One laser, multi-color nanoscopy

- STORM made easy for high throughput research

Single Molecule Localization Microscopy (SMLM) methods (PALM[1,2], PAINT[3], STORM[4-5]) have become important tools to access biological information at the nanoscale. These techniques can resolve structures with a quasi-isotropic 3D localization precision of ~10 nm[6]. However, SMLM suffers from being dependent on fluorophore photoswitching characteristics[7], which depends on the wavelength, and therefore can ad significant chromatic aberration at the nanoscale. These challenges are hampering straightforward multi-color imaging with SMLM. So far, the multicolor information has been accessed by sequential acquisitions at different excitation wavelengths, which on the other hand induce uncontrollable drifts between the different color recordings.
In this white paper we present a new technique that makes use of a single laser to excite multiple fluorophores in the same spectral range (red/far-red) coupled to a spectral demixing strategy that allows these limits to be overcome thereby permitting 3D multi-color nanoscopy with increased simplicity, speed, and reliability of output data.

SAFe RedSTORM Strategy

Different methods have been proposed by the SMLM community, based on spectral demixing algorithm[8-13]. By taking these approaches a step forward, Abbelight has been able to demonstrate an easy and robust ratiometric technique to spectrally separate the fluorescence emission contributions of red emitting dyes.

In Abbelight's set-up, the emission light is split with a long pass dichroic (for example at 700 nm) into two separate pathways before reaching the two cameras

(Fig 1); the photon counting is measured for each detection on both cameras and the calculated photon ratio, R, is related to the spectral separation; R thus represents the lambda identity card or the fluorophore identity card. Two parameters have a main role at this point of the process: the range chosen for λ-assignment and the percentage of rejected molecules. The goal is to minimise crosstalk to best separate different structures, while still rejecting as little as possible of the emission detections for a good quality reconstruction.

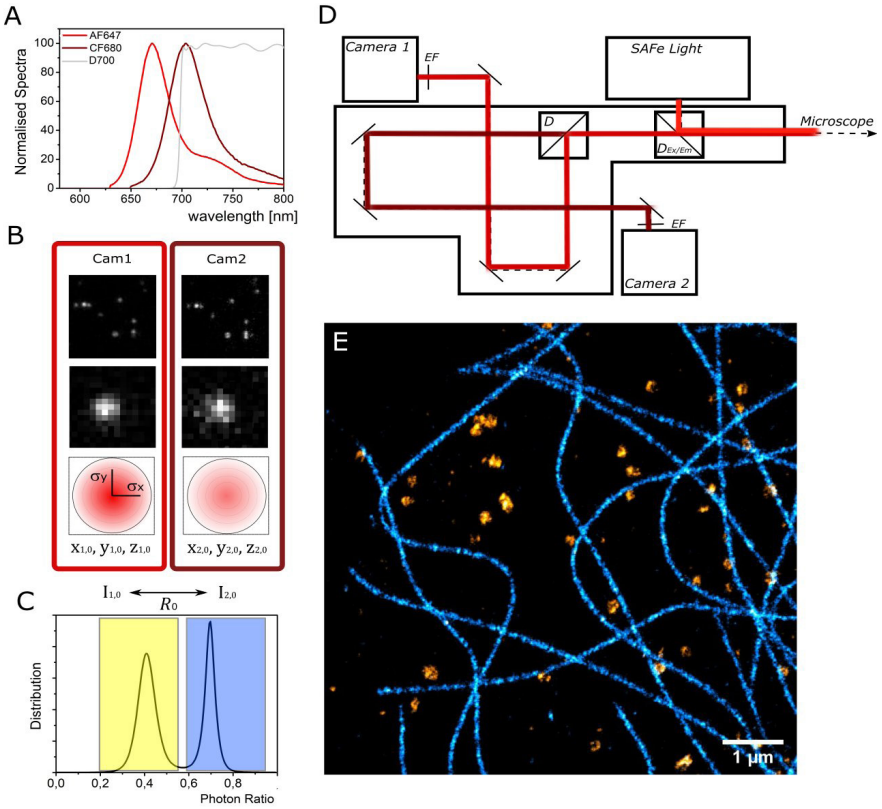

Fig 1. *Spectral Demixing Principle. Normalised spectra of two typical red dyes AF647 and CF680 are shown together with the long pass dichoic filter at 700nm to separate the two emission (**A**); example of PSF recordings on Cam1 and Cam2 (**B** top), zoom in (**B** middle) and 2D gaussian PSF representation to evaluate centre position and photon counting (B bottom); photon ratio distribution and example of ranges chosen for λ assignment (**C**); optical pathway adopted for the spectral separation: a first dichroic separate excitation from emission; the emission light is then splitted with a second long pass dichroic into the two cameras (**D**); example of image reconstruction of Cos7 cell labelled for tubulin AF647 and Clathrin CF680 (E).*

With this spectral demixing approach only one laser is required, as the fluorescent dyes used are all excited in the same spectral region **(Fig 2)**. Furthermore, chromatic aberrations are reduced and there is no need for drift correction between different excitation colors as the wavelength information is recorded in one single acquisition. The easiness and robustness of the process is such that straightforward multicolor imaging at the nanoscale is gained both in 2D and in 3D, assuring a maximal crosstalk of 10% in the worst case scenario for three dyes in 3D (with typical values kept below 1% crosstalk for two dyes in 2D).

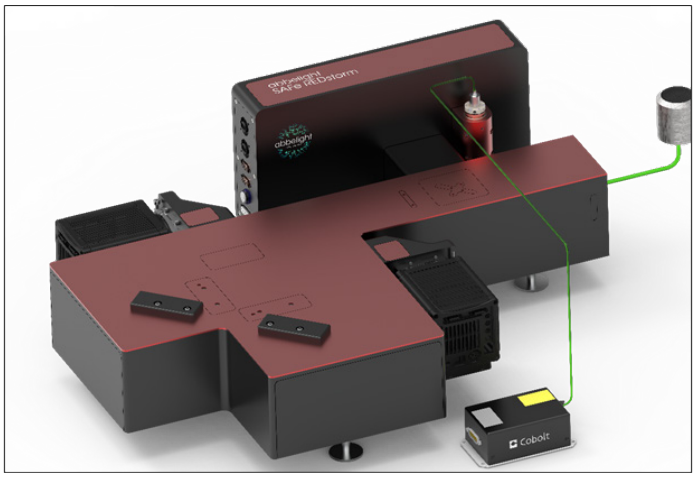

Fig 2. *Schematic illustration of the SMLM multicolor set-up using a single red laser at 640 nm coupled into the imaging set-up via single-mode fiber.*

This new spectral demixing strategy is implemented in Abbelight's dedicated system SAFe RedSTORM as well as in the highest range Abbelight SAFe360. Both systems are equipped with the SAFeLight module which integrates ASTER (Adaptable Scanning for Tunable Excitation Regions) technology[14]. This is a versatile illumination technique that generates uniform excitation in EPI, Hilo or TIRF mode over adaptable fields of view up to a 200x200 µm² field of view, 25 times larger than the standard on the market **(Fig 3).**

Lasers for SMLM

In SMLM, high irradiance (several kW/cm²) is crucial to reach a single molecule regime and hence assuring quantitative imaging at the nanoscale. It becomes even more critical while imaging simultaneously 3 fluorophores at the density needed for SMLM over large fields of view. Furthermore, higher irradiance allows an increase in the blinking rate which in turn results in faster imaging at the nanoscale.

The need for high irradiance to excite multiple fluorophores with nanoscale resolution over large field of view's (FOV) and at high speeds translates into the need for high power laser sources. In this regard, the availability of new high performance laser sources in the red spectral range is facilitating the development of simplified multicolor SMLM imaging technology with high throughput capability. The Cobolt Rogue™ CW laser at 640 nm and with 1W, is suitable for simultaneous excitation of red dyes, as AF647 and CF680. The diode-pumped laser emits a diffraction limited, single transverse mode TEM00 beam which allows efficient coupling into single-mode polarization maintaining fibers for efficient delivery into the microscope.

This laser is based on Pr3+ doped laser crystals, which is a relatively new class of diode-pumped laser sources on the market. The realization and industrialization of this laser technology has been made possible thanks to developments in pump diode technology in the visible range. An advantage with theses lasers is that they emit directly in the visible range, which means that the output power can more easily be scaled in small formats by operating the laser as multi-longitudinal mode. Another advantage with the multi-longitudinal mode operation in imaging applications is that the broader optical spectrum reduces the coherence length, which reduces the impact on image quality from speckle effects or interference from surfaces in the beam path. This is of particular importance in imaging applications where larger areas are illuminated.

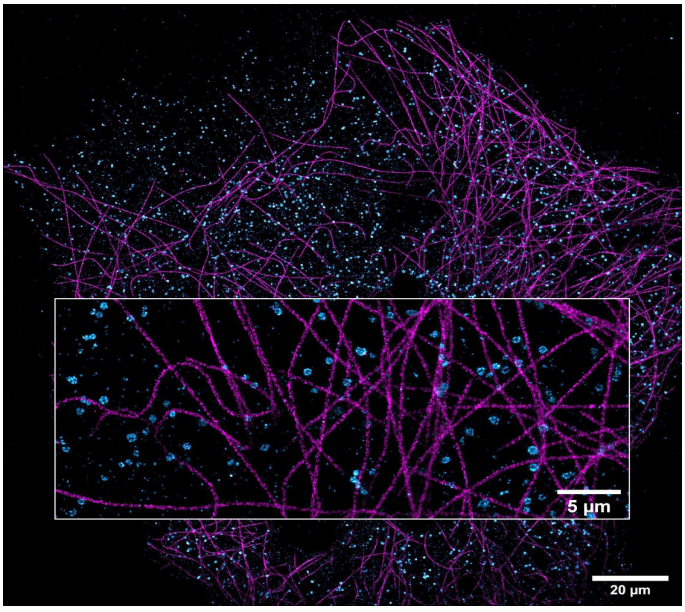

Fig 3. *Image reconstruction of a two simultaneous color acquisitions on cos7 cells labelled for tubulin-Af647 in magenta and clathrine-CF680 in cyan. Sample prepared by Karoline Friedl (NeuroCyto Lab)*

The multi-longitudinal operation in combination with low intensity noise, is fairly unusual for diode-pumped laser technology in the visible spectral range. Typically, diode-pumped CW lasers in the visible are based on intra-cavity frequency conversion and in such configurations, it is important to operate the laser on one single-longitudinal cavity mode only to avoid the intensity noise arising from chaotic fluctuations in nonlinear losses between competing modes in a multi-longitudinal mode cavity, so called "green noise".

Single-longitudinal mode (SLM, or single-frequency) operation is usually achieved by intra-cavity spectral filtering mechanisms, which to some extent reduces the output power.

As the Cobolt Rogue™ is operating at the fundamental wavelength and without frequency conversion, it is possible to operate the laser multi-longitudinal mode and still with low intensity noise **(Fig 4a)**. This means that spectral filtering is not necessary, and thus allows power scaling of the output.

By using a laser technology that emits directly in the visible, in a multi-longitudinal mode configuration and combined with an assembly technology based on high precision mounting of miniaturized optics onto thermally controlled, hermetically sealed platforms, it allows 1W output power to be achieved in a perfect TEMoo beam **(Fig 4b)** with very high level of output stability, also in varying ambient conditions, from a laser housing size of just 115x55 mm.

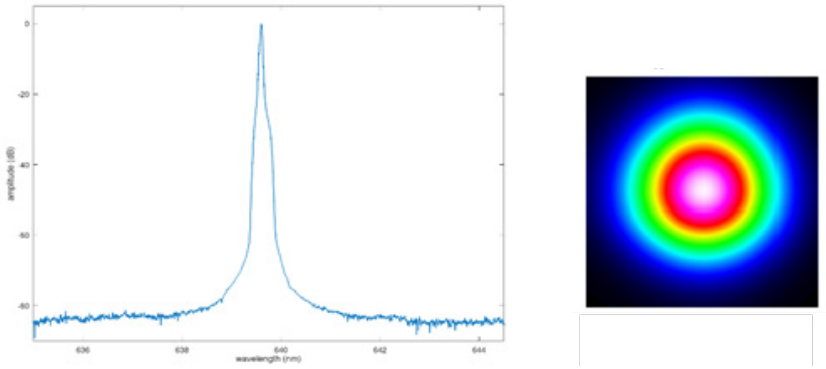

Fig 4. a) *The spectral bandwidth of the Cobolt Rogue™ laser is 0.2 nm, which translates into a coherence length of about 2 mm*
b) *TEMoo beam profile of the Cobolt Rogue™ with M2<1.1.*

Outlook

Combining the compact high power red output of the Cobolt Rogue™, delivered in a single-mode polarization maintaining fiber, with Abbelight's new product SAFe RedSTORM focused on a ratiometric approach to spectrally separate the fluorescence emission contributions of red emitting dyes has enabled the realization of a multicolor 3D nanoscopy set-up capable of delivering images at the nanoscale over fields of view up to a 200x200 µm² with a localization precision of 15 nm, which is easy to integrate and use and therefore broadly accessible.

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Abbelight *is the result of 10 years of academic research on cutting-edge detection methods in SMLM Single Molecule Localisation Microscopy. Our focus is to develop innovative instruments that matter to research and researchers. Our solutions accelerate the entire imaging workflow, from sample preparation, image acquisition to data analysis. Our aim is to equipe you with a state of the art nanoscope for you to produce reliable and reproducible data allowing to reveal and fully exploit the complexity of your data. Thus still fully immersed in the research environment, abbelight converts the most advanced technology into robust, evolutive, and accessible products.*

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About *HÜBNER Photonics*

HÜBNER Photonics *is committed to supplying high performance and innovative lasers that meet or exceed the market's expectations concerning quality, reliability and robustness. HÜBNER Photonics offers the full range of high performance Cobolt lasers,the VALO femtosecond fiber laser, the CW tunable laser C-WAVE and a full selection of C-FLEX laser combiners. Through continuous technology development, customer orientation and an ISO certified quality management system, HÜBNER Photonics has become a preferred supplier of lasers to major instrument manufacturers and leading research labs for cutting-edge applications in the areas of fluorescence microscopy, flow cytometry, Raman spectroscopy, metrology, holography, nanophotonics and quantum research. HÜBNER Photonics has manufacturing sites in Kassel, Germany and Stockholm, Sweden with direct sales and service offices in USA and UK.*