ED SUBA JR./Akron Beacon Journal

A recent study has confirmed what cat owners have long known: Cats understand when spoken to, but they choose to ignore most of what people say.

Study: Our cats understand us, but they don't really care

By KATHY ANTONIOTTI
Akron Beacon Journal

The agony of unrequited

A study by two University of Tokyo researchers, published by Springer in

stress of moving them to strange surroundings had no role in the outcome of

bred and have evolved "to follow their owner's orders, but cats have not been."

Micron Advanced Light Microscopy Course 2018

lecture 7

Live Cell Imaging

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

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

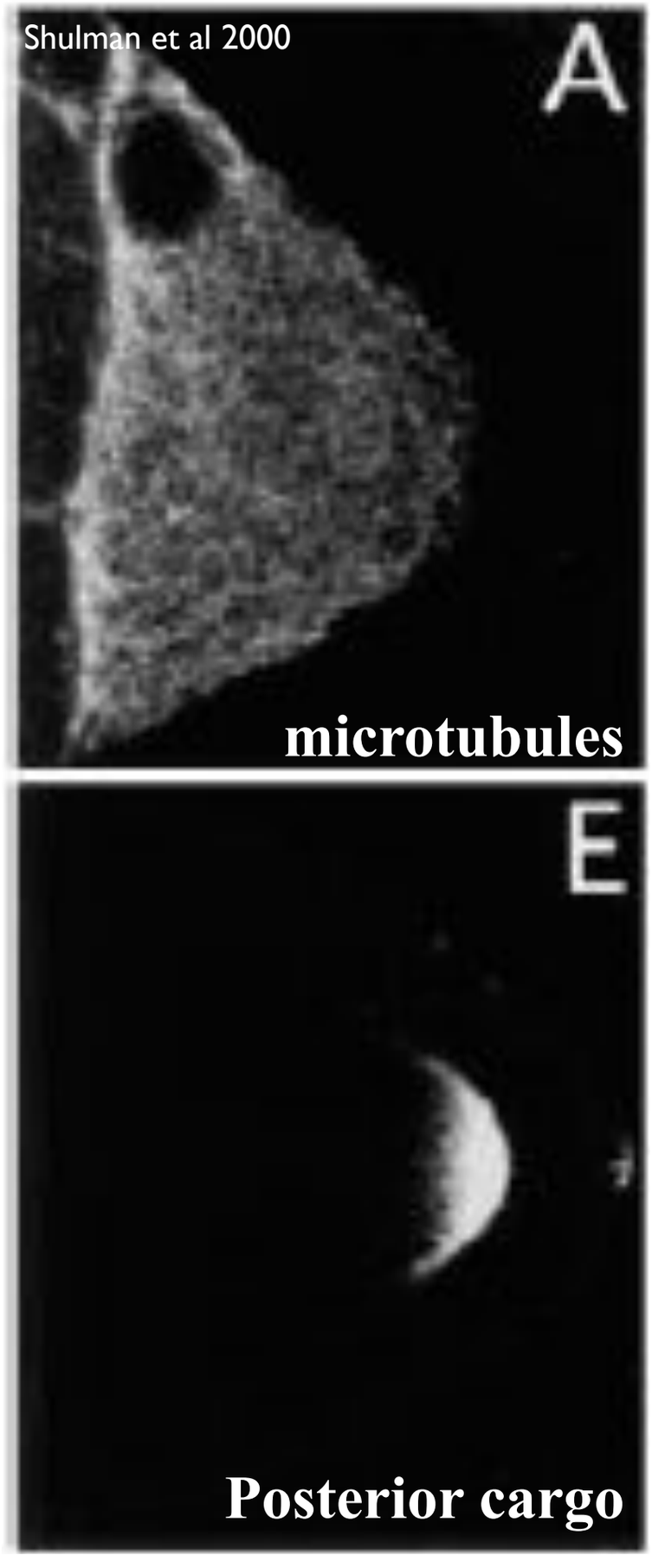
http://imgur.com/a/fhuPr

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

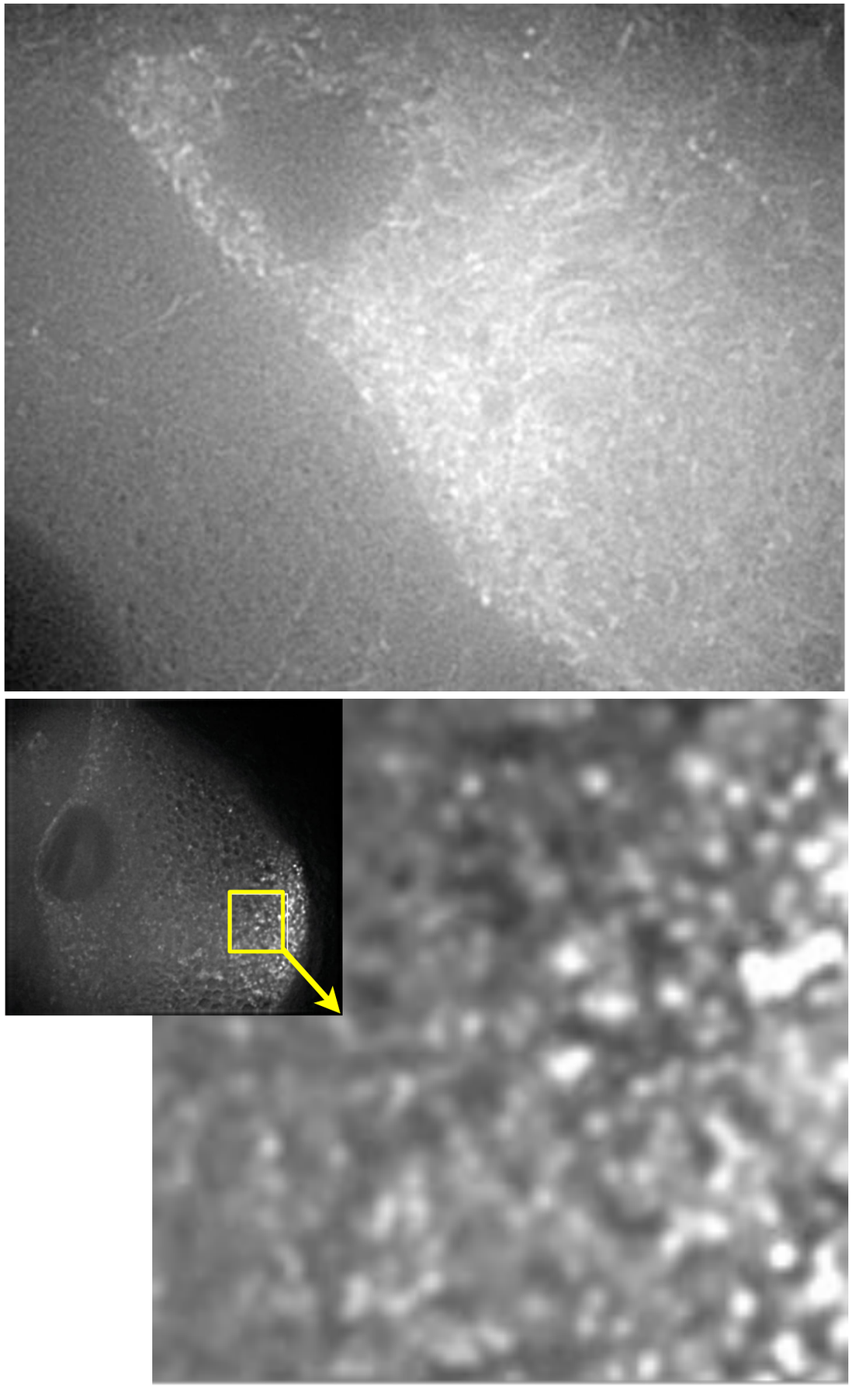
Fixed

Live

Fixed



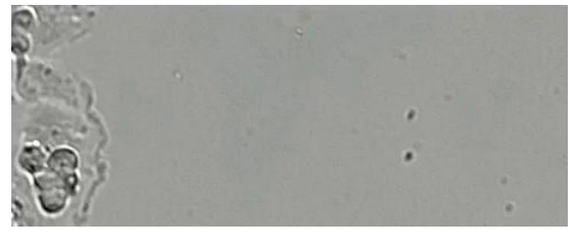
Live



Reasons for live imaging

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

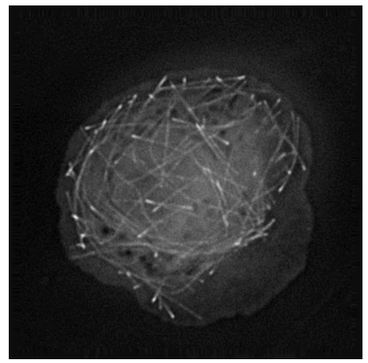
time-course of cell migration - Andrea Linford Barr lab



2) Can monitor the kinetics of dynamic processes:

- active transport vs diffusion
- Microtubule turnover

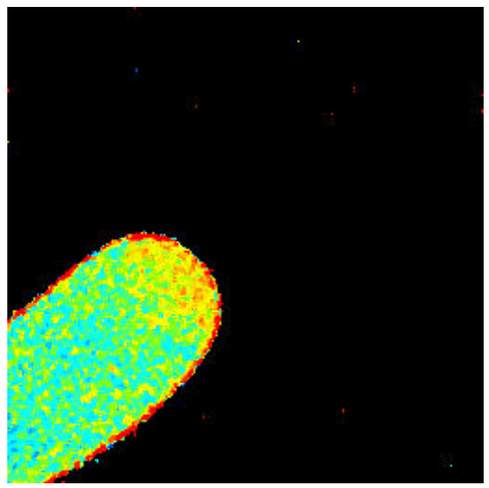
Macrophage: EB1-GFP tagged MT



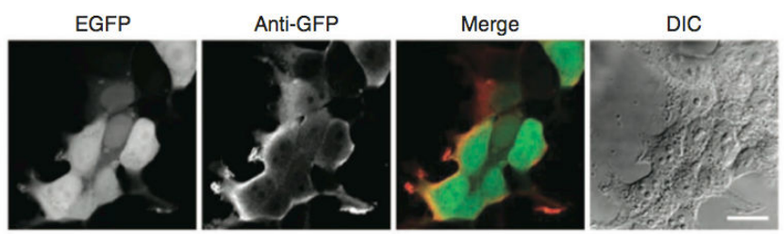
3) Can record sensitive or transient processes:

- Calcium signalling transients
- Ion gradients
- membrane potential

Calcium ratio imaging pollen tube

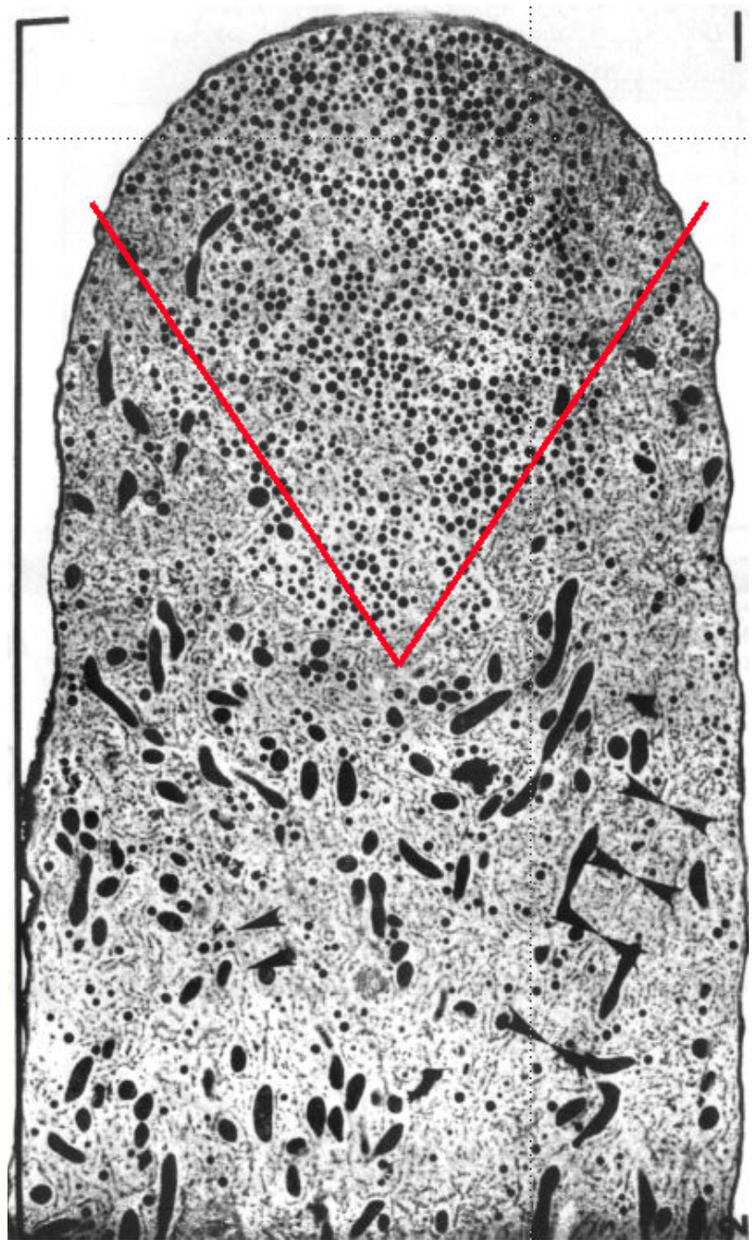


4) You can believe what you see - no fixation artefacts



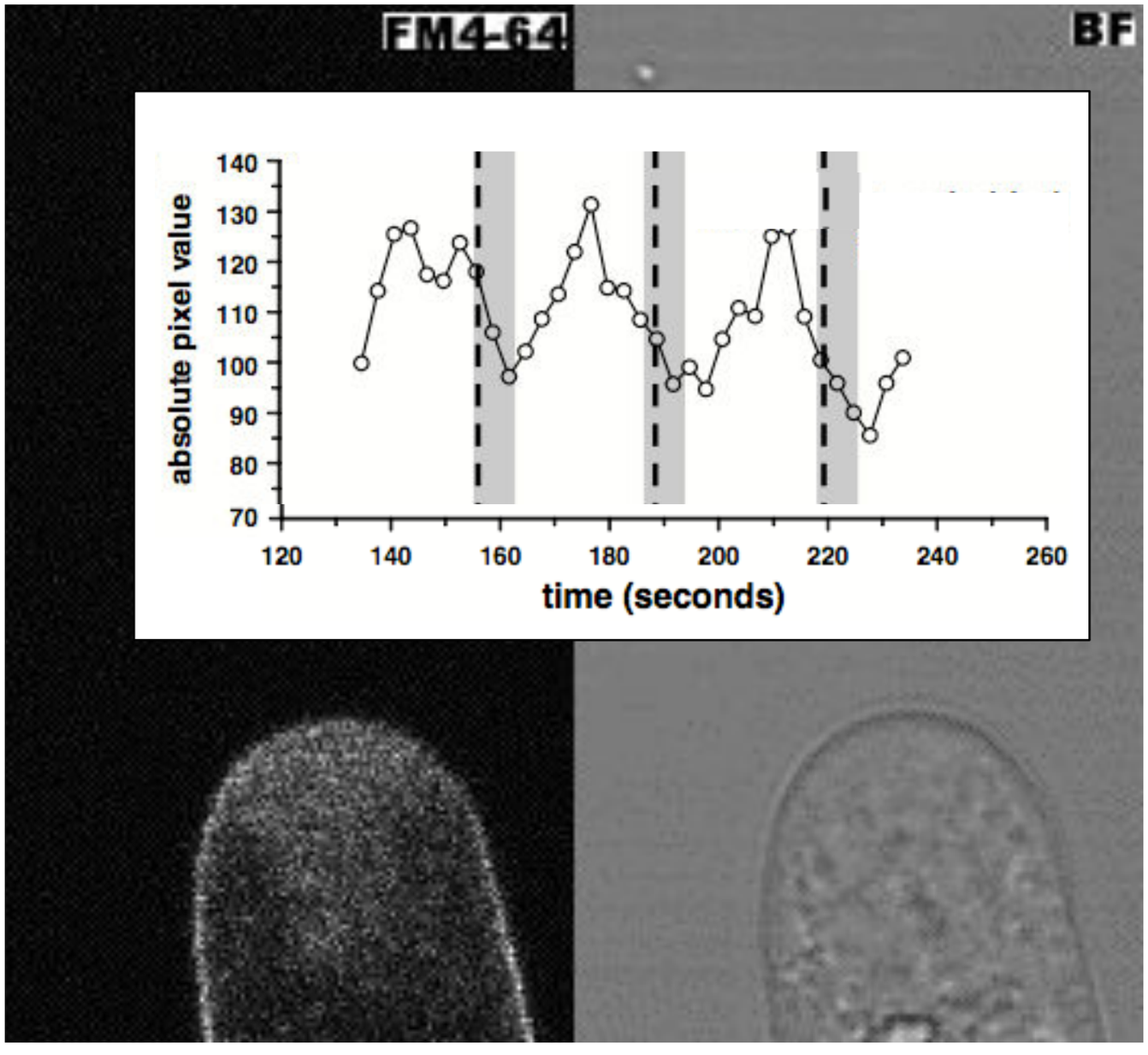
Can monitor the kinetics of dynamic processes

Fixed - EM



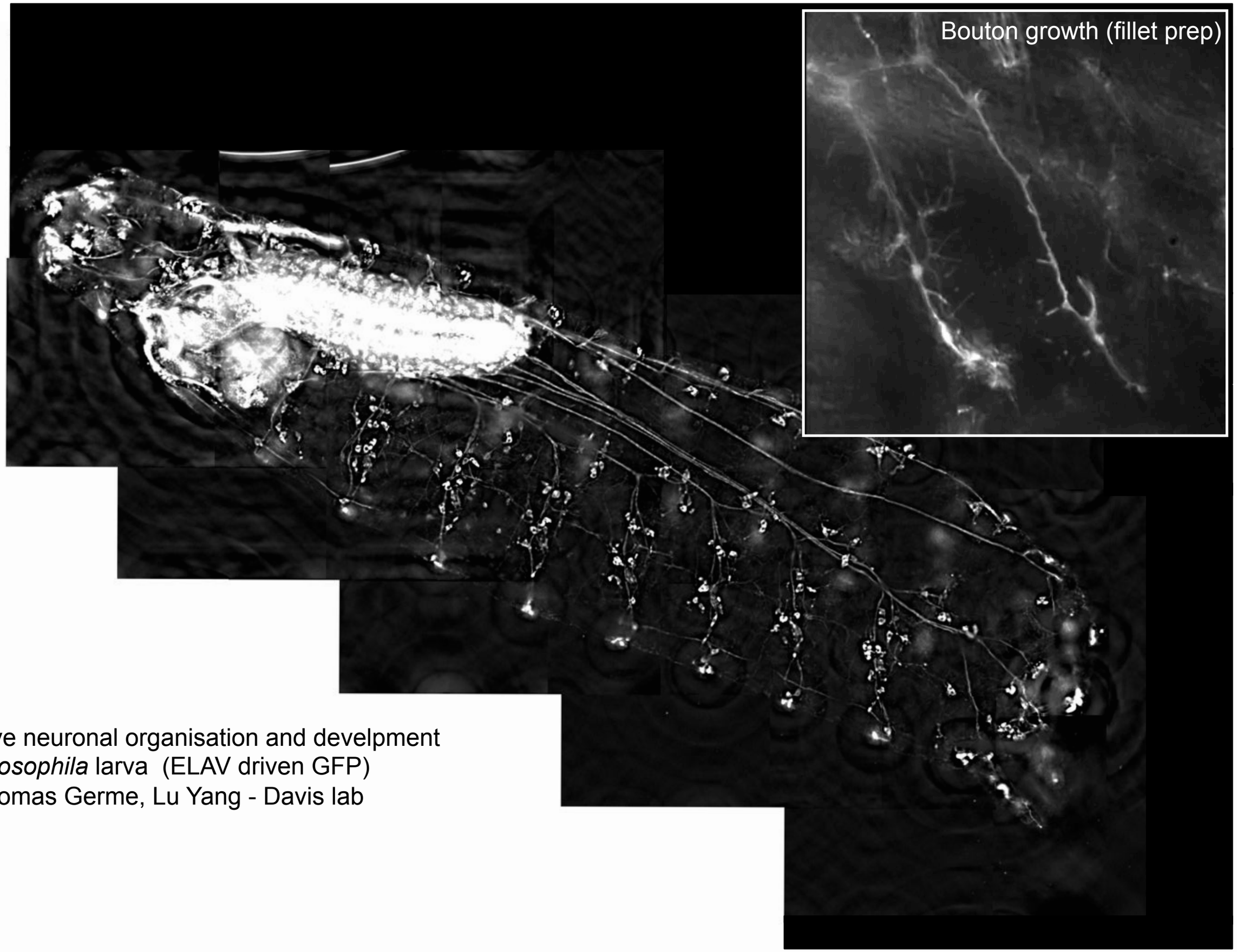
Electron Micrograph From
Lancelle,S.A.; Cresti,M.; Hepler,P.K. **(1997)** ***Protoplasma*** **196, 21-33.**

Live



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

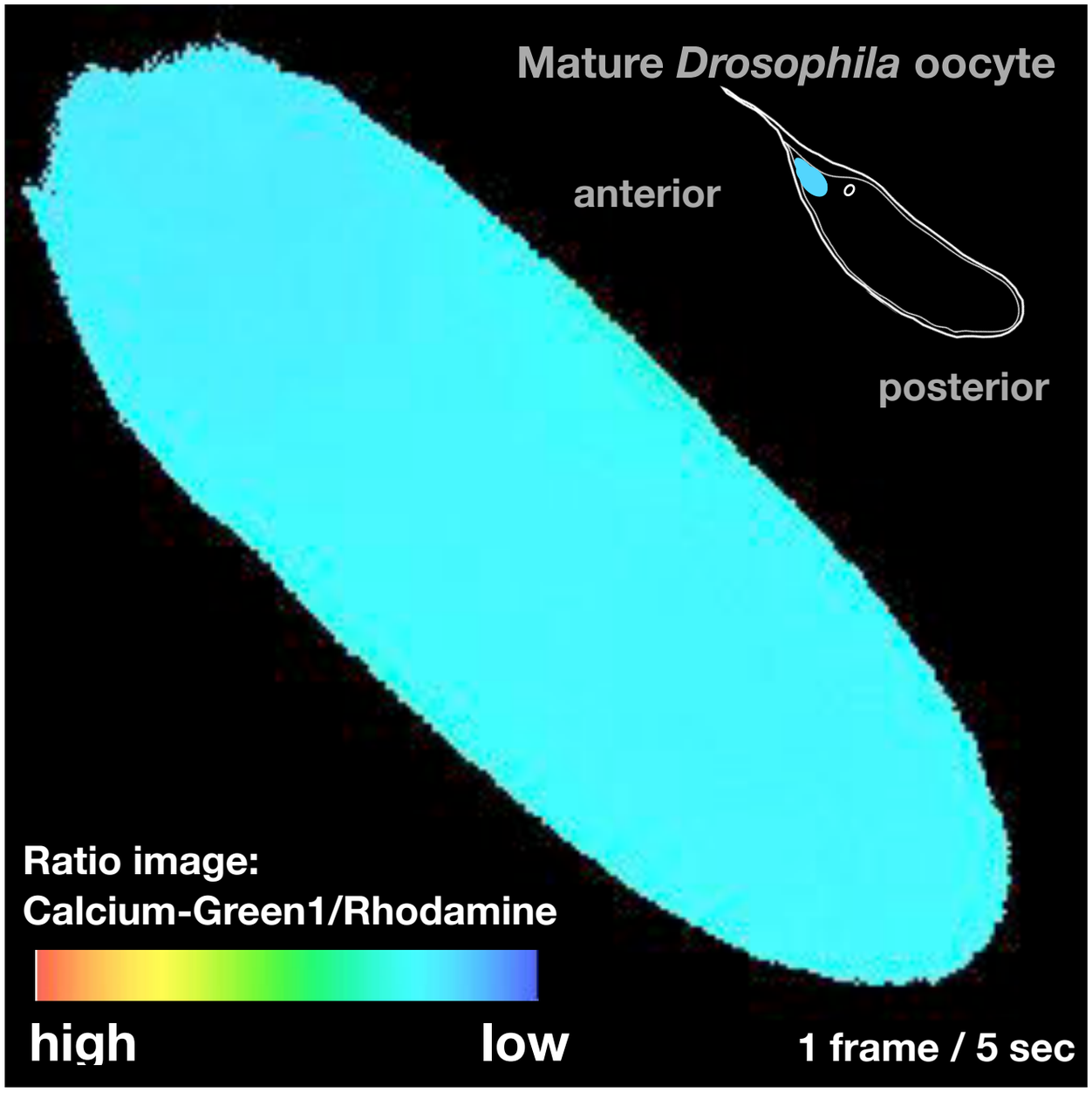
Can follow the order of sequential events in real time

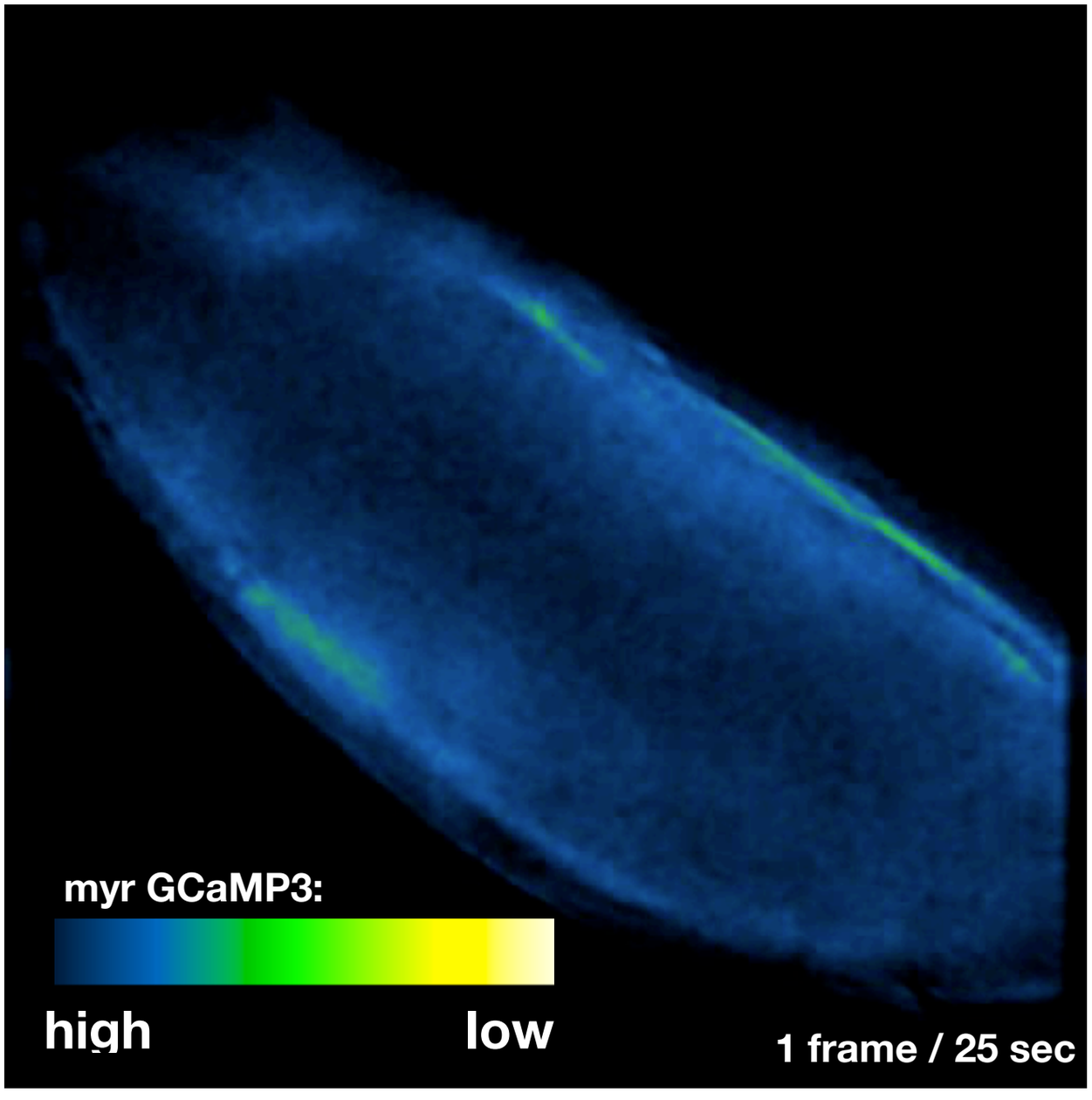


Live neuronal organisation and develpment
Drosophila larva (ELAV driven GFP)
Thomas Germe, Lu Yang - Davis lab

Can record sensitive or transient processes

Calcium transient upon activation





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

Live imaging as an experimental tool:

- Photoactivation / FRAP - for kinetics

- FCS / FCCS / RICS - correlation spectroscopies

  - for molecular kinetics and associations

- Photoactivation - cage probe release

  - stimulation / drug release

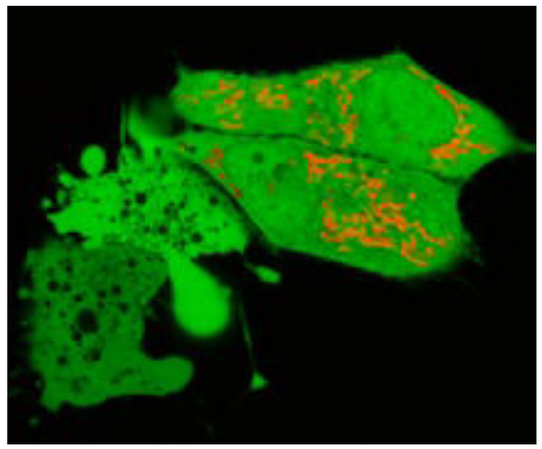
https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook/crosslinking-and-photoactivatable-reagents/photoactivatable-reagents-including-photoreactive-crosslinkers-and-caged-probes.html

Live imaging as an experimental tool:

Using light to manipulate cell behaviour:

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

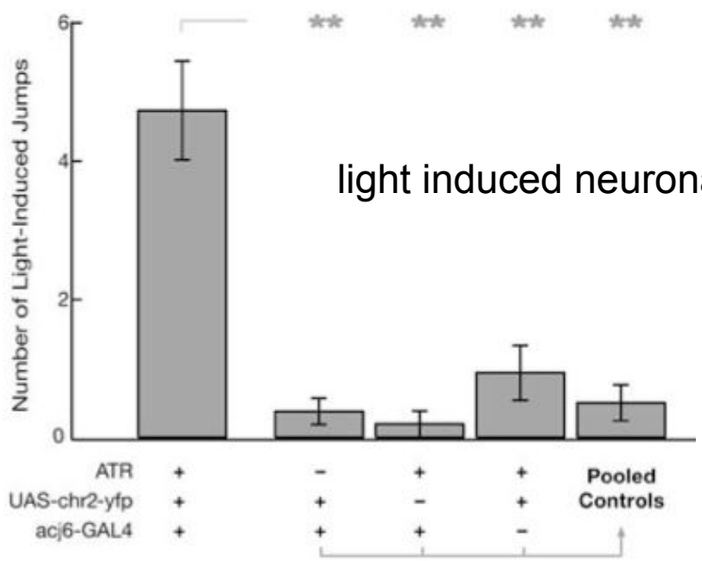
Reactive oxygen species in photochemistry of the red fluorescent protein "Killer Red"
Vegh et al, Chem. Commun., 2011,47, 4887-4889
DOI: 10.1039/C0CC05713D



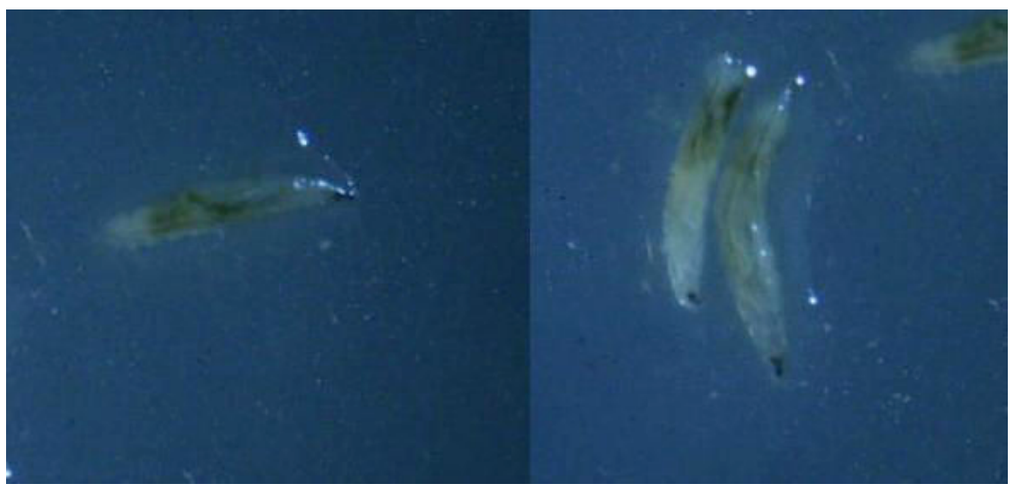
EVROGEN - Killer red expressed in mitochondria

- 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

T.Germe

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

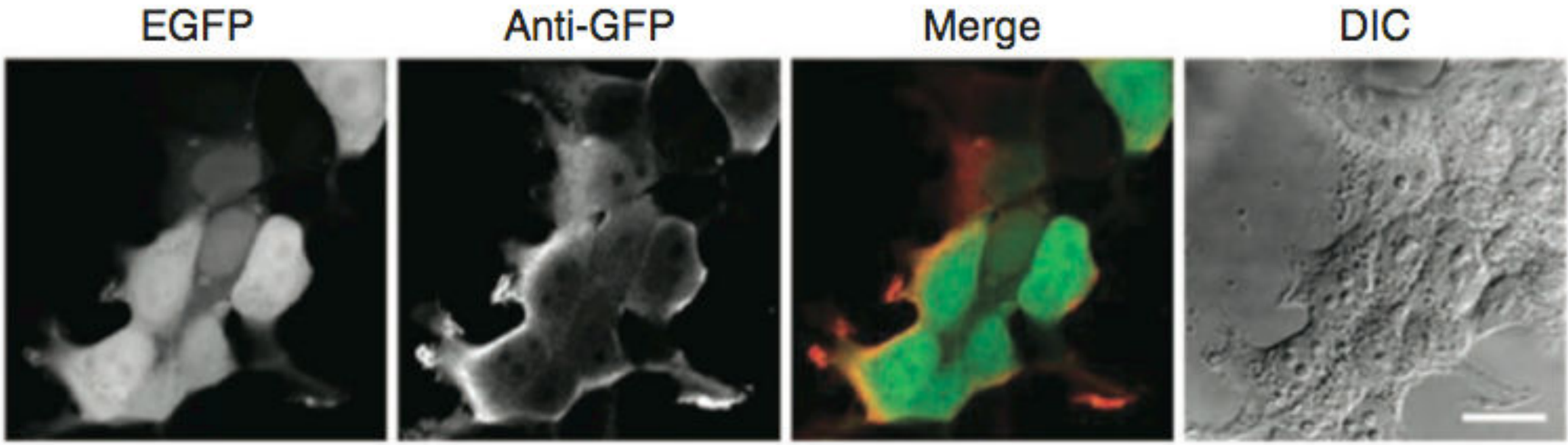
You can believe what you see mostly!

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

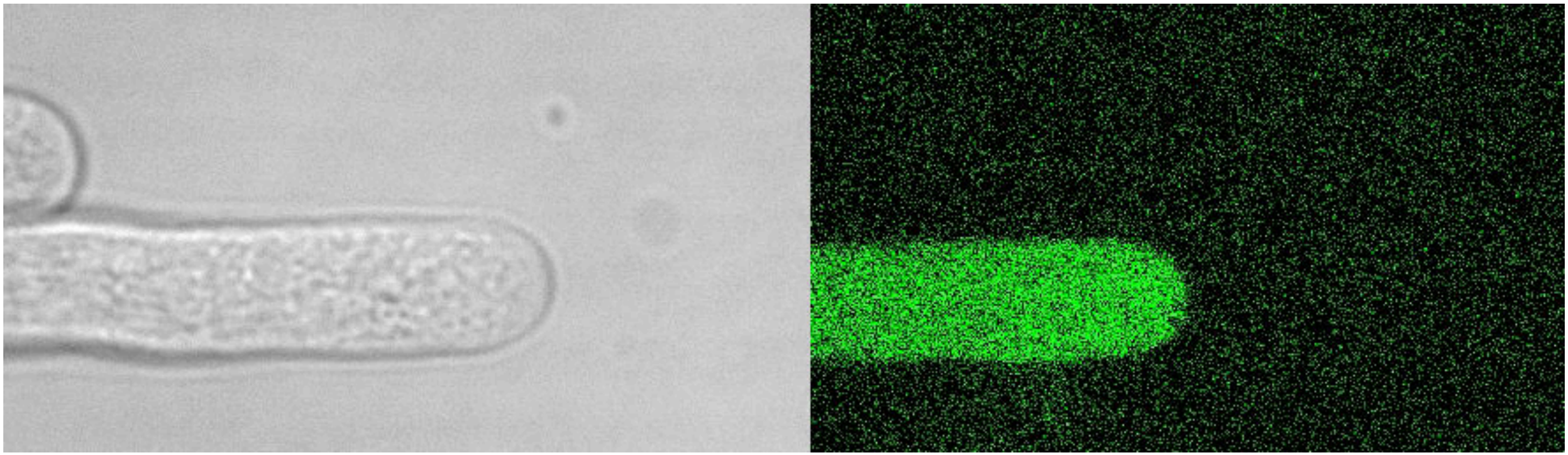
avoids fixation artefacts



But….

You can believe what you see?

Imaged cells might behave abnormally!



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

Death by imaging!

Cautionary note:

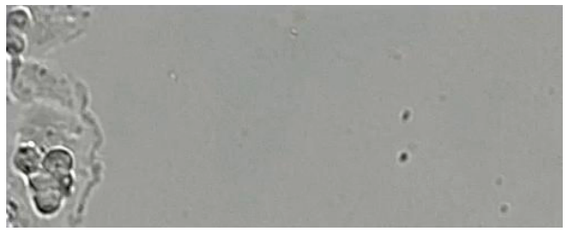
- The microscope stage is no place for a cell!

- “live cell” labels can still be toxic
- mis-expression or aberrant behaviour of GFP tagged proteins
- labelled live cells are more sensitive to imaging (photons)
- stressed live cells are more sensitive to imaging
- mutants are often more sensitive to imaging

But live cell imaging is still brilliant!

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

time-course of cell migration - Andrea Linford Barr lab



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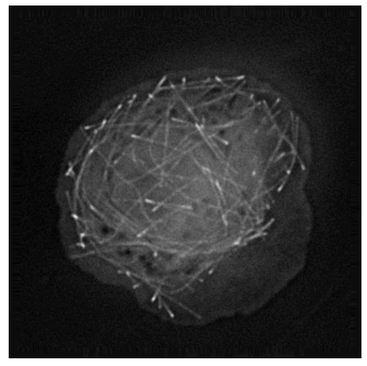

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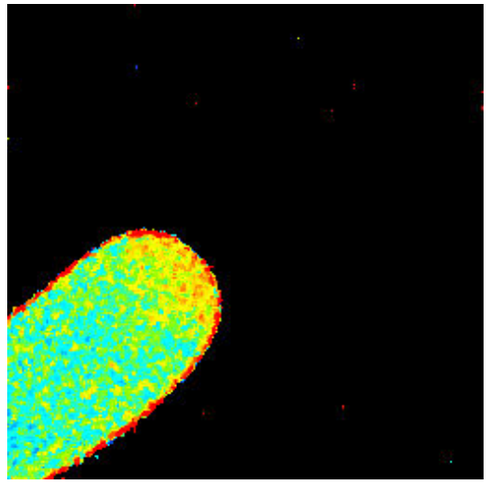

do the appropriate controls!

Do the appropriate controls:

- compare results to know behaviour e.g. expected division rate
- compare with results under less stringent imaging conditions
- compare with results from alternative approaches
- compare with different imaging modalities / labels
- check the WT behaves as expected before looking at mutants

Optimise your imaging!

Requirements for live cell imaging:

Careful balance 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
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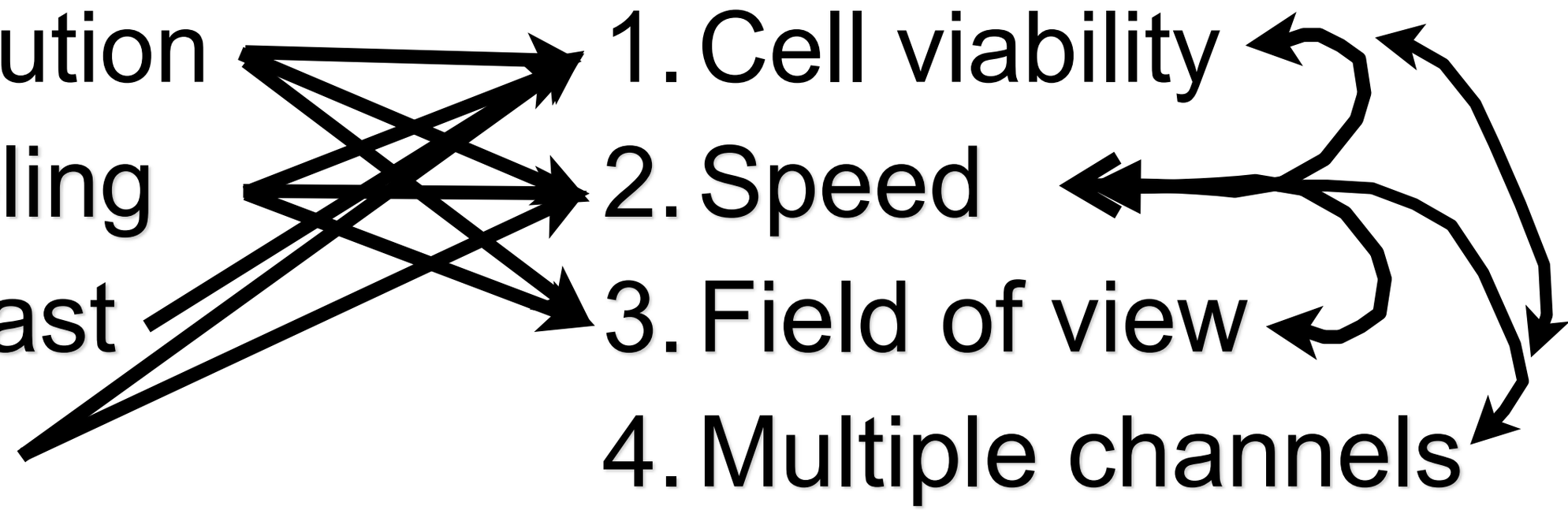
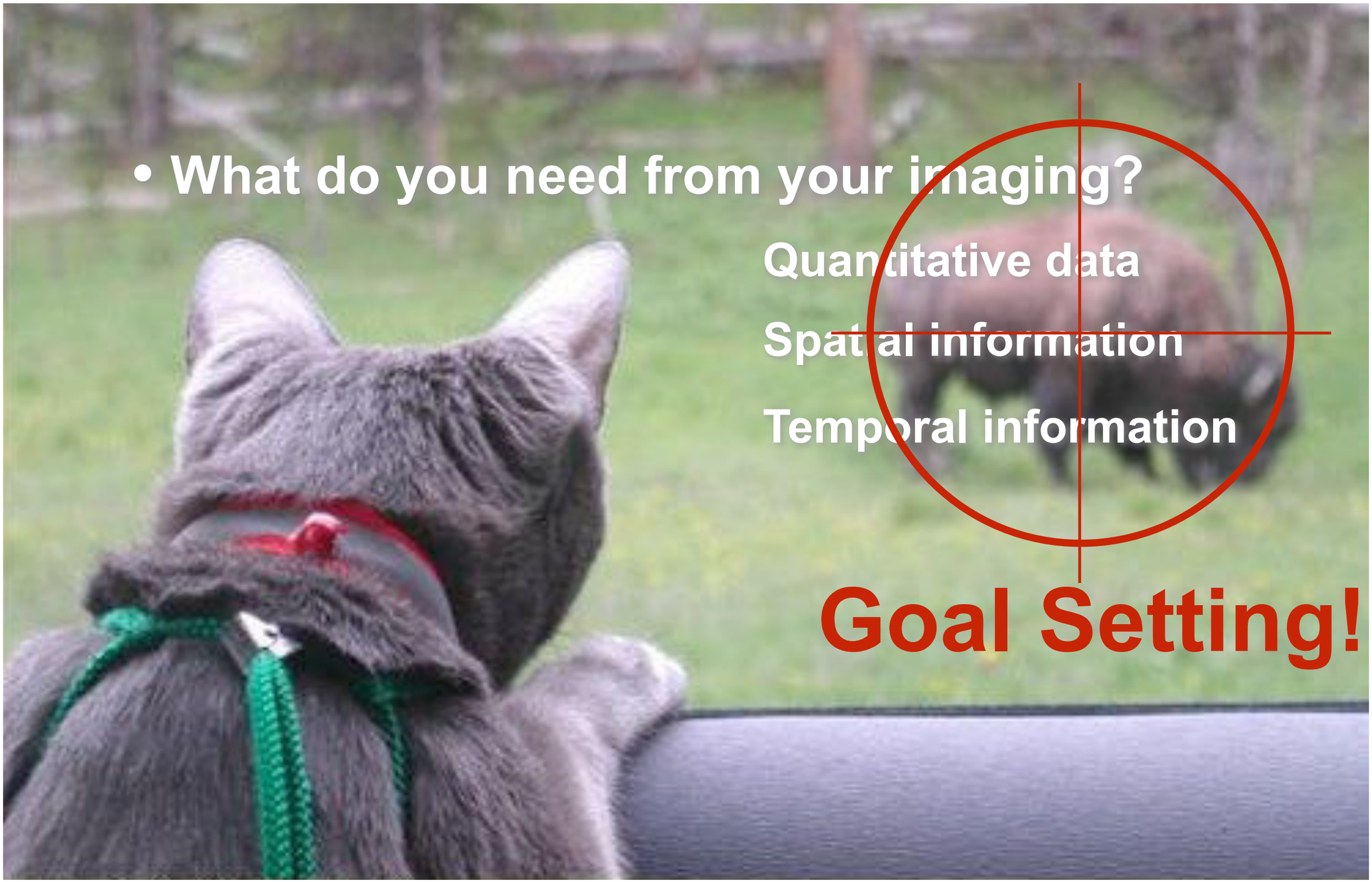


Live-cell imaging is a compromise!

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

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!

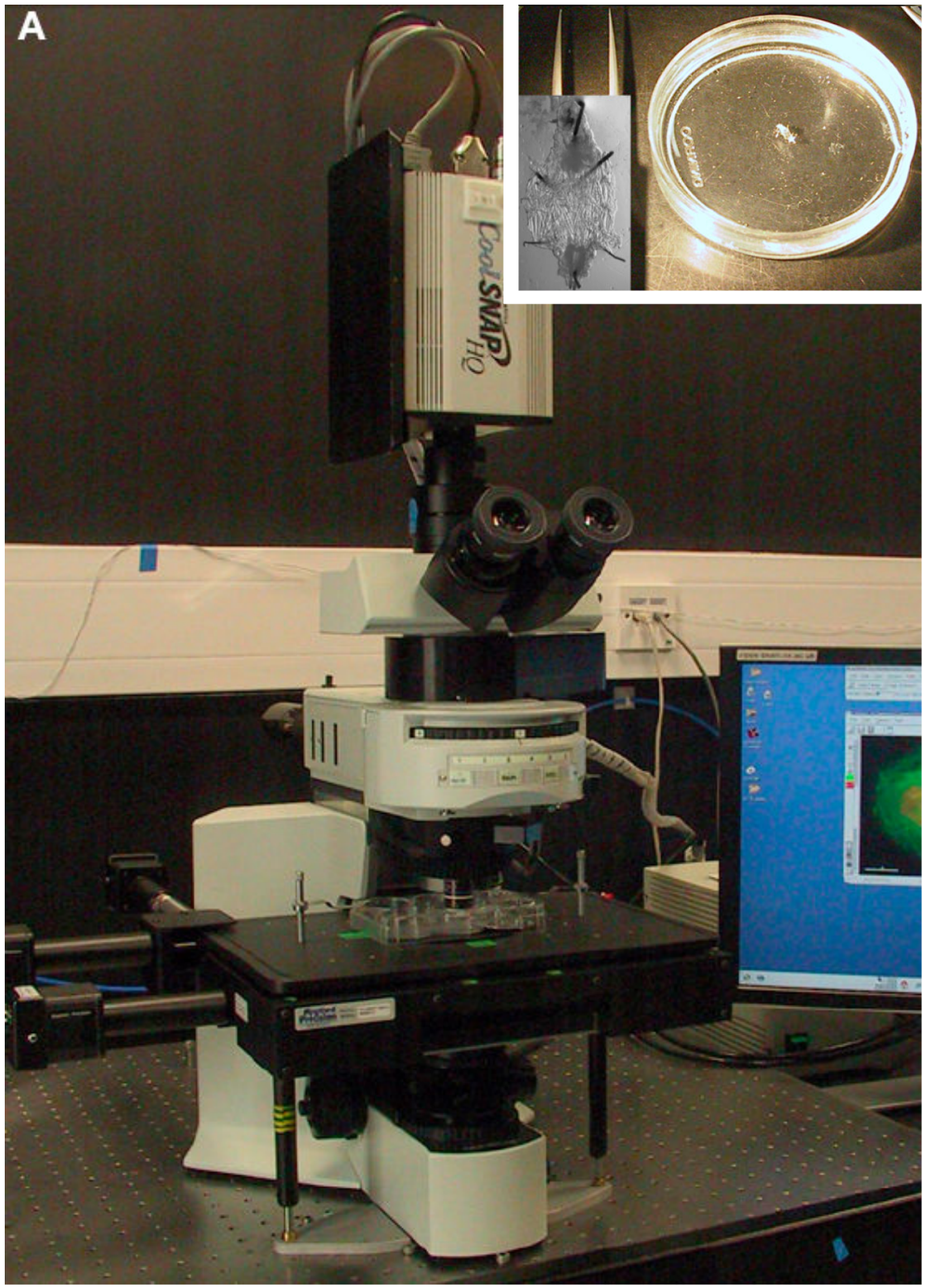
Choice of microscope stand

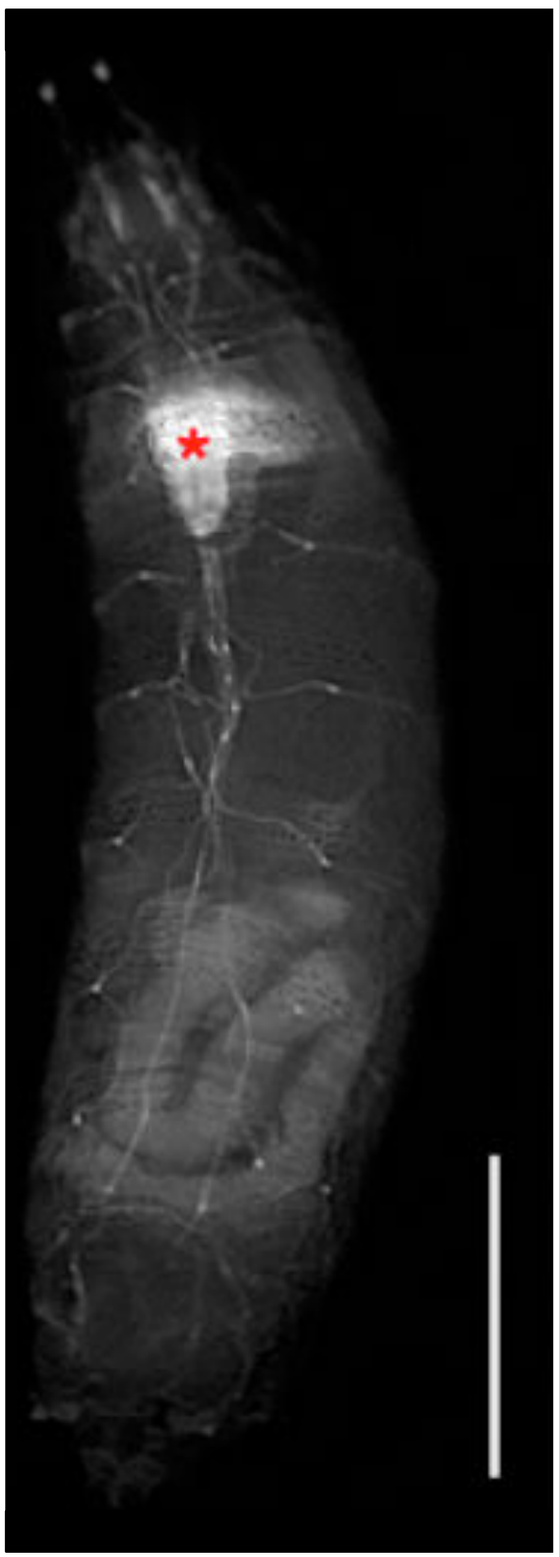
- The modern epifluorescence microscope

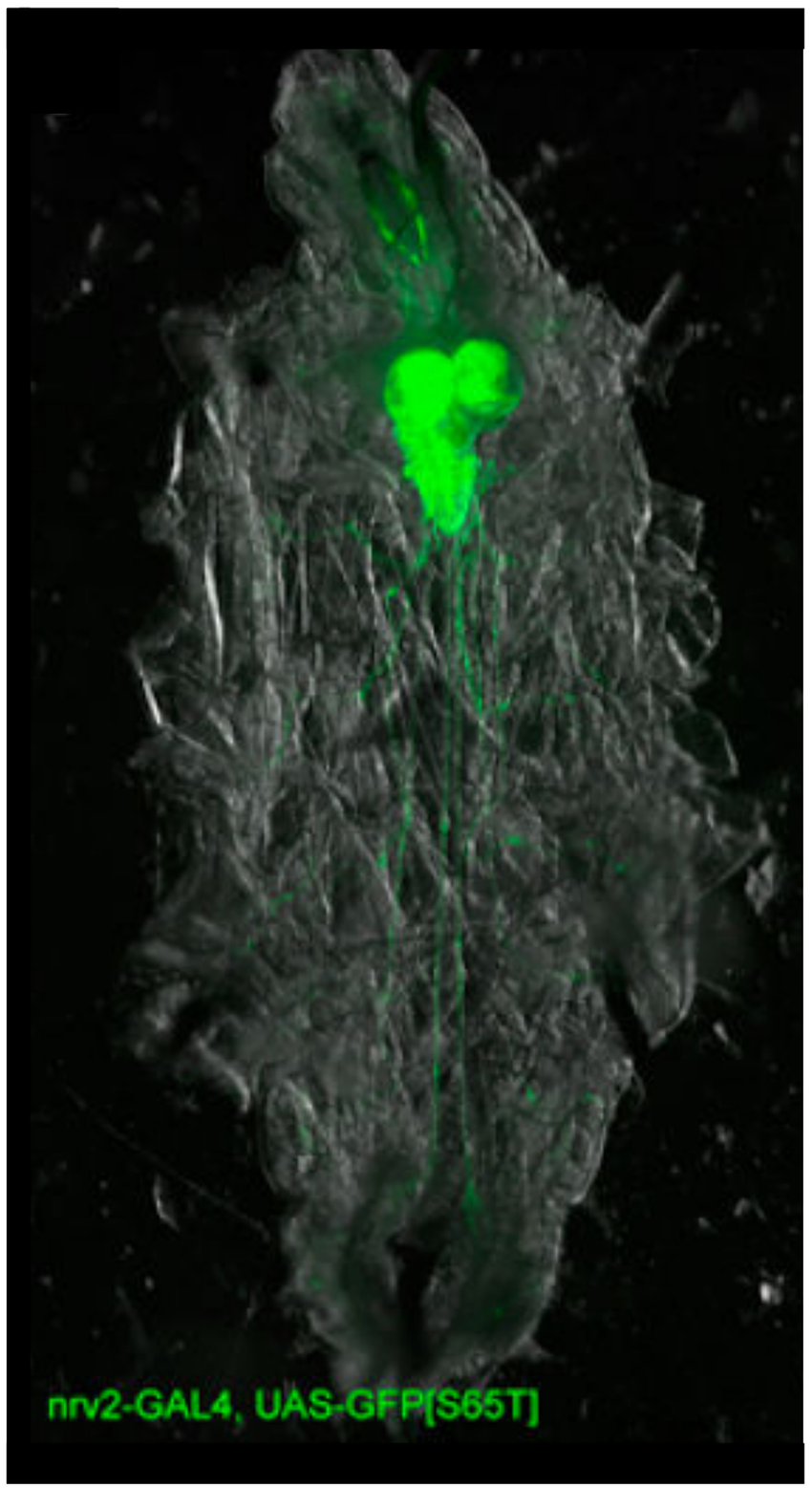
  Upright microscope (lens above specimen)

  Inverted microscope (lens below specimen)

Upright microscope - 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 / 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

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**

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

point scanning confocal

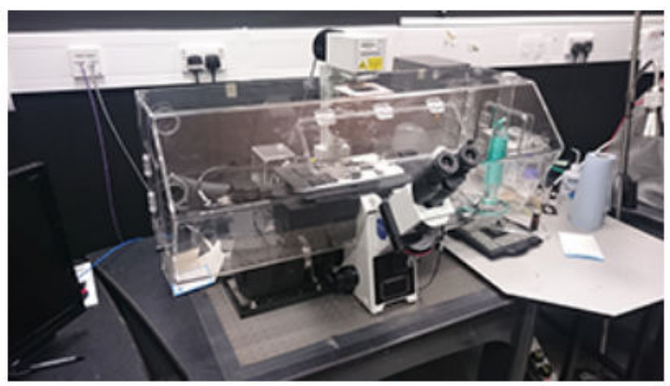
Micron OXFORD

System operational

Live Cell / DNA damage Olympus

Wide Field Decon

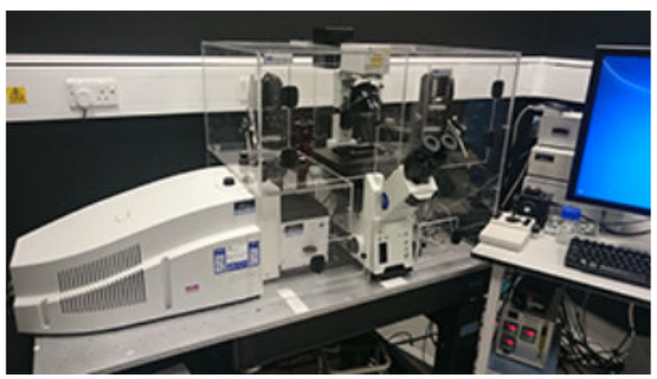
System operational



The DV core microscopes are wide-field deconvolution systems.

Spinning Disc

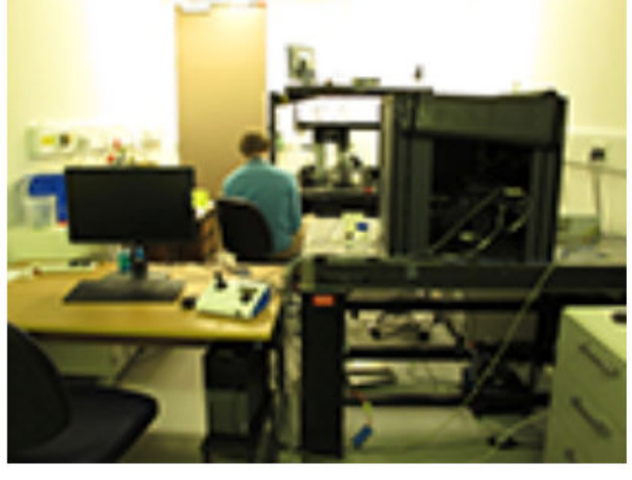
System operational



Nasmyth Perkin-Elmer spinning disk confocal.

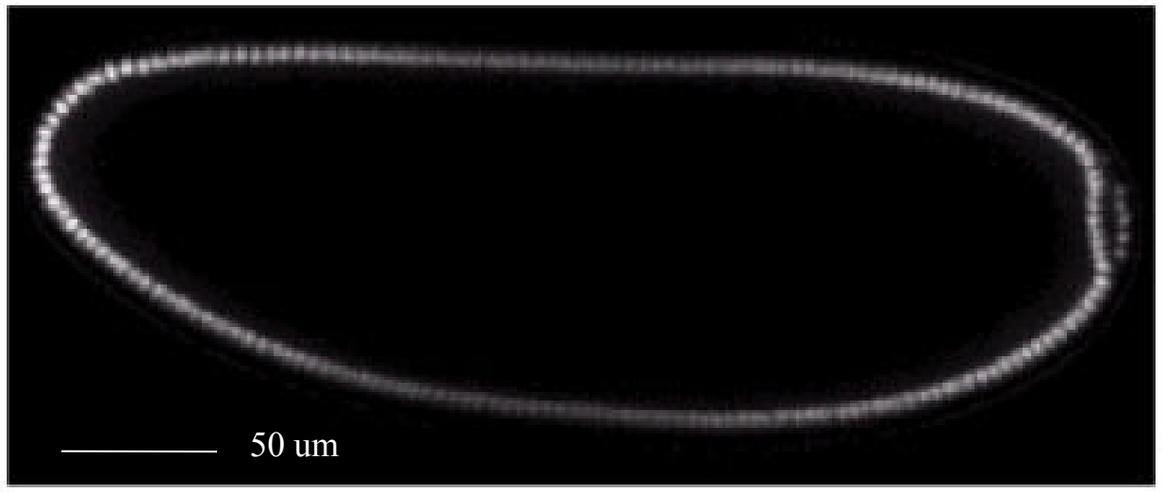
PALM/TIRF

System operational

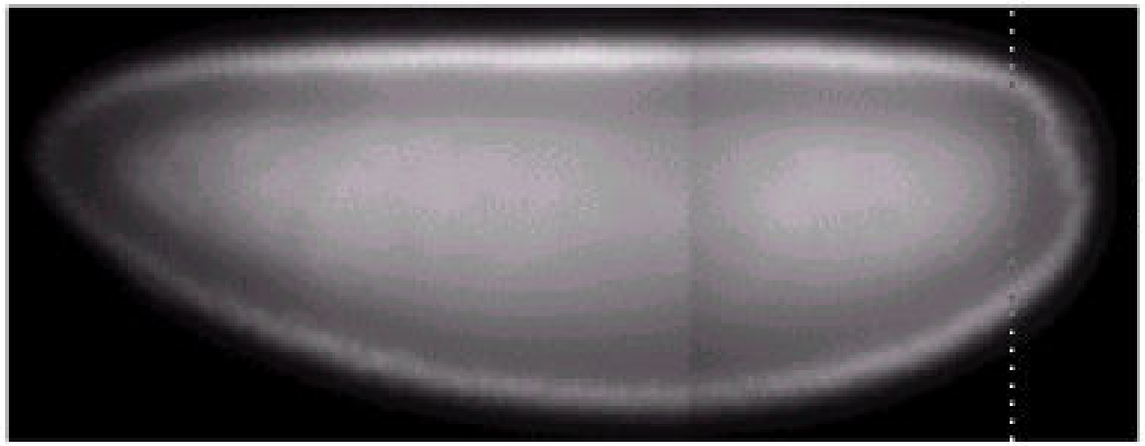


Choice of imaging technique:
Example of a thick specimen

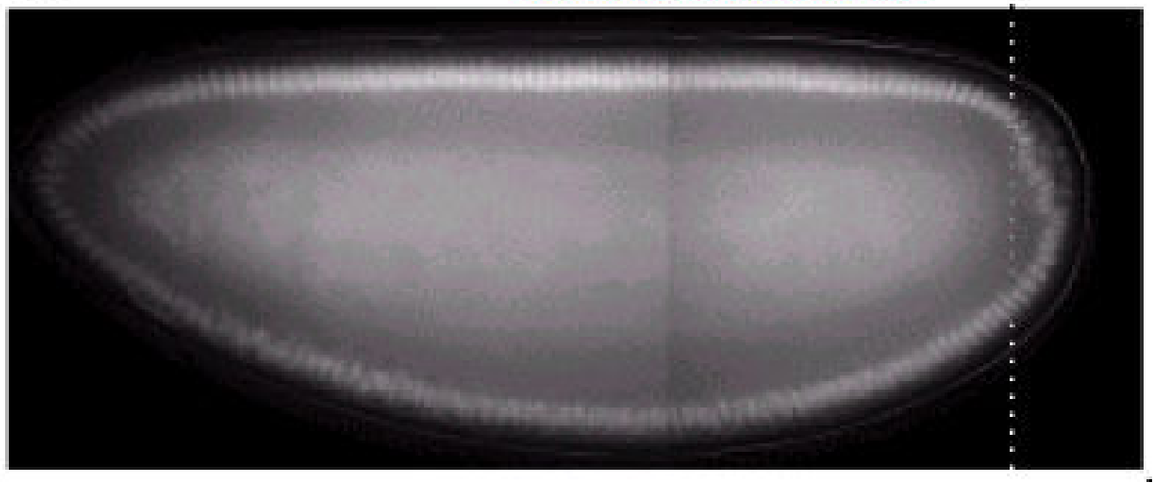
Confocal



Wide-field



WF- deconvolved



Drosophila embryo, nls GFP

Thick, bright specimen

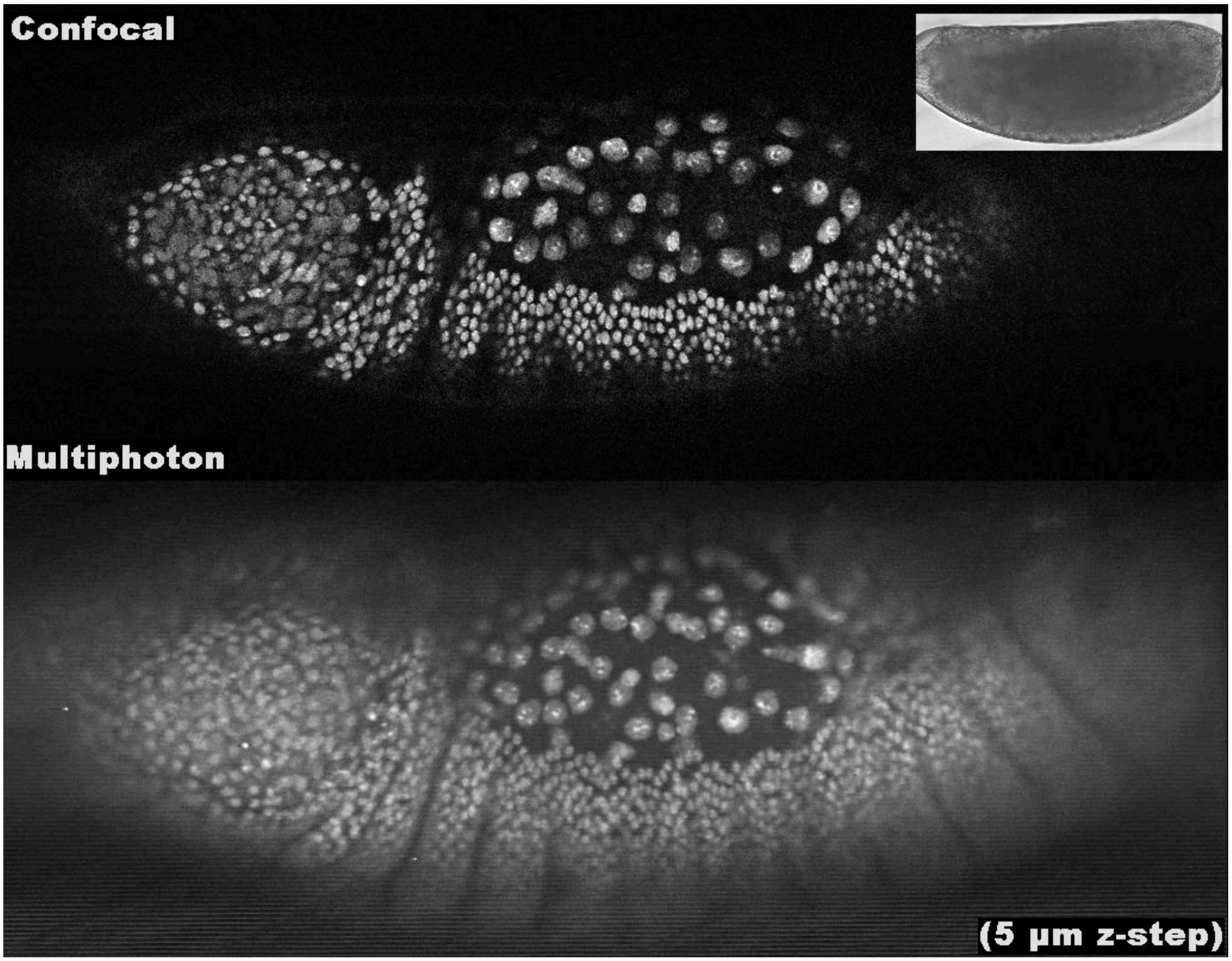
For really thick specimens

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

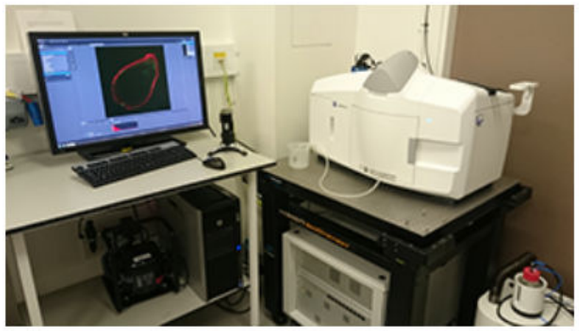
consider….

multiphoton
DLSM/SPIM - light sheet
Adaptive-optics

Multiphoton

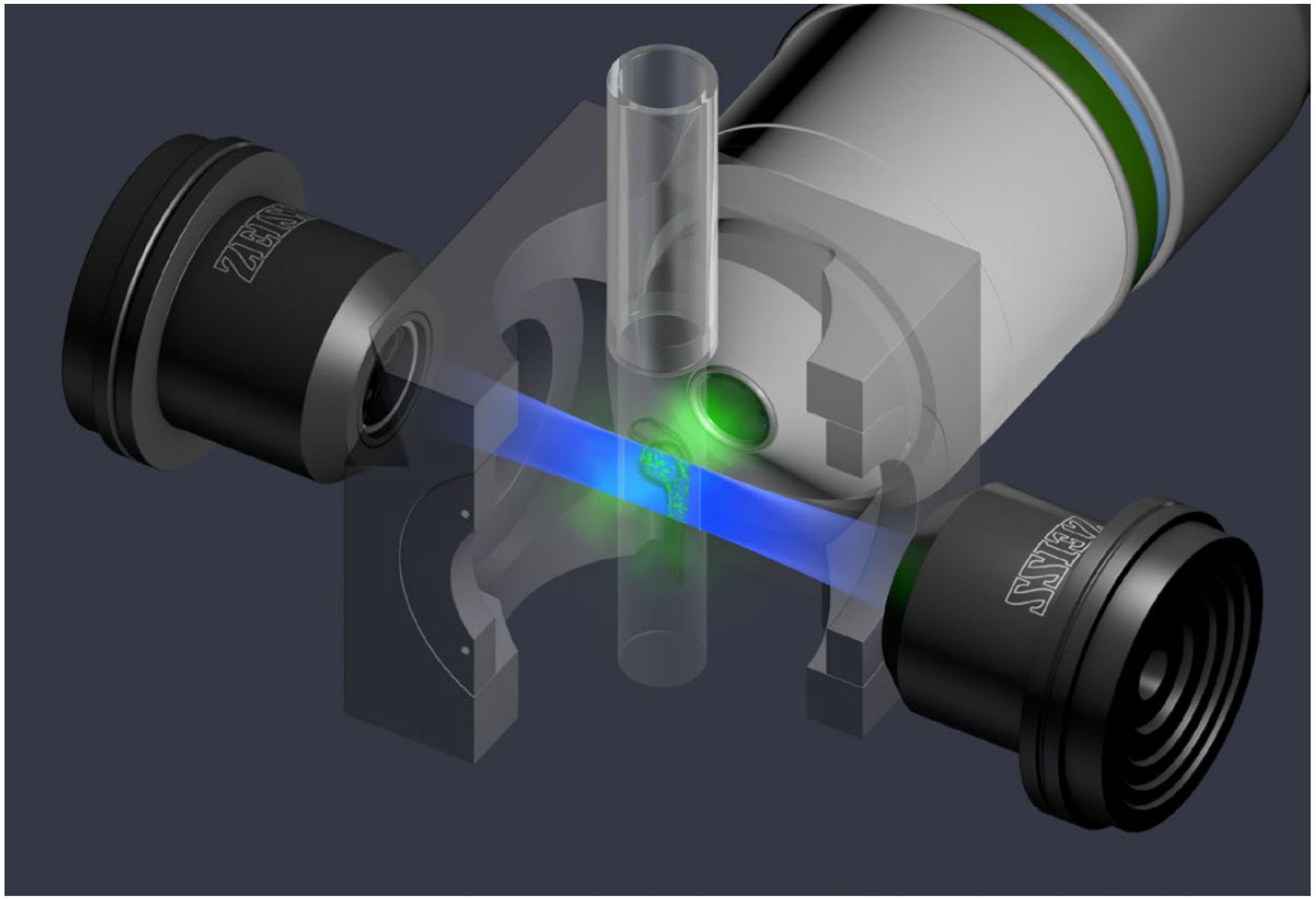


System operational



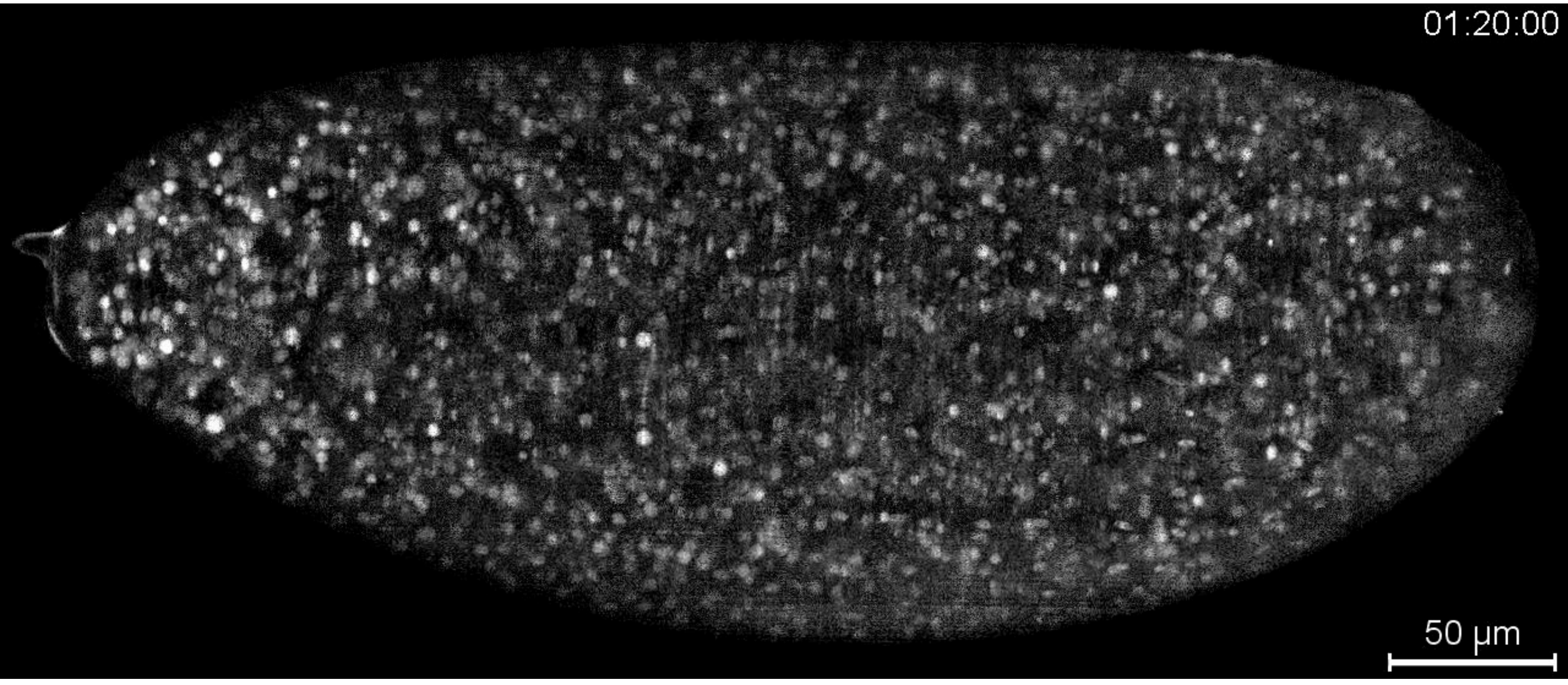
Zeiss Z1 light-sheet microscope

SPIM - Zeiss Z1
Selective Plane Imaging



http://downloads.micron.ox.ac.uk/lectures/micron_course_2014/Lecture_13_light_sheet_microscopy.pdf

Selective Plane Imaging, Light sheet microscope



Raju Tomer, Khaled Khairy, Fernando Amat & Philipp J Keller

simultaneous multiview light-sheet microscopy

https://images.nature.com/original/nature-assets/nmeth/journal/v9/n7/extref/nmeth.2062-sv1.mov doi:10.1038/nmeth.2062

If the choice is not obvious...

it’s worth trying different ones….

For live cell imaging catch every photon:

Be economical with your light budget - Hardware

- Optimised filter sets for your probes

  hard coated “ET” filter sets

  filter free “spectral” options

- Choose the best lens for the job

  Oil immersion
  water immersion

  RI matching immersion

- Optimised synchronisation of illumination, exposure and readout

  “real time” system controllers
  fast shuttering
  diode light sources

- Sensitive detectors

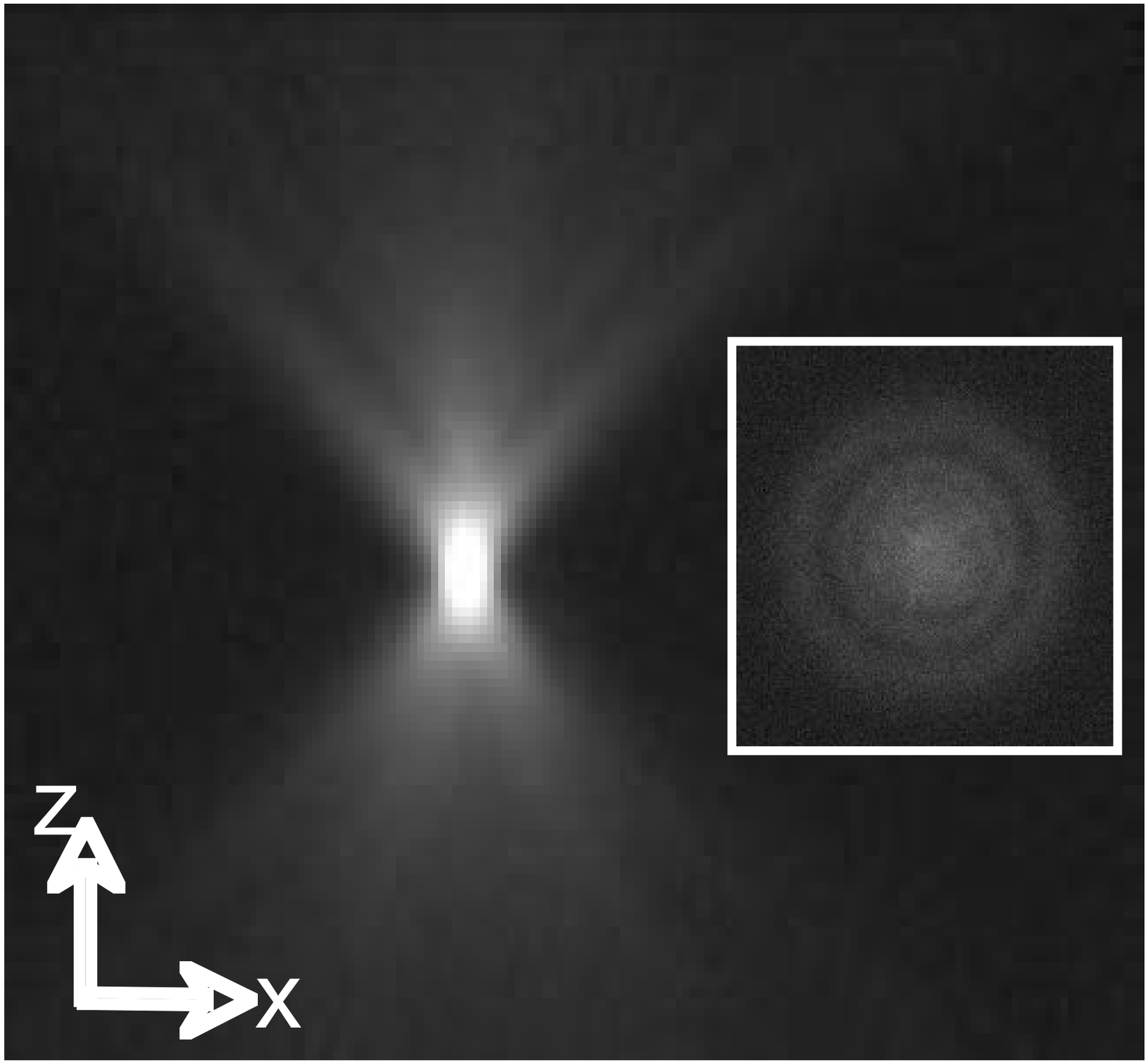
  Deep Cooled CCD’s
  EMCCD’s
  back-thinned sCMOS

  LECTURE M3

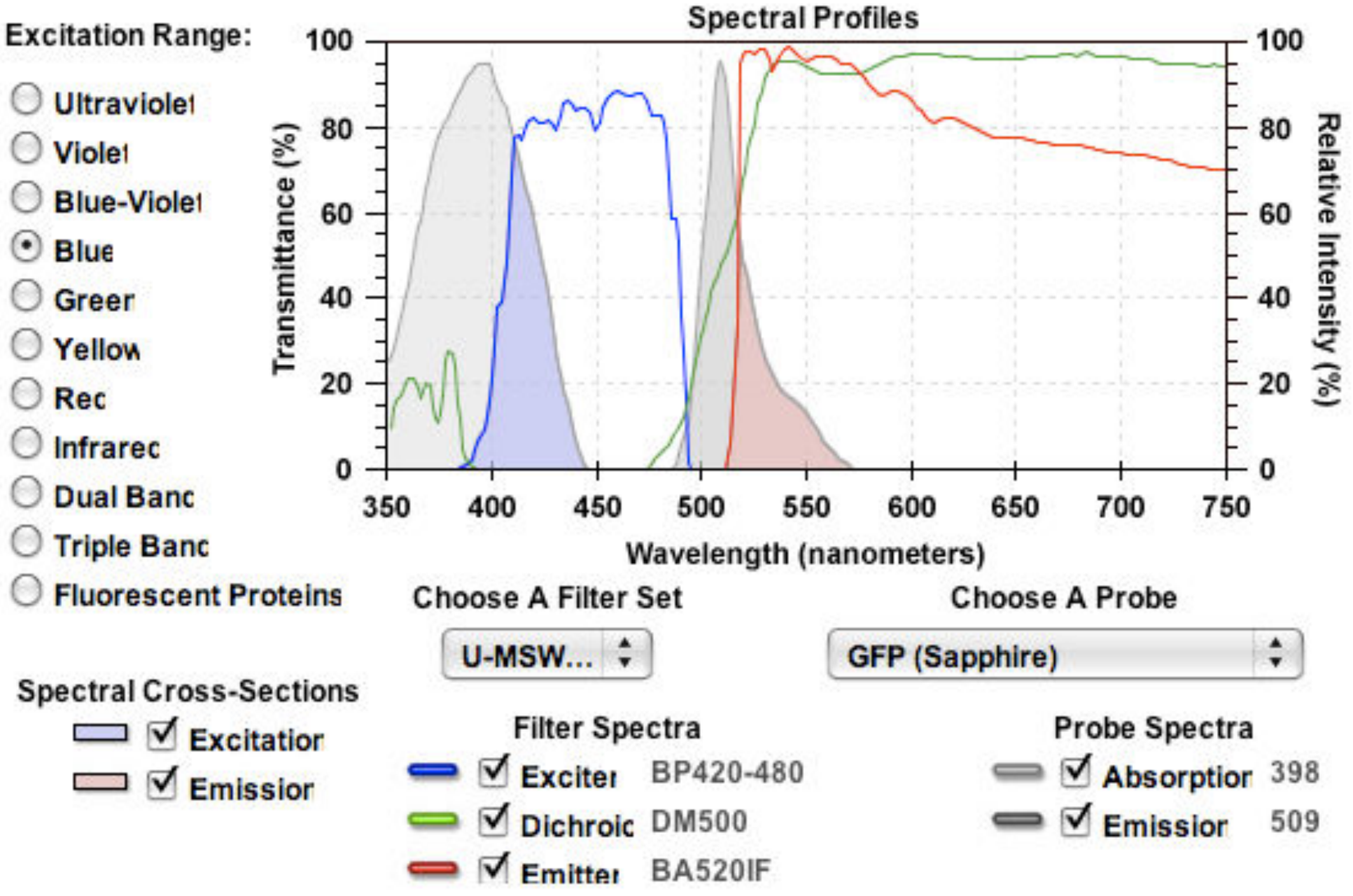
- Set up your equipment properly

  alignment
  aberration correction

Setup your imaging equipment properly:



Matching Fluorescent Probes to Filter-Sets



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

Lens choice: 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

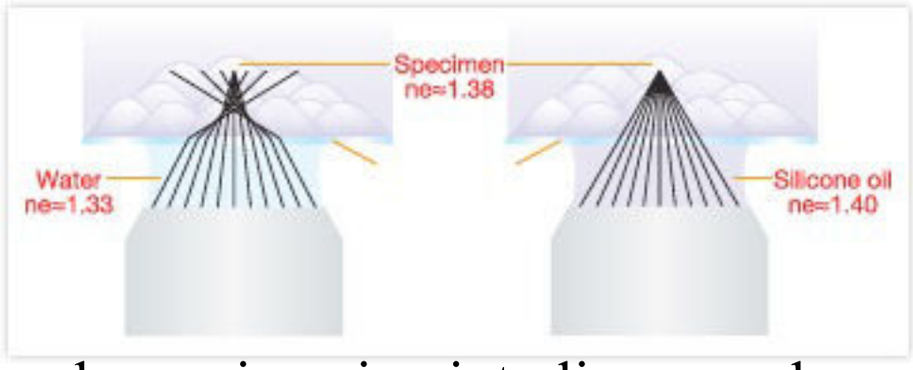
  (apo chromatic)

Lenses:

- Specialist Objectives - Water/glycerol immersion objectives

- silicone immersion objectives

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

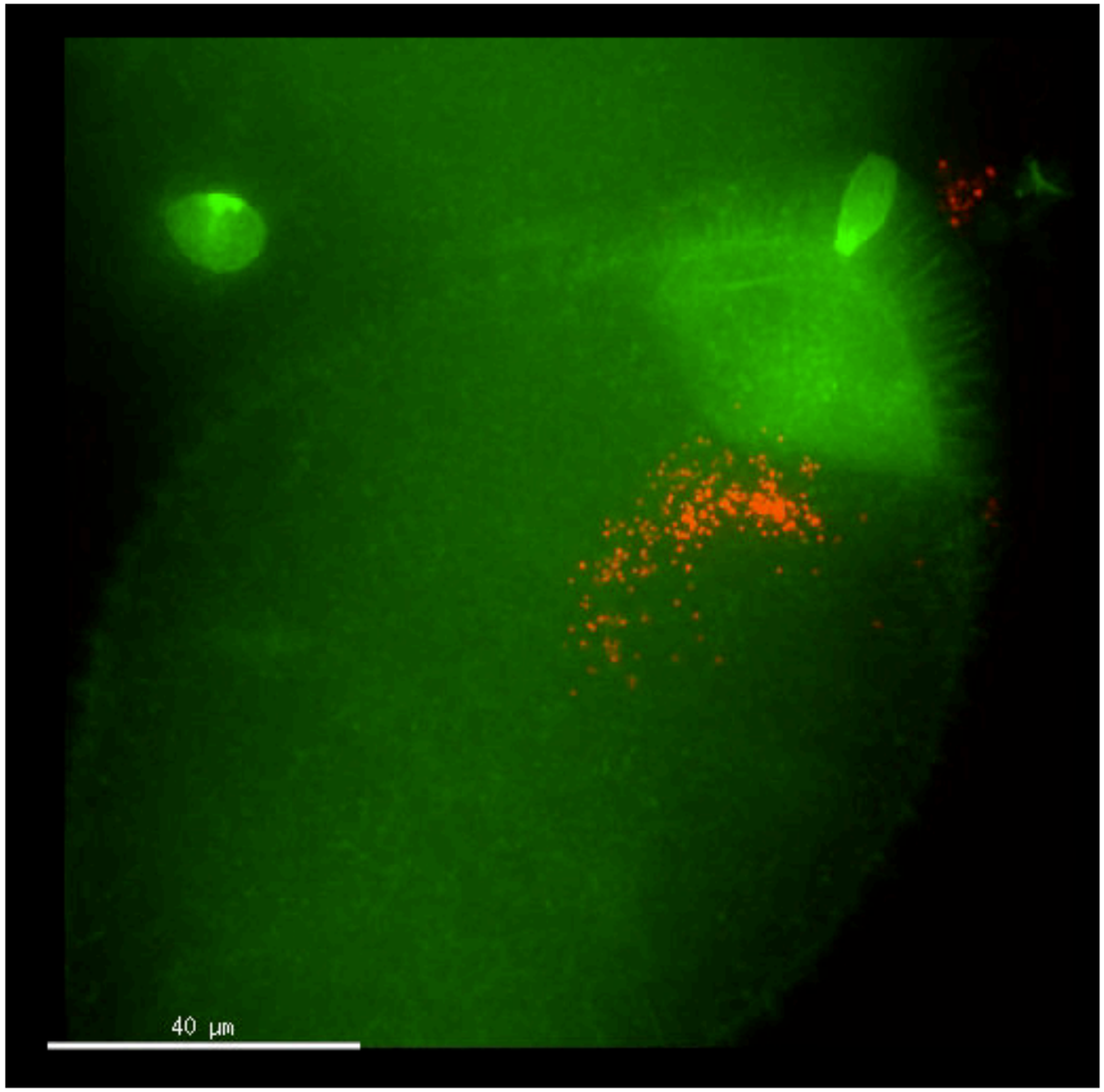


deeper imaging into live samples

Very Expensive!!

Correcting Spherical Aberration:

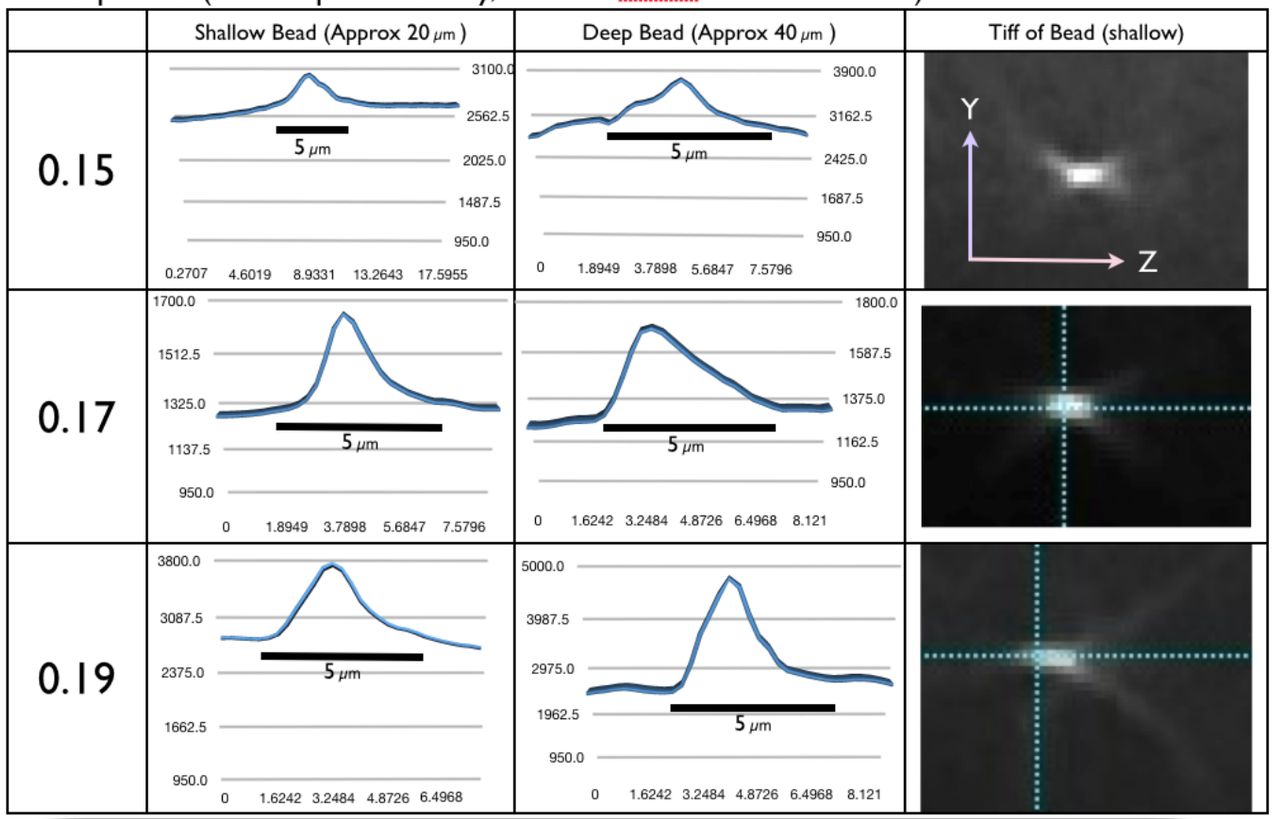
- Evaluate of spherical aberration with depth
- Explore corrective collar settings
- Automate correction



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)



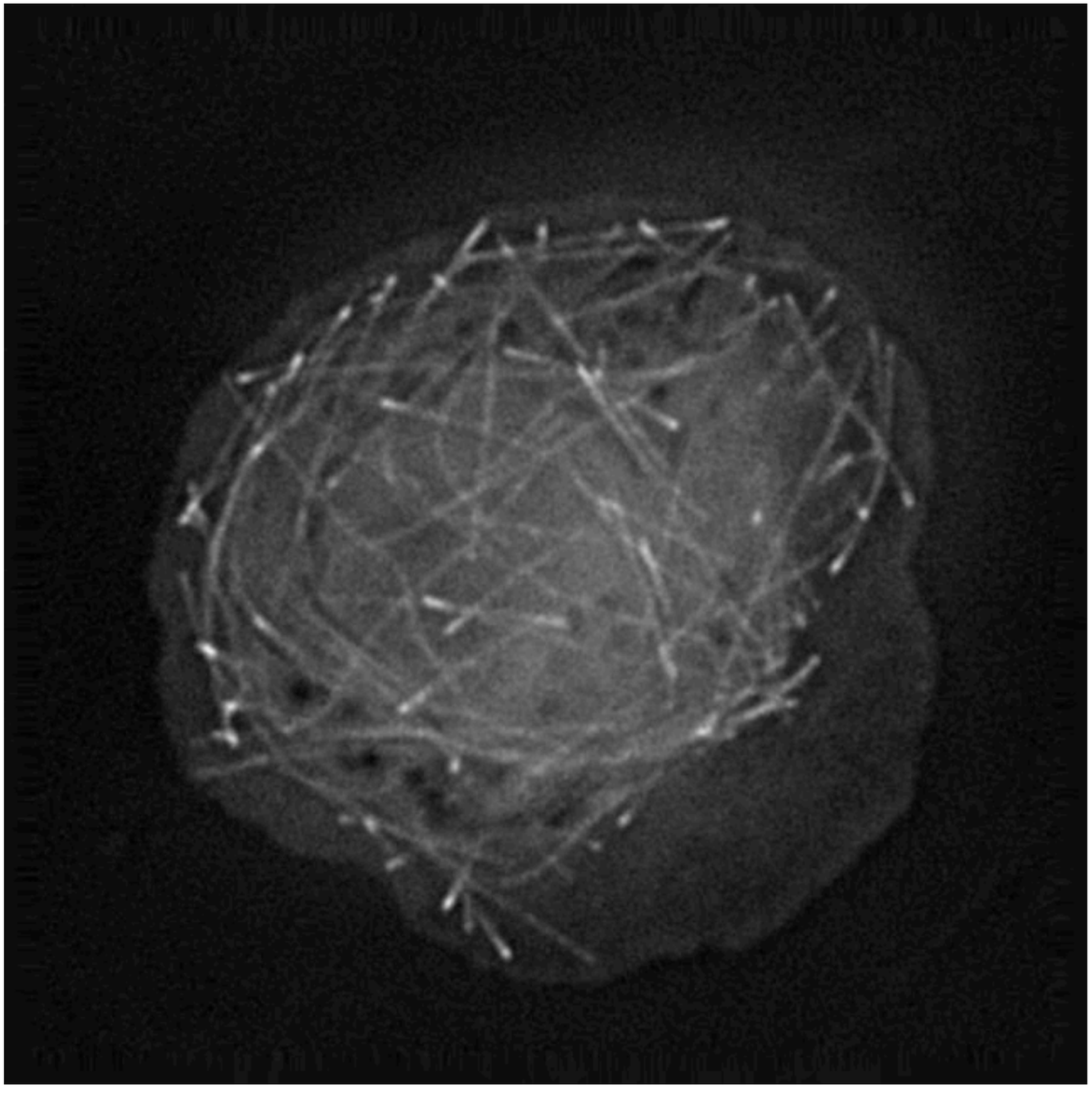
Jonathan Sturt, RMP: x60 SI lens - manual collar correction

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
- Choose good labels
- Careful specimen preparation
- Make use of denoising algorithms

Choose your dyes / labels carefully!

Lectures 4 / 5



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

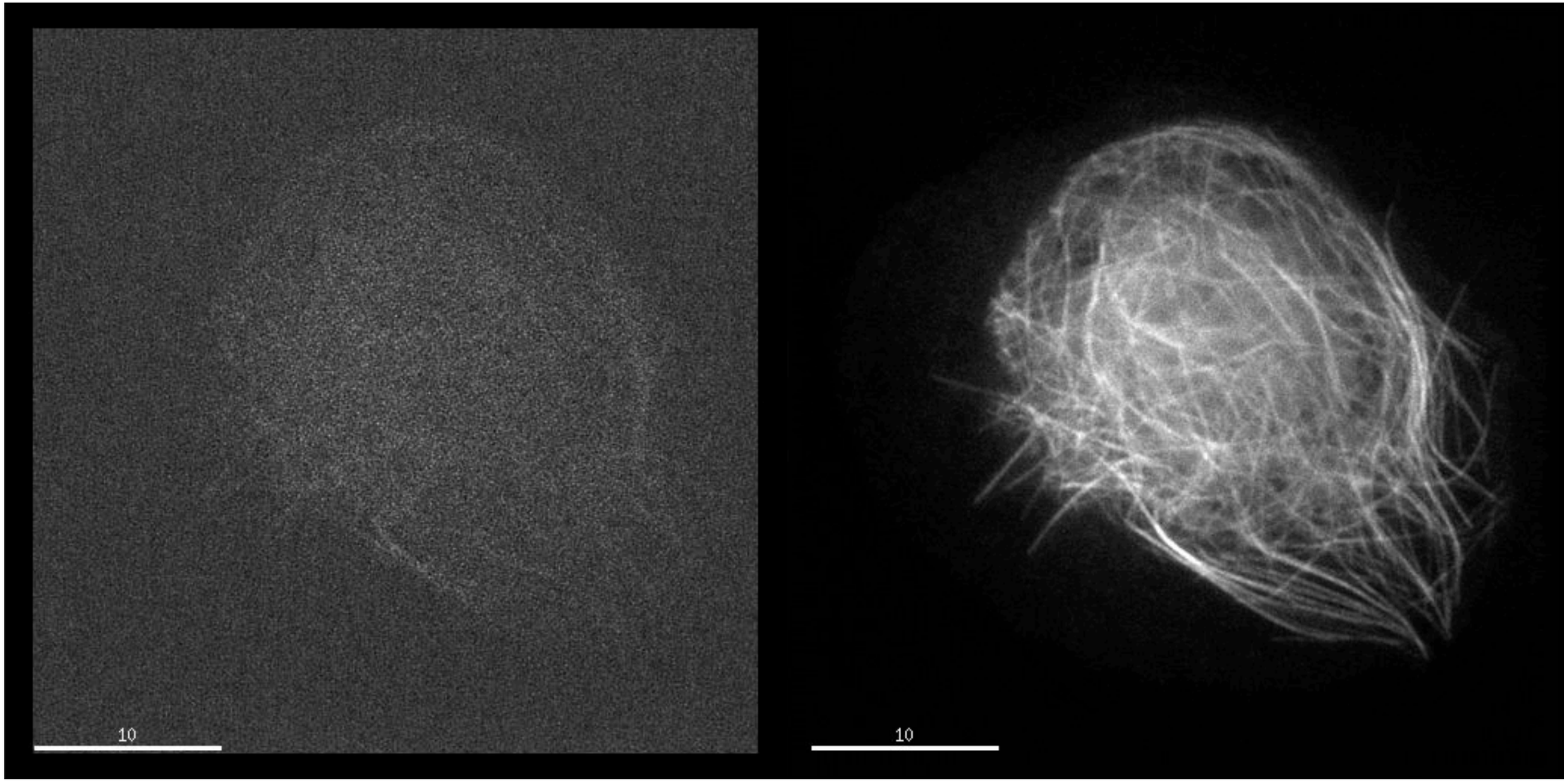
Using Image Processing:

"I want you to make me the fairest of them all."

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

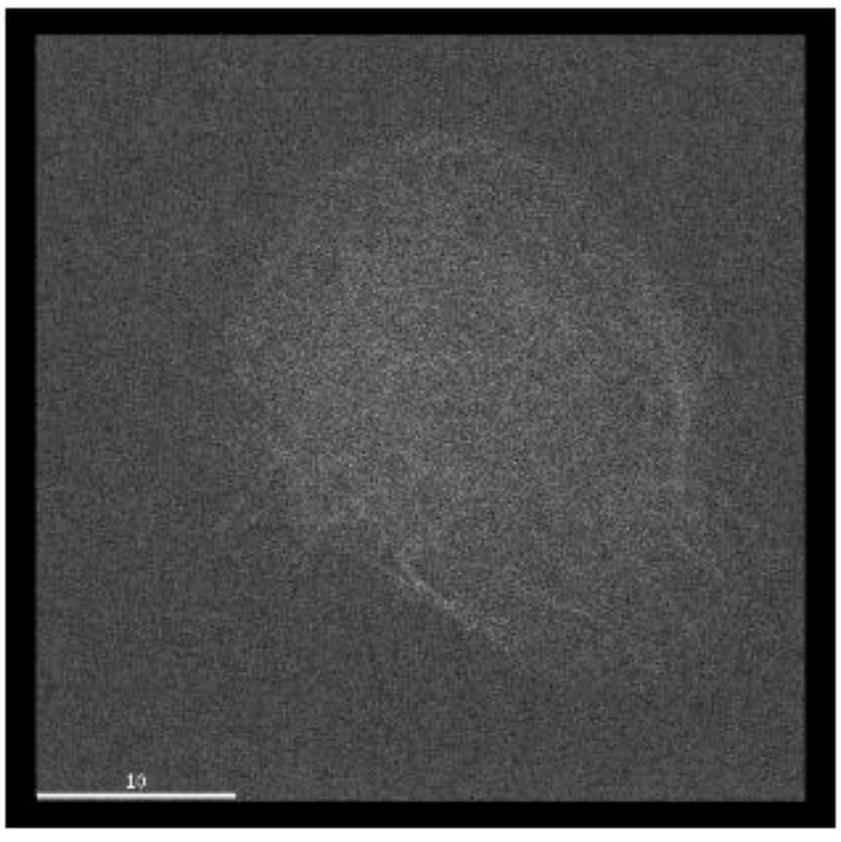
Jerome Boulanger: SAFIR Denoising software

Integrated into Priism by the John Sedat Group UCSF

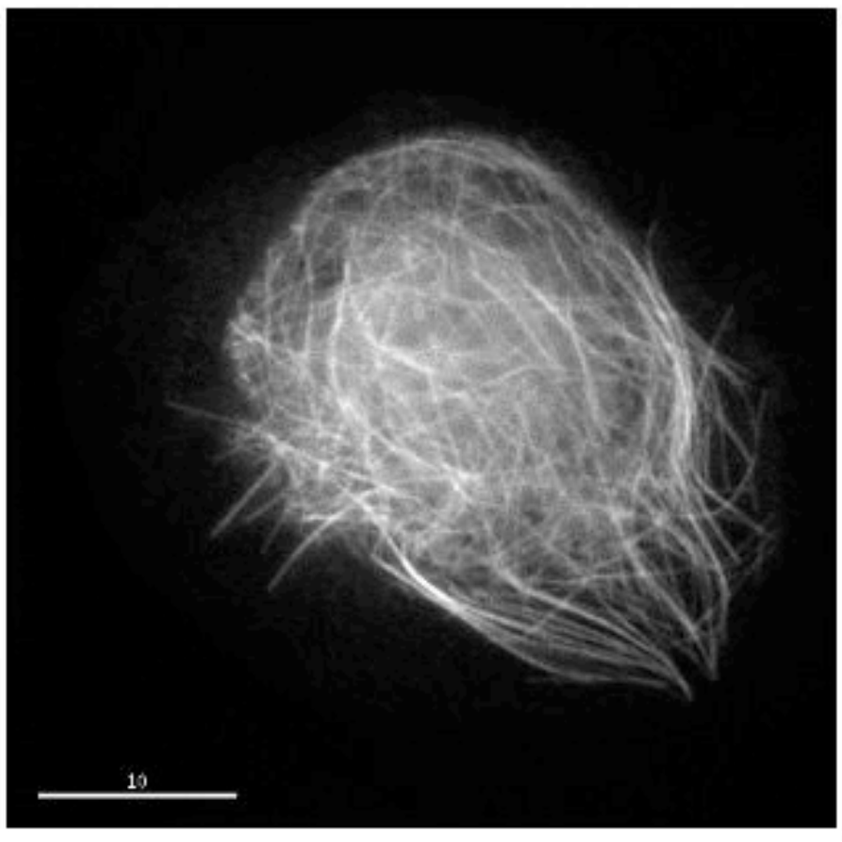
J. Boulanger, C. Kervrann, and P. Bouthemy, “Space-time adaptation for patch-based image sequence restoration,” *IEEE Trans. on Pattern Analysis and Machine Intelligence*, vol. 29, no. 6, pp. 1096ñ1102, June 2007

Denoising - imaging with 10-100 x less light!

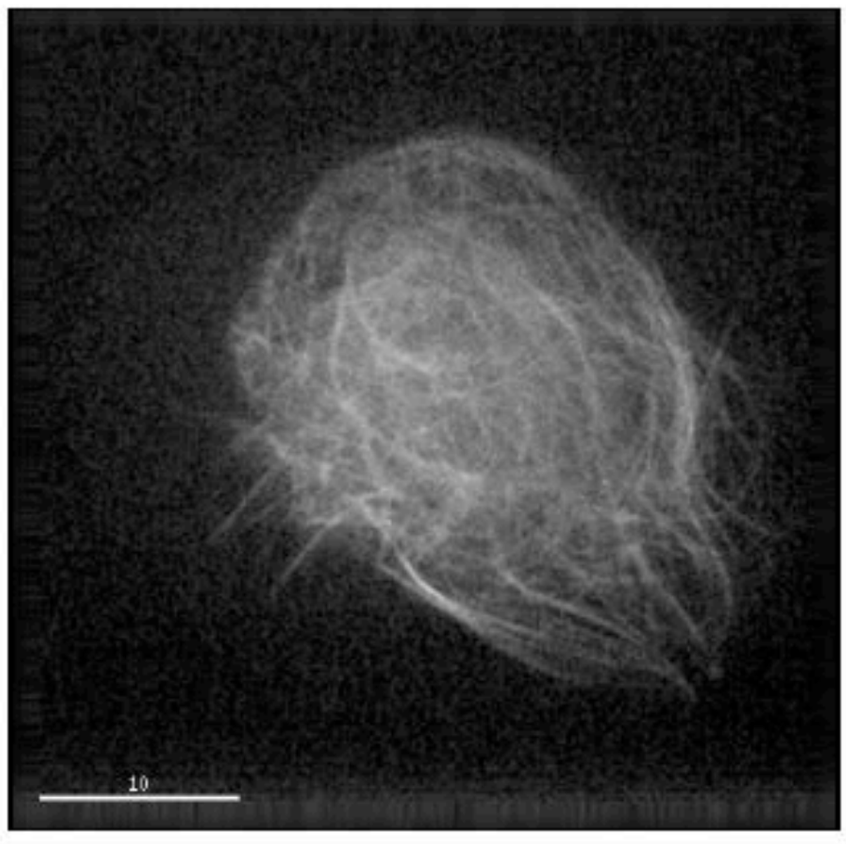
8 ms ex, 0.1% 488 laser



8 ms ex, 10% 488 laser



8 ms ex, 0.1% 488 laser - Denoised



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

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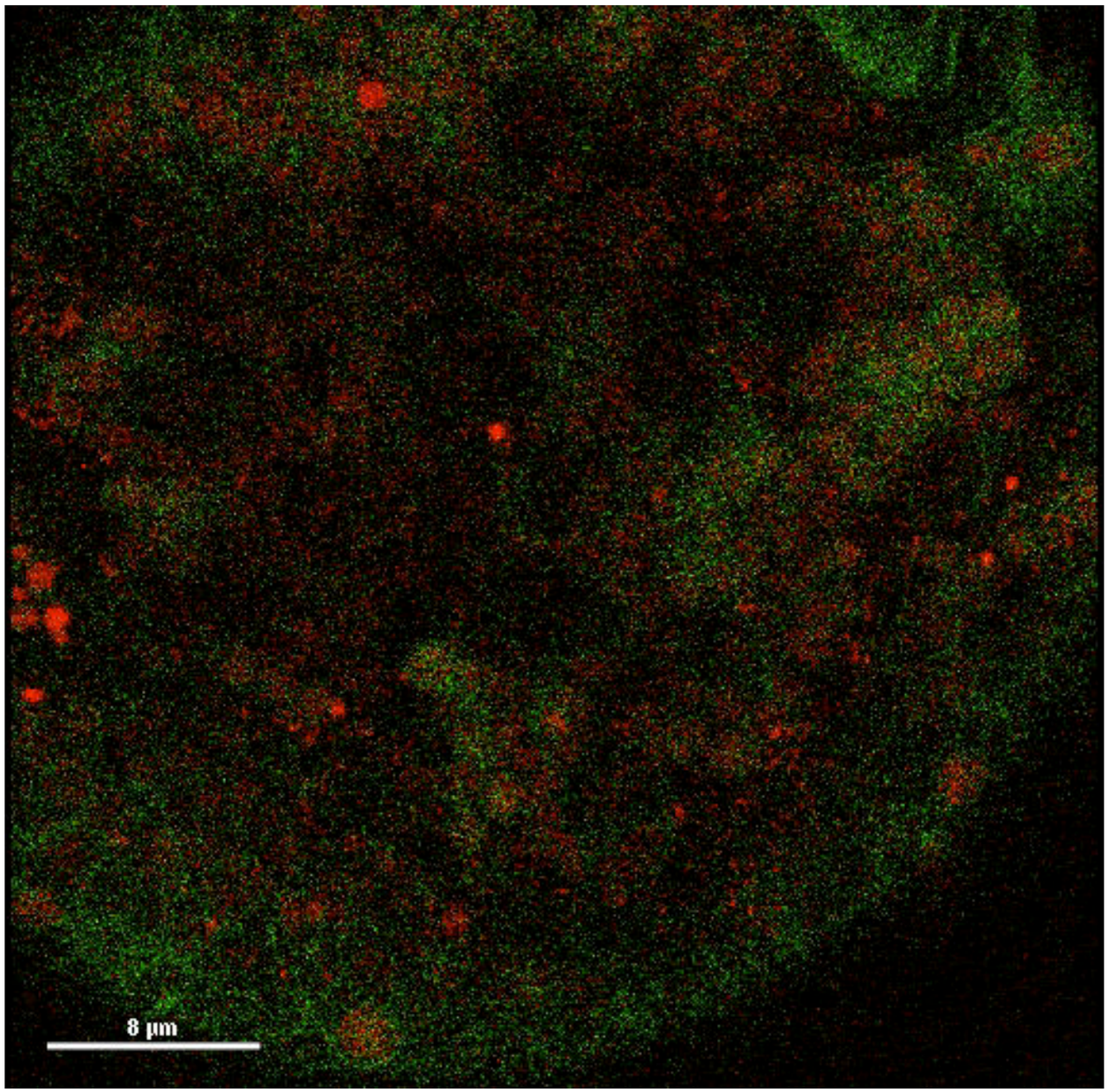
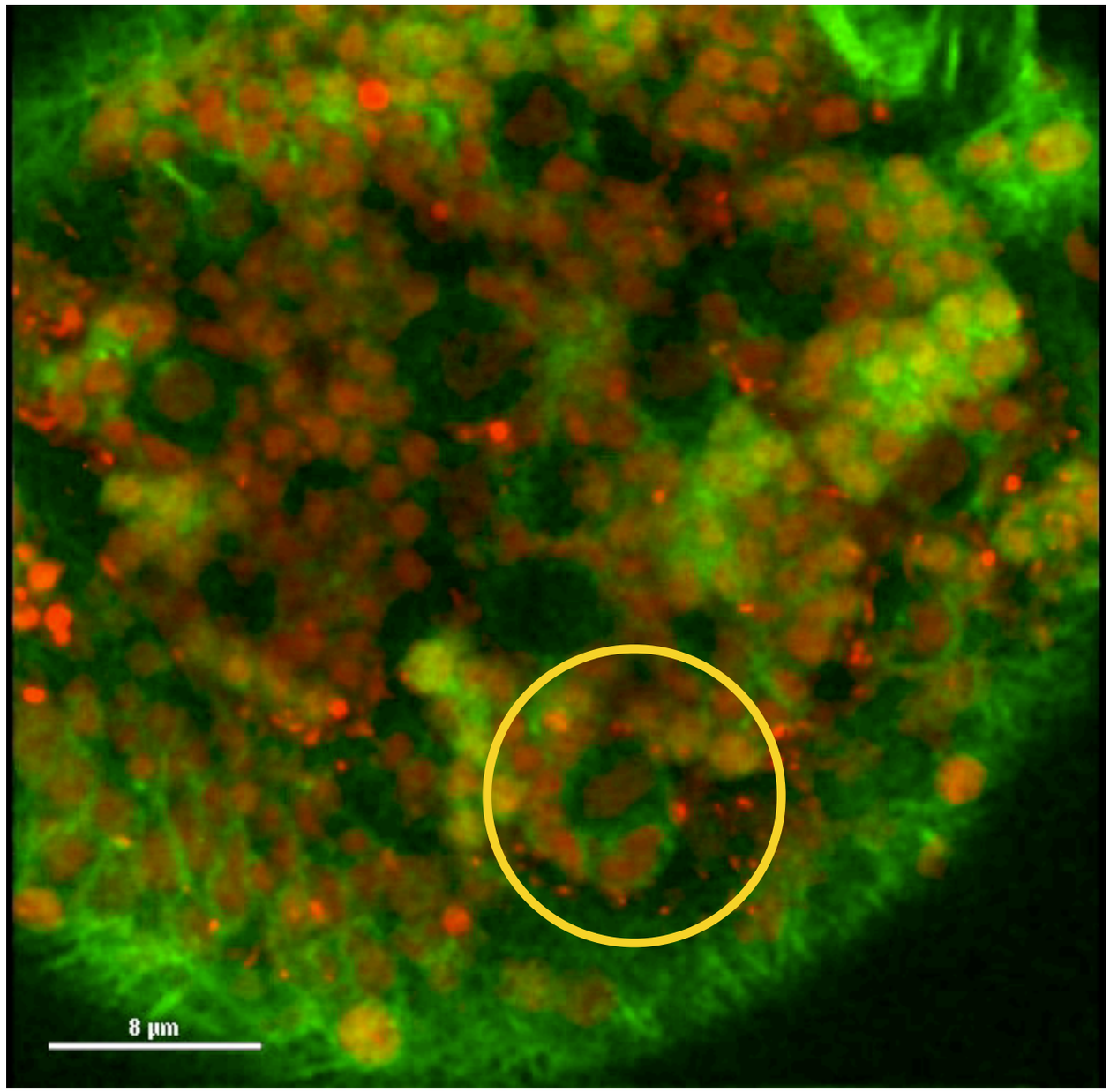
Denoising - imaging with 10-100 x less light!

Raw: low laser, no averaging, 60Z, 12 hr (43,200 images)



Patch-Based Denoising



Live *ex vivo Drosophlia* larval brain: Jupiter-GFP labeling microtubules; HisRFP nuclei (M Hailstone, RM Parton)

SUMMARY:

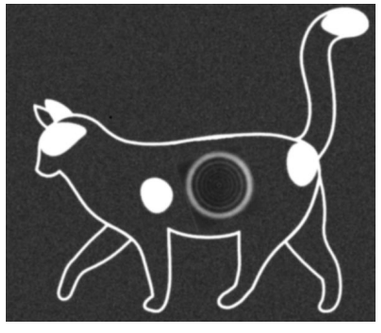
Optimise your experimental design

Select the right approach

Catch every photon

Set up your equipment properly

Correct Aberrations



END

(Dog-lover friendly format available upon request)