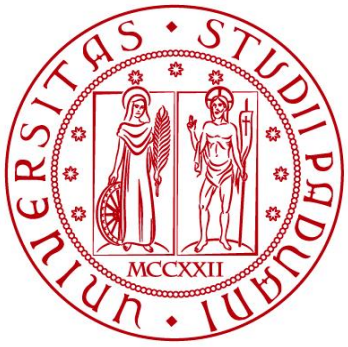
UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea in Biotecnologie



ELABORATO DI LAUREA

Development of a genetically encoded fluorescent probe for sensing molecular crowding in mitochondria

Tutor: Prof. Diego De Stefani
Dipartimento di Scienze Biomediche

Co-tutor: Dott. Massimo Vetralla
Dipartimento di Scienze Biomediche

Laureando: Alessio Ferrini

ANNO ACCADEMICO 2021/2022

TABLE OF CONTENTS

1. ABSTRACT

Molecular crowding (MC) is a phenomenon naturally occurring in all intracellular compartments due to the high concentrations of ions and biological molecules (primarily proteins) densely populating the cellular milieu. Multiple factors, including the size and broad chemical diversity of different crowders (e.g. ions, small organic compounds, polymers), as well as their complex interactions with the solvent, contribute to establish an environment with extremely variable context-dependent crowding states. Furthermore, MC continuously undergoes rapid changes, in response to both physiological processes and environmental stress, along with modifications in cellular architecture and metabolism. Given its key role in controlling protein stability, interactions and functionality, it represents a cellular parameter that needs to be reliably measured. Although FRET-based probes had already been developed and proposed as suitable and promising tools in this field, their application within the mitochondrial compartment is poorly explored. To this aim, we developed a fluorescent genetically encoded probe specifically targeted to the organelle matrix in order to monitor local MC dynamics occurring in cellular models during physiological and pathological conditions.

2. STATE OF THE ART

2.1 Molecular crowding

Molecular crowding (MC) is an event that occurs in all cellular departments (cytoplasm, organell's lume, membrane surface). Cells are highly crowded with a great variety of compounds, primarily proteins and nucleic acids as well as ions and sugars densely populating the aqueous intracellular milieu (Model et al., 2021). The concentration of molecules can vary, either increasing or decreasing in a short amount of time, thus altering the structural and chemical properties of neighboring biomolecules. Cell homeostasis is therefore a dynamic state constantly perturbed by changes in the extracellular environment. Given the numerosity and variety of compounds interacting with each other in the intracellular milieu, it is widely experimented and recognised that MC influence all molecular events within the cell, like biomolecular transport, protein folding and biochemical reactions (Leopold et al.,2019).

2.2 Exclusion Volume effect

The first and most intuitive effect that a crowder molecule exerts in a solution is known as the exclusion volume effect (EV). This phenomenon does not occur because of any specific chemical feature of the crowder, but derives solely from the steric repulsion of two molecules in a solution caused by their mutual impenetrability. Because of EV,, the presence of a molecule prevents other solutes to occupy a portion of the solution; more precisely, if we approximate the two molecules as spheres, it impedes the center of a molecule to be located in a spherical volume given by the sum of their ranges. Since less volume is accessible to other molecules, less different spatial distributions are permitted, therefore the randomness of the solute is reduced and its entropy lowers. This is paired with an increase of the free energy of the solute, which affects proteins in different ways.

On the scale of a single protein, EV can impact on both the folded-unfolded and elongated-compact conformation equilibria of a protein. In both cases, the presence of particles reducing the accessible volume, favors the transition to a more compact shape that occupies less space, a folded state (in the case of a single polypeptide) or a compact conformation (in the case of a multi-domain protein). More precisely, EV influences the hydrodynamic volume of the protein by destabilizing the extended and malleable conformations, thus indirectly stabilizing the more compact one. On the wider scale, EV impacts also on the state of association of proteins able to interact with each other. In order to contrast the aforementioned decrease of entropy, these molecules assemble forming oligomers, because the volume they occupy is less than the sum of the ones occupied by the single monomers. This phenomenon doesn't involve only

functional oligomers, but can lead to the formation of pathological misfolded protein aggregates. For the same reason, MC can also influence enzymatic activity in the media. Although enzymes with low molecular weight substrates are not significantly affected by EV, the presence of crowders can still increase the activity of enzymes that operate on larger substrates (nucleic acids, larger proteins) or that can assemble into oligomers (Kuznetsova et al., 2015). EV impacts also on reaction rates, even if the overall effect is hard to determine in theory. Although higher density may in fact make molecules at close range clutter increasing reaction rate, a slower diffusion reduces the number of effective collisions, counteracting the aforementioned effect (Model et al., 2021).

2.3 Additional crowding interactions

EV has a strong influence on protein structure and behavior in solution, but it's not able to exhaustively explain such characteristics nor to justify the discrepancy between analysis performed in vivo and in artificial polymeric solutions in vitro. In order to achieve this, different additional interactions between crowders and proteins must be taken into account.

Weak, transient, and nonspecific interactions (e.g., van der Waals, electrostatic, or hydrophobic forces) between a crowder and a protein, most prominently between their surface areas, have proved to be capable of altering the stabilizing effect of EV on compact-shaped conformations, either positively or negatively, up to to the point of inversion. Other interactions can be more strong and specific, such as interactions between two aminoacids or that involve binding sites, or not consist in steric hindrance, like interaction with enzymes tuning the folding of the protein. The complexity of crowder and protein topography is important too, as a rod-like shaped crowder (fairly common in the cytoplasm) has more surface area capable of interaction with other components compared to globular proteins and therefore are more heavily affected by the solution’s features. Another effect exerted by a crowder on a solution is the increase of viscosity, which primarily alters the diffusion rate of solutes in the media and impacts on the protein folding process by increasing the friction the polypeptide undergoes when changing conformation (in particular during translational movements). The volume difference between protein and crowder is significant, as big macromolecules alter the macro-viscosity but create spaces between molecules filled with bulk water, where smaller molecules and protein can diffuse, undergo folding and behave almost like in a not-crowded environment. One last, important effect that MC exerts on the solutes of a solution does not actually involve the solutes, but rather the solvent itself. Many polymers have been proven to be able to form, at specific salt concentration or together with a different polymer, two distinct phases in the solution, leading to changes in the properties of the solvent. The settling of these two distinct environments, comparable to two separate organic and aqueous solutions, is not due to interactions between crowders and solutes, like those mentioned before. It has been shown that the polymers can change the properties of the solvent, leading to the formation of separate phases and to an imbalance in the distribution

of solutes among themselves, caused by the modification of the soft, polar interaction the solvent can establish with the molecules. The fact that even two polymers compatible in a dry environment can form immiscible aqueous phases in water reinforces this suggestion. This means even a single polymer in a solution, when it undergoes changes such as a temperature shift or a salt concentration alteration, can significantly affect protein conformation and distribution and modify the very geometry of the solution (Kuznetsova et al., 2015).

Molecular crowding varies very little within cells of a given cell-type, given the tight regulation it is subjected to. However, it can undergo prominent variations during physiological cell events (cell division, cell differentiation, senescence) as well as environmental and metabolic stress responses (osmotic stress, starvation) and in pathological contexts (Model et al., 2021). Because of that, we can't only focus on the behavior of MC in a given cell but we must investigate how it characterizes different tissues and different physiopathological conditions. The intracellular media is far more complex and structured than any artificial in vitro solution and it constantly adapts to mutating architecture populated by an immense number of different compounds and reactions. Therefore, we can't ignore the impellent urge to study MC in vivo and implement that knowledge with our current simulations.

2.4 Intrinsically Disordered Proteins

Intrinsically Disordered Proteins (IDPs) are proteins that lack a stable 3D structure at their native state, either considering their entire amino acid sequence or some regions of the protein (IDRs). Indeed, it is not possible to attribute a tertiary structure to these proteins and they do not present stable secondary structures. These properties are sequence-determined and make IDPs very dynamic compounds characterized by different unstable possible conformations in solution. IDPs and IDRs are widely spread among organisms and viruses, with around 40% of human proteins containing stretches of around 30 amino acids which are IDRs, often flanking globular domains by at least one side. Because of their numerosity, their molecular interactions with other molecules, primarily other proteins and nucleic acids, are as significant as diversified, both in physiological macromolecules and processes and in pathogenesis.

The most prominent feature characterizing interactions of intracellular compounds and structures (e.g. the cytoskeleton) with IDPs is the latters' malleability, which allows them to bind several ligands at the same moment or the same ligand multiple times, modifying their folding and conformation accordingly to the substrates and the environment. Along the disordered region's sequence are present short sequence motifs responsible for binding with other molecules. They are generally subdivided as short linear motifs (SLiMs, 3-10 residues long, involved in directing protein-protein interactions), molecular recognition features (MoRFs, 10-40 residues long, undergo a disordered-to-ordered folding upon binding with a protein), and low-complexity sequences (characterized by short repetitions, some

of them are involved in protein-nucleic acid and protein-protein interactions). Short sequence motifs are often present in multiple copies in a single IDP or IDR, either copies of the same motif or of different ones, which allows disordered proteins to bind simultaneously different molecules, increasing gradually the avidity of the interaction. Furthermore, IDPs are frequently subject to post-translational modification, which tune the protein's solubility and affinity towards different partner molecules. (Fung et al., 2018)

2.5 Biological role of IDPs and IDRs

Interactions depending on the binding of IDRs have been proven to be fundamental to direct the formation of macromolecular complexes, such as the Nuclear Pore Complex localized in the nuclear membrane, where IDRs selectively link more rigid and structurally defined proteins, mediating the overall complex stability and solutes recognition (Fung et al., 2018). When interacting with other proteins, IDPs and IDRs may assume different behaviors. While sometimes they undergo disorder-to-order transition upon binding, assuming a stable structure, in many cases different conformations and therefore different interactions are actually possible. In other situations, the IDP maintains high flexibility and heterogeneous folding even after binding with the partner molecules presenting a dynamically shifting interface which switches conformations by involving different competitive binding sites (Wang et al., 2019).

Multivalent interactions involving IDPs are also capable of creating phase separation in aqueous solutions, leading to the formation of biomolecular condensates. Biomolecular condensates are defined as protein-rich phases separated by the hydrophilic solvent not by any membrane, like organelles, but instead by the structured interactions between the solutes. Being constituted by a liquid phase surrounded by a second liquid phase, their solute exchange rates are high and their role in cells is to isolate metabolites from the cytoplasm. The phase separation is often driven by collective, multivalent, weak interactions between IDPs and their nucleic acid or protein ligands. In those situations, it has been observed both in vitro and in vivo that the formation of oligomers caused by the interactions between charged residues of the molecules can be sufficient for phase separation (Fung et al., 2018). IDPs and IDRs have been reported to be involved also in protein-carbohydrate interactions, virus-antibody binding, enzyme catalysis, clustering of cellular receptors, microtubule interaction and stabilization, microfilament nucleation, signal transduction and gene replication and translation, but since their function can hardly be determined by a strong relation with their 3D structure our knowledge about their biological relevance is still incomplete (Fung et al., 2018) (Wang et al., 2019).

3. EXPERIMENTAL APPROACH

3.1 FRET probes

Föster resonance energy transfer (FRET) is a phenomenon occurring when two fluorescent molecules, one whose emission spectra overlaps with the absorption spectra of the other, are excited by electromagnetic radiation while at a close range of 1-100 Å. The core of the event consists of an energy transfer occurring from the highest emission frequency molecule (M1) to the lower (M2) without emission of photons through dipole-dipole interaction. In order to happen the dipoles of the molecules must be roughly parallel. As a result, excitation is transferred from M1 to M2 leading to an overall decrease of the *ratio* between intensity of fluorescent emission of M1 over M2. FRET probes consist of molecules displaying two fluorescent dyes compatible with each other for FRET linked by a sensing domain.

The latter, modifying its conformation or getting degraded following a change in the environment, alter the distance between the two dyes provoking, while under proper fluorescent stimulation, perceptible variation in the FRET *ratio.* Therefore, the efficiency of a FRET probe depends both on the FRET characteristics of the dyes and on the sensitivity of the linker domain toward a specific ligand or environment.

FRET probes consisting in fluorescent dyes attached to DNA oligomers have known extended use in a wide variety of applications both *in vitro* and *in vivo*. They have proven to be useful tools for monitoring the rate of biochemical reactions such as DNA amplification in Real Time PCR, DNA synthesis and DNA recombination, detecting DNA recombination, nuclease activity, DNA mutations. DNA-based probes' structure span from linear single stranded molecules to double stranded, harpin-like or even more complex compounds, a wide variety of dyes are viable for FRET probes (some can be associated with non-FRET quenchers) and the oligonucleotide region can assume any sequence depending on the specific application. This plethora of possible combinations allow these probes to enjoy the aforementioned versatility (Didenko et al., 2001).

3.2 Genetically encoded FRET-probes

Genetically encoded FRET-probes are engineered proteins that display fluorescent probes as reporter elements, while the sensing domain is constituted by the polypeptide itself. Over the decades they have been applied to sense intracellular concentrations of ions and metabolites as well as voltage difference between membranes and transporters activity. In these applications, they are able to sense changes in living tissues at rates down to the Hertz range (60 s−1), making them a great tool for a non-invasive monitoring of the intracellular metabolism. Part of their versatility is due to the possibility of altering the sequence of the probe in

order to fit the experiment's needs; among those modifications, the most significant consists in adding an organelle's targeting sequence, which allows the protein to be transported to specific cellular departments without losing its functionality, increasing notability their spatial accuracy and therefore making them suitable for a wide range of studies and analysis.

Another fundamental step to take in account explaining the success of genetically encoded FRET-probes was the implementation of bacterial periplasmic binding proteins (PBPs). PBSs consist of a large family of proteins capable of specifically binding various sets of molecules, such as ions, amino acids and sugars. After the ligation they undergo a drastic conformational change which leads the extremities at close range. Because of that, they are excellent candidates for the role of the sensing domain, as their interaction with the analyte can efficiently cause a FRET event between two fluorophores attached at their ends. This conformational change is usually not disrupted by adding said dyes, therefore sandwiching a PBP between two fluorescent molecules has often brought to the formation of functional FRET probes without major complications, while of course their efficiency optimization requires empirical trial and error, testing different mutated variants. The bacterial origin of the linker domain is a useful feature that helps to avoid bona fide interactions with an eukaryotic intracellular environment. Compared to other genetically encoded fluorescent sensors, like intensiometric single-fluorescent protein probes, ratiometric FRET-based sensors are better suited for quantitative imaging, since they can eliminate measurement bias from the analyte concentration, and face a less complex development process (Mayuri et al., 2021).

3.3 SED1 probe

Our probe was the SED1 (Sensor Expressing Disordered protein 1) probe developed by Cesar L. Cuevas-Velazquez's group. It features mCerulean3 as the FRET donor and Citrine as the FRET acceptor. In order to associate the FRET event with intracellular density, the chosen sensitive domain towards molecular crowding was a member of the group 4 Late Embryogenesis Abundant(LEA) proteins from the model plant Arabidopsis thaliana. Proteins of that group possess IDRs (Intrinsically Disordered Regions), domains that lack a stable and defined three-dimensional conformation and undergo a constant and rapid shift to different transient conformations. Because of their flexible, elongated form they are more susceptible to interaction with the solute compounds and consequently more sensitive to changes in solute concentrations or solvent chemical characteristics. Furthermore, it has already been observed that LEA group 4 proteins undergo a strong and reversible disorder-to-folded transition coupled with the rise of MC. This makes them excellent candidates for the role of the sensitive domain of the probe, as their behavior in crowded media brings the fluorescent dyes close to each other leading to a FRET ratio change. SED1 probe has already been reported to be able to efficiently and dynamically visualize and quantify osmolarity shifts in the yeast cytoplasm, with a particular sensitivity

towards MC induced by macromolecules and amine-based compounds. (Cuevas-Velazques et al., 2021)

Our goal was to test whether and how this probe is able to sense and monitor MC when targeted inside the mitochondrial matrix.

3.4 Methods

Figure 1: pcDNA31_AtLEA4-5_CerCit.GA plasmid coding the SED1 probe, whose sequence is flanked by HindIII and NotI restriction sites.

Our starting material was the plasmid coding for the cytoplasmic probe AtLEA. In order to direct the expressed probe to the mitochondria, we cloned the coding sequence of AtLEA in a receiving vector containing a COX8 targeting sequence repeated 4 times. The cloning was carried out cutting both plasmids with the restriction enzymes HindIII and NotI, recovering the fragments of interest through gel electrophoresis and subsequent agarase dissolution of the gel fragment containing them and executing their ligation using an insert concentration threefold the concentration of the vector. The resulting plasmid with the mitochondrial targeting sequence was used to transform competent bacteria in order to amplify it through solid and liquid bacterial growth. The bacterial colonies were pelleted and lysed. DNA was eluted and the presence and sequence of the plasmid was verified with a Sanger DNA sequencing.

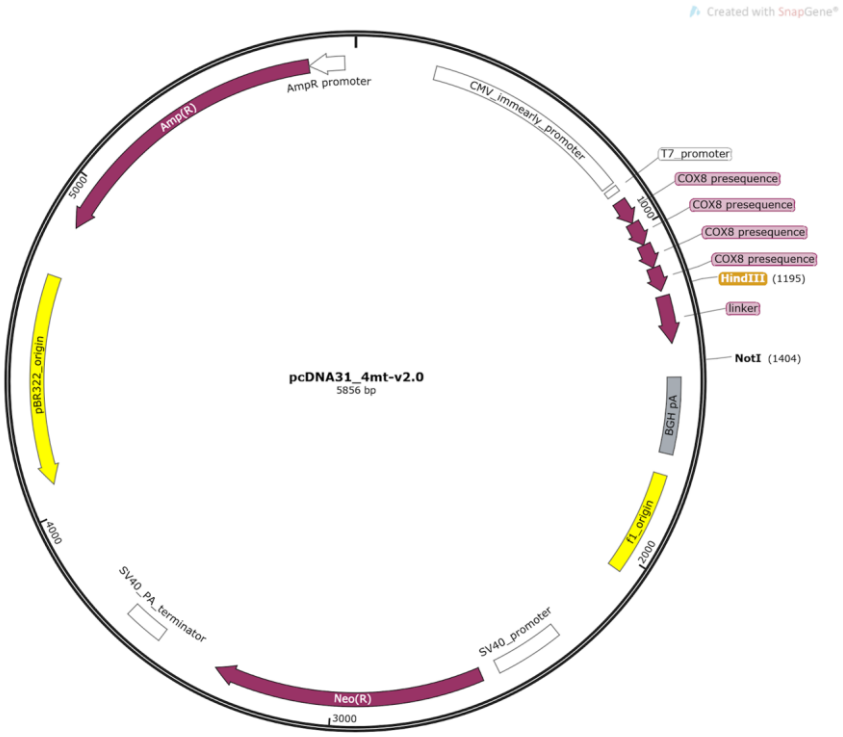


Figure 2: pcDNA31_4mt-v2.0 containing the mitochondria targeting sequence followed by HindIII and NotI restriction sites

A maxiprep following a second bacterial transformation was performed in order to assure sufficient DNA quantity for the following steps of transfection. Both standard and modified with target sequence plasmids coding the AtLEA probe were transfected in HeLa cells, which were left incubating for 2 days In order to allow the expression of the probe. We performed an asynchronous transfection using AtLEA plasmid and a reference plasmid coding an Equorin probe.

The plasmid amplifications were carried out in Top10 competent cells (Thermofisher) in Luria-Bertani terrain with ampicillin (the vector contained the proper gene of resistance). Ligation was performed with 100 ng of vector and a 3x molarity of the insert.

All the experiments were performed in HeLa cells (ATCC Number: CCL-2) cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco #41966052, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), containing penicillin (100 U/ml) and streptomycin (100µg/ml) (Euroclone). When needed, cells were seeded onto 24-mm glass coverslips and allowed to grow to 50% confluence before transfection.

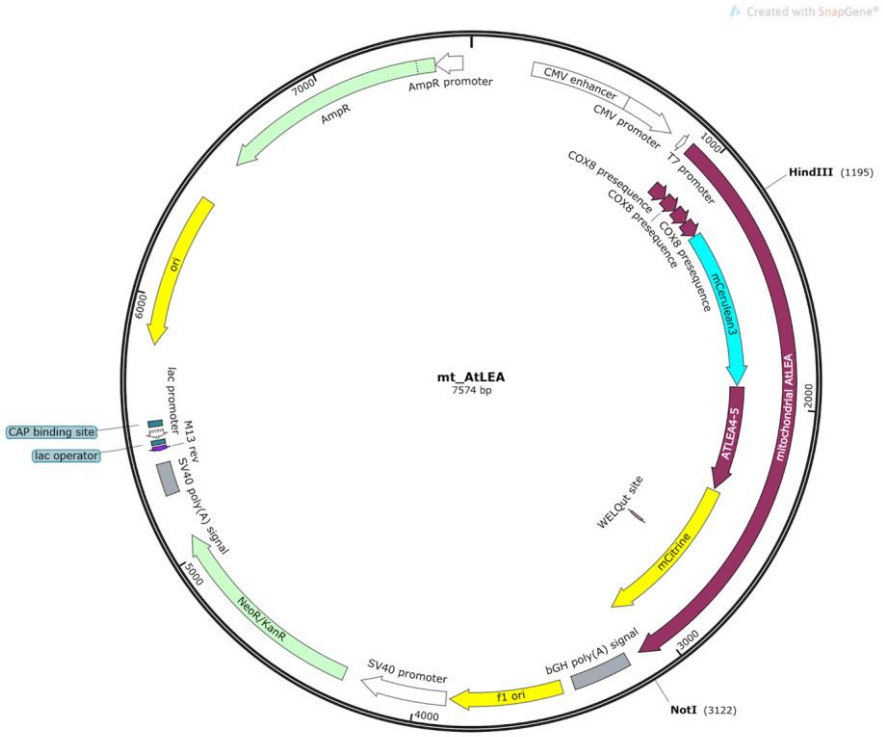


Figure 3: Plasmid containing the mitochondrial crowding probe

Widefield imaging was performed after treating the cells for a minute with a solution containing 25 mM Tris, 1 mM EGTA, 2 µM CCCP. After that they were put in a 100 µM digitonin solution (pH 7). Digitonin selectively binds cholesterol and acts as a selective permeabilizer of the plasma membrane, leading to the formation of pores and therefore the merging of the cytoplasm with the extracellular media. This step allows the direct exposure of mitochondria to solutions of a given osmolarity. FRET ratios were registered by adding distilled water and subsequent solution of sucrose of increasing concentrations (100 mM, 200 mM, 300 mM, 450 mM). We used standard widefield microscopy to test the functionality of our probe. To do this, HeLa cells were grown on 24 mm round glass coverslips at 50% confluence and transfected with the mitochondrial crowding sensor. Images were acquired on an Olympus IX73 microscope equipped with a 40x/1.3 N.A. Plan Fluorite objective. Excitation was performed with a Deltaram V high speed monochromator (Photon Technology International) equipped with a 75W Xenon Arc lamp. Images were captured with a high sensitivity Evolve 512 Delta EMCCD (Photometrics). The system is controlled by Metamorph 7.10 and was assembled by Crisel Instruments. Excitation was performed at 490 nm. Emissions were alternatively collected through 500–550 (donor channel) nm and 579-631(FRET channel) bandpass filters, using a filterwheel (Cairn Optospin). Images were acquired every 5 seconds with a fixed 200 milliseconds exposure time. All analyses were performed with the Fiji distribution of ImageJ.

4. RESULTS

We collected data from Hela cells both transfected with the cytoplasmic probe and with the mitochondrial probe using an inverted microscope. The cytoplasmic probe showed to be distributed uniformly in the whole cell, while the mitochondrial probe proved to be correctly targeted inside the mitochondria.

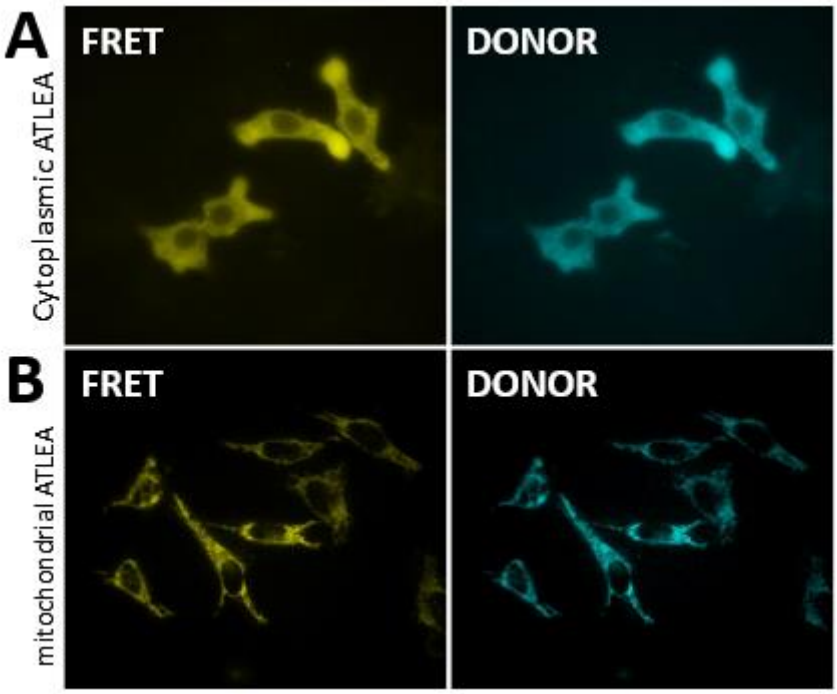


Figure 4: (A) Representative images of HeLa cells transfected with the cytosolic crowding probe. FRET and donor channels are colored in yellow and blue, respectively. (B) Representative images of HeLa cells transfected with the mitochondrial crowding probe. FRET and donor channels are colored in yellow and blue, respectively.

We permeabilized the cells in order to easily manipulate intracellular osmolarity and track the behavior of the sensor. We performed widefield imaging by exciting the donor dye, mCerulean3, and alternatively measuring fluorescence on the emission wavelength of the both the donor and acceptor dyes. We then perfused solutions with increasing osmolarity (by increasing the sucrose concentration of the medium). The probes showed an increase in the Citrine emission's intensity; however this signal by itself can't be compared to the one from other cells, as it is influenced by the quantity of probe inside a single cell, which varies in accordance to the latter's metabolism and transfection's efficency. In order to normalize the signal we took advantage of the following formula

$$FRET = \frac{F_{DA}}{F_{DD}}$$

where FDA is the emission of the acceptor and FDD is the emission of the donor. In this way our data is not affected by quantitative features of the environment but solely by the efficiency of the probe.

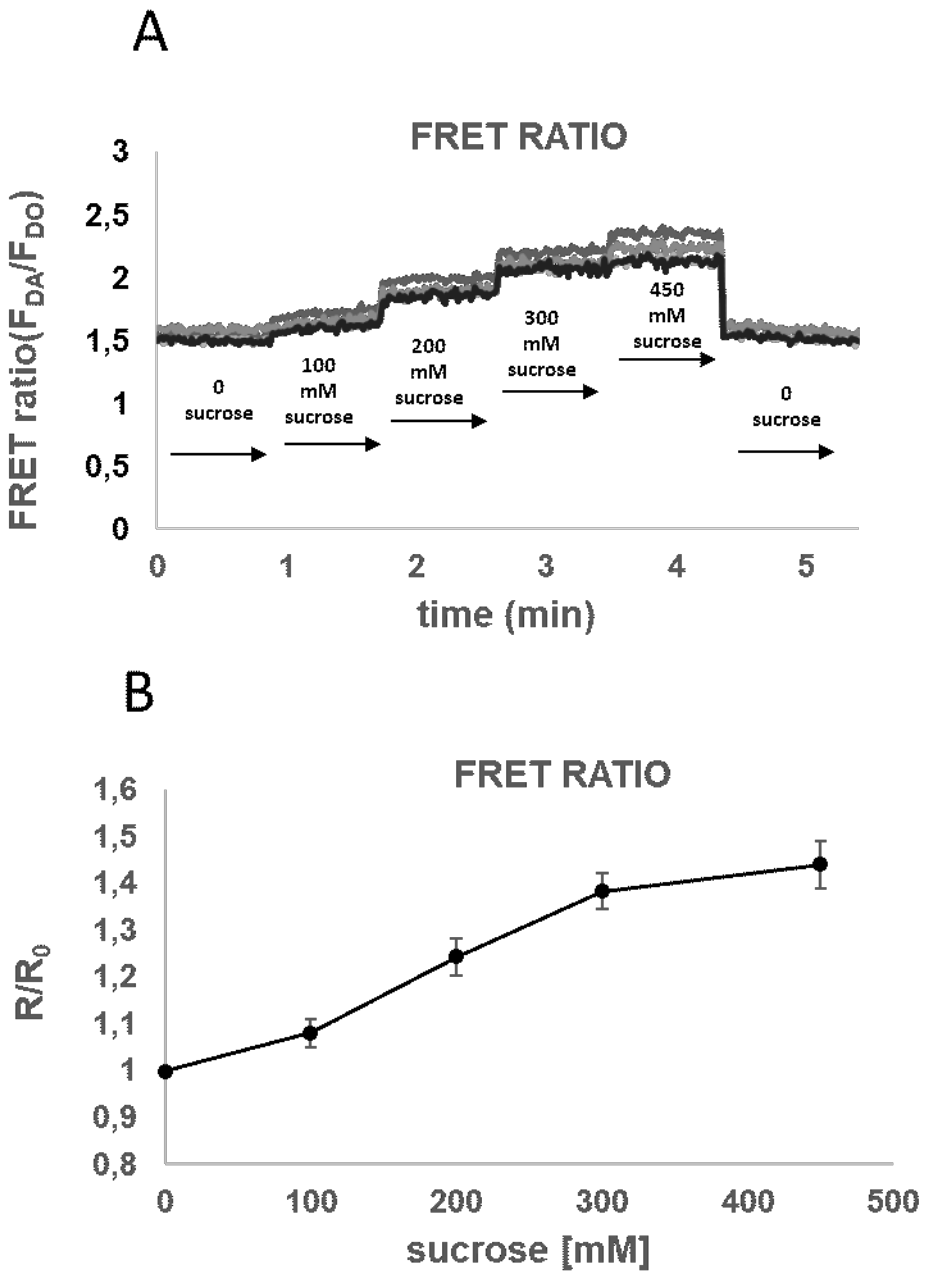


Figure 5. A: the graph shows changes in FRET ratios through time; when indicated (see arrows), cells were treated with solutions with increasing sucrose concentration. The different lines represent data collected from different cells. B: *the graph represents the variation in the FRET ratio along increasing concentrations of sucrose. The error represents the standard deviation.*

In the Figure 5A the different lines refer to different cells, all of which share a highly similar FRET ratio at every given position along the x axis, proving the sensor efficiency is independent of its amount inside the cell. The graphic shows that the FRET ratio increases noticeably and rapidly in response to each increase of sucrose

concentration and returns to the basal level when sucrose concentration returns to 0.

We plotted the recorded increases of the FRET ratio as a function of the sucrose concentration, and they showed to be directly proportional. This feature suggests the sensor is efficient and reliable in monitoring the mitochondrial crowding, as in the range from 0 to 450 mM the relation between sucrose molarity and FRET ratio is strong and consistent.

We tested the sensor's efficiency in tracking the environmental changes in mitochondria after treatment with Valinomycin, an ionophore capable of altering the membrane permeability of the mitochondria leading to an enhanced influx of potassium ions and a consequent swelling of the organelles. We registered a rapid decrease of FRET efficiency following the administration of the antibiotic, in accordance with the expected decrease of density in the mitochondrial matrix. This last experiment confirms the reliability of the probe on a functional level.

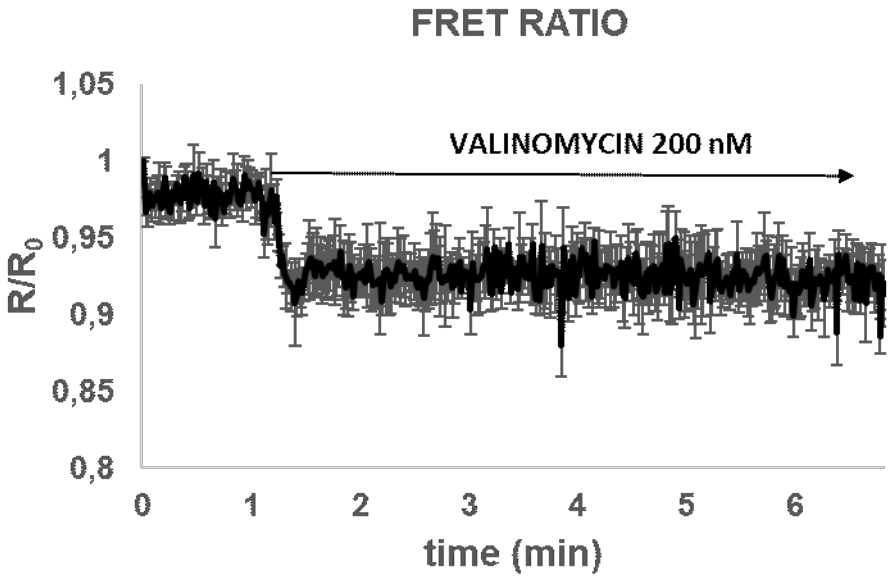


Figure 6: the graph represents the decrease of the FRET efficiency in mitochondria after treatment with valinomicin. The error represents the standard deviation.

5. DISCUSSION

The AtLEA probe has proven to be a viable and responsive tool in sensing the MC both in the cytoplasm and in the mitochondria. Under microscopy observation both versions of the probe behaved consistently with every osmotic alteration, and the mitochondrial probe appeared only inside mitochondria, proving the COX mitochondrial sequence to be reliable in directing this specific probe in the organelles.

Although preliminary, these results are promising and pave the way to future application of utmost interest. Further experiments could be performed by altering the cytoplasmic MC in various ways (e.g. osmotic stress, starvation) and investigating how organelles interact with the cytoplasm in order to adapt their metabolism to cellular demand. Given key role played by mitochondria in apoptosis, this probe could prove itself very useful in determining the relevance of MC in tuning the complex group of concerted reactions occurring cell deaths and, vice versa, whether and how those changes can influence intracellular and mitochondrial MC. It could also be used to observe the behavior of mitochondrial MC during the rise and development of pathologies or senescence, and to investigate its specific regulative role in many other cellular events, for instance exploiting cells carrying mutations affecting metabolism pathways or membrane transport. Currently we take advantage of a wide variety of probes for intracellular signals, like ions, and we believe they can be successfully combined in order to correlate multiple information in all aforementioned experiments.

Of course, compatibility between these eventual probes and our AtLEA probe should be evaluated first. Being at its first application in mitochondria, our probe should go through further validation steps to confirm that the mitochondrial environment, which carries differences in respect to the cytoplasm, the original target of the AtLEA probe designed by VELASQUEZ team, does not disrupt its reliability and function. Conditions such a pH higher than the cytosol, higher redox activity, different membrane potential could interfere with both the linking domain sensitivity and the dyes fluorescent characteristic, like for example by stabilizing the excited or grounded state of a fluorophore. Such an environment needs to be proven not impactful in the probe activity and every eventual interaction should be noted and taken into account in subsequent applications.

References

Kuznetsova, I. M., Zaslavsky, B. Y., Breydo, L., Turoverov, K. K., & Uversky, V. N. (2015). Beyond the excluded volume effects: mechanistic complexity of the crowded milieu. *Molecules (Basel, Switzerland)*, *20*(1), 1377–1409. https://doi.org/10.3390/molecules20011377

Didenko V. V. (2001). DNA probes using fluorescence resonance energy transfer (FRET): designs and applications. *BioTechniques*, *31*(5), 1106–1121. https://doi.org/10.2144/01315rv02

Sadoine, M., Ishikawa, Y., Kleist, T. J., Wudick, M. M., Nakamura, M., Grossmann, G., Frommer, W. B., & Ho, C. H. (2021). Designs, applications, and limitations of genetically encoded fluorescent sensors to explore plant biology. *Plant physiology*, *187*(2), 485–503. https://doi.org/10.1093/plphys/kiab353

Leopold, H. J., Leighton, R., Schwarz, J., Boersma, A. J., Sheets, E. D., & Heikal, A. A. (2019). Crowding Effects on Energy-Transfer Efficiencies of Hetero-FRET Probes As Measured Using Time-Resolved Fluorescence Anisotropy. *The journal of physical chemistry. B*, *123*(2), 379–393. https://doi.org/10.1021/acs.jpcb.8b09829

Model, M. A., Hollembeak, J. E., & Kurokawa, M. (2021). Macromolecular Crowding: a Hidden Link Between Cell Volume and Everything Else. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, *55*(S1), 25–40. https://doi.org/10.33594/000000319

Fung, H. Y. J., Birol, M., & Rhoades, E. (2018). IDPs in macromolecular complexes: the roles of multivalent interactions in diverse assemblies. *Current opinion in structural biology*, *49*, 36–43. https://doi.org/10.1016/j.sbi.2017.12.007

Wang, W., & Wang, D. (2019). Extreme Fuzziness: Direct Interactions between Two IDPs. *Biomolecules*, *9*(3), 81. https://doi.org/10.3390/biom9030081

Cuevas-Velazquez, C. L., Vellosillo, T., Guadalupe, K., Schmidt, H. B., Yu, F., Moses, D., Brophy, J. A., et al. (2021). Intrinsically disordered protein biosensor tracks the physical-chemical effects of osmotic stress on cells.. Nat Commun, 12 (1), 5438. https://doi.org/10.1038/s41467-021-25736-8