doi:10.1017/S1431927610055479
Microsc. Microanal. 16 (Suppl 2), 2010

Imaging Connexin And Pannexin Trafficking Structures In Situ

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We study trafficking intermediates of the gap junction protein, connexin43 (**Cx43**) and the connexin-like proteins pannexin1 (**Panx1**) and pannexin2 (**Panx2**) within cells and tissues using several different imaging modalities. Cx43 has the most ubiquitous expression and is the most well studied member of the connexin family. Mammalian pannexins are a recently discovered connexin-like family of three proteins and while their functionality is still being determined, Panx1 has been shown to be an ATP release channel important during purinergic receptor P_2X_7 signaling [1].

Distinguishing specific proteins within their cellular context is akin to highlighting individual trees or groups of trees within a densely packed forest. In order to locate proteins within cells, we use several labeling techniques that allow us to correlate light microscopic fluorescence images with electron micrographs [2]. The fluorescence images permit highlighting proteins using specific probes to elucidate the spatial arrangements within cells. Often, these tagged proteins are used for live cell imaging to study their cell cycle dynamics. Depending on our specific goals, we use (1) genetically encoded tags using one or two tetracysteine domains alone or in combination with GFP or (2) antibodies such as anti-Cx43, anti-Panx1 or anti-Panx2 antibodies for which we apply a secondary label conjugated with either quantum dots or Fluoronanogold™. Genetically appended tags that generate singlet oxygen in sufficient amounts for photooxidation and diaminobenzidine (DAB) polymerization provide the advantage of being suitable for optimal preserving preservation of ultrastructure. Optical pulse chase labeling using tetracysteine domains also provides a method for determining the relative ages of protein during its life cycle. However, correlating exogenously expressing proteins with endogenous proteins, typically with immuno-labeling, is critical.

In one study in our lab, we are interested in understanding how phosphorylation of specific serines in the Cx43 carboxy terminus (C-terminus) function as signals, signposts or gatekeepers. Cx43 moves through the cell cycle in segregated populations of (1) old versus newly synthesized proteins and (2) specific phosphoforms that are physically compartmentalized and probably are related to the sequence or hierarchy of phosphorylation events. This kind of isoform localization process has recently been termed “Spatial Cell Biology” [3]. Antibodies sensitive to the phosphorylation of specific residues are used to localize specific subsets of phosphorylated Cx43 because these structure specific antibodies are the only method for visualizing subsets of Cx43 that have been phosphorylated on known serines or tyrosines in the C-terminus [4]. We use a tetracysteine tag (called 4C) either appended on the C-terminus or inserted into the C-terminus. The latter does not interfere with binding of the scaffolding protein ZO-1 to Cx43. Correlating light microscopy and electron microscopy (CLEM), in particular with tomography (EMT), we investigate these intermediates at reasonably high electron tomographic resolution (~40-60 Å) in 3D to determine their composition and locations within the context of other cellular components.

For pannexins, we use these tools in combination with mutants and pharmacological agents to study how glycosylation affects cellular localization and trafficking. We have previously found

that unglycosylated Panx1 is found intracellularly while glycosylated Panx1 is localized to the cell surface [5]. These labeling tools were critical in establishing that pannexins do not form gap junctions. Unlike connexins, pannexins are not very tolerant of large additions to their C-terminus, such as with GFP or RFP and exogenously transfected cells often have cellular accumulations. These trafficking errors are proportional to the size of the C-terminal addition as small tags such as myc, HA or tetracysteine domains do not show trafficking errors. Because pannexins are relatively new, we also use large scale mosaic LM imaging of rodent brain to look at endogenous expression across multiple cell types to correlate tissue culture cell expression with that found in tissues.

To summarize, these two projects highlight how similar tools can be used to probe for different kinds of trafficking events related to whether they serve as gap junction channels (connexins) or single membrane channels (pannexins).

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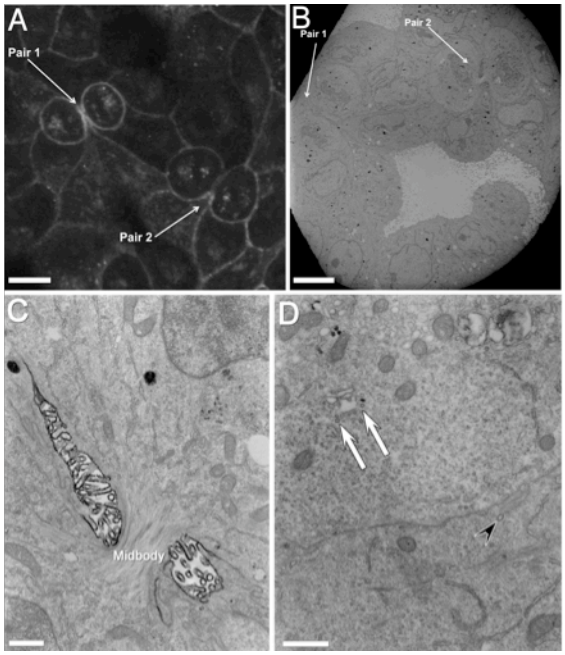

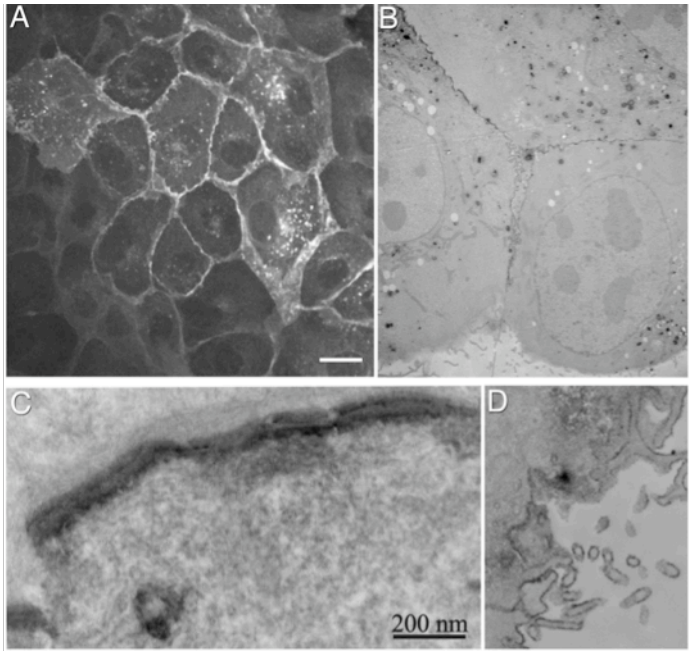

Fig. 1. (Left) Photooxidation labeling of Cx43-GFP-4C MDCK mitotic cells in telophase shows that the concentration of older Cx43 is in the membrane surrounding the midbody and not in large accumulation of vesicles. (**A**) GFP fluorescence (**B**) Same area after photooxidation observed as a thin section low power EM. (**C**) Thin section through the midbody area shows extensive staining of the plasma membrane (**D**) Close-up of cytoplasm showing staining of vesicles (white arrows) and budding of vesicles (black arrowhead). Scale bars: (A) and (B) =5 μm; (C) and (D)= 1 μm.

Fig. 2. (Right) EM localization of Panx1. (**A**) MDCK cells stably expressing rPanx1-4C were stained with ReAsH-EDT_2, fixed and imaged before photoconversion. (**B**) Low magnification EM of the same cells after DAB photoconversion. Higher magnification of junctional (**C**) and non-junctional (**D**) areas shows specific electron densities along the plasma membrane. Panx1 in junctional areas does not appear as canonical gap junctions.

https://doi.org/10.1017/S1431927610055479 Published online by Cambridge University Press