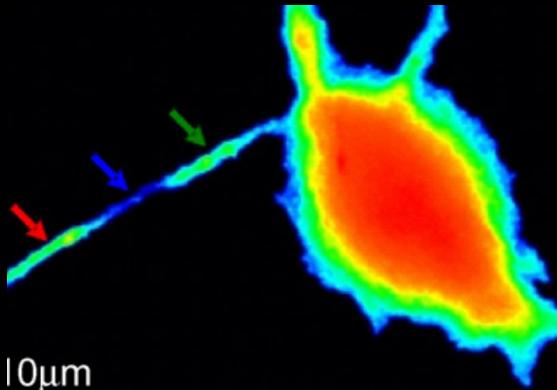




# Basics of Light and Fluorescence Microscopy & Advanced Fluorescence Imaging Techniques

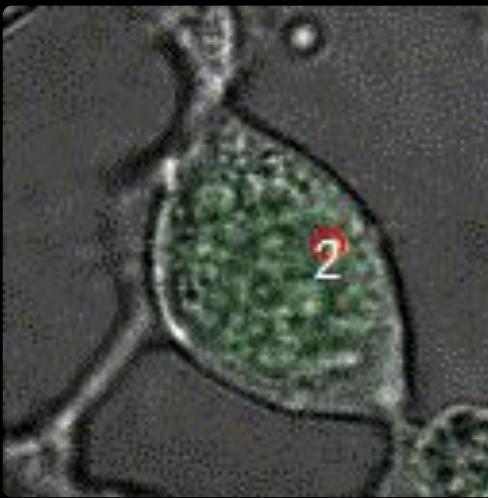
Borys Olifirov  
Uzhgorod  
07.2024

# Resolving in Space, Time, and Colors



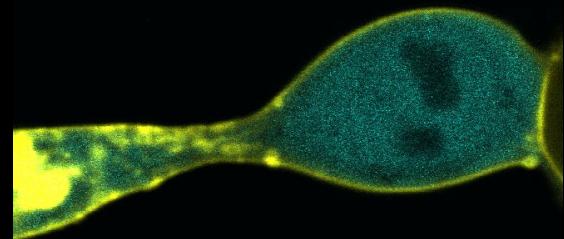
Markova et al., 2008, doi:10.1016/j.neulet.2008.06.089

↑Labeled HPCA-CFP distribution along axon  
in hippocampal neuron



Olifirov et al., 2020, unpublished

↑Ca<sup>2+</sup> transient registration with Fluo-4  
in HEK 293 cells

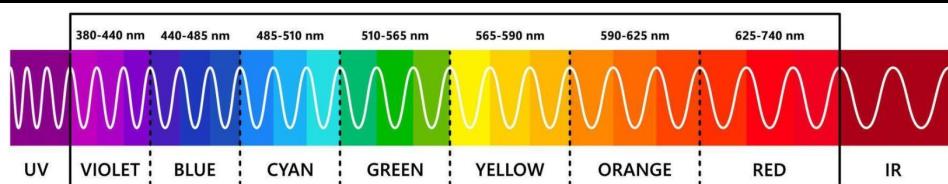


Olifirov et al., 2020, unpublished

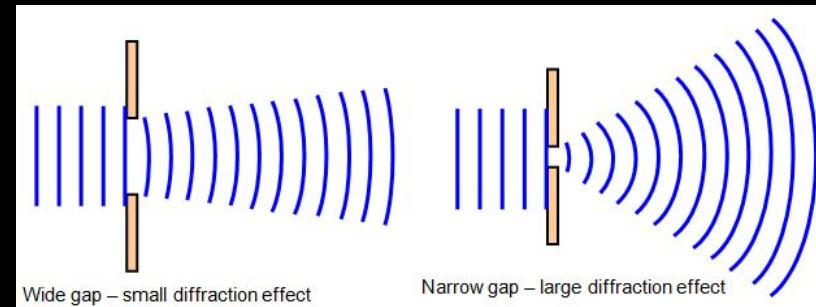
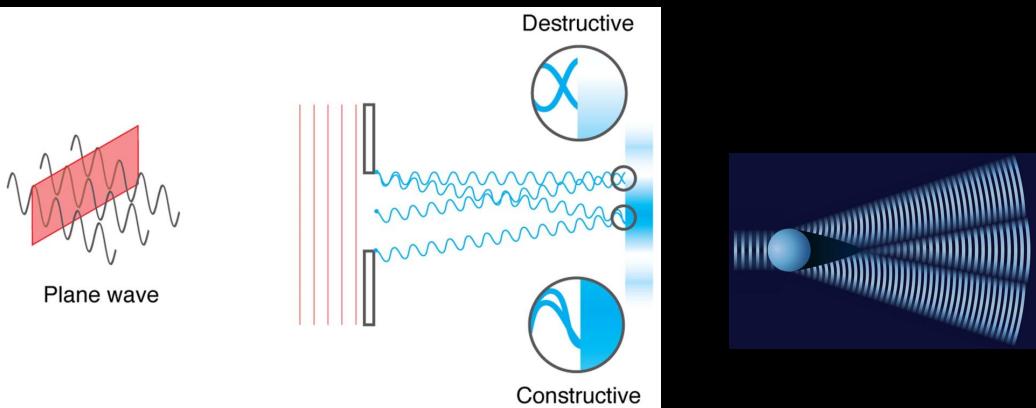
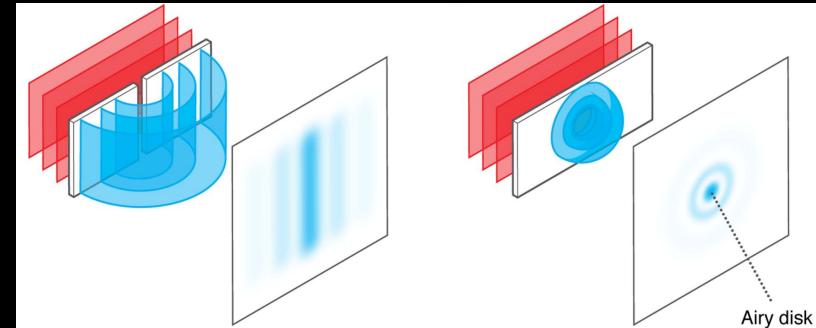
↑Co-transfection of Mem-EYFP and HPCA-ECFP  
in HEK 293 cells

# Physical & Technical Intro

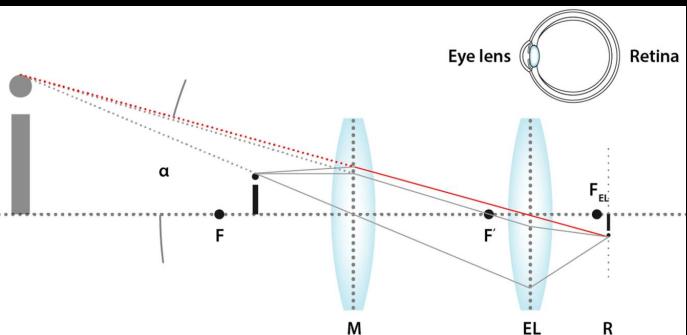
# Brief Physics of Light



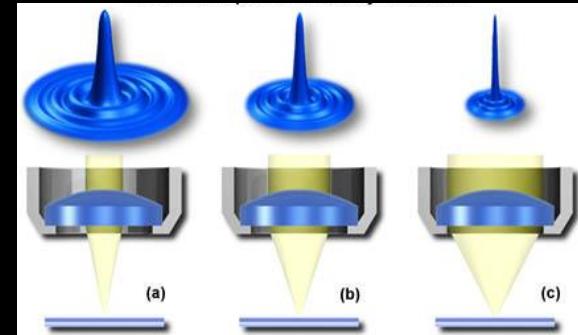
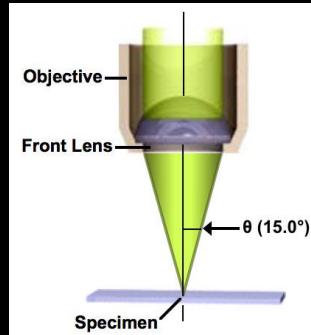
$$E = h\nu = h\frac{c}{\lambda} \quad \leftarrow \text{Energy of light}$$



# Objective

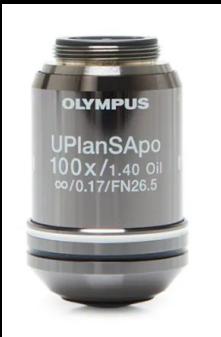


→ The size and angular aperture of objective light cones



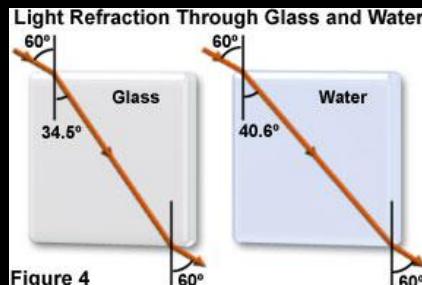
Vangindertael et al., 2018, <https://doi.org/10.1088/2050-6120/aaa0c>

↑Angular magnification by a single convex lens



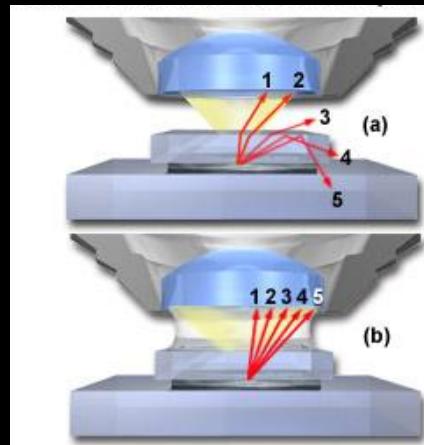
$$NA = n \cdot \sin \theta$$

↑Numerical aperture

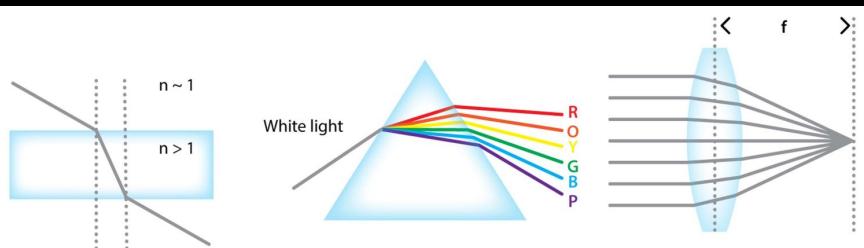


↑→ Light refraction and Immersion media advantages

[olympus-lifescience.com](http://olympus-lifescience.com)



# Abberations



Vangindertael et al. 2018, <https://doi.org/10.1088/2050-6120/aaae0c>

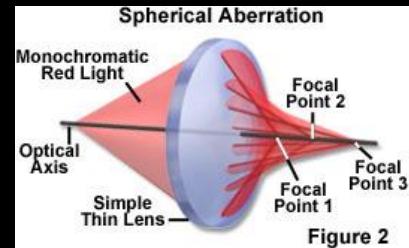


Figure 2

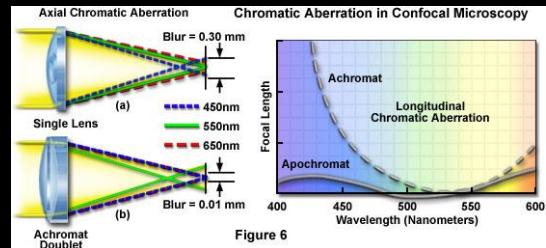


Figure 6

↑↑On-axis aberrations

↑The basic concepts of refraction and lenses

↓↓Off-axis aberrations

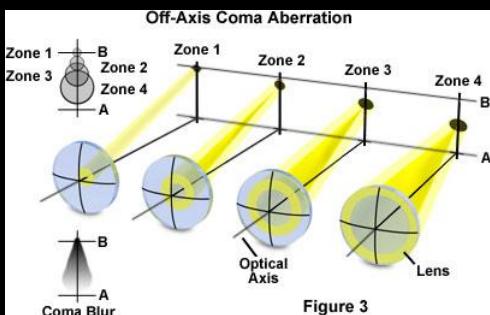


Figure 3

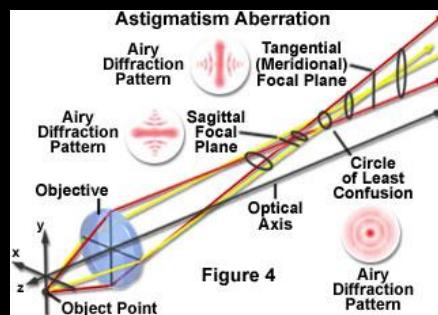


Figure 4

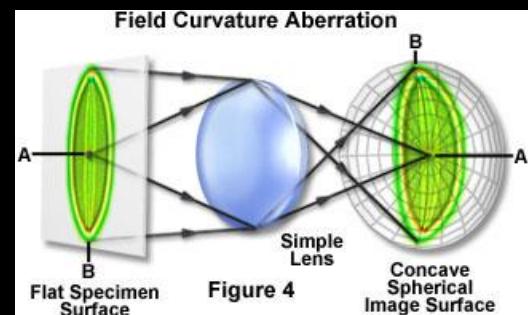
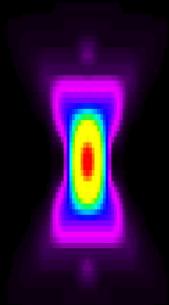


Figure 4

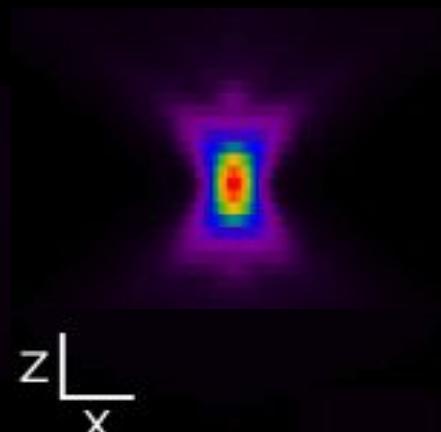
# Point Spread Function (PSF) of Optical System

Y  
X

svi.nl

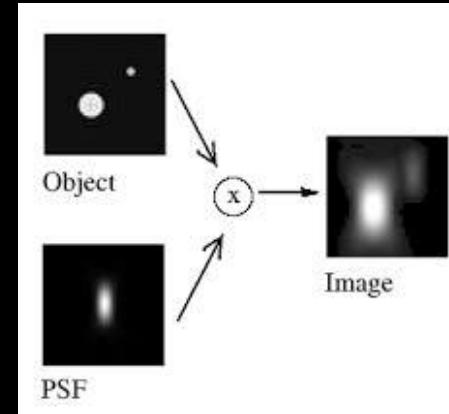


NA=1

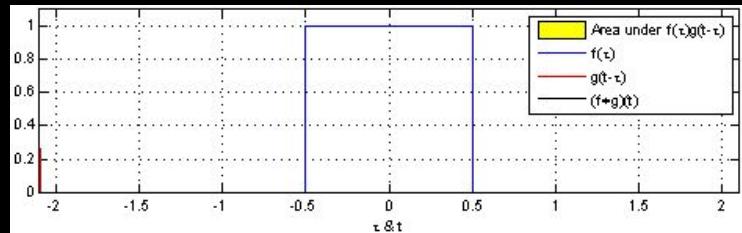


NA=1.4

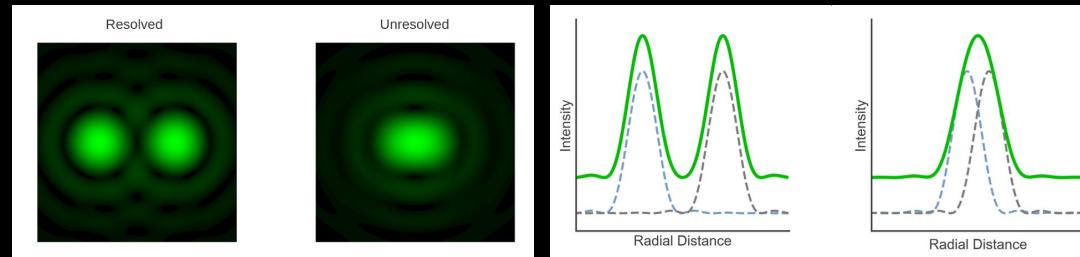
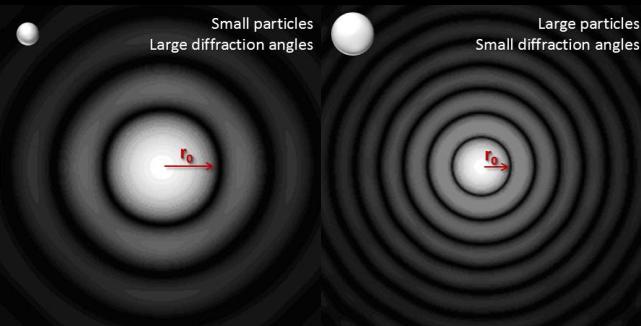
→ Convolution in microscope



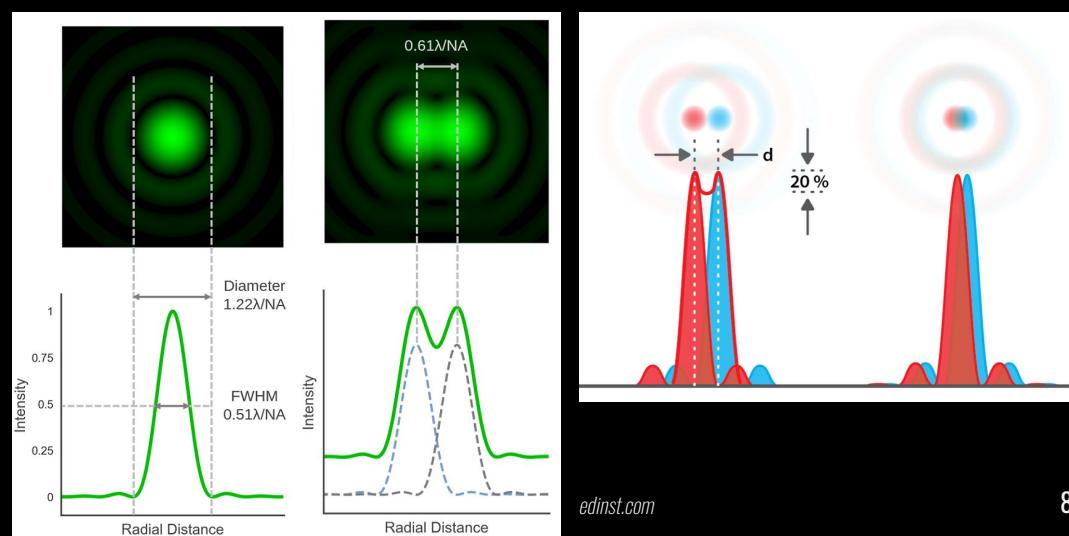
→ Convolution  
of two square pulses



# Lateral Resolution



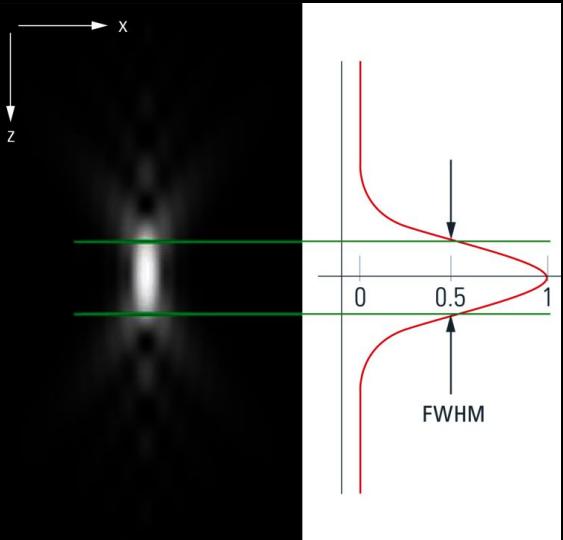
↑↓ Airy Pattern / 2D-Point Spread Function and the Rayleigh resolution limit



$$d_{xy} \approx \frac{0.61 \cdot \lambda}{NA}$$

↑Lateral resolution

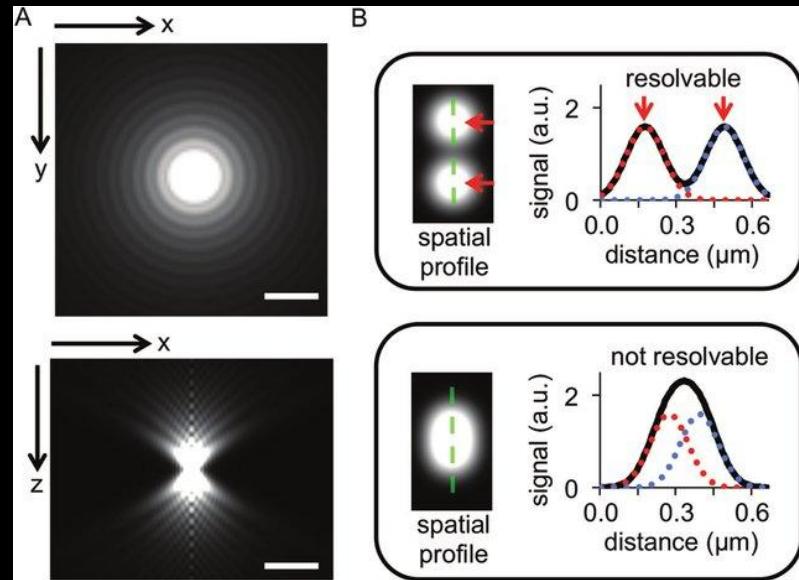
# Axial Resolution



↑PSF lateral projection

$$d_z \approx \frac{2 \cdot n \cdot \lambda}{NA}$$

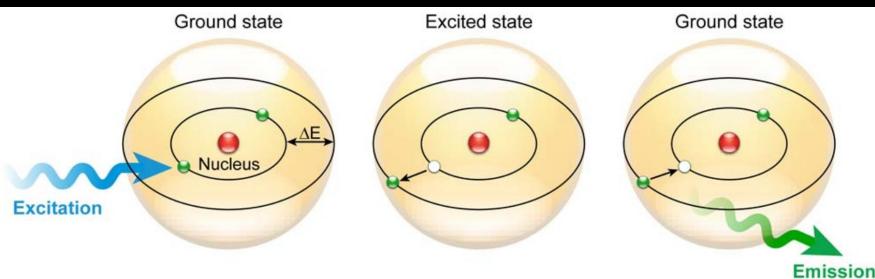
↑Axial resolution



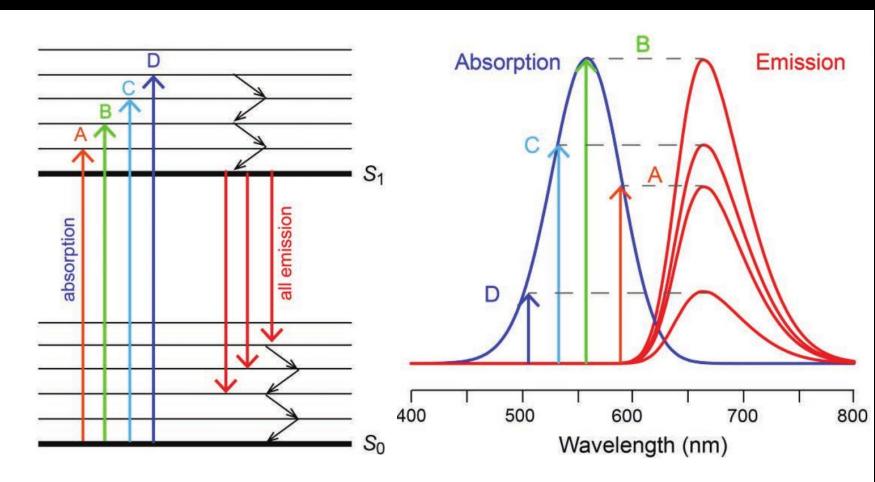
Herbert et al., 2012, doi:10.1017/S1431927612013347

↑PSF and the resolution limit

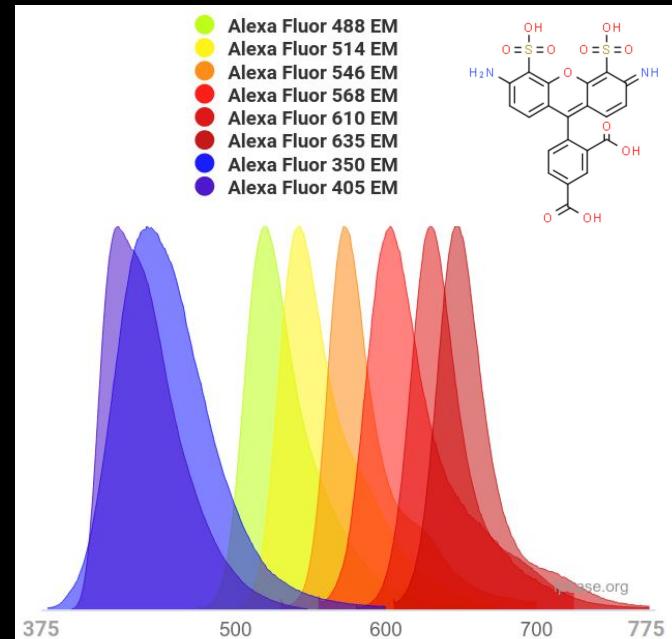
# Molecule Energy Levels and Fluorescence



← Fluorescence schematic

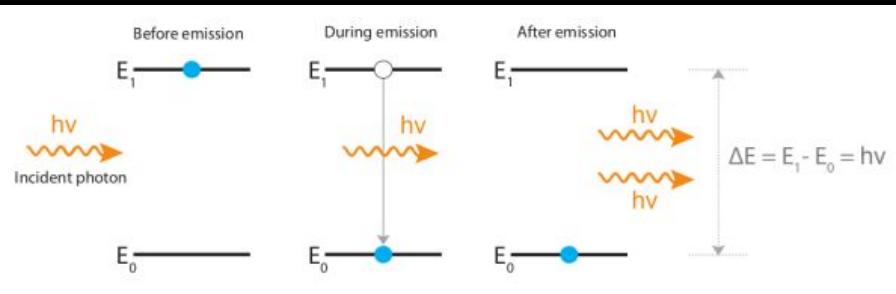


→ Emission spectra  
of several AlexaFluor  
fluorescence dyes



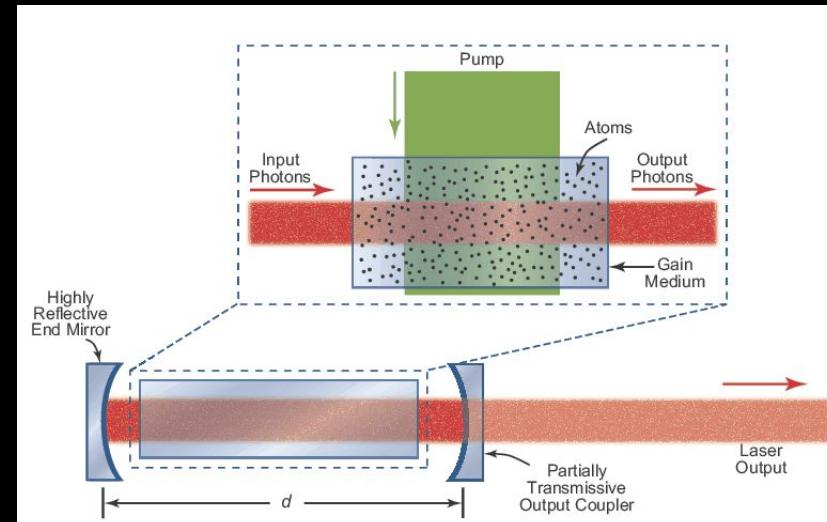
← Jablonski diagram illustrating the energy state transitions  
and spectra of fluorophore

# Stimulated Emission & Lasers

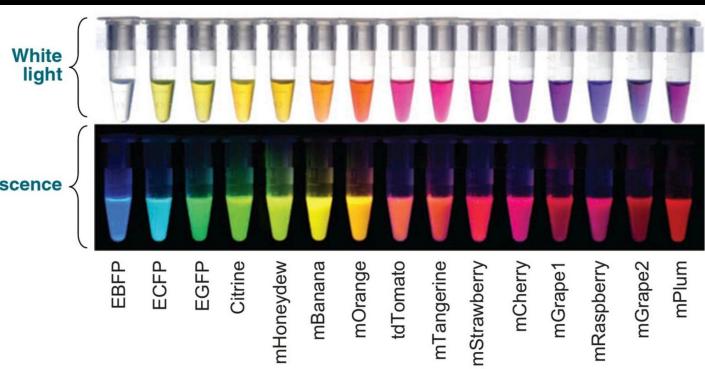


← Diagram illustrating the process of stimulated emission from left to right

→ Simplified diagram of a laser

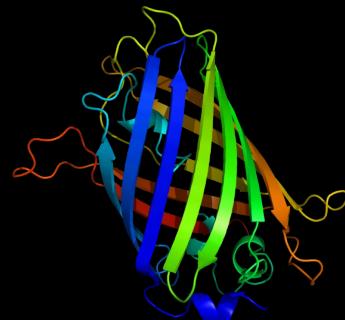


# Fluorescent Proteins

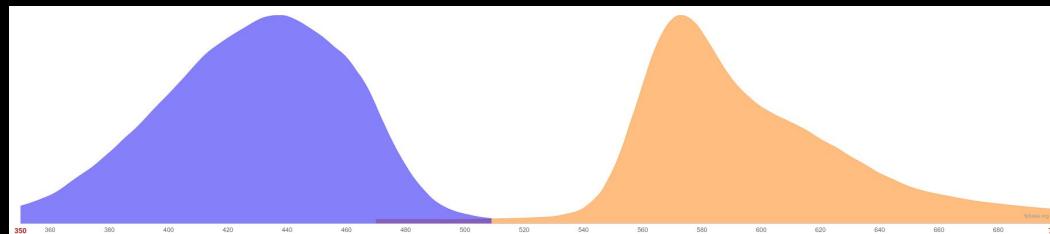
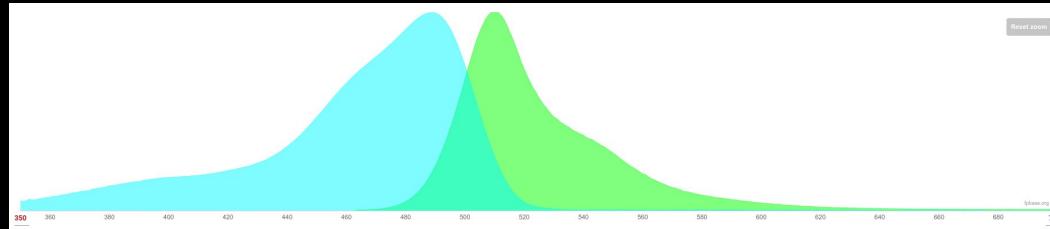


← Set of different fluorescent proteins

Tsien Lab



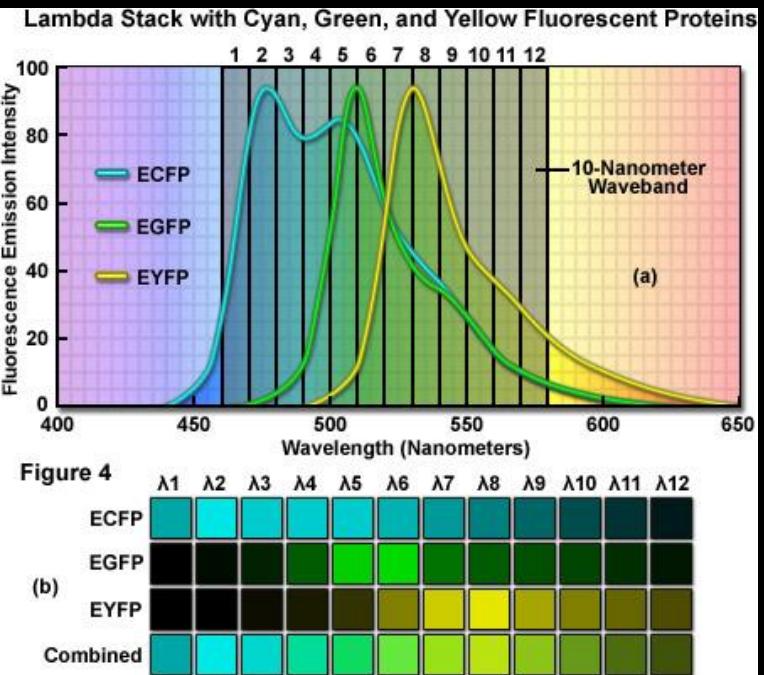
↑3D structure of green fluorescent protein



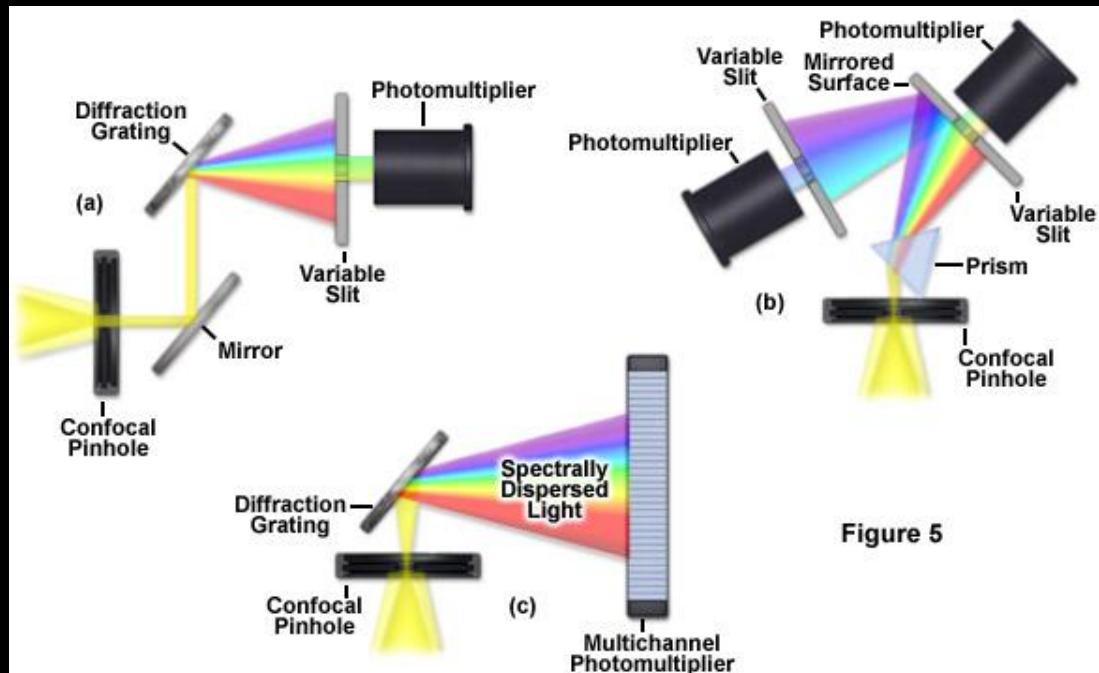
↑Excitation and emission spectra of EGFP (top)  
and LSSmOrange (bottom)

fpbase.org

# Spectral Imaging

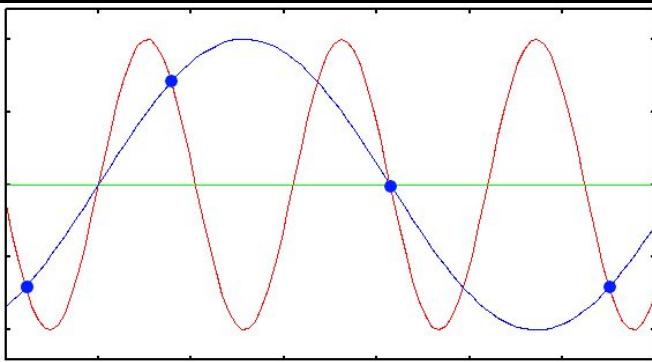


↑Distribution of fluorescence proteins intensity between multiple spectral channels



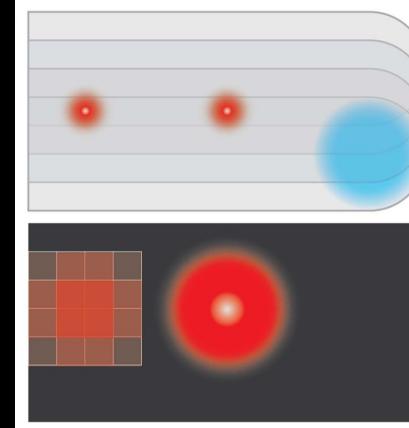
↑Spectral imaging configurations

# Spatial Sampling and Nyquist Rate

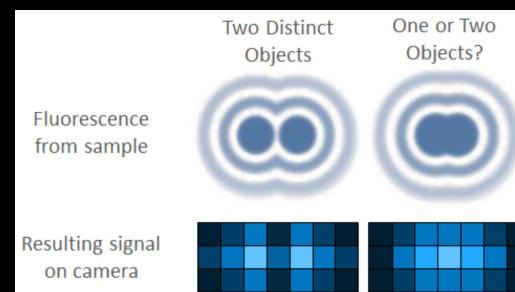
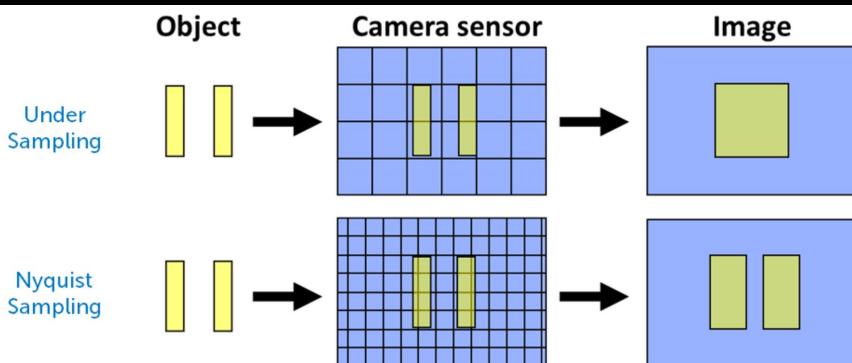
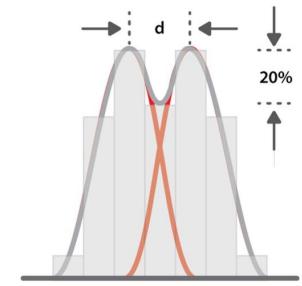


↑ Plot showing the undersampled acquisition  
of a 1D sinusoidal signal in red

→ The Shannon-Nyquist criterion states that to adequately represent a PSF in a digital manner, at least 4 by 4 pixels are required



B



# Detectors

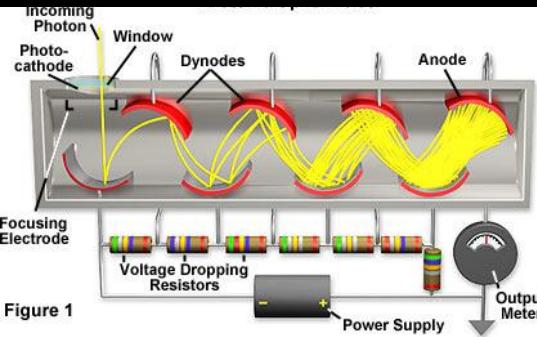
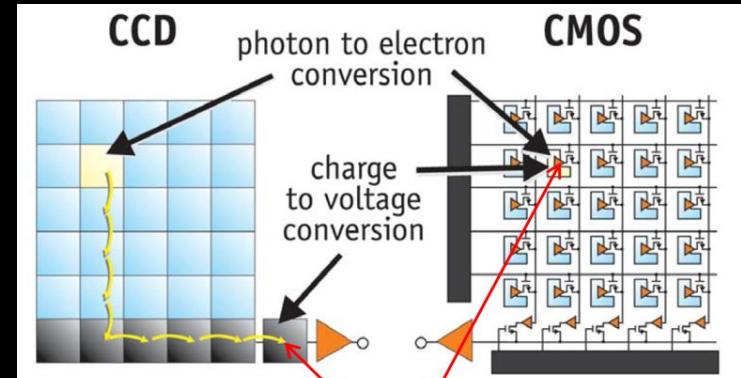


Figure 1

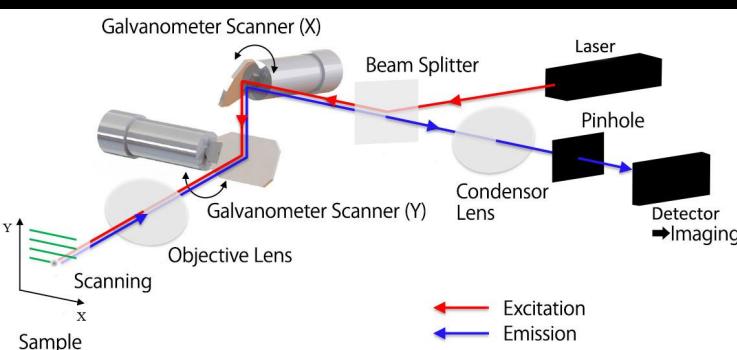
[olympus-lifescience.com](http://olympus-lifescience.com)

← Photomultipler tubes schematic



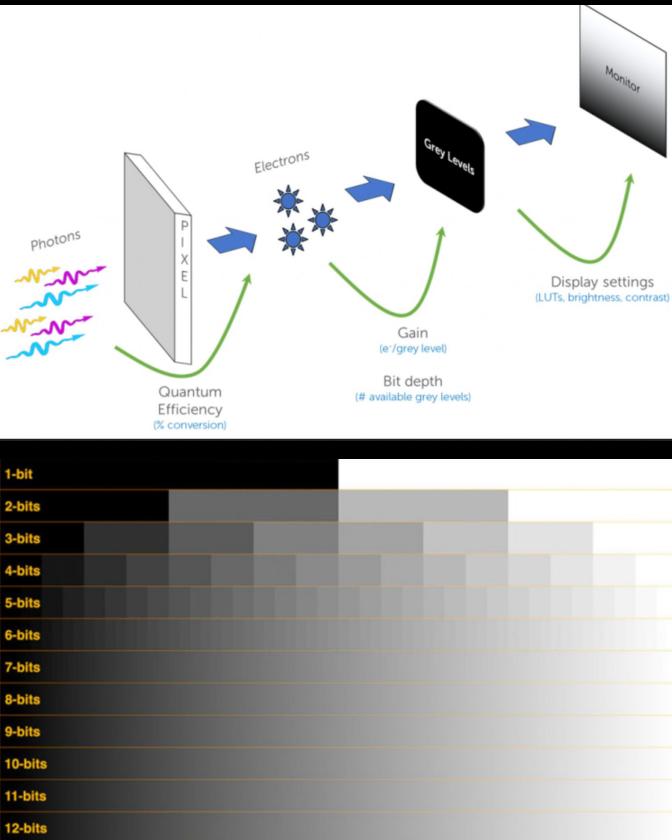
[accu-scope.com](http://accu-scope.com)

↑Photons conversions to signal in charge-coupled device (CCD) and complementary metal-oxide-semiconductor (CMOS) sensors



← CCD (left) and CMOS (right) sensors form-factors

# Bits and LUTs



← Bits per pixel and intensity levels



↑Different lookup tables (LUT)



4	6	58	144	183	53	7	3
2	34	208	239	248	212	74	5
83	138	232	242	235	249	171	3
68	223	219	174	202	229	87	4
5	152	146	47	150	223	89	4
0	99	213	121	136	110	180	4
1	115	209	252	199	216	255	38
3	161	148	231	213	213	201	51

Pixel values



LUT



4	6	58	144	183	53	7	3
2	34	208	239	248	212	74	5
83	138	232	242	235	249	171	3
68	223	219	174	202	229	87	4
5	152	146	47	150	223	89	4
0	99	213	121	136	110	180	4
1	115	209	252	199	216	255	38
3	161	148	231	213	213	201	51

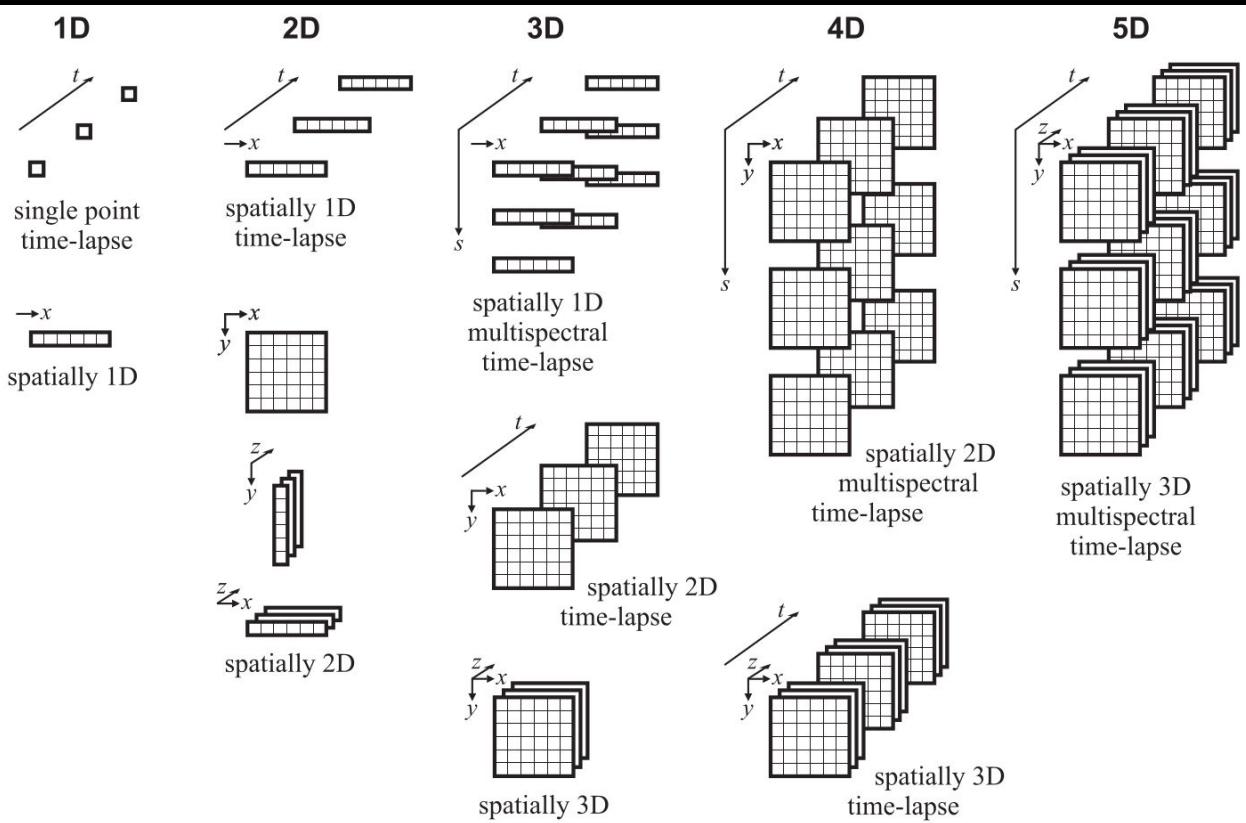
Pixel values



LUT

[qupath.readthedocs.io](http://qupath.readthedocs.io)

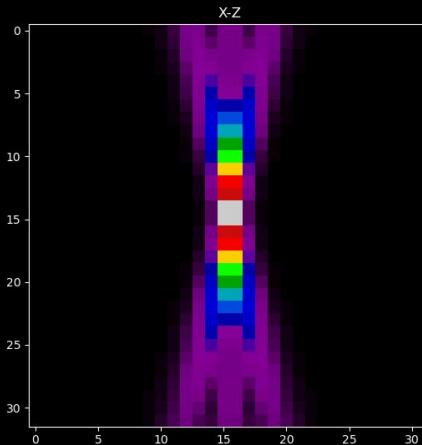
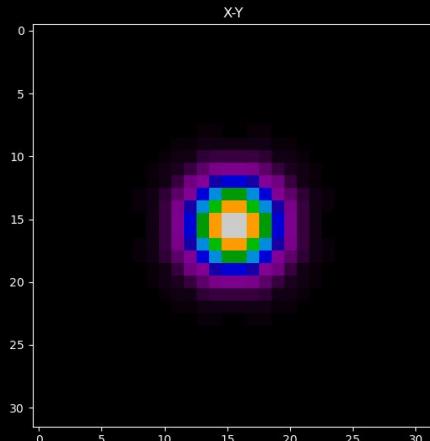
# Images as Arrays



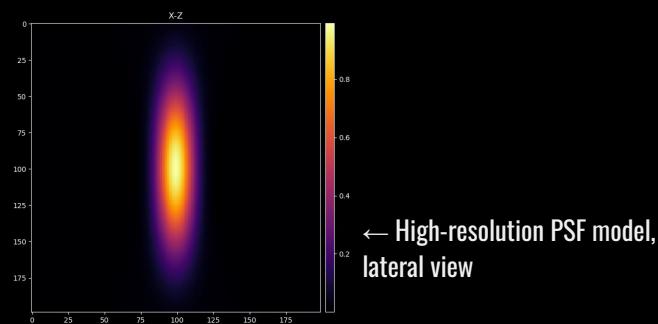
← Possible dimensional structures of image data

## Intermezzo for Deconvolution

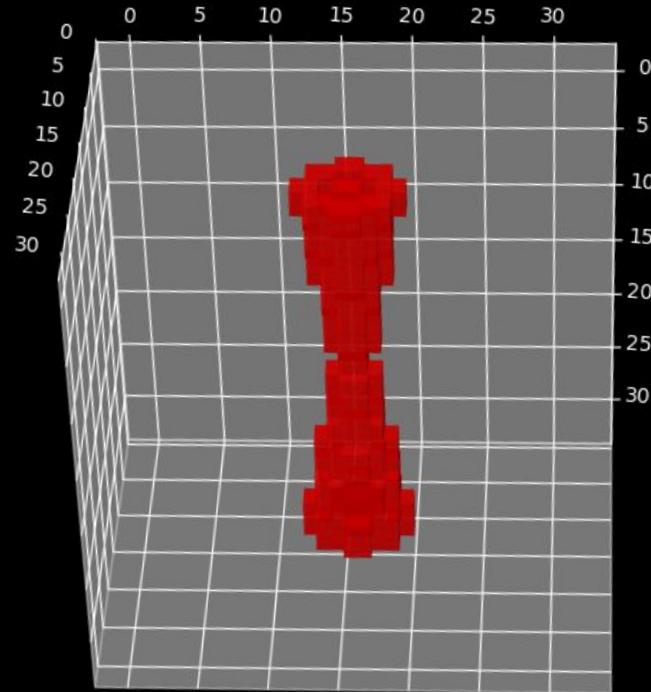
# Point Spread Function (PSF) of Optical System



↑Low-resolution PSF model, axial and lateral view

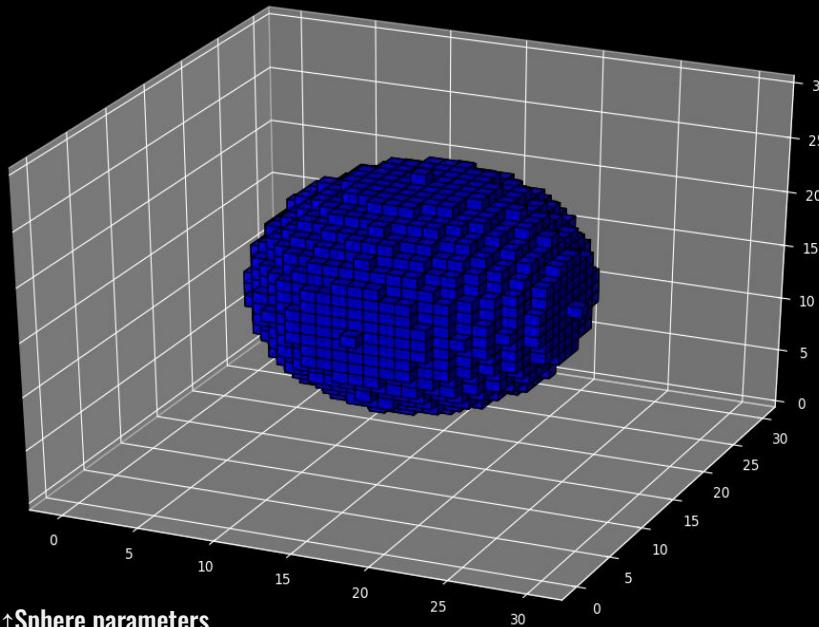


← High-resolution PSF model,  
lateral view



↑PSF model, 3D view

# Model 3D-object



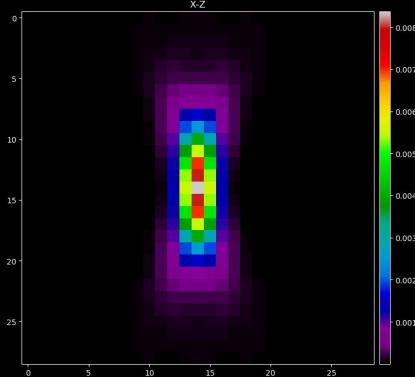
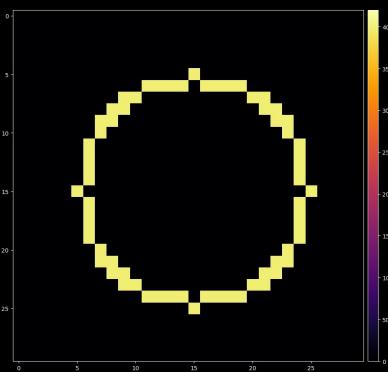
↑Sphere parameters

Shape: 30 x 30 x 30 px

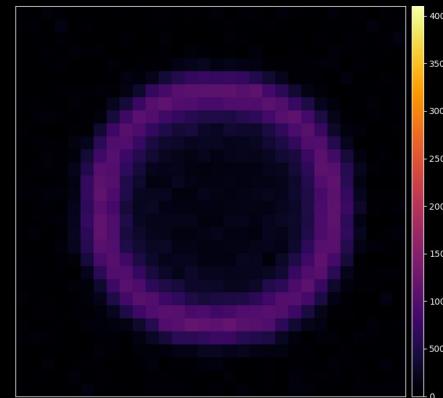
$R_{sphere}$ : 10px

Wall: 1px

# Model Object Convolution & Noise



+ NOISE =



## PSF parameters

Shape: z = 15 px, r = 15 px (30 x 30 x 30 px)

Dims: z = 1.5 um, r = 1.5 um (100 nm/px)

NA = 1.0

Ref. index = 1.333

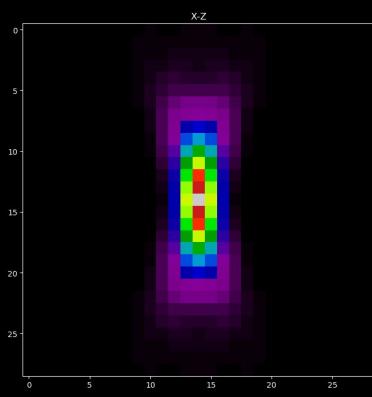
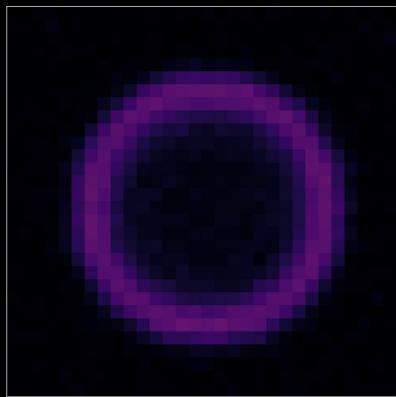
M = 60X

Pinhole radius = 0.25 mm

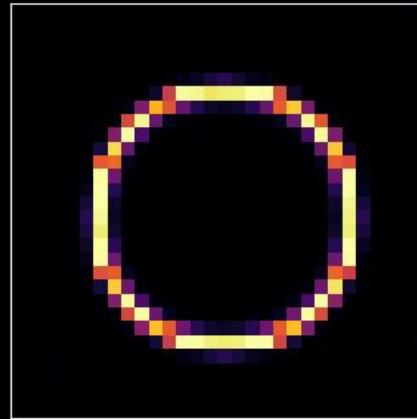
## Noise parameters

Noise generation algorithm: random gaussian noise

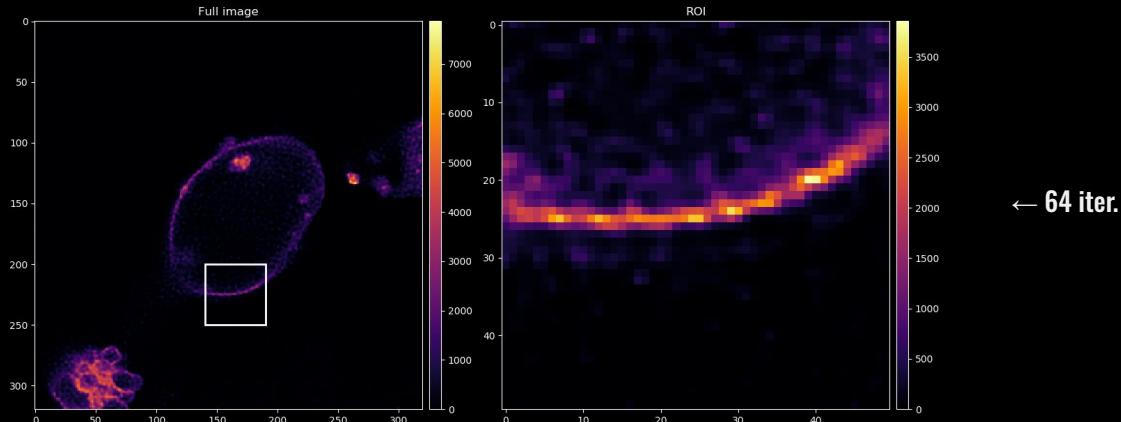
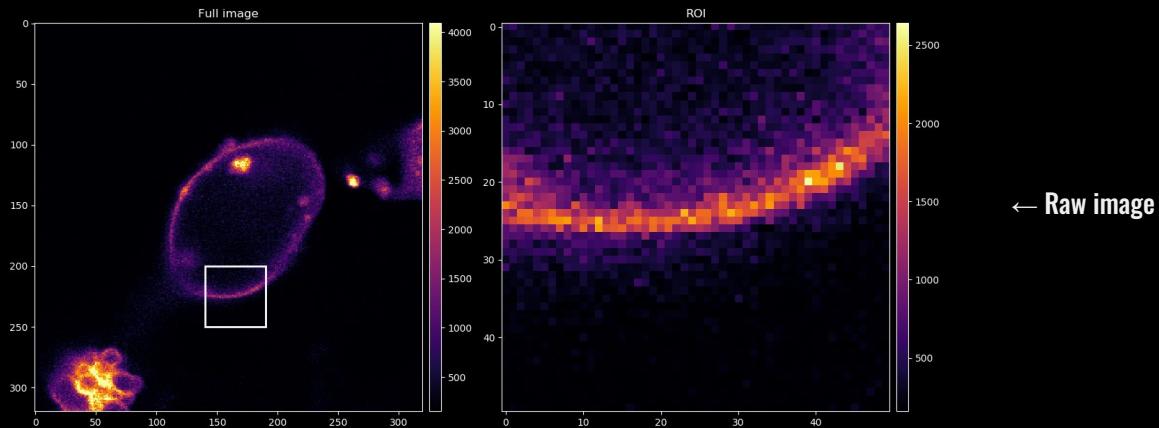
# Deconvolution



=

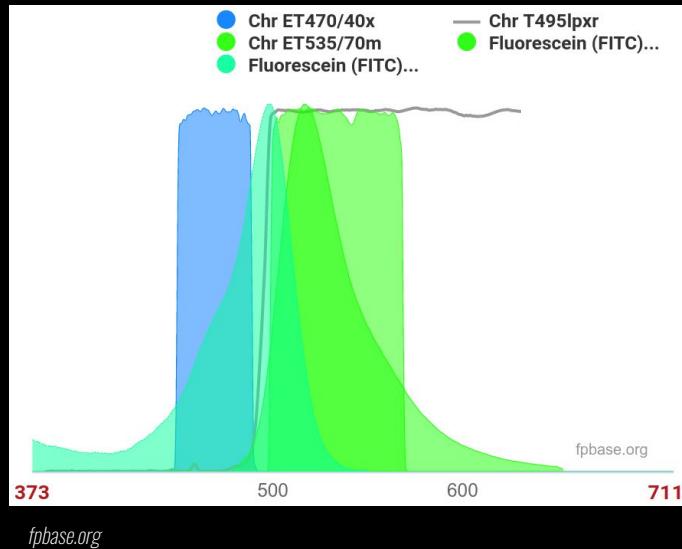
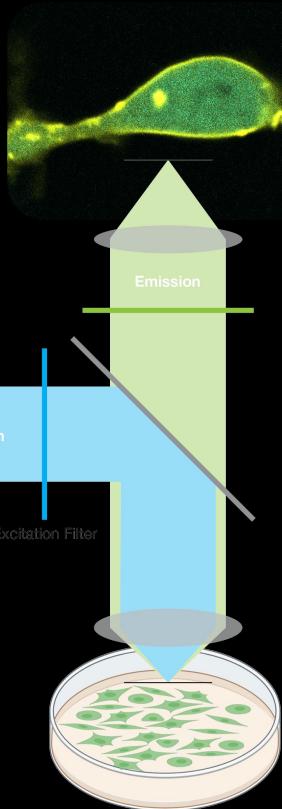


# Experimental Results

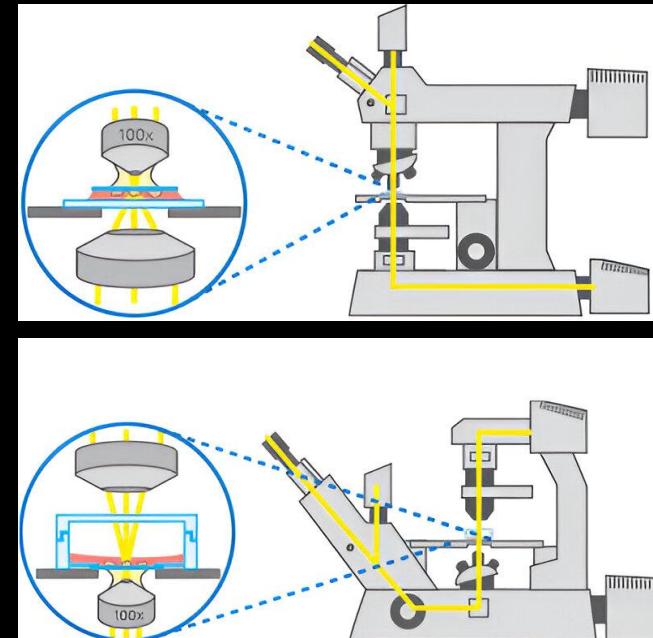


# Fluorescence Microscopy Technics

# Widefield Epifluorescence Microscopy

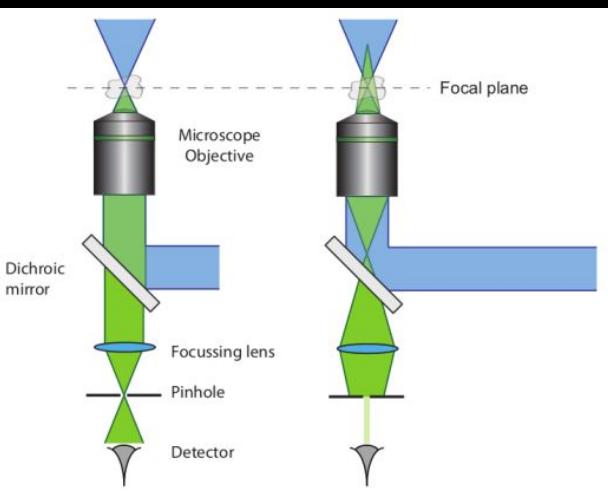
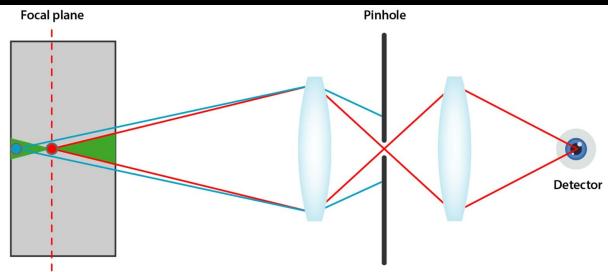


↑Set of optical elements for detection of fluorescein fluorescence



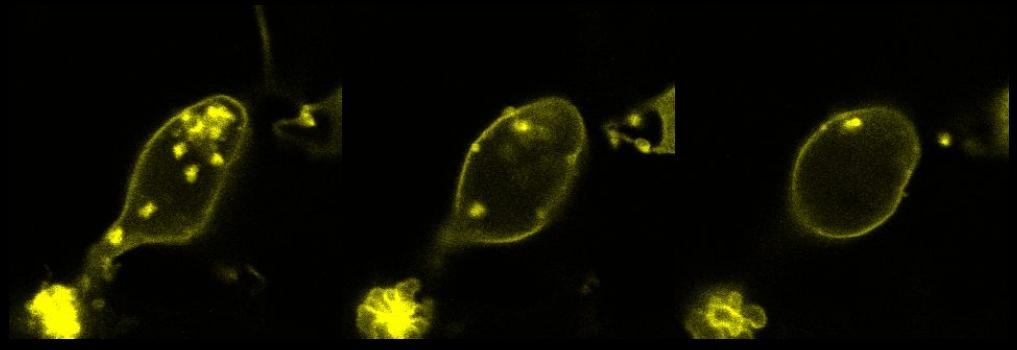
↑Optical scheme of upright (top)  
and inverted (bottom) microscopes

# Confocal Microscopy

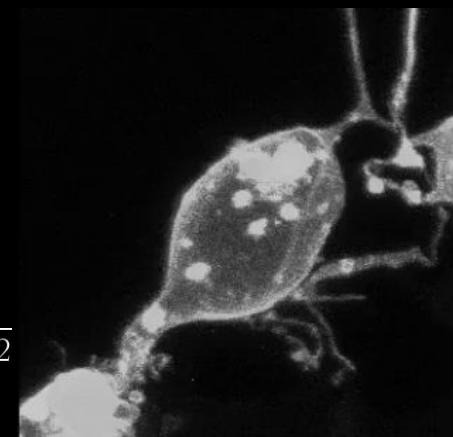


$$d_z \approx \sqrt{\left( \frac{0.88 \cdot \lambda_{ex}}{n - \sqrt{n^2 - NA^2}} \right)^2 + \left( \frac{\sqrt{2} \cdot n \cdot D_{C.A.}}{NA} \right)^2}$$

↑Axial resolution of confocal system



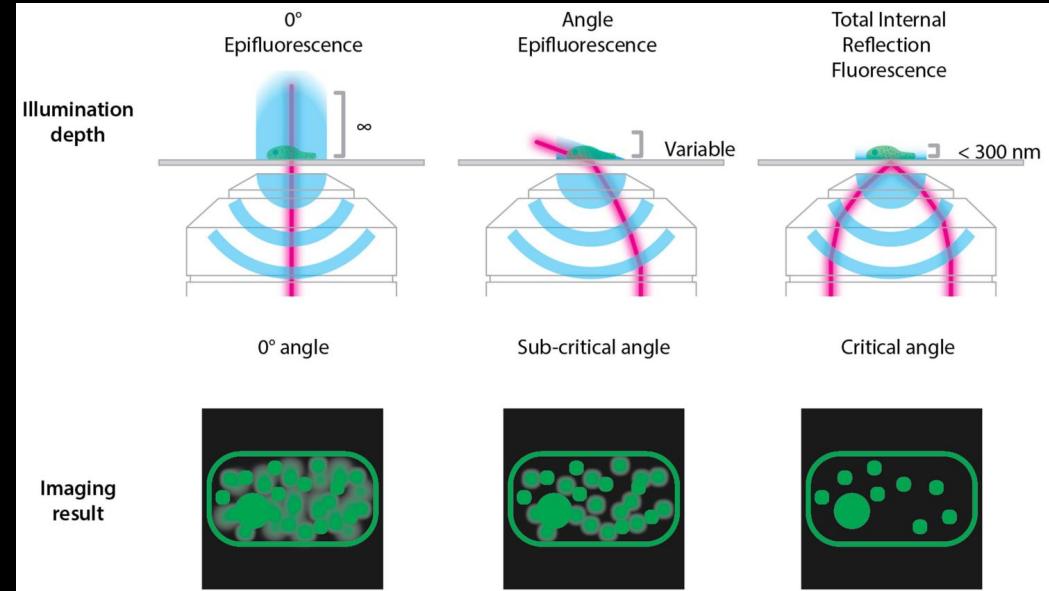
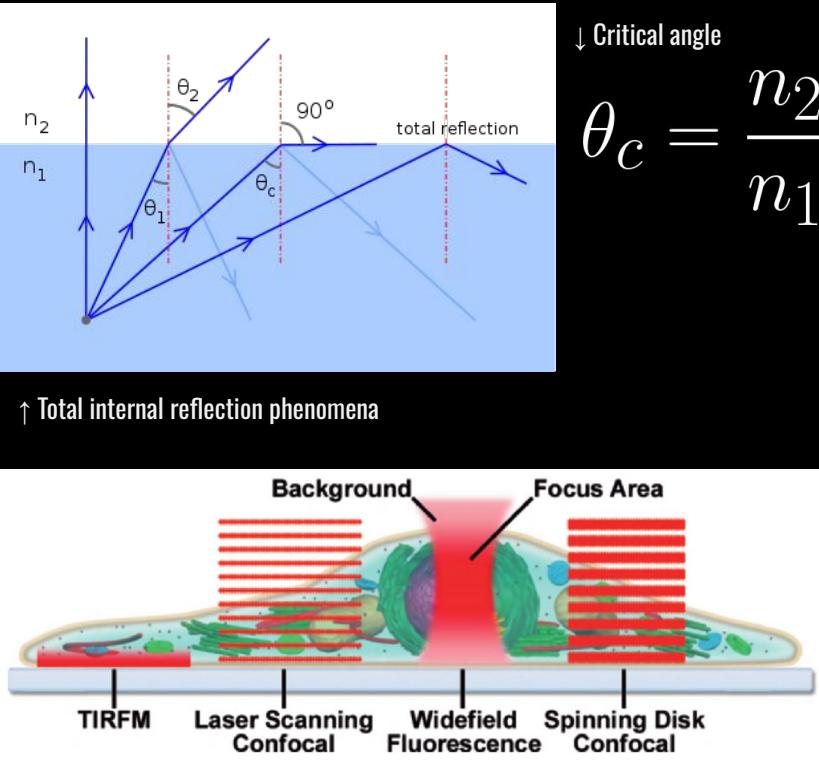
↑→ Optical sections and  
3D-reconstruction  
of z -stack.  
HEK 293 cell transfected with  
EYFP-Mem



Sheremet, 2018, unpublished

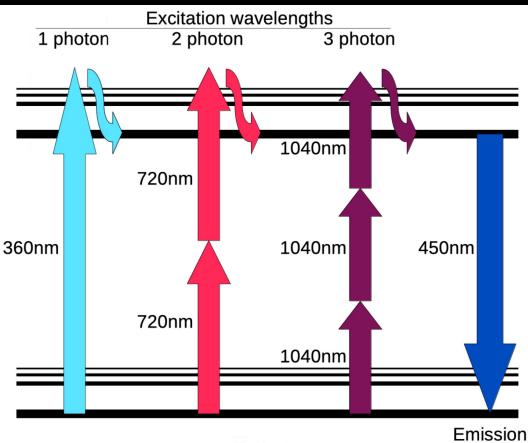
↑Confocal microscope schematic

# TIRF (Total Internal Reflection Fluorescence) Microscopy

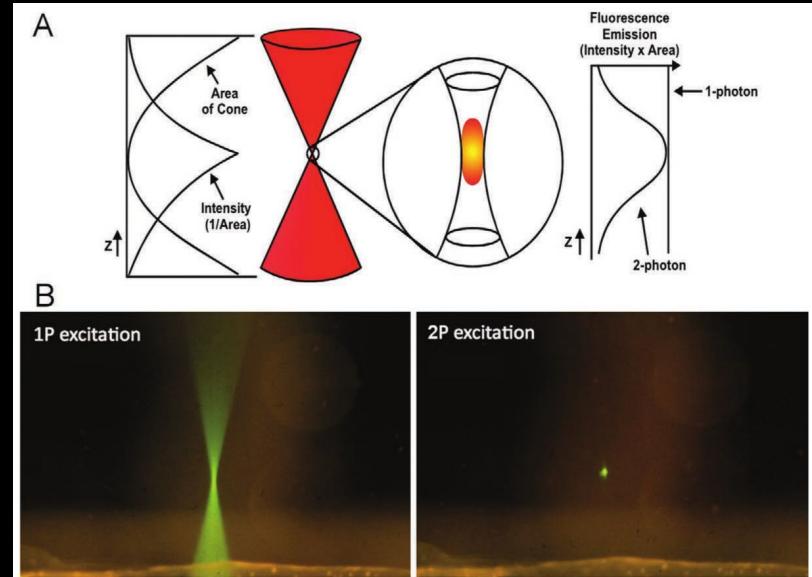
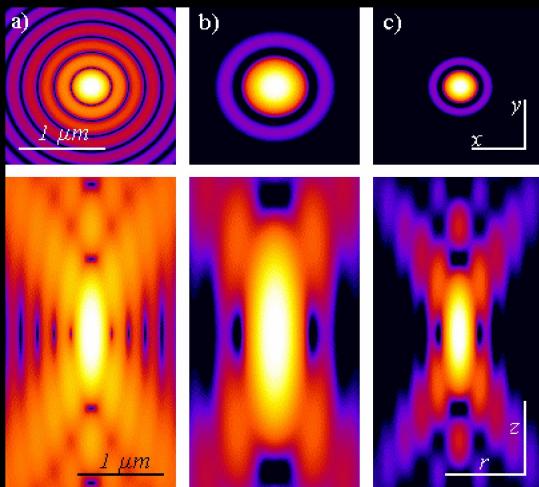


Vangindertael et al. 2018, <https://doi.org/10.1088/2050-6120/aaa0c>

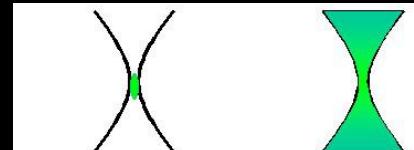
# Two Photon Excitation (2P) Microscopy



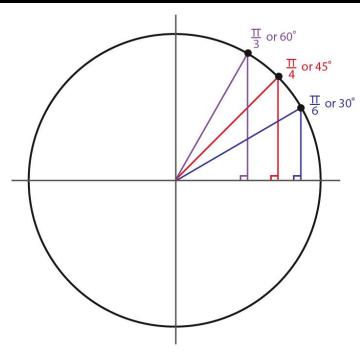
↑Jablonski diagram of multiphoton excitation



↑→  
Excitation volume



# Solid Angle & Maximal Possible NA values



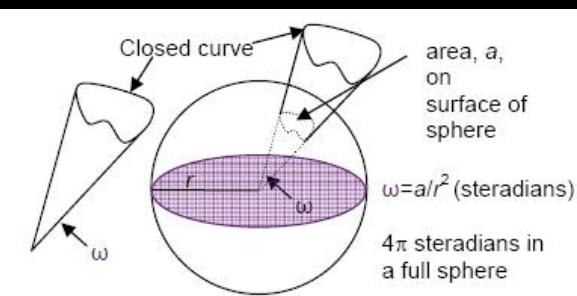
$$L = 2\pi R$$

$$\Omega = 2\theta$$

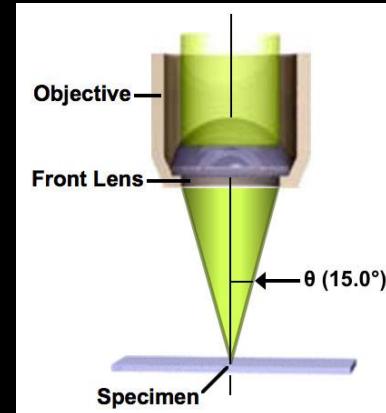
$$NA = n \cdot \sin \left( \frac{\Omega}{2} \right)$$

$$NA = n \cdot \sin 90^\circ = n$$

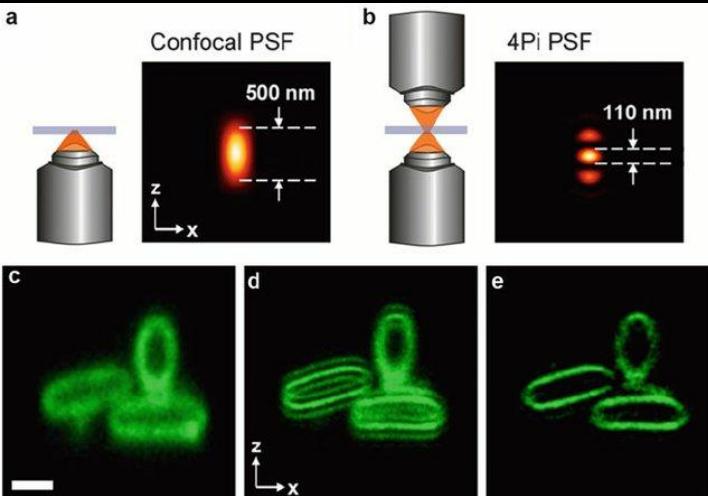
$$n_{water} = 1.33 \quad n_{oil} \approx 1.5$$



↑Solid angle definition



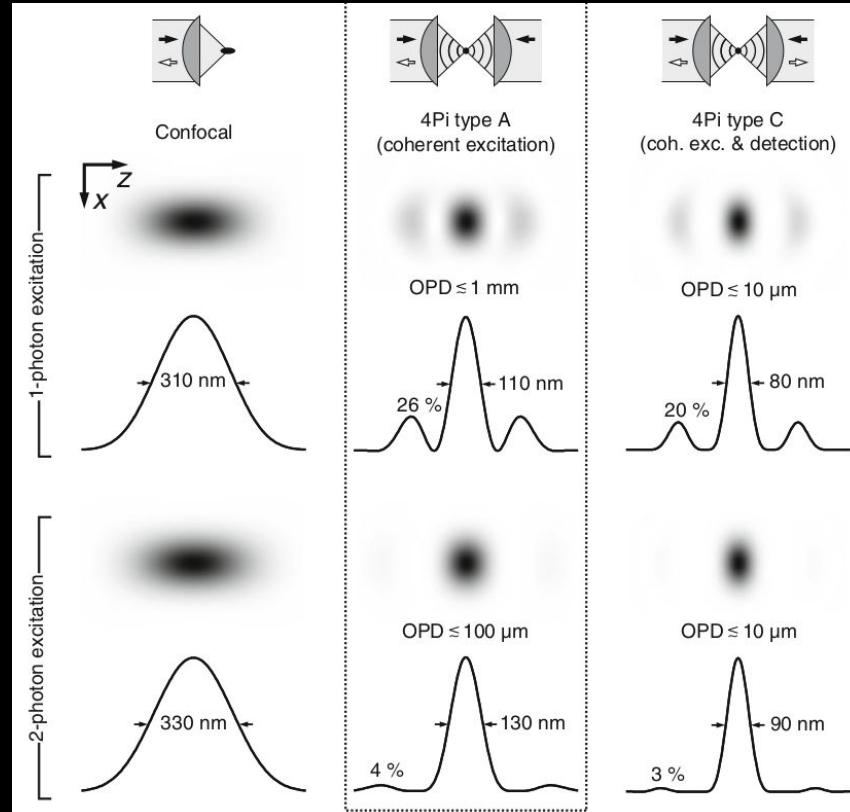
# $4\pi$ (4Pi) Microscopy



Hedde and Nienhaus, 2013, DOI 10.1007/978-94-007-5313-6\_4

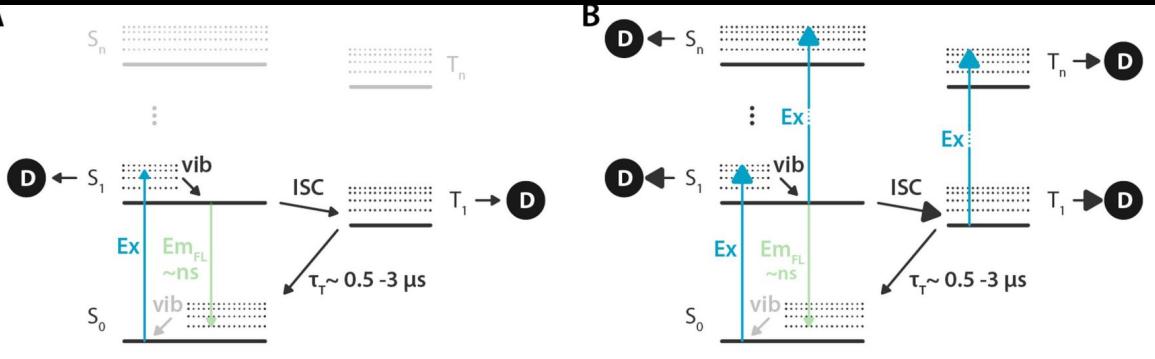
## ↑4Pi microscopy advantages

→ Overview of the modes of operation  
of a 4Pi microscope and its effective PSF size

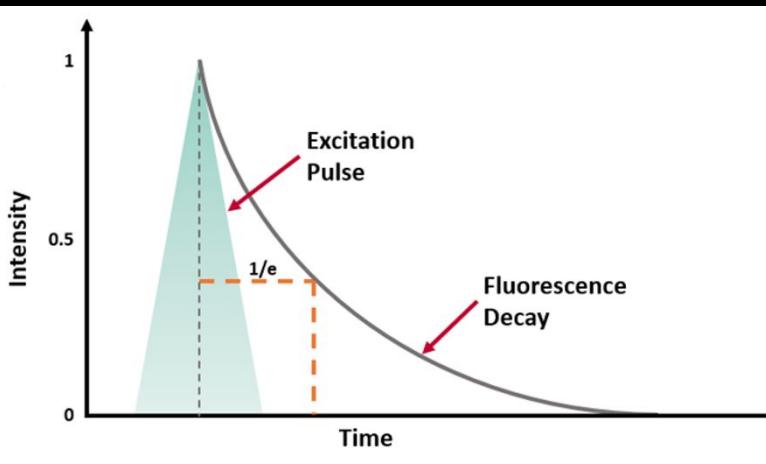


Schmidt et al., 2012, DOI 10.1007/978-1-62703-137-0\_3

# Fluorescence Lifetime. Exponential curve



← Simplified Jablonski energy diagram showing multiple relaxation pathways to exit the excited state

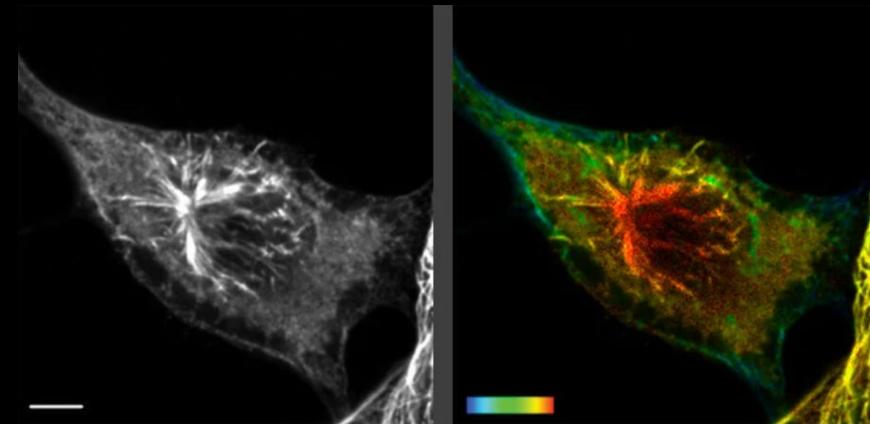
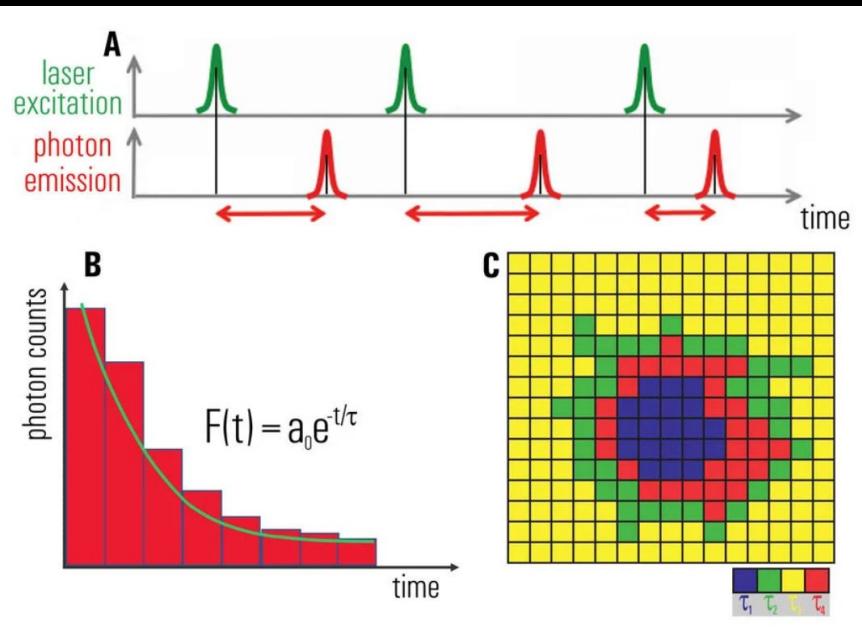


→ Fluorescence intensity decay equation

$$I(t) = I_0 e^{-t/\tau}$$

← Fluorescence intensity decay following a short excitation pulse

# FLIM (Fluorescence Lifetime Imaging Microscopy)



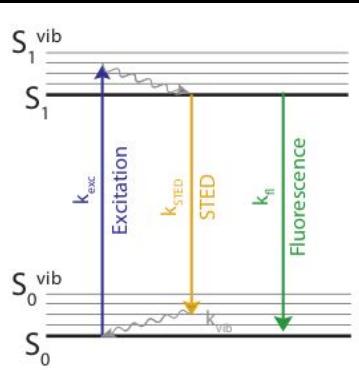
↑Intensity image vs. FLIM image.  
 $\alpha$ -tubulin and vimentin immunostaining

Leica Microsystems

↑FLIM imaging principle diagram

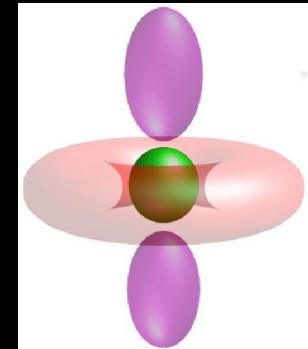
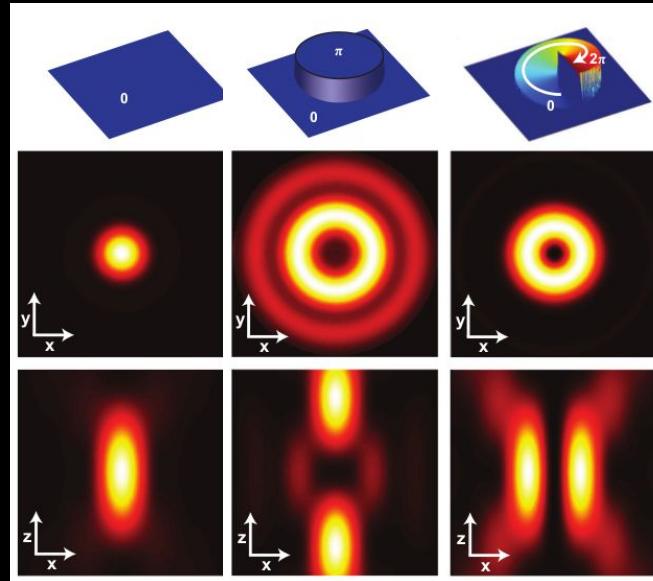
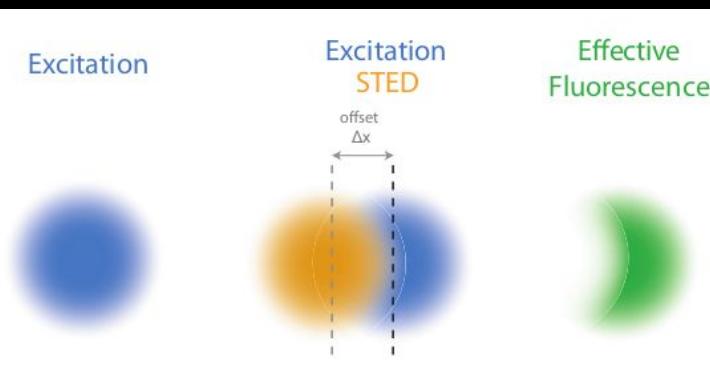
# Super-Resolution Microscopy Technics

# STED (Stimulated Emission Depletion) Microscopy

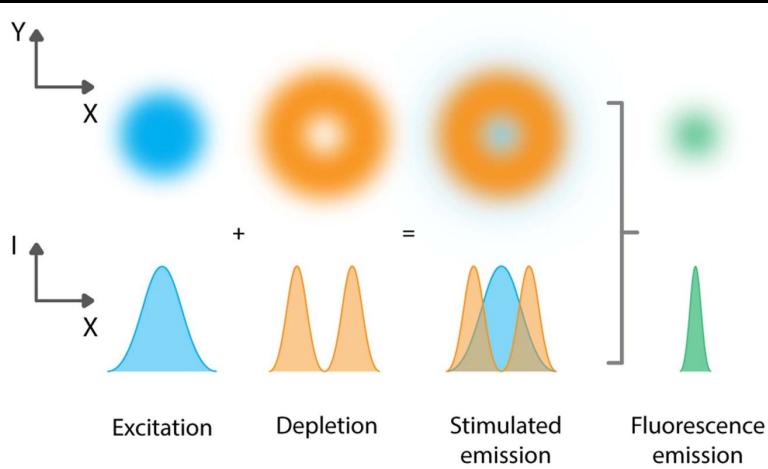


← Jablonski diagram illustrating the process of excitation, stimulated emission and fluorescence

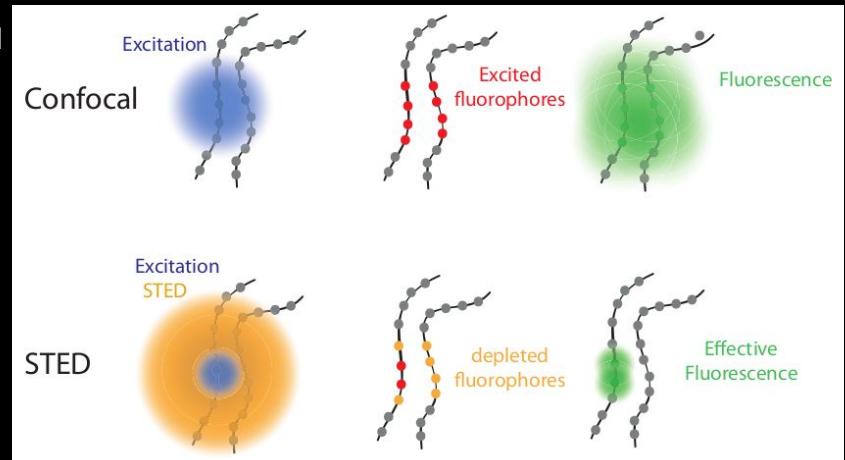
↓ Depletion to the ground state by stimulated emission in two diffraction limited spots



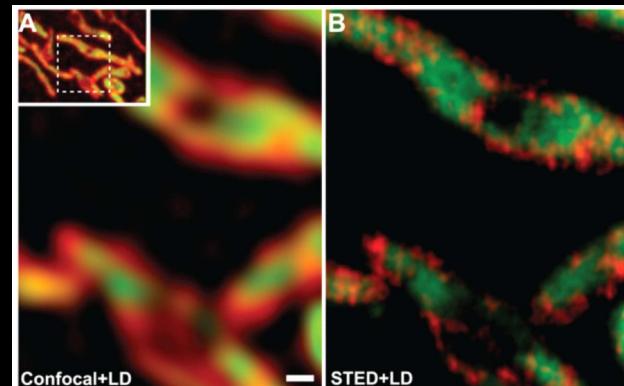
# STED (Stimulated Emission Depletion) Microscopy



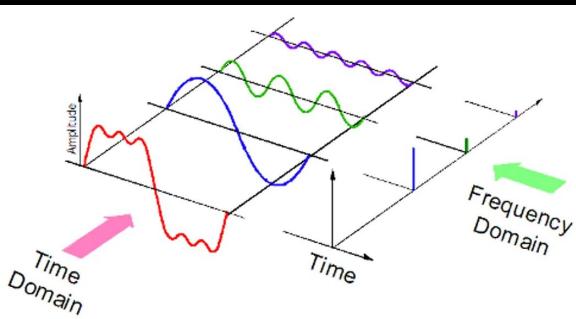
→ Illustration of the spatial resolution increase by STED



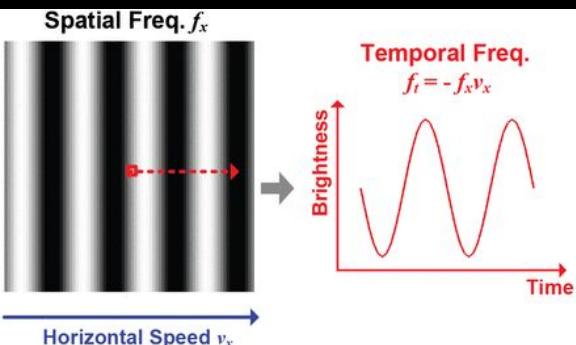
Overlapping a doughnut-shaped STED spot with a diffraction limited excitation spot will result in a confinement of the area in which fluorophores can fluoresce



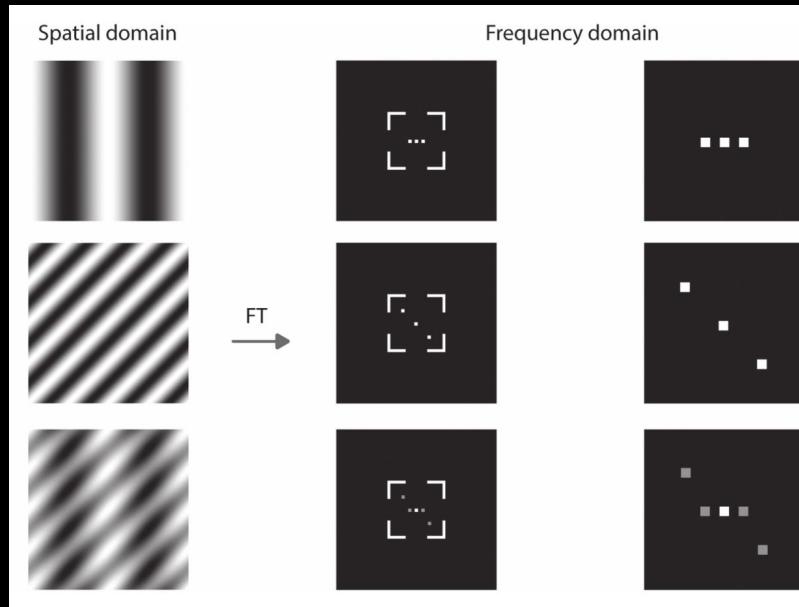
# Images in Frequency Domain



↑↓ Concepts of temporal and spatial frequencies



[sbme-tutorials.github.io/2018/cv/notes/3\\_week3.html](https://sbme-tutorials.github.io/2018/cv/notes/3_week3.html)



Vangindertael et al. 2018, <https://doi.org/10.1088/2050-6120/aaaec0>

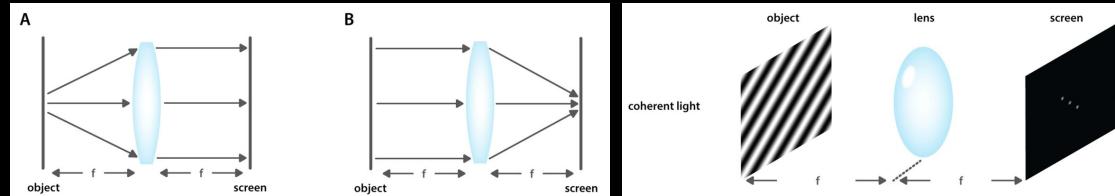
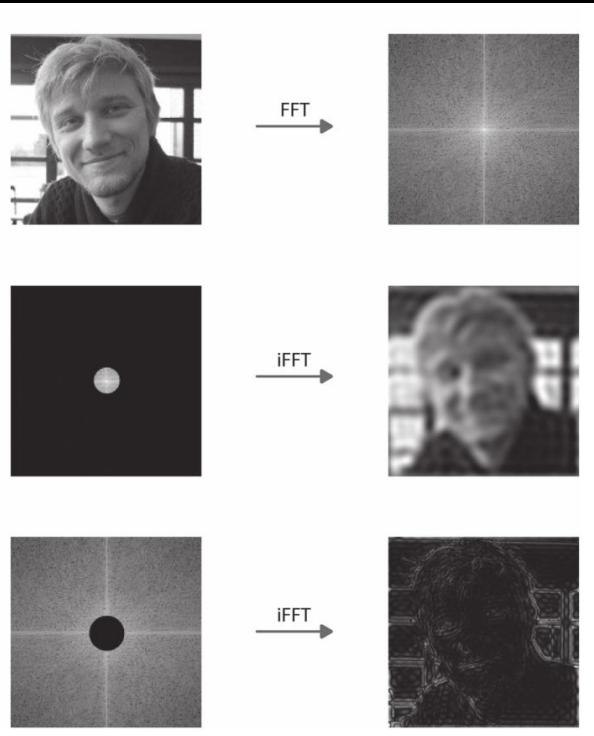
↑Fourier transform of model 2D images



$$\mathcal{F}$$

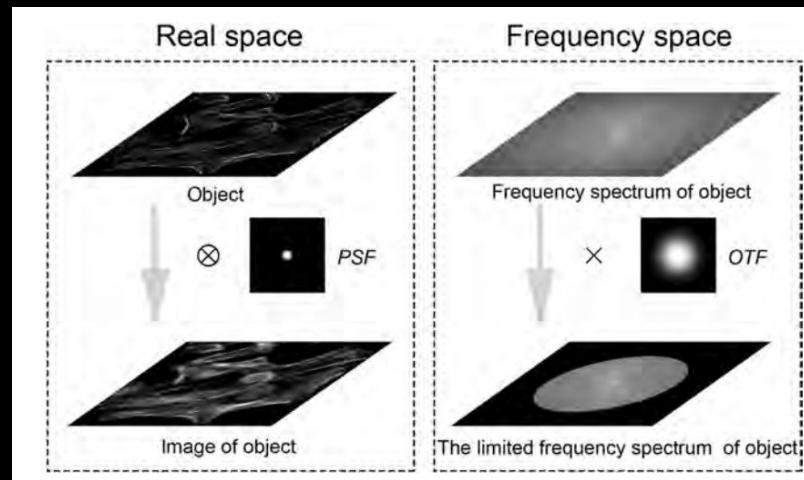


# PSF is a Cookie Filter in Frequency Domain



← Localization of different features on frequency-domain image

→ Convolution with PSF in the spatial domain corresponds to multiplication with OTF (optical transfer function) in the frequency domain

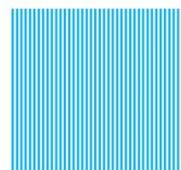
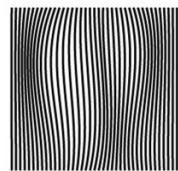


# SIM (Structured Illumination Microscopy)

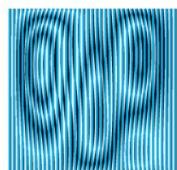
Sample

Illumination

Moiré image

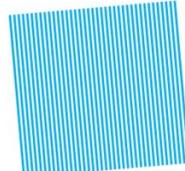


=

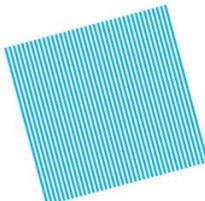
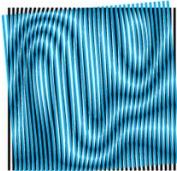


→ The concept of resolution enhancement in structured illumination

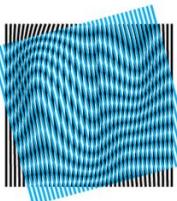
+



=



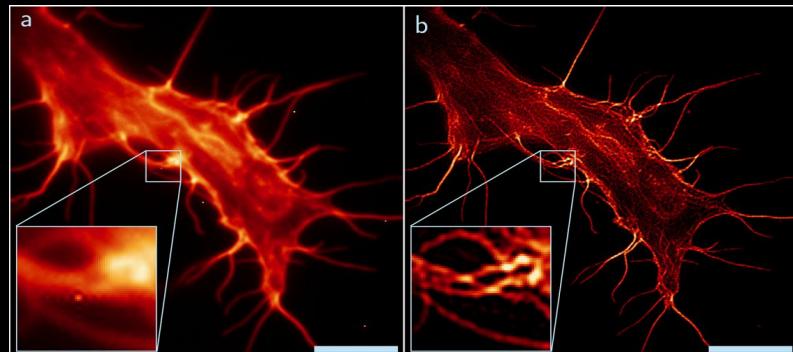
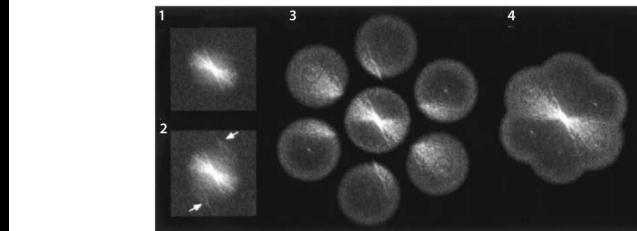
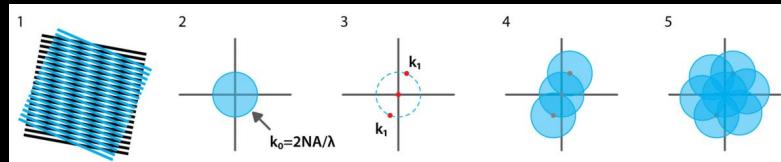
=



Vangindertael et al. 2018, <https://doi.org/10.1088/2050-6120/aaaec0>

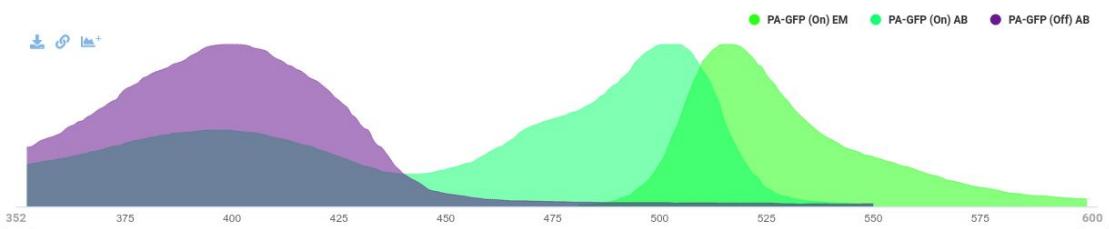
↑ Well-defined periodic illumination provokes the appearance of Moiré fringes with lower spatial frequencies

→ Actin fibers in HEK293 as seen by wide field (a) and SIM (b)



Sandmeyer et al. 2021, <https://doi.org/10.1021/acspolymers.Oc01937>

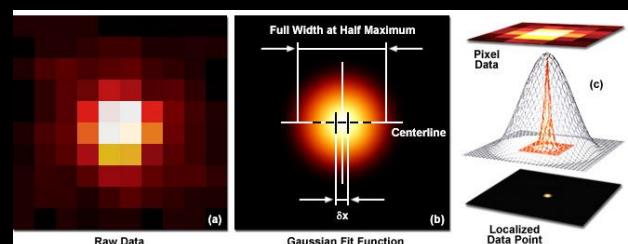
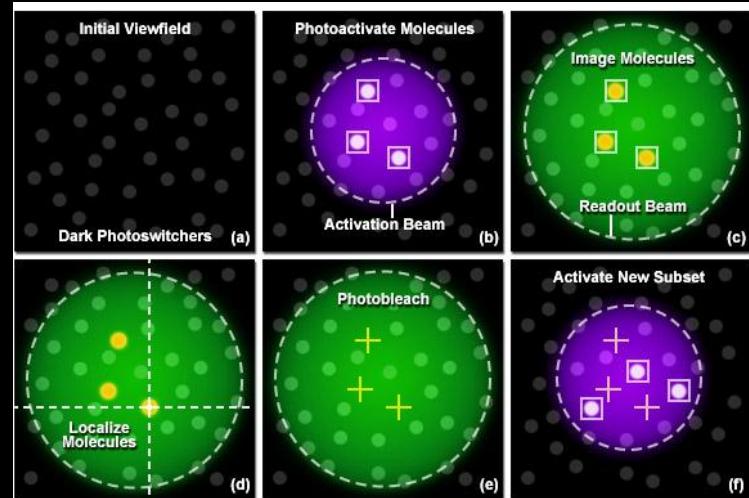
# PALM (PhotoActivated Localization Microscopy) and STORM (STochastic Optical Reconstruction Microscopy)



fpbase.org

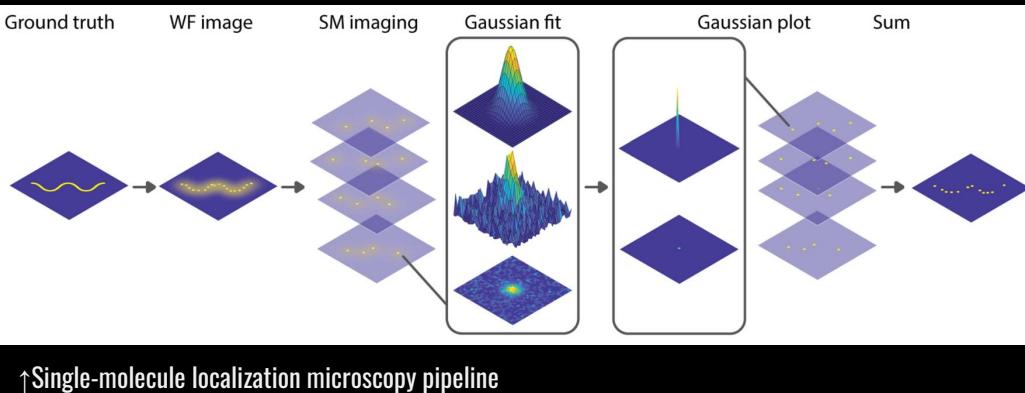
↑ Spectra of the photoactivatable green fluorescence protein (PA-GFP)

→ Principle of single-molecule localization microscopy (SMLM)

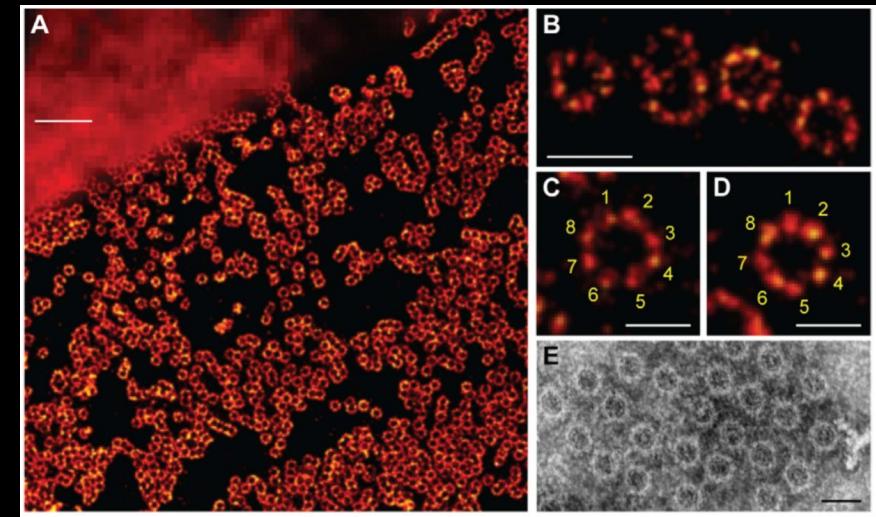


→ Fitting single-molecule pixel data to a Gaussian function

# PALM (PhotoActivated Localization Microscopy) and STORM (STochastic Optical Reconstruction Microscopy)

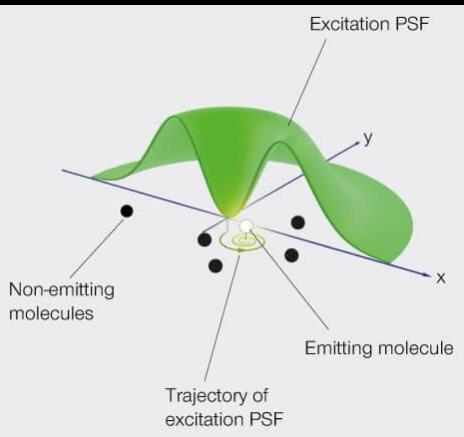


↑Single-molecule localization microscopy pipeline



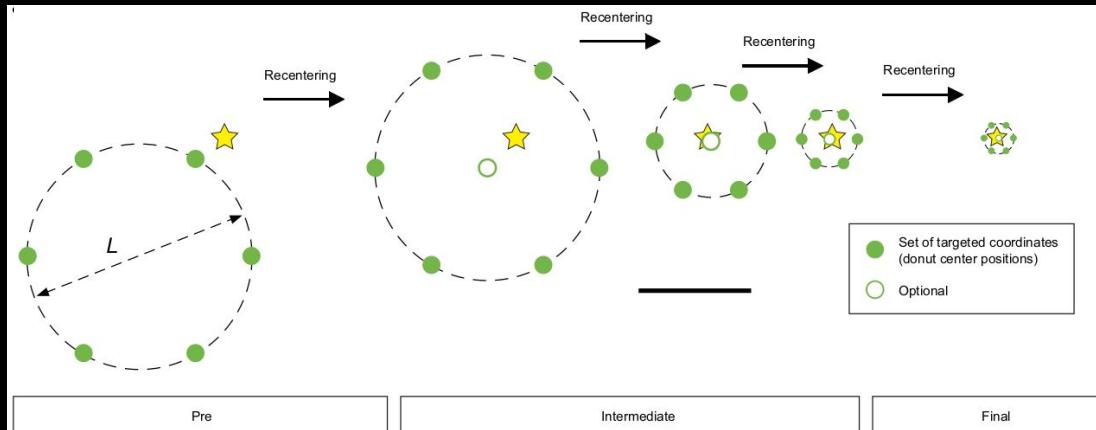
↑dSTORM visualization (A-D) and electron microscopy image (E) of nuclear pore structure

# MINFLUX (MINimal photon FLUXes) Microscopy



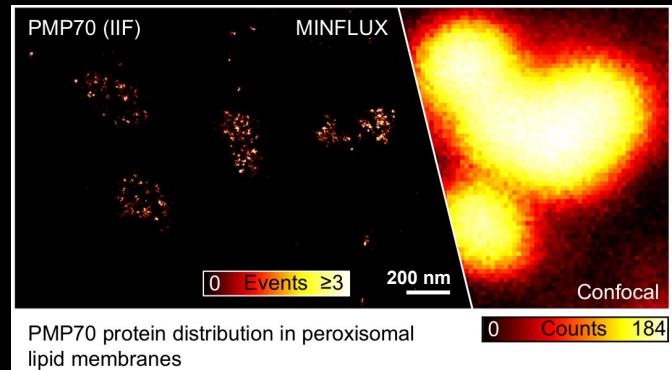
abberior.rocks

↑ Principle of MINFLUX single molecule localization



↑ Targeted coordinate patterns (TCP)  
in sequential iterations for imaging  
and fast single-molecule tracking

→ Example of MINFLUX imaging



THANK YOU!