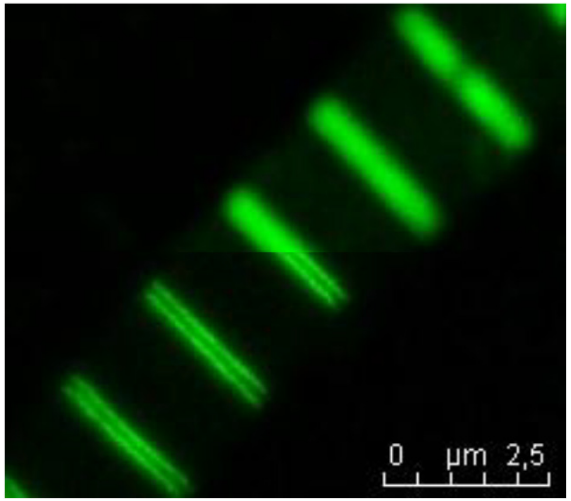
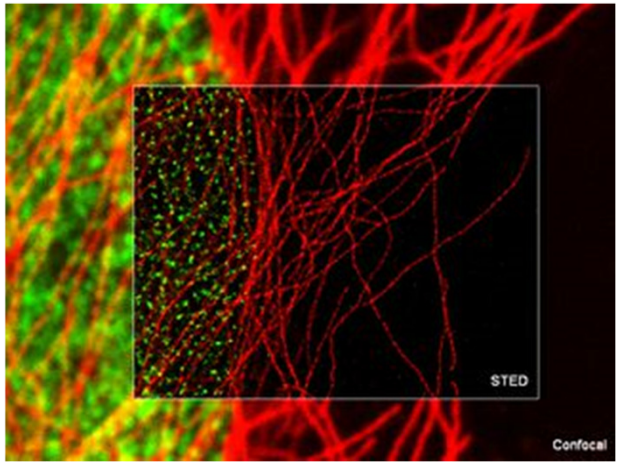
Living up to Life

Leica MICROSYSTEMS





STED 3X Sample Preparation

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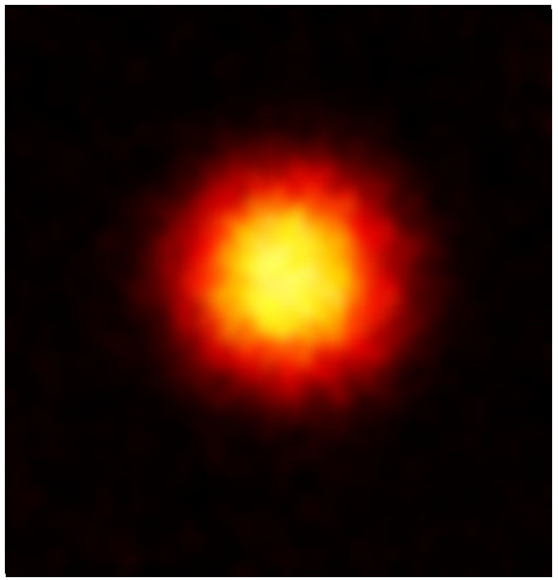
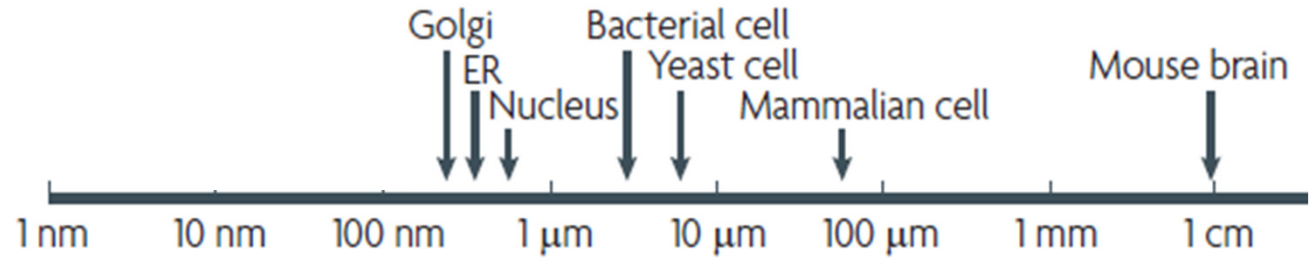
Sr. Product Specialist / Product Specialist

Application, Technology, and Training Center

Why Super Resolution?

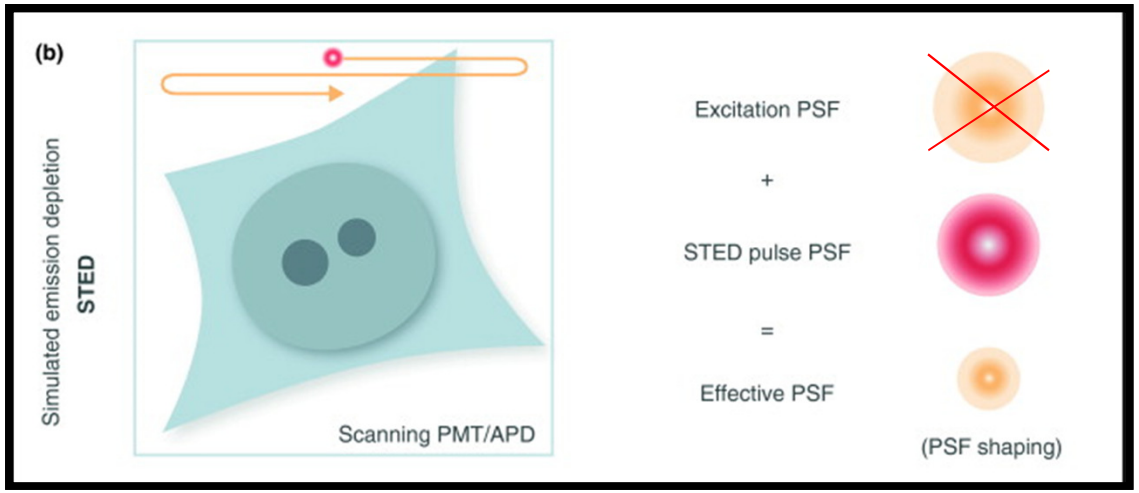
- To study details:
  - beyond the diffraction limit
  - with standard dyes/FPs
  - in 2 or more colors
  - inside cells/organisms
  - in live cells
  - with high throughput
  - on fully integrated systems





STED Microscopy – The concept

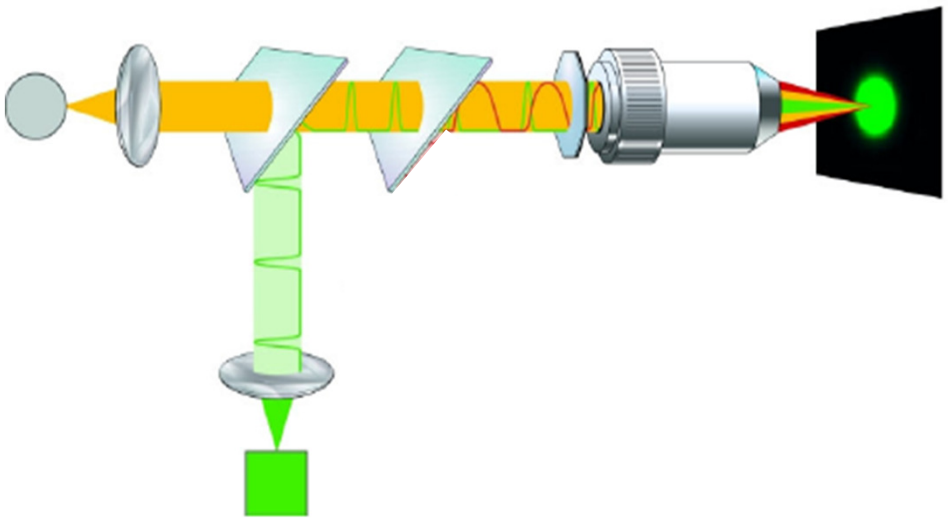
- For STED imaging, you take standard confocal microscopy and introduce a technique to reduce the excitation spot.
  - Shrinking the PSF of the microscope
  - Depleting the fluorescence emission in the outer areas of the diffraction limited spot via a process called stimulated emission.



JBC Review 2010 from Lothar Schermelleh, Rainer Heintzmann & Heinrich Leonhardt

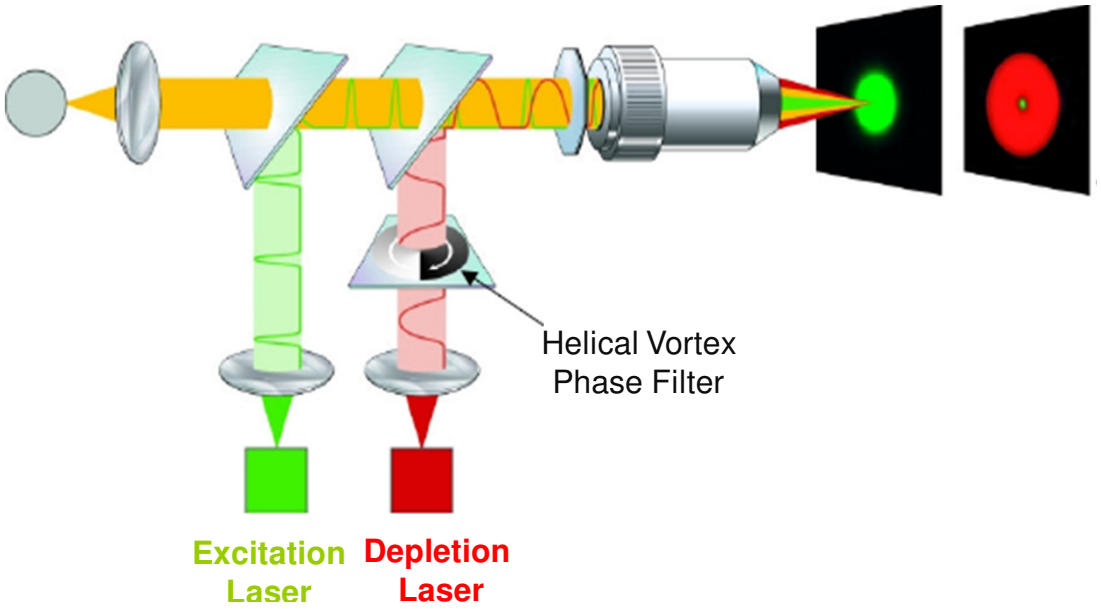
STED Microscopy – The concept

- Uses two differential methods of diffraction patterns
  - One excites (pulse laser)



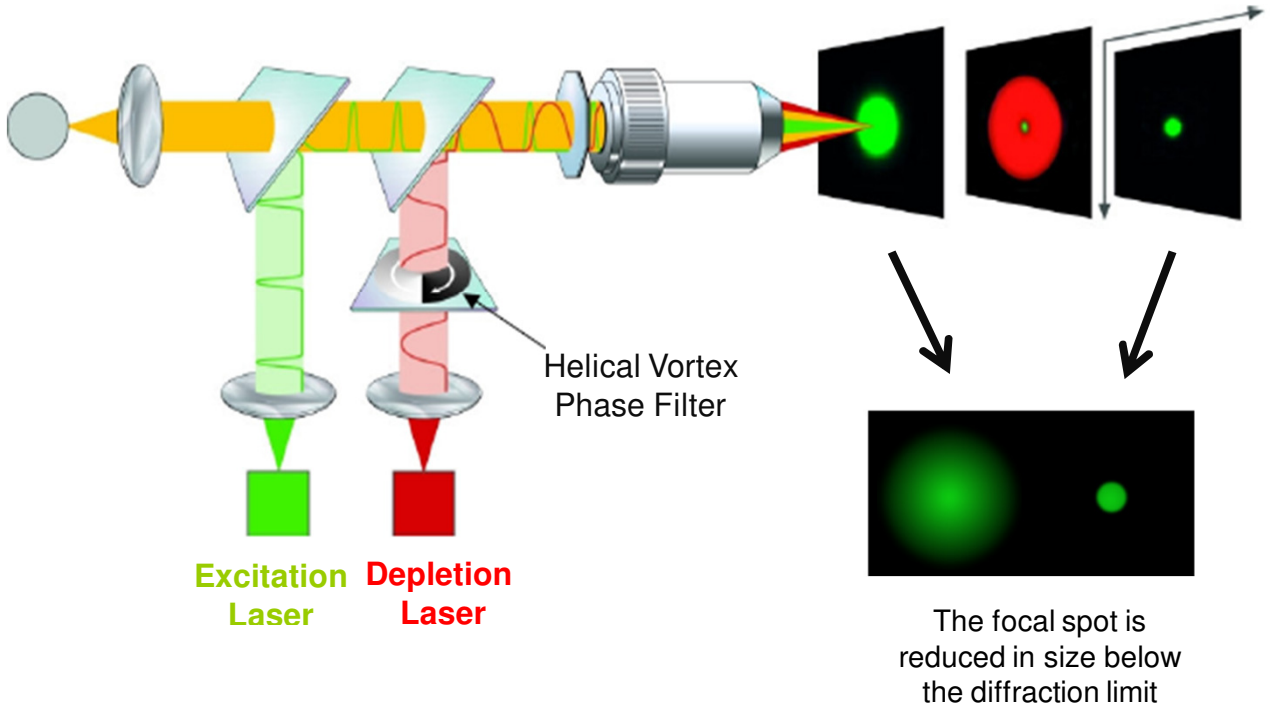
STED Microscopy – The concept

- Uses two differential methods of diffraction patterns
  - One excites (pulse laser)
  - Second, induced/forced fluorescence (stimulated emission)
  - Creating the donut shape fluorescence depletion



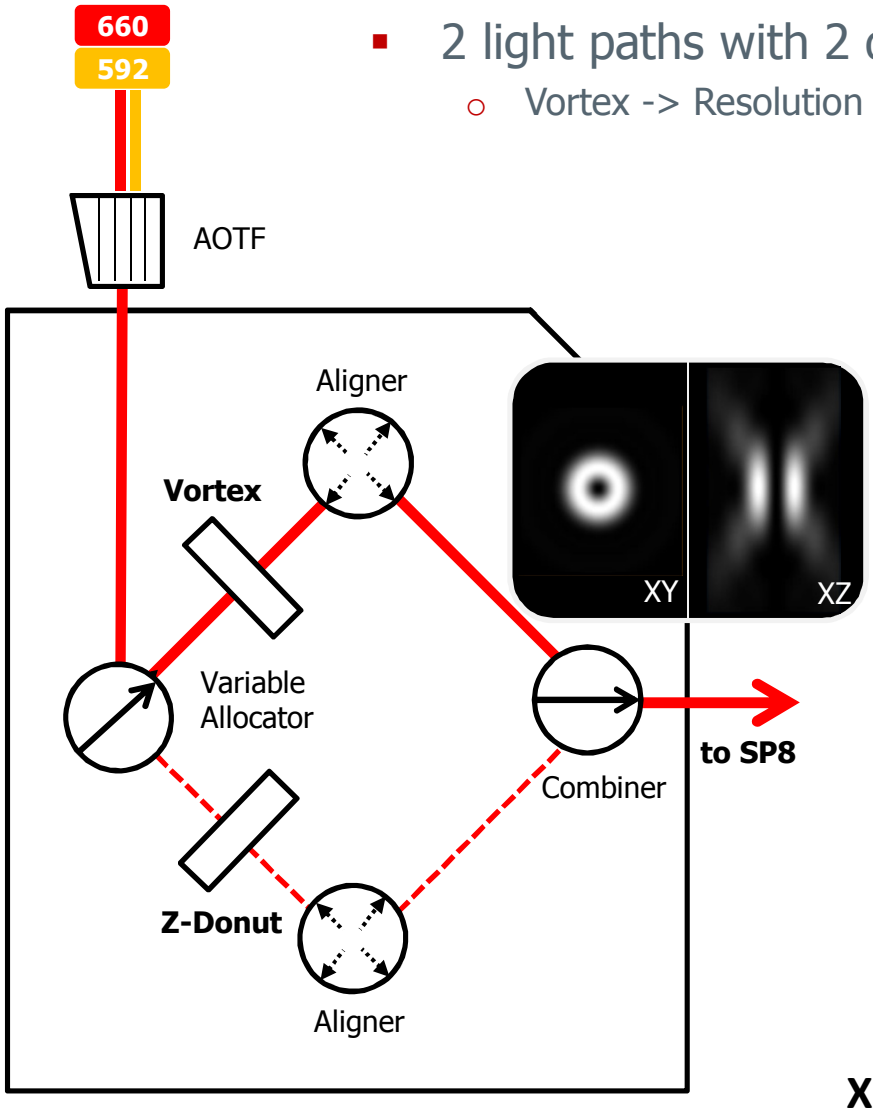
STED Microscopy – The concept

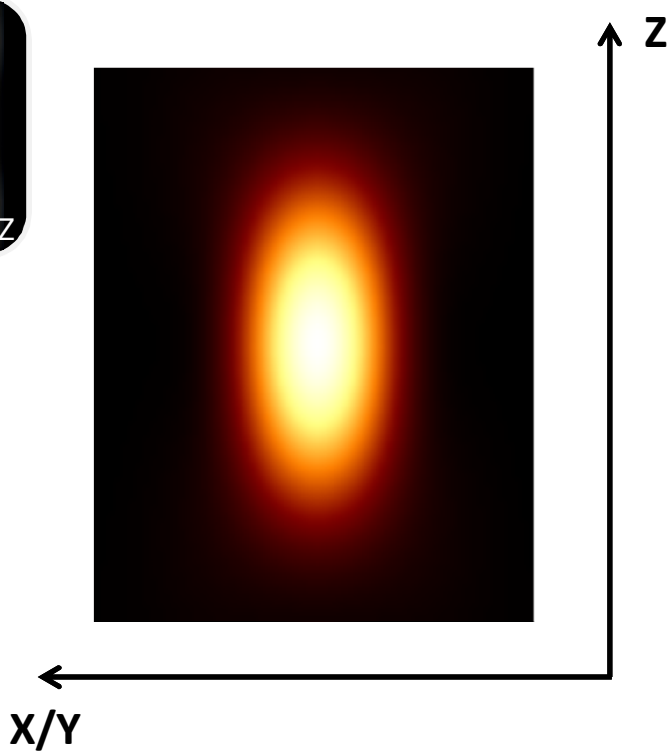
- Uses two differential methods of diffraction patterns
  - One excites (pulse laser)
  - Second, induced/forced fluorescence (stimulated emission)
  - Creating the donut shape fluorescence depletion
- Excitation and STED donut are perfectly overlaid in the focus



STED 3X Microscopy – The concept

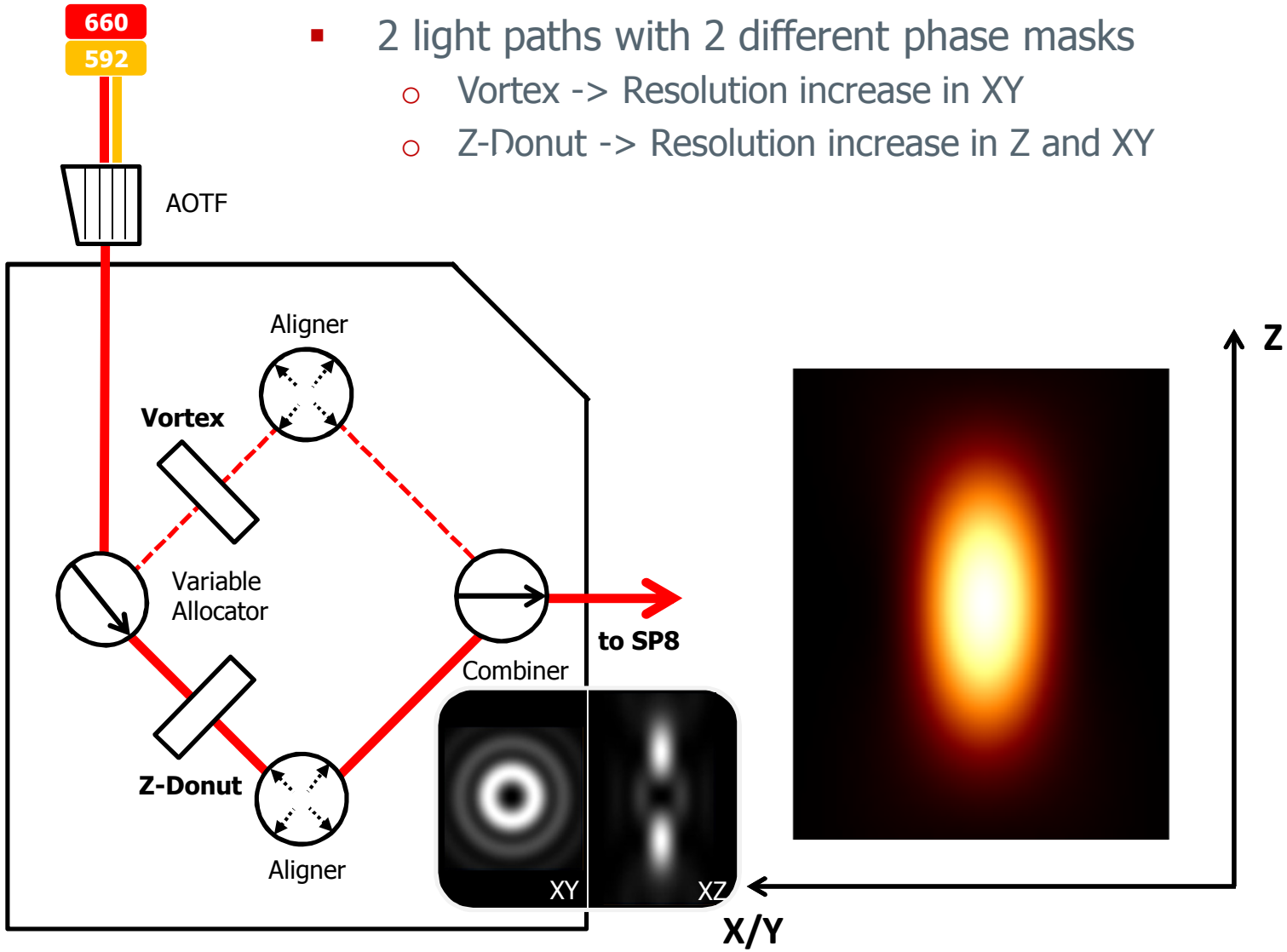
- 2 light paths with 2 different phase masks
  - Vortex -> Resolution increase in XY





STED 3X Microscopy – The concept

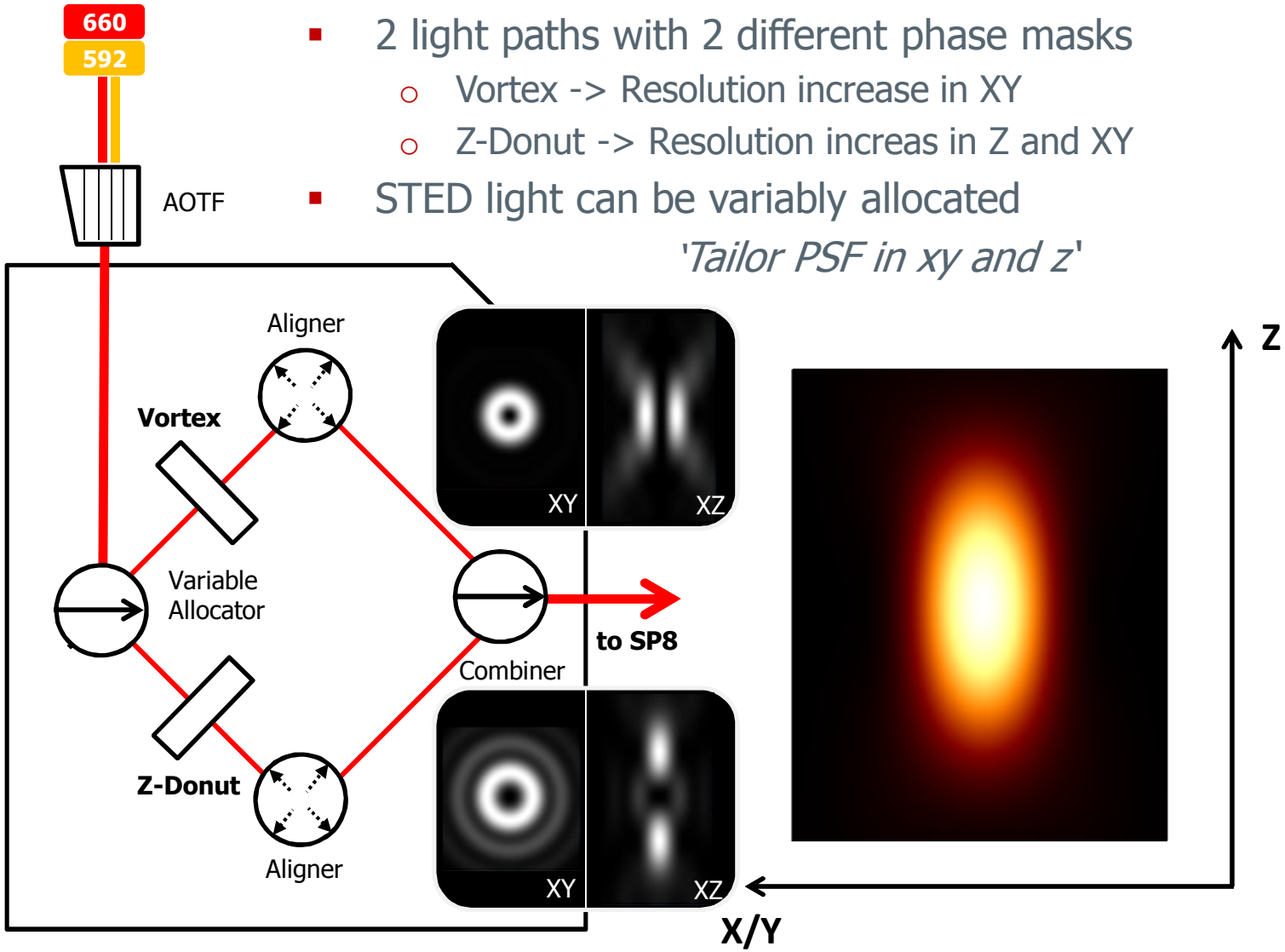
- 2 light paths with 2 different phase masks
  - Vortex -> Resolution increase in XY
  - Z-Donut -> Resolution increase in Z and XY



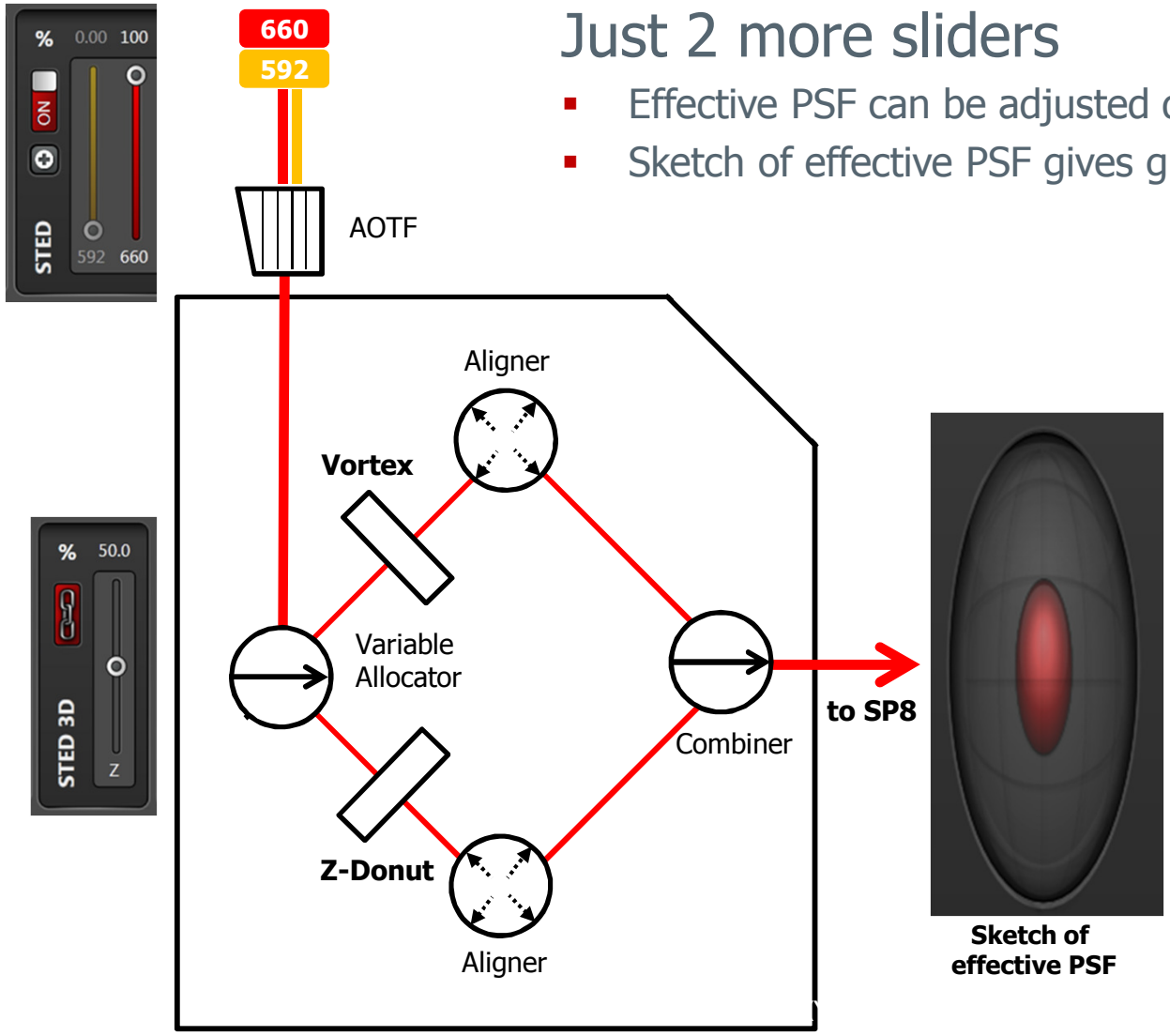
STED 3X Microscopy – The concept

- 2 light paths with 2 different phase masks
  - Vortex -> Resolution increase in XY
  - Z-Donut -> Resolution increas in Z and XY
- STED light can be variably allocated

'*Tailor PSF in xy and z*'



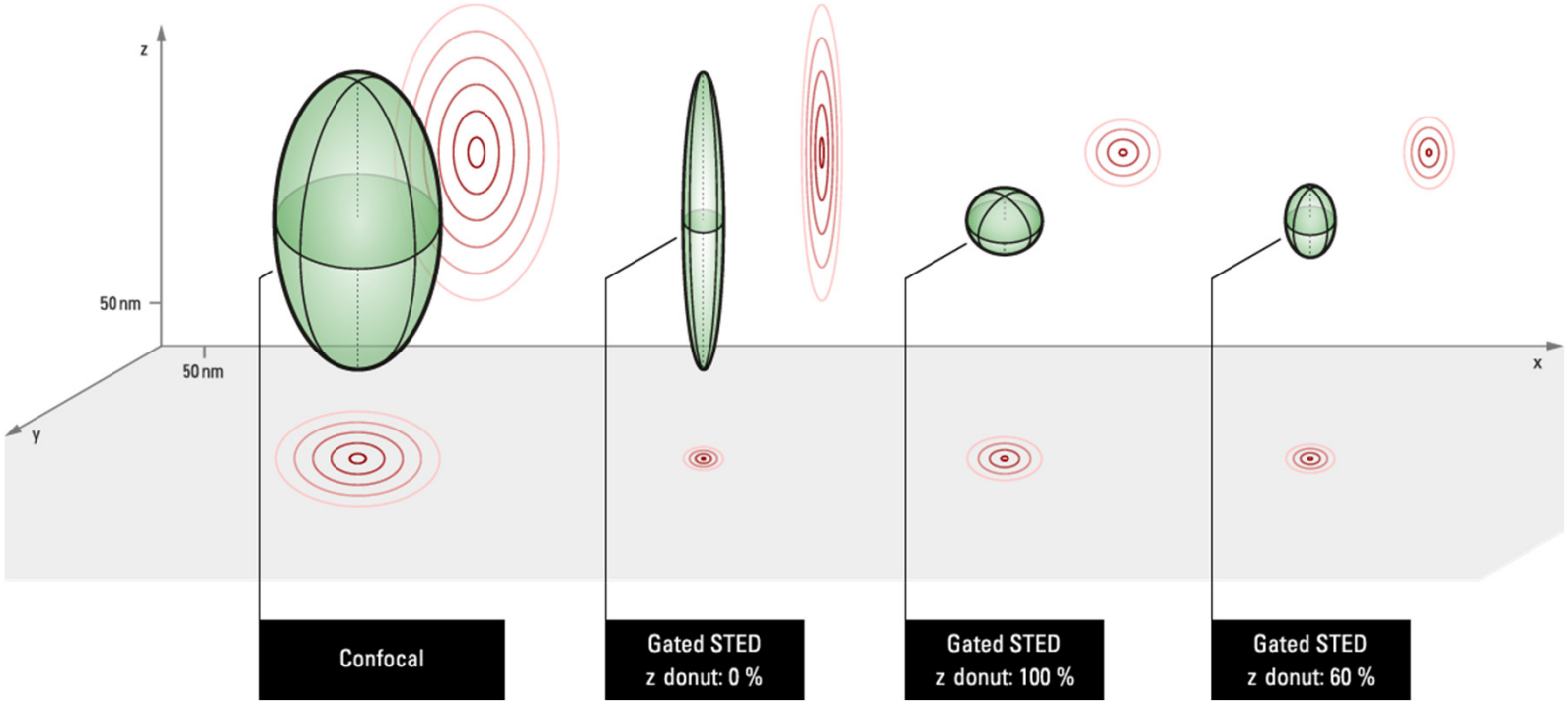
STED 3X Microscopy – The concept



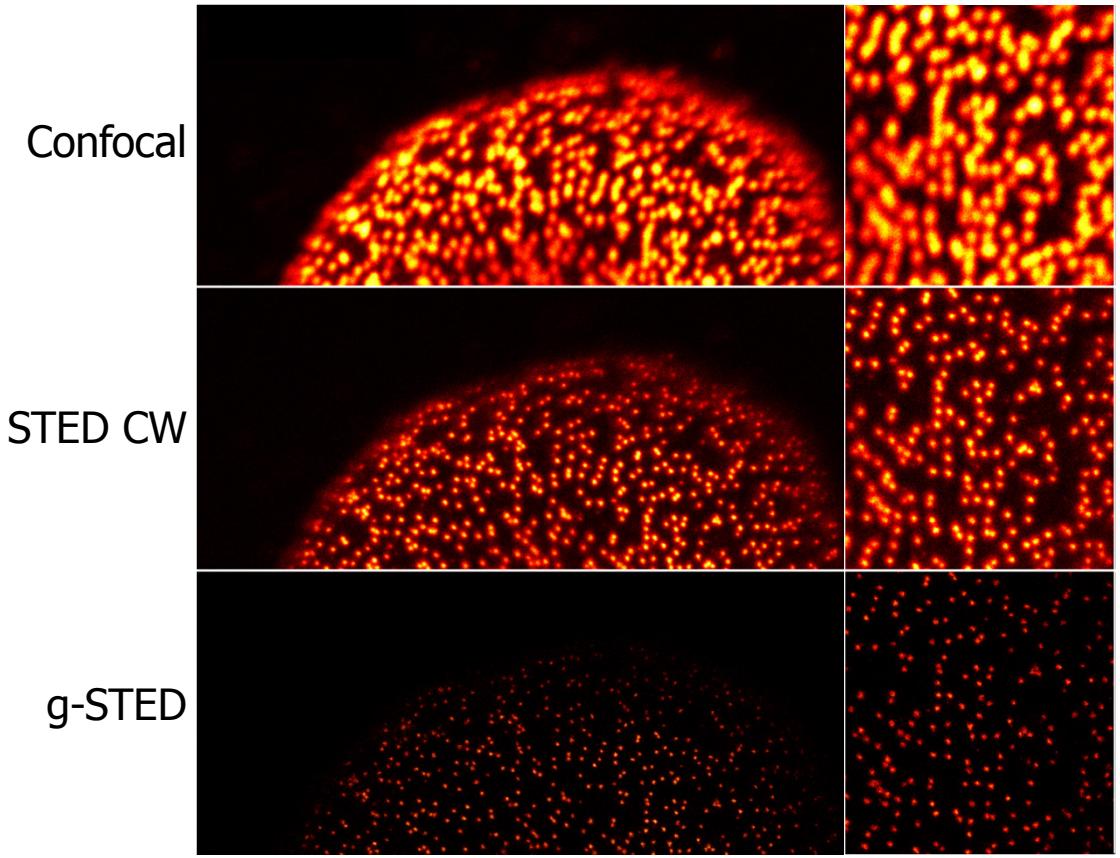
Just 2 more sliders

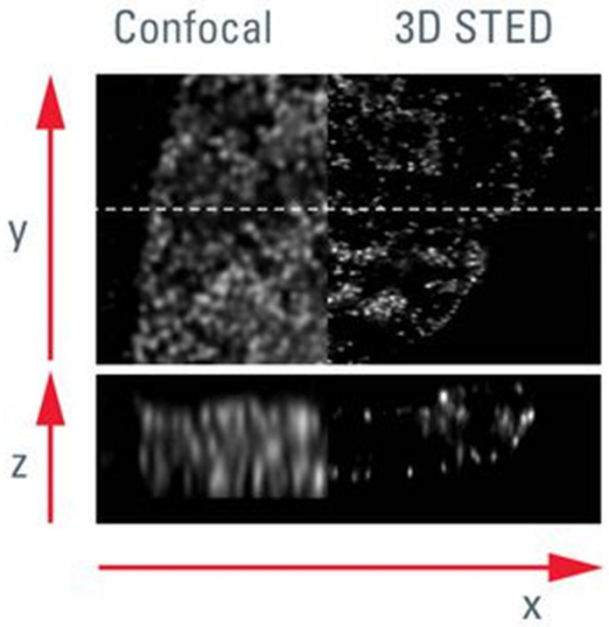
- Effective PSF can be adjusted during live scan
- Sketch of effective PSF gives guidance

STED 3X Microscopy – The concept



Confocal vs STED Images





IF: Nuclear Pore Complex; Alexa 488

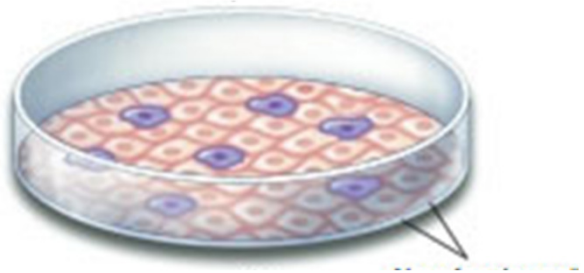
STED CW and gated STED - Summary

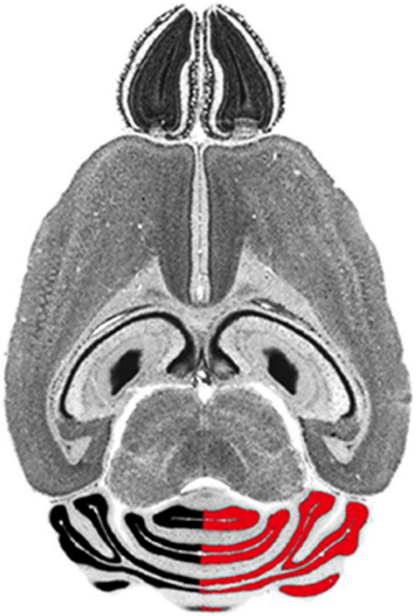
- Super-resolution method based on fluorescence confocal imaging, in which images are acquired by scanning a focused light spot over a ROI and collecting fluorescence sequentially pixel by pixel.
- Main Strengths:
  - Pure physical resolution improvement below 50nm, without any additional post-processing.
  - Intrinsic optical sectioning enabling the acquisition of Z-Stacks
  - Axial (Z) resolution improvement to ~120nm by using the 3D donut.
  - Fast image acquisition of several images per second.
  - Live cell imaging using common fluorescent proteins or other fluorescent tags.
  - Three STED depletion lasers enabling the use of a wide range of fluorescent dyes

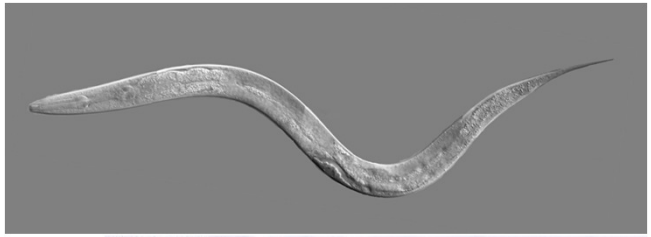
Sample choices for STED

Choice of samples

- Big variety of samples from:
  - Single cultured flat cells,
  - Tissue slices to whole animals, e.g. nematodes (*C. elegans*)
  - Insects (*D. melanogaster*).





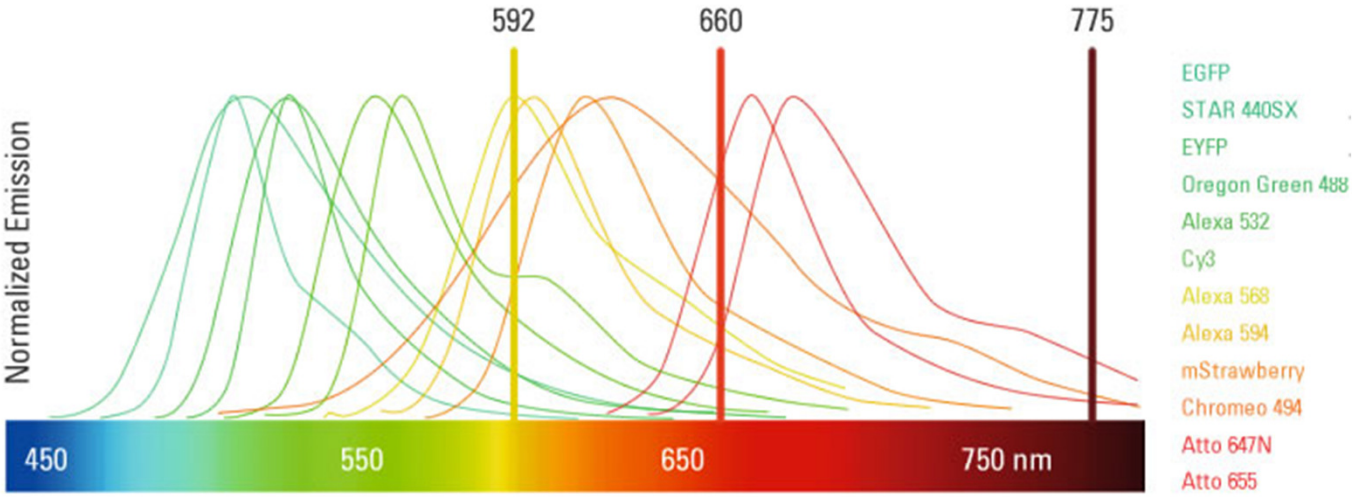


Choice of samples

- Keep in mind:
  - STED applies a specially developed STED 100x/1.4 oil objective, which has a working distance of 90µm.
  - The observed structure should be at most 80µm away from the coverglass (use #1.5 ONLY!!!), within 20µm range for optimal performance.
  - To achieve the best results, the refractive index of the mounting medium should match the refractive index of the immersion used (immersion liquid = 1.518). ProLong Diamond mounting Media is highly recommended
  - Auto-fluorescence, as well as sudden and unpredicted changes of the refractive index may influence the shape of the focal spot and consequently the performance of the microscope.
  - Also certain customers found that old or unfiltered PFA might have high background possibility with the 592 laser. Electron Microscope Grade which is already premixed produces good results (post-fixation)
  - During STED imaging, samples are irradiated strongly at a wavelength of 592 nm, 660 nm orb 775nm. It is of crucial importance that the sample is not absorbing light at this wavelength.

Choosing fluorescent dyes for STED

STED depletion lasers:

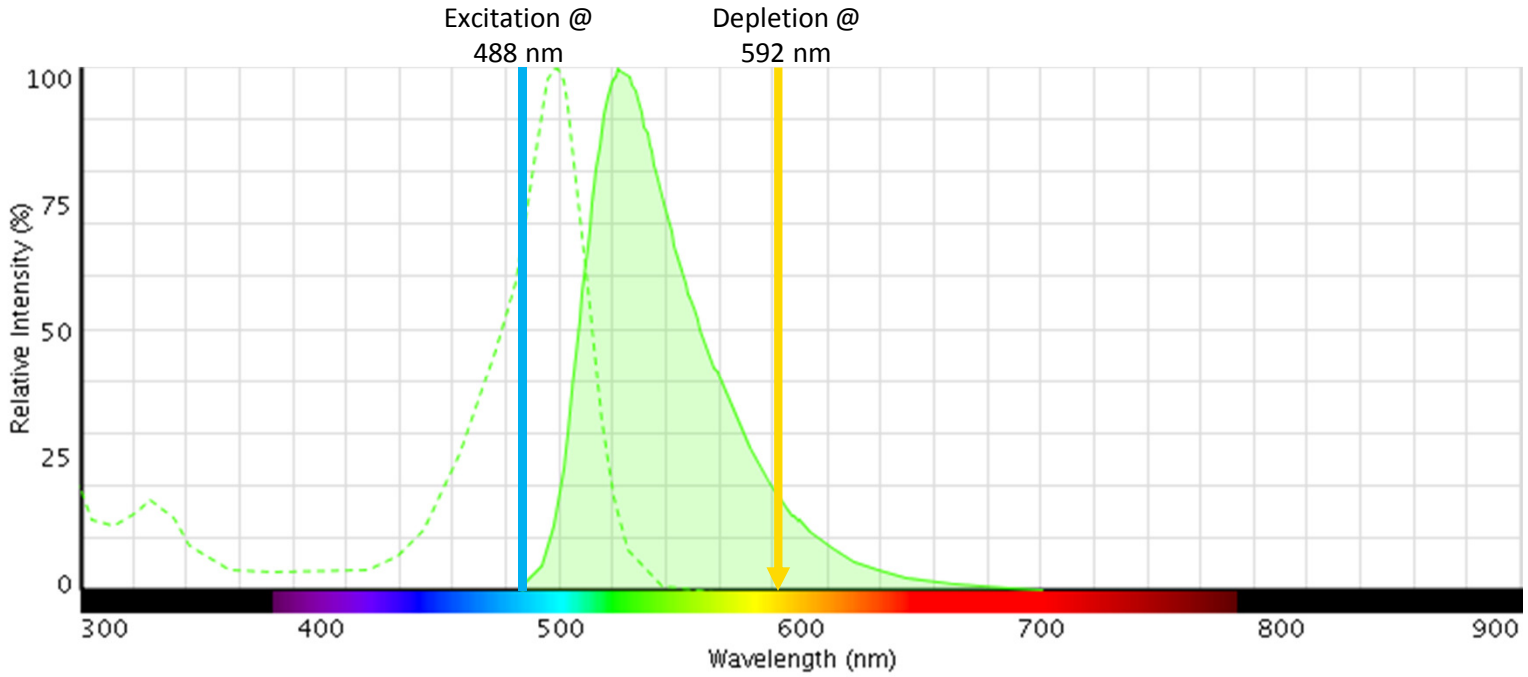


	592 GATED/CW	660 GATED/CW	775 PULSED
Strength	GFP/YFP	Multicolor	Most established spectral range
Colocalization studies	+	++	+
Photostability	+	++	++
Live cell	++	+	(+)

Leica Microsystems 2015

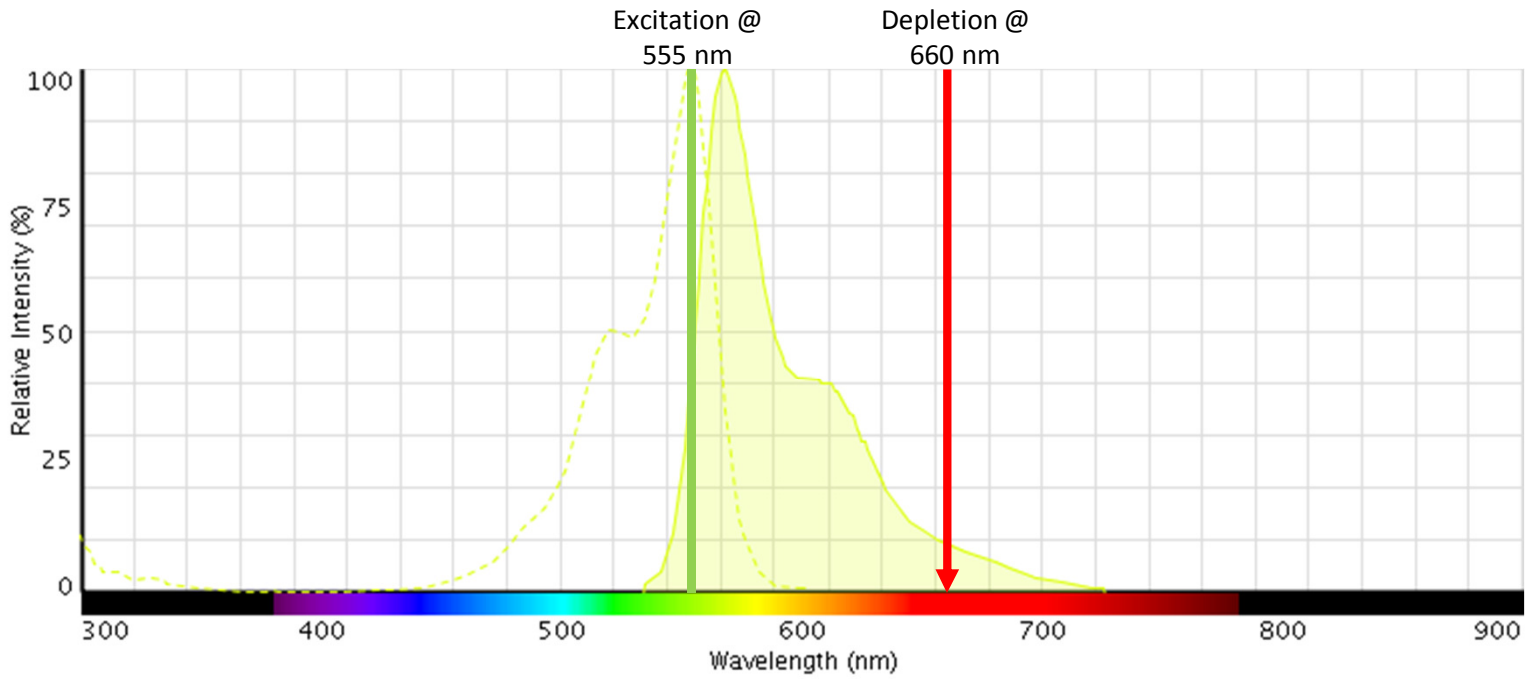
Single color STED

- For STED to work efficiently, the emission spectra needs to show significant emission at the STED wavelength.
- No excitation at the depletion laser wavelength
- Ex: **Oregon Green 488** for the **592 nm** depletion laser →



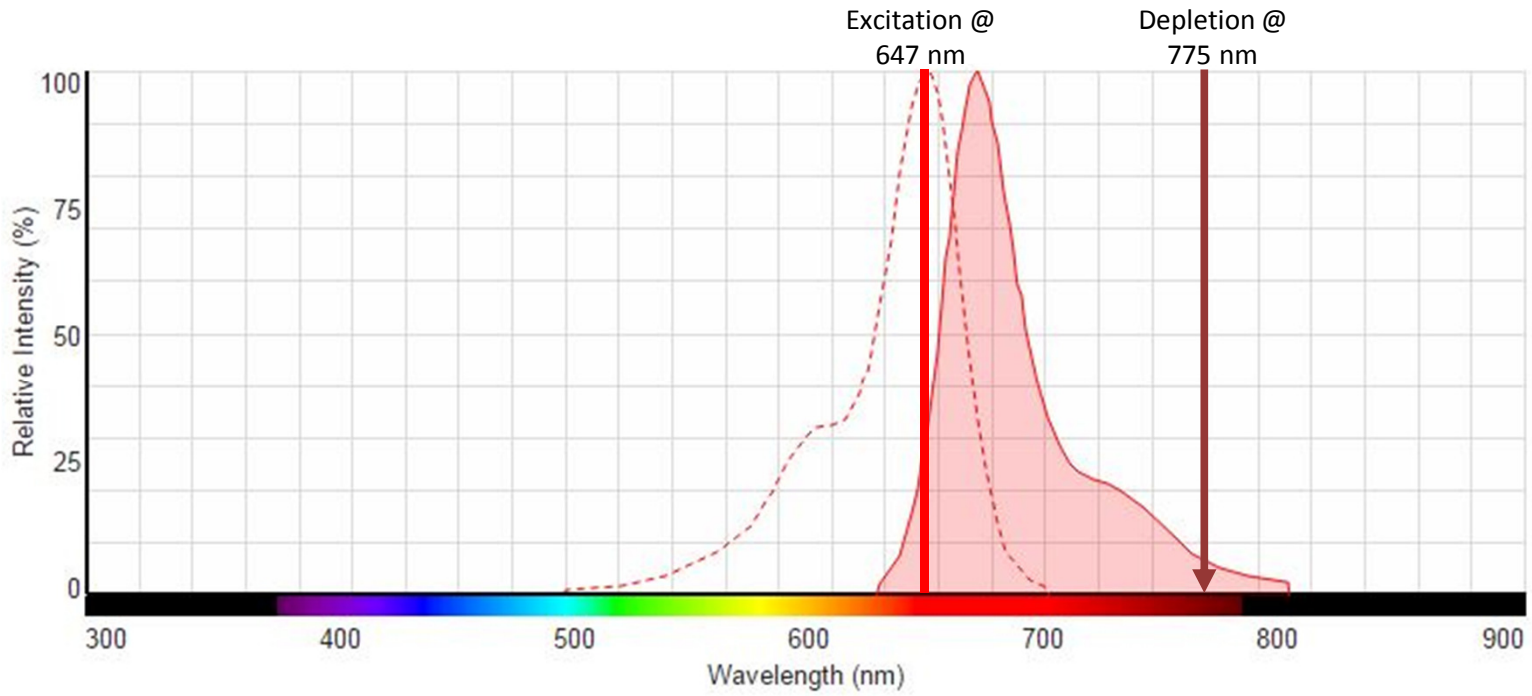
Single color STED

- For STED to work efficiently, the emission spectra needs to show significant emission at the STED wavelength.
- No excitation at the depletion wavelength.
- Ex: **Alexa 555** for the **660 nm** depletion laser →



Single color STED

- For STED to work efficiently, the emission spectra needs to show significant emission at the STED wavelength.
- No excitation at the depletion wavelength.
- Ex: **Alexa 647** for the **775 nm** depletion laser →

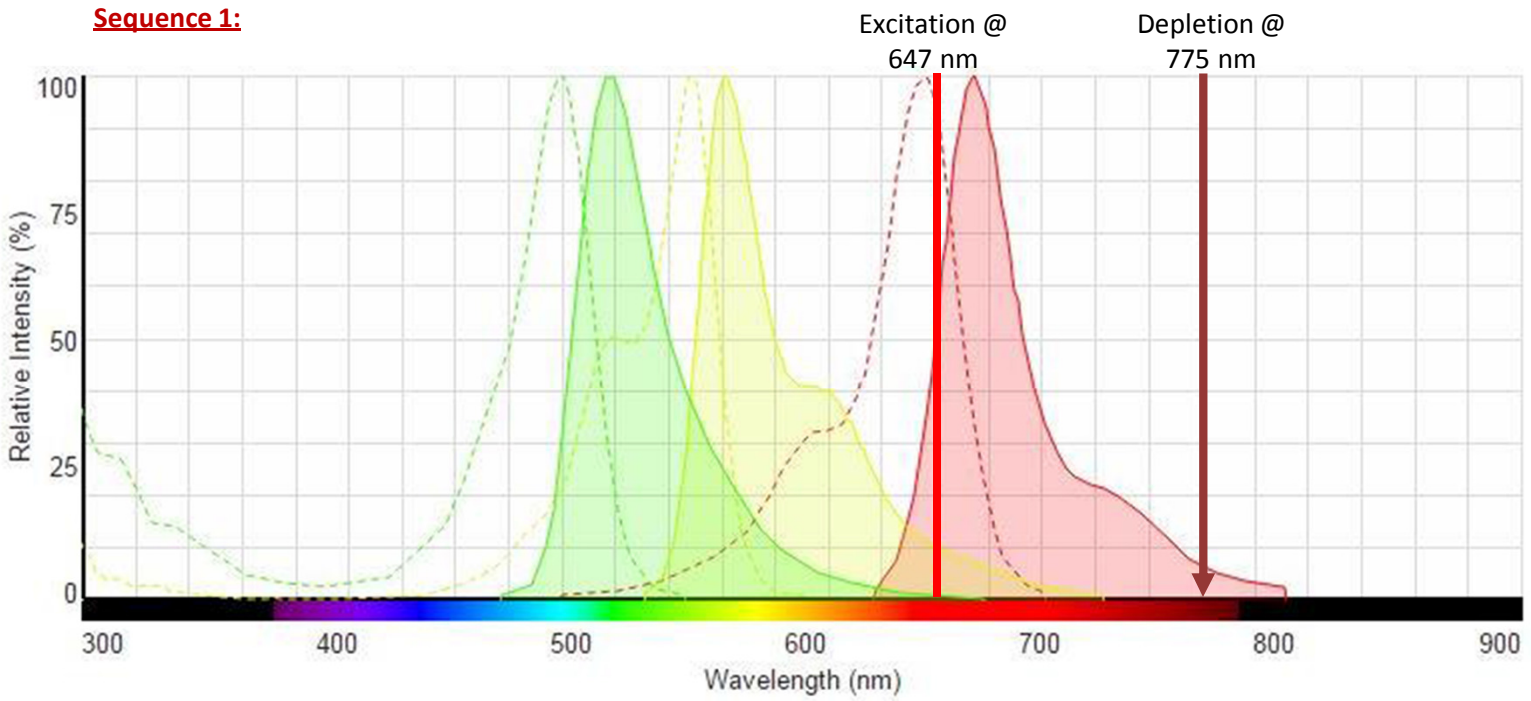


Recommended Single Dyes

- Single color for 592 nm depletion
  - DyLight 488 or 514
  - Oregon Green 488 or 514
  - AlexaFluor 488 or 514
  - ATTO 488 or 514
- Single color for 660 nm depletion
  - Alexa 532
  - ATTO 532 or 550
  - TMR/TRITC
  - Alexa 555
- Single color for 775 nm depletion
  - ATTO 647N
  - Alexa 633
  - Alexa 594

Multi-color STED

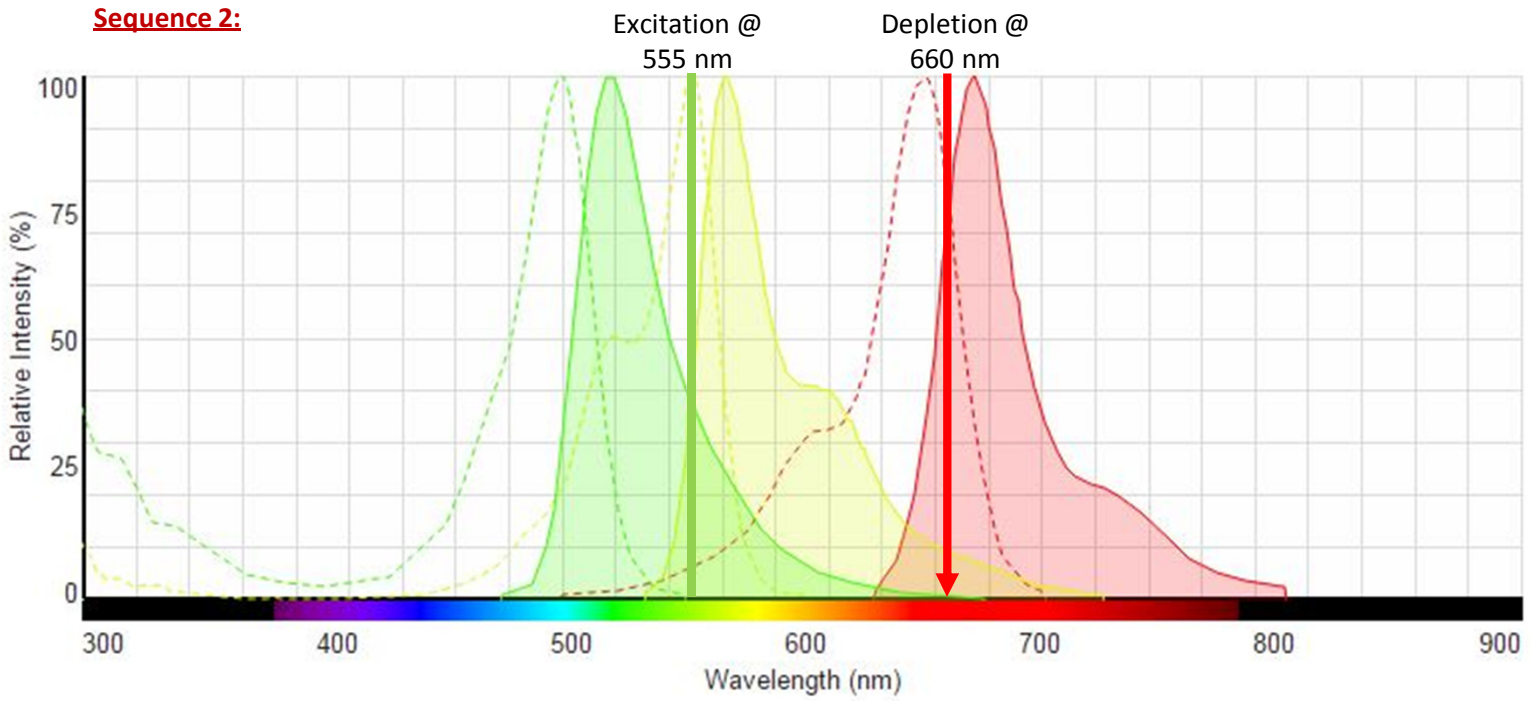
- For STED to work efficiently, the emission spectra of both dyes need to show significant emission at the STED laser wavelengths.
- Ex: **Alexa 647, Alexa 555** and **OG 488** for the **775nm, 660 nm** and **592 nm** depletion laser →



** **Rule: "Image far red → red"** ALWAYS start your sequential 775/660 nm STED scans with the LONGER WAVELENGTH dye and the 775nm depletion laser!!! The 660 nm depletion laser may bleach orange/red dyes.

Multi-color STED

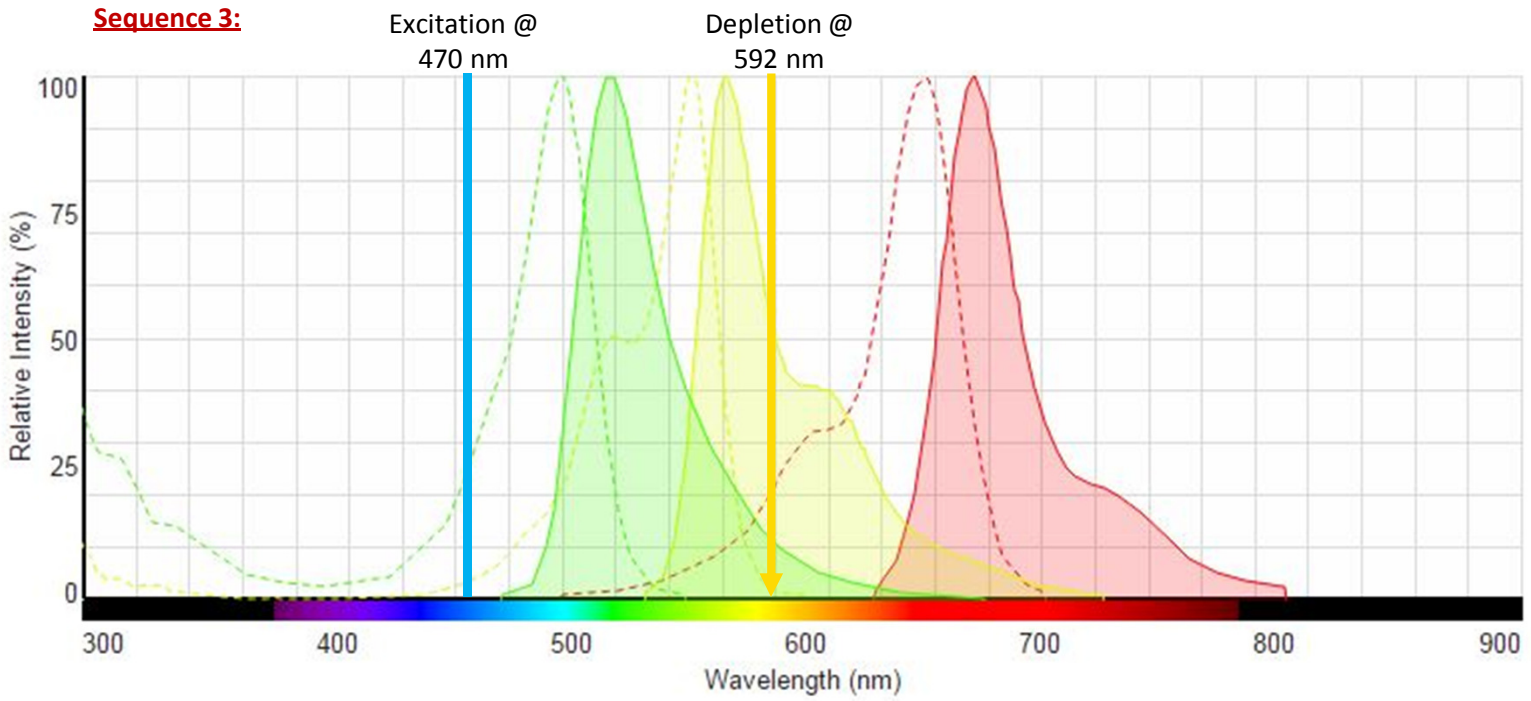
- For STED to work efficiently, the emission spectra of both dyes need to show significant emission at the STED laser wavelengths.
- Ex: **Alexa 647, Alexa 555** and **OG 488** for the **775nm, 660 nm** and **592 nm** depletion laser →



** **Rule: "Image red → blue"** ALWAYS start your sequential 660/592 nm STED scans with the LONGER WAVELENGTH dye and the 660 nm depletion laser!!! The 592 nm depletion laser may bleach orange/red dyes.

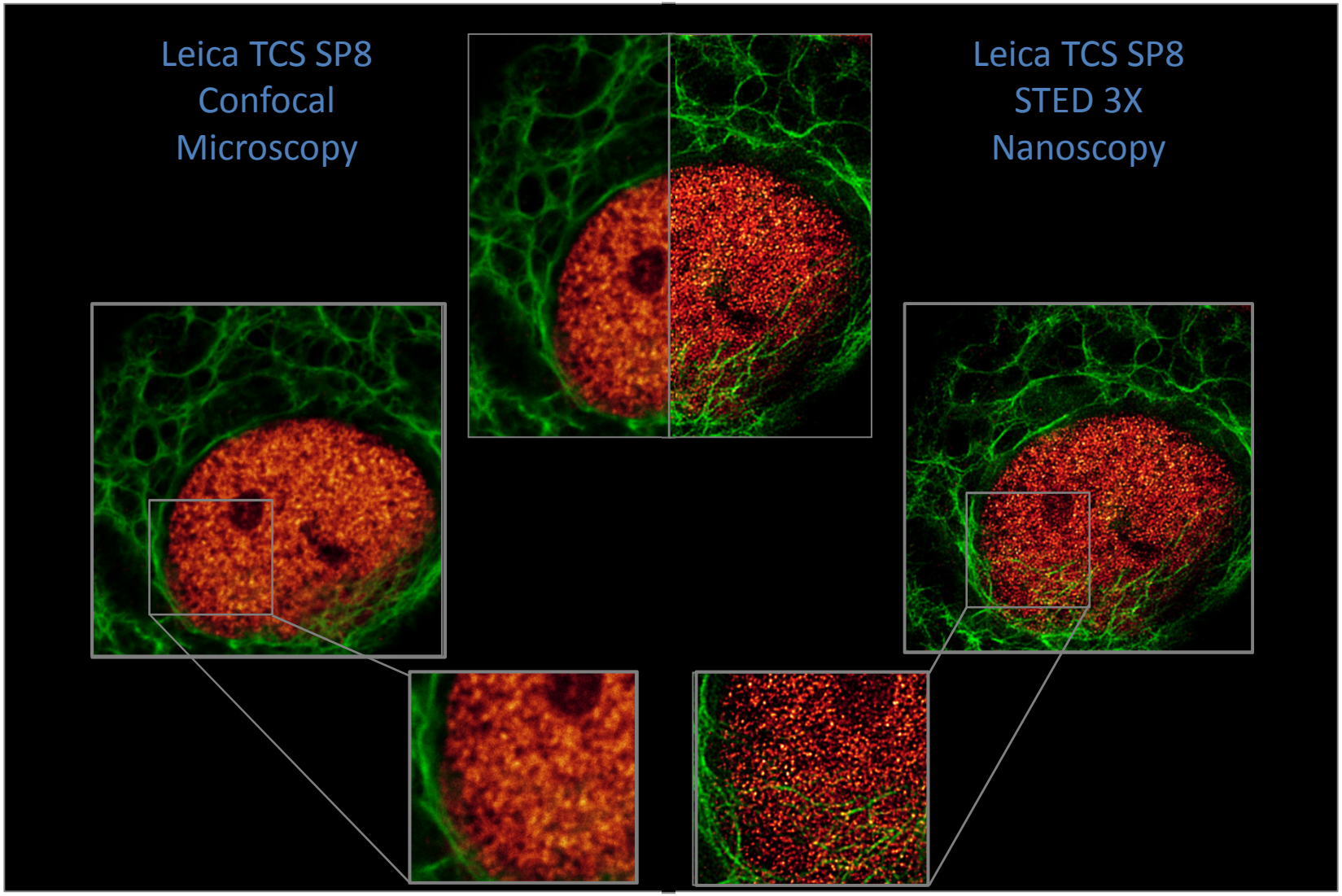
Multi-color STED

- For STED to work efficiently, the emission spectra of both dyes need to show significant emission at the STED laser wavelengths.
- Ex: **Alexa 647, Alexa 555** and **OG 488** for the **775nm, 660 nm** and **592 nm** depletion laser →



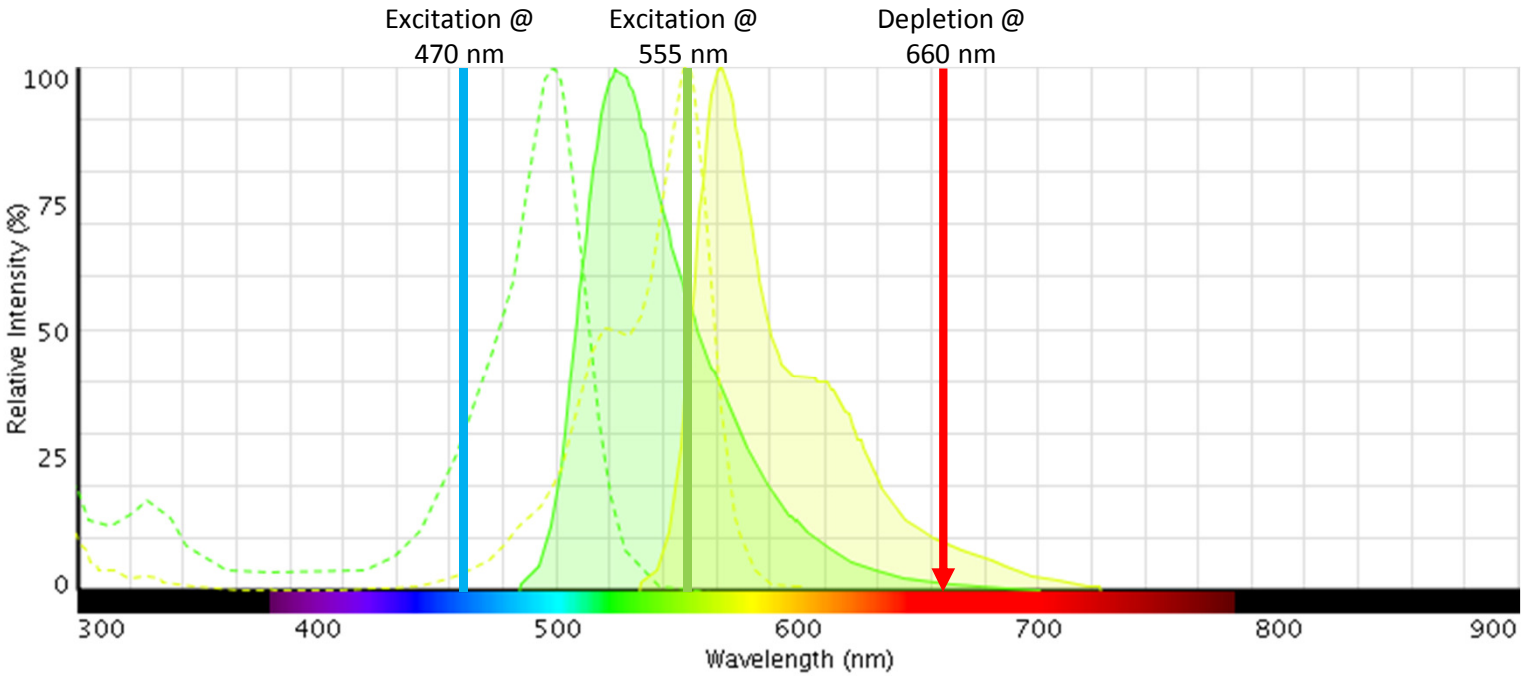
** **Rule: "Image red → blue"** ALWAYS start your sequential 660/592 nm STED scans with the LONGER WAVELENGTH dye and the 660 nm depletion laser!!! The 592 nm depletion laser may bleach orange/red dyes.

Multi-color STED real-life example!



Multi-color STED – 1 STED laser

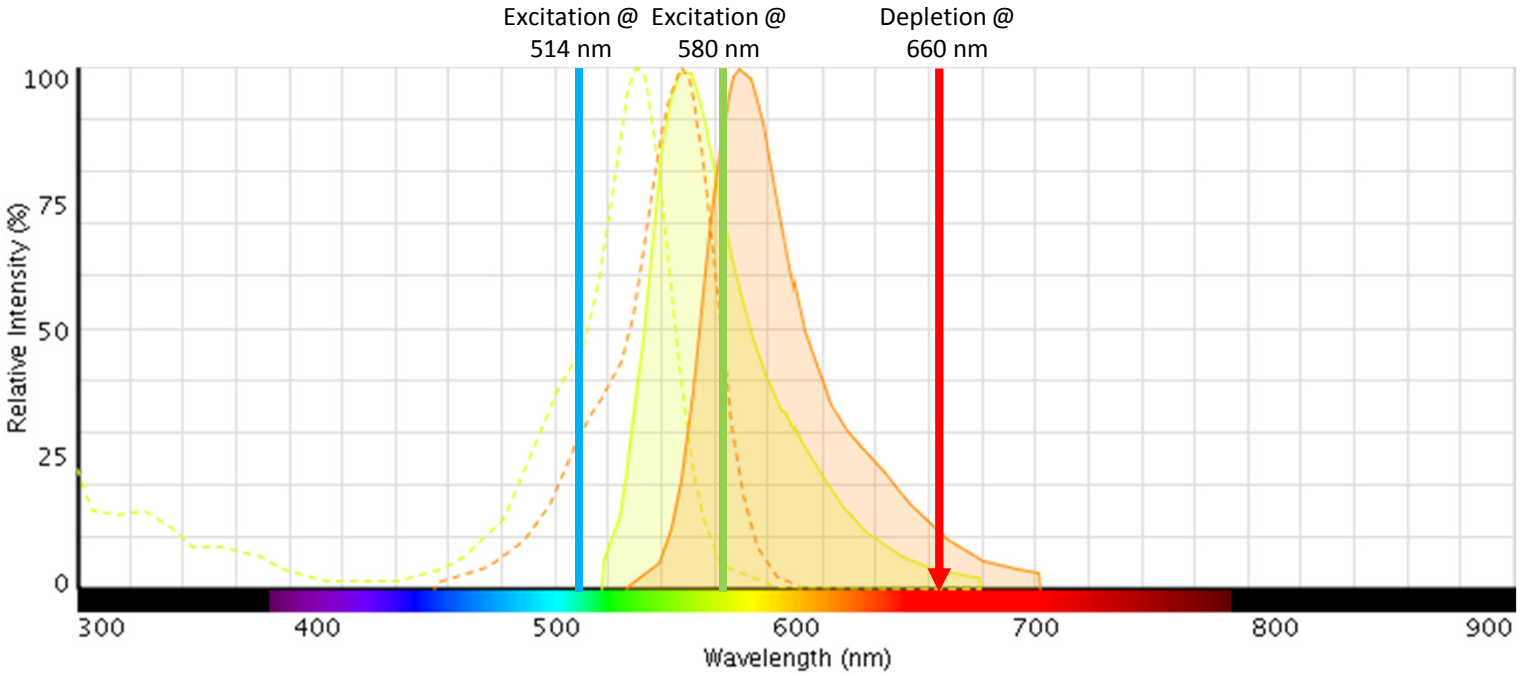
- For STED to work efficiently, the emission spectra of both dyes need to show significant emission at the STED laser wavelengths.
- Ex: **Alexa 555** and **OG 488** for the **660 nm** depletion laser →



** Dyes with similar emission spectra to OG488 will show resolution improvement with the 660nm laser, but the optimal super-resolution will be obtained with the 592nm laser.

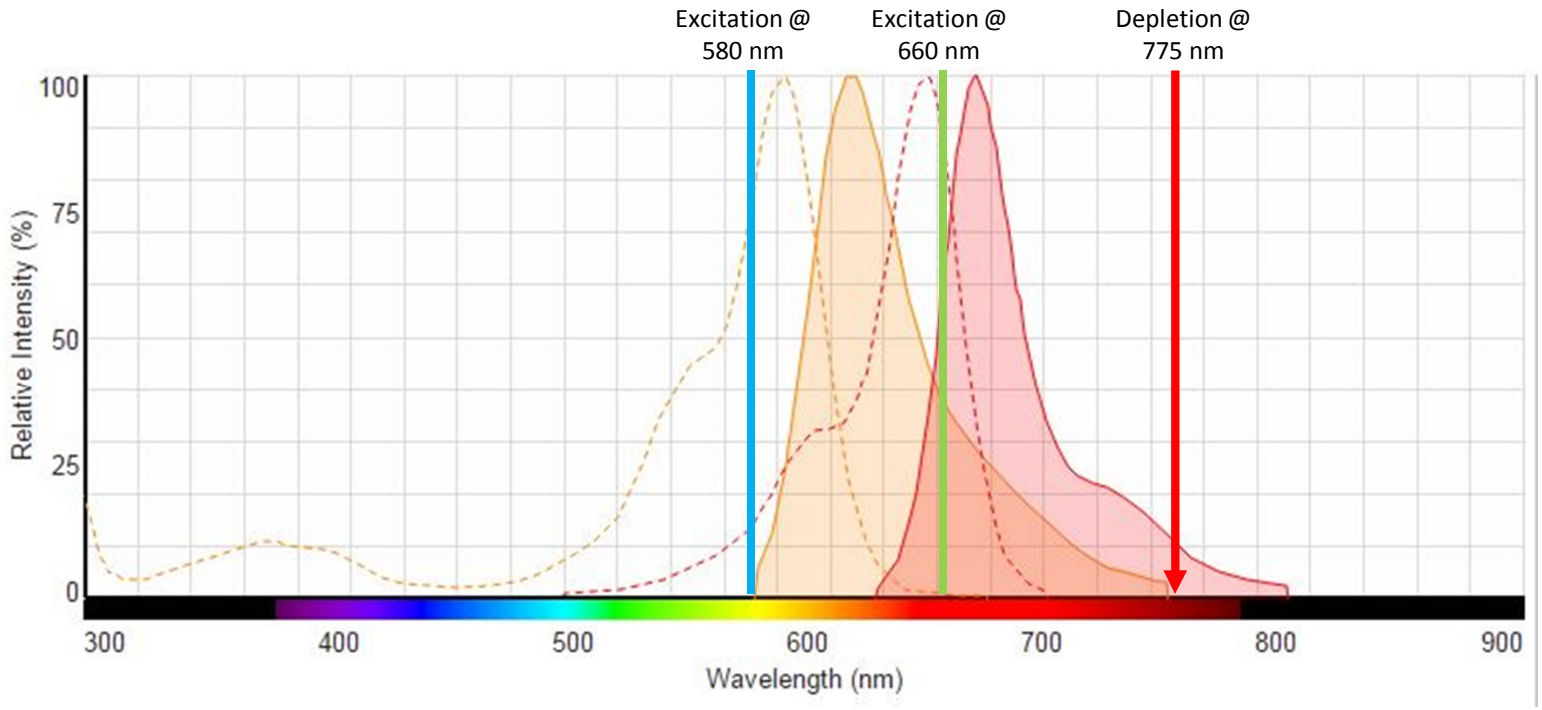
Multi-color STED – 1 STED laser

- For STED to work efficiently, the emission spectra of both dyes need to show significant emission at the STED laser wavelengths.
- Ex: **Alexa 532** and **TMR** for the **660 nm** depletion laser →



Multi-color STED – 1 STED laser

- For STED to work efficiently, the emission spectra of both dyes need to show significant emission at the STED laser wavelengths.
- Ex: **Alexa 594** and **Alexa647** for the **775 nm** depletion laser →



gated STED

- Examples of dual color acquisition for selected dyes pairs, and spectra:

STED: 592 nm, 660nm, 775nm					
Dye1			**Dye2**		
Name	**Excitation**	**Emission: e.g.**	**Name**	**Excitation**	**Emission: e.g.**
BD Horizon V500	458/470	475–510	Oregon Green 488/ Chromeo 505	514/520	523–580
Oregon Green 488	470 (WLL)	480–520	Alexa Fluor 532	545 (WLL)	555–580
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	Fluor 647	635/650	655–750

gated STED

- Examples of triple color acquisition for selected dyes pairs, and spectra:

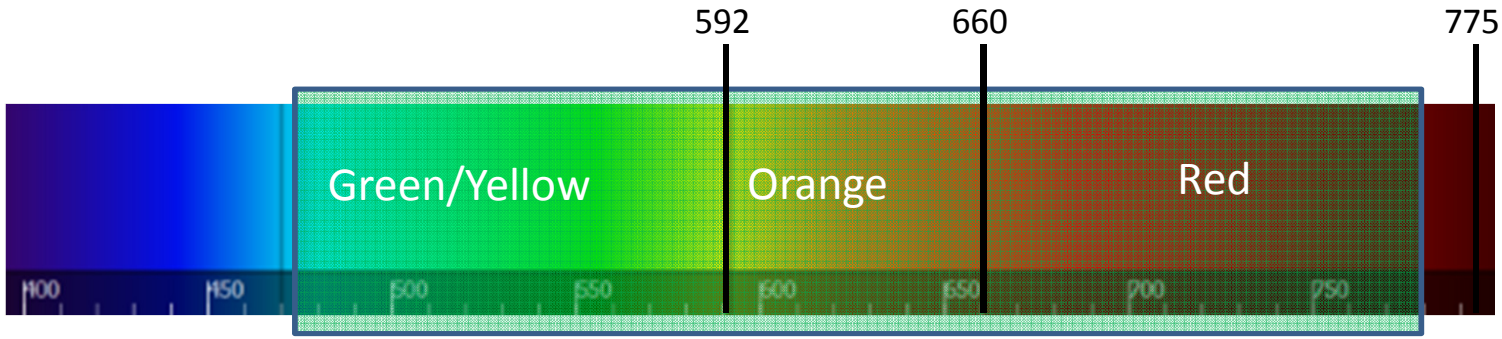
STED: 592 nm, 660nm, 775nm

Dye1			Dye2			Dye 3		
Name	**Excitation**	**Emission**	**Name**	**Excitation**	**Emission**	**Name**	**Excitation**	**Emission**
STAR 440 SX**	470	475–505	Oregon Green 488	510	515–530	Alexa Fluor 532	540	550–585
Oregon Green 488**	470	475–525	Alexa Fluor 532	532	538–550	TMR/TRITC	580	590–650
Alexa Fluor 514**	480	490–535	Alexa Fluor 546	540	545–580	Alexa Fluor 594	590	600–650
TRITC	550	560–590	ATTO 594	600	610–640	STAR 635P	660	665–750
Alexa Fluor 594	580	580–615	Alexa Fluor 633	620	625–655	Alexa Fluor 660	660	665–750
488	488	500–545	TRITC/TMR	550	560–635	635P	640	750

Choice of Fluorescent Dyes

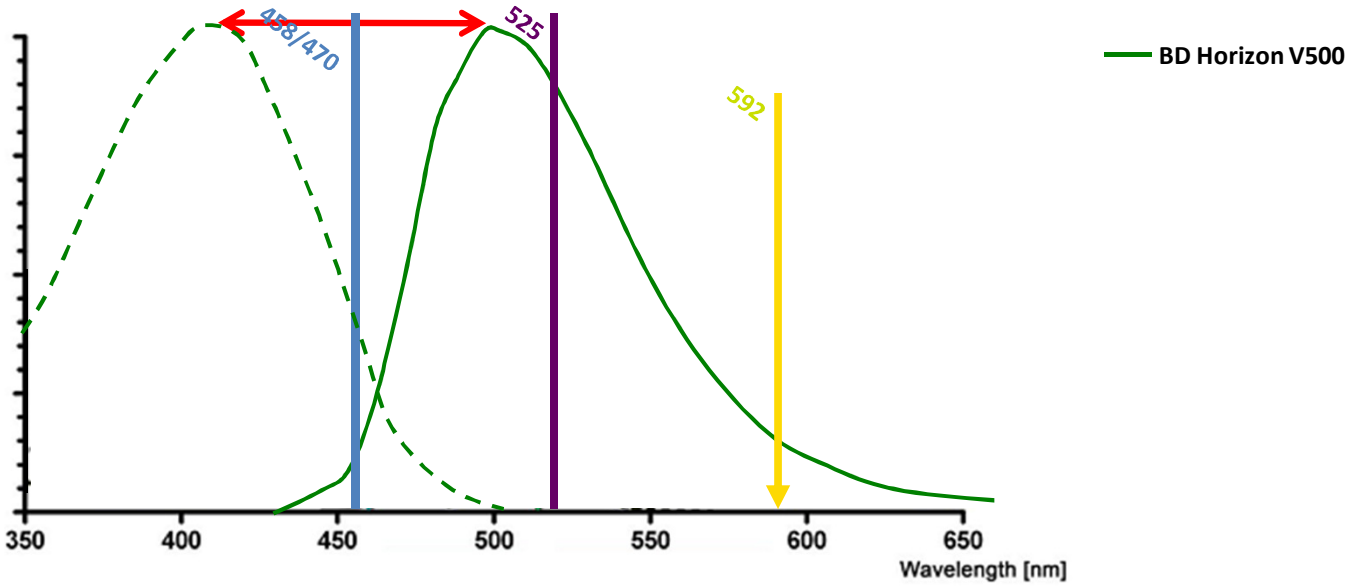
- More dyes could be use for more colors.
- Before Neuroscience (Nov. 10, 2013)
  - We were able only to use the green/yellow fluorophores

- Now
  - We are able to use not only the green/yellow fluorophores but also orange to deep red fluorophores



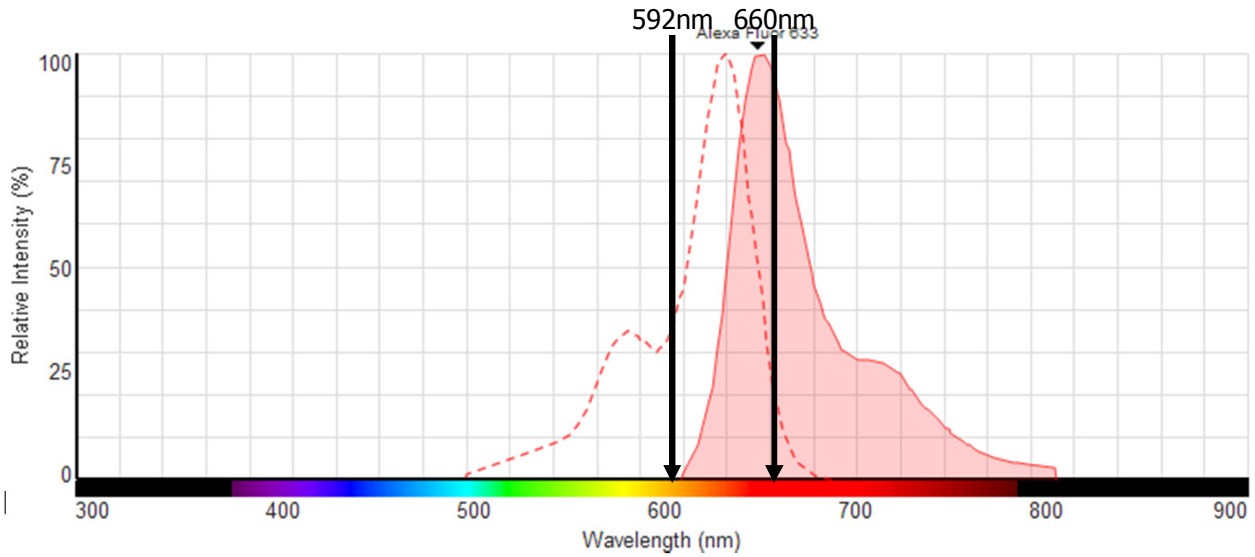
Choice of Fluorescence Dyes

- For the spectral separation of two dyes with similar emissions:
  - Different excitations are required.
  - This is realized by using large Stoke's shift dyes with absorption spectra located in the violet/blue range.



Choice of Fluorescent Dyes

- Other dyes can be used as a confocal reference stain/counter-stain.
- However:
  - The emission of these dyes, should reside outside the range of the STED detection.
  - If dyes in the orange/red range are selected (e.g. Cy5), they will absorb the strong STED depletion light and get bleached
  - Thus all reference images need to be acquired *before* the STED images.



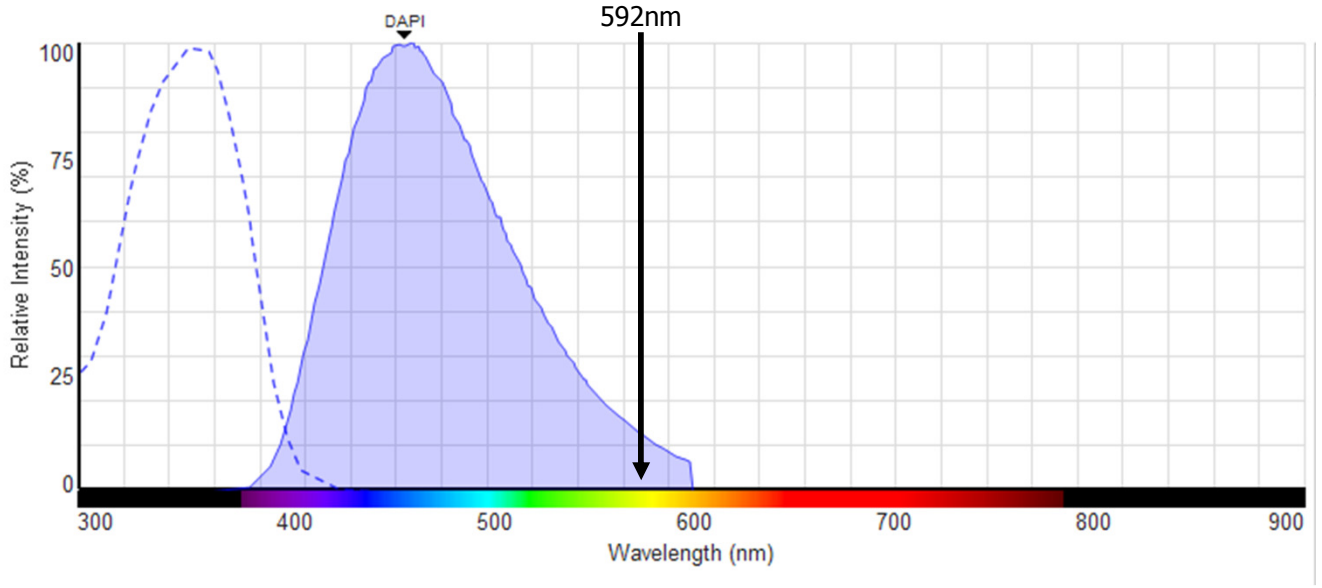
Primary and Secondary Concentrations

- Increase your normal working concentrations of both primary and secondary antibodies!
  - We recommend most concentrations be about 1:100 for primary and 1:50 – 1:200 for secondary
  - It is always better to start with higher concentrations of antibodies for the first samples, to rule out some issues with labeling density
- Examples:
  - Oregon Green 488 (1:100) *(Life Technologies)* Goat α-mouse *(O-11033)* or α-rabbit *(O-11038)*
  - Alexa Fluor 488 (1:100) *(Life Technologies)* Goat α-mouse *(A-11001)* or α-rabbit *(A-11008)*
  - Alexa Fluor 532 (1:100) *(Life Technologies)* Goat a-mouse *(A-11002)* or a-rabbit *(A-11009)*
  - Alexa Fluor 555 (1:100) *(Life Technologies)* Goat a-mouse *(A-11031)* or a-rabbit *(A-11011) or* a-chicken *(A-11041)*
  - Tetramethylrhodamine (TRITC) (1:100) *(Life Technologies)* Goat α-mouse *(T-2762)* or α-rabbit *(T-2769)*

Living cell STED imaging

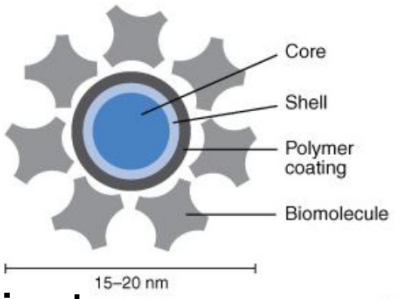
Fluorescent proteins	Excitation (nm)	Depletion (nm)
eGFP	484 (488)	592
EmGFP	487 (488)	592
eYFP	514 (514)	592 / 660
Venus	515 (514)	592 / 660
mCitrine	516 (514)	592 / 660
dsRed	558	660
mStrawberry	574	660
Other probes		
Tubulin Tracker Green	488 / 514	592
BABTA	488 / 514	592
Lifeact (Actin marker)	488 / 514 (483 / 506)	592
SiR Actin/Tubulin	652 / 674	775

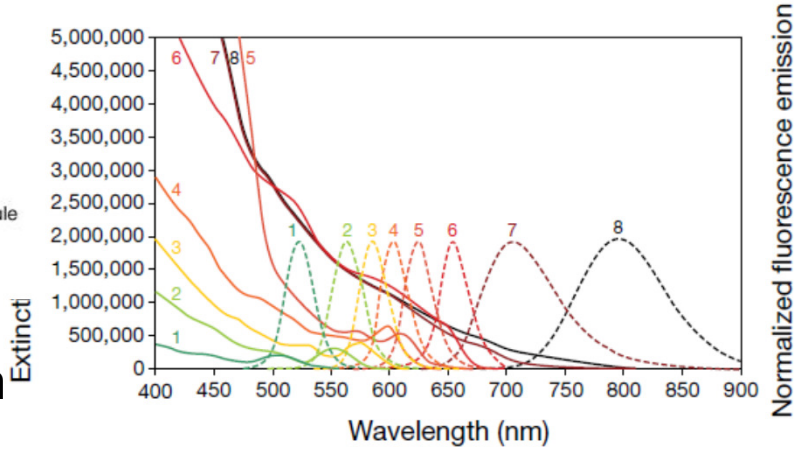
- Do not use:
  - Other fluorescence Proteins not excitable by any laser lines from the Argon laser – STED CW (458, 476, 488, 514nm) or with the WLL gSTED between 470 and 580 nm.
  - DAPI (replace with TO-PRO-3, YOYO-3, PicoGreen)
  - QDOTs or other fluorophores excited by the 405 nm laser



Nanocrystals (Quantum Dots - eFluor)

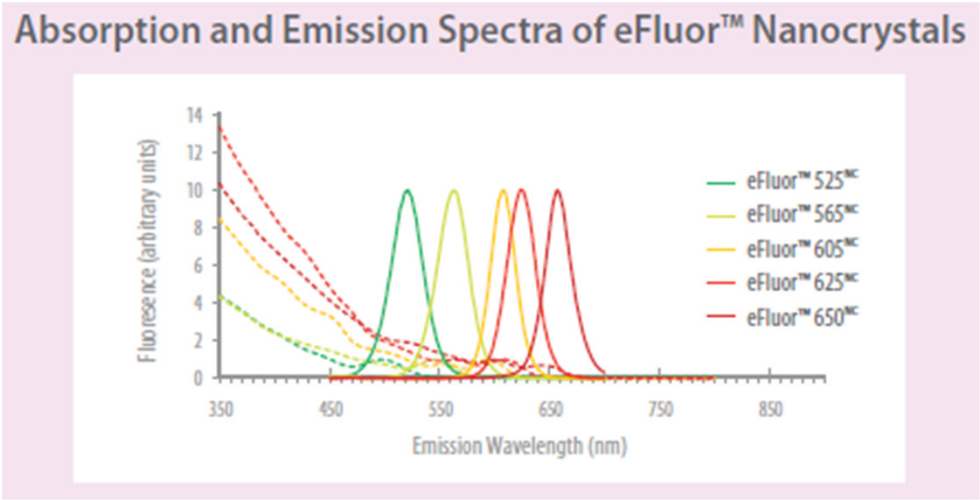
- Emission Spectrum
  - Size & composition
  - FWHM 25
- High photostability
- Large extinction coefficients
  - 130.000 – 2.900.000 $M^{-1}cm^{-1}$ @ 488nm
  - Fluorescein: 80.000 $M^{-1}cm^{-1}$
- Excitation @ 592nm 3-9% of maximum





Not a too good idea because they have a very narrow emission and broad absorption spectrum.

Up to now, they never worked



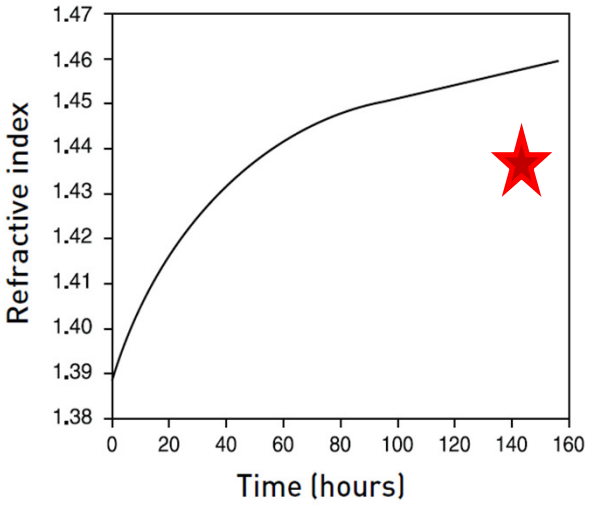
Sample Mounting

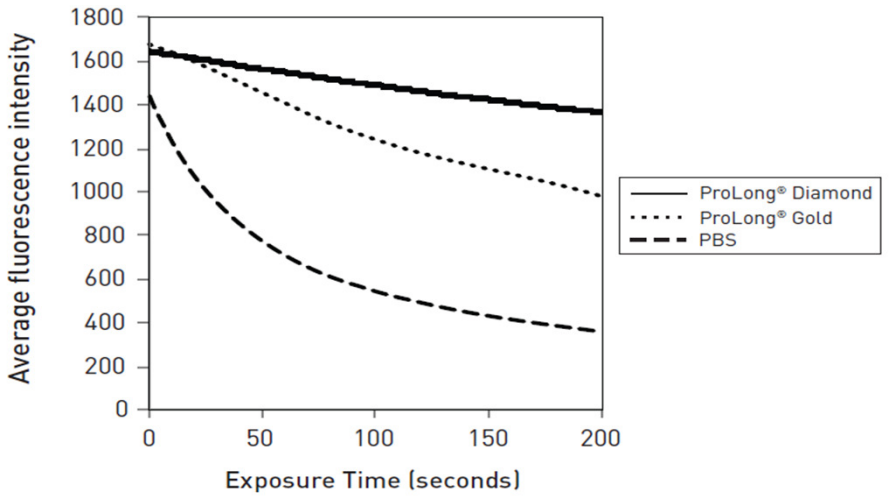
Points to keep in mind:

- **#1.5 coverslips ONLY!**
- DAPI free mounting media
- Refractive index matched mounting media (oil=1.518)
- Acid-washed coverslips are preferable to flamed coverslips
- Avoid "sealants" that can quench fluorescence or create autofluorescence (i.e. color nailpolish)

Mounting Media

- With cell culture and thin tissue sections
  - Prolong® Diamond Antifade Mountant *(Life Technologies)*
    - Protects fluorescent dyes and fluorescent protein from fading, across entire visible and IR spectrums
    - Ready-to-use liquid that cures for longer-term storage (polymerizes, so no sealant needed)
    - Ideal for Alexa Fluor® and traditional dyes, such as FITC and Cy®3, and fluorescent proteins like GFP, RFP, and mCherry
    - Mounted samples are stable for months
    - Maintains fluorescence signal—little to no quenching





Mounting Media

- With thick tissue sections
  - Thiodiethanol *(TDE, Sigma, #88559)* mixed with an anti-fade reagent has been used with good results, especially for deep imaging.
    - The TDE concentration must be gradually enhanced (up to 97%) to obtain a final refractive index of 1.514
    - Sequential steps in TDE 50%, 70% (15-30 minutes at each step), then in 97% + antifade as final mounting media must be undertaken.
    - The coverslip must be sealed using invisible nail polish or other sealants.
    - Be sure that the sealant is not quenching your fluorescence or creating any autofluorescence.
  - Prolong only if the structure of interest is close from the coverslip. (closer than 25 µm)

Microsc Res Tech. 2007 Jan;70(1):1-9.

2,2'-thiodiethanol: a new water soluble mounting medium for high resolution optical microscopy.

Staudt T, Lang MC, Medda R, Engelhardt J, Hell SW.

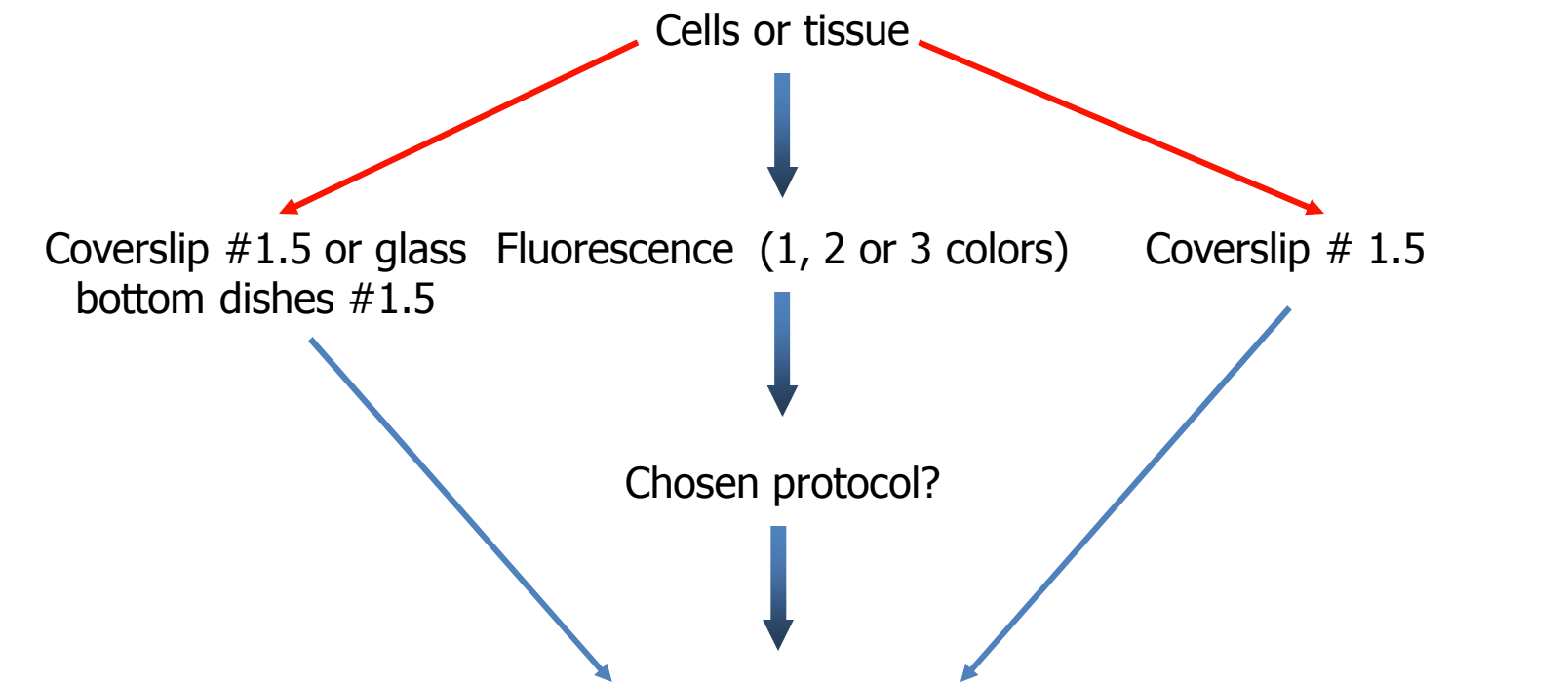
STED CW and gated STED – Mounting Media

- Thiodiethanol preparation for tissue mounting
  (TDE, Sigma #88559)

6 Well-plate → TDE 25% → TDE 50% → TDE 75%
→ TDE 80% + Antifade for GFP
→ TDE 97% + Antifade

  - 15-30 minutes incubation at each step (depending of the section thickness)
  - Mix very well the antifade (pipette in and out) with the TDE 97% prior to mount the tissue sections (eliminate the bubbles by centrifugation)
  - Use nail polish (high quality uncolored nail polish) on the #1.5 coverslip corner to keep it in place for the night (must be kept flat).
  - Seal the coverslip edges the following morning with nail polish.

General STED Workflow



- **Prolong Diamond**
- Mowiol (+/- Antifade)
- Fluoromount

- **Prolong Diamond**
- TDE + Antifade
- Mowiol (+/- Antifade)

TCS STED CW and gSTED – Tips and Tricks received by customers

- DRAQ 5 in a dilution of 1:5000 works well with TDE and ***p-Phenylenediamine*** (PPD) seems to be the most effective antifade mixed; no fading or bleaching at high depletion power.
  - At more concentrated amounts DRAQ 5 mounted with Prolong, customer noticed very strange results in the green channel; Draq gets much brighter; there is some kind of photo conversion in prolong.
  - Excitation peak ~650nm

- TDE de-polymerizes F-actin so isn't suitable for that.
  - TDE works well with any antibody (best if you post fix the sample prior to mounting).
  - However, **it does not work with reagents that can diffuse away** - e.g. phalloidin, To-Pro3, etc.

Quick Guide by Wernher Fouquet

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

by Leica Microsystems

Science Lab

TOPICS AUTHORS INSTITUTIONS EVENTS & WEBINARS CONTACT SEARCH

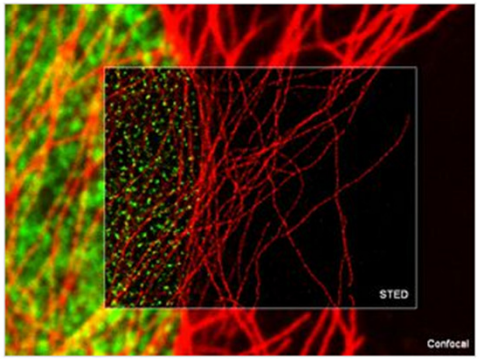
Quick Guide to STED Sample Preparation

Wernher Fouquet, Ph.D.

Leica Microsystems

November 14, 2012

This guide is intended as a quick reference guide for the most common questions regarding the preparation of samples for the Leica SP8 TCS STED, by briefly explaining the theoretical basics required from **samples for stimulated emission depletion**. It provides information about the most common **immunofluorescence labeling techniques**, **working mounting media**, **basic quality optimization procedures** and **experiment designs**. It also contains a detailed list of reagents, antibodies and disposables frequently and successfully used in super-resolution STED microscopy. In summary, this guide is meant to present the necessary knowledge, including some tips and tricks, to users that are preparing their first super-resolution microscopy sample.



Topics

Super-Resolution

Tagged as

Super-Resolution

STED

gSTED

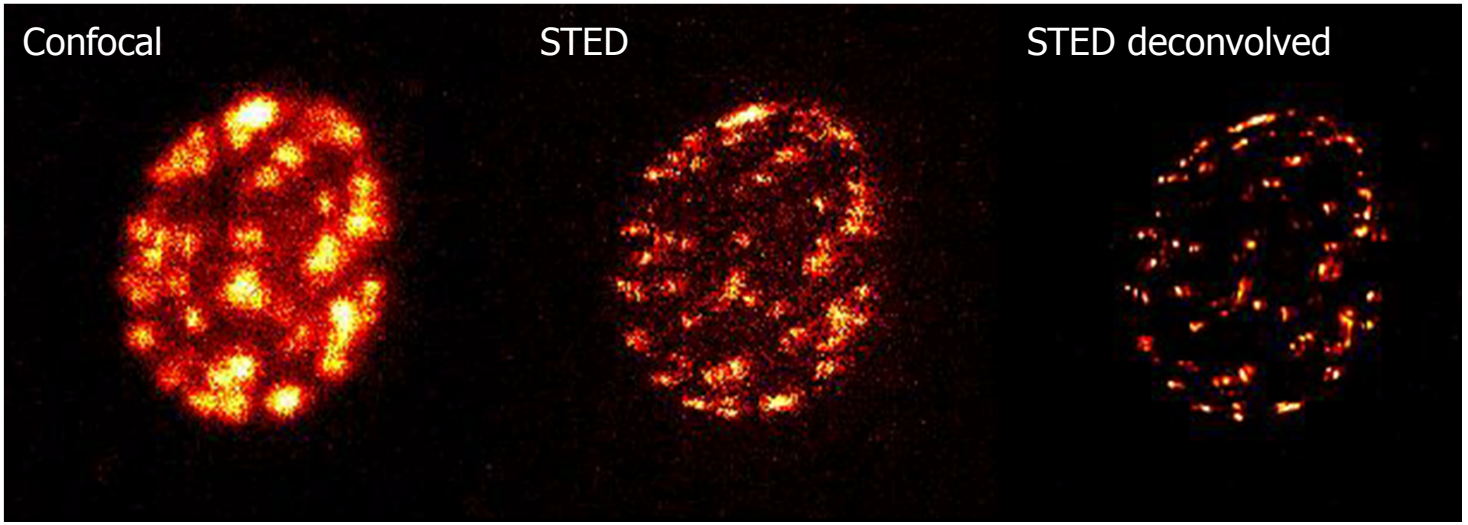
Sample Preparation

more

STED Deconvolution

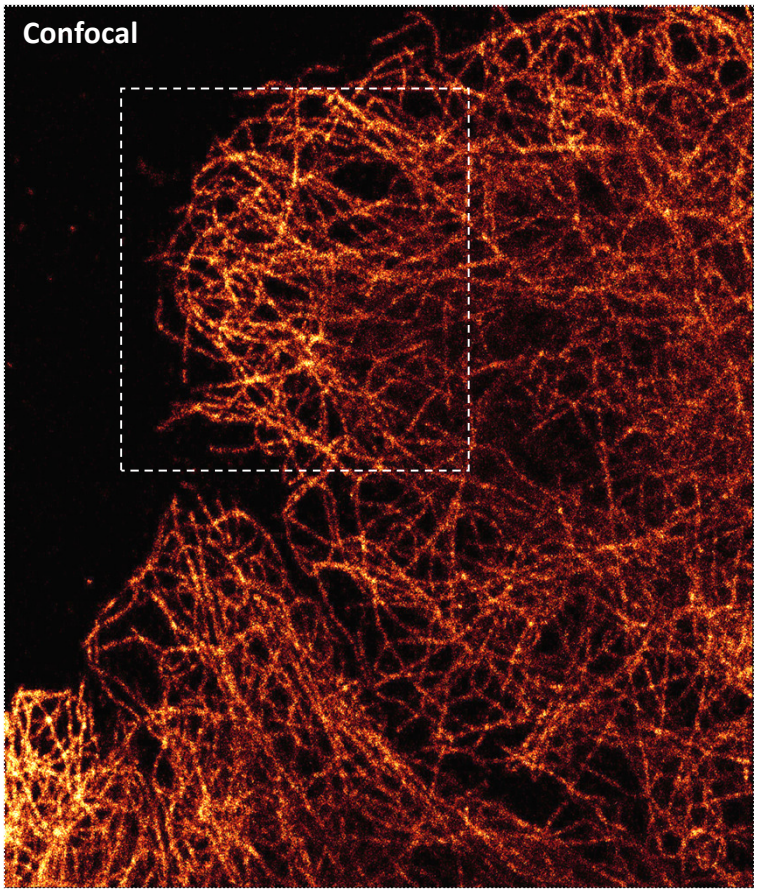
Deconvolution

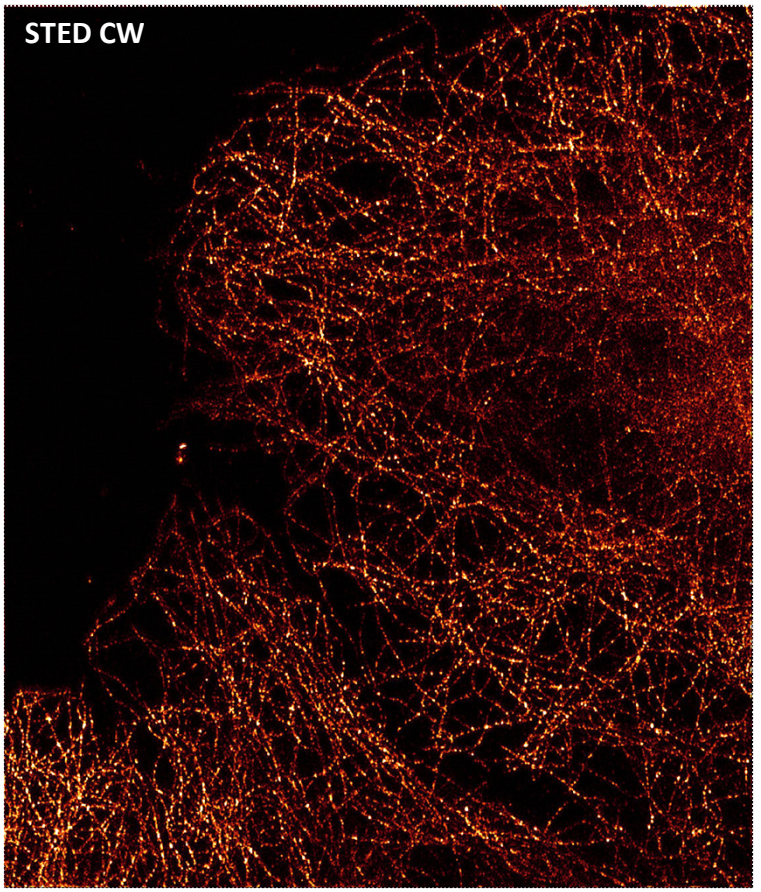
- STED Deconvolution
  - Note that deconvolution is a drastic post processing step.
  - To avoid artifacts, it should be carefully used, and the results should always be confirmed by comparing them with the original data.
  - This applies for STED data as well as for work with all other microscopic images



Courtesy of Marko KaksonenEMBL, Heidelberg, 3D projections

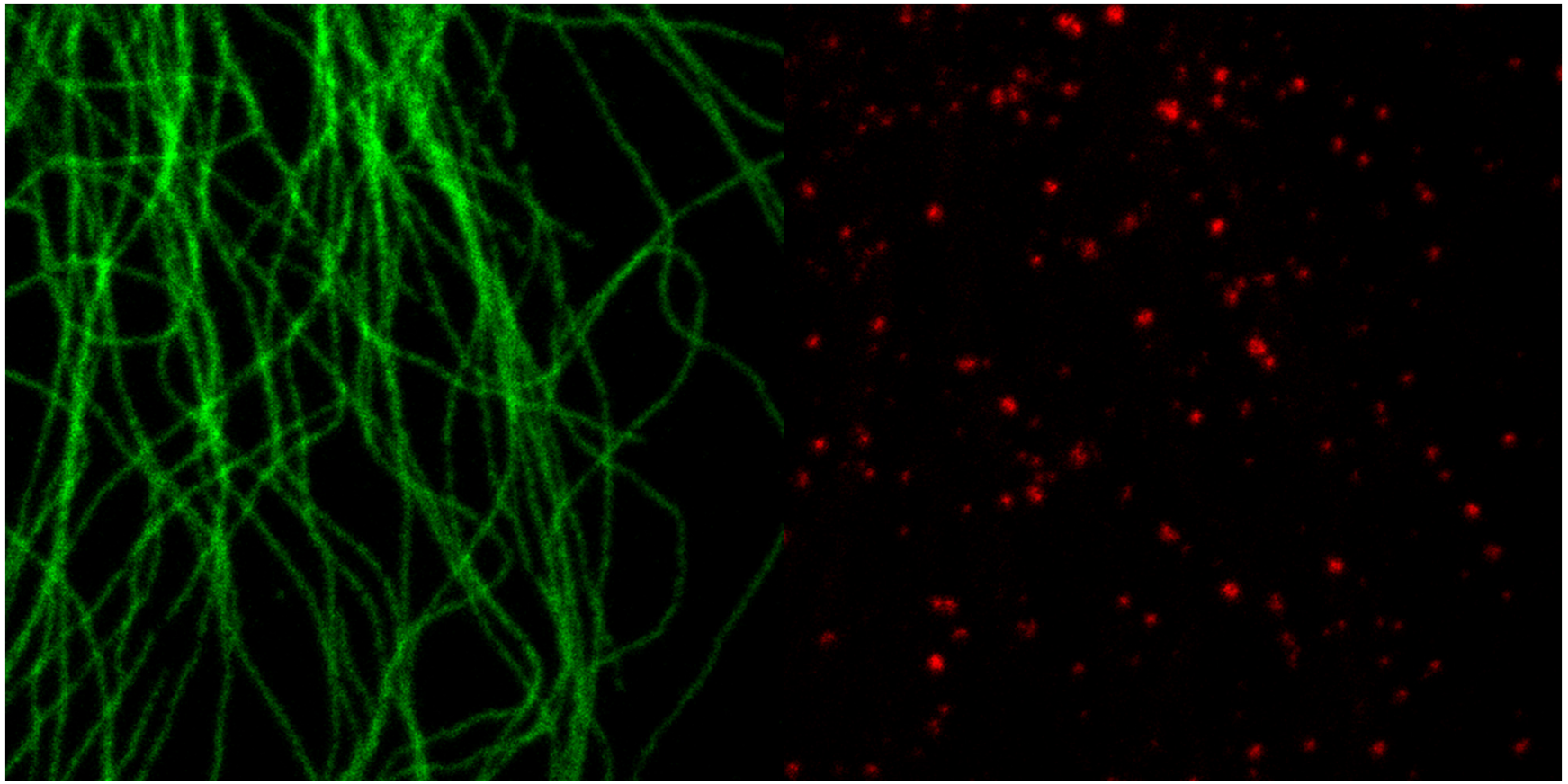
STED CW, 1 color – Deconvolved





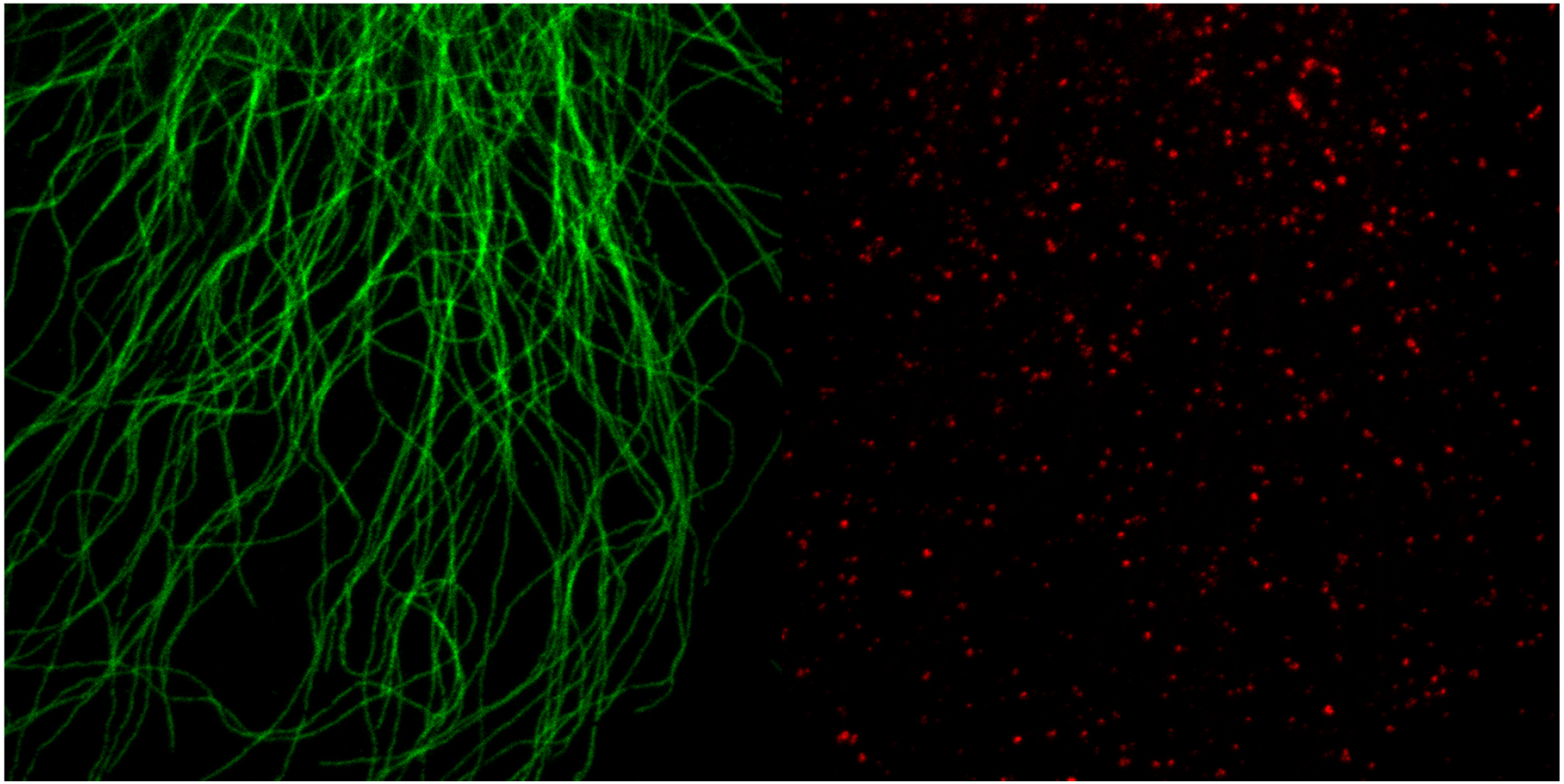
Courtesy: Myriam Gastard, Leica Microsystems, Inc
DyLight 488

Multicolor Imaging, g-STED – Raw Data



Courtesy: Rebecca Medda, University Göttingen
PTK cells: tubulin, V500; red: Clathrin, Oregon Green 488

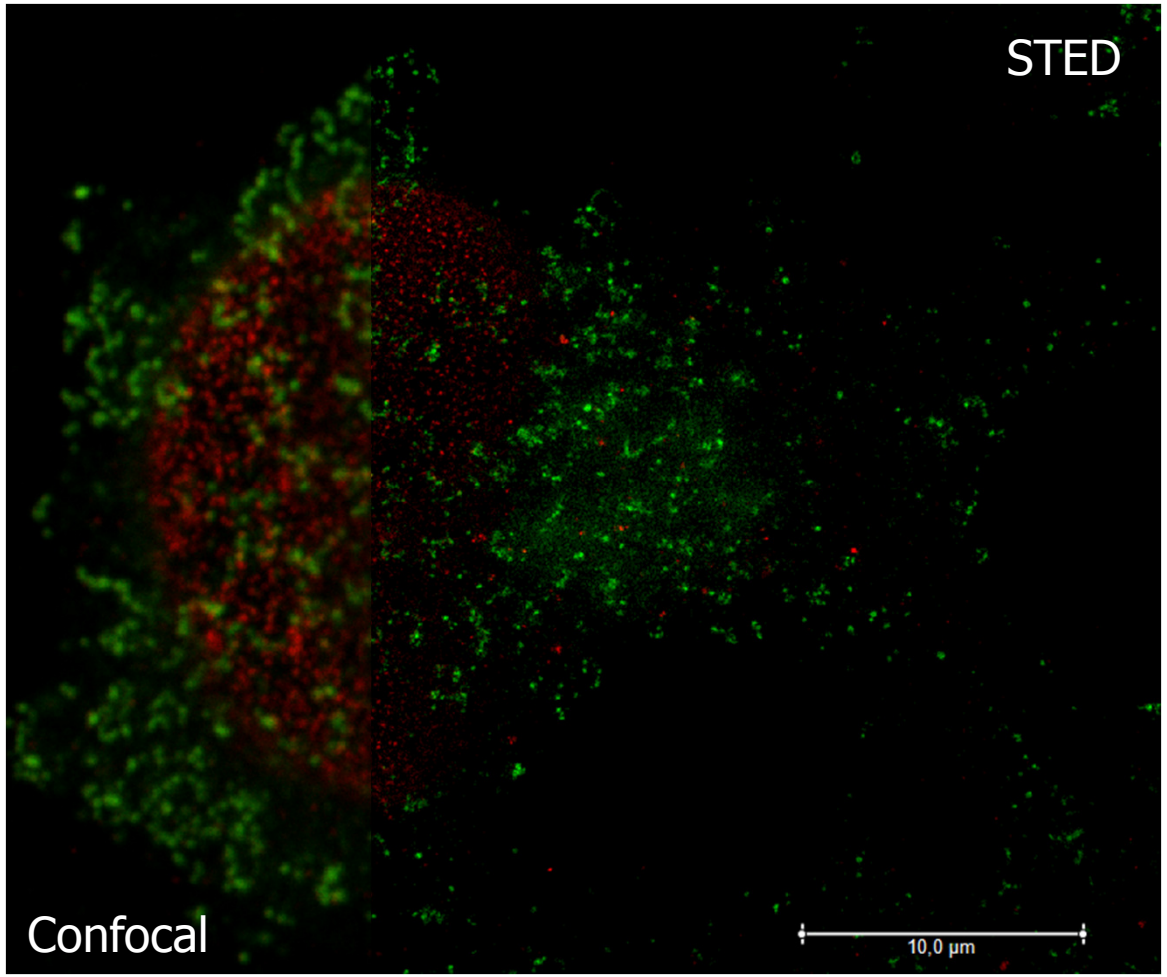
Multicolor Imaging, g-STED – Deconvolved



Courtesy: Rebecca Medda, University Göttingen
PTK cells: tubulin, V500; red: Clathrin, Oregon Green 488

Dual color gated STED with 592 – Blur filtered

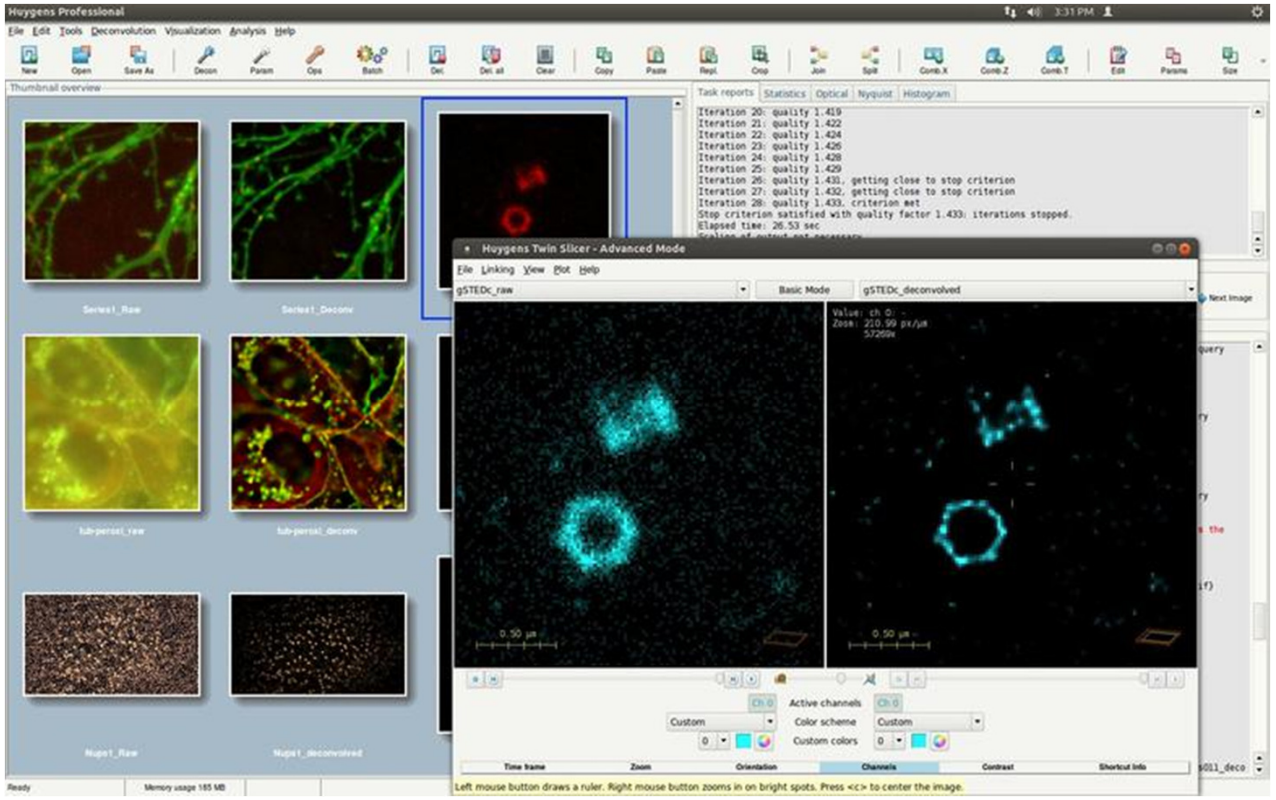
Cell line: HeLa
NUP153 Alexa 532
Clathrin TMR-Oregon Green
HeLa cells



Courtesy: Mannheim, Germany
Pixel size: 19,27 x 19,27 nm
STED power: 100% (660)
gate start: 0,8 - 1,5 ns

Huygens STED Deconvolution

- http://www.youtube.com/watch?v=8A7v3LlUCjk
- http://www.leica-microsystems.com/science-lab/huygens-sted-deconvolution-quick-guide/



Thank you!

Living up to Life

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