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CIRCOAST: A Statistical Hypothesis Test for Cellular Colocalization with Network Structures

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Abstract

Motivation: Colocalization of structures in biomedical images can lead to insights into biological behaviors. One class of colocalization problems is examining an annular structure (disk-shaped such as a cell, vesicle, or molecule) interacting with a network structure (vascular, neuronal, cytoskeletal, organelar). Examining colocalization events across conditions is often complicated by changes in density of both structure types, confounding traditional statistical approaches since colocalization cannot be normalized to the density of both structure types simultaneously. We have developed a technique to measure colocalization independent of structure density and applied it to characterizing intercellular colocation with blood vessel networks. This technique could be used to analyze colocalization of any annular structure with an arbitrarily shaped network structure.

Results: We present the circular colocalization affinity with network structures test (CIRCOAST), a novel statistical hypothesis test to probe for enriched network colocalization in 2D z-projected multichannel images by using agent-based Monte Carlo modeling and image processing to generate the pseudo-null distribution of random cell placement unique to each image. This hypothesis test was validated by confirming that adipose-derived stem cells (ASCs) exhibit enriched colocalization with endothelial cells forming arborized networks in culture and then applied to show that locally-delivered ASCs have enriched colocalization with murine retinal microvasculature in a model of diabetic retinopathy. We demonstrate that the CIRCOAST test provides superior power and type I error rates in characterizing intercellular colocalization compared to generic approaches that are confounded by changes in cell or vessel density.

Availability: CIRCOAST source code available at: https://github.com/uva-peirce-cottler-lab/ARCAS.

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Supplementary information: Supplementary data are available at Bioinformatics online.

1 Introduction

Interactions between vascular endothelial cells, which are arranged in arborized networks throughout all tissues of the body, and other cell types are instrumental in the initiation and perpetuation of a wide range of diseases, including diabetes mellitus (Ruggiero et al. 1997). Interacting cell types with vascular endothelial cells include immune cells (Gerhardt and Ley 2015), perivascular cells (Sheets et al. 2016; Motherwell et al. 2017), and stem cells (Amos et al. 2008). Modulating intercellular interactions associated with disease progression is seen as a therapeutic target for preventing or ameliorating the associated pathology (Gartner et al. 2018).
A key imaging-based measure of cell-cell interactions is intercellular colocalization, the frequency that two cell populations reside immediately adjacent to each other. Changes in intercellular colocalization suggest changes in cell-cell interactions and cellular behaviors that influence the interaction, including altered migrational capabilities, cytokine sensing, and other chemotactic behaviors. Most research in cellular colocalization has focused on intracellular interactions with point-based features, specifically whether two molecular probes codistribute (dispersed in a spatially related fashion) or associate with a particular organelle (Dunn et al. 2011). The statistics are often limited to a pixel-by-pixel analysis of correlation using Pearson’s Correlation Coefficient or Mander’s Overlap Coefficient (Zinchuk and Zinchuk 2008), or more advanced analysis techniques such as spatial point pattern analysis (Helmuth et al. 2010; Burguet and Andrey 2014) or protein-protein interaction models (Johnson et al. 2015). By contrast, there is a lack of statistical techniques to study cell-cell interactions (Payés et al. 2012) where point-based analysis is less pertinent.

### Table: Key Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ASC</td>
<td>Adipose-derived Stem Cell</td>
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<td>BMRP</td>
<td>Binomial Model of Random Placement</td>
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<tr>
<td>CDNF</td>
<td>Cell-Dilated Network Fraction</td>
</tr>
<tr>
<td>COI</td>
<td>Cell-Of-Interest</td>
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<tr>
<td>HMRP</td>
<td>Hypergeometric Model of Random Placement</td>
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<tr>
<td>CIRCOAST</td>
<td>Circular Coloc. Affinity with Structures Test</td>
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<td>ICA</td>
<td>Intercellular Colocalization Affinity</td>
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<tr>
<td>ICF</td>
<td>Intercellular Colocalization Fraction</td>
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<tr>
<td>MCMRP</td>
<td>Monte Carlo Model of Random Placement</td>
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</table>

Cell populations are known to change dramatically in disease (Kowluru and Chan 2008), which can confound metrics of colocalization. Intercellular colocalization events depend on the prevalence of the two interacting cell populations, and generic statistics cannot ascertain changes in colocalization because the data cannot be normalized to both cell populations simultaneously. This is especially problematic when there are substantial changes in vascular or cellular density between study groups or high variance between biological replicates. Here, we present an image analysis tool that statistically assesses intercellular colocalization independent of cell and network density by testing against a pseudo null distribution for random intercellular colocalization events unique to each image. By comparing the intercellular colocalization fraction (ICF), the fraction of cells colocalizing with network structures, between an experiment image compared to the distribution of ICF values derived from modeling random cell placement in the same image, changes in colocalization can be ascertained relative to random behavior. Using additional statistics to combine data across images from a single biological replicate and compare between study groups yields a process that can characterize changes in intercellular colocalization affinity (ICA), which we define as the frequency of colocalization events between two cell populations corrected for changes in cell density, cell size, and network density across study groups.

An example where large changes in cellular density are observed is diabetic retinopathy, a disease that is marked by progressive damage to the retina (Wong et al. 2016). Decreases in the densities of both blood vessels (Kowluru and Chan 2008) and pericytes (Ejaz et al. 2008), a cell type that colocalizes with and stabilizes the microvasculature, have been observed in early diabetes and are thought to initiate the degradation of the retina (Belbramo and Porta 2013). Toward cell-based therapies, previous work has shown that injecting adipose-derived stem cells (ASCs) can ameliorate microvessel loss when ASCs colocalize with blood vessels and adopt a pericyte-like morphology in the retina and other tissues (Amos et al. 2008; Mendel et al. 2013). However, it was difficult to conclude whether or not ASC colocalization with the retinal microvasculature occurred at a rate greater than random chance without a validated statistical method.

We developed and validated CIRCOAST as a tool to measure intercellular colocalization by testing for a known enriched colocalization between ASCs and the arborized networks that endothelial cells form in culture. Then, we applied CIRCOAST to determine that locally delivered ASCs significantly colocalize with the retinal microvasculature in a murine model of diabetic retinopathy. By providing a robust method for evaluating the statistical significance of cell-cell colocalization, CIRCOAST provides insight into putative mechanisms of and potential therapies for a wide range of pathologies. This method naturally extends to the colocalization analysis of any annular shaped structure (disk-shaped such as a cell, vesicle, or molecule) with any arbitrary background network structure within tissues or cells.

## 2 Methods

### 2.1 Codebase

CIRCOAST was written in MATLAB 2016b using the image processing toolbox and can be run either as source code or as compiled code with the MATLAB runtime environment version 9.1. The source code and compiled executable are available as a part of the Automated Random Cell Association Simulator (ARCAS) code repository (https://github.com/uva-peirce-cottler-lab/ARCAS). The user interface is designed with MATLAB’s graphical user interface development environment (guide), which allows the user to analyze a dataset of two or more color images, one marking the vasculature, and the others marking one or more cell types to be examined individually for enriched vascular colocalization. In this study, a confocal microscope was used to acquire a z-stack at approximately Nyquist sampling. The 3D images were then flattened with a max projection in the z axis dimension to produce a 2D RGB image.

![CIRCOAST GUI](image)

**Fig. 1. CIRCOAST GUI for Analyzing Cellular Colocalization.** (A) GUI for CIRCOAST that imports a thresholded vasculature and predicts the random cell colocalization fraction (ICF) (B), through a series of trials from a Monte Carlo Model of Random Placement (MCMRP).

### 2.2 Monte Carlo Model Development (MCMRP)

An initial Monte Carlo model of Random Placement (MCMRP) was created to simulate randomly placed cells. An input image of the network is imported into the CIRCOAST GUI, and segmented via an adjustable global threshold (Figure 1A). Image resolution, cell size, and number of
cells are set by the user, and a series of Monte Carlo simulations are performed with an agent-based model that stochastically spawns cells in an image, in which under the random cell placement paradigm, every location within the input image region has an equal chance of being selected as a site of cell placement; and once placed, the fraction of cells overlapping with the vasculature network is calculated (Figure 1A, intercellular colocalization fraction, ICF). A probability distribution for the ICF in a given image is approximated based on the thousands of Monte Carlo simulation repetitions of the random cell placement process (Detailed outline of algorithm in Supplementary Note 1).

Probing of the key parameters that influence cellular colocalization with the vasculature was undertaken via a simple program created to stochastically create a network structure resembling blood vessels in a controlled manner (Supplementary Figure 1). Dense microvascular networks have been described as having structural characteristics of interconnected wires (Longden et al. 2017). The stochastically-generated networks were created by defining a randomly seeded point cloud with an enforced minimum distance between points, and using the watershed algorithm to create a network of line segments bisecting all points. Line segments were then iteratively removed until the desired vessel network was obtained, mimicking the range of vessel density found in vascularized tissues (Extended explanation of algorithm in Supplementary Note 2).

A set of parameters defining the network structure and the cell of interest (COI) were identified (Supplementary Figure 2), including: 1) network fraction: the fraction of pixels in an image that comprise the network, 2) network length density: the length of the centerline for all network structures divided by the area of the image, 3) network radius: thickness of network orthogonal to the network centerline, 4) cell number, 5) cell diameter, and 6) cell dilated network fraction (CDNF). CDNF defines the area of the image where if the center of a COI is within that area, the COI overlaps with the network by at least one pixel and is counted as being a colocalized cell, which is captured by morphologically dilating the segmented network with the length of the radius of the COI. Therefore, with a fixed network structure, as cell diameter increases so will the value for CDNF.

The relation between each system parameter and the ICF predicted by the MCMRP was examined over a wide range of parameter values (Supplementary Figure 3). All parameters correlated with the MCMRP-derived mean ICF; except for cell number, suggesting many variables influence mean of ICF distribution under the random cell placement paradigm, but give little insight to what parameter(s) directly dictate network colocalization.

Figure 2. Network area fraction dilated by cell radius determines the random cell colocalization fraction. The mean ICF was calculated with the MCMRP over 10,000 trials with randomly selected parameters and displayed as a function of (A) cell diameter, (B) cell number, (C) network radius, (D) network fraction, (E) network length density, and (F) cell dilated network fraction (CDNF, N=2,500 images). Pearson correlation coefficient and associated 95% confidence interval and p-value are provided at the top of each scatterplot.

To determine if any of the parameters can directly predict mean ICF under the random cell placement paradigm, a dataset of 2500 simulated experiments were generated using the vessel network generator with randomly assigned parameters. The means of ICF distributions derived from the 2500 experiments were correlated with the individual parameters (Figure 2). While most parameters correlated with mean ICF, only CDNF had a correlation coefficient (r) of 1 (rounded to within 6 decimal places), suggesting that CDNF correlates almost perfectly with the MCMRP-derived mean ICF (Figure 2F). Note that in Fig. 2F; that for all intended purposes, the relationship between the CDNF values and mean ICF values is deterministic (points fall on a 45° line).

To further examine if CDNF is a unique predictor of ICF, a multivariable linear regression (MVLR) analysis was conducted with all input parameters as predictor variables and mean ICF as the response variable. Input parameter values and mean ICF values were converted to z-scores so that all of the multivariate regression model coefficients shared the same scale of measure while still preserving the underlying multivariate relationships that exist on the non-z-score scale. All predictors had insignificant p-values except for CDNF (Table 1). Furthermore, since the regression coefficients of all predictors other than CDNF were essentially equal to zero, CDNF is the only input parameter that was given any weight in the MVLR in terms of predicting the mean ICF z-score. Given that the MVLR model multiple coefficient of determination (R²) was equal to 1, we conclude that CDNF is highly and uniquely correlated with the predicted mean ICF of the MCMRP. Although the ICF in given MCMRP trial can differ from CDNF due to stochasticity, and the ICF from an acquired image may differ from CDNF from stochasticity or nonrandom cell placement, the CDNF can be used to calculate the mean ICF from random behavior in both cases.

Table 1. Multivariable regression of z-scores of input parameters versus the z-score of the ICF predicted by Monte Carlo Model of Random Placement (R² =1.00). Note that the z-score transformation preserves the underlying multivariate relationships.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>SE</th>
<th>F-statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-9.54E-16</td>
<td>1.20E-05</td>
<td></td>
<td></td>
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<tr>
<td>Cell Diameter</td>
<td>-1.96E-05</td>
<td>2.56E-05</td>
<td>-0.76</td>
<td>0.444</td>
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<tr>
<td>Cell Number</td>
<td>-5.57E-06</td>
<td>1.20E-05</td>
<td>-0.46</td>
<td>0.642</td>
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<tr>
<td>Network Rad.</td>
<td>-2.59E-05</td>
<td>4.34E-05</td>
<td>-0.60</td>
<td>0.551</td>
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<tr>
<td>Network Frac.</td>
<td>5.01E-06</td>
<td>5.74E-05</td>
<td>0.09</td>
<td>0.931</td>
</tr>
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<td>Network Len. Dens.</td>
<td>-3.24E-05</td>
<td>4.67E-05</td>
<td>-0.69</td>
<td>0.489</td>
</tr>
<tr>
<td>CDNF</td>
<td>1.000</td>
<td>5.00E-05</td>
<td>2.00E4</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Model</td>
<td>1.16E9</td>
<td>&lt;0.000</td>
<td></td>
<td></td>
</tr>
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2.3 Binomial Model Development and Validation (BMRP)

Based on the aforementioned Monte Carlo findings, CDNF was used to develop a binomial model of random placement (BMRP) for a more mechanistic and exact representation of cell colocalization under the random placement paradigm. By defining CDNF as the probability of success for a randomly placed cell colocalizing with the vasculature within an image, intercellular colocation can be modeled as a binomial stochastic process using Eq. 1 (Sokal and Rohlf 2012);
\[ f(c; n, p) = \binom{n}{c} p^c (1 - p)^{n-c}, \quad (1) \]

where \( p \) is the cell dilated network fraction (CDNF), \( c \) is the number of cells colocalizing, and \( n \) is the total number of cells in the image. The mean (\( \mu \)) and standard deviation (\( \sigma \)) of the distribution can be directly calculated using the formulas listed in Eq. 2 (Sokal and Rohlf 2012), as opposed to approximating the values based on successive MCMRP trials.

\[
\mu = n \times p, \quad \sigma = \sqrt{n \times p \times (1 - p)} \quad (2)
\]

To evaluate the BMRP, the predicted mean ICF from random cell placement was compared to the MCMRP predicted mean ICF (10,000 predicted mean ICFs of the BMRP and the MCMRP (\( p=0.850, \alpha=0.05 \)).

A paired two-tailed Student’s t-test revealed no difference between the predicted mean ICFs of the BMPR and the MCMRP (\( p=0.850, \alpha=0.05 \)). Furthermore, no systematic relationship could be detected between the discrepancy between the predicted mean ICFs of the BMPR and the MCMRP as a function of the mean ICF values, with the difference in paired values plotted against the average. Note that the blue horizontal line identifies the mean discrepancy between the 2500 pairs of BMPR and MCMRP predicted mean ICF values (mean: 1.05E-7), and the green horizontal lines identify the lower and upper 95% confidence limits [-0.00025, 0.00026] for the discrepancy between any pair of BMPR and MCMRP mean ICF values.

A paired two-tailed Student’s t-test revealed no difference between the predicted mean ICFs of the BMPR and the MCMRP (\( p=0.850, \alpha=0.05 \)). Furthermore, no systematic relationship could be detected between the discrepancy between the predicted mean ICFs of the BMPR and the MCMRP as a function of the mean ICF values, with the difference in paired values plotted against the average. Note that the blue horizontal line identifies the mean discrepancy between the 2500 pairs of BMPR and MCMRP predicted mean ICF values (mean: 1.05E-7), and the green horizontal lines identify the lower and upper 95% confidence limits [-0.00025, 0.00026] for the discrepancy between any pair of BMPR and MCMRP mean ICF values.

2.4 Hypergeometric model and Validation (HMRP)

The binomial model assumes that the random placement of cells are completely independent events, that each successive COI placed in an image can be placed anywhere. However, in practice, COIs cannot overlap because they would be counted as a single cell. Thus, at higher cell densities, there are less locations for additional cells to be added and still counted as additional cells in the image, suggesting that placement of cells are related events and there exists a maximum number of placed cells, possibly suggesting that a hypergeometric model of random placement (HMRP) could be more suitable to model this process:

\[
h(k|N, n, K) = \binom{K}{c} \binom{N-K}{n-c} \binom{n}{k} \quad (3)
\]

Where \( k \) is the number of colocalizing cells, \( n \) is the total observed number of cells in the image, \( N \) is the max number of cells that can exist in the image, \( K \) is number of colocalizing cells of the max population of cells placed in the image. The mean (\( \mu \)) and standard deviation (\( \sigma \)) of the distribution can be directly calculated using the formulas listed in Equation 4 (Sokal and Rohlf 2012):

\[
u = \frac{n \times k}{N}, \quad \sigma = \sqrt{\frac{n \times k \times (N-k) \times (N-n)}{N^2 \times (N-1)}} \quad (4)
\]

Figure 3. Discrepancy between BMRP predicted mean ICF and MCMRP predicted mean ICF. Bland Altman plot of the 2500 pairs of BMRP and MCMRP predicted mean ICF values, with the difference in paired values plotted against the average. Note that the blue horizontal line identifies the mean discrepancy between the 2500 pairs of BMPR and MCMRP predicted mean ICF values (mean: 1.05E-7), and the green horizontal lines identify the lower and upper 95% confidence limits [-0.00025, 0.00026] for the discrepancy between any pair of BMPR and MCMRP mean ICF values.

Figure 4. Discrepancy between BMRP mean ICF and MCMRP mean ICF versus the cell and network input parameter values. (A-F) Relationship between each input parameter and the discrepancy between the BMRP predicted mean ICF values and the MCMRP predicted mean ICF values. Pearson correlation coefficient and associated 95% confidence interval and p-value are provided at the top of each scatterplot.

Table 2. Multivariable linear regression of z-scored input parameters versus the z-score of the difference in mean ICF predicted by MCMRP and BMPR (R² < 0.00).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>SE</th>
<th>F-statistic</th>
<th>P-value</th>
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<tr>
<td>Intercept</td>
<td>-0.013</td>
<td>0.017</td>
<td>0.968</td>
<td>0.352</td>
</tr>
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<td>Cell Diameter</td>
<td>-0.023</td>
<td>0.036</td>
<td>1.05</td>
<td>0.305</td>
</tr>
<tr>
<td>Cell Number</td>
<td>0.017</td>
<td>0.017</td>
<td>2.74</td>
<td>0.098</td>
</tr>
<tr>
<td>Network Rad.</td>
<td>-0.0001</td>
<td>0.060</td>
<td>1.55</td>
<td>0.213</td>
</tr>
<tr>
<td>Network Frac.</td>
<td>0.048</td>
<td>0.065</td>
<td>0.54</td>
<td>0.462</td>
</tr>
<tr>
<td>CDNF</td>
<td>-0.004</td>
<td>0.070</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>1.38</td>
<td>0.217</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Theoretically, the max number of non-overlapping cells that can be found in an image is defined by the hexagonal packing of circles, which previous research has shown to have a packing ratio of 0.901 (fraction of image area covered by circles), demonstrated to be invariant to the size of the circle and bounding area (Steinhaus 1999). Yet this packing assumes perfect placement of all circles. Randomly placed non-overlapping pixelated cells may have a much lower packing ratio than this scheme, and may not be invariant to cell size since the shape of a cell is approximated in a pixelated fashion. Moreover, cells must be fully contained in the image area to contribute to the packing ratio for possibly colocalizing cells; cells that exceed the border of the image are not counted because their colocalization state with the network outside of the image cannot be determined.

We designed a Monte Carlo model of random cell placement without replacement that iteratively places cells until no more can fit in the image and then calculates the final packing ratio. Doing this for a range of cell diameters reveals that the packing ratio of randomly placed fully contained non-overlapping pixelated circles changes with cell size (p=0, Kruskal Wallis, N=100 trials, Supplementary Figure 4A) and image dimensions (p=0, Kruskal Wallis, N=100 trials, Supplementary Figure 4B). For cell diameters less than 5 pixels, distinct cells cannot be discerned at the highest density, so experiment data with that low cellular resolution is considered invalid. For cell diameters above 5 pixels in diameter, a look-up table is provided (Supplementary Table 1) for 512 by 512 image dimensions and is used for calculating the N parameter in a hypergeometric distribution:

\[ N = \frac{\text{avg}}{r^2} \]  

(5)

Where \( A \) is the pixel area of the image, \( \eta \) is the packing ratio from the look up table, and \( r \) is the pixel radius of the cell (Equation 5). The CDNF of each image is used to approximate the number of cells colocalizing (\( k \)) from the max population of cells (\( N \)), since it represents the fraction of the image where colocalization occurs:

\[ k = Np \]  

(6)

Where \( p \) is the CDNF, used also in the binomial distribution from Equation 1.

The mean ICF from the BMRP was compared to HMRP with the same dataset in Figure 3. No difference was seen in mean ICF values (\( p=0.194 \), paired t-test, \( N=2500 \), Supplementary Figure 5A). Multiple variable linear regression revealed a marginally significant relationship (p=0.194, paired t-test, \( N=2500 \), Supplementary Figure 5A). Multiple random placement of individually placed cells compared to cells placed in non-overlapping or overlapping clumps, suggesting that colocalization with the network structure is independent of self-colocalization with the COI (Supplementary Figure 6).

2.5 Statistical Pipeline

Statistical processes were created to test for: (1) enriched intercellular colocalization affinity (ICA) of a cell type with the network structure within a single image, (2) enriched ICA for a study group of images, and (3) unique ICA between two study groups. All three of these tests were conducted by examining where the observed value of the random variable is located along the null probability distribution (Supplementary Figure 7).

2.5.1 CIRCOAST Test: Testing Colocalization for Single Image

To test for enriched colocalization affinity in a given image, the network structure in the image is thresholded and segmented, dilated by the radius of the COI, and the fraction of white pixels defines the cell dilated network fraction for that image. Under the binomial stochastic model, the CDNF and cell number is used to calculate the probability of observing colocalization with the network to an equal or greater extent than what is observed in the image if colocalization occurs under random placement (Supplementary Figure 7A). Equation 3 is utilized to derive the \( p \) values for a one-tailed binomial hypothesis test:

\[ \text{CIRCOAST} p = 1 - \sum_{c}^{n} \left( \binom{n}{c} p^c (1-p)^{n-c} \right) \]  

(3)

where \( c \) is the observed number of cells colocalizing in the image, \( n \) is the total number of cells in the image and \( p \) the cell dilated network fraction (CDNF) for that image. The null hypothesis that the image exhibits a degree of colocalization no greater than what would be expected by chance is rejected if CIRCOAST \( p \) is less than or equal to 0.05.

Since intercellular colocalization is modeled as a binomial process, sufficient sampling is determined by the quantity of each cell population sampled rather than fields of view imaged. In order to ensure that sufficient sampling can be obtained for each biological replicate, the data from multiple images is pooled together by calculating the combined CDNF across images and summing total COIs found in each image. This technique was validated by comparing the CDNF and CIRCOAST \( p \) value calculated from a test image compared to splitting it into four sub images. No difference was seen between the test images and original image, confirming the validity of this technique to join colocalization information across images (Supplementary Figure 8). Notably, while this process can probe for enriched colocalization affinity in an image, it fails to directly test for enriched colocalization for a study group of multiple biological replicates (animals, culture well plates, etc.).

2.5.2 One-sample CIRCOAST Test: Testing Colocalization for a Single Study Group

We asserted that a statistical test that probes for enriched colocalization within one or more study groups requires a random variable that acts as a metric of colocalization and is scaled to reflect the degree of colocalization beyond what would be expected purely by chance. The \( p \)-value from the CIRCOAST calculated from the binomial hypothesis test in Equation 3 satisfied both of the aforementioned criteria. \( p \)-values are known to be reliable random variables suitable for hypothesis testing (Sackrowitz and Samuel-Cahn 1999). The mean CIRCOAST \( p \)-value is calculated across the joined images of each animal or subject (Supplementary Figure 7B). The null distribution of the mean CIRCOAST \( p \)-value is simulated by assuming the null hypothesis is true and approximating what the distribution of the mean CIRCOAST \( p \)-value would be under the random placement paradigm (i.e. the pseudo null distribution). When the null hypothesis is true, the \( p \)-value of a hypothesis test is a uniform (0, 1) random
variable. Therefore, to generate a pseudo null distribution for the mean binomial p-value, sets of N p-values are generated from a uniform (0, 1) distribution to match the N number of biological replicates in the experiment data, and the mean of the distribution of generated p-values is calculated. Repeating this process (10,000,000 trials) yielded the pseudo null distribution of the mean CIRCOAST p-value under the random cell placement paradigm. The percentile at which the observed mean p-value falls along the distribution of simulated mean p-values yielded a one-sample cellular colocalization affinity with network structures (1-sample CIRCOAST) p-value for enriched colocalization across the entire study group by combining the information from the unique binomial distributions found in each image.

2.5.3 Two-sample CIRCOAST Test: Testing Colocalization between 2 Study Groups

To determine if two study groups differ with respect to the frequency of cell-cell colocalization, the CIRCOAST p-values are calculated for all the images from each group and subjected to a two-sample parametric (e.g. Student’s t-test) or non-parametric (e.g. Wilcoxon Rank Sum test) test to yield an “observed” p-value (Supplementary Figure 7C). This p-value is then compared to the pseudo null distribution of p-values that are generated under the null hypothesis scenario. The pseudo null distribution is generated by way of a large number of permutations of the study group identifications; i.e. the original study group identifications are randomly assigned to the sample identification numbers and the same parametric or non-parametric two-sample test is conducted using these random study group assignments. The fraction of the two-sample permuted test p-values less than or equal to the “observed” two-sample test p-value yields the two-sample cellular colocalization affinity with network structures p-value (2-sample CIRCOAST).

2.6 Experimental Validation

In Silico Validation: a dataset of images was created to represent healthy and diseased tissue, with one study group with high injected cell and endothelial cell density to mimic healthy conditions, and the another with low vascular and injected cell density for the dropout seen in diabeteres (Supplementary Figure 9). Vessels were created with the vessel network generator program and cells randomly placed with a single run of the Monte Carlo Model of Random Placement. Correct statistical analysis should reveal no changes between study groups since the cells were randomly placed. While generic statistics revealed a change in intercellular colocalization affinity (colocalization per field of view: p=2.46e-09; colocalization per 1 mm vessel length: p=1.12e-02; fraction of injected cells colocalizing: p=6.68e-07; unpaired t-test), CIRCOAST correctly revealed no changes in colocalization between study groups (p=0.494). This dataset reveals that false a positive conclusion can be generated by incorrectly quantified cell clumps can be minimized by accounting for how the cell clusters change the mean cell area, although high emphasis should be placed on correct cell counting in experiment images. If cells are randomly missed and not counted in the quantification process, mean ICF or CIRCOAST 1-samples p values do not change (Supplementary Figure 12 F-H), corroborated by the fact that cell density does not change mean ICF with the MCMRP parameter sweeps in Supplementary Figure 3.

Cell Sources: See Supplementary Note 3.

In Vitro and In Vivo Validation: See Supplementary Note 4.

Image Acquisition, Thresholding, and Quantification: See Supplementary Note 5.

3 Results

A biologically relevant application of colocalization of annular cells with network structures is cellular colocalization with microvascular networks, which are comprised of branched networks of endothelial cells. ASCs are known to colocalize with vascular endothelial cells in vitro (Merfeld-Clauss et al. 2010) and can engraff when injected in vivo (Mendel et al. 2013). Active homing of ASCs to the vasculature is hypothesized to play a role in this intercellular colocalization, but has not been established at a cell population level. The CIRCOAST statistical pipeline is validated by testing for the enriched cellular colocalization known for ASCs and ECs in vitro compared to fluorescent microspheres for a negative control, and then used in vivo to determine if injected ASCs exhibit greater than random colocalization with the vasculature to give insight to their possible mode of therapeutic action in disease (Mendel et al. 2013). ASCs cultured with HUVECs were found to have enriched colocalization over random behavior predicted by the BMRP (Figure 5 A, B; p<1e-7 1-sample CIRCOAST, a=0.05, 1e7 trials, N=6 wells, 3 images/well). For a negative control, fluorescent microspheres cultured with endothelial cells did not exhibit unique colocalization as expected (Figure 5 C, D; p=0.235 1-sample CIRCOAST, a=0.05, 1e7 trials, N=6 wells, 3 images/well). There was a significant difference in injected cell density (Figure 5 E, -18.7%, p=2.4E-2 two-sample t-test) and endothelial cell density (Figure 4F, +74.5%, p=3.3E-3 two-sample t-test) between study groups, illustrating the need for statistical tests that are not confounded by cell or vessel density. As demonstrated in Supplementary Figure 9, changes in vascular and cell density can confound generic statistics that examine colocalization events, while the CIRCOAST takes into account these changes between study groups and effectively standardizes for both changes in vascular and cell density. Unique colocalization affinity between ASCs and microspheres with endothelial cells was detected (Fig-
Figure 5: ASCs exhibit enriched colocalization with HUVECs, while fluorescent microspheres (µSpheres) do not. (A) ASCs (red) co-cultured with HUVECs (green). (B) Distribution of simulated mean CIRCOAST p values (blue) of random colocalization of ASC group compared to observed mean CIRCOAST p value (red). (C) Fluorescent µSpheres seeded on a culture of HUVECs (scale bar 250 um). (D) Distribution of simulated mean CIRCOAST p values (blue) of random colocalization from fluorescent µSpheres compared to actual mean p value (red). (E) Injected cell density and (F) endothelial cell density between groups. (G) Distribution of p values (blue) derived from permuting CIRCOAST p values in a Wilcoxon Sum Rank Test between ASCs and µSpheres, with observed p value (red) (N=6 wells, 3 images/well).

Figure 6: Injected live and dead ASCs both exhibit enriched, but unique, intercellular colocalization affinity with the vasculature. (A) Confocal image of retinal vasculature (green, preprocessed and thresholded) and injected with live DiRlabeled ASCs (red). (B) Distribution of simulated mean CIRCOAST p values (blue) of random colocalization of ASC group compared to observed mean binomial p value (red). (C) Dead DiR-labeled ASCs in the retinal vasculature (scale bars 150 um). (D) Distribution of simulated mean CIRCOAST p values (blue) of random colocalization from dead cell group, compared to actual mean p value (red). (E) Injected cell density and (F) endothelial cell density between study groups. (G) Distribution of permuted p values of Wilcoxon sum rank test of CIRCOAST p values between study groups, with observed p value (red) (N=6 mice, 3 images/mouse).

4 Discussion

In summary, we present CIRCOAST, a tool to characterize intercellular colocalization with network structures independent of the changes in cell and vessel network density found across study groups from both in vitro and in vivo experiments. The tool was validated by probing for the previously known colocalization events observed between ASCs and endothelial cells in vitro, and used to test for enriched colocalization between these cells in vivo.

In the field of immunology, changes in cell density measured via flow cytometry or fluorescence microscopy are used as key metrics to study cell behaviors in disease (Krummel et al. 2016). These measurements report cell numbers or densities and only indirectly allude to changes in cell-cell interactions. In studies when cellular colocalization is examined more directly using microscopy, changes to cell density of either cell population can confound colocalization metrics analyzed with generic statistics: the method presented here does not have such drawbacks. Additionally, this method could be used to test for changes in colocalization within subpopulations of a single cell type denoted by unique marker expression to implicate marker expression with colocalization behavior.

Although we focused on characterizing the frequency of interactions between cells and microvessels, other static cellular network structures...
could be analyzed, such as neuron networks, glial cells, and lymphatics. Furthermore, we think that CIRCOAST could be extended beyond cell-vessel associations to study colocalization between two migrating cell populations so as to interrogate putative chemotactic behaviors. Possible applications include the study of interactions between T-cells and B-cells, which are known to be critical for T-cell activation and immune responses to infection (Janeway et al. 2001). Additionally, T-cell interactions with antigen presenting cells (e.g. macrophages, monocytes, and dendritic cells) play a significant role in homeostatic conditions and in initiating the adaptive immune response during disease (Mahnke et al. 2008). Furthermore, in vivo time lapses indicate that macrophages may preferentially interact with pericytes in a juxtacrine fashion and receive instructions for launching innate immune responses (Stark et al. 2012) and play a role in vascular remodeling (Corliss et al. 2016). Analysis of intercellular colocalization could confirm that macrophages are preferentially migrating to pericytes as part of this process. This method could also be extended to intracellular colocalization studies at high resolutions where imaged structures typically approximated as a point cloud are better approximated as an annular shape. Possible intracellular applications would include characterizing vesicle trafficking across cellular cytoskeletal components (Schuh 2011).

Although CIRCOAST serves as a new method for hypothesis testing, additional features could enhance its capability. Future work, for example, could include extending the framework to perform power analyses for experiments, along with measuring the effect sizes between groups. Continued research in using a hypergeometric model of random cell placement could yield more accurate results once the distribution’s parameters are better understood for this application. CIRCOAST is also limited to approximating cells as circular shapes, but supporting elongated cell morphologies could facilitate its use in analyzing a greater diversity of cell types and phenotypes. Furthermore, using established methods in characterizing cell morphology (Pincus and Theriot 2007), simulated cells could be designed to directly represent the heterogeneity in cell size and morphology specific to each image instead of using a single cell shape as an approximation. Currently, CIRCOAST supports the analysis of 2D images that are maximum projections of 3D confocal z-stacks, leading to the issue that cells may appear to colocalize with the projected image but may not be colocalized in the z-dimension. Extending CIRCOAST so that it is capable of analyzing 3D image volumes will allow for more accurate determination whether cells of interest are colocalizing in the z-direction, in addition to the x- and y-directions. Usage of a nuclear dye would add greater certainty quantifying structures that correspond to distinct cells, especially in the cases where cell clumps form. Indeed, this test could be extended to characterize colocalization of a cell type with itself to study homotypic interactions.

Limitations in generic statistical approaches have been implicated as a contributing factor in the crisis of reproducibility in the biomedical sciences (Nuzzo 2014). With the development and validation of CIRCOAST, we aim to provide a novel statistical method that is superior to generic hypothesis tests for studying intercellular colocalization, allowing for more robust and repeatable characterization of cell-cell interactions in 2D images of tissues.

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References


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