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Single-Molecule Tracking Photoactivated Localization Microscopy to Map Nano-Scale Structure and Dynamics in Living Spines

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Abstract

Super-resolution microscopy has rapidly become an indispensable tool in cell biology and neuroscience by enabling measurement in live cells of structures smaller than the classical limit imposed by diffraction. The most widely applied super-resolution method currently is localization microscopy, which takes advantage of the ability to determine the position of individual fluorescent molecules with nanometer accuracy even in cells. By iteratively measuring sparse subsets of photoactivatable fluorescent proteins, protein distribution in macromolecular structures can be accurately reconstructed. Moreover, the motion trajectories of individual molecules within cells can be measured, providing unique ability to measure transport kinetics, exchange rates, and binding affinities of even small subsets of molecules with high temporal resolution and great spatial specificity. This unit describes protocols to measure and quantify the distribution of scaffold proteins within single synapses of cultured hippocampal neurons, and to track and measure the diffusion of intracellular constituents of the neuronal plasma membrane.

Keywords

single-molecule tracking; photoactivated localization microscopy; PALM; STORM; live-cell imaging; super-resolution microscopy; neuron; dendritic spine; synapse; postsynaptic density; hippocampal cultures

INTRODUCTION

The transmission of signals between neurons requires the strictly regulated incorporation and positioning of receptor complexes at the postsynaptic membrane. A multitude of scaffolding and signaling molecules that are assembled into the postsynaptic density (PSD) establish the structural framework that holds and positions synaptic receptor complexes. However, although extensive molecular analysis has provided a wealth of information about the characteristics of individual constituents of the PSD (Okabe, 2007; Sheng and Hoogenraad, 2007), the small scale and high density of proteins packed together at the PSD

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INTERNET RESOURCES

Single-molecule tracking software is available from: <http://physics.georgetown.edu/matlab/>

have hampered the investigation of how the spatial organization of protein complexes at individual synapses shape synaptic signaling. Fortunately, a growing number of super-resolution imaging techniques has been developed over the past decade permitting the investigation of sub-cellular structures at a scale of tens of nanometers (Betzig et al., 2006; Hell, 2007; Hess et al., 2006; Huang et al., 2010; Rust et al., 2006; Sigrist and Sabatini, 2012). Among these techniques, photoactivated localization microscopy (PALM) is unique in its relatively easy implementation and its ability to analyze the position and mobility of single molecules within living neurons. In this Unit, we describe the use of PALM to map the distribution of intracellular synaptic molecules in live hippocampal neurons, and track individual molecules within dendritic spines.

PALM is based on the notion that although fluorescence emitted from a single molecule is diffracted by the optics of the microscope and thus essentially appears as a blur, its resulting intensity profile on the camera has a predictable distribution so that the exact position of the molecule can be estimated with nanometer precision (Fig. 1A). In PALM, single molecules are spatially isolated by stochastically photo-activating fluorescent probes from a dark to a fluorescent state or from one emission spectral state to another (such as from green to red-emitting, as in the case of Eos). Activating and localizing many thousands of molecules individually, nanometer-scale organization of biological structures can be reconstructed at a resolution far exceeding classical imaging techniques (Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006). The resulting collection of localizations can be used to reconstruct the molecular organization of cellular structures by rendering “molecular maps”, or to analyze the motion of single molecules by tracking their location over consecutive frames (Frost et al., 2010; Manley et al., 2008) (Fig. 1B). It is important to note that other single-molecule tracking techniques have been used routinely for many years (Saxton and Jacobson, 1997), but have been limited to probe the movement of proteins and other molecules on the cell surface and thus only accessible to antibodies or ligands. By harnessing the use of photoactivatable GFP-type molecules, PALM can be used to probe location of intracellular proteins in live cells. In addition, the utility of this microscopy approach has been unexpectedly broadened by the discovery that many fluorophores undergo spontaneous or light-controllable blinking in a variety of solutions amenable for live-cell imaging (Jones et al., 2011; Shim et al., 2012; van de Linde et al., 2011).

In this unit, we describe the basic hardware requirements, acquisition strategies and analysis procedures (Basic Protocol 1) to perform live-cell PALM experiments in cultured hippocampal neurons (Support Protocol 1).

BASIC PROTOCOL 1

SINGLE-MOLECULE PALM IN LIVING NEURONS

This protocol describes the basic steps for mapping and tracking mEos2-tagged proteins within live hippocampal cultures (Support Protocol 1). Other photoconvertible or photoactivatable proteins such as photoactivatable GFP (PA-GFP) can be used (Dempsey et al., 2011), but we find mEos2 (McKinney et al., 2009) and its more recent derivatives mEos3.1 and mEos3.2 (Zhang et al., 2012) outperform other photoconvertible proteins in terms of brightness and photostability. Note that the basic imaging and analysis approaches

described here can be easily adapted to image diverse fluorophores and execute a variety of single-molecule imaging experiments in live or fixed cells (Huang et al., 2010; van de Linde et al., 2011).

Materials—Hippocampal cultures, (14 - 21 DIV) (see Support Protocol 1)

Lipofectamine 2000 reagent (Invitrogen)

Opti-MEM I reduced serum medium (Invitrogen)

Purified expression plasmid expressing protein of interest tagged with photoconvertible protein
Inverted microscope (e.g. Olympus IX81)

Continuous feedback control over the focus position is critical. This is built into some microscope models, or can be added with separate accessories.

Epifluorescence light source (e.g. Arc lamp)

Oil immersion objective suitable for TIRF (e.g. 100X/1.49 NA)

Lasers:

405-nm activation laser (e.g. Coherent Cube, 50 mW)

561-nm excitation laser (e.g. Cobolt Jive; 200 mW or higher)

Acousto-optical tunable filter (AOTF, e.g. Neos)

Filter cubes in the microscope

- 1) For visualizing unconverted green Eos, a standard EGFP filter set is excellent
- 2) For photoactivation and imaging of converted red Eos, use a cube containing a dual-band excitation filter (405+561 nm), a dual-band dichroic to reflect these laser lines, and a red-pass emission filter. Bandpass filters should be designed for TIRF, with OD6, and hard coating (e.g. the ET series from Chroma)

iXon+ 897 EM-CCD camera (Andor Technology)

Additional magnification optics should be included to result in camera pixels representing 100 to 130 nm of real space. Because the iXon 897 camera has 16 μm CCD pixels, we use a 1.6 $\times$ intermediate lens built into the Olympus IX81 body, giving 100 nm pixels in the image. An equivalent alternative is a C-mount adaptor that includes a 1.6 $\times$ magnification prior to the camera.

Stimulator or timing circuit (e.g. AMPI Master-8)

iQ software version 2.4 or higher (Andor Technology)

MATLAB software (with Image Processing toolbox, MathWorks)

NOTE: The imaging system described here and in Fig. 2 is the setup used in our laboratory, but serves merely as an example to lay out the principle requirements for a microscope setup suitable for PALM imaging.

Transfect dissociated hippocampal cultures

We transfect neuronal cultures (Support Protocol 1) one to three days before imaging using Lipofectamine 2000 reagent (Invitrogen). Note that Lipofectamine 2000 yields low transfection efficiencies (<1%), usually sufficient to transfect 5 to 15 neurons per coverslip. While this is sufficient for most PALM imaging experiments, higher rates can be achieved using viral vectors.

1. For each well of cells (of a 12-well culture plate) dilute 0.5 – 1.0 µg plasmid DNA in 25 µl OptiMEM I reduced serum medium.
2. For each well of cells dilute 1.0 µl Lipofectamine 2000 reagent in 25 µl OptiMEM I reduced serum medium. Incubate at room temperature for 5 minutes.
3. Combine the dissolved Lipofectamine 2000 reagent with the DNA and incubate at room temperature for 20 minutes.
4. Add 50 µl DNA- Lipofectamine 2000 mix drop-wise to each well and gently mix by moving the plate back and forth.

Identify a region of interest and set up an imaging protocol

- 5 Before imaging, wash the coverslip containing transfected cells briefly in extracellular imaging buffer (see recipe) and place in microscope chamber containing imaging buffer.
- 6 Locate a transfected cell by excitation from a standard epifluorescence light source and monitoring fluorescence of the unconverted green state of mEos2 through the oculars.

Avoid long exposures, as mEos2 is weakly activated by light with wavelengths at least as long as 488 nm, and so the wide bandpass filters used for epifluorescence visualization will permit the activation (and depletion) of the photoactivatable marker.
- 7 Switch to camera acquisition and select a region of interest (ROI).

A smaller ROI allows higher frame rates (see below) and reduces data analysis time. Restrict excitation of the sample using the read-out laser, and only use minimal activation laser power sufficient to select the ROI and bring the sample in focus.
- 8 Set the incidence angle of the beam to ensure oblique illumination of the sample.

Optimize the angle of the beam using the TIR adjustment mirror to reduce illumination of molecules above the imaging plane, but retaining as many bright molecules as possible (Fig. 2B).
- 9 Set up an acquisition scheme.

The activation laser can be applied either continuously or in synchrony with the read-out laser. For the greatest flexibility (Fig. 2A), AOTF gating of excitation lasers can be controlled by a TTL timing source (AMPI Master-8; Fig. 2B) synchronized to the initiation of the camera frame by the “fire” or frame-start TTL pulse on the EM-CCD. In this manner, excitation pulse length (integration time) can be varied independently from the frame rate and kept as short as possible (Fig. 2C). This is ideal for tracking quickly moving molecules, since their motion during the integration time degrades the accuracy of their estimated location.

10 Set laser intensity.

Use high excitation laser power for mapping purposes. For tracking purposes, limit the excitation power and/or excitation pulse length. Set activation laser low at the start of the acquisition, increase during acquisition appropriately.

11 Set the camera exposure time and frame rate.

We generally image at 50 Hz frame rate with 10 ms laser pulses. See below for a more elaborate discussion.

12 Acquire a stack of 5,000 to 20,000 frames.

13 Export the image as a multi-layer TIF file.

Camera photoelectron calibration

An estimate of the number of photons per molecule is a useful metric to calibrate your expectations of image quality, and is generally required to calculate the precision of the localized molecules. Calibrating the number of photons per image pixel intensity unit is thus required to interpret the image in terms of photons. Some newer EM-CCD cameras can report pixel intensities to the computer already calibrated in photons/pixel, eliminating the need for this calibration step.

14 Acquire several images using the transmitted light source (room light can serve too) at intensity levels ranging from nearly dark to half the camera range. Take care that all acquisition parameters (e.g. frame rate, EM gain) are set the same as in the experiment.

15 Calculate the mean and variance of the pixel intensity within a uniformly illuminated region.

16 Plot the variance as a function of the mean. Calculate the slope of this relationship using linear regression. Use the slope value as the calibration ratio.

Single-molecule identification and localization

Note that available free software packages now provide a variety of ways to analyze data in near-real-time, with varying degrees of flexibility and accuracy.

17 Identify candidate peaks.

Band-pass filter the data, with the high frequency cut-off just above the size of one pixel to eliminate noise, and the low frequency cut-on somewhat larger than the peak diameter. The resulting images cannot be used for localization, but are very efficient for identifying the presence of peaks. In every frame, identify candidate peaks that contain at least one pixel above a defined threshold value.

18 Localize the sub-pixel position of the peak.

For every peak, perform a least-squares fitting of an elliptical Gaussian to a 9×9 pixel array surrounding the peak (Fig. 1):

$$PSF(x, y, A, w_x, w_y, B) = A * \exp \left\{ - \left[\frac{(x - x_o)^2}{2w_x^2} + \frac{(y - y_o)^2}{2w_y^2} \right] \right\} + B$$

where x_o and y_o are the coordinates of the molecule, A the amplitude of the intensity distribution, w_x and w_y are the width of the ellipse in the respective axes, and B the background signal intensity. All variables can be treated as free fitting parameters in a nonlinear least squares fit.

19 Estimate the precision of single localizations.

The localization precision is a measure of the confidence in the estimated position of a molecule and principally scales inversely with the square root of the number of photons per molecule, N , but increases with the amount of background noise, b , and the area of the microscope's point-spread function, A :

$$\sigma_{xy}^2 = \frac{Ab}{\sqrt{N}}$$

This calculation, however, ignores the effects of pixilation noise and a more complex equation was developed to calculate the localization precision based on the standard deviation of the Gaussian fit, s , incorporating the pixel size p , and reporting b as the number of noise photons per pixel (Thompson et al., 2002):

$$\sigma_{xy}^2 = \frac{s^2 + p^2/12}{N} + \frac{8\pi s^4 b^2}{p^2 N^2}$$

This is a commonly used equation, though should be applied with caution because it underestimates the true error (Thompson et al., 2002).

20 Exclude poorly localized molecules by selecting only localizations that have a high photon count (e.g. > 100) and acceptable localization precision (e.g. <25

nm). Also, in order to exclude potentially overlapping molecules or those that are in rapid motion, select only non-elliptic Gaussians, i.e. localizations with a low elliptical form (e.g. parameters from Gaussian fit $w_y/w_x < 1.3$).

21 Single-molecule tracking analysis.

Use Matlab single-particle tracking algorithms (freely available from <http://physics.georgetown.edu/matlab/>) to track localized molecules that appear in consecutive frames. Set the tracking radius, which is the maximum allowable frame-to-frame displacement, on the basis of the tracked molecule's expected diffusion coefficient D .

22 Calculate mean square displacement (MSD) and diffusion coefficient for each track.

In 2 dimensions, the relationship between the mean squared displacement (MSD) and the effective diffusion coefficient (D_{eff}) is given by: $\text{MSD} = 4D_{\text{eff}} t$, where t is the interval between observations. For freely diffusing molecules, the MSD increases linearly with time, while for confined diffusion such as within a barrier, this relationship will plateau after an initial linear rise. In both cases, however, the instantaneous D_{eff} can be determined by estimating the slope from the best linear fit on the MSD vs. time curve for the first few (4 or more) time points.

23 Render PALM images.

The spatial distribution of the collection of localized molecules can be plotted in several ways.

a. Scatter plot:

The most basic representation of the data is an X-Y scatter plot of the complete collection of localized molecules. This effectively visualizes the overall density of localized points, but also accentuates the level of background localizations due to noise, or erroneous contaminants such as dust. In this regard, it is often helpful to overlap widefield images taken prior or after the PALM image stack to estimate the level of background localizations outside the transfected cell. Color-coding the individual molecular points according to frame number can visualize lateral drift during the acquisition.

b. Density map:

To create a more interpretable image, localizations can be binned in pixels of chosen size (usually 25 – 50 nm). The pixel value is then determined by the number of molecules localized within that pixel. Thresholding the density map helps eliminate many artifacts, by eliminating pixels that contain just one localization likely to stem from noise.

c. Probability map:

Finally, images can be rendered as probability maps, where each molecule's localization is plotted as a 2D Gaussian with standard deviation σ_{xy} . The resulting pixel values are thus proportional to the probability that a molecule can be found at that location (Betzig et al., 2006).

SUPPORT PROTOCOL 1

DISSOCIATED HIPPOCAMPAL CULTURE PREPARATION

Dissociated hippocampal neurons prepared from rat embryos are a well-established and widely used model system. It allows the study of synaptic structure and function in a much more controlled environment than possible *in vivo*, and in much greater detail as neurons can be easily manipulated, recorded with neurophysiological techniques and readily visualized using imaging techniques. Here, we describe the basic steps for the culture of rat embryonic (E18) hippocampal neurons.

All animal procedures must be approved by Institutes ACUC or equivalent

Materials

#1.5 glass coverslips (Warner instruments)

12-well cell culture plates

Ammonium hydroxide

Hydrogen peroxide

Poly-L-lysine

Sterile filtered water

Hanks Plus (HBSS+, see recipe)

Pasteur pipet

Binocular dissecting microscope

Microsurgical tools

10-cm scissors

11-cm fine shanks forceps

45-tip forceps

8-cm spring scissors

Dissociation medium (see recipe)

Trypsin-EDTA

Trypsin inhibitor

DNase

Trypan Blue stain 0.4%

Hemocytometer Fluoro-2'-deoxyuridine

Uridine

Plating medium (see recipe)

Feeding medium (see recipe)

Preparing coverslips

1. Place coverslips in a heat-resistant coverslip rack.
2. Place one or more racks in a 1000-ml glass beaker.
3. Add 450 ml filtered water, 90 ml ammonium hydroxide and 90 ml hydrogen peroxide.
4. Place beaker on a hot plate stirrer and boil coverslips for 3 hours with moderate stirring.
5. Carefully pour out the hot solution and rinse coverslips extensively by filling the beaker 3 – 5 times with filtered water.
6. Rinse coverslips briefly with 96% ethanol and move the racks to a sterile culture hood.
7. Dip each coverslip in methanol, flame briefly to dry, then let cool for 5 sec before placing in 12-well culture plate.
8. Coat coverslips with 0.5 mg/ml poly-L-lysine overnight in a cell incubator at 37°C.
9. Wash coverslips 3 times with sterile water.
10. Leave the coverslips in plating medium in the incubator until you are ready to plate the cells.

Tissue dissection

11. Sterilize all dissection tools using a dry-heat sterilizer.
12. Prepare ice-cold dissection medium (see recipe) in a 60-mm dish and two 15 ml-tubes.
13. Anesthetize an E18 timed-pregnant rat with CO₂ and sacrifice by cervical dislocation.
14. Open the abdominal wall and remove all embryos.
15. Decapitate the embryo by cutting the head right behind the ears using large scissors.
16. Cut skin and skull through the middle line, and make two cuts behind the eyes using 10-cm scissors.
17. Lift skin and skull to expose the brain using a 10-cm Graefe forceps.

- 18 Move the brain into a 60-mm dish with dissection medium using a micro-spatula.
- 19 Tear off the vessel membrane outside the cortex using 11-cm fine shanks forceps and 45 tip forceps.
- 20 Expose hippocampus using 11-cm fine shanks forceps and 45 tip forceps.
- 21 Cut hippocampus along the outside line and roll it off using 11-cm fine shanks forceps and 8-cm spring scissors.
- 22 Roll and open hippocampus and cut correct region of hippocampus using 11-cm fine shanks forceps and scalpel.
- 23 Chop hippocampal tissue into small pieces and pipette into a 15-ml tube with ice-cold dissection medium.

Tissue digestion

- 24 Spin tissue down for 30 sec at 1000 rpm, and suck off supernatant with Pasteur pipet.
- 25 Rinse with 10 ml ice-cold dissection medium.
- 26 Spin down for 30 sec at 1000 rpm and suck off supernatant with Pasteur pipet.
- 27 Resuspend tissue in pre-warmed 4 ml trypsin-EDTA solution.
- 28 Digest for 5 – 8 min at 37°C, agitating slowly from time to time.
- 29 During incubation, dissolve 3 mg DNase in 3 ml trypsin-EDTA solution.
- 30 Spin cells down for 30 sec at 1000 rpm and aspirate the supernatant with Pasteur pipet.
- 31 Incubate with filter-sterilized, pre-warmed trypsin inhibitor for 3 – 5 min at 37°C.
- 32 Spin down for 1 min at 1000 rpm and aspirate the supernatant with Pasteur pipet. If cells are still stringy with DNA and the supernatant cannot be removed, add a more concentrated, filter-sterilized solution of DNase (e.g. 2 mg in 500 µl for 4 ml tissue), and incubate until supernatant can be removed (1 - 2 min).
- 33 Resuspend tissue on ice in 2 ml dissection medium.
- 34 Triturate slices very gently. Avoid air bubbles. First, use a fire polished glass Pasteur pipette, then use a fire polished Pasteur pipette with reduced diameter, and a third smaller one. Optimally, no cell chunks will be visible, and the solution will be a uniform gray.

Counting cells

- 35 Remove a 10 µl sample of cells and mix in a tube with 90 µl Trypan Blue stain 0.4%.

- 36** Pipette 10 μ l of the cell mixture into a hemocytometer. The cell number inside the 4 $\times$ 4 square will be N . The concentration of cells can then be calculated by multiplying N with 10^5 . For instance, a count of 20 cells in the 4 $\times$ 4 square means 2 million cells per ml of suspension.

Plating cells

- 37** Plate 50 - 70 thousand cells in 1 ml of plating medium (see recipe) on each coverslip.

Feeding cells

- 38** Two days after plating cells, aspirate plating medium and replace with feeding medium (see recipe).
- 39** Twice per week aspirate half the medium and replace it with fresh feeding medium.
- 40** To inhibit the proliferation of dividing non-neuronal cells, we add FUDR (1:1000 from the FUDR stock solution; see recipe) to the feeding medium, 7 – 10 days after plating.

REAGENTS AND SOLUTIONS

Hanks Plus (HBSS+)

HBSS (without Ca^{2+} , Mg^{2+})
10 mM HEPES
33.3 mM glucose
5 μ g/ml gentamycin

Dissection Medium

Hanks Plus (HBSS+)
0.3% (w/v) BSA
12 mM MgSO_4

Digestion Solution

4.2 mM NaHCO_3
25mM HEPES
137 mM NaCl
5 mM KCl
7 mM Na_2HPO_4
Adjust to pH 7.4 and filter sterilize before use.

Plating Medium

Neurobasal Medium
2% (v/v) B27 supplement
1% (v/v) GlutaMAX I
1 µg/ml Gentamycin
5% (v/v) Fetal Bovine Serum

Feeding medium

Neurobasal Medium
2% (v/v) B27 supplement
1% (v/v) GlutaMAX I
1 µg/ml Gentamycin
5% (v/v) Fetal Bovine Serum

FUDR stock solution

10 mM fluoro-2'-deoxyuridine
10 mM uridine
dissolve in H₂O and filter (0.22 µm) sterilize before use.

Extracellular imaging buffer

120 mM NaCl
3 mM KCl
2 mM CaCl₂
2 mM MgCl₂
10 mM glucose
10 mM HEPES
Adjust pH to 7.35 and filter (0.22 µm) to reduce fluorescent contaminations.

COMMENTARY**Background Information**

Signal transmission across neuronal synapses is mediated by neurotransmitter receptor complexes that are retained within the postsynaptic membrane by a diversity of scaffolding molecules that form the postsynaptic density (PSD) (Okabe, 2007; Sheng and Hoogenraad, 2007). Alterations in the number of AMPA-type glutamate receptors (AMPA-Rs) evoked by synaptic activity mediate the plastic changes in synaptic strength that underlie long-term depression and potentiation (LTD and LTP), processes that are thought to be the cellular

basis of learning and memory. Over the past decades, significant progress in the field has led to a thorough understanding of the mechanisms that permit these activity-dependent changes in synaptic receptor content (Malenka and Bear, 2004; Malinow and Malenka, 2002). However, although the details of receptor trafficking to the synapse remain under extensive study, we know little about how receptors are positioned within the synapse once they have reached the PSD. The lateral positioning of AMPARs however, is a critical determinant of the efficiency of signal transmission across synapses (MacGillavry et al., 2011). Because only AMPARs within the close vicinity of a presynaptic release event are exposed to a sufficiently high concentration of glutamate to become activated, ultimately it is the local density of receptors apposing the release site that determines the strength of a synaptic response (Franks et al., 2003; Freche et al., 2011; Lisman et al., 2007; Raghavachari and Lisman, 2004). Thus, although information on the mobility and positioning of individual molecules *within* the synapse is critical to further our understanding of synaptic physiology, we have surprisingly little insight in these processes.

Most of the information we have obtained about the organization of protein complexes at the synapse is derived from biochemical analyses and electron microscopy (EM) that require extensive isolation and fixation procedures that inevitably perturb the innate structure of the synapse and at best provide only a static snapshot of the synapse. Fluorescent light microscopy offers a huge advantage over these techniques in that it is applicable to live systems and especially confocal microscopy has been essential in visualizing the distribution and dynamic movements of proteins in neurons. The continuing development of a growing arsenal of genetically encoded fluorescent tags has added to the versatility of fluorescent microscopy by enabling the specific labeling of one or more proteins simultaneously. However, the optical resolution of conventional light microscopy is inherently limited by diffraction to about half the wavelength, or ~250 nm, preventing the investigation of the organization and mobility of proteins within the compartments of neurons that are smaller than this diffraction limit, such as synapses. Moreover, fluorescence-based measurements of protein mobility such as FRAP provide population averages of mobility, but lack the ability to track single molecules in real-time. In the past few years, several different super-resolution imaging techniques have been developed that cleverly circumvent the diffraction limit, achieving a 2 to 10-fold increase in resolution. These techniques employ different strategies and a number of excellent reviews have been published that describe the principles behind these methods and their application to neuroscience in great depth (Hell, 2007; Huang et al., 2010; Maglione and Sigrist, 2013; Sigrist and Sabatini, 2012). Of these techniques, the single-molecule localization-based super-resolution techniques PALM and STORM (Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006), are versatile tools to study the distribution (Dani et al., 2010) and dynamic behavior (Frost et al., 2010) of molecular species inside dendritic spines with nanometer accuracy.

To study the structural organization of the PSD we used live-cell PALM to measure the spatial distribution of four major PSD scaffold molecules, namely PSD-95, GKAP, Shank and Homer, within single synapses in living hippocampal neurons. Interestingly, we found that in the vast majority of PSDs these four major PSD scaffold molecules are each organized in distinctive nano-domains, 80 nm in diameter (MacGillavry et al., 2013).

Remarkably, time-lapse PALM imaging revealed that the distribution of these scaffolds changed continuously over time within single, living synapses further emphasizing the dynamic nature of the synapse interior (Kerr and Blanpied, 2012). Furthermore, two-color, single-molecule imaging revealed that the dense subdomains of PSD-95 were preferentially enriched in AMPA receptors more than NMDA receptors. We thus concluded that the PSD is organized in a highly non-uniform manner, and individual constituents are assembled into dynamically reorganizing clusters. This malleable organization is likely to dictate continual repositioning of receptors within the synapse and might facilitate the alignment of dense receptor-scaffold clusters with the presynaptic release site, determining the efficiency of synaptic transmission. Indeed, computational simulations of receptor activation showed that release over clusters of receptors evoked postsynaptic currents twice as large as elsewhere.

Mechanisms that modify the size, contents, or position of these nano-domains might thus potentiate or depress synaptic responses, even in the absence of altered receptor numbers. Especially, molecular links that physically link postsynaptic receptor-scaffold domains with the presynaptic release site would maximize the efficiency of synaptic transmission. Transsynaptic adhesion complexes, such as neuroligins and LRRTMs that can interact with PSD-95, for example, might bi-directionally influence the organization and alignment of pre- and postsynaptic elements. Future investigations into these mechanisms will rely heavily on the continual development of super-resolution imaging, especially in live cell preparations, and will undoubtedly provide unprecedented new insight in the organization and function of excitatory synapses.

Critical Parameters and Troubleshooting

Setting the activation and read-out laser power—The power of the activation laser determines the density of molecules per frame and should be optimized to activate as many molecules per frame as possible, while preventing overlap of single-molecule intensity profiles. Because over the course of the acquisition protocol the number of molecules in the inactive or dark state decreases, the activation laser power will need to be adjusted accordingly. For molecular mapping purposes, the read-out laser power is generally set as high as possible to capture as many photons per molecule within a single frame. For single-molecule tracking purposes however, the read-out laser intensity has to be considered carefully to optimally use the limited “photon budget” of fluorophores before they bleach. Generally, the read-out laser is set to a lower intensity to divide the total number of photons over multiple frames and to be able to track molecules for longer periods.

Setting the camera exposure time and frame rate—The exposure time and frame rate are critical parameters determining the final resolution of the image and the ability to discern mobility. For molecular mapping purposes maximal frame rates permit the acquisition of as many molecules as possible within short time windows and reduce the blurring of single-molecule intensity profiles which influences the localization precision. Newer generation EM-CCD cameras generally can acquire at faster frame rates than those made prior to 2011, making this an easier choice. Note that the read-out of larger areas (in the vertical direction) of the CCD camera chip can take longer than the exposure time, thus

to match the frame rate and read-out speed of the CCD it can be helpful to select a sub-region of the CCD chip for read-out.

For single-molecule tracking, the optimal acquisition parameters depend on the characteristics of the molecule that is being studied. For example, treadmilling actin may move only 10s of nm/s. In this case, long inter-frame pauses are necessary to allow molecules to move distances that can be measured accurately. On the other hand, freely diffusing molecules, such as lipid-bound tags in the cell membrane, will move significant distances even within short exposure times. Accordingly, high frame rates are desired to connect single molecules unambiguously in consecutive frames without using a low activation power that unacceptably reduces the number of molecules present per frame. In addition, any motion during the frame integration blurs the molecule, reduces the precision of its localization, and decreases accuracy of measured motion and cell morphology (Frost et al., 2012). However, frame rates that are high enough to sufficiently reduce molecular motion (<2 ms) require the most expensive cameras, and greatly limit the extent of the camera chip that can be imaged and read out quickly enough, thus reducing overall experiment efficiency. To avoid this conflict, we find it advantageous to uncouple the duration of the excitation laser from the camera frame duration (Frost et al., 2012), allowing the user to optimize these acquisition parameters individually and to match the acquisition protocol with the mobility characteristics of the protein of interest (Fig. 2). To achieve laser pulse durations of <2 ms with sufficient power for PALM generally requires either a fiber-coupled 561-nm laser of >200 mW or a 200 mW laser directed to the microscope through the air rather than a fiber optic.

Lateral drift correction—For 2D imaging as described here, the commercially available focus-tracking hardware keeps the sample positioned in the z axis within ~50 nm, which is adequate for most circumstances, particularly during oblique illumination which is less sensitive to this displacement than is TIRF illumination. However, lateral (X-Y) drift of the sample during imaging is essentially unavoidable due to thermal expansion or mechanical instability. In our systems, we find that lateral drift averages <25 nm per 3.5 min, as estimated from the displacement of 100-nm fluorescent beads adhered to a coverslip, or from fixed-cell preparations. For some experiments, this drift is of no consequence. For instance, during the brief persistence of any individual tracked molecule, lateral drift is negligible and can be ignored if the desired measure is independent of position within the cell, such as an average molecular velocity. However, because the amount of drift and its time course during an experiment are unpredictable, for most experiments such as molecular mapping, the drift must be measured and retroactively corrected for. For live-cell preparations, fiducial markers (usually gold beads, which fluoresce red and can be obtained in a variety of convenient small sizes) adhered to the coverslip work well for sample regions that are close enough to the coverslip to image both the sample and the markers in the same image stack. Fiducial marker localizations over time can then be used to measure and correct for lateral drift. For fixed preparations, we routinely use a cross-correlation drift-correction approach (Geisler et al., 2012; Mlodzianoski et al., 2011). However, it would not always be appropriate to apply this to live cell acquisitions if changes in cell morphology occurring over the course of the acquisition would potentially interfere with the correction. For brief

imaging times, the small drift may not impact measurements very severely; indeed, for most fixed cells, PSD nano-cluster area was unaffected by prior application of the cross-correlation drift correction. Newer options that are expensive but probably ideal include hardware that measures XY or XYZ position during imaging, and provides an active feedback to a nanopositioning stage (Lee et al., 2012; Pertsinidis et al., 2010), eliminating the need for *post hoc* correction.

Map resolution—Maps constructed of only a few, but precisely localized molecules will give a poor image resolution, while an image built from a high density of poorer localizations might have a reasonable image resolution. Thus, the final map resolution R_{eff} is determined by two factors, the localization precision (σ_{xy}) of the individual molecules that are used to construct the image and the density of the localizations, or average nearest neighbor distance (r_{NN}) (Gould et al., 2009).

$$R_{eff} = \sqrt{\sigma_{xy}^2 + r_{NN}^2} \quad [*Equation 4]$$

Note that r_{NN} will vary widely across the image, due both to biological structure and to the fraction of molecules that are successfully localized. Other strategies such as Fourier ring correlation (Nieuwenhuizen et al., 2013) offer alternative methods to calculate an overall map resolution.

Cell age—For the study of synaptic processes in mature neurons we generally use neurons grown for 14 - 21 days *in vitro*. Younger neurons have fewer and less matured synapses, while older cultures generally contain more glial cells attached to the coverslip with neuronal processes growing on top of these, limiting the number of transfected neurons that can be imaged using oblique illumination.

Evaluation of protein expression and targeting—Expression of exogenous proteins should always be carefully evaluated. First, correct targeting of the protein can be evaluated with confocal microscopy. Second, in specific cases, it is critical to control the absolute expression levels of the protein of interest to avoid unwanted effects on cellular processes. Using a replacement strategy where endogenous protein levels are minimized using RNAi while an RNAi resistant, fluorophore-tagged protein is expressed is often desirable. Third, if available, antibody-labeling of the endogenous protein can be used to confirm localization and quantify relative expression levels of expressed proteins.

Anticipated Results

Resolving protein distribution within individual PSDs—Scaffolding proteins within the PSD establish the structure and organization of excitatory synapses. We have used PALM to map the location of scaffold molecules within living synapses. We found that scaffolding molecules form dense, subsynaptic clusters that are enriched in AMPA-type glutamate receptors (MacGillavry et al., 2013). Mapping the distribution of other scaffolding molecules, signaling molecules and transmembrane receptors and adhesion molecules will contribute to a better understanding of the internal organization of the synapse. As an

example here, we discuss a PSD-95-mEos2 imaging experiment, providing a practical outline of a typical mapping experiment.

Cells were transfected (at DIV 13) with a plasmid that simultaneously expresses shRNAs targeting endogenous PSD-95 and a mEos2-tagged, short-hairpin resistant version of PSD-95 (shrPSD-95-mEos2). Using this replacement strategy we targeted PSD-95-mEos2 to synapses while minimizing unwanted effects of PSD-95 overexpression (Fig. 3A). A stretch of dendrite was then selected and individual molecules were activated by weak illumination with 405-nm laser and simultaneously imaged by 561-nm laser excitation. Lasers were pulsed for 10-ms triggered by the camera TTL output at a 50 Hz frame rate. Single molecules were accumulated over a period of 10,000 frames (~3 minutes) and localized. Direct comparison of the widefield image (summed TIRF image) with the rendered PALM density map reveals much more clearly delineated PSDs with PALM than with conventional widefield imaging (Fig. 3B). Synapses resolved with PALM appear smaller with complex, but well-defined borders (Fig. 3C and D). We generally achieve 100 to 500 photons per molecule (Fig. 3E). This corresponds to a localization precision of ~20 nm according to equation 3, though this is likely an underestimate of the true error. By applying a threshold to the density plots (pixels containing >1 molecule), individual synapses can be outlined (Fig. 3F), and all points within the PSD can be isolated (Fig. 3G) and plotted according to their local density (Fig. 3H). We calculate this local density value D_L for each molecule by counting the number of its neighboring molecules within a radius R . The value for R is calculated for each individual synapse as 5 times the nearest neighbor distance within the synapse, and as such normalizes for differences in the measured density between individual synapses (differences which in turn can stem from biological or experimental factors). These local density plots can then be thresholded (molecules with a local density value ≥ 40 , as derived in MacGillavry et al., 2013) to outline subsynaptic nano-domains (Fig. 3H). We find a large variation in the size of the nano-domains between individual synapses, with nano-cluster area ranging from 500 to 15,000 nm² (Fig. 3I and J).

Single-molecule tracking within dendritic spines—PALM is especially powerful as it enables tracking over time of single molecules within the cell interior, providing unprecedented insights in the dynamics of proteins within biological structures. Here, we demonstrate a tracking experiment using a mEos2-tagged protein motif that is targeted to the plasma membrane. Specifically, we fused mEos2 to the N-terminal 20 amino acids of GAP-43 containing a palmitoylation motif (based on the EYFP-mem construct from Clontech). This probe is efficiently targeted to the plasma membrane where it is able to diffuse essentially free of binding partners. Neurons (14 DIV) were transfected with this plasmid for two days before imaging. Single molecules were sparsely activated with weak 405-nm illumination and excited with low 561-nm laser power to track molecules over multiple frames (Fig. 4A). Here we collected 10,000 frames at 50 Hz with relatively short, 5-ms laser pulses to limit motion-induced blurring of the freely diffusing single-molecule intensity profiles. Individual localizations were connected in trajectories that allowed a maximal inter-frame displacement of 500 nm. Individual localizations were plotted in a scatter plot and overlaid with the deduced single-molecule trajectories (Fig. 4B). The high mobility of this probe provides a large pool of molecules that can be imaged over long time

frames as they exchange in and out of the area of the cell targeted by the excitation light. The resulting high density of localizations outlined the morphology of a stretch of dendrite and its spines, at resolution sufficient to measure spine neck widths accurately (Frost et al., 2012), thus demonstrating the use of PALM to acquire both live-cell super-resolved morphology and molecular dynamics simultaneously. For every track that persisted longer than 3 frames (23% of all localized molecules in this example) we constructed a MSD vs. time plot and derived the D_{eff} from the linear fit to the first 3 time points. Interestingly, the histogram of D_{eff} displayed a clear bi-modal distribution, suggesting there are two populations of mem-mEos2 molecules that diffuse with different mobility characteristics, a slow (mean D_{eff} = 0.04 $\mu\text{m}^2/\text{s}$) and a rapidly diffusing pool (mean D_{eff} = 0.3 $\mu\text{m}^2/\text{s}$, Fig. 4C).

Time Considerations

Coverslips, dissociated hippocampal cultures, and transfection—Coverslips are cleaned and coated on the day before making the neuronal cultures, and involves a 3 hour boiling step, with the washing and coating of the coverslips taking another 30 to 45 minutes. The culture preparation typically takes around 3 hours, including surgery, dissociation and plating of cells. Allow neurons to grow for 2 to 3 weeks for maturation of neuronal morphology. Transfect neurons 1 to 7 days before imaging, depending on expression level and efficiency of targeting to the desired cell compartment of the protein of interest. For shrPSD-95-mEos2, allowing 3 days for efficient knockdown of endogenous PSD-95 and synaptic targeting of mEos2-tagged PSD-95 results in a very modest increase of PSD-95 per synapse (1.6-fold, instead of the 10-fold overexpression without concurrent shRNA).

Imaging and analysis—We generally image cells within an hour after taking them from the incubator. It is important, although not trivial, to monitor cell health. Usually co-transfection of a cytosolic fluorescent protein that does not spectrally interfere with the photoactivatable marker (e.g. Cerulean-3) helps determining overall neuronal morphology. *Post-hoc* molecular localization typically takes 1 to 3 hours per image stack depending on the size of the file, density of imaged molecules and computer CPU.

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LITERATURE CITED

- Betzig E, Patterson G, Sougrat R, Lindwasser O, Olenych S, Bonifacino J, Davidson M, Lippincott-Schwartz J, Hess H. Imaging intracellular fluorescent proteins at nanometer resolution. *Science*. 2006; 313:1642–1645. [PubMed: 16902090]
- Dani A, Huang B, Bergan J, Dulac C, Zhuang X. Superresolution imaging of chemical synapses in the brain. *Neuron*. 2010; 68:843–856. [PubMed: 21144999]
- Dempsey GT, Vaughan JC, Chen KH, Bates M, Zhuang X. Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging. *Nat Methods*. 2011; 8:1027–1036. [PubMed: 22056676]
- Ehlers M, Heine M, Groc L, Lee M, Choquet D. Diffusional trapping of GluR1 AMPA receptors by input-specific synaptic activity. *Neuron*. 2007; 54:447–460. [PubMed: 17481397]

- Franks KM, Stevens CF, Sejnowski TJ. Independent sources of quantal variability at single glutamatergic synapses. *J Neurosci*. 2003; 23:3186–3195. [PubMed: 12716926]
- Freche D, Pannasch U, Rouach N, Holcman D. Synapse geometry and receptor dynamics modulate synaptic strength. *PLoS One*. 2011; 6:e25122. [PubMed: 21984900]
- Frost NA, Lu HE, Blanpied TA. Optimization of cell morphology measurement via single-molecule tracking PALM. *PLoS One*. 2012; 7:e36751. [PubMed: 22570741]
- Frost NA, Shroff H, Kong H, Betzig E, Blanpied TA. Single-molecule discrimination of discrete perisynaptic and distributed sites of actin filament assembly within dendritic spines. *Neuron*. 2010; 67:86–99. [PubMed: 20624594]
- Geisler C, Hotz T, Schonle A, Hell SW, Munk A, Egner A. Drift estimation for single marker switching based imaging schemes. *Opt Express*. 2012; 20:7274–7289. [PubMed: 22453409]
- Gould T, Verkhusha V, Hess S. Imaging biological structures with fluorescence photoactivation localization microscopy. *Nat Protoc*. 2009; 4:291–308. [PubMed: 19214181]
- Heine M, Groc L, Frischknecht R, Béïque J, Lounis B, Rumbaugh G, Huguier R, Cognet L, Choquet D. Surface mobility of postsynaptic AMPARs tunes synaptic transmission. *Science*. 2008; 320:201–205. [PubMed: 18403705]
- Hell SW. Far-field optical nanoscopy. *Science*. 2007; 316:1153–1158. [PubMed: 17525330]
- Hess S, Girirajan T, Mason M. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys J*. 2006; 91:4258–4272. [PubMed: 16980368]
- Huang B, Babcock H, Zhuang X. Breaking the diffraction barrier: super-resolution imaging of cells. *Cell*. 2010; 143:1047–1058. [PubMed: 21168201]
- Jones SA, Shim SH, He J, Zhuang X. Fast, three-dimensional super-resolution imaging of live cells. *Nat Methods*. 2011; 8:499–508. [PubMed: 21552254]
- Kerr JM, Blanpied TA. Subsynaptic AMPA receptor distribution is acutely regulated by actin-driven reorganization of the postsynaptic density. *J Neurosci*. 2012; 32:658–673. [PubMed: 22238102]
- Lee SH, Shin JY, Lee A, Bustamante C. Counting single photoactivatable fluorescent molecules by photoactivated localization microscopy (PALM). *Proc Natl Acad Sci U S A*. 2012; 109:17436–17441. [PubMed: 23045631]
- Lisman JE, Raghavachari S, Tsien RW. The sequence of events that underlie quantal transmission at central glutamatergic synapses. *Nat Rev Neurosci*. 2007; 8:597–609. [PubMed: 17637801]
- MacGillavry HD, Kerr JM, Blanpied TA. Lateral organization of the postsynaptic density. *Mol Cell Neurosci*. 2011; 48:321–331. [PubMed: 21920440]
- MacGillavry HD, Song Y, Raghavachari S, Blanpied TA. Nanoscale Scaffolding Domains within the Postsynaptic Density Concentrate Synaptic AMPA Receptors. *Neuron*. 2013; 78:615–622. [PubMed: 23719161]
- Maglione M, Sigrist SJ. Seeing the forest tree by tree: super-resolution light microscopy meets the neurosciences. *Nat Neurosci*. 2013; 16:790–797. [PubMed: 23799471]
- Malenka RC, Bear MF. LTP and LTD: an embarrassment of riches. *Neuron*. 2004; 44:5–21. [PubMed: 15450156]
- Malinow R, Malenka RC. AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci*. 2002; 25:103–126. [PubMed: 12052905]
- Manley S, Gillette JM, Patterson GH, Shroff H, Hess HF, Betzig E, Lippincott-Schwartz J. High-density mapping of single-molecule trajectories with photoactivated localization microscopy. *Nat Methods*. 2008; 5:155–157. [PubMed: 18193054]
- McKinney S, Murphy C, Hazelwood K, Davidson M, Looger L. A bright and photostable photoconvertible fluorescent protein. *Nat Methods*. 2009; 6:131–133. [PubMed: 19169260]
- Mlodzianoski MJ, Schreiner JM, Callahan SP, Smolkova K, Dlaskova A, Santorova J, Jezek P, Bewersdorf J. Sample drift correction in 3D fluorescence photoactivation localization microscopy. *Opt Express*. 2011; 19:15009–15019. [PubMed: 21934862]
- Nieuwenhuizen RP, Lidke KA, Bates M, Puig DL, Grunwald D, Stallinga S, Rieger B. Measuring image resolution in optical nanoscopy. *Nat Methods*. 2013; 10:557–562. [PubMed: 23624665]
- Okabe S. Molecular anatomy of the postsynaptic density. *Mol Cell Neurosci*. 2007; 34:503–518. [PubMed: 17321751]

- Pertsinidis A, Zhang Y, Chu S. Subnanometre single-molecule localization, registration and distance measurements. *Nature*. 2010; 466:647–651. [PubMed: 20613725]
- Raghavachari S, Lisman JE. Properties of quantal transmission at CA1 synapses. *J Neurophysiol*. 2004; 92:2456–2467. [PubMed: 15115789]
- Rust MJ, Bates M, Zhuang X. Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat Methods*. 2006; 3:793–795. [PubMed: 16896339]
- Saxton MJ, Jacobson K. Single-particle tracking: Applications to membrane dynamics. *Annual Review of Biophysics and Biomolecular Structure*. 1997; 26:373–399.
- Sheng M, Hoogenraad C. The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu Rev Biochem*. 2007; 76:823–847. [PubMed: 17243894]
- Shim SH, Xia C, Zhong G, Babcock HP, Vaughan JC, Huang B, Wang X, Xu C, Bi GQ, Zhuang X. Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes. *Proc Natl Acad Sci U S A*. 2012; 109:13978–13983. [PubMed: 22891300]
- Sigrist SJ, Sabatini BL. Optical super-resolution microscopy in neurobiology. *Curr Opin Neurobiol*. 2012; 22:86–93. [PubMed: 22051692]
- Thompson R, Larson D, Webb W. Precise nanometer localization analysis for individual fluorescent probes. *Biophys J*. 2002; 82:2775–2783. [PubMed: 11964263]
- van de Linde S, Loschberger A, Klein T, Heidbreder M, Wolter S, Heilemann M, Sauer M. Direct stochastic optical reconstruction microscopy with standard fluorescent probes. *Nat Protoc*. 2011; 6:991–1009. [PubMed: 21720313]
- Zhang M, Chang H, Zhang Y, Yu J, Wu L, Ji W, Chen J, Liu B, Lu J, Liu Y, Zhang J, Xu P, Xu T. Rational design of true monomeric and bright photoactivatable fluorescent proteins. *Nat Methods*. 2012; 9:727–729. [PubMed: 22581370]

Figure 1. Conceptual basis of PALM

(A) Image of a typical emission profile from a single mEos2 molecule photoactivated from its basal green state to its activated red state by a brief exposure to 405-nm light. The distribution of pixel intensities (middle) can be fit by an elliptical Gaussian function (right). The molecule's x-y position is then estimated by determining the center of the peak.

(B) Schematic representation of information obtained from PALM experiments. In a molecular mapping experiment, localizations of many of thousands of molecules are accumulated in a series of frames that can then be combined to reconstruct an image of the protein distribution in live and fixed cells. In a single-molecule tracking PALM experiment, individual molecules that appear in consecutive frames are assembled into trajectories to measure their motion in live cells.

Figure 2. Hardware configurations for PALM

(A) Hardware schematic. A typical PALM microscope setup consists of an inverted microscope equipped for TIRF imaging. Collimated lasers are launched into single-mode optic fibers via an AOTF to direct the laser beam into the TIRF port of the microscope. The beam is collimated and aligned with a collimating lens (CL), and is then steered by a translating mirror (M) so that its position on the back aperture of the objective (OBJ) can be manipulated to adjust the incident angle. The emission light is passed through the dichroic mirror (DCM) in the microscope and directed to the EM-CCD camera. For two-color imaging an image-splitting device like the Dual-View system can be placed in between the microscope and the camera.

(B) Excitation configurations to reduce out of focus fluorescence. The position where the laser beam enters the back aperture of the objective determines the angle at which the laser beam enters the sample. When a beam parallel to the optical axis enters the center of the

objective, it will propagate vertically through the sample, producing wide-field excitation of fluorophores in and above the focal point (right). When the beam is displaced away from the center of the optical axis, the objective will steer the beam with an increasingly strong incident angle into the sample. At intermediate angles this will produce “oblique illumination” of the sample, limiting the excitation of out-of-focus fluorophores (center). When the incident angle is greater than a “critical angle” determined by the refractive index change between the glass coverslip and the aqueous environment of the imaging buffer and cells, the light will undergo total internal reflection (TIR) off the glass-water interface, allowing only a non-propagating evanescent wave to excite the sample. The evanescent wave decays rapidly (within ~200 nm), so only those fluorophores that are very close to the coverslip are excited. Lack of the out-of-focus fluorescence from molecules deep in the cell greatly decreases the background fluorescence in the image, and permits much higher accuracy localization of the molecules in focus.

Figure 3. Example: mapping the distribution of PSD-95 within individual synapses

(A) Confocal image of a hippocampal neuron transfected at DIV 17 with shrPSD-95-mEos2 (green) and cerulean3 (blue) and fixed at DIV 20.

(B) Stretch of dendrite from a live neuron (DIV 16) expressing shrPSD-95-mEos2, with the lower left part showing the summed TIRF image (green) and the upper right part showing the PALM image (red). Scale bar, 500 nm.

(C and D) Examples of individual PSDs resolved with shrPSD-95-mEos2 PALM, plotted as density maps with 25-nm pixels. Scale bar, 200 nm.

(E) Histogram of number of photons collected in the image of each molecule.

- (F-G)** Example of a synapse imaged with shrPSD-95-mEos2 PALM and thresholded to outline the entire PSD. Scale bar, 200 nm.
- (G)** Scatter plot of the single-molecule localizations falling within the PSD in **(F)**.
- (H)** Each localization color-coded for its local density (see text) revealing the heterogeneous distribution of PSD-95 within an individual synapse. The border of the nano-cluster is defined by applying a fixed threshold to the local density map. Scale bar, 200 nm.
- (I)** Histogram of nano-cluster area measured with shrPSD-95-mEos2 PALM (data from 498 synapses, 26 neurons).
- (J)** Examples of local density plots of two PSDs imaged with shrPSD-95-mEos2 live-cell PALM. Scale bar, 200 nm.

Figure 4. Example: tracking the diffusion characteristics of a membrane-bound tag

(A) Series of frames from a single-molecule tracking experiment showing activation of two mem-mEos2 molecules, one which lasts for 2 frames (blue arrowhead) and one that lasts for 5 frames (red arrowhead). Scale bar, 1 μm .

(B) Scatter plot of all localized mem-mEos2 molecules in a stretch of dendrite (DIV 14). The first and last localizations of each tracked molecule are plotted in green and red respectively. Scale bar 5 μm .

(C) Histogram of diffusion coefficients for mem-mEos2 in the cell shown in A and B. Solid lines show the components of a Gaussian fit to the histogram, showing two well-separated subpopulations.