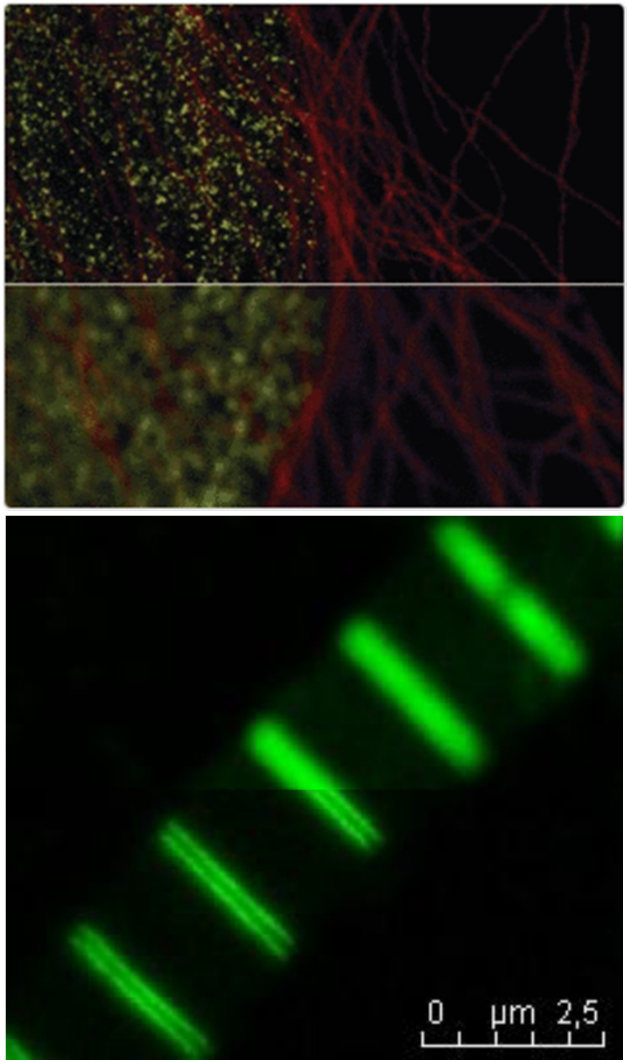


STED 3X Sample Preparation

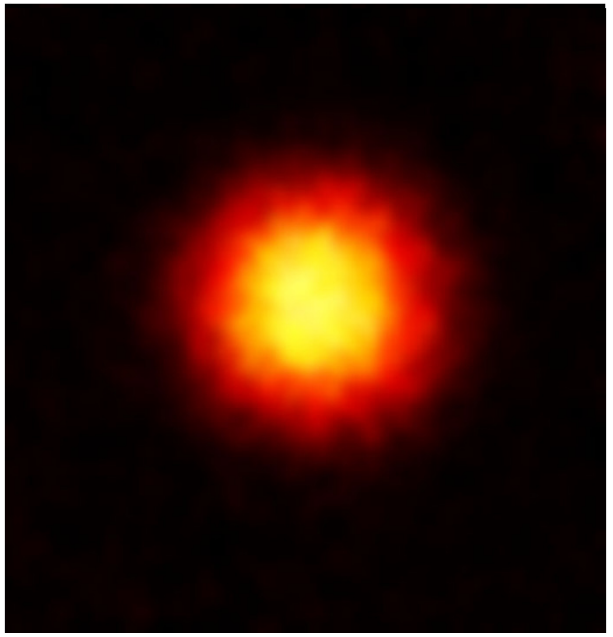
Louise Bertrand

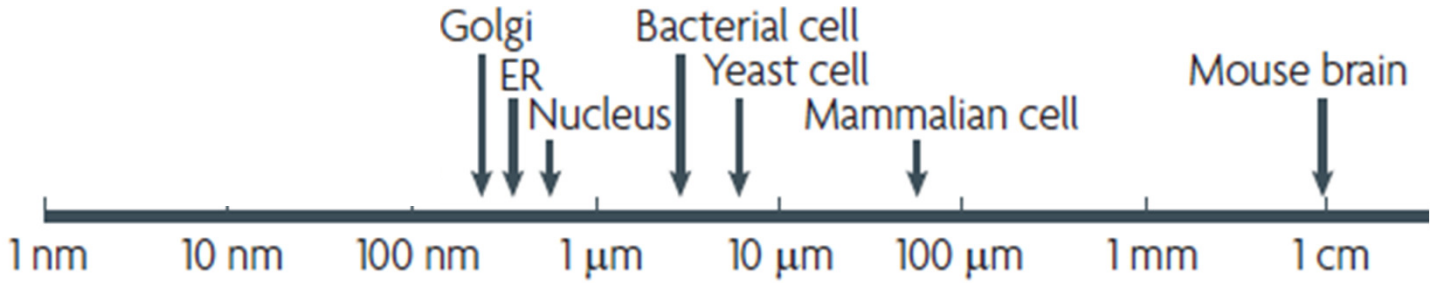
Product Specialist

Application, Technology, and Training Center

Why is Super Resolution

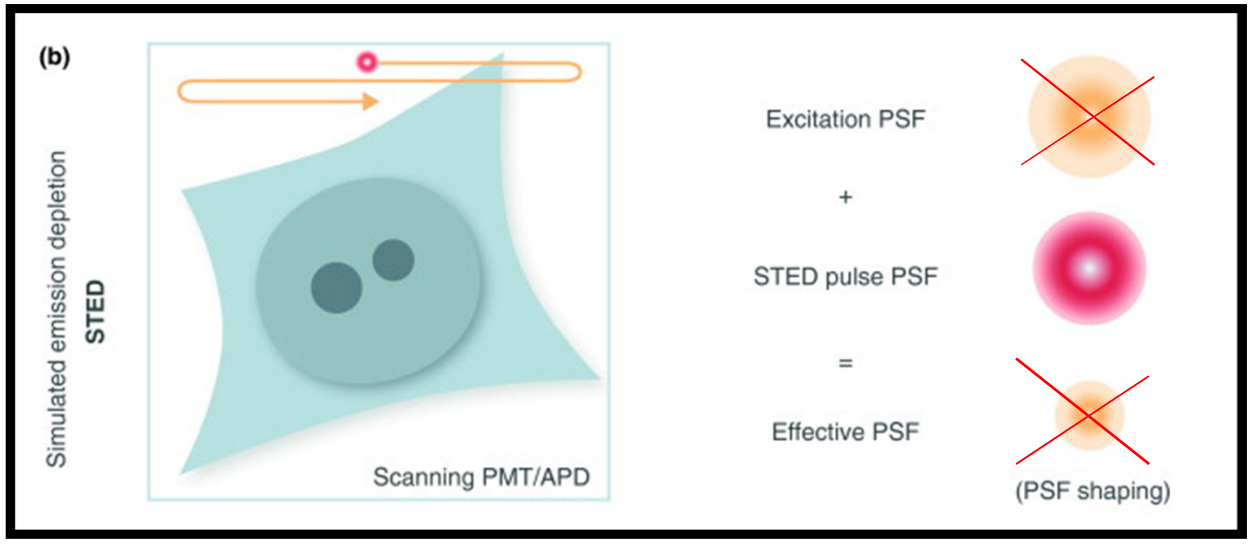
- To study details:
  - beyond the diffraction limit
  - with standard dyes/FPs
  - in 2 colors or more colors!! See quick guide.
  - inside cells/organisms
  - live
  - with high throughput
  - on fully integrated systems





STED Microscopy – The concept

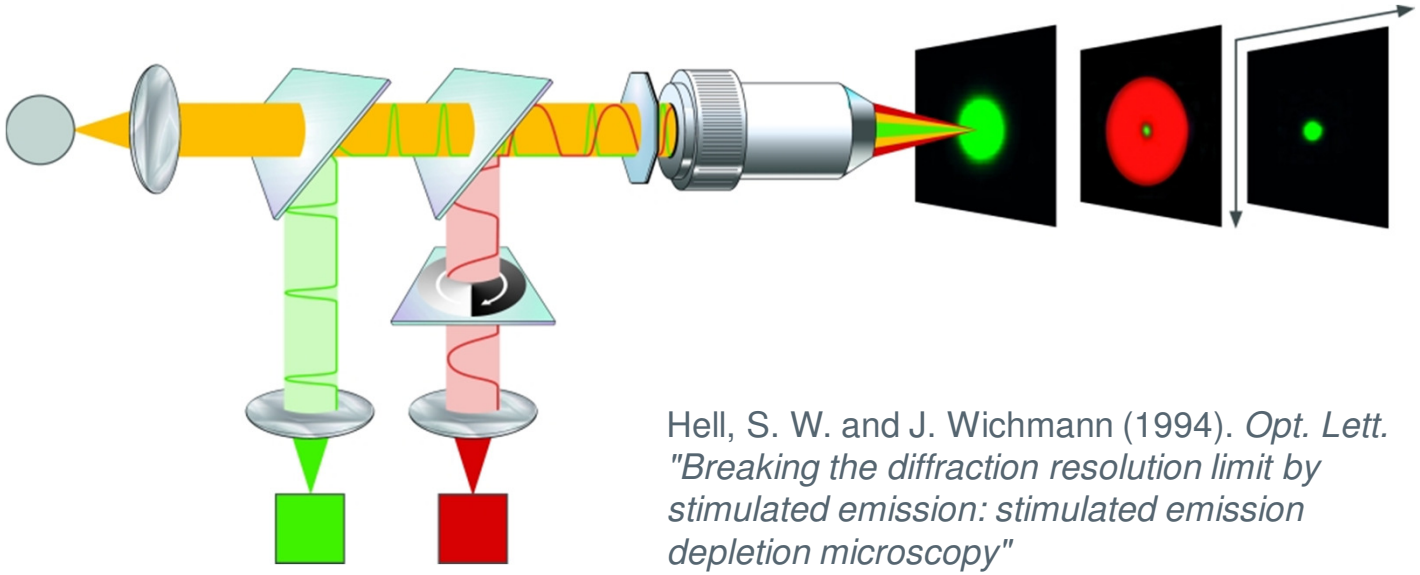
- In STED, you take standard confocal microscopy and introduced a technique to reduce the emitted spot.
  - Shrinking the PSF of the microscope
  - Depleting the fluorescence emission in the outer areas of the diffraction limited spot via a process called stimulated emission.



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)
  - Second, induced/forced fluorescence (stimulated emission)
  - Creating the doughnut shape fluorescence depletion
- Excitation and STED doughnut are perfectly overlaid in the focus



Hell, S. W. and J. Wichmann (1994). *Opt. Lett.* *"Breaking the diffraction resolution limit by stimulated emission: stimulated emission depletion microscopy"*

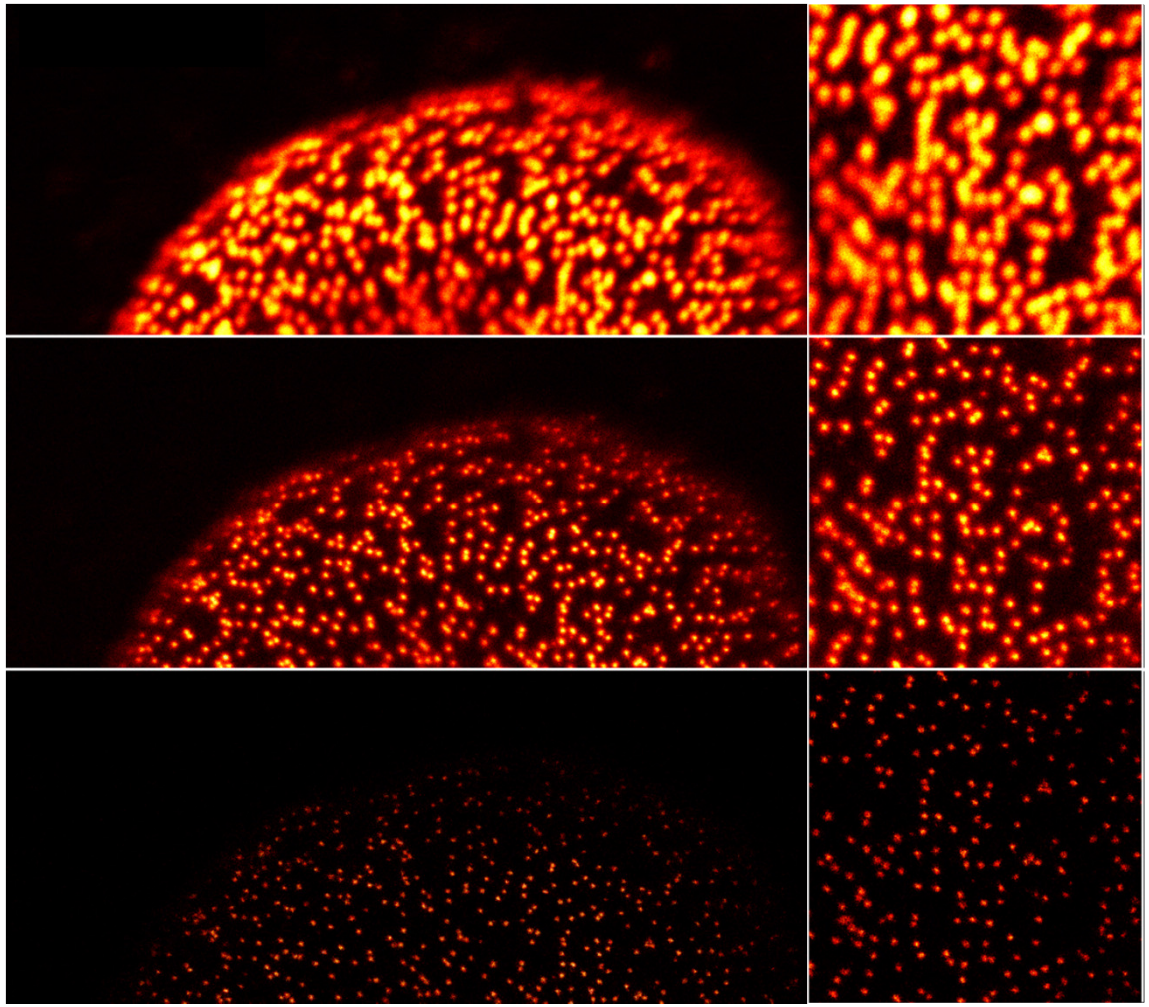
g-STED for more details

Sub 50nm resolution specified!

Confocal

STED CW

Gated STED



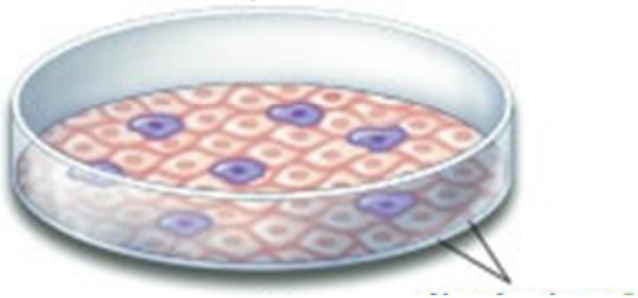
IF: Nuclear Pore Complex; Alexa 488

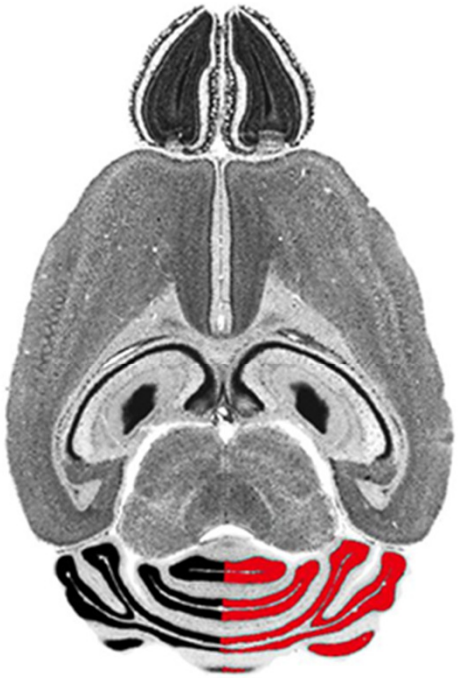
STED CW and gated STED - Summary

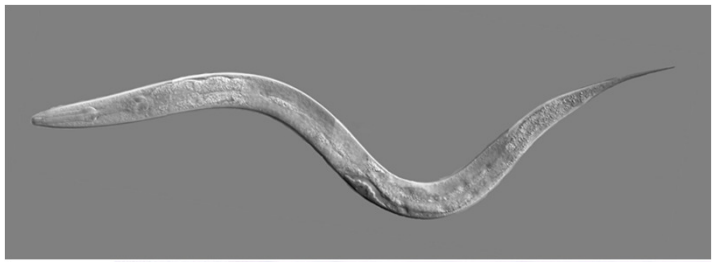
- Super-resolution method based on fluorescence confocal imaging, in which images are acquired by scanning a focused light spot over a ROI and collecting fluorescence sequentially pixel by pixel.
- Main Strengths:
  - Pure physical resolution improvement without any additional post-processing below 50nm.
  - Intrinsic optical sectioning enabling the acquisition of planes of roughly 500 nm, 3-dimensional structures, even several tens of microns deep inside the tissue.
  - Axial resolution improvement of ~120nm by using the 3D donut.
  - Fast image acquisition of several images per second.
  - Live-imaging capabilities by using either fluorescent proteins or other fluorescent tags.
  - Support for a wide range of fluorescent dyes enabled by the choice of two different STED depletion lasers for different emission ranges (592nm -green-; 660nm -yellow/orange-)

STED CW and g-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*).





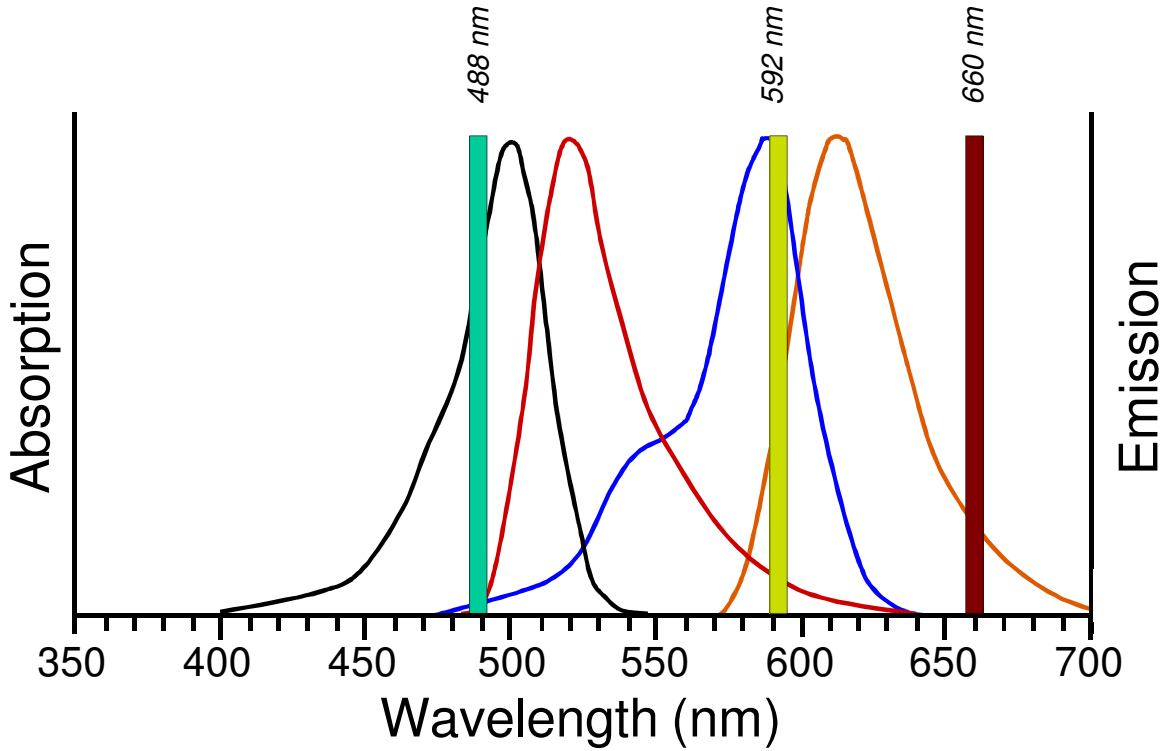


STED CW and gated STED - Choice of samples

- 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, within 20µm range for optimal performance.
  - To achieve the best results, the refractive index of the mounting medium should match the index of the immersion used (immersion liquid = 1.518)
  - Auto-fluorescence, sudden and unpredicted changes of the refractive index that may influence the shape of the focal spot and consequently the performance of the microscope. If experience with clearing solutions is available, it might be worth testing here.
  - 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 with strong light at a wavelength of 592 nm or 660 nm. It is of crucial importance that the sample is not absorbing light at this wavelength.

Choice of Fluorescence Dyes

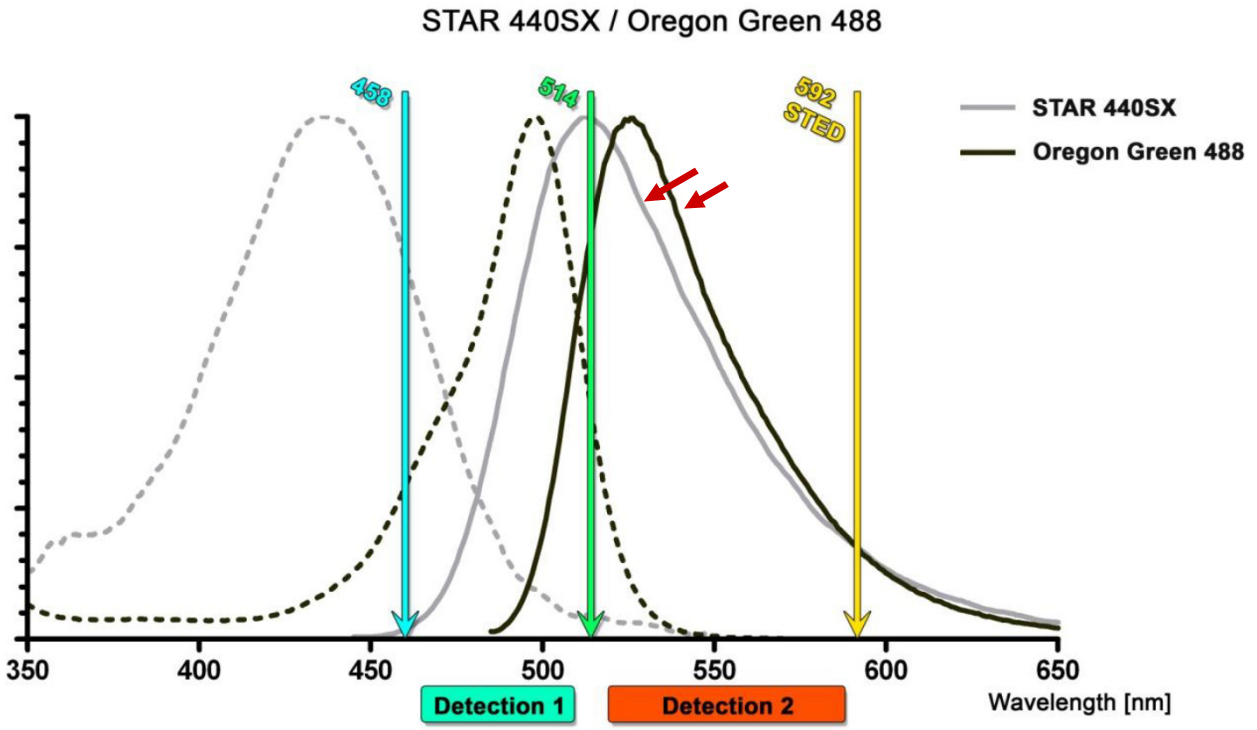
- Excitation lines
  - 458 nm
  - 488 nm — Argon laser
  - 514 nm
  - WLL: 470 to 520 nm
  - WLL: 520 to 580 nm
- Detection range
  - ~ 465 to 585 nm
  - ~ 585 to 650 nm
- Depletion line
  - 592 nm fiber laser
  - 660 nm fiber laser



No excitation @ depletion wavelength

Choice of Fluorescence Dyes

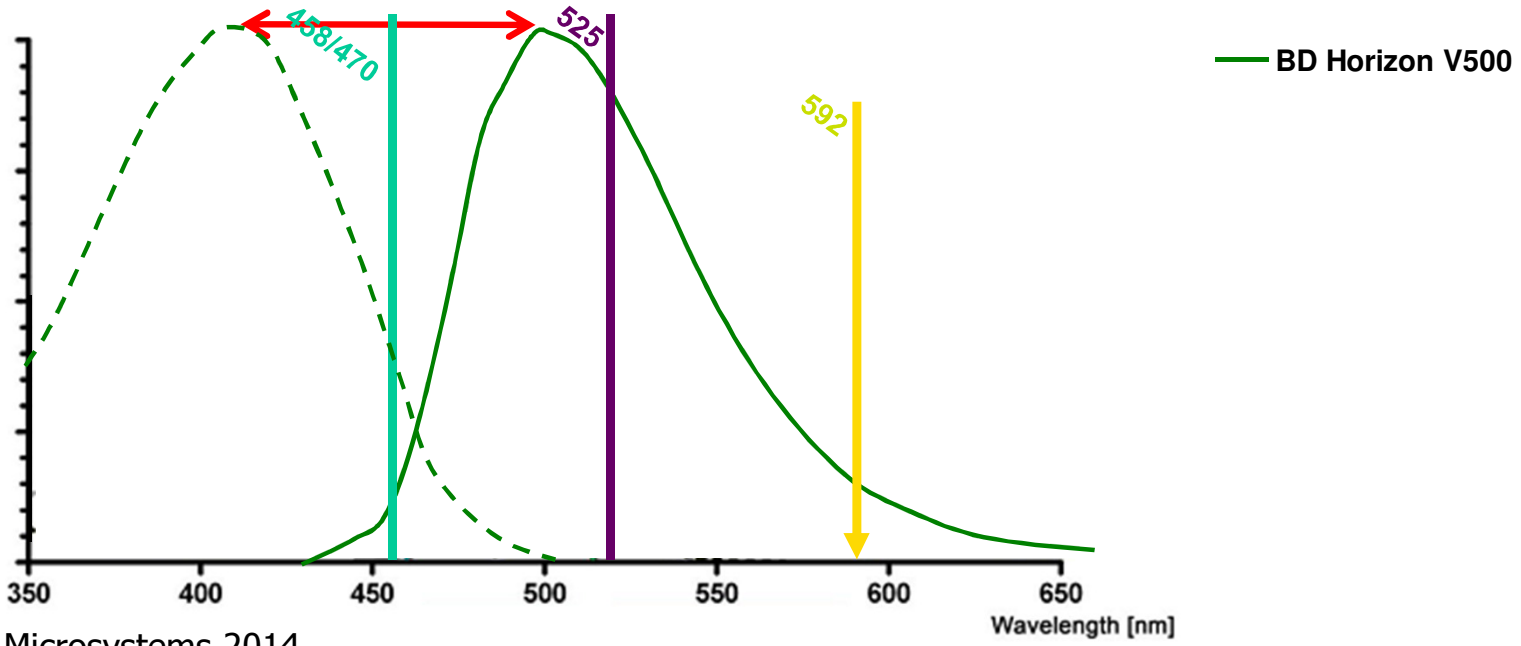
- For STED to work efficiently, the emission spectra of both dyes need to show significant emission at the STED wavelength.



Dye1 Name	Excitation	Detection	Dye2 Name	Excitation	Detection
BD Horizon V500	458/470	475 - 510	Oregon Green 488/ Chromeo 505	514/520	523 - 580
Abberior STAR 440SX	458/470	475 - 515	Oregon Green 488/ Chormeo 505	514/520	523 - 580

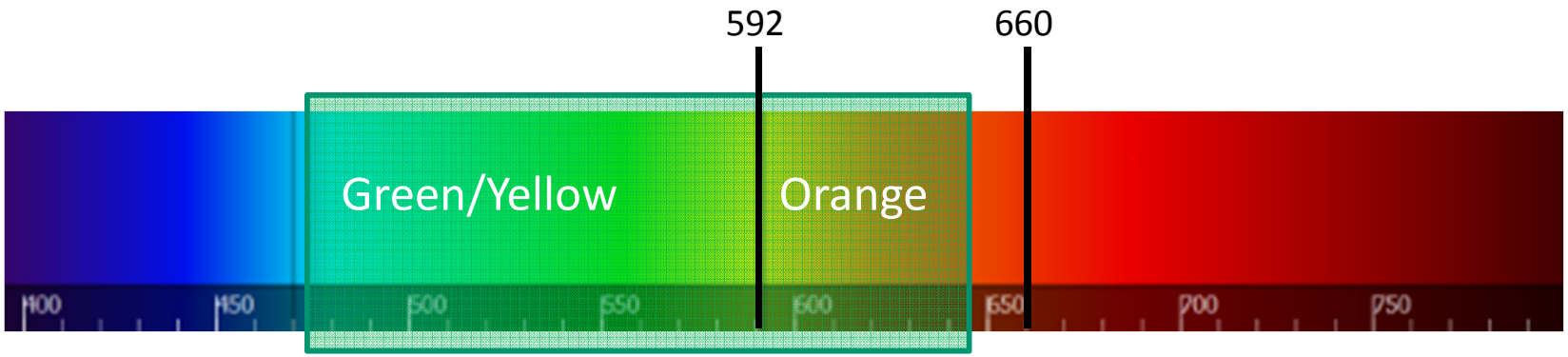
Choice of Fluorescence Dyes

- 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.



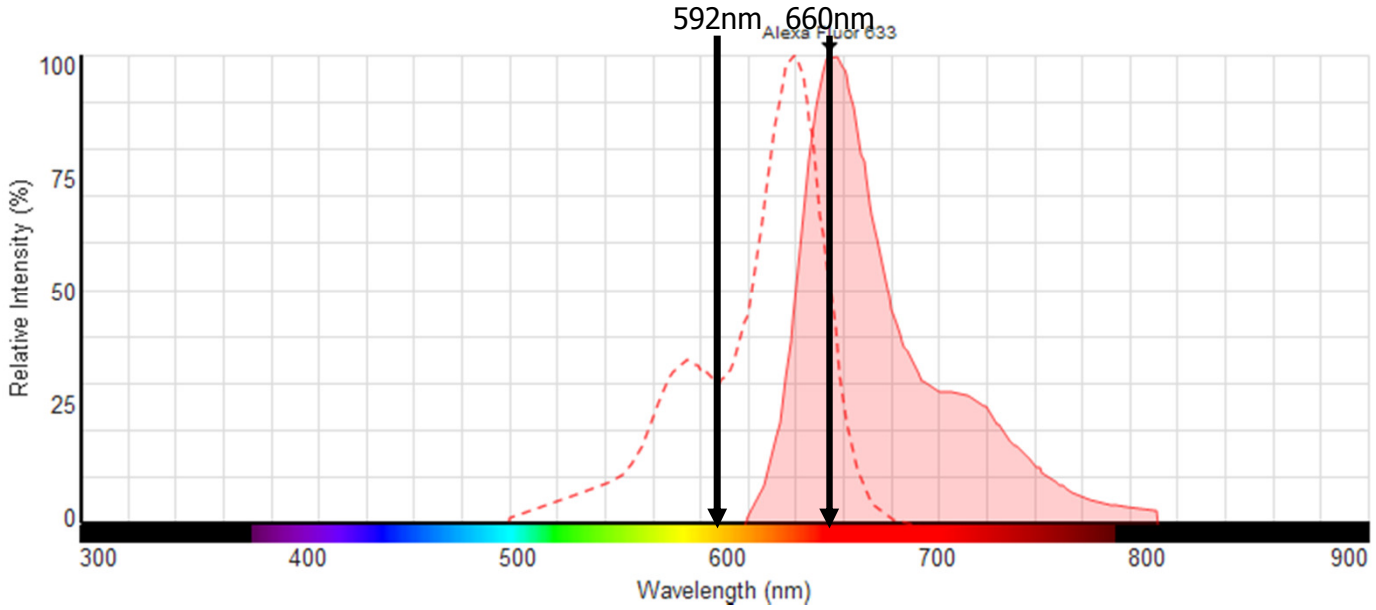
Choice of Fluorescence Dyes

- 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 the orange



Choice of Fluorescence Dyes

- More dyes could be use for more colors.
- 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), that they will absorb the strong STED depletion light and get bleached
  - Thus all reference images need to be acquired *before* the STED images.



Some working dyes for STED

Fluorophore	Excitation (nm)	Depletion (nm)	Provider	Cat. Number
Biotinylated Antibody	-	-	Jackson Immunoresearch	115-065-003 (mouse) 111-065-003 (rabbit)
BD Horizon V500**	458 / 470	592	Beckton & Dickinson	561419 (Streptavidin)
Abberior STAR 440SX**	458 / 470	592	Abberior	2-0002-003-7 (mouse) 2-0012-003-4 (rabbit)
ATTO 488	488	592	Sigma-Aldrich	62197 (mouse) 18772 (rabbit)
Abberior STAR 488*	488	592	Abberior	2-0002-006-8 (mouse) 2-0012-006-5 (rabbit)
Alexa Fluor 488*	488	592	life technologies	A11001 (mouse) A11008 (rabbit)
Chromeo 488	488	592	Active Motif	15051 (mouse) 15061 (rabbit)
FITC	488	592	Sigma-Aldrich	F0257 (mouse) F0382 (rabbit)

Some working dyes for STED

Fluorophore	Excitation (nm)	Depletion (nm)	Provider	Cat. Number
DyLight 488	488	592	Thermo Scientific	35502 (mouse) 35552 (rabbit)
Chromeo 505**	488 / 514	592	Active Motif	15050 (mouse) 15060 (rabbit)
Oregon Green 488**	488 / 514	592	life technologies	O6380 (mouse) O6381 (rabbit)
Abberior STAR 470SX**	470	660	Abberior	2-0002-004-4 (mouse) 2-0012-004-1 (rabbit)
Alexa Fluor 514**+	514	592/660	live technologies	A31555 (mouse) A31558 (rabbit)
Alexa Fluor 532**+	532	592/660	life technologies	A11002 (mouse) A11009 (rabbit)
Alexa Fluor 546**	546	660	life technologies	A11030 (mouse) A11035 (rabbit)

Some working dyes for STED

Fluorophore	Excitation (nm)	Depletion (nm)	Provider	Cat. Number
Tetramethylrhodamine/ TRITC**	554	660	life technologies	A16071 (mouse) T2769 (rabbit)
Alexa Fluor 555**	555	660	life technologies	A21424 (mouse) A21429 (rabbit)
CF 555*	550	660	Biotium Inc.	20031 (mouse) 20232 (rabbit)
Alexa Fluor 568**	568	660	life technologies	A11004 (mouse) A11011 (rabbit)
Alexa Fluor 594**ǂ	594	660	life technologies	A11032 (mouse) A11037 (rabbit)

* recommended dyes for single color experiments;
** recommended dyes for single, dual and triple color experiments
ǂ might require advanced imaging parameter settings due to higher anti-Stroke's excitation, e.g. small pinhole, low STED laser power and high gate start values.

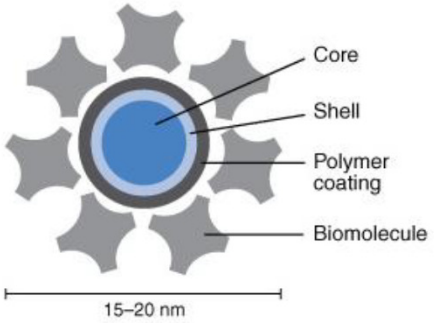
Living cell STED imaging

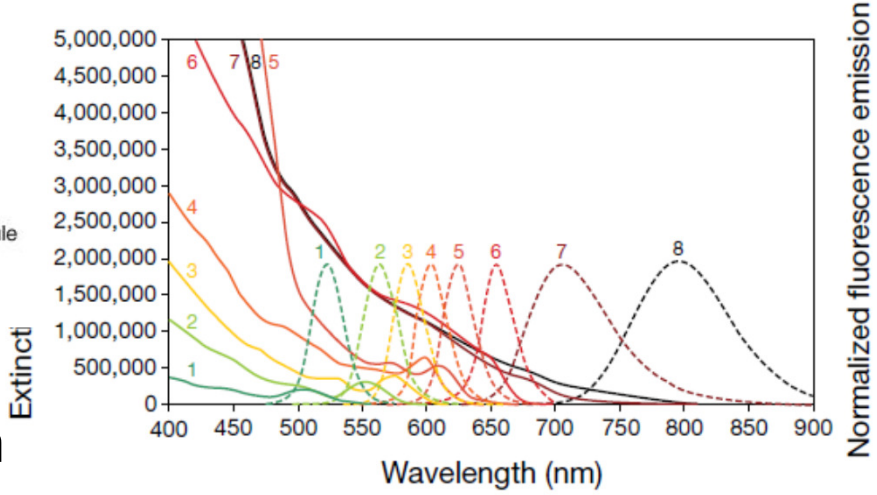
Fluorescent proteins	Excitation (nm)	Depletion (nm)
eGFP	484 (488)	592
EmGFP	487 (488)	592
eYFP	514 (514)	592 / 660
Venus	515 (514)	592 / 660
mCitrine	516 (514)	592 / 660
dsRed	558	660
mStrawberry	574	660

Other probes		
Tubulin Tracker Green	488 / 514	592
BABTA	488 / 514	592
Lifeact (Actin marker)	488 / 514 (483 / 506)	592

Nanocrystals (Quantum Dots - eFluor)

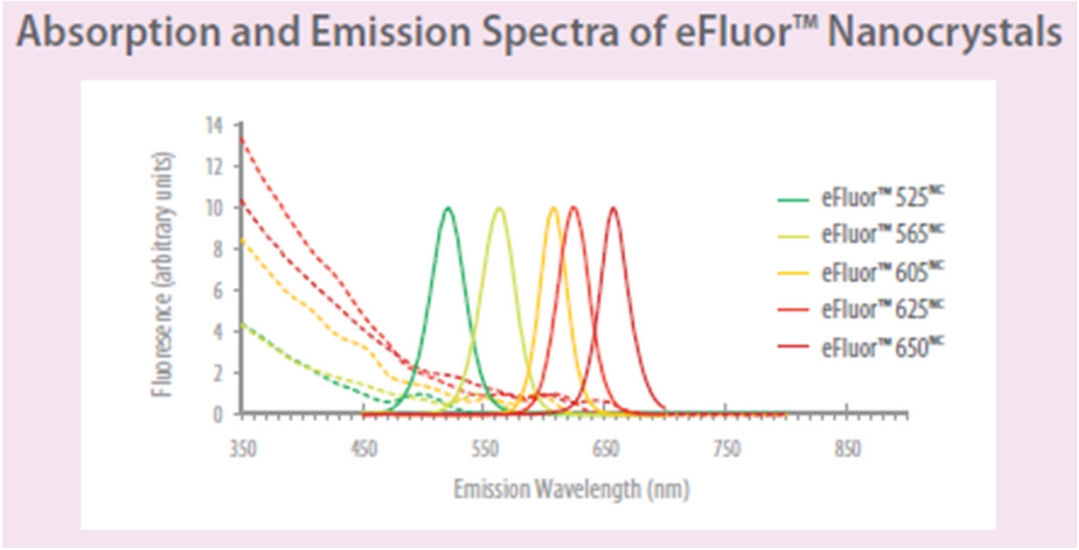
- Emission Spectrum
  - Size & composition
  - FWHM 25
- High photostability
- Large extinction coefficients
  - 130.000 – 2.900.000 $M^{-1}cm^{-1}$ @ 488nm
  - Fluorescein: 80.000 $M^{-1}cm^{-1}$
- Excitation @ 592nm 3-9% of maximum



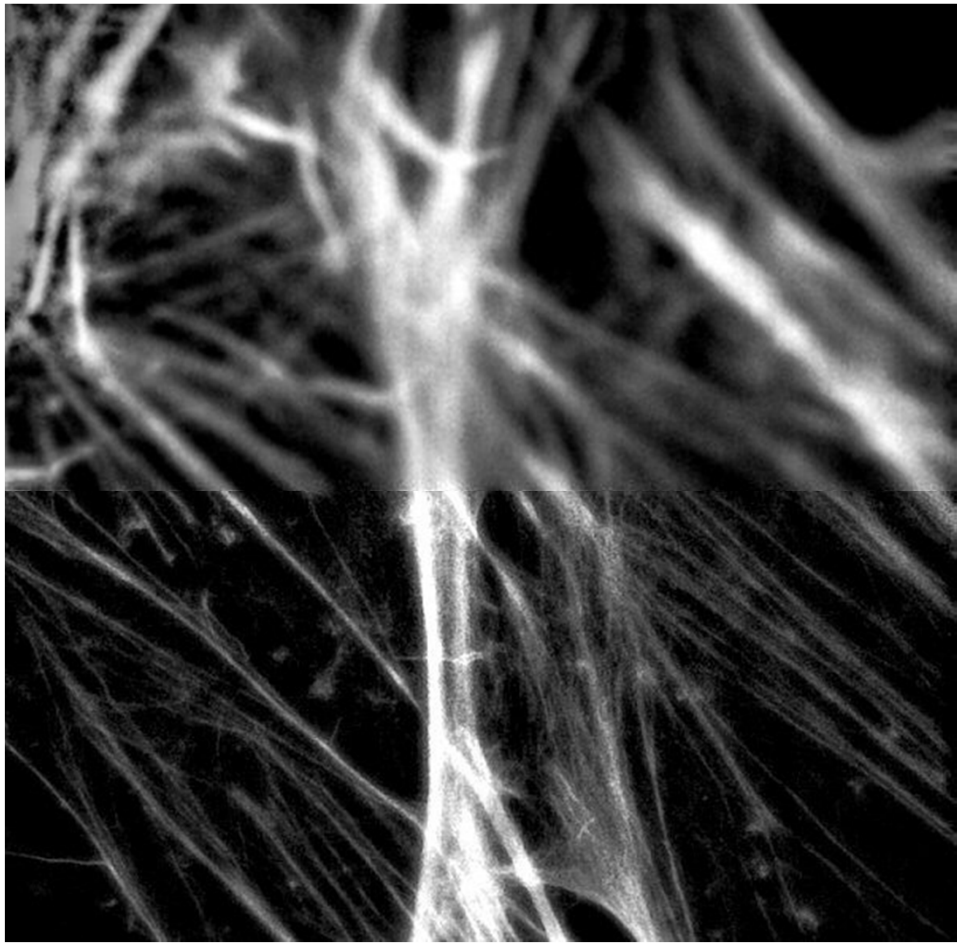


Not a too good idea because they have a very narrow emission and broad absorption spectrum.

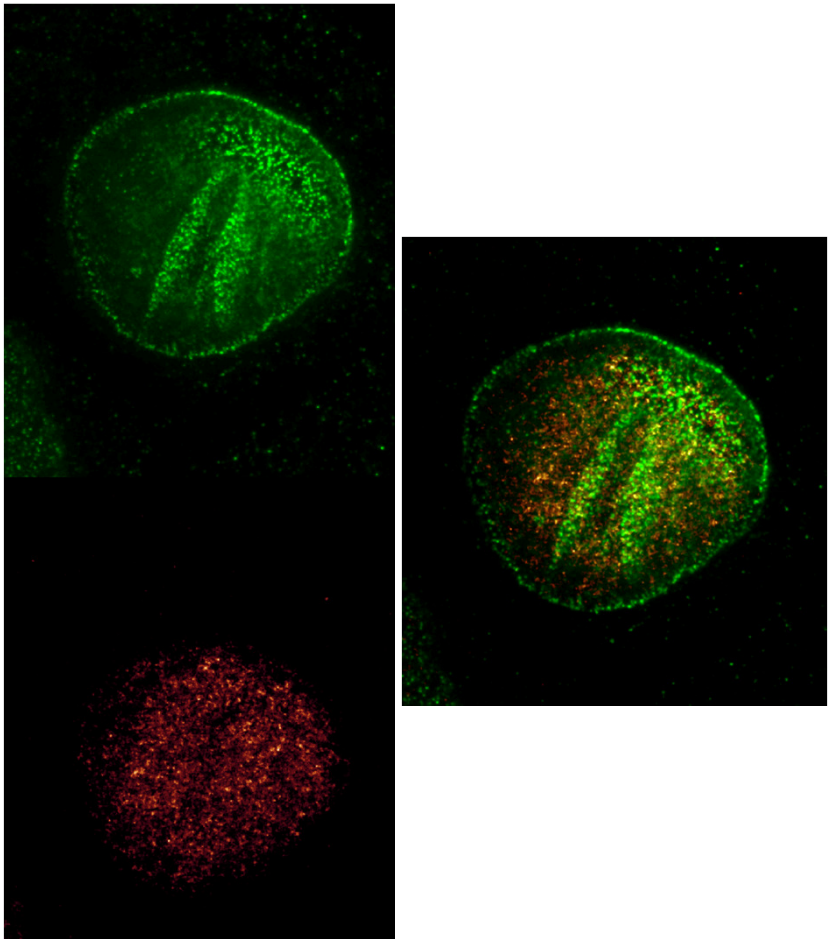
Up to now, they never worked



2 colors gated STED and STED CW



ACTIN



NUP + Ezh2

STED CW and gated STED – Secondary Antibodies

- Single color
  - DyLight 488 or 514: Highest resolution, Bright, Very good
  - Chromeo 488 or 505: Bright
  - Oregon green 488 or 514: Very bright, careful with precipitates
  - AlexaFluor 488 or 514: Not as bright or stable
  - Atto 488 or 514: Bright, careful with precipitates

- Double colors; dyes below in combination with single color dyes above
  - Abberior STAR 440SX (g-STED and STED CW)
  - Atto 425 antibody or Streptavidin conjugated (STED CW)
  - BD Horizon V500, Streptavidin conjugated (g-STED and STED CW)
  - Pacific Orange, antibody conjugated (STED CW)

STED CW and gated STED – Secondary Antibodies

- Triple colors; dyes below in combination double color matched dyes on the previous page
  - Abberior STAR 470SX
  - AlexaFluor 532, *(Alexa Fluor 546, Alexa Fluor 555)*
  - Tetramethylrhodamine (TAMRA - TRITC)
  - Alexa Fluor 568
  - Alexa Fluor 594 – Absorption end tail end at depletion laser (660) *(Work well with the 660nm depletion laser)*

STED CW and gated STED – Recommended pair

Channel 1		Channel 2	Comment
Pacific Orange (Secondary Antibodies conjugated) (STED CW)	+	DyLight488 / Alexa488 (excluding Oregon Green 488 and Chromeo 505)	Dye separation required
Atto 425 (STED CW) or Abberiror 440 SX (g-STED and STED CW)	+	DyLight 488, Chromeo 505, Oregon Green 488, Alexa 488	No dye separation required. (recommended)
V500 (Streptavidin) (g-STED and STED CW)	+	Oregon Green 488, Chromeo 505, DyLight 488.Any other green dye reported to work for STED CW.	No dye separation required (recommended)
Alexa Fluor 532 (gated STED and	+	Tetramethylrhodamine / Alexa Fluor 568	No dye separation required (recommended)
Chromeo 505 (gated STED and STED CW)	+	Tetramethylrhodamine / Alexa Fluor 568	No dye separation required (recommended)

Note: The resolution of ATTO 425 is ~100 nm, even in gated STED. ATTO 425 = 100 nm, STAR 440 = 50 nm.

STED CW and gated STED – Recommended pair

- Recommended concentration
- Channel 1:
  - BD Horizon V500 Streptavidin (1:50) *(BD #561419)* + Biotin α-mouse or Biotin α-rabbit (1:100*)* *(Jackson ImmunoResearch 115-065-003 or 111-065-003)*
  - STAR 440SX Goat α-mouse or α-rabbit, 1:50
    (*Abberior, 2-0002-003-7 and 2-0012-003-4)*
  - Pacific Orange (1:100) *(Life Technologies)* α-mouse *(P-31585)* or α-rabbit *(P-31584)*
  - Atto 425 Goat α-mouse or α-rabbit (0.2µg/ml)
    (*Rockland Immuno*)

Abberrior dyes now offered by Sigma as **NHS** (labeling of amino groups in proteins) or **maleimid** (labeling of thiol groups in proteins) activated

http://www.sigmaaldrich.com/life-science/cell-biology/detection/abberior-dyes.html

STED CW and gated STED – Recommended pair

- Recommended concentration
- Channel 2:
  - Oregon Green 488 (1:100) *(Life Technologies)* Goat α-mouse *(O-11033)* or α-rabbit *(O-11038)*
  - Alexa Fluor 488 (1:200 to 1:100) *(Life Technologies)* Goat α-mouse *(A-11001)* or α-rabbit *(A-11008)*
  - DyLight 488 (0.1 to 0.2µg/ml,) Goat α-mouse *(Rockland Immuno #610-141-121)* and α-rabbit *(Rockland Immuno #610-141- 122)*
  - Chromeo 488 (1:100 to 1:200) *(Active Motif)* α-mouse *(15051)* or α-rabbit *(15061)*
  - Atto 488 (1:200) Goat α-mouse and α-rabbit (0.2µg/ml) (*Rockland Immuno*)

Note: It is recommended to centrifuge the secondary antibody before usage so precipitation can stay in the bottom of the vial

STED CW and gated STED – Recommended pair

- Recommended concentration
- Channel 2 or 3:
  - Chromeo 505 (1:100 – 1:200) *(Active Motif)* α-mouse *(15050)* or α-rabbit *(15060)*
  - Alexa Fluor 532 (1:100) *(Life Technologies)* Goat α-mouse *(A-11002)* or α-rabbit *(A-11009)*
  - Alexa Fluor 546 (1:100) *(Life Technologies)* Goat α-mouse *(A-11003)* or α-rabbit *(A-11010)*
  - Tetramethylrhodamine (TRITC) (1:100) *(Life Technologies)* Goat α-mouse *(T-2762)* or α-rabbit *(T-2769)*
  - Alexa Fluor 555? (1:100) *(Life Technologies)* Goat α-mouse *(A-11031)* or α-rabbit *(A-11011)* *or* α-chicken *(A-11041)*

Note: It is always better to start with higher concentrations of antibodies during the first tries, to rule out some issues with labeling density

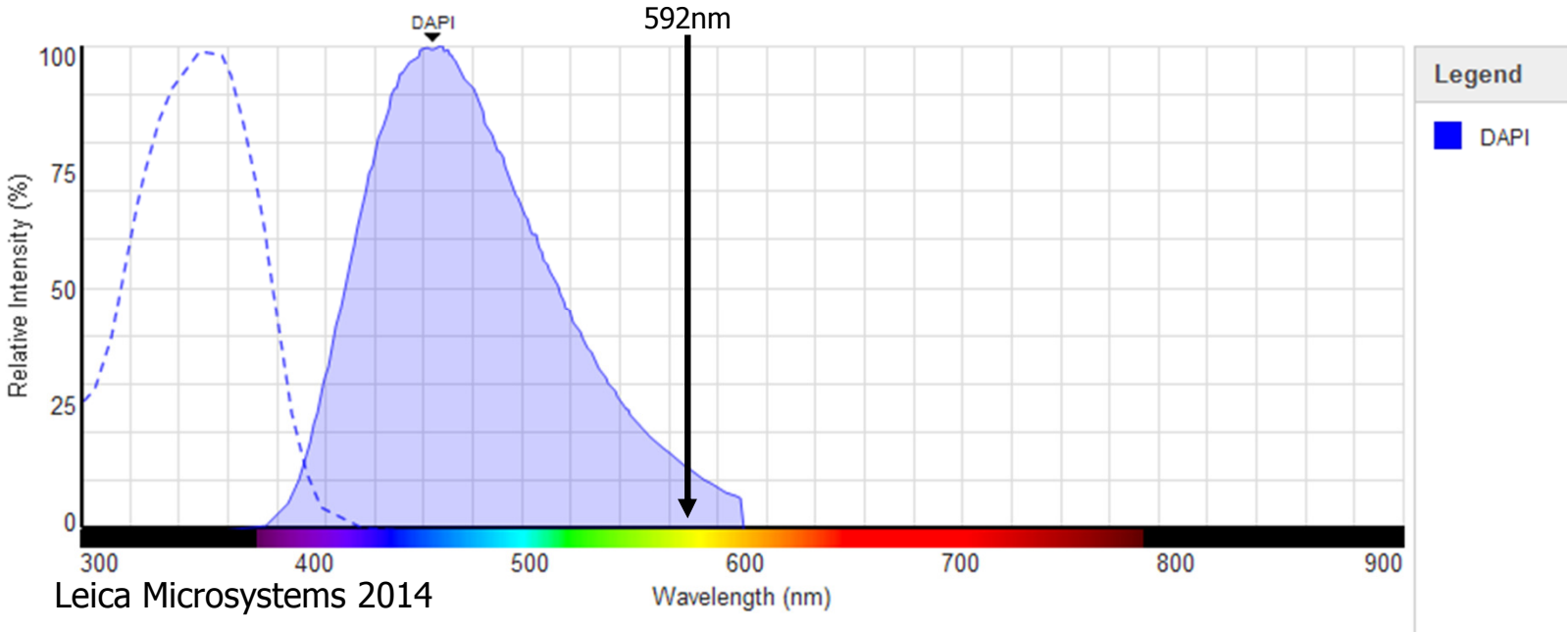
STED CW and gated STED – Fluorescent Proteins

Channel 1 (458 ex)	Channel 2 (514 ex)
mCerulean	mCitrin, mVenus
eGFP, Emerald (EmGFP)	dsRed, mRFP or mStrawberry

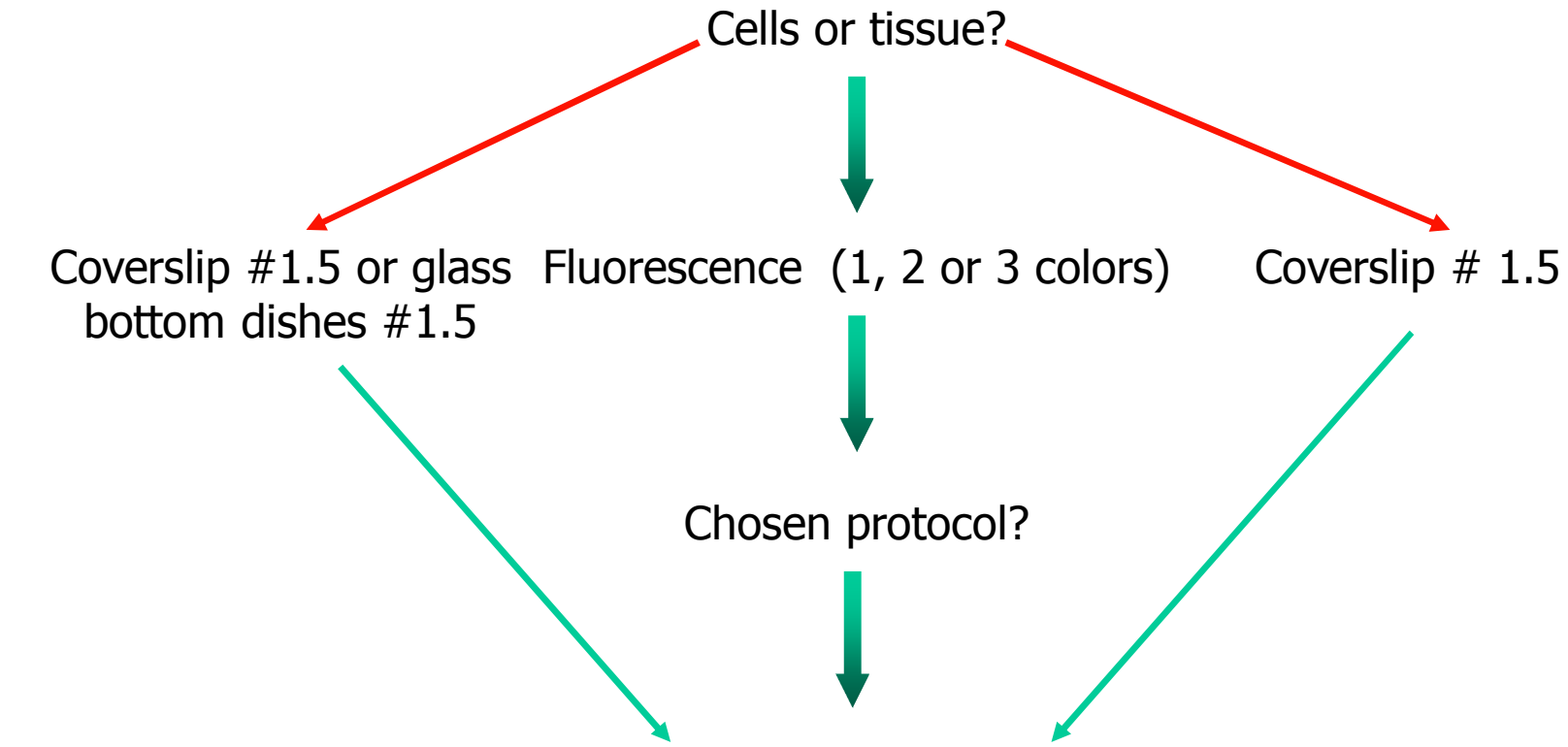
- EmGFP (488nm excitation) has shown excellent results when used on Live Cells and should be considered as first choice when possible.
- mStrawberry (574nm excitation) has performed good in first experiments.
- Other secondary antibodies or Fluorescent Proteins can be tried in STED mode as long as they:
  - Can be excited with the 470 nm line and above for gated STED
  - Have an emission tail at 592 nm or 660nm

STED CW and gated STED – Fluorescent Proteins

- 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 580nm.
  - DAPI (replace with TO-PRO-3, YOYO-3, DRAQ 5)
  - QDOTs or other fluorophores excited by the 405 nm laser



STED CW and gated STED – Remember…



- Prolong Antifade kit or Prolong Gold)
- Moviol (+/- Antifade)
- Fluoromount

- Prolong Antifade kit or *Prolong Gold)
- TDE + Antifade
- Moviol (+/- Antifade)

*Prolong Gold has strange effect with certain dyes

STED CW and gated STED – Mounting Media

- With cell culture

Best	Good	Do Not Use
Prolong + Antifade (will polymerize)	**Mowiol +/- Antifade (DABCO 2.5%)** (will polymerize)	Slowfade
Thiodiethanol (TDE, Sigma, #88559. See below about the concentration). **Add Antifade** (will stay viscous)		Vectashield

  - Prolong Antifade Kit *(Invitrogen #P7481)*, (w/o DAPI), or Prolong Gold
    - Polymerize, has been tested in STED mode
    - Gave excellent results as long as the protein of interest is located within 30µm from the coverslip.
  - Thiodiethanol *(TDE, Sigma, #88559)* has been used with excellent results.
    - The TDE concentration must be gradually enhanced (up to 97%) to obtain a final refractive index of 1.514
    - The coverslip must be sealed using invisible nail polish or other sealants.

STED CW and gated STED – Mounting Media

- With cell culture
  - Prolong Antifade Kit *(Invitrogen #P7481)*, (w/o DAPI),
    - Prolong needs to cure before to be imaged as the refractive Index increases with curing time.
    - Allow at least 24 to 48 hours for the Prolong mounting medium to cure.

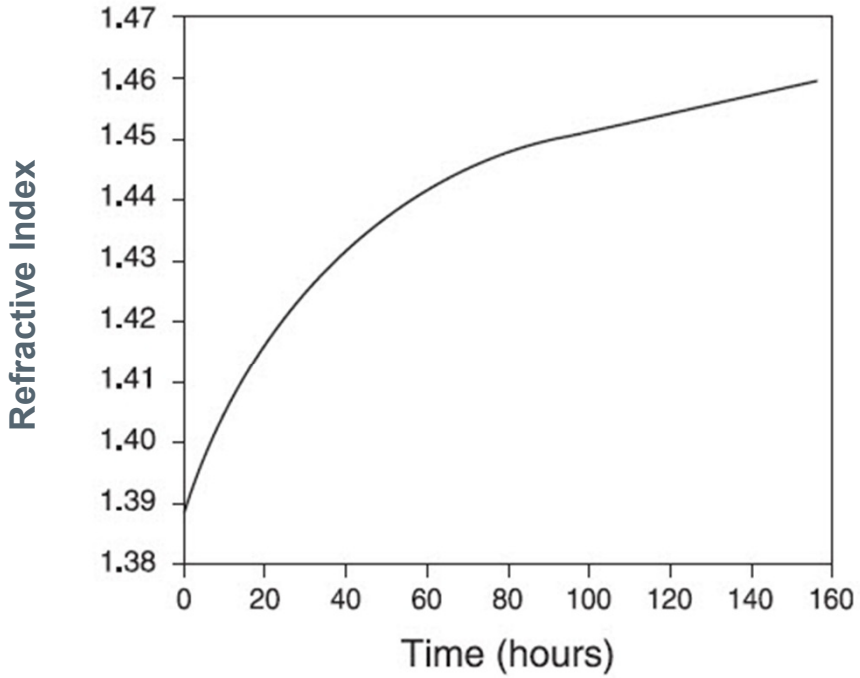


Figure 2. Increase in the refractive index of ProLong® Gold antifade reagent during the curing process.

STED CW and gated STED – Mounting Media

- With tissue sections
  - Thiodiethanol *(TDE, Sigma, #88559)* has been used with excellent 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 with antifade 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.

STED CW and gated STED – Mounting Media

- Antifade reagents
  - Most of these mounting media must have antifade freshly mixed in it.
    - **Do NOT use a solution mixed with any antifade which is older than few hours.**
    - **Very good results were obtained using Mowiol and TDE without any antifade.**
  - The proprietary antifade from Invitrogen supplied in the Prolong Kit Reagent special packaging can be used with TDE.
    - Just add 1 ml of TDE 97% to the tube (component A), mix well and centrifuge the tube to eliminate as much as possible the bubbles.
  - N-propyl gallate (NPG, 2%) while not very soluble, is non-toxic and can be used on live cells.
    - Normally, the recommended medium for the specific cells (without Phenol Red, and buffered at pH 7.4) should be used, or might be replaced with PBS.

STED CW and gated STED – Mounting Media

- Antifade reagents
  - p-Phenylenediamine (PPD), most effective antifade but can react with cyanine dyes (especially Cy2) and cleave away half of the cyanine molecule.
    - May result in weak and diffused fluorescence following storage of the stained slides. There are several reports of ultraviolet-induced yellow-fluorescence
  - DABCO (2.5%) is a well-known antifade

STED CW and gated STED – Protocol Example

- Stay as close as possible to your usual protocol

1) Cells grow on **# 1.5 coverslip**, 18 mm^2 coverslip, #1.5 in 6 wellplate, or 8 mm^2 coverslip, # 1.5 (EMS, # 72296-08**),** in 24 wellplate
2) Cells fixed with PFA 4% 10 min., RT, or methanol, 5-7 min, -20 °C (if looking at tubulin or other structural proteins, the methanol fixation is appropriate).
3) Rinse 3x in PBS.
4) Block with PBS/ 0.2% Triton X100 /Normal goat serum 10%, 15 min in rotation at RT.
5) Incubate in primary Ab in PBS/Tx/NGS 1 hr, RT.
6) Rinse 3x in PBS.
7) Block for 15 min.
8) Incubate in secondary: ***Abberior 440SX (gated STED or CW) for 2-3 hours at RT on rotation or at 4 degree C overnight on rotation, in*** **PBS/Tx/NGS. Biotinylated antibody for 1 hour at RT followed by Horizon V500 Streptavidin, 1 hr at RT**.
9) **Rinse, rinse, rinse!!!!** At least 3x in PBS, in rotation at RT.
10) Block in PBS/Tx/ normal serum (in accordance with the "second" secondary Ab species).
11) Incubate in 2nd primary Ab for 1hr at RT in PBS/Tx/NGS, followed by several rinses in PBS.
12) Block in PBS/Tx/NGS.

STED CW and gated STED – Protocol Example

- Stay as close as possible to your usual protocol

13) Incubate in **DyLight 488**, or **Chromeo 505** for 1 hr, RT
14) Rinse 3x in PBS.
15) Rince once in tap water.
16) Mount in **freshly mixed** Prolong Antifade Kit (or TDE + antifade).
17) Let cure at least overnight (at best 48 hrs to reach the maximum RI).
18) Keep the slide flat at 4 degree C and protected from the light.
19) Enjoy the STED CW imaging!

PS: The two Primaries might be incubated at the same time as long as they are against 2 different species (see Example 2). Same as for the Secondaries. Obviously, to reduce chances of cross-reactions, full separation between the two primaries must be followed as shown here.

STED CW and gated STED – Protocol Example 2

- α-Tubulin (ATTO425)+ HDAC-1 (DyLight 488)

Material

- Blocking Buffer: 10% Normal Goat Serum (NGS), (Invitrogen, #50-062Z), 0.2% Triton X-100 (Sigma #X100-1L), in PBS, pH 7.4.
- Primary Abs: Goat anti-mouse α-tubulin (Rockland Immuno Inc. #200-301-880).
  Goat anti-rabbit HDAC-1 (Rockland Immuno, Inc. #600-401-879).
- Secondary Abs: Anti-Rabbit IgG (H+L) Atto425 conjugated (Rockland Immuno Inc. #611-151-122).
  Anti-Mouse IgG (H+L), DyLight 488 conjugated(Rockland Immuno, Inc. #610-141-121).
  Note secondary Abs should be read as Anti-Host
- Mounting Media: Prolong Antifade Kit (Invitrogen, # P7481).

Procedure

- Cells grow on **# 1.5 coverslip**, 18 mm^2 coverslip, **#1.5** in 6 wellplate, or 8 mm^2 coverslip, **# 1.5** (EMS, # 72296-08**)** in 24 wellplate, until 50 to 70% cells confluence.
- Cell fixation in methanol, 7 min, at -20 ºC.
- Rinse in PBS several times.
- Block with **Blocking Buffer** (see Material for details), 15 min, at room temperature (RT) on an orbital shaker.
- Incubate in primary antibodies: Pipette *4µl/ml of the goat anti-mouse α-Tubulin tube* (*= 0.4 µg/ml final concentration*). Add *1µl/ml of the goat anti-rabbit HDAC-1 tube* (*= 0.14 µg/ml final concentration*) in blocking buffer 1 hr, RT.
- Rinse 3x in PBS.
- Block for 15 min.
- Incubate in secondary antibodies: Pipette *2µl/ml of the ATTO 425 anti-Rabbit tube.* Add *1µl/ml of the DyLight 488 anti-mouse tube,* in ***blocking buffer*** for 1 hr at RT on an orbital shaker.
- ***Rinse, rinse, rinse!!!!*** At least 3x in PBS at RT, on an orbital shaker.
- Rince once in tap water.
- Mount in **freshly mixed** *Prolong Antifade Kit* (Invitrogen, # P7481).
- Keep the slide flat at 4°C and protected from the light, for at least 24 to 48 hrs to reach the maximum Refractive Index before to do any imaging.

gated STED

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

STED: 592 nm, 660 nm

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
Abberior STAR 440SX	458/470	475 - 515	**Oregon Green 488/ Chormeo 505**	514/520	523 - 580
Alexa Fluor 532	514	520 - 565	**TMR/TRITC/ Alexa Fluor 568**	580	590 - 650
Chromeo 505	505	515 - 565	**TMR/TRITC/ Alexa Fluor 568**	580	590 - 650

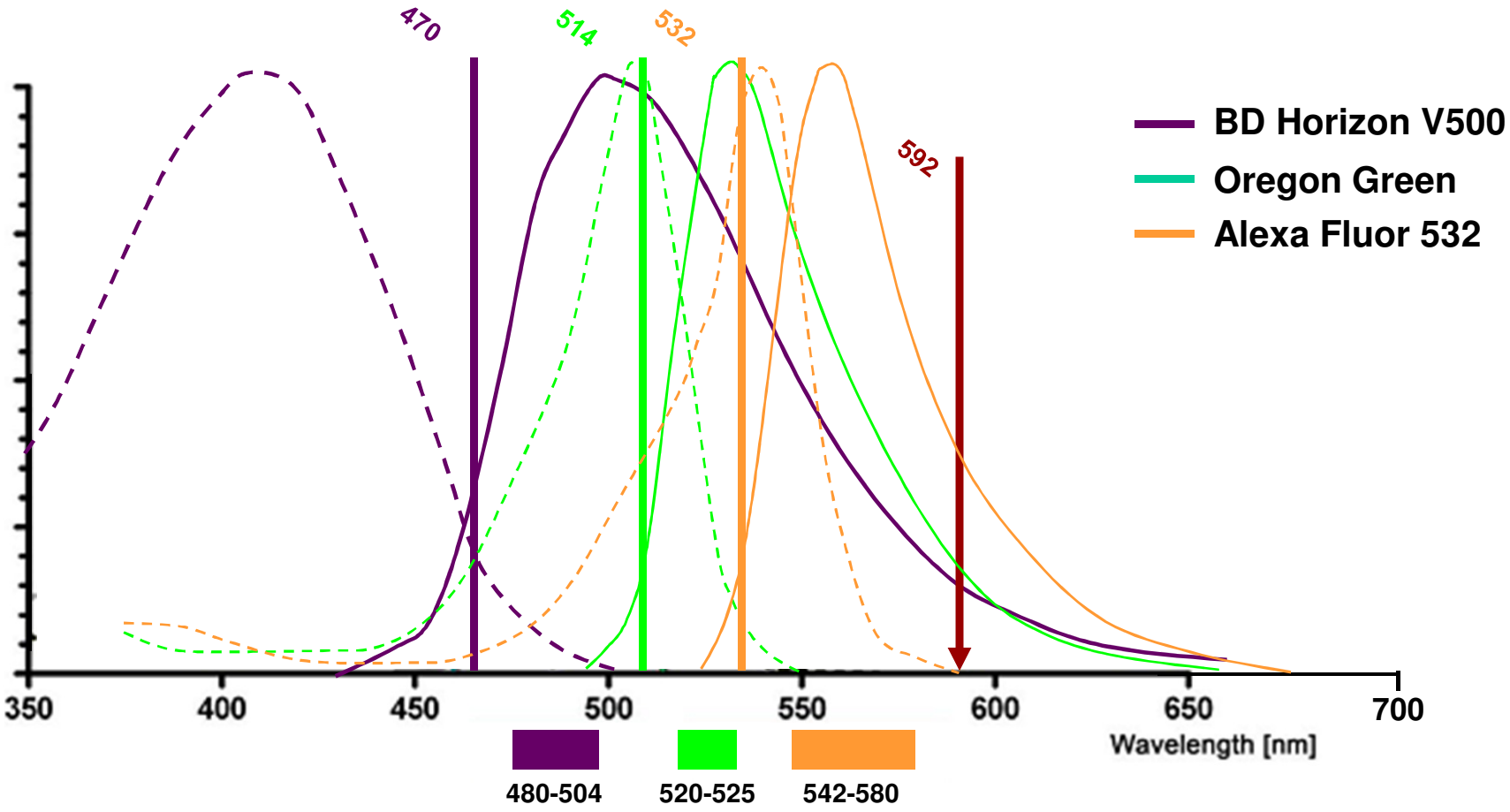
gated STED

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

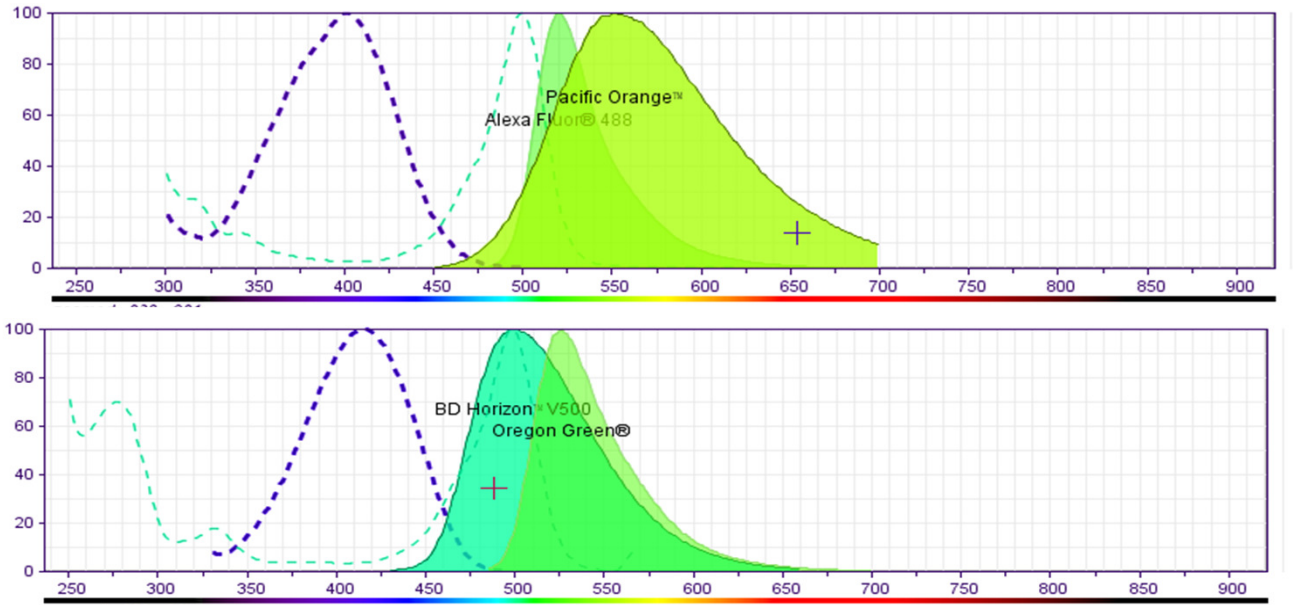
STED: 592 nm, 660 nm

Dye1			Dye2			Dye3		
Name	**Excitation**	**Emission**	**Name**	**Excitation**	**Emission**	**Name**	**Excitation**	**Emission**
STAR 440 SX**	470	475 – 505	**OG 488**	510	515 - 530	**Alexa Fluor 532**	540	550 - 585
OG 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

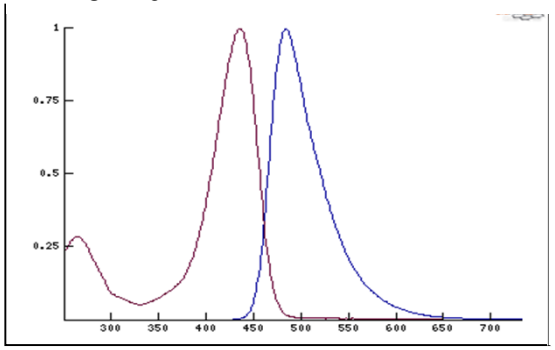
Selected spectra for 3 color gated STED



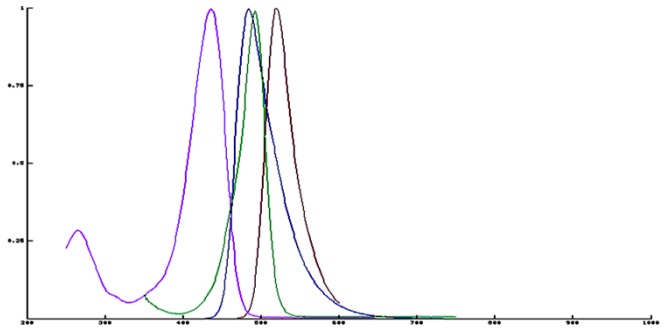
Selected spectra for gated STED



ATTO 425



ATTO 425 (Ex / Em)+ DyLight 488 (Ex / Em)

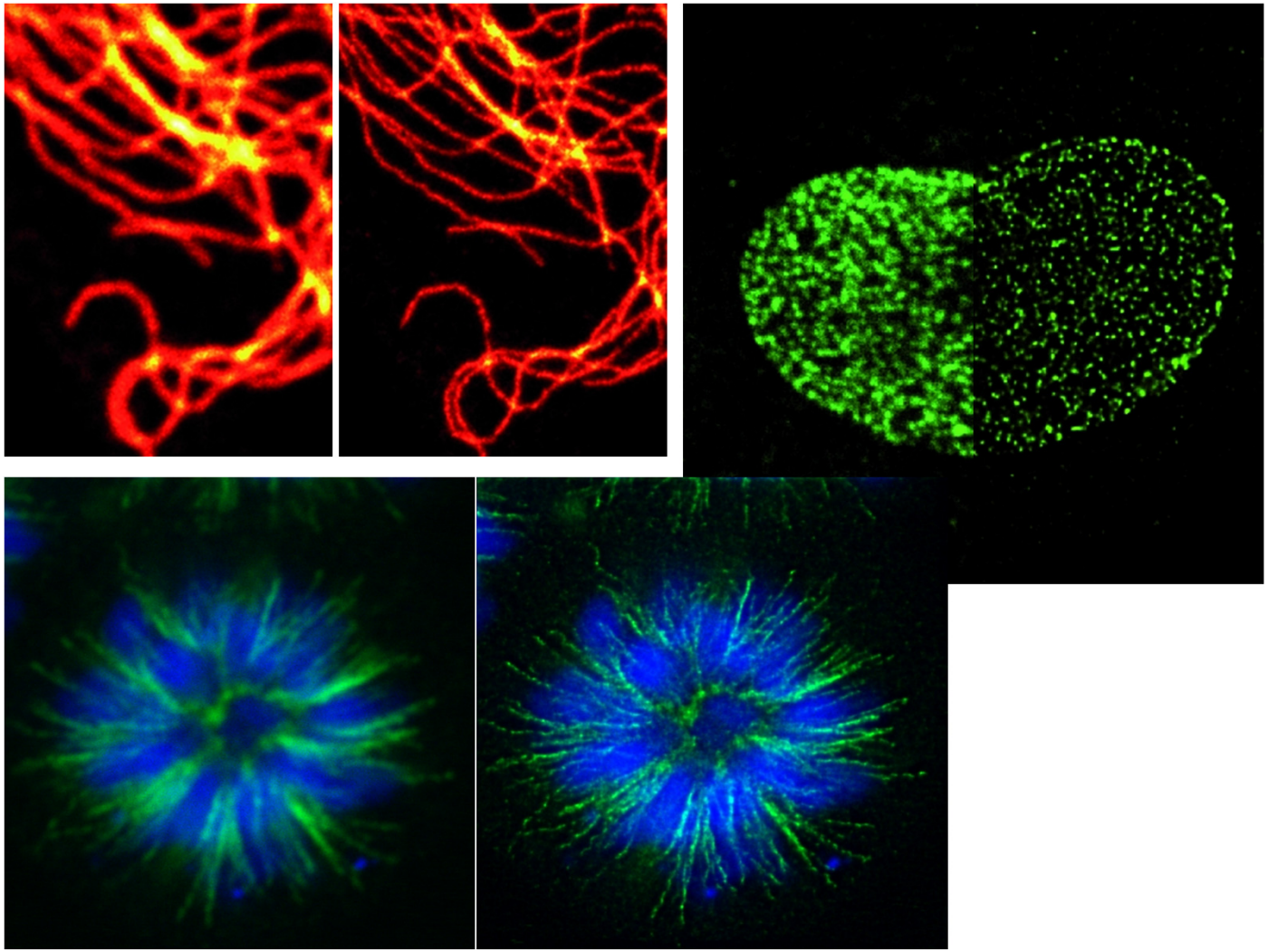


TCS STED CW and gSTED – Tips and Tricks received by customers

- 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

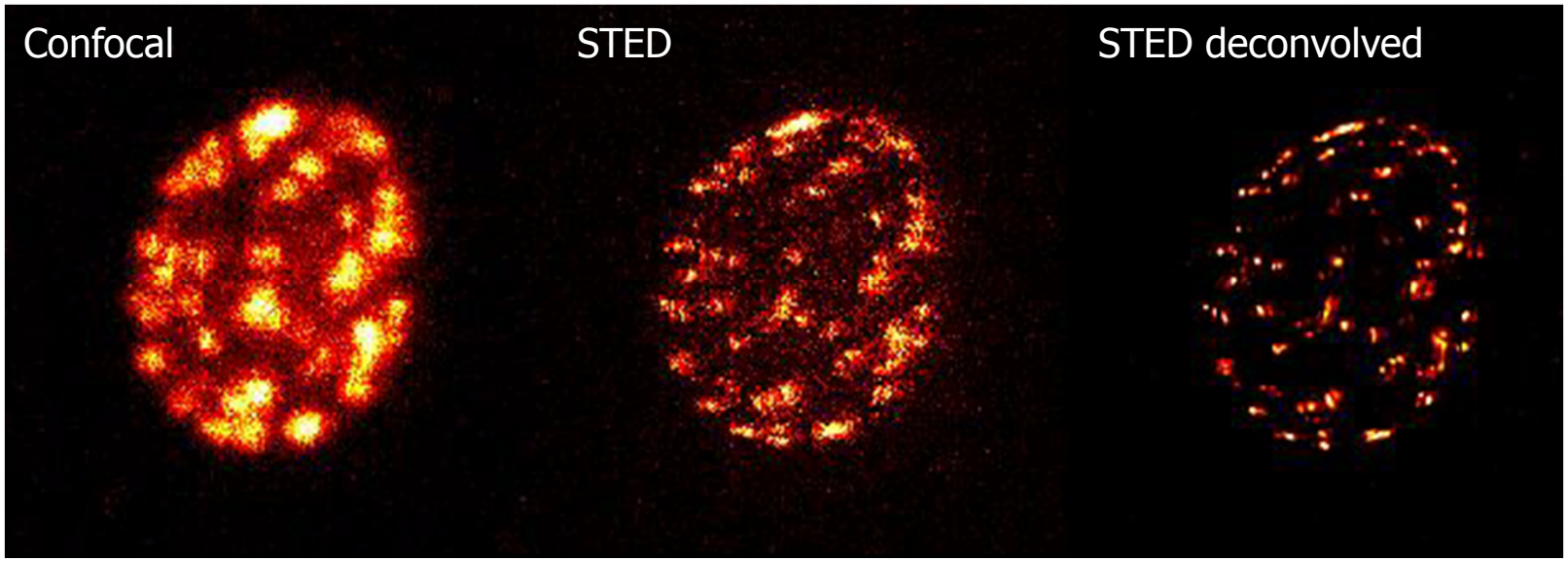
- TDE de-polymerizes F-actin so isn't suitable for that.
  - TDE works well with any antibody (best if you post fix the sample prior to mounting).
  - However, **it does not work with reagents that can diffuse away** - e.g. phalloidin, To-Pro3, etc.

TCS STED CW – Example Images



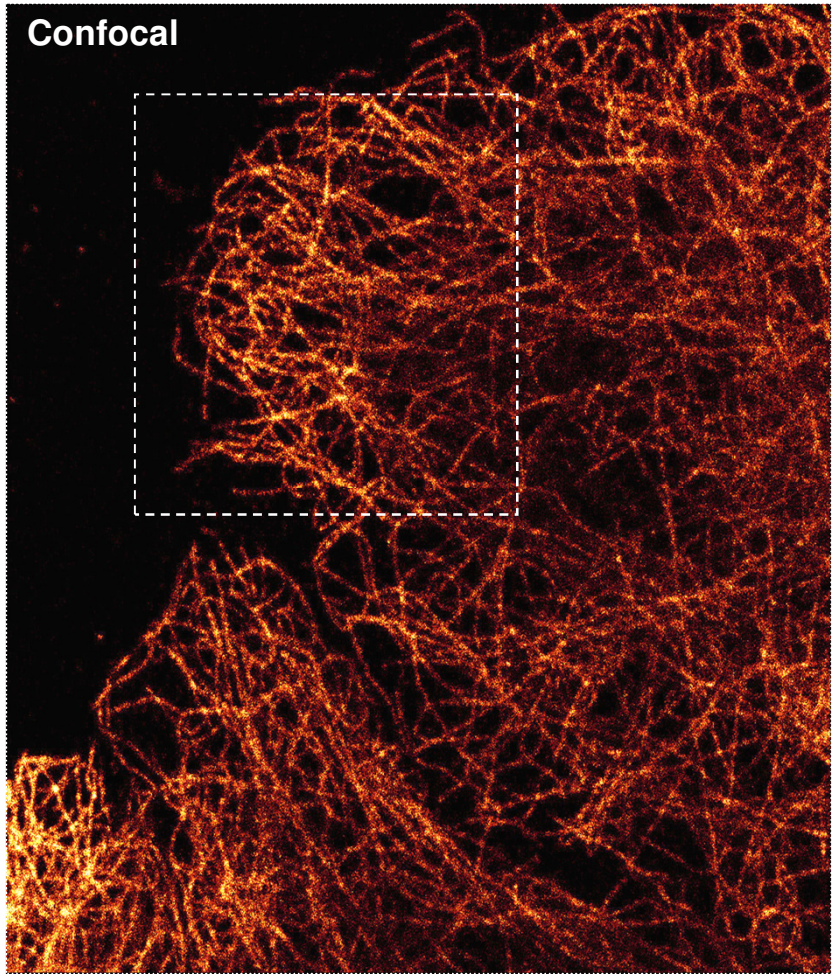
Deconvolution

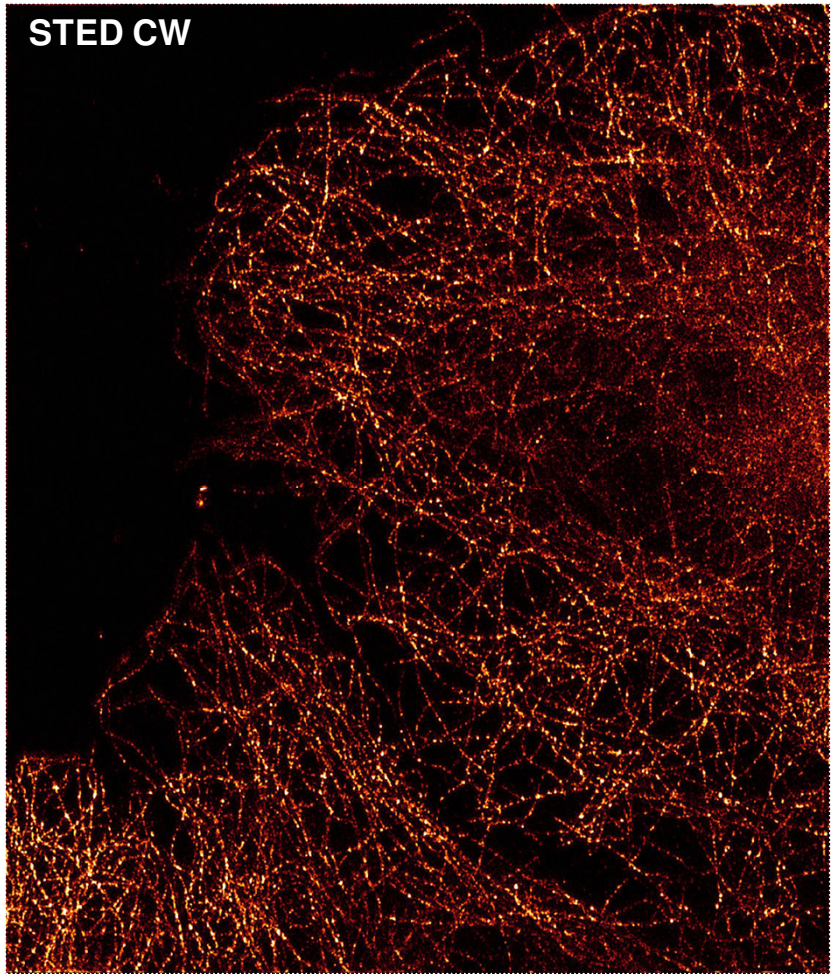
- STED Deconvolution
  - Note that deconvolution is a drastic post processing step.
  - To avoid artifacts, it should be carefully used, and the results should always be confirmed by comparing them with the original data.
  - This applies for STED data as well as for work with all other microscopic images



Courtesy of Marko KaksonenEMBL, Heidelberg, 3D projections

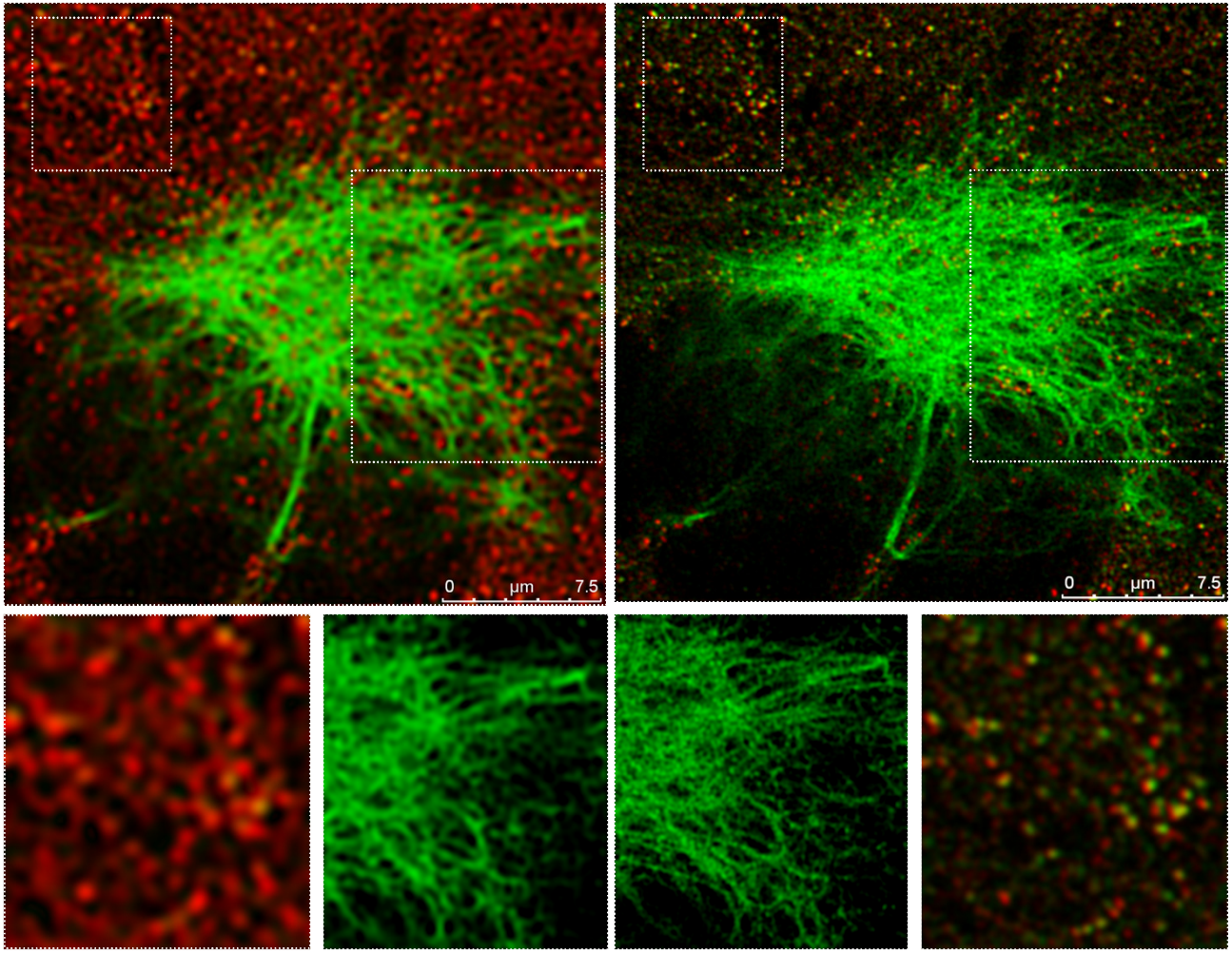
STED CW, 1 color – Deconvolved





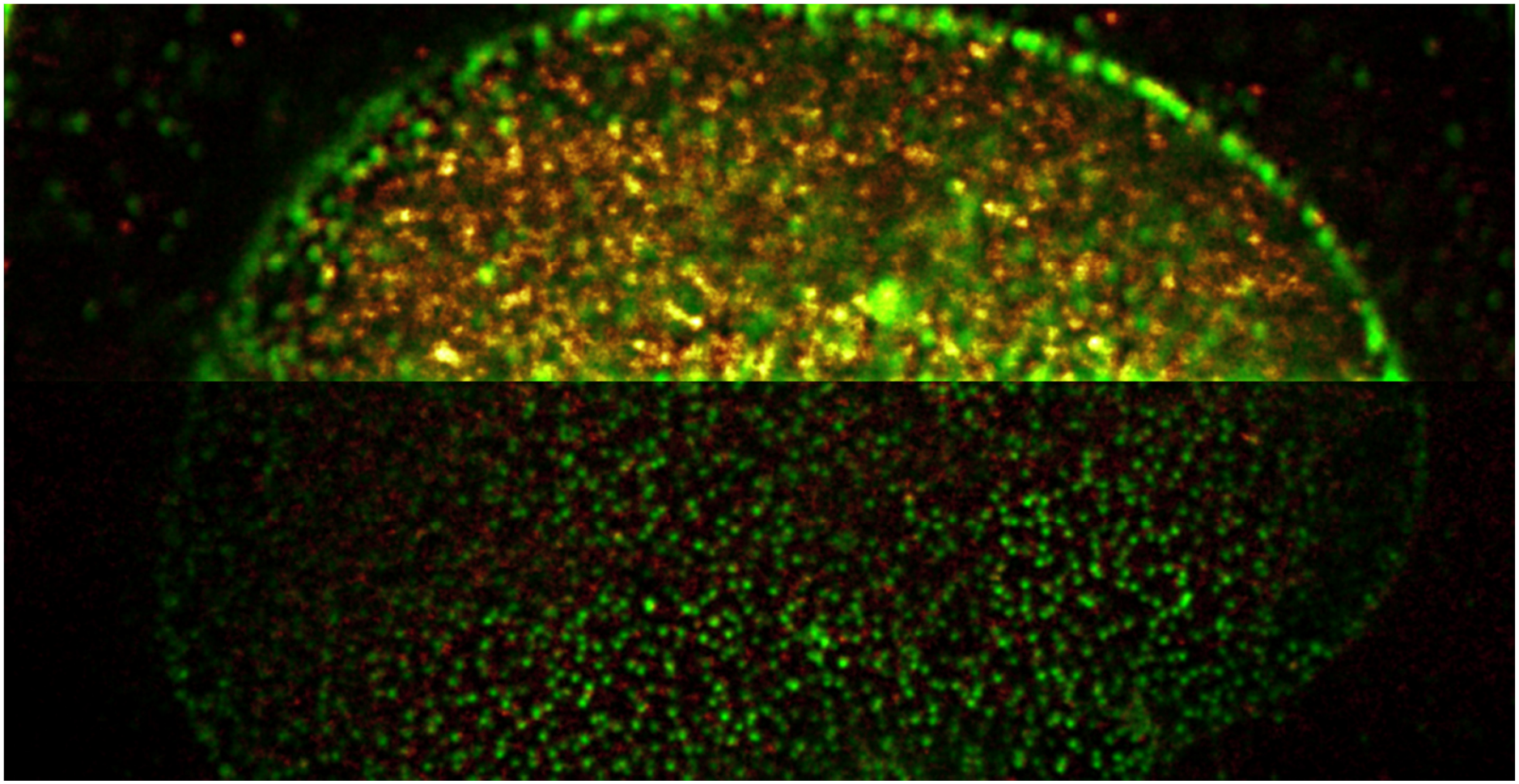
Courtesy: Myriam Gastard, Leica Microsystems, Inc
DyLight 488

STED CW, 2 colors – Deconvolved



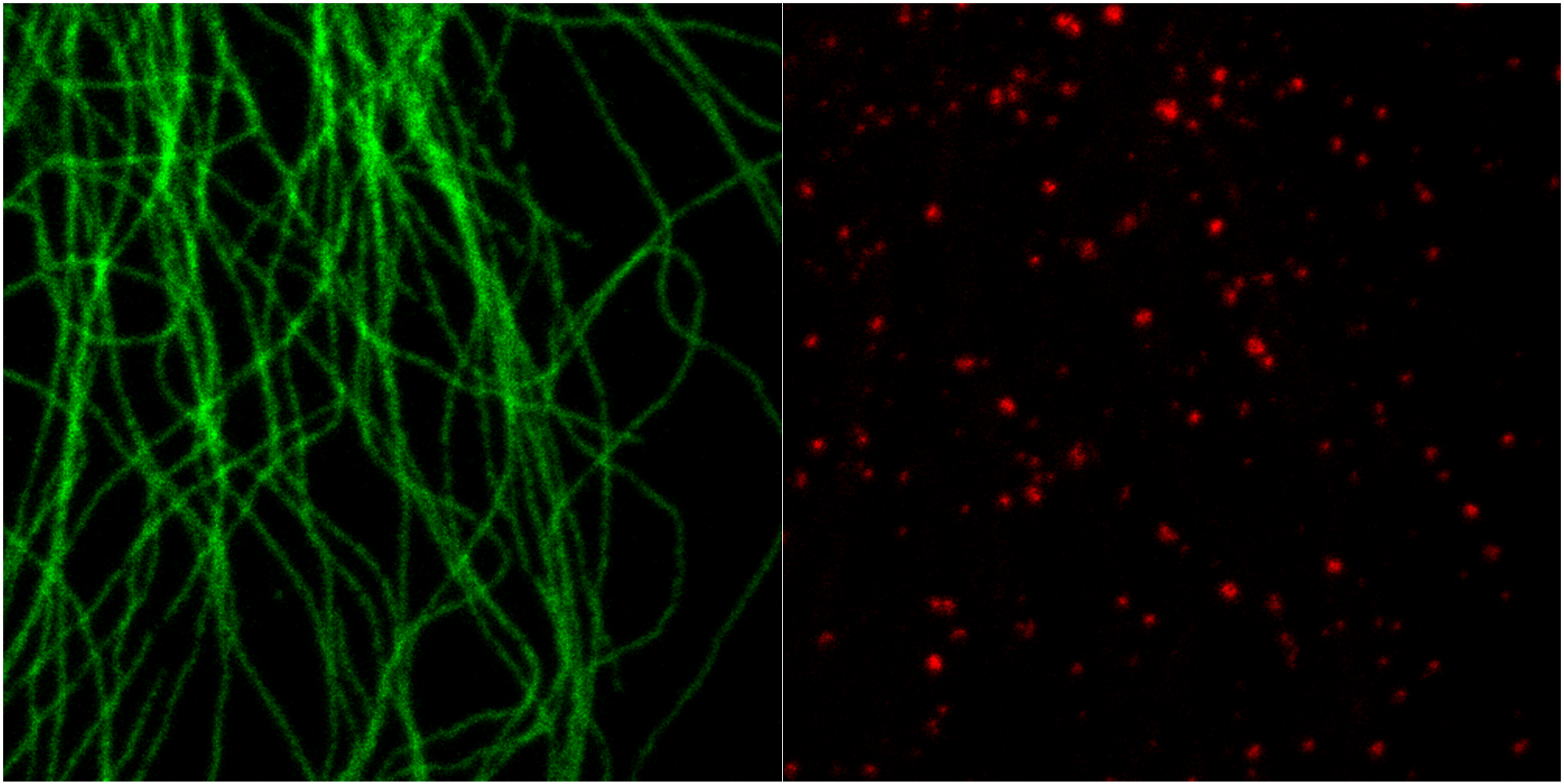
Courtesy: Myriam Gastard, Leica Microsystems, Inc
α-tubulin (Alexa Fluor 430, green); DARPP (Dylight 488, red)

STED CW, 2 colors – Deconvolved



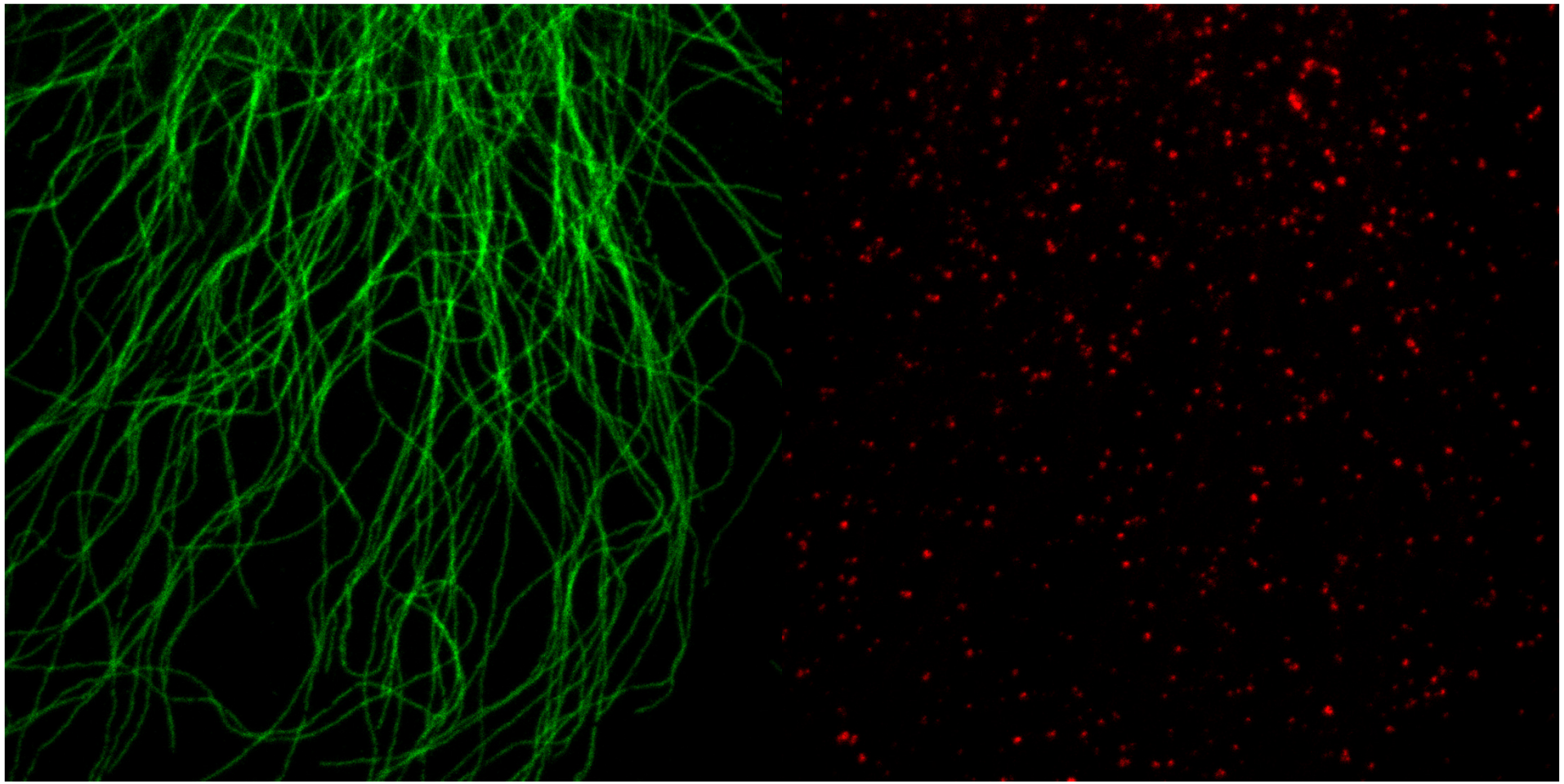
Courtesy: Myriam Gastard, Leica Microsystems, Inc
NuP (Dylight 430, green); Nuclear Kinase (Dylight 488, red)

Multicolor Imaging, g-STED – Raw Data



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

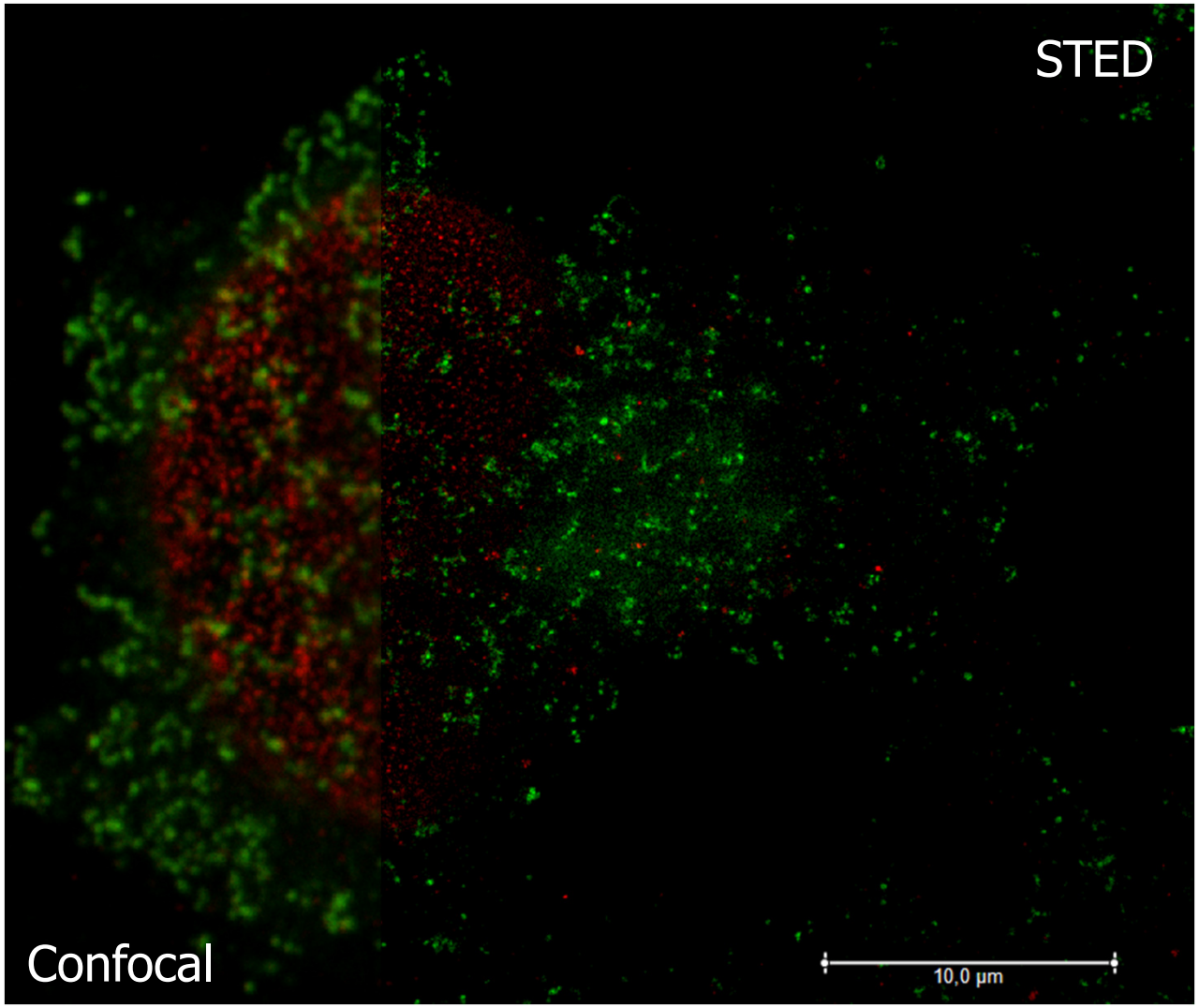
Multicolor Imaging, g-STED – Deconvolved



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

Quick Guide by Wernher Fouquet

Living up to Life

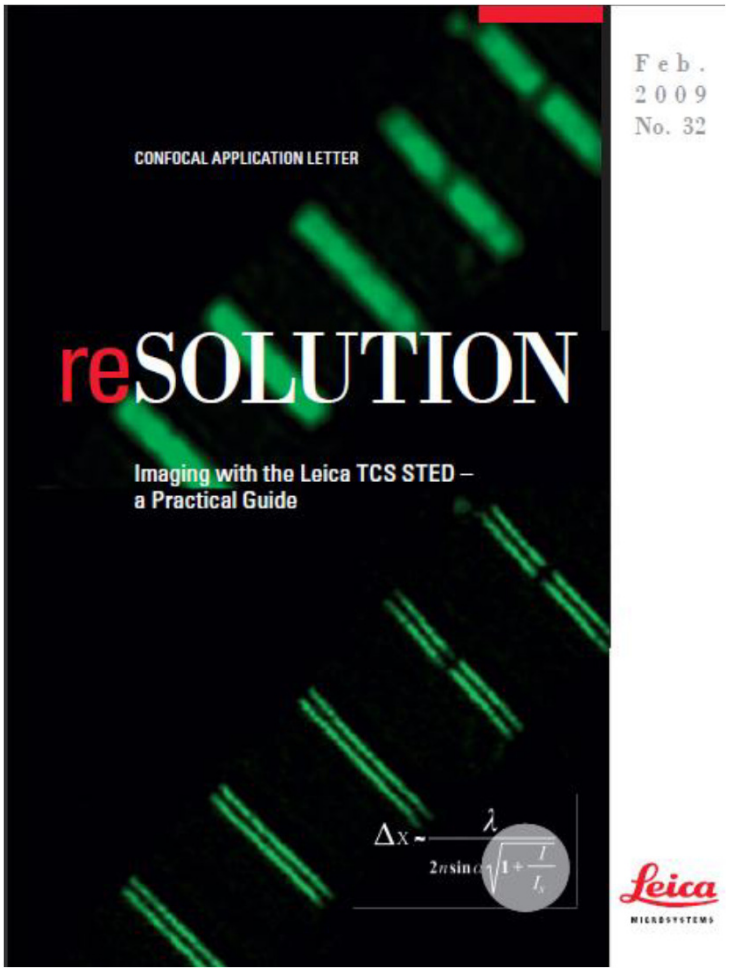
QUICK GUIDE TO STED SAMPLE PREPARATION

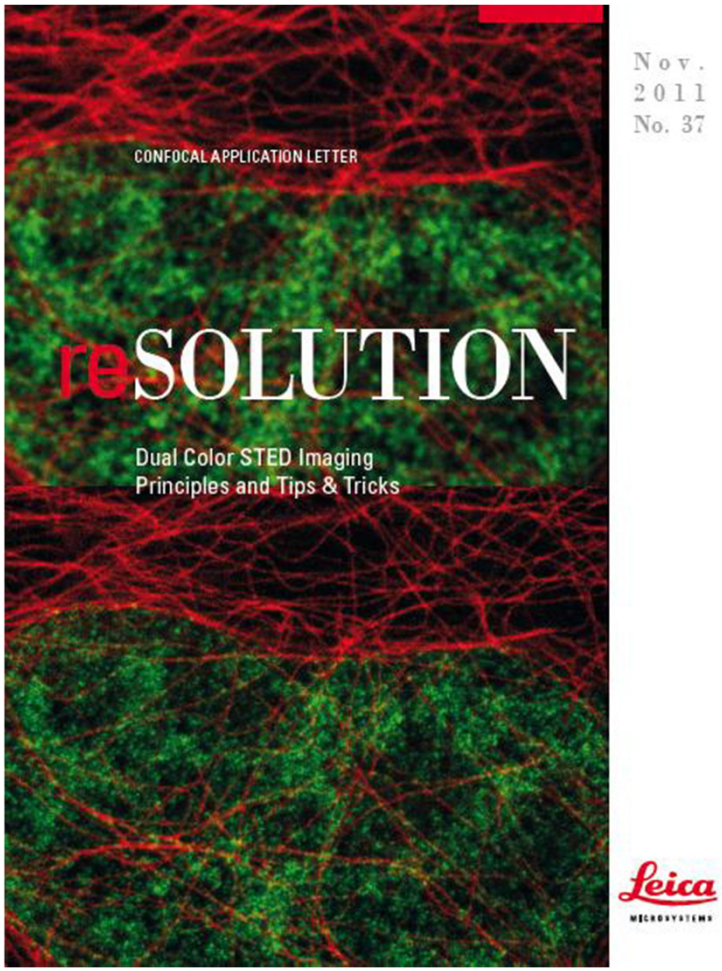
Date 2012-09-14 **Page** 2 | 9

*The guide's focus is set on the **Leica TCS SP8 STED** and **Leica TCS SP8 gated STED** microscopes*

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

Application Letters





http://www.leica-microsystems.com/fileadmin/downloads/Leica%20TCS%20SP8%20STED/Application%20Notes/

Living up to Life

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