Micron Advanced Light Microscopy Course
Introduction to Microscopy 2016

lecture 6
Live Cell Imaging

Richard M Parton - Richard.Parton@bioch.ox.ac.uk
Department of Biochemistry
University of Oxford

Live Cell Imaging

- Reasons for live cell imaging

- Requirements for live cell imaging

  Experimental design

  Choice and setup of equipment

  Collect every photon

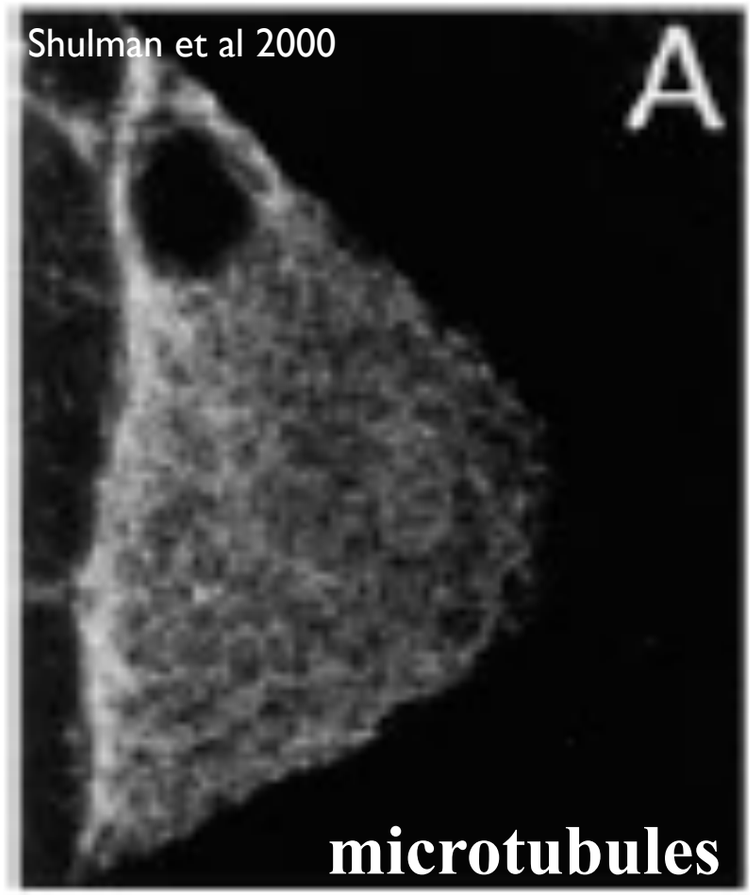
  Image processing and analysis

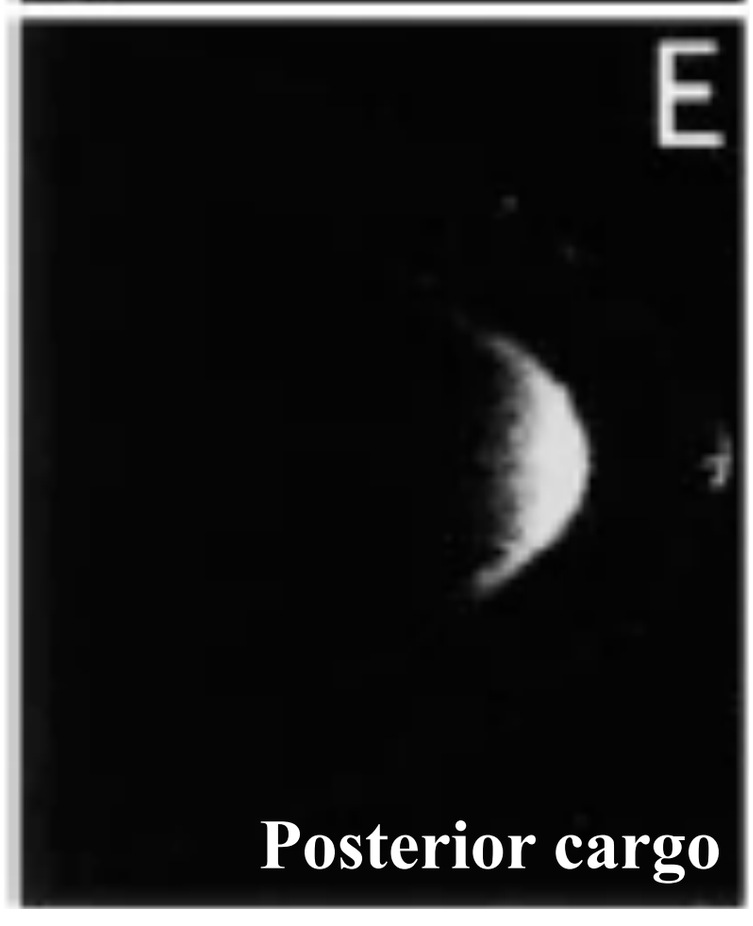
Reasons for live imaging: Fixed vs Live

http://imgur.com/a/fhuPr

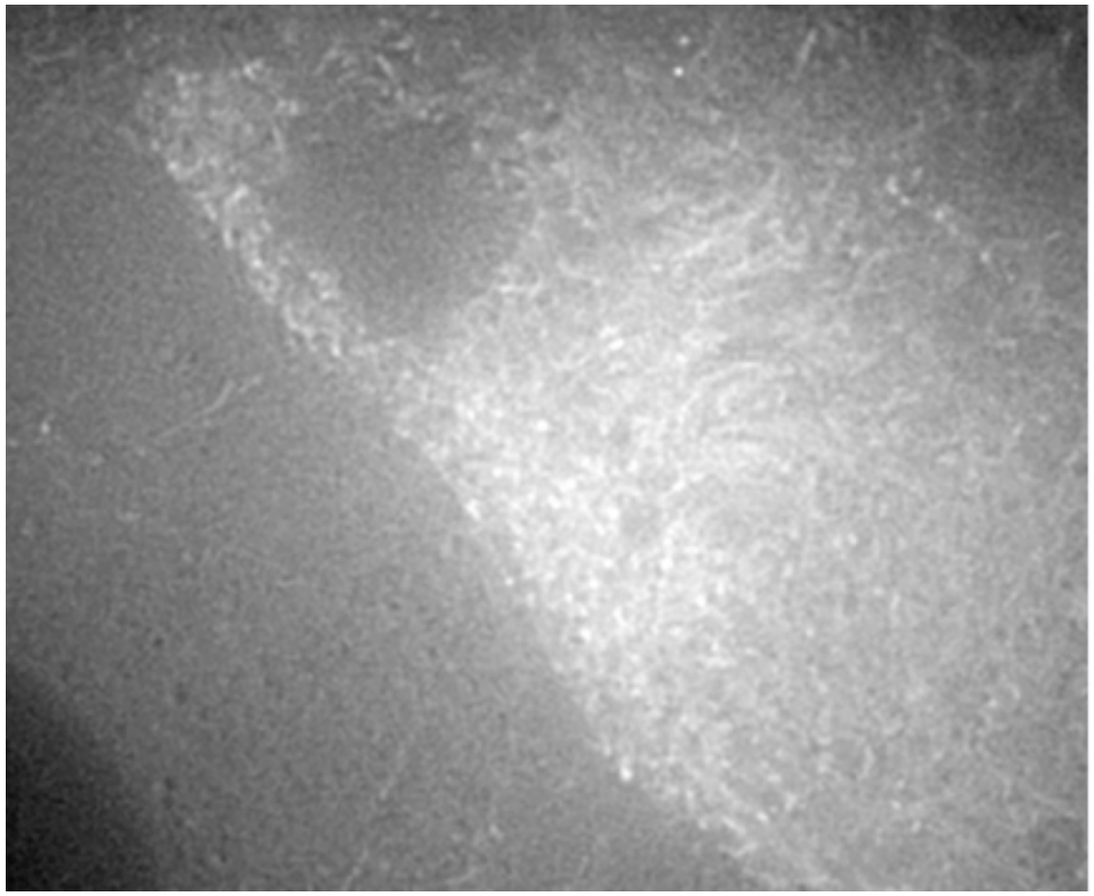
http://www.afranko.org/2014/01/calico-cat/

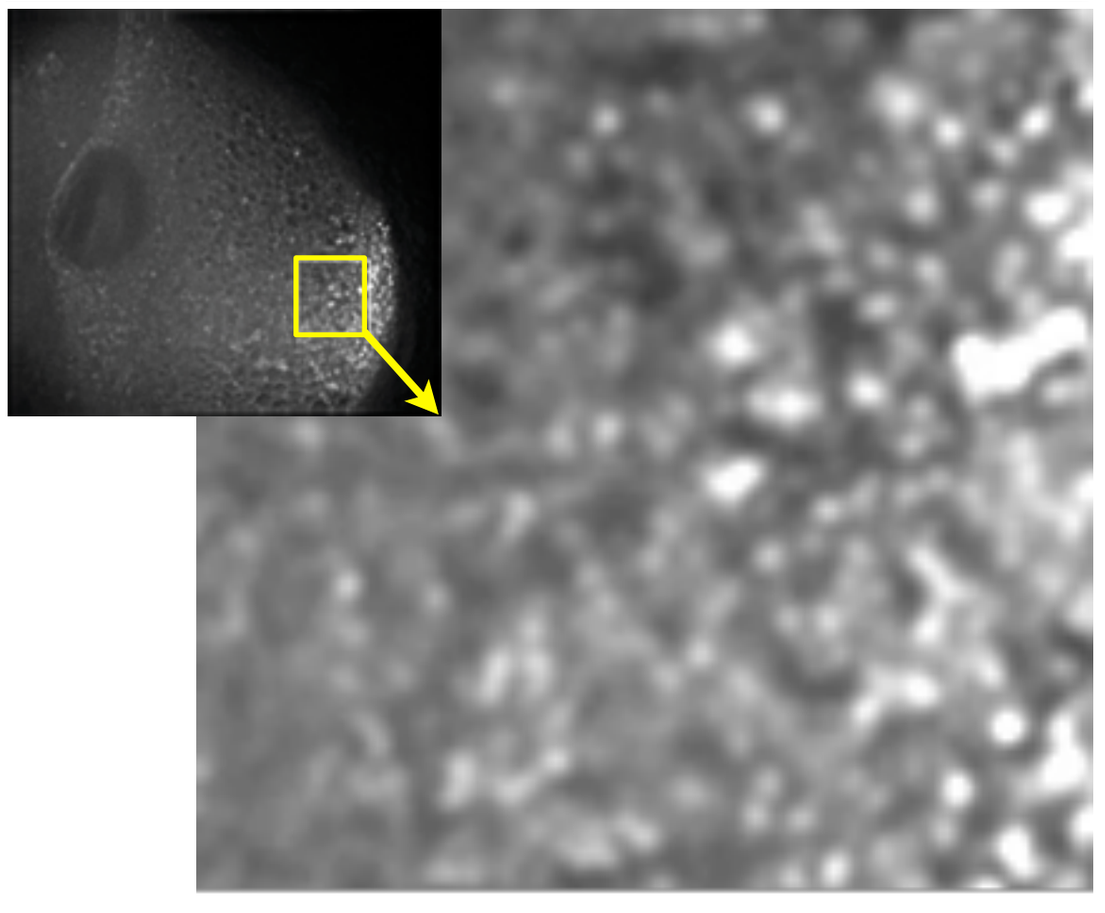
Fixed



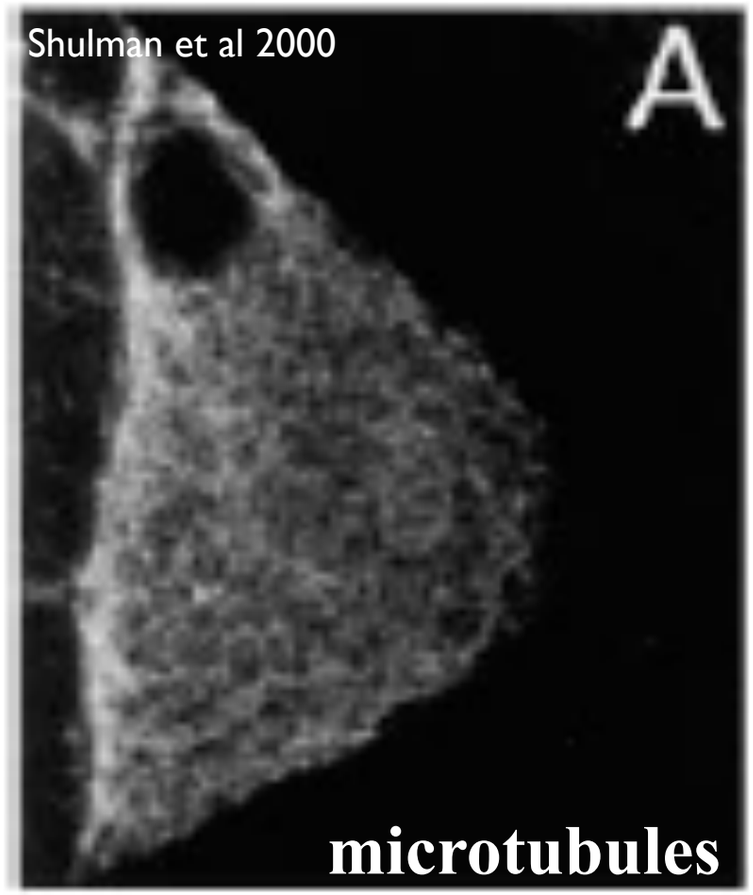


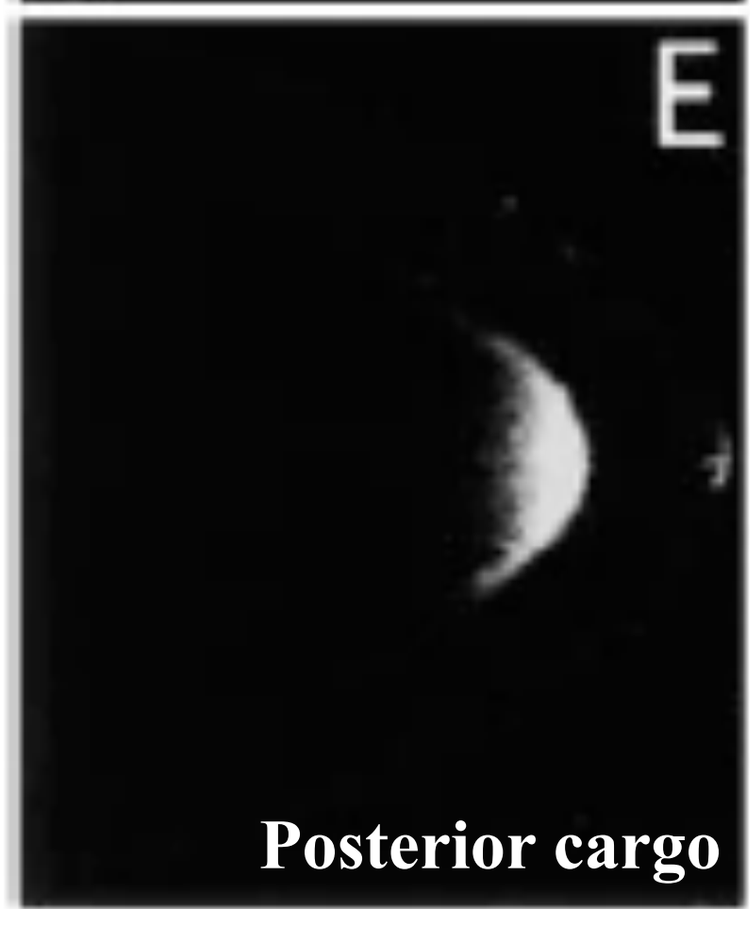
Live





Fixed





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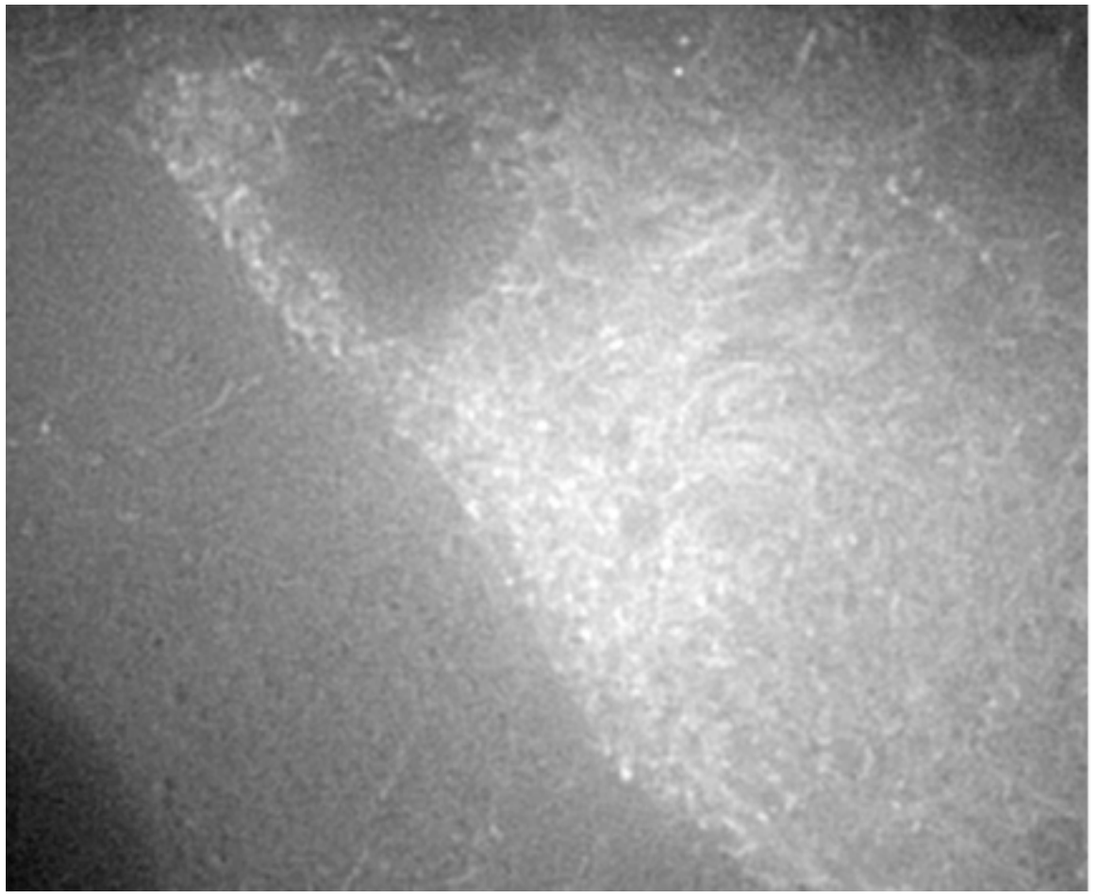

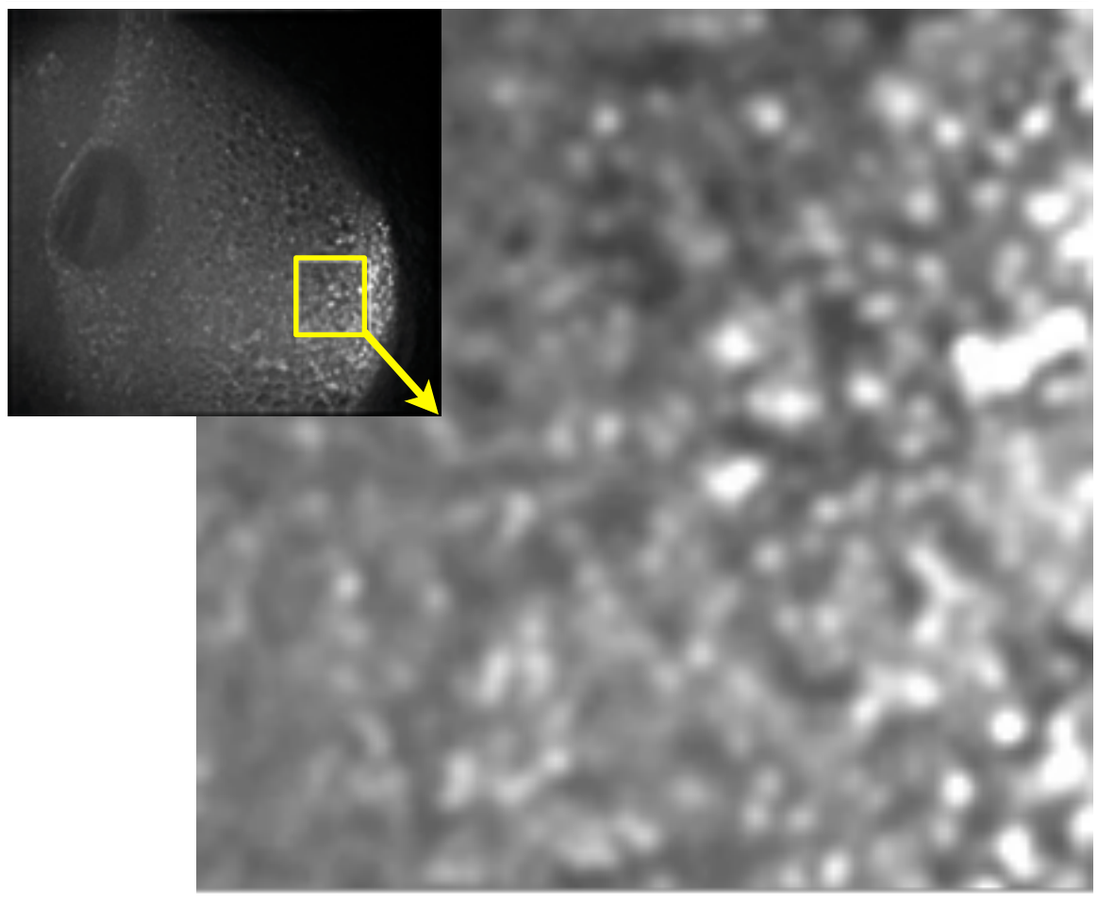

Reasons for live imaging

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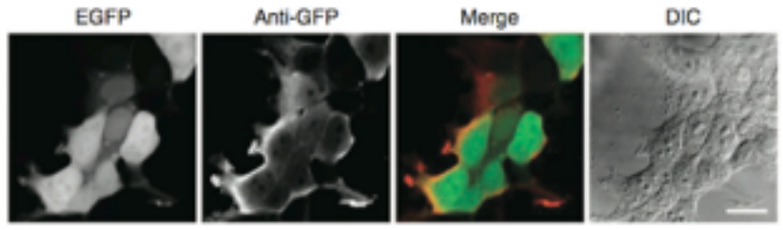
Reasons for live imaging

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



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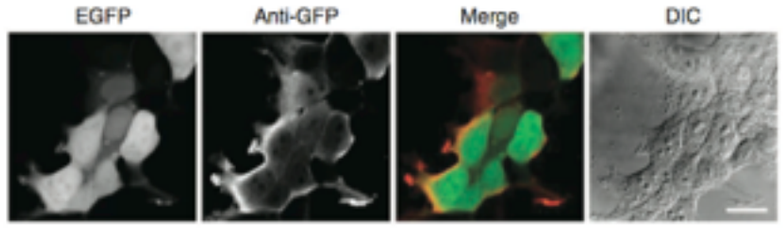
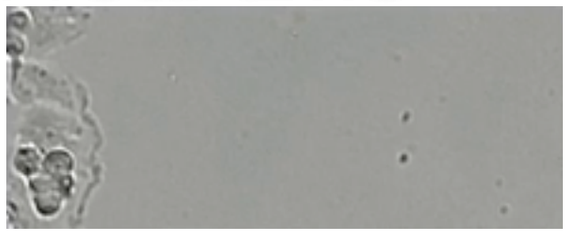


2) Can follow the order of sequential events in real time

time-course of cell migration - Andrea Linford Barr lab



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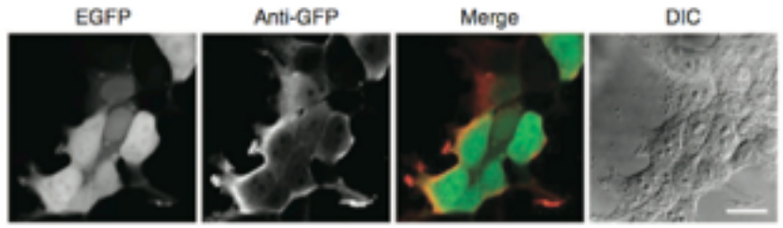

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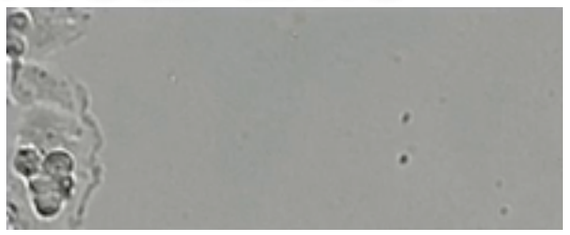
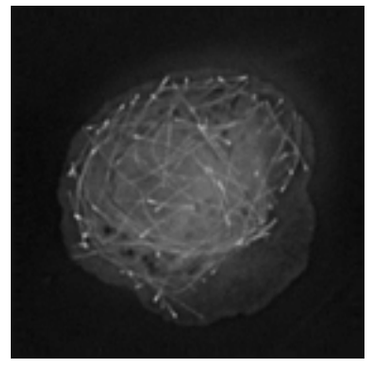


3) Can monitor the kinetics of dynamic processes:
- active transport vs diffusion
- Microtubule turnover

Macrophage: EB1-GFP tagged MT



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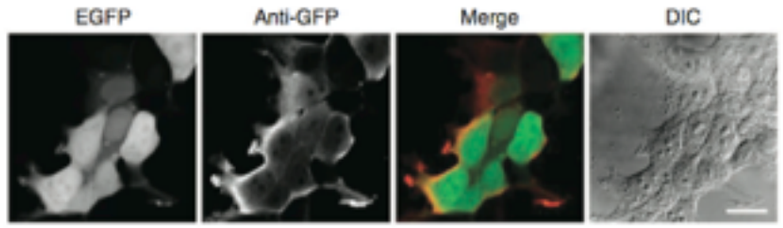

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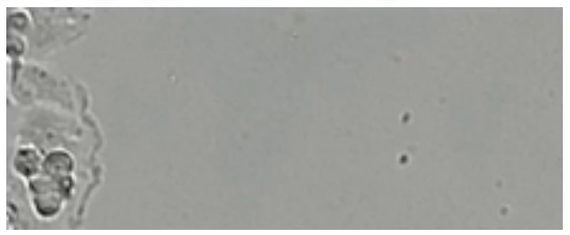

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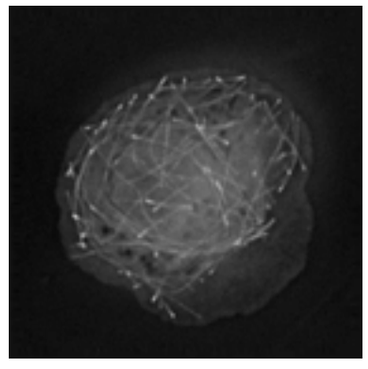
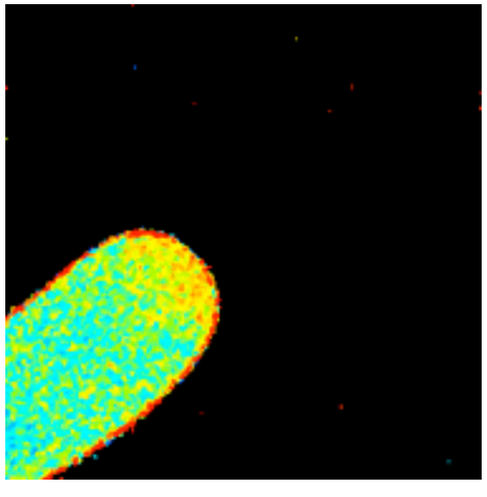


4) Can record sensitive or transient processes:
- Calcium signalling transients
- Ion gradients
- membrane potential

Calcium ratio imaging pollen tube



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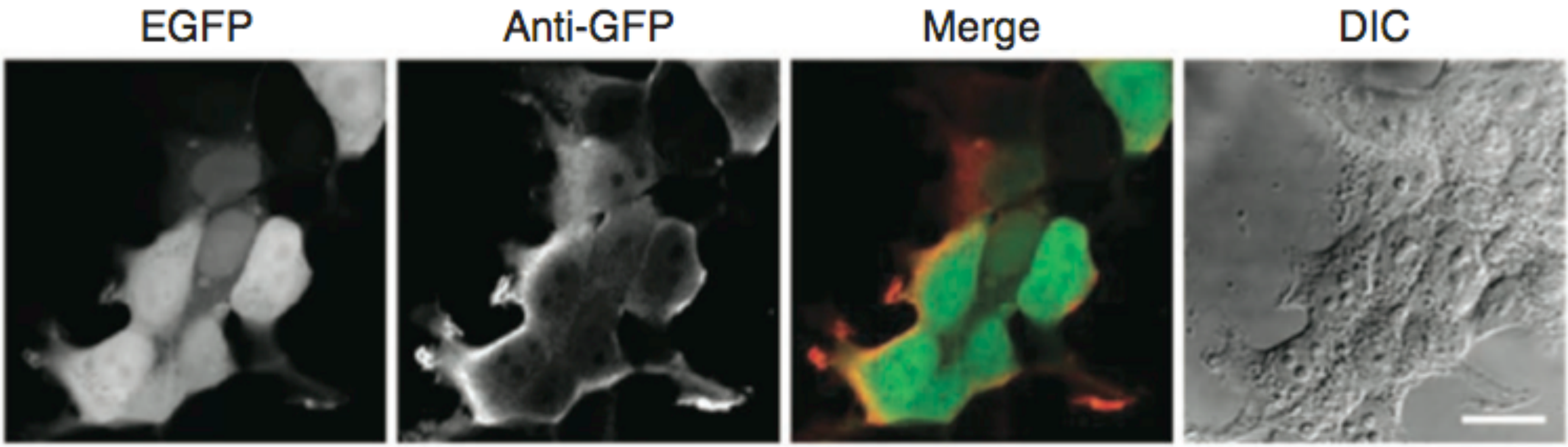
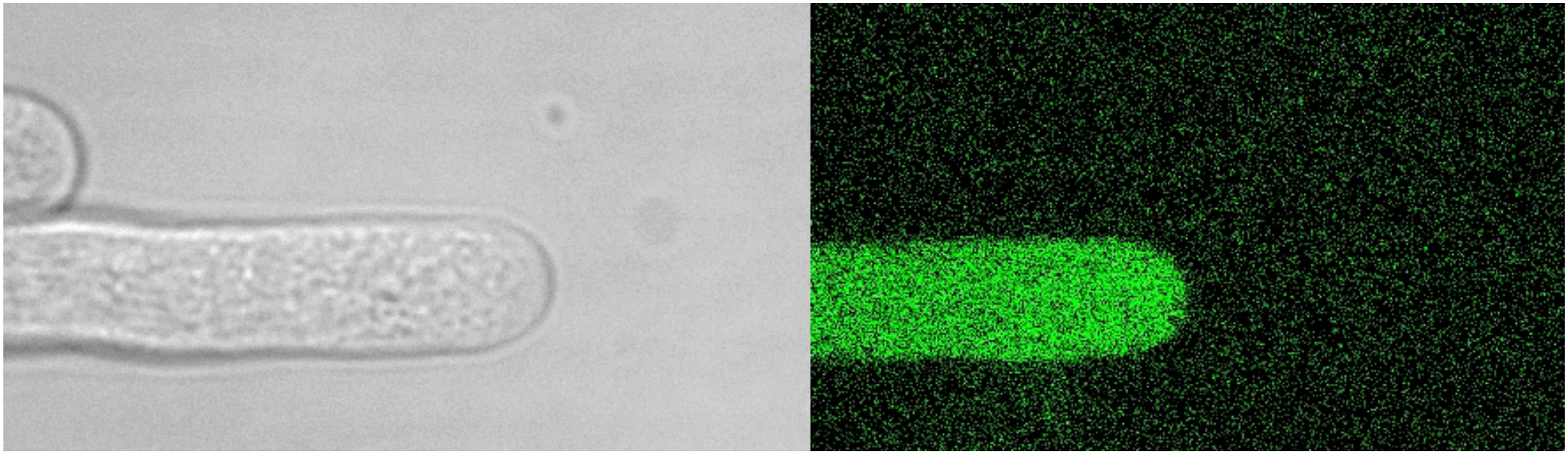
Immunolabeling artifacts and the need for live-cell imaging

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

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



Death by imaging!



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

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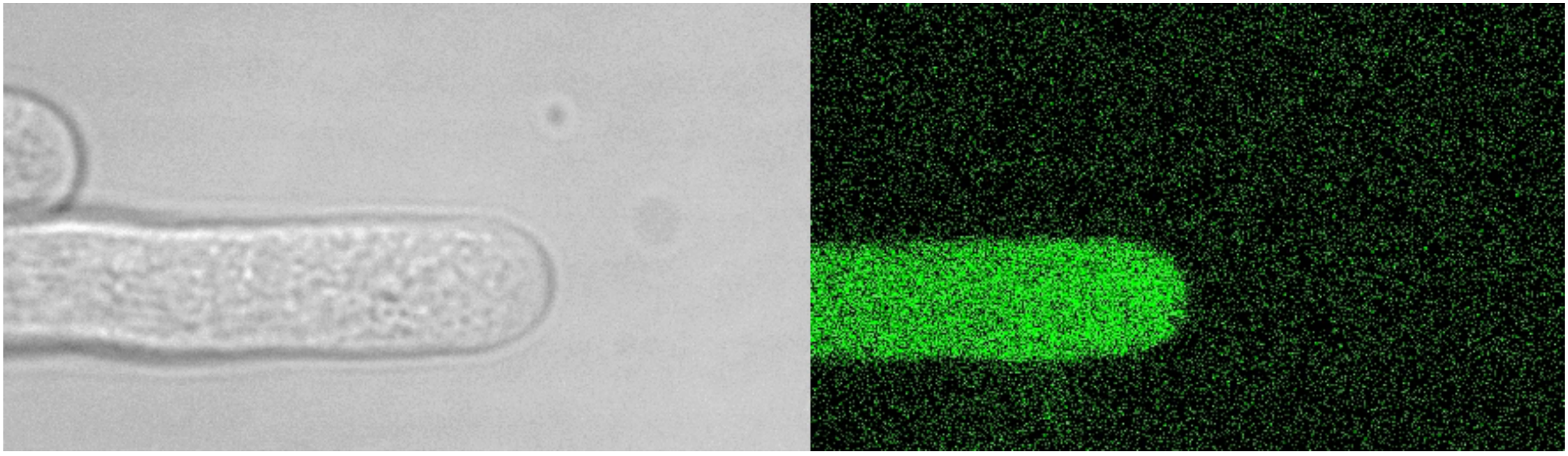

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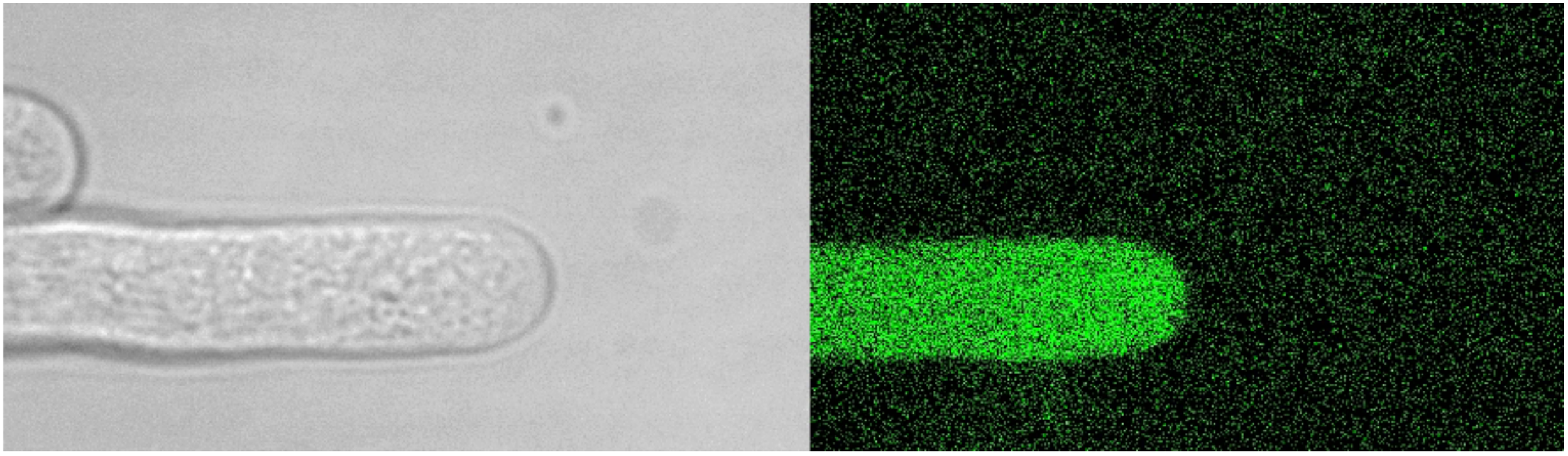

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Also:

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

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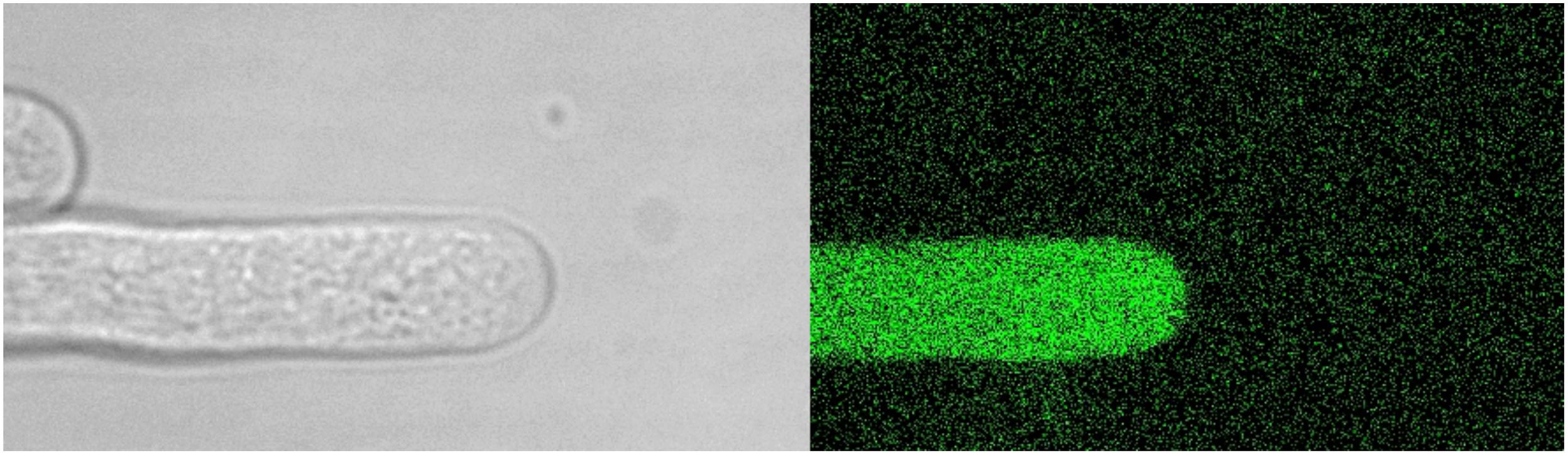

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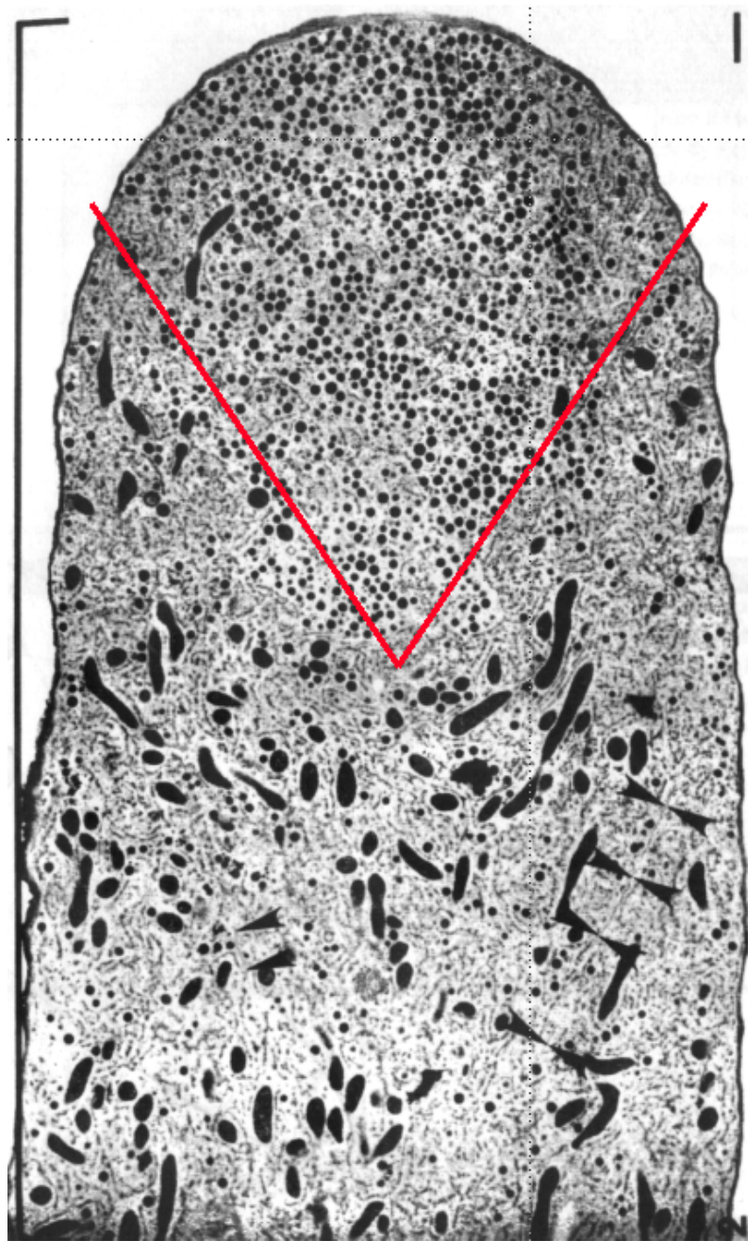
Also:

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

do the appropriate controls

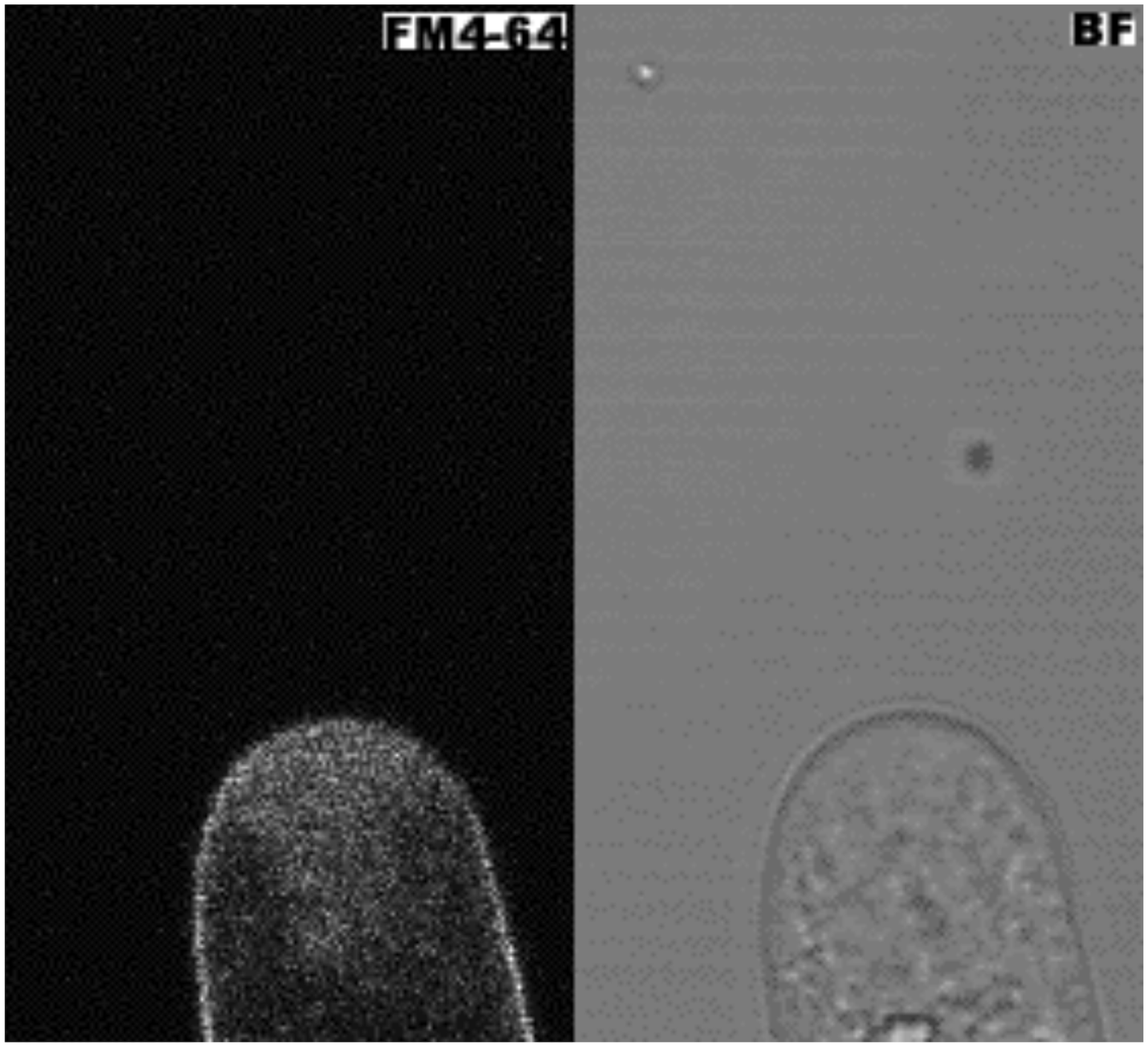
Can monitor the kinetics of dynamic processes

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

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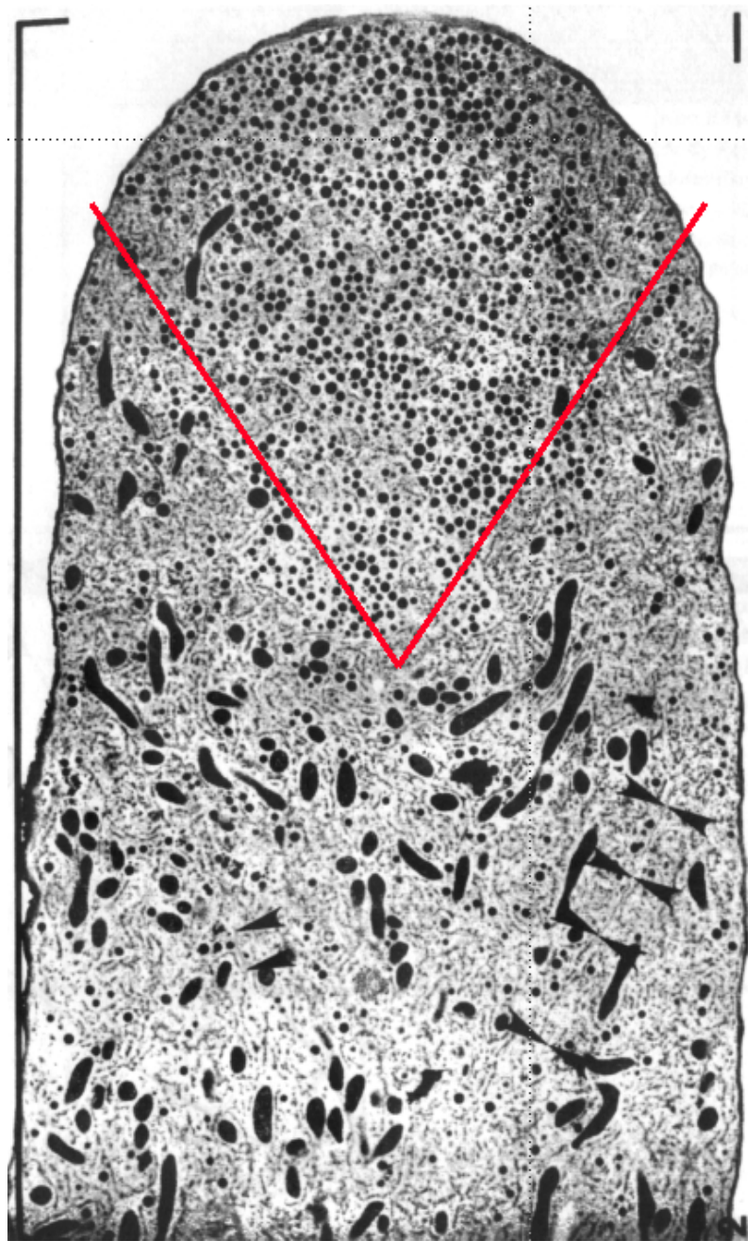

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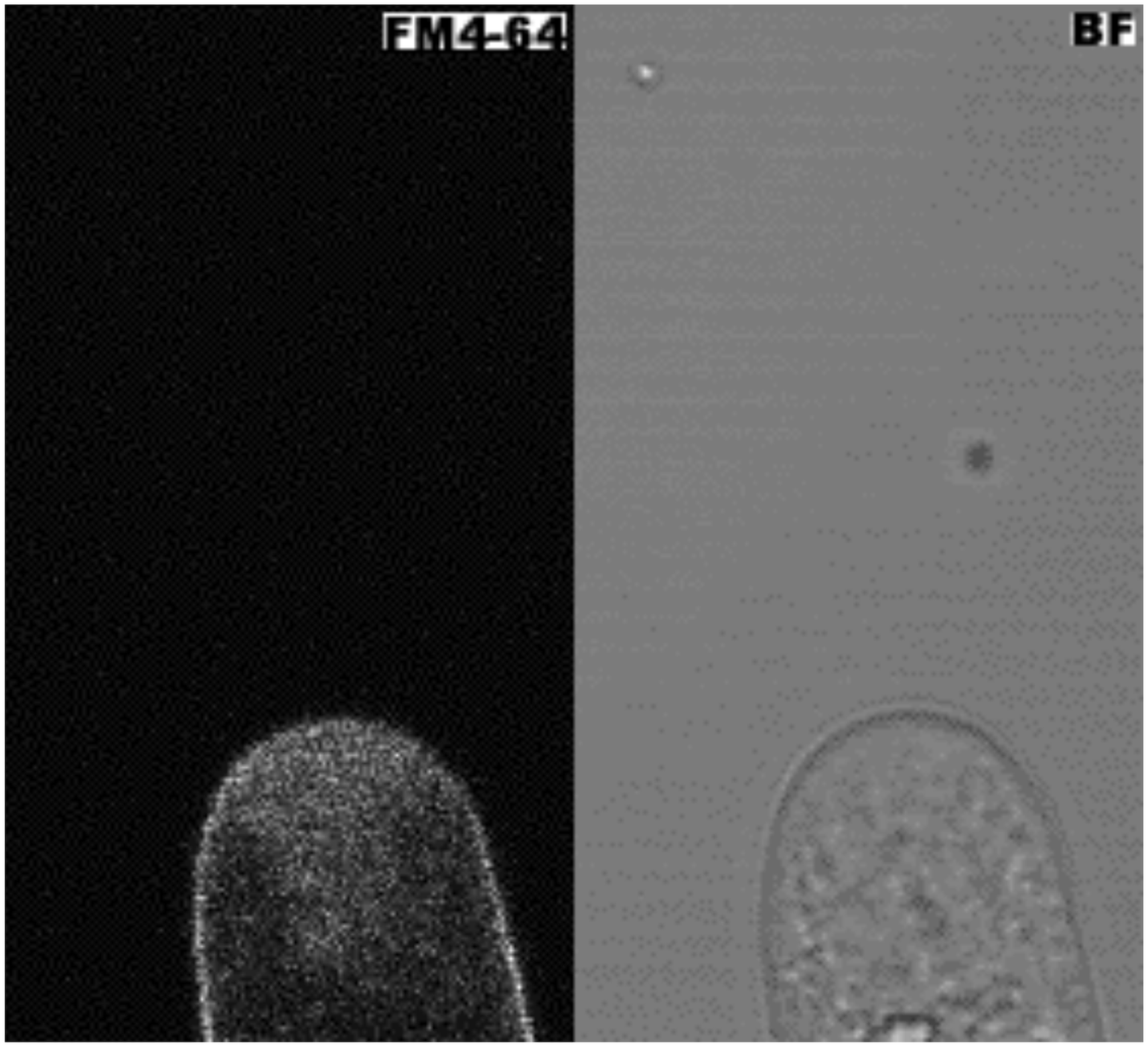

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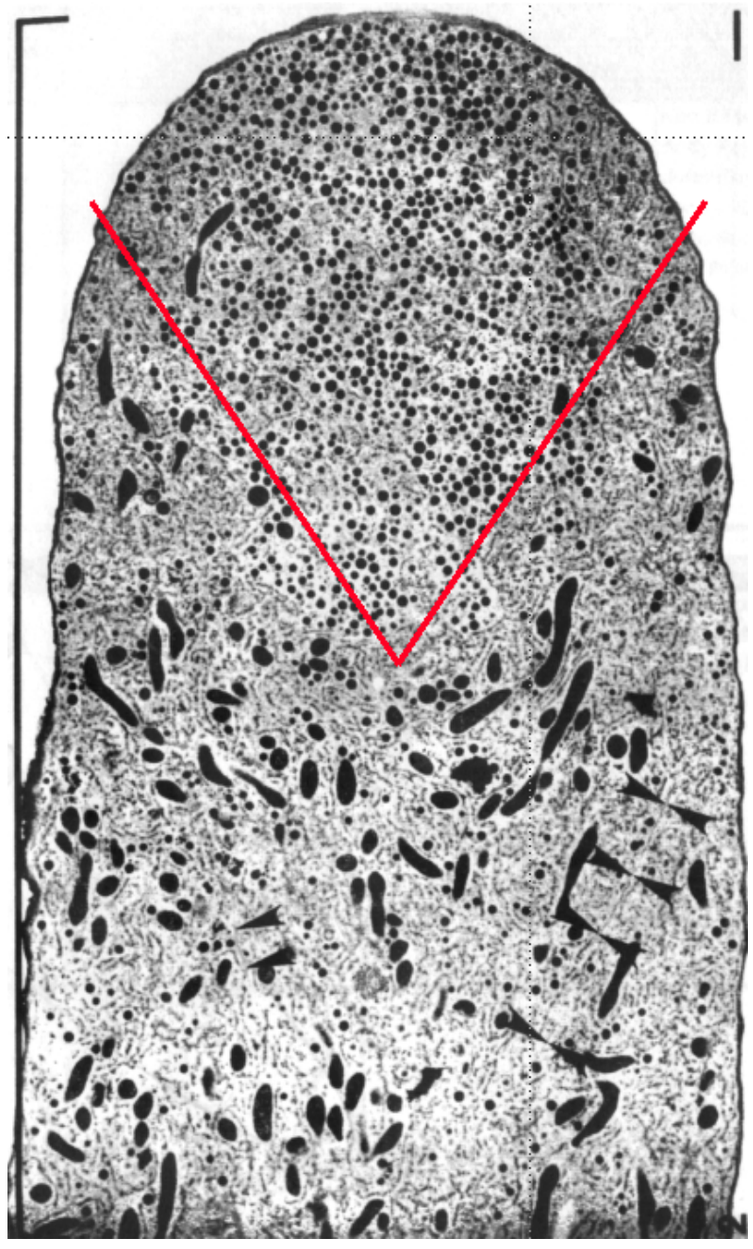

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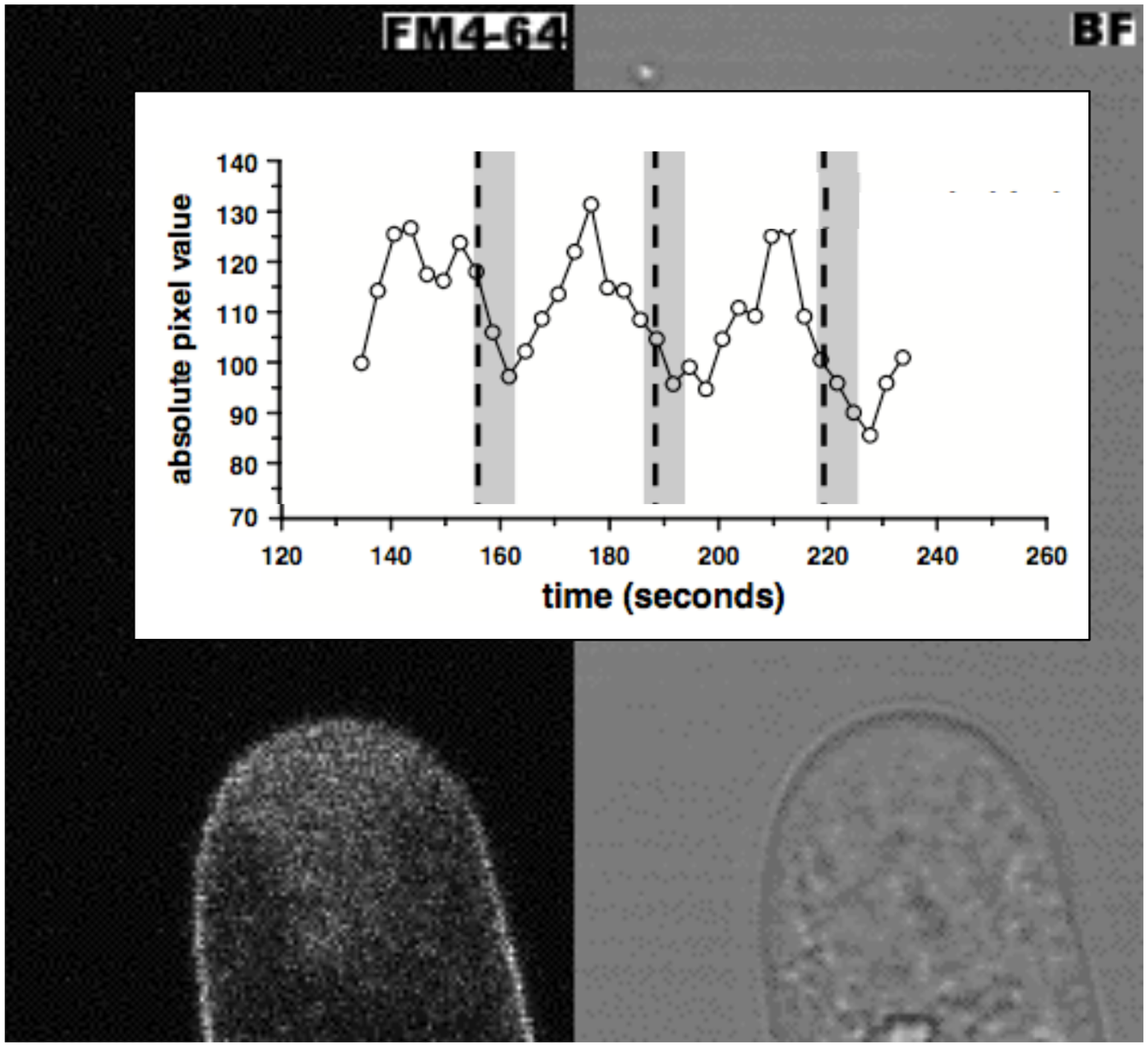

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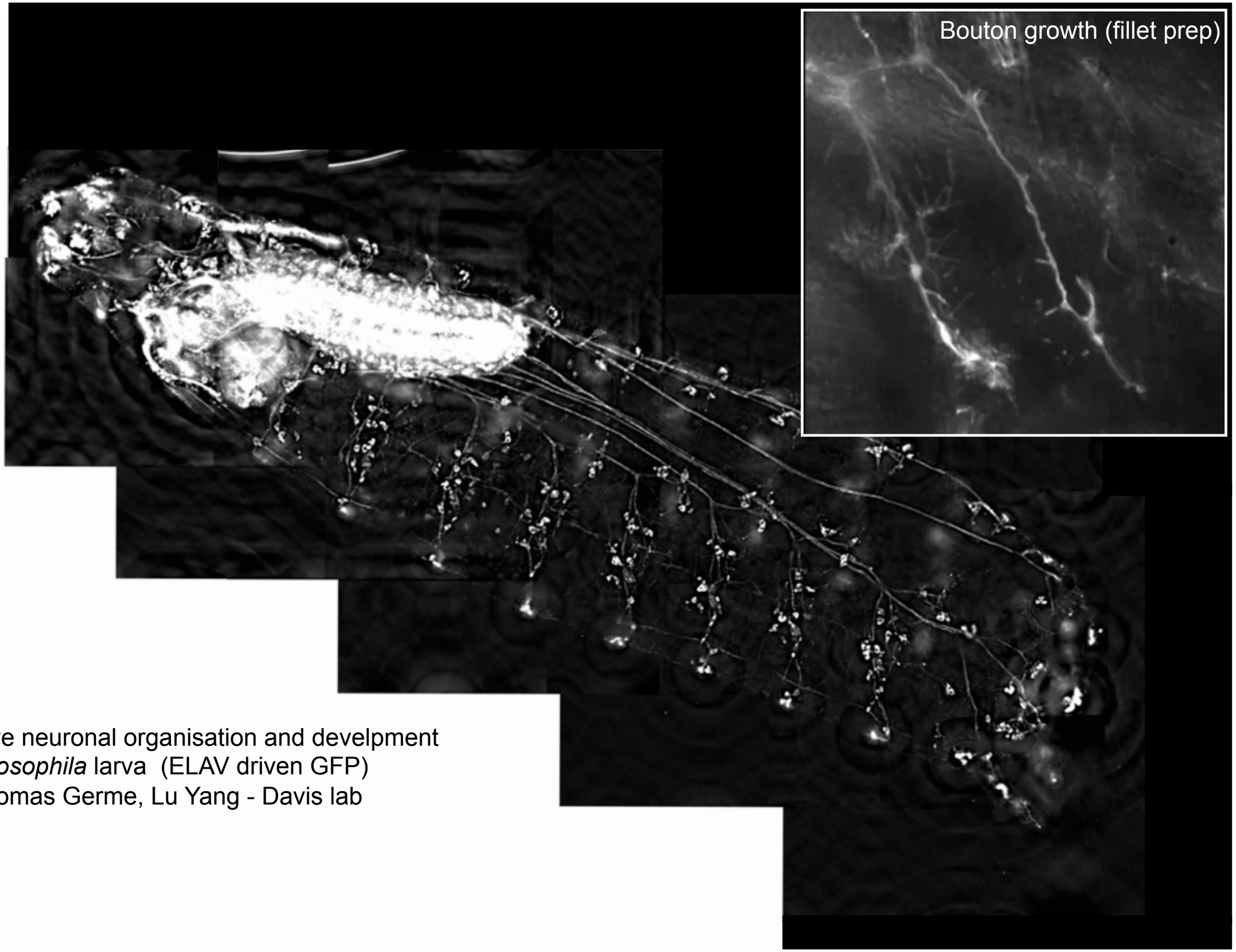

Live neuronal organisation and develpment
Drosophila larva (ELAV driven GFP)
Thomas Germe, Lu Yang - Davis lab

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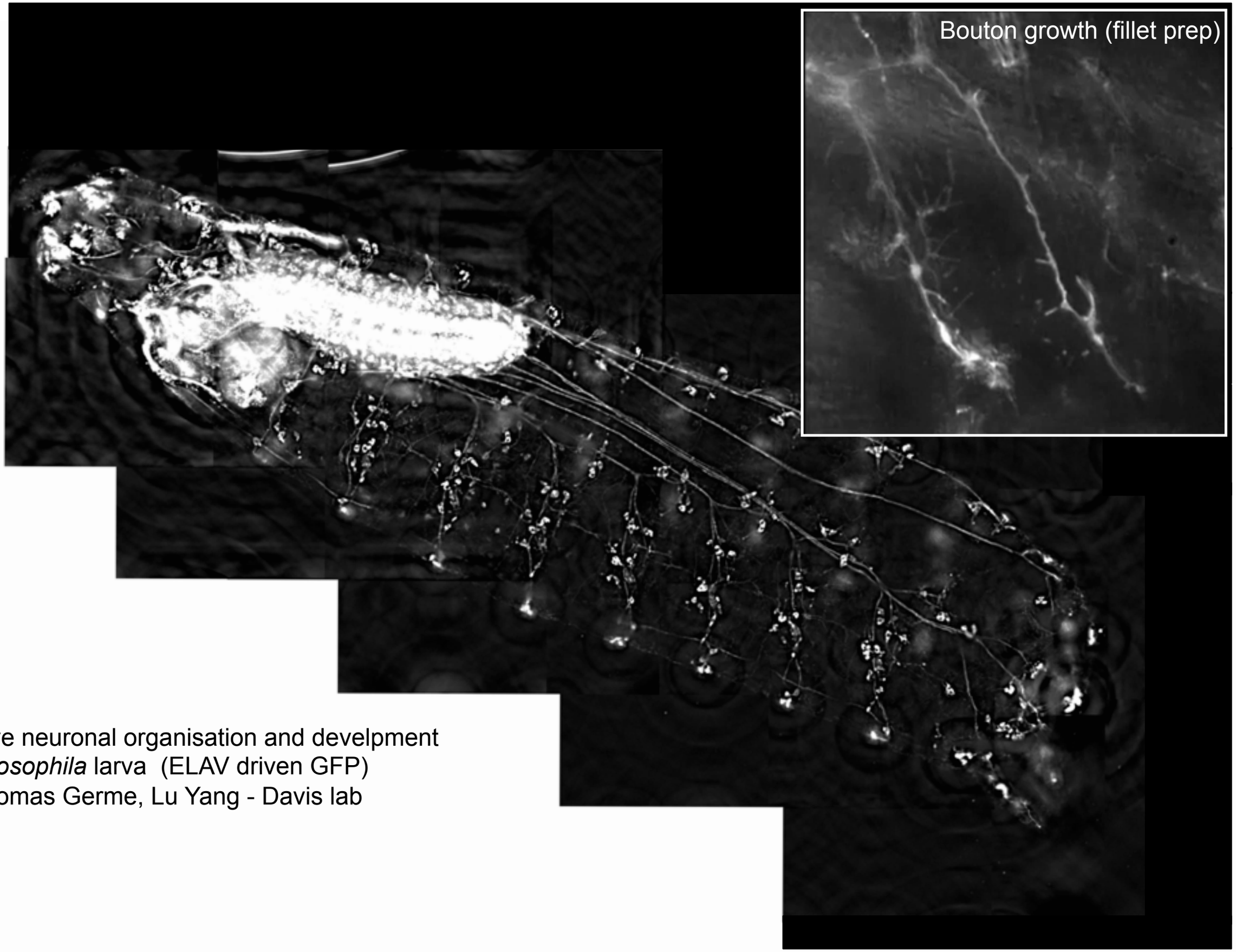

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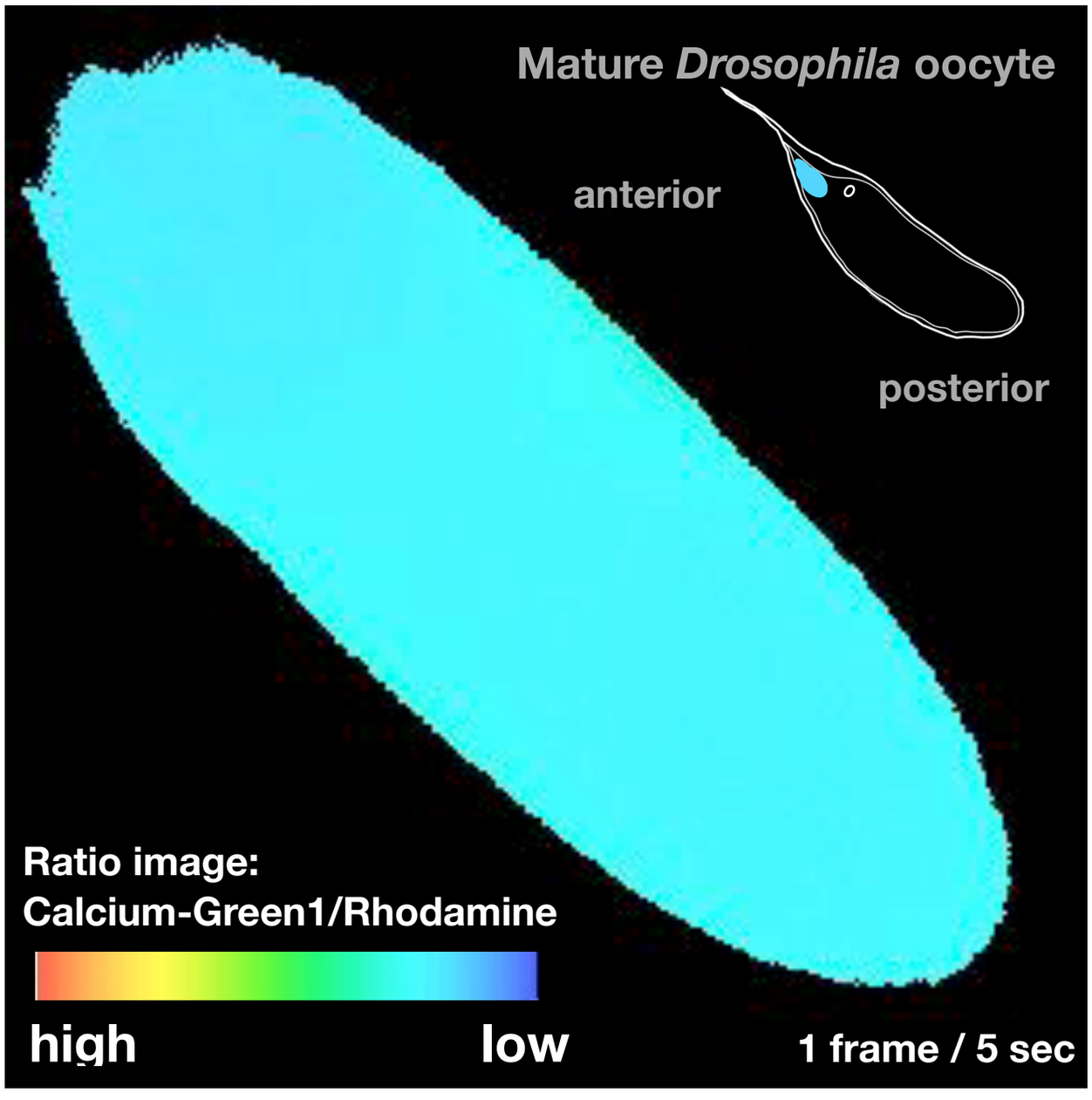
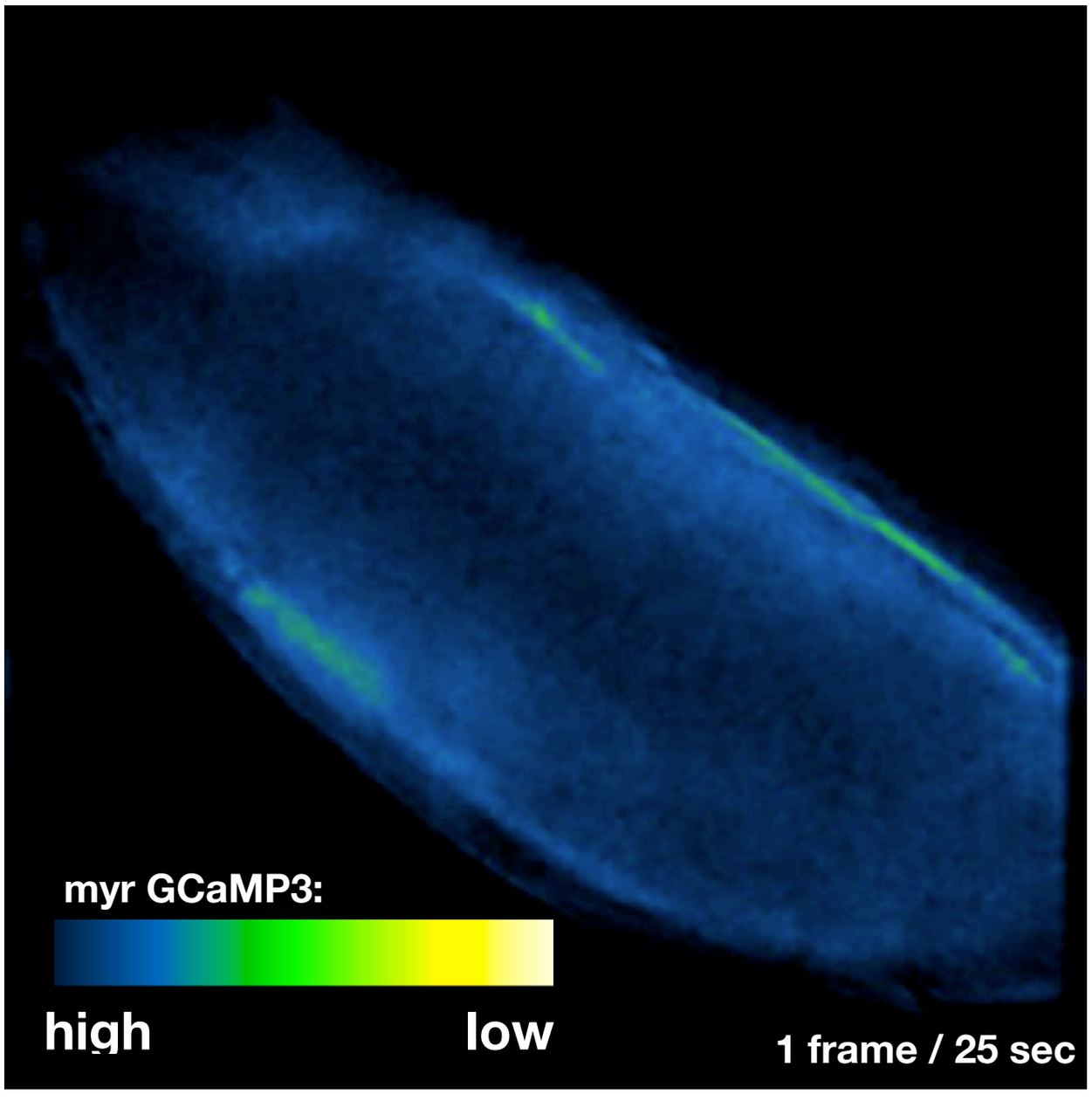
Can record sensitive or transient processes

Calcium transient upon activation





York-Andersen, A. H., Parton, R. M., Bi, C. J., Bromley, C. L., Davis, I., & Weil, T. T. (2015). A single and rapid calcium wave at egg activation in Drosophila. Biology Open, 4(4), 553–560. http://doi.org/10.1242/bio.201411296

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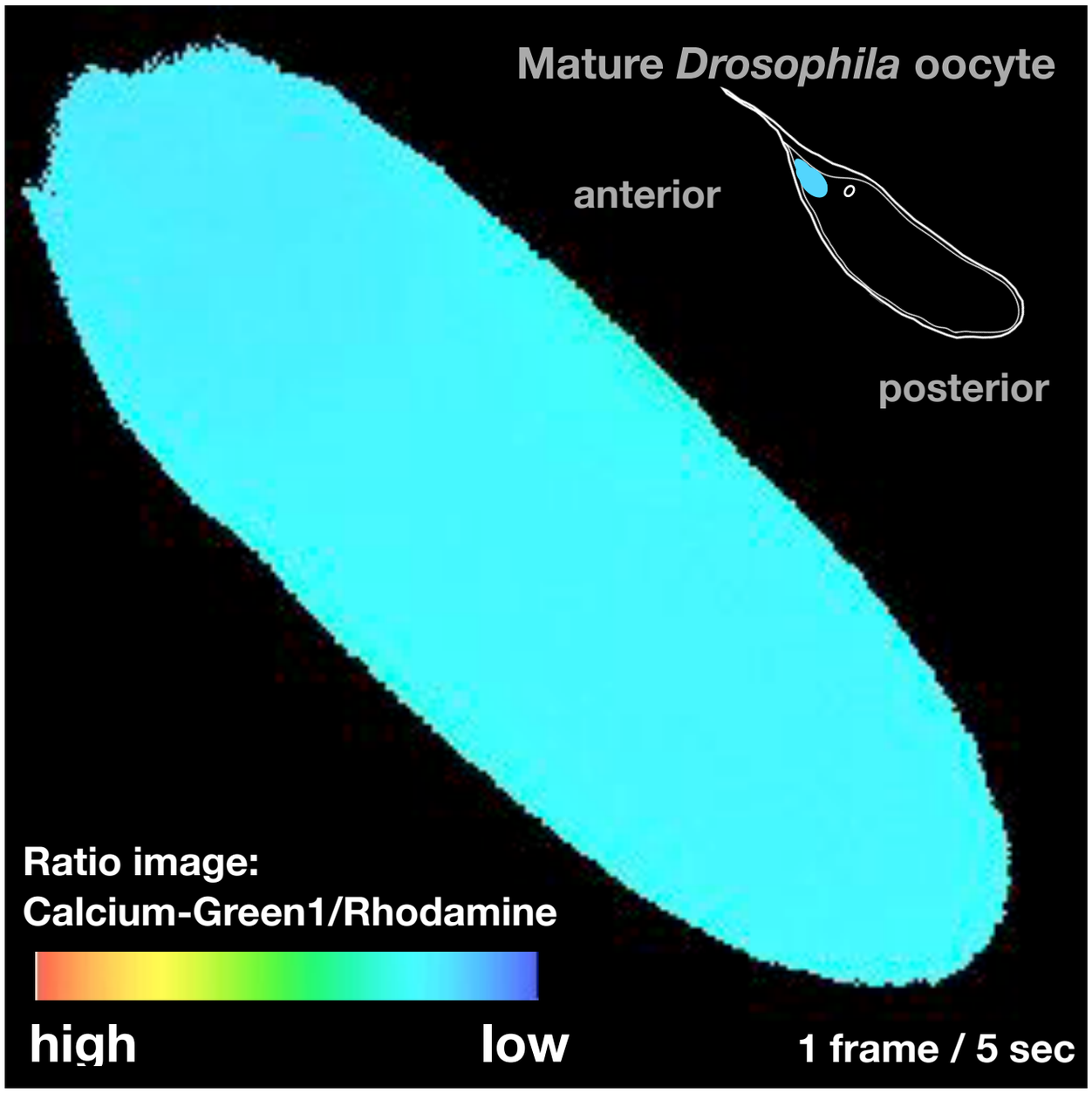

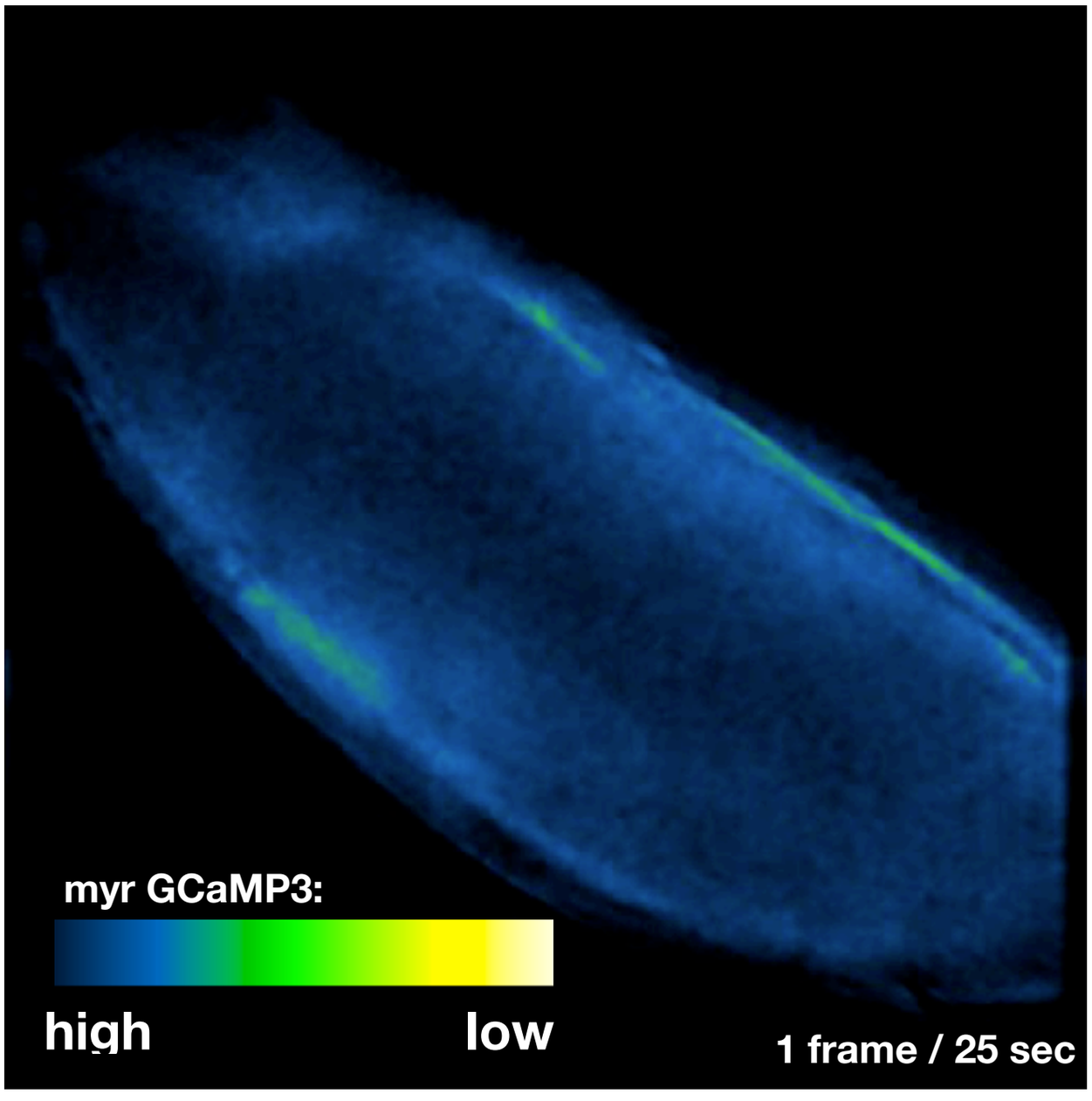

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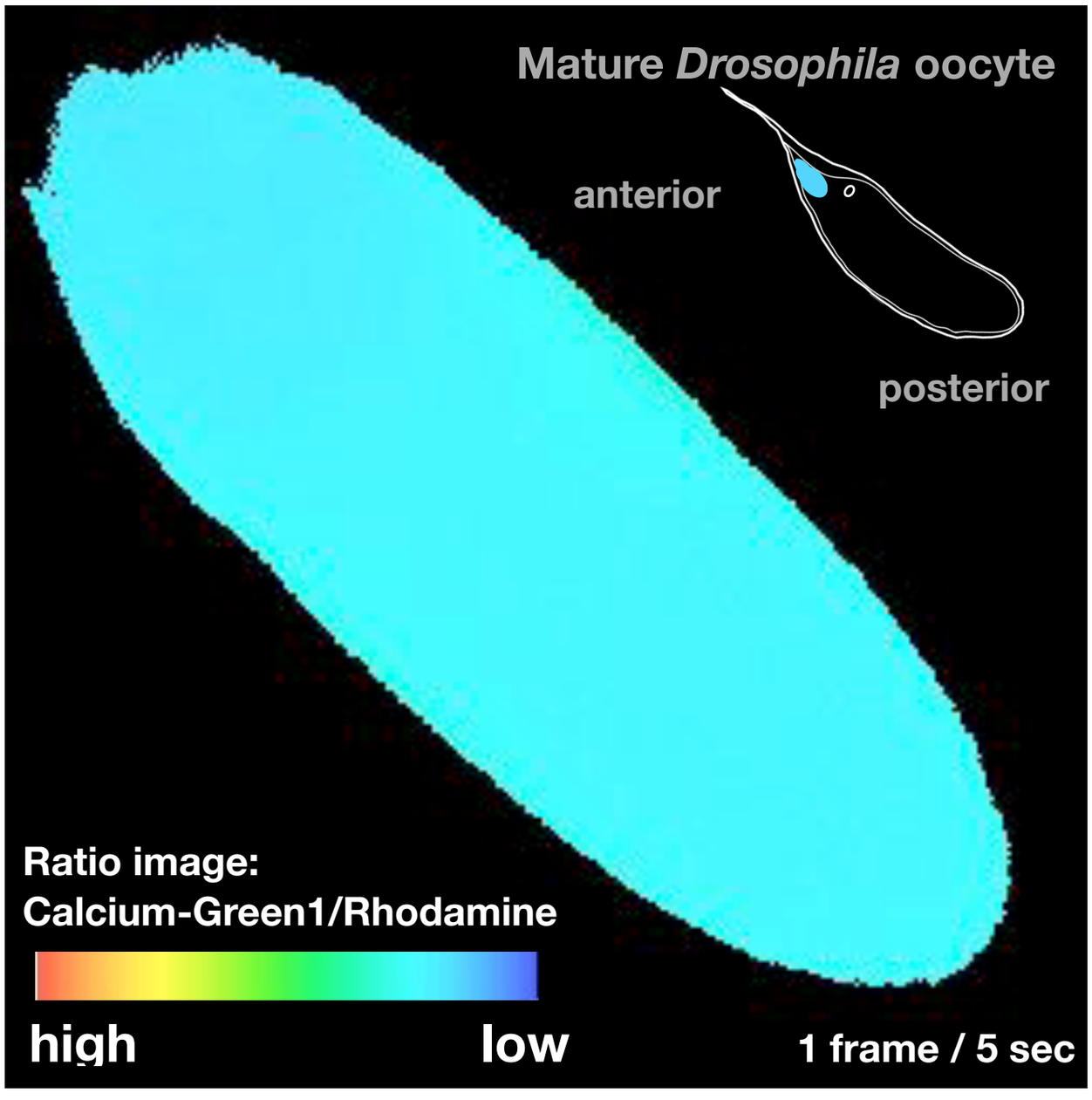

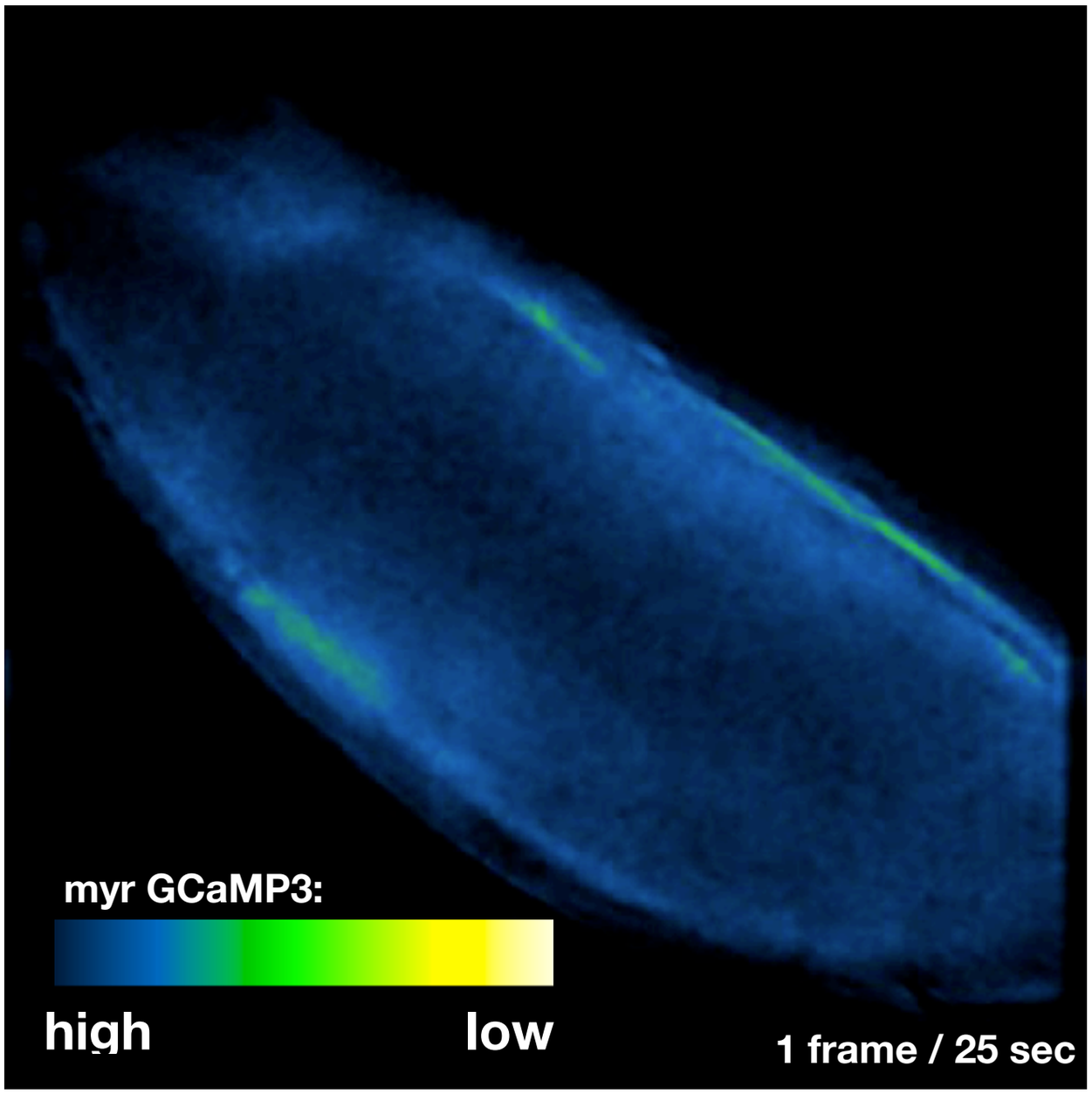

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Live imaging as an experimental tool:

Using light to manipulate cell behaviour:

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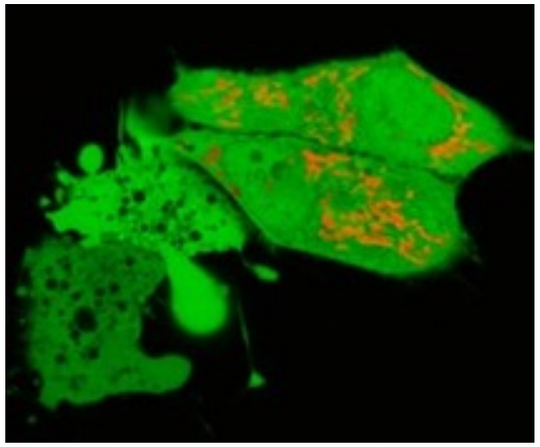
Using light to manipulate cell behaviour:

- "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

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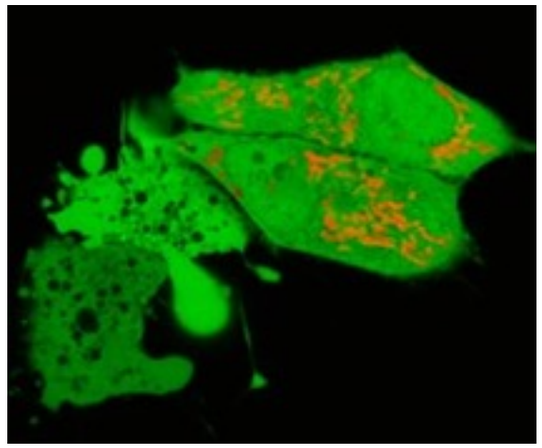
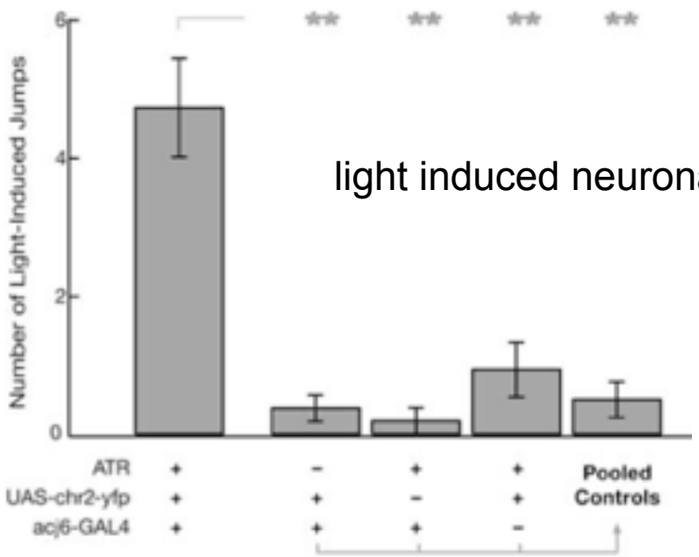


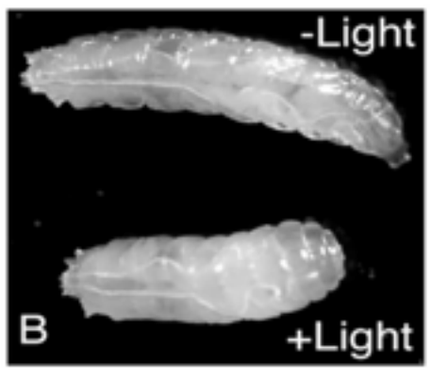
EVROGEN - Killer red expressed in mitochondria

- 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

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

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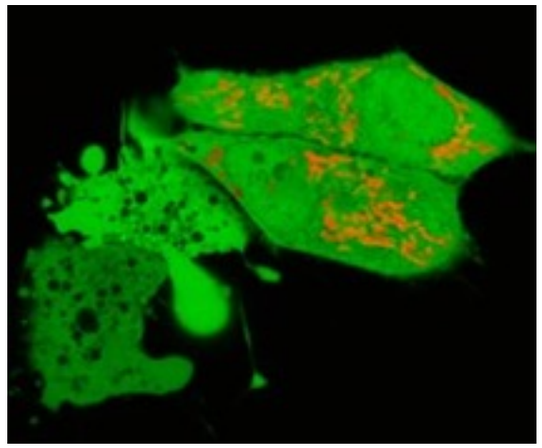

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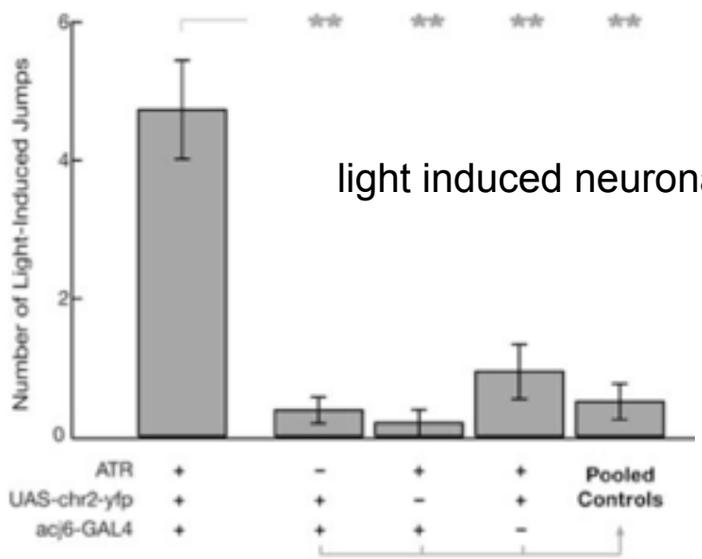
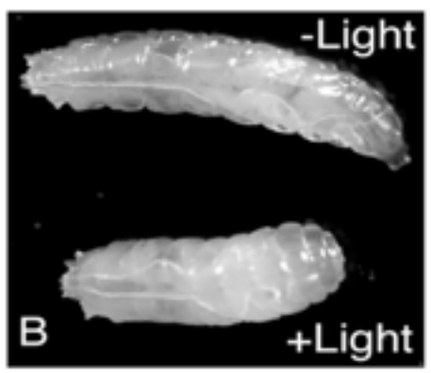
- Channelrhodopsin-2 (ChR2) photo-induced behaviour through light activation of cation-selective ion channels

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

T.Germe

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

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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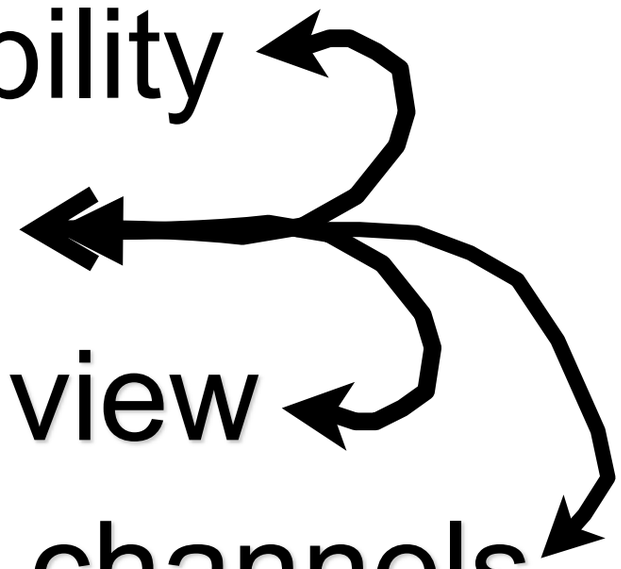

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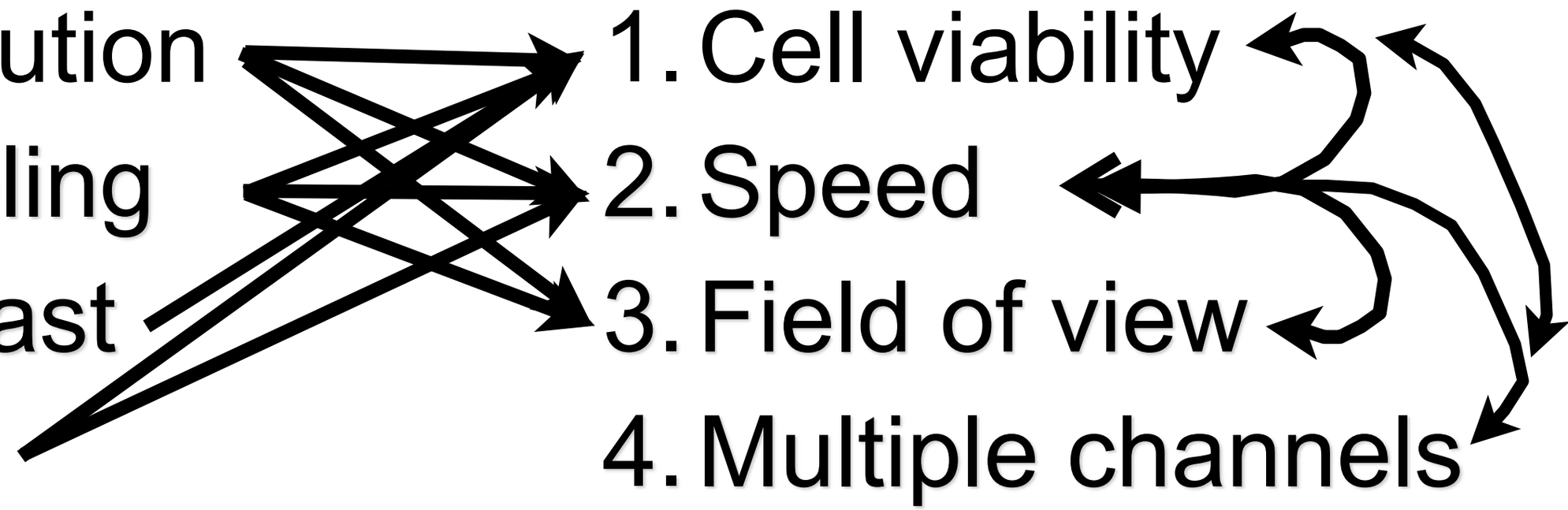

Live-cell imaging is a compromise!

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:

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

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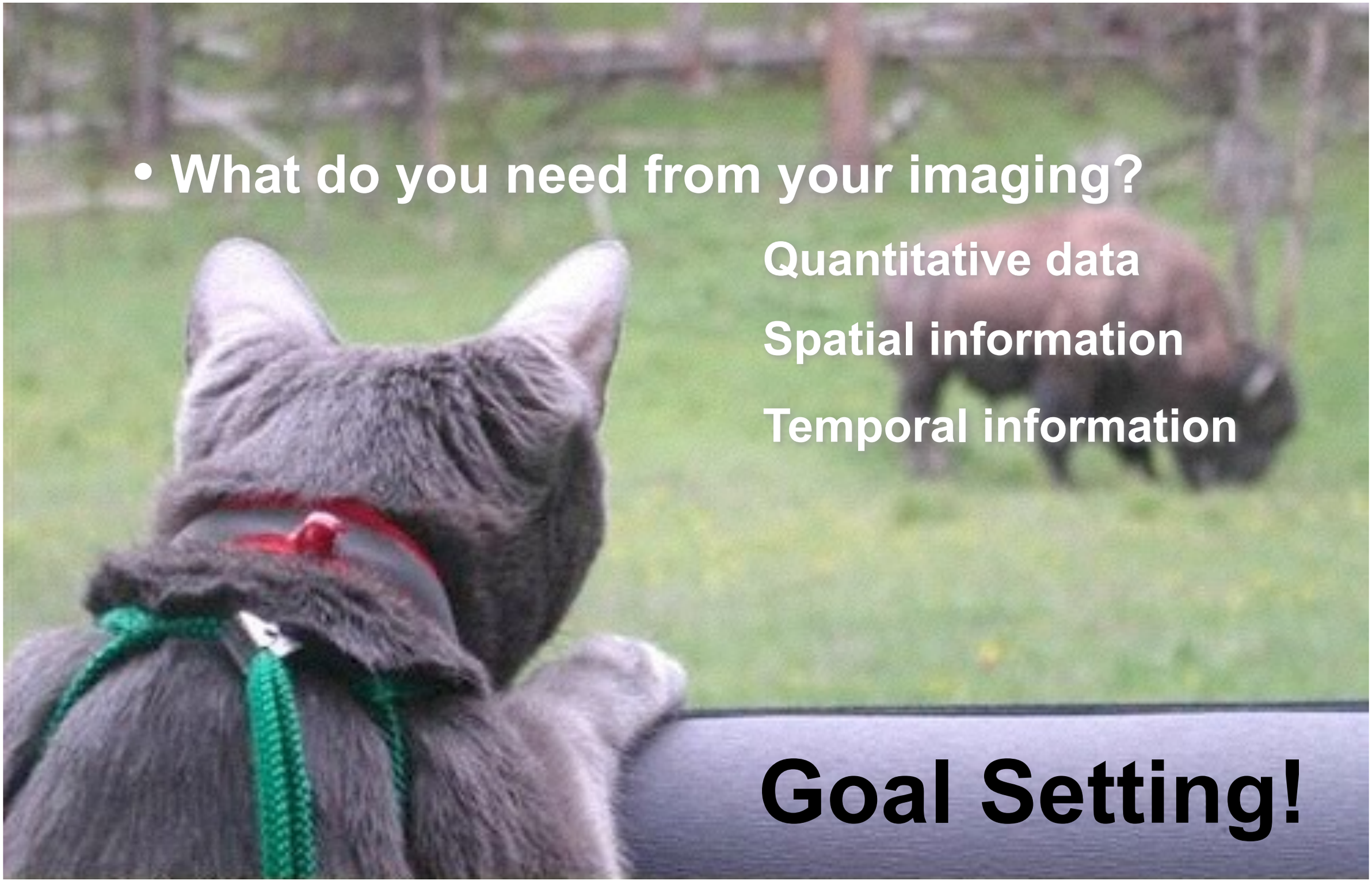

http://thecatsdiary.typepad.com/.a/6a0133f3617f23970b0147e36dbedc970b-pi

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

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There is no, one, perfect technique!

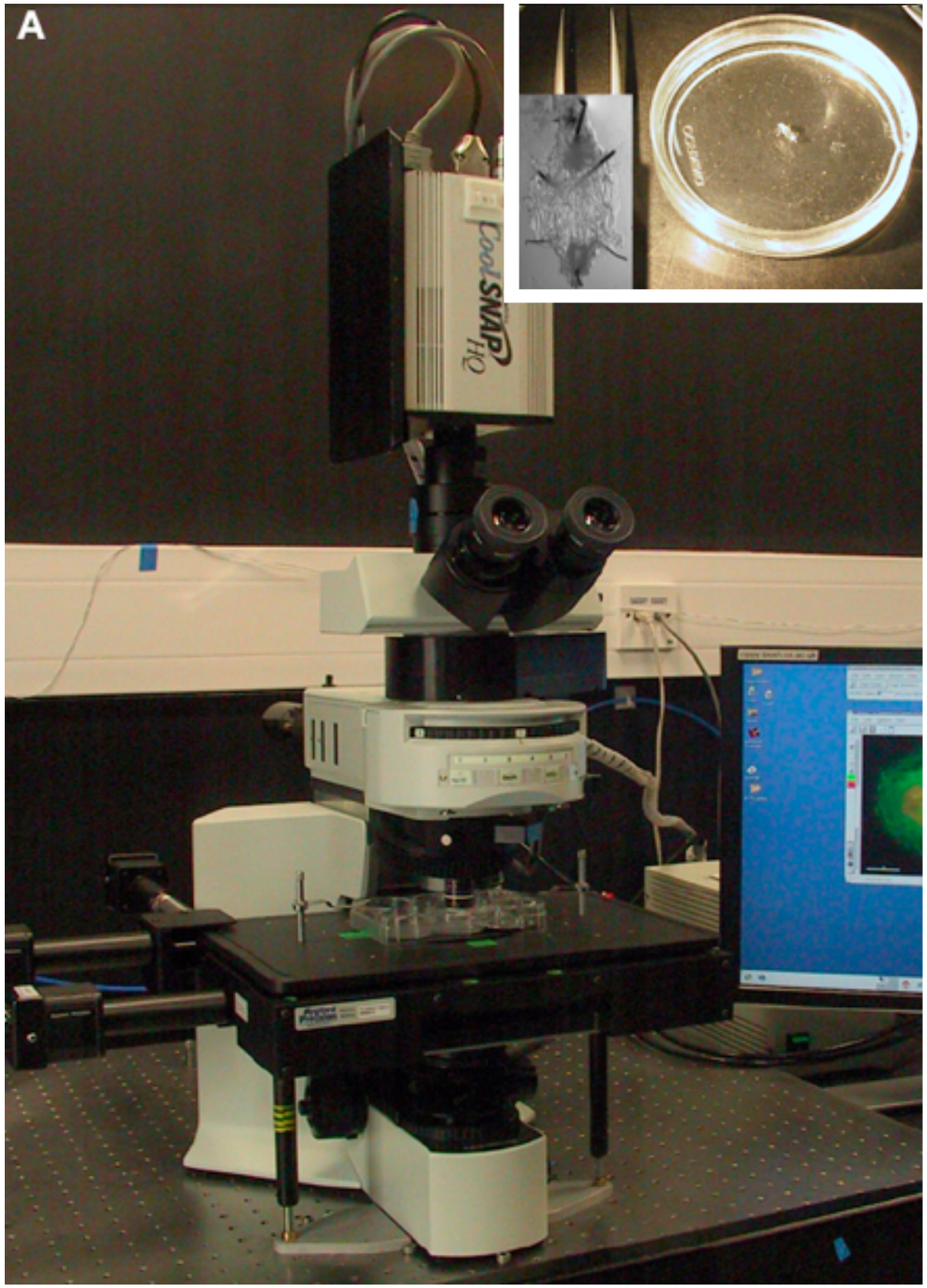
Choice of microscope stand

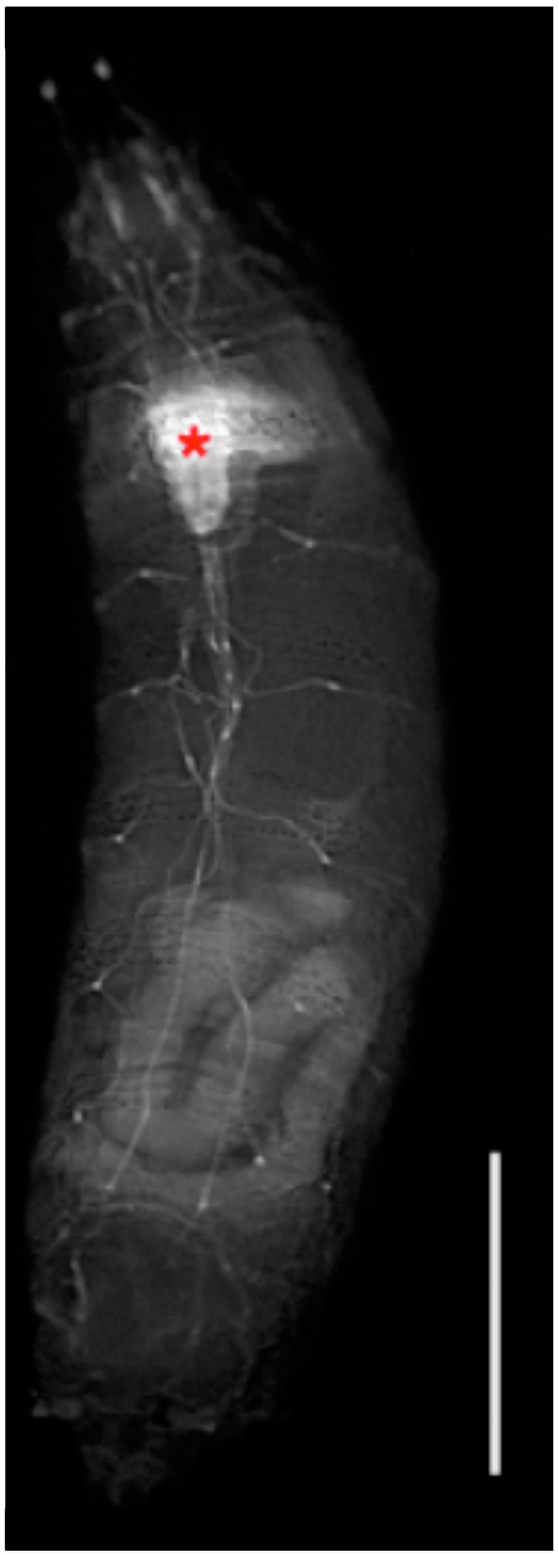
- The modern epifluorescence microscope

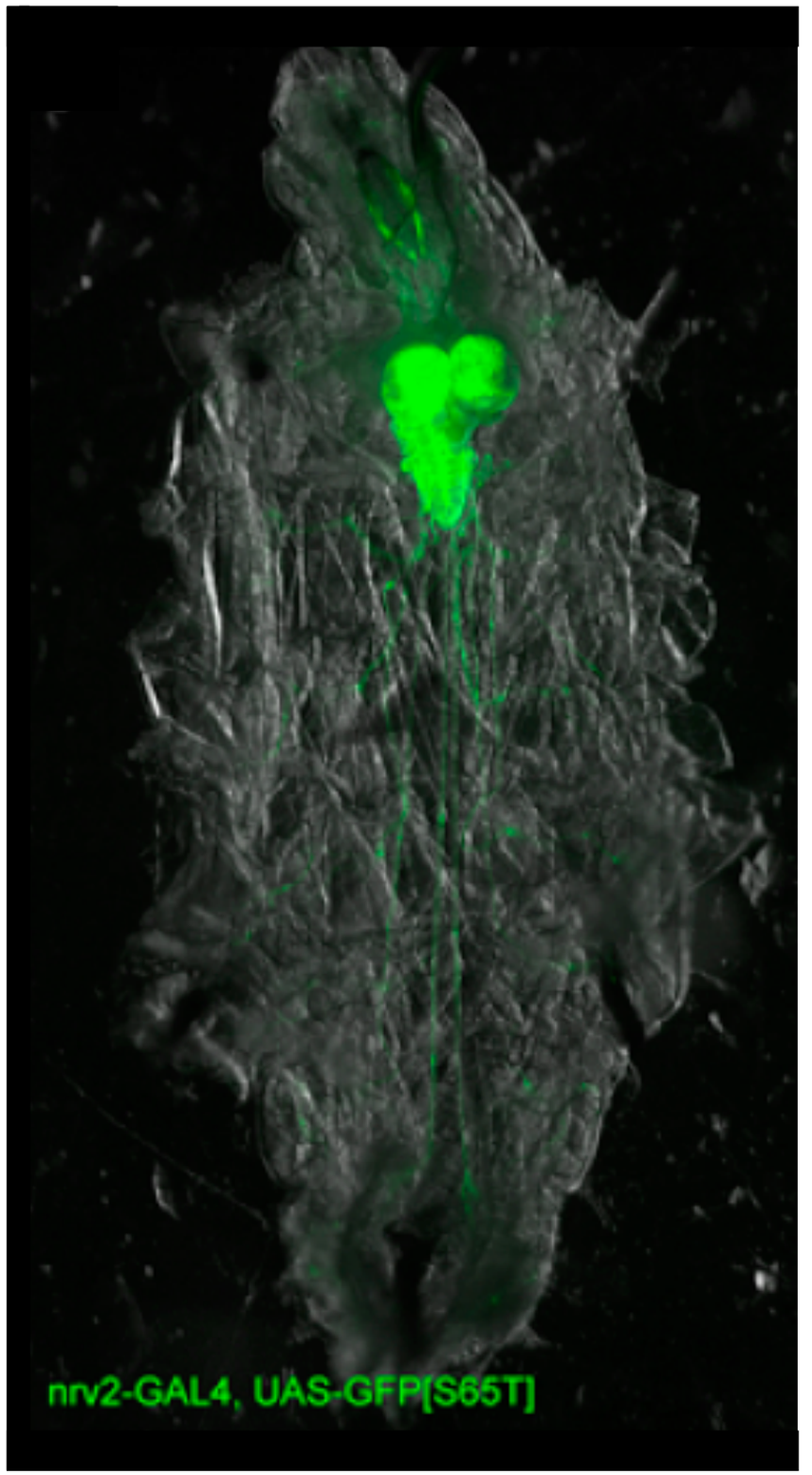
  Upright microscope (lens above specimen)

  Inverted microscope (lens below specimen)

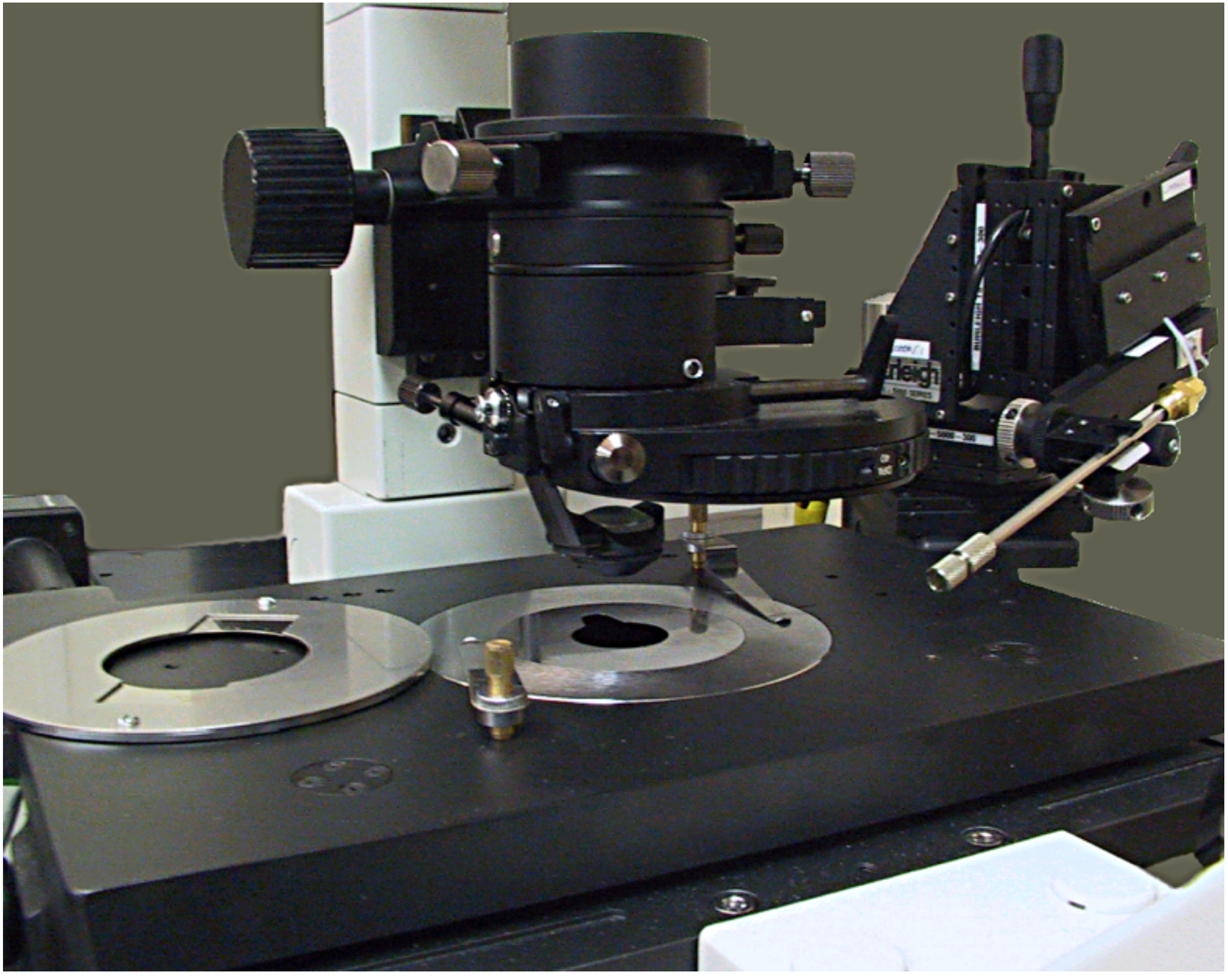
Upright microscope - larval fillet prep







Inverted microscope - injection



Inverted microscope - injection

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

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.

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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 / Spinning Disc confocal for:

Weakly fluorescent, sensitive specimens

Following fast dynamic events

Use TIRF for:

Imaging with high contrast within 100 nm of the coverslip

http://www.micron.ox.ac.uk/microngroup/facilities.php

point scanning confocal

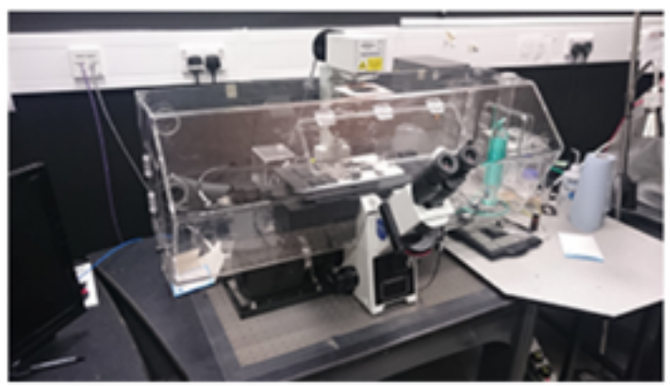
Micron OXFORD

System operational

Live Cell / DNA damage Olympus

Wide Field Decon

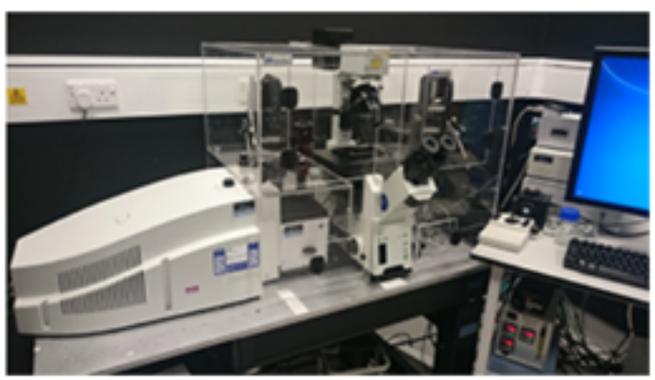
System operational



The DV core microscopes are wide-field deconvolution systems.

Spinning Disc

System operational



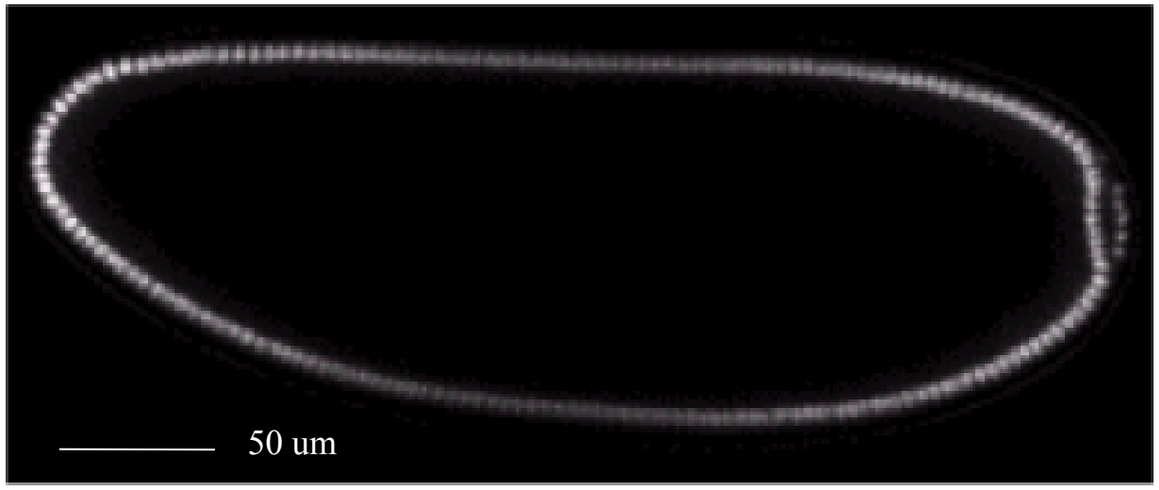
Nasmyth Perkin-Elmer spinning disk confocal.

PALM/TIRF

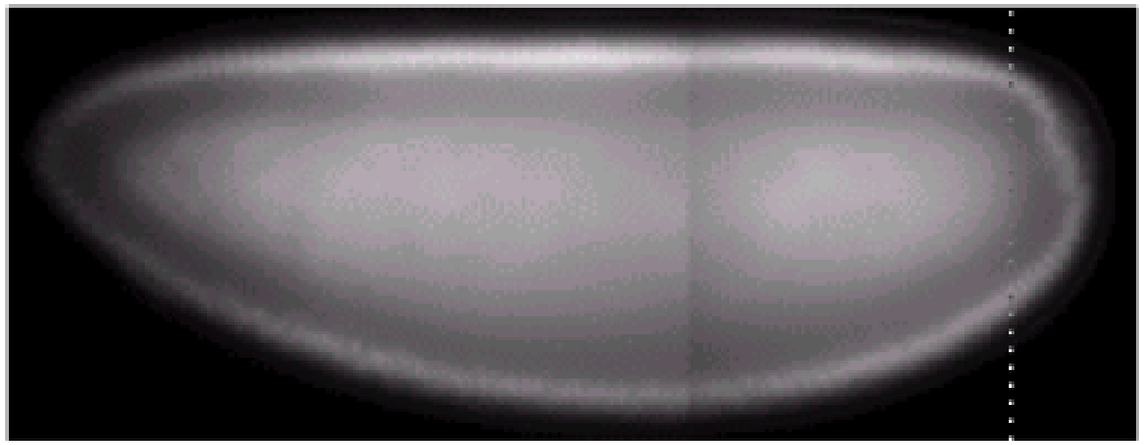
System operational

Choice of imaging technique:
Example of a thick specimen

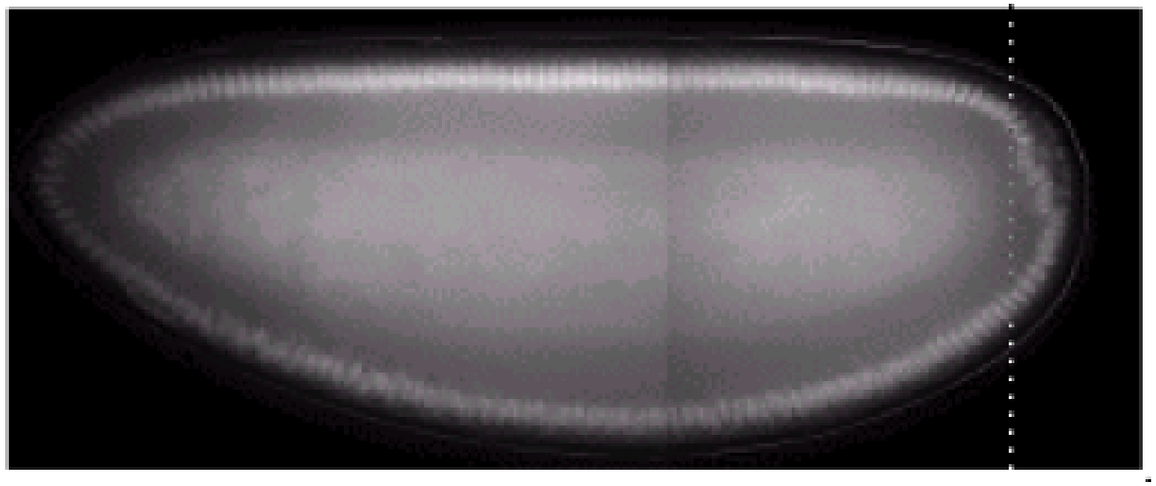
Confocal



Wide-field



WF- deconvolved



Drosophila embryo, nls GFP

Thick, bright specimen

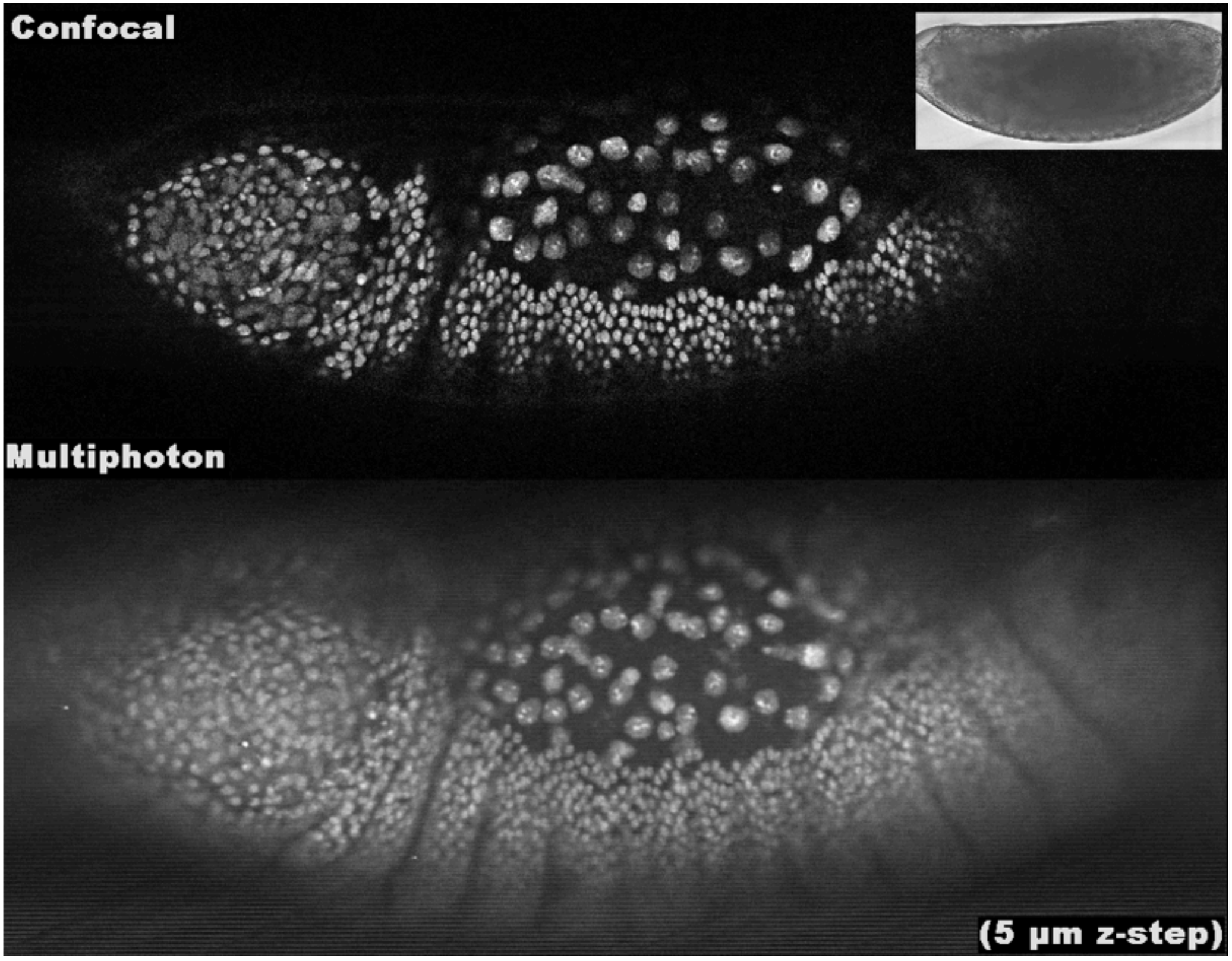
For really thick specimens

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

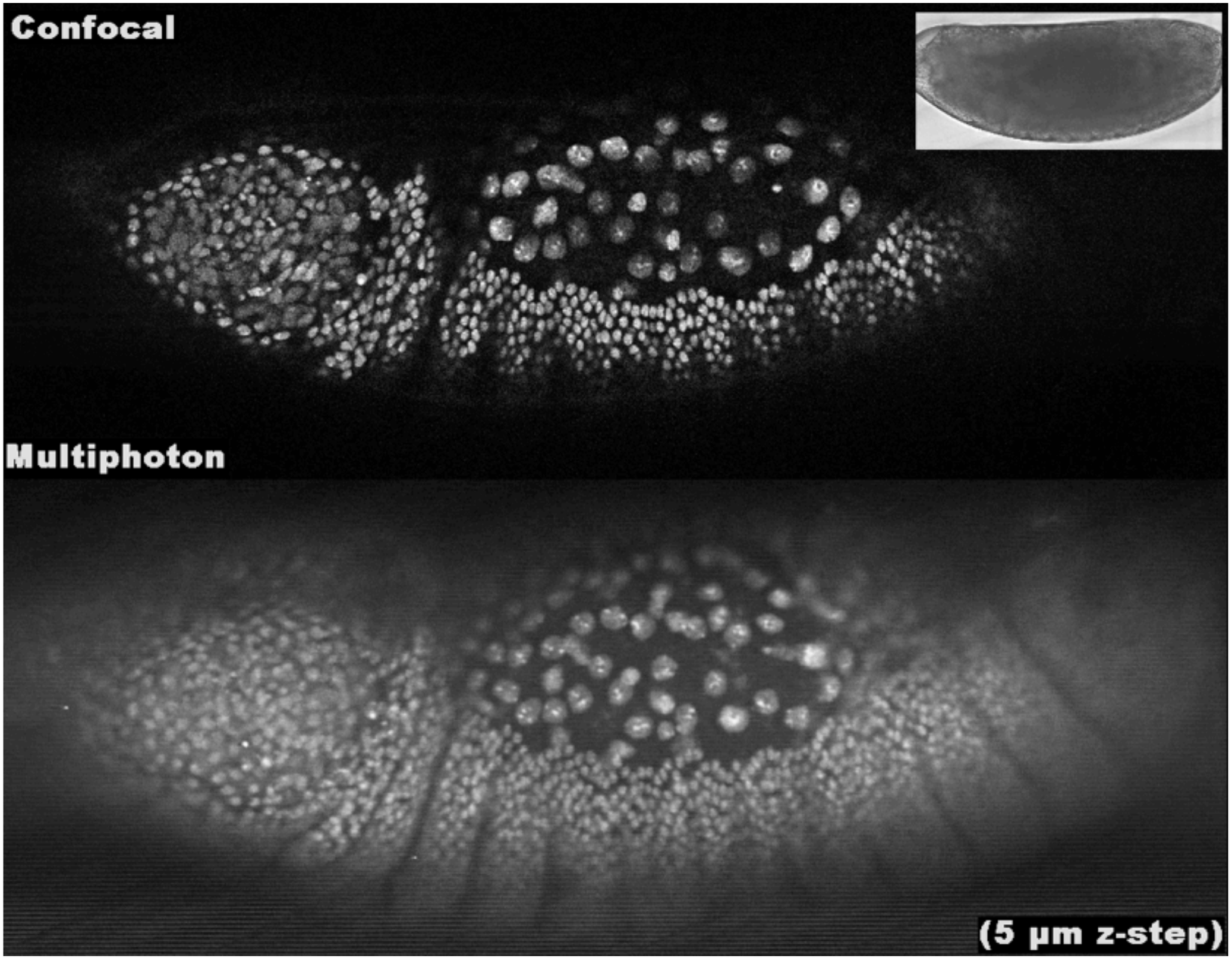
consider….

point scanning confocal
multiphoton
DLSM/SPIM
Adaptive-optics

Multiphoton

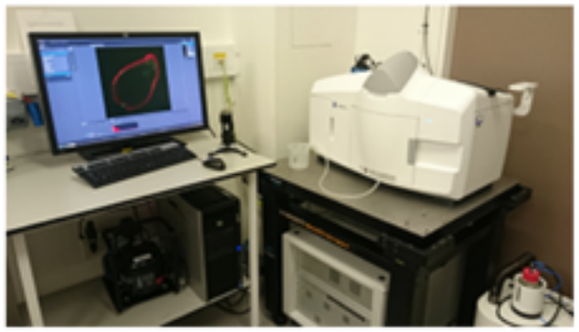


Multiphoton

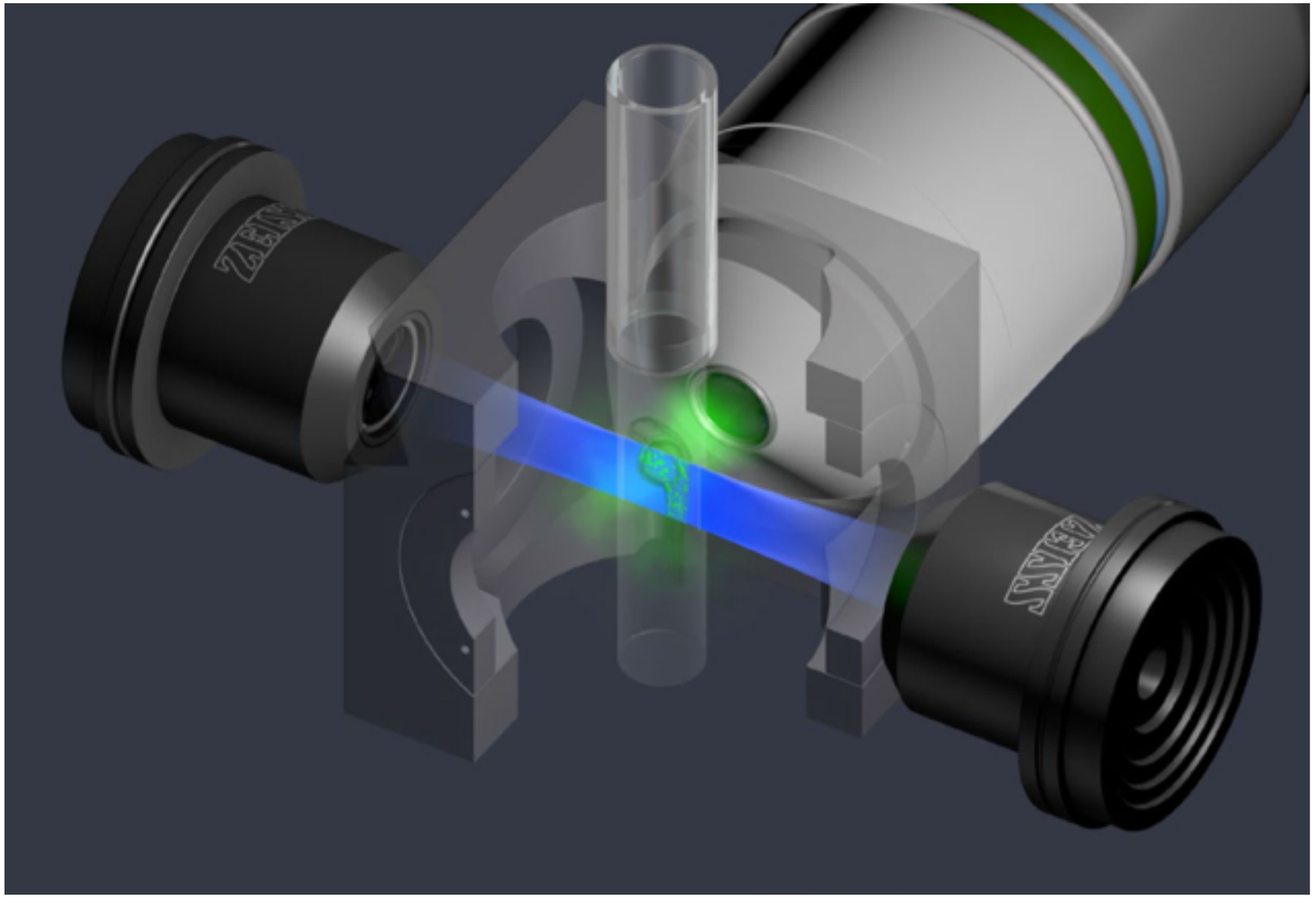


System operational

SPIM - Zeiss Z1



Zeiss Z1 light-sheet microscope



System operational

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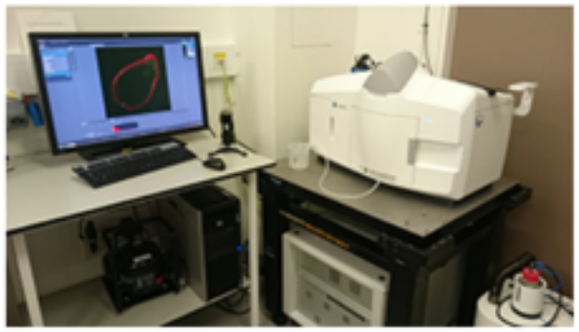

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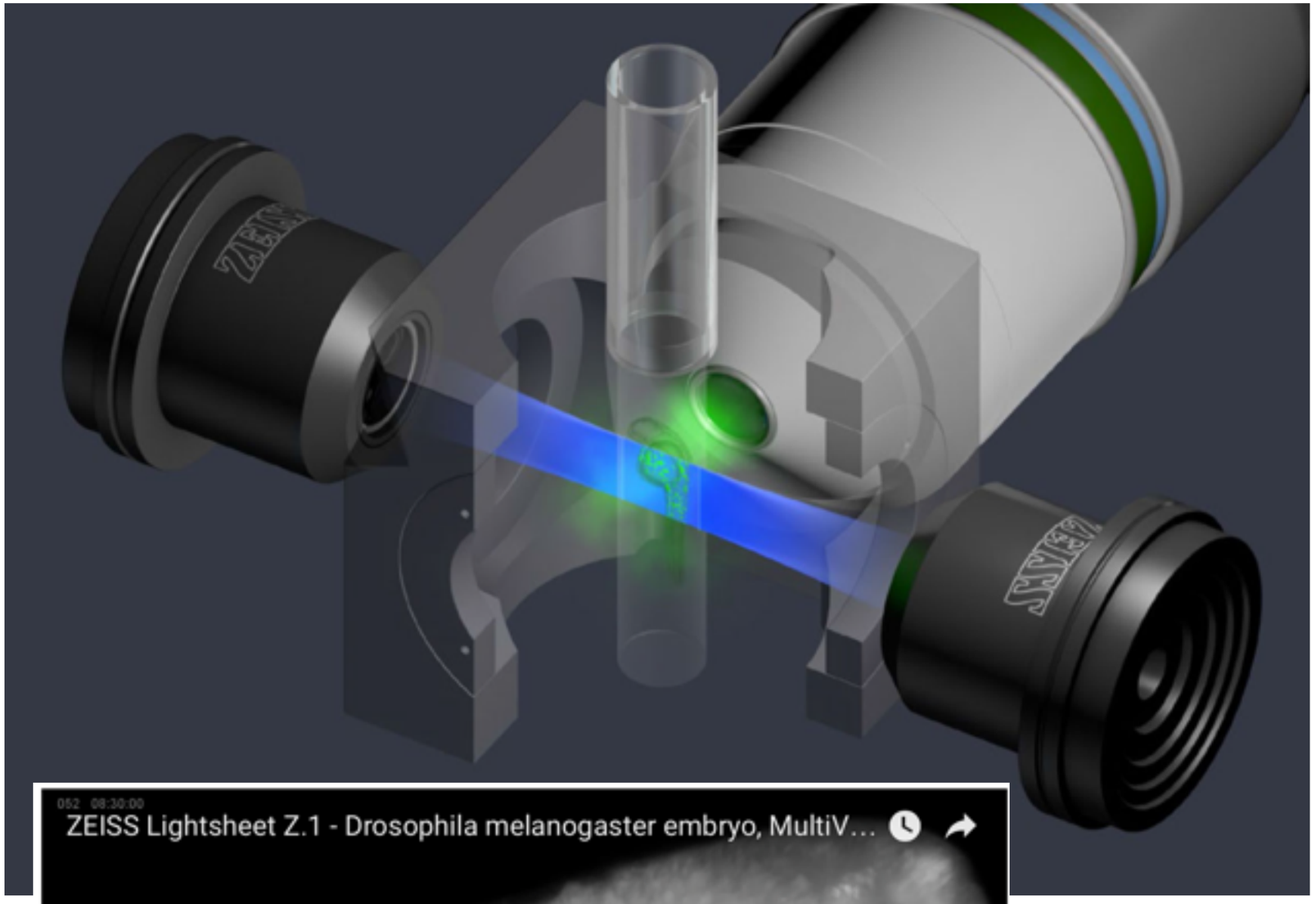


http://www.azonano.com/nanotechnology-video-details.aspx?VidID=938

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.

For live cell imaging catch every photon:

Be economical with your light budget - Hardware

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

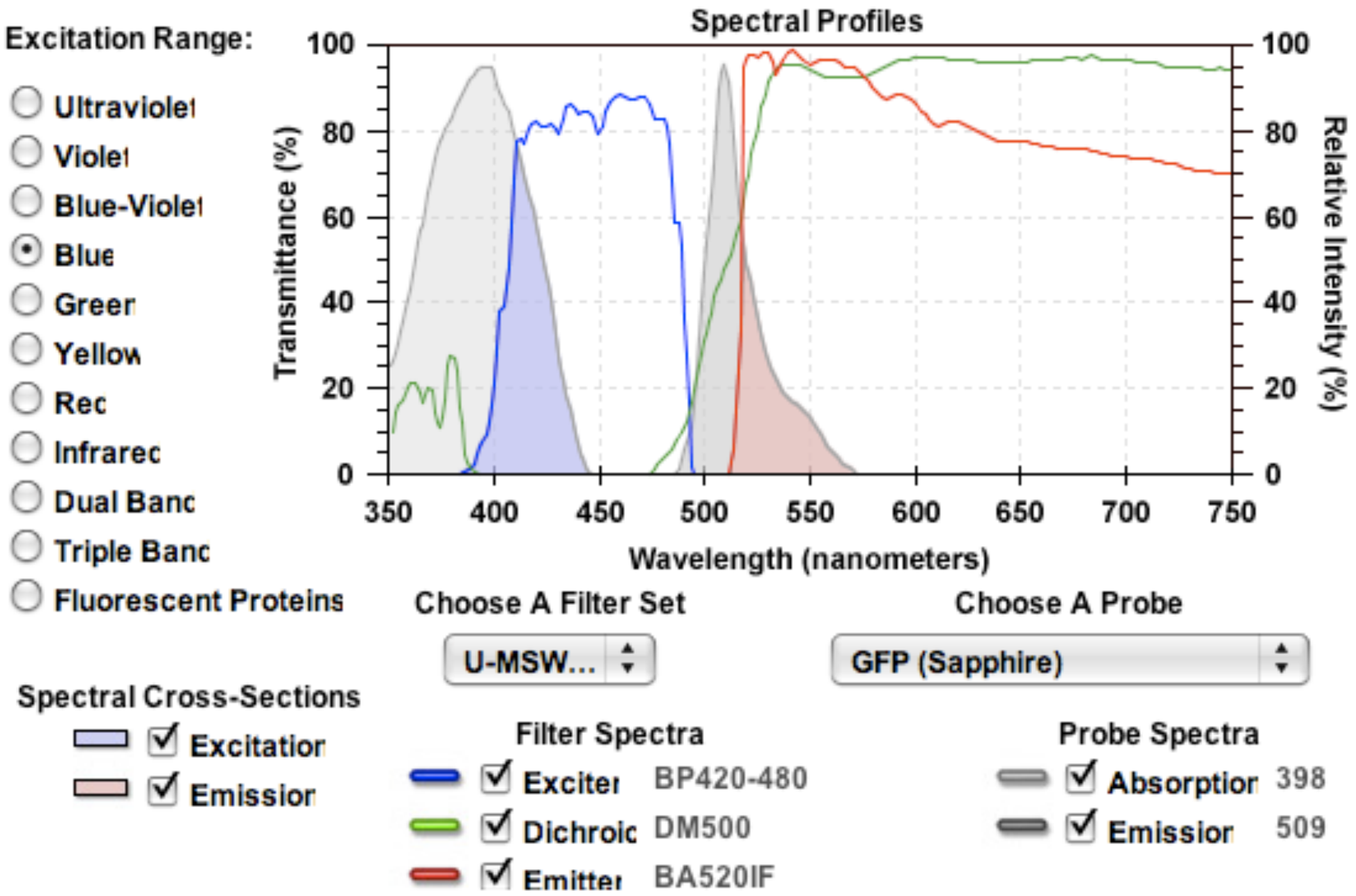
- Optimised synchronisation of illumination, exposure and readout
  "real time" system controllers
  fast shuttering
  diode light sources

- 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

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- 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

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

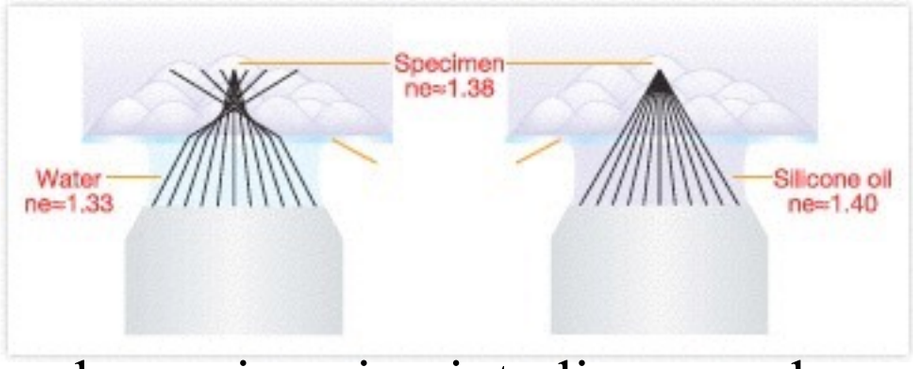
(apo chromatic)

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

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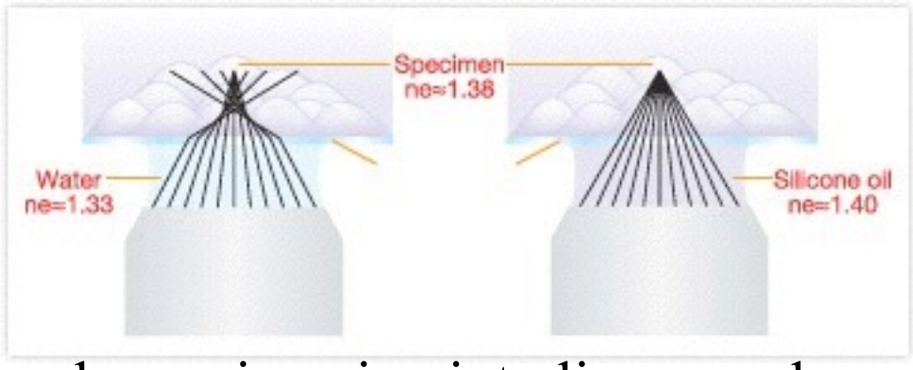
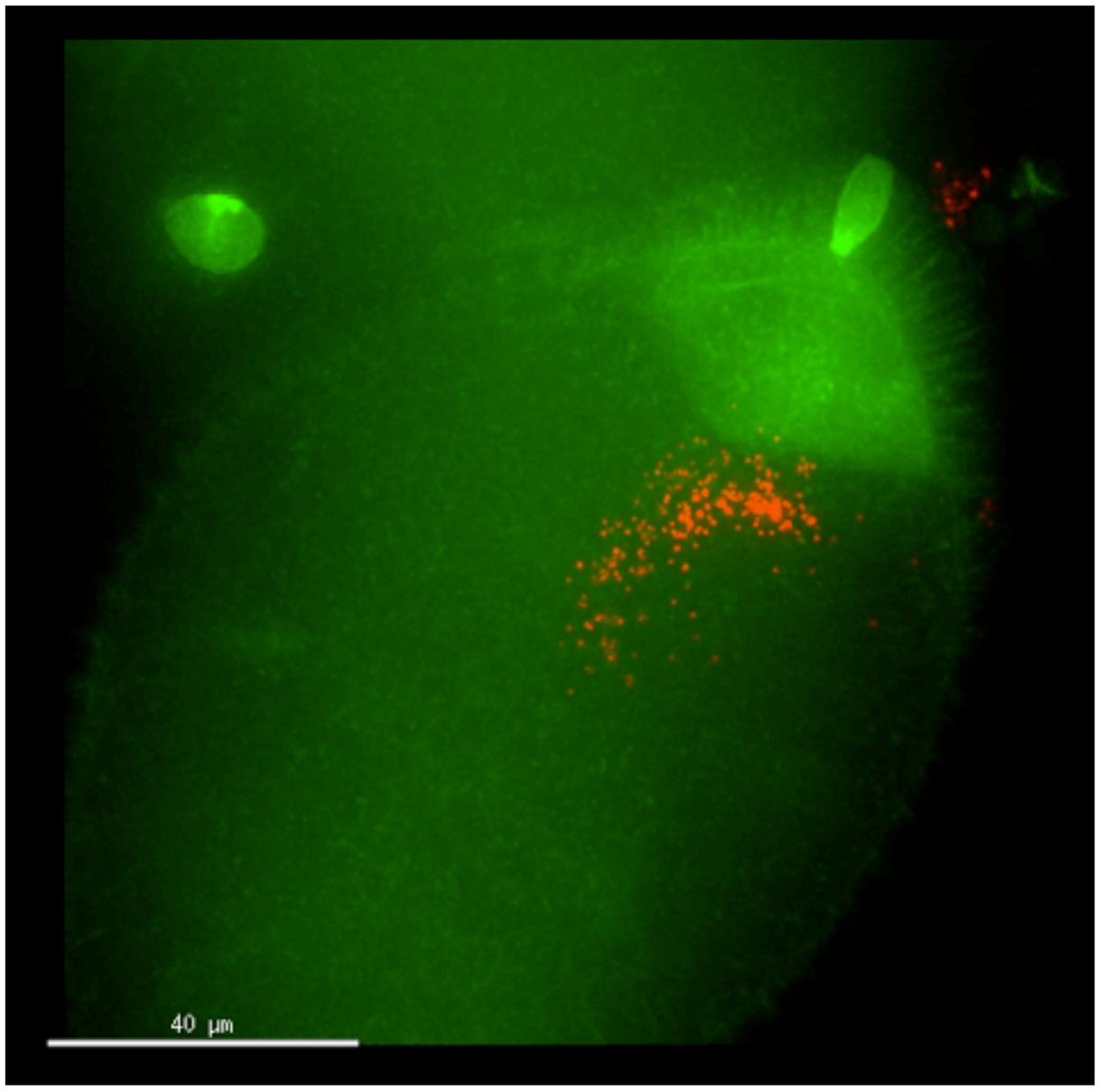


deeper imaging into live samples

Very Expensive!!

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

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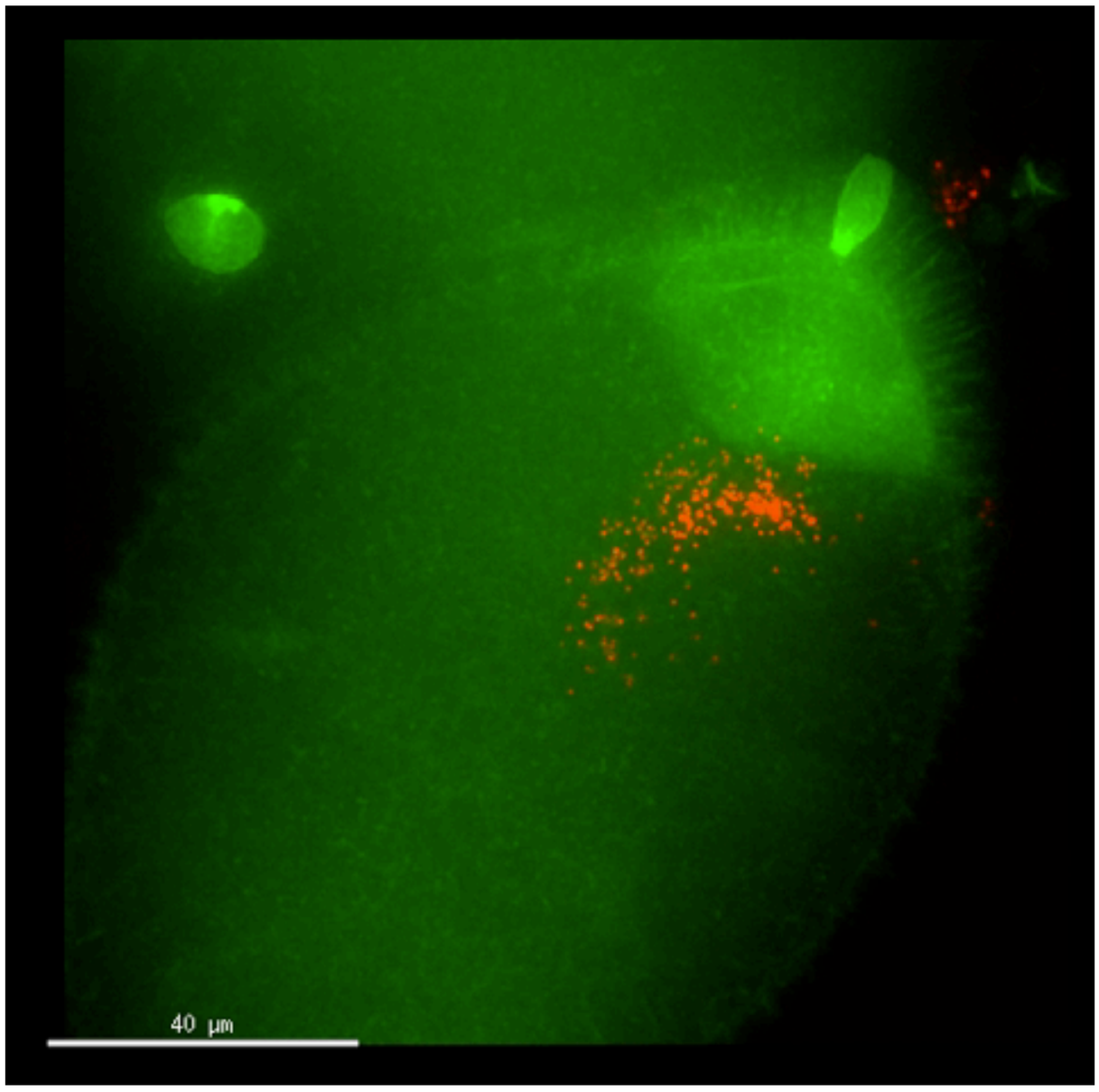

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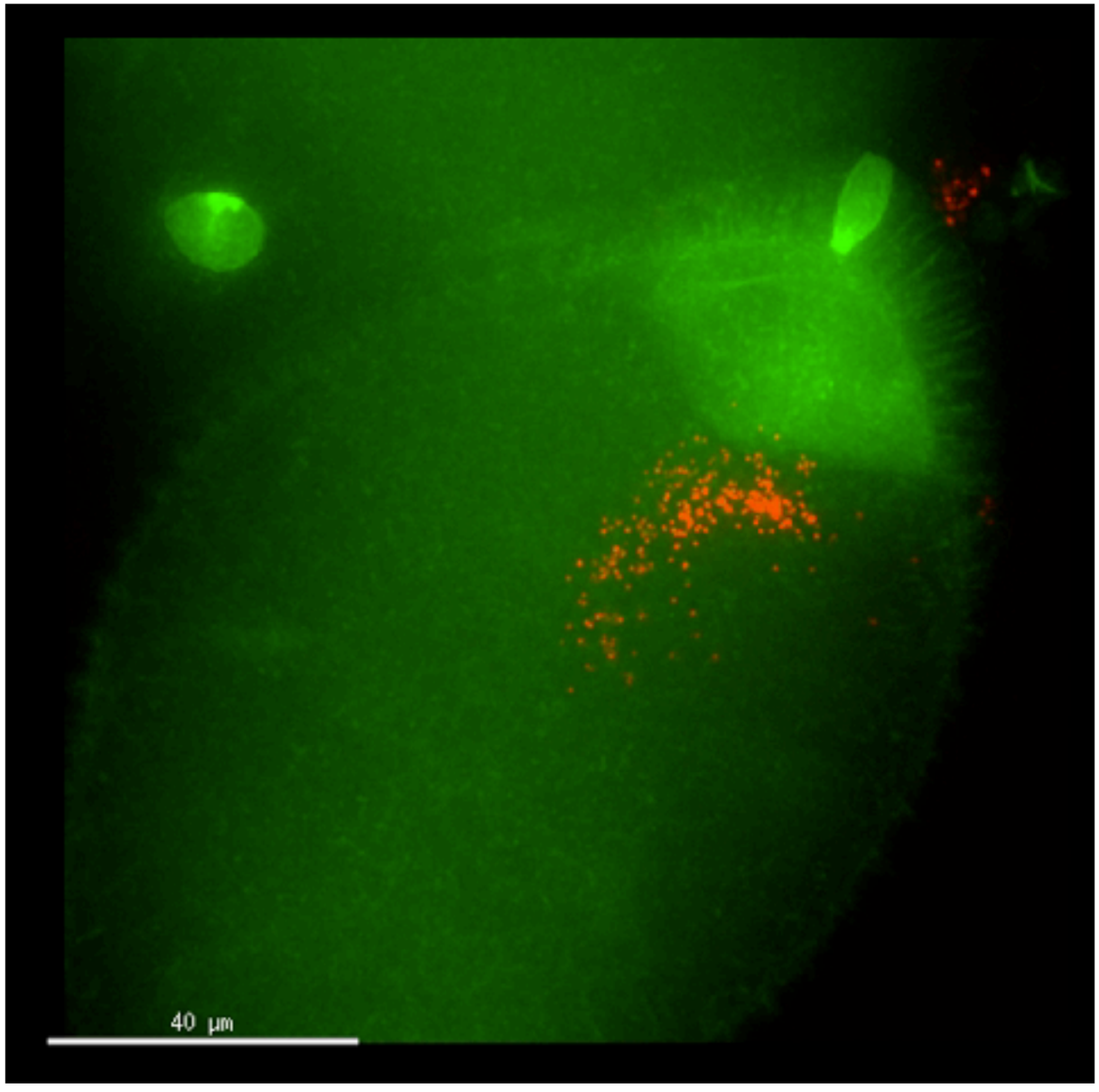

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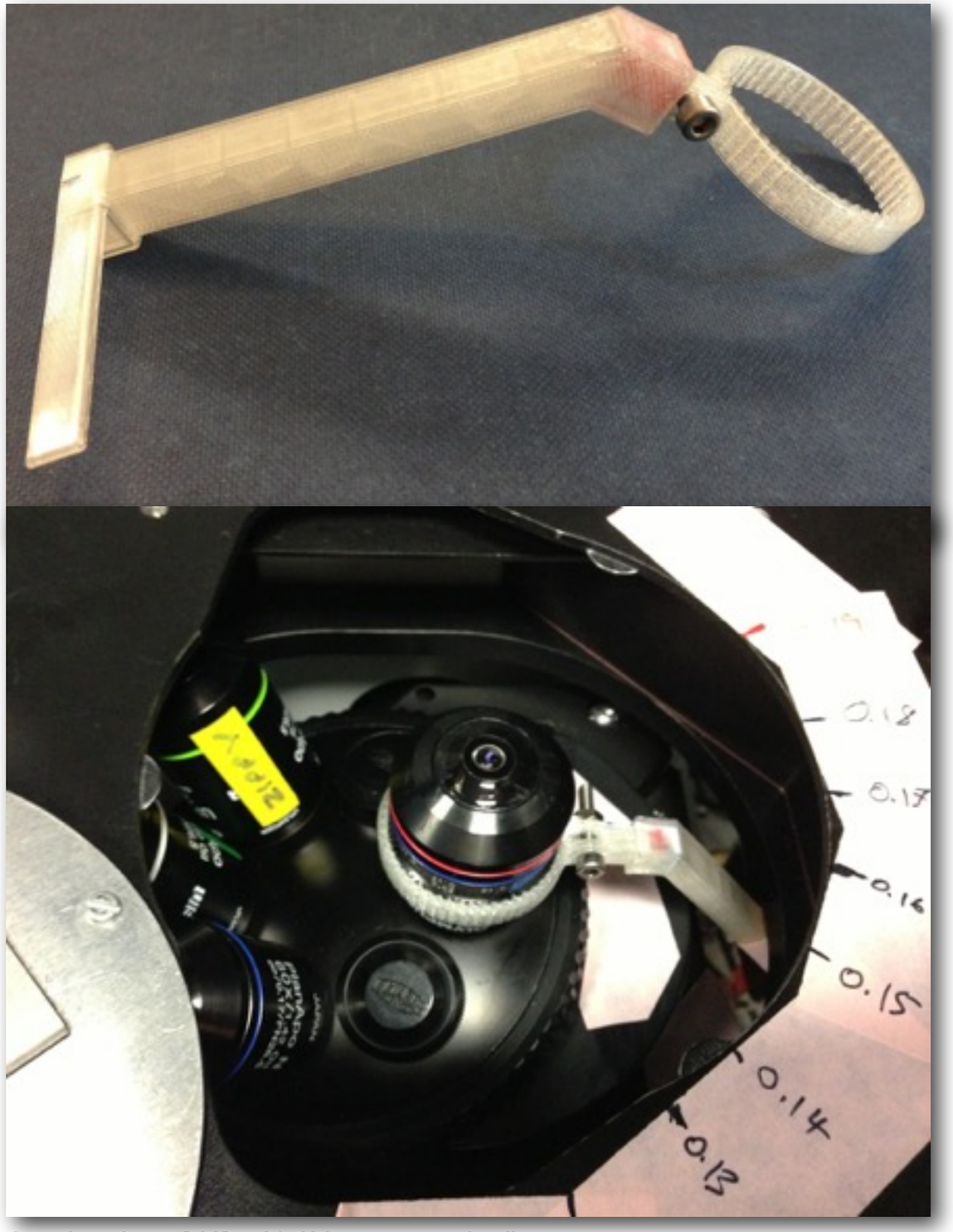

Jonathan Sturt, RMP: x60 SI lens - manual collar correction

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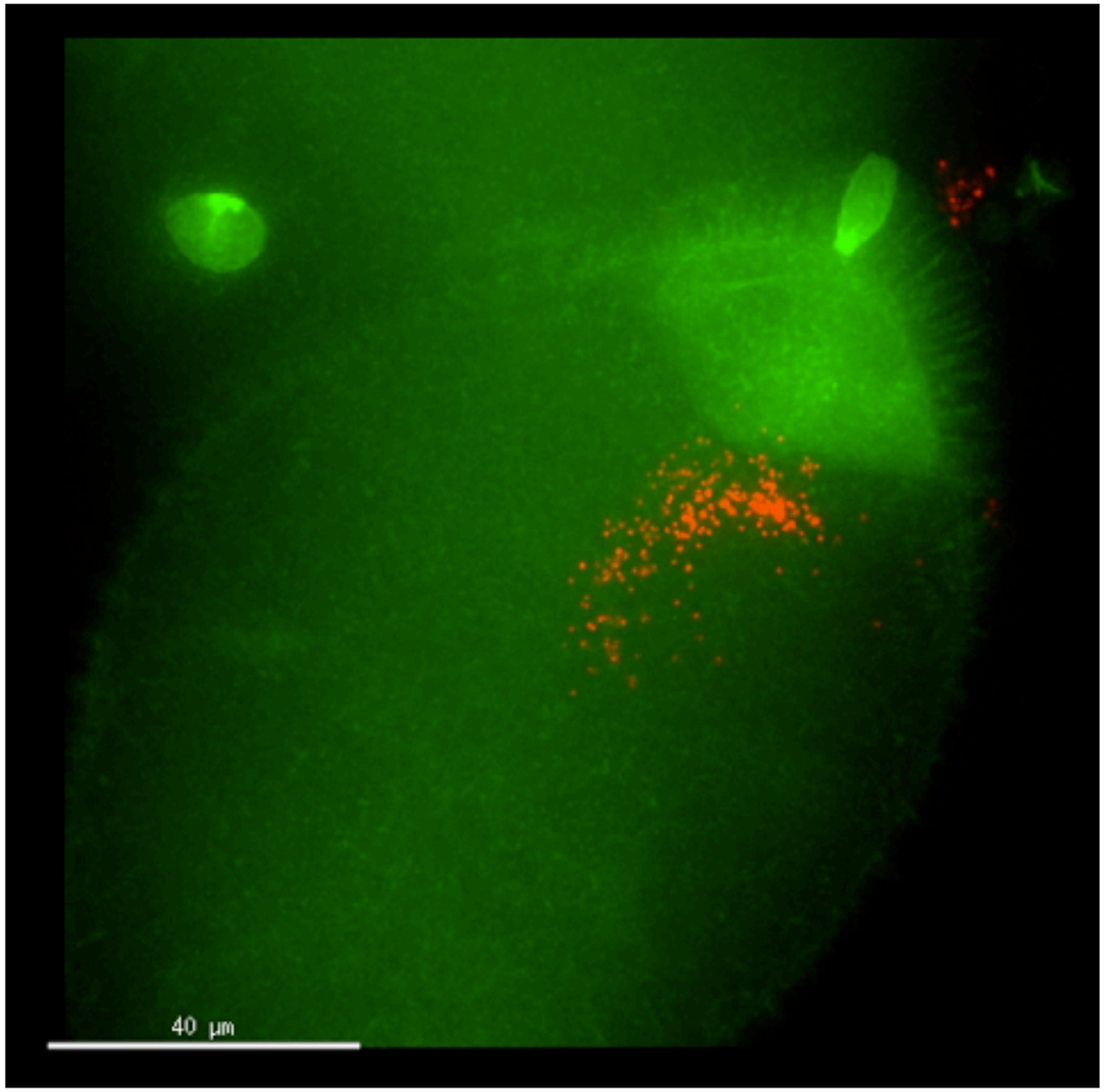
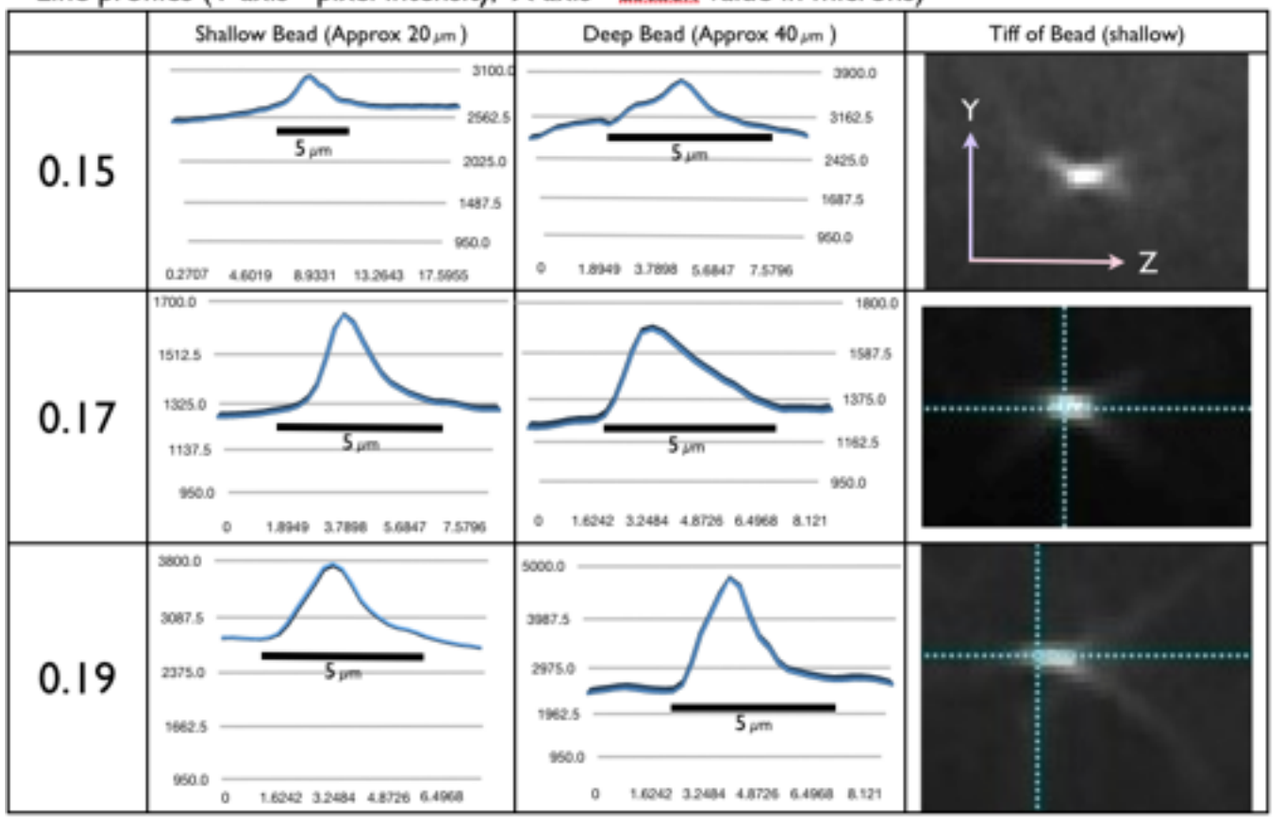


ActinGFP expressing Drosophila egg chamber injected with 100 nm red beads

Data Taken with Manual Lever

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



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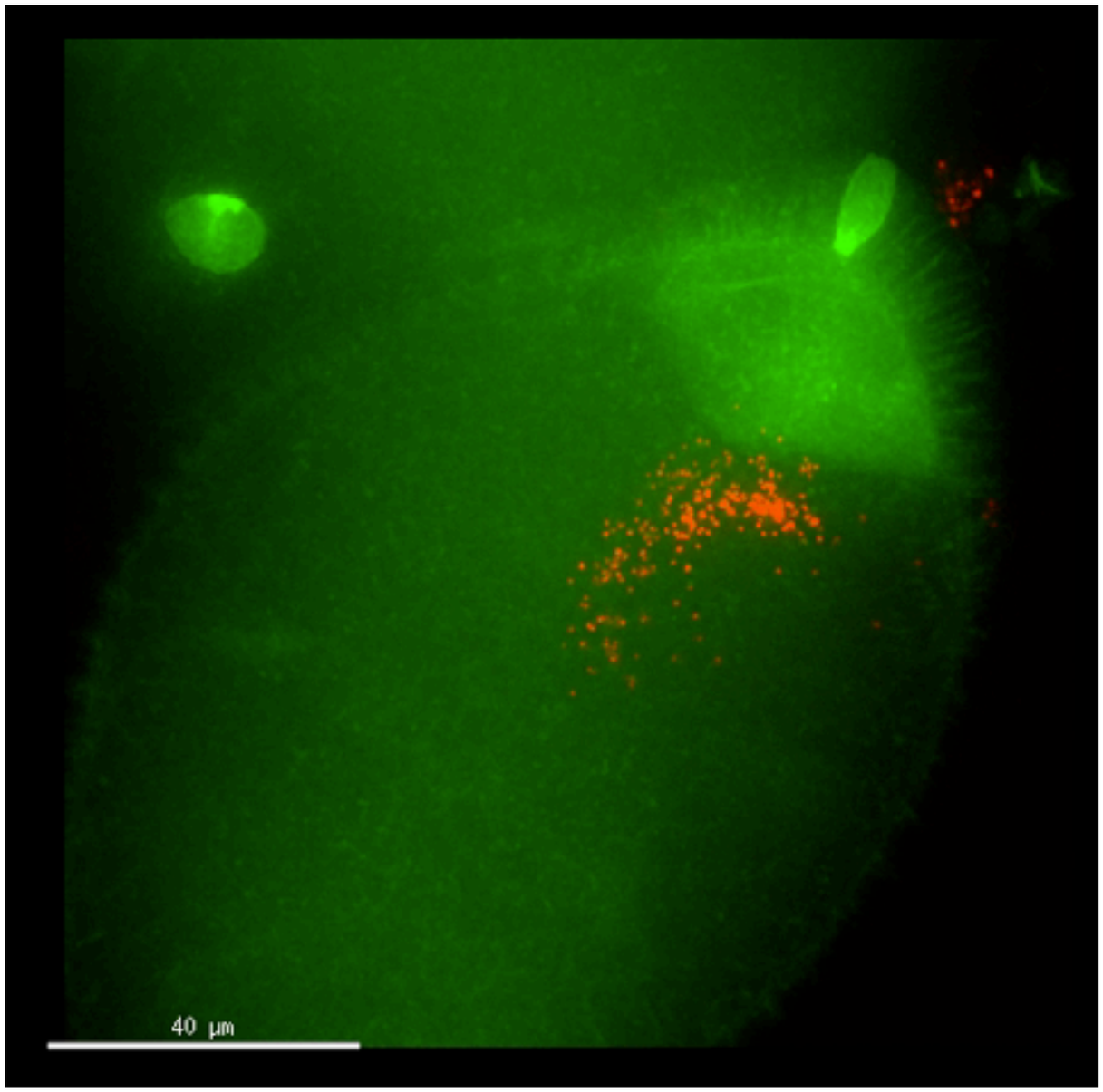


ActinGFP expressing Drosophila egg chamber injected with 100 nm red beads

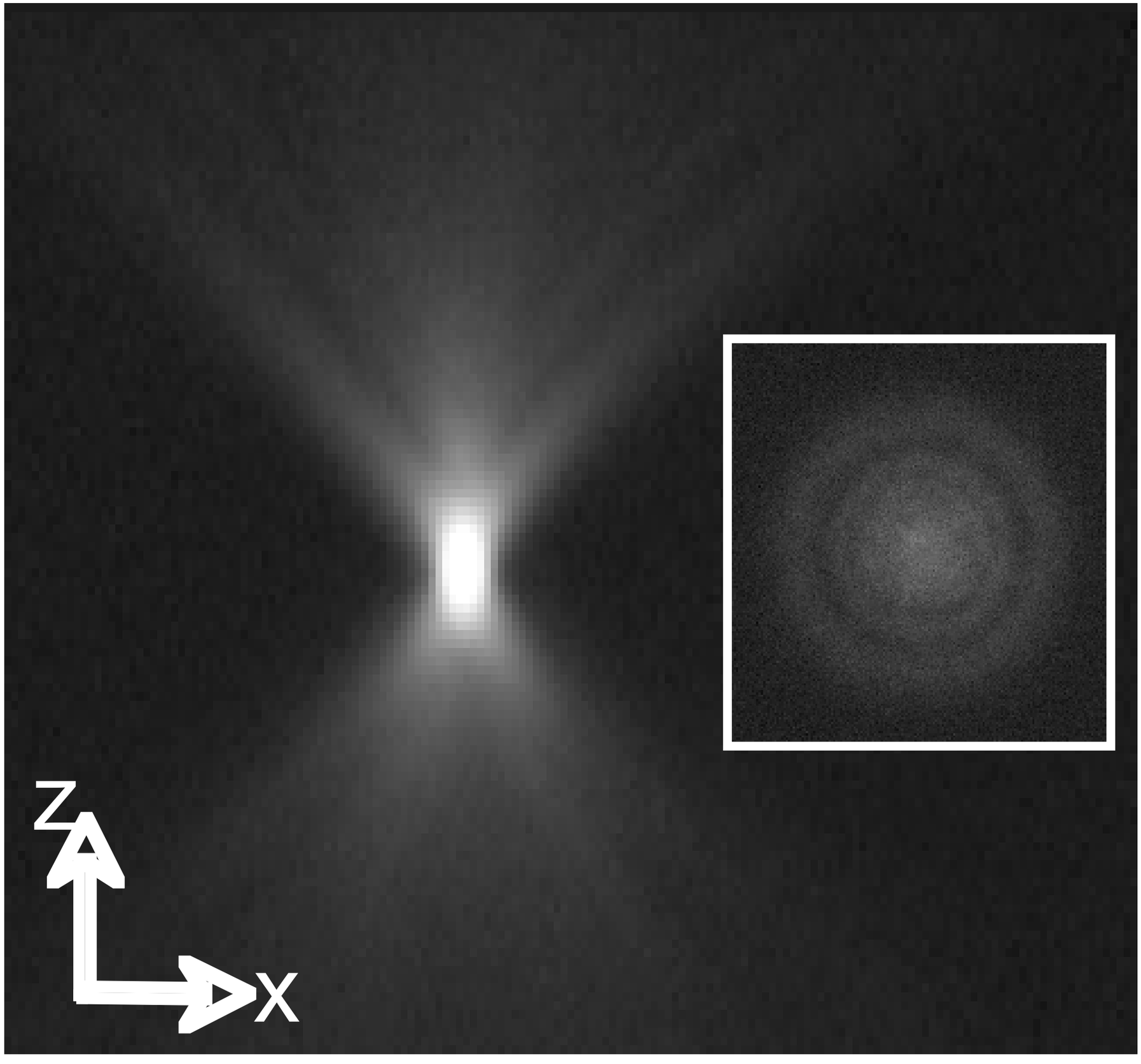
Jonathan Sturt, RMP: x60 SI lens - automated collar correction

Setup your equipment properly:

Setup your equipment properly:

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

Setup your imaging equipment properly:



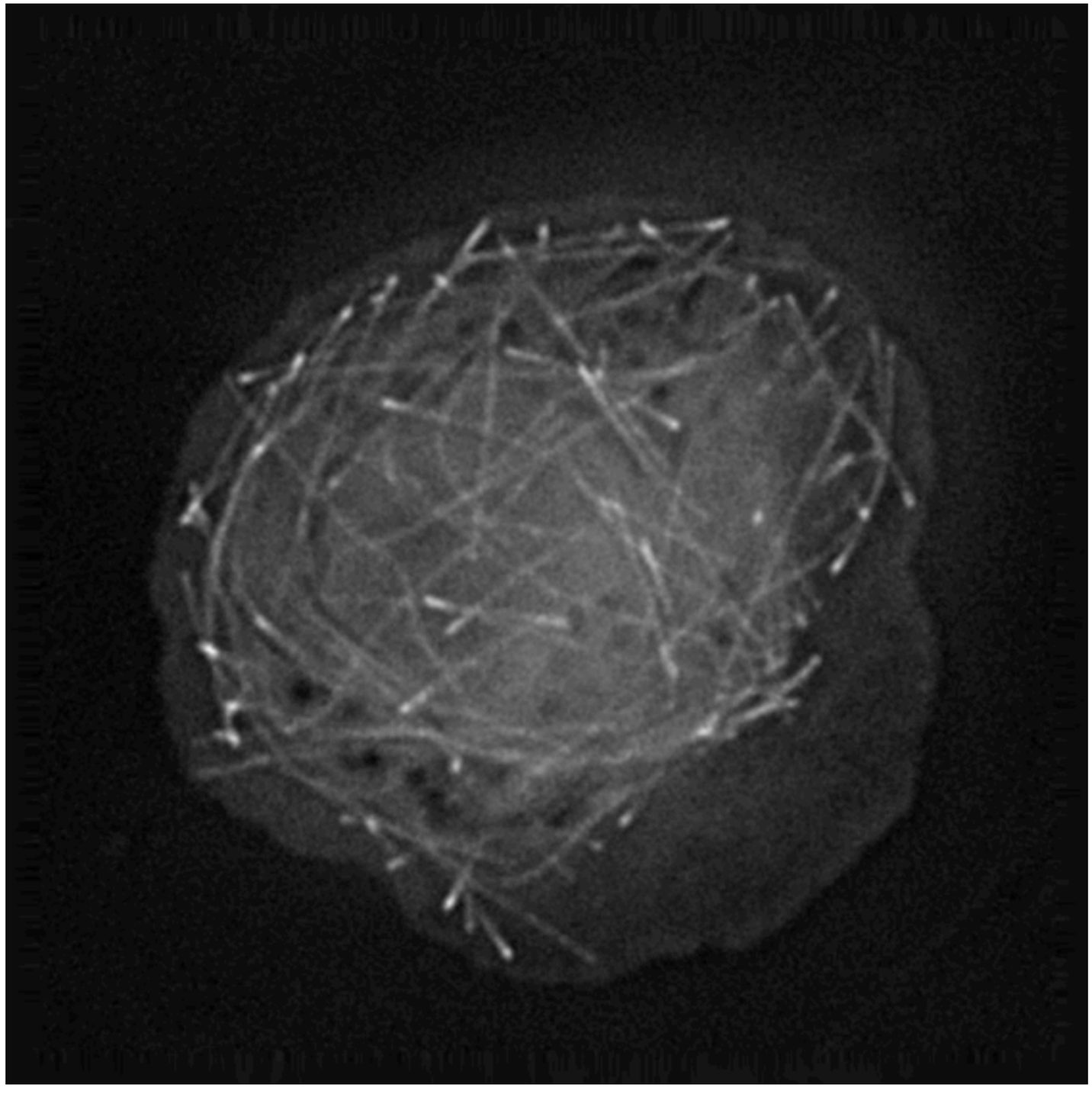
Be economical with your light budget - best practice

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

Choose your dyes / labels carefully! **Lectures 4 / 5**

Choose your dyes / labels carefully!

Lectures 4 / 5



Macrophage: GFP microtubules

Specimen Preparation:

Specimen Preparation:

- Oil objectives image best close to the coverslip

Mount the specimen appropriately

Use alternative immersion lenses

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- 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

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  Tricks to keep specimens still

- Ensure the viability of your sample

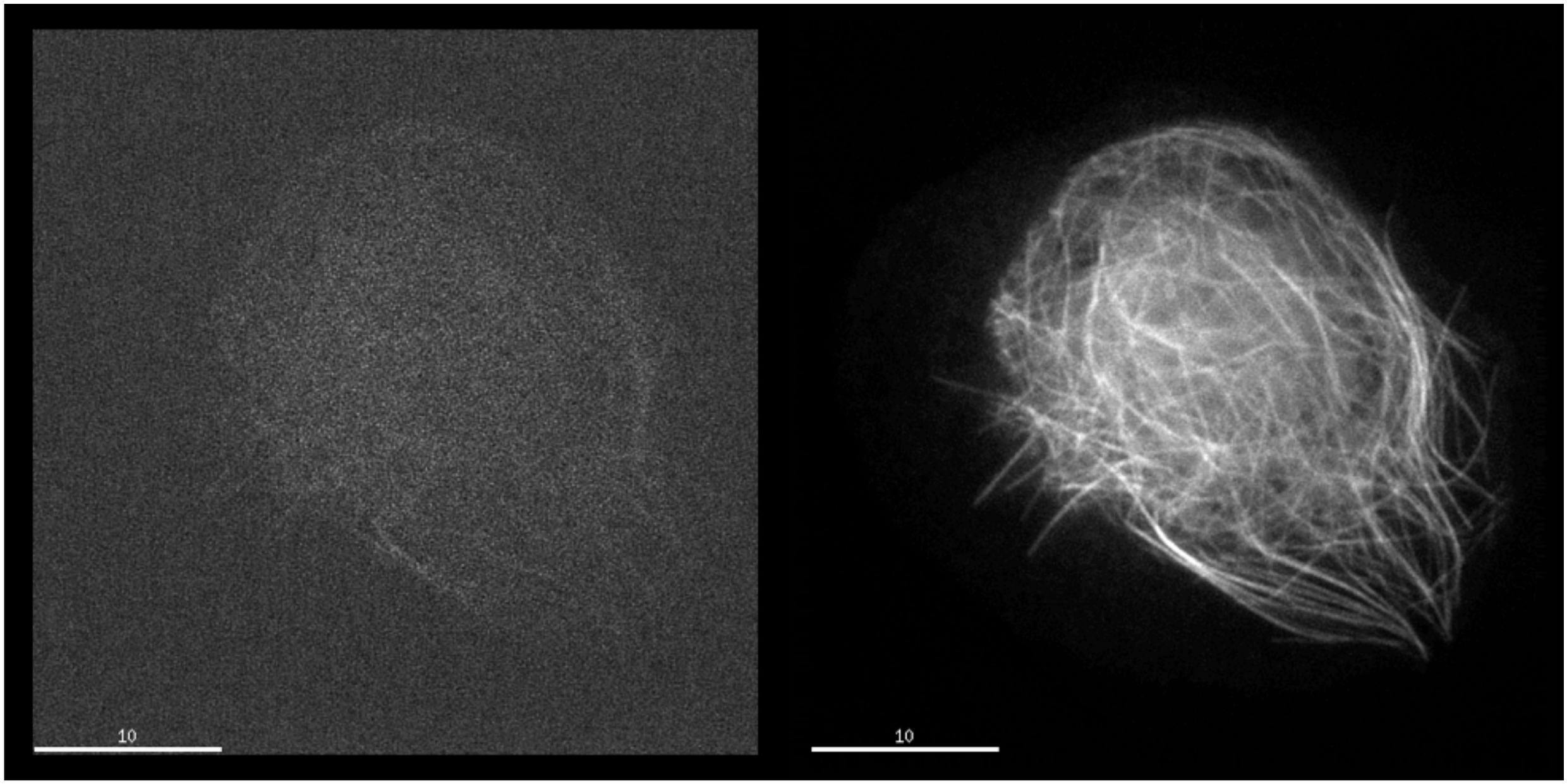
  Media / drying out

  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



Jerome Boulanger: SAFIR Denoising software

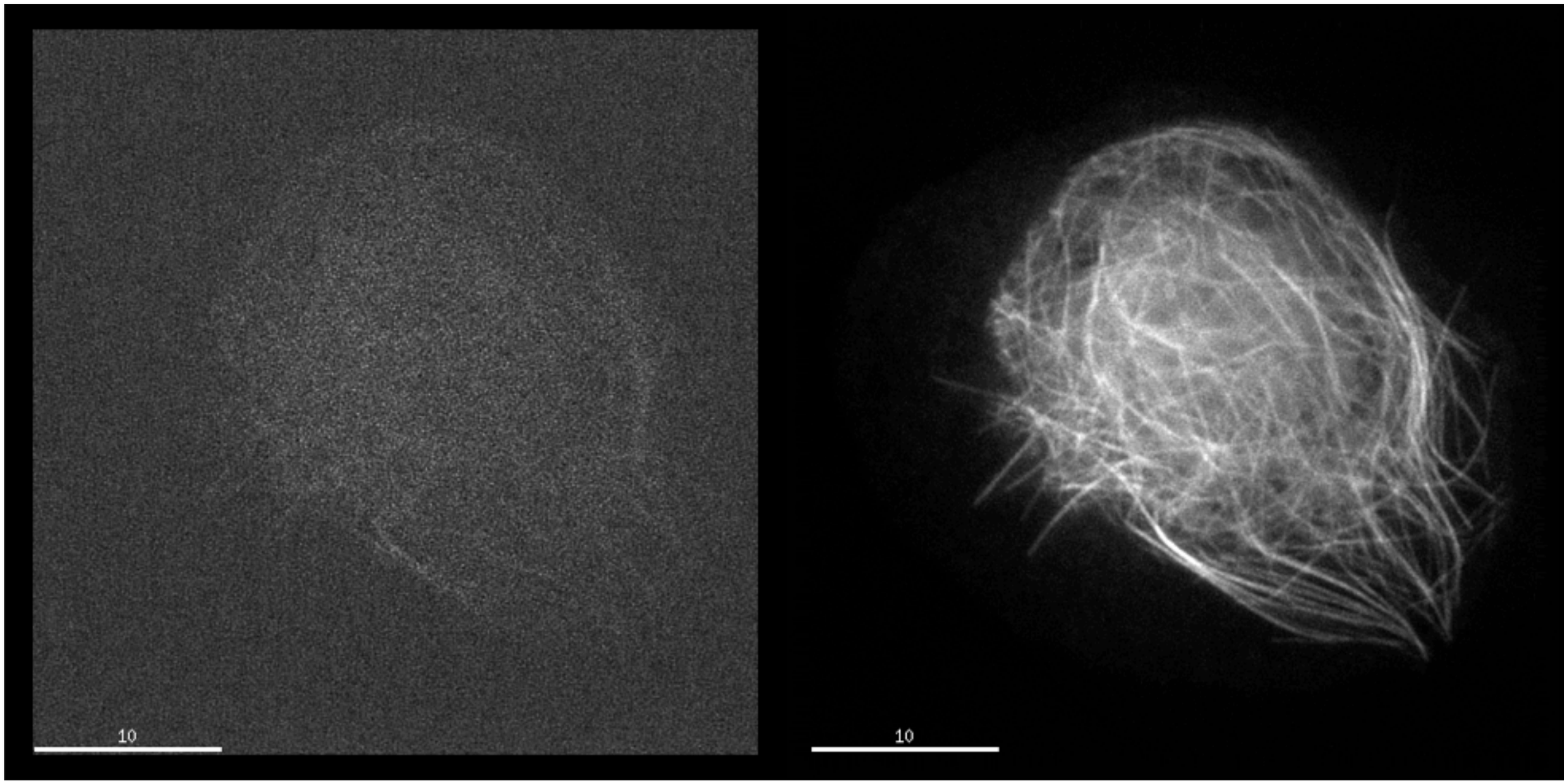
Integrated into Priism by the John Sedat Group UCSF

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

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

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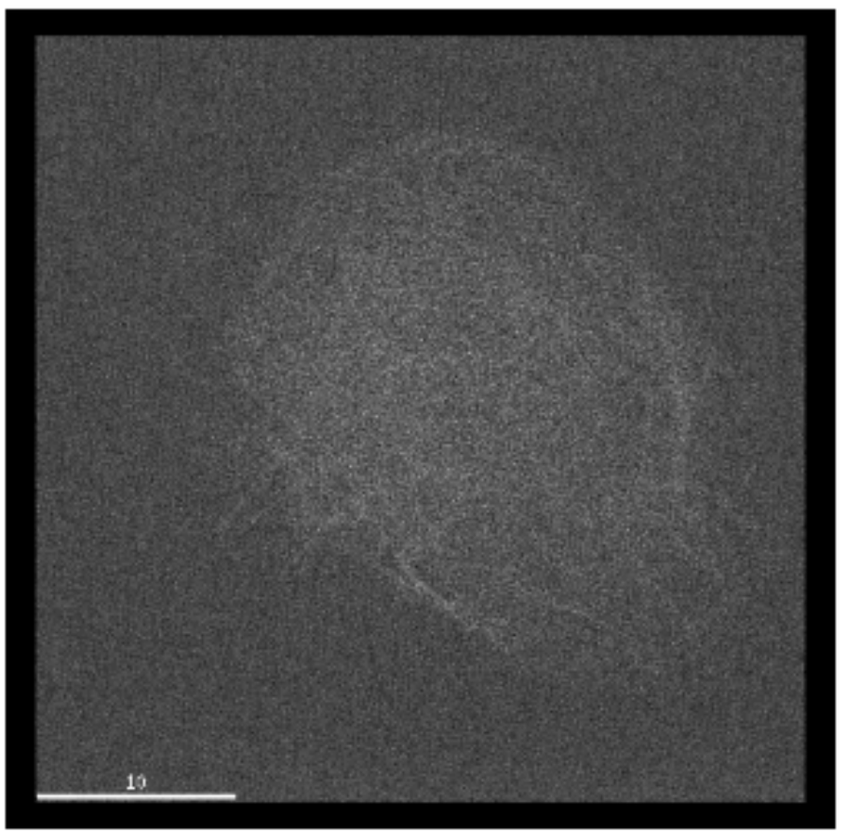
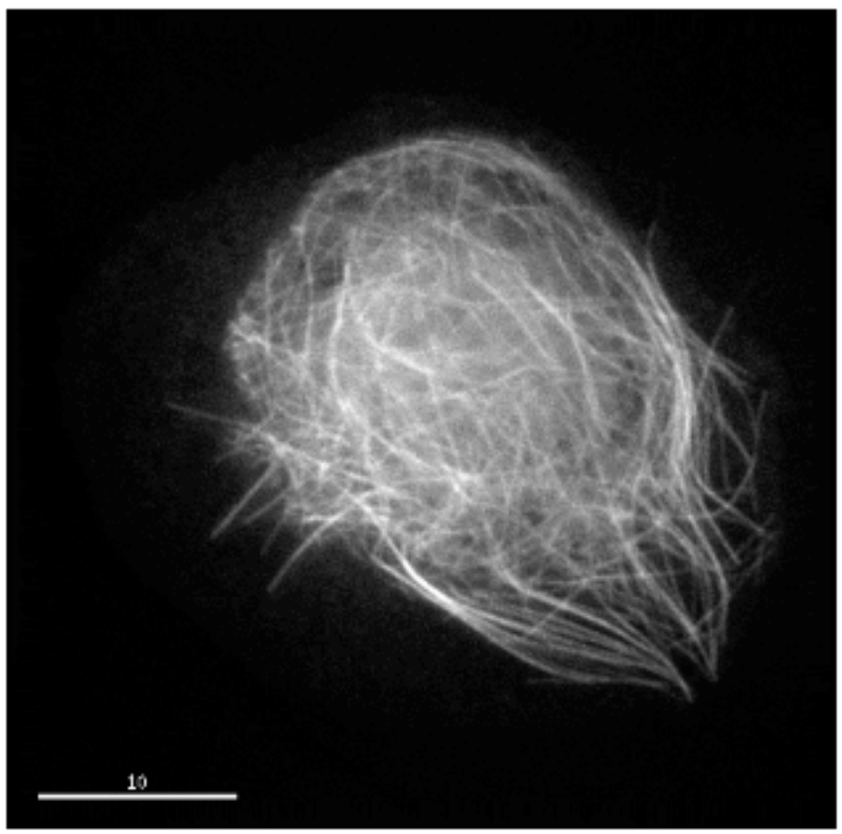
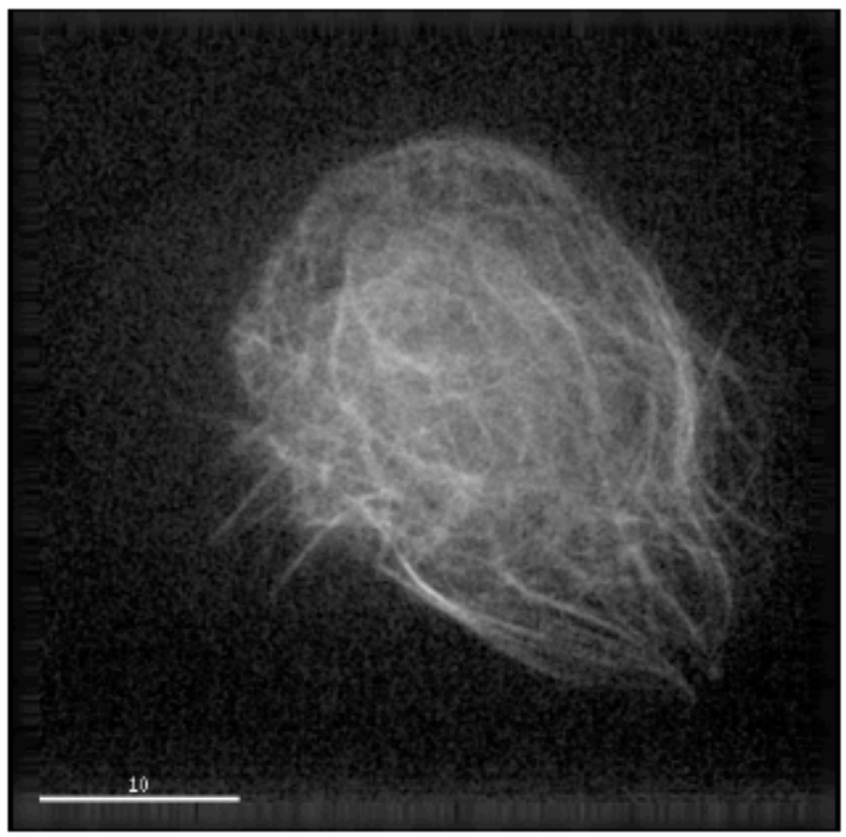


8 ms ex, 10% 488 laser



8 ms ex, 0.1% 488 laser - Denoised



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END