doi:10.1017/S143192761400806X
Microsc. Microanal. 20 (Suppl 3), 2014

New Method for Multiple Immunodetection on Resin Ultrathin Section in the Field Emission Scanning Electron Microscope

Petr Wandrol[1], Marie Vancová[2,3] and Jana Nebesářová[2,4]

[1.] FEI Czech Republic, Brno, Czech Republic
[2.] Institute of Parasitology, Biological Centre of ASCR, v.v.i, Ceske Budejovice, Czech Republic
[3.] Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic
[4.] Faculty of Science, Charles University in Prague, Czech Republic

Localization of a specific protein within the cell ultrastructure in high resolution is based on a specific bond between the molecule of interest (antigen) and an antibody conjugated to an electron dense nanoparticle clearly visible in consecutive observation in the electron microscope. Colloidal gold nanoparticles are usually used as the label. Diameter of the nanoparticle must be small enough to ensure good labeling efficiency together with good visualization of particles in cell structures, typically 6 – 12 nm. Localization of multiple molecules within the same sample area can be done by using gold particles of different sizes. However, detection of more than two molecules is difficult because of a narrow diameter range. Polydispersity of the diameter of colloidal particles can also cause problem when multiple particle sizes are applied. Several approaches of immunodetection of more than three proteins by the electron microscope were used, such as using nanoparticles of different shapes [1] or composition [2].

This abstract proposes method that doubles number of localized proteins compared to commonly used methods. Maximum number of simultaneously detected antigens with nanoparticles of various shapes in TEM is now 5 [3]. This method increases the number of simultaneously labeled antigens in cell ultrastructure up to ten. Moreover, the method allows using two antibodies conjugated with gold nanoparticles of the same diameter as well as two different primary antibodies produced in the same animal. Since the diameter of nanoparticles influences the labeling density, the method provides excellent opportunity to compare simultaneously the concentration of two antigens in the studied specimen area. The new method can be easily and widely used in contrast to the necessity of invention and standardization of new alternative labelled probes. In contrary to the current immunodetection of antigens on resin sections by the transimission electron microscope, proposed method uses scanning electron microscope (SEM) as a primary imaging device.

The general concept is to label both sides of the ultrathin section with different antibodies conjugated to same electron-dense nanoparticles and to distinguish on which side the label is by advanced imaging method, such as combination of images acquired at different energies, correlation of signals from different detectors or using images taken at different tilt angles - sort of mini-tomography series. Detailed description of the method using advanced imaging in backscattered (BSE) and transmitted (STEM) modes at two energies of the primary beam in the SEM is as follows.

The labelling is done on both sides of the ultrathin section by the same particle type (e.g. Au 6nm). Particles of the same diameter conjugated to different antibodies are used to label different antigens on either side of the section. Another pair(s) of antigens can be labeled by using different particle size (e.g. 10 or 12nm). Labeling can continue as long as suitable markers are available. Finally, contrast of the ultrastructure and sample conductivity can be increased by section staining with uranyl acetate. When

the labeled ultrathin section is prepared, two images I1 , I2 of the same area of the section are taken at two different energies and detectors. Markers on the top side of the ultrathin section are detected at the energy E1 by the detector of backscattered electrons. The energy E1 is too low to penetrate the lamella and hence labels on its top side are detected only. The E1 is typically around 1 keV. The image I1 is then thresholded to separate the labels from the background and labels are colored.

Energy E2 and the collection of transmitted electrons (STEM) are used to take the image I2. The energy E2 is high enough to penetrate through the section. The image I2 consists of the projection of both labels together with the ultrastructure. The E2 energy typically is between 15 to 30 keV depending on the thickness of the resin section. The image I2 is then thresholded as well. The original image is preserved for later correlation. Labels on the bottom side are visualized in the image I3 which is result of the subtraction of I1and I2 images. Labels are colored in the I3 image. Final image is then correlation of the I1, original I2 and I3 images and localizes both types of molecules within the ultrastructure. Complete workflow is illustrated in Figure 1.

The results are shown in Figure 2.The upper side of the Lowicryl K4M ultrathin section of salivary glands isolated from the tick *Ixodes ricinus* was labeled using anti-BSA IgG followed by secondary IgG conjugated to 10 nm gold particles. The bottom side was labeled with anti-hemoglobin IgG and protein A-10 nm gold particles.

References:

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[4] The authors acknowledge funding from the Technology Agency of the Czech Republic, project TE01020118

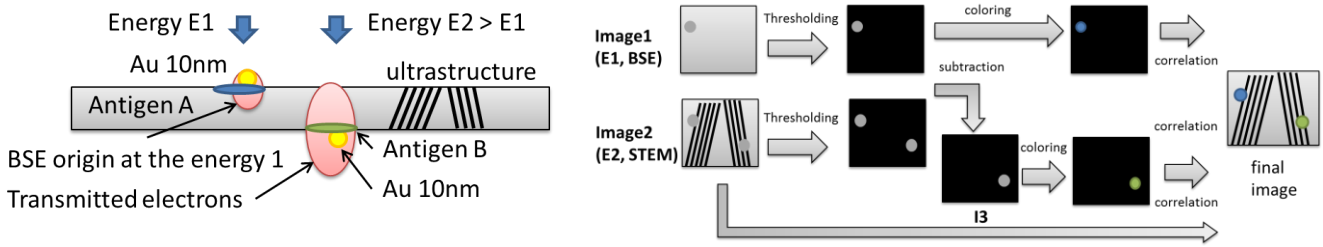

Figure 1. Ultrathin section labeled for double immuno-detection and diagram of the acquisition and processing workflow.

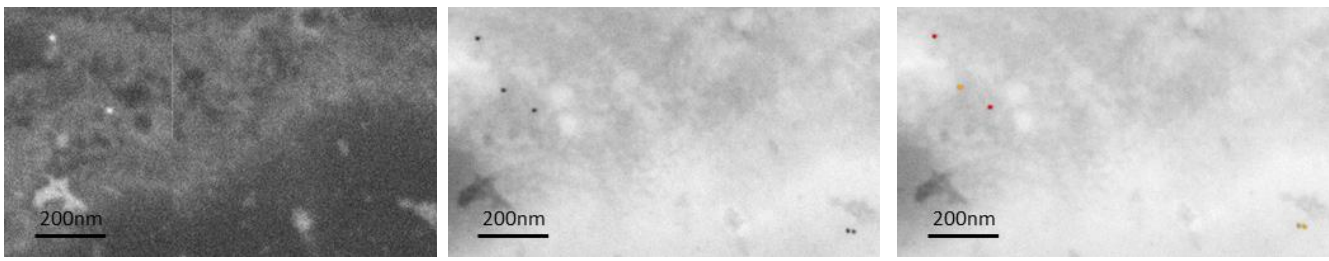

Figure 2. BSE image taken at energy of 1keV (left) – top side particles are observed; STEM image taken at energy of 20keV (middle) – particles on both sides are clearly visible; final processed image (right) showing particles on the top side (red) and bottom (yellow)

https://doi.org/10.1017/S143192761400806X Published online by Cambridge University Press