Negative Staining Electron Microscopy

Tips & Trick for the Diagnosis of Pathogens

In infectious diseases, diagnostic electron microscopy (DEM) is used for screening samples to find the pathogenic agent, e.g. viruses, bacteria or parasites [1]. For this purpose, negative staining followed by transmission electron microscopy is predominantly applied because of its simplicity, quickness and, finally, long tradition which made numerous images of pathogens available in the literature. As diagnostic result, DEM provides rather the morphological group than the actual species or strain of the pathogens found, which may already be sufficient to diagnose the disease. However, in most cases DEM is used as a rapid scouting method to restrict the number of possible pathogens involved, enabling a more focussed, target-specific diagnosis. The application of DEM is beneficial in cases in which speed of diagnosis is essential, previously unknown pathogens may be involved, specific tests are lacking, or just as an independent control [2].

Negative Staining Procedure

Negative staining is a procedure, which embeds small biological particles adsorbed on an electron transparent sample support (EM grid) in a thin and amorphous film of heavy metal salts to reveal their structural details in the transmission electron microscope (Figure). Different protocols for negative staining electron microscopy were successfully used [3], [4]. The use of small droplets for particle adsorption from a suspension and for washing and staining became the most popular procedure. The droplet protocol [5] usually comprises the following steps: EM grid pre-treatment, particle adsorption, washing, and incubation with heavy metal stain. The following short paragraphs will detail the specific requirements of each step and provide solutions to meet them.

Preparation of EM Grids

Requirement: Support films must be transparent and mechanically stable during exposure in the electron beam.

Solution: Use grids with small meshes (e.g. 300-400 mesh) and good heat conductivity (e.g. copper grids). Adding a thin (2-5 nm) carbon layer on top of the
plastic film helps to spread efficiently the thermal energy introduced by the electron beam during imaging.

**Pre-Treatment**

**Requirement:** The hydrophilic suspension must spread on the plastic film of the EM grid to allow adsorption of particles.

**Solution:** Physical charging by a glow discharge procedure (advantage: no chemicals; disadvantages: needs a machine, variable efficiency). Adsorption of a charged chemical (e.g. alcian blue; advantage: reproducibility, quickness; disadvantage: chemical modification of the sample support).

**Adsorption**

**Requirement:** Attachment of as many particles of interest at the grid surface as possible

**Solution:** Adding a drop of suspension on pre-treated grid surface (drop-on-grid). This procedure usually results in higher particle number at the grid surface than putting the grids on a sample droplet. The incubation time is a critical factor: the longer, the better. 10 min is a good compromise. To increase the particle number further, use ultracentrifugation directly onto the grid, e.g. by using an airfuge [6].

**Washing**
Requirement: Removal of salts or macromolecules, which might interfere with the staining or visibility of particles.
Solution: Transfer of EM grids with adsorbed particles successively on two to four droplets of deionized water. The procedure removes also particles of interest [6].

**Staining**

Requirement: The heavy metal staining should protect the sample structure from collapsing during drying and should provide contrast for the detection of structural features.

Solution: Incubation of the adsorbed particles with heavy metal salt solution and drying for embedding them in an amorphous film. Use at least two different stains with different capabilities to reveal different features of the particles (e.g. 1% uranyl acetate and 1% phosphotungstic acid). It is critical to produce a thin film of amorphous stain by the final blotting procedure using filter paper to reveal fine structural details.

**Microscopy**

Requirement: Finding and recognition of relevant particles.

Solution: The microscope should be aligned properly and the imaging conditions must be balanced between sufficient beam current for visibility of densely stained particles or clusters and specimen drift (with stable grids this is achieved within a wide range of conditions). An accurate magnification calibration is important, especially for the diagnosis of virus groups, because size distribution of the particles is an important diagnostic criterion. Since the grid surface is too big for an entire screening at mid and high magnification in a reasonable time, stochastic screening must be applied. The inspection of twenty meshes of a 400 mesh grid at mid magnification and in each mesh a sub-region at high magnification will result in acceptable detection probability [6] within a reasonable time (15 to 20 min). Start in the middle of the EM grid and cover all regions approximately evenly excluding regions with film deteriorations or inappropriate staining. Recognition needs knowledge about the morphology of pathogens. Studying publications, books (e.g. [7], [8]) or image collections (e.g. The VirusExplorer for DEM [9]) and the participation in regular practical training, such as lab courses or sample exchange programs (e.g. EQA-EMV training [10]), improves and conserves the diagnostic capabilities.

**References**


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