

Determination of the intensity of a single fluorophore

This protocol is used to determine intensity of one fluorophore molecules. In our case we determined the intensity GFP and Alexa 647 molecules. For GFP, this procedure was done twice for different microscopic settings corresponding to experiments with monomeric and clustered CENP-T. The protocol below was written for GFP molecules with microscope settings corresponding to experiments with clustered CENP-T. For other microscope settings, the same protocol can be applied with only modification in imaging settings. Exact imaging settings for experimenting with monomeric CENP-T are provided in the protocol “TIRF microscopy assay to study the interactions between monomeric CENP-T and soluble Ndc80”.

Equipment

- Nikon Eclipse Ti microscope equipped with 1.49NA TIRF 100 Oil objective and laser illumination that can be used in TIRF mode
- Coherent CUBE 488-nm diode laser
- Andor iXon3 EMCCD camera
- Objective heater (Biopetechs)
- Syringe pump (New Era Pump Systems, NE-4000)

Materials

- Glass coverslips (VWR, cat # 48366-067)
- Glass slides (VWR, cat # 48312-004)
- Double-sided tape (Scotch, cat # 504829)
- VALAP

Reagents

- Catalase (Sigma-Aldrich, cat # C40) is prepared at 2 mg ml⁻¹ and stored as 10-μl aliquots at -80°C.
- DTT (Thermo Fisher Scientific, cat # 15508) is prepared at 1 M in Milli-Q water, filter sterilized, and stored as 10-μl aliquots at -80°C.
- Glucose (Sigma-Aldrich, cat # G8270) is prepared at 600 mg ml⁻¹ in Milli-Q water and stored as 10-μl aliquots at -80°C.
- Glucose oxidase (Sigma-Aldrich, cat # G2133) is prepared at 10 mg ml⁻¹ and stored as 10-μl aliquots at -80°C.
- Mg-BRB80 buffer. 80 mM PIPES, 4 mM MgCl₂, 1 mM EGTA; pH 6.9. Buffer is prepared in advance and filtered, stored at -20°C for months and at +4°C for few weeks.
- CENP-T(1-242aa)-GFP-Spy-tag stored at -80°C.

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1. Record photobleaching kinetics for the fluorescently labeled protein molecules

1. Assemble several regular microscopy chambers using nonmodified glass slides, two strips of double-sided tape, and a plasma cleaned coverslips (plasma clean for 10 min at 30 W, 200-400 mTorr pressure).
2. Evaluate the dirtiness of the coverslips. For that goal:
 - 1) assemble microscopic chamber as described in (Chakraborty et al. 2018)
 - 2) perfuse the imaging buffer into the chamber (Mg-BRB80: K-PIPES 80 mM, pH 6.9, 4 mM Mg₂, 1 mM EGTA, supplemented with 10 mM DTT, 0.1 mg ml⁻¹ glucose oxidase, 20 µg ml⁻¹ catalase, 6 mg ml⁻¹ glucose, and seal the chamber with VALAP.
 - 3) focus at the surface of the coverslip and examine a number of bright spots on the coverslip. For single molecule imaging we used following settings: for Andor iXon3 camera: gain 5.0x, EM gain 999, 10 MHz readout speed, 30 msec exposure time; 50% 488 nm laser power. It is important to minimize the exposure time to reduce the probability of more than one fluorophore bleaching during one frame. For that goal, imaging with high camera readout speed (10 MHz) is reasonable, because it leads to decrease of minimal exposure time.
 - 4) if number of bright spots on the coverslip is high, we recommend to prepare new chambers and reagents. Dirty coverslip with many bright objects on the coverslip may add artifacts to the single GFP molecule analysis. If you see just few spots per imaging field as shown on Figure 1A you are good to proceed.
3. Assemble next chamber and freshly prepared dilution of CENP-T-GFP protein in imaging buffer (see “Protein preparation and protein handling” Chakraborty et al, 2018). We recommend to start with 40 pM GFP-tagged protein. Perfuse the protein into the chamber and incubate for 5 min. Wash well with imaging buffer (50 µl imaging buffer, wait for 1 min and wash again) and seal the chamber with VALAP.
4. Focus at the surface of the coverslip, evaluate the density of GFP spots. They should appear as individual spots with quantity that is significantly higher than on empty coverslip. If density of dots is too high or low, optimize the concentration of the protein to obtain fields with evenly dispersed GFP spots (Figure 1A).
5. Close the illumination shutter, move to a new field, open the illumination shutter and acquire images until all complexes have bleached.

2. Record data to build the laser illumination profile

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We found that for TIRF imaging it is very important to build laser intensity profile on the same microscopic and laser setting, as used for single molecule imaging. Any changes in laser power, introducing of different filters, or changes in TIRF angle will lead significant change in complex laser pattern on the coverslip. For that reason imaging of high concentration of fluorophore, which was suggested for epifluorescence in (Volkov et al., 2014), cannot be used in case of TIRF imaging, because it is not possible to perform imaging on the same settings, as was used for single molecules. To overcome this problem averaging of hundreds of fields with high density of GFP spots could be used. To get such data:

1. Assemble a chamber as described above at Section 1.1.
2. Add approximately 400 pM GFP-tagged protein in imaging buffer, and seal the chamber with VALAP. Optimize protein concentration to obtain high density of GFP spots (Figure 1A). Confirm, that such concentration of GFP spots does not lead to camera saturation in any point of imaging field.
3. Collect >150 images of the entire microscope field: move the stage to a new unbleached area while the illumination shutter is closed, and acquire the images immediately after opening the shutter.
4. With the closed illumination shutter and using same camera settings acquire three image to determine camera noise (CN).

3. Correct the acquired photobleaching kinetics for unevenness of laser illumination

1. Determine the camera noise as the average pixel intensity of images with closed illumination; average based on three repeats. Resulting value corresponds to CN. For all procedures with images here and below use the the Fiji (ImageJ) software (Schindelin et al. 2012).
2. Subtract CN from each image of GFP spots at high density (400 pM). Create average projection of stack containing these images (Figure 1B).
3. Filter resulting averaged image with Gaussian blur with 5 pixel radius to get the laser intensity profile (Figure 1B; $(Illum(x,y)-CN)$, where x and y correspond to pixel's coordinates).
4. Determine the maximum pixel brightness of this image ($Max(Illum-CN)$). Be aware, if this value is close to saturation values on your camera settings, it may lead to artifacts in image analysis.
5. Subtract CN from each frame of bleaching of GFP spots at low density (40 pM; $(img(x,y)-CN)$).

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6. Use the above values and image ($Illum(x,y)-CN$) to normalize the experimental image ($img(x,y)-CN$) using the following expression:

$$img^{norm}(x,y) = \frac{Max(Illum) - CN}{(Illum((x,y) - CN)} (img(x,y) - CN)$$

Use the resulting image $img^{norm}(x,y)$ for the quantitative analysis of the brightness of the fluorescent molecules.

Efficiency of image correction on unevenness of laser illumination can be verified by image intensity plots. We show GFP spots at high density before and after correction (Figure 1A, B), corresponding intensity plots shows (Figure 1C,D), that, image intensity profile is much more smooth and GFP peaks are more homogenous after correction procedure.

4. Calculate the intensity of single fluorophore molecule

1. For analysis of GFP bleaching experiment use normalized images $img^{norm}(x,y)$ from previous step of the protocol (Figure 2A).
2. Select GFP spots with a circular region (Figure 2B) and determine its integral intensity for all time frames, generating the photobleaching traces. Avoid very large aggregates causing camera saturation. The size of region can be adjusted based on the pixel size of camera, it should be a minimal size region, which completely includes the GFP spot. For our microscope settings the optimal region radius is 3 px.
3. Select circular region of the same size, as on previous step, near GFP spots (Figure 2B) to determine the background integral intensity for all time frames, generating the background photobleaching traces. Average background values for each time frame (Figure 2C).
4. Subtract the averaged background values from the photobleaching curves for each time frame (Figure 2B). Visually inspect the resulting curves and discard any curve that shows an abrupt increase in fluorescence or lack of obvious bleaching, usually we discard about 50-70% of the total number of curves.
5. 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 photobleaching curve to minimize small variations is the background levels.
6. To reduce the size of the background peak keep ~15 points after the bleaching event and smooth curves with the sliding window of 4 points (Figure 2E). We used built Savitzky-Golay filter built in Prism Graphpad software with second order polynomic function.

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7. Plot a histogram of the intensities for all time points from 20 or more photobleaching curves ($>1,000$ time points; Figure 2F). Fit the non-zero peak of the histogram with Gaussian distribution to determine the mean value of single GFP intensity. In case if histogram contains multiple non-zero peak, the equidistant Gaussians can be used for fitting, as described in (Volkov et al., 2014).
8. At the ideal situation the histogram should exhibit at least >2 distinct peaks, as it was shown for Dam1 protein complex in (Volkov et al., 2014). However, most of the proteins do not have tendency to oligomerize. As result, the final distribution of GFP intensity in bleaching experiment has just one major non-zero peak (Figure 2F). In this situation additional conformation is needed to demonstrate that one photobleaching step corresponds to single GFP molecule, but not dimers or oligomers of GFP protein. To confirm accuracy of this analysis, time of photobleaching can be estimated and compared with time resolution in bleaching experiment. These two numbers allow to estimate the probability of two GFP molecules bleaching during one time frame. For that goal:
 - 1) Measure number of GFP spots over time using $img^{norm}(x,y)$ (Figure 2G).
 - 2) Fit the resulting curve with exponential decay function: $Y = Y_0 e^{-kx}$ to determine the coefficient of the exponent k and half life time $\frac{\ln 2}{k}$.
 - 3) Compare the half life time with time resolution of your experiment to estimate the probability of bleaching of two GFP molecules at the same time. In our case, half life time of GFP molecules was about 2 sec, that is significantly higher than 30 ms interval between time frames.

The initial distribution of GFP spots intensities could also help to validate the intensity of single GFP molecule in case, when only one major non-zero peak is present. If the dominant fraction of GFP spots are single molecules, the distribution of initial brightness should be similar to distribution obtained after photobleaching analysis (Figure 2H).

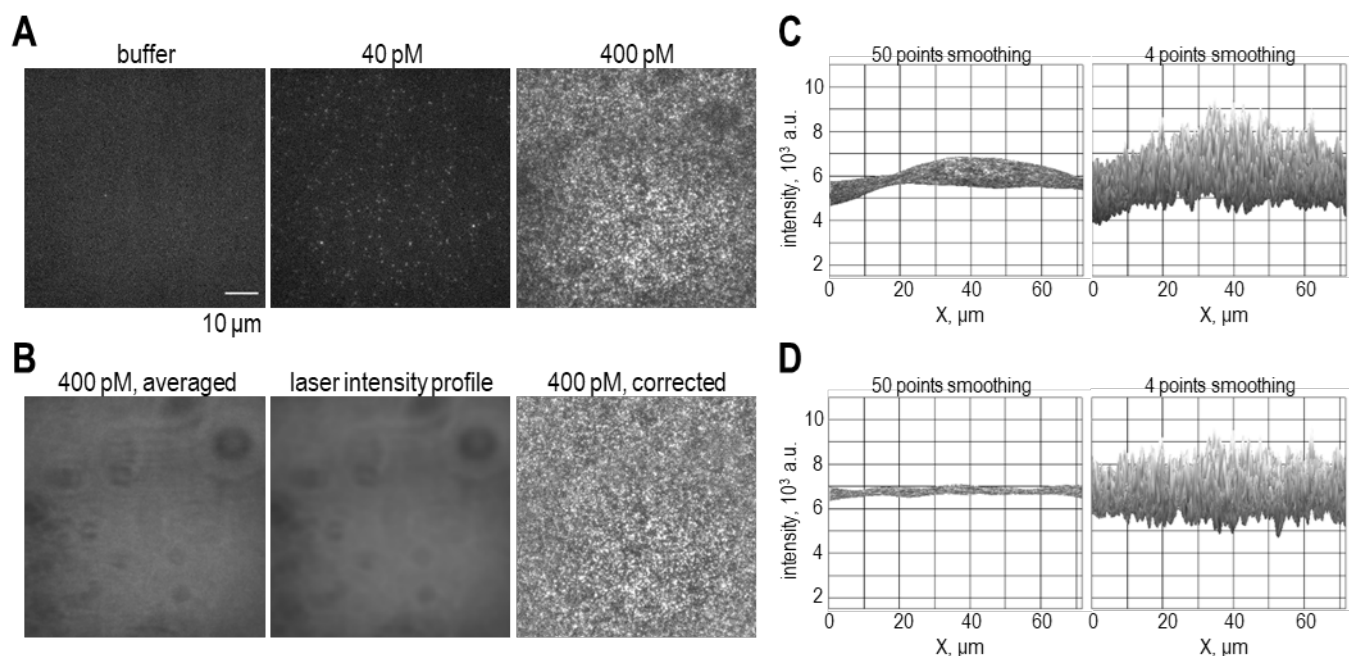
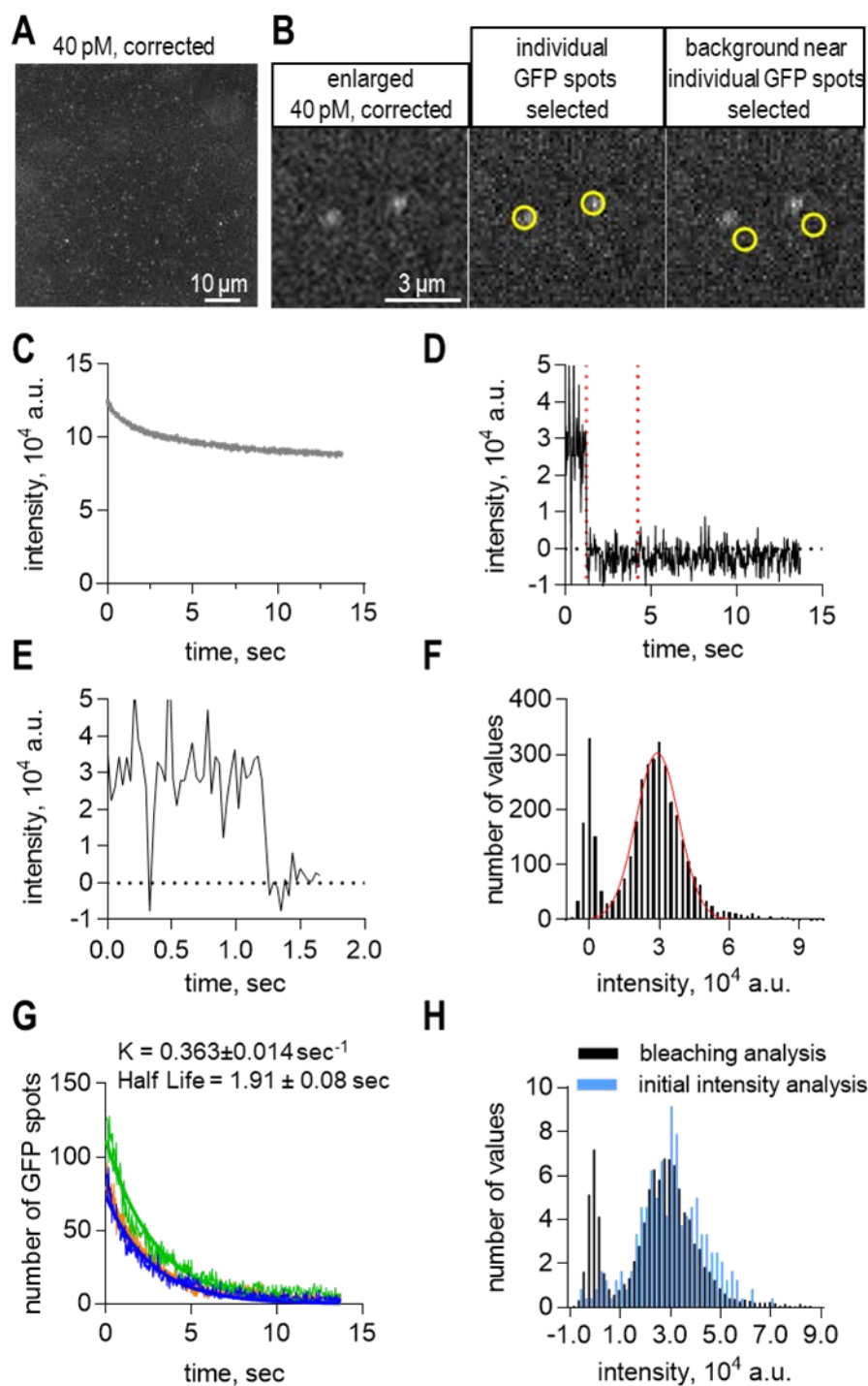


Figure 1. Illustration of correction of illumination unevenness. **A** TIRF images of GFP-labeled protein attached to the coverslip, showcasing rare fluorescent spots in the imaging buffer, as well as dispersed and highly concentrated GFP spots at the indicated concentration. **B** The left image displays the average projection of multiple fields with 400 pM GFP-labeled protein attached to the coverslip. In the center of panel B, the image is shown after the application of a Gaussian blur filter, while on the right, the result of the correction for uneven illumination is presented. **C** Surface plots illustrating the illumination profile with different levels of smoothing prior to the correction for uneven illumination. **D** Surface plots representing the illumination profile after the correction, with different levels of smoothing.

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Figure 2. Determination of fluorescence intensity of a single GFP fluorophore. **A** Representative image of a microscope field with single GFP molecules immobilized on plasma-cleaned coverslip. **B** Enlarged view of image A. Yellow circles highlight typical GFP spots used for analysis in the middle image, while yellow circles in the left image indicate typical background spots used in the analysis process. **C** Averaged intensity profile of background spots over time. **D** Example photobleaching curve for a single GFP molecule after background subtraction. **E** Photobleaching curve of a single GFP molecule after smoothing using the Savitzky–Golay filter. **F** Histogram of integral intensities collected from 60 bleaching GFP dots from 3 independent experiments. Red line is fit to Gaussian function. Peak value of $1.56 \pm 0.04 \times 10^4$ a.u. corresponds to the integral intensity of a single GFP fluorophore under our imaging conditions. **G** The plot of number of GFP spots on the image field over time, used to determine the half-life of a single GFP molecule. Different color curves corresponds to different experimental repeats. **H** Histograms illustrating the similarity between the initial GFP brightness of CENP-T-GFP spots immobilized on the coverslip, and the distribution obtained after photobleaching analysis.