Tips and Pitfalls in Microscopy

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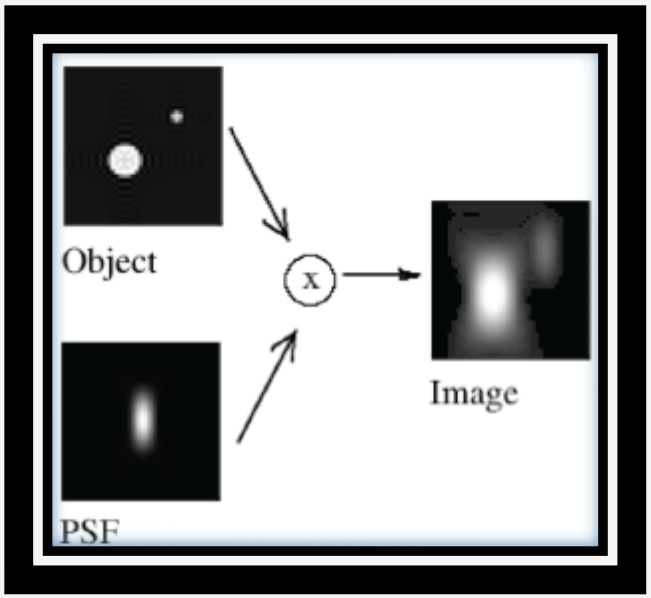
Biozentrum
Basel

Basic optics…



Images are not identical to the object

Image = Object x Point spread function

Basic optics…

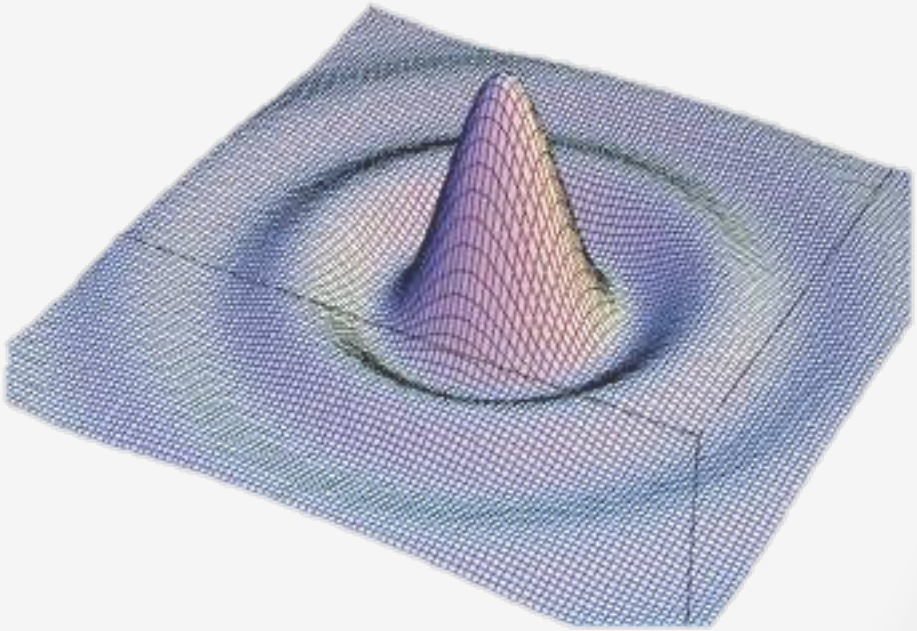
*The Airy Disk**

Caused by diffraction on objective aperture
Infinitely small self luminous points
Negative impact on resolution
(approx 200nm limit)

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

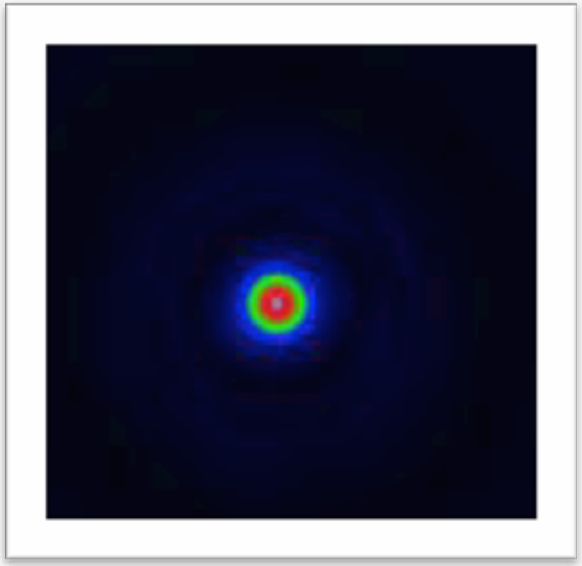


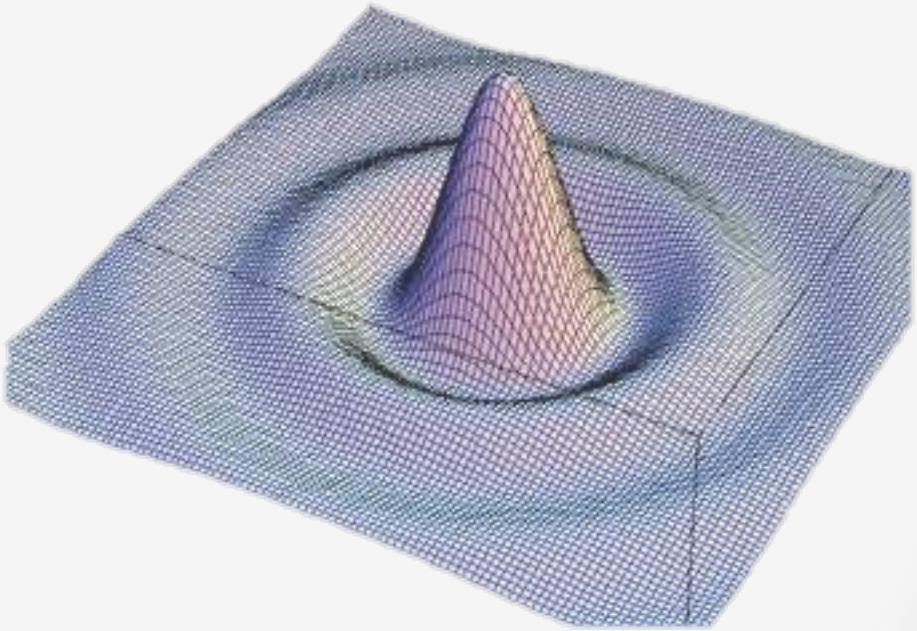
**after GB Airy, British astronomer, 1834*

Basic optics…

The Airy Disk

Rings caused by interference at optical wavefronts



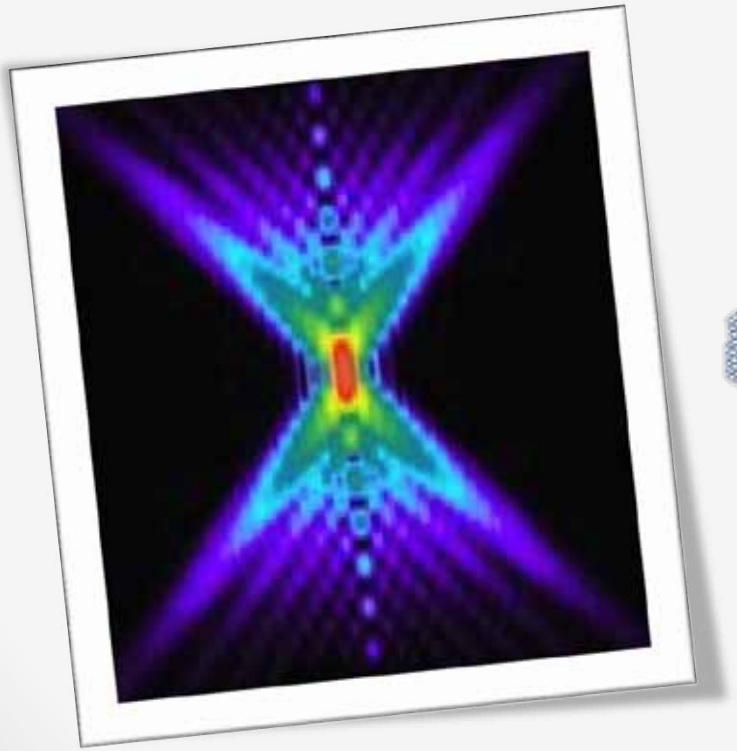


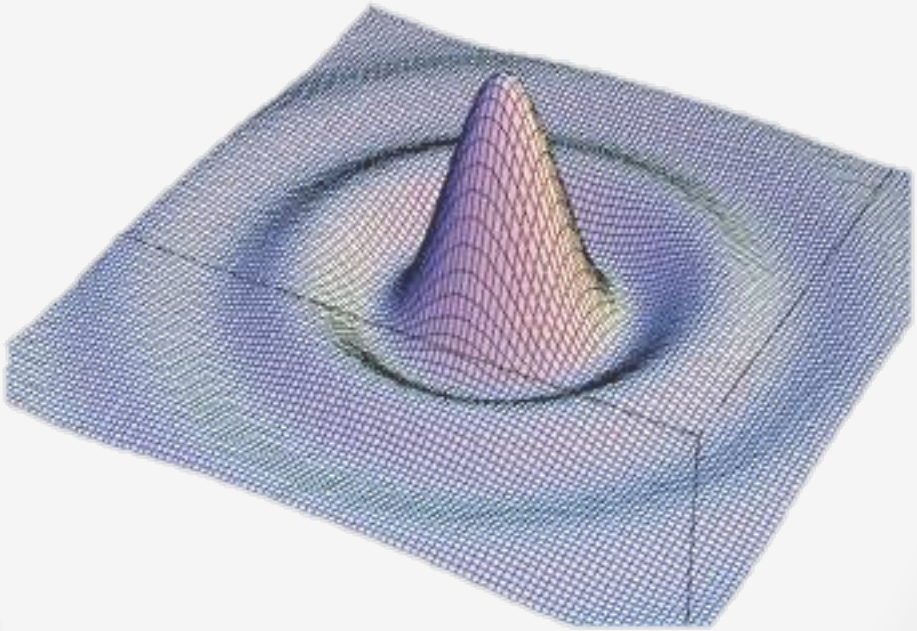
Adapted from Bob Fleming, Bio-Imaging, The Roslin Institute Edinburgh

Basic optics…

The Airy Disk

Classic Point Spread Function (PSF) of object (bead) in Z projection should be "hourglass" shape





Adapted from Bob Fleming, Bio-Imaging, The Roslin Institute Edinburgh

Basic optics…

The Airy Disk

If PSF is "wineglass"…

To improve your image:

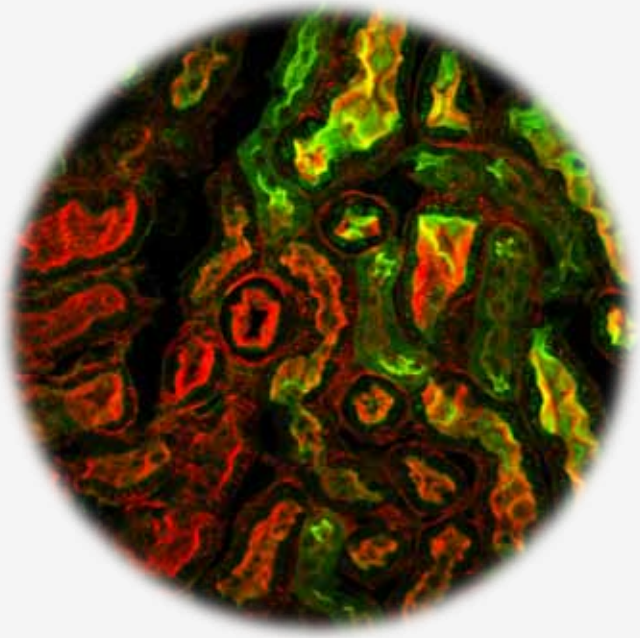
There are key steps to be aware of

Adapted from Bob Fleming, Bio-Imaging, The Roslin Institute Edinburgh

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 187 (AF)

*Except when it saturates…

Fixation of your samples

- «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

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

Preparation of your samples

- The sample is part of the optics
- The last 500 um are important

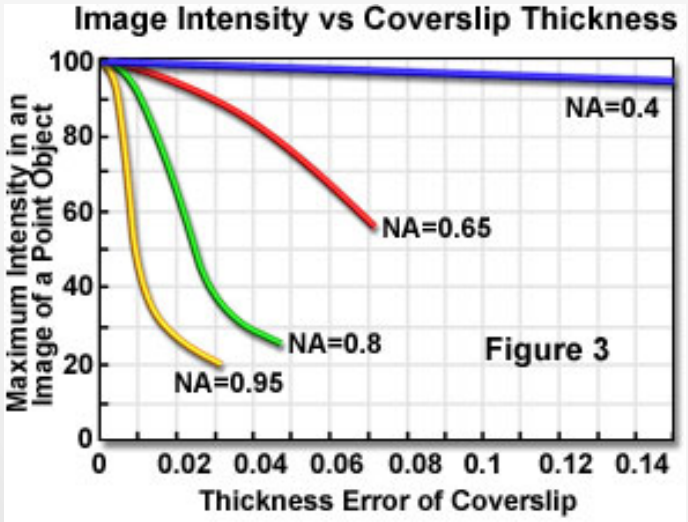
1 – coverglass

MOST COMMUN SIZE
0.17 mm = N°1.5

Preparation of your samples

- The sample is part of the optics
- The last 500 um are important

1 – coverglass (spherical aberrations)



Performance Reduction with Coverslip Thickness Variation

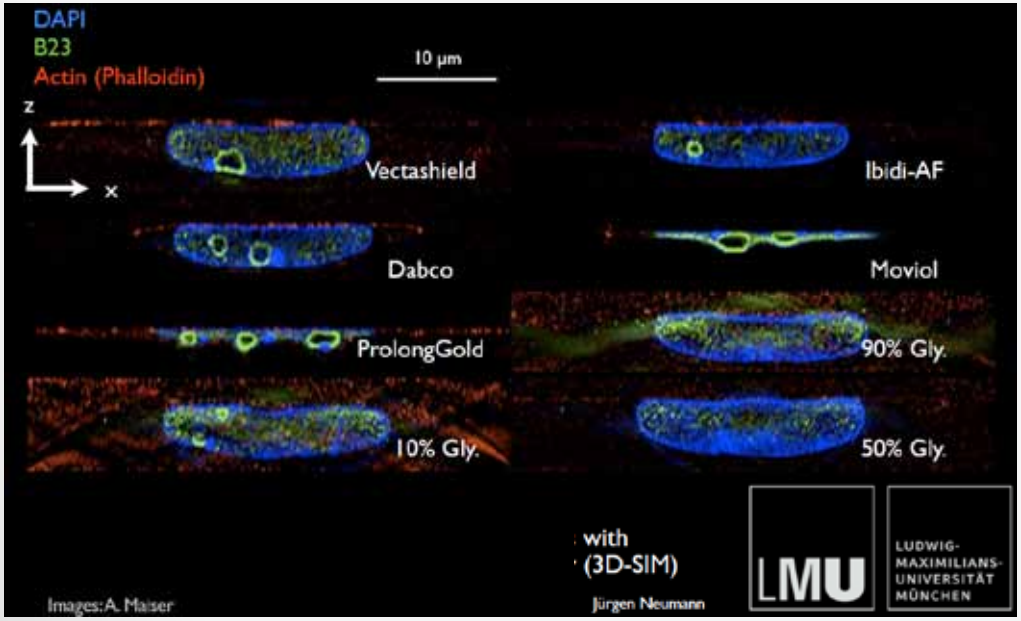
NumericalAperture	0.01 mm Deviation	0.02 mm Deviation
0.30	none	none
0.45	none	none
0.70	2 percent	8 percent
0.85	19 percent	57 percent
0.95	55 percent	71 percent

http://www.olympusmicro.com

Preparation of your samples

- The sample is part of the optics
- The last 500 um are important

2 – mounting media (avoid bubbles)



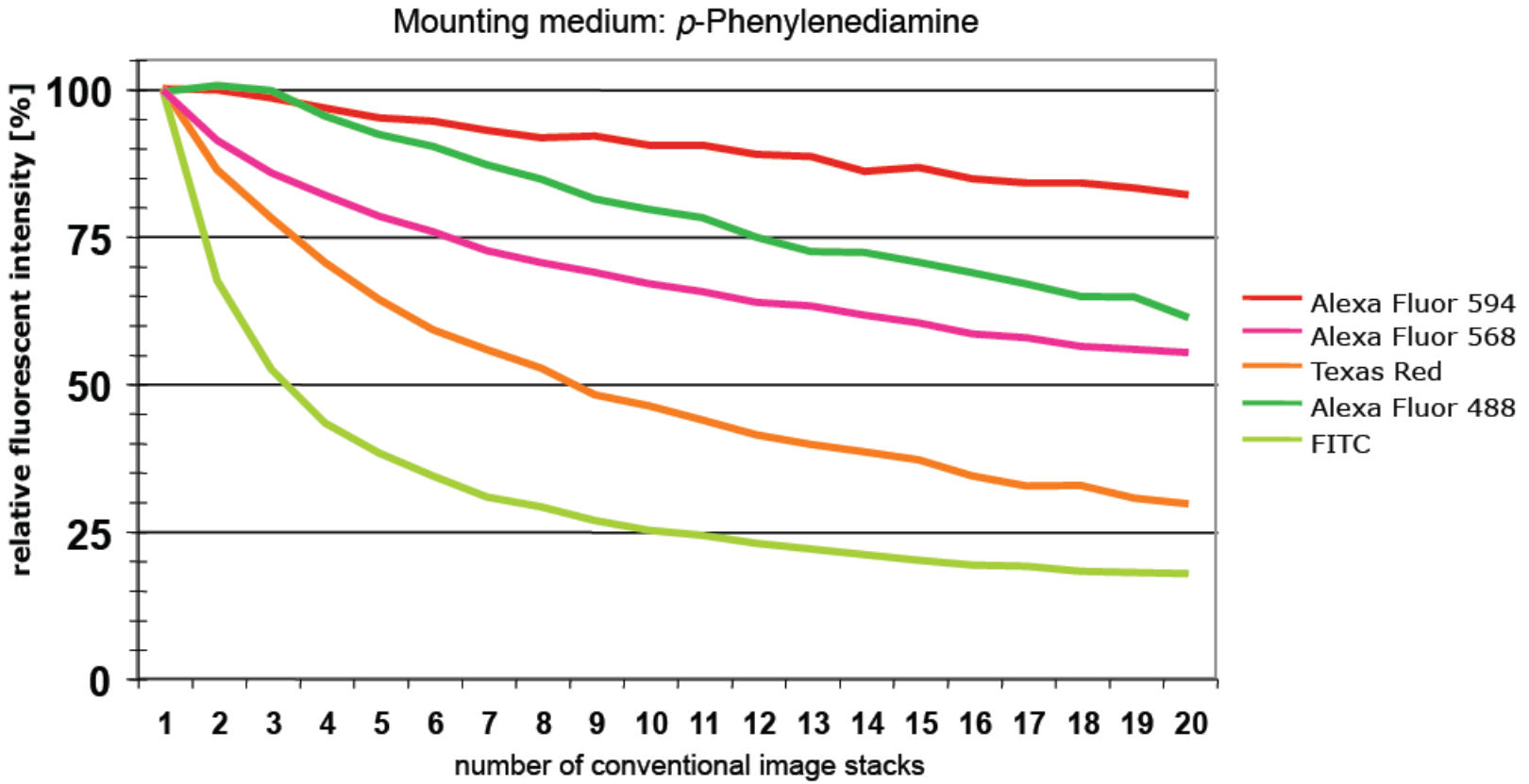
Permount (Biomeda)	1.54
glas	1.52
immersion oil	1.52
Canada balsam	1.52
DPX (Fluka)	1.52
Fluorescence Mounting Medium (DAKO)	1.47 – 1.50
Mowiol (Calbiochem)	1.49 - 1.41?
ProLong Gold (Molecular Probes)	1.47 - 1.41 (increases over time)
Kaisers glycerol jelly	1.47
Vectashield (Vector Labs)	1.46
Vectashield / 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

always check immersion medium for compatibility with your dyes!

Source: Leica

THERE IS NO «one fit all» MOUNTING MEDIA

Bleaching of various fluorophores in *p*-Phenylenediamine

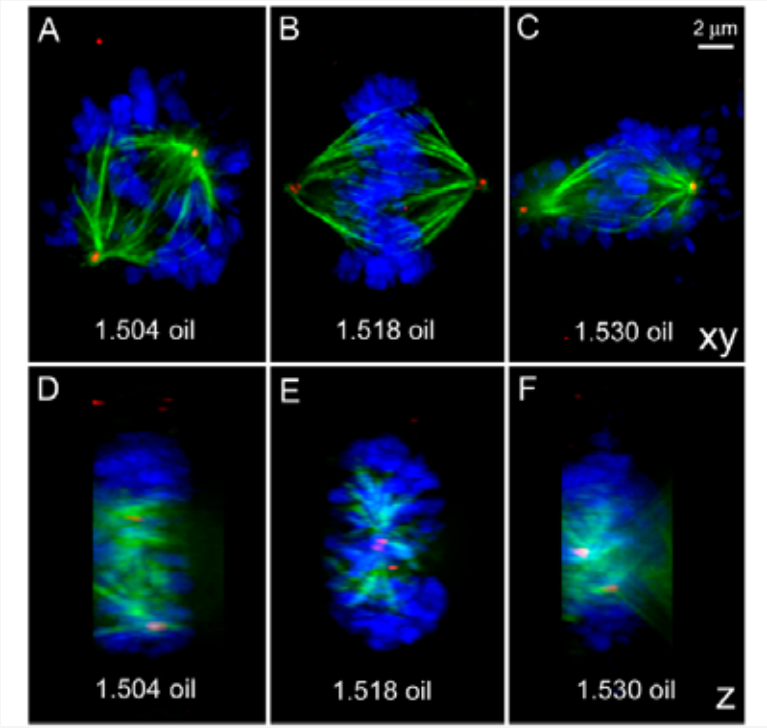


Alexa Fluor 594 and Alexa Fluor 488 are the fluorophores of choice

Preparation of your samples

- 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*

Preparation of your samples

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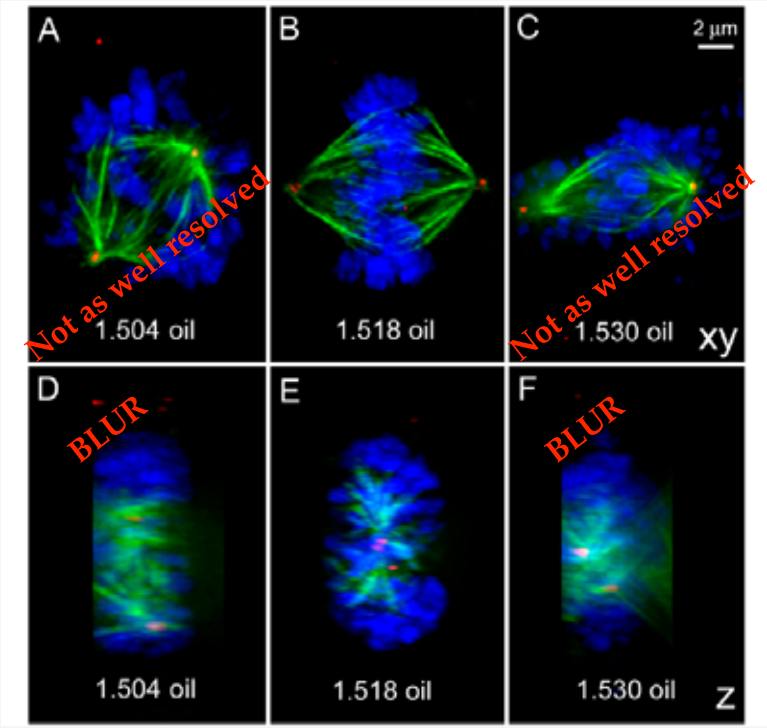

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Preparation of your samples

- The sample is part of the optics
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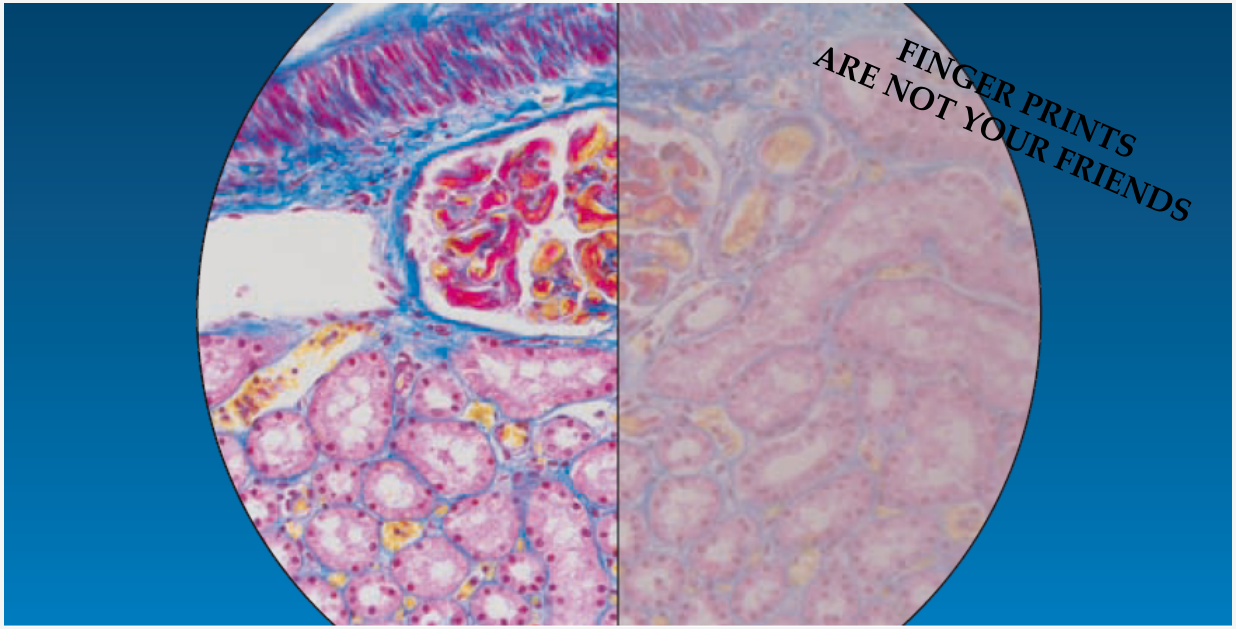
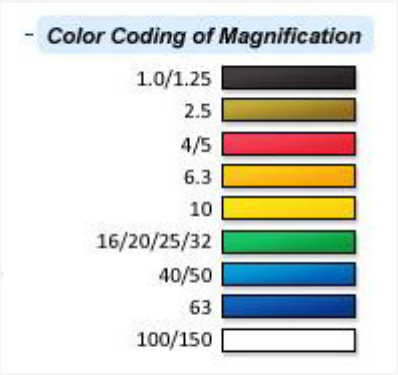
4 – Schmutz effect



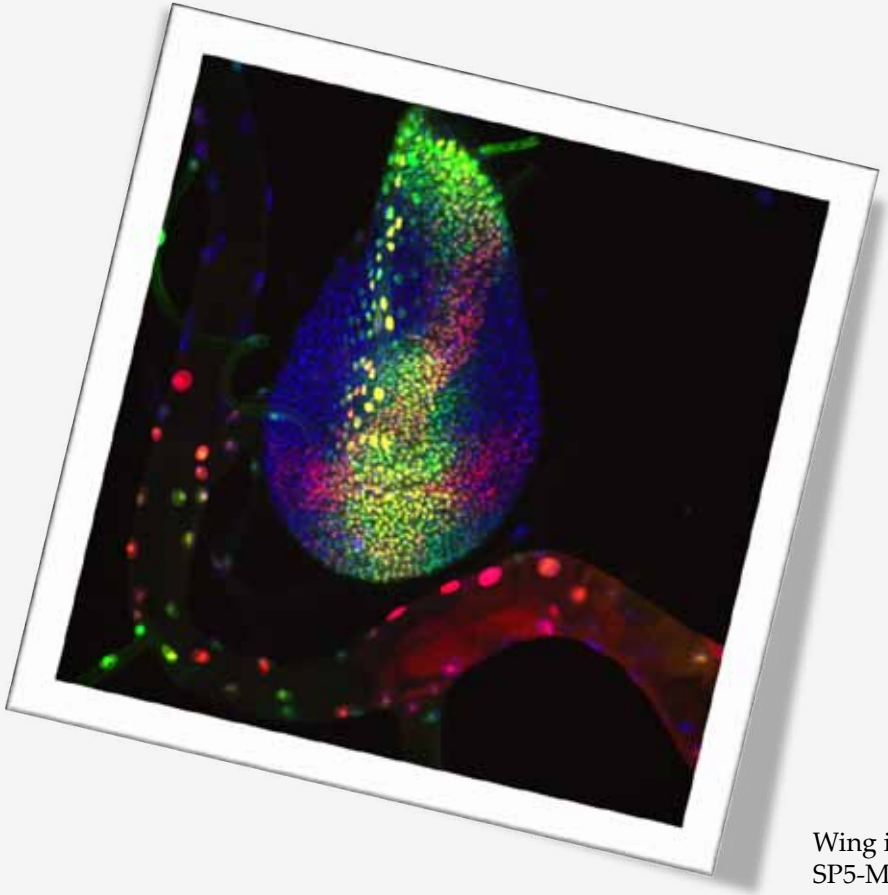
Choice of the lens

- What information can you get from the lens?



Choice of the lens

- What influences the resolution?



Wing imaginal disk taken on our confocal SP5-MP room 235 (F.Hamaratoglu)

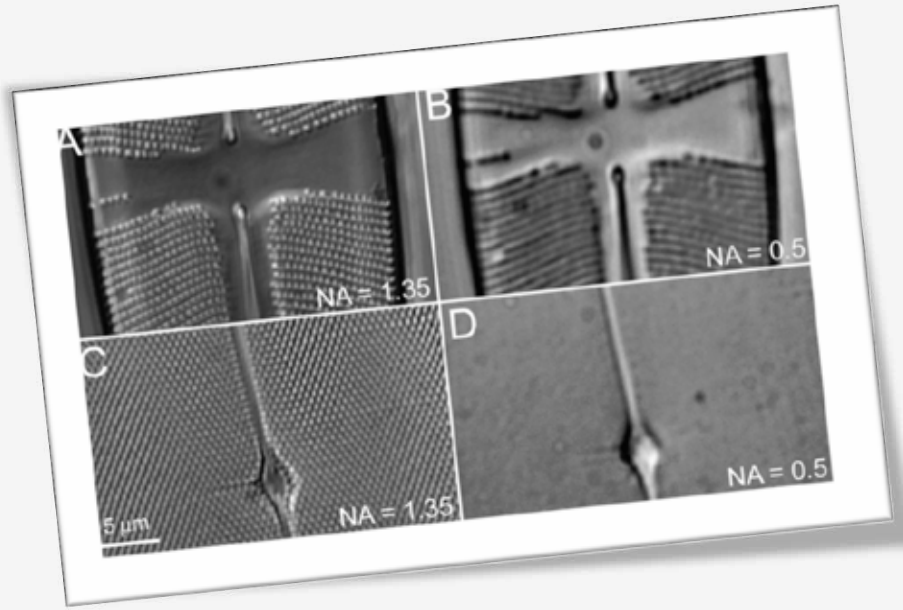
Choice of the lens

- 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*

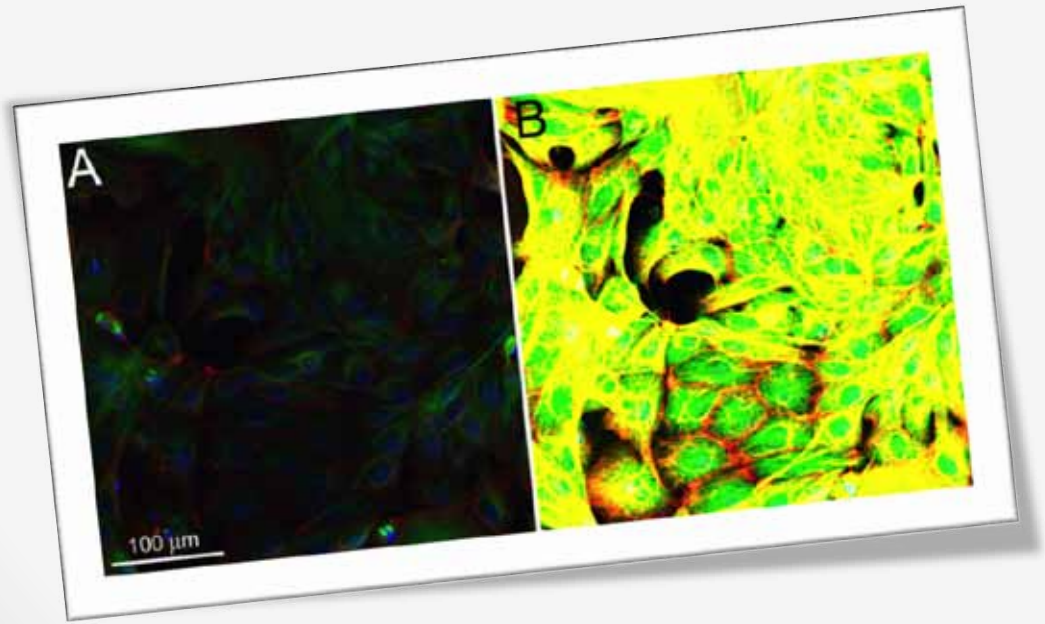
Choice of the lens

- 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*

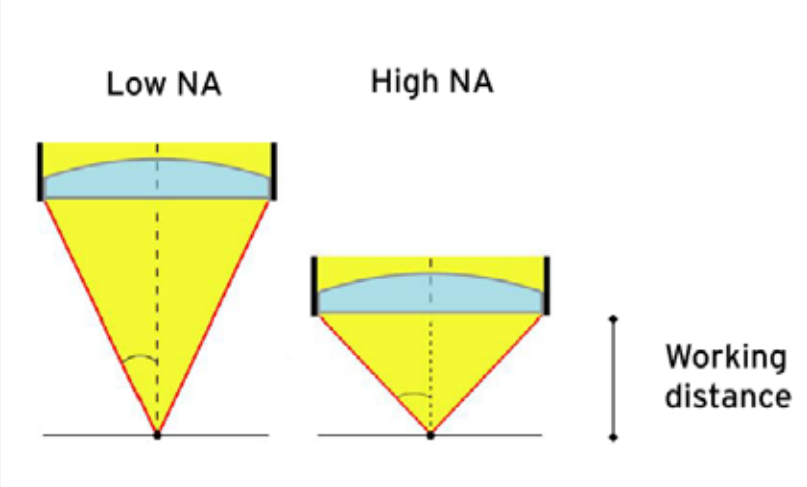
Choice of the lens

- 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?
- NA of the objective
- Good refractive index match

A refractive index mismatch gives rise to geometrical aberrations.

Choice of the lens

- What influences the resolution?
- NA of the objective
- Good refractive index match
- 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, room 186

Optimizing images

Increase signal

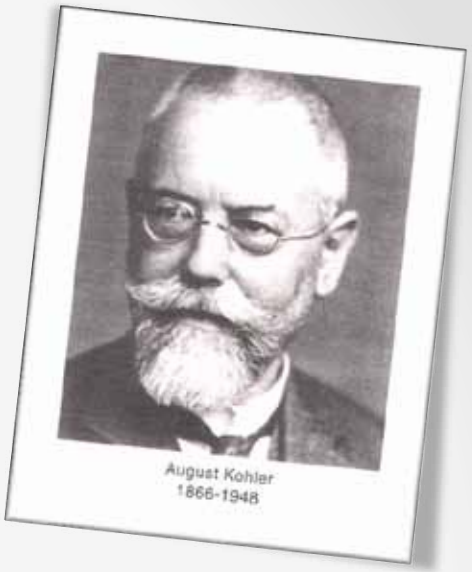
- Choose bright dyes
- Image through a clean N° 1.5 coverslip
- Mount specimen as close to the coverslip as possible
- Use high NA clean objective with the lowest magnification
- Use glycerol-based medium containing anti-bleaching
- Remove DIC Wollaston prism and analyzer from light path

Decrease background

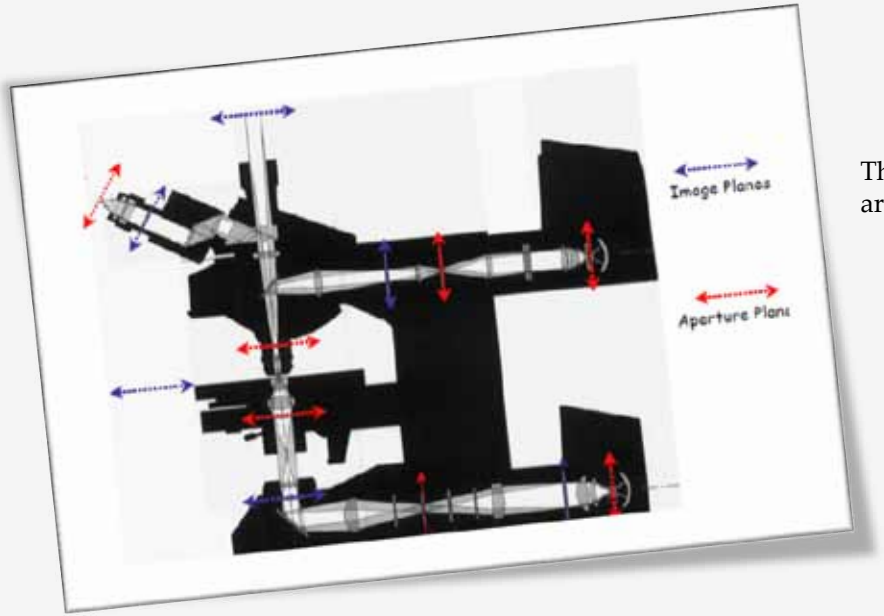
- Clean coverslip and optics
- Perfect fluorophore labeling protocol to minimize nonspecific labeling
- Turn off the room lights

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

Transmitted light



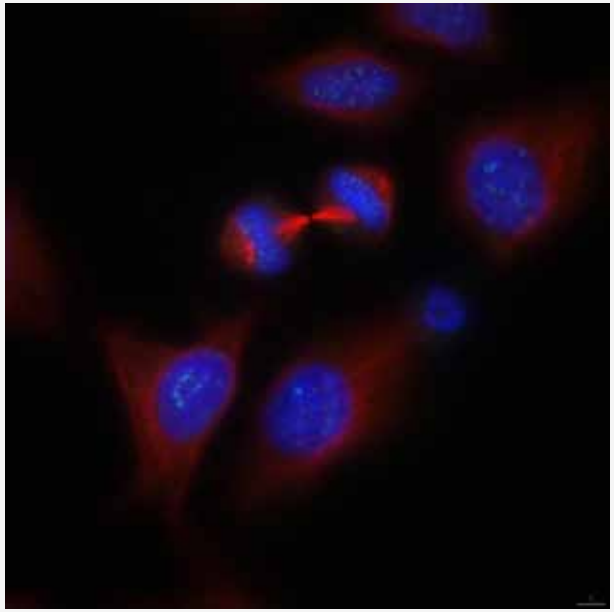
- Köhler illumination
- Bright and even illumination with good contrast and resolution

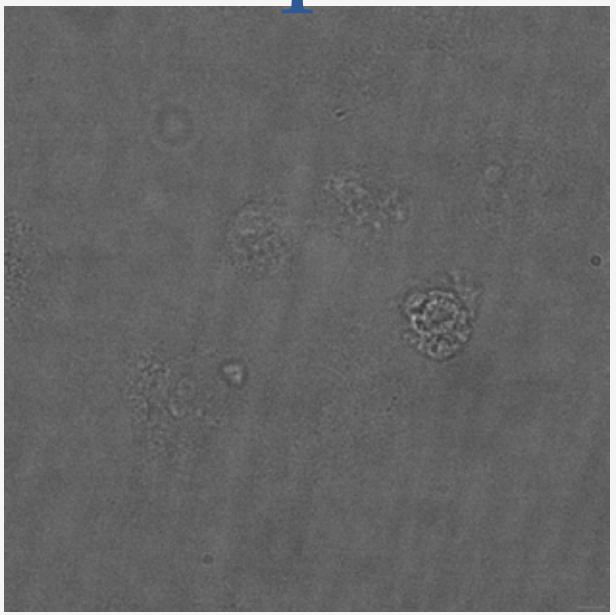


The **Image** and **Illumination** planes are aligned in the microscope

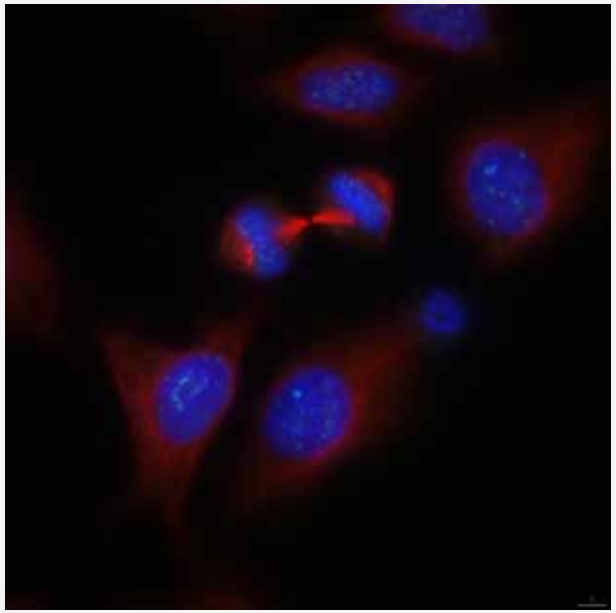
- Also important for epifluorescence

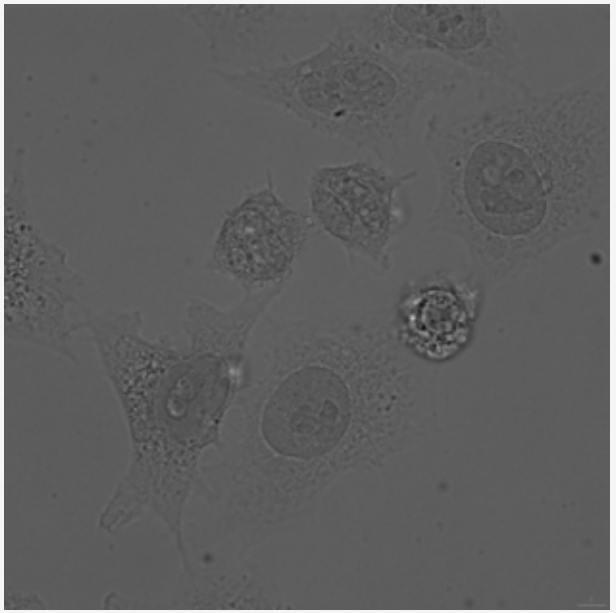
A clear example…





BEFORE



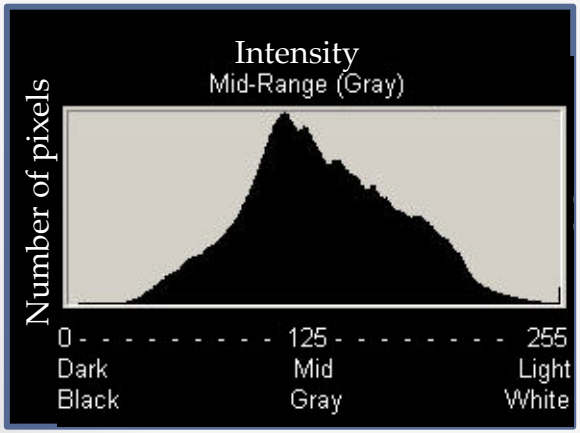


AFTER

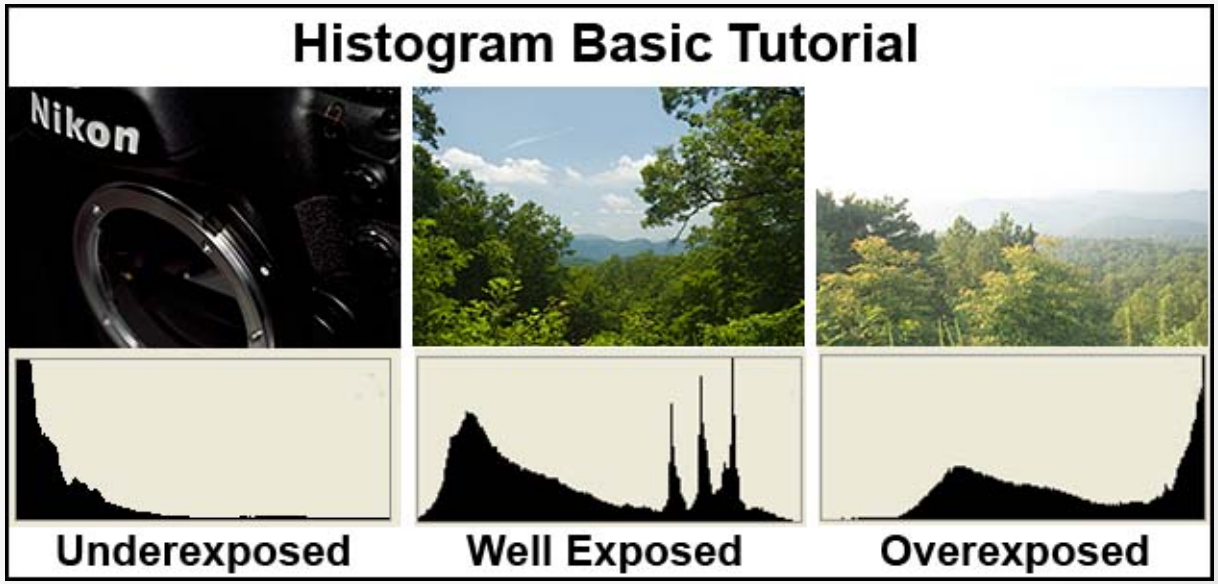
HeLa taken on our DeltaVision room 229 (AF)

Fluorescence

- The brighter the better, BUT NO SATURATION
- Otherwise, no quantification will be possible
- Check your histogram



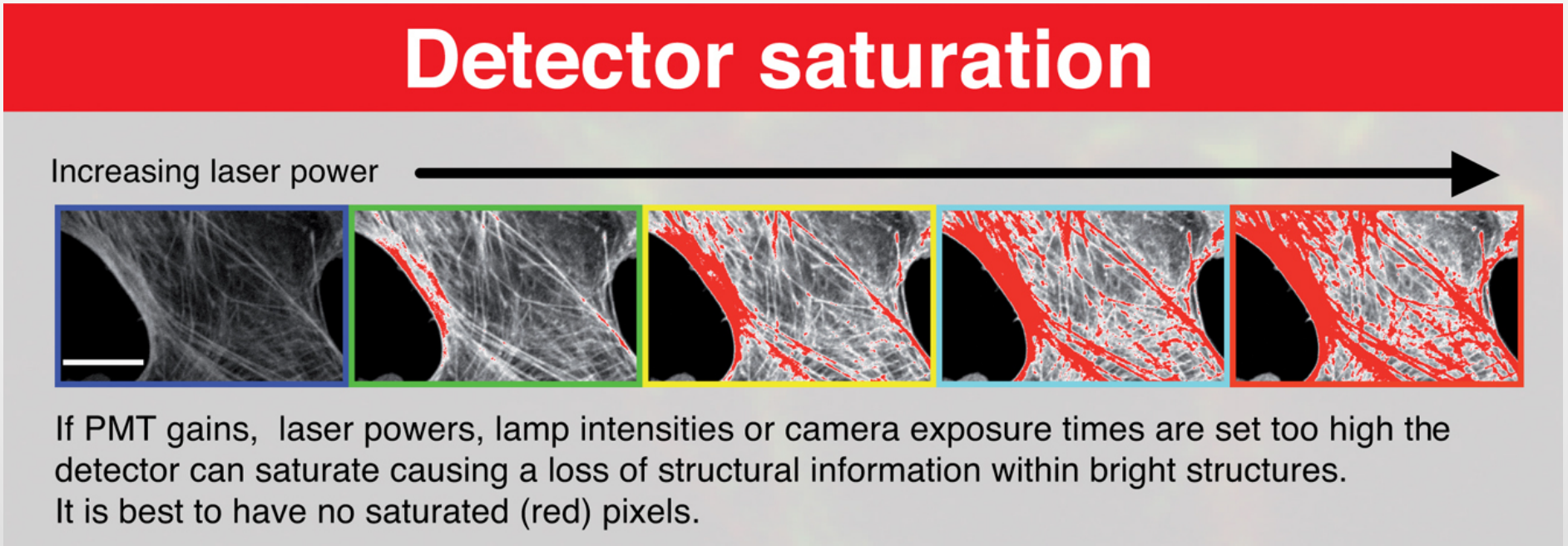
8 bit (0-255 gsl)



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

Multi-labeling

When > 1 color, cross-talk and bleedthrough can happen

- Excitation and Emission spectra are not well separated.

Do proper controls

- Single labelled controls with each fluorochrome to check all channels

Check different settings on the confocal

- Use sequential scan instead of exciting the sample with multiple laser lines at once
- Check the filter sets before choosing your fluorophores

Multi-labeling

SIMULTANEOUS

SEQUENTIAL

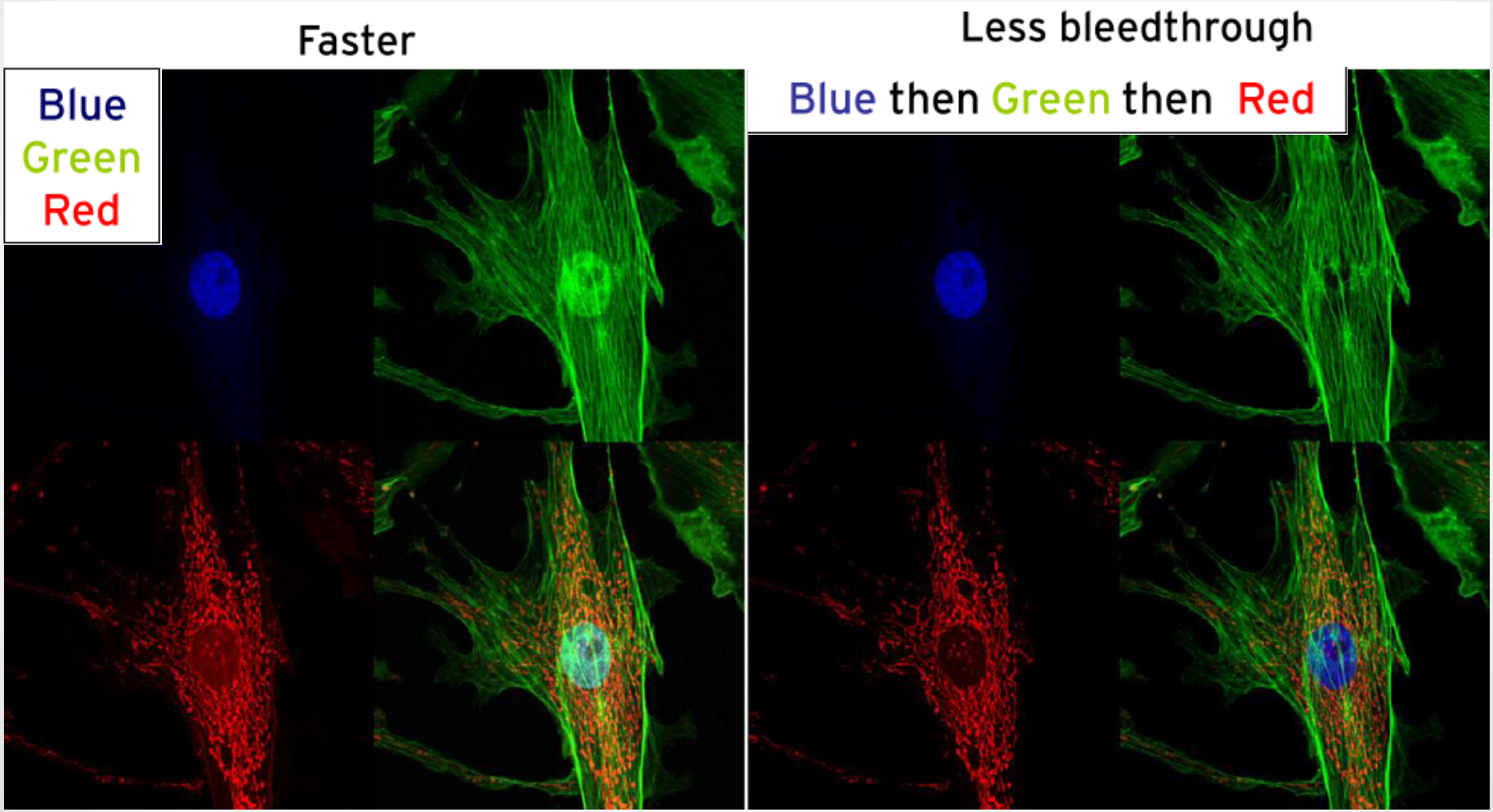
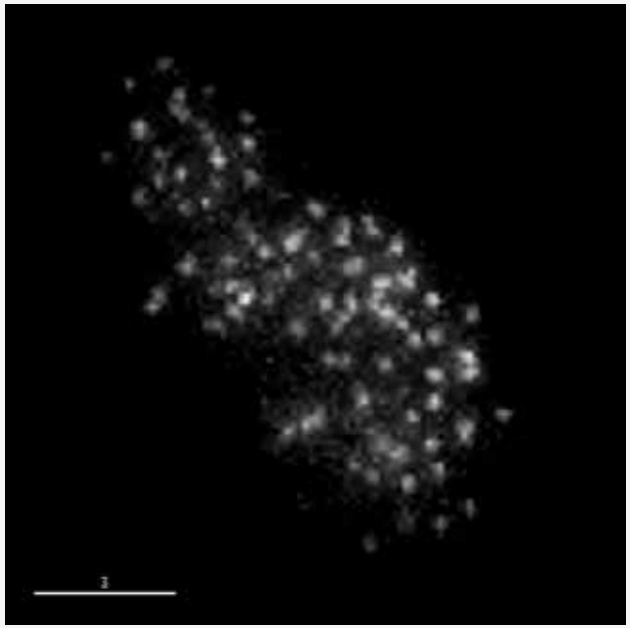
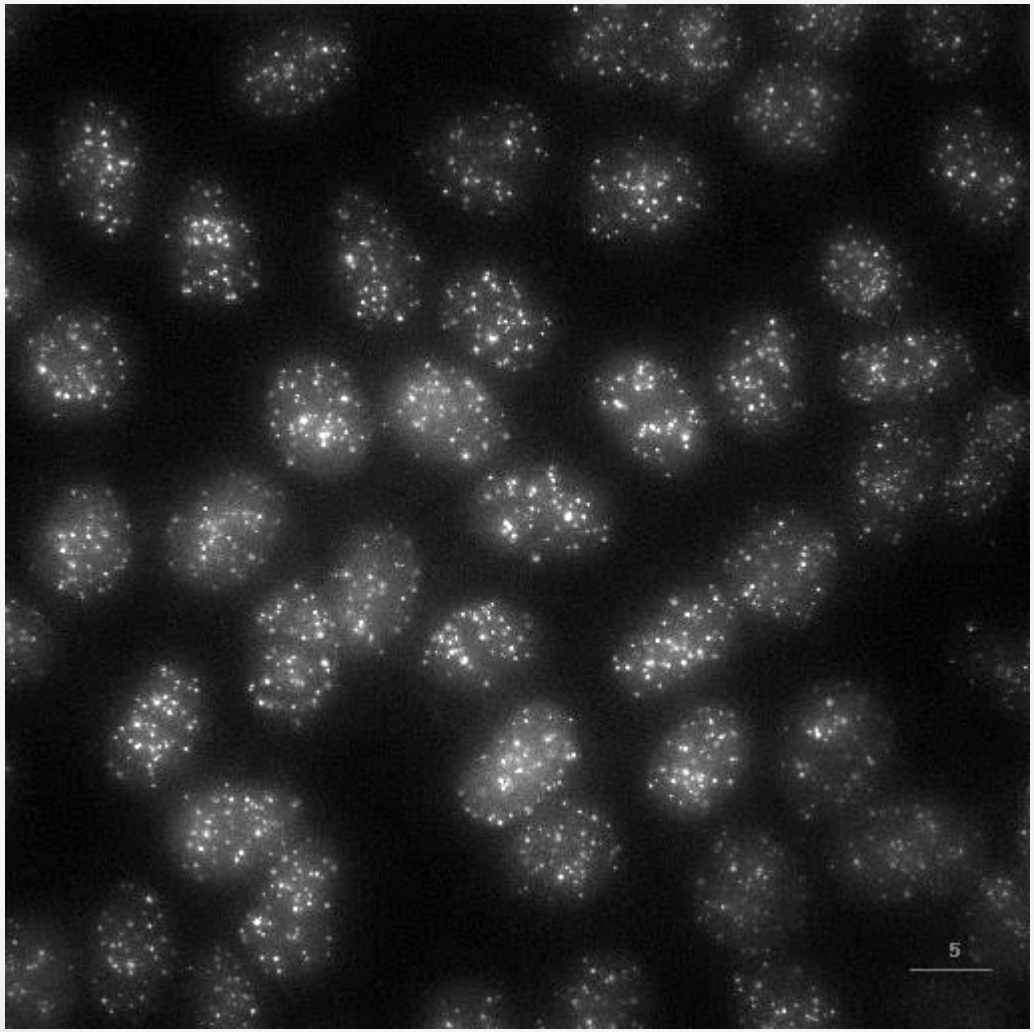


Image Acquisition for live imaging



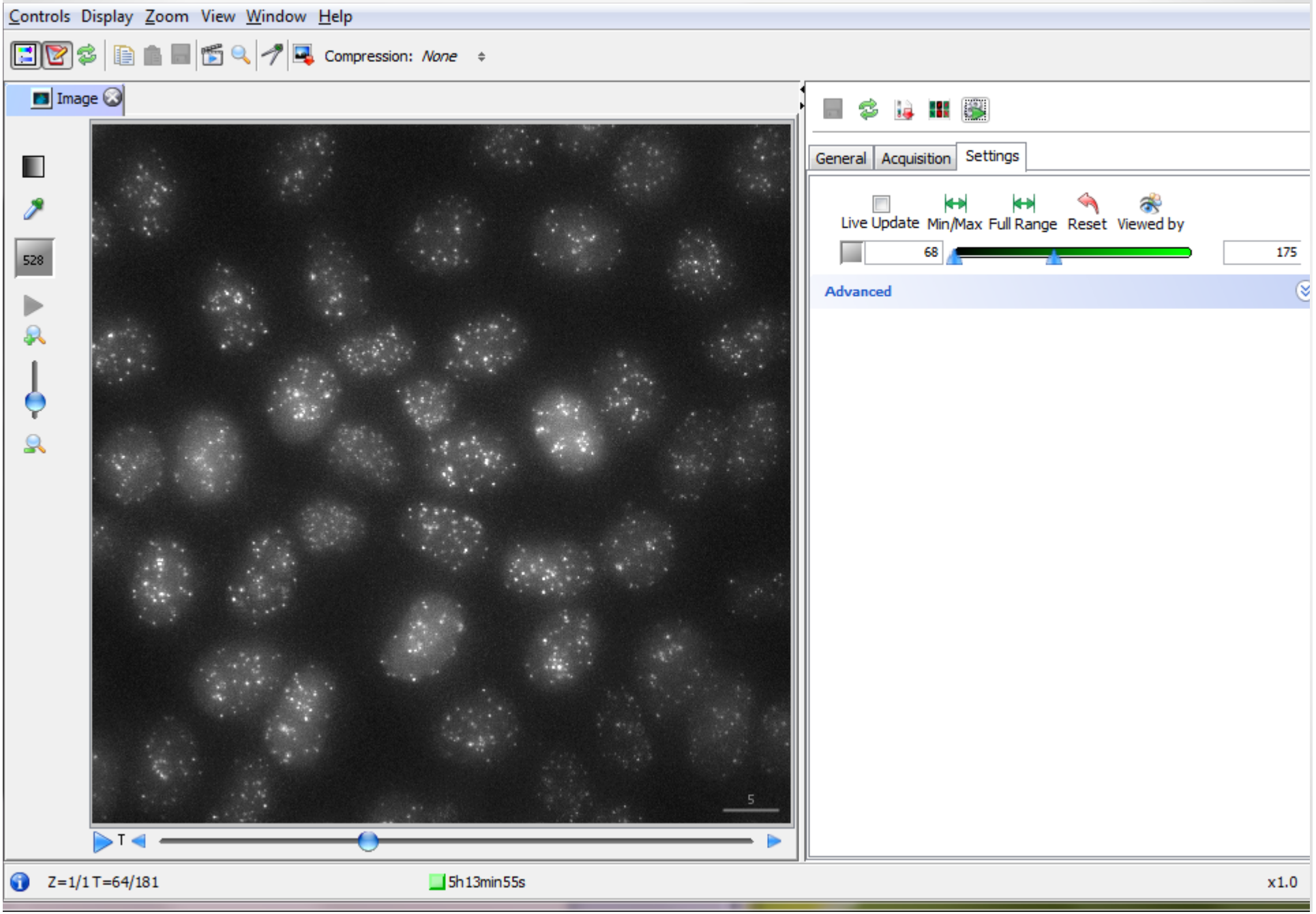
Think viability and photostability more than nice picture!



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
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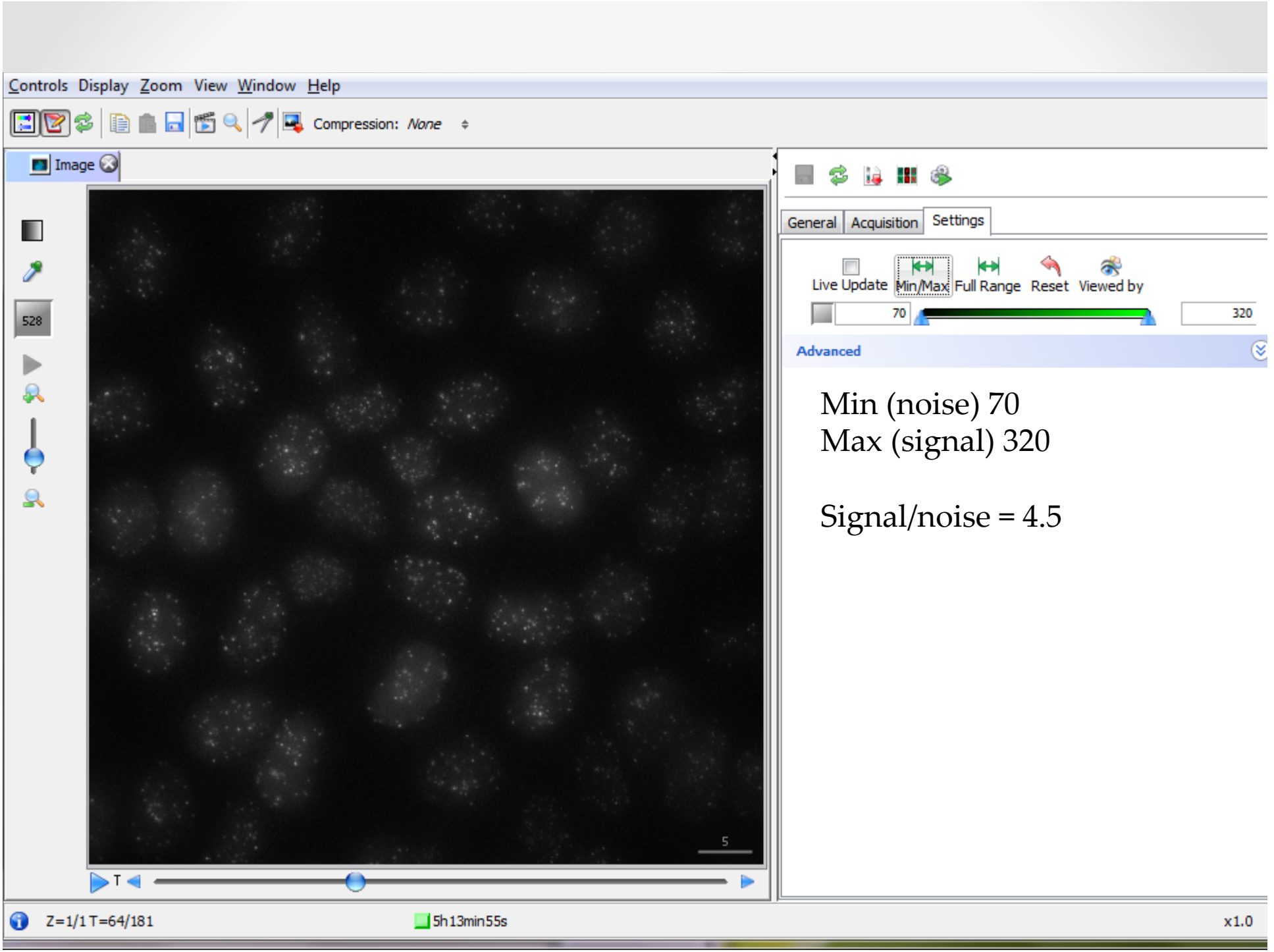
Z=1/1 T=64/181
5h13min55s
x1.0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Acquisition
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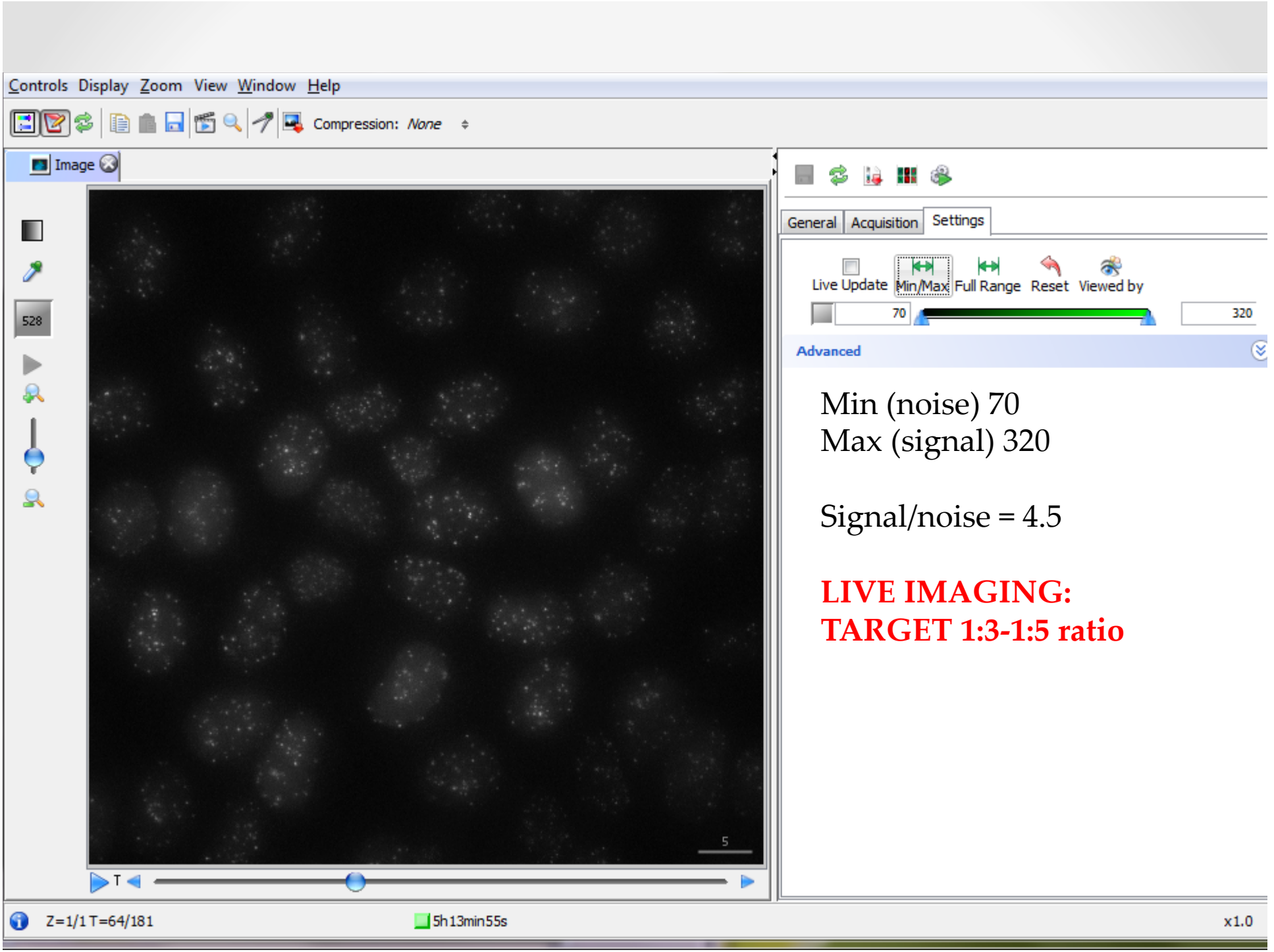
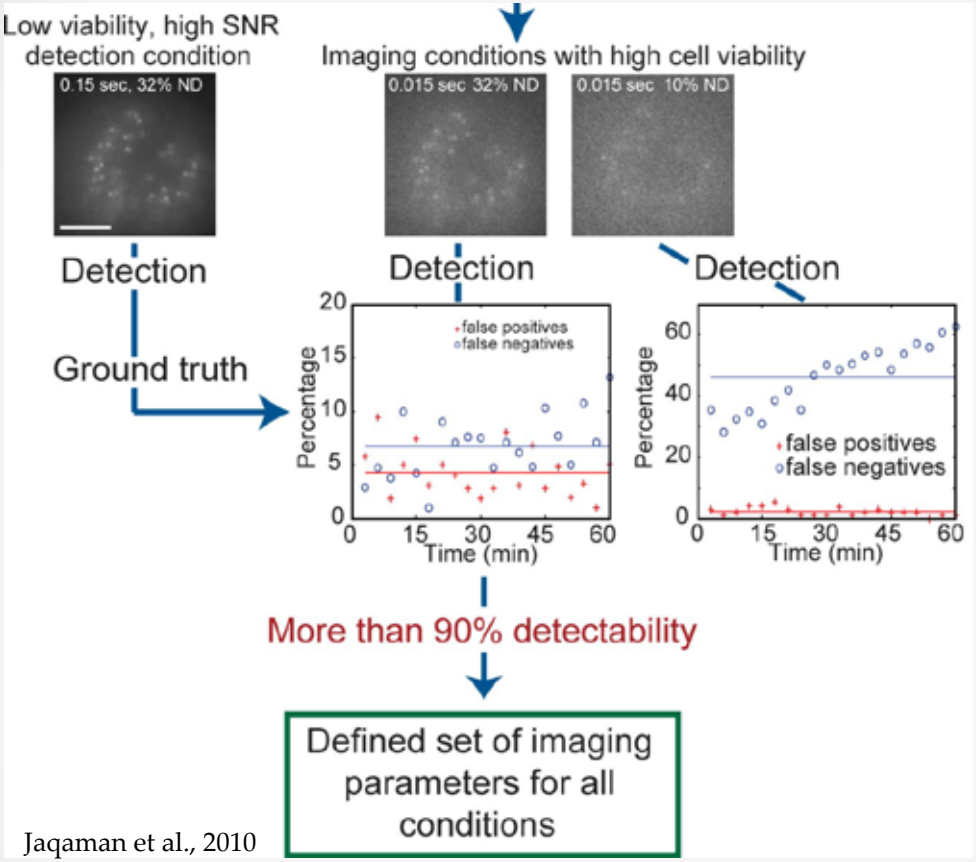
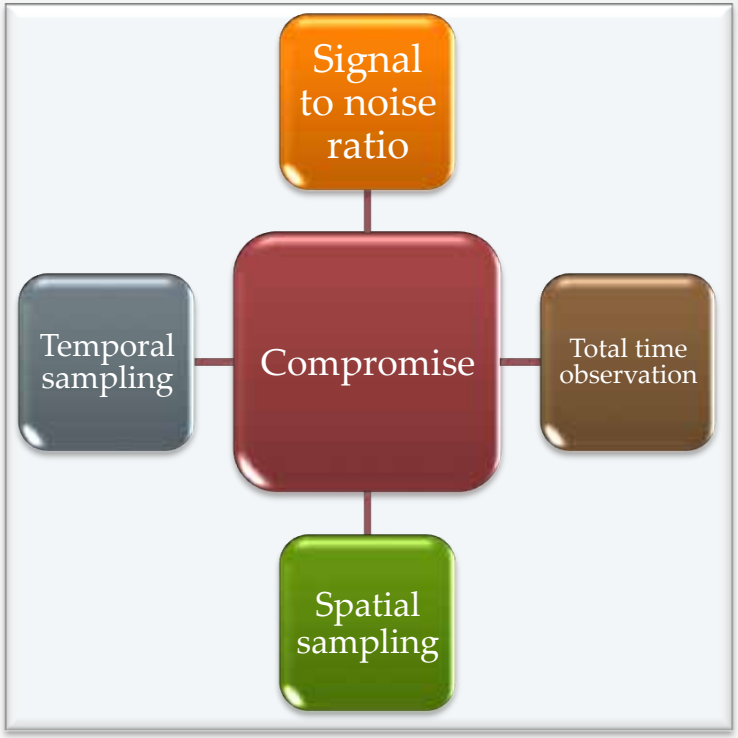
Max (signal) 320
Signal/noise = 4.5
Z=1/1 T=64/181
5h13min55s
x1.0


Controls Display Zoom View Window Help
Compression: None
Image
528
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
T
Z=1/1 T=64/181
5h13min55s
x1.0

Keep your cells alive





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

Optimizing live imaging

Increase signal

- Choose bright dyes
- Image through a clean N° 1.5 glass bottom dish
- Mount specimen as close to the coverslip as possible
- Use high NA clean objective with the lowest magnification
- Remove DIC Wollaston 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

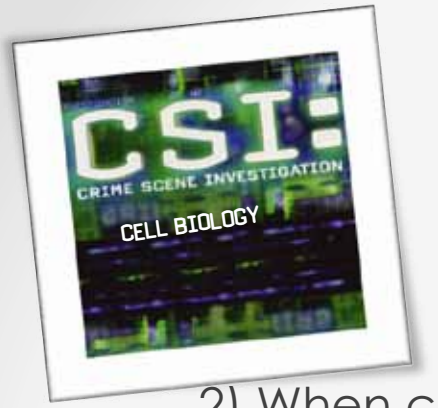
Decrease background

- Clean coverslip and optics
- Use media without phenol red for imaging
- Turn off the room lights

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

Image Processing

. . .



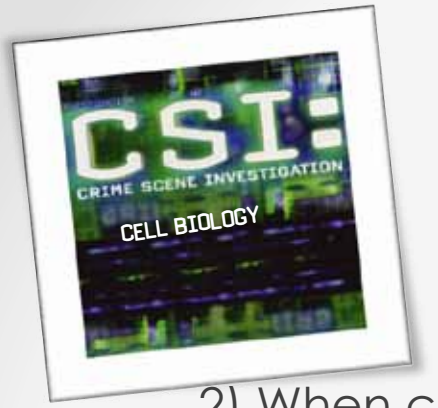
Tips for your analysis

1) Always keep the original data.

2) When converting image data from a proprietary format, save your image data as TIFF (tagged image file format) files.

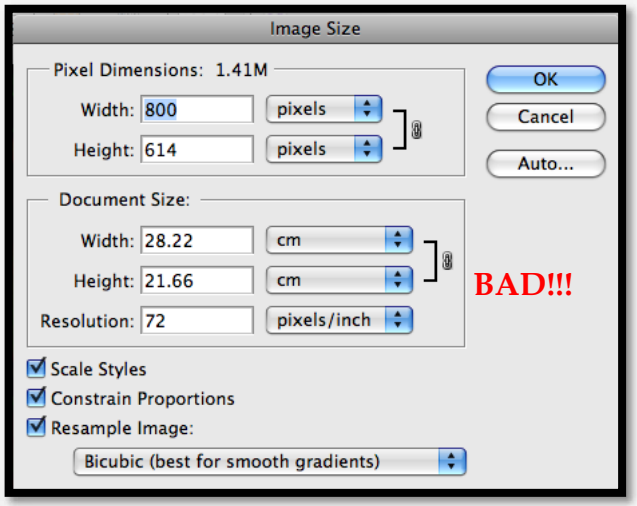
3) Simple adjustments to the entire image are usually acceptable.

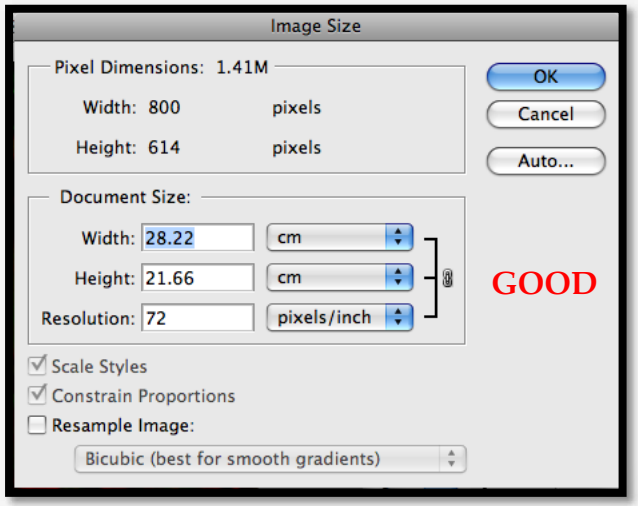
4) Acquire your images under identical conditions, and any post-acquisition image processing should also be identical.



Tips for your analysis

1) Always keep the original data.
2) When converting image data from a proprietary format, save your image data as TIFF (tagged image file format) files.
3) Simple adjustments to the entire image are usually acceptable.
4) Acquire your images under identical conditions, and any post-acquisition image processing should also be identical.
5) Be careful when you change your image size, especially in Photoshop





What to add in your M&Ms

- Manufacturer and type of system/microscope (eg Zeiss LSM700 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 CoolSNAP HQ2, 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 be required also.

Take-home messages

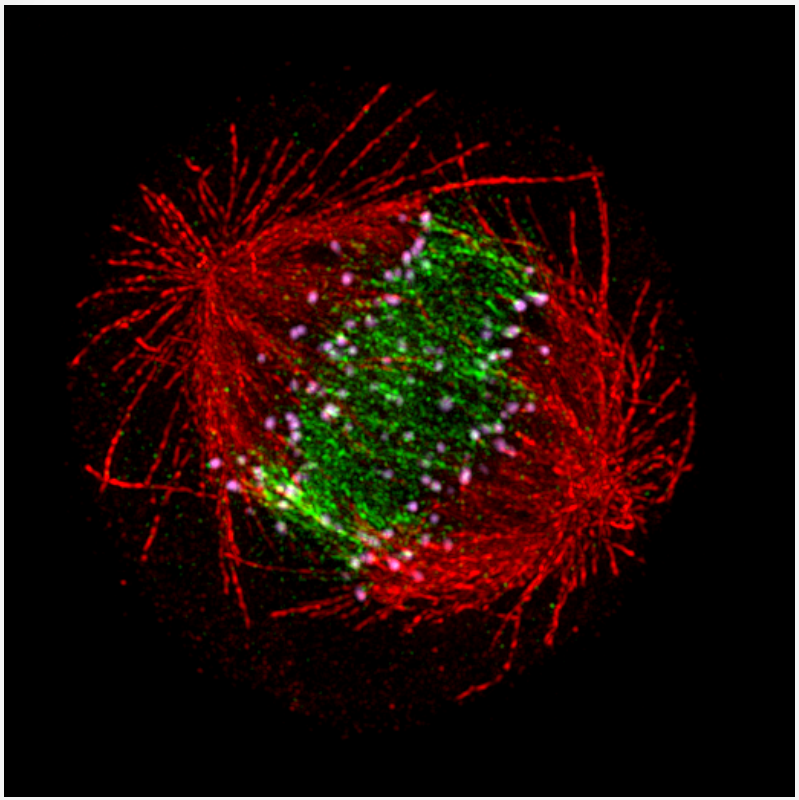
- The sample is part of the optics – Avoid sample-induced aberrations
- Try to increase signal and decrease background
- Do not overexpose your images
- Adjust exposure time, laser power (confocal), and neutral density (widefield/spinning disk) to keep your cell happier (alive and functional) for longer

• • •

Always keep your raw data and work on copies

Be ethical with your images!

Thank you!



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