**Important STED Information**

* 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.
* 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**

**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

**Coverslip requirements**

* #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 Requirements**

**With cell culture and thin tissue sections (within 25um from the coverslip)**

* 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

**With thick tissue sections (beyond 25um from the coverslip)**

* 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.

**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

**Users Experience**

* Alexa 633 is a solid STED dye with little bleaching
* 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.

Links

<https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/414/390/sted-sample-preparation-guide.pdf>

<https://www.imperial.ac.uk/media/imperial-college/medicine/facilities/film/The-Guide-to-STED-Sample-Preparation.pdf>