Quantitative analysis tools and correlative imaging applications for N-STORM

Super-resolution techniques have had a significant impact on our understanding of biological processes at the molecular level. However, one of the challenges to their broad utilization has been our limited ability to quantitatively analyze super-resolution images of complex biological tissues. In this Application Note, we highlight recent work by Dudok *et al*. utilizing Nikon's N-STORM system to develop new correlative imaging methods and quantitative analysis tools to study the mechanism of cannabinoid signaling in the brain.

Stochastic optical reconstruction microscopy (STORM) is a localization-based super-resolution technique that provides a tenfold improvement in resolution compared with conventional light microscopy[1]. Unlike immunogold electron microscopy, STORM imaging enables localization of proteins without compromising sample size, but with similar precision.

Using N-STORM to image cannabinoid receptors in brain tissue

Dudok *et al*.[2] developed an efficient tissue-processing and immunolabeling protocol for 3D-STORM and an efficient workflow that allowed for examination of cannabinoid receptor (CB_1) distribution in 7,051 individual GABAergic axon terminals in tissue sections, with localization precision of 6 nm in *xy* and 41 nm in *z*. 3D N-STORM imaging of hippocampal sections derived from CB_1^+ mice revealed high CB_1 densities on cholecystokinin (CCK)-containing GABAergic axon terminals, which formed basket-like arrays around CB_1-immunonegative pyramidal cell somata (**Fig. 1**).

Correlative patch-clamp, confocal and N-STORM imaging

To determine the nanoscale organization of cannabinoid receptors in a cell-type-specific manner, Dudok *et al*.[2] combined N-STORM with confocal imaging and patch-clamp electrophysiology. The authors first carried out whole-cell patch-clamp recordings on acute slice preparations to determine distinct spiking patterns for individual neurons. These same neurons, which were filled with biocytin during recording, were then imaged by confocal microscopy and reconstructed *post hoc* in Neurolucida to distinguish perisomatic versus dendritic interneurons. **Figure 2a–c** shows a representative voltage tracing and reconstruction of a typical perisomatic interneuron.

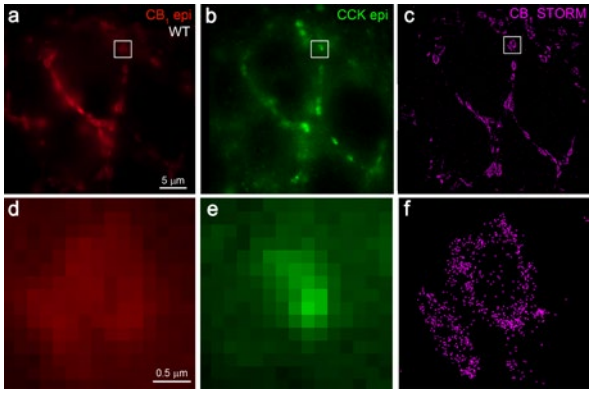

Figure 1 | Using N-STORM for nanoscale 3D tissue imaging. (**a,b**) Immunofluorescence labeling of CB_1 (**a**) and CCK (**b**), a neuropeptide used to identify CB_1-expressing interneurons, imaged with epifluorescence (epi). (**c**) CB_1 imaged with 3D N-STORM. (**d–f**) Magnified views of axon terminals in epifluorescence images of brain tissue labeled for CB_1 (**d**) and CCK (**e**) and in an N-STORM image of tissue labeled for CB_1 (**f**). The improved resolution of N-STORM enabled the visualization of two adjacent boutons, unlike the corresponding epifluorescence images, as well as localization points representing individual CB_1. WT, wild type. Figure reproduced with permission from ref. 2.

Subsequently, 20-µm sections were prepared from the acute slice and CB_1-immuostained for N-STORM imaging. Confocal microscopy was performed to identify biocytin-filled axon terminals (**Fig. 2d**), and CB_1 localization points within these cells were visualized by 3D N-STORM (**Fig. 2e,f**) and overlaid on the corresponding confocal image. For correlative confocal and N-STORM imaging, the authors used a single microscope platform (Nikon Ti-E) configured with both a confocal system (Nikon C2) and a Nikon N-STORM module. This novel combination of patch-clamp electrophysiology, confocal microscopy and N-STORM imaging enabled Dudok *et al*.[2] to acquire physiological, anatomical and nanoscale molecular-distribution information from the same neuron in a highly complex tissue.

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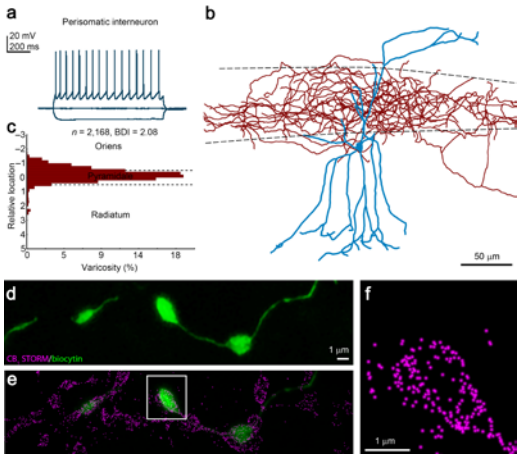

Figure 2 | Correlating N-STORM imaging with confocal microscopy and patch-clamp electrophysiology. Integrated analysis of the physiological properties and anatomical parameters was carried out along with nanoscale imaging to determine the nanoscale architecture of axon terminals in a cell-type-specific manner. Whole-cell patch-clamp recordings were performed on acute brain slices. (**a**–**c**) Voltage recording of a representative perisomatic interneuron (**a**). The same cell, which was loaded with biocytin during recording, was imaged by confocal microscopy and reconstructed with Neurolucida (**b**). On the basis of the physiological (**a**) and anatomical (**c**) measurements, the neuron was identified as a perisomatic interneuron. (**d**,**e**) After immunostaining, axon terminals of the same cell were imaged with correlative confocal microscopy (**d**) and 3D N-STORM (**e**, purple) to determine the nanoscale distribution of CB_1 in axon terminals. (**f**) Higher magnification view of CB1 localization points in the axon terminal shown in the boxed region in **e**. BDI, bouton distribution index. Figure reproduced with permission from ref. 2.

Quantitative analysis of two-color 3D N-STORM data

To determine whether CB_1 displays distinct coupling distances to effectors on the basis of cell type, Dudok *et al*.[2] performed two-color 3D N-STORM imaging for CB_1 and the protein Bassoon, a constituent of the release machinery. Two-color 3D N-STORM imaging was combined with confocal imaging to visualize CB_1 and Bassoon in identified perisomatic and dendritic interneurons (**Fig. 3a,b**). To quantitatively analyze spatial relationships between CB_1 and Bassoon, the authors measured the Euclidean distance between CB_1 and Bassoon N-STORM localization points in 3D (**Fig. 3c**). Because G protein–coupled receptors, such as CB_1, carry out their function in the plasma membrane, the authors also analyzed distance relationships between CB_1 and Bassoon along the membrane surface. Dudok *et al*. approximated the plasma membrane contour of the axon terminal by fitting a 3D convex hull to CB_1 localization points. They fitted a second convex hull to the Bassoon localization points and projected it onto the CB_1 surface. The shortest distances between each CB_1 localization point and the nearest projected Bassoon point along the CB_1 surface were calculated using an approximative mathematical algorithm developed by the authors. Both Euclidean and surface-based distance measurements revealed similar spatial relationships between CB_1 and Bassoon in both perisomatic and dendritic interneuron synapses. However, unlike CB_1, which displayed homogeneous distributions, Bassoon localized to clusters. Using density-based analysis (**Fig. 3e**,**f**), the authors determined that there were higher numbers of Bassoon clusters in perisomatic boutons than in dendritic boutons. However, the number of localization points per cluster was lower in perisomatic boutons. When the number of Bassoon localization points was normalized to the number of CB_1 localization points, perisomatic boutons displayed significantly higher receptor/effector ratios than did dendritic boutons. This difference in receptor/effector ratios may contribute to cell-type-specific cannabinoid signaling efficiency.

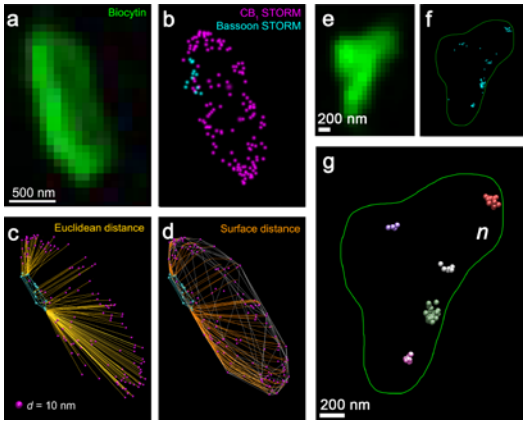

Figure 3 | Quantitative analysis of multicolor 3D N-STORM data. (**a**,**b**) Dudok *et al*.[2] developed custom analysis tools to determine distance measurements between CB_1 localization points (purple) and Bassoon localization points (blue) in dual-channel N- STORM images (**b**) of individual axon terminals (**a**) .(**c**,**d**) Euclidean distance measurements (**c**) and surface distance measurements (**d**). (**e**–**g**) Density-based measurements on single-channel biocytin- (**e**) and Bassoon-immunostained (**f**) samples used to analyze the number of Bassoon clusters and the number of localization points per Bassoon cluster *n* (**g**). Representative data from perisomatic interneuron boutons are shown. Figure from ref. 2.

Super-resolution microscopy is a powerful tool for probing the molecular landscape of cells, and the impact of this field in revolutionizing our understanding of biological processes was recently recognized with a Nobel Prize in Chemistry. However, initial implementations of many super-resolution techniques suffered from poor temporal resolution, a lack of broad contextual information and a lack of analysis tools for extracting quantitative data. Recent developments in super-resolution microscopy are expanding its technical capabilities. Nikon's newest N-STORM version 4.0 provides new integrated analysis tools, including tools for determining cluster size and distance measurements, as well as an imaging speed ten times faster than in previous versions. N-STORM can also be easily combined with other imaging modalities such as confocal microscopy and N-SIM (structured illumination microscopy) to expand the functionality of N-STORM experiments. More information about our super-resolution systems is available at our website (www.nikoninstruments.com/sr).

1. Rust, M.J., Bates, M. & Zhuang, X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **3**, 793–795 (2006).
2. Dudok, B. *et al*. Cell-specific STORM super-resolution imaging reveals nanoscale organization of cannabinoid signaling. *Nat. Neurosci*. **18**, 75–86 (2015).