Chapter 18

Large-Scale Production of Membrane Proteins in *Saccharomyces cerevisiae*: Using a Green Fluorescent Protein Fusion Strategy in the Production of Membrane Proteins

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Abstract

The production of membrane proteins in the large quantities necessary for structural analysis requires many optimization steps. The GFP-fusion-based scheme described in earlier chapters (Chapters 4, 8, and 16) facilitates these steps by allowing the selection of high yielding clones that produce detergent-stable membrane proteins. Here, we describe the experimental steps required to establish the reproducible, large-scale production and purification of membrane protein–GFP fusions using *S. cerevisiae*.

Key words: Membrane protein, Green fluorescent protein, Overexpression, *Saccharomyces cerevisiae*, Fluorescence-detection size exclusion chromatography

1. Introduction

To facilitate the high-throughput screening and purification of membrane proteins for structural and functional studies, we have developed a protocol based on fluorescent monitoring of a carboxy-terminal GFP fusion tag (1–6). Analysis of its fluorescence enables the yields of membrane protein–GFP fusions to be easily estimated in both whole cells and isolated membranes. Further, this approach facilitates the optimization of membrane protein–GFP fusion yields, as well as detergent screening and purification by fluorescence-detection size exclusion chromatography (FSEC) (7). Using this approach, membrane protein–GFP fusions that are well expressed and stable in detergent, as judged by their monodispersity by FSEC, can be selected for downstream large-scale production and purification (Fig. 1).

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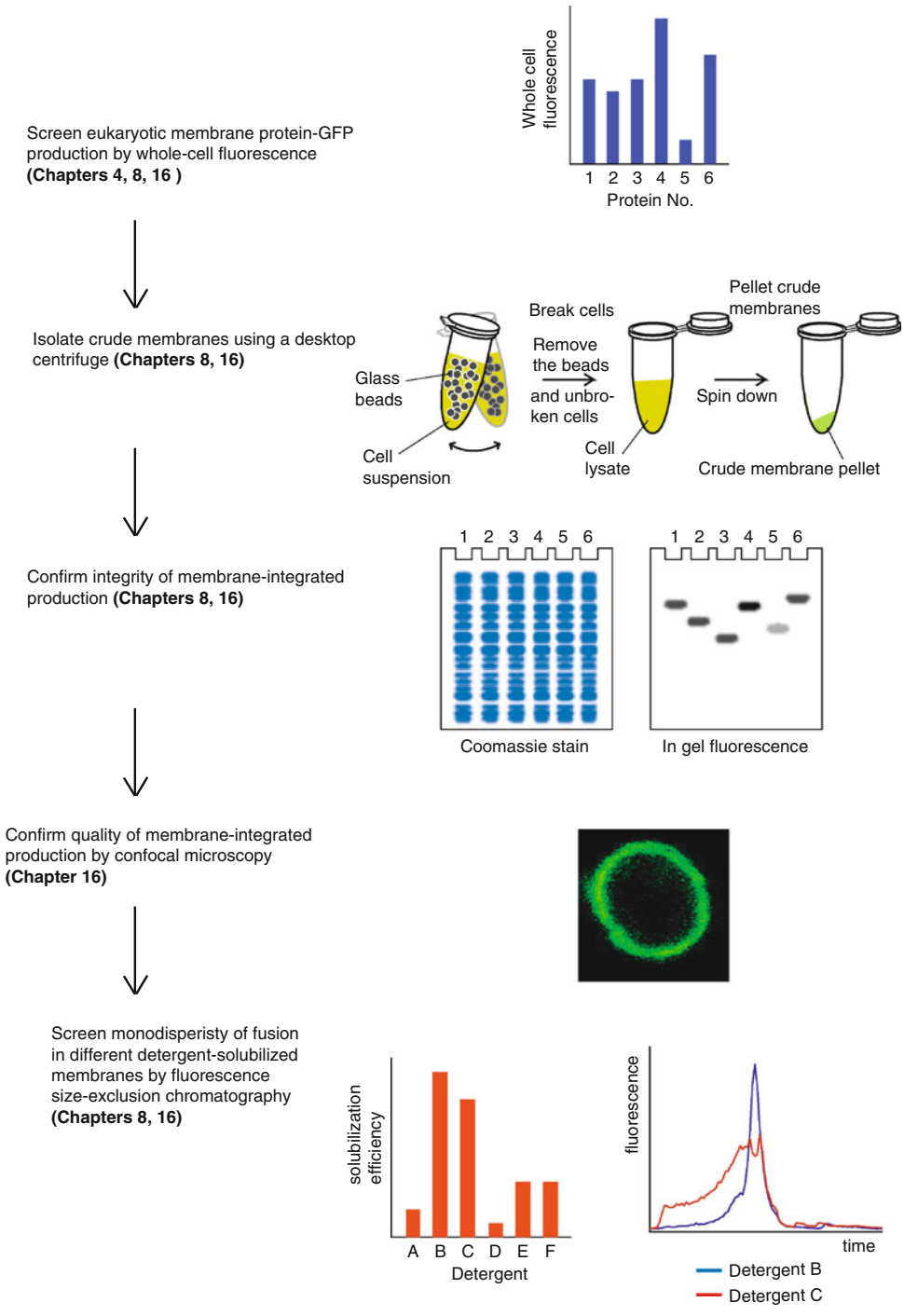

Fig. 1. Flowchart illustrating the screening process for the production and purification of eukaryotic membrane protein–GFP fusions in *Saccharomyces cerevisiae* (Figure adapted from ref. 5). Most targets are evaluated based on the quality of the FSEC trace in DDM.

This chapter describes a protocol for (1) the large-scale isolation of membranes, (2) purification of membrane protein–GFP fusions and (3) cleavage of the GFP-His_8 tag from membrane protein–GFP fusions.

2. Materials

1. Medium lacking uracil: 2 g yeast synthetic drop-out medium without uracil, 6.7 g yeast nitrogen base without amino acids and either 2% glucose (for pre-culture) or 0.1% glucose (expression culture), adjusted to a final volume of 1 L. For plates, use 2% glucose and add 20 g bacto agar.
2. 96-well black optical-bottom plates (Nunc).
3. SpectraMax M2e microplate reader (Molecular Devices).
4. Benchtop ultracentrifuge, Beckman Coulter Optima MAX series with TLA-55 and TLA 120.1 rotors (Beckman).
5. 1.5 mL polyallomer microcentrifuge tubes (Beckman).
6. Imidazole, minimum 99% (Sigma).
7. Ni-NTA superflow resin (Qiagen).
8. His-tagged TEV protease (8), stored at −80°C in buffer containing 50% glycerol, 20 mM Tris–HCl, pH 7.5, 0.3 M NaCl, and 1 mM DTT.
9. Cell resuspension buffer (CRB): 50 mM Tris–HCl, pH 7.6, 1 mM EDTA, 0.6 M sorbitol.
10. Constant Systems TS series cell disruptor (Constant Systems).
11. Membrane resuspension buffer (MRB): 20 mM Tris–HCl, pH 7.6, 0.3 M sucrose, 0.1 mM $CaCl_2$.
12. Bicinchoninic acid (BCA) protein assay kit (Pierce).
13. Phosphate-buffered saline (PBS): 1.44 g $Na_2HPO_4{\cdot}2H_2O$ (8.1 mM phosphate), 0.25 g KH_2HPO_4 (1.9 mM phosphate), 8 g NaCl, 0.2 g KCl, adjust pH to 7.4 using 1 M NaOH or HCl and adjust final volume to 1 L.
14. Equilibration buffer (EB): 1 × PBS, 10 mM imidazole pH 8.0, 150 mM NaCl, 10% glycerol (w/v), 3 × critical micelle concentration (CMC) detergent of choice.
15. Dialysis buffer (DB): 20 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 3 × CMC detergent of choice.
16. 5 mL His-trap columns (GE Healthcare).
17. Dialysis tubing, 12–14 kDa molecular weight cut-off (Spectrum labs).

18. Poly-Prep glass Econo-Column chromatography columns (Bio-Rad).
19. Superdex 200 10/300 GL Tricorn gel filtration column (GE Healthcare).
20. Centrifugal filter devices (Millipore/Vivascience).
21. 15 L or 50 L bioreactor vessels (Applikon).
22. ÄKTA FPLC system (GE Healthcare).
23. Frac-950 fraction collector with rack C (GE Healthcare).
24. Peristaltic pump P-1 (GE Healthcare).

3. Methods

3.1. Large-Scale Isolation of Membranes

1. Inoculate 10 mL medium lacking uracil containing 2% glucose with transformed yeast cells and incubate overnight in an orbital shaker at 280 rpm and 30°C. The following day, transfer the overnight culture to a 500-mL shake flask containing 150 mL medium lacking uracil with 2% glucose and incubate overnight as in the first step.
2. Dilute the 150 mL overnight culture to an OD_{600} of 0.12 into 1 L medium lacking uracil containing 0.1% glucose in a 2.5 L baffled shake flask. Incubate the culture in an orbital shaker at 280 rpm and 30°C. Induce at OD_{600} 0.6 using the parameters established in the initial optimization screens (Chapters 8 and 16). Use 10–15 L flasks for large-scale production. Alternatively, 15 L or 50 L bioreactors can be used to obtain similar yields per cell to shake flasks.
3. After a 22 h incubation, harvest the cells by centrifugation at $4{,}000 \times g$ at 4°C for 10 min. Decant the supernatant and resuspend the cell pellet in 25 mL of cell resuspension buffer (CRB) per L original cell culture.
4. Break cells using a heavy-duty cell disrupter for four passes at incremental pressures of 25, 30, 32, and 35 kpsi ($1.7–2.4 \times 10^3$ atm) at 4–15°C. Remove 100 µL cells, transfer to a 96-well plate and measure GFP fluorescence emission at 512 nm and excitation at 488 nm in a microplate spectrofluorometer.
5. Remove unbroken cells and debris by centrifugation at $10{,}000 \times g$ at 4°C for 10 min and collect the supernatant, which contains the membranes. Transfer 100 µL supernatant to a 96-well plate and measure GFP fluorescence. Calculate the yeast cell breakage efficiency by comparing GFP fluorescence to that measured in step 4 (see Note 1).
6. To collect the membranes, centrifuge the cleared supernatant at $150{,}000 \times g$ at 4°C for 120 min. Discard the supernatant

and resuspend the pellet to a final volume of 6 mL/L of original cell culture with MRB using a disposable 10 mL syringe with a 21 gauge needle. Transfer 100 µL membrane suspension to a 96-well plate and measure GFP fluorescence as outlined in step 4. Calculate the yield of membrane protein based on this fluorescence reading as well as the total protein concentration using the BCA protein assay kit following the manufacturer's instructions.

7. Freeze membranes in liquid nitrogen and store at −80°C (see Note 2).

3.2. Purification of Membrane Protein–GFP Fusions (Fig. 2)

1. Dilute the membrane suspension isolated from 10 to 15 L of culture into a 500 mL beaker to a final protein concentration of 3 mg/mL using equilibration buffer (EB). Add the detergent powder that produced the best FSEC trace (see Chapter 8) to a final concentration of 1 or 2% (w/v) depending on the CMC of the selected detergent. Incubate the mixture at 4–10°C for 1 h and use a magnetic stirrer to mix the solution.

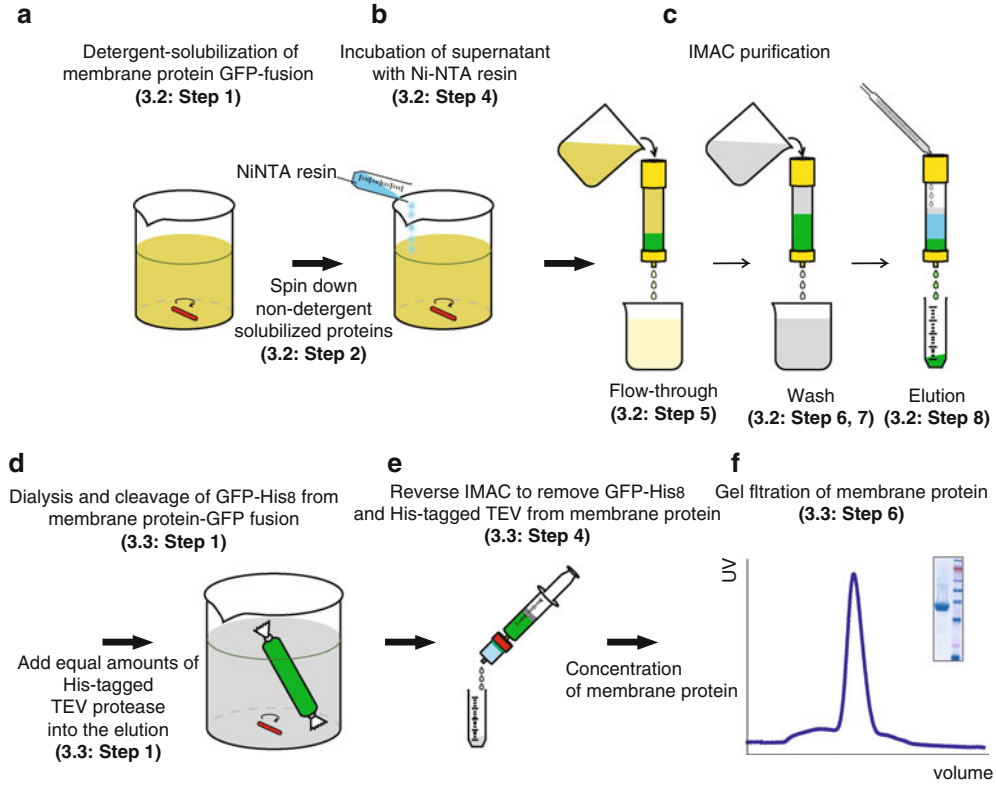

Fig. 2. Flowchart illustrating the purification of eukaryotic membrane proteins from GFP fusions (figure adapted from ref. 5). This protocol follows a standard Ni-NTA purification procedure. However, we recommend dialysis for efficient TEV cleavage and passing material through a higher capacity Ni-NTA column than in the first step (using the His-Trap™ column) to obtain very pure protein.

2. Pellet the insoluble material by centrifugation at 100,000 × *g* at 4°C for 45 min. Transfer the supernatant into an appropriately sized glass beaker. Transfer 100 μL detergent-solubilized membranes to a 96-well plate and measure GFP fluorescence as outlined in Subheading 3.1, step 4. Calculate the amount of GFP fusion as detailed in Chapter 8.
3. Use 1 mL Ni-NTA resin (2 mL of a 50% slurry) per milligram of GFP fusion to be purified (see Subheading 3.2, step 2) and equilibrate with five column volumes of EB (see Note 3).
4. Add the equilibrated Ni-NTA resin to detergent-solubilized membranes. Use a magnetic stirrer to mix the solution at 4°C for 2–3 h (see Note 4).
5. Pour the slurry into a glass Econo-Column. Transfer 100 μL of the flow-through to a 96-well plate and measure GFP fluorescence as outlined in Subheading 3.1, step 4. Compare with the reading taken in Subheading 3.2, step 2 and calculate the binding efficiency of the fusion protein to the Ni-NTA resin.
6. Wash the column with 10 column volumes of EB.
7. Add 1 M imidazole to EB to a final concentration of 30 mM and wash the column with 35 column volumes.
8. Add 1 M imidazole to EB to a final concentration of 250 mM and elute the protein from the column using 2 column volumes.
9. Transfer 100 μL eluate to a 96-well plate and measure GFP fluorescence as outlined in Subheading 3.1, step 4. Determine the amount of GFP fusion as outlined in Chapter 8. The amount of GFP fusion in the eluate should be determined by measuring the GFP fluorescence. The BCA assay measures total protein, including any contaminants.

3.3. Removal of the GFP-His_8 Tag from Membrane Protein–GFP Fusions

1. The total amount of membrane protein–GFP fusion is calculated as described in Chapter 8. For every 1 mg of membrane protein–GFP fusion, add 1 mg of tobacco etch virus (TEV) protease. The total reaction volume is typically 40 mL. Transfer the reaction mix to dialysis tubing and dialyze overnight at 4°C against 3 L of dialysis buffer (DB).
2. After overnight dialysis, inject 500 μL digested material onto a size exclusion chromatography column (SEC) and confirm by FSEC that all protein has been cleaved by the His-tagged TEV protease (see Note 5).
3. To remove the His-tagged TEV protease, cleaved GFP-His_8 tag and coeluting contaminating proteins from the reaction mix, equilibrate a 5 mL His-trap column with DB.
4. Pass the dialyzed sample through the His-Trap column at a flow rate of 2 mL/min. Collect the flow-through, which should contain the target membrane protein, into a 50 mL falcon

tube. If the membrane protein lacking a His_8-tag still binds to the resin, wash with 30 mM imidizole and collect the flow-through. Elute the bound material, which contains the cleaved GFP and His-tagged TEV protease, and analyze all fractions by SDS-PAGE (see Note 6).

5. Concentrate the flow-through from step 4 to 0.5 mL using a centrifugal concentrator. Calculate the total yield of protein using the BCA assay and adjust the concentration to 20 mg/mL, if necessary. Inject 0.5 mL onto a Superdex 200 10/300 column pre-equilibrated with the buffer used for dialysis at a flow rate of 0.5 mL/min.
6. Collect 0.5 mL fractions. Analyze the UV trace and pool the fractions containing the membrane protein. For structural work, concentrate using a 100 molecular weight cut-off (MWCO) concentrator to avoid concentrating detergent micelles, as these can inhibit crystallization (see Note 7).

4. Notes

1. Breakage efficiency should be around 80%. If lower, dilute the cells further with buffer as this increases the efficiency of breakage. Depending on the stability of the recombinant membrane protein, incubate cells with zymolase, which removes the cell wall, prior to breakage.
2. If the membranes isolated from 1 L of cells are resuspended in 6 mL MRB, the GFP fluorescent counts typically match the original whole-cell fluorescent counts. This corresponds to approximately 60% of the amount of GFP measured in whole cells, which is the fraction recovered in membranes.
3. It is worth mentioning that as the His_8-tag is carboxy-terminal to GFP, the amount of GFP that binds as a fusion is calculated at this step rather than the amount of membrane protein. Although the amount of resin used is much higher than for soluble proteins we have empirically found that this amount is necessary to ensure a good binding efficiency.
4. Batch binding of yeast-solubilized membranes improves protein recovery compared to flow loading. A 1 mL sample can be pelleted to compare fluorescence with that measured in step 2 of Subheading 3.2. Usually, binding efficiency reaches a plateau after 2 h, with only a modest gain of 10–15% after overnight incubation.
5. The most common reason for incomplete cleavage is that insufficient TEV protease is used. We typically cleave in the presence of 1 mM DTT. However, EDTA is not added as this may interfere with subsequent Ni-NTA chromatography steps.

6. When the purification is carried out for the first time, it is important to verify that the protein does not bind to the column. Once this is established, it is possible to proceed directly to the next step in all future purifications.
7. Note the concentrator flow-through should be retained in case the membrane protein–detergent complex passes through.

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