

TIRF microscopy assay to study the interactions between clustered 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 camer
- Objective heater (Biopetechs)
- 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 ₂ HPO ₄ , 1.8 mM KH ₂ PO 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.
SpyCatcher-mi3 core particles		-80 °C	nanomolar	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 CENP-T-GFP- SpyTag to 1 μ M in PBS-BSA-casein buffer.
- b.** Dilute stock of GFP-tagged Ndc80 to 1 μ M in BRB-BSA-casein buffer.
- c.** 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.
- d.** Right before addition to the microscopic chamber dilute the proteins to the final concentrations (SpyCatcher-mi3 core particles to ~100 nM in PBS buffer supplemented 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- μ L 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.** Wash assembled chamber by perfusing 150 μ L PBS at flow rate of 150 μ L/min using a syringe pump. Use these pump settings at all steps, unless otherwise pointed.
- 6.** Block exposed silanized glass surface by perfusing 100 μ L of 1% Pluronic, and incubate for 10 min.
- 7.** Wash the chamber by perfusing 150 μ L of PBS at flow rate of 50 μ L/min using a syringe pump.
- 8.** To immobilize SpyCatcher-mi3 core particles perfuse 100 μ L particles, and incubate for 10 min.
- 9.** During incubation with particles 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.
- 10.** Wash the chamber by perfusing of 150 μ L of PBS-BSA-casein.
- 11.** Perfuse 150 μ L of 200 nM CENP-T-GFP-SpyTag in PBS-BSA-casein, incubate for 20 min.
- 12.** Wash the chamber by perfusing 150 μ L of PBS-BSA-casein.
- 13.** Wash the chamber by perfusing 150 μ L 1x Imaging Buffer.
- 14.** Focus, and close the diaphragm until its image is seen in the edges of the field of view. Avoid photobleaching, to find imaging fields move the stage with closed illumination and evaluate the field by one image shot.
- 15.** Image assembled GFP-tagged CENP-T clusters in 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. Quickly check images to make sure, that there is no saturation (use the “show saturation” option in NIS Elements software). Put the cursor over a couple of clusters to reveal pixel intensity - it should be 1,000-3,000 a.u. If pixel intensity is higher, reduce EM Gain. If the image intensity is too low, make sure you remove the polarizer and all filters in the light path. Use this microscope setting for all stages of the experiment. Do not change these settings between experiments.
- 16.** After the imaging field was selected, collect 25 images of assembled GFP-tagged CENP-T clusters to evaluate their initial intensity (Figure 2).

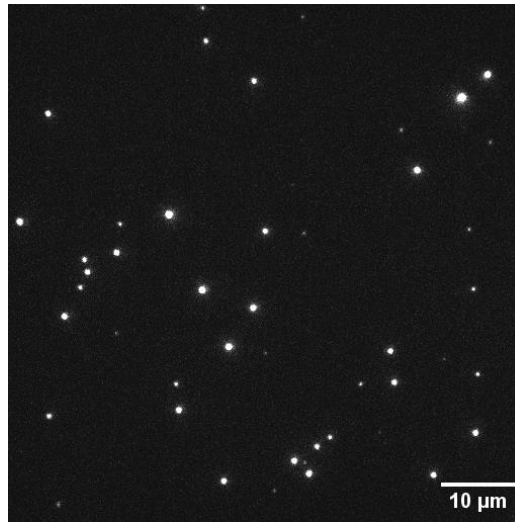


Figure 2. Example of imaging field with immobilized CENP-T^{6D} clusters.

17. Make 488 nm laser power 100% and bleach GFP on CENP-T-clusters for 30 s. This time is usually enough to bleach all GFP molecules. Collect 25 images of clusters to evaluate their initial after bleaching (Figure 3).

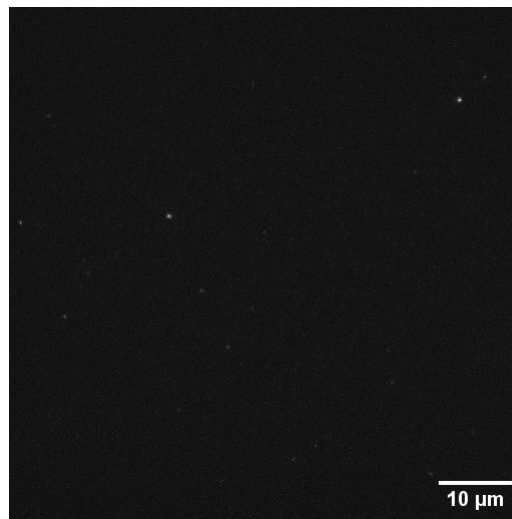


Figure 3. Immobilized CENP-T^{6D} after bleaching.

18. Turn the 488 nm laser back to 1% and collect 25 images of clusters to evaluate their intensity after bleaching.

19. 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.

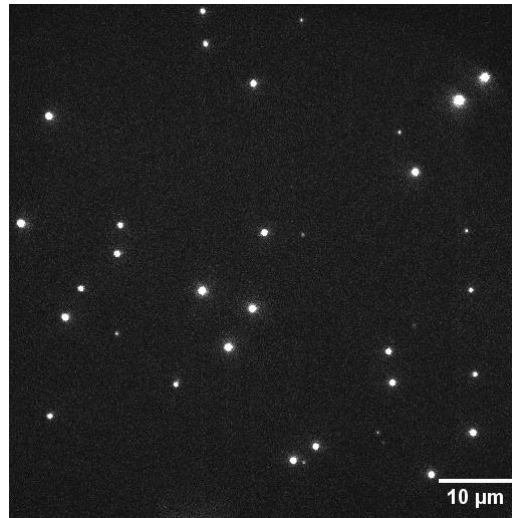


Figure 4. Bleached CENP-T^{6D} clusters after the addition of 200 nM GFP-tagged Ndc80.

20. Image binding of Ndc80 to clustered CENP-T for desired time (Figure 4). The frequency of imaging depends on the total binding time. Collect 60-120 frames total during the binding phase.

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

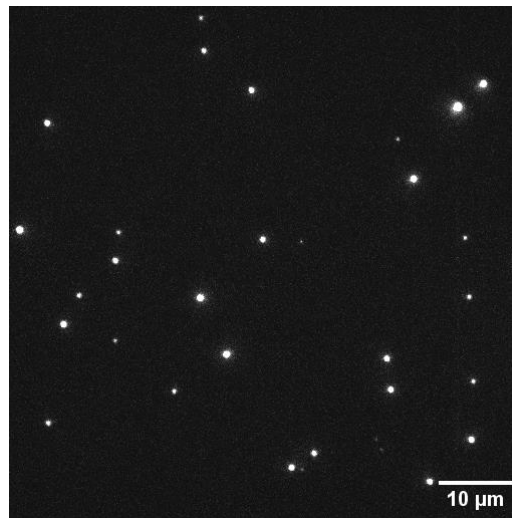


Figure 5. Bleached CENP-T^{6D} clusters with bound GFP-tagged Ndc80 after Ndc80 washout.

22. Image unbinding of Ndc80 from clustered CENP-T for 10-20 min (Figure 5). As described above frequency of imaging depends on the total unbinding time. Collect 60-120 frames total during this phase.

Data analysis

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

2. To correct the microscope stage drift in the image sequence using the "Manual drift correction" plugin available in Fiji. This plugin requires tracking the positions of a single cluster across the image stack. By executing the plugin, it generates an image stack with the stage drift corrected based on the positions of the tracked cluster.

3. To select CENP-T clusters and background nearby, use circular regions. Minimize the radius of the region while ensuring that the cluster is completely included. Based on our microscope settings, an optimal region radius of 8 pixels is recommended. For semi-automatic cluster selection, we suggest using the Fiji script provided in Appendix ('intensity_program.ijm').

4. Indicate in the script the path to the output folder and the radius of the circular regions in pixels. 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 running one part of the script be sure, that other parts are commented.

A. Clusters Selection: To select clusters automatically the noise for clusters selection should be determined. For that goal gradually increase the "noise" parameter until the script stops selecting background as clusters. In all our experiments the noise was 300. Do not change the threshold and radius between experiments. After you select the noise, the script will select circle regions around clusters. Visually inspect selected regions, and delete from Fiji ROI manager circles that are: include large aggregates, have two or more clusters in one circle, overlapping circles, or regions partially outside the image bounds (Figure 6).

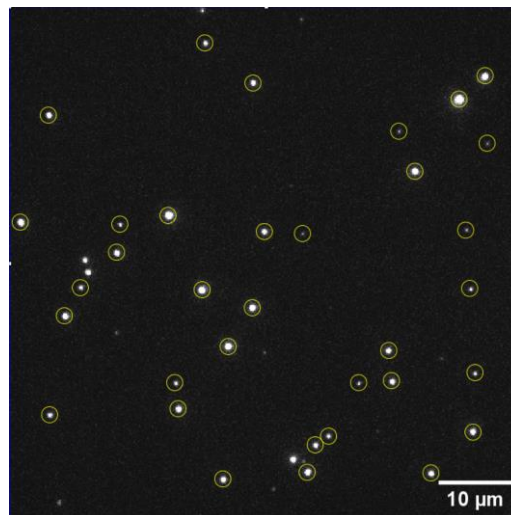


Figure 6. Example of cluster's selection.

B. Measurement of Integrated Fluorescence Intensity: In this step, the script will calculate the integral fluorescence intensity of the selected circles. The output file 'sample.csv' will contain the integral intensity, 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 right to designate them as background regions. After executing this step, visually examine the

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image to ensure the background regions are selected correctly (Figure 7). Using Fiji ROI manager manually move the position of the circles so that contain clusters and extend beyond the image borders.

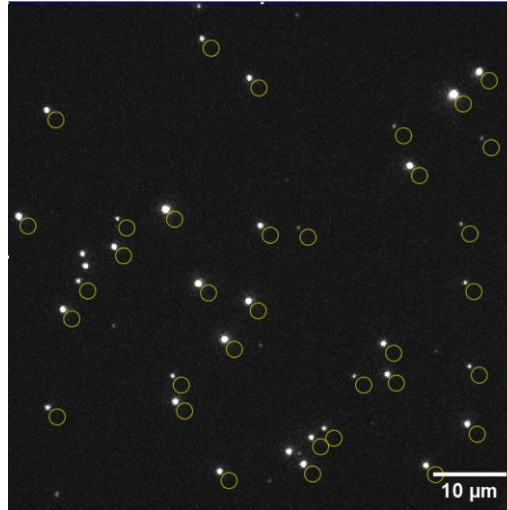


Figure 7. Example of background selection.

D. Measurement of Background Fluorescence Intensity: In the final part of the script, the background circles will be used to measure the integral intensity. First, indicate the path for the output directory and run the script. The output file ‘background.csv’ will contain integral intensity, mean, minimum, and maximum values of the pixels within each background circle.

5. Analyze resulting Excel files ‘sample.csv’ and ‘background.csv’ using any available software (MatLab, Excel).

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

7. Subtract individual integral fluorescent intensities of background from sample intensity values to get the intensity of CENP-T clusters for each time point of the experiment (Figure 8).

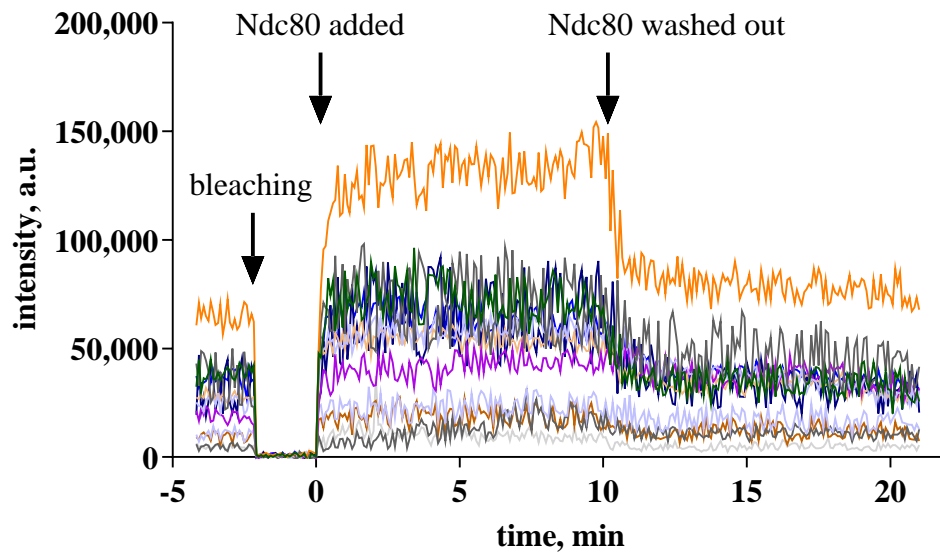


Figure 8. Examples of curves showing changes in $CENP-T^{6D}$ cluster's intensity against time. Each curve is an individual cluster.

8. For each time point, normalize the intensity of each GFP cluster by dividing it on its initial intensity (average at 25 frames before bleaching). The resulting values after the addition of Ndc80 show how many Ndc80 were bound to each CENP-T molecule within the cluster (Figure 9).

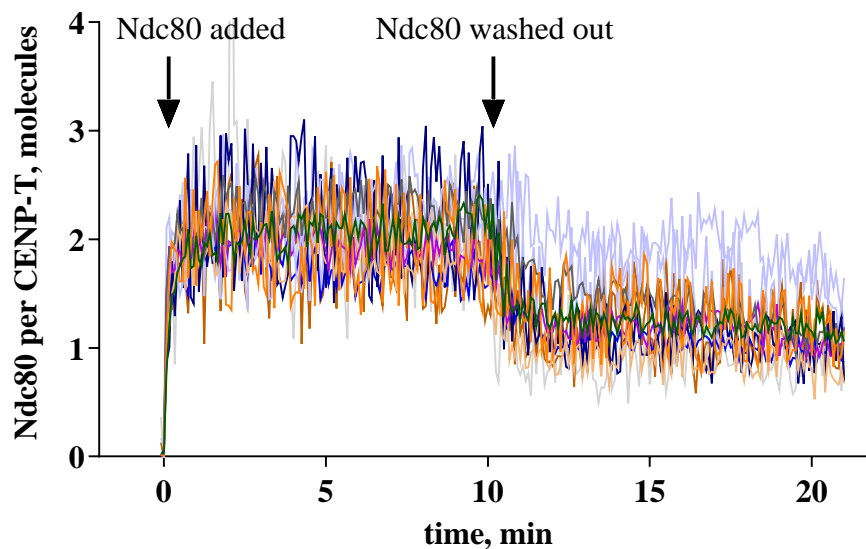


Figure 9. Examples of curves showing Ndc80 binding to clustered $CENP-T^{6D}$ molecules against time. Each curve is an individual cluster.

9. Plot the median of Ndc80 per CENP-T against time (Figure 10).

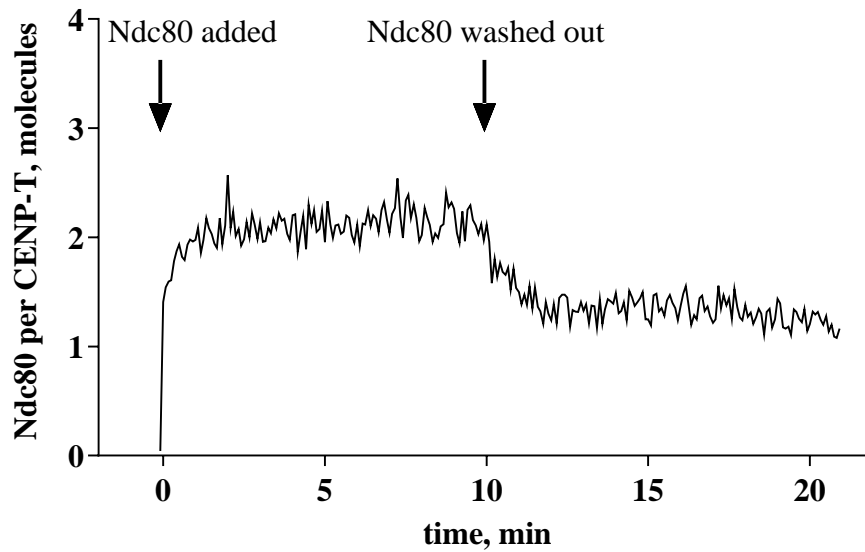


Figure 10. Median of curves shown on Fig. 9.

10. While it is not clear how to take into account photobleaching in the presence of frequently exchanging GFP-tagged Ndc80, only data after Ndc80 washout will be corrected on photobleaching. For that, divide these regions of experimental curves on the corresponding value from the photobleaching curve, obtained using the “Analysis of photobleaching rate of monomeric and clustered fluorescent molecules” protocol (Figure 11).

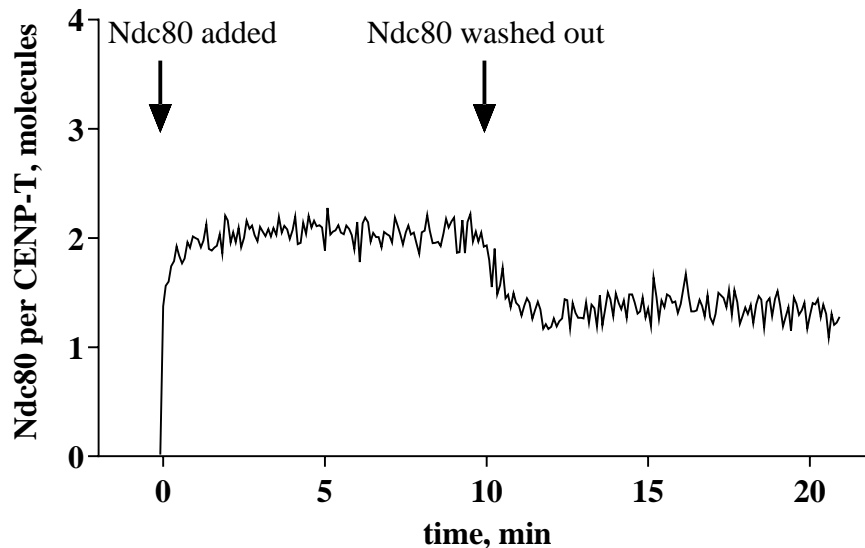


Figure 11. Curve from Fig. 10 after photobleaching correction.

11. To determine the total binding, fit the part of the curve from Ndc80 addition to washout using one-phase association exponent ($Y = (\text{Plateau}) \cdot (1 - \exp(-K \cdot x))$) and determine the Plateau level. To

determine the stable binding, fit the part of the curve after Ndc80 washout using one-phase decay exponent ($Y = (Y_0 - \text{Plateau}) \cdot \exp(-K \cdot (X - \text{IntTime})) + \text{Plateau}$), where Y_0 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 12).

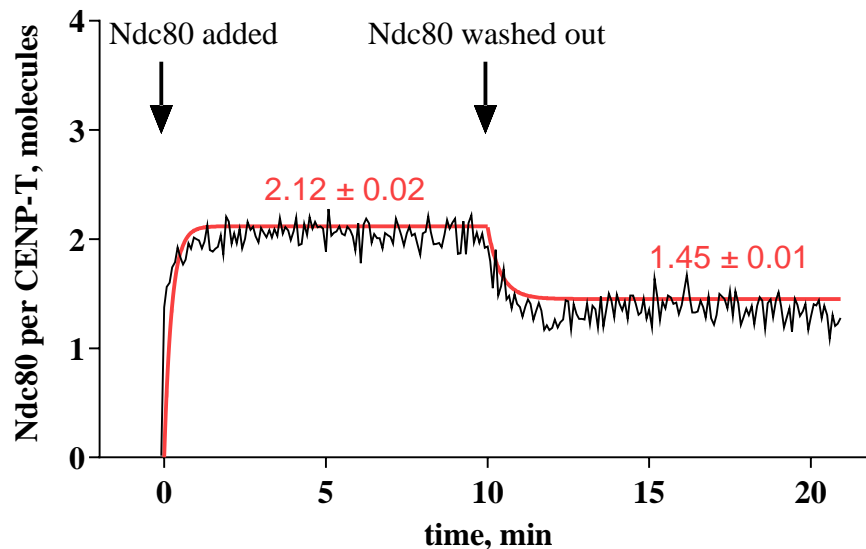


Figure 12. The Ndc80 binding curve (black) and exponential fittings (red) used to determine the total and stable binding. The values above the curve show the level of total and stable binding correspondingly.

Appendix. Fiji script (intensity_program.ijm):

```
path = "/Path/to/your/output/folder/"
radius=8;

//PART A - automatic cluster selection

noise = 300
run("Find Maxima...", "noise="+noise+" output=[Point Selection]");
roiManager("Add");
getSelectionCoordinates(xpoints, ypoints);

roiManager("Delete");
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 clusters integral fluorescence
intensity
```

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

//PART C – automatic shifting of clusters circles for background measurement

```
roiManager("translate", 10, 10);
```

//PART D – measurement of background integral fluorescence intensity

```
roiManager("multi-measure measure_all append");  
saveAs("Results", path + "background.csv");
```