Protocol: Pan-EV, Tetraspanin Trio, and user-defined protein detection

All steps are performed at room temperature. All volumes provided are per lane. Pan-EV detection can be used for size evaluation with or without permeabilization. All incubation steps should be performed in a humidity chamber. When applying reagents to the inlet use a vacuum aspirator to remove the flow through from the outlet.

Surface preparation – total time: 30 – 40 min

1. Turn on and initiate microscope temperature control to allow the microscope temperature to equilibrate prior to imaging (see image acquisition protocol).
2. Remove all kit components except for dSTORM Imaging Buffer from the fridge and freezer and place at room temperature. Place DMSO in 37°C heat block for a minimum of 10 minutes.
3. Allow unopened Assay Chip to reach room temperature. **[10 min incubation]**
4. Open the chip pouch and place the Assay Chip in a humidity chamber. Set aside inlet/outlet sealing stickers until they are needed for imaging.
   Note: it is essential that chips are placed in a humidity chamber during incubations to prevent evaporation.
5. Apply 10 µL of Surface Reagent. **[15 min incubation, rocking 30-45 RPM parallel to Assay Chip lanes]**
6. Wash with 100 µL Wash Buffer.
7. Apply 10 µL Capture Reagent. **[15 min incubation, rocking 30-45 RPM parallel to Assay Chip lanes]**
8. Wash with 100 µL Wash Buffer.

Capture and fixation – total time: 85 min

9. Apply 10 µL EV solution (Click here for EV preparation)
   [75 min incubation, rocking 30-45 RPM parallel to Assay Chip lanes]
   Note: add PS Capture Supplement (1:10) to EVs if using PS Capture
0. Wash with 100 µL Wash Buffer.
1. Apply 20 µL Fixative.

   [10 min incubation]
2. Wash with 100 µL Wash Buffer.

Staining – total time: 65 min

3. Apply 10 µL Staining Buffer

   [10 min incubation]

4. Prepare Detection Antibody dilution.

    1. Reconstitute lyophilized Pan-EV Detection reagent

        1. Centrifuge Pan-EV Detection

           to ensure the reagent is at the bottom of the tube
        2. Add 48 µL DMSO
        3. Vortex for at least 20 seconds
        4. Centrifuge to bring the reconstituted reagent to the bottom of the tube
        5. Pipet and visually inspect to ensure even distribution and no visible flakes

Note

Reconstituted Pan-EV Detection can be stored at –20°C for 2 months.

For the 4-lane chip prepare 50 µL (10 µL/lane + 25%).

Component	No target or target on surface	Luminal cargo target
Optional User-defined Detection Reagent 1-10ug/ml (647)	X µL	X µL
Permeabilization Buffer	–	30 µL
Staining Buffer	40.5- X µL	10.5 – X µL
Tetraspanin Trio Detection	8 µL	8 µL
Pan-EV Stain**	1.5 µL	1.5 µL
Final volume	50 µL	50 µL

Note: The provided Tetraspanin Trio Detection (561) is only sufficient for 3 unique user-defined Detection Antibody Dilution Mixes (one per chip being processed). If using more than 3 unique Detection Antibody Dilution Mixes,e.g., 6 different User-Defined Proteins, additional Tetraspanin Trio Detection (561) should be purchased at the ONI online store or by a purchase order.

**Add Pan-EV detection last, and do not let it sit in aqueous media for >10 min

5. Apply 10 µL Detection Antibody dilution.
   [50 min incubation, rocking 30 RPM parallel to Assay Chip lanes]
   Note: Protect from light in an opaque or foil-covered humidity chamber.
6. Wash with 100 µL Wash Buffer.
7. Apply 20 µL Fixative. **[5 min incubation]**
8. Wash with 100 µL Wash Buffer. Pause point: Chips can be stored in 4°C for a maximum of 24 hours with lanes sealed with inlet/outlet sealing stickers in a humidity chamber. Data quality will be impacted if imaging is performed after 24 hours.
   Note: If imaging more than one Assay Chip, only add dSTORM Imaging Buffer immediately prior to imaging. Preparing dSTORM Imaging Buffer – total time: 20 min
9. Remove one vial of dSTORM Imaging Buffer Part A from –20°C storage.

10. Allow dSTORM Imaging Buffer Part A to reach room temperature. **[10 min incubation]**
11. Centrifuge dSTORM Imaging Buffer Part A for 10 seconds.
12. Add 99 µL of dSTORM Imaging Buffer Part A to a fresh microtube.
13. Remove dSTORM Imaging Buffer Part B from –20°C and immediately proceed to step 24, returning dSTORM Imaging Buffer Part B to -20°C storage as quickly as possible.
14. Add 25 µL of Part B Resuspension Buffer to the vial, then put it on ice for 5 minutes. Gently mix by pipetting up and down, avoiding bubbles or air. Do not shake or vortex the vial.
15. Add 1 µL of dSTORM Imaging Buffer Part B to the fresh tube of dSTORM Imaging Buffer Part A prepared in step 23. Carefully mix the solution by pipetting, making sure not to introduce bubbles.
    Note: dSTORM Imaging Buffer Part B is viscous so pipette slowly and avoid introducing excess liquid from the outer walls of the pipet tip.
    Note: Never flick, shake, invert, or vortex dSTORM Imaging Buffer. Introducing oxygen will affect its performance.
16. Add 20 µL of prepared dSTORM Imaging Buffer to all Assay Chip lanes and seal lanes with inlet/outlet sealing stickers.
    [10 min incubation]

Note

dSTORM Imaging Buffer requires an incubation to deplete the oxygen content. During this step, allow the oxygen content to be depleted. During this step prepare the microscope, including channel mapping and setting up imaging parameters. Note: After 90 min of imaging, the dSTORM imaging buffer should be replenished.

Note

If the chip will be re-imaged, dSTORM Imaging Buffer should be replaced with 100 µL of Wash Buffer and can be stored for a maximum of 24 hours at 4°C when sealed with inlet/outlet sealing stickers inside a humidity chamber.
