



Protocol: LNP Profiler

Sample preparation: staining of lipid nanoparticles for SMLM and diffraction limited imaging

This protocol refers to ONI's Application Kit™: LNP Profiler for capturing and staining LNPs onto the surface of functionalized chips for imaging using super-resolution microscopy.

PROTOCOL

Sample preparation: staining of lipid nanoparticles for SMLM and diffraction limited imaging

Component list

Component	Quantity	Volume	Hazard	Storage upon arrival
Surface Block†	4	55 µL	N/A	❄️
Surface Reagent†	4	55 µL	N/A	❄️
Anti-PEG Capture†	4	55 µL	N/A	❄️
Wash Buffer*	1	12 mL	N/A	RT*
Cargo Detection	1	10 µL	N/A	❄️
PanLNP Detection	1	8 µL	N/A	❄️
Ligand Detection	1	10 µL	N/A	❄️
LNP Assay Chip†	4	N/A	N/A	❄️

❄️ Store at -20°C † Single-use

* Wash buffer can be stored at -20°C with all kit component, but storage at RT or 4°C is recommended for ease of use

Equipment needed

- Pipettes (p10, p20, p200, p1000)
- Pipette Tips (10, 20, 200 and 1000 µL)
- Low Bind Microcentrifuge tubes (1.5 mL or 2 mL), e.g., ThermoFisher cat # 90410 or similar
- Timer(s)
- Vortex Mixer
- Benchtop microcentrifuge
- Personal Protective Equipment: gloves, goggles, chemical waste disposal
- Aspirator or Vacuum Pump
- ONi Chip Positioner
- ONi magnets
- Humidity chamber

Components to be provided by the user

1. Sample to be tested
2. PBS without calcium and magnesium for LNP dilutions (as needed)
3. Bead slide

Experimental workflow overview



Advice before you start

- Launch CSA (CODI System App, Version 0.19.51 or higher) and allow the temperature of the microscope to reach 32°C. This will provide ample time for the system to reach optimal temperature.
 - If prompted to install a new version of CSA, proceed with the install according to the CODI prompts. Remember to reopen CSA after the install is complete.
- Up to four chips can be prepared in parallel, a pause point is after the antibody detection mix is removed.
- Chips can be stored for up to 24 hrs at 4°C prior to imaging.
- Once cargo detection has been applied to the Assay Chip, proceed to imaging immediately. Imaging should be completed within 1 hour after the cargo detection incubation is complete. Please consider that imaging using AutoLNP takes 30-50 minutes for each Assay Chip, depending on how many field of views are imaged.

Guidance for using the Assay Chip

- Assay Chips should be brought to room temperature before opening the pouch and must be used upon opening. Use the chip immediately and do not store opened chips for later use.
- ONi recommends using a designated humidity chamber such as Simport Stain Tray M918-2TM. This will provide three advantages: a humid environment to avoid evaporation from the lane inlet and outlet, protection of the sample from light, and avoiding chip movement during pipetting. Humidity chambers can be made with available lab equipment (such as slide storage boxes or dark-colored plastic freezer boxes) provided they meet the advantages listed above.
- We strongly recommend the use of a laboratory aspirator to ensure low variability and background within the assay. If you do not have access to an aspirator, use a laboratory dust-free wipe to remove excess liquid, and expect additional variability and additional background in the cargo detection channel.
 - Recommended purchasing options for laboratory aspirator:
 - Integra Biosciences™ Vacusip Aspiration System
 - Grant Bio FTA-1
 - Aspeed 2

⚠ IMPORTANT: The assay surface is VERY sensitive to bubbles or air being introduced to a lane. In case of transient introduction of bubbles to the lane, you will see increased variability in cluster counts and positivity. If a bubble remains in the lane during an incubation, the region impacted by the bubble will have higher non-specific fluorescent background in all channels, and should not be included in analysis. In order to avoid introducing bubbles, follow these directions:

- Use a p20 pipette for reagent additions, and a p200 pipette for wash steps.
- Prior to adding a reagent or Wash Buffer to a lane, ensure there is no air in the bottom of the pipette tip. If air is present, gently depress the plunger until you can no longer see the air, and a small drop of liquid is present on the bottom of the pipette tip.
- At this point, firmly place the pipette tip into the inlet (marked "IN") of the 4 lane chip. Press down firmly, and ensure the angle between the pipette tip and chip surface is approximately 90 degrees. You may experience a small amount of flex in the pipette tip as you press down, this indicates a seal has been created between the pipette tip and the chip inlet.
- If liquid spills out of the inlet, press the pipette tip down harder to ensure a full seal. Remove any spilled droplets with a lint-free lab wipe.
- Apply new reagents and Wash Buffer at a constant rate, depressing the plunger constantly until you reach the first stop.
- Do not pipette past the first stop.
- As you pipette into the chip, watch carefully for bubbles in the pipette tip, and stop pipetting immediately if a bubble is about to be introduced to the lanes.
- If bubbles are introduced, wash the lane with 100 μ L of Wash Buffer to dislodge bubbles and then reintroduce the intended reagent. If the bubble would not be washed, mark the area to avoid its imaging.
- As you pipette liquid into the inlet hole, the liquid will flow through the lane and exit through the outlet marked "OUT".
- During wash steps only, remove excess liquid as it flows out of the outlet using a vacuum aspirator, by placing it at the bottom of the outlet reservoir, and aspirating in real time ("active aspiration") as you wash out lanes. Ensure the outlet is empty before reagent addition steps
- Do not use the aspirator during the reagent additions and leave the outlet reservoir full during incubation steps. There is sufficient volume in the outlet reservoir that it won't overflow.
- Use an active aspirator during all wash steps. To perform active aspiration, once you start pipetting in the wash buffer, place the tip of the aspirator pipette into the "corner" between the outlet reservoir wall and outlet reservoir lip (See diagram on the right). Continue aspirating as you add the Wash Buffer, and cease aspirating once you have added the full 100 μ L of wash buffer.
- If a bubble is visible in the inlet after an incubation, CAREFULLY place the aspirator tip on the edge of the inlet to remove this bubble. Do not let the aspirator tip into the inlet, as this will empty the lane.
- At the end of a wash step, the outlet reservoir should appear empty and liquid should remain in the opening below the reservoir.
- If bubbles are introduced during the assay workflow and remain in the lane during a reagent incubation, mark the bubble with a sharpie or take a photo, so that the area can be avoided while imaging.
- If a lane is completely dried out (i.e., air is introduced at the end of a pipetting step, and there is no liquid in the lane), do not image this lane, as the air will negatively impact the assay, primarily impacting non-specific background. Do not include these lanes in analysis.

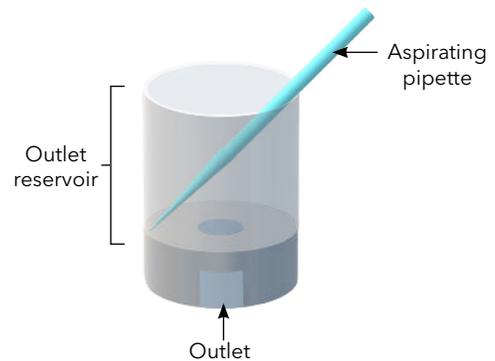


Diagram of active aspiration

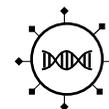
Stepwise protocol

Notes

- Remove the Assay Chip from the pouch and place it on a clean, dark surface. This will make it easier to see if any bubbles are introduced.
- At the beginning of the assay, all assay components except Cargo Detection can be removed from the fridge and freezer and held at room temperature.
- Allow all reagents to reach room temperature before using. Do not use a reagent if there is still ice in the tube.
- Reagents are meant to be used at room temperature and should not be stored on ice.
- All steps are performed at room temperature.
- All volumes provided are per lane.
- Vortex each reagent for approximately 5 seconds once thawed. Spin down in a microcentrifuge if needed.
- Use a new pipette tip for each lane when adding reagents or washing.
- Outlet reservoir should be full during reagent incubations. However, ensure that the outlet reservoir is completely empty at the end of each wash step.
- Always pipette the required solution directly into the inlet hole, marked by "In".
- Do not tilt the chip, as this can cause flow within the lane.
- Incubate the Assay Chip in a designated humidity chamber or in an appropriate alternative, as suggested in "Guidance for using the Assay Chip".

Surface preparation - total time: 40 min

1. Add 10 μ L Surface Block.
[15 min incubation in room temperature, preferably in a humidity chamber]
2. Wash with 100 μ L of Wash Buffer, using active aspiration.
3. Add 10 μ L Surface Reagent.
[10 min incubation in room temperature, preferably in a humidity chamber]
4. Wash with 100 μ L of Wash Buffer, using active aspiration.
5. Add 10 μ L Anti-PEG Capture.
[10 min incubation in room temperature, preferably in a humidity chamber]
6. Wash with 100 μ L of Wash Buffer, using active aspiration.



LNP capture - total time: 50 min

7. Dilute LNPs in Wash Buffer or 1X PBS. The exact dilution depends on the sample concentration. Final RNA concentration of dilution should be between 40 ng/mL and 100 ng/mL, assuming an encapsulation efficiency of over 50%.

We recommend performing a dilution titration across the RNA concentration range above using a full Assay Chip (four lanes), run a full LNP Profiler Assay, and select the concentration that gives around 1000-2000 LNP counts per single Field of View.

IMPORTANT NOTES:

- a. Do not vortex, flick, or spin down the LNPs. This can result in a fragmented appearance when imaging.
 - b. Remove LNPs from storage after anti-PEG capture has been washed out. Dilute LNPs immediately, and use diluted LNPs within 5 minutes of diluting.
 - c. When diluting LNPs, we recommend performing two dilution steps if diluting over 1:2000.
 - d. When mixing LNP dilutions, we recommend mixing with 50% of the total solution volume. For large dilution (i.e., 1:500 or higher), mix slowly 10 times by pipetting up and down. For small dilutions, mix slowly at least 3 times by pipetting up and down.
8. Add 10 μ L of the diluted LNPs to a single lane in the chip. Repeat for each LNP sample.
- NOTE: Use a p20 pipette with 10 μ L volume. Pipette up and down three times (down, up, down, up, down, up) prior to adding to each lane. Change the pipette tip and proceed to the next diluted LNP sample.*
- [45 min incubation in room temperature, preferably in a humidity chamber]
9. If not done at the start of the assay, remove PanLNP Detection and Ligand Detection reagents from the freezer during this incubation, and allow them to reach room temperature.
 10. Wash with 100 μ L of Wash Buffer, using active aspiration.

EXAMPLE: If diluting 1:14,000, perform the following:

- i. Prepare a single 2 mL tube with 999 μ L of Wash Buffer or PBS without Mg and Ca (tube 1)
- ii. Prepare another tube with 130 μ L of Wash Buffer or PBS without Mg and Ca (tube 2)
- iii. Once LNPs are thawed, add 1 μ L of the LNP stock to tube 1. When pipetting the 1 μ L from the stock, make sure to pipette to the second stop to ensure that the full 1 μ L is ejected, and slowly pipette up and down once to ensure LNPs are mixed.
- iv. Once LNPs have been added to tube 1, use a p1000 pipette with a 500 μ L volume, and slowly pipette up and down 10 times to mix the LNPs into solution.
- v. With a p20 or p10 pipette, take a 10 μ L volume from tube 1, pipetting up and down 2 times before pulling out the 10 μ L volume. Add this 10 μ L volume to tube 2.
- vi. Mix tube 2 with a p200 at 100 μ L volume by pipetting up and down slowly two times.
- vii. Tube 2 is your final 1:14,000 LNP dilution. Use within 5 minutes of mixing.

Staining - total time: 65 min

11. Prepare Antibody Detection Staining mix.

IMPORTANT NOTES:

- a. Ensure detection reagents are completely thawed prior to diluting.
- b. Vortex briefly (1-2 seconds) prior to use, and spin down in a microcentrifuge if needed.
- c. Add the Wash Buffer to the dilution tube first. When adding detection reagents, pipette to the second stop to ensure that reagent is completely ejected from the tip.
- d. Vortex detection reagent mix for 5-10 seconds after adding all components. Spin down in a microcentrifuge is necessary. Tapping the tube on the bench, or quickly flicking is also acceptable if liquid is sitting on the sides of the tube.

- 13. Apply 10 μ L of Antibody Detection Staining mix to each lane in your chips. [45 min incubation in room temperature, protected from light in a humidity chamber]
- 14. During this incubation, allow the Cargo Detection reagent to reach room temperature for at least 10 minutes before use. Vortex thoroughly, and spin down in a microcentrifuge after thawing.
- 15. Wash the Antibody Detection Staining mix with 100 μ L of Wash Buffer, using active aspiration.

⚠ This is a pause point. Chips can be stored at room temperature for up to four hours or in 4°C for up to 24 hours before proceeding to the next step.

12. Choose option a or b according to your experiment.

- a. PanLNP, prepare if you are only interested in LNP size and morphology.

Number of chips	Wash buffer	PanLNP detection
1	48.75 μ L	1.25 μ L
2	97.5 μ L	2.5 μ L
3	146.25 μ L	3.75 μ L
4	195 μ L	5 μ L

- b. PanLNP + Ligand, prepare if you are interested in LNP size, morphology, and the presence of a ligand on your LNPs.

Number of chips	Wash buffer	Ligand detection	PanLNP detection
1	46.75 μ L	2 μ L	1.25 μ L
2	93.5 μ L	4 μ L	2.5 μ L
3	140.25 μ L	6 μ L	3.75 μ L
4	187 μ L	8 μ L	5 μ L

16. Prepare Cargo Staining mix

- a. Prepare the Cargo Staining mix directly before you plan to image the chip. Prepare 1 chip at a time. Keep the other chips with Wash Buffer in the humidity chamber.
- b. Cargo Detection reagent can be held at room temperature for up to 4 hours, but the Cargo Staining mix has to be prepared fresh for each Assay Chip.

IMPORTANT NOTES:

- i. Always add Wash Buffer to the dilution tube first, and then add the Cargo Detection reagent.
- ii. Pipette to the second stop to ensure that reagent is completely ejected from the tip.
- iii. Cargo Detection should change the solution color to a very pale yellow. Thoroughly vortex the Cargo Staining mix until it is a single, uniform color and no Schlieren lines are visible.
- iv. Use Cargo Staining mix within 5 minutes of preparation.

Number of chips (4 lanes)	Wash buffer	Cargo detection
1	48.5 µL	1.5 µL

17. Apply 10 µL of Cargo Staining mix to each lane. [15 min incubation in room temperature, protected from light in a humidity chamber]

NOTE: During this incubation, complete experiment setup and channel mapping in AutoLNP.

- 18. Wash with 100 µL of Wash Buffer, using active aspiration. Repeat one additional time.
- 19. Add additional Wash Buffer to fill the outlet reservoir.
- 20. Seal the inlet and outlet of the chip with the provided stickers. Image immediately.

Imaging (30-50 minutes/chip)

Once your chip is ready to image, please proceed to the Nanoimager. If channel mapping has not yet been completed, complete channel mapping prior to placing your chip on the stage.

For further guidance on imaging and analysis with AutoLNP, please refer to the AutoLNP user guide.