Living up to Life

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2-color STED CW Sample Preparation

The protocol doesn't differ from any other normal immunocytochemistry, and we are encouraging you to stay as close as possible to your usual immunocytochemistry protocol. Only exchange the secondary antibody (if necessary) and test different concentrations for optimization of the signal.

For cell cultures, please grow your cells on a **#1.5 coverslip** (**0.170 mm**), **or on a cell culture chamber slide with a #1.5 glass bottom**. **Do NOT use any other coverslips thickness** as microscope objectives are corrected for # 1.5 coverslips and therefore best penetration depth will be obtained with these coverglasses. We recommend the Lab-Tek¨ II Chambered Coverglass products with **#1.5 Borosilicate** Coverglass (no commercial interest).
If you are using tissue sections, please use a # 1.5 coverslip to mount your sections.

Prepare your sample as usual with your primary antibody and the typical washing steps as well as the preincubation in PBS/normal serum/ permeabilisant (Triton X100 or other).

The **choice and combination** of the fluorescent secondary antibodies (or fluorescence protein) is important in order to be able to image your sample in STED mode using the Leica TCS STED CW.

For **single color STED CW**, the following dyes has been tested and reported to give excellent results in STED CW:

- DyLight 488
- Chromeo 505
- Oregon green 488
- Alexa 488 , Alexa 514.

For **2-color STED CW**, the following dyes have been tested and showed good results in combination with one dye from the single color STED CW list.:

- Atto 425, antibody or Streptavidin conjugated
- Pacific Orange, antibody conjugated
- BD Horizon V500, Streptavidin conjugated.

Dye conjugates:

In the following tables show the *recommended dyes and dye combinations which gives good results*.

Recommended pair for 2-color STED CW

Channel 1		Channel 2	Comment
Pacific Orange (Secondary Antibodies conjugated)	+	DyLight488 / Alexa488 (excluding Oregon Green 488 and Chromeo 505)	Dye separation required
Atto 425 (Anti-mouse)	+	DyLight 488, Chromeo 505, Oregon Green 488, Alexa 488	No dye separation required.
V500 (Strept)	+	Oregon Green 488, Chromeo 505, DyLight 488.Any other green dye reported to work for STED CW.	No dye separation required
NBD-X	+	Any other green dye reported to work for STED CW	No commercial conjugates for NBD-X so far

Recommended concentration and possible providers (no commercial interest):
We encourage people to adjust the concentration as needed

Channel 1:

- Atto 425 Goat α-mouse and α-rabbit (0.2µg/ml; *Rockland Immuno*).
- BD Horizon V500 Streptavidin (1:50; *BD* #561419) + Biotin α-mouse (1:200; *Sigma* #B6649) or Biotin α-rabbit (1:200, *Sigma*, B8895).
- Pacific Orange (1:200; *Invitrogen*), α-mouse (P-31585) or α-rabbit (P-31584).

Channel 2:

- DyLight 488 (0.1 to 0.2µg/ml,).Goat α-mouse (*Rockland Immuno* #610-141-121) and Goat α-rabbit (*Rockland Immuno* #610-141- 122).
- Oregon Green 488 (1:200; Invitrogen), Goat α-mouse (O-11033) or α-rabbit (O-11038).
- Alexa 488 (1:200 to 1:400).
- Atto 488 (1:1000).
- Chromeo 488 (1:100 to 1:200).

Fluorescent Proteins:

Channel 1 (458 ex)	Channel 2 (514 ex)
mCerulean	mCitrin, mVenus
eCFP, mCFP	eYFP, mYFP
	eGFP, Emerald (**EmGFP**)

This list is not exhaustive and other secondary antibodies or FPs can be tried in STED mode as long as ***they can be excited with the 458, 488 or 514 nm lasers AND have their peak emission wavelength around 510*** (as well as to have a tail emission at 592 nm).

EmGFP has shown excellent results when used on Live Cells and should be considered as first choice when possible.

Do Not use List:

Do NOT Use
• Other Fluorescence Proteins **not excitable by any laser lines from the Argon Laser (458, 476, 488, 494, 514nm).**
• Do **NOT** use **DAPI**
• Do **NOT** use **QDOTs**
• Do **NOT** use other fluorescence excited with the 405 nm laser

Mounting media:

If working with cell culture

Best	Good	Do Not Use
Prolong + **Antifade** (will polymerize)	**Mowiol** + **Antifade** (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), a mounting which will polymerize, has been tested in STED mode and gave excellent results as long as the protein of interest is located within 30 µm from the coverslip. It will also depend on the tissue. While brain can be imaged quite deep using visible lasers, other denser tissues may be more difficult to image at depth >30 µm. If your target is further away, then TDE is the perfect choice (see below for the concentration and preparation steps).

Thiodiethanol (TDE, Sigma, #88559) has been used with excellent results (see reference below) especially for deep imaging. The TDE concentration **must be gradually enhanced to obtain a final refractive index of 1.514**, which is reached using a TDE concentration of 97%. If using TDE, the coverslip must be sealed using invisible nail polish or other sealants. Please, be sure that the sealant is not quenching your fluorescence or creating any autofluorescence.

For tissue sections

TDE is the best selection. As described before, the TDE concentration **must be** gradually enhanced to obtain a final refractive index of 1.514, which is reached using a TDE concentration of 97% (see the reference below). Sequential steps in TDE 50%, 70% (15-30 minutes at each step), then in 97% + antifade as final mounting media must be undertaken. Here again, if using TDE, the coverslip must be sealed using invisible nail polish or other sealants. Please be sure that the sealant is not quenching your fluorescence or creating any autofluorescence.

Reference about TDE:

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.

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Prolong with antifade can be used too if the structure of interest is close from the coverslip. If you need to image further away from the coverslip, then the TDE should be tried.

Antifade reagents:

All 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.***

- The proprietary antifade from Invitrogen supplied in the Prolong Kit Reagent special packaging can be used. The antifade is coming separately in small opaque tubes, and therefore may 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. Pipette at the bottom of the tube avoid any air bubbles left.
- ***Most Live Cell experiments usually do not include any adjunction of antifade.*** Nevertheless, 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 maybe replaced with PBS.
- DABCO (2.5%) is a well-known antifade, while not as effective as the PPD, but is also less toxic.

Counterstain:

You have the possibility to counterstain and image in confocal mode your STED CW samples using a near far red dye like **Alexa, DyLight or Atto 633, 647 or 655 nm**. If using an orange-red fluorescence like Alexa, DyLight or Atto 543, 555, or 565 nm there is a possibility of crosstalk when using the Argon laser in the range of 488 and 514, therefore it would be appropriate to exchange these counterstains to the ones cited above them.

STED CW: 2-color Sequential protocol Example 1

This is just an example and we are encouraging you to stay as close as possible to your usual protocol. Only adjust the "Must Know" to your 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 PAF 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 X /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: ***Atto 425, 1 hours at RT on rotation or at 4 degree C overnight on rotation***.
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 Rinse in PBS.
12) Block.
13) Incubate in **DyLight 488**, 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 may be incubated at the same time as long as they are against 2 different species (see Example 2). Same as for the Secondaries. Obviously, if you want to reduce chances of cross-reactions, full separation between the two primaries must be followed as shown here.***

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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).
- 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.

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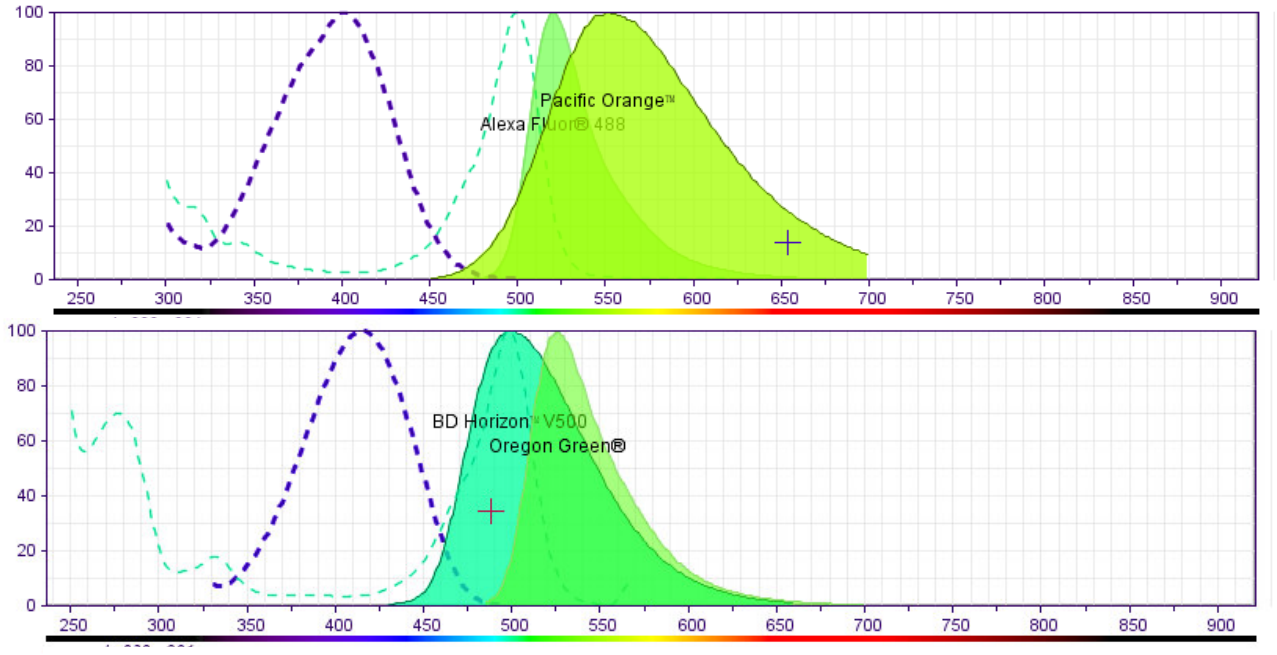
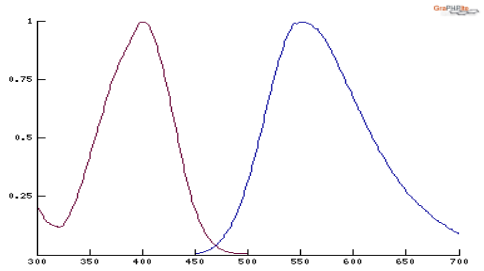
APPENDIX:

Examples of optimization of the acquisition for selected dyes pairs, and selected spectra:

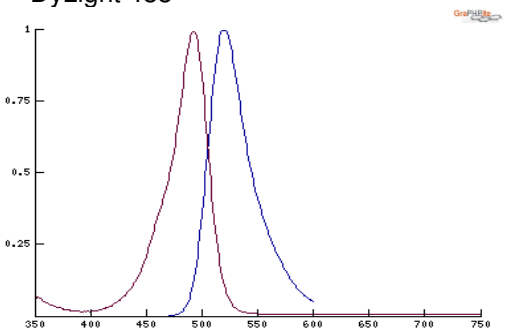
Dye1			+	Dye2		
Name	**Excitation**	**Emission**		**Name**	**Excitation**	**Emission**
Atto 425	458	465 - 510	+	DyLight 488/Oregon Green 488/ Any other 488 or 514 dyes	514	520 - 580
Pacific Orange	458	535 - 580	+	DyLight 488, Alexa 488 / Any 488 (excluding Oregon Green 488)	488 496	500 – 530 505 - 530
Pacific Orange	458	500 -580	+	Any 488 (excluding Oregon Green 488)	488 496 514	500 – 580 505 – 580 525 - 580
Horizon V500	458	465 – 500 465 - 510	+	Oregon Green 488, DyLight 488 / any 514 dyes	488 514	505 - 580 520 - 580
NBD-X	458	535 – 580 Or 500 - 580	+	DyLight 488, Alexa 488 / Any 488 (excluding Oregon Green 488)	488	500 – 530 Or 500 -580



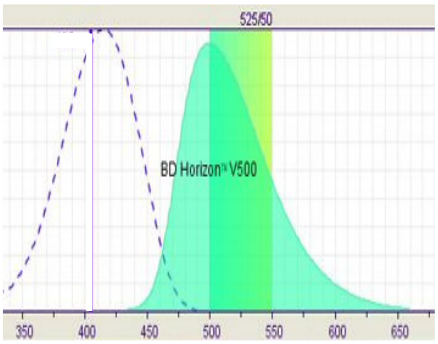
Pacific Orange



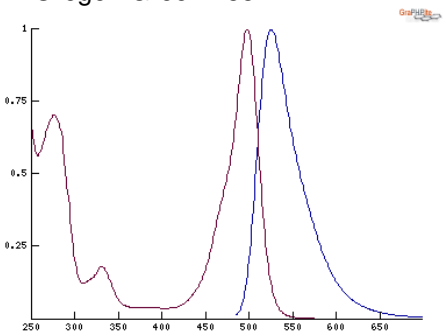
DyLight 488



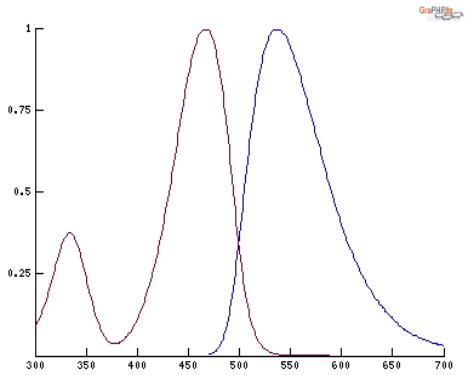
BD Horizon V500



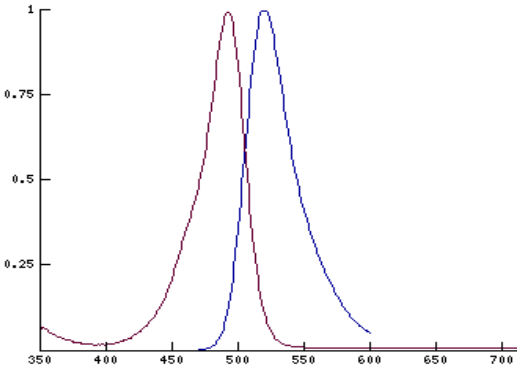
Oregon Green 488



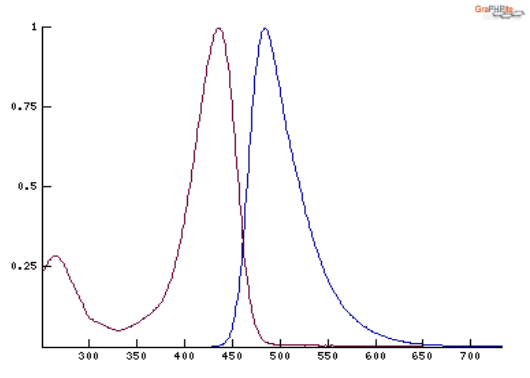
NBD-X



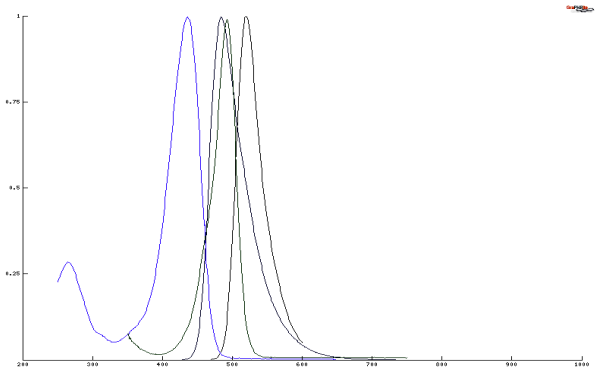
DyLight 488



ATTO 425



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



If you need any help or if you have any questions, please be sure to call me at **866-830-0735, opt 3**.

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