Chapter 5

Single Nanoparticle Tracking of Surface Ion Channels and Receptors in Brain Cells

Juan Varela, Julien Dupuis, and Laurent Groc

Abstract

Neuronal communication requires a constant adjustment of the number and subtype of neurotransmitter receptors and ion channels at the plasma membrane. Although classical fluorescence ensemble approaches have provided valuable insights into cellular and molecular pathways, their inherent limitations (e.g., average behaviors, spatial resolution) have prompted neuroscientists to use and adapt other imaging approaches. Among these, single nanoparticle tracking offers a remarkable mean to explore, at the single neurotransmitter receptor and ion channel level, the behavior of these molecular actors in living brain cells. In this chapter, we describe the procedure and experimental steps necessary to perform single nanoparticle tracking of membrane neurotransmitter receptors and ion channels in living brain cells.

Key words Single-molecule detection, Nanoparticle, Quantum dot, Neurons

1 Introduction

One important feature of neuronal connections is to adapt their strength in response to network stimuli. One way of adapting is to dynamically regulate the number and subtype of neurotransmitter receptors and ion channels at the pre- and postsynaptic levels. Over the last decade, numerous studies have explored the molecular mechanisms shaping fast and sustained changes in synaptic transmissions. The current view is that neurotransmitter receptors and ion channels cycle to and from the plasma membrane through exocytosis and endocytosis processes, respectively, and once inserted at the membrane, they diffuse at the surface of brain cells and even within the synapse through lateral diffusion. These major discoveries have been possible thanks to the development of high-resolution imaging approaches that revolutionized the cellular biology field (2014 Nobel Laureates in Chemistry). Indeed, classical fluorescence approaches using, for instance, green fluorescent protein and its variants can only provide information on the average behavior of rather large receptor clusters with diffraction-limited spatial

Rafael Luján and Francisco Ciruela (eds.), *Receptor and Ion Channel Detection in the Brain: Methods and Protocols*, Neuromethods, vol. 110, DOI 10.1007/978-1-4939-3064-7_5,

resolutions (few hundreds of nanometers). In contrast, single nanoparticle/molecule tracking techniques allow to directly measure the movements of individual surface neurotransmitter receptors and ion channels with nanometer resolutions [4]. Movements of neurotransmitter receptors and ion channels at the neuronal surface have initially been recorded by tracking submicron-sized latex particles manipulated with optical tweezers. The size of the particles however precluded access to confined compartments, such as synapses. This drawback has been overcome by replacing the bead with individual nanometer-sized fluorescent objects such as semiconductor nanocrystals (e.g., "quantum dots"), which are less bulky objects (~10–20 nm) with characteristic blinking behavior and strong photoresistance (allowing minute-long recordings). The method is based on the labeling of surface neurotransmitter receptors and ion channels with low concentrations of nanoparticle complexes through a ligand (typically an antibody) binding to an extracellular domain of the receptor/channel or to a genetically engineered tag. Single nanoparticle tracking provides an important advantage over ensemble measurements, namely, the possibility to track molecules with a position accuracy only limited by the signal-to-noise ratio at which the molecules are detected, typically below 30 nm in live cells. By removing the averaging inherent to ensemble measurements, single nanoparticle tracking yields a measure of the full mobility distribution of neurotransmitter receptors and ion channels within various cellular compartments (e.g., inside and outside synapses). This new approach and its application to the neurobiology field revealed, for instance, that neurotransmitter receptors and ion channels exchange rapidly by lateral diffusion between submicron neighboring compartments such as the synaptic, peri-, and extrasynaptic spaces. Thus, the single-molecule/nanoparticle tracking offers a unique possibility to explore, at the single neurotransmitter receptor and ion channel level, the behavior of these molecular actors in living brain cells. The following chapter specifically describes the procedure and experimental steps necessary to perform single nanoparticle tracking of membrane neurotransmitter receptor and ion channel in living brain cells.

2 Materials

2.1 Media and Buffers

The most commonly used medium for single-particle tracking applications on neurons is complete Neurobasal medium. Phosphate buffer saline (PBS) is also often used to dilute antibodies or quantum dots, or as a solvent for drugs applied in the bath.

1. Complete Neurobasal medium. To prepare complete recording medium, supplement 500 mL of Neurobasal medium (Gibco, Life Technologies, Thermo Fisher Scientific Inc., USA)

with 10 mL of 50X B-27 serum-free supplement (Gibco, Life Technologies, Thermo Fisher Scientific Inc., USA) and glutamine (Sigma-Aldrich, St Louis, USA) at a final concentration of 2 mM. Complete Neurobasal medium can be stored at 4 °C for several weeks under sterile conditions. To avoid unspecific binding of antibodies, complete Neurobasal medium is pre-heated at 37 °C on the day of experiment and supplemented with casein or bovine serum albumin (BSA; Sigma-Aldrich, St Louis, USA) at a final concentration of 1 % before use.

2. Phosphate buffer saline (PBS). When required, quantum dot-coupled secondary antibodies can be pre-diluted in PBS 1X. To obtain 1 L of PBS 1X, dilute 100 mL of PBS solution 10X (Euromedex, France) in 900 mL of ultrapure water. Filter before use and store at 4 °C.

2.2 Dissociated Neuronal Cultures

Although the procedures described here apply to any neuronal preparation, we will deliberately focus on primary hippocampal cultures which are the most commonly used in vitro model by the neuroscience community. Briefly, cultures of hippocampal neurons are prepared from embryonic (day 18) Sprague-Dawley rats as follows (for details, *see* [5]):

1. Hippocampi of embryonic day 18 Sprague-Dawley rats are dissected and transferred to a 15 mL Falcon tube filled with pre-heated HBSS medium (Gibco, Life Technologies, Thermo Fisher Scientific Inc., USA).
2. To initiate dissociation, the HBSS medium is then replaced by 5 mL of pre-heated trypsin/EDTA solution (Gibco, Life Technologies, Thermo Fisher Scientific Inc., USA) for 15 min incubation at 37 °C. Trypsin/EDTA is then removed and washed twice with 10 mL of pre-heated HBSS medium. Hippocampi are transferred in 2 mL of pre-heated HBSS, and the tissue is dissociated by gently pipetting several times.
3. Cells are plated on polylysine-precoated glass coverslips in Ø60 mm petri dishes (Falcon, Corning Inc., USA) at a density of 350×10^3 cells per dish. Coverslips are maintained at 37 °C/5 % CO_2 for 72 h in 5 mL of a 3 % horse serum-supplemented Neurobasal medium. This medium is then replaced by pre-heated and equilibrated serum-free Neurobasal medium, and cultures can be maintained at 37 °C/5 % CO_2 for up to 20 days.
4. For imaging experiments requiring the tracking of recombinant membrane proteins, neurons can be transfected with plasmids of interest at 7–14 days in vitro using the Effectene transfection kit (Qiagen, Germany) according to the manufacturer's instructions.

Warning: all procedures are performed in sterile conditions under a hood.

2.3 Quantum Dot Labeling

Single-particle tracking on neurons requires highly specific monoclonal or polyclonal antibodies targeting extracellular epitopes of the receptor or channel of interest, as well as photostable nanoparticles functionalized with secondary antibodies of which quantum dots (QDs) are the most commonly used. Bright and coated QDs are commercially available, with fluorescence emission at several different wavelengths.

1. Cell culture incubator set at 37 °C/5 % CO_2
2. Twelve-well culture plate
3. 50 mL of BSA-supplemented (1 %) Neurobasal medium
4. 1 mL of PBS 1X
5. Appropriate primary antibody and QD-coupled secondary antibody
6. 1000 μL, 200 μL, and 2.5 μL micropipettes
7. Tweezers

Warning: primary antibodies and secondary antibody-coupled QD should always be carefully kept on ice. Neurobasal medium should be pre-heated and equilibrated in the incubator before use.

2.4 Imaging Setup

There is a wide variety of microscope setups that can be adequate to perform single QD tracking (Fig. 1). The main features needed are:

1. Inverted fluorescence microscope body with appropriate excitation/emission filters adapted to the optical properties of the fluorophores
2. Vibration isolation table supporting the microscope (e.g., TMC Vibration Control, USA) to avoid drifts during acquisitions
3. Magnification objectives in the range of 60x to 100x
4. Mercury lamp
5. EM-CCD camera (e.g., Evolve, Photometrics, USA)
6. Bath heater system to maintain samples at 37 °C (e.g., TC-324B/344B Temperature Controller, Warner Instruments, USA)
7. Acquisition software (e.g., MetaMorph, Molecular Devices, USA)

3 Methods

3.1 Transfections

In order to perform the tracking of an exogenous membrane protein, a transfection needs to be done before the QD experiment is carried on. In many cases, a transfection may also be required to have a marker that may be used to allow co-localization studies (such as a synaptic protein). Whether it is a simple or a double

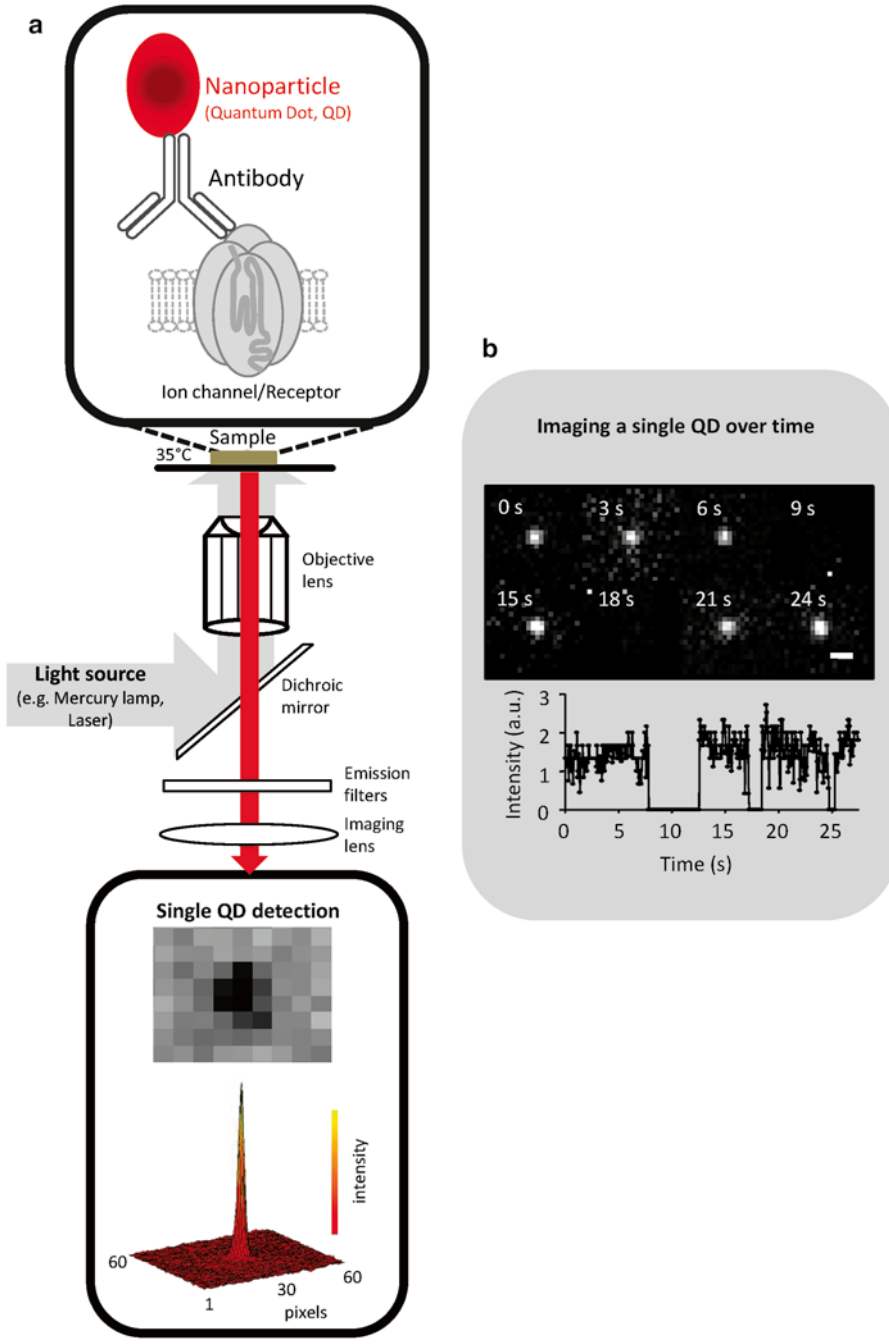

Fig. 1 Schematic microscopy setting to perform single nanoparticle tracking in live brain cells.(**a**) Description of the optic path to achieve single nanoparticle tracking of a quantum dot. The single nanoparticle is part of a complex with an antibody directed against an extracellular epitope of the ion channel/receptor of interest. (**b**) The blinking of a single quantum dot (655 nm emission) is represented. Note the fast change in luminescence over time

transfection, the QD experiment needs to be done once the transfected proteins are expressed (e.g., 24–72 h after transfection).

3.2 QD Labeling of Extracellular Epitopes of Membrane Proteins in Live Neurons

1. Equilibrate a flask of complete medium in the incubator at 37 °C and 5 % CO_2 overnight before experiment. Leave the cap of the flask slightly loose in order to permit gas exchange. In general, 50 mL is more than enough for a typical experiment.
2. One hour before starting the experiment, add bovine serum albumin (BSA) to the medium (final concentration of 1 %). The BSA decreases unspecific binding of QD. Do not agitate the flask, and just dissolve the BSA gently mixing and leaving the flask in the incubator for an hour (*see* **Note 3** for multiple staining).
3. Put in a 6-well plate 3 mL of complete medium with BSA in each well, to wash the coverslip after incubation with antibodies and QD. Leave the plate in the incubator as well.
4. Prepare primary antibody solution mixing 0.5 μL of antibody in 500 μL of medium with BSA (1:1000 dilution, but this concentration should be adjusted according to the particular molecule to be tracked).
5. Vortex the antibody solution gently.
6. Prepare functionalized QD dilution (Fab fragments coupled to QD) by diluting QD stock 1:10000 in medium with BSA. To do this, prepare a pre-dilution 1:10 in PBS, then dilute 1:1000 in the BSA medium.
7. Vortex the functionalized QD solution.
8. Stretch a piece of clean Parafilm over a flat surface to do the incubation with antibodies.
9. Put a 100 μL drop of primary antibody solution over the Parafilm.
10. Carefully grab a coverslip of your cultured neurons with a thin forceps and flip it over the antibody drop, in such a way that the cells are facing the Parafilm (and therefore embedded in antibody solution).
11. Put the Parafilm and coverslip back to the incubator for 10 min.
12. Wash the coverslip by submerging it in three of the wells containing medium with BSA for 10 s each time.
13. Repeat the incubation process but now with a drop of the QD solution, letting it for 10 min inside the incubator.
14. Wash again the coverslip three times in the wells with medium and BSA that haven't been used.

15. Place the coverslip in an imaging chamber and fill the chamber with cell medium (about 200 μL depending on the cell chamber type).
16. Place the chamber in the microscope, controlling the temperature and CO_2 as required for the desired experiment (atmosphere typically fixed at 5 % CO_2 at 37 °C).

3.3 Imaging

Single-molecule tracking can be performed in standard epifluorescence microscopes as long as the camera is sensitive and fast enough to detect individual QD at a fast sampling rate. Make sure you have the right filter sets for your labels. If the instrument has the right equipment, the imaging procedure is straightforward.

1. Choose a cell of interest according to the quality of the labeling and bright field image.
2. Make sure that the number of QD over the cell is high enough to allow a decent statistical analysis but low enough to avoid crossing of trajectories from different QD. If the QD density is too high or too low, then the incubation process needs to be adjusted changing the concentration of QD.
3. Record images of the fluorescent dyes used, as well as a bright field image.
4. Take an image stream of QD (in general a few 100 consecutive frames is enough) at a high sampling rate (at least 20 or 30 images per second).
5. Avoid imaging for long time periods, as the photodamage increases and some of the QD will be internalized. For most cell membrane receptors, imaging can be performed for about 20 min after incubation with QD.

3.4 Image Analysis

Image processing for single-particle tracking is a delicate process, and if possible, it should be performed with the assistance of imaging experts until the needed skills are acquired. Particle tracking methodologies are a research topic on itself, and there are constant improvements and new developments that are better suited for specific applications [2, 3]. There are several available commercial softwares to do automated particle tracking such as Imaris (Bitplane) or Volocity (PerkinElmer), while some laboratories prefer to develop their own codes. Another solution is to use available scripts and plug-ins that run, for example, in Matlab (MathWorks) or ImageJ (NIH).

The first step for particle tracking procedures is the automated detection of the QD in every frame of the stream. To do this, one of the most robust and precise ways is to detect intensity maxima and fit two dimensional Gaussians to obtain the position of the QD. The pointing accuracy of this procedure is mainly given by the quality of the distance traveled by the QD during and each

frame's time exposure. Due to the high signal-to-noise ratio of typical QD tracking experiments, the accuracy could reach about 30 nm [4]. If markers of interest are transfected beforehand (e.g., GFP-tagged synaptic protein), QD can be precisely located within specific membrane compartments – e.g., synaptic, perisynaptic, and extrasynaptic compartments – on each frame of a stream. Identified QD locations can then be projected on a single image to provide a high-resolution distribution of the receptor/QD complexes over the acquisition. After all QD positions are detected, software packages link individual QD positions from one frame to the following one (*see* **Note 4** for blinking reconnection).

Once the detection and tracking algorithms are applied over the QD image streams, the standard way of characterizing the behavior of a diffusing particle is by calculating the mean squared displacement (MSD).

For a trajectory of N positions ($x(t),y(t)$) with times from $t=0$ to $t=N^*\Delta t$, the MSD corresponding to the time interval $\tau = n\Delta t$ can be calculated as follows:

$$\sum_{i=1}^{N-n} \frac{\left[x\left((i+n)*\Delta t\right)-x\left(i*\Delta t\right)\right]^2+\left[y\left((i+n)*\Delta t\right)-y\left(i*\Delta t\right)\right]^2}{N-n}$$

From each MSD, the instantaneous diffusion coefficient may be calculated, measuring how much each QD moves (in μm per second). The linear fit of the first few points (say 4 or 5) of the MSD(τ) plotted against the lag time τ is proportional to the diffusion coefficient:

$$\mathrm{MSD}(\tau) = < r^2 > (\tau) = 4D\tau$$

4 Notes

1. Although still in its infancy, single-particle tracking in cultured organotypic brain slices has also recently been reported. While protocols remain very similar to those developed for dissociated neuronal cultures, we encourage the reader to refer to [1] for more details.
2. Since quantum dots of non-overlapping excitation/emission wavelengths are now available, dual-color tracking experiments can be performed simultaneously using a beam splitter (e.g., DV2 Multichannel Imaging System, Photometrics, USA) to separate acquisition channels.
3. If a particular staining needs to be done (e.g., MitoTracker Green to facilitate detection of synapses), the staining should be done according to the manufacturer's protocol, which could mean doing the staining before or after QD incubation.

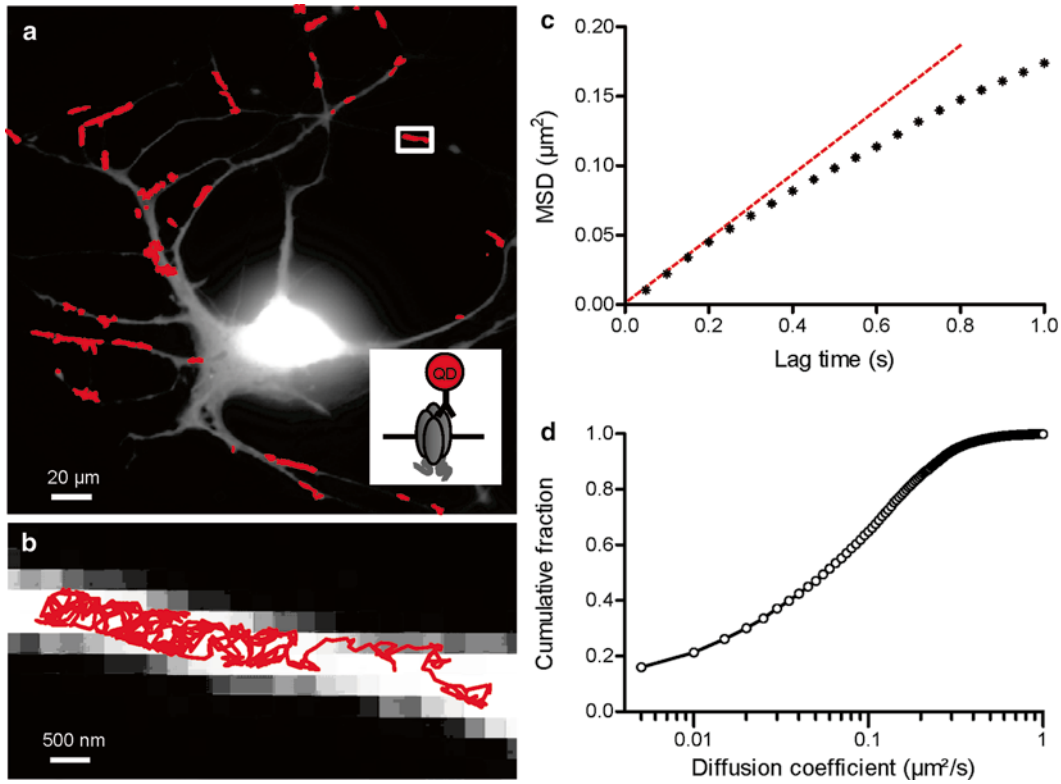

Fig. 2 Single-particle tracking on hippocampal neurons. (**a**) Trajectories of single QD-labeled surface NMDA receptors on the dendrites of a hippocampal neuron (20 Hz acquisition, 30 s duration). *Inset*, schematic representation of a receptor labeled by an antibody coupled to a quantum dot. (**b**) Representative trajectory taken from panel **a** of a single NMDA receptor on a dendrite. (**c**) Plot of the mean square displacement (MSD) versus time of the NMDA receptor surface trajectory presented in panel **b**. The instantaneous diffusion coefficient, *D*, is calculated for each trajectory from a linear fit (*red dotted line*) of the first 4 points of the mean square displacement versus time following the equation $MSD(t) = <r^2>(t) = 4Dt$.(**d**) Cumulative distribution of NMDA receptor instantaneous surface diffusion coefficients. The initial point of the distribution represents the immobile fraction of the population of receptors ($D < 0.005$ µm²/s for typical pointing accuracy)

4. A typical property of QD luminescence is the blinking (Fig. 1b). Up to a certain point, this effect can be corrected in the trajectories analysis imposing conditions to the linkage of trajectories that are adequate to the experimental settings. In particular, automated blinking correction should consider a maximum number of frames to reconnect trajectories and a maximum distance allowed for the displacement during the blink (Fig. 2).

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique, Agence Nationale de la Recherche, Conseil Régional d'Aquitaine, and Marie Curie Individual Fellowship 326442.

We thank lab members for constructive discussions and critical reading of the manuscript and the Bordeaux Imaging Center for technical support.

References

1. Biermann B, Sokoll S, Klueva J, Missler M, Wiegert JS, Sibarita JB, Heine M (2014) Imaging of molecular surface dynamics in brain slices using single-particle tracking. Nat Commun 5:3024
2. Chenouard N, Smal I, de Chaumont F, Maška M, Sbalzarini IF, Gong Y, Cardinale J, Carthel C, Coraluppi S, Winter M et al (2014) Objective comparison of particle tracking methods. Nat Methods 11:281–289
3. Deschout H, CellaZanacchi F, Mlodzianoski M, Diaspro A, Bewersdorf J, Hess ST, Braeckmans K (2014) Precisely and accurately localizing single emitters in fluorescence microscopy. Nat Methods 11:253–266
4. Groc L, Lafourcade M, Heine M, Renner M, Racine V, Sibarita JB, Lounis B, Choquet D, Cognet L (2007) Surface trafficking of neurotransmitter receptor: comparison between single-molecule/quantum dot strategies. J Neurosci 27:12433–12437
5. Mikasova L, De Rossi P, Bouchet D, Georges F, Rogemond V, Didelot A, Meissirel C, Honnorat J, Groc L (2012) Disrupted surface cross-talk between NMDA and Ephrin-B2 receptors in anti-NMDA encephalitis. Brain 135:1606–1621