Chapter 7

Probing protein–membrane interactions using optical traps

This chapter is based on a publication in preparation (see page 205).
Abstract — Membrane interactions are vital for the cell and occur in many important cellular processes such as endo- and exocitosis. The capture and fusion of synaptic vesicles to the plasma membrane of neurons is known to be mediated by several proteins including the well known SNARE complexes. Despite their importance a mechanical and physical understanding of the protein–induced membrane interactions are currently lacking. The small amount of biophysical studies on these systems is perhaps due to the limited number of biophysical tools available to study these interactions. Here we present a new experimental method that allows the characterisation of membrane-protein-membrane interactions using dual optical tweezers. This method allows the characterisation of protein–membrane interactions free in solution and gives access to many bilayer samples per single experiment. We demonstrate the method by measuring the binding strength of two membranes linked by Doc2b, an important protein in neurons inside our nerve cells. These kinds of experiments can be easily expanded to many other membrane interaction proteins, opening a whole new range of possible research questions that can now be addressed.


7.1. Introduction

Signals in the brain where long thought to be dependent on electrical signals only. However, in the early 20th century, it was discovered that neurons can communicate to each other and to non–neuronal cells by sending special chemical signals (neurotransmitters) through a small space between these cells (synaptic cleft). The neurotransmitters activate the receptors on the receiving cell in order to adapt the cell for incoming signals. After processing the signal, the receiving cell cleans up the transmitter, and prepares itself for the next signal. The neurotransmitter are packaged into synaptic vesicles, consisting out of phospholipid bilayers (figure 7.1a). In order to release its content to the receiving cell, the vesicle has to undergo a series of events from docking on the membrane (figure 7.1b), getting to a release–ready state, and finally fusing the two membranes (figure 7.1c) [169, p. 420–424].

![Figure 7.1. Simplified schematic of synaptic vesicle fusion. a. Synaptic vesicle of a lipid bilayer containing neurotransmitters. b. Docking of the vesicle to the plasma membrane. The protein Doc2b is known to bind two membranes, and enhance the fusion. c. Protein mediated fusion, mediated by the long coiled–coil domains of the SNARE proteins bound to the plasma membrane and synaptic vesicle. Note that the whole process of vesicle fusion involves many proteins, which are here omitted for clarity.](image-url)
One of the proteins involved in the process of synaptic vesicle fusion in the brain are the Doc2 (double C2 domain) proteins [170]. There are three Doc2 family proteins: Doc2a, -b, and -c, of which a, b are expressed in the brain [171]. Doc2b by itself cannot achieve vesicle fusion, but it has been shown to bind to lipid vesicles (in the presence of Ca\(^{2+}\)) and enhance the synaptic vesicle fusion by acting as a highly sensitive Ca\(^{2+}\) sensor [171–174]. However, little is known about the mechanical properties and mechanism of these proteins.

Here we present a new method to study protein mediated membrane–membrane interactions at the single molecule level. Where previous techniques removed part of the substrate by immobilising the protein to a surface (for example to a micro bead [175, 176]), we choose to i. study the interactions between two bilayers and ii. have the protein free in solution. In the experiments two beads, coated with a lipid bilayer, are brought in close contact using optical tweezers to allow protein induced bridging and/or fusion of the lipid bilayers. The force that is needed to dissociate the protein–membrane complex is investigated to reveal the molecular mechanism and binding strength of the formed protein–membrane complex. The developed assay is tested by measuring the binding strength of the C2 domain of the Doc2b protein.

### 7.2. Materials and method

#### 7.2.1. Proteins and lipid–coated beads

The Doc2b proteins where purified as described in [173]. All experiments are done in a buffer containing 25 mM NaCl, 25 mM HEPES (pH 7.4), 1 mM TCEP, and 100 \(\mu\)M CaCl\(_2\) or 3 mM EGTA.

The lipid bilayer is prepared on freshly washed and sonicated (MQ) polystyrene beads (3.84 \(\mu\)m in diameter). The (phosphatidylinerine) lipids undergo a series of treatment to promote a uniform layer and prevent
phase separation during the process. The beads and lipids are mixed and incubated, protocols as described in [177].

To check if the protocol worked and the beads are indeed homogeneously covered by a lipid bilayer, a small amount of NBD labelled phospholipids are added (Invitrogen, excitation, emission maxima at 463, 536 nm). The beads are imaged using a confocal microscope, and a continuous layer is seen in the images (figure 7.2a).

**Figure 7.2. Fluorescent images of silica beads containing NBD labeled lipids in the bilayer.** a. Images obtain in a confocal microscope that are used to check the quality of the prepared bilayer. b. Wide-field imaging of trapped beads using a combined optical trapping and fluorescence microscopy. The images are taken at the i. start and ii. end of a trapping experiment that lasted for $\approx 10$ minutes.

To check if the lipid bilayer does not get destroyed by the heat generated in the optical trap [162], the beads are imaged in an optical trap setup that is combined with fluorescence detection [122, 167]. As is seen in figure 7.2b the bilayer is still intact even after spending $\approx 10$ minutes in focus of the optical trap, indicating that the lipid bilayer is not visibly affected by the trapping laser.

### 7.2.2. Instrumentation and method

To probe the interaction of proteins between two membranes, we need to be able to both control the distance between the two membranes and measure the forces on the protein–membrane complex. We choose to
use polystyrene beads coated with a lipid bilayer as our substrate. The position of the beads is controlled with sub nanometer precision using optical tweezers, with which the force on the beads can be measured with \( \approx 0.1 \text{ pN} \) precision [106, 107, 139, 148]. To allow the protein of interest to interact with the bilayers, the two beads are brought into close proximity (figure 7.3a). The protein induced interactions between the lipid membranes are probed by retracting one of the beads at a constant velocity while we monitor the force on the stationary bead (figure 7.3b). During pulling the force will build up until the protein–membrane complex ruptures.

**Figure 7.3. Assay for probing lipid–membrane interactions using optical tweezers.** a. Two, polystyrene beads are coated with a lipid bilayer and brought into close contact in presence of the protein of interest. b. One of the beads is moved at a constant velocity (\( v \)) to probe the protein–lipid complex. The force (\( F \)) it takes before the complex breaks or dissociates is recorded on the second bead.
7.3. Results

7.3.1. Bead–bead interactions

In order to measure interactions between the two membranes, the lipid bilayers have to be brought in close proximity. However, since the beads vary in size (⌀ 4% STDEV), the distance at which the two bilayers are actually touching varies from bead to bead. Therefore, the spacing between the two beads is decreased in steps, while we probe for bridging events at each successive step. We start by placing the two beads at a distance of 100 nm, wait a few seconds and retract the first bead by about a micron to see if there are any interactions. The beads are now moved closer with increments of 5 nm and the procedure is repeated until the beads are pushed into each other by several pN’s (figure 7.4a).

To examine the interactions between the two lipid coated beads, the measured force is plotted as a function of the distance ($d$) between the centre of the two beads figure 7.4b. When the two beads approach ($d \approx 4-5 \mu m$), the force rises due to the interference of the two traps and attraction between the two beads. When decreasing the distance between the two beads to roughly the diameter of the bead, we start to push the beads into each other, which results in a sharp drop in the measured force (starting in this case around $d = 3.8 \mu m$).

Using salt concentrations above 25 mM NaCl resulted in sticking interactions between the two lipid membranes in the absence of proteins. These sticking events could be misinterpreted as a protein bridging the two membranes, or worse, obscure the signal caused by the protein interacting with the membranes. Therefore, all measurements are carried out at 25 mM NaCl or less, where little to no interactions between the beads in buffer are observed.
Figure 7.4. Method for measuring protein–membrane interactions using optical tweezers. a. The distance between the beads is decreased in steps of 5 nm. Between each step the beads are incubated a few seconds, and moved apart (at a constant speed $v$) to probe any possible bridging between the two membranes. b. Force–distance graph of a single probe event. There is a significant interference force between the two traps just before the beads start touching each other ($3.8 \mu m \approx$ the diameter of the beads). At shorter bead–bead distances the beads push each other out of the traps, giving rise to a decreasing force. While at higher distances the force lowers due to the diminished effect of the interference.
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Is it indeed possible to use this method to measure protein mediated lipid membrane interaction? To find out, we introduce the lipid binding protein Doc2b in the system and follow the measurement protocol as described in the previous section. During the gradual decrease of the distance between the two beads there is a point at which the force steeply increases as we retract the beads (figure 7.5a). This rise in force is due to the bridging of the two membranes by the protein. Upon breakage of the protein–membrane complex, the force drops and follows the force distance curve as expected. It is interesting to note that the force spike is observed before the beads touch (i.e. there is no dip observed in the curve).

To characterise the breakage force, multiple events need to be registered. Therefore, as soon as the first peak appears during the stepwise decrease of the distance between the two bead, the movement is stopped. Next, 200 back and forward traces are recorded moving from and to the position where the first peak occurred (figure 7.5b). To build up a reliable statistics, the beads are discarded after 200 events, two new beads are caught and the process is repeated. The resulting data traces are inspected and the rupture forces (clearly visible as peaks in the force) are extracted. The extracted force is filtered by its corresponding distance at which the rupture occurs. Only rupture events that occur within 10% of the diameter of the bead are taken into account. This last step eliminates false positive events.

7.3.3. Characterisation of the rupture force

The rupture forces are collected for several measurements and plotted in a histogram (figure 7.6). A clear peak in the distribution of the rupture forces is observed at 17.5 pN (bin size 5 pN, N ≈ 200 events). In order
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Figure 7.5. Membrane bridging by the Doc2b protein, measured using optical tweezers. a. Force distance curve of a single probe event in the presence of Doc2b. When retracting the left bead \((v = 5 \mu m \, s^{-1})\), the force rises while the distance between the centre of the two beads stays constant. This is due to the bridging of the two membranes by Doc2b. Around 35 pN, the bridge between the membranes breaks and the force starts to follow the force distance curve without any bridging. b. In order to obtain a statistical measurement of the membrane rupture, the force distance curve as shown in panel a is repeated 20 times. As is seen the membranes are bridged around 50% of the time, and rupture of the complex occurs at different forces.
to show the interactions measured with the Doc2b are due to specific interactions, two controls are performed. First the interactions where measured in the absence of protein, where no peaks were observed in the traces. Second as Doc2b is known to have a strong calcium dependence, and should not bind without it, we perform experiments in the absence of CaCl$_2$ and in the presence 3 mM EGTA.

To be able to compare the obtained results between the measurements in Calcium and EGTA, the interactions are probed roughly the same amount of time and plotted on the same scale in a histogram (figure 7.6 inset). The EGTA measurements show very few peaks, confirming the loss of lipid binding by Doc2b without calcium. The controls provide the proof of principle that with this new technique we are able to probe and quantify the specific interactions between two membranes of protein free in solution.

7.4. Discussion and conclusion

In this chapter we demonstrated a new method to measure protein–membrane interactions. One advantage over existing techniques is that by using two optically trapped beads, we are able to study the protein free in solution without immobilising it to a surface. In experiments done by Pyrpassopoulos et al [175], the proteins are attached to a surface immobilised pedestal bead and only a single lipid coated micro bead is used to probe the interactions. In their experiments a clear distribution in rupture forces is obtained, however, the question arises if the activity of the protein is not altered by the tags and proximity of the surface. The method described in this chapter is actually very close to the real cellular situation and allows us, for instance, to study concentration effects, rapid change of buffer conditions as well as studying the interplay of multiple membrane interacting proteins at the same time.

Atomic Force Microscopes (AFM) are also used to image membranes
Figure 7.6. Distribution of rupture forces induced by the Doc2b protein. The rupture forces are extracted from the force–time traces as described in section 7.3.2. The histograms contain empty bins at low forces because the rupture force is defined as the force peak on top of the force distance curve without any protein. The inset shows the results of rupture forces when the CaCl$_2$ is replaced by 3 mM EGDTA. Both histograms contain roughly the same amount of probing events. As is seen the amount of bridging effects in the presence of EGTA is drastically reduced compared to the bridging with Ca$^{2+}$ because, as expected, Doc2b needs calcium to bind to lipid membranes.
and the proteins that recite in the membrane [178,179]. In addition, with AFM it is also possible to probe proteins bound to the membranes [180]. However, using two optical tweezers enables us to study the interactions of a protein between two lipid bilayers. Moreover, it provides a very quick and easy method to change substrate: simply catching two new beads provides the experimenter with a clean membrane. Combined with automatising of the measurements, this opens the way to gathering large amount of data and statistics.

Using combined optical trap and fluorescence microscopy [122, 167] , enabled the visual inspection of the status of the (fluorescence) bilayer as the experiment progressed. The combination of these two techniques has strong potential as multiple proteins that govern the fusion of membranes can be labeled with different colours and simultaneously observed at work in real-time. The newly developed method allows the characterisation of one of the most important systems in our neurons (the SNARE complex) and opens up a whole new range of possible questions that can now be addressed.