Training Kit™: dSTORM

Sample preparation, image acquisition and analysis protocol

The ONI dSTORM Training Kit includes a microscopy slide with fixed mammalian cells, and reagents to label and image nuclear pores using the Nanoimager.

Use the QR code to access all resources for this product or visit: oni.bio/dstormtrainingkit_resources

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Training Kit™: dSTORM

PROTOCOL

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Component list

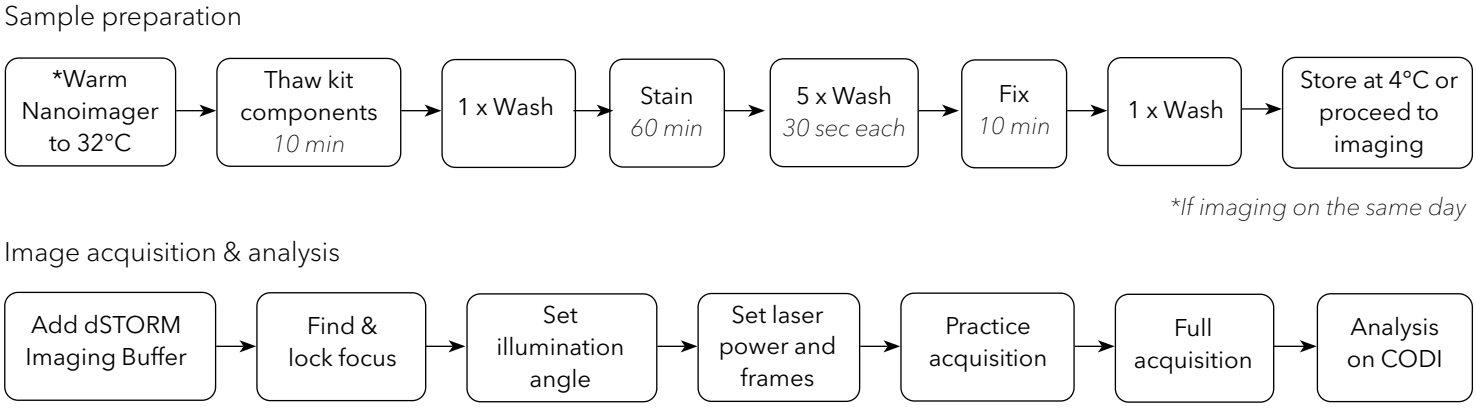
Component	Quantity	Volume	Hazard	Storage
Sample slide	1	-	N/A	❄ †
Staining solution	1	60 µL	N/A	❄ †
Fixative	1	340 µL		❄
Washing solution	1	1.5 mL	N/A	❄
dSTORM Imaging Buffer Part A	1	400 µL	N/A	❄
dSTORM Imaging Buffer Part B	1	N/A	N/A	❄ †
Part B Resuspension Buffer	1	30 µL	N/A	🌡

❄ Store at -20°C † Single-use 🌡 Store at 4°C

Equipment needed

- ☐ Pipettes (p20, and p200)
- ☐ Pipette Tips
- ☐ Vials (1.5 mL microtubes)
- ☐ Timer
- ☐ Aspirator or Vacuum Pump (optional)
- ☐ Water bath or heat block
- ☐ Lens cleaning paper
- ☐ Objective oil
- ☐ Nanoimager

Experimental workflow overview



Advice before you start

It is recommended that you read the complete protocol thoroughly before starting. Kit materials only allow for 1 experimental cycle, with imaging buffer provided for up to 4 imaging sessions. Please pay particular attention to the sample slide guidance section below, as well as the reagent handling considerations (i.e. when reagents should be removed from storage, thawed, or warmed up).

Guidance for using the sample slide

Once the slide is opened it should not be re-sealed or stored at -20ºC. Liquid should be added into each lane through the inlet hole marked "IN", taking care not to introduce bubbles into the lane. Pipette out any air at the pipette tip before pipetting liquid into the inlets, and stop pipetting once all the liquid in the tip has been pushed out. The slide inlet is optimally designed to work with 200 µL pipette tips, using either a p20 or p200 pipette. It is not recommended to use other tip sizes.

When ready (follow the Stepwise protocol on page 5), please consider the following:

- Place the slide on a clean surface. The slide pouch or a clean tissue can be used.
- In order to ensure consistent liquid delivery, insert the pipette tip into the inlet at a 90° angle, holding the pipette vertically.
- Before pushing contents out, press the pipette tip against the inlet walls until a resistance is felt to create a seal and keep light pressure while pipetting. Liquid should be seen flowing into the lane and coming out at the outlet side "OUT" of the slide lane. Add entire volumes gently in one motion, taking care not to move liquid back and forth within the lane.
- If any liquid is seen leaking from the inlet port, it means that the seal between the pipette tip and inlet port is not optimal. If this occurs, stop pipetting but do not reverse the flow of liquid by sucking back through the pipette. Gently adjust the position of the pipette to improve the seal around the pipette tip and then continue to push the remaining volume through the slide. It is safest to repeat the addition of liquid in that lane before proceeding if needed.
- It is recommended to use an aspirator connected to a vacuum or suction pump to remove excess solutions from the outlet port. This can be most easily achieved by placing the aspirator tips at the edge of the wide outlet reservoir and keeping in a fixed position as you pipette. Do not place

the aspirator tip into the central hole of the slide outlet as this will result in aspiration of all liquid from the sample lane, which will impact sample quality.

- If an aspirator is not available, liquid waste can be removed from the slide outlet by gently touching the surface of the droplet forming around the outlets using a delicate task tissue (e.g. Kimwipe) to absorb excess liquid.
- While the reagents on the slide are incubating, cover the slide with the pouch provided or a box lid, so that the slide stays clean and covered from light.
- If any air pockets or very large bubbles are observed at any step inside the slide lanes, additional liquid can be flushed through the relevant lane to push this out before proceeding with the following steps in the protocol. This is not recommended unless required, as it risks damaging the slide surface. Avoiding introduction of air bubbles during slide handling is crucial for the success of the experiment.

Important reagent handling considerations

- Assay slides should be brought to room temperature before opening. Please note that once opened, slides cannot be stored or reused.
- dSTORM Imaging Buffer Part B should be kept at -20°C at all times and should only be removed briefly before adding to dSTORM Imaging Buffer Part A, and kept on ice during handling. Then, immediately returned to -20°C.
- Following first thawing, dSTORM Imaging Buffer Part A may be aliquoted in 100 µL volumes and refrozen at -20°C if desired. If doing this, it is recommended to thaw the original stock on ice to minimize exposure to higher temperatures. Once aliquoted, each aliquot should be used once only and not refrozen.
- The Staining Solution is photosensitive and should be protected from light when not being directly handled. This also applies to the Sample Slide during and following staining.

Suggested protocol stop points

- It is recommended to image the sample on the same day as preparation, however, it is also possible to store the sample for up to 7 days prior to imaging. Ensure that the slide inlet and outlet are well sealed and then place at 4°C, protected from light.
- If planning to image the sample multiple times on different days, wash out the dSTORM Imaging Buffer after each imaging session with 150 µL Washing Solution or phosphate buffered saline prior to storing as described above.

Stepwise Protocol

All steps are performed at room temperature. It is recommended to perform imaging as soon as is convenient after sample preparation. Refer to the Sample preparation training video for support: oni.bio/dstormtrainingkit_resources

Sample preparation - total time: 85 - 95 min

Important note: if imaging on the same day as sample preparation, begin warming the Nanoimager to 32°C before beginning with step 1, or for at least 60 min prior to imaging.

1. Remove the Staining Solution, Washing Solution, and Fixative from -20°C storage and allow to reach room temperature, while protected from light. Do not remove the dSTORM Imaging Buffer from the -20°C freezer at this stage.
2. Thaw the Sample Slide unopened by allowing it to reach room temperature for 10 minutes. This is important to minimize bubble formation.
3. Open the Sample Slide pouch and place the slide on a clean, flat surface. Take care during handling not to damage or deface the glass underside of the slide. Retain inlet/outlet sealing stickers provided.
4. Gently flush 100 µL Washing Solution through the sample lane.
5. Apply 50 µL Staining Solution and incubate for 60 min, protected from light.
6. 30 minutes before the completion of the staining incubation, transfer the Fixative to 37°C water bath or heat block.
7. Wash Sample Slide five times with 100 µL Washing Solution. Wait 30 seconds between each wash.
8. Apply 50 µL warm Fixative and incubate for 10 min, protected from light.
9. Wash once with 100 µL Washing Solution.
10. EITHER proceed to step 11 for imaging, OR seal lane with stickers provided and place at 4°C, protected from light for storage.

Imaging - total time: 30-60 min

11. Thaw one vial of dSTORM Imaging Buffer Part A at room temperature for 10 minutes and transfer 99 µL dSTORM Imaging Buffer Part A to a microtube.
12. Remove dSTORM Imaging Buffer Part B from -20°C and let come to room temperature. Ensure you re-seal the plastic bag it came out of
13. Add 25 µL of Part B Resuspension Buffer to the vial. Slowly add a single drop to the center of the pellet, and the rest is applied to the sides of the vial to ensure complete dissolution of the pellet.
14. Put the rehydrated vial on ice for 5 minutes.
15. Gently mix the re-hydrated pellet by pipetting up and down. The vial can be removed from the ice bucket for this process. **Do not introduce bubbles or air. Do not shake or vortex the vial**.

    Important note: Keep dSTORM Imaging Buffer on ice while handling, store immediately at -20°C after use.
16. Add 1 µL of the resuspended Part B to the 99 µL of Part A in the new microtube and mix gently.
17. Apply 50 µL dSTORM Imaging Buffer solution through the sample lane, first removing the lane-sealing stickers if applicable.

    Important note: do not use a KimWipe on the objective lens of the Nanoimager, but ultrafine lens cleaning paper.

At this point, open the Image Acquisition & Analysis training video which will introduce you to the Nanoimager software (NimOS) and guide you through the acquisition and analysis.

The NimOS software is accessed from the desktop of the laptop connected to the Nanoimager.

Finding & locking focus

18. In the Nanoimager software (NimOS), activate the 520 nm transillumination laser to 50% power and ensure the focus laser is active.

19. Starting at 0 µm, move the stage Z position down by clicking inside the Z text field and using the arrow keys (hold Control + Down arrow key to increase speed of movement), until the highest contrast image is observed in the top right focus laser window and the slide surface comes into rough focus in transillumination. Note that there may or may not be cells present in the exact position initially imaged.

20. Once you have found rough focus, turn off the Transillumination and activate the 640 nm laser at 5% power and move the stage in X and Y directions, by clicking the camera image and using the arrow keys, to locate the cells of interest. Ensure the cells are in focus, run "set focus reference" calibration, then activate Z-lock to fix the focus.

    Note: lower laser powers can be used for this step if sample bleaching is observed. The Z-lock button is activated when it reads "Stop Z-Lock".

21. Once the Z-lock is engaged, fine adjustments can be made by adjusting the Z-offset position. In this case, the basal side of the sample (i.e. the side in contact with the imaging surface) is in sharp focus.

Setting the illumination angle

22. Start by setting the illumination angle at 45° and slowly increase half a degree at a time. Adjust the illumination angle such that the focal plane including the basal side of the sample is bright but that the out-of-focus background is reduced. This is typically between 50° and 55°, depending on the Nanoimager system.

    Tip! The optimal illumination angle for imaging nuclear pores is slightly less stringent than the maximum at which the sample is still visible. An effective way to determine this optimum is to increase the angle to the point at which illumination of the sample is no longer visible and then reduce this by ~3°. This is 2° less than the highest contrast image, and will result in some background blur around the outside of the nucleus. If poor blinking is seen with the imaging settings described below, this may be caused by too stringent an illumination angle, and so further reducing the angle may be beneficial.

Setting the laser power, frame rate and frame numbers

23. Increase the 640 nm laser power until good blinking is achieved. This is typically between 100-150 mW (typically around 50% power for most systems). Deactivate the 640 nm laser.

    Note: It is recommended to move to another field of view, as the original field will have partially photobleached through exposure to high laser power.The laser power determined in this step is applicable to all fields of view and does not need to be re-established.

24. In Acquisition control, set the exposure time to 30 ms and number of frames to 20,000.

25. Enter the folder and filenames into their respective fields (on previous versions of NimOS these are labeled data and acquisition tags).

Practice acquisition

26. Activate the 640 nm laser at low power to find a new cell, focus on the bottom of the nucleus using the Z-offset. When ready, turn the laser power back up to imaging power (e.g. from 5 to 50%) and quickly click 'Acquire' to begin acquisition. We recommend using an initial practice acquisition to optimize the illumination angle and Z-offset to obtain the best blinking.

27. Adjust the illumination angle up and down until your dSTORM spots have a good signal-to-noise ratio with fast and dense blinking.

    Note: If the illumination angle is set too high, the blinking slows down. In this case, it is recommended to lower the illumination angle by 2° to increase blinking speed.

28. During acquisition, it is possible to switch to the 'Analyze' tab to see the super-resolution image building up in real-time.

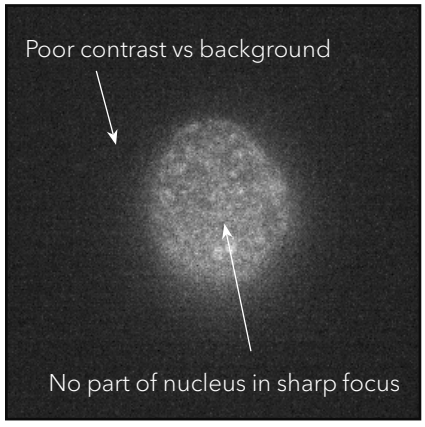
    Note: During the initial acquisition localizations may form in lines, this is due to thermal drift. This may require the Nanoimager and sample temperature to stabilize, and can also be corrected during data analysis.

Full acquisition

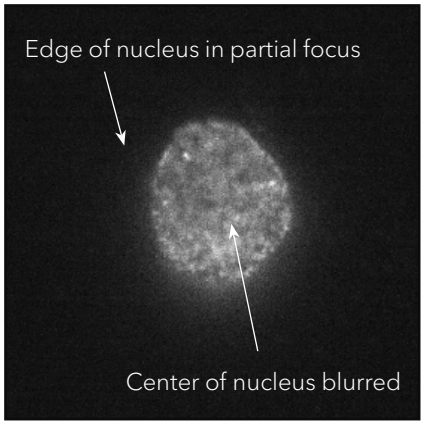
29. Reactivate the 640 nm laser to the lower laser power, aim to find a big and round nucleus, adjust the Z-offset and illumination if needed to obtain maximum brightness at the bottom of the nucleus.
30. To save a diffraction-limited or 'normal' resolution image, set the exposure time to 100 ms and number of frames to 1, change the filename and click 'Acquire'.
31. To obtain the dSTORM image, change the filename name, and adjust the exposure time to 30 ms and the number of frames to 20,000.
32. Ensure the 640 nm laser is back at the imaging power (around 150 mW), turn on the laser , and click 'Acquire'.

Examples of correct imaging setup

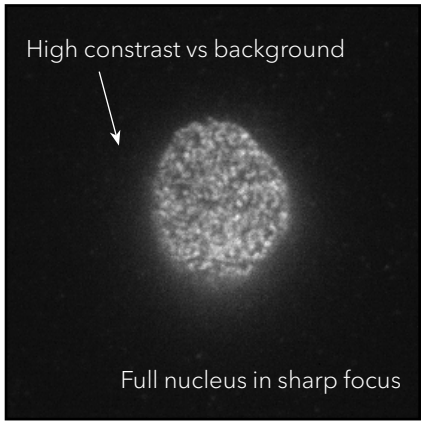
Focal position too low



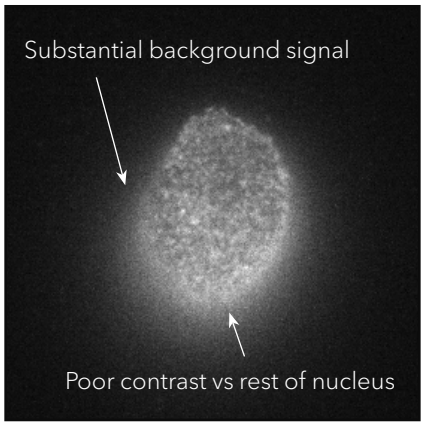
Focal position too high



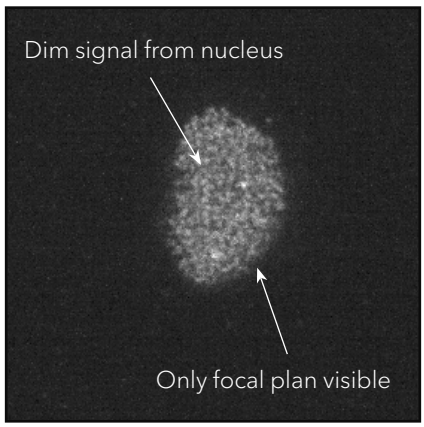
Correct focal position



Illumination angle too low



Illumination angle too high



Correct illumination angle

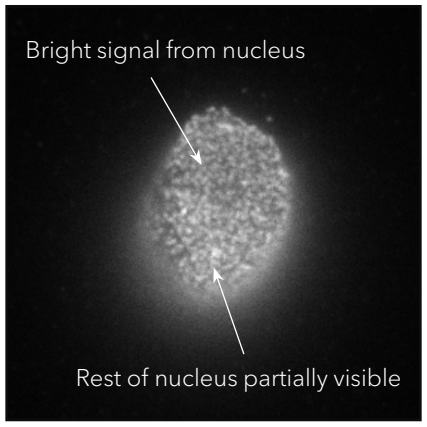


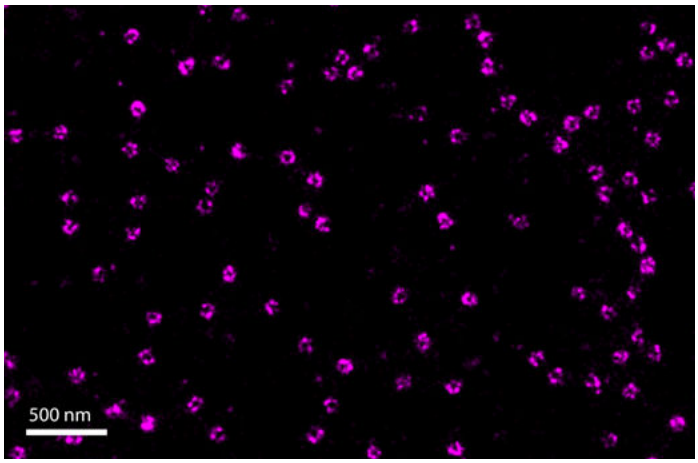
Image Analysis - total time: 5 - 10 min per dataset

CODI is ONI's cloud-based data analysis program, which is operated in your web browser. It can be accessed through: alto.codi.bio You can sign up either with your email or a Google account. You will upload your final dSTORM dataset using the CODI Desktop Uploader, then, use re-configured settings to apply drift correction and basic single-molecule filters, and finally perform localization clustering.

- Sign into CODI, click upload dataset and install the CODI Desktop Uploader if not yet installed. Otherwise, the Upload function in CODI can be used.
- Go to Collaborations, the space where you can organize datasets and share them with other users. Click the new Collaboration button and name it 'dSTORM Training Kit'.
- Log into the CODI Desktop Uploader, open the file explorer, select the folder containing your data, and select the Nuclear Pore dSTORM Image. Then select the dSTORM Training Kit Collaboration. Click Upload and then the red Upload button without checking the include raw data box. This will upload all files for any given acquisition, with exception of the .tif file.
- Once the data is optimized, you can access your dataset on the cloud from the browser on any computer. Go to CODI in your browser and open Collaborations. Open the relevant dataset by clicking on the thumbnail.
- Click 'Add a new analysis tab' on the left-hand side menu, select the 'Clustering' App and load the default 'dSTORM training kit' settings.
- Click the 'Run all steps' play button at the bottom to start the analysis. This includes drift correction, basic single-molecule filters and clustering analysis with parameters optimized in-house by the ONI team.
- Icons on the left will turn green as each tool is applied. Once the settings have finished running, you can zoom into your image, inspect individual nuclear pores, and you should even be able to resolve the ring-like structure of the nuclear pores.
- To see the improvements after analysis, you can toggle the drift correction button on/off, and, under the filtering tab, see the settings that allow you to exclude localizations based on five different parameters. These settings have been optimized for the dSTORM Training Kit. For more information about localization filtering and the individual filters, please see the CODI How to Guide.
- Go to the Clustering tool tab. Clustering is a method to group together localizations that are near to each other to identify biological structures for further analysis. There are several different algorithms that can be used for clustering, which you can learn about in the CODI How To Guide, or by visiting our website Learning section at oni.bio. For nuclear pores, we are using DBSCAN.
- Each nuclear pore has been grouped as an individual cluster. We can analyze several different features and view them as a histogram for all the pores in the field of view. For example, you can see the cluster area, the number of localizations in each cluster, or the radius of the cluster. For more information on each of these measurements, please refer to the How To Guide or visit the website Learning section.
- Finally, it is recommended to compare the image of the same cells that you acquired with normal (diffraction-limited) resolution. You can full screen the CODI image with the button in the bottom left of the window. Open the TIFF file for the normal resolution image, for instance, using FIJI or Image J.
- In the image with normal resolution, you cannot resolve individual nuclear pores from each other, whereas in the dSTORM image you can resolve pores from each other as well as see the ring-like shape of the pores, even though this is less than 100 nm across. Amazingly, the nuclear pore is about the same size as a single pixel when you look at it with normal resolution.

Troubleshooting notes

- Good blinking is essential for the resolution of individual nuclear pores and their structures. Good blinking is characterized by rapid flashing of individual bright spots that are roughly uniform in size and intensity, and that completely disappear to background levels between blinks. It should not be possible to discern any meaningful cellular structures by eye during blinking. Note that it typically takes 100-300 frames for all fluorophores to begin blinking effectively, and so during this time individual spots may not be visible. These frames should be filtered out of the final data.
- Poor blinking is characterized by spots that persist for several seconds, spots that are clearly heterogeneous in size and/or intensity, or being able to still discern cellular structures by eye during blinking.
- The most likely causes of poor blinking are an illumination angle that is too high and/or a laser power that is too low. Troubleshoot this by systematically reducing illumination angle and increasing laser power and observe if blinking improves. If poor blinking is observed even at laser powers >200 mW and illumination angles >10° below the point at which sample illumination is lost, then incorrect illumination settings are likely not the cause of poor blinking. In such a case, try preparing fresh dSTORM Imaging Buffer by mixing both components again. Otherwise, poor quality imaging buffer is the most likely cause so please contact us via: https://oni.bio/contact
- Good photostability of the fluorophores is also needed to resolve nuclear pore structures effectively. If the sample exhibits good photostability the overall density of blinks should not obviously decrease by eye in a short period, however will gradually drop over the course of the acquisition. Poor photostability is characterized by a rapid drop in the number of blinking events within 2000-5000 frames. Poor photostability is most likely due to high levels of oxygen within the sample, which indicates either poorimaging buffer quality, or incorrect handling of the buffer during preparation (reminder - take care not to introduce excess oxygen into the solution by flicking or shaking the tube).
- If needed, replace the imaging buffer with a fresh stock and repeat imaging. If the imaging buffer is good but poor photostability is still observed, ensure that the laser power is not too high. Reassess the laser power to determine if there is a lower power that still gives effective blinking but induces less photobleaching.



- 20,000 frames is the recommended acquisition length to get excellent nuclear pore morphology, however pores should be visibly evident after 3,000-5,000 frames. If pores are not visibly evident after 10,000 frames it is likely that blinking and/or staining is poor. Acquisitions can be extended beyond 20,000 frames if more localizations are desired, though typically this will not significantly improve visible pore morphology.

  Note: if there is drift during acquisition pores may not be clear by eye until post-processing drift correction is performed and is not inherently an indicator of poor blinking.
- If either the signal background on the imaging surface or between nuclear pore complexes is high, this is most likely due to poor washing following staining. Ensure that the full wash volume is flushed through at each step; that liquid is not transferred from the lane inlet to the tube containing PBS; and that the 30 s wait period is observed after each wash step.

Access all videos through the product Resource Hub:
oni.bio/dstormtrainingkit_resources

Additional information on dSTORM sample preparation:
https://pages.oni.bio/dstorm-sample-preparation-workflow

Contact us
https://oni.bio/contact

ONI

QUICK GUIDE

Training Kit™: dSTORM

Training Kit™: dSTORM

This quick protocol refers to ONI's Training Kit for dSTORM imaging, which provides a sample slide for customers to stain and image nuclear pores using the Nanoimager. Use the QR code or link below to access the stepwise protocol and accompanying training videos. All steps are performed at room temperature.

Equipment needed

- Pipettes (p20 and p200)
- Pipette tips (200 µL)
- Vials (1.5mL microtubes)
- Timer
- Aspirator or vacuum pump (optional)
- Water bath or heat block
- Lens cleaning paper
- Nanoimager

Sample Preparation - 85 - 95 mins

1. If imaging on the same day, warm the Nanoimager to 32°C for at least 60 min prior to imaging
2. Allow the Fixative, Staining, and Washing Solutions to reach room temperature. Keep dSTORM Imaging Buffer in the -20°C freezer
3. Thaw the Sample Slide unopened at room temperature **[10 min incubation]**
4. Open the chip pouch and place on a clean surface (chip pouch or tissue). Retain provided inlet/outlet sealing stickers
5. Gently flush 100 µL Washing Solution through the lane
6. Apply 50 µL Staining Solution and protect from light **[10 min incubation]**
7. 30 min before completion of step 6, place the Fixative to 37°C water bath or heat block
8. Wash Sample Slide 5x with 100 µL Washing Solution **[30 sec incubation between each wash]**
9. Apply 50 µL Fixative, protect from light **[10 min incubation]**
10. Wash once with 100 µL Washing Solution
11. Proceed to imaging or seal lane with stickers provided and place at 4°C, protected from light for storage

Sample Preparation - 85 - 95 mins

12. Ensure the Nanoimager is stably warm at 32°C
13. Remove dSTORM Imaging Buffer Part A from -20°C and thaw at room temperature. Transfer 99 µL dSTORM Imaging Buffer Part A into a microfuge tube
14. Resuspend dSTORM Imaging Buffer Part B in 25 µL Part B Resuspension Solution
15. Add 1 µL dSTORM Imaging Buffer Part B to the 99 µL dSTORM Imaging Buffer Part A and gently mix by pipetting to the new microtube containing 99 µL dSTORM Imaging Buffer Part A
16. Apply 50 µL of the dSTORM Imaging Buffer mix to the sample lane and seal inlet and outlet with stickers
17. If necessary, clean the microscope objective lens with appropriate ultrafine cleaning tissue dipped in 100% ethanol
18. Apply 1-2 drops of immersion oil onto the objective lens and place your sample on the Nanoimager stage
19. In the NimOS software, focus on your sample, and set Z-lock to fix using the focus laser
20. Make fine adjustements using the Z-offset to find the basal side of the nucleus
21. In Illumination control: set an illumination angle, normally between 50-55°. Increase the 640 nm laser power until good blinking is achieved. This is typically between 100-150 mW
22. In Acquisition control: set the exposure time to 30 ms and number of frames to 20,000
23. Enter folder and filenames, click 'Acquire'
24. See full protocol for details on optimizing imaging conditions and signal-to-noise ratio (QR code below or visit oni. bio/dstormtrainingkit_resources)

Image Analysis - 5 - 10 mins per dataset

25. Sign into CODI (alto.codi.bio) and upload all files for any given acquisition, with exception to the .tif file. It is recommended to use the Desktop Uploader function
26. Open the relevant dataset, click 'Add a new analysis tab', select the 'Clustering' App and load 'dSTORM training kit' settings
27. Click 'Run all steps' button at the bottom. Analysis includes drift correction, single-molecule filters and clustering. It is recommended that the user optimizes parameters for their dataset
28. Data can be inspected by zooming in and observing the ringlike structure characteristic of nuclear pores

oni.bio/dstormtrainingkit_resources