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Quantitative analysis of the molecular size of fluorescent molecules and clusters

The rationale for this approach is to determine the number of molecules in fluorescently labeled particles by finding the ratio of integral fluorescent intensity of the multimolecular clusters to the intensity of a single fluorophore. This approach can be applied to different fluorophores. Here we show the application of the protocol on the example of GFP-tagged CENP-T molecules clustered on the mi3 core.

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.

1. Determine the coefficient of transition between different laser powers

While each of mi3-based clusters was expected to contain about 60 GFP molecules (Bruun et al. 2018), it is impossible to measure their intensity without camera saturation on the same microscope settings, as single GFP molecules. The laser power can be decreased to reduce signal from GFP clusters, however, the dependency of illumination intensity from laser power should be analyzed separately. In epifluorescence mode, the intensity of laser illumination linearly depends on laser power in the range from 1-100% in our microscope system (data not shown), but we found that this is not the case for TIRF imaging. To estimate the coefficient of transition between different laser power settings the calibration curve may be plotted using two different technics.

For experiments with unclustered CENP-T, the quantification of single GFP intensity and Ndc80 binding experiments were done on identical imaging and laser settings. So, the laser power conversion coefficient is not needed. Thus, this part of the protocol can be skipped.

1. Prepare the microscopy chamber and assemble CENP-T-GFP clusters as described in protocol “TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80”.
2. Adjust the microscope settings. Similarly to experiments with single GFP molecules we were using the TIRF imaging mode. However, to test laser power settings in 1-100% range, a wide range of gray values is needed. For that goal, the 1 MHz camera readout speed (16 bit images) is more reasonable, compared to 10 MHz used for photobleaching analysis (14 bit images). The recommended setting for Andor iXon3 camera: gain 5.0x, no EM gain, 1 MHz readout speed, 300 msec exposure time.
3. Focus at the surface of the coverslip and collect 10-30 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. Repeat step 3 for all desired laser power settings (Figure 1A).
5. Using Fiji select CENP-T clusters and background nearby with a circular regions, as described in protocol “TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80”.

6. Radius of this region should be minimized, but completely includes the cluster. For our microscope settings the optimal region radius is 6 px.
7. Measure the to the sample and background integral intensities, and subtract the median background intensity from all sample values.
8. Plot resulting distribution of clusters intensities for desired laser power settings (Figure 1B). With increase of laser power the distribution of clusters intensity is shifting to higher values. Median of these distributions can be used to recalculate the transition coefficient between different laser settings (Figure 1C).

2. Determine the size of mi3-based clusters

1. Prepare the microscopy chamber and assemble CENP-T-GFP clusters as described in protocol "TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80".
2. All microscope and camera settings, except the laser power, should be identical to settings used for determining of single GFP fluorescence intensity (for Andor iXon3 camera: gain 5.0x, EM gain 999, 10 MHz readout speed, 30 msec exposure time). Adjust the laser power to visualize the GFP-tagged CENP-T clusters without camera saturation. In our case, laser power was reduced from 50% (used for single GFP fluorescence intensity analysis) to 1%.
3. Focus on the surface of the coverslip and collect several 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. Collect data to build the laser illumination profile. For that goal the separate chamber with high concentration of clusters can be used, analogously, as it was described for single GFP molecule analysis (protocol "Determination of the intensity of a single fluorophore") or soluble GFP protein taken at density that will not lead to camera saturation at current microscope settings. That is important to wash out the clusters or protein from the microscopic chamber. The soluble pool of fluorescence protein would affect the resulting profiles. Finally, the laser intensity profile for adjusted laser power laser should be obtained.
5. Correct the acquired CENP-T clusters on unevenness of laser illumination, as described in protocol "Determination of the intensity of a single fluorophore" (Figure 2A).
6. Using Fiji select CENP-T clusters and background nearby with a circular regions, as described in protocol "TIRF microscopy assay to study the interactions between clustered CENP-T and soluble Ndc80". It is recommended to minimize the radius of the region while ensuring that the cluster is completely included. Based on our microscope settings, an optimal region radius of 6 pixels is recommended (Figure 2B).

7. Measure the integral fluorescent intensity of areas surrounding CENP-T clusters and corresponding background areas.
8. Plot the distributions of sample and background integral intensities (Figure 2C), calculate median background intensity and subtract the median background intensity from all sample values (Figure 2D).
9. Calculate the number of GFP molecules in the mi3-based clusters by dividing their fluorescent intensity by the intensity of the single GFP molecule and multiplying on the coefficient of transition between different laser powers (Figure 2E). In our case coefficient of transition between 1 and 50% laser power was 31.2 ± 1.3.

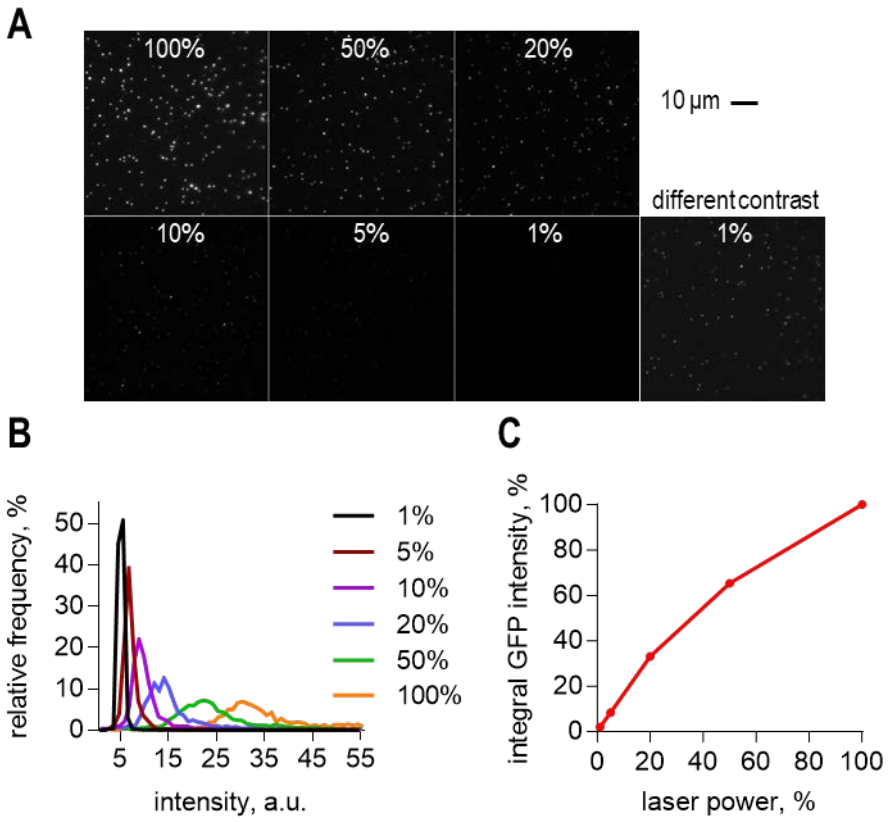

Figure 1. Determination of transition coefficient between different laser powers. A representative images used to calculate the transition coefficient. Images were acquired with the indicated laser power. **B** Distributions of fluorescence intensities of clusters, obtained at different laser powers as indicated. **C** The calibration curve showing the normalized intensity of GFP-tagged CENP-T clusters at various laser power settings. Each point represents the mean ± SEM, N = 3.

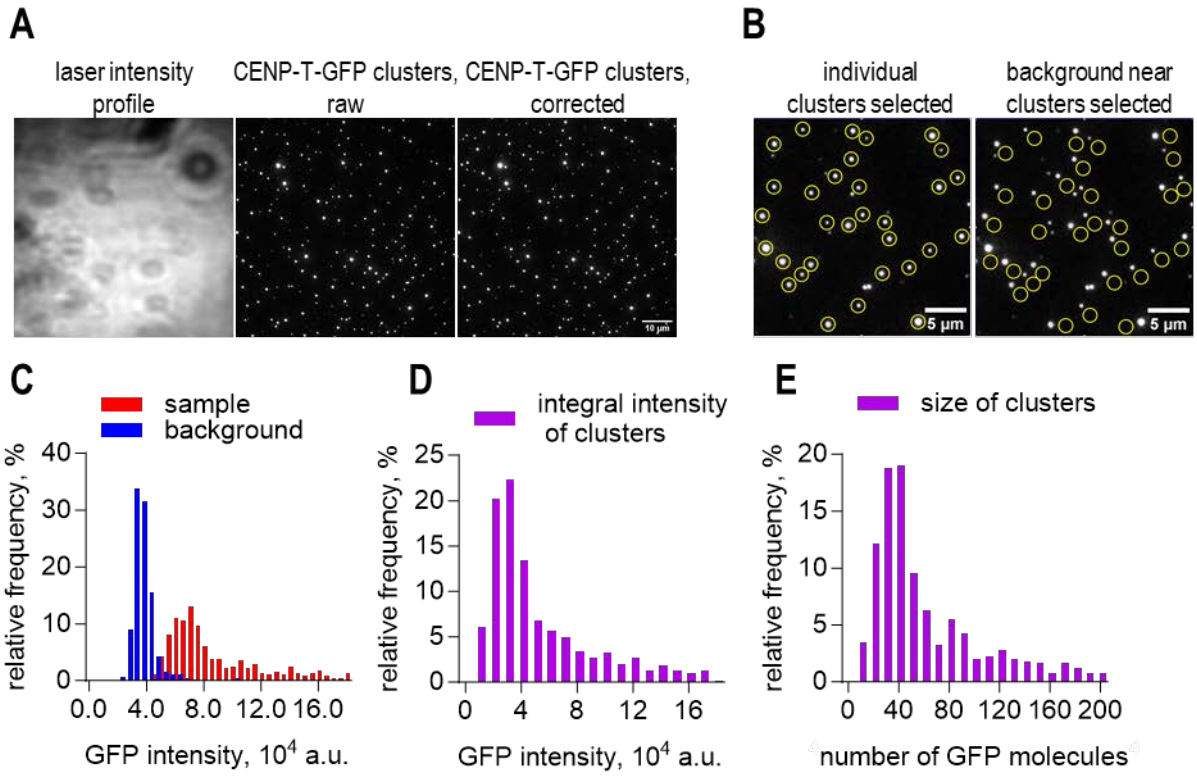

Figure 2. Illustration of fluorescence and size distributions of CENP-T-GFP-mi3 clusters. **A** Examples of laser illumination profile and CENP-T-GFP clusters before and after correction on laser profile. **B** Example of particles and background selection. **C** Distributions of background and cluster fluorescence intensities. **D** The distribution of CENP-T-GFP clusters fluorescence after background subtraction. **E** Distribution of cluster sizes in number of GFP molecules per cluster.