

Advanced Microscopy Course 2013

Lecture 7:

Live Cell Imaging (Fluorescence)

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Live Cell Imaging

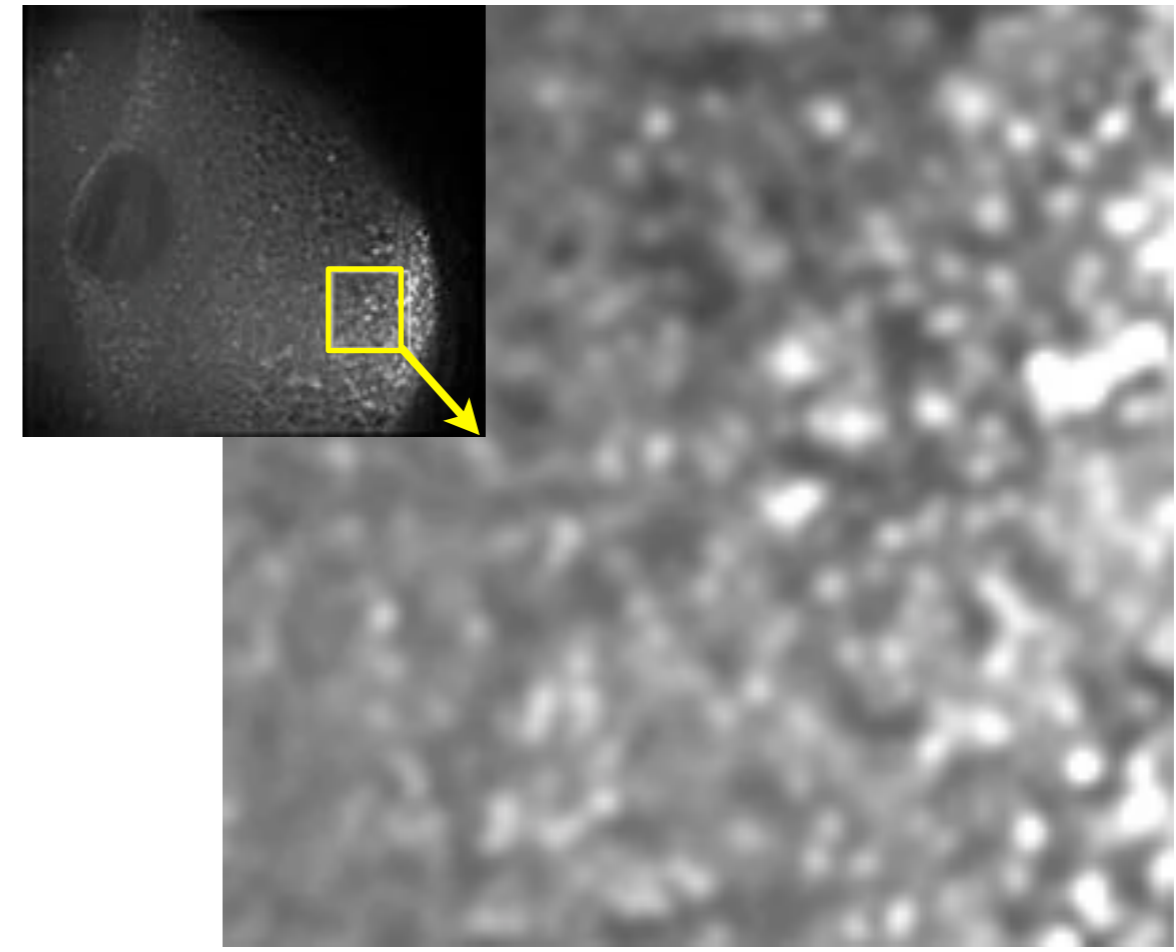
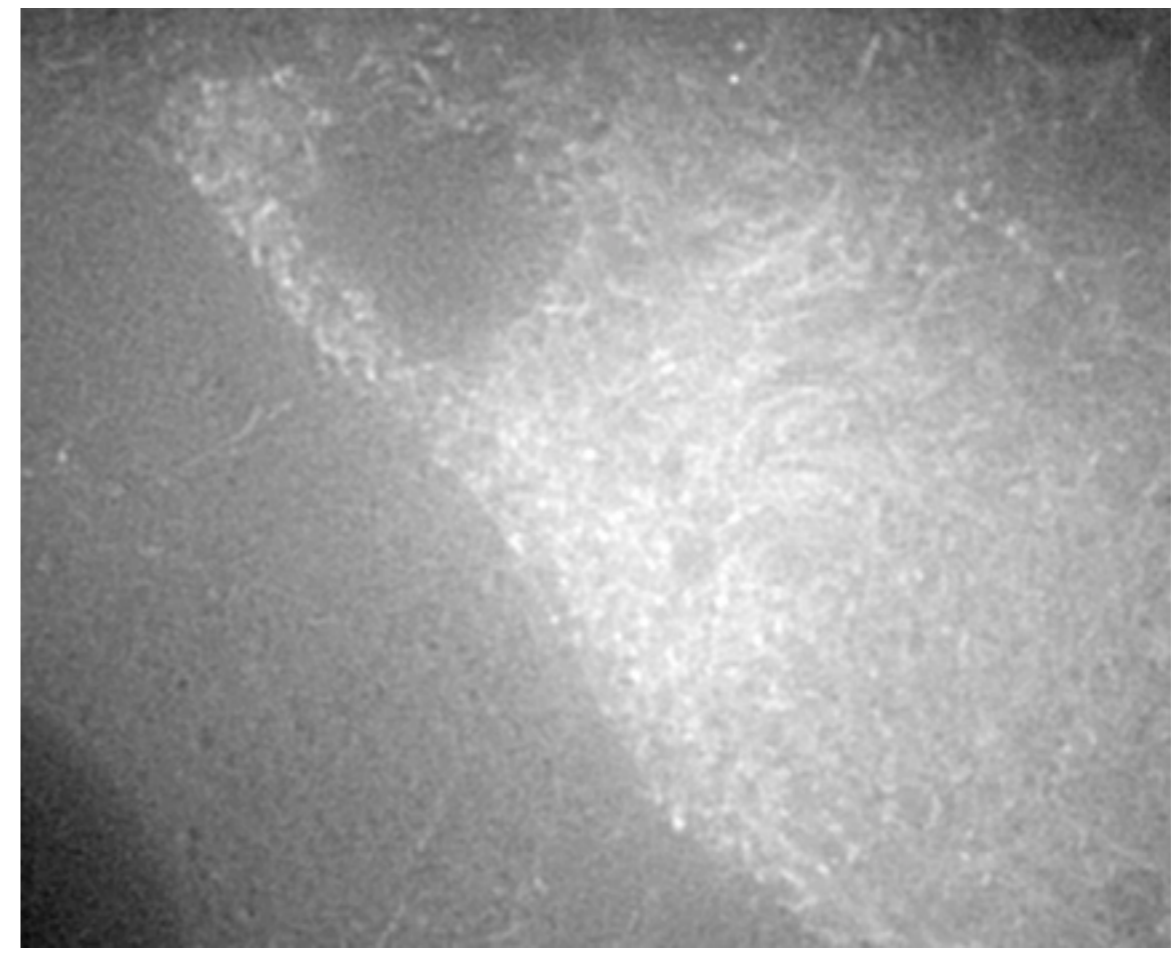
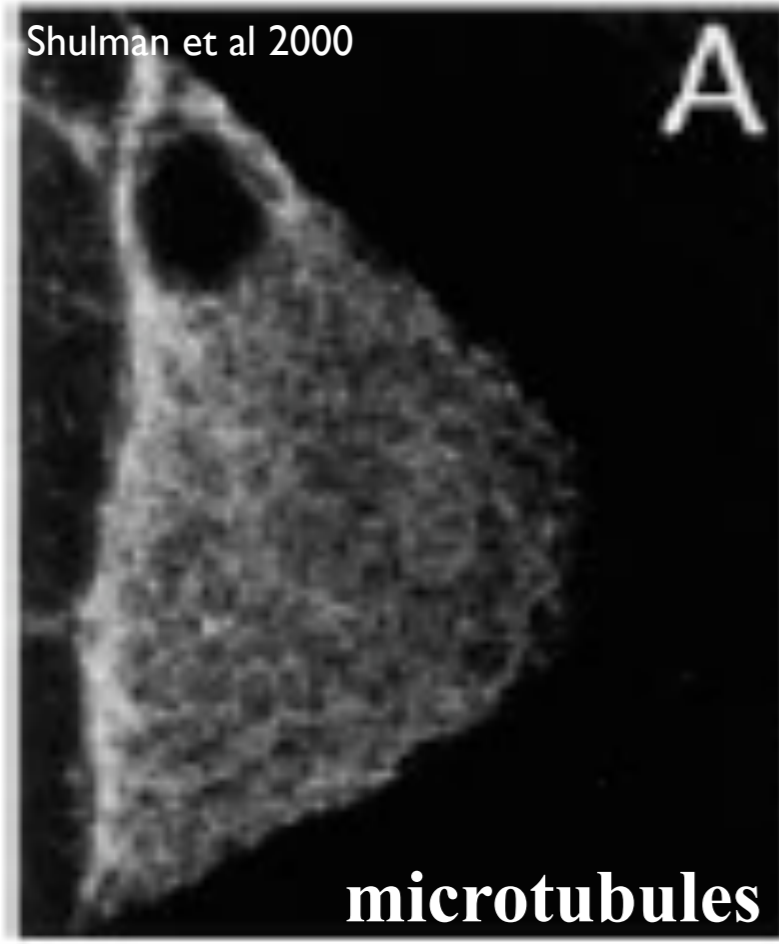
- Reasons for live cell imaging
- Requirements for live cell imaging
 - Experimental design
 - Choice and setup of equipment
 - Collect every photon
 - Image processing and analysis

Reasons for live imaging: Fixed vs Live



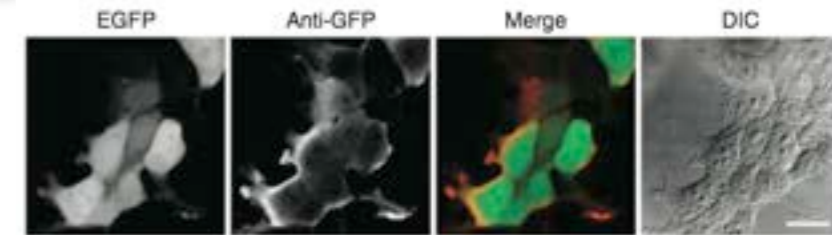
Fixed

Live



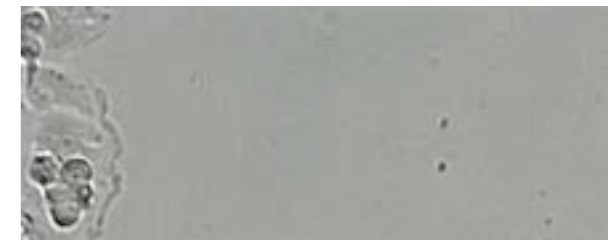
Reasons for live imaging

1) You can believe what you see - no fixation artifacts



2) Can follow the order of sequential events in real time

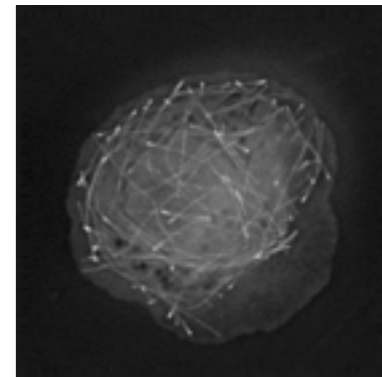
time-course of cell migration - Andrea Linford Barr lab



3) Can monitor the kinetics of dynamic processes:

- active transport vs diffusion
- Microtubule turnover

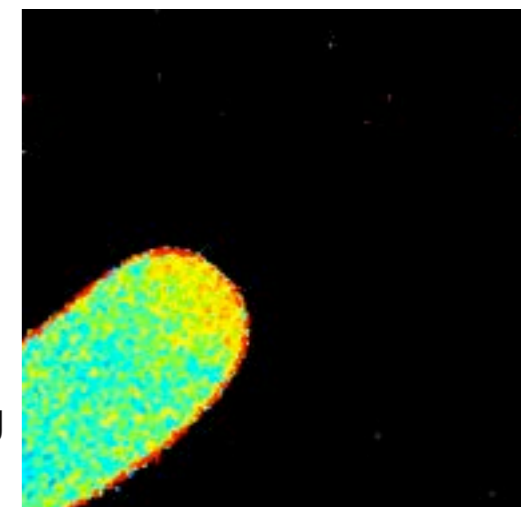
Macrophage:
EB1-GFP
tagged MT



4) Can record sensitive or transient processes:

- Calcium signalling transients
- Ion gradients
- membrane potential

Calcium ratio imaging
pollen tube



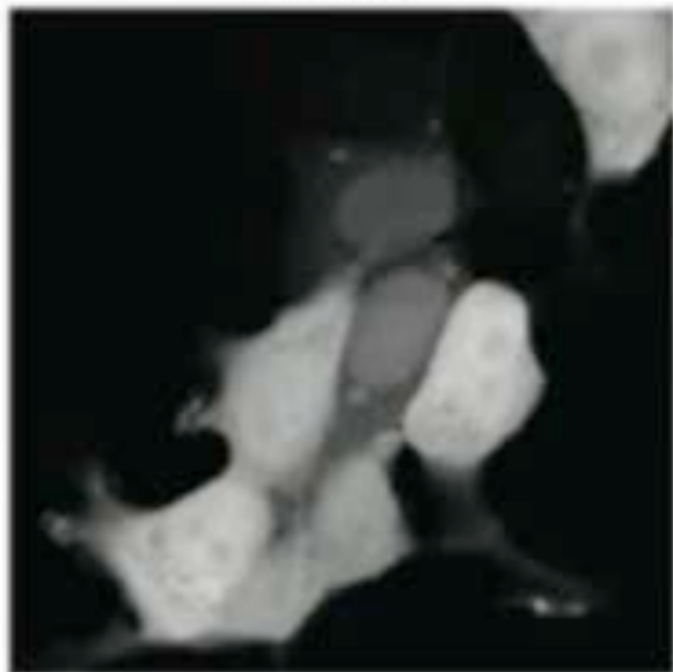
You can believe what you see - no fixation artifacts

Immunolabeling artifacts and the need for live-cell imaging

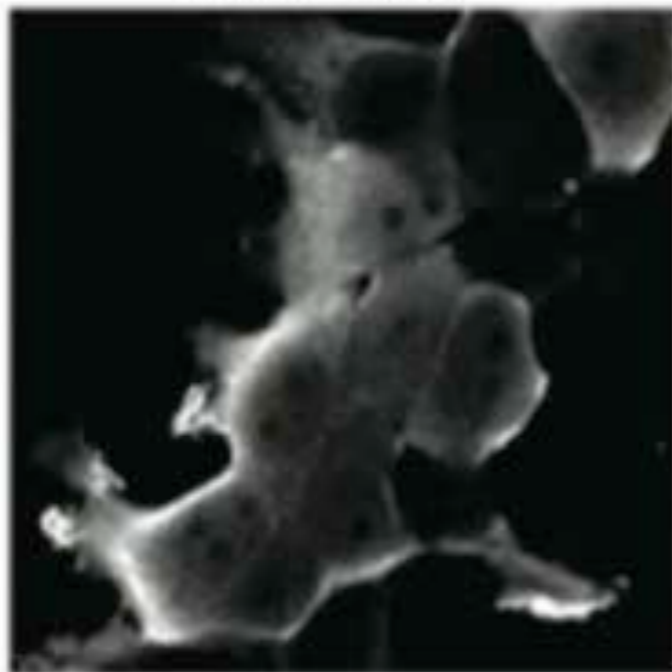
Ulrike Schnell, Freark Dijk, Klaas A Sjollema & Ben N G Giepmans

Nature Methods, 9(2), 152–158. doi:10.1038/nmeth.1855

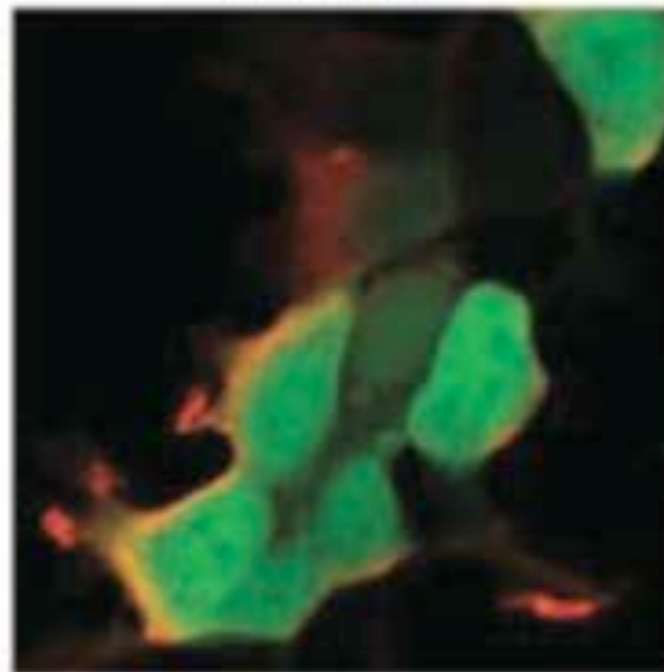
EGFP



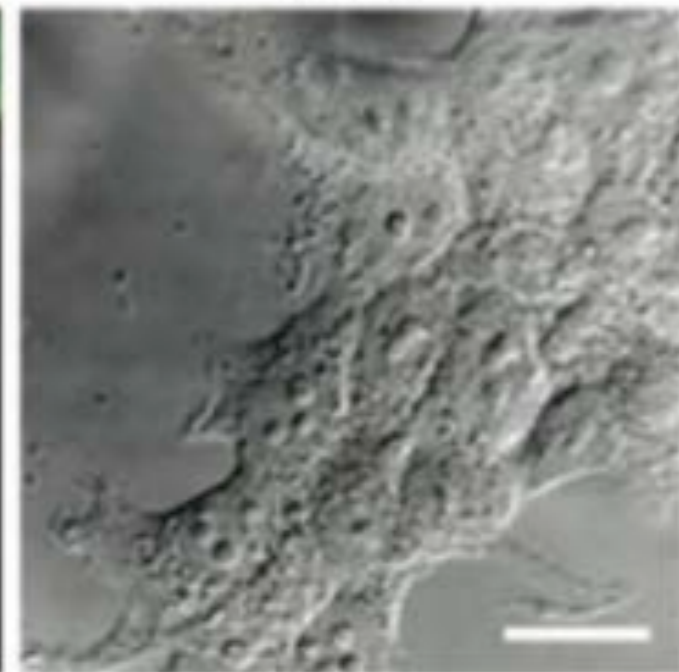
Anti-GFP



Merge



DIC

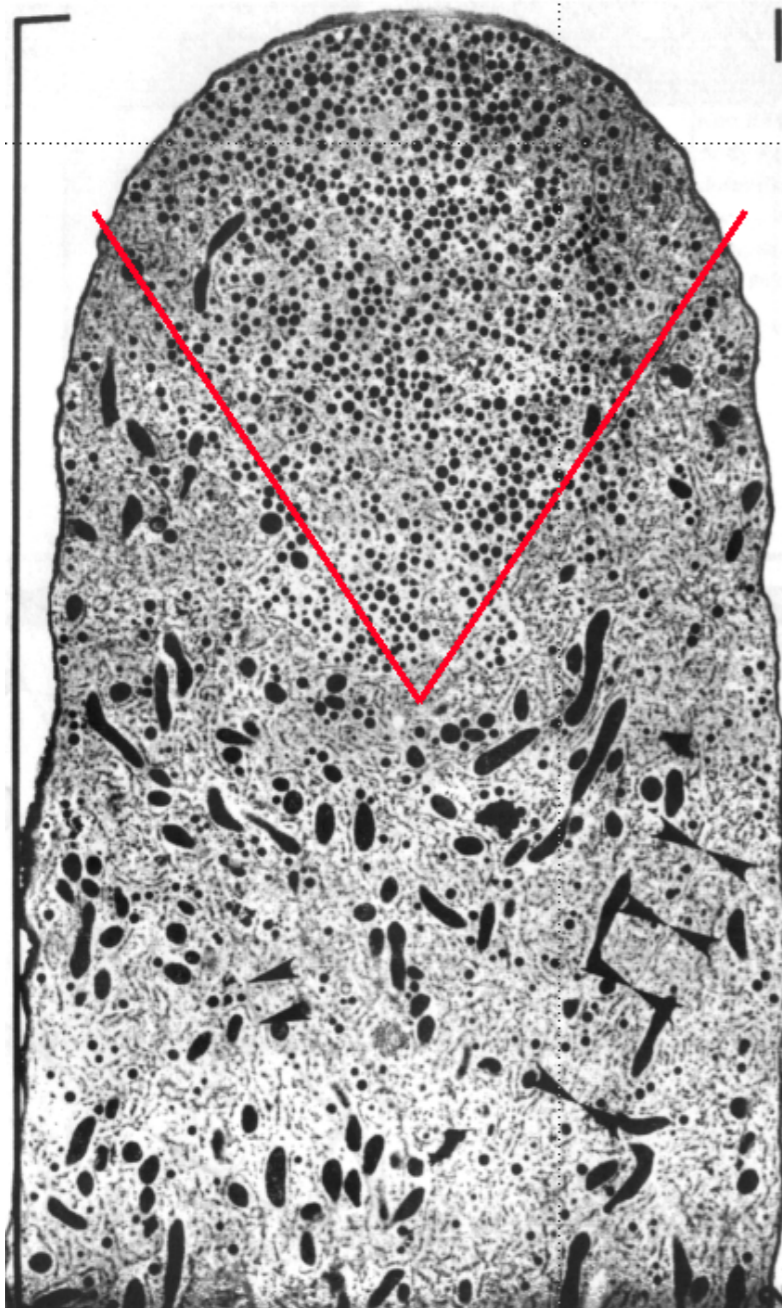


** Eva - lecture 4 - sample prep and minimizing artifacts **

** Errin - lecture 17 / Rainer - lecture 15 EM, correlative light and EM, super-precision microscopy **

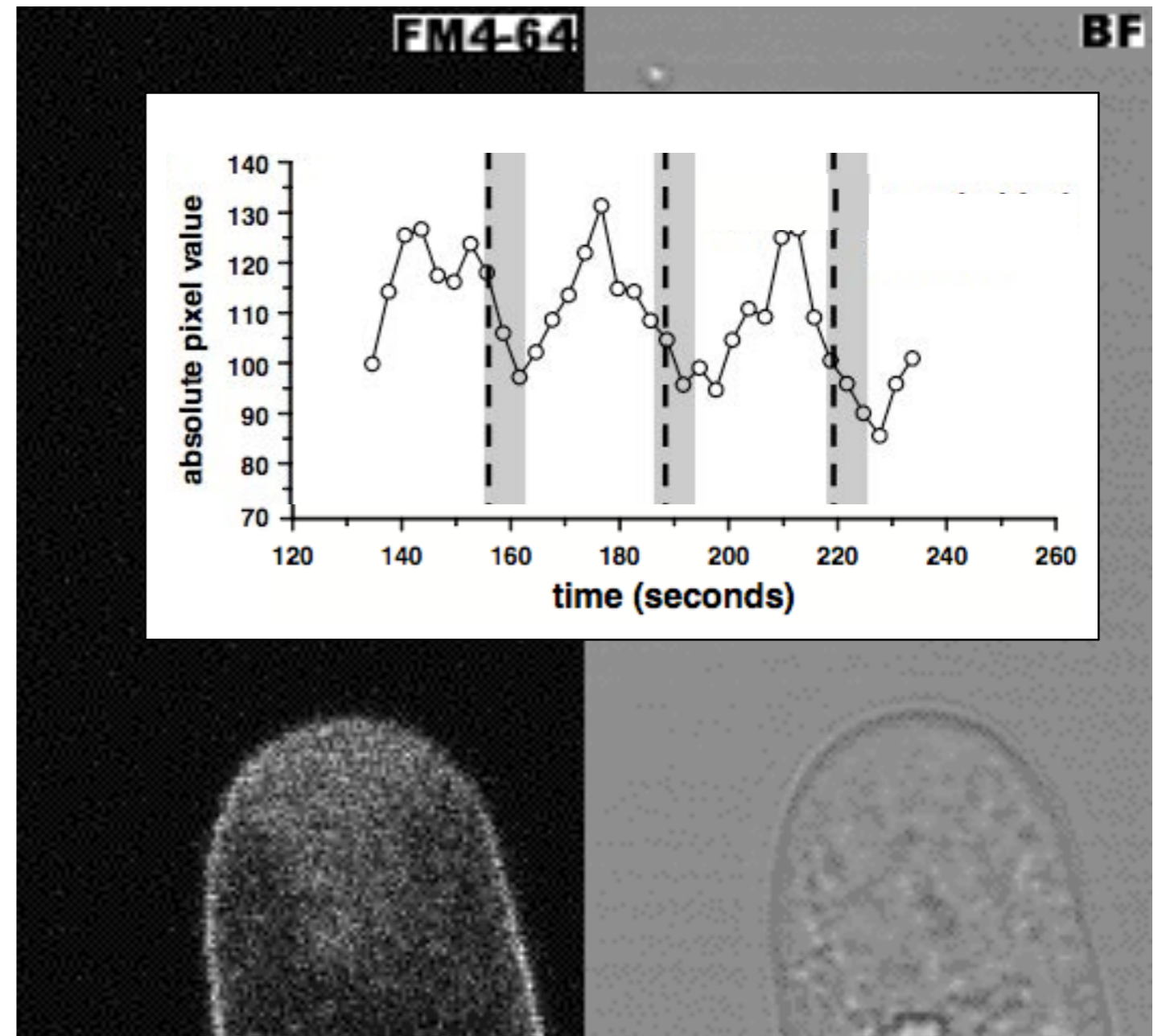
Can monitor the kinetics of dynamic processes

Fixed - EM



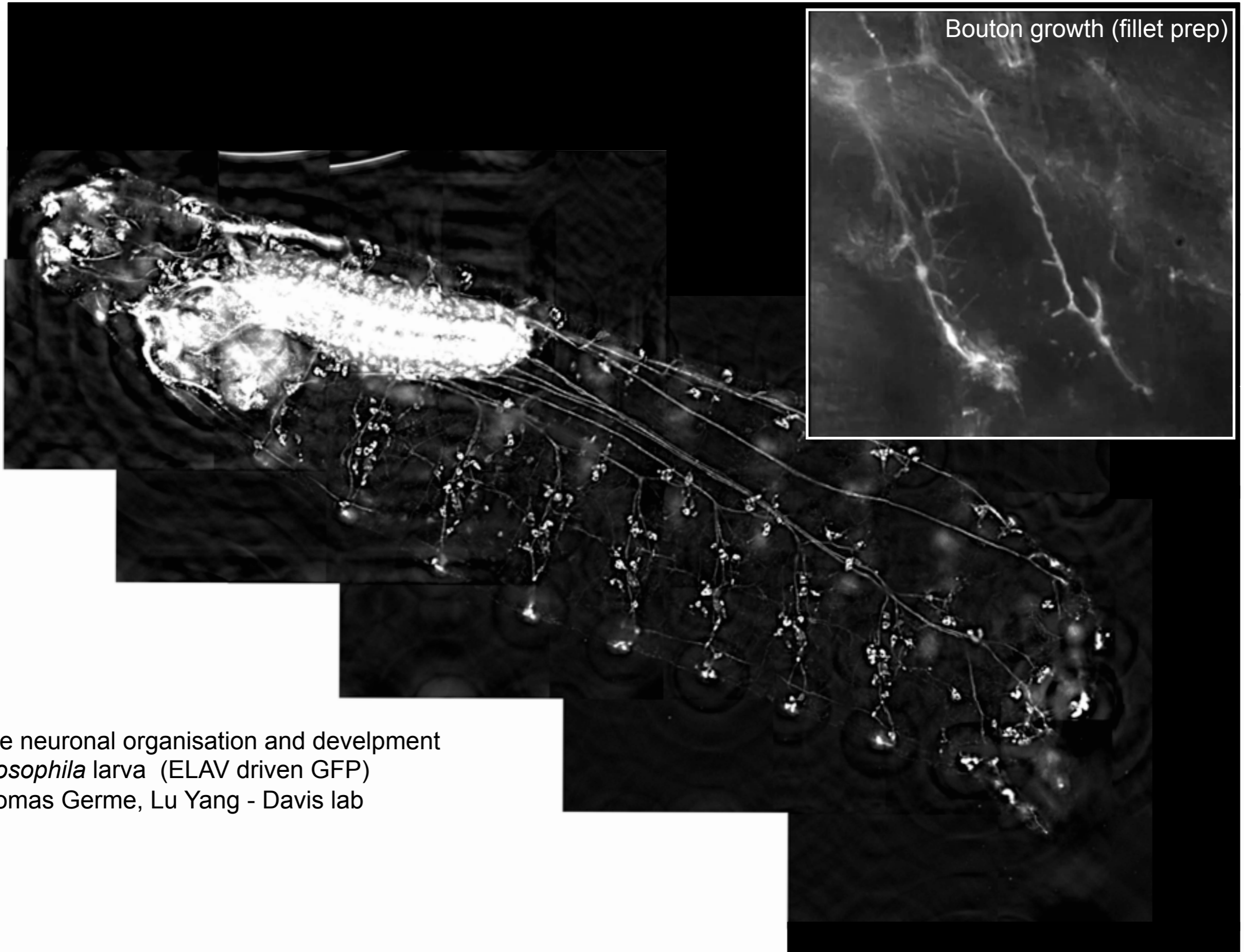
Electron Micrograph From
Lancelle, S.A.; Cresti, M.; Hepler, P.K. (1997)
Protoplasma 196, 21-33.

Live



FM4-64 labelling of the plasma membrane
and apical vesicles in a living pollen tube
Parton *et al*, 2001. JCS

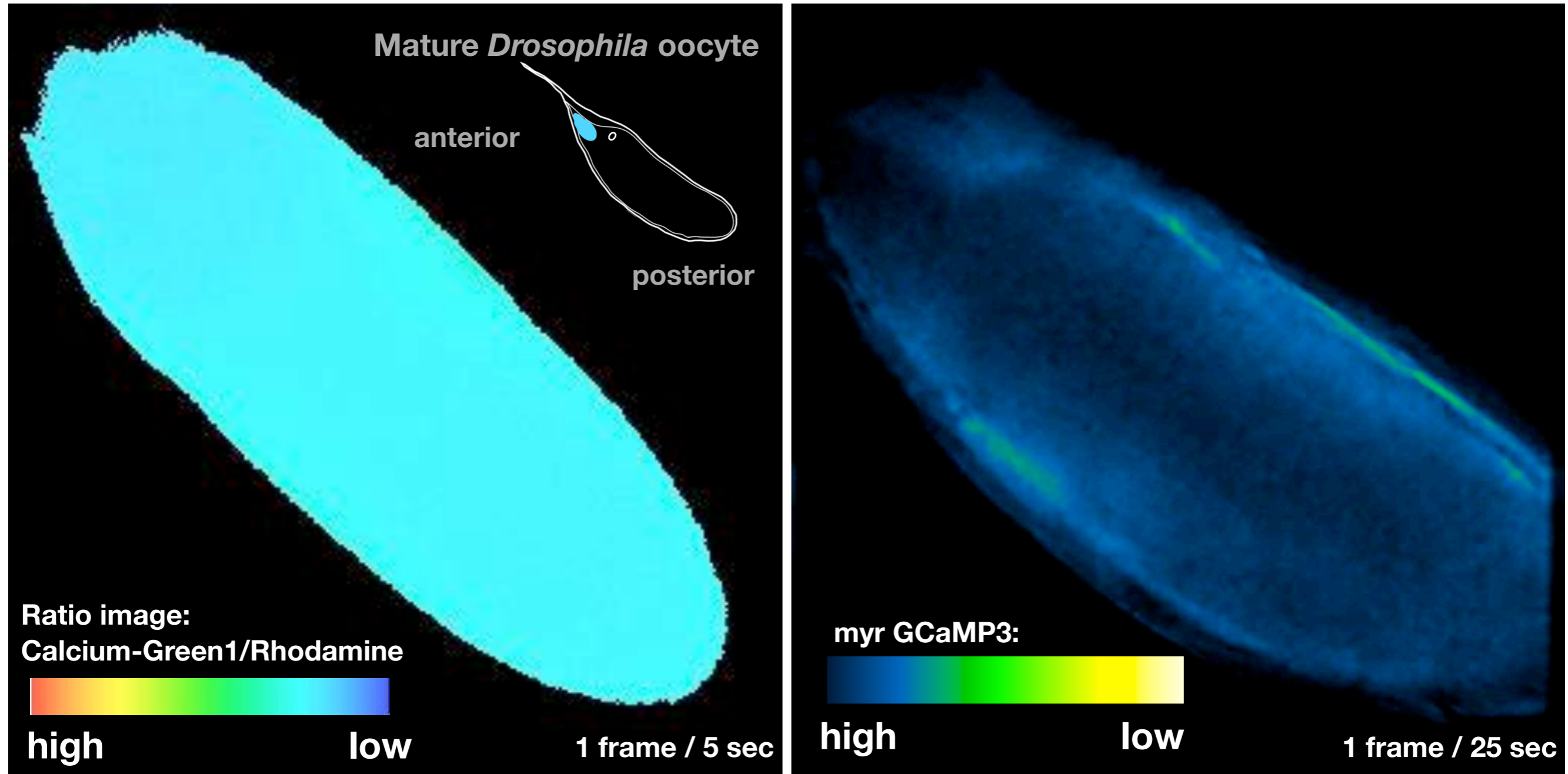
Can follow the order of sequential events in real time



Live neuronal organisation and development
Drosophila larva (ELAV driven GFP)
Thomas Germe, Lu Yang - Davis lab

Can record sensitive or transient processes

Calcium transient upon activation



Claire Bromley, Richard Parton, Tim Weil: Davis Lab

Requirements for live cell imaging:



Careful Balancing of Conflicting Interests

What is important in microscopy?

1. Resolution
2. Sampling
3. Contrast
4. Noise

What is also important in live-cell imaging?

1. Cell viability
2. Speed
3. Field of view
4. Multiple channels

What is important
in microscopy?

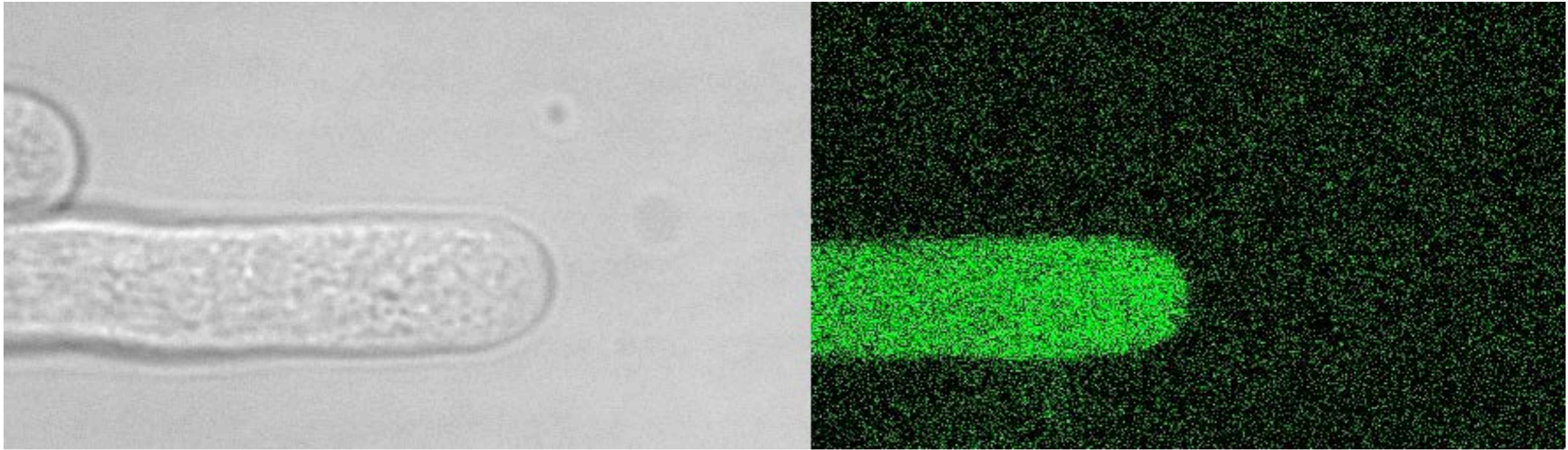
1. Resolution
2. Sampling
3. Contrast
4. Noise

What is also important
in live-cell imaging?

1. Cell viability
2. Speed
3. Field of view
4. Multiple channels

Live-cell imaging is a compromise!

Death by imaging!



Cytoplasmic GFP in a living *Lilium* pollen tube imaged by multiphoton (800 nm)

Also:

- mis-expression or aberrant behaviour of GFP tagged proteins
- stressed live cells behave abnormally

do the appropriate controls

Requirements for live cell imaging:

1. Optimise your experimental design
2. Choose your technique carefully
3. Set up you imaging equipment properly
4. Correct Spherical Aberration
5. Collect every photon

Optimise your experimental design:

- What do you need from your imaging?
 - » Qualitative
 - » Quantitative
 - » Spatial information
 - » Temporal information



Goal Setting!

Choice of equipment and technique:

Depends upon:

- 1) What you want to see - experimental design
- 2) Your experimental material
- 3) What is available
- 4) Your budget

There is no, one, perfect technique!

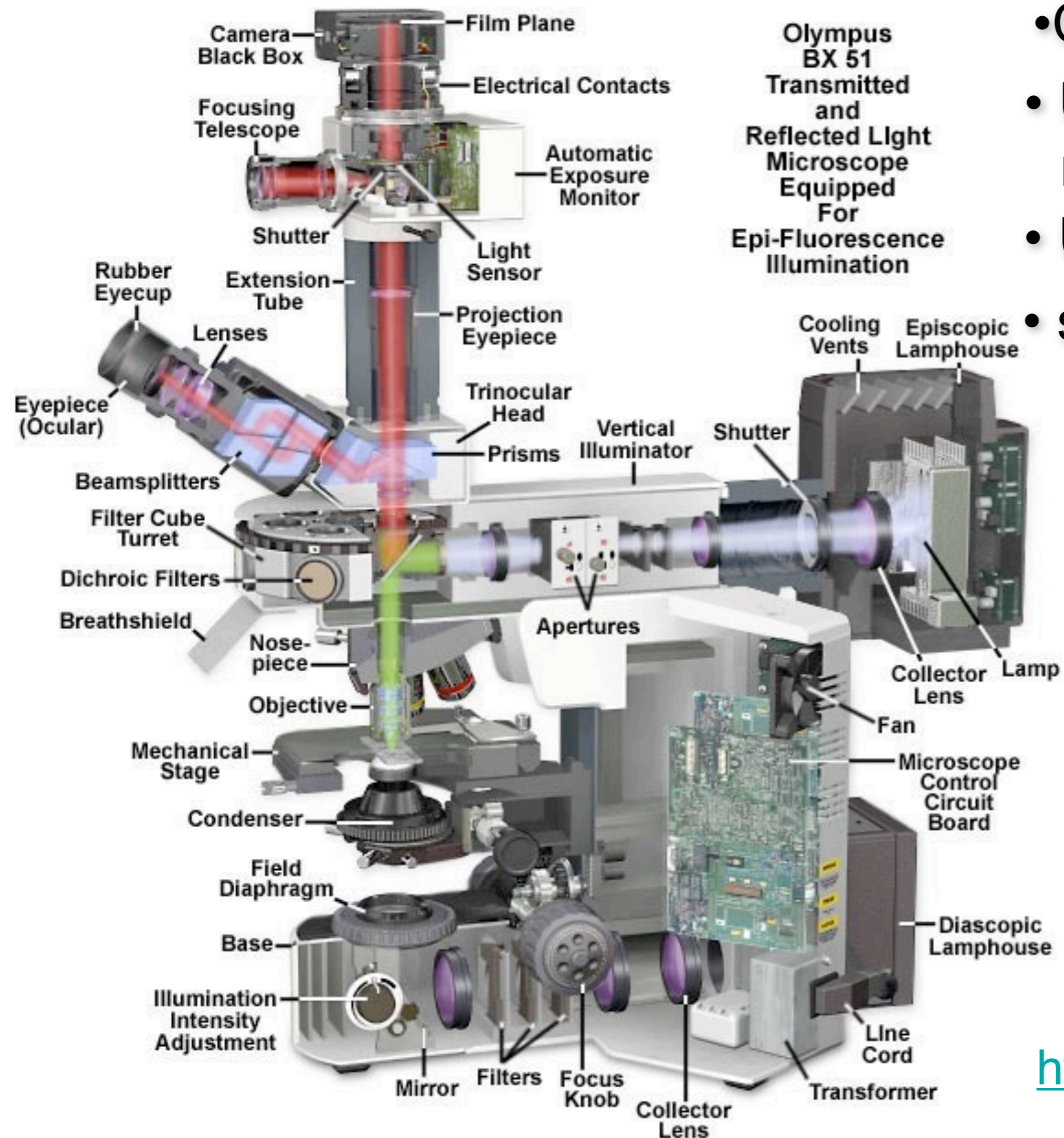
Choice of microscope stand

- The modern epifluorescence microscope

Upright microscope (lens **above** specimen)

Inverted microscope (lens **below** specimen)

Upright microscope design



- Cheaper
- Use with thick or opaque material
- Use with dipping objectives
- stable stand for manipulation

<http://www.olympusmicro.com/>

Upright microscope design

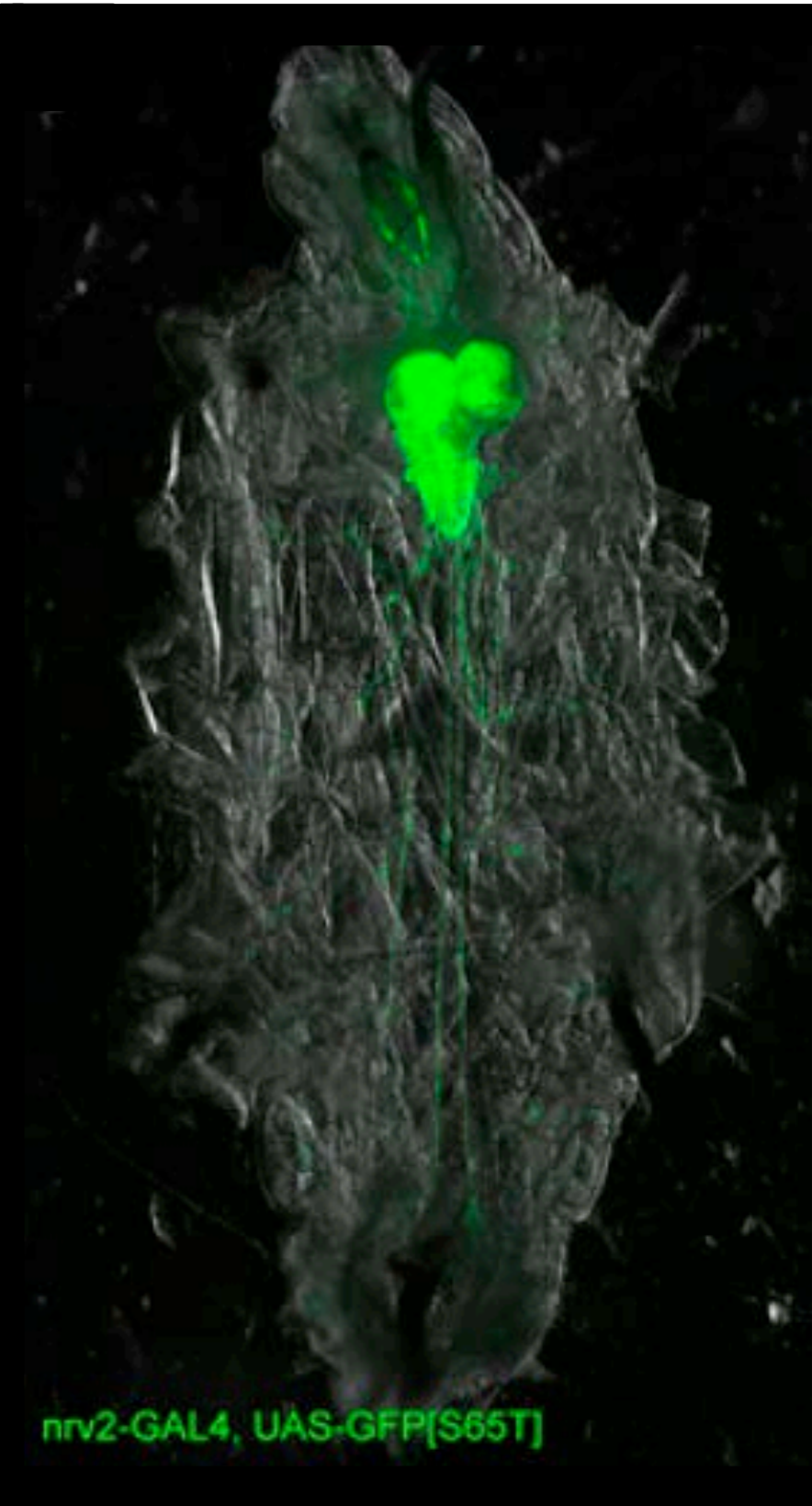
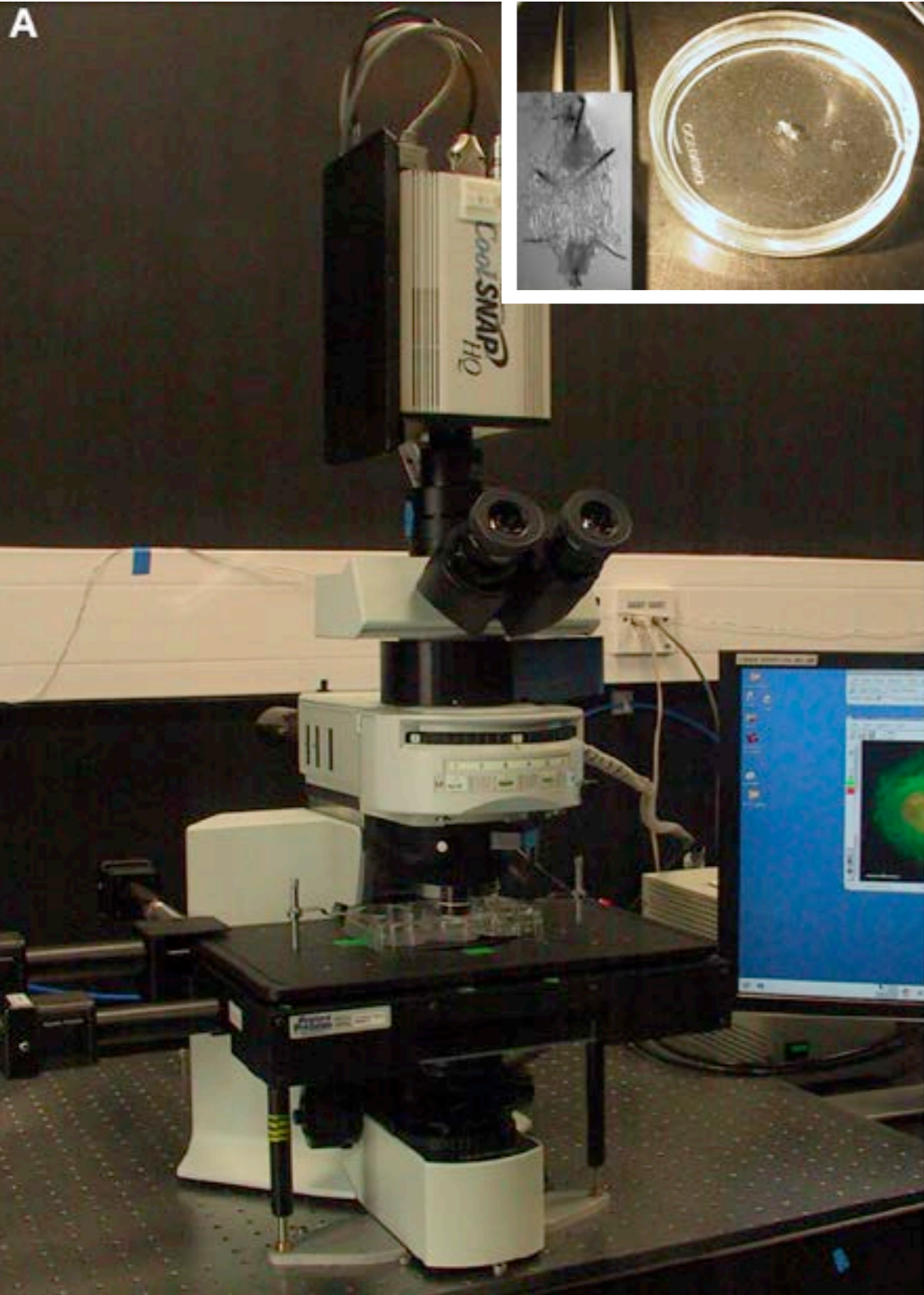
 Psi Scientific

PatchPro 6000



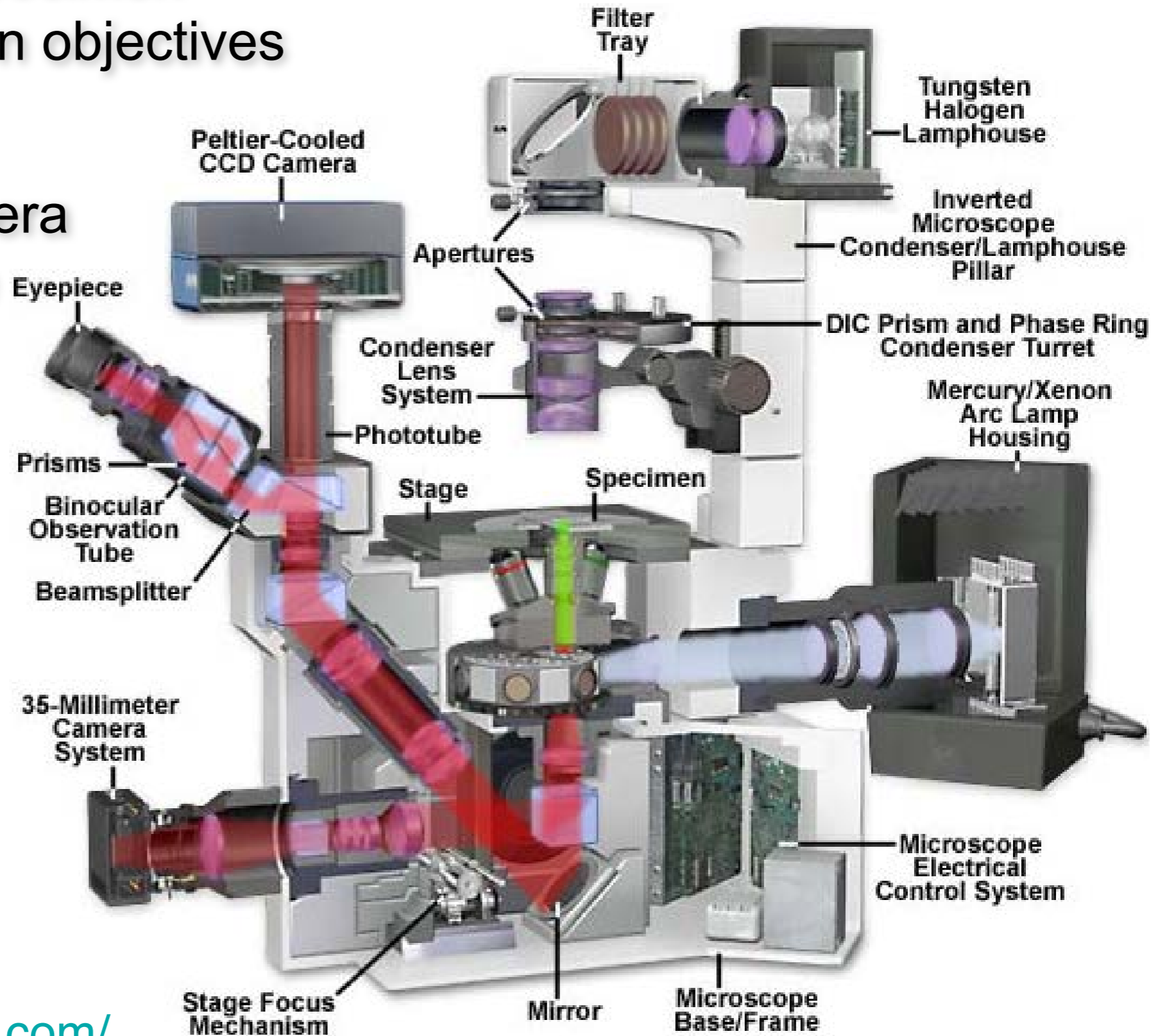
- stable stand for electrophysiology

Upright microscope - larval fillet prep

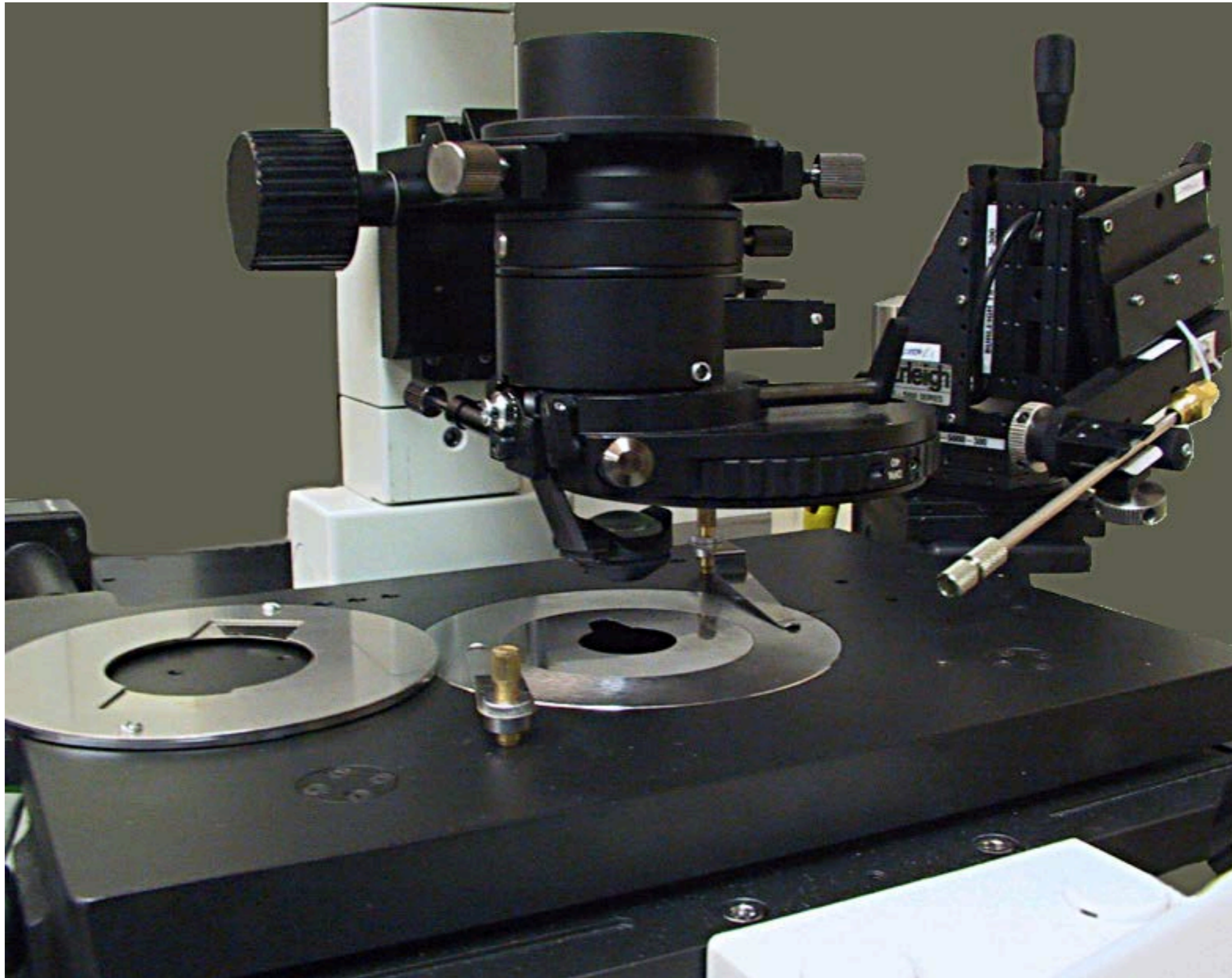


Inverted microscope design

- Easy access to the specimen
- Good for oil immersion objectives
- Convenient side port
- Possible second camera on bottom port or side port



Inverted microscope - injection



There is a microscope stand for everything....



Thorlabs - B scope: www.thorlabs.de/newgrouppage9.cfm?objectgroup_id=6611

The Thorlabs scope is set up to rotate about an axis that is in the plane of focus. So you can be looking at a cell and then, while imaging, rotate the scope (since it's motorized) and still keep looking at the same thing, just from a different angle.

be inventive..

Lenses: http://www.olympusamerica.com/seg_section/uis2/seg_uis2.asp

- **Low mag, Low NA air objectives** (x4 - x40 dry, to 0.95 NA):
 - Can image deep, long working distance (mm)
 - Wide field of view
 - Low resolution
 - Low mag leads to undersampling
- **Dipping, Water, multi-immersion objectives** (x20 - x100 to 1.0 NA):
 - Can image relatively deep, working distance (200 μ m - mm)
 - Reduced field of view
 - Increased resolution
 - High mag options for better sampling



Lenses: http://www.olympusamerica.com/seg_section/uis2/seg_uis2.asp

- High mag, High NA oil objectives (x40 - x150 oil, 1.35 to 1.45 NA):
 - Problems imaging deep, short working distance (170 μm)
 - Prone to spherical aberration
 - High resolution
 - Good light efficiency (High NA)
 - High mag allows appropriate sampling
 - Often highly corrected, flat field (plan), colour corrected (apo chromatic)



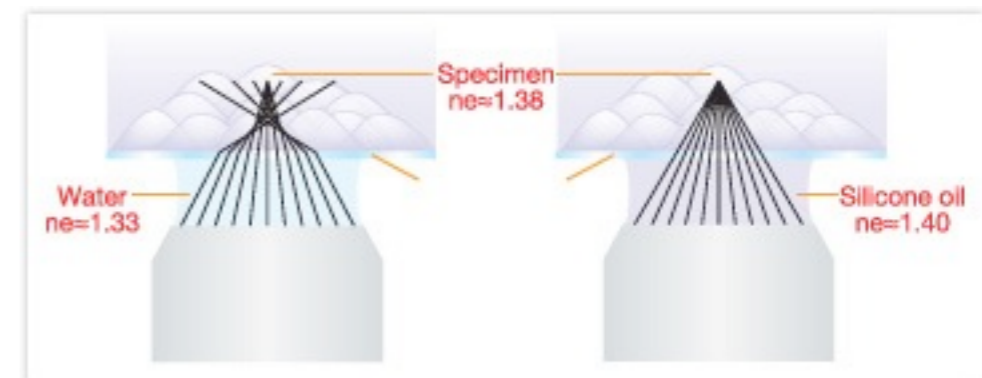
Lenses:

- Specialist Objectives - Water/glycerol immersion objectives



- silicone immersion objectives

http://www.olympusamerica.com/seg_section/seg_silicone_oil_objectives.asp



deeper imaging into live samples

Very Expensive!!

Specimen Preparation:

- Oil objectives image best close to the coverslip

Mount the specimen appropriately

Use alternative immersion lenses

- Vibration / movement can degrade imaging

Adhere cells to substrates

Tricks to keep specimens still

- Ensure the viability of your sample

Media / drying out

Temperature / CO₂

Choice of imaging technique

Use a confocal for:

Bright, thick specimens with low contrast

To generate high resolution 3D image reconstructions

Easy simultaneous multichannel imaging

Use wide-field deconvolution for:

Weakly fluorescent, sensitive specimens

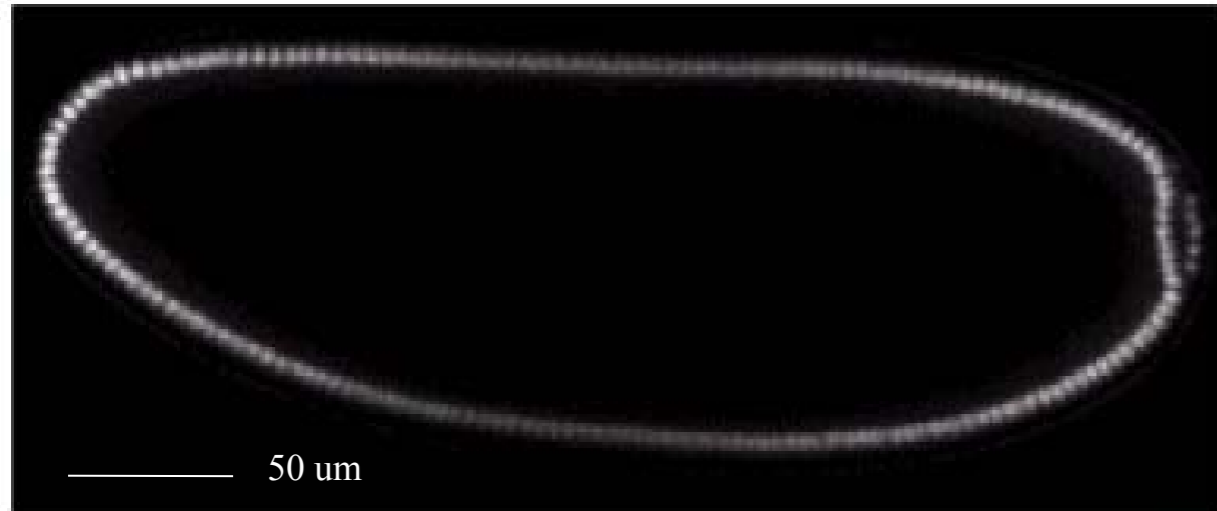
Following fast dynamic events

Use TIRF for:

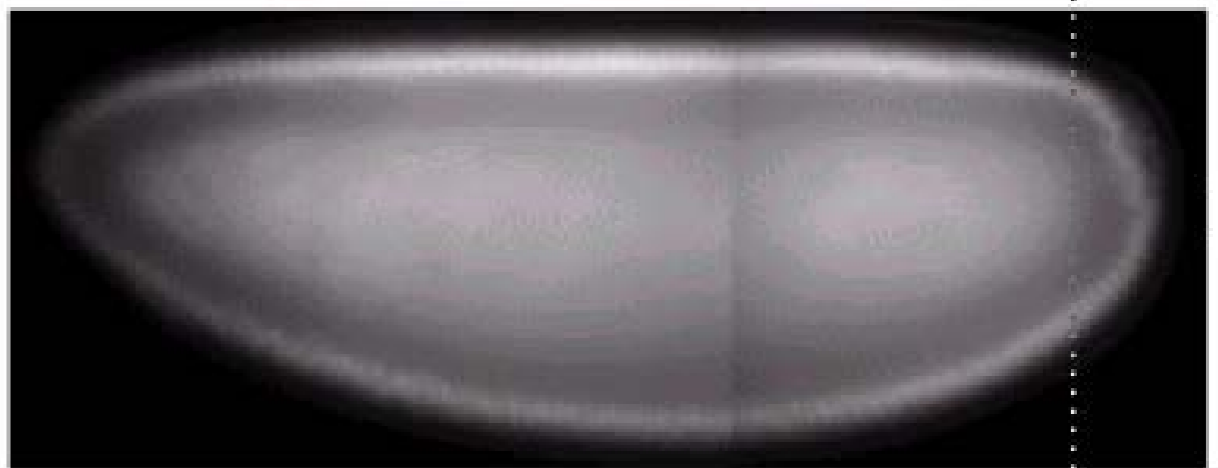
Imaging with high contrast within 100 nm of the coverslip

Choice of imaging technique: Example of a thick specimen

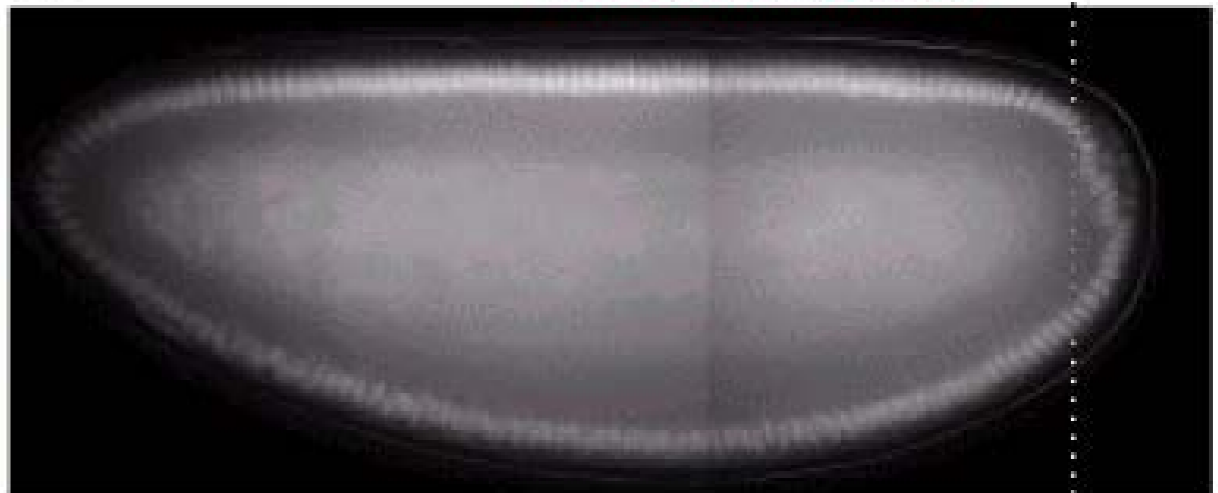
Confocal



Wide-field



WF- deconvolved



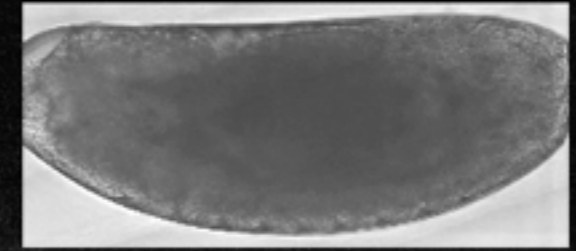
Drosophila embryo, nls GFP
Thick, bright specimen

For really thick specimens consider
point scanning confocal or multiphoton



Multiphoton

Confocal



Multiphoton

(5 μm z-step)

can be combined with SPIM (DLSTM)

Confocal vs Widefield Deconvolution

Confocal (optical configuration)

- **Discards out-of-focus light** using a pinhole in the light path
- **Less sensitive - throws away light, generally poorer signal to noise**
- **More convenient - immediate high contrast images, even with **single Z sections**.**
- **Electronic zoom**
- **Deals well with **strong but diffuse signal with a lot of out-of-focus light** (low contrast)**
- **Confocal images can be deconvolved as well**

Widefield Deconvolution (processing)

- **Reassigns out-of-focus light to its point of origin**
- **More sensitive (and quantitative) - Better signal to noise ratio**
- **Less convenient - requires time consuming (post acquisition) calculations, **best with multiple Z sections**.**
- **Better for **point sources** of light and **weak signals****

If the choice is not obvious...



it's worth trying them all.

Live imaging as an experimental tool:

F* techniques to measure protein interactions and dynamics:

- **FRAP** (Fluorescence Recovery After Photobleaching)
- **Photo-activation** (PA-GFP)
- **FRET** (Fluorescence Resonance Energy Transfer)
- **FLIM** (Fluorescence Lifetime IMaging)
- **FCS** (Fluorescence Correlation Spectroscopy)

Live imaging as an experimental tool:

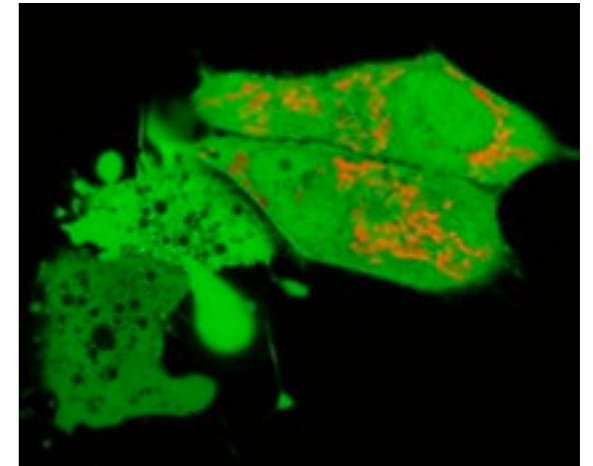
Using light to manipulate cell behaviour:

- **“Killer red”** genetically encoded photosensitiser
CALI = chromophore assisted light inactivation

Reactive oxygen species in photochemistry of the red fluorescent protein “Killer Red”

Vegh et al, Chem. Commun., 2011,47, 4887-4889

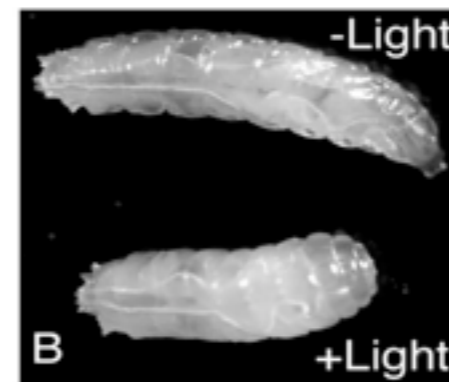
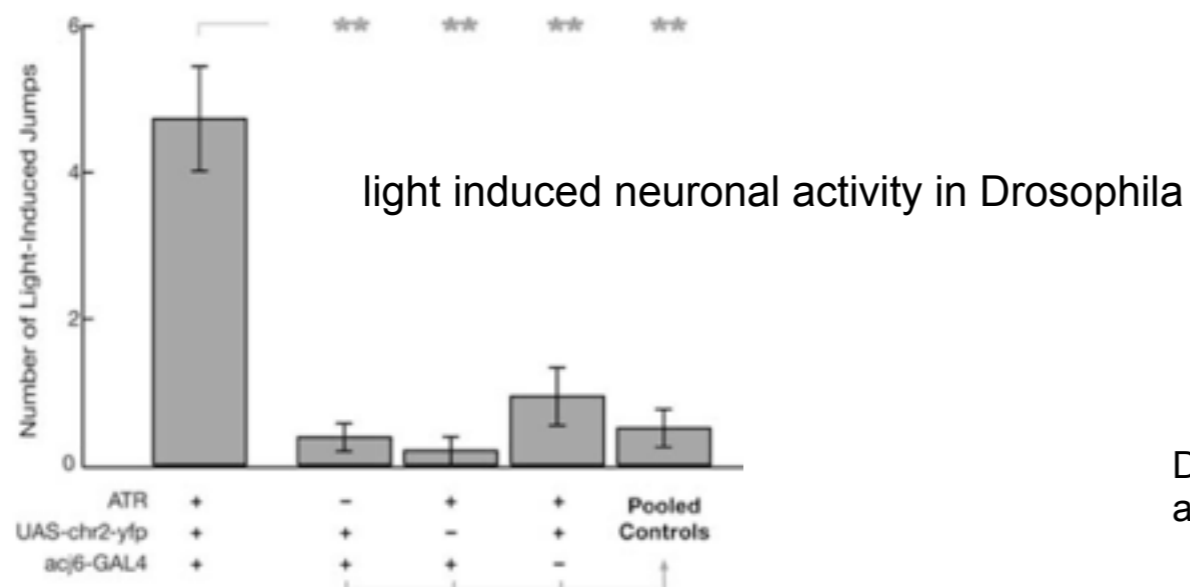
DOI: 10.1039/C0CC05713D



EVROGEN - Killer red expressed in mitochondria

- **Channelrhodopsin-2 (ChR2)** photo-induced behaviour through light activation of cation-selective ion channels

Zimmermann, G., et al. (2009). Manipulation of an Innate Escape Response in Drosophila: Photoexcitation of acj6 Neurons Induces the Escape Response. PLoS ONE, 4(4), e5100. doi:10.1371/journal.pone.0005100.g005



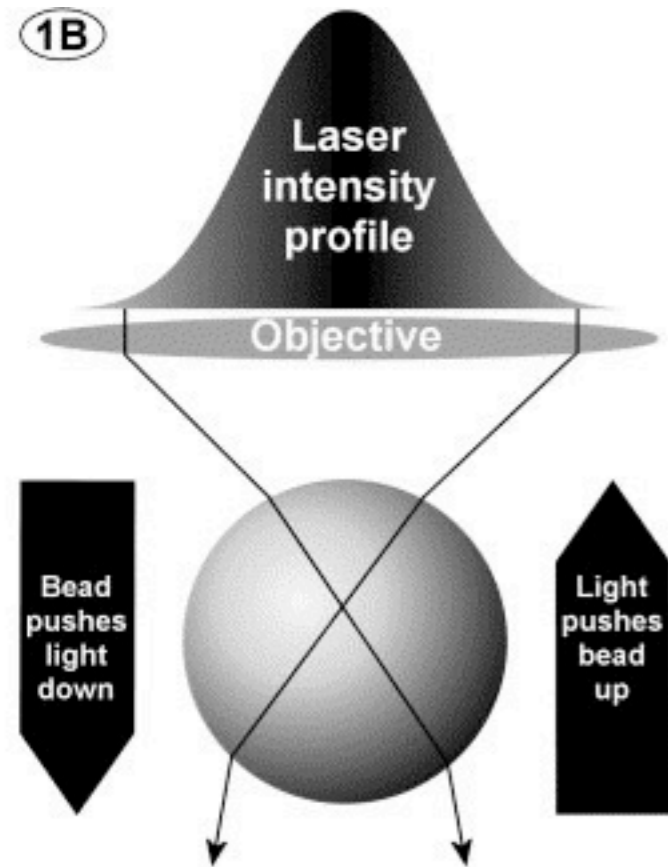
D42-GAL4 motor neuron driver and three copies of UAS-chr2::yfp

“Photoexcitation of acj6 neurons is sufficient to induce a startle response”

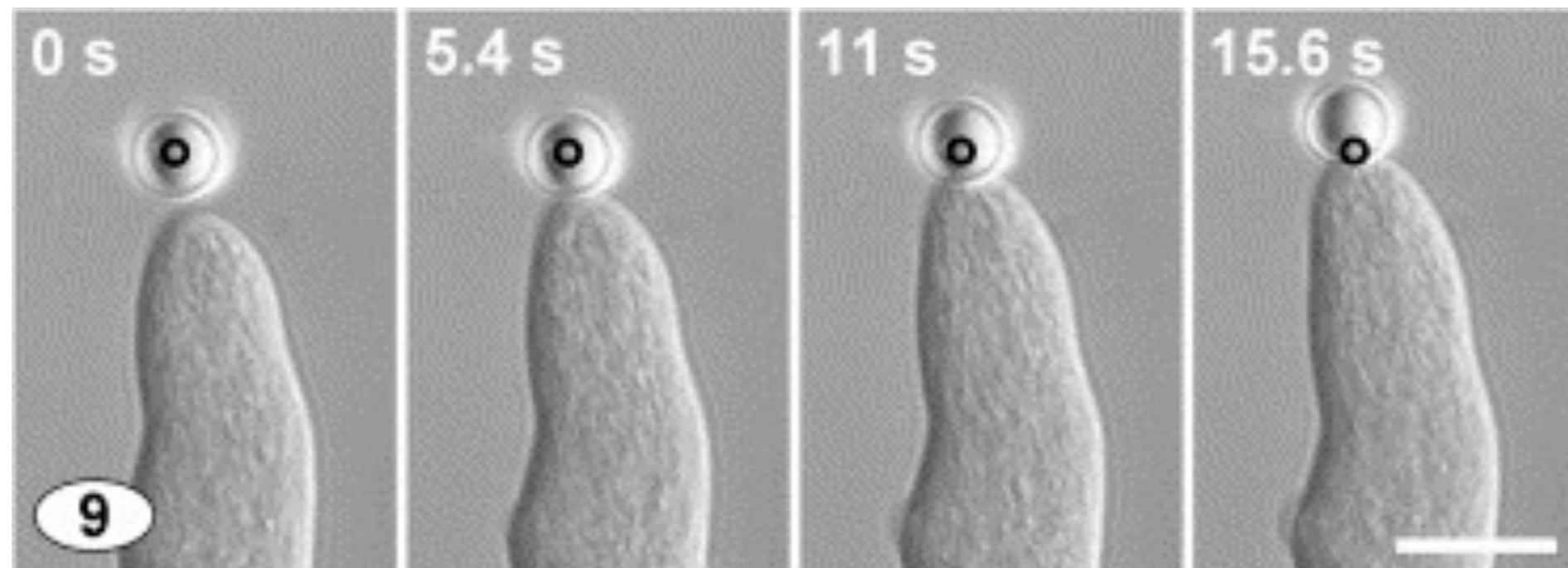
Live imaging as an experimental tool:

Using light to manipulate cell behaviour:

laser tweezers (optical trap)



manipulating the behaviour of fungal hyphae



output laser power (70 mW) was used in this experiment, which equates to a trapping force of 19 pN

Graham D. Wright et al., Fungal Genetics and Biology. [Volume 44, Issue 1](#), January 2007, Pages 1–13

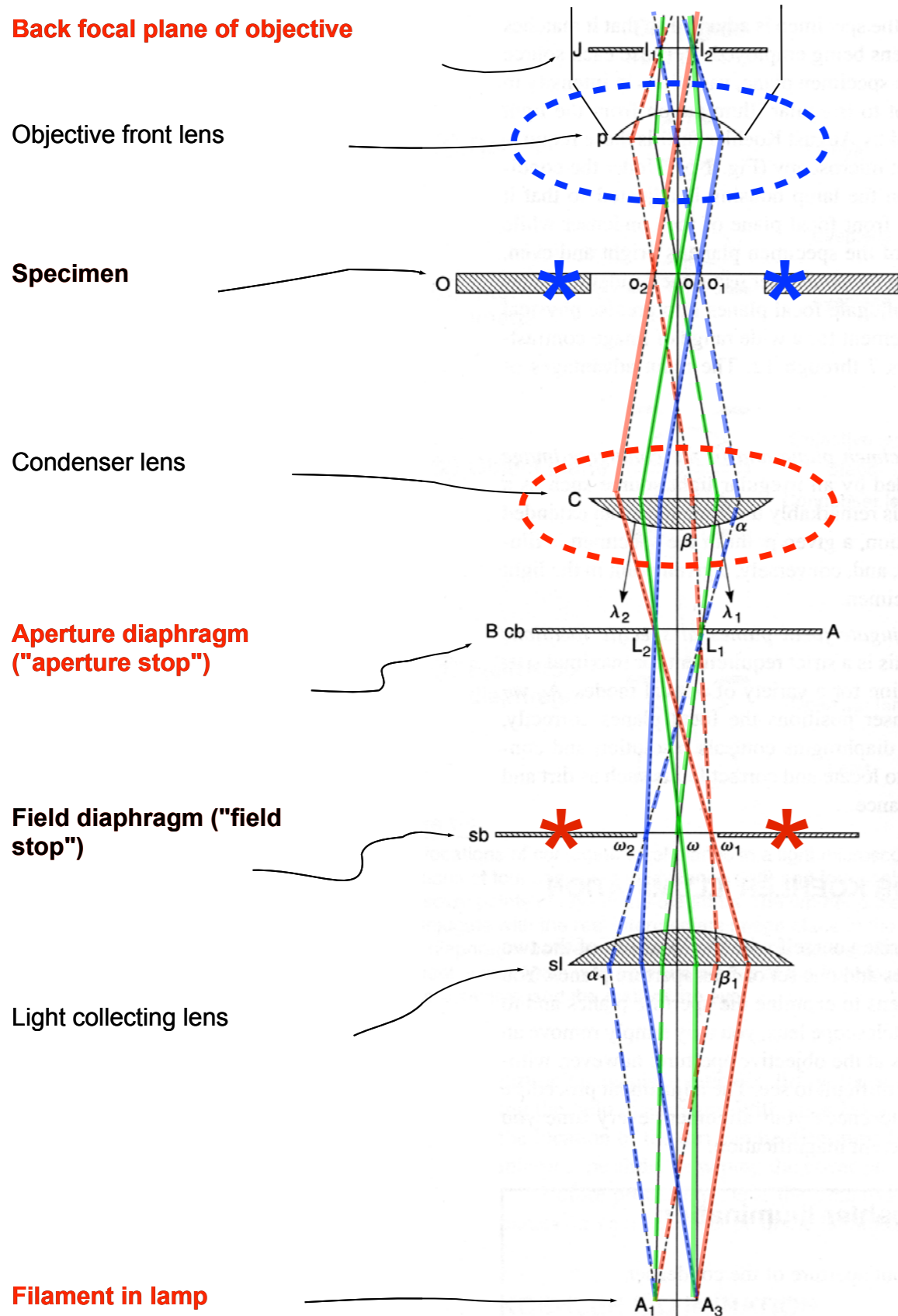
<http://dx.doi.org/10.1016/j.fgb.2006.07.002>

Setup your imaging equipment properly:



Koehler alignment:

CONJUGATE PLANES - bright field



Conjugate Planes in the Optical Microscope

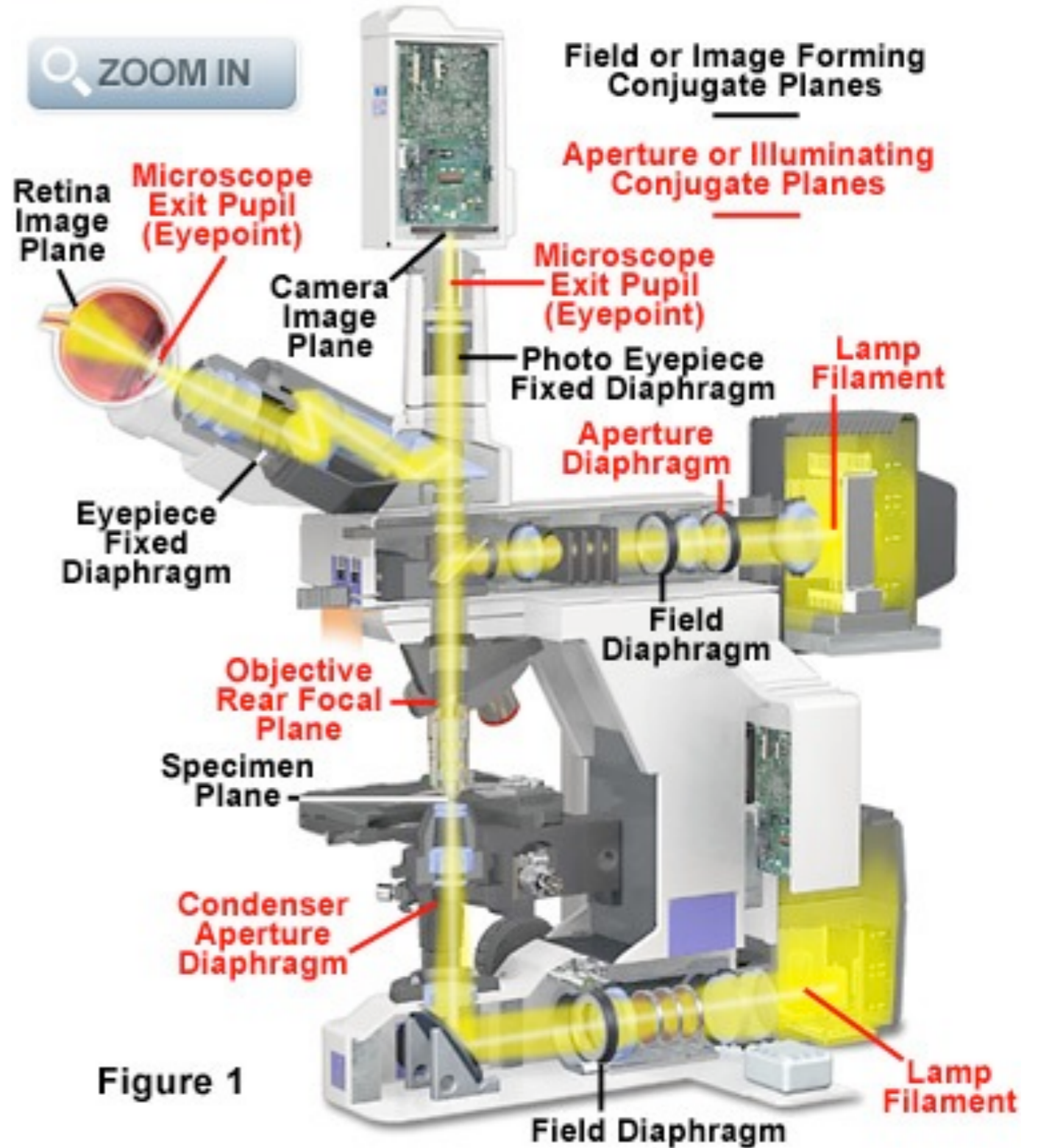
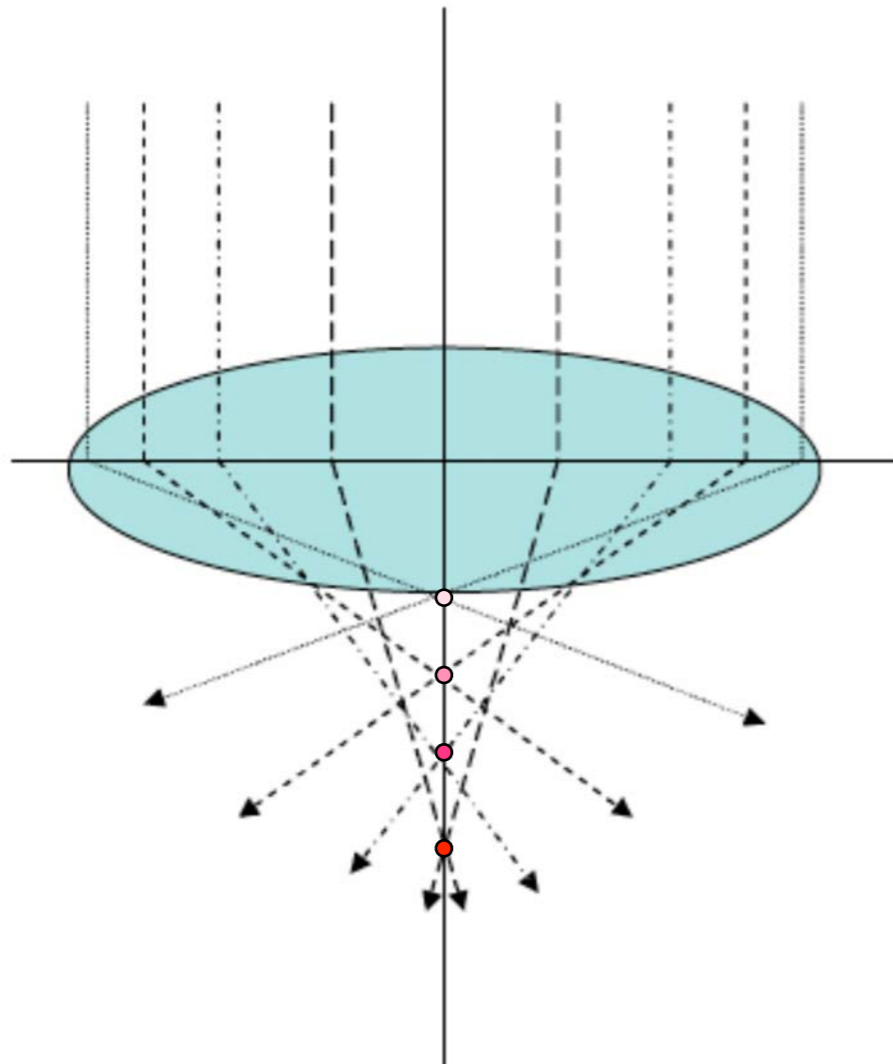


Figure 1

Correct Spherical Aberration:

Spherical aberration (SA) - beams passing through different parts of the lens brought to different focal points

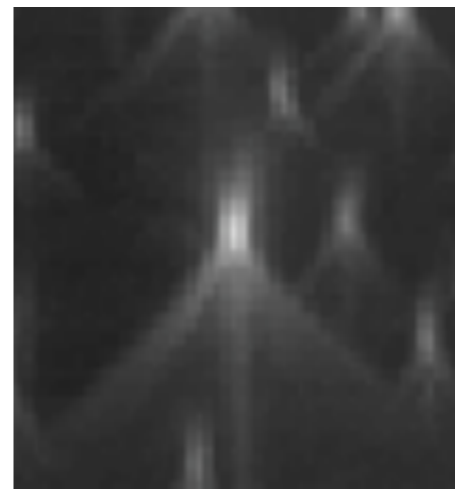


Confocal

- “In focus” light is blocked by the pinhole

Wide field

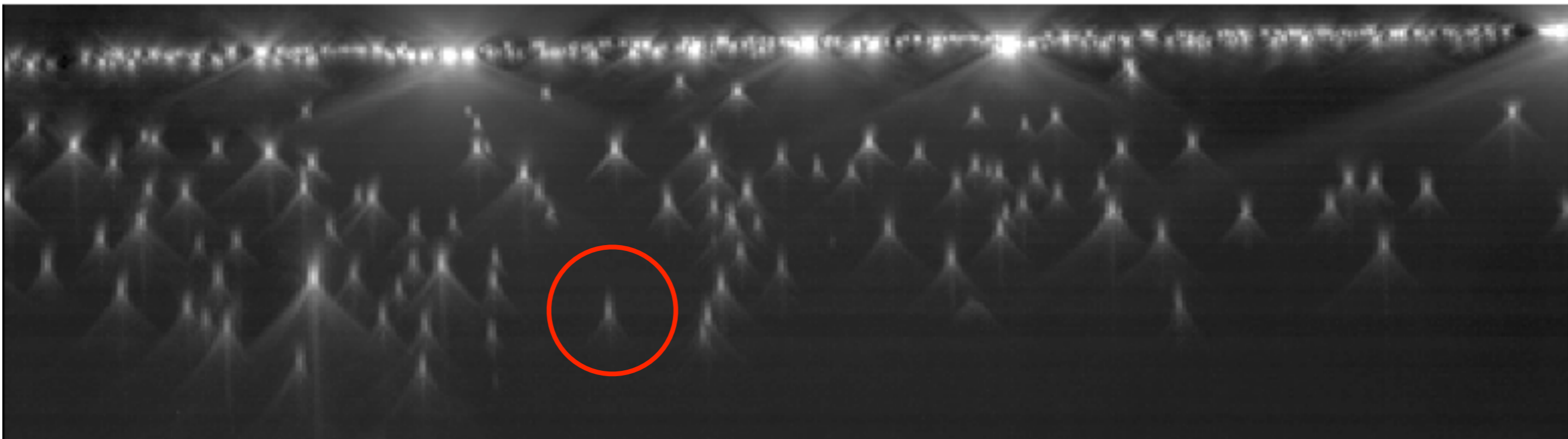
- Detail is “smeared” in Z



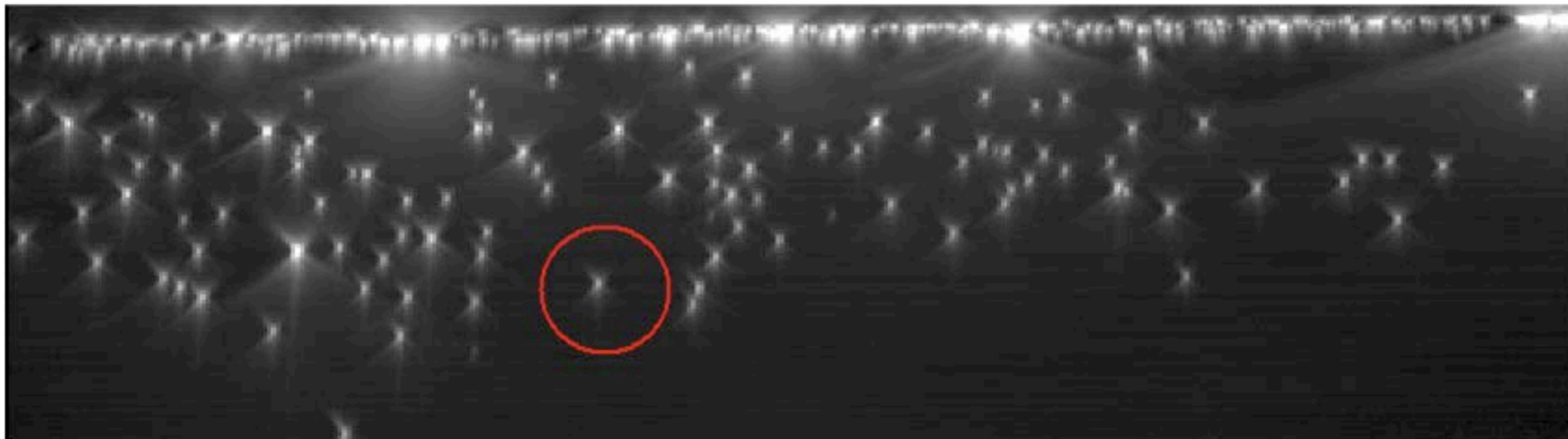
SA results from refractive index mismatch along the optical path:
glass 1.514; oil 1.33-1.534; air; 1.0; water 1.33; cell 1.35-1.6; 70% glycerol 1.47

Correct empirically using beads:

Spherically aberrated



Corrected Increased signal and resolution!

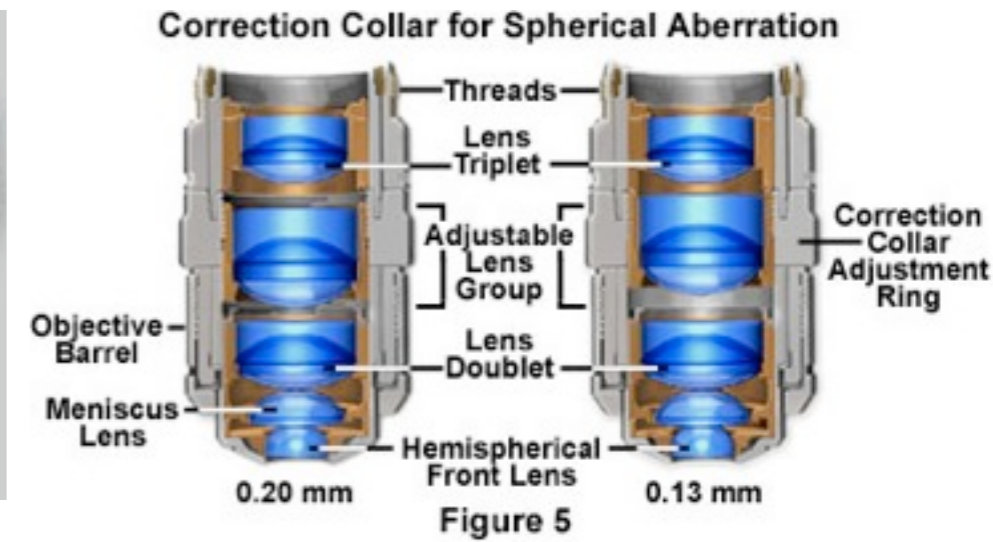


Correcting Spherical Aberration:

- Immersion oils

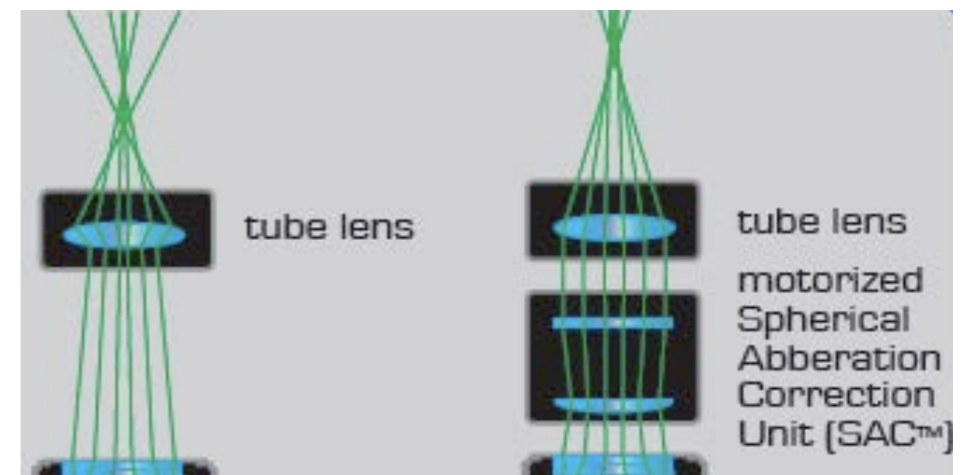
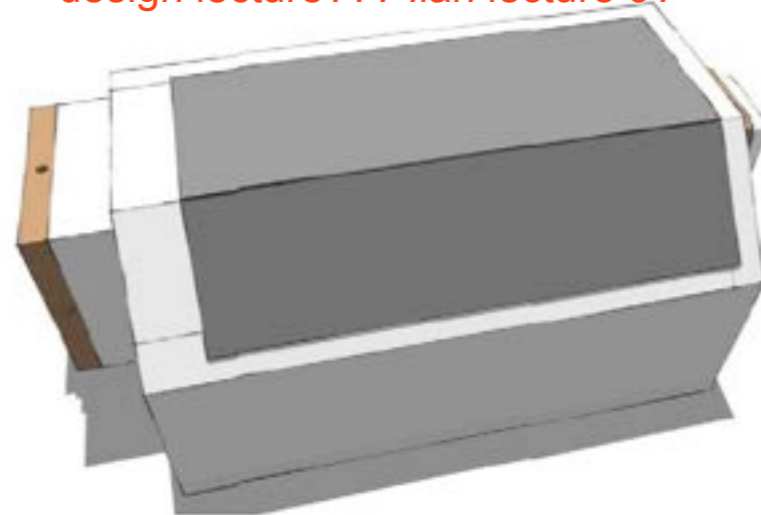


- Objective SA correction collar



- Adaptive optics

mention adaptive spherical aberration correction - refer to custom experimental design lecture??? Ilan lecture 9?



Collect every photon:

- Properly setup your equipment
- Correct spherical aberration
- Use bright field to minimise photodamage
- Reduce fluorescence exposure
- Use the best fluorochromes
- Optimise filter-sets
- Use sensitive detectors (lecture 10)



Matching Fluorescent Probes to Filter-Sets:

Covered in lecture 4 - Eva Wegel

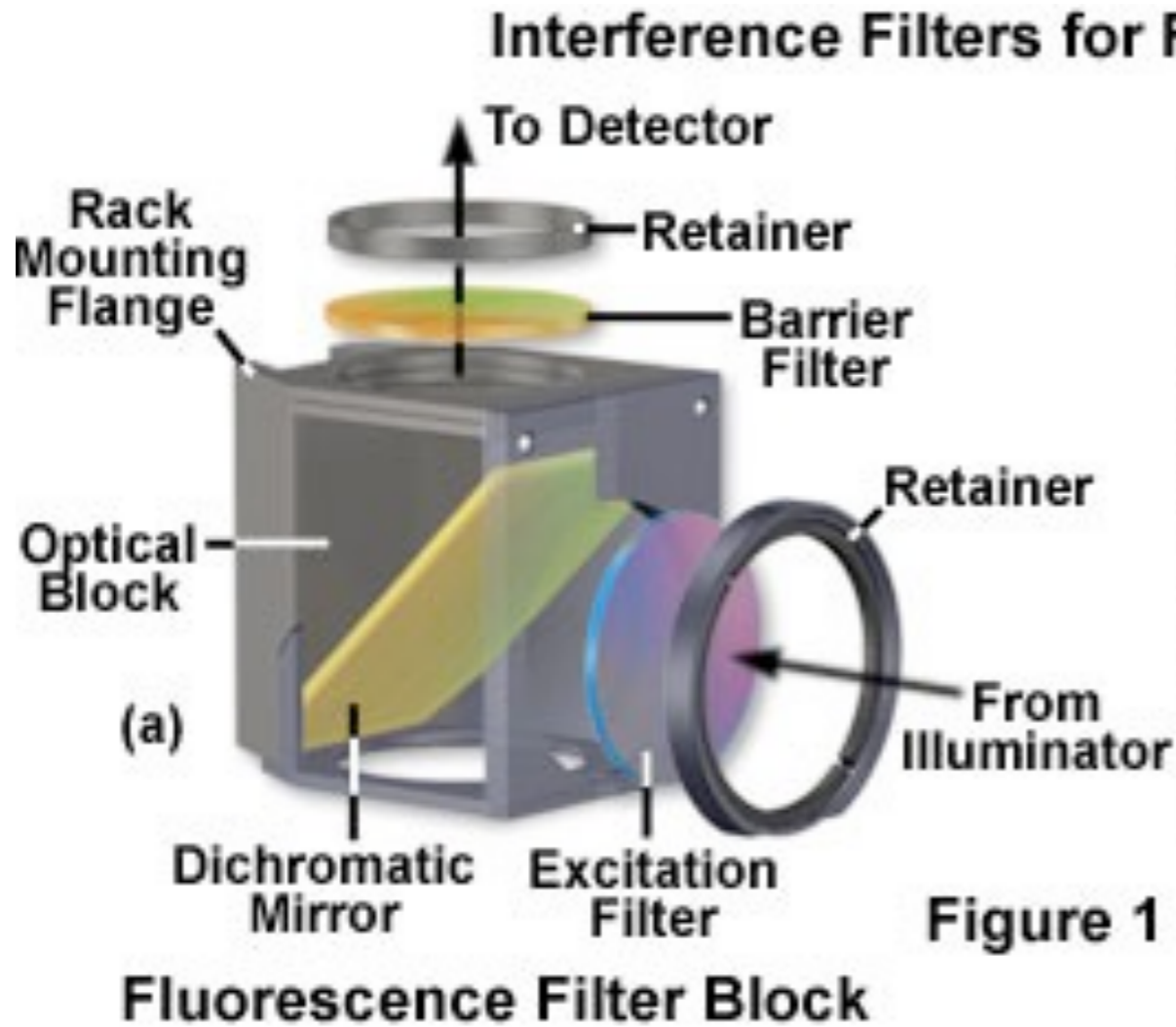
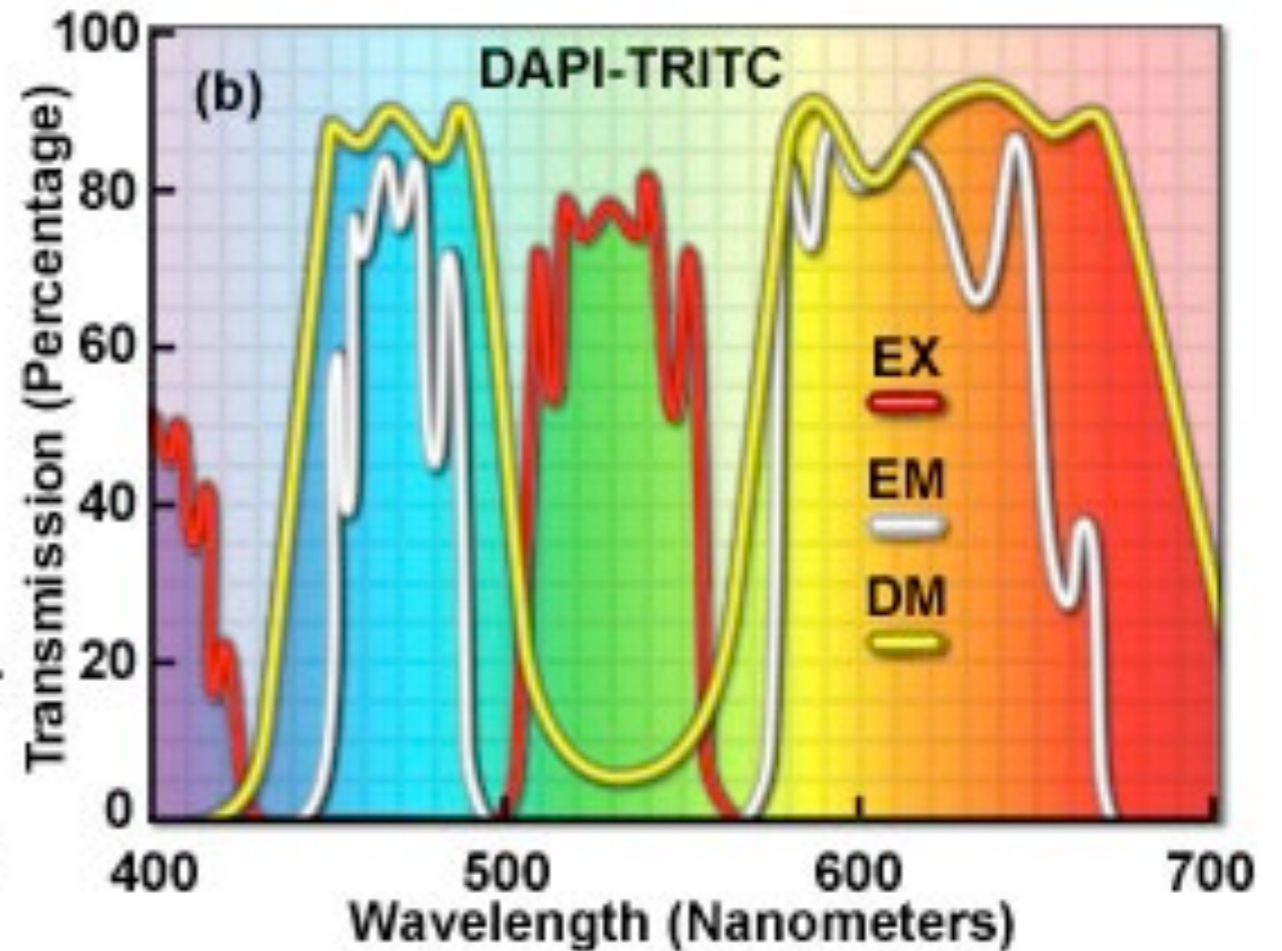
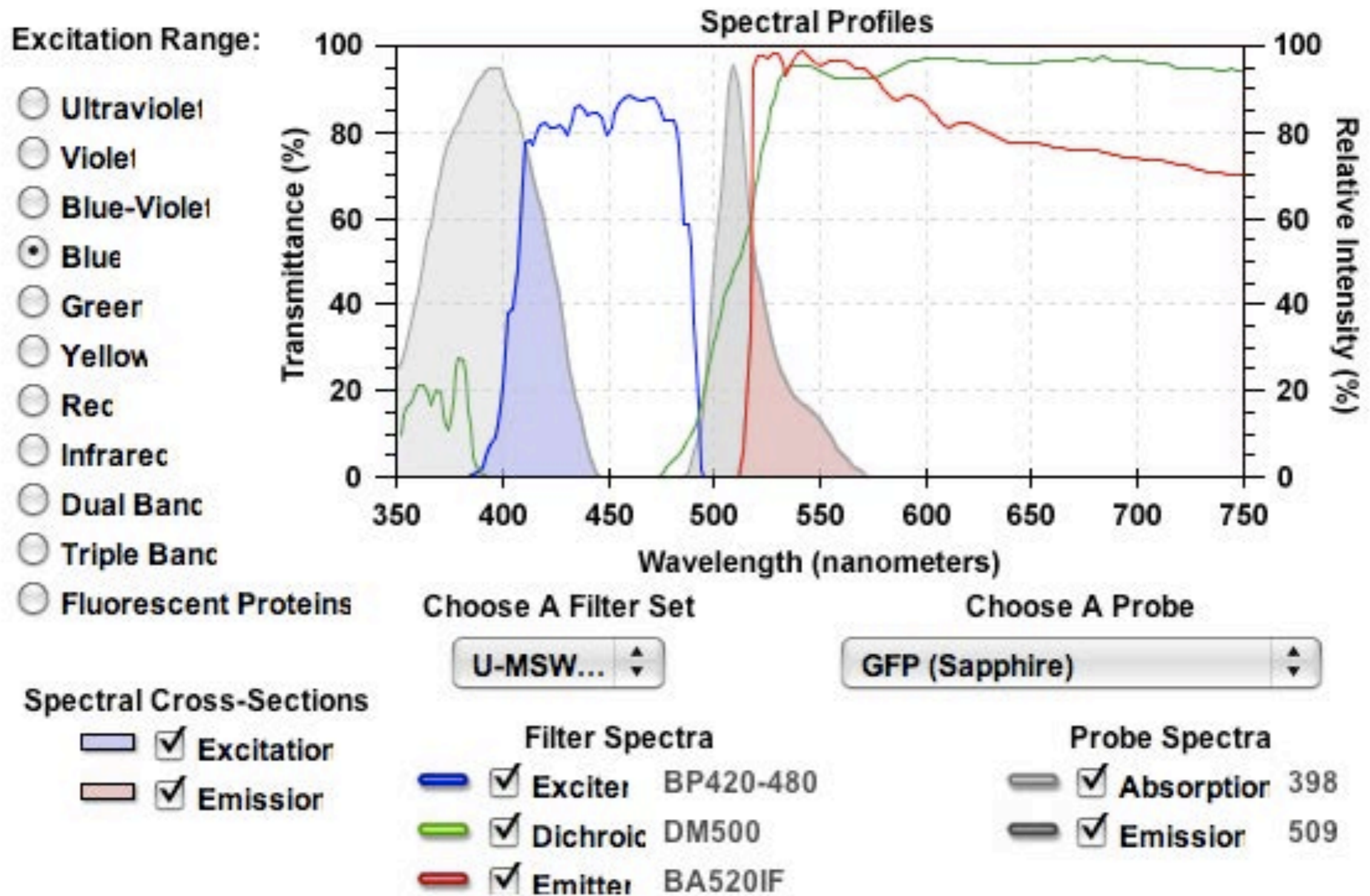


Figure 1

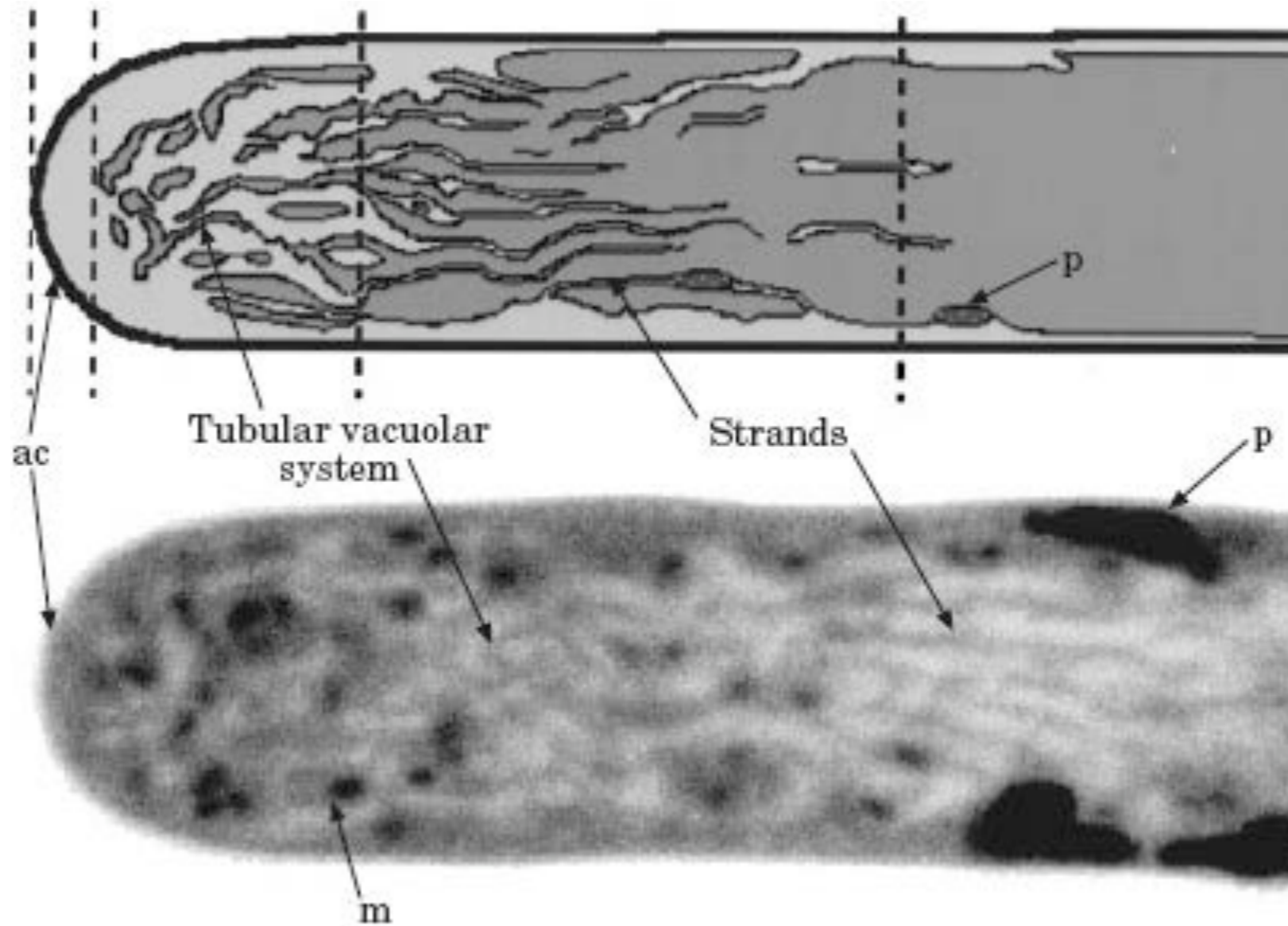


Matching Fluorescent Probes to Filter-Sets



Loading dyes into living cells:

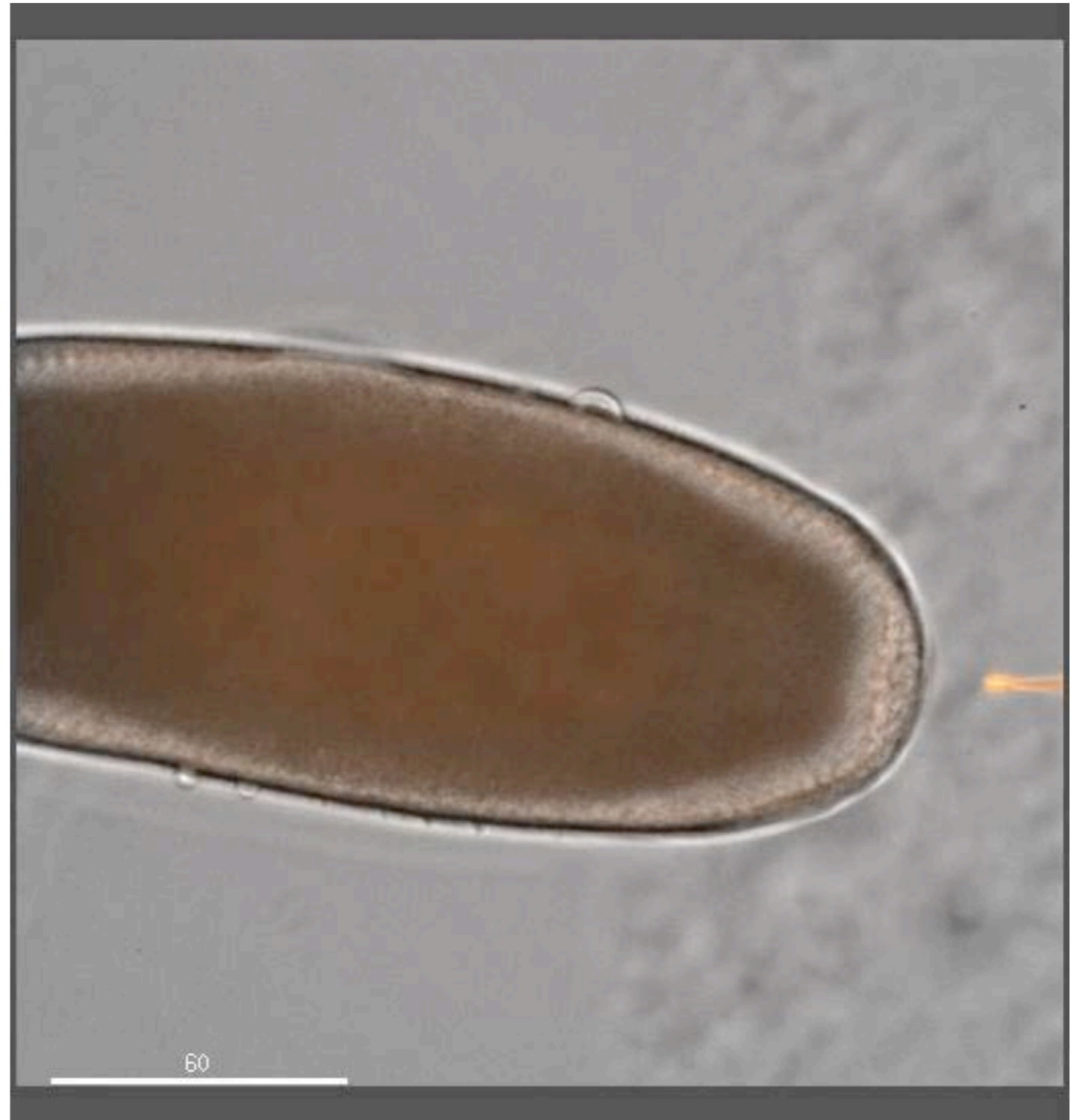
- Cell permeant dyes



Nile Red: lipid stain
Rhizoid of fern gametophyte

Injection into cells:

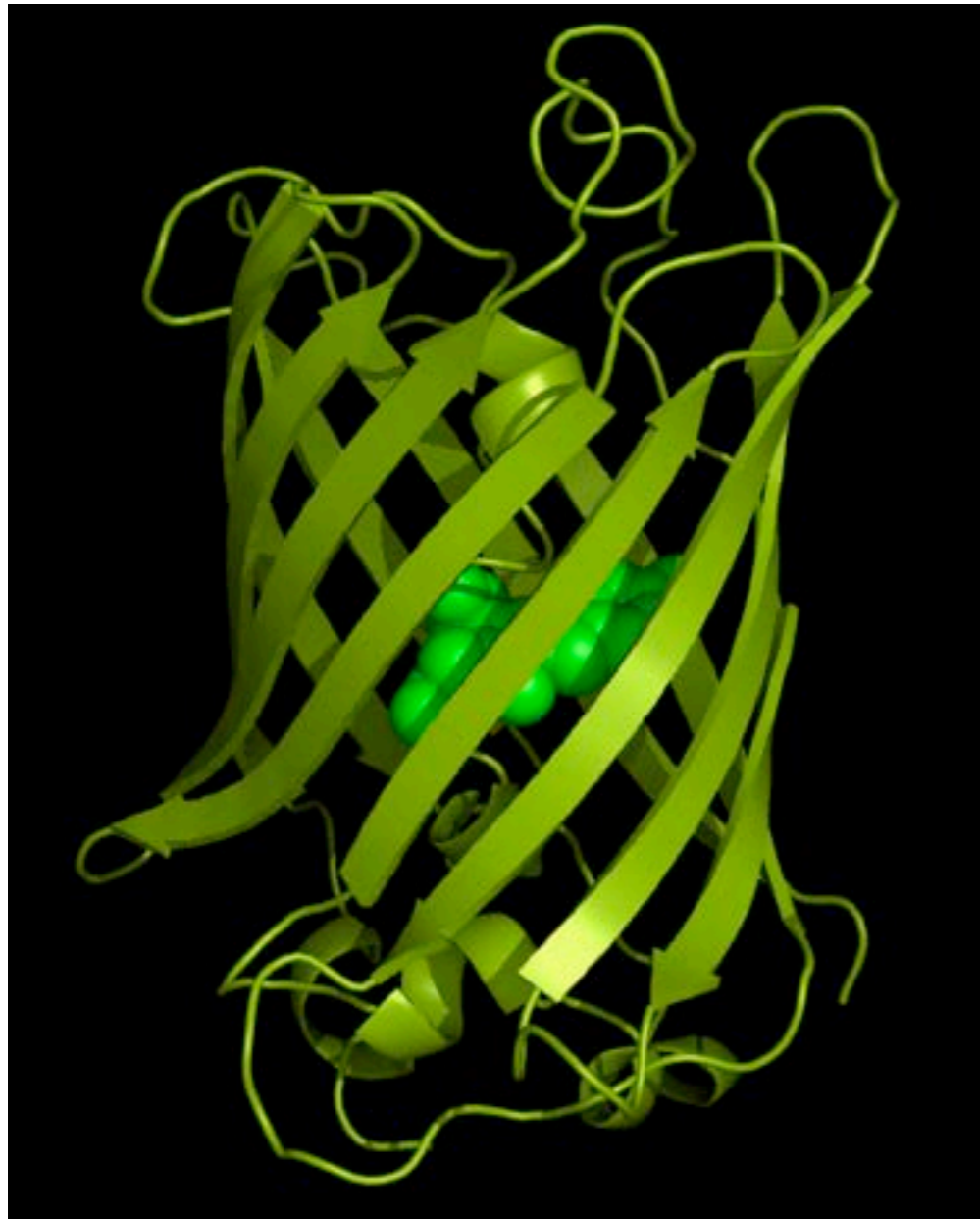
- Virtually any probe
- Technically difficult
- Expensive equipment
- Not all cell types amenable
- Potentially damaging



Injection into a *Drosophila* embryo

Transgenic GFP:

Covered in lecture 6 - Mark Howarth



beta-barrel in light green
fluorophore in bright green

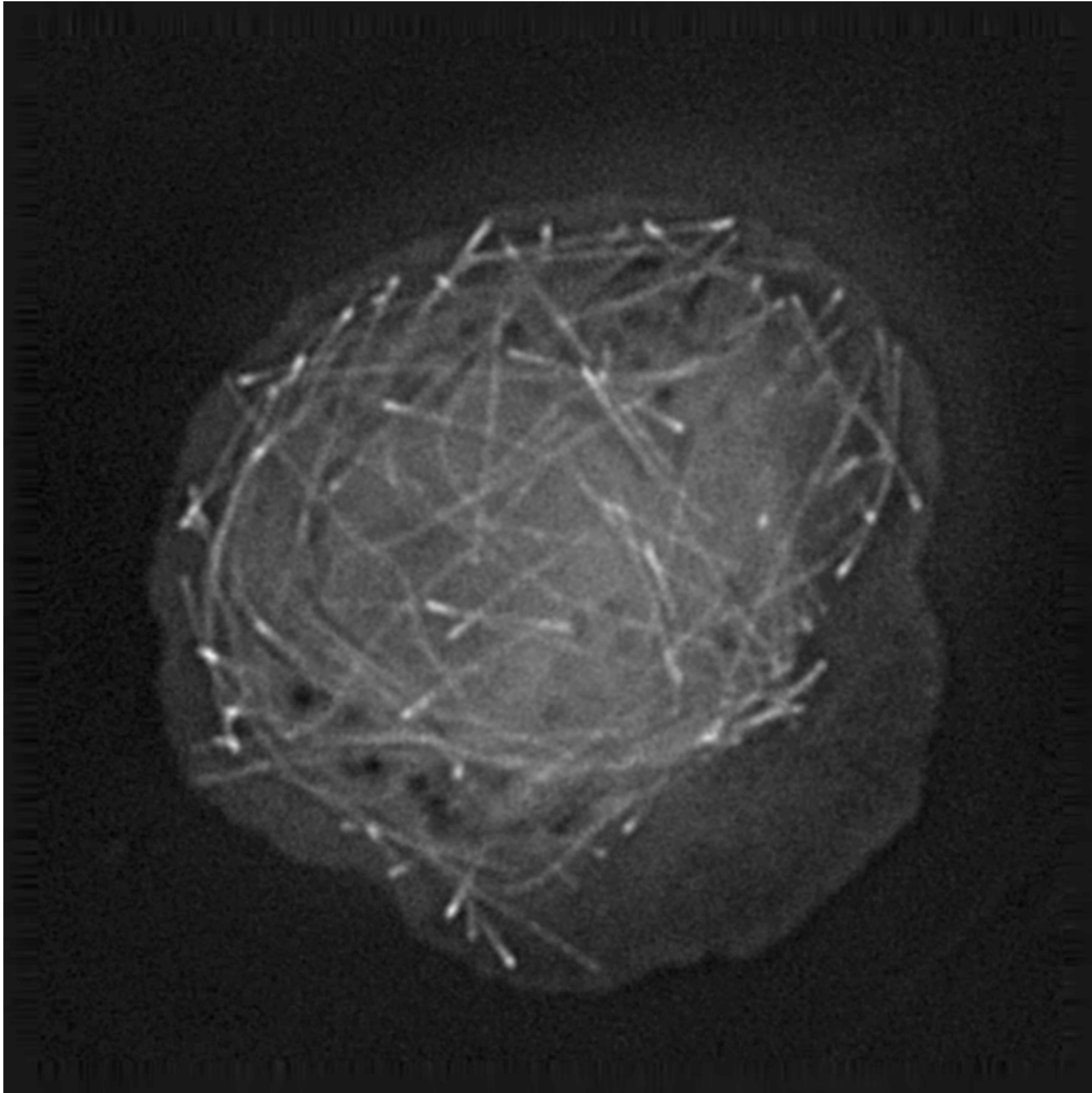
(Ser-65, Tyr-66, Gly-67)

- GFP is inherently fluorescent
- 238 AA; 27 KDa; 4nm - dominated by an 11 stranded beta-barrel
- Fluorochrome forms by the post-translational oxidisation and cyclisation of residues 65 to 67 during folding
- Can be expressed in many organisms
- GFP can be functionally expressed as N or C terminal conjugates to other proteins
- Protein traps are preferable to over-expression lines

Scientists hope to use the GM animals in the study of HIV/Aids

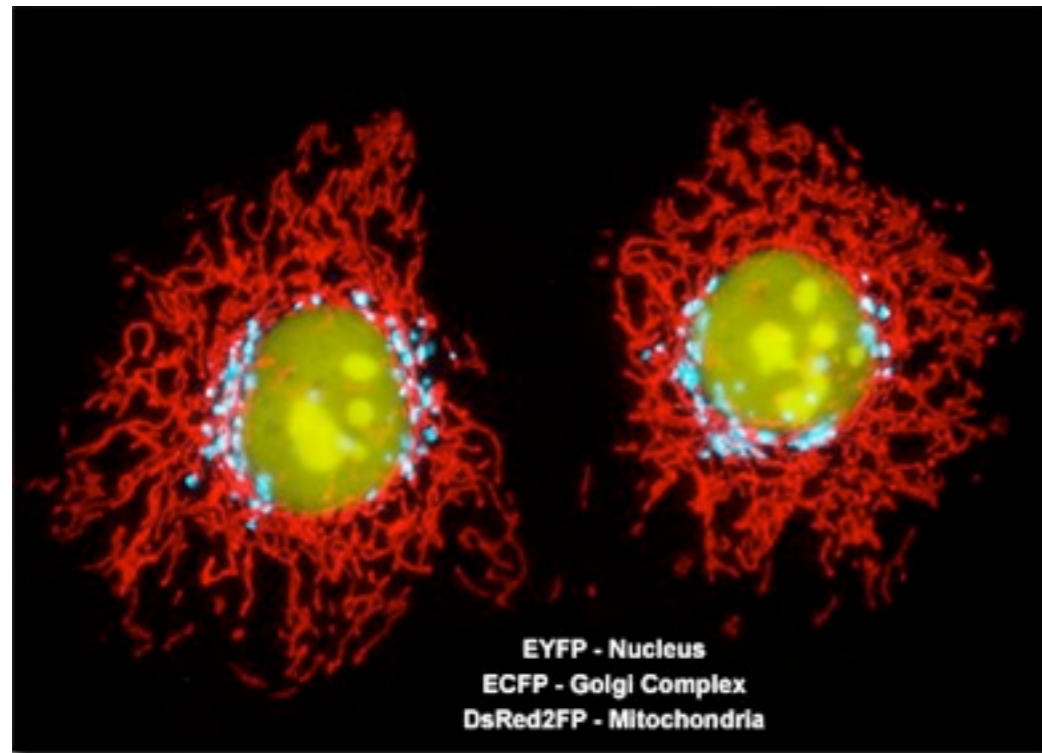


Eric Poeschla, Mayo Clinic



Macrophage: GFP microtubules

Extending the palette of fluorescent proteins

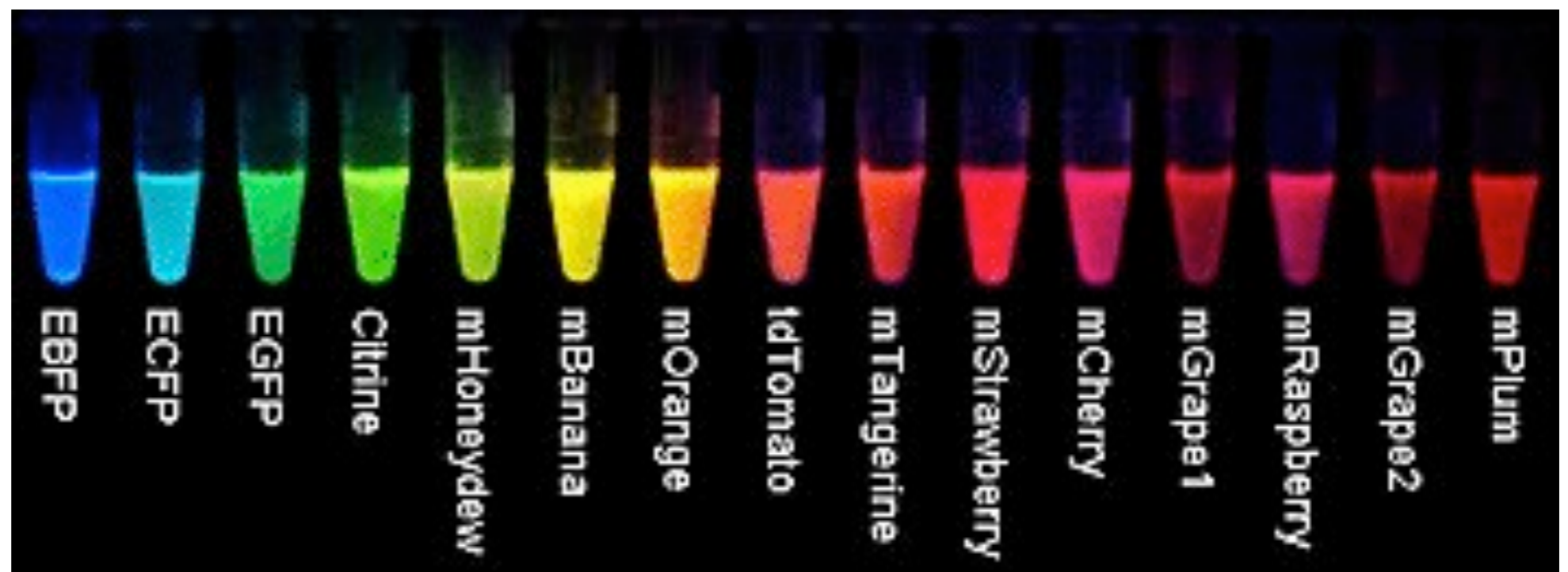


EYFP = enhanced Yellow Fluorescent Protein (GFP derivative)

ECFP = enhanced Cyan Fluorescent Protein (GFP derivative)

DsRed2FP = Red Fluorescent Protein (coral protein, unrelated to GFP, and not monomeric)

Changing the properties of GFP and RFP by genetic engineering



Include Tsien reference!

Post acquisition image processing:

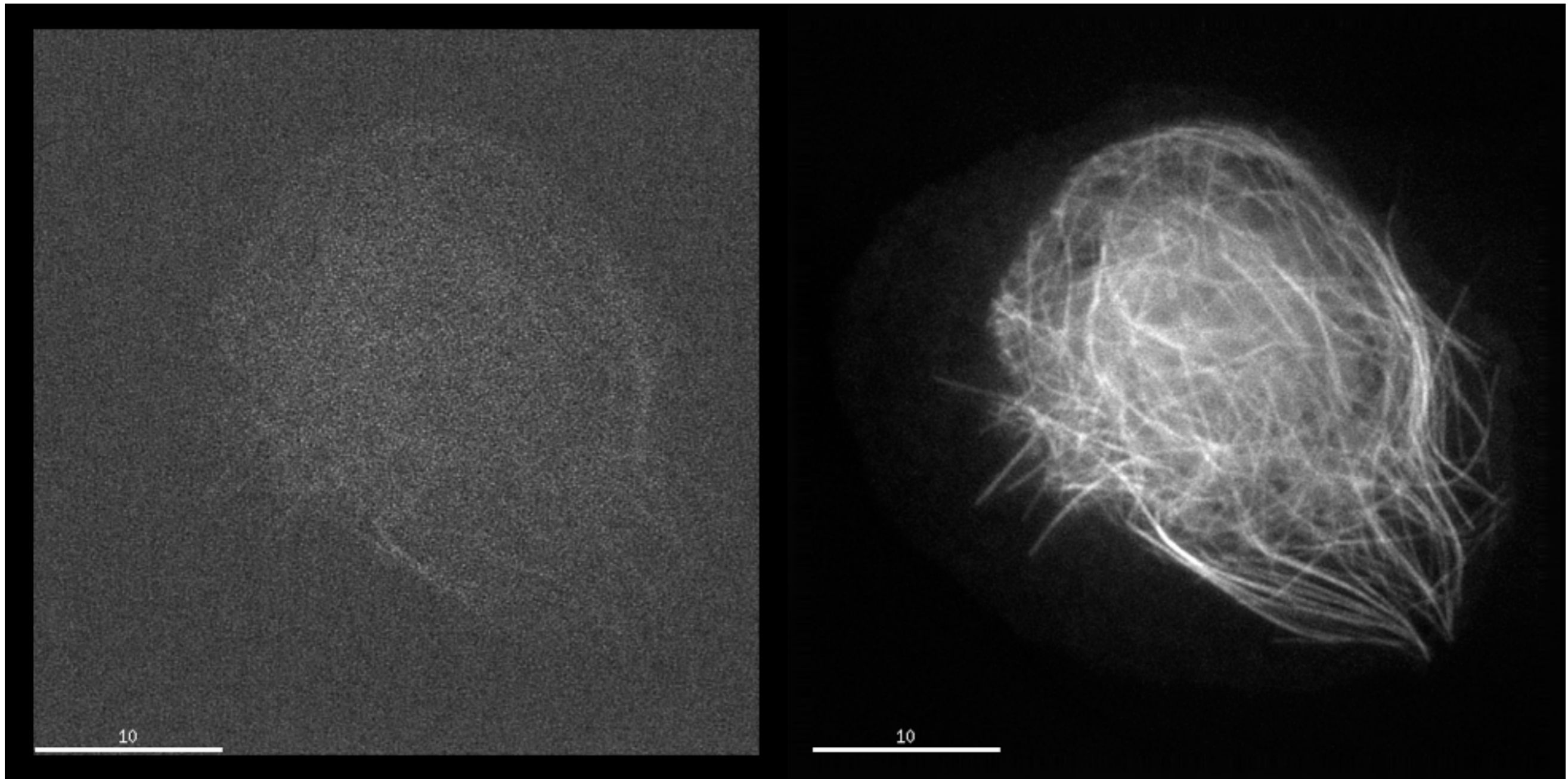


- Deconvolution
- **Denoising**
- Segmentation
- Tracking
- Quantitation

Denoising - imaging with 10-100 x less light

8 ms exposure, 0.1% 488 Laser power

8 ms exposure, 10% 488 Laser power



Live Macrophage: Jupiter-GFP labeling microtubules; 7Z, 3 stacks per second

Jerome Boulanger: SAFIR Denoising software

Integrated into Priism by the John Sedat Group UCSF

J. Boulanger, C. Kervrann, and P. Bouthemy, "Space-time adaptation for patch-based image sequence restoration," *IEEE Trans. on Pattern Analysis and Machine Intelligence*, vol. 29, no. 6, pp. 1096ñ1102, June 2007

Reference Material:

<http://www.olympusmicro.com/>

Live Cell Imaging, (2010) 2nd Edition Eds Goldman, Swedlow, Spector.
Cold Spring Harbour Press.

END

