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Rapid Assessment of Membrane Protein Quality by Fluorescent Size Exclusion Chromatography

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Abstract

During membrane protein structural elucidation and biophysical characterization, it is common to trial numerous protein constructs containing different tags, truncations, deletions, fusion partner insertions, and stabilizing mutations to find one that is not aggregated after extraction from the membrane. Furthermore, buffer screening to determine the detergent, additive, ligand, or polymer that provides the most stabilizing condition for the membrane protein is an important practice. The early characterization of membrane protein quality by fluorescent size exclusion chromatography provides a powerful tool to assess and rank different constructs or conditions without the requirement for protein purification, and this tool also minimizes the sample requirement. The membrane proteins must be fluorescently tagged, commonly by expressing them with a GFP tag or similar. The protein can be solubilized directly from whole cells and then crudely clarified by centrifugation; subsequently, the protein is passed down a size exclusion column, and a fluorescent trace is collected. Here, a method for running FSEC and representative FSEC data on the GPCR targets sphingosine-1-phosphate receptor ($S1PR_1$) and serotonin receptor ($5HT_{2A}R$) are presented.

Introduction

Size exclusion chromatography (SEC), also known as gel filtration chromatography, is commonly used in protein science[1]. During SEC, proteins are separated based on their hydrodynamic radius, which is a function of the protein size and shape[2]. In brief, this separation is achieved by applying the protein samples under flow to a packed bed of porous beads that act as a molecular sieve. The beads used are often cross-linked agarose with a defined range of pore sizes to allow proteins to either enter or be excluded from the pores of the beads[3,4,5,6,7]. Proteins with smaller hydrodynamic radii spend a greater proportion of time within the pores and, thus, flow through the packed bed at a slower rate, whereas larger proteins spend a greater proportion of time outside the beads (the excluded volume) and move through the packed bed at a faster rate. SEC can be used as a protein purification step when a preparative column is used[1]. When an analytical

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column is used, SEC can be used to analyze the protein quality and properties[2]. For example, protein aggregates that may be present in a sample and indicate poor quality protein tend to be very large, meaning they only travel in the excluded volume and, thus, are eluted from the column at the earliest point; this volume is referred to as the column void or void volume. Furthermore, molecular weight standards can be used to calibrate the column, allowing an estimated molecular weight of the protein of interest to be interpolated from a standard curve.

Typically, the protein absorbance at 280 nm is used to monitor the protein elution from a size exclusion column. This restricts the use of SEC as an analysis tool until the protein of interest is largely free from contaminating proteins, for example, at the last step of protein purification. However, fluorescent SEC (FSEC) utilizes a protein of interest that is fluorescently labeled. Therefore, a fluorescent signal can be used to specifically monitor the elution of the protein of interest in the presence of other proteins or even crude mixtures[8,9]. Furthermore, as fluorescent signals are highly sensitive, successful analysis can be performed on samples with extremely low protein quantities. The protein of interest is often fluorescently labeled by including a green fluorescent protein (GFP) or enhanced GFP (eGFP) tag in the expression construct. The fluorescent signal can then be monitored by excitation at 395 nm or 488 nm and detecting the fluorescent emission at 509 nm or 507 nm for GFP or eGFP, respectively[10].

The benefit of using a fluorescent signal to monitor protein elution from an SEC column makes FSEC a valuable tool for analyzing membrane protein samples when the expression levels are particularly poor in comparison to soluble proteins. Crucially, the quality and properties of membrane proteins can be analyzed directly following solubilization from crude lysates without the requirement to optimize the purification process first[11,12]. For these reasons, FSEC can be used to rapidly analyze the membrane protein quality while exploring the different factors that may be required to improve the behavior of the membrane protein in solution. For example, it is common to trial numerous constructs containing different tags, truncations, deletions, fusion partner insertions, and stabilizing mutations to find one that is not aggregated after extraction from the membrane[13,14]. Furthermore, buffer screening to determine the detergent, additive, ligand, or polymer that provides the most stabilizing condition for the membrane protein can define the best buffer composition for protein purification or for providing stability for downstream uses, such as biophysical assays or structural characterization.

Thus, the overall goal of the FSEC method is to collect an SEC column elution profile for a target membrane protein of interest. Furthermore, as fluorescence is used, this SEC trace is collected at the earliest possible point in the optimization of the constructs and conditions prior to any lengthy purification. The FSEC trace can be used as a comparative tool to judge the likelihood of success of purifying a membrane protein with different buffer conditions or membrane protein constructs. In this way, the collection of FSEC profiles can be used as a quick iterative process to arrive at optimal construct design and buffer composition prior to spending effort generating the quantities of pure protein required for other analysis methods.

Protocol

1. Detergent and buffer preparation for FSEC

1. Prepare a detergent stock solution.

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1. To prepare a 20 mL stock solution, weigh 4 g of dodecyl maltoside (DDM) powder and 0.4 g of cholesteryl hemisuccinate (CHS) powder, and make it to 20 mL with laboratory-grade distilled H_2O.

2. After adding all the components, mix with end-over-end inversion at 4 °C until the components are completely solubilized. Overnight end-over-end mixing at 4 °C is recommended.

3. Aliquot and store the detergent stocks at −20 °C until use. If the stock needs to be used immediately, store the detergent stock on ice.

   NOTE: The standard detergent stock used in this work was a 20% (w/v) DDM and 2% (w/v) CHS mixture (see **Table of Materials**). Different detergents can be used (e.g., lauryl maltose neopentyl glycol; LMNG), or the use of detergent-free extraction with polymers such as styrene-maleic acid (SMA) can be tested. This needs to be decided when designing the experimental conditions to be tested.

2. Prepare a solubilization buffer.

   1. Prepare a solubilization buffer by combining the correct weight or volume of the components to achieve a final concentration of 100 mM HEPES, 200 mM NaCl, 20 % (v/v) glycerol, and 1x protease inhibitor cocktail (see **Table of Materials**) in a beaker.

      NOTE: In the present study, the preparation of 50 mL of solubilization buffer was sufficient for processing five samples.

   2. Add a 0.7 volume (e.g., 35 mL if making 50 mL of buffer) of laboratory-grade distilled H_2O to the beaker.

   3. Mix on a magnetic stirrer, and using a pH meter, adjust the buffer pH to 7.5 by the dropwise addition of concentrated NaOH.

   4. Using a measuring cylinder, top up the buffer to the final required volume with laboratory-grade distilled H_2O.

3. Prepare the SEC running buffer.

   1. Prepare the SEC running buffer by combining the correct weight or volume of the components to achieve a final concentration of 100 mM HEPES, 150 mM NaCl, and 10% (v/v) glycerol in a beaker.

      NOTE: In the present study, preparing 600 mL of SEC buffer was sufficient for running five samples.

   2. Add a 0.7 volume (e.g., 420 mL if making 600 mL of buffer) of laboratory-grade distilled H_2O to the beaker.

   3. Mix on a magnetic stirrer, and using a pH meter, adjust the buffer pH to 7.5 by the dropwise addition of concentrated NaOH.

   4. Using a measuring cylinder, top up the buffer to the final required volume with laboratory-grade distilled H_2O.

   5. Filter the SEC buffer through a bottle-top 0.45 µm pore filter under a vacuum (see **Table of Materials**).

   6. Once the buffer has passed through the filter, degas it by leaving it under the vacuum until no more bubbles appear when shaken.

   7. Add a final concentration of 0.03% (w/v) DDM and 0.003% (w/v) CHS to the SEC buffer by adding the required volume of the detergent stock prepared in step 1 (e.g., 0.9 mL if making 600 mL of SEC buffer).

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8. Pre-chill the buffer before use.

NOTE: Different buffers can be used depending on the conditions tested. For example, if testing the effect of different detergents on the protein of interest, a buffer with the same detergent as the one used to solubilize the protein would ideally need to be made. If testing detergent-free conditions with SMA, detergent should be omitted from the SEC buffer completely. However, see the discussion section for more details about protocol modifications for detergent screening.

2. Sample preparation for FSEC

1. Prepare the cell pellets.

NOTE: The starting point for the assay is harvesting the cell pellet from a suspension cell expression culture of the GFP-tagged (or other fluorescently labeled) protein of interest. The exact timings and conditions for harvest will depend on the protein being expressed, the cell line being used, the conditions that the cells that have been grown in, and the method by which protein expression has been induced. These details are beyond the scope of this protocol. In this study, 0.5-1 g of Sf21 cell pellet was used per condition to be tested, corresponding to 25-50 mL of culture 2-3 days after infection with approximately 4 x 10^6 viable cells/mL of a 1:20 dilution of P2 baculovirus. Please note that the protocol described here has been tested and found to work equally well with similar wet weights of cell pellets from other eukaryoticcell lines (e.g., HEK293E).

   1. When the cells from the suspension cultures are ready to harvest, transfer 25-50 mL culture aliquots to 50 mL conical tubes.

   2. Counterbalance the tubes, and use a benchtop centrifuge in a swing-out bucket (see **Table of Materials**) at 2,000 x *g* for 15 min at room temperature to pellet the cells.

   3. Remove and discard the culture supernatant by gently tipping it away, or use a 50 mL pipet with a pipet filler if the cell pellet is particularly loose.

   4. If the cells will be used immediately for analysis, place the cell pellet on ice, and proceed directly to step 5. If the cells are to be saved for use at a later stage, freeze the cells by placing them at -80 °C.

   5. If the cell pellet has been stored at -80 °C, rapidly thaw it by incubating it at room temperature for 15 min or until the sample is no longer frozen. Move the sample immediately to ice following this step.

2. Resuspend and solubilize the sample.

   1. Add 2 mL of the solubilization buffer (step 1.2) to the cell pellet.

   2. Incubate with end-over-end inversion at 4 °C for 15-30 min until homogenous.

   3. Add premixed detergent stock (step 1.1) (e.g., 100 µL of 20% DDM/2% CHS stock) for a final concentration of 1% DDM/0.1% CHS.

   4. Solubilize for 30 min with end-over-end inversion at 4 °C.

   NOTE: If desirable, multiple conditions can be tested in parallel. The number of parallel samples that can be processed at once will depend on the available system for running the SEC experiment. In the setup described here, it was possible to process up to five samples at a time.

3. Perform a low-speed centrifugation step.

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1. Centrifuge the sample in a pre-chilled (4 °C) benchtop centrifuge in a swing-out bucket at 2,000 x *g* for 15 min.

4. Perform a high-speed centrifugation step.
   1. Carefully transfer the supernatant from the low-speed centrifugation to ultra-centrifugation tubes (e.g., 0.5-2 mL tubes) by using a blunt-ended needle attached to a 5 mL syringe, being careful not to disturb the pellet from the low-speed spin.
   2. Balance pairs of tubes to within 0.05 g, and place them in a fixed-angle ultracentrifugation rotor (see **Table of Materials**).
   3. Centrifuge at 4 °C for 30 min at 250,000 x *g*.

3. Size exclusion chromatography (SEC)

1. Prepare the fast protein liquid chromatography (FPLC) system, and equilibrate the column.
   1. Prepare the system following the manufacturer's instructions (see **Table of Materials**), for example, by filling the system with SEC buffer and purging the pumps of air.
   2. Connect the SEC column to the FPLC, ensuring no air enters the column. This is accomplished by applying back pressure to the SEC column with a syringe filled with water (attached to the bottom of the column) in order to perform a drop-to-drop connection with the flow path of the FPLC system.
   3. Pre-equilibrate the SEC column by washing it first in 1.5 column volumes (36 mL for a 24 mL column) of laboratory-grade distilled and filtered H_2O, followed by 1.5 column volumes of SEC buffer at the recommended flow rate and pressure for the column (see **Table of Materials**).

   NOTE: The FPLC system used in this study to perform FSEC was in a cold environment and had a five-position loop valve and a six-position plate fraction collector fitted. This setup allows five samples to be loaded and run sequentially down the same column in an automated fashion without requiring manual intervention between the runs. For running the SEC experiments, a commercially available pre-packed 24 mL column was used (see **Table of Materials**), which contained a resin that allowed proteins in the molecular weight range of 10-600 kDa to be resolved. If the protein of interest is particularly large, an alternative column matrix could be used instead, allowing the separation of proteins up to 5,000 kDa in molecular weight. Please note the presence of detergent/lipid micelle will increase the overall size of the membrane protein by >150 kDa, depending on the protein and detergent used.

2. Apply the sample to the column, and run the SEC experiment.
   1. Transfer the supernatant from the high-speed centrifugation step to a 1 mL syringe using a blunt-ended needle attached to the syringe. This allows for sample recovery from the centrifuge tube without disturbing the pellet.
   2. Set the sample loop to load. Overfill a 500 µL sample loop by injecting 600-700 µL of the sample from the syringe into the loading port. Depending on the system used, this loading step can be programmed into the method to ensure no mistakes are made.

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3. During the method, inject the sample from the loop into the column by emptying it with 4 mL of SEC buffer at the recommended flow rate and pressure for the column (see **Table of Materials**).
4. Run the column at the same flow rate until 1.5 column volumes (36 mL for a 24 mL column) of the buffer have passed down.
5. At 0.25 of the column volume (6 mL for a 24 mL column), start collecting 0.2 mL fractions in order to collect 90 fractions.

   NOTE: As the void volume of the column is expected to be 0.3 column volumes, beginning the collection of fractions immediately prior to this ensures that the elution of all the protein is monitored, including any protein present in the void volume.

4. Fluorescent trace collection and analysis

1. Transfer the samples from the fraction collector (step 3.2.5) to a 96-well plate, and read the fluorescent signal.
   1. Before taking a fluorescence reading, dilute the collected samples. Using a multi-channel pipette, transfer 90 µL of laboratory-grade distilled H_2O from a reservoir to each well of an opaque flat bottom 96-well plate (see **Table of Materials**).
   2. If fractions were collected into a 96-well block during step 3.2.5, use a multi-channel pipette to transfer 10 µL of the SEC fractions from the block to the opaque flat bottom 96-well plate, and mix by pipetting up and down. Otherwise, if SEC fractions were collected into individual tubes during step 3.2.5, transfer 10 µL of each fraction to the opaque flat bottom 96-well plate one by one, pipetting up and down each time to mix the samples.
   3. Place the opaque flat bottom 96-well plate into the plate reader, and measure the fluorescence. If GFP is the fluorescent label (used here), set the excitation as close as possible to 488 nm, and detect the fluorescent emission as close as possible to 507 nm.

      NOTE: The dilution required before taking the fluorescence reading will depend on the total amount of protein of interest present in the expression culture and the sensitivity of the plate reader being used. In the examples shown in this study, the samples were diluted 10-fold in water. As a suggestion for a starting point, the fractions should be diluted 5-10-fold in water or buffer before detection. If the recorded FSEC trace signal is particularly low, smaller dilutions or even an undiluted sample can be used instead. The device used for detection in this study was a plate reader capable of excitation at 488 nm and detecting fluorescent emission at 507 nm (see **Table of Materials**).
2. Plot the FSEC traces.
   1. Export the data from the plate reader in a raw format (raw text or a comma-separated values file). These raw data will be plotted on the Y-axis of the FSEC trace.
   2. For the X-axis, calculate the volume at which each fraction was collected. The first well needs to be the elution volume at which the first fraction was collected to account for the fractionation delay (6 mL in this example). The fractions after this must sequentially increase by the collected fraction volume (0.2 mL in this example).

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3. Once the X-axis and Y-axis data have been calculated, copy and paste the data into the graphing software (see **Table of Materials**) in order to plot the fluorescent signal in each well against the volume at which it eluted from the column.

   NOTE: **Figure 1** shows a schematic representation of the steps required to run an FSEC experiment.

3. Analyze the FSEC traces.

   1. Assess the amount of protein elution at the void volume (approximately 8 mL for a 24 mL column), which indicates that the protein is very large in size and is likely unfolded/aggregated.

   2. Assess the amount of protein eluting in any later peaks, which indicate folded protein. This is expected to be between 10 mL and 16 mL for a 24 mL column depending on the protein size (when associated with a detergent/lipid micelle). Pay close attention to the peak shape, particularly if it is a broad or split peak spanning greater than 3-4 mL (for a 24 mL column), as this indicates a polydisperse sample.

   3. Calculate the ratio between the monomeric peak height and the void peak height by dividing the maximum signal recorded in the monomer peak by the maximum signal in the void peak.

      NOTE: This value represents the monodispersity index and allows a quantitative measure of protein quality; larger values indicate the best possible quality, whereas values below 1 indicate problematic samples, as they have more aggregated protein than folded protein.

   4. If multiple FSEC runs are to be compared and the most important feature is the amount of monomer in each case, plot the traces as the raw signal recorded by the plate reader (e.g., RFU).

      NOTE: If a comparison of the amount of monomer compared to the unfolded protein is more important, the signal must be normalized to a percentage of the total signal using the minimum and maximum readings in the trace, which will accentuate differences in the ratio between aggregated and monomeric protein between the traces.

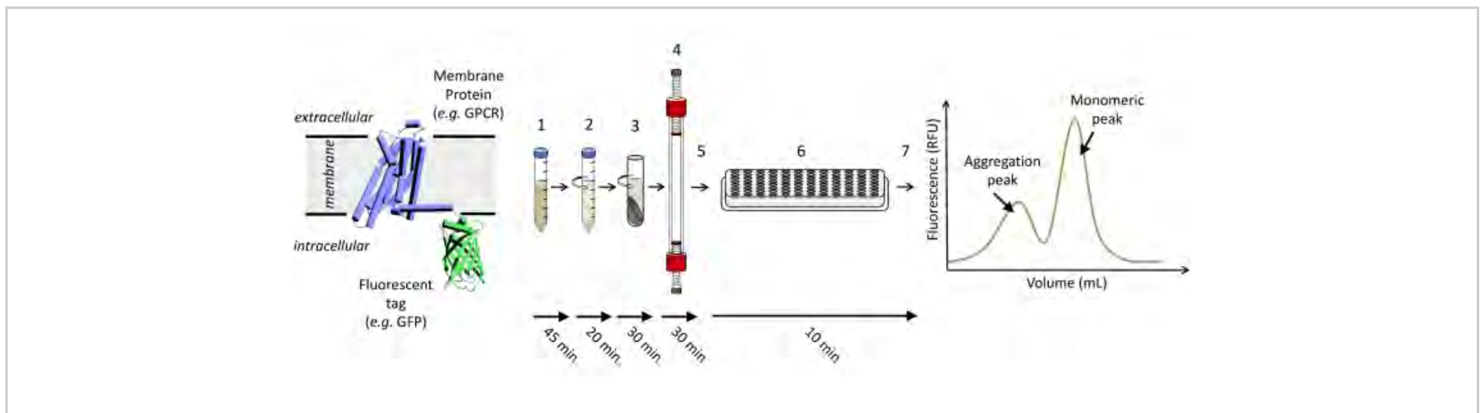

Figure 1: Schematic representation of the steps required to run an FSEC experiment. (1) Cells that express the fluorescently tagged protein of interest are solubilized. (2) The crude solubilization is clarified first with a low-speed spin, followed by (3) a high-speed spin. (4) The clarified sample supernatant is loaded and run on an appropriate SEC column,

and (5) the fractions are collected. (6) Samples of the fractions are transferred to a 96-well plate, and a GFP-fluorescent signal is detected using a plate reader to (7) plot the FSEC trace. Please click here to view a larger version of this figure.

Representative Results

First, the dynamic range and lower limits of eGFP detection for the plate reader used in this study were investigated. A purified eGFP standard of known concentration was diluted in a 50 µL final volume to 50 ng·µL^{-1}, 25 ng·µL^{-1}, 12.5 ng·µL^{-1}, 6.25 ng·µL^{-1}, 3.125 ng·µL^{-1}, 1.5625 ng·µL^{-1}, 0.78125 ng·µL^{-1}, and 0.390625 ng·µL^{-1}, and the fluorescence was read using an excitation of 488 nm and an emission of 507 nm (**Figure 2**). This experiment indicated that the plate reader had a lower detection limit of 30 ng of eGFP-labeled protein per well and a dynamic range of up to 500 ng of eGFP-labeled protein per well before signal saturation. Using the value for the lower limit and assuming that the protein elution is confined to 0.33 of the column volume, as little as 1.28 µg of eGFP labeled protein of interest is required for SEC column loading in order for a detectable FSEC signal to be observed.

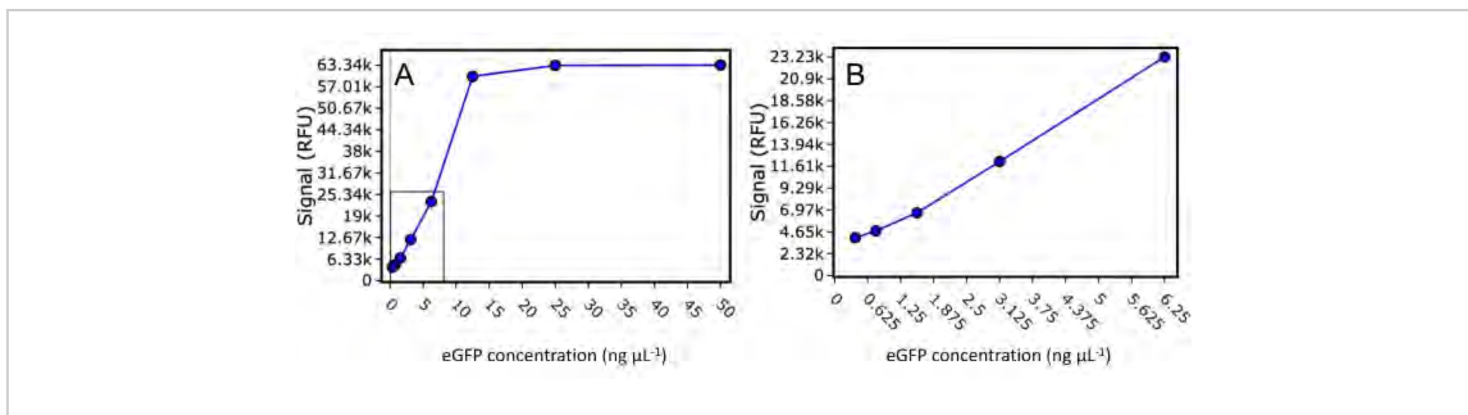

Figure 2: eGFP standard curve. Scatter graph of the fluorescent signal for purified eGFP standard diluted to 50 ng·µL^{-1}, 25 ng·µL^{-1}, 12.5 ng·µL^{-1}, 6.25 ng·µL^{-1}, 3.125 ng·µL^{-1}, 1.5625 ng·µL^{-1}, 0.78125, and 0.390625 ng·µL^{-1}. (**A**) All dilutions are displayed, including those with the saturated signal. (**B**) A zoomed-in scatter graph including only the standards that fall into the dynamic range of the plate reader. Please click here to view a larger version of this figure.

Secondly, the 24 mL column used for this study was calibrated with molecular weight standards. Using the same buffer and running conditions as used for the FSEC analysis, the molecular weight standards blue dextran (>2,000 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (43 kDa) were individually injected and run through the column, and elution traces were collected at 280 nm absorption. The elution volumes recorded were 8.9 mL, 12.4 mL, 15.2 mL, 16.9 mL, and 18 mL, respectively. When these elution volumes were converted to K_{av} (**Equation 1**) and plotted against log molecular weights, a standard curve could be fit. This allowed the molecular weight of the GPCRs tested in this study to be estimated by interpolation of the standard curve (**Figure 3**). For example, the FSEC trace of the GPCR serotonin receptor 2A ($5HT_{2A}R$) after solubilization in the detergent DDM indicated an elution volume of 13.4 mL. This $5HT_{2A}R$ elution volume falls between those elution volumes recorded for ferritin and

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aldolase and provides an estimated molecular weight of approximately 300 kDa. The $5HT_{2A}R$ construct used in this study is approximately 50 kDa (including the eGFP tag), meaning that if $5HT_{2A}R$ is assumed to be monomeric, 250 kDa of molecular weight could be attributed to the DDM detergent/lipid micelle. The equation for the conversion of elution volumes is as follows (**Equation 1**):

$$K_{av} = \frac{V_e - V_0}{V_c - V_0} \quad \text{(Equation 1)}$$

where V_e is the elution volume, V_0 is the column void volume, and V_c is the total column volume.

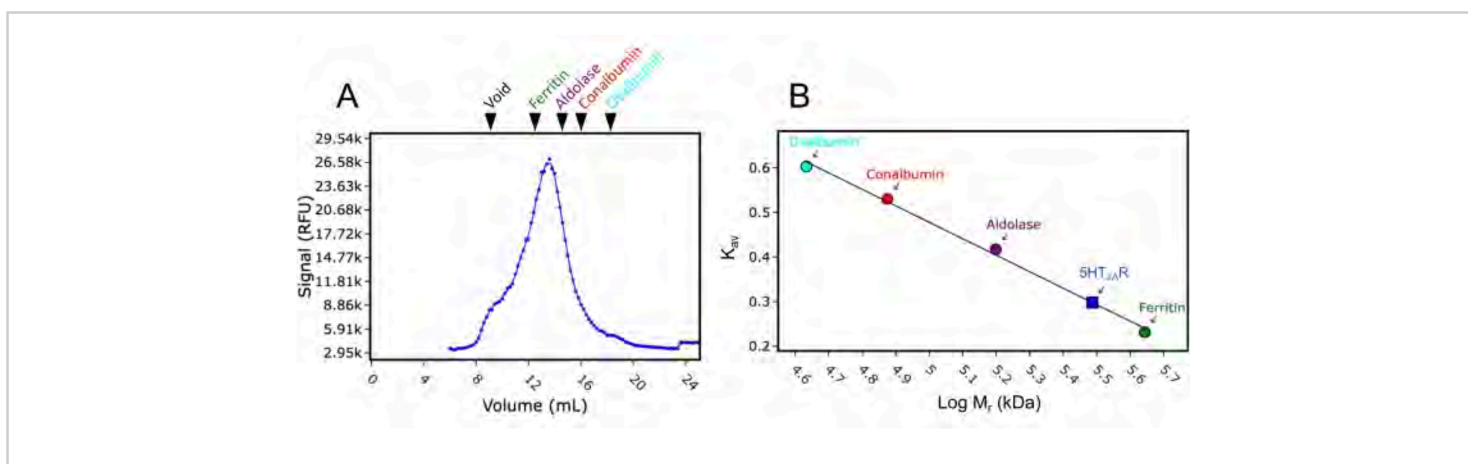

Figure 3: Calibration curve of the SEC column using molecular weight standards. (**A**) A representative FSEC trace of $5HT_{2A}R$ solubilized in DDM, with the relative elution positions of the molecular weight standards blue dextran (Void), ferritin, aldolase, conalbumin, and ovalbumin marked. Ferritin, aldolase, conalbumin, and ovalbumin are colored in green, purple, red, and cyan, respectively. (**B**) The molecular weight calibration curve using the elution positions of the standards after conversion to K_{av} (**Equation 1**) plotted against the log molecular weight (M_r). The M_r of $5HT_{2A}R$ in DDM was interpolated from the curve using K_{av} and is displayed on the curve (blue square). Please click here to view a larger version of this figure.

FSEC was then used to assess the quality and properties of the GPCR sphingosine-1-phosphate receptor ($S1PR_1$)[15]. Insect cells expressing GFP-tagged human $S1PR_1$ were processed for FSEC as described in the protocol (**Figure 1**).

Firstly, the optimal membrane extraction conditions were explored by testing the detergents DDM and LMNG against detergent-free extraction with SMA (**Figure 4A**). The monodispersity index was used to assess the quality of the protein sample and the ratio between the protein in the void (~8 mL column retention) compared with the monodispersed sample (14-15 mL column retention). The sample solubilized in LMNG displayed a superior FSEC profile with a better monomer peak shape and a lower protein aggregate peak, indicating that solubilization and purification in LMNG were the most stabilizing conditions for this membrane protein. In contrast, the sample solubilized in DDM had a relatively poorer FSEC profile, with a larger aggregate peak and a broader monomeric peak, indicating polydispersity in the sample.

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Secondly, the effect of ligand addition on the FSEC profile was investigated by adding sphingosine-1-phosphate (S1P) to the sample during solubilization. In this instance, DDM was used as the solubilization reagent, and the FSEC traces of $S1PR_1$ in the presence and absence of S1P were compared (**Figure 4B**). The sample solubilized in the presence of S1P showed a superior FSEC trace with reduced aggregation. This indicated that purification in the presence of a ligand was advantageous for improving the protein sample quality by stabilizing the receptor in solution but was also advantageous also as a surrogate marker of protein activity, as the results suggested the protein was correctly folded and competent for ligand binding.

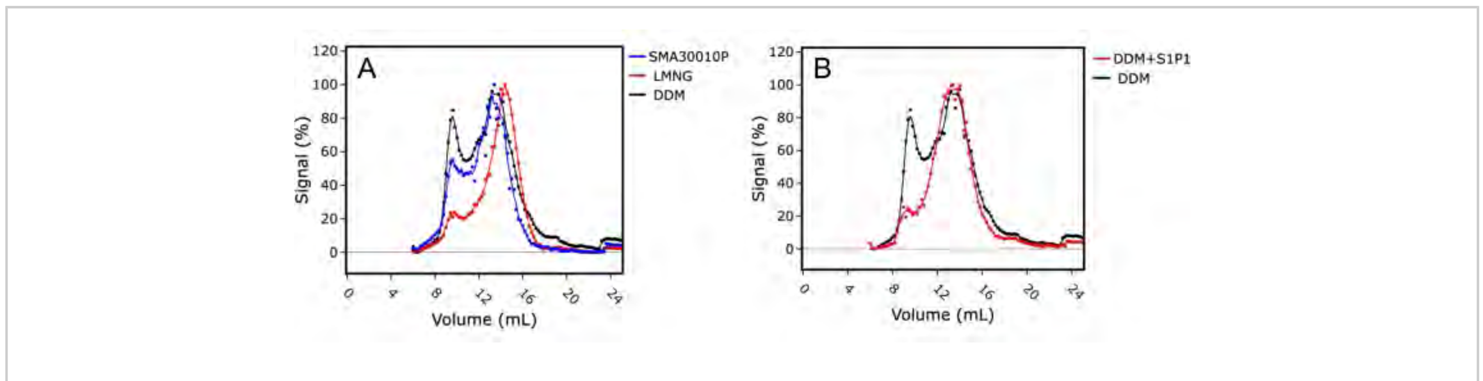

Figure 4: FSEC using a crude extract of $S1PR_1$ indicating optimal membrane extraction conditions and ligand binding. (**A**) Comparison of the FSEC traces of $S1PR_1$ solubilized in styrene-maleic acid co-polymer (SMA; blue), lauryl maltose neopentyl glycol (LMNG; red), or dodecyl maltoside (DDM; black). (**B**) Comparison of the FSEC traces of $S1PR_1$ solubilized in DDM in the presence (red) or absence (black) of the agonist sphingosine-1-phosphate (S1P). Please click here to view a larger version of this figure.

FSEC was also used to investigate the long-term stability of $5HT_{2A}R$ under different conditions. GFP-tagged human $5HT_{2A}R$ was solubilized from insect cell membranes in either detergent (DDM) or SMA polymer and analyzed by FSEC at several time points after storage at 4 °C or room temperature (**Figure 5**). Following several days of incubation, $5HT_{2A}R$ in DDM displayed a significant drop in the monomeric peak height at either temperature, and significant increases in the aggregate peak were observed. In contrast, $5HT_{2A}R$ in SMA lipid particle (SMALP) did not show a significant drop in the monomeric peak height over the course of the experiment, indicating that the protein in SMALP remained stable for longer, even at unfavorable temperatures. This may be important when considering protein preparations for downstream biophysical applications such as surface plasmon resonance (SPR) experiments for which there is a requirement for the sample to be stable and active over a long time period for the successful completion of the binding experiments.

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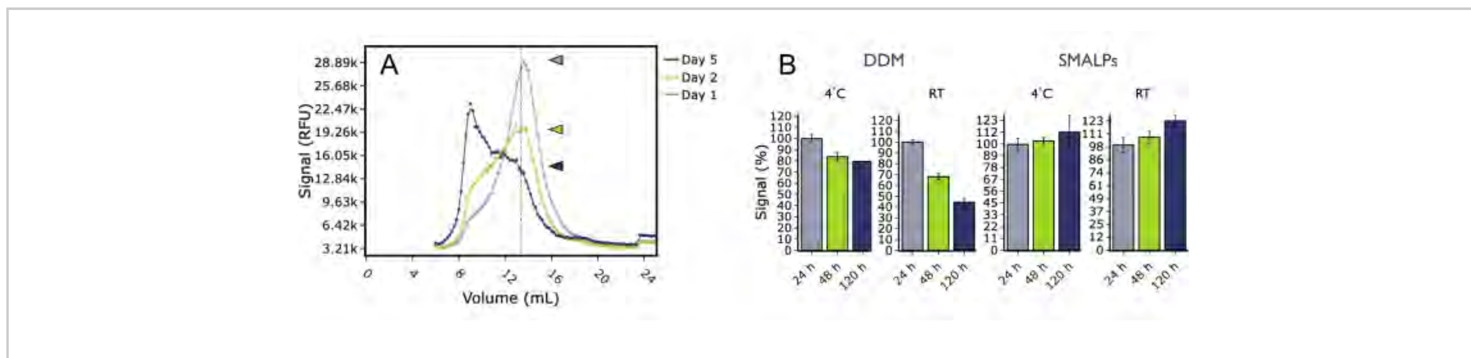

Figure 5: Effect of time and temperature on the quality of 5HT$_{2A}$R extracted from the membrane using either DDM or SMALP, as analyzed by FSEC. (**A**) Representative FSEC traces of 5HT$_{2A}$R solubilized in DDM and stored at room temperature for 1 day (grey), 2 days (green), or 5 days (blue). (**B**) Histograms of the normalized monomeric peak height for DDM samples stored at 4 °C (blue) or RT (green) compared to SMALP samples stored at 4 °C (grey) or RT (black). Error bars are representative of the SEM. Please click here to view a larger version of this figure.

Discussion

The generic systematic approaches for condition screening with FSEC that are presented here allow the fast optimization of solubilization and purification parameters for the production of membrane proteins. This means that stable and functionally active membrane proteins can be rapidly produced for biophysical and structural studies. Furthermore, FSEC can be run using laboratory equipment that is likely already in place in membrane protein labs, and thus, there is no requirement for the purchase of a specialist instrument for running the assays.

Critical steps

The time taken between the point of solubilization from cells in detergent to the point at which the sample is passed down the SEC column (steps 2.1.5-3.2.5) are time critical, and there must be no pauses between these steps. All the steps should be conducted at 4 ˚C or on ice, and the time taken to perform these steps needs to be kept to the minimum possible. These time and temperature constraints are necessary in order to record the FSEC profile for the membrane protein before any potential unfolding or degradation. After the membrane protein has been solubilized, there is a greater risk of unfolding, aggregation, and degradation, even at 4 ˚C. Ideally, any samples for which the FSEC traces are to be compared should pass down the SEC column in the same length of time after the solubilization step. In practice, this is difficult, particularly if the samples are passed sequentially down a single column, but it is possible to collect up to five SEC traces within 3 h of one another, and in this time frame, there should not be significant degradation.

Troubleshooting

If, on performing the FSEC experiment, there is low or no fluorescent signal, it is possible the membrane protein of interest has not expressed the chosen cell line, has very low expression of the chosen cell line, or has not been solubilized in the chosen detergent. If the samples were diluted before collecting the fluorescence signal and recording the FSEC trace, a simple first step would be to try a lower dilution or no dilution of the SEC fractions. If this still does not yield an

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interpretable FSEC trace, the expression and solubilization of the protein should be checked.

The analysis of protein expression can be achieved by checking the fluorescence of the sample after step 2.2.2. If there is a very low or no fluorescent signal from this sample (e.g., a signal very close to the background), there is likely an issue with the protein expression. Steps can be taken to improve the expression levels of the membrane protein, such as switching to an alternate cell line or adjusting the growth conditions, the induction of expression, and the time between the induction/infection/transfection and the harvest. However, particularly poor protein expression can indicate an unstable membrane protein and, thus, a poor construct choice.

If the expression has been checked and there is a clear fluorescent signal above the background prior to FSEC, the solubilization efficiency can be checked by measuring the remaining fluorescent signal of the sample after step 2.4.3 (soluble membrane protein) in comparison to the sample after step 2.2.2 (total protein). It is common for the solubilization efficiency to be 20%-30% and still allow for the successful analysis and purification of the membrane protein. However, if the solubilization efficiency is less than 20%, a different detergent for solubilization or different solubilization conditions may be required. If attempts to improve the solubilization are not successful, this can indicate a particularly unstable membrane protein and, thus, a poor construct choice.

If a very late eluting peak is observed in the FSEC trace (e.g., 18-24 mL), this indicates that the fluorescent protein has a protein molecular weight that is much lower than expected. This can be caused by the membrane protein of interest being degraded, resulting in "free" GFP. One should check if the protein is intact before and after solubilization using in-gel GFP fluorescence. If the protein of interest does appear to be degrading or being proteolyzed, the amount of protease inhibitor can be increased twofold to fourfold. However, a high sensitivity to proteases or degraded protein even before solubilization can indicate a particularly unstable protein and, thus, a poor construct choice.

Modifications and further applications of FSEC

Commonly, the fluorescent tag that is used in FSEC is GFP or eGFP, as described in this protocol. However, many different fluorescent protein tags are available. The choice of the fluorescent tag to be used depends on having a plate reader that can achieve the correct excitation and emission parameters to record the fluorescent signal for the selected fluorescent tag and having a fluorophore with little to no change in quantum yield in different environmental conditions. Furthermore, FSEC is not restricted to fluorescent proteins but can also work equally well with a protein that has been labeled with a fluorescent dye. For example, an NTA dye could be used, which would favorably bind to histidine-tagged membrane protein constructs. Furthermore, either a fluorescently labeled antibody chemically labeled with a fluorescent dye and specific for binding the membrane protein of interest or a purification tag included in the membrane protein construct could indirectly label a target for FSEC.

When performing detergent screening using FSEC, a choice can be made regarding whether the buffer used to run the SEC column should contain the matching detergent that the protein has been solubilized in or whether a standard detergent should be used across all the runs. A more accurate representation of the behavior of the protein will be obtained if the whole experiment is performed with the matching detergent throughout. However, it can be time-consuming and wasteful of detergent if the column must be

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re-equilibrated in a new detergent before each run is carried out. Furthermore, as the main purpose of detergent screening is to compare traces, the trends will remain in the traces even if the conditions are not ideal. Thus, a compromise can be reached whereby the protein is solubilized in the detergent of interest but the column is run in a standard buffer with a single detergent across all the runs (e.g., DDM)[11], which can save time and detergent consumables.

By modifying the FLPC equipment used, the throughput of the FSEC protocol can be significantly increased, and the sample requirement can be minimized. For example, an FPLC or HPLC system could be equipped with an autosampler, a smaller bed volume analytical column (such as a 3.2 mL analytical SEC column), and an in-line fluorescent detector for monitoring continuous FSEC traces directly from the column. The resulting setup would allow more FSEC runs to be carried out in a shorter period of time and remove the manual plotting step, thus allowing a greater number of conditions to be tested in a shorter time frame. Furthermore, the sample requirement would be further reduced, as fewer samples would have to be prepared and loaded onto the FSEC column for each run. This would open up possibilities for reducing the expression cultures to a plate-based format, as such little material would be required for the analysis.

Strengths and weaknesses of the FSEC compared to other methods

A disadvantage of FSEC is that the membrane protein constructs need to be designed to introduce the fluorescent label, and on introduction, there is a small possibility that the placement of the label could interfere with the function or folding of the membrane protein of interest. In addition, the FSEC protocol, as described here, monitors the characteristics of a membrane protein in the presence of cell lysate, which is a crude mixture of proteins. The behavior of a membrane protein in this environment may be different than when the membrane protein of interest is subjected to a preparative SEC column at the end of purification when fully isolated from other proteins. Furthermore, FSEC provides a somewhat qualitative measure of protein quality. However, by converting the FSEC trace to a monodispersity index, as described in step 4.3.3 of the protocol, a quantitative measure of protein quality can be obtained.

FSEC is not the only method that can be used in the early analysis of membrane protein constructs, solubilization conditions, and purification buffer composition. The alternative approaches have both advantages and disadvantages over FSEC. For example, fluorophore-based thermostability assays exist, particularly the use of the dye 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM)[16,17]. The advantage of this method is that, unlike FSEC, which provides a qualitative measure of protein quality, thermostability assays provide a quantitative measure in the form of a relative melting temperature. Furthermore, there is no requirement to introduce a fluorescent tag on the protein construct. However, the disadvantages of thermostability assays compared with FSEC are that purified protein must be used and that the assay is not compatible with all protein constructs, as it relies on the advantageous positions of native cysteine residues in the folded protein.

Another method that has similarities to both FSEC and fluorophore-based thermostability assays is an assay that measures the temperature sensitivity of a membrane protein. In this assay, the protein is challenged with different temperatures, and the protein that remains in solution after centrifugation is detected. Detection in this method has

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been conducted in several ways, including measuring the fluorescence in solution[18], the fluorescence of an SDS-PAGE gel band[19], or the signal intensity in a western blot[20]. However, a significant disadvantage of these approaches is that the assay is very labor-intensive and prone to high noise in the results, as each individual temperature point must be collected independently.

Finally, several more advanced biophysical techniques can be used to assess membrane protein quality in a similar manner to FSEC, for example, flow-induced dispersion analysis[21], microscale thermophoresis[22], or SPR. Although very powerful approaches, the disadvantage of these methods is the requirement for highly specialized instruments to run the analyses.

In conclusion, FSEC provides an invaluable tool for use in membrane protein production campaigns, and although it is not the only option, it has several distinct advantages over other methods, as listed above. The cross-validation of the results by orthogonal assays is always recommended, and none of the methods discussed above are mutually exclusive of one another.

Disclosures

Peak Proteins is a contract research organization that provides protein expression, purification, mass-spectrometry, and structural determination for a fee.

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