RE-SCAN CONFOCAL MICROSCOPY: SCANNING TWICE FOR BETTER RESOLUTION AND HIGH SENSITIVITY

CARACTERIZATION AND APPLICATIONS IN MULTICOLOR MEASUREMENTS

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In confocal microscopy, the pinhole is influencing the axial resolution, the lateral resolution and the signal to noise ratio (SNR) of the final image. When the pinhole is almost closed, the lateral resolution of confocal is $\sqrt{2}$ times better than widefield. Users do not often set the pinhole so small because of the severe reduction in SNR. When the pinhole is bigger than 1 A.U., the gain in lateral resolution of confocal compared to widefield is lost.

We developed a new optics-only super-resolution technique, Re-scan Confocal Microscopy (RCM), based on standard confocal microscopy, extended with an optical unit (re-scanner) that projects the emitted light directly on a camera with double angular amplitude compared to the first scanning unit. As a result, the width of the spot is improved by a factor of $\sqrt{2}$ relative to diffraction limited resolution. With a 100 nm fluorescent bead sample, the FWHM is reduced from 250 nm in widefield down to 170 nm in RCM (Fig.1). The extra-resolution is obtained by using a very sensitive state-of-the-art detector (sCMOS or EMCCD) offering extra sensitivity and good opportunities for live cell imaging.

We quantify how the pinhole is influencing the performances of RCM and we compare it with confocal. For RCM, the lateral resolution is independent from the pinhole size: it is not necessary anymore to close the pinhole down to almost zero diameter to improve the lateral resolution. Measurements performed at different pinhole sizes show how the axial resolution of RCM improves with 10% compared to confocal. We compare the SNR of confocal and RCM for the same light dose and same pinhole size and we show how the detector doubles the SNR in RCM compared to confocal.

We show how the RCM can be equipped with different excitation laser lines and emission filters, allowing imaging of multicolor samples in a subsequent frame-by-frame manner. In this imaging mode, we show how RCM is capable of imaging 3D multicolor stacks and measuring FRET interactions between TagGFP and TagRFP during Caspase-3 mediated apoptosis in HeLa cells (Fig.2).

If imaging frame-by-frame is too slow for a particular biological application, we show how the scanning and the re-scanning units can be separately controlled to direct the emission light coming from different excitations in different areas of the camera, allowing the imaging of two colors with a time difference now reduced down to the time to image a single line. For these imaging settings, we show how RCM is capable of fast and sensitive ratiometric quantification of intracellular pH (pH$_i$) in live yeast cells expressing cytosolic pHluorin. Because of the high speed of the imaging, RCM captures fast pH change when glucose is added to starved yeast (Fig.3).


Fig. 1: Widefield (left) vs RCM (right) imaging of 100 nm beads. Scale bars are 100 nm.

Fig. 2: FRET interaction (graph) in HeLa cells before (left) and after (right) apoptosis.

Fig. 3: Ratio image of pH, of starved yeast before (left), during (center) and after (right) glucose addition.