# Nearest Neighbor-Based Spatial Analysis of Fluorescence Microscopy Data Reveals Increased Association of $Na_V1.6$ with Cardiac Dyads in Mouse Model of Type-2 Diabetes

Andrew M. Soltisz[1,2*], Heather Struckman[2], Przemysław Radwański[1-3] and Rengasayee Veeraraghavan[1,2]

[1.] Department of Biomedical Engineering, The Ohio State University, Columbus, OH, United States.
[2.] Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH, United States.
[3.] Division of Pharmacy Practice, College of Pharmacy, The Ohio State University, Columbus, OH, United States.
* Corresponding author: soltisz.5@osu.edu

Diabetes affects more than 6% of the world population and can lead to diabetic cardiomyopathy (DCM), a condition characterized by heart failure (HF), increased arrhythmia burden, and eventually, sudden cardiac death [1]. Cardiomyocytes under DCM exhibit severe calcium ($Ca^{2+}$) mishandling, as evidenced by increased sarcoplasmic reticulum (SR) $Ca^{2+}$ sparking [2]. However, little is known of the structural remodeling which underpins this physiology. Previous studies in diabetic mice have linked pathological increases in late sodium ($Na^+$) current, mediated by the voltage-gated $Na^+$ channel subtype 1.6 ($Na_V1.6$), to arrhythmogenic $Ca^{2+}$ release events [3], as well as increased reverse current of the sodium-calcium exchanger (NCX) [4]. We hypothesize that, in DCM, $Na_V1.6$ channels relocalize at dyads and cause abnormally high $Na^+$ intracellular concentrations near dyadic NCX, leading to excessive reverse NCX current near ryanodine receptors (RyR2) and subsequent SR $Ca^{2+}$ misfiring. Here, we aim to assess this hypothesis by determining if the the spatial association of $Na_V1.6$ relative to dyads is altered in diabetic mice.

Confocal microscopy was used to examine ventricular tissue samples from wild-type (WT, $n = 6$) and 36-week-old type-2 diabetic mice (db/db, $n = 4$), induced by homozygous mutation of the db/db gene, which were immunofluorescently labeled for $Na_V1.6$ and the dyad marker, RyR2. The spatial association of the two signals were assessed from the resulting images by comparing their observed and random nearest neighbor distance distributions [5-7]. Briefly, each protein's image signal was segmented and localized to the nearest voxel of the other protein (**Fig1**). The same signals were then randomized to simulate a distribution with no spatial relationship. The observed and random distributions were then compared, where their significant difference indicates non-random spatial association of the proteins (**Fig2**).

Spatial analysis indicated that both mice genotypes exhibit statistically significant attraction between $Na_V1.6$ and RyR2 (**Fig1**), however, the diabetic mice exhibited an increased association compared to WT. Additionally, the distance of significant attraction is reduced in diabetic mice (**Fig2**).

Despite the low sample size, $Na_V1.6$ is significantly more colocalized with RyR2, and therefor cardiac dyads, in diabetic mice, thus suggesting a potential mechanism for arrhythmogenesis. Future work aims to elaborate on these findings by structurally investigating NCX and L-type $Ca^{2+}$ channels, as well as corroboration through electrophysiological studies of $Na^+$ and $Ca^{2+}$ currents.

CrossMark
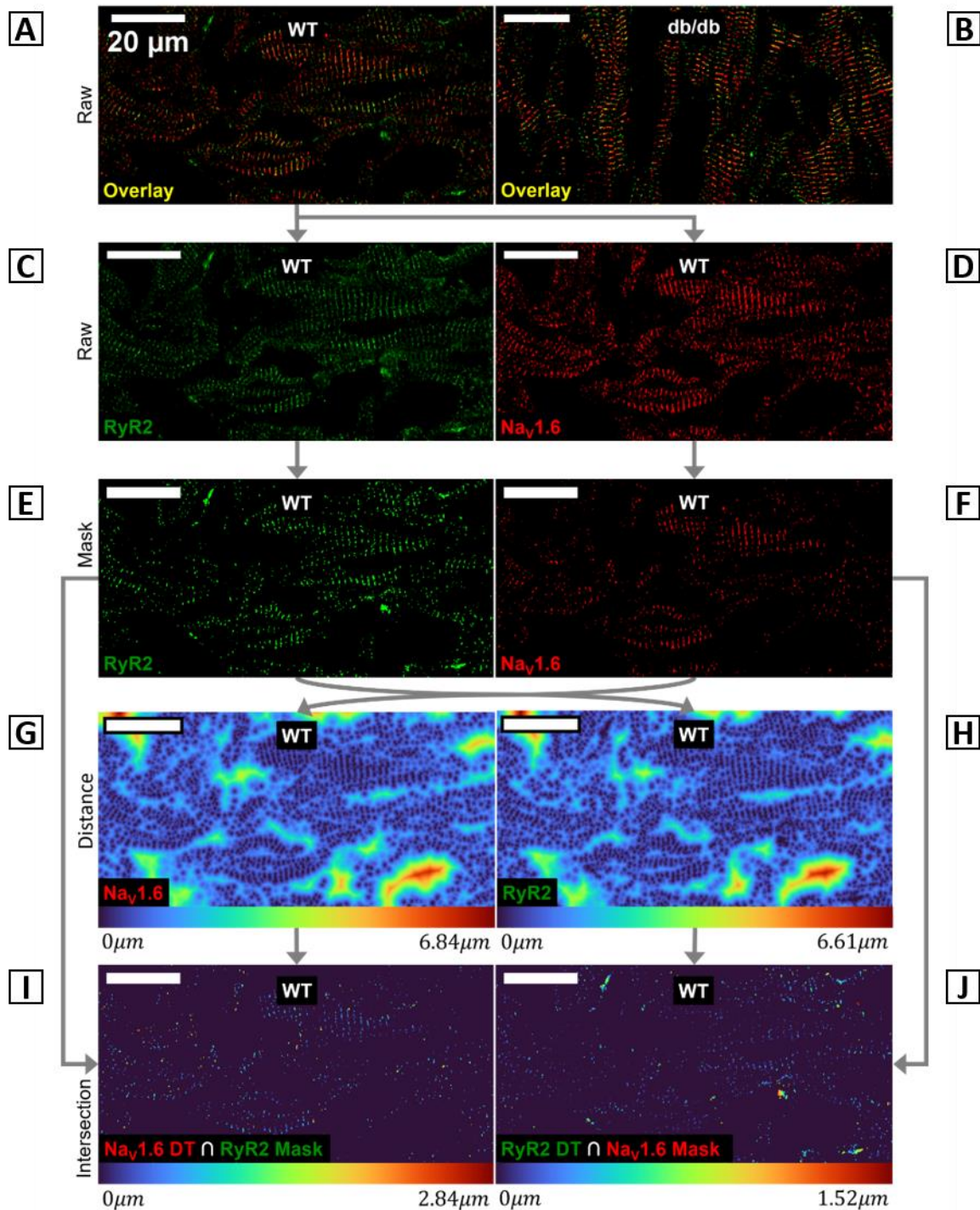
**Figure 1.** <u>Measuring Nearest Neighbor Distances</u>: RyR2 and Na$_V$1.6 are immunofluorescently labeled and imaged in ventricle sections from both WT and db/db mice (A-B). Proteins are then segmented from their parent image channel (C-D) using intensity-based thresholding to create masks which identify positive signal (E-F). The distance transformation (DT) of each mask is computed which generates a new image indicating the distance of each pixel to the nearest protein-positive pixel from its parent mask (G-H). Nearest neighbor distances between protein signals are measured as the intersection of one protein's mask with another's DT.
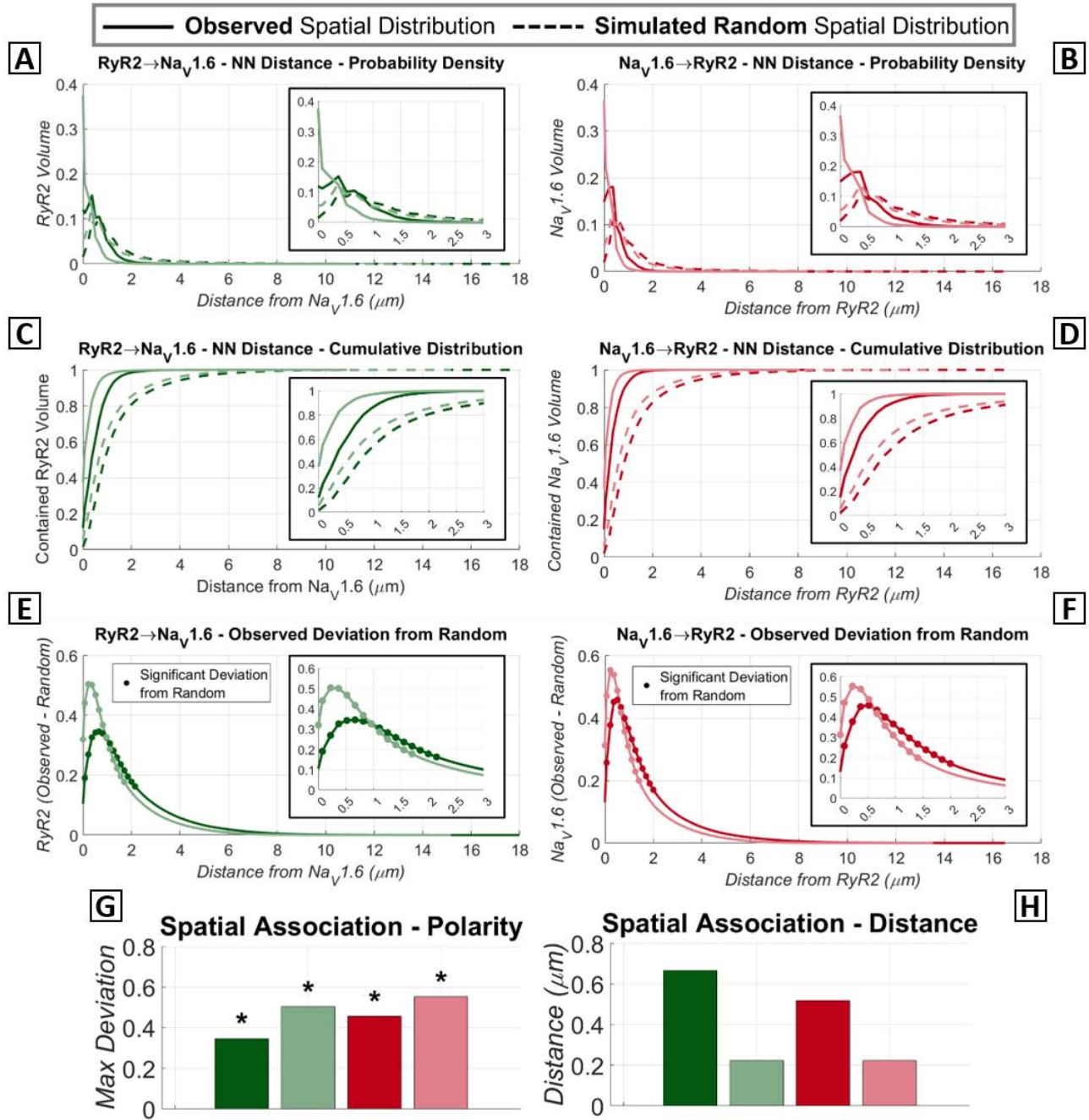
**Figure 2.** <u>Spatial Analysis Results</u>: Nearest neighbor distances from mask-DT intersections represent the observed spatial distribution (solid lines) between two signals while all distances from a DT represent the random distribution (dashed lines) of a signal. The empirical probability density function of these distances (A-B) is cumulatively summed to produce their empirical cumulative distribution function (CDF) (C-D). Deviation of the observed CDFs from their random counterparts indicate non-random spatial distributions (E-F). Statistically significant deviation is determined using the two-sampled Kolmogorov-Smirnov test, where the sign of the test statistic, the maximum deviation between CDFs (G), indicates repulsion (negative), attraction (positive), or no relationship (zero) of the two signals. The x-intercept of the maximum deviation indicates the distance at which non-random spatial association occurs (H).

References:

[1] LA Grisanti, Frontiers in Physiology **9** (2018), p. 1. doi:10.3389/fphys.2018.01669

[2] SM Zhao et al., Cardiovascular Diabetology **13**(1) (2014), p. 1. doi:10.1186/1475-2840-13-75

[3] X Jin et al., European Journal of Pharmacology **857** (2019), p. 172444.
doi:10.1016/j.ejphar.2019.172444

[4] S Litwin et al., Ann. N.Y. Acad. Sci. **779** (1996), p. 451. doi:10.1111/j.1749-6632.1996.tb44820.x

[5] PJ Diggle, Biometrics **35**(1) (1979), p. 87. doi:10.2307/2529938

[6] BD Ripley, Journal of the Royal Statistical Society: Series B (Methodological) **39**(2) (1977), p. 172.
doi:10.1111/j.2517-6161.1977.tb01615.x

[7] MNM van Lieshout and AJ Baddeley, Statistica Neerlandica **50** (1996), p. 344. doi:10.1111/j.1467-9574.1996.tb01501.x