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Analysis of photobleaching rate of monomeric and clustered fluorescent molecules

The objective of this protocol is to determine the photobleaching rate of fluorescent molecules in kinetics assays. Photobleaching rate should be determined to estimate the corresponding error for selected imaging settings. Also, for some kinetics experiments the photobleaching correction is necessary to accurately determine the interaction stoichiometry. Our approach can be applied to various fluorophores. Here, we demonstrate our approach on photobleaching of monomeric and clustered CENP-T-GFP.

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 (Bioptechs)
- Syringe pump (New Era Pump Systems, NE-4000)

Materials

- Reusable glass slide with tubing (Volkov et al. 2014)
- Glass slides (VWR, cat # 48312-04) silanized as described in (Volkov et al. 2014)
- Double-sided tape (Scotch, cat # 504829)
- Sealant – silicone rubber (Smooth-On)

Reagents

- BSA (Sigma A7638) is prepared at 100 mg/mL and stored as 100-µL aliquots at -80°C.
- Catalase (Sigma-Aldrich, cat # C40) is prepared at 2 mg/mL and stored as 10-µL aliquots at -80°C.
- Casein (Sigma 5890) is prepared at 8 mg/mL and stored as 100-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 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 and stored as 10-µL aliquots at -80°C.

- Mg-BRB80 buffer. 80 mM PIPES, 4 mM $MgCl_2$, 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.
- PBS buffer. 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4; 140 mM NaCl, 2.7 mM KCl; pH 7.2 Buffer is prepared in advance and filtered, stored at -20°C for months and at +4°C for few weeks.
- Pluronic F127 (Sigma P2443) is prepared at 1% and stored as 1-mL aliquots at room temperature for a week.
- CENP-T(1-242aa)-GFP-Spy-tag.
- SpyCatcher-mi3 core particles.
- SNAP- SpyCatcher.

I. Analysis of photobleaching rate of monomeric CENP-T-GFP

1. Prepare the microscope chamber and immobilize CENP-T-GFP molecules to the coverslip as described in the protocol “TIRF microscopy assay to study the interactions between monomeric CENP-T and soluble Ndc80”. As described in this protocol, use the objective heater set at a temperature of 32°C.

2. Wash the chamber by perfusing 150 μL 1x Imaging Buffer (for more details see the protocol “TIRF microscopy assay to study the interactions between monomeric CENP-T and soluble Ndc80”).

3. Focus, and find imaging field by one image shot to avoid photobleaching.

4. Image the photobleaching of GFP-CENP-T molecules in stream-acquisition mode (no offset between imaging frames) for 1 min. Use the settings identical to those in “TIRF microscopy assay to study the interactions between monomeric CENP-T and soluble Ndc80”: TIRF mode, 1 MHz readout speed, 50 EM Gain, 5x Conversion Gain, 300 ms exposure on the Ardor iXon3 EMCCD camera; 20% 488 nm laser power.

5. To analyze the data use the Fiji (ImageJ) software.

6. Open the image sequence.

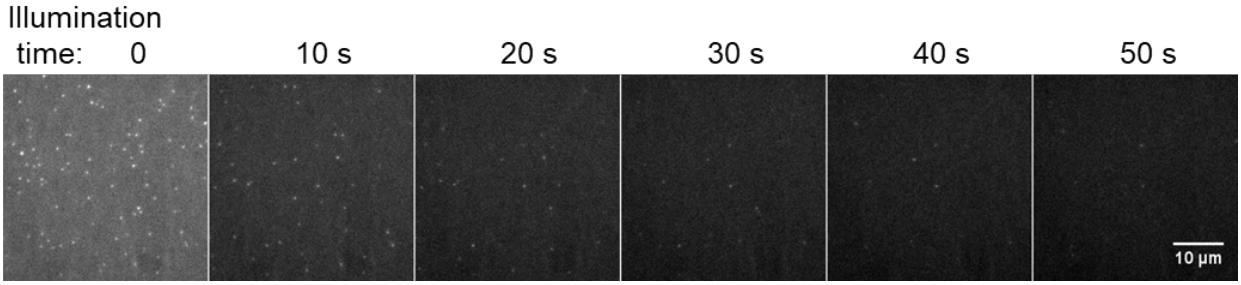

Figure 1. Examples of GFP-CENP-T spots after different illumination time.

7. Note, that there is a continuous background bleaching over time (Figure 1). To remove the impact of background use the script provided below. It removes the median background for each time frame. So, the resulting image sequence has the same background without any photobleaching (Figure 2).

```
for (i=1; i<=nSlices; i++) {
    setSlice(i);
    getStatistics(area, median);
    run("Select None");
    run("Subtract...", "value="+median);
    run("Restore Selection");
}
```

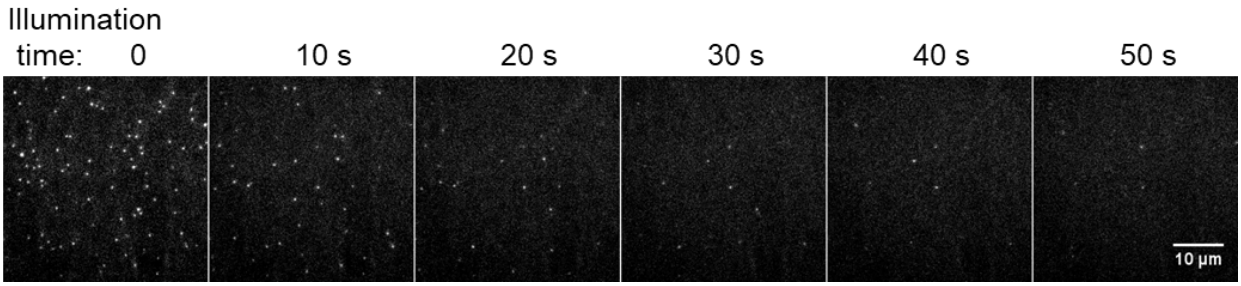

Figure 2. Background corrected examples of GFP-CENP-T spots after different illumination time.

8. Next, in the corrected image sequence count the number of GFP spots per field at all time points. It can be done using the script provided below. It selects the spots above the noise threshold. To determine correct "noise" value gradually increase the "noise" parameter until the script stops selecting background as spots. In all our experiments the noise was 1,000.

```
for (i=1; i<=nSlices; i++) {
  setSlice(i);
  run("Find Maxima...", "noise=1000 output=[Count]");

  }
```

9. Using any available software (MatLab, Excel, Prizm) plot the number of GFP spots against illumination time (Figure 3).

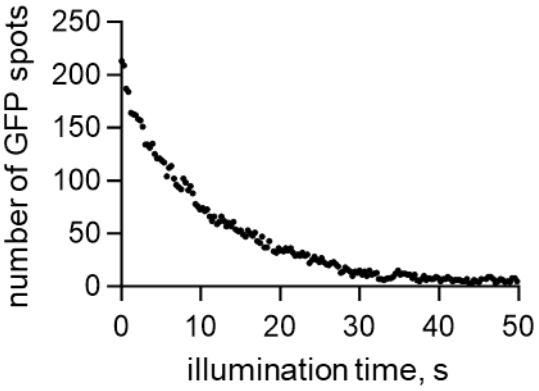

Figure 3. Example curve showing number of GFP spots against illumination time.

10. To remove variability between experiments normalize the curve by dividing it by the initial number of GFP spots (Figure 4).
11. Fit the resulting curve with exponential decay function (Figure 4): $Y = Y_0 e^{-kx}$ to determine the coefficient of the exponent *k* and half life time $\frac{ln2}{k}$.

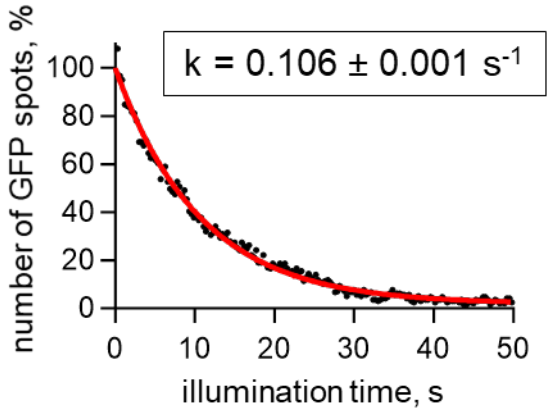

Figure 4. Example curve showing the percent of remaining GFP spots against illumination time. Black dots – experimental points from one independent experiment, red – exponential decay fitting.

12. To estimate the probability of bleaching multiply coefficient k on the total illumination time in Ndc80-binding experiment. In our case, k = 0.106 and total illumination time was 2x300 ms = 600 ms, so the probability of bleaching 6.4%.

II. Analysis of photobleaching rate of clustered CENP-T-GFP

1. Prepare the microscope chamber and assemble CENP-T-GFP clusters as described in the protocol "TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80". As described in this protocol, use the objective heater set at a temperature of 32°C.

2. Wash the chamber by perfusing 150 μL 1x Imaging Buffer (for more details see the protocol "TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80").

3. Focus, and find imaging field by one image shot to avoid photobleaching.

4. Image the photobleacing of GFP-CENP-T clusters for 30 min (Figure 5). For that, use the same microscope settings as in experiment "TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80": TIRF mode, 10 MHz readout speed, 300 EM Gain, 5x Conversion Gain, 30 ms exposure on the Ardor iXon3 EMCCD camera; 1% 488 nm laser power. Similarly to most Ndc80 binding experiments, take an image every 5 sec, and keep illumination off between imaging frames.

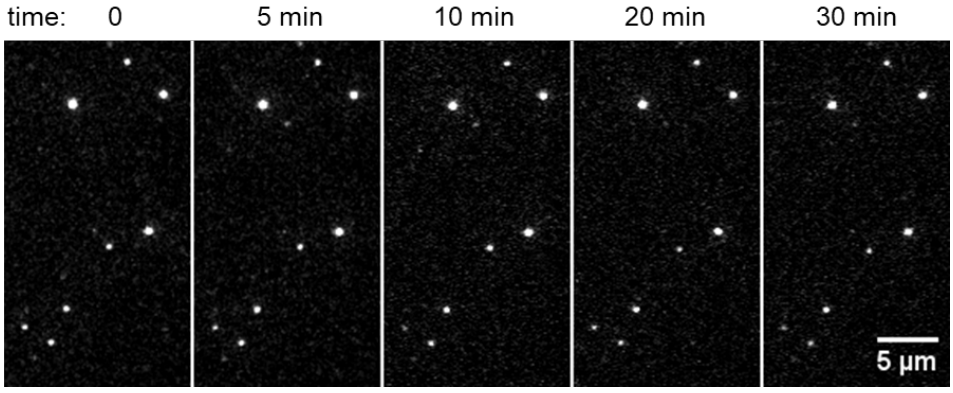

Figure 5. Time-lapse showing changes in fluorescence intensity of CENP-T-GFP clusters during the experimental time.

5. To analyze the data use the Fiji (ImageJ) software.

6. Correct the microscope stage drift, as described in protocol “TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80”.

7. Select CENP-T clusters and background nearby using circular regions, as described in protocol “TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80”.

8. Measure the integral fluorescence intensity of clusters and background, as described in protocol “TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80”.

9. Analyze resulting Excel files using any available software (MatLab, Excel).

10. Remove all samples and related background values that correspond to large clusters with a mean intensity close to the camera saturation. On our settings, the threshold for this step was 15,000 a.u.

11. Subtract individual integral fluorescent intensities of background from sample values to get the intensity of CENP-T clusters for each time point of the experiment (Figure 6).

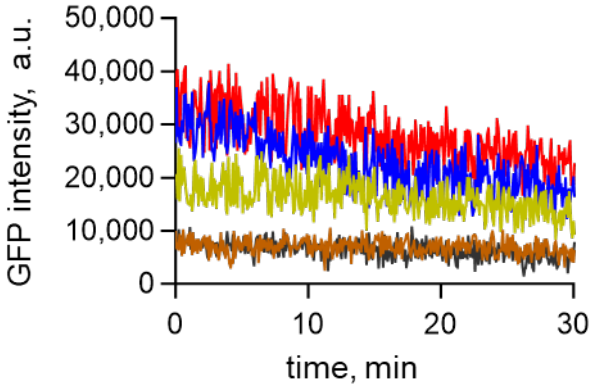

Figure 6. Example curves showing changes in GFP intensity of CENP-T-GFP clusters against time due to photobleaching. Each curve is an individual cluster.

12. For each time point, normalize the intensity of each GFP cluster by dividing it by its initial intensity (average first 5 frames; Figure 7).

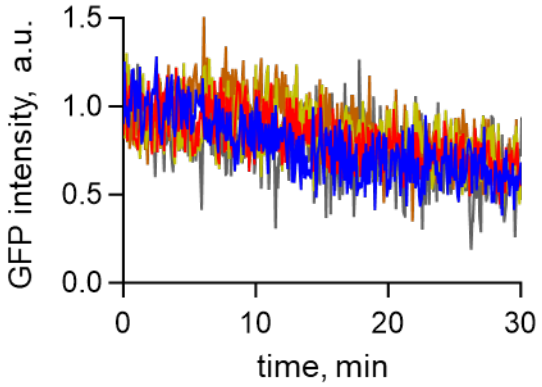

Figure 7. Example normalized curves showing changes in GFP intensity of CENP-T-GFP clusters against time due to photobleaching. Each curve is an individual cluster.

13. Plot the median of normalized intensity against time (Figure 8).

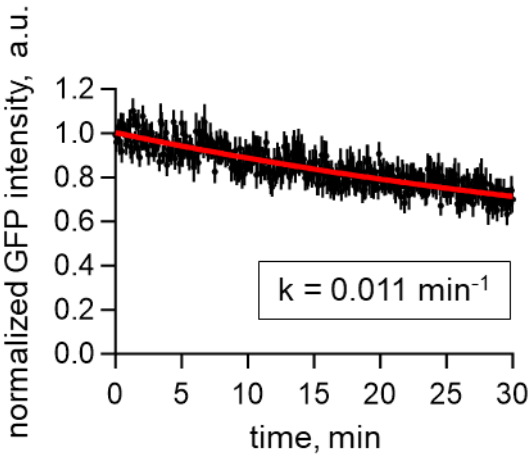

Figure 8. The median of normalized bleaching curves from one independent experiment with n = 32 clusters. Experimental data – black curve, fitting with exponential decay – red.

14. Fit the resulting curve with exponential decay function (Figure 8): $Y = Y_0 e^{-kx}$ to determine the coefficient of the exponent *k*.

15. Generate the photobleaching curve as exponential decay curve with coefficients determined on step 14.

15. The photobleaching correction procedure is described in protocol "TIRF microscopy assay to study the interactions between clustered CENP-T and soluble

Ndc80". It can be only applied, if there is no exchange of bound Ndc80 molecules on CENP-T clusters.

While a fraction of bound Ndc80 molecules associate with clustered CENP-T weakly, and undergo exchange in the presence of soluble Ndc80, photobleaching correction cannot be applied for the binding stage of the experiment. Instead, we can just estimate the upper limit on photobleaching during this phase. For that, multiply coefficient *k* by the duration of the Ndc80 binding phase. In our case, $k = 0.01\ min^{-1}$, and the typical time for the binding phase was 10 min. So, less than 10% of GFP-tagged molecules bleach during the binding phase. For longer experiments with binding times of 30 and 60 min, the frequency of imaging was reduced to keep the same total illumination time, as in 10 min experiments (120 frames total). So, the estimation of the upper limit of bleached Ndc80 molecules is the same < 10%.