Negative Staining Standard Operating Procedure

version 1.0

Authors: Protocols from diverse groups assembled and combined by Christina Zimanyi

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1. Purpose

1.1. Prepare a negative stained grid of a sample of interest on a continuous carbon grid.

2. Supplies & Equipment

- ❑ PPE (BSL-1)
  - ❑ Laboratory Coat
  - ❑ Nitrile Gloves
  - ❑ Goggles / Safety Glasses
- ❑ Chemicals/Reagents
  - ❑ Ethanol (for cleaning tweezers)
  - ❑ Heavy metal stain
  - ❑ Distilled or MilliQ (UltraPure) Water
  - ❑ Sample buffer (Avoid phosphate buffers if possible. They can cause crystallization and precipitation of heavy metal stains. If you can't avoid phosphate buffer, add extra water wash steps)
  - ❑ Sample
- ❑ Filter Paper (Whatman #1 or #4)
- ❑ Pipette and Tips
- ❑ Parafilm
- ❑ Self-closing tweezers: negative action, or positive action with clamps, at least 1 pair
- ❑ Tissues or Kimwipes
- ❑ Plasma cleaner or glow discharger
- ❑ Metal grid pedestal or glass slide for plasma cleaner/glow discharger
- ❑ Continuous carbon (e.g. 20 nm) EM Grids
- ❑ Grid Storage Boxes
- ❑ Timer

3. Procedure

3.1. Prepare negative stain solution

3.1.1. Prepare your stain ahead of time, using either a commercially available solution or making ready to use aliquots for your staining session **(see site specific instructions)**. Protocols for preparing stains are not part of this SOP.

Note: Multiple stains can be used for this SOP. The most popular, particularly for single particle work, are uranyl stains: 2% uranyl acetate (UA) or 2 % uranyl formate (UF). Commonly used non-uranyl stains include 2% phospotungstic acid (PTA), 2% ammonium molybdate, and commercial products such as "NanoW", and "UranyLess EM Stain". Choose a single stain to start and only consider switching if necessary for troubleshooting.

Safety note – Uranyl salts are radioactive and must be handled accordingly. Other heavy metal stains such as tungsten or vanadium are not radioactive but must still be handled as hazardous. Be familiar with the SDS and proper handling protocols of the stains you are working with.

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3.2. Prepare your sample

3.2.1. You will need ~3 µL of sample per grid. Dilute your sample in a suitable buffer to an appropriate concentration. The ideal concentration for negative staining is empirical. Many purified samples look good near a concentration of 20 ng/µL (0.02 mg/mL). If imaging a new sample, it is recommended to make serial dilutions (for example: 0.2 mg/mL, 0.02 mg/mL and 0.002 mg/mL) and make one grid for each concentration. Once you see which concentration looks best, you can fine tune to a more optimal concentration if needed.

3.3. Plasma clean grids

3.3.1. Place as many grids as you need, **carbon foil side up**, on a grid holding pedestal or glass slide. The carbon side will be matte or grey. The opposite side, or mesh side, will be shiny and metallic in color (Figure 1).

Figure 1. A continuous carbon grid will look matte and grey on the foil side and shiny and metallic on the mesh (grid bar) side. Also note the placement of the tweezer, touching only the very outer edge of the grid.

3.3.2. Glow discharge the grids using the recommended recipe for continuous carbon on the instrument you are using **(see site specific instructions)**. Use the grids within ~30 minutes of glow discharge.

3.4. Prepare negatively stained grids

3.4.1. Pipette 2 drops of distilled water and 3 drops of stain on a strip of parafilm. Drops of ~5-20 µL are recommended (Figure 2). Buffer can be used in place of water for sensitive samples, but water is recommended and generally leads to better stain quality.

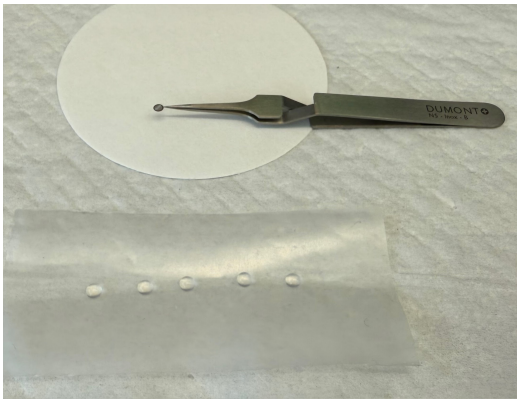

Figure 2. Five 20 µL drops are lined up on a strip of parafilm for blotting. A grid, held by self-closing tweezers, and a clean filter paper are ready.

3.4.2. Pick up a grid with self-closing tweezers, carbon side facing up (Figure 2).

3.4.3. Apply 3 µL sample onto the carbon side of the grid and incubate for ~30 seconds.

3.4.4. Touch the edge of the grid briefly on filter paper to blot away liquid from the side, then immediately touch the carbon side of the grid to the first drop of water. Don't let the grid dry between steps.

3.4.4.1. Make sure the carbon side is facing down when touching the grid to the droplets.

3.4.5. Repeat the blotting and touching of subsequent drops without any incubation time between each step, until the final drop of stain. Hold the grid in the final stain drop for ~30 seconds.

3.4.6. Blot away the final stain drop from the grid and allow the grid to dry on the tweezers before transferring to a grid box for storage.

3.4.7. Repeat for each grid. Between grids, wipe the tweezer clean with distilled water and then ethanol and wipe dry with a Kimwipe.

3.4.8. Allow grids to dry for ~30 min before loading them onto a microscope. Optionally, store the grid box in a vacuum desiccator to dry further if not imaging right away.

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3.5. Clean up

3.5.1. Use a Kimwipe to absorb any left-over water, buffer, or stain. Dispose of the Kimwipe and parafilm in a hazardous waste container **(see site specific instructions).**

3.5.2. Clean tweezers with water and ethanol and return to their storage space.

3.5.3. Dispose of blot paper **(see site specific instructions)**.

3.5.4. Take any leftover sample or buffer tubes with you and leave the bench as you found it

4. Waste Disposal

4.1. Follow facility procedure for proper disposal of biohazardous and chemical waste **(see site specific instructions).**

4.2. Biohazardous waste will be collected in designated bins lined with red biohazard bags.

4.3. Chemical hazardous waste will be segregated by hazard class (e.g. flammable, corrosive) and state (e.g. solid, liquid), appropriately labelled, and placed in the laboratory's hazardous waste cabinet.

5. Appendix 1: Adaptations to the protocol

5.1. The general protocol is forgiving to modification and many variations can produce grids with good stain. You can be creative, but all follow the general workflow:

1) Adsorb particles to the carbon **2)** Wash **3)** Apply stain **4)** Dry

Some examples of common adjustments include:

5.1.1. Leave the grid stationary on the tweezer, and instead of touching the grid to a drop of water or stain, pipette 3-4 µL onto the grid and blot from the side with a filter paper (i.e. move water, stain, and blot paper to the grid instead of the grid to the water, stain, and blot paper).

5.1.2. Use larger (20-50 µL) drops of water and stain on a small multi-well plate and transfer the grid from drop to drop without blotting dry between each step.

5.1.3. Instead of touching the grid to filter paper to blot away drops of liquid, use your wrist to "flick" the drops onto the filter paper.

5.1.4. The volumes used, number of steps of washing and staining, and incubation times with sample and the final stain drop can all be modified as needed if staining does not look good with the initial protocol.

5.2. Suggested starting protocols for staining bacteria

5.2.1. A general protocol for bacteria, adapted from J. Sanchez, UW-Madison Cryo-Electron Microscopy Research Center:

5.2.1.1. Apply 10 µL of a fresh bacterial culture (OD_{600} 0.6 – 0.8) onto glow a discharged grid and incubate for 1 minute.

5.2.1.2. Gently blot the sample from the side with Whatman #1 filter paper.

5.2.1.3. Repeat the entire process 2 times.

5.2.1.4. Apply 4 µL of distilled water (or other buffer solution) and blot with filter paper to remove the excess.

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5.2.1.5. Repeat 2 times.
5.2.1.6. Apply 4 µL of 2% uranyl acetate stain and incubate for 45 seconds.
5.2.1.7. Blot with filter paper to remove the excess stain.
5.2.1.8. Apply 4 µL of distilled water and blot with filter paper to remove the excess water.
5.2.1.9. Let the grid(s) air dry for 5 minutes.

5.2.2. A protocol for looking at bacteria with intact flagella, adapted from J. Wang, New York Structural Biology Center
5.2.2.1. Grow bacterial culture overnight at 30°C with gentle shaking.
5.2.2.2. Dilute overnight cultures 1:100 in LB +antibiotic and grow at 30°C for 4-6 hours (the timing of culture growth affected the observation of intact flagella, and may be different with different bacteria. Start by checking at an OD_{600} around 0.6-0.8).
5.2.2.3. Gently centrifuge (4000 xg) the culture and resuspend in an equal volume of buffer or milliQ water.
5.2.2.4. Apply 3 µL of the resuspended cell culture onto a glow discharged grid and incubate for 1 minute.
5.2.2.5. Gently blot the excess solution using filter paper.
5.2.2.6. Apply 3 µL of 1% uranyl acetate and incubate for 1 minute.
5.2.2.7. Gently blot away the excess stain using filter paper.
5.2.2.8. Let the grid(s) dry for ~5 min.

6. Appendix 2: Troubleshooting

6.1. If you **can't distinguish particles** on the grid at any concentration attempted, your concentrations are too high (there is so much material on the carbon there is no room for stain) or too low (there is stain but no particles). Confirm your sample concentration by UV-VIS spectrophotometry or another assay that works for your sample. You can try to further lower the concentration by dilution. If you cannot practically increase the sample concentration, you can use a longer incubation time in step 3.4.3 to get more adsorption to the grid. If you need to increase the time to more than a couple of minutes, keep the grid in a high humidity environment to avoid evaporation and drying of the full drop on the grid.

6.2. **Poor stain quality/visible stain crystallization:** At times, stored stains may have a buildup of crystals and/or other debris that will interfere with negative stain grid preparation. These steps are used to reduce the likelihood of stain crystal artifacts:

6.2.1. Store uranyl acetate stain in a black tube to reduce light exposure when possible

6.2.2. Store uranyl formate stain at -80°C and use aliquots only one time.

6.2.3. Filter the stain and the water or buffer used for washing with 0.1 µm or 0.2 µm syringe filters.

6.2.4. Centrifuge the stain aliquot and buffer in a mini-centrifuge for 15 min, 15,000 rpm, at room temperature (RT). Note: for the transfer, remove only the upper portion of the centrifuged solution to minimize transfer of any stain or buffer crystals.

6.2.5. If you suspect your buffer components are interfering with the stain, try diluting the sample the into water just before applying onto the grid.

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