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Review

Methods for studying mammalian aquaporin biology

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Abstract

Aquaporins (AQPs), transmembrane water-conducting channels, have earned a great deal of scrutiny for their critical physiological roles in healthy and disease cell states, especially in the biomedical field. Numerous methods have been implemented to elucidate the involvement of AQP-mediated water transport and downstream signaling activation in eliciting whole cell, tissue, and organ functional responses. To modulate these responses, other methods have been employed to investigate AQP druggability. This review discusses standard *in vitro*, *in vivo*, and *in silico* methods for studying AQPs, especially for biomedical and mammalian cell biology applications. We also propose some new techniques and approaches for future AQP research to address current gaps in methodology.

Keywords: aquaporins; methods; in vitro; permeability; inhibitors; cell behaviors

Introduction

Aquaporins (AQPs), transmembrane protein channels responsible for facilitating passive water flux across cell membranes, are critical for regulating cell shape, cell volume, and fluid homeostasis in nearly all organisms. Thirteen AQP isoforms (AQP0–AQP12) have been identified in mammals [1], some of which are classified as aquaglyceroporins that transport glycerol in addition to water (AQP3, 7, 9, and 10) or super AQPs (AQP11 and AQP12) that have a unique cysteine residue in their pore-forming asparagine-proline-alanine (NPA) box [2]. AQPs are known to drive water-essential processes such as tear production, saliva secretion, renal reabsorption, and vaginal lubrication, but they also have other nonobvious functions in cell migration, cell invasion, proliferation, angiogenesis, and neuroexcitation. Growing evidence indicates AQP involvement in the pathophysiology of multiple clinical conditions including cancer, endometriosis, glaucoma, and epilepsy [3, 4], thus prompting further investigation into AQP-mediated processes and the manipulation of AQPs in the design of therapeutics.

Over the past 30 years, the diverse cellular roles of AQPs and their mechanisms in physiological and pathophysiological systems have been studied *in vitro, in vivo,* and *in silico.* Although the research performed with these methods has provided a foundational understanding of AQPs, further study is still warranted to clarify AQP-mediated biological processes. *In vitro* methods may be used to induce or measure AQP expression and subcellular localization or to analyze AQP function in regulating cell behaviors. Several *in vivo* models drive many of the pre-clinical efforts in understanding the relationship between AQPs and disease progression. Finally, there are several *in silico* methods that can elucidate AQP structure, function, and interactions with other molecules and proteins, as well as model the resulting cell behavior. The goal of this review is to collate and discuss established *in vitro*, *in vivo*, and *in silico* methods for studying mammalian AQPs in biomedical science and engineering applications. A condensed collection of these methods is depicted in Fig. 1. We also suggest some new techniques and models that can be applied for future AQP-related research.

In vitro methods for studying AQPs

Once AQPs were discovered in 1992 by Peter Agre *et al.* [5, 6], X-ray crystallography [7], nuclear magnetic resonance (NMR) spectroscopy [8], and 3D electron microscopy [9] techniques defined the structures of AQP isoforms. Since then, the majority of work conducted on AQP biology and druggability has been conducted *in vitro*; this prevalence occurs because *in vitro* models allow for the fine-tuned manipulation of experimental conditions, greater accessibility in observing mechanistic changes, and overall ease of use. Moreover, *in vitro* techniques are sometimes preferred over *in vivo* experiments for their practicality, cost–effectiveness, and ethicality. A summary of *in vitro* AQP methods and example references is detailed in Table 1.

Expression and localization

Since AQP localization and expression are essential to the roles they play in the regulation of cellular processes, it is worth identifying their alterations across healthy and disease states. Here, we summarize traditional methods for measuring and modulating AQP expression and subcellular distribution.

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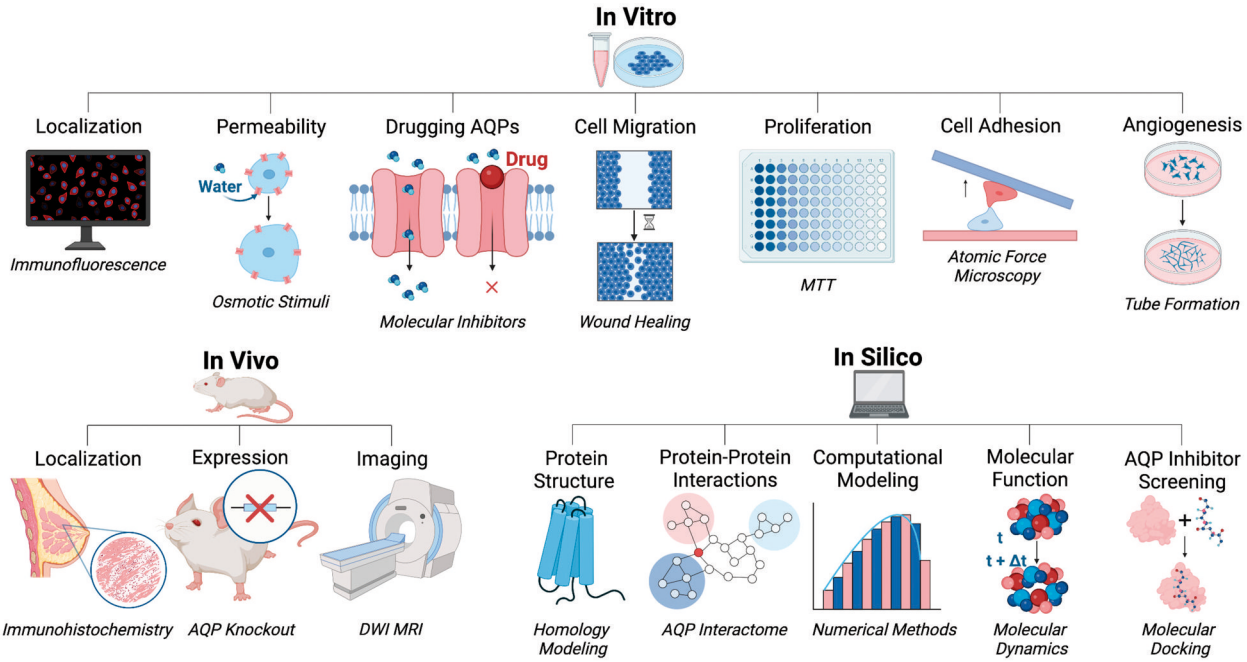

Figure 1. Graphical summary of *in vitro*, *in vivo*, and *in silico* methods for AQP biology as discussed in this review.

AQP expression

AQP genes are commonly knocked down using RNA interference. siRNA transfections and shRNA lentiviral vector transductions are common techniques for gene knockdowns [109, 110] as they are low-cost and quick to perform compared to a knockout. Full AQP knockouts have been created primarily in mice and will be discussed in 'Observing AQP expression and activity *in vivo*' section. To induce or increase AQP expression, cells can be transfected with a constructed overexpression vector [76]. Western blots and RT-PCR/RT-Qpcr [14, 91–111] are used to quantify AQP protein and gene expression levels, respectively. These studies continuously add to a wide range of healthy and pathophysiological microenvironmental components that have been shown to alter AQP expression [83, 112–115].

AQP localization

The ability to visualize the localization of AQPs, which primarily exist on the plasma membrane or within the cytoplasm, can lend additional insight into AQP abundance, trafficking, and function with respect to cell behavior. Immunofluorescence (IF), known as immunocytochemistry (ICC) for cells *in vitro*, is the gold standard method to visualize protein localization. ICC can be employed for cells situated in different environments, including 2D culture and 3D culture such as within microfluidic devices [16]. Although IF produces qualitative data, multiple parameters can easily be quantified from microscopy images. For example, the mean fluorescence intensity (MFI) of a region of interest (ROI) is often used as a proxy for relative protein expression in a sample compared to a control. Measuring MFI values and selecting an ROI are easily accomplished using the Fiji-ImageJ software [116]. Additionally, the nuclear to cytoplasmic MFI ratio is one metric of subcellular AQP distribution that can be informative for cells expressing intranuclear AQPs, a phenomenon that has been previously reported [17]. The main disadvantage of ICC is that it does not allow for dynamic visualization of AQP stimulus/response trafficking since the samples must be fixed. Instead, transfecting cells with a construct to tag AQPs with a fluorescent protein are the standard technique for imaging live subcellular AQP distribution over time [15, 19, 20]. Furthermore, observing the co-localization of AQPs with other markers associated with cellular trafficking can provide insight into the mechanisms underlying subcellular distribution [22, 117].

In addition to observing AQP localization, it is possible to induce a particular subcellular distribution if needed. AQPs are predominantly found in the cytoplasm or on the cell surface, which is often a result of trafficking activities that are regulated by molecules such as arginine vasopressin (AVP), adenosine 3′,5′-cyclic monophosphate (cAMP), protein kinase C (PKC), and protein kinase A (PKA) [118]. These molecules use phosphorylation to regulate the endocytosis, storage, degradation, or exocytosis of AQPs in response to a stimulus. For example, redistribution of AQP1 to the cell membrane has been observed in human umbilical vein endothelial cells (HUVECs) upon exposure to osmotic stimuli, which was immediately mediated by PKC and then mediated by calcium signaling in the following minutes [21]. Similarly, AVP can induce a translocation of AQP2 to the membrane during dehydration [23] or AQP2 internalization in other instances [22, 117]. Subcellular AQP distribution patterns may differ across different tissues, and due to dynamic trafficking activities, AQPs are generally not solely situated in one localization [25, 48, 119–121]. Even AQP6, which is predominantly found intracellularly [122], has recently been reported in discrete areas of the plasma membrane [123].

Analyzing the AQP function

AQPs are traditionally known for their function in transmembrane water transport to regulate cell volume and support a wide variety of physiological phenomena, but some have been shown to play a second role in the activation of multiple signaling pathways such as Ras/Raf [14], MAPK/p38 [124], Wnt [50], and PI3K/Akt [125, 126]. To fully understand the impact of AQPs in biological systems, it is important to have methods to characterize both water permeation and signaling mechanisms.

Table 1. *In vitro* methods

Study area	Goal	Method	Example Ref(s)
Expression & Localization	AQP Expression	siRNA or shRNA lentiviral vector transfections	[10–13]
		Overexpression vector transfection	[14, 15]
	AQP Localization	IF	[16–18]
		Fluorescently labeled AQPs	[15, 19, 20]
		Osmotic stimuli	[21]
		AVP	[22, 23]
AQP Function	Permeability Assays	Proteoliposomes	[24]
		Stopped-flow spectrophotometry	[25–29]
		X. laevis oocyte swelling	[30, 31]
		Fluorescence (calcein)	[32, 33]
		Epithelial assays	[34]
		Yeast freeze–thaw challenge	[35, 36]
		Ion conductance using electrophysiology	[37–42]
		Cell swelling with glycerol gradient	[25, 27–29]
		H_2O_2 fluorescent label	[26]
	Modulating AQP activity	Small molecule inhibitors	[43]
		Heavy metal inhibitors	[44]
		Bumetanide derivative	[45, 46]
		Plant-based compound inhibitors	[46, 47]
		Drug AQP translocation	[48]
		Modlate ion conductance	[26, 38, 41]
		Optogenetics	[49]
	AQP–Protein Interactions	co-IP	[50–54]
		PDAs	[45, 55]
		Y2H screening	[56]
		Cell swelling assays	[53, 56, 58]
		Cross-linking mass spectrometry	[59–62]
		Overlay assays	[63, 64]
		Microscale thermophoresis	[27, 65, 66]
		Proximity ligation assays	[66, 67]
	AQP–Lipid Interactions	X-ray crystallography/electron crystallography	[68–71]
	Phosphorylation Site Identification & Characterization	Liquid chromatography-mass spectrometry	[72–74]
		Radioactively labeled ATP & immunoprecipitation	[53, 56]
		Phospho-specific antibodies	[72]
		Functional mutant AQPs	[23, 67, 75]
Cell Behavior	Migration & Invasion	Wound healing	[12, 66, 76]
		Transwell invasion	[12, 66, 76]
		2D random migration[a]	[77]
		Monolayer incorporation[a]	[78]
		Spheroid invasion	[47]
		3D random invasion[a]	[79]
		Confining microfluidic devices	[16, 80–82]
		Actin cytoskeleton visualization using ICC	[11, 18, 76, 83, 84]
		Fluorescently labeled filamentous actin probe	[15, 85]
		Western blotting for invasive markers	[76, 83, 85, 86]
		Protrusion analysis	[15, 76, 85, 87–89, 90]
	Proliferation	AlamarBlue	[13, 83]
		Bromodeoxyuridine	[91]
		MTT	[92]
		Cell Counting Kit 8	[93]
		Crystal violet assays	[47]
	Apoptosis	Caspase-3 activity assay	[94–96]
		Flow cytometry with a viability dye & annexin V	[47, 97]
		Terminal deoxynucleotidyl transferase dUTP nick end labeling	[96, 98, 99]
		Western blotting for apoptotic factors	[95, 98, 99, p. 4, 100]
		Swelling assay	[94]
		Mitochondrial membrane potential analysis	[94, 100]
	Cell Adhesion	Crystal violet staining	[83]
		AFM	[10]
		ICC visualization of junction proteins	[18, 101]
		Dispase-based dissociation	[101]
		Phenotyping cell–cell junctions[a]	[102]
		Fluorescence-based microtubule polymerization	[103]
	Angiogenesis	Tube formation	[69, 81, 88]
		HUVEC cell assays	[13, 104–107]
		Western blotting for angiogenic factors	[98]
		IF	[108]

[a] Assay that, to our knowledge, has not been employed yet to study AQPs.

Permeability assays

Permeability assays leverage an osmotic gradient to measure AQP-mediated water flux. For benefits, limitations, and further details of each AQP permeability assay, we direct the reader to other reviews [127, 128].

Stopped-flow

Water permeation through AQP pores can be measured in AQP-containing proteoliposomes using stopped-flow spectrophotometry. In this technique, the scattered light intensity correlates with the change in vesicle volume after an osmotic shock. Although this method requires a challenging sample preparation process, Yue *et al*. devised an *Escherichia coli* Cell-Free Protein Synthesis (CFPS) system that does not require cells to generate AQPs, circumventing the need for an additional purification step [24]. The CFPS system efficiently synthesizes a large quantity of AQPs that are then inserted into the liposomes in order to study water permeability. In addition to proteoliposomes, AQP-expressing yeast cells have also been used with stopped-flow measurements [26].

Cell-based permeability assays

More commonly, cell swelling assays are utilized to measure permeability. Usually, human AQPs are heterologously expressed in *Xenopus laevis* oocytes, and the cell volume is monitored with videomicroscopy—such as phase contrast, confocal or fluorescence microscopy—during a hypo-osmotic shock [30]. The oocytes do have other endogenous membrane proteins [24], but the advantage of this model organism is its low intrinsic water and glycerol permeability [129]. Using fluorescence microscopy techniques, cell volume can be measured with fluorescent dyes that undergo quenching during cell swelling or shrinking from an osmotic shock [130]. To achieve high-throughput measurements, Fenton *et al*. adapted a calcein-quenching-based assay for a plate reader that can be employed for a variety of adherent cell types: briefly, the calcein-AM fluorophore was loaded into cells and cell volume changes were calculated from plate reader fluorescence intensity readings [32, 131]. A step-by-step protocol for this assay has been written by Kitchen *et al*. [132]. Similarly, Mola *et al*. developed an automated cell-based AQP inhibitor screening assay, which utilizes a microplate reader to make dynamic fluorescence measurements of cell volume; the assay was validated using calcein-AM-loaded primary astrocytes and fibroblasts that strongly express AQP4 and AQP1, respectively [33].

Epithelial permeability assays, where an epithelial monolayer on a permeable surface is mounted in an Ussing chamber, are also used to measure water transport [34]. An osmotic gradient is applied to one compartment and the fluid height is measured in a capillary tube connected to the other compartment; alternatively, a fluorescent dye is added to the hyperosmotic compartment and the rate at which the fluorescence intensity decreases is measured. This method is best for characterizing AQP-mediated cellular water permeability in epithelial cell monolayers [129].

Yeast has also been used as a permeability measurement tool: AQPs of any origin are heterologously expressed in yeast cells and a freeze–thaw challenge is applied [35, 36]. The survival of the yeast post-challenge depends on AQP-mediated water flux, making this an effective and simple inhibitor screening method.

Measuring permeability of unconventional permeants

In addition to facilitating water flux, certain AQP isoforms are capable of transporting ions, with the most notable example being AQP1. AQPs are known to form a quaternary tetrameric structure, resulting in a central pore between the monomers. The role of this central pore has been a topic of debate in the AQP biology field, but it is the current understanding that the central pore of AQP1 acts as a cGMP-gated ion channel [133–135]. Ionic conductance is typically measured with electrophysiology techniques, including two-electrode voltage clamp [37–39], black lipid membrane [40, 136], and patch-clamp experiments [39, 41]. Furthermore, some AQPs can transport small solutes, such as glycerol and hydrogen peroxide (H_2O_2) [42]. To measure glycerol permeability, a cell swelling assay on the *X. laevis* oocytes can be performed by replacing the sodium chloride in the culture medium to glycerol at the same tonicity. The cell volume increase represents a solute influx first, followed by water influx [10, 130, 137]. The stopped-flow light scattering method is another technique used to measure glycerol permeability in cells under an applied glycerol gradient [25, 27–29]. H_2O_2 flux has been measured under live cell imaging after transfecting cells with a construct containing a fluorescent tag such as the HyPer biosensor [11, 29, 45] or the H_2O_2-specific small molecule indicator Peroxy Yellow 1 Methyl-Ester [11]. Similarly, live cell imaging can be performed on cells incubated with the cell-permeant indicator 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA) that fluoresces upon oxidation [26]. In the same study, yeast cells expressing human AQPs, H_2O_2 uptake was measured with an electrochemical assay which measures its conversion to oxygen [26].

Modulating AQP activity

Modulating the activity of AQPs by blocking their transport functions or their trafficking is desirable for *in vitro* experiments as well as for future clinical applications in treating AQP-mediated diseases. The following subsections describe current strategies for reducing AQP activity.

Drugging AQP-mediated water permeation

The majority of putative AQP inhibitors are small molecules, including tetraethylammonium (TEA), acetazolamide, antiepileptic drugs, TGN-020, and phloretin, which generally work by blocking water transport through AQP pores. Heavy metal compounds such as nickel chloride, copper compounds, and silver are used as cytotoxic, nonspecific AQP water inhibitors. Bumetanide derivative AqB013 [45] and plant-based compound bacopaside II [30] have shown AQP1 water channel inhibiting abilities. Additionally, AuPhen is capable of significantly reducing glycerol permeability in AQP3. A detailed description of these inhibitors and their putative AQP binding sites can be found in other reviews [127, 128]. Unfortunately, these inhibitors range in efficacy for a limited number of human, mice, or rat AQP isoforms and produce variable outcomes across different permeability assays [127]. The lack of specific, effective, and nontoxic AQP inhibitors means that further high-throughput screening is necessary [138]. Once more AQP inhibitor candidates are identified, multiple permeability assays should be performed reproducibly to validate that they remain robust under a variety of test conditions. A discussion on computational screening methods is included in ‘AQP drug screening’ section.

Targeting AQP translocation

Aside from blocking water flux with AQP inhibitors, cell water permeability could be controlled by drugging signaling pathways that regulate AQP trafficking to the cell surface, such as PKC-dependent pathways for AQP1, and cAMP- and PKA-dependent pathways for AQP5 [118]. Recently, trifluoperazine blocked AQP4 localization to the blood-spinal cord barrier in astrocytes by

inhibiting calmodulin. Since calmodulin likely binds directly with AQP4 to induce its translocation, trifluoperazine successfully neutralized the hypoxia-driven increase in astrocyte water flux [48].

Drugging AQP-mediated ion conductance

Lastly, a group of AQP-targeting drugs that modulate ion conductance through the putative central ion pore of AQP1 has been identified. The central ion channel is of interest due to its potential involvement in volume regulation and signal transduction [139], and indeed, AQP1 ion conductance has been shown to facilitate human colon cancer cell migration [37]. To attenuate this migratory behavior, ion conductance through the central pore can be blocked with the bumetanide derivative, AqB011, alone or in conjunction with one of the aforementioned AQP (water flux) inhibitors such as bacopaside II [37, 46]. In another study, AqB013 and AqB050, which are also AQP1 drugs, inhibited HUVEC tube formation via induction of apoptosis and impairment of cell migration [45]. Finally, work by Palethorpe *et al.* showed that bacopasides I and II can synergistically inhibit breast cancer cell growth, migration, and invasion by blocking AQP1-mediated ion and water transport [47].

Optogenetics to modulate AQPs

Optogenetic techniques have recently emerged as tools for the precise manipulation of protein function and localization in specific, photosensitive cells using light [140]. While optogenetics has not been widely employed for AQP modulation, they have been used to control light-gated ion channels that create an osmotic gradient and trigger a water influx or efflux through AQP1 [49]. Future investigations should continue to explore optogenetics as a means to control AQP expression, localization, and function in cells.

AQP-protein and AQP–lipid interactions

Although individual AQP monomers are functional water channels, they often interact with each other to form tetramers: each monomer transports water and the role of the central pore in between the monomers is still controversial. AQPs of the same or different isoforms may combine to form these tetramers [65]. AQPs may interact with other proteins, such as ion channels that establish osmotic gradients to drive water transport or upstream or downstream molecules in signaling pathways. AQPs can trigger several pathways, including Ras/Raf/ERK [14, 66], MAPK/p38 [124], Wnt/β-catenin [50], and PI3K/Akt [125, 126]. On the other hand, upstream signals regulate cell water permeability by triggering AQP membrane translocation or internalization [67, 141]. Identification of protein–protein interactions (PPIs) is valuable for understanding AQP activities within the cell, and the regulatory mechanisms behind these activities can be further elucidated by studying AQP post-translational modification sites. AQP–lipid interactions are also critical in biological membranes, as these interactions impact both integral protein function and lipid organization. AQP–lipid interactions are much less characterized than AQP–protein interactions due to our incomplete knowledge of atomic-level AQP isoform structures but are worth studying due to their impact on membrane properties.

AQP–protein interaction detection methods

Of the *in vitro* techniques for extracting AQP binding partners, co-immunoprecipitation (co-IP) [50, 51, 52, 103], pull-down assays (PDAs) [63, 142, 143], and yeast two-hybrid (Y2H) screening [56] are the gold standards. Co-IP and PDA involve isolating the protein of interest along with their putative binding partners from a cell lysate. They are usually used in conjunction with IF to ensure that the proteins are co-localized in the native cell, and with mass spectrometry to identify the protein complexes. Y2H employs a reporter gene and a transcription activator with an activating domain and a DNA binding domain. In addition to co-IP and IF [53], cell swelling assays have been used to characterize AQP collaboration with ion channels, especially transient receptor potential vanilloid 4 [53, 57, 58]. Other less commonly used methods that have been employed to reveal AQP–protein interactions include cross-linking mass spectrometry [59–62], overlay assays [63, 64], microscale thermophoresis [48, 141, 144, 145], and proximity ligation assays [143, 145, 146]. The AQP interactome, including binding partners and detection method(s) used, has been thoroughly discussed in other reviews [65, 147, 148]. Computational PPI methods can be found in 'Identification of AQP–protein and AQP–lipid interactions' section.

Identification of post-translational modification sites on AQPs

Once putative binding partners are identified, their interactions with AQPs can be further characterized by determining likely phosphorylation sites via a phosphoproteomic analysis. Knowledge of AQP phosphorylation sites lends a greater mechanistic understanding of how AQPs receive regulatory signals from other proteins, eventually resulting in altered water permeability or subcellular localization [118]. These regulatory events could be targeted in order to control AQP activities or downstream signaling pathways. AQP phosphorylation sites are already well-defined experimentally and are tabulated on bioinformatics databases [72, 149, 150]. Phosphoproteomic analyses are usually carried out by various phosphopeptide enrichment methods. For example, a prominent study combined immobilized metal affinity chromatography and liquid chromatography–mass spectrometry (LC–MS) neutral loss scanning to enrich phosphopeptides from rat inner medullary collecting duct (IMCD) tissues treated with AVP, leading to the detection of four phosphorylation sites on AQP2 [72]. After advancements in the sensitivity and accuracy of mass spectrometers, these results were confirmed over a decade later using LC–MS in a similar experimental setup [73]. Another group used an *in vitro* phosphorylation assay in which rat tissue homogenate was incubated with radioactively labeled ATP and either PKA or PKC. The phosphorylated proteins were then immunoprecipitated with an AQP1 or AQP4 antibody, respectively, showing that AQP1 is phosphorylated by cAMP-dependent PKA [151] and that AQP4 is phosphorylated by PKC in the presence of a PKC activator [151, 152]. The same assay was also utilized to study AQP7 phosphorylation by PKA [143]. These interactions can be further quantified by measuring protein levels using phospho-specific antibodies; Hoffert *et al.* used an AQP2 antibody that recognized site S256 phosphorylation and found that samples treated with AVP showed an increased phospho-AQP2 abundance compared to the control [72].

In addition to phosphorylation sites, some AQP isoforms contain ubiquitylation [67], glycosylation [75], SUMOylation [153], and acetylation [74] sites. These sites have been identified similarly to phosphorylation sites; for example, an AQP3 SUMOylation site was found by wild-type α-lytic protease digestion of murine tissues and subsequent mass spectrometry [153], and an AQP3 acetylation site was identified using liquid chromatography–tandem mass spectrometry on rat IMCD samples [74]. AQP2 ubiquitylation and N-glycosylation were studied using mutant forms in Madin–Darby canine kidney cells [67, 75]. This

method of using site-directed mutagenesis on AQPs is widely used to understand how the loss-of-function of a PPI (phosphorylation, ubiquitylation, glycosylation, acetylation, etc.) of interest affects AQP regulation and cell behavior [23].

AQP–lipid interaction characterization methods

The AQP–lipid interactions that have been studied thus far come from X-ray crystallography and electron crystallography, which allows for imaging of atomic-level structures of AQP–lipid complexes [7]. In particular, the structure of AQP0 has been solved by electron crystallography, so its integration and organization with various lipids have been observed in detail [68–71]. Structures obtained from crystallography are often then used in computational simulations as described in 'Identification of AQP–protein and AQP–lipid interactions' section .

Understanding cell behavior with respect to AQP activity

AQPs have gained significant interest for their roles in cell activities such as fluid homeostasis, proliferation, migration, invasion, angiogenesis, and apoptosis. These functions are fundamental to healthy processes including kidney water reabsorption, brain-water homeostasis, neuroexcitation, development, and wound healing; and are also crucial for disease processes like cancer metastasis, tumor growth, seizures, and endometriosis development [3, 4]. Having a wide variety of *in vitro* cell-based assays enables us to study the involvement of AQPs in both healthy and pathologic cell states. Although this is not a comprehensive review of cell-based assays, here we discuss common techniques used in AQP studies and highlight those that are particularly relevant to AQP regulation of cellular processes. These experiments are typically performed with AQP knockdowns or overexpressions, AQPs treated with inhibitors, or functionally mutated AQPs, along with controls.

Cell migration and invasion

AQPs have been scrutinized for their involvement in the migration and invasion of healthy and diseased cells. Specifically, AQPs are responsible for facilitating rapid water fluxes that allow cells to change volume and shape and provide space for actin polymerization. AQPs co-localize with ion channels that establish an osmotic gradient at the leading edge of a cell, thereby inducing a water influx that creates space for actin to polymerize. Similarly, an AQP-mediated outflux of water at the trailing edge of the cell can help the membrane retract [154]. When studying the impacts of AQPs on cell motility, the most simplistic experiment that countless studies have included is a wound healing or gap closure assay, where collective cell migration is measured by the change over time in the width of a scratch or gap made in a monolayer. In parallel, many researchers use transwell invasion assays in which individual cells invade from an upper chamber through a semipermeable membrane into a lower chamber containing a chemoattractant. To give a few examples, knocking down AQP1 [66], AQP3 [76], and AQP5 [12] expression resulted in reduced wound closure and number of invaded cells for gastric cancer cells, endometrial carcinoma cells, and nonsmall cell lung carcinoma cells, respectively.

Although wound healing and transwell invasions are useful low-cost methods, they only provide insight into the involvement of AQPs in directed cell migration; it is unknown whether AQPs have a significant role in undirected single-cell migration. Given that AQPs facilitate rapid cell volume changes and polarize to the leading and trailing edges of moving cells, studying 2D random migration may shed light on the contribution of AQPs to innate cell motility. This can be achieved with time-lapse tracking of individual cells with altered AQP expression or function; this would yield data on cell speed, directional persistence, mean square displacement, and morphological parameters. Another simple AQP-mediated cell invasion method could be an incorporation assay [78], which has not yet been employed for studying AQPs. Briefly, invasive cells such as MDA-MB-231s are stained and seeded on top of an endothelial or epithelial monolayer; the percentage of cells that are "incorporated" and disappear into the monolayer is quantified. This method could be used to characterize the effect of the AQPs in the invading cells or in the cell monolayer. In other words, do AQPs enhance the capability of the invading cells to disrupt the monolayer, and/or do the AQPs in the monolayer affect its barrier function? This assay can provide a preliminary answer to this question. Moreover, the method is relevant to AQP biology because the invading cells have to deform and squeeze through cell–cell junctions in the monolayer, representing a form of confined migration which AQPs may facilitate.

In addition to these basic migration assays, it is important to use models that are more representative of the *in situ* physiological microenvironment that cells experience (and which AQPs may help them traverse) during healthy and disease processes. A spheroid invasion assay more closely mimics how cells can metastasize away from a primary tumor, for example. One group found that treating breast cancer cells with bacopasides I and II, putative AQP1 inhibitors, significantly reduced MDA-MB-231 triple-negative breast cancer spheroid invasion into the surrounding matrix [47]. Heterotypic spheroids with leader and follower cells would be particularly fascinating for studying AQP-mediated invasion; follower cells can become invasive and travel through microtracks made by malignant leader cells [155]. Spheroid invasion assays would shed light on whether AQP expression enhances the ability of leader cells to proteolytically degrade the extracellular matrix or the ability of follower cells to deform and migrate through pre-made tracks. And, similar to the 2D random migration assay, there have been no reports of the role of AQPs in undirected cell invasion: a random invasion assay on cells evenly distributed within a 3D matrix such as collagen or Matrigel without a chemoattractant could be informative.

Stroka *et al.* also utilized a more physiologically relevant 3D model for cell migration in confined spaces that exist *in vivo*, such as tissue microtracks. Within a polydimethylsiloxane microchannel device, they blocked actin polymerization and myosin II-mediated contractility and administered osmotic shocks at the front or back of the cell. They found that water permeation drives tumor cell migration in confined spaces in the absence of actin polymerization and myosin II-mediated contractility, a phenomenon termed the "Osmotic Engine Model" [16]. In light of these findings, it makes sense to use methods that further clarify how AQPs might enable confined migration of invasive cell types, especially since AQPs facilitate rapid cell shape changes. Although several different models of confinement exist [156], they have not been widely employed to study AQPs. However, three papers after the establishment of the Osmotic Engine Model have reported research on confined migration with respect to AQPs. Two studies similarly performed time-lapse imaging of confined cell migration in a microfluidic device: one of them studied live AQP4 localization [80], and the other measured migration speed during an AQP5 knockdown [81]. The third investigation used an electro-osmotic microfluidic system that applies precise osmotic gradients to cells in microchannels, leading

them to find that knocking down AQP4 resulted in a decreased confined cancer cell migration speed [82]. Broader use of different types of confined migration models across more AQP isoforms will help elucidate the influence of AQPs in enabling cells to move and deform through tight spaces.

Apart from directly studying cell migration and invasion, AQP expression has been linked to other hallmarks of invasion such as actin cytoskeleton rearrangement, focal adhesion dynamics, and epithelial–mesenchymal transition (EMT) [154]. The standard for studying the actin cytoskeleton and focal adhesion kinase organization is ICC [18, 50, 76, 84, 85, 157], but for unfixed cells, actin dynamics are usually observed by transfecting cells with a fluorescently labeled filamentous actin probe [15, 85], such as LifeAct. In many AQP studies, expression levels of cytoskeletal proteins and EMT markers have been measured with simple western blots [76, 83–86, 157], although this type of data can only provide correlations with AQP expression. A more AQP-relevant hallmark of cell invasiveness would be to visualize water-induced membrane protrusions, which multiple cell motility studies have done using microscopy techniques [15, 76, 84, 85, 87–90]. One study quantified bleb-like protrusions and filopodia from fluorescence microscopy images and found that overexpression of AQP9 in HEK-293 cells promoted the formation of membrane protrusions and therefore directed actin polymerization [15]. Similarly, knocking down AQP1 in rat gastric epithelial cells impaired lamellipodia formation during wound repair [87]. Continued use of membrane protrusion analyses with respect to other AQP isoforms and migratory cell types would help diversify our understanding of AQPs' function in the formation of migratory membrane structures. Future work could also determine how inhibiting AQP water flux impacts cell morphology.

Cell proliferation, apoptosis, adhesion, angiogenesis, and more

AQPs have been implicated in other cell behaviors, including proliferation, adhesion, angiogenesis, and apoptosis. AQPs facilitate volume changes necessary for cell division, and some isoforms participate in proliferative signaling pathways such as Ras/Raf/ERK [14] and MAPK/p38 [124]. Moreover, aquaglyceroporins transport glycerol, an important intermediate for ATP production and biosynthesis [158]. The alamarBlue [13, 83], BrdU [91], MTT [92], CCK-8 [93], and crystal violet assays [47] have been widely used to understand the effect of AQP expression on cell proliferation. Most often, AQP knockdowns have been correlated with reduced cell proliferation [13, 83, 91, 93]. But of the studies on AQPs and proliferation, there have been markedly fewer that utilize the two AQP modulation strategies other than expression: inhibitors and mutants. Performing more thorough testing of cell proliferation with respect to AQP water/glycerol permeability and AQP phosphorylation would lend a more holistic understanding of which mechanism(s) different AQP isoforms use to impact proliferation. Along with proliferation assays, dynamic AQP localization should be analyzed during cell division via live imaging of fluorescently labeled AQPs to gain more clues on their role in proliferation.

AQPs are linked to apoptosis as well by allowing a water efflux during the apoptotic volume decrease (AVD); after AVD, which is driven by an efflux of K^+, AQPs are thought to be inactivated to allow intracellular K^+ concentrations to drop enough for the activation of apoptotic enzymes [94]. However, the relationship between AQPs and cell death is unclear due to mixed results [10, 94, 95, 97–100, 159, 160, 163] and should be further investigated. There are several ways apoptosis can be characterized in AQP experiments, including a caspase-3 activity assay [94–96], flow cytometry with a viability dye and annexin V [97, 163], a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay [96, 98, 99], western blotting for apoptotic factors [95, 98–100], a swelling assay for measuring a reduced cell water permeability post-AVD [94], and a mitochondrial membrane potential analysis [94, 100]. Like proliferation, most of these methods are performed in the context of an AQP knockdown; however, the cell swelling assay is the most direct way to measure the effect of the AQP water transport function on apoptotic shrinkage. Jablonski *et al.* used flow cytometry to perform a cell swelling size distribution analysis after a hypotonic insult on shrunken (apoptotic) cells and healthy cells. The shrunken cells were significantly less water-permeable when compared to healthy cells, supporting the idea that AQPs may become inactivated after AVD to enable apoptotic enzyme activation. Since the AQP inactivation does not seem to be a result of AQP degradation or removal from the cell membrane [94], future use of mutant AQPs (with modified phosphorylation sites) in the apoptosis methods listed above would clarify the mechanism of this inactivation. The wide variety of findings in the literature on AQP and apoptosis might be partially due to the fact that the timing of the biochemical events during cell death varies significantly depending on the cell type, drug concentration, apoptotic stimulus, and exposure time [161]. More systematic and time-dependent experimentation is needed to understand how these parameters might affect the function of AQPs in apoptosis, especially if their function changes during the course of cell death.

Less prominent but still fascinating is how AQPs have been implicated in a variety of other cellular processes. For example, cell adhesion onto a Matrigel-coated surface with respect to AQP expression has been estimated with absorbance readings after crystal violet staining of washed and fixed samples [83]. Atomic force microscopy (AFM) with a tipless cantilever can quantify the work required to overcome cell–cell adhesion and has shown that AQP knockdown cells have weaker cell–cell adhesion [10]. Cell aggregation and expression of membrane-associated junctional proteins are other metrics for intercellular adhesion, which can be visualized with ICC [18, 101]. Conversely, cell–cell dissociation has been observed with video microscopy of migrating epithelial cell sheets and quantified with a dispase-based dissociation assay [101].

Related to cell–cell adhesion is junction phenotype, which is especially relevant in the blood–brain barrier. Our lab has developed a novel Junction Analyzer Program (JAnaP) for phenotyping cell–cell junctions from IF images as an indicator of endothelial barrier integrity [102]. This method has not yet been used to investigate the effect of AQP expression or function on endothelial junction presentation and would be informative given that some AQPs are involved in cell–cell adhesion and junction formation [162, 163]. JAnaP is not limited to endothelial cells—epithelial monolayers can be analyzed as well. In the same vein, AQPs have been studied in the context of paracellular permeability in epithelial cells. Cell–cell junctions are anchored to cytoskeletal components including microtubules, and thus a cytoskeletal rearrangement can alter the permeability between the junctions. Microtubule stability has been linked to AQP5 expression via ICC visualization of microtubules, soluble and insoluble microtubule extraction, and a fluorescence-based microtubule polymerization assay [162].

Finally, AQP expression has been linked to angiogenesis in multiple cell types, and investigations have most commonly used tube formation assays [12, 13, 163, 105]. These are often

performed in parallel with other cell migration, invasion, and proliferation assays [13, 163, 105–107] using endothelial cells such as HUVECs. The relationship between AQPs and other angiogenesis promoters, such as erythropoietin [108] and vascular endothelial growth factor [98], can also be examined using previously discussed western blotting, IF, or PPI detection methods. Again, these studies mainly provide some correlative insight on the effect of AQP expression on angiogenic cell behavior, but not AQP function. Using functional AQP mutants and AQP inhibitors in these methods would demonstrate the impact of AQP-triggered signaling cascades and AQP water blockade on angiogenesis, respectively.

In vivo methods for studying AQPs

Though *in vitro* methods are important for identifying the specific effects of individual cues on cells, it is essential to understand the translatability of these findings into complex *in vivo* systems. Here we provide an abridged compilation of the numerous ways *in vivo* models can be used to study AQP expression, AQP-induced cellular function, AQP-targeted drug development, and AQP-based anatomical imaging modalities.

Observing AQP expression and activity *in vivo*

The most simplistic studies conducted *in vivo* plainly observed AQP expression or localization in samples from animal models or patients, typically via immunohistochemistry [164–169]. Building upon this baseline, physiological AQP expression and localization can be compared to pathological tissue samples excised from patients or animal models. Common disease states studied for pathological changes in AQP dynamics include cancer (colorectal [170], lung [171], liver [96], breast [172]), diabetes [173, 174], arthritis [175], and chronic inflammation [176–181]. Other animal models can be employed to identify changes in AQP expression during events like development [182, 183].

Additionally, the opposite could be done, where AQPs are knocked out of *in vivo* models, and the functional changes that accompany this knockout can be observed. This idea was epitomized by much of the work done by Dr. Alan Verkman: their lab developed numerous AQP isoform knockout models and conducted experiments to identify differences among them [131, 184–188]. To our knowledge, murine knockout models have been created for the majority of AQP isoforms (AQP0, 1, 2, 3, 4, 5, 7, 8, 9, 11, and 12) [184–194], with additional models across other species. By observing the functional changes that occur amidst these knockout models, AQPs have been shown to regulate numerous functional pathways in the brain [184, 195–198], immune system [185, 191], the urinary tract [193, 199, 200], diabetes [194, 201–204], development [131, 205], and more [206, 207]. A functional correlation between AQPs and other pathways can be determined by developing knockout models of AQP-related proteins or regulators [208–213]. Changes in AQP functionality in the absence of these regulators provide a more holistic perspective of the complex interplay of signaling *in vivo*.

AQP modulation *in vivo*

After using the above methods to identify the abundant functional roles of AQPs across different systems, the next step is to identify if these roles can be modulated *in vivo*. Using molecular [214–218], siRNA [219], and immuno-based drugs [220], researchers have successfully inhibited AQP functionality or expression. In many cases, this inhibition reversed the burden and symptoms of the pathological system in question. Additionally, other molecular drugs [221] or viral vectors [222] have been used to increase the expression of AQPs, thereby enhancing their function. For instance, an adenoviralus vector with cDNA to increase AQP1 expression enhances saliva secretion in Wistar rats with hypofunctional salivary glands.

AQPs applied to imaging

Finally, AQP's water transport function is a property that can be appropriated for the use of anatomical imaging. Diffusion-weighted imaging (DWI) uses a pair of pulsed magnetic field gradients to relate nuclear spin to diffusion; this can relate to water, as molecules with the ability to diffuse more appear darker. In cells, this diffusion is related to the water's ability to move intracellularly and extracellularly, regulated by AQPs [223]. By tagging specific cells to overexpress AQP isoforms, DWI magnetic resonance imaging (MRI) is capable of tracking their dynamic behaviors *in vivo* in a noninvasive manner [224–226]. Aside from DWI analysis of MRI, other MRI methods have been used to identify functional AQP characteristics, like the tagging of ^{17}O water molecules analyzed by JJ vicinal coupling proton exchange MRI [227] or AQP4 glymphatic drainage following gadoteric acid through t1 mapping [212]. Fluorescent, positron emission tomography, and hyperspectral imaging can also be utilized to identify the functional properties of AQPs [228, 229].

In silico methods for studying AQPs

While *in vitro* and *in vivo* methods have provided us with a wealth of knowledge on AQP expression and function in biological systems, most of our understanding of AQP structure and function on a molecular level is due to *in silico* work. Computational models and simulations have the obvious benefits of saving time and laboratory resources, but they are also the most effective way to handle large amounts of data. Molecular dynamics (MD) and molecular docking, while computationally expensive, have revealed crucial information regarding AQP structures, the mechanism of water transport, AQP drug candidates and their putative binding sites, and PPIs. Stochastic and deterministic mathematical cell models have also begun to shed light on the impact of AQPs on cell activities.

AQP structure and water flux

Much of our current understanding of AQP structure stems from X-ray crystallography and electron microscopy, but the structures of AQP3 [43], AQP7 [230], AQP9 [231], AQP11, and AQP12 [232] have additionally been predicted using structural bioinformatics *in silico* technique called homology or comparative modeling. This involves building a 3D model of a protein from its amino acid sequence and is compared to the known structures of a similar protein. More recently, machine learning has also been implemented in sequence-based AQP prediction [233, 234]. However, most of our initial knowledge of AQPs and AQP-mediated water flux comes from MD, a computational method in which the movements of atoms and molecules are simulated according to Newton's equations of motion. Extensive MD simulations have been performed to elucidate AQP folding [9], proton exclusion from AQP pores [9, 235, 236], gating capabilities [237–239], ion conductivity [238, 240], voltage sensitivity [241, 242], lipid interactions [243, 244], permeability [28, 245–250], effects of mutations [251, 252], effects of phosphorylation [253, 254], and suppression of water flux by inhibitors [231, 255, 256]. For a more thorough review of MD simulations for the AQP study, we direct the reader to other reviews [257, 258]. Hand-in-hand with MD is

meta-dynamics, which estimates the free energy of a system and simulates rare events, particularly accelerating the sampling of protein conformations within an MD simulation [259]. Metadynamics has been used to simulate water, glycerol, and H_2O_2 transport through AQP3 [260, 261], and to study the effects of electric fields on AQP4 [262, 263].

AQP–molecular interactions

Once protein structures are defined, *in silico* methods can provide much quicker insight into how AQPs interact with other molecules compared to *in vitro* methods. Predicting AQP–ligand interactions allows for expedited drug screening; AQP–protein interactions shed light on AQP regulation and signaling activities; and AQP–lipid interactions elucidate key aspects of plasma membrane properties and how they affect AQP function.

AQP drug screening

Over time, the revelation that AQPs play key roles in several diseased cell behaviors has driven the search for AQP drugs that can block water transport, glycerol permeation, or ion conductance. *In vitro* methods for drug validation can be tedious and time-consuming. Molecular docking is a computational tool that has been widely used to expedite the screening of AQP inhibitors [30, 231, 264–267], predicting how small molecules will fit with a protein at the atomic level. In most cases, the protein and ligand are prepared and docked using automated docking software such as AutoDock. Once inhibitors are identified with molecular docking, binding modes may be further analyzed with MD simulations [256, 264].

Identification of AQP–protein and AQP–lipid interactions

Molecular docking can predict PPIs in addition to protein–ligand interactions and has been used to model binding between AQP5 and ezrin [146]. While it would be desirable to discover more AQP–protein binding partners using molecular docking, it remains a challenge to manage the heavy computational cost of docking two proteins while capturing their conformational dynamics. However, a simpler method exists for a broader detection of putative PPIs; predictions can be made based on consensus sites in amino acid sequences [268] that can be compared using software such as ProteinPrompt [269]. To predict AQP–lipid interactions, structural AQP data from crystallography can be used in MD simulations. For example, MD studies have investigated lipid organization around AQP0—an isoform whose structure has been solved from crystallography—to better understand membrane properties, protein mobility, and cholesterol-mediated tetrameric assembly [70, 71].

Computational and mathematical cell models

Another *in silico* approach to studying AQPs is to model them within cells. To our knowledge, few studies have developed computational models showing the interplay of AQPs and cell processes. One group ran simulations of 2D neural crest cell migratory streams involving cell speed and filopodia dynamics associated with an AQP1 overexpression or downregulation [270]. Another study built a multiscale compartmental model of AQP2 trafficking via the vesicular transport system in renal principal cells. Their numerical simulations incorporate membrane agents, filaments, vesicles, and a rule-based reaction system for the chemical entities [271]. Numerical methods have also been used to model AQP1 in ion and fluid transport across parotid duct cells [272], AQP4 in brain swelling in meningitis [273], and AQP4 supramolecular assembly into orthogonal arrays within cell membranes [274]. Future work modeling AQPs in intracellular signaling pathways would elucidate how AQPs can trigger downstream cascades affecting cell migration, proliferation, differentiation, etc.

Concluding remarks and outlook

In this review, we cover frequently used *in vitro*, *in vivo*, and *in silico* methods for studying AQP expression, localization, function, effects on cell behavior, and more. While these methods have earned us a great deal of basic knowledge of AQP structure and functions, they still have their limitations in their robustness, reproducibility, and physiological relevance. Hopefully, future method design will include more sensitive permeability assays, more reproducible inhibitor screening, further characterization of AQPs within *in vivo* systems, and wider use of computational modeling tools. Since much of AQP biology research is performed *in vitro*, there is also a need for more physiologically relevant models that help clarify the role of AQPs in both healthy and pathologic cell behaviors. Addressing these areas for improvement for current methods, exploring new uses of existing methods, and designing novel methods may well lead to the discovery of more effective AQP-targeting therapeutics and a greater understanding of the impact of AQPs in biological processes.

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Figure 1 was created using BioRender.com.

Disclaimers

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Authors' contributions

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Data availability

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