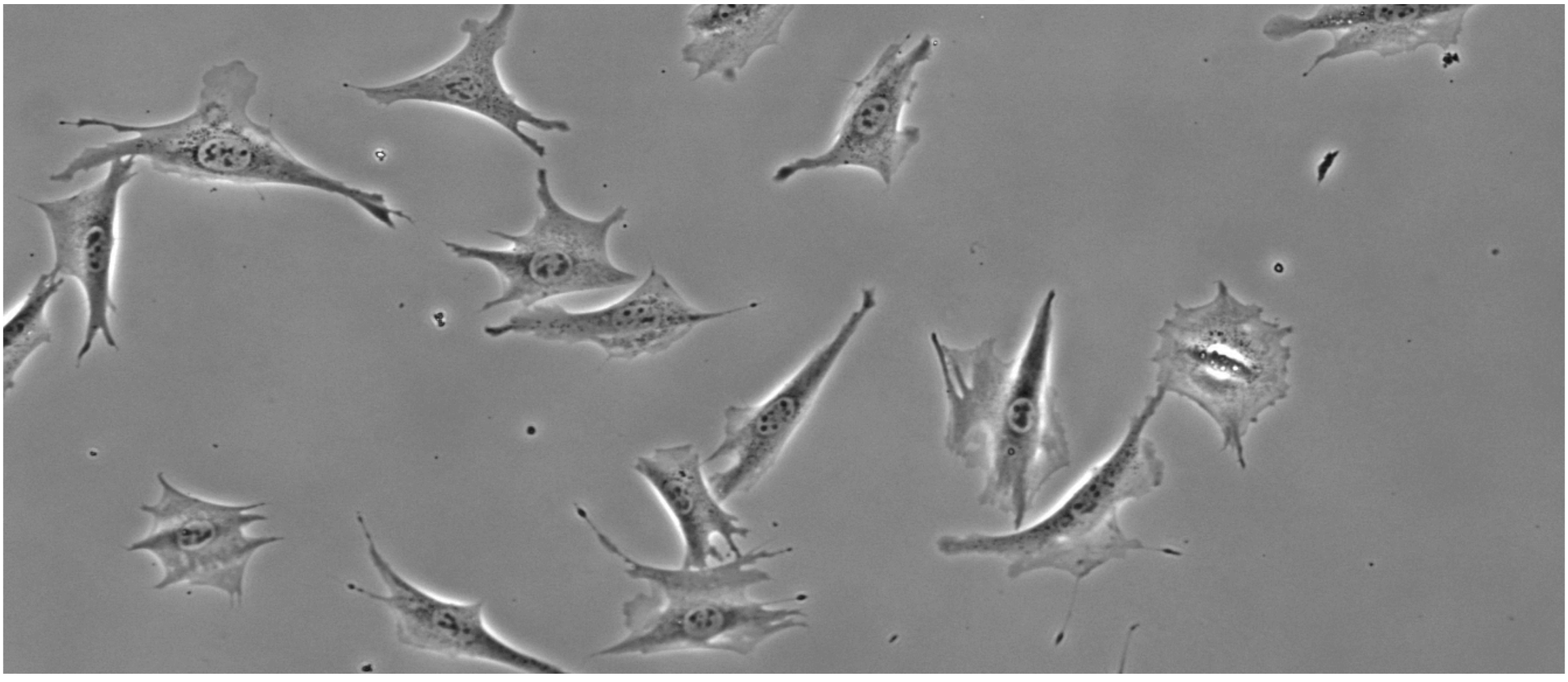
Live-cell Imaging: Liven up your data!



Nadia Halidi

Imaging Facility Manager

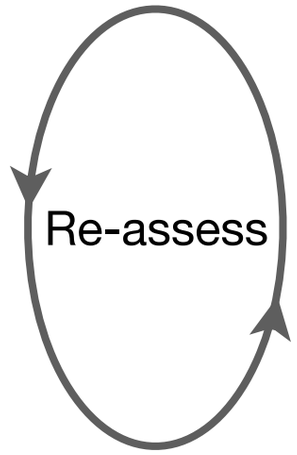
@ Micron Advanced Bioimaging Unit

Designing a microscopy experiment

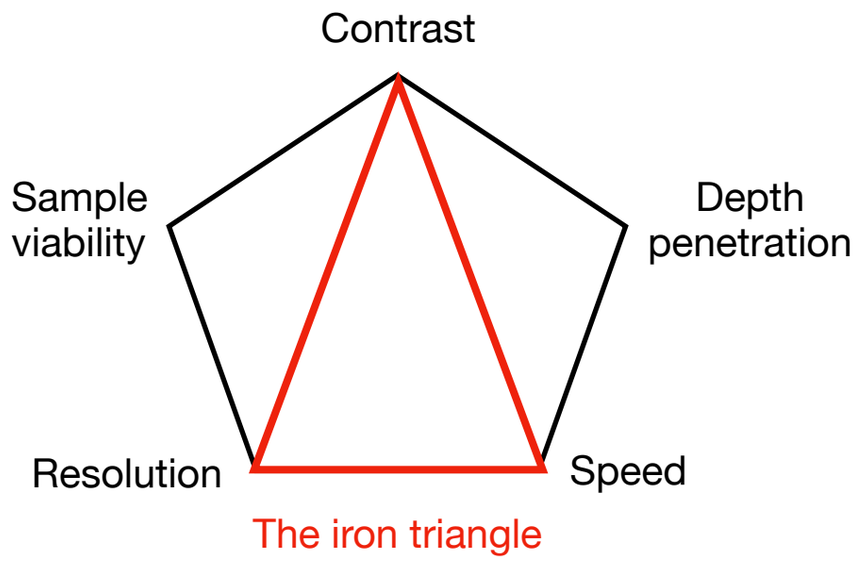
Micron OXFORD

Hypothesis → Experimental planning → Sample preparation → Image Acquisition → Processing & analysis → Data visualisation & Interpretation

- **Informative results** — what kind of informative results are needed to test the hypothesis
- **Required data** —what data are required to produce the informative results
- **Required controls** —what controls are required to support the informative results
- **Parameters** —what are the experimental parameters dictated by the data
- **Microscope selection** — which instruments aligns with the experimental parameters
- **Data management and storage**
- **Data analysis** — what tools are required to extract the informative results

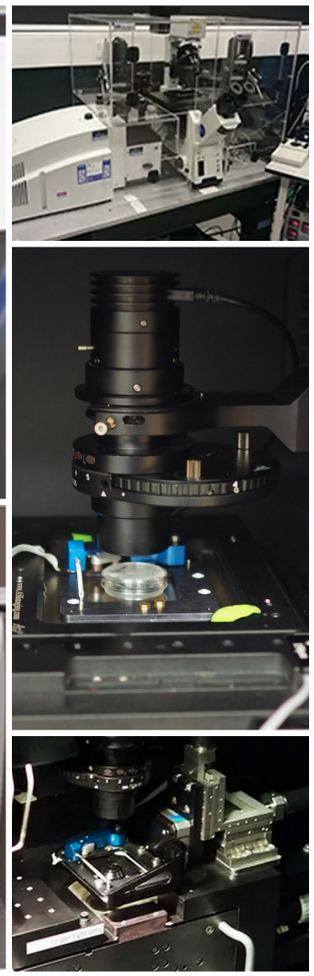


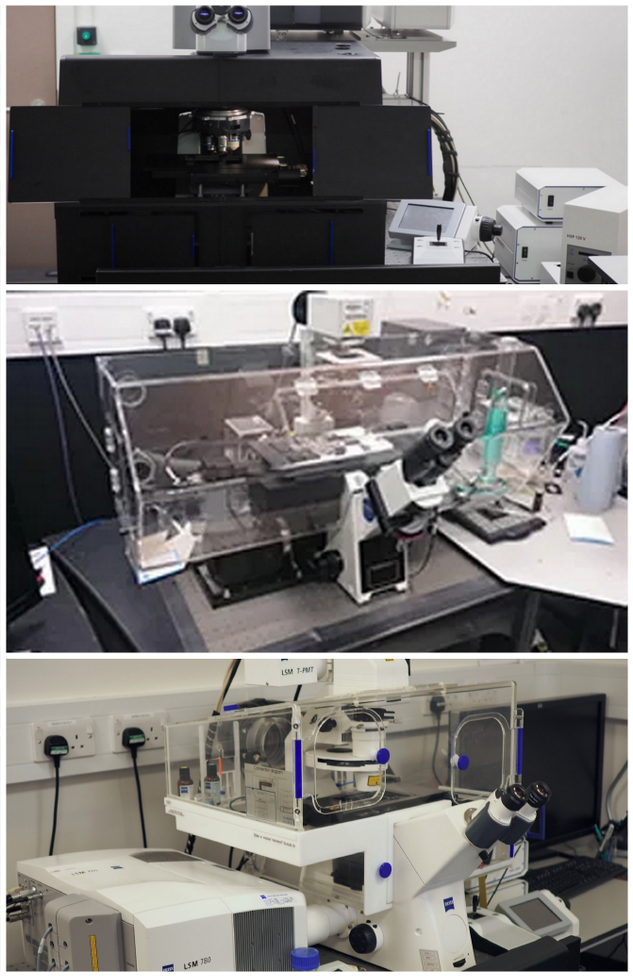
The limiting factor is: Photon budget.

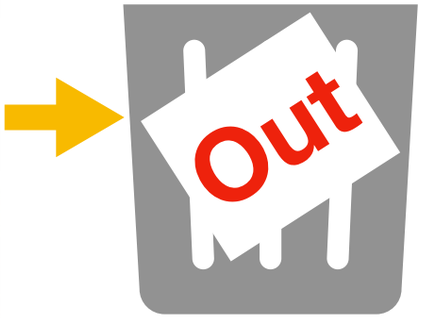


The iron triangle

GIGO!

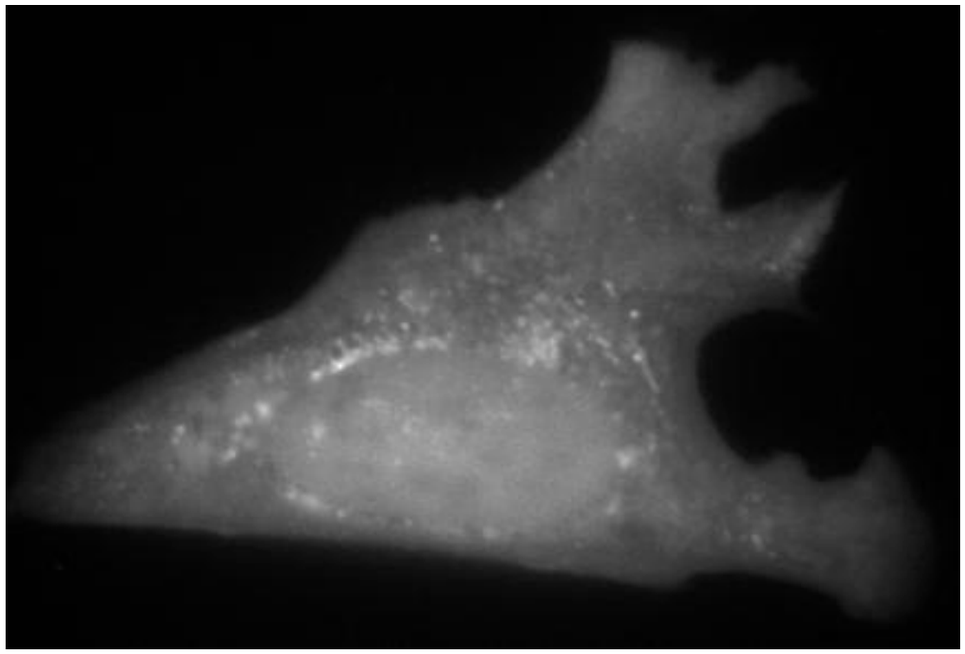






Let's see some interesting experiments!

A



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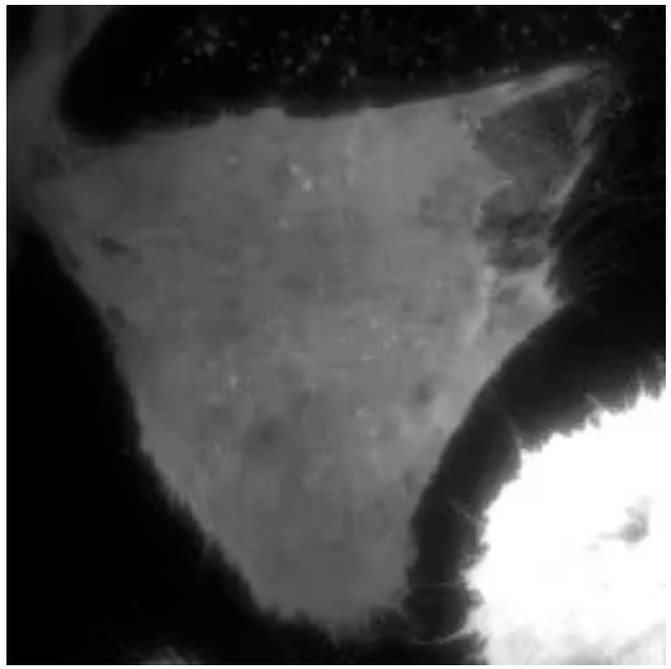
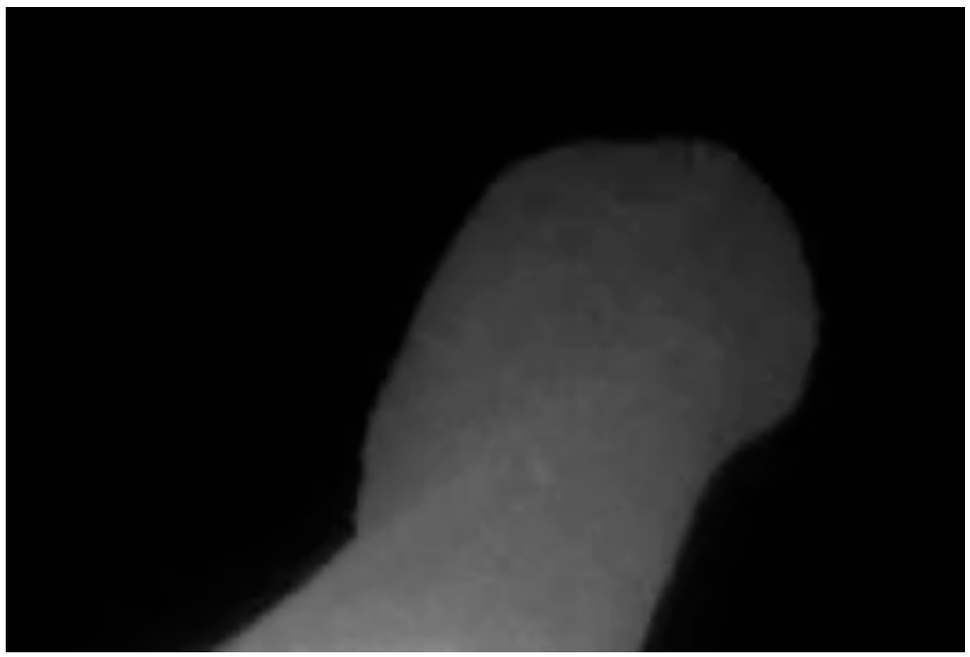
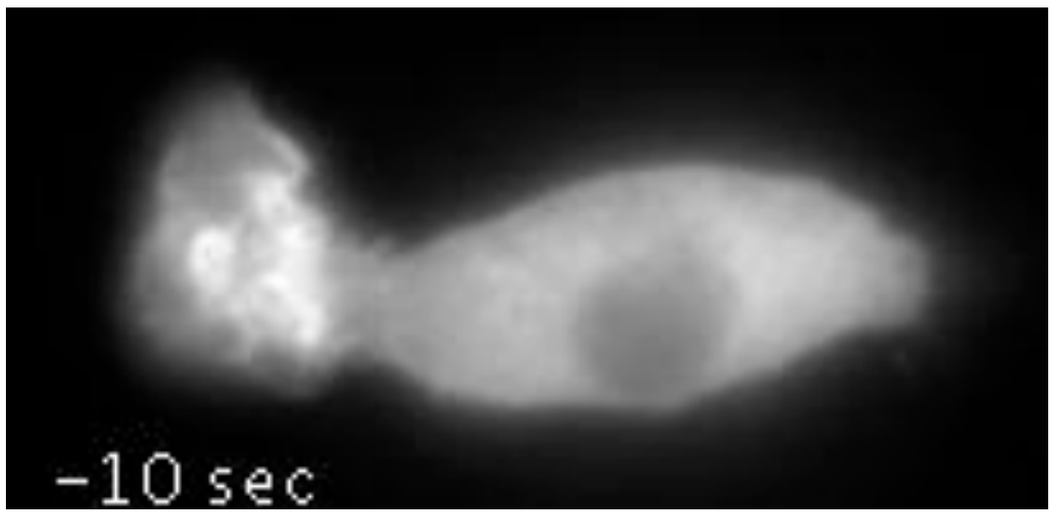


C

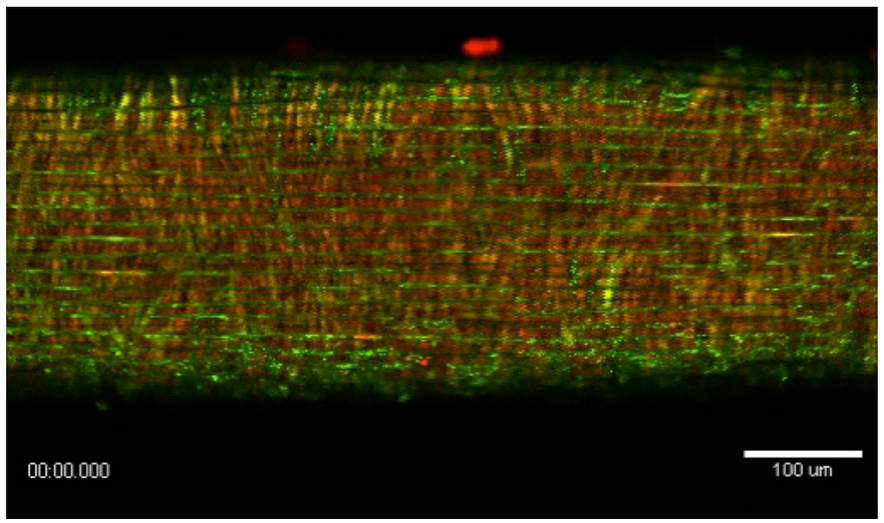


D

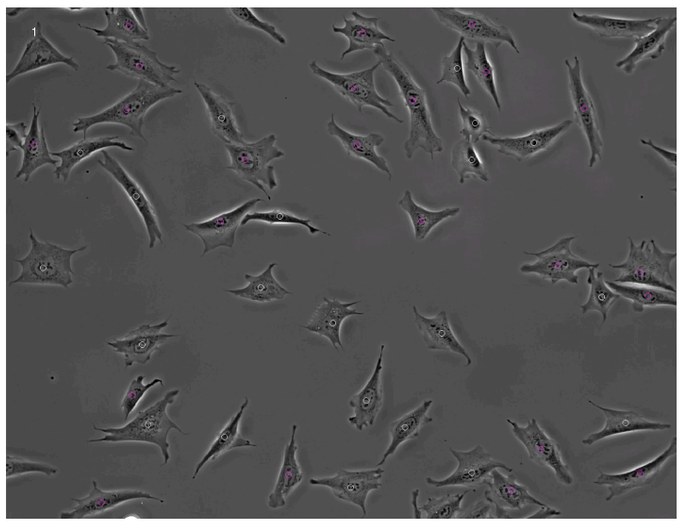


Not mine!

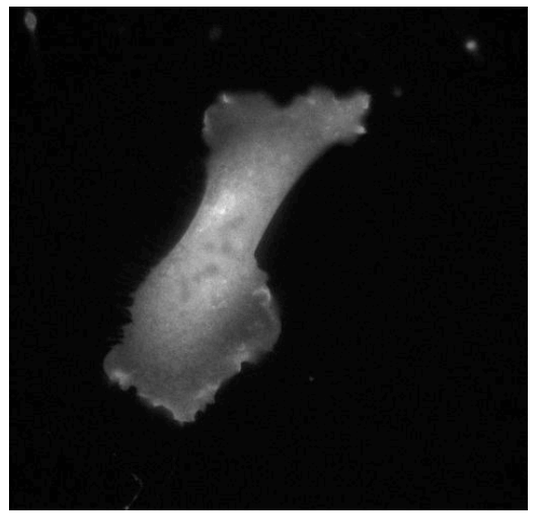
Live-cell imaging - Why?



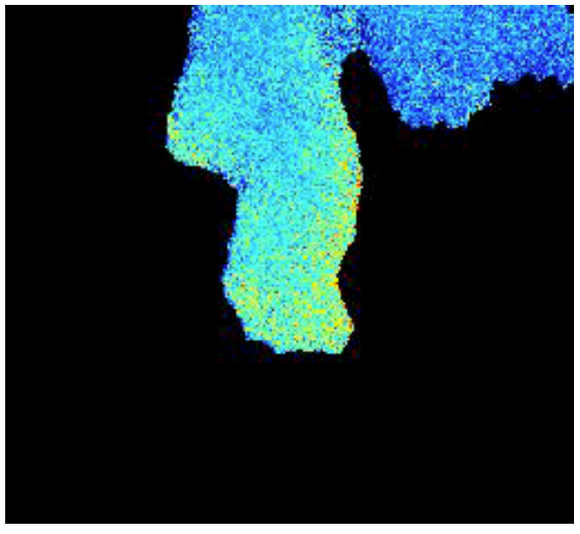
Calcium wave propagation in an arterial strip
(*Seppey et al. 2010*)

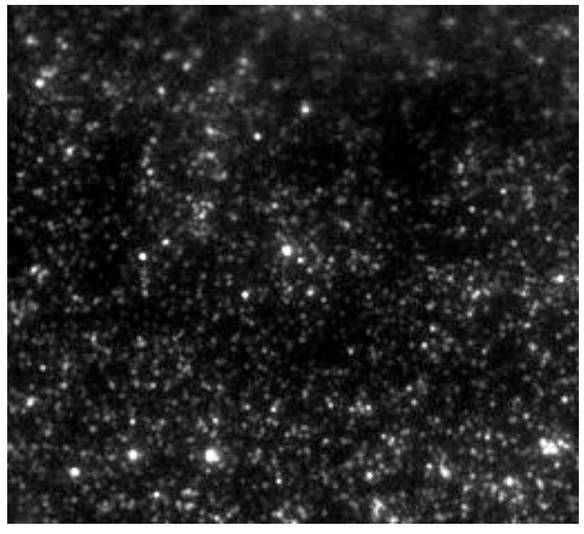


Cell migration on substrates
(*Halidi, unpublished data*)

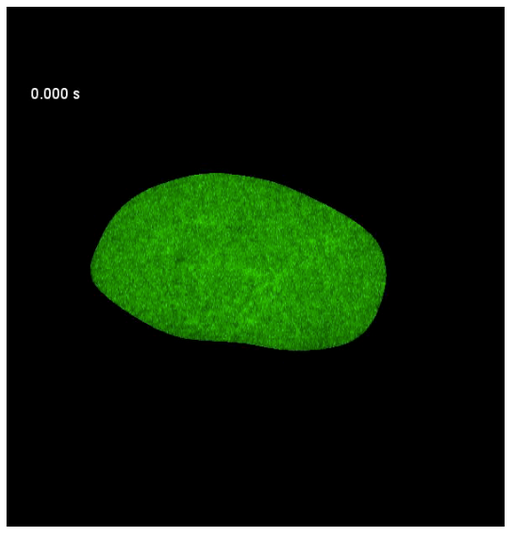


RhoA FRET-biosensors activity
in MEFs on soft substrates
(*Halidi, unpublished data*)

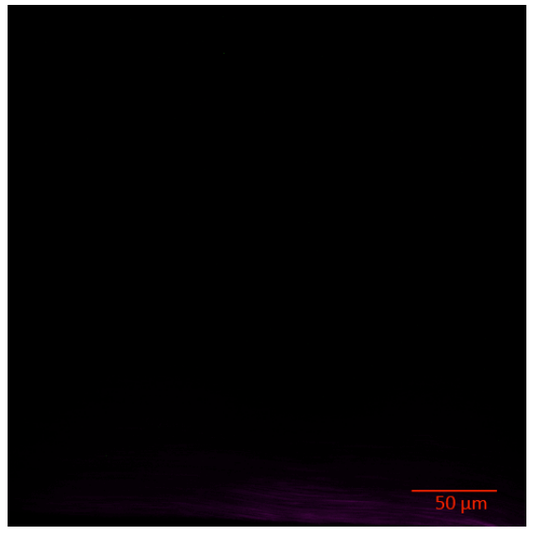




Simultaneous Rac1 FRET-based biosensors activity and
traction force microscopy (*Halidi, unpublished data*)



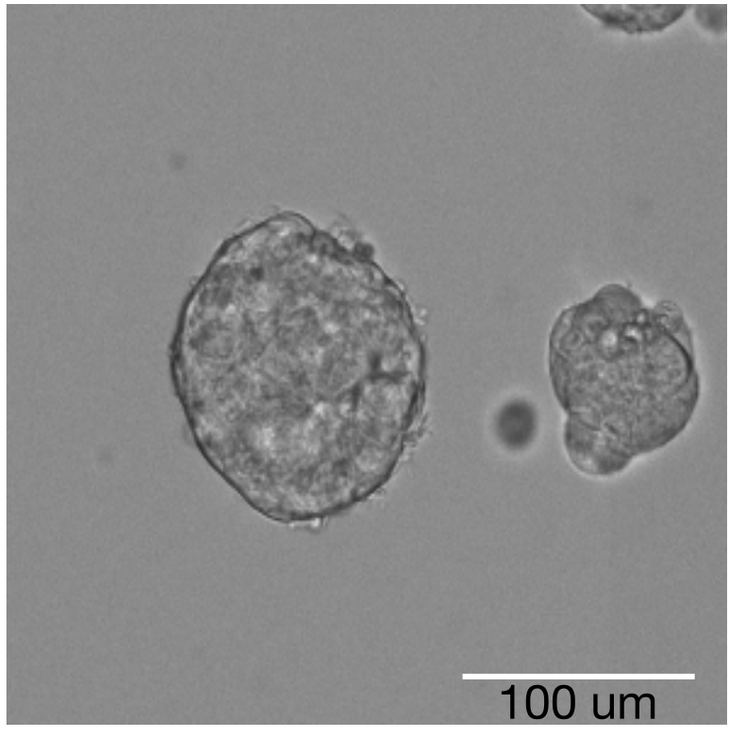
FRAP in U2OS cells transfected with
GFP-tagged MLLT1
(*Moustakim et al. 2018*)



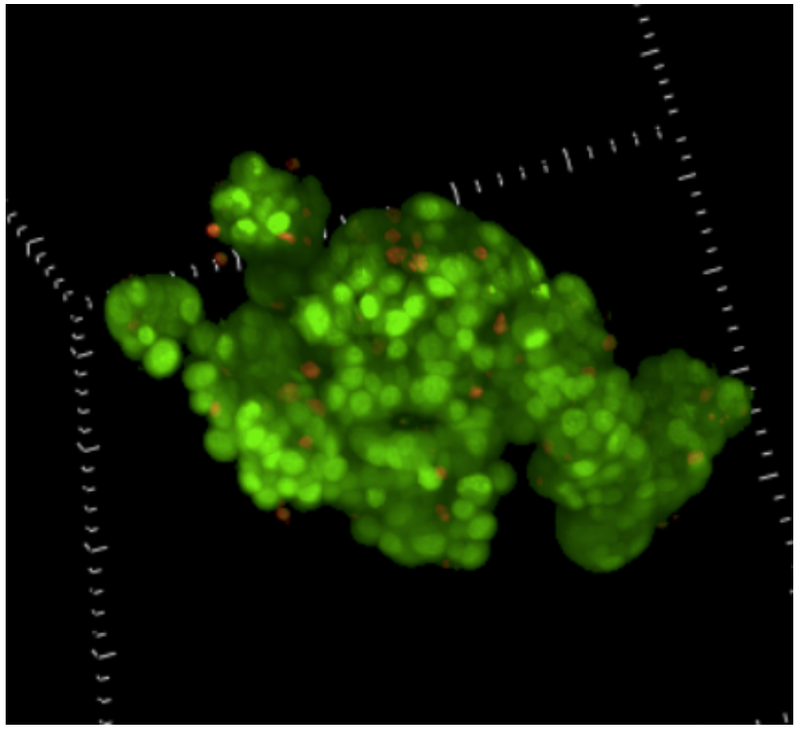
Second harmonic generation
in tendon
(*Halidi & Lagerholm, unpublished data*)

Track cellular and sub-cellular processes in real time

Live-cell imaging - Why?



A mammosphere formation of MCF7 cells in growth media, brightfield

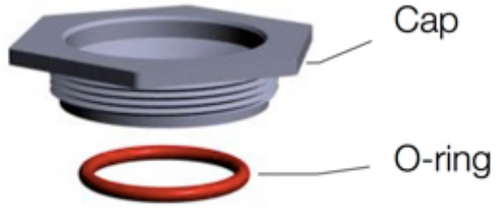


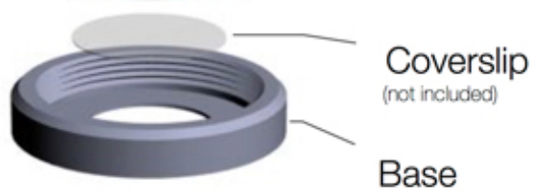
A tumoroshpere formation of MCF7 cells in agarose gel, lightsheet

Overview

- Sample preparation (mounting, staining, media)
- Choosing a microscope (inverted vs upright)
- Maintaining live cells on the microscope stage
- Efficiency of detection
- Photobleaching & Phototoxicity
- Data processing and analysis through examples

Sample preparation: Mounting options





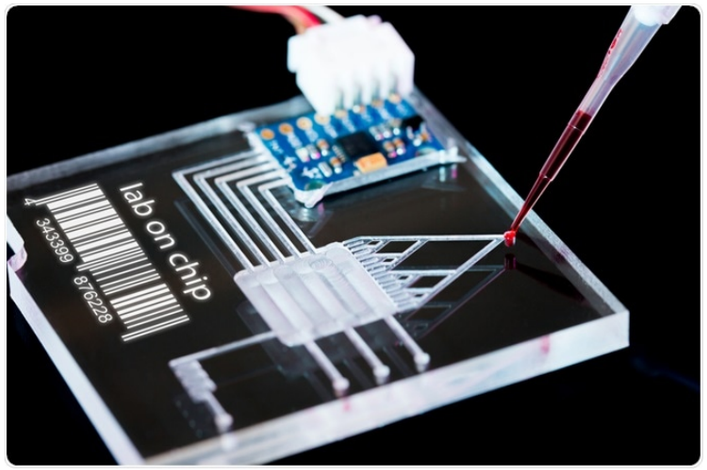
airekacells.com

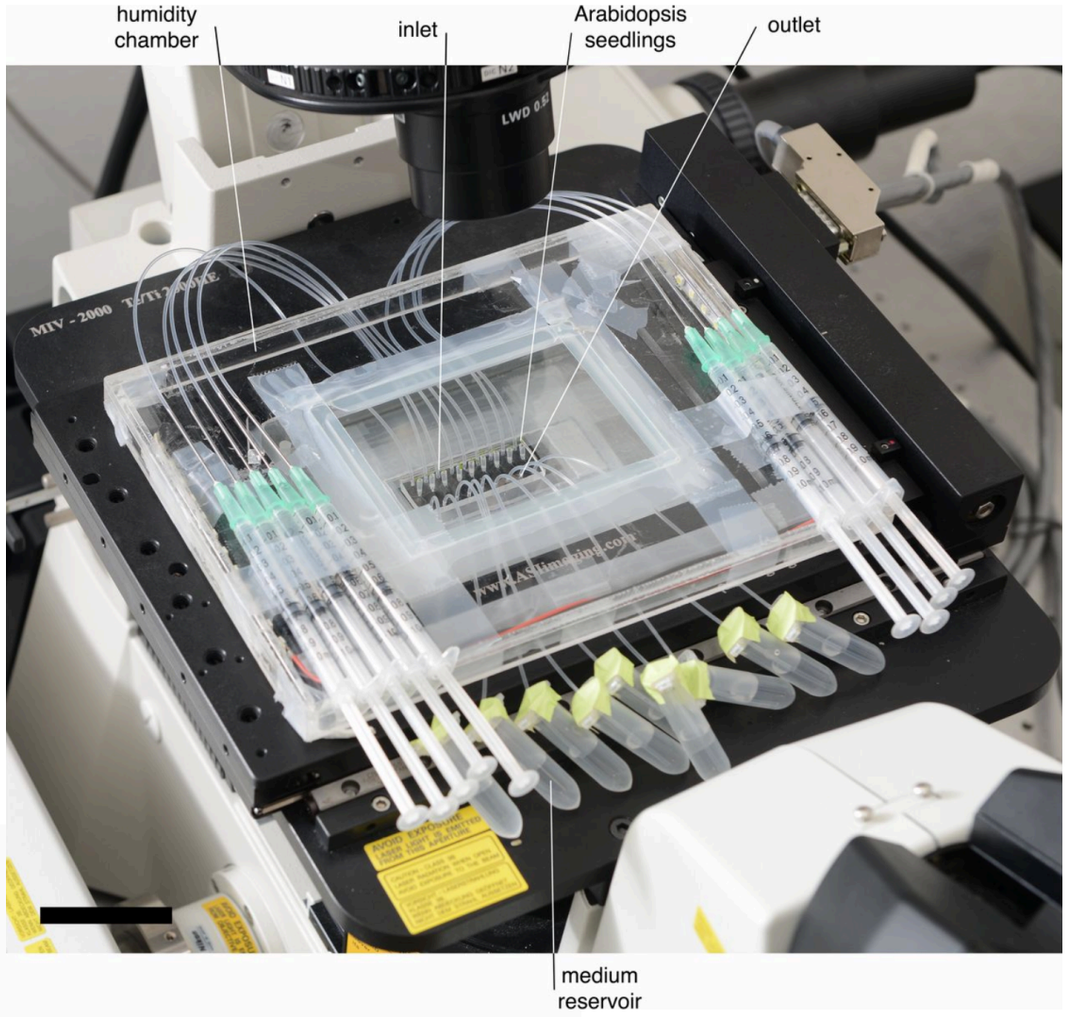
Fluorodish.com

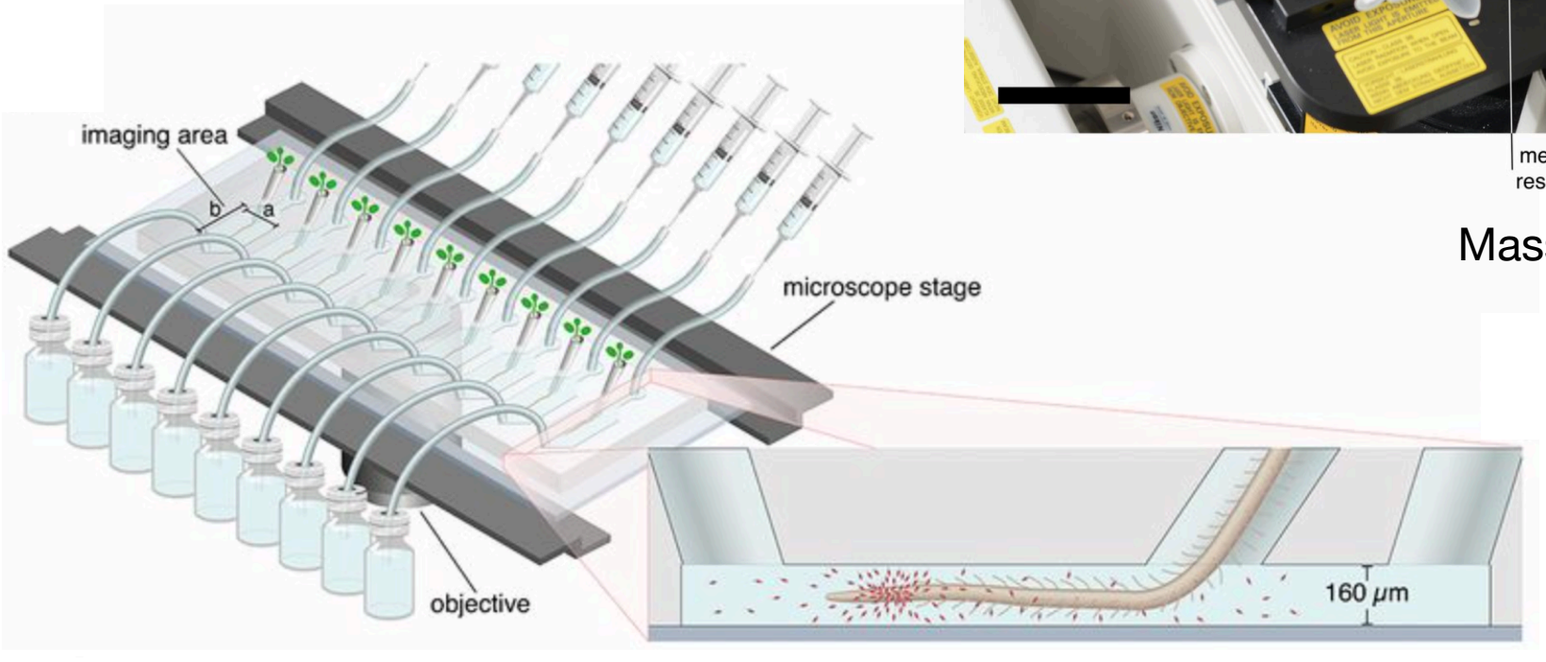
Thin coverslip #1.5 thickness or 170 um

Sample preparation: Mounting options

Microfluidics setups



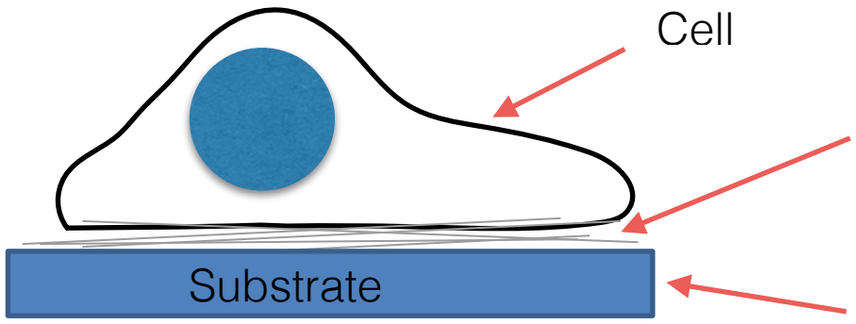




Massalha et al. PNAS 2017

Sample preparation: Mounting options

Extracellular matrix (ECM) proteins coating and coated plates

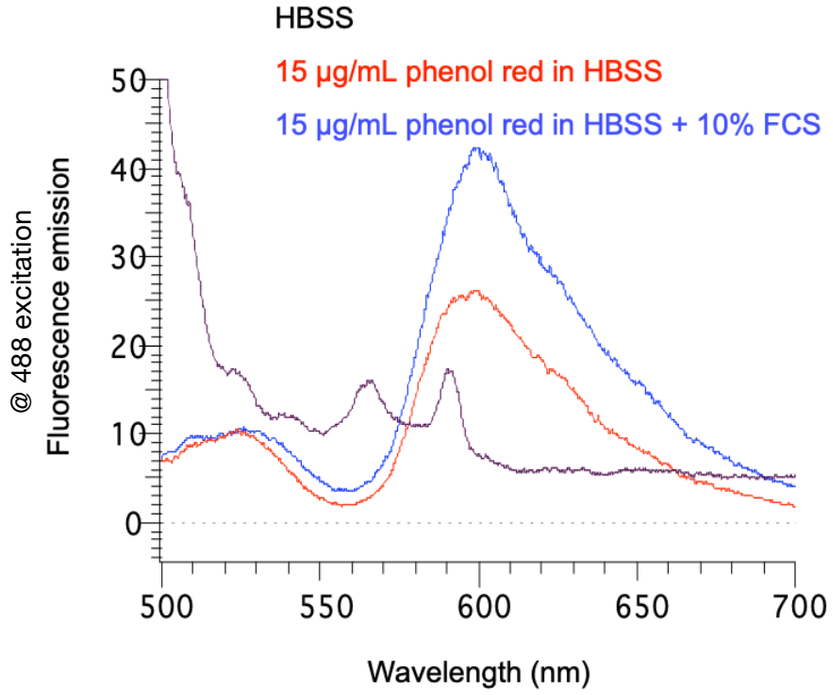


ECM: e.g. fibronectin, collagen, laminin

Glass, plastic, or silicon substrates of variable stiffness

Media options and considerations

- Avoid media w/ autofluorescence properties (phenol red, serum proteins)



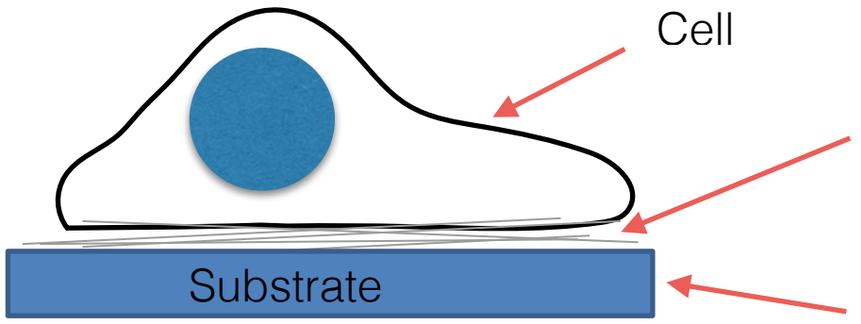
From Iain Johnson, Microscopic Imaging Core @ Wake Forest University

Source	Organism/Tissue	Ex (nm)	Em (nm)
Flavins	CHO cells	380, 460	520
	Rat hepatocytes	468	525
	Neural cells (rat, bovine)	488	540-560
	Goldfish inner ear	450	540
	Periplaneta americana	<350	530
NAD(P)H	Rat cardiomyocytes	395	509
	S. cerevisiae	366	440-470
	CHO cells	360	440-450
Lipofuscins	Medulla (rat, human, rhesus monkey)	460-490	520
	Rat heart	450-490	550
	Muscle, myocardium, hepatocytes	360	540-560
	Human brain	435	481-673
	Rat liver	345	430
	Rat retina	390-490	>510
Collagen and elastin	Aorta, coronary artery (human)	476	>515
	Skin (human)	442	470-520

Analytical Biochemistry, 2001, 291:175

Sample preparation: Mounting options

Extracellular matrix (ECM) proteins coating and coated plates

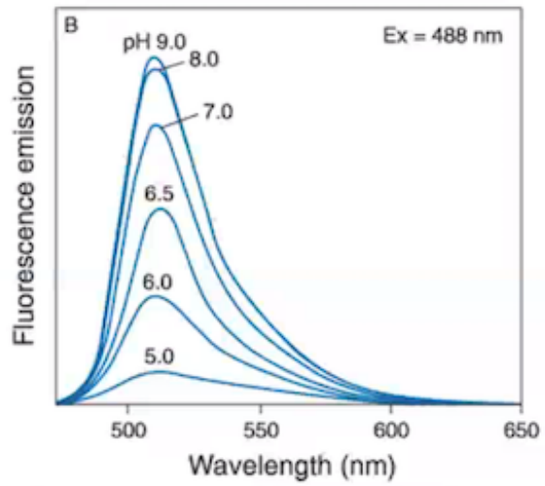


ECM: e.g. fibronectin, collagen, laminin

Glass, plastic, or silicon substrates of variable stiffness

Media options and considerations

- Avoid media w/ autofluorescence properties (phenol red, serum proteins)
- CO_2-dependent media —> requires CO_2 in the atmosphere
- CO_2-independent media —> requires buffers e.g. HEPES buffered media or Leibovitz L 15



Fluorescein pH sensitivity

From yesterday's Fluorescent dyes and Proteins talks!

Sample preparation: Staining options

- Fluorescent protein tags
- Fluorescently tagged ligands
- Fluorescent antibodies to extracellular epitopes
- Cell permeant small molecule fluorophores (e.g. dyes, DNA stains)

See yesterday's Mark Howarth lecture: Fluorescent Dyes and Proteins

Brightness

Photostability

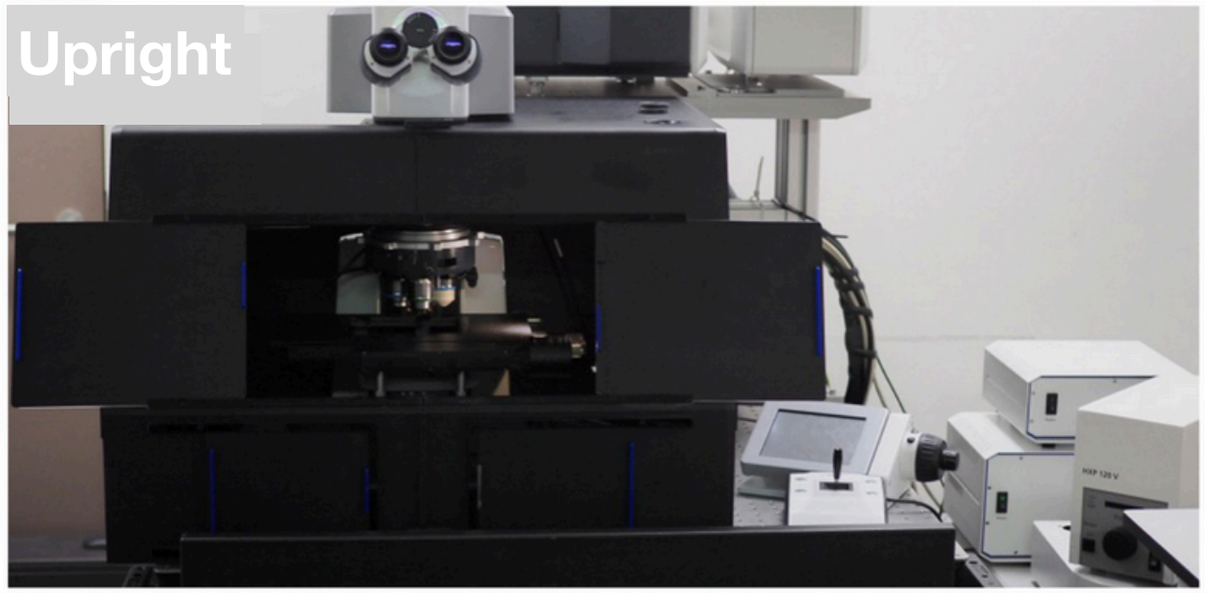
Fluorescent proteins: mTagBFP2, EGFP, tdTomato, iRFP, cerulean, citrine, mcherry, mKate2

Multi-color experiments: DAPI (Alexa 405, Alexa/Atto 488, Alexa 568, Alexa 647)

Live cell nuclear dyes: Hoechst, SYBR safe DNA stain (replacing Ethidium Bromide)

Cell tracker dyes, Vybrant DiI, CM-DiI, DiO and DiD cell-labeling

Choosing a microscope: Upright or Inverted?



Things to considerations:

- Samples mounted on a multi-well plate
- Samples won't grow on glass bottom dishes
- Sample thickness
- Need access to samples (e.g. addition of drugs, inhibitors)
- Environmental control is important
- Location of what we want to detect (adhesion sites —> TIRF)

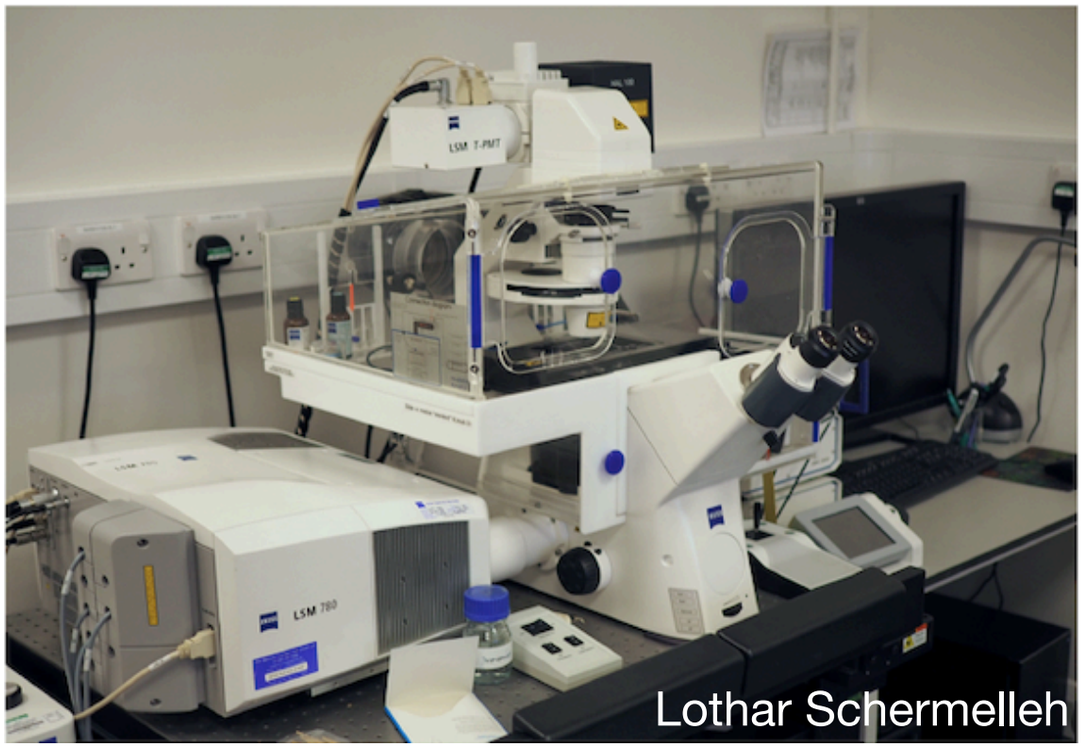
Maintaining live cells on the microscope stage

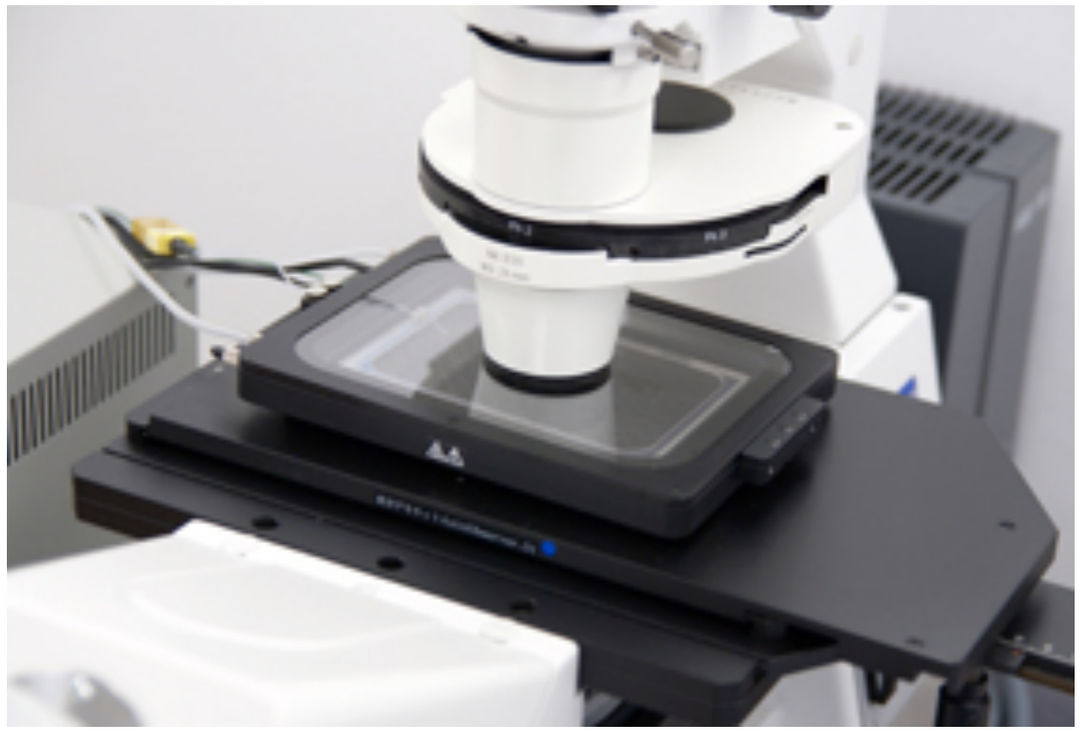
Environmental control:

Temperature

CO_2

Humidity





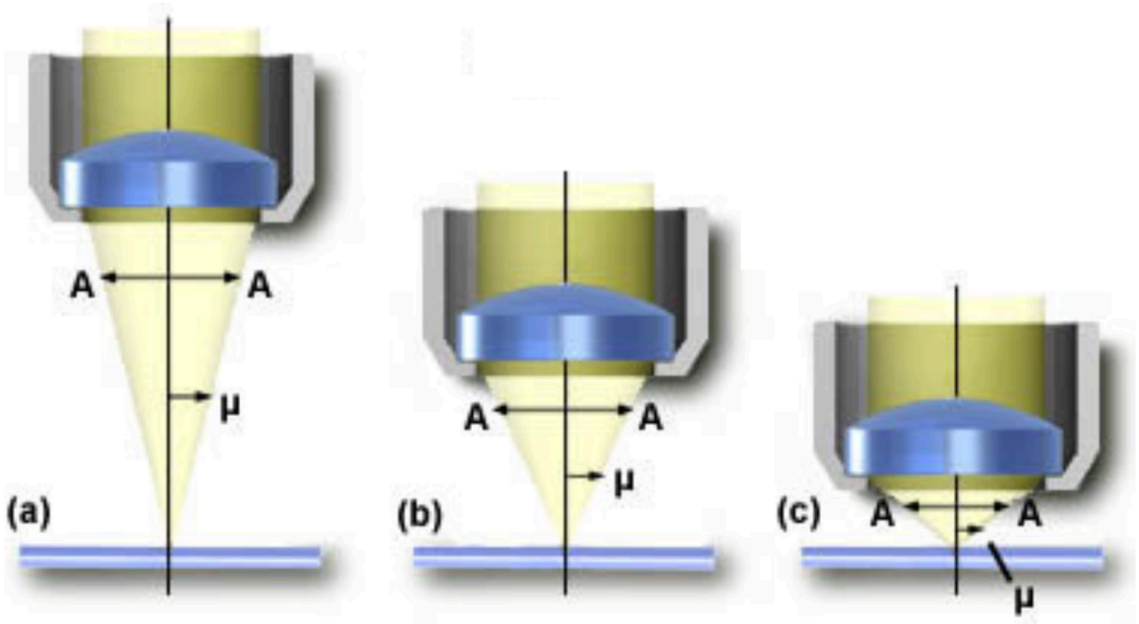
Ready to image? … Wait!

The efficiency of detection depends mainly on:

1. The objective
2. The filter set
3. The detector

Efficiency of detection: The objective

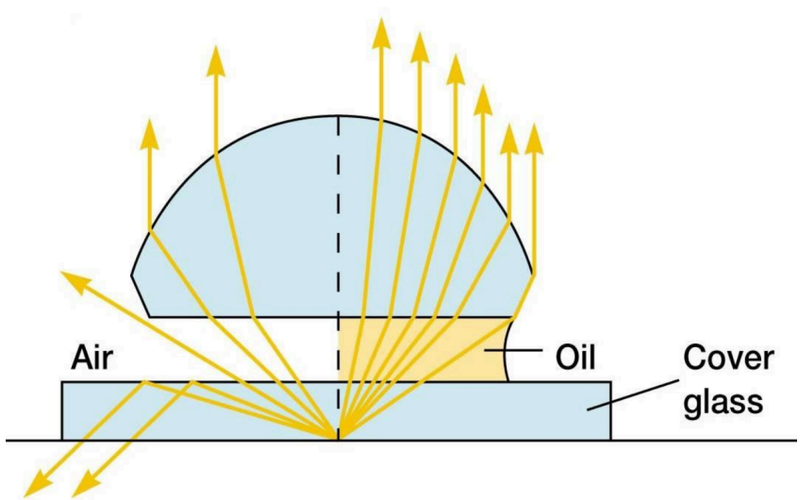
- The objective determines the special resolution and controls the amount of light gathered from the specimen. (look for NA)



https://micro.magnet.fsu.edu/

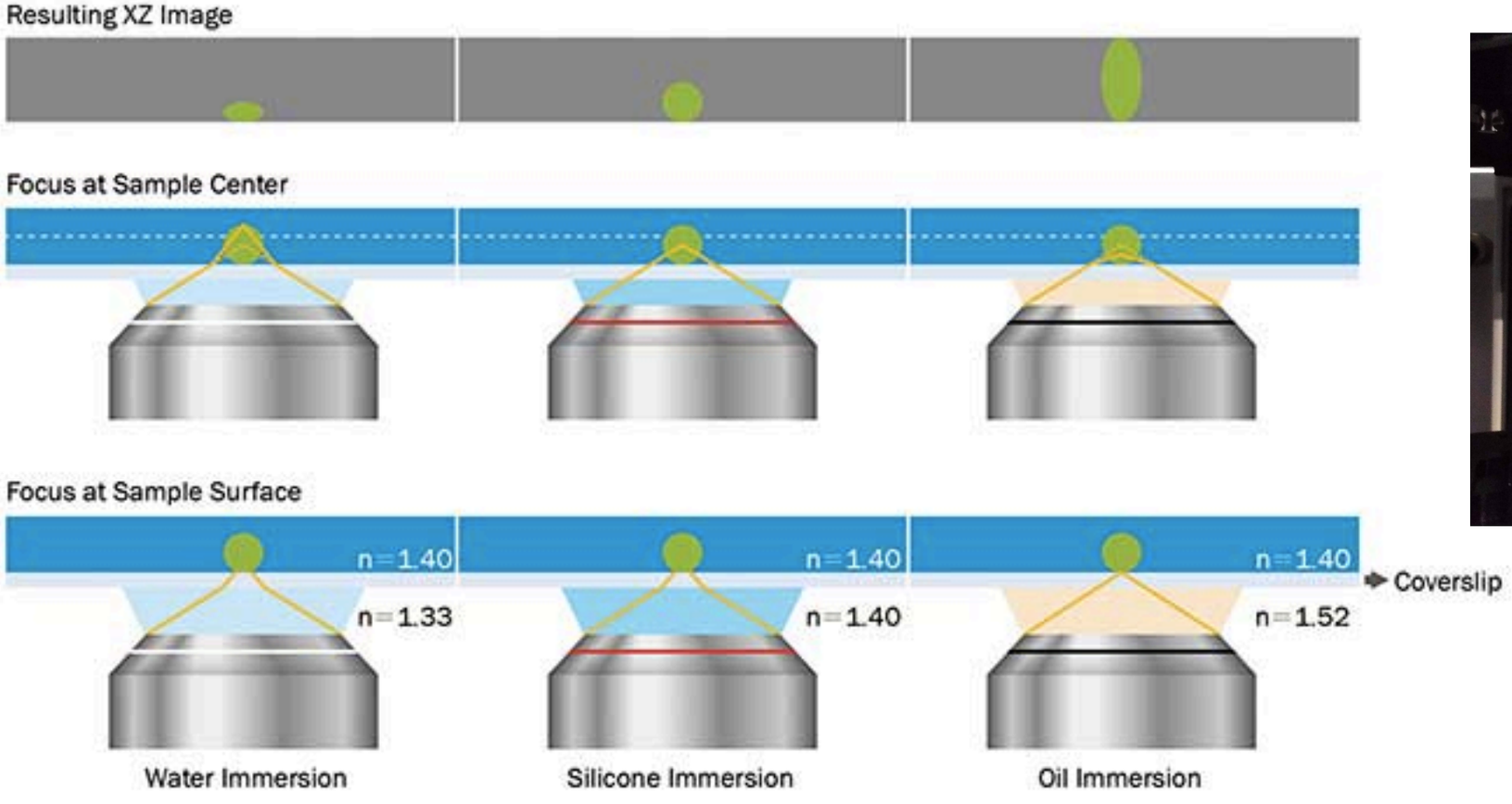
Numerical aperture (NA) = n **x** sin μ

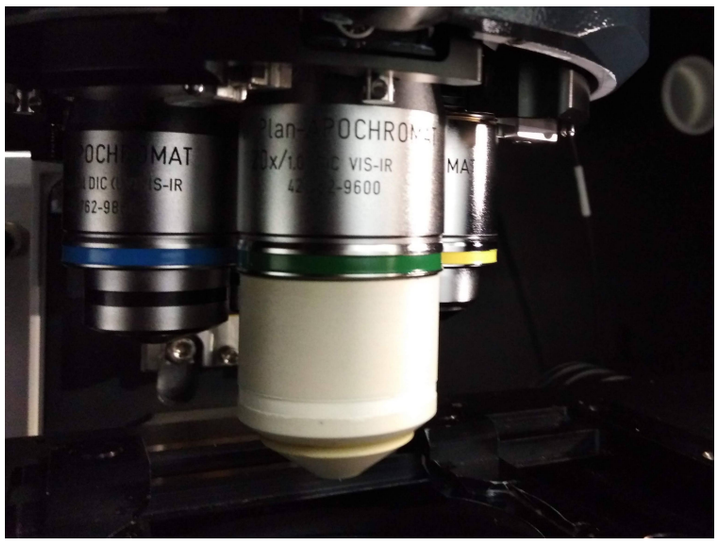
(a) μ = 7° NA = 0.12
(b) μ = 20° NA = 0.34
(c) μ = 60° NA = 0.87



Efficiency of detection: The objective

- The objective determines the special resolution and controls the amount of light gathered from the specimen. (look for N.A.)
- Avoid refractive index mismatches between the sample and the immersion oil.





https://www.olympus-lifescience.com

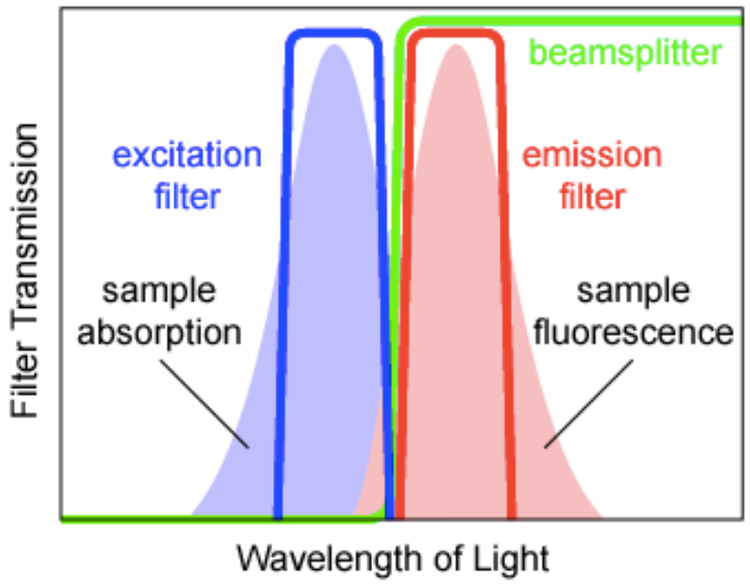
Efficiency of detection: The objective

- The objective determines the special resolution and controls the amount of light gathered from the specimen. (look for N.A.)
- Avoid refractive index mismatches between the sample and the immersion oil.
- Working distance
- Field of view
- Number of optical corrections in the lens design

https://www.olympus-lifescience.com

Efficiency of detection: The filter sets

- Know your fluorescent protein absorption and emission spectra
- What filters are there on the system (preferably narrow bandpass)



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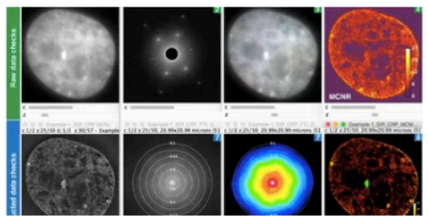
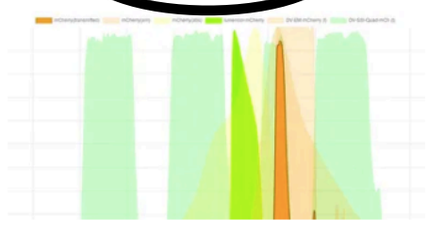
SIMcheck



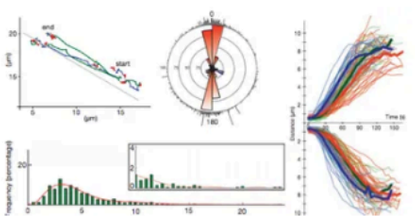
SIMcheck is a suite of ImageJ plugins enabling users to identify and avoid common problems with 3D-Structured Illumination Microscopy (3D-SIM)

SPEKcheck



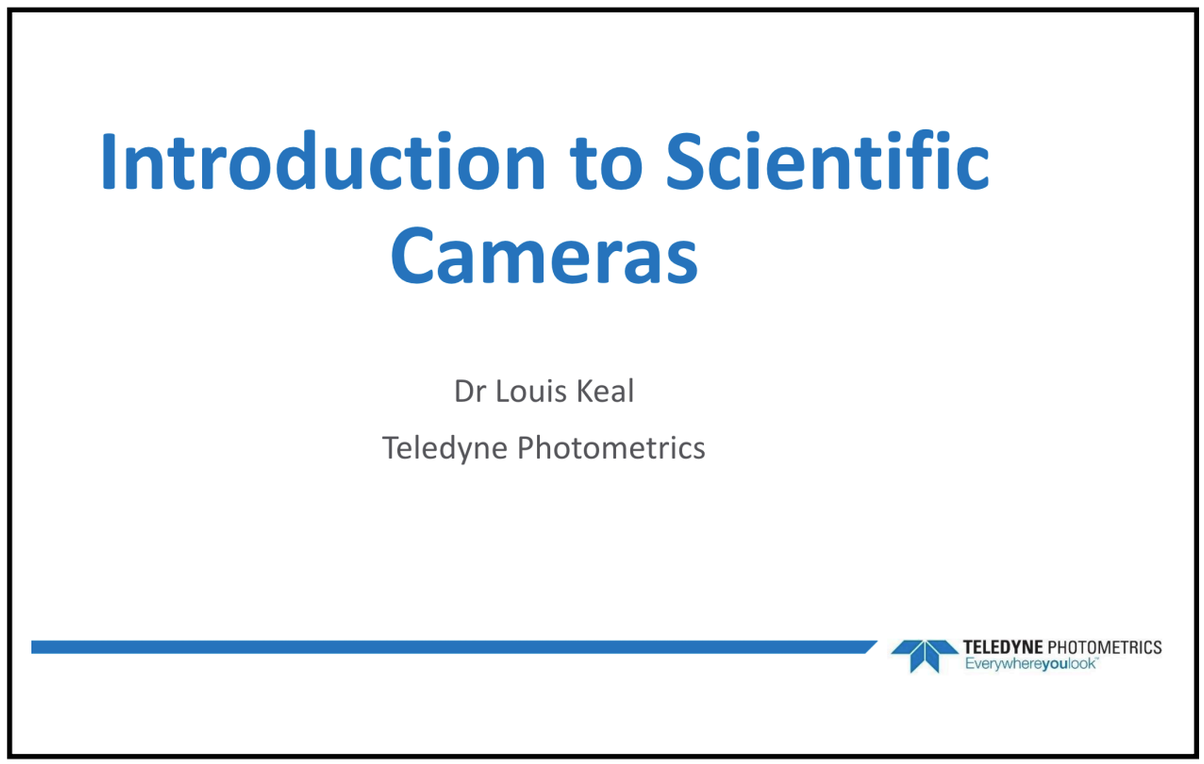
Advanced fluorescence imaging methods require careful matching of excitation sources, dichroics, emission filters, detectors, and dyes to operate at

Particle Stats



The study of dynamic cellular processes in living cells is central to biology and is particularly powerful when the motility characteristics of

Efficiency of detection: The detectors



Widefield & Spinning disk confocal cameras

CCD

EMCCD

sCMOS

Scanning confocal detectors

PMT

Gallium Arsenide Phosphide (GaAsP)

The final image always boils down to signal-to-noise!

Ready to image? … Wait!

The efficiency of detection depends mainly on:

1. The objective
2. The filter set
3. The detector

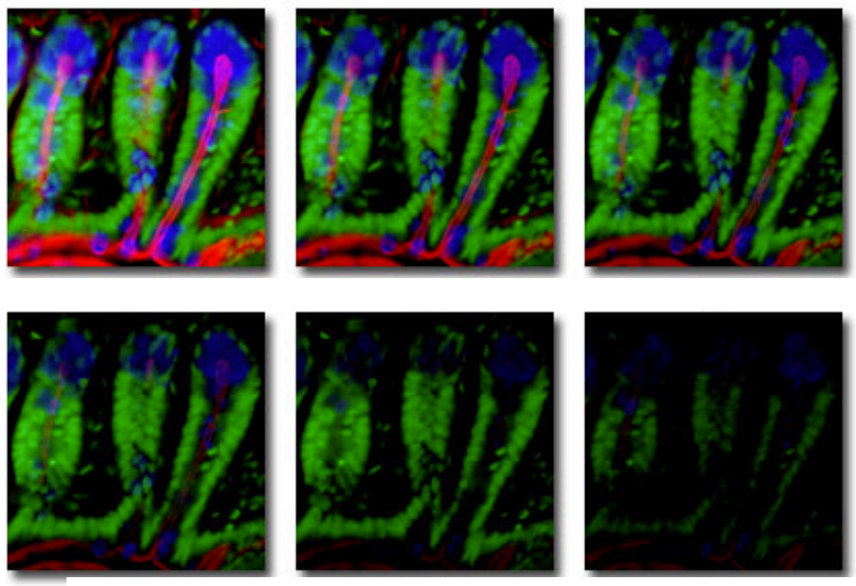
… but also

4. Correcting focus drift
5. Stage control
6. Imaging multi color w/ spectral detection & linear unmixing

Photobleaching & Phototoxicity

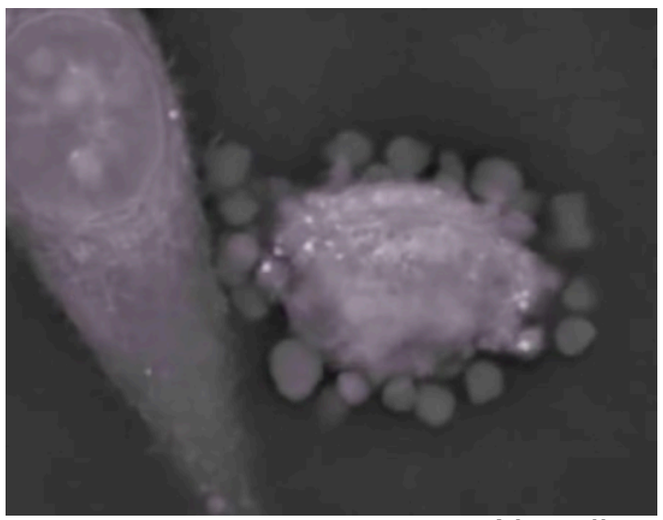
Micron OXFORD

Photobleaching —> dye not happy!

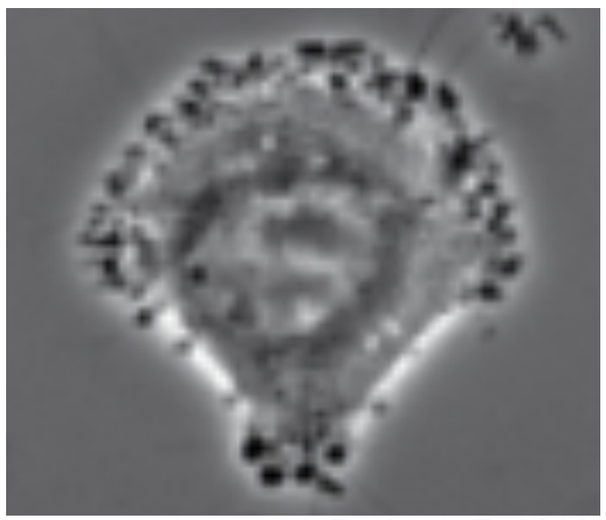


micro.magnet.fsu.edu

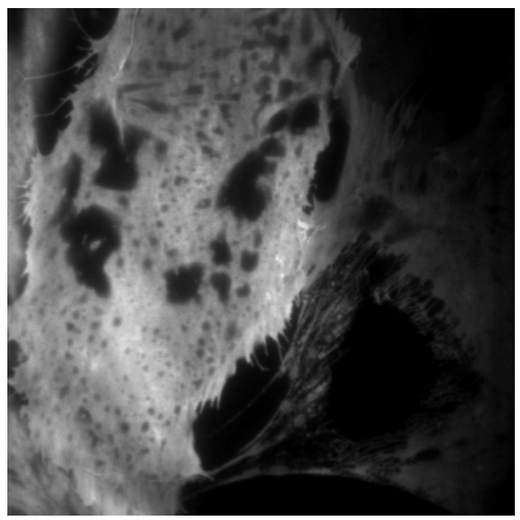
Phototoxicity —> cells not happy!



Nanolive



Derivery E, 2008



Reactive oxygen species (ROS)

E.g., peroxides, superoxides, hyrax radical and single oxygen.

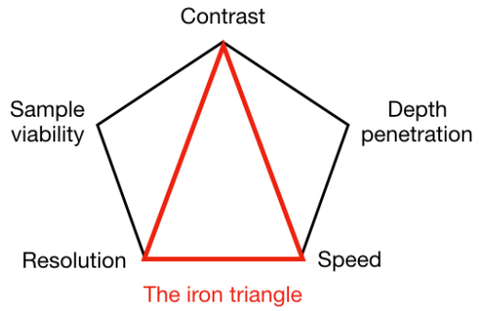
Excessive ROS can:

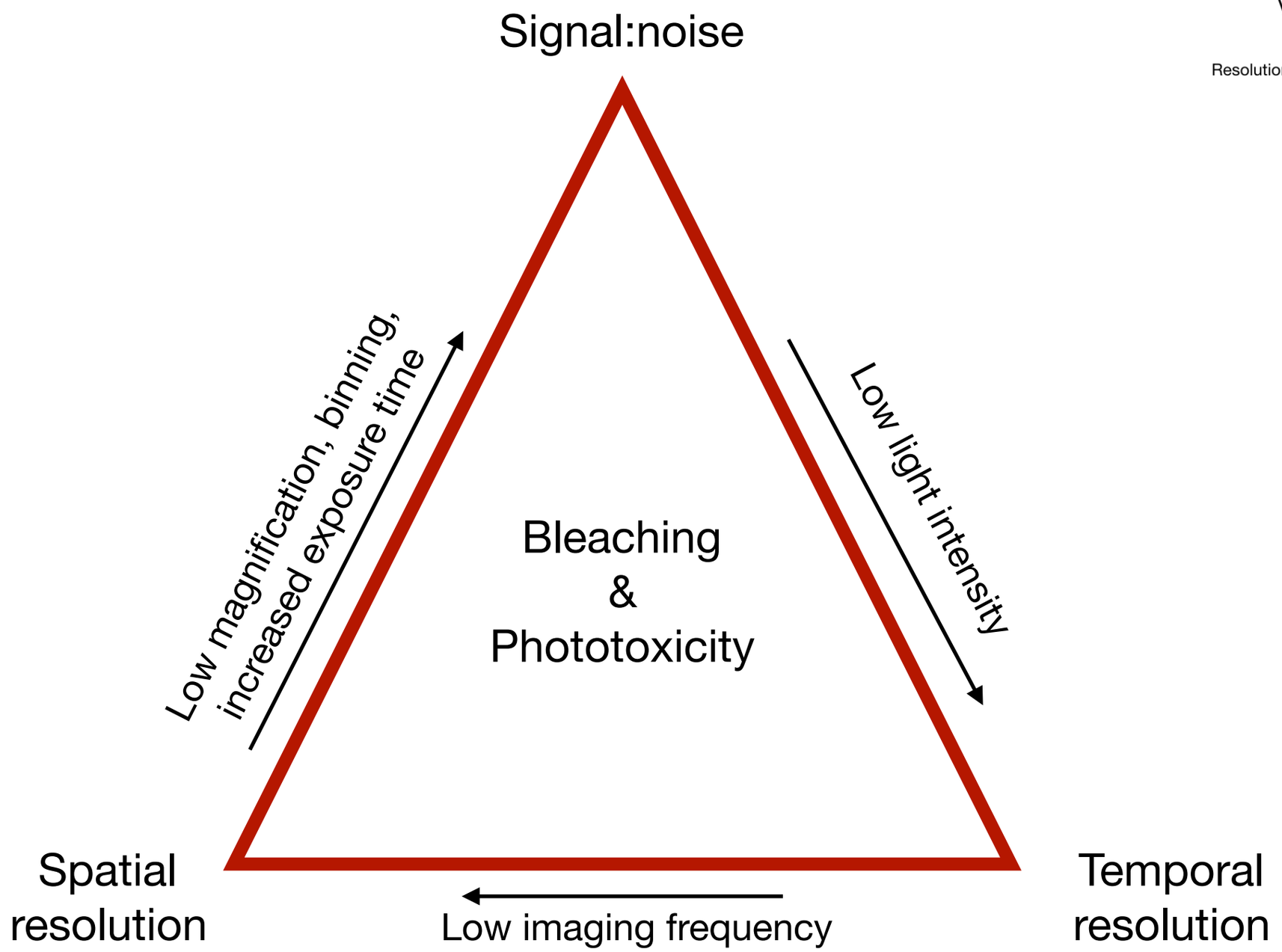
- Damage of the lipids, DNA, RNA and proteins
- Induce apoptosis

Literature:

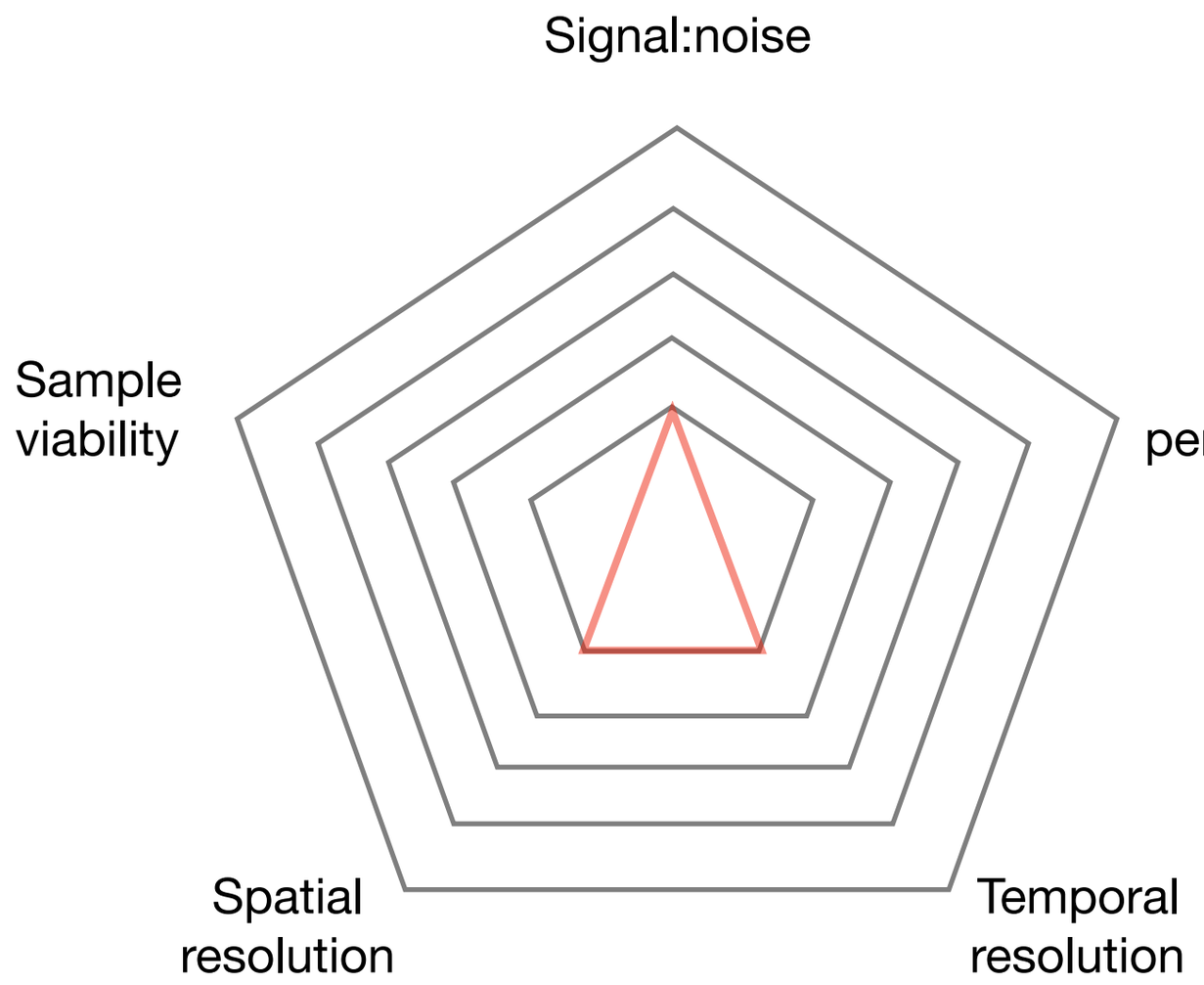
- DNA Damage and Oxygen Radical Toxicity. Imlay et al. Science 1988, 240:1302
- Cross-talk between calcium and reactive oxygen species signaling. Yan et al. Acta Pharmacologica Sinica (2006, 1745:)

The iron triangle





Now which microscope should I use?



3D imaging and optical sectioning:

WF deconvolution

Spinning disk confocal

Scanning confocal

Multi-photon

lighsheet

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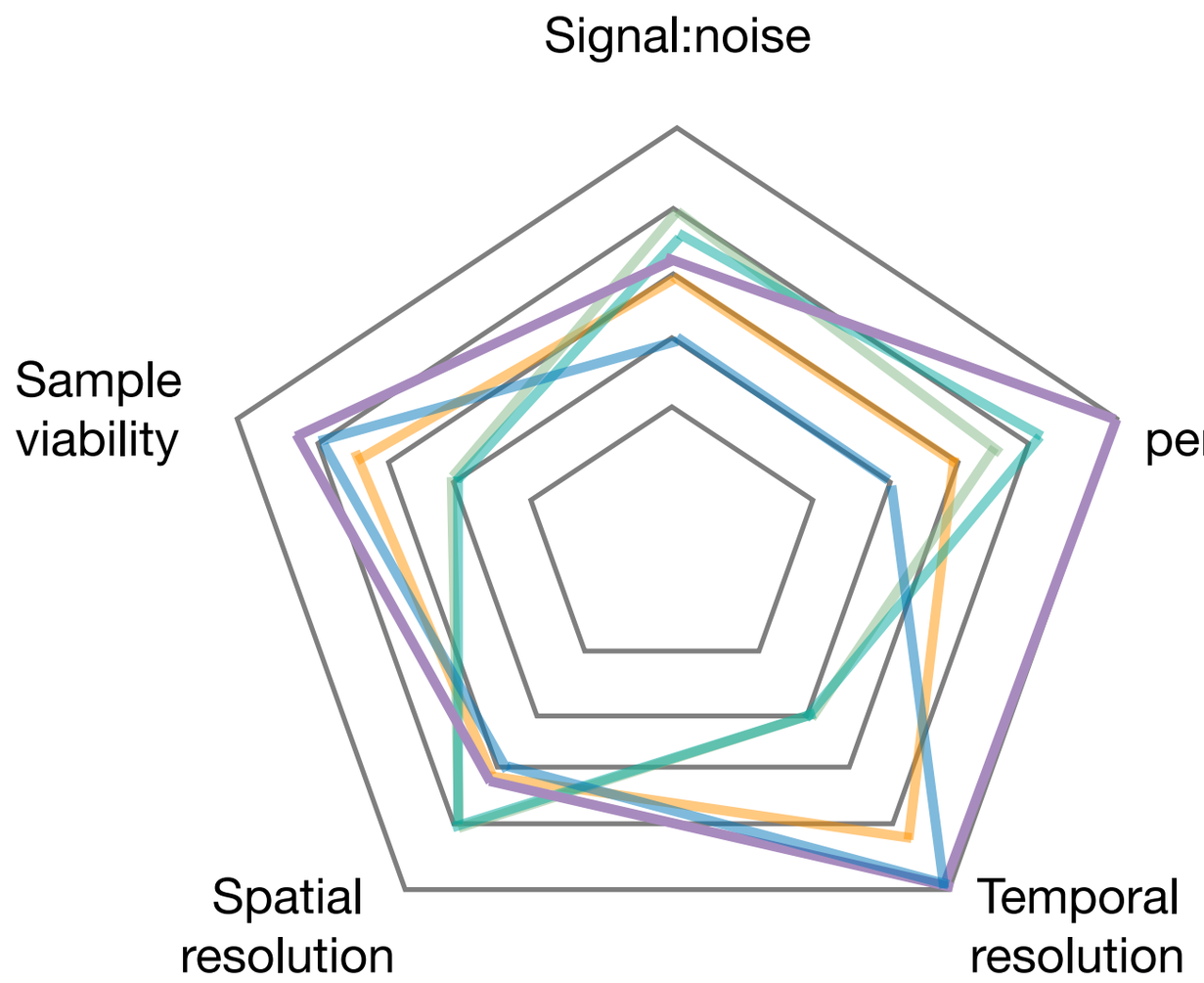

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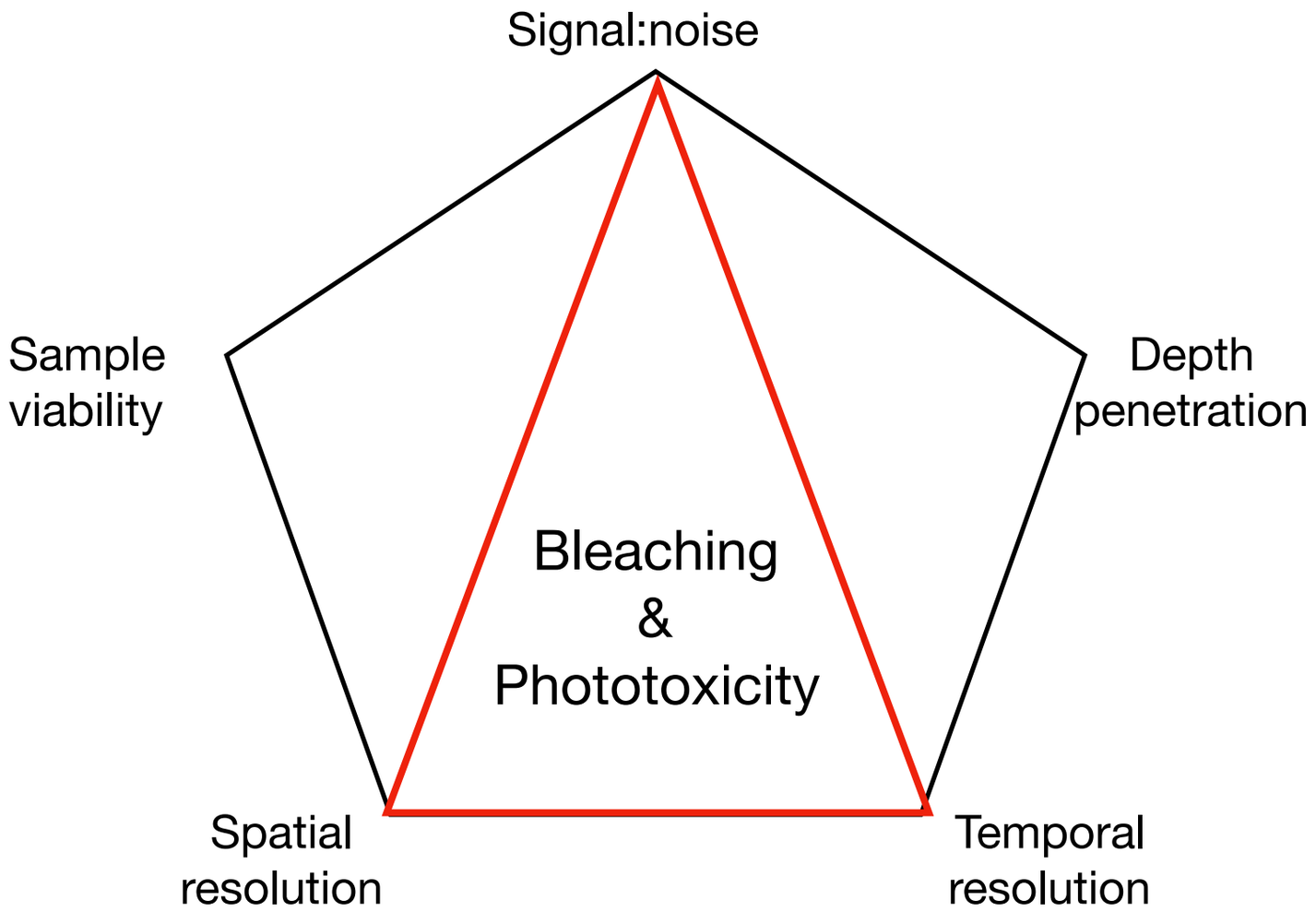


The limiting factor is: Photon budget.

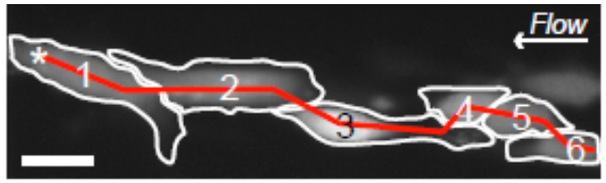
Recommended reading: Jonkman et al. Nature Protocols 2020, 1585–1611

Data processing and analysis through examples

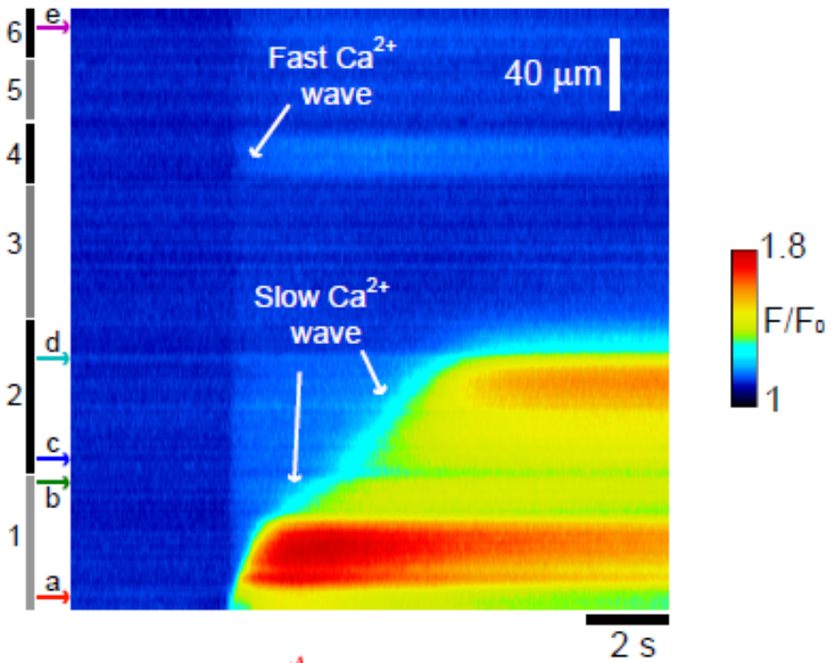
I have images! YAAAAY! …. so what now?

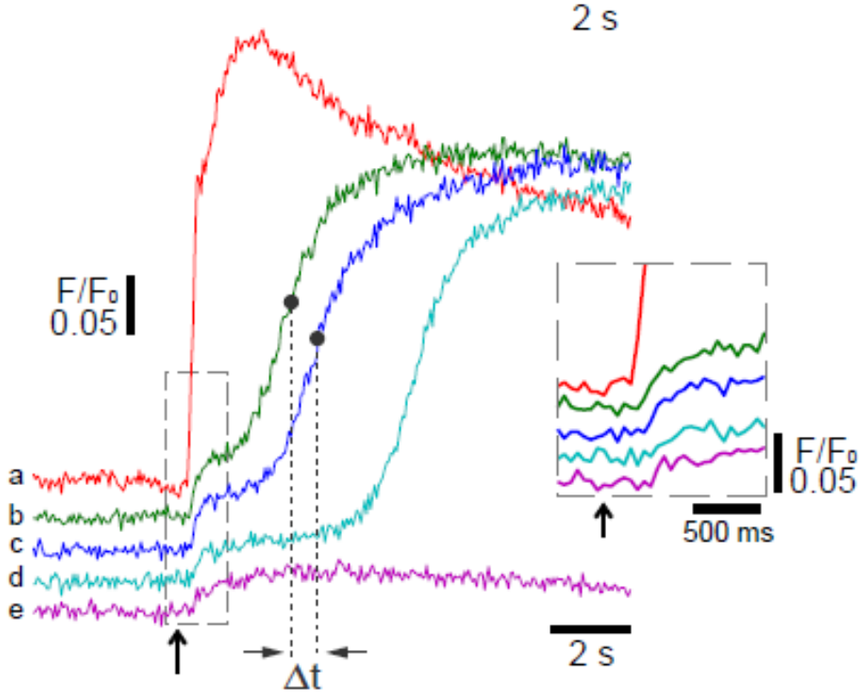
Be quantitative!

Fast dynamics:
Propagation of intercellular Ca^{2+} waves



Primary SMCs grown on µCP collagen lines.





Fast Ca^{2+} wave → 2310 ± 210 µm/s

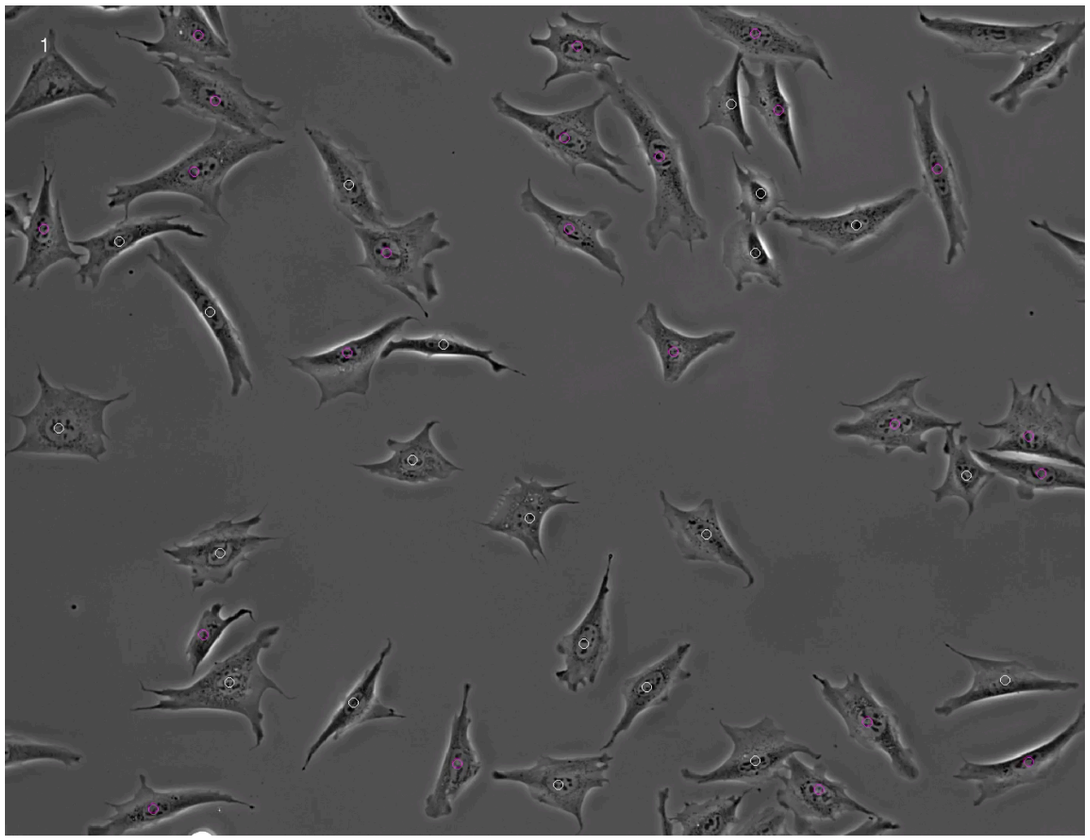
Slow Ca^{2+} wave → **1)** 19.8 ± 1.6 µm/s

2) 21.4 ± 2.2 µm/s → 28%

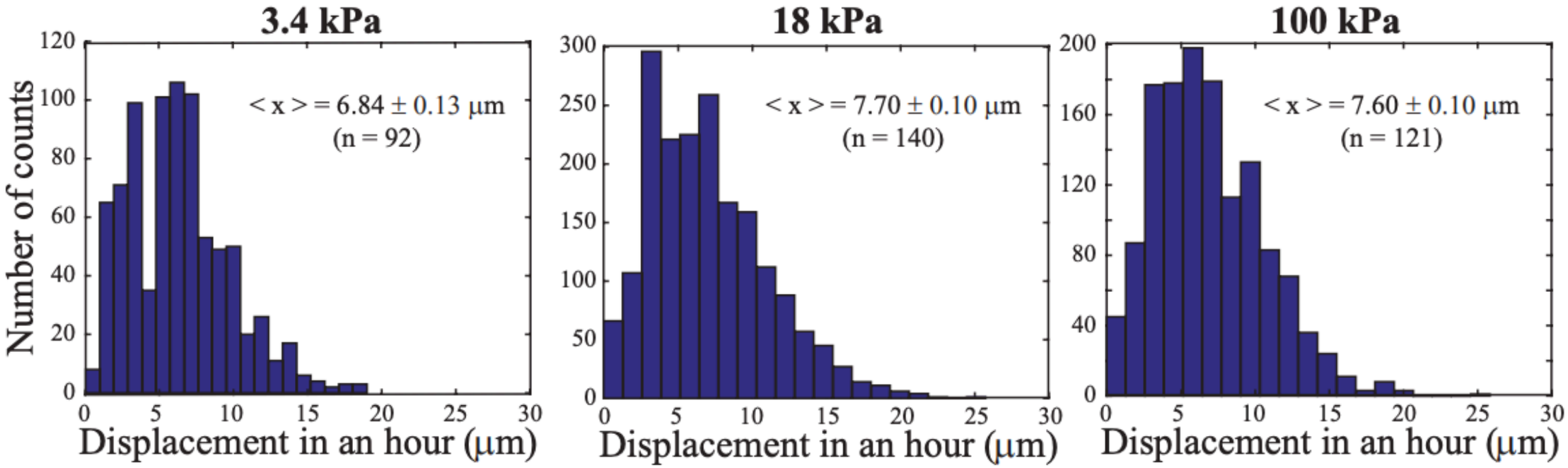
Transjunctional delay $\Delta t = 0.84 \pm 0.16$ s

Halidi et al.Cell Calcium, 2011

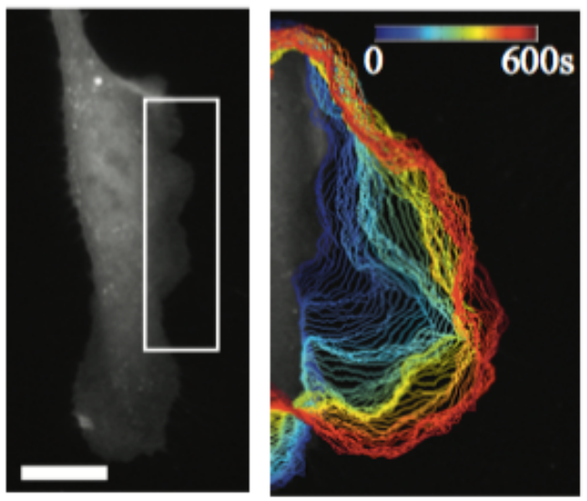
Tracking cell migration on variable stiffness

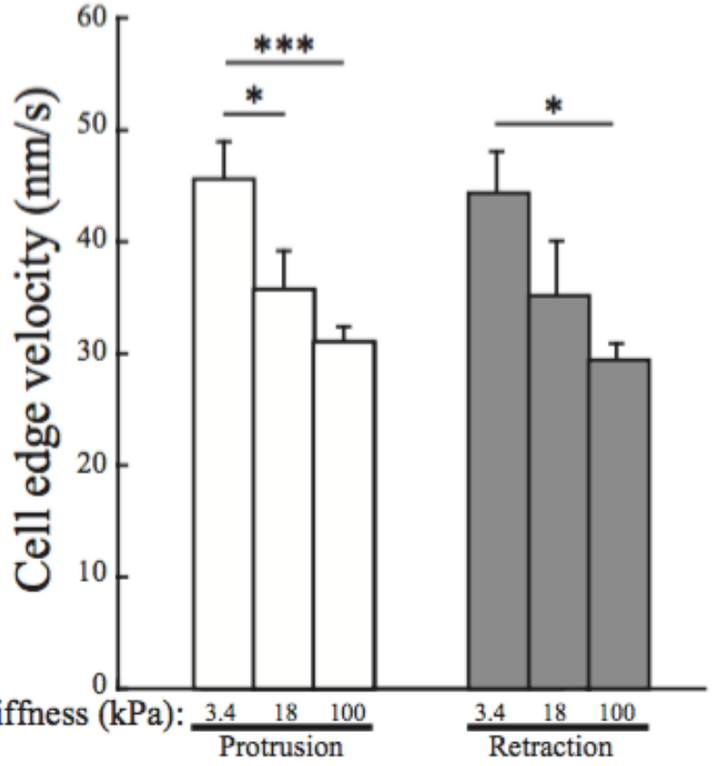


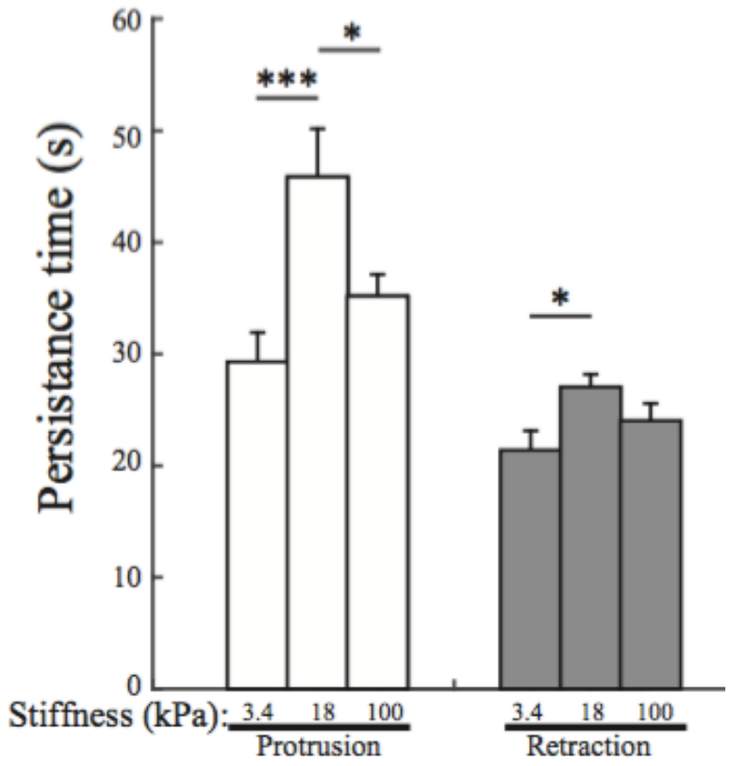
Cell migration on substrates
(*Halidi, unpublished data*)

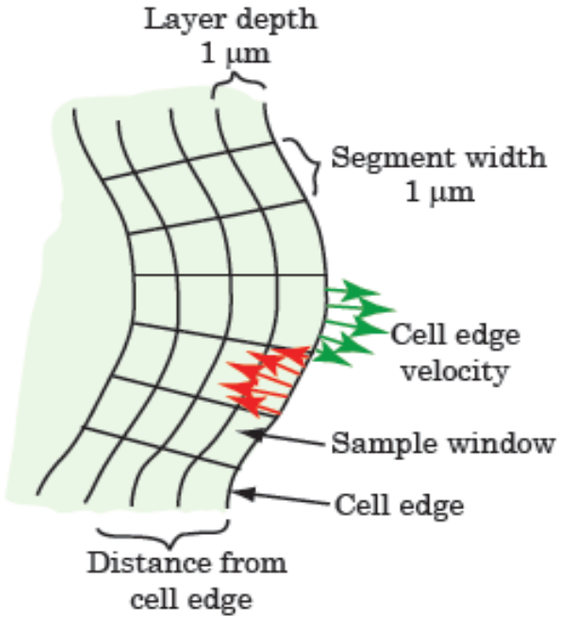


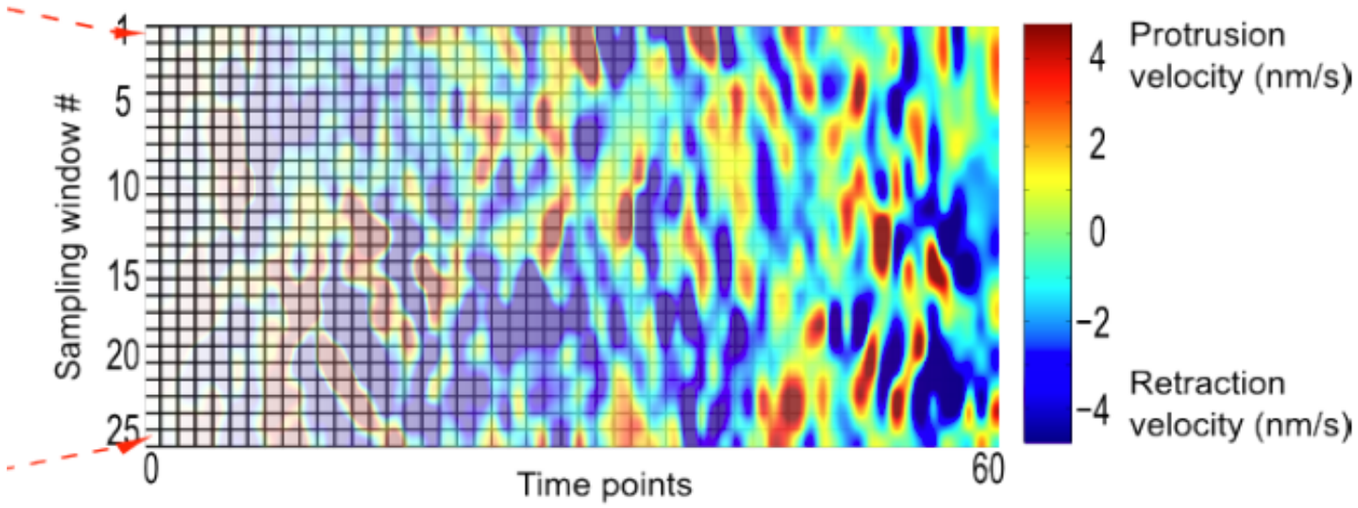
Morphodynamics





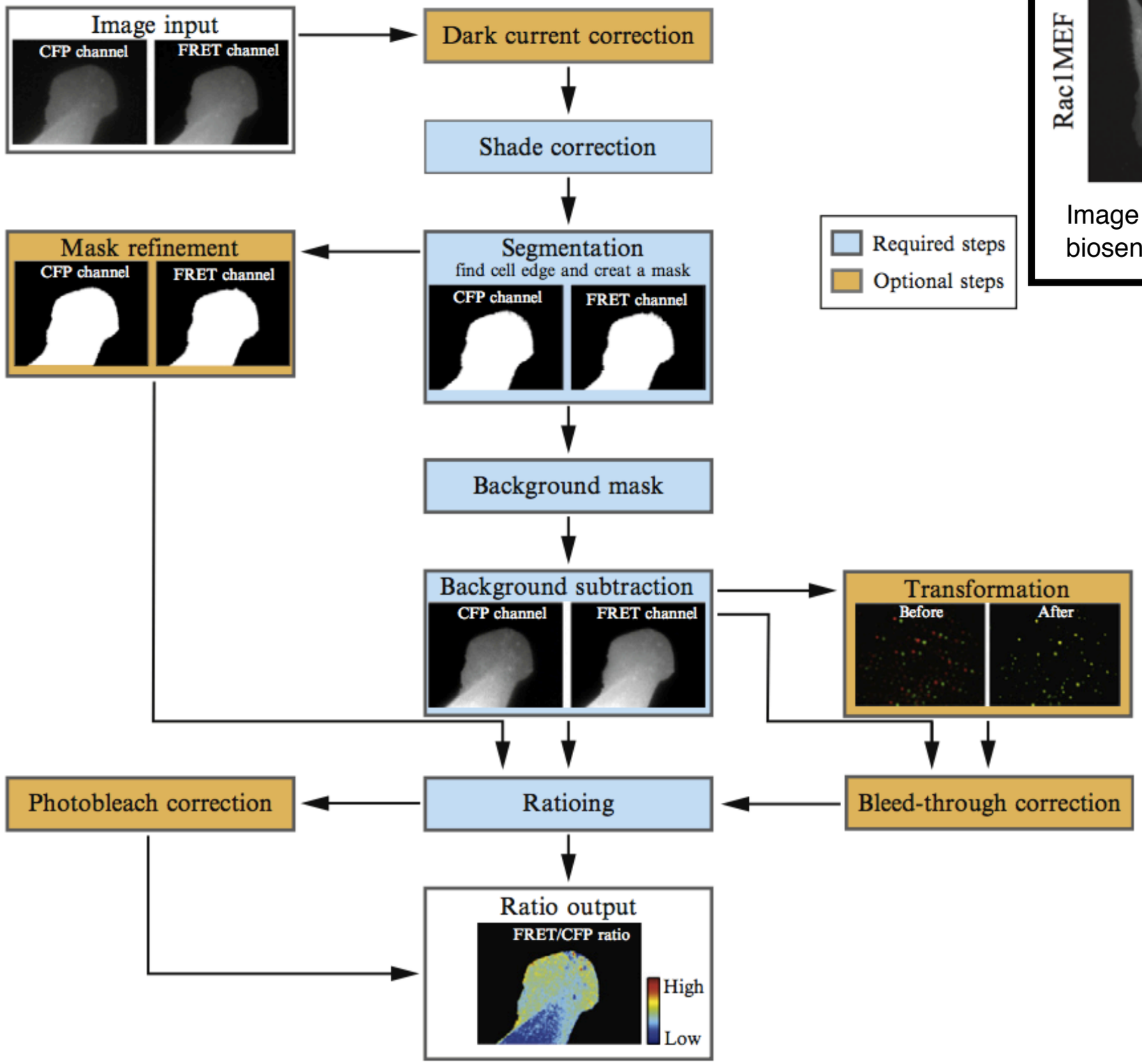






Halidi, unpublished data

FRET experiment



Required steps
Optional steps

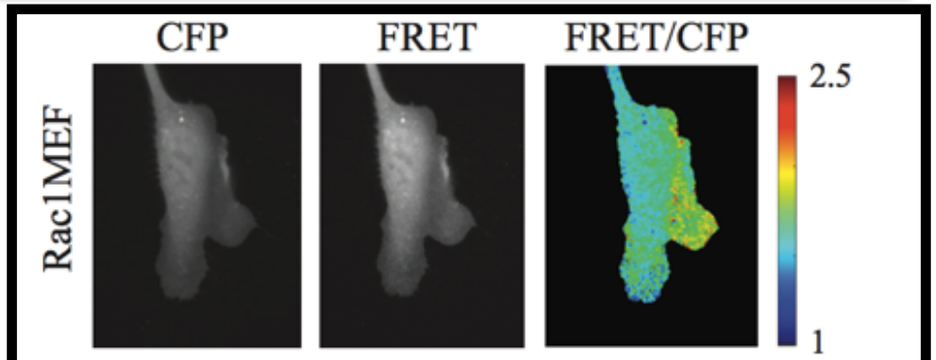


Image corrections and processing for FRET-based biosensor read- outs of signaling activities

Biosensors software downloadable:
http://www.utsouthwestern.edu/labs/danuser
Vilela et. al. 2013 Methods in Enzymology

FRAP experiments

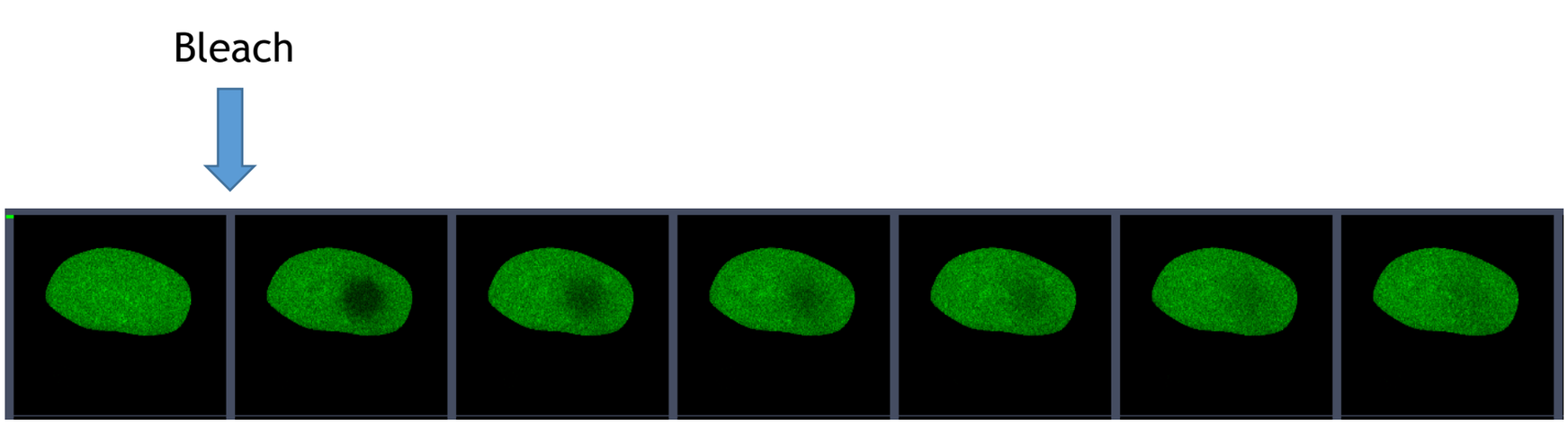
Acquire Images
In different Rois

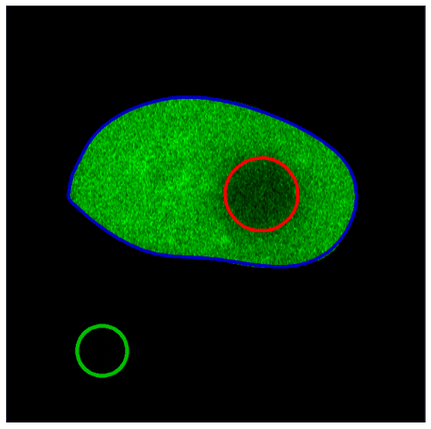
Visualize Raw Data

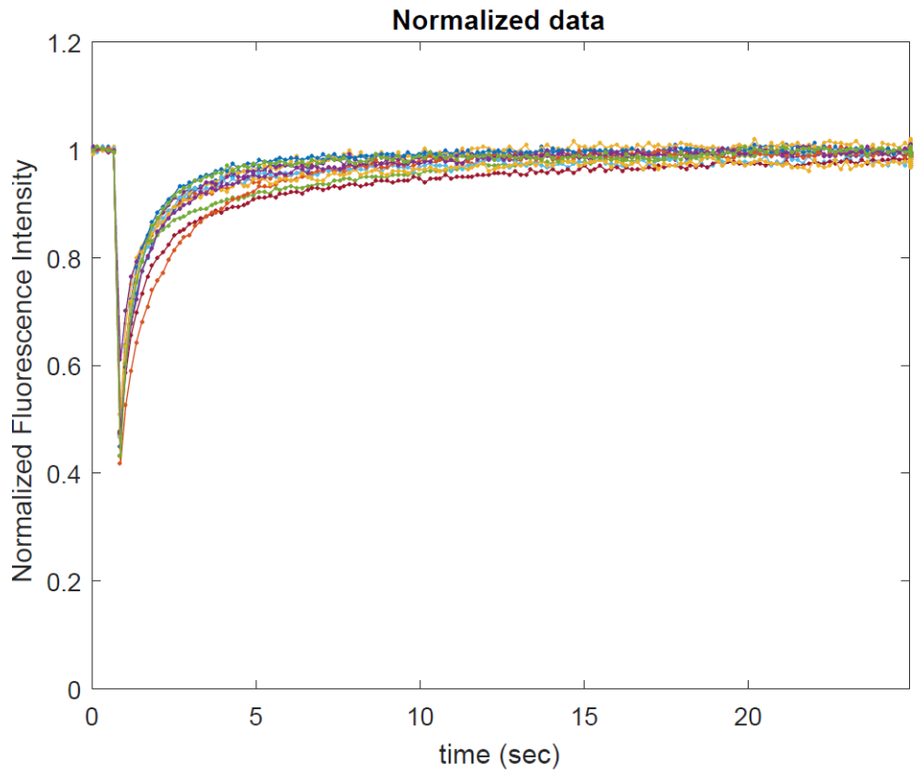
Correct for bg & bleaching

Normalize Data

Curve fitting







Moustakim et al. 2018

Instruments at Micron Imaging Facility

Fluorescence Systems

Widefield fluorescence microscopes
Personal DeltaVision
DeltaVision Core
DeltaVision Elite
DeltaVision Elite 37°C

Scanning confocal systems
ZEISS LSM 780
ZEISS LSM 880 inverted w/ Airyscan
ZEISS LSM 880 upright w/ Airyscan
Olympus FV1000
Olympus FV1200
Olympus FV3000

Spinning disk confocal systems
PerkinElmer UltraVIEW

Lightsheet systems
ZEISS lightsheet Z.1

Super-resolution Systems

Structured illumination
DeltaVision OMX V2
DeltaVision OMX V3

Photoactivated localization
Bespoke PALM/TIRF

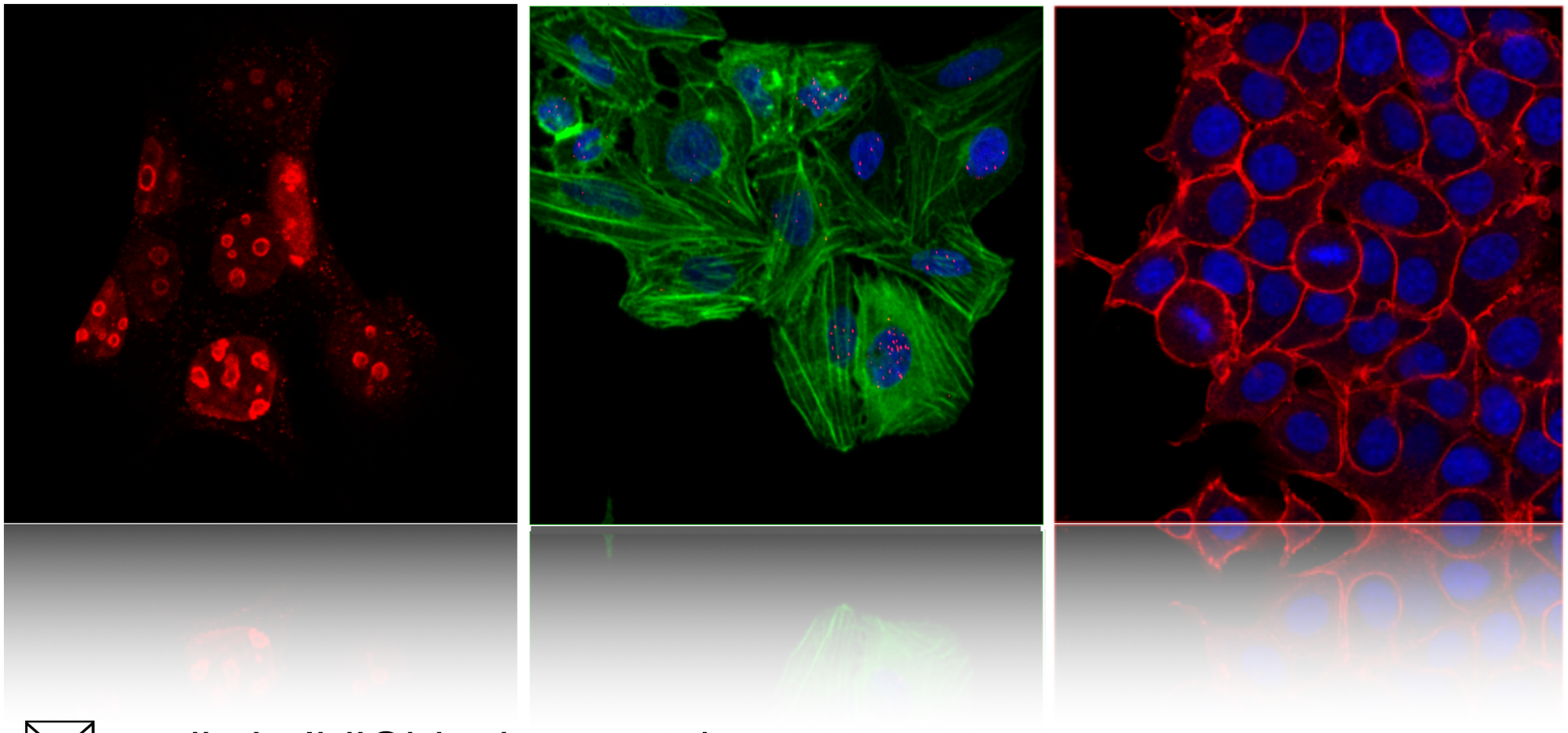
Stochastic optical reconstruction
Nanoimager

Image Analysis Suite

OME database
Image processing and analysis softwares

FIJI/ImageJ	Imaris
Arivis	SoftWorx
MatLab	Volocity
Chromagnon	Zen blue

Thank you!



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