

Protocol 9: Recording the photobleaching kinetics for the fluorescently labeled protein molecules.

9.1. Assemble regular microscopy chamber using a non-modified glass slide, two strips of double-sided tape, and a clean coverslip, which can be prepared using the entire Protocol 2, or only steps 2.1-2.6 of this protocol.

9.1.1. Add approximately 50 nM protein in motility buffer, wash briefly with motility buffer and seal the chamber with VALAP. Optimize protein concentration to obtain the field with evenly dispersed spots, representing single molecules and their small aggregates (trimers and tetramers, which may form spontaneously in the solution or may appear when several single molecules are close together and cannot be resolved). This step is very important for obtaining a multi-peak distribution of photobleaching steps and accurate determination of a step size (see below).

9.1.2. Minimize the illumination laser intensity at which the individual fluorescent spots are still visible; with lower illumination the photobleaching time is extended, so longer photobleaching traces can be obtained. Also minimize the exposure time to reduce the probability of more than one fluorophore bleaching during one frame. The recommended setting for Andor iXon3 camera: gain 5.0x, EM gain 999, 10MHz readout speed, 50-100ms exposure time.

Focus at the surface of the coverslip, close the illumination shutter, move to a fresh field, open the illumination shutter and acquire images until all complexes have bleached (thereafter referred to as $Img(x,y)$).