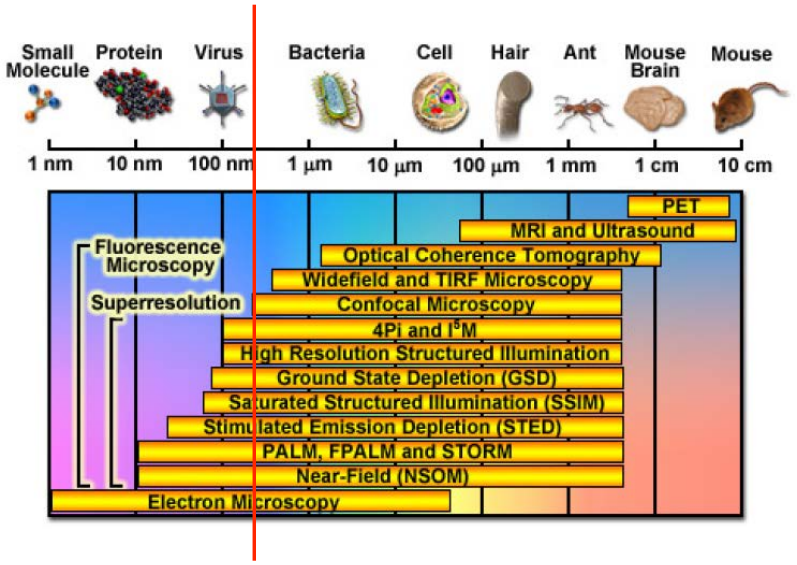
MRC Laboratory of Molecular Biology

Super-Resolution Microscopy

Jonathan Howe

Spatial Resolution of Biological Imaging Techniques



Optical Resolution Limit!
~200-250 nm

Image from Zeiss Website

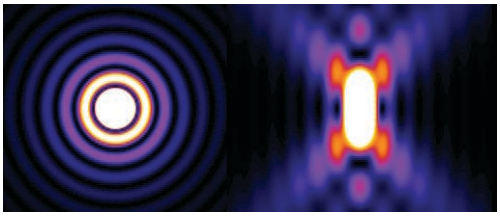
Diffraction Limited Imaging

Image of Point Source – Point Spread Function (PSF)

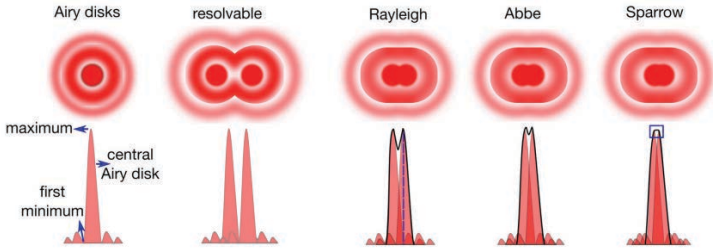
XY plane XZ plane

Point Source
e.g. Single
Fluorophore (~1 nm)

Lens



Airy Pattern



PSF Image from PSF Generator (BIG, EPFL). Resolution Criteria Image from Sezkin, Journal of Physics, 2017

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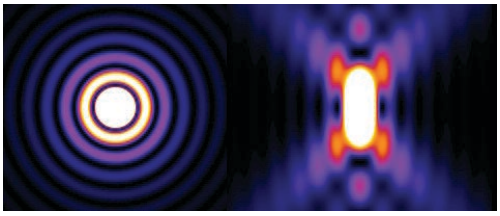
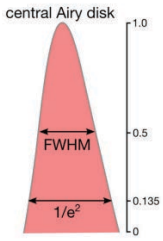


Airy Pattern

Lateral (XY) Resolution ≈ PSF_{FWHM} ≈ 200-250 nm

Axial (Z) resolution is typically 2-3 times worse i.e. 500-700 nm.

FWHM – Full-Width at Half Maximum



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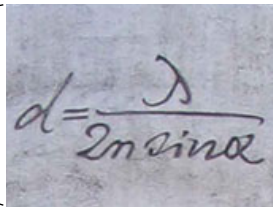
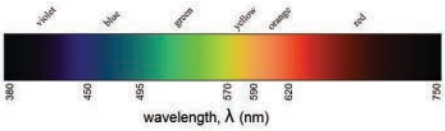
Lateral (XY) Resolution Limit



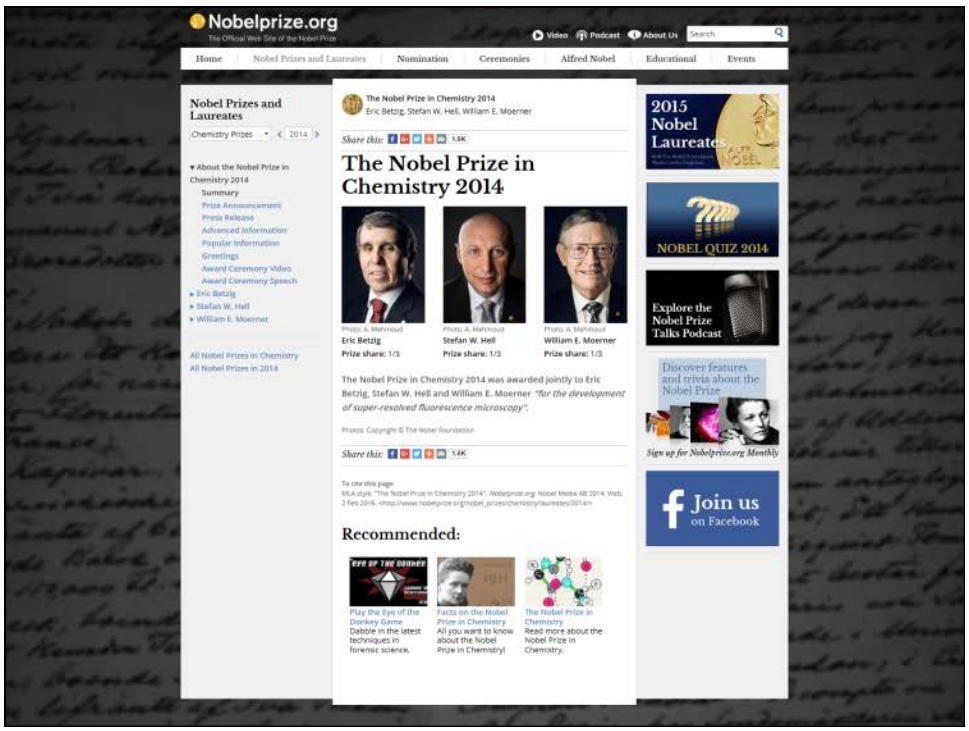


At best λ is 400-500 nm

nSinα = NA (Numerical Aperture) ≈ 1

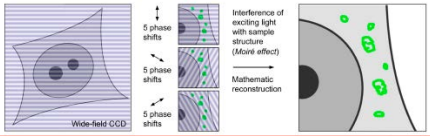
Therefore,
d (lateral [XY] resolution) ≈ λ/2 ≈ 200-250 nm

Images from Wikipedia , Universiy of Texas Website and Hell, Nature Methods, 2009.



Super-resolution Microscopy Techniques

- Structured Illumination Microscopy (SIM). Sample is excited with patterned illumination, which highlights different objects at different times.



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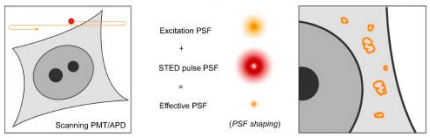
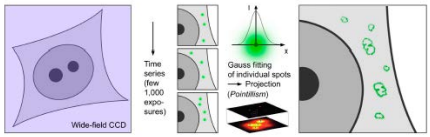


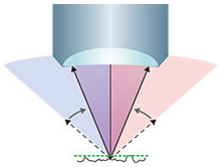
- Localisation Microscopy (LM) e.g. PALM and STORM. Fluorophores are randomly activated, imaged and bleached. Localisation of the fluorophores builds up a super-resolution image over time.



Schermelleh *et al.*, JCB, 2010.

Structured Illumination Microscopy (SIM)

$d \approx \lambda/4NA$



- The patterned illumination light and high frequency structures in the sample interfere with each other to form a third pattern (moiré fringes) that have a lower spatial frequency and can therefore be captured by the objective lens.
- The interdependency of the 3 patterns is used to compute the unknown super-resolution information in the sample.

pattern 1 (grid) *pattern 2 (sample)* *pattern 3 (Moiré)*

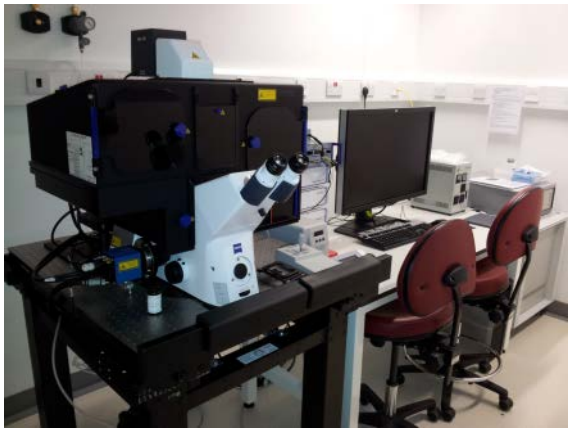
- Acquiring raw images with patterned excitation at 3 (2D) or 5 (3D) phases and 3-5 orientations and processing all acquired images using computer algorithms, a super-resolution image can be generated (2-fold improvement in XYZ resolution). 9-25 images per Z plane - bleaching!

XY resolution: 100-130 nm
Z resolution: 250-350 nm

Images from Nikon website.

Zeiss Elyra S.1

- 4 lasers (50 mW 405 nm, 100 mW 488 nm, 100 mW 561 nm, 150 mW 640 nm).
- 100x 1.46 NA oil objective.
- 63x 1.4 NA oil objective.
- 60x 1.2 NA water objective.
- Pco-edge sCMOS camera capable of 100 fps.
- 5 gratings (23 µm, 28 µm, 34 µm, 42 µm and 51 µm).
- Up to 4 colours in one sample is realistically possible.
- Resolution improvement is limited by wavelength of label. Blue/green dyes better than red/far red – Also true for diffraction-limited imaging!



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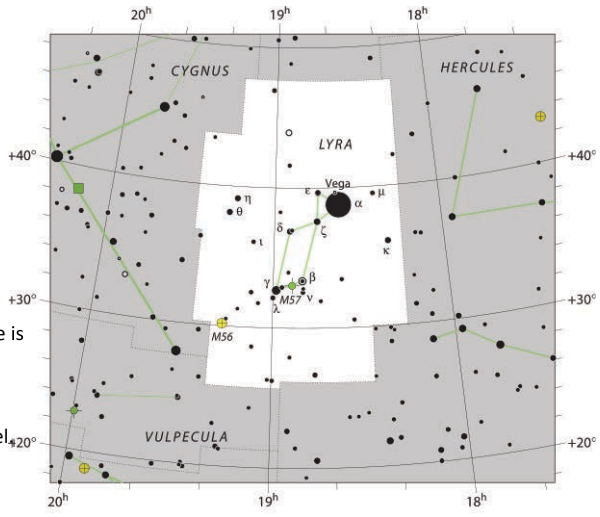
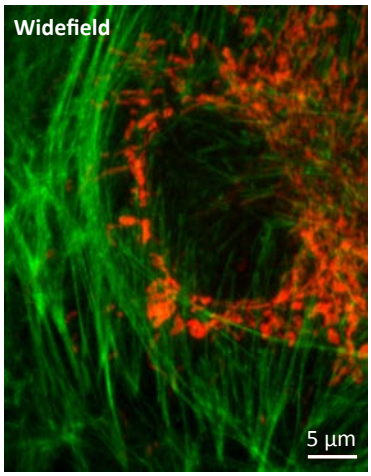
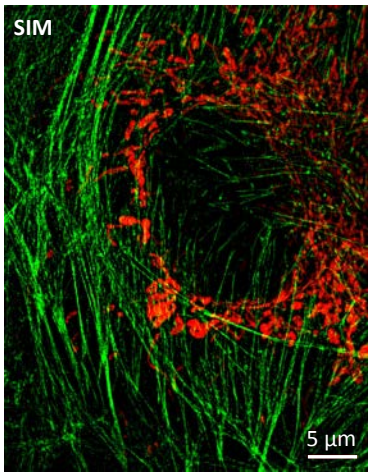


Image from Wikipedia.

2-Colour SIM Example: Actin and Mitochondria

- Bovine Pulmonary Artery Endothelial (BPAE) Cells.
  - Alexa Fluor 488 Phalloidin (Actin).
  - MitoTracker Red (Mitochondria).

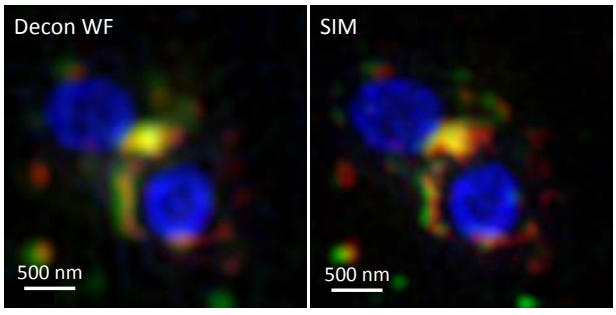




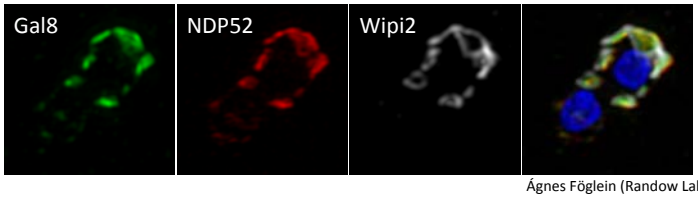
Nick

3/4-Colour SIM: Microdomains of Autophagy Markers

- HeLas infected with Salmonella:
  - mCherry-Salmonella (Blue).
  - Galectin8 (Alexa 488, Green).
  - ATG13a (Alexa 647, Red).



- HeLas infected with Salmonella:
  - Galectin8 (Alexa 488, Green).
  - NDP52 (Pacific Blue, Red).
  - Wipi2 (Alexa 647, White).
  - mCherry Salmonella (Blue).



Ágnes Föglein (Randow Lab)

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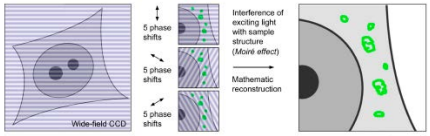

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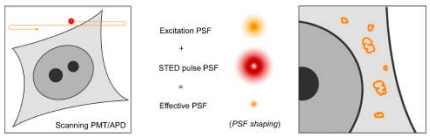

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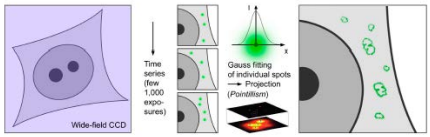
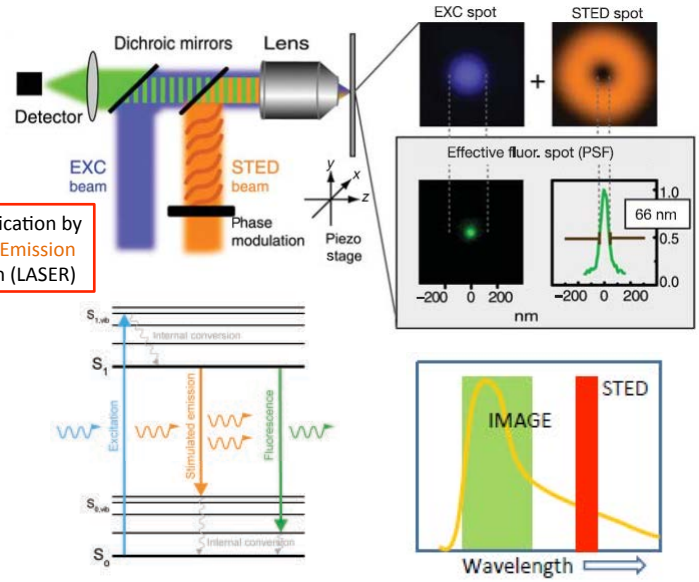


Schermelleh *et al.*, JCB, 2010.

Stimulated Emission Depletion (STED) Microscopy

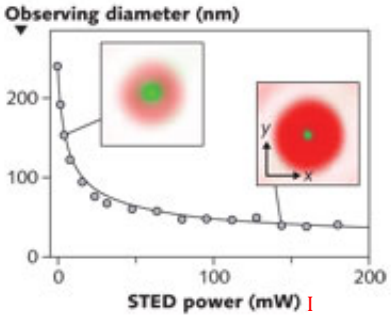
Light Amplification by Stimulated Emission of Radiation (LASER)

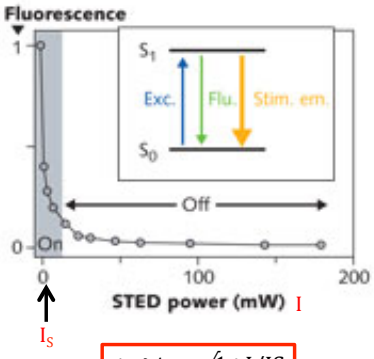


Images from nanobiophotonics.mpibpc.mpg.de and Willig *et al.*, Nature Letters, 2006.

Stimulated Emission Depletion (STED) Microscopy

- Resolution increases as the intensity of the STED beam is turned up.
- I = STED laser power.
- I_S = Saturation intensity (STED power needed to quench emission by 50% - fluorophore specific).





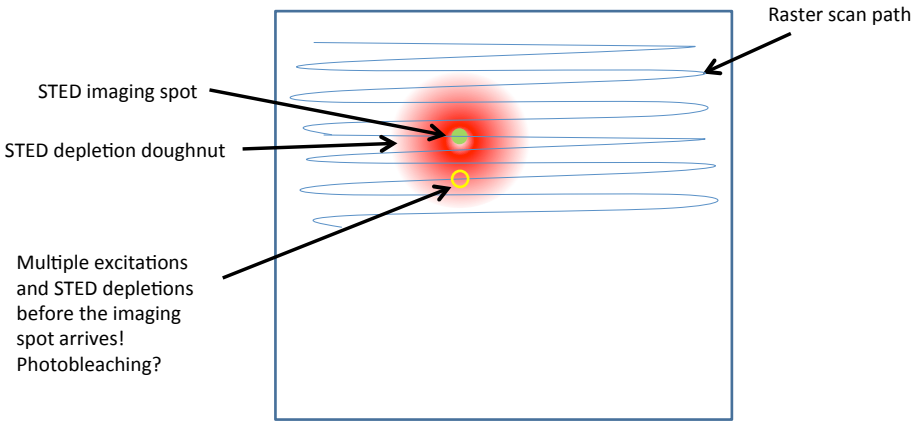
XY resolution >10 nm (25-80 nm typical)
Z resolution same as confocal

$$d \approx \lambda / 2NA \sqrt{1 + I/IS}$$

Image from laserfocusworld.com

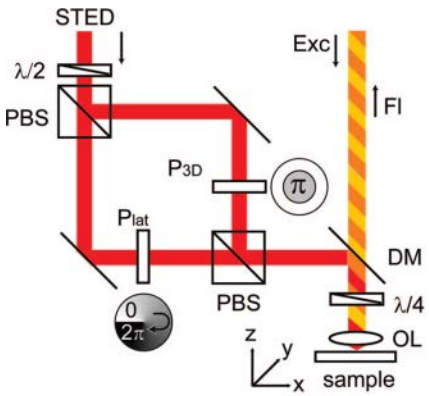
Stimulated Emission Depletion (STED) Microscopy

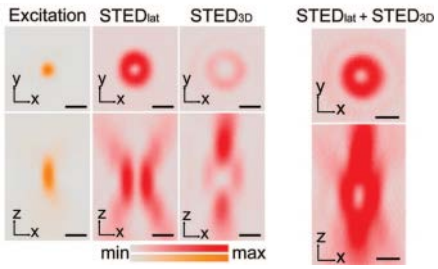
- The STED image is built up by raster scanning the super-resolution central spot. Because it is much smaller than a regular confocal spot the image pixels must also be smaller.



Improving Axial (Z) Resolution - 3D STED

- With an alternatively shaped STED beam, additional axially-shifted intensity lobes can be created that quench the axial extension of the PSF as well.





Z resolution ~125 nm

Harke *et al.*, Nano Letters, 2008

Leica TCS SP8 STED 3X

- Essentially just a standard Leica TCS SP8 confocal plus a few extra bits:

+ Tuneable pulsed supercontinuum "white light" laser (470-670 nm).

+ High power 592 nm, 660 nm and 775 nm STED lasers.

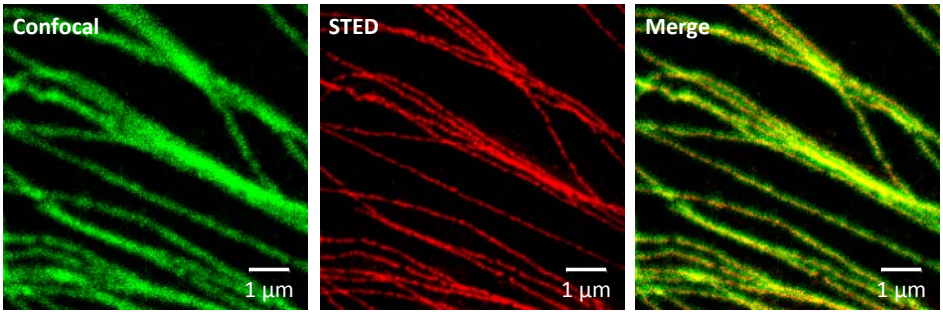
+ 3D STED alignment unit.

+ Gated detectors.

- Scan time scales with frame size as with confocal.
- However, pixel density must be higher (20 nm pixels) to capture super-resolution information.
- Therefore, scan time is considerably slower (~16-fold).
- Resonant scanner.

1-Colour STED Example (Microtubules)

- HeLa Cells.
  - Primary Ab: Rat Anti-Alpha Tubulin.
  - Secondary Ab: Anti-Rat-Oregon Green 488.



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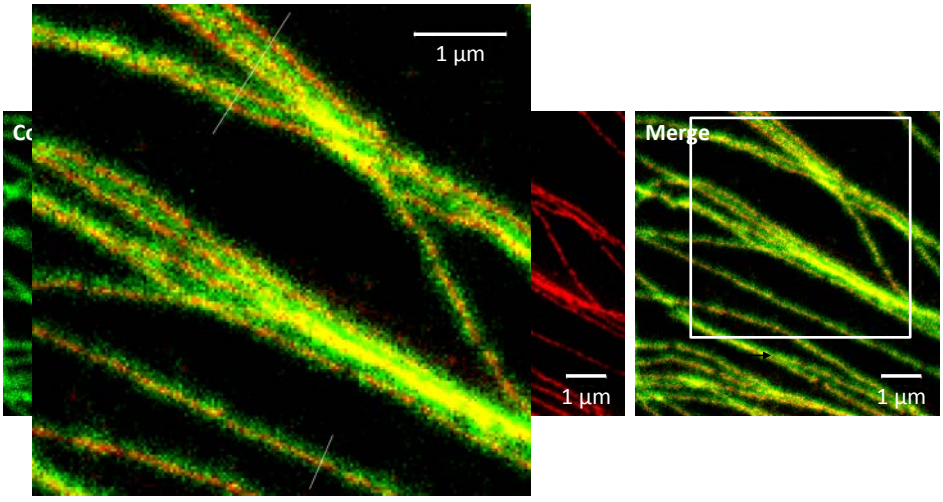

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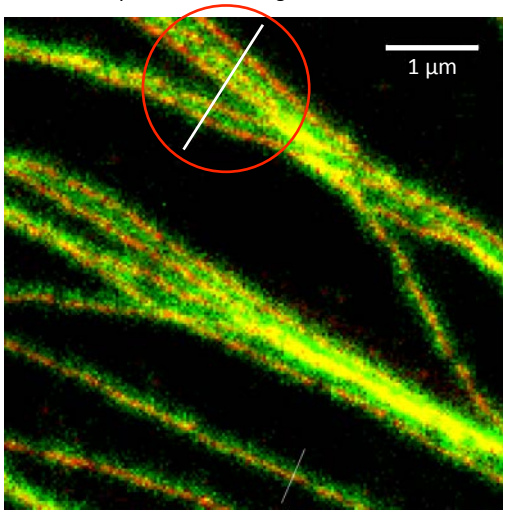

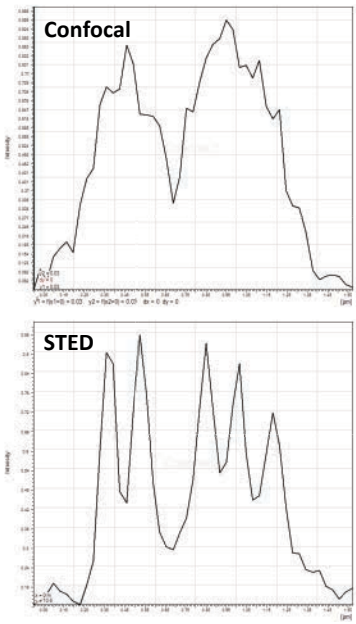

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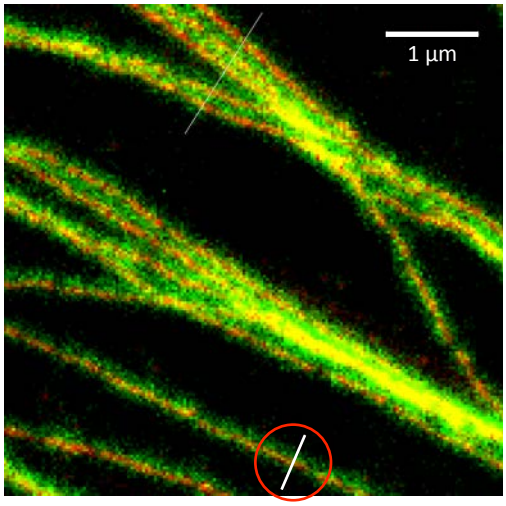

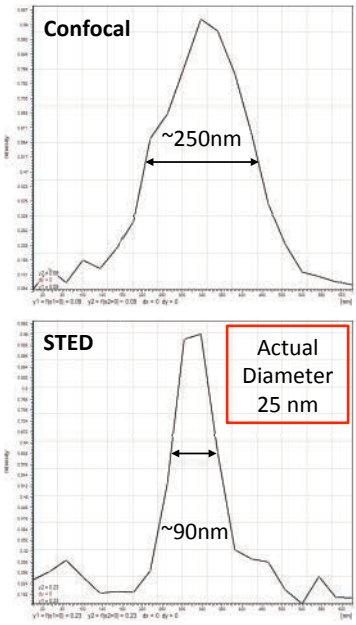

2-Colour STED: Example 1 (Primary Neurons)

- Primary Rat Hippocampal Neuron Culture (Jake Watson – Greger lab).
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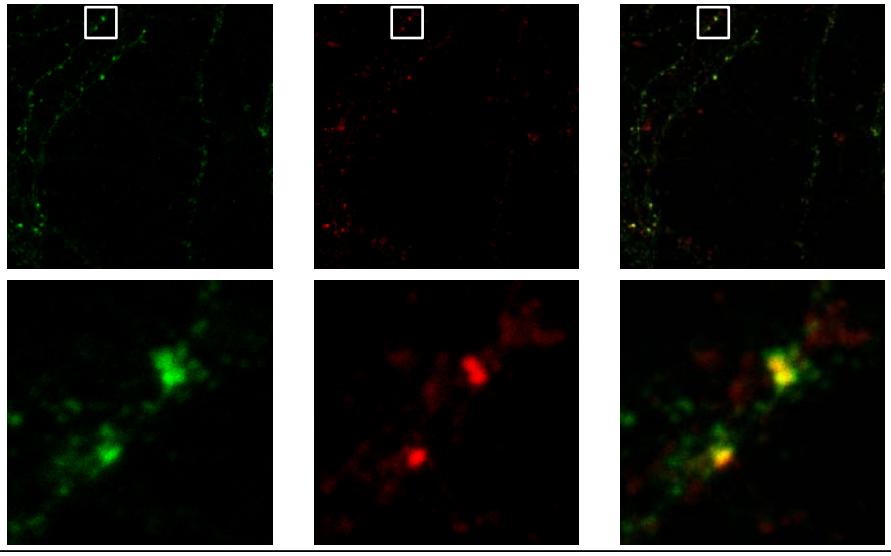
  - Secondary Abs: Anti-Rabbit-Alexa 488 + Anti-Mouse-Alexa 568.



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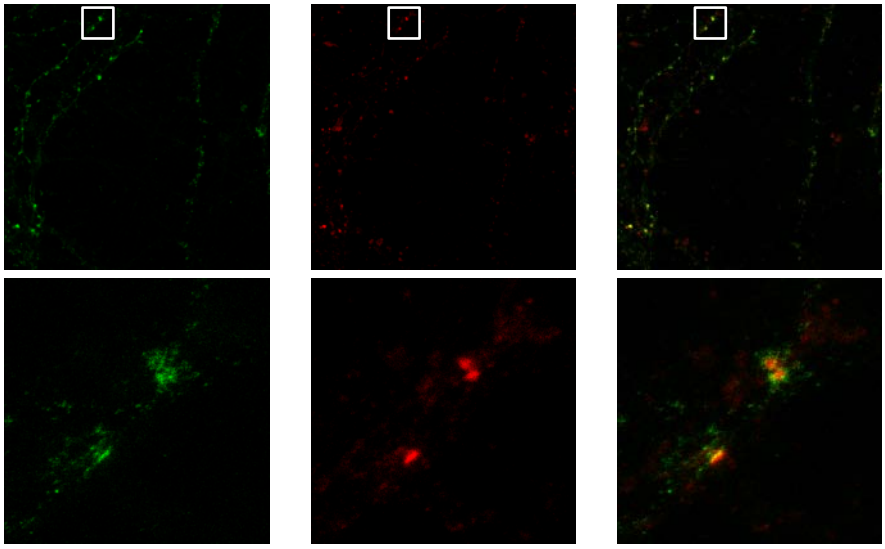
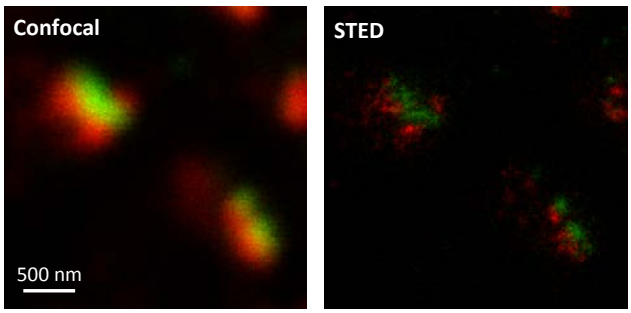


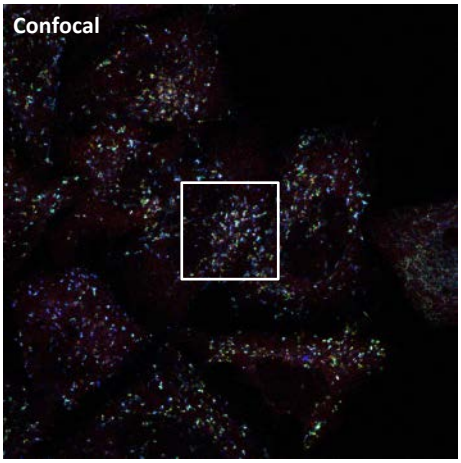
2-Colour STED: Example 2 (More Primary Neurons)

- Primary Rat Hippocampal Neuron Culture.
  - PSD95 (Alexa 488, Green)
  - Bassoon (Alexa 568, Red)



Jake Watson (Greger Lab)

3-Colour STED: Example (Recycling Endosomes)



Sophie Bruesegem (Formally of Seaman Lab, CIMR)

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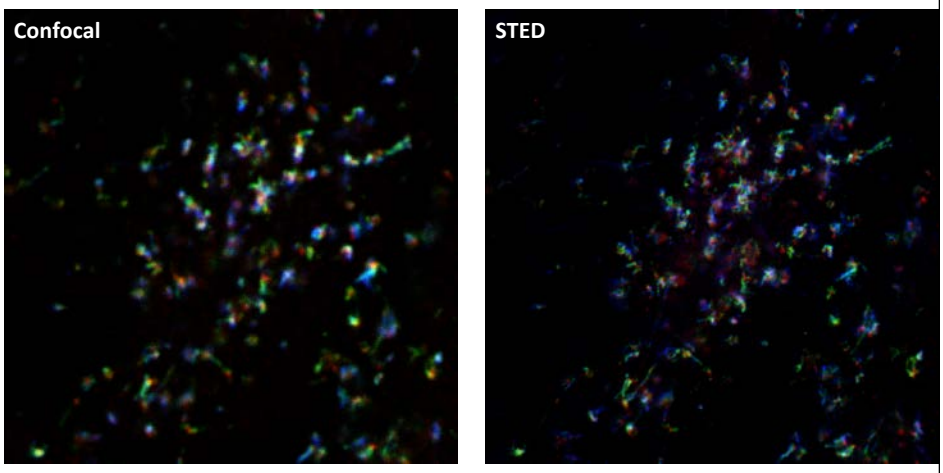

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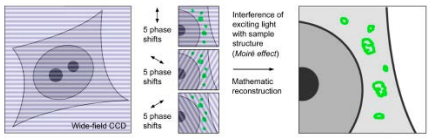

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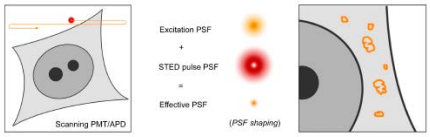
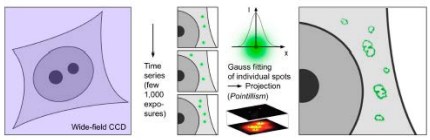
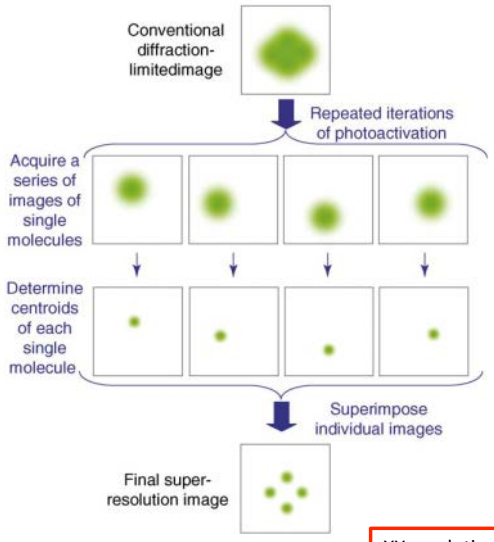


- Localisation Microscopy (LM) e.g. PALM and STORM. Fluorophores are randomly activated, imaged and bleached. Localisation of the fluorophores builds up a super-resolution image over time.



Schermelleh *et al.*, JCB, 2010.

Localisation Microscopy (LM)



XY resolution ~10 nm (25-40 typical)
Z resolution ~50 nm

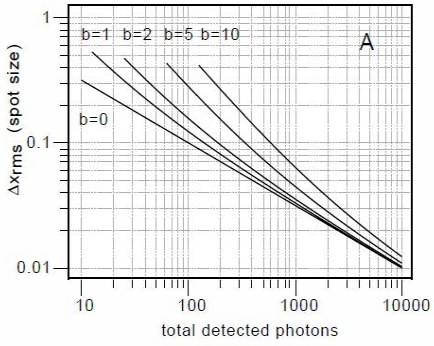
Zemer Gitai, Current Opinion in Microbiology, 2009

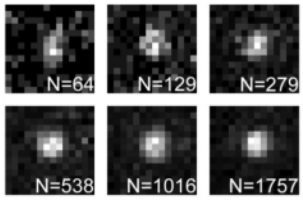
Photons and Precision

- An image taken by a camera contains several noise factors including photon noise, the effect of pixelation and fluorescent background.
- Localisation precision scales as the inverse square root of the number of collected photons.

$$\sigma_{\mu_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/12}{N} + \frac{8\pi s_i^4 b^2}{a^2 N^2}\right)}$$

N = number of photons collected
a = pixel size
b = background
s = SD of the PSF

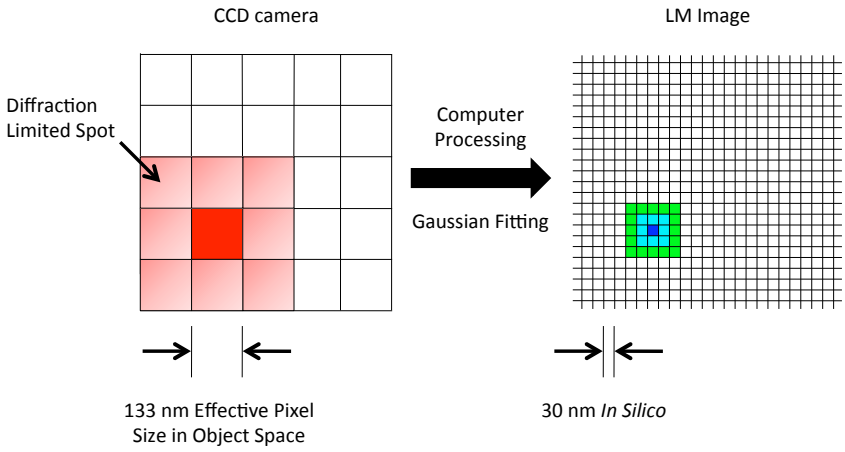




$FWHM_{LOCALISATION} \approx FWHM/\sqrt{N}$

Thompson *et al.*, Biophysical Journal, 2002.

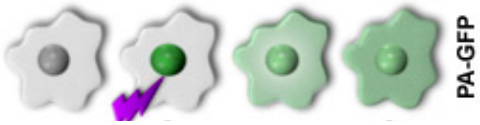
Localisation Microscopy (LM)



$$FWHM_{LOCALISATION} \approx FWHM/\sqrt{N}$$

Photoactivation Localisation Microscopy (PALM)

- Optical highlighter fluorescent proteins used as switchable probes. 2 useful types for PALM: -
- Photoactivatable fluorescent proteins:
  - Initially non-fluorescent (dark) and can be switched on by illumination at a specified wavelength e.g. PAGFP, PATagRFP and PAmCherry.



- Photoconvertible fluorescent proteins:
  - Emission spectra can be shifted (usually to longer wavelength) by illumination at a specified wavelength e.g. Kaede, Eos and Dendra (all green-to-red).

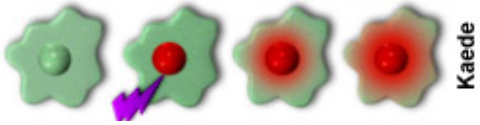
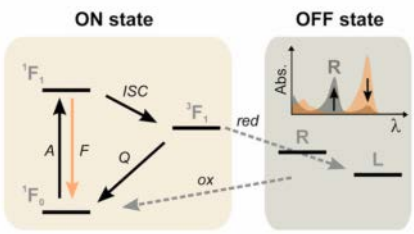
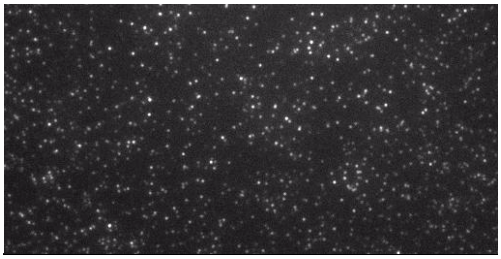


Image modified from Olympus website.

STochastic Optical Reconstruction Microscopy (STORM)

- A large number of commercially available fluorophores can be used as photoswitches in the presence of millimolar concentrations of reducing thiols e.g. β-ME, MEA or GSH (live cells).
- The triplet state is reduced by the thiol to form a long-lived intermediate radical anion (off state). The fluorescent state of the fluorophore is recovered through oxidation by molecular oxygen, which is facilitated by irradiation with purple/blue light.
- Carbocyanine dyes (Cy5, 5.5, 7, Alexa 647) are far more easily oxidised and therefore an enzymatic oxygen scavenging system is required to prolong lifetime in the off state (glucose oxidase and catalase).



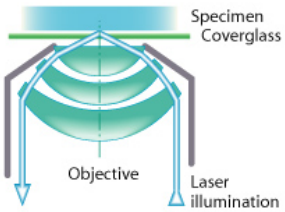


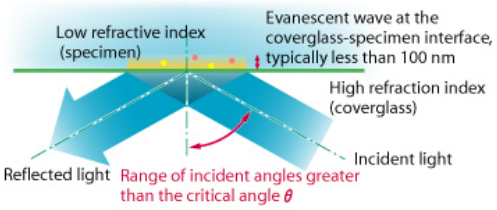
Van de Linde *et al.*, Chem. Biol., 2013

Nikon N-STORM

- TIRF illuminator attached to a Nikon Ti inverted microscope body.
- 4 lasers (30 mW 405 nm, 90 mW 488 nm, 90 mW 561 nm, 170 mW 647 nm).
- 100x 1.49 NA TIRF objective.
- 60x 1.27 NA water objective.
- Andor iXon3 897 EMCCD camera capable of up to 35 fps.
- Perfect Focus System (PFS) to reduce Z-drift during acquisitions, which can take up to 1 hour.
- XY-drift correction in software by cross-correlation.
- Cylindrical lens in detection path allows 3D position mapping over 1 µm range.

Total Internal Reflection Fluorescence (TIRF) Microscopy

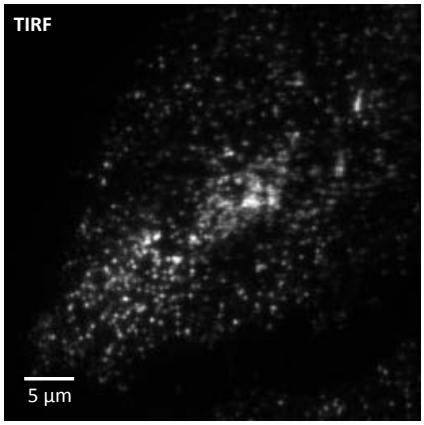


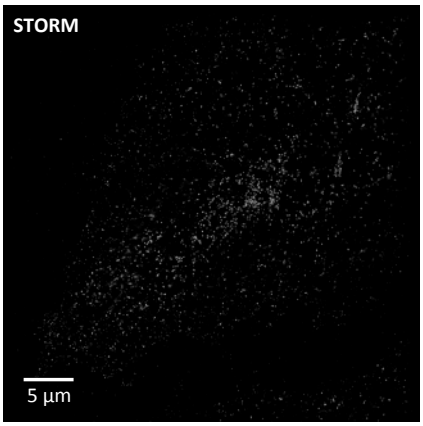


TIRFM Image from Nikon website.

1-Colour STORM: Example 1 (Caveolae)

- HeLa Cells.
  - Primary Ab: Rabbit Anti-Cavin1.
  - Secondary Ab: Anti-Rabbit-Alexa Fluor 647.





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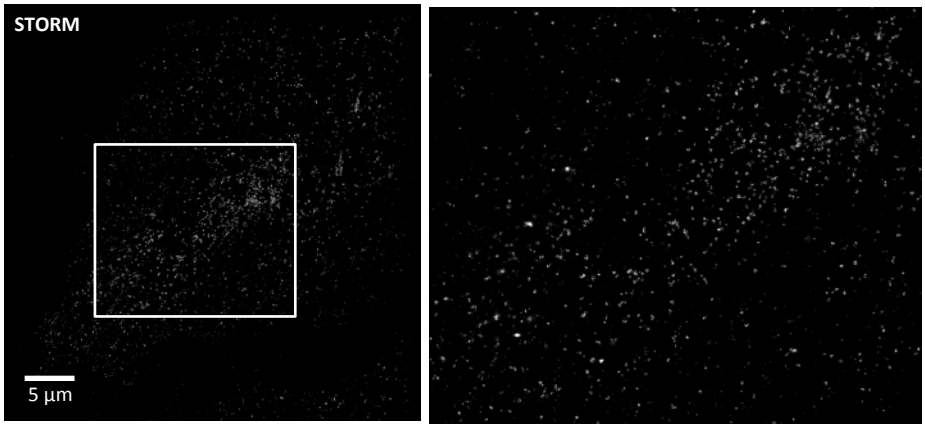

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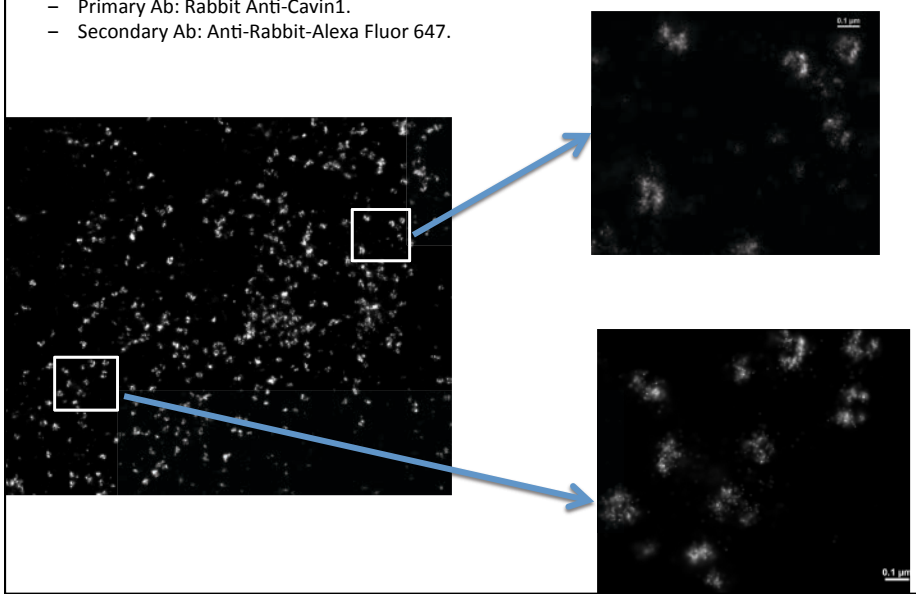
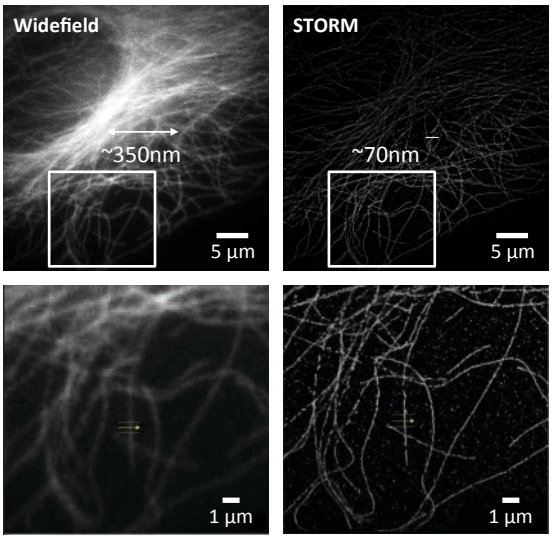


1-Colour STORM: Example 2 (Microtubules)

- HeLa Cells.
  - Primary Ab: Rat Anti-Alpha Tubulin.
  - Secondary Ab: Anti-Rat-Alexa Fluor 647.



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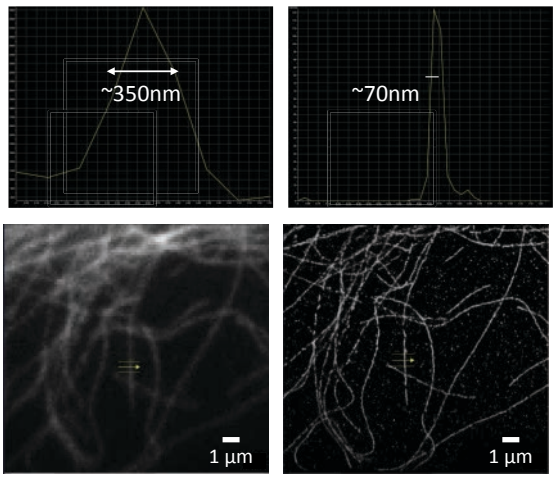

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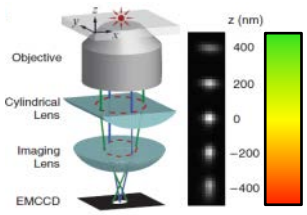
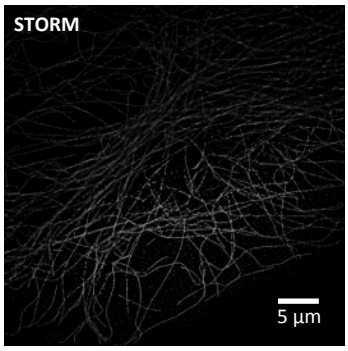
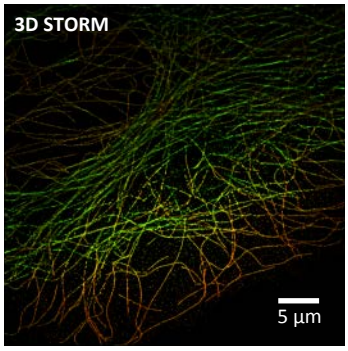


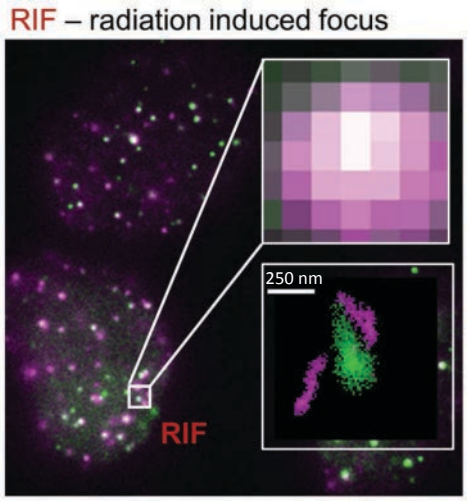
Huang *et al.*, Science, 2008





2-Colour STORM Example (Radiation-Induced Damage)

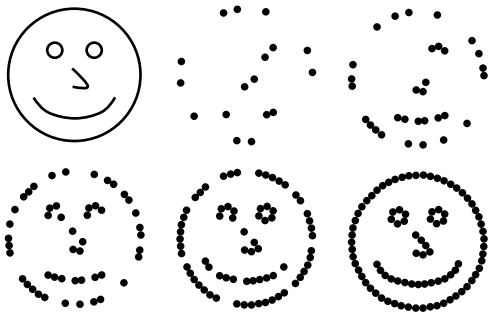
- HeLa cells
  - RPA (CF 568, Green).
  - Rad51 (Alexa 647, Magenta).

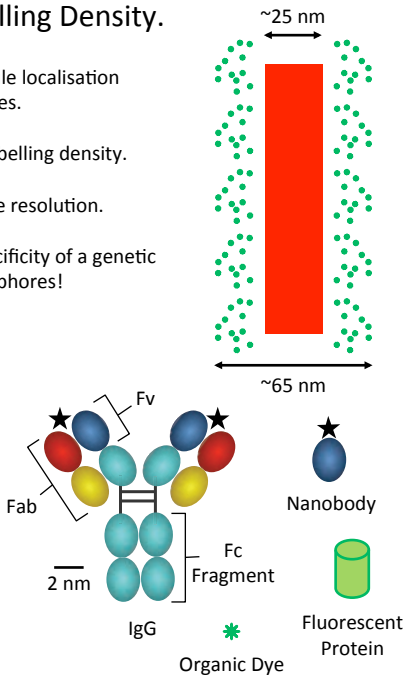


Kalina Haas (Formerly of Venkitaraman Lab, MRC Cancer Unit)

Label Size and Labelling Density.

- Photon number limits the precision of single molecule localisation and signal-to-noise for all super-resolution techniques.
- The resolution of the final image also depends on labelling density.
- The physical size of the label can limit the achievable resolution.
- Ideally, we would like to label proteins with the specificity of a genetic fusion but with the signal intensity of organic fluorophores!





Comparison of Super-resolution Methods

	SIM	STED	LM
Detector	Wide-field EMCCD/ sCMOS camera	Scanning PMT/APD	Wide-field EMCCD/ sCMOS camera
Lateral (XY) Resolution (nm)	100-130	25-80	25-40
Axial (Z) Resolution (nm)	250-350	125	50
Temporal Resolution	ms-sec	ms-min	s-min
Multiplexing	4 colours, regular fluorophores	3 colours, restricted by number of STED lasers	2/3 colours, not trivial
Postprocessing	Yes, 9-25 raw images per z-plane, risk of artefacts	No	Yes, 1000+ images per z-plane

- Always image through no. 1.5 (170 µm) cover glasses, ideally low-tolerance (± 5 µm).
- If possible use phenol red-free culture medium.
- Use high refractive index mounting media e.g. Prolong Diamond or 2,2'-thiodiethanol (TDE). Ideally with antifade compounds to limit bleaching.
- If immunostaining, consider increasing antibody concentrations to ensure high labelling density. Also, increase number and duration of washes to reduce non-specific labelling.

Acknowledgements

- Light Microscopy Facility
- Nick Barry
- Jérôme Boulanger
- Ben Sutcliffe
- James Manton

- Ágnes Föglein (Formally of Randow Lab).

- Jake Watson (Greger Lab).

- Sophie Bruesegem (Formally of Seaman Lab, CIMR)

- Kalina Haas (Formally of Venkitaraman Lab, MRC Cancer Unit)

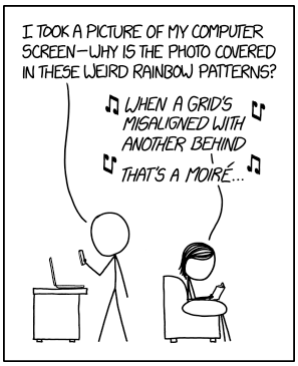


Image from xkcd.com