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Indirect Correlative Light and Electron Microscopy (*iCLEM*) Coupled with Computational Modeling Reveals the Nanoscale Basis of Functional Heterogeneities within the Heart

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Purpose of Study: During each heartbeat, electrical impulses flow through the heart to precisely synchronize billions of cardiac muscle cells. These signals propagate much faster in atrial myocardium than in ventricular myocardium and this has been attributed to micro- through millimeter scale structural heterogeneities. However, this view has not well explained differences in the two chambers' susceptibility to arrhythmia (rhythm disturbances). Previous studies from us [1, 2] and others [3, 4] suggest that these functional heterogeneities are determined by nanoscale organization of specialized structures that connect cardiac muscle cells, known as intercalated discs (ID). The ID is a complex, heterogeneous structure that affords electrical (gap junctions; GJ) and mechanical (adherence junctions [AJ], desmosomes [Des]) coupling between cardiomyocytes. Electrogenic proteins underlying the action potential upstroke (cardiac sodium channels [$Na_V1.5$], inward-rectifier potassium channels [$K_{ir}2.1$] and sodium potassium ATPase [NKA]) enriched within ID nanodomains are emerging as vital machinery for cardiac impulse propagation. ID structure is thus a critical determinant of cardiac conduction.

We undertook a comprehensive quantitative assessment of ID structure and molecular organization from the microscale to the nanoscale via a novel *indirect* correlative light and electron microscopy [*iCLEM*] approach. *iCLEM* affords a low-cost, high throughput option with extensive quantitative capabilities. This approach leverages the high imaging throughput of confocal microscopy, STORM single molecule localization microscopy, and transmission electron microscopy (TEM) and exploits structural fiducials / landmarks identifiable via both light and electron microscopy to correlate measurements. This approach is enabling us to systematically investigate the structural underpinnings of electrical propagation differences between atrial and ventricular myocardium in the healthy heart.

Research Methods: Murine hearts were isolated and preserved for assessment of ultrastructure (TEM) and molecular organization (STORM) as previously described [2, 5]. Paired sets of TEM images of the ID were obtained at 6,000x, 10,000x, and 20,000x magnification on a FEI Technai G2 Spirit transmission electron microscope. Morphometric quantification was performed using ImageJ. Stochastic optical reconstruction microscopy (STORM), a single molecule localization technique, was used to assess the spatial organization of landmark structural proteins - connexin43 for gap junctions, N-cadherin for adherens junctions, and desmoglein for desmosomes – as well as populations of key electrogenic proteins – specifically, the cardiac sodium channel 1.5 ($Na_V1.5$), the inward-rectifier potassium channel ($K_{ir}2.1$), and the sodium potassium ATPase (NKA) – in relation to the landmark

structural proteins. Spatial protein organization was quantified by STORM-based relative localization analysis (STORM-RLA[6]). Sub-diffraction confocal microscopy (sDCI) was used to assess the organization of the electrogenic proteins at the whole cell scale between ID and non-ID regions. Spatial protein organization was quantified by morphological object localization (MOL [7]; a distance transformation-based spatial analysis approach) for microscale information.

Findings: TEM uncovered structural differences from the micro- through nano-scales including key factors that may underlie faster atrial conduction: larger interplicate regions and more numerous GJs with associated perinexi. Confocal microscopy revealed ID enrichment of $Na_V1.5$, $K_{ir}2.1$ and NKA with more intense immunosignals in atrial myocytes than ventricular. STORM defined the $Na_V1.5$, $K_{ir}2.1$ and NKA distribution relative to AJ, Des, and GJ: In the ventricle, $Na_V1.5$ associated most closely with GJ (median intercluster distance: 117 nm), $K_{ir}2.1$ with Des (151 nm), and NKA with both GJ (165 nm) and AJ (150 nm). Next, percent of each electrogenic protein localized within 100 nm from ID junctions: 35% of $Na_V1.5$ around GJs, 49% of $K_{ir}2.1$ around Des and 33% and 39% of NKA near GJ and AJ respectively. Protein organization within atria ID had some notable differences: $Na_V1.5$, $K_{ir}2.1$ and NKA was shifted closer to GJs, $Na_V1.5$ to Des, and $K_{ir}2.1$ and NKA to Ncad.

Implications: In the broader context, this demonstrates the utility of the *iCLEM* approach in robust assessment of complex and subtle differences between biological samples. In the context of cardiology, these data provide the first ever comprehensive quantitative picture of ID ultrastructure and molecular organization. Functional implications of these nanoscale structural differences will be elucidated by implementation into our recently published 3D finite-element computational model. By incorporating such structural detail into computational models of electrical signal propagation, we are uncovering previously unappreciated structure-function relationships that determine the regularity of the heart's rhythm. These predictions, along with functional imaging studies of electrical signal spread in the heart, are providing the basis for the development of novel classes of anti-arrhythmic drugs.

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