

A new approach for correlated confocal and superresolution microscopy visualizes the distribution of presynaptic CB₁ cannabinoid receptors at the nanoscale

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Summary

How synapse-specific differences in signaling protein density determine the qualitative and quantitative properties of synaptic transmission is a fundamental question in neuroscience. However, our understanding of the molecular parameters underlying synaptic processes remains limited due to methodological difficulties. Therefore, in the present study, we exploited whole-cell patch-clamp recordings, confocal microscopy and STORM superresolution microscopy to allow the integrated analysis of physiological and morphological properties of a single neuron with quantitative molecular imaging within its axon terminals. Importantly, the whole experimental and analysis process can be carried out in a few days and reveals the localization of synaptic proteins with excellent sensitivity and specificity, ultra-high spatial resolution, and in large sample sizes. We first employed methodological improvements for STORM superresolution imaging in mouse hippocampal sections, which revealed the subcellular distribution of CB₁ cannabinoid receptors with a fluorophore localization precision of 6 nm and 41 nm in the lateral and axial dimensions, respectively. The specificity of CB₁ immunolabeling in STORM images was validated by using CB₁ knockout animals. To make physiological characterization and anatomical classification possible in combination with superresolution imaging, CB₁-positive GABAergic interneurons were filled with during electrophysiological recording and their morphology was visualized by confocal microscopy. To provide a cellular context for the 3D coordinates of CB₁ localization points obtained by STORM, confocal microscopy was used to detect labeled axon terminals, and then CB₁ distribution within the selected axon terminal was imaged by STORM. To facilitate the correlative visualization and analysis of confocal and STORM microscopy data, we also developed a new software with an easy-to-use graphical user interface. A correlation analysis of confocal and STORM images demonstrated that 3-D STORM provides a reliable quantitative readout of protein abundance in HEK cells and in GABAergic axon terminals. Finally, we applied new algorithms allowing nanoscale measurements of relative CB₁ density and intermolecular distances along the surface of GABAergic axon terminals. Taken together, our new methodology including improvements for brain tissue processing and imaging, as well as new software tools for data analysis represents a powerful approach for cell type-specific nanoscale molecular investigations in association with physiological and anatomical characterization in intact brain circuits.

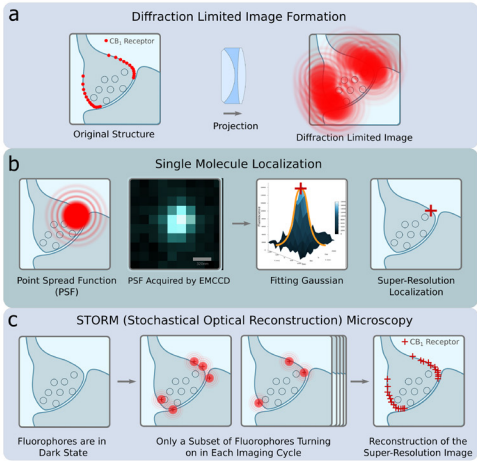


Figure 1. The principle behind STORM super-resolution microscopy. In light microscopy, resolving close emitters is impossible because of their overlapping diffracted projections (a). However, the localization of a single light source can be determined very precisely by fitting a Gaussian function on the diffracted image of the emitter (b). In Stochastic Optical Reconstruction Microscopy, fluorophores are reversibly converted into a non-excitable form. By a low-probability, stochastic return from this dark state, a sufficiently low density of single emitters can be maintained in each imaging frame to allow single-molecule localization of each active fluorophore. By capturing several frames, the original structure can be reconstructed from the localizations (c).

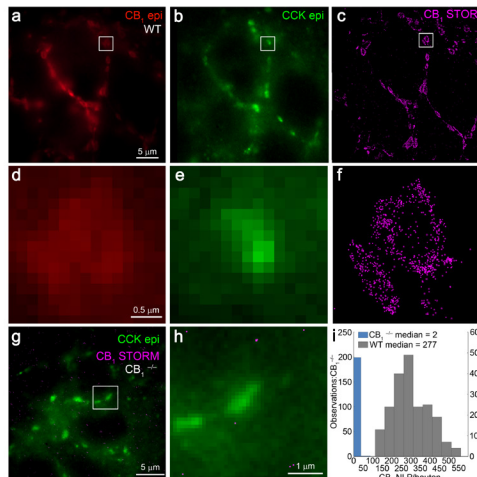


Figure 2. 3D-STORM imaging of CB₁ receptors on hippocampal GABAergic axon terminals (a) Immunofluorescence labeling of CB₁ receptors in the CA1 stratum pyramidale of CB₁^{-/-} mice showed basket-like arrangement of CB₁-positive axon terminals encircling a CB₁-immunonegative pyramidal cell somata. (b) These axon terminals could also be visualized by immunofluorescence staining for the neuropeptide CCK. (c) STORM imaging of CB₁ receptors revealed identical basket-like orientation of the same perisomatic GABAergic axon terminals. (d, e) Magnifying the epifluorescence images allows to delineate the morphological structure of the respective profiles or the precise nanoscale position of CB₁ receptors. (f) In contrast 3D-STORM microscopy made it possible to reliably discern individual CB₁-positive boutons located adjacently to one another, as well as to reveal the nanoscale position of CB₁ receptors. (g, h) To validate the specificity of our approach, hippocampal sections from littermate CB₁^{-/-} mice were also processed and imaged under the same conditions. In these sections, we found an almost complete absence of STORM localization points representing CB₁ within CCK-immunolabeled boutons. (i) Histogram of CB₁ NLP in individual CB₁-positive varicosities from CB₁^{-/-} (n = 3 animals; n = 208 axon terminals) or CB₁^{+/+} (n = 2 animals; n = 200 axon terminals) mice.

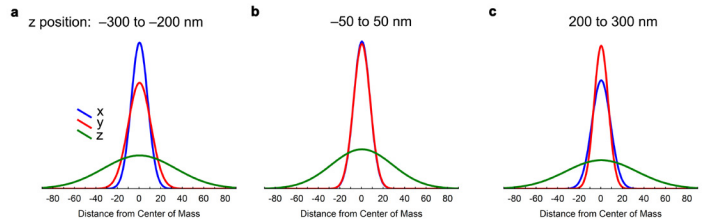


Figure 5. Quantitative evaluation of the localization precision of STORM imaging in brain sections (a-c) Localization precision displayed only a modest variation (1 nm or 5 nm at x-y or z directions, respectively) as a function of axial position ie. at different z distances from the focal plane. Clusters resulting from the multiple blinking of isolated fluorophores in the STORM images of CB₁-immunostaining were identified, and the distribution of the distances of individual localization points from the center of mass of the clusters was plotted.

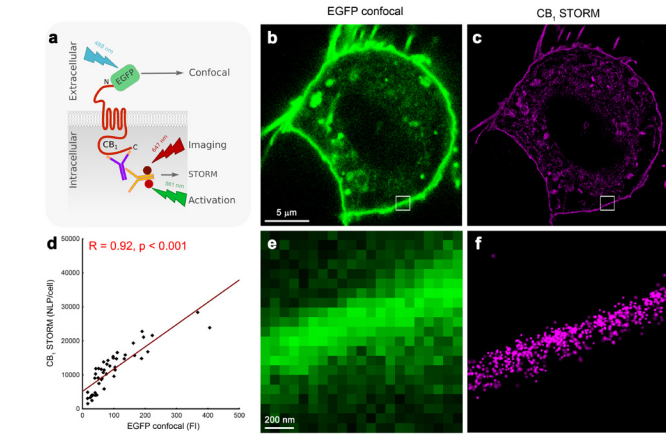


Figure 4. Nanoscale localization precision and relative quantification of CB₁ receptors by 3D-STORM imaging (a) Schematic representation of the experimental paradigm for combined confocal/STORM imaging in HEK cells. (b) Confocal image of a representative HEK cell reveals CB₁ receptor accumulation within the plasma membrane. (c) At lower magnification, STORM superresolution image of the same HEK cell depicts a similar subcellular CB₁ localization pattern as confocal imaging. (d) To study whether the Number of Localization Points (NLP) obtained by STORM imaging and measured in a given anatomical profile reliably predicts relative protein quantity, randomly selected HEK cells (n = 46) expressing an EGFP-CB₁ fusion construct at various expression levels were imaged by both confocal and STORM microscopy. Intrinsic EGFP fluorescence intensity (FI) values were measured by confocal microscopy and determined by subtracting mean background level (assessed from 3-3 Regions of Interest (ROIs), located in the extracellular blank regions (n=138) from every image) from the mean pixel intensity. The strong positive linear correlation between the two imaging readouts confirms that NLP values reliably predict relative protein quantity (n = 46 cells, Spearman's rank order correlation, p < 0.001). (e) The high magnification confocal image of the boxed plasma membrane segment in b illustrates the diffraction-limited blurred nature of the EGFP fluorescence signal. (f) In contrast, at higher magnification, the nanoscale localization precision of fluorophores representing CB₁ receptors by STORM imaging results in crisp visualization of the same boxed plasma membrane segment compared to the confocal image shown in e.

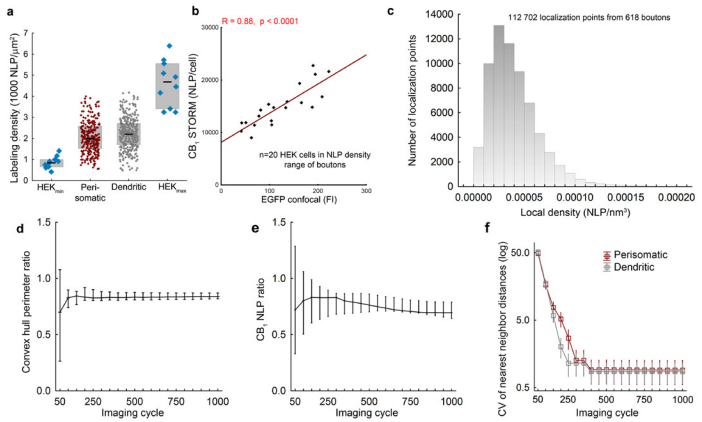


Figure 5. CB₁ density distribution on axon terminals of identified interneurons can be reliably determined under the specific imaging conditions used throughout the study (a) The local density of localization points per bouton, measured as NLP within 200 nm from each CB₁ localization point, falls within the range of local densities measured in plasma membrane regions from EGFP-CB₁-expressing HEK cells with the weakest and the strongest expression levels. Note that the vast majority of individual boutons also fell in this density range between the two extreme HEK cells. Graphs show raw data and median±IQR. (b) HEK cells (n=20), which exhibited a plasma membrane localization density value falling within the localization density range of interneurons (mean±SD) were identified from the randomly selected HEK cell population (n=46) shown in Fig. 4. Note the highly linear positive correlation between intrinsic fluorescence intensity and NLP within this population (Spearman's rank order correlation, p<0.0001). (c) The distribution of local densities (neighboring localizations within 50 nm 3D distance from each localization point) on the boutons from (a) shows a heavy right tail indicating that the signal is not likely to be saturated. (d) The different size of perisomatic and dendritic boutons is evident regardless of the duration of the imaging (significant at every time point after 250 cycles). See the poster by Dudok et al on board C29 for details about the cell type-specific comparison. (e) Similarly the difference was significant at every time point afterwards (n=16 cells/cell type, Mann-Whitney U test, p<0.05). The same approach was used to compare the number of CB₁ localization points per bouton between interneuron types. (f) The variance of nearest-neighbor distances between CB₁ localization points within boutons was constant in both cell types after 400 cycles into imaging, indicating that running the imaging for up to 1000 cycles for each image provides reliable measurement of CB₁ distribution within GABAergic axon terminals.

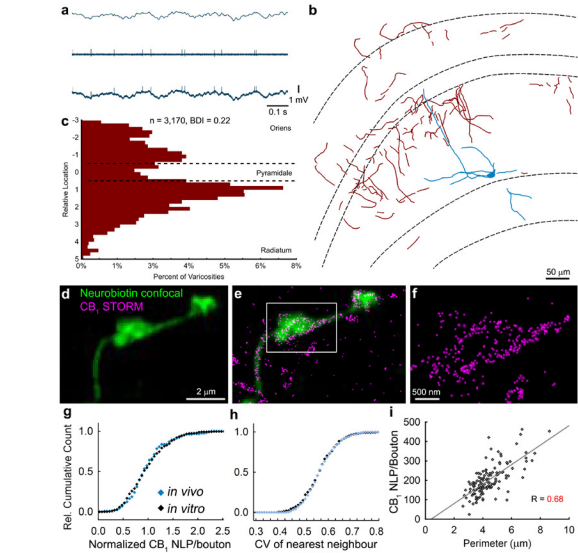


Figure 6. Combination of in vivo single neuron recording in a behaving mouse with post-hoc anatomical characterization and molecular imaging at the nanoscale (a) Example traces from juxtacellular recording of a CA1 interneuron in an anesthesia-free mouse running on a spherical treadmill. A 1 sec-long recording, filtered for local field potential, and spikes, and the raw trace are shown below each other, respectively. (b) After the recording, the cell was filled with neurobiotin. Morphological analysis of the labeled neuron was performed by confocal microscopy. Part of the axonal arbor (red), as well as its soma and some dendrites (blue), were reconstructed from four consecutive 20 µm thick sections by Neurolucida. (c) Maximum intensity projection of a deconvolved confocal image stack of individual axon terminals from the same in vivo recorded interneuron. (e) CB₁-immunolabeling on these identified axon terminals is visualized by overlaying the corresponding confocal and STORM images. (f) Magnification of the boxed region of the STORM image in e illustrates the intraterminal distribution of CB₁ localization points at the nanoscale level and reveals a qualitatively similar pattern as that observed in the case of in vitro-labeled interneurons in Figure 3. (g) Cumulative probability plots of normalized CB₁ NLP found on individual terminals of in vivo- or in vitro-filled dendritic interneurons (See the poster by Dudok et al on board C29 for details on in vitro filled neurons). The distribution of data obtained from the two experimental preparations were identical (Kolmogorov-Smirnov test, p > 0.1). Data were collected from single CB₁ STORM imaging of 129 boutons from one in vivo, and of 141 boutons from 5 in vitro-filled cells and were normalized to the mean NLP value of the respective cell. (h) The variance of CB₁ nearest neighbor distances is also comparable under the different experimental conditions indicating similar CB₁ distribution at the nanoscale level in boutons of in vivo- or in vitro-filled dendritic interneurons (Kolmogorov-Smirnov test, p > 0.1). (i) In line with the in vitro findings, at the single bouton level, the number of CB₁ localization points correlates linearly with bouton size (n = 129 axon terminals, Spearman's rank order correlation, p < 0.001).

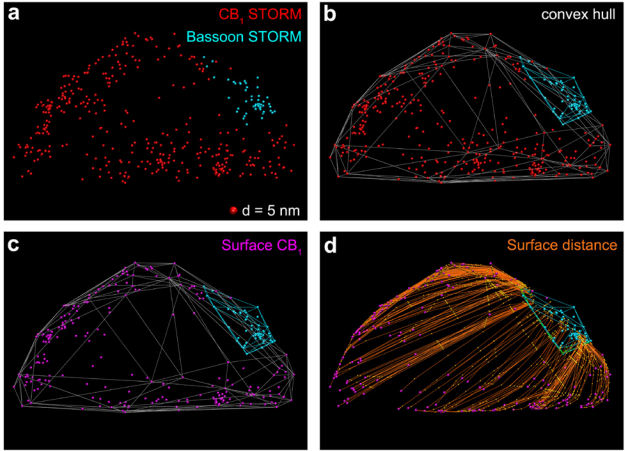


Figure 9. Visualization of the localization coordinates of the STORM signal by 3D rendering and calculation of membrane surface distances (a) 3D positions of STORM localization points are illustrated as atoms in Visual Molecular Dynamics. (b) First, a convex hull was fitted on the localization points, giving an approximation of the structure surface. (c) Next, the distance of each localization point from the convex hull was measured, and only localizations within 80 nm from the surface were included in further analysis. (d) Finally, the distance of each localization point from the active zone was measured as the shortest route on the polyhedron surface.

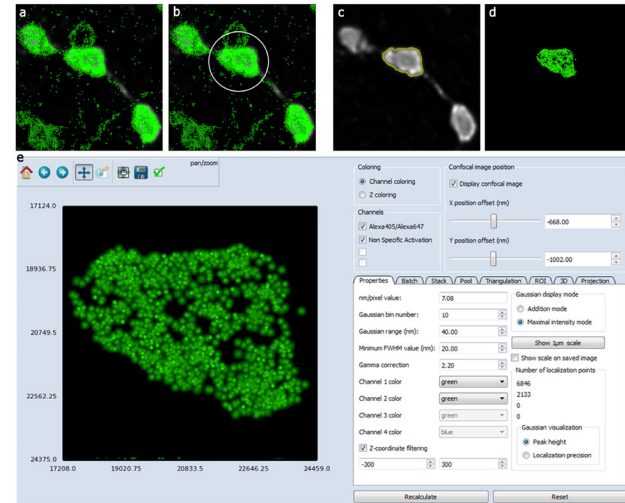


Figure 10. VividSTORM: an integrated software for the correlated analysis of confocal and STORM microscopy data (a-d) Illustration of the analysis workflow in VividSTORM. (e) STORM and confocal images of CB₁ immunostaining and biocytin cell fill, respectively, recorded from the same field of view, are opened. (b) A structure is selected for analysis by placing a circular ROI. (c) The outlines of the confocal image of the structure are automatically detected by MACWE (Morphological Active Contour Without Edges) algorithm. (d) Localization points from the area of the structure are selected for further analysis and visualization. (e) Snapshot of the VividSTORM GUI with gaussian visualization of localization points.

Results and Conclusions

1. STORM imaging provides a versatile and fast tool for quantitative molecular profiling in a cell-type specific manner and allows combination of physiology, anatomy and molecular biology with an unprecedented speed and experimental flexibility.

2. The experimental and analysis methods presented here facilitate the application of correlated confocal and STORM microscopy for the investigation of molecules of interest within any labeled cell type or subcellular domain.

Acknowledgements:

