ONBI Advanced Microscopy Course 2014

Week1 - Principles of light microscope

lecture 8

Live Cell Imaging

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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

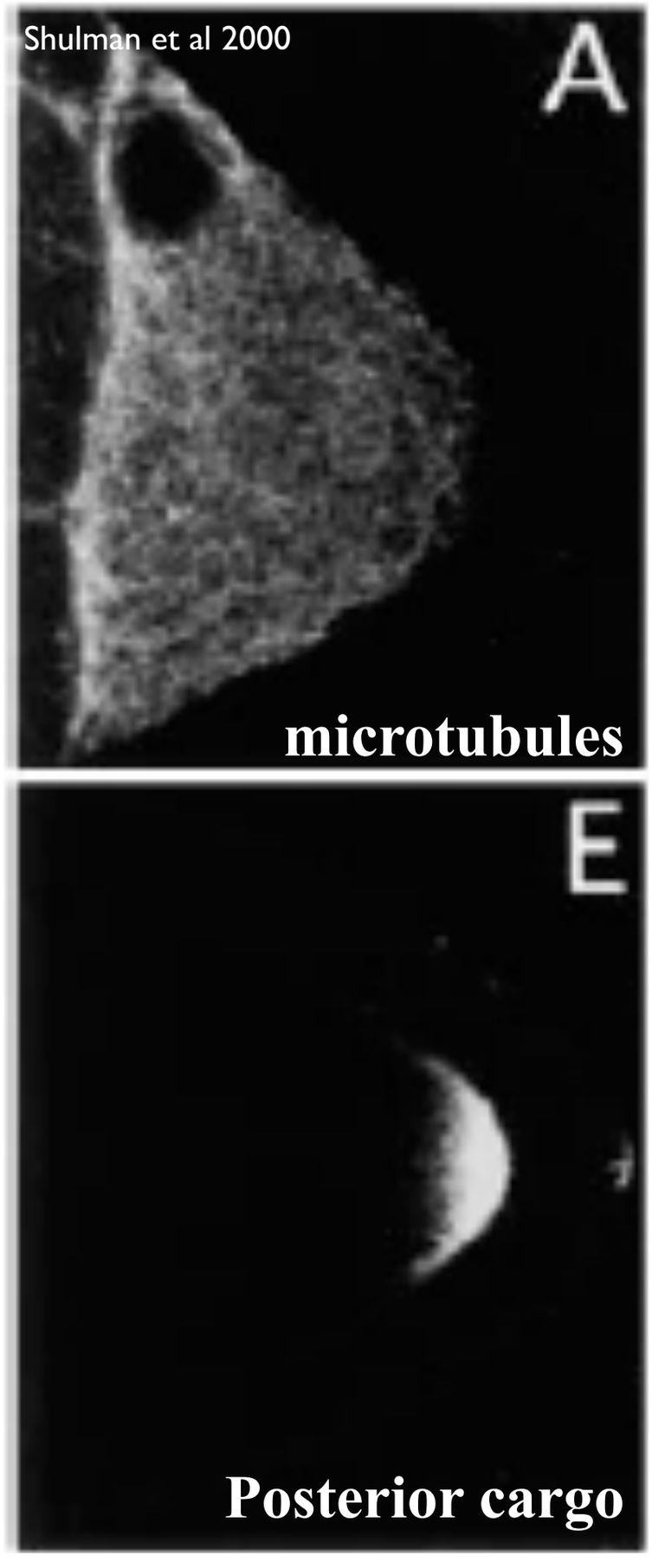
  Image processing and analysis

Reasons for live imaging: Fixed vs Live

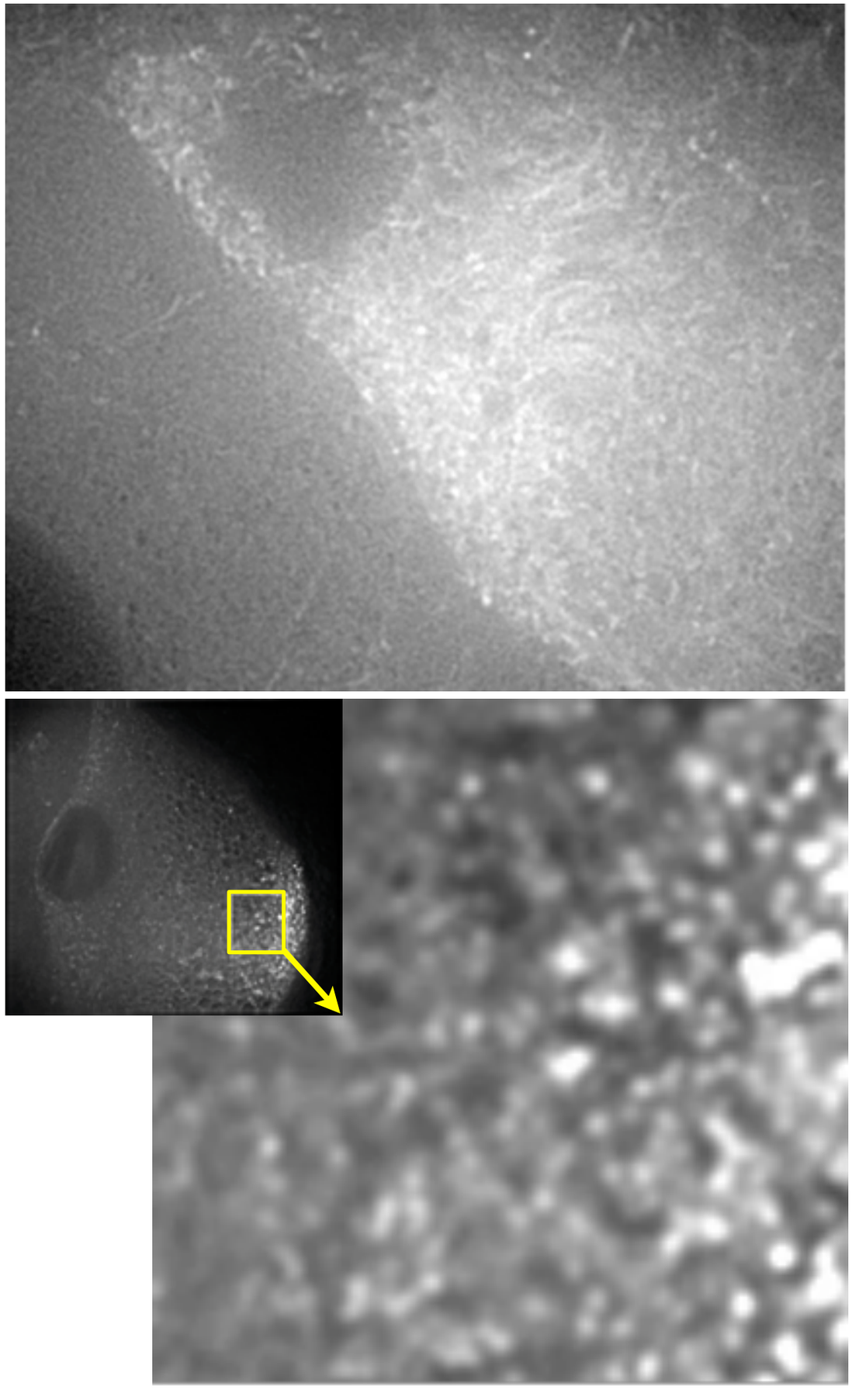
http://imgur.com/a/fhuPr

http://www.afranko.org/2014/01/calico-cat/

Fixed

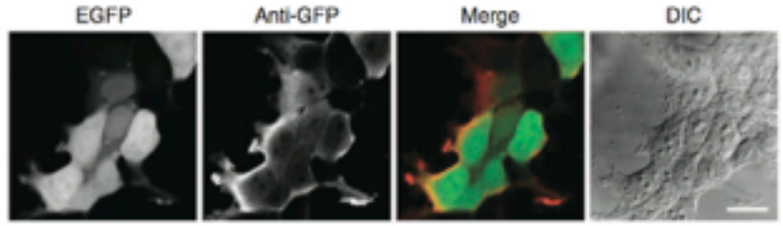


Live



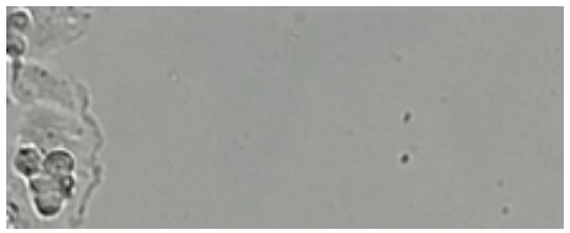
Reasons for live imaging

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



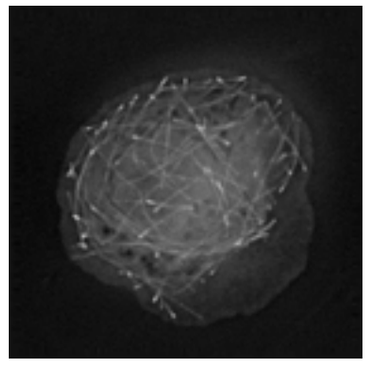
2) Can follow the order of sequential events in real time

time-course of cell migration - Andrea Linford Barr lab



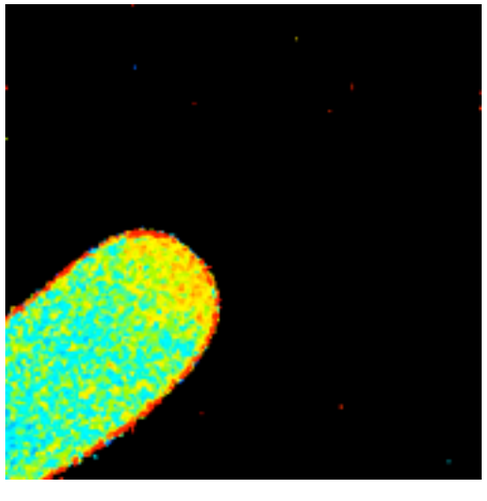
3) Can monitor the kinetics of dynamic processes:
- active transport vs diffusion
- Microtubule turnover

Macrophage: EB1-GFP tagged MT



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

Calcium ratio imaging pollen tube

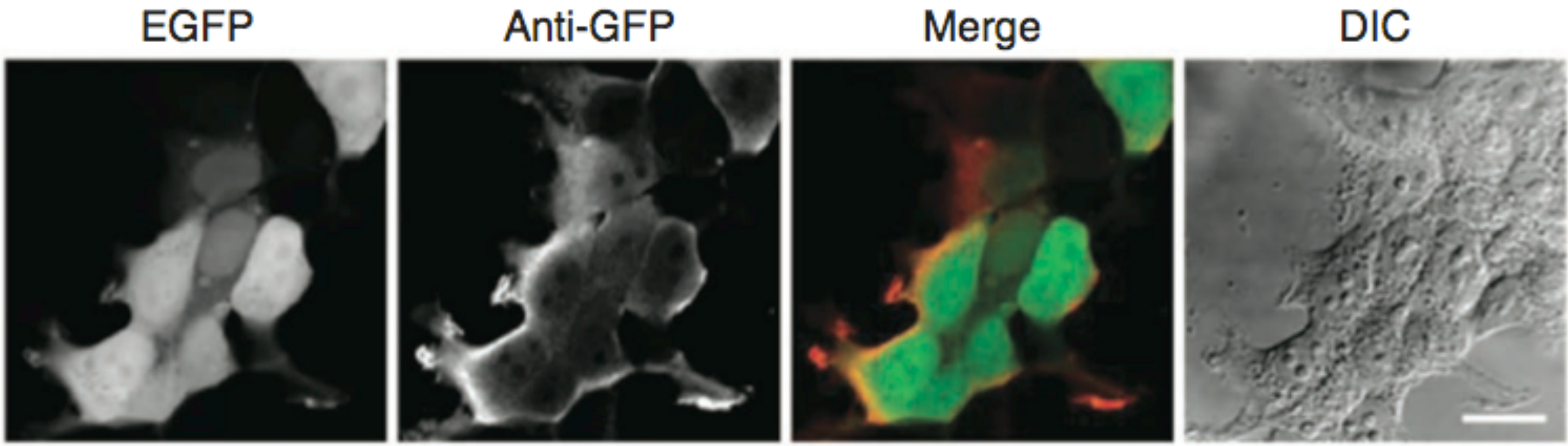


You can believe what you see - no fixation artifacts

Immunolabeling artifacts and the need for live-cell imaging

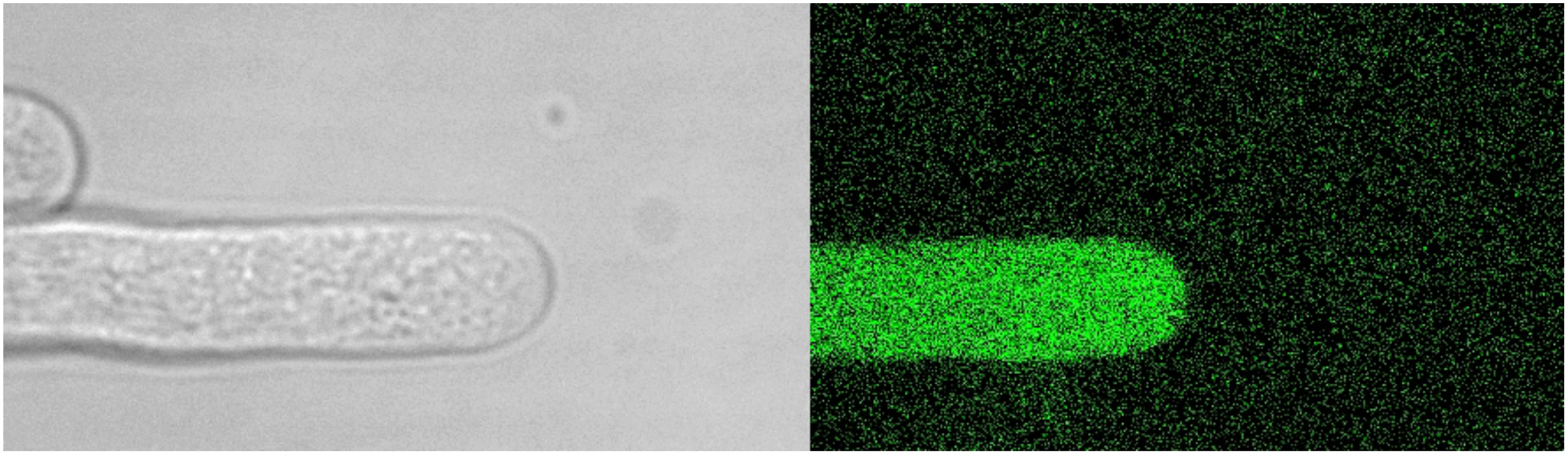
Ulrike Schnell, Freark Dijk, Klaas A Sjollema & Ben N G Giepmans

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



** Eva - lecture 4 - sample prep and minimizing artifacts **
** Errin - lecture 19 / Rainer - lecture 15 EM, correlative light and EM, super-precision microscopy **

Death by imaging!



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

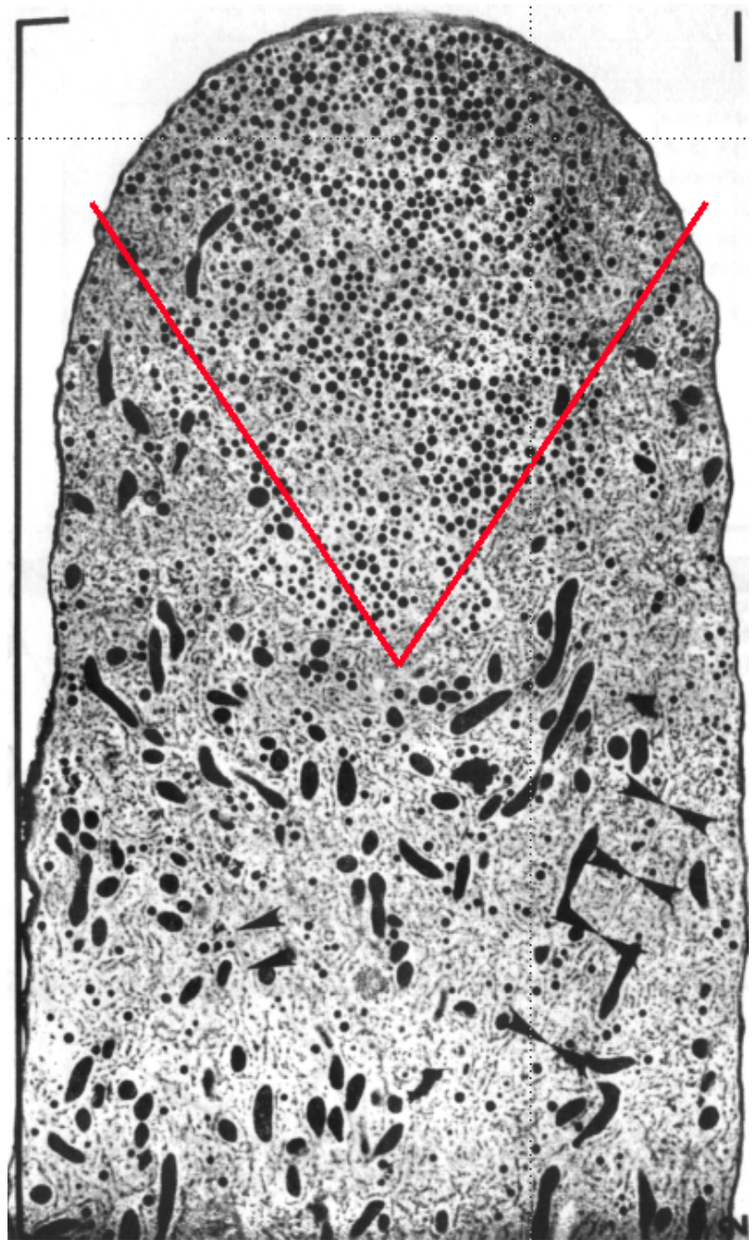
Also:

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

do the appropriate controls

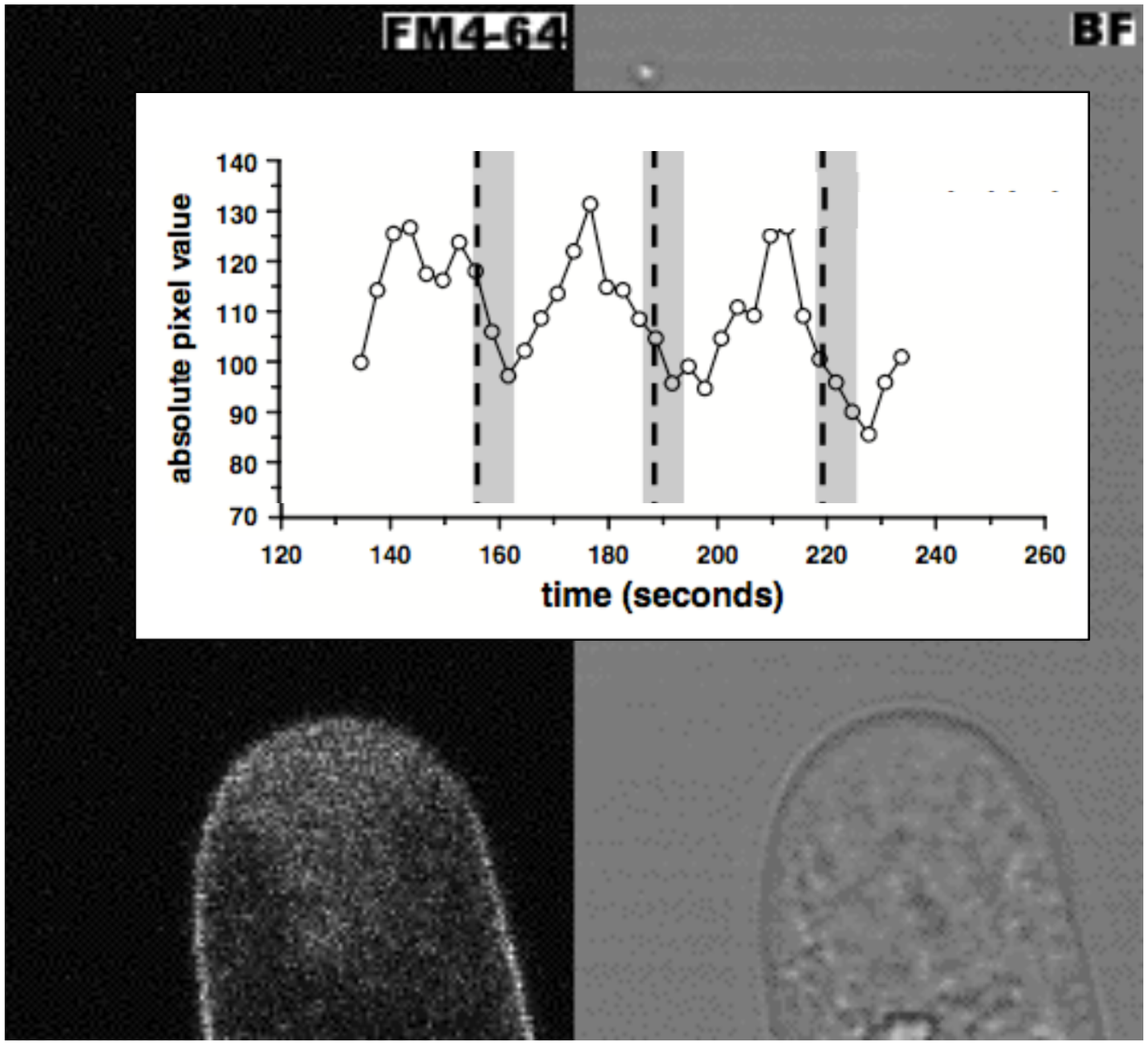
Can monitor the kinetics of dynamic processes

Fixed - EM



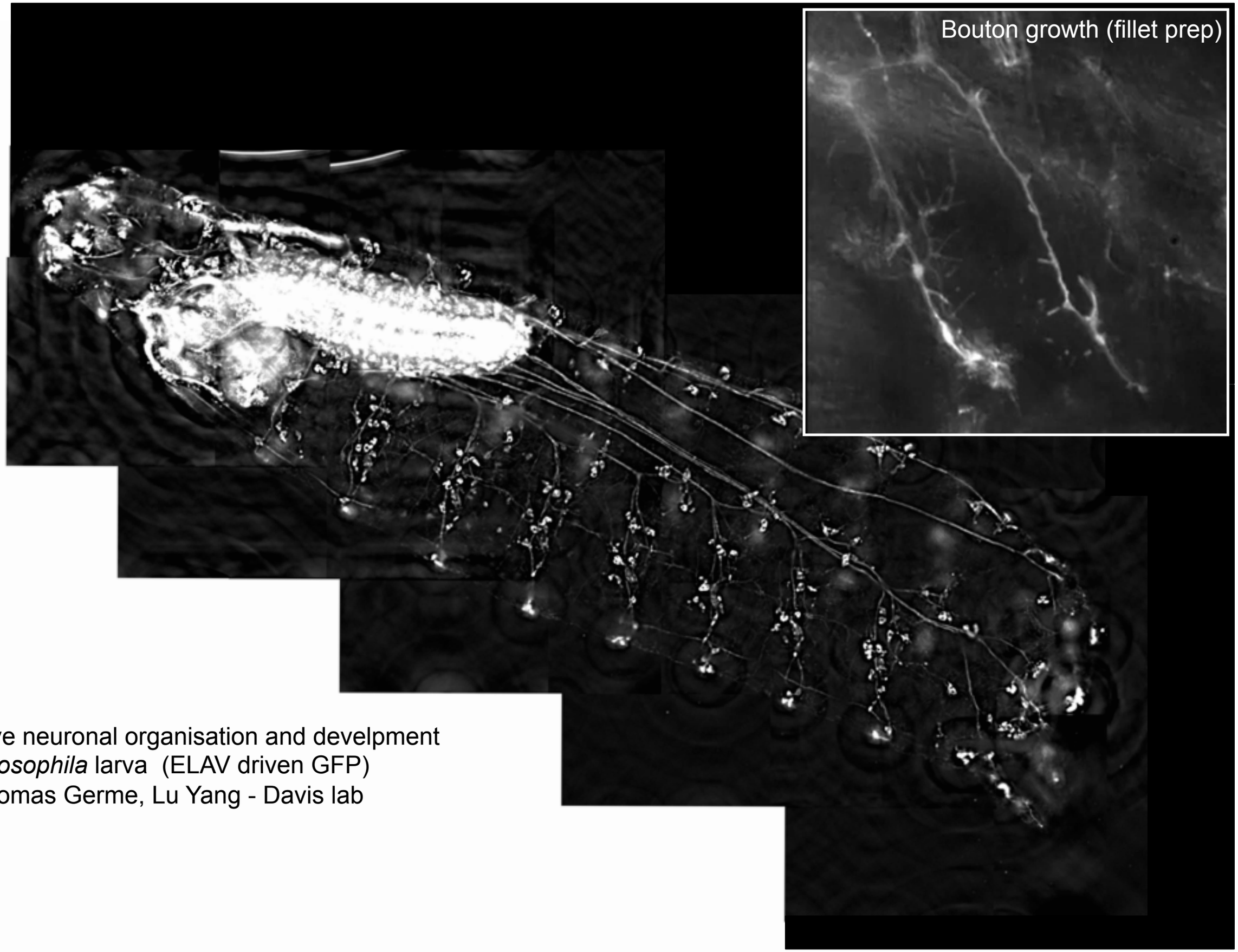
Electron Micrograph From
Lancelle,S.A.; Cresti,M.; Hepler,P.K. **(1997)** ***Protoplasma*** **196, 21-33.**

Live



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

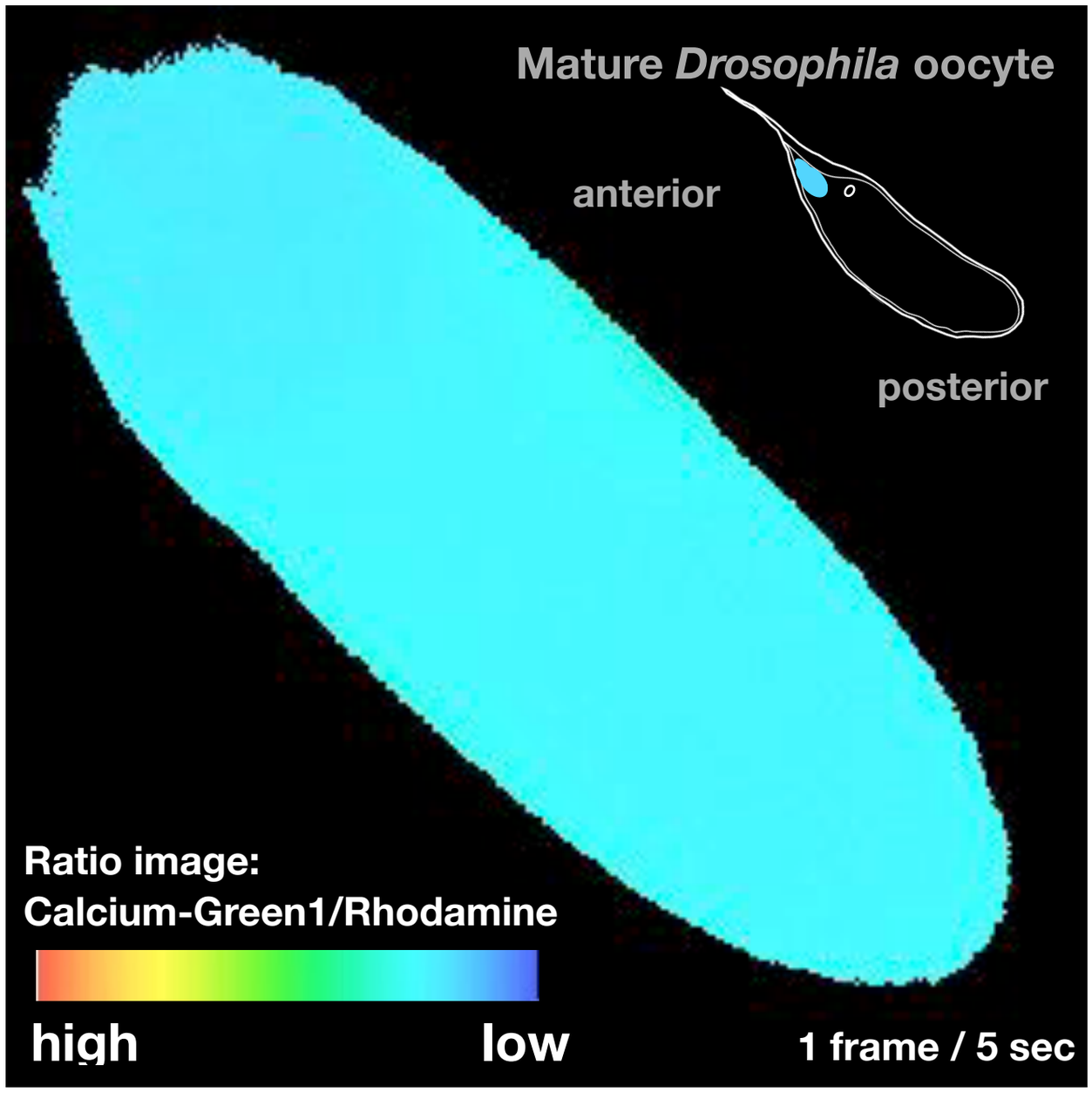
Can follow the order of sequential events in real time

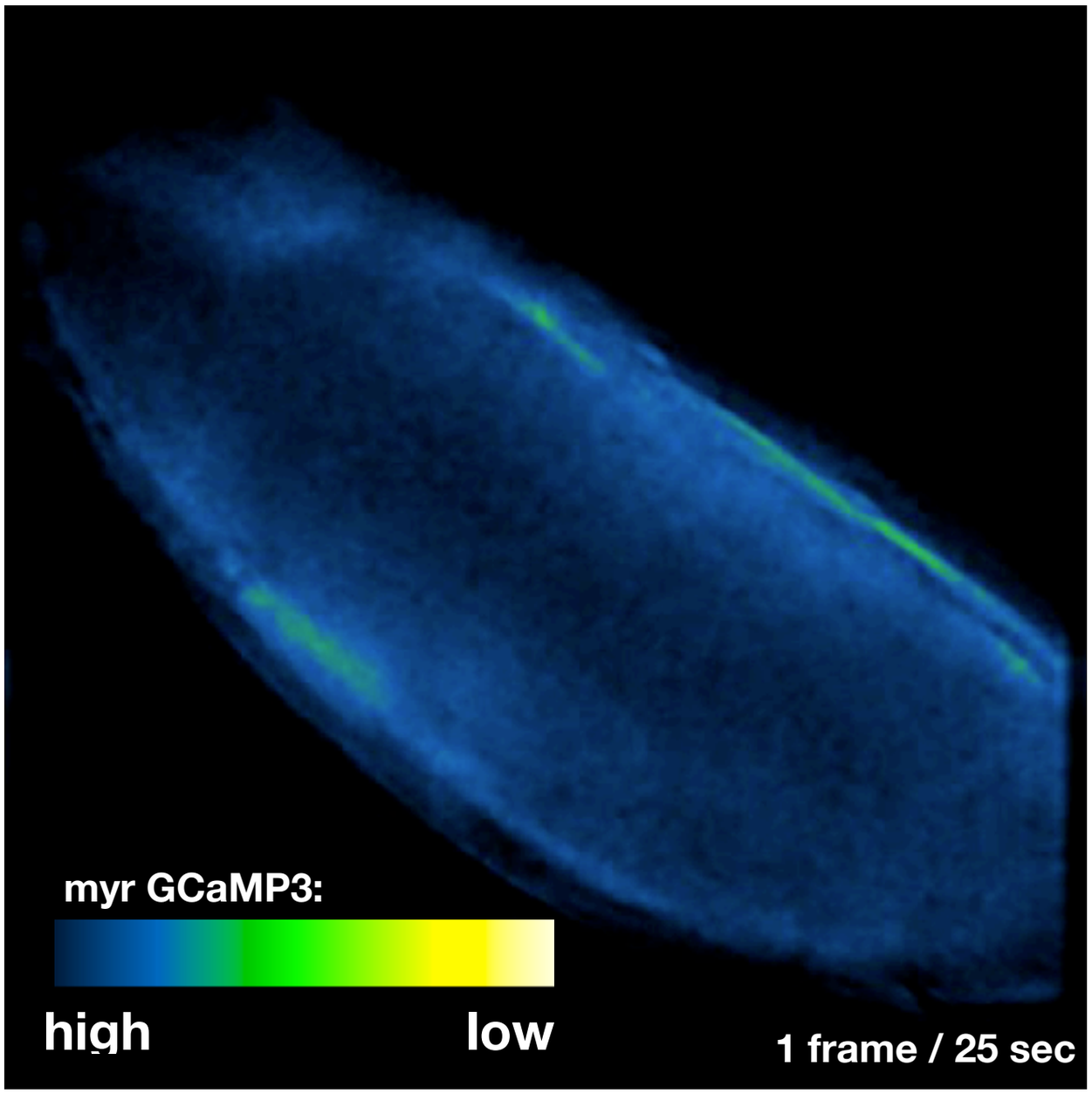


Live neuronal organisation and develpment
Drosophila larva (ELAV driven GFP)
Thomas Germe, Lu Yang - Davis lab

Can record sensitive or transient processes

Calcium transient upon activation





Claire Bromley, Richard Parton, Tim Weil: Davis Lab

Requirements for live cell imaging:

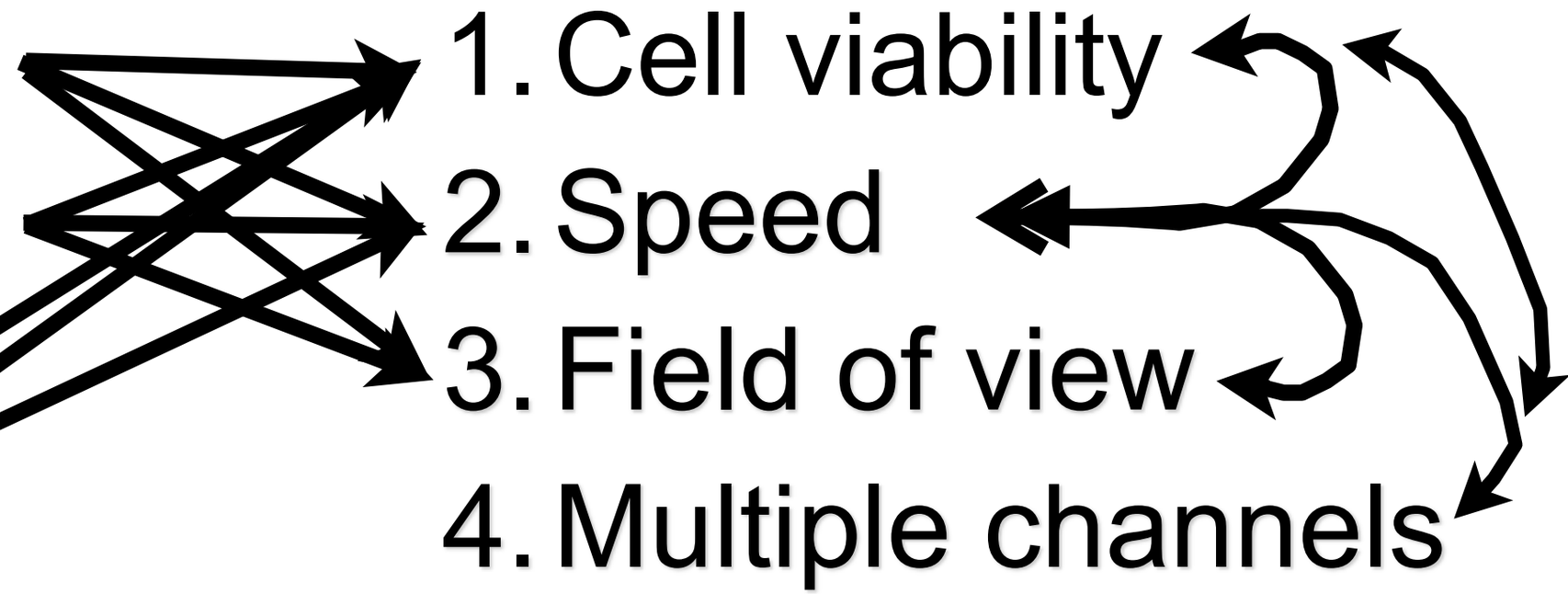
Careful Balancing of Conflicting Interests

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



What is important in microscopy?

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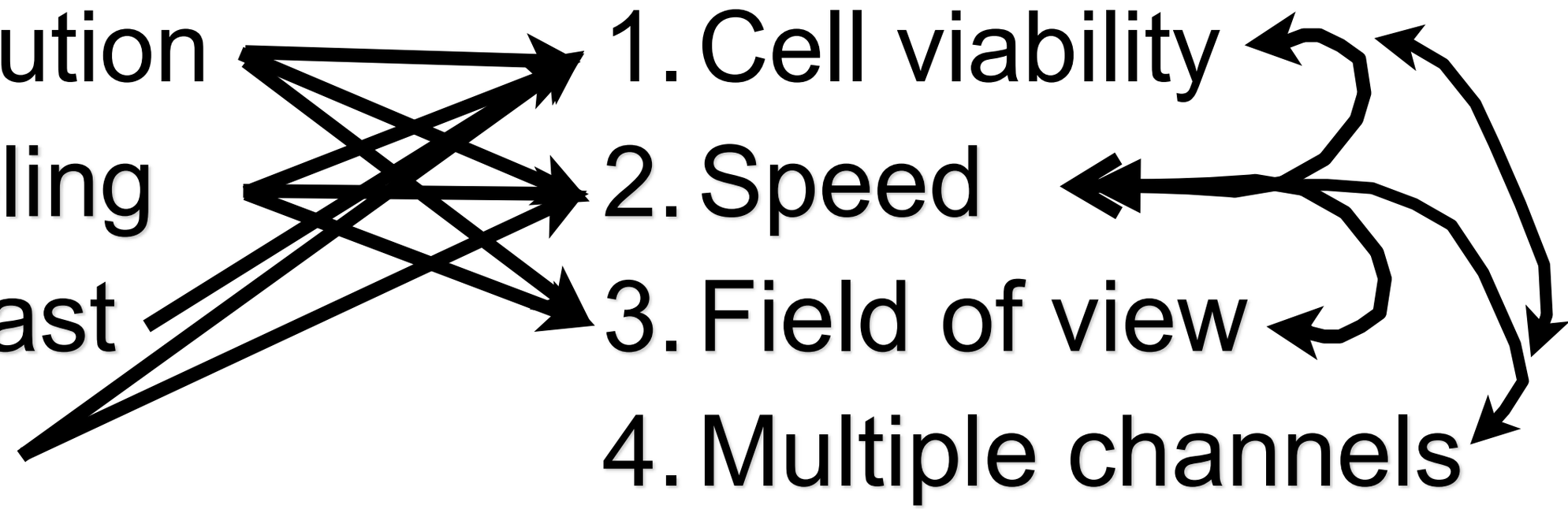


Live-cell imaging is a compromise!

Requirements for live cell imaging:

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

Optimise your experimental design:

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

http://thecatsdiary.typepad.com/.a/6a0133f3617f23970b0147e36dbedc970b-pi

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, purfect technique!

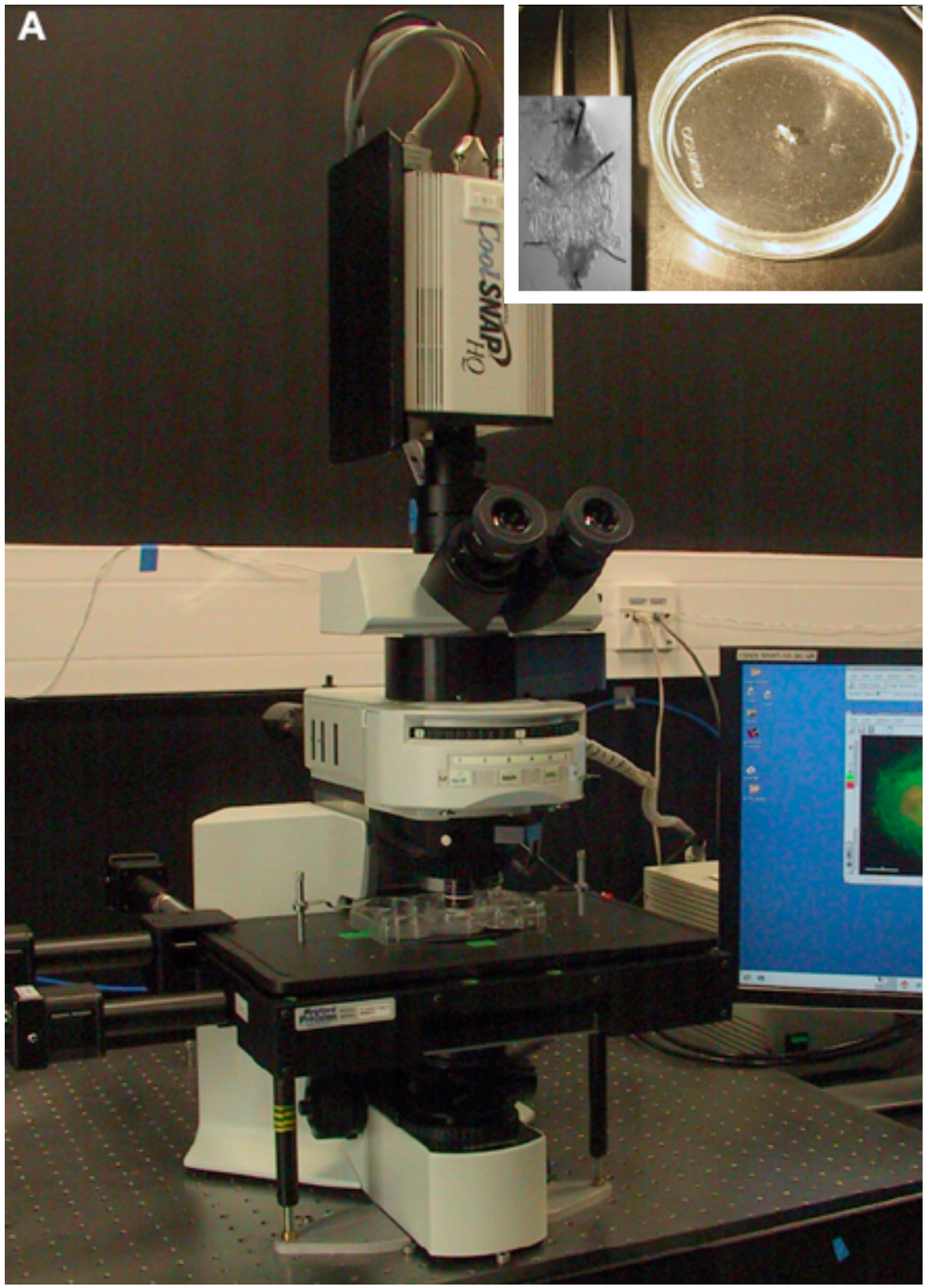
Choice of microscope stand

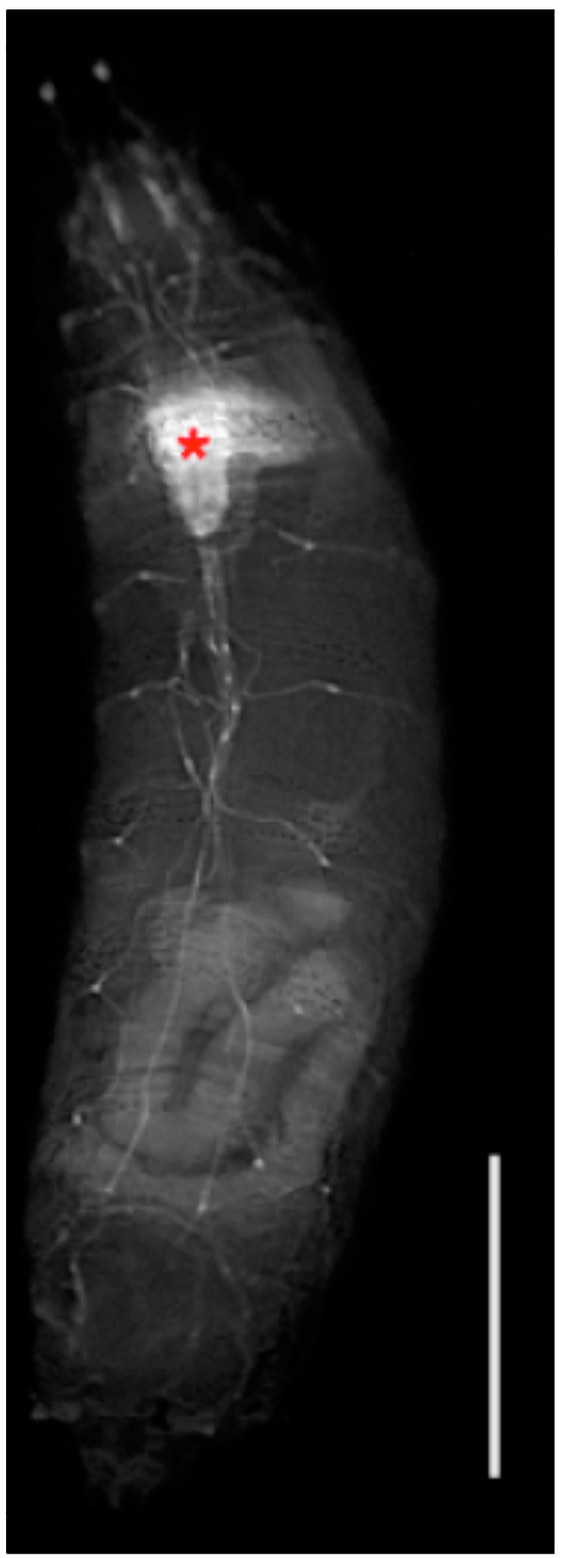
- The modern epifluorescence microscope

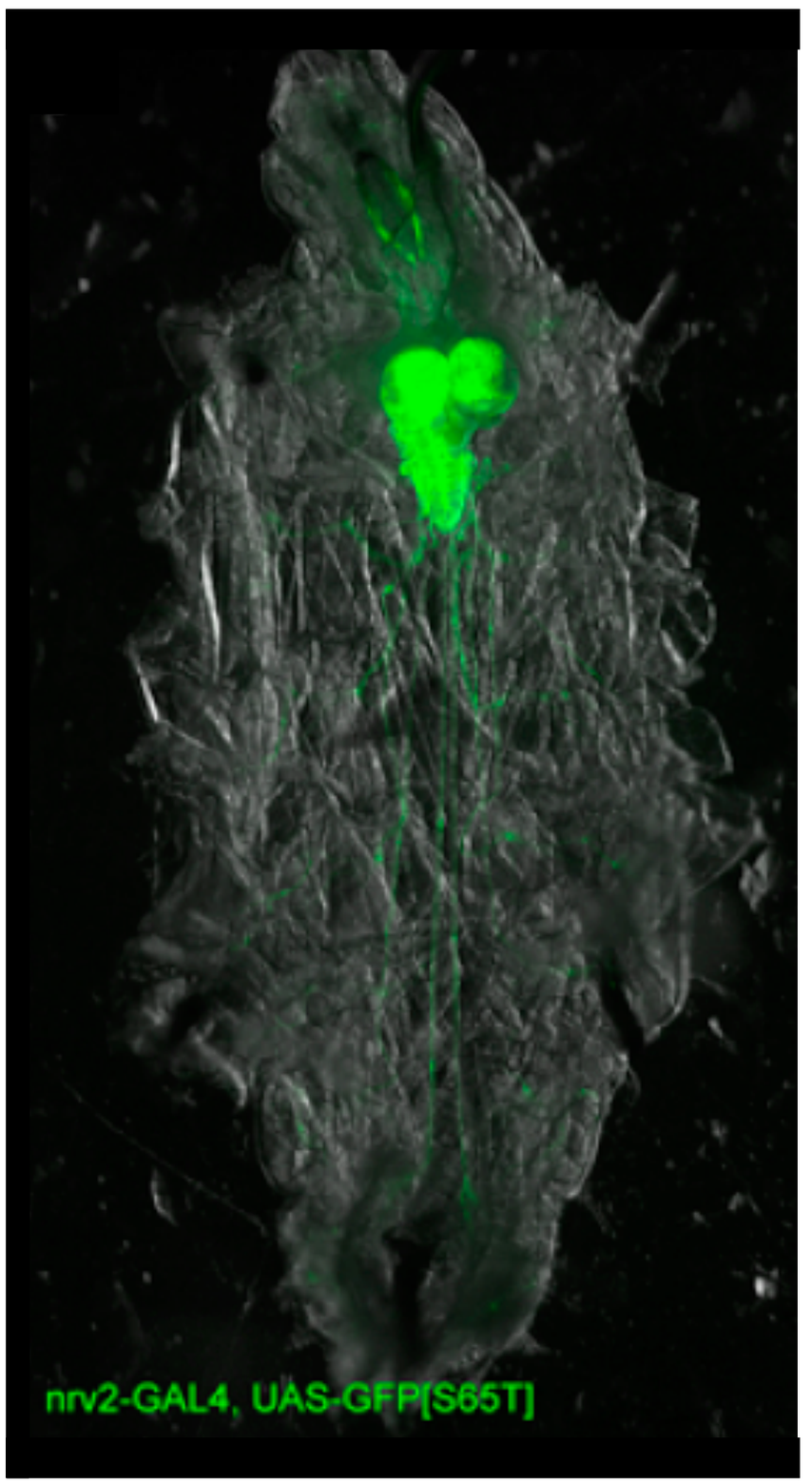
  Upright microscope (lens above specimen)

  Inverted microscope (lens below specimen)

Upright microscope - larval fillet prep

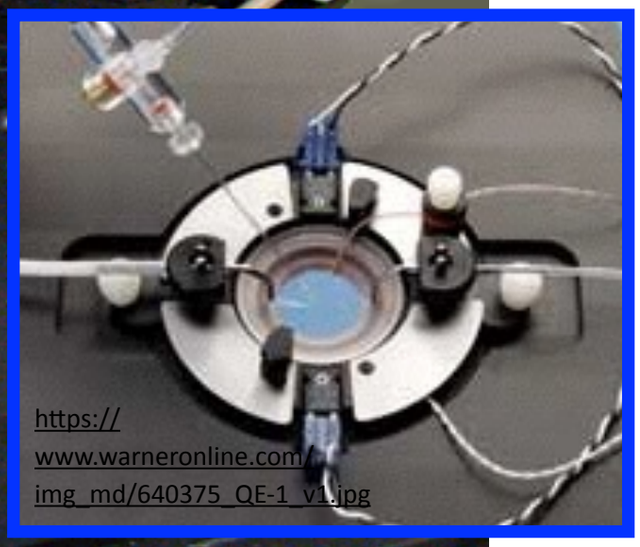






Inverted microscope - injection

http://www.liveassay.com/catalogue-2/images/47/single-dish-black-big-72dpi-301.jpg?240,300,1,100,-1493687186



https://www.warneronline.com/img_md/640375_QE-1_v1.jpg

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..

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

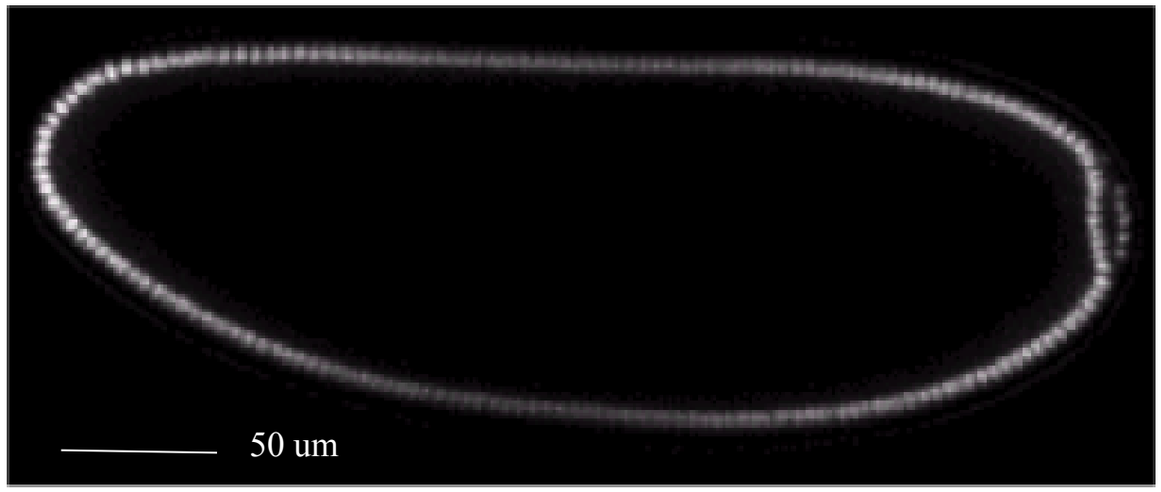
Following fast dynamic events

Use TIRF for:

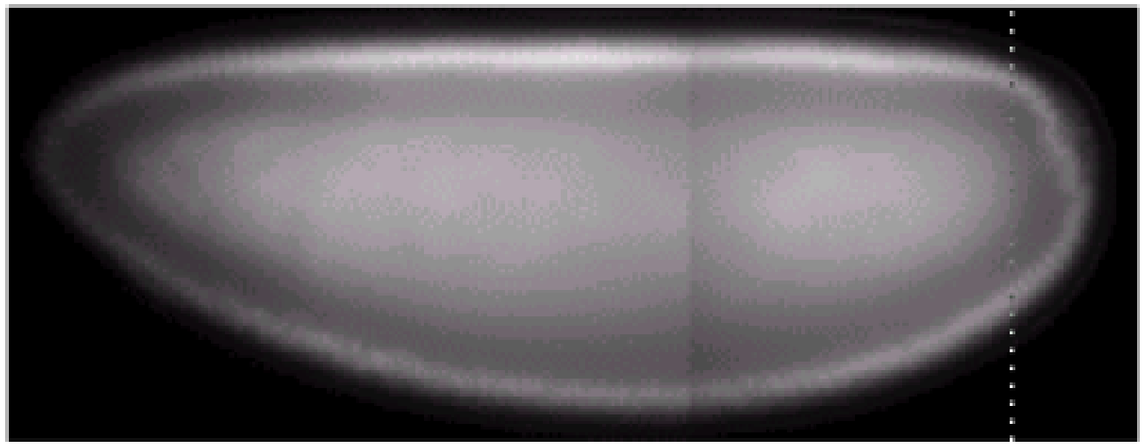
Imaging with high contrast within 100 nm of the coverslip

Choice of imaging technique:
Example of a thick specimen

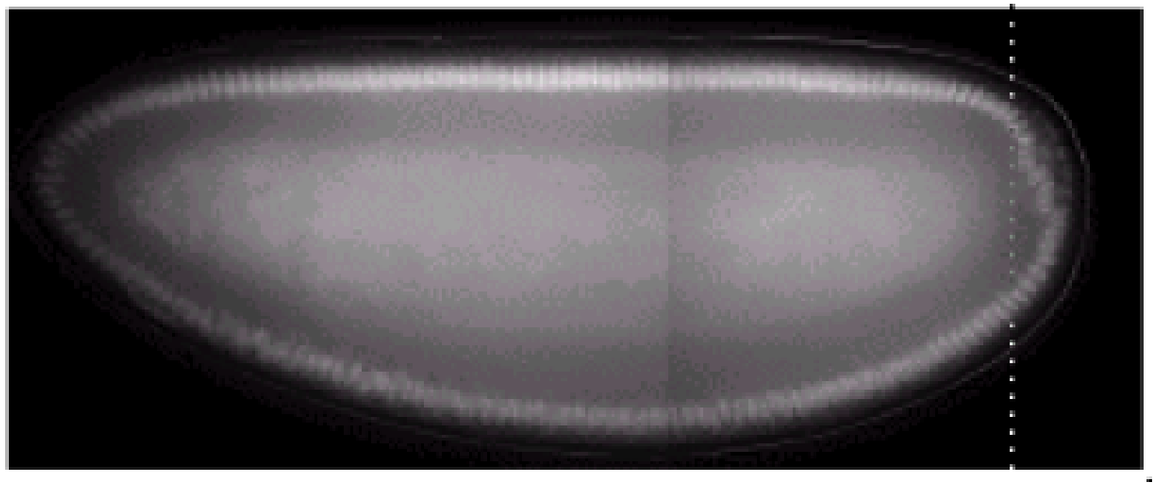
Confocal



Wide-field



WF- deconvolved



Drosophila embryo, nls GFP

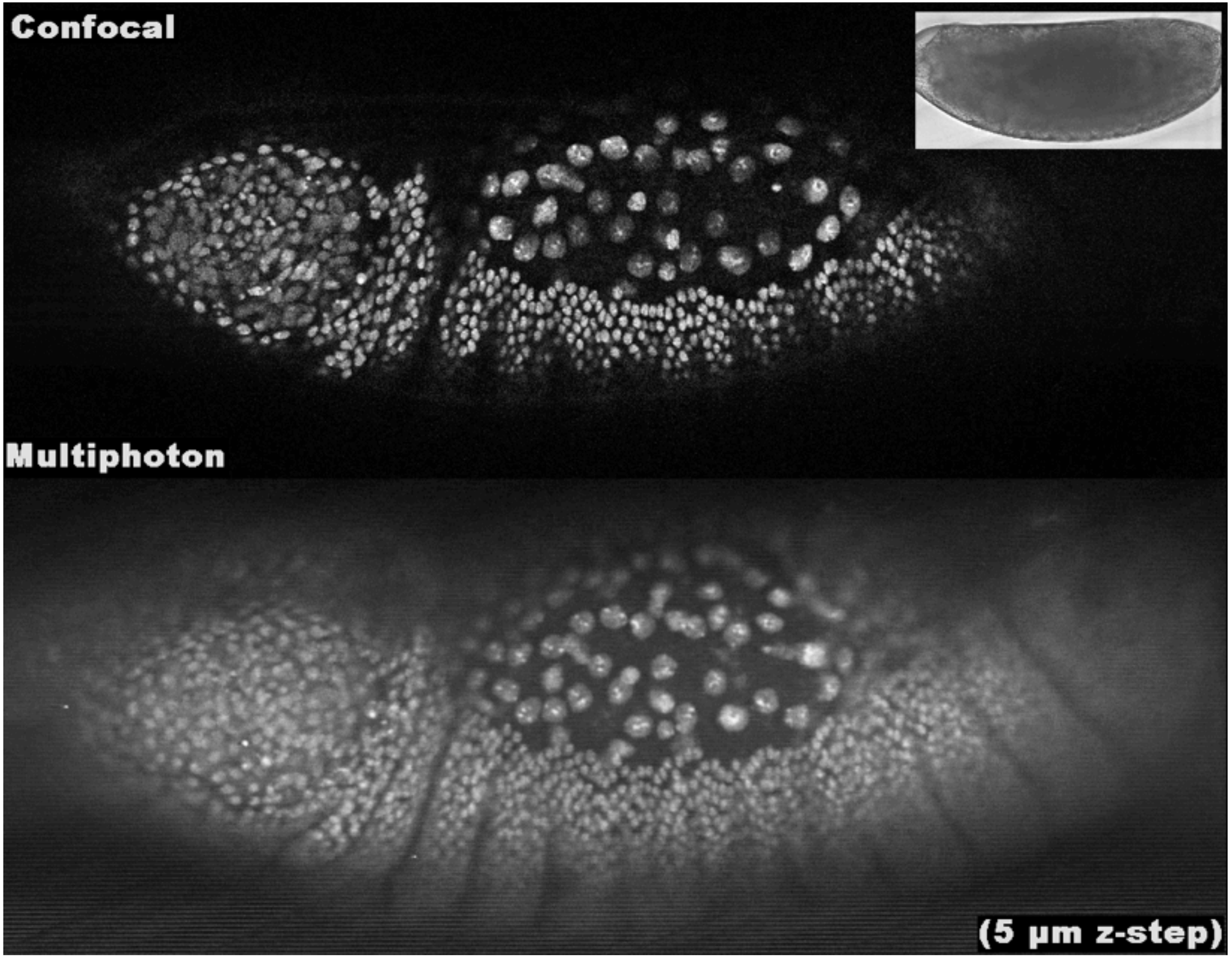
Thick, bright specimen

For really thick specimens consider point scanning confocal or multiphoton

http://animalzfun.blogspot.co.uk/2012/09/fat-cats-awesome-photographs.html

or DLSM *lecture 13*

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**

Alan - lecture 8 - confocal and multiphoton

If the choice is not obvious...

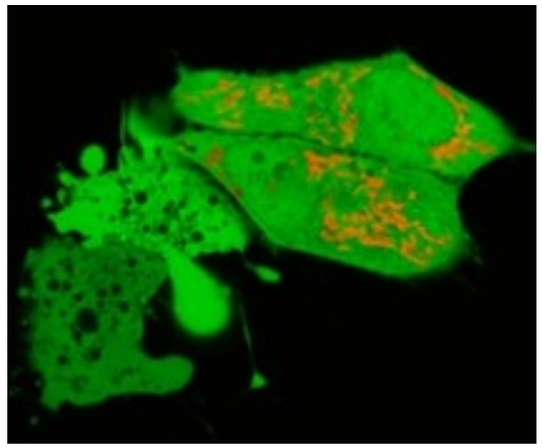
it’s worth trying them all.

Live imaging as an experimental tool:

Using light to manipulate cell behaviour:

- "Killer red" genetically encoded photosensitiser CALI = chromophore assisted light inactivation

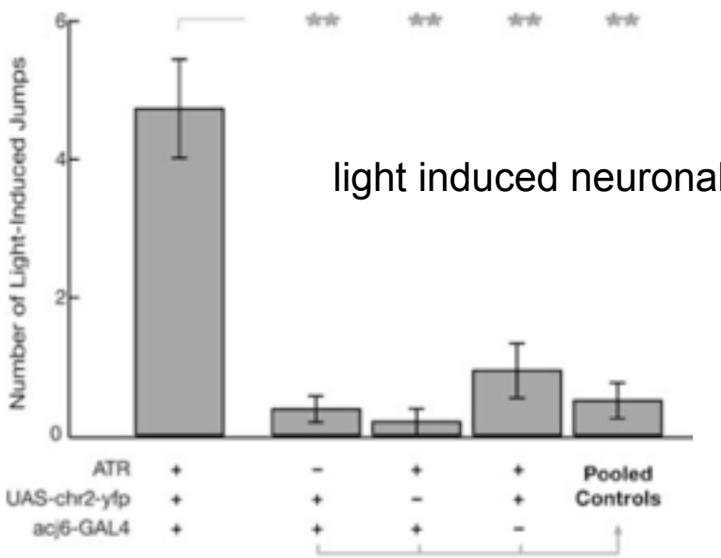
Reactive oxygen species in photochemistry of the red fluorescent protein "Killer Red"
Vegh et al, Chem. Commun., 2011,47, 4887-4889
DOI: 10.1039/C0CC05713D



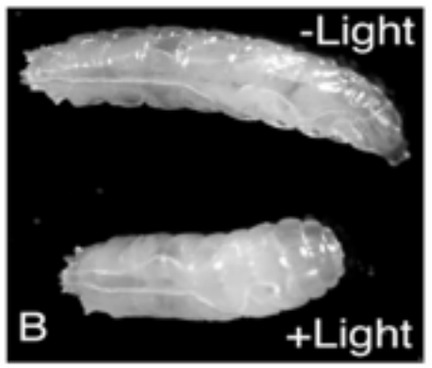
EVROGEN - Killer red expressed in mitochondria

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

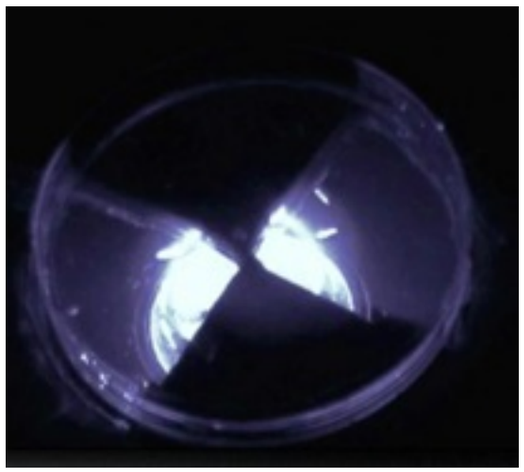
Zimmermann, G., et al. (2009). Manipulation of an Innate Escape Response in Drosophila: Photoexcitation of acj6 Neurons Induces the Escape Response. PLoS ONE, 4(4), e5100. doi:10.1371/journal.pone.0005100.g005



light induced neuronal activity in Drosophila



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



Ruhr-Universitaet-Bochum via PhysOrg

"Photoexcitation of acj6 neurons is sufficient to induce a startle response"

For live cell imaging collect every photon:

Be economical with your light budget - hardware

- Sensitive detectors **Deep Cooled CCD's**
  EMCCD's

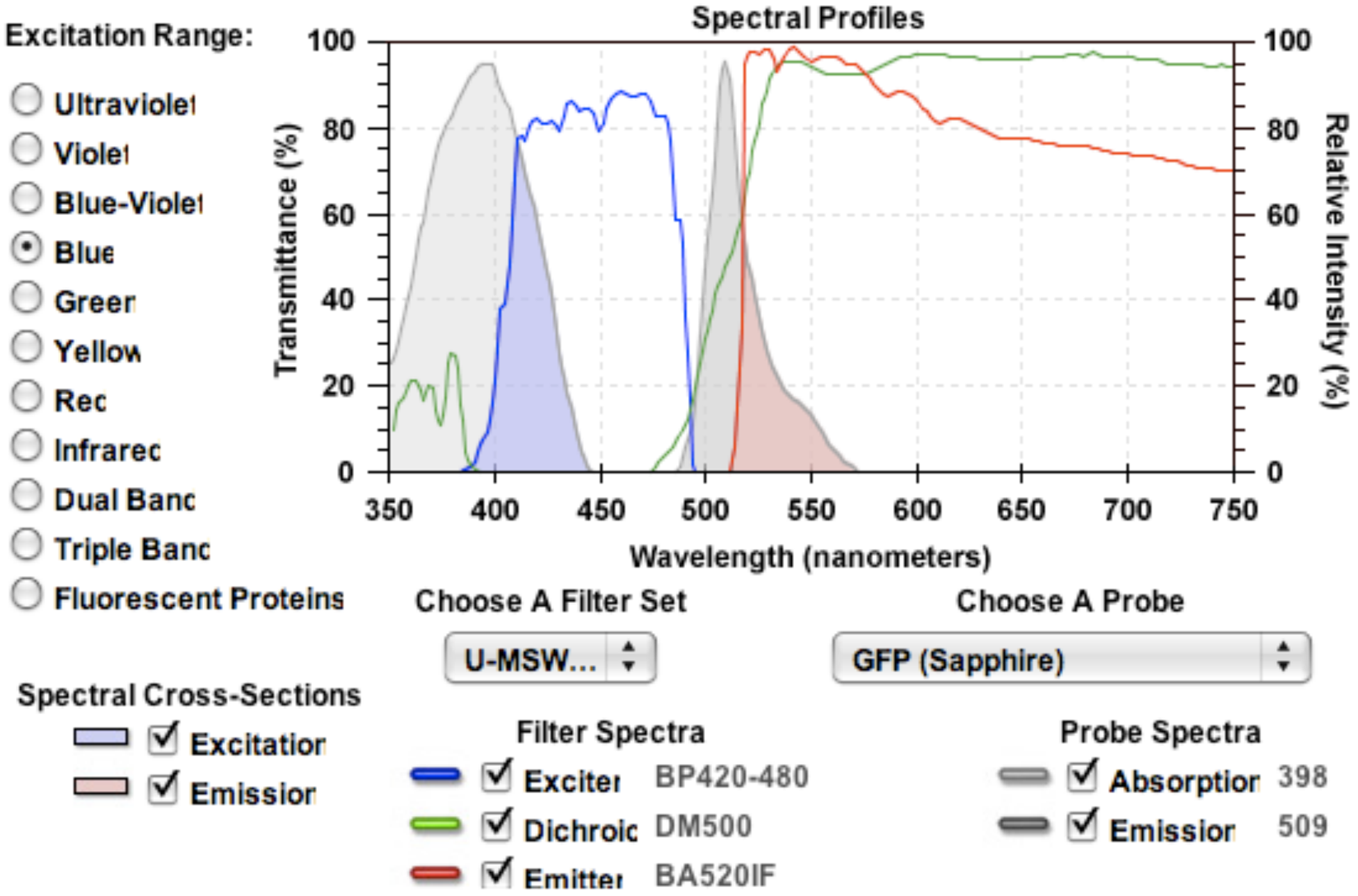
- Optimised synchronisation of illumination, exposure and readout
  "real time" system controllers
  fast shuttering
  diode light sources

- Optimised filter sets for your probes
  hard coated "ET" filter sets
  filter free "spectral" options

- Choose the best objective for the job **Oil immersion**
  water immersion
  RI matching immersion

- Set up your equipment properly

Matching Fluorescent Probes to Filter-Sets



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

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

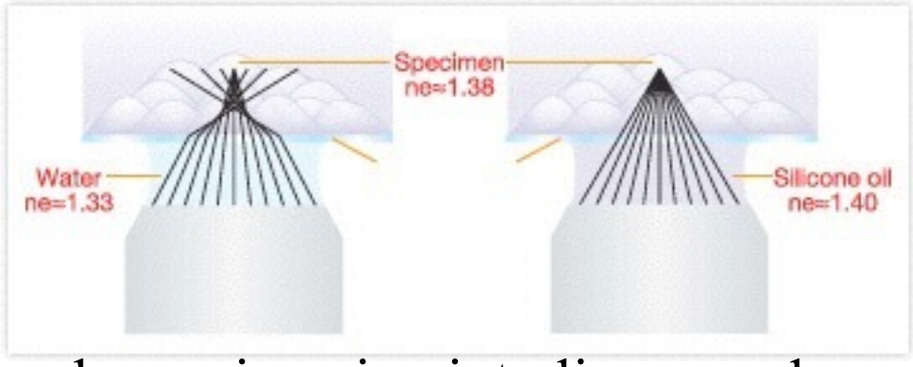
  (apo chromatic)

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

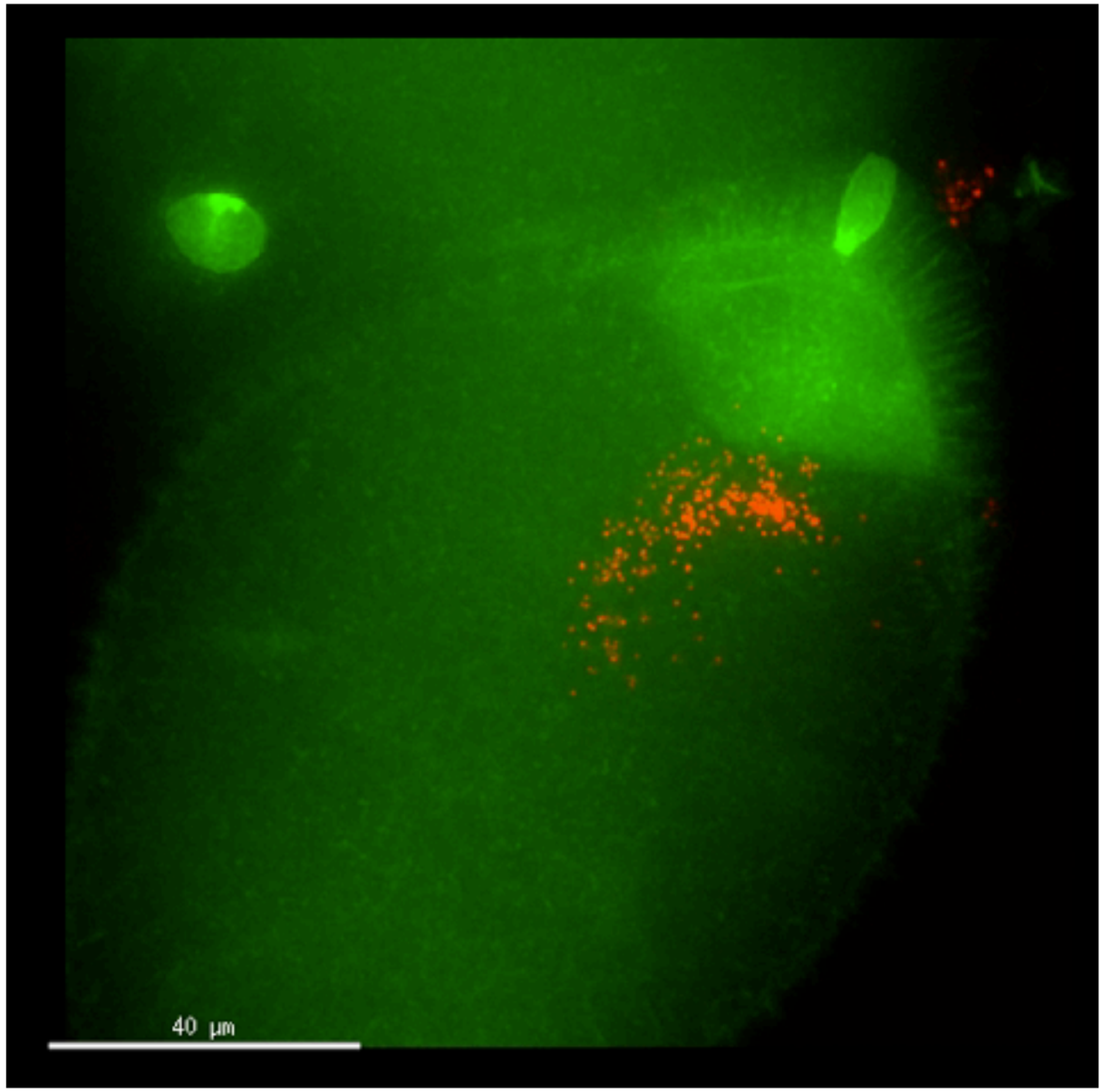
Very Expensive!!

Setup your imaging equipment properly:

http://ecx.images-amazon.com/images/I/61XmwpJvskL._SL1500_.jpg

Correcting Spherical Aberration:

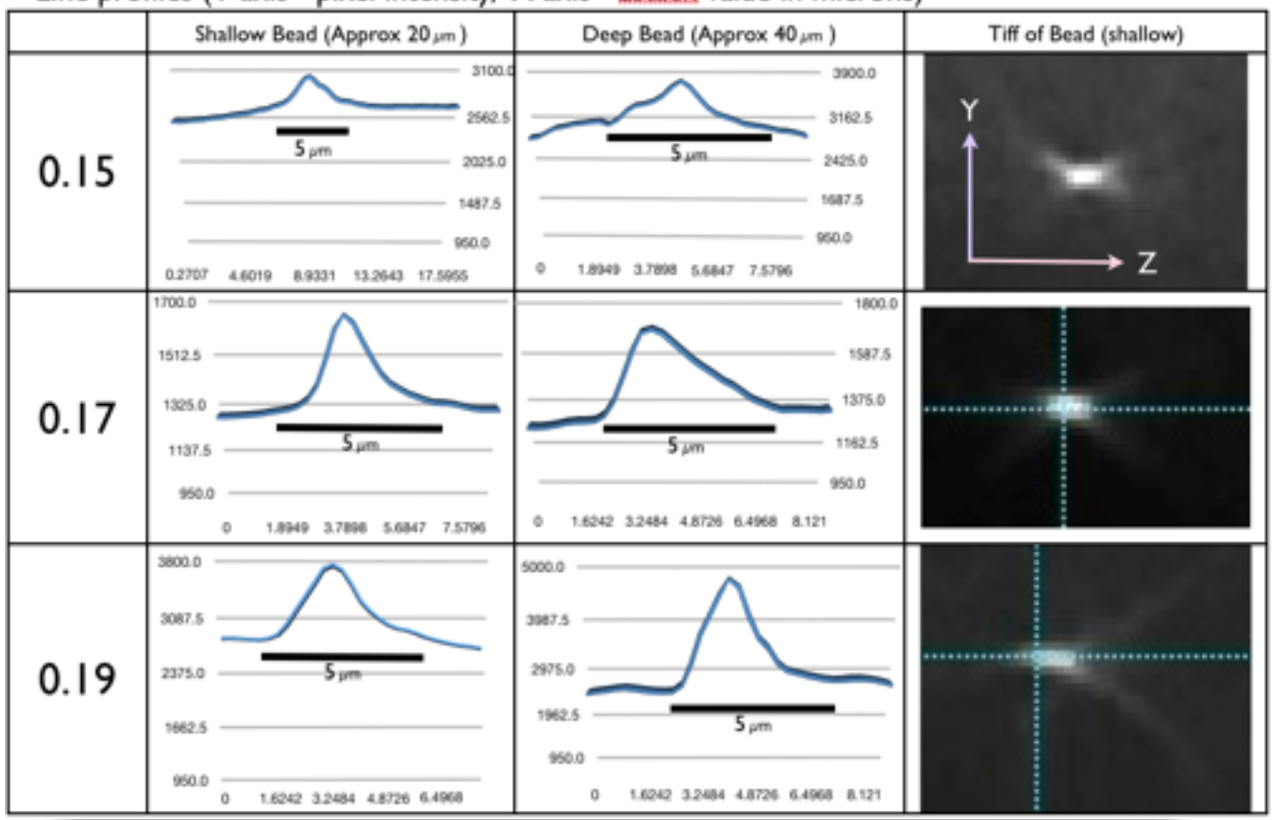
- Evaluate of spherical aberration with depth
- Explore corrective collar settings
- Automate correction



ActinGFP expressing Drosophila egg chamber injected with 100 nm red beads

Data Taken with Manual Lever

Line profiles (Y axis - pixel intensity, X axis - zstack value in microns)



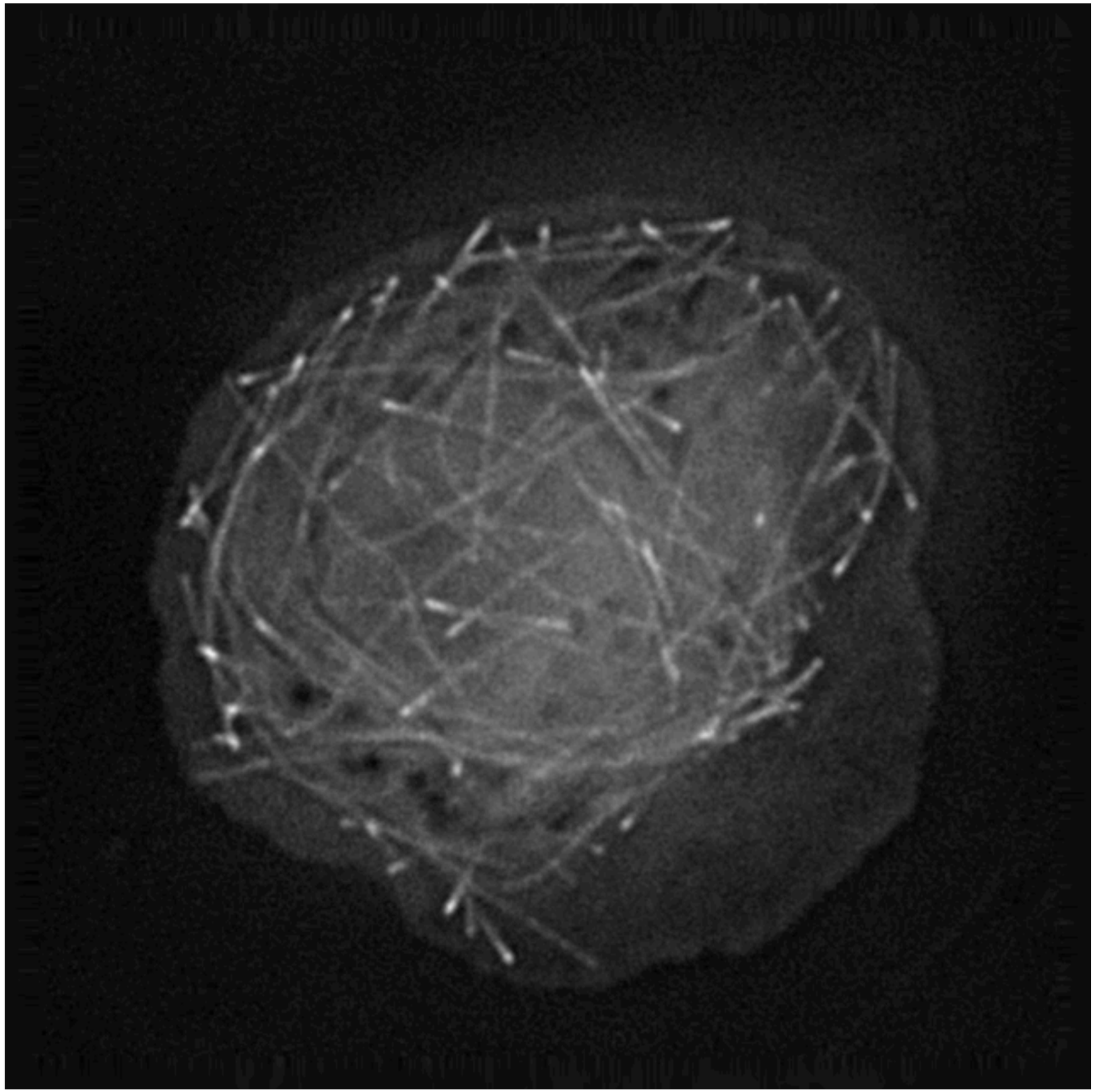
Jonathan Sturt, RMP: x60 SI lens - manual collar correction

Be economical with your light budget - best practice

- Close down the field iris to cover just the region of interest
- Use bright-field to minimise light exposure
- Correct spherical aberration
- Choose good labels
- Careful specimen preparation
- Make use of denoising algorithms

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

Eric Poeschla, Mayo Clinic



Macrophage: GFP microtubules

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

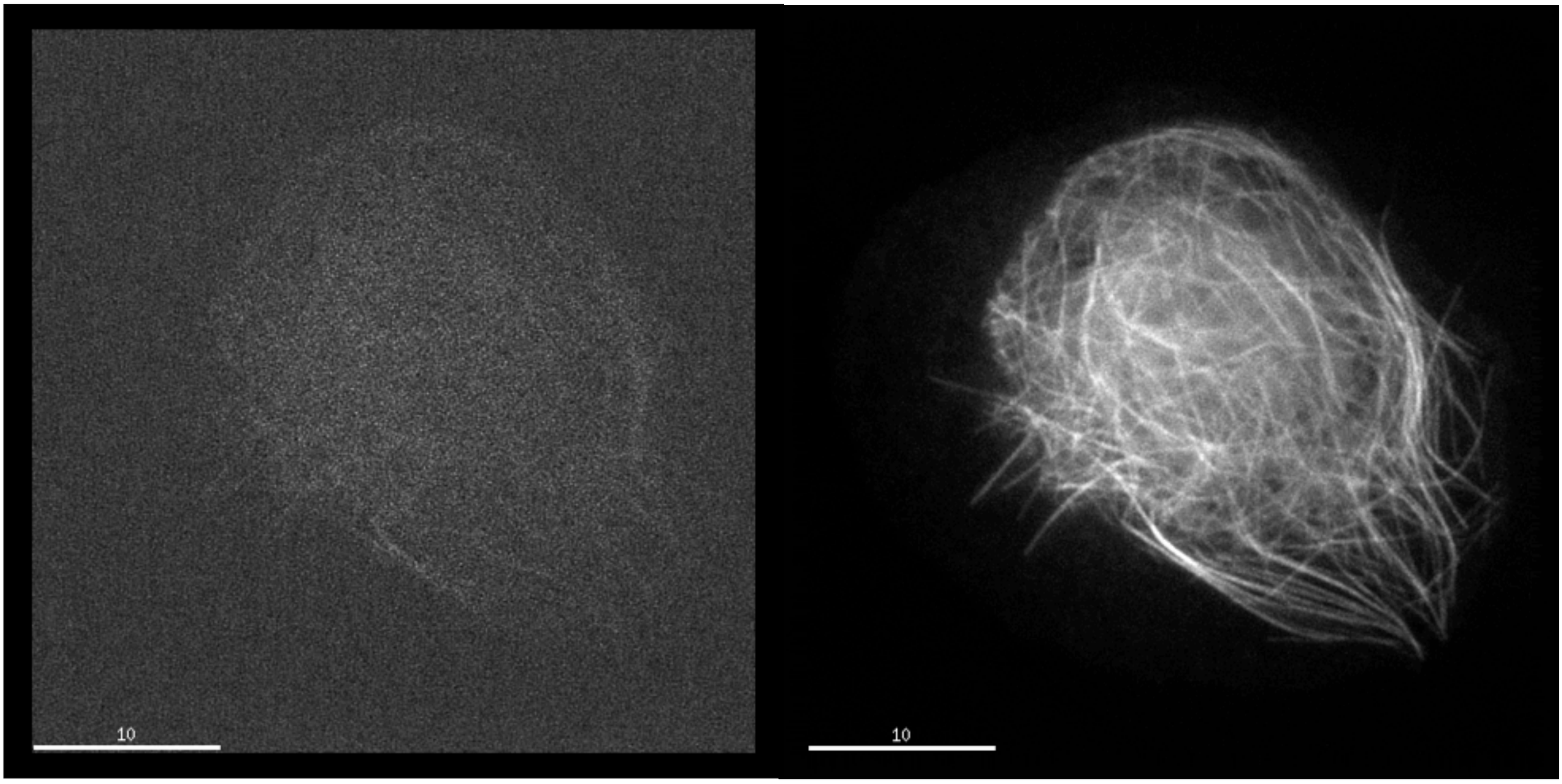
  Media / drying out

  Temperature / CO2

Denoising - imaging with 10-100 x less light

8 ms exposure, 0.1% 488 Laser power

8 ms exposure, 10% 488 Laser power



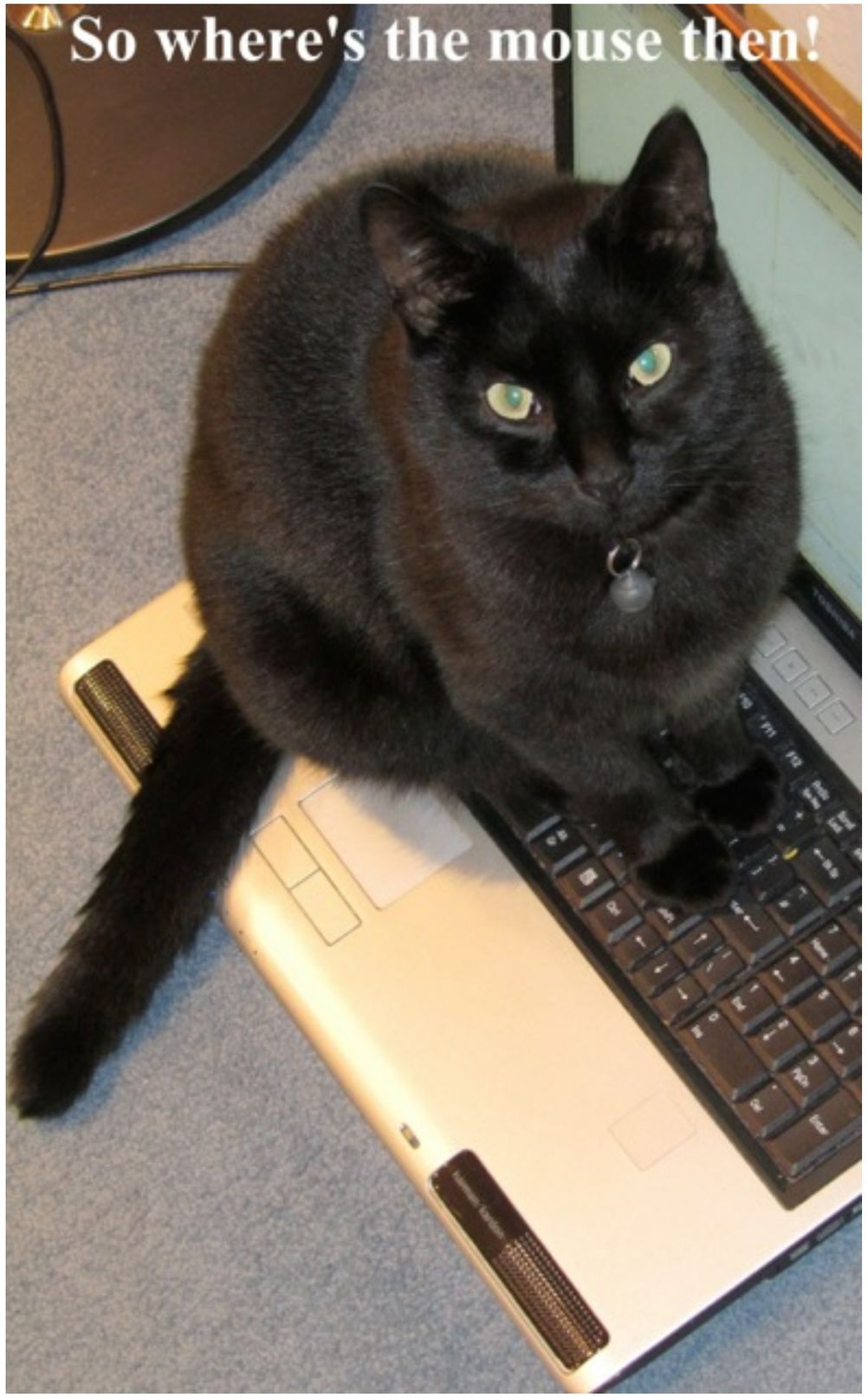
Live Macrophage: Jupiter-GFP labeling microtubules; 7Z, 3 stacks per second

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

END



Reference Material:

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

http://www.olympusmicro.com/
General info on microscope components, setup and use

http://www.olympusmicro.com/primer/digitalimaging/deconvolution/deconartifacts.html
Deconvolution artefacts