NEUROPHOTONICS

PERSPECTIVE

Molecular mapping of neuronal architecture using STORM microscopy and new fluorescent probes for SMLM imaging

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ABSTRACT. Imaging neuronal architecture has been a recurrent challenge over the years, and the localization of synaptic proteins is a frequent challenge in neuroscience. To quantitatively detect and analyze the structure of synapses, we recently developed free SODA software to detect the association of pre and postsynaptic proteins. To fully take advantage of spatial distribution analysis in complex cells, such as neurons, we also selected some new dyes for plasma membrane labeling. Using Icy SODA plugin, we could detect and analyze synaptic association in both conventional and single molecule localization microscopy, giving access to a molecular map at the nanoscale level. To replace those molecular distributions within the neuronal three-dimensional (3D) shape, we used MemBright probes and 3D STORM analysis to decipher the entire 3D shape of various dendritic spine types at the single-molecule resolution level. We report here the example of synaptic proteins within neuronal mask, but these tools have a broader spectrum of interest since they can be used whatever the proteins or the cellular type. Altogether with SODA plugin, MemBright probes thus provide the perfect toolkit to decipher a nanometric molecular map of proteins within a 3D cellular context.

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1 Introduction

Imaging neuronal architecture has been a recurrent challenge over the years. The use of Golgi technique by Ramón y Cajal paved the way for the first characterization of neuronal architecture using microscopy on fixed brains. Indeed, metallic impregnation with silver salts provided an opportunity to see and reconstruct the dendritic architecture of various types of neurons in the depth of the nervous tissue. Although Golgi staining is still used in widefield microscopy, its use in confocal microscopy remains limited for 3D analysis. The use of fluorescent labeling in conjunction with 3D microscopy led to the production of large amounts of published data that are on the way to being classified and accessible through various infrastructures or free repositories (eBrains, Zenodo, etc.). This huge amount of data and their accessibility raise the question of potential new automated, unbiased statistical analysis.

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2 Colocalization and Coupling Analysis in Conventional and Super-Resolution Microscopy

To analyze the structure of synapses quantitatively, we recently developed, in association with statisticians, free software to detect the association of pre- and postsynaptic proteins. This software, called SODA for standard object distance analysis, makes it possible to identify and measure the spatial distribution of either clusters (conventional microscopy) or single molecules (single molecule localization microscopy) and provides the distance of association when those are statically found associated.[1,2] SODA (available here; see Ref. 3) can be used in conventional microscopy (confocal, widefield, and video microscopy) or in super-resolution microscopy, such as SIM, STED, or even SMLM (PALM or STORM). From the mathematical point of view, SODA will analyze the cellular shape and spot density (Fig. 1) to evaluate the expected spatial distribution using Ripley's function. If clusters are more frequently associated than the expected random distribution, then they are identified as associated spots. As a proof of concept, we analyzed the distribution of three synaptic molecules named synapsin, homer, and PSD-95 using SIM microscopy. Using only 15 pictures, we were able to analyze about 50,000 synapses and identify that the distance between a synapsin and post-synaptic PSD95 cluster was 107 ± 73 nm while the PSD-95-homer was 64 ± 48 nm. Beyond raw distances, this system allows to detect in a quantitative manner any morphological variations that may occur in various mutants, physiological conditions or in certain synaptopathies.

SODA can be used either with sparse labeling ($>30-100$ objects per image)[4] or with high-density labeling as in single-molecule localization microscopy[1,2] where several thousands of localizations can be retrieved. The only limitation of SODA is the need to get the cell boundary to correctly evaluate the object's density. In contrast to methods using Voronoï tesselation[5] that

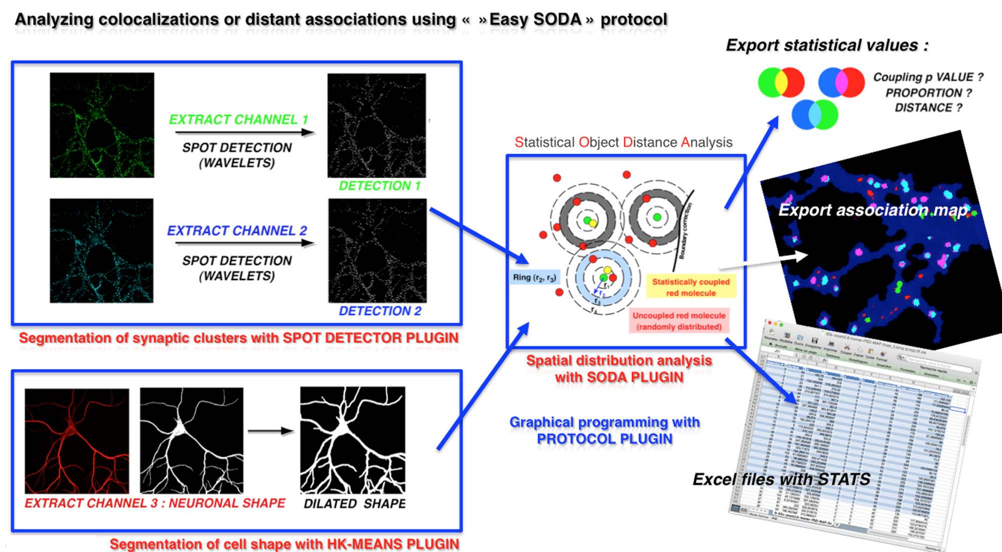

Fig. 1 Workflow of the Easy SODA protocol available in Icy Software. Colocalization or distant association can be analyzed using the user-friendly Easy SODA protocol that is freely available in Icy Software. This protocol is a graphical programming automatization routine that allows analysis of synaptic proteins' distribution within neuronal cell shape. Here, neurons are labeled with two proteins (green and cyan channels) that are distributed in clusters. Clusters are segmented using wavelet segmentation through "spot detector" plugin. Cell shape is extracted using a MAP2 stain, and segmentation is done using the "Hierarchical KMeans" plugin. Cluster distribution within the cellular mask is then analyzed through the "SODA" plugin using Ripley's analysis. Statistical associations are detected if any, and the proportion of associated clusters with their distance is provided with a *p*-value indicating the statistical robustness of the association. If many pictures are analyzed in batch mode, all results can be exported to Excel files. A molecular map is exported for each picture with an association color code. For example, if we take a red-green spot analysis: isolated green spots remain green, green spots associated with red are cyan, isolated red spots remain red, and red spots associated with green are pink. Localization of significant associations is thus visible at a glance over the cell mask (here in deep blue).

are limited up to now two-dimensional analysis, SODA can be used in 3D, which is an added value to analyze thick 3D volume STORM images. Because SODA does not rely on any overlap methods, it is far less sensitive to high-density false positive colocalization artifacts.[1,2]

SODA can also be used for all other associations (either direct or distant) in neurosciences[6–10] and even outside this field like, for example, in cell biology,[11–18] virology,[12] in bacteria[19] or plants.[20] Its use has been highlighted in several reviews.[20–22]

3 Imaging Plasma Membrane in Live or Fixed Cells

3.1 Optimizing Live Plasma Membrane Imaging with MemBright Probes

In order to be able to place the molecules within the cell shape, and in collaboration with chemists, we have selected new membrane probes capable of revealing the cell shape and imaging fine structures, such as dendritic spines.[23]

These membrane probes, named MemBright, upon insertion in the membrane, emit fluorescence with narrow emission peaks, allowing correlation with other conventional fluorescence for multi-color labeling. A family of seven members is now available and can be used all over the fluorescence spectrum (from 480 and 750 nm). Upon incubation with living cells, these probes insert directly into the membrane through a lipid anchor and thus reveal the cell shapes without the use of any transfection or viral vectors (Fig. 2). This strategy thus makes it possible to reveal all neurons and/or glial cells in a few minutes without any toxicity.

The big advantage of MemBright probes is also its fluorogenic property. Indeed, MemBright probes are non-fluorescent within media and become fluorescent when reaching the plasma membrane. It means that the probe can be let within the cell culture media without having a fluorescence background under the microscope. Letting the probe in the cell chamber during live acquisition allows the perpetual replacement of the probes if bleaching occurs, thus leading to persistent bright labeling of the membrane.

MemBright probes are more efficient when incubated on cells in the absence of serum to avoid any titration of the probes by serum fat. In neurons and glial cells, we usually incubate MemBright probes in Krebs Ringer solution at a concentration of 200 nM at 37 deg under the microscope. The absence of serum optimizes the labeling, and the absence of phenol red lowers the fluorescence background. Fluorescence on the plasma membrane appears very fast within a few minutes. To avoid saturation of the plasma membrane with a huge amount of lipids, it is crucial not to use a high concentration of probes. MemBright probes are sufficiently bright to be used at the nanomolar range, whereas other commercial probes have to be used at the micromolar range. Moreover, it should be stressed that illumination of any fluorescent probes may induce the production of reactive oxygen species that can imbalance the intracellular redox state and be

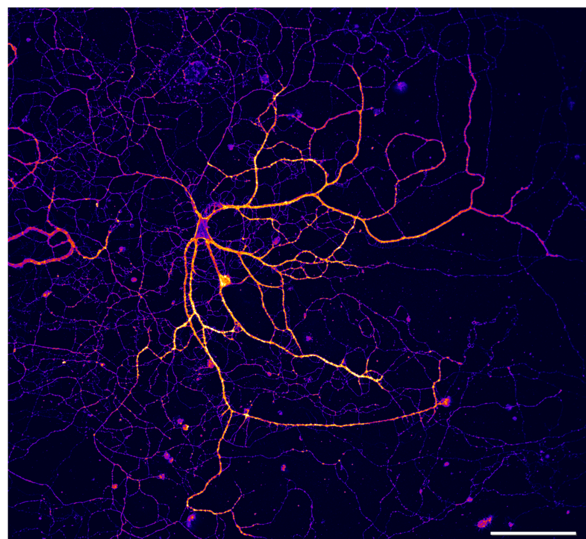

Fig. 2 Confocal image (20×) of hippocampal neurons after 2 months in a culture labeled with Cy3.5-MemBright probe reveals dendritic and axonal branch complexity. Scale bar: 100 μm.

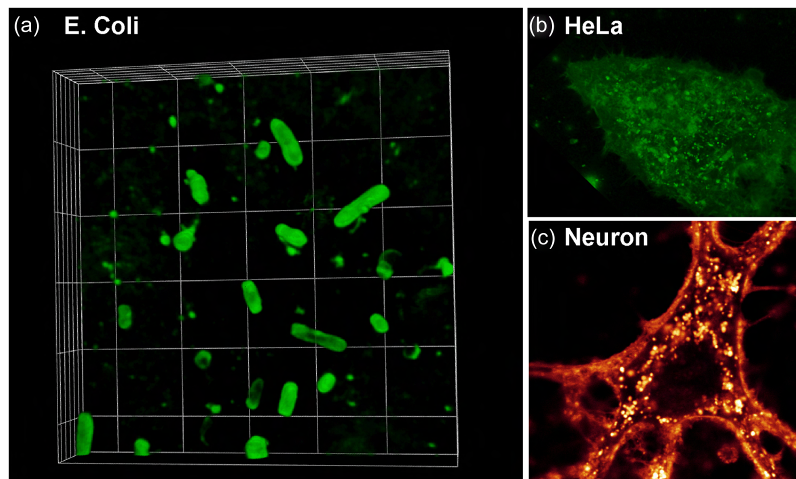

Fig. 3 Confocal images (93×) of various cell types labeled with Cy3.5-MemBright probe. (a) 3D rendering of *E.coli* bacteria incubated overnight with MemBright probes. (b) 3D rendering of HeLa cells incubated 30 min with MemBright. (c) Confocal section of hippocampal neurons incubated several hours with MemBright, revealing intracellular vesicles.

deleterious to the cell.[24] Thus, it is also a good practice to minimize illumination time and frequency, to the minimal amount needed to the right sampling of a biological event. Previously, we could follow neuronal growth over time during 13 h, imaging every 2 min with low laser power (0.2% of an 561 laser line of an Elira PS1), without any detrimental effects.[23] At last, we have shown that MemBright probes are resistant to permeabilization when fixed properly with a mixture of 4% paraformaldehyde-0.2%glutaraldehyde, and can thus be combined with conventional immunolabeling with primary and secondary antibodies.[23] MemBright staining can be correlated either to live antibody staining or with immunochemistry on fixed samples. We could show double labeling of the plasma membrane and live L1-CAM endocytosed antibodies. We also showed that intracellular vesicular transporter VGLUT could be revealed by immunochemistry within 3D neuronal cell shape reconstructed using MemBright. This property thus allows identifying surface or internal protein locations using MemBright counterstaining to visualize cell shape. We have selected MemBright probes for their stability at the plasma membrane and their slow endocytosis. However, on long-term incubation, they will be finally endocytosed and can thus be used to label endocytic pathways [Fig. 3(c)].[25]

3.2 Imaging Plasma Membrane of Various Cell Types

We originally showed that MemBright probes could be used in various cell types, such as epithelial cells in culture (HeLa cells or KB cells),[23,26–28] and dissociated hippocampal neurons,[23] hippocampal astrocytes.[23] We could also use MemBright probes to label live brain (hippocampus, cortex, and cerebellum) or liver slices, allowing the labeling in depth and imaging using confocal or two photons microscopy.[23] Since our first paper in 2019, MemBright probes have been used by several other labs and cited in more than 60 articles and 39 reviews.[29–57] It has been used in B lymphocytes,[58] in A431 cells,[59] and reused in neuronal cells to label growth cone and initial segments of hippocampal neurons,[60] presynaptic terminals,[29] and post-synaptic compartments.[53] It has also been used to label apoptotic bodies (AB), microvesicles (MV), and small EV (sEV) isolated from MIN6 pancreatic beta cells exposed to inflammatory, hypoxic, or genotoxic stressors.[61] Since we were asked several times if MemBright could be used on bacteria, we did recently *E. coli* live labeling with MemBright – CY3.5. As shown in Fig. 3(a), the bacteria 3D shape can be efficiently labeled in live.

3.3 Imaging Extracellular Vesicles

MemBright has been widely adopted by the extracellular vesicles community[51] to track extracellular vesicles both *in vitro* or *in vivo*[61–81] in hippocampal[63] or cortical neurons,[79] zebrafish,[62,67,72] breast cancer cells or tumours,[66,71,80] myotubes,[82] and red blood cells.[74,76]

Particle size distribution and zeta potential analysis of EVs derived from A375 cells using nanoparticle tracking analysis (NTA) showed that EVs labeled before and after labeling by MemBright have almost no change in size and only a slight shift of zeta potential.[75] Due to its ease of use and brightness, MemBright has thus been widely used to label exosomes. However, it should be stressed that MemBright is not specific to extracellular vesicle labeling. MemBright will be able to label any membrane in contact with the probe. That means that membrane debris trails left behind by migrating cells will be labeled, whatever the nature of the membrane (EVs or not). Any membranous organelles (tubules, endosomes, lysosomes, synaptosomes, etc.) that would have been retrieved by ultracentrifugation can be labeled when incubated with MemBright. Some controls are thus needed before labeling to ensure that the fraction is homogeneous and not contaminated by different organelles.

Hyenne et al.[62] show that MemBright can be used in pulse-chase experiments and that some CD63-GFP EVs can be labeled with MemBright, while others are not.[62] Sung et al. concluded that MemBright can label exosomes as well as plasma membrane-derived EVs, but that MemBright does not label all exosomes.[47] Using a pulse-chase experiment, it is expected that not all endosomal compartments will be labeled. Indeed, only those deriving from the plasma membrane exposed to the MemBright at a time t will be visible. It is likely that a proportion of EVs that were generated before or after incubation with MemBright and that are then stored in the cell will not be labeled by the MemBright wave. It is therefore essential to properly calibrate the labeling time incubation and the chase time to observe the desired events.

4 Imaging Plasma Membrane with Single Molecule Localization Microscopy

MemBright probes can be used in conventional and super-resolution microscopy (Fig. 4 and Video 1) and thus make it possible to observe the molecular distribution of synaptic proteins in

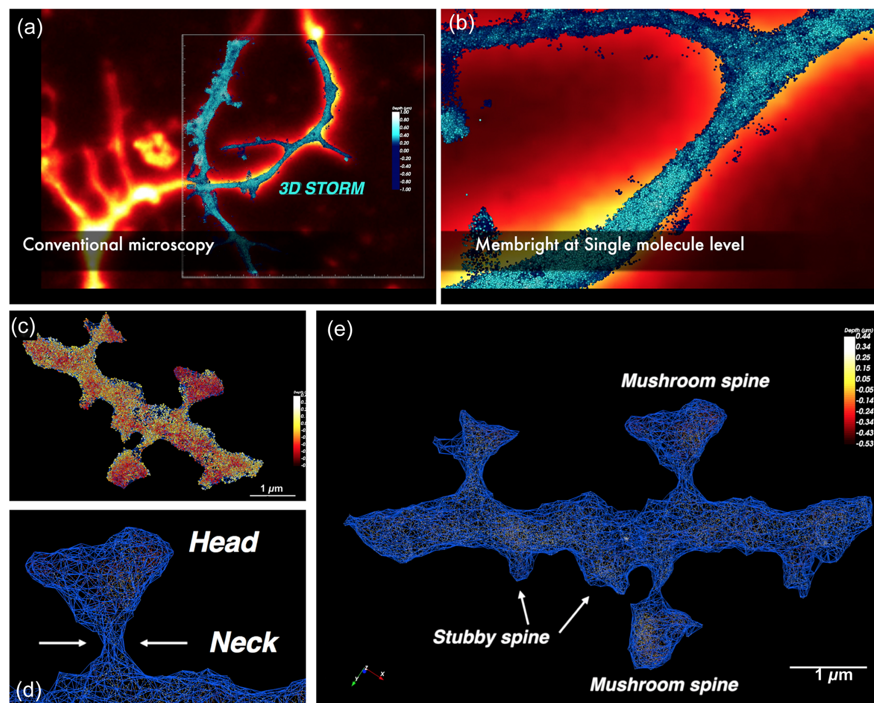

Fig. 4 STORM imaging of hippocampal neurons labeled with Cy3.5-MemBright probe and imaged in conventional widefield microscopy [fire LUT in (a)] and in STORM microscopy (blue spheres in rectangle). (b) Magnification of the plasma membrane 3D STORM image shows the single molecule organization of MemBright all over the plasma membrane. Light blue localizations are closer and deep blue are deeper. (c)–(e) Example of stubby and mushroom dendritic spines in 3D STORM. All the localizations found in (c) (published previously in a different form in Ref. 3) can be used to reconstruct the 3D shape of the spine in a wireframe. This 3D shape can then be used for volumetric estimation or fine measurements of the spine neck.

correlation with the structural morphology at the nanoscale using STORM. Altogether with the SODA plugin, MemBright probes thus provide the perfect tools to access a nanometric molecular map of various proteins within the 3D cellular context. These high-resolution techniques were set up on fixed samples, but it is expected that the need for super-resolution imaging with small molecular probes to allow imaging of living samples will be growing and will stimulate the development of new molecular probes. Indeed, chemical development will be required in the next years, since molecular probes are still up to now far beyond the recent development in instrumentation that can reach a resolution of 3 to 4 nm.[83] One interesting track will be probably the development of self-blinking dyes[84–86] or the development of new convertible fluorophores[28] for live SMLM. We are now working on a photoswitchable version of the MemBright that would be able to be photoswitched without any reducing buffer, to be used in live single molecule localization microscopy.[87] Moreover since MemBright has been used a lot in the extracellular vesicle community, it indicates that membrane dyes are of great interest for people working on vesicular trafficking. One other challenge, in the next years, would be to develop new MemBright probes devoted to fast internalization, to be able to decipher different vesicular pathways with various colors.

5 Appendix: Supplementary Information

Video 1 Conventional and STORM imaging of hippocampal neurons labeled with Cy3.5-MemBright to label dendritic spines in 3D at the nanoscale level (MP4, 60.9 MB [URL: https://doi.org/10.1117/1.NPh.11.1.014414.s1]).

Disclosures

The authors have no competing interests to declare and are not financially interested or remunerated for MemBright probe sales.

Code and Data Availability

Tutorial and download concerning SODA plugin and user-friendly "Easy SODA protocol," can be found on Icy Software website here: https://icy.bioimageanalysis.org/protocol/easy-soda-2-colors-1-image/

Sample image is also available here on Zenodo: https://zenodo.org/record/4323312-.Y_3tqx1CdHQ

A French Tutorial on Ripley's function and Icy SODA plugin can be found on YouTube: https://www.youtube.com/watch?v=7yVp73s-4TA

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