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

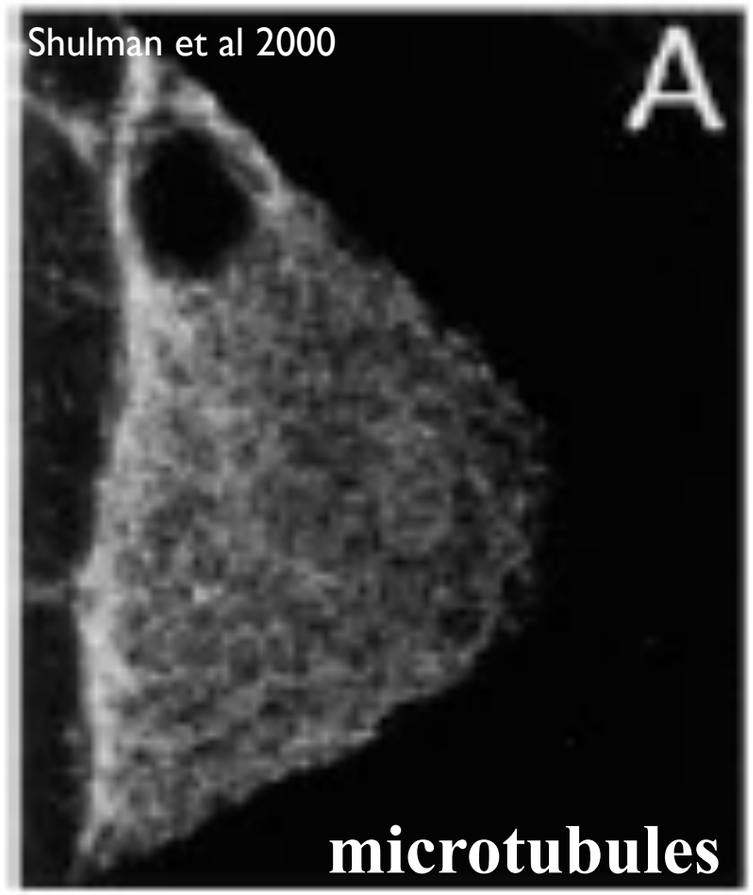
  Choice and setup of equipment

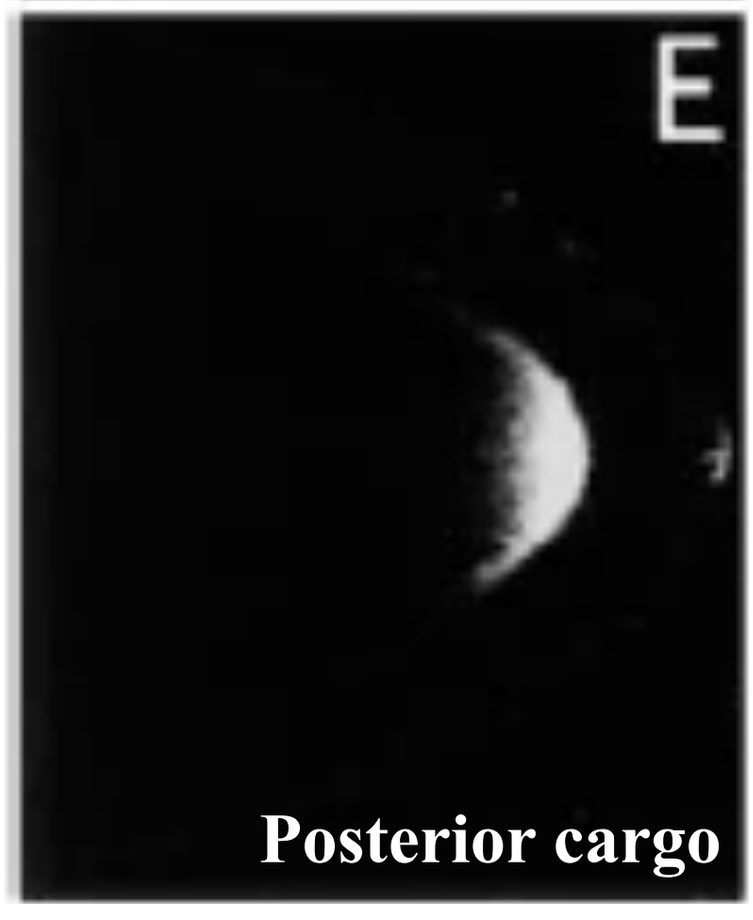
  Collect every photon

  Image processing and analysis

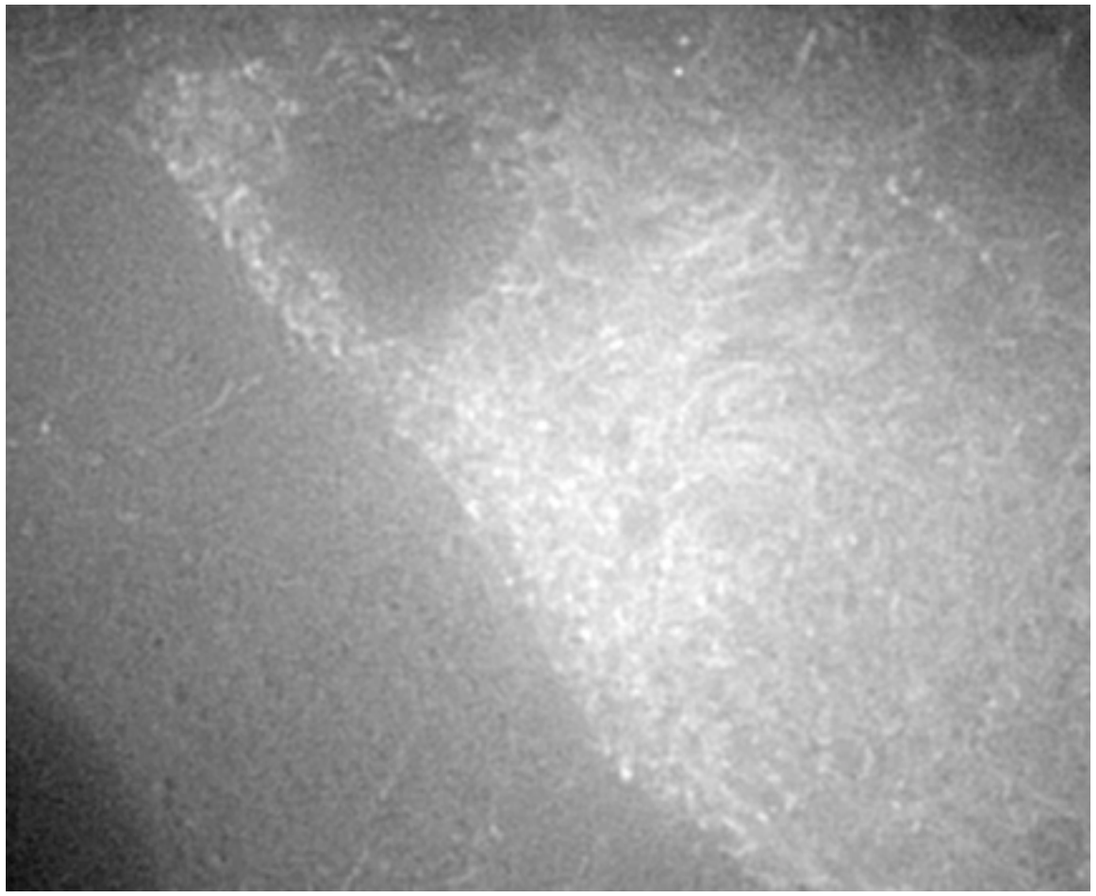
Reasons for live imaging: Fixed vs Live

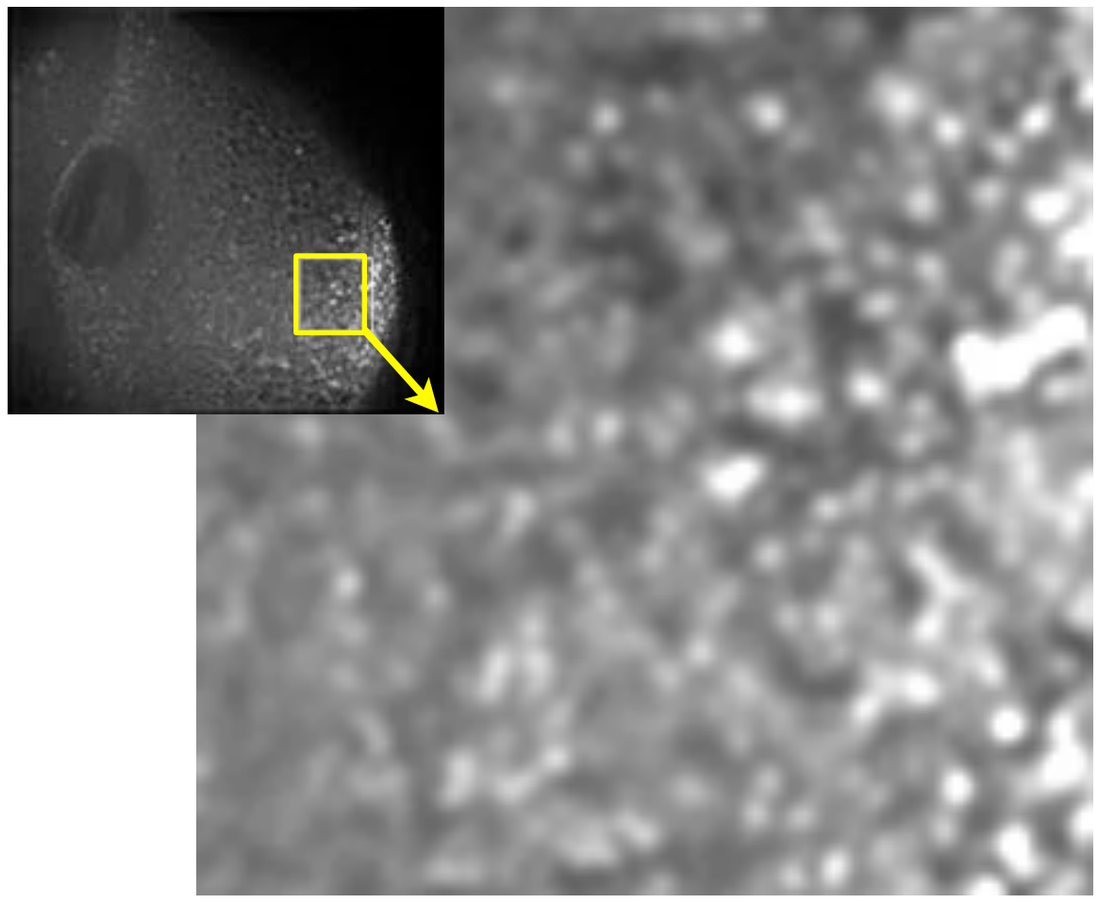
Fixed





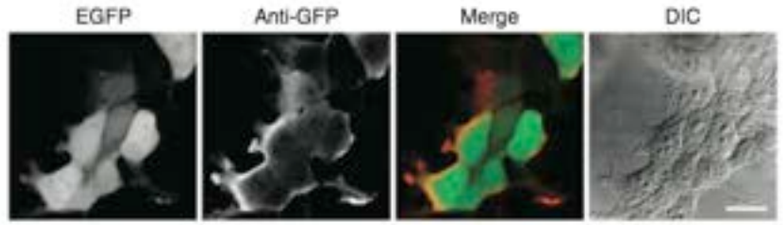
Live





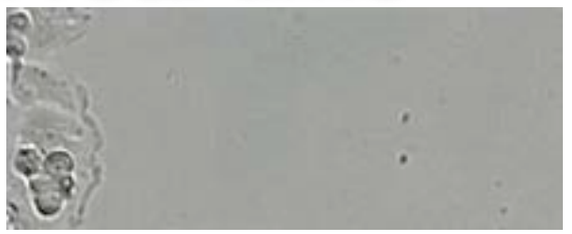
Reasons for live imaging

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



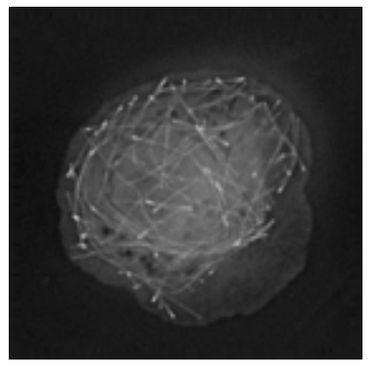
2) Can follow the order of sequential events in real time

time-course of cell migration - Andrea Linford Barr lab



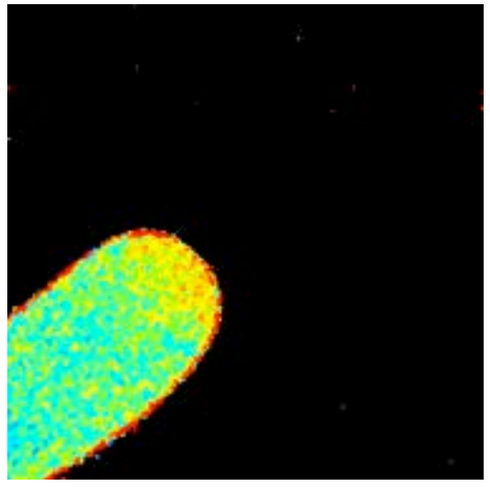
3) Can monitor the kinetics of dynamic processes:
- active transport vs diffusion
- Microtubule turnover

Macrophage: EB1-GFP tagged MT



4) Can record sensitive or transient processes:
- Calcium signalling transients
- Ion gradients
- membrane potential

Calcium ratio imaging pollen tube

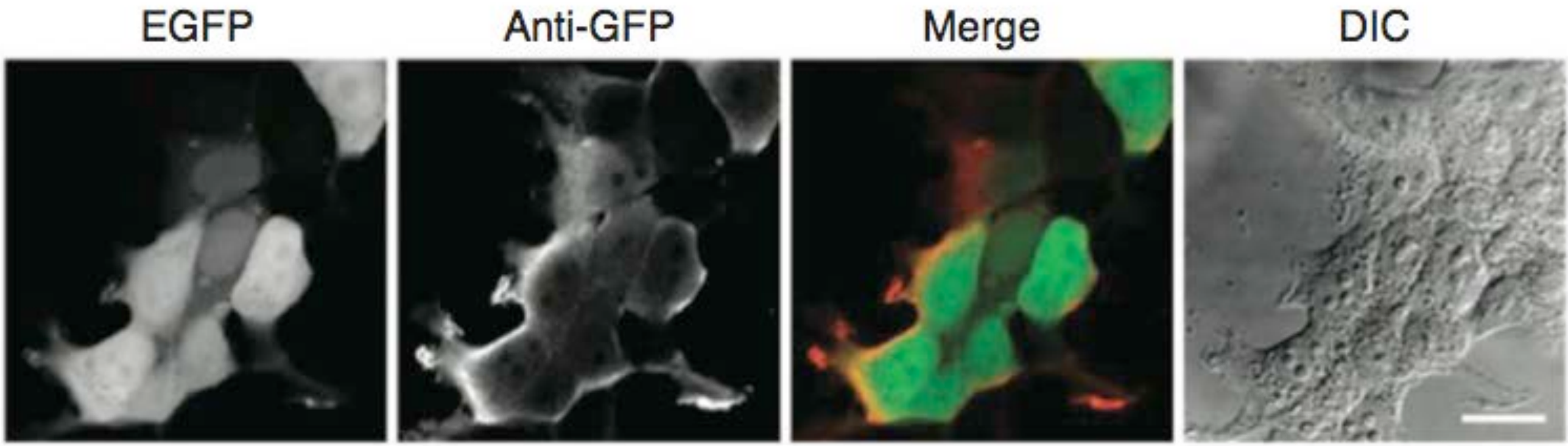


You can believe what you see - no fixation artifacts

Immunolabeling artifacts and the need for live-cell imaging

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

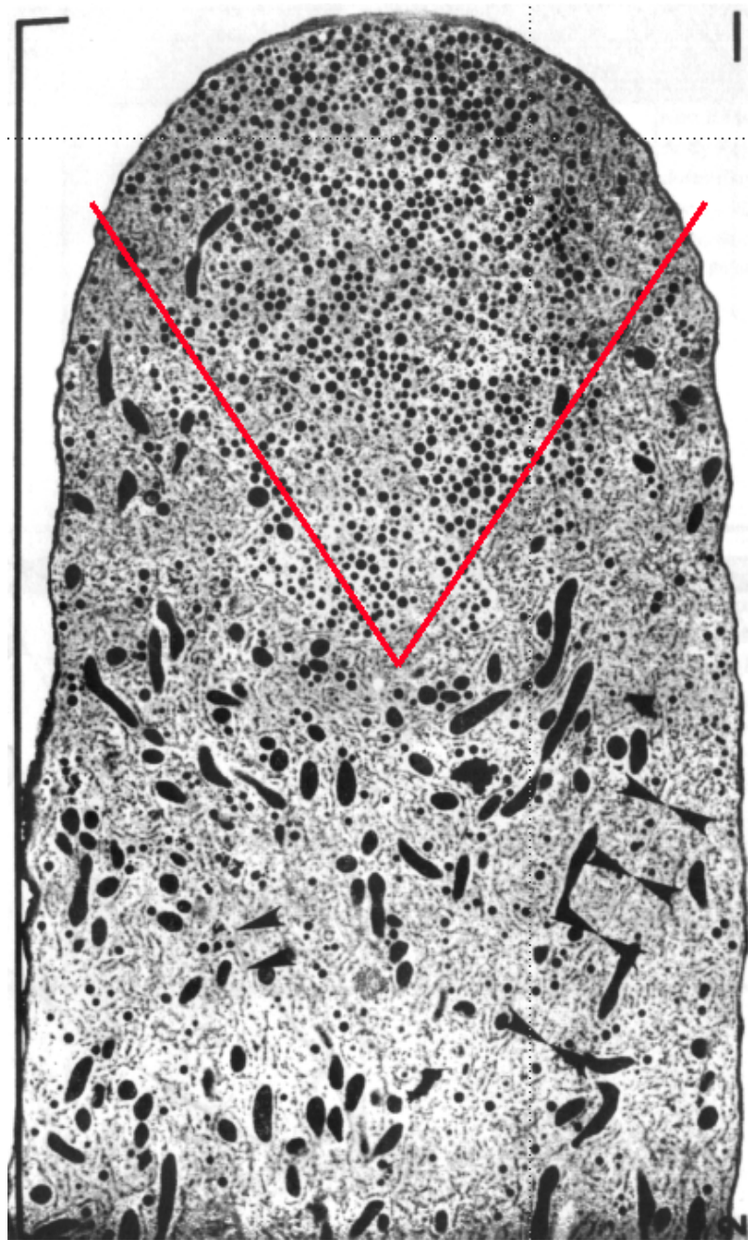
Nature Methods, 9(2), 152–158. doi:10.1038/nmeth.1855



** Eva - lecture 4 - sample prep and minimizing artifacts **
** Errin - lecture 17 / Rainer - lecture 15 EM, correlative light and EM, super-precision microscopy **

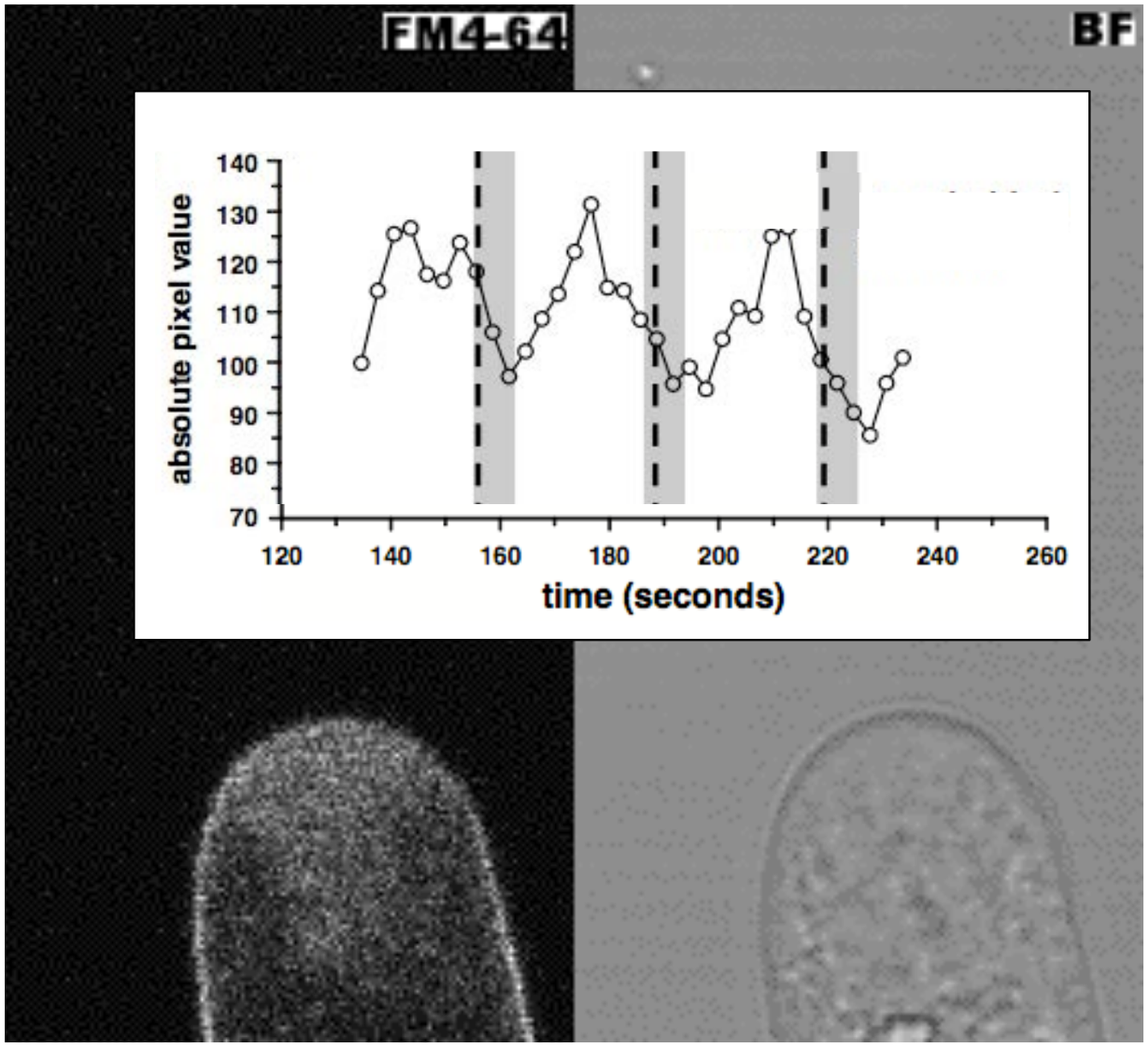
Can monitor the kinetics of dynamic processes

Fixed - EM



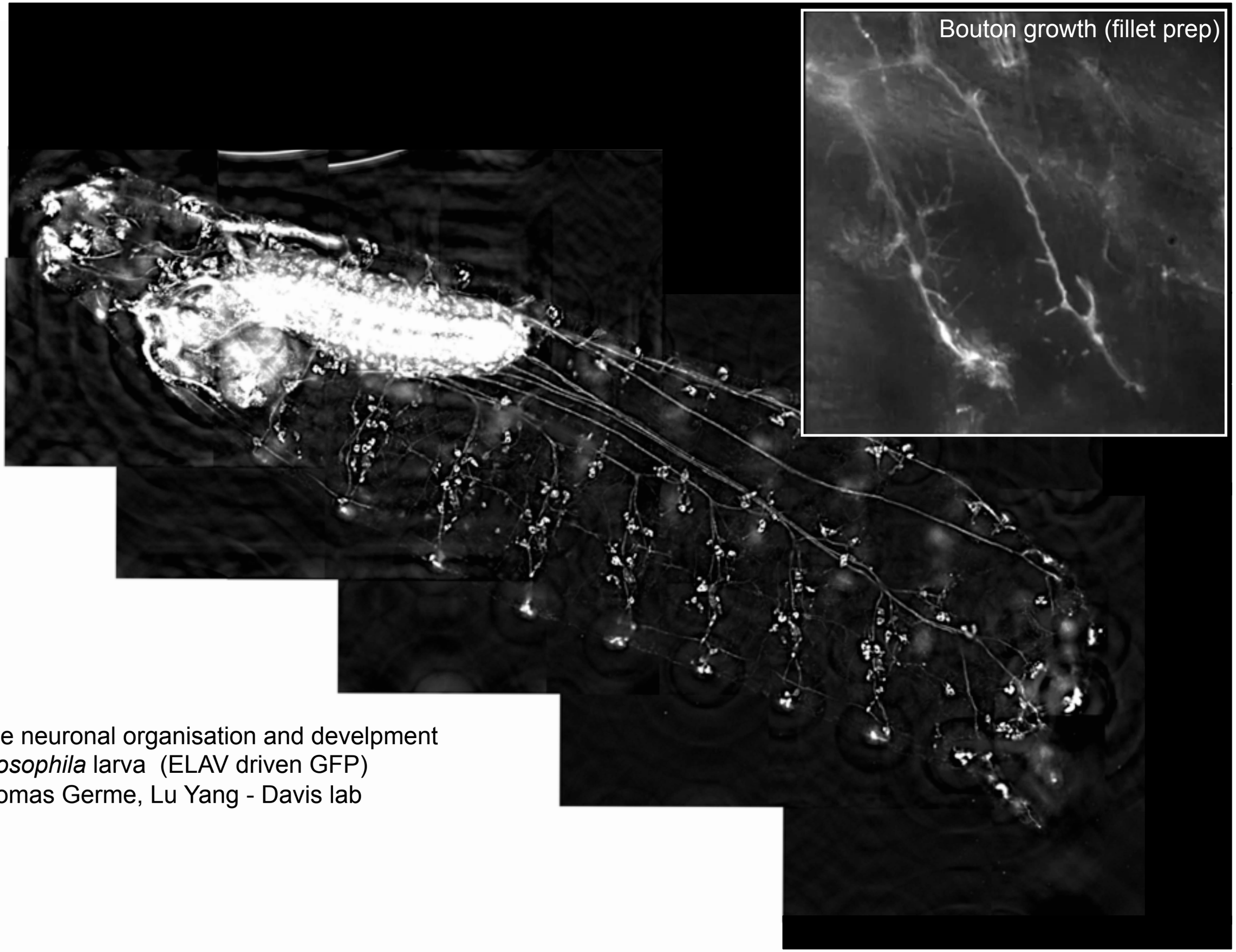
Electron Micrograph From
Lancelle,S.A.; Cresti,M.; Hepler,P.K. **(1997)** ***Protoplasma*** **196, 21-33.**

Live



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

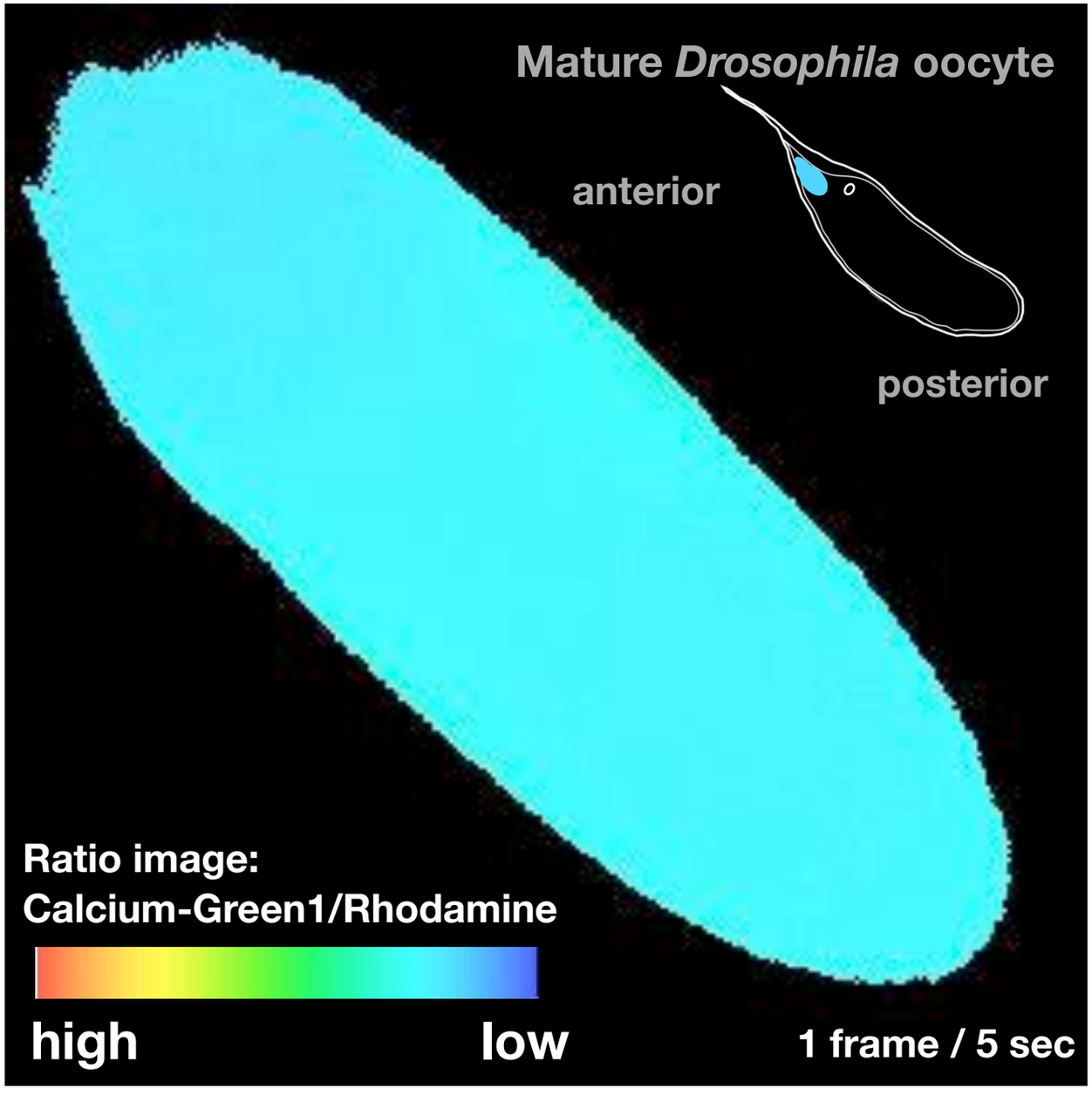
Can follow the order of sequential events in real time

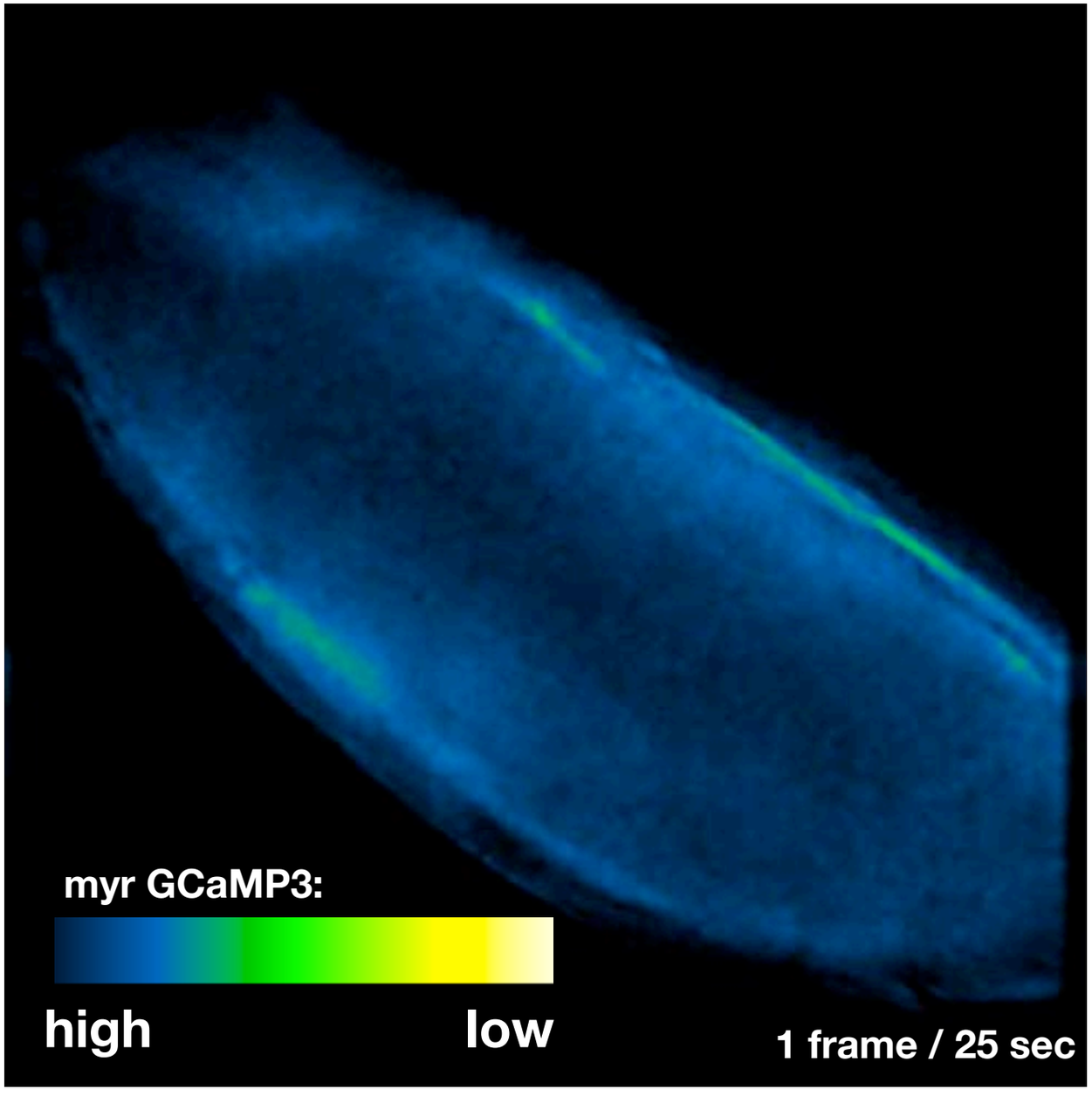


Live neuronal organisation and develpment
Drosophila larva (ELAV driven GFP)
Thomas Germe, Lu Yang - Davis lab

Can record sensitive or transient processes

Calcium transient upon activation





Claire Bromley, Richard Parton, Tim Weil: Davis Lab

Requirements for live cell imaging:

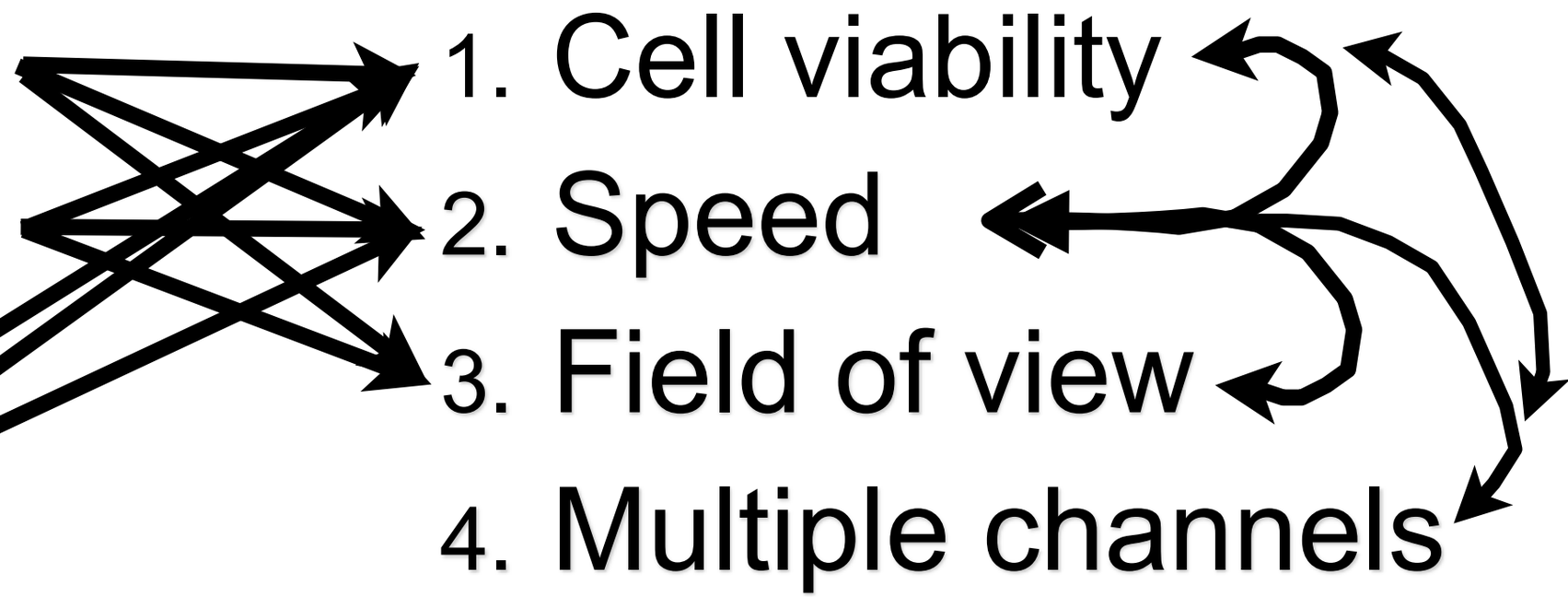
Careful Balancing of Conflicting Interests

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



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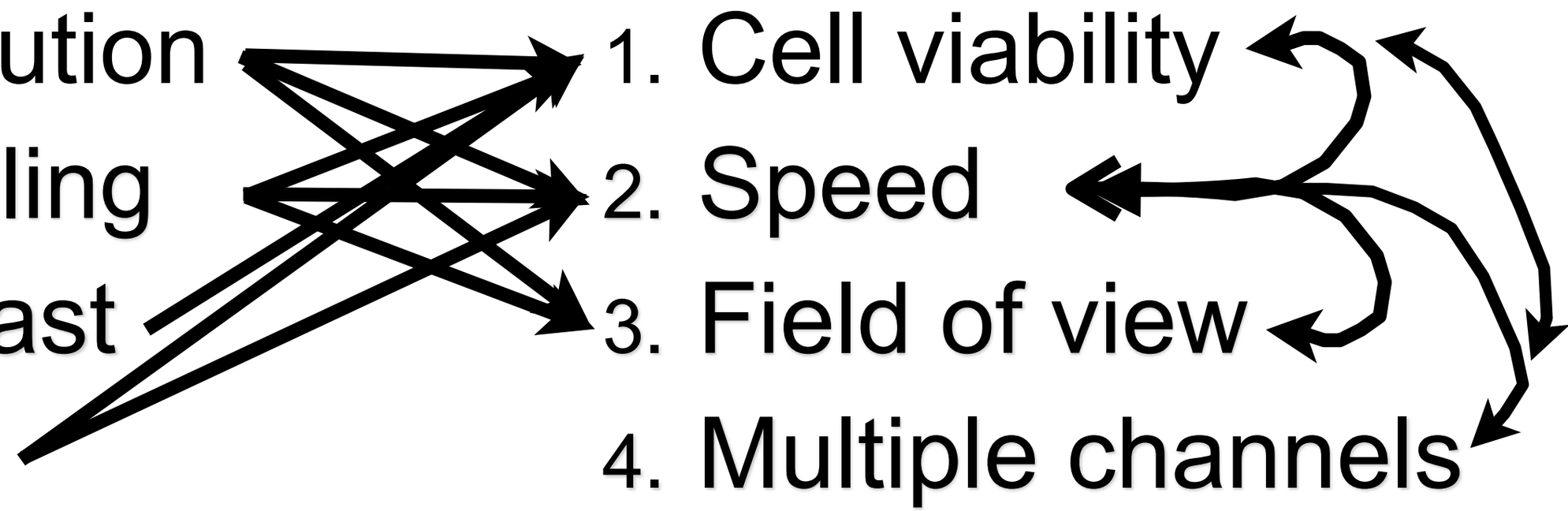
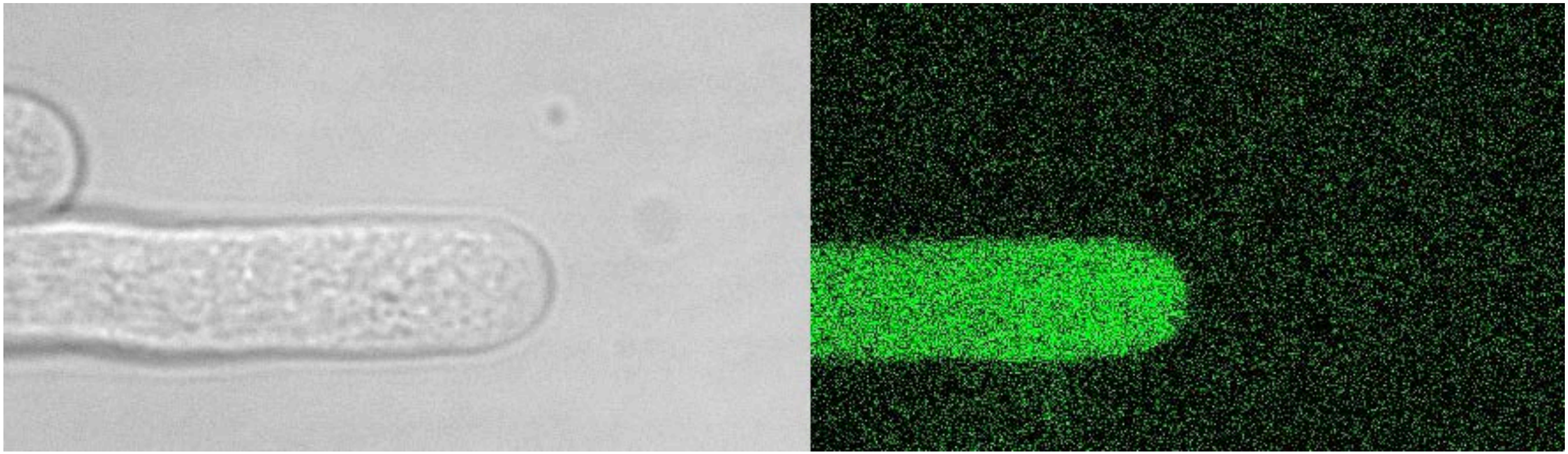


Live-cell imaging is a compromise!

Death by imaging!



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

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!

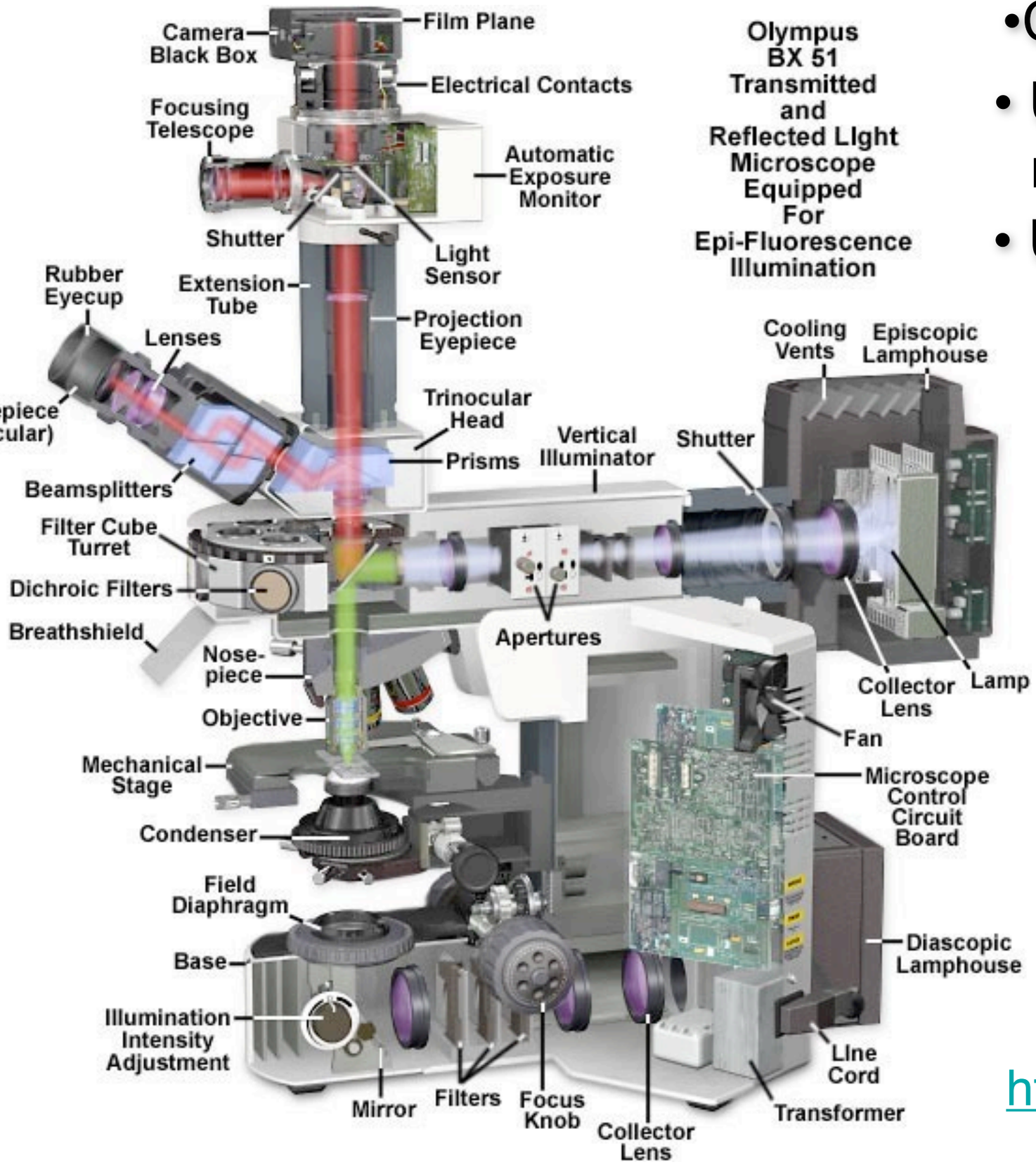
Choice of microscope stand

- The modern epifluorescence microscope

  Upright microscope (lens above specimen)

  Inverted microscope (lens below specimen)

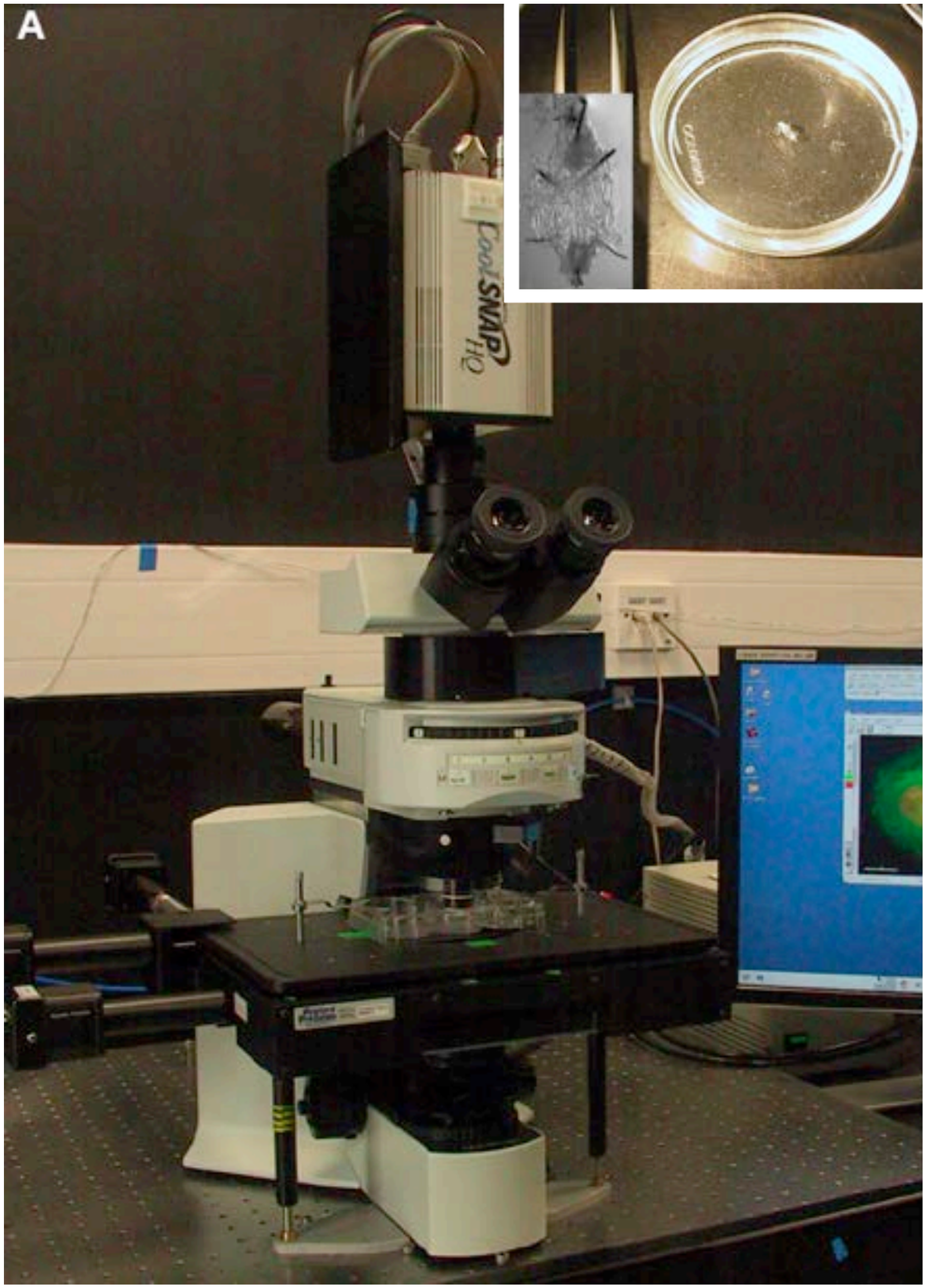
Upright microscope design

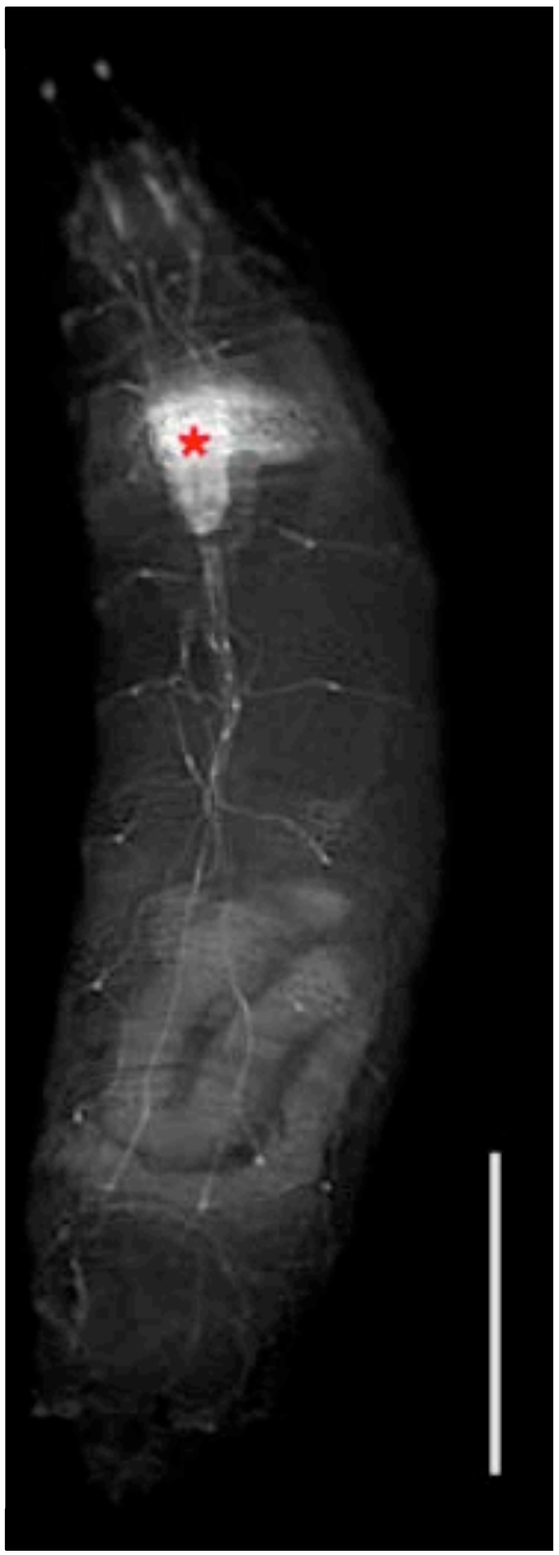


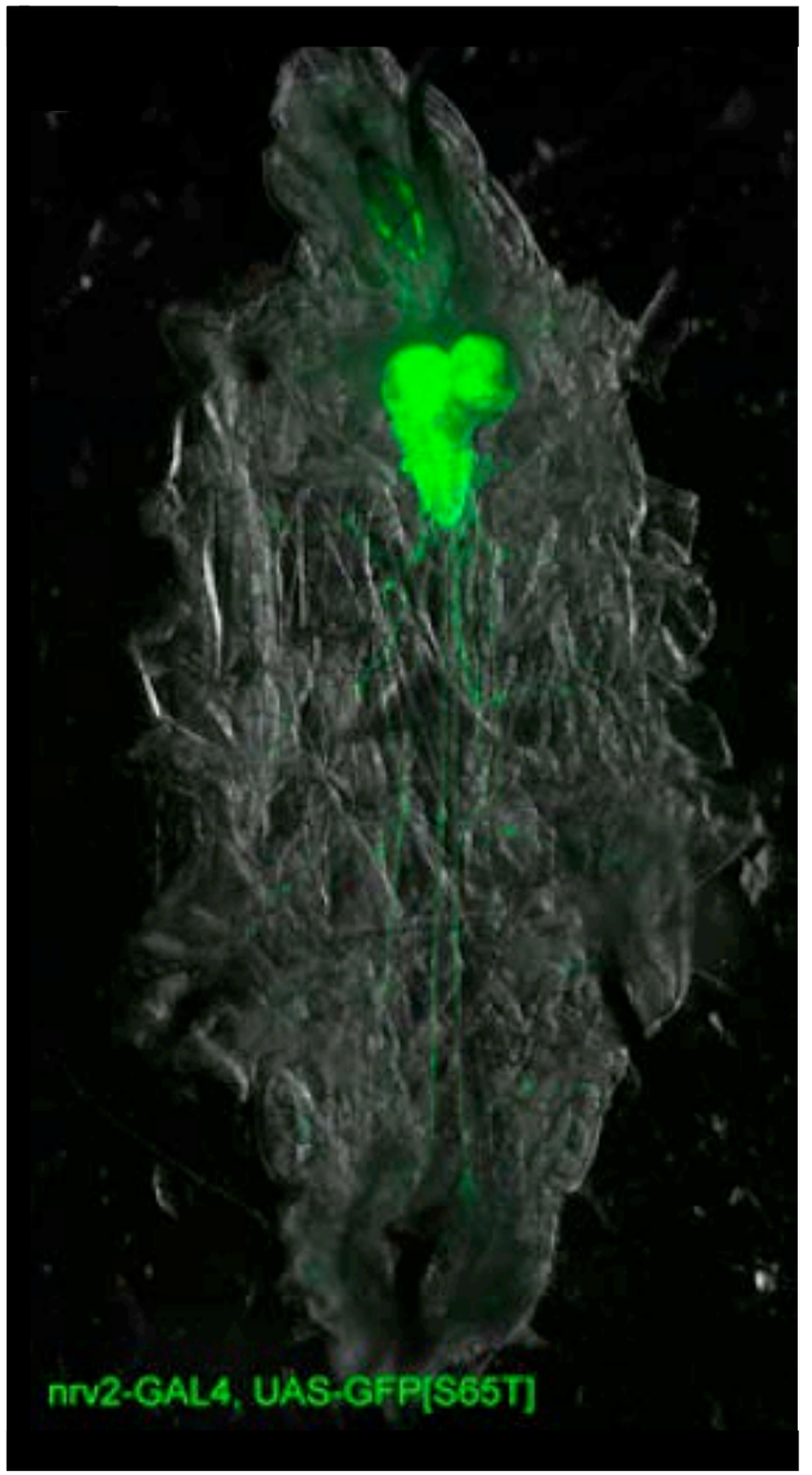
- Cheaper
- Use with thick or opaque material
- Use with dipping objectives

http://www.olympusmicro.com/

Upright microscope - larval fillet prep

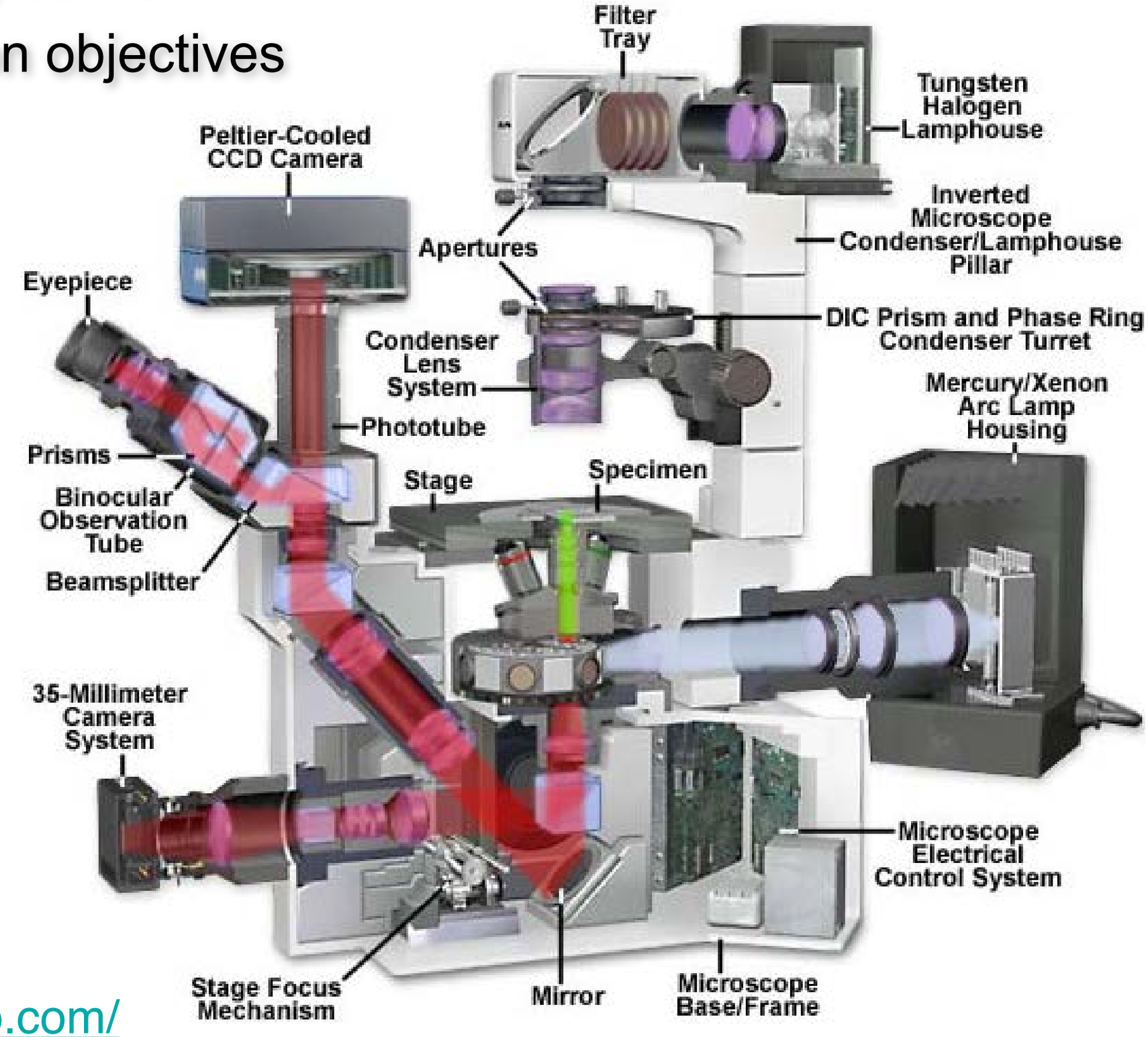






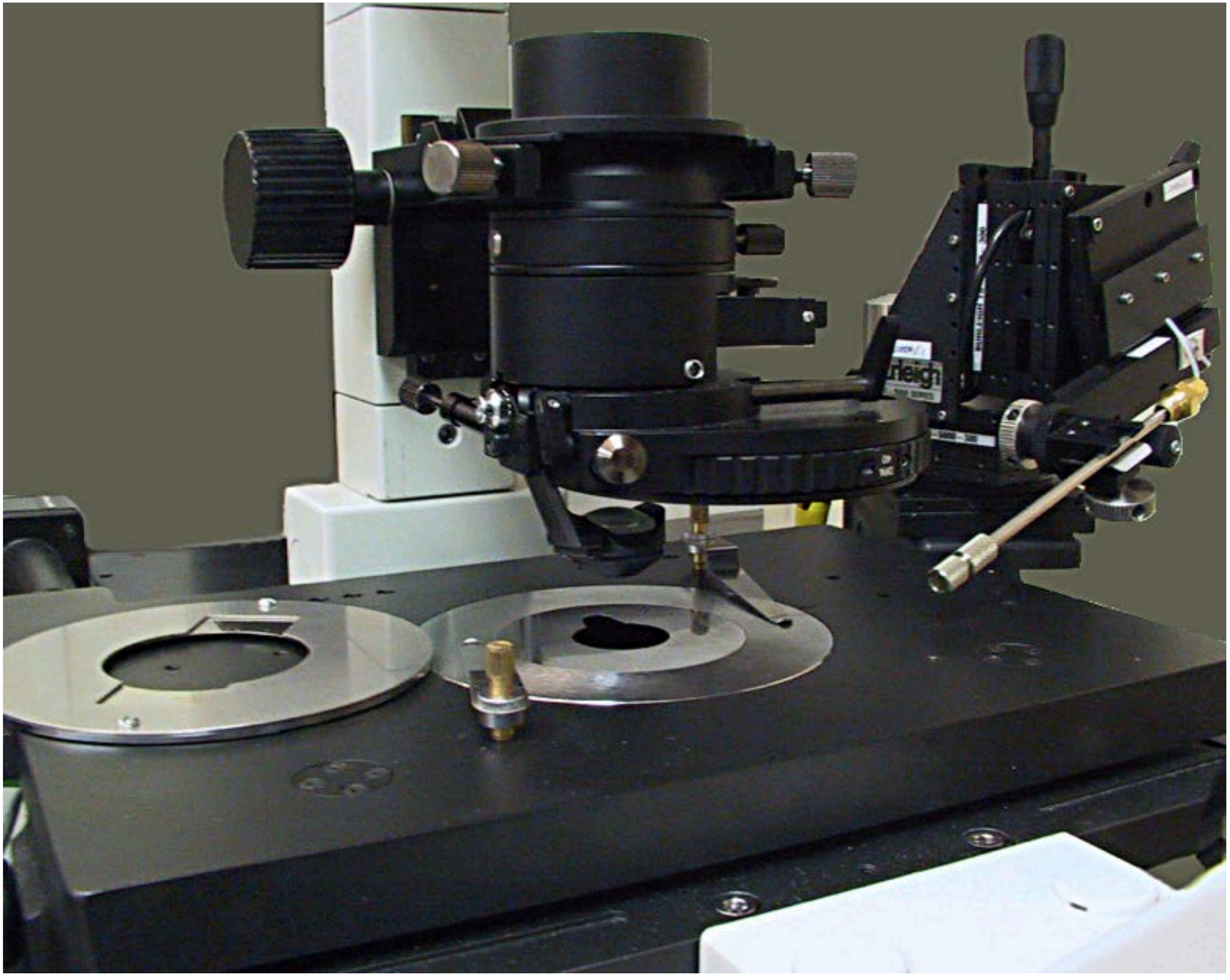
Inverted microscope design

- Easy access to the specimen
- Good for oil immersion objectives
- Convenient side port



http://www.olympusmicro.com/

Inverted microscope - injection



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 um - 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 um)

  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)

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 / CO_2

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

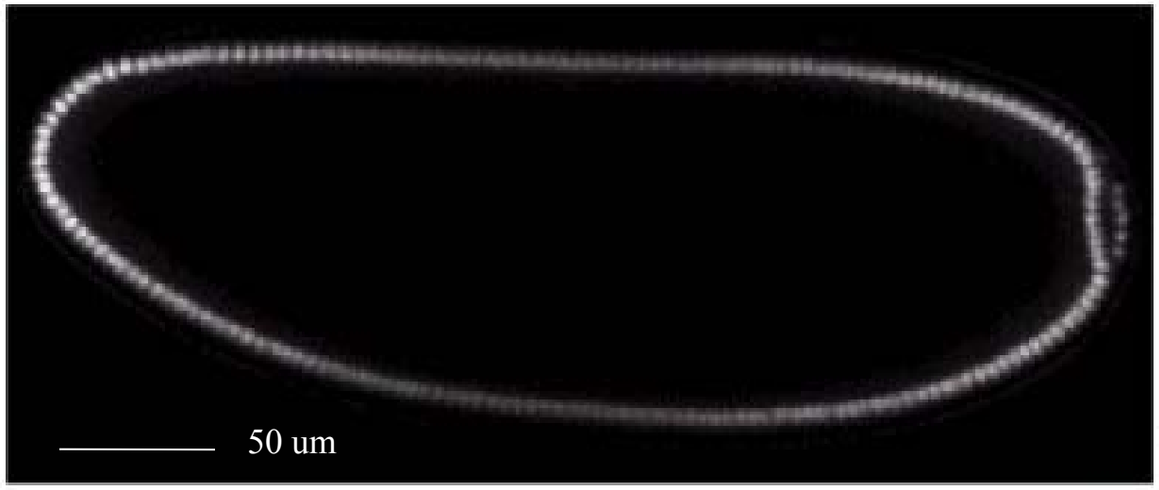
Following fast dynamic events

Use TIRF for:

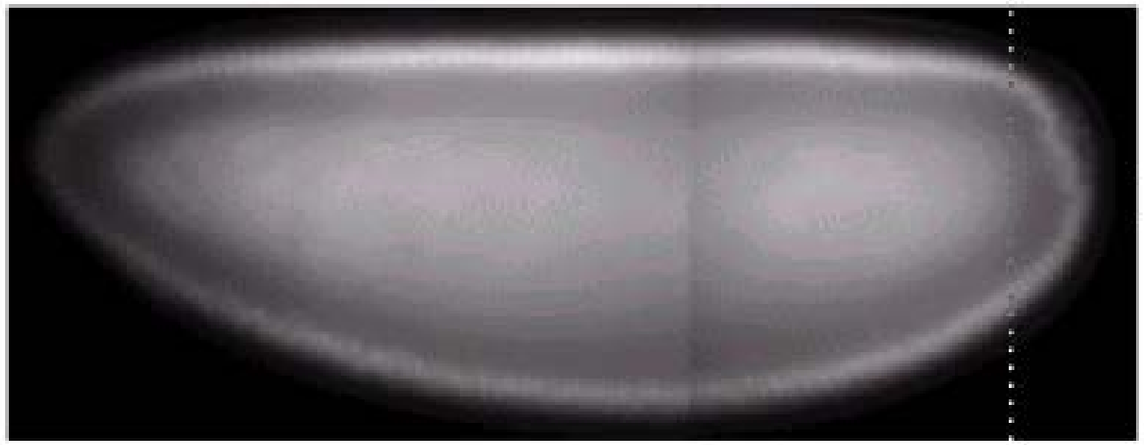
Imaging with high contrast within 100 nm of the coverslip

Choice of imaging technique:
Example of a thick specimen

Confocal



Wide-field



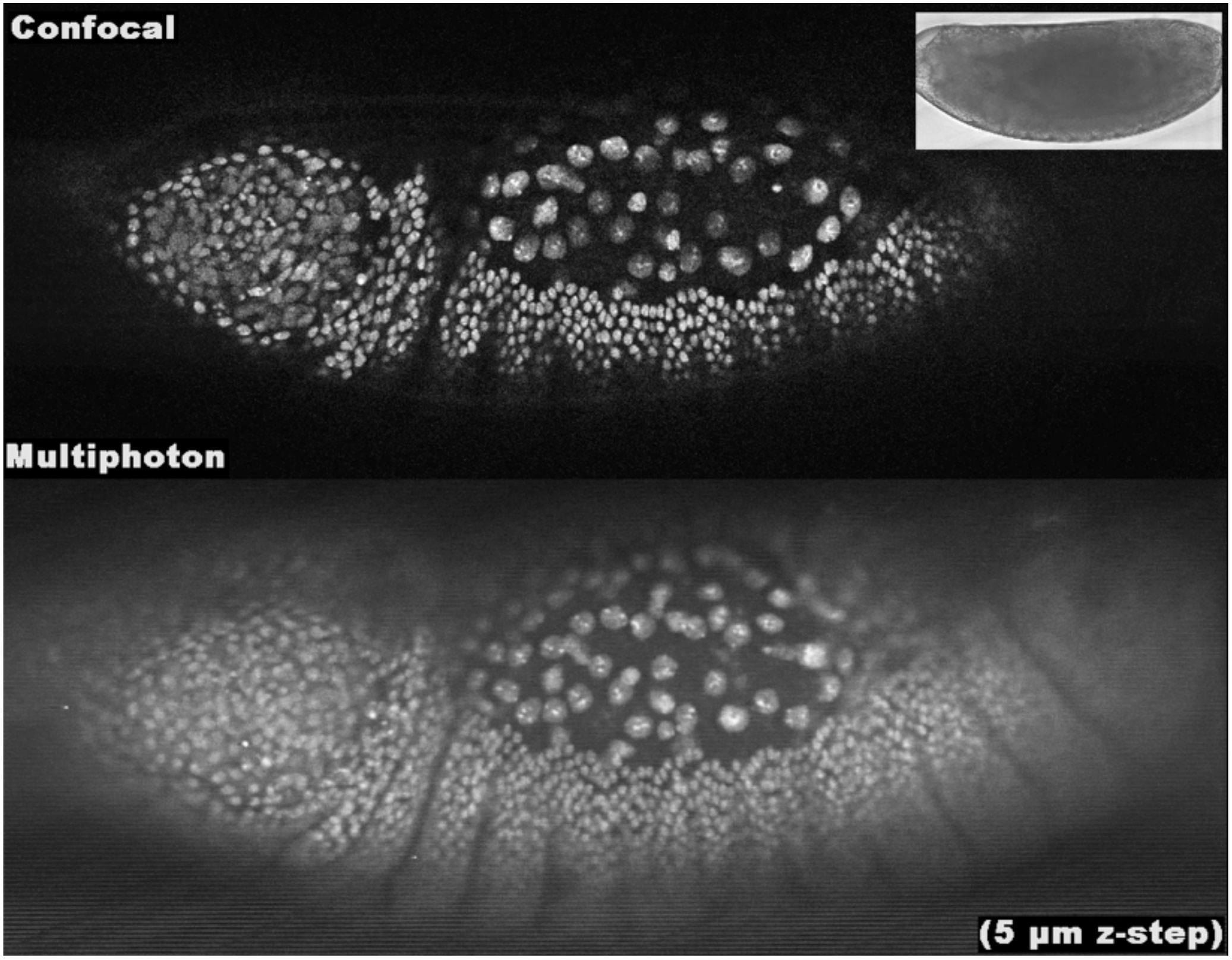
WF- deconvolved

Drosophila embryo, nls GFP

Thick, bright specimen

For really thick specimens consider point scanning confocal or multiphoton

Multiphoton



Confocal 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

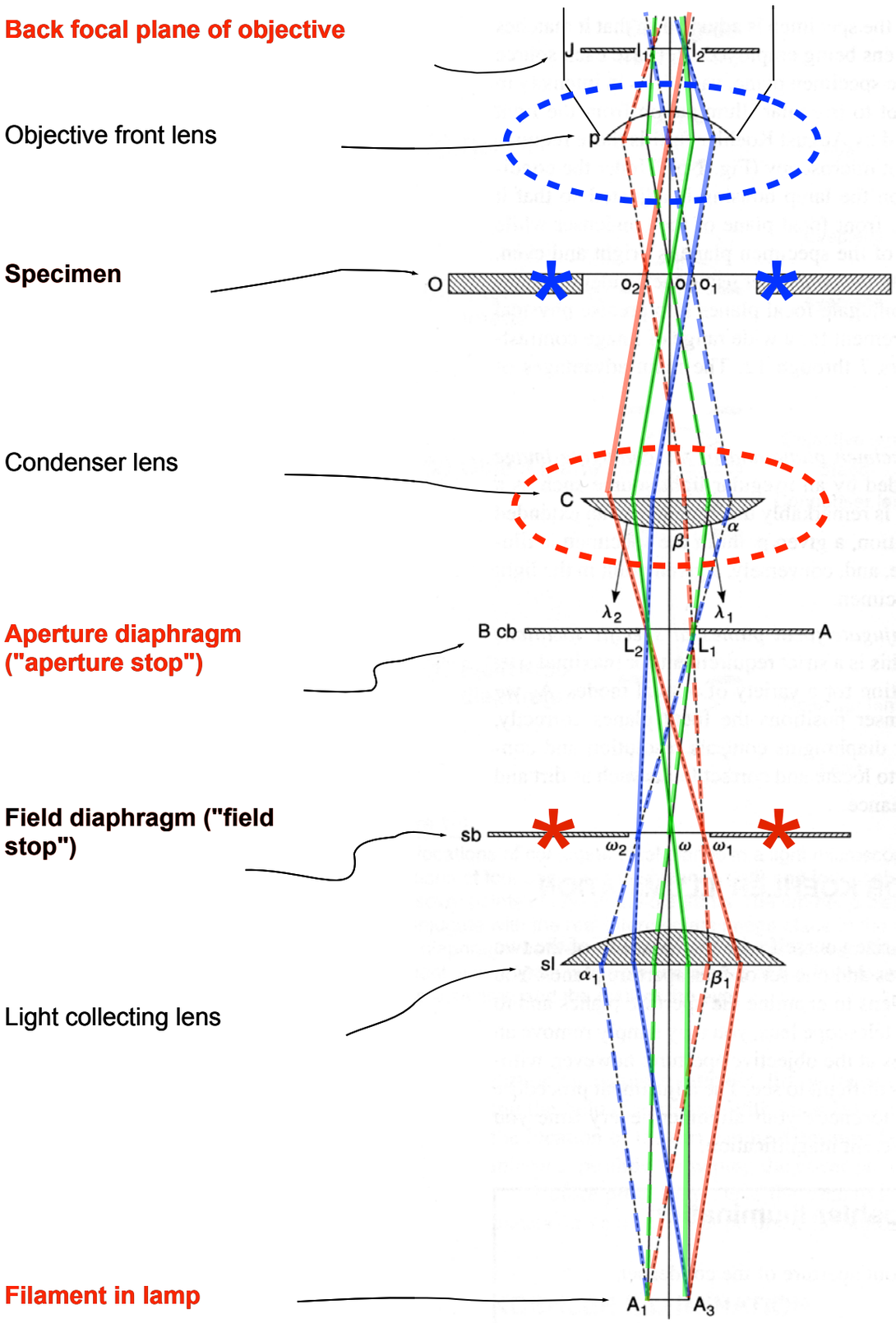
- FRAP (Fluorescence Recovery After Photobleaching)
- Fhoto-activation (PA-GFP)
- FRET (Fluorescence Resonance Energy Transfer)
- FLIM (Fluorescence Lifetime IMaging)
- FCS (Fluorescence Correlation Spectroscopy)

Ian - lecture 11 - confocal and multiphoton

Setup your imaging equipment properly:

Koehler alignment:

CONJUGATE PLANES - bright field



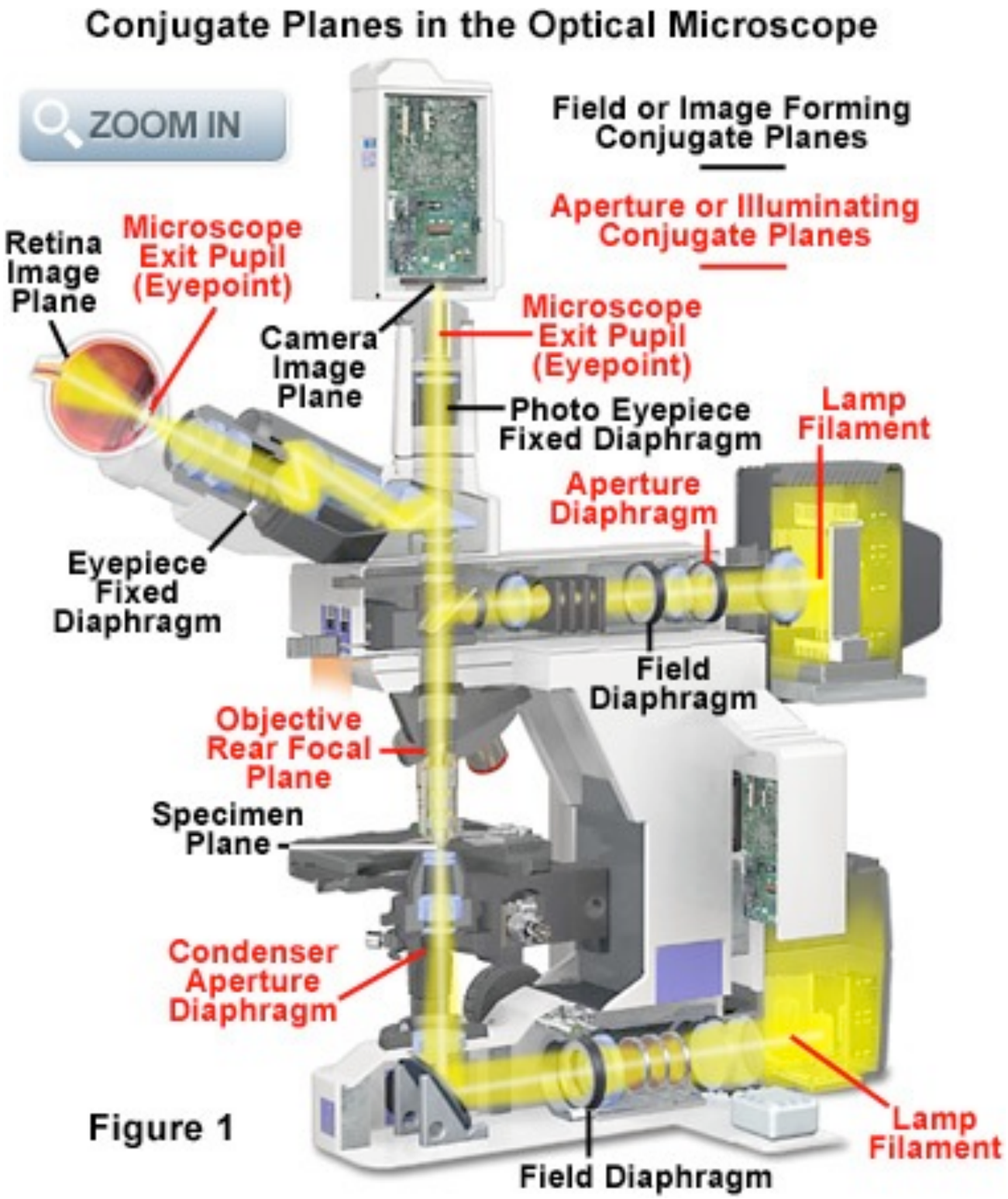
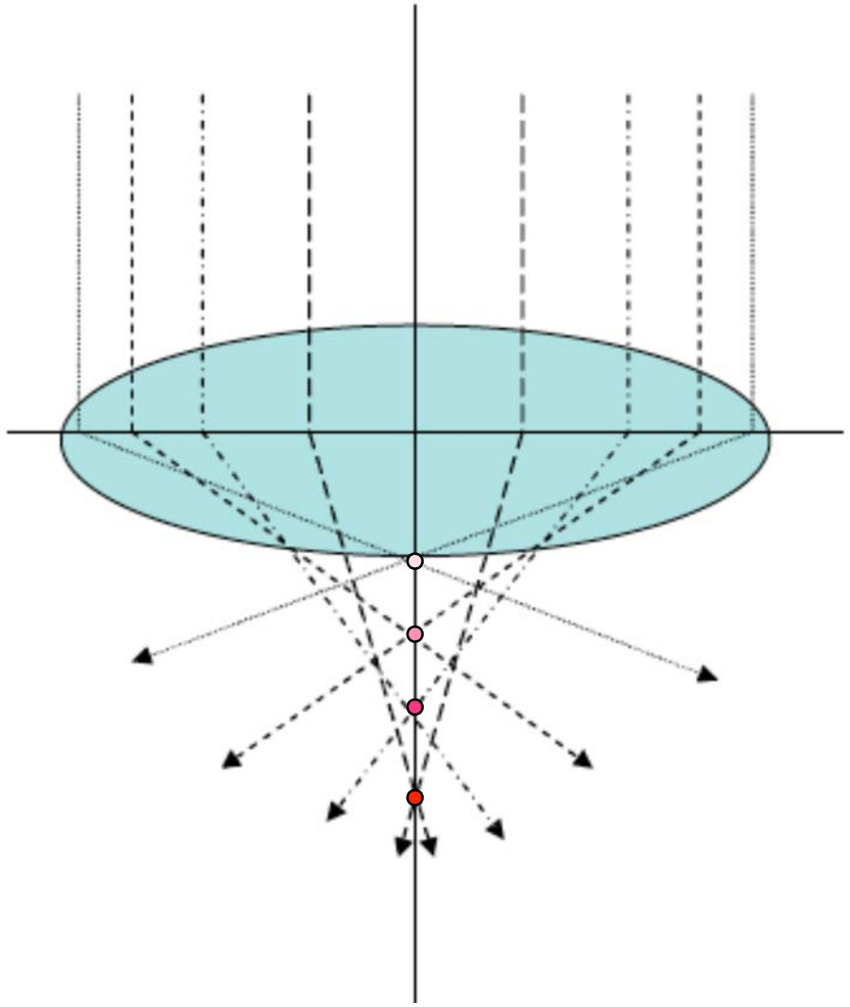


Figure 1

Correct Spherical Aberration:

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

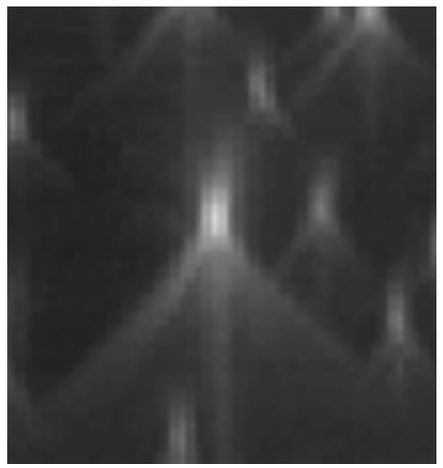


Confocal

- “In focus” light is blocked by the pinhole

Wide field

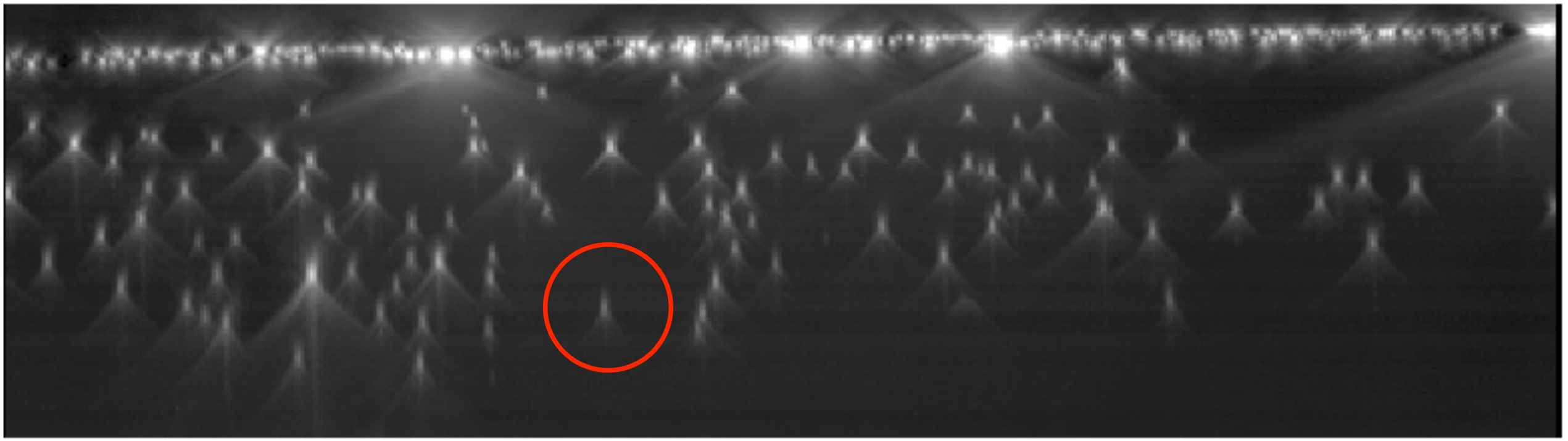
- Detail is “smeared” in Z



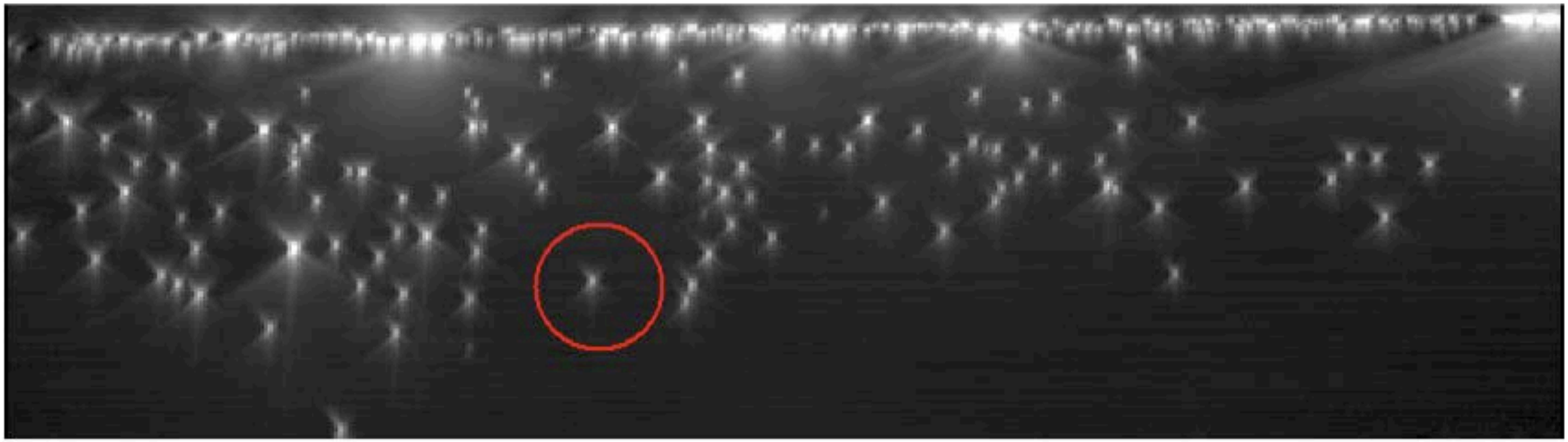
SA results from refractive index mismatch along the optical path:
glass 1.514; oil 1.33-1.534; air; 1.0; water 1.33; cell 1.35-1.6; 70% glycerol 1.47

Correct empirically using beads:

Spherically aberrated



Corrected Increased signal and resolution!



Correcting Spherical Aberration:

- Immersion oils

- Objective SA correction collar

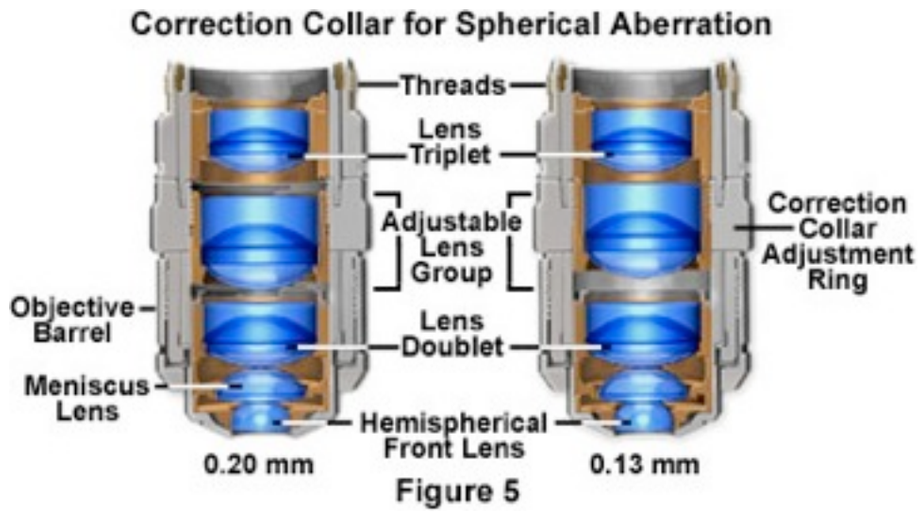
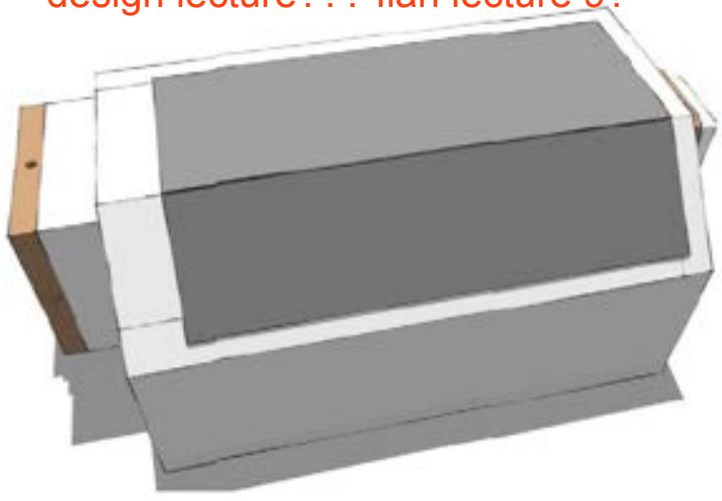
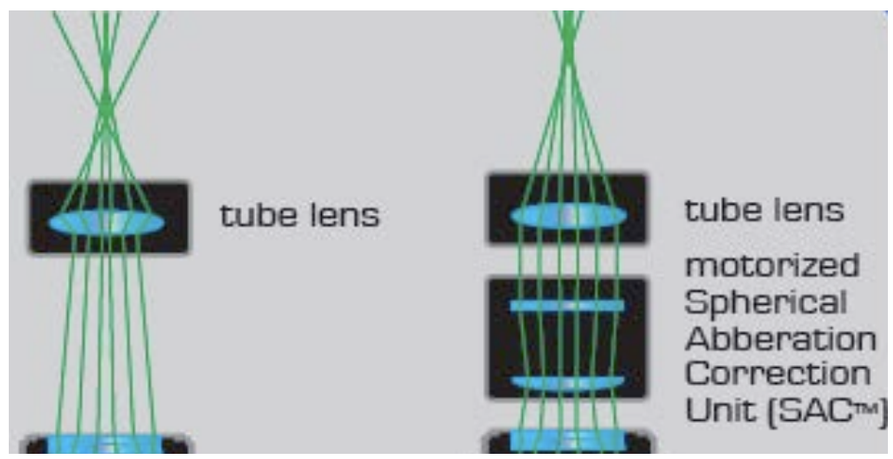


Figure 5

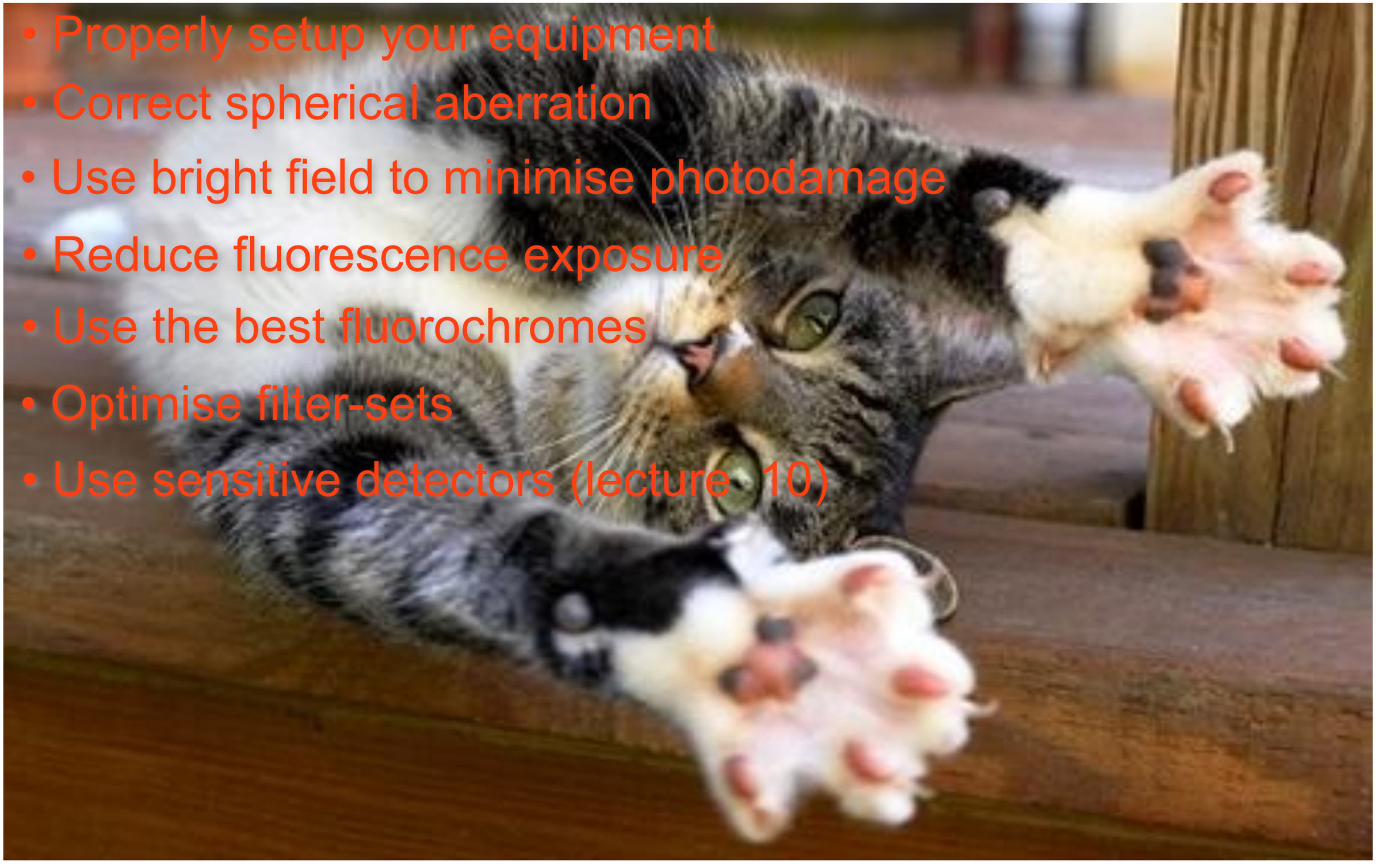
- Adaptive optics

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

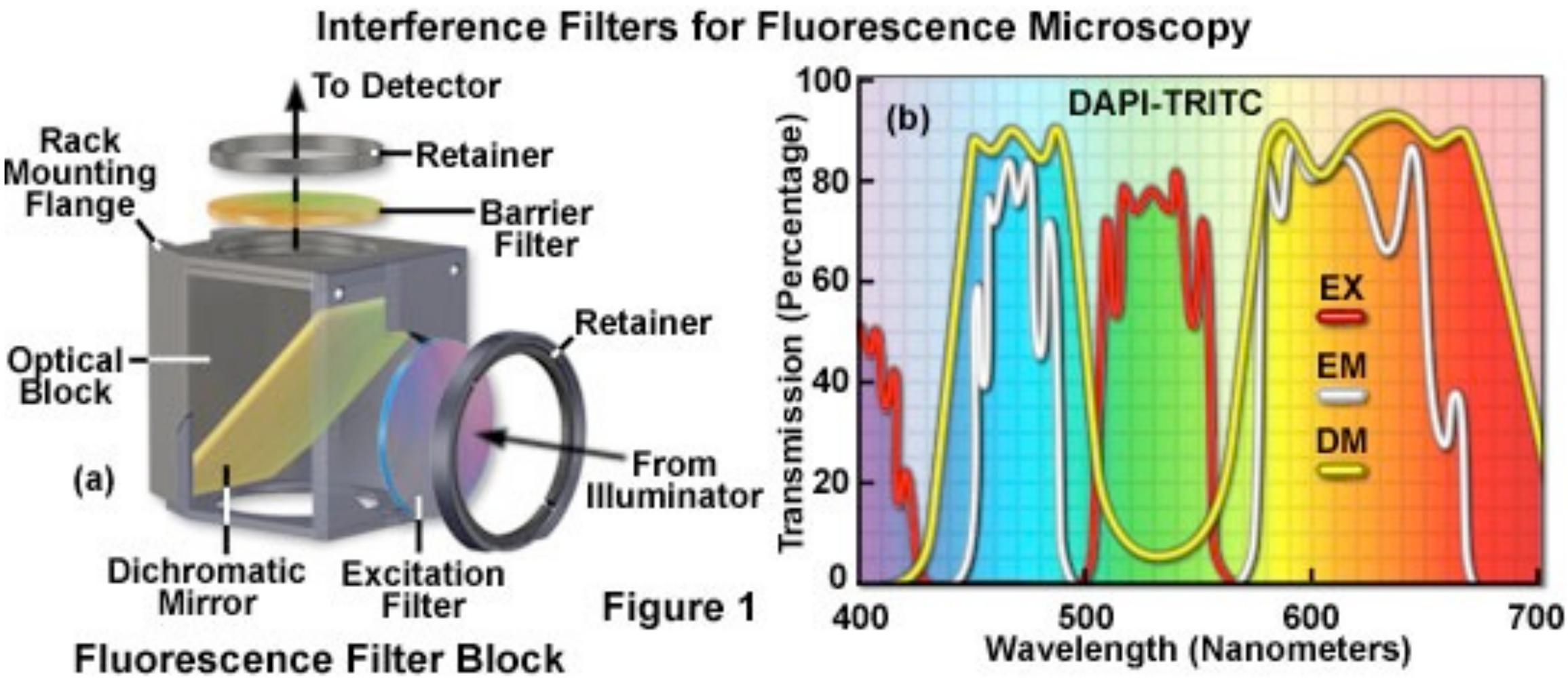
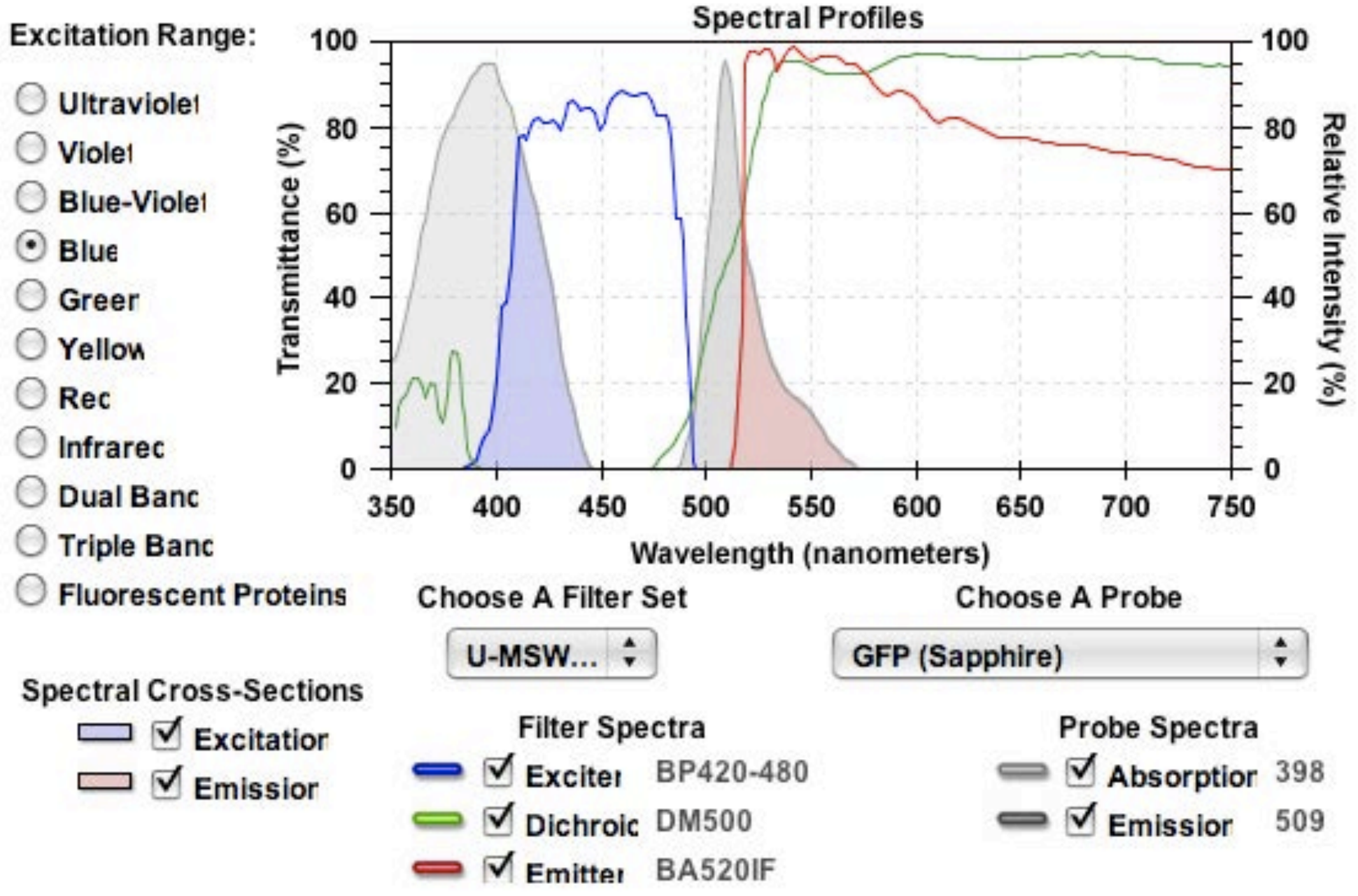


Figure 1

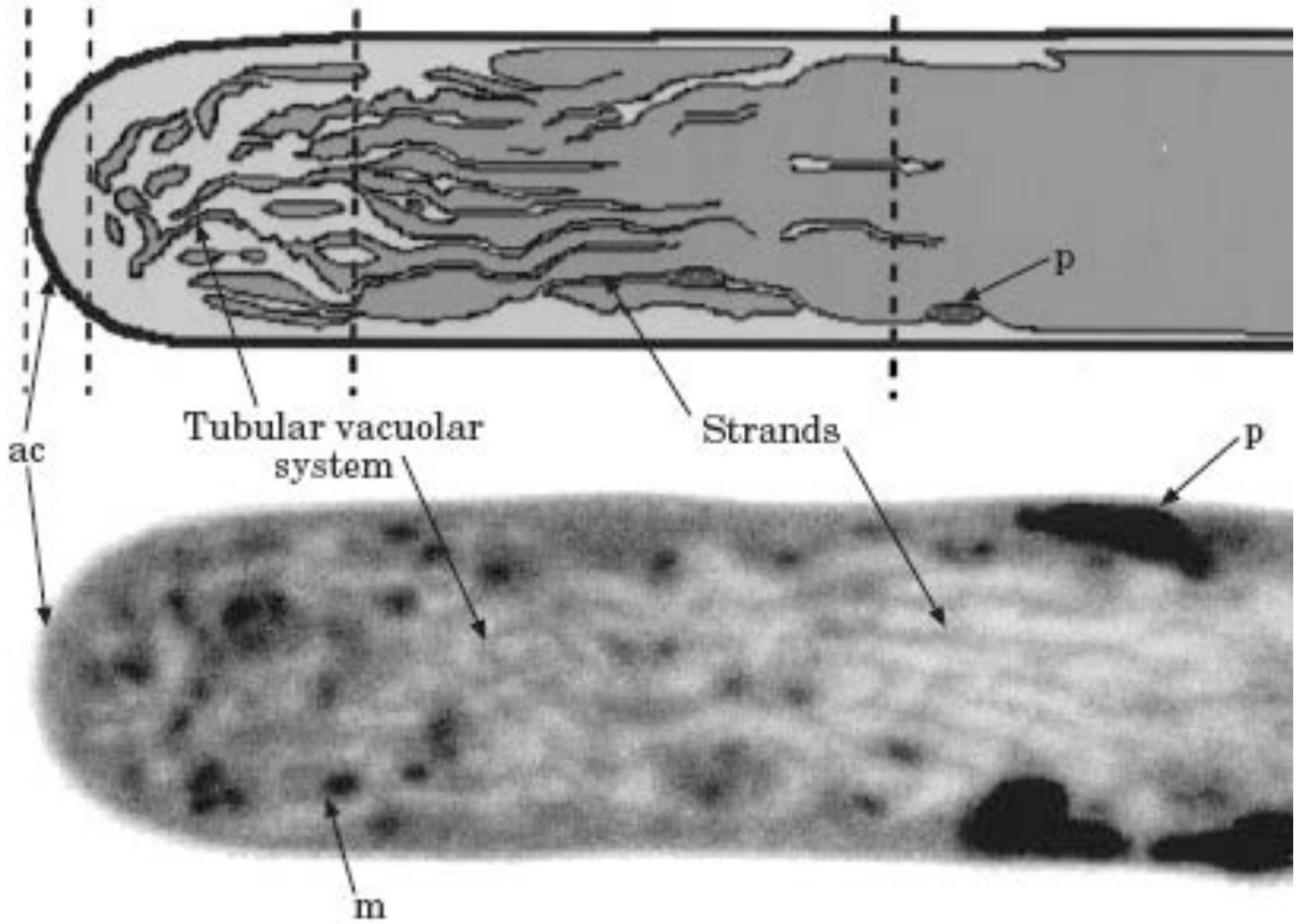
Matching Fluorescent Probes to Filter-Sets



http://www.olympusmicro.com/primer/java/fluorescence/matchingfilters/index.html

Loading dyes into living cells:

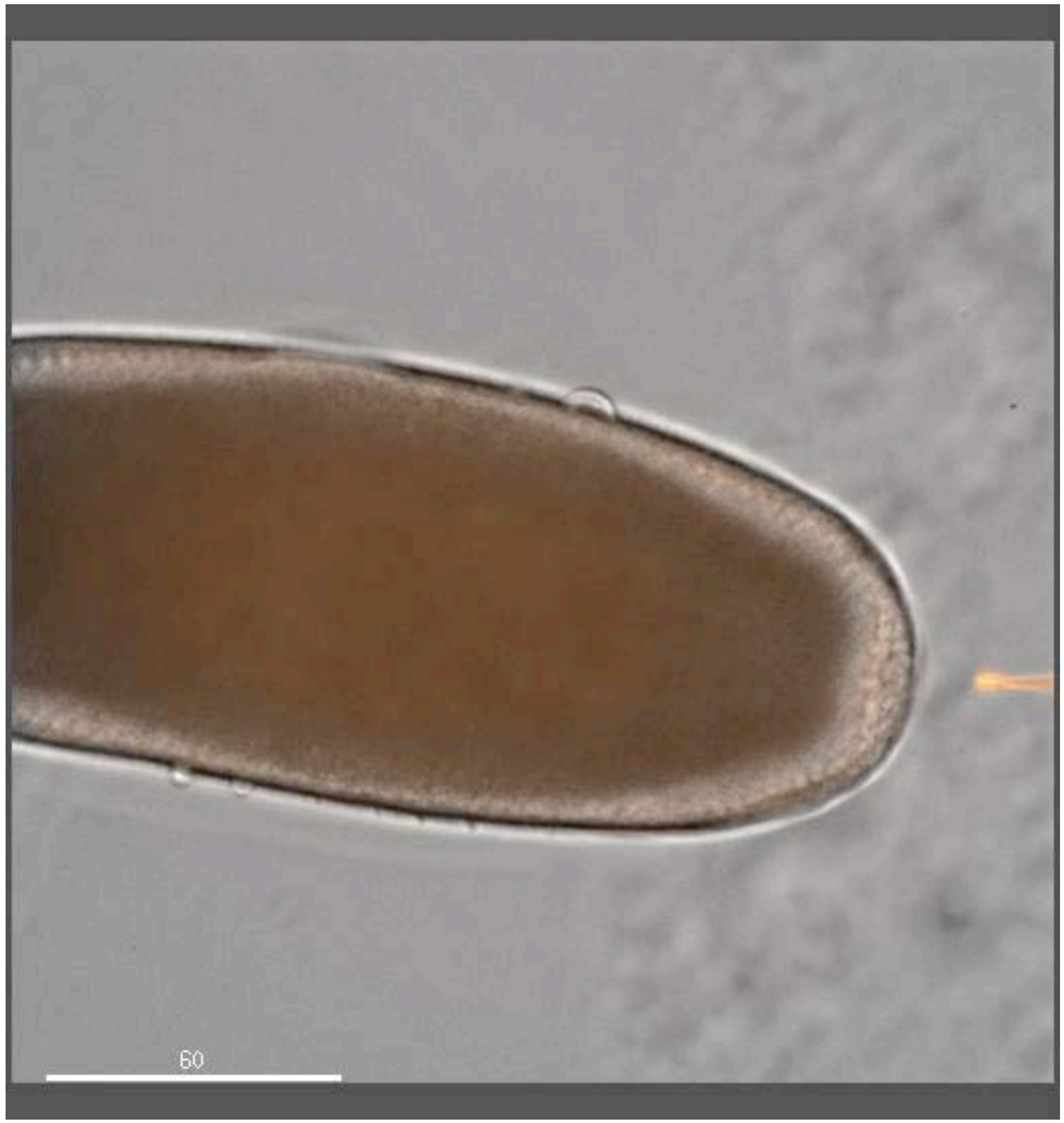
- Cell permeant dyes



Nile Red: lipid stain
Rhizoid of fern gametophyte

Injection into cells:

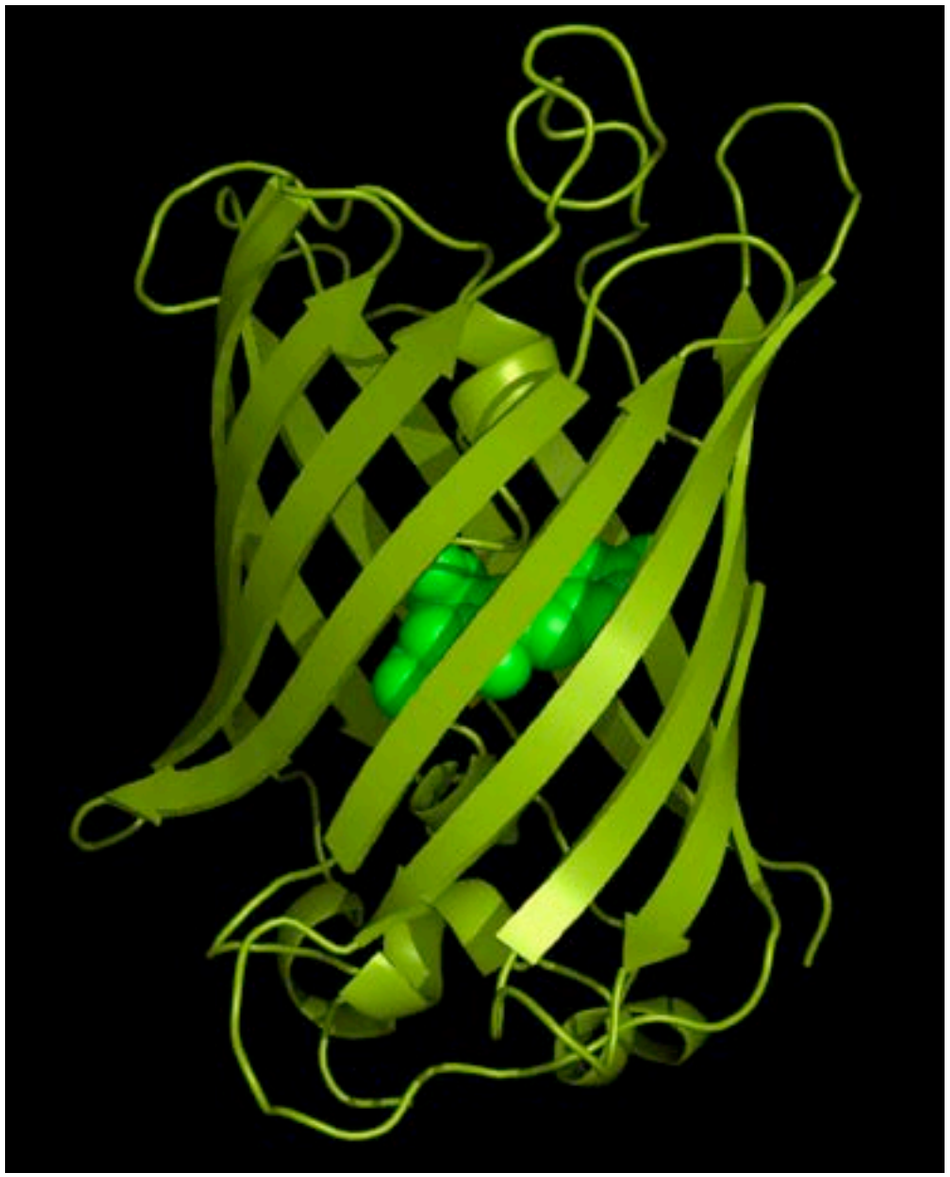
- Virtually any probe
- Technically difficult
- Expensive equipment
- Not all cell types amenable
- Potentially damaging



Injection into a Drosophila embryo

Transgenic GFP:

Covered in lecture 6 - Mark Howarth

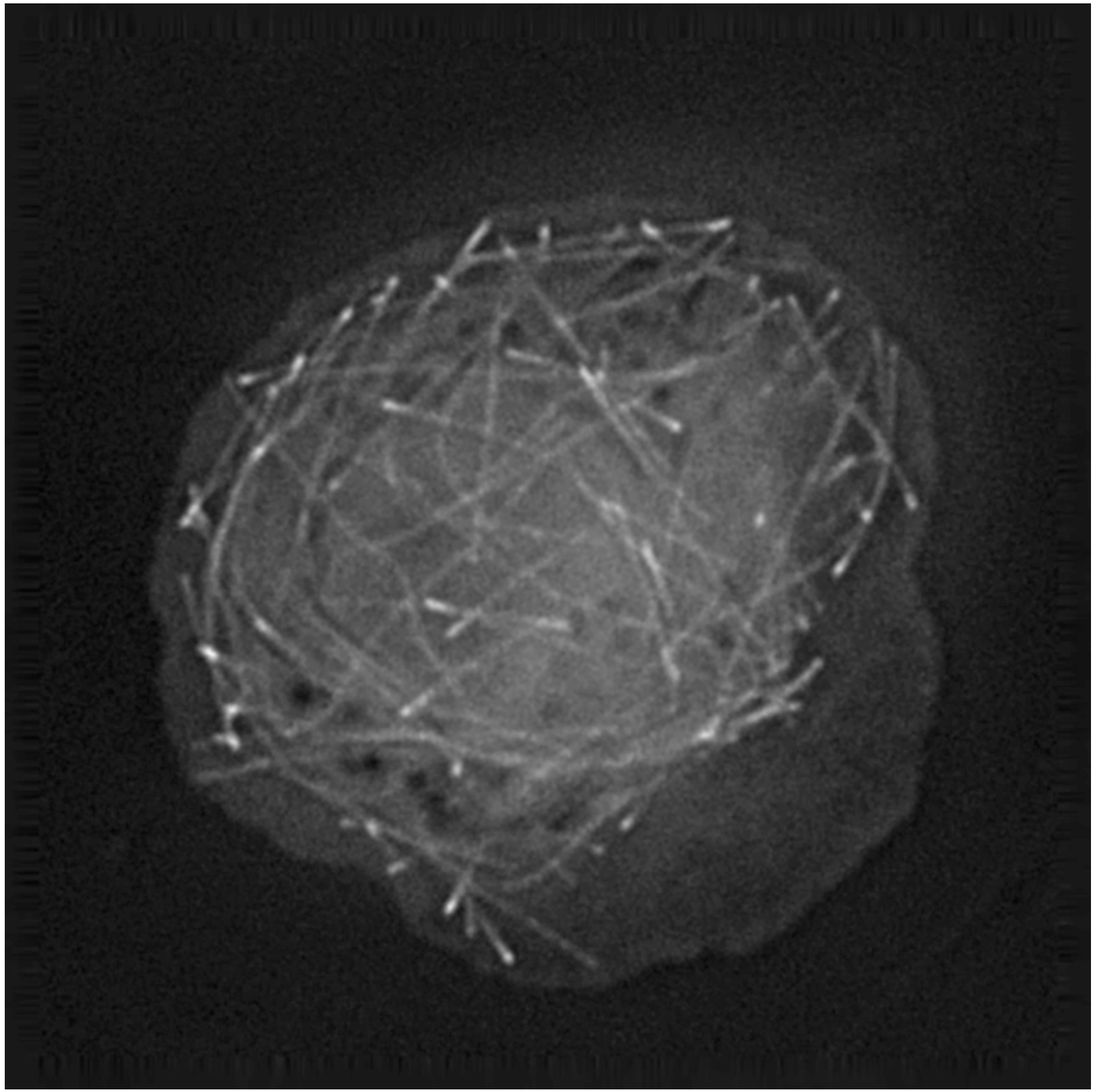


beta-barrel in light green
fluorophore in bright green

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

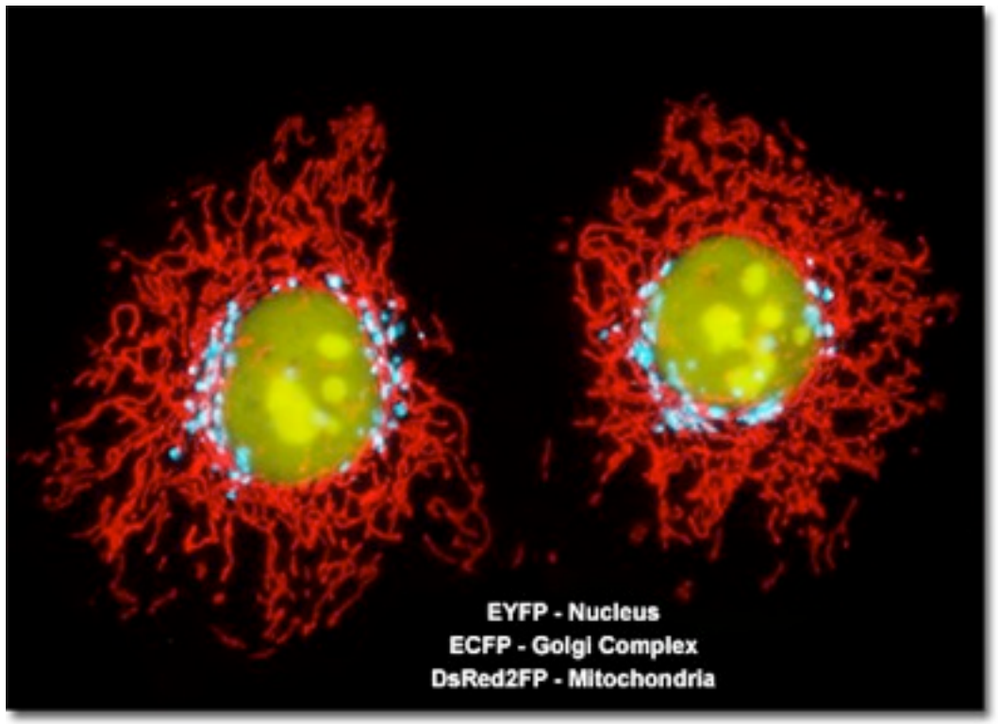
- GFP is inherently fluorescent
- 238 AA; 27 KDa; 4nm - dominated by an 11 stranded beta-barrel
- Fluorochrome forms by the post-translational oxidisation and cyclisation of residues 65 to 67 during folding
- Can be expressed in many organisms
- GFP can be functionally expressed as N or C terminal conjugates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

http://www.nature.com/nbt/journal/v14/n10/pdf/nbt1096-1219.pdf



Macrophage: GFP microtubules

Extending the palette of fluorescent proteins



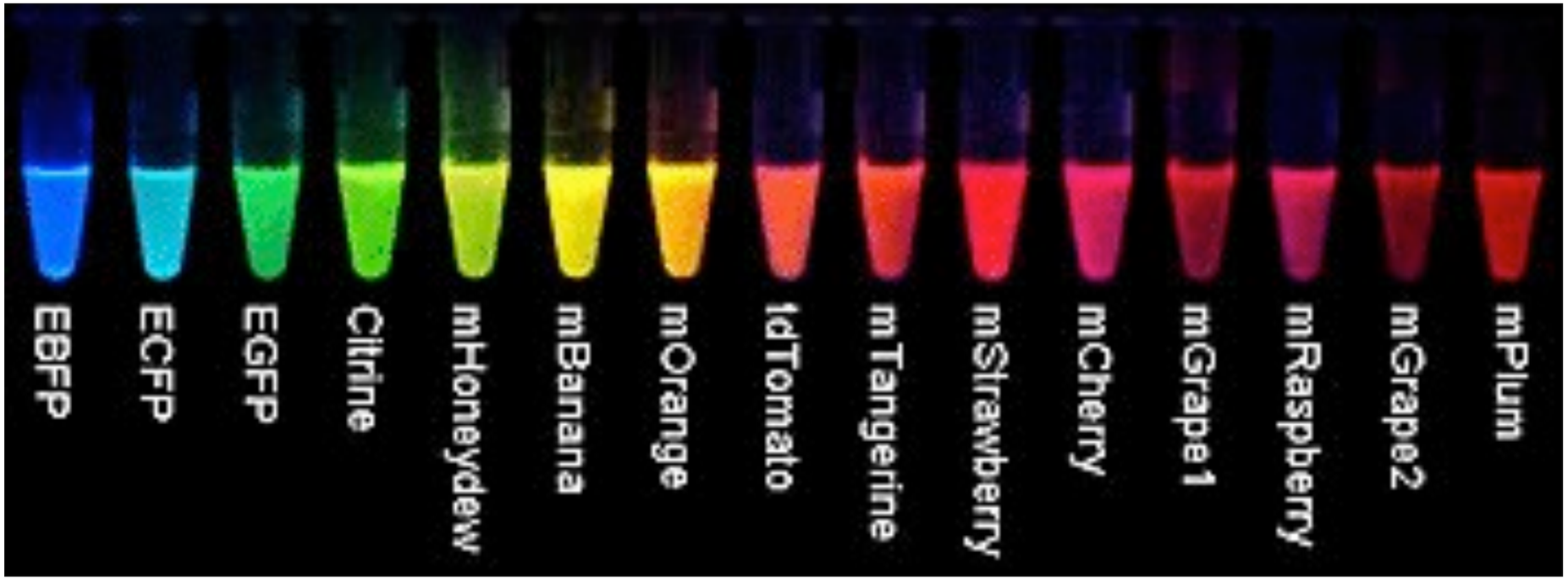
EYFP = enhanced Yellow Fluorescent Protein (GFP derivative)

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!



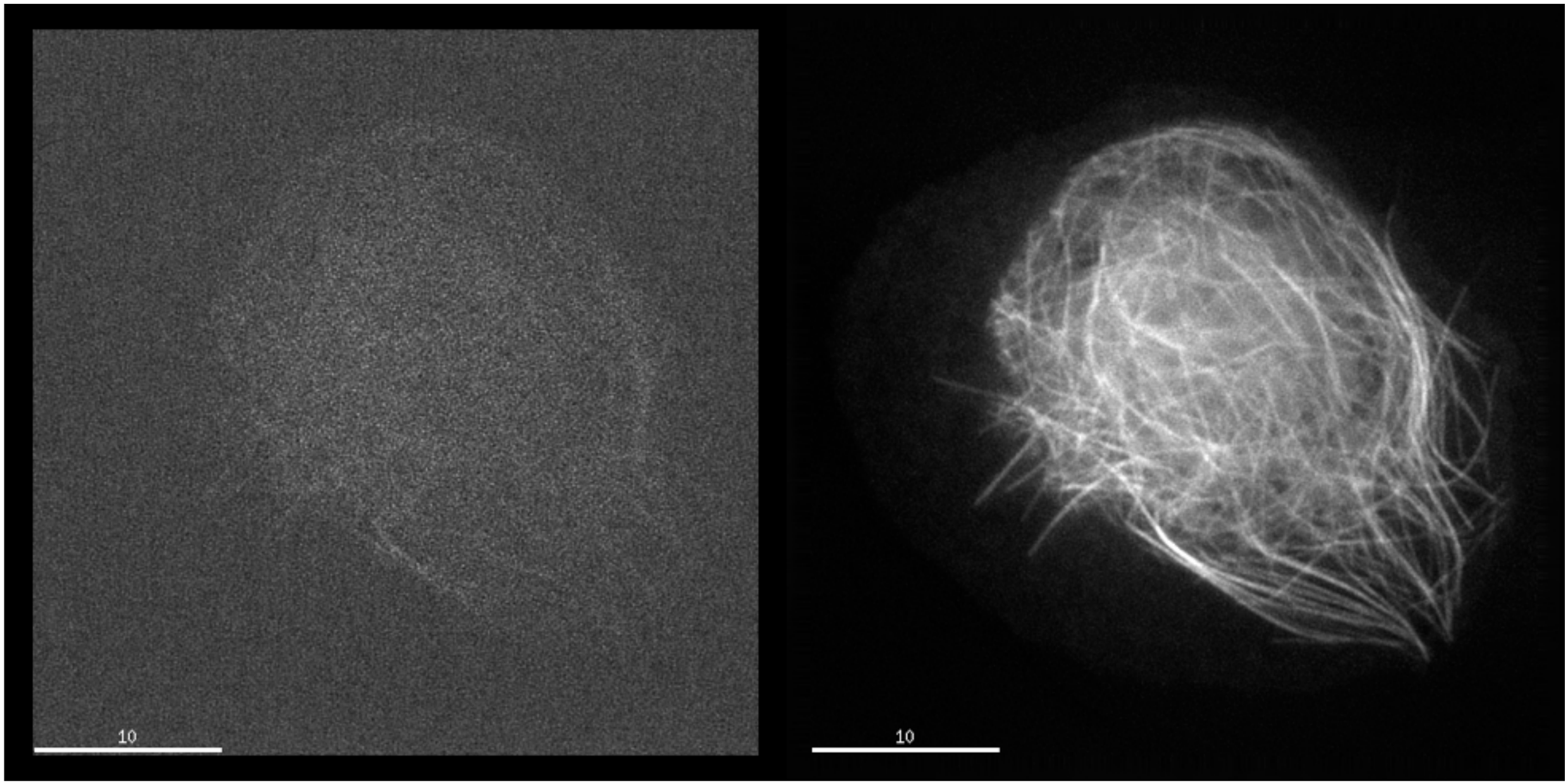
Post acquisition image processing:

- 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.

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