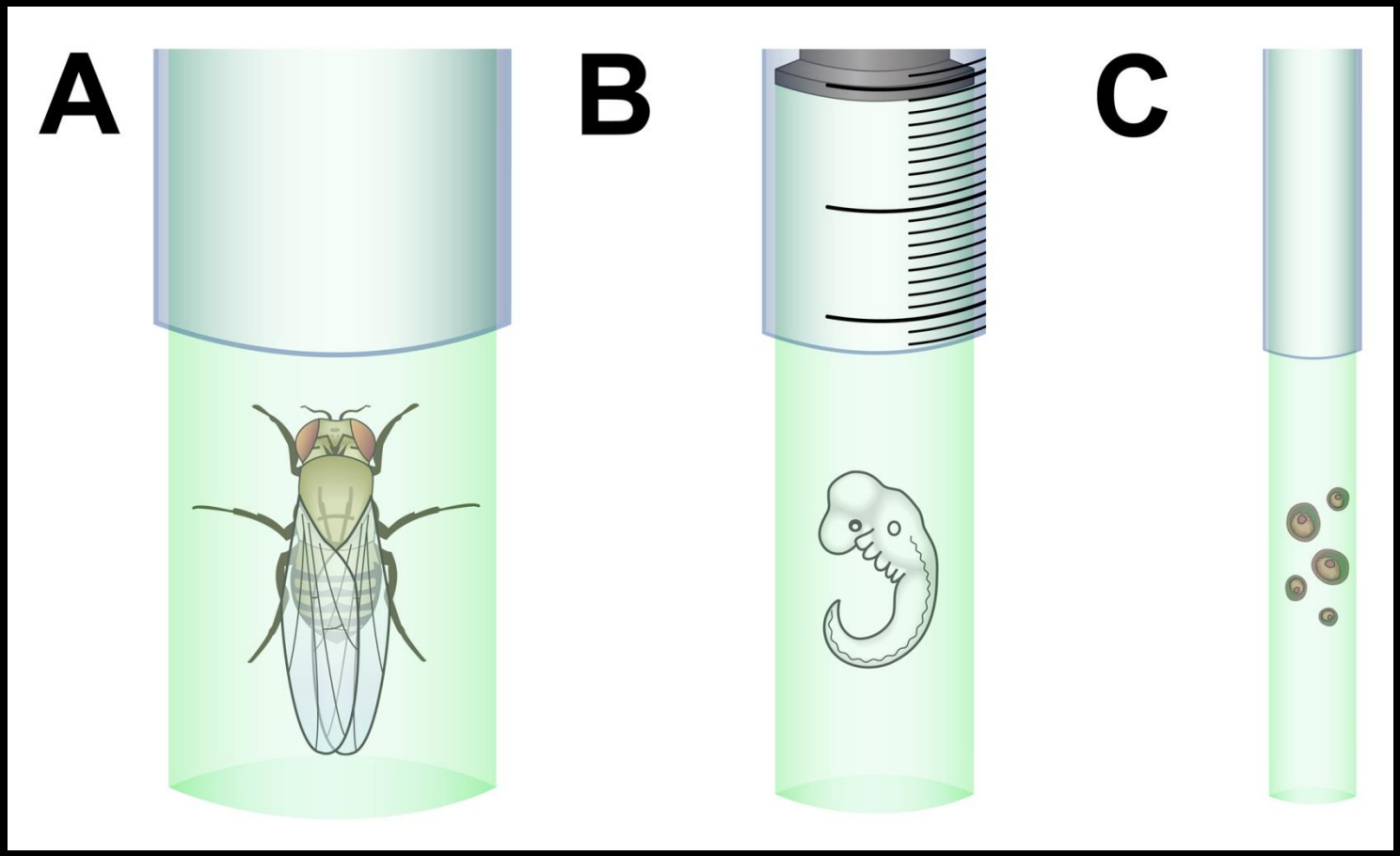
# A few more points…

- Media (Air, water, PBS, oil…)
- Fixed or alive (Temperature, pH, drugs, anesthesia…)
- Labelling (Dyes, diffusion, penetration, washing…)
- Clearing (Penetration…)
- Accesibility (Size ratio (chamber, objective)…)
- Time (Stability, movements…)

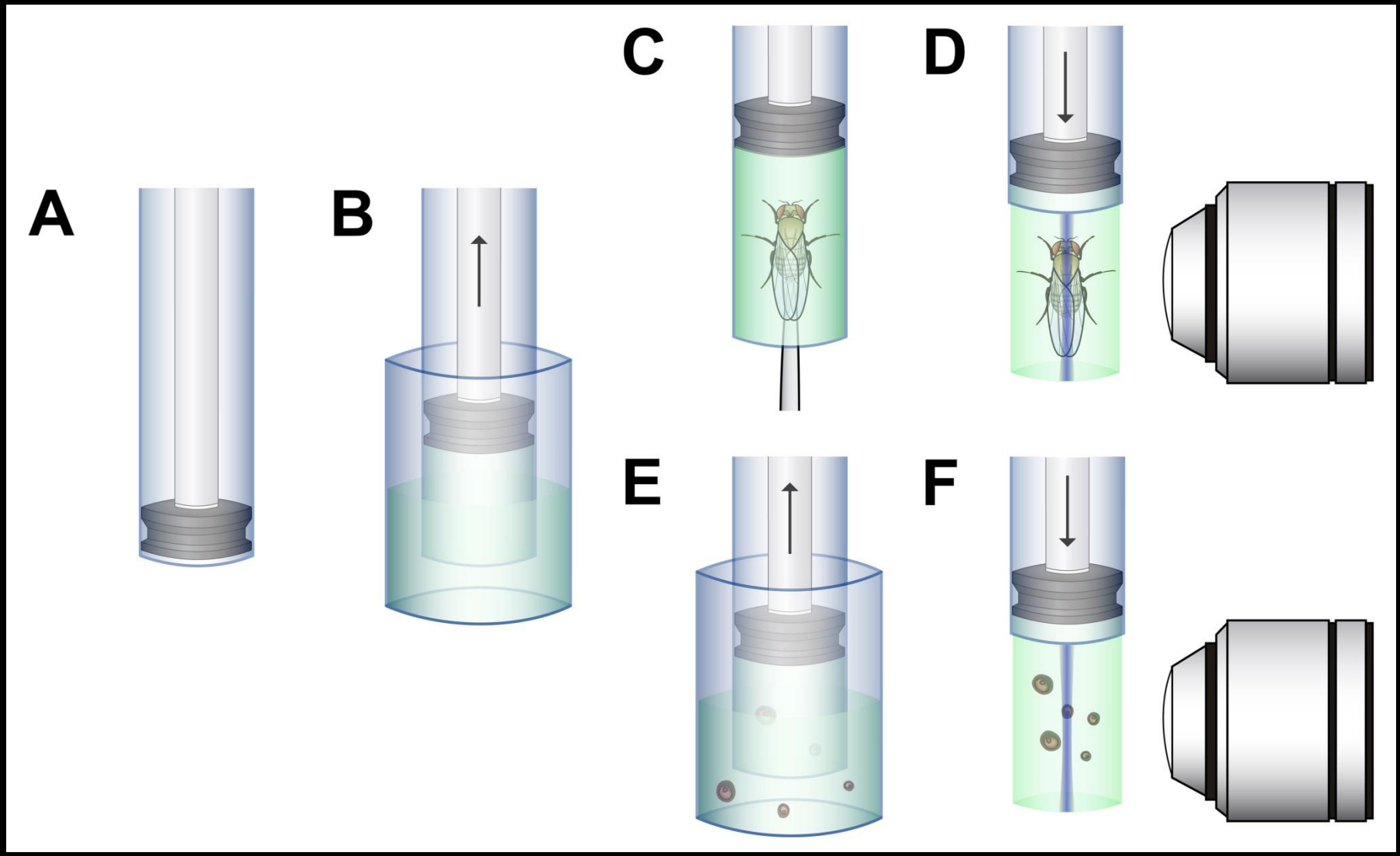
# Mounting techniques



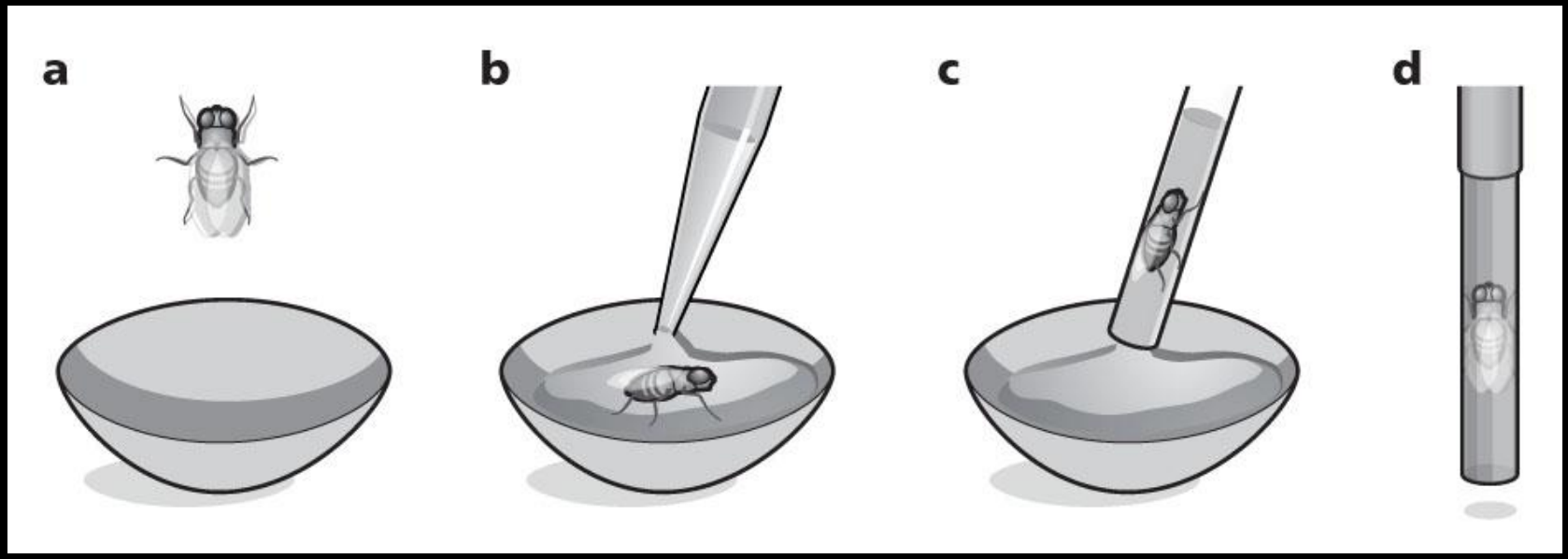
# Embedded samples



# Embedded samples



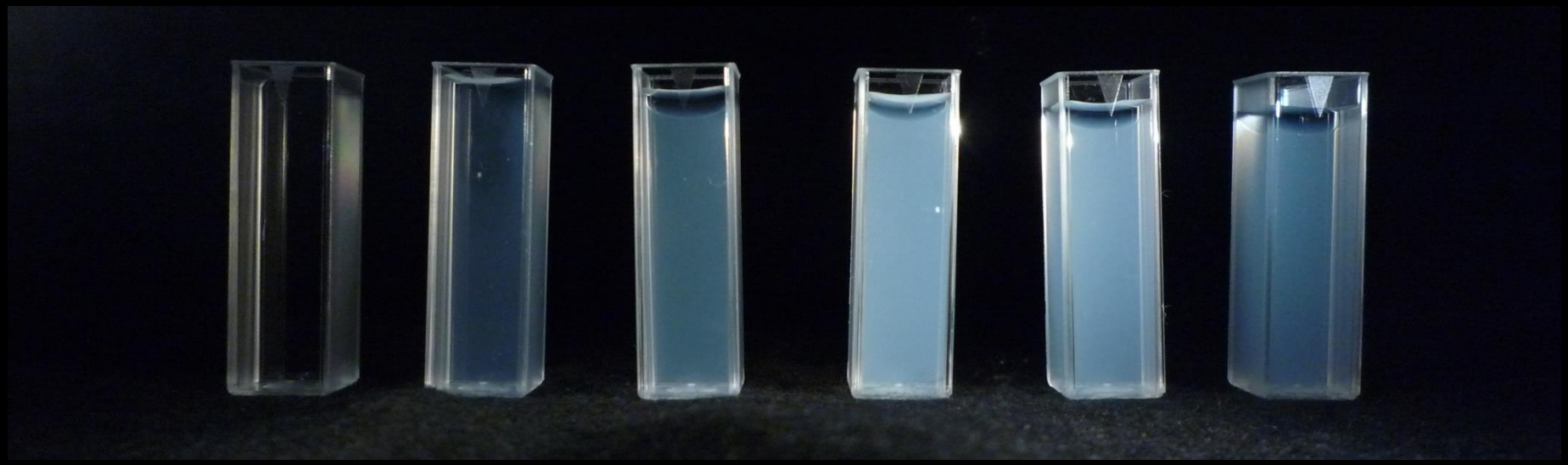
# Embedded samples

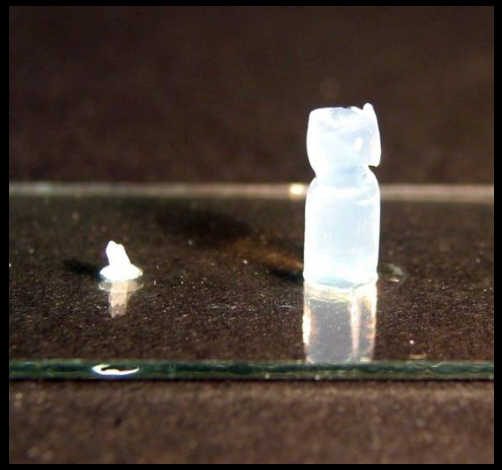



***d. melanogaster*  pupa mounting**

- dissection
- self alignment
- dissected organs, embryos…

# Gelling agent related problems





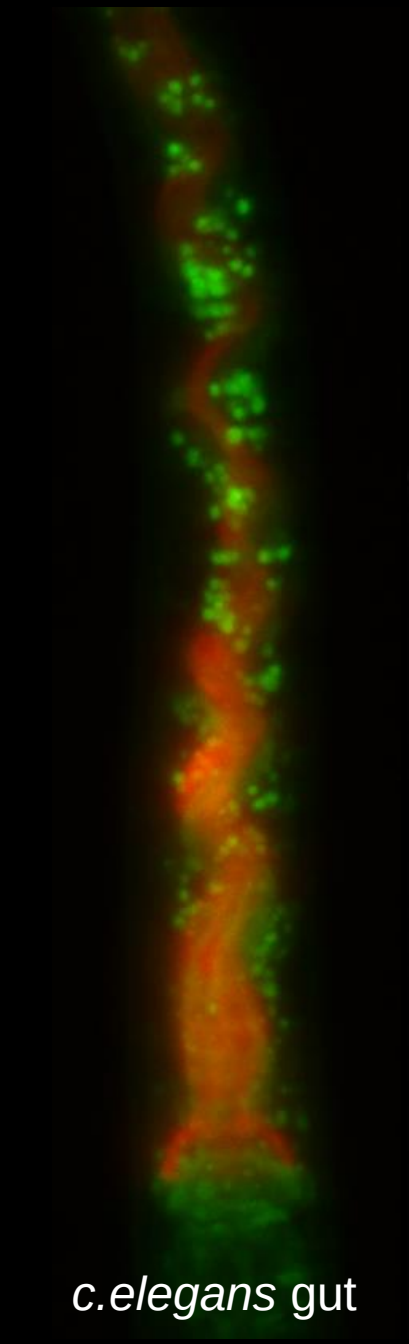
**-Optical properties**
- light sheet penetration
- detection limitations
- aberrations

**-Gel properties**
- Ashes
- Structure
- Diffusion

**-Physical properties**
- melting temperature
- gelling temperature
- air drying
- gelling stability

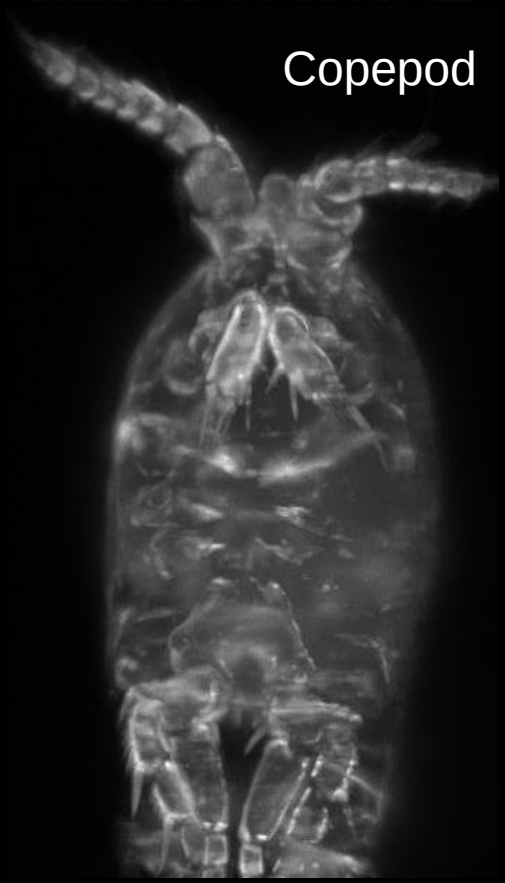
**-Preparation**
- purity
- air content
- homogeneity

# Embedded samples

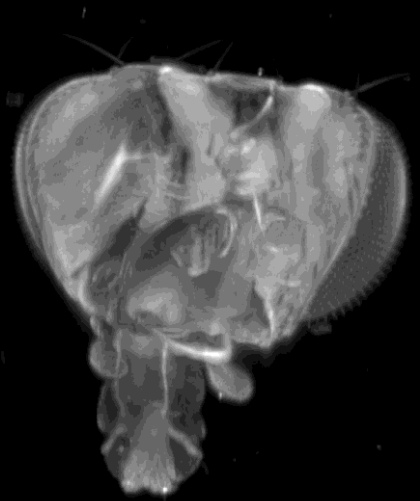


*c.elegans* gut

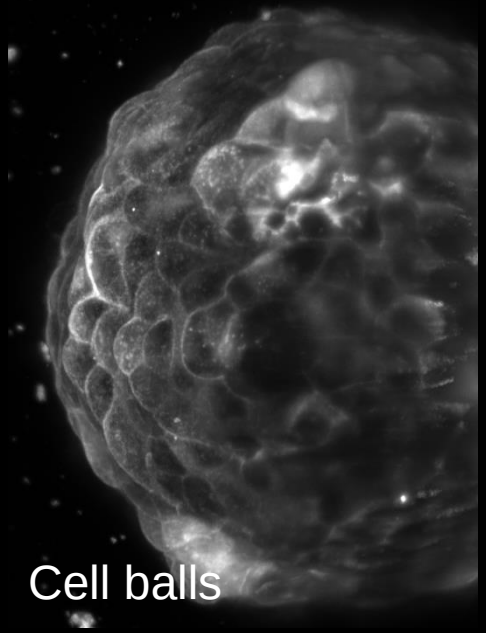
*S.cerevisiae*



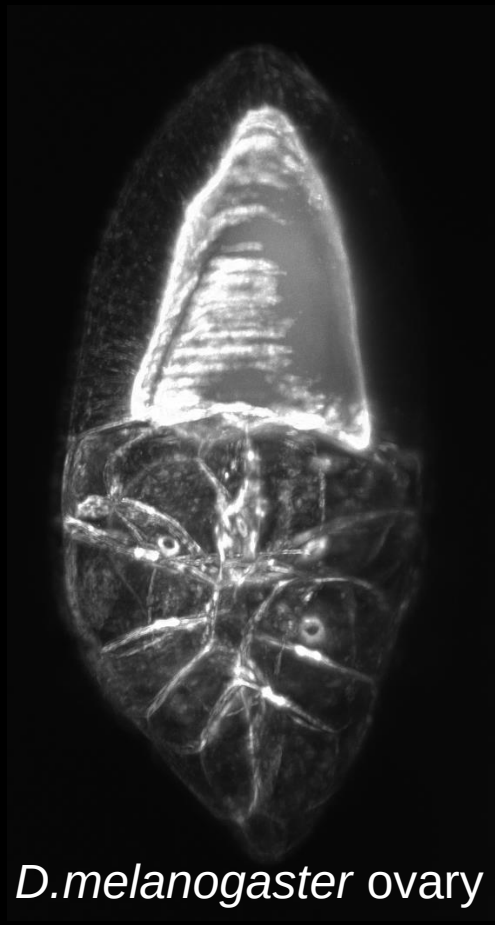
Copepod



*D.melanogaster* head

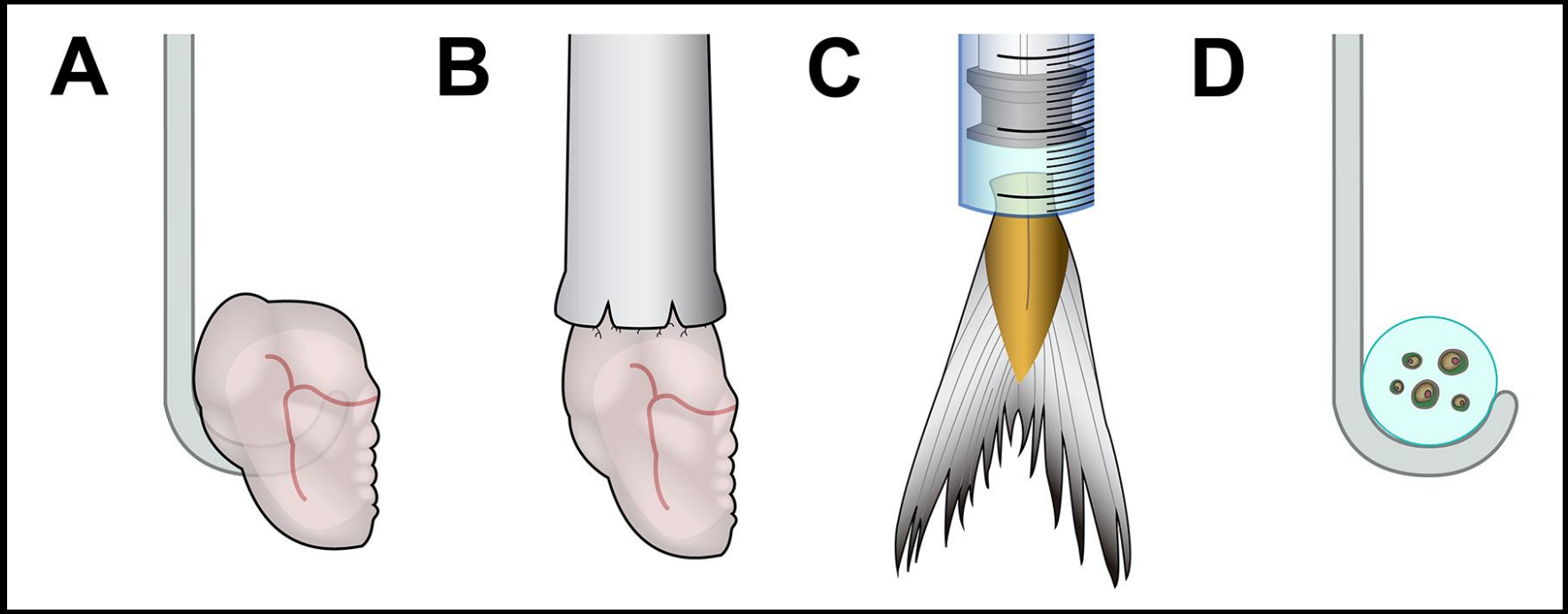


Cell balls

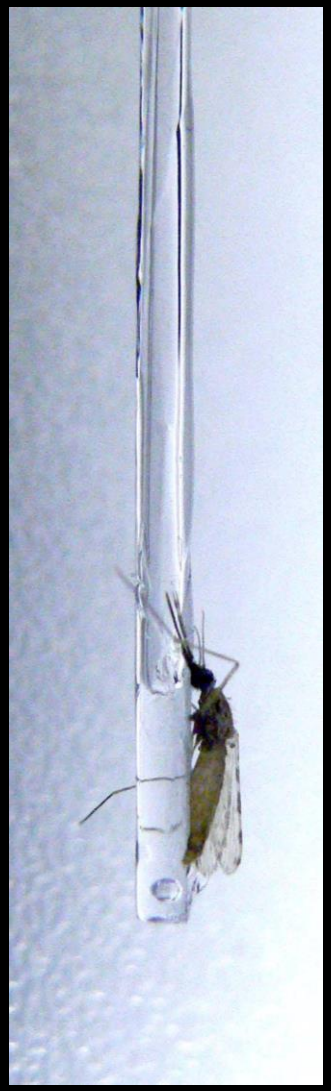


*D.melanogaster* ovary

. Fish embryos
. Cell cysts
. Spheroids
…

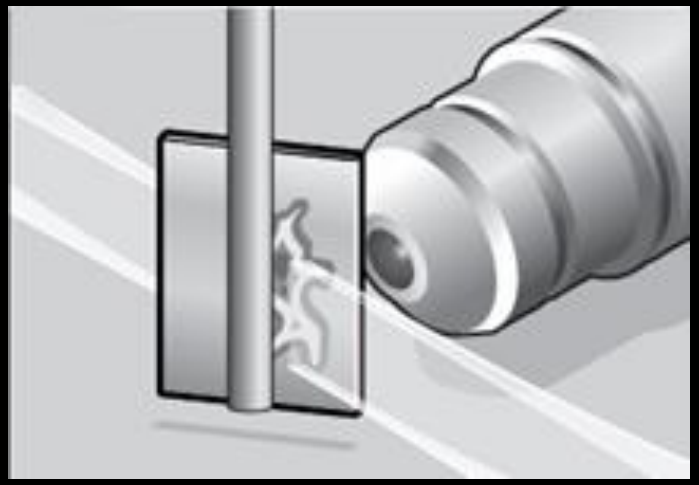

A
B
C
D

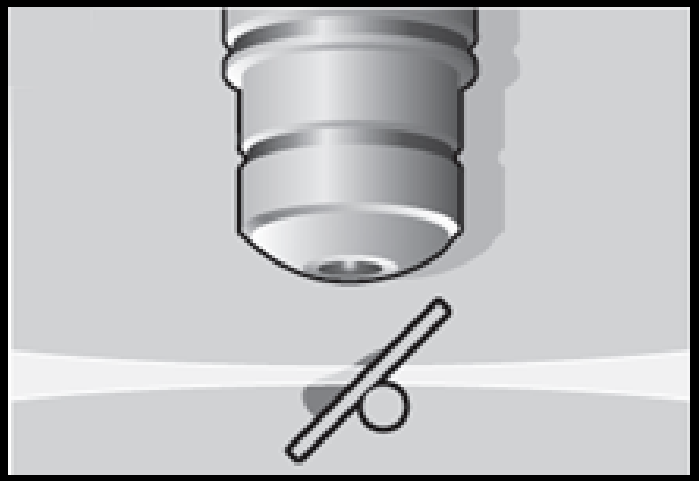
# Hanging samples



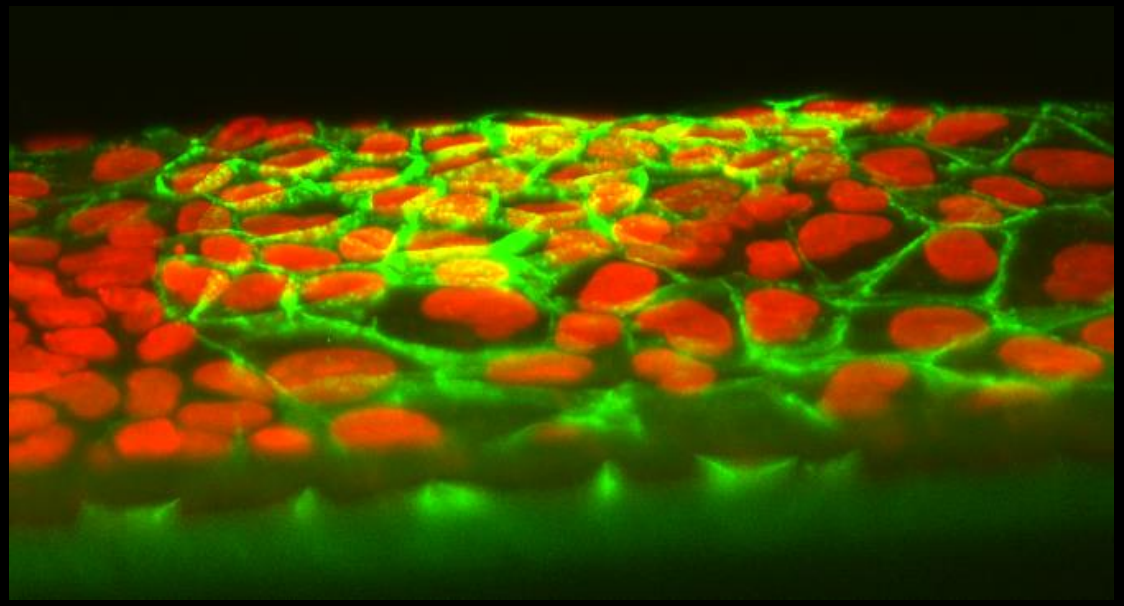
- Stability (rotation…)
- Contact
- Accesibility
- Live samples?

# Flat samples

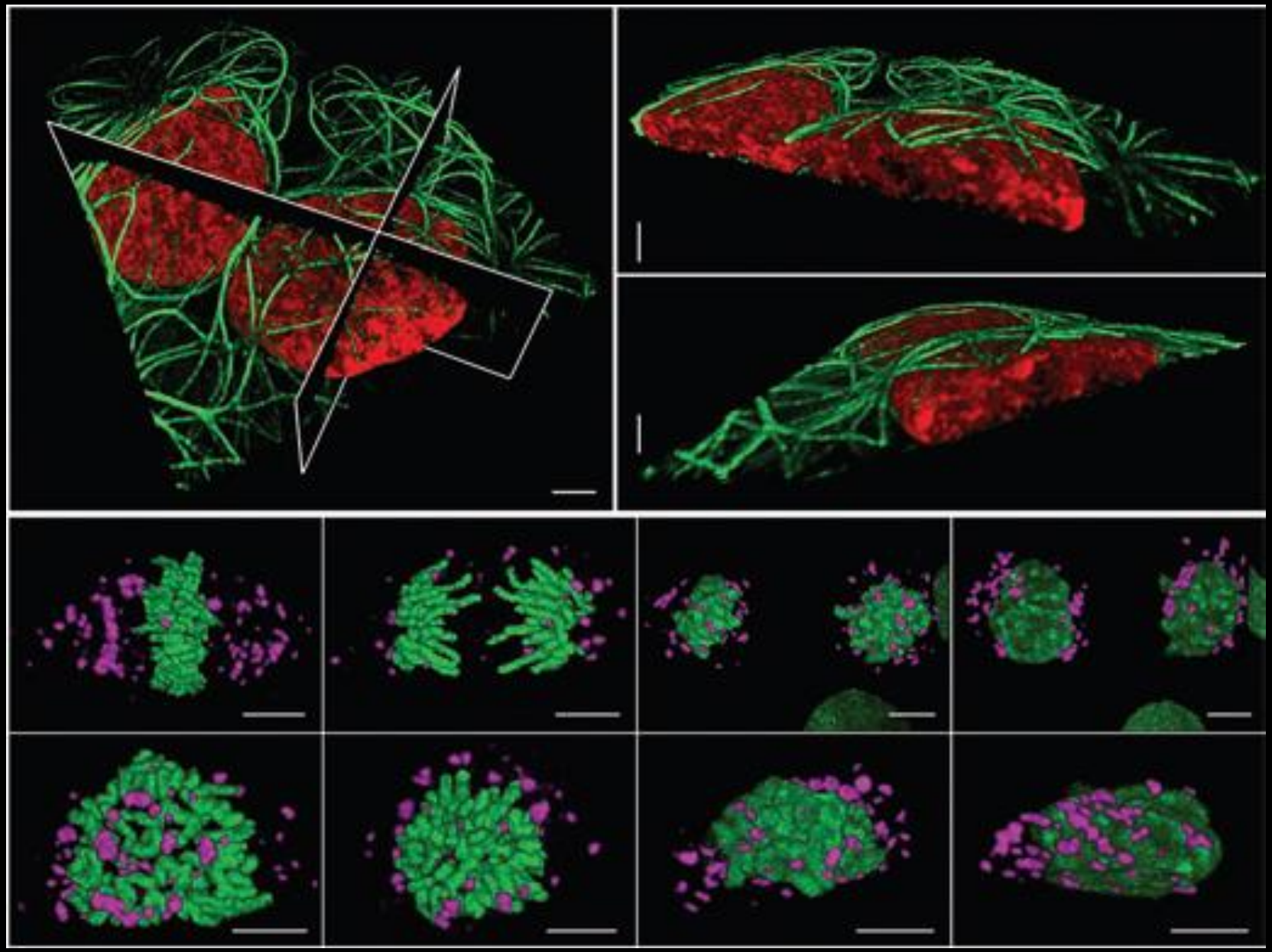




- Accesibility
- Rotation
- Contact

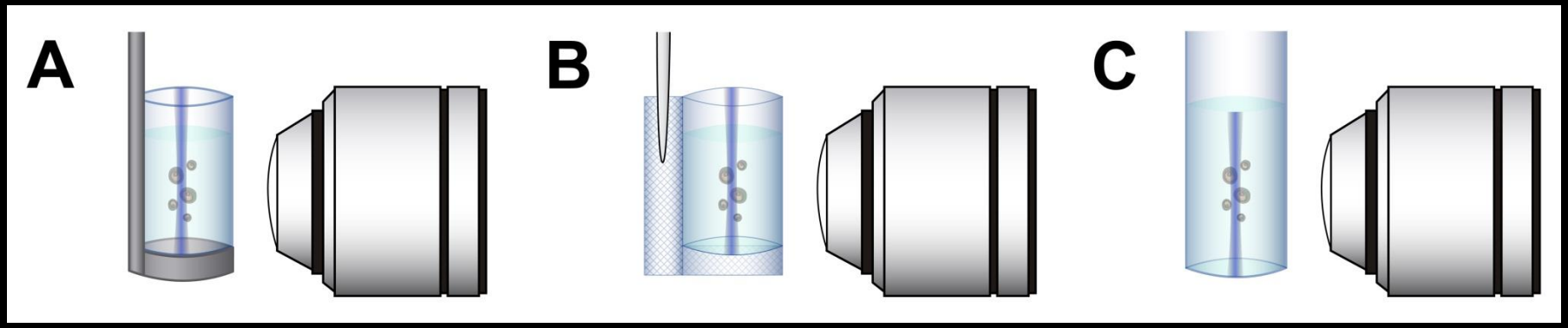


# Flat samples

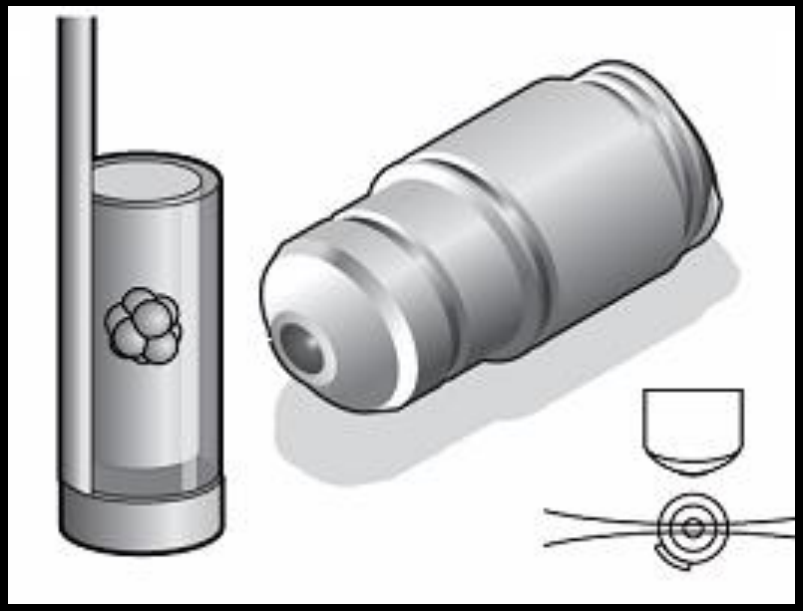


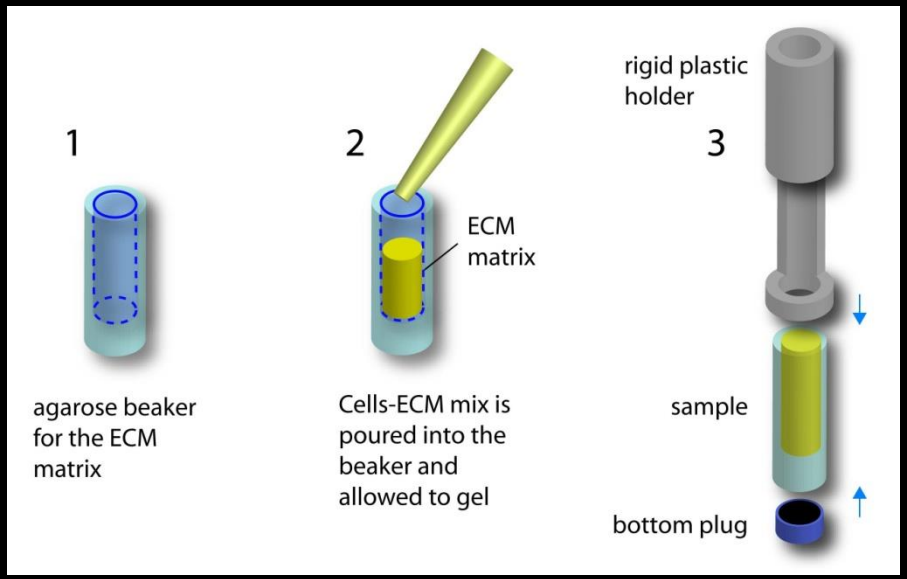
Planchon et al., 2011

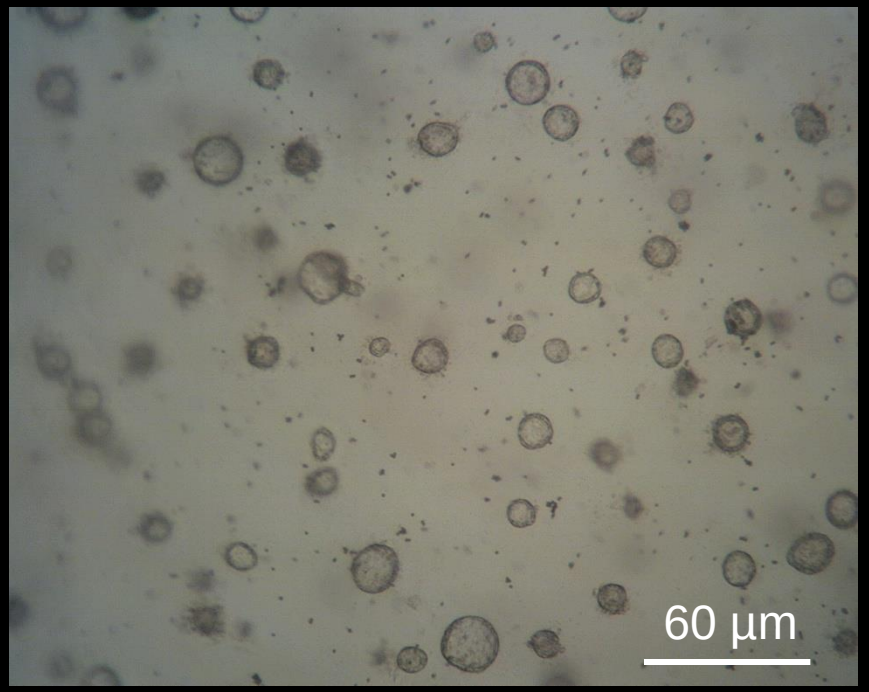
# Enclosed samples



# Enclosed samples

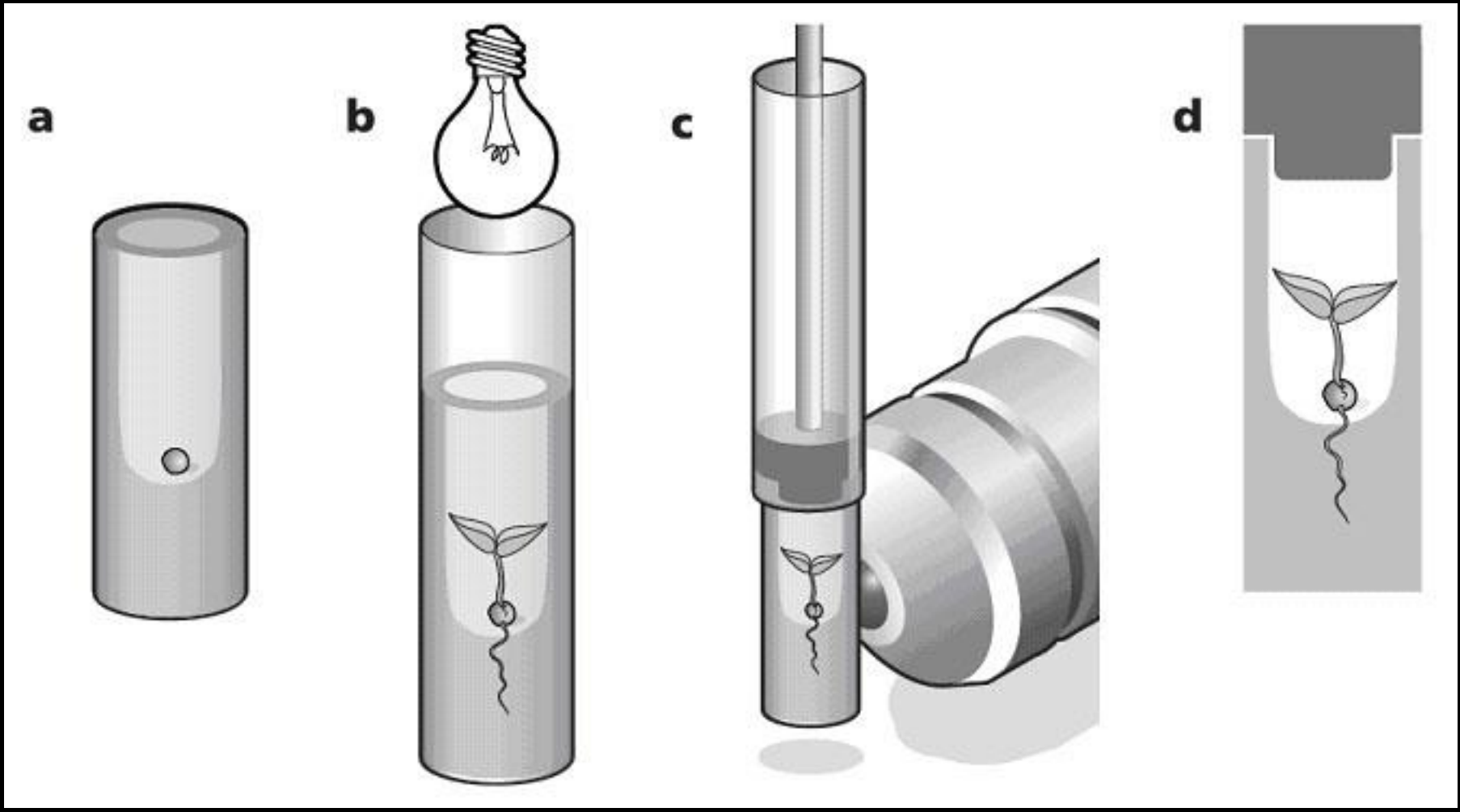





MDCK cells cultured for 10 days in matrigel, inside an agarose beaker within Matrigel (37°C, 5% $CO_2$)

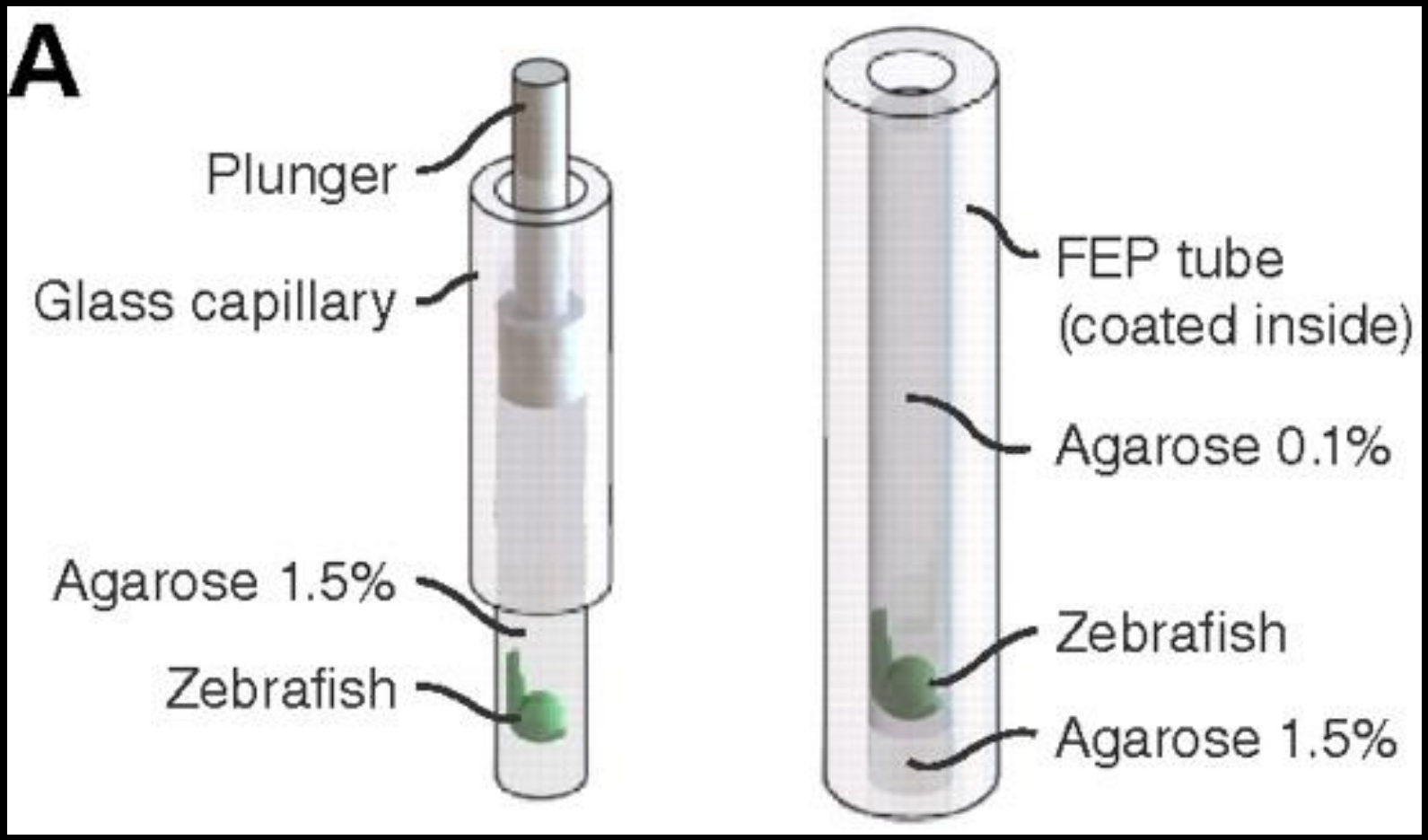
# Enclosed samples




**A. thaliana**

- development and growth
- stability

# Enclosed samples




Kaufmann, A et al, Development 139, 3242-3247 (2010)

# Enclosed samples

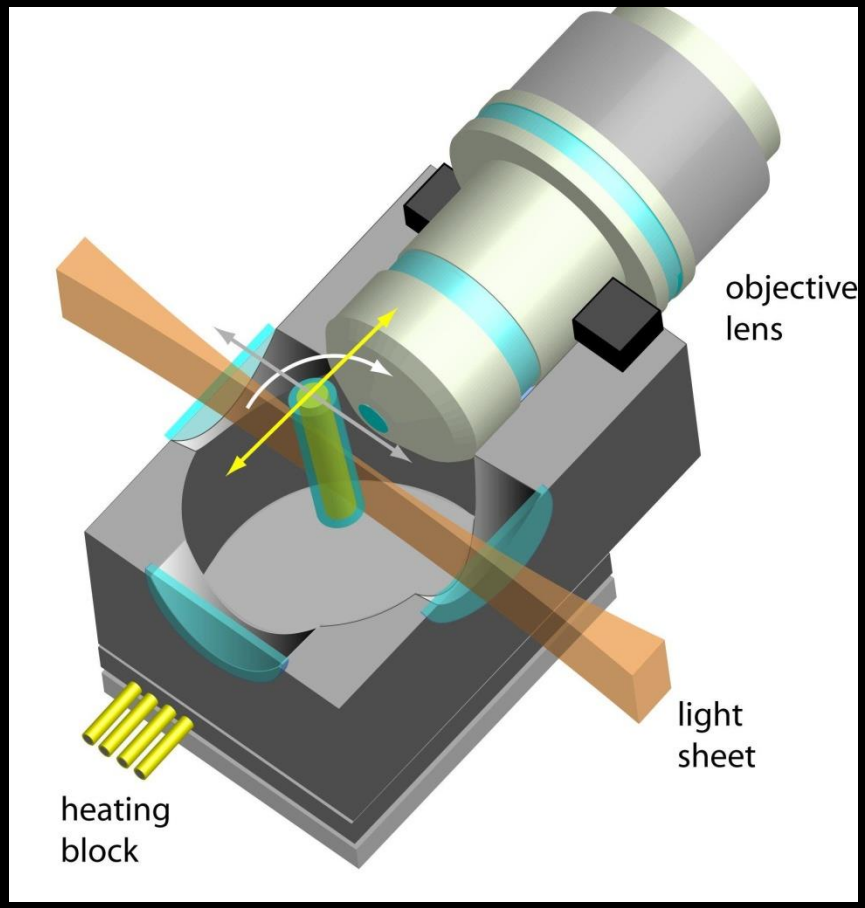
- Diffusion
- Accesibility
- Rotation
- Assays

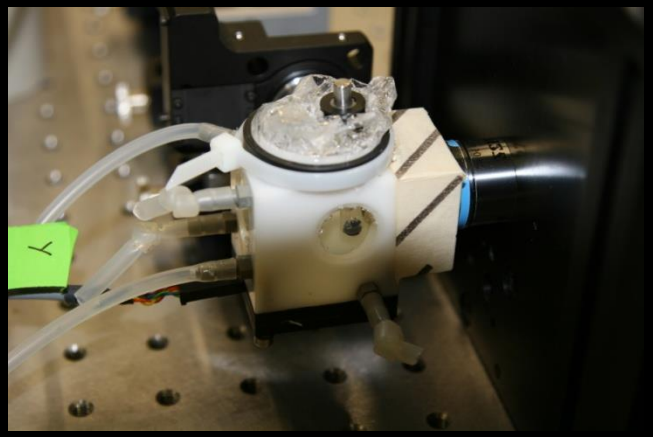
Keller et al, Nat. Meth, 2007

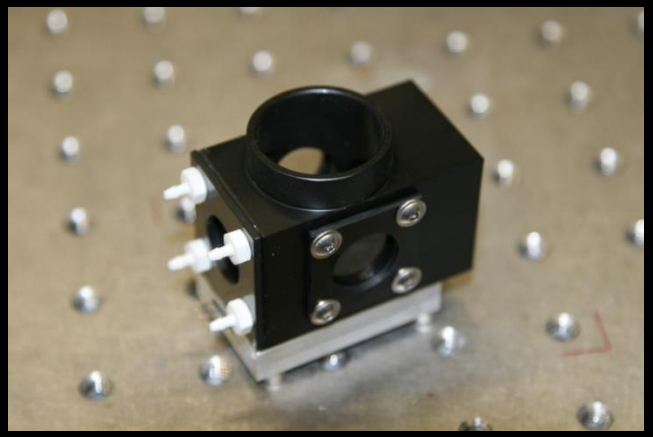
# Cell biology applications

- Three dimensional growth conditions
- Support (scaffolds, matrices)
- Protocols
- Imaging fixed and live cells
- 3D culture chamber

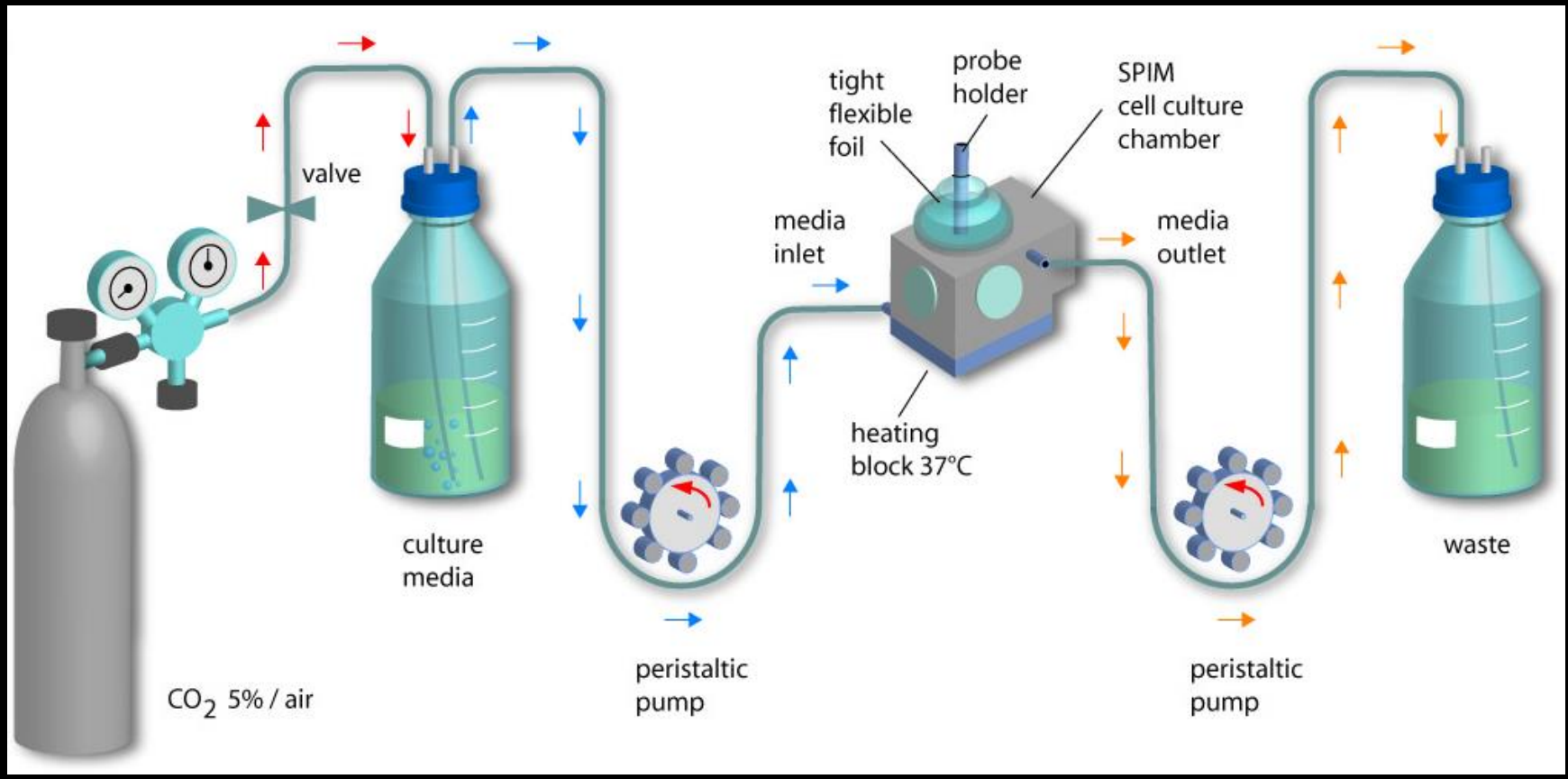
# SPIM-compatible perfusion chamber



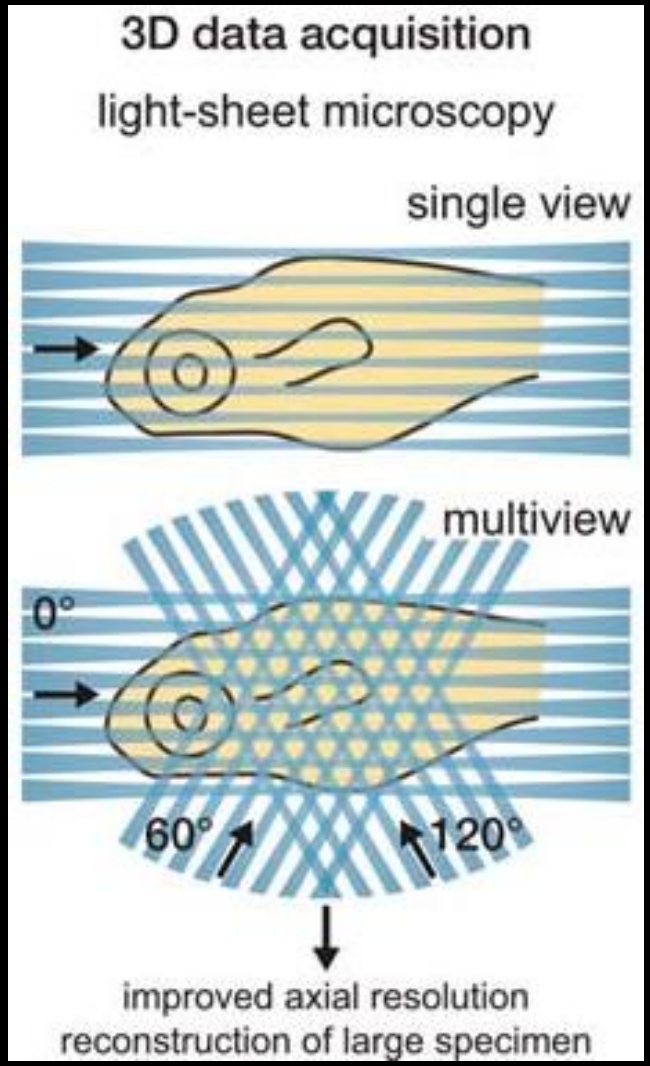


# Environmental control of the 3D culture



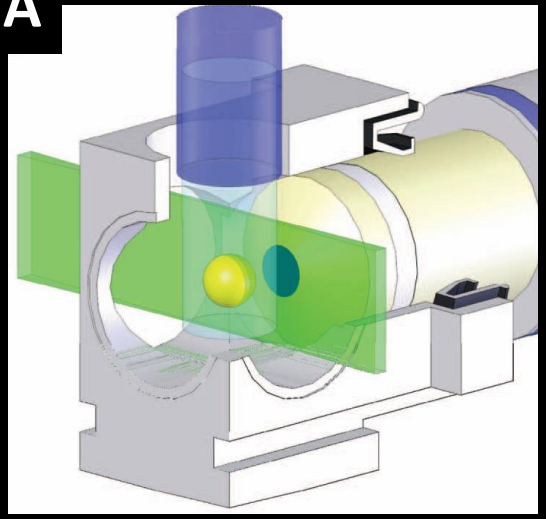
# Image processing

- Stacks...
- Multiple stacks...
- Channels
- Time, gravity and movement
- Data deluge
- Registration
- Fusion
- Add-ons
- Artefacts (stripes, blurring)

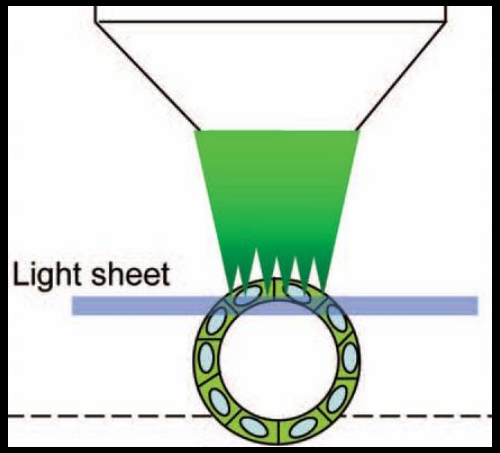


# Image processing

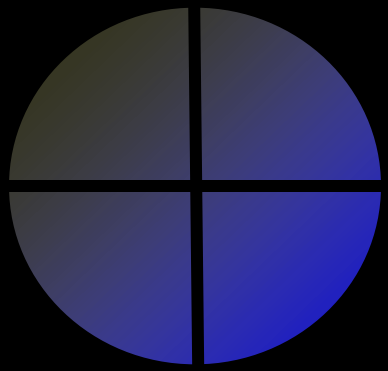
A



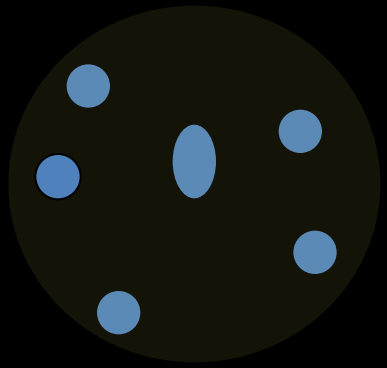
B



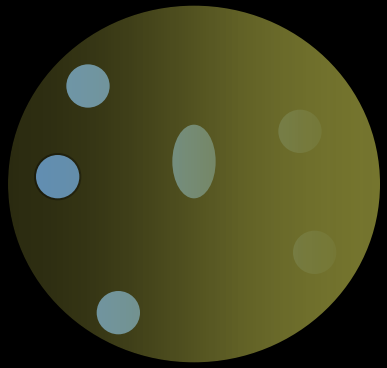

C



D



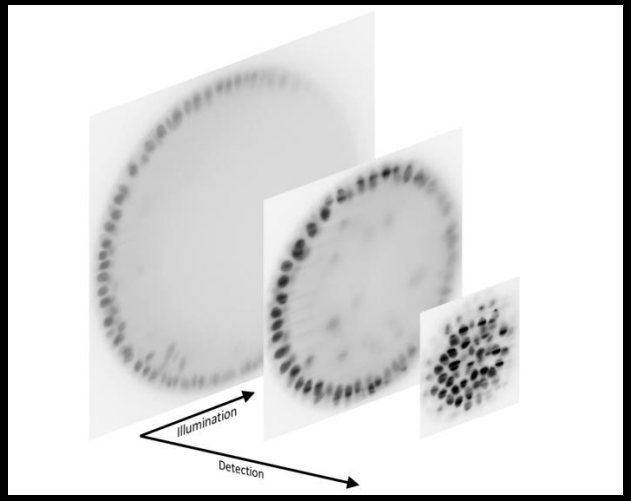
E



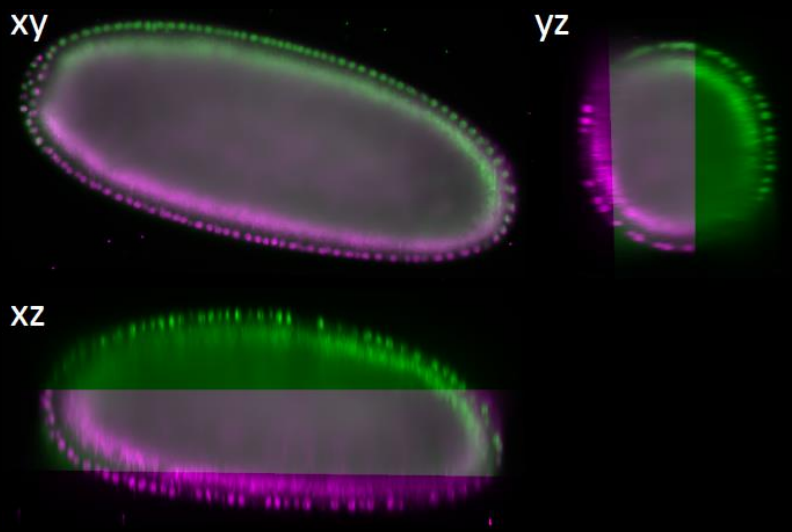
F

G

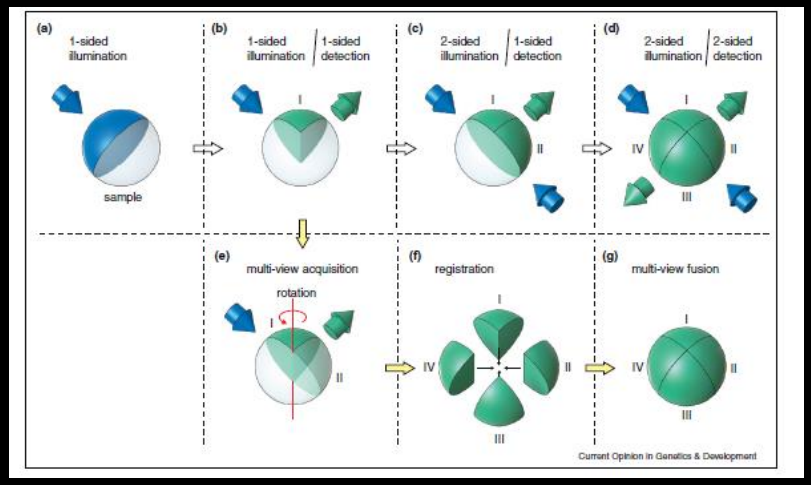
# Image processing




1. Signal Degradation

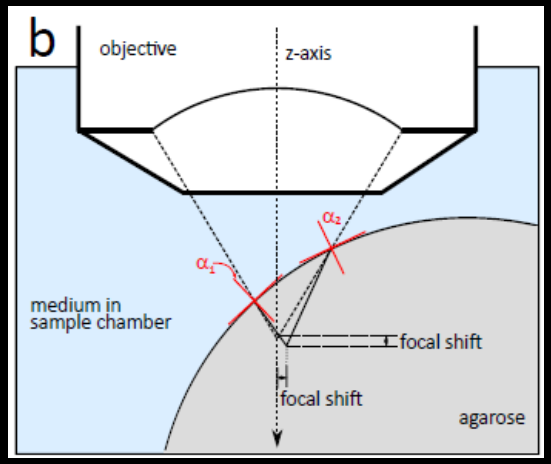



2. Limited Overlap




3. Varying orientations of the optical sections

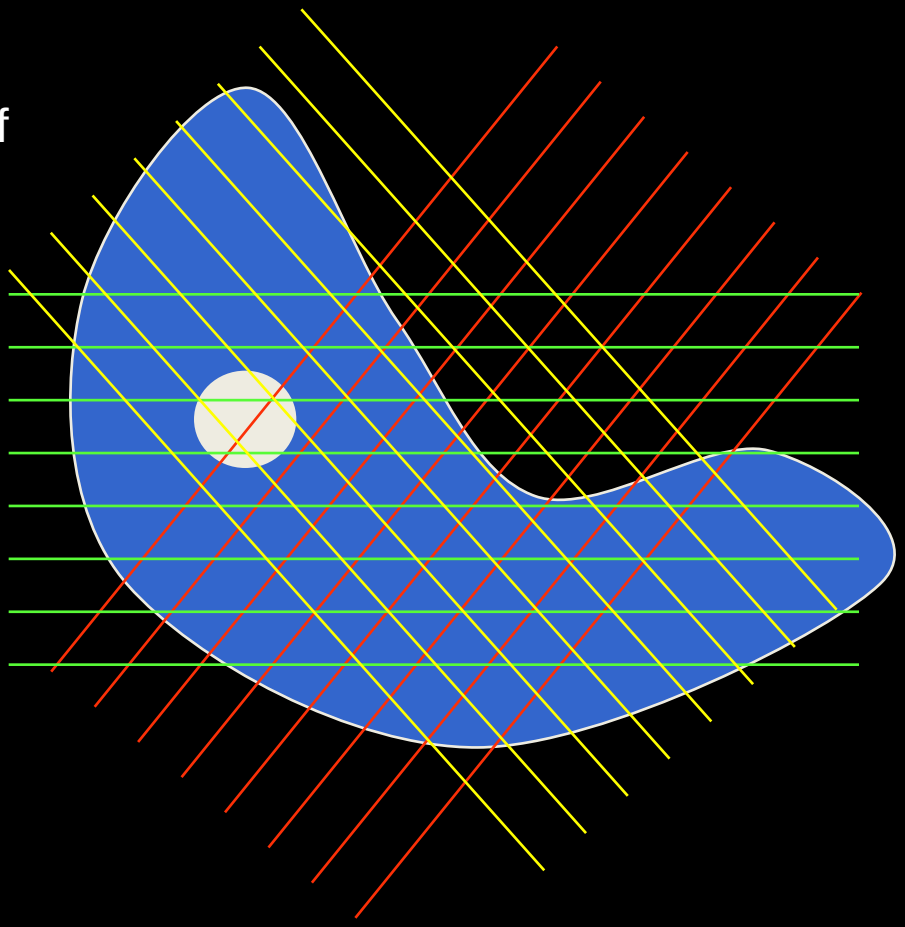
4. Development of the specimen




5. Scaling introduced by refractive index change

# Multi-View

- opaque/complex sample
- rotation to access ROIs
- compensate absorption
- combination allows reconstruction of details invisible in single stack, but require huge amounts of data (up to several hundred GByte) and heavy processing

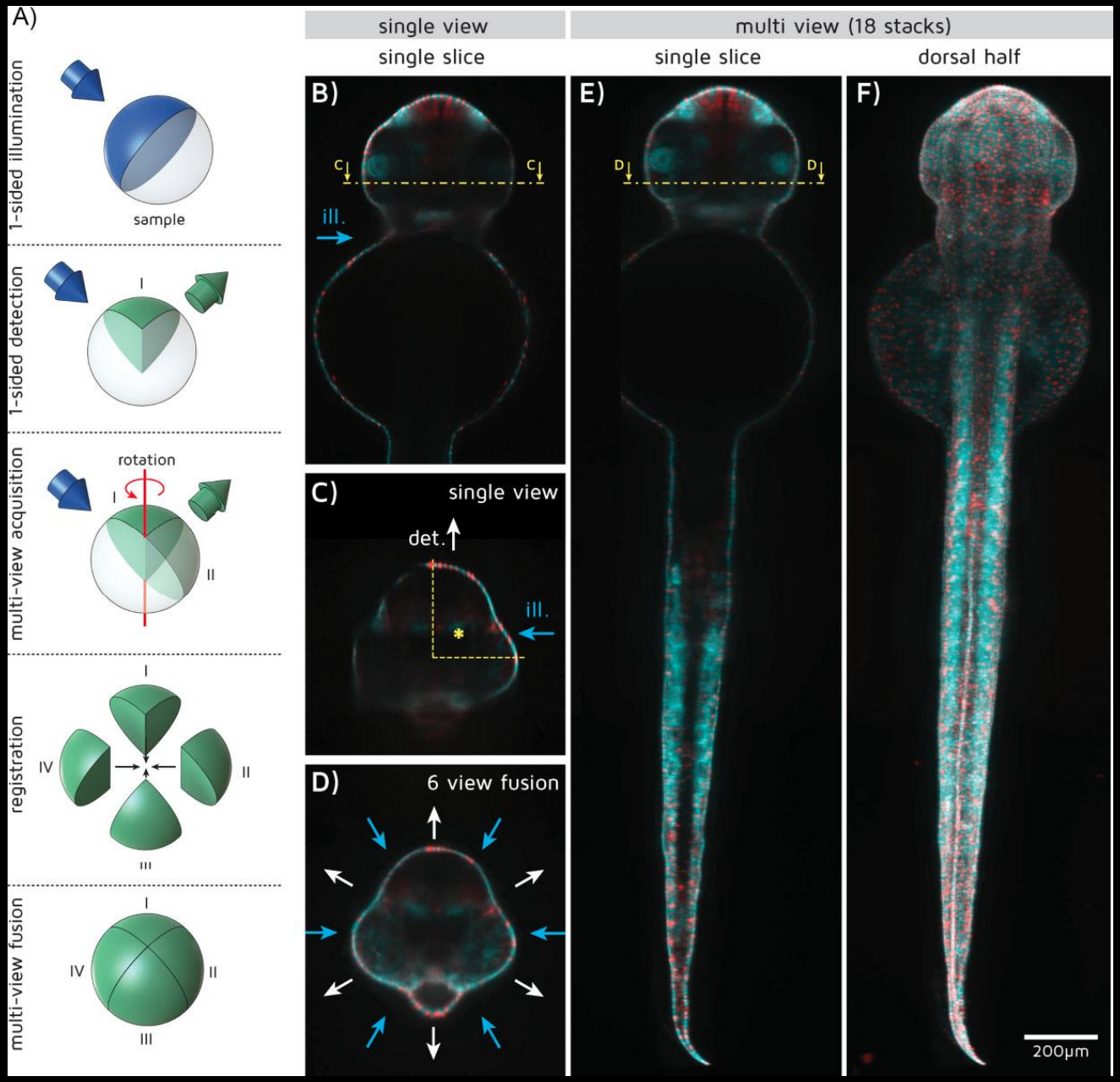


# A Drosophila experiment…

- Multi-View acquisitions
- Multi-Channel acquisitions
- Time-lapse acquisitions
  - Preview
  - Compression?
- Acquisition speed is very high over 24 hours
  - 5-50 fps @ 1 Megapixel (soon 4-8 Megapixel)
- 0,5-4 TB raw data per experiment
  - 100s of terabytes of data

**Welcome to Big Data Land!!!!**

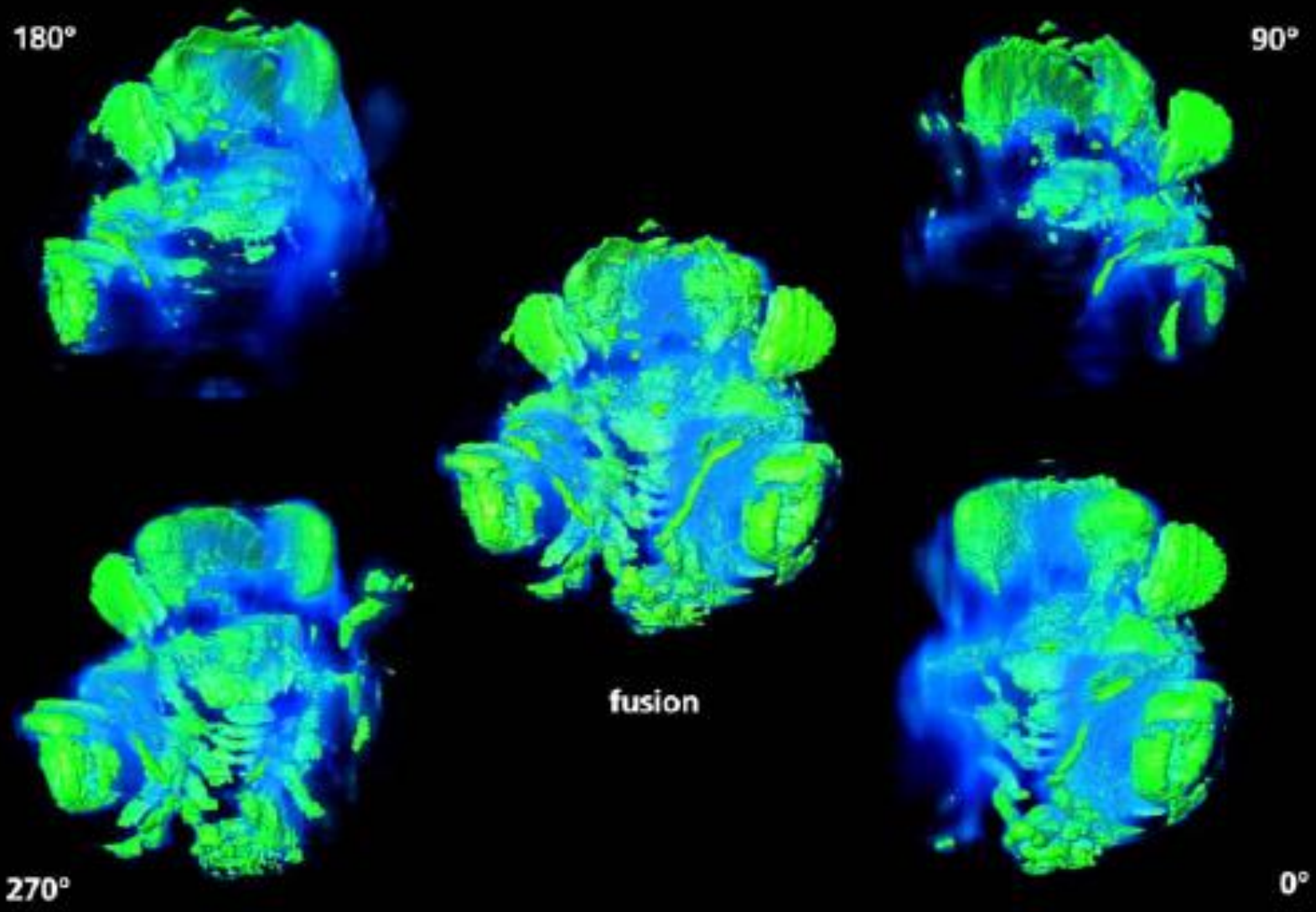
# Multi-View



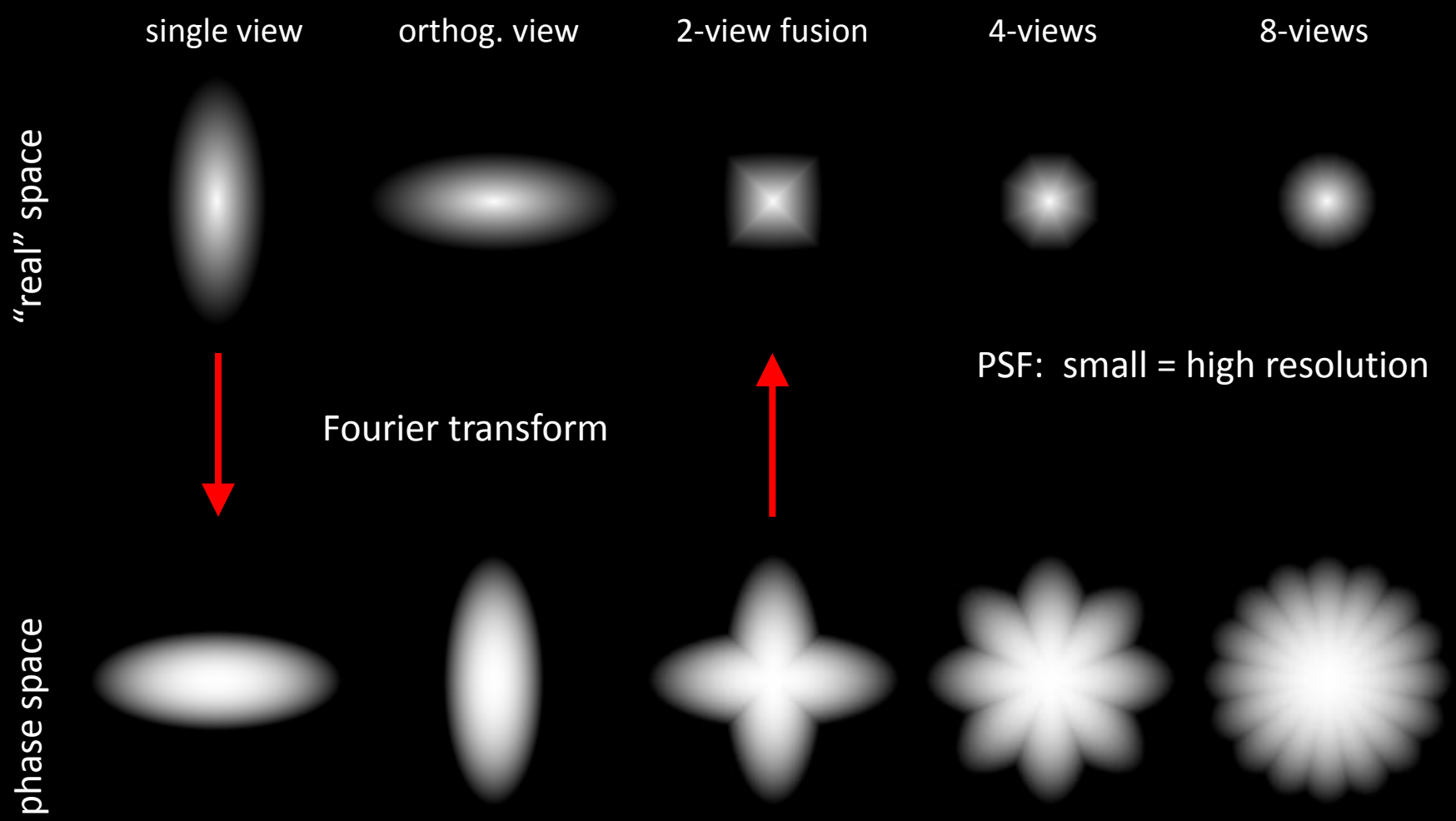

Nuclei in red
Membranes in cyan

Jan Huisken
Bioessays 34: 406–411

# Examples of recombination



# SPIMage fusion



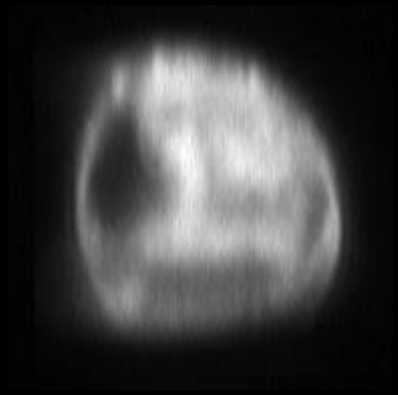

**Swoger, Huisken & Stelzer, Opt Lett, 2003**
**Verveer, …, Stelzer, Nature Methods, April 2007**
**Swoger, … & Stelzer, Opt Expr, 2007**

# SPIMage processing: pollen

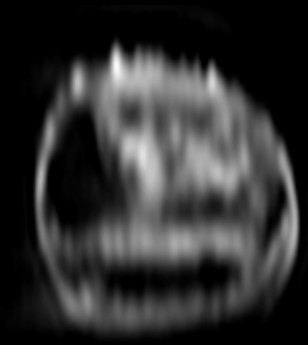
Autofluorescence of a Paper Mulberry pollen

Slices from 3D data sets

Figure by J. Swoger.



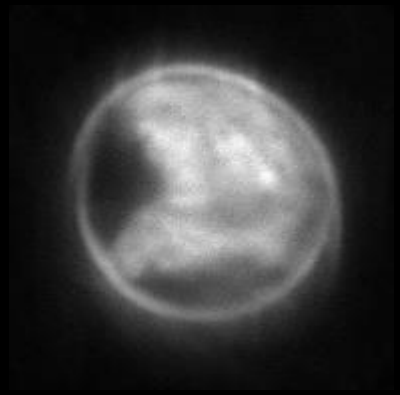
Raw, single view



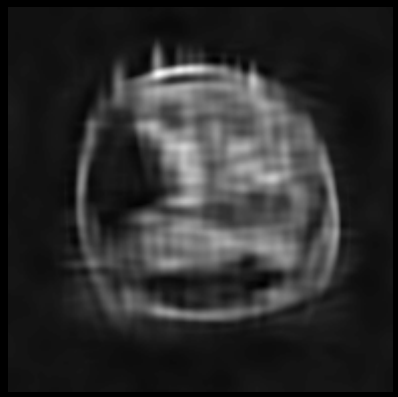
Deconvolved, single view

Images are

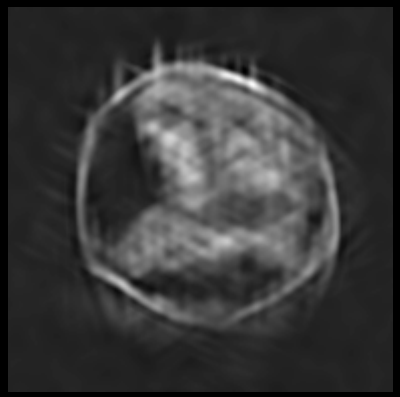
~ 20 μm square



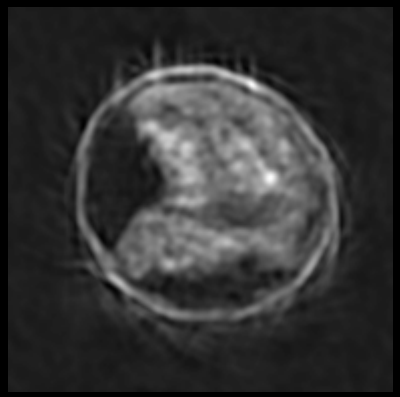
Fusion, 18 views (no deconvolution)



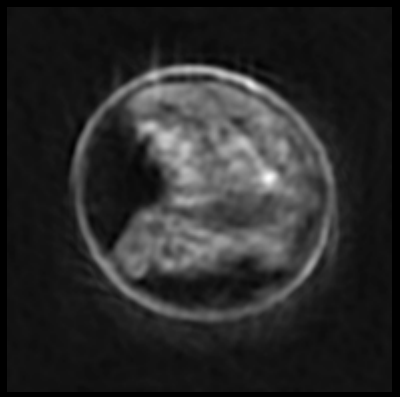
Fusion, 2 views



Fusion, 3 views



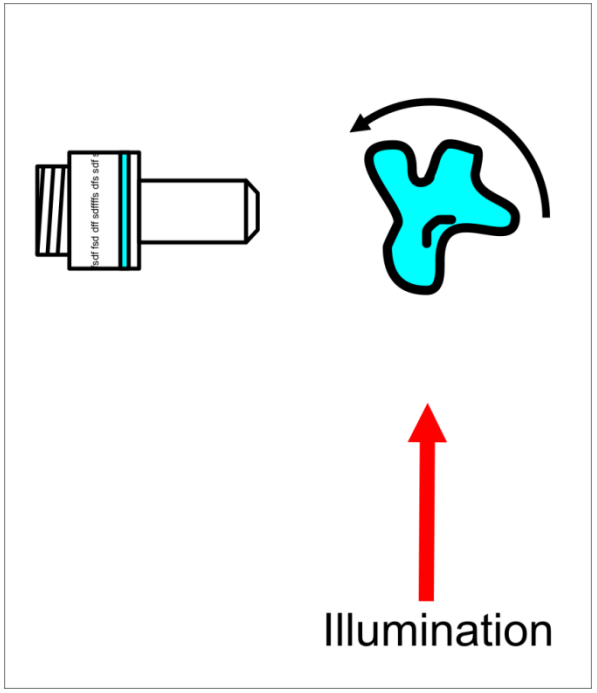
Fusion, 6 views

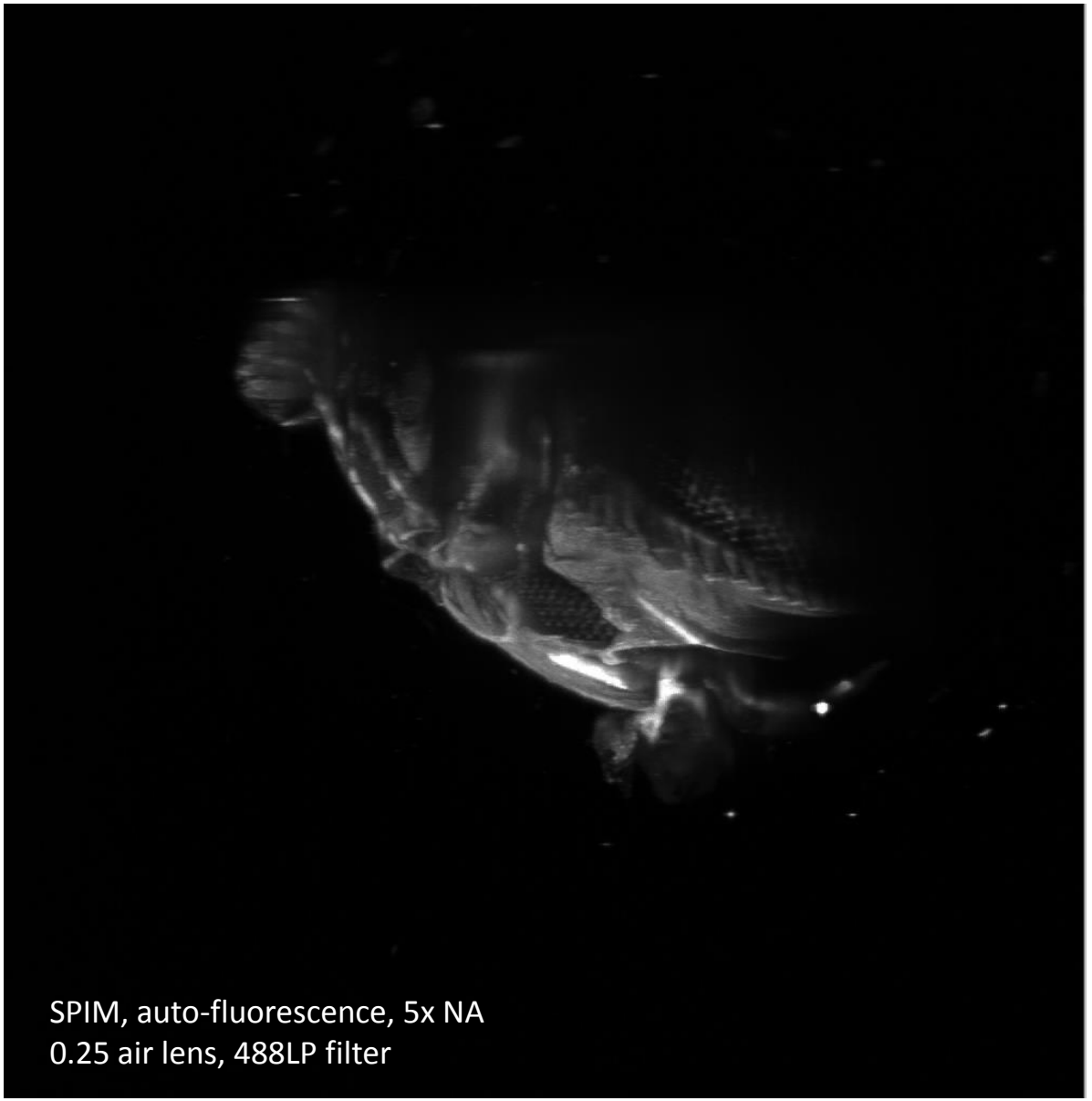


Fusion, 18 views

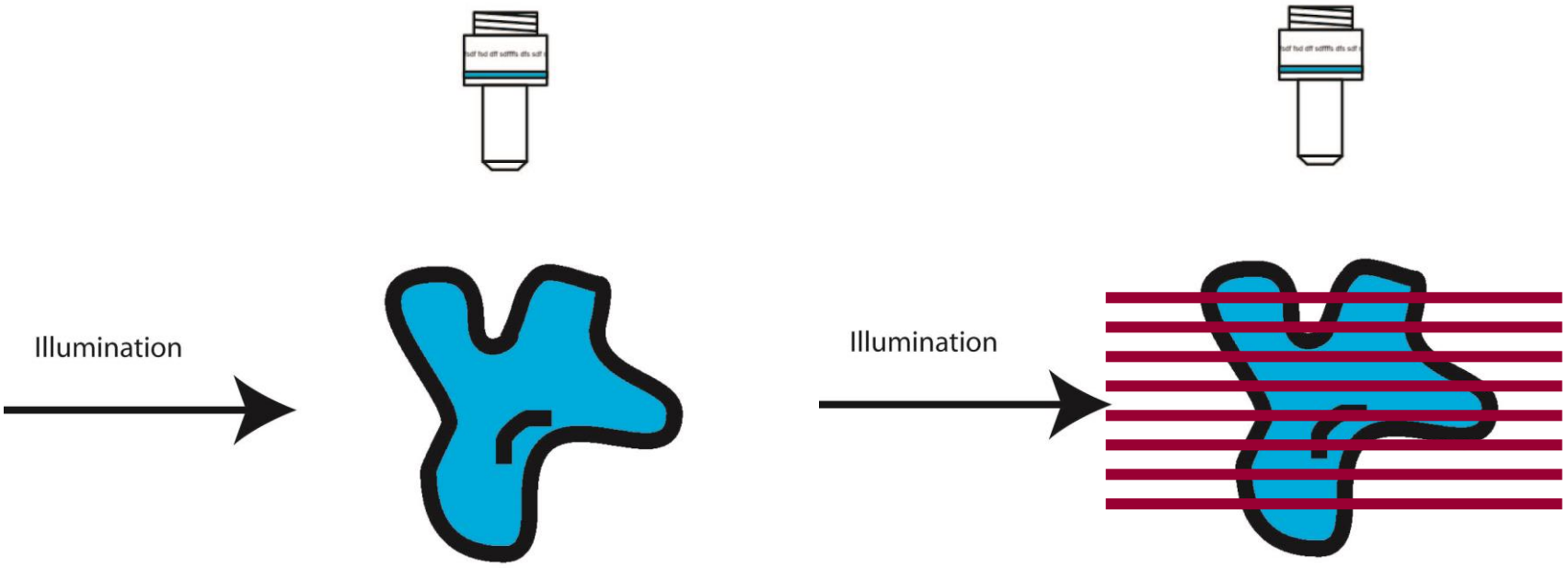
**Swoger, Huisken & Stelzer, 2003**
**Swoger, … & Stelzer , Opt Expr, 2007**

# A real example (single view)

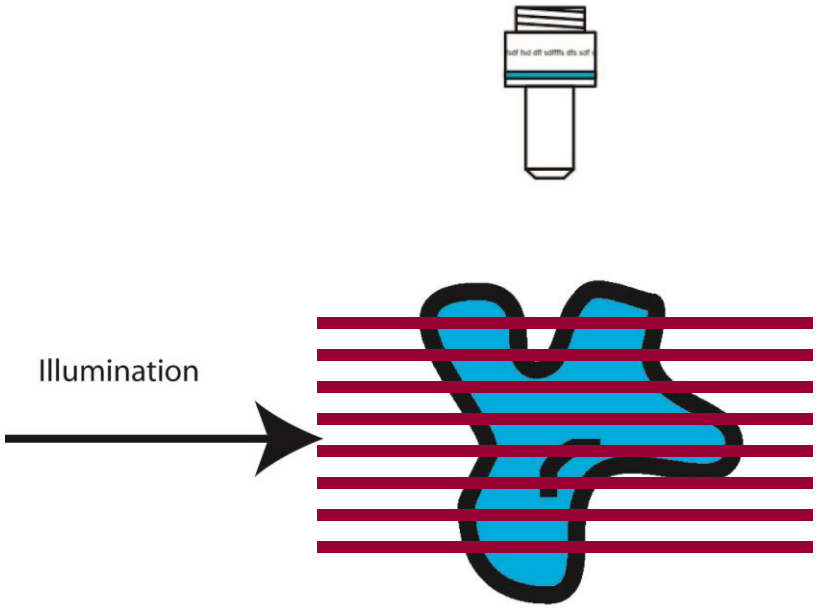


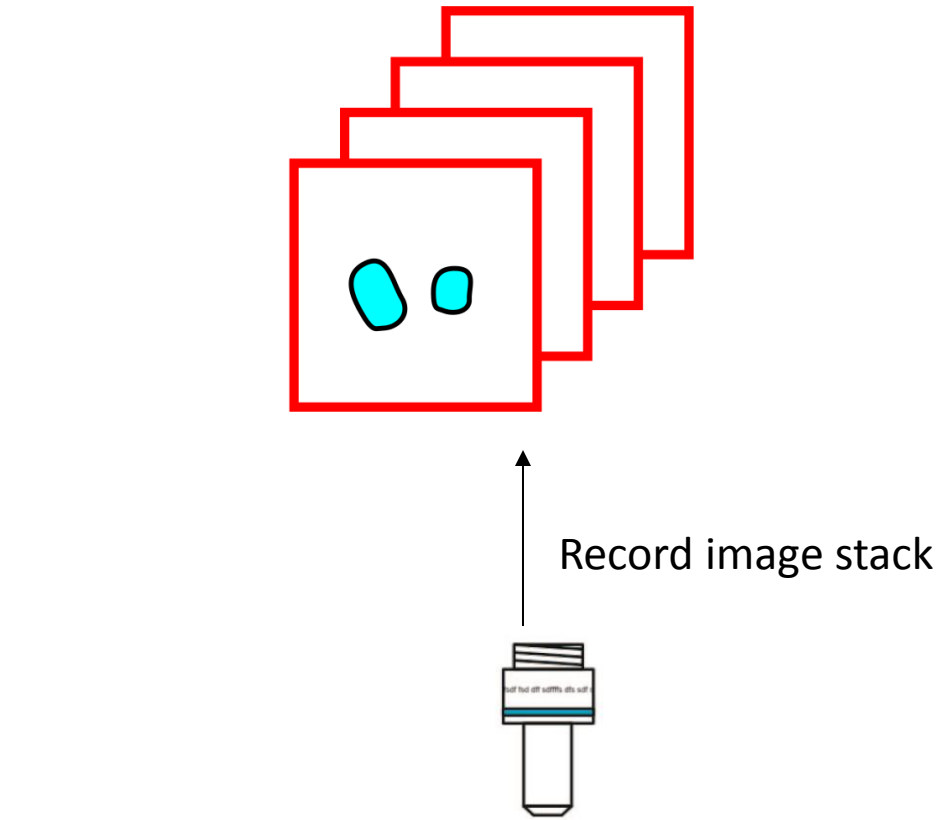
# Multi-view SPIM



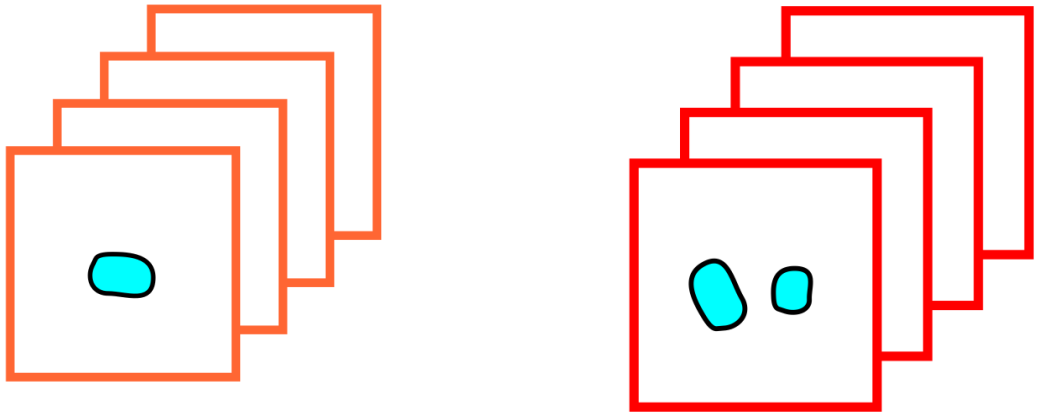
# Multi-view SPIM



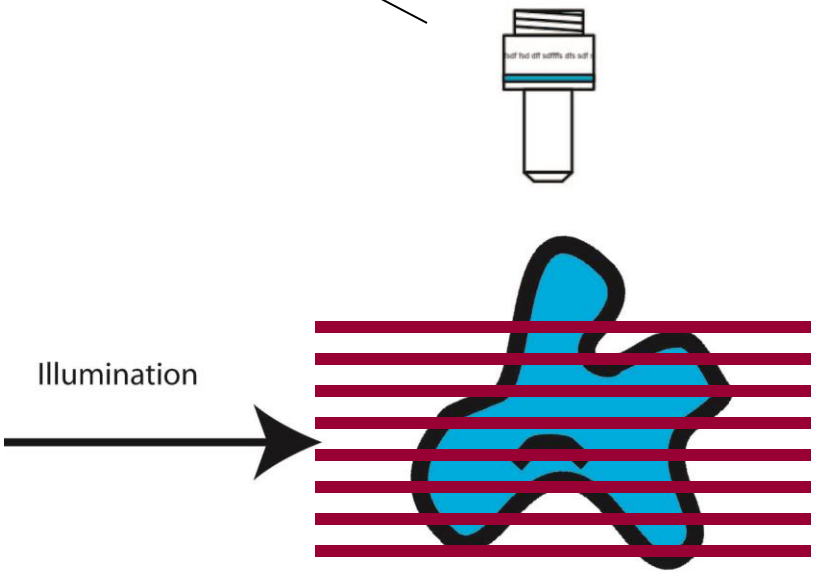
# Multi-view SPIM



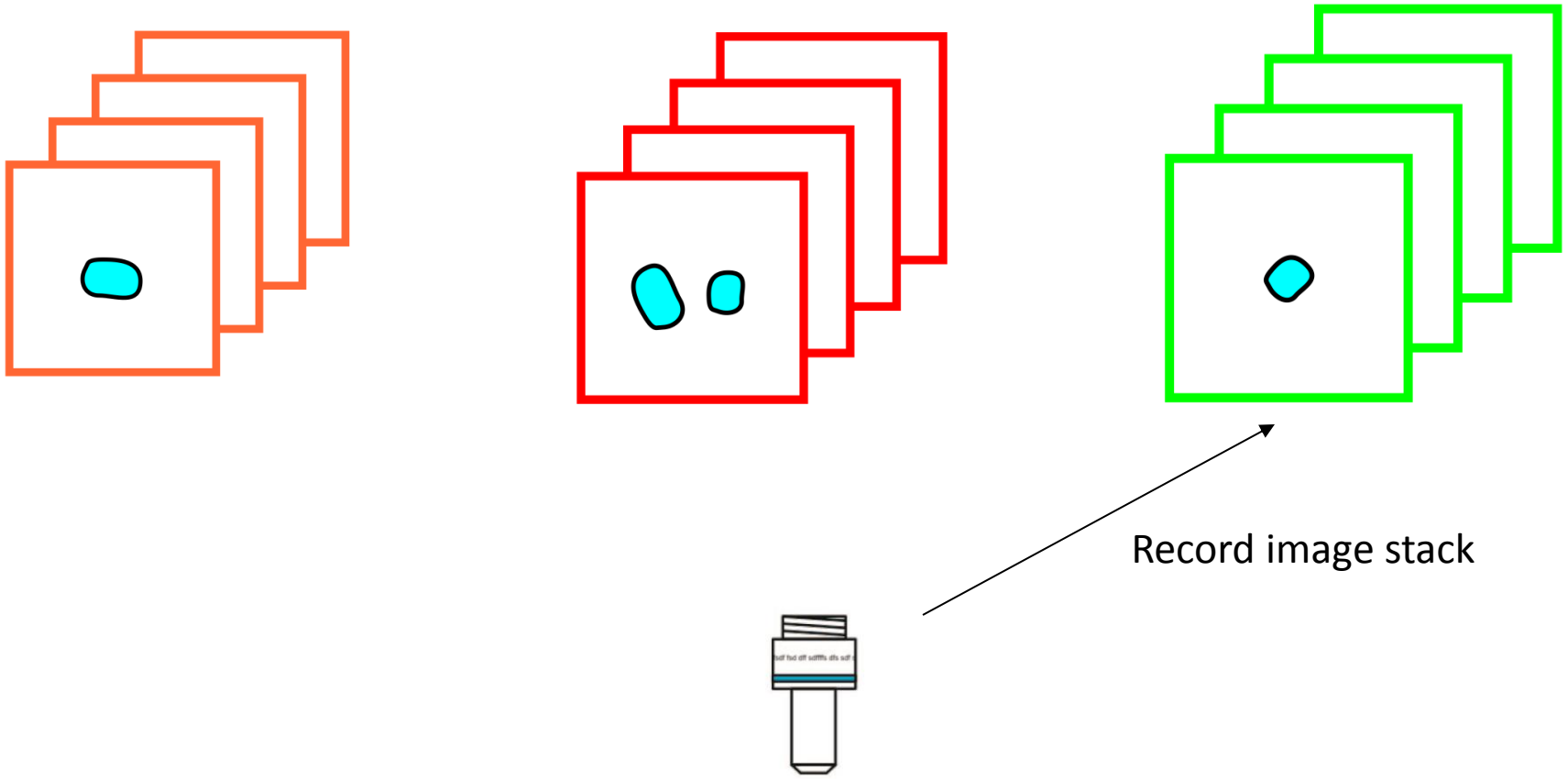
# Multi-view SPIM



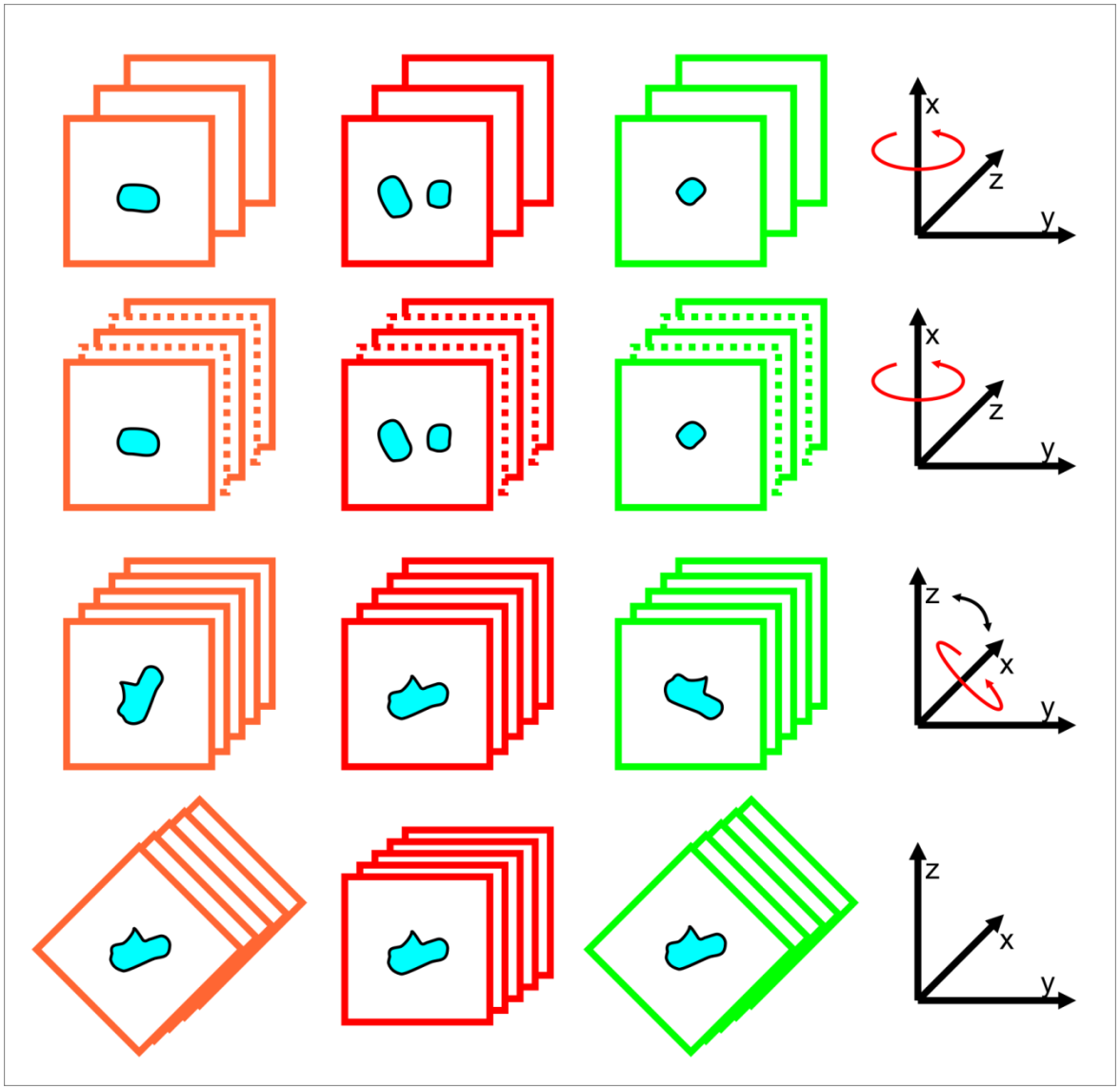
Record image stack



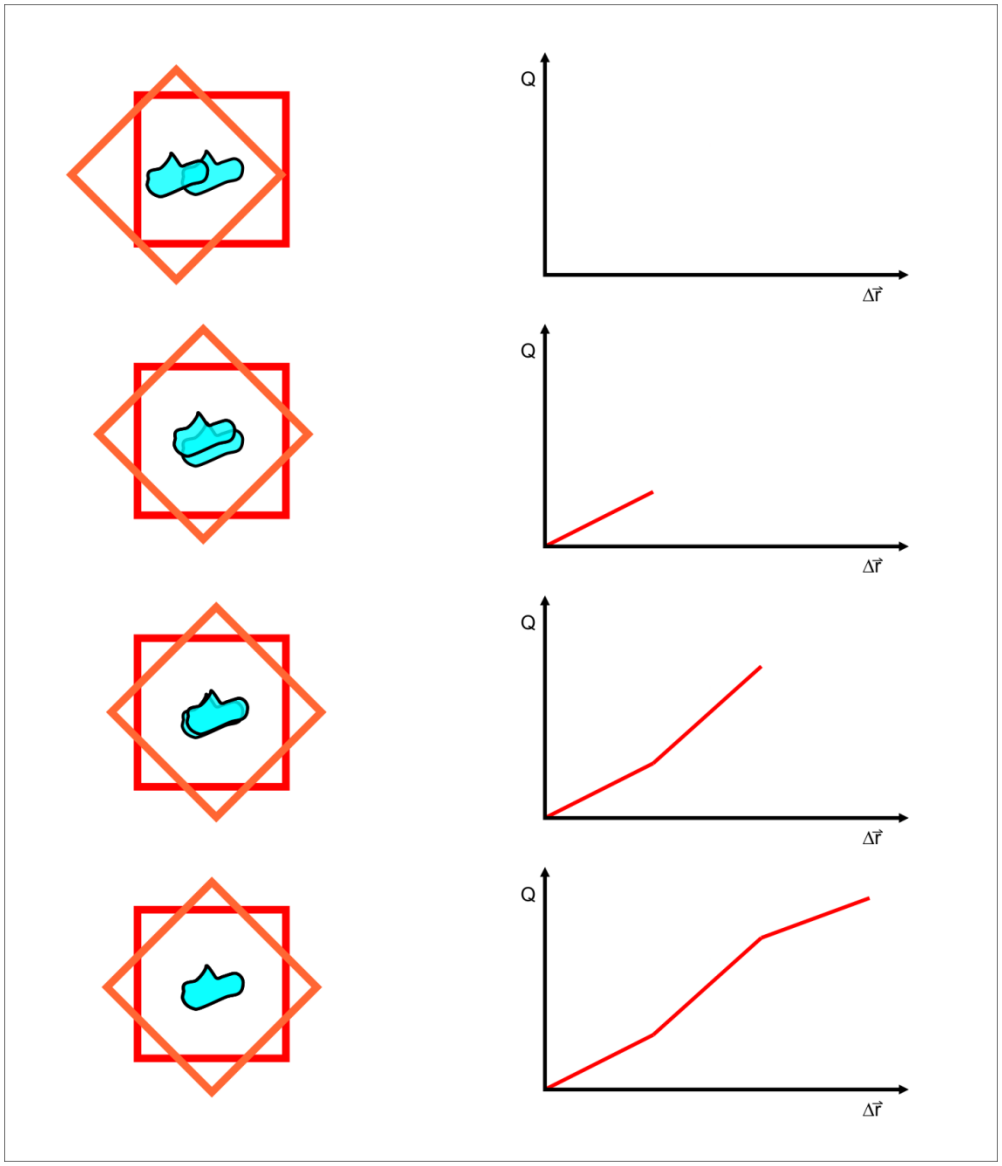
# Multi-view SPIM



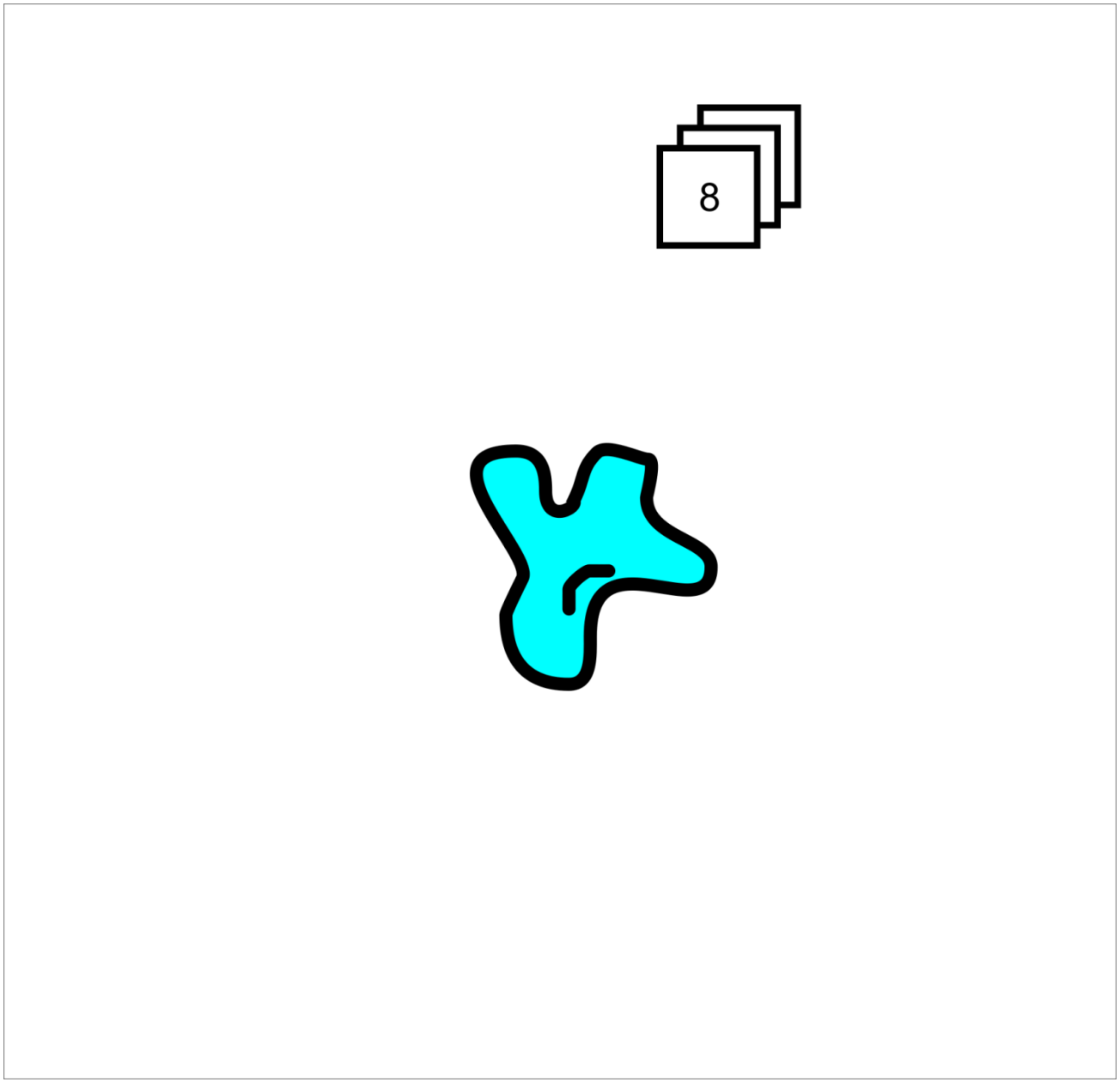
# Pre-processing



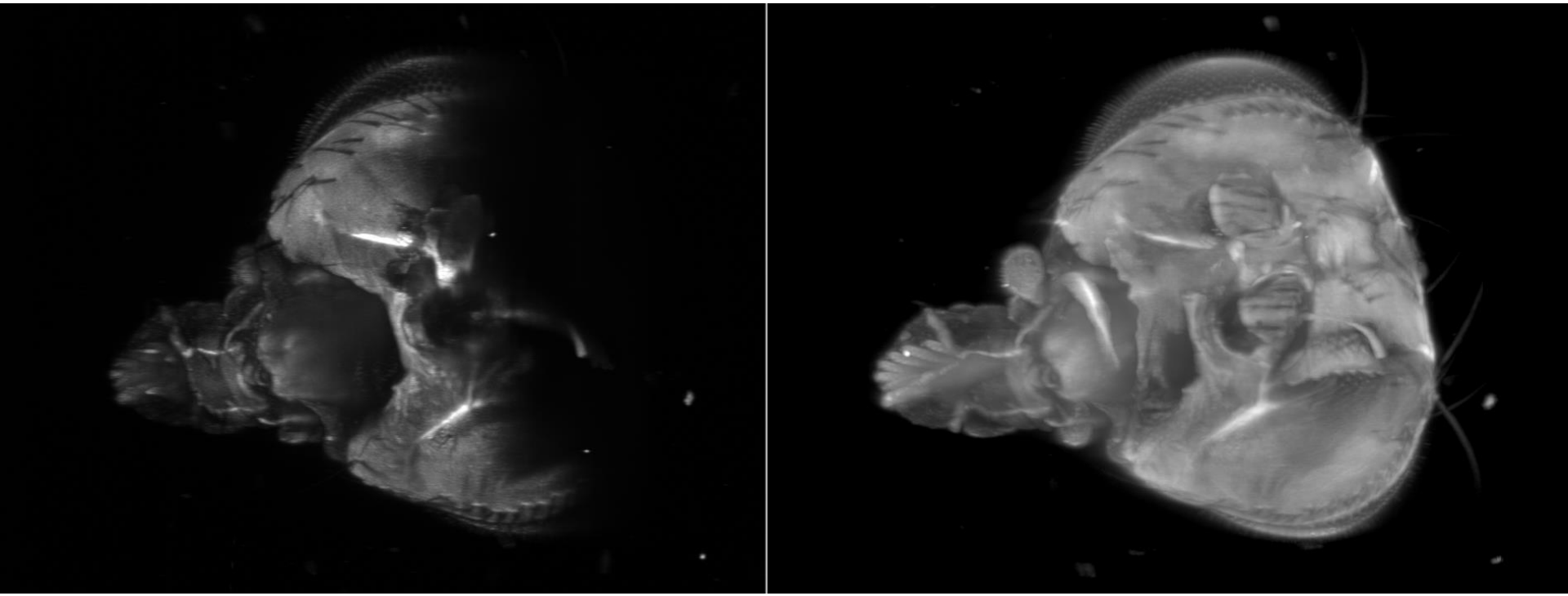
# Image registration



# Multi-view image alignment



# Example



# Registration fusion

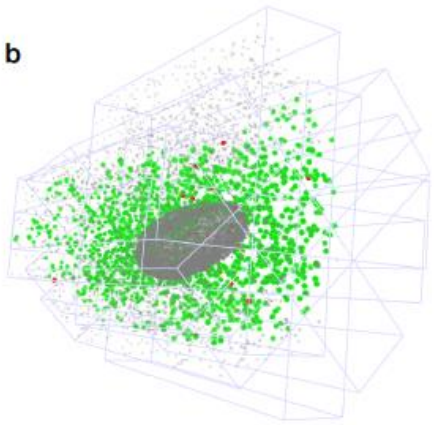
**Intensity based**
- No embedding necessary
- Sample independent

- Typically slow
- Hard to cope with developing samples
- Result hard to verify automatically

P. Shaw et al., in *Biophysical Journal* ***55***, 1989.
C. J. Cogswell et al., in *Proceedings of SPIE*, 1996.
R. Heintzmann et al., in *Analytical Cellular Pathology* ***20***,
R. Heintzmann et al., in *Journal of Microscopy* ***206***, 2002
J. Huisken et al., in *Science* ***305***, 2004.
J. Swoger et al., in *Optics Express* ***15***, 2007.
P. Verveer et al., in *Nature Methods* ***4***, 2007.

**Bead based**
- Very fast
- Sample independent
- Easy use with developing samples
- Automatic verification

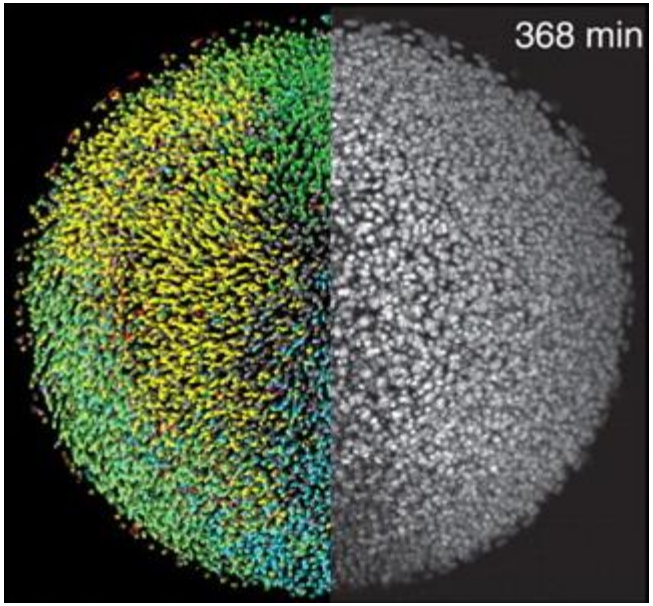
- Embedding in rigid medium




**S. Preibisch**, S. Saalfeld, J. Schindelin, P. Tomancak, in *Nature Methods* ***7, 2010.***

**Segmentation based**
- Potentially fast
- Automatic verification possible
- No embedding necessary

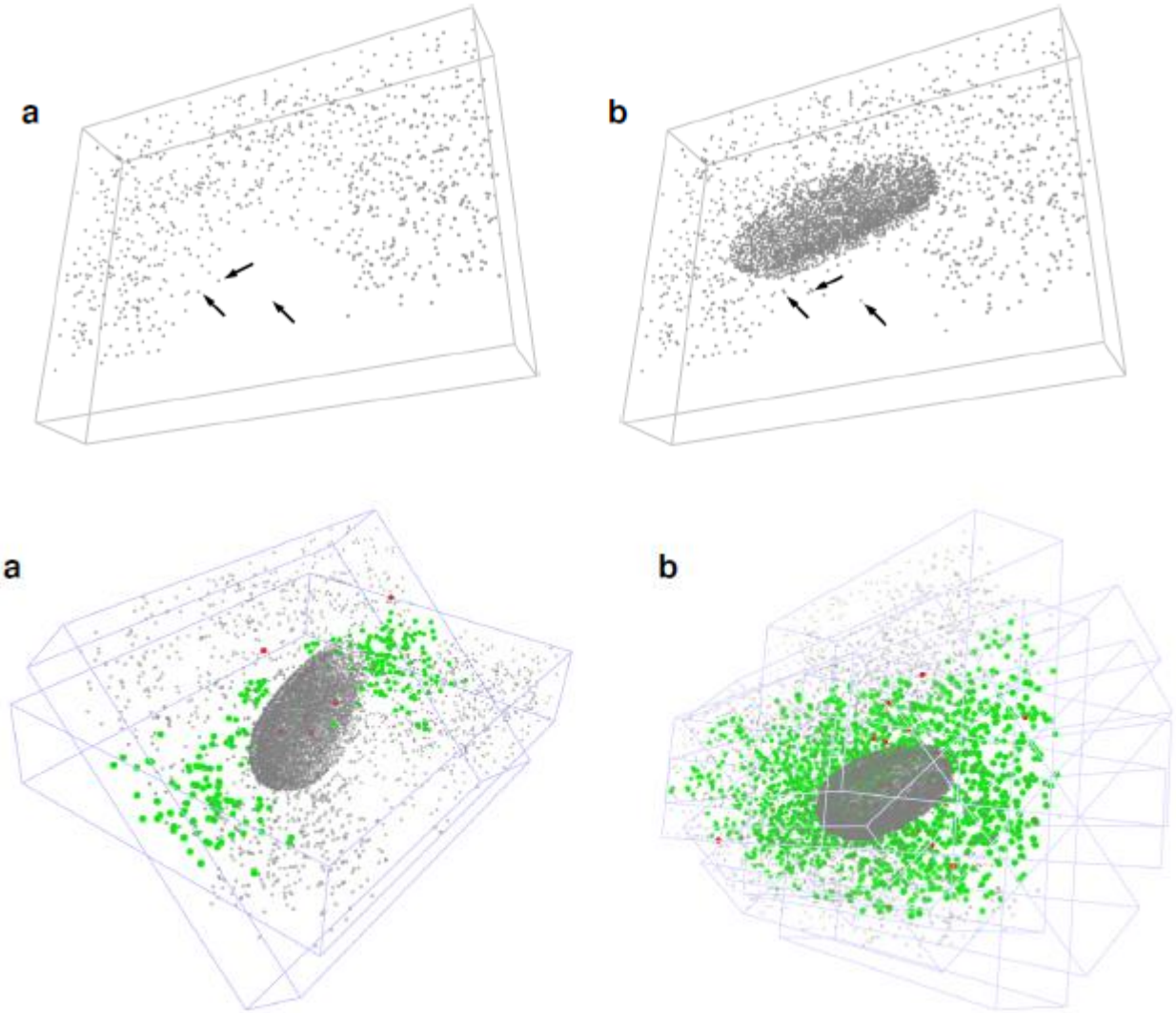
- Staining dependent
- Hard to cope with developing samples




P. Keller et al., in S*cience*, 2008..
P. Keller et al., in *Nature*
**S. Preibisch** et al., in *IEEE ISBI*, 2008.

# Bead based Registration Framework

**Preibisch S.**, Saalfeld S., Schindelin J., Tomancak P., “Software for bead-based registration of selective plane illumination microscopy data”, *Nature Methods* ***7****(6)*, 2010.



# What to remember…

- **Which LISH do you have?**
- **Which sample do you want to image?**
- **Can you mount it?**
- **Plan your data flow!!!**
- **Plan your image processing!!! (scale it down first!!!)**
- **Enjoy!!!**