

Quantitative analysis of the molecular size of fluorescent molecules and particles

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 particles 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. A coherent CUBE 488-nm diode laser provides excitation for GFP visualization. Images are acquired on an Andor iXon3 EMCCD camera.
- Plasma cleaner Harrick Plasma PDC-32G

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 8 mg/mL and stored as 10- μ L aliquots.
- DTT (Thermo Fisher Scientific, cat # 15508) is prepared at 1 M in Milli-Q water, filter sterilized, and stored as 10- μ L aliquots.
- EGTA (Sigma-Aldrich, cat # E4378) is prepared at 0.2 M in Milli-Q water, pH is adjusted to 8.0 with NaOH or KOH. The solution is filter sterilized and stored as 10-mL aliquots at -20°C.
- Glucose (Sigma-Aldrich, cat # G8270) is prepared at 600 mg/mL in Milli-Q water and stored as 10- μ L aliquots.
- Glucose oxidase (Sigma-Aldrich, cat # G2133) is prepared at 10 mg/mL and stored as 10- μ L aliquots.
- MgCl₂ (Thermo Fisher Scientific, cat # M33-500) is prepared at 1 M in Milli-Q water, filter sterilized, and stored as 1–10-mL aliquots at -20°C.
- PIPES (Sigma-Aldrich, cat #1851) is prepared at 0.5 M in Milli-Q water and pH is adjusted to 6.9 with KOH. The solution is filter sterilized and stored at room temperature for several months or at -20°C as 10–50 mL aliquots.

Proteins

- CENP-T (1-242aa)-GFP-Spy-tag
- SpyCatcher-mi3 core particles

I. Determine the intensity of a single fluorophore

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. 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 1 A 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.** 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 I.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; $(img(x,y)-CN)$).
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 polynomial function.
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).

II. Determine the molecular size of the fluorescent mi3-based particles

1. Determine the coefficient of transition between different laser powers

While each of mi3-based particles was expected to contain about 60 GFP molecules (Bruun et al. 2018), it is not possible 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 particles, 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 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 coefficient of transition between different laser power settings the calibration curve may be plotted using two different technics.

1. Particles based method.

1. Assemble regular microscopy chamber using a nonmodified glass slide, two strips of double-sided tape, and a plasma cleaned coverslip.
2. Add mi3-based particles to the chamber and allow them to adsorb on the coverslip for 10 min.
3. Wash the chamber with imaging buffer and seal with VALAP. Optimize particles concentration to obtain the field with evenly dispersed objects (Figure 3A).
4. 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.
9. 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.
10. Repeat step 9 for all desired laser power settings.
11. Select mi3-based particles and background nearby with a circular regions. Radius of this region should be minimized, but completely includes the particle. For our microscope settings the optimal region radius is 5-8 px.
12. Measure the to the sample and background integral intensities, and subtract the median background intensity from all sample values.
13. Plot resulting distribution of particles intensities for desired laser power settings (Figure 3B). With increase of laser power the distribution of particles intensity is

shifting to higher values. Median of these distributions can be used to recalculate the transition coefficient between different laser settings.

2. Soluble fluorophore method

1. Assemble regular microscopy chamber using a nonmodified glass slide, two strips of double-sided tape, and a plasma cleaned coverslip.
2. Add about 1 μM GFP protein in imaging buffer to the chamber, seal with VALAP, and allow protein to adsorb on the coverslip for 10 min.
3. Suggestion about microscope settings are similar to particles based method.
4. 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.
5. Repeat step 4 for all desired laser power settings (Figure 3C).
6. For each laser power settings measure the integral intensity of imaging field, average these values between different imaging fields.
7. Subtract the camera noise values multiplied on area of imaging (in our case it was $\text{CN} \times 512 \times 512 \text{ px}^2$) from integral intensity values.
8. Resulting values can be plotted as function of laser power and used to recalculate the transition coefficient between different laser settings.

For both methods, that we tested, results were consistent and dependency of GFP intensity from laser power deviates from linear function (Figure 3D).

2. Determine the size of mi3-based particles

1. Assemble the microscopic and attach GFP-tagged mi3-based particles to the coverslip as described in section II.1.1.1-3.
2. All microscope and camera settings, except the laser power, should be identical to settings used for single GFP molecule analysis. Adjust the laser power to visualize the mi3-based particles without camera saturation. In our case laser power was reduces from 50% (used for single GFP molecule analysis) to 1%.
3. Focus at 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 high concentration of mi3-based particles can be used, analogously, as it was described for single GFP molecule analysis (Section I.2) 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 particles or protein from the microscopic chamber. 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 mi3 particles on unevenness of laser illumination, as described in section I.3 (Figure 4A).
6. To select mi3-based particles and background nearby, use circular regions as shown in Figure 4B. It is recommended to minimize the radius of the region while ensuring that the particle is completely included. Based on our microscope settings, an optimal region radius of 5-8 pixels is recommended. For semi-automatic particle selection, we suggest using the ImageJ script provided in Appendix 1('brightness_program.ijm'). To start the program, first, indicate the path for output directory at line 2. Then, assign value 1 to the parameter "frame" at line 3, it indicates number of frame that is currently analyzed. After switching to the new imaging frame of the same data set increase this parameter on 1. Each part of the script (labeled as A-D below) should be run consequently. For that goal, uncomment parts of the script one by one using "" symbols. When run one part of the script be sure, that other parts are commented.
 - A. Particle Selection: To select particles automatically the noise for particles selection should be determined. For that goal gradually increase the "noise" parameter at line 1 of the script from 400 until the script stops selecting background as particles. In case of shown example noise was 1400 (Figure 4). Do not change the threshold and radius between experiments. After you select the noise, the script would select circle regions around particles with pixel brightness above this threshold. The radius of circle is adjustable in the program. For our experiments we use 8 px radius. Visually inspect selected regions, delete from ImageJ ROI manager circles that are: include large aggregates, has two or more particles in one circle, overlapping circles, or regions partially outside the image bounds, see example images Figure 5B.
 - B. Measurement of Integrated Fluorescence: In this step, the script will calculate the integral fluorescence of the selected circles. The output file 'sample0.csv' will contain integral fluorescence, mean, minimum, and maximum values of the pixels within each circle.
 - C. Background Region Selection: The script will shift the circles 10 pixels down and 10 pixels to the left to designate them as background regions. After executing this step, visually examine the image to ensure the background regions are selected correctly. Using ImageJ ROI manager manually delete the position of the circles so that contain particles and extend beyond the image borders (see example images Figure 5B).
 - D. Measurement of Background Fluorescence: In the final part of the script, the background circles will be used to measure the integral fluorescence. The output file 'bcg0.csv' will contain integral fluorescence, mean, minimum, and maximum values of the pixels within each background circle.

7. Plot the distributions of sample and background integral intensities (Figure 6A), calculate median background intensity and subtract the median background intensity from all sample values (Figure 6B).
8. Calculate the number of GFP molecules in the mi3-based particles 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 6C). In our case coefficient of transition between 1 and 50% laser power was 35.7.
9. Particles aggregates could interact differently with your protein of interest. Also, small particles could increase the noise of the final curve. To address their potential impact, we recommend applying a brightness cutoff to initial particles brightness in kinetics experiments. To determine cutoff bounds, fit fluorescence distribution with Gaussian (Figure 6D). We suggest excluding particles with integral fluorescence outside the range mean integral fluorescence $\pm 1.5\sigma$. In case of current microscope setting and used CENP-C-eGFP-mi3 particles the range $11.8 \cdot 10^3 - 41.7 \cdot 10^3$ a.u.

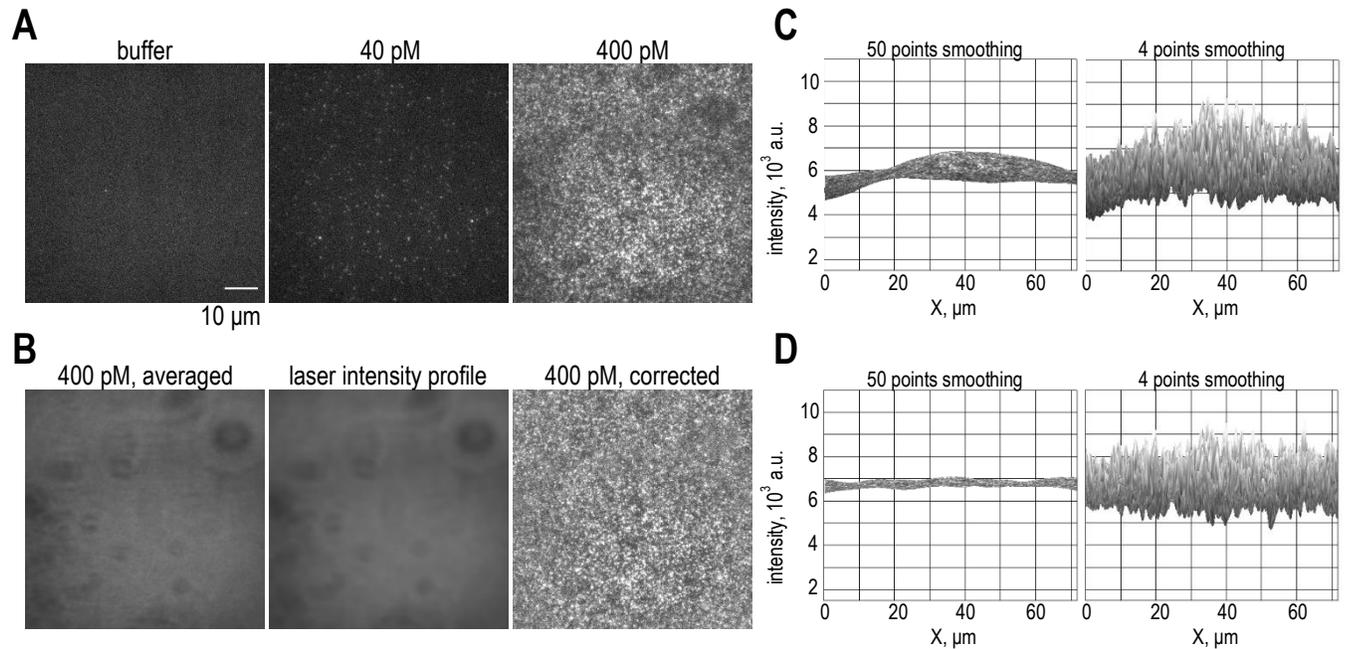


Figure 1. Illustration of correction of illumination unevenness. **A** TIRF images of eGFP-labeled protein attached to the coverslip, showcasing rare fluorescent spots in the imaging buffer, as well as dispersed and highly concentrated GFP spots at the indicated concentration. **B** The left image displays the average projection of multiple fields with 400 pM eGFP-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.

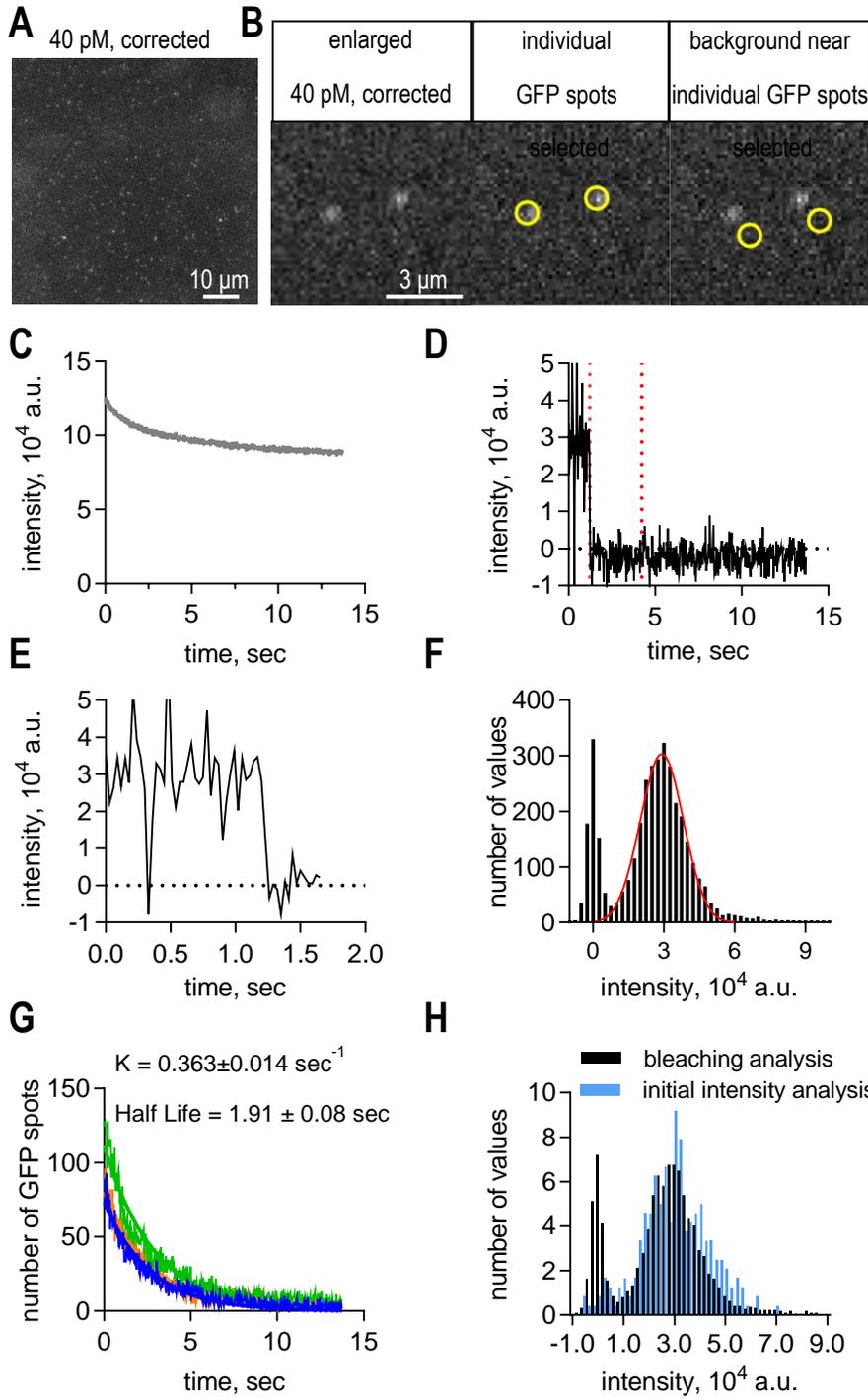


Figure 2. Determination of fluorescence intensity of a single GFP fluorophore. **A** Representative image of a microscope field with single GFP molecules immobilized on plasma-cleaned coverslip. **B** Enlarged view of image A. Yellow circles highlight typical GFP spots used for analysis in the middle 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.

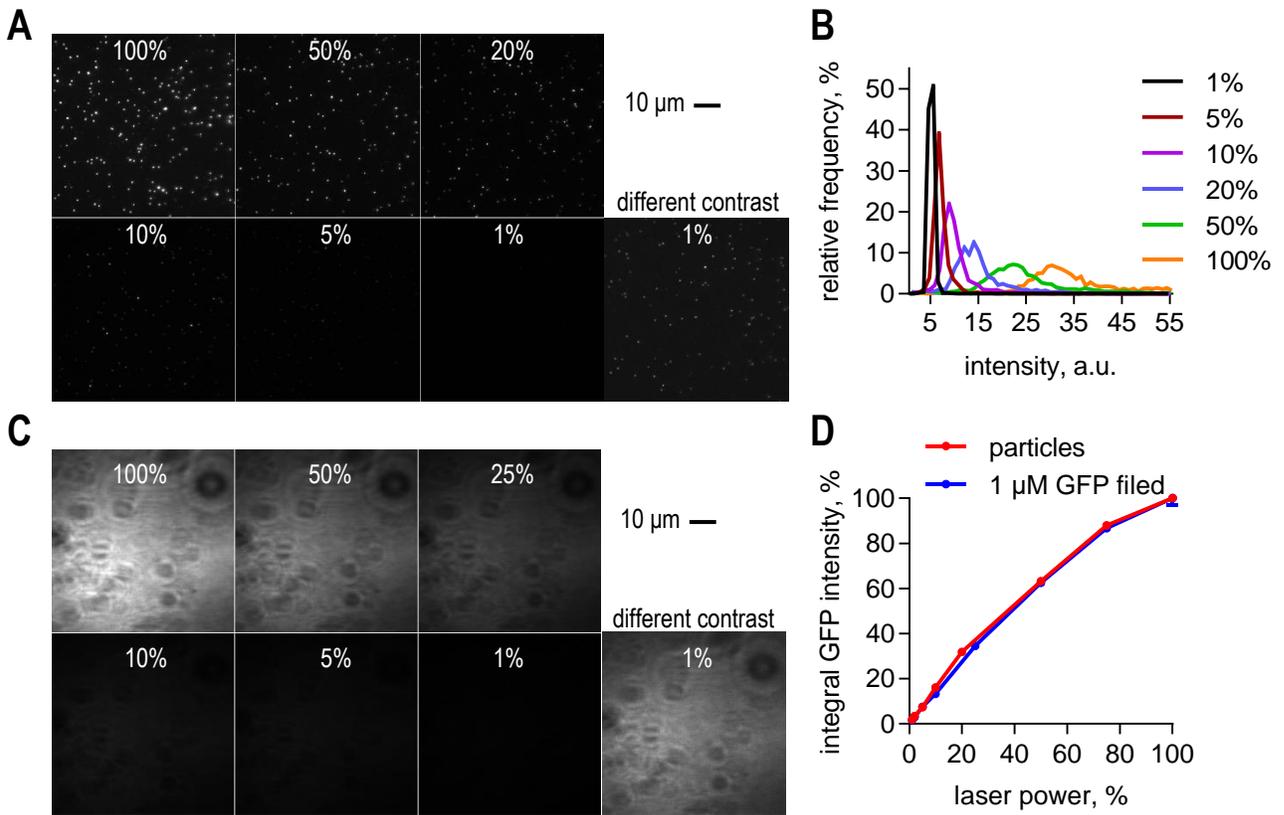


Figure 3. Determination of transition coefficient between different laser powers. **A** representative image used to calculate the transition coefficient through a particle-based method. Images were acquired with the indicated laser power. **B** Distributions of fluorescence intensities of particles, obtained at different laser powers as indicated. **C** Representative images used to calculate the transition coefficient using the soluble fluorophore method. Images were captured with the indicated laser power. **D** Dependency of integral GFP fluorescence versus laser power for determining the transition coefficient. The curves were obtained using both the particle-based method and the soluble protocol method. This dependency is utilized for determining the transition coefficient.

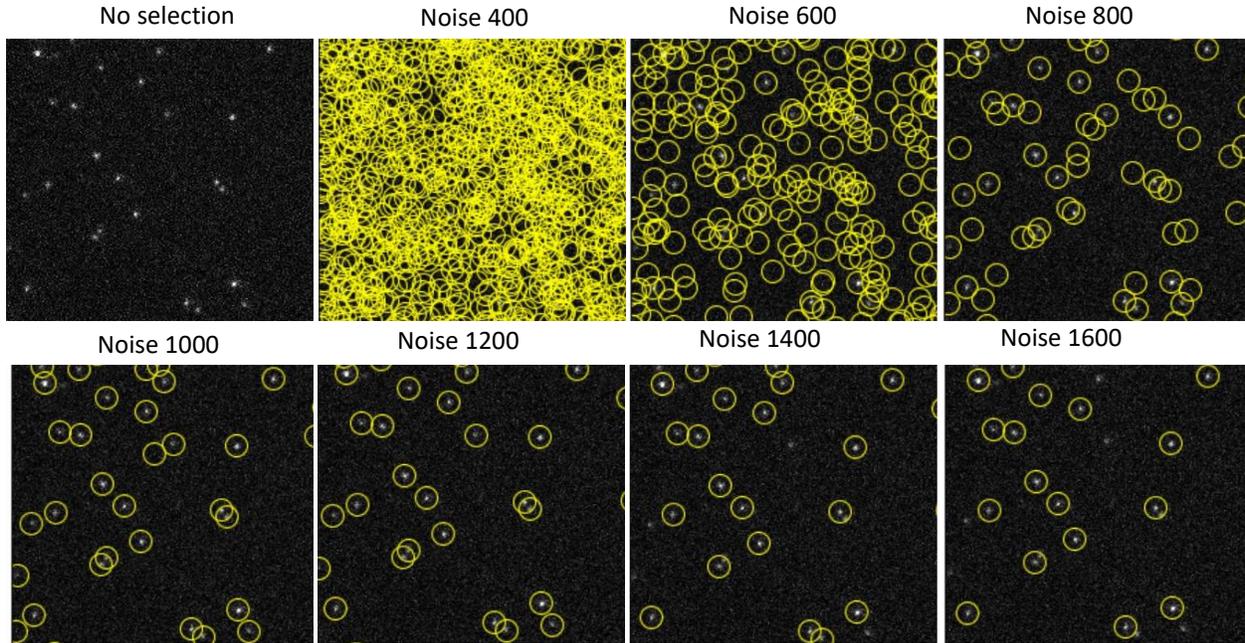


Figure 4. Optimization of the noise parameter for automatic particle selection. Yellow circles indicate the region selections performed by the script using different noise parameters.

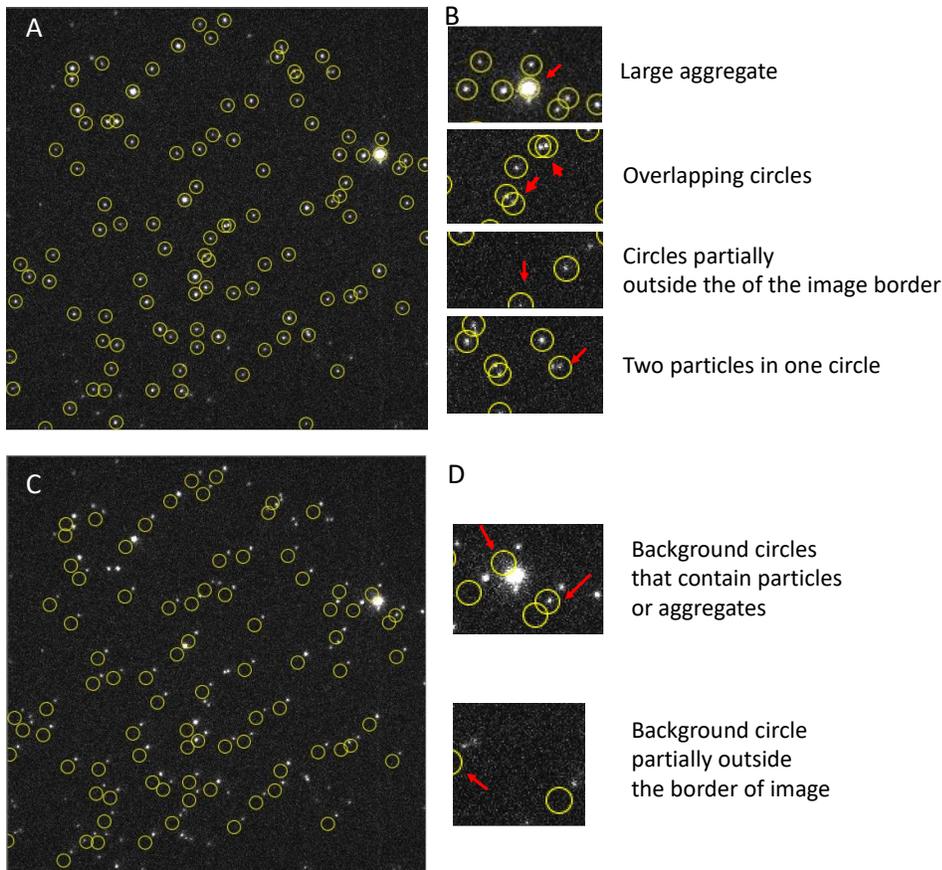


Figure 5. Representative image used for particle size analysis. A Yellow circles highlight particles automatically selected using a script with a threshold of 1400. **B** Enlargements of the image in A reveal regions within particles that should be excluded based on the exclusion criteria, indicated by red arrows. **C** Yellow circles indicate automatically selected background circles. **D** Enlargements of the selections in C identify background regions that should be removed according to the exclusion criteria, as denoted by red arrows.

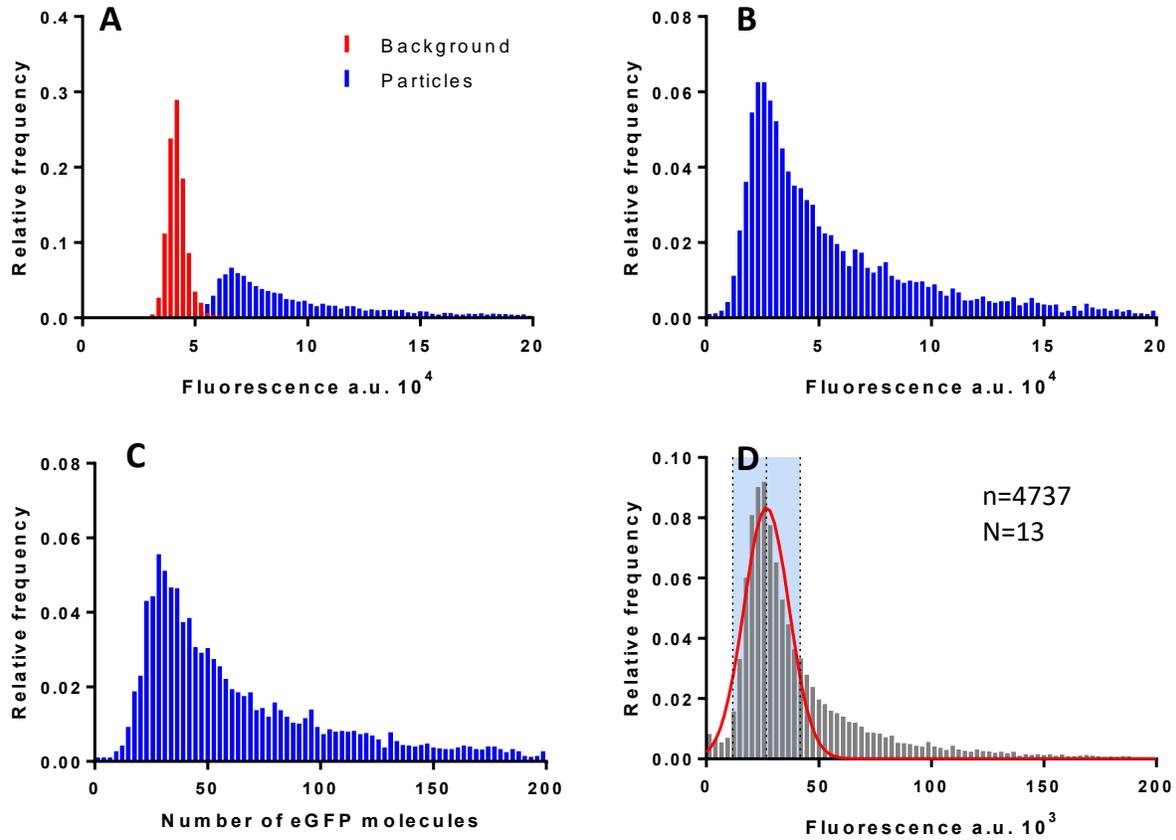


Figure 6. Illustration of fluorescence and size distributions of CENP-C-eGFP-mi3 particles. A Representative distributions of background and particle fluorescence. **B** The distribution of particles fluorescence after background subtraction. **C** Distribution of particle sizes in number of eGFP molecules per particle. **D** The distribution of particle fluorescence used to determine the cutoff range. The red curve corresponds to the Gaussian distribution fitting, while the pale blue region indicates the 1.5σ range based on the fitting.

Appendix 1. Image J script listing(brightness program.ijm):

```
noise = 1400
path = "/Path/to/your/output/folder/"
frame = 1

//PART A - automatic particles selection

run("Find Maxima...", "noise="+noise+" output=[Point Selection]");
roiManager("Add");
getSelectionCoordinates(xpoints, ypoints);

roiManager("Delete");
radius=8;
for (i=0; i<lengthOf(xpoints); i++) {
    makeOval(xpoints[i]-radius, ypoints[i]-radius, 2*radius, 2*radius);
    roiManager("Add");
}

//PART B - automatic measurement of particles integral fluorescence

run("Clear Results");
roiManager("Show All");
roiManager("multi-measure measure_all append");
saveAs("Results", path+"sample"+frame+".csv");
run("Clear Results");

//PART C - automatic shifting of particles circles for background
measurement

roiManager("Save", path + "/RoiSet"+frame+".zip")
roiManager("translate", -10, 10);

//PART D - measurement of background integral fluorescence

roiManager("multi-measure measure_all append");
saveAs("Results", path+"bcg"+frame+".csv");
roiManager("Save", path + "/RoiSet_bcg"+frame+".zip")
```

Bruun, T. U. J., A. C. Andersson, S. J. Draper and M. Howarth (2018). "Engineering a Rugged Nanoscaffold To Enhance Plug-and-Display Vaccination." ACS Nano **12**(9): 8855-8866.

Chakraborty, M., E. V. Tarasovetc and E. L. Grishchuk (2018). "In vitro reconstitution of lateral to end-on conversion of kinetochore-microtubule attachments." Methods Cell Biol **144**: 307-327.