Microsc. Microanal. 8 (Suppl. 2), 2002
DOI 10.1017.S1431927602100341

Microwave Processing and Pre-embedding Nanogold Immunolabeling for Electron Microscopy

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The introduction of microwave assisted processing for electron microscopy (EM) Login and Dvorak [1], Giberson et al. [2], Giberson and Demaree [3] has dramatically reduced the amount of time required for sample preparation from days to hours. In addition, the introduction of the cold spot and variable wattage controller by Giberson (Ted Pella, Inc.) has provided a means of regulating specimen temperature during processing steps. We have applied the microwave technology to immuno-labeling of the green fluorescent protein (GFP).

Transiently transfecting cells with GFP-fusion proteins and subsequent imaging by live confocal microscopy is a powerful method for studying the function of proteins *in vivo*. However, because many GFP tagged structures are below the resolution limit of the confocal microscope, we expanded our observation to the Electron Microscope (EM) level. Paupard et al. [4] combined microwave fixation with LR White embedding and post-immunolabeling with GFP antibodies on *C. elegans*. In this study we used commercially available anti-GFP antibodies (Roche, Indianapolis, IN) and Nanogold labeled secondary antibodies (Nanoprobes, Stony Brook, N.Y) for pre-embedding immuno-labeling of hippocampal neurons in culture. We have found that microwave processing dramatically shortens all steps of the immuno-labeling procedure, including incubation in both in primary and secondary antibodies. It also speeds up the permeabilization step to allow better access of antibodies while maintaining structural integrity. This method combines the use of the latest in microwave technology combined with the use of ultra small gold labels to obtain excellent ultrastructural preservation and good labeling density in 3 hours.

Hippocampal neurons (div 7-9) were transfected with the PLC-δ PH domain fused to GFP (PH-GFP). This PH domain binds specifically and with high affinity to phosphatidylinositol 4,5-biphosphate (PIP2). As observed by confocal microscopy, PH-GFP labeled the plasma membrane of cell bodies and neuronal processes. Weaker diffuse cytoplasmic staining was observed as well. Accordingly, at the electron microscope level, the silver enhanced gold label was seen on the neuronal plasma membrane and, at a lower density, in the cytoplasm. Heavier labeling was observed in many small processes, at neuronal branch points and synaptic contact areas. Interestingly, no labeling was seen on the synaptic vesicles. Control (untransfected cells) cells showed no labeling. As an additional control, neurons were also transfected with a GFP fusion to a PH domain having a point mutation in the binding site to PIP2 (PHM-GFP). In this case, at the light level the GFP fluorescence appeared to be uniformly distributed throughout the neurons. At the electron microscope level, the immunolabeling was dispersed throughout the cell cytoplasm, with a heavier concentration of silver enhanced gold in the cell nucleus. This highly specific immunostaining of GFP-fusion proteins and the well-preserved cellular ultrastructure, combined with live imaging of transfected cells, can provide essential information for deciphering the function of biologically important molecules.

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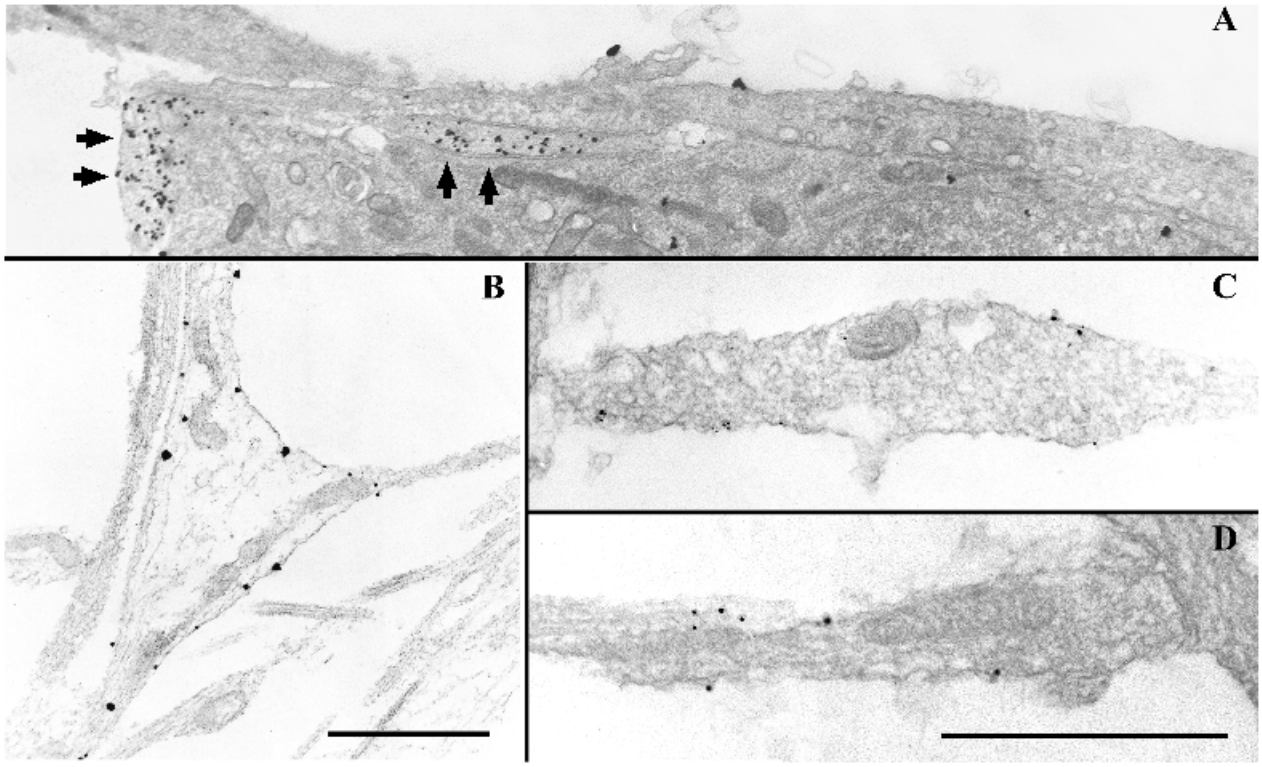

A. Small process of a hippocampal neuron 14 days *div* immuno-labeled with anti-GFP (PHM-GFP) primary and Nanogold conjugated secondary antibodies. Silver intensification.
B. Hippocampal neuron showing specific membrane labeling for anti-GFP (PH-GFP), unlike the PHM-GFP staining pattern. Scale bar 1 micron. Same magnification in A and B.
C. Membrane of an axonal varicosity of hippocampal neuron immuno-stained for anti-GFP (PH-GFP). Synaptic vesicles fill the process.
D. Synaptic varicosity of hippocampal neuron immuno-stained for anti-GFP (PH-GFP) shows silver enhanced gold particles along the membrane. Scale bar 1 micron. Same magnification in C and D.

https://doi.org/10.1017/S1431927602102194 Published online by Cambridge University Press