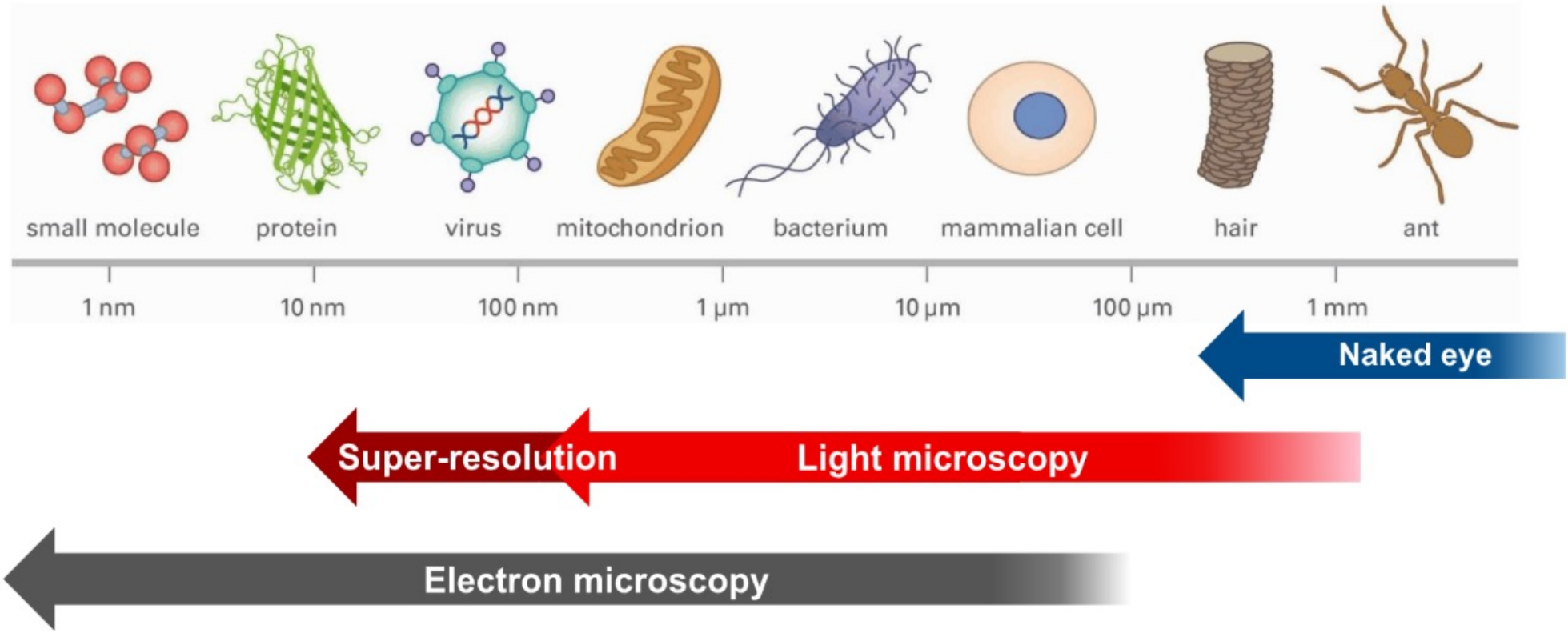
Tips and Pitfalls in Microscopy

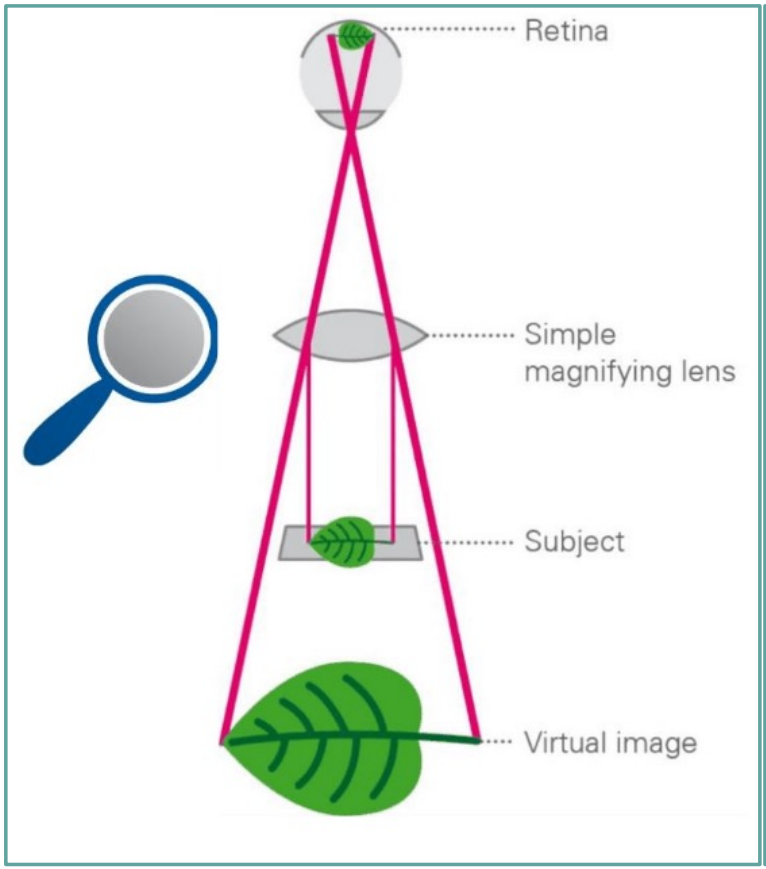
ALEXIA FERRAND
IMAGING CORE FACILITY
BIOZENTRUM
BASEL

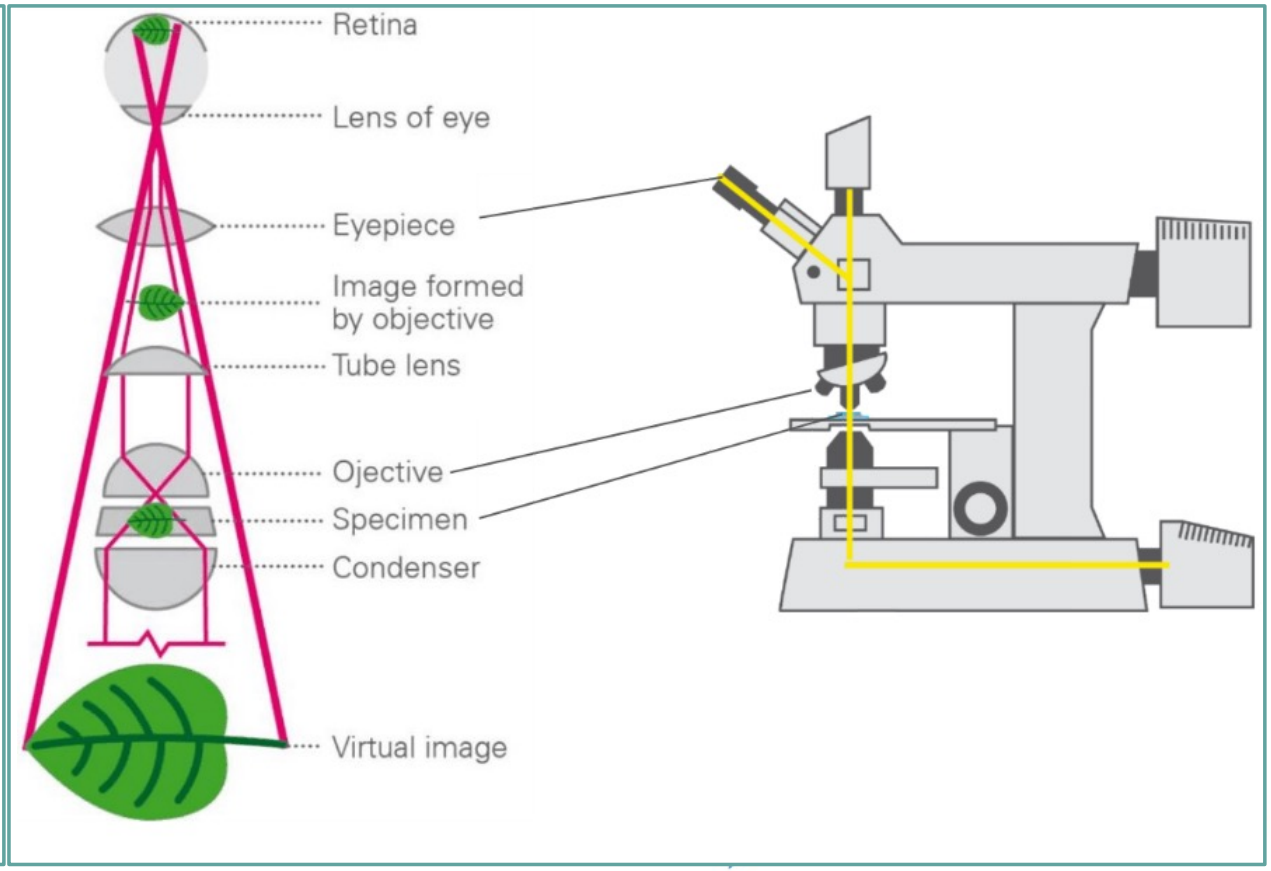
Why use a microscope?



Why use a microscope?

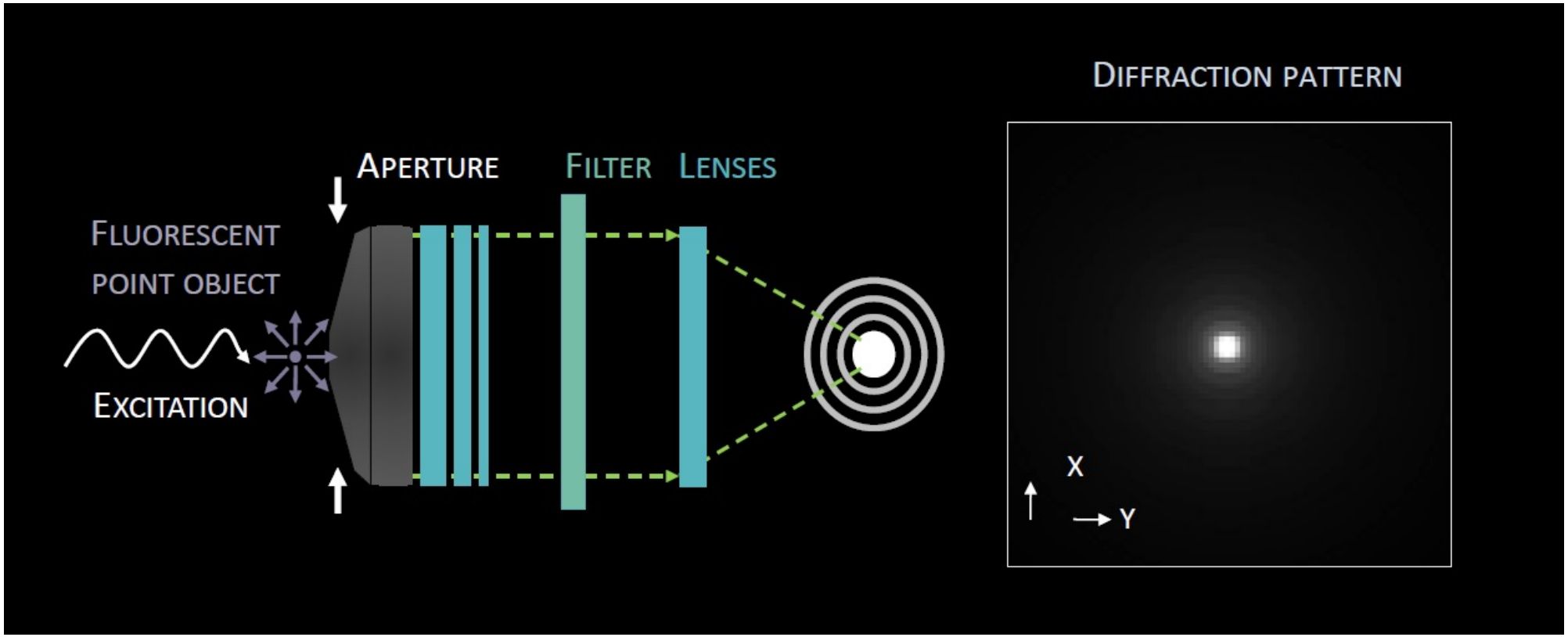
Magnification





Why use a microscope?

Resolution

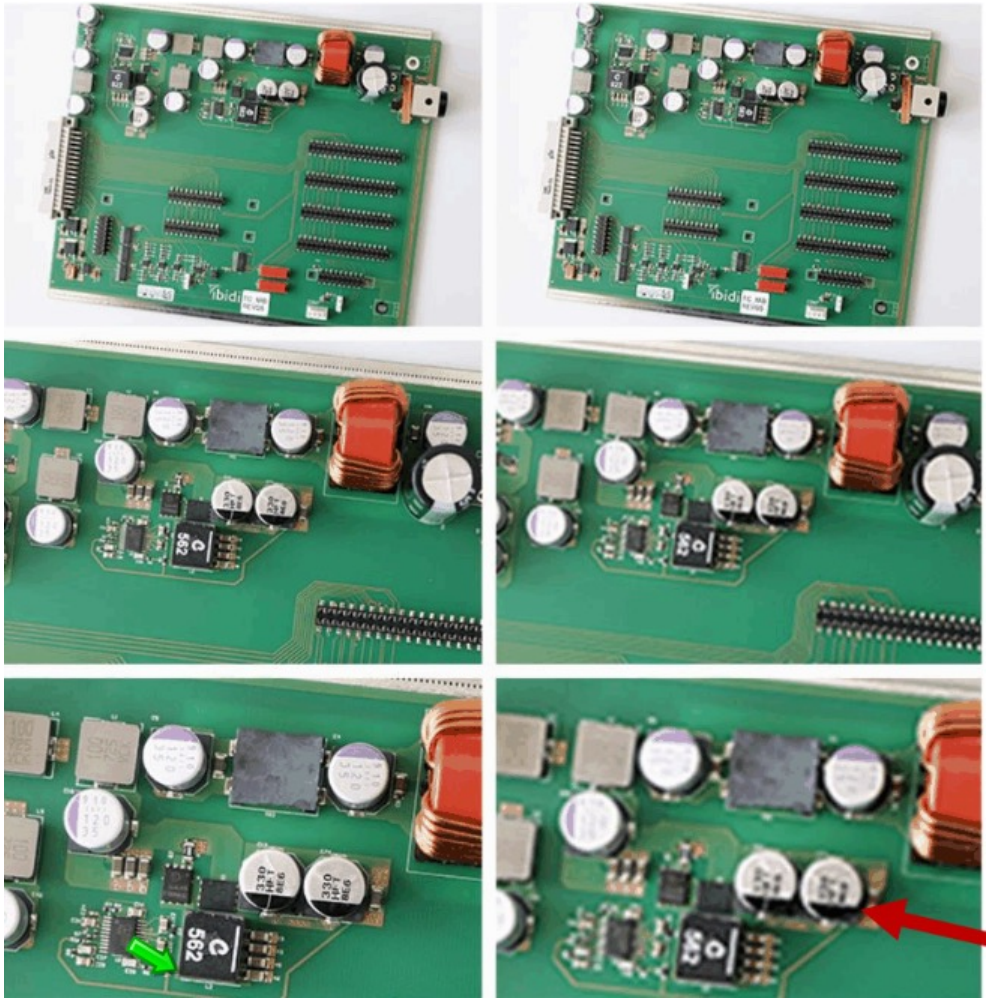


Why use a microscope?

Resolution: Smallest distance between 2 point sources such that their diffraction patterns show a detectable drop in intensity between them.

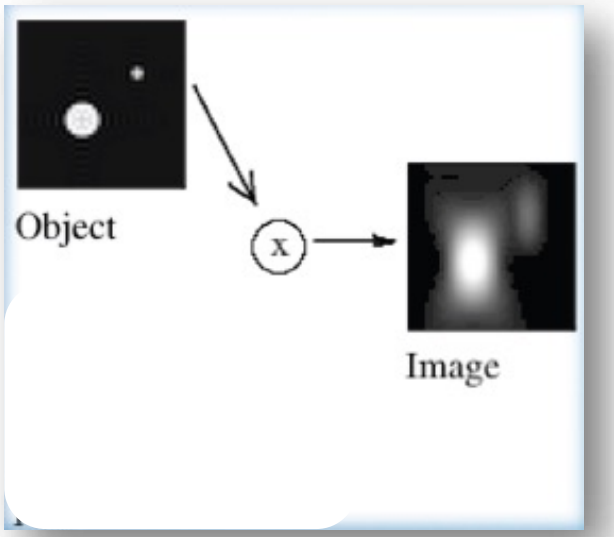
Why use a microscope?

Magnification versus Resolution:



Magnification can be meaningless if the necessary resolution is lacking.

Basic optics…

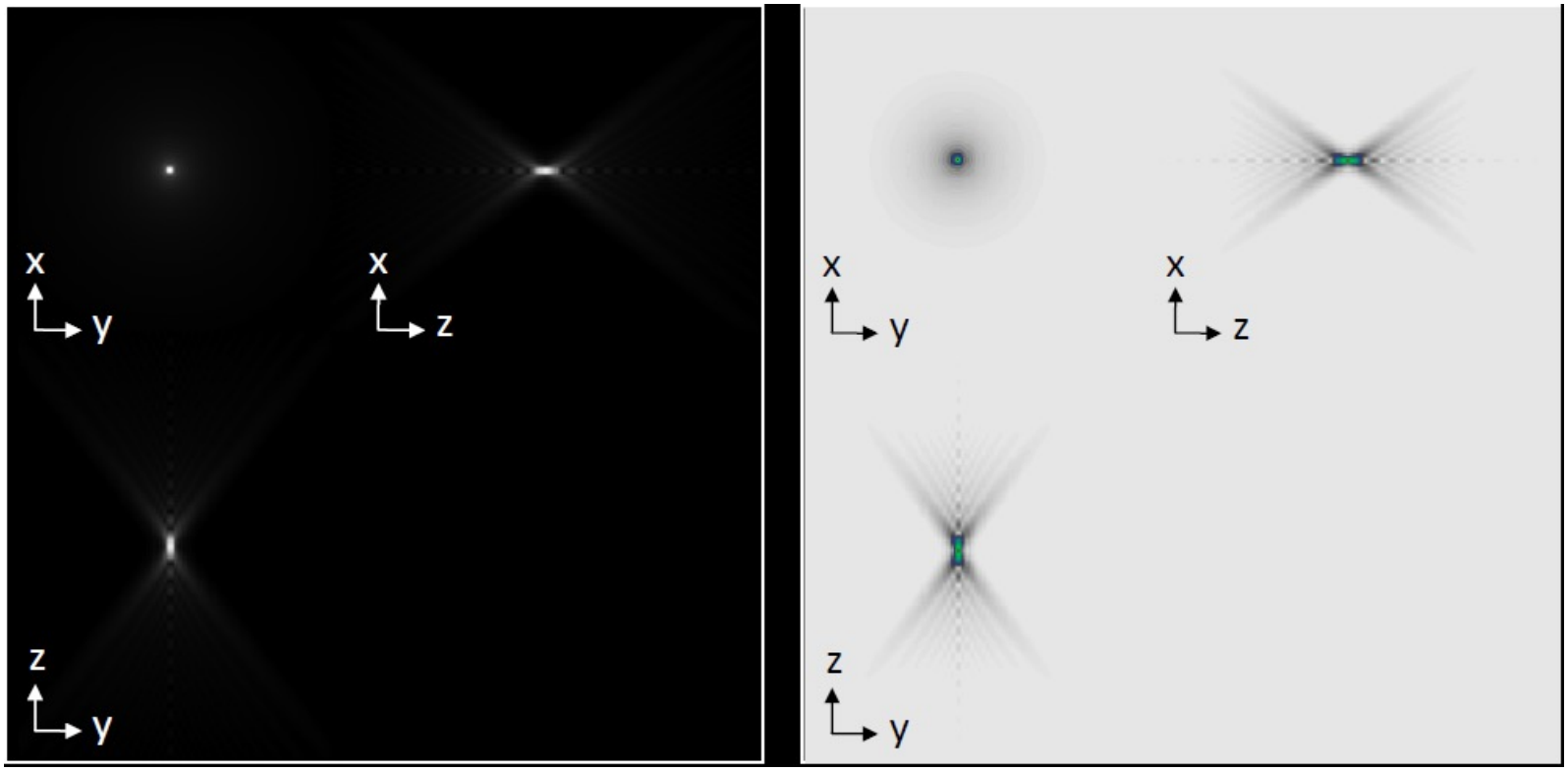


Images are not identical to the object

Image = Object x Point Spread Function

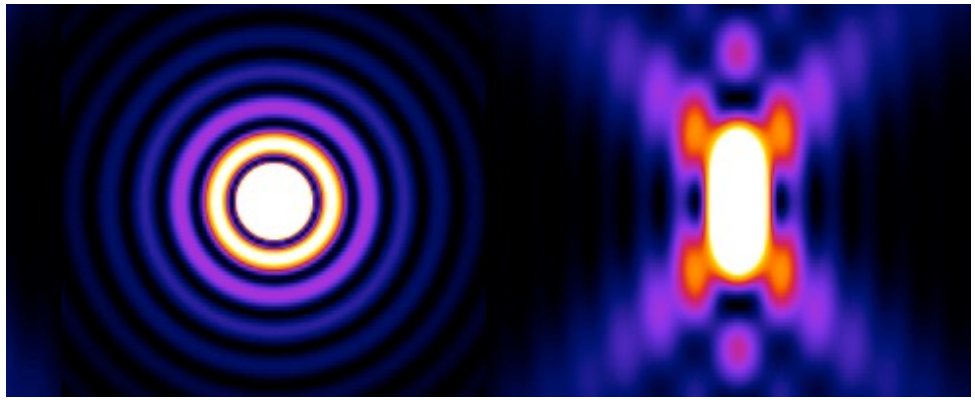
Basic optics…

- **Point Spread Function:** Describes the response of an imaging system to a point source or point object.



Basic optics…

- The Airy Disk*: Caused by diffraction on objective aperture
- Negative impact on resolution



resolution= 0.61 x λ / n sin α

Where: n sin α = NA

n = RI of medium

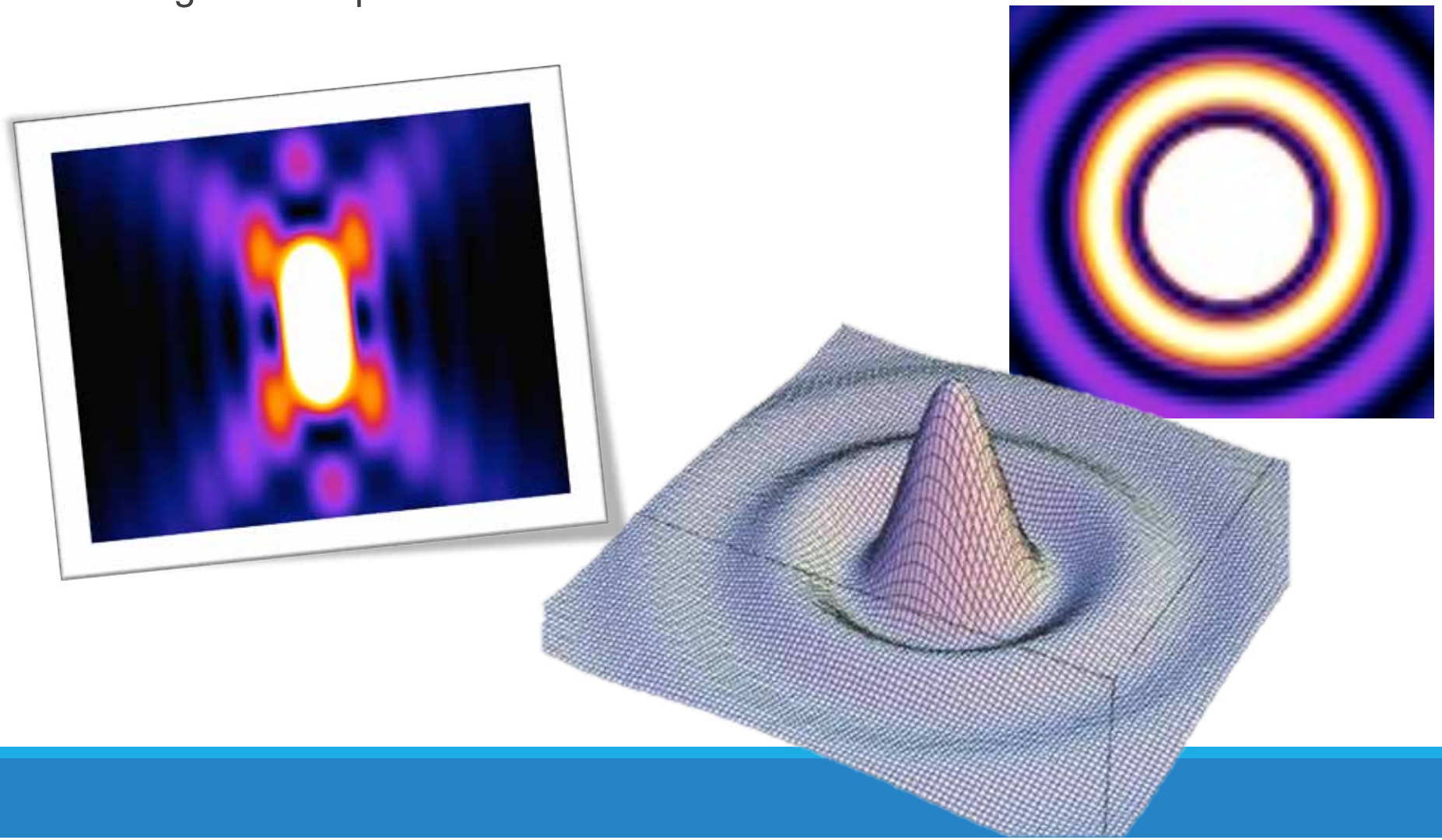
α = 1/2 objective collection angle

λ = wavelength of light (Emission)

**after GB Airy, British astronomer, 1834*

Basic optics…

- **Point Spread Function:** A good PSF of an object (bead) should have an "hourglass" shape.



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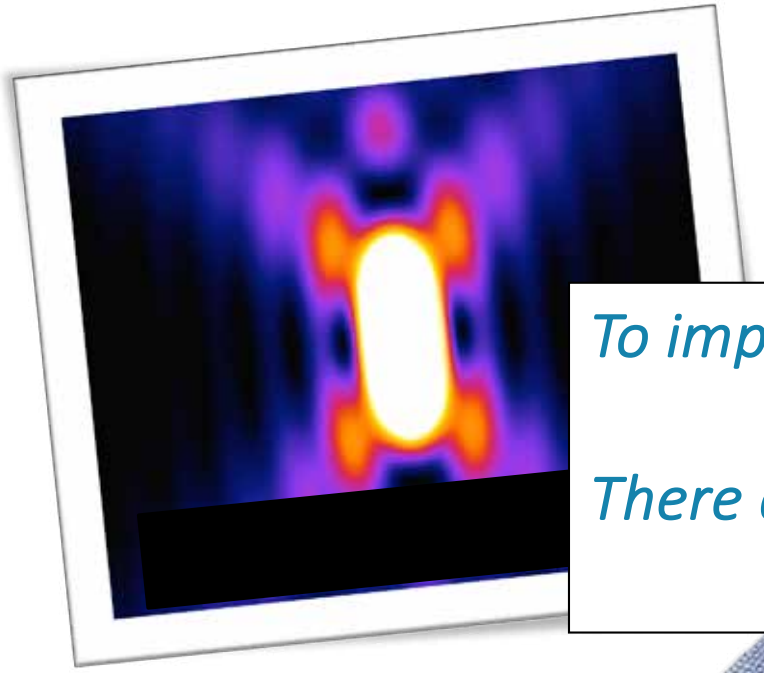
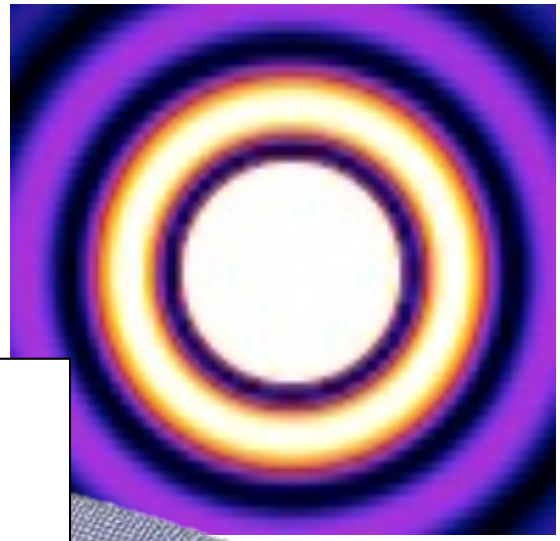
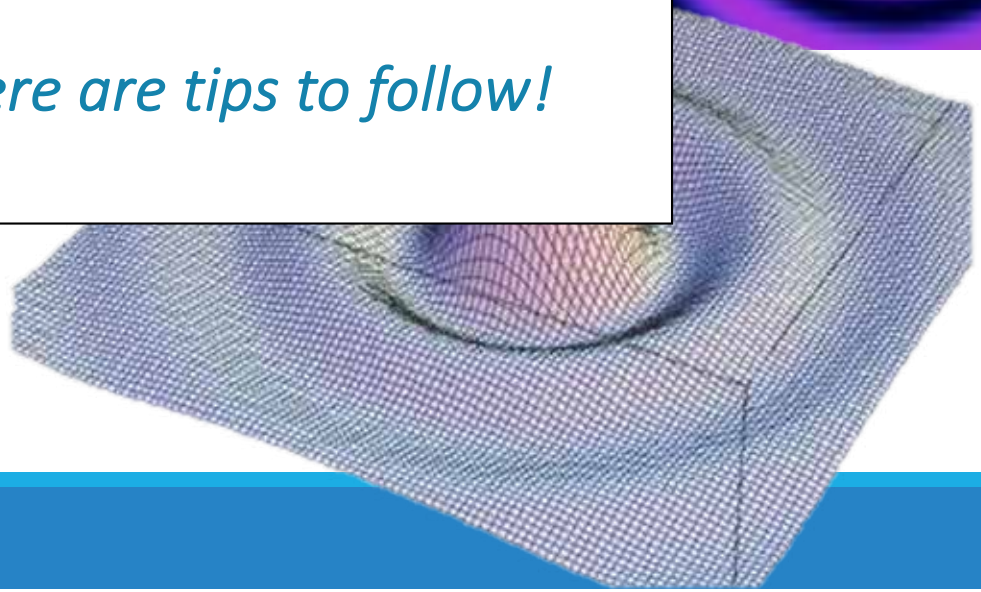






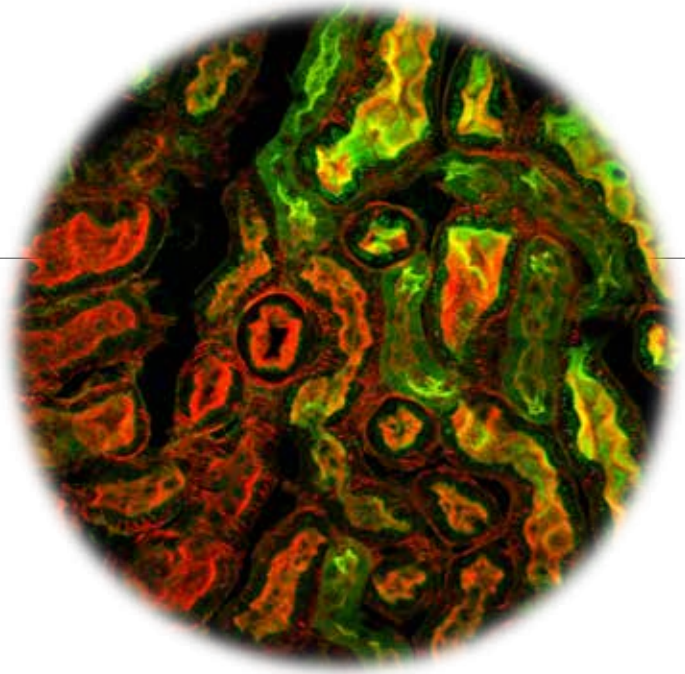
To improve your images

There are tips to follow!

Outline

- Image Acquisition for fixed samples
- Image Acquisition for live imaging
- Image Processing

Image Acquisition for fixed samples



The brighter, the better!*

Image taken on our confocal SP5-Matrix room 07.079 (AF)

*Except when it saturates...

SAMPLE Fixation

- «Standard» Fixation: PFA
- Alternative: 100% MeOH
- Methanol works nicely for microtubules and centrosomes
- For fluorescent proteins, DON’T USE MeOH!
- Also poor results with phalloidin when using MeOH

Immuno-Staining

- Blocking step with BSA or serum
- Primary Ab
  - 30 min-1h (cells)
  - 1h – days (tissues sections/organs)
- Secondary Ab
  - 30 min-1h
- Washing steps
  - Many short ones better than couple long ones

- Store in the dark at 4°C or -20°C depending on your mounting media.

Careful experiment planning

Do proper controls	• Single labelled controls with each fluorochrome to check all channels (Pos/Neg)
When > 1 color, cross-talk and bleedthrough can happen	• Make sure that Excitation and Emission spectra are well separated. • Use sequential scan if needed • Check the filter sets before choosing your fluorophores

Careful experiment planning

https://www.biozentrum.unibas.ch/imcf/links-downloads

Careful experiment planning

Antibodies, fluorescence dyes, fluorescent proteins, filters:

- BenchSci - A.I. Driven Antibody Search
- Fluorescence spectra viewers:
  - FPbase - a database of fluorescent proteins.
  - SearchLight (Semrock)
  - Fluorescence SpectraViewer - Plot and compare spectra and check the spectral compatibility for many fluorophores.
  - Table of Fluorochromes @ Iowa State University - check for excitation and emission maxima of most of the current fluorochromes.
- Fluorescent Biosensor Database - Repository of genetically encodable fluorescent biosensors.

Biology resources:

- The Human Protein Atlas - Database of proteins' distribution in cells, tissues and organs.
- Allen Brain Map - Brain atlases and advanced tools for neurobiologists.
- Image Data Resource (IDR) - Public repository of image datasets from published scientific studies.
- BioNumbers - Search tool for molecular biology associated numbers

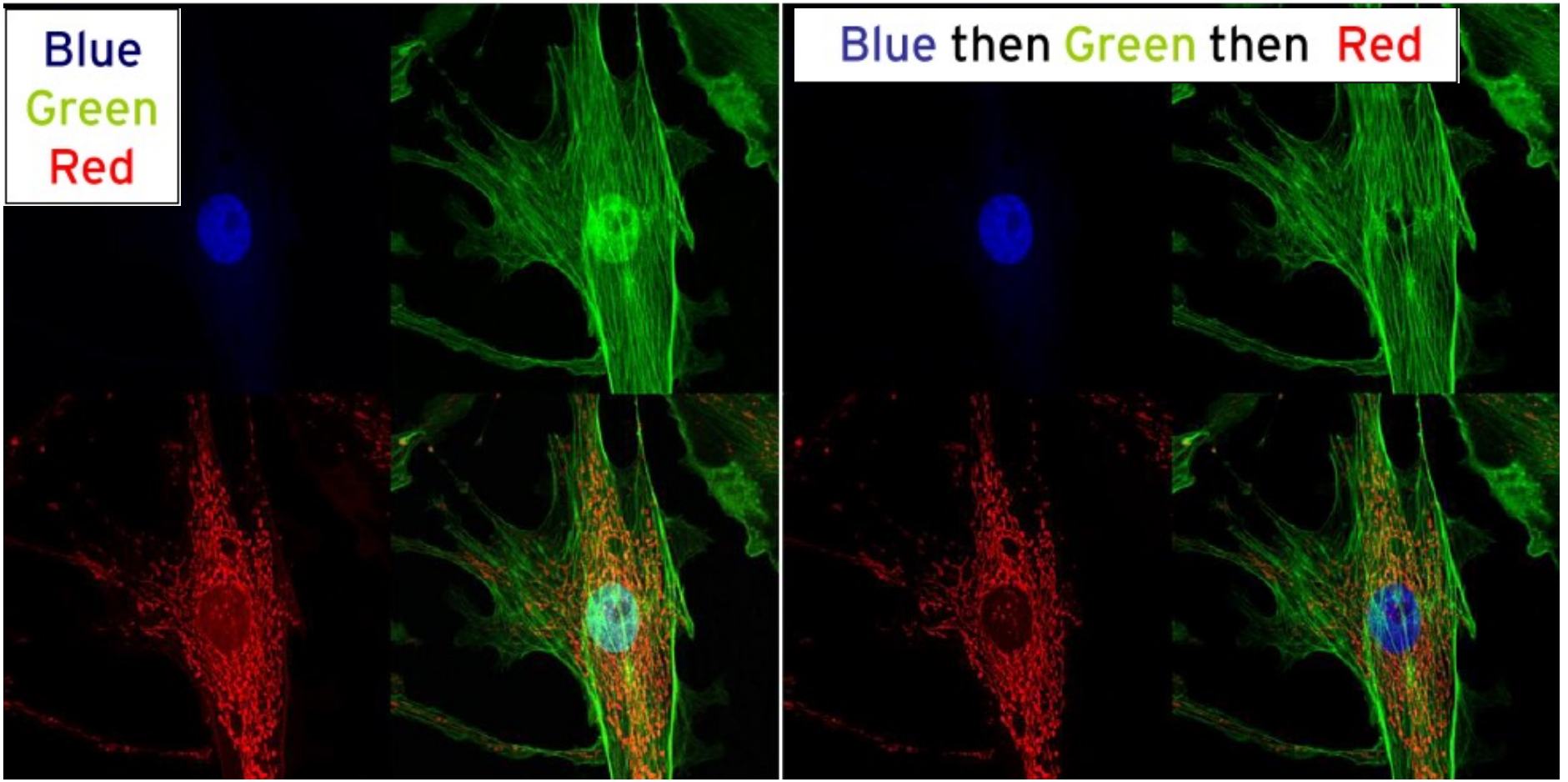
Multi-labeling

SIMULTANEOUS

Faster

SEQUENTIAL

Less bleedthrough



Sample Preparation

The sample is part of the optics

The last 500 um are important

1 – coverglass

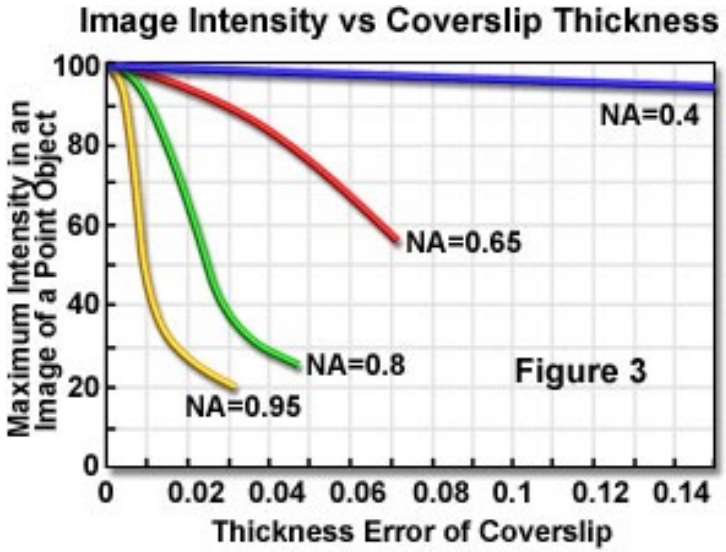
MOST COMMUN SIZE
0.17 mm = N°1.5

Sample Preparation

The sample is part of the optics

The last 500 um are important

1 – coverglass (spherical aberrations)



Performance Reduction with Coverslip Thickness Variation

NumericalAperture	0.01 mm Deviation	0.02 mm Deviation
0.30	none	none
0.45	none	none
0.70	2 percent	8 percent
0.85	19 percent	57 percent
0.95	55 percent	71 percent

http://www.olympusmicro.com

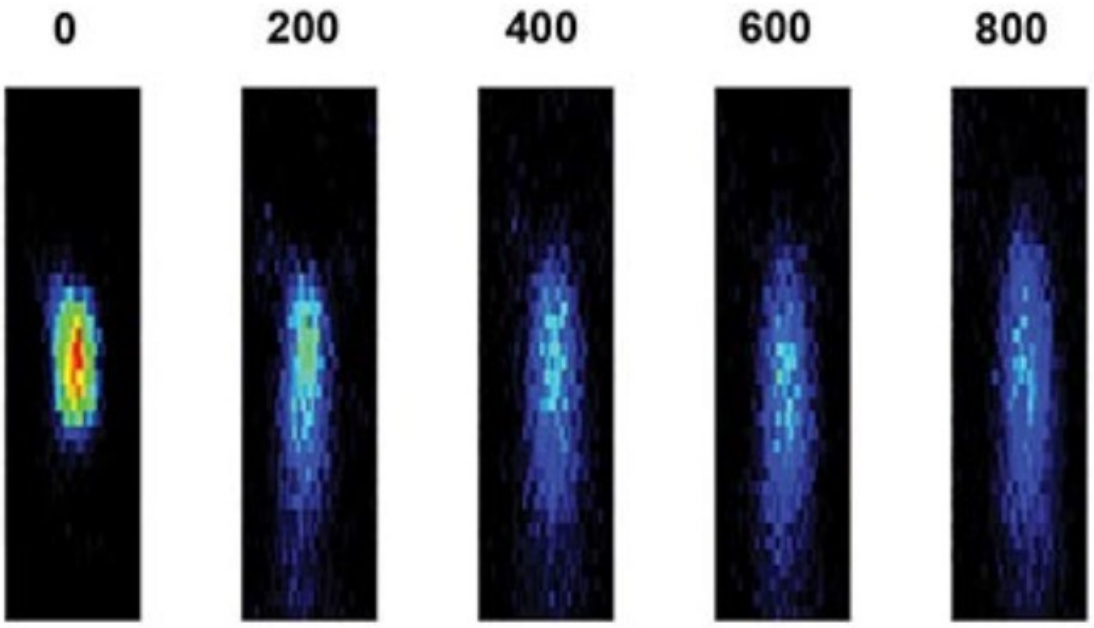
Sample Preparation

The sample is part of the optics

The last 500 um are important

1 – coverglass (spherical aberrations)

Grow cells directly on the coverglass
//
Mount your specimen on the coverglass



Sample Preparation

The sample is part of the optics

The last 500 um are important

2 – mounting media (avoid bubbles)

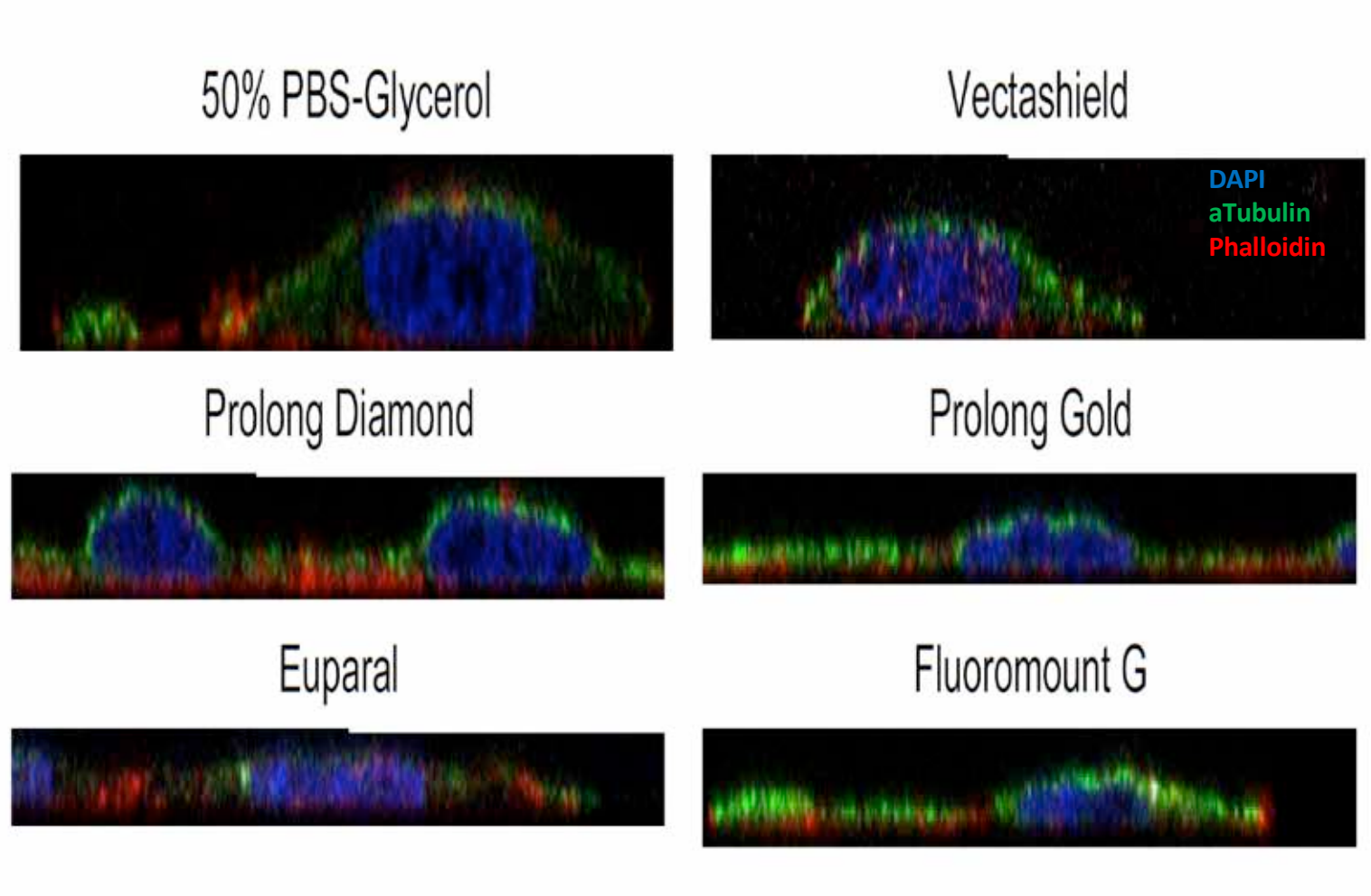
THERE IS NO «one fits all» MOUNTING MEDIA

Permount (Biomeda)	1.54
glas	1.52
immersion oil	1.52
Canada balsam	1.52
DPX (Fluka)	1.52
Fluorescence Mounting Medium (DAKO)	1.47 – 1.50
Mowiol (Calbiochem)	1.49 - 1.41?
ProLong Gold (Molecular Probes)	1.47 - 1.41 (increases over time)
Kaisers glycerol jelly	1.47
Vectashield (Vector Labs)	1.46
Vectashield / glycerol + 2% DABCO	1.46
glycerol / water in different ratios	1.47 – 1.33
glycerol 100%	1.47
glycerol 90% (w/w)	1.46
glycerol 80% (w/w)	1.45
Aquatex (Merck)	1.40
albumin/water	1.42 - 1.33
Fluoromount-G (Southern Biotech Assoc.)	1.39
Gelmount (Biomeda)	1.36
water	1.33
air	1.00

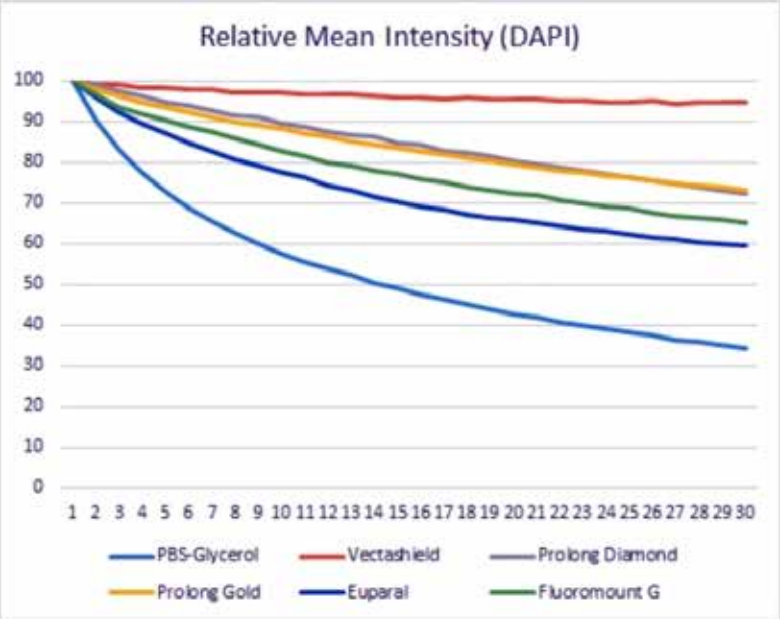
Source: Leica

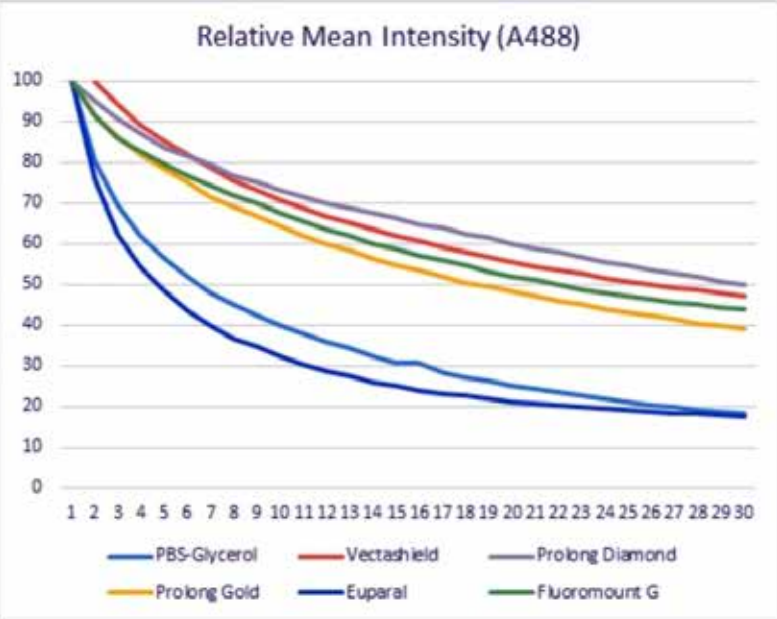
Preserve 3D information

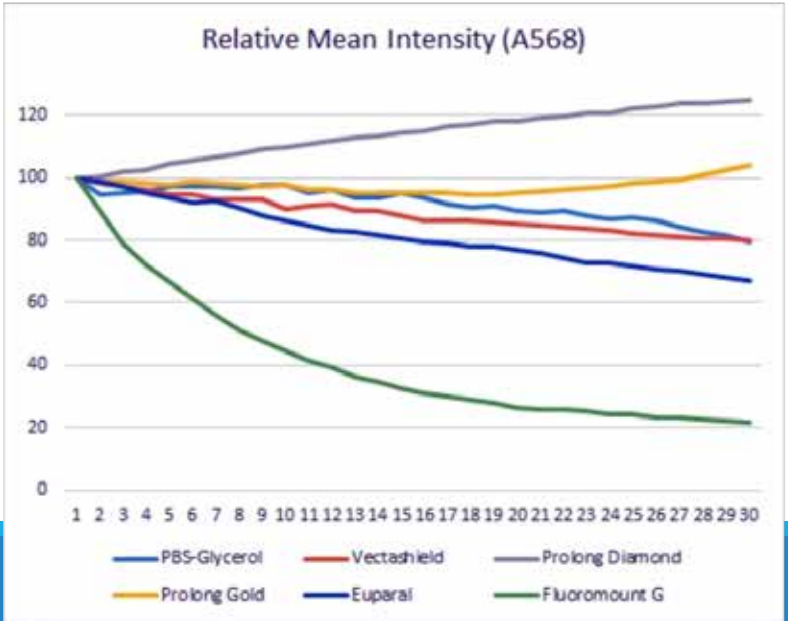
Some mounting media can flatten the sample

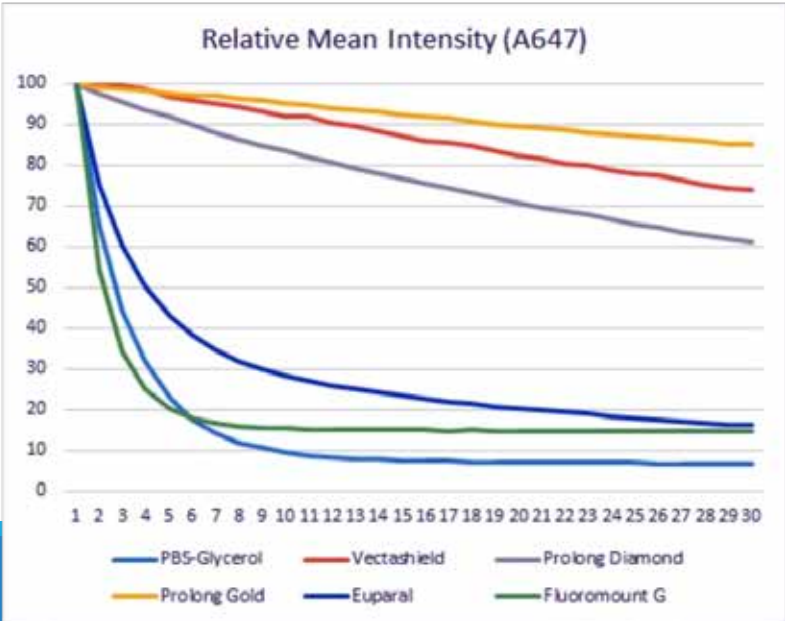


Which mounting media?







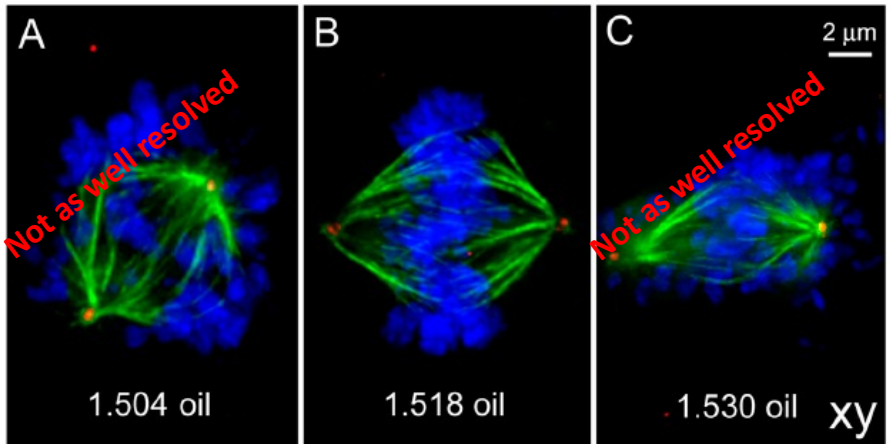


Sample Preparation

The sample is part of the optics

The last 500 um are important

3 – matching the refractive indexes (spherical aberrations)



U Plan-Apo 100X / 1.35 NA objective
Microtubules, **centrosomes** and **DNA** are stained. *Seeing is believing? A beginners' guide to pratical pitfalls in image acquisition. AJ North, JCB, 2006*

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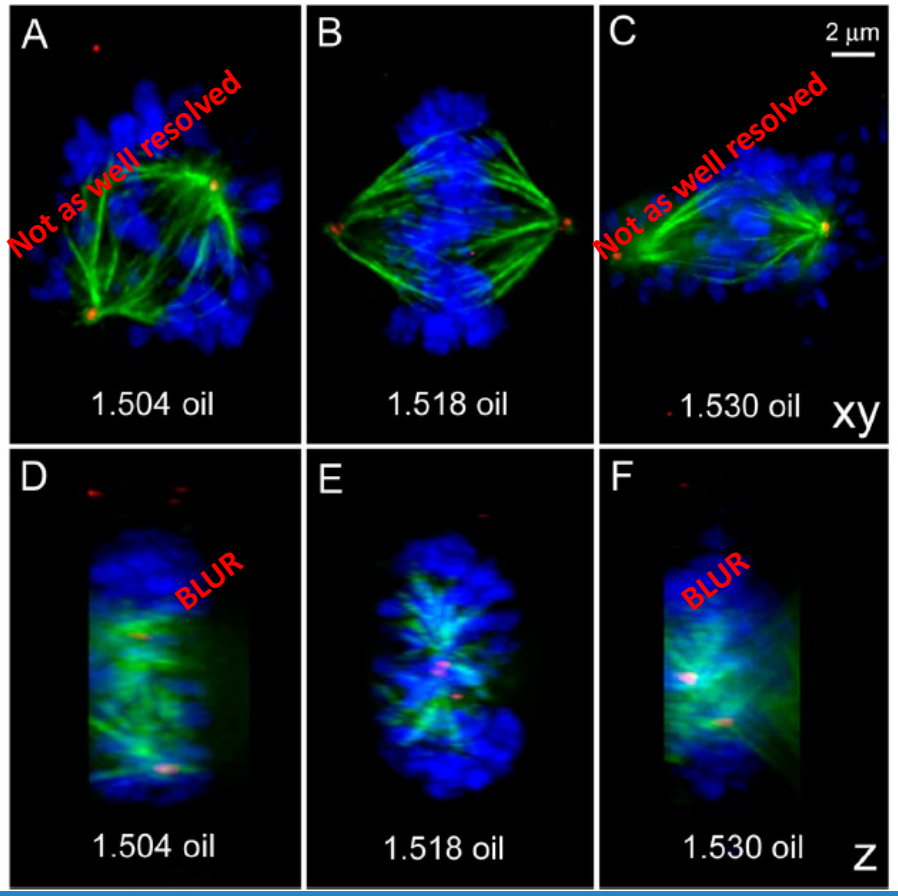

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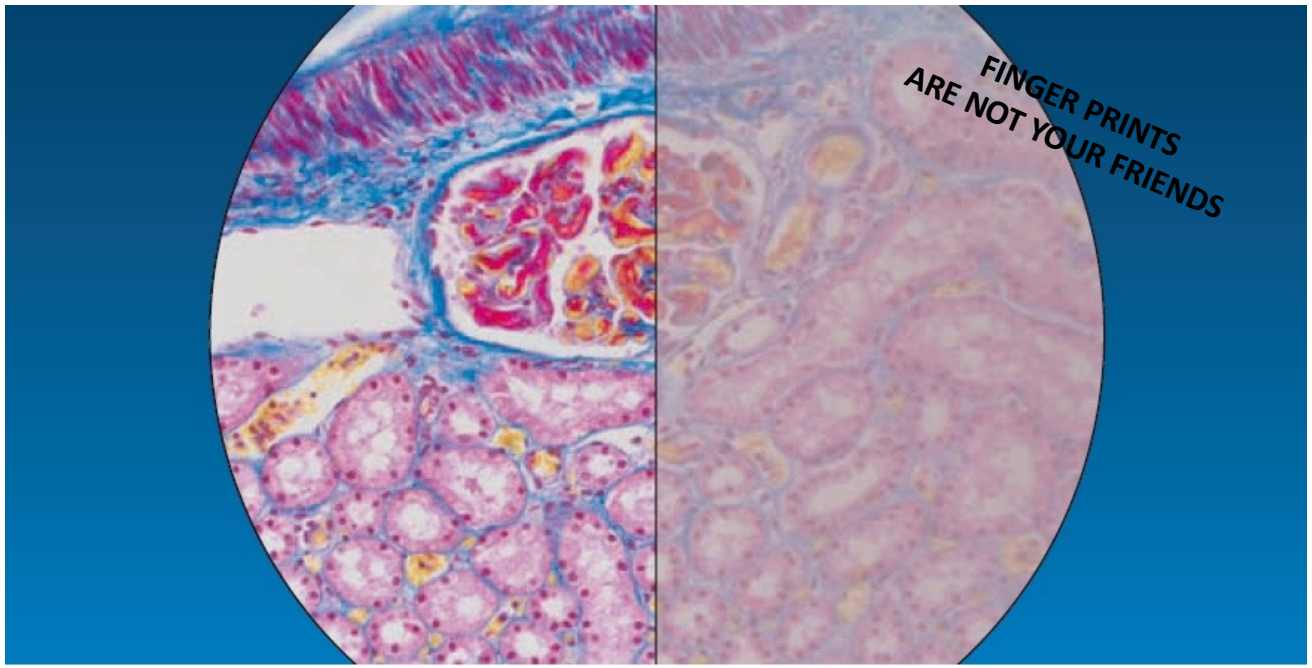
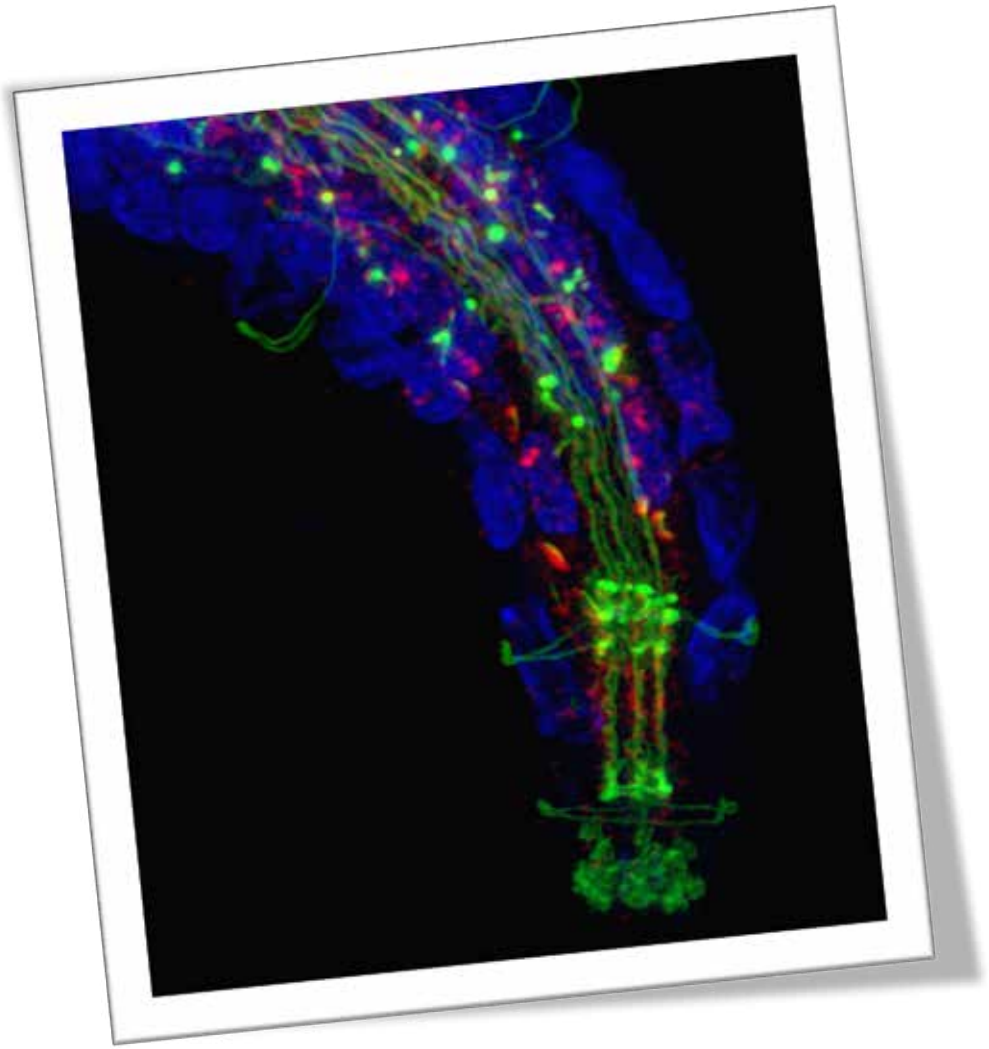
4 – Schmutz effect



What information can you get from the objective lens?

Choice of the lens

What influences the resolution?

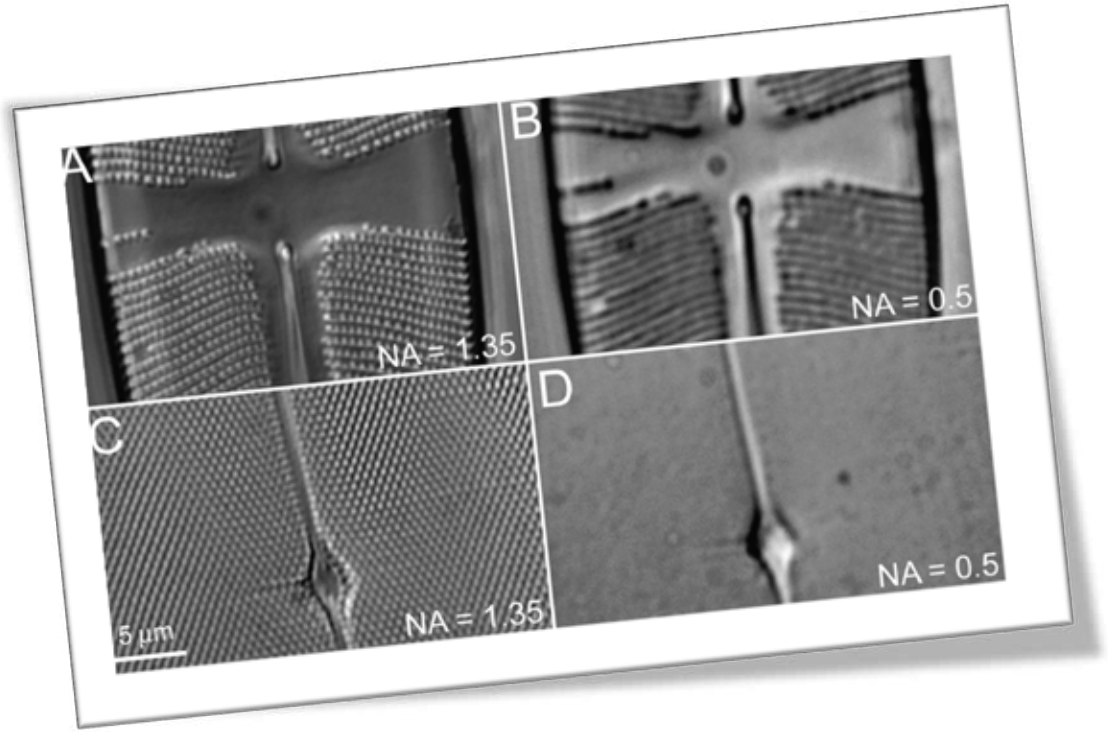


C. elegans larvae taken on a Zeiss Elyra 7 Lattice SIM (P. Ray and A. Ferrand)

Choice of the lens

What influences the resolution?

- **NA of the objective (Light gathering ability)**
  - **The higher the NA, the better the details,**

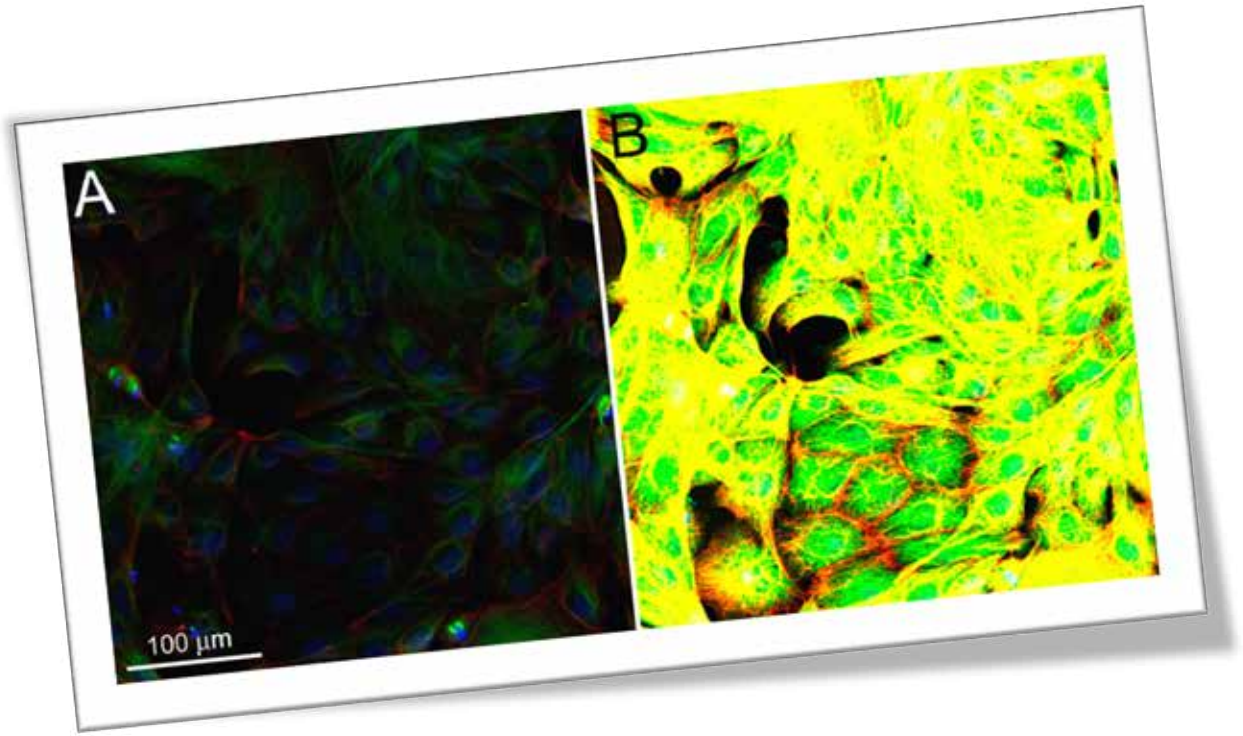


Diatoms captured using the same 100X lens with an adjustable NA (0.5 – 1.35). *Seeing is believing? A beginners' guide to pratical pitfalls in image acquisition. AJ North, JCB, 2006*

Choice of the lens

What influences the resolution?

- **NA of the objective (Light gathering ability)**
  - The higher the NA, the better the details,
  - And the **higher the intensity**

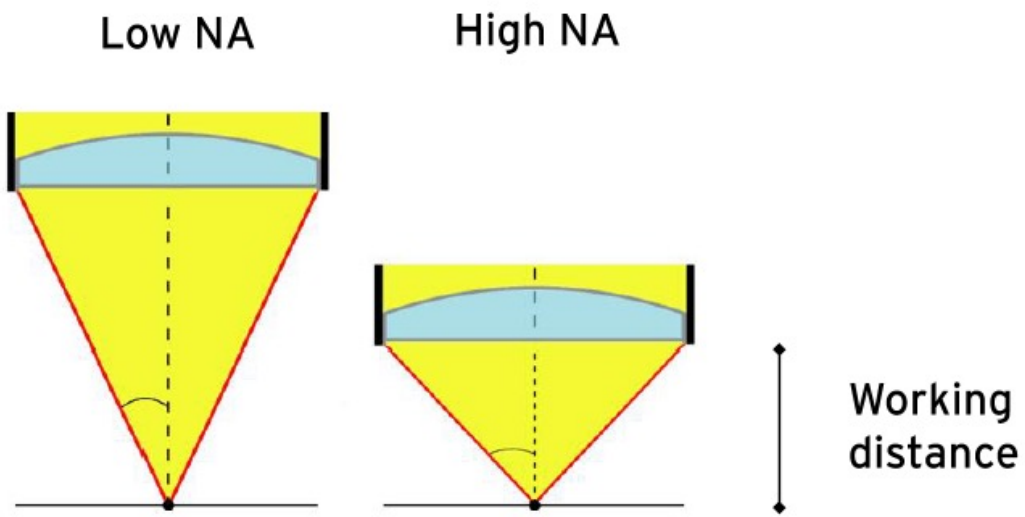


MDCK epithelial cells imaged with a confocal using identical settings. A uses a 10X / 0.25 NA. B uses a 10X / 0.45 NA. *Seeing is believing? A beginners' guide to pratical pitfalls in image acquisition. AJ North, JCB, 2006*

Choice of the lens

What influences the resolution?

- **NA of the objective (Light gathering ability)**
  - The higher the NA, the better the details,
  - And the higher the intensity
  - **A high NA reduces the working distance**



Choice of the lens

What influences the resolution?

- **Good refractive index match**

A refractive index mismatch gives rise to geometrical aberrations.

Choice of the lens

What influences the resolution?

- **Pixel size**

Question

What objective will have the best resolution?

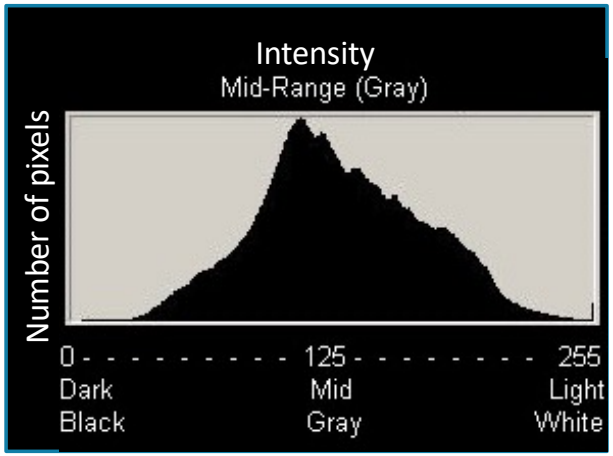
- 60X, 1.42 NA, zoom 1.6
- 60X, 1.4 NA, zoom 1.6
- 100X, 1.4 NA, zoom 1

Drosophila brain with Neurotactin in red and GFP-tubulin clone in green. Sample N. Riebli, Image W. Büttner taken on our LSM 700 inverted confocal.

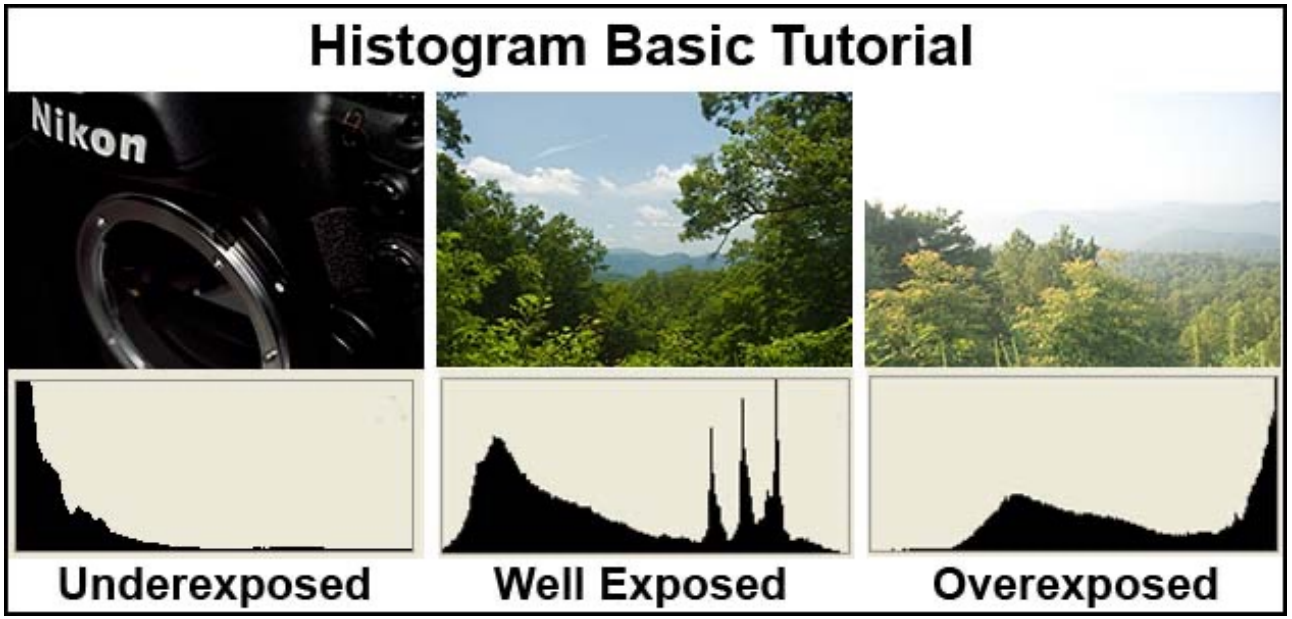
Fluorescence

The brighter the better, BUT NO SATURATION

- Otherwise, no quantification will be possible
- Check your histogram



8 bit (0-255 gsl)

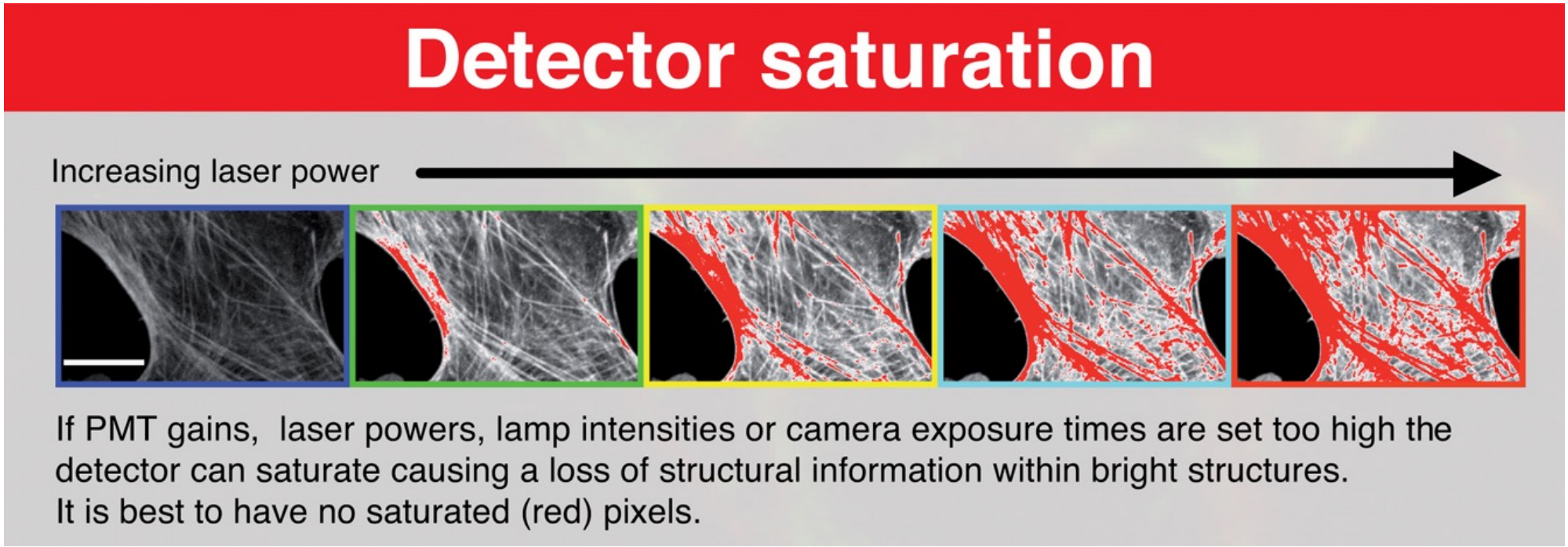


http://www.digitaldarrell.com/article-understandingyourdigitalcamerashistogram.asp

Fluorescence

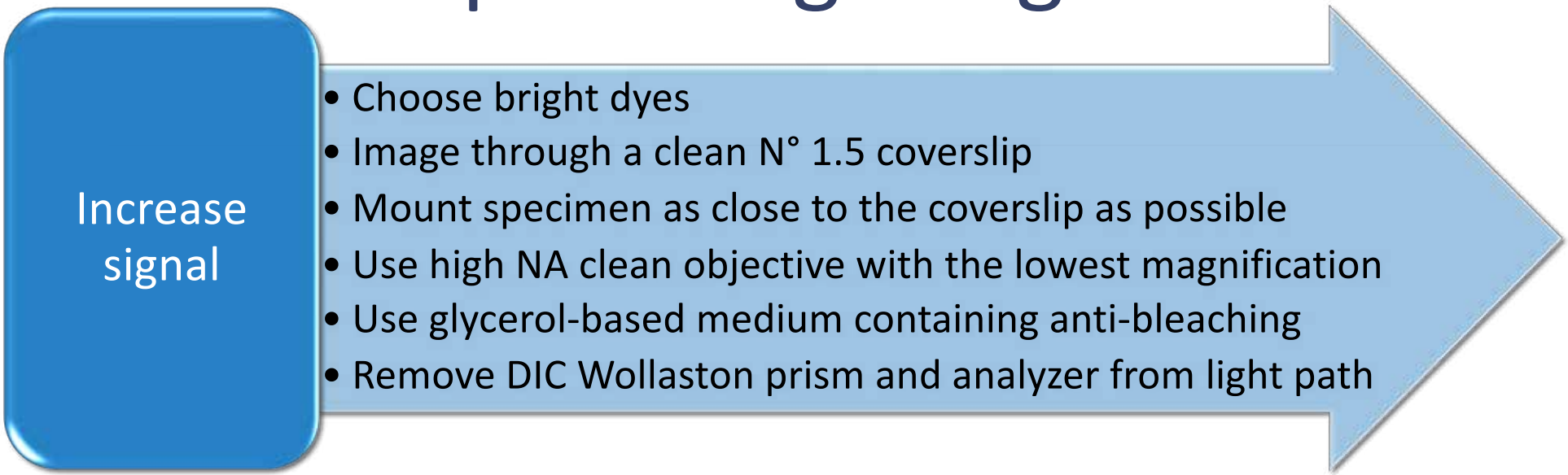
The brighter the better, BUT NO SATURATION

- Otherwise, no quantification will be possible
- Use the visual help from the acquisition software



Fluorescence Microscopy – avoiding the pitfalls. CM Brown, JCS, 2007

Optimizing images



Adapted from Accuracy and precision in quantitative fluorescence microscopy. JC Waters, JCB, 2009

Optimizing images

Increase signal

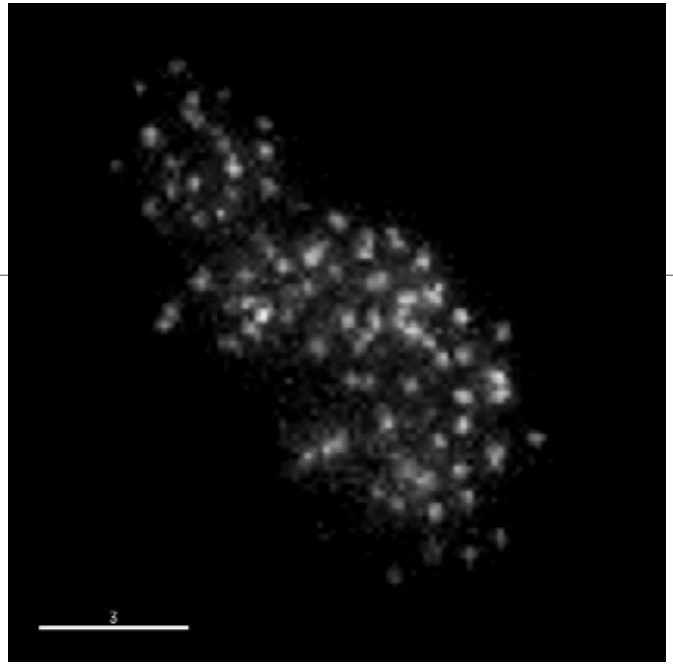
- Choose bright dyes
- Image through a clean N° 1.5 coverslip
- Mount specimen as close to the coverslip as possible
- Use high NA clean objective with the lowest magnification
- Use glycerol-based medium containing anti-bleaching
- Remove DIC Wollaston prism and analyzer from light path

Decrease background

- Clean coverslip and optics
- Perfect fluorophore labeling protocol to minimize nonspecific labeling
- Turn off the room lights

Adapted from Accuracy and precision in quantitative fluorescence microscopy. JC Waters, JCB, 2009

Image Acquisition for live imaging



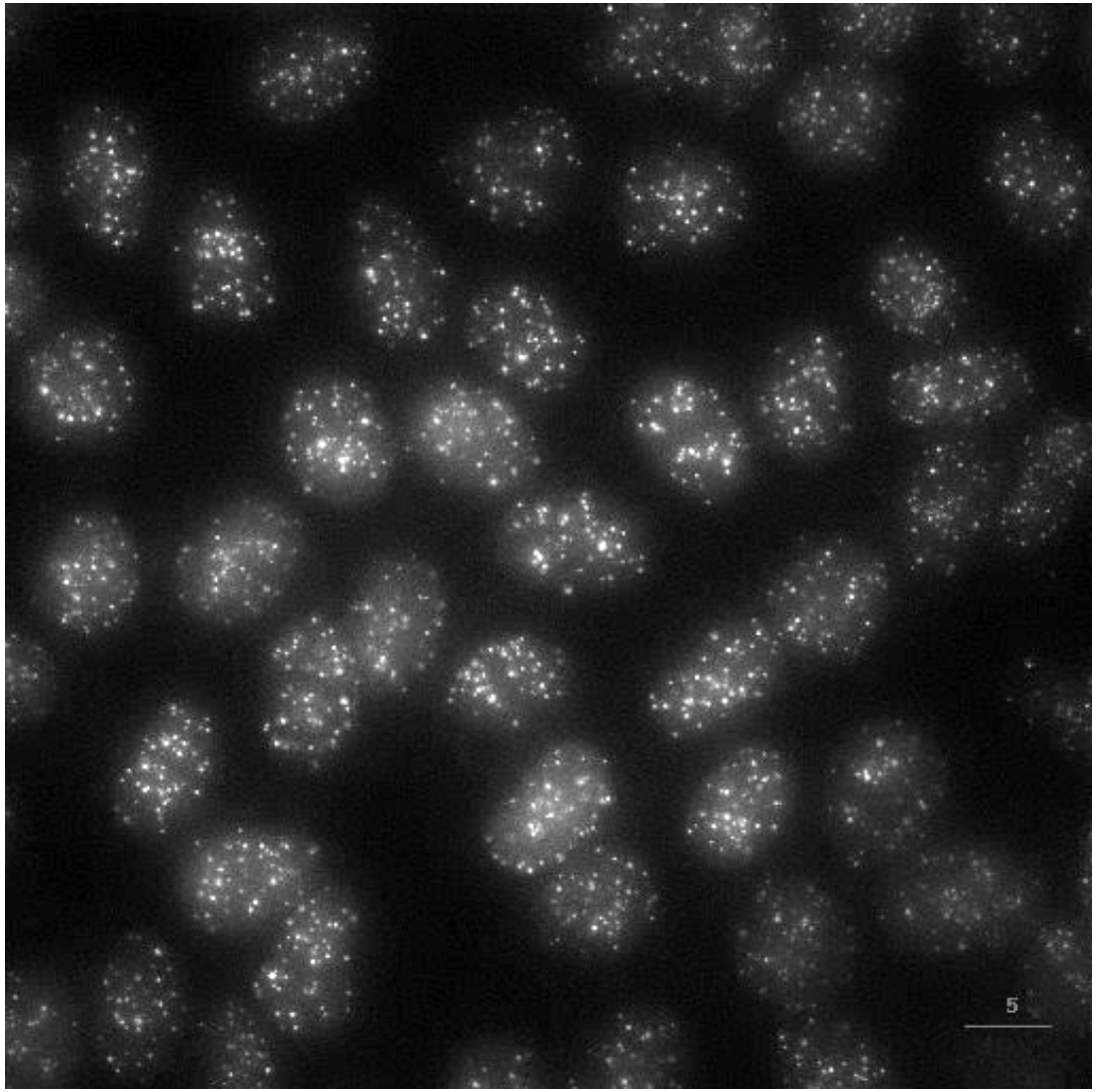
Think viability and photostability more than nice picture!

Careful experiment planning

- The previous rules apply
  - Controls
  - Fluorescent proteins matching the microscope configuration
  - No crosstalk/Bleedthrough
  - Sample preparation in appropriate dishes (#1.5 thickness)
  - Choice of the objective

- Extra Challenges
  - Bleaching
  - Phototoxicity
  - Autofluorescence (biological material, material and/or media) influencing the SBR
  - Controlled environment (Temperature, Humidity, pH, CO_2)

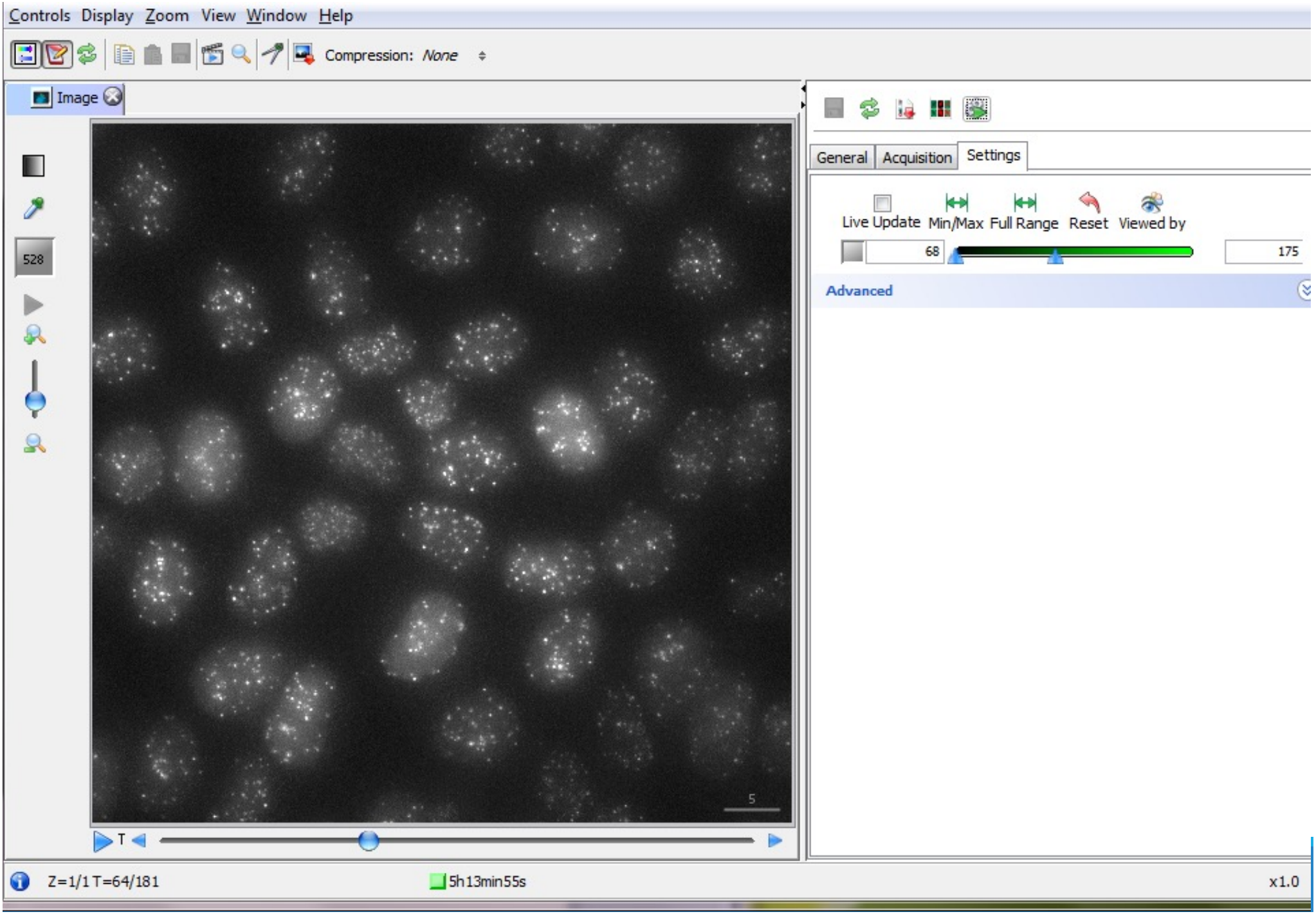
Live imaging

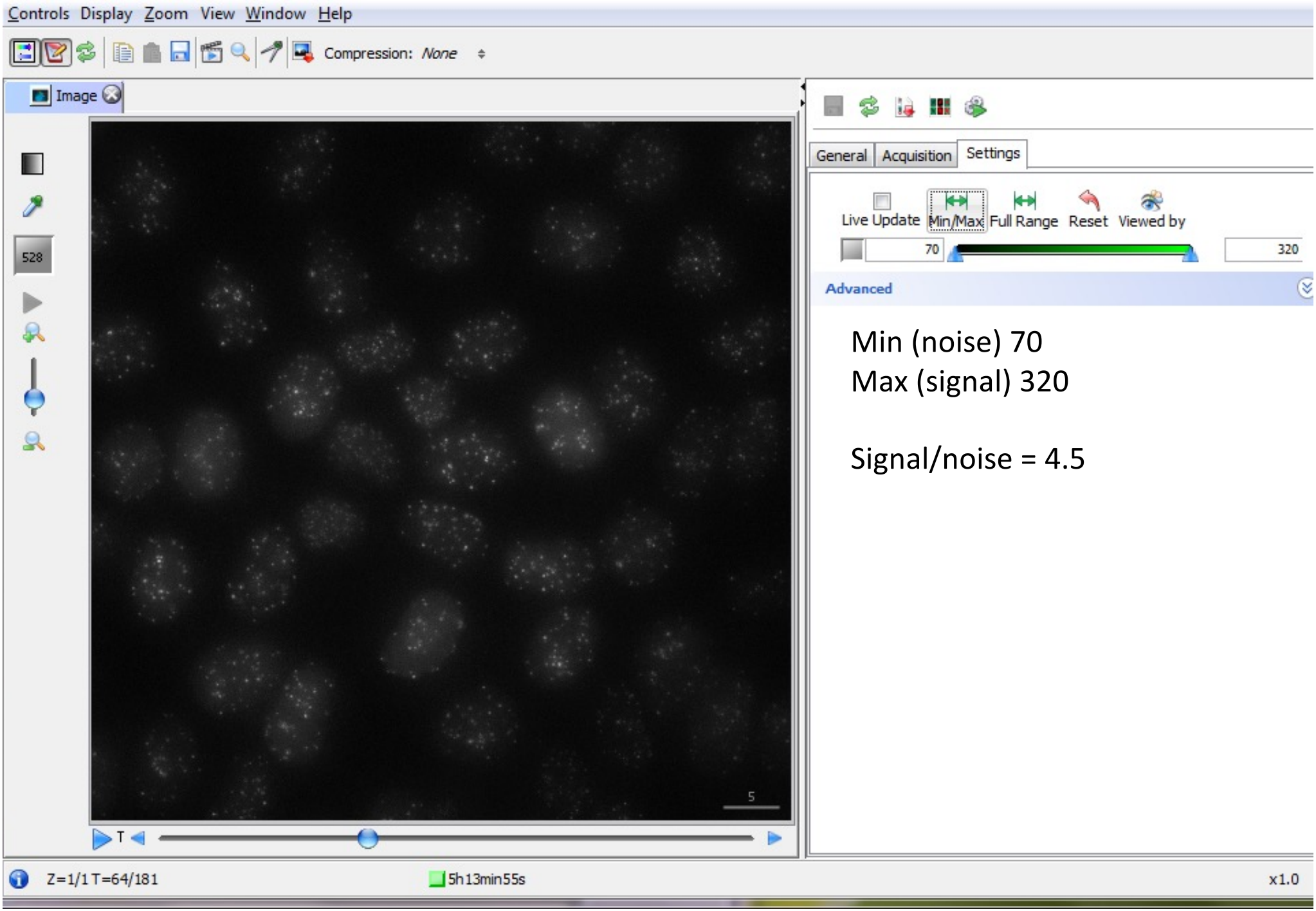


HeLa GFP-CENP-A cell line
Overnight movie using a widefield DeltaVision Core 12 bit camera (0-4095 gsl)

Neutral Density 32%
Exposure time 25 ms

Normal mitosis timing under those conditions.


Controls Display Zoom View Window Help
Compression: None
Image
528
T
5
General
Acquisition
Settings
Live Update
Min/Max
Full Range
Reset
Viewed by
68
175
Advanced
Z=1/1 T=64/181
5h13min55s
x1.0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528
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5
General
Acquisition
Settings
Live Update
Min/Max
Full Range
Reset
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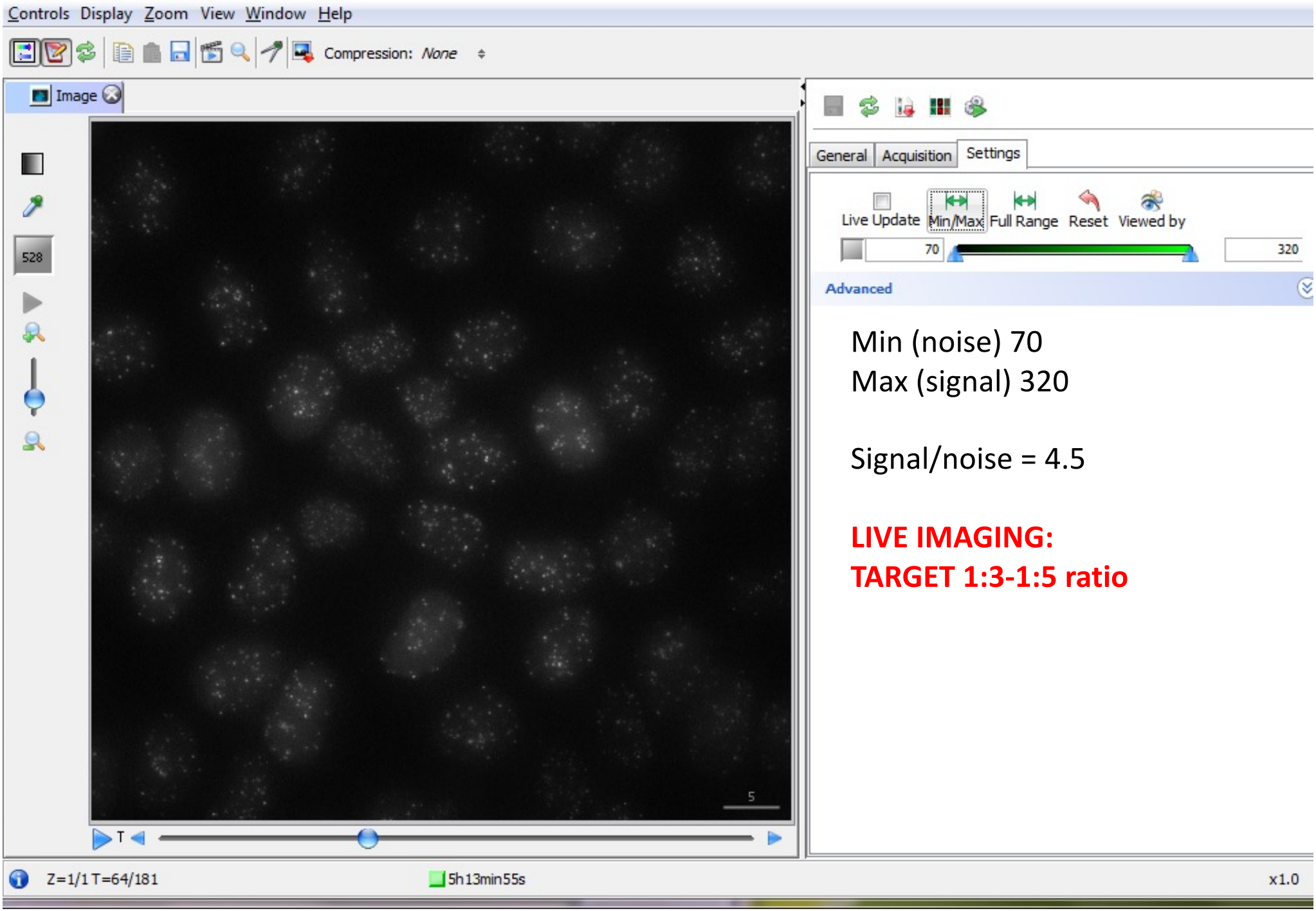
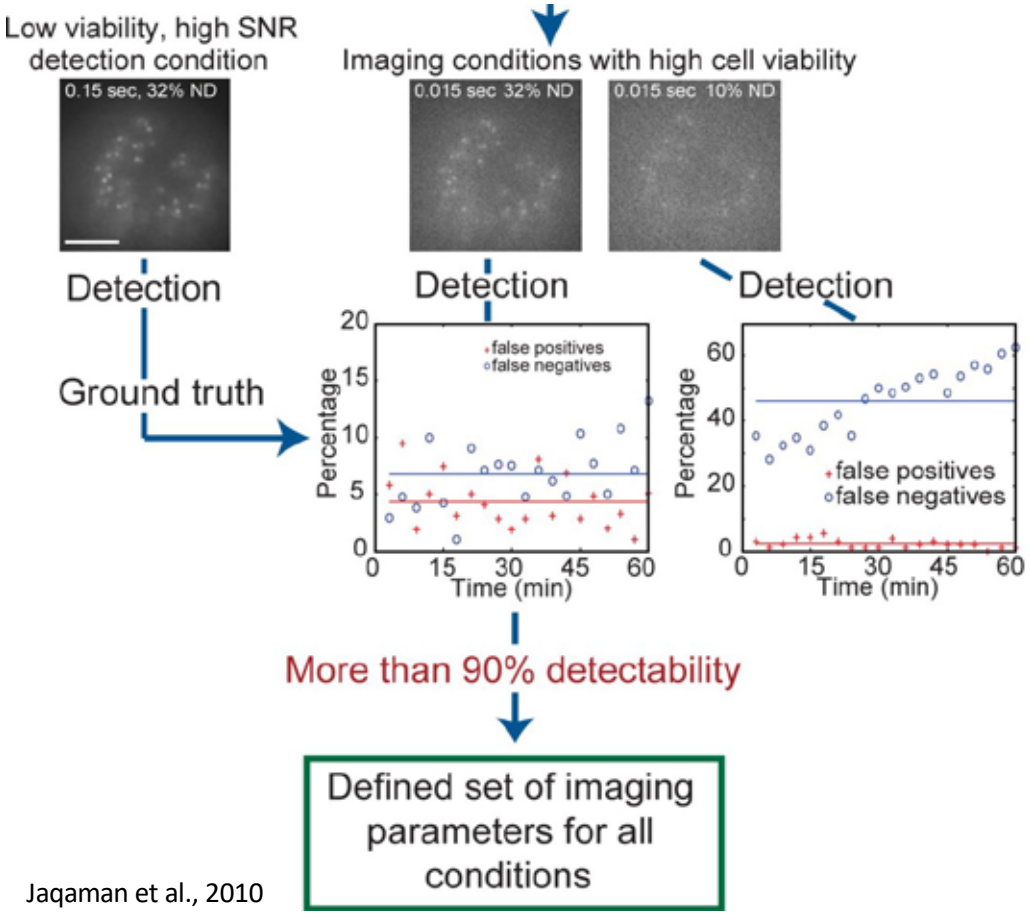
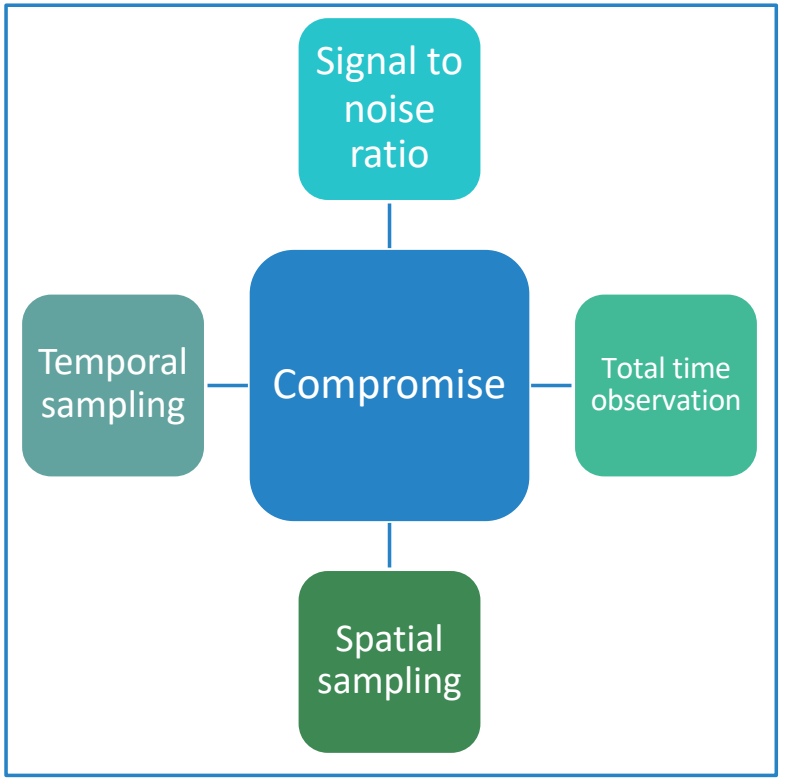
70
320
Advanced
Min (noise) 70
Max (signal) 320
Signal/noise = 4.5
Z=1/1 T=64/181
5h13min55s
x1.0


Controls Display Zoom View Window Help
Compression: None
Image
528
T
5
General
Acquisition
Settings
Live Update
Min/Max
Full Range
Reset
Viewed by
70
320
Advanced
Min (noise) 70
Max (signal) 320
Signal/noise = 4.5
LIVE IMAGING:
TARGET 1:3-1:5 ratio
Z=1/1 T=64/181
5h13min55s
x1.0

Keep your cells alive



Jaqaman et al., 2010



- And also, keep your cells in focus: do not hesitate to contact us to show you the autofocus options

More information:

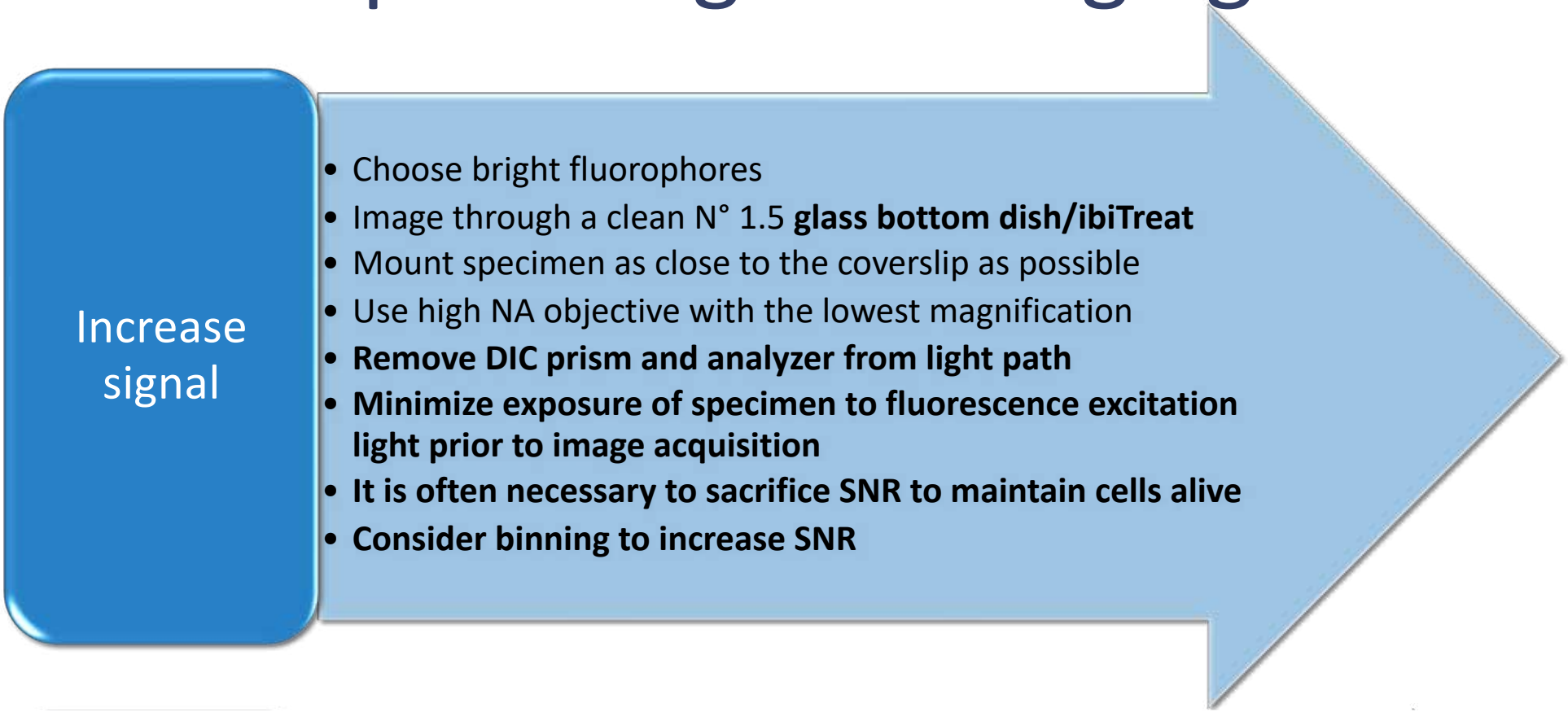
Prospects & Overviews

Phototoxicity in live fluorescence microscopy, and how to avoid it

Jaroslav Icha [1)†], Michael Weber [2),†], Jennifer C. Waters [2)] and Caren Norden [1)*]*

Bioassays 2017

Optimizing live imaging



Adapted from Accuracy and precision in quantitative fluorescence microscopy. JC Waters, JCB, 2009

Optimizing live imaging

Increase signal

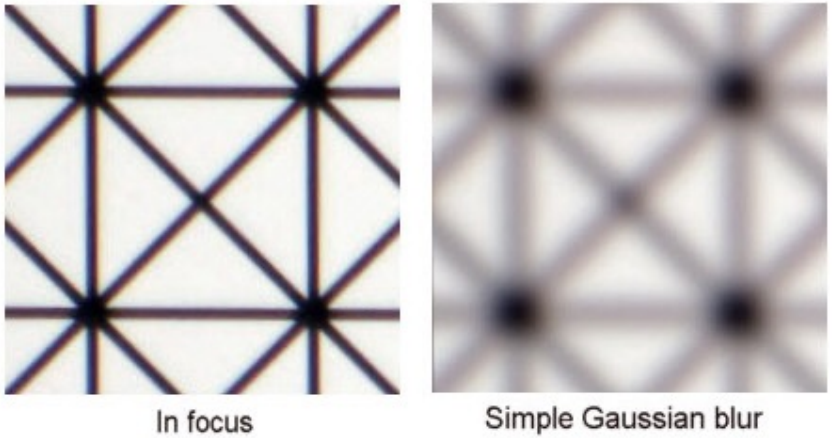
- Choose bright fluorophores
- Image through a clean N° 1.5 **glass bottom dish/ibiTreat**
- Mount specimen as close to the coverslip as possible
- Use high NA objective with the lowest magnification
- **Remove DIC prism and analyzer from light path**
- **Minimize exposure of specimen to fluorescence excitation light prior to image acquisition**
- **It is often necessary to sacrifice SNR to maintain cells alive**
- **Consider binning to increase SNR**

Decrease background

- Clean coverslip and optics
- Use media **without phenol red** for imaging (i.e. DMEM GFP, Evrogen)
- Turn off the room lights

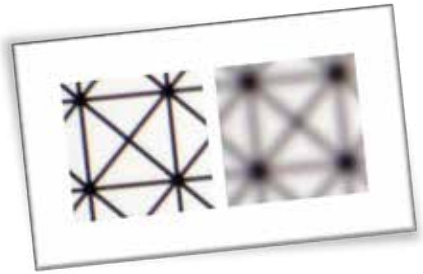
Adapted from Accuracy and precision in quantitative fluorescence microscopy. JC Waters, JCB, 2009

Image Analysis and Processing



In focus

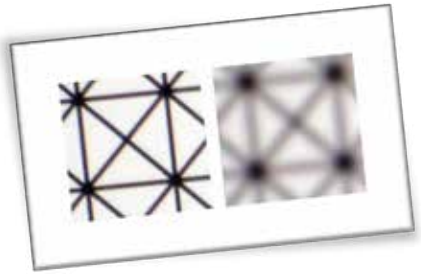
Simple Gaussian blur



Tips for your analysis

- Always keep the original data.
- When converting image data from a proprietary format, save your image data as TIFF (tagged image file format) or ome.tiff (lossless compression).
- Simple adjustments to the entire image or cropping are usually acceptable.
- Acquire your images under identical conditions, and any post-acquisition image processing should also be identical.

For more details, please read: Digital Images Are Data: And Should Be Treated as Such, by Douglas W. Cromey



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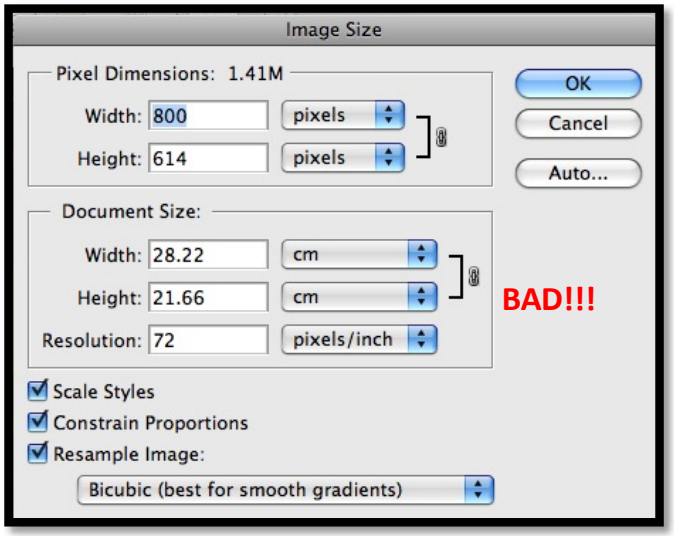

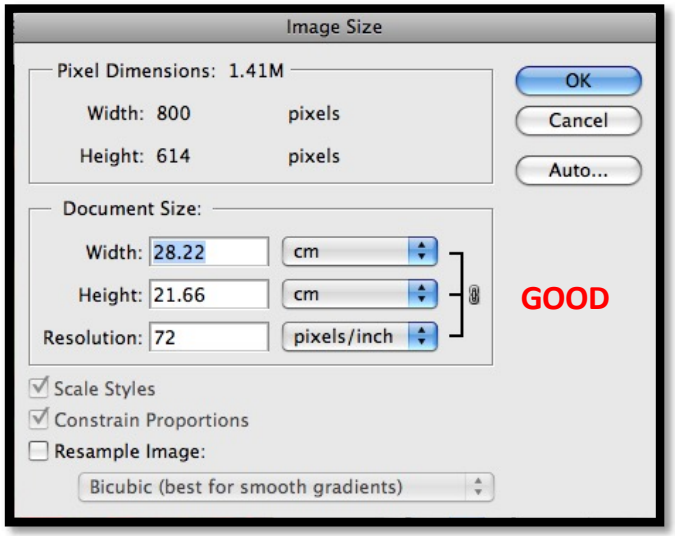

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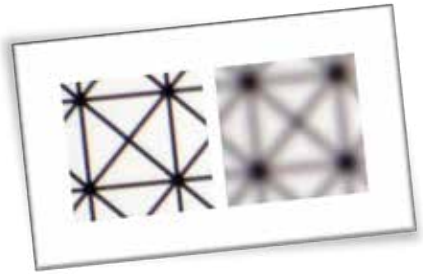

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- Be careful when you change your image size, especially in Photoshop
- Use of software filters to improve image quality is usually not recommended for biological images.
- Cloning or copying objects into a digital image, from other parts of the same image or from a different image, is very questionable.

For more details, please read: Digital Images Are Data: And Should Be Treated as Such, by Douglas W. Cromey

What to add in your M&Ms

Manufacturer and type of microscope (eg. Zeiss LSM880 confocal mounted on an Axio Observer microscope stand)

Objective used (eg. 63x 1.4 NA oil Plan-Apochromat)

Illumination light source, wavelengths of excitation and emission (eg. 488 nm line of Argon laser with a longpass 500nm filter)

Camera manufacturer and model (eg. Prime 95B, Photometrics)

Software for acquisition (eg. LASAF 2.6) and image acquisition settings (eg. ND, exposure time, binning, interval in t and z)

Details of any image processing or analysis routine– raw images may also be required

Quantitative imaging

- Avoid Bias – the difference needs to be statistically relevant
- Illumination of the field of view needs to be homogenous
- Avoid focus drift in a timelapse
- Be careful of laser power/illumination fluctuation
- Avoid Photobleaching
- Be careful with mounting your samples
- In a full set of experiments, make sure that there are no software updates
- Stats: Nature has a collection of short articles called 'Statistics for Biologists' (https://www.nature.com/collections/qghhqm)

More information:

nature protocols

REVIEW ARTICLE

https://doi.org/10.1038/s41596-020-0313-9

Check for updates

Tutorial: guidance for quantitative confocal microscopy

James Jonkman[1✉], Claire M. Brown[2], Graham D. Wright[3], Kurt I. Anderson[4] and Alison J. North[5]

Take-home message

- The sample is part of the optics – avoid sample-induced aberrations
- Try to increase signal and decrease background
- Do not overexpose your images
- For live images, adjust exposure time, laser power (confocal), and neutral density (widefield/spinning disk) to keep your cell happier (alive and functional) for longer

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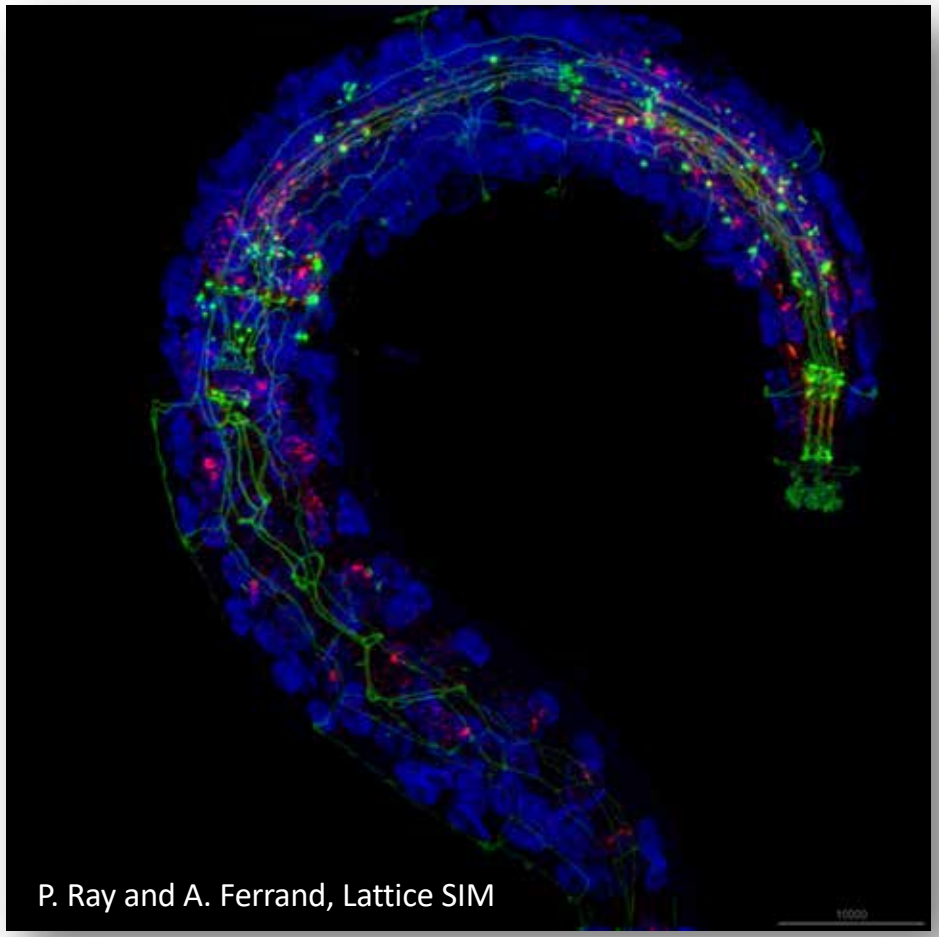
- Always keep your raw data and work on copies
- Be ethical with your images!

Thank you!



Alexia Ferrand
Imaging Core Facility
Biozentrum
University of Basel
alexia.ferrand@unibas.ch
www.biozentrum.unibas.ch/imcf

This lecture found some inspiration from the work of others:
Dr C. Janz (Ibidi), Dr L. Gelman (FMI Basel)
B. Fleming (Roslin Institute Edinburgh)
Jonkman et al., Nature Protocols 2020