Biological (Far-field) Microscopy
Beyond the Diffraction Limit of Light

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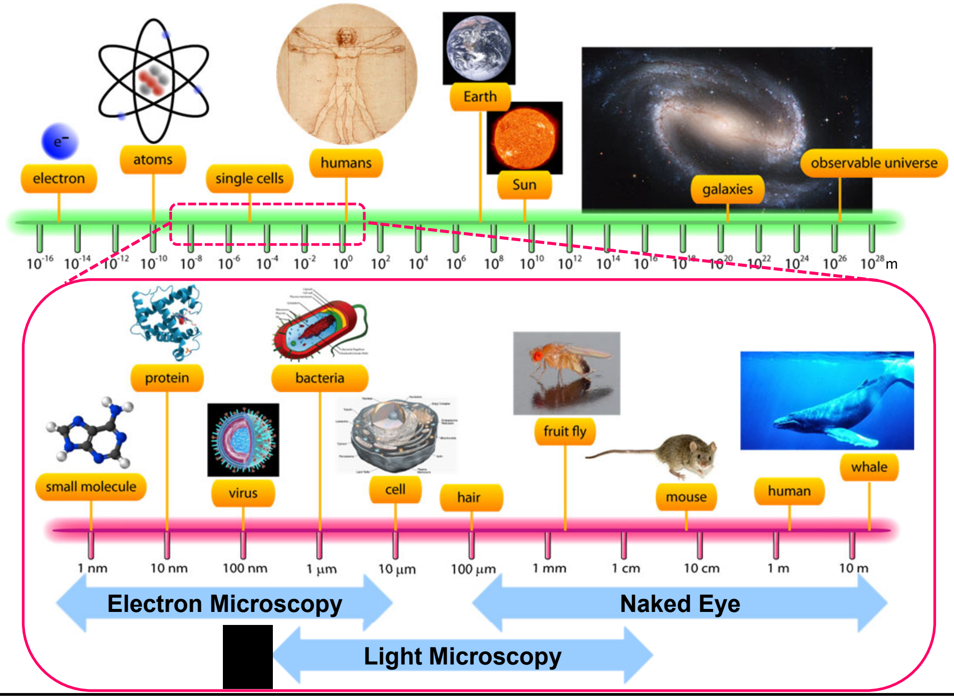
Yale University
School of Medicine



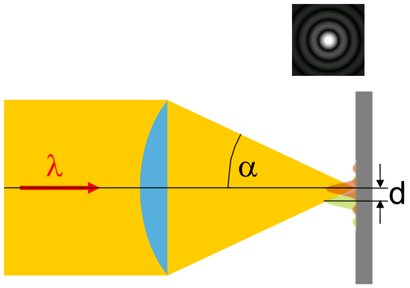
[redacted]

The Diffraction Limit

Ernst Abbe
(1872)

diffraction limit

structures smaller than half a wavelength cannot be resolved.



cf. Airy disk radius:

$$r_0 \approx 0.61 \frac{\overset{\text{wavelength}}{\lambda}}{\underbrace{\underset{\text{refractive index}}{n} \sin \underset{\text{aperture angle}}{\alpha}}_{\text{numerical aperture (NA)}}}$$

Resolution in Far-field Microscopy

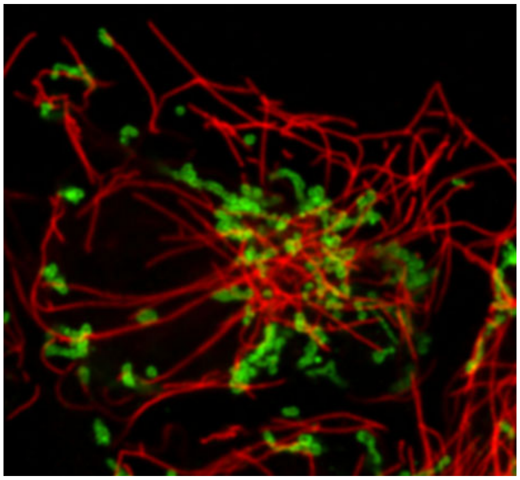
$$r_0 \approx 0.61 \frac{\lambda}{n \sin \alpha}$$

	λ	d_{min}
light	~ 500 nm	~ 250 nm
X-ray	~ 2 nm	~ 25 nm
electron	~ 0.001 nm	~ 0.1 nm (>2 nm)

Why Fluorescence Light Microscopy?

Advantages

- Wide variety of labels and targets
    - Specific
    - Multi-color
    - Sensitive
- Imaging live specimens
- 3D imaging inside samples
- Easy sample preparation compared to EM



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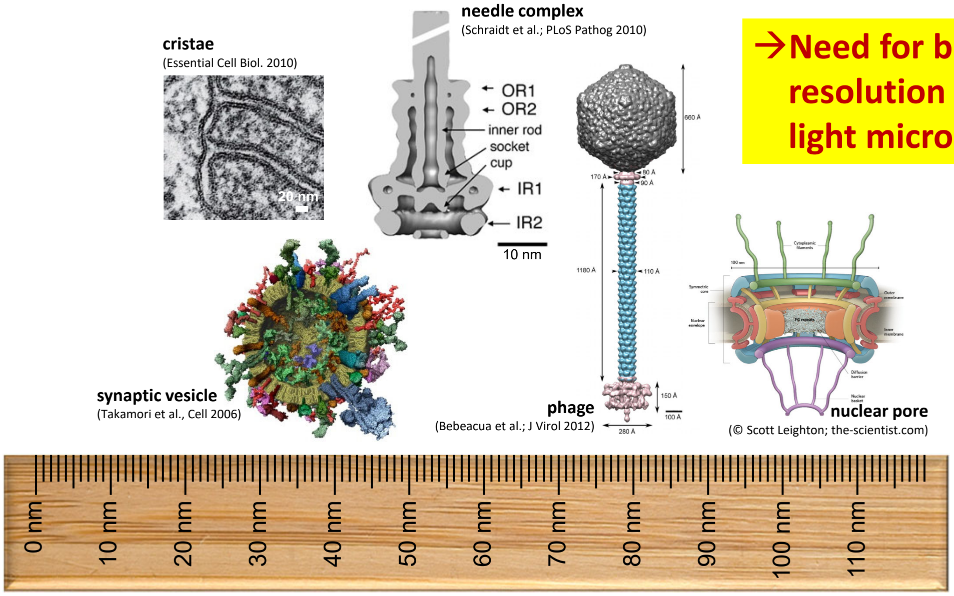
A Fascinating World Below 100 nm

→Need for better resolution in light microscopy



cristae
(Essential Cell Biol. 2010)

needle complex
(Schraidt et al.; PLoS Pathog 2010)

synaptic vesicle
(Takamori et al., Cell 2006)

phage
(Bebeacua et al.; J Virol 2012)

nuclear pore
(© Scott Leighton; the-scientist.com)

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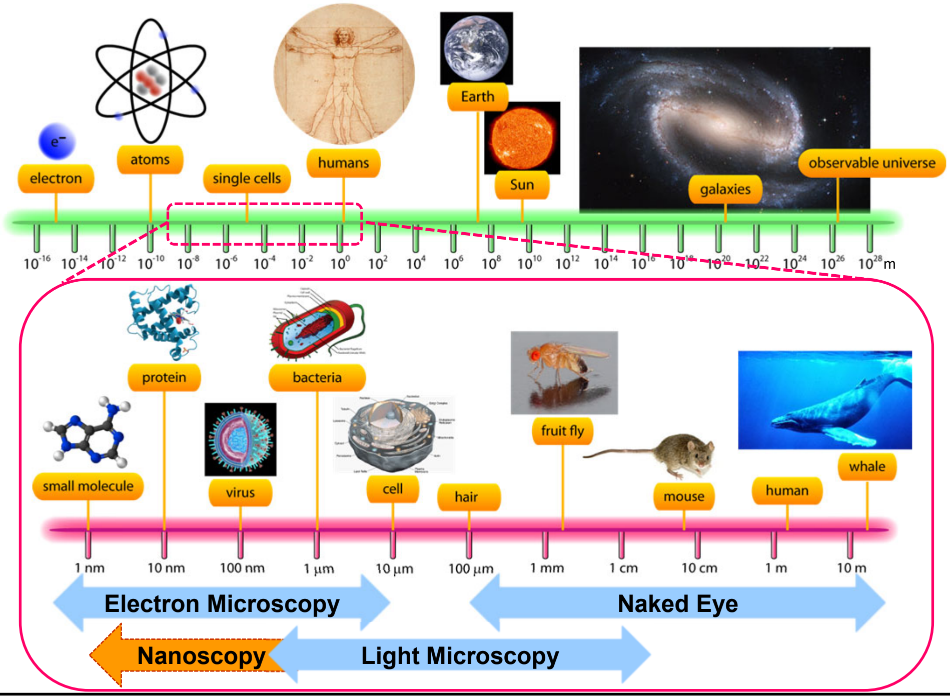


Overview of Super-resolution (Far-field Fluorescence) Microscopy

Resolution improvement

Light

Pushing the resolution within the diffraction limit

- 4Pi microscopy
- Structured Illumination Microscopy (SIM)
- Image-scanning microscopy, iSIM, AiryScan, …

} ~2x

Sample-light interaction

Breaking the diffraction limit of light

- Spatially targeted off-switching techniques (STED, …)
- Single-molecule localization techniques (PALM/STORM/PAINT/… and MINFLUX)


2014

} ~5-10x (50x)

Sample

Making the sample bigger

- Expansion Microscopy

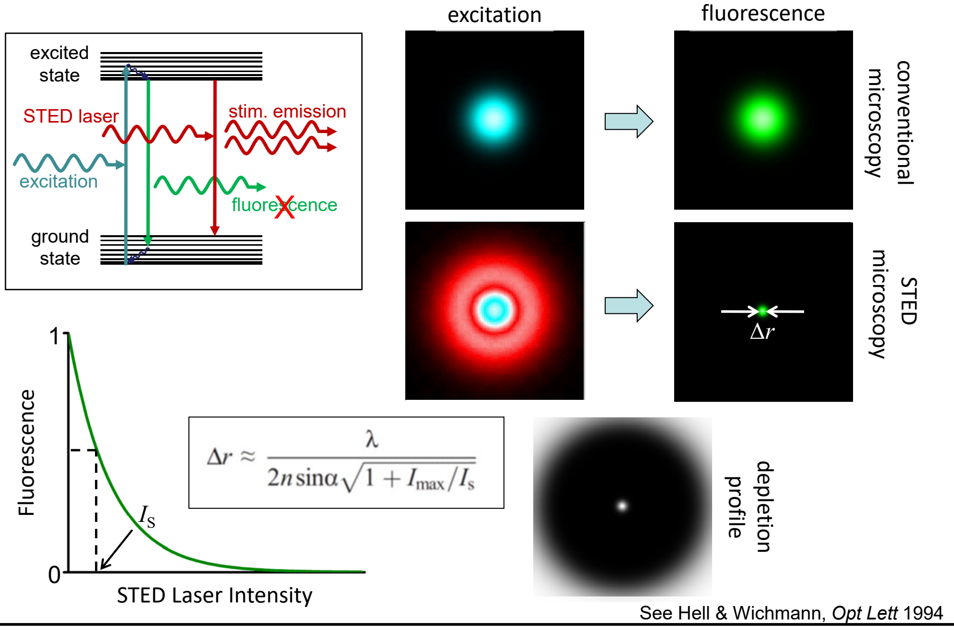
} ~4-20x

STED Microscopy

(or: STED ‘Nanoscopy’)

Breaking the diffraction limit by
targeted off-switching of molecules

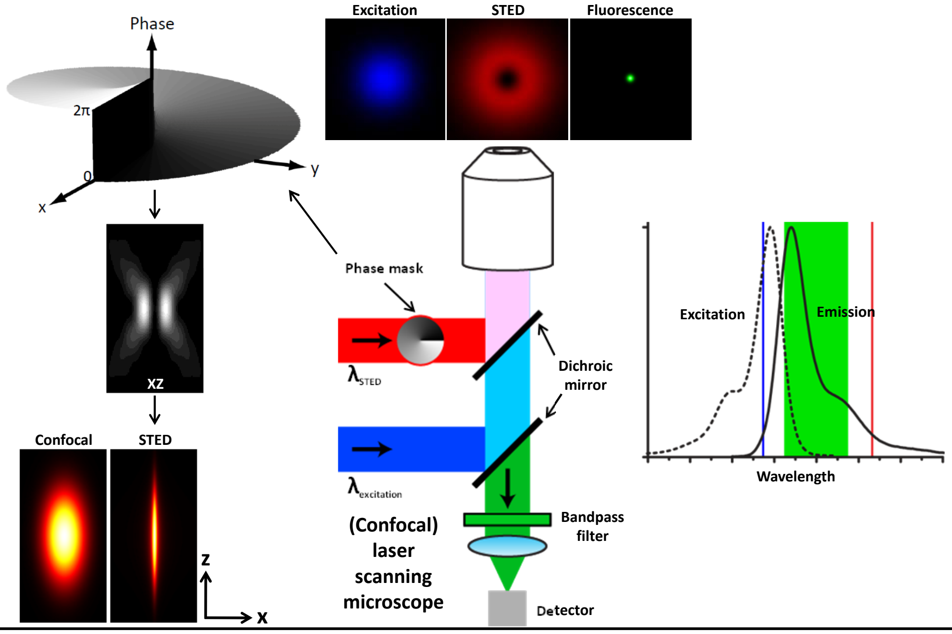
Principle of STED Nanoscopy



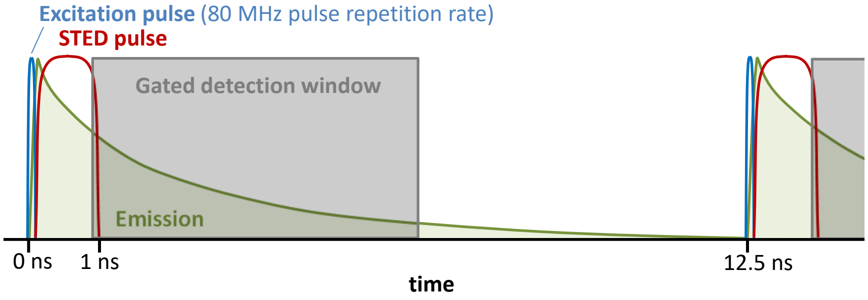
$$\Delta r \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+I_{\max}/I_s}}$$

See Hell & Wichmann, *Opt Lett* 1994

A Basic STED Nanoscope

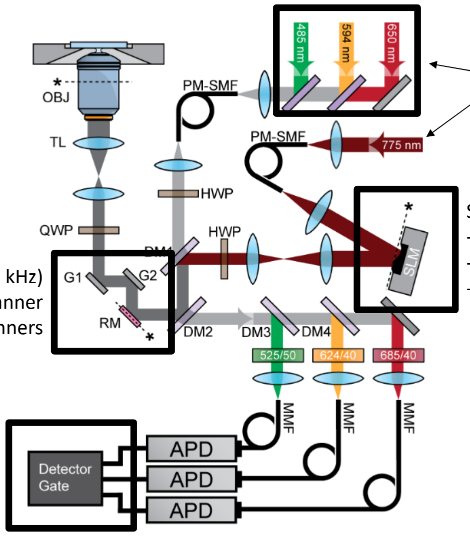


Pulsed Lasers and Gating in STED Nanoscopy



- STED laser power concentrated to time point when it is most needed
- Gating suppresses background
- Gating avoids detecting fluorescence that was emitted before STED pulse could act

A Custom-built STED Nanoscope for Live-cell Imaging



pulsed

SLM provides:
- Phase mask
- Alignment
- Aberration correction

X: Fast (16 kHz) resonant scanner
Y: 2 galvo scanners

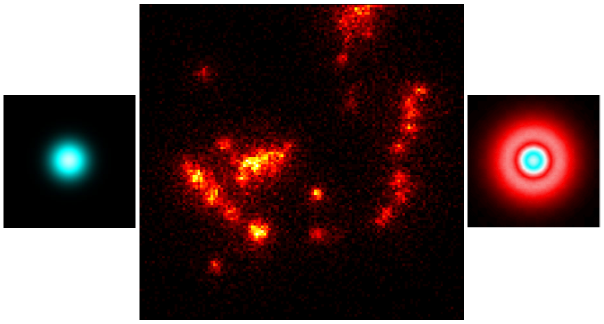
Gould *et al.*, Optics Letters (2013)
Gould *et al.*, Optics Express (2012)

Pulsed lasers and gated detection: detect fluorescence only *after* STED laser illumination

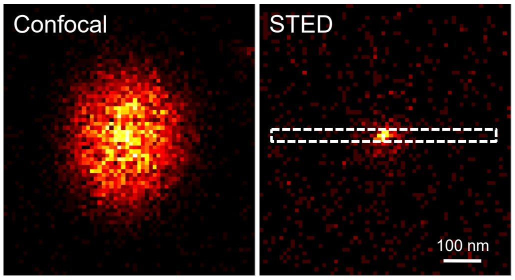
inspired by: Laser lines: Gottfert *et al.*, Biophysical Journal (2013)
SLM configuration: M.O Lenz *et al.*, Journal of Biophysics. (2014)

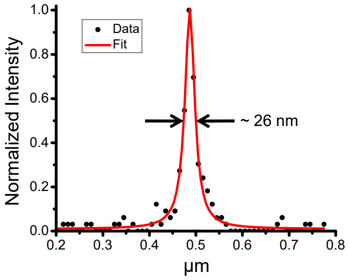
Example of STED Microscopy

Confocal STED



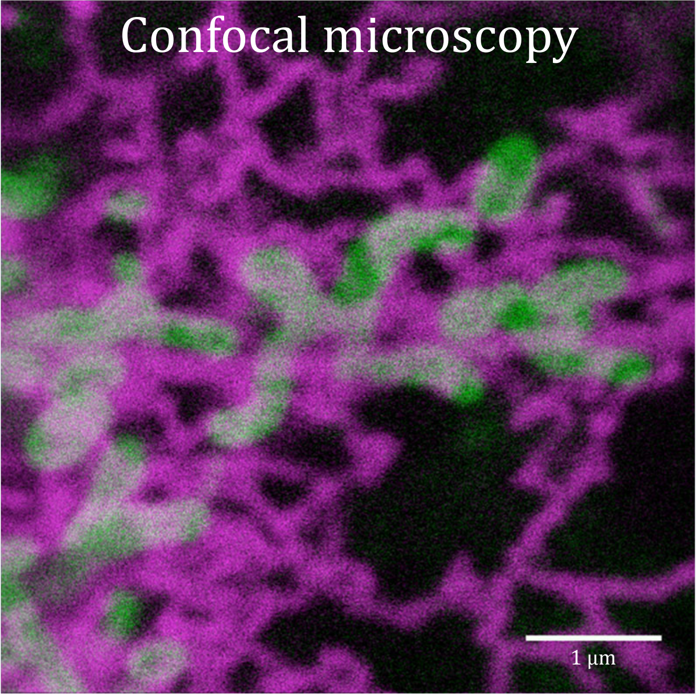
20 nm beads





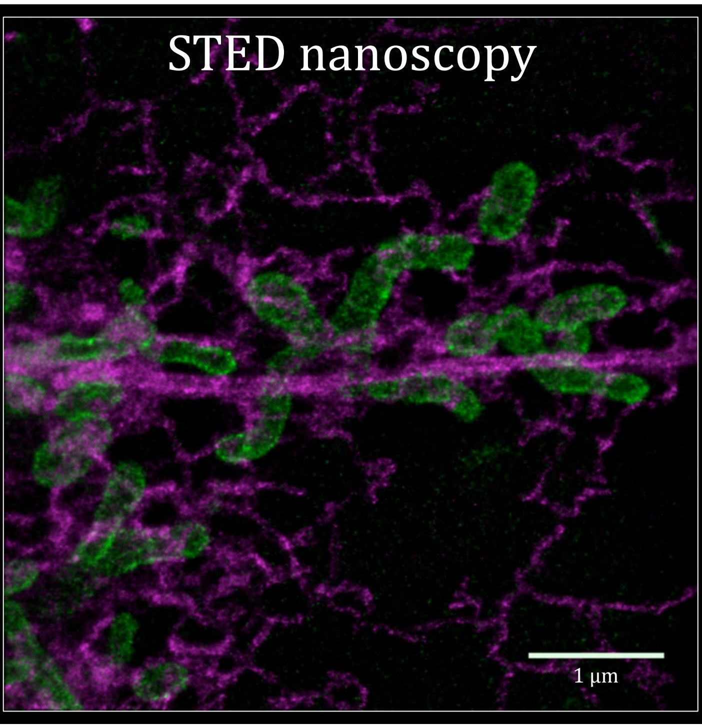
Travis Gould

Confocal microscopy



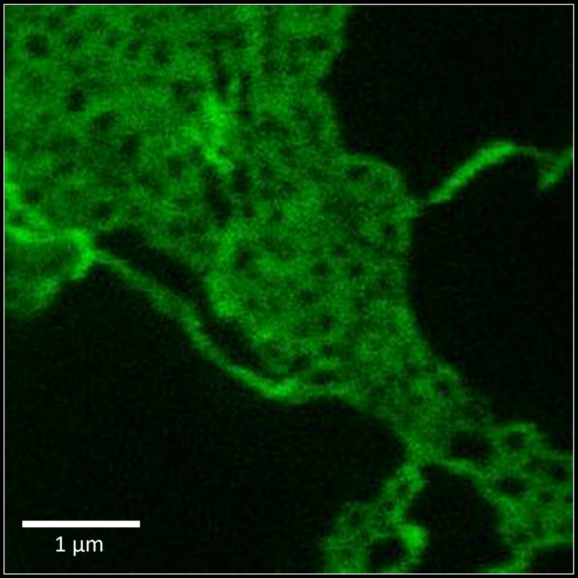
Emil Kromann

STED nanoscopy



Emil Kromann

Dynamics of Micro-fenestrations in ER Sheets



Lena Schroeder

COS7 cell at 37 °C

SNAP-KDEL BG-SiR

4 frames/second

Bleaching compensation applied

Collaboration with Shirin Bahmanyar

3D STED Microscopy for Deep-tissue Live-animal Imaging

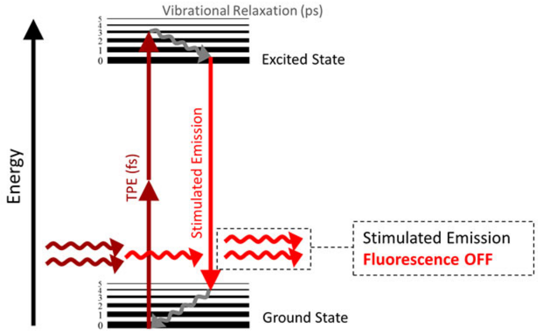
Challenge: Scattering and wavefront aberrations

Solution:

- ☐ **2-Photon Excitation**
  → reduced scattering (proportional to λ^{-3} to λ^{-4})
- ☐ **Far-red Dyes**
  → reduced scattering in detection beam path
- ☐ **Adaptive Optics**
  → reduced optical aberrations
- ☐ **Long Working Distance Objective**

Velasco et al., Optica 2021

2-photon STED Microscopy



Moneron & Hell, *Opt. Express* (2009)
Ter Veer, …, Nagerl, *Methods Mol. Biol.* (2017)
Velasco et al., *Opt. Lett.* (2015)

Mary Grace Velasco

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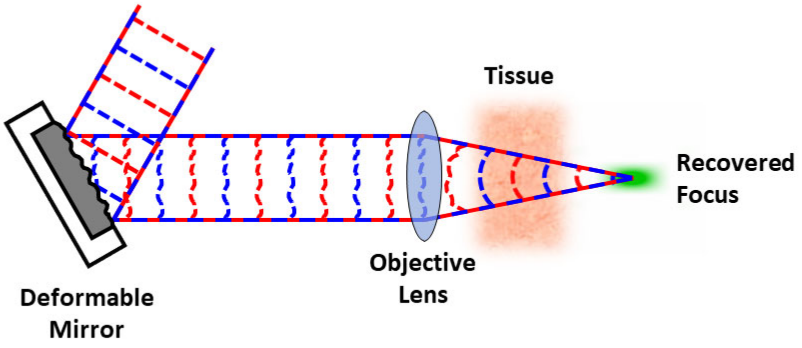
Aberration Correction



Review: Booth, *Light: Science & Applications* (2014)

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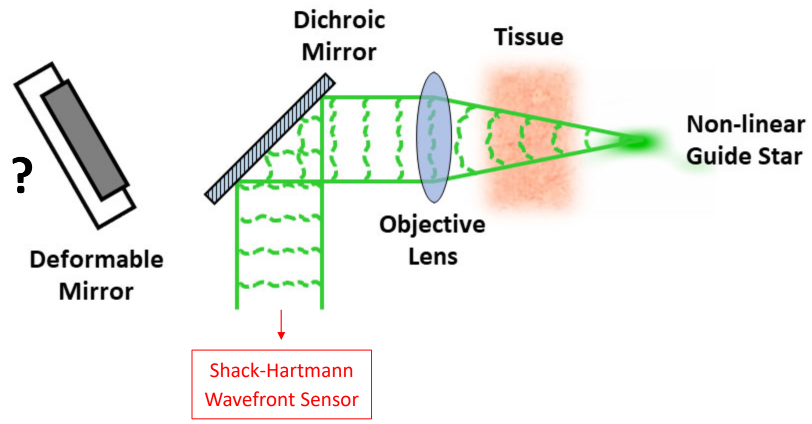
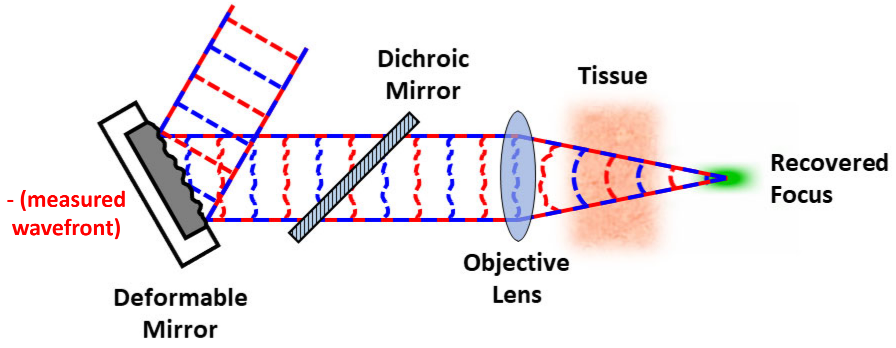
Aberration Correction

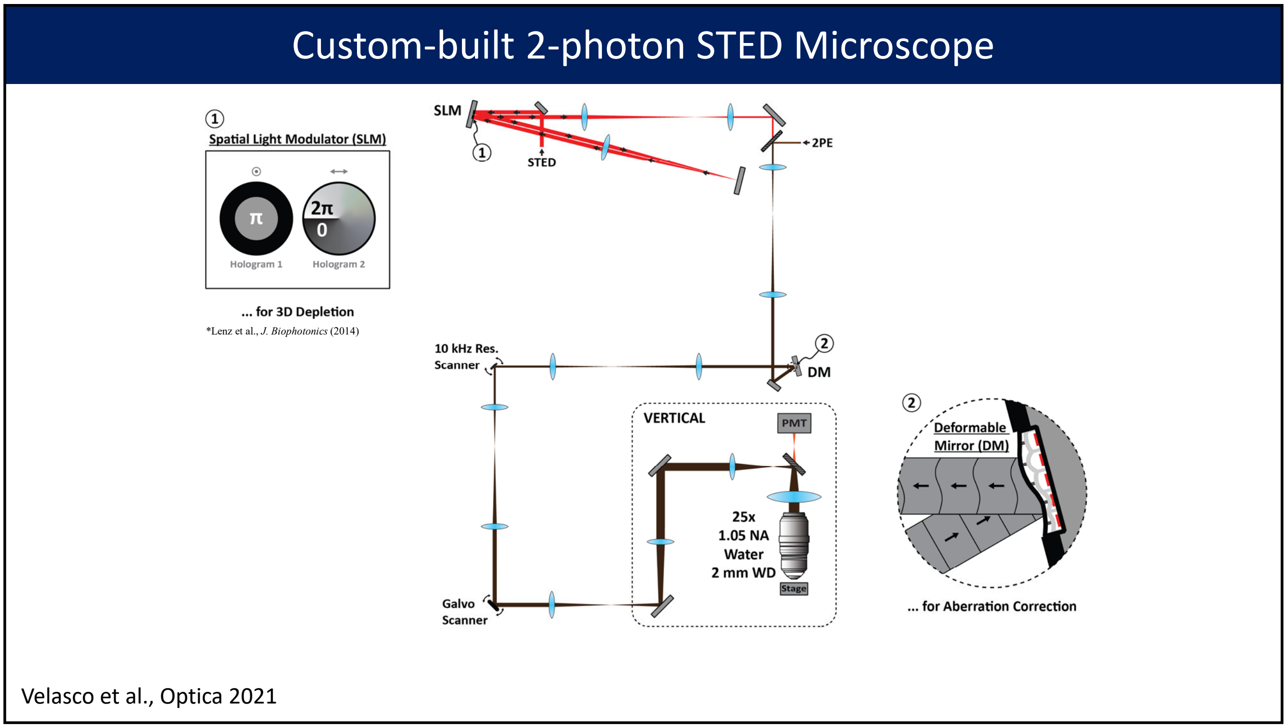


Aviles-Espinosa et al., *Biomed. Opt. Express* (2011)

Wang et al., *Nature Methods* (2014)

Aberration Correction

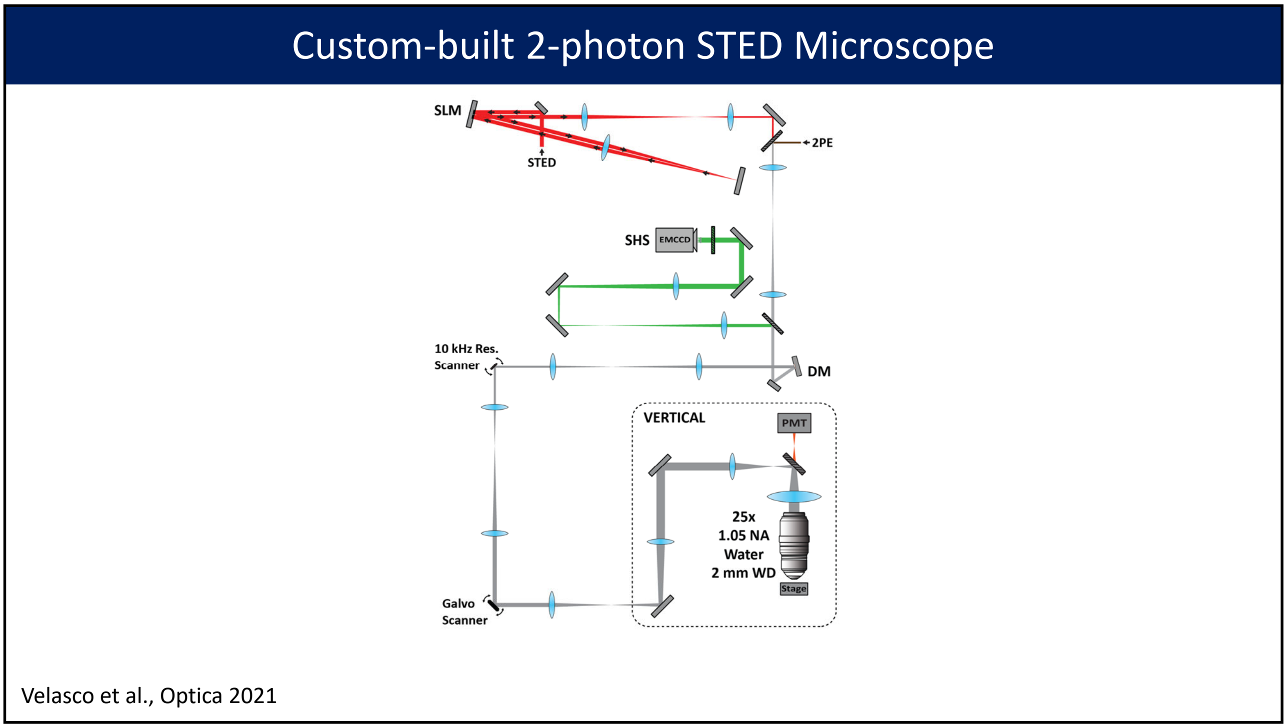




Custom-built 2-photon STED Microscope

*Lenz et al., *J. Biophotonics* (2014)

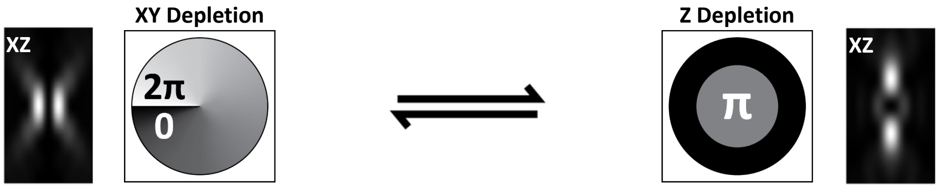
Velasco et al., Optica 2021



Custom-built 2-photon STED Microscope

Velasco et al., Optica 2021

Resolution Quantification with 1.05 NA Objective



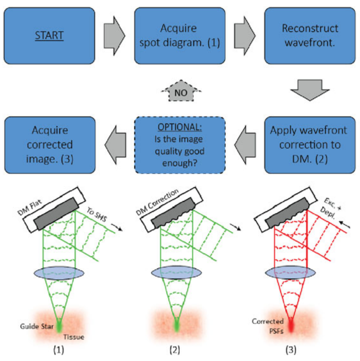
PSF size	XY Depletion	XYZ Depletion	Z Depletion	2PE
XY	**70 nm**	**133 nm**	247 nm	**354 nm**
Z	(1,131 nm)	**454 nm**	**151 nm**	**1,131 nm**

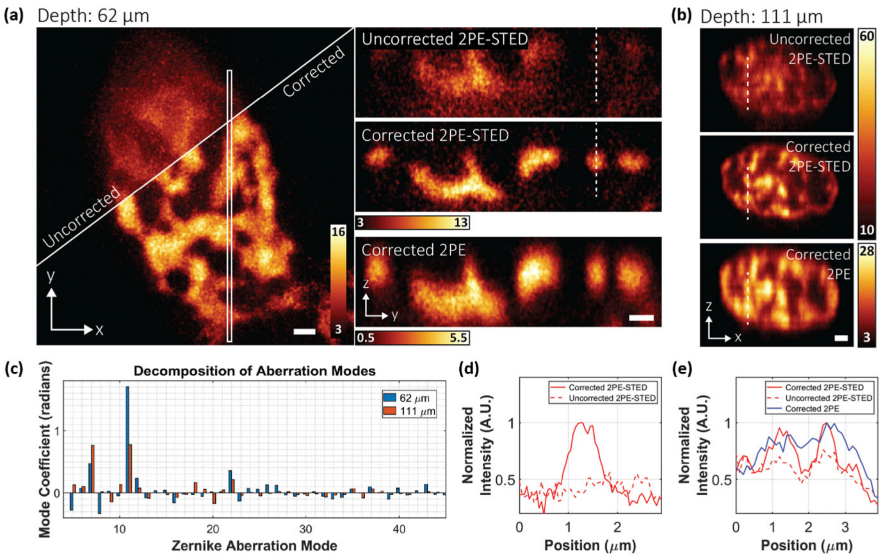
n = 70 – 160 measurements

NEP fitting: Barentine et al., *Biophys. J.* (2018)

Velasco et al., Optica 2021

Aberration Correction

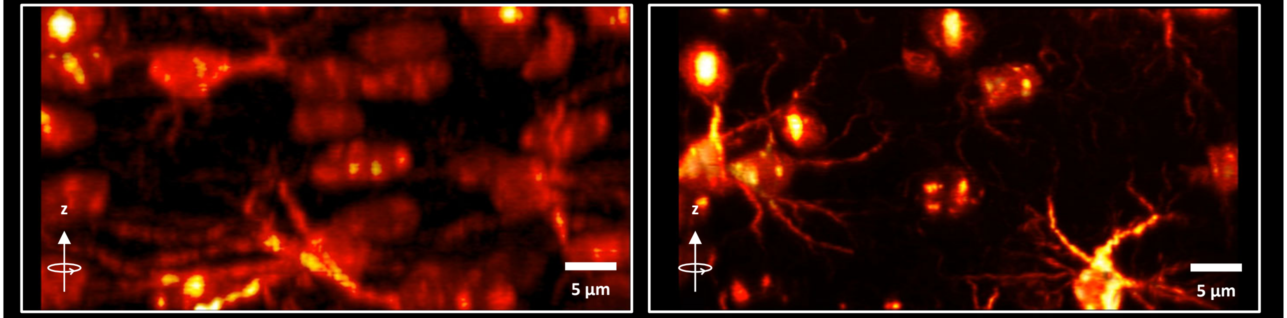




Velasco et al., Optica 2021

3D STED Microscopy In Mouse Brain Tissue Section

Combination: STED Microscopy
2-Photon
Adaptive Optics



2-Photon

2-Photon STED

Astrocytes in mouse brain section
Anti-GFAP ATTO594

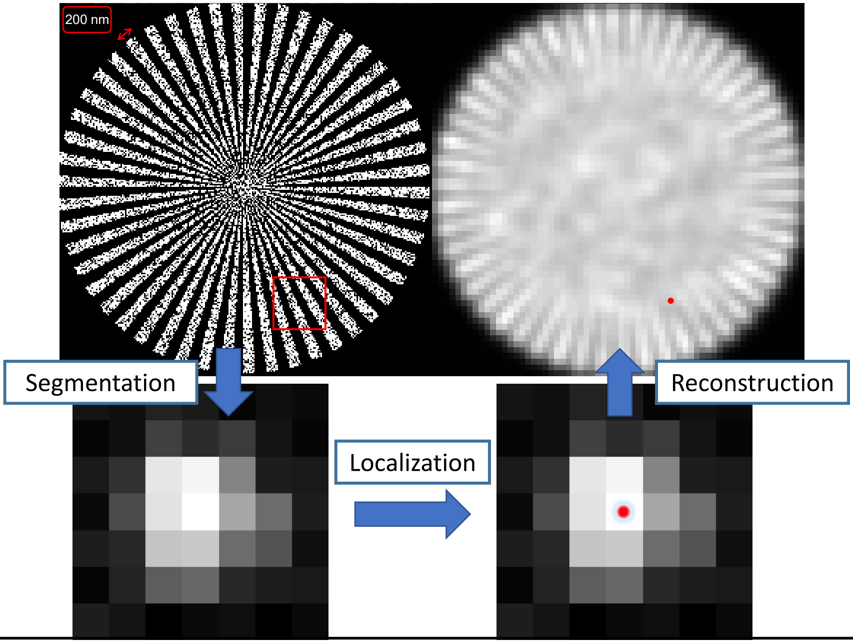
Unpublished
Mary Grace Velasco, Mark Lessard
Bewersdorf Lab

Single-molecule Localization Microscopy

(PALM/STORM/FPALM/…)

Breaking the diffraction limit by
stochastically switching of individual molecules

Single-molecule Localization Microscopy



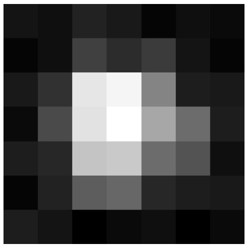
Segmentation

Localization

Reconstruction

Fang Huang

Single-Molecule Localization



For isolated molecules, determine the center of the diffraction-limited image to find its position.

Typically done by fitting a model function to the image.

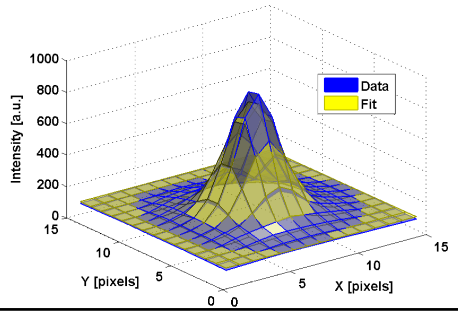
The precision depends primarily on S/N.

Simple approximation: $\sigma_{loc} \approx \frac{\sigma_{PSF}}{\sqrt{N}}$

More accurately:

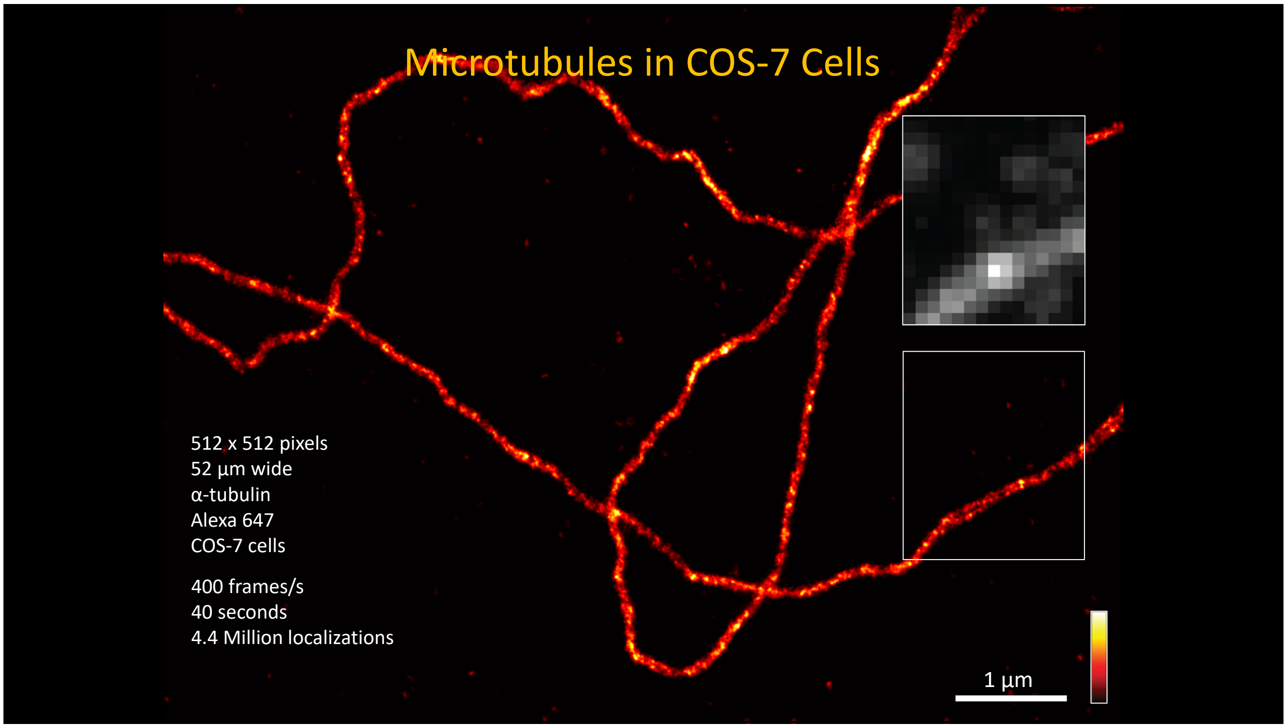
$$\sigma_{loc} \approx \sqrt{\frac{\sigma_{PSF}^2 + a^2/12}{N}\left(1 + 4\tau + \sqrt{\frac{2\tau}{1+4\tau}}\right)}$$

with: $\tau = \frac{2\pi b(\sigma_{PSF}^2 + a^2/12)}{N a^2}$



σ_{PSF}: PSF width
N: signal photons
a: pixel size
b: background photons per pixel

Rieger & Stallinga ChemPhysChem 2014



Microtubules in COS-7 Cells

512 x 512 pixels
52 µm wide
α-tubulin
Alexa 647
COS-7 cells

400 frames/s
40 seconds
4.4 Million localizations

3D Single-molecule Switching Nanoscopy

3D SMS resolution:

XY	**~20-30 nm**
Z	**~50-80 nm**

4Pi-SMS/iPALM:

XY	**~20 nm**
Z	**~10-20 nm**

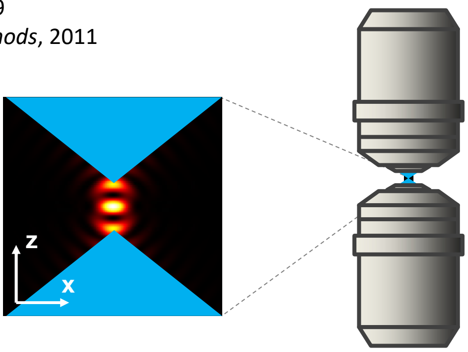
iPALM: Shtengel *et al. PNAS*, 2009
4Pi-SMS: Aquino *et al. Nature Methods*, 2011

Astigmatism:
Huang *et al. Science*, 2008
Biplane:
Juette *et al. Nature Methods*, 2008
Double Helix:
Pavani *et al. PNAS*, 2009
Phase Ramp:
Baddeley *et al. Nano Research*, 2011
…

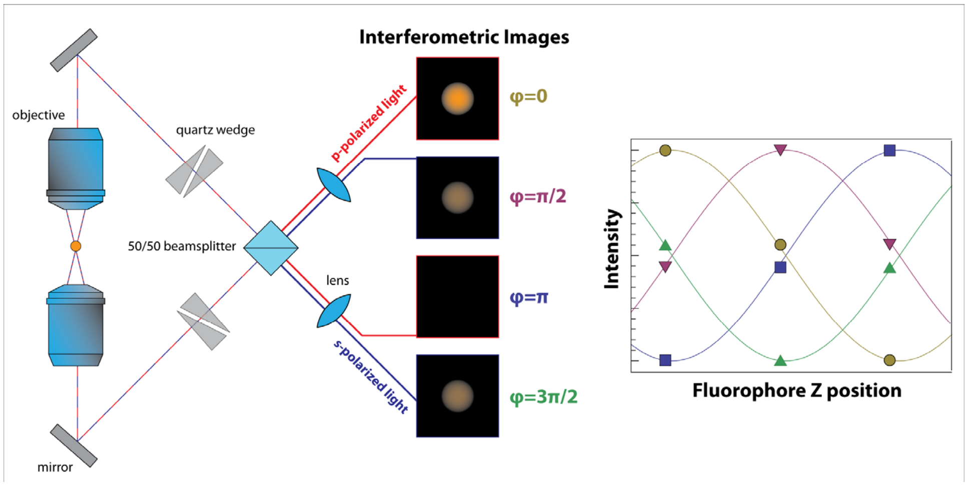
~~Reason: elongated PSF~~

Solution: 4Pi detection

S.W. Hell 1992
Gugel, Bewersdorf *et al. Biophys. J*, 2004



4Pi-SMS Nanoscopy

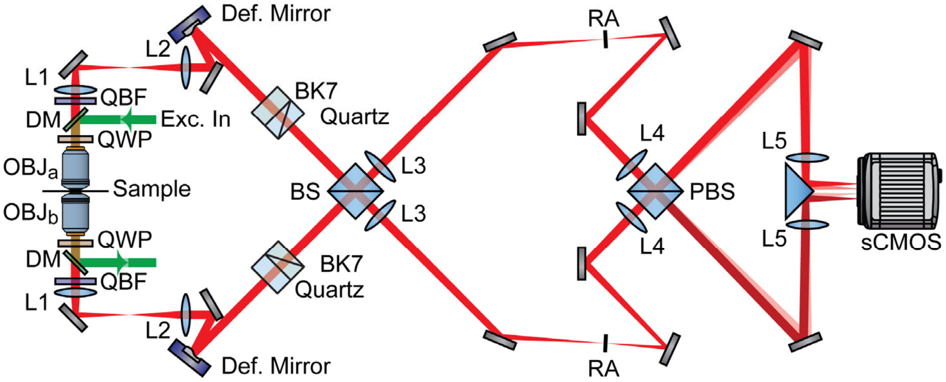


Shtengel, G. et al. *PNAS* **2009**, 9, 3125-30
Aquino, D. *et al. Nat. Methods* **2011**, *8*, 353-359

George Sirinakis

4Pi-SMS Nanoscopy

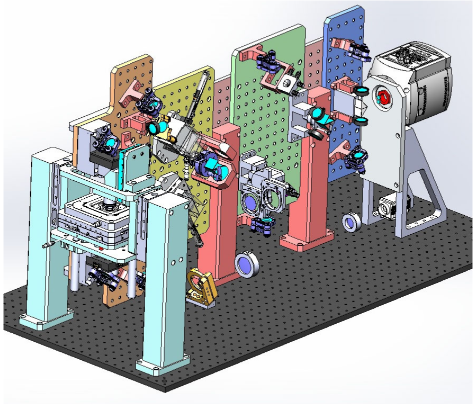
20 x 20 x 10 nm resolution
Adaptive optics enables >5 μm thick samples

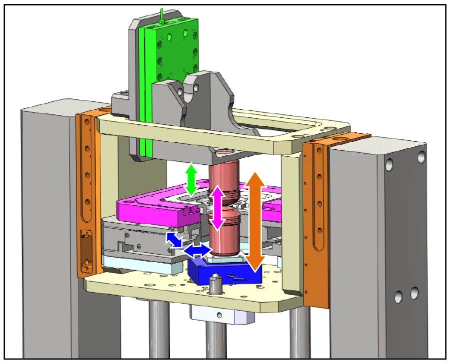


George Sirinakis, Edward Allgeyer

4Pi-SMS Nanoscopy

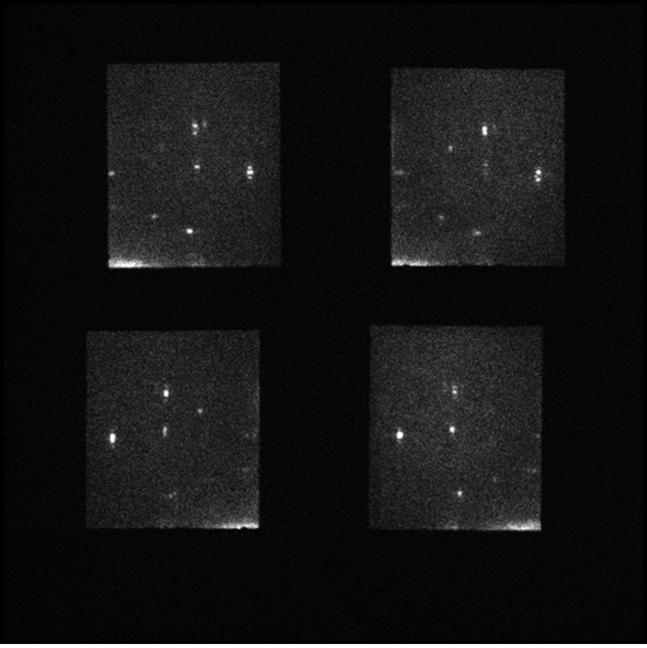
20 x 20 x 10 nm resolution
Adaptive optics enables >5 µm thick samples

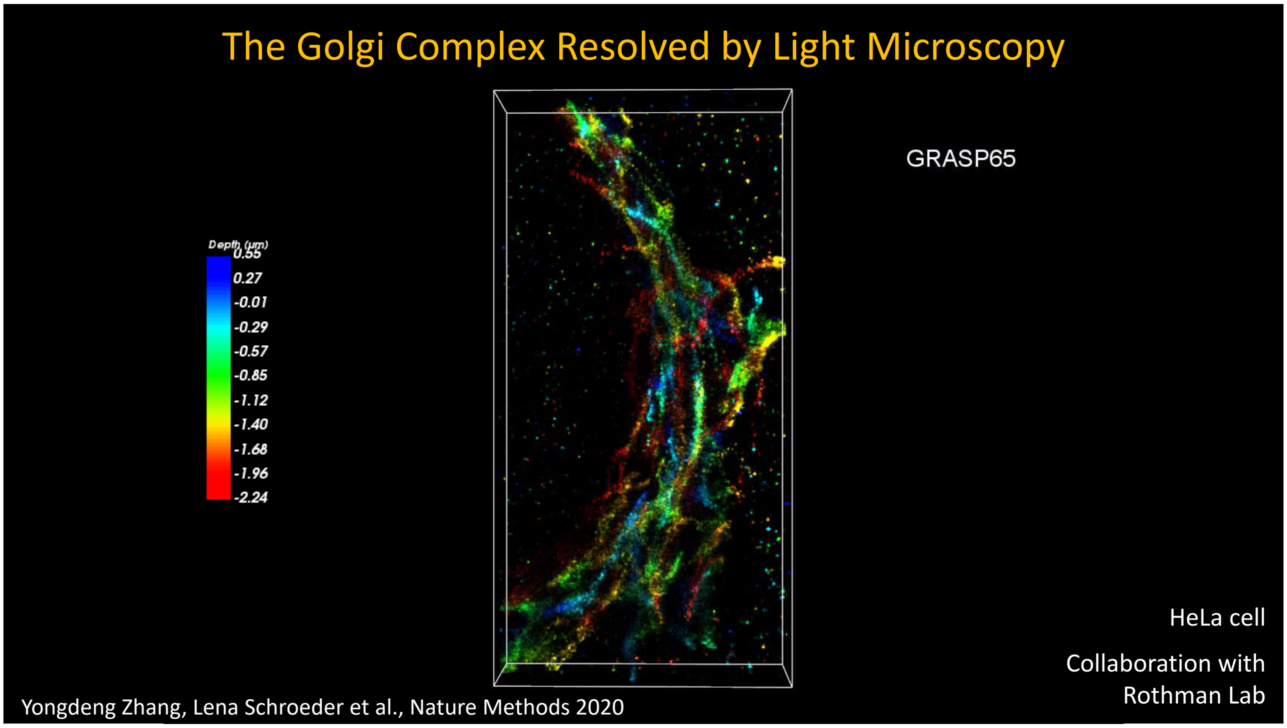




George Sirinakis, Edward Allgeyer

4Pi-SMS raw data





The Golgi Complex Resolved by Light Microscopy

HeLa cell

Collaboration with
Rothman Lab

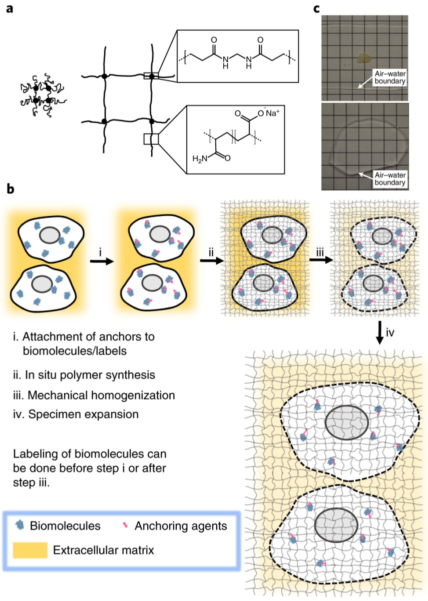
Yongdeng Zhang, Lena Schroeder et al., Nature Methods 2020

Expansion Microscopy

Enlarge the sample to resolve
originally small structures

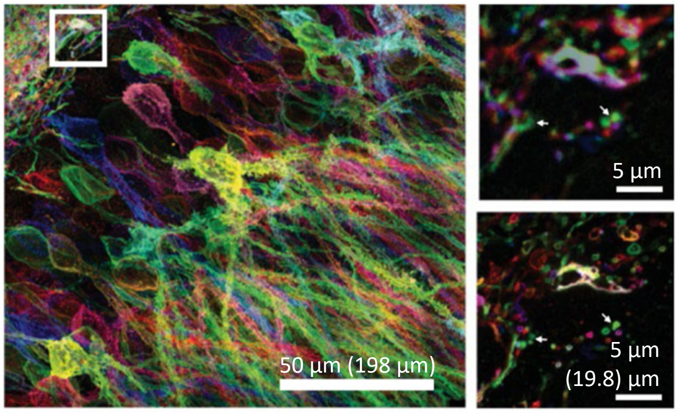
Expansion Microscopy

- Invented 2015
  - Chen, Tillberg, Boyden, Science 347:543-548
- Sample is physically expanded 4-5x in a hydrogel
- Initially: only specific proteins
  - Probes cross-linked to gel
  - all proteins degraded
    → requires labeling before expansion process, which is not ideal (crowded environment; labels also expand)
- 2016: protease-free approach (MAP, U-ExM)
  - Proteins not degraded anymore
    → post-expansion labeling possible
- 2017: iterative expansion (i-ExM)
  - Up to 20x (~4.5 x 4.5) expansion
    → makes very small structures visible; but degraded all proteins (see problems above)

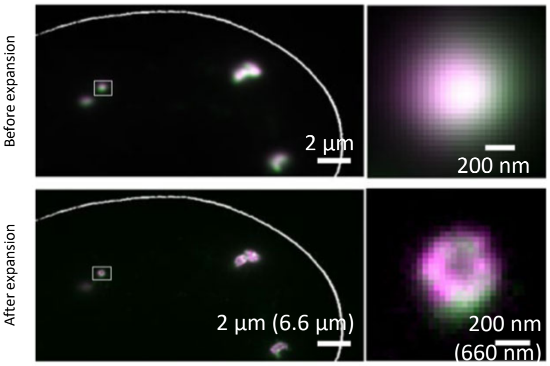


Wassie, Zhao & Boyden, Nat. Meth. 2019

Expansion Microscopy Examples



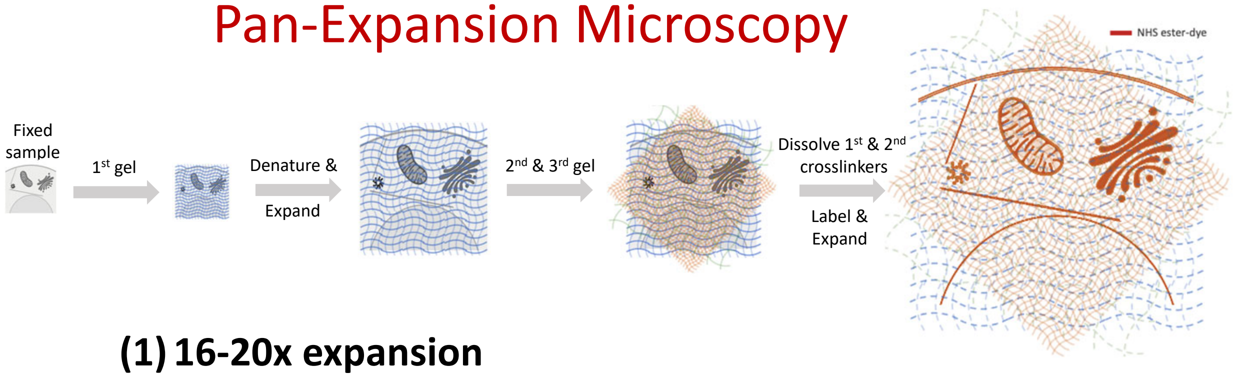
mouse hippocampus expressing virally delivered Brainbow3.0 epitopes



smFISH of NEAT1 lncRNA in the nucleus of a HeLa cell

Wassie, Zhao & Boyden, Nat. Meth. 2019

Pan-Expansion Microscopy



(1) **16-20x expansion**
(2) **Protein retention through polymer entanglement**
(3) **Pan-stainings reveal ultrastructural context**

→ CLEM-like images with a confocal microscope

M'Saad & Bewersdorf, Nat. Comm. (2020)

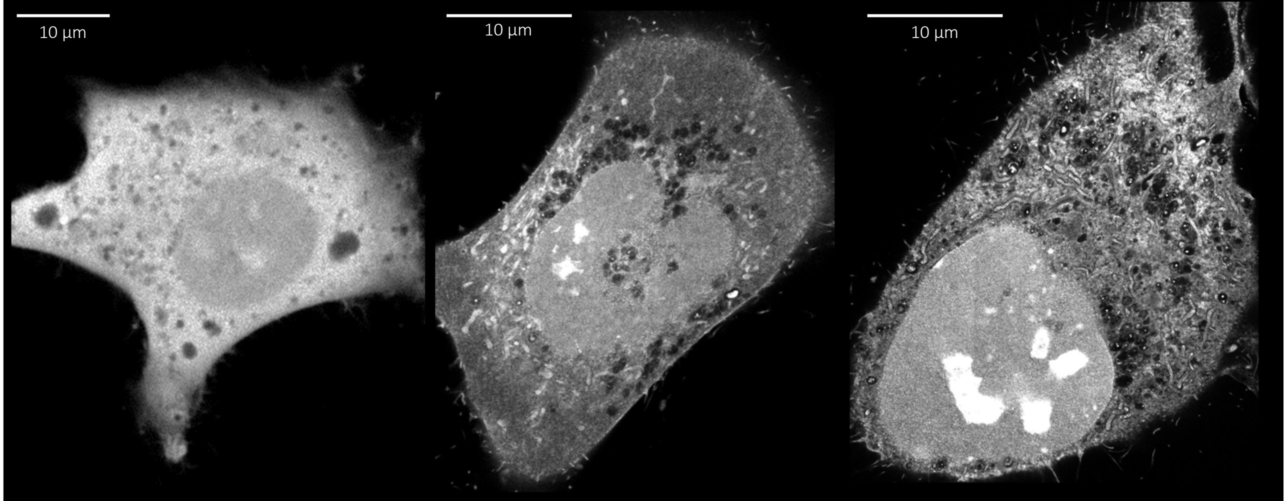
CLEM: Correlative Light Electron Microscopy

Pan-staining Reveals Structural Details After 16-fold Expansion

No expansion

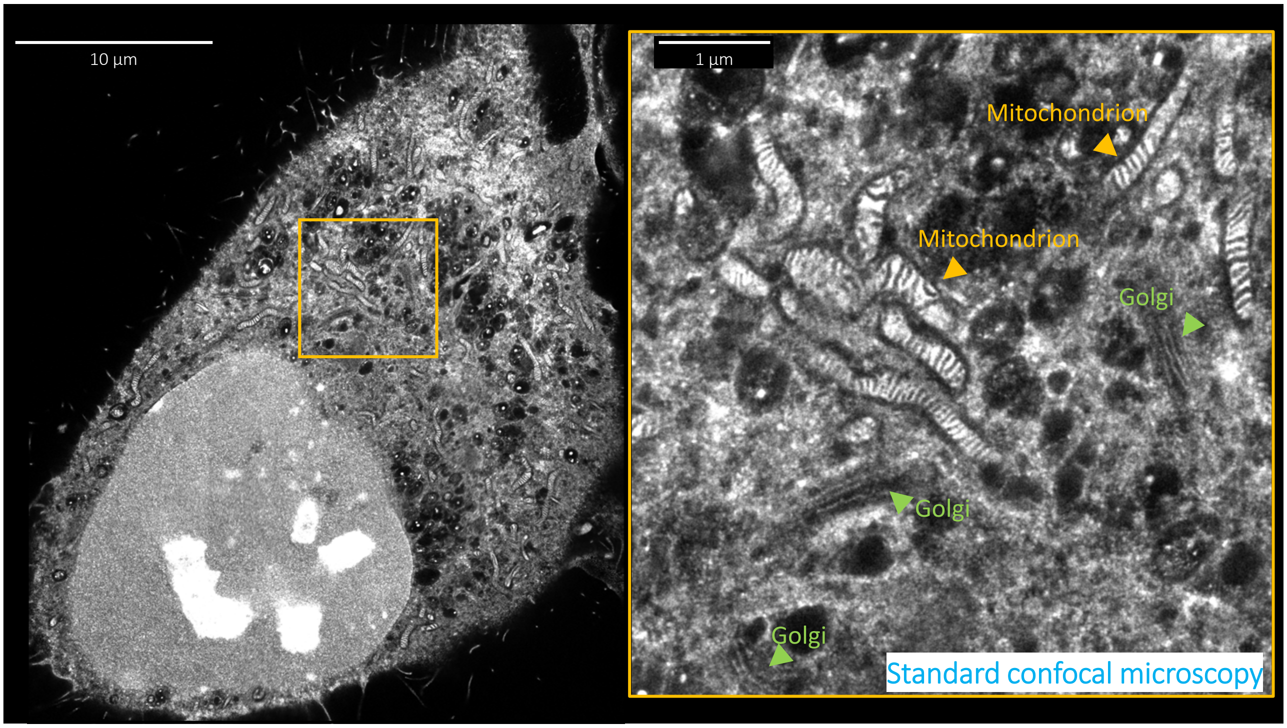
4-fold expansion

16-fold expansion



NHS ester
63X/1.2 NA water objective; confocal microscopy

M'Saad & Bewersdorf, Nat. Comm. (2020)



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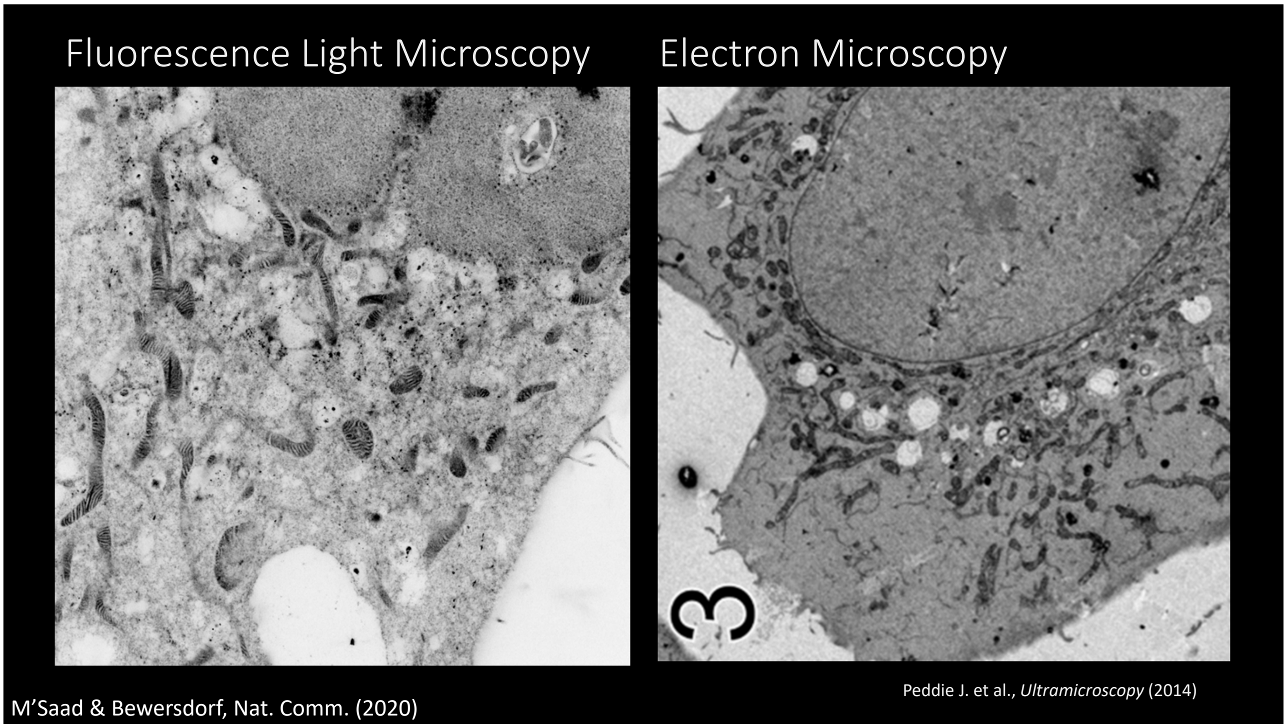

M'Saad & Bewersdorf, Nat. Comm. (2020)

Peddie J. et al., *Ultramicroscopy* (2014)

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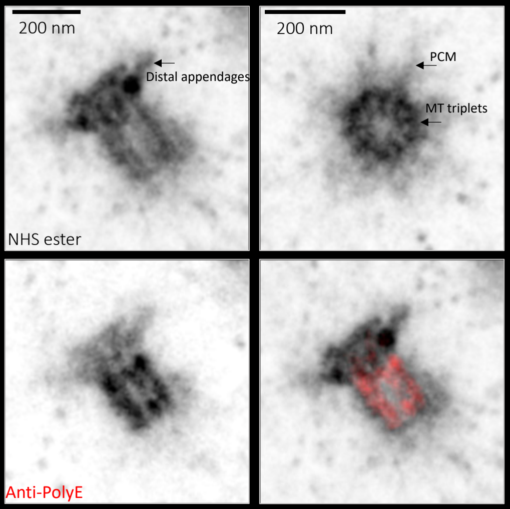
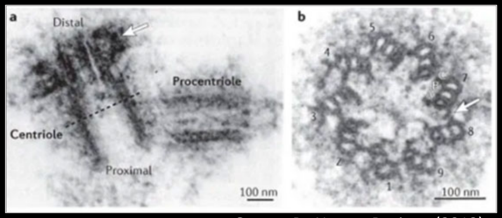
Pan-ExM reveals centriole ultrastructure



Comparison: Electron Microscopy



Gonczy P., *Nature Reviews* (2013)

63X/1.2 NA water objective; confocal microscopy

M'Saad & Bewersdorf, Nat. Comm. (2020)

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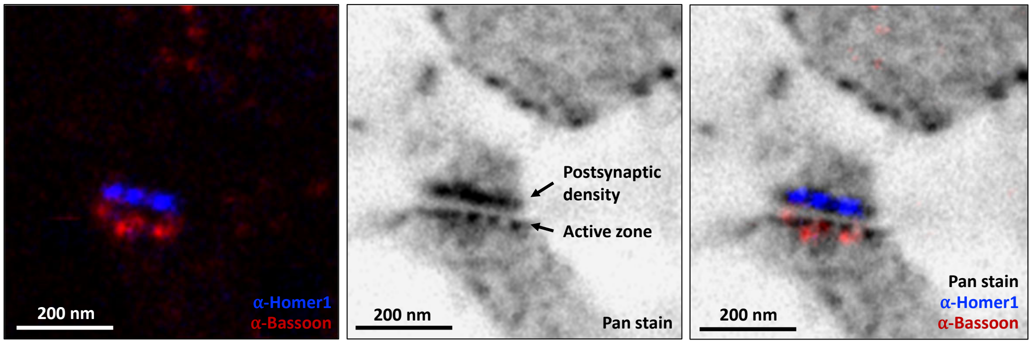
Synaptic Organization in Cultured Neurons



scale bar corrected for expansion factor

M'Saad et al., *unpublished*

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Acknowledgements

Current:
- Kenny Chung
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- Phylicia Kidd
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- Ons M'Saad
- Florian Schueder
- Yuan Tian

Former:
- Andrew Barentine
- Edward Courchaine
- Xiang Hao
- Fang Huang
- Emil Kromann
- Mark Lessard
- Yu Lin
- Timy Phan
- Bennett Rollins
- Lena Schroeder
- Helen Sun
- Mary Grace Velasco
- Yongdeng Zhang

Cell Biology, Yale
- Jim Rothman
- Felix Rivera-Molina
- Derek Toomre
- Jeeyun Chung
- Pietro de Camilli
- Zhao Zhang
- Nathan Williams
- Yang Yang
- Chenxiang Lin

Mol., Cell. and Dev. Biology, Yale
- Tom Pollard
- Holly Merta
- Shirin Bahmanyar

Microbial Pathogenesis, Yale
- Maria Lara-Tejero
- Jorge Galan

Genetics, Yale
- Miao Liu
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