Biooptics Newsletter 11/2019

Biooptics Core Facility-in general

The Biooptics Core Facility of the MUI exists since early 2009 and is located in the CCB, Innrain 80-82, first floor, rooms M01.370 and M01.381. We are currently hosting four microscopes in-house, a neuron-tracing instrument in cooperation with the Department of Pharmacology at Peter-Mayr-Strasse 1a and a 2-photon microscope in cooperation with the Department of Physiology, Schöpfstrasse 41. You can find more information on the official website.

SP8 STED upgrade (10/2019)

After many delays and problems, the STED upgrade is now up and running since middle of October 2019. This newsletter will therefore be exclusively dedicated to comments on the new system (upgrade).

In order to use the pulsed 775 STED depletion laser, far-red STED chromophores are required. In addition to the planned 775 nm STED laser and the new 93x Glycerol STED objective (both were planned), we have additionally received a 3D STED upgrade (not planned but needed for the other upgrade). The 3D STED upgrade enables superresolution along the z-axis using both the 775 and/or the 592 STED laser.

This upgrade results **in highly improved bleach properties**, since the overall energy transferred to the sample is much lower (pulsed and far-red STED light compared to continuous and orange STED light). Second the glycerol STED objective in combination with the 775 far-red laser enables **STED-imaging of thick material** such as **tissue sections** (20-50 µm and above). **Multi-color STED** will be easier to perform as well. For 775 STED, far-red STED fluorophores are required. For immunofluorescence, typical chromophores are StarRed, Star 635, Star 635P (Abberior) and Alexa 594 or Star ORANGE (Abberior). Some **small aliquots of secondary antibodies** (mouse/rabbit) are be **available from the core facility**, for free, as long as supply lasts; thereafter they will be sold for the purchase price in small aliquots. If you are interested in live-cell imaging, I recommend checking the Spirochrome dyes (SiR-Dyes, Tebu-Bio, Spirochrome, tested by me already) and also the Abberior LIVE dyes (not tested).

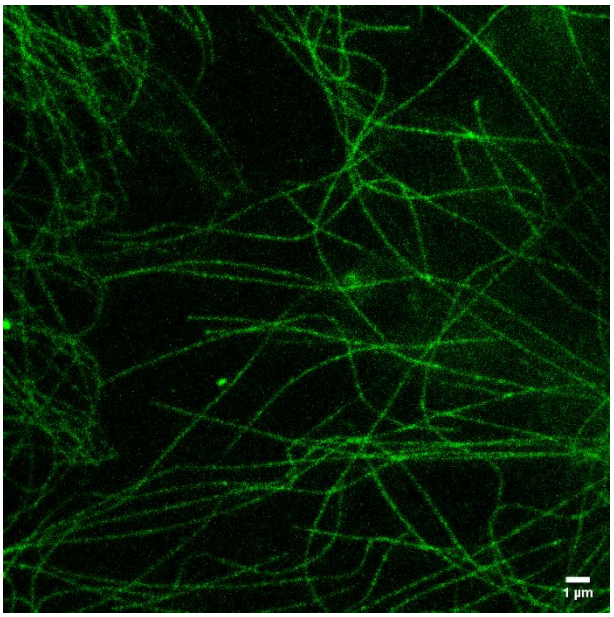
Preparing a fixed sample for 775 STED

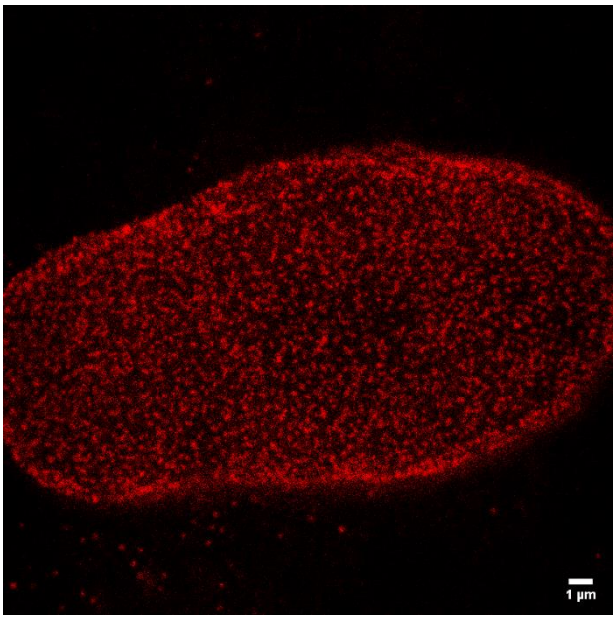
In order to use the 775 Laser for STED the following basic procedure is recommended: Prepare and fix your sample essentially as for indirect immunofluorescence. In the ideal case your sample should be directly on top of a high-precision No1.5 coverslip - not on the slide! Adapt (!) your indirect IF protocol by using saturating amounts of primary antibody, clearly more antibody is needed than you would typically need for confocal. Increase also the staining times. Use STED dyes on the secondary antibody: Best choice: **STAR Red or Star 635P,** Alexa647 and Atto647N have been used in the literature but are certainly not optimal; possible second dyes (for double STED labelling): Atto 594, Alexa 594 (tested!), possibly the best (not yet tested): STAR Orange.

Mounting/embedding Medium: Any mounting medium for 775 STED MUST not absorb any light at 775 nm (!). Tested and good options are ProLong Diamond (solidifying, Thermo-Fisher) or the Abberior mounting media (either liquid or solidifying). Simple Mowiol can be used as well but doesn't have very good anti-bleach properties.

2D STED with 775 nm

If you are mostly interested in 2D information about your sample, 2D STED will be the best choice. In this case you can also expect the best resolution in the xy direction. All the energy of the 775 STED laser donut will be applied in xy only. There won't be any STED laser applied along the z-axis. Expected resolution will be 35 nm in xy, resolution in z: similar to confocal around 800 to 1000 nm. The required pixelsize **in xy will be <20 nm** in order to recover the available resolution. This approach will work well for intrinsically flat samples, since out of focus light along the z-axis will have a negative impact on the resolution in xy. Even if you are interested in 2D information it still can be useful to take a few (!) z-sections (4 to 5) at a **z-distance of 150 nm.** These additional z-planes can be used in the Huygenssoftware in order to improve the resolution in the xy direction even more (2D STED deconvolution). Image 1: 2D 775 nm STED of microtubules of a Cos7 cell, Alexa 594. Image 2: 2D 775 nm STED of a Cos7 cell, Nuclear Pore Complex, stained with Star635P.

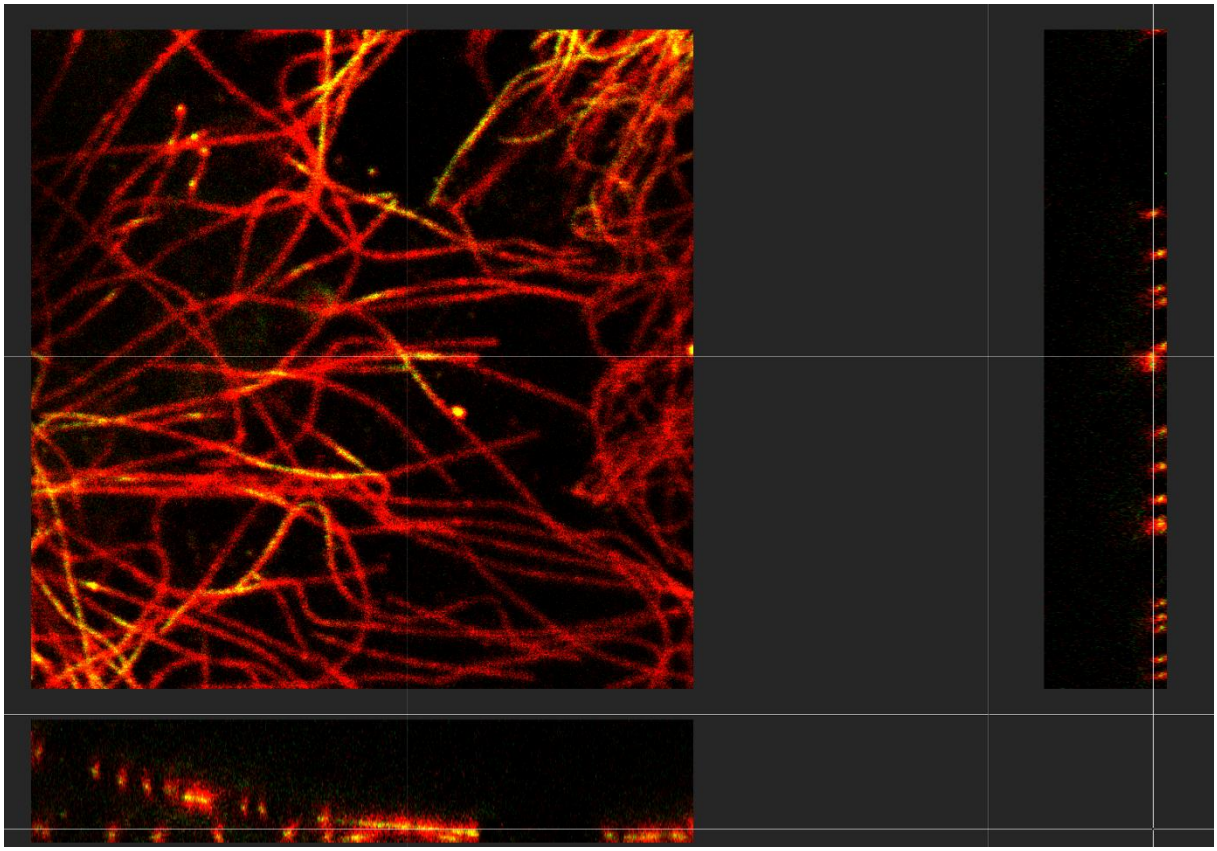




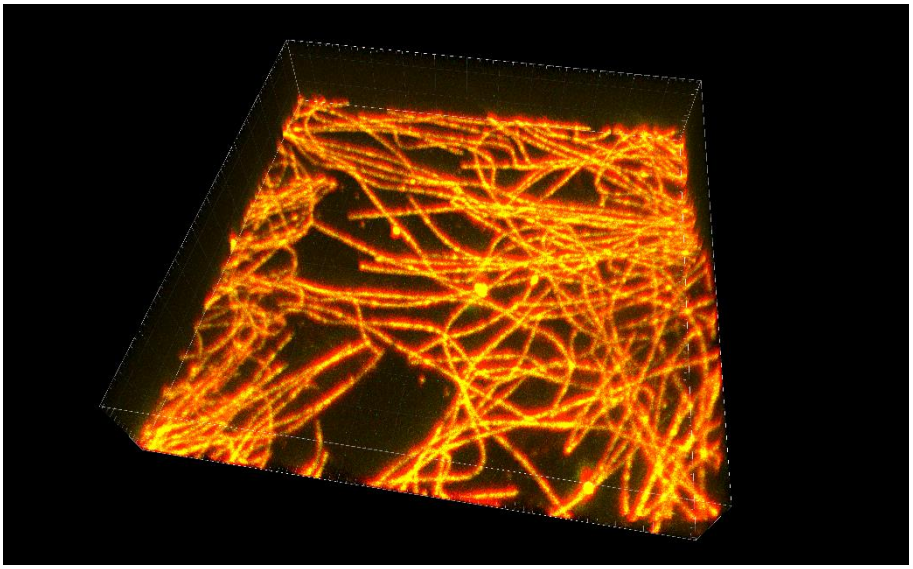
3D STED with 775 nm

If your sample is clearly 3D and/or you are interested in 3D information, you should go for 3D STED. In 3D STED the 775 nm STED laser is applied onto the sample as 2 donuts, one in xy and one in z-direction. Hence, the energy of the STED laser is split over the optical axes, resulting in some loss of resolution in xy and an improvement in z. This 3D STED option is very powerful and it provides you with an excellent tool against the (intrinsically) bad z-resolution in confocal microscopy. In our system it is also possible to tune the amount of z- vs xy STED light to be applied from 0% z (= 2D STED) to 100% z- STED light (= 3D STED). You can also decide for e.g. 2.5D STED. The final resolution will also depend on this z-donut setting; for full 3D STED it is expected to be around 60 nm for xy and slightly lower for z.

In order to visualize this different 3D performance of 3D STED vs Confocal I did a confocal scan in parallel to a 3D STED (100%) using again Alexa594 stained microtubules**. Red=confocal, low z-resolution**;

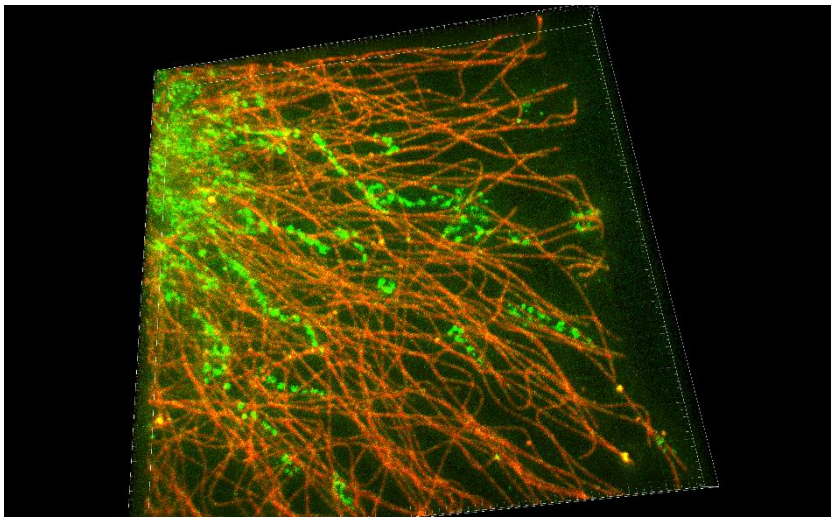


green=3D STED, high z-resolution: Only the part of the MTs which is really “in focus” is seen in green in this ortho-section view. Also note the elongated structures in red in the two smaller images (xz and yz views). Image 2: Same image in 3D view. In general, green structures (3D STED) are thinner (=have higher resolution) than the red ones (Confocal).



2 color STED using the 775 STED laser with two dyes

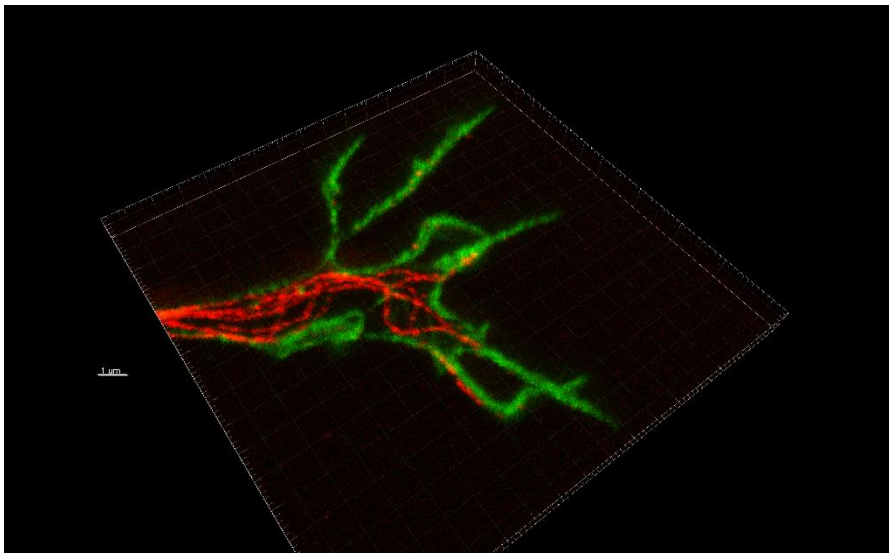
There are now 2 possible strategies for doing multi-color STED experiments. There more commonly used and easier one is to use two different dyes that can both be used with one STED laser, such as the 775 STED laser. It is easier to setup and to perform than a two-STED laser experiment. For this (as discussed above) both dyes need to be suitable for the 775 laser. The two dyes – such as Alexa 594 and STAR 635P - can be imaged in a sequential scan setup between lines or frames (even stacks). Linear unmixing ("dye separation") may be required for a good separation- it is the preferred approach nevertheless. In addition, a simultaneous scan using two Detectors (HyDs) is also possible. Again the user has full freedom to either decide for 2D or 3D STED.



Example: Cos7 cell with MTs (A594) and the mitochondrial Marker Tom20 (STAR 635P), sequential scan between lines, 3D STED for both dyes.

Advanced: 2 color STED using the 592 AND the 775 STED Laser

In this approach the sequence of the overall imaging procedure is very critical. It is mandatory to first image with the 775 Laser and then with the 592 STED Laser- not vice versa! Any (even short) exposure of the far-red 775 fluorophores to the 592 STED laser will immediately bleach all far-red fluorescence. For z-stacks the switch between the dyes needs to be done "between stacks". In case of failure, a new cell/structure needs to be found on the slide, re-imaging is not possible. Suitable dyes are green STED dyes such as Oregon Green 488 plus far-red dyes such as STAR RED. Example (in cooperation with Rüdiger Schweigreiter, Neurobiochemistry): Growth cone of a sensory neuron (DRG): Actin was stained with Phalloidin-OregonGreen 488, MTs were stained with AB and STAR Red. Microtubules were imaged first (complete zStack, 3D STED); thereafter the ActinOG488 was imaged (complete z-stack, 3D STED).



Final remarks

During the setup of the upgrade also the **old 592 STED laser has been realigned extensively**. The performance of 592 STED is clearly better than before (less bleaching, than it used to be; but certainly more bleaching than with the 775 Laser).

The 93 x glyerol objective together with the 775 laser enables imaging of **thick samples such as thick tissue sections.** I was not able to test it since I had no sample around, but it is described to work very well. All scientists interested in such thick samples should consider to try it on the new setup - including those who failed in the past with the 592 Laser + oil objective – it will certainly be a lot better.

In the Huygens software, we are currently able to process 2D STED images only; I have requested the 3D STED option and hope to get it soon. All images in this newsletter have not been processed with Huygens (this would result in a further improvement).

Please contact me for any further questions regarding this newsletter or for any introductions to the new upgrade on the microscope.

Contact and further information

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