Interactions between lipid bilayers and membrane proteins can be revealed by recording the fluorescence of tryptophan residues and collisional quenching by bromine atoms bound to lipid fatty acid chains. These important interactions are far easier to detect using fluorescence quenching than with other biophysical methods as for example X-ray crystallography. By choosing the tryptophan site and location of bromine on the fatty acid chain, high structural specificity is obtained. In this application note some practical tips are given while further practical considerations can be found in the literature.

Membrane proteins make up about a third of all proteins. They transport molecules and information over the membrane and their importance is proven by the fact that they provide the majority of drug targets. The lipid bilayer is not only a passive solvent for membrane proteins but often specific interactions between lipid molecules and proteins exists. They may have structural importance or even modulate the activity of a protein and disturbances can cause disease.

Lipid molecules are often not resolved in X-ray structures of membrane proteins therefore other techniques need to be employed to measure lipid-protein interactions. Here, an easy method is described where tryptophan residues are used as fluorescence probes which can be introduced at locations where a lipid-protein interaction is suspected. Sometimes in existing crystal structures it is not clear where exactly lipids can contact or ab initio data for predicted transmembrane helices can be obtained to predict the topology of a membrane domain. The membrane protein is reconstituted into lipid bilayers with and without bromine bound to the fatty acid chains. Bromine atoms will quench the fluorescence of tryptophan if it is located at the lipid-protein interface.

As an example, a quenching experiment on the mechanosensitive channel of small conductance (MscS) from Escherichia coli is presented here. Interaction of lipids with bacterial mechanosensitive channels is particularly interesting because they are able to sense directly the tension in the membrane which is still not fully understood.

Emission spectra were recorded in an FLS980 Fluorescence Spectrometer equipped with double excitation and emission monochromators. Calcite polarisers were used in the excitation and emission, while a photomultiplier tube detector (Hamamatsu, R928P) with 1 s dwell was utilised. The FLS980 provides a flexible and sensitive instrument to record the emission that allows for optimising the settings, while polarisers are convenient to repress effects interfering with the fluorescence of tryptophan. For this reason, the polarisers are set to 90° at the excitation and 0° at the emission to minimise the effect of light scattering.

Tryptophan residues are used as a probe in this method so that site-directed mutagenesis is often required to remove native tryptophans and introduce them in other positions. The abundance of tryptophan is often low in proteins and it can in many cases be replaced by other aromatic amino acids, phenylalanine or tyrosine, without loss of function. While native tryptophan residues can be used, this method is much more powerful if the location of the probe can be freely selected.

It is necessary to purify the membrane protein, which is often the most difficult part of the experiment, but often established for other biochemical and biophysical studies. Typically, a few mg will be sufficient for a series of experiments. Dodecylmaltoside has been used for the purification of proteins intended for this experiment. The concentration of the membrane protein was determined by UV-Vis spectroscopy. The required protein concentration may vary as the quantum yield of the tryptophan can vary considerably dependent on the environment. The stock solution of MscS I150W had a concentration of 47 µM and MscS N207W 34 µM which resulted in final concentrations for the measurements of 0.48 µM and 0.44 µM, respectively. An extinction coefficient of 15.93 mM cm⁻¹ was used that was calculated from the primary sequence using a protein identification tool (ProtParam).

The lipids used in this experiment were brominated in-house under a fume hood. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and its brominated equivalent (BrOC) were used. A known amount of lipid as chloroform solution is added to a glass tube, the chloroform is carefully removed in a nitrogen stream and then by desiccation, and resolved in a buffer containing cholate by sonication.

Lipid and protein solutions were mixed at a ratio of 100:1 (mol/mol) and allowed to equilibrate for 15 min at room temperature. The mixture is then diluted 20 times into a buffer without detergent for reconstitution of the protein into lipid bilayers directly in the fluorescence cuvette. Samples are equilibrated for 5 min.
Standard stirred fluorescence cuvettes (Hellma, 109.004F) and custom-made cuvettes were used with 4x4mm light paths to minimise the required sample volume to 0.6 ml but still allow stirring. The temperature was kept constant at 20°C.

The fractional quenching (FrQ) was calculated as \( FrQ = \frac{F_0 - F}{F_0} \), where \( F_0 \) is the fluorescence intensity of samples reconstituted in non-brominated lipids and \( F \) in brominated lipids. The methods followed here are graphically presented in the inset of Figure 2.

\[ \text{Fractional quenching} \ (FrQ) = \frac{F_0 - F}{F_0} \]

The crystal structure of MscS shows unusual “paddles”, see inset of Figure 3, that seem to be located in the membrane and are presumably important for tension sensing. MscS is a homo-heptamer with “paddles” on the membrane domain and in addition a large cytosolic domain.

However, it is not clear if lipids can penetrate between the paddles and if the paddles are completely immersed in the lipid bilayer. A functional tryptophan-free version of MscS was labelled with tryptophan in the gaps between the paddles at position I150W and as negative control on the cytosolic domain at N207W far away from the membrane.

The raw spectra in Figure 1 show clearly the Raman signal of water around 330 nm. Control spectra of lipid samples without MscS were subtracted from the raw spectra and are displayed in Figure 2. Then, the fractional quenching was calculated over the whole spectrum, see Figure 3.

The excitation wavelength was set to 295 nm to select tryptophan fluorescence and avoid excitation of tyrosines. Still some effect of tyrosines in the region 300 nm-320 nm might be seen.

A fractional quenching of \( \sim 0.5 \) can be seen for I150W proving that lipids penetrate into the gaps between the paddles. The control at N207W shows no quenching.

The fractional quenching for I150W shows hardly any wavelength dependence. Only at higher wavelengths a drift is seen due to the low total intensities of the spectra here (Figure 1A and B) and thus higher inaccuracy. For N207W, some quenching is seen below 320 nm which is most likely caused by tyrosine quenching (Y75 is probably exposed to lipids) despite excitation at 295 nm. Therefore, we usually report data for 340 nm which are not influenced by tyrosine fluorescence or the Raman signal but still have high intensities in the raw data.

The presented experiment can be extended to further investigate a) the number of lipid binding sites, b) the relative binding constants for lipids, and c) the depth-dependent fluorescence quenching in the membrane. In particular, the experiments can include:

a) In addition to samples at 0% and 100% brominated lipid, intermediate mixtures can be prepared (e.g. in 10 or 20% steps) that reveal the concentration dependence of the quenching. This concentration dependence of quenching can be interpreted and analysed as the number of binding sites at the tryptophan location.

b) Different lipids can be taken as non-brominated and brominated constituents and concentration dependent experiments can be performed. If the number of binding sites is known, one can calculate the binding constant of one lipid relative to the other.

c) The bromine can be bound at different positions along the fatty acid chain of the lipid and the quenching is measured for these different positions. With the distribution analysis one can then determine how deep a certain tryptophan is located within the membrane.

The experimental procedure is shown in the inset of Figure 2.

\[ \text{Fractional quenching} \ (FrQ) = \frac{F_0 - F}{F_0} \]

Figure 1: Emission spectra of brominated and non-brominated lipids with lipid-only controls. The samples were excited at 295 nm with \( \Delta \lambda_{\text{exc}} = 3 \) nm and \( \Delta \lambda_{\text{em}} = 7 \) nm, \( \text{Pol}_{\text{exc}} = 90^\circ \), \( \text{Pol}_{\text{em}} = 0^\circ \), step = 1 nm, \( t_{\text{integr}} = 1 \) s.

Figure 2: Emission spectra of the reconstituted protein samples with 100% of non-brominated lipids or 100% of brominated lipids were obtained. Emission spectra of controls which contain lipids but no protein were subtracted. A summary of the experimental procedure is shown in the inset.

Figure: Fractional quenching of the samples calculated from the corrected spectra in Figure 2 in the integrated software package F980. Inset: Crystal structure of MscS11 showing the two independently labelled positions, I150W and N207W (negative control). The approximate position of the membrane is indicated (orange bars).
REFERENCES


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