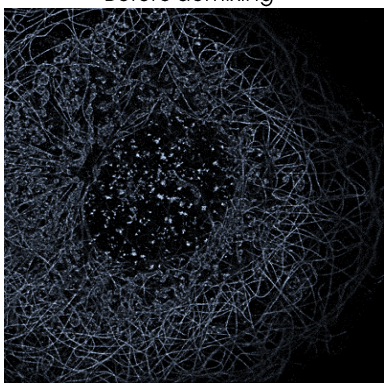
How to obtain simultaneous multicolor nanoscopy with one laser

Single-molecule localization microscopy is particularly adapted to observe colocalization between different small biological structures. There are two methods to obtain multicolor images: acquiring different colors sequentially or using dichroic cubes to allow simultaneous multicolor imaging in spectral demixing.
Spectral demixing uses a single laser to excite two or three spectrally close fluorophores. This technique eliminates several problems, including color cross-talk, chromatic aberration effects, and problems with drift between sequential color acquisitions.
Here, we describe sample preparation and algorithms developed to do spectral demixing.

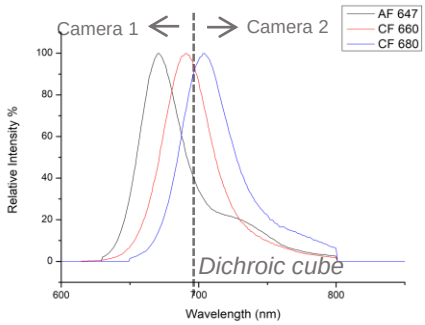
Before demixing



In practice

What do I need to do Far red Spectral Demixing ?

- Excitation with 1 laser (640nm)
- Dichroic cube (700 nm)
- Smart Kit, abbelight buffer
- Standard commercial fluorophores (AF647, CF660, CF680)
- A SMLM microscope with 2 cameras, to measure the intensity ratio between the 2 cameras
- A software including spectral demixing analysis



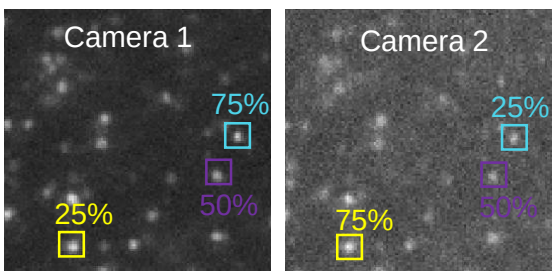
The far-red dyes emission is spectrally separated by a dichroic cube and localized on each camera.

What type of fluorophores can I use?

The three far-red dyes AF647, CF660 and/or CF680, which are all highly performing in terms of brightness and duty-cycle in the same conventional blinking buffer, can be used for single-molecule spectral demixing.

How can I isolate individual dyes ?

The dichroic cube splits the fluorescence intensity of each fluorophore differently. AF647 is split 75% - 25% between short (camera 1) and long (camera 2) wavelength channels; CF660 is split 50%-50%; and CF680 is split 25% - 75%.



Intensity ratio:

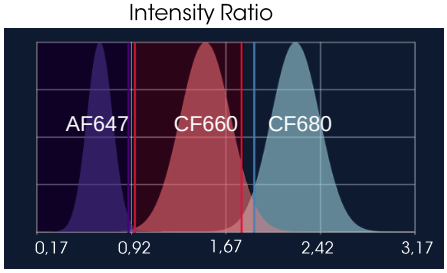
75%-25% → AF 647

50%-50% → CF 660

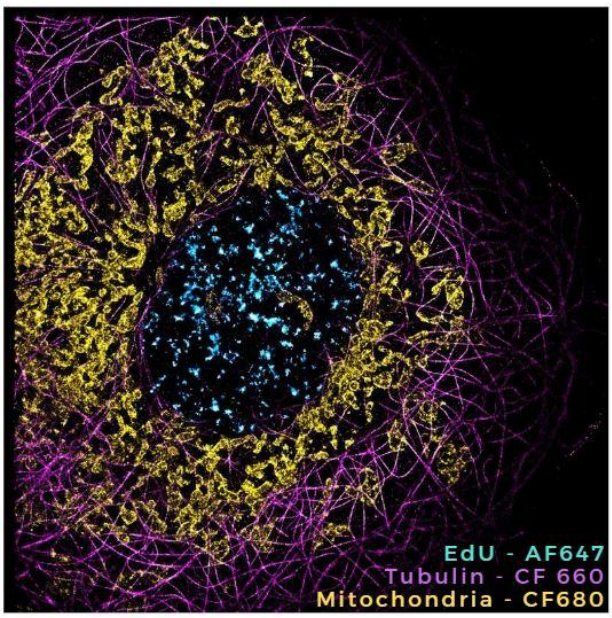
25%-75% → CF 680

To determine the characteristic intensity ratio of each fluorophore, a calibration is required.
For example, for AF647, we use a sample stained only with AF647 and we measure the intensity ratio between the 2 cameras. This gives us the intensity ratio characteristic of AF647.

Intensity Ratio



After demixing



Do you want to know if spectral demixing could help your project? Discuss with our experts: **contact@abbelight.com**