EV Profiler 2

Manual sample preparation protocol

For the characterization of extracellular vesicles (EVs) using the Nanoimager.

TABLE OF CONTENTS

Component list - EV Profiler 2 (900-00079)

Component	Quantity	Volume	Hazard	Storage
Assay Chip	3	-	N/A	❄†
Surface Reagent	3	55 µL	N/A	❄†
Wash Buffer	1	12 mL	N/A	🌡
PS Capture	x	55 µL	N/A	❄†
PS Capture Supplement	1 x	16 µL	N/A	🌡
anti-CD81 Capture	x	55 µL	N/A	🌡†
anti-CD63 Capture	x	55 µL	N/A	🌡†
anti-CD9 Capture	x	55 µL	N/A	🌡†
Tetraspanin Trio Capture	x	55 µL	N/A	🌡†
Fixative	1	1.1 mL	☠ ◆	🌡
Permeabilization Buffer	3	55 µL	N/A	🌡
Staining Buffer	3	400 µL	N/A	🌡
Tetraspanin Trio Detection (561)	1	25 µL	N/A	🌡
Pan-EV Detection (488)	1	0.2 nmol	N/A	🌡
DMSO	1	55 µL	!	🌡
dSTORM Imaging Buffer Part A	3	400 µL	! ◆	❄
dSTORM Imaging Buffer Part B	3	25 µL	N/A	❄
EV Standard Control	x	30µg	N/A	🌡

❄ Store at -20°C

🌡 Store at 4°C

† Single-use

x Dependent on customer-selected configuration

Component list - EV Profiler 2: Tetraspanin Profiling (900-00084)

Component	Quantity	Volume	Hazard	Storage
Assay Chip	3	-	N/A	❄†
Surface Reagent	3	55 µL	N/A	❄†
Wash Buffer	1	12 mL	N/A	🌡
PS Capture	x	55 µL	N/A	❄†
PS Capture Supplement	1 x	16 µL	N/A	🌡
anti-CD81 Capture	x	55 µL	N/A	🌡†
anti-CD63 Capture	x	55 µL	N/A	🌡†
anti-CD9 Capture	x	55 µL	N/A	🌡†
Tetraspanin Trio Capture	x	55 µL	N/A	†
Fixative	1	1.1 mL		🌡
Permeabilization Buffer	3	55 µL	N/A	🌡
Staining Buffer	3	400 µL	N/A	🌡
anti-CD81 Detection (647)	1	15 µL	N/A	🌡
anti-CD63 Detection (561)	1	10 µL	N/A	🌡
anti-CD9 Detection (488)	1	10 µL	N/A	🌡
dSTORM Imaging Buffer Part A	3	400 µL		❄
dSTORM Imaging Buffer Part B	3	25 µL	N/A	❄
EV Standard Control	x	30µg	N/A	🌡

❄ Store at -20°C

🌡 Store at 4°C

† Single-use

x Dependent on customer-selected configuration

PROTOCOL

Equipment needed

- ☐ Pipettes (p2.5, p20, and p200)
- ☐ Pipette tips (10 µL, 20 µL 200 µL)
- ☐ Vials (1.5 mL microtuwbes)
- ☐ Timer(s)
- ☐ Humidity chamber e.g. Staintray™ 10 Slides Staining System (M918-1)
- ☐ Vortex mixer
- ☐ Personal protective equipment: gloves, goggles, biohazard bag
- ☐ MilliQ water
- ☐ Laboratory rocker or rotator
- ☐ Aspirator or vacuum pump (optional)
- ☐ Lens cleaning paper
- ☐ Objective oil

Components to be provided by the user

1. EV sample to be tested
2. User-defined antibody against EV surface or luminal targets of interest conjugated to dSTORM-compatible fluorophores (Alexa Fluor 647® (AF647), or other far red dSTORM compatible fluorophore), in selected configurations.
3. Isotype-control antibodies conjugated to the same fluorophores, in selected configurations.

Overview

The EV Profiler 2 kit provides a fast, user-friendly system for the characterization of EVs. EV Profiler 2 uses a combination of functionalized surface chemistry, biochemical capture and detection molecules, and is compatible with the ONI Nanoimager and ONI's cloud-based analysis platform, Collaborative Discovery software (CODI), to streamline sample preparation, imaging, and analysis.

User-defined protein, Pan-EV, and Tetraspanin Trio Detection (EV Profiler 2 (900-00079))

The protocol for user-defined protein and Pan-EV Detection enables the identification of the EV population, characterization of EV size, combined CD81, CD63, and CD9 presence (1-color), and surface or luminal user-defined protein detection on human derived EVs. This protocol can also be used with either Tetraspanin Capture or phosphatidylserine (PS) Capture. The user shall provide the primary antibody for user-defined protein detection, and a matched isotype control, fluorescently labeled with dSTORM compatible fluorophore, such as AF647 or other far red dSTORM compatible fluorophore.

Tetraspanin detection (EV Profiler 2: Tetraspanin Profiling (900-00084))

The protocol for Tetraspanin Detection (3-color) is designed to characterize CD81, CD63, and CD9 expression on human-derived EVs. It can be used with either tetraspanin-specific EV capture (Tetraspanin Capture) or phosphatidylserine EV capture (PS Capture).

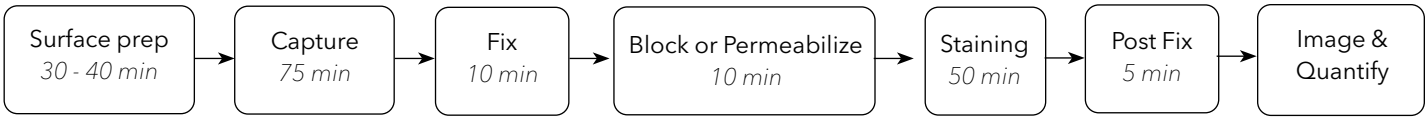
Quality control

Each lot of the EV Profiler 2 components is tested against predetermined specifications to ensure consistent product quality.

Intended use

EV Profiler 2 kits are intended for Research Use Only. The kits are intended for use by professional users with training in basic laboratory techniques. Care should be taken in the handling of products. We recommend that users adhere to the MISEV guidelines provided by the International Society of Extracellular Vesicles.

Experimental workflow overview



Advice before you start

Before you begin, choose your capture and detection methods.

EV Profiler 2 configurations:

Protocol	Capture options	Detection	Jump to
1	anti-CD81 anti-CD63 anti-CD9 Tetraspanin Trio - (anti-CD81+ CD63 + CD9) PS Capture User-defined	Optional User-Defined (647) Tetraspanin Trio (561) Pan-EV (488)	pg. 8
2	anti-CD81 anti-CD63 anti-CD9 Tetraspanin Trio - (anti-CD81 + CD63 + CD9) PS Capture User-defined	anti-CD81 (647) anti-CD63 (561) anti-CD9 (488)	pg. 10

- Considerations for capture selection:
  - Select anti-CD81, anti-CD63 or anti-CD9 Capture if you want to enrich in a specific tetraspanin. If the EV source is known to be enriched in an individual tetraspanin, isolating with that individual tetraspanin for capture can help isolate that EV sub-population. Otherwise, combined tetraspanin capture can be used to isolate a broad range of EVs.
  - PS Capture may be used with EVs enriched in phosphatidylserine (e.g. cancer-derived EVs). PS Capture is useful when you do not want to enrich a protein biomarker.
  - If phosphatidylserine (PS) content is unknown, we suggest comparing PS Capture to anti-CD81, anti-CD63, anti-CD9 (individual or combined) capture.
  - To select a user-defined capture molecule, ensure that the target of interest is membrane-associated and accessible. To check for compatibility with EV Profiler 2, test a lane with the selected staining protocol but without EVs: analyze with the defined analysis parameters, and confirm fewer than 100 clusters per field of view in negative control lanes. For more detailed information regarding capture molecule selection, see page 7.
- Considerations for detection selection:
  - Detecting with the Pan-EV (488) and Tetraspanin Trio allows for the assessment of EV sizing and EV shape, as well as characterization of tetraspanin-negative EVs. Additionally, this configuration allows for detection of protein targets in the 647 channel. Follow the provided protocol to assess surface or luminal protein cargo associated with EVs. For more detailed information regarding capture molecule selection, see page 7.
  - Detecting with anti-CD81 (647), anti-CD63 (561), and anti-CD9 (488) allows for the biotyping of EVs, and can aid in the understanding of EV populations. This can be useful in down-selecting EV capture and detection for future experiments, for characterizing new EV sources, or EV isolation methods.
- To maximize reproducibility and lane coverage, we recommend agitation on a laboratory rocker or oscillatory shaker. When rocking, Assay Chips lanes should be parallel to the direction of agitation.
- Prepared EV samples should be purified prior to deposition onto the Assay Chip. If assistance is needed regarding suggested EV purification for dSTORM imaging, please see

our webinar on EV preparation. We suggest size exclusion chromatography purified EVs or differential centrifugation EVs.

- If using the EV Standard Control, reconstitute the lyophilized EV Standard Control by gently adding 30 µL MilliQ H_2O dropwise on the tube sides, taking care not to introduce bubbles to the solution. Allow to rehydrate for 10 minutes. Do not vortex EVs. Centrifuge to ensure all EVs are in the bottom or the tube. The reconstituted EV Standard Control should be used fresh, however, it can be stored at 4°C for up to 5 days.
- The E1 EV Standard Control (800-00014) may be used as a positive control because they are known to be enriched with PS and are captured efficiently with PS Capture.
- Dilute EVs to 10^8-10^{10} EVs / mL for deposition onto the Assay Chip.
    - Once reconstituted EV Standard Control is a 20X solution. If using EV Standard Control, dilute the reconstituted EVs in Wash Buffer (e.g., 1 µL EV Standard Control in 19 µL Wash Buffer).
- PS Capture Supplement is needed:
    - If using PS Capture and user-supplied EVs
- PS Capture Supplement is not needed:
    - If using antibody based capture (including Tet. Trio Capture).
    - If using PS Capture with EV Standard Control reconstituted in Wash Buffer.
- PS Capture Supplement is a 10x concentrated solution. Dilute 1:10 in your EV sample (e.g. 2.5 uL of supplement per 25 uL EVs).
    - If EVs are diluted at least 1:5 in Wash Buffer (i.e., 10uL of EVs into 40uL of Wash Buffer), there is no need to add additional capture supplement.

Guidance for using the Assay Chip

- Assay Chips should be brought to room temperature before opening and must be used upon opening.
- Do not store opened chips for later use.
- Once the Assay Chip is removed from the packaging, incubate it in a humidity chamber. This reduces the risk of evaporation and bubble introduction during the protocol.
    - ONI recommends using a designated humidity chamber such as Simport Stain Tray M918-2™. 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.
- Liquid should always be added to the inlet hole makered "IN".
- To create a seal and ensure constant liquid delivery, insert the pipet tip at a 90° angle, hold the pipet vertically, and press down firmly before you begin dispensing liquid. If liquid spills out of the inlet, press the pipette tip down harder to ensure a full seal.
- Ensure that liquid is flowing into the chip lane by watching the meniscus during liquid deposition.
- It is important to minimize the introduction of bubbles when pipetting. To reduce bubbles, we suggest using p20 or p200 pipette tips, only pipetting to the first stop, and keeping consistent pressure until the pipette tip has been removed from the inlet (to reduce backflow).
- As you pipet liquid into the inlet hole, the liquid will flow through the lane and exit through the outlet marked "OUT". We suggest removing excess liquid as it flows out of the outlet using a vacuum aspirator, by placing it gently next to the outlet hole. If you do not have access to an aspirator, use a laboratory dust-free wipe to remove excess liquid. Regardless of method, ensure there is minimal spillover from one lane outlet to another.

  Note: Do not insert the aspirator directly into the outlet hole, this will result in liquid being completely removed from the lane.
- Lanes should contain liquid throughout the entire experiment. If there are bubbles within the lane, or liquid has been inadvertently removed, wash the lane with 100 µL of Wash Buffer to dislodge bubbles and reintroduce the intended liquid.

Important reagent handling considerations

- Do not vortex EVs.
- At the beginning of the assay, all assay components (except dSTORM Imaging Buffer) can be removed from the fridge and freezer.
- Prepare detection dilutions immediately prior to use.

Storage of EV Standard Control

- The reconstituted EV Standard Control can be stored for a maximum of 5 days at 4°C with minimal loss of EVs.
- We do not recommend long-term storage.

Storage of Pan-EV Detection

- Once reconstituted Pan-EV Detection should be stored at –20°C for a maximum of two months.
- We do not recommend long-term storage.

Guidance for using alternative capture molecule

The EV Profiler 2 Assay Chips can be used with any biotinylated capture molecule. An alternative biotinylated capture molecule can be selected to isolate EV subpopulations via a capture modality not provided in the kit.

- Optimization of user-defined capture molecules should include the following steps:
  - Assess capture molecule specificity: To determine specificity, stain tissue culture cells that express the target and a negative control (e.g., knockout or peptide blocking) with a fluorescent-labeled version of the capture molecule. Ideally, the negative controls should include less than 10% of the density of the positive control samples; however, this threshold should be predetermined by the user.
  - Assess capture molecule compatibility with EV Profiler 2 assay: When using a new biotinylated capture molecule, ensure that at least one lane with the capture molecule does not contain EVs and that less than 100 clusters are detected in the EV negative lane when stained in the same manner as EVs.
  - Optimize background: If negative control lanes with the new capture molecule result in high background, 0.5%-2.5% BSA can be included in the biotinylated capture molecule dilution.
  - Optimize capture efficiency and reproducibility: For the most efficient capture, start with 70 nM of biotinylated capture molecule and test 20% higher and lower concentrations for ideal capture efficiency. A 75 min incubation time is recommended, but 20% shorter and longer incubation times can also be tested for ideal capture efficiency and reproducibility.

Guidance for using alternative detection molecule

Surface or luminal user-defined molecules can be assessed in the 647 channel with the Pan-EV (488) and Tetraspanin Trio (561).

- Optimization of user-defined detection molecules should include the following steps:
  - Assess detection molecule specificity: To determine specificity, stain tissue culture cells that express the target and a negative control (e.g. knockout or peptide blocking) with a fluorescent-labeled version of the detection molecule. Ideally, the negative controls should include less than 10% of the density of the positive control samples, however, this threshold should be predetermined by the user.
  - Optimize degree of labeling: Molecules (antibodies, proteins etc.) can be purchased fluorescently labeled or can easily be labeled by the user. Use a dSTORM compatible dye that fluoresces in the 647 channel (e.g, AF647). The number of fluorophores per protein can be modulated by increasing the molar ratio of dye to protein during the conjugation. Please reach out to ONI if support is needed for antibody labeling.
  - Optimize concentration: For most efficient detection, start with 5 µg/mL of labeled antibody. The antibody or the detection molecule can be titrated around 1-10 µg/mL. Use negative control lanes (no permeabilization for internal cargo, isotype controls for surface cargo) to guide detection molecule concentration.
  - Optimize laser power: The laser power recommended in this protocol was optimized for AF647. If the user-defined detection molecule is not AF647, titrate the 647 laser power to ensure fluorophore blinking throughout the acquisition. Optimal laser power and number of frames for the user-defined molecule can be determined using the CODI analysis software. The optimal laser power will minimize the localization precision (increased precision leading to a decreased numerical value) and maximize the photon count while still capturing several localizations per frame over the entirety of the imaging period. This can be determined by observing the number of localizations per frame, which should have a steep decline in the first frames leading to a blinking steady state for the remainder of the imaging time.

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 (see page 6).

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.
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 (step 27).

   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 (see page 5-6 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
10. Wash with 100 µL Wash Buffer.
11. Apply 20 µL Fixative

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

Staining - total time: 65 min

13. If detecting a luminal target, apply 10 µL Permeabilization Buffer Otherwise, apply 10 µL Staining Buffer

    [10 min incubation]
14. Prepare Detection Antibody dilution.
    a. Reconstitute lyophilized Pan-EV Detection regent
       i. Centrifuge Pan-EV Detection to ensure the reagent is at the bottom of the tube
       ii. Add 40 µL DMSO
       iii. Vortex for at least 20 seconds
       iv. Centrifuge to bring the reconstituted reagent to the bottom of the tube

    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	Volume
Staining Buffer	39.5 - X µL
User-defined Detection Reagent 1-10 µg/mL (647)	X µL
Tetraspanin Trio Detection (561)	8 µL
Pan-EV Detection** (488)	2.5 µL

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

15. 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.

16. Wash with 100 µL Wash Buffer.

17. Apply 20 µL Fixative.

    [5 min incubation]

18. 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

19. Remove one vial of dSTORM Imaging Buffer Part A from –20°C storage.

20. Allow dSTORM Imaging Buffer Part A to reach room temperature.

    [10 min incubation]

21. Centrifuge dSTORM Imaging Buffer Part A for 10 seconds.

22. Add 99 µL of dSTORM Imaging Buffer Part A to a fresh microtube.

23. 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.

24. Add 1 µL of dSTORM Imaging Buffer Part B to the fresh tube of dSTORM Imaging Buffer Part A prepared in step 23.

    Note: dSTORM Imaging Buffer Part B is viscous so pipette slowly and avoid introducing excess liquid from the outer walls of the pipet tip.

25. Carefully mix the solution by pipetting, making sure not to introduce bubbles.

    Note: Never flick, shake, invert, or vortex dSTORM Imaging Buffer. Introducing oxygen will affect its performance.

26. 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.

Protocol: Tetraspanin Detection

All steps are performed at room temperature. All volumes provided are per lane. 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 (see detailed instructions on page 6).

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.
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 (step 27).

   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 (see page 5-6 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 (see page 7-8).
10. Wash with 100 µL Wash Buffer.
11. Apply 20 µL Fixative

    [10 min incubation]
12. Wash with 100 µL Wash Buffer.
13. Apply 10 µL Staining Buffer.

    [10 min incubation]
14. Prepare Detection Antibody dilution:

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

Component	Volume
Staining Buffer	42 µL
anti-CD81 Detection (647	4 µL
anti-CD63 Detection (561)	2 µL
anti-CD9 Detection (488)	2 µL

15. 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.

16. Wash with 100 µL Wash Buffer.

17. Apply 20 µL Fixative.

    [5 min incubation]

18. Wash with 100 µL Wash Buffer.

    Pause point: Assay can be paused here. 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

19. Remove one vial of dSTORM Imaging Buffer Part A from –20°C storage.

20. Allow dSTORM Imaging Buffer Part A to reach room temperature.

    [10 min incubation]

21. Centrifuge dSTORM Imaging Buffer Part A for 10 seconds.

22. Carefully mix the solution by pipetting, making sure not to introduce bubbles.

    Note: Never flick, shake, invert, or vortex dSTORM Imaging Buffer. Introducing oxygen will affect its performance.

23. Add 99 µL of dSTORM Imaging Buffer Part A to a fresh microtube.

24. Remove dSTORM Imaging Buffer Part B from –20°C and immediately proceed to step 25, returning dSTORM Imaging Buffer Part B to –20°C storage as quickly as possible.

25. Add 1 µL of dSTORM Imaging Buffer Part B to the fresh tube of dSTORM Imaging Buffer Part A prepared in step 23.

    Note: dSTORM Imaging Buffer Part B is viscous so pipette slowly and avoid introducing excess liquid from the outer walls of pipet tip.

26. Mix gently using a pipette, making sure not to introduce bubbles.

27. 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, 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.