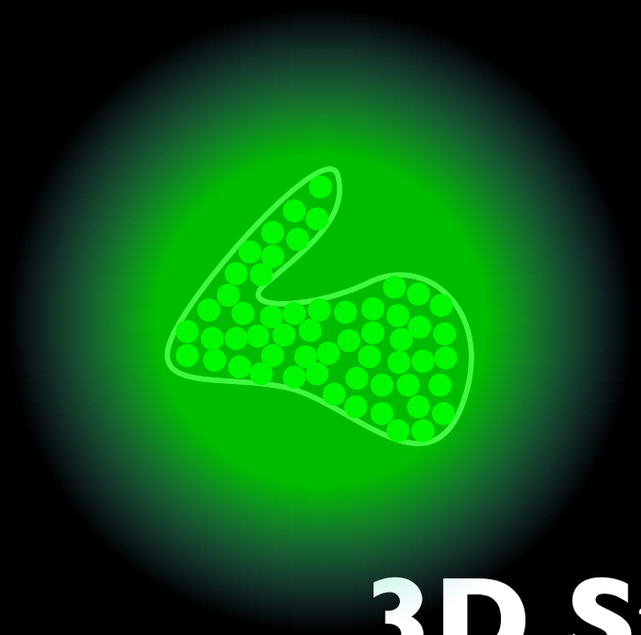
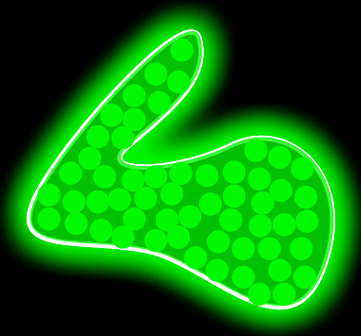




3D Structured Illumination Microscopy

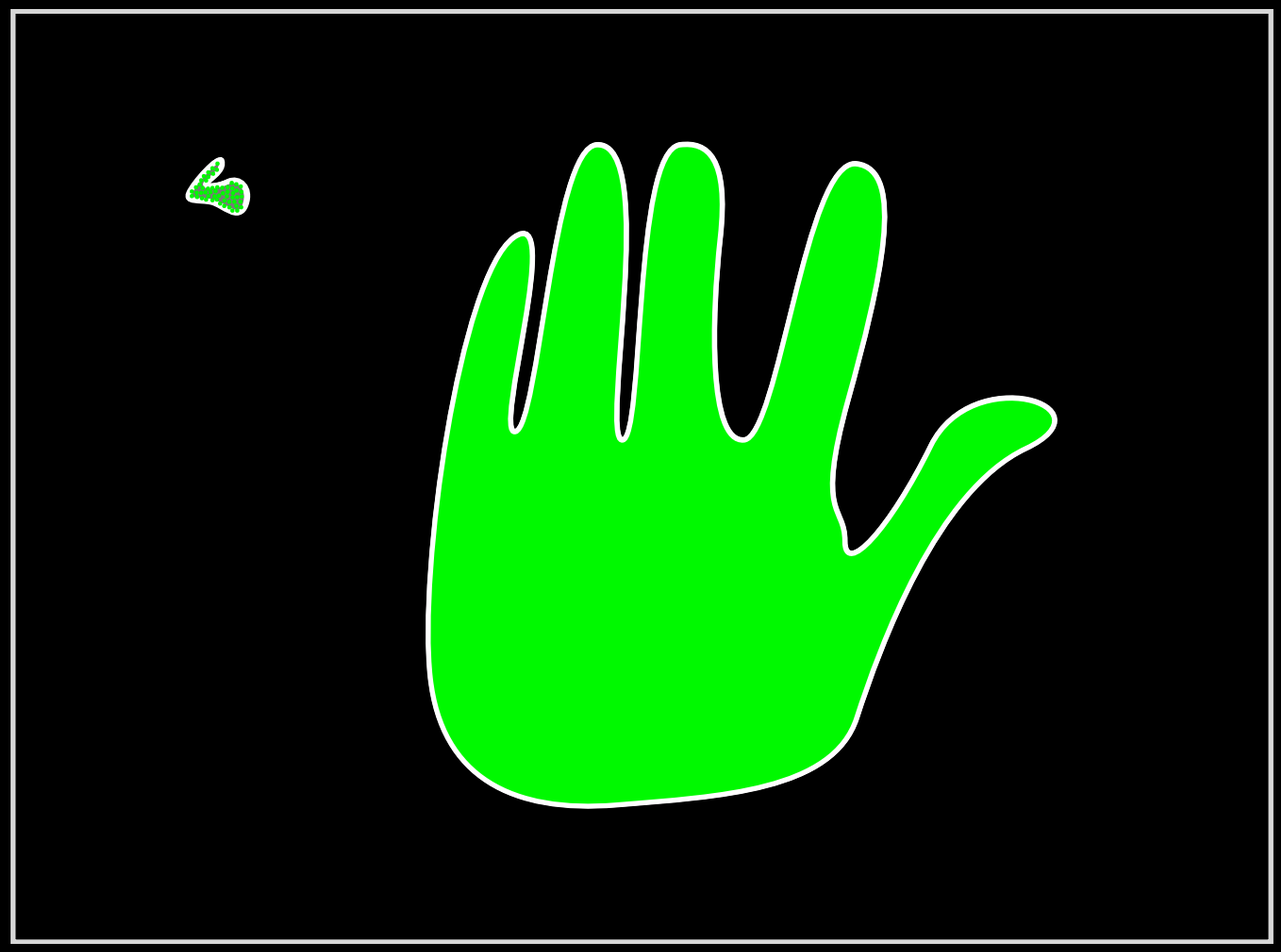
Julio Mateos Langerak

November 2014

BioCampus Montpellier

MRI Montpellier RIO Imaging

The resolution problem
Causes of the "blur"



The resolution problem
Causes of the "blur"

The resolution problem
Causes of the "blur"

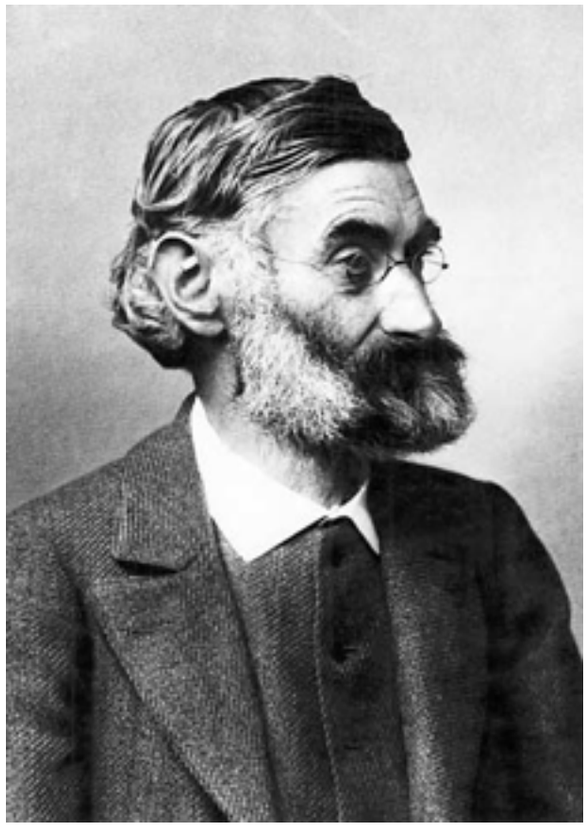
- Optical aberrations: *Aim to use perfect optics*
- Optical properties of sample environment:
  Careful choice of coverslip, staining quality, mounting and immersion medium
- Signal to noise ratio: *Strong (high QE) dyes, better cameras/PMTs,...*
- Out of focus light: *Confocal microscopy, de-convolution*
- Light scattering: *Sample close to objective*
- Diffraction of light… Is there something to do?

The resolution problem
Causes of the "blur"

- Optica
- Optica
  Carefu
- Signal
- Out o
- Light s
- Diffrac

medium

...

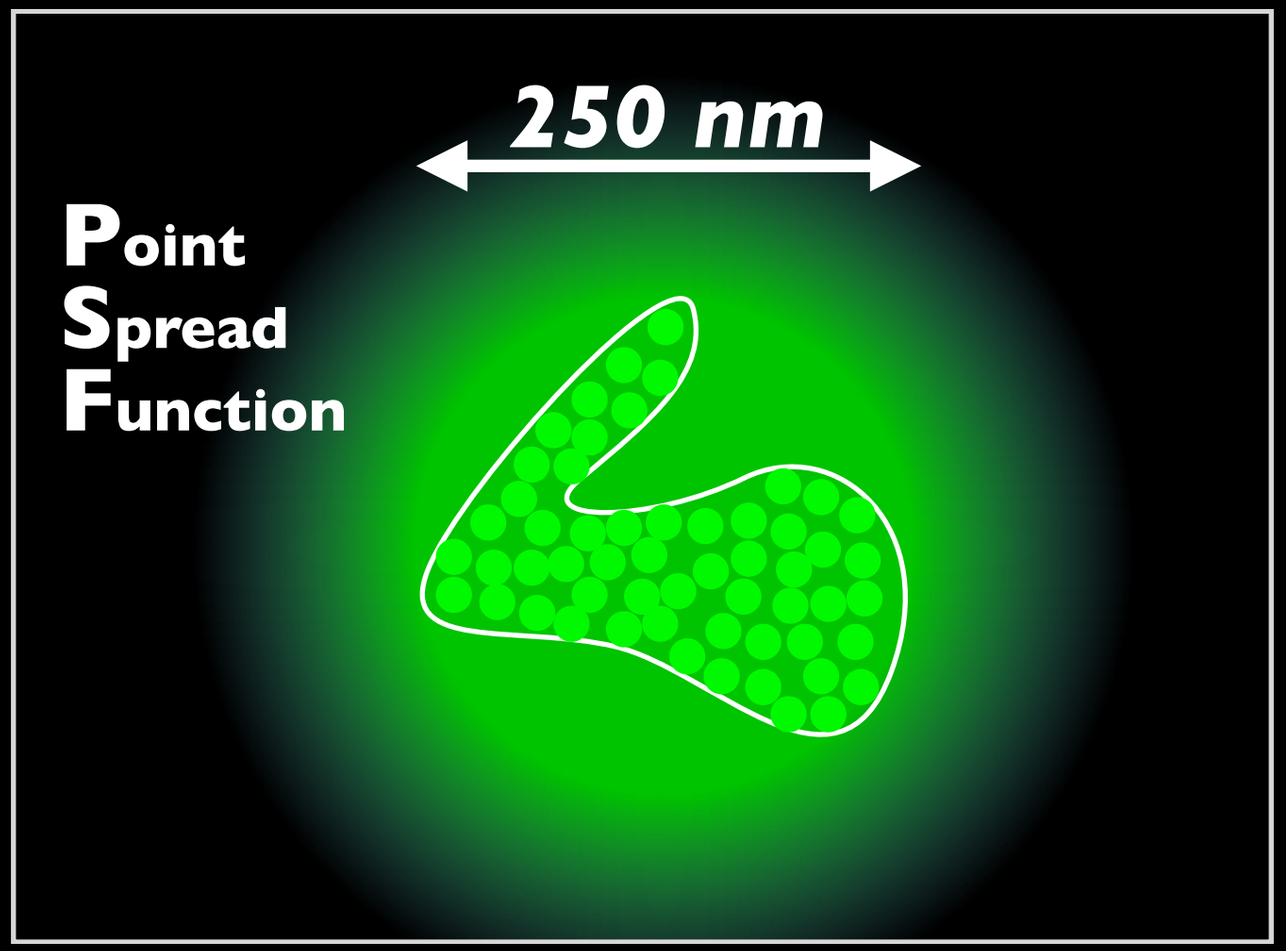


Ernst Abbe's limit

$$\text{resolution} = \frac{0.61\lambda}{NA}$$

Braking the diffraction barrier

The point spread function describes the response of an imaging system to a point source or point object



Braking the diffraction barrier

Keep in dark state some of the fluorophores within your PSF, so you know where light comes from

SIM / 3D-SIM

STED

PALM / STORM

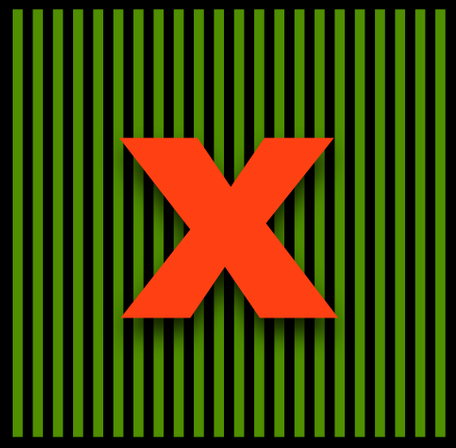
Structured Illumination Microscopy

Projects a structured light pattern onto the sample that interacts with the fluorescent probes to generate interference patterns. By modulating the illumination pattern, collecting and reconstructing the subsequent images you can extract information on the sub-diffraction structures.

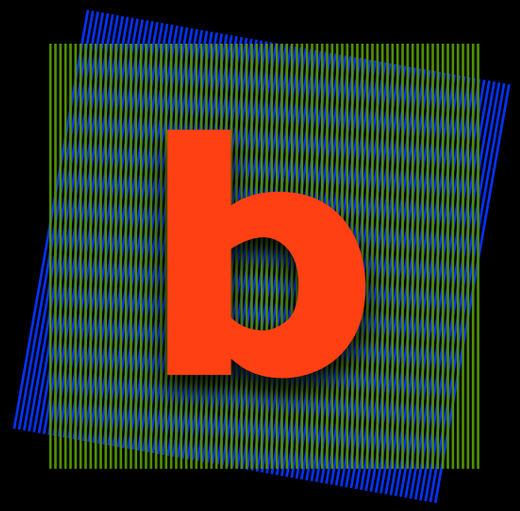
Super-Resolution
J Mateos Langerak
November 2014

The Moire effect

Your sample



What you see under the microscope



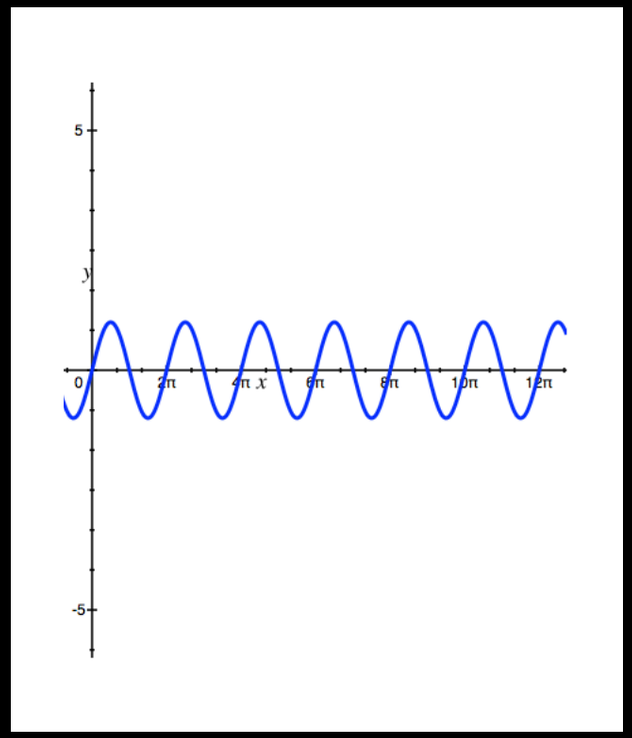
The excitation pattern

$$x = a * b$$

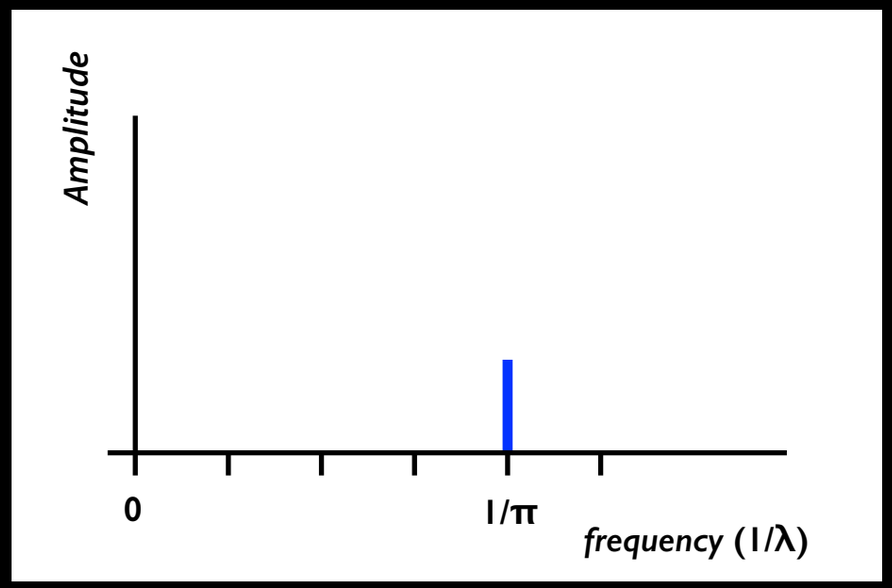
the Fourier space in 1D

$y = \sin x$

Real space



Fourier space



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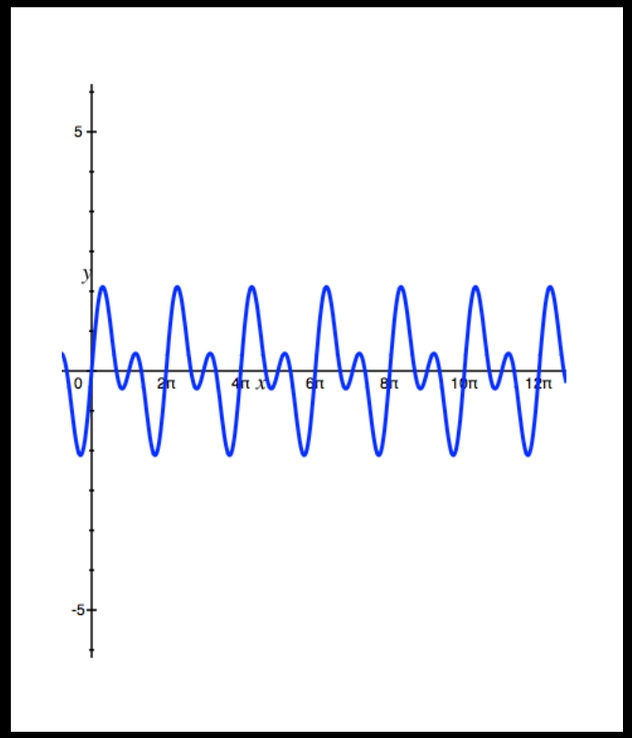
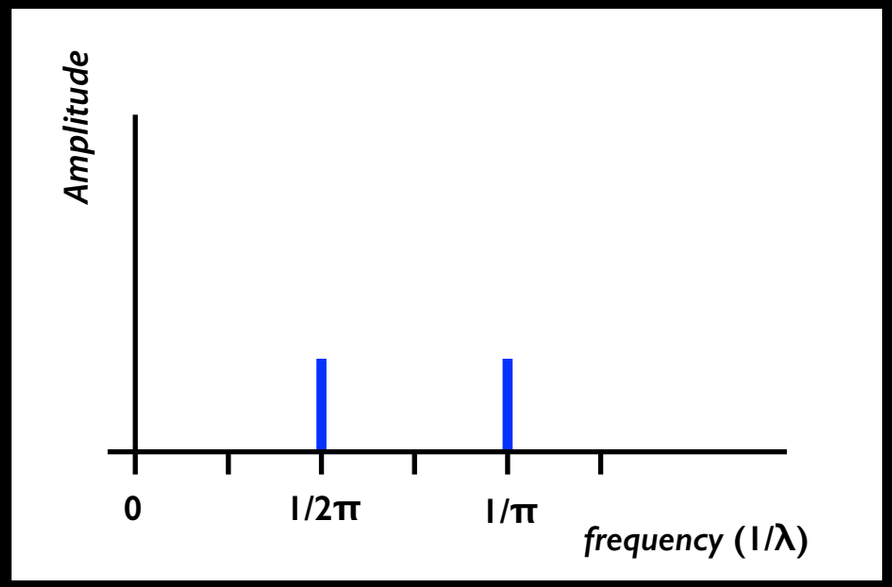
the Fourier space in 1D

$$y = \sin x + \sin 2x$$

Real space



Fourier space



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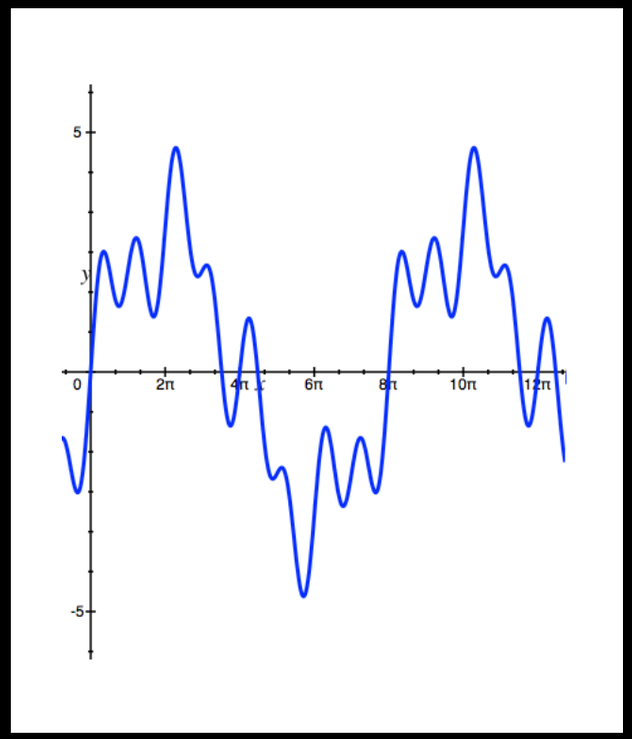
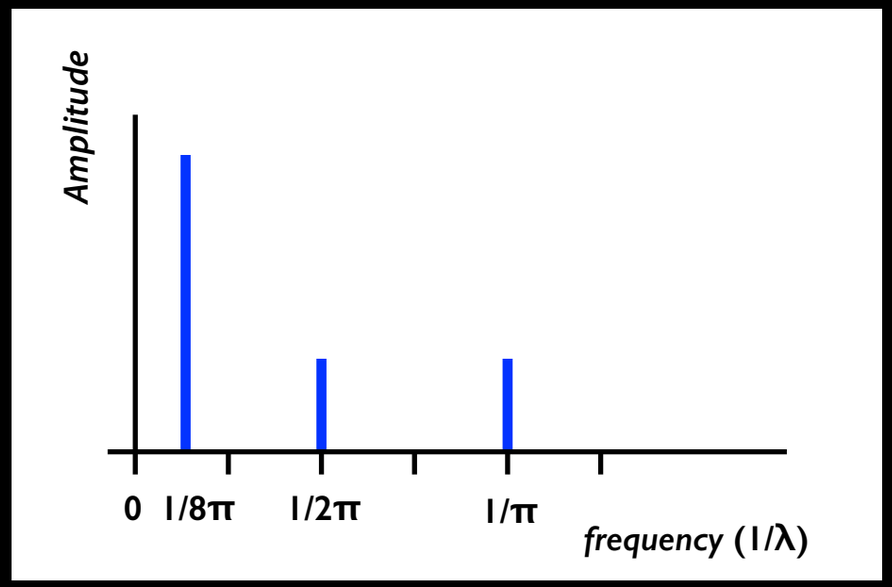
the Fourier space in 1D

$$y = \sin x + \sin 2x + 3 \sin x/4$$

Real space



Fourier space



Super-Resolution
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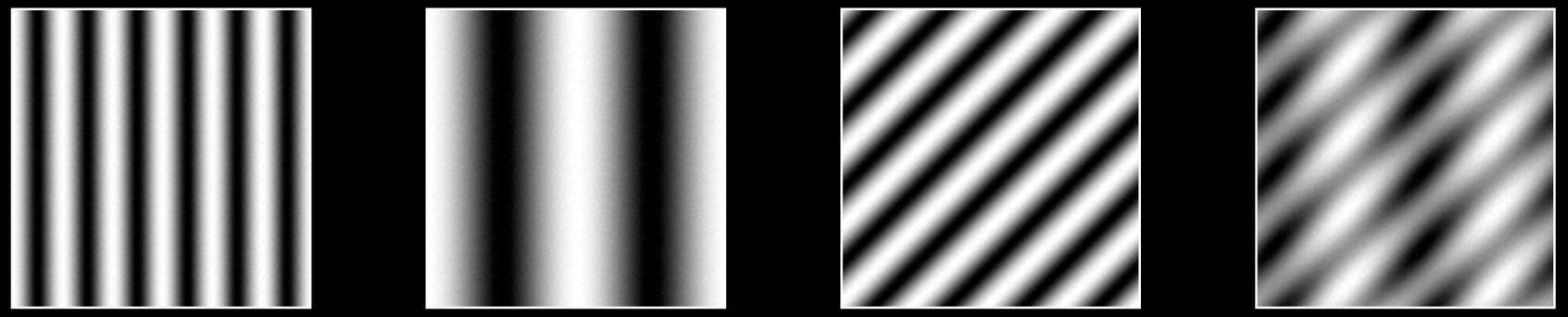
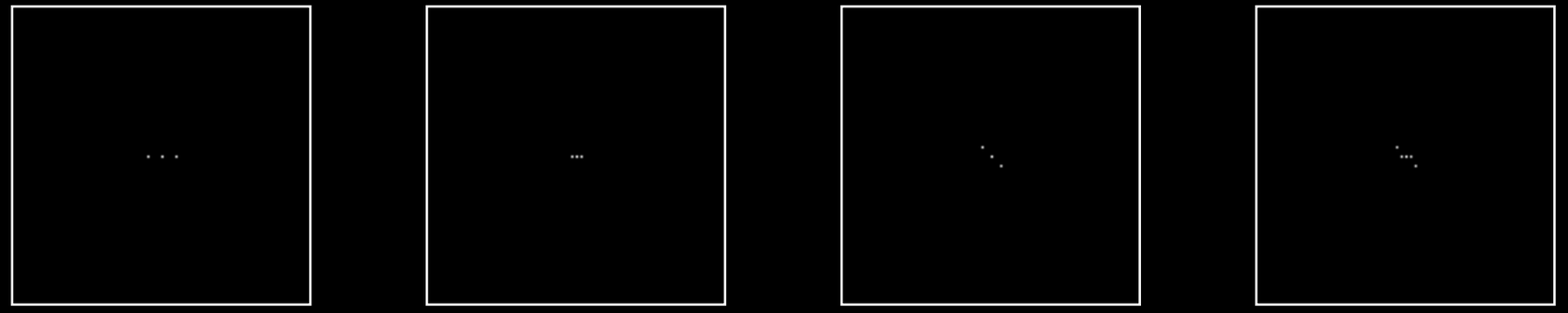
the Fourier space in 2D

Real space



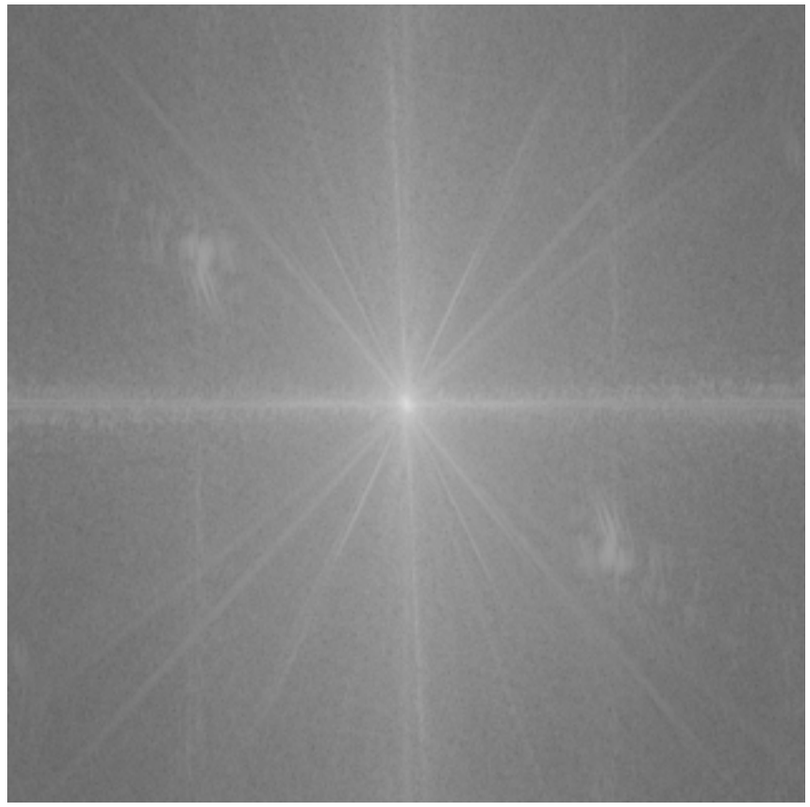
Fourier space



the Fourier space in 2D

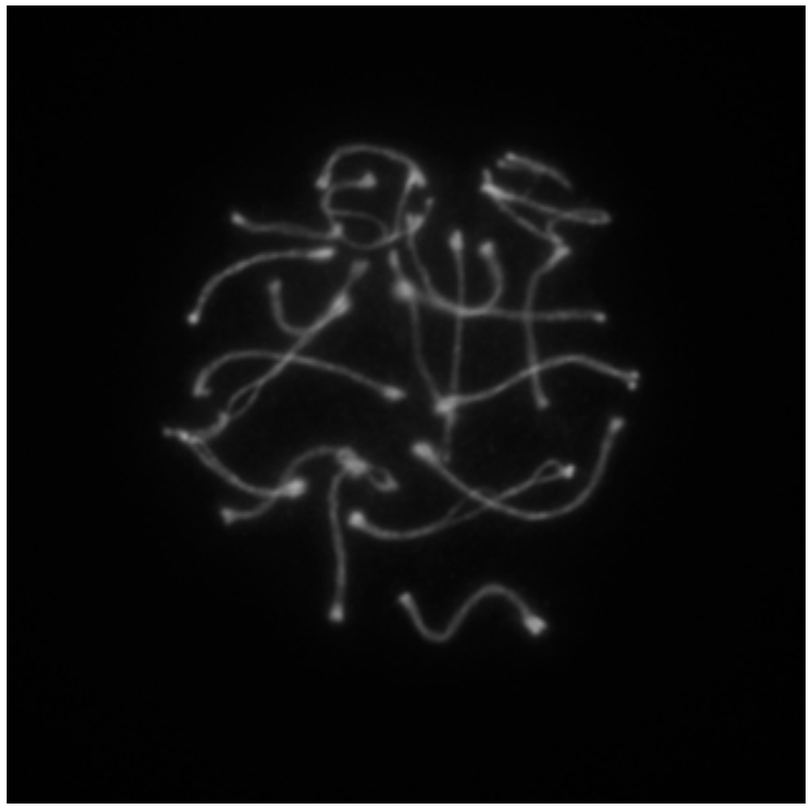
Real space

Fourier space

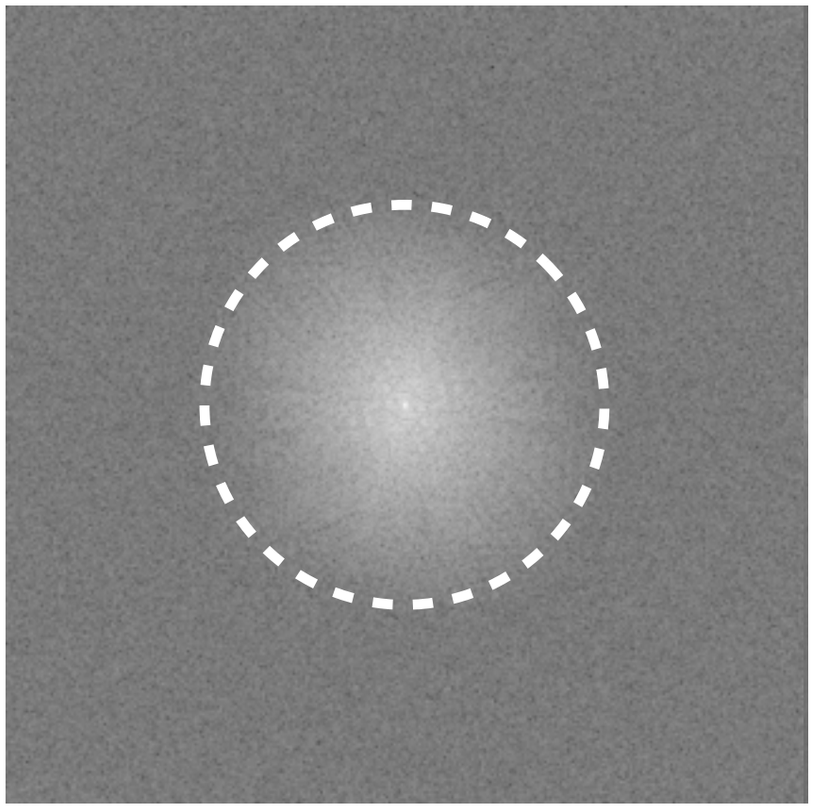


the Fourier space in 2D

Real space

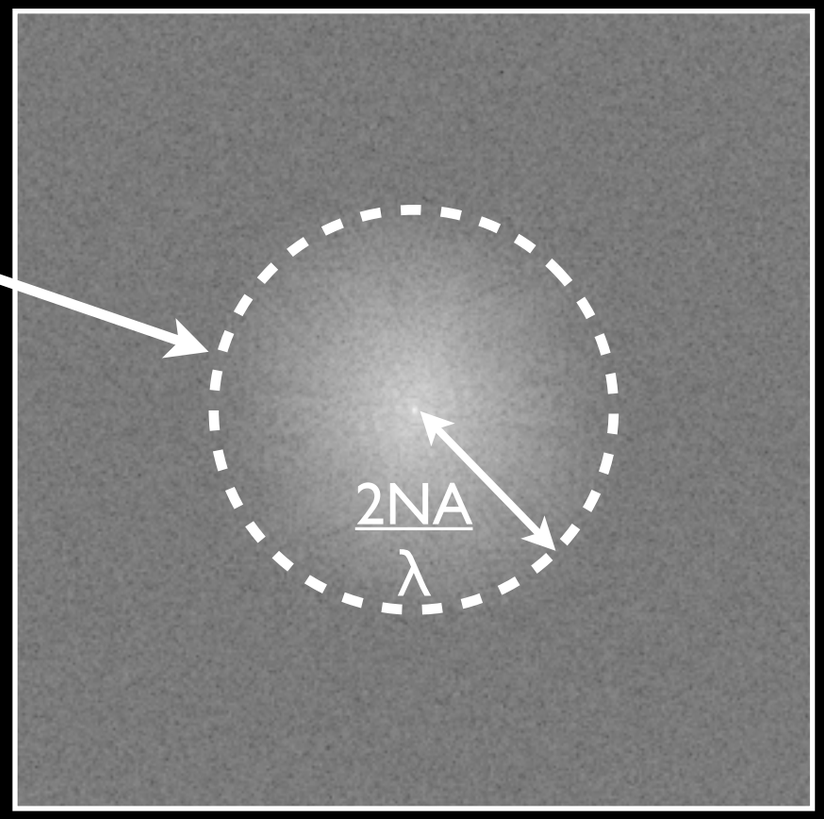


Fourier space



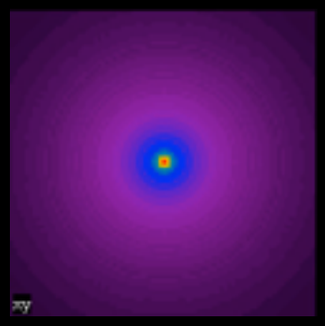
The resolution limit is manifest in the Fourier space

Manifestation of the resolution limit

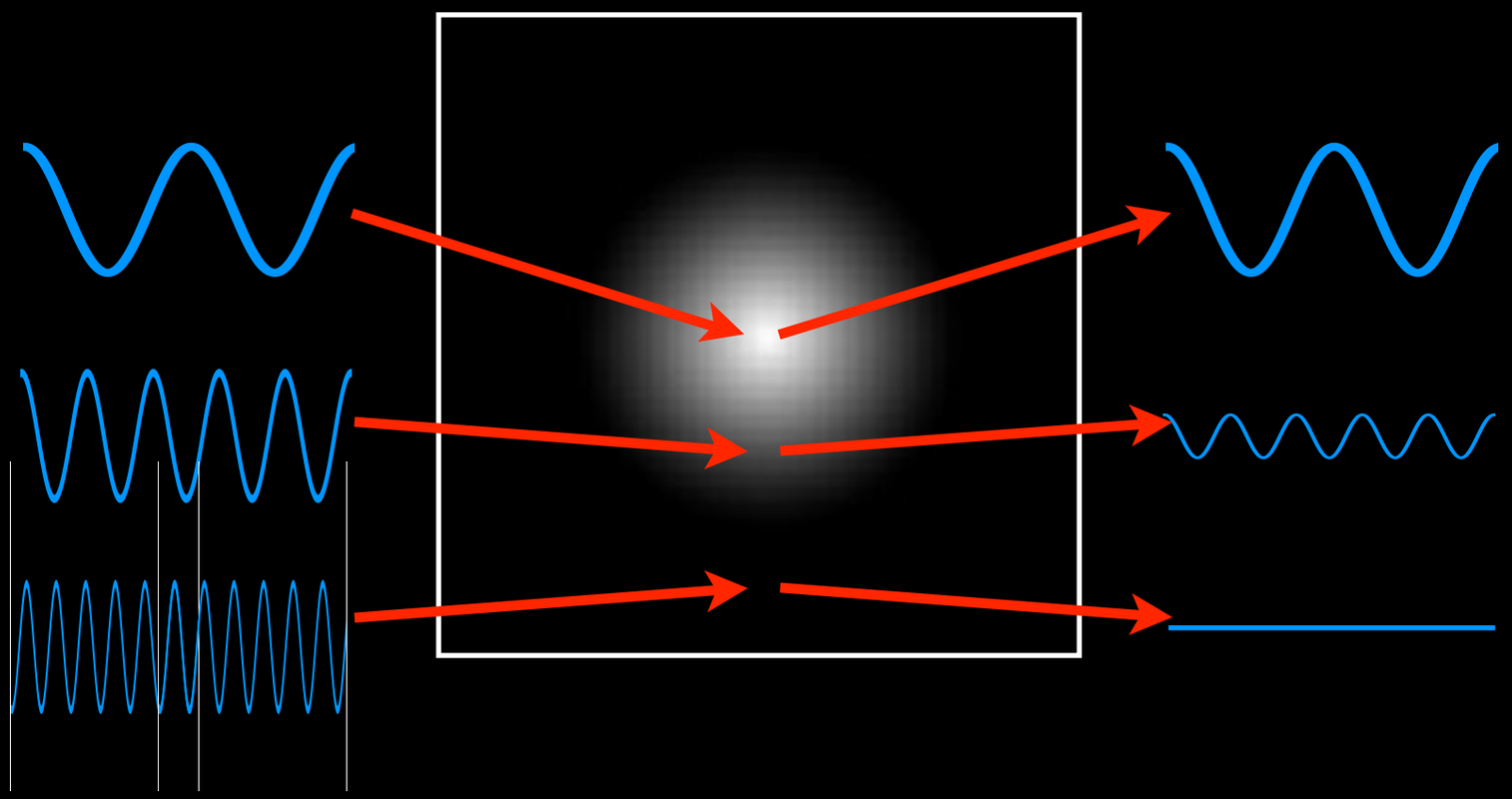


Optical Transfer Function

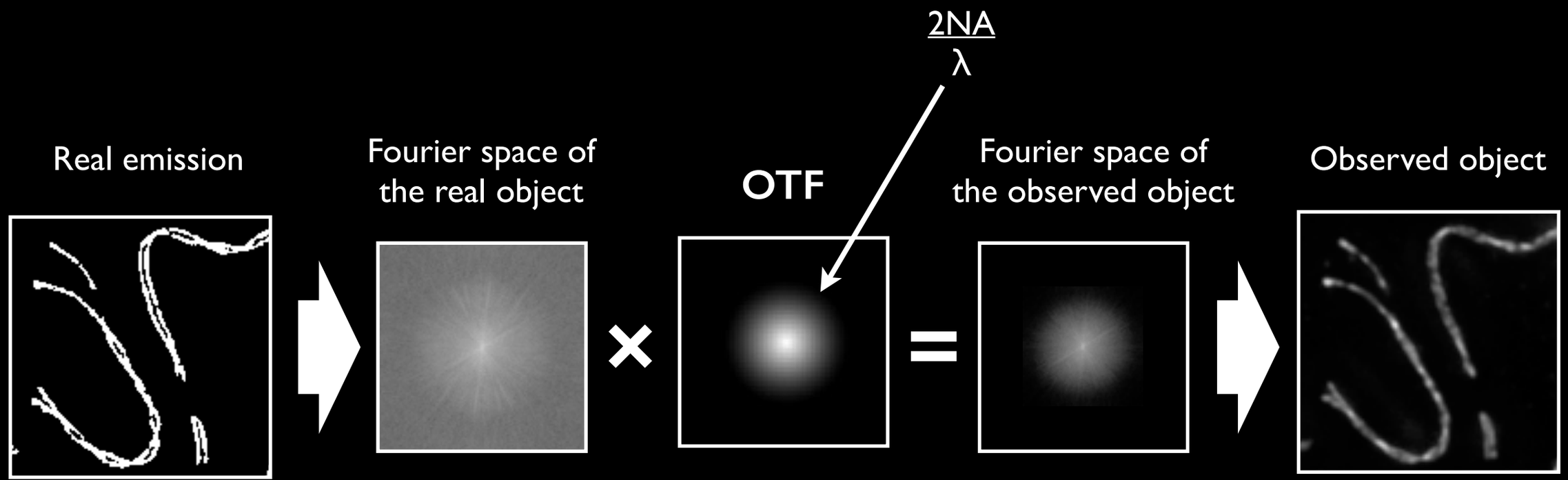
PSF



OTF



Optical Transfer Function

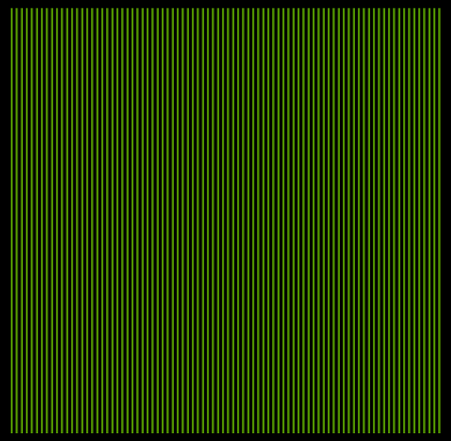


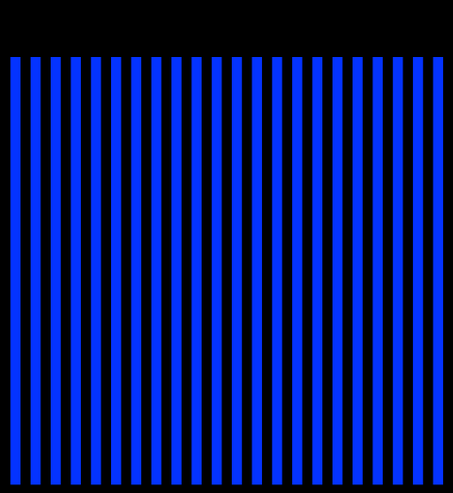
$$(E \otimes H)(r) = D(r)$$

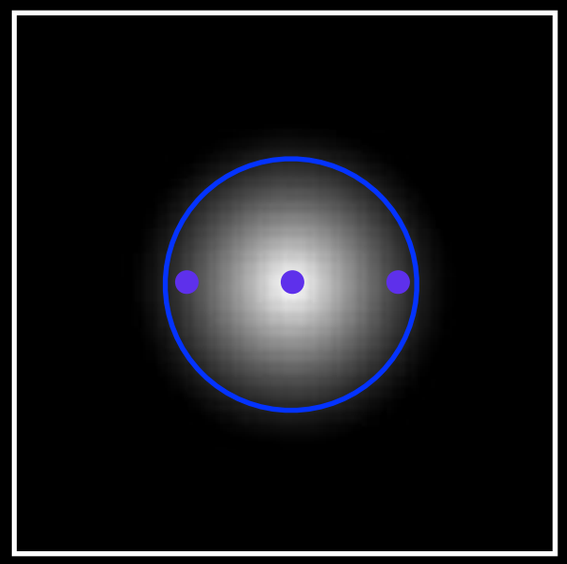
$$\tilde{E}(k)\,\tilde{H}(k) = \tilde{D}(k)$$

$$\tilde{E}(k)\,O(k) = \tilde{D}(k)$$

Super-resolution: extending the Optical Transfer Function







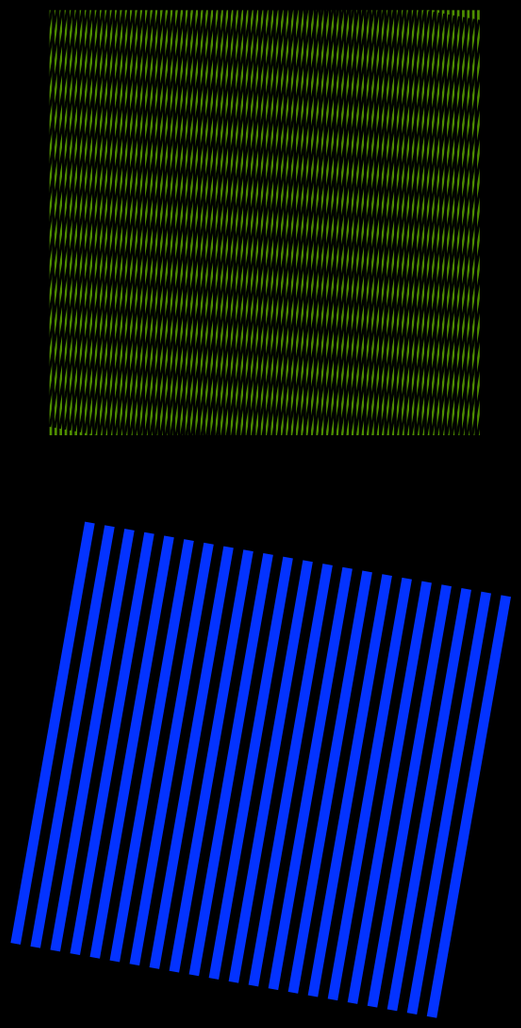
$$\tilde{E}(k)\, O(k) = \tilde{D}(k)$$

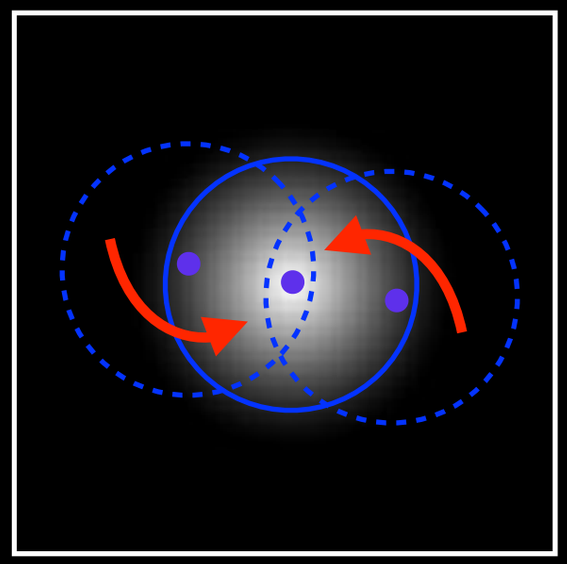
$$E(r) = S(r)\, I(r)$$

$$\tilde{E}(k) = (S \otimes I)(k)$$

The OTF is not only valid for the emission light but also for the excitation

Super-resolution: extending the Optical Transfer Function





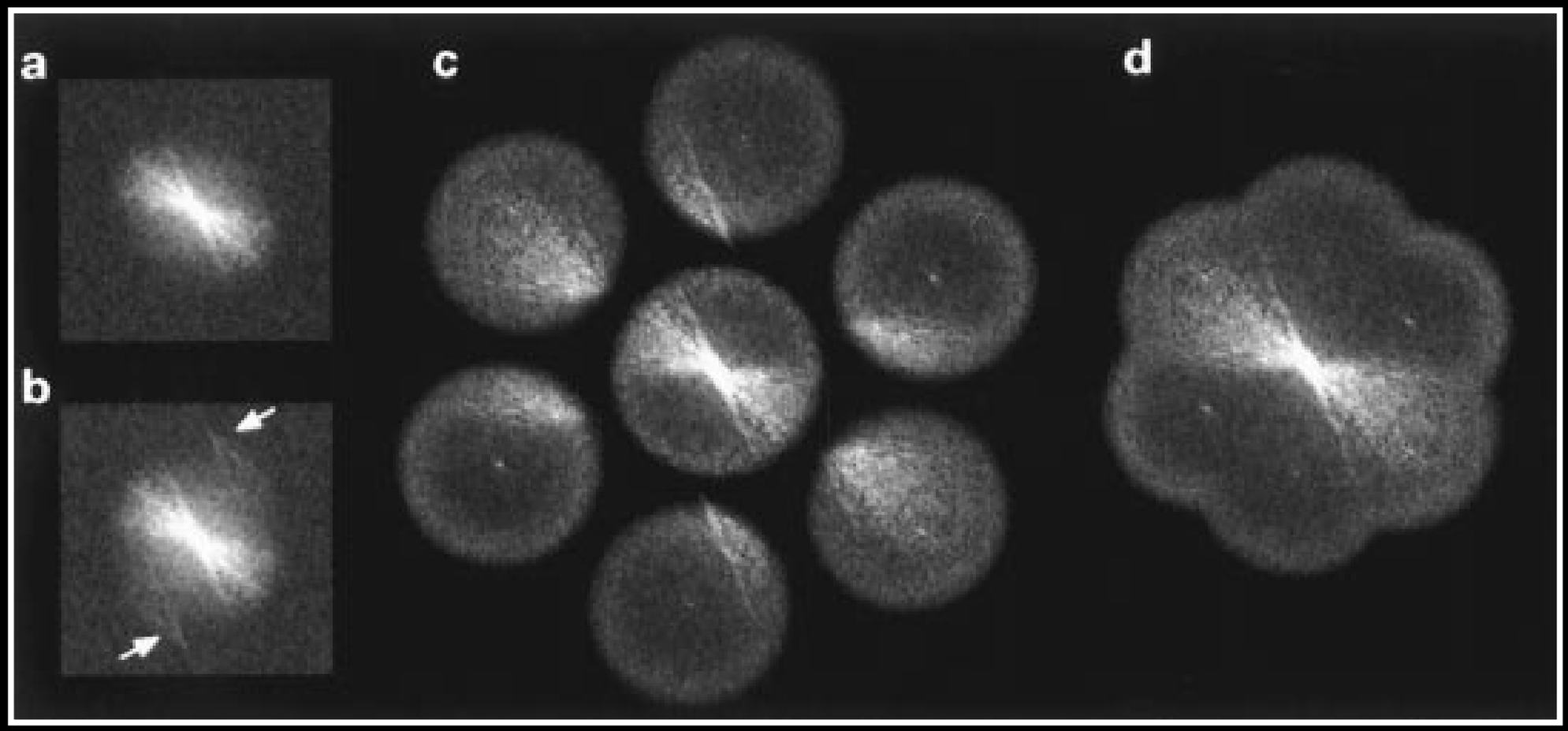
$$\tilde{E}(k)\, O(k) = \tilde{D}(k)$$

$$E(r) = S(r)\, I(r)$$

$$\tilde{E}(k) = (S \otimes I)(k)$$

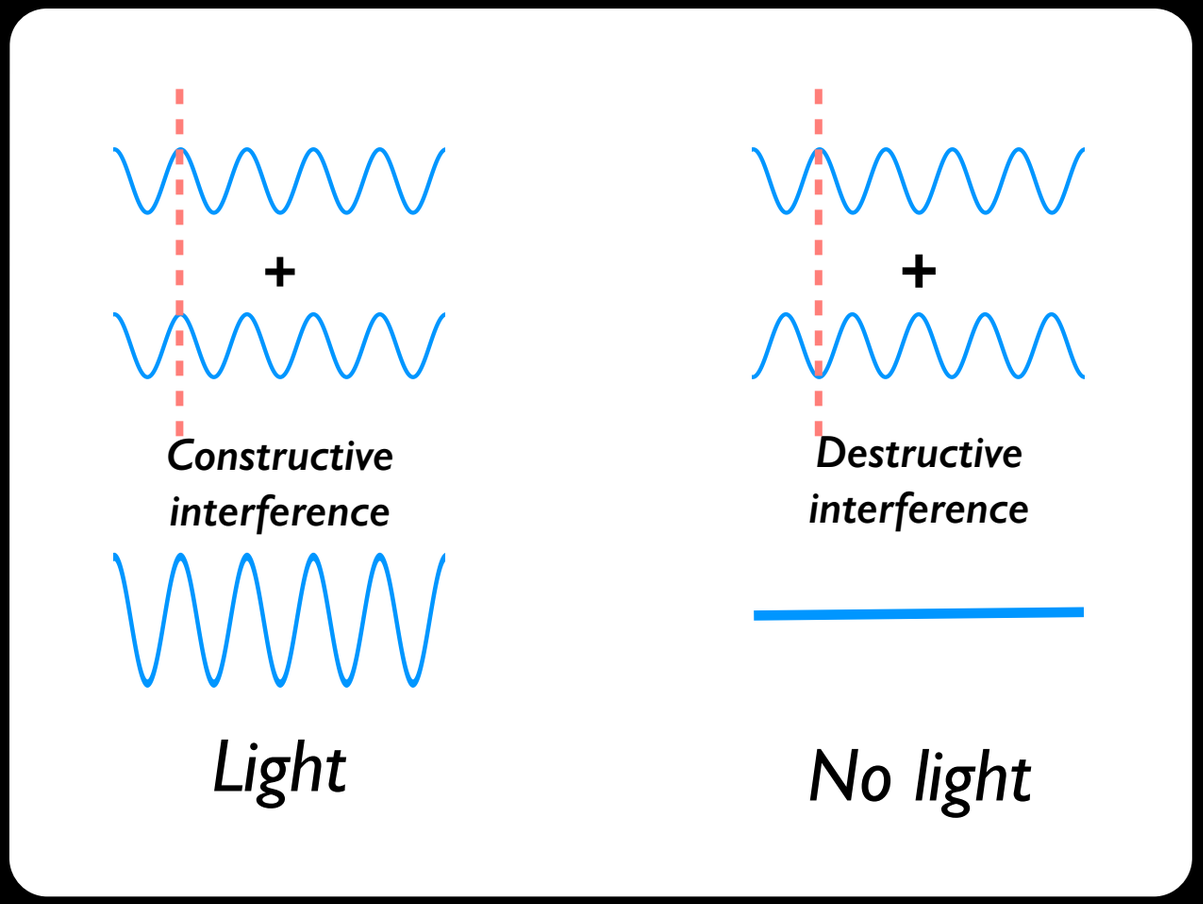
The OTF is not only valid for the emission light but also for the excitation

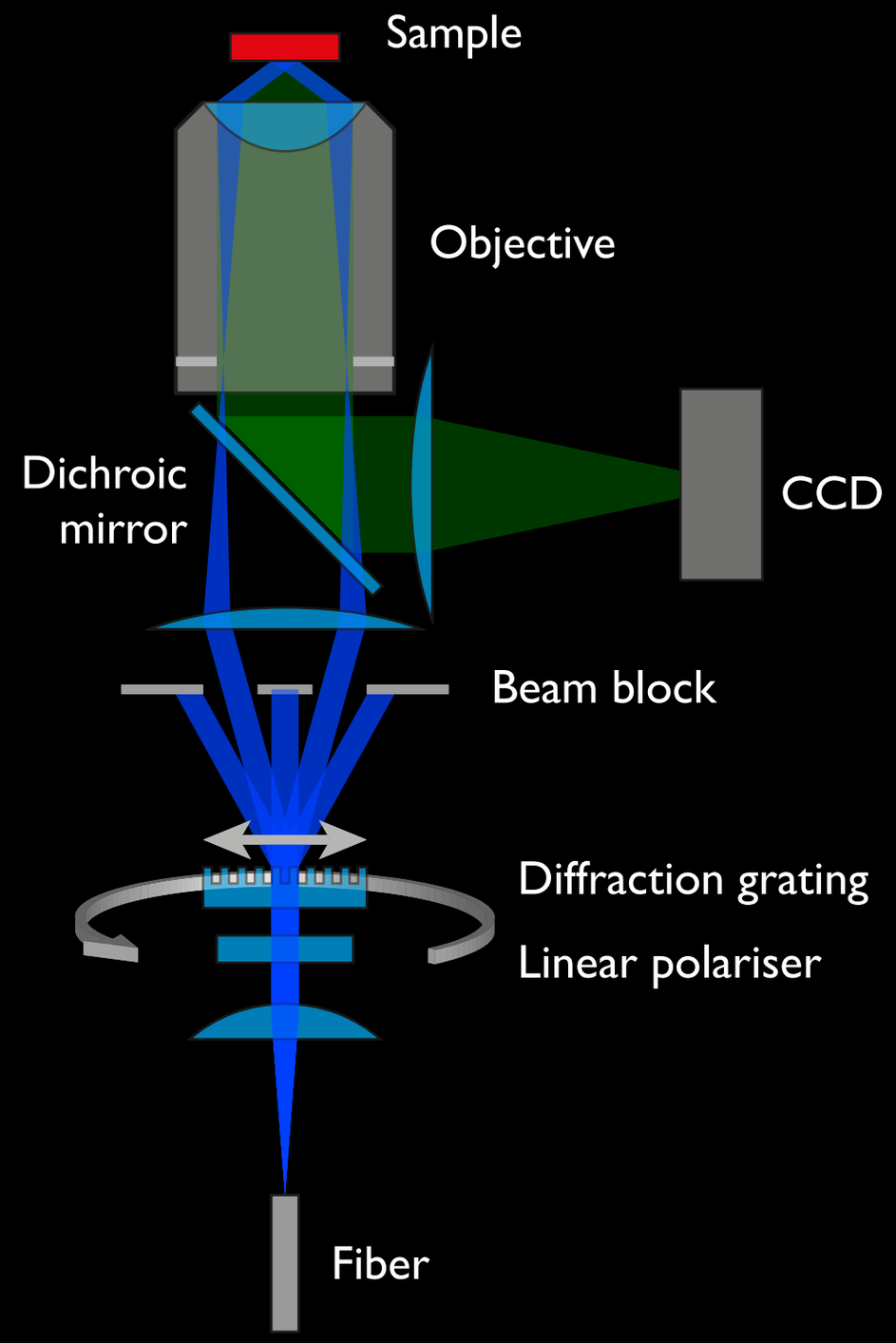
“Extending” the Optical Transfer Function



M.G.L. GUSTAFSSON et al. Journal of Microscopy, Vol. 198, Pt 2, May 2000, pp. 82-87.

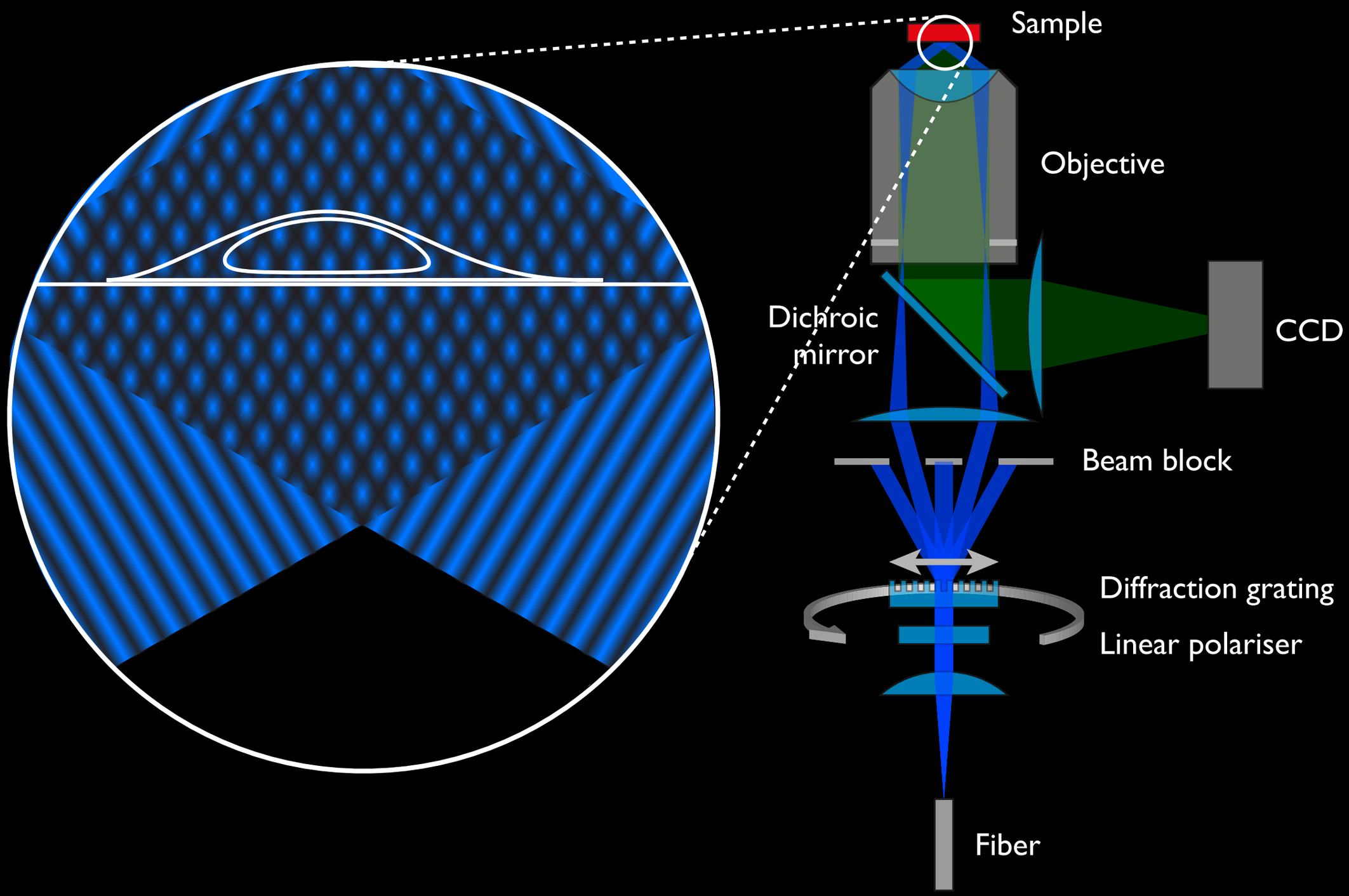
Microscope design





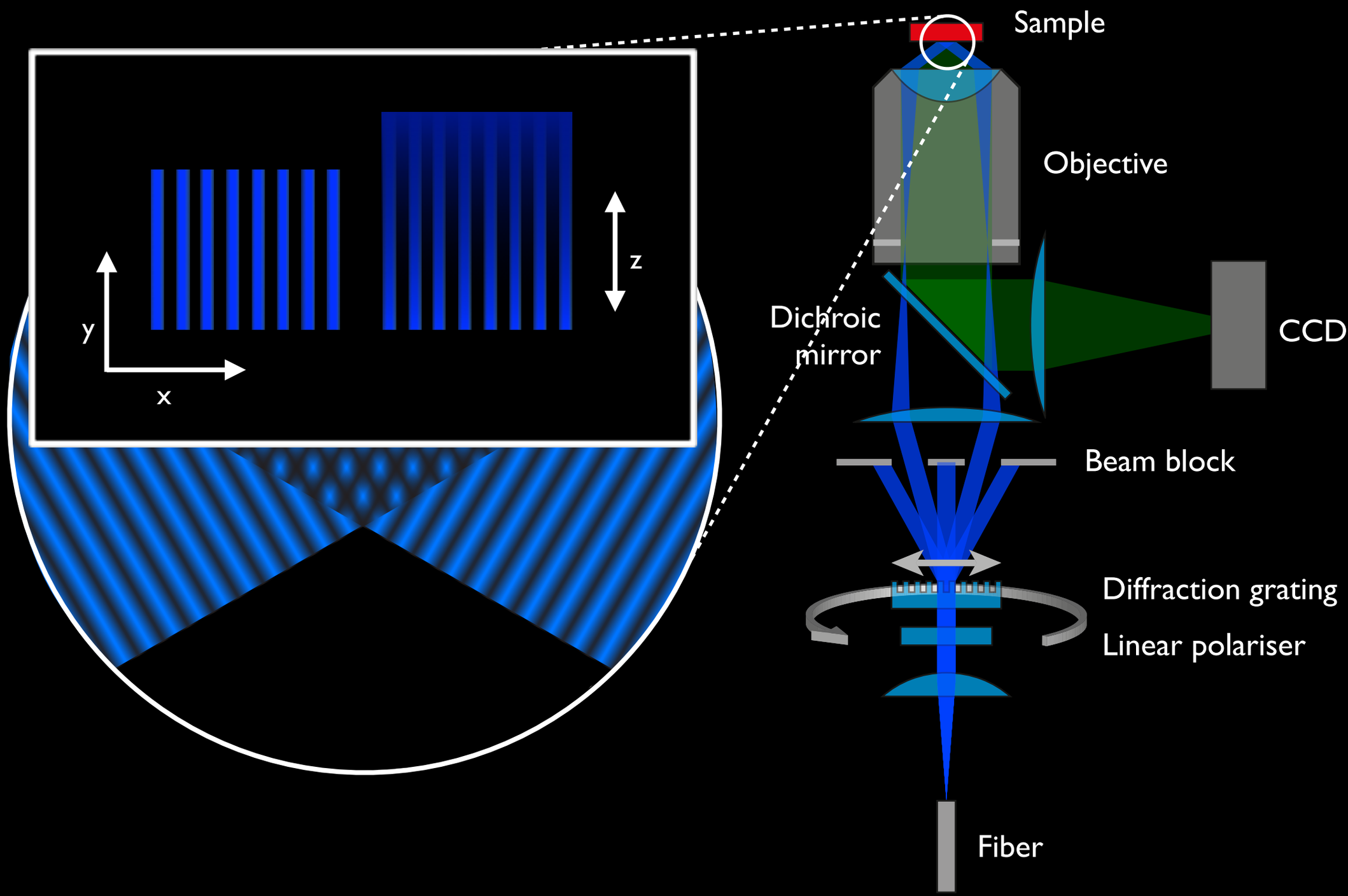
3D-SIM
J Mateos Langerak
November 2013

Microscope design

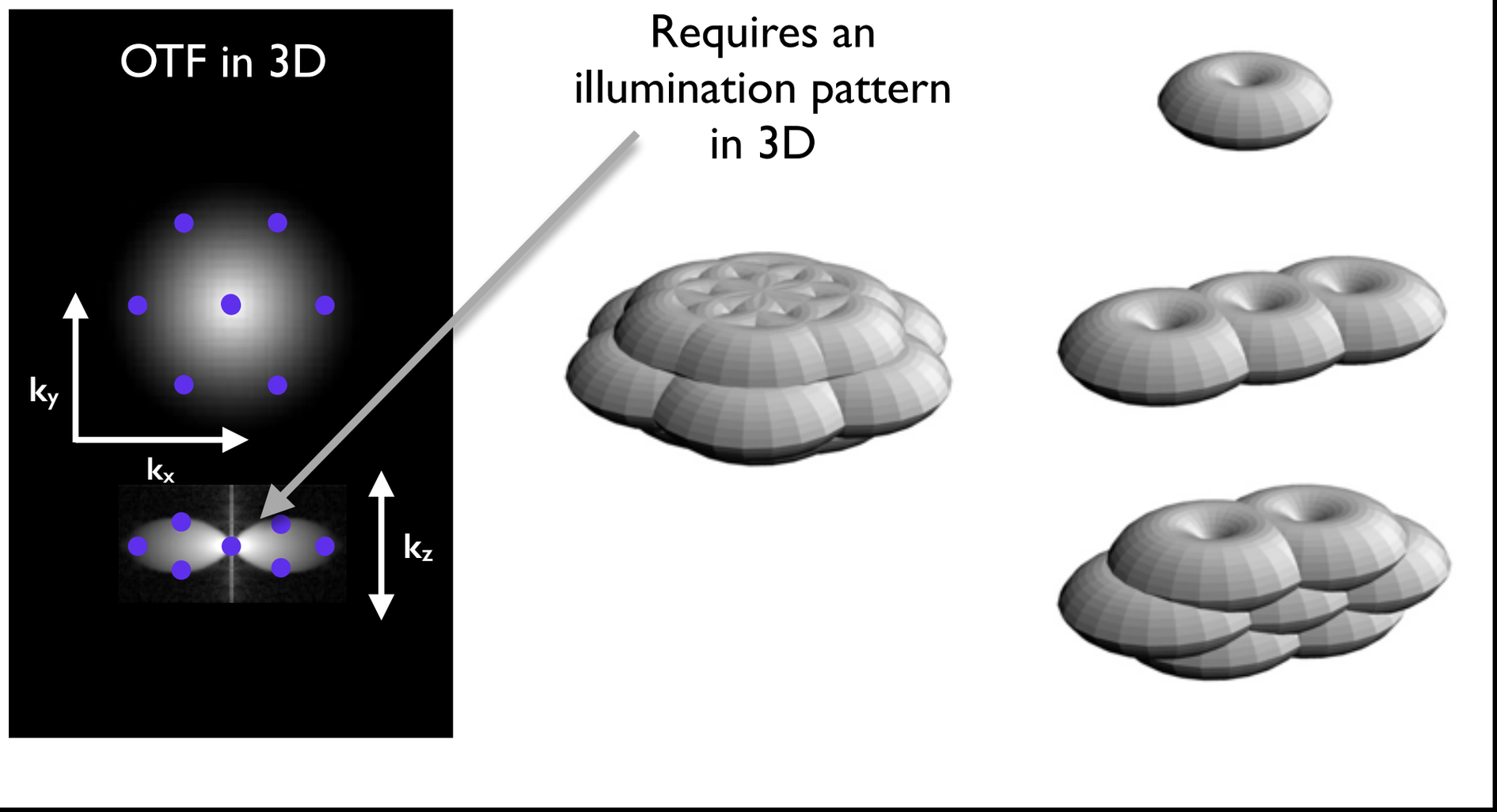


3D-SIM
J Mateos Langerak
November 2013

Microscope design



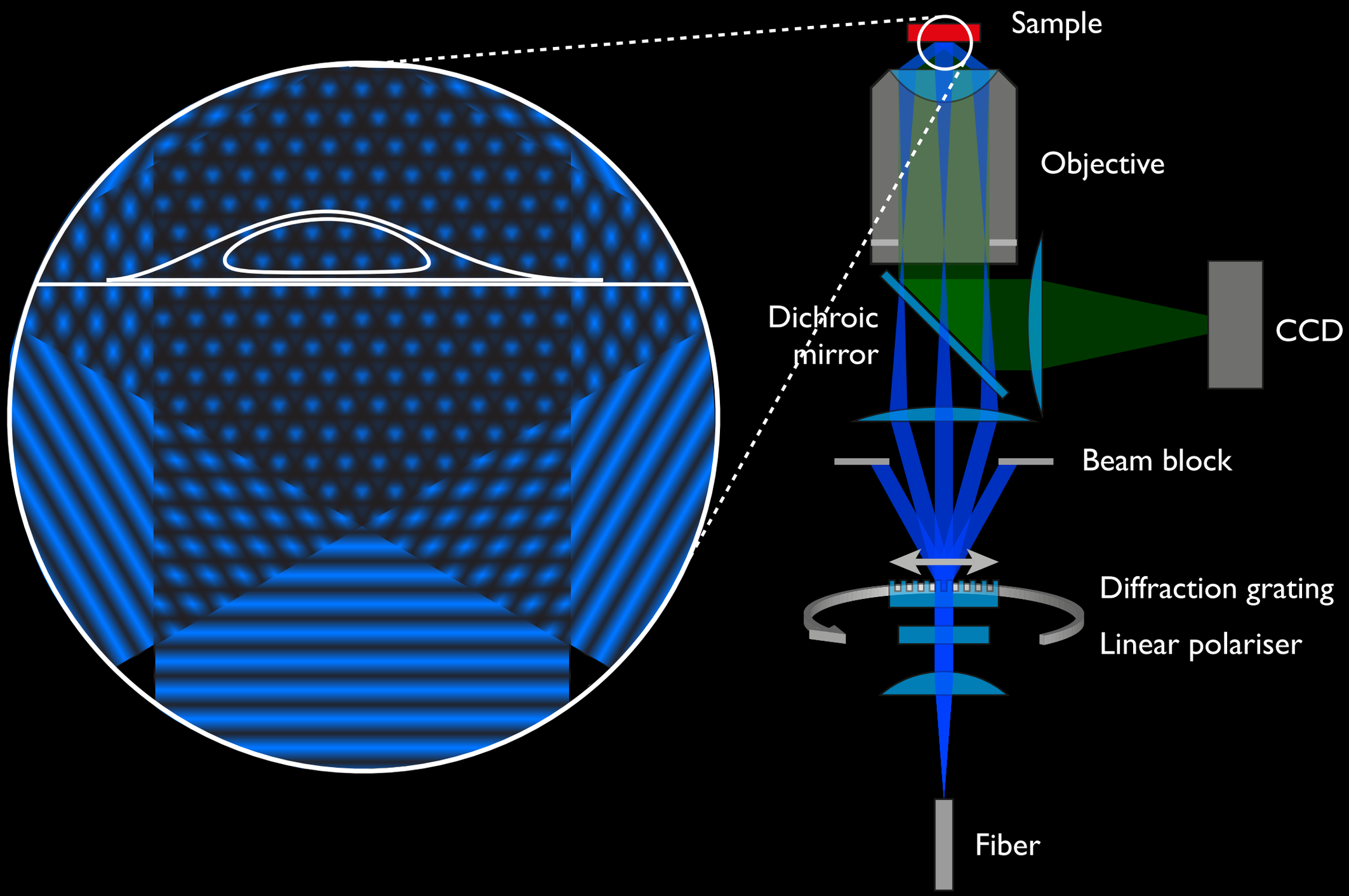
3D-SIM Structured Illumination in 3D



M.G.L. GUSTAFSSON et al. Biophysical Journal. Volume 94 June 2008 4957-4970

3D-SIM
J Mateos Langerak
November 2013

Microscope design



3D-SIM
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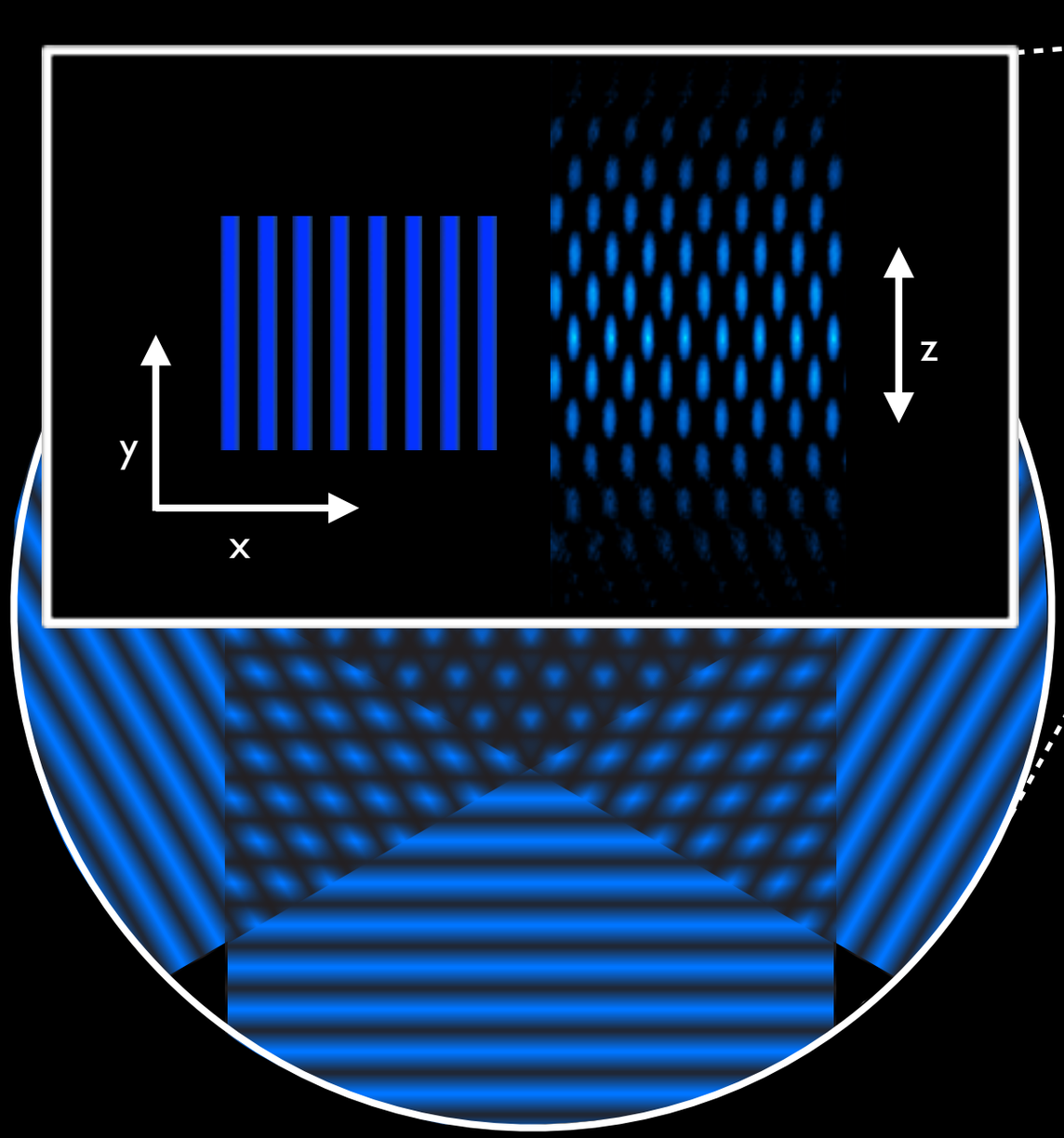
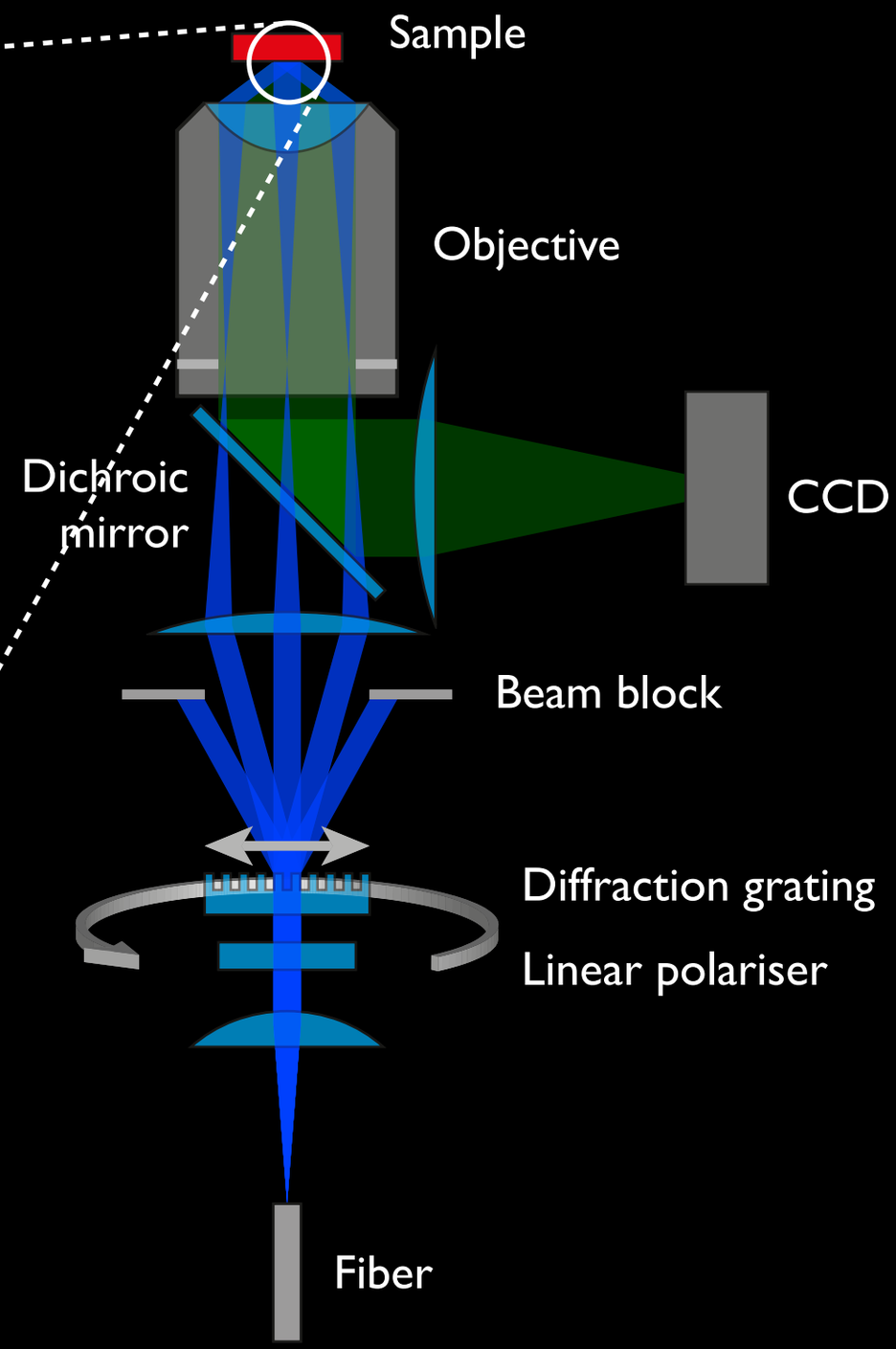
November 2013

Microscope design





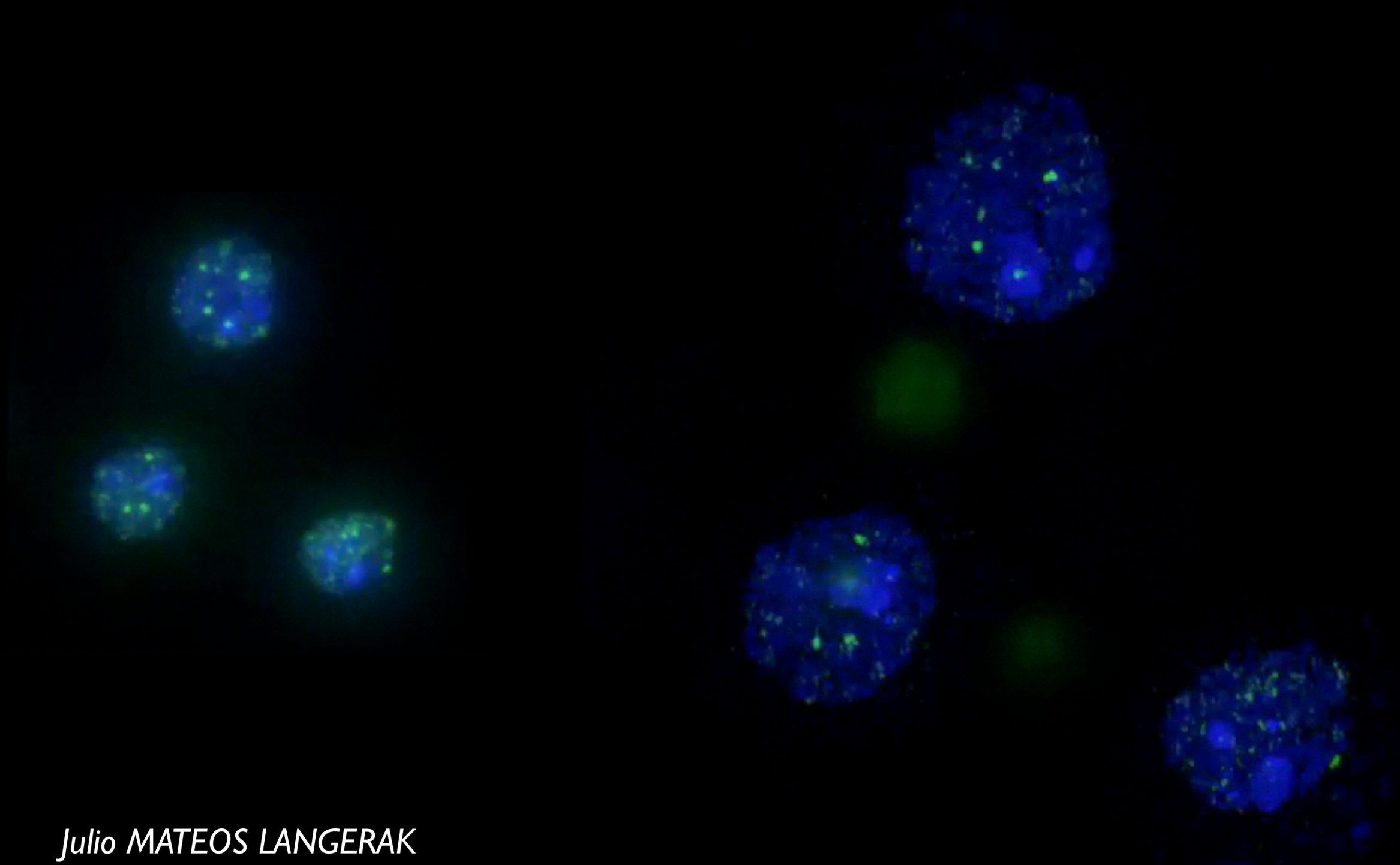
Structured Illumination Microscopy.
What do you need?

- Neat staining: Strong and well contrasted. Good signal/noise
- Bleaching resistant fluorophores: Intensities between all 15 images per optical slice must be comparable. Recommended Alexas and ATTOs
- Near perfect optical properties of sample:
  - High quality coverslips
  - Good embedding medium
  - Sample close to the objective. Ideally less than 10 µm

Structured Illumination Microscopy.
What do you get?

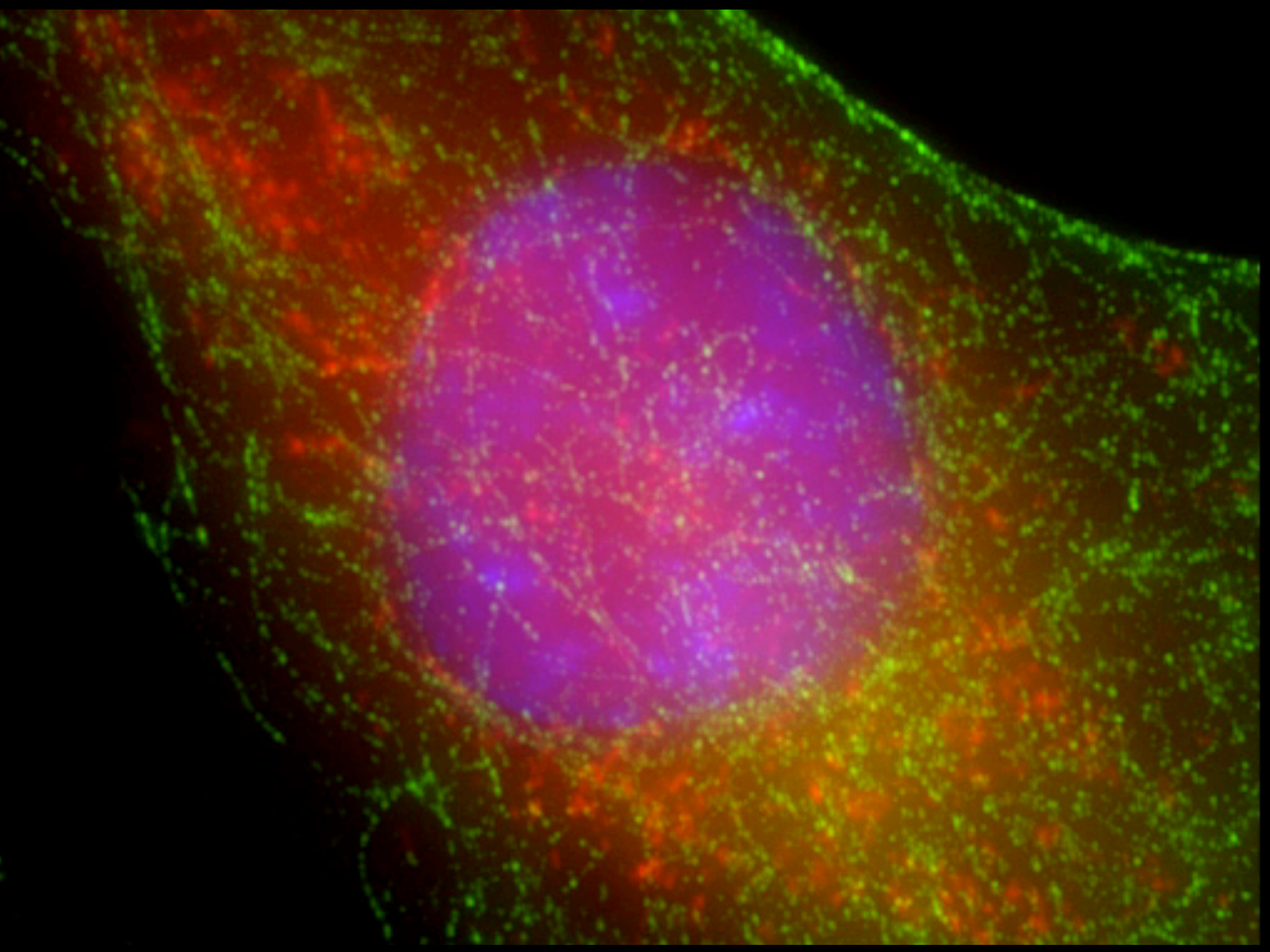
- High resolution: 100-130 nm X-Y and ~300 nm Z
- Relatively fast: one to several seconds per stack
- Up to three simultaneous colours without loss of time
- Relatively easy to implement
- Possible to do life imaging under certain conditions

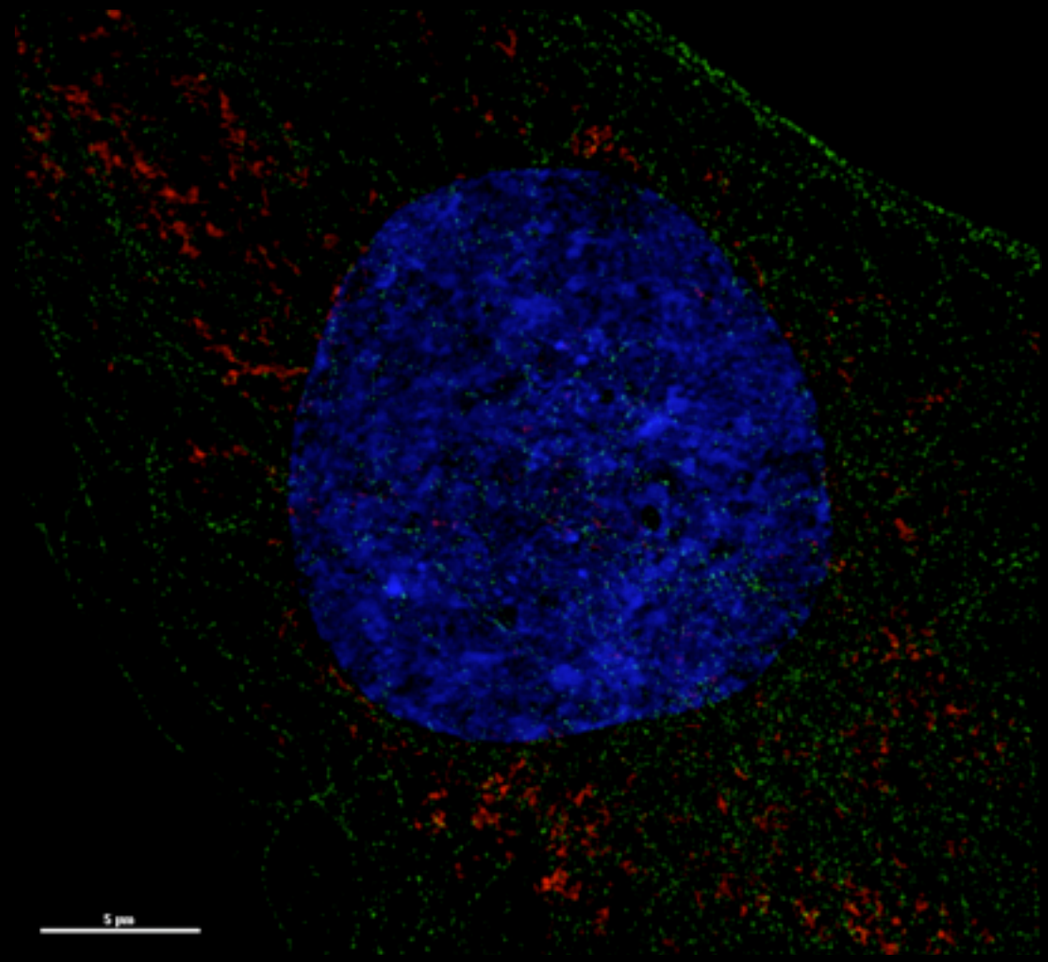
3D-SIM Examples



Julio MATEOS LANGERAK

3D-SIM Examples

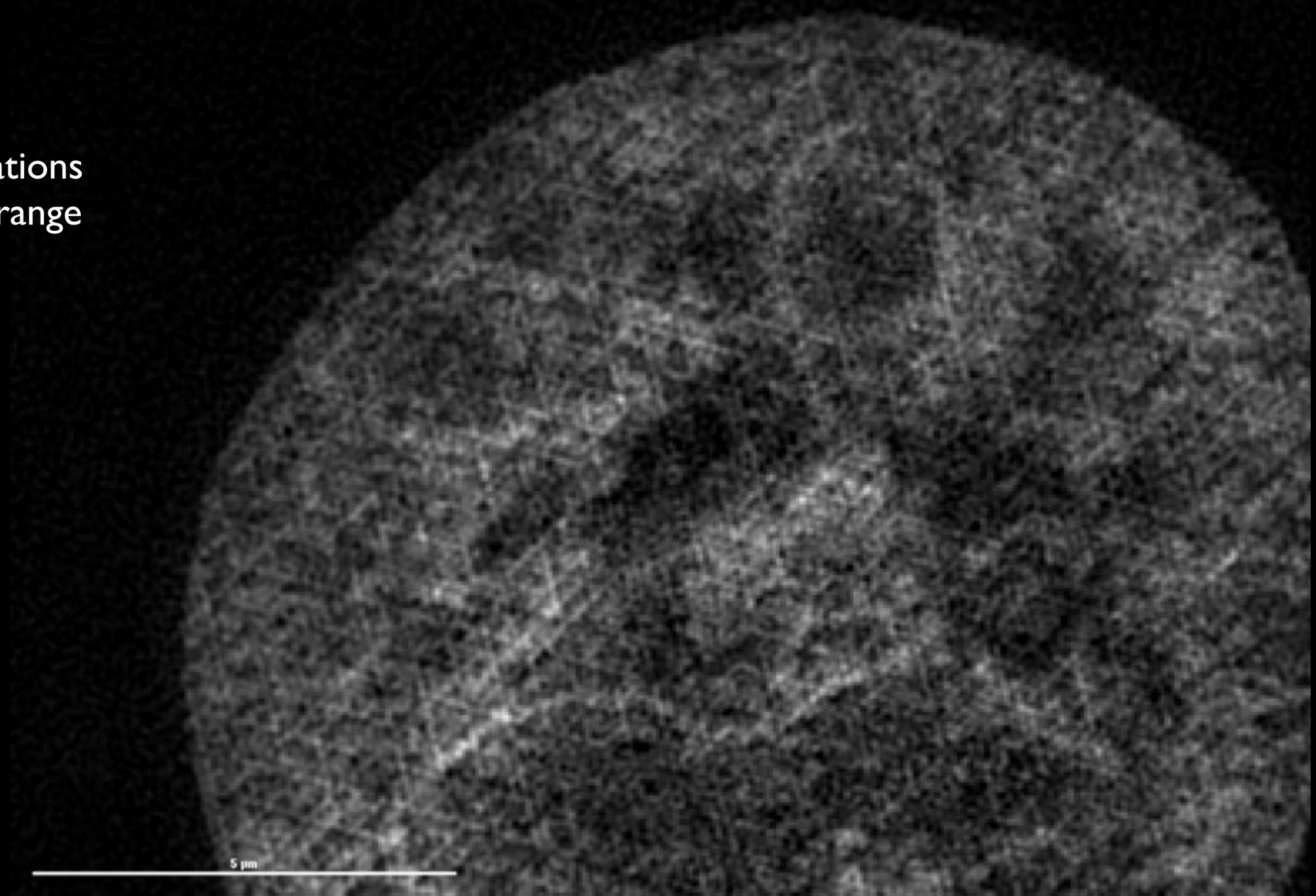




Krzysztof ROGOWSKI

Acquisition and Troubleshooting

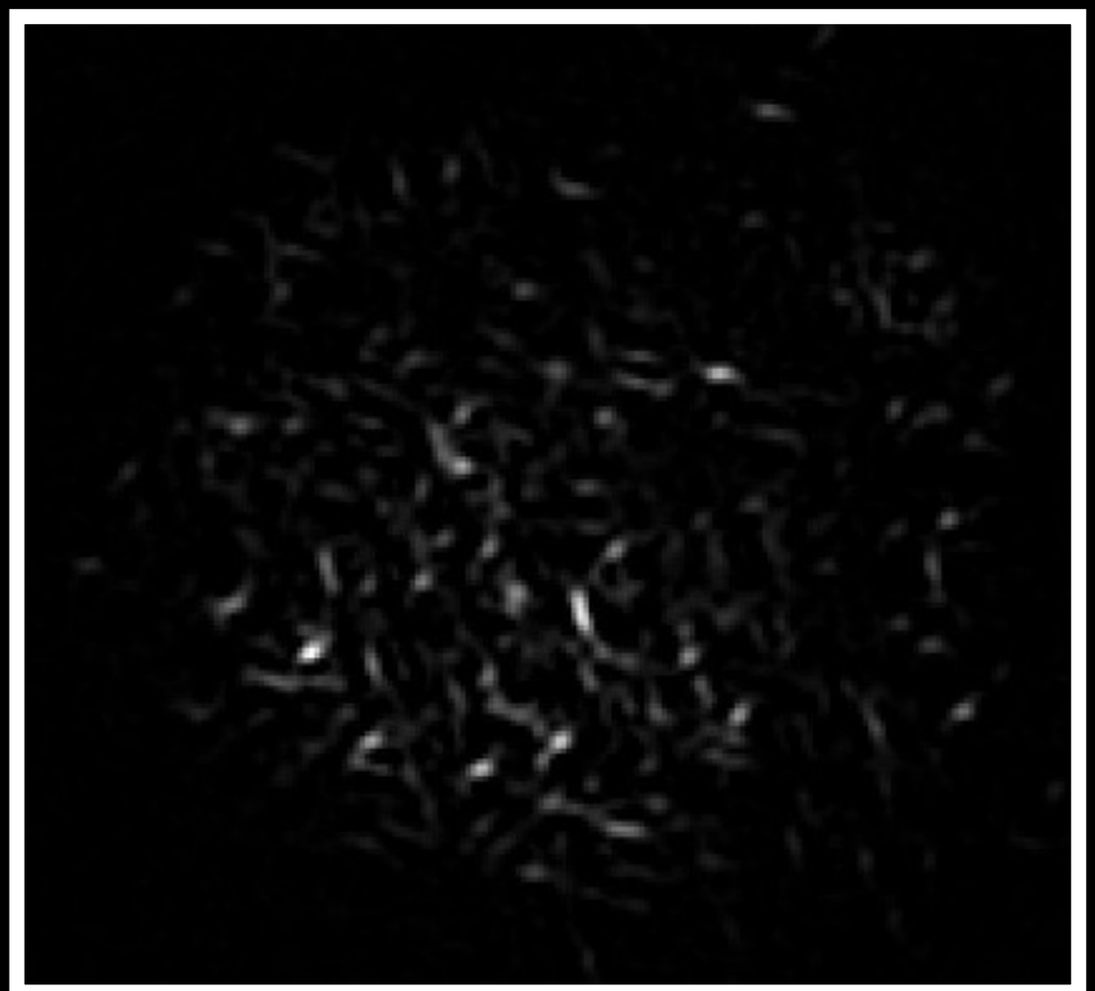
Mesh artefacts
- Bleaching
- Poor S/N
- Spheric aberrations
- Low dynamic range



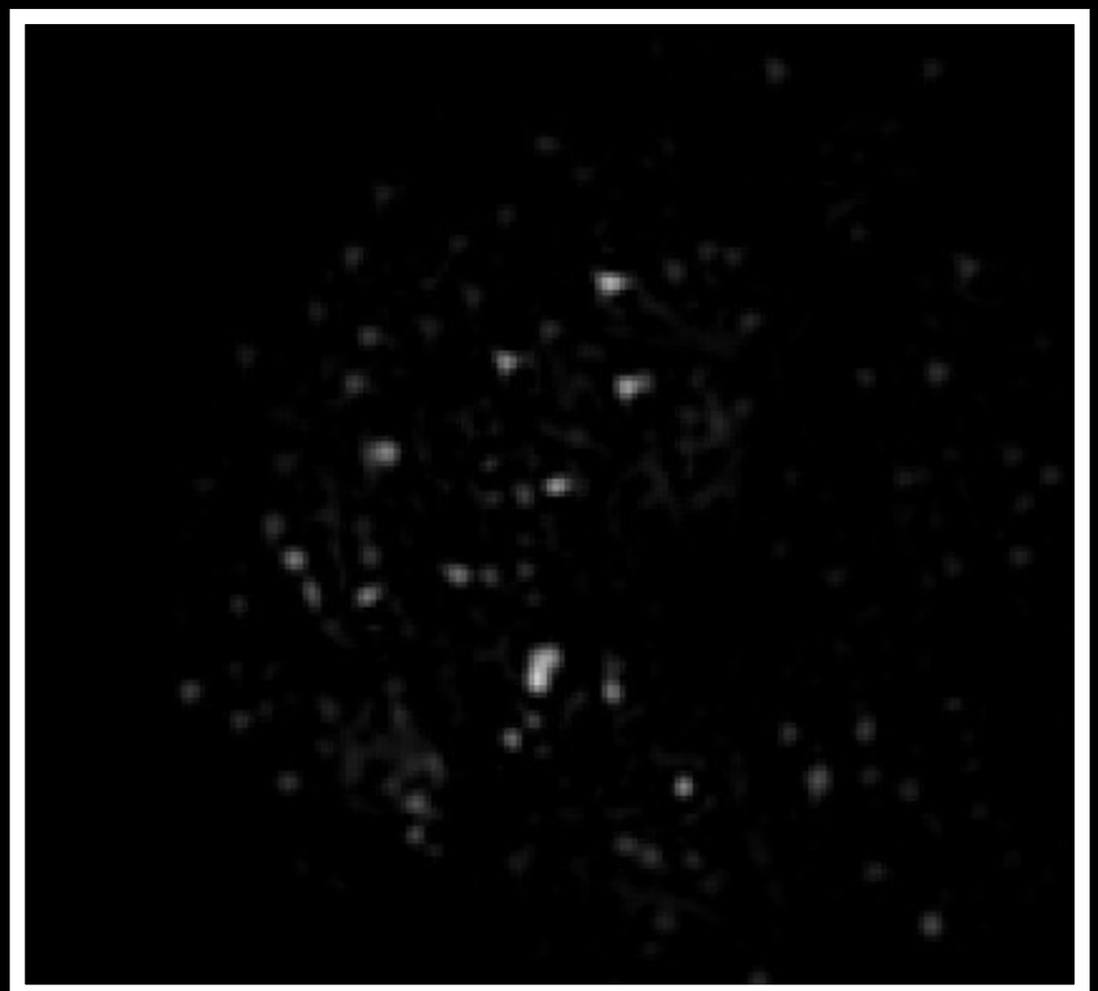
Super-Resolution
J Mateos Langerak
November 2014

Acquisition and Troubleshooting

With DRIFT



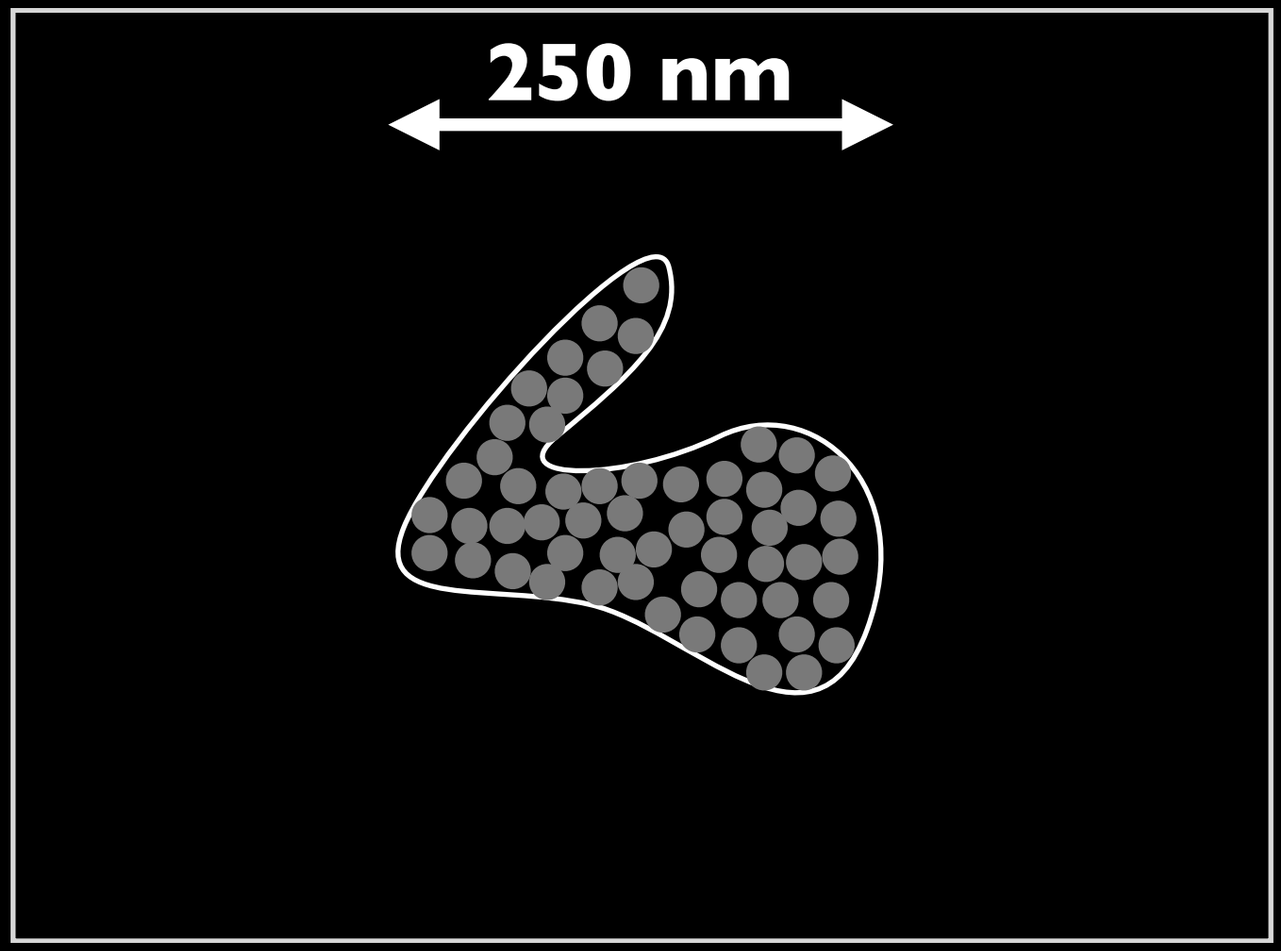
No DRIFT



STimulated Emission Depletion Microscopy (STED)

Fluorophores are excited by a focused light beam. An additional depletion beam around the PSF is used to bring molecules back to the ground state by a process called stimulated emission. Thereby the effective PSF is reduced.

STimulated Emission Depletion Microscopy (STED)



Super-Resolution
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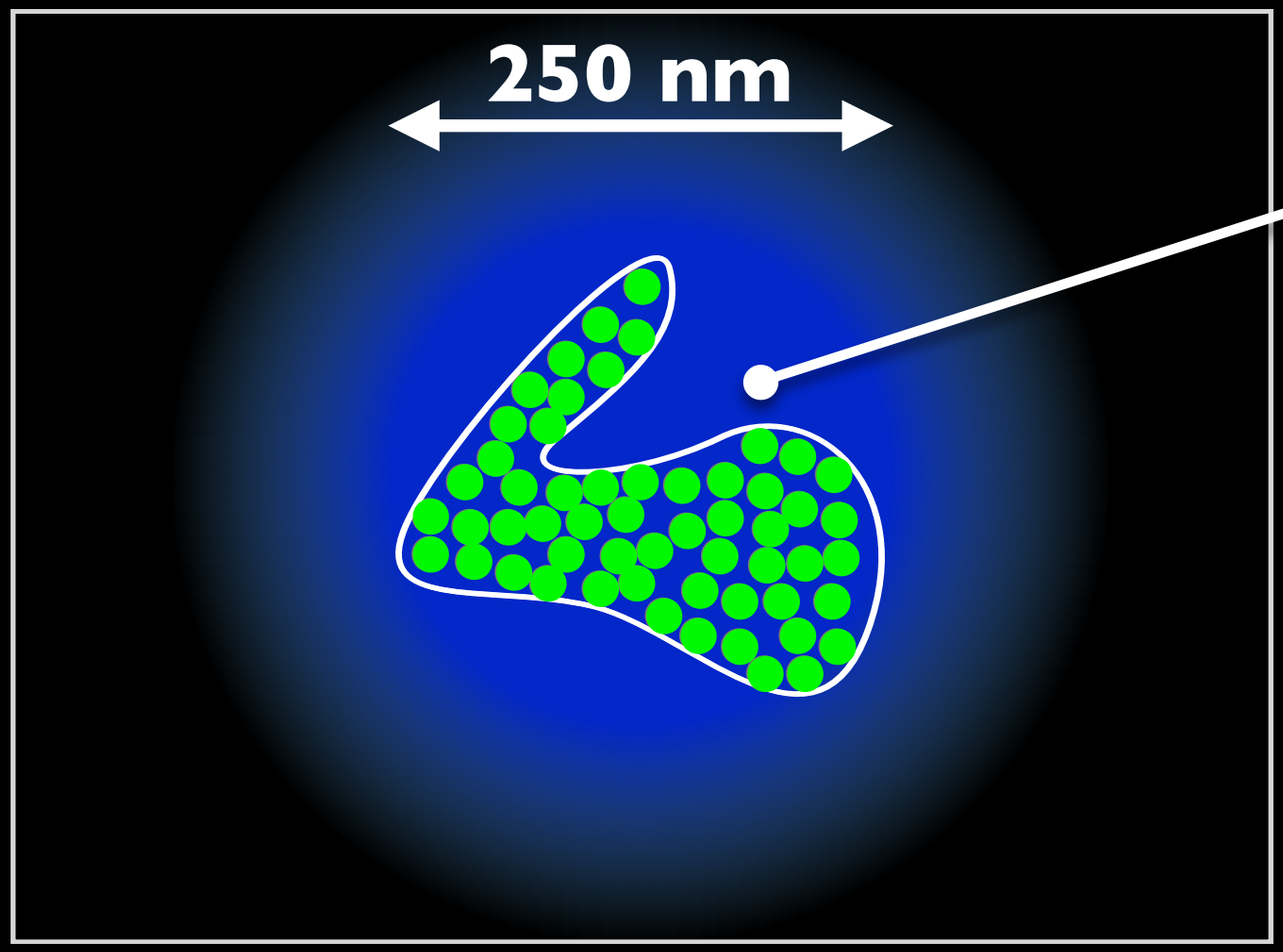
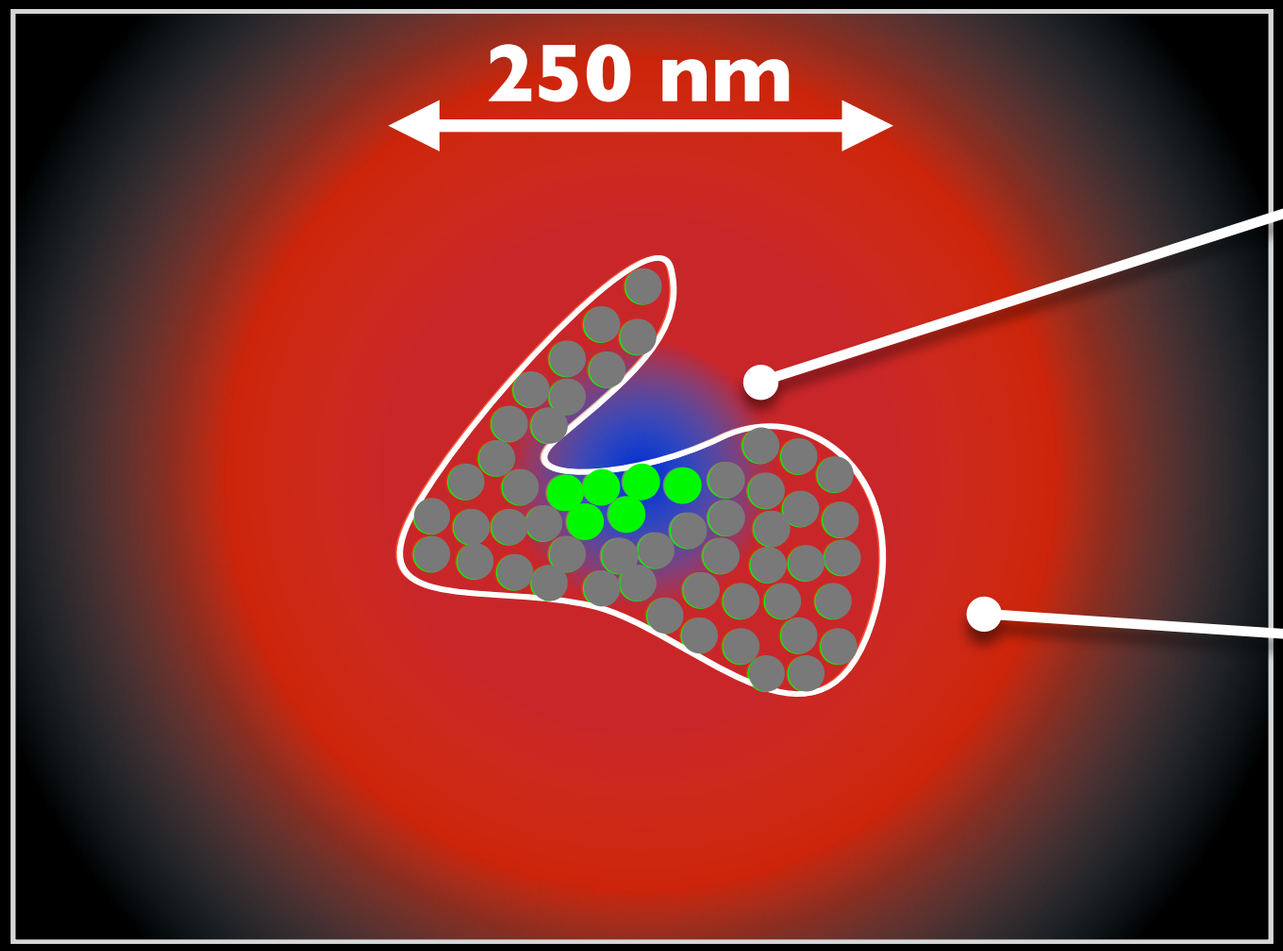
November 2014

STimulated Emission Depletion Microscopy (STED)

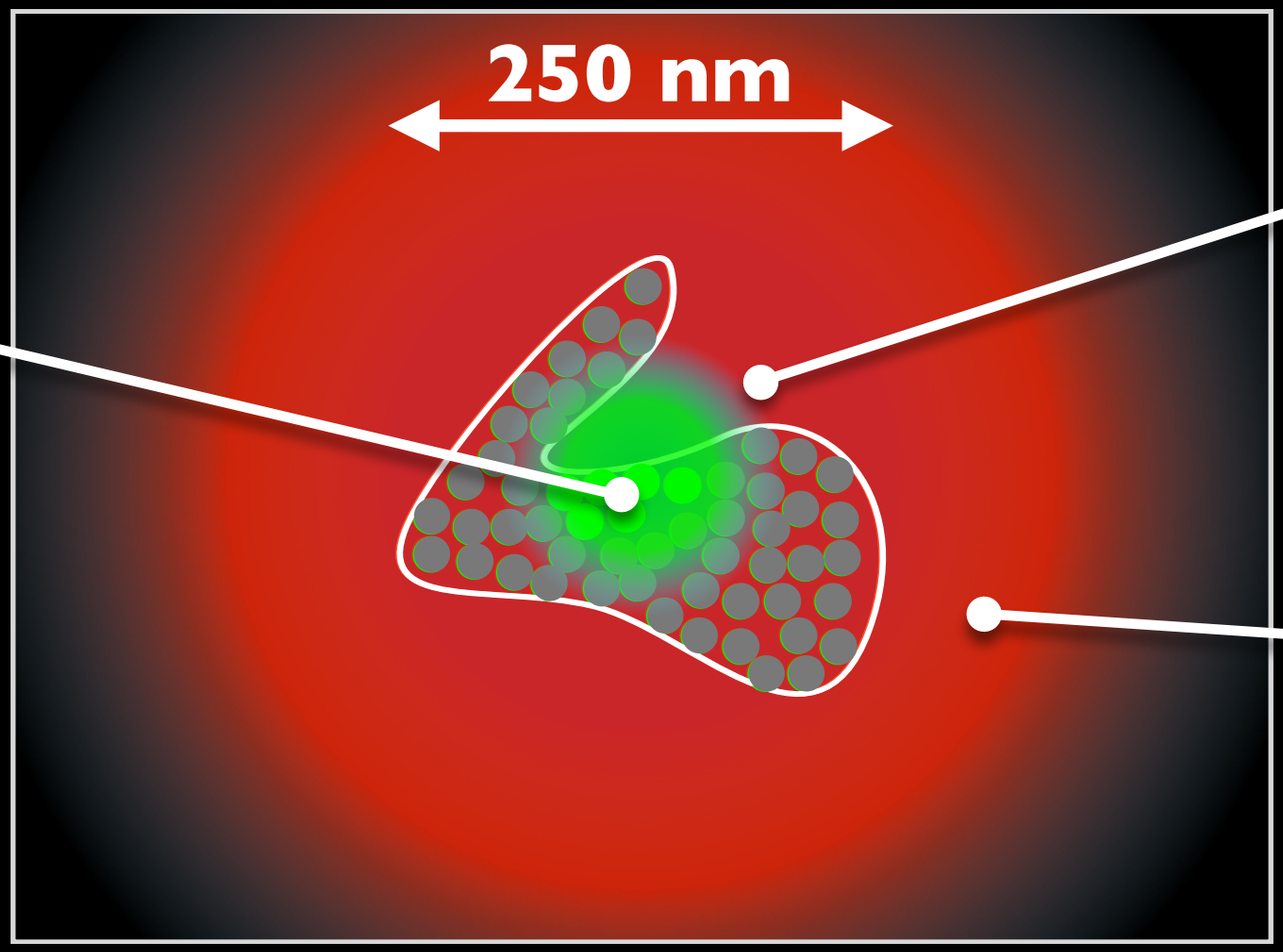


STimulated Emission Depletion Microscopy (STED)



Super-Resolution
J Mateos Langerak
November 2014

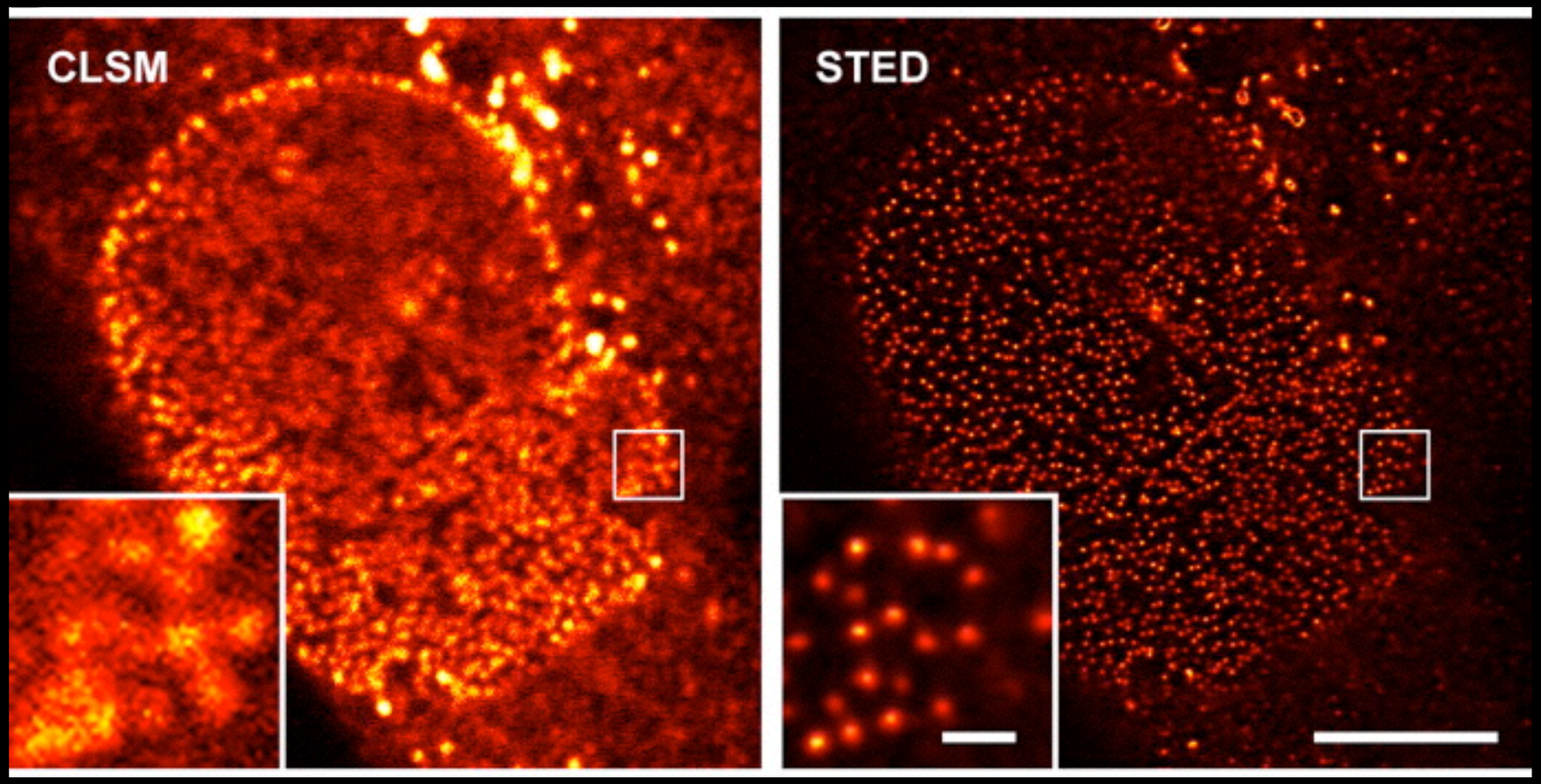
STimulated Emission Depletion Microscopy (STED)



STimulated Emission Depletion Microscopy (STED). What do you get?

- Higher and tunable X-Y resolution: ~40 nm
- Confocal Z resolution: ~500 nm
- STED 3x: improved Z resolution at the cost of X-Y resolution
- Up to two colors
- Confocal setup: less out of focus emission
- Need highly efficient dyes and with the right spectra
- Not suited for life experiments

STimulated Emission Depletion Microscopy (STED). What do you get?



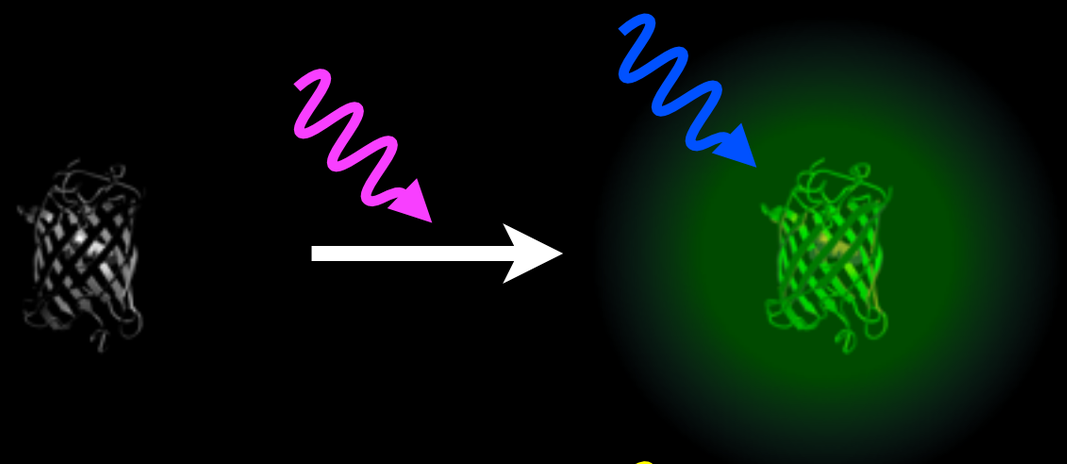
Schermelleh et al. 190 (2): 165

Microscopy based on Single Molecule Localization

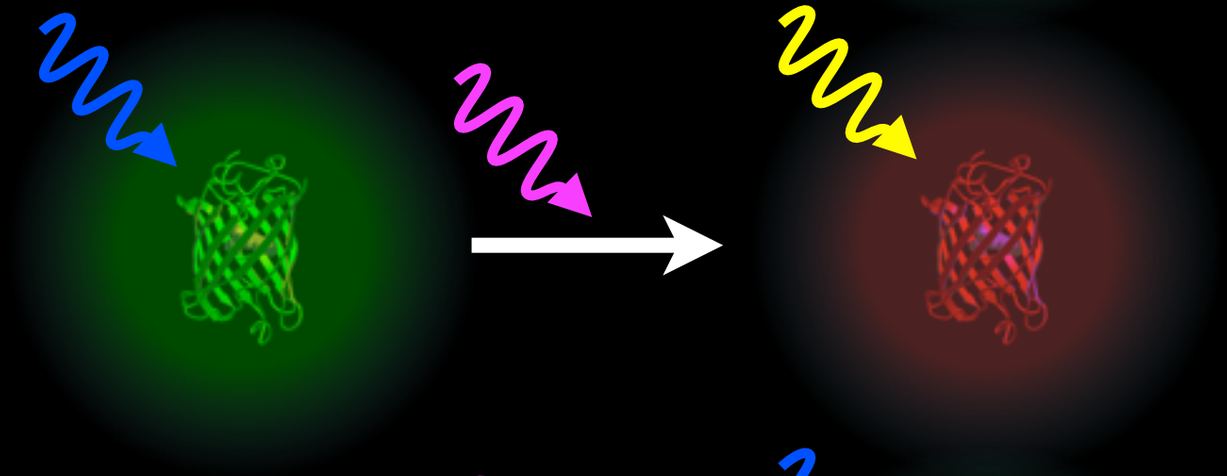
Photo-switching of fluorophores allows temporal separation of molecules that overlap in space. After multiple iterations of the photo-switching, a super-resolution image is constructed from the localizations of many fluorophores.

Single Molecule Localization fluorophores

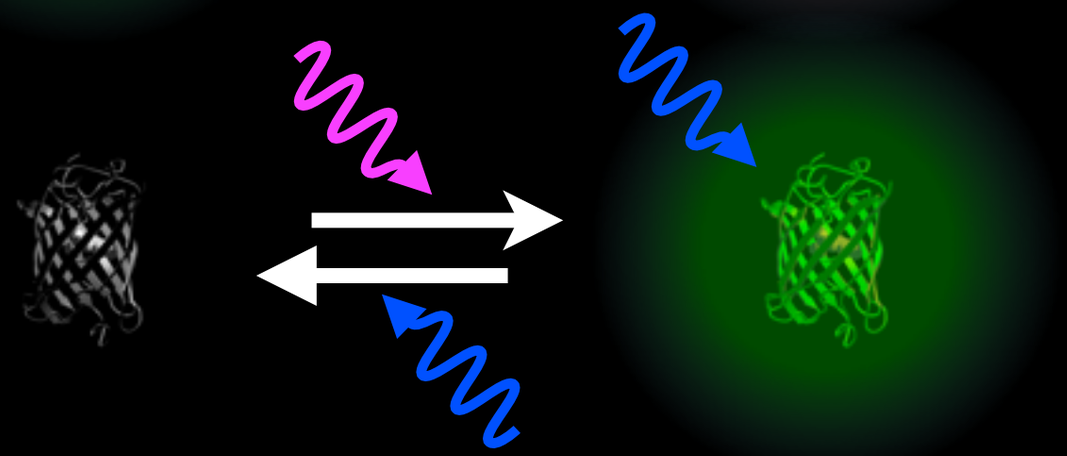
- Photoactivable



- Photoconvertable

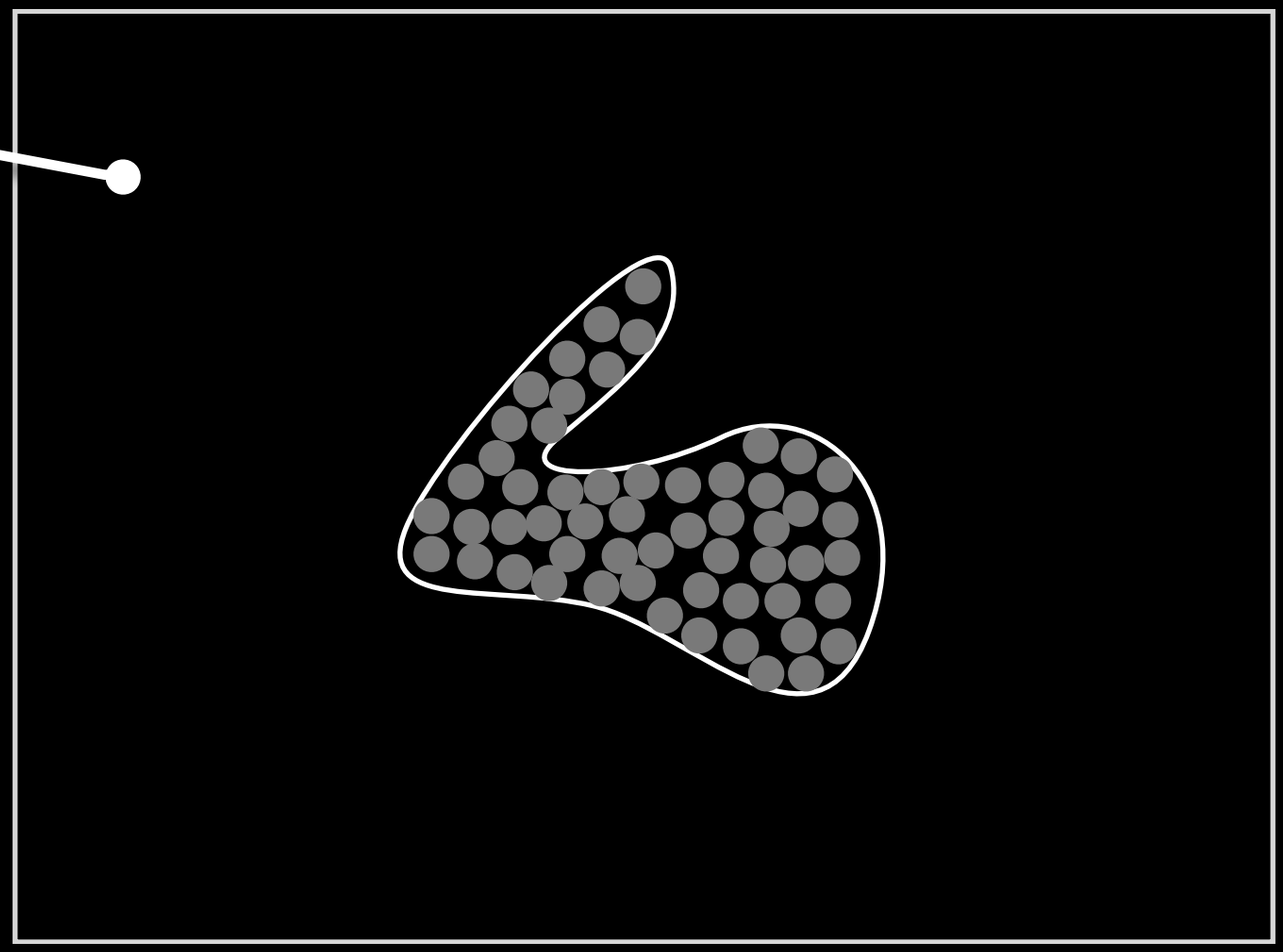


- Photoswitchable



Microscopy based on Single Molecule Localization

Fluorophores are brought to a dark state



Super-Resolution
J Mateos Langerak
November 2014

Microscopy based on Single Molecule Localization

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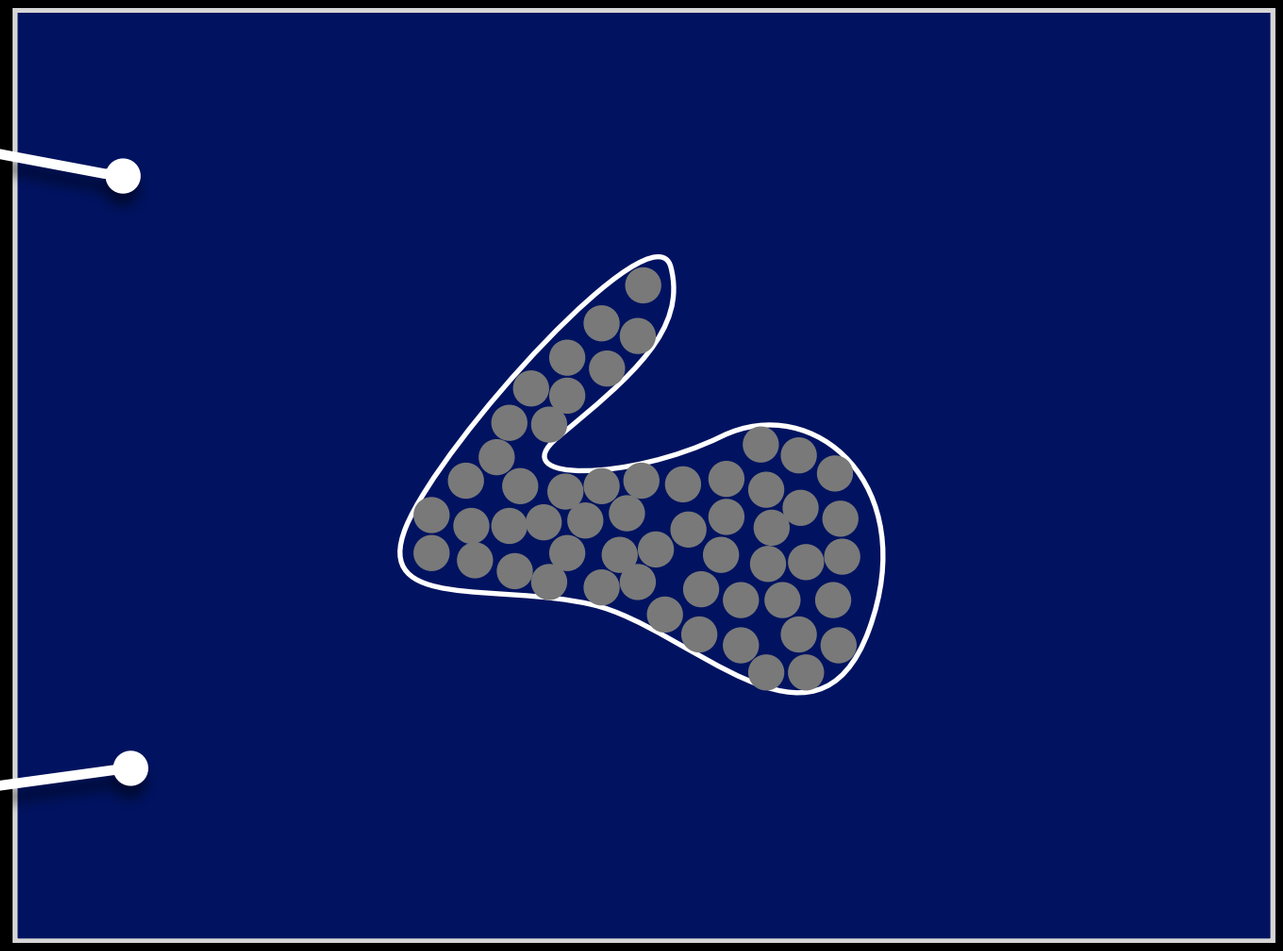

Fluorophores are re-activated with weak illumination

Microscopy based on Single Molecule Localization

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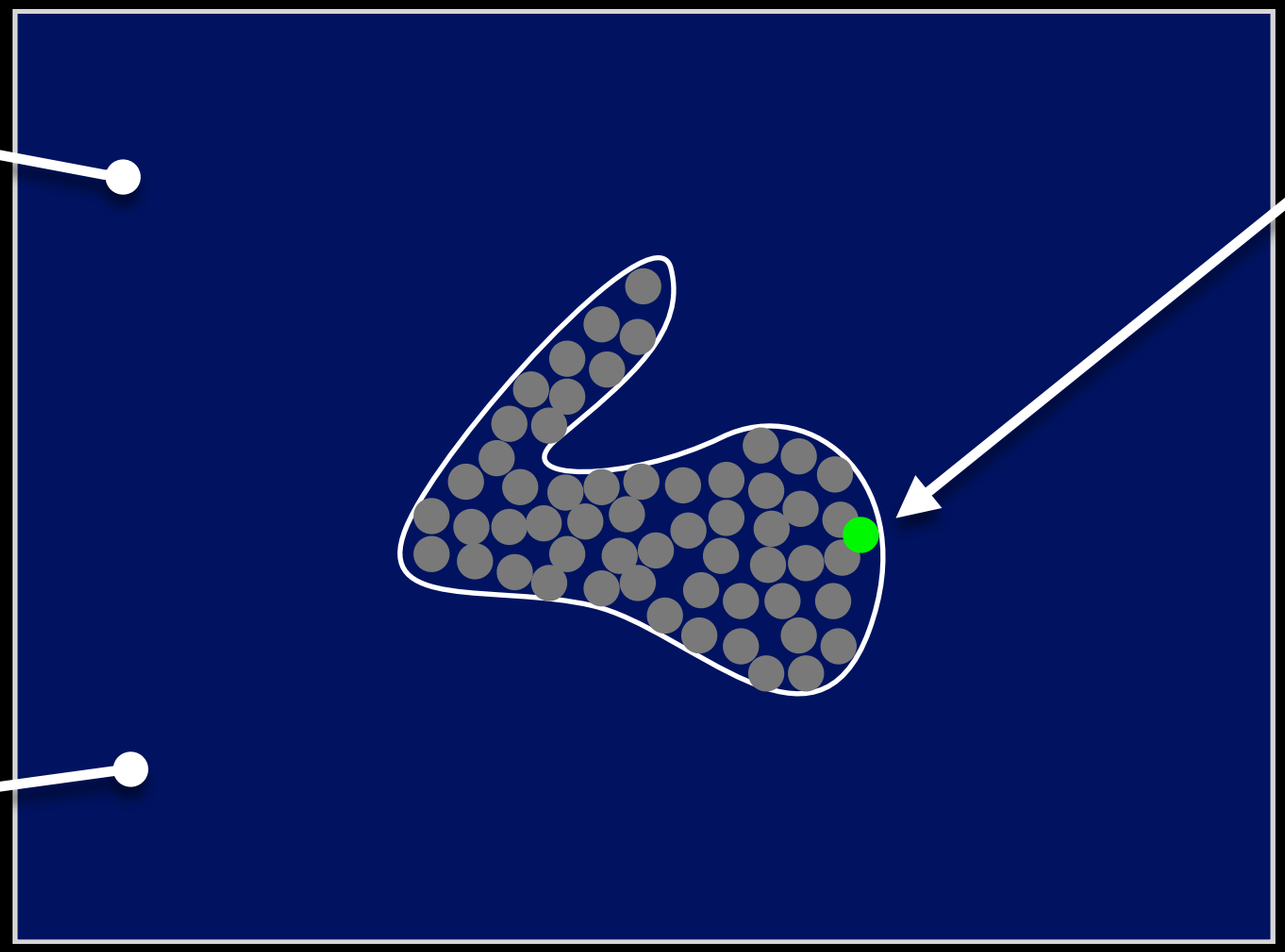

Every time a single fluorophore within the PSF is activated

Fluorophores are re-activated with weak illumination

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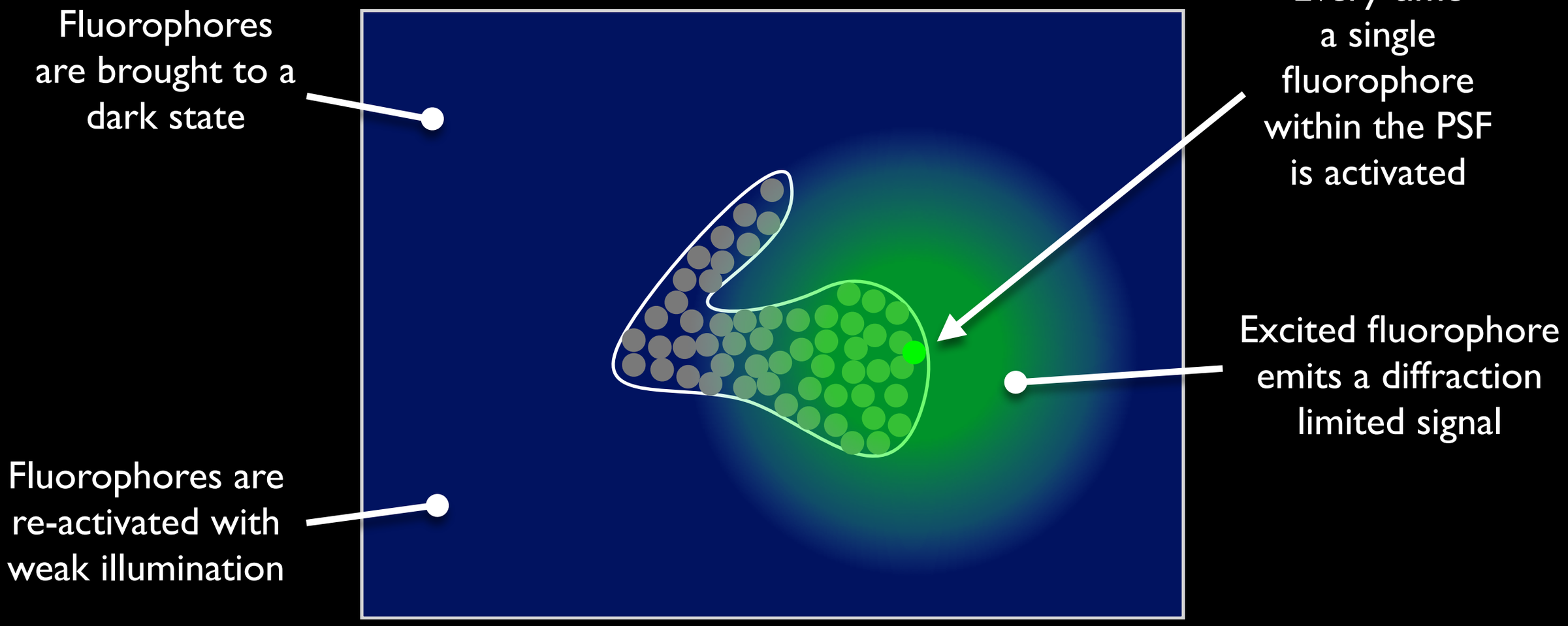
Microscopy based on Single Molecule Localization



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Microscopy based on Single Molecule Localization

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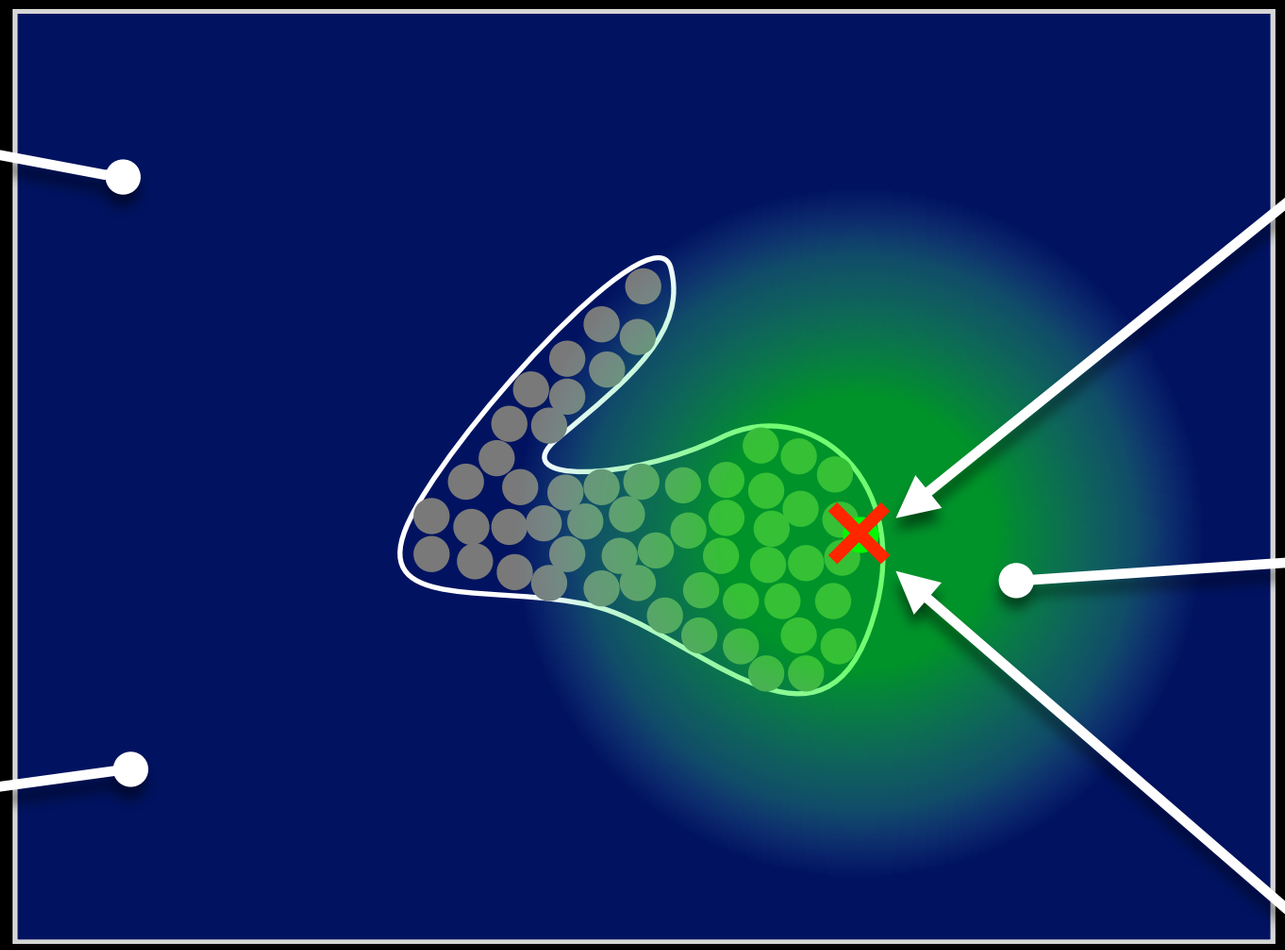

Every time a single fluorophore within the PSF is activated

Excited fluorophore emits a diffraction limited signal

Fluorophores are re-activated with weak illumination

Because we know it is a single event we can calculate the position with high precision

Super-Resolution
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Microscopy based on Single Molecule Localization

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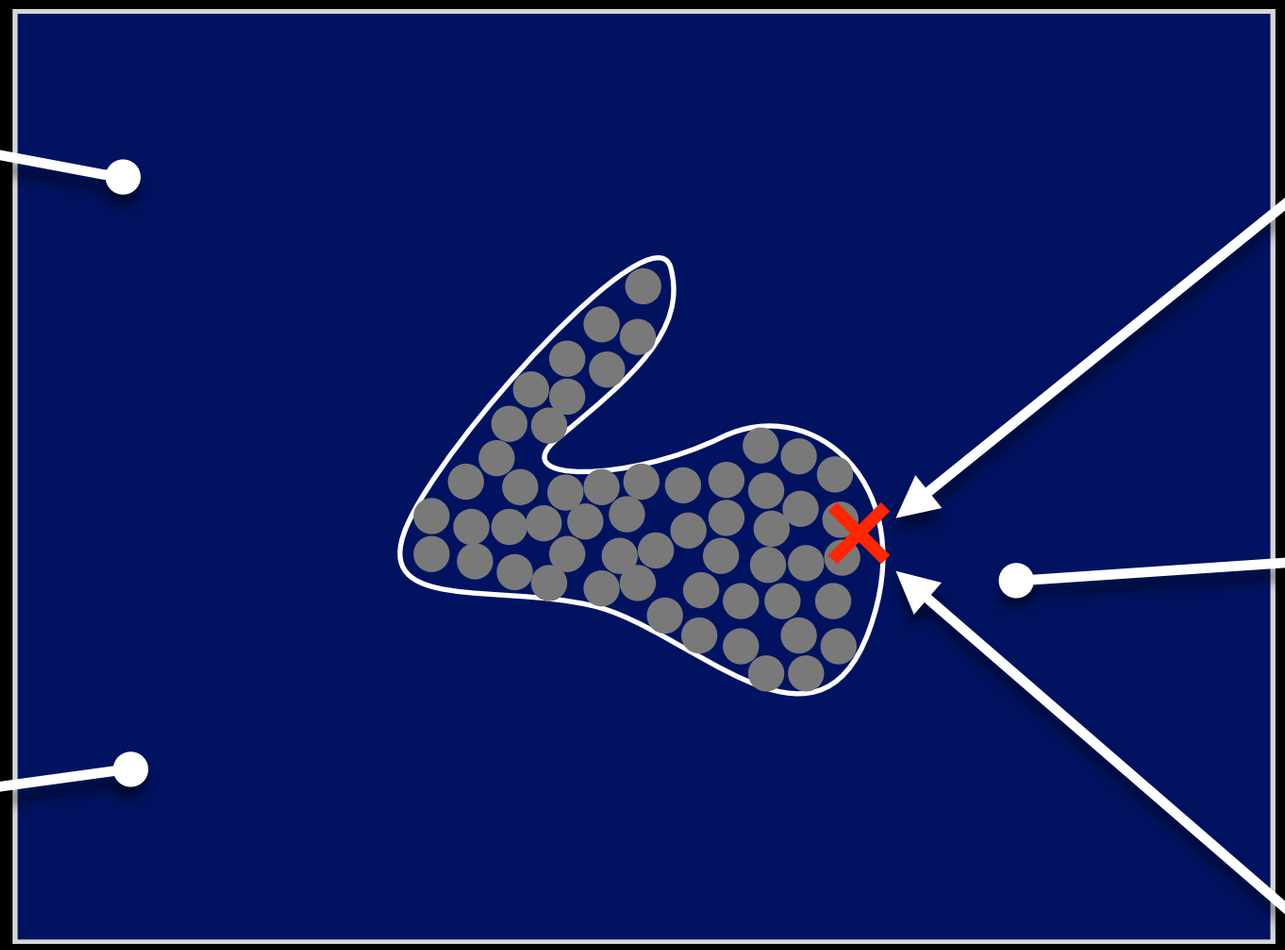

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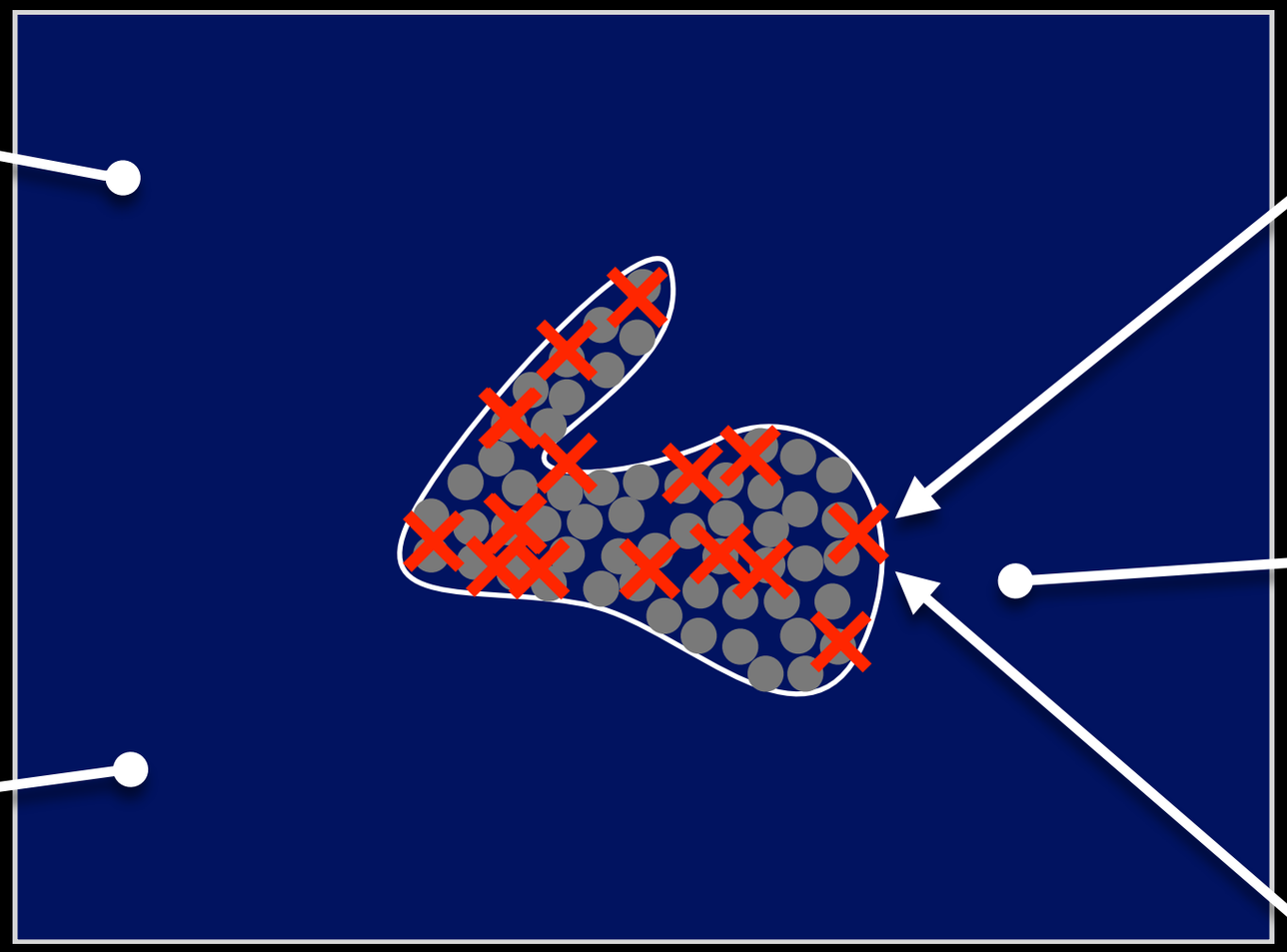

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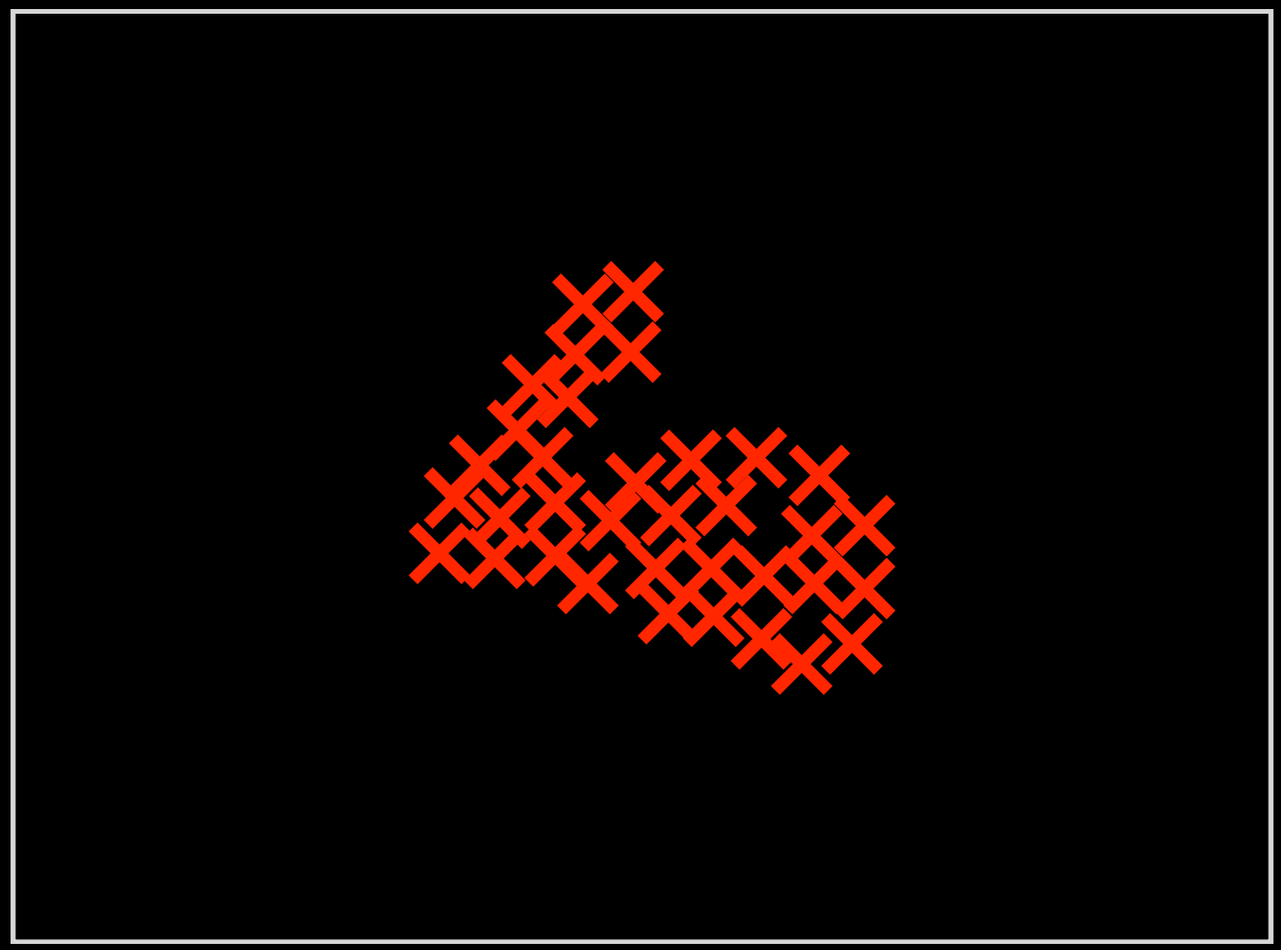
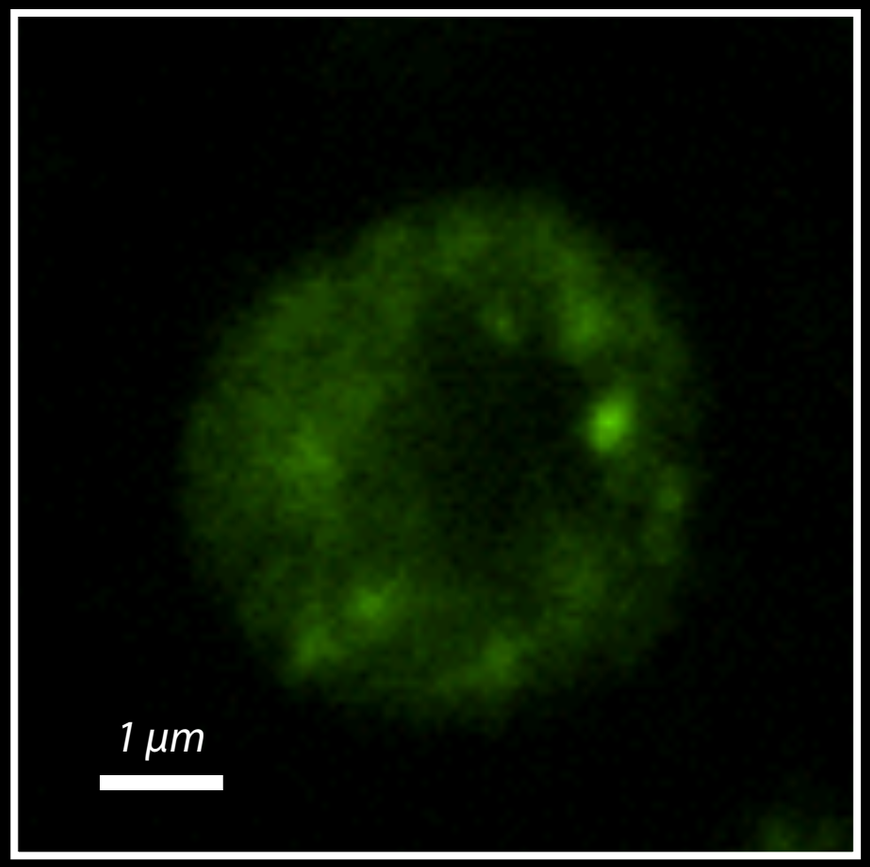


Microscopy based on Single Molecule Localization. What do you get?

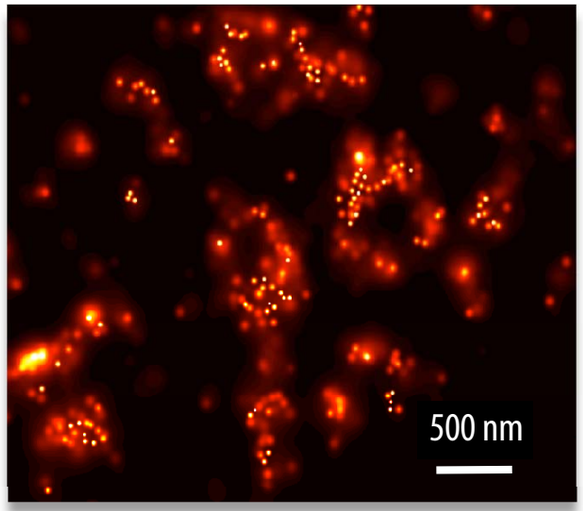
- Very high X-Y "resolution": ~30 nm
- Limited capabilities in 3-D
- Up to two colors
- Need "special" dyes, mounting media
- With exceptions, not suited for life imaging

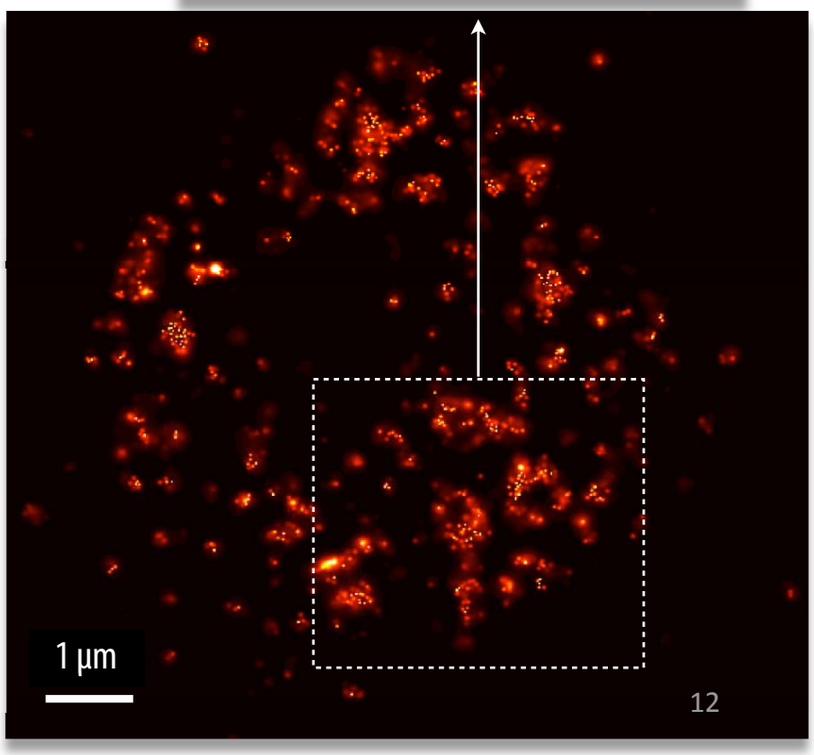
Microscopy based on Single Molecule Localization. What do you get?

WideField

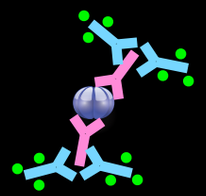


STORM

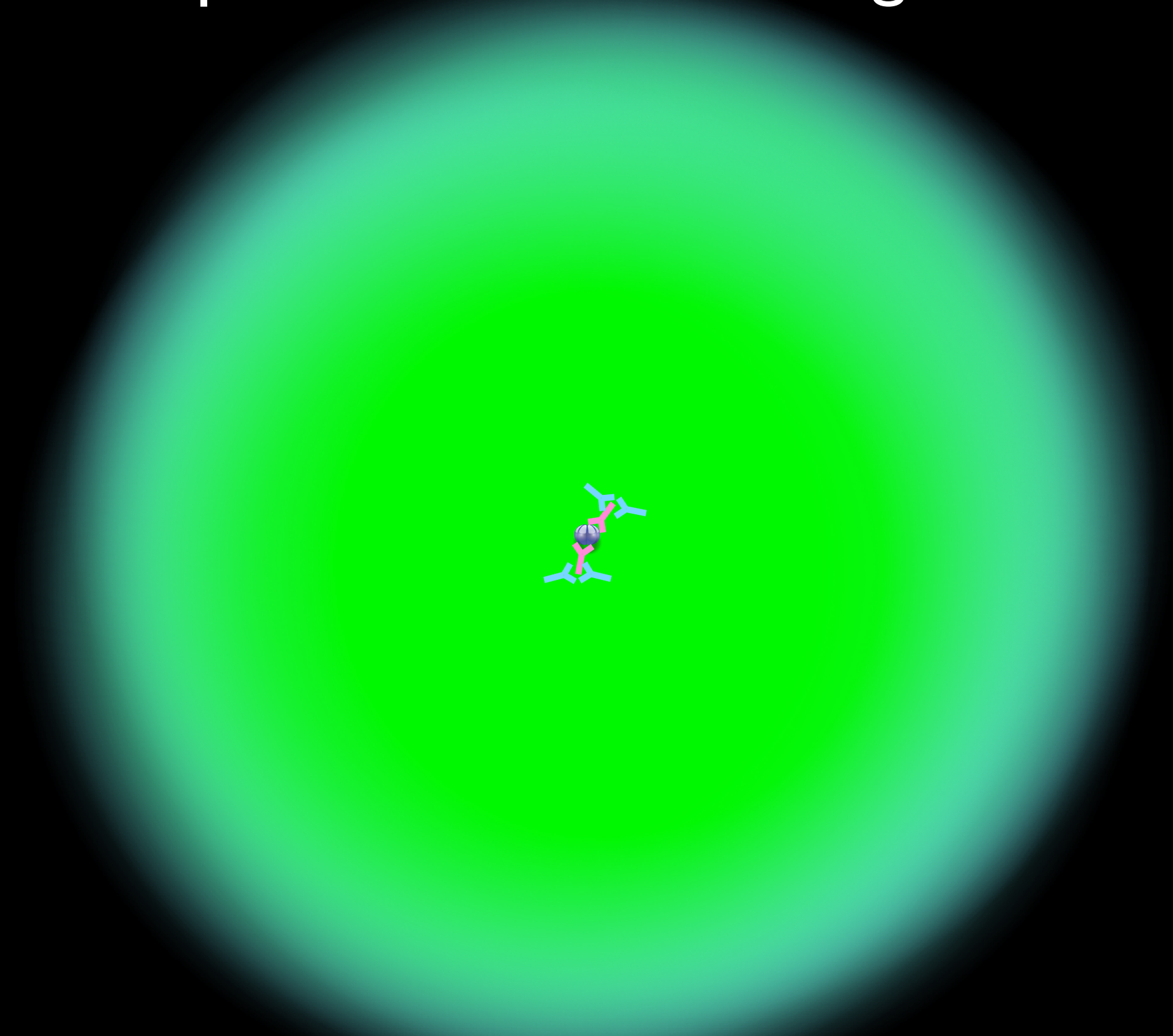




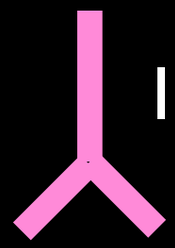
Super Resolution: labeling



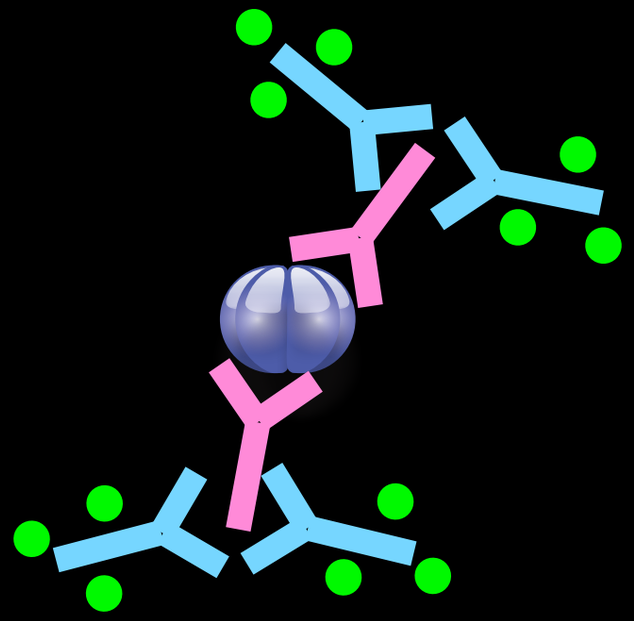
Super Resolution: labeling



Super Resolution: labeling



10 nm!



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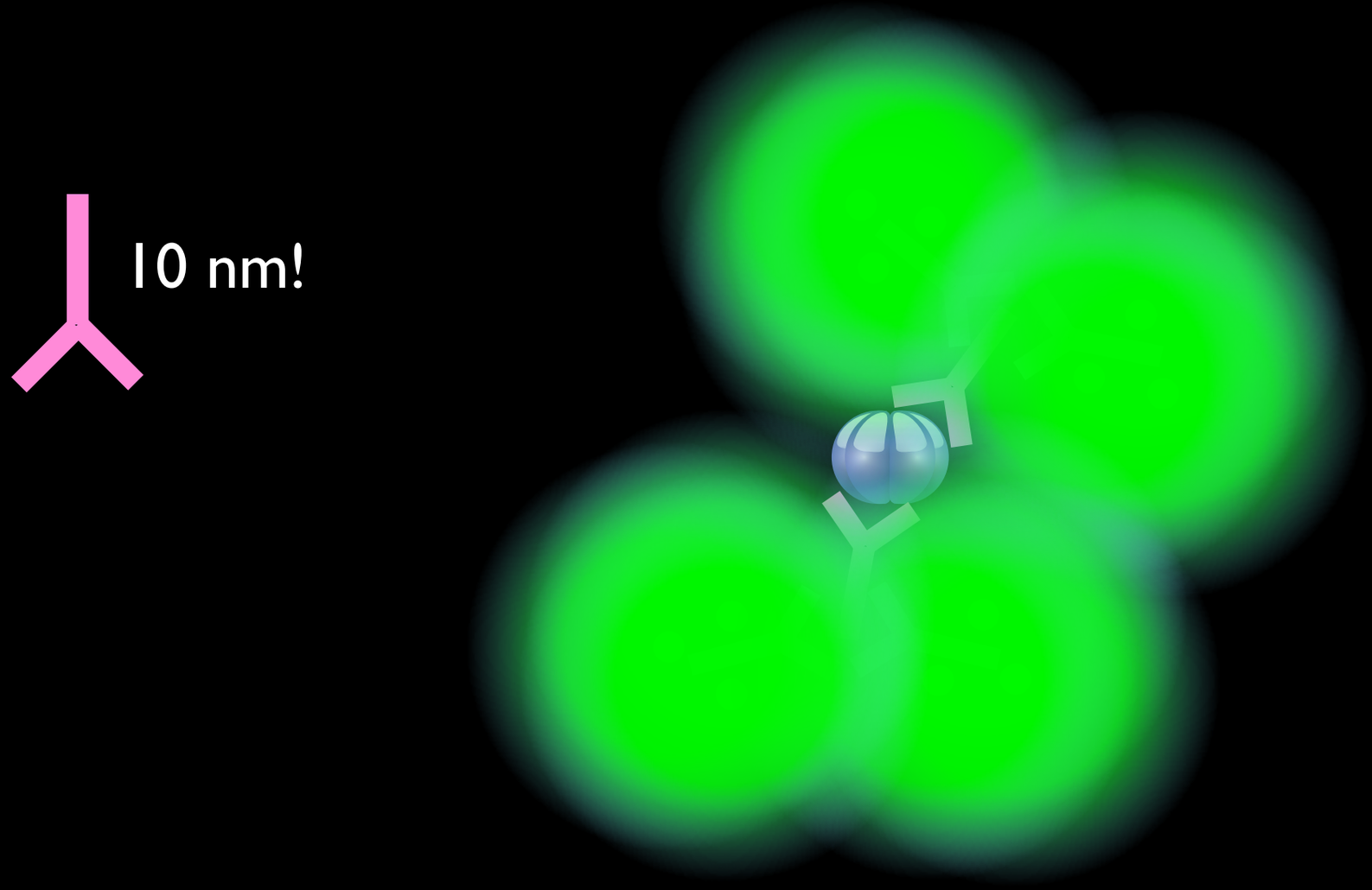

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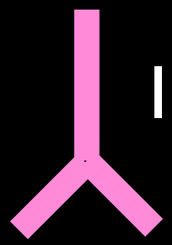

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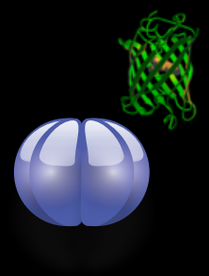
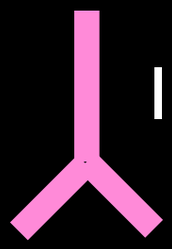
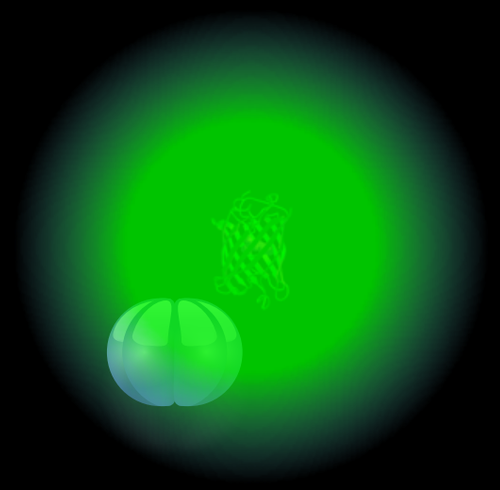


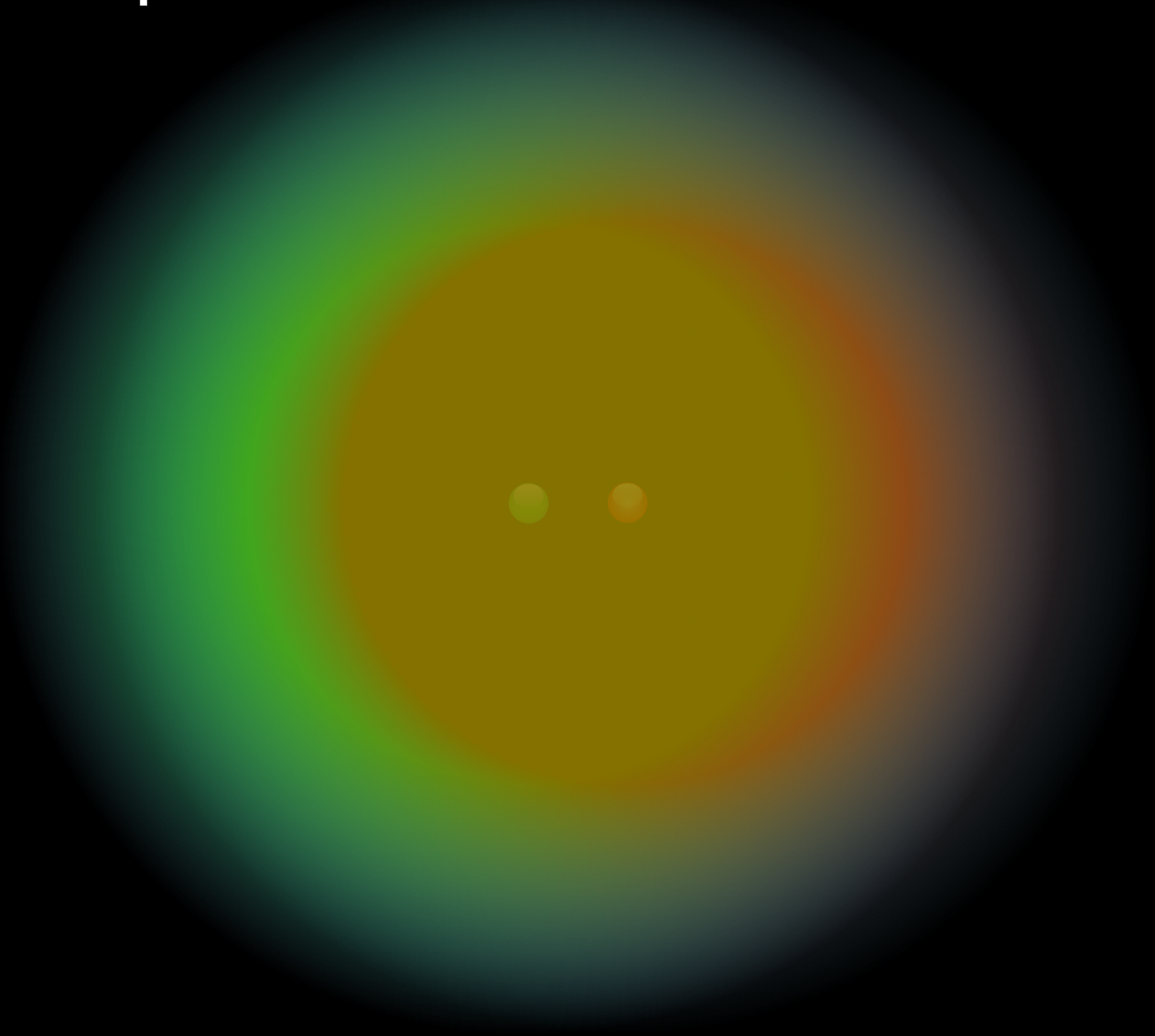
Super Resolution: labeling



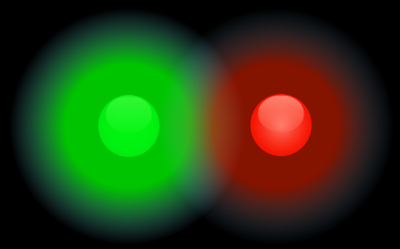
10 nm!



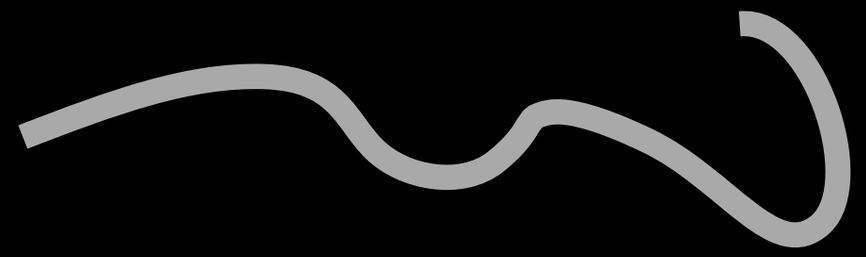
Super Resolution: co-localization



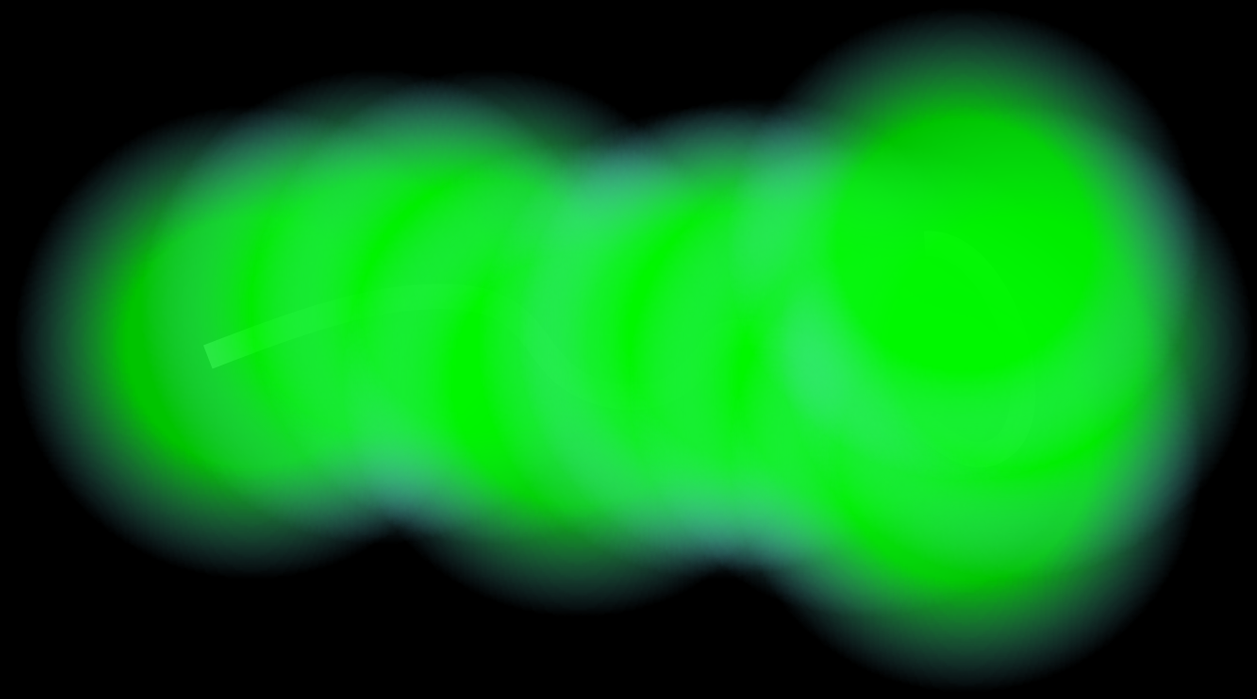
Super Resolution: co-localization



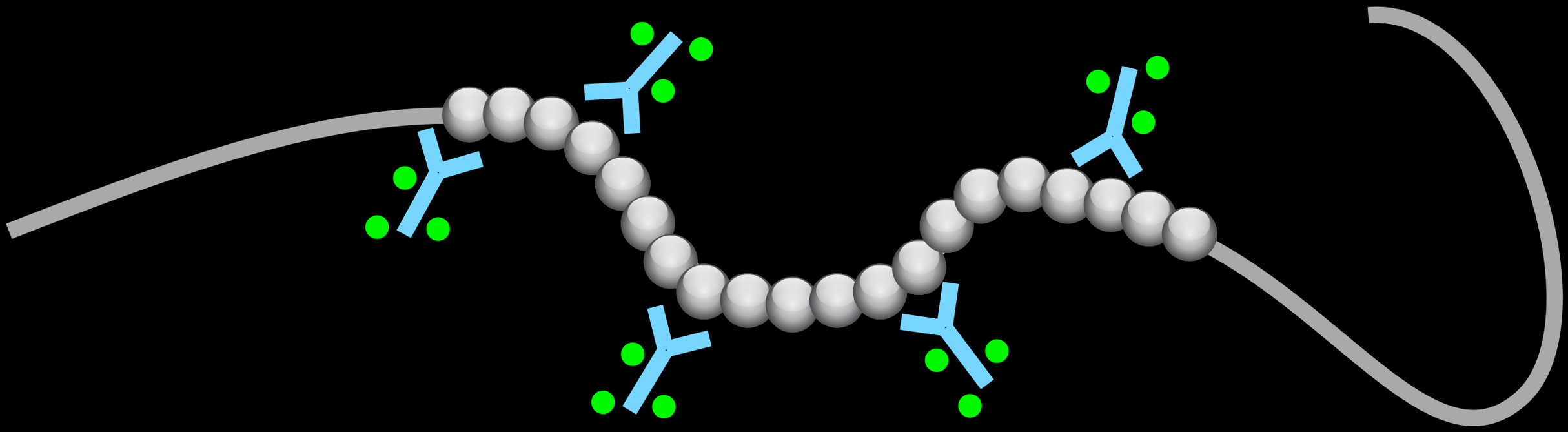
Super Resolution: labeling density



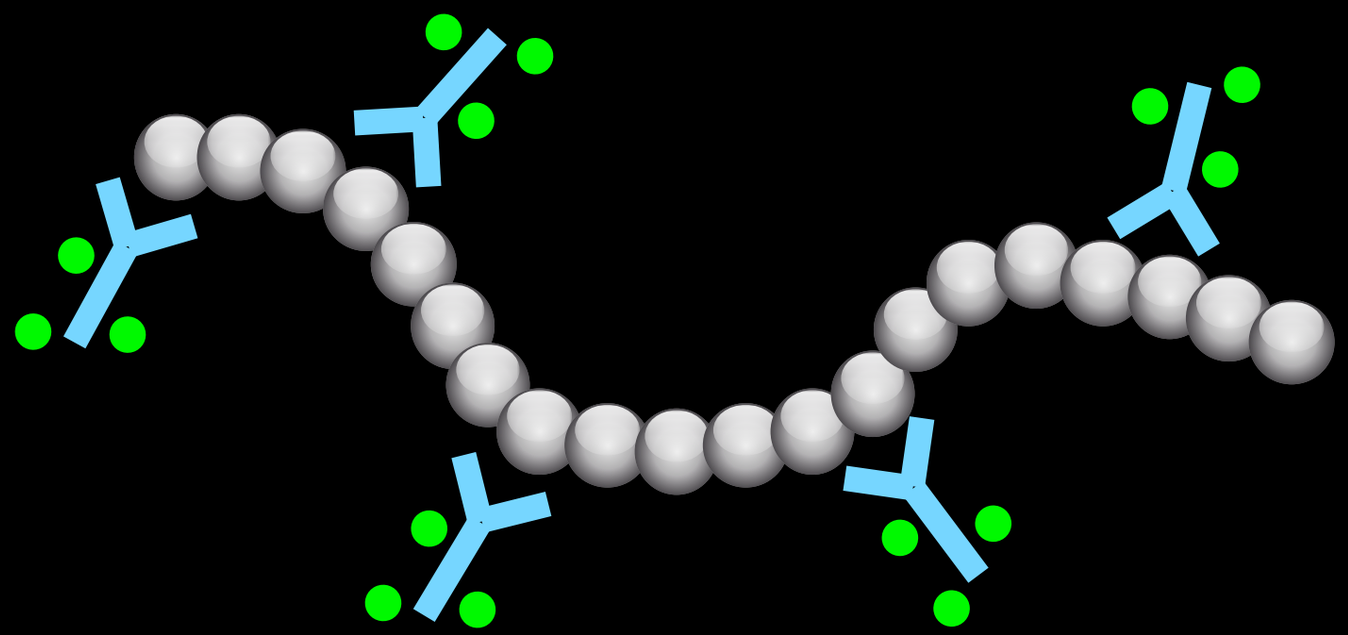
Super Resolution: labeling density



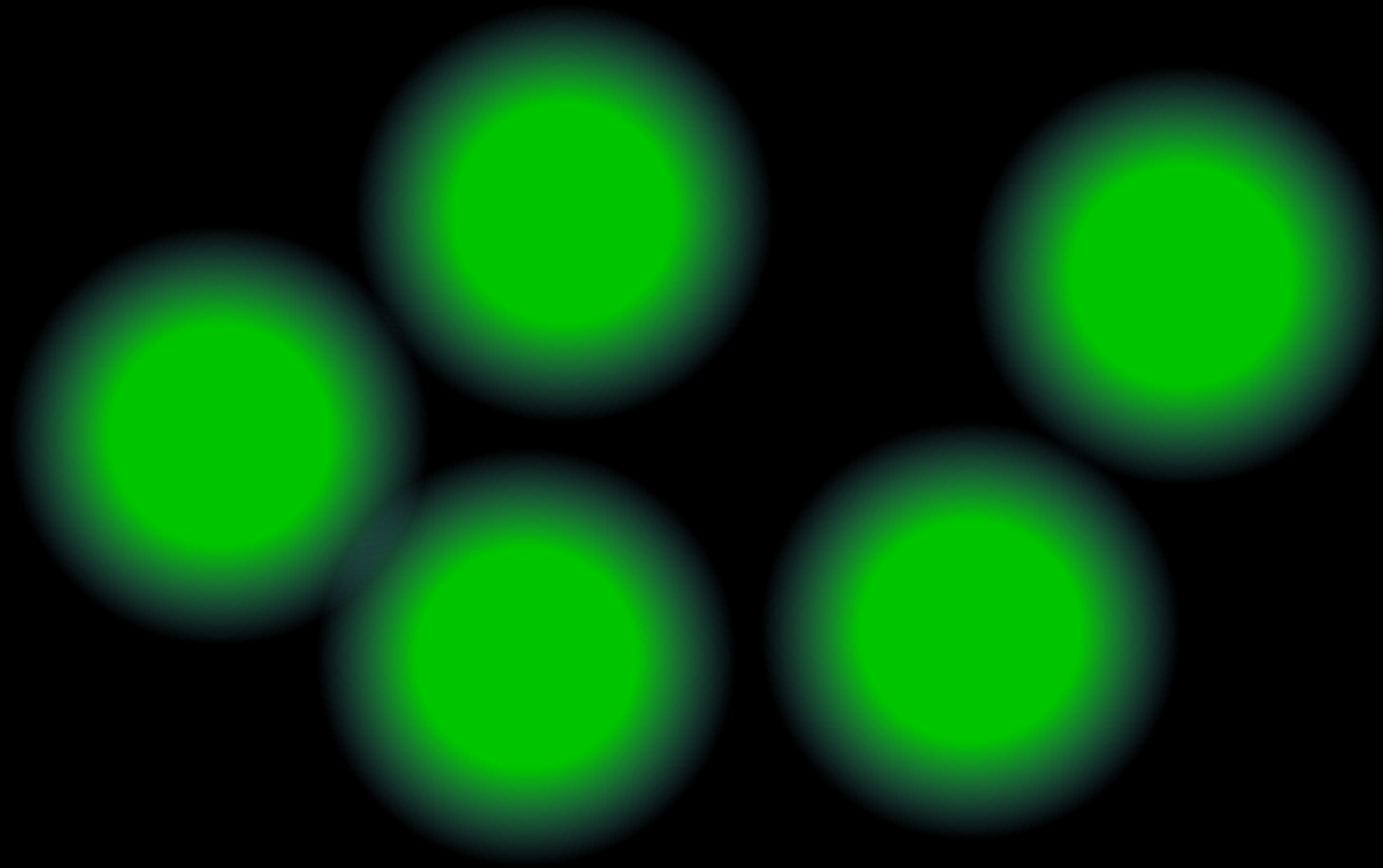
Super Resolution: labeling density



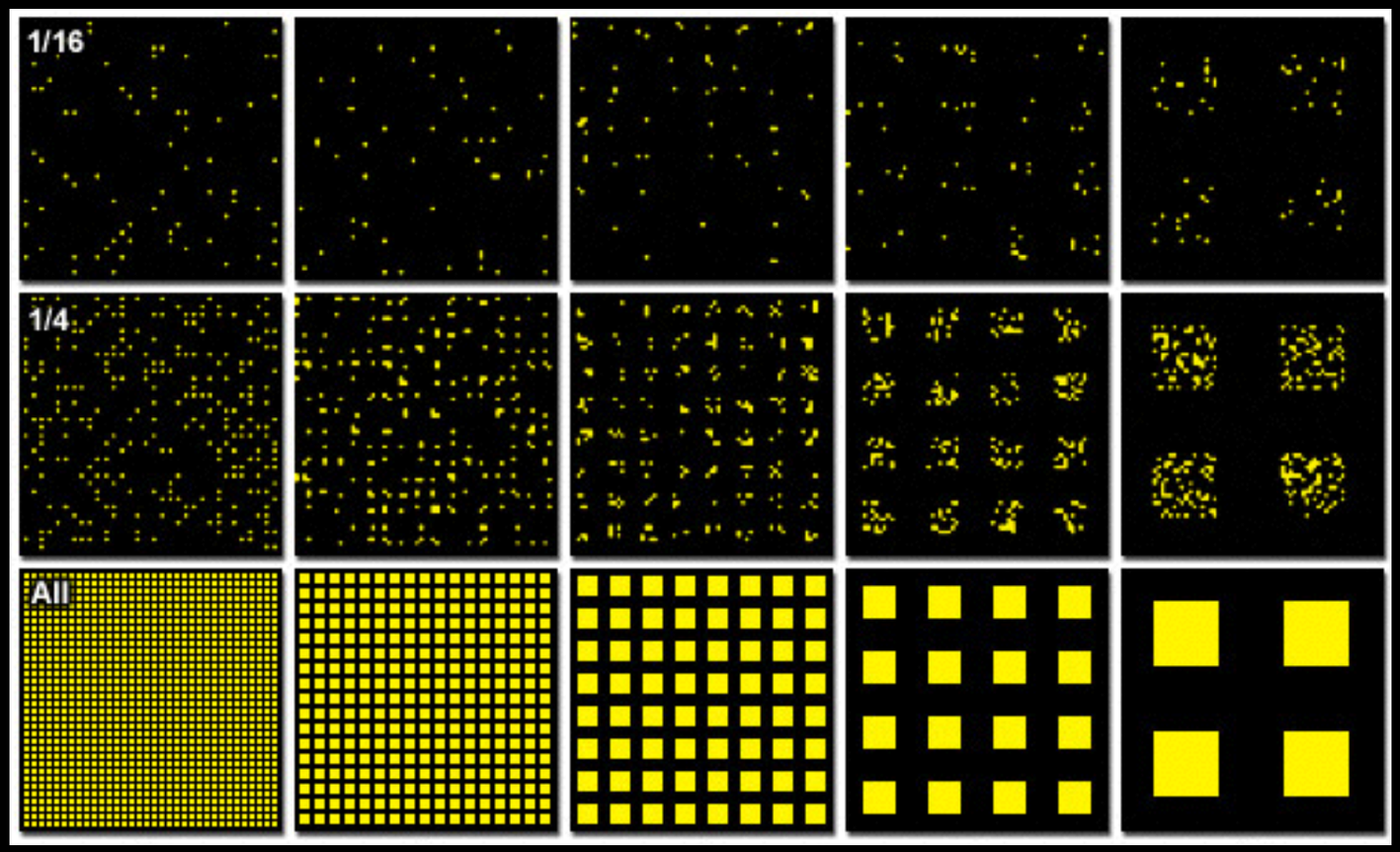
Super Resolution: labeling density



Super Resolution: labeling density



Localization precision is not resolution: labeling density



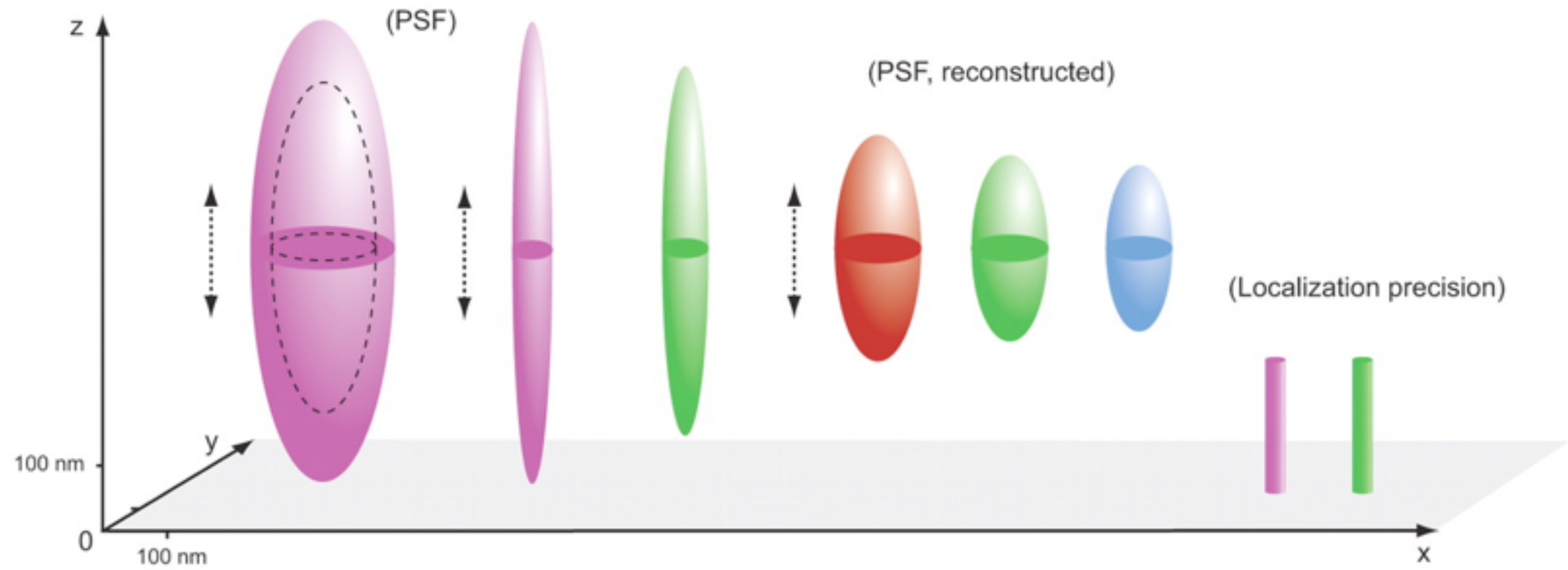
Sample preparation

- Be clean with your sample
- Use the proper coverslips: #1.5H = 170 ± 5 µm
- Plate your cells or other biologic material on the coverslip
- Optimise fixation and labelling protocols to get strong and contrasted stainings: play with Abs concentrations, incubation temperature and time and washes
- Be aware of the mounting medium. Let it embed
- Be aware of the fluorophores
- STORM requires special mounting media
- ...

Contact the specialist before doing the experiment

Super Resolution: PSFs

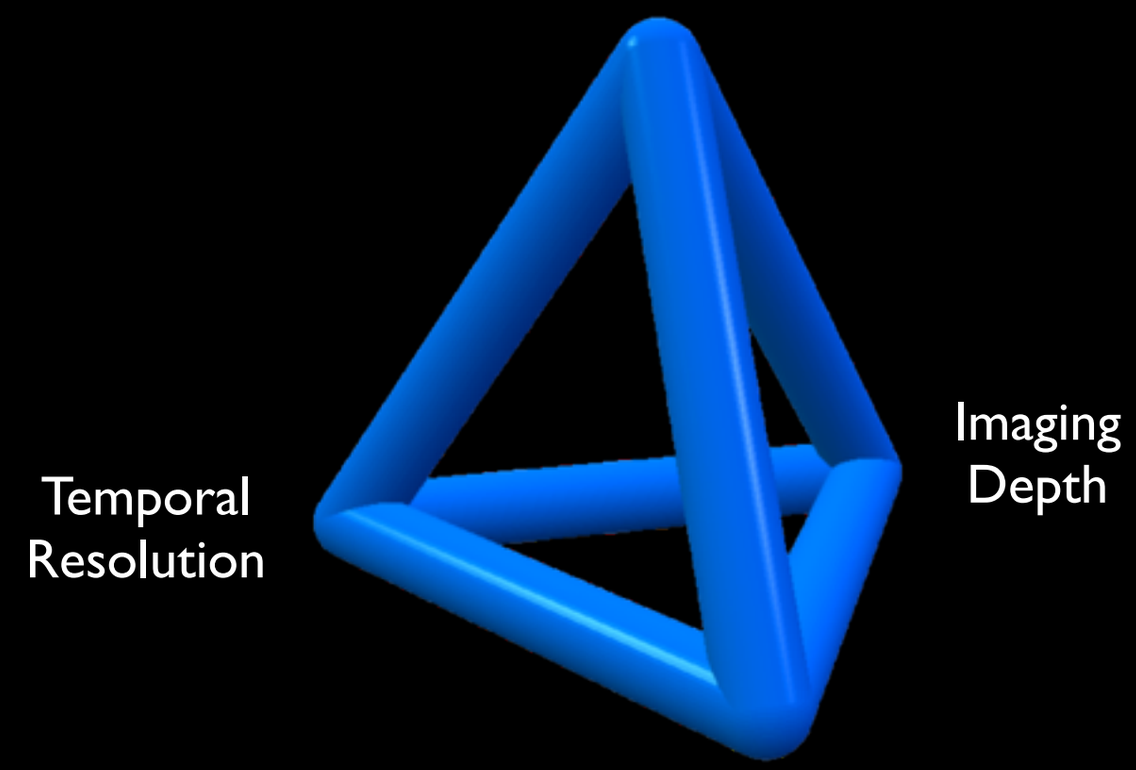
	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λ_{em} [nm]	460-670	670	520	620	520	460	670	520



~$D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
~D_z [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
~$V_{x,y,z}$ [*10^{-3} µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

Schermelleh et al. 190 (2): 165

Super Resolution: a matter of photon budget management

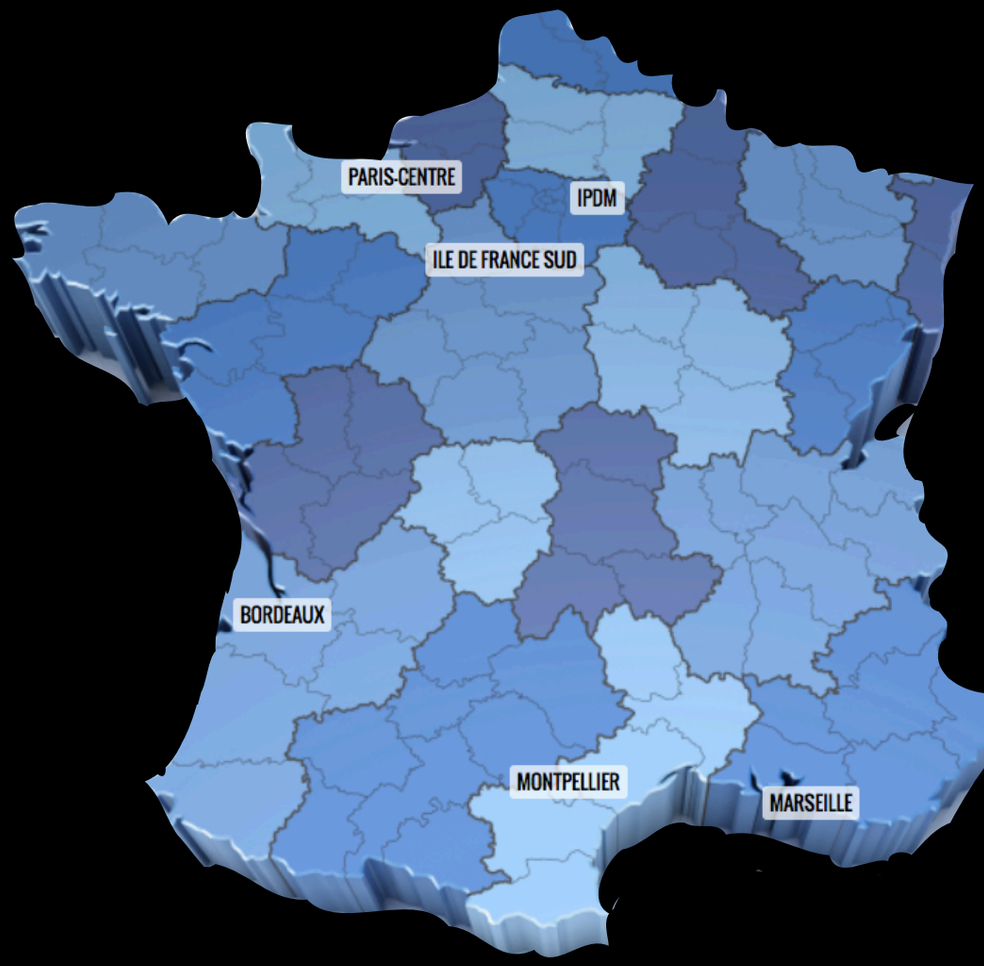


Super Resolution: a matter of photon budget management

	CLSM/WF	SIM/3D-SIM	STED/3xSTED	PALM/STORM
X-Y Resolution	180-250 nm	80-130 nm	60-80 nm	20-40 nm (Precission)
Z Resolution	500-700 nm	250-350 nm	250-500 nm	With limitations 140 nm in TIRF
Time Resolution	100 msec - 2D 5 sec - 3D	100 msec - 2D 5 sec - 3D	30 sec - 2D 10 min - 3D	20 sec - 2D 15 min - 3D
Dyes	Any	Most conventional dyes	Special dyes	Special dyes
Simultaneous wavelengths	>3	>3	2	2
Live-cell imaging	Yes	Restricted	Very Restricted	Very Restricted

Where can I do super-resolution?

FRANCE-BIOIMAGING



Montpellier RIO Imaging

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