Analysis of conditional colocalization relationships and hierarchies in three-color microscopy images

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***Short Abstract* — Colocalization analysis of multicolor microscopy images is a cornerstone approach in cell biology. However, almost all colocalization analyses are designed for two-color images. This limits their applicability and the type of information that they reveal, leading to underutilization of multicolor microscopy images. We developed an approach, termed "conditional colocalization analysis," for analyzing the colocalization relationships between three molecular entities in three-color microscopy images. Going beyond the question of whether colocalization is present or not, it addresses the question of whether the colocalization between two entities is influenced, positively or negatively, by their colocalization with a third entity.**

I. Background

Colocalization analysis provides information on the localization of molecules within various subcellular compartments and allows the interrogation of known molecular interactions in their cellular context, shedding light on their function. However, almost all colocalization analyses are designed for two-color images [1-3], which limits the type of information that they reveal. In order to better utilize multicolor microscopy images, it is necessary to develop colocalization analysis tools that go beyond two colors.

II. Approach

To analyze three-color microscopy images, we developed a novel approach termed "conditional colocalization analysis." In this approach, we calculated colocalization measures that reflect the positive or negative influence of one molecular entity on the colocalization of two other molecular entities. Referring to the three object types as target (T), reference (R) and condition (C), the question that the analysis aimed to address is: *How much is the colocalization of target objects with reference objects influenced by target and/or reference colocalization with condition objects?*

The first step of our analysis is to detect and/or segment the objects in the different channels. Then, we divide the population of T and R objects into colocalized or not with C objects. This is done based on the nearest neighbor distance between each T and R object and C objects. Then, we calculate the fraction of T objects colocalizing with R objects for the different subsets of T and R (based on their colocalization with C). In both cases, objects are considered colocalized if the distance between them is below the colocalization radius, for which we employ a range of values in order to obtain robust results. We assess the significance of the calculated conditional colocalization measures by abolishing the spatial relationships between the different object types. Our analysis is applicable to both punctate and non-punctate three-color images.

III. Validation and Biological Application

We benchmarked our approach using simulated data mimicking experimental data for different combinations of punctate and non-punctate objects. We also applied it to biological positive control (molecules known to interact) and negative control (molecules not known to interact) datasets. Lastly, we applied conditional colocalization analysis to investigate the colocalization relationships between a cell surface receptor and its downstream adapter in the context of membrane microdomains.

IV. Conclusion

Our tests demonstrated that conditional colocalization analysis is able to accurately assess colocalization relationships in simulated data and in the biological positive control, and to correctly identify no significant colocalization in the biological negative control. Applying conditional colocalization analysis to the receptor VEGFR2 and its downstream adapter TSAd revealed that the colocalization relationship between VEGFR2 and TSAd varies depending on their location at the plasma membrane. In particular, it is enhanced at focal adhesions (FAs) and clathrin coated structures (CCSs). Yet the two membrane domains exhibit differential interplay with VEGFR2-TSAd colocalization. In the case of FAs, VEGFR2 and FAs mutually enhance each other's colocalization with TSAd. In the case of CCSs, TSAd and CCSs mutually enhance each other's colocalization with VEGFR2. This application highlights the unique information on molecular interactions that can be revealed by conditional colocalization analysis.

Acknowledgements: This work was funded by NIH/NIGMS (R35 GM119619), including a Diversity Supplement to JVL and the Welch Foundation (I-1901)

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