Immunogold detection of synaptic membrane proteins on grid-glued double replica pairs

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Neuronal synapses are key to brain information processing, learning and memory. However, a complete understanding of neurotransmitter receptor distribution in post-synaptic membranes remains elusive. Immunolabeling studies at the ultrastructural level are a prerequisite to dissect the roles of neuronal synapse membrane proteins. Traditional immuno-electron microscopy methods, such as pre- and post-embedding immunolabeling, are hampered by limited antibodies accessibility, making visualization of all molecules present in individual synapses difficult. Yet, information on the total distribution and numbers of membrane proteins is essential to interpret their roles in individual synapses. Thus, a different approach is needed.

In freeze-fracture replica immunolabeling (FRIL), lightly fixed tissue or cells are high pressure frozen and then fractured at lipid bilayers revealing P-face (protoplasmic side) and E-face (extracellular side) membrane leaflets. Coating these exposed faces with a thin carbon layer captures membrane proteins, which are retained in the carbon replica, even after the remaining tissue is removed with detergent. These fine carbon replicas are floated in drops of antibody and wash solutions and finally with gold-conjugated secondary antibodies, then examined by TEM or SEM to identify and localize specific proteins of interest.

FRIL has two distinct advantages over the traditional immunolabeling methods; firstly, the whole synaptic active zone can be revealed by the fracture, and secondly, the membrane protein epitopes are exposed and detectable with a high sensitivity by immunogold labeling. Thus, both two-dimensional localization and quantitation of membrane proteins at the synaptic membrane are feasible. One challenge of the FRIL technique is the fragility of the replicas. Fragmented replicas often make interpretation difficult as protein localization must be mapped to histological context to discern regions of interest. In addition, the fracture plane captures the membrane proteins to either P-face or E-face sides of the membrane. Thus, immunolabeling on a single replica will only reveal membrane proteins allocated to either P- or E- face of individual synapses, while those allocated to the corresponding E- or P-face must be detected on the complementary replica. Thus, a count of gold particles on mirrored replica pairs, with appropriate synapse matching, allows a total count of the gold-labelled protein, providing a full and more accurate picture of membrane protein distribution at the synapse. Until now, such work required considerable technical skill.

Here we describe a new modification of the original FRIL technique. After freeze-fracture and carbon shadowing, we keep double replica pairs intact by gluing them to EM grids using a UV-cured glue. This procedure is more technically robust as the grid-supported replica can be handled easily and is tolerant to rigorous washing. In double replicas pairs, we can identify reference areas for orientation, finding both sides of matched synapses. Then, using antibodies to intracellular and extracellular epitopes of the same molecule, we can estimate total protein numbers and their distribution. We will describe this "**grid-glue FRIL technique**" in detail using, as an example, a glutamate receptor quantitation on cerebellar molecular layer replica pairs.

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