Negative Stain Grid Preparation

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Generic protocol for negative stain grid preparation

Think of this as a “try this first” pipeline for new samples. Sample concentration and type of stain tend to be the most important variables, then additives like detergents or crosslinking should be tried next.

Overview at a glance:

Step 1:
Prepare serial four-fold dilutions of your sample, covering at least two order of magnitude in sample concentration. Typical ceiling for sample concentration is ~ 1 mg/mL.

Step 2:
Prepare negative stain solution. Uranyl stains are most popular as a first pass, especially for single particle work. Consider filtering (0.1 μm or 0.2 μm syringe filters) and centrifuging (5-15 min, 15,000 rpm, and at room temperature (RT)) your stain to reduce crystals and debris.

Step 3:
Glow discharge grids. Standard thickness carbon on a 200 copper mesh is a popular support.

Step 4:
Place grid in self closing tweezers. Use a microscope or magnifying lens to help you touch only the edge of the grid with the tweezers.

Step 5:
Set up 2x 20 μL drops of buffer and 3x 20 μL drops of stain on a strip of parafilm. Apply 3 μL of sample to carbon side of grid and incubate for 1 minute.

Step 6:
Side blot to remove sample but do not let grid dry out. Touch carbon face to a 20 μL drop of buffer and immediately side blot again without drying. Repeat one more time without pause with a fresh drop of buffer.

Step 7:
Side blot to remove buffer and then touch carbon face of grid to a 20 μL drop of stain. Repeat one more time without pause with a fresh drop of stain.

Step 8:
Finally, release grid so it floats on top of a final drop of stain. Ideally only the carbon side of the grid is touching the stain solution. Incubate for 1 minute.

Step 9:
Side blot to remove stain. This is the only step where you will be blotting to complete dryness.

Step 10:
Transfer to a grid box. Store grid box in a vacuum desiccator.

Step 11:
Clean up after yourself. Strive to leave the workspace better than you found it please.

Step 12:
Store grids in vacuum desiccator for at least 30 minutes before loading into microscope.

Generic protocol for negative stain grid preparation

Detailed example protocol:

Step 1:
Prepare serial four-fold dilutions of your sample, covering at least two orders of magnitude in sample concentration. The typical ceiling for sample concentration is ~ 1 mg/mL, and it may be necessary to go down to 0.001 mg/mL. Use a buffer that is compatible with the stain that you will be using. No phosphate for uranyl stains. This protocol will use uranyl acetate, since a decent version of that stain is commercially available as a pre-mixed solution and it appears to be reasonably stable.

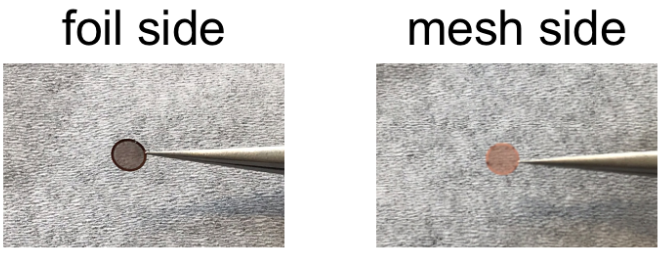
If you are a good experimentalist and understand the importance of a positive control, you can use beta-galactosidase (Sigma # G5635-1KU) with the following buffer: 50 mM NaCl, 10 mM HEPES acid, 10 mM sodium HEPES base, 2 mM $MgCl_2$, 1 mM TCEP, final pH ~ 7.0.

- Prepare 20 μL of sample at 0.5 mg/mL
- Place 15 μL of buffer in three other microfuge tubes.
- Withdraw 5 μL of sample from 0.5 mg/mL stock and add to the first 15 μL buffer tube
- Mix with pipette, then withdraw 5 uL and repeat dilution series to last tube.
- Do not exchange pipette tip in between dilutions
- This gives a concentration series of 0.5, 0.125, 0.03, 0.008 mg/mL

Step 2:
Prepare negative stain solution. Uranyl stains are most popular as a first pass, especially for single particle work. Here we will just use the commercial 2 % uranyl acetate stain from EMS. At times, stored UA stain (or other 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 artifact.

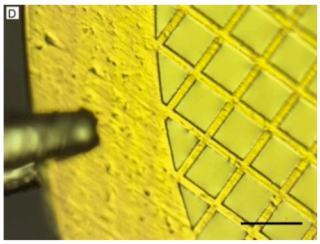
1. Remove an aliquot of stain from the pre-made UA stain reagent bottle. Use 0.1 μm or 0.2 μm syringe filters and filter the stain and the planned wash buffer.
2. Use the mini-centrifuge and centrifuge the stain aliquot and buffer for 15 min, 15,000 rpm, and at room temperature (RT).
3. Transfer the stain to a black tube to reduce light exposure when possible. Note: for the transfer, remove only the upper portion of the centrifuged solution to minimize transfer of any stain or buffer crystals.

Step 3:
Glow discharge grids. We will use a standard thickness carbon film with a 200 mesh copper support (EMS # CF200-Cu). Refer to the glow discharge protocol for the instrument that you plan to use. The iridescent grey side is the carbon foil onto which you will apply your sample. The shiny metal side is the copper support mesh and you want to avoid applying sample, buffer or stain to that side.

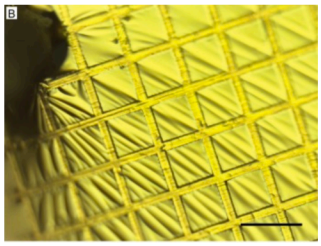


Step 4:
Place grid in self closing tweezers. Use a microscope or magnifying lens to help you touch only the edge of the grid with the tweezers. Below is a good example from Passmore and Russo, 2016 (but that's a "gold on gold" foil, so the colors don't match your carbon/copper grids here).

good, tip of tweezers only touch rim of grid



bad, tweezers touch and damage foil



Step 5:
Set up 2x 20 μL drops of buffer and 3x 20 μL drops of stain on a strip of parafilm. Apply 3 μL of sample to carbon side of grid and incubate for 1 minute. Here is a short overview video of the approach: https://youtu.be/wffPeZMejm0

Step 6:
Side blot to remove sample but do not let grid dry out. Touch carbon face to a 20 μL drop of buffer and immediately side blot again without drying. Repeat one more time without pause with a fresh drop of buffer.

Step 7:
Side blot to remove buffer and then touch carbon face of grid to a 20 μL drop of stain. Repeat one more time without pause with a fresh drop of stain.

Step 8:
Finally, release grid so it floats on top of a final drop of stain. Incubate for 1 minute.

Step 9:
Side blot to remove stain. This is the only step where you will be blotting to dryness. Anything that has touched stain must go in a dedicated stain waste container.

Step 10:
Transfer to a gridbox. Store gridbox in a vacuum desiccator.

Step 11:
Clean up after yourself. It is absolutely essential that any stain solutions and items that have touched stain are disposed of in a dedicated stain waste container.

Step 12:
Store grids in vacuum desiccator for at least 30 minutes before loading into microscope.

Negative Stain Protocol for Bacteria

This is a basic protocol that has been used for a number of bacterial species, in particular, excellent results have been achieved for *Caulobacter crescentus*. Protocol prepared by J. Sanchez, 12/6/2020

Reagents and materials:
Bacterial organism
Wash buffer (water or buffer solution)
Stain (2% UA). Other stains may be used, such as 1% Phosphotungstic Acid (PTA), and should be tested during initial negative stain experiments to determine best conditions for the organism being studied.
Tweezers
Grids (300 mesh carbon copper only grids)
Grid storage box
Centrifuge and storage tubes
Syringe and either 0.1 μm or 0.2 μm syringe filters
Parafilm
Whatman #1 filter paper

Stain and Wash Buffer Preparation: At times, stored UA stain (or other stains) and buffer solutions 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 and wash buffer crystal artifact.

4. Remove an aliquot of stain from the pre-made UA stain reagent bottle. Use 0.1 μm or 0.2 μm syringe filters and filter the stain and the planned wash buffer.
5. Use the mini-centrifuge and centrifuge the stain aliquot and buffer for 15 min, 15,000 rpm, and at room temperature (RT).
6. Transfer the stain to a black tube to reduce light exposure when possible. Note: for the transfer, remove only the upper portion of the centrifuged solution to minimize transfer of any stain or buffer crystals.

Grid Glow Discharging: Refer to the glow discharge protocol for the instrument that you plan to use.

1. Use either a static dissipation gun or a U-electrode antistatic system to remove charge effects from tools and grids.
2. Place grids (we used coper/carbon 300 mesh) in the instrument you plan to use. Follow the protocol for its use.
3. Turn off system and slowly release vacuum to minimize disruption of the grids.

Grid Preparation with Sample and Stain:

1. Apply 10 μl of a fresh bacterial culture (OD_{600} 0.6 – 0.8) onto glow a discharged grid for 1 minute. Gently blot the sample from the side with Whatman #1 filter paper. Repeat the entire process 2 times.
2. Apply 4 μl of distilled water (or other buffer solution) and blot with filter paper to remove the excess. Repeat the entire process 2 times.
3. Apply 4 μl of 2% UA stain for 45 seconds and blot with filter paper to remove the excess stain.
4. Apply 4 μl of distilled water and blot with filter paper to remove the excess water.
5. Let the grid(s) air dry for 5 minutes.
6. Store the prepared grids in an empty grid box and record the position of the grids. Grid box may be stored in a vacuum desiccator prior to and after imaging on the microscope. Prepared grids should be stored in vacuum desiccator for at least 30 minutes before loading into the microscope.
7. Dispose of all UA stain contacted materials in a dedicated stain waste container.

Negative Stain Protocol for Viruses

This is a basic protocol that has been used for a number of viruses, in particular, excellent results have been achieved for respiratory syncytial virus. Protocol prepared by Z. Ke, 01/26/2014

Reagents and materials:
Virus suspension
Wash buffer (water or buffer solution (HBSS))
Stain (2% Sodium Phosphotungstate (SPT). Other stains may be used, such as 2%UA, and should be tested during initial negative stain experiments to determine best conditions for the organism being studied.
Tweezers
Grids (400 mesh carbon copper only grids)
Grid storage box
Centrifuge and storage tubes
Syringe and either 0.1 μm or 0.2 μm syringe filters
Parafilm
Whatman #1 filter paper

Stain and Wash Buffer Preparation: At times, stored SPT stain (or other stains) and buffer solutions 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 and wash buffer crystal artifact.

1. Remove an aliquot of stain from the pre-made SPT stain reagent bottle. Or make a fresh aliquot, preferred for any Phosphotungstate derivative stain. Use 0.1 μm syringe filters (preferred) and filter the stain and the planned wash buffer.
2. Use the mini-centrifuge and centrifuge the stain aliquot and buffer for 5 min, 15,000 rpm, and at room temperature (RT).
3. Transfer the stain to a black tube to reduce light exposure when possible. Note: for the transfer, remove only the upper portion of the centrifuged solution to minimize transfer of any stain or buffer crystals.

Grid Glow Discharging: Refer to the glow discharge protocol for the instrument that you plan to use.

1. Use either a static dissipation gun or a U-electrode antistatic system to remove charge effects from tools and grids.
2. Place grids (we used coper/carbon 400 mesh) in the instrument you plan to use. Follow the protocol for its use.
3. Turn off system and slowly release vacuum to minimize disruption of the grids.

Grid Preparation with Sample and Stain:

1. Apply 4 μl of a fresh virus suspension (know the titer of the virus) onto a glow discharged grid for 2 minutes. Gently blot the sample from the side with Whatman #1 filter paper.
2. Immediately after blotting, apply 4 μl of 1X HBSS buffer (or other buffer solution) for 30 seconds and blot with filter paper to remove the excess.
3. Immediately after blotting, apply 4 μl of 2% SPT stain for 45 seconds and blot with filter paper to remove the excess stain.
4. Let the grid(s) air dry for 5 minutes.
5. Store the prepared grids in an empty grid box and record the position of the grids. Grid box may be stored in a vacuum desiccator prior to and after imaging on the microscope. Prepared grids should be stored in vacuum desiccator for at least 30 minutes before loading into the microscope.
6. Dispose of all SPT stain contacted materials in a dedicated stain waste container.

Negative Stain Protocol for Purified Flagella

This is a basic protocol that has been used for a number of appendages purified from bacterial species, in particular, excellent results have been achieved for *Caulobacter crescentus*. Protocol prepared by J. Sanchez, 12/6/2020

Reagents and materials:
Purified flagella (or other object) from bacterial organism
Wash buffer (water or buffer solution)
Stain (2% UA). Other stains may be used, such as 1% Phosphotungstic Acid (PTA), and should be tested during initial negative stain experiments to determine best conditions for the material being studied.
Tweezers
Grids (300 mesh carbon copper only grids)
Grid storage box
Centrifuge and storage tubes
Syringe and either 0.1 μm or 0.2 μm syringe filters
Parafilm
Whatman #1 filter paper

Stain and Wash Buffer Preparation: At times, stored UA stain (or other stains) and buffer solutions 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 and wash buffer crystal artifact.

1. Remove an aliquot of stain from the pre-made UA stain reagent bottle. Use 0.1 μm or 0.2 μm syringe filters and filter the stain and the planned wash buffer.
2. Use the mini-centrifuge and centrifuge the stain aliquot and buffer for 15 min, 15,000 rpm, and at room temperature (RT).
3. Transfer the stain to a black tube to reduce light exposure when possible. Note: for the transfer, remove only the upper portion of the centrifuged solution to minimize transfer of any stain or buffer crystals.

Grid Glow Discharging: Refer to the glow discharge protocol for the instrument that you plan to use.

4. Use either a static dissipation gun or a U-electrode antistatic system to remove charge effects from tools and grids.
5. Place grids (we used coper/carbon 300 mesh) in the instrument you plan to use. Follow the protocol for its use.
6. Turn off system and slowly release vacuum to minimize disruption of the grids.

Grid Preparation with Sample and Stain:

1. Place 3- 50 μl drops of sterile water and 2- 50 μl drops of 2% UA on parafilm
2. Apply 4 μl of sample onto glow discharged grid for 40 sec
3. Gently blot the sample (Whatman #1 filter paper).
4. Immediately touch grid to the 1st drop of water and gently blot off water, repeat 2 more times.
5. Immediately touch grid to the 1st 2% UA drop and gently blot off with water.
6. Touch grid to the last 2% UA drop and hold for 40 sec (do not immerse grid), blot off the stain.
7. Let the grid(s) air dry for 5 minutes.
8. Store the prepared grids in an empty grid box and record the position of the grids. Grid box may be stored in a vacuum desiccator prior to and after imaging on the microscope. Prepared grids should be stored in vacuum desiccator for at least 30 minutes before loading into the microscope.
9. Dispose of all UA stain contacted materials in a dedicated stain waste container.