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Correlative Microscopy for Localization of Proteins In Situ: Pre-embedding Immuno-Electron Microscopy Using FluoroNanogold, Gold Enhancement, and Low- Temperature Resin

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Immuno-electron microscopy (immuno-EM) is a technique that has been used widely to determine sub cellular localization of proteins. Different approaches are available for immuno-EM: pre-embedding method, post-embedding, and cryosectioning (Tokuyasu “style”). Here we describe a pre-embedding technique that allows the labeling of a target protein in situ , retention of fluorescence signal in plastic, and its localization at the EM level in a given cellular context. The procedure can be technically challenging and labor intensive: it requires optimization of fixation protocols to better preserve the cellular morphology and screening of compatible antibodies. Nevertheless, immuno-EM can be a powerful localization tool.

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1. For the localization of intracellular proteins such as Cx43, this protocol necessitates the use of a permeabilizing agent. In general, the use of detergents, although useful to facilitate the penetration of antibodies into cells, is detrimental for the preservation of the overall ultrastructure, which is why we recommend performing the permeabilization step at 4 °C. For Triton X-100 we recommend not to exceed a concentration of 0.1 %. Several types of detergents are available and can be used as alternative to Triton X-100. For example, saponin is gentler with the membranes and is reversible and can be used to increase membrane permeabilization. Digitonin is another alternative. We recommend testing a series of dilutions with 0.1 % being the highest to assess the lowest concentration of detergent that will achieve a good labeling.
2. The optimal primary antibody dilution should be determined empirically for each antibody used. Also, the primary antibody incubation time can be different depending on the antibody used. Some might require a longer incubation time at 4 °C; others might work well only at room temperature. Whenever possible we recommend working at 4 °C in order to better preserve the ultrastructure.
3. The quenching step with glycine is important to inactivate unreacted aldehyde groups, which may be present after glutaraldehyde fixation. It does not affect negatively the ultrastructure preservation.
4. The size of the final gold particles depends of the time of application. Based on the target of interest the experimenter should adjust the time of gold enhancement.

1. NRK cells plated on 35 mm glass-bottom dishes (MatTek Corporation, Ashland, MA) pre-coated with poly-d-lysine are grown in Dulbecco's modified Eagle's medium (Mediatech, Inc., Manassas, VA) supplemented with 10 % FBS in a humidified 5 % CO₂ incubator at 37 °C. Cells should be ~70–90 % confluent to be used for the experiment.
2. Buffered solutions. Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium, no phenol red, 1×, pH 7.0 (LifeTechnologies). Phosphate-Buffered Saline (PBS), pH 7.0.
3. Antibodies. Primary antibody: rabbit polyclonal anti-Cx43 (dilution 1:400; Sigma, Cat. # C6219). Secondary antibodies: Alexa 488-FluoroNanogold goat anti-rabbit (dilution 1:100; Nanoprobes, Inc., Yaphank, NY) or FITC goat anti-rabbit (dilution 1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).
4. Fixative. Prepare a 4 % (w/v) paraformaldehyde (PFA) solution in PBS. To make a volume of 50 ml: heat up 40 ml of ddH₂O at 60 °C with a stirring hot plate in a fume hood; turn off the hot plate and dissolve 2 g of PFA prills (Electron Microscopy Sciences) and while stirring add 25 µl of 5 N NaOH. Filter the solution in a graduated cylinder using a filter paper. Add 5 ml of 10× PBS. Adjust pH to 7.4. Adding ddH₂O bring the volume to 50 ml.
 - 4 % PFA/PBS + 0.1 % glutaraldehyde: add 200 µl of 25 % glutaraldehyde solution in 50 ml of freshly prepared PFA.
 - 2 % PFA/PBS: follow same procedure as 4 % PFA/PBS adjusting the quantity of PFA prills according to the desired concentration.
 - 2 % PFA/PBS + 0.1 % glutaraldehyde: add 200 µl of 25 % glutaraldehyde solution in 50 ml of freshly prepared PFA.
 - 2 % glutaraldehyde/PBS: add 1 ml of 25 % glutaraldehyde solution in 11 ml PBS (1×), to obtain a total volume of 12 ml.
5. Quenching solution. 50 mM glycine in PBS. Dissolve 188 mg of glycine in 50 ml of PBS (1×).
6. Blocking/permeabilizing buffer. 1 % Bovine Serum Albumin (BSA), 5 % Normal Goat Serum (NGS), 0.1 % Triton X-100, 20 mM glycine, 1 % fish gelatin in PBS. Dissolve 0.5 g of BSA, 2.5 ml of NGS, 50 µl of Triton X-100, 0.075 g of glycine, and 500 µl of fish gelatin in 50 ml of PBS (1×).
7. Working buffer. Dilute blocking buffer 1:10 in PBS (1×).
8. Gold enhancement EM kit (Nanoprobes, Inc., Yaphank, NY). The mixture should be prepared just before use.
9. Ice-cold ethanol solutions: 20–50–70–90–100 %.
10. LR White resin (London Resin Company, Berkshire, England).
11. Aclar Embedding Film (Electron Microscopy Sciences).
12. 200 mesh copper grids (Electron Microscopy Sciences).
13. Diatome diamond knife for ultrathin sections (Electron Microscopy Sciences).

Fixatives are extremely hazardous and should be prepared and handled in a fume hood. Gloves and protective eyewear should be used at all times. PFA is a small fixative molecule and penetrates more rapidly into cells. Glutaraldehyde, though slower to penetrate, is a bifunctional crosslinker and therefore stabilizes the cellular components more efficiently. While PFA is typically used for light-level immunocytochemistry, allowing for overall antigen preservation, glutaraldehyde is a superior fixative for EM but can decrease the antigenicity significantly. For these reasons the ideal fixative for this method is a combination of the two to reach a balance between ultrastructure preservation and antigen retention.

Perform a fixation series of paraformaldehyde and glutaraldehyde dilutions, and assess where the fluorescence signal is lost.

- 1 Remove the culture medium from the MatTek dishes containing the cells and wash three times with HBSS pre-warmed up at 37 °C. Fix cells with pre-warmed fixative at 37 °C (4 % PFA, 4 % PFA + 0.1 % glut, 2 % PFA, 2 % PFA + 0.1 % glut) for 5 min at room temperature followed by 30 min over ice. All solutions and steps from this point on are utilized and performed at 4 °C for the preservation of the ultrastructure (see Safety Warnings).
- 2 Remove fixative and wash cells three times for 2 min each in ice-cold 1× PBS. Note: during any washing step the cells should never become dry to avoid irreversible damage to morphology.
- 3 Incubate in blocking buffer for 1 h at 4 °C (see Guideline 1).
- 4 Incubate in primary antibody diluted in blocking buffer at 4 °C for 1 h on a rocking platform set for gentle agitation (see Guideline 2).
- 5 Rinse cells with Working Buffer, six times for 2 min each at 4 °C.
- 6 Incubate in secondary antibody diluted in blocking buffer at 4 °C for 1 h on a gentle rocker, protected from light. Remember: keep cells in the dark from now on, to preserve fluorescence.
- 7 Rinse cells with Working Buffer, six times for 3 min each at 4 °C.
- 8 Wash cells with ice-cold 1× PBS, three times for 3 min each at 4 °C.

- 9 Check staining with a fluorescence microscope; proceed with EM processing only if good signal and specificity is observed. An example of the fluorescence immunolabeling for Cx43 in NRK cells is shown below.

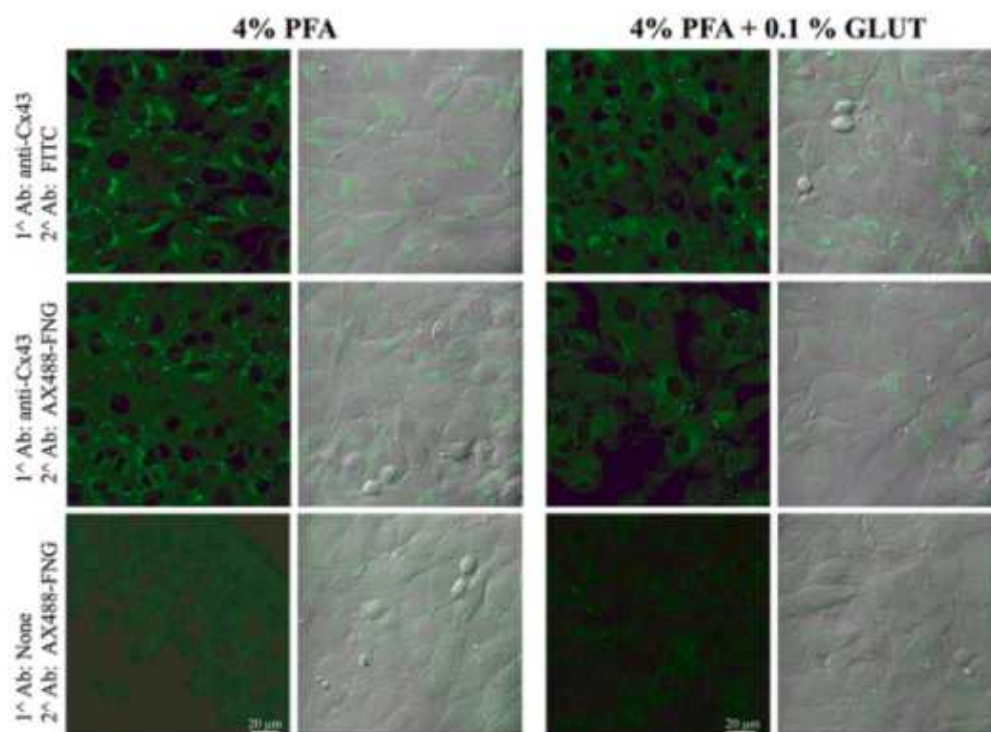


Fig. 1 Immunostaining of endogenous Cx43 in NRK cells. Two different fixative conditions were tested using a rabbit polyclonal anti-Cx43 antibody, a secondary goat anti rabbit Alexa Fluor 488-FluoroNanogold and a goat anti-rabbit FITC antibody. Gap junctions are clearly visible under all conditions tested, indicating the primary antibody tolerates the glutaraldehyde fixation. As control, primary antibody was omitted and no labeling was observed

Fig. 1

- 10 Postfix with 2 % glutaraldehyde in PBS for 10 min at 4 °C.
- 11 Remove fixative and rinse three times for 2 min each in ice-cold 1× PBS. Then wash cells for 5 min in quenching solution at 4 °C to remove aldehydes (see Guideline 3).
- 12 Rinse three times for 5 min each in ddH₂O at 4 °C.

- 13 *Gold enhancement*: incubate cells in the Gold Enhancement kit for 1–5 min to intensify gold particles. The reaction is light insensitive so it can be carried out under normal room lighting.

The kit is composed of 4 components: the enhancer (A), the activator (B), the initiator (C), and the buffer (D).

Equilibrate the solutions at room temperature. Right before use, combine equal volumes of A and B first, wait 5 min then add equal amounts of C and D (see Guideline 4).

- 14 Thoroughly rinse three times for 5 min each in ddH₂O at 4 °C.

- 15 *Dehydration* (ethanol series): dehydrate in increasing concentration of ethanol: 20, 50, 70, 90, and 2× 100 % ice-cold ethanol for 2 min each. Then rinse with 100 % ethanol at room temperature for 2 min.

- 16 *Infiltration and embedding*:

- Remove 100 % ethanol and replace with a 50:50 mixture of low-temperature resin (LR White): ethanol 100 % for 20 min at room temperature.

- Remove the mixture and replace with 100 % LR White for 1 h. MatTek dish can be stored overnight in unpolymerized resin at 4 °C.

- The following day allow three more changes in 100 % of LR White within an hour.

- 17 *Cold-cure procedure*: In a glass vial add 10 ml of LR White and one drop of accelerator. Mix well but avoid introducing any air to the solution and immediately add to cells. Cover with a piece of Aclar Embedding film, avoiding any air bubbles. Place MatTek dish inside an aluminum dish with ethanol on ice to keep the temperature low. Keep it on ice until the resin polymerizes (30 min to an hour).

Store at 4 °C.

- 17.1 At this point the cells can be imaged at the LM level: the signal of the fluorescent label is retained after the embedding procedure and the enhanced gold particles can be viewed by transmitted light. An example is shown in below.

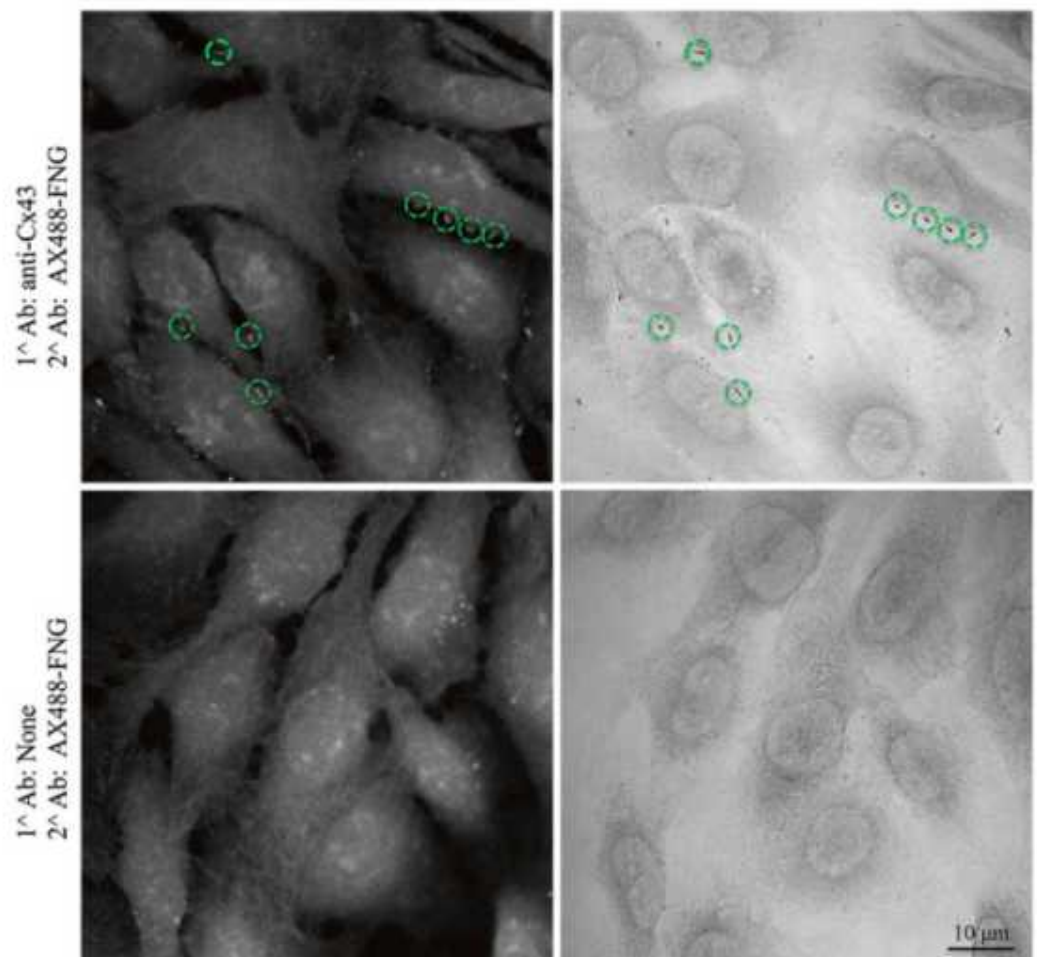


Fig. 2 Fluorescence preservation and gold labeling of endogenous Cx43 in NRK cells embedded in LR White resin. Following immunolabeling and gold intensification, cells embedded in LR White resin retained the Alexa Fluor 488 fluorescent signal (*green circles, left column, top row*). Transmitted light images (*right column*) show the corresponding gap junction puncta (*green circles*) visible as *darker spots* due to the gold intensification step. No labeling was observed in the control condition when the primary antibody was omitted (*bottom row*)

Fig. 2

- 18 Sectioning: choose a region containing labeled cells and mount and trim the LR-White block. Cut ultrathin sections (70–90 nm) and collect them on EM copper grids. Dry grids on filter paper and store them in a grid case at room temperature. Examine the ultrathin sections by TEM (see Fig 3).

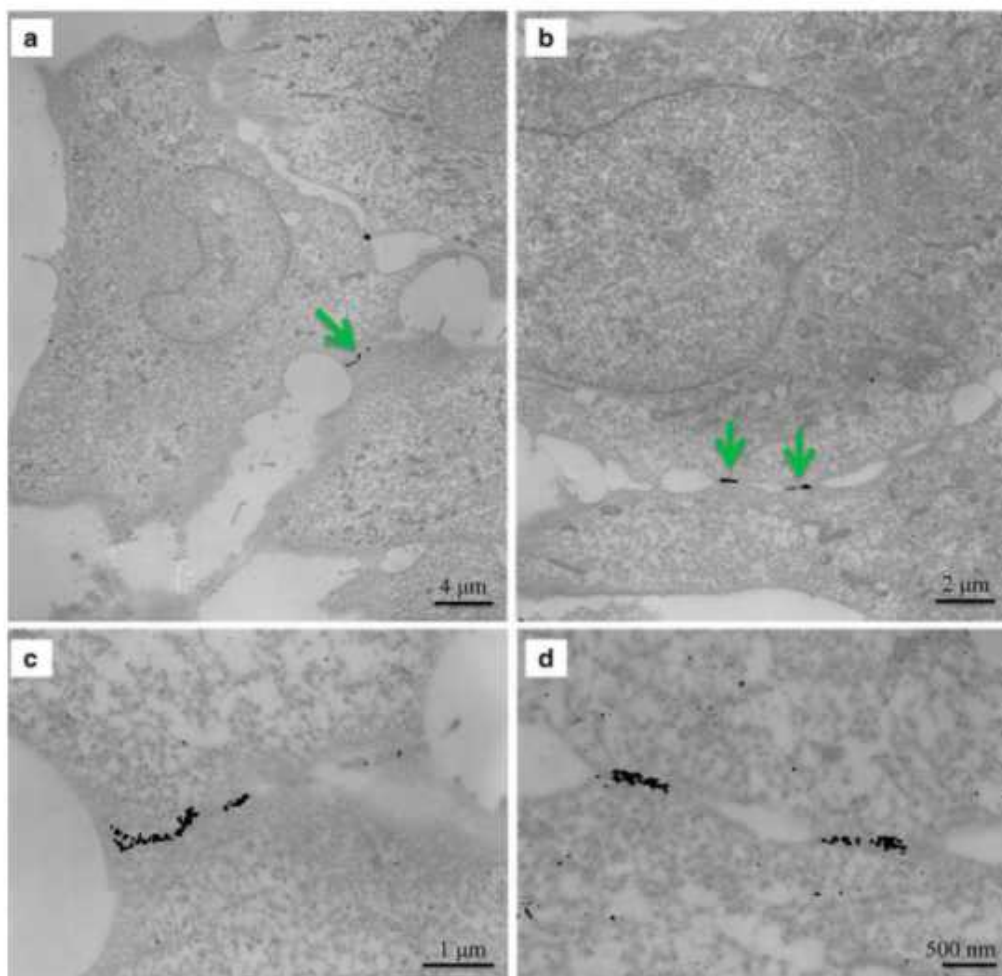


Fig. 3 Labeling of gap junctions after gold intensification. Electron micrographs showing gap junctions labeled by electron-dense gold particles (*green arrows in a and b*). Corresponding areas are shown at higher magnification in **c** and **d**