A density-based enrichment measure for assessing colocalization in single-molecule localization microscopy data

Aske L. Ejdrup 1, Matthew D. Lycas 1, Niels Lorenzen 1, Ainoa Konomi 1, Freja Herborg 1, Kenneth L. Madsen 1 & Ulrik Gether 1

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Dual-color single-molecule localization microscopy (SMLM) provides unprecedented possibilities for detailed studies of colocalization of different molecular species in a cell. However, the informational richness of the data is not fully exploited by current analysis tools that often reduce colocalization to a single value. Here, we describe a tool specifically designed for determination of co-localization in both 2D and 3D from SMLM data. The approach uses a function that describes the relative enrichment of one molecular species on the density distribution of a reference species. The function reframes the question of colocalization by providing a density-context relevant to multiple biological questions. Moreover, the function visualize enrichment (i.e. colocalization) directly in the images for easy interpretation. We demonstrate the approach’s functionality on both simulated data and cultured neurons, and compare it to current alternative measures. The method is available in a Python function for easy and parameter-free implementation.
results in a summary statistic between 0 and 1 that provides rather little information on where the interaction takes place. Coloc-Tesselere12 works in 3D and is density based, but likewise it outputs a summary statistic that reduces colocalization to a single value without further context to the value.

In this work, we present a function that describes relative enrichment of one molecular species on the density distribution of a reference species. The algorithm is inspired by the pair-correlation function13 and the Voronoï tessellation of Coloc-Tesselere12. It reframes the question of colocalization by providing a density-context relevant to numerous biological questions. The tool is parameter-free and outputs a unitless ratio measure based on the average distance to neighboring proteins. Further, the algorithm can visualize enrichment directly in the images for qualitative interpretation, making colocalization information from super-resolution microscopy interpretable by scientists without advanced microscopy expertise. It works in both 2D and 3D and is available as a python function for further open development and easy implementation in a processing pipeline across operating systems and file formats. We envision this tool broadly used in future studies of the cellular nano-architectural organization due to its intuitive, unitless representation of colocalization, its contextual insight when protein distribution is heterogenous, its ability to generate informative qualitative images, and its easy and parameter-free implementation.

**Results**

Tessellation-based assessment of relative enrichment

Voronoï tessellation partitions a plane into discrete regions around seeds with each region containing the area closest to its seed (Fig. 1). The method can be applied to SMLM data, where each molecular localization represents a seed, and has proven itself a robust and efficient tool to assess local protein density14–18. Previously, Levet and colleagues proposed a colocalization analysis based on tessellation12. Building on their approach, we propose a method that integrates tessellation and conceptual elements of the pair-correlation function13, with the aim to unfold new biological insight by deriving a continuous measure of colocalization in the context of density. Our method calculates the enrichment of one primary molecular species across the density distribution of a reference molecular species. The result is a histogram of densities for the reference species with a score for each bin, indicating how enriched the primary species is near reference species with the corresponding density. The two species can be interchanged depending on the biology of interest.

To illustrate the method, we simulated two randomly distributed species of localizations (Fig. 1a). First step is to split the assigned reference species into separate regions by Voronoï tessellation (Fig. 1b). We refer to these as reference regions. Regions with no outer neighbor are considered edge regions, denoted with dashed lines and are not included in the analysis. Localizations of the primary species are then superimposed in their original positions (Fig. 1c), and the heart of the method is to quantify the observed number of primary localizations for each reference region, and compare it to the expected amount:

$$\text{relative enrichment} = \frac{\text{primary}_{\text{observed}}}{\text{primary}_{\text{expected}}}$$

(1)

The expected number of primary localizations is calculated for each region as per Eq. (2), by dividing the individual region area with the total area of all regions, and then multiplying that number with the total number of observed primary localization:

$$\frac{\text{primary}_{\text{expected}}}{\text{primary}_{\text{total}}} = \frac{\text{area}_{\text{region}}}{\text{area}_{\text{total}}}$$

(2)

To exemplify this, the relative enrichment (RE) is calculated for the region denoted with an asterisk on Fig. 1c (Eq. 1). This region has an average of 0.24 μm², with the total area of all nine included regions at 1.3 μm². That is 18.5% of the total area, and with 20 total primary localizations, Eq. 2 tells us that the expected occurrence is 3.7 localizations. As only one primary localization is found inside that region, Eq. (1) gives us a RE score of 0.27. For two completely uniform distributions, each region of the reference species would have an average RE of 1, and as such, an RE below 1 indicates a depletion. Conversely, an RE above 1 indicates an enrichment of the primary species in the vicinity of the associated reference localization. When plotting all the regions of the reference species color-coded by their RE, the heterogenous distribution of the primary species relative to the reference species becomes evident (Fig. 1d).

Larger dataset and scoring across densities

A typical SMLM image contains on the order of thousands to millions of localizations. To distill information from a wealth of RE scores in a density context, our method bins reference localizations by their region size, and assess the mean enrichment score per bin. To demonstrate this, we simulated normally-distributed, overlapping clusters of each species, slightly off-set from one another, and added
random background noise (Fig. 2a). The RE score for each region was assessed as described earlier (Fig. 2b), and an illustrative RE-color-coded map of reference regions is shown on Fig. 2c. Following this procedure, we uniformly binned regions by the logarithm of their area. This generates a histogram that visualizes the density distribution of the reference species (Fig. 2d). For each bin, we additionally plot the mean RE of the primary species in reference regions of that size interval. This results in a plot that shows the relative enrichment of one molecular species for a given density of a reference molecular species. This presentation of RE by reference region size provides a density context for assessing colocalization, without reducing it to a one-dimensional phenomenon.

Binning regions by area is the most apparent choice, as RE is computed from area. However, area is not an immediately intuitive measure. Therefore, we introduce binning by distance to neighbors, which is a more intuitive parameter, directly correlated to the size of the tessellated area. As complex polygons have no direct radius, mean distance to nearest neighbors is an excellent alternative, that provides a one-dimensional measure for local density. Fortunately, nearest-neighbor distance (NND) scales rather robustly with region area (Fig. 2e), with the added advantage of higher resolution in the densest localizations, due to the concave shape of the correlation. As shown on Fig. 2f, binning by NND rather than area produce a similar result, but with a more intuitive unit on the x-axis.

To show an example with no colocalization, we once again simulated two species with different distributions: one clustered and one uniform (Fig. 2g). The irregular distribution in one species does not distort the result, and this situation still yields an average RE score of 1 across densities (Fig. 2h), as the method accounts for the area covered when computing enrichment. This is true regardless of which species is chosen as reference (Fig. 2a). While some of the smaller regions might score high in RE, this is counteracted by a low score for the majority, and the two will even out in the binning when no specific colocalization is present. This is illustrated by the color-coded plot for RE score by region (Fig. S1b). The bimodal distribution of the reference species is also apparent from the histogram of NND, showing the usefulness of mapping densities when assessing colocalization.

To assess the outcome of various scenarios, we simulated data and calculated the RE values. Three pairs of random distributions with increasing densities were simulated first (Fig. S2a-c), which all yielded an RE of 1 across local densities. We also simulated a set of randomly distributed as well as anti-colocalized clusters with background noise in both (Fig. S2d, e). Akin to the random distributions, random clusters resulted in an RE of 1 across localization densities, whereas anti-colocalized clusters gave a lower RE score for the high-density localization, i.e. the clusters, and an RE score above one for the non-clustered portion. Finally, we simulated two identical random distributions (perfect colocalization of each pair, Fig. 2f). Here, the RE by design yields the inverse of the mean density of the color coded for each bin, RE score plotted on left-hand y-axis, and relative bin distribution plotted on right-hand y-axis. The two will even out in the binning when no specific colocalization is present. This is illustrated by the color-coded plot for RE score by region (Fig. S1b). The bimodal distribution of the reference species is also apparent from the histogram of NND, showing the usefulness of mapping densities when assessing colocalization.

Coltharp and colleagues point out that careful consideration should be put into the selected region size when assessing spatial organization. This can significantly impact the colocalization score of many methods, and lead to unwarranted conclusions if neglected. While region size can impact absolute RE values, the scoring across density bins in this method carries information about this spatial distribution and calculated for each bin. RE score plotted on left-hand y-axis, and relative bin distribution plotted on right-hand y-axis. The two will even out in the binning when no specific colocalization is present. This is illustrated by the color-coded plot for RE score by region (Fig. S1b). The bimodal distribution of the reference species is also apparent from the histogram of NND, showing the usefulness of mapping densities when assessing colocalization.
Assessing colocalization of synaptic release proteins

Molecular architecture is highly important for the correct functioning of cells. Especially in neuronal synapses, where various proteins coordinate docking, priming and fusion during synaptic vesicle release at astounding precision and speed. To showcase the insight supplied by applying our relative enrichment measure, we imaged two proteins central to vesicle fusion: mammalian uncoordinated 18 (Munc18-1) and syntaxin-1 (Stx1). Stx1 has multiple functions and is involved in both neuronal maintenance as well as vesicle docking and fusion. In contrast, Munc18-1 exclusively orchestrates the assembly of release machinery. Evidence from previous studies in neuroendocrine and neuronal cells suggest the two proteins colocalize at docked vesicles and disperse following vesicular neurotransmitter release. We harvested hippocampal neurons from rat pups, fixated the cultures, stained for the two proteins and imaged with direct stochastic optical reconstruction microscopy (dSTORM) (see methods for detailed

Fig. 3 | Visualising RE across densities for larger images. a Representative image of Stx1 and Munc18-1 in primary hippocampal neurons acquired with dSTORM. Molecular localizations visualized by gaussian representation. Scale bar is 4 µm. b Zoom on single varicosity from (a), with both Gaussian representation (left) and individual localizations (right). Scale bar is 400 nm. c Voronoï-regions for tessellation of either Munc18-1 (left) or Stx1 (right). Individual localizations of opposite species are plotted beneath. Same scale as (b). d Munc18-1 regions binned by nearest neighbour distance, with mean Stx1 RE value for each bin as line plot. RE score on left-hand y-axis, and relative bin distribution on right-hand y-axis. e Stx1 as reference. f Localizations of the reference species color-coded by RE score for Munc18-1 (left) and Stx1 (right).
Validation of RE-scoring in 3D. a Simulation of a single docked vesicle with three molecular species: a vesicle membrane-bound, a SNARE-like and a lumenal. Grid is 50 by 50 nm. b Representative simulation of twenty vesicles. Half docked at the membrane and associated with SNARE-like molecules, and half in the intracellular space without SNARE association. Grid is 200 by 200 nm. c 2D projection of representative simulation in (b). Scale bar is 100 nm. d Vesicle Voronoï regions binned by nearest neighbour distance in 2D, with mean RE value of each bin for both AZ and cargo as line plot. Shaded area indicates S.E.M., n = 20. Scale bar is 200 nm. e Simulated vesicle localizations from (b) color-coded for their relative enrichment of either SNARE (top) for lumenal (bottom) localizations. Grid is 200 by 200 nm. f Vesicle Voronoï regions binned by nearest neighbour distance in 3D, with mean RE value of each bin for both AZ and cargo as line plot. Shaded area indicates S.E.M., n = 20.

 protocol). Under basal conditions, the two proteins appear to form nanoclusters (Fig. 3a, b). This is in line with findings showing that both proteins exist in clusters at resting conditions 12. Next, we assessed colocalization with our proposed method. As relative enrichment can be examined bidirectionally, we alternated the appointed primary and reference species: colocalization of primary Stx1 to reference Munc-18 (Fig. 3c, left) or colocalization of primary Munc-18 to reference Stx1 (Fig. 3c, right). Both species showed a propensity to colocalize with the densest clusters of the opposing species, as maximal enrichment is at localizations with less than 10 nm on average to adjacent molecules of the same species (Fig. 3d, e). However, the relative enrichment was higher for Munc-18 on Stx1, possibly because Stx1 is in functional excess relative to Munc18-1, as Stx1 has multiple independent roles in neuronal maintenance and neurotransmitter release 20. This relative over-enrichment of Munc18-1 on Stx1 as compared to vice-versa can also be illustrated by color-coding the reference species based on individual RE score, as previously shown (Fig. 3f). Here, we plot the individual localizations rather than the associated regions, as the border between extracellular space and cells is harder to distinguish in these more complex neuronal structures when using Voronoï regions and to avoid visual domination by less dense regions (Fig. S3a). Next, we treated the primary hippocampal cultures with either N-methyl-D-aspartate (NMDA) to activate NMDA-type ionotropic glutamate receptors and stimulate vesicular neurotransmitter release or with tetrodotoxin (TTX), a sodium channel blocker, to prevent vesicular release (Fig. 3g). As Munc18-1 was more tightly associated with Stx1 during basal conditions, we focused on this enrichment-directionality. Upon stimulating release with NMDA, enrichment of Munc18-1 on Stx1 dropped in the densest Stx1 localization (Fig. 3h), in line with their proposed role in the synaptic vesicle fusion 22. In a mirror image, blocking synaptic vesicle release with TTX increased the propensity of the two proteins to colocalize, consistent with an increase in vesicular docking or priming. This effect can again be visualized by plotting the reference species, Stx1, color-coded by RE score (Fig. 3i, S3b). We observed no significant redistribution of Stx1 after treatment (Fig. S3c), matching previous findings when analyzed with tessellation 22. But tessellation may not uncover all reorganizational nuances. Results from applying the same analysis with primary and reference species reversed is shown in Fig. S3d, e.

Lastly, we compared our results to the other tessellation-based method, Coloc-Tesseler 12. This method calculates either Spearman or Mander’s coefficient, and while both can be computed in two ways similarly to our analysis, they are not directly comparable. When comparing the coefficient values of NMDA and TTX treated cells (Fig. 3j), neither of the four coefficients showed a significant difference, in contrast to both our proposed method and literature findings.

Validation on a simulated 2D and 3D vesicle example

As membranes are relatively flat on a mesoscopic level, 2D models of plasma membrane-bound proteins, and by extension 2D images, capture a good part of the relevant biology. Some molecules, however, reside in the cytoplasm or on the membrane of different organelles. To fully capture their spatial distribution, analyses need to incorporate z-positions from 3D images. The same is true in thicker tissue samples. To test the 3D capabilities of our method, we simulated vesicles at an active zone. Three different molecular species were simulated: a plasma membrane-bound active-zone-like (AZ) species, i.e. SNAP-25, a species bound to the vesicle membrane, i.e. synaptophysin, and a cargo species in the lumen of the vesicle, i.e. neuropeptides (Fig. 4a). As input to our analysis, we generated ten images with twenty vesicles in each, all of which contained the cargo molecule but only half were docked and associated with a cluster of the AZ molecule (Fig. 4b). As most 3D SMLM techniques have a higher uncertainty in the z-plane, the random jitter representing technical uncertainty was doubled for this axis. To mimic the results that similar biology would yield with 2D SMLM, we projected the simulated 3D images on to a 2D plane (Fig. 4c).

When assessed in 2D, using the vesicle species as reference, the AZ and cargo species have equal RE score across the vesicle density distribution (Fig. 4d). They both appeared as highly enriched around the densest vesicle membrane localizations and fell below an RE score of 1 outside of the vesicles, indicating under-enrichment in the surrounding space. In contrast, a different distribution was resolved when analyzed in 3D (Fig. 4e). Here, a high degree of colocalization between the cargo and vesicles species was still evident, whereas the enrichment of the AZ species was reduced as expected, as apparent from the RE score (Fig. 4f). Based on the histogram of vesicle NND, we can see a preferential colocalization of the cargo to the densest localizations of the vesicle, whereas the AZ species peaks at the intermediate density, suggesting a more peripheral association. This becomes evident when plotting the vesicle localization color-coded for their RE score of the two species.
The AZ species clearly enriches the bottom portion of vesicles near the membrane, whereas the cargo species highlights the center of all vesicles. This difference is much clearer in 3D as compared to the 2D visualization (Fig. S4a, b). Analysis of these simulated data underscores the importance of a 3D-compatible colocalization analysis for certain biological contexts.

While 3D data provides a greater amount of information than 2D, the extra dimension requires significantly more processing power. However, the relative enrichment function has acceptable processing times as compared to Coloc-Tesseler. For a two-color 3D SMLM image with half a million localization in each channel, loading and processing took on the order of 30 to 60 seconds for the relative enrichment function across the computers we tested, whereas Coloc-Tesseler for comparison finished in 5 to 10 seconds.

**Investigating protein trafficking itineraries**

Protein trafficking is an important element in cell biology, where 3D information is often needed to draw a conclusion. To investigate whether our method can inform us of trafficking itineraries, we tested it on experimental data of endocytosis of two different transmembrane proteins, the dopamine (DAT) and noradrenaline transporter (NET). The two transporters mediate clearance of their respective substrates, dopamine and noradrenaline, and play an important role in shaping the spatiotemporal profile of dopaminergic and noradrenergic neurotransmission. Interestingly, DAT and NET display differences in subcellular localization and trafficking itinerary.

**Fig. 5 | Neurotransmitter transporter localization to early endosomes.**

a. Representative top view of image with EEA1 and DAT in PC-12 cells acquired with astigmatic dSTORM. Molecular localizations visualized by gaussian representation. Scale bar is 0.5 μm. b. Representative top view of image with EEA1 and NET in PC-12 cells acquired with astigmatic dSTORM. Molecular localizations visualized by gaussian representation. Scale bar is 0.5 μm. c. Mean distribution of transporter and EEA1-positive structure depth. Normalized to peak transporter depth. Arrows indicate the differential intracellular accumulation of NET and EEA1. d. EEA1 Voronoï regions binned by nearest neighbour distance in 2D, with mean RE value of each bin for both DAT (n = 7 images) and NET (n = 8 images) as dots. Error bars area indicates S.E.M. e. Side view of black box from (a) and (b). Top panel shows both species, and bottom panel EEA1 localization color-coded by relative enrichment (RE) score. Dashed lines indicate plasma membrane depth as assessed by median transporter location in z. Scale bar is 200 nm. f. Top view of image with VMAT2 and DAT from primary midbrain cultures acquired with astigmatic dSTORM. Molecular localizations visualized by gaussian representation. Scale bar is 4 μm. j. Zoom on boxes marked on (i) and (k) showing representative structures. Scalebars are 200 nm.
Indeed, only one of four measures showed significant midbrain cultures of dopaminergic neurons. These neurons express visualization of our method.

In a context, compared to the quantitative output and the qualitative single colocalization values provided by these methods offered no association of the two transporter species to EEA1. Moreover, the putative early endosomes, were significantly more enriched with NET than DAT (Fig.5g). This provides clear evidence that the higher steady-state median transporter position in the z-axis, EEA1-positive structures of NET and DAT in EEA1-positive structures (Fig.5c), an intracellular accumulation is present for NET, but not for DAT, matching the expected distribution.

We first assessed if a differential colocalization pattern to EEA1 could be resolved for NET and DAT in 2D images. This was done by applying our method without depth (Fig.5d). We used EEA1 as the reference species, and while both transporters showed slight enrichment in the denser endosome localizations, indicating some association, there was no difference between the relative enrichment of NET and DAT in EEA1-positive structures. To assess if 3D information changes the result, we included depth information in the images (Fig. 5e, f, upper panels). Defining plasma membrane depth as the median transporter position in the z-axis, EEA1-positive structures were primarily intracellular, but proximal to the surface (Fig.5c).

Additionally, a qualitative assessment indicated more NET in these cases for transporter localization to early endosomes or vesicles, where neither NET nor DAT have free roam of the cytoplasm and must reside in the membrane of organelles. To use the absolute RE values in instances like these, careful thought must be put into the biology. In addition to the density context provided by the RE plot, the scoring of each individual reference localization allows for semi-quantitative visualization of the co-organization that can aid deciphering of the underlying biology. The raw data can be visualized in color-coding based on RE score, with either polygon plots or scatter plots, as shown throughout the study. This visualization tool is a strong hybrid between qualitative and quantitative approaches, that relies on the scoring of each individual localization. It is inspired by Malkusch and colleagues, and distinguished the method from ones that are stardardized to terminals and whose impact require in-depth knowledge of the tool to fully grasp.

In this work, we have presented an alternative approach to colocalization analysis. Here the co-organization of two molecular species is assessed in a density context relevant to a wealth of emerging biological questions, as heterogenous organization of plasma membrane as well as cytoplasmic proteins has become apparent. A parameter-free computation of the RE score provides the fold-above-expected occurrence of a primary species, across the density distribution of a reference species. We recommend binning by average distance to nearest neighbor, rather than by region size, as the distance is more easily interpretable than area or volume. The method is bilaterally applicable, as the species can be freely interchanged based on the biological question. It is worth mentioning that interpretation of the absolute RE score can be more complicated in 3D analyses - particularly when molecules are restricted to certain structures. This is the case for transporter localization to early endosomes or vesicles, where neither NET nor DAT have free roam of the cytoplasm and must reside in the membrane of organelles. To use the absolute RE values in instances like these, careful thought must be put into the biology. In addition to the density context provided by the RE plot, the scoring of each individual reference localization allows for semi-quantitative visualization of the co-organization that can aid deciphering of the underlying biology. The raw data can be visualized in color-coding based on RE score, with either polygon plots or scatter plots, as shown throughout the study. This visualization tool is a strong hybrid between qualitative and quantitative approaches, that relies on the scoring of each individual localization. It is inspired by Malkusch and colleagues, and distinguished the method from ones that are standardized to terminals and whose impact require in-depth knowledge of the tool to fully grasp.

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Methods

Ethical Statement

The experiments were conducted in accordance with the Animal Experimentation Inspectorate, Denmark. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Relative Enrichment Function

First step of calculating relative enrichment (RE) is selecting a reference and a primary species. The reference species then segments the total image into regions by Voronoi tessellation, where each region contains an equal number of points belonging to the elected primary species, with the number of expected occurrences, \(e_{ref} \), inside the region, where \(P\) denotes the full set of indices belonging to the elected primary species, with the number of expected occurrences, \(RE = \frac{e}{n_{obs}}\) (Eq. 1). The number of expected occurrences is calculated by dividing the area of the region with the total area analyzed and multiplying by total number of primary localizations, \(L_p = \frac{area_{region}}{n_{total}} \cdot L_{total}\) (Eq. 2).

Primary hippocampal cultures

Hippocampal neurons from E19 (embryonic) Wistar rat embryos of mixed gender (Charles River, Wilmington, MA) were plated on 15 mm coverslips using 6 well plates in at a density of approximately 100,000 cells/well. The coverslips were sterilized with UV and first coated with poly-L-lysine (Gibco) overnight and then coated with Neurobasal media (Gibco) supplemented with 4% FBS (Gibco), 1% Glutamax (Gibco), 100 U/mL penicillin, and 10 mg/mL streptomycin (Invitrogen) overnight. The rat brains were removed and placed in ice-cold dissection media: HBSS (Gibco) supplemented with 30 mM glucose, 10 mM HEPES (Gibco) (pH 7.4), 1 mM sodium pyruvate (Gibco), 100 U/mL penicillin, and 10 mg/mL streptomycin (Sigma). The brains were cut in half by sagittal incision. To access the hippocampus, each hemisphere has the medial section facing up and the corpus callosum was removed. After isolation, the hippocampi were treated with sterile filtered papain solution (Worthington) at 37°C for 20 min, triturated with two different diameter fire-polished Pasteur pipettes 10 times each, and filtered through a 70 μm cell strainer to remove cell debris. Density was calculated by a hemocytometer. The cells were seeded in Neurobasal media supplemented with 4% FBS, 2% B27 supplement (Gibco), 1% Glutamax (Gibco), 100 U/mL penicillin, and 10 mg/mL streptomycin (Invitrogen). After 24 h of seeding, the growth media was substituted with serum and glutamate-free media and cultured for 12–14 days in vitro (DIV). The cultures were stored at 35°C with 5% CO2. Media was changed every 3–4 days by replenishing half the media with fresh growth media.

Primary midbrain cultures

Primary midbrain cultures were prepared essentially as described by Rahbek-Clemmensen and colleagues. Briefly, the ventral tectal area and substantia nigra were surgically isolated from P1 to P3 Wistar rats (Charles River, Germany) and treated with papain (16 mM NaCl, 5.4 mM KCl, 26 mM NaHCO3, 2 mM NaH2PO4, 1 mM MgSO4, EDTA, 25 mM glucose 1 mM cysteine, 0.5 mM Kynurate, and Papain 20 U ml−1) at 37°C for 25 min. Next, we generated a single cell suspension by triturating gently, followed by spinning at 100 x g for 10 min and seeding in heated SFIC (50% (v/v) modified Eagle’s medium (MEM), 40% (v/v) Dulbecco’s modified eagles medium (DMEM), 10% (v/v) F-12 (all from Invitrogen) supplemented with 1% (v/v) heat-inactivated calf serum (FBS, 2.5 mg ml−1, Invitrogen), 0.35% (w/v) D-glucose, 0.5 mM glutamine, 5 mM Kynuric acid, Penicillin, Streptomycin, liquid catalase (0.05%), and DiPorzio61 (containing insulin, transferrin, superoxide dismutase, progesterone, cortisol, Na2SeO3, and T3) on 1M KOH suctioned coverslips (160-180 μm in thickness) coated with Poly-D-Lysine and pre-seeded with a glia cell monolayer. A few hours after seeding we added glia-derived neurotrophic factor. Cell proliferation was inhibited by adding 5-Fluorodeoxyuridine after 6-7 days. The cultures were imaged after 8–16 DIV.

Heterologous cell cultures

PC-12 cells are derived from neuroendocrine chromaffin cells, and were maintained in Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum, 5% horse serum and 1% penicillin/streptomycin.

Transfection

PC-12 cells were transfected in T25 flasks the after seeding 1 mio. cells in fresh media. 3 μl of lipofectamine was added to 100 μl of Opti-MEM and 1 μl of 1 μg/μl of DNA to another 100 μl of Opti-MEM. The lipofectamine mixture was left for 5 min. to form micelles and after another 25 min. with the DNA-mixture added, the combined solution was added to the T25 flasks and left at 37°C O/N. After the following day the flasks were washed with PBS, cells dissociated with trypsin and split into six- or twelve-well plates with coverslips coated with poly-L-ornithine.

Immunocytochemistry

The cell samples were fixed in paraformaldehyde (3%) and washed three times in saline (20 mM) and NH4Cl (50 mM) in PBS. Subsequently, the cells were washed in blocking buffer (5% Donkey serum, 1% BSA in PBS), and incubated in blocking permeabilization buffer (blocking buffer with saponin (0.2%)). Primary antibody was added in blocking buffer for 60 min, followed by 3–5 min incubation in blocking buffer. Secondary antibody was applied in blocking buffer for 45 min, and the sample was incubated 2x for 5 min in blocking buffer. Samples were washed in PBS twice and post-fixed in paraformaldehyde (3%) for 15 min. Samples were washed twice in glycine (20 mM) and NH4Cl (50 mM) in PBS and stored in PBS at 4°C until imaging.

Antibodies

We used the following primary antibodies for staining: Anti-Stx1 at 1:500 (Sypmatic Systems, I1001), anti-Munc18-1 at 1:500 (Sypmatic Systems, I1002), anti-HDAT at 1:1000 (Sigma-Aldrich, MAB369), anti-HetNet at 1:1000 (Mab Technologies, NET71), anti-EEA1 at 1:1000 (Abcam, Ab2900) and anti-VMAT2 at 1:4000 (kindly provided by Dr. Garry Miller, Columbia University, New York). Secondary antibodies with conjugated AF568 or AF647 were added in 1:400 dilution.

dSTORM

For dSTORM we used a buffer containing β-mercaptoethanol and an enzymatic oxygen scavenger system (10% (w/v) glucose, 1% (w/v) beta-mercaptoethanol, 2 mM cyclooctatetraene, 50 mM Tris-HCl (pH 8), 10 mM NaCl, 34 μg mL−1 catalase, 28 μg mL−1 glucose oxidase). The imaging was performed with an ECLIPSE Ti-E epifluorescence/TIRF microscope (Nikon, Japan) equipped with 405 nm, 488 nm, 561, and 647 nm lasers (Coherent, California, USA). For dual-color imaging was performed with an ECLIPSE Ti-E epi microscope (Nikon, Japan) equipped with 405 nm, 488 nm 561, and 647 nm lasers (Coherent, California, USA).
secondly by an extra filter to decrease noise (561 nm Longpass, Edge Basic, F76-561, AHF). A motorized piezo stage controlled by a near-infrared light-adjusted perfect focus system (NIKON) was applied to the system to reduce any sample drift over time in the z-direction. Dual-color dSTORM images were constructed from 10,000 frames for each color, taken at a 10 ms frame rate, with each color alternating by frame. Photons were collected with an iXon3 897 EM-CCD camera (Andor, United Kingdom). Laser powers used were 2.3 kW cm² for 364 nm, 1.0 kW cm² for 488 nm and for 561 nm. The 405 nm laser was used to incrementally increase blinking behavior at power <0.1 kW cm². For 3D-dSTORM, a cylindrical lens was placed before the camera to impart astigmatism. A reference z-stack of fluorescent beads (TetraSpeck) was acquired with 25 nm intervals spanning 2 μm before each imaging session. Localizations from dSTORM videos were fitted with 3D-DAOSTORM⁴⁵, with a background sigma of 8, maximum likelihood estimation as the fitting error model, 20 peak identification iterations, an initial sigma estimate of 1.5 and a threshold adjusted to each imaging session. A linear fit in both the x and y-plane was subtracted to account for coverslip tilt.

**Statistical analysis**

Choice of statistical analysis is presented in the legends associated with each figure, and where specified multiple testing was corrected for using the Bonferroni-Holmes correction. All p-values are individual images or simulations. Statistical analyses were carried out in Python 3.6.10 with the open-source python packages SciPy v1.5.2, Numpy v1.18.1, and Seaborn v0.11.0, and linear models in Statsmodels v0.12.2. Images or simulations. Statistical analyses were carried out in Python for using the Bonferroni-Holmes correction. All data are plotted as outliers. No statistical methods were used to predetermine sample sizes.

**Data availability**

The processed data generated in this study are provided in the Source Data file. Due to size the raw dSTORM images are not included, but available from the corresponding author within two weeks upon request. Source data are provided with this paper.

**Code availability**

All custom python functions and working examples with data from the simulated 2D and 3D vesicle example are available online at: https://github.com/Ejdrup/relative-enrichment/releases/tag/v0.1.0 or at DOI: 10.5281/zenodo.6627703. The figure-specific analyses are available from the corresponding author within two weeks upon request.

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Author contributions
A.L.E. developed the method with help from M.D.L. and N.L. Experiments were performed by A.L.E., N.L., M.D.L. and A.K.. Simulations and analyses were carried out by A.L.E. A.L.E. wrote the manuscript. A.L.E., M.D.L., F.H.H., K.L.M. and U.G. edited the manuscript. All authors reviewed and critically evaluated the manuscript. K.L.M. and U.G. supervised the project.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Aske L. Ejstrup or Ulrik Gether.

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