Analysis 2. Determining the intensity of single fluorophore.

2.1 Using normalized images $Img^{norm}(x,y)$ and any image processing software select a fluorescent spot with a circular region (5-6 pixels in diameter) and determine its integral intensity for all frames, generating the photobleaching traces. Avoid very bright dots (> 5-fold brighter than the dimmer ones).

2.2. Using the same circular region tool, select at least 3 spot-free areas, generate the corresponding photobleaching traces, average and fit with exponential decay function.

2.3. Tabulate this background intensity curve to match the experimental time points and subtract it from the photobleaching curves.

2.4. Smooth the photobleaching curves (average with the sliding window of 3-5 points). Visually inspect the resulting curves and discard any curve that shows an abrupt increase in fluorescence or lack of obvious bleaching.

2.5. For each of the remaining curves (usually 50-70% of the total number of curves), visually select the final plateau, when the fluorescent spot has bleached. Shorten this segment to leave only ~ 100 points and average these intensities. Subtract this value from the shortened photobleaching curve to minimize small variations is the background levels and to reduce the size of the background peak (below).

2.6. Plot a histogram of the intensities for all time points from 20 or more photobleaching curves (>1,000 time points). The histogram should exhibit at least 4 distinct peaks (see Note 2).

2.7. Fit the histogram with equidistant Gaussian distribution using MatLab, Mathematica or similar software:

$$\sum_{i=0}^{N} A_i\, e^{-\frac{(x-i\,d)^2}{2\sigma_i^2}}$$

where A_i and σ_i, d and N are fitting parameters. Parameters A_i and σ_i correspond to amplitude and width of i-th peak; d is distance between peaks; N is integer number, which corresponds to the total number of peaks in the distribution. If centers of the first 3 or more peaks show a visually good match to the fitted line, the distance between these peaks (parameter d) corresponds to a fluorescent intensity of a single fluorophore.

Note 1: Number of examined dots should be increased if the microscopy system exhibits significant vibrations or there is another source of noise (e.g. unstable laser beam).

Note 2. It is essential to obtain > 3 peaks for accurate analysis with equidistant Gaussian fit. If fewer peaks are obtained, the false (e.g. double) step size could be obtained when the

illumination conditions are not optimal, e.g. when the dots bleach too fast and single steps are not resolved well.