Advanced Light Microscopy Course, GSK Cancer Engineering

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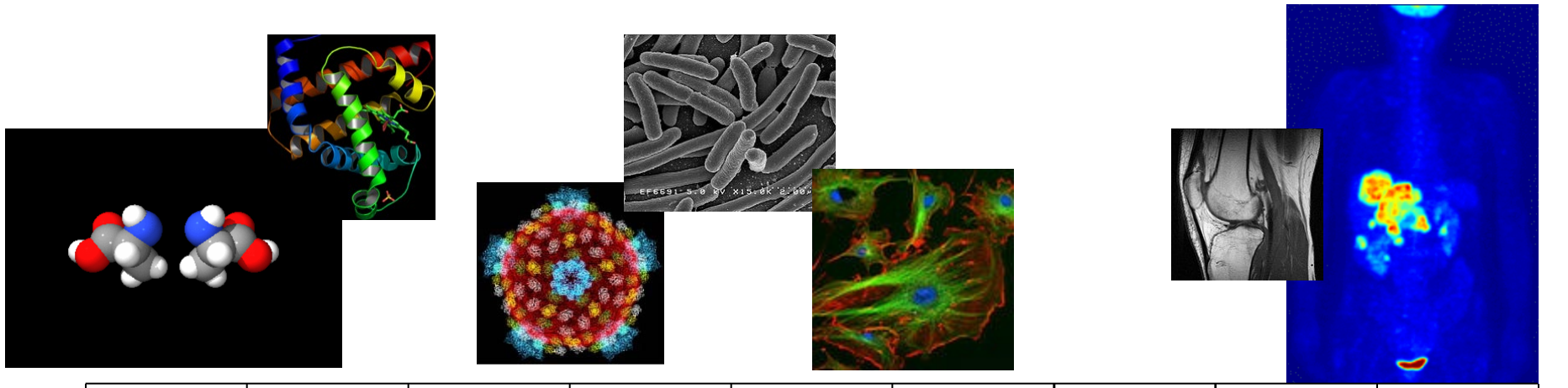
Imaging across spatial scales



0.1nm 1nm 10nm 100nm 1μm 10μm 100μm 1mm 1cm 1m

NMR
Crystallography

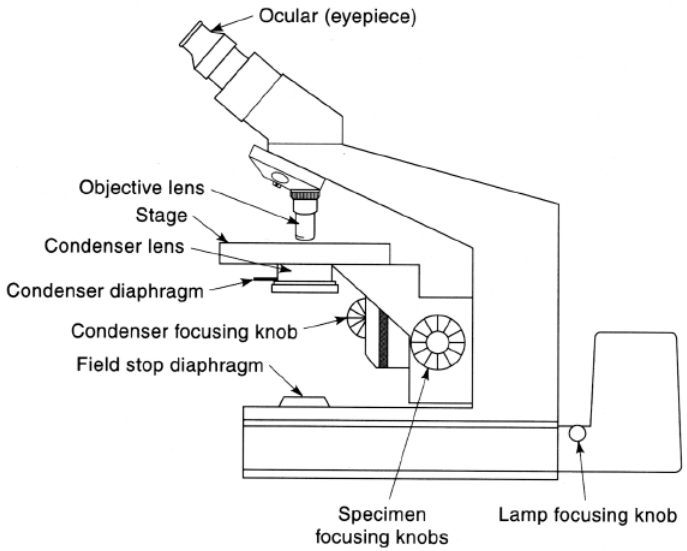
Electron-microscopy

Optical Microscopy

MRI
PET
CT

What a microscope needs to do

- Magnify things
- Resolve points which are close together
- Collect as much light as possible (esp. for fluorescence)
- Do all of the above while introducing as little distortion as possible



Also, for imaging live biological samples

- **Speed is important**
- **Must not disturb biological process**

https://downloads.micron.ox.ac.uk/lectures/onbi_2014-2015/week-1/ONBI_Lecture_03_imageformation_resolution.pdf

Image position and magnification depend on lens curvature (focal length) and on the physical distance from the object to the lens

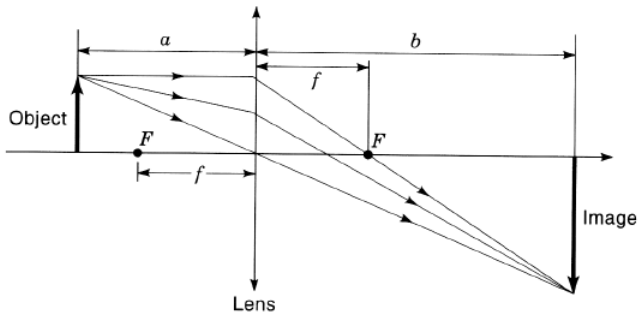


Figure 4-3

Geometrical optics of a simple lens. The focal length *f*, focal point *F*, object-lens distance *a*, and lens-image distance *b* are indicated.

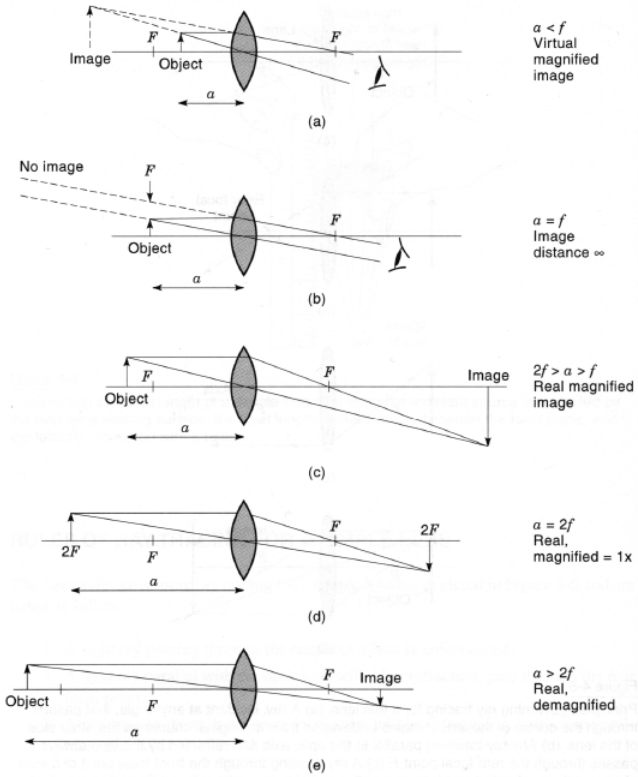


Figure 4-6

Object-image relationships. (a) $a < f$; (b) $a = f$; (c) $2f > a > f$; (d) $a = 2f$; (e) $a > 2f$.

https://downloads.micron.ox.ac.uk/lectures/onbi_2014-2015/week-1/ONBI_Lecture_03_imageformation_resolution.pdf

All simple lenses have associated aberrations

- Still may encounter: *chromatic aberration* on cheap microscopes (prism effect--but can be reduced by using monochromatic light), *spherical aberration* when imaging deep into samples (e.g. embryos, even when the objective is "corrected"), *field curvature* when using bright lenses for fluorescence (but this is not a problem if you're imaging cells only in the center of the field)

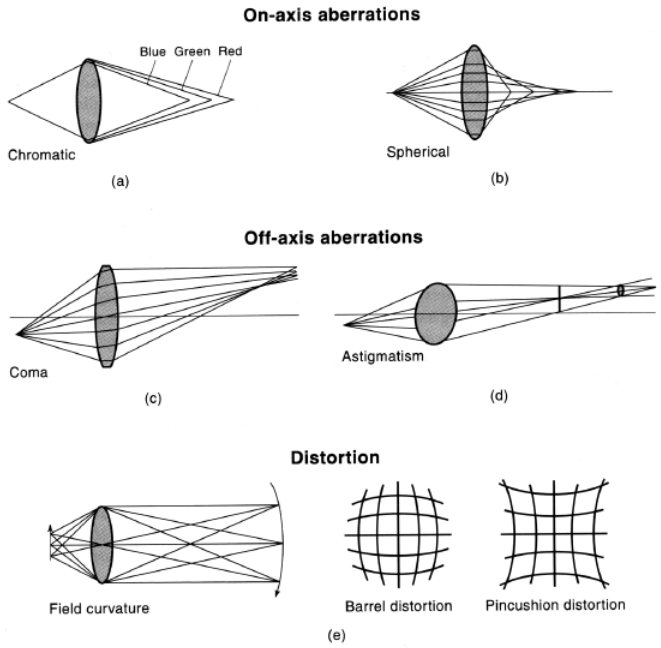


Figure 4-8
Aberrations of a simple lens. (a) Chromatic aberration: Parallel incident rays of different wavelength are focused at different locations. (b) Spherical aberration: Incident rays parallel to the optic axis and reaching the center and the periphery of the lens are focused at different locations. (c) Coma: Off-axis rays passing through the center and periphery of the lens are focused at different locations. (d) Astigmatism: An off-axis aberration causes waves passing through the vertical and horizontal diameters to focus an object point as a streak. (e) Distortion and field curvature: The image plane is curved and not planar. So-called barrel and pincushion distortions produce images that are not high in fidelity compared to the object.

More lens elements = better correction, but also possibly less light throughput

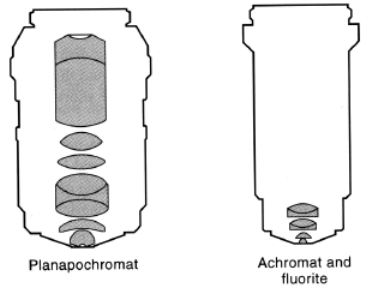


Figure 4-9
Objective lens designs. Two popular lenses for fluorescence microscopy are shown. Apochromatic lenses may contain 12 or more lens elements to give bright, flat images with excellent color correction across the visual spectrum. Fluorite lenses have fewer lens components and produce sharp, bright images. These lenses exhibit excellent color correction and transmit UV light.

Achromat = corrected for 2 colors

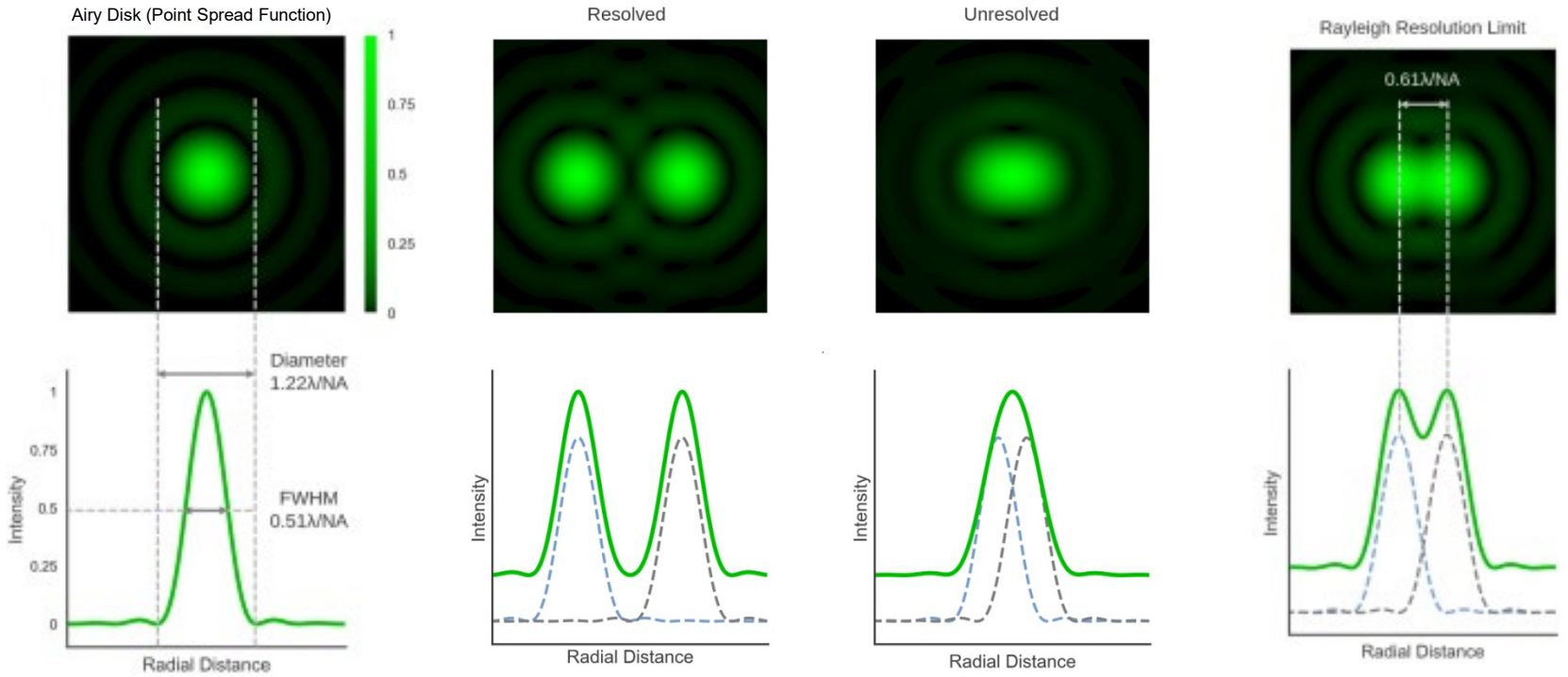
Apochromat = corrected for 3 colors

Plan = flat-field (although not always to full limits of field of view)

TEST BEFORE BUYING!!

https://downloads.micron.ox.ac.uk/lectures/onbi_2014-2015/week-1/ONBI_Lecture_03_imageformation_resolution.pdf

Resolution

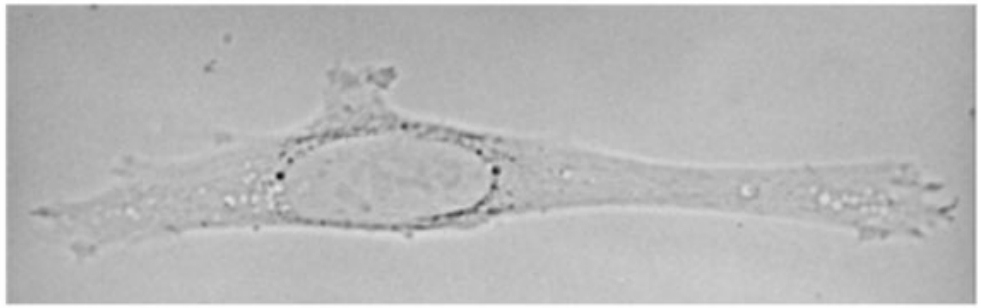


https://www.edinst.com/blog/the-rayleigh-criterion-for-microscope-resolution/

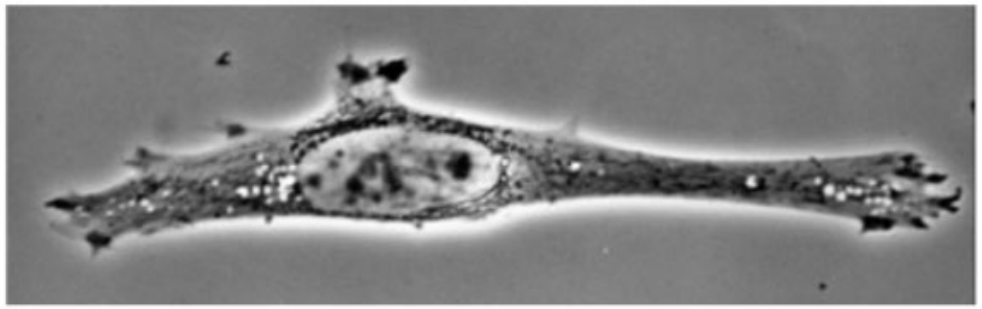
LABEL-FREE IMAGING

Contrast Enhancing Techniques

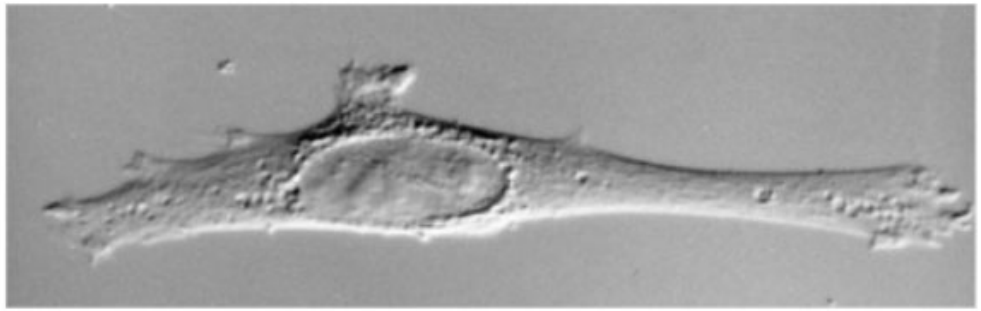
Bright Field



Phase Contrast



Differential Interference Contrast



Phase Contrast & DIC

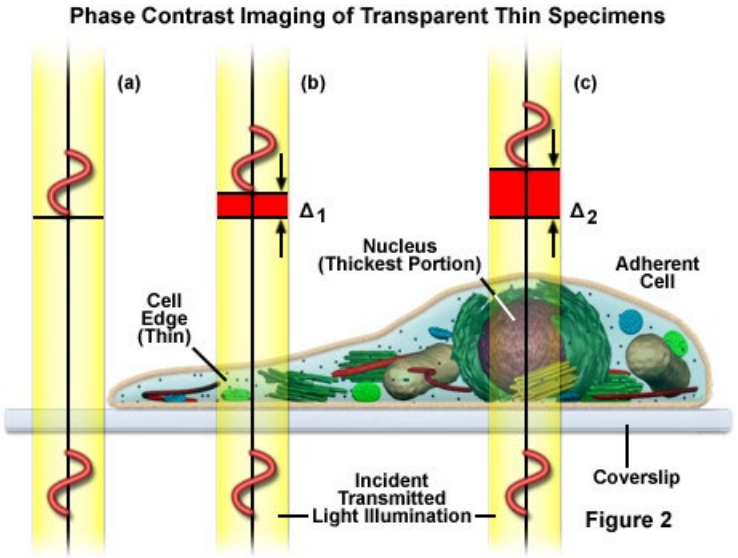


Figure 2

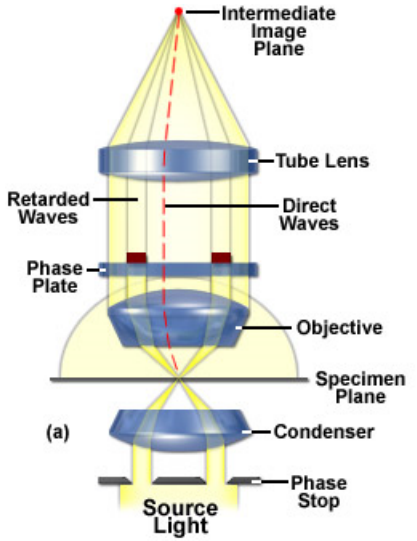


Figure 3

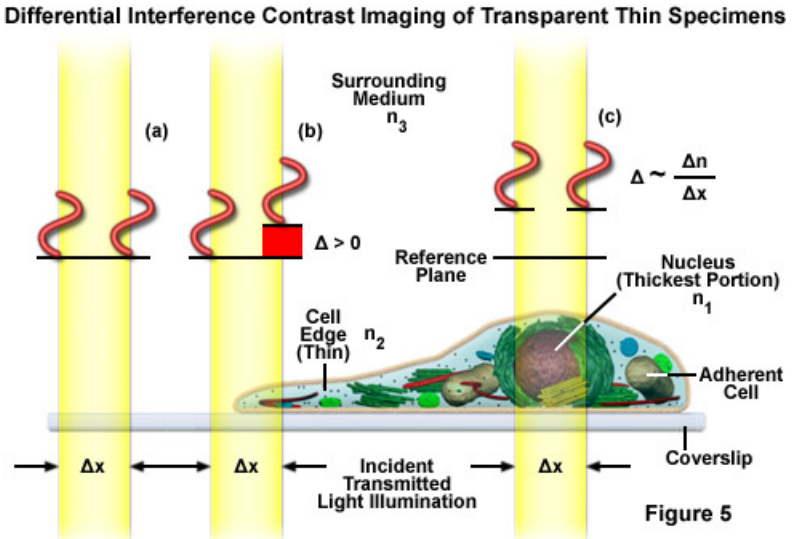


Figure 5

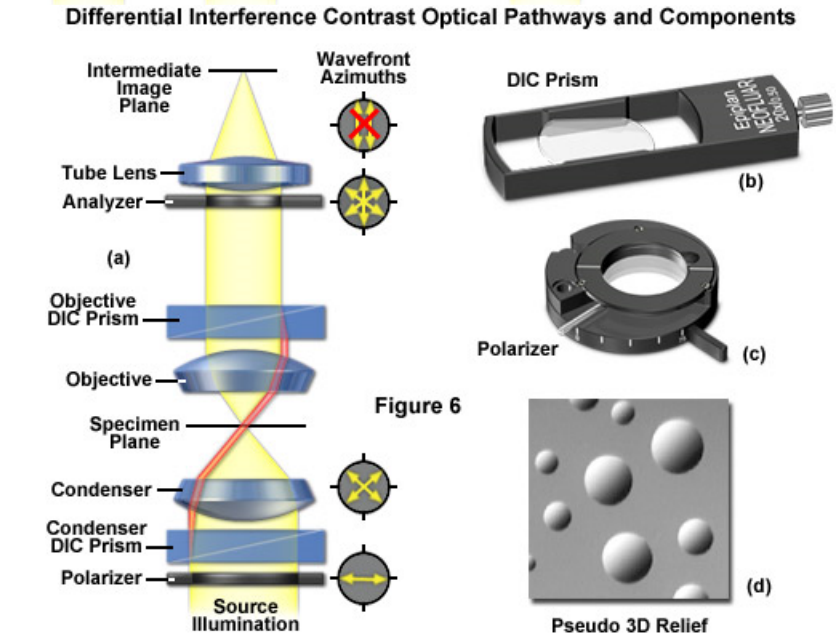
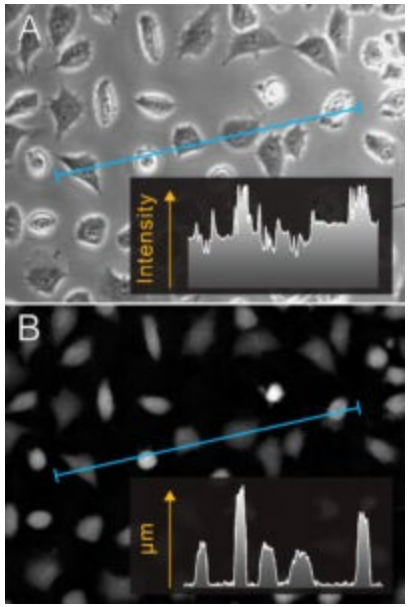
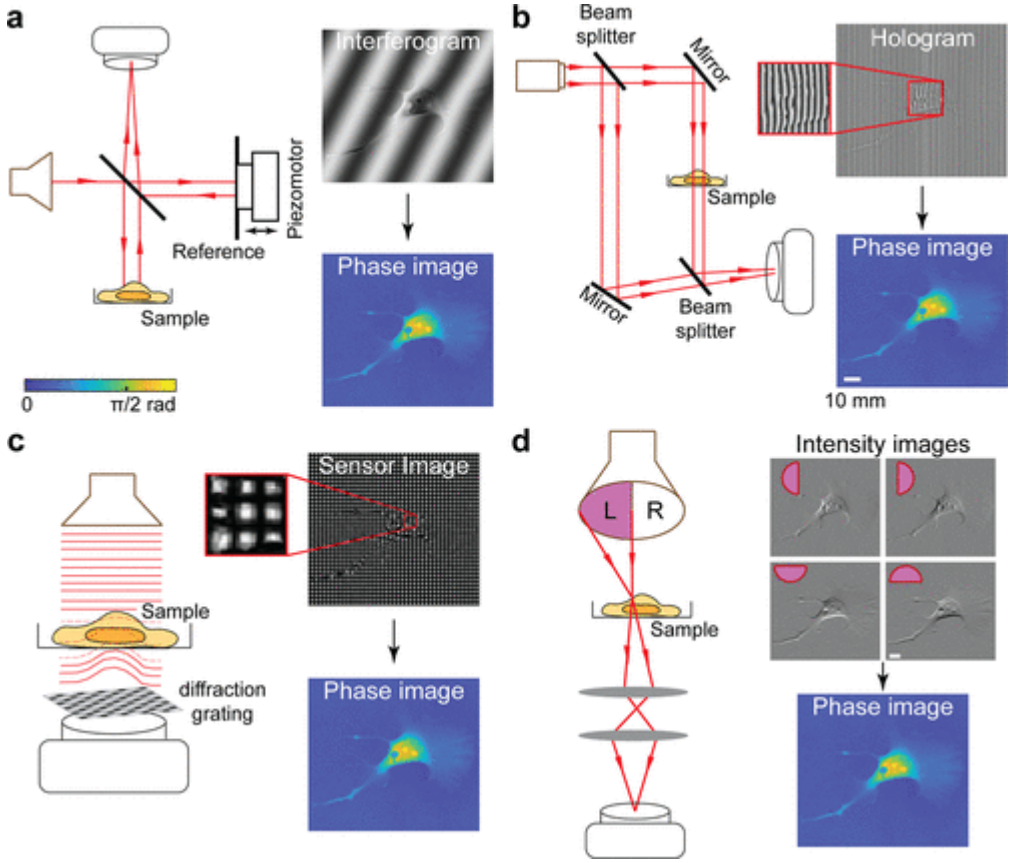


Figure 6

Quantitative Phase Imaging

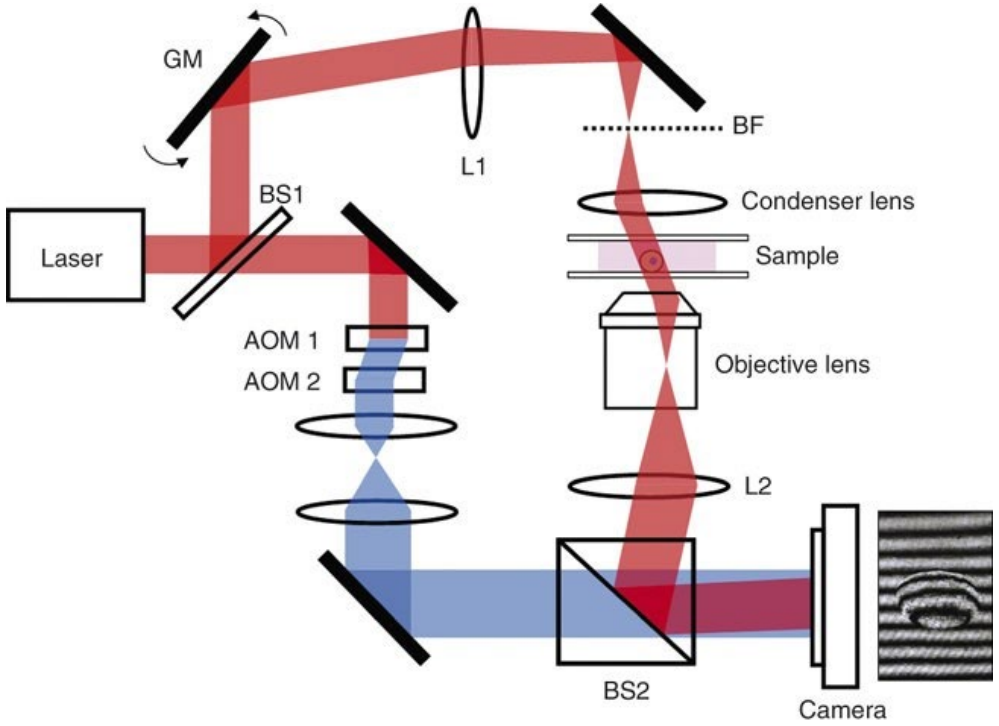


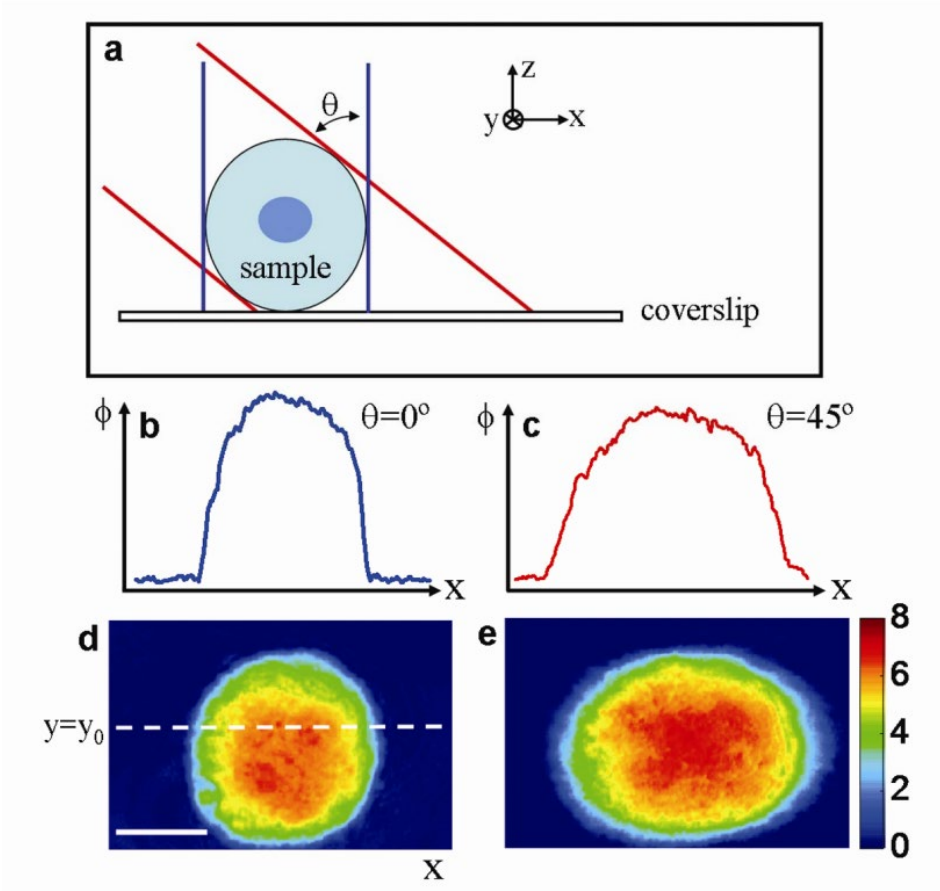


https://youtu.be/Nj21OJrJqcU

ACS Nano 2022, 16, 8, 11516–11544 https://doi.org/10.1021/acsnano.1c11507

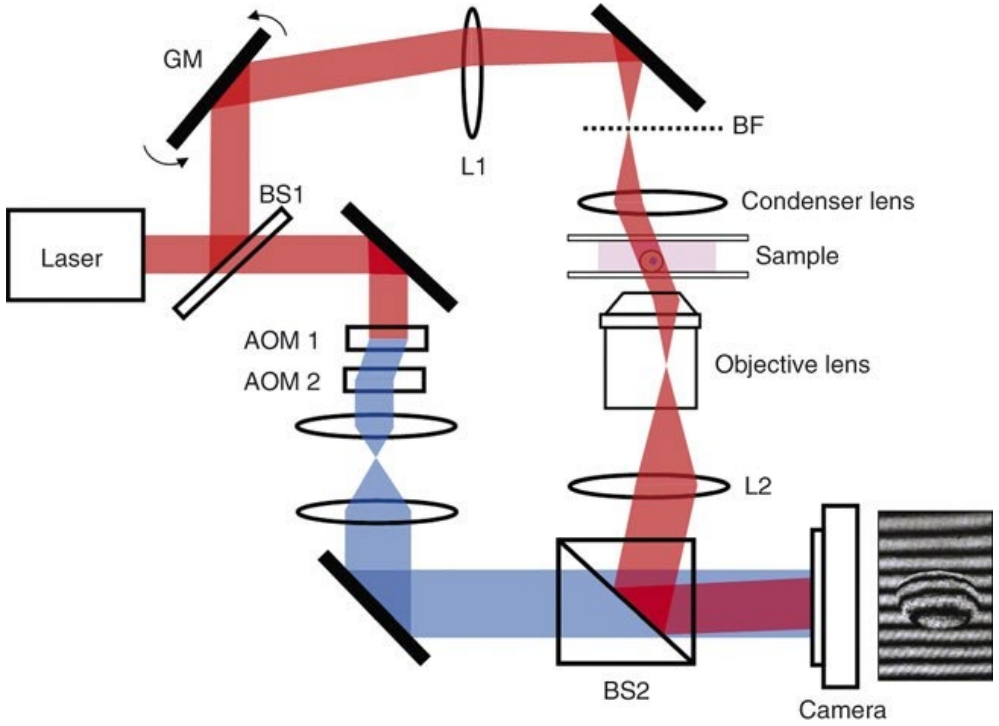
Quantitative Phase Tomography

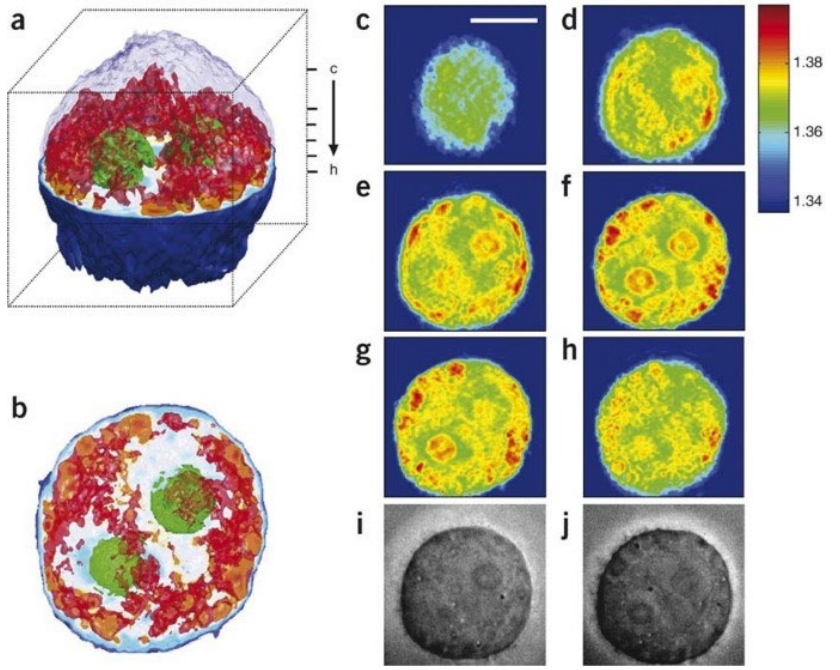


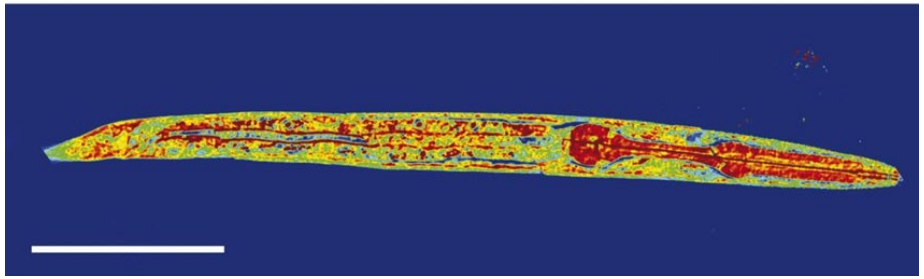


Nat Methods **4**, 717–719 (2007). https://doi.org/10.1038/nmeth1078

Quantitative Phase Tomography







Nat Methods **4**, 717–719 (2007). https://doi.org/10.1038/nmeth1078

FLUORESCENCE IMAGING

Fluorescence Basics

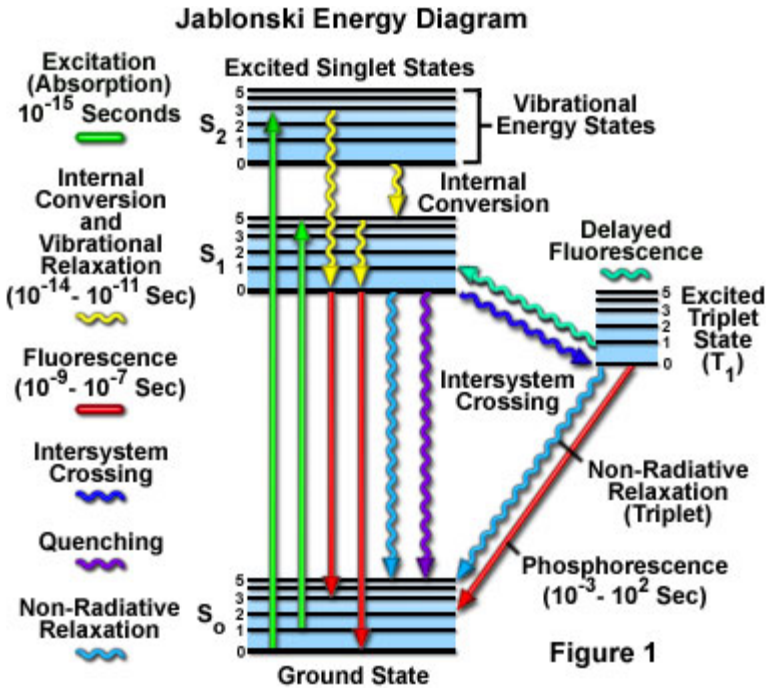
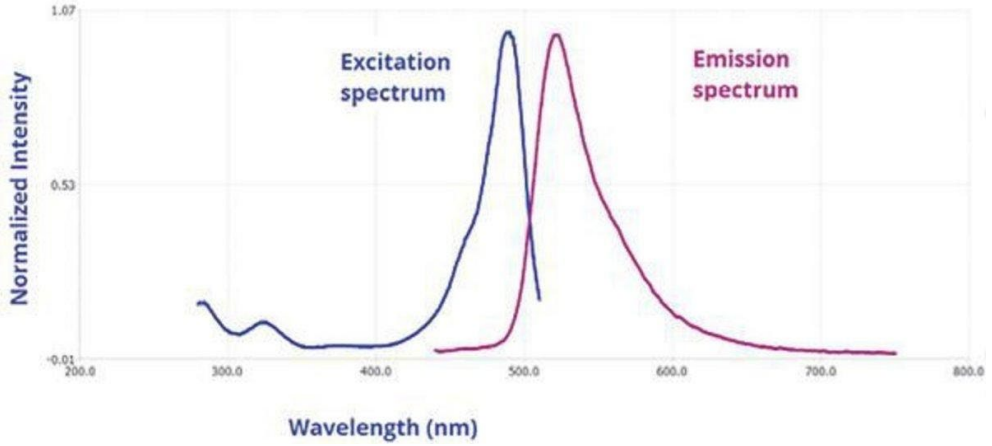


Figure 1

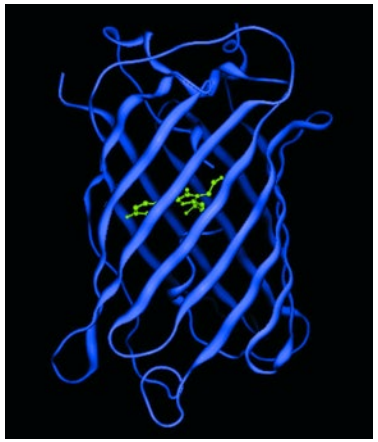
https://micro.magnet.fsu.edu/primer/java/jablonski/jabintro/index.html



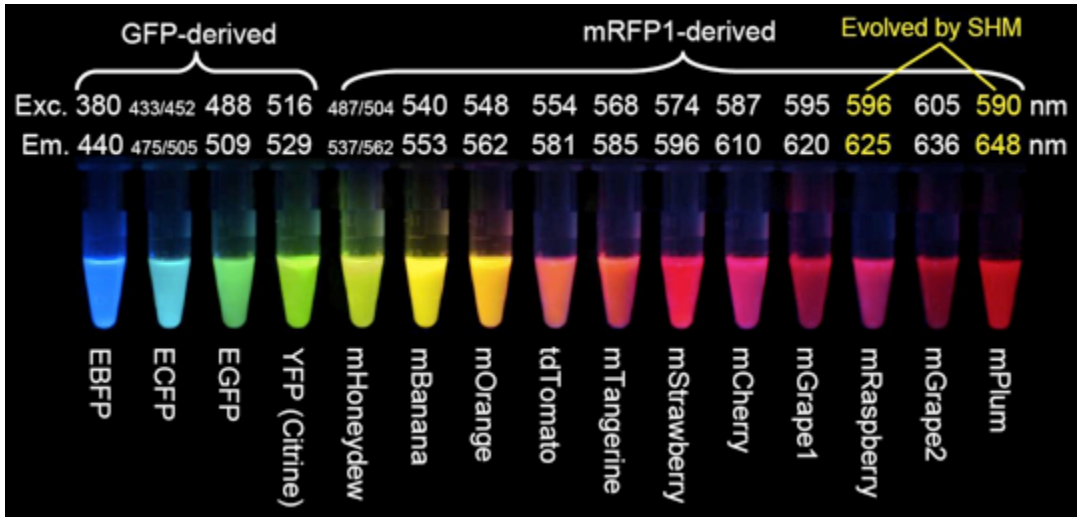
https://www.horiba.com/int/scientific/technologies/fluorescence-spectroscopy/what-is-fluorescence-spectroscopy/

Fluorescent Probes

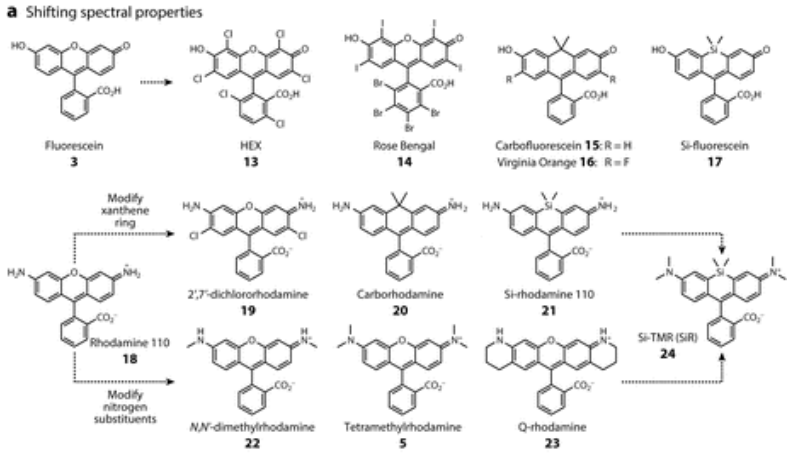
Fluorescent proteins

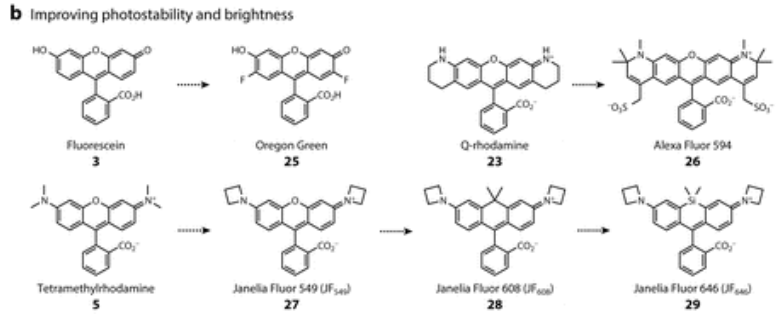


GFP



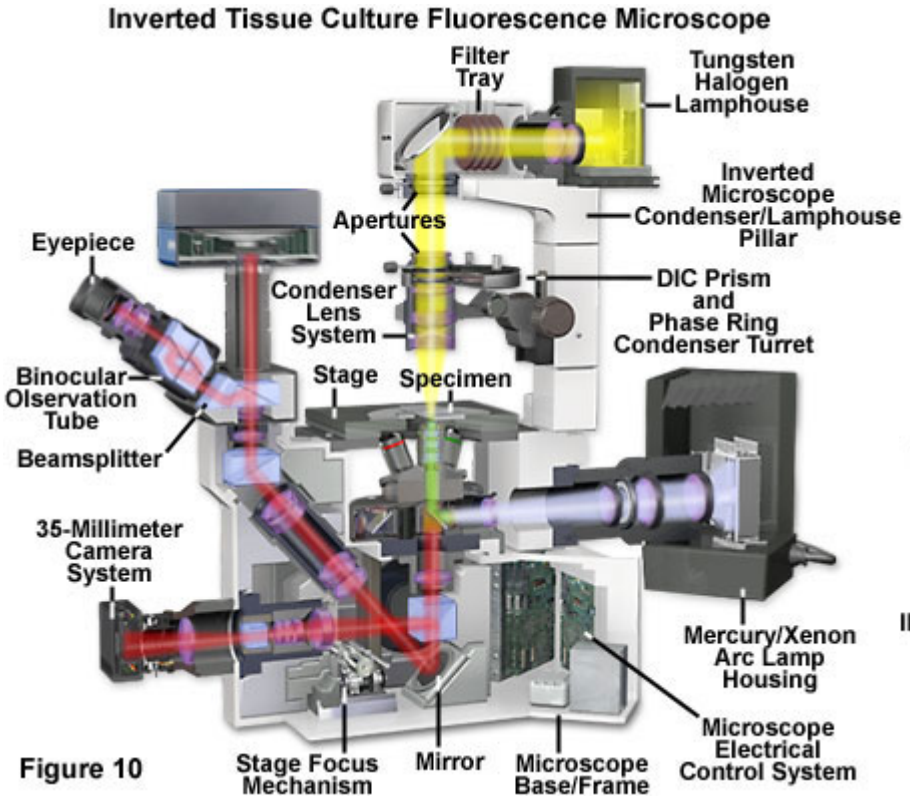
Organic Dyes

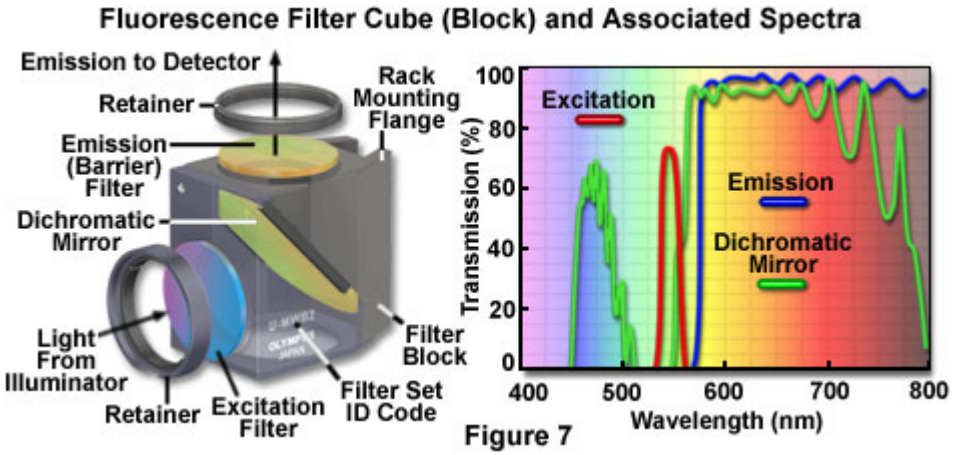




Lavis LD. 2017.
Annu. Rev. Biochem. 86:825–43

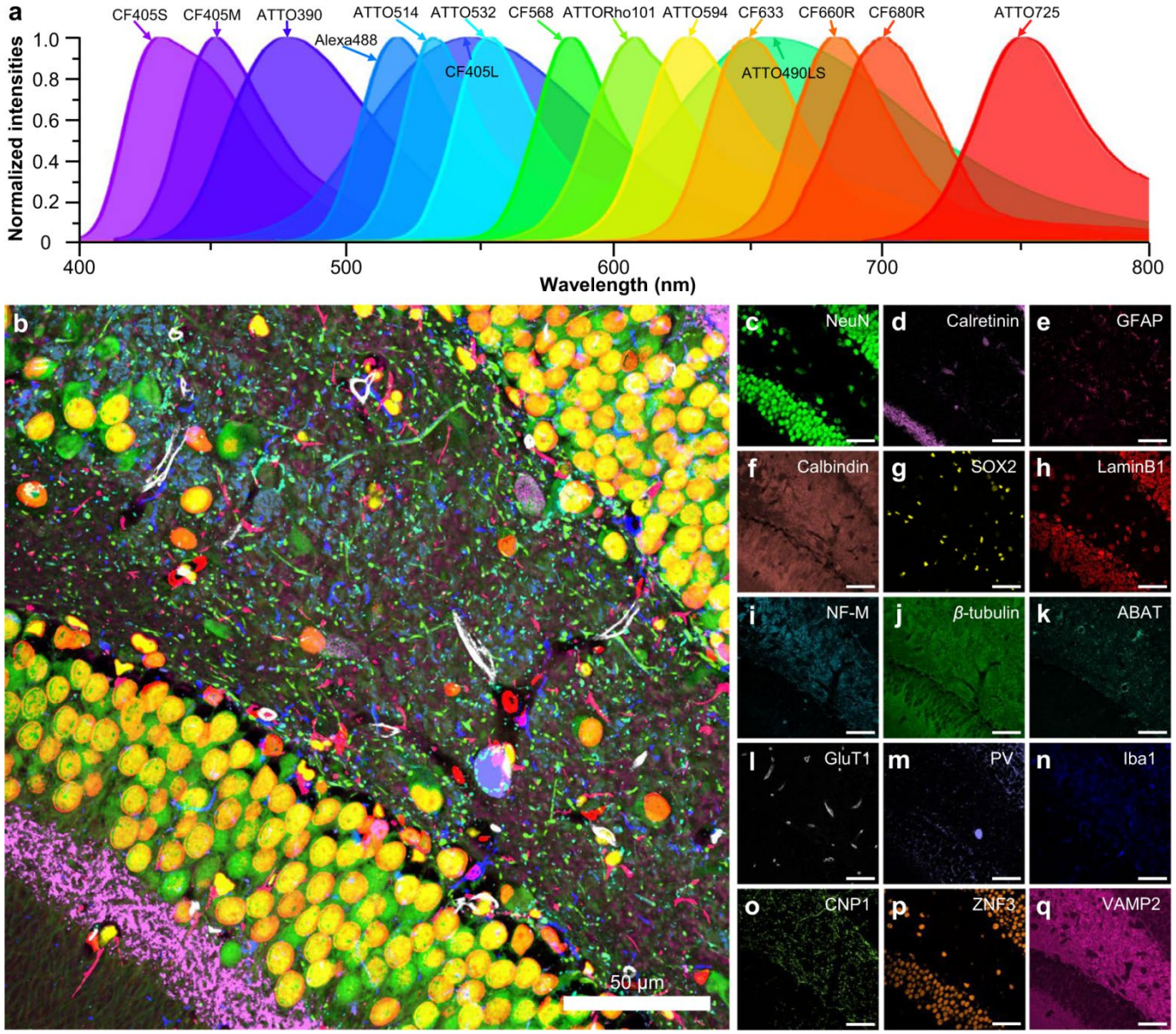
Anatomy of Fluorescence Microscope





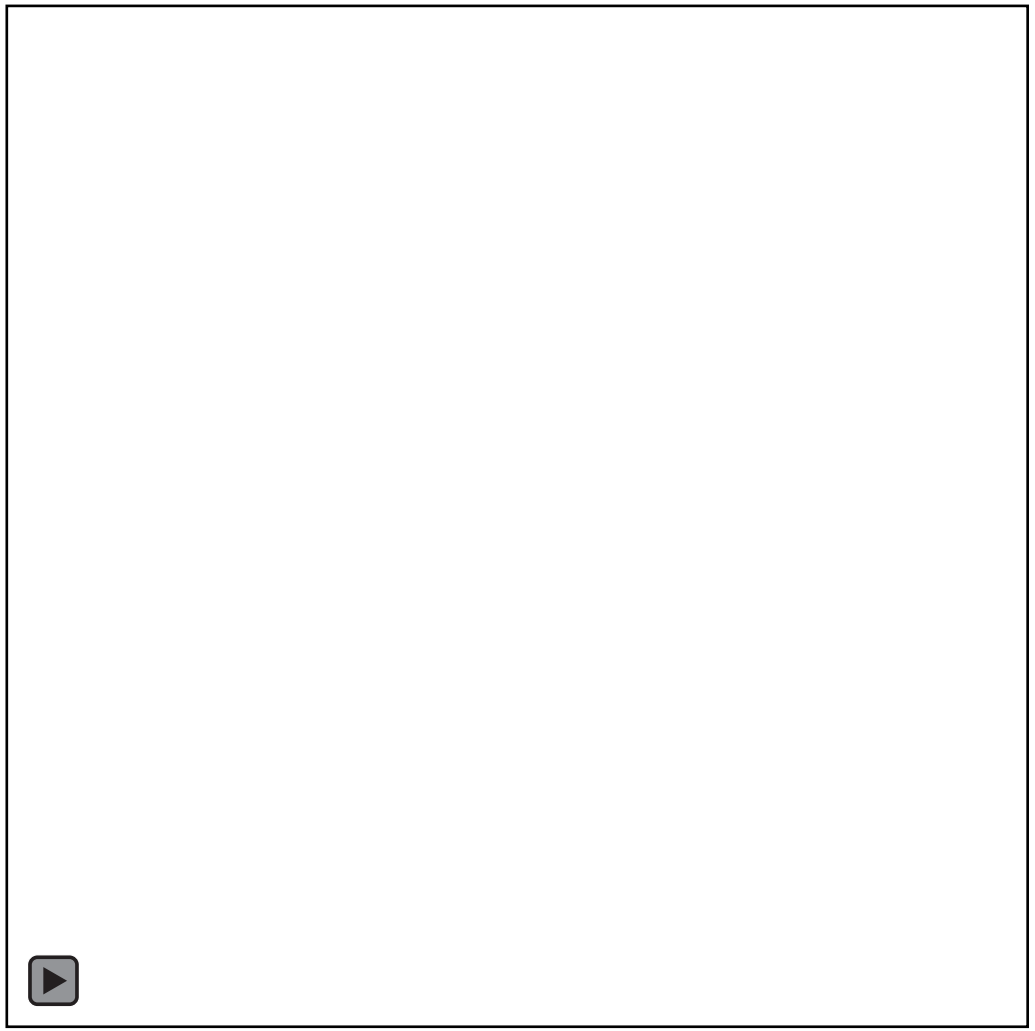
https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/fluorescence/anatomy/fluoromicroanatomy/

Multi-color Imaging



Seo, J., Sim, Y., Kim, J. *et al.* . *Nat Commun* **13**, 2475 (2022)

Multi-color Imaging

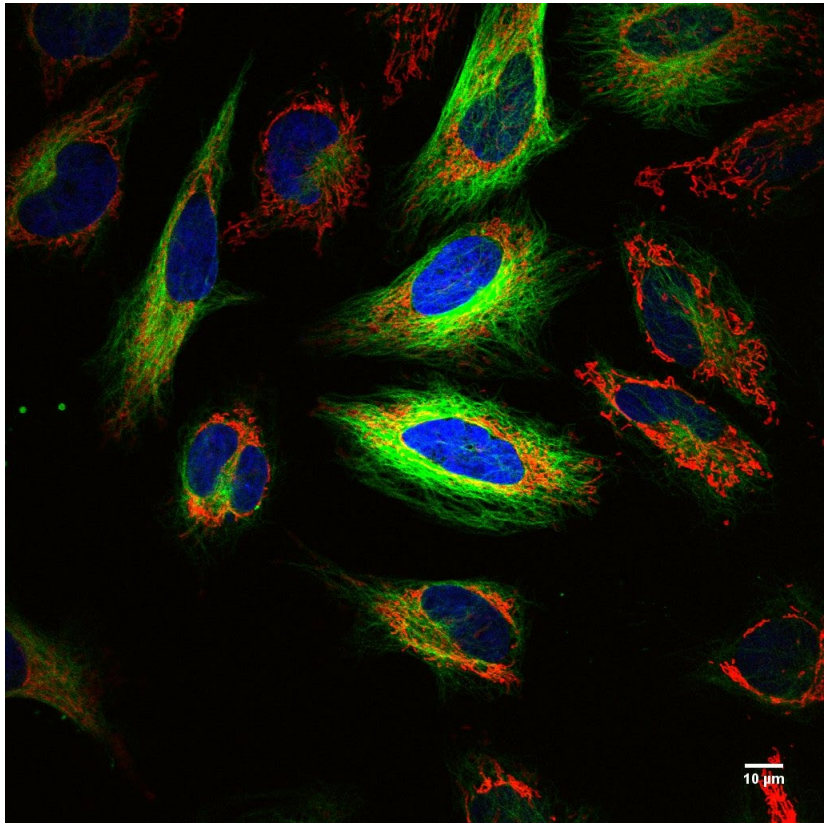


6-color LLS imaging of organelles in COS7 cell

Valm, A., Cohen, S., Legant, W. *et al.* *Nature* **546**, 162–167 (2017).

ENHANCED RESOLUTION AND SUPER-RESOLUTION IMAGING

Image formation in fluorescence microscope



wikipedia

$$I(\boldsymbol{r}) = U(\boldsymbol{r}) \otimes [E(\boldsymbol{r})c(\boldsymbol{r})]$$

$I(\boldsymbol{r})$:*Image*
$U(\boldsymbol{r}) = PSF(\boldsymbol{r})$: *Point-spread Function*
$E(\boldsymbol{r})$: *Excitation Intensity*
$c(\boldsymbol{r})$: Fluorophore distribution in sample

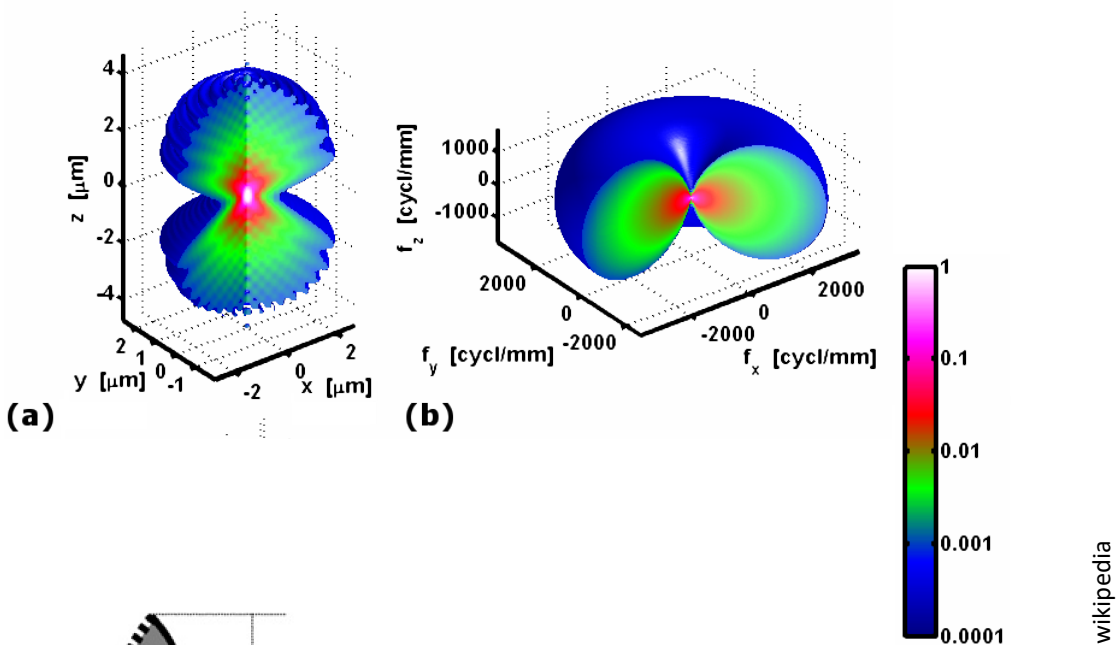
Wide-Field Imaging

$E(\boldsymbol{r})$=const

$I(\boldsymbol{r}) = PSF(\boldsymbol{r}) \otimes c(\boldsymbol{r})$

PSF

OTF



wikipedia

$OTF(\boldsymbol{k}) = FT(PSF(\boldsymbol{r}))$

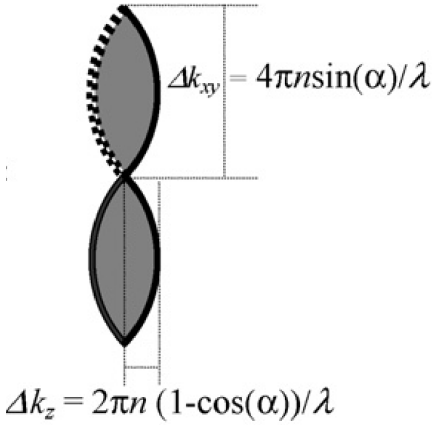
OTF(k=0)=0

No optical sectioning

OTF support

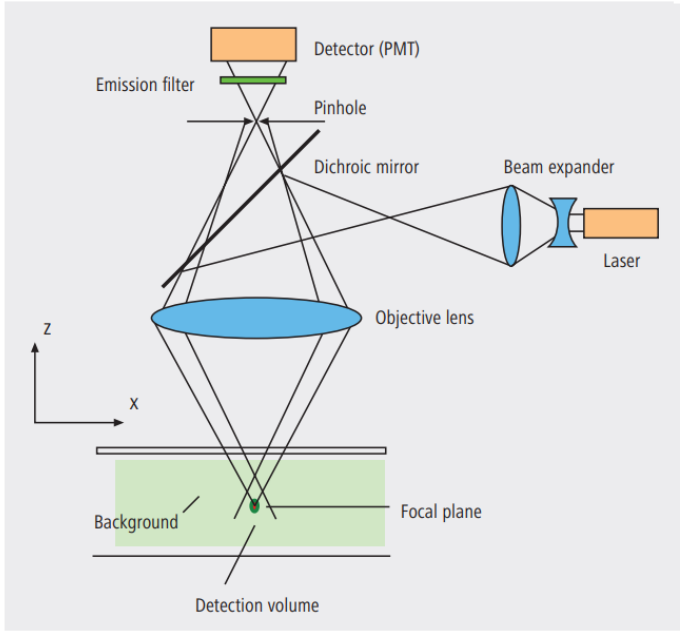
kmax

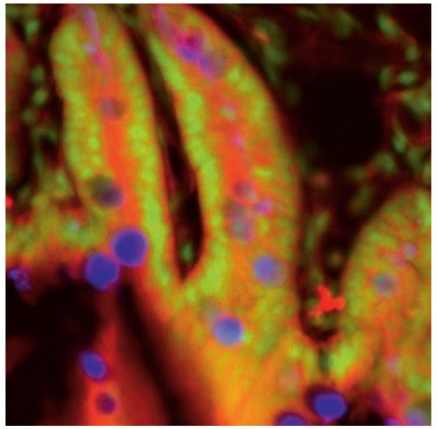
NA=nsin(α)



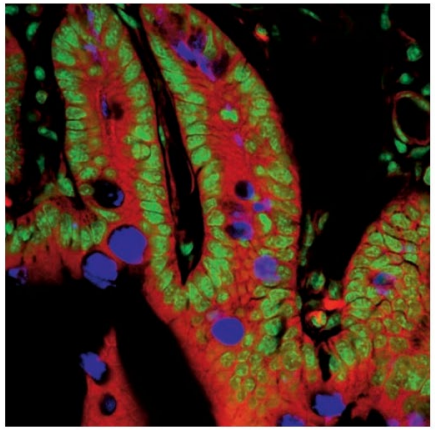
R. Heintzmann, C.J.R. Sheppard / Micron 38 (2007) 145–149

Confocal Microscopy





Epifluorescence



Confocal

Image on pinhole plane $\mathbf{s}$, given beam on sample at $\mathbf{r}$

$$I(\boldsymbol{r},\boldsymbol{s}) = \int d\boldsymbol{r}'\, U(\boldsymbol{r}-\boldsymbol{r}'+\boldsymbol{s})[E(\boldsymbol{r}-\boldsymbol{r}')c(\boldsymbol{r}')]$$

Confocal image, integrate over pinhole (radius = a):

$$I_{conf}(\boldsymbol{r}) = \int d\boldsymbol{s} * I(\boldsymbol{r},\boldsymbol{s}) =$$

$$\int_{s=0}^{a} dsU(\boldsymbol{r}-\boldsymbol{r}'+\boldsymbol{s}) \int d\boldsymbol{r}'\,[E(\boldsymbol{r}-\boldsymbol{r}')c(\boldsymbol{r}')] =$$

$$PSF_a(\boldsymbol{r}) \otimes [E(\boldsymbol{r})c(\boldsymbol{r})]$$

Confocal PSF and OTF

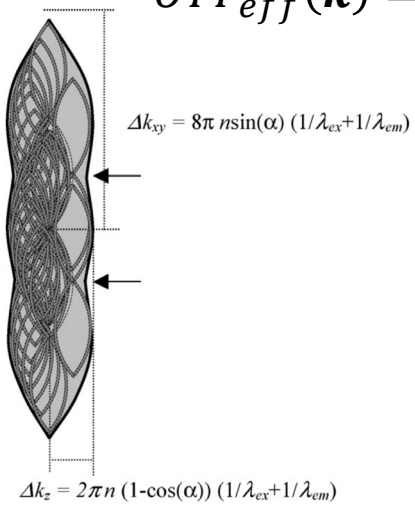
For small pinhole (radius = a->0):

$$I_{conf}(\boldsymbol{r}) = \int d\boldsymbol{s} * I(\boldsymbol{r}, \boldsymbol{s}) =$$

$$\int d\boldsymbol{r}' U(\boldsymbol{r} - \boldsymbol{r}')E(\boldsymbol{r} - \boldsymbol{r}')c(\boldsymbol{r}')$$

$$PSF_{eff}(\boldsymbol{r}) = PSF_{det}(\boldsymbol{r}) * PSF_{excit}(\boldsymbol{r})$$

$$OTF_{eff}(\boldsymbol{k}) = OTF_{det}(\boldsymbol{k}) \otimes OTF_{excit}(\boldsymbol{k})$$

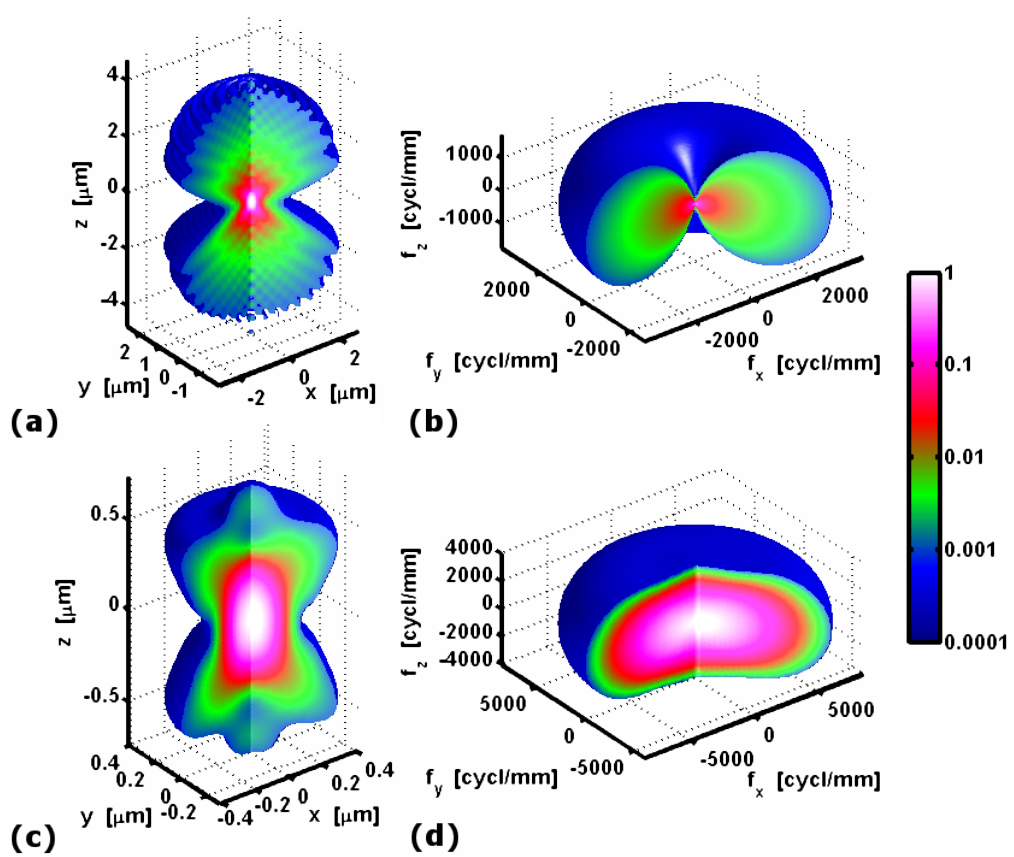


R. Heintzmann, C.J.R. Sheppard / Micron 38 (2007) 145–149

PSF OTF

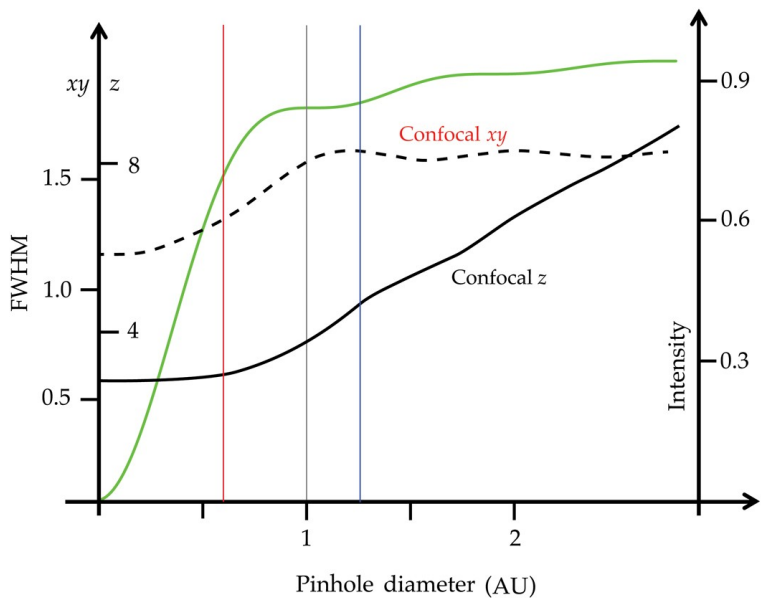
Wide-Field

Confocal



wikipedia

Confocal performance vs. pinhole diameter



Axial:

$$FWHM_{tot,axial} = \frac{0.64 \cdot \bar{\lambda}}{(n\text{-}\sqrt{n^2\text{-}NA^2})} \quad (7)$$

If NA < 0.5, equation (2) can be approximated by:

$$\approx \frac{1.28 \cdot n \cdot \bar{\lambda}}{NA^2} \quad (7a)$$

Lateral:

$$FWHM_{tot,lateral} = 0.37 \frac{\bar{\lambda}}{NA} \quad (8)$$

Axial:

$$FWHM_{ill,axial} = \frac{0.88 \cdot \lambda_{exc}}{(n\text{-}\sqrt{n^2\text{-}NA^2})} \quad (2)$$

n = *refractive index of immersion liquid*
NA = *numerical aperture of the objective*
λ_{exc} = *wavelength of the excitation light*

If NA < 0.5, equation (2) can be approximated by:

$$\approx \frac{1.77 \cdot n \cdot \lambda_{exc}}{NA^2} \quad (2a)$$

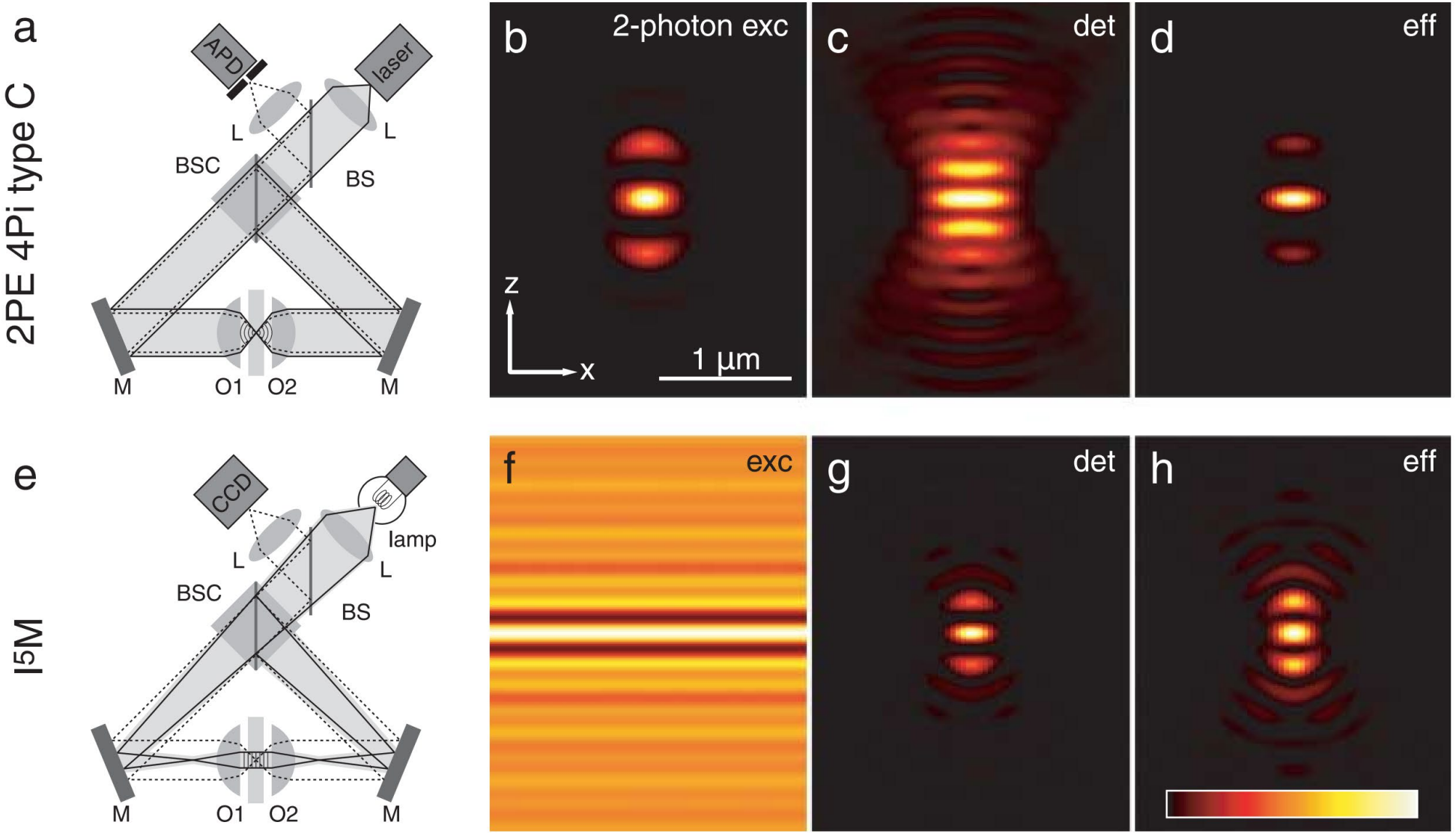
Lateral:

$$FWHM_{ill,lateral} = 0.51 \frac{\lambda_{exc}}{NA} \quad (3)$$

Theoretically 1.4x better resolution
But at expense of signal!

Borlinghaus, R., Kappel, C. *Nat Methods* (2016).

Increase in axial resolution – 4Pi/I5M



Journal of Microscopy, Vol. 222, Pt 2 May 2006, pp. 105–117

Image Scanning and Photon reassignment microscopy

Image on pinhole plane **s**, given beam on sample at **r** (4D data)

$$I(\boldsymbol{r},\boldsymbol{s}) = \int d\boldsymbol{r}' \, U(\boldsymbol{r}-\boldsymbol{r}'+\boldsymbol{s})E(\boldsymbol{r}-\boldsymbol{r}')c(\boldsymbol{r}')$$

$U_{eff}(r)$, peak is between r+s and r.

For photons detected at s, most likely emitter position is at r+s/2.
Reconstruction:

- shift signal to most probable position
- sum over s

Mueller and Enderlein, PRL 104, 198101 (2010)

Image Scanning and Photon reassignment microscopy

$$I_{IMS}(\boldsymbol{r}) = \int d\boldsymbol{s} * I(\boldsymbol{r} - \boldsymbol{s}/\boldsymbol{2}, \boldsymbol{s}) = \int d\boldsymbol{r}' \int d\boldsymbol{s}\, U(\boldsymbol{r} - \boldsymbol{r}' + \boldsymbol{s}/\boldsymbol{2}) E(\boldsymbol{r} - \boldsymbol{r}' - \boldsymbol{s}/2) c(\boldsymbol{r}')$$

Substitute: $(s = 4v - 2r + 2r')$

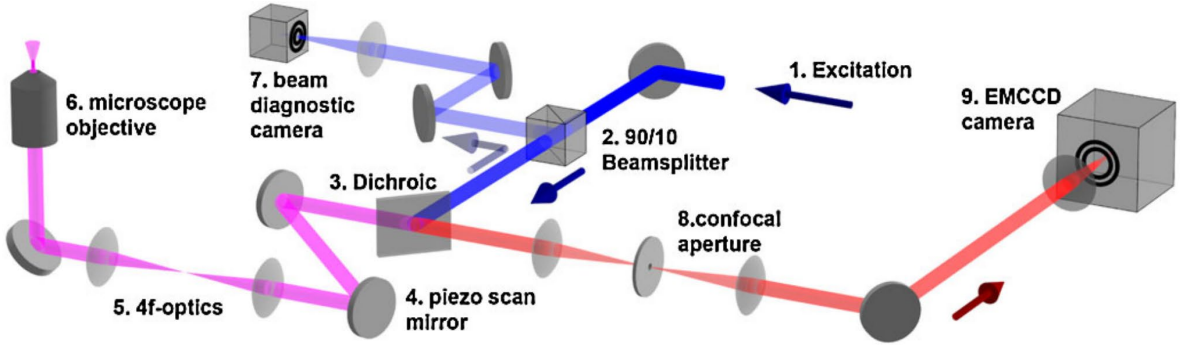
$$U_{IMS}(\boldsymbol{r}) = 4 \int d\boldsymbol{v}\, U(\boldsymbol{2v}) E(2\boldsymbol{r} - \boldsymbol{2v})$$

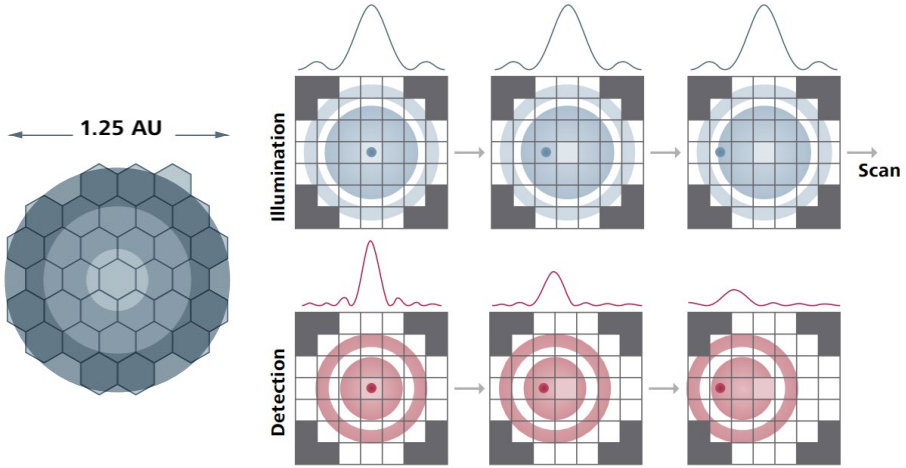
$$\widetilde{U_{IMS}}(\boldsymbol{k}) = 2\tilde{U}\left(\frac{\boldsymbol{k}}{2}\right) * \tilde{E}\left(\frac{\boldsymbol{k}}{2}\right)$$

2x resolution "attenuation factor"

Mueller and Enderlein, PRL 104, 198101 (2010)

Image Scanning Microscopy



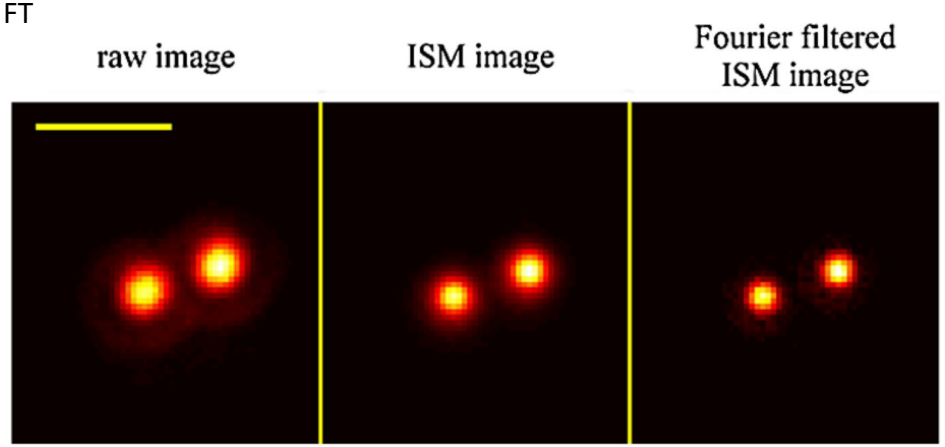


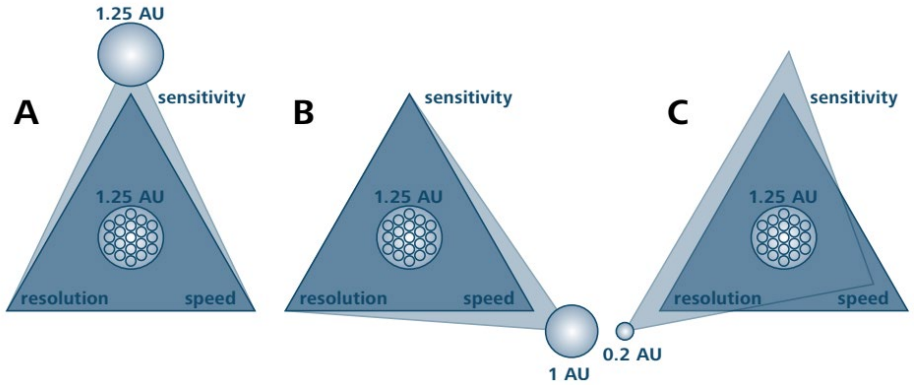
Measure: $I_{IMS}(\boldsymbol{r})$

Fourier Transform: $\widetilde{I_{IMS}}(\boldsymbol{k})$

Wiener filter: $\widetilde{I_{IMS}}(\boldsymbol{k})W(\boldsymbol{k})$, $W(\boldsymbol{k}) = \frac{\tilde{U}\left(\frac{\boldsymbol{k}}{2}\right)}{\epsilon+\tilde{U}\left(\frac{\boldsymbol{k}}{2}\right)*\tilde{E}\left(\frac{\boldsymbol{k}}{2}\right)}$ ϵ=0.01

Reverse FT

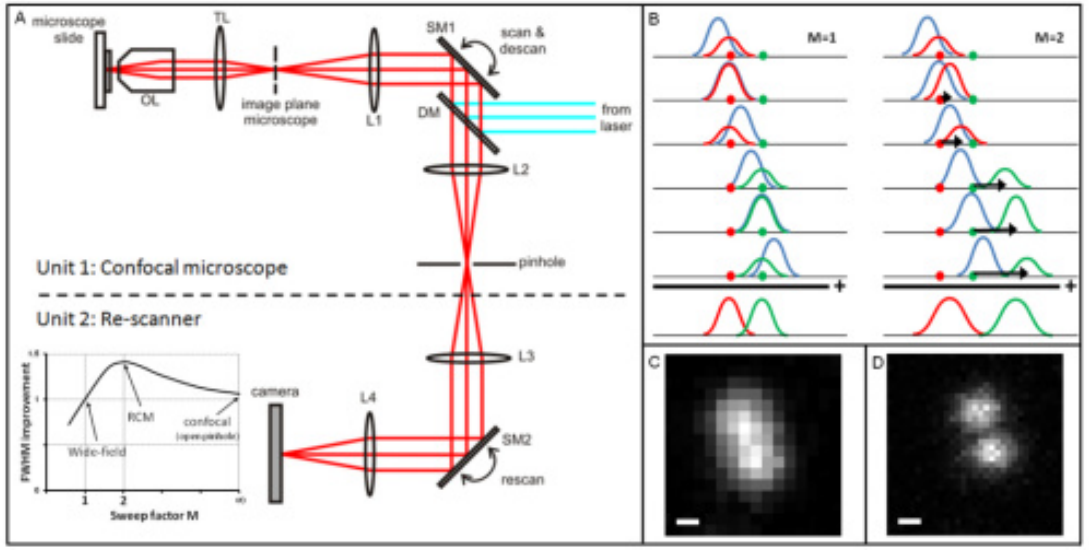


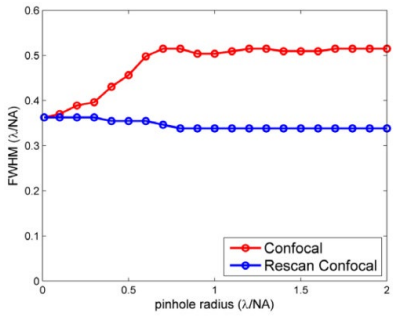


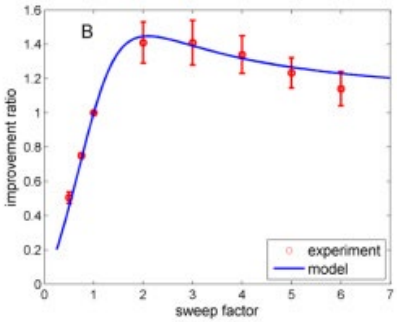
Mueller and Enderlein, PRL 104, 198101 (2010)

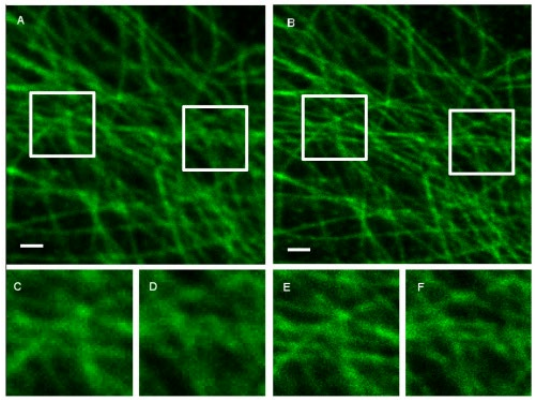
Zeiss Airyscan Technology Notes

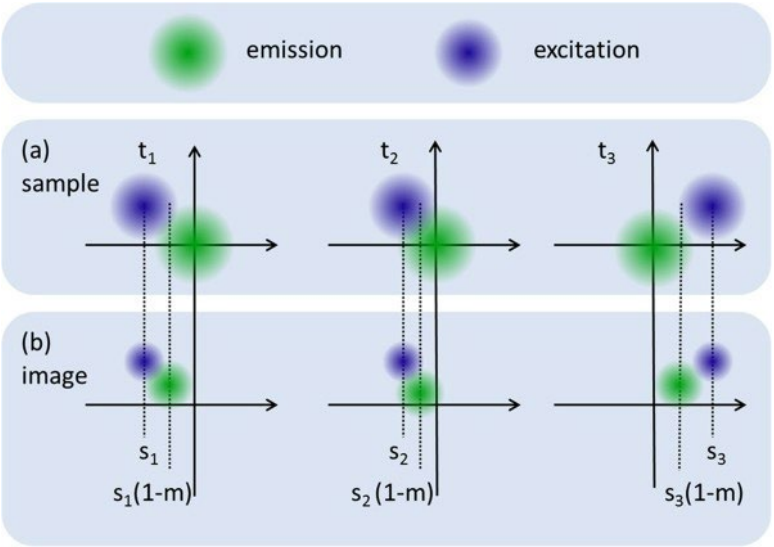
Optical Photon Reassignment Microscopy

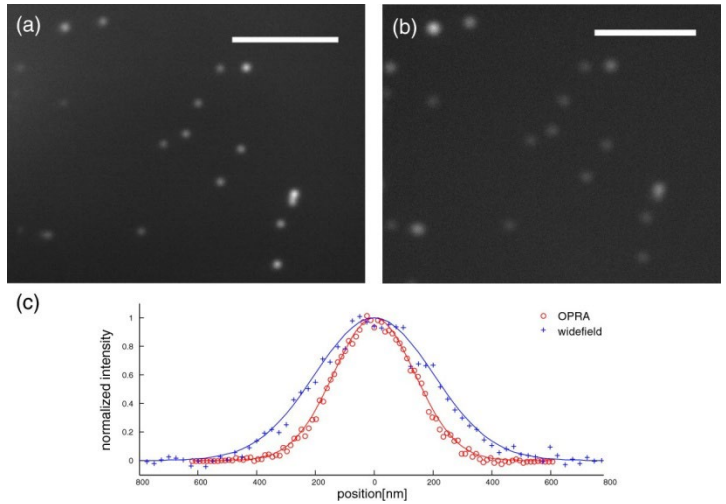








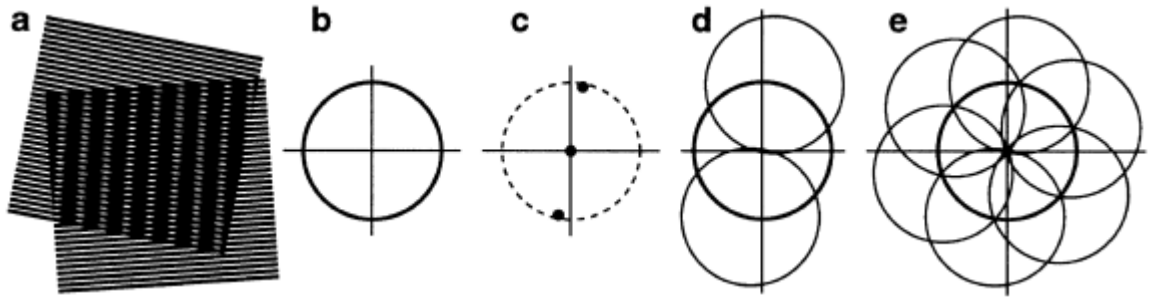


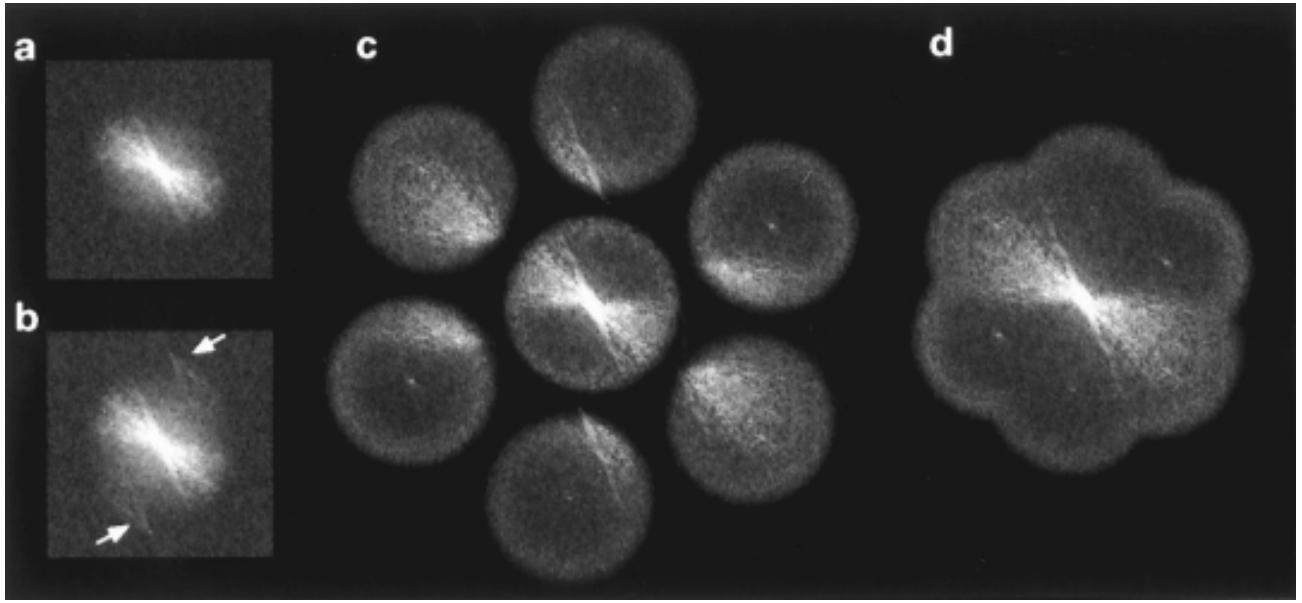


Biomed. Opt. Express 4, 2644-2656 (2013)

Roth, S., Sheppard, C.J., Wicker, K. *et al. Opt Nano* (2013).

Structured Illumination Microscopy





Images with additional information

Individual Information components

Final reconstruction

Illumination

$$I_{\theta,\varphi}(r) = \frac{I_0}{2}[1 + \cos(2\pi k_\theta \cdot r + \varphi)]$$

Image

$$D(r) = (s(r) \cdot I_{\theta,\varphi}(r)) \otimes h(r)$$

$$D_{\theta,\varphi}(k) = [\mathrm{S}(k) \otimes I_{\theta,\varphi}(k)] \cdot H(k)$$

$$= \tfrac{I_0}{2}[S(k) \cdot H(k) + \tfrac{1}{2}e^{-i\varphi} S(k - k_\theta) \cdot H(k) + \tfrac{1}{2}e^{i\varphi} S(k + k_\theta) \cdot H(k)]$$

Information previously inaccessible

Acquire 3 images (ϕ=0, 2π/3, 4π/3), solve linear system

$$D_{\theta,\varphi_1}(k) = \tfrac{I_0}{2}[S(k) \cdot H(k) + \tfrac{1}{2}e^{-i\varphi_1} S(k - k_\theta) \cdot H(k) + \tfrac{1}{2}e^{i\varphi_1} S(k + k_\theta) \cdot H(k)]$$

$$D_{\theta,\varphi_2}(k) = \tfrac{I_0}{2}[S(k) \cdot H(k) + \tfrac{1}{2}e^{-i\varphi_2} S(k - k_\theta) \cdot H(k) + \tfrac{1}{2}e^{i\varphi_2} S(k + k_\theta) \cdot H(k)]$$

$$D_{\theta,\varphi_3}(k) = \tfrac{I_0}{2}[S(k) \cdot H(k) + \tfrac{1}{2}e^{-i\varphi_3} S(k - k_\theta) \cdot H(k) + \tfrac{1}{2}e^{i\varphi_3} S(k + k_\theta) \cdot H(k)]$$

$$\begin{bmatrix} S(k) \cdot H(k) \\ S(k - k_\theta) \cdot H(k) \\ S(k + k_\theta) \cdot H(k) \end{bmatrix} = \frac{2}{I_0} M^{-1} \begin{bmatrix} D_{\theta,\varphi_1}(k) \\ D_{\theta,\varphi_2}(k) \\ D_{\theta,\varphi_3}(k) \end{bmatrix} \quad M = \begin{bmatrix} 1 & \frac{1}{2}e^{-i\varphi_1} & \frac{1}{2}e^{i\varphi_1} \\ 1 & \frac{1}{2}e^{-i\varphi_2} & \frac{1}{2}e^{i\varphi_2} \\ 1 & \frac{1}{2}e^{-i\varphi_3} & \frac{1}{2}e^{i\varphi_3} \end{bmatrix}$$

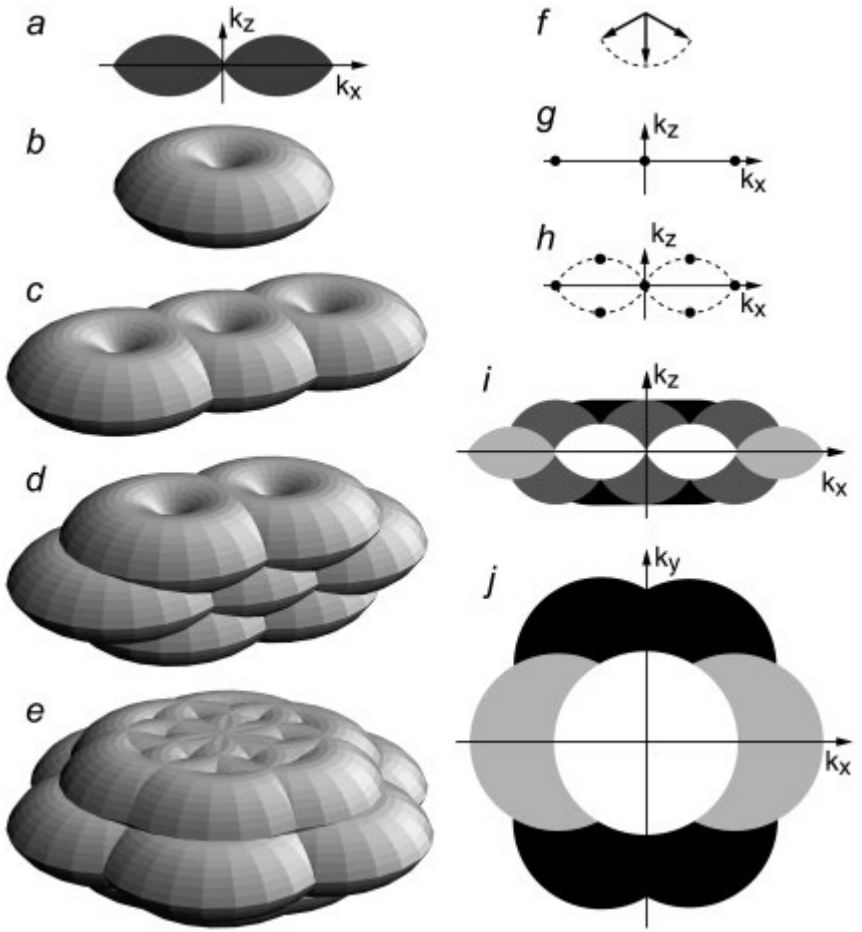
Reconstruct by shifting components in Fourier Space

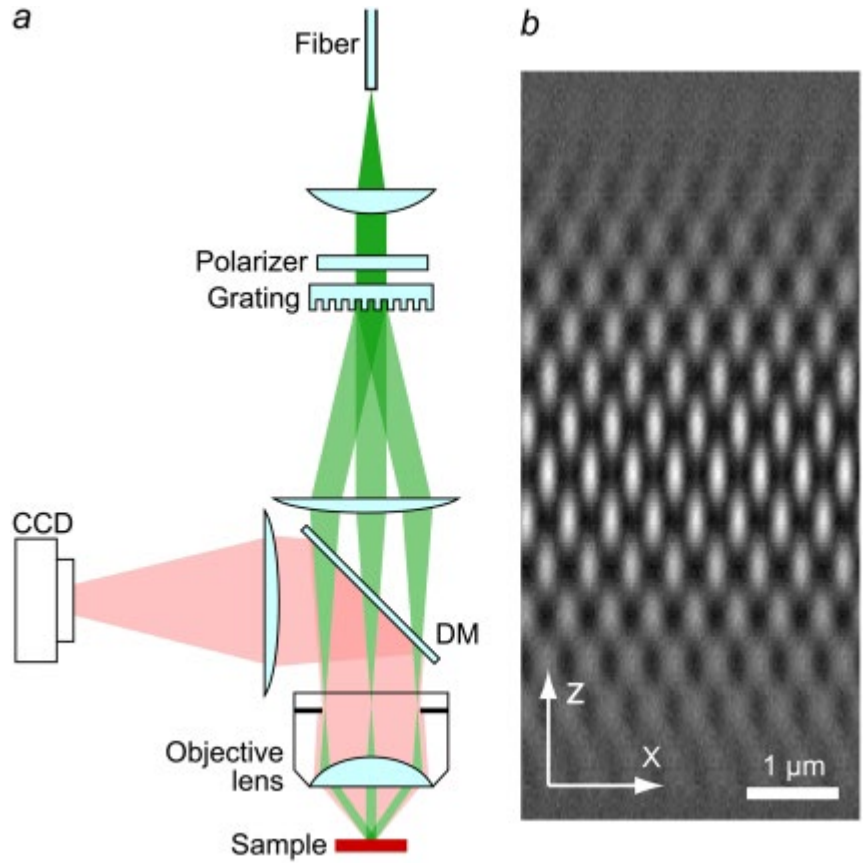
$$\widehat{S}(k) = \frac{\sum_{\theta,m} H_m^*(k + mk_\theta)\widehat{D}_{\theta,m}(k + mk_\theta)}{\sum_{\theta',m'} |H_{m'}(k + m'k_{\theta'})|^2 + w^2} A(k)$$

$$\widehat{D}_{\theta,m}(k + mk_\theta) = S(k + mk_\theta) \cdot H(k), \quad \text{m=-1,0,1}$$

Gustafsson, J Microscopy, 2000

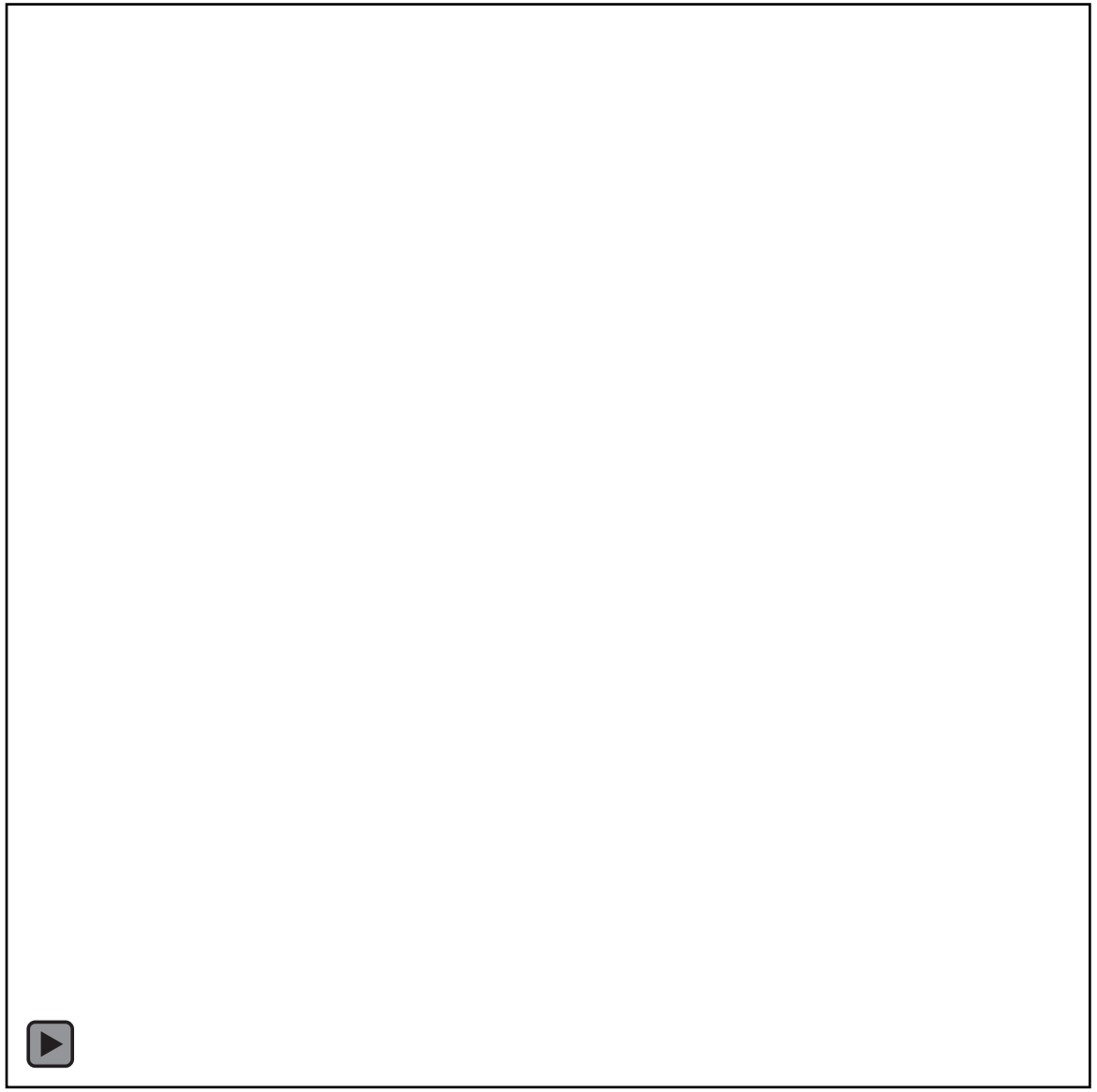
3D Structured Illumination Microscopy (SIM)





Gustafsson et al, Biophys J, 2008

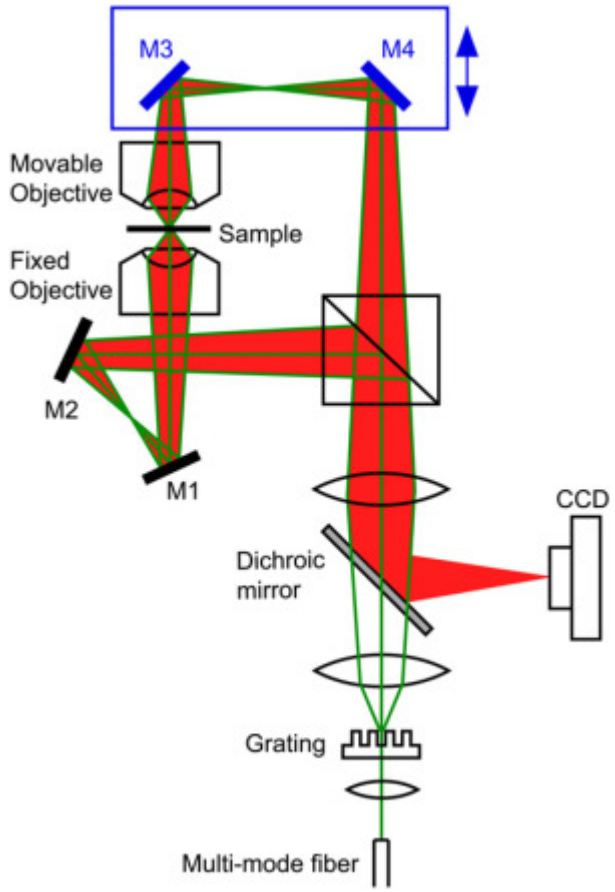
Structured Illumination Microscopy

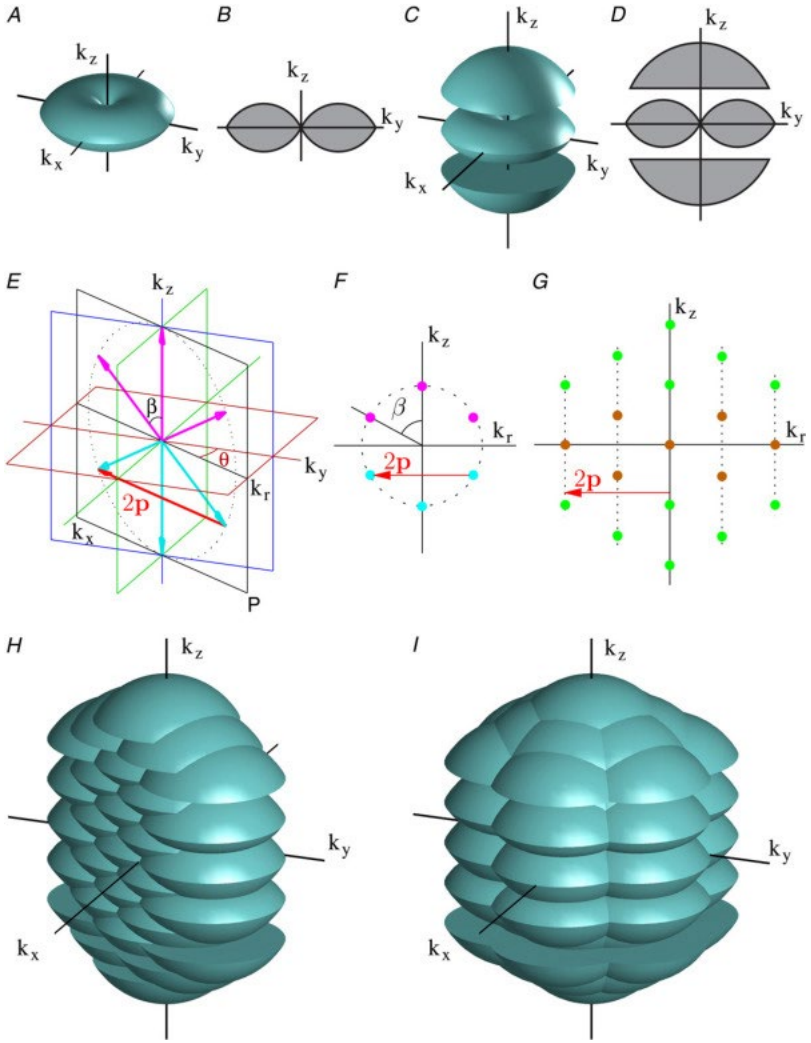


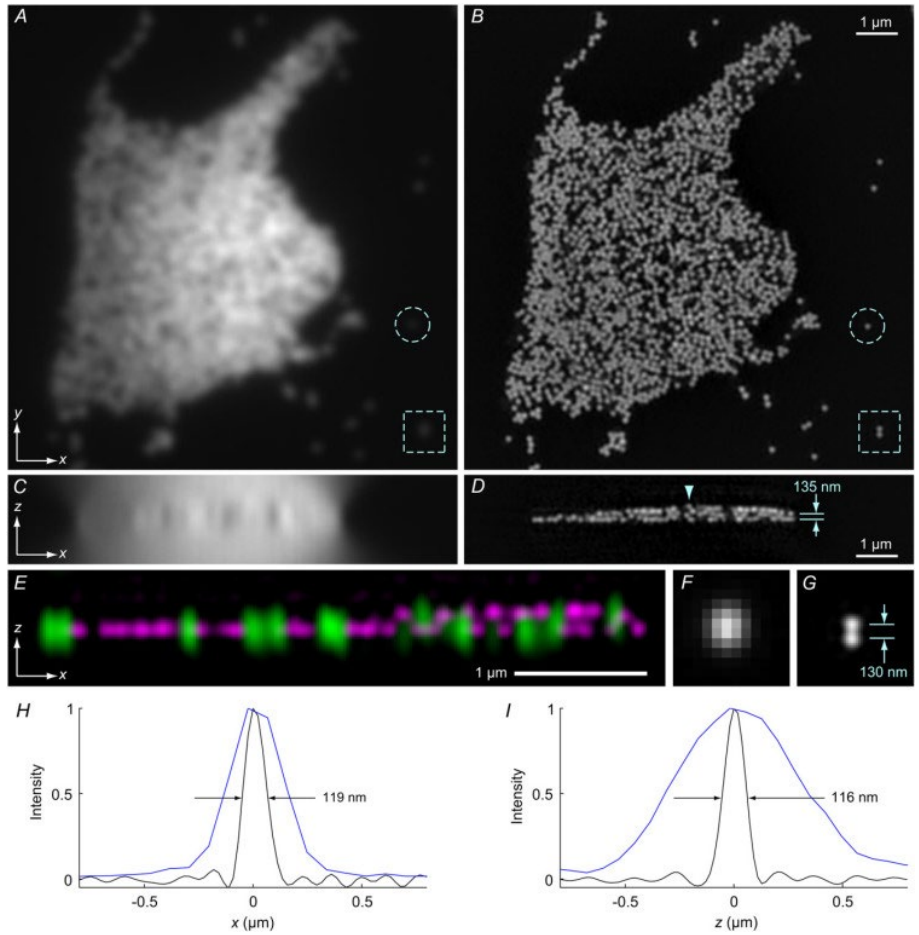
Caveolae dynamics in a COS-7 cell

Li et al, Science, 2015

I5S (SIM + 4Pi)

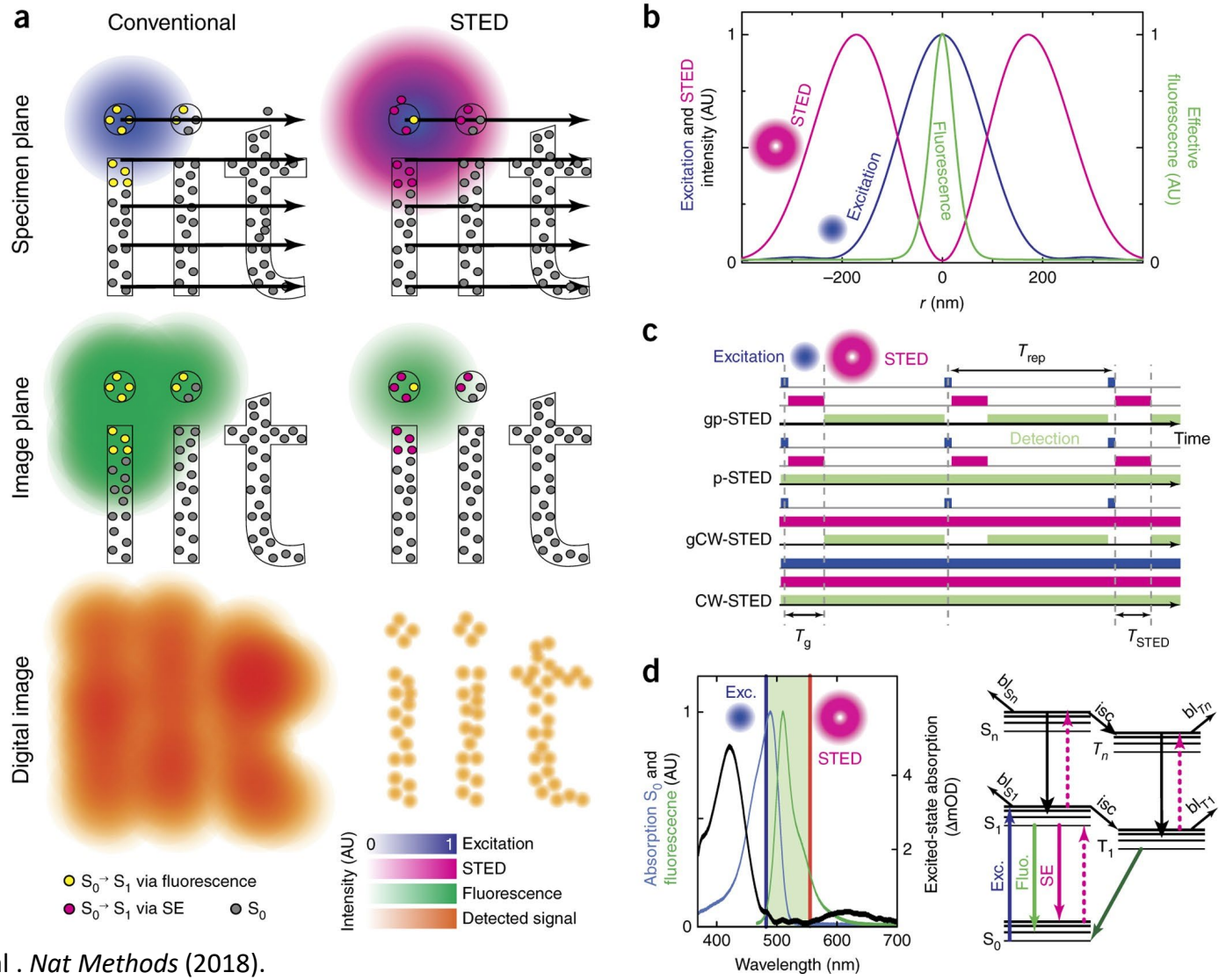






Shao et al, Biophys J, 2008

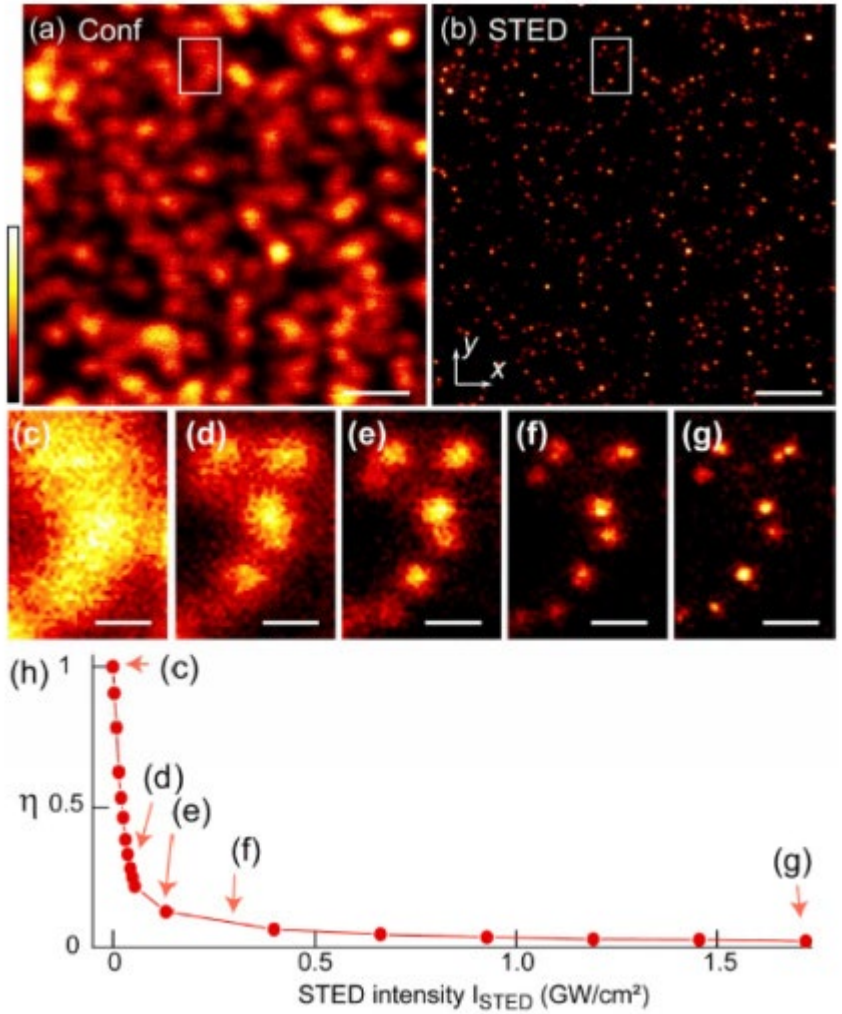
Stimulated emission depletion (STED) microscopy



Vicidomini, G., et al . *Nat Methods* (2018).

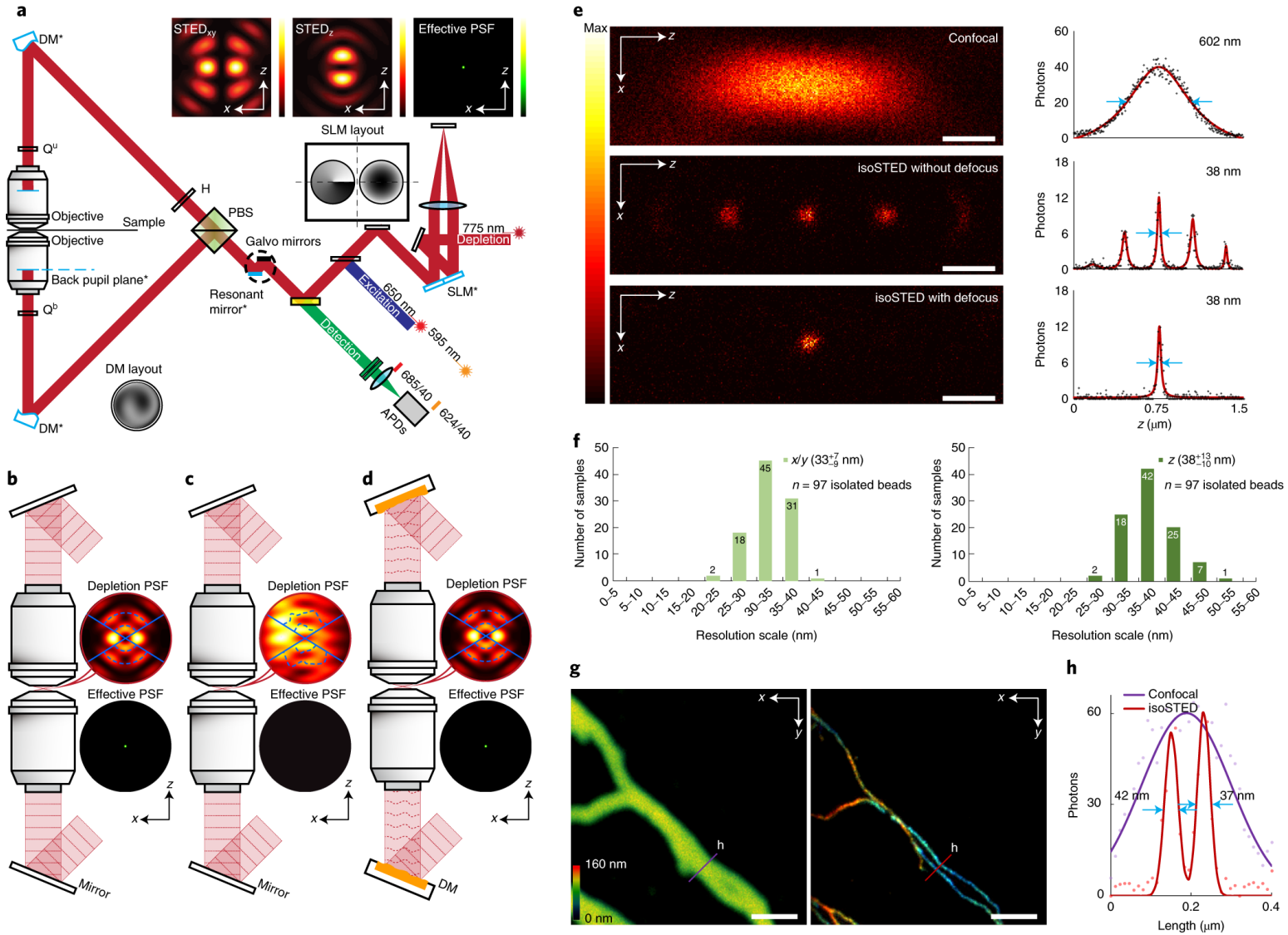
Resolution scaling in STED microscopy

$$d \approx \frac{\lambda}{2NA\sqrt{1+\zeta}}, \zeta = I_{STED}/I_{STED}^{Sat}$$



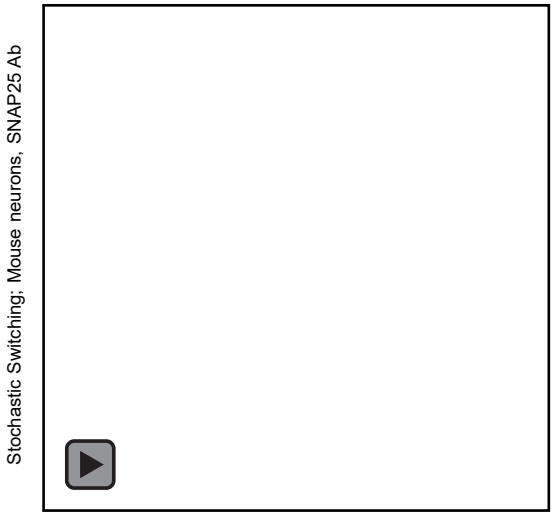
Harke et al, Opt Express (2008)

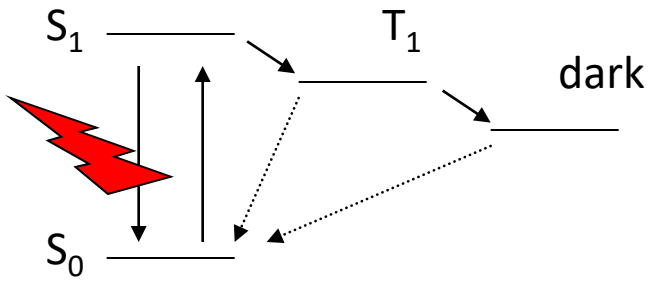
Combination of STED with 4Pi interferometry

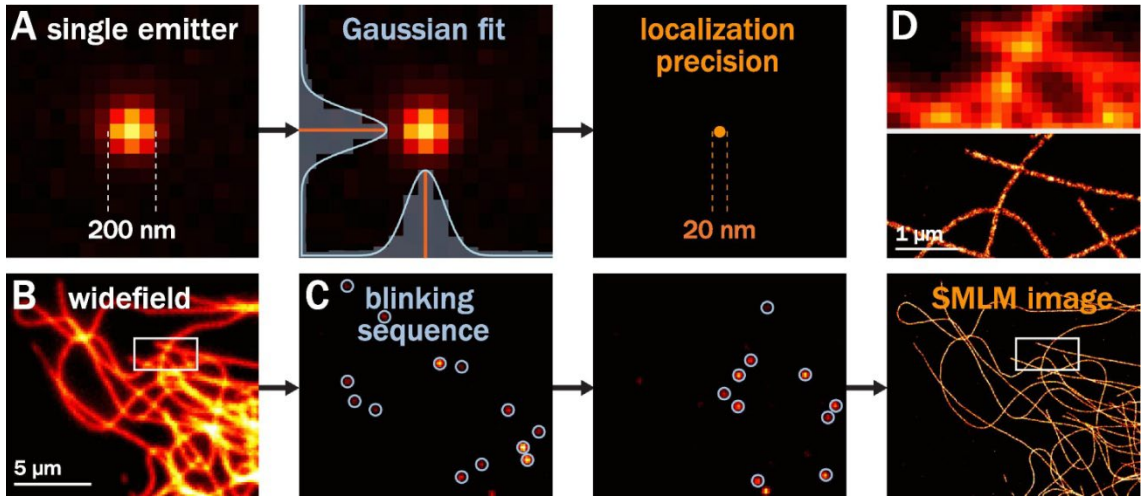


Hao, X., et al . *Nat Methods* (2021).

Single-molecule localization microscopy (SMLM)

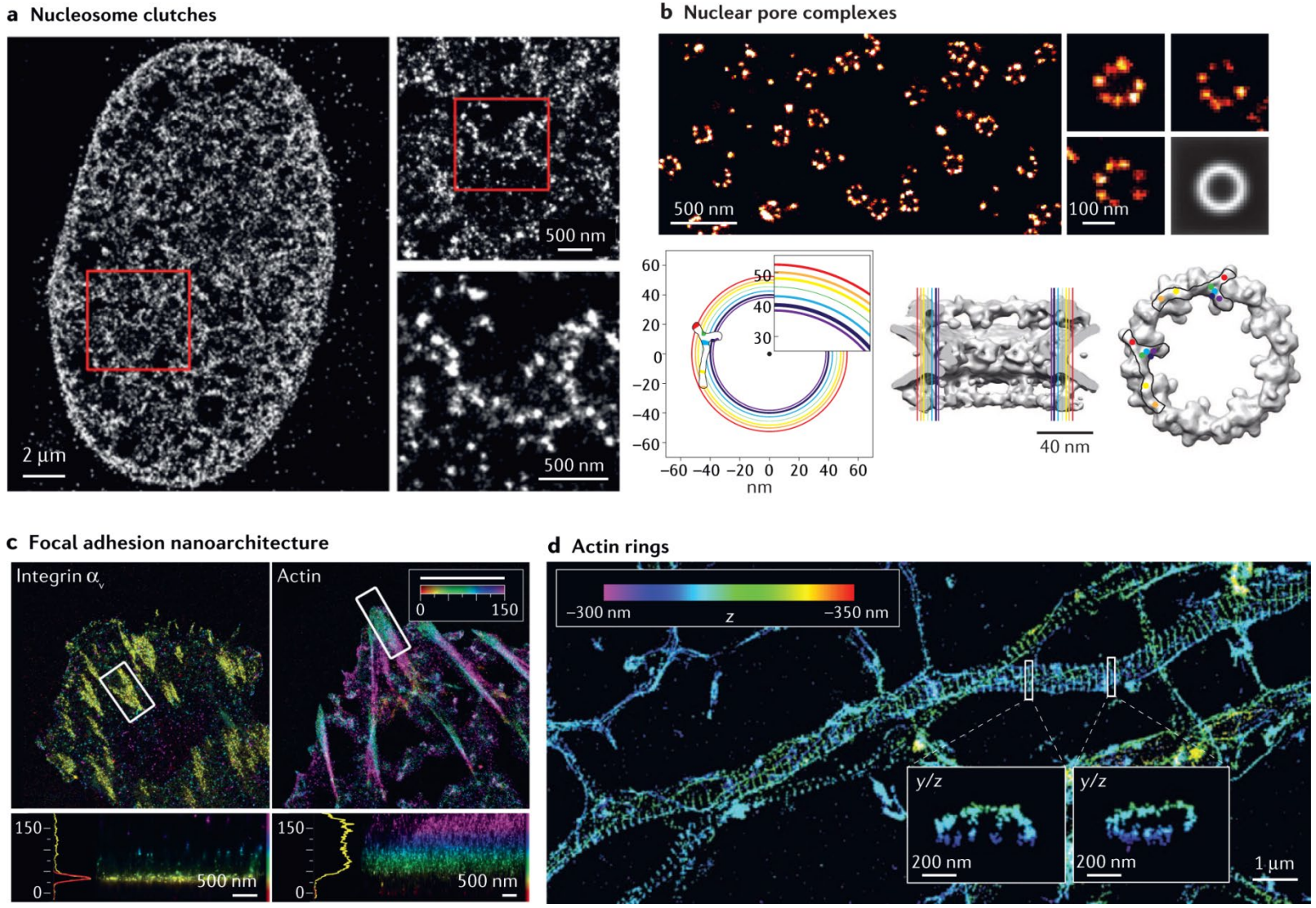






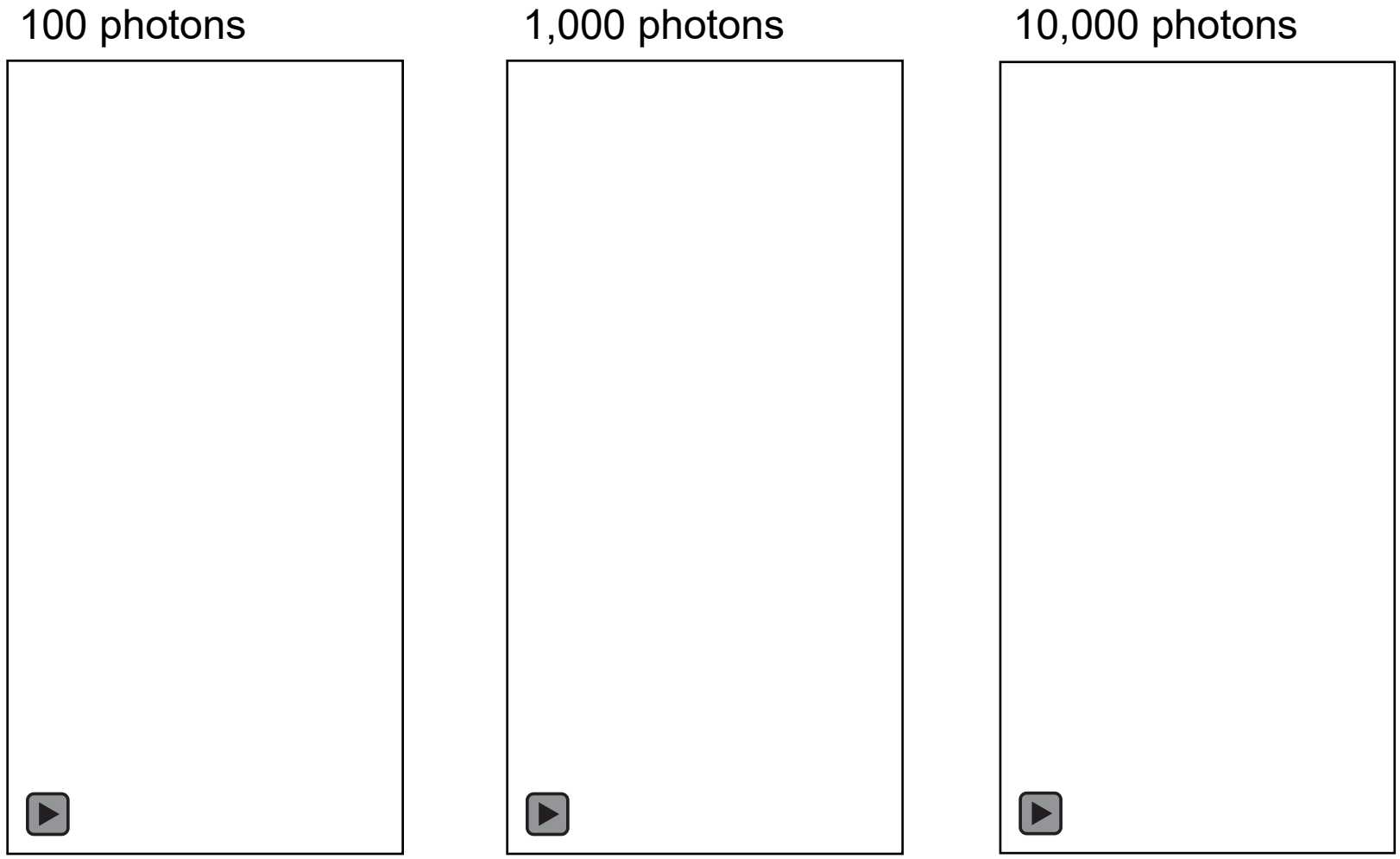
Jimenez et a. *Methods* (2020).

Discoveries by SMLM



Lelek et al. *Nat Rev Methods Primers* (2021).

Image-based Single-molecule Localization

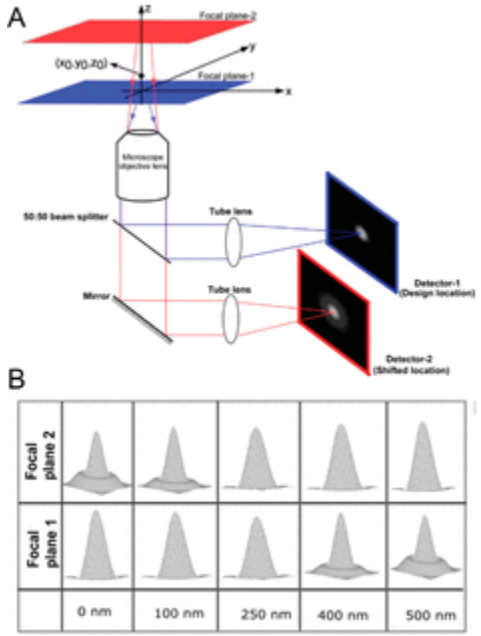


Fundamental limit: **signal-to-noise** (“uncertainty principle”)

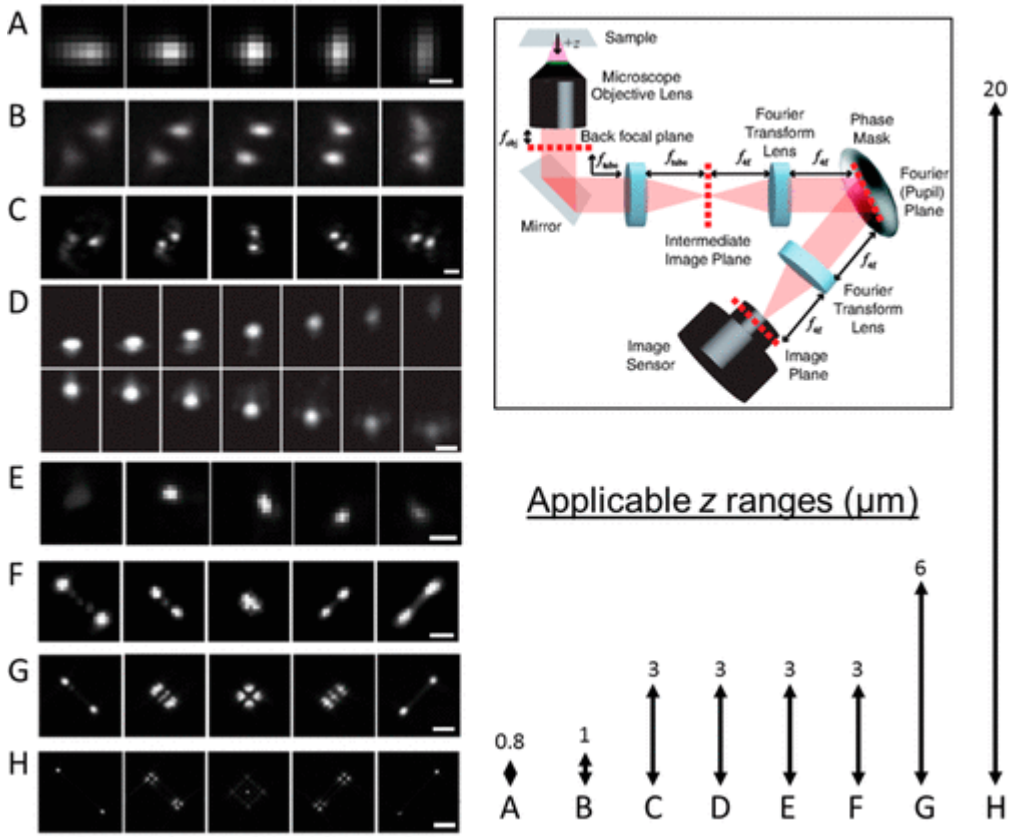
Theory: Thompson et al (2002); Molecular motor tracking: Yildiz et al (2003).

3D Localization approaches

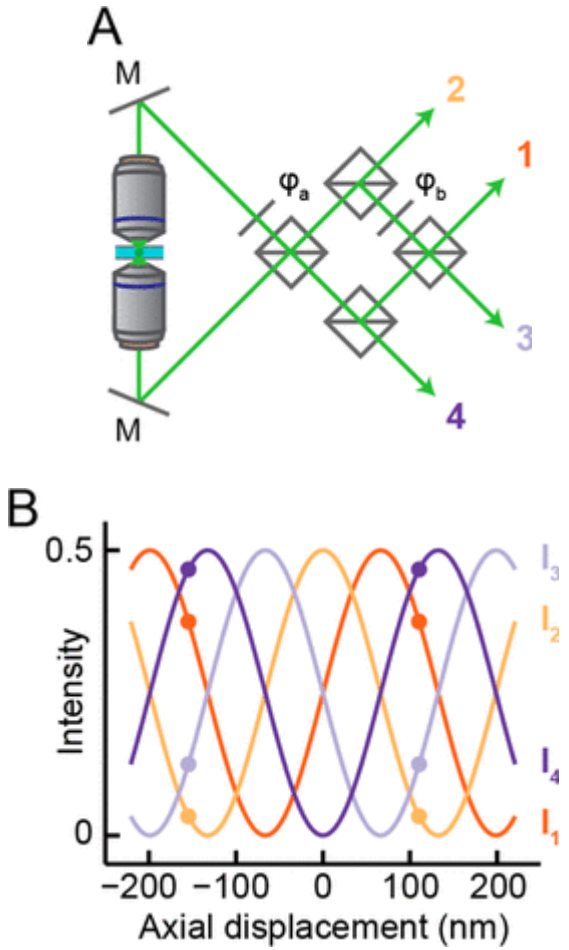
Bi-Plane



PSF Engineering

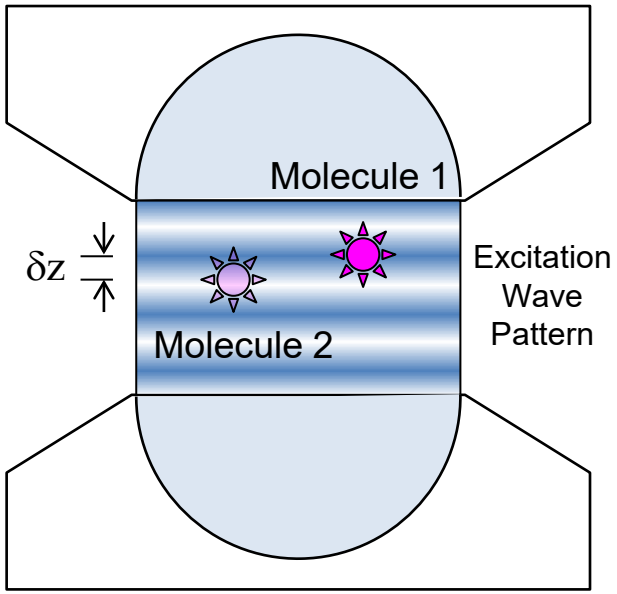


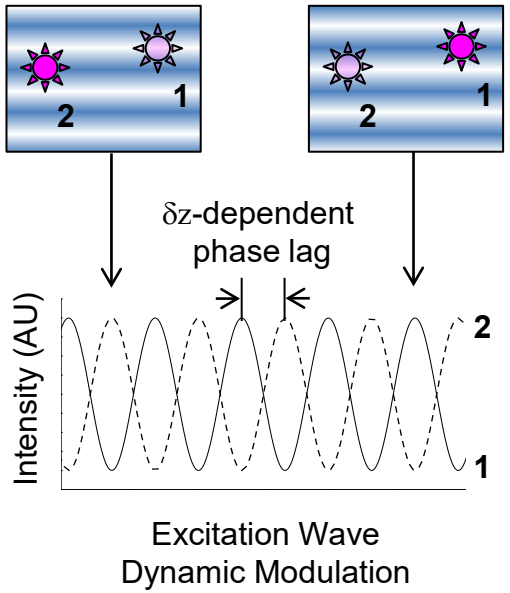
Interferometry

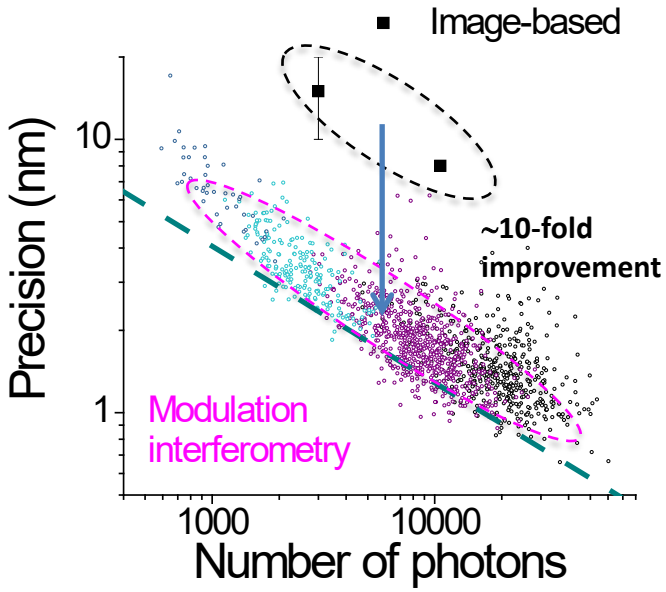


Chem. Rev. 2017, 117, 11, 7244−7275

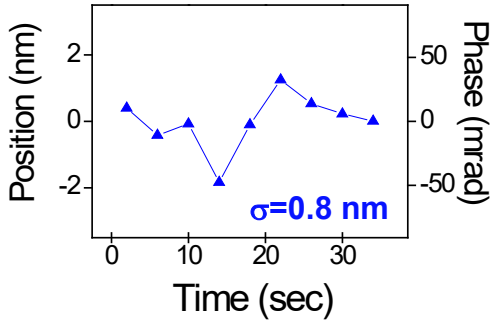
Advanced Techniques: Modulation-enhanced Localization





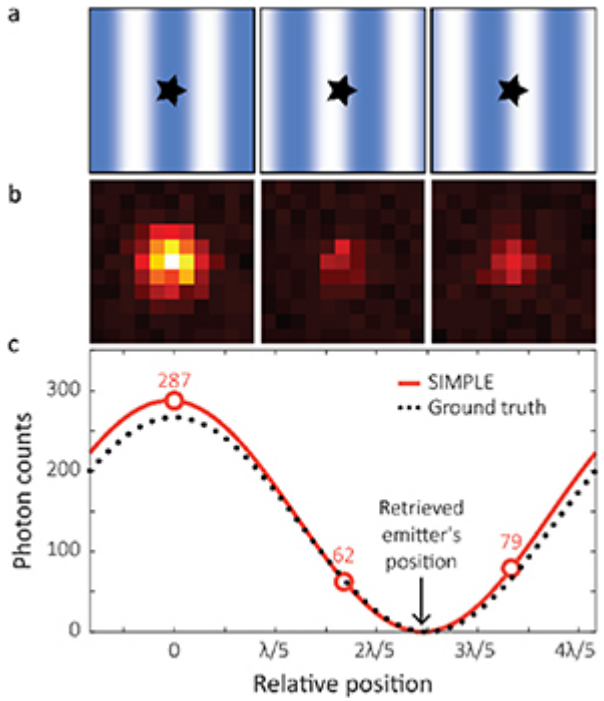


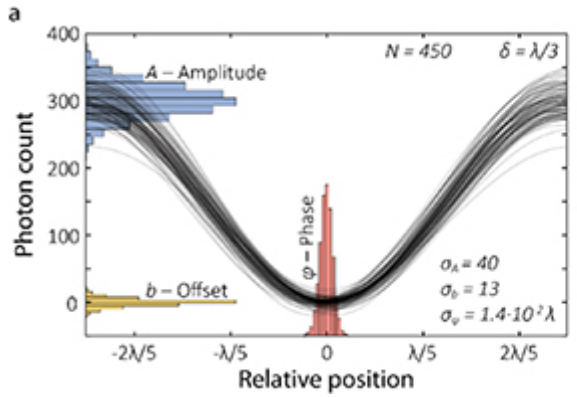
~1 nm 3D localization precision (1σ)

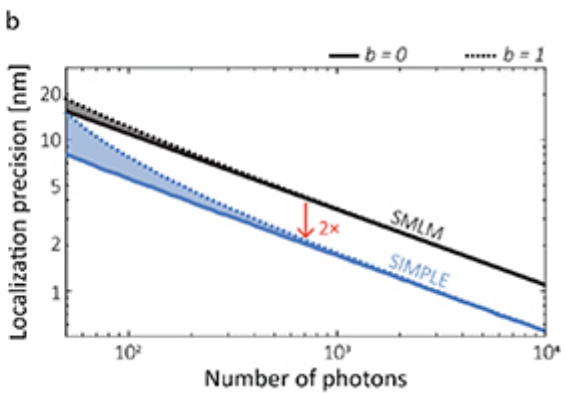


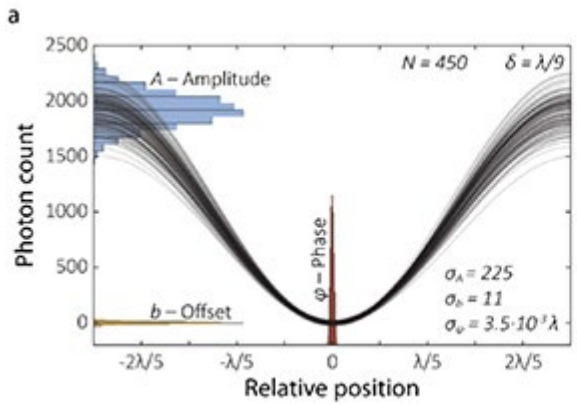
Wang *et al.*, **Cell** (2016)

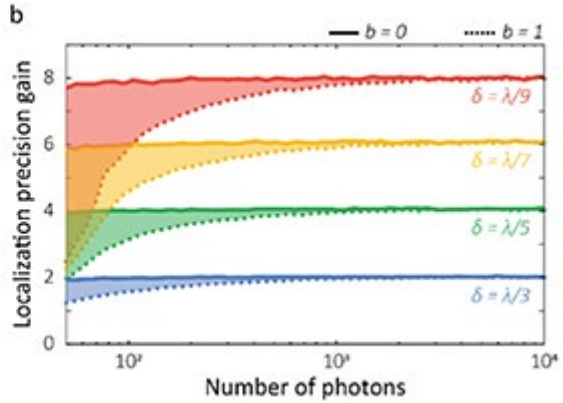
Modulation enhanced localization







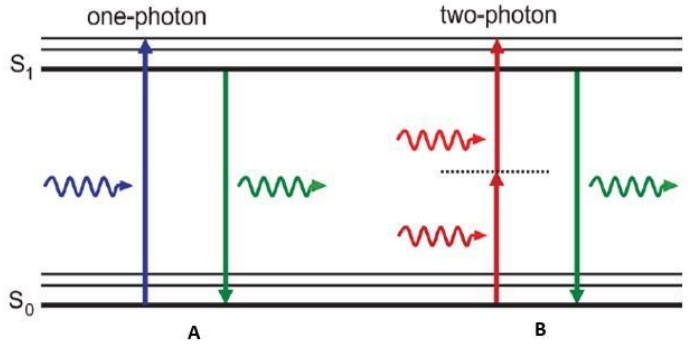


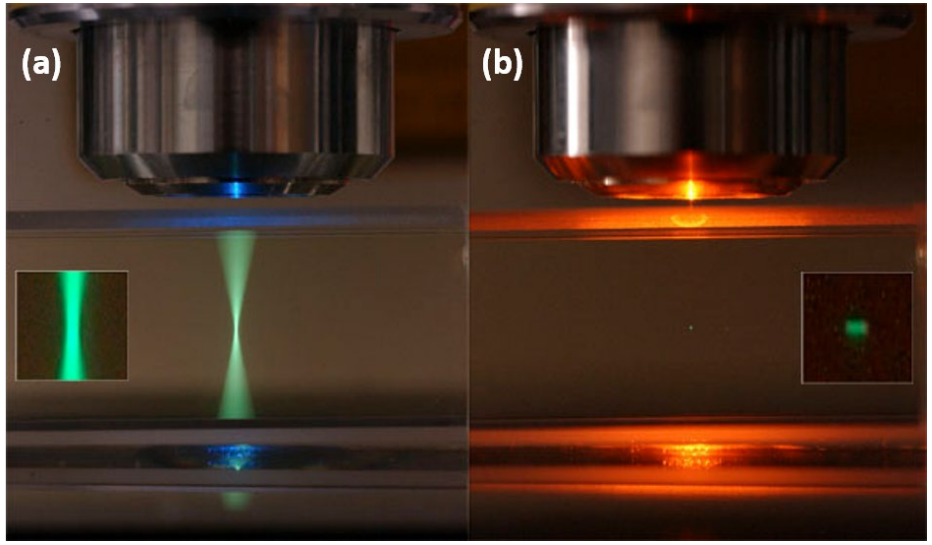


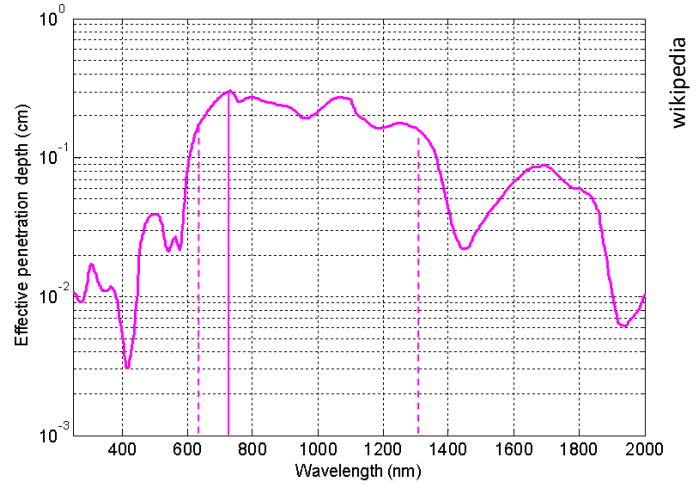
Loïc Reymond *et al* 2020 *J. Phys. Photonics* **2** 041001

IMAGING LIVE CELL AND MULTI-CELLULAR SYSTEMS

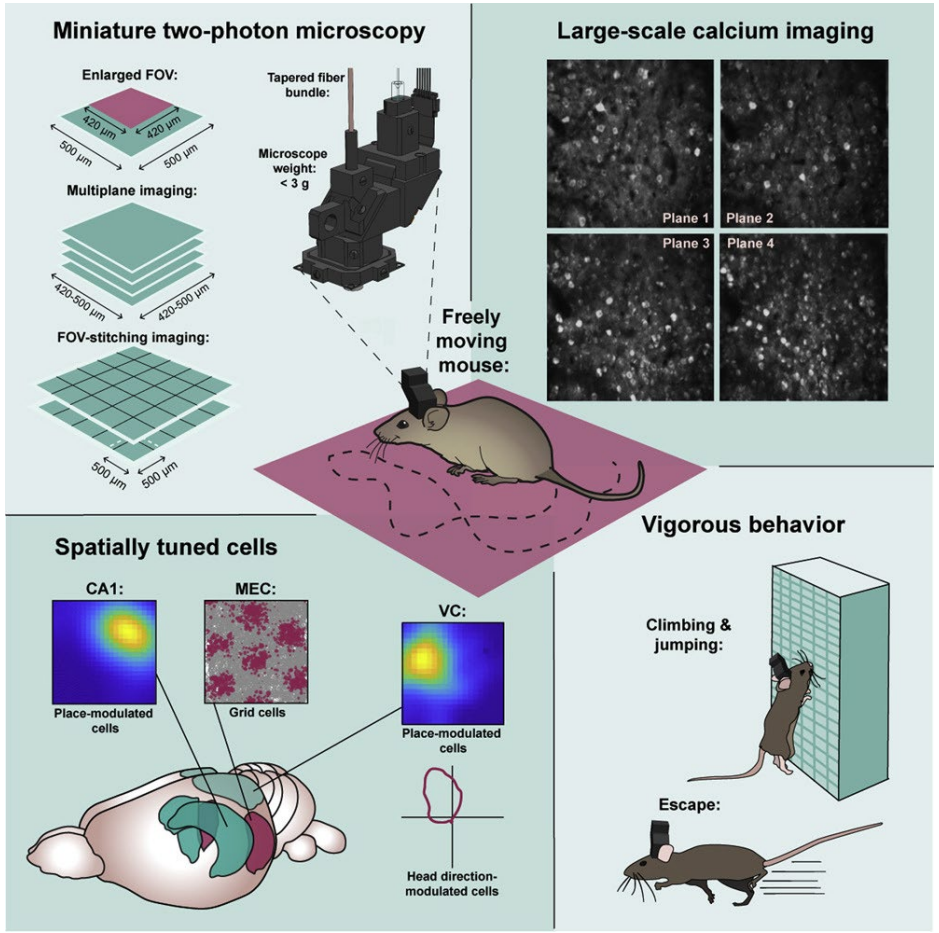
Multi-photon Microscopy

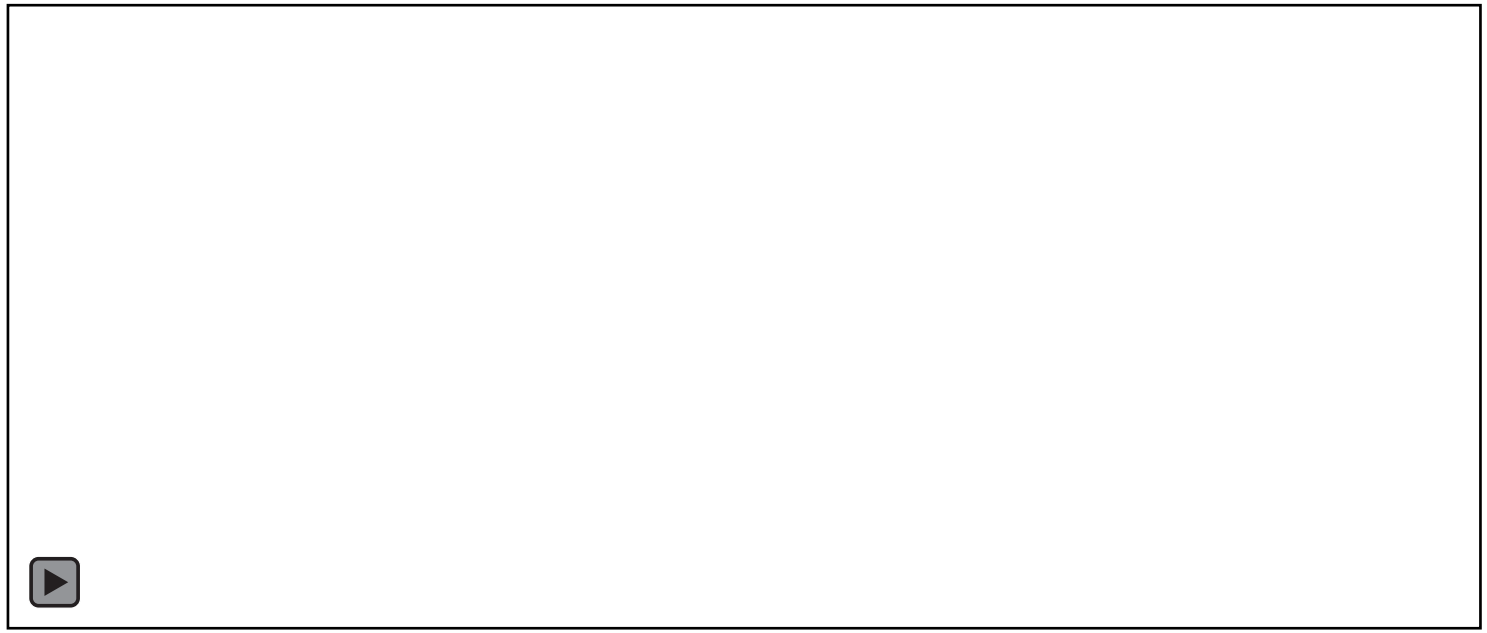






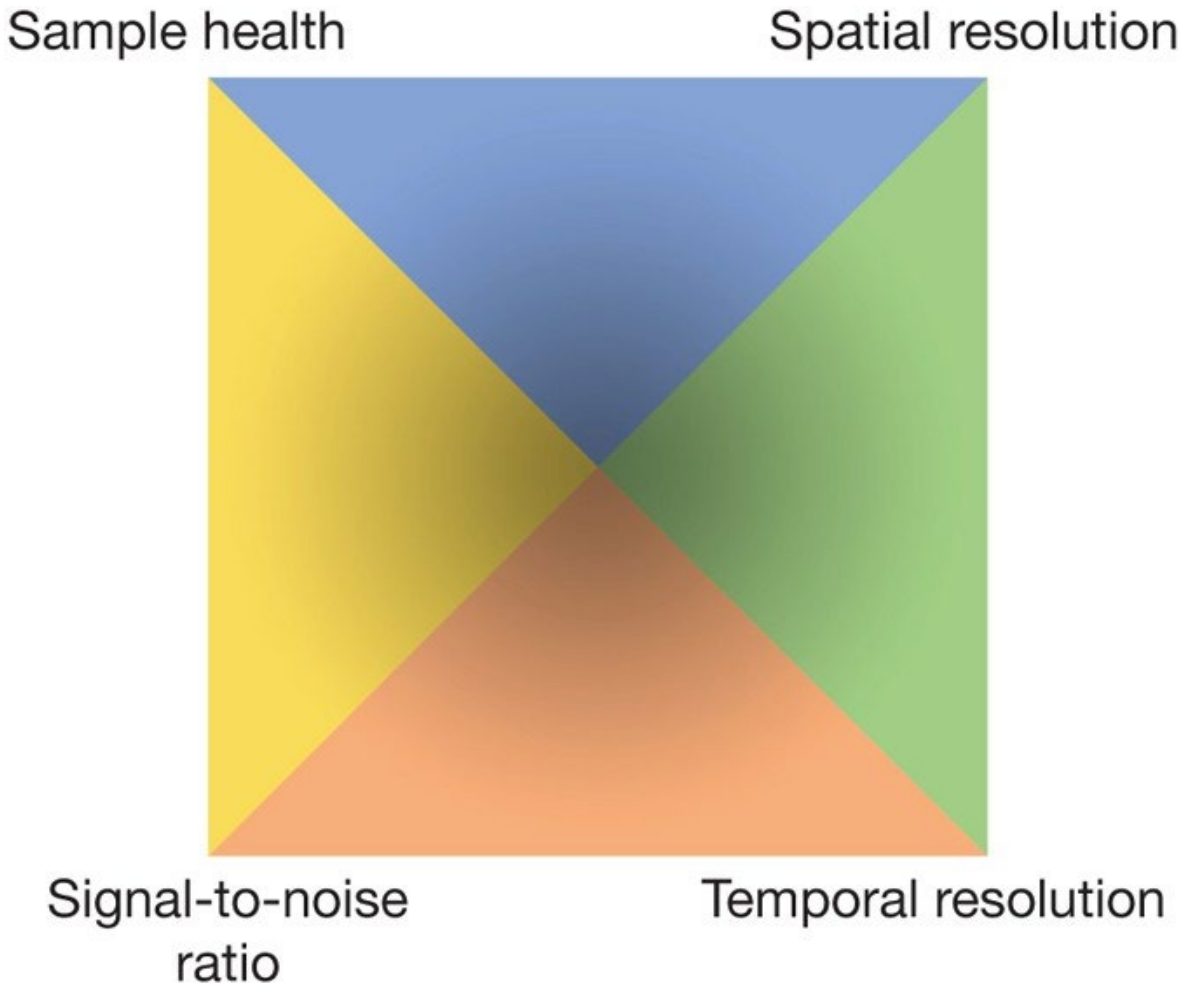
Multi-photon Microscopy





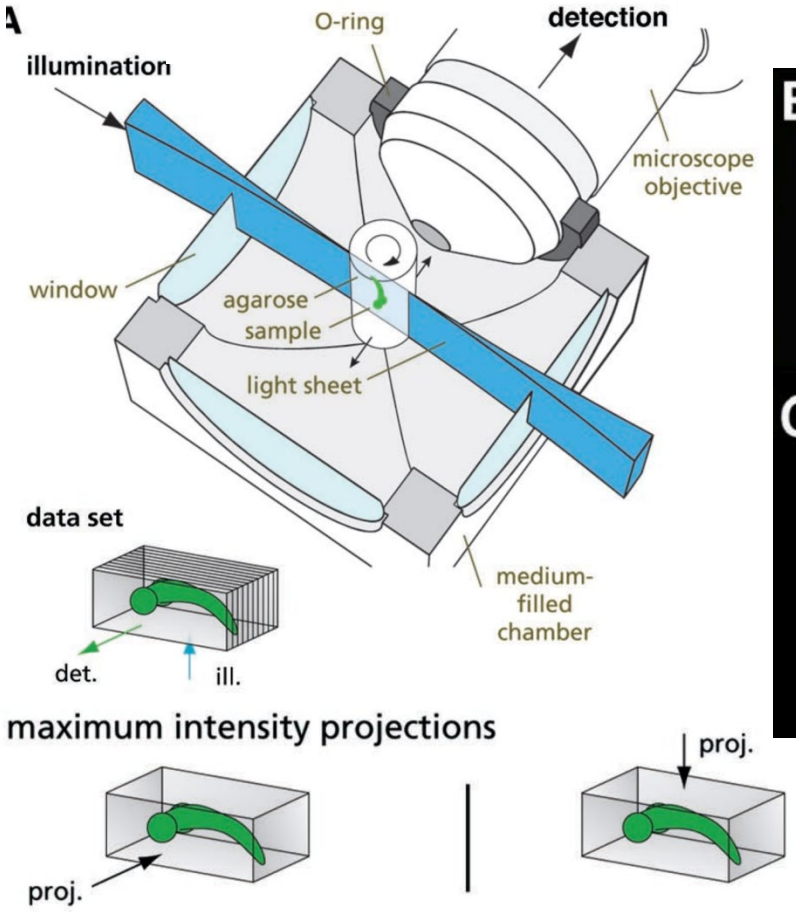
Zong et al, Cell (2022)

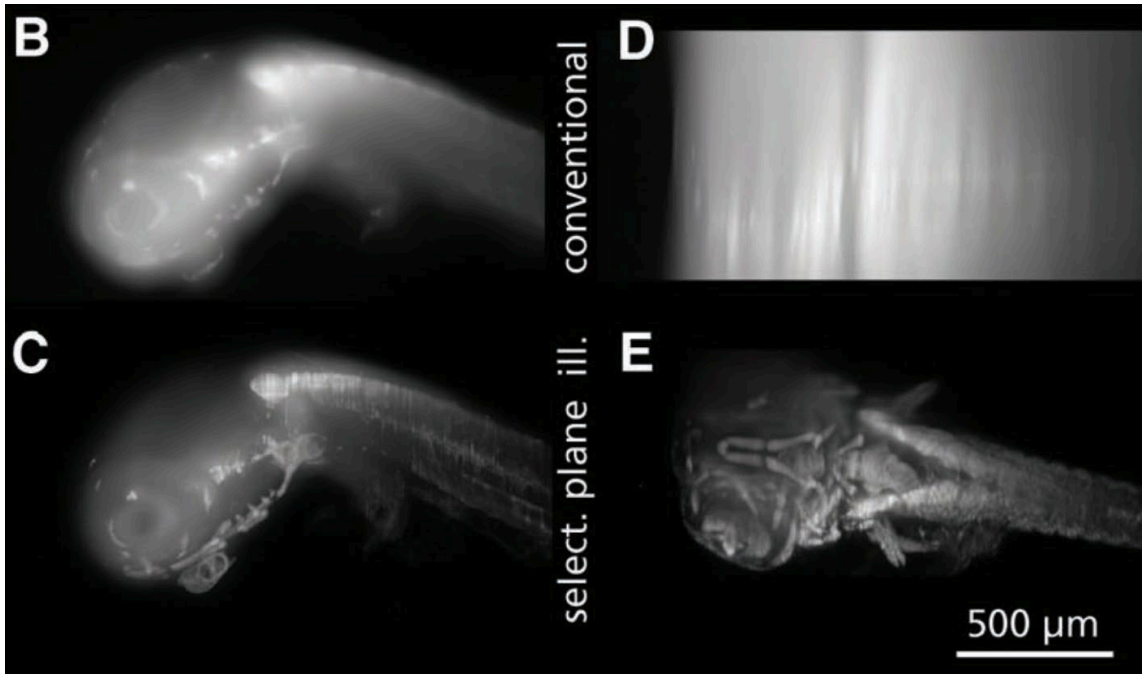
Trade-offs in imaging live biology



Laissue et al. *Nat Meth* (2017).

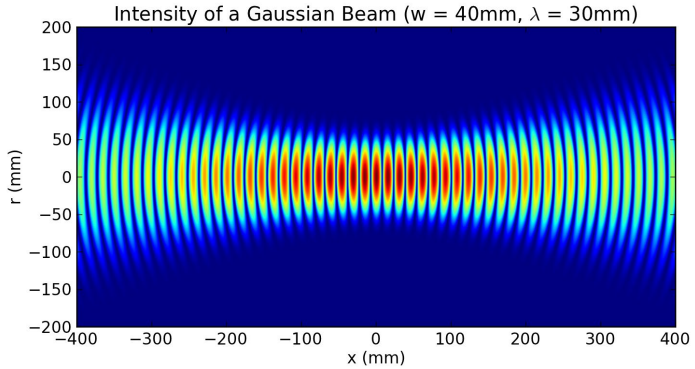
Selective-plane Illumination Microscopy (SPIM)

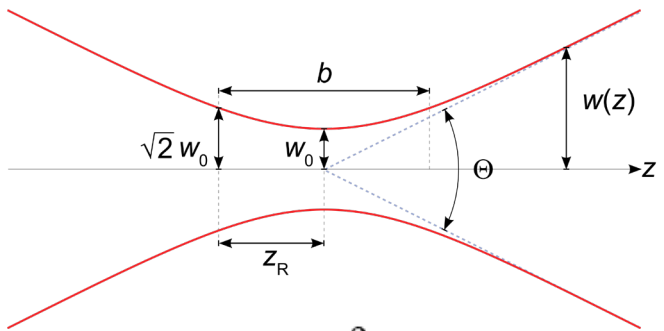




Huisken et. al. Science (2004)

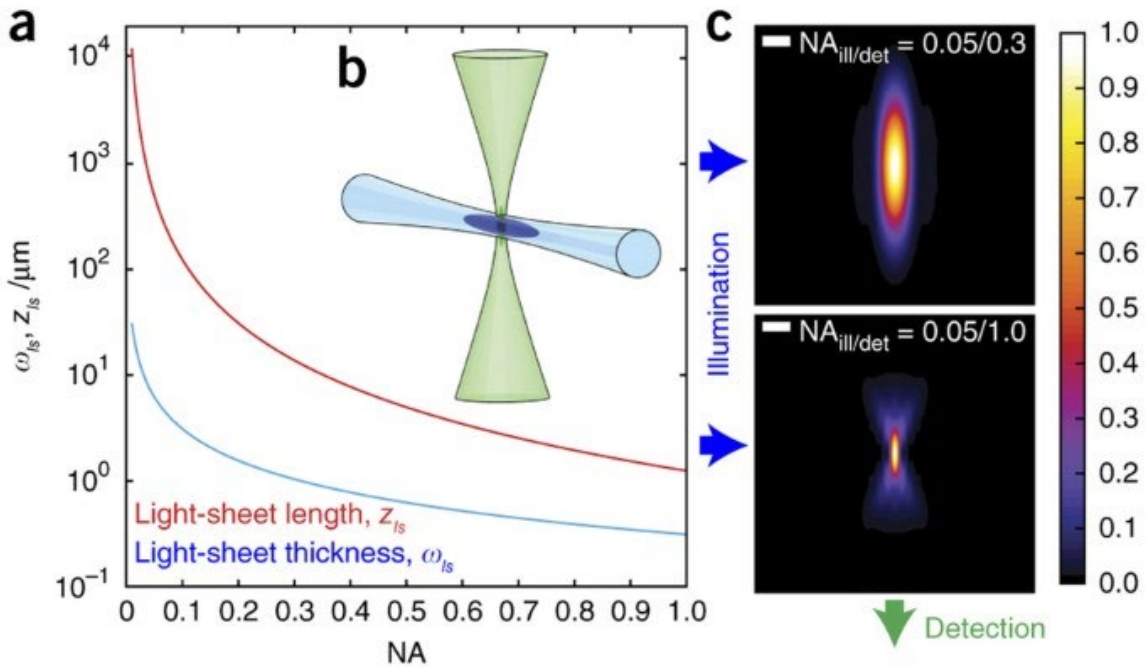
Gaussian Beam propagation





$$z_{\mathrm{R}} = \frac{\pi w_0^2 n}{\lambda}$$

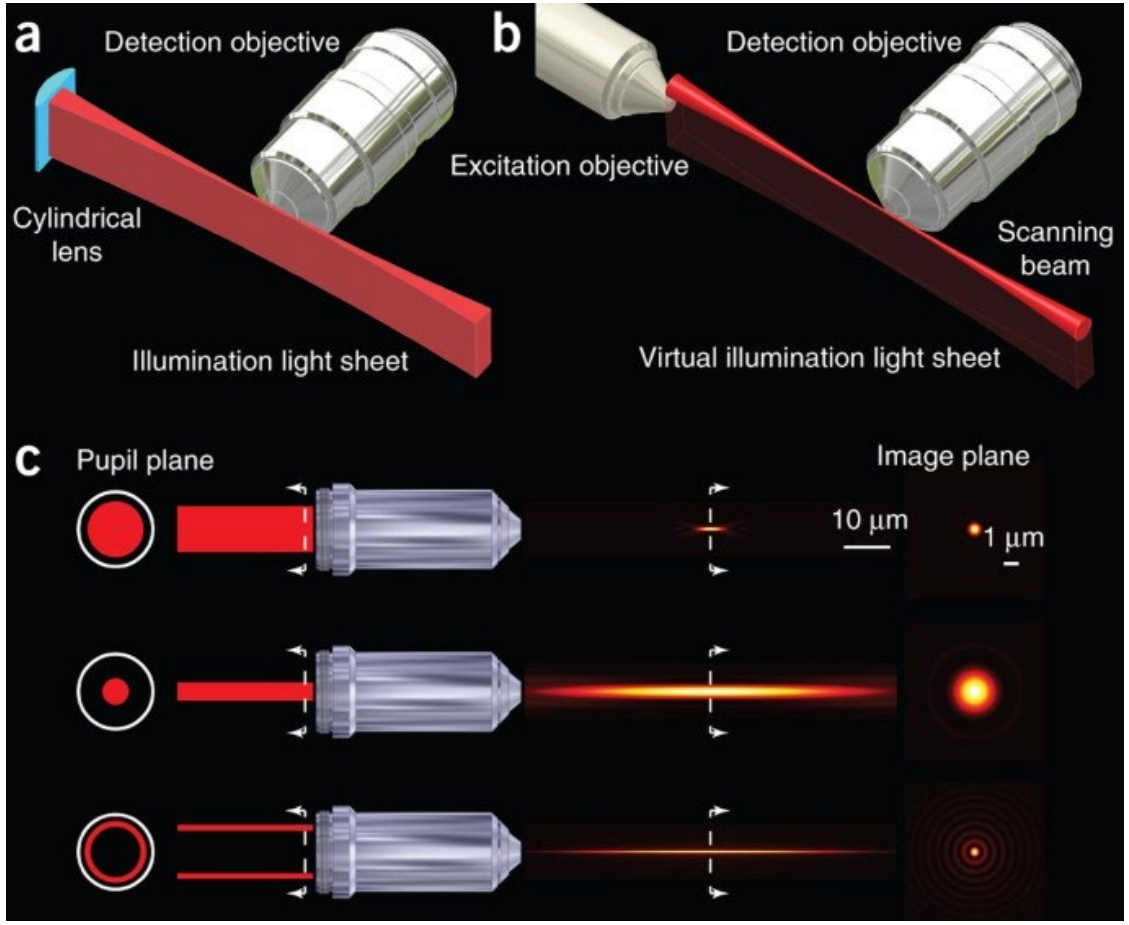
Trade-off between spatial resolution and FOV

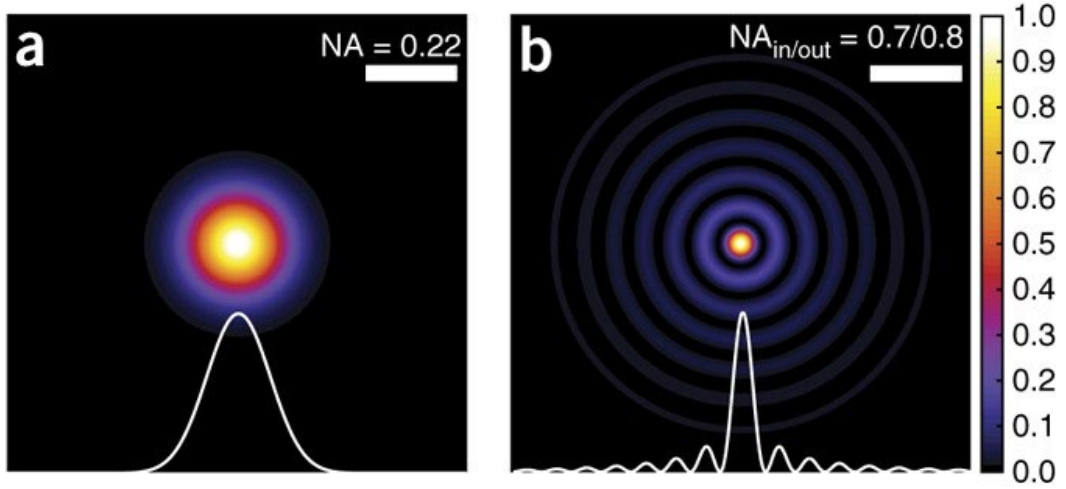


Source: wikipedia

Nature Methods **volume 14**, pages360–373 (2017)

SPIM with “Non-diffracting” Bessel beams

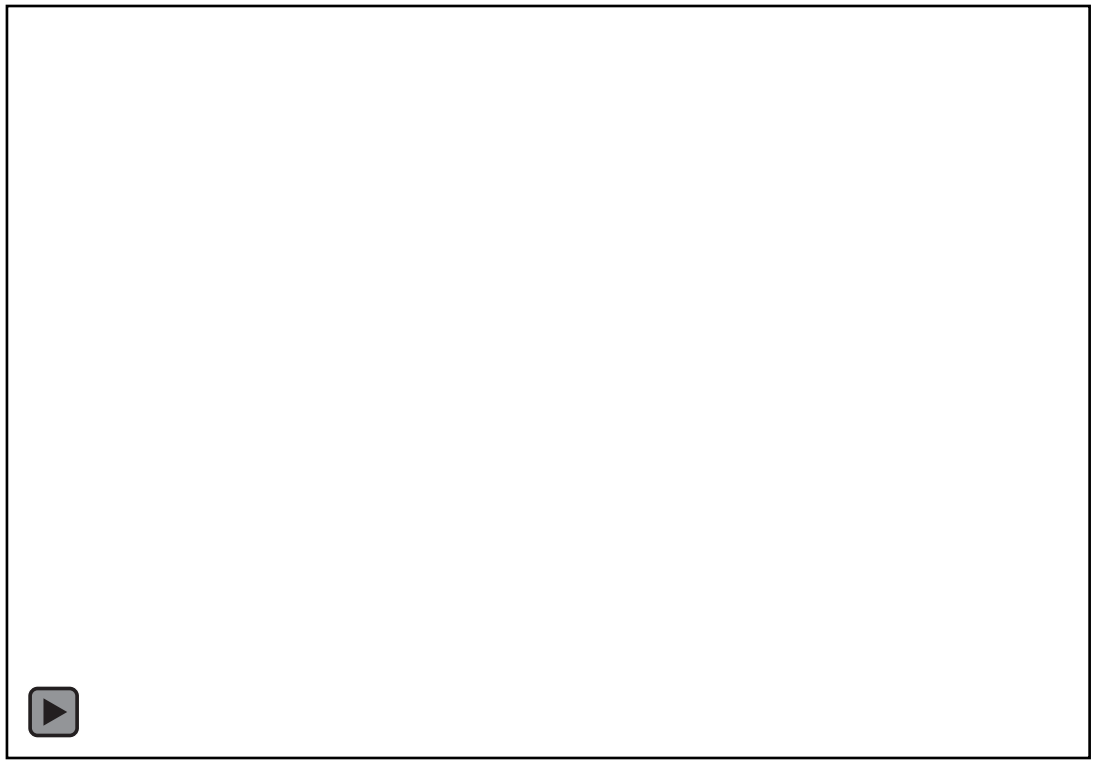


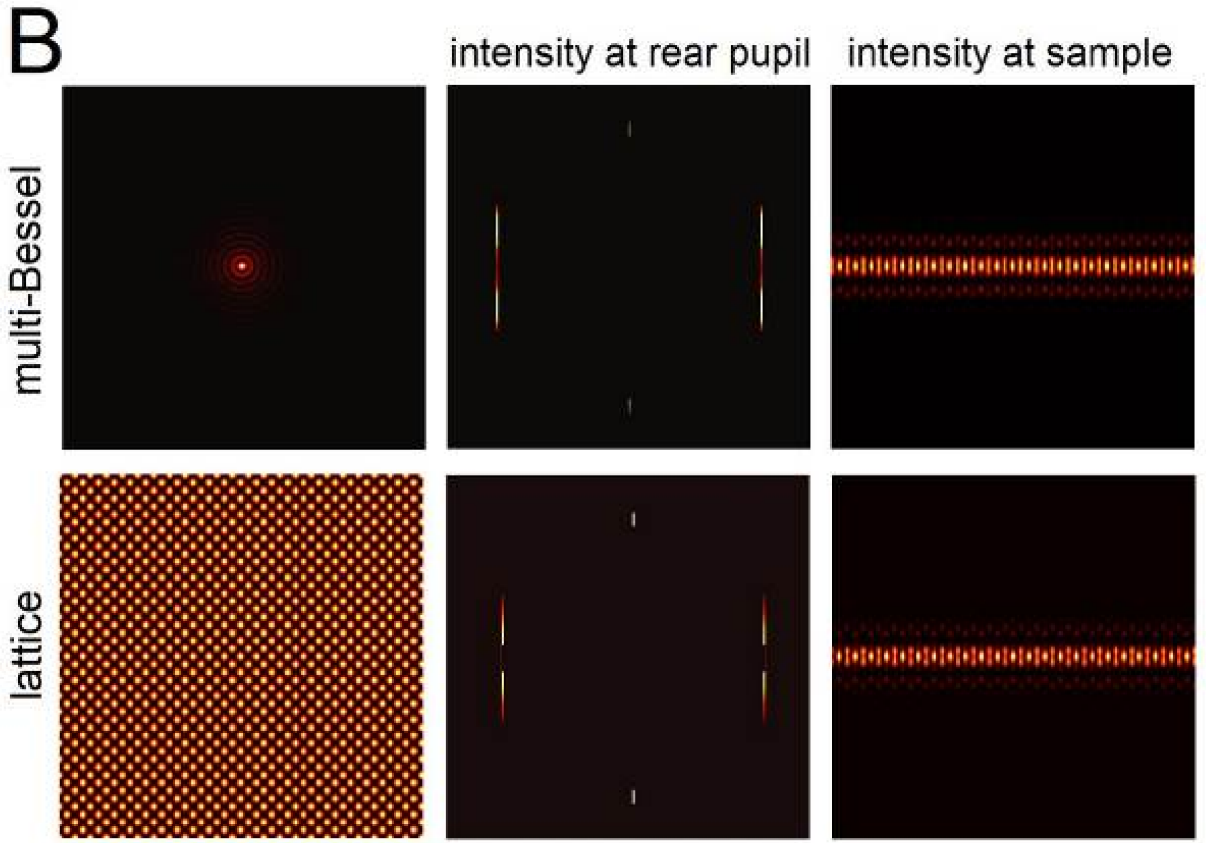


Problem: significant side-lobes and out-of-focus excitation

Nature Protocols **volume 9**, pages1083–1101 (2014)

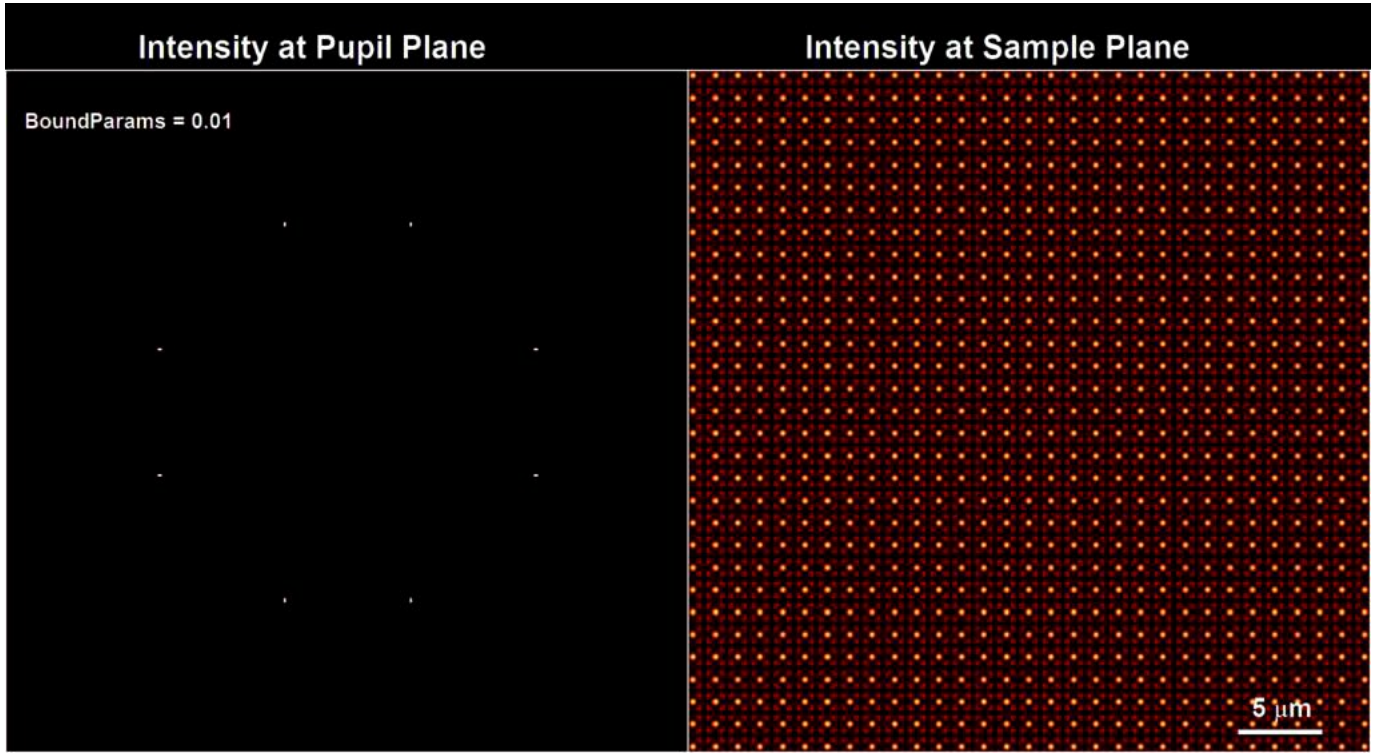
Bessel Beam Arrays and Bounded Optical Lattices





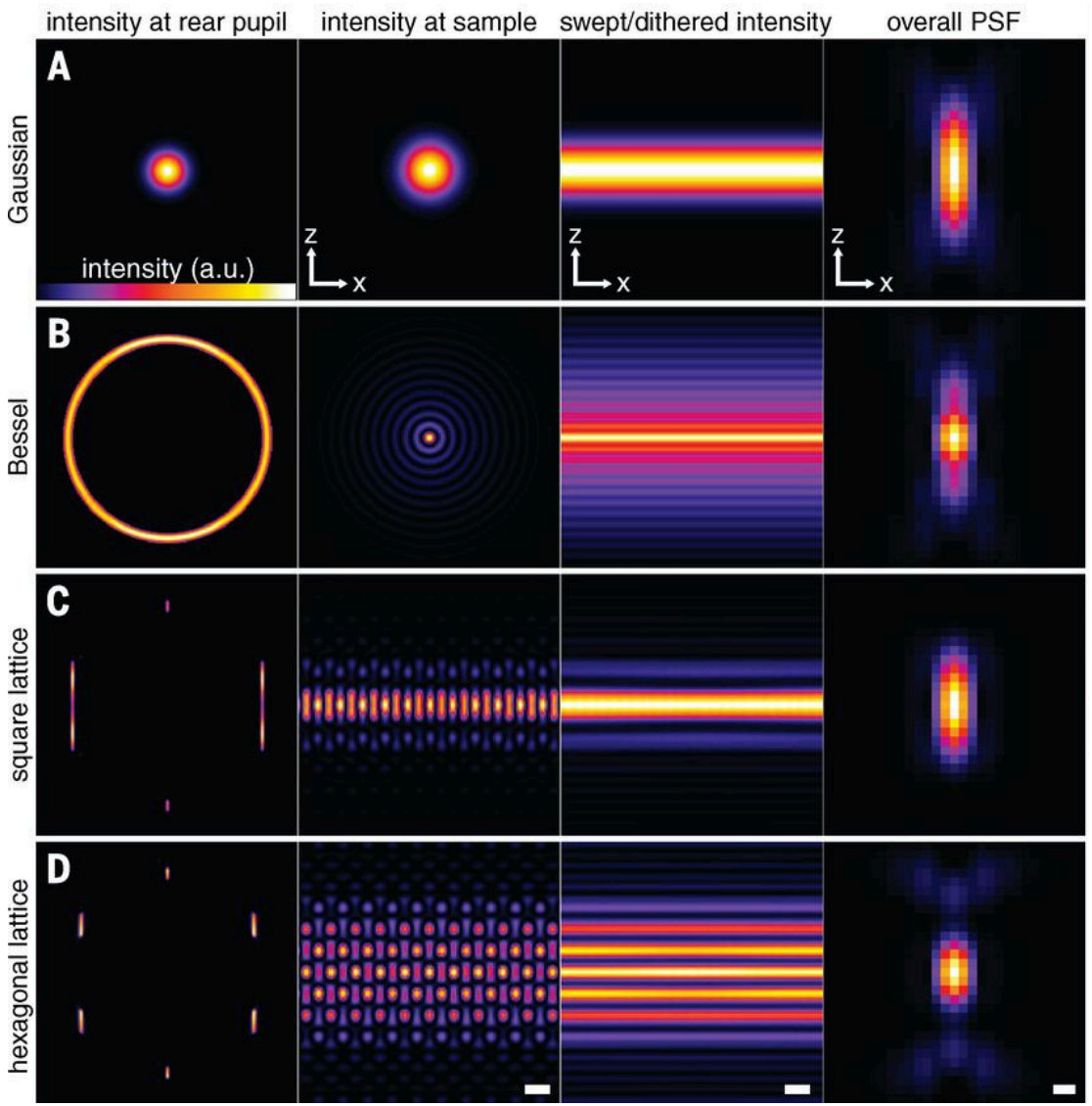
Chen et al, Science (2014)

Bounded 2D Optical Lattices



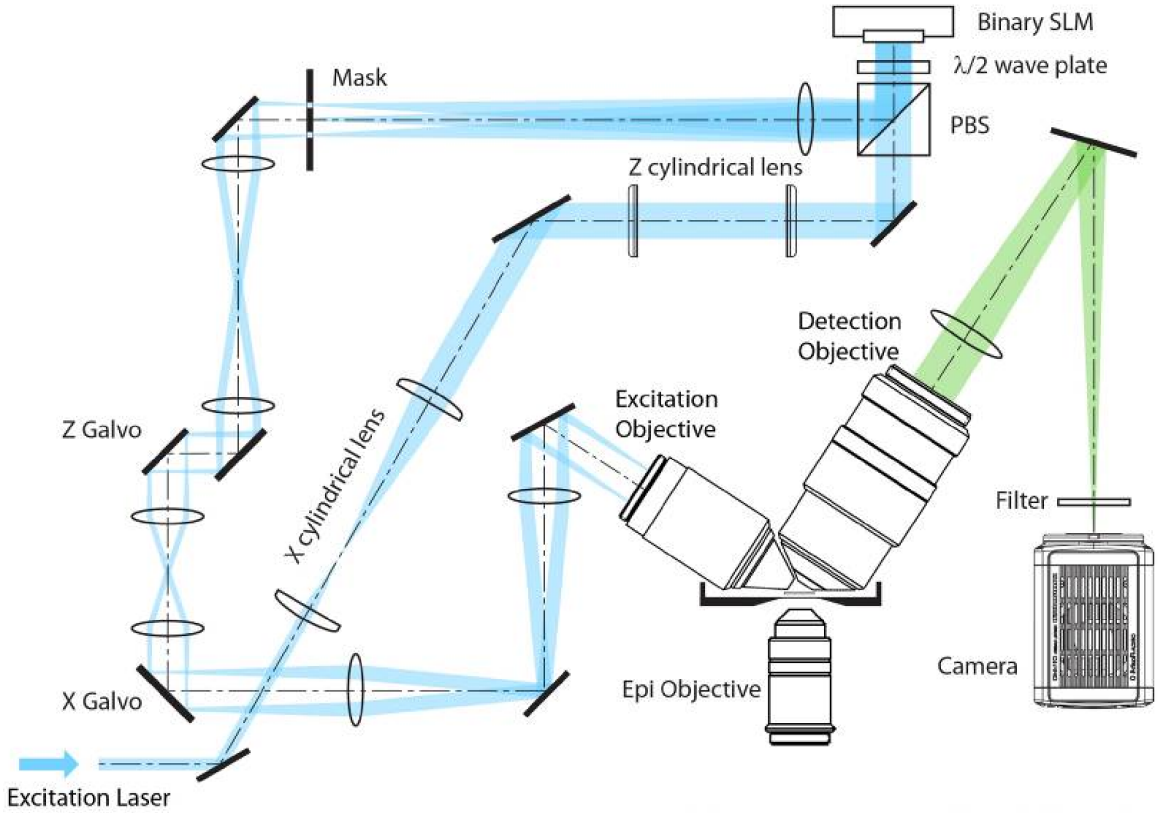
Chen et al, Science (2014)

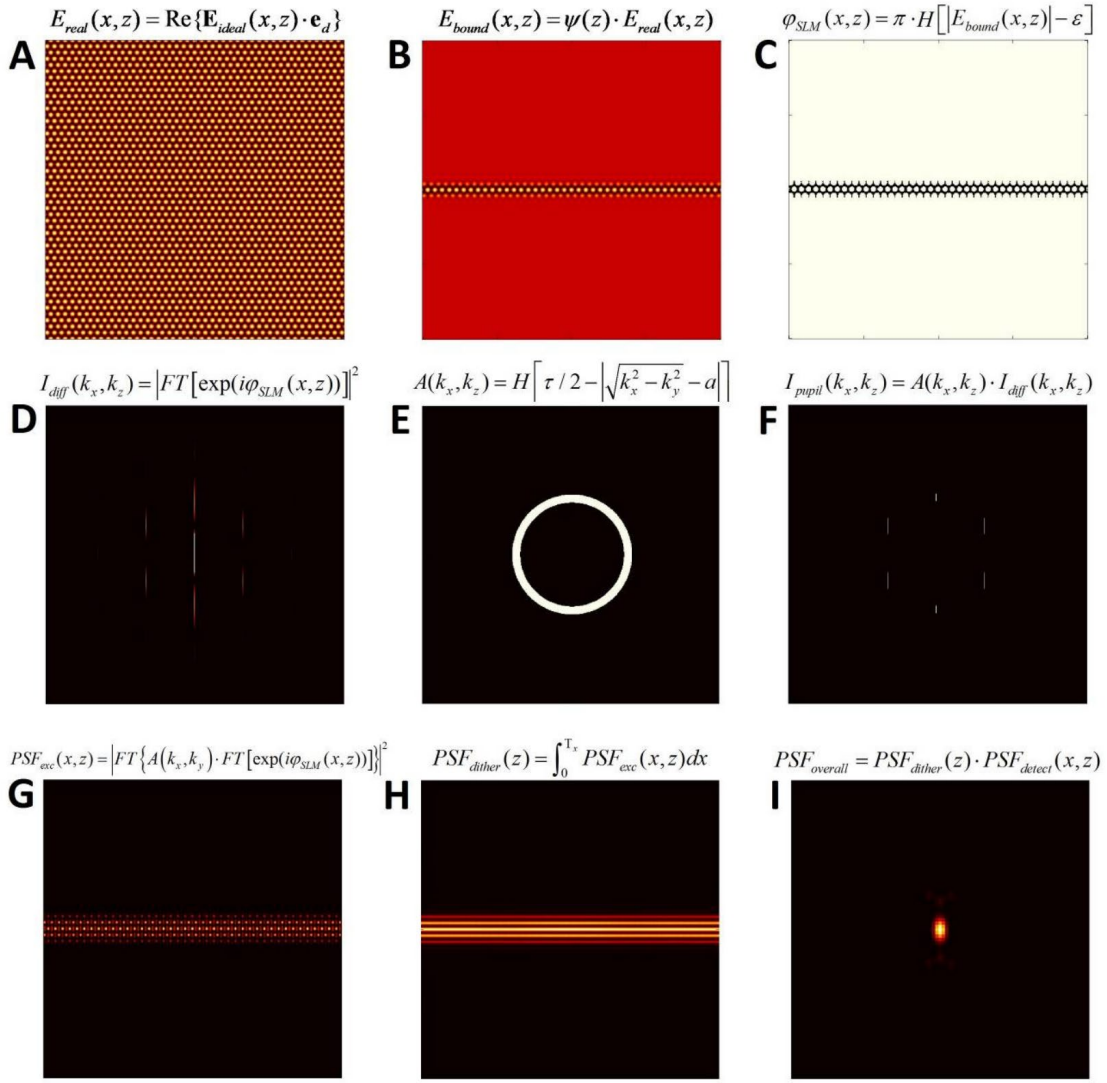
SPIM with Bounded Optical Lattices (Lattice Light-Sheet Imaging



Chen et al, Science (2014)

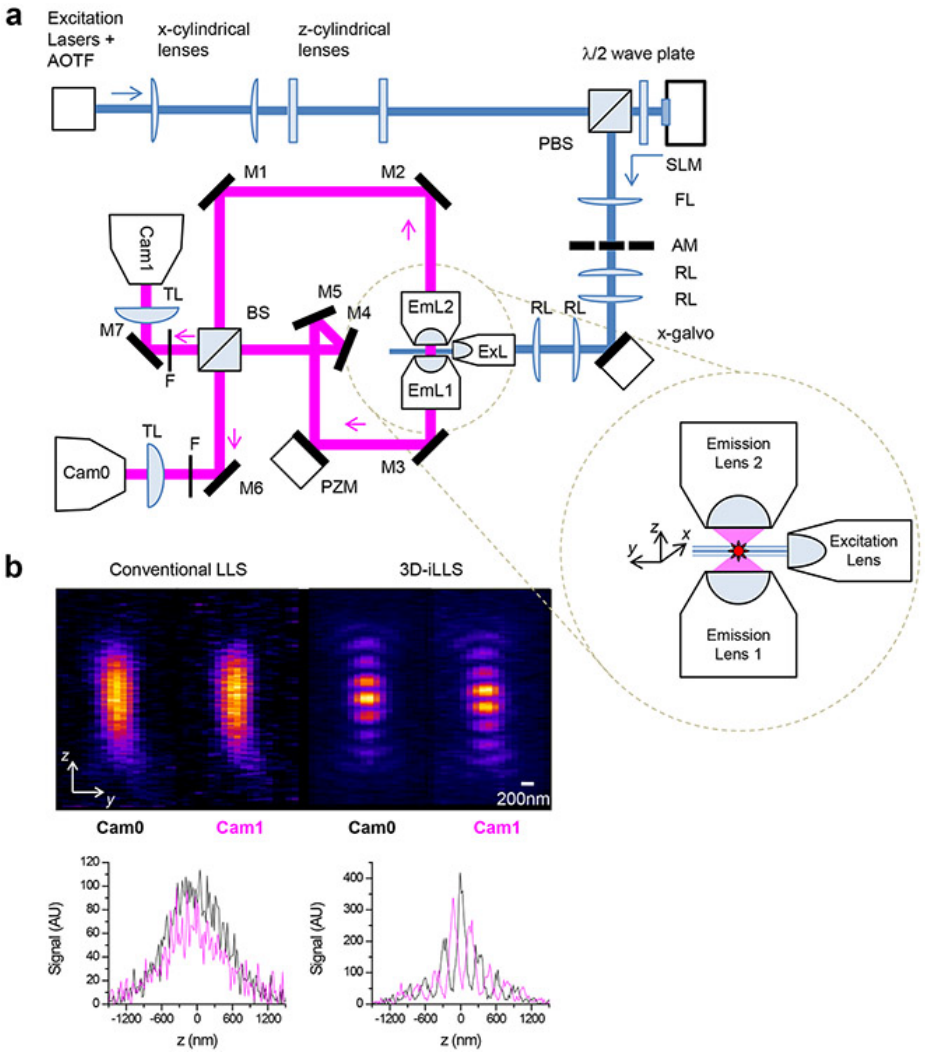
Lattice Light-Sheet Imaging

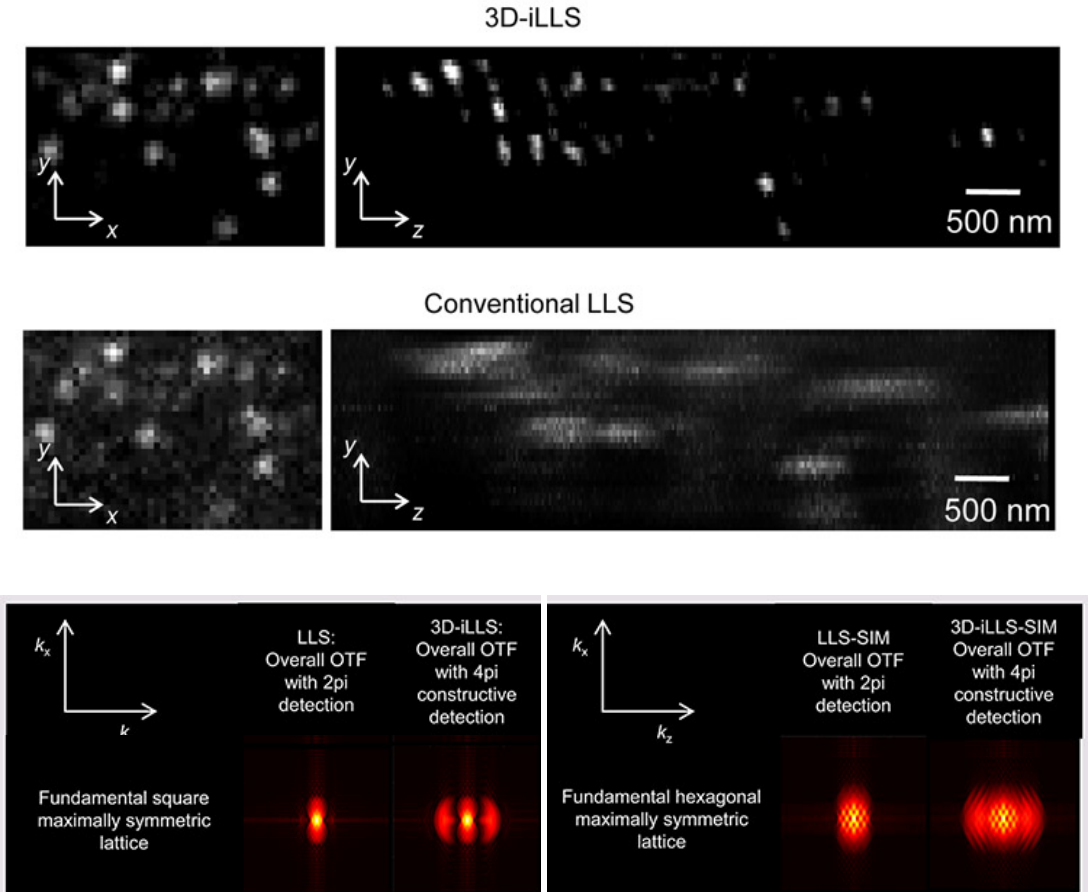




Chen et al, Science (2014)

3D Interferometric LLS Imaging

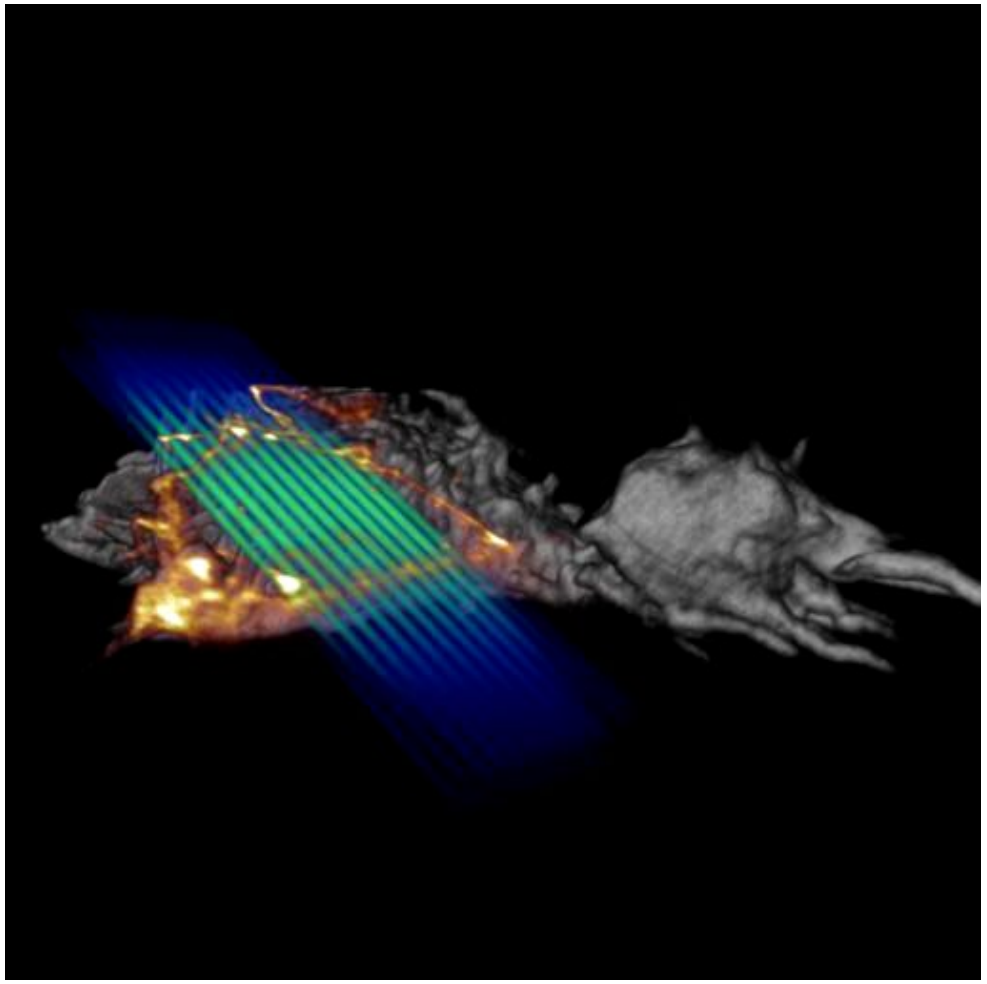




Cao et al., Nat Biotech (2021)

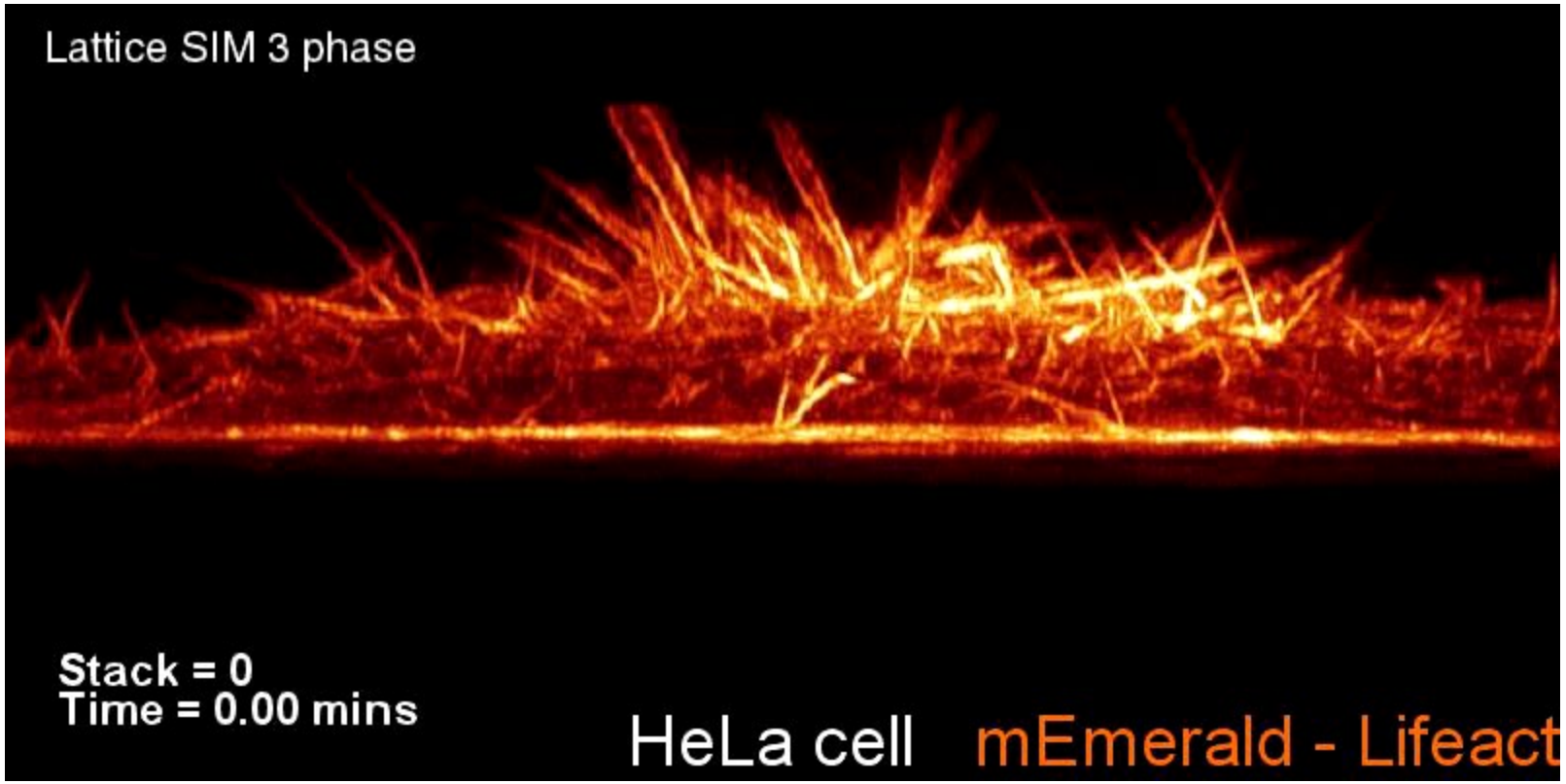
Increased 3D resolution and OTF support

Selective-plane Illumination Microscopy (SPIM)



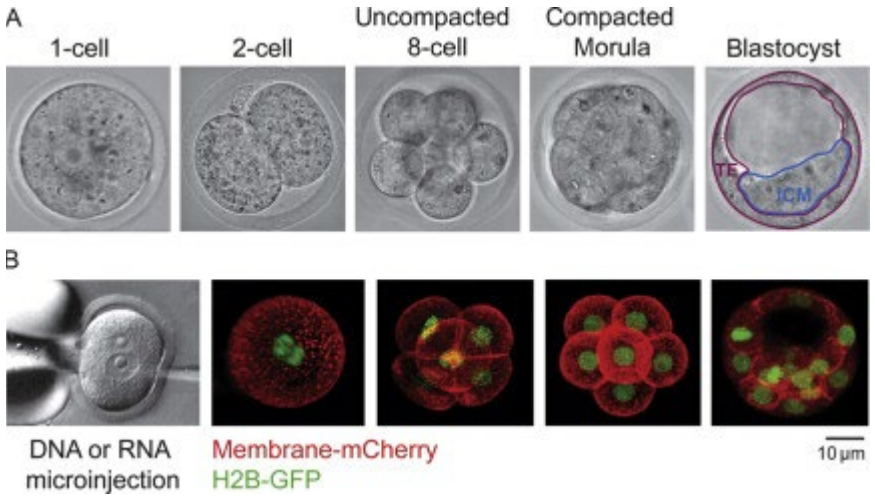
Chen et al, Science (2014)

Selective-plane Illumination Microscopy (SPIM)



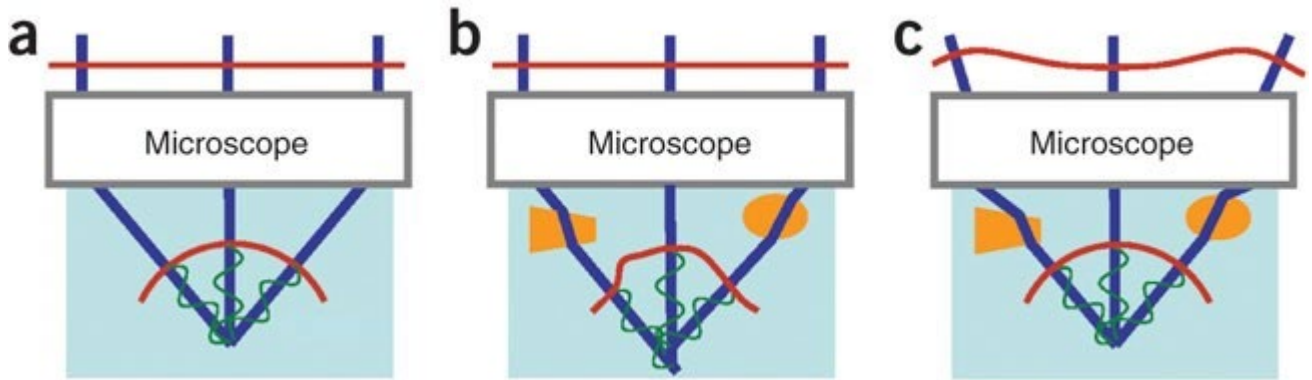
Chen et al, Science (2014)

Image Degradation in thick samples



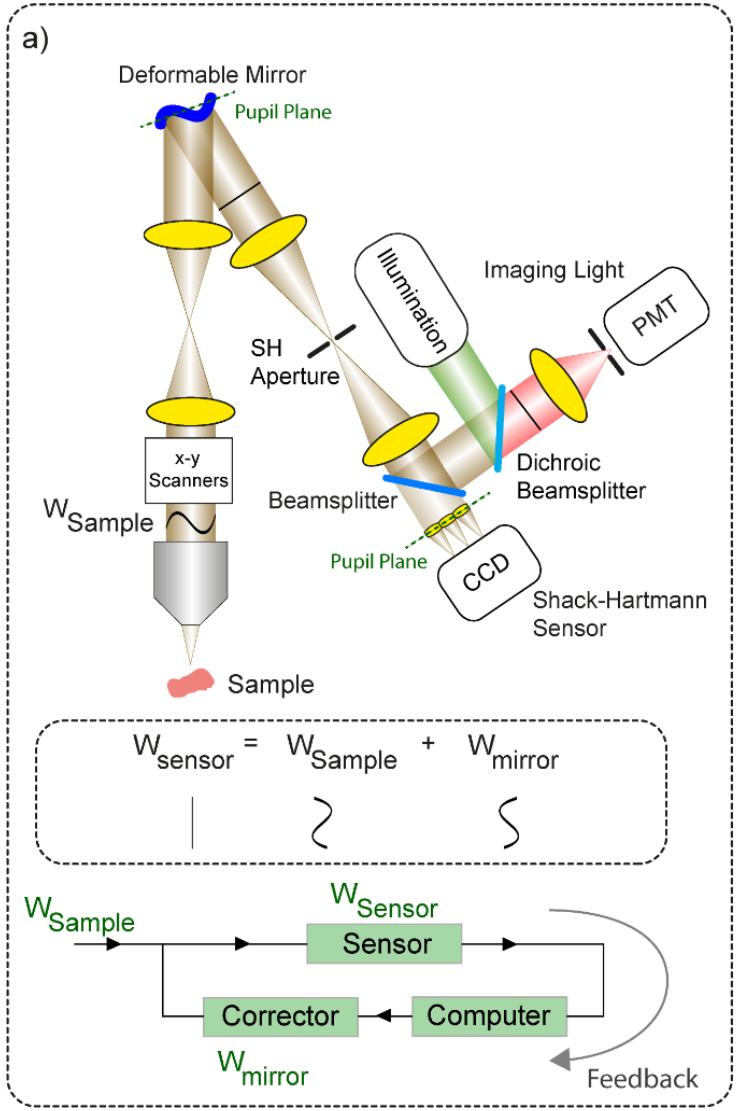
White and Plachta, Curr Top Dev Bio 2015

Source: Flickr

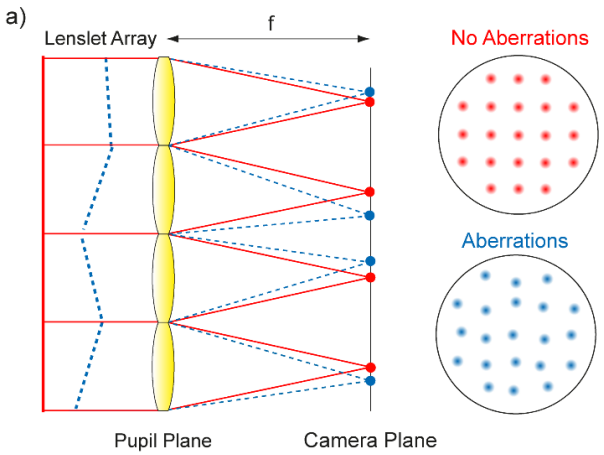


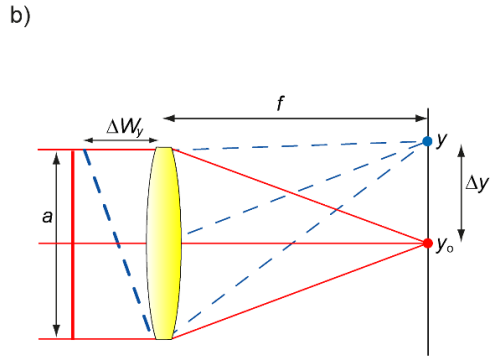
Ni et al, Nat Meth (2010)

Closed-loop AO with direct wavefront sensing



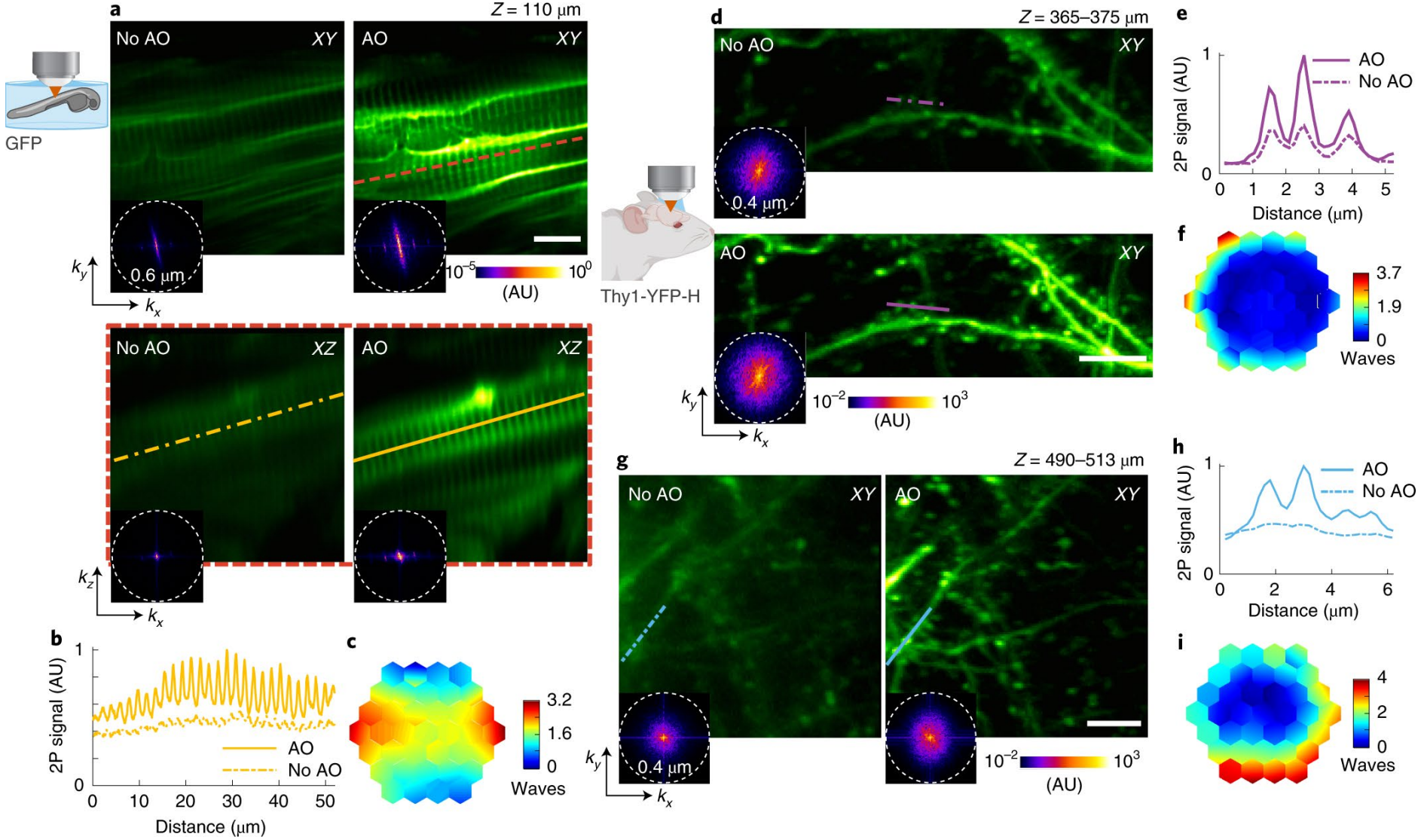
Shack-Hartmann wavefront sensor





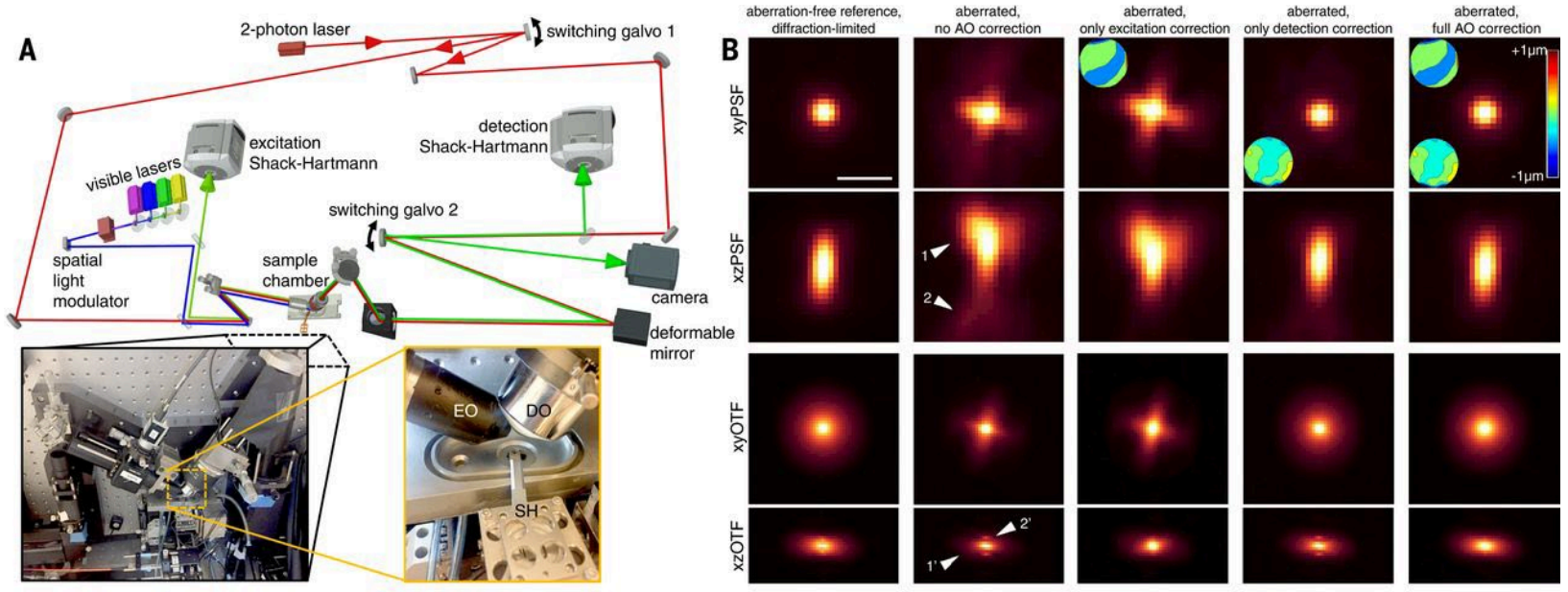
aomicroscopy.org

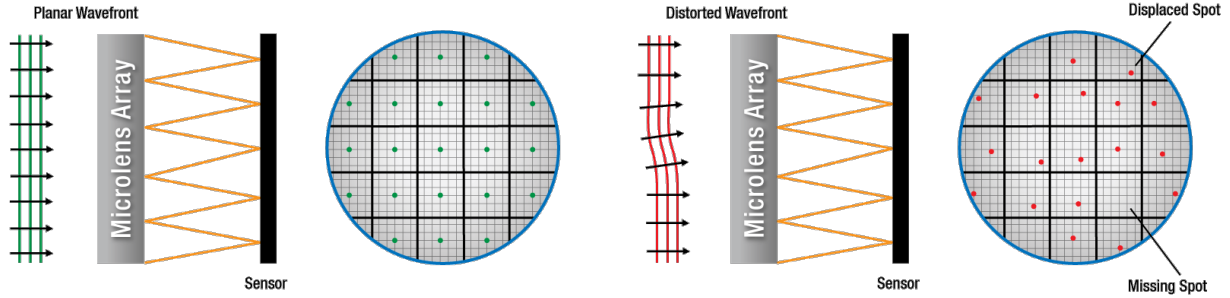
Adaptive Optics Imaging



Rodríguez, C., Chen, A., Rivera, J.A. *et al.* . *Nat Methods* (2021).

Adaptive Optics Imaging (Wide-field)

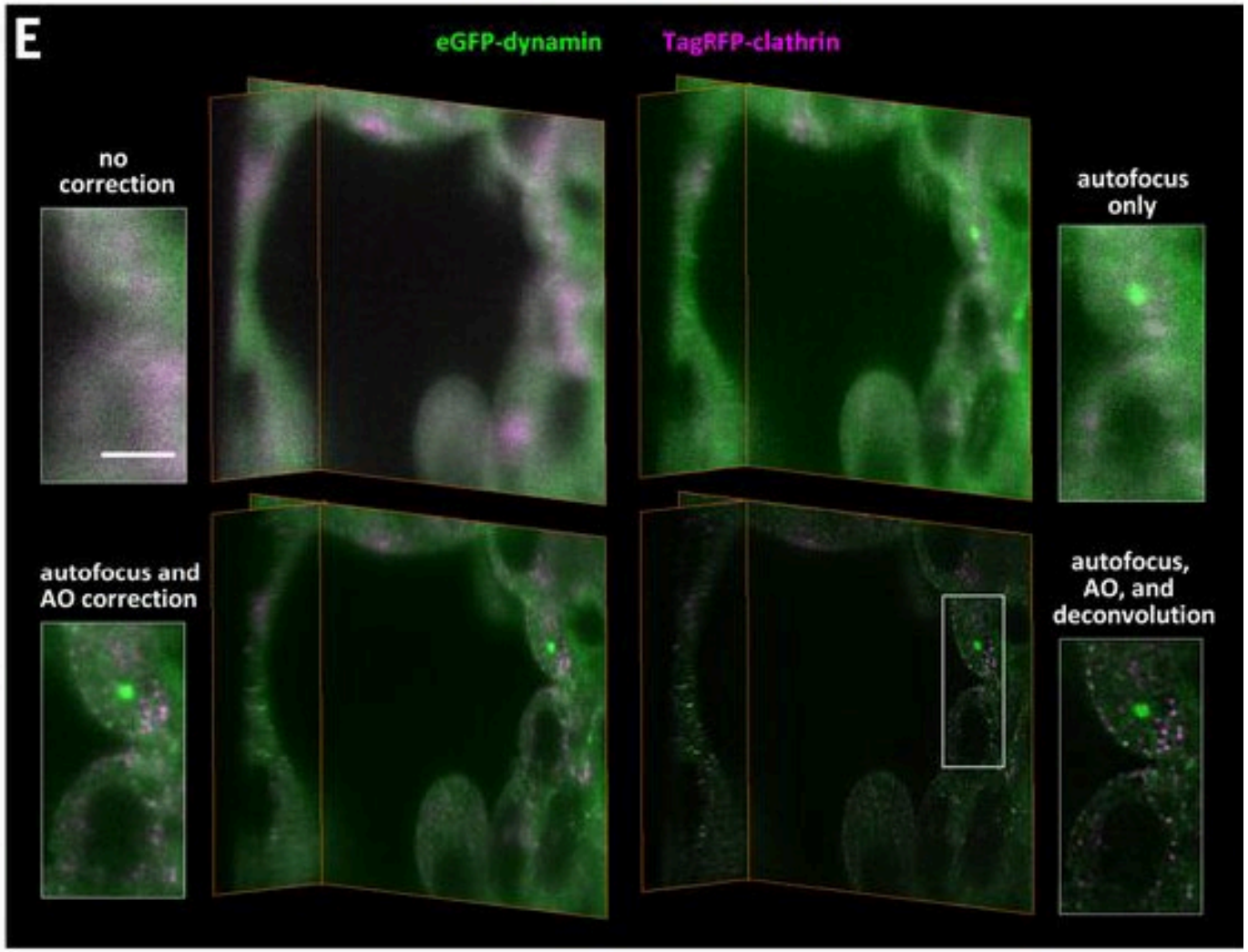




Liu et al, Science (2018)

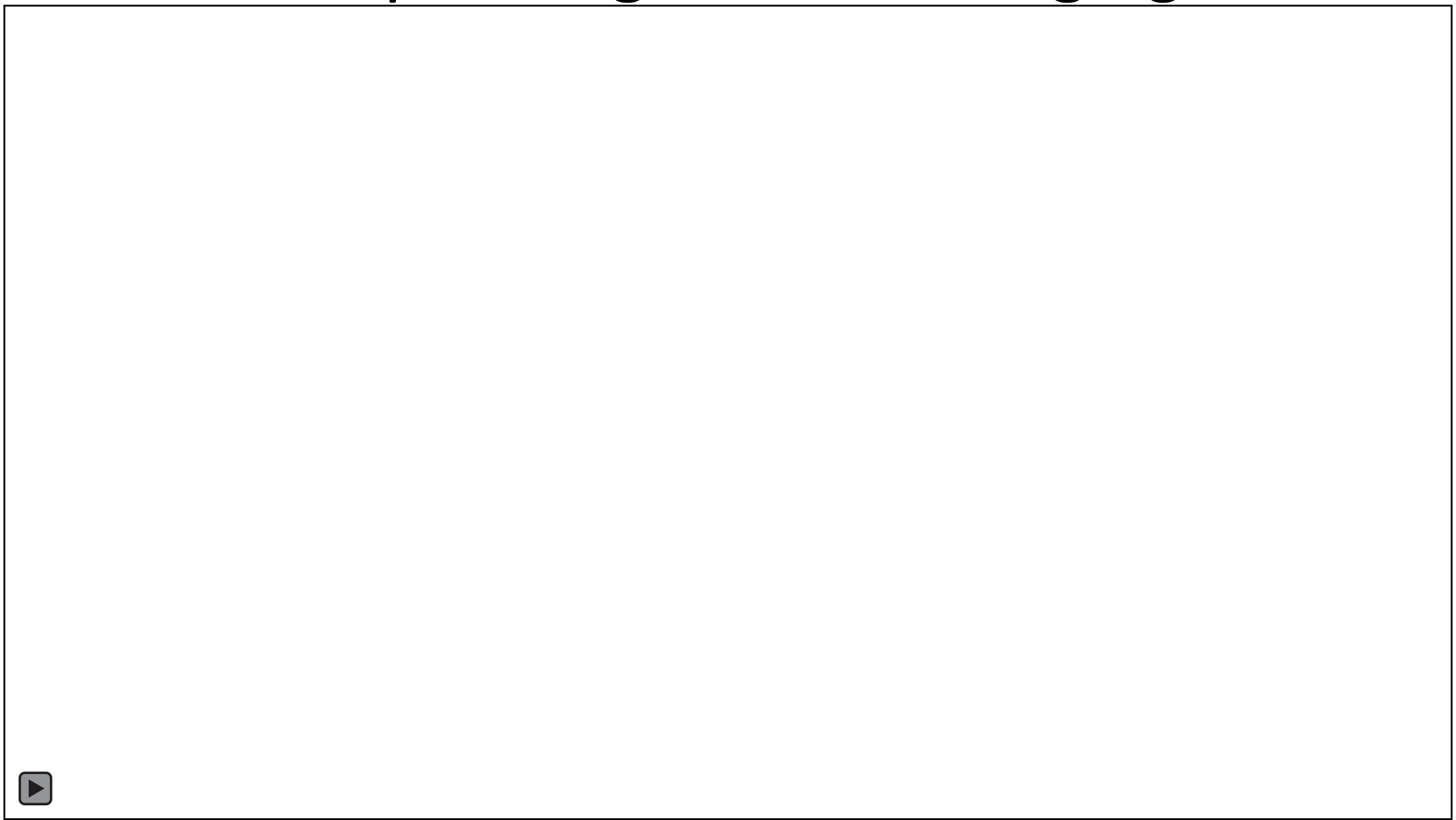
Thorlabs.com

Adaptive Optics Imaging



Liu et al, Science (2018)

Adaptive Light-sheet Imaging



McDole et al, Cell (2018)