Endomembrane-Associated Pre-embedding Immunogold Labeling

Zuzana Nichtova[1], Celia Fernandez[2], Bartok A[1], Sergio De La Fuente[1,2], Gyorgy Hajnoczky [1], Shey-Shing Sheu[2], Gyorgy Csordas[1]

[1]Dept of Pathology, Anatomy & Cell Biology, TJU, Philadelphia, PA, USA, [2]Dept of Medicine, TJU, Philadelphia, PA, USA

Introduction

Transmission electron microscopy + immunogold labeling (IG) represents a unique technique allowing to study ultrastructure of the cells and related topology of proteins of interest. Visualization of specific proteins using electron-dense markers, colloidal gold or smaller Nanogold® particles, conjugated to secondary antibody helps better understanding of the correlation of cellular processes with protein localization. IG can be applied pre-and post-embedding. Post-embedding IG is applied on ultrathin resin-embedded sections with good ultrastructural preservation; however, the resin itself limits the penetration of antibodies, resulting in high failure rate in the visualization of native endogenously expressed proteins. Pre-embedding IG, is applied on whole cells that are permeabilized, providing effective antibody penetration and epitope labeling throughout the cell, but membrane ultrastructure can be compromised.

Aim

To optimize the pre-embedding IG in stable cell lines and primary adult cardiomyocytes to get the most effective visualization of endomembrane/associated proteins: endo/sarcoplasmic reticulum Ca^{2+} release channels IP_3 receptors and ryanodine receptors (IP3R, and RyR2) and the mitochondrial fission protein Drp1.

Methods

2 plated cell types were used:

- DT40 (chicken B lymphocyte cell line) with all three IP3R isoforms ablated (TKO) and rescued by transient transfection with the rat type 3 IP3R-Flag (Fig. 1-3) for visualization IP3 receptors
- primary mouse ventricular myocytes
  - from wild type (C57BL/6) mice (WT) for in situ visualization of RyR2
  - mice with tamoxifen-inducible (MerCreMer) cardiomycyte-specific ablation of Drp1 and their control to visualize Drp1 (re)distribution. To this end, cardiomyocytes were electrically paced under stimulation by the beta adrenergic agonist isoproterenol or without isoproterenol and extracellular calcium.

Cells were first fixed with 5 % paraformaldehyde followed by quenching with glycine, permeabilization, blocking, primary and secondary antibody incubation steps and finally silver or gold enhancement of the nanogold particles as shown in the table below. To optimize labeling we varied the following parameters:

- to reduce the background labeling to minimum, we used different blocking and dilution buffers (goat serum, GS vs. bovine serum albumin BSA) in variable concentration
- to get the best signal for labeling of required proteins, we used different kind of secondary antibodies with variable dilutions
- to get optimal size of immunogold particles, we used different enhancements (silver vs. gold) with different development timing

The conditions, which provided the best IG labeling, are highlighted in red rectangles.

Results

IP3R distribution in chicken DT40 cell lines deficient in all 3 IP3R isoforms and rescued by mammalian IP3R

- **DT40 IP3RTKO cell transfected with OMM-YFP: Anti-GFP**

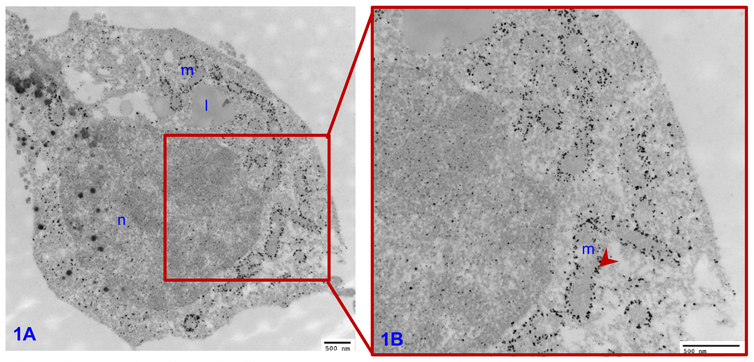
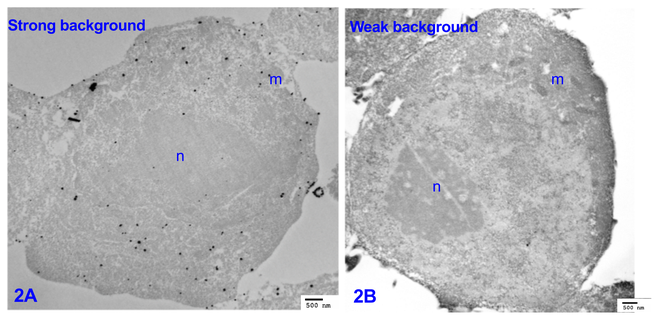
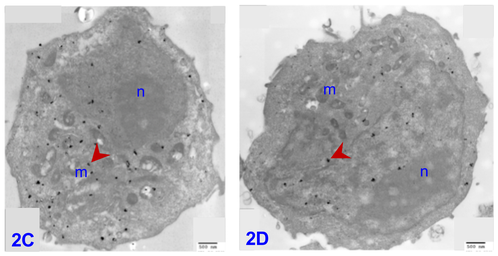


Fig. 1 Transfected DT40 TKO cell overview (A) and detail of the area in the red rectangle (B). The outer mitochondrial membrane-targeted YFP is labeled with anti-GFP and the IG particles decorate the periphery of mitochondrial cross-sections. m – mitochondria, l – lipid drop, n – nucleus, arrowheads – immunogold particles.

- **IP3RTKO: AntiFlag**



- **IP3RTKO rescued with rIP3R3-FLAG : Anti-FLAG**



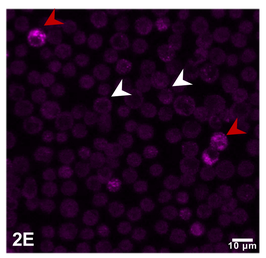


Fig. 2: IP3RTKO DT40 cells (A, B) and TKO cells rescued with FLAG-tagged rat IP3R3 (rIP3R3-FLAG) (C, D, E) after using antiFlag primary antibody. There is cell-to-cell variance in background labeling. **A** – TKO cell with strong background labeling, **B** – TKO cell with no/minimal background labeling.

C,D rIP3R3-FLAG rescues – representative cells with stronger (**C**) and weaker (**D**) anti-Flag IG labeling (red arrowheads). Note that nucleoplasmic location is not expected for IP3R. **E** – Anti-Flag immunofluorescence visualized via confocal microscopy. Note that most cells are not (white arrowheads) or just faintly labeled and only a few cells out of >100 on the representative field show strong labeling (red arrowheads). For TEM technique to find such cells with good expression is much more difficult (CLEM would be needed). Limitations of TEM in these cases is the size of grids used for TEM (<100 cells per grid), and also the thickness of the cell segment imaged is much smaller in TEM (~1 µm vs. 65 nm in confocal microscopy vs. TEM, respectively).

Native ryanodine receptors (RyR2) in primary mouse cardiomyocytes

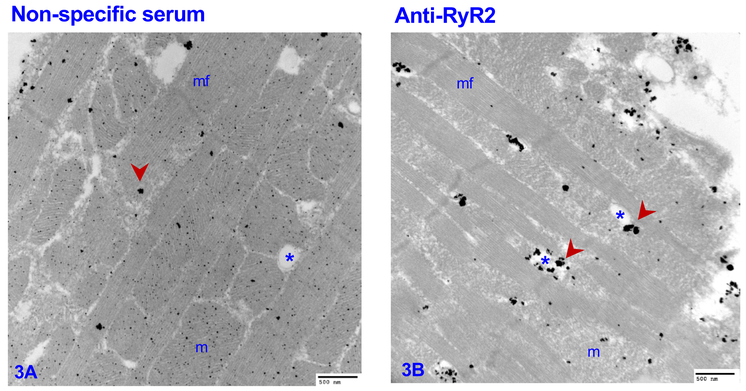
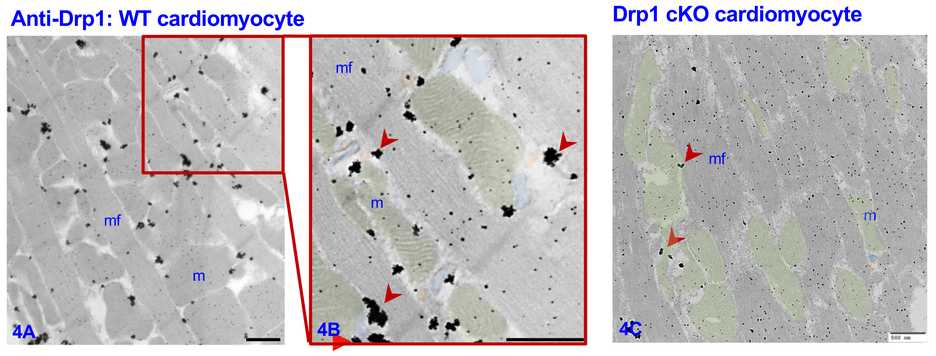


Fig. 3: Longitudinal sections of adult primary mouse ventricular cardiomyocytes. **A** – cardiomyocyte labeled without using primary antibody - only few randomly distributed gold particles (red arrowheads). **B** – cardiomyocyte labeled with anti-RyR2 primary antibody (red arrowheads) concentrated predominantly in dyadic microdomains (junctions of t-tubules and SR terminal cisternae, *); m – mitochondria, mf – myofibrils.

Mitochondrial fission protein Drp1 in primary mouse cardiomyocytes



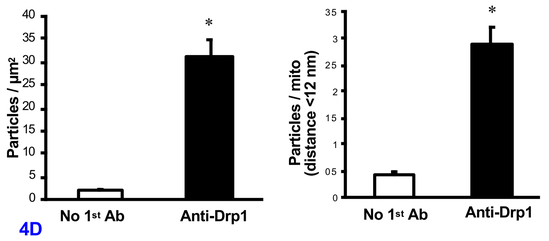


Fig. 4: **A** - Anti-Drp1 antibody labeling distribution in control cardiomyocyte; **B** – detail from red square in 'A'. Gold particles are predominantly localized in close proximity to mitochondria (green shade) and dyadic microdomains formed by T-tubule (blue shade) and junctional sarcoplasmic reticulum (orange shade). **C** - Anti-Drp1 antibody labeling in Drp1-KO cardiomyocyte. Note the presence of only few gold particles (red arrowhead). **D** - Summarized data showing several fold more Drp1-positive particles and mitochondria-associated in samples with Drp1 antibody versus no-primary antibody controls in control cardiomyocytes.

Calcium signaling activity-dependent re-localization of Drp1 in WT cardiomyocytes

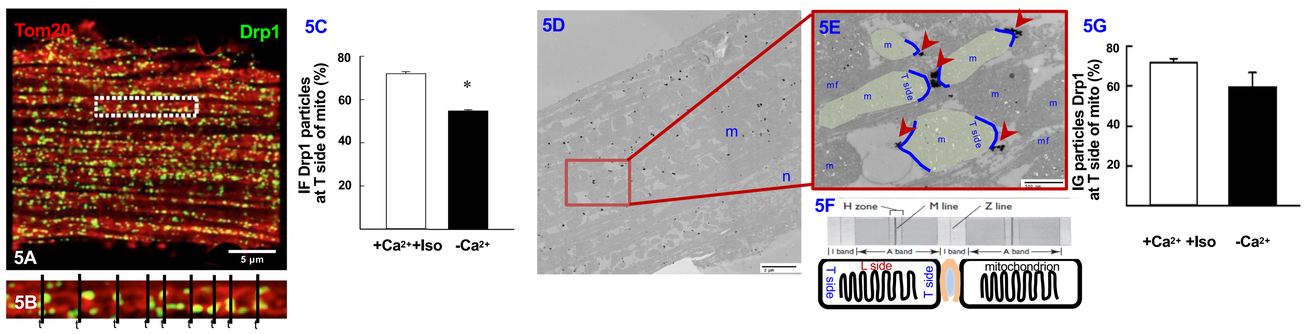


Fig. 5: Drp1 distribution in WT cardiomyocytes subjected to electric pacing in calcium-free extracellular medium (-Ca2+) or in the presence of calcium and isoproterenol (+Ca^{2+}, +Iso). **A** - Drp1 and Tom20 (OMM protein) immunofluorescence distribution in a cardiomyocyte that has been paced (2 Hz) in the presence of calcium (2 mM) and isoproterenol, visualized via super-resolution microscopy (Zeiss LSM880 Airyscan). **B** – Detail from A (dashed rectangle). The Tom20 (OMM) labeling shows mitochondria as ring-like structures. Note the predominant alignment of Drp1 labeling with the transversal side (t, black lines) of mitochondria. **C** – Quantitation of Drp1 immunofluorescence (IF) colocalization with the transversal side (quarter). Note the significant increase upon high calcium signaling activity (+Ca^{2+}, +Iso). (Means + S.E., n= 7 cells) **D** – TEM image of immunogold labeling of Drp1 in a paced cardiomyocyte (+Ca^{2+}, +Iso). **E** – Detail from D showing Drp1 IG particles localization. **F** - Schematics illustrating the transversal (T side) and longitudinal side (L side) of mitochondria in relation to myofibrils. **G** – Quantitative analysis of the fraction of Drp1 IG particles located at the T side (quarter) of the intermyofibrillar mitochondria. Note the overall high propensity of location at the T-side, but also the lack of difference between –Ca^{2+} and +Ca^{2+}, +Iso conditions. n = 4 cells/condition.

PERMEABILIZATION & 1st BLOCKING	1° ANTIBODY dilution buffer	PERMEABILIZATION & 2nd BLOCKING	2° ANTIBODY	2° ANTIBODY dilution buffer	ENHANCEMENT
5% GS + 0.1% saponin	1% GS	1% GS	Nanogold® (Nanoprobes)	1% GS	HQ Silver (Nanoprobes)
5% GS + no saponin	1% BSA + 0.1% Triton-X		1:50, 1:100		
2.5% GS + 0.1% saponin		5% GS	FluoroNanogold™	1% GS	GoldEnhance™ (Nanoprobes)
3% BSA + 0.2% Triton-X			1:50, 1:100 1:200	1% GS+5% non-fat dried milk	(3,5,7,10,12 minutes)
				1% BSA	
				1% non-fat dried milk	
				1% GS + 0.1 % Triton-X	

Conclusions

- In situ TEM visualization of proteins via immunogold is an important tool but prone to various false positive reactions that need to be carefully controlled and/or corrected for.
- To suppress non-specific binding of the primary antibodies in both cell types, the best results were reached with using combination of BSA and GS as blocking and antibody dilution buffers.
- For enhancing the 1.4 nm Nanogold conjugates we found the combination of FluroNanogold and Gold enhancement to be the most consistent and manageable with a developing time of 3 minutes.
- Besides non-specific protein binding, cross-reaction(s) by the primary antibody need to be carefully tested on a knock-out model if available (e.g. IP3RTKO in DT40 cells and Drp1 cKO in the cardiomyocytes). model with overexpression of protein (GFP in DT40 cells) or well known localized protein (RyR2 in cardiomyocytes localized in dyadic microdomains).
- Because of the unavoidable occurrence of the false positive background reactions and in the lack of an intensity threshold that can be applied in the case of immunofluorescence, qualitative changes have to be confirmed with quantitative (corrective) analysis.

Acknowledgements: Grant support: R01 HL122124 to GC and SSS, R01 HL137266 to SSS