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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 (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	CAT#	storage	Concentration	Notes
PBS buffer		+4 °C	1x	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.
Mg-BRB80 buffer		+4 °C	1x	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.
BSA	Sigma A7638	-80 °C	100 mg/mL	2 aliquots (100 µL each) thaw on ice, spin. Keep on ice.
casein	Sigma 5890	-80 °C	8 mg/mL	2 aliquots (100 µL each) thaw on ice. Keep on ice.
glucose oxidase (GO)	Sigma G2133	-80 °C	10 mg/mL	2 aliquots (10 µL each) thaw on ice, spin. Keep on ice.

catalase (CAT)	Sigma C.40	-80 °C	2 mg/mL	2 aliquots (20 µL each) thaw on ice, spin. Keep on ice.
Glucose (G)	Sigma G8270	-80 °C	600 mg/mL	2 aliquots (10 µL each) thaw on ice, spin. Keep on ice.
Dithiothreitol (DTT)	Invitrogen 15508-013	-80 °C	1 M	3 aliquots (10 µL each) thaw on ice, spin, Keep on ice. Take fresh aliquot every 3-4 h.
Pluronic F127	Sigma P2443	Room temperature	1%	Pluronic F-127 is prepared as a 1% solution in BRB80. Stored at room temperature no longer than 2 weeks.
SNAP-SpyCatcher		-80 °C	micromolar	1 aliquot thaw on ice, keep on ice.
CENP-T-GFP- SpyTag		-80 °C	micromolar	1 aliquot thaw on ice, spin, keep on ice.
GFP-tagged Ndc80		-80 °C	micromolar	1 aliquot thaw on ice, keep on ice.

Experimental procedure

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

PBS-BSA-casein:	**BRB-BSA-casein:**	**2x Imaging Buffer (dilute with BRB-BSA-casein to get 1x):**
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:

a. Dilute stock of SNAP-SpyCatcher to 1 μM in PBS buffer supplemented with 2 mM DTT.

b. Dilute stock of CENP-T-GFP- SpyTag to 1 μM in PBS-BSA-casein buffer.

c. Dilute stock of GFP-tagged Ndc80 to 1 μM in BRB-BSA-casein buffer.

d. Ultracentrifuge CENP-T and Ndc80 proteins at 156,000 *g* for 15 min at 4 °C. Take supernatant avoiding the bottom of the tube, transfer on pre-chilled Eppendorf tube, and keep on ice and dark.

e. Right before addition to the microscopic chamber dilute the proteins to the final concentrations (100 nM SNAP-SpyCatcher in PBS with 2 mM DTT; 3 nM CENP-T in PBS-BSA-casein buffer; 200 nM Ndc80 in 1x Imaging Buffer).

3. Prepare the microscope:

Here, all fluorescent imaging was performed using a Nikon Eclipse Ti microscope equipped with a 1.49xNA TIRF 100x Oil objective. Excitation for visualizing GFP-proteins in TIRF modes was provided by coherent CUBE 488-nm diode laser (Coherent). Images were captured using an Andor iXon3 EMCCD camera.

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 the 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 the silanized coverslip (Figure 1).

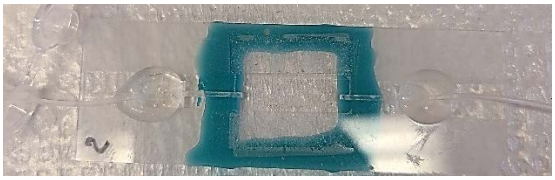

Figure 1. Assembled flow chamber lying coverslip down.

5. To functionalize the coverslip surface, perfuse 100 µL of 100 nM SNAP-SpyCatcher in PBS buffer supplemented with 2 mM DTT, and incubate for 10 min. To introduce solutions into the chamber use a syringe pump with a flow rate of 150 µL/min. Use these pump settings at all steps, unless otherwise pointed.

6. During incubation with SNAP-SpyCatcher place the chamber on the microscope and carefully make contact with oil on the objective. The objective heater should be ON, all other steps of the protocol will be done at 32°C. Preheat all solutions that will be introduced to the microscopic chamber up to 32°C by placing them on the heat block for 20 seconds. Use a heating block with a tube holder field with water.

7. Wash the chamber by perfusing 150 µL of PBS.

8. Block exposed silanized glass surface by perfusing 100 µL of 1% Pluronic, and incubate for 10 min.

9. Wash the chamber by perfusing 150 µL of PBS at flow rate of 50 µL/min using a syringe pump.

10. Wash the chamber by perfusing 150 µL of PBS-BSA-casein.

11. Focus, and close the diaphragm until its image is seen in the edges of the field of view. Image the coverslip before immobilization of CENP-T protein to make sure, that the coverslip is clean and does not have bright dots in the GFP-channel. Use TIRF mode. Settings on the Ardor iXon3 EMCCD camera: 1 MHz readout speed, 50 EM Gain, 5x Conversion Gain, 300 ms exposure; 20% 488 nm laser power. If you have many bright dots on the coverslip, try a different coverslip or prepare a fresh preparation of silanized coverslip.

12. Find the necessary amount of imaging fields without unspecific fluorescent dots (Figure 2). If necessary, to reduce the number of fluorescence dots per field you can bleach them with 100% laser power for ~10-20 s. Save positions of these fields using NIS Elements Software. A number of imaging fields depends on the number of time points, that you want to capture for the Ndc80-binding curve. In our experiments, it was usually ~15 fields.

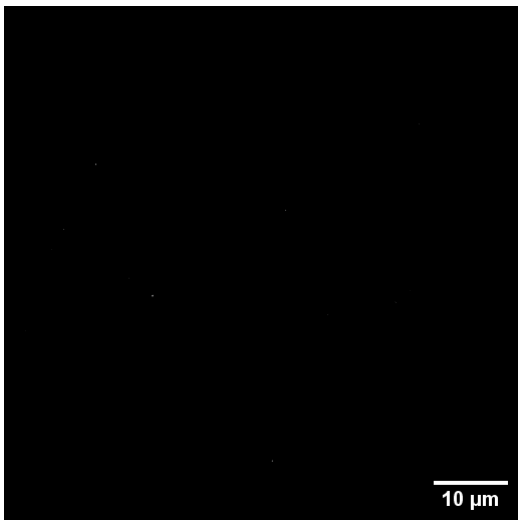

Figure 2. Example of clean coverslip before the addition of CENP-T-GFP.

13. Perfuse 150 µL of 3 nM CENP-T-GFP-SpyTag in PBS-BSA-casein, incubate for 5 min.

14. Wash the chamber by perfusing 150 µL of PBS-BSA-casein.

15. Wash the chamber by perfusing 150 µL 1x Imaging Buffer.

16. Avoid photobleaching, and image each of the fields selected in step 12 by one image shot. These images contain information about the initial fluorescence of immobilized CENP-T-GFP molecules (Figure 3).

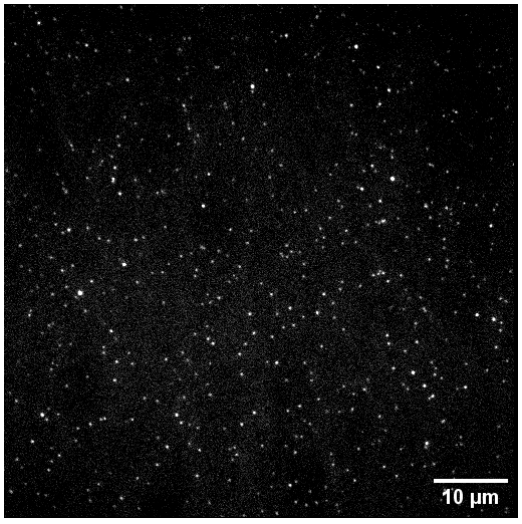

Figure 3. Example of imaging field with immobilized CENP-T-GFP molecules. The image contrast is the same as in Fig. 2.

17. Not touching the chamber perfuse 200 µL of 200 nM GFP-tagged Ndc80 in 1x Imaging Buffer using a syringe pump at a flow rate of 900 µL/min. Immediately start the stopwatch.

18. Image binding of Ndc80 to CENP-T at desired time points (Figure 4). To avoid photobleaching, image each of the selected fields just once after Ndc80 addition. Every time select a new imaging field from the preselected set of fields (step 12).

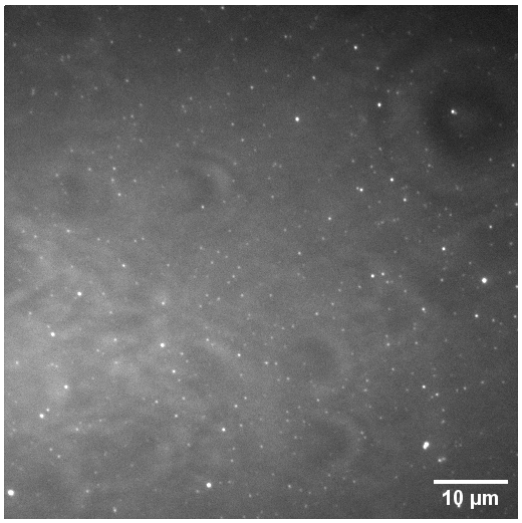

Figure 4. Example of imaging field with immobilized CENP-T-GFP molecules after addition of 200 nM Ndc80. The image contrast is different than in Figs 2 and 3.

19. Not touching the chamber perfuse 300 µL of 1x Imaging buffer with a syringe pump at a flow rate of 900 µL/min. Continue washing at a flow rate of 10 µL/min.

20. Image unbinding of Ndc80 from CENP-T for 20 min (Figure 5). To avoid photobleaching, image each of the selected fields just once after Ndc80 addition. Every time select a new imaging field from the preselected set of fields (step 12).

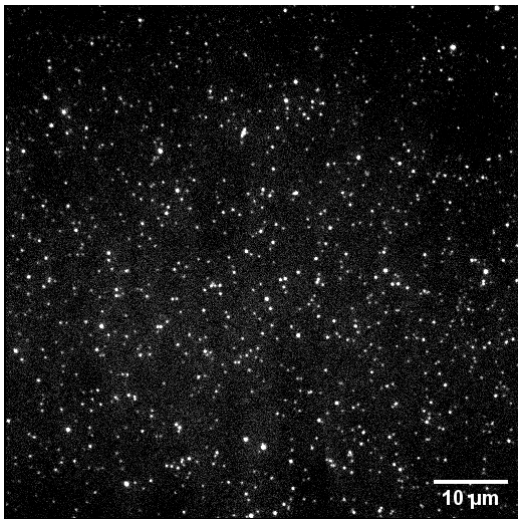

Figure 5. Example of imaging field with immobilized CENP-T-GFP after incubation with 200 nM Ndc80 and washout of soluble Ndc80. The image contrast is the same as in Figs 2 and 3.

Data analysis

1. To analyze the image sequences obtained, use Fiji (ImageJ) image processing software.

2. Correct the microscope stage drift manually in Fiji for every pair of images before/(after Ndc80 binding). For that, select one GFP molecule on both images and measure its relative displacement between images. Shift one of the images on the measured displacement along the horizontal and vertical axis to perfectly overlap images.

3. To select the GFP-tagged CENP-T molecule and background nearby, use circular regions. Minimize the radius of the region while ensuring that the molecule is completely included. Based on our microscope settings, an optimal region radius of 3 pixels is recommended.

4. For manual selection of single GFP-molecules use Time Series Analyzer V3 in Fiji. It draws circular regions of indicated size around dots selected by mouse click. Select >30 GFP dots per each time point (pair of images).

5. Select GFP spots randomly on the first image frame (before Ndc80 addition), avoid large aggregates, overlapping circles, or regions located close to the image bounds (Figure 6). If a GFP-tagged CENP-T molecule detached during the experiment, and is not present on the second image frame, do not consider it for further analysis.

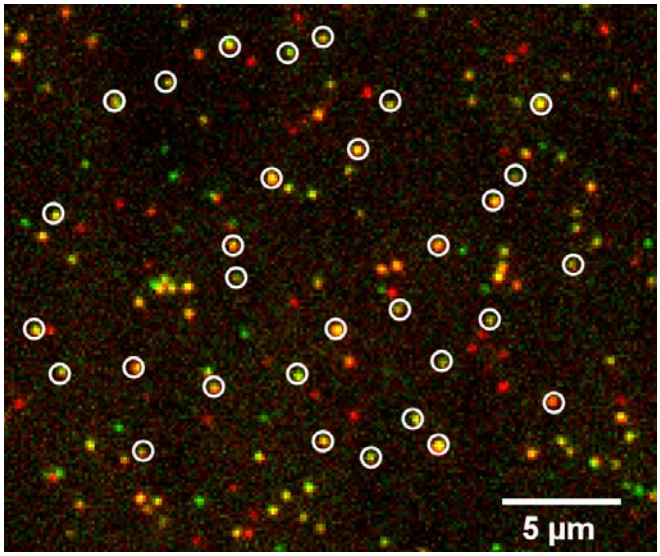

Figure 6. Example of GFP-spots selection. The image is an overlay of the same field images taken before Ndc80 binding (green) and after Ndc80 binding (red). White circles show the regions in which the integral fluorescence intensity was measured.

6. Measure the integral fluorescence intensity in the area surrounding the CENP-T molecule (3-pixel radius) on both images.

7. Manually translate regions near the selected molecule to collect individual background intensities (Figure 7).

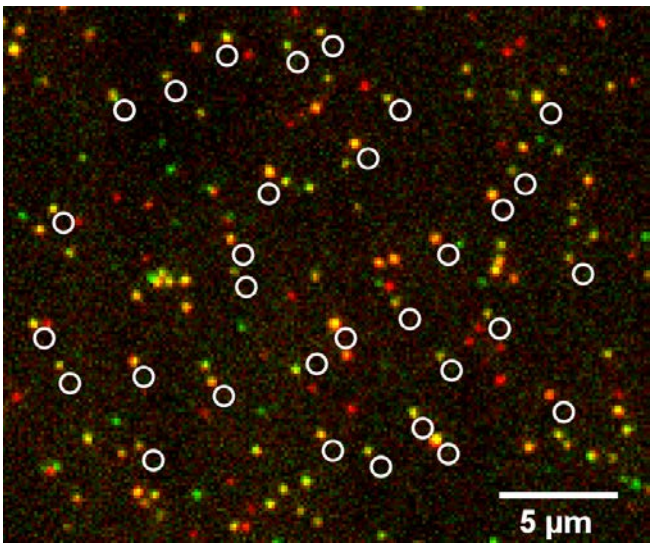

Figure 7. Example of background selection. The image is an overlay of the same field images taken before Ndc80 binding (green) and after Ndc80 binding (red). White circles show the regions in which the integral fluorescence intensity was measured.

8. Measure the integral fluorescence intensity in the background area on both images.

9. Analyze results using any available software. We used Excel. Subtract individual integral fluorescent intensity of backgrounds from sample intensity values to get the integral intensity of the GFP spot for both time points of the experiment.

10. Calculate the increase of GFP fluorescence on CENP-T, which is associated with Ndc80 binding. For that, for each GFP-spot subtract the integral intensity of CENP-T-GFP from the intensity after Ndc80 binding.

11. Normalize the increase of integral intensity obtained in step 10 by dividing it on the initial integral CENP-T-GFP intensity. The resulting values show the number of Ndc80 molecules bound per each CENP-T molecule.

12. Repeat steps 2-11 for all time points.

12. Plot the median number of Ndc80 per CENP-T molecule against time (Figure 8).

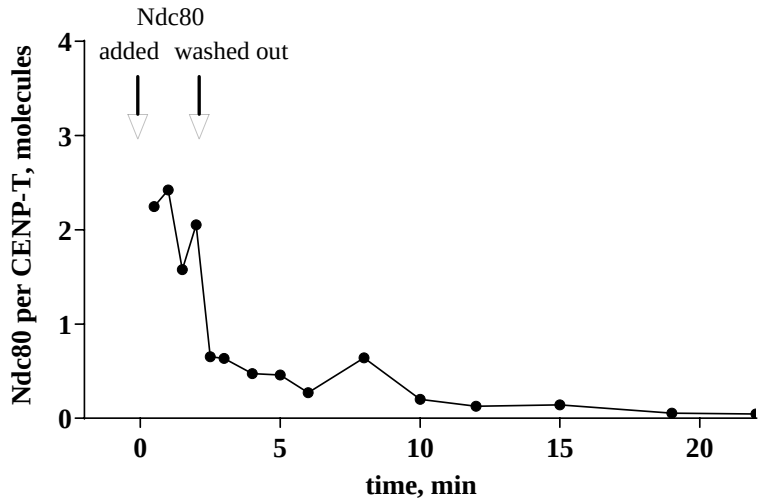

Figure 8. Ndc80 binding curve. In this experiment 200 nM of Ndc80 was added at time point 0 and washed out at 2 min. Each dot shows the median number of Ndc80 molecules per CENP-T against time based on n=30-40 molecules.

13. To determine the total binding, fit the part of the curve from Ndc80 addition to washout using one-phase association exponent (Y= (Plateau)*(1-exp(-K*x))) and determine the Plateau level. To determine the stable binding, fit the part of the curve after Ndc80 washout using one-phase decay exponent (Y=(Y0 - Plateau)*exp(-K*(X-IntTime)) + Plateau), where Y0 is equal to total binding level, and IntTime is the time when Ndc80 was washed out. The stable binding is determined as the Plateau level from this fitting (Figure 9).

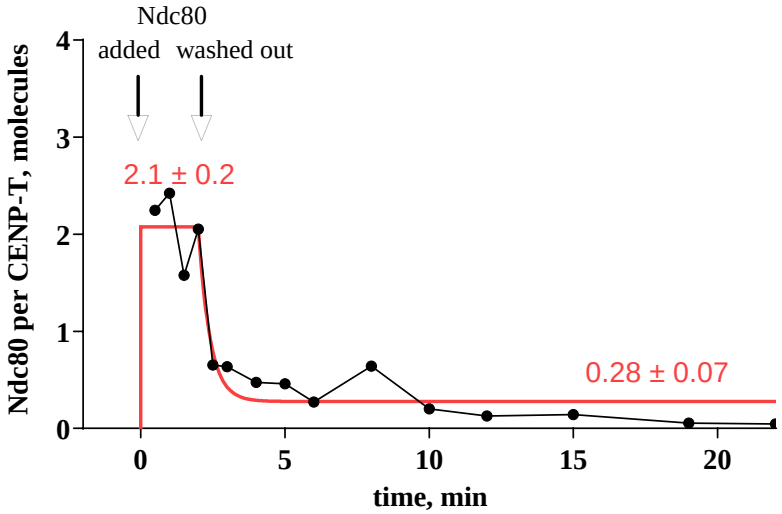

Figure 9. The Ndc80 binding curve (black) and exponential fittings (red) used to determine the total and stable binding. The values above the curve show the level of total and stable binding correspondingly.