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Dissecting the Connexin43 Vesicular Transport Pathway by Super-Resolution Microscopy

Michael J. Zeitz[1], Carissa C. James[1,2], and James W. Smyth[1,3]

[1.] Virginia Tech Carilion Research Institute and School of Medicine, Roanoke, Virginia, USA.
[2.] Graduate Program in Translational Biology, Medicine, and Health, Virginia Tech, Blacksburg, Virginia, USA.
[3.] Virginia Polytechnic Institute and State University, Department of Biological Sciences, Blacksburg, Virginia, USA.

Gap junctions comprised of the protein connexin43 (Cx43, gene name *GJA1*) facilitate the rapid propagation of action potentials through the working myocardium that is necessary for each heartbeat [1]. Altered localization of Cx43 occurs in most forms of heart disease and underlies the electrical disturbances responsible for arrhythmogenesis and sudden cardiac death. Cx43 experiences a relatively short half-life of under 3 hours. This rapid turnover has exposed the need for understanding regulation of the intracellular vesicular transport pathway as a critical component of pathological gap junction remodeling [2]. Six Cx43 protamers oligomerize intracellularly at the trans-Golgi network (TGN) to form connexon hemichannels [3, 4]. Cx43 hemichannels are then transported to the cell surface via the cytoskeleton where they form continuous intercellular conduits upon coupling with connexons on apposing cells [5, 6]. Oligomerization represents a key regulatory step in the lifecycle of ion channels yet many of the factors influencing the temporal and spatial formation of Cx43 hemichannels remain unknown.

Recently, it was reported that *GJA1* mRNA is subject to translation initiation events within its coding sequence, resulting in the generation of N-terminally truncated Cx43 isoforms [7]. The most predominantly expressed of these, GJA1-20k, was localized to the endoplasmic reticulum (ER)/Golgi apparatus and was found to positively regulate full-length Cx43 (GJA1-43k) trafficking and gap junction formation. We hypothesize that GJA1-20k facilitates GJA1-43k trafficking through the ER/Golgi prior to releasing the full-length protein for hemichannel assembly at the TGN. Dissection of the various compartments within the vesicular transport pathway is challenging biochemically and conventional microscopy lacks the resolution to address such questions. We utilize stochastic optical reconstruction microscopy (STORM) in fixed cells and photoactivated localization microscopy (PALM) to ask where GJA1-20k and GJA1-43k interact and how manipulation of GJA1-20k expression alters Cx43 gap junction formation.

Cells devoid of endogenous Cx43 expression were transfected with constructs encoding *GJA1* (expressing both GJA1-20k and GJA1-43k) or constructs encoding just GJA1-20k alone. Following fixation 16 hours post transfection, cells were labeled using antibodies directed against C-terminus of Cx43 together with markers of specific vesicular transport pathway compartments. Secondary detection was performed using the fluorophores AlexaFluor647 and AlexaFluor750 for STORM imaging. We find both GJA1-43k and GJA1-20k clustering with the endoplasmic reticulum resident protein disulfide isomerase (PDI) enzyme (Figure1A). Upon turning to the TGN however, and consistent with our hypothesis, GJA1-20k alone displays significantly lower clustering with the marker TGN38 in comparison to GJA1-43k (Figure 1B, n=3). These data are complemented by studies utilizing Cx43 fusion proteins to the photoconvertable fluorophore mEOS3.2 for PALM imaging at the single-molecule level. Through genetic mutation of *GJA1* we can limit GJA1-20k expression and subsequently analyze effects on Cx43 trafficking in real time

(Figure 2). Taken together, these data indicate that interaction between GJA1-20k and GJA1-43 is restricted to earlier compartments of the vesicular transport pathway. For the first time, we can dissect the special and temporal details of Cx43 isoform interaction at 20 nm resolution. It is our hope that these studies will provide mechanistic insight necessary to develop therapeutic interventions harnessing GJA1-20k expression and restoring normal electrical coupling to diseased hearts.

References:

[1] Gros, D.B and H.J. Jongsma, Bioessays **18** (1996), p. 719-30.
[2] Smyth, J.W., *et al*, J Clin Invest **120** (2010), p. 266-79.
[3] Das Sarma, J., F. Wang, and M. Koval, J Biol Chem **227** (2002), p. 20911-8.
[4] Musil, L.S. and D.A. Goodenough, Cell **74** (1993), p. 1065-77.
[5] Smyth, J.W., *et al*, Circ Res **110** (2012), p. 278-89.
[6] Shaw, R.M., and Fay A.J., *et al*, Cell **128** (2007), p. 547-60.
[7] Smyth J.W., and R.M. Shaw, Cell Reports **5** (2013), p. 611-8.

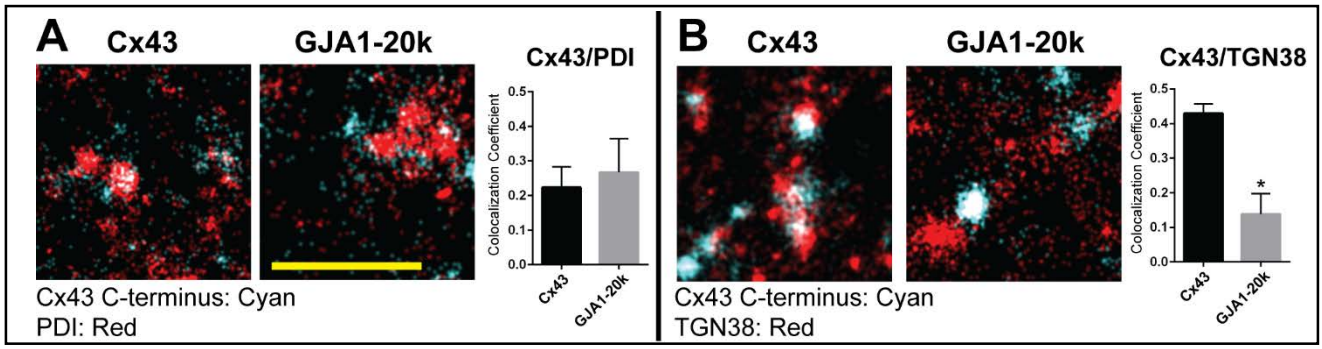

Figure 1. STORM of Cx43 isoforms together with vesicular transport pathway markers. Cells expressing GJA1-43k and GJA1-20k (Cx43) or GJA1-20k alone labeled for the Cx43 C-terminus (cyan) and with PDI (A, red) or TGN38 (B, red). Scale bar 1.5 μm.

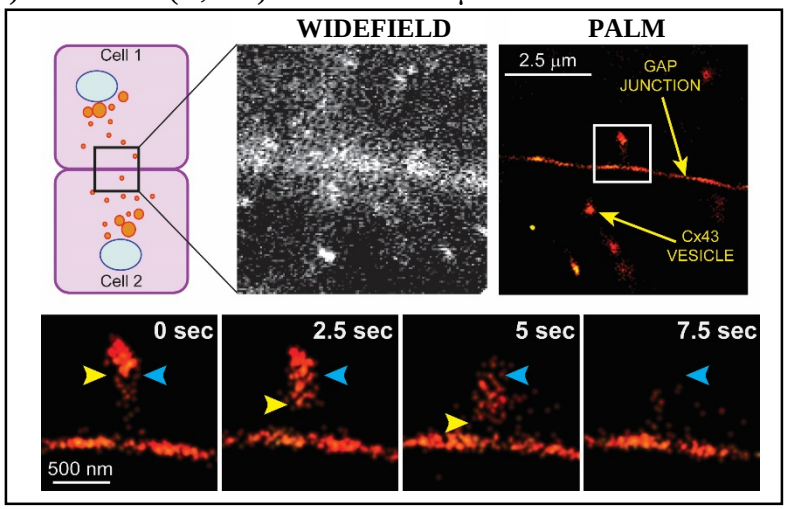

Figure 2. Real-time visualization of Cx43 vesicular transport and fusion using PALM. Cells expressing Cx43-mEos3.2 were subjected to photoconversion at 405 nm and activated mEos3.2 detected using 561 nm excitation. Top panels illustrate resolution of widefield and PALM data. Bottom panels are compiled localization data from PALM at 2.5 sec intervals during a vesicular fusion event at the cell-cell border. Scale bars 2.5 μm (top panels), 500 nm (bottom panels).

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