INTRODUCTION

Negative staining is an easy, rapid, qualitative method for examining the structure of isolated organelles, individual macromolecules and viruses at the EM level. However, the method does not allow the high resolution examination of samples – for this more technically demanding methods, using rapid freezing and sample vitrification are required. Also, because negative staining involves deposition of heavy atom stains, structural artefacts such as flattening of spherical or cylindrical structures are common. Nevertheless, negative staining is a very useful technique because of its ease and rapidity, and also because it requires no specialized equipment other than that found in a regular EM laboratory.

Principle negative stains:

Stain	Normal pH range for use
Sodium (K) phosphotungstate (PTA)	5 - 8
Uranyl acetate	4.2 – 4.5
Sodium silicotungstate	5 - 8
Ammonium molybdate	5 – 7
Methylamine tungstate	6 - 7

Ideally, the negative stain should not react with the specimen in a 'positive staining' manner (i.e. it should not bind to the specimen). However, uranyl ions will bind to proteins and sialic acid carboxyl groups and to lipid and nucleic acid phosphate groups. One effect of this is to induce aggregation of the material.

Samples should be suspended in a suitable buffer (e.g. 10 mM HEPES or PIPES), in 1% ammonium acetate, or in distilled water. It is best not to use phosphate buffer or PBS as they may contaminate the grid with salt residues that have to be washed off after staining resulting in a loss of contrast. Uranyl salts, in particular, react with phosphate ions to produce a fine crystalline precipitate that obscures the specimen. [The precipitation of uranyl ions by phosphate ions is also a potential problem when using uranyl acetate as a third fixative/en bloc stain during processing of specimens for TEM].

Fixed or unfixed samples may be used. With unfixed specimens there is the potential problem of changes occurring due to osmotic shock (or to changes in ionic composition) since most negative stains are made up in distilled water. Also there may be safety implications when examining unfixed bacterial or viral samples.

To fix samples spin them down, remove the supernatant and replace it with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.0). Immediately re-suspend the sample in the fix and leave for a minimum of 1 h at 4˚C. (If samples are left in a pellet they will much harder to put back into suspension disperse after fixation). After fixation, the samples should be gently pelleted, washed and re-suspended in distilled water or a suitable buffer.

NEGATIVE STAINS

SODIUM (POTASSIUM) PHOSPHOTUNGSTATE (PTA)

PTA is one of the most commonly used negative stains although it does have a significant disruptive effect on many membrane systems. PTA does not act as a fixative and can destroy some viruses. It is also known to interact with lipoproteins and cause the formation of 'myelin figures'. However, it can be used at physiological pH, and is less likely to precipitate with salts and biological media.

NEUTRAL PHOSPHOTUNGSTIC ACID

A 1- 3% solution of neutral PTA (buffered to pH 7 using sodium hydroxide) is a useful stain for many samples but is especially good for viruses that dissociate at low pH. The stain produces less contrast than the uranyl acetate.

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URANYL ACETATE
A 1% to 3% solution of uranyl acetate dissolved in distilled water (pH 4.2 to 4.5) can be used to negatively stain many types of samples. The stain should be filtered through a 0.22 µm filter that has been pre-rinsed with large volumes of double distilled water. The filtered stain should be stored in the dark at 4˚C and can be used for >1 year.

Uranyl acetate solutions also act as a fixative for viruses. The advantage of uranyl acetate and uranyl formate is that they produce the highest electron density and image contrast as well as imparting a fine grain to the image. The finer grained image produced is particularly useful for smaller particulate specimens.

Because stain has a low pH it is not recommended for use with specimens that are unstable in acid conditions. Also, the stain precipitates at physiological pH and in the presence of many salts and great care is need when using it.

SODIUM SILICOTUNGSTATE
Sodium silicotungstate (1-5%) provides good contrast and is also useful because it produces a particularly fine grain: thus good for small particles and individual molecules.

AMMONIUM MOLYBDATE
Used as a 1-2% solution in distilled water with the pH adjusted with ammonium or sodium hydroxide to pH 7.0. Do not exceed pH 7.0 as crystallization may occur during drying. A 2% solution of ammonium molybdate is particularly useful for staining osmotically sensitive organelles. While this negative stain seems to give the best results for many types of specimen, it does produce a lower electron density than other stains.
(This stain has also been used to negatively stain thawed, thin cryosections of fixed cells.)

METHYLAMINE TUNGSTATE
Used as a 2% solution in distilled water (usually pH 6.5). Solutions of methylamine tungstate do not keep so it is best to make up small quantities of stain freshly before each experiment. This stain does not damage delicate structures as much as PTA and has been found to be useful for negative staining macromolecules, viruses and membranes. Contrast is not as good as uranyl acetate but the resolution is good and the material wets the grid film and specimens well.

OTHER STAINS
Other less common stains include: gold thioglucose, lanthanum acetate, lithium tungstate, sodium zirconium glycollate, tungstoborate, uranyl acetate, aluminium formate, uranyl formate, uranyl oxalate, and uranyl sulphate.

NEGATIVE STAINING METHODS

There are a number of methods for preparing samples for examination by negative staining; the method used depends on either the sample to be stained or the method of choice in the laboratory. Below are outlined some of the simpler methods, with more specialized ones listed at the end.

Method A
Single-droplet method I
1. Prepare a 2% aqueous solution of the stain (and adjust the pH to 7.0 with 1M KOH, if required).
2. Mix equal quantities of sample and stain (10-20 µl of each will be enough).
3. Place a drop of this mixture onto a formvar grid held by tweezers. Leave for ~20 sec and then remove almost all the solution with filter paper.
4. Air dry. Some methods advocate a wash in distilled water after drying. In practice this is usually only necessary when using a buffer that becomes crystalline when dried or when the sample is too thick on the grid. If this is done dry the grid again before viewing.

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Single-droplet method II
1. Glow discharge formvar-carbon coated grids just before use to increase their hydrophylicity.
2. Place 1-3 µl of the sample on the grid (This is sufficient to cover the grid surface).
3. After ~10 sec slowly pipette 20 µl of stain on to sample, while gently absorbing the stain from the opposite side using a wedge of filter paper. The staining procedure should take ~30-60 sec.
4. After absorbing as much stain as possible allow the grid to dry and examine the grid as soon as possible, preferably the same day. If there are problems with stain precipitation, or with general stain background then rinses prior to staining may be necessary (see below).

Method B
Sequential two-droplet method
1. A suspension of particles/organelles is made in a suitable buffer or in distilled water.
2. Place a drop (~5-10 µl) of the suspension on to a formvar grid.
3. When the suspension has partly dried the grid is washed by touching it three times to the surface of a drop of distilled water.
4. Remove excess water by touching the grid to a filter paper.
5. A small drop of stain is then applied to the grid.
6. After 10 seconds the excess stain is removed by touching the edge to a filter paper.
7. Dry the grid at room temperature.

Method C
Tim Mitchison's lab at Harvard has found that this straightforward negative staining procedure works well with a range of specimens were there is a lot of salt or sucrose/glycerol in the buffer and washing the sample is required.

There are several methods for washing the grid surface prior to applying stain. One method is to apply sample to the grid, allow adsorption for ~10 sec, then hold the grid at a downward angle and drop 2-3 large drops of rinse solution (either ddH2O + 5 mM EGTA for removing interfering salts/buffer components/sucrose/glycerol or warm BRB80 for removing unpolymerized tubulin) over it and then apply the stain. Alternatively, washing can be done by placing the grid sample-side down on a series of large droplets of rinse solution or by slowly drawing the grid, sample-side down, over the surface of a large droplet of rinse solution. The stain can then be applied as above. Dilute samples can be concentrated on the grid by adsorption for longer times (1-3 min).

NOTES

1. If the stain fails to spread and/or forms dense masses in which the particles are completely buried, the addition of a trace of serum albumin may correct the problem.

2. If the stain spreads too widely (too pale a background), either use a higher concentration of the stain, or leave more of the stain or sample+stain on the grid (i.e. do not absorb off too much of the solution(s)).

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