FRET-nanoscopy: A hybrid approach with Ångström resolution.

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Introduction The ultimate goal of nanoscopy is to deliver molecular resolution. We present a hybrid FRET-nanoscopy approach where we make the transition from 2D to 3D at a molecular scale. We use localisation on two-channel STED (colocalization, cSTED) images to resolve xy-projections with high precision (<4 nm) at any length scale (seamless resolution). Under these conditions we obtain accurate FRET distances[1] independent of the molecular orientation. Using Pythagoras' formula we make the transition into 3D molecular resolution on short (<12 nm) scales. **Method** FRET spectroscopy requires the presence of a donor (atto594) and acceptor (atto647N) dye. We deplete both using a single depletion lase r (775 nm) resulting in a super-resolved image (fig 1a). We determine the center for the donor and acceptor spot using localization and obtain the distance. Next, Efficiency and lifetime FRET indicators are collected for each super-resolved spot. We apply correction factors and ensemble averaging to obtain accurate FRET-based distances under STED conditions (<5 Å). Uniquely, FRET measures distances independent of molecular orientation (hypotenuse), whereas localization measures the projected xy-distance (adjacent), using Pythagoras' theorem they synergistically combine to determine the angle of the protein. **Results** We apply FRET-nanoscopy on a rectangular origami platform labelled with two FRET pairs separated by 75nm (fig 1a). A pair showing no FRET (14.4 nm, bottom) and a pair showing FRET (5.2 nm, top). All dyes on this construct are imaged and localized, yielding a localisation precision of 4 nm. By particle averaging we are able to increase our precision to 4 Å, matching our platform-grid based prediction. Lastly we apply our approach to the protein hGBP1 *in vitro*, which undergoes a conformational change to an extended state upon activation (fig 1b)[2], inaccessible to either STED or FRET alone. Using cSTED, we measure the most likely donor and acceptor distance to be 28 nm (fig 1c).

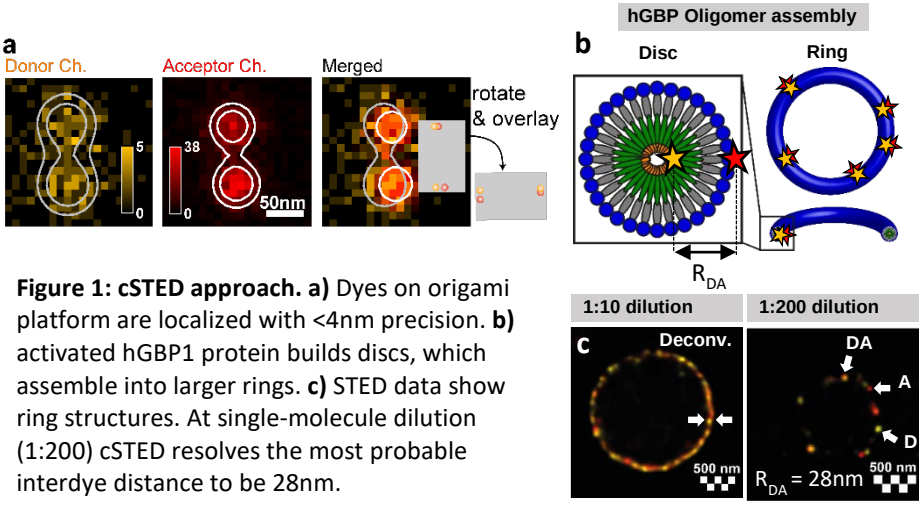

Figure 1: cSTED approach. a) Dyes on origami platform are localized with <4nm precision. **b)** activated hGBP1 protein builds discs, which assemble into larger rings. **c)** STED data show ring structures. At single-molecule dilution (1:200) cSTED resolves the most probable interdye distance to be 28nm.

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