3-Color STED Nanoscopy: Linear Unmixing by Fast Pattern-Matching

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Fluorescence super-resolution microscopy techniques such as stimulated emission depletion (STED) have proven to be a big breakthrough since their experimental inception. Since these techniques are becoming standards, the demand for super-resolved images of multi-stained samples is growing. Whereas for two-color STED protocols and hardware are becoming more accessible, adding another color is still challenging, highly increasing the complexity of the whole method. Although different solutions exist to overcome this limit, they have pre-requirements that are mandatory to achieve the unmixing.

Here, we show the application of a method called Fast Pattern-Matching (FPM). FPM is a spectrally based linear unmixing method that requires no prior knowledge of each dye's properties. Like current linear unmixing techniques, it employs the differences in excitation and emission spectra from every dye. However, with FPM all relevant information is collected by acquiring the multi-color image using multiple excitation sources and detectors. This way unmixing can be achieved using the information contained within the multi-color image itself, without references [1]. Up to three dyes were successfully unmixed using this method, resulting in reasonable residual cross-talk values. Furthermore, results yield proof that FPM is sensitive enough to unmix dyes in STED images with very similar spectra such as Abberior STAR 635P and ATTO 647N.

The FPM approach is a purely post-processing analysis method. Thus, it can be easily implemented into existing acquisition and analysis routines in confocal microscopy. It integrates well with STED microscopy and does not require additional prerequisite other than the need of Pulsed Interleaved Excitation mode. A simple turn-key system equipped with multiple excitation lasers and a single STED laser, and two detectors is enough to obtain multicolor STED images with a single image scan.

With the implementation of FPM, using a combination of pulsed excitation lasers together with two SPAD detectors it was possible to achieve 3-color STED images of a DNA Origami sample (GATTA-STED Nanoruler, GATTAQuant) with a resolution down to 40 nm.

[1] M. Gonzalez Pisfil et al., "3-Color STED Nanoscopy: Linear Unmixing by Fast Pattern-Matching". *Manuscript in preparation,* (2019).