

Correlated confocal and super-resolution imaging by VividSTORM

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Single-molecule localization microscopy (SMLM) is rapidly gaining popularity in the life sciences as an efficient approach to visualize molecular distribution with nanoscale precision. However, it has been challenging to obtain and analyze such data within a cellular context in tissue preparations. Here we describe a 5-d tissue processing and immunostaining procedure that is optimized for SMLM, and we provide example applications to fixed mouse brain, heart and kidney tissues. We then describe how to perform correlated confocal and 3D-superresolution imaging on these sections, which allows the visualization of nanoscale protein localization within labeled subcellular compartments of identified target cells in a few minutes. Finally, we describe the use of VividSTORM (<http://katonalab.hu/index.php/vividstorm>), an open-source software for correlated confocal and SMLM image analysis, which facilitates the measurement of molecular abundance, clustering, internalization, surface density and intermolecular distances in a cell-specific and subcellular compartment-restricted manner. The protocol requires only basic skills in tissue staining and microscopy.

INTRODUCTION

Physiological and pathophysiological processes are often associated with quantitative changes in the number and position of key signaling molecules. These molecular alterations usually occur only in specific cell types of a given organ, and they are typically restricted to certain subcellular compartments. The recent development of SMLM represents a revolutionary step in our ability to measure molecular changes at the nanoscale level^{1–4}. However, SMLM imaging alone does not visualize the respective cellular and subcellular environment from which the molecular data are gathered. Therefore, the combined use of an additional imaging modality may be highly beneficial by providing a cell-specific context for nanoscale molecular imaging and thereby facilitating functional interpretation of quantitative molecular observations. To this end, we recently developed a new approach for correlated confocal and stochastic optical reconstruction microscopy (STORM) superresolution imaging, and we applied it to the study of molecular changes in specific neuron types in the mouse brain⁵.

The brain is the most complex organ in the body, with hundreds of molecularly, morphologically and functionally distinct cell types distributed in numerous brain regions^{6–9}. Nerve cells convey information through their chemical synapses, and the precise activity-dependent regulation of synaptic strength is indispensable for brain circuits to operate their various functions. To dynamically control synaptic plasticity, individual synapses also exploit thousands of different proteins integrated into highly sophisticated molecular machineries^{10,11}. Therefore, a central question of neuroscience research concerns the elucidation of the molecular mechanisms underlying synaptic transmission. However, the immense structural complexity of the nervous system and the overwhelming molecular diversity of synapses represent major bottlenecks. This is important, because the molecular plasticity underlying physiological and pathophysiological mechanisms of synaptic transmission often happens at specific synapses belonging to certain cell types^{12,13}. However, methods that

routinely allow the quantitative monitoring of molecular distribution within synapses of identified neurons are still lacking.

For decades, immunogold electron microscopy (EM) has been the primary method of choice in the life sciences to interrogate nanoscale molecular distribution^{14,15}. Although EM has the necessary resolution to localize target proteins in relation to subcellular structures such as chemical synapses in the brain, serious limitations to experimental flexibility are posed by the need to dehydrate and embed samples and the limited number of molecular tags available. Fluorescence microscopy, in contrast, permits a wide array of experimental designs, including the use, in any combination, of genetically and virally delivered markers and tags, cell type-specific promoters, compartment-specific dyes, biochemical sensors and multiplex detection of several labels, in live or fixed tissue. However, the precision of molecular localization in fluorescence microscopy is constrained by the diffraction limit.

The recent advent of superresolution microscopy introduced methods that surpass the diffraction limit of fluorescence imaging^{16,17} and enabled experimental approaches that combine the high resolution previously exclusive to EM with the versatility of fluorescence microscopy¹⁸. SMLM is one of the main super-resolution approaches, and it relies on determining the precise position of isolated images of single fluorophores¹⁹. One of its branches, STORM³, exploits the photoswitching behavior of certain organic dyes to achieve temporal separation of otherwise overlapping emitters. Single-labeling STORM experiments, however, reveal molecular localizations without the cellular and subcellular context, rendering the functional interpretation of the molecular information very difficult.

Development of the protocol

To provide a means for positioning the nanoscale localization data within complex brain circuits, we have recently implemented the combination of STORM with confocal imaging of biocytin-filled neurons labeled individually during whole-cell patch-clamp

recording^{5,20}. To obtain high-resolution STORM images of our proteins of interest, we have built on a procedure, which was previously used for STORM microscopy in fixed brain sections²¹, and we have modified this procedure for free-floating immunostaining to maximize the sensitivity and specificity of antibody binding. We were able to achieve sequential imaging of the same field of view by confocal microscopy (to visualize single-cell labeling) and by STORM (to visualize nanoscale molecular distribution) on a single, commercially available microscope setup, and we developed the required software tools to analyze the corresponding image pairs in a correlated manner.

By using this modified protocol, we have achieved fluorophore localization precision of $\sigma = 6$ nm at the lateral dimension and of 41 nm at the axial dimension at a 5- μ m depth in brain sections⁵. We have also demonstrated that the number of localization points (NLPs) in a STORM image is a robust measure for relative quantification of protein abundance⁵.

To the best of our knowledge, the correlated analysis of pixel luminescence and molecule localization data has not been introduced in any publicly available software. To address this issue, we have implemented the analysis algorithms used earlier^{5,20} in VividSTORM, a stand-alone software with graphical user interface (GUI), which can readily open, visualize and analyze corresponding conventional optical microscopy images and molecule lists. By following the analysis workflow presented earlier⁵, the localization points (LPs) in the STORM molecule list belonging to the labeled subcellular structures on the overlaid confocal image are selected. On these data sets, a number of analysis functions can be run, in order to measure the number and density of LPs, inter-molecular distance distribution, density of LPs along 3D surfaces, clustering and internalization²⁰.

Overview of the procedure

Here we describe a protocol (Fig. 1) for correlated confocal and STORM super-resolution microscopy, which enables quantitative molecular imaging at sub-cellular domains of specific cells at the nanoscale level. The protocol follows the experimental procedure that we have described earlier^{5,20}. Importantly, this

protocol allows the study of any target protein in a cell type-specific manner in association with physiological and pathophysiological processes. Although the protocol is optimized for brain tissue, its basic features are generally applicable to any other tissue type or cell culture preparation. To facilitate cell type- and compartment-specific nanoscale molecular imaging, we also describe VividSTORM (<http://katonalab.hu/index.php/vividstorm>), which is the first software that is specialized in the correlated analysis of pixel intensity-based and localization microscopy data. VividSTORM incorporates the image analysis functions used in previous publications^{5,20} in a stand-alone software with a GUI. Figure 1 summarizes the key stages of the method. First, tissue is fixed, the cellular labeling is developed, and the sample is re-sectioned in Steps 1–15. Second, immunostaining is performed and sections are mounted on coverslips during Steps 16–23. Acquisition of correlated image pairs using confocal and STORM microscopy is detailed in Steps 24–33. Finally, image processing and analysis is described in Steps 34–45.

Applications of the method

The core concept of our approach is to obtain quantitative nanoscale molecular localization data and cell- and subcellular

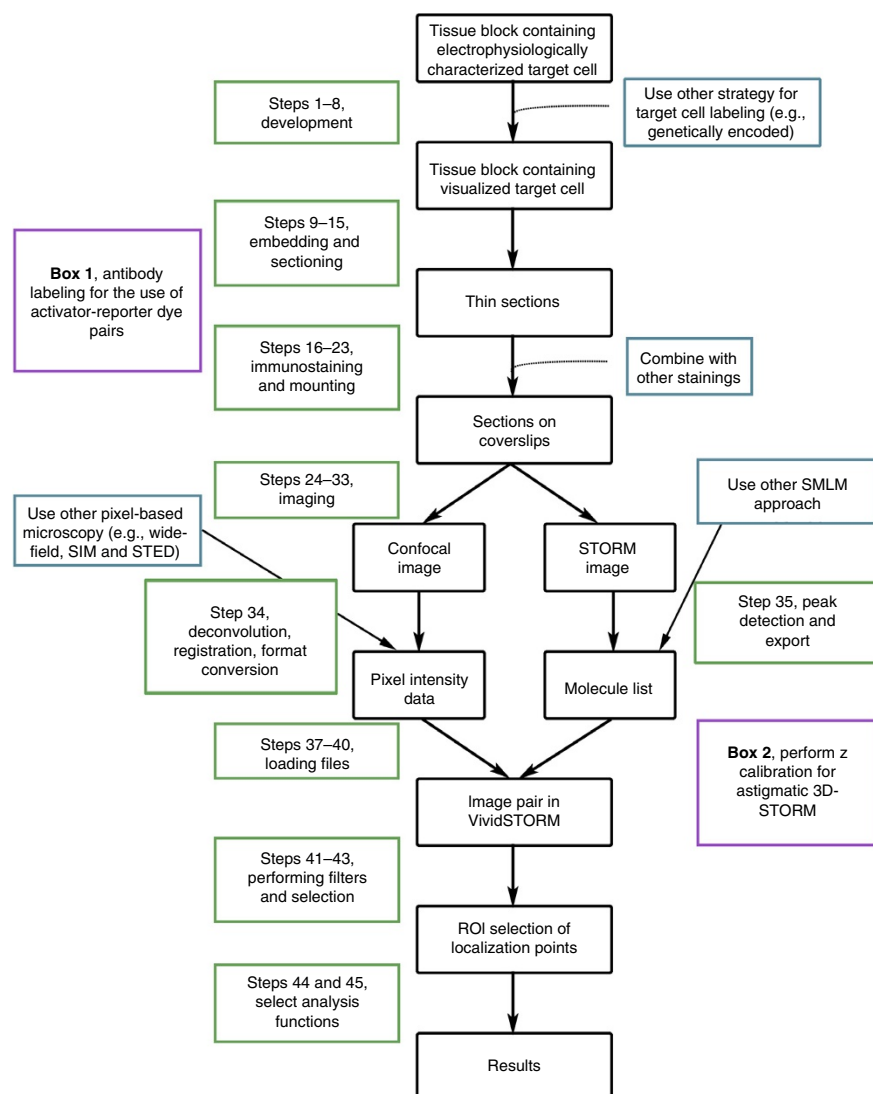
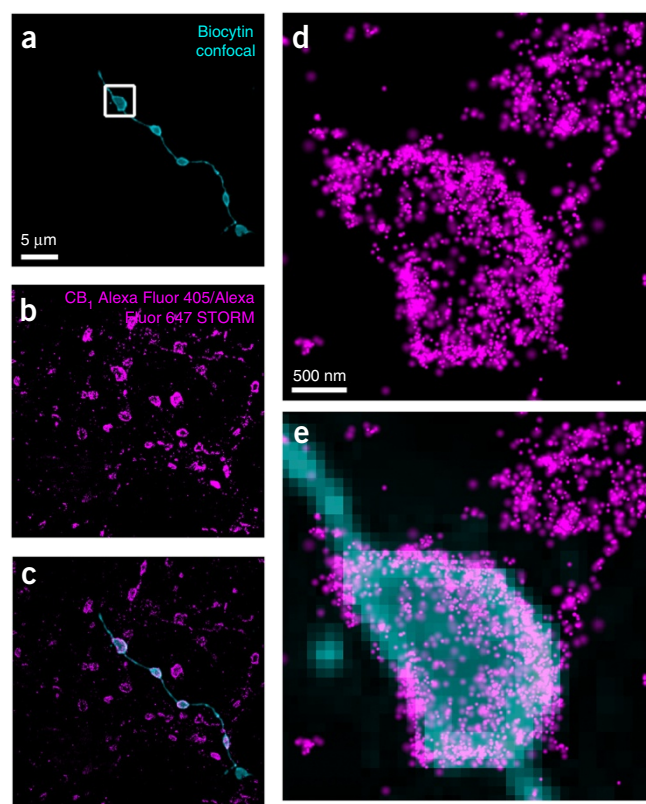


Figure 1 | Overview of the procedure.

This protocol describes sample preparation together with correlated imaging and analysis for conventional and single-molecule localization microscopy in biological tissues. The flowchart shows separate stages of the protocol (black) connected with specific steps (green). As tissue processing, staining, imaging and analysis can be independently performed, the protocol is open to modifications at several points (blue). Some steps are not necessary for the general application of the protocol; these are discussed in boxes (purple).

Figure 2 | Visualization of the nanoscale distribution of presynaptic CB₁ receptors on labeled axon terminals of identified neurons by activator-reporter STORM imaging. (a) Hippocampal GABAergic interneurons were filled with biocytin during whole-cell patch-clamp electrophysiological recording in acute slice preparation, and then the biocytin labeling was developed with Alexa Fluor 488–streptavidin according to PROCEDURE Steps 1–8. The maximal intensity projection of the deconvolved confocal image shows the axon of the filled neuron with an array of axon terminals appearing as varicosities. (b) Immunostaining against the CB₁ cannabinoid receptor was visualized using a secondary antibody coupled with Alexa Fluor 405–Alexa Fluor 647 activator-reporter dye pairs, and imaged in normal STORM mode according to PROCEDURE Steps 9–33. (c) The corresponding confocal and rendered STORM images from the sample are overlaid, identifying the localization points that belong to the biocytin-filled axon. (d,e) The enlarged view of the STORM (d) and overlay (e) images from the boxed region in a demonstrates high CB₁ receptor density on the target axon terminal. The experiment was approved by the Hungarian Committee of the Scientific Ethics of Animal Research.



compartment-specific anatomical information from the same sample, and to analyze the two in a correlated manner. As a large variety of cell labeling, molecule labeling, and microscopy techniques are available, this concept can be extended generally, and it can be used in a number of life science applications. In our previous study, we used *in vitro* single-cell biocytin labeling during whole-cell patch-clamp recording in acute brain slices and *in vivo* single-neuron labeling during juxtacellular recording in an awake mouse, which were followed by immunostaining against our proteins of interest, and then by confocal and STORM imaging⁵ (Fig. 2). However, each of these steps can be substituted by a number of alternative methods. To provide cellular context for the superresolution image, cell type-specific labeling can be achieved using immunostaining against neurochemical markers, or cell type-specific expression of genetically encoded fluorescent probes. Region-specific labeling of cells can be obtained through the injection of viral vectors or tracers, or via *in utero* electroporation. Compartment-specific labeling can also be performed by immunostaining against the appropriate markers, or by using selective fluorescent dyes. Any of these labeling methods can be then imaged by a microscopy approach with sufficient resolution, such as deconvolved confocal, structured illumination microscopy (SIM) or stimulated emission depletion (STED) microscopy.

Apart from immunostaining, the molecule of interest can also be labeled by genetically encoded fluorescent tags, SNAP tags or intrabodies. The superresolution SMLM detection of labeling, even in multiple channels, can be achieved by using the appropriate photoactivated localization microscopy (PALM)¹, fluorescence PALM (FPALM)², STORM³ or direct STORM (dSTORM)²² methods. Although we have implemented this protocol on fixed brain samples, a number of alternative methods are viable on live samples²³. Importantly, the STORM method itself has been demonstrated to be suitable for multichannel detection^{24,25}, and for live-cell imaging²⁶ of labeled proteins²⁷, membrane dyes²⁸ or fluorescent proteins²⁹. Detailed protocols for imaging of cell cultures have been published for both dSTORM and PALM imaging methods^{30–32}. Moreover, the VividSTORM analysis software, which is implemented using free libraries in Python and made available with open-source code, can be used as a platform by any researcher for developing additional visualization and analysis functions. As novel correlative methods based on super-resolution

microscopy emerge, such as STORM combined with EM³³ and live-cell imaging³⁴, the correlated analysis of separate microscopy modalities offered by VividSTORM may prove to be useful. Taken together, our method is open to a wide array of modifications and extensions for future applications.

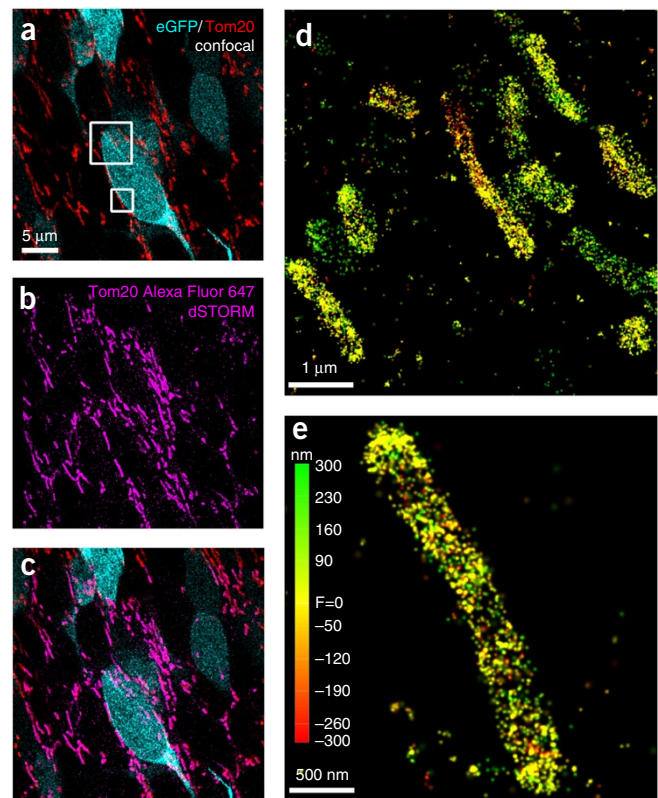
Advantages and limitations

The comparison and advantages of different superresolution microscopy techniques are reviewed extensively elsewhere^{16,18,19,23,35,36}. The main advantage of STORM on biological tissue is its unsurpassed single-molecule localization accuracy, and, consequently, the possibility of high-resolution analysis of molecular distribution. In contrast, the main limitations of STORM are (i) the Nyquist resolution for discerning structural features strongly depends on labeling density; (ii) the low number of currently available dyes that can be used optimally with STORM; and (iii) the strong dependence on molecular labeling methods.

Although STORM is a robust and efficient method for the imaging of nanoscale molecular distribution, the challenges of quantification, multicolor acquisition, 3D detection and correlated image analysis have not been solved comprehensively to date. Thus, these aspects of STORM experiments require thorough planning and consideration.

Quantitivity. A major goal for many experiments using SMLM is the quantification of molecular abundance. Although the current protocol is not optimized for absolute quantification (i.e., counting the actual number of molecules in a region), others have introduced calibration methods to estimate absolute molecule numbers from SMLM data^{37,38}. However, we showed earlier

Figure 3 | Visualization of mitochondria in genetically labeled brain cells by dSTORM imaging. **(a)** Radial glial progenitor cells in the embryonic mouse brain were transfected with an eGFP-expressing plasmid via *in utero* electroporation. Afterward, the brains were fixed and processed for immunostaining against the mitochondrial outer membrane protein Tom20 using commercially available Alexa Fluor 647-conjugated secondary antibodies according to PROCEDURE Steps 9–23. Imaging and analysis were performed according to PROCEDURE Steps 24–33. The deconvolved confocal images of intrinsic eGFP fluorescence and Tom20-specific immunostaining in radial glial cells are shown together. **(b)** The same immunolabeling for Tom20 was subsequently imaged in dSTORM mode as well. **(c)** The corresponding confocal and rendered dSTORM images of the sample are overlaid. **(d,e)** Enlarged views of the top **(d)** and bottom **(e)** boxed regions from **a** show the STORM image with localization points color-coded for *z* position. Individual mitochondria are clearly discernible. Note the sharp outlines of the Tom20 signal, resulting from the excellent localization precision of the dSTORM imaging. The experiment was approved by the Hungarian Committee of the Scientific Ethics of Animal Research.



that the raw NLPs in a STORM experiment using antibody labeling can be used as a relative quantitative readout of protein abundance⁵—i.e., to determine the fold difference in molecule numbers between two sample groups under the same experimental conditions within a certain dynamic range. However, it is important to note that a number of factors can potentially confound the quantitivity, such as epitope accessibility, fluorophore nanoenvironment and, specifically for STORM, the density of blinking events. To draw relative quantitative conclusions on molecular abundance, users should validate that the measurement is carried out in the linear range of the immunostaining and imaging.

Multicolor acquisition. The method presented in this protocol for multichannel STORM imaging using activator-reporter dye pairs²⁵ offers substantial advantages, as the reporter dye is the same (Alexa Fluor 647) for each channel. Consequentially, there is no difference between channels in the chromatic aberration, sensitivity and localization precision, and there is no need to switch filters between channels, thus allowing simultaneous acquisition of both. These circumstances result in a perfect spatial alignment of the molecule lists of each channel without the need for further correction steps. However, because of the continuous low-rate spontaneous activation of reporter dyes, cross talk between channels does occur. An algorithm to correct for this cross talk has been published^{21,25}, and it has been included in the STORM analysis software used in this protocol (Nikon's NIS-Elements N-STORM module). However, as the algorithm relies on the measured local density in each channel to estimate the likelihood of a LP belonging to a given channel, it works reliably only for relatively high labeling densities, and for spatially nonoverlapping channels. However, the algorithm suppresses low-density signal and increases high-density signal at sites of overlap, and it reduces resolution²⁵. For this reason, we do not recommend using dye pairs with the same reporter for multichannel STORM imaging in colocalization studies, or for quantitative measurements of abundance.

Another possibility to achieve multichannel detection is to use reporter dyes of different wavelengths, and to separate the channels either optically^{24,39} or by spectral unmixing⁴⁰. Although these methods offer more reliable separation of the channels, they

also raise the necessity for warp correction to counter the effect of different wavelengths, and, in the case of sequential imaging, alignment between channels. Finally, in our experience, none of the tested dyes emitting at shorter wavelengths (Atto488, Alexa Fluor 488, Cy3, Cy3B, Atto565 and Alexa Fluor 561, data not shown) performed comparably to Alexa Fluor 647 (or Cy5 or DyLight649) in terms of localization precision and sensitivity. However, we note that using higher-power lasers and imaging larger numbers of cycles may compensate for the inferior blinking properties of alternative dyes, as we have previously demonstrated using multicolor imaging of Alexa Fluor 488 and Alexa Fluor 647 in dSTORM mode⁵. Moreover, as the number of dyes tested for SMLM increases⁴¹, we expect rapid development in the quality of true multicolor STORM imaging.

3D detection. For 3D detection, we use astigmatic 3D-STORM⁴² in this protocol, which relies on collecting single-molecule images distorted by placing a cylindrical lens in the light path. Thus, after calibrating the system, the *z* position relative to the focal plane can be estimated from the parameters of the ellipticity of the point spread function (PSF) with a localization precision of ~40 nm in the axial dimension⁵. The advantage of this approach is the high *z*-resolution and the fact that there is no need to scan the sample. However, some limitations also arise. As the image of an emitter at a distance from the objective focal plane is spread over a larger area, the peak intensity is decreased, reducing the probability of detection. As a consequence, the *z*-range of one 3D-STORM image is limited to 600- to 800-nm thickness, and the sensitivity to detect blinking events within this range deteriorates quickly at increasing distance from the focal plane. It is possible, however, to compensate these limitations

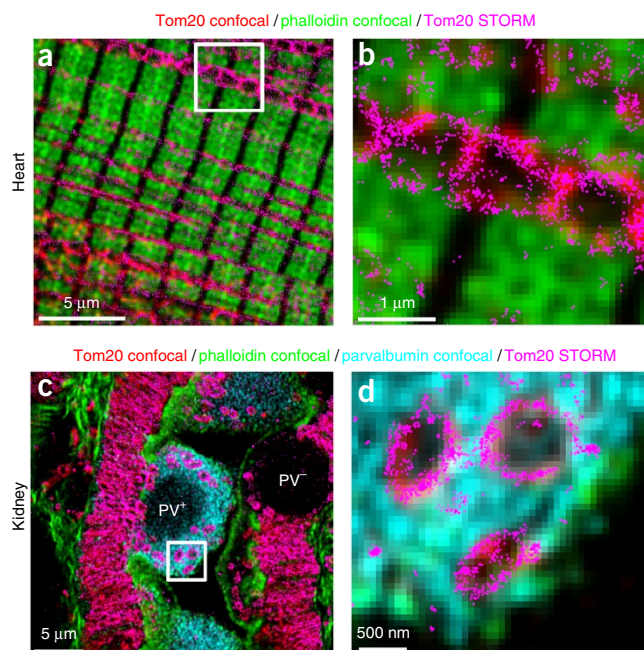
Figure 4 | Immunostaining and STORM imaging in heart and kidney tissue. A mouse was perfused in accordance with the approval of the Hungarian Committee of the Scientific Ethics of Animal Research, and the heart and kidneys were removed and processed, immunostained and imaged according to PROCEDURE Steps 9–45. **(a)** Tom20-specific immunostaining and Alexa Fluor 568–labeled phalloidin were used to visualize the mitochondria in the sarcomeres of cardiomyocytes in a 20- μ m-thick section of ventricular tissue. The confocal and STORM images were overlaid in VividSTORM. **(b)** High-magnification view of the boxed region reveals three individual intermyofibrillar mitochondria strictly ordered in a row. **(c)** Parvalbumin (PV)-specific immunostaining in the kidney cortex labels a subset of epithelial cells of the distal convoluted tubule of nephrons, which are embedded between PV-immunonegative epithelial cells of proximal convoluted tubules. **(d)** The enlarged view of the boxed region in **c** depicts three individual mitochondria from a selected PV-expressing cell, whose Tom20-containing outer membrane is nicely visualized with STORM imaging.

by acquiring z-stacks of partially overlapping 3D-STORM images^{42,43}, and/or by implementing biplane detection⁴⁴.

Correlation with other imaging modalities. For the correlative application of superresolution microscopy with other imaging methods, several approaches have been published. STORM has been used in combination with EM^{33,45}, with confocal microscopy⁴⁶ and with live-cell imaging followed by automated immunostaining in microfluidic devices³⁴. The main goal of the aforementioned approaches is to observe the same signal at different scales, and at different time points, with increased resolution. The key advantage of the approach used in this protocol lies in the correlated analysis of single-molecule data with structural context obtained from conventional images, in a workflow that preserves the individual LPs. Thus, using VividSTORM, quantitative measures of molecular abundance and nanoscale distribution can be obtained from specifically labeled cellular and subcellular compartments in the complex tissue environment.

Experimental design

Immunostaining. Although in theory a number of immunostaining protocols could be implemented for correlated confocal and super-resolution microscopy, our experience shows that acquiring single-molecule images from tissue with sufficiently high signal-to-noise ratio (S/N) can be difficult. This is a crucial issue for STORM, as the number of photons detected over background from a single blinking event determines both sensitivity and localization precision. Thus, contrary to conventional wide-field or confocal imaging, poor S/N cannot be compensated for by increasing staining density, exposure time or frame averaging. Although others have published convincing results with STORM on cryosections²¹ or cryo-ultramicrotome sections³⁷ of fixed tissue, in our hands, samples prepared by routine immunostaining protocols (thick vibratome sections stained in a free-floating manner and subsequently mounted on glass slides, or cryostat sections that have been stained and slide mounted) were not optimal for STORM imaging. The mounting of free-floating sections left a substantial amount of mounting medium between the specimen and the cover glass, which decreased the S/N of the image obtained by the oblique illumination strategy used in STORM. Staining of cryostat sections, in contrast, resulted in poor antibody penetration, and it consequently decreased the contrast of the staining, compared with sections stained in a free-floating manner.



With the immunostaining protocol presented here, vibratome sections are cut to 20 μ m, which is thinner than usual. After free-floating immunoreaction, the sections are mounted and dried on coverslips, and they are covered right before imaging. Thus, the distance of the emitting fluorophores from the coverslip is minimized, yielding higher peak intensities and S/N of the single-molecule images. In parallel, this configuration allows effective excitation with high-incidence oblique (near-total internal reflection fluorescence (TIRF)) illumination, which, together with the reduced thickness of the section, produces low background fluorescence. Because of the resulting high S/N, we were able to obtain images for up to 10- μ m depth into the tissue with sufficient resolution and detection sensitivity (Figs. 2 and 3). Moreover, although it is most frequently applied in the neuroscience field, STORM has been used successfully in other organs, such as the heart⁴⁷ and the kidney⁴⁸. Importantly, the present protocol can also be used on these tissue types without any modification (Fig. 4).

Imaging modality. Successful application of the protocol requires precise assignment of molecule localizations to the investigated target (cellular profile or organelle). The size of the target determines the most suitable method of visualization: a wide-field fluorescence image (with or without deconvolution) might be adequate for profiles that are considerably larger than the diffraction limit ($\sim$ 250 nm), such as cell bodies (>10 μ m). For smaller targets, such as axon terminals of neurons ($\sim$ 1 μ m), high-resolution deconvolved confocal images are required to delineate the borders of the structure without introducing errors due to diffraction, such as overestimation of the size of the target (Supplementary Fig. 1). For even smaller structures or those with elaborate morphology, such as dendritic spines (<1 μ m), or when the target molecule is present at high densities outside the subcellular profile, the resolution of conventional microscopy might not be sufficient, especially in the z dimension. In such experiments, consider the combination of STORM with STED or SIM, or use STORM imaging of the cell membrane⁴³ to achieve maximal resolution. Similarly, the nature of the experiment and the size

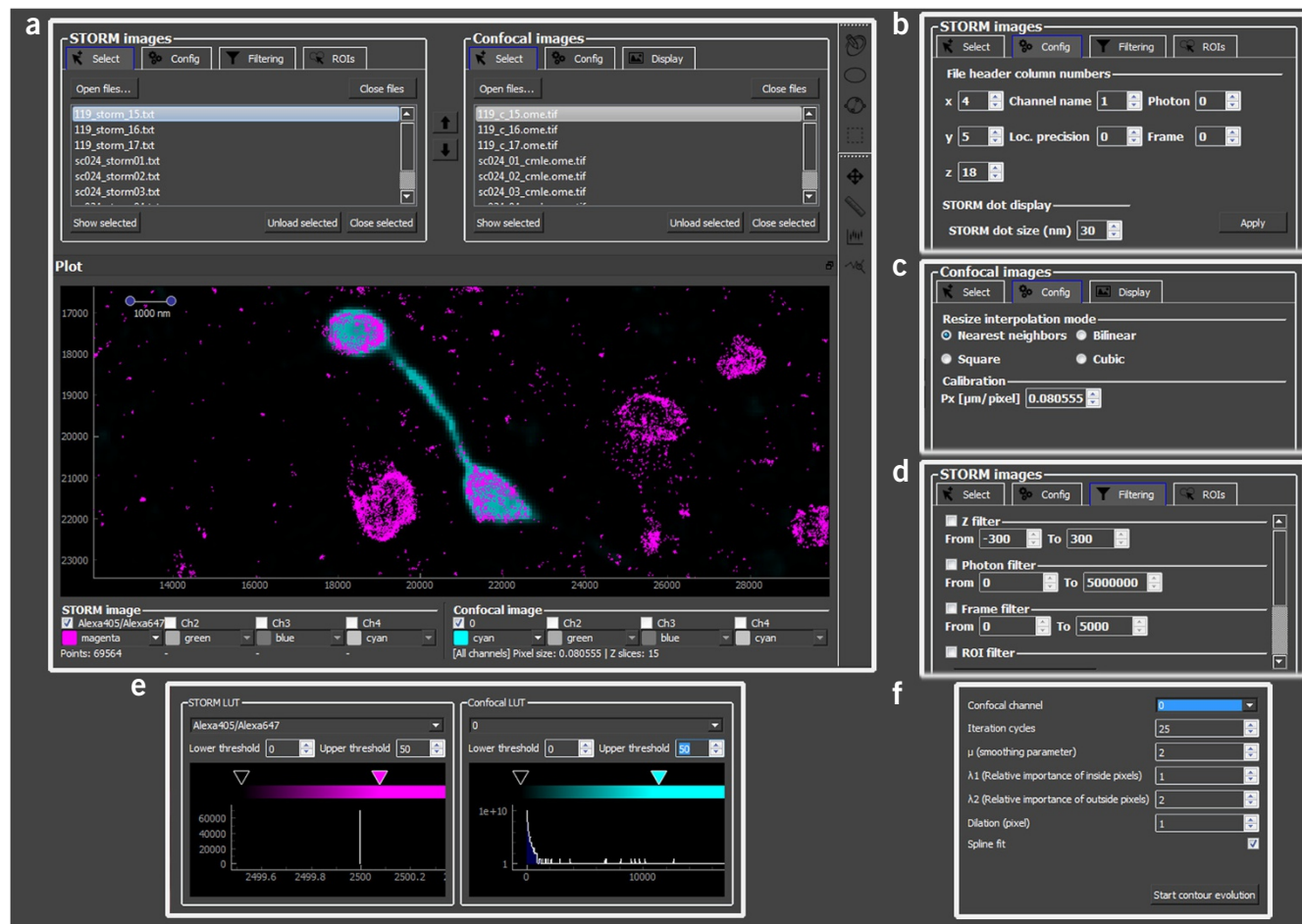


Figure 5 | Overview of the GUI in VividSTORM. **(a)** Starting the program opens three windows. Molecule lists and pixel-based images are loaded into the 'STORM images' window and the 'Confocal images' window, respectively. The 'Plot' window displays the overlay of the active channels from both selected images. The user can pan and zoom the plot using the mouse, toggle the display of channels and select colors for each channels. **(b)** The molecule list is parsed from any tab-separated text file using the 'Config' tab. **(c)** The interpolation mode and calibration of the confocal image are modified in the 'Config' tab of the 'Confocal images' section. **(d)** The STORM data are filtered for various criteria, such as z position, number of detected photons or local density, using the 'Filtering' tab. Only localization points that pass all active filters are included in the analyses. **(e)** The LUT tool is used to change the displayed intensity of each STORM and confocal channel. **(f)** The active contour selection tool automatically outlines labeled structures in the confocal image. The localization points within the selected region are passed for the analysis functions.

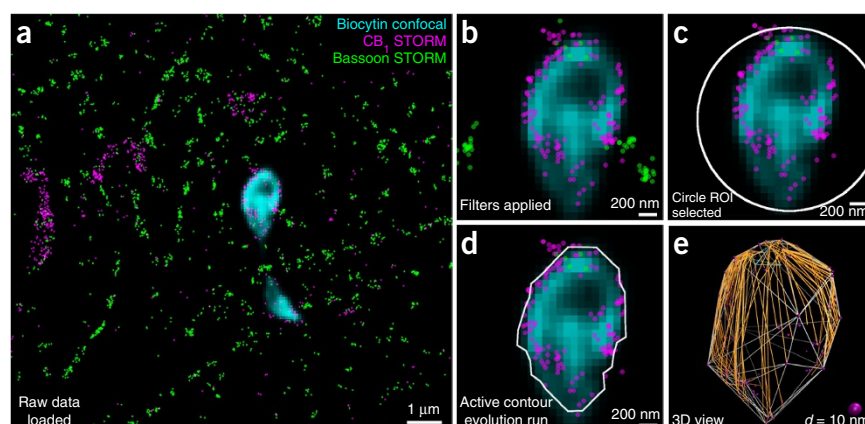
of the target structure determine the required accuracy of the registration of the correlated pixel-based and single-molecule image pairs. For applications in which both images are acquired on the same microscope, such as the examples shown in this protocol, manual alignment can be sufficient. However, when the two images are collected on separate setups, or when the pattern of the staining does not facilitate manual alignment, the use of fiducial markers might be necessary for image registration^{34,46}.

STORM strategy. There are two alternative choices for STORM imaging, which require the use of different types of secondary antibodies. Both are equally viable for imaging immunostained brain tissue, but each has different strengths and challenges. The more straightforward approach is dSTORM²², which uses commercially available secondary antibodies and can be used for multicolor experiments⁴⁰. In the presence of imaging buffer with appropriate reducing agent and reactive oxygen scavenging system, the dye molecules switch between the dark 'off' state to the fluorescent 'on' state spontaneously. This phenomenon can

be facilitated by illumination at a wavelength shorter than that of the excitation. We have found that dSTORM using Alexa Fluor 647-conjugated secondary antibodies and 405-nm activation is easy to implement and gives excellent results (Fig. 3). However, it is important to note that commercially available antibody preparations come with several fluorophores bound to each immunoglobulin protein, and that the fraction of activated molecules per frame is very low.

The second approach for STORM is based on activator-reporter dye pairs bound to the same secondary antibody³. In this case, the STORM imaging consists of cycles of one pulse of activation followed by multiple frames of excitation of the reporter fluorophore. In this protocol, we use Alexa Fluor 405–Alexa Fluor 647 and Cy3–Alexa Fluor 647 dye pairs with 405-nm and 561-nm activator lasers, respectively. With this method, antibodies carry one reporter dye molecule on average, and the reporter can be activated at a higher density compared with dSTORM, using illumination near the absorption maximum of the activator dye. We have previously shown that, using activator-reporter

Figure 6 | Analysis of inter-molecular distances by VividSTORM. Screenshots from an example workflow using VividSTORM are shown. Each program step required for the procedure is listed as the name of the button or checkbox in the GUI, italicized below. The sample was prepared as in **Figure 2**, according to PROCEDURE Steps 1–33, in accordance with the approval of the Hungarian Committee of the Scientific Ethics of Animal Research. (a) A dual-channel 3D-STORM image of immunostaining against CB₁ cannabinoid receptors (magenta) and the presynaptic active zone protein bassoon (green) is loaded, together with the corresponding confocal image of biocytin-labeling (cyan) outlining an axon terminal of the recorded neuron (*Select files, Display channels*). The two images are manually aligned (*Dragging mode*).



(b) To measure the distances from the synaptic active zone, the local density filter function of VividSTORM was first applied (*Local density filter*), removing low-density STORM signal. Second, bassoon localization points outside of the selected active zone (*Circle ROI*) were removed using the ROI filter (*ROI filter*). (c,d) After selecting the starting circle (c) (*Circle ROI*), the active contour tool was used (d) to filter localization points restricted to the area of the bouton (*Active contour*). The 'surface distance between channels' analysis was run on the selected localization points (*Analysis tool*). In this analysis, a convex hull is fitted onto the points from channel A (CB₁). All displayed molecules within the active contour ROI are included in the analysis and in the fitting of the convex hull. Next, the distance of each molecule from the nearest molecule in channel B (bassoon) is determined along the surface of this hull. The program saves the numerical results of the analysis and creates a graphical representation in an interactive 3D view (*Display plots*). (e) The coordinates of CB₁ localization points (magenta), convex hull edges (silver) and minimal distance trajectories (orange) were saved in .pdb file format (*Export to .pdb*) and then rendered in a separate software, Visual Molecular Dynamics (VMD; *Load molecule, adjust graphical representations, render*). The 3D view is slightly rotated for clarity.

dye pairs, the NLPs correlate with protein quantity in a linear manner over a wide range of labeling densities, and that there is no false-positive clustering of the signal⁵. Multiplex staining can be performed with different dye pairs, allowing for multichannel STORM detection. It was shown that under certain conditions (see limitations above in the 'Multicolor acquisition' section), up to three STORM channels sharing the same reporter dye can be separated²⁵, as can up to six channels using multi-parameter detection⁴⁹.

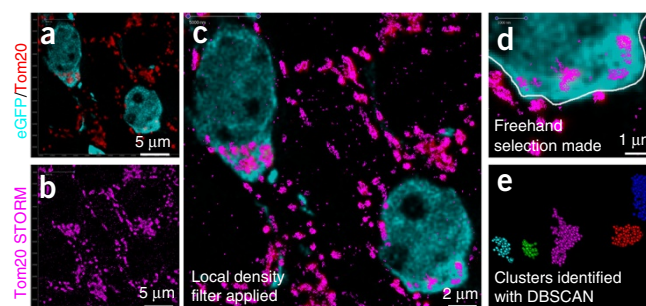
Although the variability of the obtained quantitative results depends greatly on the specific experiment, as a crude reference we note that in a typical experiment measuring the NLPs per axon terminal we observe a coefficient of variation (CV) of 30–40%. This predicts that at least 40 data points per group are required to detect a 20% effect size with 0.8 power and 0.05 alpha in a two-sided *t*-test.

STORM image analysis. Image analysis in the present protocol consists of three major phases: (i) generation of molecule lists; (ii) identification of LPs belonging to the selected neuronal profile;

and (iii) actual measurement of biological significance performed on these LPs. The first step in the analysis of the images acquired in an SMLM experiment is the identification of blinking events and fitting a Gaussian function on each single-molecule image to determine the centroid position. In this protocol, we use the NIS-Elements N-STORM module supplied with the N-STORM system by Nikon for this purpose. The latest version of this software is based on the 3D-DAOSTORM algorithm^{50,51}, which features detection of overlapping peaks, and it is also available as a free stand-alone program. A number of additional software programs are suitable for performing this step, such as Localizer⁵², LivePALM⁵³, Quickpalm⁵⁴ or RapidSTORM⁵⁵.

The second step, segmentation of the STORM image, is not trivial, because SMLM—as opposed to wide-field or point-scanning microscopy methods—generates a list of 2D or 3D coordinates as an output, instead of pixel intensities. These types of data, however, offer novel possibilities for analysis, as highlighted by a number of recent discoveries using STORM and PALM for the quantification of intermolecular distance distribution^{5,21},

Figure 7 | Cluster analysis by VividSTORM. Screenshots from an example workflow using VividSTORM are shown. Each program step required for the procedure is listed as the name of the button or checkbox in the GUI, italicized below. (a) Brain slices were processed according to PROCEDURE Steps 9–33 as in **Figure 3** in accordance with the approval of the Hungarian Committee of the Scientific Ethics of Animal Research. Confocal images of eGFP fluorescence (cyan) and immunostaining against the mitochondrial protein Tom20 (red) were taken (*Select files, Display channels*). (b) Tom20-specific immunostaining is also imaged in 3D-dSTORM mode (magenta; *Display channels*). (c) The corresponding confocal and STORM images are overlaid in VividSTORM, manually aligned (*Dragging mode*), and the local density filter is applied on the STORM data (*Local density filter*). (d) To analyze the number and size of individual mitochondria in transfected neuroblast cells, a freehand ROI containing the cell body of the eGFP-expressing cell was selected (*Freehand ROI*). The localization points located within the ROI were passed for cluster analysis (*Analysis tool*). (e) The resulting clusters (identified with DBSCAN), each representing individual mitochondria, are displayed in different colors in an interactive 3D view (*Display plots*), and the numerical results are saved for subsequent statistical analysis.



clustering^{5,29,37,56,57}, molecule numbers³⁷, orientation²¹ and trafficking^{58,59}. By using the VividSTORM GUI (Fig. 5), the pixel intensity data (obtained, e.g., from a deconvolved multi-channel confocal z-stack) and the SMLM data (collected as 2D or 3D molecule lists of multiple channels) can be loaded and visualized simultaneously. As the next major step, a 2D ROI defined by the labeling in the confocal image can be used to filter only those LPs that belong to the selected subcellular profile (Fig. 6). To facilitate the unbiased selection of the ROI, we have included in VividSTORM the morphological active contour without edges algorithm⁶⁰, which determines the outline of a structure iteratively based on intensity gradients and shape, without relying on threshold parameters. Finally, the LPs within this ROI can be analyzed with one click using the built-in measurement

functions including molecule abundance, distribution (Fig. 6), density, clustering (Fig. 7) and internalization. Taken together, correlated confocal and STORM microscopy by VividSTORM allows rapid visualization and efficient analysis of nanoscale molecular organizational properties within identified subcellular domains belonging to selected target cells.

Controls. As with any immunolabeling-based method, it is crucial to emphasize that each antibody preparation is a unique agent, and a proper negative control is necessary for every assay. To confirm that the STORM LPs indeed represent the target protein, we exploit tissue samples from knockout mice lacking the target protein (Supplementary Fig. 2). We propose this validation approach as the most convincing negative control⁶¹.

MATERIALS

REAGENTS

- 2-Mercaptoethylamine (MEA, also known as cysteamine, Sigma-Aldrich, cat. no. 30070-10G) **! CAUTION** This compound is toxic on contact or inhalation. Wear protective gloves, and be sure to handle MEA in a fume hood.
- Acetone (Sigma-Aldrich, cat. no. 179973-1L)
- Acute mouse hippocampal slices or other tissue samples obtained according to specific laboratory protocols. Slice preparation protocols have been documented extensively elsewhere^{62–64} **! CAUTION** All procedures involving animal experiments should follow approved institutional and governmental animal protocols and comply with the relevant guidelines and regulations of the local animal ethics committee.
- Agarose (for routine use, Sigma-Aldrich, cat. no. A9539-25G)
- BSA (Sigma-Aldrich, cat. no. A2153-10G)
- Alexa Fluor 488–conjugated streptavidin (Jackson ImmunoResearch, cat. no. 016-540-084), required for biocytin- or neurobiotin-labeled samples
- Catalase (catalase from bovine liver, aqueous solution, Sigma-Aldrich, cat. no. C30-100MG) **▲ CRITICAL** Catalase activity is substantially impaired upon freezing; do not substitute it with lyophilized enzyme.
- Dulbecco's PBS (DPBS; Sigma-Aldrich, cat. no. D8537-1L)
- Glucose (D-glucose; Sigma-Aldrich, cat. no. G8270-1KG)
- Glucose oxidase (Sigma-Aldrich, cat. no. G2133-10KU)
- Nail polish (clear)
- Paraformaldehyde (PFA, TAAB, cat. no. P001) **! CAUTION** PFA is toxic, an irritant, flammable and corrosive. It is very hazardous on skin or eye contact or inhalation. Wear gloves and use it in a fume hood.
- Primary antibody against molecule of interest—e.g., rabbit anti-CB₁ (from Dudok *et al.*⁵), 1:1,000, or rabbit anti-Tom20 (Santa Cruz Biotechnology, cat. no. s-11415) 1:500. These antibodies work very robustly and are recommended as positive controls
- Secondary antibody (labeled, not necessary if using custom-labeled antibodies), appropriate target species (Jackson ImmunoResearch, Alexa Fluor 647 AffiniPure donkey anti-rabbit IgG (H+L), cat. no. 711-605-152)
- Sodium azide (Sigma-Aldrich, cat. no. S2002-25G) **! CAUTION** Sodium azide is toxic on skin or eye contact; wear gloves and use respiratory protection.
- Sodium phosphate dibasic (Na₂HPO₄ dihydrate, Sigma-Aldrich, cat. no. 30435)
- Sodium phosphate monobasic (NaH₂PO₄, Sigma-Aldrich, cat. no. 71500)
- TetraSpec 0.1-μm beads (Molecular Probes, T7279)
- Triton X-100 (Sigma-Aldrich, cat. no. T98787-50ML)
- Vectashield (Vector Laboratories, cat. no. H-1000)

EQUIPMENT

- Coverslips, 24 × 40 mm (Thermo Scientific, Menzel-Glaser, no. 1.5)
- Deconvolution software (SVI, Huygens Professional 4.2.1)
- Filter paper (Whatman qualitative filter papers, 70 mm diameter, cat. no. 1002-070)
- Glass microscope slides, 76 × 26 mm (Menzel-Glaser)
- Metal plate for chilling glass slides on ice (minimum 10 × 10 cm)
- Microcentrifuge (Heraeus Biofuge Pico)
- Microwave oven

- Molds for agarose embedding. These were custom-made by etching a 14-mm-diameter circular hole in the middle of a square plastic plate, 35 mm × 35 mm, thickness 3 mm. Alternatively, the molds could be 3D-printed
- Orbital shaker (Biosan OS-10)
- Pasteur pipettes
- Piezo stage controller (Mad City Labs, Nano-Drive). This is only required for the calibration of astigmatic 3D-STORM
- Stereomicroscope with episcopic and diascopic illumination (Optech SL Dual)
- Tissue culture dishes for suspension culture, 12 and 24 well (Greiner Bio-One CELLSTAR 12- and 24-well suspension culture plate, cat. nos. 665 102 and 662 102, respectively)
- Vibrating microtome (Leica VT1000S)
- Workstation for acquisition and analysis: we have used an HP Z800 workstation (CPU Xeon X5670 (2.93 GHz, 6 cores), memory 12 GB, Windows 7, 64 bit). To increase the speed of the analysis with NIS-Elements, the most important parameters for the computer are the number of cores of the CPU, the size of the cache of CPU and the size and access speed of the memory

Microscope system with confocal and STORM capabilities

- Nikon Ti-E with C2 and N-STORM is used in this protocol. The required features for this protocol are given immediately below (with the components we have used in parentheses)
- Vibration isolation table (Supertech, STable)
- Motorized microscope stage (Nikon, Ti-E)
- Focus stabilizing system (perfect focus system, PFS)
- Confocal scan head (Nikon, C2)
- High-sensitivity, high-resolution back-lit electron multiplying CCD camera (EMCCD; Andor, iXon Ultra 897)
- Cylindrical lens inserted at the camera mount
- Laser TIRF illuminator (with a beam-focusing lens to increase intensity at the center of the field)
- Laser board with acousto-optical tunable filters (AOTFs) that allow switching the beam between TIRF and confocal light paths, or dual laser boards
- Lasers, 405, 488 and 561 nm (Melles Griot 56RCS/S2780, Coherent sapphire 488-200 CW and Coherent sapphire 561-100 CW)
- High-power 647-nm laser (300 mW, MPB Communications VFL-P-300-647) **▲ CRITICAL** Obtaining high illumination intensity is crucial for this protocol to perform well. The efficiency of coupling the laser and the extent of beam focusing determines the minimum necessary power of the laser unit. The estimated required power density for good-quality STORM imaging is between 2 and 10 kW/cm².
- High-numerical-aperture (high-NA) 100× oil-immersion objective (Nikon CFI SR Apochromat TIRF 100× oil, 1.49 NA)
- STORM filter cube (allowing simultaneous excitation with the 405 and 647-nm laser lines (and optionally the 488- and 561-nm lines as well), and detection of Alexa Fluor 647 fluorescence—e.g., Nikon N-STORM filter cube)

- Software to control confocal and STORM image acquisition (NIS-Elements AR 4.3 with N-STORM 3.4 is used in this protocol)

REAGENT SETUP

Glucose solution, 50% (wt/vol) Heat 50 ml of ddH₂O to boiling and add 50 g of D-glucose. When it has dissolved, bring the volume to 100 ml with ddH₂O. Store the solution at 4 °C for up to 4 weeks.

Agarose, 2% (wt/vol) Add 2 g of agarose per 100 ml of ddH₂O, and heat the mixture in a microwave oven until the agarose has dissolved, stirring regularly. Bring to the final volume with ddH₂O. Before cooling, divide the solution into aliquots in 15-ml Falcon tubes, and store them at –20 °C for several months. Agarose can be refrozen multiple times.

Fixative (4% (wt/vol) paraformaldehyde in 0.1 M PB) Heat 100 ml of ddH₂O to 65 °C and add 100 µl of 2 M NaOH and 8 g of paraformaldehyde (PFA). Stir the solution on a heated plate until the solution becomes clear. Filter it through paper and make up the volume to 100 ml with ddH₂O, and then to 200 ml with 0.2 M PB (see Reagent Setup below). Divide the solution into aliquots in 15-ml Falcon tubes and store them at –20 °C for several months. Before use, defrost it slowly at room temperature (RT); 21 °C; do not use a water bath to accelerate defrosting. Avoid freezing multiple times.

▲ **CRITICAL** Keep the temperature of the solution <65 °C at all times.

! **CAUTION** Handle PFA under a fume hood.

Glucose oxidase–catalase solution, component of imaging buffer Spin down 40 µl of catalase solution in an Eppendorf tube at 10,000g for 1 min at RT.

Transfer 32 µl of the clear supernatant to a new Eppendorf tube. Add 93 µl of Dulbecco's PBS (DPBS) and 10 mg of glucose oxidase. Vortex the mixture well, and spin down the solution at 10,000g for 1 min at RT. Remove debris from the surface of the supernatant. This solution can be stored at 4 °C for up to 2 weeks. Use the clear supernatant.

Imaging medium As used in Dani *et al.*²¹, this solution is prepared immediately before covering the samples for STORM imaging. In an Eppendorf tube, add 80 µl of DPBS, 10 µl of 2-mercaptoethylamine (MEA; see Reagent Setup below) solution, 10 µl of 50% (wt/vol) glucose solution and 1 µl of glucose oxidase–catalase solution, and aspirate it several times to mix. ▲ **CRITICAL** The optimal imaging medium depends on the fluorophore; the medium used here works well for Alexa Fluor 647, but it may need to be optimized for other fluorophores.

2-Mercaptoethylamine (MEA) solution, component of imaging buffer Add 77 mg of MEA to 1 ml of 1/3 molar HCl, and vortex the mixture. This solution can be stored at 4 °C for up to 2 weeks. ! **CAUTION** Handle MEA under a fume hood.

Phosphate buffer (PB) This buffer contains 0.1 M Na₂HPO₄ and 0.1 M NaH₂PO₄ at pH 7.4. To prepare this solution, make 800 ml of 0.2 M Na₂HPO₄ and 200 ml of 0.2 M NaH₂PO₄ in separate beakers. By continuously monitoring the pH, start adding the NaH₂PO₄ to the Na₂HPO₄ while the pH reaches 7.4. The solution can be stored at RT for 2 weeks (optionally, it can be autoclaved). Before use, dilute the

Box 1 | Labeling of secondary antibodies ● TIMING 1 h

This protocol is adapted from the N-STORM sample preparation protocol provided by Nikon Instruments for the generation of activator-reporter dye-labeled secondary antibodies used in immunostaining in tissue sections.

MATERIALS

Reagents

- Alexa Fluor 647–N-hydroxysuccinimide (NHS) ester (succinimidyl ester; Molecular Probes, cat. no. A-20006). Dissolve the dyes in DMSO at a concentration of 0.2 mg/ml. Divide the solution into aliquots and store them at –20 °C.
- Alexa Fluor 405–NHS ester (succinimidyl ester; Molecular Probes, cat. no. A-30000).
- Cy3 mono-reactive dye pack (GE Healthcare, cat. no. PA23001), optional, only required for dual-channel STORM imaging.
- DMSO (Sigma-Aldrich, cat. no. D8414-50ML).
- G-25 column (Illustra NAP-5 Sephadex G-25 columns, GE Healthcare, cat. no. 17-0853-01).
- Secondary antibody, unlabeled, appropriate target species—e.g., AffiniPure donkey anti-rabbit IgG (H+L; Jackson ImmunoResearch, cat. no. 711-005-152).
- Sodium bicarbonate (NaHCO₃, Sigma-Aldrich, S5761).

Equipment

- Spectrophotometer for UV-visible absorption measurement (e.g., Bio-Rad SmartSpec Plus spectrophotometer and trUView cuvettes).

PROCEDURE

1. Add the required amount of dye to 50 µl of the unlabeled secondary antibody. Recommended volumes: 4 µl of Alexa Fluor 405 and 0.6 µl of Alexa Fluor 647. For multichannel immunolabeling, prepare additional secondary antibodies with Cy3 as activator, and use 1.5 µl of 0.2 mg/ml dye for the labeling mixture.
2. Add 0.1 M NaHCO₃ (6 µl of 1 M solution), and vortex the mixture.
3. Wrap the mixture in tin foil, and incubate it for 30 min at RT on an orbital shaker. During incubation, equilibrate a NAP-5 column by running three column-volumes of DPBS.
4. Add 140 µl of DPBS to the labeling mixture to stop the reaction.
5. Apply the mixture to the column prepared in step 3.
6. Wash with 550 µl of DPBS.
7. Elute with 300 µl of DPBS.
8. Dilute some of the eluent 5× with ddH₂O (e.g., 15 µl eluent + 60 µl water), and measure absorbance on a spectrophotometer against water at 280, 401 (or 550 when using Cy3) and 650 nm.
9. Calculate dye and antibody concentrations using Beer-Lambert's law considering the extinction coefficients and CF280 values of the dyes provided by the manufacturer. Ideally, Alexa Fluor 405 or Cy3 (activators) to antibody concentration ratio should be 2–3, and Alexa Fluor 647 (reporter) to antibody ratio should be <1. The typical yield using this protocol is 250 µl of 120 µg/ml labeled antibody, which is enough for 30 immunostaining reactions.
10. After the absorbance measurement is complete, add 5 mg of BSA and 3 µl of 20% (wt/vol) sodium azide to the labeled antibody, which can be stored at 4 °C for several months. Protect it from light.
11. Regenerate and store the columns according to the manufacturer's instructions.

Box 2 | Calibration for 3D-STORM and multicolor dSTORM ● TIMING 3 h

This protocol is specific for the Nikon Ti-E microscope with N-STORM.

Calibration is best done at night or at times when thermal and mechanical instabilities are minimal. The microscope and the stage controller should be switched on several hours before calibration (and generally should be continuously turned on at all times). The temperature of the room should be stable and identical to the temperature during experiments.

1. Turn on all required components. Place the piezo stage insert in the microscope and set the piezo controller voltage in the specified range. Clean the objective.
2. To prepare the antibody sample, dilute 10 μl of commercially available Alexa Fluor 647-labeled antibodies in 300 μl of DPBS, and spread them on a clean 24 $\times$ 40-mm coverslip. Leave the coverslip covered from dust and light for 25 min, and then rinse it with distilled water and leave it to dry at RT (covered to protect from dust and light).
3. To prepare the bead sample, dilute 2 μl of TetraSpeck 100-nm beads in 300 μl of DPBS. Spread the sample on a second coverslip, incubate it, rinse it and dry the sample (covered to protect from dust and light).
4. Pipette 20 μl of DPBS onto two clean slides, and cover one with the antibody-coated coverslips and the other with the bead-coated coverslip (avoid bubbles). Seal them with a thin layer of nail polish. Leave them to dry for 10 min on the microscope stage to equalize the temperature.
5. Place the sample with beads in the piezo stage, and find the focus using the EMCCD image and the microscope z-motor. Remove the cylindrical lens from the light path and take a z-stack of the beads (3 μm range, 125 nm step size) and check the shape of the PSF. Adjust the temperature correction collar on the objective, refocus and take a new z-stack until the PSF is symmetrical and well-focused (see x-y maximal intensity projections of bead images in **Fig. 8a**).
6. Place the sample with antibodies in the piezo stage, and find the focus (this specimen is very dim, so noting the z position in focus with the beads from the previous step is useful). Find a field of view with evenly distributed antibodies (lacking aggregates). Switch to the filter cube you need to calibrate for, and place the cylindrical lens back into the light path. Engage the z-calibration module, and turn on the live monitoring of the axial ratio. Increase the laser power to achieve continuous blinking of the sample.
7. Focus with the piezo until the axial ratio is the closest to 1. Keep monitoring, and adjust the piezo if necessary. Wait until the baseline is stable for at least 2 min.

▲ **CRITICAL STEP** This is a crucial point for a successful calibration. If a stable baseline is difficult to establish, then turn off air conditioning temporarily.

8. Once the baseline is stable, run the z-calibration protocol. Open the .nd2 image file acquired by the calibration module and analyze it with peak settings that allow detection of blinking even at greater distances from the coverslip. Screenshots from the STORM module GUI at different z positions from a successful calibration are shown in **Figure 8b**. Yellow rectangles indicate the detected molecules in the given frame.

9. Click the 'Auto calibrate' function in the z-calibration window, and inspect the resulting curves with frame averages. The result of a successful calibration is shown in **Figure 8c**. Note that the baseline values before and after the z-stepping protocol are similar, that the calibration curves are steep and that they fit the data points well in the ± 400 -nm range. Repeat the calibration if necessary.

10. If the system will be used for multicolor dSTORM, additional calibration will be necessary to align STORM images taken at different wavelengths. This is automatically performed in NIS-Elements, known as Warp correction. To use this function, repeat the z calibration (**Box 2**, steps 7–9), using TetraSpeck beads as the sample. Run the z calibration with all available wavelengths enabled, with low-power excitation to get good-quality images of the beads (avoid saturation). The quality of the z calibration here is not important, as only the x-y positions of the beads will be used. Load the nd2 file, and click the 'XY Warp Calibrate' button. The system will store the transformations and automatically perform this correction on every analyzed image. Antibody-derived curves are substantially better than bead-derived curves, because the physical size of the bead is ignored for the fitting. Thus, one should avoid performing the autocalibrate function for z calibration on the bead data.

The coordinates of LPs in the.txt molecule lists exported from NIS Elements are provided with and without drift and warp corrections. Identified by column headers, the X, Y and Z columns are the raw positions, whereas Xc–Zc are drift-corrected, Xw–Yw are warp-corrected, and the Xwc and Ywc values are modified by both corrections. With default settings, VividSTORM reads the Xwc, Ywc and Zc coordinates. It is important to note that blinking events that last for more than one frame appear only once in the exported molecule list (in the first frame of detection).

required amount of 0.2 M buffer two times with ddH₂O to obtain the final concentration.

Secondary antibody (optional) If you are planning to use custom-labeled antibodies with activator-reporter dye pairs, prepare secondary antibodies according to **Box 1**. Prepare Alexa Fluor 405- and Alexa Fluor 467-labeled antibodies for single channel, and additionally Cy3- and Alexa Fluor 467-labeled antibodies for dual-channel, STORM imaging. The secondary antibodies can be stored at 4 °C for several months in the presence of 20% sodium azide.

Tris-buffered saline (TBS) This buffer contains 0.05 M Tris and 0.9% (wt/vol) NaCl at pH 7.4. Add 6.61 g of Trizma hydrochloride and 0.97 g of

Trizma base to 950 ml of ddH₂O. Check the pH and adjust it to 7.4 if necessary. Add 9 g of NaCl and complete it to 1,000 ml. This solution can be stored at RT for 2 weeks (optionally, it can be autoclaved).

EQUIPMENT SETUP

Calibrate STORM setup for 3D-STORM (Only required for 3D-STORM; **Box 2** and **Fig. 8**) Enter the camera calibration information (gray value to photon count) in the software from the data sheet supplied by the camera manufacturer (optional). Align the confocal and electron-multiplying charge-coupled device (EMCCD) cameras. Perform the physical alignment (rotation) of the two cameras using a calibration slide. Next, place a suitable sample (e.g., TetraSpec beads) in the microscope, and take an

Figure 8 | Calibrating the setup for STORM imaging. See **Box 2** for detailed description of the calibration. (a) Maximal intensity x-z view of a bead at different temperature correction collar settings. The collar was adjusted in equal steps (2 mm on the objective circumference) between the two endpoints. (b) Recognized blinking events at different z positions in the z-calibration file are shown with yellow rectangles. (c) Example results of a high quality z calibration.

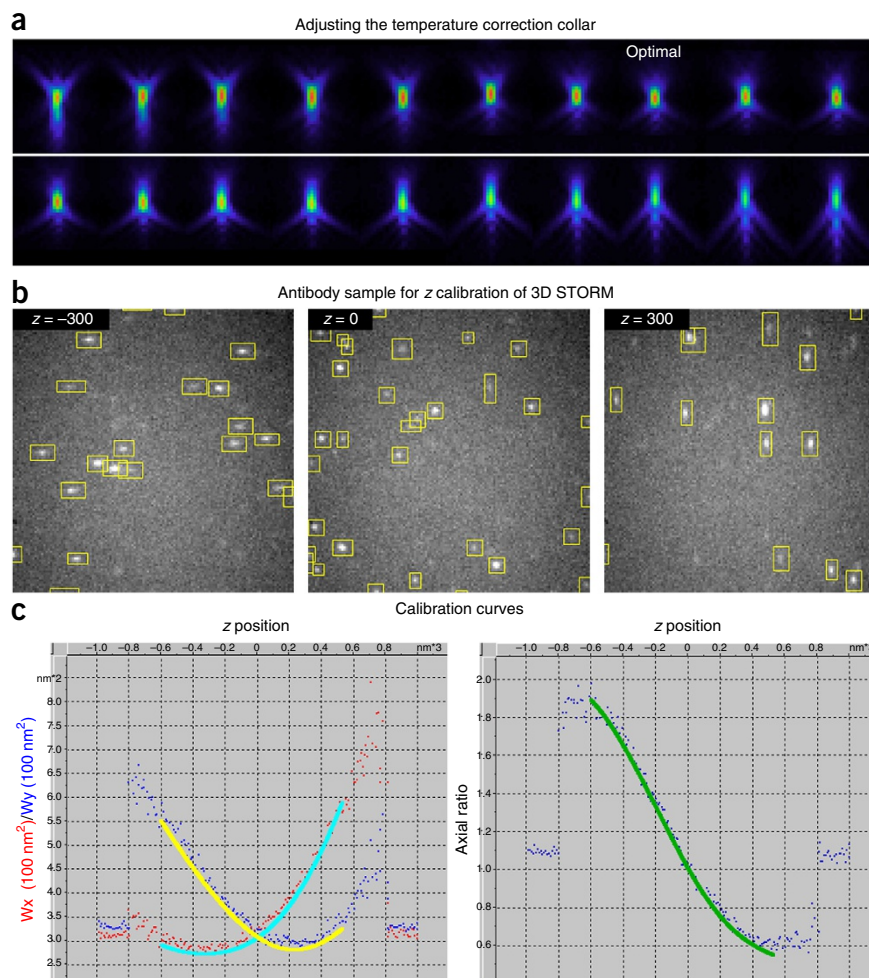


image using the objective, filter cube, cylindrical lens and EMCCD camera settings used for STORM imaging. Make sure that stage drift is minimal. Switch to the confocal camera, and take images at the same wavelength. Adjust the scan area settings until the field of view is most similar to the EMCCD field. The pixel size of the confocal and the CCD image should be different with appropriate sampling—e.g., the 256 × 256 pixel image of the EMCCD ROI (160 nm/pixel) can be matched with the 512 × 512 pixel confocal scan area (80 nm/pixel). Final alignment of the EMCCD and confocal images will be performed after acquisition, if necessary. Open the acquired confocal and EMCCD images in ImageJ, and then resize the EMCCD image to fit the size of the confocal image. Perform fitting using manual three-point affine transformation using the TurboReg plug-in⁶⁵, and then save the transformation parameters. See the ‘Convert_Stacks’ script at <http://katonalab.hu/index.php/vividstorm> (ImageJ/FIJI toolset) for help with applying the saved transformation on all confocal image stacks.

PROCEDURE

Fixation and development of specimen ● TIMING 1 or 2 d

▲ **CRITICAL** Steps 1–9 are specific for processing neurons filled by biocytin during whole-cell patch-clamp recording in 300-μm-thick hippocampal slices. See Dudok *et al.*⁵ and Lee *et al.*⁶⁶ for details on the acute slice electrophysiology. Begin from Step 10 if a different method of labeling cells or organelles has been used.

▲ **CRITICAL** All procedures involving animal experiments should follow approved institutional and governmental animal protocols and comply with the relevant guidelines and regulations of the local animal ethics committee.

1| After whole-cell patch-clamp recording and dye-filling, place the acute slice preparation in 2 ml of RT 4% (wt/vol) PFA in a 12-well plate, and incubate it for 24–48 h at 4 °C.

2| Wash the slices in PB for 10 min at RT. Place the plate onto an orbital shaker (120 r.p.m.) for this and each subsequent washing and incubation step.

■ **PAUSE POINT** Fixed slices can be stored for 1–2 weeks at 4 °C. Supplement the storage buffer with 0.05% (wt/vol) sodium azide.

3| Wash the slices by replacing the buffer with 1 ml of fresh TBS (three washes of 20 min each, at RT).

4| Wash the slices twice in TBS containing 0.5% (vol/vol) Triton X-100 for 30 min per wash to permeabilize membranes. Note that using nonionic detergents can prevent immunostaining for certain antigens. Optimize the membrane permeabilization protocol for each antibody.

5| Incubate the sections in TBS containing a 1:1,000 ratio of Alexa Fluor 488:streptavidin for 3 h.

PROTOCOL

6| Wash the sections in TBS (three washes of 20 min each at RT) and PB (two washes of 10 min each at RT).

7| Mount the sections on glass slides, with the side containing the filled cell facing upward. Remove excess buffer with filter paper. Cover each slide with Vectashield and seal it with nail polish.

■ **PAUSE POINT** Slides can be stored at 4 °C for 1–2 weeks.

8| Image the cell for reconstruction and morphological analysis. Typically, a confocal z-stack recorded using a 20× 0.75 NA objective at 300 × 300 × 1,000 nm/pixel resolution is adequate for the reconstruction of cellular processes.

? TROUBLESHOOTING

Tissue preparation and immunostaining ● **TIMING 2 d**

9| If samples were processed according to Steps 1–8, remove the slices from the slides by cutting away the nail polish with a blade and lifting up the coverslip carefully, and return them to the tissue culture plate. Wash in PB (two washes for 10 min each). If the samples have been processed differently from Steps 1–8, resume staining at this point. Make sure that all traces of fixative have been completely removed by extensive washing in PB.

▲ **CRITICAL STEP** No further steps for membrane permeabilization are included in the protocol. We recommend adding 0.3% (vol/vol) Triton X-100 to the blocking solution in Step 17, if the slices were not permeabilized during an earlier step.

10| In preparation for agarose embedding, heat an aliquot of frozen 2% (wt/vol) agarose in a microwave oven until it has dissolved. Vortex the boiling agarose until the solution becomes clear. Repeat the heating if necessary.

! **CAUTION** Loosen the cap regularly to keep pressure from building up. Use protective gloves, and be careful to avoid burns.

11| Transfer the section to a glass slide, with the labeled side of the section facing upward. Remove excess buffer with filter paper. Place the slide on a prechilled (–20 °C) metal plate resting horizontally on the surface of ground ice (**Supplementary Fig. 3a**).

12| As soon as the glass slide cools down, quickly place the embedding mold on the specimen, and fill it with agarose using an inverted glass Pasteur pipette. Cover the mold immediately with a second glass slide and leave until the agarose sets (2–5 min).

▲ **CRITICAL STEP** It is important to work quickly to prevent the tissue section from drying out.

13| Once the agarose has solidified, remove the block from the mold.

■ **PAUSE POINT** Blocks can be stored for several hours in PB at 4 °C until sectioning (the agarose will slowly start to dissolve at RT).

14| Fill the required number of wells of a 24-well tissue culture plate with PB containing 1% (wt/vol) BSA, and replace it with 1 ml of PB after 10 min. Adding BSA is optional, and it prevents sections from sticking to the wall of the plate.

15| Trim the blocks from Step 13 so that a small amount of agarose is present around the slice (**Supplementary Fig. 3b**). Section on a vibratome in PB, and collect the sections in PB in a pretreated 24-well tissue culture plate (from Step 14). Although this protocol gives good results for sections sized 10–50 µm, we generally recommend using a section thickness of 20 µm. Thinner sections are more difficult to handle, but STORM imaging can be performed to a depth of up to 10 µm without loss of quality. Thus, in thick sections, a smaller fraction of the tissue can be imaged.

■ **PAUSE POINT** The sections can be stored at 4 °C for several weeks if 0.05% (wt/vol) sodium azide is added to the storage buffer, taking the cumulative storage time from previous steps into account.

16| Transfer the sections for immunostaining into BSA-treated wells containing 0.5 ml of TBS using the tip of a glass Pasteur pipette bent over flame. Avoid staining of more than four sections within the same well. Wash in TBS (two washes, 20 min each at RT). For this and Steps 16–21, replace the solutions by carefully removing the buffer from the well using a clean glass Pasteur pipette, while avoiding touching the sections. Avoid drying the sections. After adding the fresh solution, make sure that the sections are not sticking to the well or floating on the surface of the buffer. Return the plate to the shaker for each step.

17| Replace TBS with 1% (wt/vol) BSA in TBS for blocking. Incubate the plate for 45 min at RT (if Step 4 was omitted, add membrane permeabilization agent at this step; e.g., 0.3% (vol/vol) Triton X-100).

18| Incubate the plate in primary antibody (or antibodies) in TBS at RT on a shaker overnight. Optimize the blocking solution and primary antibody concentration for each target protein. If the experiment involves multiplex immunostaining, include other primary antibodies at this step. As a control for specificity, run two parallel experiments: one using knockout mouse tissue and one omitting primary antibody (**Supplementary Fig. 2**).

19| Wash the plate in TBS (four washes 15 min each at RT).

20| Incubate the sections on the plate in secondary antibody (or antibodies) in TBS for 4 h at RT on a shaker. For dSTORM, use commercial antibodies at a 1:400 dilution. For STORM with activator-reporter dye pairs, use custom-made antibodies at 2 µg/ml. If the experiment involves additional staining, include other secondary antibodies or dyes at this step.

21| Wash in TBS (two washes of 15 min each at RT) and PB (two washes of 10 min each at RT).

? TROUBLESHOOTING

22| Prepare coverslips by dipping them in acetone and air-drying them. Clean the tools used for mounting with 70% (vol/vol) ethanol and ddH₂O to remove dust particles. Transfer the sections one by one to a Petri dish containing clean, debris-free PB. Under a stereomicroscope, remove agarose from around the tissue, and trim the brain ROI with a scalpel. Smaller-sized tissue pieces are easier to mount and more stable to image.

23| Rinse an inverted glass Pasteur pipette with PB and transfer the tissue to the center of a cleaned coverslip in a small drop of PB. Under a stereomicroscope, remove excess buffer carefully while keeping the section flat and unfolded. Remove all of the buffer gradually using a glass Pasteur pipette (**Supplementary Fig. 3c,d**). Avoid contamination of the coverslip with any debris.

■ **PAUSE POINT** Store the coverslips dry at 4 °C until imaging. The dried sections can be stored for several weeks, but experiments with quantitative comparisons should be performed on samples stored for the same length of time.

STORM imaging ● TIMING 30–180 min

24| Turn on the imaging setup. Prepare fresh imaging medium, as described in Reagent Setup.

25| Place a 25-µl drop of imaging medium onto the center of a clean slide. Place one end on the coverslip on the slide and, with a tweezer, gently lower the coverslip onto the slide so that the medium covers the entire section under the coverslip without forming any bubbles. Seal it with clear nail polish, and dry it for 10 min on the microscope stage.

▲ **CRITICAL STEP** The optimal imaging medium depends on the fluorophore; the medium used here works well for Alexa Fluor 647 but may need to be optimized for other fluorophores.

26| Insert the specimen in the microscope, and focus on the ROI with the objective used for STORM imaging. After turning on the imaging laser at minimal power, the immunostaining should be visible in the far-red channel. To check that the fluorophore can be switched, increase the laser power to maximum; the sample should bleach within seconds, and spontaneous blinking of fluorophores should be clearly visible. Upon brief pulses of illumination with low-power 405-nm laser, the density of blinking events should transiently increase. See **Supplementary Video 1** as an example of the desired blinking behavior.

? TROUBLESHOOTING

27| Optimize the microscopy parameters for STORM imaging (Steps 27–30); note that the region of the sample used for the optimization will be bleached, and thus will be lost for quantitative imaging. Set the laser TIRF illuminator incidence angle to optimal S/N (**Fig. 9a**). Insert the cylindrical lens in the light path for astigmatic 3D-STORM.

28| Turn the imaging laser (647 nm) to maximal power, and allow the sample to bleach. Blinking should be visible at this stage. Set the camera parameters for optimal S/N. On the Nikon N-STORM system with the Andor DU-897 EMCCD, it is generally not advisable to use exposure times longer than 30 ms, considering the duration of the dye blinking events. The EM gain should be set to the highest possible value without detectable electron noise or saturation, typically 300 (**Fig. 9b**). The readout gain should be set to the highest possible value without saturating peak intensities, typically 2.4. Binning should be avoided.

29| After optimizing the illumination and camera settings, move to a new field of view of the sample and illuminate with maximal power from the imaging laser. Engage the activator laser at minimal power, and increase the power gradually until blinking becomes sufficiently dense, but not extensively overlapping (**Fig. 9c**). The optimal intensity highly depends on

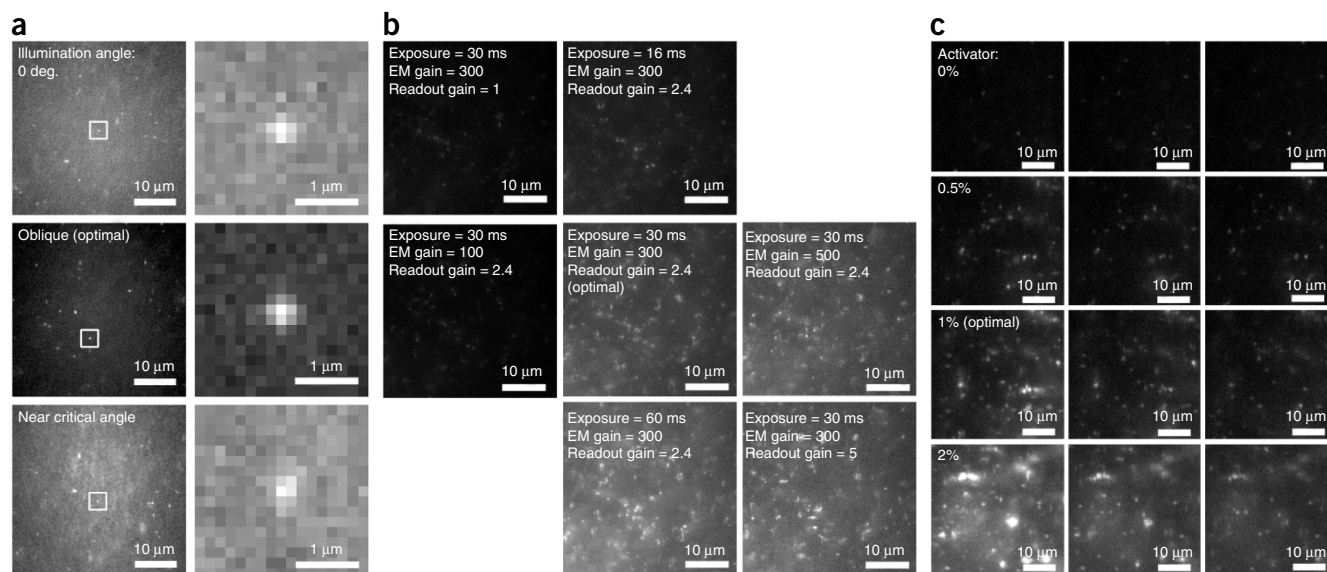


Figure 9 | Optimizing steps for STORM imaging in tissue preparations. **(a)** A hippocampal section containing CB₁ immunostaining with secondary antibodies labeled with Alexa Fluor 405–Alexa Fluor 647 activator-reporter dye pairs prepared according to PROCEDURE Steps 1–23 in accordance with the approval of the Hungarian Committee of the Scientific Ethics of Animal Research. The sample is imaged continuously with 647-nm excitation at maximal laser power. All panels show images with a gamma correction of 1.5. Single image frames and enlarged views of single-molecule blinking events are shown with different incidence angle settings of the TIRF illuminator of the microscope. After optimizing the incidence angle (PROCEDURE Step 27), low background intensity and high peak signal can be obtained. **(b)** The exposure time, EM gain and readout gain of the camera are optimized according to PROCEDURE Step 28 under the imaging conditions set up in **a**. **(c)** Using the camera settings from **b**, STORM imaging is engaged in a second, previously unbleached field of view, using one activator (405-nm excitation) and three reporter (647-nm excitation with maximal power) frames per cycle. The reporter frames after activation from representative cycles are shown with different activator laser powers (PROCEDURE Step 29). **(d)** A third, previously unbleached area is imaged under the conditions optimized through **a–c** for 10 min. The graphs show the time course of the number of localization points (NLPs) per frames (black), the cumulative NLPs (red) and the cumulative NLPs per elapsed time (gray) during the imaging. The NLPs per frame are decreased to 50% after 3,000 frames (or 750 cycles, or 90 s), and the cumulative NLPs per time are decreased to 50% after 8,000 frames (or 2,000 cycles, or 4 min).

the density and pattern of the staining, so use a sample area that is similar to the area of interest for the purpose of the experiment. On the Nikon N-STORM system, typically 10–30% of 405-nm laser power can be used for dSTORM imaging with the STORM filter cube. For STORM with activator-reporter dye pairs, typically 0.5–2% of 405-nm laser power is optimal. For multicolor imaging, ensure that the first frames after activation have substantially more blinking events than later frames (**Fig. 9c**), as only the former will appear as specific signal. Different blinking densities may be optimal for different peak detection algorithms, so test-run the peak detection phase with the given settings before proceeding with imaging. Always use the same illumination, camera and activation settings for every image throughout an experiment for the consistency of results.

30 | Optimize imaging parameters, such as activation mode and number of frames. For dSTORM, we recommend using continuous activation mode (both the activator and imaging lasers are continuously on) together with a filter cube that blocks fluorescence excited by the activator laser, such as the STORM filter cube. For STORM with activator-reporter dye pairs, we recommend using sequential activation mode (the activator and imaging lasers are turned on in separate frames). This mode is also viable when the filter cube transmits fluorescence excited by the reporter laser (such as dual or quad channel cubes). The optimal number of frames per image largely depends on the specific experiment. As a starting point, we recommend 5,000 frames in continuous mode, or 1,000 cycles of one activator (only activator laser is on) and three reporter (only imaging laser is on) frames per channel per cycle for continuous mode. The time course of detected LPs as a function of imaging time is shown in **Figure 9d**.

31| Under 488-nm illumination, find the labeled cell using the live EMCCD image. Set the profile of interest to the center of the field of view (note that the illumination is not homogeneous in the field of view). Engage the focus stabilizing system (Perfect Focus System, PFS) on the microscope, and set the focus.

? TROUBLESHOOTING

32| Switch to the confocal light path, and take a z-stack of 1.5- μ m range relative to the focal position set by the PFS. For deconvolution, we have found that 80 by 80 by 150 nm (in the *x*, *y* and *z* dimensions, respectively) resolution is sufficient. Avoid saturated pixels in the image. Use the field of view setting calibrated in Equipment Setup. Image all relevant channels. It is recommended to image the far-red channel as well, because it greatly helps with aligning the confocal and STORM images later. When using custom-made secondary antibodies with activator-reporter dye pairs, it is not recommended at this point to use excitation that could bleach the activator dye (405 or 561 nm for Alexa Fluor 405 and Alexa Fluor 561, respectively), as this will greatly reduce activation rate and introduce variability. However, these channels can still be imaged after taking the STORM image.

33| Switch back to the EMCCD light path, and run STORM imaging with the settings optimized in Steps 28–30. For increased consistency, illuminate the specimen with maximal imaging laser power (>2 kW/cm² in the field of view) until the image is no longer saturated—i.e., most of the fluorophores are in the ‘off’ state (1–2 s) before starting the STORM imaging.

? TROUBLESHOOTING

Image processing ● TIMING 30 min

34| Deconvolve the confocal images. This is necessary as the confocal image will be used to provide structural information for the super-resolution image, and thus the effect of light scattering on overestimating profile sizes has to be reduced. We have used the classic maximum likelihood estimation (CMLE) algorithm in the Huygens Professional software by SVI, which generally gives good results with default settings (**Figs. 2a** and **3a**). The refractive index of the imaging medium for this purpose can be considered to be equal to water (1.338). Convert the stacks to OME (.ome.tif) image format after deconvolution. If reference image pairs were previously recorded, or fiducial markers were used, then transform the stacks to align with the STORM image. See the ‘Convert_Stacks’ script at <http://katonalab.hu/index.php/vividstorm> (ImageJ/FIJI toolset) for help with batch conversion. The script also performs affine transformation of the confocal image using TurboReg to match the EMCCD, if reference image pairs have been recorded; see Transformation_calibration.pdf at <http://katonalab.hu/index.php/vividstorm> for detailed instructions.

35| Run peak detection on STORM images. We have used the NIS-Elements N-STORM module, which is based on the 3D-DAOSTORM algorithm^{50,51}. Spherical aberration is corrected in this algorithm using a model based on numerical simulations⁶⁷ to correct the calibration curve as a function of imaging depth, which is measured as the distance from the coverslip and stored in the STORM image files. The model has some refractive index-dependent empirical coefficients that are stored in the software. Chromatic aberrations are corrected by using individual z-calibration curves for each wavelength, and by warp calibration (**Box 2**). Load the 3D calibration data. Open an image, and inspect several single-molecule images with the peak statistics tool to assess a threshold for the peak intensity to avoid detection of noise. A typical threshold is 1,000 levels. Finally, run the analysis on each file and export the resulting molecule lists in text format. For 3D STORM, we recommend turning the ‘Fit overlapping peaks’ option off, as it does not yield better results but takes one or two orders of magnitude more time to run, depending on the images. For 2D storm experiments with high labeling density, using overlapping peaks fitting might be helpful. Typical results are shown in **Figures 2b** and **3b**. Use the ‘Batch_STORM’ and ‘Export_STORMdata’ scripts at <http://katonalab.hu/index.php/vividstorm> (Python) for help with batch analysis and export. Note that the NIS-Elements STORM module also performs image-based correction for sample drift in *x*, *y* and *z*. If necessary, perform cross talk correction on multicolor STORM data after the peak analysis. Use the same calibration and threshold settings for every image of the same experiment.

Image analysis with VividSTORM ● TIMING variable

36| Download VividSTORM (<http://katonalab.hu/index.php/vividstorm>) and run the program.

37| Load files containing molecule lists (from Step 35) using the ‘Open files’ dialog in the ‘STORM images’ section of the GUI (**Fig. 5a**). The default settings for reading molecule lists are set up for STORM data exported from the NIS-Elements STORM module. These settings can be changed in the ‘Config’ tab in the STORM section (**Fig. 5b**). Set the number of the column containing each data type, or 0 to omit (*x* and *y* coordinates are always necessary). Molecule lists containing up to four channels can be handled, and LP coordinates are expected in nanometers.

38| Load files containing microscopy images (from Step 34) using the 'Open files' dialog in the 'Confocal images' section. Microscopic image stacks of up to four channels in .ome.tif format can be opened.

39| Click the file names to open the fields of interest (**Fig. 5a**). One molecule list and one microscopy image can be opened at the same time. Use the 'config' tab from the Confocal section to set the pixel size, if calibration was not included in the metadata (**Fig. 5c**). Use the 'Display' tab to slice through a confocal z-stack. Use the arrow buttons to open the previous or next file pair from the list.

40| Align the image pair. Without registration of the image pairs, only small parts of the image can be brought to alignment, because the EMCCD camera and confocal scan head will almost certainly have slightly different zoom and rotation. Use 'Dragging mode' to register the two images by translation. This tool displays a cross-correlation value between a confocal and a STORM channel if the channels are selected in the registration tool. If fiduciary beads are included in the sample, use the Image registration tool (**Supplementary Fig. 4** and **Supplementary Methods**). If beads were not imaged, the user can still assign landmarks in the image manually.

? TROUBLESHOOTING

41| To filter LPs on the basis of z position, local density, ROI selection or number of photons, check the respective filters in the 'Filtering' tab and click 'Apply' (**Fig. 5d**).

42| Use the checkboxes above channel names to set the displayed channels. Use the look-up tables (LUT) tool to adjust the brightness of each channel (**Fig. 5e**).

43| Make a selection using the freehand, ellipse or circle ROI tools. If a circle ROI is specified, an active contour ROI based on the specified confocal channel can also be evolved enabling unbiased and automatic ROI selection of the subcellular compartment (**Figs. 5f** and **6b**). The active contour uses the morphological active contour without edges (MACWE) algorithm⁶⁰ to iteratively determine the outline of a structure without relying on thresholding (**Supplementary Video 2**). This function still requires the adjustment of parameters to use for images of different resolution and profiles of different structural complexity (see the VividSTORM user manual at <http://katonalab.hu/index.php/vividstorm>, also provided as the **Supplementary Manual**). However, the major advantage of the active contour selection over thresholding is that the resulting contour does not follow a border of pixels with equal or similar intensity, and thus biological structures with uneven labeling (holes and noise) can be detected with better accuracy. Moreover, through many images captured during an experiment, the same setting can be used to detect target profiles with varying intensity and S/N. Nevertheless, users need to be aware that a high smoothing parameter may mask small-scale object features.

44| Select the analysis runner tool. Molecules will be processed for analysis on the basis of the active filtering and ROI settings. Only molecules that pass filtering and those that are present within the selected ROI will be evaluated. If no ROI is selected, then all molecules that pass filtering will be used.

45| Check the measurements to run, and specify parameters where required. See **Box 3** for the list of currently available functions. Click the 'run' button to run each checked analysis. The results will be saved in the active working directory. Certain analysis functions also generate graphical representation of the results, which is shown in interactive 3D views (**Figs. 6** and **7**).

46| To modify the implementation of functions, or to add more analysis functions, consult the VividSTORM user guide for description of the program structure. A custom analysis function with placeholder code is included in the source code. See **Box 3**, Dudok *et al.*⁵ and the VividSTORM manual (**Supplementary Manual** and at <http://katonalab.hu/index.php/vividstorm>) for the complete description of features and functions, and the detailed explanation of each measurement function. Screenshots from the intermediate steps of two example analysis workflows are shown in **Figures 6** and **7**.

Evaluating the quality of images ● TIMING 30 min

47| Calculate localization accuracy. The easiest way to assess localization accuracy is to inspect the lateral localization accuracy value in the STORM files in .txt format (or by using the quality control analysis function in VividSTORM). This value is calculated by the NIS-Elements N-STORM software according to ref. 68, and it describes the precision of the positioning of the LPs. Importantly, however, this is not equal to image (Nyquist) resolution, as the size of the smallest resolvable feature

Box 3 | List of analysis functions available in VividSTORM

See refs. 5,20 for applications and detailed description of each type of analysis. All returned values are saved into the 'Results' output file. Each optional output data set is saved in a separate file. Functions marked with 'slow' may take some time to run, depending on the number of processed LPs.

- **Measurements**
 - NLPs per channel: returns the NLPs in each STORM channel, along with the perimeter and area of the active ROI if an ROI is selected.
 - Convex hull: returns the 2D perimeter and area, and 3D surface and volume of a convex hull fitted on the LPs in each channel. It displays the resulting hulls and the LPs in 2D and 3D.
- **Quality control**: returns the mean lateral localization accuracy and mean number of neighbors (within a 160-nm 3D Euclidean distance) per channel.
- **Cluster analysis**
 - DBSCAN: performs density-based clustering on the LPs from each channel. It returns the number of clusters, total NLPs in clusters and the total volume of clusters per channel. Optionally, it returns the parameters (NLPs, volume and length) of individual clusters, and exports the data to .pdb format. It displays a 3D view of the clusters.
- **Internalization**
 - Distance from surface (slow): fits a convex hull on one channel and measures the distance of LPs in the specified channel from the hull surface. It returns the number of LPs within the specified threshold (in nm). Optionally, it returns the distance value for each LP.
 - Distance from center of mass: calculates the d/r for each LP, where d is the distance from the center of mass of all of the LPs in the specified channel, and r is the estimated radius of the signal (radius of a circle with the area of the 2D convex hull fitted on the data). It returns the mean d/r . Optionally, it returns the values for each LP.
- **Minimum distances**
 - Euclidean distance, within channels: returns the mean and variance of nearest neighbor distances per channels. Optionally, it returns the nearest neighbor distance for each LP.
 - Euclidean distance, between channels: returns the mean and variance of nearest neighbor distances of points in one STORM channel from the points in the other STORM channel. Optionally, it returns the values for each LP. Reference channel can also be confocal data. In this case, a virtual STORM channel is made from the confocal signal on the basis of distance and intensity thresholds.
 - Surface distance, between channels (slow): fits a 3D convex hull onto LPs in one channel. It measures the minimum surface distance between the projections of points from two specified channels along this surface. It returns the mean and variance of distances. Optionally, it returns values for each LP, and it exports the data (LPs, convex hull edges and minimum distance trajectories) in .pdb format. It displays a 3D view of the results.
- **Surface density**
 - Measure surface density (slow): fits a convex hull onto LPs in one channel. It picks random coordinates with a specified density on the hull surface. It measures the densities of LPs from the specified channel within the given radius from each random point. It returns the mean and variance of the measured LP densities. Optionally, it returns the density at each random point.
 - Measure surface density distribution relative to reference (slow): fits a convex hull onto LPs in one channel. It picks random coordinates with the specified density on the hull surface. It measures the densities of LPs from the specified channel within the given radius from each random point, and the surface distance of random points from LPs of the reference channel. It returns the mean and variance of the measured LP densities. Optionally, it returns the distance and density values for each random point.
- **Export data**
 - Export coordinates: saves the LPs included after the channel, filter and ROI settings in the original format of the loaded STORM data (using the header and respective lines from the original file).
 - Export to .pdb: saves the LPs in .pdb format for visualization purposes in Visual Molecular Dynamics (VMD) software. Each channel is assigned to a separate chain identifier in the .pdb file.

also depends on labeling density^{2,69}. Localization accuracy values for high- and low-quality images are shown in **Supplementary Figure 5a–c**. Under the conditions in our experiments, values <10 can be considered optimal. The axial localization precision is more difficult to determine; it can be calculated as the dispersion of the distribution of z coordinates of repeated measurements of the same fluorophore⁵.

48 | Check whether labeling density is sufficient for the planned analysis. As a quick measure of average local density, the mean number of neighbors within a 160-nm (1 pixel) radius can be determined easily using VividSTORM. For the CB₁- and Tom20-specific labeling presented in this protocol, the mean local density was between 10 and 30 neighbors within 160 nm, whereas the typical NLPs per frame was <50 (0.03 NLPs/μm²), although the optimum value highly

depends on the distribution of the labeling. Optimizing activator laser power and increasing the number of collected frames per image increases localization density. However, in most cases it is not advisable to aim much higher than one LP per fluorophore, as this will introduce apparent clustering. In addition, elevating the activator laser power to a range where LPs start to overlap (**Fig. 9**) does not necessarily increase the number of detected points in the whole image (because of faster decline of blinking), whereas it does decrease the mean localization accuracy because of elevated background (**Supplementary Fig. 5d,e**). To see whether the number of frames is sufficient, use the frame filtering function in NIS-Elements or VividSTORM. If features of the image disappear by filtering out the last frames (by, e.g., 500 frames a time), it might be useful to collect more frames.

49 | Check the distribution of z coordinates. This can be done easily by plotting the z coordinates from a STORM .txt file (exported from NIS-Elements, or from VividSTORM using the ‘Save Coordinates’ analysis option) as a histogram. **Supplementary Figure 5f** shows a typical z distribution in a single-channel DSTORM experiment. Note that z-rejected molecules are not shown (the fraction of z-rejected molecules can be up to 50% even with an excellent z calibration), and note the slightly asymmetric shape of the distribution caused by spherical aberration above the focal plane.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
8	Cell fill is not visible	Issues with biocytin-containing intracellular solution, fixative or development procedure	The cell fill should be visible under green fluorescence in at least the top 100 μm of the section. If not, use your standard development procedure as a positive control to identify the problem
21	Immunostaining is not visible	The staining protocol is not compatible with the primary antibody	Mount a section in Vectashield and inspect it in a fluorescence or confocal microscope. Note that this section will not be suitable for subsequent STORM imaging. The immunostaining and the cell fill should be visible in the respective channels. Staining using custom-labeled antibodies should be visible faintly in both the reporter and the activator channels. If the staining quality is not adequate, optimize parameters for fixation, membrane permeabilization, blocking and antibody dilution. Use an antibody that worked robustly under the given circumstances as a positive control
		Staining using custom-labeled secondary antibody is not performing adequately	Use a commercial secondary antibody as a positive control to identify the problem
26	Sample does not bleach within a few seconds	Issues with the imaging medium	Compare it with a sample covered in PBS as a negative control. Samples in imaging medium should bleach substantially faster. Prepare fresh imaging medium from fresh components if necessary
		Incidence angle is not optimal	Bring the laser beam back to the center of the objective. Optimize the angle for S/N later, after the expected bleaching and blinking behavior has been confirmed
		Staining is too dense	Use a secondary antibody labeled with Alexa Fluor 647 dye dried on a coverslip as positive control. If the dye, but not the tissue, bleaches quickly with the same microscope settings and imaging medium, reduce the antibody concentrations
		Laser power is not high enough	Set the imaging laser power to maximum. Align the laser board if necessary. Center the beam in the TIRF illuminator if necessary. The calculated power density at the sample should reach 2.5–9 kW/cm ² . Upgrade to a more powerful laser, or to a stronger beam-focusing lens in the TIRF illuminator, if necessary

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	Individual blinking events are not resolved	Camera settings are inadequate	Set the exposure time to 50 ms or faster. Turn off any frame averaging or integration options. Turn off any binning options
		Activator laser is too powerful	Set activator laser AOTFs to the minimal value at which light passes through. Insert a neutral density filter in the beam if necessary
31	PFS cannot be set	Sample is too far from the coverslip	The focal plane should be within 15 μm of the coverslip, and it should be maintained steadily by the PFS. After turning off and on repeatedly, the PFS should always return to the same position. Check whether the section is mounted and dried flat on the coverslip (Supplementary Fig. 3d), and that there are no pieces of debris or bubbles in the medium on or near the section
		Bubbles in the immersion oil or medium	Check for bubbles, and clean the objective if necessary
		Slide is not resting steadily on the stage	With the objective in escape position, ensure that the slide is not rocking in the stage insert. Make sure that the slide is resting on its glass surface, not on the nail polish or on the coverslip
33	Lags in acquisition occur (laser illumination is turning off for brief periods)	Saving of data is too slow	The imaging should run seamlessly, and the entire experiment should be complete in the time calculated from the exposure time and the total number of frames (a few percent overhead is acceptable). Save on a fast local drive with enough free storage space
		Real-time analysis is slowing down the computer	Increase the threshold value for real-time analysis. Turn off real-time visualization of calculated localizations once the imaging parameters are established
40	It is impossible to align images	Lack of registration	Align the two cameras and calibrate for affine transformation according to Equipment Setup. Apply the transformation on the images according to Step 34. Use the dragging tool to see a measure of cross-correlation between the image pair (see VividSTORM user guide). If necessary, use the registration tool on the confocal and STORM image of the same staining, or use fiduciary beads (Supplementary Fig. 4)
		Drift in the STORM image	Make sure to read the drift-corrected coordinates from the STORM file (Step 35 and Box 2). Improve setup stability or use bead-based drift correction if necessary

● TIMING

The entire protocol can be performed in 4–7 d, depending on the time of fixation, and on the number of samples. The timing information provided refers to the approximate time required to perform the procedure on the first image pair. Processing additional images takes significantly less time.

Day 1: Steps 1–2, preparation of cellular labeling: 6–8 h for whole-cell patch-clamp recording, and placing the specimen in fixative: 24–48 h

Day 2 (or day 3, for longer fixation): Steps 3–8, development of labeled cell: 6 h, and imaging of cellular morphology: 30–60 min

Day 3 (can also be performed on day 2): Steps 9–18, sectioning the specimen: 30–60 min per sample, starting the immunostaining protocol: 90 min and incubation in primary antibody: 12–18 h

Day 4: Steps 19–23, completing the immunostaining and mounting the sections: 7 h

Day 5: Steps 24–33, imaging: 30–180 min per sample

Day 6 (can also be performed on day 5): Steps 34–46, image processing and analysis: time depends on the number of images and the type of analysis

Steps 47–49, evaluating the quality of images: 30 min (can be done anytime after image processing)

Box 1, labeling of secondary antibodies: 1 h

Box 2, calibration for 3D-STORM and multicolor dSTORM: 3 h

ANTICIPATED RESULTS

The combination of single-cell labeling with immunostaining and STORM imaging is an efficient method for visualizing any protein of interest in identified subcellular domains of individual neurons in brain tissue, with nanoscale precision (**Fig. 2**). The approach introduced in this protocol can be generalized for a number of alternative cell labeling and imaging methods, as exemplified by the adaptation of *in utero* electroporation for the genetic labeling of cells (**Fig. 3**), and to various types of tissue, as shown by immunostaining of heart and kidney sections (**Fig. 4**). A control experiment using tissue from knockout mice to identify background by primary and secondary antibodies is shown in **Supplementary Figure 2**. The free, open-source and easy-to-use VividSTORM software (**Fig. 5**) is a versatile tool for advanced analysis of SMLM data from the GUI, without the requirement for prior experience in programming. **Figure 6** demonstrates how VividSTORM is used to measure intermolecular distances within identified axon terminals in dual-channel STORM data, using the unbiased active contour selection of the labeled profile. **Figure 7** shows an example of how the DBSCAN clustering algorithm is applied to measure the number and size of mitochondria within labeled cells. The expected lateral single-molecule fluorophore localization precision of the STORM images acquired using this protocol is 8–12 nm (**Supplementary Fig. 5a**).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS L.B. and B.D. designed and performed STORM experiments, developed the protocol and analysis tools, prepared the figures and wrote the manuscript. V.M. and A.H. developed analysis and visualization tools and coded the software. Z.I.L. prepared tissue samples and performed immunostaining. I.K. conceived the method, supervised the project and wrote the manuscript.

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