UNIVERSITAT ROVIRA i VIRGILI

UNIVERSITY OF GOTHENBURG

PUTATIVE PARTNERS FOR HUMAN AQUAPORIN 4 IN *SACCHAROMYCES CEREVISIAE*

Oriol Ruiz i Isant

FINAL DEGREE PROJECT BIOTECHNOLOGY

In cooperation with the Department of Biochemistry and Molecular Biology,
University of Gothenburg

Supervisors: Kristina Hedfalk and Jessica Glas

Academic Tutor: Ricardo Román Cordero Otero

Tarragona, June 2022

Jo, Oriol Ruiz i Isant , amb DNI 45721689N, sóc coneixedor de la guia de prevenció del plagi a la URV *Prevenció, detecció i tractament del plagi en la docència: guia per a estudiants* (aprovada el juliol 2017) (http://www.urv.cat/ca/vidacampus/serveis/crai/que-us-oferim/formacio-competencies-nuclears/plagi/) i afirmo que aquet TFG no constitueixen cap de les conductes considerades com a plagi per la URV.

Tarragona, 1 de juny de 2022

Acknowledgements

I would like to begin this project by expressing my gratitude to everyone who has helped in its realisation, as well as to each and every person who has encouraged me to be where I am today.

Firstly, I would like to thank Prof. Kristina Hedfalk for giving me the opportunity to join their team during the second semester of this year. This internship has allowed me to obtain the results used in this project but has also allowed me to obtain experience when it comes to working in a laboratory. I would also like to thank her for always being helpful, especially in the last few months. None of this would have been possible had she not given me the opportunity at the beginning of this school year.

My sincere gratitude to Jessica Glas for her constant helpfulness in the laboratory as well as in all the doubts that I could have had. Had it not been for her, the results in this project would not have been obtained. I would also like to thank her for always being attentive and guiding me in how a laboratory works.

I would also like to thank Prof. Ricardo Román Cordero Otero for helping me in the writing of this project. All his corrections have allowed me to improve this manuscript and, had it not been for him, this project would not be where it is now.

My sincere gratitude also to Raquel Delgado Gil. She has been working on the same project and with her I have shared most of the internship. This project would have been harder without you.

Last, a special thanks to my partner, Ruben, for providing his help every time it was needed and for his involvement in this project.

Index

Lundberg Laboratory

This project was written thanks to the data obtained from the internship performed at Lundberg Laboratory, which hosts part of the Department of Chemistry and Molecular Biology at the Göteborg University. More specifically, I was under the supervision of Kristina Hedfalk (PI) and Jessica Glas (PhD student).

Lundberg Laboratory, with the address Medicinaregatan 9C, 413 90 Göteborg, is a major centre for cellular and molecular research. In the case of the group where the internship was performed, the primary research focus is on integral membrane proteins, more specifically on aquaporins: structure, function, and regulation.

Summary

Aquaporin 4 (AQP4) is the most abundant aquaporin in the brain. Loss of AQP4 polarisation in the end-feet of astrocytes has been associated with many pathologies. It is believed that mislocalisation of AQP4 may happen due to protein-protein interactions. Therefore, this project focuses on screening a cDNA library from human brain mRNA to find new possible interaction partners of AQP4. To achieve this, bimolecular fluorescence complementation and fluorescence-activated cell sorting are used to achieve a higher-throughput screening. Two combinations have been tested, N-terminally tagged AQP4 or C-terminally tagged AQP4, combined with C-terminally tagged cDNA library. However, no positive hits have been found up to this date.

Key words

Aquaporin 4 (AQP4), Bimolecular Fluorescence Complementation (BiFC), Fluorescence-Activated Cell Sorting (FACS), protein-protein interactions.

1. Introduction

1.1. Aquaporins (AQPs)

Aquaporins (AQPs) are transmembrane proteins that have been found in all living organisms, from bacteria to humans (Azad et al., 2021; Clément et al., 2020). The function of AQPs is to mediate the passive transport of water through osmotic gradients, all the while excluding the passage of charged molecules (Li, C. et al., 2017; Li, S. et al., 2020). AQPs are essential to maintaining water homeostasis. Water diffusion is an essential mechanism in all living organisms (Abir-Awan et al., 2019; Clément et al., 2020). AQPs not only transport water molecules but can also mediate the transportation of small, uncharged molecules (e.g. glycerol, urea) through concentration gradients (Abir-Awan et al., 2019; Li, C. et al., 2017). Up to this day, thirteen isoforms of aquaporins (AQP0-AQP12) have been identified in humans (Li, C. et al., 2017; Li, S. et al., 2020).

Despite many possible classifications, AQPs are divided into three subfamilies: [1] classical or orthodox AQPs (AQP1, AQP2, AQP4, AQP5, AQP6, AQP8), which are water-selective, although some can also transport urea and other solutes; [2] aquaglyceroporins (AQP3, AQP6, AQP7, AQP9, AQP10), which can also transport small, uncharged molecules such as glycerol; [3] and super-AQPs, subcellular or unorthodox AQPs (AQP11, AQP12), which have low homology with other AQPs (Azad et al., 2021; Clément et al., 2020; Li, S. et al., 2020; Magouliotis et al., 2020). The exact physiological role of both AQP11 and AQP12 is yet to be fully understood, as well as their function in the control of water permeability (Ahmed et al., 2021). However, they are believed to be involved in both the regulation of water transport inside the cell and in the control of organelle volume due to their cytoplasmatic location (Magouliotis et al., 2020).

There exist two water flow routes between tissues in the human body: transcellular water flow, which AQPs mediate, and paracellular flow. The fact that AQPs allow rapid water transport due to the osmotic difference between membranes makes them a key element in regulating transcellular water flow (Geng et al., 2017).

1.1.1. Structure and mechanism of action of aquaporins

Even though thirteen isoforms of AQPs have been identified in humans (Li, C. et al., 2017; Li, S. et al., 2020), they all share standard structural features and their primary sequence is 30-50% identical to other classes (Azad et al., 2021; Li, C. et al., 2017). Aquaporins have a tetrameric hourglass-like structure (Ahmed et al., 2021). The

functional unit of aquaporins is a homotetramer made from identical monomers of approximately 30-kDa (Clément et al., 2020; Li, C. et al., 2017; Li, S. et al., 2020). In addition, in the centre of the homotetramer, a fifth pore is generated due to the interactions between monomers. This pore is primarily hydrophobic and has been suggested to conduct gasses (Azad et al., 2021; Roche et al., 2017). Monomers can vary in size depending on the AQP type, ranging between 26 and 34kDa in mammalians (Ahmed et al., 2021). Each monomer independently functions as a water channel and consists of six α-helix transmembrane domains (1–6). Furthermore, monomers consist of five loops (A–E), three of them extracellular (A, C, E) and two intracellular (B, D) (Li, C. et al., 2017; Li, S. et al., 2020). Both loops B and E contain the classical asparagine-proline-alanine (NPA) motif, which can be found in all AQPs (Ahmed et al., 2021; Azad et al., 2021; Magouliotis et al., 2020). Both NPA motifs have been found to partake in proton exclusion (Azad et al., 2021). Finally, both hydrophilic N and C-termini are located in the cytoplasm (Li, C. et al., 2017; Li, S. et al., 2020).

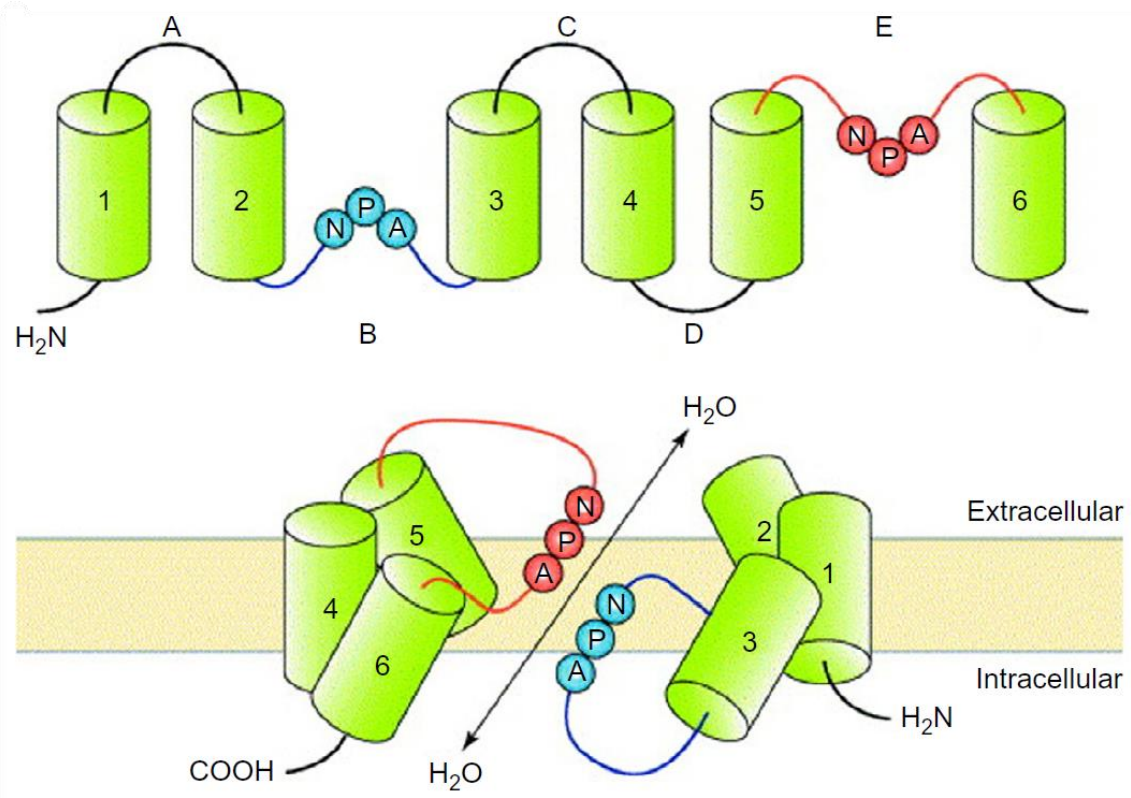

Figure 1. General molecular structure of aquaporins. Extracted from Li S. et al (Li, S. et al., 2020).

The six α-helixes surround and form the aqueous pore in each monomer, allowing water transport at different permeability rates (Geng et al., 2017; Li, C. et al., 2017; Magouliotis et al., 2020). In the case of classical AQPs, the constriction site, made from an aromatic residue plus an arginine at the extracellular side of the channel, has a diameter of 2.8-3.0 Å. Thus, only water molecules are allowed through (Azad et al., 2021; Li, C. et al., 2017). Water molecules are forced to pass one at a time and orientate themselves inside the electrical field found in the channel. On the other hand, aquaglyceroporins have a much larger selectivity filter in the constriction site. In consequence, aquaglyceroporins allow bigger molecules to pass through the channel (Li, C. et al., 2017).

Despite some AQPs allowing larger molecules through the pore, protons are repelled when trying to cross the pore. This is explained through two processes: [1] there are two positive charges (one in the side chain in loop E and a partial one in a conserved histidine residue) which repel protons; and [2] water molecules can establish hydrogen bonds which cause a transient dipole reorientation. This last process also generates a barrier against proton transport across AQPs (Geng et al., 2017).

1.1.2. Regulation of aquaporins

Aquaporins can be modulated through posttranslational modifications (PTMs) such as phosphorylation. They can modulate the localisation, activity, interaction partners, and stability of AQPs (Li, C. et al., 2017; Li, S. et al., 2020).

Water transport can be mediated through two different processes: gating, a conformational change, or trafficking, alteration of the density of aquaporins in a particular membrane (Li, C. et al., 2017; Magouliotis et al., 2020; Roche et al., 2017). The latter has more levels of regulation, including transcriptional and translational regulation. It also involves the use of shuttles between the target membrane and storage vesicles found inside the cell. PTMs are thus crucial in this process, helping with the redistribution of AQPs (Li, C. et al., 2017). Finally, another long-term regulation involves the AQP abundance in the cell, regulated by systemic hormones, local molecules, and other signals such as pH (Li, C. et al., 2017; Magouliotis et al., 2020).

Even though it can happen in other parts of the protein, in many cases, both posttranslational modifications and protein-protein interactions happen in the C-terminus of the aquaporin. Particularly, it is typical for the C-terminus of most AQPs to harbour phosphorylation sites, which further modulate protein-protein interactions (Roche et al., 2017).

1.1.3. Health implications of aquaporins

Since AQPs allow the transport of water and other small, uncharged solutes (glycerol, CO_2, etc.), they have an essential physiological role (Ahmed et al., 2021; Azad et al., 2021). Therefore, if mutations or dysregulations happen, it is highly likely that they will lead to pathophysiological phenotypes. Pathophysiological phenotypes have been associated with severe diseases, that can be non-infectious or infectious (Azad et al., 2021).

Functionally impaired AQPs are involved in many non-infectious diseases, such as cancer, neurological disorders, cardiac diseases, and others. AQPs have a highly possible implication in regulating the cell volume, and therefore they have become potential drug targets. If cell volume regulation fails, it can result in imbalanced homeostasis and in an important alteration of cell physiology. Since cells alter their volume and shape using water, it has been seen that AQPs might be crucial in controlling homeostasis in infectious diseases (Azad et al., 2021).

1.2. Aquaporin 4 (AQP4)

Several isoforms of aquaporins (AQP1, 3, 4, 5, 7, 8, 9, and 11) exist in the central nervous system. However, AQP1, AQP4, and AQP9 have a higher expression level in the brain (Azad et al., 2021; Mader et al., 2019).

Like all other aquaporins, aquaporin 4 (AQP4) consists of a tetrameric structure and is highly similar to AQP1 (Ahmed et al., 2021). AQP4 is predominantly located in the central nervous system (CNS), highly expressed in glial cells, and allows the transport of both water and CO_2 (Li, C. et al., 2017; Li, S. et al., 2020). AQP4 is found at the highest concentration in the end-feet of astrocytes, with a perivascular distribution. Such astrocytes, along with blood vessels, form the blood-brain barrier (BBB) (Abir-Awan et al., 2019; Azad et al., 2021; Clément et al., 2020). However, AQP4 has also been found in ependymal cells and osmosensory areas of the hypothalamus (Azad et al., 2021). Consequently, AQP4 is an important site of action for many drugs, especially those

treating bipolar disorders, cerebral oedema, and media temporal lobe epilepsy (Ahmed et al., 2021). This is why AQP4 has been the most studied aquaporin in the CNS ever since its discovery (Clément et al., 2020). The gene that codifies for AQP4 is located on chromosome 18q and has an open reading frame of 301 amino acids with a 94% identity to rat AQP4. The AQP4 gene consists of 4 exons encoding 127, 55, 27, and 92 amino acids, separated by introns of 0.8, 0.3, and 5.2 kb (Li, C. et al., 2017).

There exist two isoforms of AQP4: 'M1'-AQP4, which has the initiation of translation at M1, being the longer isoform (323 amino acids); and 'M23'-AQP4, which starts translation at M23, being the shorter isoform (301 amino acids) (Mader et al., 2019; Verkman et al., 2017). Both isoforms are generated through alternative splicing. Furthermore, AQP4 tetramers can be either homotetramers (all M1 or all M23) or heterotetramers (combining the two isoforms) (Verkman et al., 2017).

Localisation of AQP4 depends on two C-terminus motifs: a dileucine-like motif and a tyrosine motif. However, aquaporin 4 can be relocated to the apical membrane instead of the basolateral membrane, where it usually relies on, due to mutations in any of the two existing motifs (Azad et al., 2021).

Compared to other aquaporins, AQP4 appears to have the highest water permeability. This consideration is supported by AQP4's location in the CNS, which requires tight control of water localisation to maintain optimal conditions (Abir-Awan et al., 2019). Many different processes, including phosphorylation as a key one, regulate AQP4 water permeability (approximately $24 \cdot 10\text{-}14$ cm^3/s) (Geng et al., 2017; Li, C. et al., 2017). AQP4 has several potential phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), phosphoglycerate kinase (PKG), casein kinase (CK), and calcium/calmodulin-dependent protein kinases (CaMK). Phosphorylation of Ser111 and Ser180 indicate water transport by gating, whereas phosphorylation of the C-terminus by PKC is essential for Golgi transition (Li, C. et al., 2017). Even though mercury can inhibit most mammalian AQPs' functions, AQP4 is not inhibited by $HgCl_2$ (Geng et al., 2017; Magouliotis et al., 2020; Verkman et al., 2017). In consequence, when first discovered, it was named mercury-insensitive water channel (MIWC) (Mader et al., 2019).

1.2.1. Health implications of AQP4 in the central nervous system (CNS)

Control of water levels in the central nervous system (CNS) is of great importance since 80% of the brain's weight is water. Cerebral oedema, found in CNS disorders, can eventually lead to neurologic dysfunction or even death caused by the progressive accumulation due to high water content. Although many aquaporins are present in the brain, the focus is on AQP1, AQP4, and AQP9. Our primary concern is on AQP4, exclusively expressed by astrocytes but with a high abundance in all brain structures (Magouliotis et al., 2020).

As stated before, AQP4 is mainly found at the end-feet of astrocytes. However, there can be a loss of polarity if AQP4 is mislocalised or broadly distributed in all of the astrocyte's membrane (Mader et al., 2019).

Many studies have proven AQP4 implication in different diseases. For example, AQP4 is important in cognition and memory in a study with AQP4-deficient mice. Furthermore, and even though other studies could not replicate the same results, a study by *Zhou J et al.* showed that AQP4-deficient mice have an impaired blood-brain barrier (BBB). Finally, AQP4-deficient mice also showed more pronounced phenotypes in certain injuries (Mader et al., 2019).

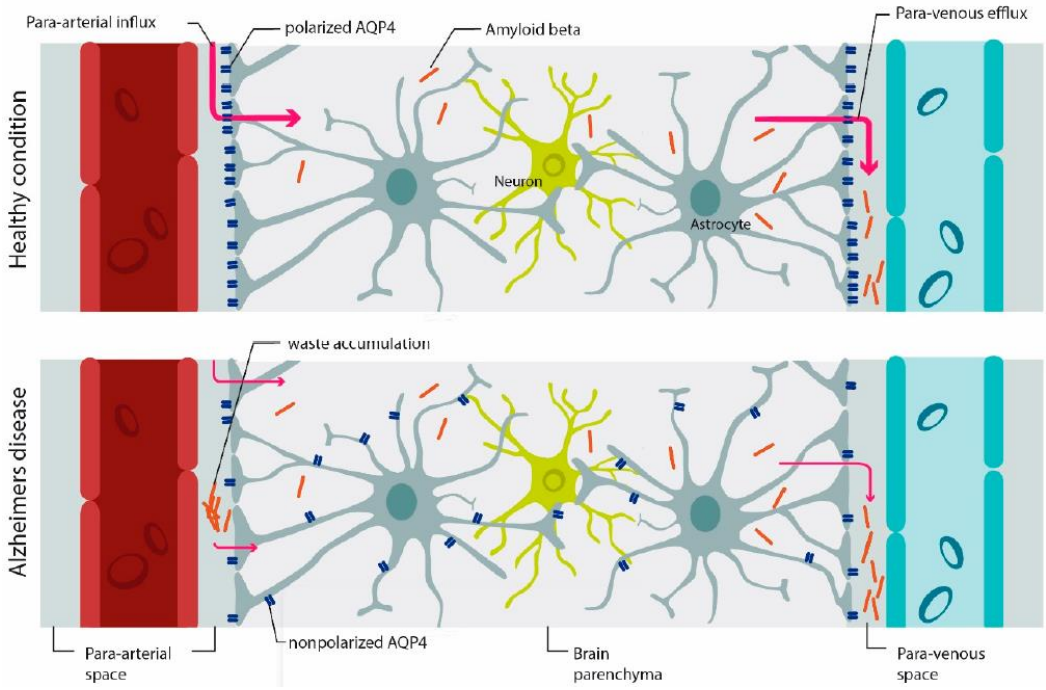

Figure 2. Differences in AQP4 location in the healthy brain versus in pathological conditions such as Alzheimer's disease. In the first case, AQP4 has polarised expression, mainly expressed on the astrocyte end-feet; in the second case, AQP4 polarisation is reduced, being involved in different diseases. Image extracted from Mader S. et al. (Mader et al., 2019).

In cerebral oedema and ischemia, expression of AQP4 is increased, correlated with the release of inflammatory cytokines (Abir-Awan et al., 2019). During trauma or ischemia, failure of Na^+, K^+, and Cl^- pumps happens after a state of hypoxia, and this ends up causing dysregulation of the osmolality (Abir-Awan et al., 2019; Ahmed et al., 2021). Afterwards, this will result in an increased intracranial pressure (ICP) and cerebral oedema caused by water flows into astrocytes (Abir-Awan et al., 2019). However, *Ahmed T et al.* described that AQP4 deficiency could also play a role in brain oedema (Ahmed et al., 2021).

Up to date, there is still a lack of therapeutic agents able to inhibit or enhance water transport through the regulation of aquaporins, and there are only limited options in other cases (Abir-Awan et al., 2019; Magouliotis et al., 2020). In cytotoxic oedema models, AQP4 KO mice were protected, leading us to believe that AQP4 inhibition might be beneficial in such cases (Abir-Awan et al., 2019; Roche et al., 2017). In vasogenic oedema, however, AQP4 KO mice had worse outcomes since they could not remove excess water from the brain tissue. This leads us to the conclusion that any inhibition of the function of aquaporin 4 needs to be acute (Abir-Awan et al., 2019).

1.3. Bimolecular Fluorescence Complementation (BiFC) as a tool to study protein-protein interactions of aquaporin 4

Protein-protein interactions (PPIs) are fundamental for the cell, participating in many different functions, including signalling cascades, metabolism, physical motion, trafficking, posttranslational modifications, energy transduction, and environmental communication (Roche et al., 2017; Schmitz et al., 2021; Sjöhamn et al., 2016). Should such interaction be disrupted, autoimmune diseases or cancer, among others, could appear (Schmitz et al., 2021). It is estimated that, in humans, there can be up to 650.000 different protein integrations, which are essential in the transmission of biological information (Ventura, 2012).

Aquaporin 4 is a membrane protein, and most methods to study PPIs have been developed for soluble proteins (Schmitz et al., 2021; Sjöhamn et al., 2016). Despite possible adaptation of most methods in membrane proteins, a substantial downside is a tendency to overinterpret results, deriving in false positives (Sjöhamn et al., 2016).

Bimolecular Fluorescence Complementation (BiFC) is a protein-fragment complementation assay (PCA) (Ventura, 2012). In this method, a fluorescent protein such as YFP is divided into two fragments (154 and 85 residues) and further fused to the two proteins of interest. After production in a suitable host, if the two target proteins do not interact, the YFP fragments will not assemble, and there will be no fluorescence. On the other hand, should both proteins interact, the YFP fragments would assemble and mature in vivo, giving rise to a fluorescent signal (Kojima et al., 2011; Sjöhamn et al., 2016). Using the YFP proteins also has two essential benefits: [1] it allows for weaker PPIs to last longer since the assembly of both fragments is almost irreversible as well as insensitive to other interaction competitors; and [2] it provides fluorescence, a convenient signal to detect, which increases sensitivity and allows cell localisation of the signal using confocal microscopy (Hua et al., 2018; Sjöhamn et al., 2016; Ventura, 2012; Weber-Boyvat et al., 2015). Furthermore, the target proteins are not required to be found in the nucleus and do not require any exogenous substrate that could damage the organism or perturb homeostasis, giving it advantages over other methods (Kojima et al., 2011; Ventura, 2012).

Consequently, BiFC can be used to study membrane proteins, allowing efficient in vivo and high-throughput screening. The obtained data can be analysed to differentiate between random interactions and proper-formed complexes, and the protein does not need to be purified to be analysed (Schmitz et al., 2021). However, some critical aspects of BiFC are the distance and orientation of the complex, which can result in interaction impairment. Furthermore, the length of the linker is also critical, which affects how likely it is for the YFP fragments to mature into a complex, since they are not sterically equivalent and can have steric clashes (Schmitz et al., 2021; Ventura, 2012). Another drawback of this method is that dynamic studies cannot be performed due to the stabilisation caused by both fragments coming in contact (Weber-Boyvat et al., 2015). Finally, even though not likely, the two YFP fragments may interact with each other through random collisions, although the two proteins of study do not interact. If this happens, the fluorescence obtained from this random interaction will also contribute to the total detected fluorescence(Kodama et al., 2012).

Therefore, it is essential to add a negative control that will allow the determination of the contribution of the non-specific fluorescence (Kodama et al., 2012). The most valid negative control are non-constructive, pair-specific mutated proteins that prevent the YFP fragments' interaction (Kodama et al., 2012; Schmitz et al., 2021).

Bimolecular Fluorescence Complementation (BiFC) has been shown to function in many types of cells, from bacteria to mammals and including yeast (Morell et al., 2008; Weber-Boyvat et al., 2015). *Kojima T. et al.* demonstrated that this methodology could be applied to detect protein-protein interactions in yeast cells, even if they were artificial or human proteins (Kojima et al., 2011). In this case, the best system to produce both proteins of interest was *Saccharomyces cerevisiae*. This organism allows for visualizing the targets and further purifying while obtaining an acceptable yield at the end of the process (Sjöhamn et al., 2016). Furthermore, other studies, such as that made by *Bjørkskov FB. et al.*, have demonstrated that *S. cerevisiae* is an optimal system to express human aquaporins with high membrane density (Bjørkskov et al., 2017). Finally, *S. cerevisiae* allows the whole process to be performed in the same organism (Sjöhamn et al., 2016).

1.4. Flow cytometry (FC) and Fluorescence-Activated Cell Sorting (FACS)

Flow cytometry (FC) allows the rapid analysis and classification of multiple cells or particles as they go through one or many lasers in a single-manner while being suspended in a salt-based solution (Büscher, 2019a, 2019b). FC has many different applications, including immunology, virology, and molecular biology, among all others. Furthermore, a significant application is the sorting of individual cells with the desired properties (Büscher, 2019a).

Analysis of cells is based on multiple parameters, including: [1] geometric properties, which refer to the size of the particle (diameter, volume…); [2] physiological properties, related to membrane potential or vitality; and [3] quantities of molecules such as DNA, RNA, proteins, enzymes, amongst others (Büscher, 2019b). The flow cytometer will identify what is known as "events", meaning that the instrument has identified a single particle, whether that is done rightly or wrongly (Givan, 2011).

A flow cytometer consists of three essential parts: fluidics, optics, and electronics. Firstly, the fluidics system consists of a "sheath" fluid or hydraulic system which focuses the sample into the interrogation point, a minimal volume, where lasers are aimed.

Secondly, the optical system consists of excitation and collection optics (lasers and photomultiplier tubes or PTMs, respectively), which generate the light signals that are then analysed. Furthermore, there is also a system of dichroic filters which allow each fluorochrome to be analysed individually. Finally, the electronic system converts the instrument's response into digital signals, which are then read by a computer (Büscher, 2019a).

At the interrogation point, particles are analysed for visible light scatter as well as for one (or more) fluorescence parameters. For the first parameter, analysis is performed in two different directions. The first one, also known as Forward Scatter (FSC), goes in the forward direction and indicates the relative size of the cell; the second one, also known as Side Scatter (SSC), is measured at 90º or orthogonally to excitation, and gives information about the complexity of the cell's interior (Büscher, 2019a; Givan, 2011). The second parameter, fluorescence, is also analysed at 90º or orthogonally and is independent of the light scatter (Büscher, 2019a). To analyse signals, there are two types of lenses: in the same direction as the laser (to analyse FSC) and orthogonal to the laser (to analyse SSC, as well as fluorescence with wavelength-specific filters) (Givan, 2011). In order to analyse a cell or a particle with fluorescence light, it needs to be modified to express fluorescent proteins or labelled with fluorescently tagged antibodies (Büscher, 2019a, 2019b). The most commonly used fluorescent protein is green fluorescent protein (GFP), which was cloned to obtain yellow fluorescent protein (YFP) (Büscher, 2019a). When analysing such signals, the fluorescence intensity is related to the abundance of the studied molecule (Givan, 2011).

Certain flux cytometers, also known as cell sorters, have been optimised to collect individual samples for further analysis. This optimisation follows the incorporation of the cell sorting mechanism. In this case, the user can select/gate on a sample for the desired parameters to be separated from all the rest. This separation is performed first by generating drops which contain individual cells through a high-frequency oscillation of the stream. Then, drops are given a positive or a negative charge and are then passed through metal deflection plates where they deviate into a specific collector (Büscher, 2019a, 2019b). Specifically, Fluorescence-Activated Cell Sorting (FACS) can separate individual cells from the rest of the population based on fluorescent signals from the optics system in the flow cytometer.

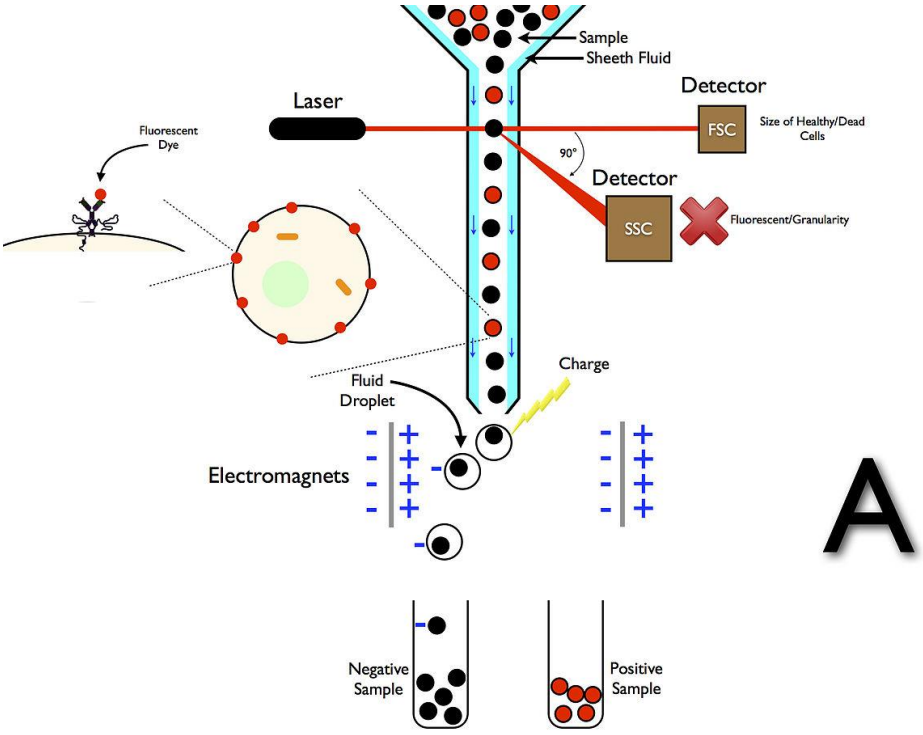

Figure 3. Fluorescence-Activated Cell Sorting (FACS) mechanism. Image extracted from Wikimedia Commons. Retrieved May 17, 2022, from https://commons.wikimedia.org/wiki/File:Fluorescence_Assisted_Cell_Sorting_(FACS)_A.jpg

1.4.1. Applying BiFC in Fluorescence-Activated Cell Sorting (FACS)

Initially, Bimolecular Fluorescence Complementation was quantified through fluorescence microscopy, which was highly time-consuming. However, the introduction of flow cytometry allowed for further benefits such as a higher-throughput screening or the possibility of sorting out cells of particular interest (Hua et al., 2018; Morell et al., 2008; Schmitz et al., 2021; Sjöhamn et al., 2016; Ventura, 2012).

BiFC-FACS coupling has been previously used to validate protein interactions by analysing the intensity and frequency of fluorescence compared to the corresponding positive and negative controls (Morell et al., 2008).

In comparison to other techniques, BiFC-FACS offers different advantages. In the case of FRET, where a fundamental problem is background fluorescence, this problem is avoided because fluorescence should not be happening unless interacting partners occur. Furthermore, when compared to the yeast two-hybrid assay, BiFC-FACS offers the advantage of not needing to transport the target proteins into the nucleus or other cellular compartments (Morell et al., 2008).

Not everything is benefits, and some challenges of such a method can be unspecific background fluorescence, also known as autofluorescence, present in many particles naturally (Givan, 2011; Schmitz et al., 2021). This can make it challenging to differentiate it from actual molecular interactions, which can lead to false positives (Schmitz et al., 2021).

1.3.2. Calmodulin as a known interaction partner of aquaporin 4

Calmodulin (CaM) is a eukaryotic protein whose primary function is to act as a receptor of the intracellular Ca^{2+} concentration, with a molecular weight of around 17kDa (Ishida et al., 2022). After calmodulin has bound calcium ions, the following conformational changes allow it to interact with other proteins, as is the case of aquaporin 4 (Ishida et al., 2022; Kitchen et al., 2020).

CaM has been associated with binding to membrane-spanning channels, and aquaporins have calmodulin-binding domains (CBD). Up to date, only AQP0, AQP4, and AQP6 have been proven to interact with calmodulin. In the case of AQP4, recent reports support that the binding of calmodulin happens in response to CNS changes, leading to cytotoxic oedema. Furthermore, it has been proven that AQP4 has CBDs at both its cytosolic N- and C-termini (Ishida et al., 2022). In the case of the C-terminus, a putative binding site for calmodulin has been identified between residues 256 and 275 (Kitchen et al., 2020).

In a study made by *Kitchen P. et al.*, they showed that the interaction of both proteins causes a conformational change in the aquaporin, localising it on the cell surface. Therefore, there is a CaM-dependence on the localisation of the aquaporin 4 in the cell (Kitchen et al., 2020).

2. Hypothesis and Objectives

Aquaporin 4 has many implications for the central nervous system's health, especially if depolarised. For the depolarisation of aquaporin 4 to happen, the implication of regulatory proteins might be essential. Therefore, to better understand how such a process happens, we hypothesised that there might be, at least, one interaction partner of AQP4 in the human brain.

To screen for interaction partners in the brain, Bimolecular Fluorescence Complementation (BiFC) along with Fluorescence-Activated Cell Sorting (FACS) was used. Interactions were studied on *Saccharomyces cerevisiae* cells transformed with two plasmids: one including the human AQP4 gene and another including a fragment of a self-made cDNA library obtained from a human brain mRNA sample.

In consequence, the main objective of this project is to screen a cDNA library to try to find an interaction partner of human aquaporin 4 in the central nervous system.

The secondary objectives of this project are:

- To establish the best transformation efficiency in *S. cerevisiae* cells.
- To establish the best conditions for FACS analysis.

3. Materials and Methods

3.1. Plasmid design and studied combinations

To apply the BiFC methodology, the YFP protein was split into two non-fluorescent fragments: Y_N and Y_C. Gateway cloning was used to introduce both the YFP fragment and the gene of interest into the plasmids used in this project. Previously to the start of this project, the Y_N fragment was N-terminally fused to the human AQP4 gene in one plasmid, and it was also C-terminally fused to the human AQP4 gene in another plasmid. Furthermore, the Y_C fragment was fused to the C-terminus of the cDNA library in a third plasmid. For the control samples, the Y_N fragment of the YFP was fused to the N-terminus of both AQP0 and AQP0ΔC; then, the Y_C fragment of the YFP was fused to the N-terminus of CaM. In the case of the plasmids containing the AQP4 gene, the AQP0 gene, and the AQP0ΔC gene, a uracil marker was introduced; on the contrary, in the plasmids containing the cDNA library and the calmodulin, a histidine marker was introduced.

In this project, two combinations were evaluated: [1] N-terminally tagged AQP4 with C-terminally tagged cDNA library (Y_N-AQP4 + cDNA-Y_C); and [2] C-terminally tagged AQP4 with C-terminally tagged cDNA library (AQP4-Y_N + cDNA-Y_C). Furthermore, three controls were used: [1] N-terminally tagged AQP0 with N-terminally tagged calmodulin (Y_N-AQP0 + Y_C-CaM) as a positive control; [2] N-terminally tagged AQP0ΔC with N-terminally tagged calmodulin (Y_N-AQP0ΔC + Y_C-CaM) as a negative control – truncating the C-terminus of the AQP0 hinders its interaction with CaM); and [3] N-terminally tagged AQP4 with N-terminally tagged calmodulin (Y_N-AQP4 + Y_C-CaM) as a positive control.

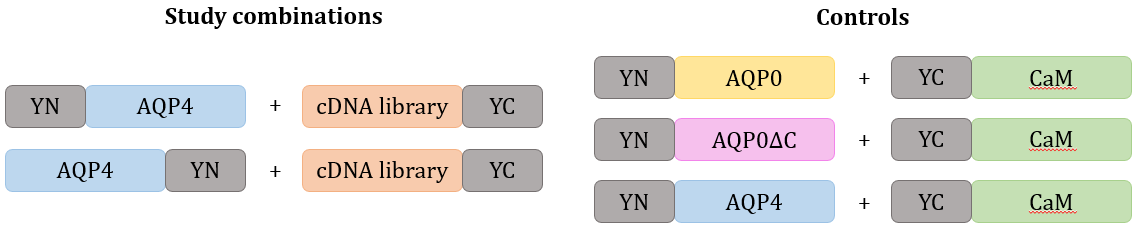

Figure 4. Schematic structures of the combinations studied in the project (left) as well as of the controls (right). Top to bottom, study combinations involve N-terminally tagged aquaporin and C-terminally tagged aquaporin, both with C-terminally tagged cDNA library; on the other hand, controls involve N-terminally tagged AQP0, AQP0ΔC, and AQP4, all with N-terminally tagged calmodulin.

3.2. *Saccharomyces cerevisiae* transformation

Saccharomyces cerevisiae cells were chemically transformed using LiAc. The first step was to prepare overnight cultures of 3-5mL YPD media with the used cell strain. The following morning, the first step was to inoculate 50mL of YPD media with an OD_{600} = 0.25, letting it grow until OD_{600} = 0.7-1.0. Once the desired OD was reached, cells were centrifuged at 3000 rpm for 5 min, resuspending the pellet in 25mL of sterile water. Afterwards, cells were again centrifuged at 3000 rpm for 5 min, resuspending the pellet in 1mL of 100mM LiAc. Cells were then centrifuged at 15000 rpm for 15 seconds with the subsequent removal of the supernatant. Cells were then resuspended in 400µL 100mM LiAc, obtaining an approximate concentration of $2 \cdot 10^9$ cells/mL. Then, 50µL of cell suspensions were pipetted into sterile Eppendorf tubes, followed by centrifugation at 6000 rpm for 15 seconds. The supernatant was discarded, and in the following order, we added 240µL PEG4000 (50% w/v), 36µL 1M LiAc, 5µL of 10mg/mL freshly denatured salmon sperm DNA, and 70µL of plasmid DNA (0.1-10µg). The pellet was resuspended, followed by a 30-minute incubation at 30ºC, then heat shock in a water bath at 42ºC for 25 minutes. Finally, cells were spun down at 8000 rpm for 15s and resuspended in 200-400µL of sterile water to be further plated on -HIS -URA plates. Such media would allow us to distinguish colonies transformed with both plasmids, as each of them allows the synthesis of one of the two molecules.

3.3. Fluorescence microscopy

To confirm intracellular BiFC complexes in control samples, fluorescence microscopy was used on the cell cultures. Overnight cultures were diluted to 2mL with an OD_{600} = 0.2 in 50mL Falcon tubes and incubated at 30ºC until they reached an OD_{600} = 0.5. Afterwards, cells were centrifuged at 3000g for 5min and resuspended in 10-20µL H_2O.

The images were obtained at an inverted Zeiss Axio Observer. The objective used was a Plan-Apochromat 100x/1.40 oil DIC M27. Excitation of the YFP protein was performed at 508nm, and the emitted fluorescence light was captured at 524nm. The collection and processing of the data were performed using the Zeiss Zen Blue software (Schmitz et al., 2021). Finally, image merging was performed using the ImageJ software.

3.4. FACS analysis

FACS analysis was performed twice to first check for the intensity of the signal and later check for the signal's frequency. Freshly transformed colonies were combined in -HIS -URA medium and grown overnight. The following morning, the sample was diluted into an OD_{600} = 0.2 and taken to an OD_{600} = 0.5 to be analysed. The first analysis was performed to check for the intensity of the signal. This analysis allowed us to sort out individual cells showing high-intensity signals, which were then regrown in 500µL of -HIS -URA medium for 5-6 days. After such, each sample was centrifuged at 8.000g for three and a half minutes, and the pellet was plated into -HIS -URA plates. The following day, each colony was inoculated into -HIS -URA medium and left to grow overnight. Afterwards, each sample was diluted into an OD_{600} = 0.2 and taken to an OD_{600} = 0.5 to be analysed, obtaining the frequency of the YFP signal.

In the case of the controls used in this experiment (AQP0ΔC + CaM as a negative control and AQP0 + CaM and AQP4 + CaM as positive controls), freshly transformed individual colonies were used to inoculate 2mL of -HIS -URA medium and left to grow overnight. The following morning, each sample was taken into an OD_{600} = 0.2 and taken to an OD_{600} = 0.5. Afterwards, each sample was introduced into the FACS and analysed to obtain the percentage of cells showing a YFP signal and the median value of the YFP signal.

3.5. Processing of positive hits

Cells that showed intensity and frequency values above the negative control threshold were preserved in optimal conditions. A glycerol stock was prepared from the overnight culture of the single colony (500µL of culture + 500µL of 50% glycerol solution) and kept at -80ºC until further analysis.

To analyse the cDNA fragment responsible for the positive hit, the first step would be to eliminate the unwanted plasmid, containing the human AQP4 gene, from *S. cerevisiae*. This would be done by plating the culture in -URA medium, so that cells would not need the histidine plasmid and kick it out of the cell independently. Furthermore, the target plasmid would be isolated from *S. cerevisiae* cells following the *Easy Yeast Plasmid Isolation Kit* from *Takara*, and further transformed into *E. coli* cells following heat-shock transformation. Finally, a plasmid prep would be performed to extract the plasmid from the *E. coli* culture, and it would be sent for sequencing.

3.6. Statistical analysis

The statistical analysis was performed using the SPSS software, and One-Way ANOVAs were performed to evaluate the significance of the results. For the One-Way ANOVA, equal variances were assumed (LSD). Results were considered to be statistically significant if $p < 0.05$.

4. Results

4.1. Establishment of controls

4.1.1. Fluorescence microscopy

The formation of intracellular BiFC complex in the controls was first evaluated using fluorescence microscopy. In Figure 5, the results of the three controls can be observed. In the first row, AQP0 + CAM, it is clear that both the intensity and the frequency of the YFP signal were high. Furthermore, the fluorescence was predominantly on the membrane, confirming the BiFC complex presence. The second row accounts for the negative control results (AQP0ΔC + CaM), showing little to no fluorescence whatsoever. Moreover, the fluorescence was distributed amongst the cell, indicating that it might be autofluorescence and not the actual YFP signal. Finally, the third row indicates the results for the second positive control, AQP4 + CaM, which showed high intensity and frequency of YFP signal, as well as a good localisation inside of the cell.

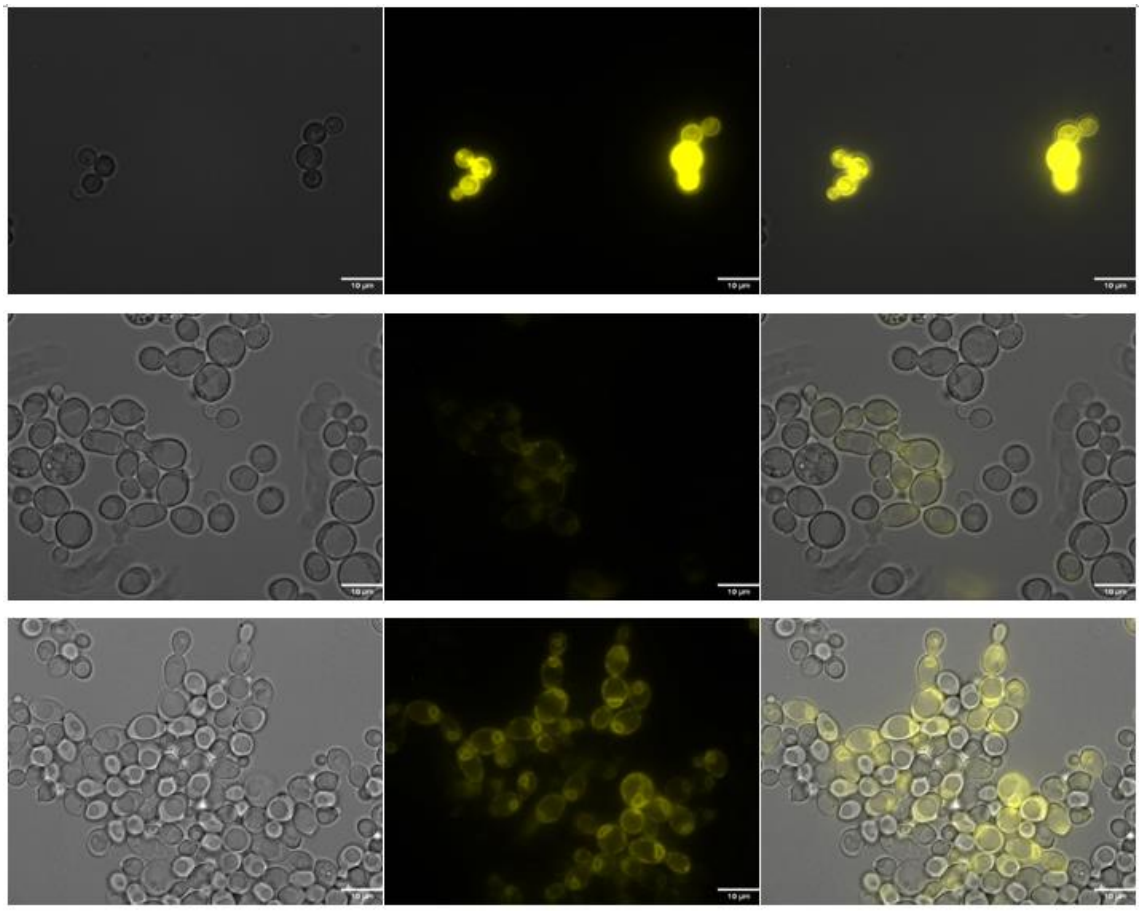

Figure 5. Brightfield microscopy, fluorescence microscopy, and merged pictures of AQP0 + CaM, AQP0ΔC + CaM, and AQP4 + CaM, respectively.

4.1.2. FACS analysis

The second step was to establish the intensity and frequency values of the control samples. This was performed to obtain the threshold values that would help us distinguish positive hits. Analysis of the three controls (AQP0 + CaM, AQP0ΔC + CaM, and AQP4 + CaM) through the FACS was done, involving 3 transformation events (N = 3) with, at least, 10 transformants in every event (n = 10) for each of the controls.

Plotted values for the intensity of the YFP signal can be seen in Figure 6. In this case, similar values were expected since the intensity of the YFP complex is the same independently of the percentage of cells with active fluorescence. However, AQP4 + CaM showed statistically significant values when compared to both AQP0 + CaM ($p < 0.001$) and AQP0ΔC + CaM ($p < 0.001$). On the other hand, no statistical difference was shown between AQP0 + CaM and AQP0ΔC + CaM ($p = 0.227$).

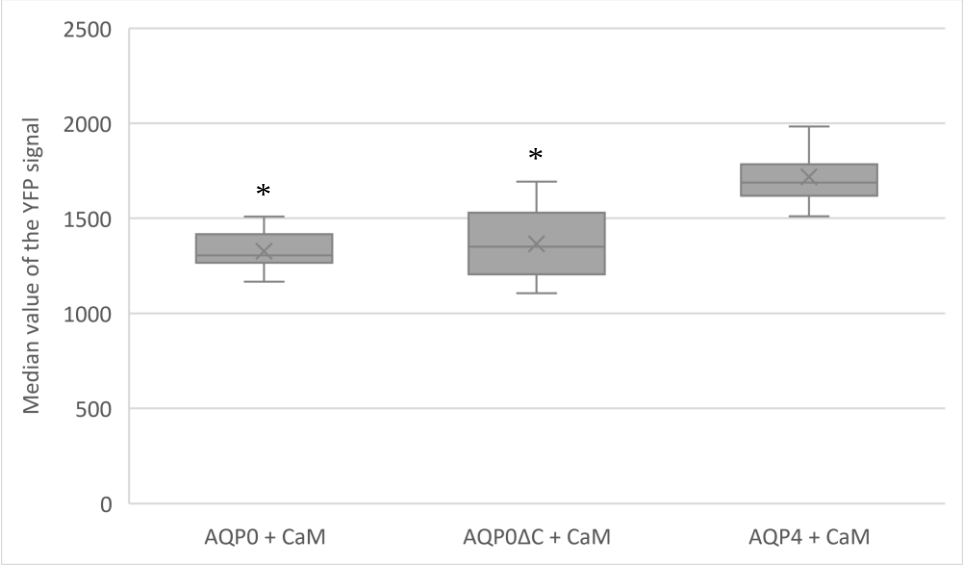

*Figure 6. Median values of the YFP signal for AQP0 + CaM, AQP0ΔC + CaM, and AQP4 + CaM. * indicates statistical differences when compared to the positive control (AQP4 + CaM).*

Furthermore, plotted values for the frequency of the YFP signal can be seen in Figure 7. It is clear in this case that the negative control shows the lowest percentage of cells showing a YFP signal, as was expected; meanwhile, the positive control AQP4 + CaM shows the highest percentage. In this case, all values are statistically significant when compared to each other ($p < 0.001$).

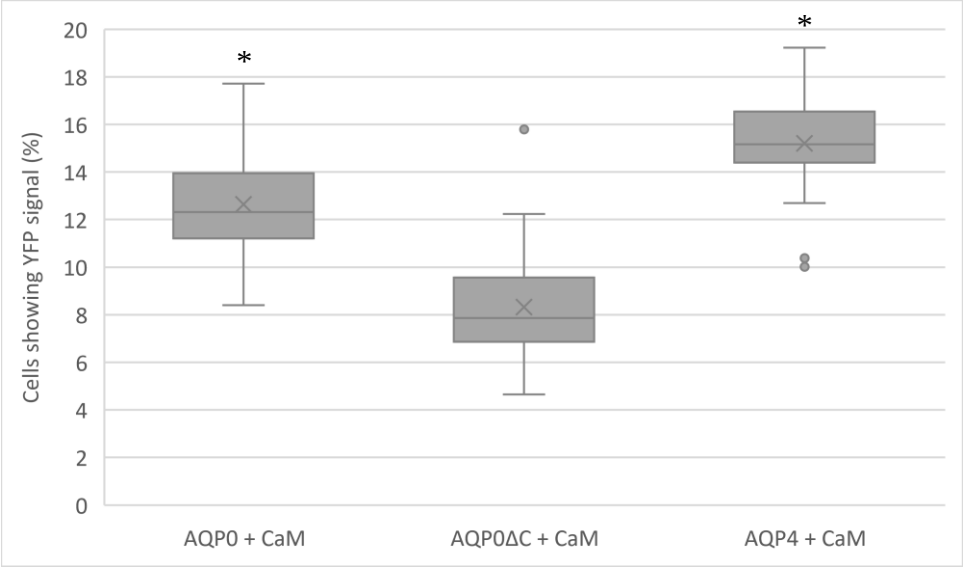

*Figure 7. Percentage of cells showing YFP signal for AQP0 + CaM, AQP0ΔC + CaM, and AQP4 + CaM. * indicates statistical differences when compared to the negative control (AQP0ΔC + CaM).*

Lastly, values of intensity and frequency of the YFP signal can be seen in Table 1.

Table 1. Values of intensity (in arbitrary units) and frequency (%) of the YFP signal for the controls. In all cases, values are calculated as median ± SEM.

	AQP0 + CaM	AQP0ΔC + CaM	AQP4 + CaM
Intensity (arbitrary units)	12.64 ± 0.38	8.32 ± 0.38	15.20 ± 0.32
Frequency (%)	1327.45 ± 15.96	1366.23 ± 28.79	1717.64 ± 20.52

4.2. N-terminally tagged AQP4 and C-terminally tagged cDNA library

The first part of the process was to establish the optimal DNA amount for the transformation. The protocol was optimised to obtain the largest number of colonies, with many small and large colonies. Since the outcome of the protein-protein interaction is not fully understood in *S. cerevisiae* cells, it could be possible that a successful interaction caused harm to the cell growth. Therefore, and even though larger colonies grow faster and better, it could also be that small colonies are the ones harbouring actual interactions.

The first transformations were performed using between 100 and 1,000ng of DNA of each of the plasmids. It was determined that 500ng of both plasmids allowed us to obtain the largest number of large colonies while still obtaining a considerable quantity of smaller colonies.

Moreover, it was also essential to determine the optimal sample preparation for the FACS analysis. Two conditions were prepared to achieve such: the first was to analyse the overnight culture; meanwhile, the second required overnight cultures to be diluted into an OD_{600} = 0.2 and further grow into an OD_{600} = 0.5. Analysis of different samples allowed us to see no difference in the amount of fluorescence for the intensity analysis in the FACS.

4.2.1. First transformation event

Analysis of the first transformation event samples in the FACS revealed that only a small percentage of cells showed a YFP signal that could be distinguished from the background fluorescence. Therefore, the set gate was fixed for a likely positive hit. Afterwards, ten individual cells were sorted out to be further analysed for signal frequency.

Out of the ten individual cells that had been sorted out, only six of them showed significant growth after six days of incubation. These individual colonies were regrown overnight and further analysed the following morning. These findings showed no frequency of fluorescence whatsoever in either of the samples.

4.2.2. Subsequent transformation events

To force more DNA into the cells and see whether higher amounts of DNA would end up causing YFP signal to show, three further transformations were performed using 1000ng and 2000ng of each of the plasmids. Still, 500ng were also used for each of the transformation events. In all the previous cases, the transformation was successful, and there were both small and large colonies. However, no YFP signal could be detected in either of the FACS analysis preparations (overnight or regrown).

4.2.3. N-terminally tagged AQP4 and C-terminally tagged cDNA library – Summary

As a summary for the first studied combination (N-terminally tagged AQP4 and C-terminally tagged cDNA library), and even though transformation efficiency was good, the FACS analysis revealed no YFP signal in any of the cases. Summarised values for the first combination can be seen in Table 2.

Table 2. Transformation and FACS analysis results for the N-terminally tagged AQP4 combined with the C-terminally tagged cDNA library.

Amount of DNA (µg)	Transformation events	Highest number of colonies	Highest transformation efficiency (col/µg)	YFP signal frequency (%)
0.5	6	27	54	No fluorescence
1	4	33	33	No fluorescence
2	4	80	40	No fluorescence

4.3. C-terminally tagged AQP4 and C-terminally tagged cDNA library

4.3.1. First transformation event

Considering the transformation efficiency values from the previous combination, the first transformations were evaluated at 500ng and 1000ng of DNA from each plasmid. In this case, however, the transformation had lower efficiency. Still, both larger and smaller colonies were sought after.

As with the previous combination, it was also important to determine the optimal sample preparation for the FACS analysis. The same two preparations were studied: overnight cultures and regrowing overnight cultures to an OD_{600} = 0.5. In the first transformation event, it was demonstrated that there was no difference between the two.

FACS analysis of the few obtained colonies revealed no frequency of the YFP signal in any of the colonies.

4.3.2. Subsequent transformation events

In order to try to enhance the transformation efficiency for the studied combination, subsequent transformation events were performed trying different amounts of DNA (either higher, lower, or in-between the previous transformation). Tested concentrations were 200ng, 500ng, 1000ng, 1500ng, and 2000ng.

FACS analysis of the obtained colonies revealed no frequency of the YFP signal in any of the colonies. Furthermore, no difference was observed between the two different sample preparations.

4.3.3. C-terminally tagged AQP4 and C-terminally tagged cDNA library – Summary

Results for the second combination of study (C-terminally tagged AQP4 and C-terminally tagged cDNA library) can be seen in Table 3. As is apparent, transformation efficiency was considerably low, especially compared to the previously studied combination. Furthermore, after the FACS analysis, we determined that no YFP signal was coming from any of the colonies, neither on the overnight culture nor the regrown to OD_{600} = 0.5.

Table 3. Transformation and FACS analysis results for the C-terminally tagged AQP4 combined with the C-terminally tagged cDNA library.

Amount of DNA (µg)	Transformation events	Highest number of colonies	Highest transformation efficiency (col/µg)	YFP signal frequency (%)
0.2	2	1	5	No fluorescence
0.5	4	1	2	No fluorescence
1	4	2	2	No fluorescence
1.5	2	0	0	No fluorescence
2	2	1	0.5	No fluorescence

5. Discussion

In summary, the FACS analysis of the controls showed the expected intensity and frequency results. In the case of intensity, both AQP0 + CaM and AQP0ΔC + CaM showed statistically similar values, even though AQP4 + CaM showed statistically significant higher values. In the case of the frequency: AQP0ΔC + CaM showed a lower percentage of cells showing YFP signal when compared to the two positive controls. More specifically, comparing AQP0 + CaM and AQP0ΔC + CaM, differences were expected since the binding of calmodulin to AQP0 occurs through the C-terminus (Kreida et al., 2018).

Furthermore, the two studied combinations (Y_N-AQP4 + cDNA library-Y_C and AQP4-Y_N + cDNA library-Y_C) showed similar results in YFP signal, both in intensity and in frequency, but transformation efficiency was highly different. In the case of the Y_N-AQP4 + cDNA library-Y_C combination, transformation efficiency was high despite the analysed colonies not showing YFP signal whatsoever; alternatively, in the case of the AQP4-Y_N + cDNA library-Y_C combination, transformation efficiency was almost non-existent, and the small number of analysed colonies showed no YFP signal at all.

Each cDNA library plasmid contains one fragment from a human brain tissue sample, and, as a consequence, it might be possible that it is not a complete protein but a small part that is introduced into it. Therefore, even if a specific full protein might be an interaction partner of AQP4, a small fragment of it might not form such interaction. This is why, in our project, the aim was to perform high-throughput screening of transformed *S. cerevisiae* cells. Combining BiFC with FACS would allow us to study the YFP signal of as many colonies as possible. The results that we have obtained, specifically on the second studied combination (AQP4-Y_N + cDNA library-Y_C) are not enough to determine whether there exists an interaction partner of AQP4 in the human brain: less than ten colonies have been analysed in the whole experiment. On the other hand, the first combination (Y_N-AQP4 + cDNA library-Y_C), having higher transformation efficiency, allowed us to screen larger amounts of colonies. Therefore, future studies should focus on screening more colonies from each combination, as well enhancing transformation efficiency, specifically on the second combination.

It is true, however, that previous studies on interaction partners of AQP4 have shown that such interactions tend to happen on the hydrophilic extensions of the aquaporin, more specifically on the C-terminus of such (Sjöhamn et al., 2014). One example could be the study by *Madrid R et al.*, where they identified a 30-amino-acid cassette in the cytoplasmic C-terminus of AQP4 that allows interaction with clathrin-adaptor protein complexes and regulates basolateral delivery and surface expression (Madrid et al., 2001; Sjöhamn et al., 2014). This study, however, was performed in epithelial cells and not in brain cells (Madrid et al., 2001). Another example of C-terminal interaction would be the one presented in the study by *Connors N et al.*, where they found that rat AQP4 could interact with DGC proteins in the rat retina (Connors et al., 2006; Sjöhamn et al., 2014). Finally, another study by *Neely J et al.* stated that rat AQP4 could also interact through the C-terminus with the PSD95-Discs large-ZO1 (PDZ) domain of syntrophin, a component of the dystrophin protein complex (Neely et al., 2001; Sjöhamn et al., 2014). Such results would enhance the fact that the first combination (Y_N-AQP4 + cDNA library-Y_C) should be more successful during the screening. This is justified by the fact that the C-terminus of the aquaporin is not blocked by the Y_N, as well as by the fact that it follows the same structure as the positive control (AQP4 + CaM – Y_N-AQP4 + Y_C-CaM).

However, other studies have also reported binding of the AQP4 through the N-terminus. One example would be the study published by *Illarionova N et al.*, which identified a segment in the N-terminus of the AQP4 (Lys27 and Trp30) which generates an interaction site for both the catalytic subunit of the Na,K-ATPase as well as with the metabotropic glutamate receptor (mGluR5) (Illarionova et al., 2010; Sjöhamn et al., 2014). On the other hand, these findings can also support the second combination studied in this project (AQP4-Y_N + cDNA library-Y_C), even though it would seem less likely than the first combination.

There are different reasons why no fluorescence was obtained in any combination, which this discussion will go through.

It is important to note that *Saccharomyces cerevisiae* is a heterologous system; meanwhile, the proteins in this study were of human origin. This can bring quite a few incompatibilities to the experiment. Firstly, some proteins can be produced in an immature form, which might require maturation inside the cell (Kim et al., 2019; Murozuka et al., 2013). However, human proteins can require posttranslational modifications, which might not be found in other organisms (Murozuka et al., 2013).

Secondly, additional proteins can be necessary for a protein-protein interaction to occur. Our experiment expected that only one cDNA fragment would be incorporated into the plasmid. Consequently, any interactions requiring additional proteins should not happen, but that does not mean they do not exist (Horstman et al., 2014). Thirdly, *Horstman A et al.* found differences in the BiFC efficiency between species (Horstman et al., 2014). Therefore, if done correctly and including additional necessary proteins, studies in human cells might allow the obtaining of positive hits.

Another crucial factor to consider is the incubation conditions of the sample. *Horstman A et al.* stated that incubation conditions seem to have a more significant impact on BiFC than the vector itself. For example, an adverse effect of the temperature of the culture on the YFP complementation has been noticed in specific cases. In the case of mammalian cells, incubating them for four hours at lower temperatures prior to reading enhanced the fluorescence signal (Horstman et al., 2014). This, consequently, might need to be studied in *Saccharomyces cerevisiae* to enhance the obtained YFP signal. Another piece of advice given by *Horstman et al.* is to measure the BiFC signal at different times after transformation. This should be done to avoid fluorescence saturation, which might influence the signal-to-noise ratio. However, it is also mentioned that the use of flow cytometry, where many cells are analysed, might overcome this issue (Horstman et al., 2014).

Different aspects should also be considered, focusing more on the plasmid construction. The sequence and the length of the linker found between the YFP fragment and the protein of study (AQP4 or cDNA library) is important for YFP complementation. It has been previously described that the linker can affect the flexibility or the folding of the proteins of study, which is essential for the protein-protein interaction (Horstman et al., 2014). The linker sequence (GGPGGGHQTS-LYKKAGF) has 17 amino acids in our case. It is possible that introducing either a larger or a shorter sequence might help form protein-protein interactions.

It is also important to note that the binding of the YFP fragment to the protein of interest can hinder the complementation either by steric clashes or by affecting the folding of the AQP4 or the cDNA library fragment (Horstman et al., 2014). For an interaction to happen, it has been calculated that the two fragments of the YFP should be no further than 10nm, approximately, which, again, might cause topological constraints between the two proteins of study (Kim et al., 2019). Furthermore, both proteins of study should have enough dynamic flexibility to associate and form the protein complex (Kerppola, 2006).

There are eight possible combinations between the proteins of study and the YFP fragments in any bimolecular fluorescence complementation assay. In our case, two of the combinations have been studied, both involving a C-terminal tagging of the cDNA library with the Y_C fragment of the YFP. *Kim Y et al.* have previously expressed that the C-terminal tagging of proteins might cause difficulties in folding or even in protein expression. Furthermore, it has also been mentioned that nonmature proteins might need to suffer a cleavage on the C-terminus for maturation. This process might eliminate the YFP fragment (Kim et al., 2019). Both affirmations might also be causes for the absence of YFP signal in the studied combinations. Moreover, two other combinations are being studied at the same time in our research group (Y_N-AQP4 + Y_C-cDNA library and AQP4-Y_N + Y_C-cDNA library), and the latter has shown detectable YFP signals (with increasing frequencies of 2.27%, 2.97%, 6.63%, 8.14%, and 18.78%) without being C-terminally tagged, reinforcing the previously mentioned causes for lack of fluorescence.

6. Conclusions

To sum up, bimolecular fluorescence complementation seems to be a suitable method for studying protein-protein interactions in various organisms, including *Saccharomyces cerevisiae*. This method can be even more useful when coupled to fluorescence-activated cell sorting, allowing for higher-throughput screening.

However, even if the analysis of the controls revealed positive results, with statistical significance between the frequencies, the screening of the actual samples revealed no potential protein-protein interactions. A higher number of colonies were analysed in the case of the first combination of study, N-terminally tagged AQP4 and C-terminally tagged cDNA library. Nonetheless, no positive hits were obtained. On the other hand, the number of analysed colonies was low for the second combination of study, C-terminally tagged AQP4 and C-terminally tagged cDNA library. Again, no positive hits were obtained.

The high number of proteins that can be obtained in a cDNA library from human brain mRNA, and the fact that it is more likely that only fragments of the protein entered the plasmid, makes screening for protein-protein interactions a challenging process. Thus, in conclusion, the aim of future projects should be focused on: first, enhancing transformation efficiency; and second, analysing as many colonies as possible.

7. Bibliography

Abir-Awan, M., Kitchen, P., Salman, M. M., Conner, M. T., Conner, A. C., & Bill, R. M. (2019). Inhibitors of mammalian aquaporin water channels. *International Journal of Molecular Sciences*, *20*(7). https://doi.org/10.3390/ijms20071589

Ahmed, T., & Ghafoor, S. (2021). Aquaporins; systemic, functional and therapeutic correlations in health and disease. *Journal of the Pakistan Medical Association*, *71*(4), 1228–1233. https://doi.org/10.47391/JPMA.01-063

Azad, A. K., Raihan, T., Ahmed, J., Hakim, A., Emon, T. H., & Chowdhury, P. A. (2021). Human Aquaporins: Functional Diversity and Potential Roles in Infectious and Non-infectious Diseases. *Frontiers in Genetics*, *12*(March). https://doi.org/10.3389/fgene.2021.654865

Bjørkskov, F. B., Krabbe, S. L., Nurup, C. N., Missel, J. W., Spulber, M., et al. (2017). Purification and functional comparison of nine human Aquaporins produced in Saccharomyces cerevisiae for the purpose of biophysical characterization. *Scientific Reports*, *7*(1), 1–21. https://doi.org/10.1038/s41598-017-17095-6

Büscher, M. (2019a). Flow Cytometry – An Overview. *Current Protocols in Cytometry*, *87*(1), 1–16. https://doi.org/10.1002/cpcy.52

Büscher, M. (2019b). Flow Cytometry Instrumentation – An Overview. *Current Protocols in Cytometry*, *87*(1), 1–8. https://doi.org/10.1002/cpcy.52

Clément, T., Rodriguez-Grande, B., & Badaut, J. (2020). Aquaporins in brain edema. *Journal of Neuroscience Research*, *98*(1), 9–18. https://doi.org/10.1002/jnr.24354

Connors, N. C., & Kofuji, P. (2006). Potassium channel Kir4.1 macromolecular complex in retinal glial cells. *Glia*, *53*(2), 124–131. https://doi.org/10.1002/glia.20271

Geng, X., & Yang, B. (2017). Transport characteristics of aquaporins. *Advances in Experimental Medicine and Biology*, *969*, 51–62. https://doi.org/10.1007/978-94-024-1057-0_3

Givan, A. L. (2011). Flow Cytometry: An Introduction. *Methods in Molecular Biology*, *699*(1). https://doi.org/10.1007/978-1-61737-950-5

Horstman, A., Tonaco, I. A. N., Boutilier, K., & Immink, R. G. H. (2014). A Cautionary note on the use of split-YFP/BiFC in plant protein-protein interaction studies. *International Journal of Molecular Sciences*, *15*(6), 9628–9643. https://doi.org/10.3390/ijms15069628

Hua, Y., Ju, J., Wang, X., Zhang, B., Zhao, W., Zhang, Q., Feng, Y., Ma, W., & Wan, C. (2018). Screening for host proteins interacting with Escherichia coli O157:H7 EspF using bimolecular fluorescence complementation. *Future Microbiology*, *13*(1), 37–58. https://doi.org/10.2217/fmb-2017-0087

Illarionova, N. B., Gunnarson, E., Li, Y., Brismar, H., Bondar, A., Zelenin, S., & Aperia, A. (2010). Functional and molecular interactions between aquaporins and Na,K-ATPase. *Neuroscience*, *168*(4), 915–925. https://doi.org/10.1016/j.neuroscience.2009.11.062

Ishida, H., Vogel, H. J., Conner, A. C., Kitchen, P., Bill, R. M., & MacDonald, J. A. (2022). Simultaneous Binding of the N- and C-terminal Cytoplasmic Domains of Aquaporin 4 to Calmodulin May Contribute to Vesicular Trafficking. *Biochimica et Biophysica Acta - Biomembranes*, *1864*(2).

Kerppola, T. K. (2006). Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells (Nature Protocols (2006) (1278-1286) 10.1038/nprot.2006.201). *Nature Protocols*, *1*(3), 1278–1286.

Kim, Y., Jung, J. P., Pack, C.-G., & Huh, W.-K. (2019). Global analysis of protein homomerization in Saccharomyces cerevisiae. *Genome Research*, *29*(1), 135–145. https://doi.org/10.1074/jbc.M409203200

Kitchen, P., Salman, M. M., Halsey, A. M., Clarke-Bland, C., MacDonald, J. A., et al. (2020). Targeting

Aquaporin-4 Subcellular Localization to Treat Central Nervous System Edema. *Cell*, *181*(4), 784-799.e19. https://doi.org/10.1016/j.cell.2020.03.037

Kodama, Y., & Hu, C. D. (2012). Bimolecular fluorescence complementation (BiFC): A 5-year update and future perspectives. *BioTechniques*, *53*(5), 285–298. https://doi.org/10.2144/000113943

Kojima, T., Karasawa, S., Miyawaki, A., Tsumuraya, T., & Fujii, I. (2011). Novel screening system for protein-protein interactions by bimolecular fluorescence complementation in Saccharomyces cerevisiae. *Journal of Bioscience and Bioengineering*, *111*(4), 397–401. https://doi.org/10.1016/j.jbiosc.2010.12.013

Kreida, S., Roche, J. V., Olsson, C., Linse, S., & Törnroth-Horsefield, S. (2018). Protein-protein interactions in AQP regulation-biophysical characterization of AQP0-CaM and AQP2-LIP5 complex formation. *Faraday Discussions*, *209*, 35–54. https://doi.org/10.1039/c8fd00065d

Li, C., & Wang, W. (2017). Molecular biology of aquaporins. In *Advances in Experimental Medicine and Biology* (Vol. 969). https://doi.org/10.1007/978-94-024-1057-0_1

Li, S., Li, C., & Wang, W. (2020). Molecular aspects of aquaporins. In *Vitamins and Hormones* (1st ed., Vol. 113). Elsevier Inc. https://doi.org/10.1016/bs.vh.2019.08.019

Mader, S., & Brimberg, L. (2019). Aquaporin-4 Water Channel in the Brain and Its Implication for Health and Disease. *Cells*, *8*(2), 90. https://doi.org/10.3390/cells8020090

Madrid, R., Le Maout, S., Barrault, M. B., Janvier, K., Benichou, S., & Mérot, J. (2001). Polarized trafficking and surface expression of the AQP4 water channel are coordinated by serial and regulated interactions with different clathrin-adaptor complexes. *EMBO Journal*, *20*(24), 7008–7021. https://doi.org/10.1093/emboj/20.24.7008

Magouliotis, D. E., Tasiopoulou, V. S., Svokos, A. A., & Svokos, K. A. (2020). Aquaporins in health and disease. In *Advances in Clinical Chemistry* (1st ed., Vol. 98). Elsevier Inc. https://doi.org/10.1016/bs.acc.2020.02.005

Morell, M., Espargaro, A., Aviles, F. X., & Ventura, S. (2008). Study and selection of in vivo protein interactions by coupling bimolecular fluorescence complementation and flow cytometry. *Nature Protocols*, *3*(1), 22–33. https://doi.org/10.1038/nprot.2007.496

Murozuka, E., Hanisch, S., Pomorski, T. G., Jahn, T. P., & Schjoerring, J. K. (2013). Bimolecular fluorescence complementation and interaction of various Arabidopsis major intrinsic proteins expressed in yeast. *Physiologia Plantarum*, *148*(3), 422–431. https://doi.org/10.1111/ppl.12000

Neely, J. D., Amiry-Moghaddam, M., Ottersen, O. P., Froehner, S. C., Agre, P., & Adams, M. E. (2001). Syntrophin-dependent expression and localization of Aquaporin-4 water channel protein. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(24), 14108–14113. https://doi.org/10.1073/pnas.241508198

Roche, J. V., & Törnroth-Horsefield, S. (2017). Aquaporin protein-protein interactions. *International Journal of Molecular Sciences*, *18*(11), 1–23. https://doi.org/10.3390/ijms18112255

Schmitz, F., Glas, J., Neutze, R., & Hedfalk, K. (2021). A bimolecular fluorescence complementation flow cytometry screen for membrane protein interactions. *Scientific Reports*, *11*(1), 1–9. https://doi.org/10.1038/s41598-021-98810-2

Sjöhamn, J., Båth, P., Neutze, R., & Hedfalk, K. (2016). Applying bimolecular fluorescence complementation to screen and purify aquaporin protein:protein complexes. *Protein Science*, *25*(12), 2196–2208. https://doi.org/10.1002/pro.3046

Sjöhamn, J., & Hedfalk, K. (2014). Unraveling aquaporin interaction partners. *Biochimica et Biophysica Acta - General Subjects*, *1840*(5), 1614–1623. https://doi.org/10.1016/j.bbagen.2013.11.012

Ventura, S. (2012). Bimolecular Fluorescence Complementation: Illuminating Cellular Protein Interactions. *Current Molecular Medicine*, *11*(7), 582–598. https://doi.org/10.2174/156652411800615117

Verkman, A. S., Smith, A. J., Phuan, P. wah, Tradtrantip, L., & Anderson, M. O. (2017). The aquaporin-4 water channel as a potential drug target in neurological disorders. *Expert Opinion on Therapeutic*

Targets, *21*(12), 1161–1170. https://doi.org/10.1080/14728222.2017.1398236

Weber-Boyvat, M., Li, S., Skarp, K.-P., Olkkonen, V. M., Yan, D., & Jäntti, J. (2015). Bimolecular Fluorescence Complementation (BiFC) Technique in Yeast Saccharomyces cerevisiae and Mammalian Cells. *Membrane Trafficking: Second Edition, Methods in Molecular Biology*, *1270*, 277–288. https://doi.org/10.1007/978-1-4939-2309-0

Self-assessment

When it comes to the self-assessment of this project, I need to evaluate it from two perspectives: the one involving all the laboratory work, and the other involving the writing of the manuscript.

Firstly, these past few months at Lundberg Laboratory have allowed me to obtain experience when it comes to laboratory work. Even though the laboratory work was tutorized by Kristina Hedfalk and Jessica Glas, I have gained self-confidence about performing laboratory techniques. At Lundberg Laboratory, I have performed techniques that I had already done before, such as Western-Blotting or yeast cultures. This internship has allowed me to improve my capabilities in such techniques. Furthermore, I have also learnt about new techniques I had only studied before, like using flow cytometry and, more specifically, fluorescence-activated cell sorting.

On the other hand, the writing of this project has helped me improve my abilities in searching for an adequate bibliography for a project as well as for extracting the essential parts of an article. I feel like I have improved my knowledge of aquaporins and membrane proteins in general, but also in bimolecular-fluorescence complementation and flow cytometry. In this case, the writing of the project was supervised by Ricardo Román Cordero Otero, who has been more than helpful in pointing me in the right direction for the writing of this project.

Finally, and in summary, I feel like this project, involving internship and writing, has allowed me to grow as somebody who wants to develop a career in the investigating branch of science, and I could not be more thankful to everybody who has helped me, even if just a bit, through this whole experience.