

Outline

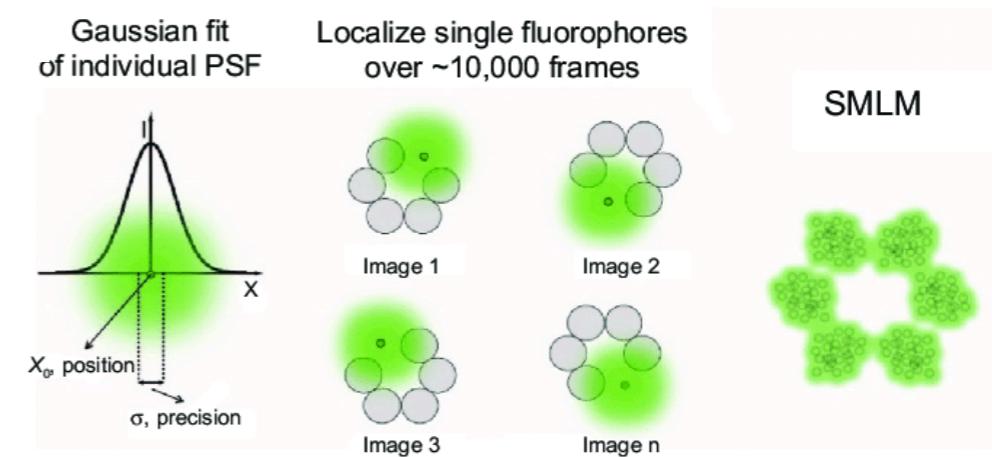
1. Introduction
2. Single molecule super resolution microscopy
3. Structured illumination super resolution microscopy
4. Case study

Outline

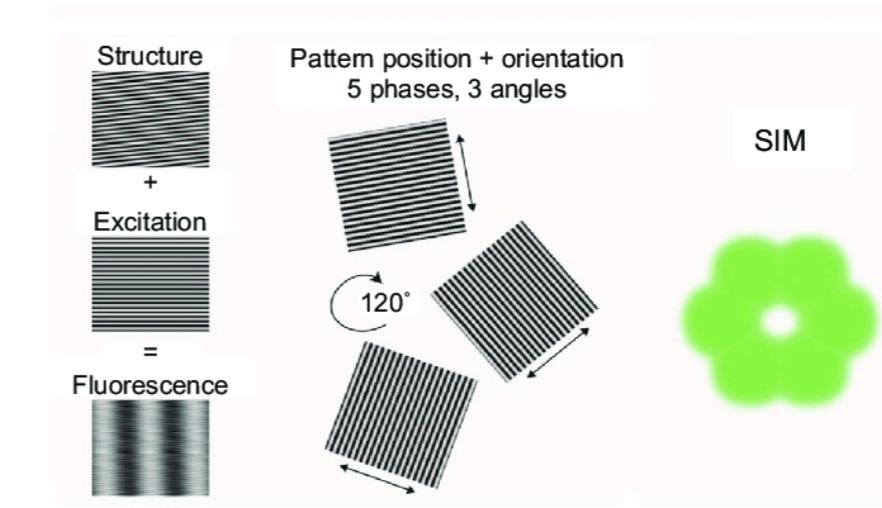
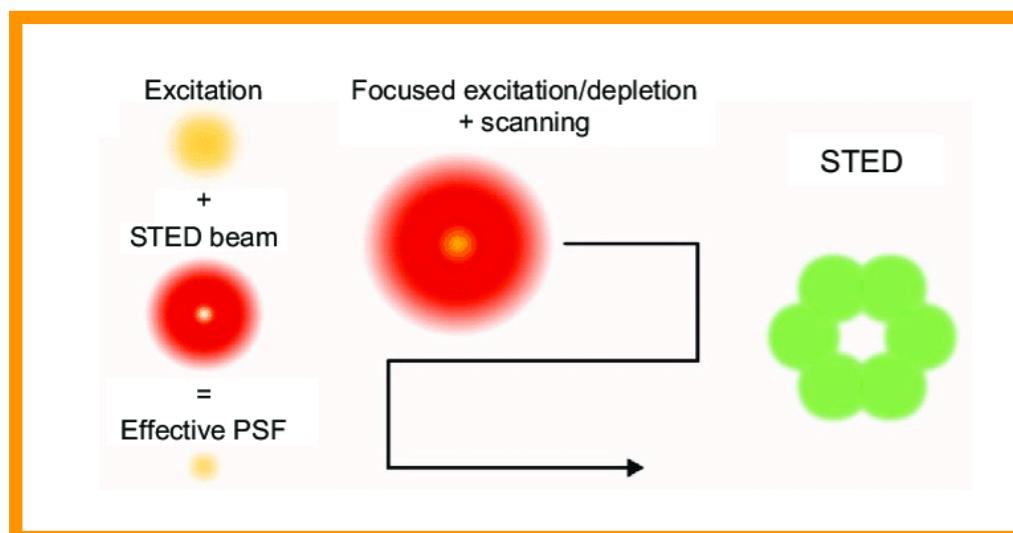
Problem : molecules within 200nm are not discernable

Solution : Keep some of them dark

SMLM —> Stochastic



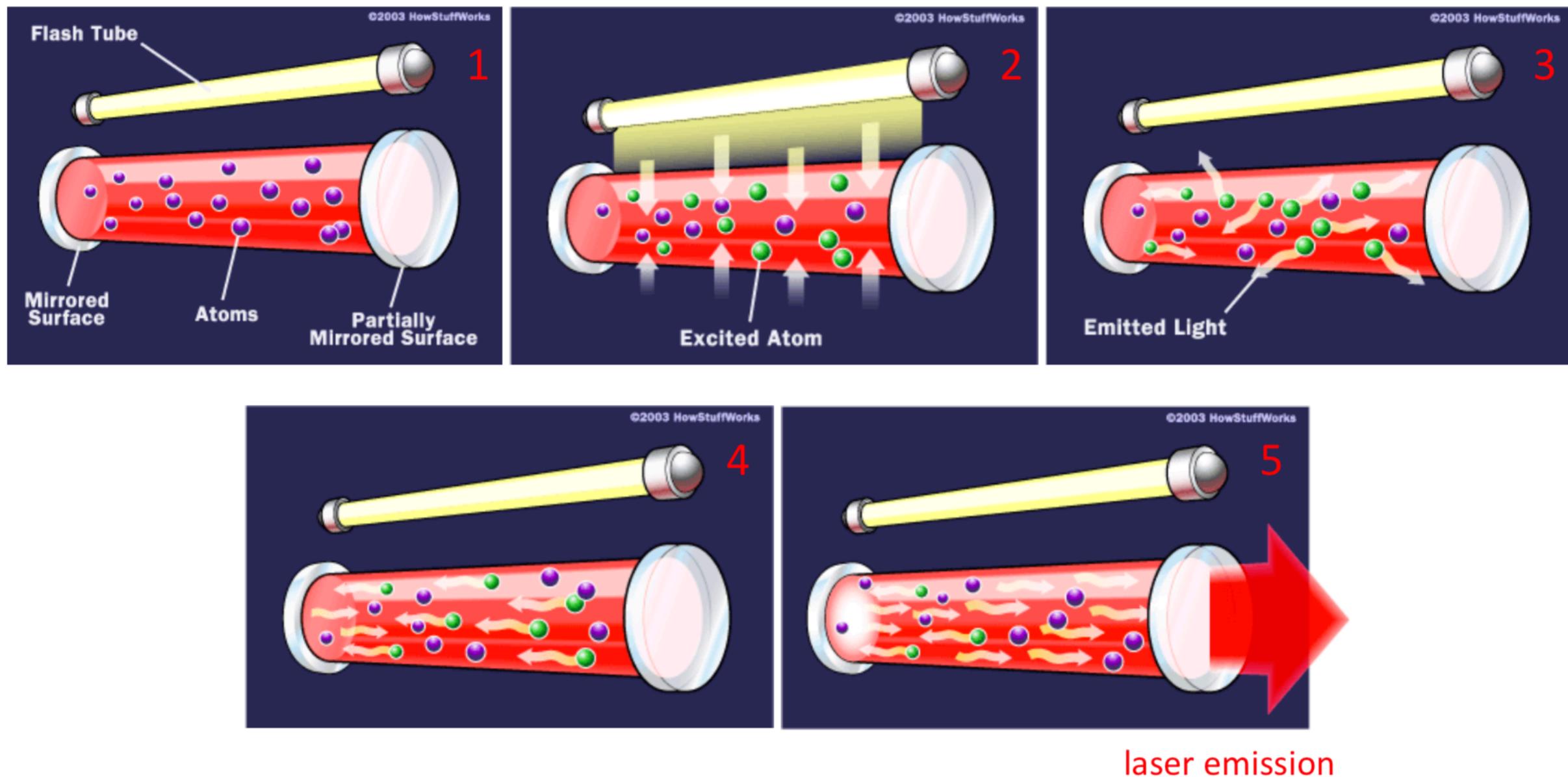
Structured illumination —> Deterministic



STimulated Emission Depletion (STED)

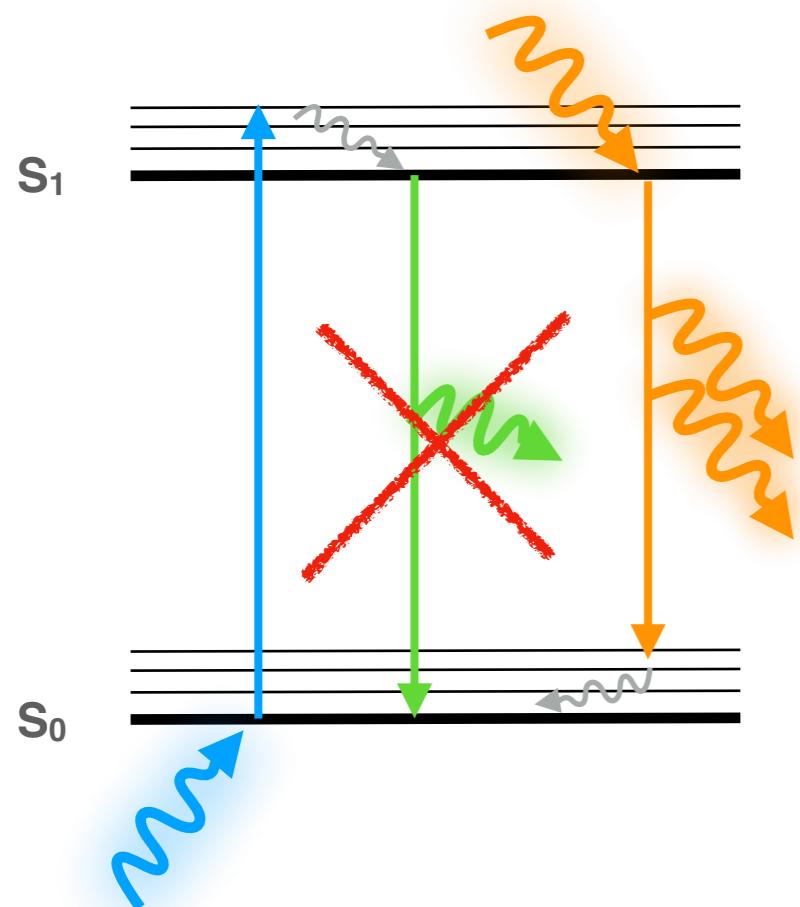
1. Stimulated emission and STED principle
2. Shape light as a donut
3. Tuning resolution and image sampling
4. Conditions with fluorophores
5. Conditions with lasers / detectors
6. Conditions with sample preparation

Light Amplification by Stimulated Emission of Radiation



Stimulated Emission

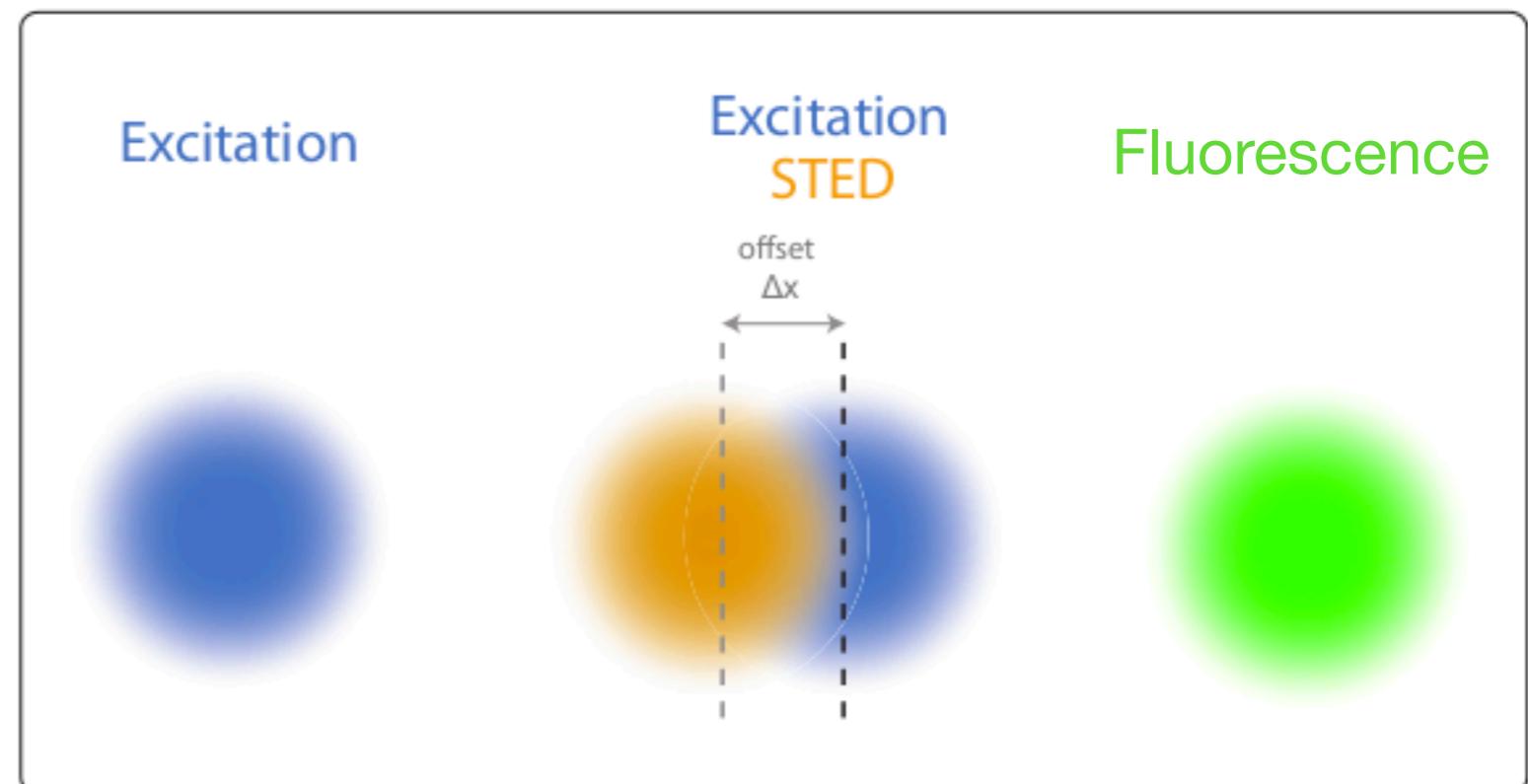
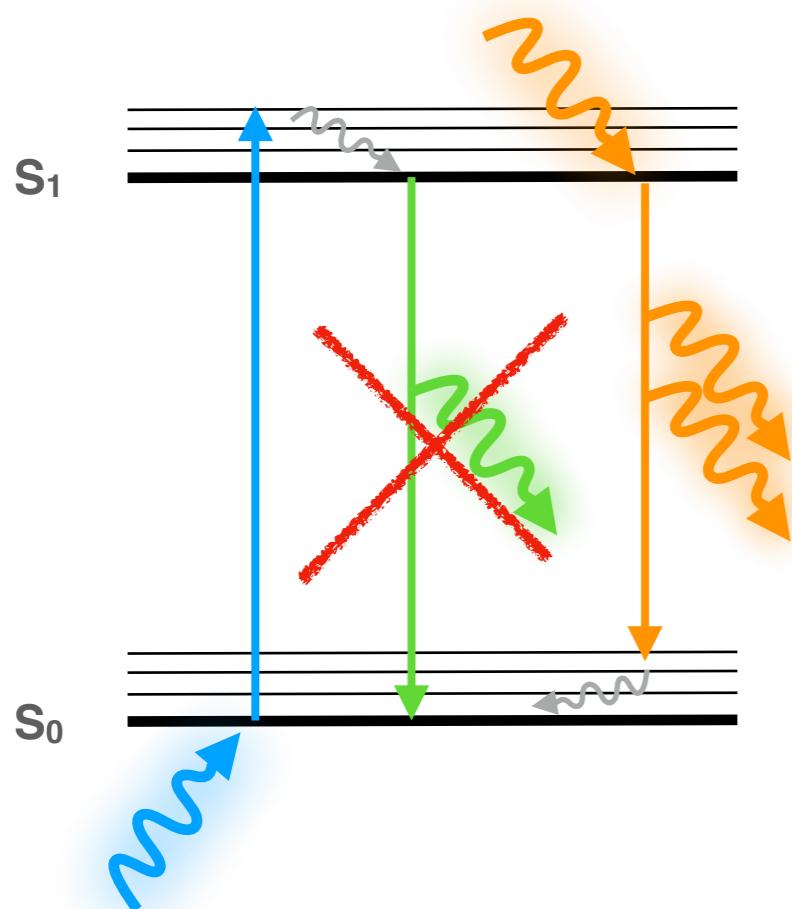
Jablonski diagram



- 1** Excitation
- 2** Internal conversion
- 3** Fluorescence
- 4** Stimulated emission

Stimulated Depletion

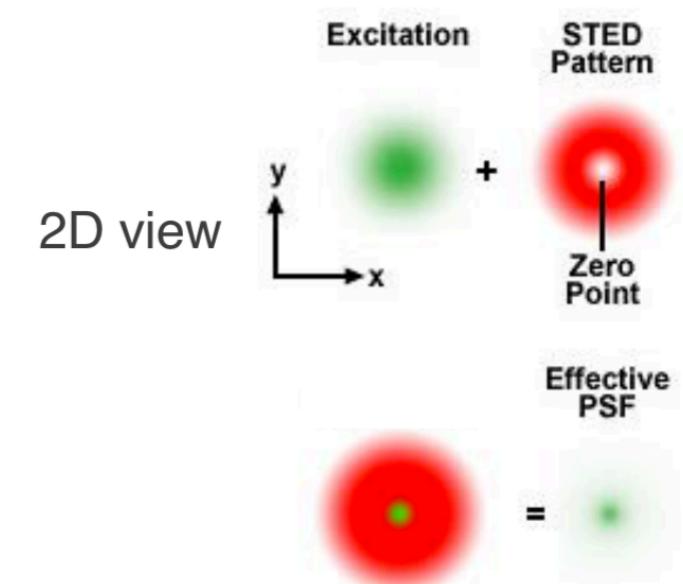
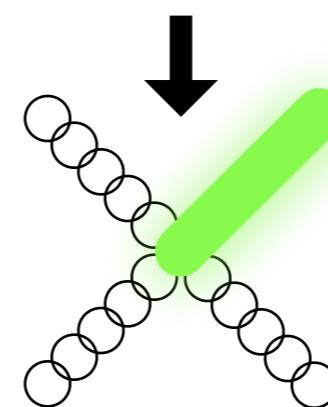
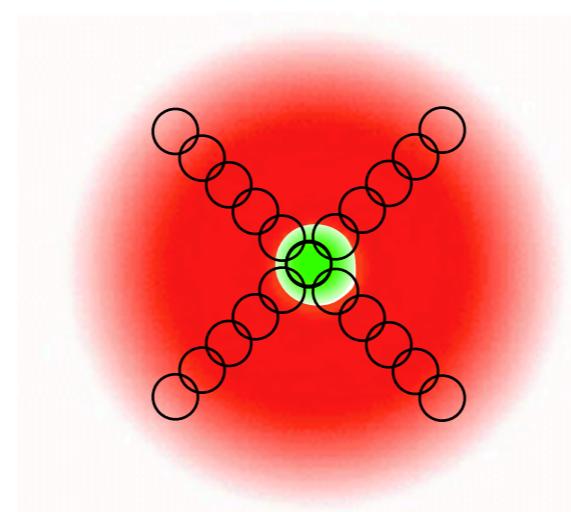
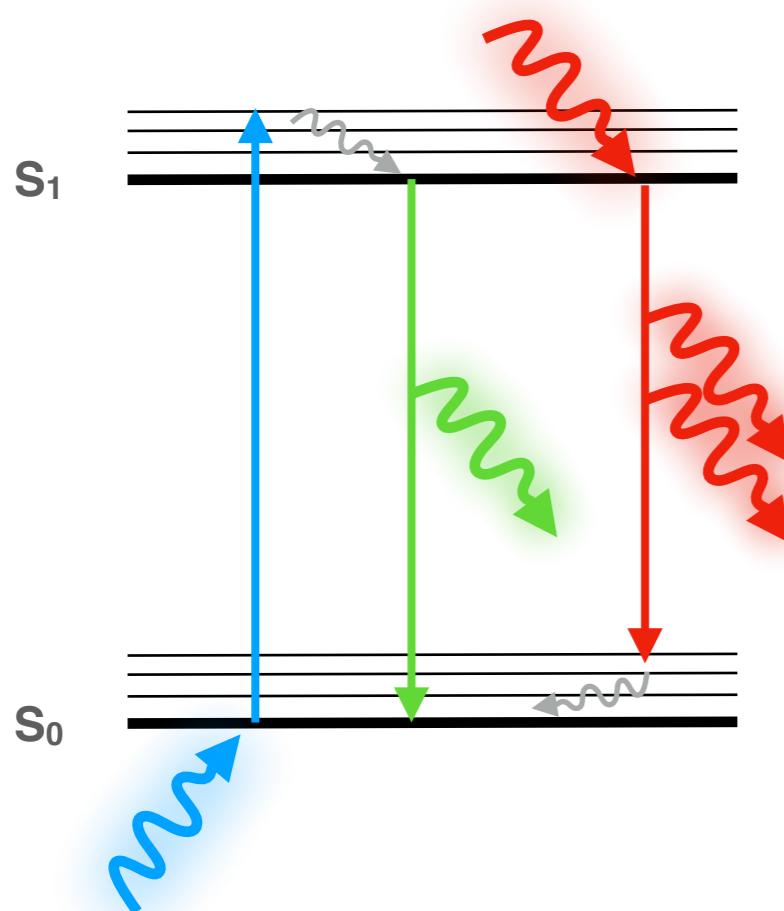
Jablonski diagram



Effective
excitation
area

Confining excitation beyond the diffraction limit

Jablonski diagram

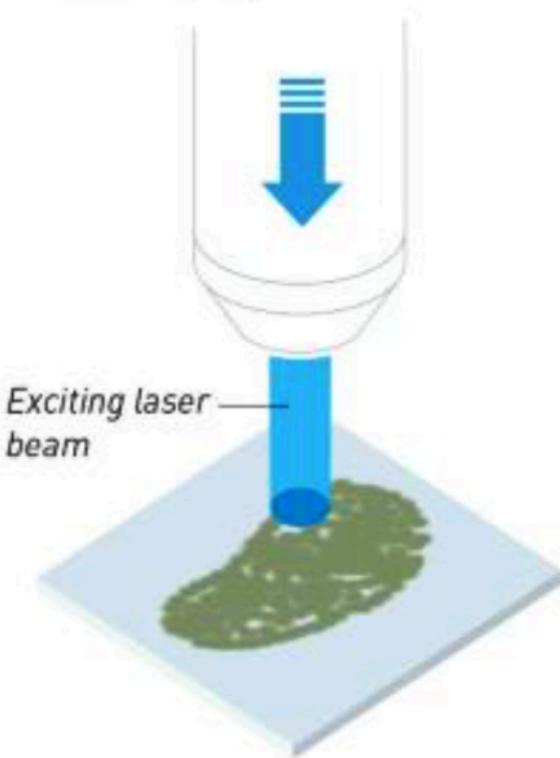


High intensities saturate the SE transition and keep virtually all the fluorophores in the ground state (OFF state), except those located in a region around the ‘zero’-intensity point

Stimulated Depletion

The principle of STED-microscopy

Regular optical microscope



STED-microscopy

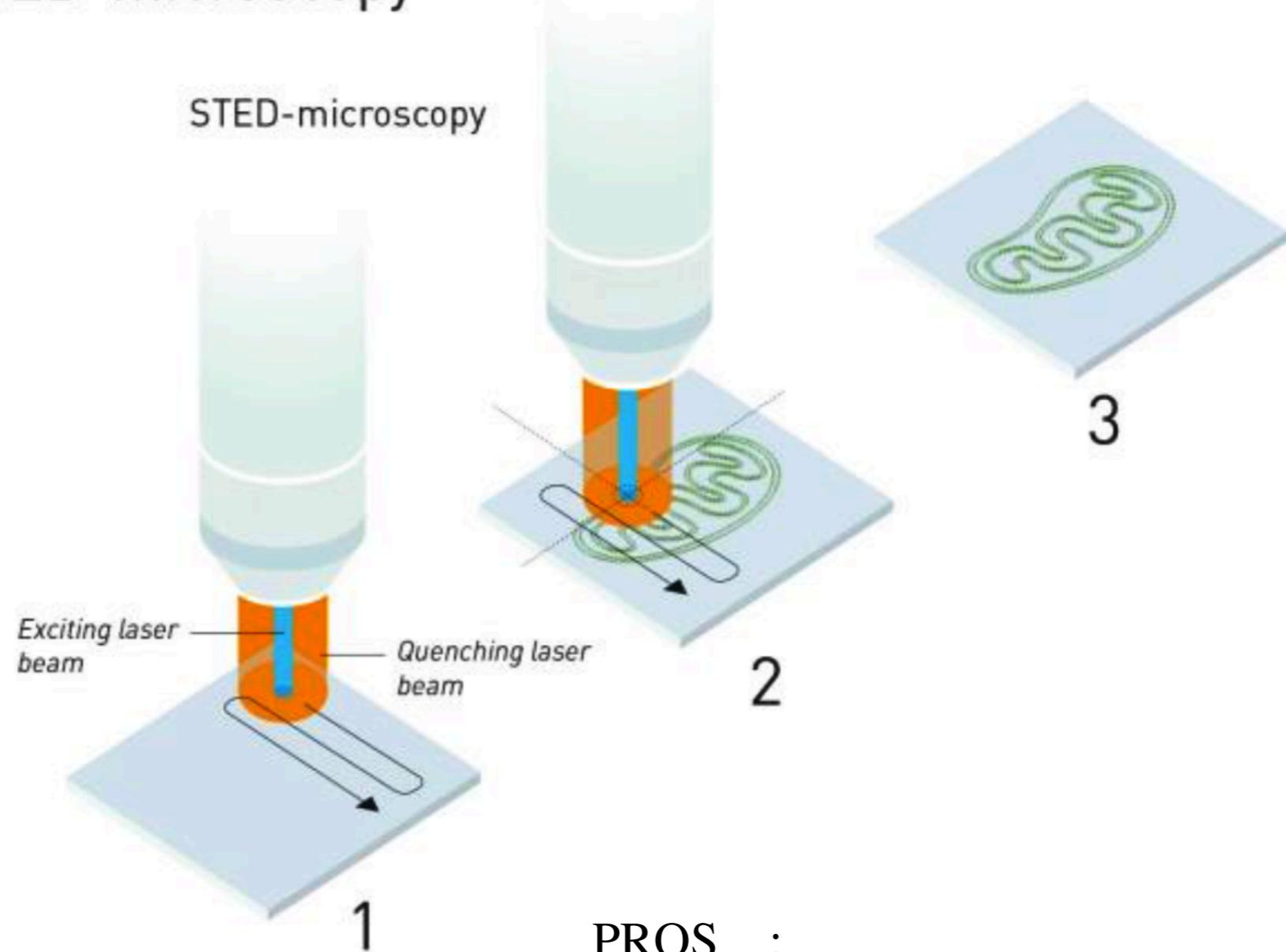
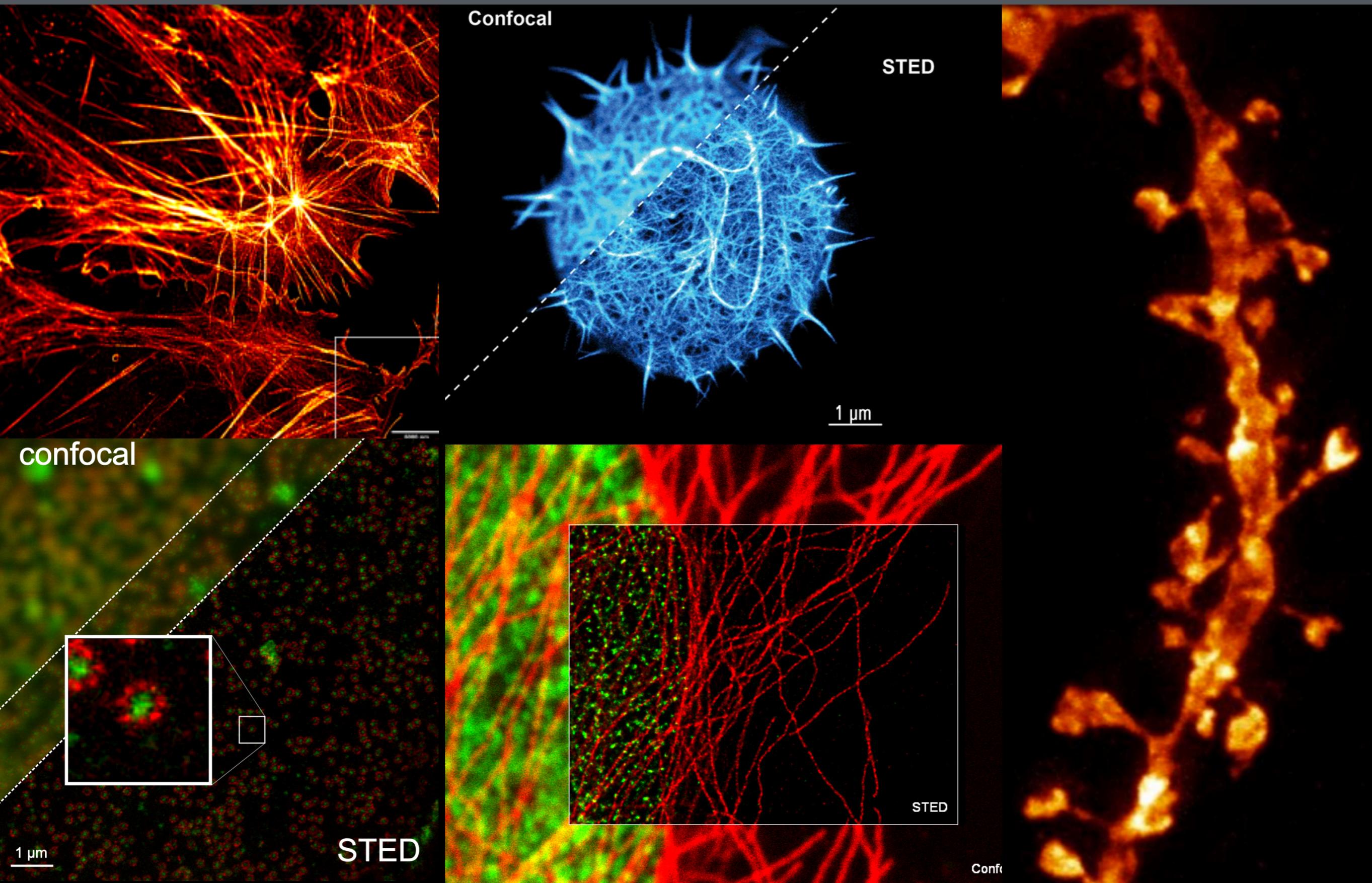


Illustration: © Johan Jarnestad/The Royal Swedish Academy of Sciences

PROS :

- Confocal-based
- No need for specialised fluorophores
- No need to computationally reconstruct images

Confining excitation beyond the diffraction

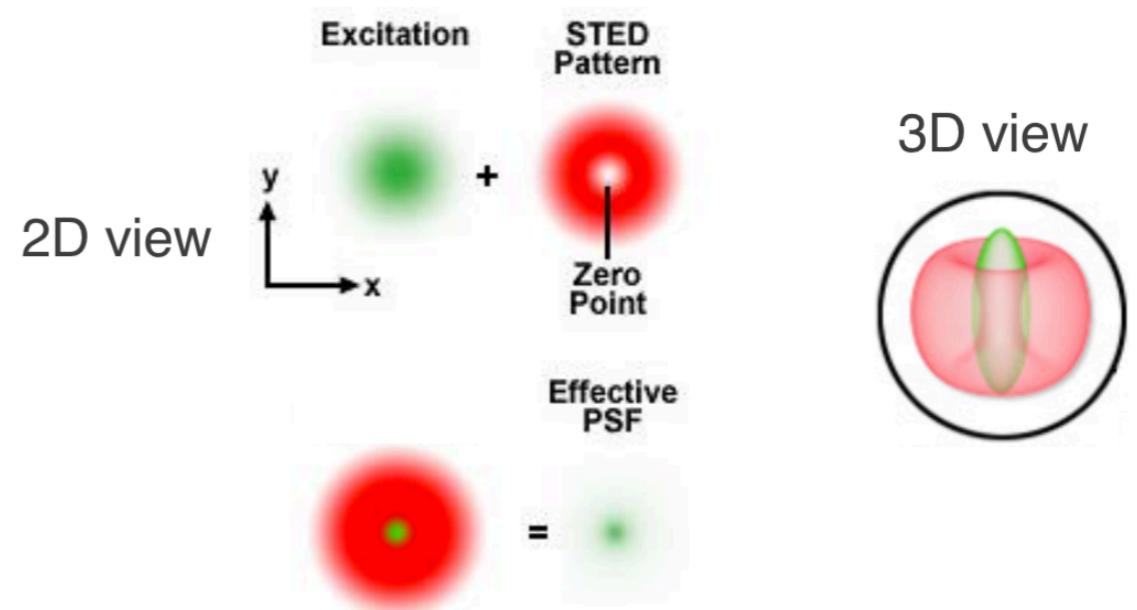


How to shape light into a donut

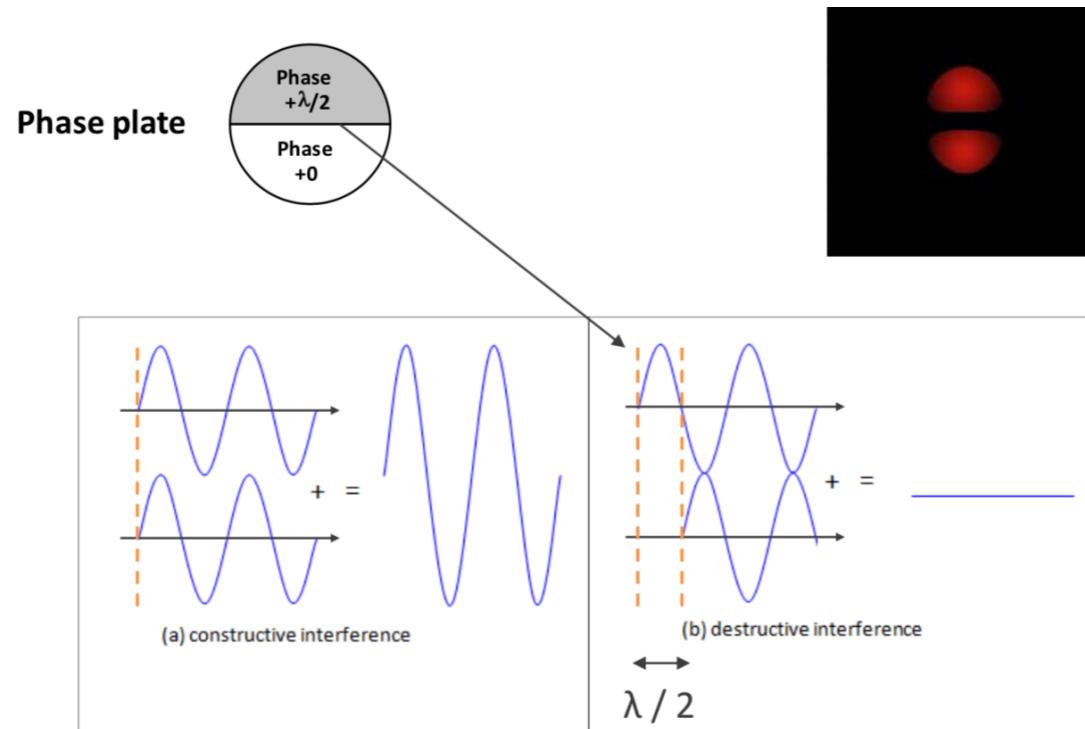
Shape light to create a donut:



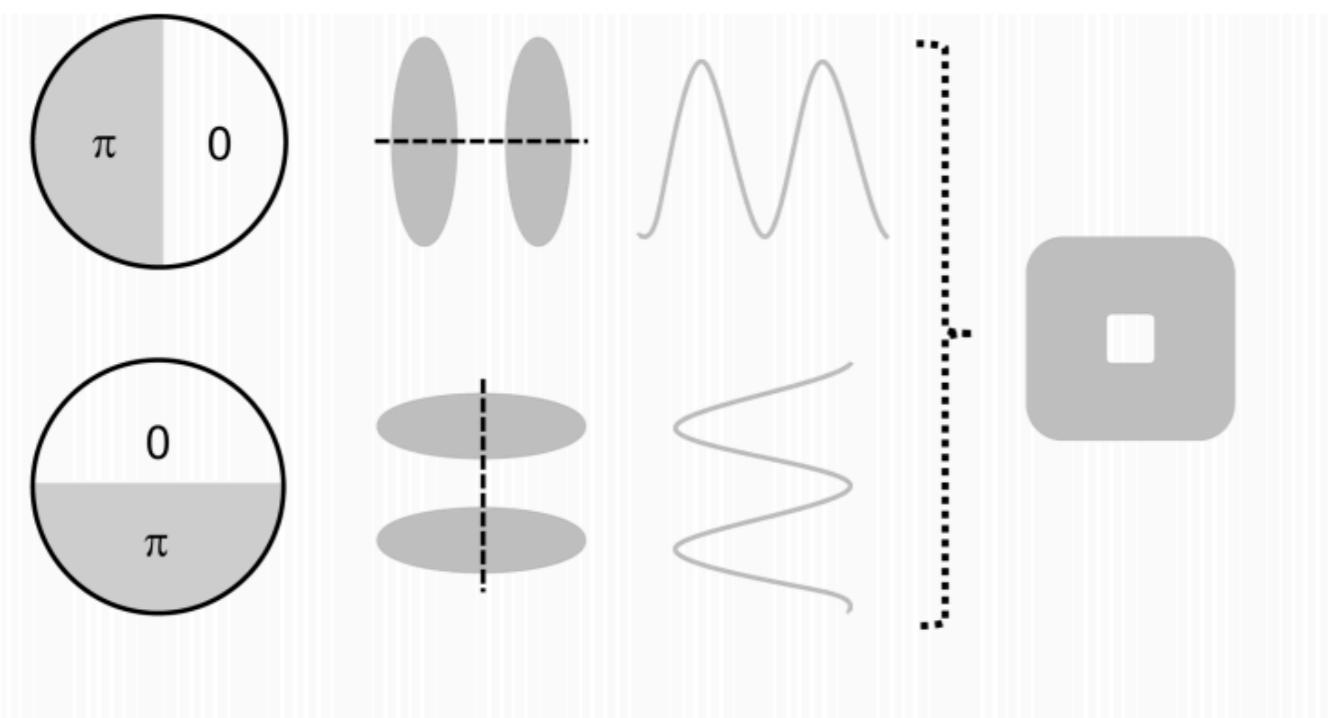
- ✓ Two half-phase plate
- ✓ Vortex / spiral phase plate
- ✓ Spatial Light Modulator (SLM)



Lateral phase-plates



Source: <http://www.schoolphysics.co.uk/>

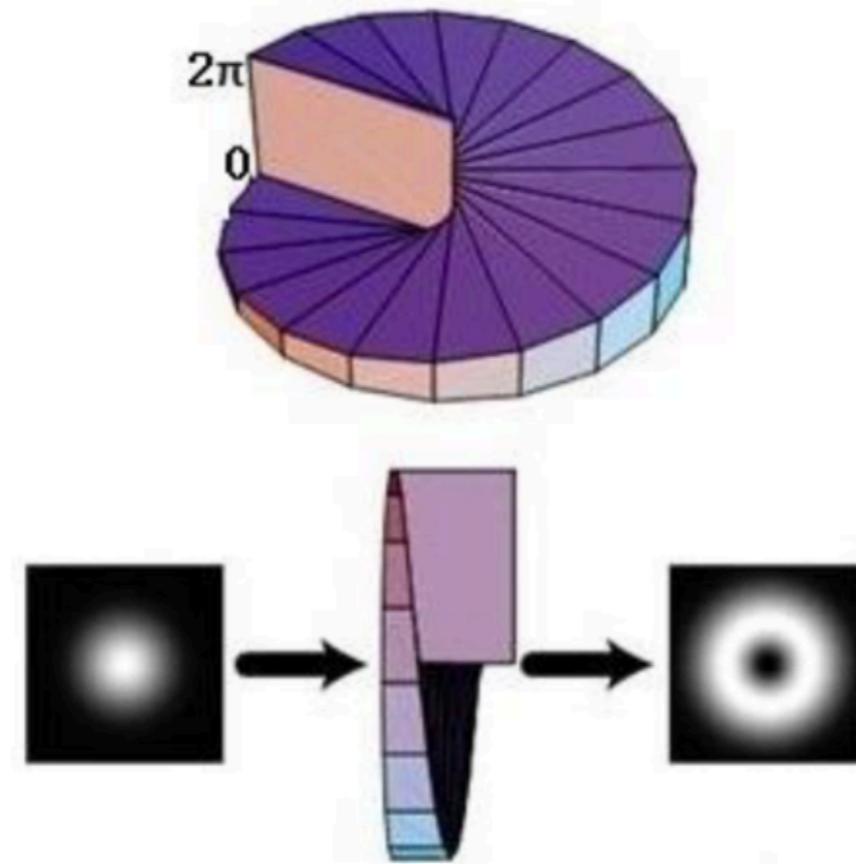


cross-section of the STED beam is optically delayed from left-to-right as well as from below-to-above

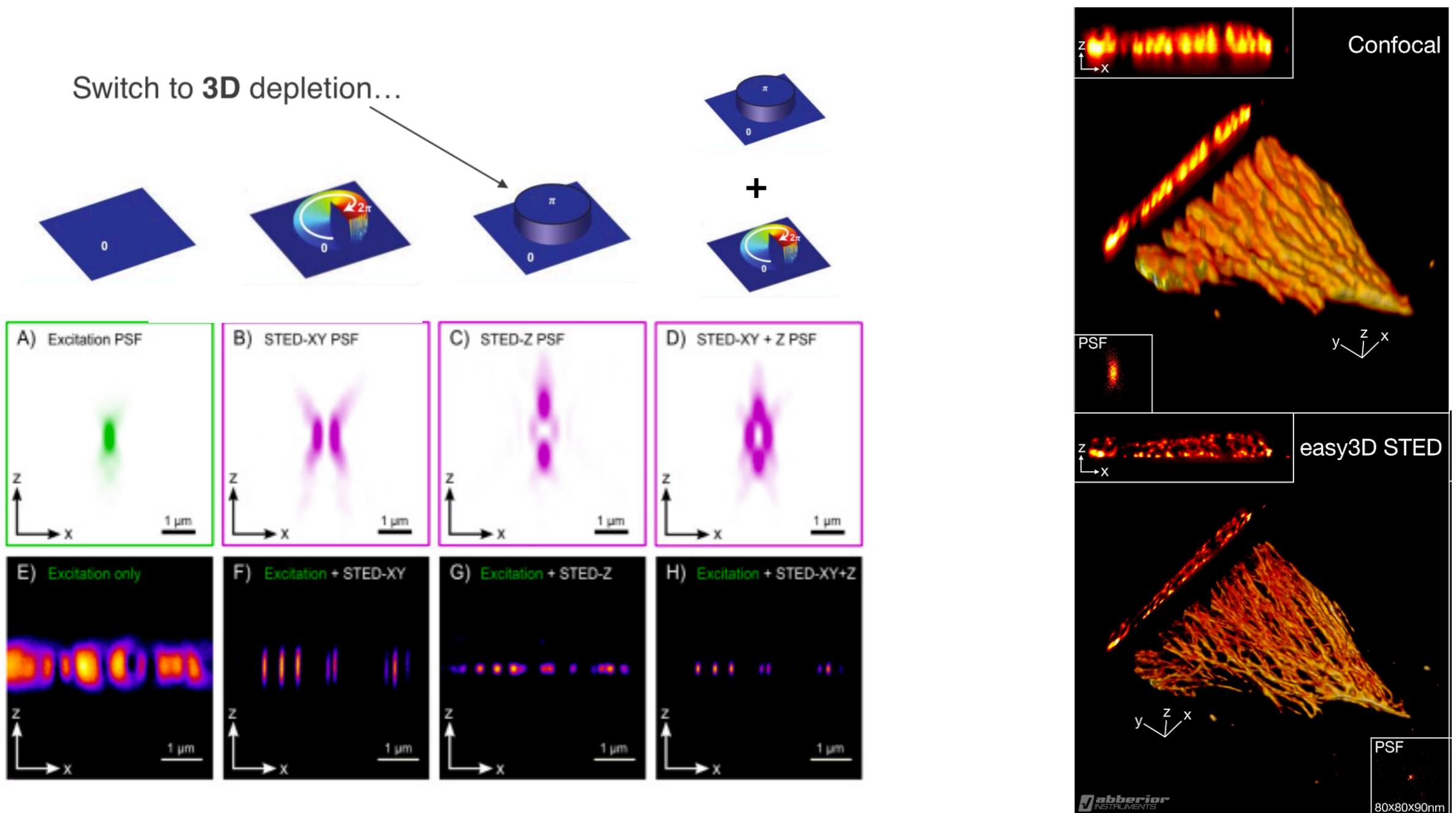
Vortex phase plate

Figure 2.5 Optical Vortex Creation

Courtesy of Courtil and O'Holleran, 2007

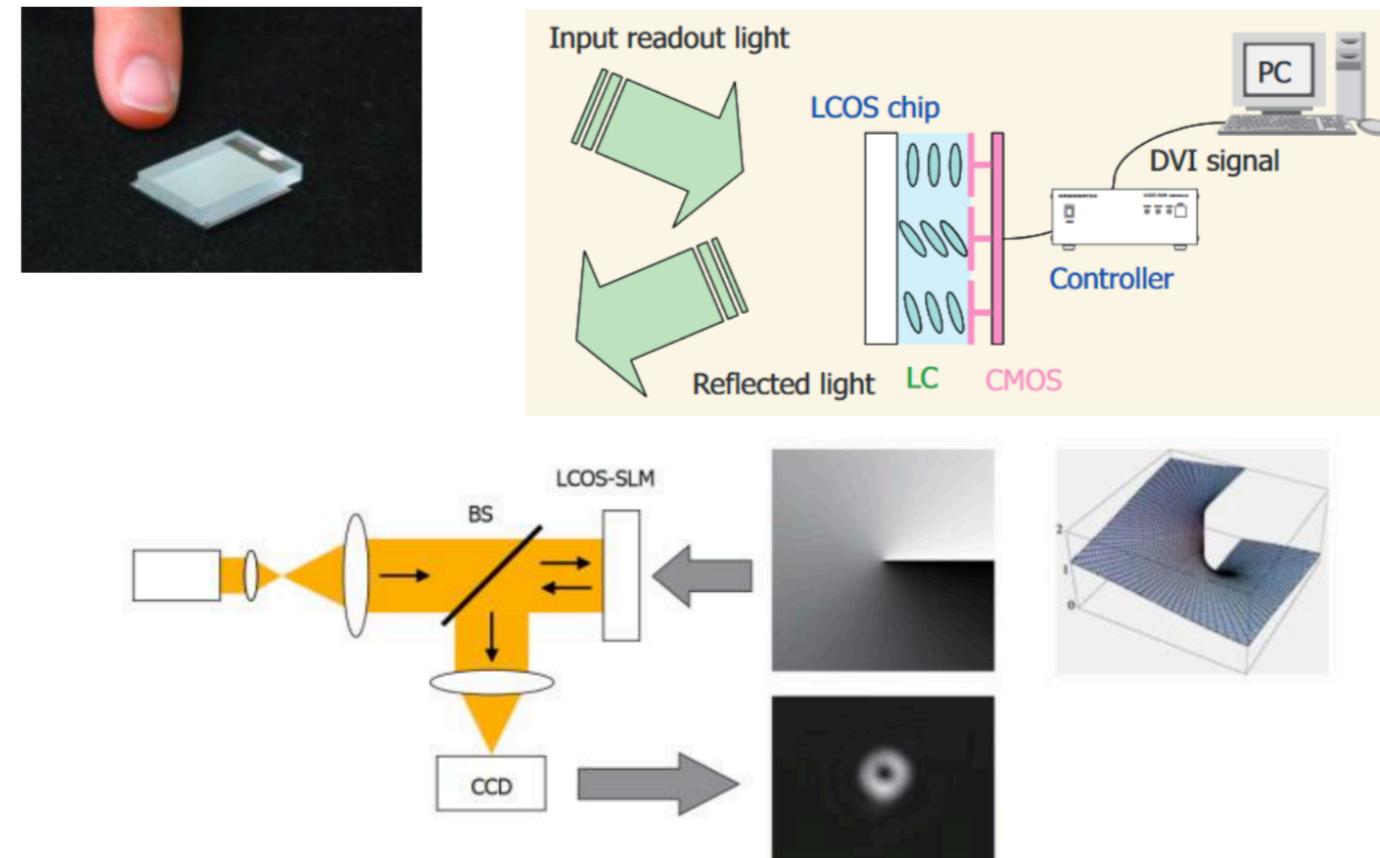


Vortex phase plate 3D



Spatial light modulator (SLM)

Liquid Crystal On Silicon - Spatial Light Modulator (LCOS-SLM)



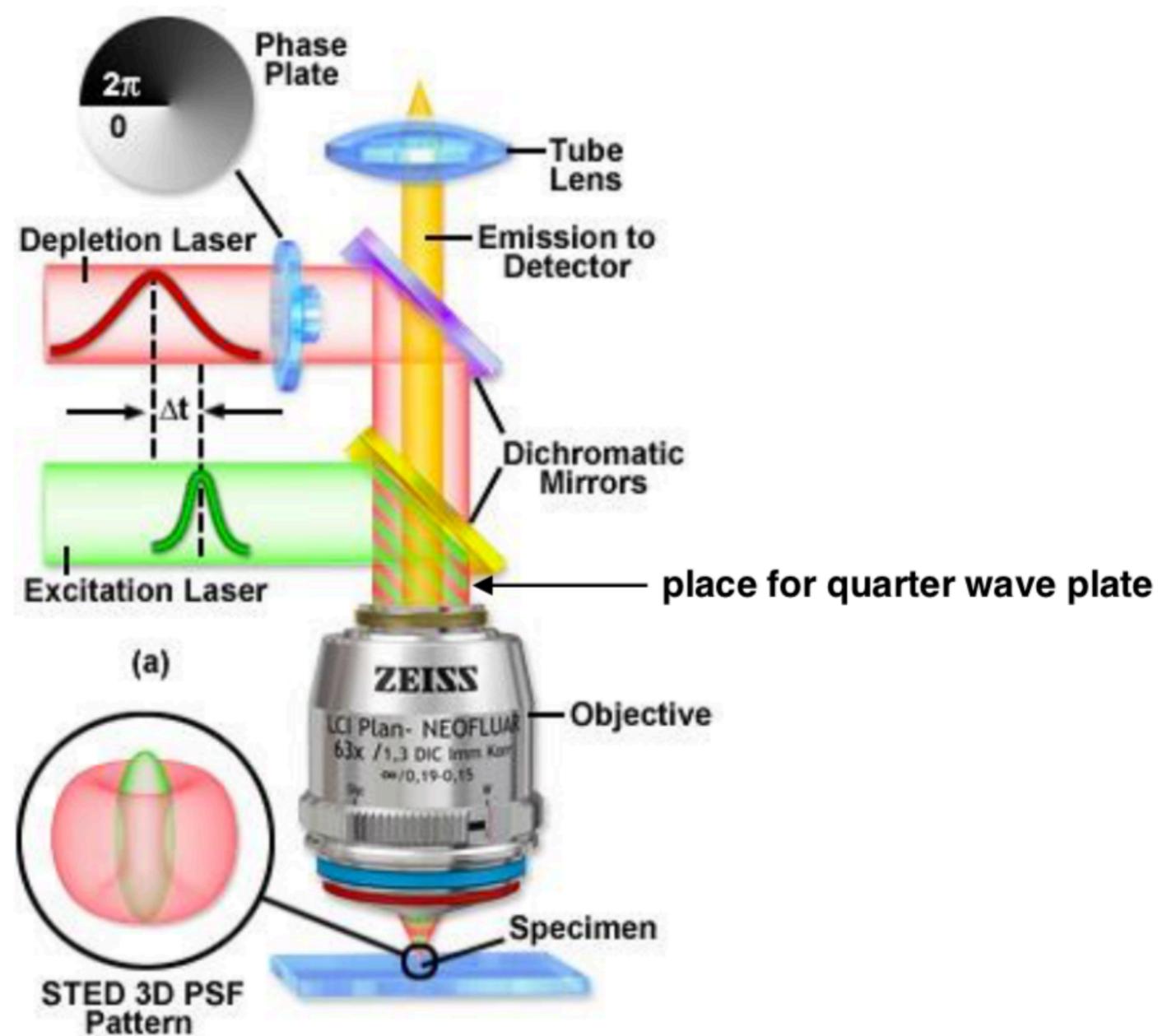
More expensive but more flexible

—> allow fine-tuning and correcting aberration of the laser beam wave-fronts and allows imaging deeper into, for example, layers of highly scattering biological tissue

Source: <http://www.hamamatsu.com>

STED - implementation

STimulated Emission Depletion setup with vortex plate



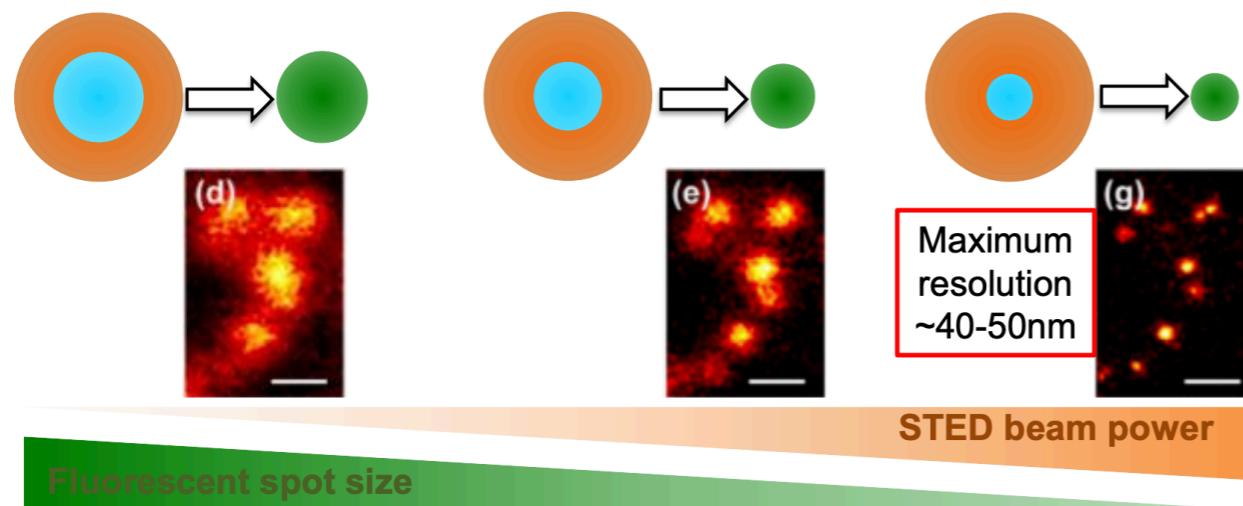
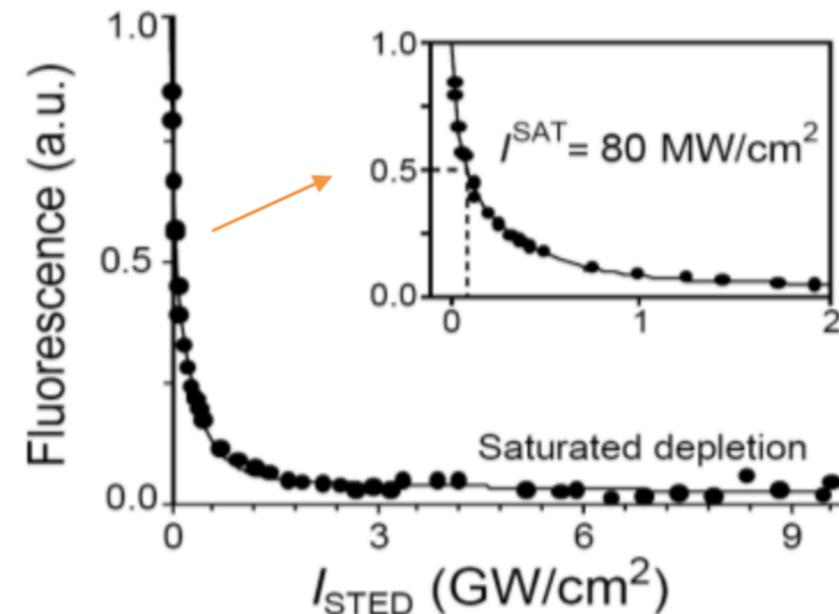
STED resolution

STED resolution depends on depletion laser intensity

STED

$$\Delta xy = \frac{0.5 \times \lambda}{NA \times \sqrt{1 + \frac{I^{max}}{I^{sat}}}}$$

NA = Numerical Aperture
 λ = emission wavelength
 I^{max} = Max STED laser intensity
 I^{sat} = STED intensity for which we have 50% of initial fluorescence depleted (depends on fluorophore)



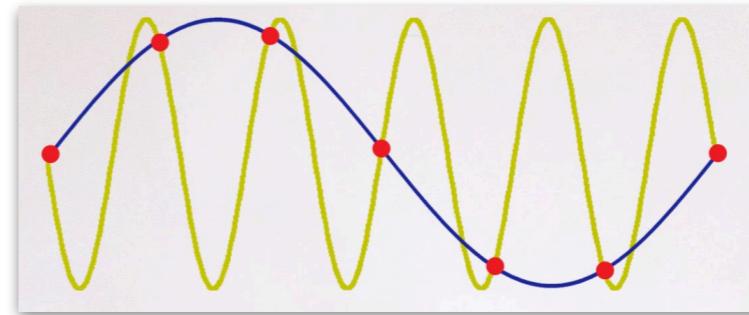
Resolution is determined by the size of the donut ‘hole’

Higher STED beam power → smaller hole

Sampling

Nyquist theorem

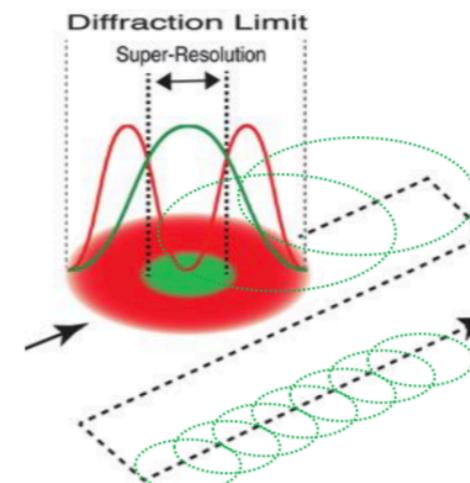
To capture the full information, the sampling frequency needs to be at least twice the highest sample frequency



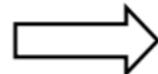
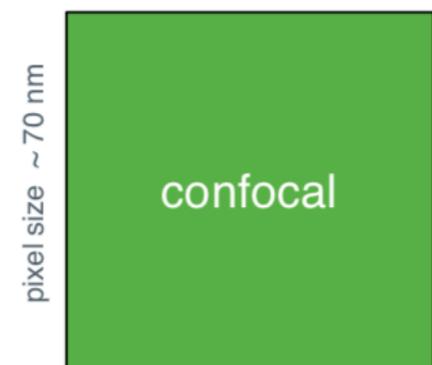
STED resolution depends on depletion laser intensity...

STED

$$\Delta xy = \frac{0.5 \times \lambda}{NA \times \sqrt{1 + \frac{I^{max}}{I^{sat}}}}$$



pixel size ~ 70 nm



Increasing the resolution by a factor of ~ 4 implies an acquisition ~ 16 times longer.

pixel size ~ 20 nm

STED	STED	STED	STED
STED	STED	STED	STED
STED	STED	STED	STED
STED	STED	STED	STED

Longer acquisition time!!

Fluorophores

Requirements on fluorophores

Fluorophores

General requirements:

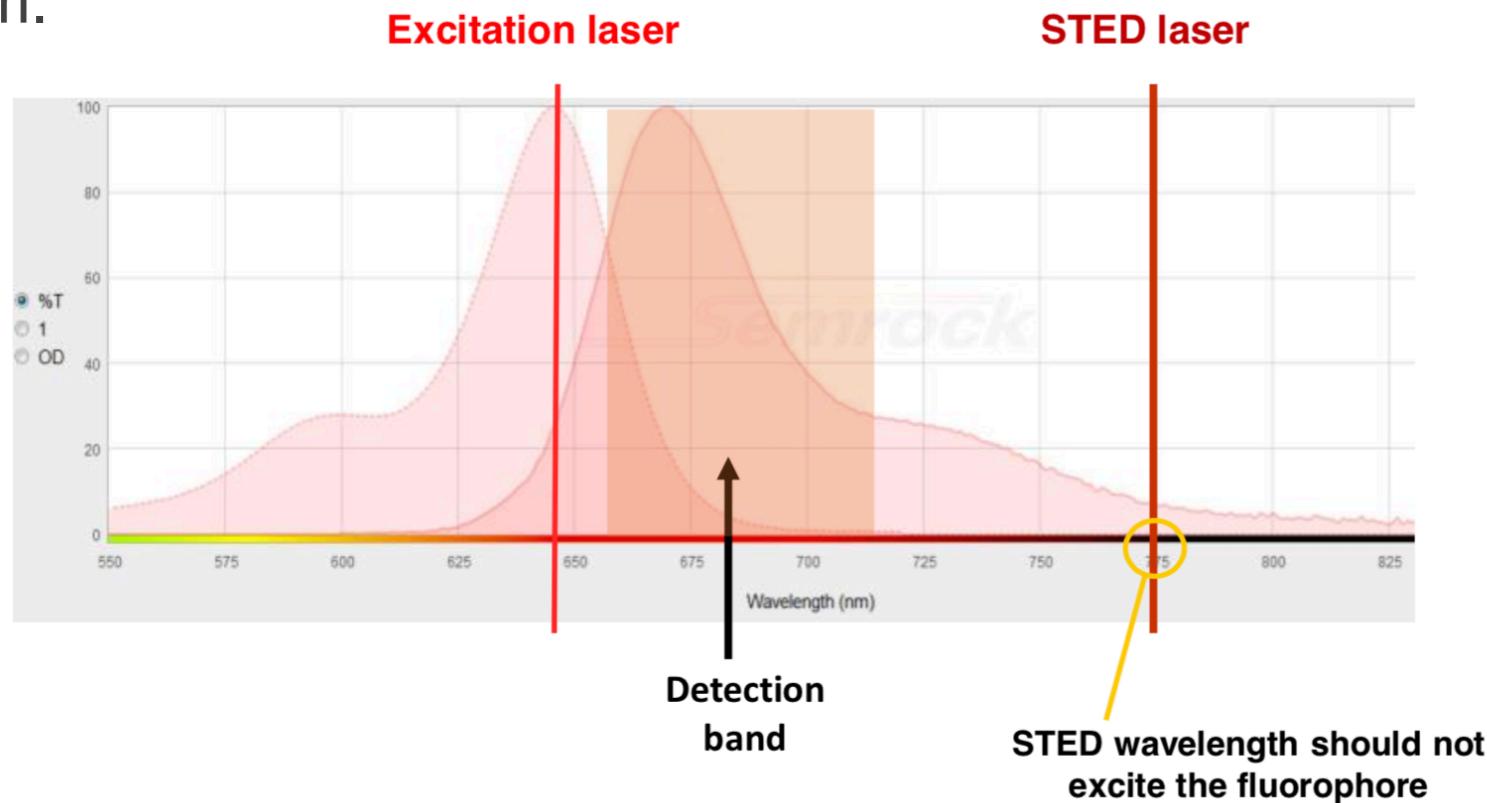
- ✓ High cross section (=probability) for stimulated emission
- ✓ No excitation at depletion wavelength

STED

$$\Delta xy = \frac{0.5 \times \lambda}{NA \times \sqrt{1 + \frac{I_{max}}{I_{sat}}}}$$

NA = Numerical Aperture
 λ = emission wavelength
 I_{max} = Max STED laser intensity
 I_{sat} = STED intensity for which we have 50% of initial fluorescence depleted (depends on fluorophore)

wavelengths close to the peak of the fluorophore's emission spectrum improve the SE cross-section but increase the probability of exciting the fluorophores with the STED beam.



The STED wavelength must coincide with the emission spectrum but not the excitation spectrum of your fluorophore!

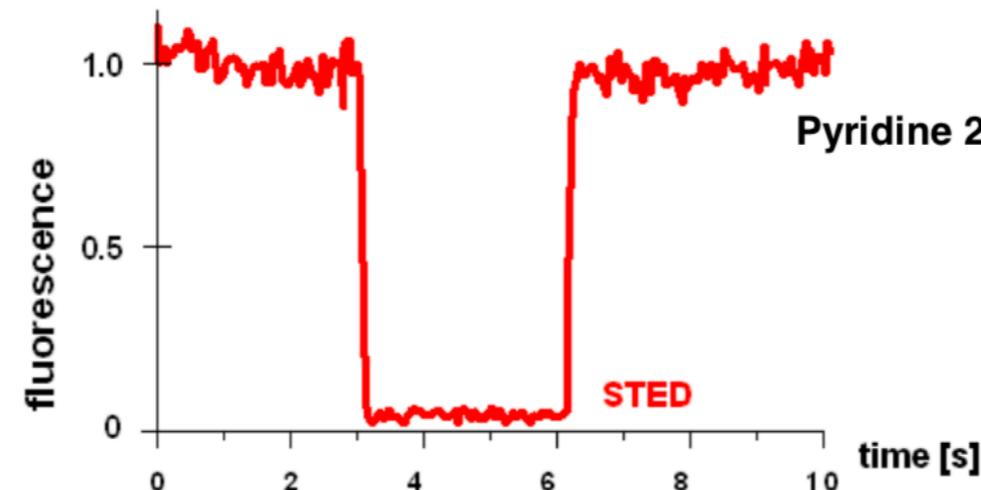
Fluorophores

General requirements:

- ✓ High cross section (=probability) for stimulated emission
- ✓ No excitation at depletion wavelength
- ✓ Brightness (Quantum Efficiency + Extinction coefficient)
- ✓ Photostability at depletion and excitation wavelength

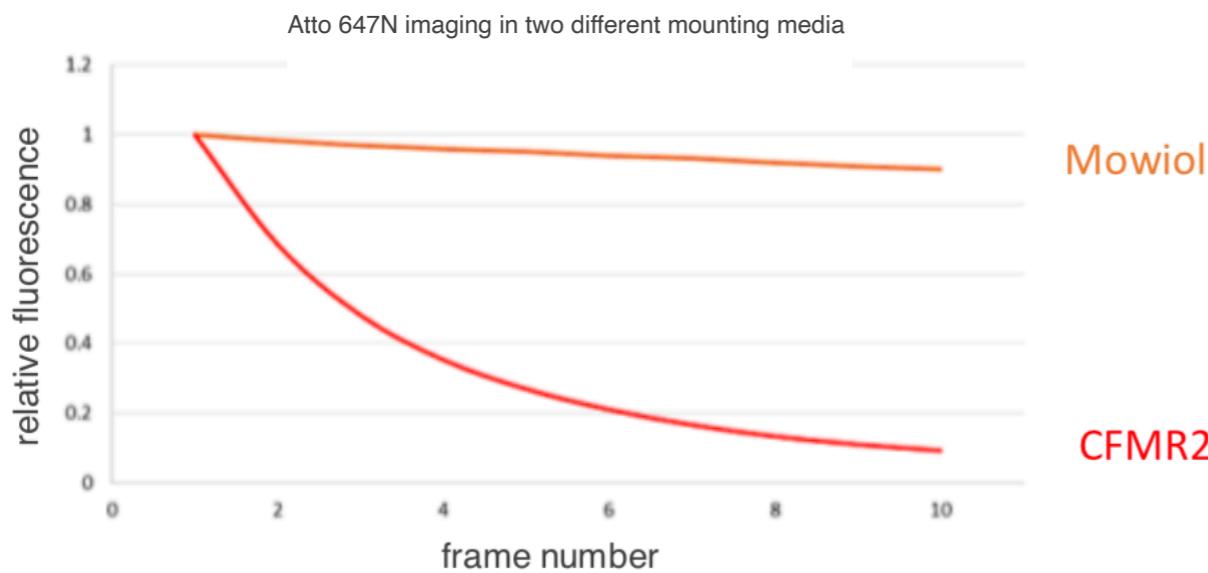
Fluorophores

Fluorescence Depletion by STED is not a photobleaching process...



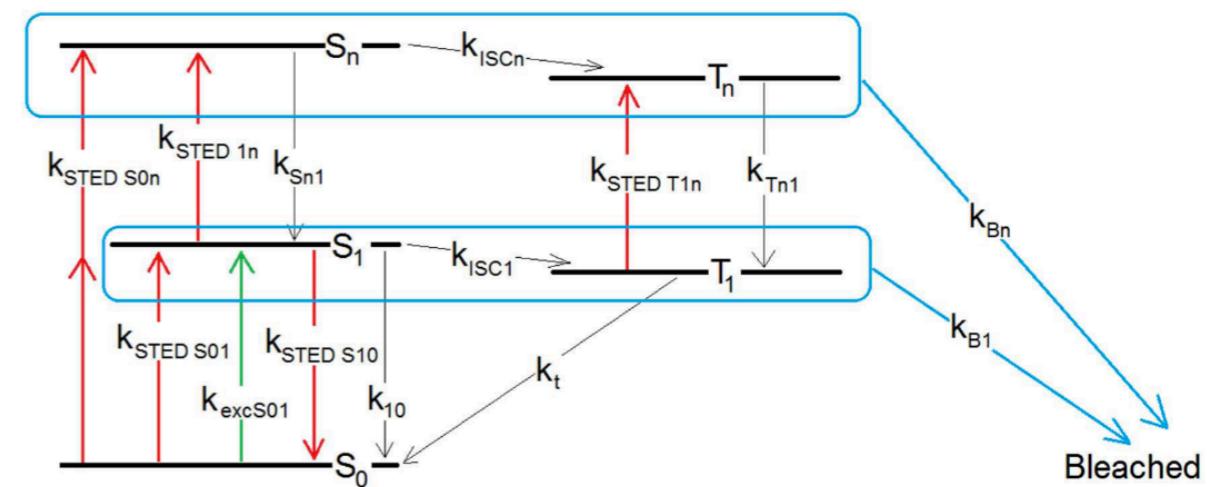
... but photobleaching happens!

STED laser sends fluorophores to different electronic states leading to bleaching



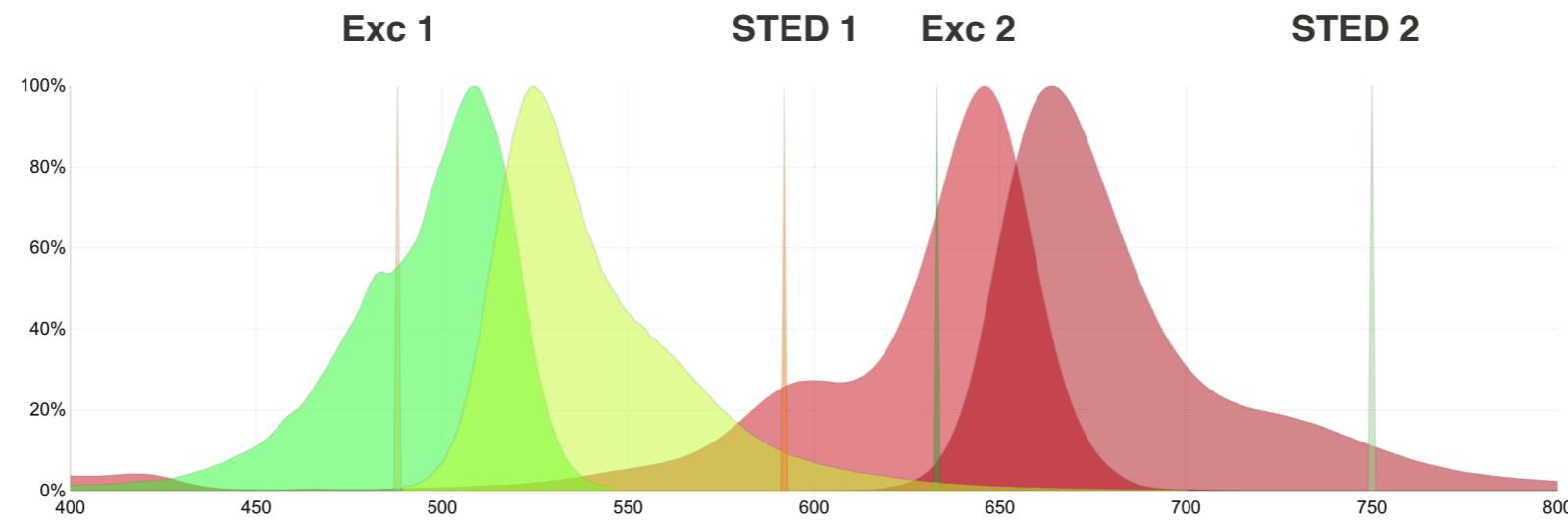
Mowiol

CFMR2

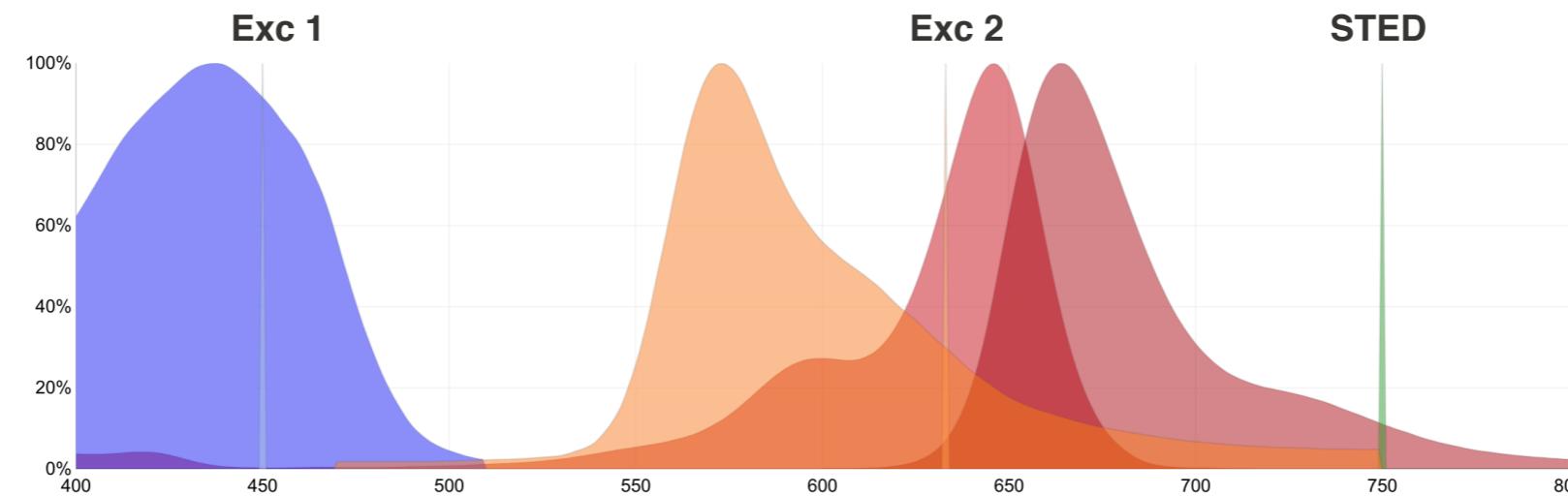


Multicolor

2 colors with two **STED** lines



2 colors with a single **STED** line



Multicolor

2 colors with a single **STED** line

3 colors with a single or two **STED** lines

STED: 592 nm, 660 nm or 775 nm

Dye 1			Dye 2		
Name	Excitation	Emission: e.g.	Name	Excitation	Emission: e.g.
BD Horizon V500	485/470	475–510	Oregon Green 488/Chromeo 505	514/520	523–580
Oregon Green 488	470 (WLL)	480–520	Alexa Fluor 532	545 (WLL)	550–580
Alexa Fluor 532	514	520–565	TMR/TRITC/Alexa Fluor 568	580	590–650
Alexa Fluor 514/Oregon Green 488	505	515–565	TMR/TRITC/Alexa Fluor 568	580	590–650
ATTO 594/Alexa Fluor 594	532/590	600–630	STAR 635P	635/650	655–750
TMR/TRITC	532/550	560–630	ATTO 647N/Alexa Fluor 647	635/650	655–750

Dye 1			Dye 2			Dye 3		
Name	Excitation	Emission	Name	Excitation	Emission	Name	Excitation	Emission
STAR440-SX**	470	475–505	Oregon Green 488	510	515–530	Alexa Fluor 532		
Oregon Green 488**	470	475–525	Alexa Fluor 532	532	538–550	TMR/TRITC		
Alexa Fluor 514**	480	490–535	Alexa Fluor 546	540	545–580	Alexa Fluor 594		
TRITC	550<	560–590	ATTO 594	600	610–640	STAR 635P		
Alexa Fluor 594	580	580–615	Alexa Fluor 633	620	625–655	Alexa Fluor 660		
Oregon Green 488	488	500–545	TRITC/TMR	550	560–635	STAR 635P		

<https://nanobiophotonics.mpibpc.mpg.de/dyes/>

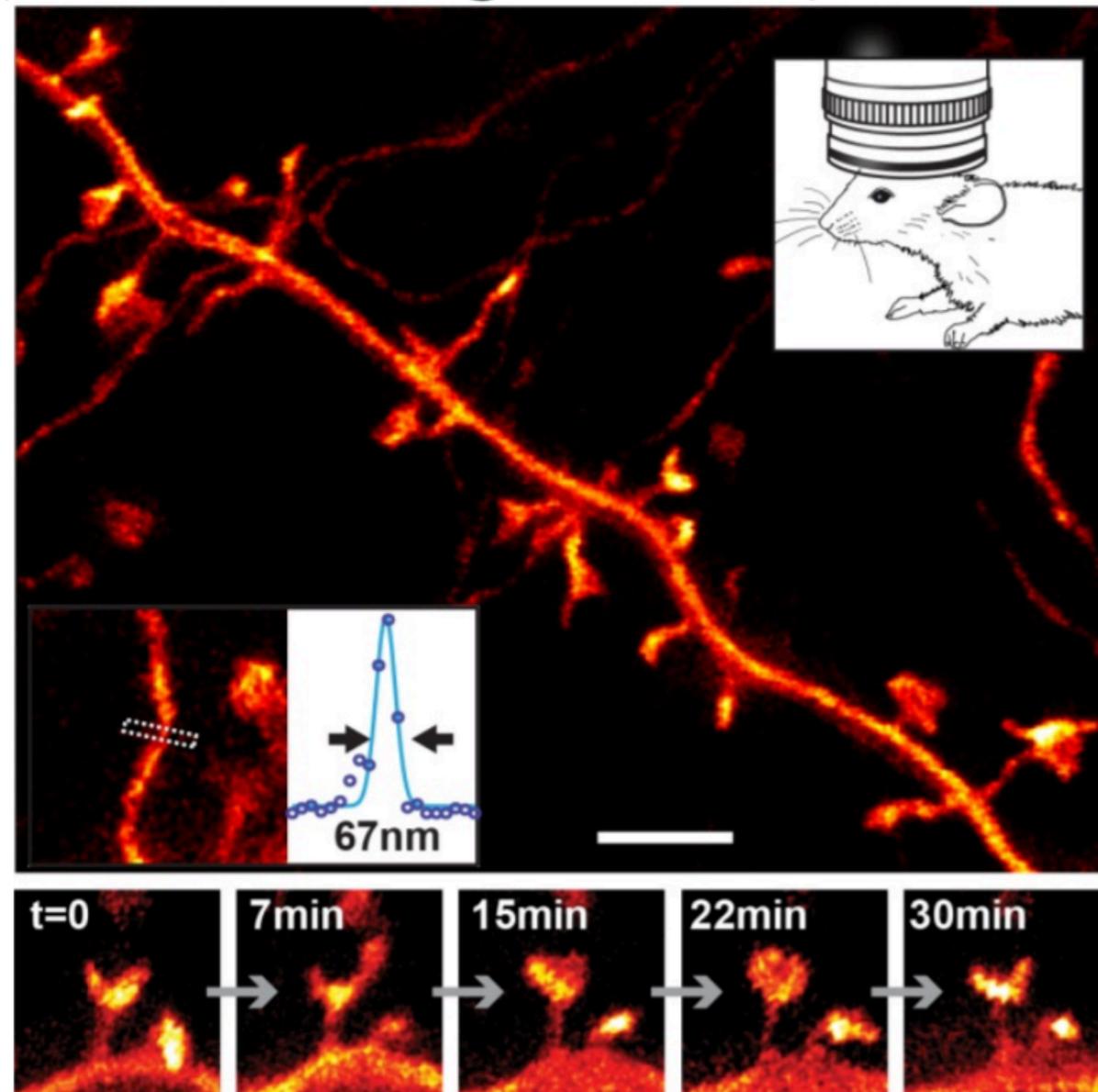
<https://www.leica-microsystems.com/science-lab/quick-guide-to-sted-sample-preparation/>

Live STED (Fluorescent proteins)

Live Mouse

YFP

(Science Berning et al 2012)



Challenging!

- > phototoxicity
- > photodamage

These depend on many parameters:

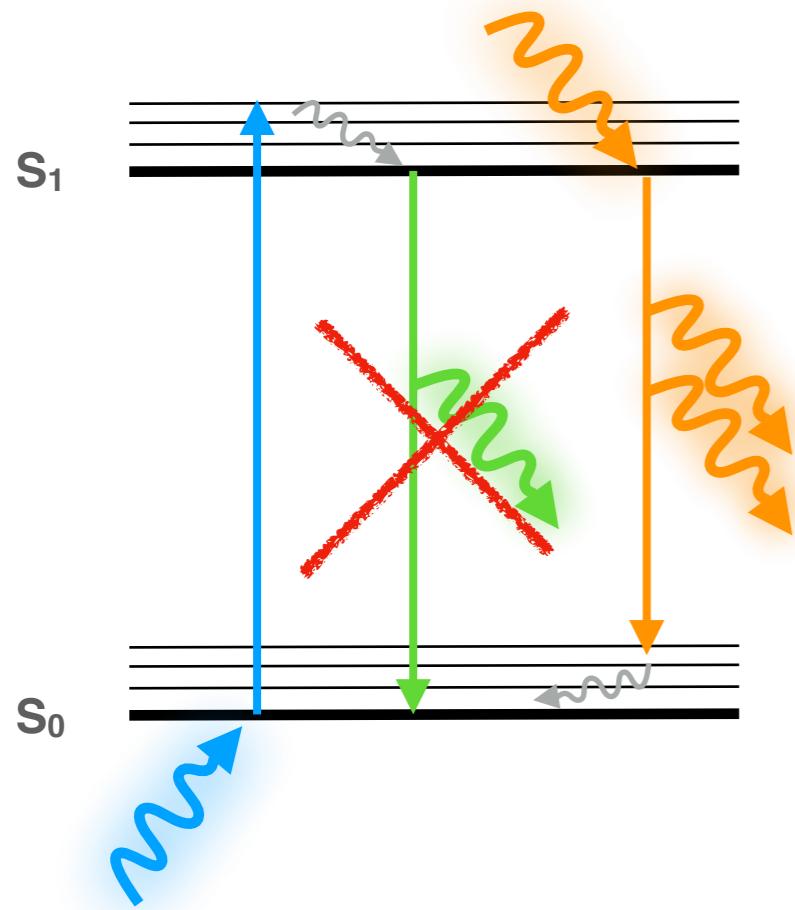
- wavelength of the STED beam,
- the time of irradiation,
- the resolution needed,
- the investigated area
- the specimen itself

Lasers

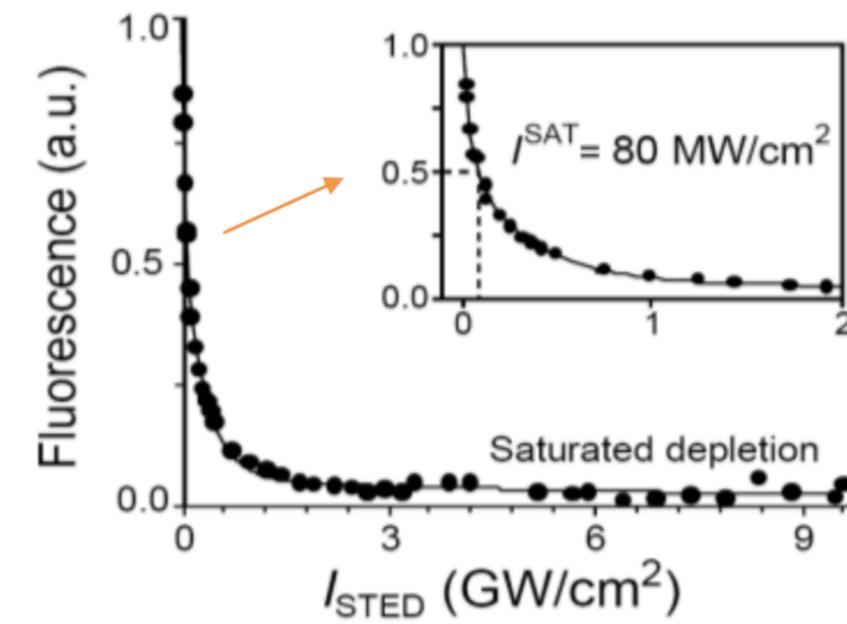
Requirements on laser excitation

Stimulated Emission

Jablonski diagram



STED resolution depends on depletion laser intensity



Depletion laser should have (very) high intensity in order to saturate depletion

1 Excitation ~ fs

2 Internal conversion ~ ps

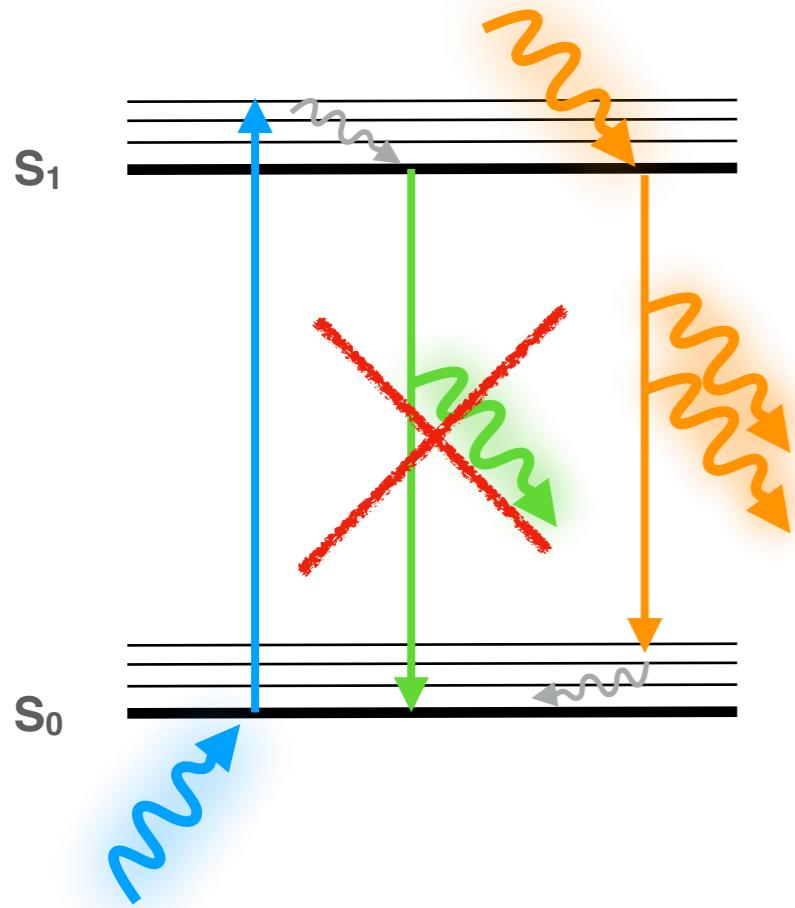
3 Fluorescence ~ ns

4 Stimulated emission

Excitation and depletion laser should be synchronized in space and time

Stimulated Emission

Jablonski diagram



To efficiently force a fluorophore to the OFF state,
SE has to win the competition with spontaneous emission, which typically occurs within a few nanoseconds after the excitation event (fluorophore's excited-state lifetime).

This short temporal window and the small cross-section of SE demand a high flux of stimulating photons.

Laser requirements

1. peak intensity much greater than 10 MW/cm^2 (the saturation intensity) to obtain significant resolution enhancement;
2. high-repetition rate (tens of MHz) for fast imaging;
3. few hundreds picosecond pulse width to efficiently quench fluorophores and reduce photobleaching;
4. narrow spectral width to generate a high quality ‘zero’-intensity point;
5. ideally, wavelength tunability to match the spectra of many fluorophores.

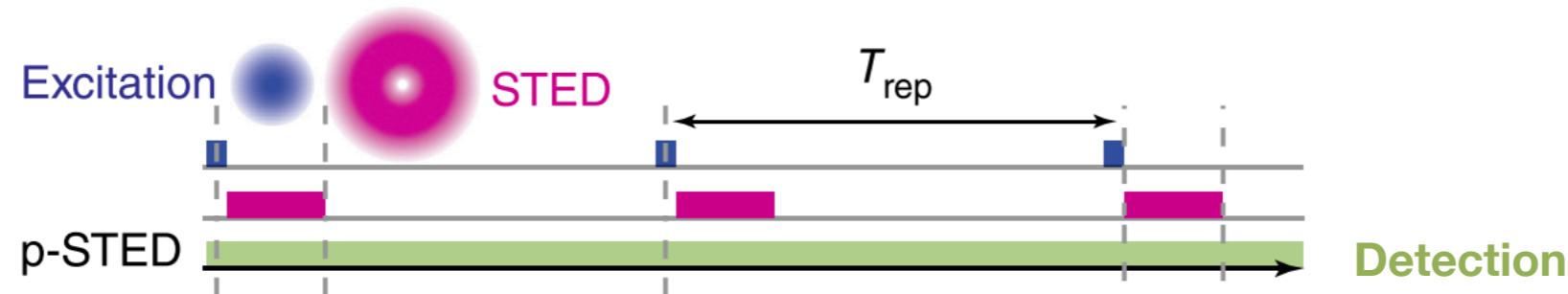


© CanStockPhoto.com - csp55304212



Pulsed STED

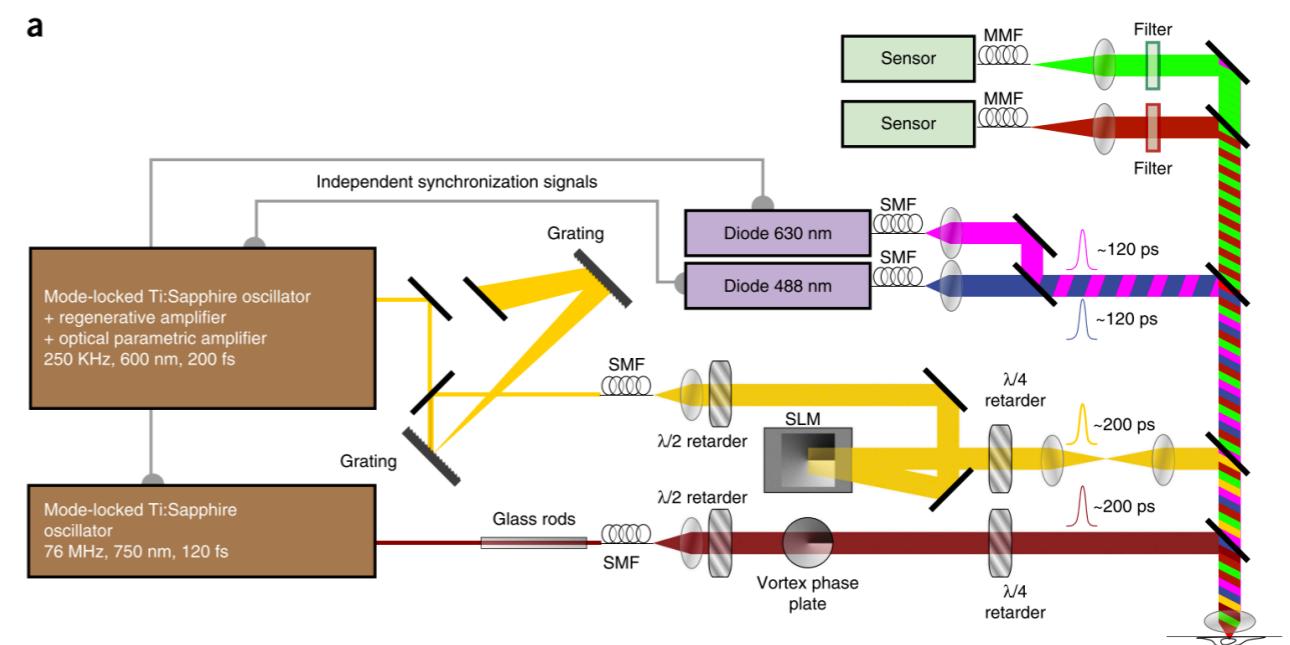
- Pulsed STED (first demonstration of STED):



Excitation pulses are followed immediately by the depletion pulses

At the beginning of the 2000s, most STED microscopes used Ti:Sapphire lasers as STED beams, whose pulses required stretching to guarantee a few hundred picosecond pulse width and conversion to the visible range.

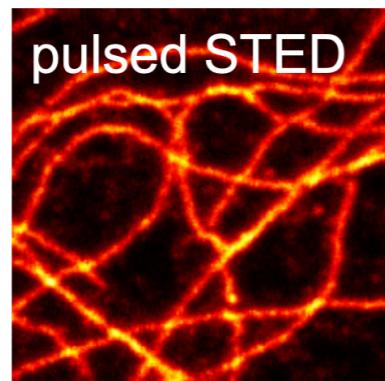
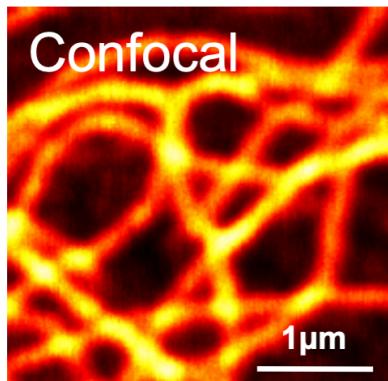
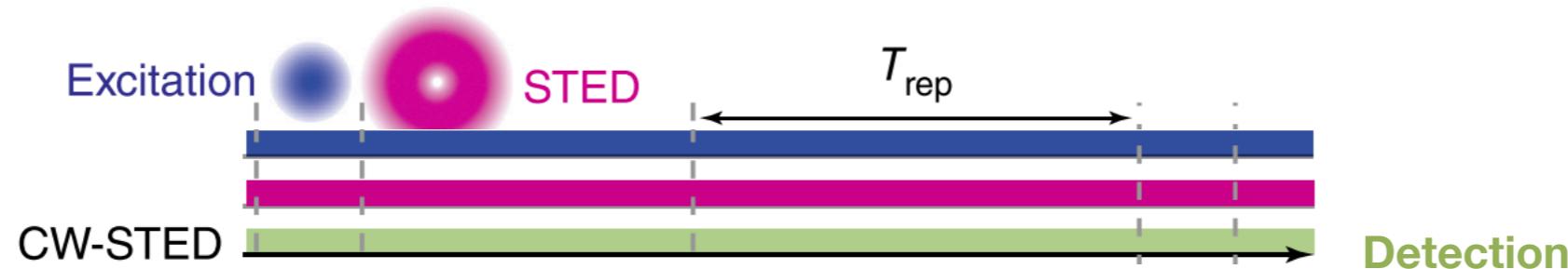
A second pulsed diode laser electronically synchronized with the Ti:Sapphire laser provided the excitation beam.



→ very complex and expensive instruments

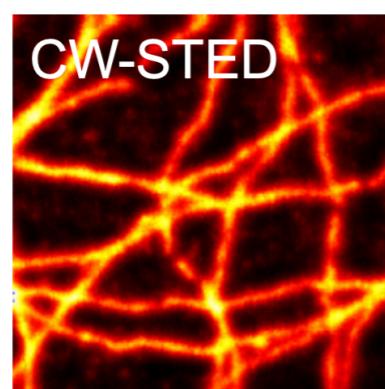
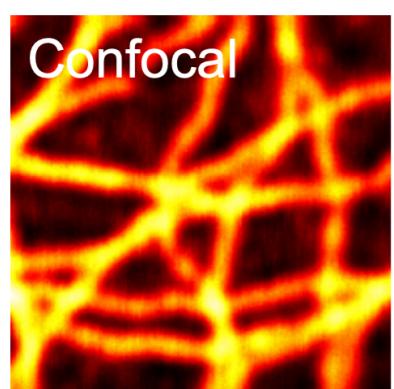
Continuous STED

- Continuous STED:



Microtubules filaments in fixed PtK2 cells labelled with ATTO647N. Pulsed STED beam: 760nm, 80MHz and 45 mW

→ Affordability and elegant simplicity of the implementation



Microtubules filaments in fixed PtK2 cells labelled with ATTO647N. CW STED beam: 760nm and 320 mW

BUT
Better performance has so far been achieved using pulsed STED!

Gated STED

“Use time information to extract spatial information”

How far away a thunderstorm is?

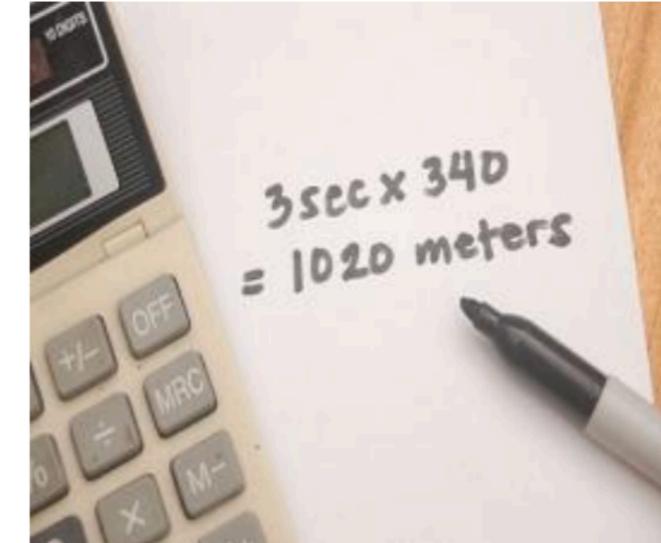
Watch the sky for a flash of lightning.



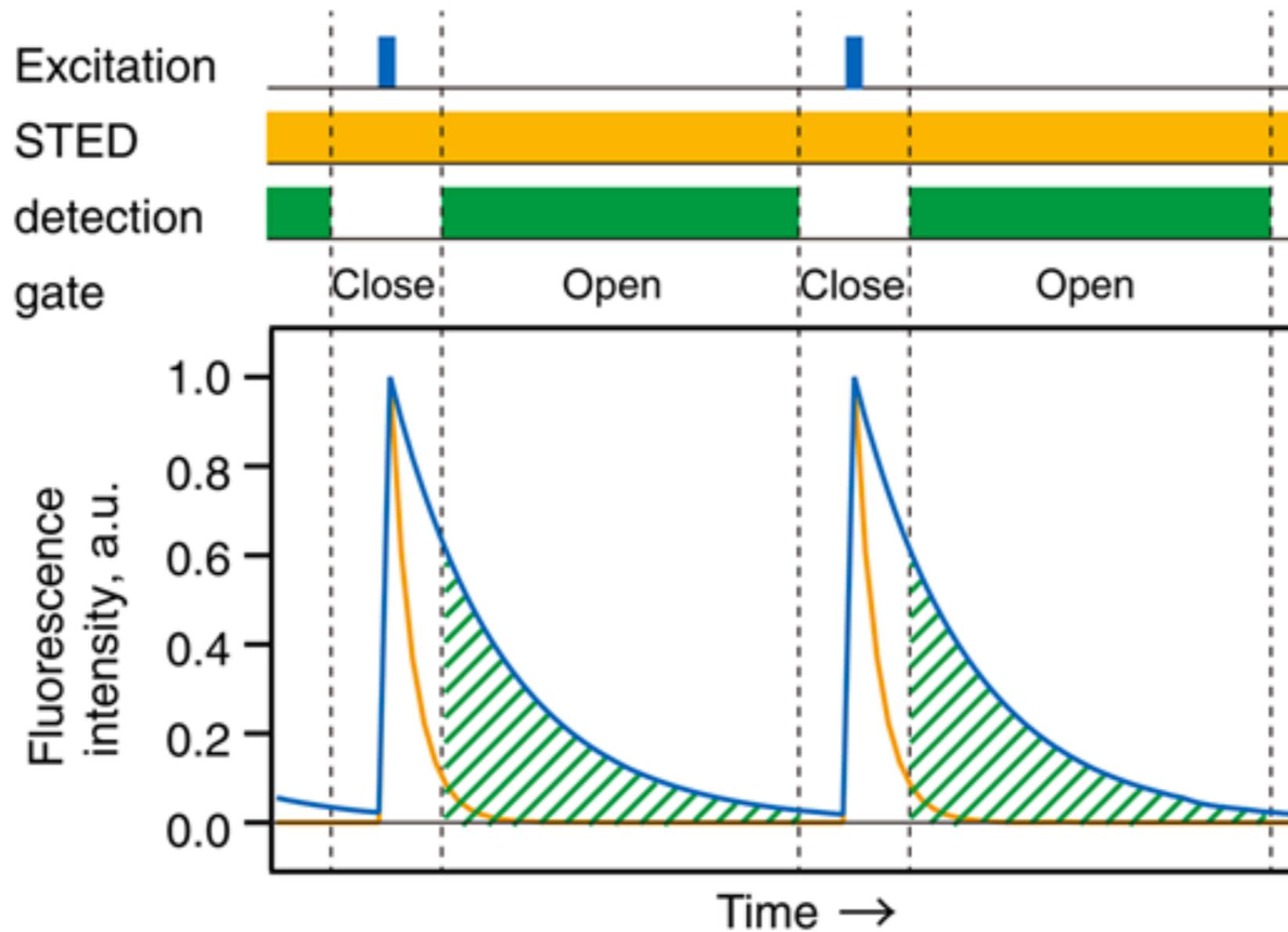
Count the number of seconds until you hear thunder.



Multiply the seconds you counted by 340 meters.



Gated STED



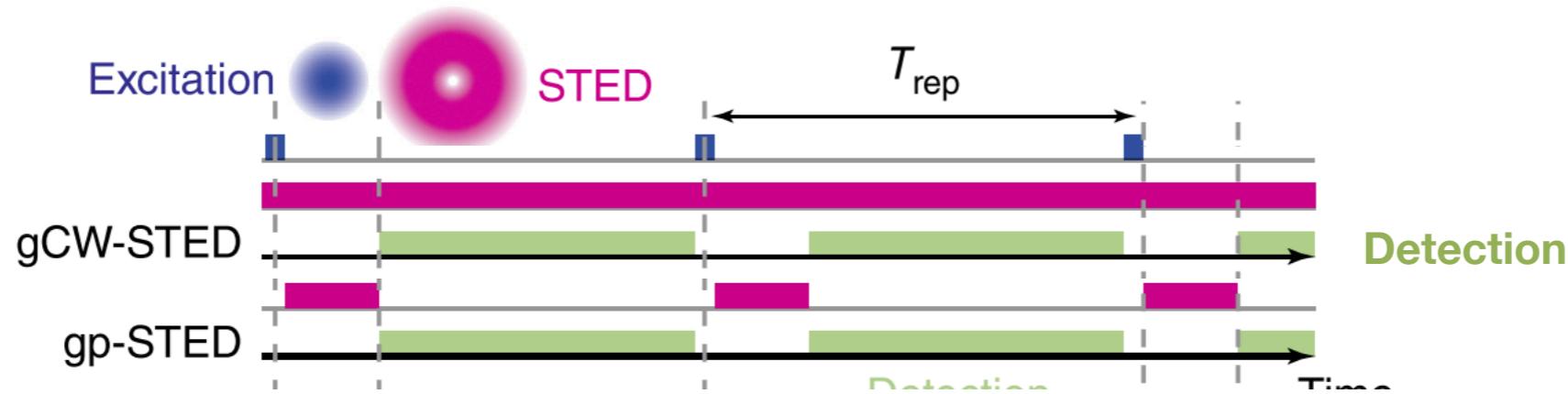
**Send a short
excitation
pulse**

**Wait for undesired
molecules to be
turned off**

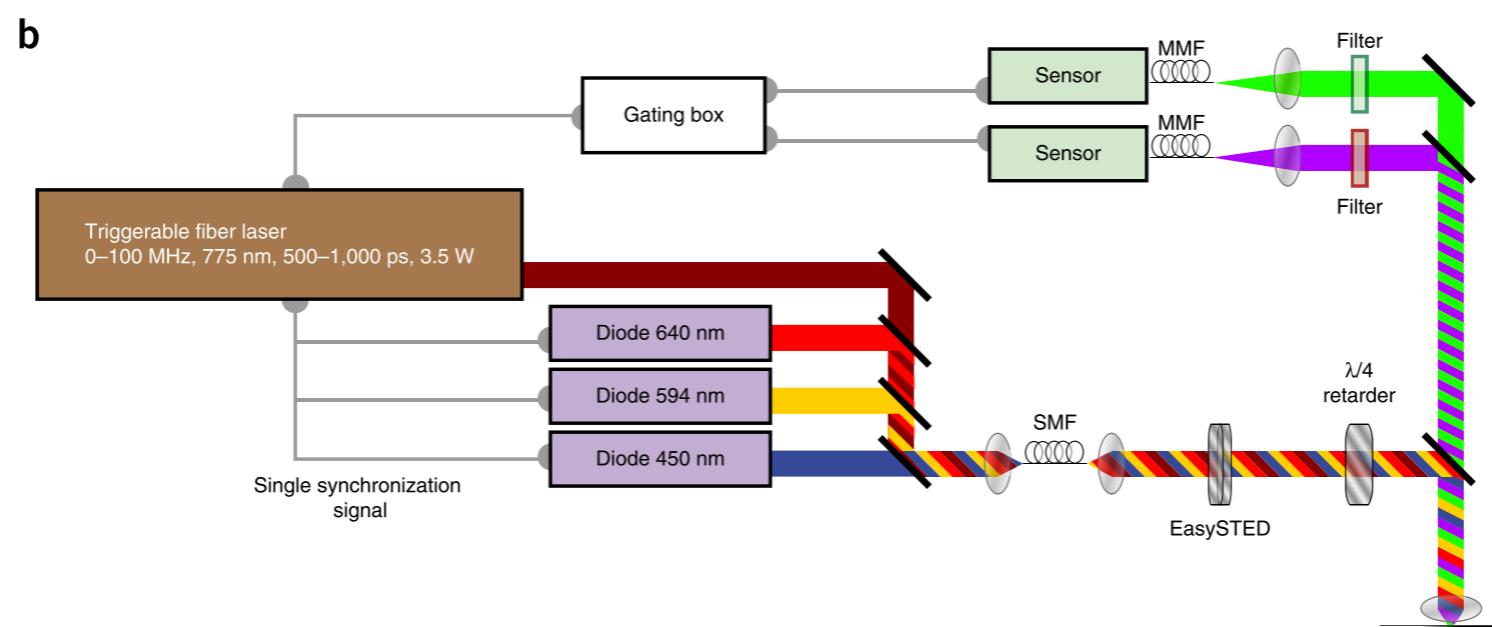
**Collect photons
from the remaining
ones**

Gated STED

- Gated STED:



Continuous/pulsed STED + Excitation pulses synchronised with detection (with a delay)



→ g-STED provides the sharpest images with the lowest peak intensity!

Sample preparation



- Coverslips should be $1.5H$ = thickness is $170 \pm 5 \mu\text{m}$
- Mounting medium should have a RI close to glass and/or immersion oil (usually 1.518)
ex: Vectashield = 1.45, Prolong Gold = 1.44 (after 48 h), Mowiol = 1.49
- Imaging depth in sample should be preferably $< 10\text{-}15 \mu\text{m}$
- Staining may have to be adapted: labelling density has to be high enough to reach super-resolution

Summary

PROS

- Confocal-based – fast scanning over small regions, good penetration depth, multicolour
- No need for specialised fluorophores
- STED laser power tunes resolution
- No need to computationally reconstruct images

CONS

- Very high laser intensities required for highest resolutions (photobleaching, phototoxicity...)
- Beams must remain aligned

++

- STED-FCS
- STED-Expansion microscopy
- MiniFlux