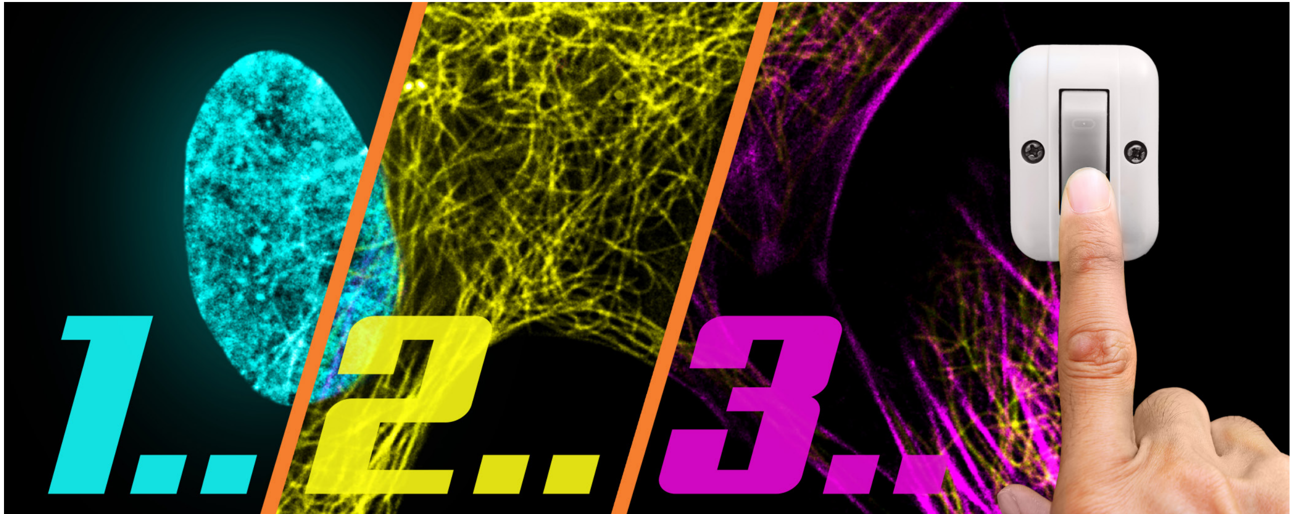
Multi-color single-STED-laser imaging
with abberior STAR and abberior LIVE dyes



Multi-color imaging of cells and tissue is essential for many biomedical research questions. Due to the small size of many subcellular organelles, their structures and dynamic interactions can only be visualized and analyzed using superresolution light microscopy. Stimulated Emission De-excitation (STED) microscopy is one such technique which enables multi-color superresolution imaging in fixed (Hell et al. 1994; Saal et al. 2017) and in living cells (Stockhammer and Bottanelli, 2020). Multi-color STED imaging has been demonstrated in fixed cells providing useful biological information but does not allow to study cellular dynamics. The biggest challenge for multi-color STED imaging in living cells has been the lack of suitable dye combinations and gentle imaging routines. The *abberior* LIVE 460L overcomes this hurdle. This long-Stokes shift dye is optimized for STED and easily combinable with multiple live-cell dyes for three-color imaging in living cells.

- abberior LIVE 460L and STAR 460L are long Stokes shift dyes opitimized for STED microscopy
- the combination of abberior LIVE 460L with STED dyes emitting in the red and far-red spectra allows multi-color live-cell STED imaging

Strategies for Multi-color STED imaging

Several strategies for multi-color STED imaging have been proposed over the last few decades. The first implementation of the technique accomplished two-color imaging by relying on pairs of excitation and STED beams of different wavelengths suited to the dyes present in the specimen (Donnert et al. 2007; Bückers et al. 2011). To expand this concept to three-colors, the popular 775 nm de-excitation line for red and far-red labels, is often combined with a de-excitation line at 595 nm for green fluorescent proteins such as GFP, YFP or mNeonGreen. Although effective, the use of a STED laser in the yellow spectrum can lead to increased phototoxicity in living cells (Wäldchen et al. 2015). Further, the use of two STED wavelengths requires sequential scanning. The red fluorophores with red shifted excitation and STED lasers should be imaged first, before the green fluorophores relying on green-shifted wavelengths. This is due to the fact, that the green-shifted STED laser can excite and photo bleach the red fluorophores. This ultimately reduces imaging to a single timepoint and prolongs imaging time.

Later, two-color (Göttfert et al. 2013) and multi-color (Winter et al. 2017) STED imaging with a single STED laser was established. Here, spectrally different fluorophores were excited with different excitation lines and were detected in spectrally similar detection win-

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dows. However, they were all de-excited using the same STED laser. The main advantage of this strategy is the inherent coalignment of the color channels.

This strategy can be extended to further channels. In addition to the above-mentioned combination of regular fluorophores with narrow excitation and emission bands, long-Stokes shift dyes which exhibit a very wide gap or spectral shift between their excitation and emission spectra can be used. In practice, they are combined with regular fluorophores in the red and far-red range. The combined dyes are excited sequentially with excitation lasers of different wavelengths, but all are sharpened with just one STED laser. This makes them suitable for application in multi-color STED microscopy.

abberior STAR 460L for multi-color imaging using long-Stokes shift dyes

abberior now offers a novel long-Stokes shift dye called *abberior* STAR 460L. The dye is most efficiently excited in the range of 440 - 490 nm and emits between 560 - 680 nm, allowing de-excitation between 750 - 800 nm (Figure 2). These characteristics make *abberior* STAR 460L an optimal dye for multi-color STED experiments using a single STED wavelength. Indeed, for three color STED imaging in fixed cells, *abberior* STAR 460L can be combined with the *abberior* STAR RED and *abberior* STAR ORANGE, and all three dyes may be sharpened by STED at 775 nm.

Recommended for: fixed-cell	Recommended for: live-cell	Excitation		STED Laser		Comment
abberior STAR GREEN	×	488	▲ (blue)	595	▼ (orange)	Interleaved scanning required
abberior STAR ORANGE	×	561	▲ (yellow)	775	▼ (brown)	
abberior STAR RED	×	640	▲ (red)			
abberior STAR 460L	*abberior* LIVE 460L	440 488	▲ (blue)	775	▼ (brown)	Excellent combination for three-color STED
abberior STAR ORANGE	*abberior* LIVE 550	561	▲ (yellow)			
abberior STAR RED	*abberior* LIVE610	640	▲ (red)			

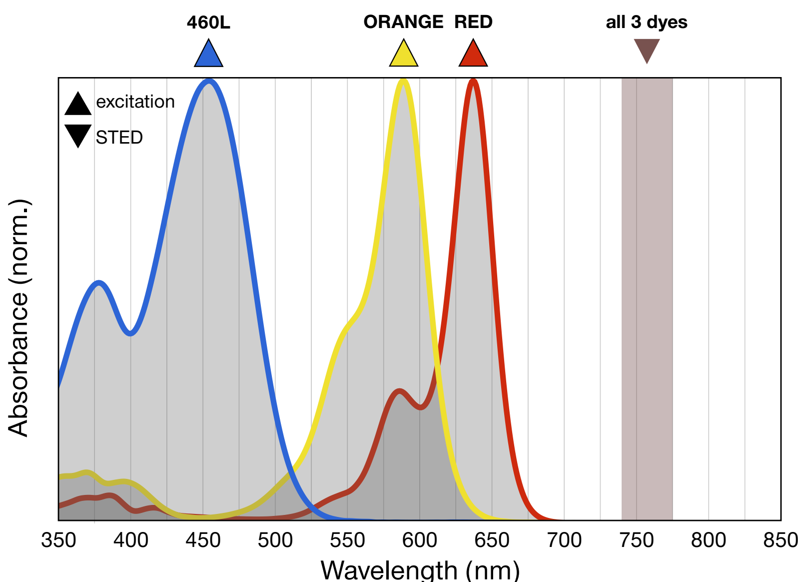

Figure 1 & 2. *Dye combinations for multi-color STED imaging.*

The table shows suitable dye combinations for three-color staining in fixed cells (abberior STAR dyes) and living cells (abberior LIVE dyes) with exciation and STED implementations. The excitation spectra of the abberior STAR460L shows that the dye is most efficiently excited between 440 - 490 nm and therefore combinable with the abberior STAR ORANGE (excited between 550 - 610 nm) and the abberior STAR RED (excited between 630 - 650 nm). These characteristics allow de-excitation with one STED laser between 750 - 800 nm.

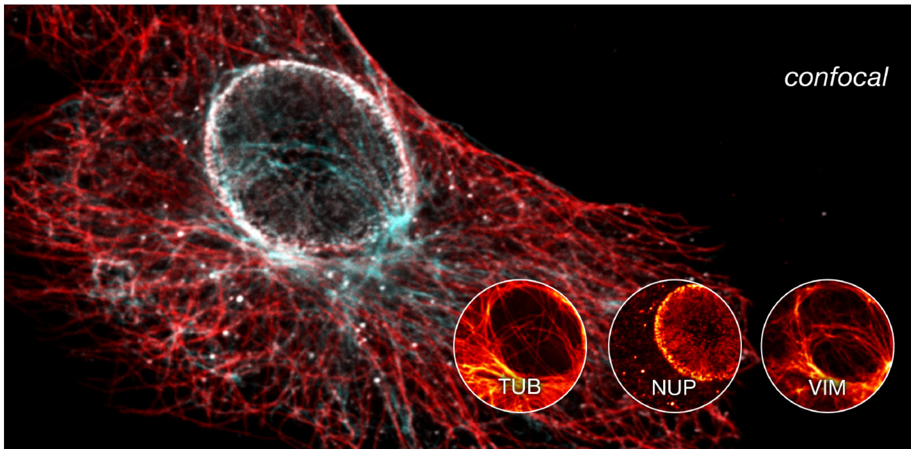

Figure 3. Multi-color confocal image with the abberior STAR 460L dye. *Fixed mammalian cells were stained with the abberior STAR 460L dye to visualize tubulin (TUB), abberior STAR RED to show the nuclear pore complex (NUP) and abberior STAR ORANGE to stain vimentin (VIM). The confocal images were acquired with a STEDYCON in confocal mode.*

Confocal imaging incorporating *abberior* STAR 460L

For three-color imaging, fixed mammalian cells were stained with *abberior* STAR 460L specific to tubulin, *abberior* STAR RED specific to the nuclear pore complex, and *abberior* STAR ORANGE specific to vimentin. The samples were then imaged with an *abberior* STEDYCON microscope in confocal mode. The STAR 460L dye was excited at 488 nm and detected between 575 - 625 nm, the STAR RED dye was excited at 640 nm and detected between 650 - 700 nm and the STAR ORANGE dye was excited at 561 nm and detected between 575 - 625 nm. No crosstalk was visible in the individual channels in the confocal image (Figure 3), despite the detection windows of STAR 460L and STAR ORANGE being similar.

STED imaging incorporating *abberior* STAR 460L

The performance and behavior of the *abberior* STAR 460L dye in STED microscopy was also verified. Specimens were prepared as described in Figure 3 and were imaged using an *abberior* STEDYCON microscope in STED mode. As before, no crosstalk was detected between the individual channels in the STED images (Figure 4).

These results demonstrate that crosstalk-free multi-color confocal and STED imaging is easily achieved using *abberior* STAR 460L combined with STED-compatible red and far-red dyes such as *abberior* STAR RED and *abberior* STAR ORANGE.

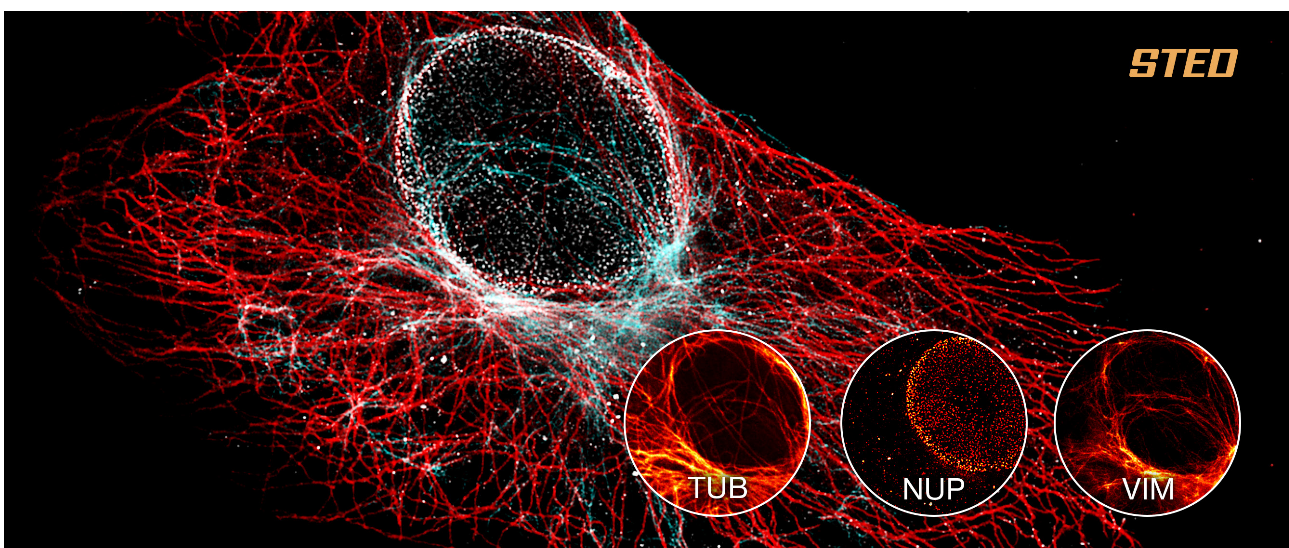

Figure 4. Multi-color STED image with the abberior STAR 460L dye. *Fixed mammalian cells were stained as described in Figure 3. STED images were acquired using a STEDYCON in STED mode.*

Multi-color imaging in living cells incorporating *abberior* LIVE 460L

Two-color live-cell STED imaging was demonstrated using SNAP-tag® based labelling methods (Pellett et al. 2011). SNAP-tags® are genetically encodable protein tags that can be combined with cell-permeable (and therefore live-cell compatible) organic fluorophores. They are therefore well-suited for live-cell (superresolution) imaging. In general, multi-color superresolution imaging can provide a clearer picture of the spatial distribution of two or more proteins. In living cells, the added advantage is that the interaction between these proteins may be studied in real-time. While three-color STED imaging in fixed cells is relatively straightforward, similar experiments in living cells have proven to be a challenge. And yet, superresolution multi-color live-cell imaging is indispensable to fully understand biological processes (Pellett et al. 2011).

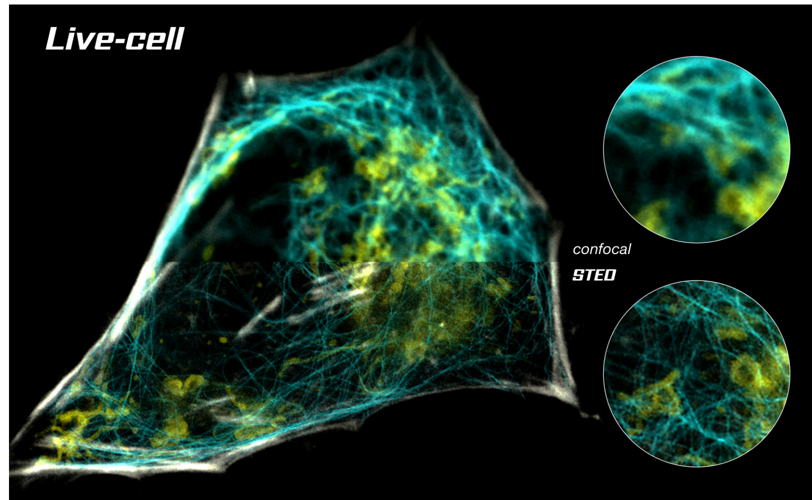

Figure 5: Multi-color STED image with the abberior LIVE 460L dye. *Living mammalian cells were stained with the abberior STAR 460L dye to visualize mitochondria (yellow), abberior LIVE 610 to show tubulin (cyan) and abberior LIVE 550 to stain actin (grey). The images were acquired with a STEDYCON.*

One of the main bottlenecks hindering the application of three-color STED imaging in living cells has been the lack of suitable fluorophores. To help overcome this hurdle, *abberior* developed the *abberior* LIVE 460L dye, a novel long-Stokes shift dye that is optimized for STED and live-cell imaging. Worth noting is that LIVE 460L can be easily combined with live-cell dyes in the red and far-red range for three-color imaging (see Figure 1) and due to the long-Stokes Shift of LIVE 460L, all three dyes can be sharpened using a single STED wavelength. This eliminates the need for multiple STED lasers, which in turn ensures that all color channels are inherently aligned. Further, it eliminates the need for sequential scanning, thus minimizing photobleaching and phototoxicity.

To demonstrate, superresolution multi-color live-cell imaging, mammalian cells were stained with *abberior* LIVE 460L to visualize mitochondria, *abberior* LIVE 610 to show tubulin, and *abberior* STAR 550 to stain actin (Figure 5). The stained cells were maintained in a DMEM GFP medium without phenol red (DMEMgfp-2, Evrogen) and were imaged using the *abberior* STEDYCON.

abberior LIVE 460L, *abberior* LIVE 610, and *abberior* LIVE 550 dyes are an ideal combination for three-color live-cell microscopy. All three dyes can be de-excited simultaneously using the same 775 nm STED laser. This routine together coupled with the superior brightness and photostability of *abberior* LIVE dyes lead to high resolution images while keeping the cells alive and healthy for long time-lapse measurements.

To allow for a broad spectrum of applications, the novel 460L long-Stokes shift dye is available as an *abberior* STAR dye conjugated to various secondary antibodies, streptavidin, phalloidin or neutravidin or as a cell-permeable *abberior* LIVE dye conjugated to jasplakinolid to stain actin, or Hoechst for DNA labelling. Further, *abberior* LIVE 460L is also available as a SNAP-tag®-reactive ligand.

This broad spectrum of dye derivatives facilitates various combinations with different *abberior* STAR and *abberior* LIVE dyes for various multi-color configurations. The use of long-Stokes shift dyes provides an easy-to-use approach for high-quality, three-color STED imaging in fixed and living cells under mild imaging conditions. This in turn allows for the precise analysis of the structure, position, and dynamic interactions of multiple proteins, and subsequently the improved understanding of cellular and molecular processes in cells.

Abbreviations

GFP green fluorescent protein

STED stimulated emission de-excitation

YFP yellow fluorescent protein

Got questions?

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