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Binding of fluorescent proteins to mi3-based particles with dual color proteins

I. Experimental procedures

Take aliquots from:

Reagents	where	Cat	Con.	Notes
1. PBS buffer	+4 °C			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. Protocol "10xPBS Dulbeecco's modified".
2. BRB80 buffer	+4 °C			80 mM PIPES, 4 mM $MgCl_2$, 1 mM EGTA, 10 mM NaCl; pH 6.9 Buffer is prepared in advance and filtered, stored at -20°C for months and at +4°C for few weeks. Protocol "Stock solution for BBB80" by Katya T 07/17/21.
3. 2x Particles dilution buffer	-20 °C			50 mM Tris-HCl pH 8.0, 300 mM NaCl, 40 % glycerol. Stored in 500 µl aliquots at -20 C.
4. BSA	-80 °C	Sigma A7638	100 mg/ml	1 aliquot (100 µl) thaw on ice, spin. Keep on ice. Protocol "BSA stock" by Nikita 04/05/2011.
5. casein	-80 °C	Sigma 5890	8 mg/ml	1 aliquot (100 µl) thaw on ice. Keep on ice. Lab protocol "Casein stock".
6. Glucose oxidase (GO)	-80 °C	Sigma G2133	10 mg/ml	1 aliquot (10 µl) thaw on ice, spin. Keep on ice. Protocol "Lab reagents stock protocol" by Katya T and Anna 07/05/2018
7. Catalase (CAT)	-80 °C	Sigma C.40	2 mg/ml	1 aliquot (20 µl) thaw on ice, spin. Keep on ice. Protocol "Lab reagents stock protocol" by Katya T and Anna 07/05/2018
8. Glucose (G)	-80 °C	Sigma G8270	600 mg/ml	1 aliquot (10 µl) thaw on ice, spin. Keep on ice. Protocol "Lab reagents stock protocol" by Katya T and Anna 07/05/2018
9. Dithiothreitol (DTT)	-80 °C	Invitrogen 15508-013	1 M	1 aliquot (10µl) thaw on ice, spin, Keep on ice. Take fresh aliquot every 3-4 h. Protocol "Lab reagents stock protocol" by Katya T and Anna 07/05/2018.
10. Pluronic F127	RT	Sigma P2443	1%	Pluronic F-127 is prepared as a 1% solution in BRB80. Stored at room temperature no longer than 2 weeks. The protocol: "Preparation of Pluronic F127" by Katya T 06/29/21

11. mi3-based particles	-80 °C		nanomolar	1 aliquot thaw on ice, spin, keep on ice.
12. Protein of interest fused to eGFP-SpyTag	-80 °C		micromolar	1 aliquot thaw on ice, spin, keep on ice. ONLY if you are assembling particles on the coverslip.
13. kinetochore proteins	-80 °C		micromolar	1 aliquote aliquot thaw on ice, spin, keep on ice.

1. Prepare PBS-BSA-casein, BRB-BSA-casein and Imaging 2x buffers by following recipes:

PBS-BSA-casein	**BRB-BSA-casein**	**2x Imaging Buffer (final concentration of 1x Imaging buffer in experiment, after dilution with BRB-BSA-Casein)**
900 µl PBS	900 µl BRB80	820 µL BRB-BSA-casein
2 µl DTT (2 mM final concertation)	2 µl DTT (2 mM final concertation)	18 µL DTT (10 mM final concertation)
40 µl BSA (4 mg/ml final concertation)	40 µl BSA (4 mg/ml final concertation)	40 µl BSA (4 mg/ml final concertation)
63 µl casein (0.5 mg/ml final concertation)	63 µl casein (0.5 mg/ml final concertation)	63 µl casein (0.5 mg/ml final concertation)
		20 µL catalase (20 µg/ml final concertation)
		20 µL glucose oxidase (100 µg/ml final concertation)
		20 µL glucose (6 mg/ml final concertation)

2. Prepare proteins:

   eGFP labeled protein: dilute stock to 1 µM.

   Alexa 647 labeled protein: dilute stock 2 to 500 nM in BRB-BSA-casein buffer. Ultracentrifuge eGFP labeled and Alexa 647 labeled proteins using TLA100 rotor at settings: 60,000 rpm, 15 min, 4°C. Take supernatant avoiding the bottom of the tube, transfer on pre-chilled Eppendorf tube, and keep on ice and dark. Right before addition to the microscopic chamber dilutes the proteins to the desired concertation in Imaging buffer.

   If you are using with pre-assembled eGFP labeled mi3-based particles: dilute stock 5 times: 25 µL stock + 135 µL of particles dilution buffer (the dilution provided only for CENP-C-eGFP-mi3 particles).

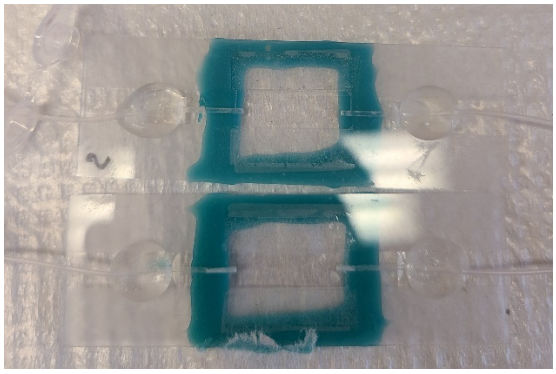
   If you are working with not assembled mi3 based particles, prepare components for assembly:

   Protein of interest-eGFP-SpyTag: dilute stock to 1 µM in PBS-BSA-casein buffer.

   Spy-Catcher-mi3: dilute stock to 2 µM PBS buffer supplemented with 2 mM DTT . Spin at settings: 16,000 g, 10 min, 4°C. Take supernatant avoiding bottom of the tube, transfer on pre-chilled Eppendorf tube. Before addition to the microscopic chamber dilute to 200 nM PBS buffer supplemented with 2 mM DTT.

3. Wipe the surface of the microscope slide with Windex solution and put the oil drop on the microscope slide. Preheat the microscope: put a drop of immersion oil on an ethanol-cleaned glass microscope slide, place the slide to the microscope stage and secure it with holders. Make sure, that the oil is in contact with the objective. Turn on the objective heating at 32°C, and keep at least for 30 min.

4. Prepare flow chamber using a reusable slide with tubing:
    a. Attach a silanized coverslip over a reusable glass slide with tubing using spacers made from two strips of double-sided tape (two layers), generating about 15-µL flow chamber.
    b. Cut off excess tape.
    c. Cover the sealant up the perimeter of silanized coverslip. The resulting chambers lying coverslip down is shown below:



5. Wash assembled chamber with 150 µL of PBS, use a syringe pump with a flow rate 150 µl/min. Use these pump settings at all steps, unless otherwise pointed.

6. Block exposed silanized glass surface by perfusing 100 µL of 1% Pluronic at 150 µl/min rate, and incubate 10 min.

7. Wash chamber with 150 µL of PBS, use syringe pump with flow rate 50 µl/min.

8. <u>If you are working with preassembled particles</u>:

    a. Perfuse 100 µl of particles through the chamber and incubate the chamber for 10 min.
    b. Wash the chamber with 150 µL PBS-BSA-casein.

    <u>If you are assembling on the coverslip</u>:

    a. Immobilize mi3-Spy-catcher particles by perfusing of 100 µL Mi3 particles (diluted 1/100 in PBS with DTT). Incubate Mi3 particles for 10 min.
    b. Wash the chamber by perfusing of 150 µL of PBS-BSA-casein.
    c. Perfuse 150 µL of 200 nM Protein of interest-eGFP-SpyTag in PBS-BSA-casein, incubate for 20 min.
    d. Wash the chamber by perfusing of 150 µL of PBS-BSA-casein.

9. During incubation with particles place the chamber on the microscope and carefully make a contact with oil on the objective. Objective heater should be ON, all other steps of the protocol

will be done at 32°C. Wait 10 min for temperature equilibrate. Preheat all solutions that will be introduced to the microscopic chamber up to 32°C by placing them on the heat block for 20 s. Use heating block with tube holder field with water.

10. Wash chamber by perfusing 150 µL Imaging Buffer.

11. Focus, close the diaphragm until its image is seen in the edges of field of view. Avoid photobleaching, to select imaging fields move stage with closed illumination, evaluate field by one image shot. If more than one imaging field will be captured, save stage positions using NIS elements software.

12. Image GFP-tagged mi3-based particles. Use TIRF mode. Settings on the Ardor iXon3 EMCCD camera: 10 MHz readout speed, 300 EM Gain, 5x Conversion Gain, 30 ms exposure; 1% 488 nm laser power. For second fluorophore in our case Alexa 647 use following settings for Andor iXon3 EMCCD camera: 10 mHz readout speed, 999 EM Gain, 5x conversion Gain, 100 ms exposure; 1% of 640 nm laser power. Quickly check images to make sure, that there is no saturation (use "show saturation" option in NIS elements software). Put the cursor over couple particles to reveal pixel brightness - it should be 1,000-3,000 a.u. If pixel intensity is higher, reduce EM Gain. If image intensity is too low, make sure you removed polarizer and all filters in light path. Use this microscope settings for all stages of the experiment. Do not change these settings between experiments.

13. Take 25 images of selected fields with mi3-based particles to evaluate their initial brightness. Here and below make sure, that illumination shutter is closed between time frames. From now on imaging should be done in two channels.

14. Make 488 nm laser power 100% and bleach GFP on particles for 30s. This time is usually enough to bleach all GFP molecules. This step is only necessary if you study binding of eGFP labeled protein together or consequentially with Alexa 647 labeled protein. In case if you study binding of Alexa647 labeled protein alone skip this step.

15. Turn 488 nm laser back to 1% and collect 25 images of bleached particles.

16. Do not touching the chamber perfuse 200 µL of pre-warmed kinetochore proteins with syringe pump at speed 900 µl/min.

17. Image binding of kinetochore proteins to mi3-based particles for desired time. Frequency of imaging depends on total binding time. Collect 60-120 frames total during binding phase.

18. Do not touching the chamber perfuse 300 µL of Imaging buffer with syringe pump at speed 900 µl/min. Continue wash with low rate 10 µl/min.

19. Image unbinding of kinetochore proteins from mi3-based particles for desired time. As described above frequency of imaging depends on total unbinding time. Collect 60-120 frames total during

this phase.

20. Some experiments contain 2 phases of binding and unbinding of eGFP labeled proteins. For example, eGFP labeled mi3-based particles are fist incubated with Alexa 647 labeled protein, than Alexa 647 labeled protein is washed out and eGFP labeled protein is added, then washed out. For such experiments repeat steps 16-19.

II. Data analysis

1. Calculate integral intensity of particles in GFP and Alexa67 channels, as described in "Quantification of the photobleaching rate for fluorophores" section IV, steps from 1-4. Be sure, that you applied the fluorescence cut off as described there.
2. To visualize and process brightness measurements you can use any data analysis software of your choice (e.g. Excel, Prism Graphpad, Origin Pro, Python). First, using preferred software import generated files from the previous step. Second, subtract the individual background values from the particle photobleaching curves for each time frame. Visually inspect the resulting curves and discard any curve that shows an abrupt decrease/increase in fluorescence (Figure 1A,B). See examples of individual curves included in the analysis on Figure 1C.
3. Because in this protocol we are analyzing images acquired in different channels it is important to convert fluorescence from a.u. to a number of molecules, thus we will be able to compare data between channels. For that goal, divide fluorescence intensity by the value of a corresponding single fluorophore fluorescence. For fluorophores and settings used in "Binding experiment with mi3-based particles" the values for a single eGFP fluorophore is 202.65 a.u. and for a single Alexa 647 – 623.45 a.u. These values were calculated by diving single molecule fluorescence obtained at single molecule settings by the coefficient of transition between different laser power settings. It is important that single molecule and particles microscopic settings differ only in laser power. To determine the intensity of a single molecule fluorophore use the protocol "Quantitative analysis of the molecular size of fluorescent molecules and particles" section I "Determine the intensity of a single fluorophore" with modified microscopic settings. For GFP channel use: gain 5.0x, EM gain 300 (in the original protocol it was 999), 10 MHz readout speed, 30 msec exposure time, 50% laser power. For Alexa 647 use: gain 5.0x, EM gain 999, 10 MHz readout speed, 100 msec exposure time, 50% laser power. To determine the transition coefficient between laser power settings for single molecules and particles use section II.1 "Determine the coefficient of transition between different laser powers".
4. Calculate the initial size of mi3-based particles in a number of GFP molecules, by averaging the first 25 time-points before bleaching of mi3-particles. Normalize experimental curves corresponding to individual particles on their size value.

5. To take into account bleaching of GFP and Alexa 647 molecules, data should be normalized on the bleaching exponent. While it is not clear how to take into account bleaching in the presence of GFP- or Alexa 647 labeled proteins, only data after protein washout should be normalized. For that, divide these regions of experimental curves on the corresponding value from the photobleaching curve, obtained using “Quantification of the photobleaching rate for fluorophores” protocol.
6. Average resulting curve from different particles, to plot the final graph showing binding and unbinding of fluorescently the labeled proteins over time (Figure 2).

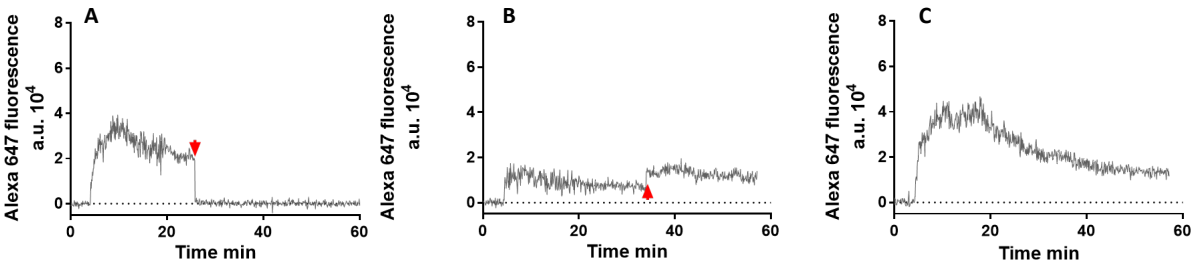


Figure 1. Examples of experimental curves showing binding and unbinding of Alexa647 labeled Mis12/KNL1 to CENP-C-eGFP-mi3 particles. In these experiments 100 nM of Alexa647 labeled Mis12/KNL1 was added at 2.5 min, and unbound protein was washed out at 18.5 min. Each curve corresponds to one particle. **A** shows a curve excluded from the analysis due to an abrupt decrease of fluorescence. This event is indicated with red arrow. **B** shows curve excluded from the analysis due to an abrupt increase of fluorescence. This event is indicated with an arrow. **C** shows representative curves included to the analysis.

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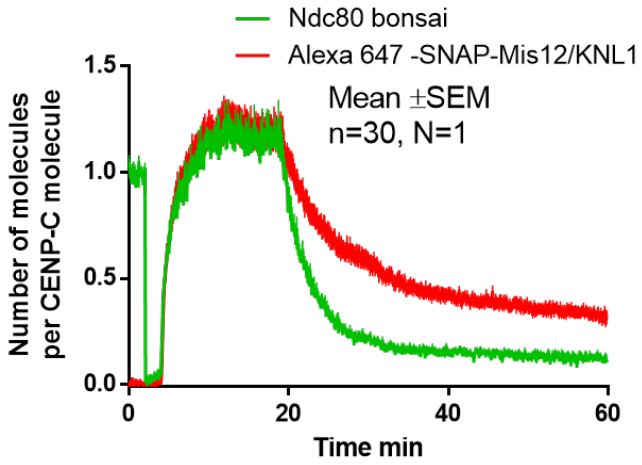

Figure 2. The result of experiment analysis of Alexa 647-SNAP-Mis12/KNL1 and GFP-tagged Ndc80 binding to CENP-C-eGFP-mi3 particles. In this experiment concertation of Alexa 647-SNAP-Mis12/KNL1 was 100 nM, GFP-tagged Ndc80 – 200 nM. Proteins were added at 4 min and removed at 19 min.