2 Imaging Single Receptors with Quantum Dots

Sabine Lévi,[1] Maxime Dahan,[2] and Antoine Triller[3]

[1]*INSERM UMR-839, IFM, Paris, France;* [2]*Laboratoire Kastler Brossel, CNRS UMR8552, ENS, Paris, France;* [3]*INSERM U497, ENS, Paris, France*

ABSTRACT

This chapter describes a highly sensitive approach for tracking the motion of membrane molecules over extended time periods with single-molecule resolution. This technique uses nanometer-sized quantum dots linked to the extracellular part of the proteins to be followed. Single-fluorophore epifluorescence imaging then reveals the membrane diffusion of the particle of interest.

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INTRODUCTION

Quantum dot staining is easy, fast, and nontoxic and has low background fluorescence. Quantum dots (QDs) come in many colors and wide absorption spectra, yet have narrow emission spectra, facilitating multicolor detection. Moreover, QDs, which are ~10–20 nm in diameter, intermediate between latex beads (500 nm) and conventional fluorophores (~1–4 nm), can still access confined cellular domains such as the synaptic cleft. The photostability of QDs significantly extends the recording period duration (see the section, "Using Quantum Dots to Measure GlyR Diffusion"). Another key feature of QDs is their strong fluorescence. With an integration time of 75 msec, the spots are detected with a signal-to-noise ratio of ~50 (an order of magnitude more than that of traditional fluorophores). As a result, the lateral resolution with which individual QD spots can be localized reaches 5–10 nm, well below the 40 nm achieved with Cy3 (Dahan et al. 2003). Furthermore, QDs are brighter than conventional fluorescent molecules such as Cy3 and therefore require less excitation light. They can be excited with a mercury lamp instead of a laser beam, which is required for single Cy3 detection. The laser beam excites only a small region of the field compared to the mercury lamp, which excites the entire field equally and therefore permits imaging of more QDs per field. Consequently, multiple individual fluorescent spots can be observed simultaneously. In addition, the same QD probe can be used both at the optical and the electron microscope levels.

There are two main limitations to QD staining. First, QD blinking makes the analysis of long trajectories more complex and time-consuming, requiring sophisticated software. Second, particular attention should be given to the size of the QDs and the control of stoichiometries.

QD staining, as described in this chapter, has been used successfully in cultured neurons to follow the membrane diffusion of individual glycine receptors (Dahan et al. 2003; Charrier et al. 2006; Calamai et al. 2009); type A GABA receptors (Lévi et al. 2008; Bannai et al. 2009); AMPA receptors (Ehlers et al. 2007; Groc et al. 2007, 2008; Heine et al. 2008; Frischknecht et al. 2009); NMDA receptors (Groc et al. 2006; Michaluk et al. 2009); cannabinoid receptors (Mikasova et al. 2008); and lipid raft markers, glycophosphatidylinositol-anchored green fluorescent protein (GPI–GFP), and cholera toxin (Renner et al. 2009).

Protocol

Labeling Neuronal Membrane Receptors with Quantum Dots

Two methods are presented for labeling neurons with the primary antibody of choice along with a secondary anti-Fab antibody that is either biotinylated or directly coupled to the desired quantum dot. The behavior of QD-labeled molecules can then be followed within the cell using epifluorescence imaging.

IMAGING SETUP

This procedure used an inverted microscope (Olympus, IX70 or 71) equipped with a 60× objective (numerical aperture [NA] = 1.45; Olympus). QD-605 and FM 4-64 were detected using a mercury arc lamp (excitation filter 525DF45) and appropriate emission filters (595DF60 and 695AF55 from Omega Filters). For Cy3 excitation, to achieve single-dye detection, the sample was illuminated with a frequency-doubled YAG crystal laser at 532 nm (~0.5 kW/cm^2). For detection of single QDs, the sample was illuminated with a mercury lamp. Real-time QD and Cy3 recordings were obtained at 13 Hz and 10 Hz, respectively, using a CCD camera (Micromax 512EBFT, Cascade+128, Roper Scientific or ORCA II ER, Hamamatsu Photonics) with up to approximately 1000 consecutive frames under Metaview (Universal Imaging). Single-molecule trajectories were analyzed with custom-written routines (Bonneau et al. 2005) in MatLab (MathWorks). Similar algorithms are freely available (Jaqaman et al. 2008; Sergé et al. 2008).

MATERIALS

CAUTION: See Appendix 6 for proper handling of materials marked with <!>.
See the end of the chapter for recipes for reagents marked with <R>.

Reagents

Antibody, primary
Biotinylated secondary Fab antibody (Jackson ImmunoResearch Laboratories) or
QD F(ab′)2 secondary IgG antibody (Invitrogen)
Casein (1× for labeling Method B)
FM4-64 (*N*-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl)hexatrienyl)pyridinium dibromide), 1 μM in 40 mM KCl <!>
MEM medium for quantum dot imaging <R>
Use this medium at 37°C for incubations, washes, and imaging.
Neurons for labeling
QD streptavidin conjugate solution (for labeling Method A) <R>

Equipment

Imaging setup, as described above

EXPERIMENTAL METHOD

1. Label neurons using one of the following two methods:

   Method A:

   i. Incubate the neurons for 5 min with a high dilution (~1–10 μg/mL) of primary antibody to label a small number of molecules.

   ii. Wash the neurons three times without waiting, and then incubate them for 5 min in ~10 μg/mL biotinylated secondary Fab antibody.

   iii. Incubate neurons for 1 min in ~0.5–2 nM QD streptavidin conjugate solution.

   Method B:

   i. Mix 5 nM of the primary antibody with 30 nM QD F(ab′)2 secondary IgG antibody for 30 min at room temperature and then for an additional 15 minutes with 1x casein to block nonspecific binding.

   ii. Incubate neurons for 10 min at 37°C with 0.06 nM of the pre-coupled QD-antibody.

   iii. Rinse off excess antibody.

   If sufficient primary antibody is available, the primary antibody can be bound directly to QD (IgG/QD ratio = 1:1) with the QD antibody conjugation kit (Invitrogen) in a fast (few hours) and specific (coupling of thiols to maleimide groups) manner.

2. Label presynaptic boutons for 30 sec with 1 μM FM4-64 in 40 mM KCl.

   See *Troubleshooting*.

3. Wash the cells, mount the coverslip in a recording chamber, and image the cells for up to 30 min in the imaging medium.

   In our experiments, we perform QD real-time imaging at 13 Hz for approximately 500 consecutive frames. Time-lapse recording is preferred in experiments intended to determine dwell times in compartments (e.g., synaptic) where the molecules might reside for long durations. We often capture a single image of the cell morphology with transmitted light and of the synaptic marker (FM4-64, Venus::gephyrin, or DsRed::Homer1c) with fluorescent light before the QD image sequence.

TROUBLESHOOTING

Problem (Step 2): The diffusion coefficients of the molecule of interest may be changed by neuronal activity stimulation.

Solution: Neuronal activity regulates the neurotransmitter receptor's lateral diffusion properties (Tardin et al. 2003; Groc et al. 2004; Ehlers et al. 2007; Lévi et al. 2008; Bannai et al. 2009). Therefore, the KCl-induced FM4-64 synaptic vesicle loading may change the diffusion coefficients of the molecule of interest. As an alternative, one can use markers that do not require activity stimulation to be integrated into synaptic boutons (e.g., Mitotrackers; Invitrogen). Another option is to lipotransfect neurons with the main components of the inhibitory and excitatory postsynaptic differentiations, such as Gephyrin and Homer1c recombinant proteins tagged with GFP variants.

DISCUSSION

Interpretation of Imaging Data

After imaging, the trajectory is reconstructed from the image sequence of a single QD identified by the intermittency in its fluorescence emission. QDs are classified as synaptic upon co-localization with a synaptic marker, such as glycine receptor (GlyR; see below) (e.g., see Fig. 2). For each QD, we calculate the mean square displacement (MSD), diffusion coefficient (D), confinement area, transition between compartments, and dwell time within a compartment. The MSD is determined from

$$\mathrm{MSD}(n\tau)=\frac{1}{N-n}\sum_{i=1}^{N-n}\left[\left(x((i+n)\tau)-x(i\tau)\right)^2+\left(y((i+n)\tau)-y(i\tau)\right)^2\right], \quad (1)$$

where τ is the frame acquisition time, N is the total number of frames, and n and i are positive integers with n determining the time increment. For simple two-dimensional (2D) Brownian mobility, the MSD as a function of time is linear with a slope of $4D$. If the MSD as a function of time tends to a constant value L, the diffusion is confined in a domain of size L. The diffusion coefficient (D) is determined by a fit on the first four points of the MSD as a function of time, with $\mathrm{MSD}(n\tau) = 4Dn\tau + b$, where b is a constant reflecting the spot localization accuracy. The area in which diffusion is confined can be estimated by fitting the MSD as a function of time to

$$\mathrm{MSD}(n\tau)=\frac{L^2}{3}\left(1-\exp\left(-\frac{12Dn\tau}{L^2}\right)\right)+4D_{\mathrm{mac}}n\tau, \quad (2)$$

where L^2 is the confined area in which diffusion is restricted and D_{mac} is the diffusion coefficient on a long time scale.

Using Quantum Dots to Measure GlyR Diffusion

GlyR lateral diffusion was studied in cultured neurons using a single-particle tracking (SPT) approach (Dahan et al. 2003). Data obtained with Cy3 fluorophore coupled directly to primary antibody (Fig. 1A) were compared with data obtained using QD-605-streptavidin conjugates (Fig. 1B,C). Cy3- and QD-tagged receptors were detected in extrasynaptic and synaptic regions. Individual Cy3 molecules were identified by their single-step bleaching. Cy3-GlyR could be tracked only for short durations (~2.5 sec in Fig. 1A, white arrow). In contrast, the photostability of QDs allowed QD-GlyR trajectories to be visualized (Fig. 1B,C) for unprecedented durations (20 min) (Fig. 1C; Movie 2.1). Single QDs were identified by the random intermittency of their fluorescence emission (Nirmal et al. 1996). For example, one QD (white arrow in Fig. 1B) temporarily disappeared after 31.5 sec of recording. Long imaging duration enabled the observation of exchanges between extrasynaptic and synaptic domains, in which a GlyR alternated between free and confined diffusion states, respectively (white arrow in Fig. 1B). Silver-intensified and gold-toned QDs were detected using transmission electron microscopy (EM) with the same QD immunolabeling protocol (Fig. 1D). EM analysis provided evidence that QD-GlyR could access the core of the synapse (Fig. 1D). QD trajectories were reconstructed from recordings with custom "Sinema" software written in MatLab (Bonneau et al. 2005). Exploratory maps of trajectories indicated that individual QDs can exchange between extrasynaptic and synaptic compartments (e.g., blue and green trajectories, Fig. 2A). QDs diffused over broad areas of the extrasynaptic plasma membrane, whereas the QD exploratory map is reduced at synapses.

As exemplified (Fig. 2B) for the QD trajectory shown in Figure 2A, the average diffusion coefficient (D) of the QD was lower within the synaptic area (Fig. 1, orange arrows). The plots of mean square displacement function (MSD) versus time were linear and negatively bent in the extrasynaptic and synaptic membrane, respectively (Fig. 2C), indicating a stronger confinement at synapses. This reflects local molecular interactions.

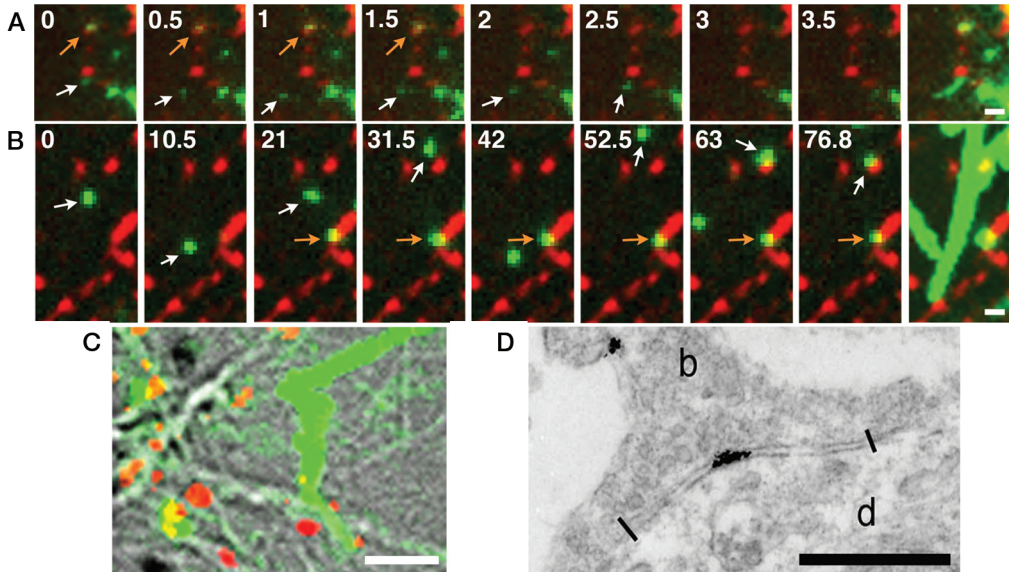

FIGURE 1. Comparison of lateral GlyR motion analyzed with Cy3 and QD probes. GlyRs were detected in cultured neurons with Cy3 (*A*) or QD-streptavidin (*B*,*C*). (Green) GlyR spots; (red) FM4-64-labeled synaptic boutons. (*A*,*B*) Images were extracted from a sequence of 35 and 1024 images with an acquisition time of 100 msec for Cy3 and 75 msec for QD. The time after the start of recording (in seconds) is indicated on each image. The last image is a maximum projection of the entire stack of images corresponding to the GlyR trajectory. (*C*) Projection of time-lapse recording (1 Hz, 20 min) of QD-GlyR trajectory (green) overlaid with FM 4-64 staining (red) and bright-field image. (*A*–*C*) Cy3 and QD diffused rapidly in the extrasynaptic region (white arrows), whereas synaptic GlyRs were stable (orange arrows). Note the short membrane surface explored by Cy3-GlyR compared with QD-GlyR. Long QD imaging duration enabled the observation of a synaptic entry (white arrow in *B*). (*D*) EM detection of QD-GlyR within the synaptic cleft. d, Dendrite; b, synaptic bouton. The edges of the cleft are outlined. Scale bars, 1 µm (*A*,*B*); 5 µm (*C*); 500 nm (*D*).

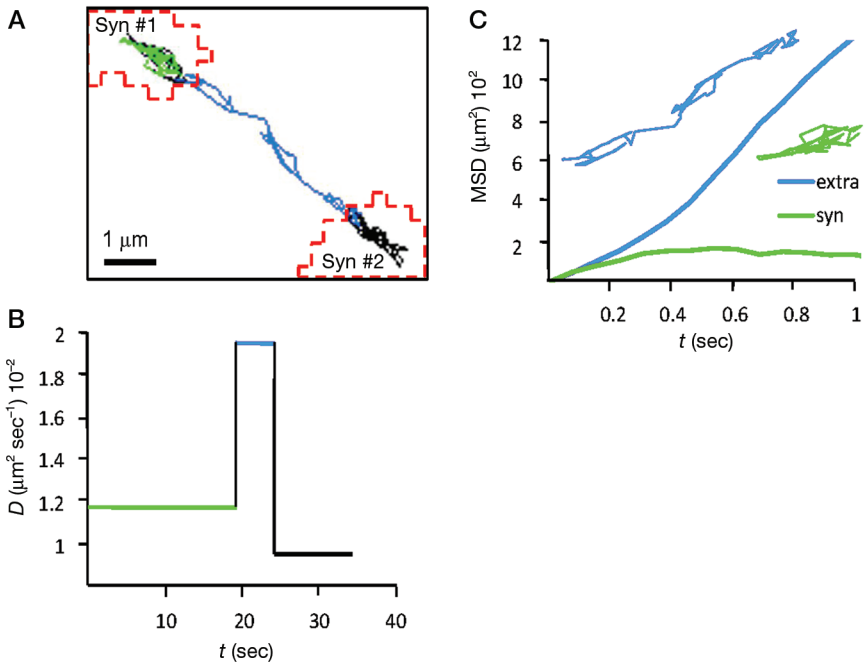

FIGURE 2. QD trajectory analysis. (*A*) Example of surface exploration by an itinerant GlyR-QD visualized on a reconstructed trajectory. QD trajectories in synaptic areas #1 (Syn #1) and #2 (Syn #2) are green and black, respectively, and FM4-64-stained synapses are red. Note the large surface area explored by the GlyR-QD outside (blue) the synaptic areas. Scale bar, 1 µm. (*B*) Average diffusion coefficients of the QD shown in *A* during its extrasynaptic (blue) and synaptic journey (green and blue). Note the changes in the QD diffusion coefficient when exiting synapse #1 or entering synapse #2, since there is one sudden increase and one drop. (*C*) Time-averaged MSD function of individual QD shown in *A* during its exploration of extrasynaptic (blue) and synaptic (green) loci during a recording sequence. The same QD displayed an extrasynaptic linear MSD curve and a synaptic negatively bent MSD curve, characteristic of random walk and confined movement, respectively.

RECIPES

Recipes for reagents marked with <R> are included in this list.

MEM Medium for Quantum Dot Imaging

Prepare MEM Eagle medium (Eagle 1959) without phenol red but containing:

$NaHCO_3$	4 mM
HEPES	10 mM
Glucose	6 g/L
Glutamine	2 mM
Na^+ pyruvate	1 mM
B27 supplement (Invitrogen)	1×

QD Streptavidin Conjugate Solution

QD 605 streptavidin conjugate (Invitrogen)	~0.5–2 nM
Borate buffer (pH 8–8.5)	50 mM
Sucrose	215 mM

The sucrose in the solution increases the osmolarity of the buffer to a physiological level (300 mosmol), whereas the borate buffer minimizes nonspecific QD labeling.

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MOVIE LEGEND

Movies are freely available online at www.cshprotocols.org/imaging.

MOVIE 2.1. Diffusion and stabilization of single QD-GlyRs (green). Time-lapse recording (1200 images at 1 Hz; acquisition time, 75 msec). Synapses are labeled with FM4-64 (red).