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Supporting Material

Multi-Image Colocalization and its Statistical Significance

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Extending the voxel, Manders colocalization formulae

Voxel colocalization:

Assuming that we have three channels R, G and B, colocalization can be measured as follows: The number of above threshold voxels labeled with the red (n_R) and green (n_G) and blue (n_B) fluorophores, and having intensities I_R, I_G and I_B is given by:

$$n_R = \sum_k^{n_p} R_k\,; \quad n_G = \sum_k^{n_p} G_k\,; \quad n_B = \sum_k^{n_p} B_k$$

$$J_k = \begin{cases} 0, & I_J < J_{threshold} \\ 1, & I_J \geq J_{threshold} \end{cases}, \quad J = R, G, B$$

where n_p is the number of voxels within the ROI.

The number of colocalized voxels, $n_{RGB_{coloc}}$ containing above-threshold signal from all fluorophores is:

$$n_{RGB_{coloc}} = \sum_k^{n_p} R_k\ G_k\ B_k$$

The percentage of colocalized red voxels C_{RGB}, the percentage of green colocalized voxels C_{GBR}, and the percentage of blue colocalized voxels C_{BRG} are given by:

$$C_{RGB} = 100\,\frac{n_{RGB_{coloc}}}{n_R}; \quad C_{GBR} = 100\,\frac{n_{RGB_{coloc}}}{n_G}; \quad C_{BRG} = 100\,\frac{n_{RGB_{coloc}}}{n_B}$$

Colocalization between two of the three labels is measured as before:

$$n_{RG_{coloc}} = \sum_k^{n_p} R_k\ G_k\ ,\ B_k = 0; \quad C_{RG} = 100\,\frac{n_{RG_{coloc}}}{n_R}; \quad C_{GR} = 100\,\frac{n_{RG_{coloc}}}{n_G}$$

with similar formulae for C_{RB}, C_{BR}, C_{GB} & C_{BG}. Although nine different values can be derived from a voxel-based triple colocalization, there are only four independent measures: $n_{RGB_{coloc}}$, $n_{RG_{coloc}}$, $n_{RB_{coloc}}$ and $n_{GB_{coloc}}$. Note that when calculating the colocalization between two of the three colors we exclude the triple colocalizations. This metric can be extended to four fluorophores, resulting in one quadruple, three triple, and six double colocalizations with their various combinations.

Intensity-based colocalization

The only intensity based measure that can be easily translated to three or more fluorophores are the Manders' coefficients. In this case we sum the intensities of the colocalized voxels in each channel:

$$I_{RGB_{coloc}} = \sum_{k}^{n_p} I_R\, R_k\, G_k\, B_k\,; \quad I_{GRB_{coloc}} = \sum_{k}^{n_p} I_G\, R_k\, G_k\, B_k\,; \quad I_{BRG_{coloc}} = \sum_{k}^{n_p} I_B\, R_k\, G_k\, B_k$$

where:

$$J_k = \begin{cases} 0, & I_J < J_{threshold} \\ 1, & I_J \geq J_{threshold} \end{cases} \;,\; J = R, G, B$$

and n_p is the number of voxels within the ROI. The sum of the above-threshold voxels is given by:

$$I_{J_{tot}} = \sum_{k}^{n_p} I_J \;,\; J = R, G, B$$

to produce three Manders' coefficients:

$$M_{RGB} = \frac{I_{RGB_{coloc}}}{I_{R_{tot}}}; \qquad M_{GBR} = \frac{I_{GBR_{coloc}}}{I_{G_{tot}}}; \qquad M_{BRG} = \frac{I_{BRG_{coloc}}}{I_{B_{tot}}}$$

The intensities above can be written as the product of the number of voxels multiplied by their mean intensity, so:

$$M_{RGB} = \frac{n_{RGB_{coloc}}\, \overline{I_{RGB_{coloc}}}}{n_R\, \overline{I_R}} = \frac{C_{RGB}}{100}\, \frac{\overline{I_{RGB_{coloc}}}}{\overline{I_R}}$$

Similar equations can be written for the other coefficients. Clearly the Manders' coefficients will only differ from its voxel counterpart if the mean intensity of the colocalized voxels of a particular color differs from the mean intensity of all of the voxels of that color.

Dual colocalization with three colors is simply an extension of the original Manders (5) equations:

$$I_{RB_{coloc}} = \sum_{k}^{n_p} I_R B_k \ , \ G_k = 0 \ ; \qquad I_{BR_{coloc}} = \sum_{k}^{n_p} I_B R_k \ , \ G_k = 0$$

$$M_{RB} = \frac{I_{RB_{coloc}}}{I_{R_{tot}}} \ ; \qquad M_{BR} = \frac{I_{BR_{coloc}}}{I_{B_{tot}}}$$

There are similar equations for the other coefficients; M_{RG}, M_{GR}, M_{GB} and M_{BG}:

$$I_{RG_{coloc}} = \sum_{k}^{n_p} I_R G_k \ , \ B_k = 0 \ ; \qquad I_{GR_{coloc}} = \sum_{k}^{n_p} I_G R_k \ , \ B_k = 0$$

$$M_{RG} = \frac{I_{RG_{coloc}}}{I_{R_{tot}}} \ ; \qquad M_{GR} = \frac{I_{GR_{coloc}}}{I_{G_{tot}}}$$

$$I_{BG_{coloc}} = \sum_{k}^{n_p} I_B G_k \ , \ R_k = 0 \ ; \qquad I_{GB_{coloc}} = \sum_{k}^{n_p} I_G B_k \ , \ R_k = 0$$

$$M_{BG} = \frac{I_{BG_{coloc}}}{I_{B_{tot}}} \ ; \qquad M_{GB} = \frac{I_{GB_{coloc}}}{I_{G_{tot}}}$$

The nine Manders' coefficients generated by three-color colocalization are independent of one another.

Calculating the statistics for the Cluster Diameter and Inter-Cluster Distance

There are a number of ways to calculate statistics for this data: First by plotting the cumulative distribution function (CDF; Figs. 6 & S1). We plot the upper and lower simulation envelopes, which represent the minima and maxima of the simulated cluster diameters (dotted lines), as well as the data (solid line). If the data line goes outside the envelope then the cluster diameter is not CSR for those distances that are outside the envelope. Second, we can generate two statistics from the data: The median D_C from the measured data can be ranked on the distribution of the medians from the simulated data giving a p value. We can also define a statistic *u* to be the sum of squared differences between the observed CDF ($\hat{G}_i(D_C)$) and the mean of the CDF's from the simulations (9):

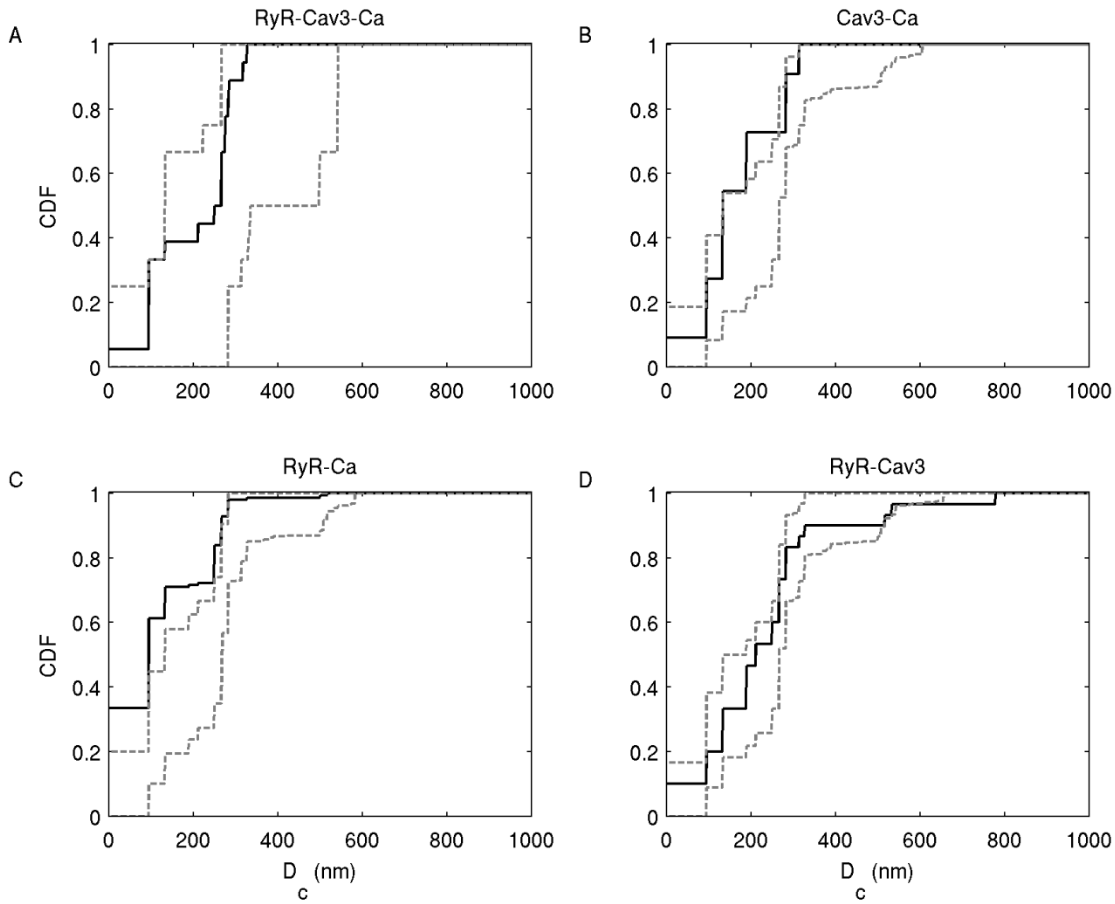
$$u_i = \int \left\{ \hat{G}_i(D_C) - \bar{G}_i(D_C) \right\}^2 dD_C \ , \ i = 1..n$$

where n is the number of simulations and

$$\bar{G}_i\,(D_C) = \frac{1}{(n-1)} \sum_{j \neq i} \hat{G}_j\,(D_C)$$

which generates a distribution $u_1 \ldots u_n$. We calculate the statistic u_o for the measured data using the mean of all the simulations and rank it in the distribution, yielding a p value.

Fig. S1



Cumulative distribution functions for the diameter of the colocalized clusters. The black solid line represents the measured data and the gray dotted lines are the 95% limits of the simulated distribution functions. Solid lines that fall outside these limits are not spatially random. Shown are A) RyR-Cav3-Ca_v1.2; B) Cav3-Ca_v1.2; C) RyR-Ca_v1.2; D) RyR-Cav3.