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FRIL is for the Tenacious: Maintaining Rigor and Reproducibility

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Freeze-fracture replica immunogold labeling (FRIL) is a high-resolution immunocytochemical technique for visualizing, identifying, and mapping intramembrane proteins (IMPs) to ultrastructurally-identified membrane domains in complex tissues. Although light microscopic immunocytochemistry (LMIC) allows simultaneous use of different fluorophores bound to antibodies from multiple animal species, allowing separate detection, quantification, and subcellular mapping of multiple immunologically-distinct proteins over histological dimensions (Fig. 1A), spatial resolution in LMIC is limited by wavelength of the fluorophores to 100-300 nm. Moreover, detection of labeled proteins in conventional 5-10 µm-thick tissue slices is limited to ≥100 molecules, limited primarily by detection of florescence "signal" above autofluorescence "noise" [1]). Consequently, multiple "controls" are required for all LMIC investigations. In addition to confirmation of specificity of labeling of purified proteins in Western blots, additional "controls" required: ***1)*** demonstration that similar fluorescent labeling did not occur in specimens in which the target protein had been knocked out (ko control), and ***2)*** documentation that all labeling was abolished when the primary antibody was omitted while including the secondary antibodies (control for non-specific binding of secondary antibodies on other tissue components).

In contrast, because FRIL is based on whether or not immunogold beads bind to and are restricted to multiple examples of the target ultrastructural feature (*i.e.*, ultrastructural target specificity; Figs. 1B,C), FRIL demonstration of target specificity eliminates the need for a separate "ko control", primarily because one can neither visualize nor label structures that are no longer present, because non-specifically bound immunogold beads ("noise") are identified by other approaches (next item). Ultrastructural specificity of labeling in FRIL also documents that the component proteins remain adsorbed to the replicated ultrastructural feature. However, because the most common artifacts unique to FRIL result from the high molecular-adsorptivity of the platinum/carbon replica itself, a completely different set of approaches and controls must be implemented for FRIL:

each FRIL sample, the SNR and approximate LE must be determined at first viewing, and samples with excessive noise must be discarded. Typically, structures examined to date by FRIL (*e.g.*, gap junctions, glutamate receptor clusters) have LE's of 1:3 to 1:100, with LE of 1:10 considered optimal because high LE's (*i.e.*, > 1:3) using gold beads that are larger than 10 nm may obscure target IMPs or pits (Fig. 1B), whereas LE's < 1:30 (Fig. 1B, *arrow*) often result in failure to detect IMP arrays consisting of only a few IMPs.

Proteins dissolved by SDS washing and readsorbed to the Pt/C replica and then immunogold labeled provide a major source of labeling "noise". To minimize protein readsorption to the highly-adsorbent replica, non-specific binding sites must be blocked by efficient "blocking" buffers applied both during and after SDS washing, and in particular, during labeling with both primary and secondary antibodies. "Blocking buffers" routinely contain either non-fat dry milk or fish gelatin digest, dissolved in buffer [2]. In freeze-fracture replicas, labeled structures (*e.g.*, neuronal gap junctions) typically occupy only about 0.0001% of the replica surface, with ≈1,000,000-times more area available for non-specific labeling than for specific labeling. Thus, with noise at ≤ 1 gold bead per square micrometer, there is always much more total "noise" than "signal" on each FRIL replica, even at a high LE of 10,000:1. However, this "noise" is

easily discriminated from the "signal" found on labeled structures, where dozens of gold beads are present, such as on gap junctions (Fig. 1B). At an LE of 1:10-1:30, 10-30 gold beads on a gap junction of 300 connexons *(*or 0.03 μm^2) is easily detectable above random noise. Moreover, this optimum LE of 10-30 gold beads on a 0.03 μm^2 gap junction, with modest background ($1/\mu m^2$), translates into a SNR of 1,000:1 to 3,000:1.

A current limitation of FRIL is the 20-30-nm "radius of uncertainty" of labeling imposed by the double-antibody bridge that links the gold bead to the target protein (Fig. 1D; [1]). This does not pose a problem for large clusters of IMPs in which most of the antibodies fall well within the perimeter of the labeled structure (Fig. 1B,C), but can pose a problem when the target structures are small (Fig. 1C, *circle*), form linear arrays (*e.g.*, tight junctions [1, 3] or K_V1 channels and Cx29 "rosettes" [4]), or are present in only a few copies. Even then, trigonometric considerations result in >70% of such labels falling within 10-nm of the target protein (Fig. 1D).

By attending to these caveats, combined LMIC / FRIL provides the most powerful immunocytochemical tool available for identifying, characterizing, and subcellular mapping of diverse classes on intramembrane proteins. Ongoing developments are expected to extend FRIL approaches to the analysis of cytoplasmic, nucleoplasmic, and extracellular matrix proteins, lectin-binding sites, and to the detection and localization of various RNAs [6].

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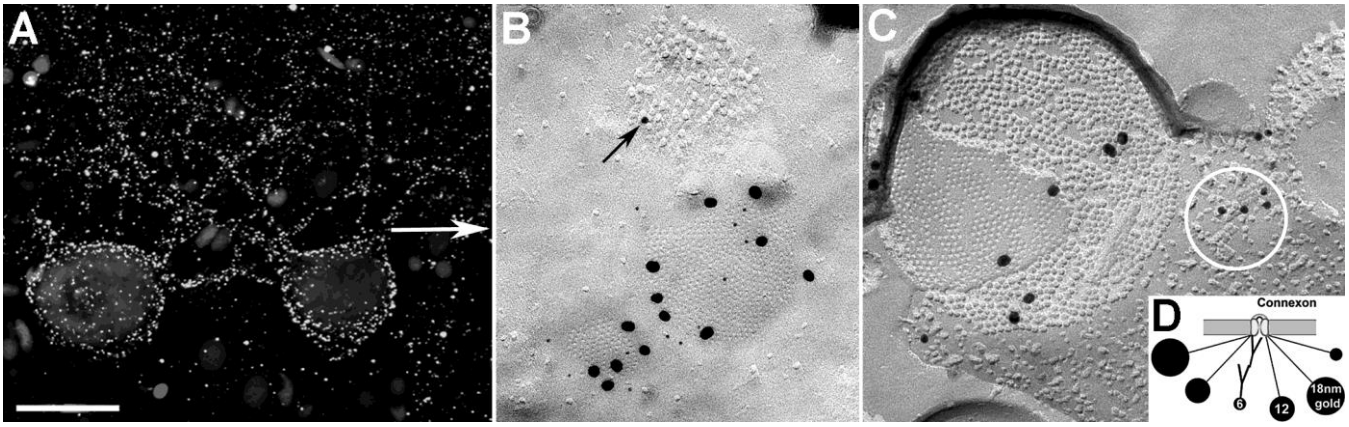

Figure 1. Comparison of LMIC *vs.* FRIL labeling. ***A***, NeuroTrace® 640/660 Deep-Red Fluorescent Nissl (motor neuron soma), with connexin 35 (mab 3043/Alexa Flour 488) immunoreactive puncta associated with neuronal somatic and dendritic plasma membranes in mosquitofish spinal cord. ***B***, Two mosquitofish neuronal gap junctions labeled for Cx36 by 6-nm and 18-nm gold beads, and NMDA glutamate receptors labeled by 12-nm gold beads (*arrow*) (see [5]. ***C,*** Gap junction between astrocytes, weakly labeled with monoclonal antibody to Cx43 (LE ≈ 1:100; six 18-nm gold beads), with nearby square arrays *(circle*) labeled by polyclonal antibody to AQP4 (12-nm gold). ***D.*** Radius of uncertainty of immunogold labeling in 3-dimensional space (Modified from [1]).

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