National Center for CryoEM Access and Training

NYSBC NEW YORK STRUCTURAL BIOLOGY CENTER

Negative Staining Protocol version 1.0

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1. Purpose

1.1. Prepare a negative stained grid of a sample of interest on a continuous carbon grid. This protocol works well for samples like ribosomes and apoferritin.

2. Supplies & Equipment

- ❑ PPE (BSL-1)
  - ❑ Laboratory Coat
  - ❑ Nitrile Gloves
  - ❑ Goggles / Safety Glasses
- ❑ Chemicals/Reagents
  - ❑ Ethanol (for cleaning tweezers)
  - ❑ Heavy metal stain (i.e. NanoW, 2% uranyl acetate, 2% uranyl formate, etc.)
  - ❑ Distilled Water
  - ❑ Sample buffer (Avoid phosphate buffers if possible. They can cause crystallization and precipitation of the heavy metal stains. If you can't avoid phosphate buffer, add extra water wash steps)
  - ❑ Sample (3 µL per grid)
- ❑ Whatman Filter Paper (#1 or #4)
- ❑ Pipettemen and Tips
- ❑ Parafilm
- ❑ Negative action tweezers, or positive action tweezers with clamps, at least 1 pair
- ❑ Plasma cleaner or glow discharger
- ❑ Continuous carbon (e.g. 20 nm) EM Grids (for example: https://www.emsdiasum.com/microscopy/products/grids/carbon.aspx - Carbon Film on Square Mesh Grids)
- ❑ Grid Storage Boxes
- ❑ Timer

3. Procedure:

3.1. Prepare your sample

- The ideal concentration for negative staining is empirical. Many samples look good near a concentration of 20 ng/µL (0.02 mg/mL).

3.1.1. Dilute your sample in a suitable buffer to an appropriate concentration. If looking at a new sample, try making three dilutions: 0.2 mg/mL, 0.02 mg/mL and 0.002 mg/mL and make one grid of each. Once you see which concentration looks best, you can fine tune to a more optimal concentration if needed.

3.2. Plasma clean grids

- This step makes the carbon surface hydrophobic so that liquid droplets spread evenly over the grid

3.2.1. Place as many grids as you need, **carbon side up**, on a grid holding pedestal or glass slide

- The carbon side will be matte or grey. The opposite side, or grid bar side, will be shiny and metallic in color)

3.2.2. Glow discharge the grids using the recommended recipe for continuous carbon on the instrument you are using. Use the grids within ~30 minutes of glow discharge.

3.3. Prepare grids

Safety note – Heavy metal stains such as uranyl acetate and uranyl formate 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.

3.3.1. Cut a strip of parafilm and place it down on the bench (a light crease down the center of the long dimension of the strip can help keep the drops centered).

3.3.2. Pipette 3x 20 µL drops of distilled water and by 2x 20 µL drops of stain onto the piece of parafilm.
- Buffer can be used in place of water for sensitive samples, but water is recommended and generally leads to better stain quality.

3.3.3. Pick up a grid with the tweezers, carbon side facing up.

3.3.4. Apply 3 µL sample onto carbon side of grid and incubate for 30 seconds.

3.3.5. Touch the edge of the grid briefly on Whatman paper to blot away liquid, then immediately touch the carbon side of the grid to the first drop of water

3.3.6. Repeat blotting and picking up of subsequent drops without any incubation time, until the final drop of stain. Hold the grid in the final stain drop for 30 sec.
3.3.6.1. Make sure carbon side is facing down when touching the grid to the droplets.
3.3.6.2. You might or might not pick up the full 20 µL droplet due to water tension – either is fine.

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

3.3.8. Allow grids to dry for ~30 min before loading them onto a microscope.

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

3.4. Clean up

3.4.1. Use a kimwipe to absorb any left-over water, buffer, or stain. Dispose of the kimwipe and parafilm in a hazardous waste container.
3.4.2. Clean tweezers with water and ethanol and return to their storage space
3.4.3. Dispose of blot paper
3.4.4. 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.
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. Additional Resources

- This the "side blot method" which is shown in the video in the paper attached at minute 1:08: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5912373/
- This is another good reference for negative stain protocols: https://pmc.ncbi.nlm.nih.gov/articles/PMC389902/