Published in final edited form as:

Proc SPIE. 2009 March 3; 7489: . doi:10.1117/12.816900.

QUANTUM DOT SINGLE MOLECULE TRACKING REVEALS A WIDE RANGE OF DIFFUSIVE MOTIONS OF MEMBRANE TRANSPORT PROTEINS

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Abstract

Single particle tracking (SPT) provides information about the microscopic motions of individual particles in live cells. We applied SPT to study the diffusion of membrane transport proteins in cell plasma membranes in which individual proteins are labeled with quantum dots at engineered extracellular epitopes. Software was created to deduce particle diffusive modes from quantum dot trajectories. SPT of aquaporin (AQP) water channels and cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels revealed several types of diffusion. AQP1 was freely mobile in cell membranes, showing rapid, Brownian-type diffusion. The full-length (M1) isoform of AQP4 also diffused rapidly, though the diffusion of a shorter (M23) isoform of AQP4 was highly restricted due to its supermolecular assembly in raft-like orthogonal arrays. CFTR mobility was also highly restricted, in a spring-like potential, due to its tethering to the actin cytoskeleton through PDZ-domain C-terminus interactions. The biological significance of regulated diffusion of membrane transport proteins is a subject of active investigation.

Keywords

single particle tracking; quantum dots; aquaporins; CFTR; diffusion; mathematical modeling

1. INTRODUCTION

The most complete description of the movement of individual molecules is afforded by single particle tracking (SPT). Advances in microscopy instrumentation and fluorescence labeling methods allow tracking of individual membrane proteins and lipids in living cells with spatial resolution generally better than 20 nm. SPT involves the labeling of target molecules with fluorophores, such as quantum dots (Qdots), green fluorescent protein or organic dyes, or probes such as gold beads that are visible with transmitted light. Serial *x,y*-positions of many individual labeled particles are measured over time by a camera detector to give a set of single particle trajectories.

This review is focused on our studies of the diffusion of membrane transport proteins, including aquaporin (AQP) water channels and cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels. Following a description of conventional and new methods to analyze SPT data, we review some key experimental observations on AQP and CFTR diffusion made by quantum dot SPT. Our findings address fundamental issues in membrane biology about anomalous and restricted diffusion in membranes, and demonstrate that membrane proteins can manifest a wide range of modes and mechanisms of diffusion. The potential biological relevance of membrane transport diffusion is discussed in the final section of this review.

2. METHODS

2.1 Deducing Information about Diffusion from Single Particle Trajectories

Figure 1A shows a schematic of different diffusion modes, including simple 'Brownian' diffusion, as well as several examples of 'anomalous' diffusion. Anomalous diffusion may take on several forms, such as confined diffusion, where particles diffuse normally but are confined within an impenetrable barrier, or convective diffusion, where particles diffuse in a flow field. Diffusion in cell membranes may also be altered by immobile barriers, lipid rafts, or specific interactions with neighboring proteins. These various diffusion modes can be simulated,¹ but practically it is difficult to distinguish between different modes of anomalous diffusion in a live cell membrane.

Generally, the first step in analysis of experimental single particle trajectories is computation of mean squared displacement (MSD), $\langle r^2(t) \rangle$ for each trajectory:

$$\langle r^2(n\delta t) \rangle = \frac{1}{N-n} \sum_{j=0}^{N-n-1} \left([x(j\delta t+n\delta t) - x(j\delta t)]^2 + [y(j\delta t+n\delta t) - y(j\delta t)]^2 \right) \quad (1)$$

where δt is the temporal resolution of the acquisition, $x(j\delta t)$, $y(j\delta t)$ is the particle coordinate at time $t = j\delta t$, N is the total number of frames recorded for an individual particle, and $n = 0, 1, 2, \dots, N-1$. A linear least-squares fit to the first three points of the MSD vs. t curve, $\langle r^2(t) \rangle_{1-3}$, gives the microscopic diffusion coefficient, D_{1-3} :

$$\langle r^2(t) \rangle_{1-3} = 4D_{1-3}t + \text{offset}. \quad (2)$$

An 'offset' in the MSD curve, resulting from uncertainty in the position of the centroid of the particle, is subtracted and the first 25% of the resulting curve is fitted using a weighted Levenberg-Marquardt non-linear least squares fitting algorithm to a combined quadratic, polynomial and exponential function with fitting parameters $a_1, a_2, a_3 = 0$:

$$\langle r^2(t) \rangle_{\text{fit}} = a_1 t^2 + a_2 [1 - \exp(-a_3 t)]. \quad (3)$$

The fit is weighted by the variance in the MSD at each time step. Figure 1B shows examples of combined MSD vs. t curves for simulated simple, confined, and convective diffusion. The range of an individual particle at specific time t is then computed as:

$$\text{range}(t) = \langle r^2(t) \rangle_{\text{fit}}^{1/2}. \quad (4)$$

Immobile particles are generally defined as those with a range less than the positional accuracy of the measurement, typically 15–20 nm, which depends on frame rate and optical configuration. Diffusion results are often reported in the form of a cumulative probability distribution of diffusion coefficients or ranges (Figure 1C), where P is defined as the probability that a particle's diffusion coefficient or range at a given time is less than or equal to a given value. Figure 1C shows cumulative probability distributions for simulated simple and confined diffusion. In all cases the diffusion coefficient was set at $0.1 \mu\text{m}^2/\text{s}$, with particles confined to areas of $0.1 \mu\text{m}^2$, $0.25 \mu\text{m}^2$, $0.5 \mu\text{m}^2$ or $1 \mu\text{m}^2$, or not confined. Though the distributions of diffusion coefficients are nearly identical (Figure 1C, *left*), the distribution of ranges clearly separates the populations (Figure 1C, *right*).

In cases where differences in range or diffusion coefficient may not be sufficient to separate differently diffusing species, it becomes useful to determine the relative abundance of diffusional modes, such as Brownian vs. anomalous diffusion. This can be done from analysis of the shape of the MSD vs. t curves. The relative deviation (RD) is defined as the ratio of the experimental MSD at a given frame to that from a line extrapolated from the initial slope of the MSD,² with $RD < 1$ indicating restricted diffusion and $RD > 1$ indicating directed diffusion (Figure 1B). At the n th step of a trajectory with total length N :

$$RD(N, n) = \frac{\langle r^2(n\delta t) \rangle_{\text{fit}}}{4D_0 n\delta t} \quad (5)$$

where $4D_0$ is the initial slope of $\langle r^2(t) \rangle_{\text{fit}}$. Because of experimental noise, however, even a particle undergoing Brownian diffusion generally has an MSD that differs from linearity, so a major challenge is to distinguish between true anomalous diffusion and simple statistical variations in Brownian diffusion. For an ensemble of particles undergoing Brownian diffusion, the distribution of RD at specified frame n broadens as total trajectory length N is reduced. We therefore generated Brownian trajectories using experimentally relevant trajectory lengths to establish the effective cut-off values of RD at 50 frames. The central 95 % of calculated RD values for an ensemble of trajectories were taken to represent statistical variations in Brownian motion, and not anomalous diffusion.³ Figure 1D maps the 2.5th and 97.5th percentiles of $RD(N, 50)$ for Brownian diffusion as a function of N . A linear least-squares fit to the 2.5th percentile points may be used to define the lower boundary for free diffusion, with trajectories having $RD(N, 50)$ below this line classified as restricted. To increase the reliability of this method, only trajectories longer than 200 steps are generally considered for analysis.

If a set of particles is classified as confined, whether by range or RD analysis, the potential function $V(r)$ acting on confined channels can be determined by nonlinear least-squares regression to the radial particle density distribution $d(r)$ according to the Boltzman distribution:

$$d(r) = d(0) \exp(-V(r)/k_B T) \quad (6)$$

where r is the distance from the potential origin, k_B is the Boltzman constant, and T is absolute temperature.⁴ We consider four different potentials where V_0 is the potential strength, and r_c is the radius of confinement: 1) 'hard-wall potential', where particles are confined by an impermeable wall [$V(r) = 0 (r < r_c)$ and $\infty (r > r_c)$]; 2) 'spring potential', where particles are tethered to a spring-like force-producing mechanism [$V(r) = V_0 r^2$]; 3) 'cone potential', where particles are trapped by a softer, viscoelastic-like potential [$V(r) = V_0 r$]; and 4) ' r^4 potential', which is harder than a spring potential but softer than a hard-wall potential [$V(r) = V_0 r^4$]. Experimentally, $d(r)$ is determined from serial x, y coordinates in individual trajectories:

$$d(r) = N(r) / \pi \left([r + \Delta r/2]^2 - [r - \Delta r/2]^2 \right) \quad (7)$$

where Δr is the distribution resolution and $N(r)$ is the number of particles with radial distance r_p in the range $r - \Delta r/2 < r_p < r + \Delta r/2$. $d(r)$ is normalized by $d(0)$ for fitting to Eq. 6.

2.2 Labeling and Data Acquisition

Several methods have been described to selectively attach Qdots to a protein of interest in the plasma membrane. Our experimental strategy has been to utilize antibody-mediated

attachment of Qdots, which requires an appropriate endogenous or engineered extracellular epitope. In the case of AQPs and CFTR, no antibodies against suitable endogenous epitopes have been described. As such, external epitopes for antibody targeting have been engineered into the proteins of interest. In the case of the aquaporins, we used a c-myc epitope (10 amino acids) inserted in the second extracellular loop (Figure 2A). This position was chosen because it is well removed from any glycosylation sites and the highly-conserved NPA-NPA motif that serves as the selectivity filter for the channel (Figure 2B). For CFTR, a triple-HA tag (27 amino acids) was inserted into the fourth extracellular loop, between glycosylation sites. In each case, the inclusion of these external epitopes does not disrupt membrane targeting, glycosylation or channel activity.^{5–9} Following transfection into various cell types, the surface-exposed epitope is conjugated to a primary antibody, and subsequently labeled with a secondary-antibody-linked Qdot. Alternatively, a biotinylated primary or secondary antibody can be used, followed by a streptavidin-coated Qdot. The fluorescence of Qdots on the cell surface can be excited between 390–460 nm. A series of images is acquired by a cooled EM-CCD camera at the desired rate, the centroid of each particle determined, and trajectories constructed. Blinking of individual Qdots is accounted for during trajectory constructions. Data obtained using Qdot labeling strategies can be validated using alternative labeling methods such as monovalent antibody fragments (Fab fragments) and organic dye molecules such as Cy dyes.

3. EXPERIMENTAL RESULTS AND DISCUSSION

3.1 Unrestricted Brownian Diffusion of AQP1 Water Channels

Aquaporin-1 (AQP1) is an integral membrane protein that facilitates osmotic water transport across cell plasma membranes in epithelia and endothelia, where it plays an important role in the urinary concentrating system, fluid secretion in the eye and brain, and angiogenesis.¹⁰ AQP1 is constitutively active and assembles in membranes as a stable tetramer. Each monomer (MW ~ 30 kDa) consists of 6 membrane-spanning α -helical segments forming a twisted barrel (Figure 2B) that forms the aqueous pore.^{11–13}

We characterized long-range motions of AQP1 in cell membranes using time-lapse image acquisition at 1 Hz over 6 min, and short-range diffusion by continuous acquisition at 91 Hz over 6 s.³ Time-lapse acquisition at 1 Hz provided a good overview of the long-range motions of AQP1, while the faster rate of acquisition was necessary to characterize the modes of diffusion of individual tetramers and to accurately determine diffusion coefficients. Examples of Qdot trajectories from time-lapse SPT are shown in Figure 2C. Cells were co-transfected with GFP in order to readily identify cell boundaries. AQP1 diffused more rapidly and covered a much larger area in COS-7 than in MDCK cells. The range (Eq. 4) of AQP1 at 1 min was ~2 μm in COS-7 cells, compared to ~0.9 μm in MDCK cells, with approximately linear MSD vs. t plots over 5 min (Figure 2D). In all measured cell types < 1% of Qdot-labeled AQP1 was immobile, with average D_{1-3} of $\sim 8 \times 10^{-10} \text{ cm}^2/\text{s}$ in COS-7 cells, $\sim 3 \times 10^{-10} \text{ cm}^2/\text{s}$ in MDCK cells, and $\sim 7 \times 10^{-10} \text{ cm}^2/\text{s}$ in primary mouse astrocytes. We concluded by RD analysis (Eq. 5, Figure 1D) that > 75 % of AQP1 diffusion in COS-7 cells was Brownian, and > 66 % of AQP1 diffusion was Brownian in MDCK cells and primary astrocytes under normal conditions.^{3, 6} No change was found in the free or restricted fractions of AQP1 upon disruption of the cytoskeleton by latrunculin. However, a large fraction of AQP1 became significantly slowed and restricted by addition of cyclodextrin in MDCK cells, likely due to obstruction by solid-like lipid or protein clusters resulting from cholesterol depletion. We concluded that AQP1 is a largely non-interacting protein, whose diffusion is determined by the concentration of obstructions in the plane of the membrane. These results demonstrate long-range non-anomalous diffusion of AQP1 in cell membranes, challenging a prevailing view of universally anomalous diffusion of

integral membrane proteins, and providing evidence against the accumulation of AQP1 in lipid rafts.^{14, 15}

3.2 Restricted Diffusion of AQP4 Water Channels in Membrane Arrays

Aquaporin-4 (AQP4) is a water-selective plasma membrane transport protein expressed in glial cells, where it is involved in brain water balance, glial cell migration, and neural signal transduction.¹⁶ Our lab discovered that AQP4 is the protein responsible for formation of orthogonal arrays of particles (OAPs).^{17, 18} OAPs are cobblestone-like square arrays of intramembrane particles that have been seen by freeze-fracture electron microscopy (FFEM) for more than 30 years in cell plasma membranes from brain and other tissues.¹⁹ The AQP4 transcript contains alternative translation initiation sites,²⁰ yielding a full-length 'long' (M1) isoform (AQP4-M1) of ~34 kDa, and a 'short' (M23, translation begins at Met23) isoform (AQP4-M23) of ~31 kDa.^{21, 22} Both isoforms of AQP4 function as water channels²¹ and form stable tetramers. The relative abundances of AQP4 isoforms are tissue specific, with rat brain containing an approximate 3:1 ratio of AQP4-M23 to AQP4-M1.²³ FFEM in cells transfected with AQP4-M1 vs. AQP4-M23 shows that only the shorter form assembles into large OAPs of >100 particles, while AQP4-M1 tetramers are largely dispersed as individual tetramers²⁴ (Figure 3A, *left*). Recently, alternative methods have been developed to detect OAPs that are less technically challenging than FFEM. Using fluorescent antibody labeling, we are able to detect OAPs in fixed cells by total internal reflection fluorescence microscopy (TIRFM).⁶ Nearly all of the observed fluorescence from AQP4-M23 comes from distinct, diffraction-limited puncta, corresponding to dense OAPs, whereas the fluorescence from AQP4-M1 is fairly uniform over the cell surface (Figure 3A, *middle*). OAPs may also be detected by Blue-native gel electrophoresis (BN-PAGE).²⁵ In this technique, a non-denaturing detergent is used to separate on a gel AQP4 tetramers from higher-order AQP4 complexes. The resulting immunoblot shows a diffuse band migrating at > 1200 kDa for AQP4-M23, suggesting large AQP4 complexes, along with a smaller band at ~300 kDa, corresponding to AQP4 tetramers, while AQP4-M1 shows a dense tetramer band and a faint band at ~600 kDa, but no very high molecular weight bands (Figure 3A, *right*).

The methods illustrated in Figure 3A, while informative and useful, still require cell fixation, fractionation, or lysis with detergents. We reasoned that SPT would be a superior approach to these static, invasive methods, as SPT can be done on live cells, providing dynamic data on AQP4 in OAPs in real time. As expected, SPT of Qdot-labeled AQP4 is able to readily distinguish between AQP4 molecules in rapidly diffusing individual tetramers and those that are relatively immobilized by accumulation in OAPs (Figure 3B, *left*). Trajectories from AQP4-M1 and AQP4-M23 acquired at 91 Hz over 6 seconds show very different behaviors (Figure 3B, *right*). AQP4-M1 diffusion is rapid and largely Brownian, whereas the diffusion of AQP4-M23 is highly restricted. The MSD vs. *t* plot computed from many Qdot trajectories for AQP4-M23 shows the characteristic negative curvature expected for confined or anomalous diffusion (Figure 3C), with the median diffusion coefficient and range at 1 second being an order of magnitude lower than that of AQP4-M1 (Figure 3C, 3D).

Due to the remarkable immobility of AQP4-M23 and rapid diffusion of AQP4-M1, we have exploited AQP4 diffusion as a 'read-out' of OAP assembly in live cells to investigate questions regarding the biophysics and determinants of OAP formation.^{6, 26} The role of specific domains within AQP4 isoforms was investigated by measurement of the diffusive behavior of a large number of AQP4 mutants. One example is shown in Figure 3E, in which the diffusive behaviors of serial deletion mutants of AQP4-M1 was measured to investigate whether specific residues between Met1 and Met23 are responsible for preventing OAP formation. The deletion mutants are named for the position of the N-terminal methionine

relative to Met1 in full-length AQP4-M1. Deletion of up to 16 residues from the N-terminus (M1 through M16) has little effect on AQP4 diffusion (Figure 3E, *left*), indicating the presence of few, if any, OAPs formed by these mutants. Deletion of Cys17, however, results in a large fraction of AQP4 with restricted diffusion. Note that the cumulative distribution of ranges at 1 second clearly indicates the presence of two differently diffusing populations of M17, M18 and M19. The percentage of AQP4 mutants in OAPs can be estimated using the 95th percentile of ranges for AQP4-M23 at 1 second (*dashed line*).²⁶ Using this criterion, 42–48% of M17 is found in OAPs. Further deletions increase the percentage of AQP4 in OAPs, with 52–64% for M18 and M19, and 84–96% for M20 and M21 (Figure 3E, *middle*). The breakdown of diffusive modes by RD analysis is qualitatively similar to estimations by range, with AQP4-M23 exhibiting largely anomalous diffusion, AQP4-M1 largely Brownian diffusion, and deletion mutants showing an increase in anomalous fraction as more residues are removed from the N-terminus (Figure 3E, *right*).

3.3 Restricted Diffusion of Actin-Associated CFTR Chloride Channels

The relatively common genetic disease cystic fibrosis is caused by mutations in CFTR, a cAMP-regulated chloride channel expressed in the apical membrane of epithelial cells in tissues including the airways, and gastrointestinal and reproductive tracts. CFTR is a polytopic glycoprotein of 1480 amino acids that contains two transmembrane domains, two nucleotide binding domains and a regulatory domain that becomes phosphorylated to open the channel. The C-terminus of CFTR (Asp-Thr-Arg-Leu) is a consensus class I PDZ-binding domain that was shown to interact with EBP50 (ezrin-radixin-moesin binding phosphoprotein of 50 kDa) and proposed to tether CFTR to the actin cytoskeleton through ezrin and possibly other proteins.²⁷ The C-terminus of CFTR has also been demonstrated to interact with further PDZ domain proteins including NHERF2/E3KARP, CAP70/PDZK1, and CAL/GOPC/FIG/PIST. Several distinct roles for CFTR–PDZ interactions have been proposed, including apical polarization/targeting, direct regulation of CFTR channel gating, and assembly of CFTR-protein complexes. However, no consensus for the role PDZ interactions has been reached.²⁷

To study CFTR mobility and interactions at the single molecule level, we performed SPT of Qdot-labeled CFTR, which allowed direct visualization of plasma membrane CFTR at very low membrane density as found in native, CFTR-expressing cells.²⁸ MSD *vs.* *t* plots derived from trajectories of Qdot-labeled CFTR-3HA expressed in COS-7 cells show downward curvature, indicating confined CFTR diffusion (Figure 4A), with similar MSD *vs.* *t* plots for the other cell types (including human bronchial epithelial cells). The deduced mean diffusion coefficient (D_{1-3}) for short-range CFTR motions of mobile CFTR-3HA is $\sim 5 \times 10^{-11}$ cm²/s, in the range found for other membrane proteins, but with a high degree of confinement. Control experiments were performed at higher frame rates and using alternative labeling strategies to verify our initial observations. To investigate the role of the CFTR C-terminus in its confined diffusion, several experimental strategies were used including investigation of the diffusion of naturally occurring CFTR mutants that lack the C-terminus PDZ-binding motif (CFTR-3HA-Δ26 and –Δ70),⁷ overexpression of dominant negative PDZ domain proteins that do not interact with ezrin, and pharmacological disruption of the actin cytoskeleton. Taken together, these studies provide direct *in vivo* evidence that CFTR is highly confined in the plasma membrane in nonpolarized cells and epithelia, and that its confinement involves C-terminus interactions with EBP50, ezrin and actin.

To investigate the confinement of CFTR further, we developed and applied analysis algorithms to characterize the potential function that restricts the diffusion of CFTR.⁴ Single particle diffusion was simulated in two dimensions in an arbitrary potential $V(r)$ consisting

of random and potential gradient-driven motions (Figure 4B, 4C). Algorithms were applied and verified for detection of potential-driven diffusion, and for determination of $V(r)$ from radial particle density distributions, taking into account experimental uncertainties in particle position and finite trajectory recording. For CFTR diffusion, SPT data fits closely to a spring-like attractive potential ($V(r) = V_0 r^2$) but not to other $V(r)$ forms such as hard-wall or viscoelastic-like potentials (Figure 4D, 4E). As such, this technique formally distinguishes spring-like tethering of CFTR by the actin cytoskeleton as the mechanism of confinement from alternative physical barriers, such as 'picket fence corrals'.¹⁴ The 'spring constant' k determined from SPT data is 2.6 ± 0.8 pN/ μm , and not altered significantly by modulation of skeletal protein architecture by jasplakinolide (Figure 4E). However, k is reduced by a low concentration of the actin fragmenting compound latrunculin, supporting the involvement of actin in the spring-like tethering of CFTR. Notably, this data provided the first measurement of actin elasticity that does not involve application of an external force, such as with laser tweezers.

4. POTENTIAL BIOLOGICAL RELEVANCE AND FUTURE DIRECTIONS

The AQP and CFTR examples indicate that membrane proteins exhibit a wide range of diffusive motions in live cell membranes. An important question is the biological significance of regulated membrane diffusion. In the case of AQPs such as AQP1, rapid membrane diffusion may be required for their movement into lamellipodia in migrating cells, where they serve to facilitate water influx during cell membrane protrusions.²⁹ There has been much speculation about the biological significance of assembly of AQP4 into OAPs, including facilitated water permeability,³⁰ cell-cell adhesion^{31, 32} and AQP4 localization to foot-processes of glial cells.³³ For CFTR, we found that phosphorylation of CFTR, which increases its chloride conductance, did not alter its mobility, indicating that CFTR complexation is constitutive and that PDZ-interactions probably maintain the channel in a context that can be readily activated. Naturally occurring CFTR mutants that lack the C-terminus are not immobilized by phosphorylation, suggesting that CFTR complexation is not a prerequisite for activation.^{7, 34, 35} Further, the finding of similar diffusion of metabolically stable PDZ-binding domain mutants of CFTR (CFTR- $\Delta 26$) and rapidly degraded mutants (CFTR- $\Delta 70$) suggests that C-terminal interactions do not have a role in CFTR degradation.^{34–36} As such, the functional consequences of CFTR immobilization are not clear at the present time. It is possible that channel immobilization affects downstream events such as endocytosis and sorting. In neurons, the immobilization of receptors has been proposed to regulate several processes including synaptogenesis, long-term potentiation and long-term depression.³⁷ Further studies will be needed to investigate these various possibilities.

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Figure 1.

Approaches to analyze trajectories from SPT measurements. A. Schematic of different types of diffusion occurring in cell membranes. B. Mean-squared displacement curves showing Brownian, restricted and directed diffusion. C. Cumulative probability distributions of rates and ranges from simulated free and confined diffusion. Diffusion coefficients (*left*) from all simulations (free and confined) set at $0.1 \mu\text{m}^2/\text{s}$. Ranges (*right*) for 200 trajectories of simulated unrestricted diffusion (black), and diffusion confined to areas of $0.1 \mu\text{m}^2$ (red), $0.25 \mu\text{m}^2$ (green), $0.5 \mu\text{m}^2$ (blue) and $1 \mu\text{m}^2$ (orange). D. Plot of $RD(N,50)$ vs. N for simulated free (non-anomalous) diffusion with $N = 100, 200, 300, 400, 500$ and 600 . Solid circles indicate 2.5th and 97.5th percentiles.

Figure 2.

Brownian diffusion of AQP1 water channels in cell membranes. A. Schematic diagram of a c-myc tagged AQP molecule, showing transmembrane domains (pink) and inserted c-myc sequence (blue) in the second extracellular loop. B. Qdot-labeled AQP1 monomer: The c-myc epitope was inserted between residues Thr120 and Gly121 (red), in the extracellular loop (yellow), between transmembrane helices H4 and H5 (green), well removed from NPA-motifs (blue). C. Trajectories from time-lapse SPT acquired at 1 Hz (total time 6 min) overlaid onto fluorescence images of GFP in the cytoplasm of a COS-7 cell (*left*) and a MDCK cell (*right*). D. MSD vs. t plots from all time-lapse SPT ($n > 300$).

Figure 3.

Restricted diffusion of intramembrane array-associated AQP4 water channels. A. Examples of static methods of detecting AQP4 arrays: Freeze-fracture electron micrographs of the plasma membrane P-face of COS-7 cells expressing c-myc-tagged AQP4 isoforms (*left*). Total internal reflection fluorescence micrographs of Alexa-labeled AQP4 isoforms in COS-7 cells, with inset showing expanded $4 \times 4 \mu\text{m}$ area (*middle*). Immunoblots following Blue-native gel electrophoresis of cell lysates from COS-7 cells transfected with AQP4 isoforms (*right*). B. Schematic showing the organization of AQP4 tetramers (*left*) and representative single particle trajectories (*right*) of Qdot-labeled AQP4 molecules in the plasma membranes of COS-7 cells expressing AQP4-M1 (*top*) or AQP4-M23 (*bottom*). Each grey cylinder represents one AQP4 tetramer. A subset of AQP4 molecules are labeled with quantum dots (red) for single particle tracking. C. Combined MSD vs. t plots and averaged diffusion coefficients for AQP4-M1 (grey) and AQP4-M23 (black) in COS-7 cells. D. Cumulative probability distribution of ranges at 1 second ($P(\text{range})$) for AQP4-M1 (grey) and AQP4-M23 (black), with dashed lines indicating median range. E. $P(\text{range})$ for indicated AQP4 truncation mutants upstream of Met23: M16 (red), M17 (green), M18 (dark blue), M19 (light blue), M20 (orange), M21 (purple), AQP4-M23 (black) and AQP4-M1 (grey). Dashed line indicates 95th percentile of the range of AQP4-M23 (*left*). Estimated percentage of indicated AQP4 mutants in OAPs based on the 95th percentile of range for AQP4-M23 (*middle*). Estimated percentage of indicated AQP4 mutants exhibiting Brownian (white) or restricted (grey) diffusion based on RD analysis, with immobile fraction in black (*right*).

Figure 4.

Restricted diffusion of actin-associated CFTR chloride channels. A. MSD vs. t plots for CFTR-3HA in MDCK cells (solid line), for immobilized Qdots (dotted line) and for CFTR mutant CFTR- $\Delta 26$ that lacks its C-terminal PDZ binding domain (dashed line). Plots are averaged for 16–38 individual trajectories and data was acquired at 62.5 Hz. B. Simulation of confined diffusion in a potential: profiles of the four potential functions used in this study (left) as well as representative single particle trajectories (right, centers marked by circles). The steepness of the potential decreases in the order hard-wall, r^4 , spring-like (r^2) and cone (r). C. Normalized radial particle density functions [$d(r)/d(0)$], open circles represent computed $d(r)$ from simulations, and solid lines denote the Boltzmann distribution functions [$\exp(-V(r)/k_B T)$]. D. Determination of $V(r)$ for confined diffusion of CFTR. Particle distributions and corresponding $d(r)/d(0)$ for two CFTR molecules in control cells (top) and in cells treated with latrunculin (bottom) are shown. E. Mean $d(r)/d(0)$ and best fits to spring potential shown for control cells ($n = 65$ trajectories, $k = 2.6 \pm 0.8$ pN/ μm) and cells treated with jasplakinolide ($k = 2.4 \pm 0.8$ pN/ μm , $n = 42$ trajectories) or latrunculin ($k = 1.9 \pm 0.6$ pN/ μm , $n = 27$ trajectories). Inset shows histogram of spring constant (k) for control cells.