Fig 2a. Single molecules diffuse in and out of a confocal volume of approximately 1 μm3 constructed by focusing the lasers into a diffraction limited spot and using lenses and a pinhole to select light in a thin focal plane. Alternating laser excitation (ALEX) 20,000 times a second between the donor (green) and acceptor (red) allows excitation of either dye multiple times per molecule as it transits the confocal volume. b. A typical time trace for an smFRET experiment, as per C, green emission under green excitation is shown in green (DexDem), red emission under green excitation shown in red, and red emission under red excitation is shown in purple. Encircled bursts from left to right are acceptor only, donor only, and doubly labelled. c.Equations for E and S. E\* is the apparent FRET efficiency (see next section), it is a ratiometric measure which scales with distance. S, stoichiometry, is a measure of the presence of either dye, which can be assessed from the acceptor excitation of ALEX. d. A 2D ES histogram showing dye stoichiometry against FRET efficiency. Donor only molecules appear with low E\* but high S, and acceptor only molecules appear with low S. Doubly labelled molecules appear with intermediate S.

smFRET techniques typically achieve the high signal to noise ration needed to detect single molecules by using a reduced detection volume. This can be done using total internal reflection (TIRFM) for immobilised molecules, or using a diffraction limited spot for freely diffusing molecules as in the smfBox. By alternating the excitation laser between donor and acceptorwavelegths, not only can FRET efficiency be measured (under donor excitation), but apparent dye stoichometry can also be accessed. In this way, a low FRET efficiency molecule can easily be distinguished from a donor in the absence of an acceptor, which may arise from incomplete labelling or photobleaching

**Fig 3.** Schematic of how accurate FRET works. **a.** The ideal situation; the donor is excited by a laser and emission is separately detected from both the donor and acceptor with equal efficiency. **b.** The problem; the acceptor can be directly excited by the laser due to the shorter wavelength tail of its absorption spectrum and the donor can leak emission into the acceptor detection channel due to the longer wavelength tail of its fluorescence spectrum. Furthermore, detection efficiencies and quantum yields are not equal. **c.** The solution; in addition to exciting the donor, the acceptor is also excited (at a longer wavelength) to distinguish the presence of either dye and gain access to comparable quantities to calculate correction factors. The amount of direct excitation of the acceptor with the donor excitation laser is corrected for by Delta. Beta relates the quantity of excitation of each dye by its corresponding laser (dependent on the relative excitation efficiencies and laser powers). Gamma relates the relative emission and subsequent detection efficiencies of each dye. Αlpha corrects for the emission of the donor into the acceptor channel.

The alignment tool will show incoming light in photons per millisecond on either APD without alternating the laser or plotting histograms. This means it can display a much higher incoming photon count without crashing. To align the emission pathway, place a fluorescent sample (~1 uM) on the coverslip which will emit in both channels when excited with the donor excitation laser (we use free cy3B), then maximise the emission intensity reported by iteratively adjusting the positions of the APD lenses (L6 and L7), the pinhole, and if needed M3 and L4.