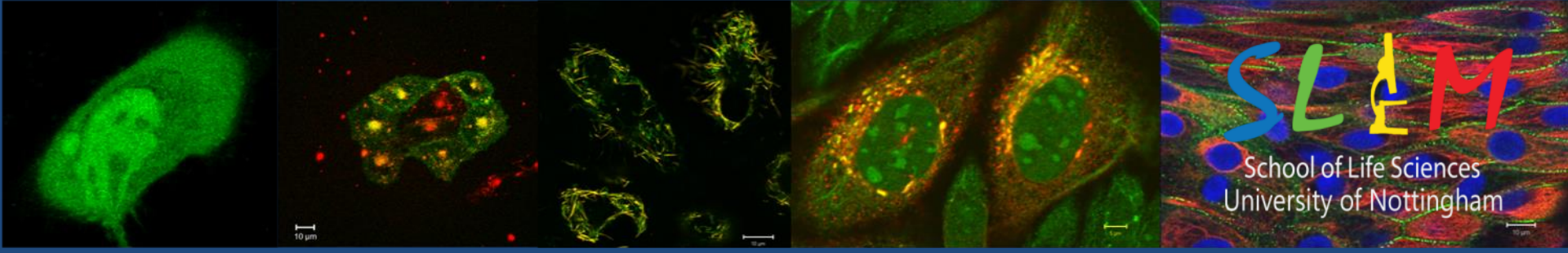

Wide field microscopy, resolution and restoration.

Chris Gell. Jan. 2018

Widefield images.

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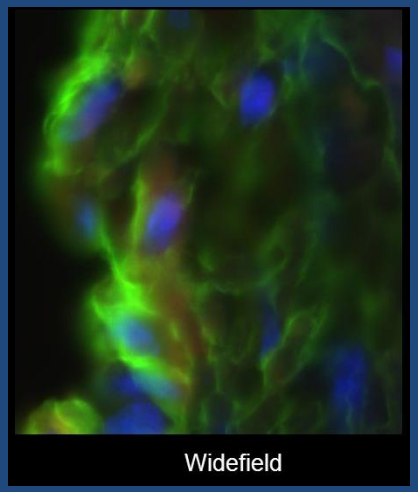
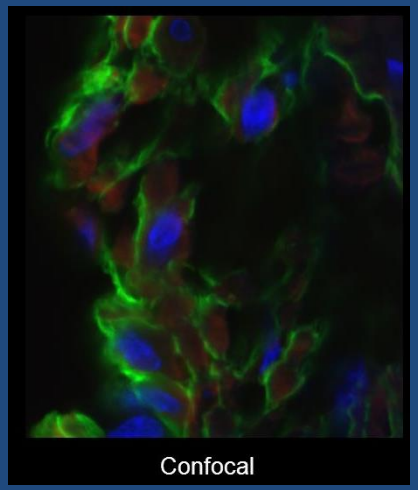
What is the resolution, in practice, of a microscope?

What is a wide field microscope?

Why are the images blurry?

What can we do about it?


Widefield


Confocal

20 um thick section (rat intestine, 60x/1.4NA)

Fundamentals for a good image: Resolution and contrast.

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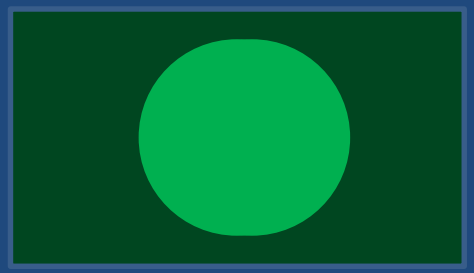
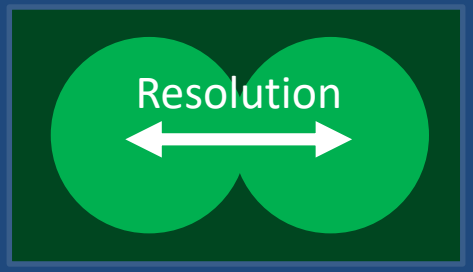
Resolution





Contrast

Theoretical resolution *vs* 'real' measurements

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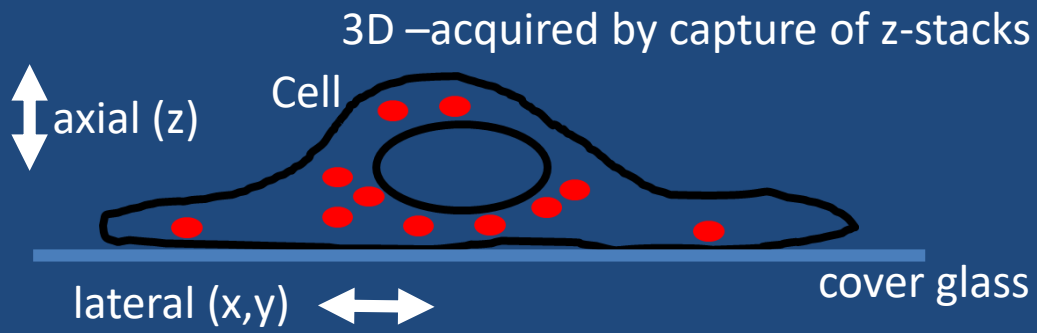
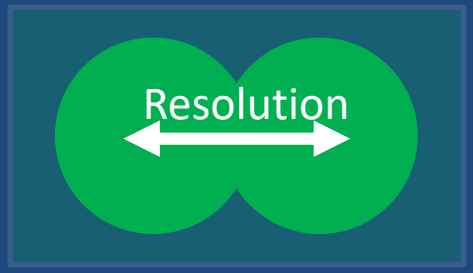
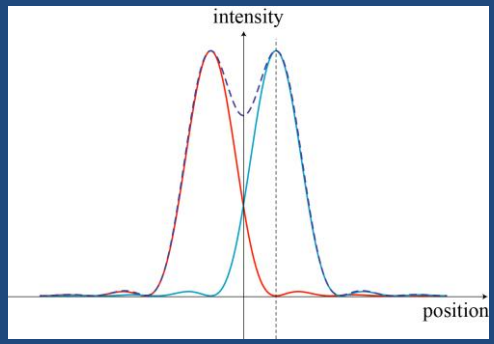
Lateral resolution (nm)	Object (nm)
	Theoretical	FWHM
WF	250	
Confocal	170	



Axial resolution (nm)	Object (nm)
	Theoretical	FWHM
WF	590	
Confocal	420	





Maximum resolution is dependant on wavelength and numerical aperture (assuming no aberrations). Axially also more on n.

wf. $lateral = 0.61\lambda$ / NA

conf. $lateral = 0.37\lambda$ / NA

wf. $axial = 2\lambda n / NA^2$

conf. $axial = 1.4\lambda n / NA^2$

NA – numerical aperture = 1.4
n – refractive index = 1
λ - wavelength = 594 nm

Theoretical *vs* 'real' resolution

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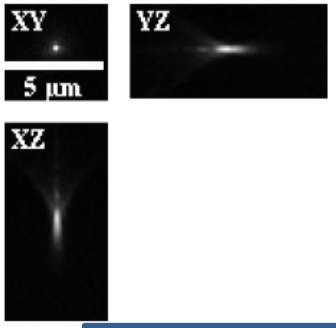
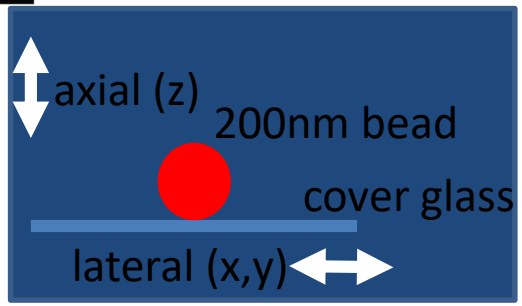
18 January 2019 11:08
PSF profiler report on C1-20170125_60x_res_02_R3D.ome_crop.ome - C=0-1

Profile view:





Microscope infos:

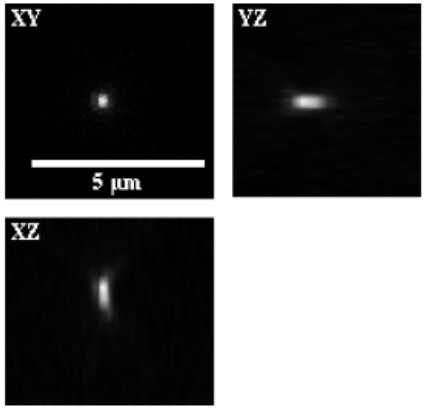
Microscope: WideField
Wavelength: 594.0 nm
NA: 1.42
Sampling rate: 0.106x0.106x0.2 µm

Resolution table:

	FWHM	Theoretical resolution
x	0.25 µm	0.255 µm
y	0.267 µm	0.255 µm
z	1.39 µm	0.589 µm

January 17, 2017 10:02 AM
PSF profiler report on TRITC

Profile view:



Microscope infos:

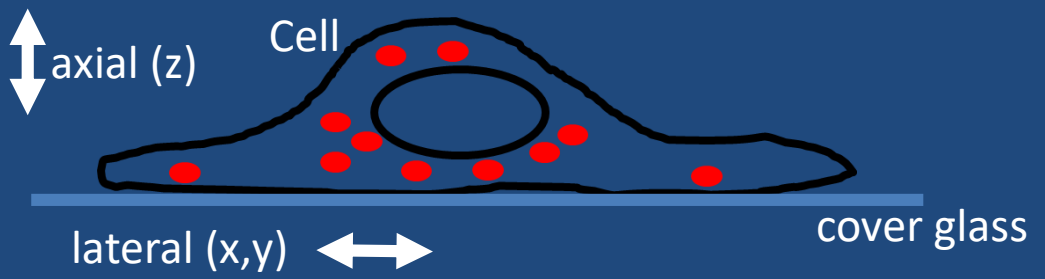
Microscope: Confocal
Wavelength: 590.0 nm
NA: 1.4
Sampling rate: 0.054x0.054x0.361 µm
Pinhole: 1.0 Airy Units

Resolution table:

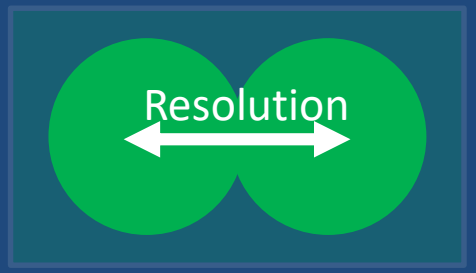
	FWHM	Theoretical resolution
x	0.214 µm	0.169 µm
y	0.296 µm	0.169 µm
z	0.404 µm	0.421 µm

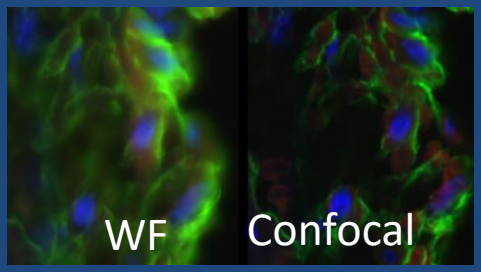
You need contrast to get good resolution.

Object FWHM Lateral (nm)	
WF	260
Confocal	250



Object FWHM Axial (nm)	
WF	1400
Confocal	400





Maximum resolution assumes ideal conditions (esp. SNR/SBR) – 'perfect' contrast.

Wide field microscopes have reduced contrast which leads to an apparently poorer resolution.

Contrast

What is a widefield microscope?

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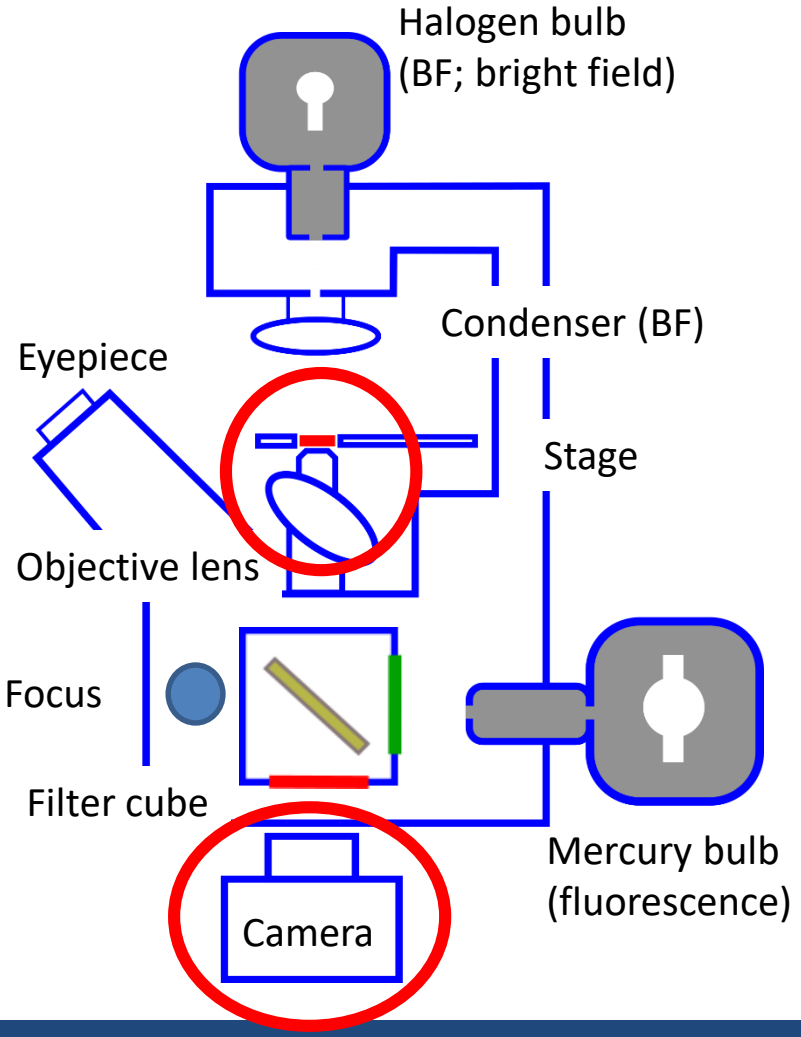

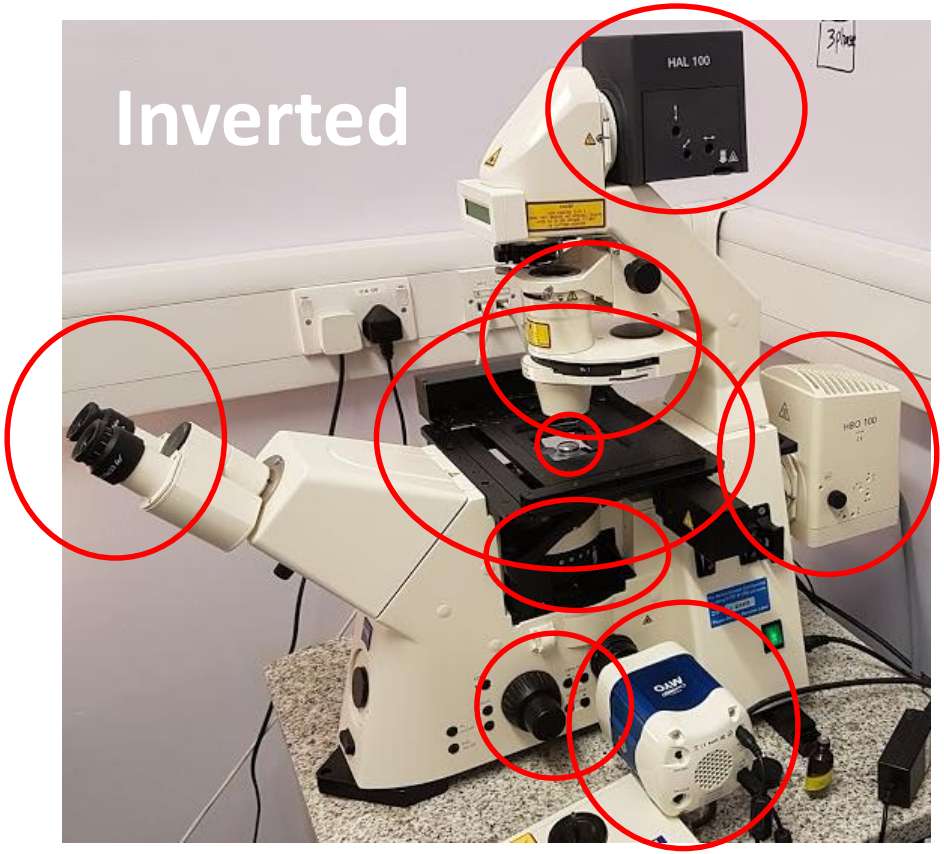

What is a widefield microscope?

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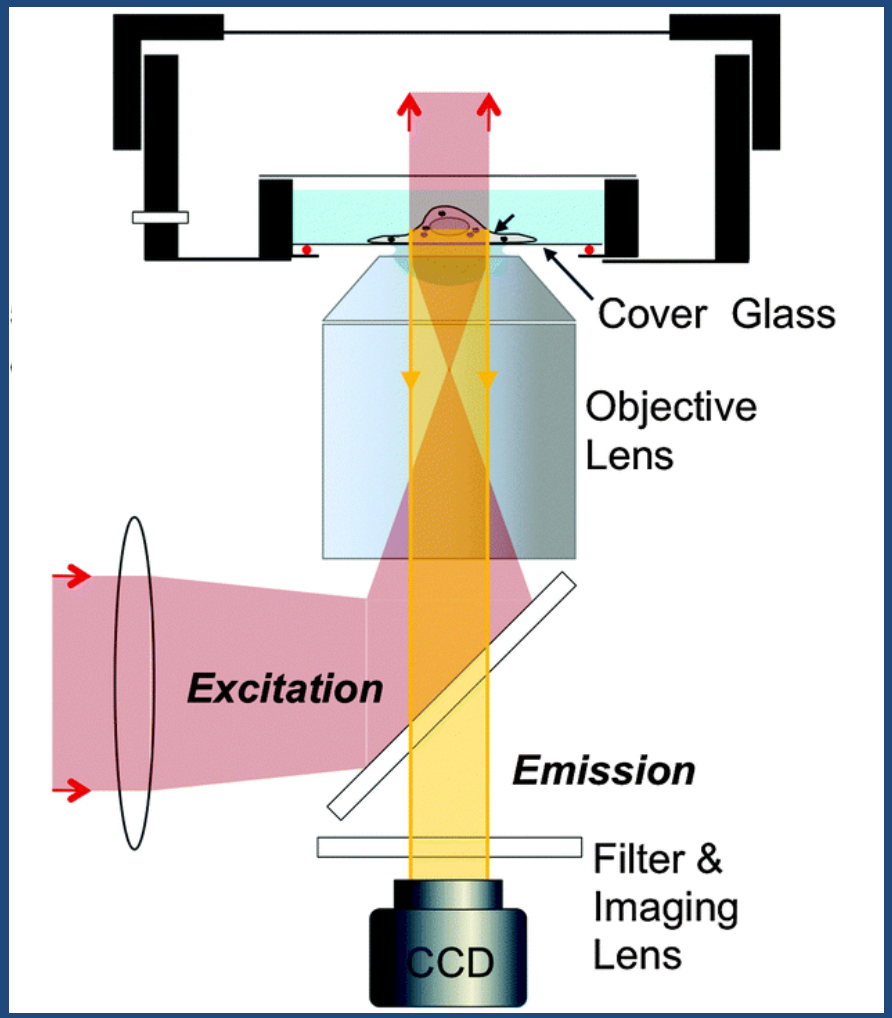
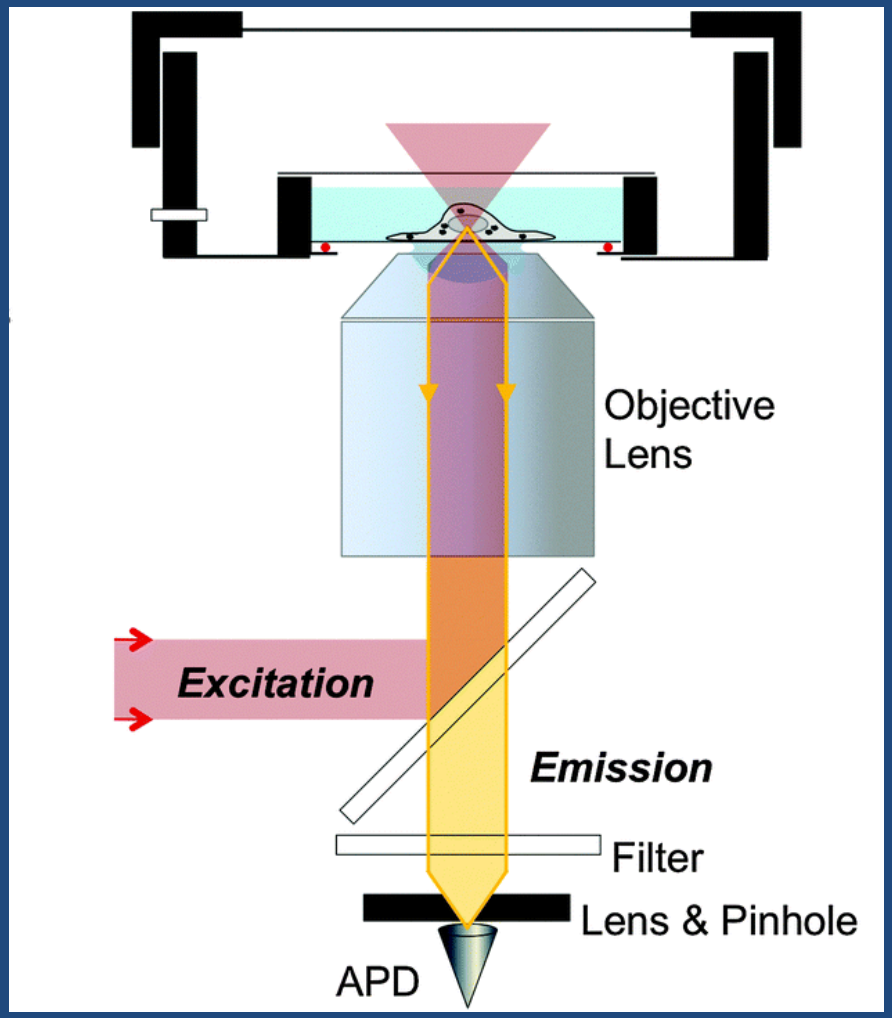
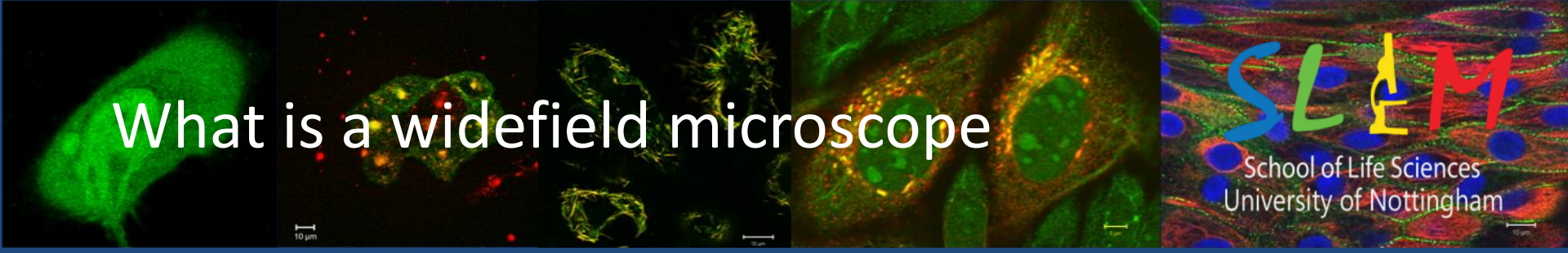
Widefield



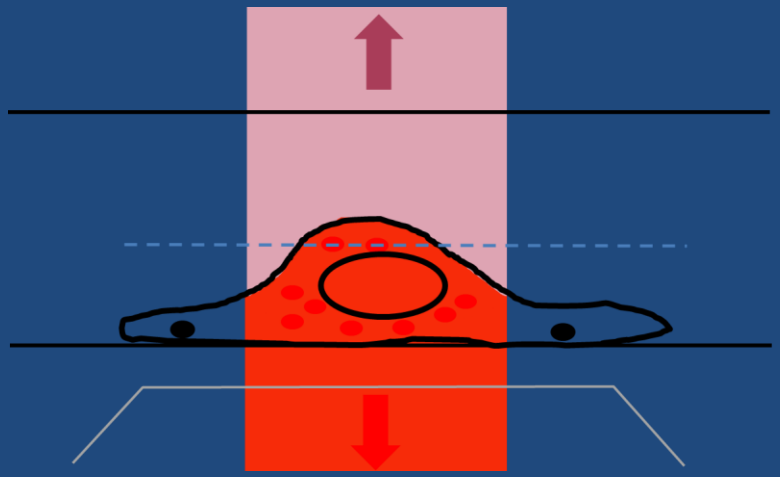
Confocal



What is a widefield microscope

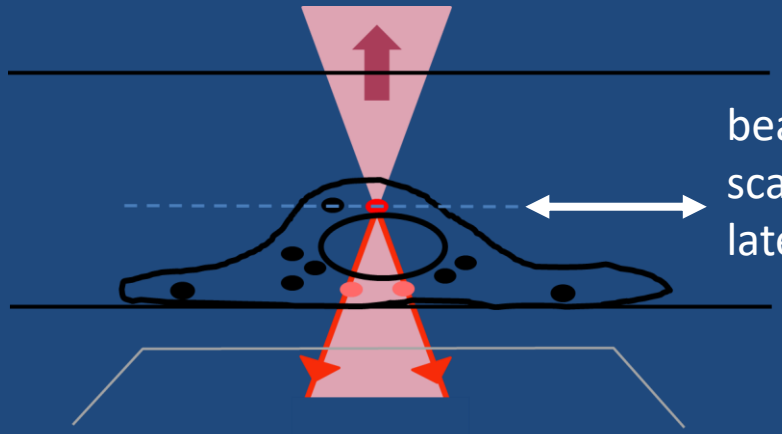


Wide field

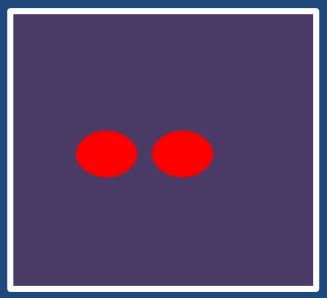


Direct to camera

Confocal



Scanned to a detector *via* a pinhole



Same issue for the beads

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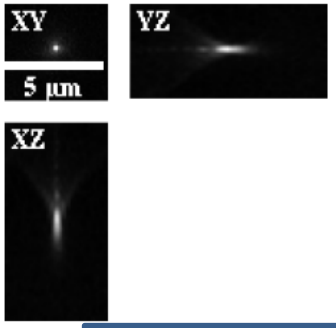
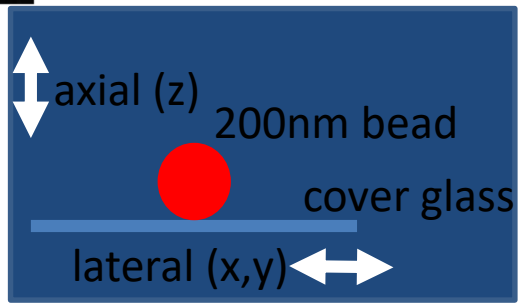
18 January 2019 11:08
PSF profiler report on C1-20170125_60x_res_02_R3D.ome_crop.ome - C=0-1

Profile view:





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January 17, 2017 10:02 AM
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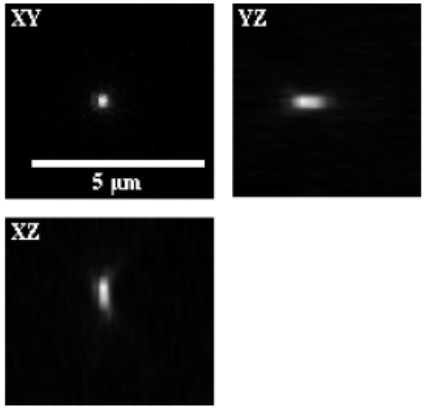

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Resolution table:

	FWHM	Theoretical resolution
x	0.214 µm	0.169 µm
y	0.296 µm	0.169 µm
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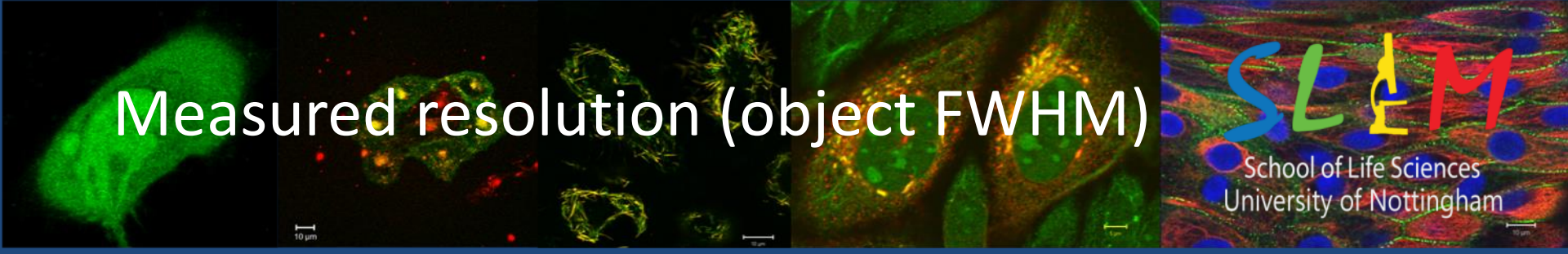
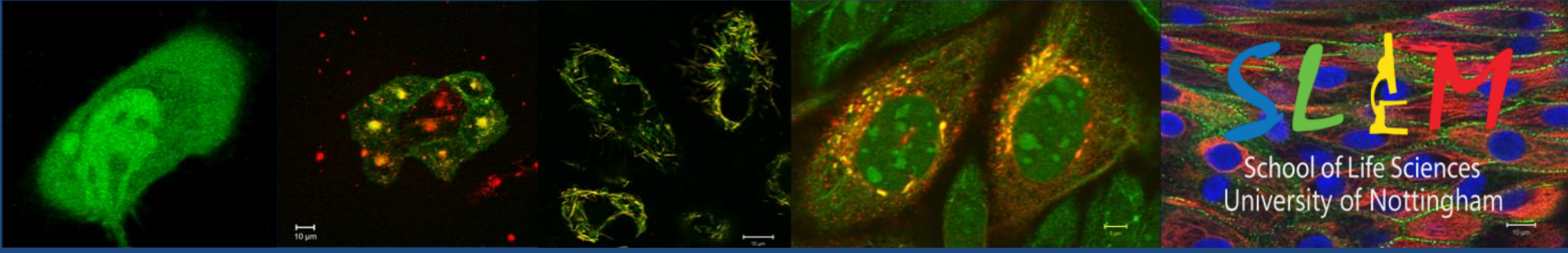
Measured resolution (object FWHM)



Object FWHM Lateral (nm)	
WF	260
Confocal	250

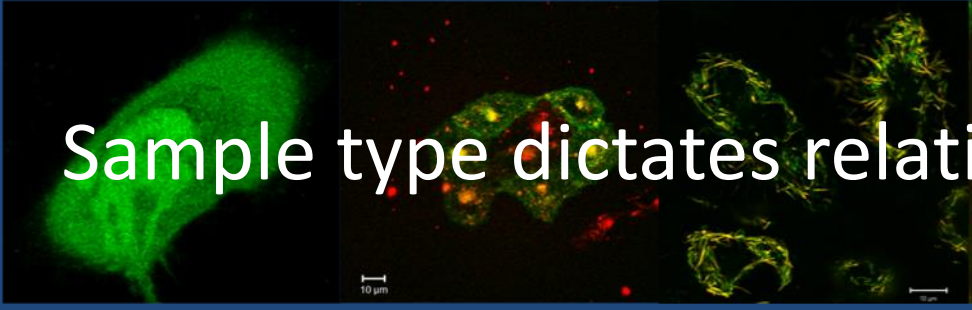
Object FWHM Axial (nm)	
WF	1400
Confocal	400

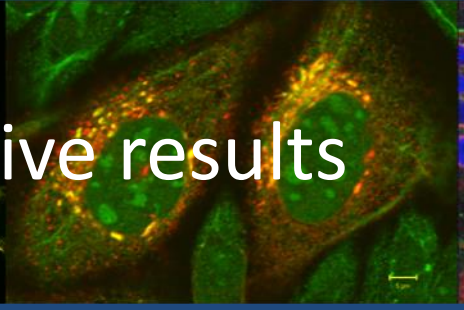
The ‘poor’ practical (measured) resolution in wide field is largely due to a lack of contrast as signal is collected from a ‘wide’ volume of the sample.



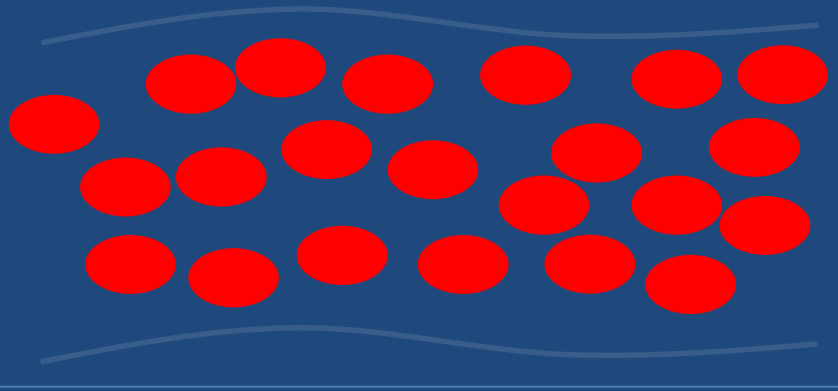
Anything we can do to improve WF performance?

Sample type dictates relative results





Thick and dense – difficult via wide field



cover glass

Disperse monolayer – better results via WF

- reduce background
- brighter stains
- lower wavelength, higher NA, higher n mounting/immersion (ignoring aberrations)

Contrast in widefield fluorescence.

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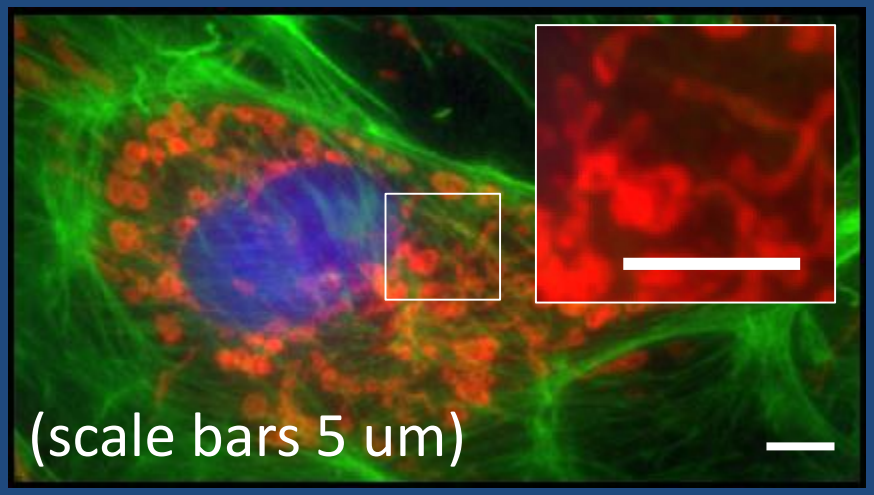
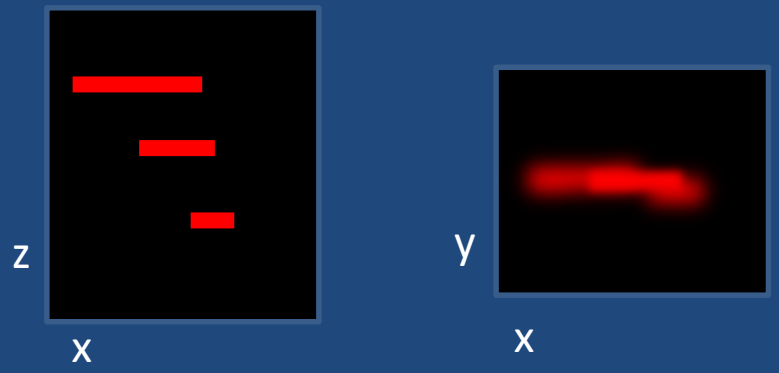
Wide field





- Fluorescence from nearby moieties (mostly above and below plane of focus): **secondary fluorescence**.
- This reduces contrast, thereby reducing effective resolution.
- **Also makes data much less quantitative.**

BPAE cells with MitoTracker Red, Alexa Fluor 488 Phalloidin, and DAPI (Chris Gell).

Restoration (aka deconvolution) can ameliorate this.

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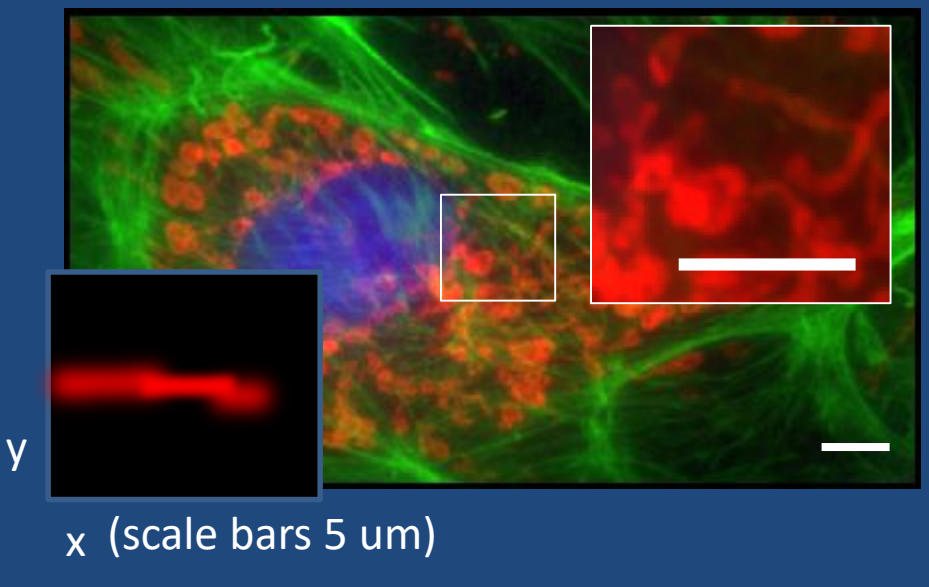
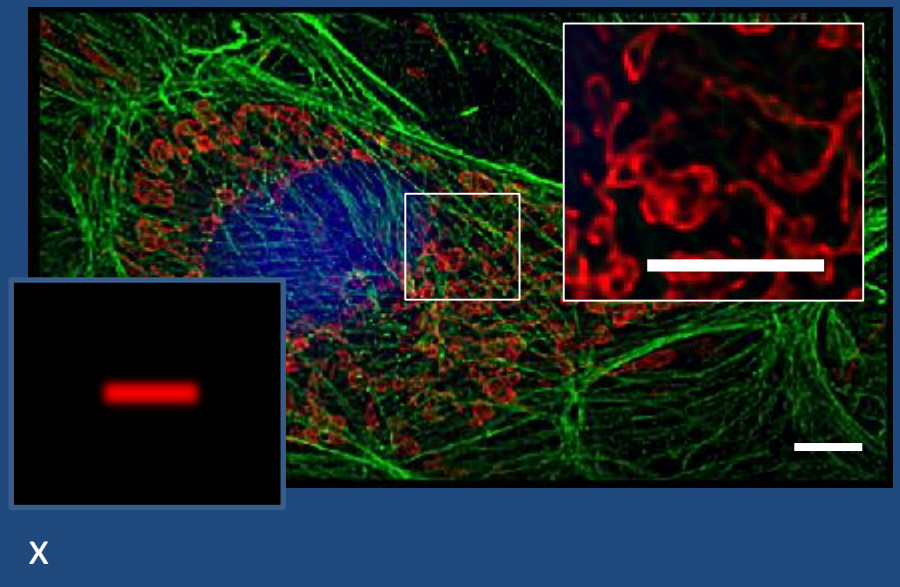
Wide field fluorescence *vs* Wide field restoration

Wide field



(scale bars 5 um)

Restored (in Huygens pro)



More resolution (contrast) and quantitative results.

BPAE cells with MitoTracker Red, Alexa Fluor 488 Phalloidin, and DAPI (Chris Gell).

Image deconvolution

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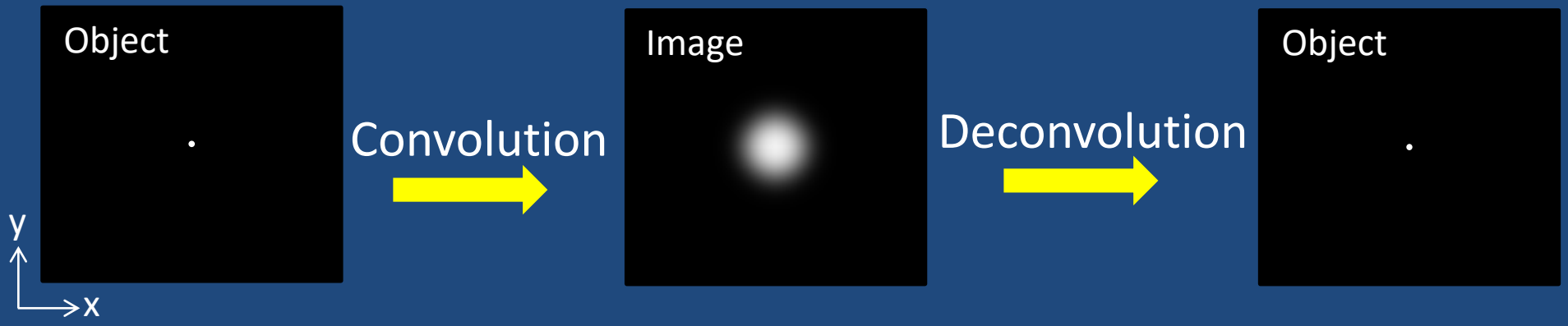
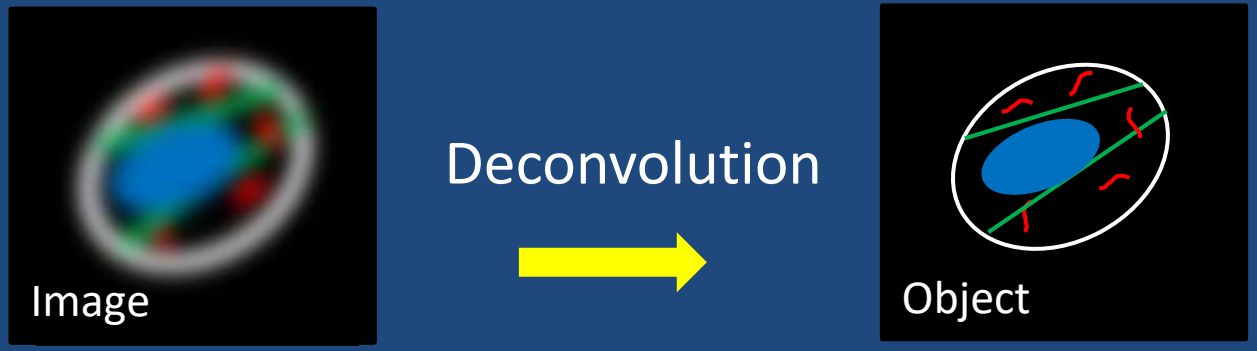
A microscope is a diffraction limited imaging system.
Diffraction of light (and other effects) leads to “blur”.



Knowing the system response to a point light source (PSF), the object can restored from image.



Iterative deconvolution

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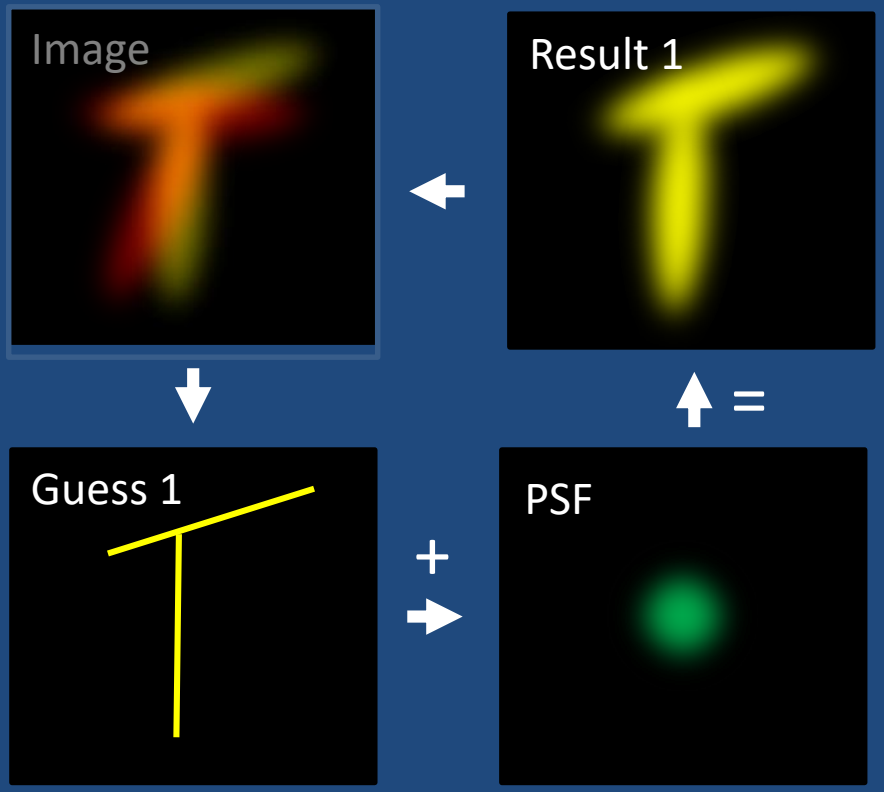
Iteration 1



Iterative deconvolution

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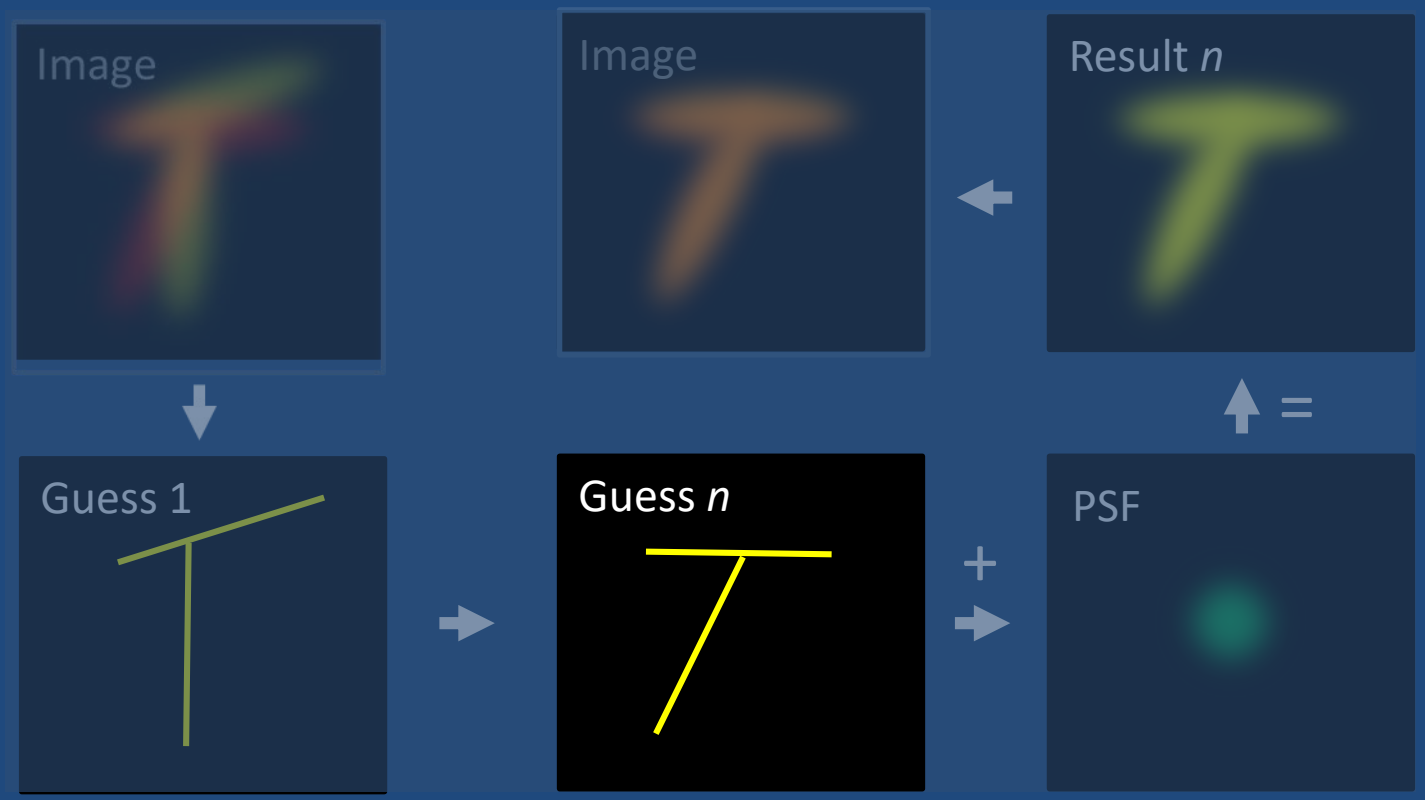
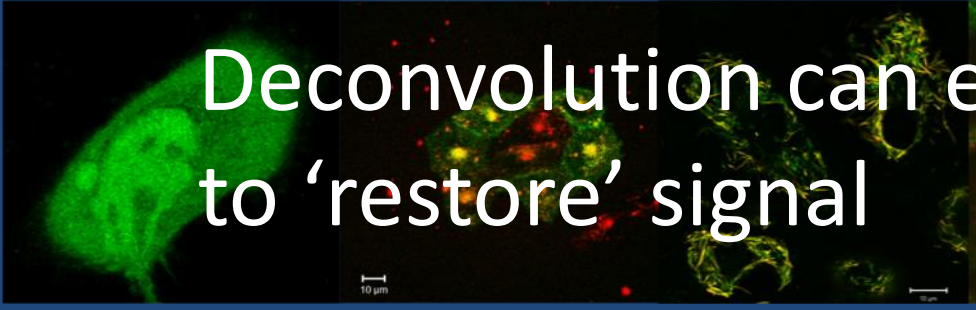
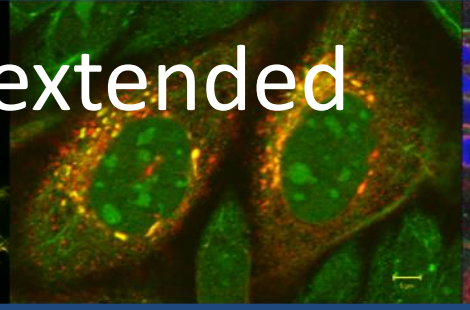
Iteration *n*



Deconvolution can extended to ‘restore’ signal





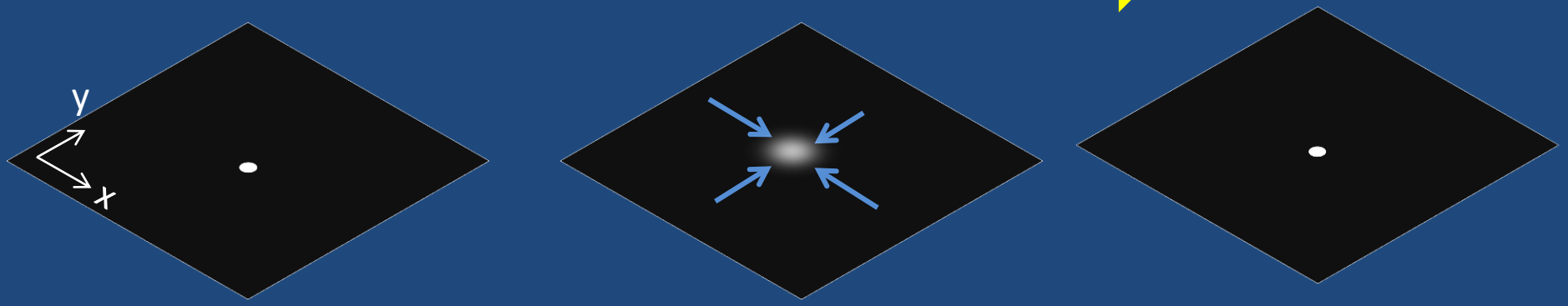
Real Object

Measured

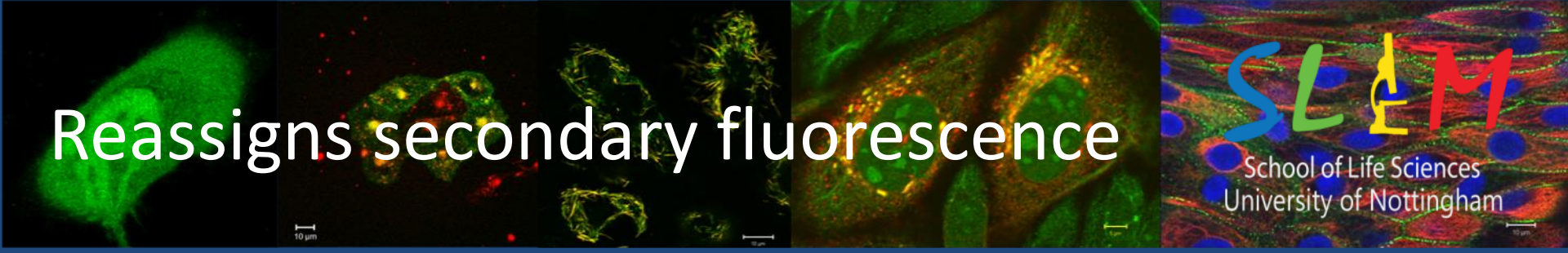
Restored

Convolution

Restoration



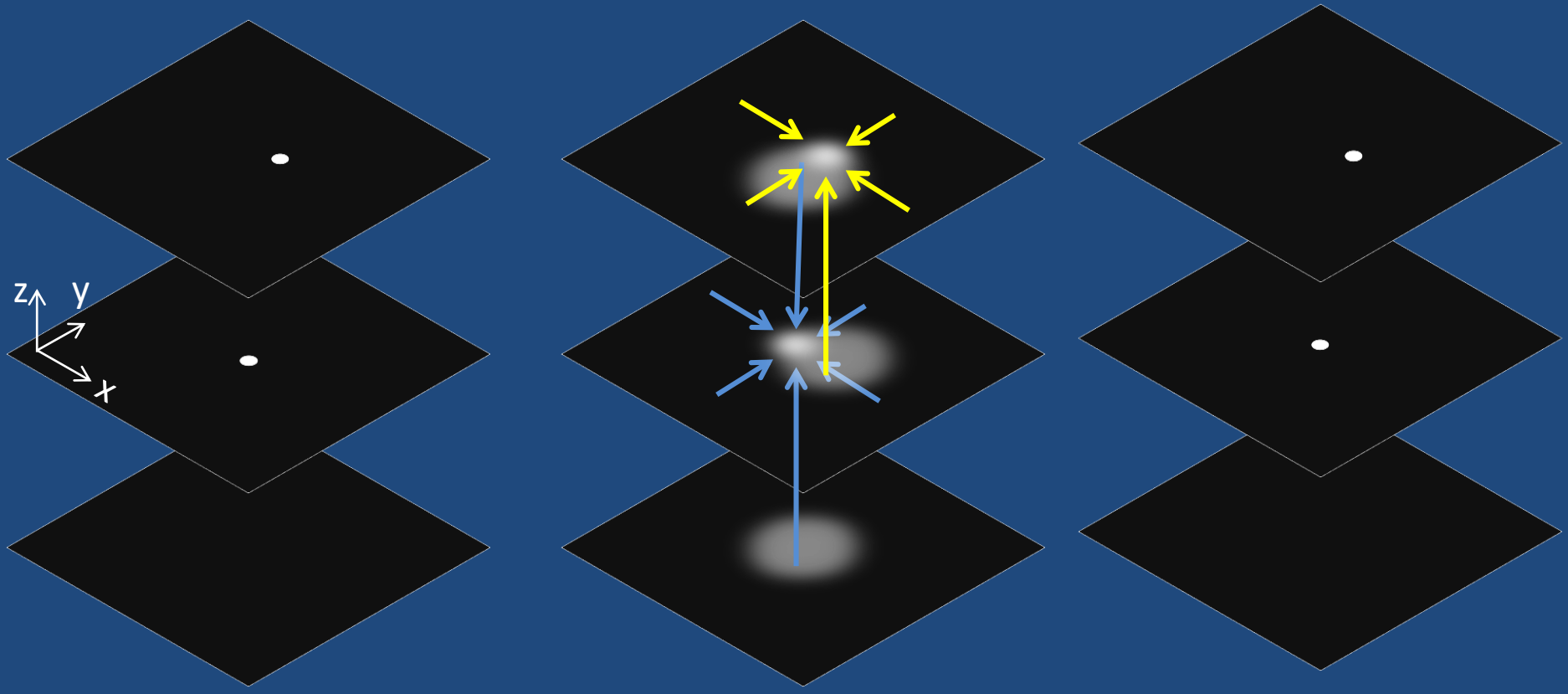
Reassigns secondary fluorescence



Real Object

Measured

Restored



Quantitative results, enhanced contrast.

Deconvolution/Restoration Methods

Slower

Blind de-blurring.	Imprecise. No reassignment. Removes information. Affected by noise and aberrations.
Non-blind iterative.	Needs a measured PSF. Potentially very precise (but often not). Affected by aberrations.
Adaptive iterative blind.	No measured PSF. Good with aberrations. Very computer intensive.

Accuracy

Does it restoration really improve resolution?

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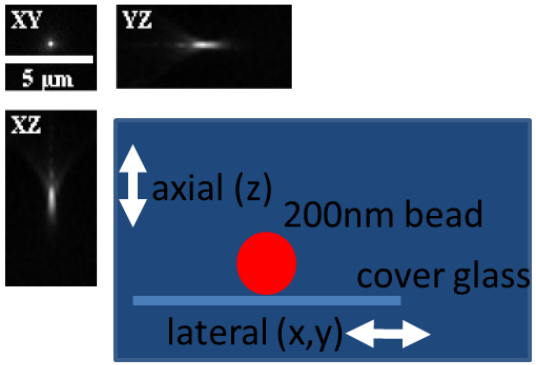
18 January 2019 11:08
PSF profiler report on C1-20170125_60x_res_02_R3D.ome_crop.ome - C=0-1

Profile view:



Microscope infos:

Microscope: WideField
Wavelength: 594.0 nm
NA: 1.42
Sampling rate: 0.106x0.106x0.2 µm

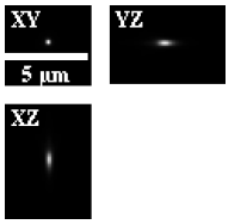
Resolution table:

Unrestored

	FWHM	Theoretical resolution
x	0.25 µm	0.255 µm
y	0.267 µm	0.255 µm
z	1.39 µm	0.589 µm

18 January 2019 11:07
PSF profiler report on C1-20170125_60x_res_02_R3D.ome_crop_decon_2.ome - C=0-1

Profile view:



Microscope infos:

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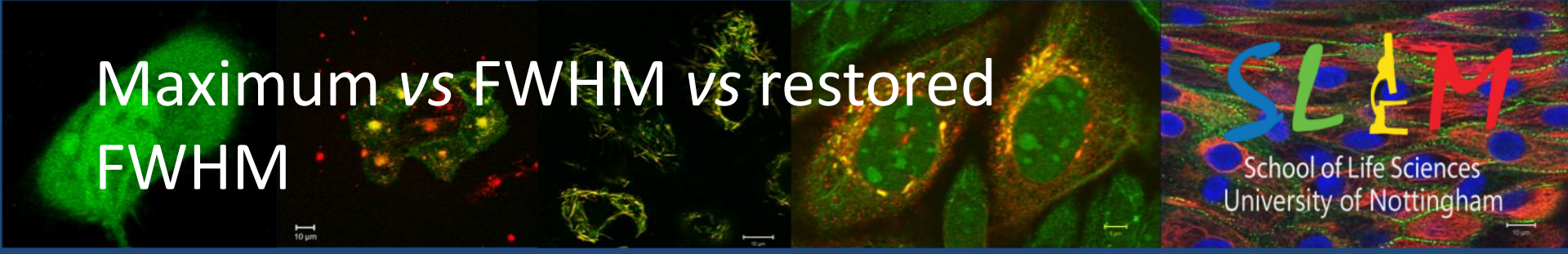
Resolution table:

Restored

Maximum *vs* FWHM *vs* restored FWHM



Lateral resolution (nm)		Object FWHM (nm)	
	Theoretical	FWHM	FWHM (Restored)
WF	250	260	205
Confocal	170	240	-

Axial resolution (nm)		Object FWHM (nm)	
	Theoretical	FWHM	FWHM (Restored)
WF	590	1400	652
Confocal	420	400	-

The ‘poor’ resolution in wide field is really a contrast issue. Restoration can help.

Practical considerations for restoration

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- Data collection (setting a good exposure, correct sampling).
- Sample (staining, structure).
- Understanding of parameters (RI, NA, coverslip parameters, wavelengths, x-y-z voxel sizes).
- Which algorithm / software?
- Time consuming, but off-line.
- Do you actually need it?!

Huygens

Fiji (ImageJ)

Restored Images – contrast, reduced background.

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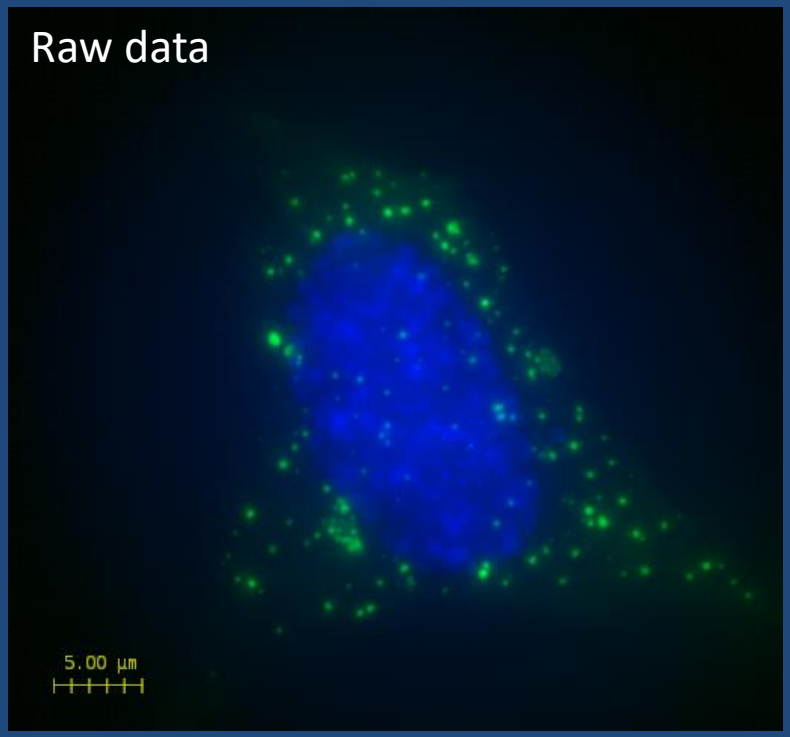
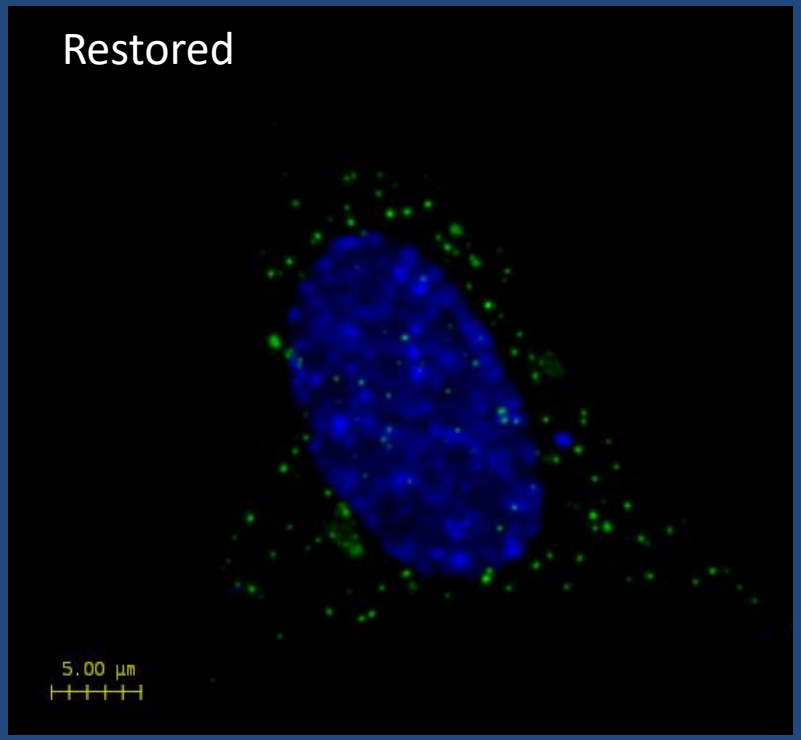
Z-stack viewed in ‘extended focus’ (MIP), fixed cell.





p62 in NSC34 cells (Alice Goode/Rob Layfield/Chris Gell).

Intensity quantitation

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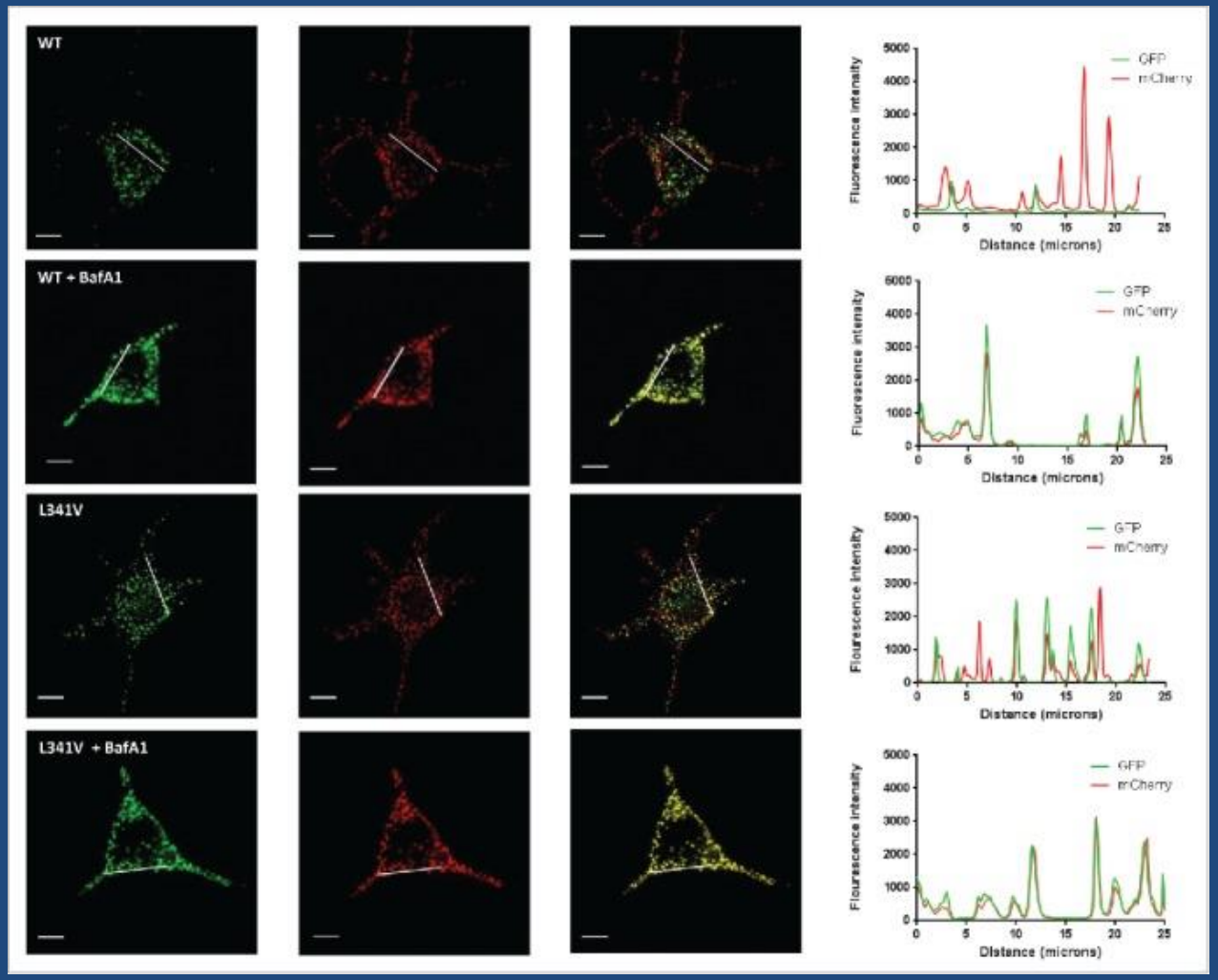
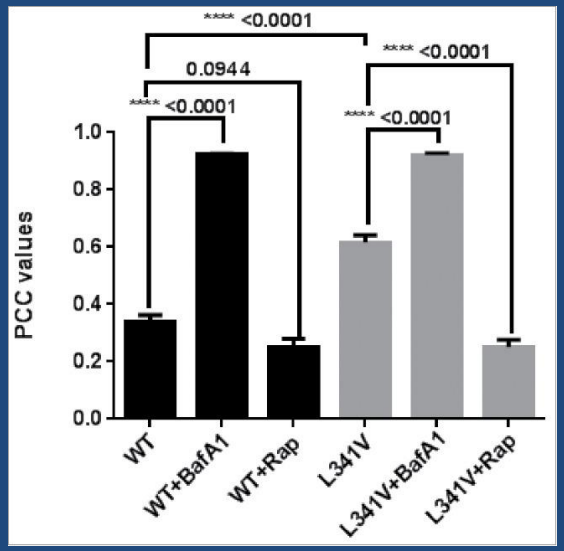
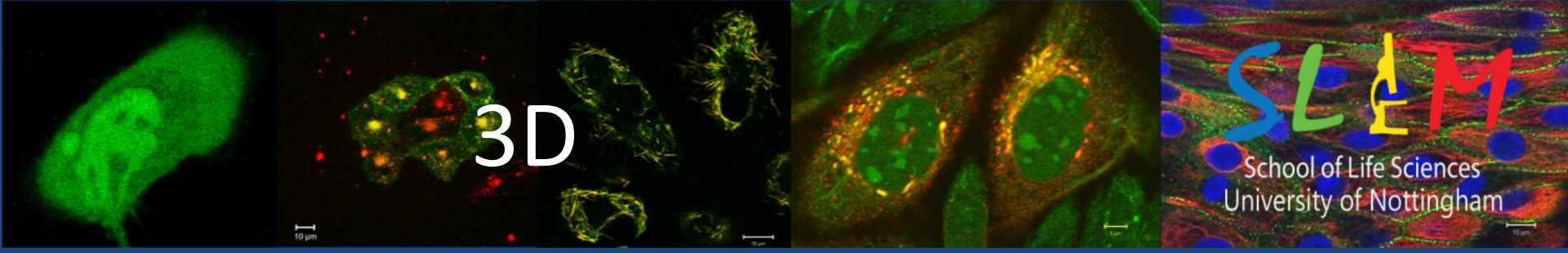
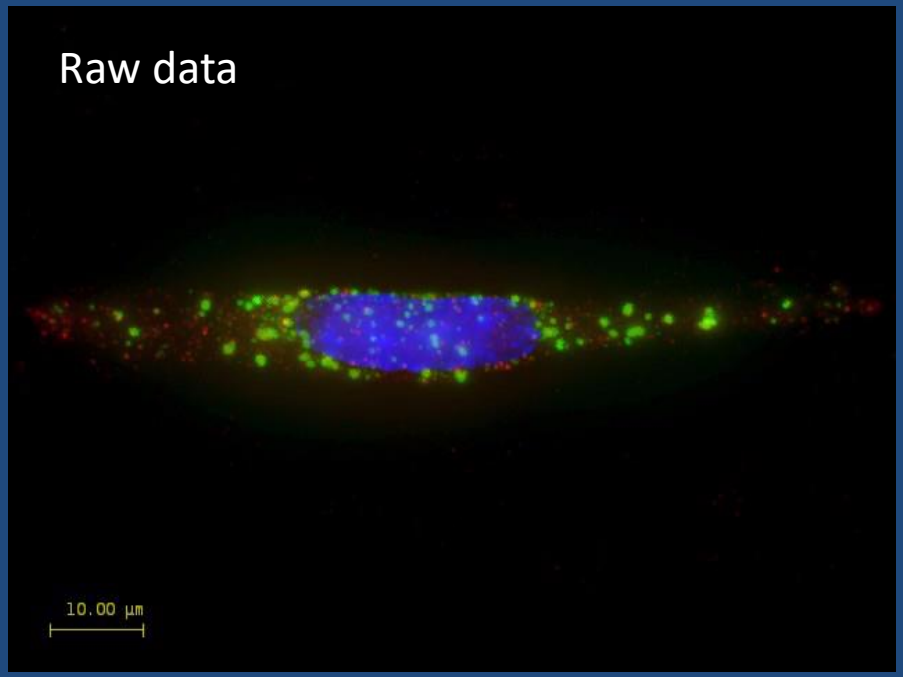
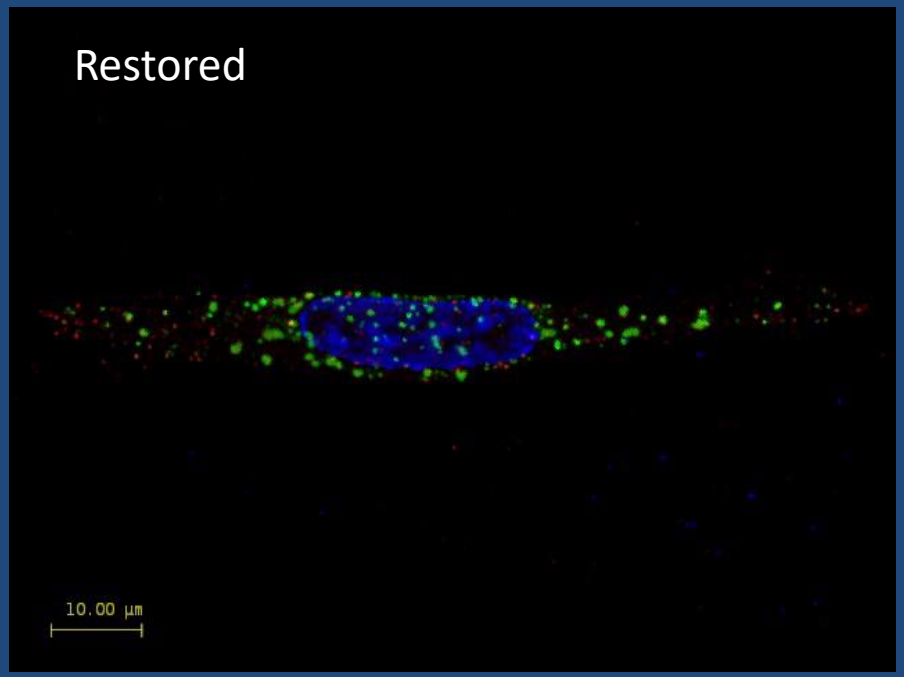






Z-stack viewed in 3D, fixed cell.





Proteins p62 and LC3 in NSC34 cells (Alice Goode/Rob Layfield/Chris Gell).

Background suppression

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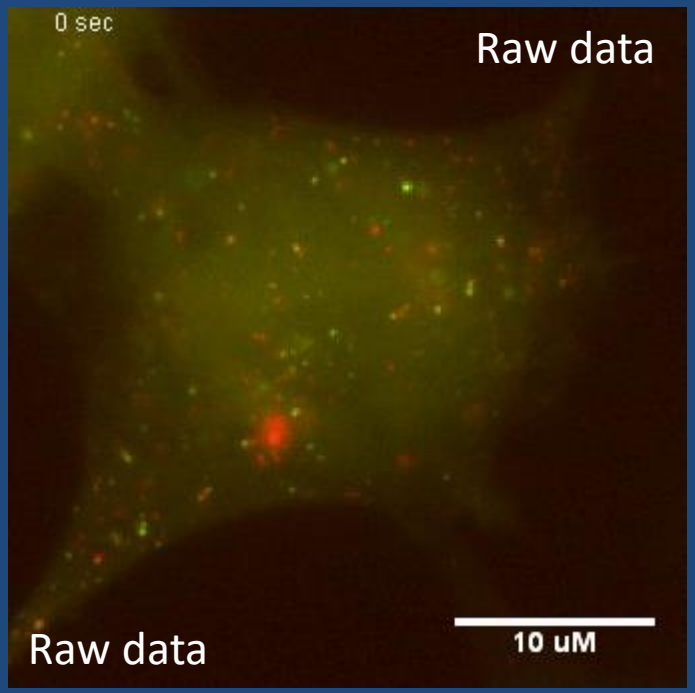
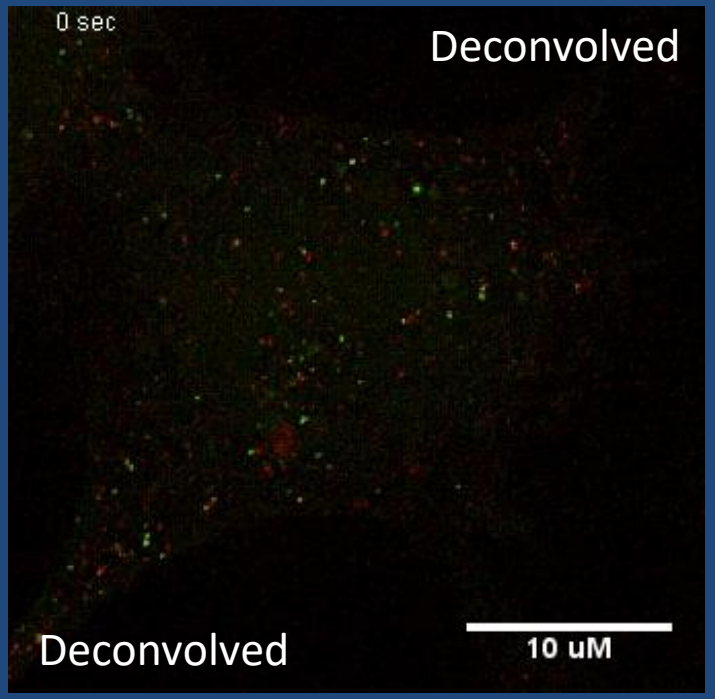
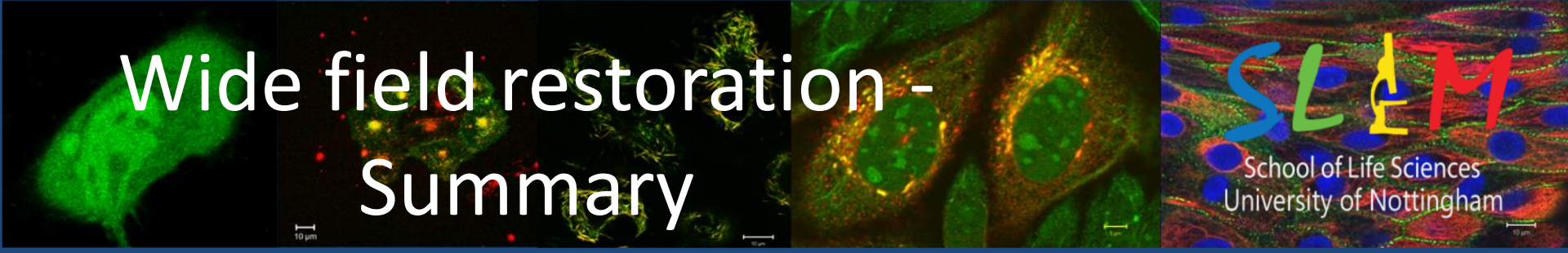
Single slice, time lapse, live cell.



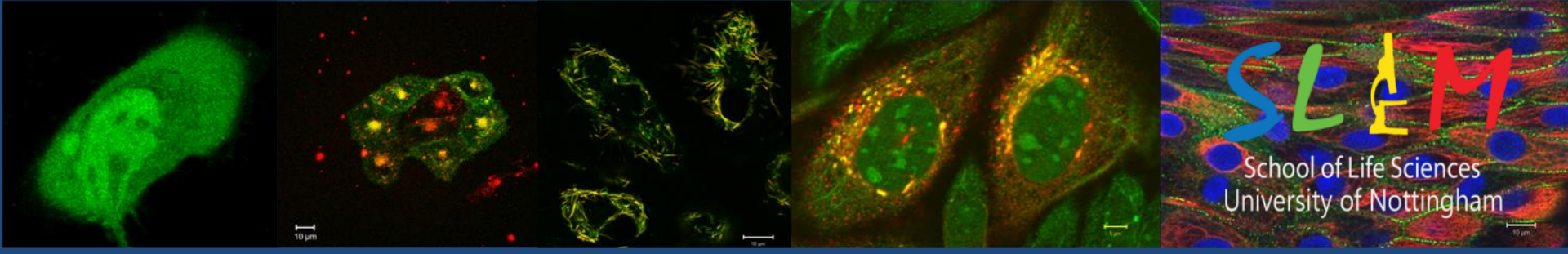


MEF cells stably transfected with mCherry-GFP-LC3 as a marker of autophagy (Alice Goode/Rob Layfield/Chris Gell).

Wide field restoration - Summary



- Wide field fluorescence microscopes collect light from a large volume, typically this results in low contrast.
- Maximum resolution (in any conventional scope) is defined by NA and λ, but is limited by contrast (and aberrations).
- Contrast is affected by many things: Labelling, lens, sample thickness, filters, camera etc. (Anything that affects SNR/SBR.)
- Restoration is a post-acquisition method to increase image information content.
- Need to acquire z-stacks to be a useful method.
- Images need structure – useless for diffuse staining (eg. cytosolic).
- Still limited by image quality (noise, aberrations): Structures must be visible!
- Can also be used to improve confocal data (esp. if pinhole was opened).
- Very useful for some subsequent analyses (eg. co-localisation, intensity quantitation, more accurate spatial measurement).



"Deconvolution is not photoshopping,
it's a systematic error correction."

Someone on Twitter

More information:

https://www.ibiology.org/speakers/jeff-lichtman/

4 – talks: Huygens Wavelets, resolution (2), PSF

https://www.ibiology.org/talks/deconvolution-microscopy/

David Agard UCSF