ALMA MATER STUDIORUM · UNIVERSITÀ DI BOLOGNA

Scuola di Scienze
Corso di Laurea Magistrale in Fisica

DEVELOPMENT OF A FLUORESCENT-BASED SINGLE LIPOSOME ASSAY FOR STUDYING CALCIUM TRANSPORTER LMCA1

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Sessione: III

Anno Accademico: 2014/2015

Acknowledgements

The experiments of this work were carried out in the Bio-nanotechnology and Biomedicine Laboratory under the supervision of Prof. Dimitrios Stamou. I would like to thank first Dimitrios for letting me be part of this research for the preparation of the thesis of my degree in Applied Physics. I would like to thank also my Italian supervisor Prof. Gastone Castellani who let me carry out the experiments of my thesis in a foreign country. It is a pleasure to thank my daily supervisor Mads P. Møller for introducing me to the laboratory and for supervising me throughout the entire project with a big amount of patience, professionalism and constant availability. I am thankful also to Garima Ghale for different tips and meaningful discussions along the way. I would like to thank all the members of the "transporter team" and I would also like to acknowledge Artù Breuer and Samuel Walsh for helping me to solve issues with the TIRF microscope and for advising me about Matlab programming. I am grateful to all the members of Stamou Lab for letting me see how you "make science" but also for all the fun moments. It was also a great opportunity to grow up as a person.

Thanks also for the big support from my family in Italy and from my international friends I met in Copenhagen.

Abstract

The morphological and functional unit of all the living organisms is the cell. The transmembrane proteins, localized in the plasma membrane of cells, play a key role in the survival of the cells themselves. These proteins perform a variety of different tasks, for example the control of the homeostasis. In order to control the homeostasis, these proteins have to regulate the concentration of chemical elements, like ions, inside and outside the cell. These regulations are fundamental for the survival of the cell and to understand them we need to understand how transmembrane proteins work. Two of the most important categories of transmembrane proteins are ion channels and transporter proteins. The ion channels have been depth studied at the single molecule level since late 1970s with the development of patch-clamp technique. It is not possible to apply this technique to study the transporter proteins so a new technique is under development in order to investigate the behavior of transporter proteins at the single molecule level.

This thesis describes the development of a nanoscale single liposome assay for functional studies of transporter proteins based on quantitative fluorescence microscopy in a highly-parallel manner and in real time. The transporter of interest is the prokaryotic transporter *Listeria Monocytogenes* Ca^{2+}*-ATPase1* (*LMCA1*), a structural analogue of the eukaryotic calcium pumps *SERCA* and *PMCA*. This technique will allow the characterization of LMCA1 functionality at the single molecule level. Three systematically characterized fluorescent sensors were tested at the single liposome scale in order to investigate if their properties are suitable to study the function of the transporter of interest. Further studies will be needed in order to characterize the selected calcium sensor and pH sensor both implemented together in single liposomes and in presence of the reconstituted protein LMCA1.

Sommario

L'unità morfologica e funzionale di tutti gli organismi viventi è la cellula. Le proteine transmembrana, localizzate nella membrana plasmatica delle cellule, svolgono un ruolo chiave per la sopravvivenza delle cellule stesse. Le funzioni svolte da queste proteine sono diverse, una delle più importanti è il controllo dell'omeostasi. Al fine di svolgere questo compito, tali proteine devono regolare la concentrazione di elementi chimici, quali ioni, all'interno e all'esterno della cellula. Questo tipo di processi di regolazione, fondamentali per la sopravvivenza della cellula, può essere compreso dallo studio funzionale delle proteine transmembrana. Due dei maggiori sottogruppi di proteine transmembrana sono rappresentati dai canali ionici e dalle proteine di trasporto. I canali ionici sono stati profondamente studiati a livello di singola molecola dalla fine degli anni settanta del novecento, grazie allo sviluppo della tecnica del patch-clamp. Tale tecnica non ha avuto successo per lo studio delle proteine di trasporto, così una nuova tecnica è in fase di sviluppo al fine di studiare il comportamento delle proteine di trasporto a livello di singola molecola.
Questa tesi descrive lo sviluppo di un assay nanometrico a liposoma singolo per lo studio funzionale delle proteine di trasporto. Tale assay è basato sulla microscopia a fluorescenza quantitativa e permette delle analisi in tempo reale e altamente parallelizzate. La proteina di nostro interesse è Listeria monocytogenes Ca^{2+}-ATPase1 (LMCA1), un analogo strutturale delle pompe eucariotiche del calcio SERCA e PMCA. Questa tecnica permette la caratterizzazione delle funzioni di LMCA1 a livello di singola molecola. Tre sensori fluorescenti caratterizzati sistematicamente sono stati testati con l'assay a liposoma singolo per verificare se le loro proprietà fossero adatte allo studio della proteina di trasporto d'interesse. Ulteriori studi saranno necessari per la caratterizzazione dei sensori di calcio e pH selezionati attraverso l'implementazione contemporanea degli stessi in liposomi e in presenza della proteina ricostituita LMCA1.

Contents

1 List of abbreviations

ATP Adenosine triphosphate

CCCP Carbonyl Cyanide m-Chlorophenyl Hydrazone

DTT Dithiothreitol

FRET Förster Resonance Energy Transfer

LMCA1 *Listeria monocytogenes* Calcium ATPase 1

LUVs Large Unilamellar Vesicles

MLV Multi Lamellar Vesicles

ND Neutral density (filter)

PLL-*g*-PEG Poly-L-lysine-grafted poly-ethylene-glycol

PMCA Plasma Membrane Ca^{2+}-ATPase

SERCA Sarco(Endo)plasmic Reticulum Ca^{2+}-ATPase

SUVs Small Unilamellar Vesicles

TIRF Total Internal Reflection Fluorescence

2 Introduction

In this thesis the characteristics and the aims of the fluorescent-based single liposome assay that is under development for functional studies of the calcium-proton antiporter LMCA1 at the single molecule level are explained. In the introduction section it is given an insight on the important role of membranes in biology with a

focus on homeostasis regulation (Section 2.1) and on the proteins that allow this regulation (Section 2.2). The most important assays and methods for studying ion channels and transporter proteins are explored (Section 2.3) and the transporter protein of interest is described (Section 2.4).
In chapter 3 the general feature of the single liposome assay is presented, focusing on the potentials given by exploiting liposomes (Section 3.1) and fluorescence probes (Section 3.2) in the assay. Finally the characteristics of the assay for studying LMCA1 at the single molecule level are described (Section 3.3).
In chapter 4 the experimental setup is described. The liposome preparation and the preparation of the support surfaces (flow cells) are explained (Section 4.1 and 4.3) and the experimental setups for spectrofluorometric experiments and TIRF microscopy experiments are shown (Section 4.2 and 4.4). In the last section the image analysis procedure is summarized (Section 4.5).
The experimental analysis and the results are presented in chapter 5, in which the characterization is divided by type of fluorophore implemented in the single liposome assay. In each section it was first presented the analysis of average emitted signal by all the liposomes and then the focus was shifted to analysis of signal emitted by individual liposomes.
In the last chapters 6 and 7 conclusions and future perspectives of this research can be found. List of materials and additional figures can be found in Appendix.

2.1 Biological membranes and transmembrane proteins

Cells are separated from the external environment by a plasma membrane. This membrane consists of amphipathic phospholipid molecules that form a phospholipid bilayer, where the polar heads of these molecules separate the hydrophobic coupled tails from the aqueous cytosolic and extracellular environments. This lipid bilayer forms a barrier that is relatively impermeable to most water-soluble molecules and it creates disequilibrium. This disequilibrium is fundamental for the cell to perform all the chemical reactions that are needed to convert the provided energy into a usable form and to fulfill the tasks necessary for survival. Even though the plasma membrane is impermeable, the cells are open thermodynamic systems, which mean they can exchange matter and energy with the external environment across the plasma membrane. In order to communicate with the outside environment, a number of transmembrane proteins are embedded in the bilayer.[1]
Transmembrane proteins have three regions or domains that can be defined: the domain into the bilayer, the extracellular domain outside the cell and the intracellular domain inside the cell. Even though a cell membrane is somewhat fluid, the orientation of transmembrane proteins does not change. These proteins play several roles in the functioning of cells. Receptors are useful for signaling to the cell what the

external environment contains. Linkers connect adjacent cells together by membrane junctions or anchor cells to extracellular matrix. Ion channels and transporter proteins are associated with controlling the exchange of materials across the membrane, allowing or preventing the passage of molecules or ions.[2,3]

2.2 Ion channels and transporter proteins

Ion channels let ions pass through the membrane according to the electrochemical gradient generated by a different concentration of ions inside and outside with respect to the cell membrane. Ion channels differ from porins located into the outer membrane of bacteria, mitochondria or chloroplasts as the latter are relatively large (their diameter can be approximately between 14 and 26 Å in the case of chloroplasts)[4a] and less selective compared with ion channel (their diameter can go down to 5 Å)[4b]. It is actually the selectivity the main difference between ion channels and porins. Due to the presence of a selectivity filter inside the structure of the channels, they are able to let pass only particular ions through the membrane with a transport rate 10^5 times greater than the fastest rate mediated by any known transporter protein. Another important distinction between ion channels and porins is that ion channels are gated. This means they are not constantly opened but they alternate opened states to closed states. Mostly the gate opens in response to a specific stimulus, for example a change in the voltage across the membrane, mechanical stress and binding of a ligand or of a neurotransmitter.[4c] When a stimulus happens, it modulate the macroscopic ionic conductances by influencing the probability that the single channels will be in their open state, not by modulating the single channel conductance. As a result the response of a single channel to repeated stimuli is still stochastic but if a large population is analyzed the response can be predictable. [5]

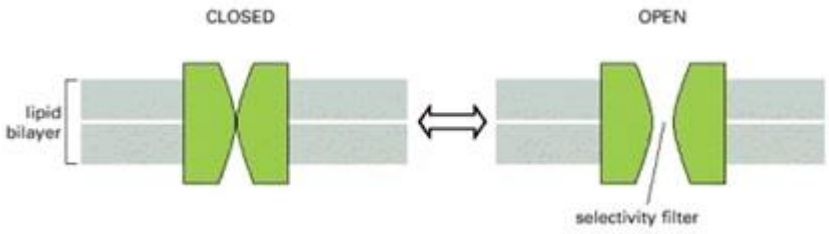

Figure 1 Cross section of opened or closed state of an ion channel. Here, a cross section of a channel protein is shown. It forms a hydrophilic pore across the lipid bilayer only in the "open" conformational state. Polar groups are aligned along the wall of the pore, while hydrophobic amino acid side chains interact with the lipid bilayer (not shown). In the opened conformation, the selectivity filter narrows to atomic dimensions in one region (the selectivity filter), where the ion selectivity of the channel is largely determined. (Molecular Biology of the Cell. 4th edition - Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002).

Transporter proteins have a function similar to that of ion channels. They bind specific solutes and transfer them across the lipid bilayer by undergoing conformational changes. One of the differences between ion channels and transporter proteins is the fact that transporters can carry a solute not only "downhill" following the electrochemical gradient but also "uphill" against the electrochemical gradient, using energy provided by ATP hydrolysis, by a downhill flow of another solute (such as Na^+ or H^+) or by light. The passage of the solute through the membrane by transporter proteins is based on different steps: the solute molecule binds to the transporter, the transporter undergoes conformational changes and finally the molecule is free to leave the transporter on the other side of the membrane. Since the passage of solutes depends on conformational changes of the transporter, the rate of transport goes from 10^0 to 10^4 ions or molecules per second, much slower compared to the one of ion channels.[4d] The transporters that carry solutes "downhill" can be called carriers while the ones that carry the solutes "uphill" using an extra source of energy can be called pumps. [4c]

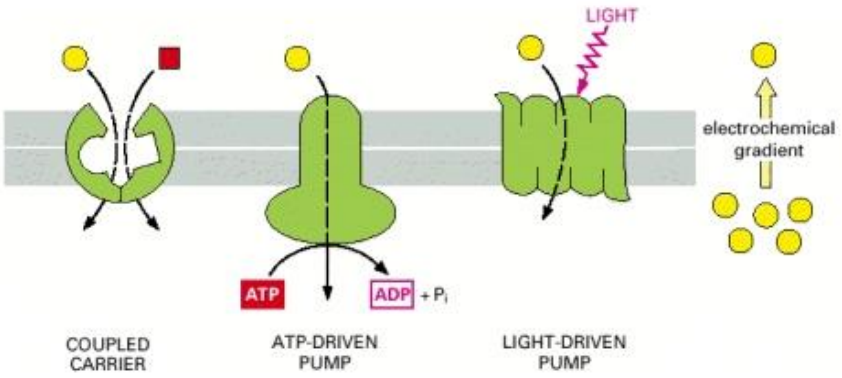

Figure 2 Three ways of driving active transport. "The actively transported molecule is shown in yellow, and the energy source is shown in red". (Molecular Biology of the Cell. 4th edition - Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002.)

2.3 Methods for studying ion channels and transporters

Ion channels are involved in a variety of fundamental physiological processes and represent a class of attractive drug target. Many techniques and assays were developed for studying ion channels. Nowadays ion channels can be studied in bulk and at the single molecule level in a high throughput manner and the available techniques are able to provide pharmacologically relevant data. [6,7] High throughput screening methods include ligand binding assay, flux-based assay, fluorescence-based assay, and automated electrophysiological assay. [8,9]

The first technique that allowed an insight on the behavior of single ion channel is the patch-clamp technique, developed by Neher and Sakmann (1976). This method is based on the electric isolation of a membrane's patch from the external solution and

on the resolution of currents through single ion channels. A glass micropipette filled with electrolyte is sealed on the plasma membrane of a cell where ion channels are located. The glass pipette represents the recording electrode and another electrode is put into the bath around the cell as a reference ground electrode, as it is shown in Figure 3. Since the diameter of the pipette's tip is of the order of micrometers, the number of ion channels inside the patch is very small and it is possible to reach diameters that can select just one single channel. The recorded current flow represents the current that passes through the ion channels.[10] As it is possible to see from Figure 4, the difference of current between opened and closed state can be around 5 pA and the noise is relatively low.

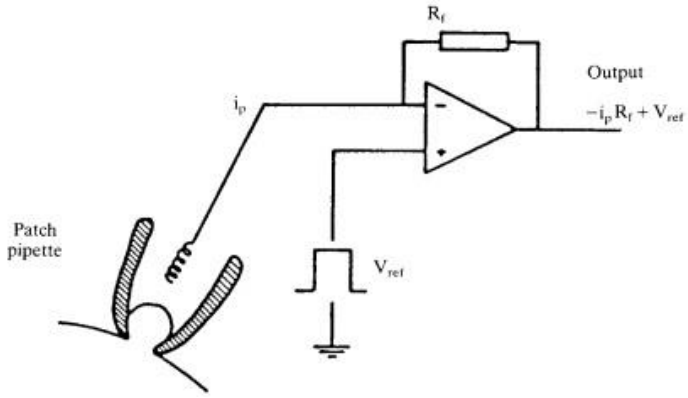

Figure 3 Example of a patch-clamp circuit. A high gain operational amplifier is connected to the circuit so that the current flowing through the ion channel is measured as a voltage drop across the resistor R_f. The resistance of R_f should be near 50 GΩ in order to decrease the noise. (Patch clamp techniques for single channel and whole-cell recording - DAVID OGDEN and PETER STANFIELD).

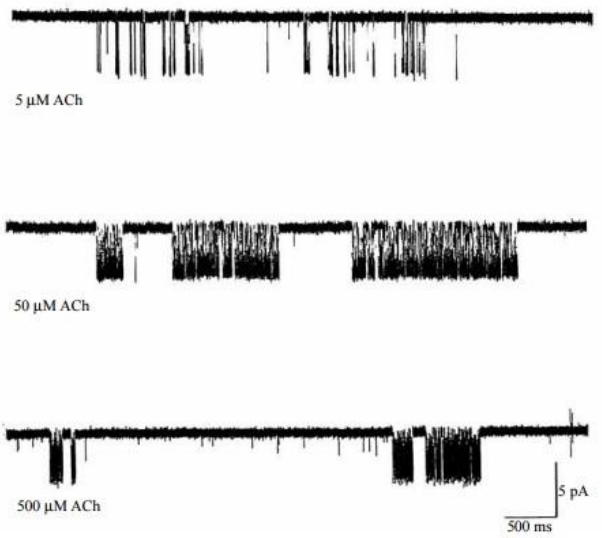

Figure 4 Examples of data recorded from patch clamp experiment. The current that flows through single channel molecules can be recorded using the patch-clamp method. The opening and closing of the gate produces two distinct current levels: a background-current when the gate is closed and a larger current when the gate is open. The probability that the gate opens depends on the control mechanism, for example in a chemically controlled channel on the concentration of the ligand. The data in the figure show rat skeletal neuromuscular junction single channel activated by acetylcholine. (Patch clamp techniques for single channel and whole-cell recording; DAVID OGDEN and PETER STANFIELD)

The conventional patch clamp technique was improved in order to have higher throughput and experimental conditions that could decrease the systematic error so new patch clamp techniques were developed, such as planar or lateral patch clamp.[11] Furthermore the technique was coupled with fluorescence assays that allow to have an insight on the intracellular side of channels and to directly investigate conformational changes and ionic currents across the membranes.[12,13,14]

On the other hand, transporter proteins play important physiologic roles such as nutrient uptake, secretion of proteins or signal molecules, exclusion of waste or exogenous compounds and energy transduction. As it is for ion channels, different techniques were developed in order to describe their properties. The patch-clamp technique was also used to study transporters at the single molecule level but the signal to noise ratio was too low due to the low transport rate of these proteins. It was only possible to notice "channels-like" behaviors on the analyzed transporters. [15,16]

The three most commonly used membrane assays for studying the large family of ABC transporters are the nucleotide trapping assay, the ATPase assay and the vesicular transport assay.[17]

The nucleotide trapping assay indirectly measures the rate of transport measuring the amount of nucleotide trapped in the binding site of the transporter. Usually the catalytic cycle of transport and the ATP hydrolysis involve the formation of a transition state complex. This complex contains the occluded nucleotide in the nucleotide binding site, which can be stabilized using trapping agents. This is not an high throughput method and it is mostly used to have an insight into the molecular mechanism of the transport process.[17]

The ATPase assay is based on the principle that compounds interacting with the transporter modulate ATPase activity of the latter. This assay indirectly measures the activity of the transporters measuring the amount of inorganic phosphate released by the enzyme during the ATP hydrolysis.[18a] The ATPase assay is one of the most widely used screening tools in the pharmaceutical industry. One of the problems of this assay is related to the fact that some compounds are translocated very slowly by the transporter, which results in a slow rate of ATP hydrolysis that do not yield detectable amount of inorganic phosphate.[17]

The vesicular assay measures the substrate carried into vesicles which have the ATP binding site and the substrate binding site of the transporter facing the buffer outside. Substrates of the transporter are taken up into the vesicles and the quantity of the transported molecules can be determined by different techniques after rapid filtration of the vesicles from the incubation solution. This assay type has the advantage of measuring the actual disposition of the substrate across the biological membrane but it works only for compounds with a low permeability to the vesicles' membrane.[17]

All the previously explained assays for studying transporters are not focusing on the single molecules but give information on an ensemble of transporter molecules. On the one hand it can be interesting to study emergent properties of ensembles as for

example the ones characterized by organized complexity because it is known that these properties arise as an interaction among smaller or simpler parts that do not exhibit such properties.[18b] On the other hand the average signal that comes from an ensemble of transporter proteins is characterized by an indistinguishable overlapping series of events and it is necessary to have a single molecule approach in order to discover a certain behavior that could be masked.[19]

In the recent years different single molecule assays were developed for studying transporter proteins at the single molecule level. Atomic force microscopy was used to probe topology, conformational changes and initial substrate-carrier interactions of Na^+-glucose co-transporter (SGLT1) in living cells on a single-molecule level.[20] In another research, molecular transporters of the cell-penetrating-peptide (CPP) type were studied. The transporter was fluorescently labeled and the interaction of the molecule with the plasma membrane of Chinese Hamster Ovary (CHO) cells was imaged.[21] Also other recent researches developed liposomes-based arrays for rapid and massively parallel single-molecule studies of activity of transporters.[22,23]

2.4 Transporter of interest: Listeria Monocytogenes Ca^{2+} ATPase 1

The assay that is under development aims to characterize the calcium pump LMCA1 found in the pathogenic bacterium *Listeria Monocytogenes*. *Listeria Monocytogenes* is a gram-positive facultative intracellular pathogen and a leading cause of listeriosis with high mortality rates (~30%). The organism survives diverse conditions such as low temperature, low pH and high salt concentrations.[24] The homeostasis of this bacterium, as it happens for other prokaryotes and also eukaryotes, is kept by membrane pumps. In particular LMCA1 maintains the intracellular Ca^{2+} concentration in the µM range. This pump belongs to the subgroup II of the P-type ATPase family, it shares a 38% sequence identity with SERCA and a 29% sequence identity with PMCA.[25] Since LMCA1 shares part of the sequence with SERCA, it can be considered its homologue. As it is possible to see from Figure 5, the pH optimum of LMCA1 is around 9 and the calcium affinity of LMCA1 is in the µM range. LMCA1 is an antiporter: it carries calcium ions out of the cell and it carries protons inside, with a stoichiometry of 1 Ca^{2+} : 1 H^+ per ATP hydrolyzed.[26]

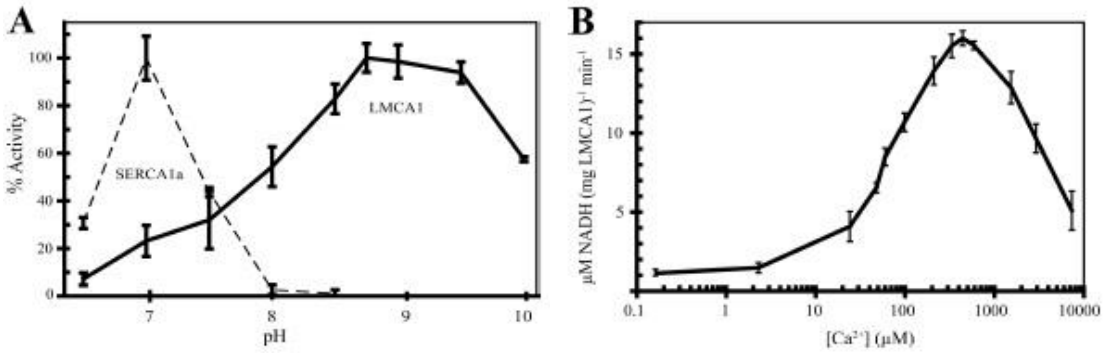

Figure 5 Activity of LMCA1 as a function of pH or calcium concentration.
A) ATPase activity of LMCA1 and SERCA1a (dashed line) as a function of pH. B) ATPase activity of LMCA1 as a function of Ca^{2+} concentration measured at pH 7.5. [26]

It is important to investigate calcium pumps for their role both in bacteria and humans. Calcium is one of the most important carriers of information for virtually all processes important to cell life, both for eukaryotic and prokaryotic cells. It has been seen that different pathologic conditions can be related to a dysfunction on the regulation of calcium concentration.[27] In particular the cardiac SERCA2a, an isoform of SERCA, regulates the calcium concentration in the heart muscles and it has a crucial role in contraction-relaxation processes of the heart. A decrease in expression level of this pump can lead to a variety of pathological conditions. [28,29,30]

3 Single liposome activity assay

In order to study the function of LMCA1 at the single molecule level, a fluorescent-based single liposome assay is being developed exploiting the possibilities given by liposomes coupled with fluorescent techniques, which make use of fluorophores sensitive to the substrate translocated by the pump itself.

3.1 Potentials of an assay based on single liposomes

Liposomes are synthetic vesicles composed at least of one lipid bilayer. There are different types of liposomes, according to size and number of lipid bilayers: SUVs, LUVs or MLVs.[31] In particular SUVs can be considered as small nanoreactors that can be used to perform a variety of different experiments at the nanoscale. Under certain conditions they have the ability to sustain electrochemical gradients and keep a spherical shape. They are made of lipids, this makes them an ideal biocompatible environment for reconstitution of transmembrane proteins. Also water-soluble molecules, for example biologically relevant molecules like DNA, can be encapsulated in their lumen to allow the investigation of their properties. They can be an ideal model system for studying processes that take place both in the lumen or on the lipid

membrane. In the past years different researches used single liposome assays, for example for the investigation of fusion processes of synaptic vesicles to presynaptic membrane in neurons and for studies about lipid membrane curvature dependent protein-recruitment. One of the most important and useful properties of an assay based on liposomes is the possibility of high throughput and a great potential for single molecule measurements. [32,33]

Different procedures can be followed to make SUVs. The one followed in this thesis is based on the rehydration of thin lipid films.[34,35] The liposomes are prepared mixing all the components of the liposomes' membrane in a vial. These are dried from the solvent with a nitrogen flow and with incubation in vacuum. After the drying process a thin film is formed on the bottom of the cuvette. Rehydration buffer containing the elements to be encapsulated is poured into the vial in order to form the liposomes, which are still multilamellar and with a high size heterogeneity. A freeze-thawing process performed on liposomes decreases multilamellarity, it increases the loading capacity and it breaks down the largest liposomes. It is even possible to extrude the liposomes through a polycarbonate membrane to select their diameter between 30 nm and 200 nm or larger sizes. It is necessary to fix the liposomes on a support in order to exploit the high throughput possibilities of the assay and to perform experiments on the single liposomes. A layer of biotinilated PLL-g-PEG or BAS is used to cover a glass support that represents the surface on which the fixed liposomes will be analyzed at the microscope. In this way the surface is passivated, in order to reduce unspecific binding.[32] This process makes also sure that liposomes adhere on the surface and prevents their deformation.[36] After the passivation process, NeutrAvidine or StreptAvidine are flushed into the chamber and they are attached to the surface by biotin anchors. Subsequently Liposomes are incubated into the chamber and they bind to NeutrAvidine/StreptAvidine tetramers by biotin anchors contained into liposomes' membranes.[37,38]

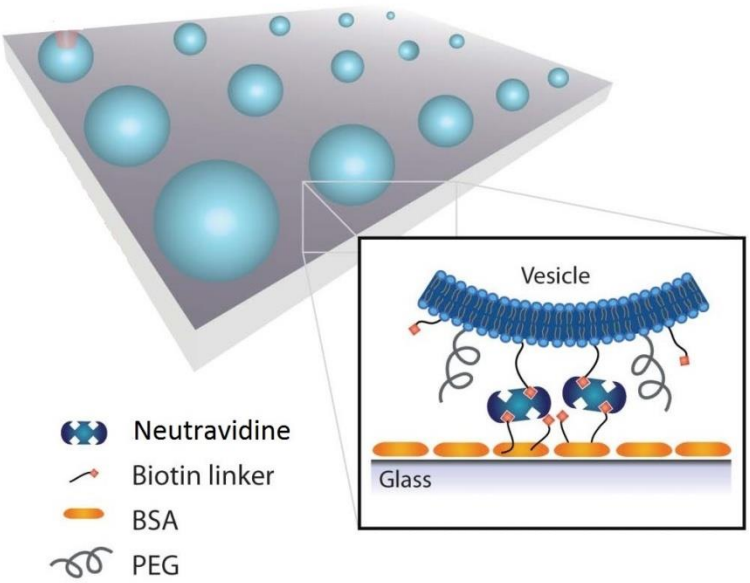

Figure 6 Array of liposomes attached to the passivated surface. The surface is passivated using BSA or PEG in order to avoid unspecific binding and to ensure that liposomes keep their shape. Liposomes are anchored to the surface by NeutrAvidine or StreptAvidine proteins. These proteins are linked both to liposomes and to the surface by biotin linkers. In this way liposomes are fixed on space and time. Since fluorophores can be embedded in liposomes, these can be analyzed at the TIRF microscope for investigation of biologically relevant molecules present into the lumen or into the membrane of the liposomes.[32]

3.2 Single liposome assay coupled with fluorescence techniques

Fluorescent dyes can be encapsulated in the lumen of liposomes or embedded into the membrane of liposomes. In this way the liposomes attached to passivated surface can by analyzed with TIRF microscopy. In the micrograph of Figure 7 is possible to see an example of immobilized liposomes recorded with TIRF microscopy technique. The liposomes are seen as diffraction limit spots and their intensity varies proportionally to the concentration of substrate at which the fluorophores are sensitive.[32] Analyzing the intensity of the liposomes is possible to have quantitative results regarding for example the concentration of the substrate present inside the lumen. The density of attached liposomes can be determined in order to keep them separated and to avoid overlapping liposomes. Using single liposome assays is possible to select, in one single frame, up to about 1000 not overlapping liposomes. It is possible to gain information about the average response of all the liposomes after an exchange of external conditions. On the same time it is possible to have an insight on the response of each single liposome, comparing their properties with each other and with the average response.

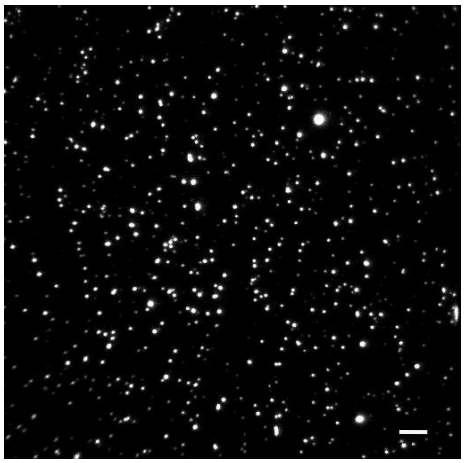

Figure 7 Micrograph of single liposome assay.
The liposomes, attached to the passivated surface, are seen as diffraction limited spots. It is possible to select each one of them and analyze their signal upon changes of the external buffer. These changes could lead to activation of reconstituted proteins that could allow us the study of their activity measuring the intensity changes of individual liposome. (scale bar of 10 μM).

3.3 Single liposomes activity assay for LMCA1

The lipids composition of the liposomes' membrane and the buffer are both chosen trying to simulate an environment in which the pump works. The transporter protein LMCA1 is supposed to be located into the membrane of liposomes after the reconstitution. The proteins can be oriented inward and outward. The ATP will be provided into the buffer outside so only transporters with an ATP-binding site that has access to ATP will work. In this conditions, when ATP is provided H^+ will be pumped out of the liposomes while Ca^{2+} ions will be pumped inside. In order to gain information about the activity of the pump, it is possible to take advantage of both the changes of pH and changes of Ca^{2+} concentration employing a pH sensor and a Ca^{2+} sensor. In order to make sure we are analyzing liposomes containing single molecules, the liposomes that show an activity should be a small percentage (around 10%) of the total number of attached liposomes during each activity measure. Since the pump has its maximum activity around pH 9, the activity experiments have to be performed in alkaline conditions so the pH sensor should be sensitive to changes between pH 6 and pH 10. Since the two fluorophores will have to be used together, they will have to be chosen considering their spectral properties: the excitation and emission peaks have to be at different wavelengths. Also when two types of fluorophores are employed in the same liposomes, it is necessary to keep the two types separated from each other to avoid cross talking. This phenomenon is a consequence of FRET: one excited fluorophore can transfer its energy to another fluorophore if they are close enough and if their spectra are overlapping. For this reason the pH sensor is conjugated to lipid and employed in the membrane of the liposomes while the calcium sensor is encapsulated into the lumen. Upon proton and calcium translocation, an accumulation

of positive charges inside the liposomes can build an electrochemical gradient that can modify the activity of the pump. In order to avoid this problem, ionophore valinomycin will be used to let get out from the lumen one K^+ for each Ca^{2+} pumped in.

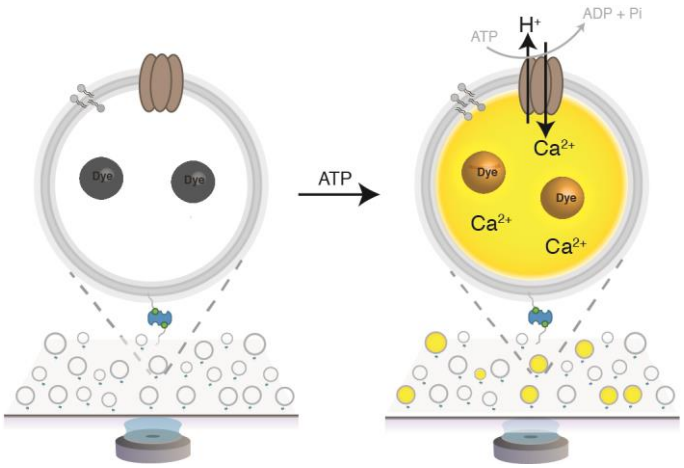

Figure 8 Single liposomes assay for functional studies of LMCA1. The liposomes are attached to the passivated surface and contain both fluorophores sensitive to calcium encapsulated into the lumen and lipid-coupled pH sensors on the membrane. After injection of ATP, pumps start pumping Ca^{2+} ions inside the lumen and H^+ outside into the external buffer. The fluorophores change their intensity according to Ca^{2+} or H^+ concentration changes. (In the Figure is shown only a change in intensity of calcium sensor). It is possible to record the intensity changes over time with a TIRF microscope and gain information about the activity of the transporter protein.

The aim of this project is the characterization of different fluorescent sensors to be employed in the single liposome assay for functional studies of the transmembrane protein LMCA1. Three different fluorescence sensors were previously characterized in bulk: the calcium sensors Fluo-5N, Oregon Green Bapta-5N and the pH sensor Snarf-DOPE. All of them showed suitable properties for studying LMCA1. We characterized them at the single liposome level to verify if these fluorophores could be employed for the investigation of LMCA1 with the single liposome assay.

4 Experimental setup and apparatus

4.1 Preparation of liposomes

The liposomes were prepared using the thin lipid film rehydration method.[34] The desired amount of lipids and possible membrane dye that will compose the liposome' membranes were poured in a clean glass vial using chloroform-resistant pipettes. The glass vials were left under nitrogen flow for approximately 10 minutes in order to have a complete evaporation of the solvent chloroform and until the formation of a thin film on the bottom of the vials was completed. Then the vials were left at least for one hour in a vacuum pump in order to be sure that the solvent was completely evaporated. If membrane dye was present, they were covered with aluminum paper during the process to avoid photobleaching. After the incubation in the vacuum pump, the dried thin film was rehydrated with a rehydration buffer of the desired pH, which was containing the dye that was supposed to be encapsulated. The rehydration buffer composition was always: Tris buffer (20 mM) at pH 8.7, KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM) and it contained also the dye to be encapsulated. DTT is a reducing agent that stabilizes the liposomes and the protein on their surface. It is supposed to be added fresh in every solution because it has a short life-time and it is sensitive to air oxidation. The volume of rehydration buffer had to be equal to the total volume of lipids and membrane dye used to form the lipid film. The rehydrated solution was pipetted in an eppendorf tube and then vortexed for 30 seconds. After vortexing, the tubes were dipped in liquid nitrogen for 1:30 minute and in hot water (50° C) for 5 minutes, for 10 times. The process of freeze-thawing was supposed to break down the biggest liposomes, to reduce the multilamellarity and to increase the dye encapsulation rate. Straight after the freeze-thawing process, the solution with liposomes were pipetted in small eppendorf tubes (20 µL), that were frozen in liquid nitrogen and then stored in the -20° C fridge, covering them with aluminum paper.

4.1.1 Snarf-DOPE containing liposomes

Snarf-DOPE liposomes were prepared with the lipid composition in which the protein is supposed to work during the activity measurements. The liposomes composition in molar percentage was:

E. coli polar lipids	74.3 %
Egg yolk PC	24.7 %
BT-PEG2000-DSPE	0.5 %
Snarf-DOPE	0.5 %

The total amount of rehydration buffer used was 500 µL and there was not any dye to be encapsulated. The vials containing free Snarf-DOPE dye were prepared by Camilla Thorlaksen.

4.1.2 Oregon Green Bapta-5N containing liposomes

Initially the Oregon Green Bapta-5N liposomes were prepared with the same composition used for Snarf-DOPE liposomes (E. Coli + egg yolk). Using this lipid composition and with a dye concentration of 100 µM in the rehydration buffer, 62% of liposomes resulted empty. Due to this poor dye encapsulation efficiency it was decided to use the following composition in molar percentage:

DOPC (850375C)	99.3 %
BT-PEG2000-DSPE	0.5 %
ATTO655-PE	0.2 %

The total amount of rehydration buffer used was 500 µL and it was containing Oregon Green Bapta-5N. Different concentrations of dye in the rehydration buffer were used: 250 µM, 100 µM, 50 µM, 25 µM, 10 µM. With this composition and with a dye concentration of 100 µM in the rehydration buffer, 25% of liposomes resulted empty.

4.2 Spectrofluorometric measurements

In order to characterize the dyes in bulk, spectrometric measurements were performed using a Horiba Jobin Yvon FluoroMax-4 spectrofluorometer, keeping the temperature at 25°C with a Wavelength Electronics LFI-3751 thermoelectric temperature controller. With a spectrofluorometer it is possible to record emission and excitation spectra of a fluorescent solution in order to investigate its fluorescent properties. As it is possible to see from the scheme in Figure 9 this instrument can have a xenon lamp as a source of exciting light. Monochromators are used to select both the emission and the excitation wavelengths. The gratings in the monochromators are used to decrease stray light. The excitation light passes through the shutter and reaches a beam splitter. Part of the excitation light is reflected to a reference cell,

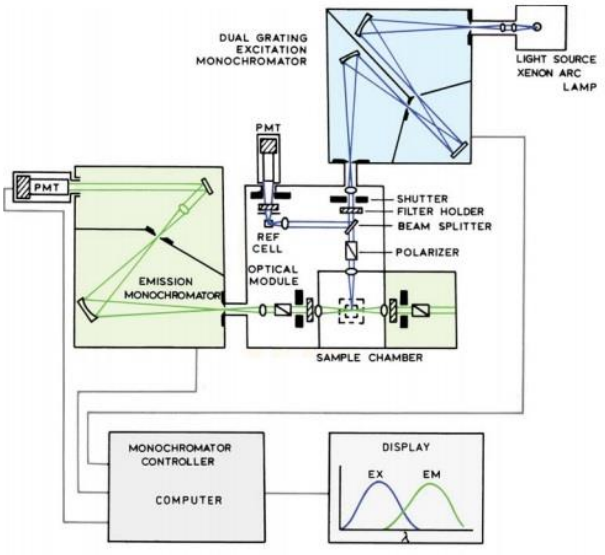

Figure 9 Scheme of a Spectrofluorometer.
A monochromator selects the wavelength of the excitation light. Part of it goes to the reference cell, part goes into the sample chamber. The light emitted by the sample passes through a second monochromator and then is collected, converted in digital signals and displayed on a monitor.

which generally contains a stable reference fluorophore. It is possible to correct the changes in the intensity of the arc lamp dividing the intensity from the sample by the intensity from the reference fluorophore. The light that passes through the sample goes through the second monochromator and finally arrives to the detector. The fluorescence is detected with photomultiplier tubes and quantified with the appropriate electronic devices, using counts per second (CPS) as a unit of measure.[39] In each experiment the sample solutions were added to a 1500 µL quartz cuvette, mixing the solution with a pipette to ensure homogeneity. The cuvette was cleaned before each experiment rinsing 5 times with milliQ and 5 times with methanol and then drying under nitrogen flow. The concentration of dye and the slit width were chosen in order to keep the counts range of the spectrofluorometer in the acceptable range between 100000 and 2 millions counts.

4.2.1 Snarf-DOPE - spectrofluorometric measurement

The measurements to obtain a pH-calibration curve for the pH sensor Snarf-DOPE were performed on 1 mL solution containing: Snarf-DOPE mock liposomes (about 100x dilution), Tris buffer (20 mM) at different pH values (from pH 4 to pH 12), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM), CCCP (5 µM) and Valinomycin (62.5 nM). To ensure the stabilization of the signal, 3 minutes of incubation were waited, monitoring the intensity of the signal with kinetic measurements. Every measure for each pH value was repeated in triplicate, each time preparing from scratch the solution to be injected in the cuvette. The cuvette was cleaned after each measure rinsing 5 times with milliQ and 5 times with methanol and then drying under nitrogen flow. The excitation wavelength used was 532 nm, the emission wavelengths range was from 550 nm to 700 nm. The slit width was 8 nm both for excitation and emission.

4.2.2 ATTO655-DOPE - spectrofluorometric measurement

The mock liposomes used in this experiment were fabricated by Mateusz Dyla from the Molecular biology and genetics Department, Aarhus University, Denmark. The liposomes composition was:

E. coli polar lipids	74.66 %
egg yolk PC	24.89 %
DOPE-cap-biotin	0.30 %
ATTO655-PE	0.50 %

and the rehydration buffer used contained: Tris-HCl pH 8.5 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM).
The measurements to obtain excitation and emission spectra for the fluorescent dye ATTO655-DOPE were performed on 1 mL solution containing: ATTO655-DOPE mock liposomes (about 200x dilution), Tris buffer pH 8.7 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM), Ca^{2+} (25 µM), ionomycin (10 nM) and valinomycin (62,5 nM). Four

emission spectra were collected setting the following excitation wavelengths: 640 nm, 532 nm, 491 nm, 405 nm. The emission wavelengths range was from 650 nm to 800 nm. The excitation spectrum was collected setting an excitation wavelength range from 400 nm to 670 nm and an emission wavelength at 679 nm that corresponds to the emission peak of the dye in these conditions. The slit width was 4 for excitation and 3 for emission.

4.3 Preparation of flow cells

In order to perform the experiments at the TIRF microscope, a support where to fix the fluorescent liposomes was needed. Flow cells were used since it is possible to fix on them the liposomes and quickly exchange the external buffer. Flow cells were prepared assembling together cleaned glass slides (thickness 170 ± 10 µm, Assistant, Sondheim, Germany) and a sticky-slide VI 0.4 (Ibidi, Germany). Glass slides were cleaned by 2 sonications in 2% Helmanex, 2 in milliQ water and 1 in methanol storing them in methanol. The glass slide was dried from Methanol under nitrogen flow and then it was put inside a plasma cleaner (PDC-32G; Harrick Plasma) for 3 minutes under vacuum. Right after the cleaning, the glass slide was put on a sticky-slide and then the tubing was inserted ("thick" tubing: TYGON standard, 1.6/4.8 mm, IDEX Health & Science GmbH, United States – "thin" tubing: Polytetrafluoroethylene, 1.0x0.5 mm, Bohlender GmbH, Germany). The flow cell was connected to a pump and a 200 µL of 1.0 g/l pll-PEG-Biotin/pll-PEG (1:100) solution was flushed into the chambers and incubated for 30 minutes. Finally 2 mL of HEPES buffer was flushed into the chambers and the flow cell was stored in a fridge at 4 °C.

Before each experiment the chamber was flushed with 1 mL of HEPES buffer and 200 µL of 0.1 g/l NeutrAvidin in HEPES buffer. After incubation of 10 minutes, 2 mL of sample buffer were flushed into the chambers. The liposomes were immobilized on the surface flushing with 250 µL of sample with the concentration needed for the experiment. Depending on the desired density of liposomes, a different incubation time was waited. After the incubation the non-bound vesicles were flushed away flushing again with 2 mL of sample buffer.

4.4 TIRF microscopy

TIRF microscopy is a technique based on an induced evanescent wave of excitation. The depth of this evanescent wave determines the limit of penetration of light over the cover slip and it allows a focus only on the sample extremely close to the surface, on a range of some nanometers. The evanescent wave is generated only when the incident light becomes totally internal reflected at the glass-sample interface. This happens for a particular incident angle called critical angle. This technique is very useful to investigate processes that occur close to the plasma membrane. In this case it was necessary in order to have a high temporal resolution and to reduce the background noise.[40] The microscope used had four different lasers with emission wavelengths of 405 nm, 491 nm, 532 nm and 640 nm. It was possible to use the microscope both in single view or dual view. When used in single view, the camera collects the light coming from the sample without additional filters, except the excitation filter that cuts the wavelengths of the laser. When used in double view, the light that comes from the sample, after the excitation filter, passes through a system of mirrors that contains an additional dichroic mirror. This dichroic mirror splits the signal in two parts, sending the light above a certain wavelength (640 nm in our case) to one half of the camera and the light below to the other half. The microscope settings (laser power, exposure time, presence of additional ND filters and type of ND filters) were determined in order to quantify and minimize photobleaching. The percentage of average signal decreased due to photobleaching in each experiment was 10% of the total average signal. Photobleaching measurements were carried out in the same condition in which the experiment was supposed to be performed.

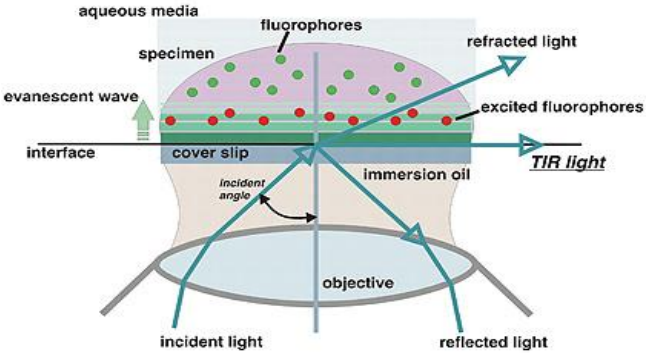

Figure 10 Total Internal Reflection Fluorescent Microscopy. The sample is excited by the incident light. When light is totally internally reflected, the evanescent wave is generated and it excites only fluorophores immediately above the cover slip, decreasing the background noise.

4.4.1 Snarf-DOPE - TIRF microscopy measurement

The mock liposomes containing membrane dye Snarf-DOPE used for the pH titration were fabricated by Camilla Thorlaksen. The liposomes composition in molar percentage was:

E. coli polar lipids	74.3 %
egg yolk PC	24.7 %

BT-PEG2000-DSPE	0.5 %
Snarf-DOPE	0.5 %

and the rehydration buffer used contained: Tris-HCl pH 8.5 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM).

- *pH titration*

The liposomes (30x dilution) were incubated for 15 minutes then 2 mL of sample buffer at pH 12 (Tris-HCl (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1mM), CCCP (5 µM), Valinomycin (62.5 nM)) were used for flushing away the non-bound liposomes. The right position of the sample was chosen and after 3 minutes of incubation, 5 images were recorded. Sample buffer at pH 11 was flushed and after 3 minutes of incubation, 5 images were recorded. This process was repeated also for the pH values in the range between pH 10 and pH 4 in order to obtain the calibration curve. The excitation wavelength of the laser was 532 nm (20% laser power), the exposure time was 500 ms, the microscope was set on double with a cutoff wavelength of 640 nm, and a neutral density filter 3.0 was used to decrease the power of the laser. The experiment was repeated in triplicate.

4.4.2 Oregon Green Bapta-5N - TIRF microscopy measurement

- *Investigation of self-quenching effect*

The liposomes (200x dilution) were incubated for 5 minutes then 2 mL of sample buffer (Tris-HCl pH 8.7 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1mM)) were used for flushing away the non-bound liposomes. The right position of the sample was chosen and a movie of 10 minutes was recorded in order to see if an increase of signal could indicate the presence of self-quenching effect for the encapsulated dye. This experiment was repeated for liposomes containing different concentration of Oregon Green Bapta-5N: 250 µM, 100 µM, 50 µM, 25 µM, 10 µM. The excitation wavelength of the laser was 491 nm (15% laser power) for the Oregon Green Bapta-5N channel and 640 nm (8% laser power) for the ATTO655 channel, the exposure time was 500 ms, the microscope was set on single view and a neutral density filter 2.0 was used to decrease the power of the laser.

4.4.3 Fluo-5N - TIRF microscopy measurement

The mock liposomes containing encapsulated Fluo-5N (they will be called *full* liposomes) were fabricated by Mateusz Dyla from the Molecular biology and genetics Department, Aarhus University, Denmark. The liposomes composition in molar percentage was:

E. coli polar lipids	74.66 %
egg yolk PC	24.89 %
DOPE-cap-biotin	0.30 %
ATTO655-PE	0.50 %

and the reconstitution buffer used contained: Tris-HCl pH 8.5 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM), Fluo-5N (1 mM).

Mock liposomes without any encapsulated dye (they will be called *empty* liposomes) were fabricated by Mateusz Dyla from the Molecular biology and genetics Department, Aarhus University, Denmark. The liposomes composition in molar percentage was:

E. coli polar lipids	74.66 %
egg yolk PC	24.89 %
DOPE-cap-biotin	0.30 %
ATTO655-PE	0.50 %

and the rehydration buffer used contained: Tris-HCl pH 8.5 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM).

- *Ca^{2+} titration*

The *full* liposomes (200x dilution) were incubated for 10 minutes then 2 mL of sample buffer (Tris-HCl pH 8.5 (20 mM), KCl (200 mM), DTT (1mM)) were used for flushing away the non-bound liposomes. The right position of the sample was chosen, 5 images were recorded both on the fluo-5N channel and on the ATTO655 channel and then sample buffer containing ionomycin (10 µM) was flushed. After 3 minutes of incubation 5 images were recorded on the fluo-5N channel then sample buffer with ionomycin (10 µM) and calcium (1 µM) was flushed. This process was repeated for all the different calcium concentrations needed to obtain the calibration curve: 1 µM, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 200 µM, 300 µM and 500 µM. The excitation wavelength of the laser was 491 nm (15% laser power) for the Fluo-5N channel and 640 nm (8.5% laser power) for the ATTO655 channel, the exposure time was 500 ms, the microscope was set on single view and a neutral density filter 2.0 was used to decrease the power of the laser.

- *Investigation of cross emission of ATTO655*

In this experiment were used both the *empty* liposomes and the *full* liposomes. The *empty* liposomes (200x dilution) were incubated around 10 minutes then 2 mL of sample buffer (Tris-HCl pH 8.5 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM), valinomycin (62.5 nM)) were used for flushing away the non-bound liposomes. The right position of the sample was chosen and fifteen images of empty liposomes were

recorded: five on the ATTO655 channel and ten on the Fluo-5N channel. Subsequently the *full* liposomes were incubated and after about 10 minutes 2 mL of sample buffer were used for flushing away the non-bound liposomes. Again fifteen images were recorded as previously in the same position. The excitation wavelength of the laser was 491 nm (55% laser power) for the Fluo-5N channel and 640 nm (15% laser power) for the ATTO655 channel, the exposure time was 500 ms, the microscope was set on double view and a neutral density filter 3.0 was used to decrease the power of the laser. The experiment was repeated in duplicate.

- *Unspecific binding to the membrane of liposomes*

The *empty* liposomes (200x dilution) were incubated for 10 minutes then 2 mL of sample buffer (Tris-HCl pH 8.5 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM), valinomycin (62.5 nM)) were used for flushing away the non-bound liposomes. The initial part of the experiment was performed to check if the injection of calcium could modify the signal. The right position of the sample was chosen and fifteen images of the initial condition were recorded: five on the ATTO655 channel and ten on the Fluo-5N channel. 200 µL of sample buffer with calcium (500 µM) were flushed. Ten images on the Fluo-5N channel were recorded. In the second part of the experiment the system was brought back to the initial conditions. Fifteen images of the initial condition were recorded: five on the ATTO655 channel and ten on the Fluo-5N channel. 200 µL of sample buffer with free Fluo-5N (100 µM) were flushed. Ten images on the Fluo-5N channel were recorded. 200 µL of sample buffer with calcium (500 µM) were flushed. Ten images on the Fluo-5N channel were recorded. The excitation wavelength of the laser was 491 nm (45% laser power) for the Fluo-5N channel and 640 nm (15% laser power) for the ATTO655 channel, the exposure time was 500 ms, the microscope was set on double view and a neutral density filter 3.0 was used to decrease the power of the laser. The experiment was repeated in triplicate.

4.5 Image analysis

All the images recorded with the TIRF microscope were first preprocessed with ImageJ or FIJI and subsequently analyzed with Igor's routines. The preprocessing stage was useful firstly to increase the signal to noise ratio: images of the sample were recorded minimum in triplicates and then averaged so as the background noise could be decreased. Secondly it was possible to align the images recorded throughout all the experiment and correct the drifting effect: all the images were aligned using translations and rotations according to the brightest one of the recorded stack. This aligning process could be done manually or using the specific plug-in of ImageJ: StackReg. During this stage the images were also rearranged in the right order, cut or combined in order to be analyzed.

Then the preprocessed images were analyzed using one of the particle tracking and analyzing routines written in Igor: NGPA by S. M. Chrsitensen or TAMT by M. Tuktus. In particular TAMT was used to analyze the images of Snarf-DOPE while NGPA was used for the rest of the images. Both the routines perform a 1D or 2D gaussian fitting on intensity peaks over a user defined threshold. The integrated intensity represents the fluorescent signal from the immobilized liposomes. It is possible to accept or reject the results according to user defined parameters such as minimum size, circularity and fitting error. It is also possible to colocalize signal between two different channels, for example this is necessary to analyze data from a ratiometric experiment or this is useful when more than one dye is employed into the liposomes. In our case it was useful to colocalize signal emitted by membrane dye (ATTO655) and encapsulated dye (Oregon Green Bapta-5N or Fluo-5N): in this way it was possible to exclude those liposomes that do not colocalize such as empty liposomes (their signal comes only from ATTO655) and conglomerates of free dye unspecifically bound to the surface (their signal comes only from Oregon Green Bapta-5N or Fluo-5N). Once the liposomes are selected on the first frame, the program can keep track of them through a series of images. Finally it is possible to plot the intensity of each liposome versus time or versus other quantities such as pH values or ions concentrations. Whit TAMT it was also possible to correct the images for the background using three different methods: “rolling ball” algorithm, gaussian filtering and low-frequency filtering.

5 Data analysis and results

The main goal of the single liposome assay for functional studies of LMCA1 is to allow an insight on the activity of the pump at the single molecule level employing fluorescent sensors that are sensitive to concentration changes of the substrate translocated across the liposomes' membrane by the pump itself. The liposomes are immersed in an aqueous medium in which other elements such as ions can be flushed during the experiments. Both the composition of liposomes' membrane and aqueous buffer were chosen in order simulate in the best way possible the biological environment in which the protein naturally works. In the following sections the characteristics of three different fluorescence sensors will be discussed and it will be described their suitability to the single liposome assay.

5.1 Characterization of Snarf-DOPE for the single liposome assay

The compound Snarf-DOPE consists of the pH sensor Carboxy Snarf-1 conjugated to lipid DOPE. This compound was synthesized for our use by Thomas Pomorski. [41] Carboxy Snarf-1 is a cell impermeable pH sensor. It has an excitation peak at 532 nm while it has two emission peaks: one at 583 nm and one at 640 nm. The presence of two peaks is due to the fact that this dye has two fluorescent states: monoanion and dianion. Upon changes of pH it exhibits an emission wavelength shift from 583 nm to 640 nm. The presence of two peaks can be useful in order to perform ratiometric experiments. It was decided to conjugate Carboxy Snarf-1 to lipid DOPE in order to obtain Snarf-DOPE and to use it as a membrane dye. The presence of Snarf-DOPE into the membrane of liposomes will allow us the measurement of pH changes of the environment in which liposomes are immersed. In the following sections properties of Snarf-DOPE will be shown, both in bulk and in the single liposomes assay. CCCP, a protonophore that translocates protons across the lipid bilayer, was used during the experiments in order to equilibrate the concentration of protons inside and outside the liposomes upon changes of buffer at different pH values.

5.1.1 Bulk characterization

In order to verify if the properties of Snarf-DOPE in bulk could suite the single liposome assay for studying LMCA1, spectrofluorometric experiments were performed. The response of Snarf-DOPE to pH changes was investigated as so to calculate the fold increase of the intensity ratio. Snarf-DOPE liposomes were dissolved into sample buffer containing CCCP and valinomycin and its intensity was measured upon changes of pH. For details on the exact protocol used to perform the pH titration see Section 4.2.1 . As it is possible to notice from Figure 11-A, the dye exhibits two emissions peaks: one more prominent at 583 nm and the other one at 650 nm. As far as the pH

increases, the peak at 583 nm increases while the peak at 650 nm decreases, undergoing a fluctuation in the intensity of emitted light. It was possible to calculate the intensity ratio curve in Figure 11-B dividing the intensity at 583 nm by the intensity at 650 nm for all the different pH values.

As it is possible to see in Figure 11-B, the intensity shift is localized between pH 6 and pH 10, which is the range in which LMCA1 is supposed to have high activity. The intensity ratio increases of I_1 = (3.3 ± 0.2) units in this range. A sigmoidal fit was performed on the intensity ratio curve and it was found a $pK_{a\ bulk}$ = (9.3 ± 0.1). The function used to fit the intensity ratio curve is a sigmoidal function defined as follow:

$$f(x) = base + \left\{ \frac{max}{1 + e^{\left(\frac{(xhalf - x)}{rate}\right)}} \right\}$$

The parameter that represents the pK_a of the intensity ratio curve is xhalf, defined as the x at which the function has a value corresponding to half of the maximum saturation value.

Since the value of $pK_{a\ bulk}$ is in a range in which LMCA1 has a high activity and the intensity ratio increases of I_1 = (3.3 ± 0.2) units, Snarf-DOPE has the right bulk properties to be tested also with the single liposome assay for studying LMCA1.

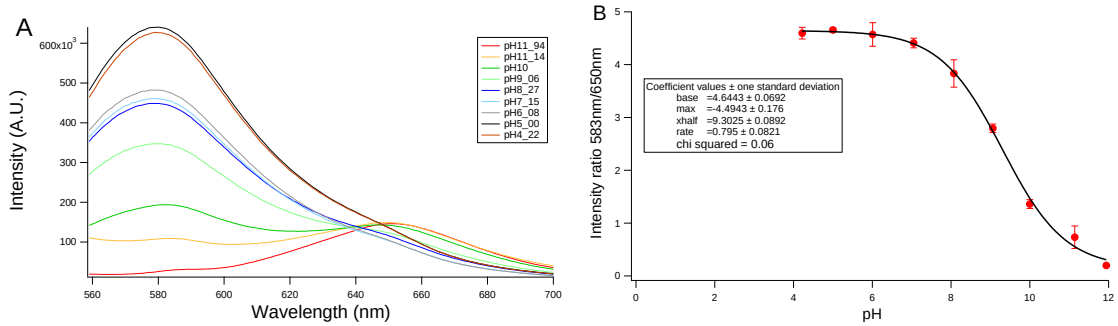

Figure 11 Spectrofluorometric measurements of liposomes containing membrane dye Snarf-DOPE

A) Emission spectra for different pH values showing the presence of two peaks at the wavelength of 583 nm and 650 nm. Every spectrum is an average of three different spectra.

B) Intensity ratio profile was determined by dividing the emission peak at 583 nm with the corresponding emission peak at 650 nm. The curve was fitted with a sigmoidal function from which it is possible to obtain the average $pK_{a\ bulk}$ = (9.3 ± 0.1). Each data point was obtained as an average between three experiments. The error bar is the standard deviation. The liposomes where diluted about 100x in a solution of Tris buffer 20 mM at different pH containing: 1 mM $MgCl_2$, 200 mM KCl, 1 mM DTT, 5µM CCCP and 62.5 nM valinomycin.

5.1.2 Single liposomes level characterization: calibration curve and pK_a distribution

In order to characterize Snarf-DOPE at the single liposomes level, the liposomes containing Snarf-DOPE were attached to the surface of a flow cell, following the procedure explained in Section 4.3 . In order to quantify the response of the

fluorophore to pH changes, a ratiometric experiment was performed. It was necessary to select two different emission ranges using the dual view of the microscope: a lower range in the interval of wavelengths (582 ± 37.5) nm and an higher range in the interval of wavelengths over 650 nm. It is possible to see from Figure 12 an example of a micrograph showing liposomes during the ratiometric experiment: the upper part of the image represents the emission signal in the range [582 ± 37.5] while the lower part represents the emission in the range over 650 nm.

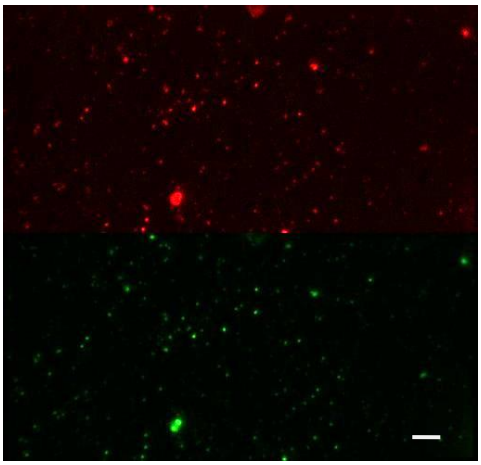

Figure 12 Micrograph showing liposomes attached to the passivated surface during the pH titration. In the upper half, in red, the 650 nm channel. In the lower half, in green, the 583 nm channel. The analysis was performed only on liposomes colocalized between these two channels. (Scale bar = 10 μM).

Liposomes were colocalized between the 650 nm channel and the 583 nm channel (over 80 % colocalization rate). During the analysis each colocalized liposome was fitted with a two dimensional gaussian function: the intensity of each liposome is represented by the area underlying the fitting function. Only fits with an error on the intensity lower than a tenth of the estimated value were accepted. The average emission signal of the two different channels was plotted as a function of the pH values where the intensity was normalized to the intensity at pH 12, as it is shown in Figure 13-A,B. The average intensity ratio is shown in Figure 13-C. It was calculated dividing each data point of 583 nm channel by the corresponding data point of 650 nm channel. A sigmoidal fit was performed on the average intensity ratio in order to calculate the average $pK_{a\ microscope\_avg}$ = (8.0 ± 0.1). Snarf-DOPE responded to pH changes also in the single liposome assay: the intensity ratio increases of I_2 = (5.5 ± 0.4) units between pH 10 and pH 6. Both the *$pK_{a\ microscope\_avg}$ value* and the *increase of the intensity ratio* I_2 in the pH range between pH 10 and pH 6 suggest that Snarf-DOPE can be a good candidate for functional studies of LMCA1 because this fluorophore can sense pH changes in an alkaline environment in which activity experiment will be performed.

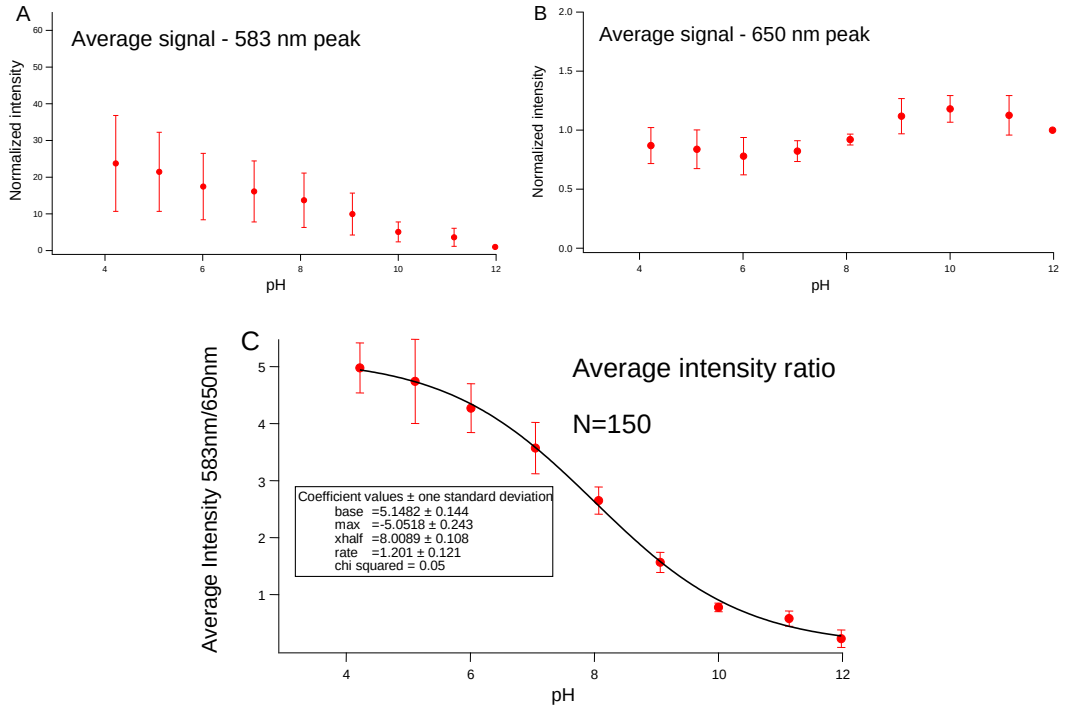

Figure 13 Ratiometric measurement at microscope of liposomes containing Snarf-DOPE. The average signal from three different experiments is shown as a function of the pH.
A) Average signal collected in the range of (582 ± 37.5) nm. B) Average signal collected over 650 nm. Both A) and B) are normalized by the intensity at pH 12. C) Ratio between the two previous averaged signal. 150 liposomes were analyzed. The intensity ratio curve was fitted with a sigmoidal function from which it is possible to obtain the average $pK_{a\ microscope\_avg}$ = (8.0 ± 0.1). It is possible to notice that the intensity ratio increases of I_2=(5.5 ± 0.4) units between pH 10 and pH 6. The experiment was repeated three times. The error bars represent the standard deviation.

The fluorophore was characterized at the single liposome level in order to evaluate the heterogeneity of the fluorescent response of individual liposomes. In particular the aim was to establish a one-to-one correspondence between intensity ratio values and pH values and to verify if this conversion could be valid for all the liposomes or a local conversion for each liposome was necessary. The distribution of intensity ratio values of every single liposome was plotted for each pH value as so to have an indication of the heterogeneity of the response of the single liposomes, as it is possible to see in Figure 14. It is possible to notice that the widths of every distribution cover different pH values. In the graph of Figure 15 the average value of each distribution plotted against the corresponding pH value is shown and the error bar corresponds to the width of the corresponding gaussian distribution. It is already clear from Figure 15 that it is not possible to establish a one-to-one correspondence between intensity ratio values and pH values. For example given an intensity ratio value of 3 units, this can correspond to three different pH values: pH 6, pH 7 and pH 8. Both Figure 14 and Figure 15 are based on the intensity value of single liposomes and show a certain heterogeneity among all the liposomes.

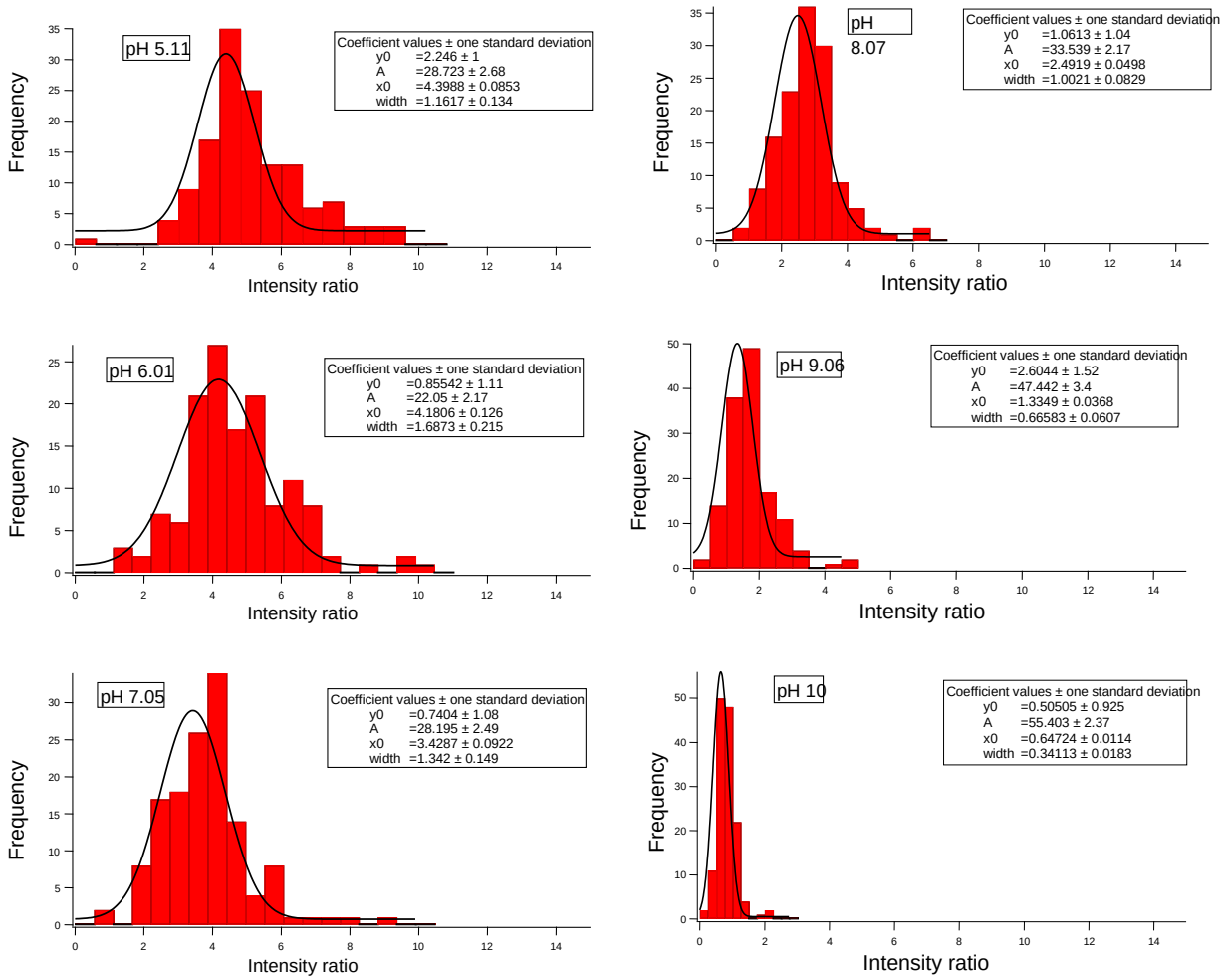

Figure 14 Distribution of intensity ratio at different pH values for the single liposomes containing Snarf-DOPE

The distributions of the intensity ratios of the single liposomes were plotted for the pH values of interest and each one was fitted with a gaussian function. The distributions have a standard deviation around 1: the response of the liposomes to pH changes is heterogeneous.

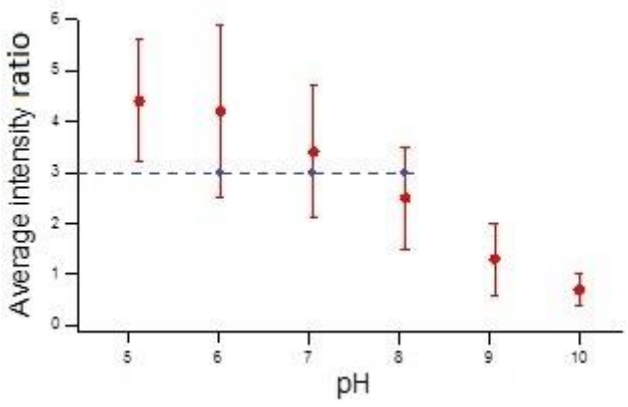

Figure 15 Distribution of intensity ratio for the single liposomes containing Snarf-DOPE

In the figure is shown the mean of every distribution with the relative standard deviations as a function of the pH. The distributions show a relatively high heterogeneity on the response to pH changes of the single liposomes. For example, the intensity of 3 units corresponds to three different pH values, as shown from the blue dashed line.

Every single liposome was characterized performing a sigmoidal fit of each trace. The pK_a of the fluorescent dye into the membrane of each liposome was estimated fitting the trace of each liposome with a sigmoidal function, as it is shown in Figure 16-A. Only the fits with an absolute error on the pK_a lower than one fifth of the estimated value were accepted. A distribution of all the estimated pK_a is shown in Figure 16-B. The average $pK_{a\ microscope\_single}$ = (8.0 ± 0.9) is in a range that is still suitable for studying LMCA1 with the single liposome assay but its uncertainty covers almost three different pH values, suggesting that there is a high heterogeneity among the liposomes.

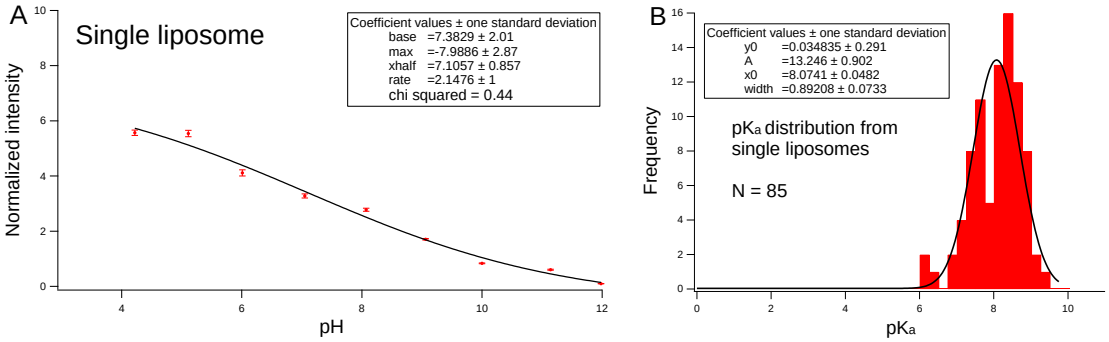

Figure 16 A) Example of a single liposome trace - B) Distribution of the pK_a
A)Example of one of the selected traces fitted with sigmoidal function. The error bars on each data point represent the error of the gaussian fitting done during the image analysis. A trace was accepted only if the error on the estimated value was less than one tenth of the estimated value itself.
B)The histogram shows the distribution of the pK_a for every single liposome. 85 liposomes were analyzed. The pK_a was calculated fitting every single trace of intensity ratio with a sigmoidal function and selecting only the fits with an absolute error on the pK_a lower than one fifth of the estimated value. The histogram of the distribution was fitted with a gaussian function. The calculated average $pK_{a\ microscope\_single}$=(8.0 ± 0.9) is in a range that is appropriate for studying LMCA1. The width of the distribution of the pK_a suggests that there is heterogeneity in the response to pH changes for individual liposomes, so a local calibration is needed.

Both from the estimated intensity values and widths of Figure 15 and from the distribution of pK_a in Figure 16 it is possible to state that the response of liposomes to pH changes is heterogeneous. Due to this heterogeneity, it is not possible to globally convert an intensity ratio value into only one pH value. The conversion from intensity ratio values into pH values has to be done locally for each single liposome so a local calibration is necessary.

5.1.3 Summary of the properties of Snarf-DOPE

Snarf-DOPE was tested with the single liposome assay in order to describe its properties at the single liposome level. The pH titration performed at the spectrofluorometer showed the presence of two emission peaks at 583 nm and 650 nm. It was possible to calculate the intensity ratio as a function of pH values and estimate a $pK_{a\ bulk}$ = (9.3 ± 0.1). The fold increase of more than 4 units in the range between pH 10 and pH 6 and the value of the $pK_{a\ bulk}$ both suggest that Snarf-DOPE has the right bulk properties for studying LMCA1 with the single liposome assay because

this fluorophore can sense pH changes while the pump will use ATP to transport calcium into the liposomes and protons outside.

The dye was successfully implemented in the single liposome assay and the average fold increase upon pH changes at the microscope I_2 = (5.5 ± 0.4) is higher than the one at the spectrofluorometer I_1 = (3.3 ± 0.2). This could be because when the titration is performed at the spectrofluorometer the measured intensity is an average intensity of the solution in which liposomes are dissolved whereas with the measures recorded at the microscope it is possible to discard the liposomes that did not respond. A pH titration allowed the measure of an average intensity ratio: $pK_{a\ microscope\_avg}$ =(8.0 ± 0.1). From the distribution of the pK_a of single liposomes it was estimated the $pK_{a\ microscope\_single}$ = (8.0 ± 0.9). The estimated $pK_{a\ microscope\_single}$ value suggests that Snarf-DOPE is sensitive to pH changes in the right pH range for studying LMCA1. The fold increase I_2 = (5.5 ± 0.4) in the right range of pH values makes Snarf-DOPE suitable for the single liposome assay. The width of the distribution of $pK_{a\ microscope\_single}$ suggests that the conversion from intensity ratio values to pH values is not valid for all the liposomes and it is necessary to locally convert the intensity ratio values into pH values. The low statistic due to the small allowed error on the data fit can be improved replacing the long pass filter at higher wavelengths with a band pass filter that select only the light close to the peak, increasing the signal to noise ratio.

5.2 Characterization of Oregon Green Bapta-5N in the single liposomes assay

Oregon Green Bapta-5N is a low affinity calcium sensor dye. It has an excitation peak at 491 nm and an emission peak at 521 nm. From previous bulk experiments, Oregon Green Bapta-5N appeared to be suitable with the single liposome assay for studying LMCA1. It was found experimentally by Simon Bo Jensen a K_d of about 31 µM, which is in the right range for studying LMCA1. We wanted to employ this dye as encapsulated dye in order to measure calcium concentration changes inside the lumen of the liposomes. In the following section some of the properties of Oregon Green Bapta-5N will be discussed at the single liposome level.

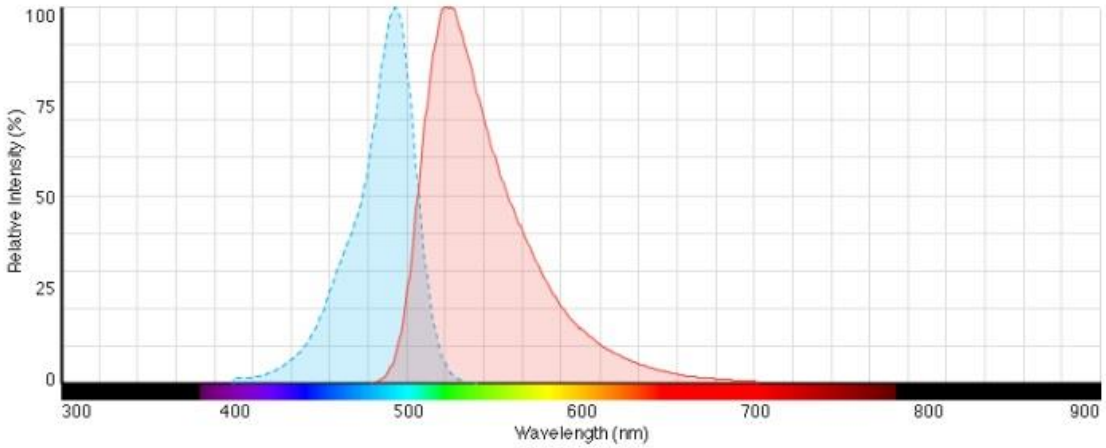

Figure 17 Excitation and emission spectrum of Oregon Green Bapta-5N. Excitation peak at 491 nm in blue, emission peak at 521 nm in red. (Graph originally from "Life technologies")

5.2.1 Single liposomes level characterization: presence of self-quenching effect

In order to describe the properties of Oregon Green Bapta-5N at the single liposomes level, liposomes containing encapsulated dye were fabricated and attached to the surface of a flow cell as explained in Sections 4.3 . The presence of self-quenching effect was seen during photostability measurements performed on the liposomes containing Oregon Green Bapta-5N. Quenching refers to a process that decreases the fluorescence intensity of a given substance. For instance FRET is based on quenching: a donor dye in excited state transfers energy to an acceptor quencher dye. This interaction is extremely dependent on the distance of the two dyes. In the case of self-quenching donor and acceptor are the same dye and the distance between the molecules of dye depends directly on the concentration of the dye itself.[42,43,44] The presence of self-quenching can represent a problem for the single liposome assay because it can influence the data analysis of activity measurements. During activity measurements the active traces should show an increase of signal due to calcium pumped inside the lumen of liposomes by LMCA1. In presence of self-quenching, if a trace shows an increase of signal, this could be either due to activity of LMCA1 or self-quenching and it could be not possible to distinguish among the two. Self-quenching can be removed decreasing the concentration of encapsulated dye. There is a limit on the dilution of dye since below a certain concentration the encapsulation rate is too low and It becomes difficult to localize liposomes on the surface.

Experimentally it is possible to recognize the presence of self-quenching looking at the profile of a photostability measurement: first the signal increases due to the self-quenching effect then after a certain period of time, depending on the type of dye and on the experimental settings (exposure time and intensity of laser light), the signal starts to decrease due to photobleaching. An experimental example of self-quenching is shown in Figure 18-A. In order to reduce self-quenching, liposomes containing Oregon Green Bapta-5N were fabricated with different concentration of encapsulated

dye: 250 µM, 100 µM, 50 µM, 25 µM, 10 µM. If the concentration of encapsulated dye in the rehydration buffer is 10 µM, the average signal from all the liposomes does not show self-quenching, as shown in Figure 18-B.

It was necessary to investigate the presence of self-quenching also at the single liposomes level. Analyzing the single liposomes traces from sample containing a dye concentration of 10 µM, it was seen that 4% still show self-quenching since the signal was increasing at least over 1.1 folds, as it is possible to see in Figure 19 (in this experiment 16 out of 400 vesicles showed self-quenching). As stated before the goal of the single liposomes assay would be to measure the activity of single molecules of LMCA1. In order to have high probability of presence of single LMCA1 molecules into the liposomes membrane, the percentage of liposomes that show activity during an activity measurement should be around 10%. If Oregon Green Bapta-5N is employed, 4% of traces will show self-quenching. This means that among the active traces, 30% will increase the signal due to self-quenching and not because of LMCA1 activity.

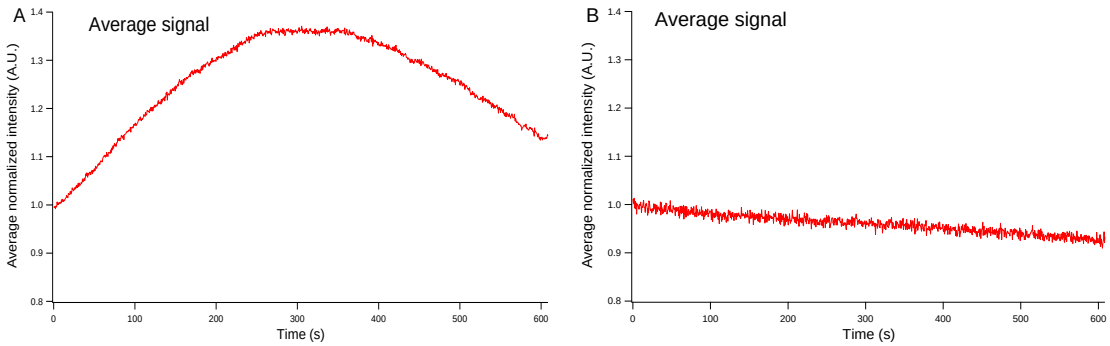

Figure 18 Self-quenching effect disappears decreasing the concentration of encapsulated OregonGreenBapta-5N. Average signal

Average signal from colocalized liposomes containing Oregon Green Bapta-5N at concentrations of A) 50 µM and B) 10 µM. It's possible to notice that self-quenching effect is still present at concentration of 50 µM looking at the shape of the curve. It disappears decreasing the concentration to 10 µM, when the signal decreases due to photobleaching.

From the calibration curve of Oregon Green Bapta-5N in Figure 20 we can find that a fold increase of 1.2 folds corresponds to a calcium concentration of about 25 µM. Given these conditions, it will not be possible to investigate activity of the pump at least below 25 µM on 30% of the active traces because it is not possible to distinguish if the fold increase is due to self-quenching or due to an increased calcium concentration into the lumen of the liposomes. Therefore even if Oregon Green Bapta-5N represented a good candidate from bulk experiments, it was decided to discard it as a calcium sensor for the single liposomes assay.

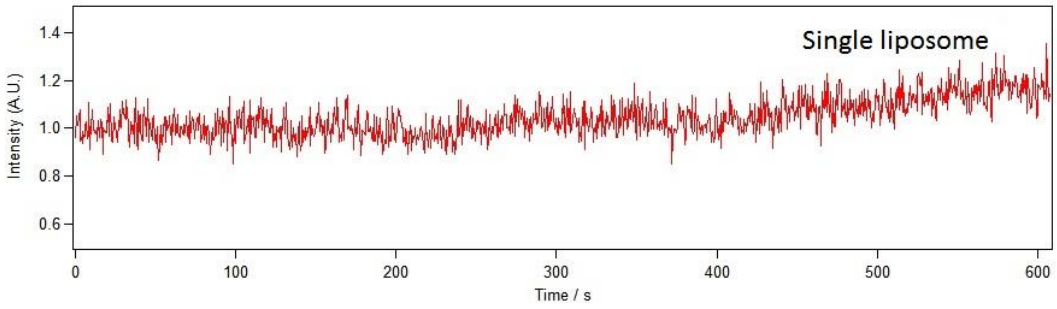

Figure 19 Self-quenching effect from a trace of a single liposome containing encapsulated OregonGreenBapta-5N 10 µM.
Example of a single trace from the liposomes at a concentration of 10 µM. The intensity is normalized by the average of the intensity of the first 10 frames. At this concentration 4% of the single traces still show self-quenching with an increase of signal up to 1.2 folds.

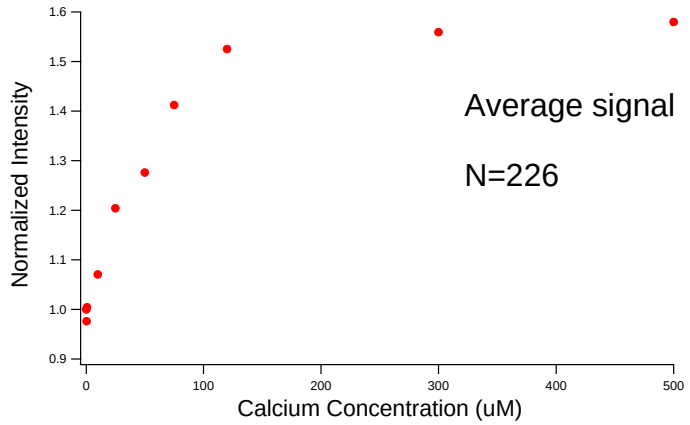

Figure 20 Calcium titration on liposomes containing Oregon Green Bapta-5N.
The graph shows the fold increase in the average signal coming from all recorded liposomes containing Oregon Green Bapta-5N. An increase of 1.2 folds corresponds to a calcium concentration of 25 µM. From figure 18 it is possible to see that self-quenching is responsible for an increase up to 1.2 folds. From these results it is possible to conclude that, employing Oregon Green Bapta-5N it is not possible to distinguish if the signal is increased because of self quenching or because of activity of LMCA1 when we are below 25 µM. The experiment was performed once and the intensity was normalized by the intensity at 0 µM calcium.

5.2.2 Summary of the properties of Oregon Green Bapta-5N

From bulk experiments, Oregon Green Bapta-5N showed right properties to be employed as a calcium sensor for the single liposomes assay. When encapsulated into liposomes, the dye showed self-quenching. It was possible to remove self-quenching from the average signal decreasing the concentration of encapsulated dye. Analyzing the single traces, self-quenching was still present in 4% of the liposomes. Since the percentage of traces showing self-quenching was too high compared with the expected percentage of active traces (10%) it was decided that the dye's properties did not suite the single liposome assay for functional studies of LMCA1. Also it is important to consider that when it was used a dye concentration of 10 µM, that is much lower compared with the initial one of 700 µM, the encapsulation rate was half (more than

1000 vesicles were colocalized when the dye concentration was 700 µM while 400 were colocalized when the dye concentration was 10 µM) and also this represents a problem if Oregon Green Bapta-5N would be used in the single liposome assay because the statistic would be low since many liposomes would not contain dye or the dye contained would not be enough to allow the detection of its fluorescence.

5.3 Characterization of Fluo-5N in the liposomes assay

Fluo-5N is a low affinity calcium sensor dye that increases its emission as the concentration of calcium increases. It has an excitation peak at 494 nm and an emission peak at 516 nm. From previous experiments in bulk or at the single liposome level performed by Mads Møller, Fluo-5N resulted suitable for being employed in the single liposome assay. Here we wanted to investigate different properties of the liposomes containing encapsulated Fluo-5N. First the heterogeneity in response to calcium of every single liposome was quantified. Secondly we wanted to evaluate the presence of cross emission between Fluo-5N in the lumen of the liposomes and ATTO-655 in the membrane of the liposomes. Finally we wanted to exclude the possibility of unspecific binding of Fluo-5N to the membrane of liposomes. Ionomycin, a calcium ionophore, was used during the experiments in order to equilibrate the concentration of calcium ions inside and outside the liposomes upon changes of buffer at different calcium concentrations.

5.3.1 Single liposomes level characterization

- *Calibration curve and K_d distribution*

In order to characterize Fluo-5N at the single liposomes level, the liposomes containing Fluo-5N were attached to the surface of a flow cell, following the procedure explained in Section 4.3 . Fluo-5N was successfully implemented in the single liposome assay, and the average fold increase of liposomes upon changes of calcium concentration is of 11 folds, as it is possible to see from Figure 21. The intensity was normalized to the intensity of 0 µM calcium. From the graph it is possible to notice also that the average K_d is higher than the expected value (90 µM). This can be due to the fact that the dye has a different K_d when encapsulated in these liposomes. Despite the higher K_d, his value is still in a useful range for the investigation of the properties of the transporter LMCA1.

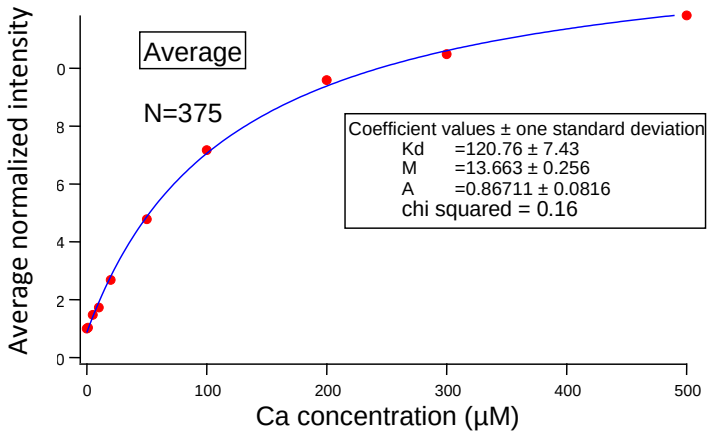

Figure 21 Calcium titration on liposomes containing Fluo-5N.
The data points represent the average intensity of liposomes containing Fluo-5N in response to varying Ca^{2+} concentration. The data were fitted with a Michelis Menten binding function with a translation factor. The intensity was normalized by the intensity at 0 µM calcium. The liposomes where diluted about 200x in a solution of Tris-HCl pH 8.5 (20 mM), KCl (200 mM), DTT (1mM).

During the analysis each colocalized liposome was fitted with a two dimensional gaussian function and only fits with an error on the intensity lower than a tenth of the estimated value were accepted. The signal to noise ratio was enhanced averaging recorded images of the same conditions. A fit was performed on the average intensity in order to calculate the average K_d=(120.0 ± 0.1). The function used to fit the intensity ratio curve is a Michelis Menten defined as follow:

$$I(c) = A + \left(\frac{\mathrm{M} \cdot \mathrm{c}}{k_d \cdot \mathrm{c}}\right)$$

A is a translational factor that describes the initial intensity of the fluorophore in absence of calcium, M represents the maximum intensity of the fluorophore when saturation is reached. Both the estimated *K_d value* and the *fold increase* suggest that Fluo-5N can be a good candidate for functional studies of LMCA1.
The fluorophore was characterized at the single liposome level in order to evaluate the heterogeneity of the fluorescent response of individual liposomes. The distribution of intensities of every single liposome was plotted for each calcium concentration value as so to have an indication of the heterogeneity of the response of the single liposomes, as it is possible to see in Figure 22.

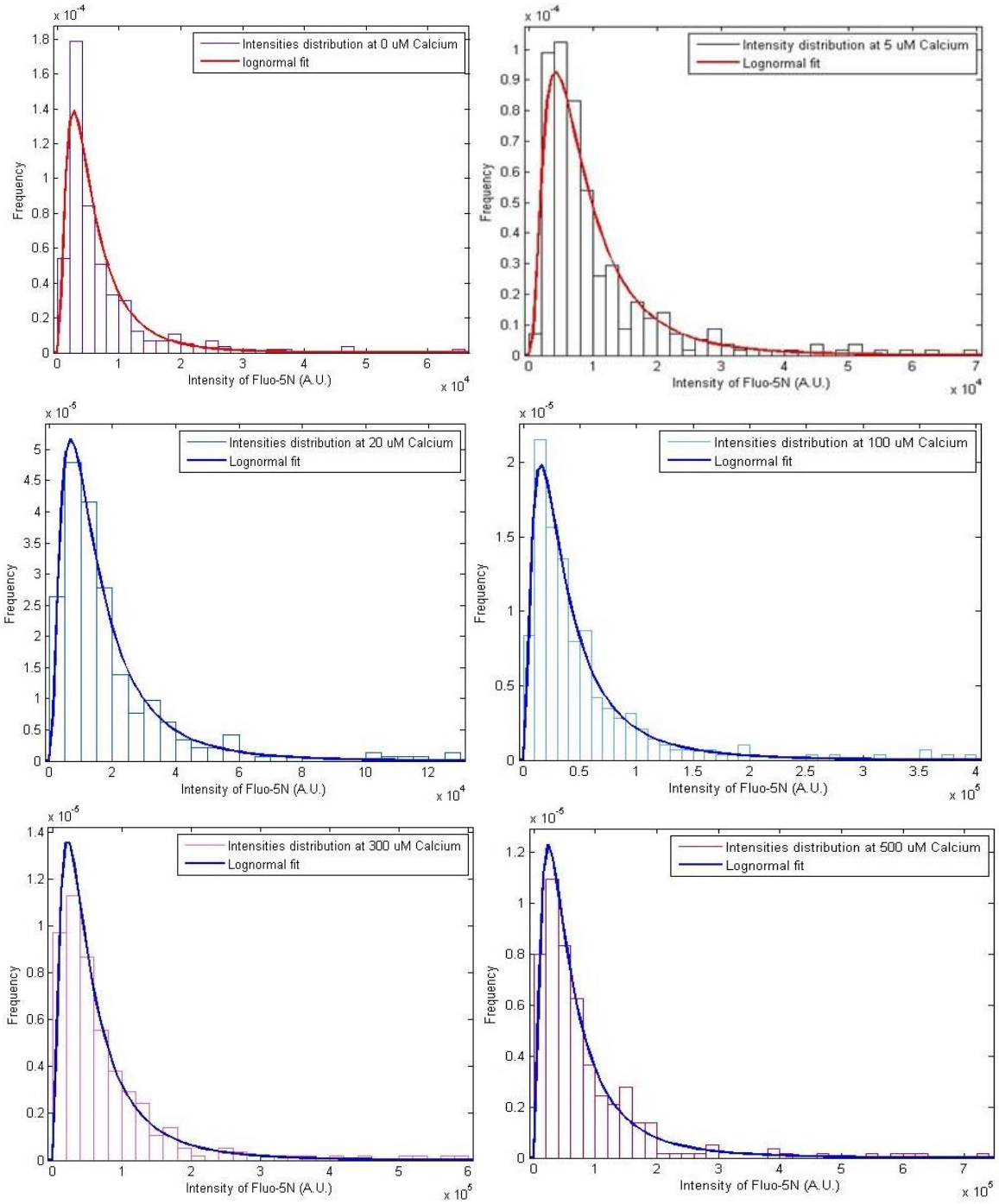

Figure 22 Distribution of intensities from Fluo-5N containing liposomes in presence of different calcium concentrations.

It was plot the distribution of the intensities of the liposomes containing Fluo-5N for each calcium concentration used during the titration; in particular the figures show the distributions of the intensities in presence of 0 µM, 5 µM, 20 µM, 100 µM, 300 µM and 500 calcium concentration. The distributions follow a lognormal distribution. From the results in the table it is possible to notice a high heterogeneity in the response of the single liposomes to calcium concentration changes.

A fit was performed on each single liposome trace in order to estimate the K_d in each vesicle as it is shown in Figure 23-A. Only the fits with an absolute error on the K_d lower than one fifth of the estimated value were accepted. A distribution of all the estimated K_d is shown in Figure 23-B. The average K_d = (99 ± 54) is in a range that is

suitable for studying LMCA1 with the single liposome assay but its uncertainty suggests that there is a high heterogeneity among the liposomes.

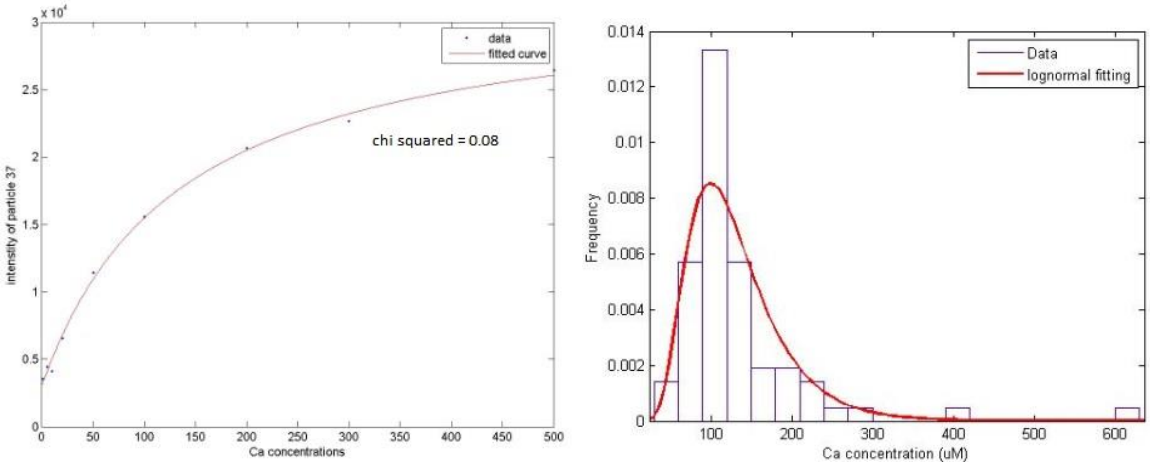

Figure 23 Example of a single liposome trace. Distribution of the K_d. N=70.
A)Example of one of the selected titration curves relative to a single liposome and fitted with a Michelis Menten function. The error on each data point was chosen to be 10 times less than the measured value in order for the trace to be accepted.
B)The histogram shows the distribution of the K_d of the single liposomes. The K_d has been calculated fitting every titration curve with a Michelis Menten function and selecting only the fits with an absolute error on the K_d lower than one third of the measured value. The histogram of the distribution has been fitted with a gaussian function. The calculated average K_d=(99 ± 54) it's in a range that is good for studying LMCA1. As it is possible to see from the distribution, there is heterogeneity in response to calcium concentration changes for individual liposomes.

Both from the data of Figure 22 and from the distribution of K_d in Figure 23 it is possible to state that the response of liposomes to pH changes is heterogeneous. It is necessary to take into account this heterogeneity when activity measurements will be performed in order to compare the active traces.

- *Investigation of cross emission of ATTO655*

The liposomes containing encapsulated Fluo-5N were fabricated also with membrane dye ATTO655. ATTO655 is a fluorescent label that is commonly used and designed for high sensitivity applications. It has an excitation peak at 655 nm and an emission peak at 680 nm in 0.1 M phosphate pH 7,0.[46] In the single liposome assay ATTO655 is used for colocalization. Since ATTO655 and Fluo-5N have excitation peaks and emission peaks at different wavelengths, it is possible to record images of attached liposomes first when only ATTO655 is emitting and then when only Fluo-5N is emitting. The emission of ATTO655 identifies the membrane of liposomes while the emission of Fluo-5N is related to lumen of liposomes. It is possible to colocalize the two recorded emissions in order to select only those ROIs containing liposomes that emits both in the ATTO655 channel and in the Fluo-5N channel.
Some ROIs in which there is signal only in the Fluo-5N channel could be found: this can be due to conglomerates of free Fluo-5N attached to the surface. It is also possible to find some ROIs in which there is signal only in the ATTO655 channel: this is due to

liposome that does not contain encapsulated Fluo-5N. It is possible to have also another issue related to the emission of ATTO655. As stated before, Fluo-5N and ATTO655 have different excitation peaks: ATTO655 can be excited with 640 nm laser light while Fluo-5N can be excited with 491 nm laser light. Since the excitation spectrum of fluorescent dyes has a certain distribution, ATTO655 can be excited also with 491 nm laser light: this may represent a problem if the emission of ATTO655 is comparable with the emission of Fluo-5N when the sample is excited at 491 nm. We wanted to quantify how much of the total emitted signal is emitted by ATTO655 and how much is emitted by Fluo-5N, when the sample is excited at 491 nm in the single liposome assay.

An excitation spectrum of ATTO655 was recorded, correcting for the signal of the buffer in which the liposomes containing only ATTO655 (*empty* liposomes) were dissolved. From the corrected excitation spectrum of Figure 24 we can see that ATTO655 can be excited at 491 nm, and the intensity at 491 nm is 2% of the intensity corresponding to 640 nm. The emission was chosen at 679 nm that correspond to the emission peak in the emission spectrum measured in the same conditions, as it can be seen in Appendix B.2.

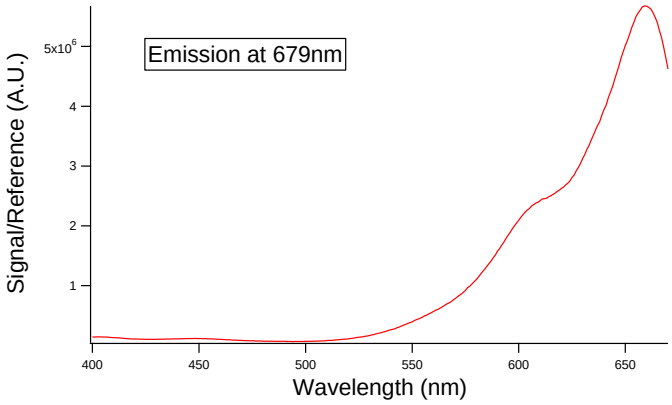

Figure 24 Excitation spectrum of liposomes containing ATTO655-DOPE.
Excitation profile with emission at 679 nm of a solution of tris buffer 20 mM containing ATTO655-DOPE liposomes 200x diluted, KCl 200 mM, $MgCl_2$ 1 mM, DTT 1 µM and Valinomycin 65.2 nM. The intensity of the dye at 491 nm is 2% of the intensity at 640 nm, suggesting that a small percentage of ATTO655 can be excited at the same wavelength at which the Fluo-5N is excited. The spectrum was corrected subtracting the excitation profile of the dummy buffer. Each data point is a average of three measures.

The emission signal of *empty* liposomes and the one of liposomes containing both ATTO655 and Fluo-5N (*full* liposomes) were recorded exciting both at 491 nm. It was possible to compare directly the average intensity of all the selected *empty* liposomes with the average intensity of all the selected *full* liposomes because both the two types of liposomes were incubated in the same cell. The background was corrected subtracting its average value from the recorded images. In order to calculate the average value of the background, 10 background ROI's were selected from the recorded images and then averaged. In Figure 25-A it is possible to see the average

intensity emitted by the *empty* and the *full* liposomes. The signal emitted by the *full* liposomes is composed of the signal emitted by ATTO655 and Fluo-5N. If this signal is compared with the signal emitted by *empty* liposomes, it is possible to conclude that the majority of the signal emitted by *full* liposomes (68%) is emitted by ATTO655. This problem can be solved in two different ways: 1) the concentration of membrane dye ATTO655 can be diluted until its emission when excited at 491 nm becomes neglectable, 2) the emitted light can be filtered, selecting only the emission range of Fluo-5N that is below 640 nm. It is possible to select an emission range below 640 nm using the dual view of the TIRF microscope. In Figure 25-B average intensities of *empty* and *full* liposomes are shown when the dual view setup is used. In this case when the sample is excited at 491 nm, the emission of ATTO655 is completely filtered and all the recorded signal is related to emission of Fluo-5N.

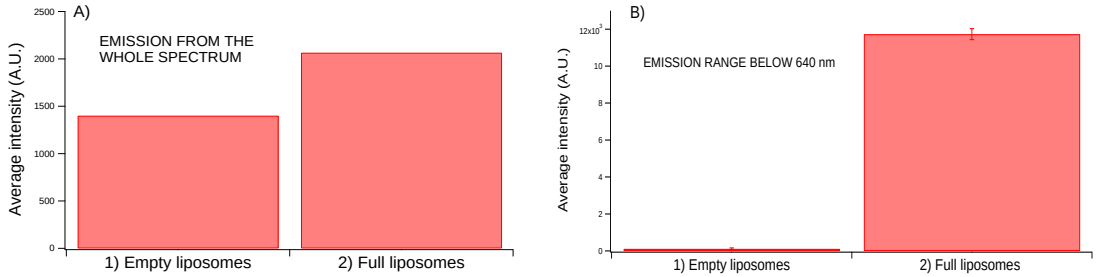

Figure 25 Comparison between signal coming from empty liposomes (1) and full liposomes (2) for two different filtering setup.
The emitted light was collected from the whole spectrum in A) and from below 640 nm in B). In both cases the liposomes were incubated in the same flowcell. The excitation was at 491 nm and the signal from the background was corrected. It is possible to notice that when the signal is collected from the whole spectrum, 68% of the signal coming from full liposomes is emitted by ATTO655 while using the other setup the signal coming from ATTO655 is neglectable.

We can conclude that when Fluo-5N and ATTO655 are employed together respectively as encapsulated dye and membrane dye in the single liposome assay, it is necessary to consider the cross emission effect of ATTO655. The percentage of signal emitted by ATTO655 is the majority of the emitted signal when the sample is excited at 491 nm. Since during activity measurements we are interested in intensity changes on the emission of Fluo-5N, it is possible to filter the extra signal emitted by ATTO655 selecting the emission range below 640 nm and collecting only the light emitted by Fluo-5N.

- *Unspecific binding to the membrane of liposomes*

The single liposome assay exploits fluorophores sensitive to substrates that are translocated by LMCA1 in order to measure its activity. As explained before in Section 3.2 a combination of two fluorophores would be preferred: an encapsulated dye sensitive to calcium concentration changes and a membrane dye sensitive to pH changes. When the encapsulated fluorophore is excited, we can measure its intensity

and correlate it to a certain calcium concentration. If during the encapsulation process the fluorophore not only is encapsulated into the liposomes but sticks also to the external side of the membrane then the emitted signal will not describe only the concentration of calcium into the lumen and it will have to be corrected for the signal emitted by unspecifically bound dye. We wanted to investigate the presence of unspecific binding of Fluo-5N to the membrane of liposomes and possibly quantify the percentage of its emission.

In order to perform this measure, *empty* liposomes were attached to the surface of a flow cell as explained in Section 4.3, subsequently they were incubated with 100 µM Fluo-5N. Finally the sample was incubated also with 500 µM calcium so as the presence of Fluo-5N stick to the membrane would be more highlighted. Dual view of the microscope was used to select the emission range: the upper part collect light emitted above 640 nm and corresponds to emission of ATTO655, lower part collect light emitted below 640 nm and corresponds to emission of Fluo-5N. In Figure 26 is shown a micrograph of *empty* liposomes recorded after the incubation of Fluo-5N and in presence of calcium. It is possible to see that the liposomes are visible only when the membrane dye ATTO655 is excited. If we excite the sample in the Fluo-5N channel, we cannot record any significant emission.

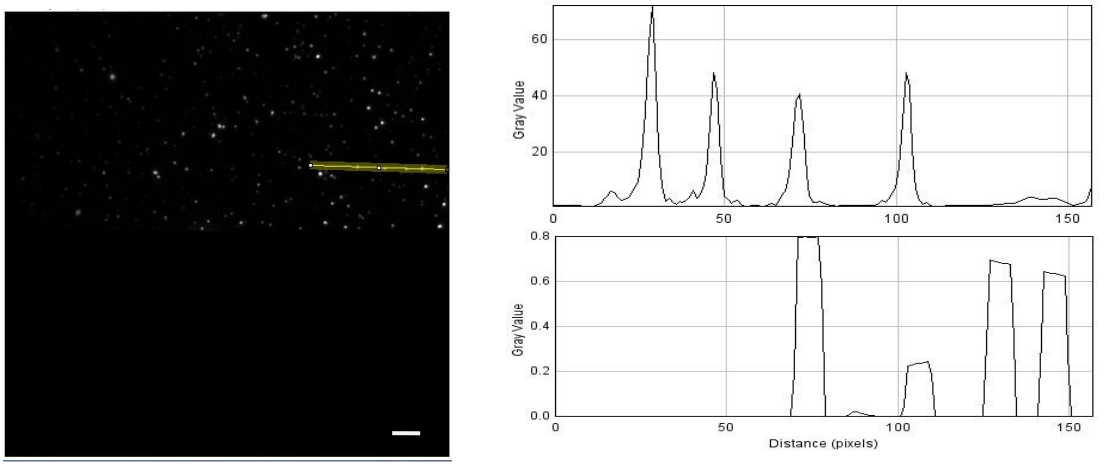

Figure 26 Micrograph showing *empty* liposomes excited at two different wavelength in presence of calcium and Fluo-5N. Profiles of two corresponding positions of the micrograph.
The upper part shows liposomes excited at 640 nm, collecting the light above 640 nm (ATTO655 channel). The lower part shows liposomes excited at 491 nm, collecting the light below 640 nm (Fluo-5N channel). From the intensity plots it is possible to see that the liposomes are emitting only in the ATTO655 channel, while in the Fluo-5N channel it is not possible to recognize any liposome. It is possible to conclude qualitatively that Fluo-5N does not bind to the membrane of liposomes. (Scale bar = 10 µM).

Looking at the Figure 26 it is already possible to conclude qualitatively that Fluo-5N does not bind to the membrane of liposomes. In order to have also quantitative results, we analyzed the images recorded in the Fluo-5N channel. Since it was not possible to select any liposome in the Fluo-5N channel, the coordinates of the ROIs containing liposomes were taken from the ATTO655 channel then the analysis was performed in the images recorded in the Fluo-5N channel. As it is possible to see from

Figure 27, the average intensity calculated in the selected ROIs does not change either after incubation with free Fluo-5N or in presence of both calcium and Fluo-5N. This is an evidence that Fluo-5N does not bind to the membrane of liposomes. It was possible also to quantify the percentage of background signal as 99% of the total emitted signal, as expected from the qualitative analysis of the micrograph in Figure 26.

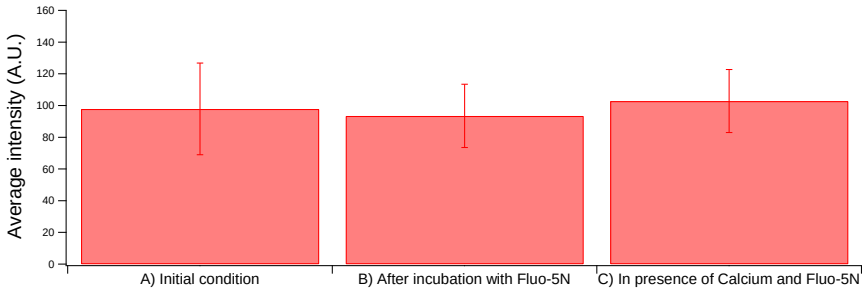

Figure 27 Average intensity of liposomes upon incubation with Fluo-5N and Calcium.
Sample tested: liposomes containing only membrane dye ATTO655. Sample was excited at 491 nm and emission signal was collected below the wavelength of 640 nm. The background was corrected. A) The sample was attached to the surface and the emission signal was collected. B) The emission signal was then collected in the same position, after incubation with Fluo-5N 100 µM. C) Finally the emission signal was collected in the same position after incubation with calcium 500 µM. There is no appreciable average increase of signal meaning that Fluo-5N does not stick to the external part of the membrane. The intensity shown represent 1% of the total recorded signal, the rest is background. The experiment was repeated three times and the error bars represents standard deviation of the mean.

5.3.2 Summary of the properties of Fluo-5N

The liposomes containing Fluo-5N were tested at the microscope in the single liposome assay. The calcium titration allowed the measure of the intensity response of the dye upon changes of calcium concentrations. It was possible to calculate the distribution of the K_d from which is possible to conclude that liposomes have an high heterogeneity in response to calcium concentration changes. It is necessary to take into account this heterogeneity during activity measurements. It was investigated the unspecific binding of Fluo-5N to the external part of the liposomes' membrane. The cross emission of ATTO655 was individuated and it was quantified the percentage of the signal emitted from ATTO655 as the majority of the emitted signal when no filters are used to select the emission range. The effect can be removed filtering the emitted light in order to collect only the signal emitted by Fluo-5N. It was also investigated the presence of unspecific binding to the external membrane of liposomes and it was seen that Fluo-5N does not bind unspecifically.

6 Conclusions and Outlook

In this thesis the development of a single liposome assay for functional studies of transporter proteins LMCA1 was presented. The assay can represents a novel technique for studying transporters based on quantitative fluorescence. It exploits the properties of the employed fluorophores to monitor the activity of the protein and it gives the possibility to have an insight into its activity at the single molecule level.

Three different fluorophores were characterized in order to decide if they were suitable with the single liposome assay for studying LMCA1. Exploiting the characteristics of the assay it was possible to analyze both average signal coming from all the liposomes and the signal coming from each single liposome. In this way it was possible to characterize the properties of the fluorophores at the single liposome level and quantify the level of their heterogeneity.

Snarf-DOPE represents a good candidate for the single liposome assay from bulk experiments. It was successfully implemented as membrane dye into liposomes. From the analysis of the average signal coming from all liposomes, it shows a good response to pH changes with an average fold increase of around (5.5 ± 0.4) units in the pH range of interest. The average pK_a is in a good range for studying the transporter LMCA1. The heterogeneity of the liposomes was quantified analyzing the signal from each single liposome. The response to pH changes at the single liposome level is heterogeneous and a local calibration is needed. It will be possible to implement additional filters to the dual view of the microscope in order to enhance the signal to noise ratio and increase the number of analyzed liposomes per experiment. In this way it could be possible to have higher statistics and arrive to even more robust conclusions about the properties of Snarf-DOPE.

Oregon Green Bapta-5N containing liposomes were analyzed at the microscope in the single liposome assay. The fluorophore sensitive to calcium previously showed good bulk properties for the single liposome assay and it was successfully implemented into liposomes. The self-quenching effect was removed decreasing the concentration of the fluorophore. Again the analysis of the average signal was masking properties of the single liposomes: even if the average signal did not show the presence of self-quenching, analyzing the signal emitted by single liposomes it was seen that self-quenching was still present in 4% of the liposomes. It was decided to discard Oregon Green Bapta-5N since it wasn't possible to decrease the concentration of encapsulated dye even more without losing signal to noise ratio.

Fluo-5N was a good candidate from bulk experiments and it was successfully implemented in liposomes as encapsulated fluorophore. Unspecific binding of Fluo-5N to the membrane of liposomes was investigated and quantified: there is no significant unspecific binding to the external part of the liposomes' membrane. The problem of cross emission between ATTO655 and Fluo-5N was presented and solved. It was also

quantified the heterogeneity of the response of liposomes to calcium concentration changes, investigating the K_d of the fluorophore encapsulated in each liposome.

Two fluorophores resulted compatible with the single liposome assay among the three candidates: the calcium sensor Fluo-5N and the pH sensor Snarf-DOPE. Future experiment will have to be performed in order to establish the potencies of these fluorophores directly with the protein LMCA1. In particular the two fluorophores can be implemented together into liposomes in order to monitor the activity of LMCA1 in real time measuring pH changes and calcium concentration changes. When the two liposomes will be implemented together, it will be necessary to check also the presence of FRET between the two.

The assay presented represents a good possibility for studying LMCA1 at the single molecule level but its potential is not limited to this transporter protein. It will be possible to use this assay also for studying other transporters at the single molecule level, as it was used already for studying the transporters AHA2 and ClC-ecl1. This is possible because the characteristics of the assay can be tuned to match different needs: it is possible to change fluorophores to match a different translocated substrate and the dimension of the liposomes can be controlled by extrusion. It is also possible to monitor increase in intensity due to a single ion entrance into the liposomes' lumen, exploiting the TIRF microscopy technique possibilities. It is also interesting to underline the possibility of the assay of analyzing average signal coming from all the liposomes but mainly from each single liposome. In this way effects that could be masked by the overlap of many different signal can be discovered and investigated.

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8 Appendix

A - Materials

Abbreviation	Name	MW (g/mol)	Purchased
Lipids			
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine	786.11	Avanti Polar Lipids
DOPE-cap-biotin	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt)	1105.48	Avanti Polar Lipids
Egg PC	L-a-phosphatidylcholine (Egg,Chicken) (Chloroform)	770.123	Avanti Polar Lipids
E. coli Polar Lipid Extract	E.coli PE 67 %, E. coli PG 23.2 % and E. coli CA 9.8 %	798.637	Avanti Polar Lipids
BT-PEG2000-DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (ammonium salt)	3016.8	Avanti Polar Lipids
Fluorophores			
Fluo-5N	Fluo-5N® pentapotassium salt, cell impermeant	958.0582	Life Technologies
Atto655-DOPE	N-(ATTO 655)-1,2-Dioleoyl-sn-glycaro-3-phosphoethanolamine	1366	ATTO-TEC
Snarf-DOPE		1197.48	Department of Plant and Environmental Sciences (Gerdi Christine Kemmer)
Oregon Green Bapta-5N	Oregon Green® 488 BAPTA-5N, Hexapotassium Salt, cell impermeant	1159.28	Life Technologies
Oregon Green Bapta-2	Oregon Green® 488 BAPTA-2, AM, cell permeant	1751.4454	Life Technologies
Chemicals			
Valinomycin	Valinomycin	1111.32	Sigma-Aldrich
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone	204.64	Sigma-Aldrich
Ionomycin	Ionomycin	709.00	Sigma-Aldrich

$CaCl_2$	Calcium chloride dehydrate	147.01	Sigma-Aldrich
KCl	Potassium Chloride	74.56	Sigma-Aldrich
$MgCl_2$	Magnesium Chloride	203.2	Sigma-Aldrich
NaOH	Sodium Hydroxide	40.0	(Martinez Lab, KU)
HCl	Hydrogen Chloride	36.46	(Martinez Lab, KU)
DTT	Dithiothreitol	154.25	(Nissen Lab, AU)
Proteins			
NeutrAvidin	NeutrAvidin® biotin-binding protein		Life Technologies
Buffers			
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid). 15mM		Sigma-Aldrich
Tris base	Tris(hydroxymethyl)aminomethane 10 mM		Sigma-Aldrich
Tris with ions	20 mM Tris base, 200 mM KCl, 1 mM MgCl2, pH 3-12		
Solvents			
$CHCl_3$	Chloroform, CHROMASOLV®, for HPLC		Sigma-Aldrich
CH_3OH	Methanol, CHROMASOLV®, for HPLC		Sigma-Aldrich
CH_3CH_2OH	Ethanol		Sigma-Aldrich
MilliQ	Ultra pure water, 18.2 MΩ		
Detergents			
Helmanex	Helmanex® III		Sigma-Aldrich
Polymers			
Pll-PEG	Poly-L-lysine-grafted (20 kDa) poly-ethylene-glycol (2 kDa)		SuSoS
Pll-PEG-biotin	Poly-L-lysine-grafted (20 kDa) poly-ethylene-glycol (2 kDa) and PEG-Biotin (3.4 kDa). The % of Biotin functionalized PEG is 15-25%.		SuSoS

B - Additional properties of liposomes containing Fluo-5N and ATTO655

B.1 Calcium leakage

As previously explained, the lipid bilayer of cells is particularly impermeable to ions, which allows cells to regulate salt concentrations and pH using different ion channels or pumps. Liposomes are nanoreactors whose membranes simulate the lipid bilayer of cells, where we can reconstitute the protein LMCA1. By adding ATP to the system, the reconstituted pumps start to transport calcium inside the lumen of the liposomes. We can measure the activity of the pump measuring the increased signal coming from the encapsulated Fluo-5N. We wanted to quantify and characterize calcium leakage across the liposomes' membranes of our assay, because if this effect is present the concentration of calcium into the liposomes does not change only due to activity of the pump but also due to passive leakage. Also it is known from other studies that even if the lipid bilayer is almost impermeable to all ions, a certain leakage is still present. [45]

- *Experimental conditions*

The *full* liposomes (200x dilution) were incubated for 10 minutes then 2 mL of sample buffer (Tris-HCl pH 8.5 (20 mM), KCl (200 mM), $MgCl_2$ (1 mM), DTT (1 mM)) were used for flushing away the non-bound liposomes. The right position of the sample was chosen and two movies were recorded. During the first movie a solution of sample buffer containing calcium (100 µM) was flushed into the chamber. During the second movie a solution of sample buffer containing calcium (100 µM) and ionomycin (10 µM) was flushed into the chamber. The position was kept along all the experiment. The excitation wavelength of the laser was 491 nm (15% laser power) for the Fluo-5N channel and 640 nm (8.5% laser power) for the ATTO655 channel, the exposure time was 500 ms, the microscope was set on single view and a neutral density filter 2.0 was used to decrease the power of the laser. The experiment was repeated a second time using a sample buffer without $MgCl_2$.

- *Results*

We performed the calcium leakage experiment monitoring the response of liposomes to calcium concentration changes in presence and in absence of ionomycin. A solution of sample buffer containing 100 µM of calcium was added to the chamber where liposomes were previously attached to the surface. We decided to add 100 µM of calcium because this is the concentration corresponding to the K_d of the fluorophore. The solution was first injected in absence of ionomycin: in this situation the only way for calcium to enter into the liposomes was due to passive leakage through the liposomes' membrane. After a certain amount of time we injected the same solution in

presence of ionomycin. In this case ionomycin allowed equilibration of the system and the calcium could enter into the liposomes through ionomycin. We monitored the signal coming from the liposomes during both the injections. In Figure 28 is reported the average signal from all liposomes during one calcium leakage experiment. It is possible to notice an increase of intensity after the first injection in absence of ionomycin and a higher increase of intensity after the second injection in presence of ionomycin.

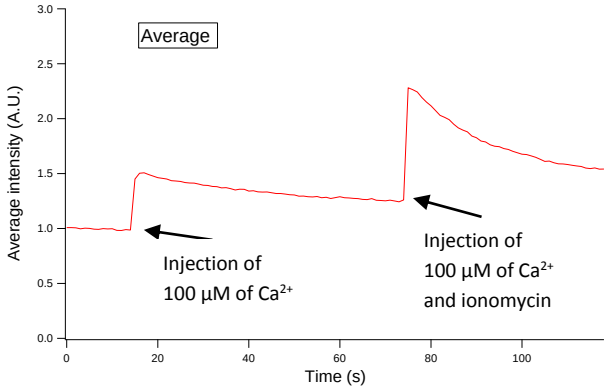

Figure 28 Average signal during calcium leakage experiment. The signal from fluo-5N encapsulated in liposomes was collected: after 2 minutes of recording Tris buffer containing 100 µM of Ca^{2+} was flushed then after 12 minutes buffer containing 100 µM of Ca^{2+} and 10 µM of ionomycin was flushed.

Analyzing the behavior of single liposomes it was possible to notice the presence of 4 different behaviors. The percentage of the four behaviors is shown in Figure 29. The majority of the selected liposomes (~ 65%) are tight to calcium, since we could see an increase of signal only in presence of ionomycin. A small amount of liposomes (~ 10%) are leaky to calcium since there was an increase of signal in absence of ionomycin, which kept decreasing until the end of the experiment. A certain amount of liposomes (~ 20%) showed an intermediate behavior: their signal increased after injection of calcium both in presence and in absence of ionomycin. There is a very small percentage (~ 1%) of liposomes that did not respond to any calcium concentration changes.

The intermediate behavior can be due to different factors. First it might be due to a partial equilibration: after the first injection in absence of ionomycin, calcium diffuses passively through the liposomes membrane but the concentration gradient is partially kept until ionomycin is introduced in the system and then the equilibration is completed. Secondly it might be also that to Fluo-5N is stick to the external part of the membranes: the dye could respond to calcium injection without need calcium to enter into the liposomes. This second hypothesis can be excluded from the results of the unspecific binding showed in Section 5.3.1 .

We can conclude that the majority of liposomes are completely tight to calcium and that the leaky ones show two different behaviors: a small amount is completely leaky,

a bigger amount shows a partial equilibration. Future experiments can use these results to describe the activity of LMCA1 taking into account calcium leakage.

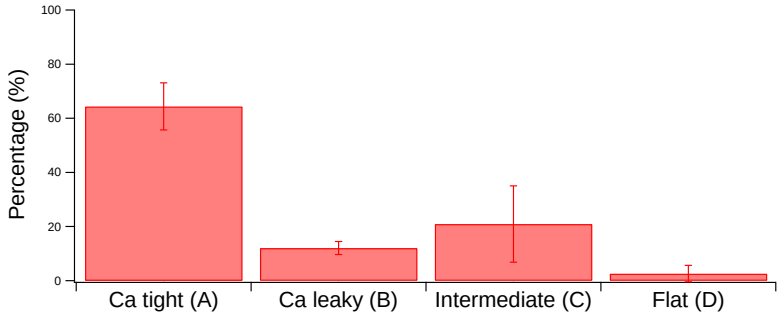

Figure 29 Percentage of four different behavior followed by traces of single liposomes containing Fluo-5N

From the graph emerges that a majority of the liposomes are tight to Ca^{2+}(A), about 10% are leaky (B) and about 20% show an intermediate behavior (C) that could suggests a partial equilibration. The rest (D) don't respond to calcium both in presence and in absence of ionomycin. The experiment was repeated two times and the error bars are the standard deviation of the mean percentage.

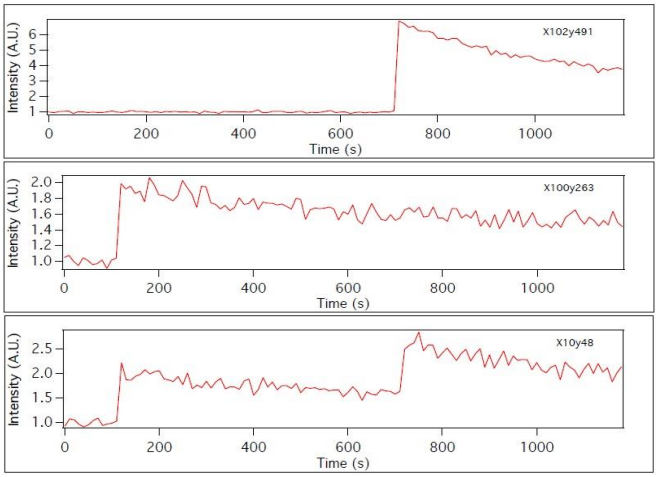

Figure 30 Single liposomes traces showing three different behaviors during calcium leakage experiment.

The first graph shows an example of liposome tight to calcium: the signal increases only in presence of ionomycin. The second graph shows the trace of a liposome leaky to calcium: signal increase in absence of ionomycin after injection of calcium. The third graph shows the partial equilibration: signal increases both in absence and in presence of ionomycin. After 120 s calcium is injected, after 720 s calcium and ionomycin are injected.

B.2 Emission spectrum of ATTO655-DOPE containing liposomes in bulk

In order to record the excitation profile of ATTO655, it was necessary to establish the position of the emission peak of the fluorophore. The emission profile was determined experimentally and the emission peak was found to be at 679 nm as can be seen on Figure 31.

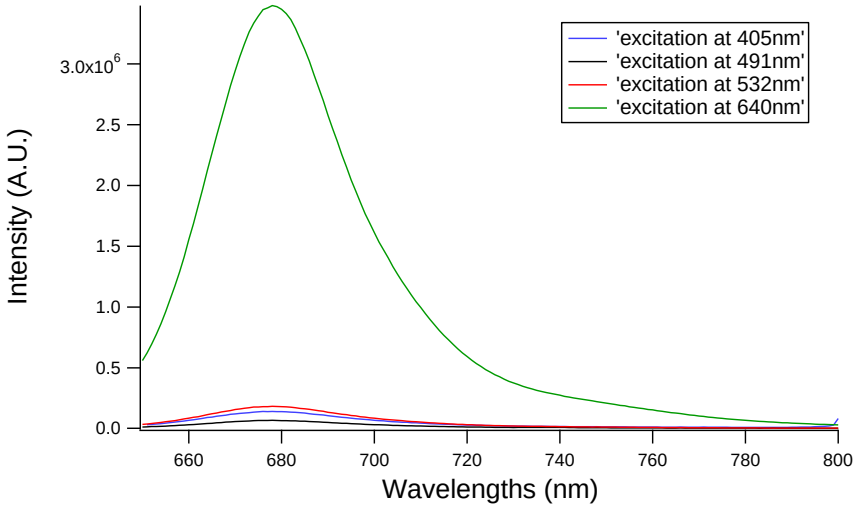

Figure 31 Emission spectrum of liposomes containing ATTO655-DOPE
Emission profile with four different excitation wavelength of a solution of tris buffer 20 mM containing ATTO655-DOPE liposomes 200x diluted, KCl 200 mM, $MgCl_2$ 1 mM, DTT 1 µM and Valinomycin 65.2 nM. The emission peak was found experimentally at 679 nm for all four excitation wavelengths. It is possible to notice that the biggest amplitude is reached when the dye is excited at 640 nm. The dye is excited also at 491 nm, even if the intensity of the peak is 2% of the intensity of the peak when the excitation is 640 nm.

The emission peak was used in order to measure the excitation profile of liposomes containing ATTO655-DOPE.

C - Spectrometric measurements of Oregon Green Bapta-2 in bulk

Oregon Green Bapta-2 is a calcium sensor with higher affinity to calcium respect to Oregon Green Bapta-5N. It has a excitation peak at 492 nm and an emission peak at 523 nm. It was tested in bulk in order to verify its compatibility with the single liposome assay.

- *Experimental conditions*

1) The measurements to obtain a calcium calibration curve for Oregon Green Bapta-2 were performed on 1 mL solution containing: free Oregon Green Bapta-2 (0.5 µM), Tris buffer (10 mM, pH 9 and pH 7). The excitation wavelength used was 488 nm, the emission wavelengths range was from 500 nm to 650 nm. The slit width was set at 1 for both emission and excitation. Each spectrum is an average of three measures. When the calcium titration was performed in buffer at pH 9, the following calcium concentrations were used: 10 nM, 50 nM, 0.5 µM, 1 µM, 2.5 µM, 5 µM, 10 µM, 90 µM, 200 µM, 500 µM. When the calcium titration was performed in buffer at pH 7, the following calcium concentrations were used: 0.5 µM, 1 µM, 50 µM, 100 µM, 200 µM.

2) The measurement performed in order to compare the fold increase of Oregon Green Bapta-2 was performed on 1 mL solution containing: free Oregon Green Bapta-2 (0.5 µM), either Tris buffer (10 mM, pH 8) or milliQ water. The excitation wavelength used was 488 nm, the emission wavelengths range was from 500 nm to 650 nm. The slit width was set at 1 for both emission and excitation. The measurements in water or tris were repeated three times. The concentration of calcium used to saturate the fluorophore was 500 µM.

- *Results*

Even upon changes of pH, the fold increase of the free fluorophore in bulk was up to only 1.5 folds as it is possible to see from Figure 32, while from literature it was expected a fold increase of about 100 folds.[47] In order to establish if the weak fold increase was due to buffer composition, the fluorophore was diluted also in water and it was saturated with 500 µM of calcium. In Figure 33 the fold increase after injection of 500 µM calcium both in tris buffer pH 8 and water is compared. It is possible to notice a bigger fold increase when the dye is diluted in water, suggesting that the fluorescent properties of the dye are inhibited when it is diluted in tris. Since the buffer composition couldn't be changed in order to suite the fluorophore and the fold increase was too weak, it was decided that the fluorophore didn't suite the single liposome assay.

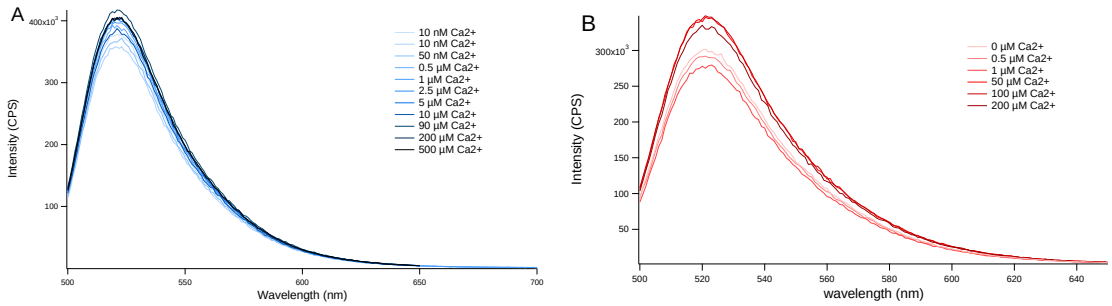

Figure 32 Calcium titration of free Oregon Green Bapta-2

Emission profiles of Oregon Green Bapta-2 in solution with Tris buffer 10 mM at two different pH values: A) pH 9, B) pH 7. In both the experiments the fluorophore was diluted to a concentration of 0.5 µM in the cuvette. It's possible to notice that the fold increase is around 1.5 and 1.1 folds, respectively.

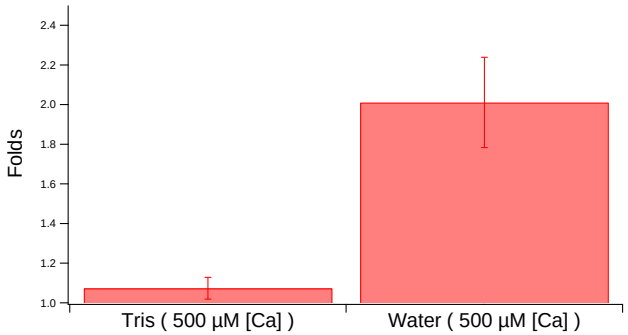

Figure 33 Response of free Oregon Green BAPTA-2 diluted in two different solutions: tris buffer (pH 8) and water.

The dye in the two solutions has been saturated with a Ca^{2+} concentration of 500 µM. The fold increase of the signal when the dye is diluted in water is 2 compared with the lower fold increase when diluted in buffer. This suggests an interaction of the dye with the buffer that prevents it to fluoresce.

D - Shift of the pK_a of Snarf-DOPE

In Sections 5.1.1 and 5.1.2 the pK_a of Snarf-DOPE was shown both for liposomes in bulk and in the single liposome assay. It is possible to notice a shift of the average pK_a value between the experiment in bulk and the experiment at the microscope: $pK_{a\ bulk}$ = (9.3 ± 0.1), $pK_{a\ microscope\_avg}$ = (8.0 ± 0.1). This can be due to the fact that the intensity ratio is calculated in two different ways. The intensity ratio of the bulk data is calculated dividing the intensity value corresponding exactly to the peak at 583 nm by the intensity value corresponding exactly to peak at 650 nm. At the microscope it is not possible to collect the emission signal corresponding exactly to the two emission peak wavelengths. Each intensity data point can be mathematically described as a definite integral of the emission spectrum: the intensity of the 583 nm peak is a definite integral from ~ 544.5 nm to ~ 619.5 nm, the intensity of the 650 nm peak is a definite integral from ~ 650 to infinity. The limits of the integrals do not refer to a specific wavelength because the used filters do not cut the light exactly at a specific wavelength. In the following graph the intensity ratio from bulk data was calculated integrating the emission spectra of Snarf-DOPE and dividing the corresponding calculated intensities. The extremes of the integrals are [550 - 620] nm for the peak at 583 nm and [650 - 700] nm for the peak at 650 nm. These extremes were chosen to be similar to the filters used at the microscope. It is possible to see from the graph the estimated pK_a = (9.0 ± 0.1) that is decreased compared to the $pK_{a\ bulk}$ = (9.3 ± 0.1) even if the value is still not comparable with $pK_{a\ microscope\_avg}$ = (8.0 ± 0.1). This could be due to the fact that the extremes of the integrals used to calculate the intensities did not represent exactly the filters used during the microscope measures.

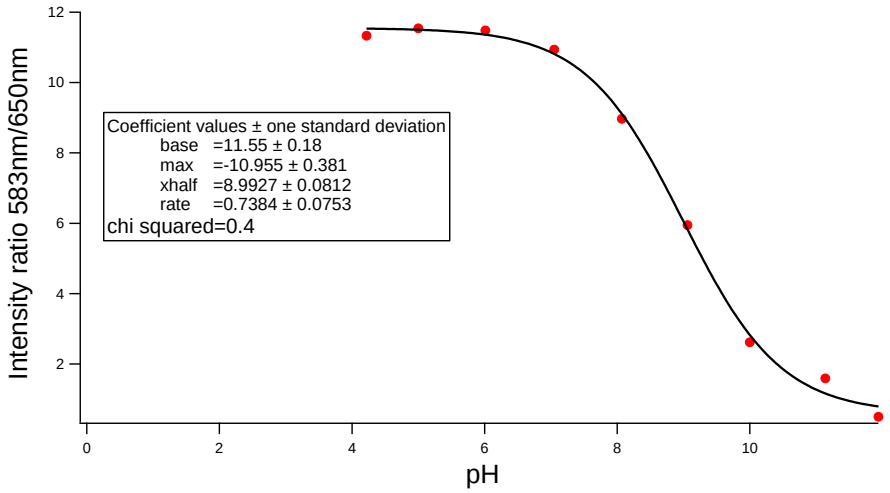

Figure 34 intensity ratio curve from spectrofluorometric measurements of liposomes containing membrane dye Snarf-DOPE. The intensity of the peak at 583 nm was calculated integrating the emission spectrum in the interval [550 - 620] nm while the peak at 650 nm was calculated integrating the emission spectrum in the interval [650 - 700] nm. The intervals were chosen to be similar to the band filters used during the experiment at the microscope.