Published in final edited form as:

Methods Enzymol. 2012 ; 504: 341–354. doi:10.1016/B978-0-12-391857-4.00017-3.

Live-Cell Imaging of Aquaporin-4 Supramolecular Assembly and Diffusion

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Abstract

Aquaporin-4 (AQP4) is a water channel expressed in astrocytes throughout the central nervous system, as well as in epithelial cells in various peripheral organs. AQP4 is involved in brain water balance, neuroexcitation, astrocyte migration, and neuroinflammation and is the target of pathogenic autoantibodies in neuromyelitis optica. Two AQP4 isoforms produced by alternative splicing, M1 and M23 AQP4, form heterotetramers that assemble in cell plasma membranes in supramolecular aggregates called orthogonal arrays of particles (OAPs). OAPs have been studied morphologically, by freeze-fracture electron microscopy, and biochemically, by native gel electrophoresis. We have applied single-molecule and high-resolution fluorescence microscopy methods to visualize AQP4 and OAPs in live cells. Quantum dot single particle tracking of fluorescently labeled AQP4 has quantified AQP4 diffusion in membranes, and has elucidated the molecular determinants and regulation of OAP formation. The composition, structure, and kinetics of OAPs containing fluorescent protein-AQP4 chimeras have been studied utilizing total internal reflection fluorescence microscopy, single-molecule photobleaching, and super-resolution imaging methods. The biophysical data afforded by live-cell imaging of AQP4 and OAPs has provided new insights in the roles of AQP4 in organ physiology and neurological disease.

1. Aquaporin-4 (AQP4) and Orthogonal Arrays of Particles

Aquaporin-4 (AQP4) is a water-transporting, integral membrane protein cloned by our lab (Hasegawa *et al.*, 1994). AQP4 is expressed at the plasma membrane in astrocytes throughout the central nervous system (CNS), in epithelial cells in kidney, stomach, lung and exocrine glands, and in skeletal muscle (Frigeri *et al.*, 1995). Phenotype analysis of knockout mice lacking AQP4 (Ma *et al.*, 1997) has defined the roles of AQP4 in brain water balance (Manley *et al.*, 2000; Papadopoulos *et al.*, 2004), astrocyte migration and glial scar formation (Auguste *et al.*, 2007; Saadoun *et al.*, 2005), neuroexcitation (Binder *et al.*, 2006; Padmawar *et al.*, 2005) neurosensory signaling (Li and Verkman, 2001; Lu *et al.*, 2008), and neuroinflammation (Li *et al.*, 2011). AQP4 is also involved in the multiple sclerosis-like disease neuromyelitis optica (NMO), where pathogenic antibodies against AQP4 cause astrocyte damage, neuroinflammation, and demyelination, which leads to paralysis and blindness (Jarius *et al.*, 2008; Lennon *et al.*, 2005).

AQP4 monomers, each of ~30 kDa molecular size, contain six, membrane-spanning helical domains surrounding a narrow aqueous pore that confers water selectivity (Ho *et al.*, 2009). Like other aquaporins, AQP4 is present as tetramers in membranes. AQP4 is expressed in two isoforms produced by alternative splicing: a long (M1) isoform with translation initiation at Met-1, and a short (M23) isoform with translation initiation at Met-23 (Jung *et al.*, 1994; Lu *et al.*, 1996; Yang *et al.*, 1995). As discussed further below, the M1 and M23 isoforms associate in membranes as heterotetramers. AQP4 tetramers can further assemble into supramolecular aggregates called orthogonal arrays of particles (OAPs), which are square arrays of intramembrane particles seen in cell membranes by freeze-fracture electron

microscopy (FFEM; Landis and Reese, 1974; Rash *et al.*, 1974; Wolburg *et al.*, 2011). Our lab discovered that AQP4 is the major constituent of OAPs based on the appearance of OAPs in AQP4-transfected cells (Yang *et al.*, 1996) and their absence in AQP4 knockout mice (Verbavatz *et al.*, 1997). As discussed further below, the M23 isoform of AQP4 forms OAPs, which can incorporate with AQP4-M1 by heterotetramer formation (Furman *et al.*, 2003; Silberstein *et al.*, 2004). The biological function of OAPs remains unclear, with proposed involvement of AQP4 OAPs in water transport (Fenton *et al.*, 2009; Silberstein *et al.*, 2004), cell–cell adhesion (Hiroaki *et al.*, 2006; Zhang and Verkman, 2008), and membrane polarization (Noell *et al.*, 2009). Pathogenic autoantibodies in NMO bind preferentially to AQP4 OAPs (Crane *et al.*, 2011a).

2. Approaches to Image AQP4 and OAPs

Figure 17.1A shows FFEM in cells transfected with AQP4-M1 (left) versus AQP4-M23 (right). While AQP4-M1 is largely dispersed as individual tetramers, AQP4-M23 assembles into large OAPs of >100 particles. AQP4 aggregates, which represent OAPs, have also been studied by blue native gel electrophoresis (BN-PAGE), in which AQP4 tetramers are separated from higher order AQP4 aggregates using a nondenaturing detergent. By BN-PAGE with AQP4 immunoblot, AQP4-M1 is seen as a dense tetramer band and a faint band at ~600 kDa, whereas M23-AQP4 is seen as diffuse band migrating at >1200 kDa, corresponding to large AQP4 aggregates, along with a smaller band at ~300 kDa, corresponding to AQP4 tetramers (Fig. 17.1B). FFEM and BN-PAGE, while informative, require cell fixation or detergent solubilization, precluding measurements in live cells.

Total internal reflection fluorescence microscopy (TIRFM) allows visualization of fluorescently labeled AQP4 tetramers and OAPs. Figure 17.1C shows TIRFM of cells expressing green fluorescent protein (GFP)-AQP4 chimeras. AQP4-M1 is fairly uniformly distributed over the cell surface, whereas AQP4-M23 is seen as distinct, diffraction-limited puncta, corresponding to dense OAPs. As discussed below, TIRFM of fluorescently labeled AQP4 is useful to investigate AQP4/OAP composition, structure, and kinetics.

Single particle tracking (SPT) provides an incisive single-molecule approach to study AQP4/OAP dynamics and assembly. In one implementation of SPT, a subset of AQP4 molecules is labeled at their extracellular surface by quantum dots, utilizing an antibody against an engineered extracellular epitope on AQP4, or an NMO autoantibody against native extracellular epitopes (Fig. 17.1D, left). We found that inclusion of a Myc or HA epitope in its second extracellular loop did not affect AQP4 expression, trafficking, or function (Crane *et al.*, 2008). The movement of individual quantum dots, consequent to AQP4 diffusion, is recorded by wide-field fluorescence microscopy, allowing reconstruction and analysis of single particle trajectories. We initially applied SPT to study the diffusion of a different aquaporin, AQP1, finding long-range free diffusion over a wide variety of conditions, indicating that AQP1 exists in the plasma membrane largely free of specific interactions (Crane and Verkman, 2008). In applying SPT to AQP4, we reasoned that individual AQP4 tetramers should be mobile, whereas AQP4 in large OAPs should be relatively immobile. Figure 17.1D (center) shows remarkably greater mobility of AQP4-M1 than AQP4-M23. SPT data can be quantified by a variety of approaches to resolve diffusion mechanisms and potential interactions (Jin and Verkman, 2007; Jin *et al.*, 2007). Figure 17.1D (right) quantifies the large difference in AQP4-M1 versus AQP4-M23 diffusion from mean squared displacement (MSD) and cumulative probability analyses. As reported, analysis of SPT data showed that AQP4-M1 diffuses freely, with diffusion coefficient $\sim 5 \times 10^{-10} \text{ cm}^2/\text{s}$, covering $\sim 5 \text{ }\mu\text{m}$ in 5 min. AQP4-M23 diffuses only $\sim 0.4 \text{ }\mu\text{m}$ in 5 min. Whereas actin modulation by latrunculin or jasplakinolide did not affect AQP4-M23 diffusion, deletion of the AQP4 C-terminal Post synaptic density protein (PSD95),

Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 (ZO-1) (PDZ)-binding domain increased its range by approximately twofold over minutes. Biophysical analysis of short-range AQP4-M23 diffusion in OAPs indicated a spring-like potential with a restoring force of ~ 6.5 pN/ μm . As described below, SPT has been applied to identify the molecular determinants of AQP4 assembly.

3. AQP4 Diffusion and OAPs Studied by Quantum Dot Single Particle Tracking

Figure 17.2A shows the amino acid sequence of AQP4, with indicated sites of translation initiation, epitope insertion, and PDZ-domain interactions, as well as the residues involved in OAP assembly. From measurements on AQP4 mutants and chimeras, we concluded that OAP formation by AQP4-M23 involves hydrophobic intermolecular interactions of N-terminal AQP4 residues just downstream of Met-23, and that the inability of AQP4-M1 to form OAPs results from nonspecific blocking of N-terminal interactions by residues just upstream of Met-23 (Crane and Verkman, 2009a). Our study involved the generation of serial deletion mutants at the AQP4-M1 N-terminus. Deletions of up to 16 residues had little effect on diffusion. Further truncation, however, resulted in a large fraction of AQP4 with restricted diffusion (examples shown in Fig. 17.2B). Continued deletions up to Met-23 further increased the percentage of AQP4 in OAPs, whereas truncations or certain mutations downstream of Met-23 produced progressive loss of OAPs. Also, AQP1, which does not itself form OAPs, was induced to form OAPs upon replacement of its N-terminal domain with that of AQP4-M23. These findings defined the molecular determinants of AQP4 OAP assembly.

In a follow-on study, we investigated M1/M23 interactions and regulated OAP assembly by quantum dot SPT in live cells expressing differentially tagged AQP4 isoforms, and in primary glial cell cultures in which native AQP4 was labeled with a monoclonal recombinant NMO autoantibody (Crane *et al.*, 2009). Diffusion of AQP4-M1 and AQP4-M23 were measured individually at different M23:M1 ratios. Upon coexpression with AQP4-M1, the diffusion of AQP4-M23 increased significantly, whereas expression of AQP4-M23 produced a marked reduction in AQP4-M1 diffusion (Fig. 17.2C, left). The similar diffusion of the two isoforms indicated a high level of interaction. Quite different from the experimental data are the dashed curves in Fig. 17.2C (right), which are the predicted curves if AQP4-M1 and AQP4-M23 do not comingle. Further, two-color SPT and BN-PAGE showed that mutants of AQP4-M23 that do not themselves form OAPs (M23-F26Q and M23-G28P) fully coassociated with native AQP4-M23 to form large immobile OAPs. In primary astrocyte cell cultures, which coexpress the M1 and M23 AQP4 isoforms, differential regulation of OAP assembly by palmitoylation, calcium, and protein kinase C activation was found. These results indicated the coassembly of AQP4-M1 and AQP4-M23 in OAPs, and the regulation of OAP assembly in astrocytes by specific signaling events.

Having established regulated assembly of AQP4 in OAPs, we carried out biophysical studies showing rapid and reversible temperature-dependent assembly into OAPs of certain weakly associating AQP4 mutants (Crane and Verkman, 2009b). By TIRFM, M23-F26Q, and M23-G28P formed few, if any OAPs at 37 °C, but assembled efficiently in OAPs at 10 °C (Fig. 17.4D). The computed free energy from temperature-dependence data was -13 ± 2 kcal/mol for OAP formation of M23-F26Q below 30 °C. OAP assembly by M23-F26Q (but not of native AQP4-M23) could also be modulated by reducing its membrane density. Interestingly, OAP assembly and disassembly occurred within seconds or less following temperature changes. These data further support the paradigm that AQP4 OAPs are dynamic, regulated supramolecular structures.

In related studies, quantum dot SPT was applied in investigations of NMO autoantibody binding to AQP4-M1 versus AQP4-M23 (Crane *et al.*, 2011a) and to Arg-19 mutants of AQP4-M1 that have been associated with NMO (Crane *et al.*, 2011b). In other studies, the biology of a third, longer isoform of AQP4, called AQP4-Mz, was studied by quantum dot SPT (Rossi *et al.*, 2011a). AQP4-Mz is expressed in rat, with translation initiation 126-bp upstream from that of AQP4-M1. However, AQP4-Mz is not expressed in human or mouse because of in-frame stop codons. It was found that Mz, like M1, diffused rapidly in the cell plasma membrane and did not form OAPs. However, when coexpressed with M23, Mz associated in OAPs by forming heterotetramers with M23. Therefore, AQP4-Mz is unable to form OAPs on its own but able to associate with M23 AQP4 in heterotetramers.

4. OAP Dynamics and Structure Studied with GFP-AQP4 Chimeras

The ability of GFP-labeled AQP4-M23 to form OAPs allows visualization of OAP dynamics and structure in live cells (Tajima *et al.*, 2010). Figure 17.3A (left) shows TIRFM of cells expressing GFP-labeled AQP4-M23. The distinct, well-demarcated spots correspond to individual OAPs. Because the actual sizes of OAPs are generally less than the x, y -spatial resolution of TIRFM, most OAPs appear as diffraction-limited fluorescent spots whose intensity is proportional to the number of GFP-AQP4 molecules in the OAP. Figure 17.3A (right) shows individual OAP trajectories from TIRFM time-lapse imaging done over 3 h. Most trajectories show slow Brownian motion with various reorganization events, such as OAP fusion and fission, occurring over tens of minutes. An example of OAP fission is shown in Fig. 17.3A (bottom), where a single fluorescent spot separates into at least four distinct fluorescent spots. Analysis of OAP trajectories gave a median diffusion coefficient of $\sim 10^{-12}$ cm²/s.

TIRFM allows determination of the size distribution of AQP4 OAPs from computation of area-integrated, background-subtracted spot intensities. To study the dependence of OAP size on M23:M1 ratio, cells were transfected with GFP-labeled AQP4-M1 and AQP4-M23 at different ratios under conditions of low expression to enable visualization of individual fluorescent spots. OAP size was deduced from spot-integrated fluorescence intensities referenced to GFP standards (Jin *et al.*, 2011). Figure 17.3B shows TIRFM (top) and number histograms (bottom) of single-spot fluorescence. The number histogram showed $\sim$ fourfold greater intensity of individual M1 tetramers than monomeric GFP, as expected. Higher spot fluorescence intensities, corresponding to larger AQP4 aggregates (OAPs), were seen at greater M23:M1 ratios, with considerable heterogeneity seen at all ratios. These data agreed well with predictions of a mathematical model of AQP4 OAP assembly based on random heterotetrameric association of AQP4-M1 and AQP4-M23, inter-tetramer associations between AQP4-M23 and AQP4-M23 (but not between AQP4-M1 and AQP4-M23 or AQP4-M1), and a free-energy constraint limiting OAP size.

Another prediction of the AQP4 OAP model is that OAPs consist of an AQP4-M23 enriched core decorated by an AQP4-M1 enriched periphery. To test this prediction, cells were transfected with GFP-labeled AQP4-M1 and (untagged) AQP4-M23. Experiments were done at high M23:M1 ratio in order to generate large OAPs of size greater than the TIRFM x, y -resolution (diffraction limit). Figure 17.3C shows high magnification TIRFM and corresponding line scans of two large AQP4 aggregates in which GFP-AQP4-M1 fluorescence was greater at the periphery than at the core.

5. Single-Molecule Analysis Shows AQP4 Heterotetramers

Single-molecule analysis of GFP intensity and photobleaching demonstrated that AQP4-M1 and AQP4-M23 associate in heterotetramers (Tajima *et al.*, 2010). The idea is that a homotetramer containing four GFP-labeled AQP4 molecules would undergo multistep

photobleaching as each of the four GFPs are sequentially bleached, and have four times the intensity of a GFP monomer. If a tetramer consists of one GFP-labeled AQP4 molecule and three unlabeled AQP4 molecules, then photobleaching would occur in a single step and the spot intensity would be the same as that of a GFP monomer. Figure 17.4A shows the implementation of this approach, with multistep photobleaching and high spot intensity of GFP-labeled AQP4-M1, but single-step photobleaching and low spot intensity in the presence of excess unlabeled AQP4-M1. To investigate heterotetramer formation, photobleaching and intensity analysis were done on cells expressing GFP-labeled AQP4-M1 coexpressed with an excess of unlabeled AQP4-M1 or the non-OAP-forming AQP4-M23 mutant, M23-G28P. For these studies, it was necessary to use an M23 mutant that cannot form OAPs because single-spot photobleaching and intensity analysis can be applied only to physically distinct AQP4 tetramers (and not to AQP4 in OAPs). We found single-step photobleaching and low spot intensity for GFP-labeled AQP4-M1 together with excess of the AQP4-M23 mutant, providing evidence that AQP4-M1 and AQP4-M23 are able to form heterotetramers.

6. Photobleaching Reveals Post-Golgi Assembly of OAPs

Fluorescence recovery after photobleaching of GFP-AQP4 chimeras confirmed that AQP4 OAPs are present in cell plasma membranes but not in endoplasmic reticulum or Golgi (Rossi *et al.*, 2011b; Tajima *et al.*, 2010). Figure 17.4B shows confocal fluorescence micrographs of cells expressing GFP-labeled AQP4-M1 and AQP4-M23 at the cell surface. Following photobleaching of a circular spot, recovery was seen for AQP4-M1 but not for AQP4-M23, in agreement with the conclusion that AQP4-M1 is present mainly as tetramers and AQP4-M23 as OAPs. Similar photobleaching measurements in endoplasmic reticulum or Golgi-targeted AQP4-M1 and AQP4-M23 showed unrestricted and rapid diffusion of both isoforms, indicating absence of OAPs. The conclusions from photobleaching measurements were confirmed by BN-PAGE and freeze-fracture electron microscopy. It was found that AQP4 OAP formation in plasma membranes but not in Golgi was not related to AQP4 density, pH, membrane lipid composition, C-terminal PDZ-domain interactions, or α -syntrophin expression. However, fusion of AQP4-containing Golgi vesicles with (AQP4-free) plasma membrane vesicles produced OAPs, suggesting the involvement of plasma membrane factor(s) in AQP4 OAP formation. In investigating additional possible determinants of OAP assembly, we discovered membrane curvature-dependent OAP assembly, in which OAPs were disrupted by extrusion of plasma membrane vesicles to ~110 nm diameter but not to ~220 nm diameter. AQP4 supramolecular assembly in OAPs is thus a post-Golgi phenomenon involving plasma membrane-specific factor(s). Post-Golgi and membrane curvature-dependent OAP assembly may be important for vesicle transport of AQP4 in the secretory pathway and AQP4-facilitated astrocyte migration.

7. Super-Resolution Imaging of AQP4 OAPs

Though nearly all OAPs in cells coexpressing AQP4-M1 and AQP4-M23 are smaller than the diffraction limit of conventional light microscopy, many OAPs are suitable for super-resolution imaging by photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), or other super-resolution methods. Figure 17.4C shows a PALM image of a chimera containing AQP4-M23 and GFP that can undergo photoactivation (PA-GFP). Various other fluorescent protein-AQP4 chimeras can be generated for multicolor PALM for simultaneous detection of AQP4-M1 and AQP4-M23. STORM imaging is possible as well, using fluorescently labeled antibodies against engineered extracellular epitopes on AQP4, as described above, or recombinant monoclonal NMO autoantibodies directed against extracellular epitopes on native AQP4 (Crane *et al.*, 2011a).

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Figure 17.1.

Approaches to visualize AQP4 and OAPs. (A) Freeze-fracture electron micrographs of the plasma membrane P-face of COS-7 cells expressing the M1 and M23 isoforms of AQP4. (B) AQP4 immunoblot following Blue-native gel electrophoresis of cell lysates from AQP4-expressing COS-7 cells. (C) Total internal reflection fluorescence micrographs of GFP-AQP4 chimeras. (D) (left) Schematic showing the organization of AQP4 tetramers (left) and examples of single particle trajectories of Qdot-labeled AQP4 molecules in the plasma membrane of AQP4-expressing COS-7 cells. Each cylinder represents one AQP4 tetramer in which a subset of AQP4 molecules are labeled with quantum dots (red) for single particle tracking. (Center) Combined mean squared displacement (MSD) versus time plots and averaged diffusion coefficients for AQP4-M1 (gray) and AQP4-M23 (black) in COS-7 cells. (Right) Cumulative probability distribution of range at 1 s ($P(\text{range})$) deduced from SPT measurements, with dashed lines indicating median range. Adapted from Crane *et al.* (2008) and Tajima *et al.* (2010).

Figure 17.2.

Quantum dot single particle tracking reveals determinants of AQP4 OAP assembly. (A) AQP4 sequence and topology showing site of GFP or epitope (myc) insertion in the second extracellular loop. Black: Met-1 and Met-23 translation initiation sites; blue: residues where mutations did not affect OAP assembly; red: mutations disrupt OAPs; pink: mutations mildly disrupt OAPs; yellow: mutations reduce plasma membrane expression; green: C-terminal PDZ-binding domains. (B) $P(\text{range})$ for indicated AQP4 truncation mutants. (C.) OAP modulation by coexpression of M1-AQP4 and M23-AQP4. (Left) $P(\text{range})$ for cells transfected with M23 only (black) or M1 only (gray), or cotransfected with M23 (red) and M1 (green) at M23-to-M1 ratios of 1:1. (Right) $P(\text{range})$ comparing M1/M23 cotransfection (solid) versus "separate" (dashed), computed by summing $P(\text{range})$ curves for separate transfections. (D) TIRFM of Alexa-labeled AQP4 in cells expressing M23-F26Q or M23-G28P and fixed at 4 or 37 °C. Adapted from Crane and Verkman (2009a, 2009b) and Crane *et al.* (2009).

Figure 17.3.

OAP dynamics and structure revealed by TIRFM of GFP-AQP4 chimeras. (A) TIRFM image (left) showing distinct fluorescent spots in cells expressing M23-AQP4, corresponding to OAPs, with deduced single OAP trajectories over 3 h shown at the right. (Bottom) High magnification of boxed region showing spontaneous OAP disruption events; trajectories of original OAP (black) and daughter OAPs (red, green, yellow, blue) shown at the right. (B) U87MG cells were transfected with GFP-M23 and GFP-M1 AQP4 at indicated ratios. Representative TIRF micrographs show fluorescent spots (top). Deduced number histograms of single-spot fluorescence (background-subtracted, area-integrated intensities), proportional to OAP size, shown at the bottom. Unity represents the intensity of monomeric GFP. (C) U87MG cells were transfected with GFP-M23 and (untagged) M1 AQP4 at a ratio of 20:1. TIRFM of two large AQP4 aggregates (left), showing relative concentration of fluorescence at the periphery. Line profiles (dashed white lines at the left) shown at the right. Adapted from Tajima *et al.* (2010) and Jin *et al.* (2011).

Figure 17.4.

Photobleaching and super-resolution imaging of AQP4 OAPs. (A) Single molecule step-photobleaching and intensity analysis shows AQP4 heterotetrameric association. (Top) Serial TIRFM images of recombinant monomeric GFP-labeled AQP4-M1 showing multistep loss of fluorescence. (Bottom, left) Single-spot (background-subtracted) integrated fluorescence intensity histograms for GFP-labeled AQP4-M1 without or with excess unlabeled AQP4-M1. (Right) Single-spot intensities as a function of time during continuous illumination, showing single versus multistep photobleaching. (B) Diffusion of GFP-labeled M1 and M23 AQP4 at the plasma membrane in live cells. Arrowheads indicate bleached area. Adapted from Tajima *et al.* (2010). (C) Super-resolution image of AQP4 OAPs. PALM image of AQP4-M23 chimera containing PA-GFP at its C-terminus. Inset: TIRFM (non-super-resolution) of area in white box.