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Preparation of Recombinant Membrane Proteins from *Pichia pastoris* for Molecular Investigations

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ABSTRACT^[A1]^[A2]

Pichia pastoris is a eukaryotic microorganism reputed for its ability to mass-produce recombinant proteins, including integral membrane proteins (IMPs), for various applications. This chapter details a series of protocols that progress towards the production of IMPs, their extraction and purification in presence of detergents, and their eventual reconstitution in lipid nanoparticles. These *P. pastoris*-oriented basic procedures can be further optimized in order to deliver IMP samples compatible with a number of structural and/or functional investigations at the molecular level. Each protocol provides a number of general guidelines, technical hints and specific recommendations, and is illustrated with case studies corresponding to several representative mammalian IMPs.

BASIC PROTOCOL 1: PRODUCTION OF MEMBRANE PROTEINS WITH A *P. PASTORIS* RECOMBINANT CLONE

BASIC PROTOCOL 2: PREPARATION OF WHOLE MEMBRANE FRACTIONS

ALTERNATIVE PROTOCOL 2: PREPARATION OF YEAST PROTOPLASTS

BASIC PROTOCOL 3: EXTRACTION OF MEMBRANE PROTEINS FROM WHOLE MEMBRANE FRACTIONS

BASIC PROTOCOL 4: PURIFICATION OF MEMBRANE PROTEINS

ALTERNATIVE PROTOCOL 4A: PURIFICATION OF MEMBRANE PROTEINS FROM YEAST PROTOPLASTS

ALTERNATIVE PROTOCOL 4B: PROTOPLASTING, SOLUBILIZATION AND PURIFICATION OF MEMBRANE PROTEINS AT ONCE

BASIC PROTOCOL 5: RECONSTITUTION OF DETERGENT-PURIFIED MEMBRANE PROTEINS IN LIPID NANOPARTICLES

Key words: *Pichia pastoris*, Recombinant expression, Integral membrane proteins,

Protoplast, Detergent, Extraction, Purification, Lipid particles reconstitution

HOW TO PREPARE RECOMBINANT MEMBRANE PROTEINS FROM *PICHIA PASTORIS*

INTRODUCTION

As a methylotrophic yeast, *Pichia pastoris* combines the simplicity of manipulation and genetic engineering of a unicellular organism, with the sophisticated physiology and cell organization of a eukaryotic host, as well as a peculiar and strongly regulated methanol-dependent metabolism (Macauley-Patrick et al. 2005; Ahmad et al. 2014; Gasser and Mattanovich 2018). These features make it ideally suited to the mass production of a large variety of proteins and compounds, fitting the quality, regulatory and cost requirements in a number of industrial fields, including food, pharmaceutical, biotechnology and chemical industries (Mattanovich et al. 2014; Spohner et al. 2015; Love et al. 2018; Zhu et al. 2019). *P. pastoris* is also a system of choice for difficult-to-express proteins, and in particular for eukaryotic integral membrane proteins (IMPs) that are investigated for functional and structural studies or for screening applications. IMPs assume a large panel of functions that are essential for the cells' homeostasis and integrity (sensing and transport of molecules and ions, signal transduction, energy conversion, lipid metabolism, cell-to-cell communication, etc...). As these biological processes are finely tuned, the corresponding IMPs are generally poorly abundant in their natural environment and have to be recombinantly overexpressed in order to recover workable amounts. In addition, they necessarily require the hydrophobic environment of a lipid bilayer (or a membrane mimicry) to achieve the proper spatial folding responsible for their specific function. Overall, these critical aspects strongly contribute to the challenge in producing and studying IMPs at the molecular level. As a strong illustration of the high versatility and potency of *P. pastoris*, hundreds of these demanding IMPs have been successfully produced with this system (numerous references in Alkhalfioui et al. 2011, much

more since), dozens of them in quality and quantity compatible with 3D structure resolution studies (updated list available at <https://blanco.biomol.uci.edu/mpstruc/>).

Complete general guidelines, detailed protocols and optimization strategies can be found for the expression and production of IMPs with *P. pastoris* (Bornert et al. 2012; Logez et al. 2012; Singh et al. 2012a; Singh et al. 2012b; Hartmann et al. 2016). The aim of this protocol is to detail a set of procedures we routinely apply to generate the panel of IMP samples for further investigation. These Basic and Support protocols describe a series of preparative methods, from whole cells and membrane fraction preparations down to the isolation of proteins maintained in detergents or reconstituted in lipid nanoparticles. Each one is exemplified with representative results we obtained for a variety of eukaryotic IMPs. Starting from a yeast clone of interest (see Bornert et al. 2012 for upstream cloning and selection strategies), Basic Protocol 1 presents a standard and straightforward procedure for the production of the recombinant IMP in a 2 liter baffled flask culturing format accessible to every commonly equipped biology lab. This culturing volume often yields recombinant IMPs in the low milligram range, *i.e.* amounts compatible with a number of biochemical and biophysical analyses, [A3][A4] and can be easily upscaled to 10-12 liters.

Basic Protocol 2 details a robust method to efficiently disrupt the very resistant membrane envelope of *P. pastoris* and to generate whole membrane preparations. While this protocol is rather classical, Alternative Protocol 2 depicts an alternative processing [A5][A6] of the yeast cells where a treatment with a Zymolyase enzyme mixture leads to the formation of protoplasts, *i.e.* yeast cells devoid of their surrounding cell wall.

Basic Protocol 3 is focused on the extraction of IMPs from membrane samples with detergent compounds. The procedure is technically rather straightforward but also represents a crucial step as it aims at identifying the best experimental condition leading to effective membrane solubilization without IMP destabilization, which is always a protein-dependent process.

When this procedure is set up, detergent-solubilized IMPs can be then isolated following various chromatography purification approaches.

Basic Protocol 4 details a two-step purification strategy that we routinely apply as a starting point before IMP-specific optimizations and adjustments. In the two subsequent protocols, IMP solubilization and purification is achieved from yeast protoplasts, either sequentially (Alternative Protocol 4A) or concomitantly (Alternative Protocol 4B), with the double aim to reduce the procedure time frame and to improve the quality of the purified sample.

Finally, a protocol aiming at replacing IMPs in a membrane-mimicking lipid environment is exemplified with a human G protein-coupled receptor self-assembling into nanodiscs, *i.e.* nanoscale phospholipid bilayers stabilized by membrane scaffold proteins.

The protocols presented in this chapter are mainly focused on preparative approaches. As further discussed in the Commentary section, the various types of IMP samples generated with these procedures are then ready to be evaluated by a panel of analytical methods qualifying their activity, their purity, their homogeneity, their topology, their dynamics and every other parameters allowing to investigate their structure and their function at the molecular level.

BASIC PROTOCOL 1: PRODUCTION OF MEMBRANE PROTEINS WITH A *P. PASTORIS* RECOMBINANT CLONE

This production step presupposes an existing yeast clone expressing the IMP of interest. If such a clone is not available, preliminary and substantial work has to be done for the isolation of a *Pichia pastoris* recombinant clone. A full chapter of *Current Protocols in Protein Science* (Bornert et al. 2012) completed by another recent report (Hartmann et al. 2016) are

dedicated to this issue. Briefly, this includes the design and cloning of the desired sequence in a dedicated *P. pastoris* expression vector, the integrative transformation of *P. pastoris* and the phenotypic selection of recombinant clones. These clones are then screened in expression-inducing growth conditions performed in a small-scale culturing format in order to select the most valuable clones. These ideally correspond to the best balance between high level protein production and properly folded/active proteins. Once the best-behaving clone and the optimal expression conditions are identified, the production can start following the present protocol. Even if a number of alternative and valuable expression strategies have been described (Fischer and Glieder 2019), the immense majority of the IMPs produced with *P. pastoris* are expressed under a methanol-induced regimen based on the strongly regulated P_{AOXI} promoter (see Logez et al. 2012 and Ahmad et al. 2014 for a detailed description). In these conditions, yeast are first cultured in a glycerol-containing medium where recombinant protein expression is repressed, until reaching an appropriate cell density. Yeast are then transferred into a methanol-containing medium to induce the IMP production.

Baffled shake flasks are the system of choice to produce significant amounts of IMPs (in the milligram range) in reasonable volumes – usually between 2 to 10 L of culture. Importantly, flasks designed to favor optimal gas exchanges are highly recommended, such as the Ultra Yield™ flasks from Thomson and their AirOTop™ seals that include a 0.2 µm re-sealable and sterile membrane barrier.

For larger volumes of production, bioreactors are adapted to optimize production and proper folding. Parameters such as aeration, temperature, pH or media composition can be tightly regulated with those systems, with a number of fed-batch and co-feeding strategies available. Such approaches and their recent developments and applications for heterologous protein production can be found in several references (Singh et al. 2012b; Liu et al., 2019).

The procedure detailed below describes a standard condition for a 2 L production in shaken baffled flasks. As outlined in Bornert et al. 2012, a number of parameters may be adjusted such as the temperature and the duration of the methanol induction phase, the media composition and the addition of chemical chaperones such as DMSO and/or specific ligands. These protein-dependent optimizations might have a critical impact on the production outcome by increasing the proportion of properly folded and active IMPs as already shown (André et al. 2006).

Materials^[A7]^[A8]

An isolated recombinant clone freshly streaked on a YPD agar plate (see recipe in Reagents and Solutions)

BMGY medium (see recipe in Reagents and Solutions)

BMMY medium (see recipe in Reagents and Solutions)

Phosphate-buffered saline (PBS) (Euromedex, FR, ref. ET330)

2.5 L baffled-flasks (*i.e.* Ultra Yield, Thomson, ref. 931136-B)

30°C and 22°C shaking incubators

1 L sterile centrifuge bottles

50 mL conical disposal tubes

Spectrophotometer

Superspeed centrifuge

1. Inoculate 500 mL of freshly prepared BMGY medium in a 2.5 L baffled flask with a fresh recombinant colony isolated on a YPD agar plate. Incubate overnight on a shaker at 250 rpm, 30°C.

2. On the next day, measure OD_{600} of the culture. Dilute the cells into 1 L fresh BMGY medium to achieve an OD_{600} of about 2.5 (about 10^8 cells/mL) and split the culture into 2 baffled flasks. Incubate on a shaker at 250 rpm, 30°C.

When measuring cell turbidity at OD_{600} , be aware of the linearity limit of the method / instrument. We usually fix this limit at 0.3 OD and dilute the measured sample accordingly.

3. When the culture reaches ~ 10 OD_{600} (about 4 to 5 hrs later), pellet the cells by centrifugation in sterile a 1L bottle for 5 min at $2,000 \times g$, room temperature.
4. Decant the supernatant and resuspend the cell pellets with 2 L fresh BMMY medium. Split into 4 baffled flasks and incubate 18 to 24 hrs in a shaker at 22°C, 250 rpm.
5. After induction, harvest the cells by centrifugation in 1L bottles for 10 min at $3,000 \times g$, 4°C.
6. Decant the supernatant and wash each cell pellet (corresponding to 1 L of culture) with 200 mL PBS, pH 7.4. Split in 4 x 50 mL conical tubes. Centrifuge the suspension for 10 min at $3,000 \times g$, 4°C.
7. Discard the supernatant and weigh the cell pellet.

The yeast pellet can either be snap-frozen in liquid nitrogen and stored at -80 °C or kept on ice to be directly utilized for membrane preparation (see Basic Protocol 2) or for protoplasts preparation (see Alternative Protocol 2).

In this format, each conical tube content corresponds to 250 mL of culture. The wet cell pellet in each tube is expected to be between 6 to 8 gr. [A9][A10]

This protocol can be applied to other culturing formats, ranging from 50 mL conical tubes to baffled-flasks of volumes up to 5 liters. For an optimal aeration during induction, it is important to maintain a 1:5 ratio between the volume of the yeast culture and the total volume of the selected vessel.

BASIC PROTOCOL 2: PREPARATION OF WHOLE MEMBRANE FRACTIONS

Pichia pastoris cells are surrounded by a thick protective cell wall containing β -1,3-glycan, β -1,6-glycan, chitin and mannoproteins (Cabib and Arroyo, 2013), so a robust cell lysis method is required. Methods of choice usually include vigorous mechanical shaking with microbeads because they are very efficient and compatible with various sample volumes and tubes. The widest-known and simplest method is to lyse the cell membranes through several cycles of vigorous shaking and ice-cooling phases. To achieve reproducible cell lysis efficacy, programmable devices are recommended, such as the TissueLyser from Qiagen or the FastPrep24 from MP Biomedicals, which are suitable for volumes up to 50 mL, or the more sophisticated Dyno Mill agitators, that can handle larger sample volumes. Besides mechanical shaking techniques, pressure-based instruments are also efficient for the lysis of *P. pastoris* cells. In addition to the well-known French press, several cells disruptors are available from Constant Systems that can handle samples from 1 to hundreds of mL with pressure application from 1 to 2.7 kbars.

Whatever the lysis method, addition of protease inhibitors is strongly recommended to prevent the degradation of recombinant proteins from the release of intracellular proteases upon cell disruption.

Following the cell lysis step, large debris and unbroken cells are removed by low speed centrifugation and the resulting supernatant is subsequently ultracentrifuged. The membrane pellet is then recovered in an appropriate buffer with a Potter homogenizer to constitute a whole membrane preparation. Optionally, several steps of stringent washes and ultracentrifugation may be applied to optimize the removal of membrane-associated proteins, resulting in samples enriched with the IMP of interest (such a protocol is exemplified in Bornert et al., 2012). Finally, the cell lysis efficacy can be evaluated with the determination of total protein concentration (typically by BCA -Thermo Scientific ref. 23225 - or Bradford –

BioRad ref. 5000006 - assay^{[A11][A12]}) and the expression level of the protein of interest in the membrane preparation can be assessed by Western blot analysis.

The protocol described below is a bead-based disruption method we routinely apply for the preparation of membranes of *P. pastoris* overexpressing various IMPs we are producing.

Materials

Yeast cell pellets issued from 1 L of culture (*i.e.* 4 pellets from Basic Protocol 1)

TNGE buffer (see recipe in Reagents and Solutions)

TNG buffer (see recipe in Reagents and Solutions)

Acid-washed glass beads (425- to 600- μ m diameter; Sigma-Aldrich, ref. G8772-1KG)

High-speed benchtop homogenizer (*e.g.*, FastPrep 24 device from MP Biomedicals)

Ultracentrifuge-compatible bottles

Ultracentrifuge equipped with an appropriate fixed-angle rotor

50 mL Potter homogenizer

15 mL conical disposable tubes

Additional reagents and equipment for the determination of protein concentration (Olson and Markwell, 2007), *e.g.*, BCA assay

Additional reagents and equipment for SDS-PAGE (Gallagher, 2012), electrotransfer (Goldman et al. 2015), and immunoblot analyses (Olson and Markwell, 2007)^{[A13][A14]}

Lyse the yeast cells

1. Resuspend each yeast pellet of about 7 g obtained in Basic Protocol 1 with 25 mL of ice-cold TNGE buffer.
2. Add 10 mL of acid-washed glass beads and proceed as follows:

- a. Place the tubes on the cell breaker device and proceed to cell lysis by alternating shaking and cooling steps [A15] (3 cycles of 40 sec each at 6 m/sec on a FastPrep 24).
 - b. Centrifuge the samples for 5 min at $5,000 \times g$, 4°C , and collect the supernatant in a separate flask.
 - c. Dissolve the remaining pellet in up to 25 mL of ice-cold TNGE buffer and repeat steps 2a to 2c for two additional rounds.
3. Centrifuge the collected supernatants for an additional 5 min at $5,000 \times g$, 4°C .

Recover the whole membranes fraction

4. Transfer the supernatant from step 3 into appropriate tubes and separate the sample using an ultracentrifuge for 30 min at $100,000 \times g$, 4°C .

Carefully weigh the bottles and check that the rotor is properly balanced.

5. Discard the supernatant and resuspend the pelleted whole membranes in 45 mL of ice-cold TNE buffer using a 50 mL Potter homogenizer until the suspension is homogenous. Split the membrane preparation into 5 conical tubes of 10 mL each. Store at -80°C .

The membrane preparations can be directly used for further analyses and downstream processing of the IMP of interest. When stored at -80°C they are usually stable for several months.

6. Determine the protein concentration of membrane preparation as described in UNIT 3.4 (e.g., BCA assay).

When using a FastPrep homogenizer in the presented experimental format, protein concentrations usually reach around 10 mg/mL, i.e. about 500 mg for 4 cell pellets obtained from 1L of culture.

7. Evaluate the production level of the IMP of interest by a standard immunoblot technique (see Bornert et al. 2012) and, ideally, by a specific activity assay if available.

ALTERNATIVE PROTOCOL 2: PREPARATION OF YEAST PROTOPLASTS

P. pastoris is a very efficient overexpression system that very often generates important amounts of recombinant proteins. However, a variable but significant proportion of misfolded and/or aggregated proteins are often produced and co-purified when using standard IMP protocols. Even if this phenomenon is still poorly characterized in the context of membrane protein production, it is widely recognized that overexpression overwhelms the cell biosynthesis and translocation machineries and often elicits improper IMP folding^{[A16][A17]} (Vogl et al. 2014). Such events cause a number of stresses and responses with various outcomes including the retention of misfolded proteins within the secretory pathway and the formation of protein aggregates before their eventual degradation (Buck et al., 2015; Schlebach and Sanders, 2014).

In this context, we found strongly useful in some cases to preferentially purify specific membrane sub-fractions, in particular the plasma membrane, where properly folded IMPs are the most often located. This strategy is highly valuable for the extraction and purification of a number of IMPs as presented in Alternative Protocol 4 below. It requires cells compatible with such fractionation approaches, however, and requires weaker yeasts devoid of their thick cell wall, such as protoplasts.

The procedure presented here is based on the degradation of the protective cell wall of *P. pastoris* by glucanase enzymes (*e.g.*, glucanases from snail digestive juice, Zymolyase or Lyticase from microbial sources), resulting in protoplast cells. These can then serve as a starting material for several applications including IMP activity assays performed on whole cells, subcellular fractionation approaches (von Hagen J and Michelsen, 2013^{[A18][A19]}) or the direct extraction of IMPs with detergents as further described in Alternative Protocol 4 below. The following proportions are given for a cell pellet of about 6-8 g, obtained from 250 mL of *P. pastoris* culture (as described in Basic Protocol 1) and can be upscaled proportionally.

Materials

Yeast cell pellet (from Basic Protocol 1)

SED buffer (see recipe in Reagents and Solutions)

CG buffer (see recipe in Reagents and Solutions)

1 M sorbitol (Sigma, ref. S6021)

200 U/mL Zymolyase® 20 T (Amsbio, ref. 120491-1)

50 mL conical disposable tubes

Superspeed centrifuge

1. Resuspend the cell pellet in 50 mL of milli-Q water. Split in 2 tubes and fill to 50 mL with milli-Q water.
2. Centrifuge for 5 min at $5,000 \times g$, 4 °C.
3. Discard the supernatant and wash each pellet with 50 mL of SED buffer.
4. Centrifuge for 5 min at $5,000 \times g$, 4 °C.
5. Discard the supernatant and wash each pellet with 50 mL of 1 M sorbitol.
6. Centrifuge for 5 min at $5,000 \times g$, 4 °C.
7. Discard the supernatant and resuspend the cell pellet in the appropriate volume (*ca.* 40 mL) of CG buffer in order to reach a cell concentration of about 80 g/L.
8. Add 20 U of Zymolyase® per gram of initial cell paste.
9. Incubate for 15-30 min at room temperature under gentle agitation.

The amount and/or the duration of Zymolyase® treatment may impact the integrity of proteins and thus have to be adjusted to the IMP of interest.
10. Harvest the protoplasts by centrifugation for 5 min at $750 \times g$, 4 °C with minimal acceleration and deceleration in order to avoid protoplast bursting.
11. Carefully discard the supernatant using a pipette.

12. Protoplasts are then ready for detergent solubilization and IMP purification as described in Alternative Protocols 4A and 4B.

BASIC PROTOCOL 3: EXTRACTION OF MEMBRANE PROTEINS FROM WHOLE MEMBRANE FRACTIONS

When planning the investigation of IMPs isolated in aqueous solutions, a critical challenge is to determine the experimental conditions that allow its efficient extraction from membranes without destabilizing its structure and impairing its function. This crucial step is realized by the use of amphiphilic molecules, most generally detergents, though very recent detergent-free approaches using amphiphilic polymers (not developed in this article) are developing (Bada Juarez et al. 2019; Overduin and Klumperman 2019).

As often stated in biochemistry, IMP solubilization and detergent selection are still regarded as an art rather than a science. Step-by-step guidelines and very useful tips can be found in excellent comprehensive reviews and book chapters (Seddon et al. 2004; Tate 2010; Duquesne et al. 2016) to help setting and optimizing the solubilization condition of your IMP of choice. In particular, key concepts such as the *critical micellar concentration* (CMC - which represents for each detergent the concentration above which it forms micelles) are detailed. These are important to know because they condition the way each detergent is used to extract IMPs and to maintain them in solution.

If no information is already available for the solubilization of the targeted IMP, the initial step usually consists in screening a panel of extraction conditions varying a number of parameters such as the choice and concentration of the detergent, the ionic strength, the membrane protein concentration and the addition of stabilizing compounds (Champeil et al. 2016; Kotov et al. 2019). The ideal combination is hardly predictable as it depends on the nature of the IMP, on its local lipid environment in the membrane where it stands, and on the

physicochemical properties of the chosen detergent. The choice of detergent is often guided by its efficient extraction capacity, which is frequently correlated with unfolding or instability issues for the IMP of interest. Therefore, as far as a specific functional test is available, solubilization conditions are evaluated both in term of IMP yields and protein activity and stability.

Here is presented a general protocol for IMP extraction from *P. pastoris* whole membranes. From our own experience, a simple initial screening, including a small number of representative detergents and conditions, often leads to the successful extraction for a panel of eukaryotic IMPs. When a satisfying solubilization condition is identified, it can then be directly transposed to a preparative scale.

Materials

Membrane suspension sample from Basic Protocol 2

Solubilization Buffer **S0** (see recipe in Reagents and Solutions)

10X concentrated detergent solutions (*i.e.* for the detergents mentioned in Figure 1, 20% (w/v) for OG and 10% (w/v) for OGNG, DM, DDM, LMNG, Fos12 and Fos14) (Anatrace)

Benchtop tube rotation device (*e.g.*, RotoFlex tube rotator)

Ultracentrifuge-compatible microtubes

Benchtop ultracentrifuge suitable for microtubes

Disposable pestle for microtubes

Additional reagents and equipment for the determination of protein concentration (UNIT 3.4), *e.g.*, BCA assay

Additional reagents and equipment for SDS-PAGE (Gallagher, 2012), electrotransfer (Goldman et al. 2015), and immunoblot analyses (Olson and Markwell, 2007)^{[A20][A21]}

1. In the ultracentrifuge-compatible microtubes, dilute the membrane suspension to 2 mg/mL in 1 mL of the Solubilization Buffer S0 supplemented with the selected 1X detergent solutions.
2. Incubate for 30 min at RT on a benchtop rotator device.
3. Ultracentrifuge for 30 min at 100,000 x g, 4 °C.
4. Carefully transfer the supernatant containing the solubilized material to a new microtube.
5. Resuspend the remaining membrane pellet in 1 mL of Solubilization Buffer S0 with the disposable pestle.
6. Evaluate the IMP solubilization efficiency by comparing the content of the supernatant and the resuspended pellet samples with a standard immunoblot technique (see Bornert et al. 2012) and ideally by a specific activity assay if available.

Figure 1 exemplifies a typical immunoblot analysis issued from a solubilization screening experiment conducted on a human 4TM cell surface receptor expressed in *P. pastoris*.

Membrane preparations were incubated in presence of 7 representative detergents, namely *n*-octyl- β -D-glucopyranoside (OG), 2,2-dihexylpropane-1,3-bis- β -D-glucopyranoside (OGNG), *n*-decyl- β -D-maltopyranoside (DM), *n*-dodecyl- β -D-maltopyranoside (DDM), 2,2-didecylpropane-1,3-bis- β -D-maltopyranoside (LMNG), *n*-dodecylphosphocholine (Fos12) and *n*-tetradecylphosphocholine (Fos14). These data particularly highlight the differential extraction potentials of the detergents used here, some of them yielding low (i.e. OG, OGNG) to medium (DM, DDM, LMNG) or high (Fos12, Fos14) amounts of solubilized receptor.

Figure 2 illustrates how beneficial the combination of an immunodetection and an activity assay may be to select an optimized solubilization condition. *P. pastoris* membranes expressing the human ADRA2B GPCR were solubilized with a selection of 12 detergents and

the resulting solubilized fractions were analyzed through a Western blot and a specific radioligand binding assay. In this representative example, the most efficient extraction obtained with detergents from the Fos-choline series correlates with the complete loss of detectable activity, thus suggesting a strong denaturing effect of these molecules. On the opposite, the relatively low immunoblot signal observed with CHAPS corresponds to the highest amount of ligand binding solubilized ADRA2B.

BASIC PROTOCOL 4: PURIFICATION OF MEMBRANE PROTEINS

Strategies for the purification of membrane proteins are numerous and are roughly similar to those developed for soluble proteins. They are mainly relying on fusion tag-based techniques and/or on methods exploiting the intrinsic properties of the protein and are detailed in several and comprehensive reviews and book sections (Lórenz-Fonfría et al., 2011; Pandey et al. 2016; Smith, 2017). One main concern for IMPs however is related to the presence of the solubilizing detergent that may directly impact the purification strategies and yields, and the techniques to (not) use. For instance, tag sequences spatially too close to a transmembrane domain may be buried in the detergent micelle after solubilization, thus being poorly accessible to the affinity chromatography support. Similarly, any ion-exchange based chromatography may turn inefficient if the IMP of interest is solubilized with a charged detergent.

The purpose of this section is not to list and detail the multitude of purification strategies and the technique combinations available in the literature, especially as they are often tailored and optimized for each IMP of interest. Instead, we describe a simple and standard two-step purification protocol that we routinely apply as a first intention procedure, and that we further refine if needed. It consists in a first immobilized metal affinity chromatography (IMAC)

exploiting the 10His tag N- or C-terminally fused to our recombinant IMPs, followed by a size exclusion chromatography (SEC) step.

This protocol starts with the solubilized material obtained following Basic Protocol 3. The relatively high concentration of detergent that is usually required at this step to efficiently destabilize membranes and to extract IMPs could affect the folding and stability of proteins during purification and may interfere with the subsequent analyses. Therefore, detergent concentration is usually decreased in the running buffers during purification. A minimum detergent concentration (above CMC) is however crucial to maintain during all purification steps to avoid micelle dissociation and protein precipitation. Furthermore, the addition of stabilizing compounds such as glycerol, specific ligands or lipid derivatives during the purification process often reveals highly valuable.

When using IMAC resins, a number of options exists, including the choice of the immobilization approach (batch incubation or flowing through packed resins), the binding capacities and the physicochemical properties of the resin (bead size and chemistry), the metal ions that are grafted (Ni^{2+} or Co^{2+}), the elution strategy (linear gradient or fixed concentration steps of imidazole). Here again, the choice is important and dependent on the protein itself, on the objective sought, and on the optimizations that can be achieved.

The following protocol is designed for an automated purification on FPLC instruments (*e.g.* ÄKTA Purifier or ÄKTA Pure devices from GE Health Care) which is particularly crucial for the SEC step where the flow rate and the pressure need to be tightly controlled. In addition, the volume of the solubilized sample to be processed is often quite significant (from 50 mL to several hundreds of mL when upscaling) and the use of a sample pump connected to the FPLC instrument is highly recommended.

Here we present a typical protocol we use for the routine purification of TRPV4, a tetrameric calcium channel that we extract and purify with the LMNG detergent in presence of cholesteryl hemisuccinate (CHS) as a stabilizing compound.

Material

10 mL of membrane preparation at about 10 mg/mL (see Basic Protocol 2)

Solubilization buffer **S1** (see recipe in Reagents and Solutions)

Buffer **A1** (see recipe in Reagents and Solutions)

Buffer **B1** (see recipe in Reagents and Solutions)

SEC running buffer **GF1** (see recipe in Reagents and Solutions)

0.22 μ m membrane filters and a corresponding filter holder (*e.g.* reusable filter holder with receiver from Nalgene, ref. DS0320)

Ultracentrifuge-compatible bottles

Ultracentrifuge equipped with an appropriate fixed-angle rotor

Automated FPLC purification system (*e.g.* ÄKTA Purifier, GE Healthcare, or equivalent)

1 mL prepacked nickel affinity chromatography column (*e.g.* HisTrap HP 1 mL, GE Healthcare, ref. 17524701)

SEC column (*e.g.* Superdex 200 Increase 10/300, GE Healthcare, ref. 28990944)

Additional reagents and equipment for SDS-PAGE (Gallagher, 2012), electrotransfer (Goldman et al. 2015), and immunoblot analyses (Olson and Markwell, 2007)^{[A22][A23]}

Solubilize the membrane preparation sample

1. Add 10 mL of membrane preparation (obtained in Basic Protocol 2) in the solubilization buffer S1 in order to have a final protein concentration of 2 mg/mL.
2. Incubate for 30 min at RT under gentle agitation.

3. Separate the solubilized fraction by ultracentrifugation for 30 min at $100,000 \times g$ at 4 °C.
4. Spare 50 µL of the solubilized IMPs (supernatant) and store it at 4 °C for further analyses.

Proceed to IMAC purification

5. Add imidazole to the solubilized IMPs to a final concentration of 25 mM.
6. Filter the resulting suspension using a 0.22 µm membrane filter and keep on ice.
7. Equilibrate a 1 mL prepacked nickel affinity column with at least 10 column volumes (CV) of buffer A1 at 1 mL/min (same flow rate in the following steps).
8. Inject the filtered solubilized IMPs onto the column, either with a sample pump or with a superloop depending on the volume to inject.
9. Proceed to a first washing step with 10 CV of buffer A1.
10. Proceed to a second washing step with 10 CV of 30 % of buffer B1 (*i.e.* 150 mM imidazole) and collect 500 µL fractions.

Beware that the imidazole concentrations applied in this washing step and in the following elution step, are optimal for the purification of our TRPV4 construct. If you set up your IMP purification for the first time, we strongly recommend managing the elution by applying a linear gradient of imidazole (over 25 CV for instance) before optimizing the procedure to a multistep scheme.

11. Proceed to a second elution step with 10 CV of 100 % buffer B1 (500 mM imidazole) and collect 500 µL fractions.
12. Spare 50 µL of every fractions of interest and proceed to SDS PAGE analyses including Coomassie blue staining and Western Blotting (see Bornert et al. 2012).

Proceed to SEC purification

13. Equilibrate the SEC column (*e.g.* Superdex 200 Increase 10/300, GE Healthcare) with at least 2 CV of SEC running buffer GF1 at 0.5 mL/min.

14. Depending on the results of the SDS PAGE analyses, inject the appropriate fraction onto a SEC column. Set the flow rate at 0.3 mL/min and collect 500 µL fractions.

The maximal volume that can be injected on a Superdex 10/300 column is 500 µL. If the fractions of interest from the IMAC have a greater volume, they can be concentrated with appropriate centrifugal filters (e.g. Vivaspin concentrators from Sartorius or Amicon concentrators from Merck).

15. Spare 50 µL of every fractions of interest and proceed to SDS PAGE analyses as in 12.

Figure 3 is a typical illustration of the purification profile obtained for TRPV4 extracted from *P. pastoris* membranes following the presented protocol. These data exemplify an optimized IMAC 2-steps elution strategy that allows the separation of an almost pure and homogenous IMP.

ALTERNATIVE PROTOCOL 4A: PURIFICATION OF MEMBRANE PROTEINS FROM YEAST PROTOPLASTS

As mentioned in Alternative Protocol 2, the actual overexpression of IMPs with the *P. pastoris* system often results in the obtention of properly folded, functional proteins and a variable proportion of their misfolded and/or aggregated counterparts that are stacked in the membranes of intracellular compartments. When preparing whole membrane fractions for further solubilization and purification purposes, these different populations of IMPs are mixed together and are often co-purified in affinity chromatography approaches through the tag sequence they have in common. The following SEC step then separates these different populations according to their size, but sometimes with limited success due to co-elution effects or to amplified aggregation events during the purification process. In these cases, alternative methods are needed to better isolate these unwanted aggregates. During IMAC purification (see Basic Protocol 4, steps 5 to 11)^{[A24][A25]}, since the aggregated forms

potentially interact more strongly with the resin support due to their higher number of tags, finely tuned differential elution strategies may prove useful but are often challenging to set up.

Another valuable option requires applying the solubilizing detergent solution on whole cells devoid of their thick protective cell wall, *i.e.* on protoplasts. In these conditions, the detergent likely extracts IMPs according to their relative accessibility and in a kinetic mode, favoring the solubilization of proteins located in the plasma membrane or in close proximity [A26] over those stacked in inner compartments. The method has been successfully applied to several eukaryotic (mainly human) IMPs we expressed in *P. pastoris* including GPCRs, ion channels and enzymes (Hartmann et al. 2017; Vasseur et al. 2019; and unpublished work).

[A27][A28] Besides the fact that this alternative technique is much faster than standard cell lysis and membrane preparation procedures, a significantly reduced proportion of protein aggregates were released for several of the investigated IMPs.

The following protocol describes the extraction of an engineered version of the TREK1 potassium channel from yeast protoplasts [A29][A30] and its subsequent purification in conditions similar to those described in Basic Protocol 4.

Material

1 yeast protoplast pellet (from Alternative Protocol 2)

Solubilization buffer **S2** (see recipe in Reagents and Solutions)

Buffer **A2** (see recipe in Reagents and Solutions)

Buffer **B2** (see recipe in Reagents and Solutions)

SEC running buffer **GF2** (see recipe in Reagents and Solutions)

0.22 µm membrane filters and a corresponding filter holder (*e.g.* reusable filter holder with receiver from Nalgene)

Ultracentrifuge-compatible bottles

Ultracentrifuge equipped with an appropriate fixed-angle rotor

Automated FPLC purification system (*e.g.* ÄKTA Purifier, GE Healthcare, or equivalent)

1mL prepacked nickel affinity chromatography column (*e.g.* HisTrap HP 1 mL, GE Healthcare)

SEC column (*e.g.* Superdex 200 Increase 10/300, GE Healthcare)

Additional reagents and equipment for SDS-PAGE (Gallagher, 2012), electrotransfer (Goldman et al. 2015), and immunoblot analyses (Olson and Markwell, 2007)^{[A31][A32]}

1. Carefully resuspend the freshly prepared yeast protoplasts (see Alternative Protocol 2) in solubilization buffer S2 to reach a cell concentration of about 80 g/L.

2. Incubate 30 min at RT under very gentle agitation.

3. Ultracentrifuge for 30 min at $100,000 \times g$, 4 °C.

4. Recover the supernatant containing the solubilized fraction and continue with the instructions of Basic Protocol 4, starting from step 4 with the following adjustments:

- Use a 1 mL HisTrap HP column pre-equilibrated in 4% buffer B2 (20 mM imidazole).
- Successively wash with 10 CV of 4 % buffer B2 (20 mM imidazole), 10 CV of 10 % buffer B2 (50 mM imidazole) and 10 CV of 20 % buffer B2 (100 mM imidazole).
- Elute with 10 CV of 100 % buffer B2 (500 mM imidazole).

Figure 4 illustrates the purification profile obtained for an engineered TREK1 channel. In this example, the purification procedure starting from yeast protoplasts helped us to significantly reduce the presence of aggregated channels compared to the standard protocol starting from whole membrane preparations. Further mass spectrometry and *in vitro*

processing analyses performed on the final purified fraction (not shown here) reveal that the three different bands visible on the SDS-PAGE correspond to differentially glycosylated forms of the channel.

ALTERNATIVE PROTOCOL 4B: PROTOPLASTING, SOLUBILIZATION AND PURIFICATION OF MEMBRANE PROTEINS AT ONCE

The present protocol is a very convenient variation of previous Basic Protocol 4A whereby the protoplasting and solubilization processes are performed simultaneously. This procedure combines the advantages of avoiding the manipulation of the delicate and easily breakable protoplasts prior to solubilization and of reducing time required for the whole purification process. [This protocol has proven very successful in our hands for several IMPs^[A33]^[A34] (unpublished work), and the procedure and results presented here for a 1TM human lipase^[A35]^[A36] are a typical illustration of its effectiveness. In this example, the serine hydrolase activity of this IMP is irreversibly inhibited by standard protease inhibitors so their use is withheld during the purification process. The present strategy is further adapted to this enzyme as the short lapse of time between extraction and purification limits degradation events by proteases.

This particular example also brings a demonstrative illustration on how the choice of the resin support may be helpful. Actually, in order to further prevent proteolysis events, we found that adding 2 mM EDTA in the solubilization buffer was highly beneficial. Therefore, a special Ni Sepharose resin bearing a chemistry designed for minimized Ni-leakage (*i.e.* HisTrap Excel from GE HealthCare) adapted well to this relatively high concentration of EDTA.

Material

Yeast cell pellet (from Basic Protocol 1)

SED buffer (see recipe in Reagents and Solutions)

1 M sorbitol

Solubilization buffer **S3** (see recipe in Reagents and Solutions)

200 U/mL Zymolyase® 20 T (Amsbio, UK)

10 % (w/v) DDM solution

Buffer **A3** (see recipe in Reagents and Solutions)

Buffer **B3** (see recipe in Reagents and Solutions)

SEC running buffer **GF3** (see recipe in Reagents and Solutions)

50 mL conical disposable tubes

Superspeed centrifuge

Ultracentrifuge-compatible bottles

Ultracentrifuge equipped with an appropriate fixed-angle rotor

0.22 µm membrane filters and a corresponding filter holder (*e.g.* reusable filter holder with receiver from Nalgene)

1 mL prepacked nickel affinity chromatography column (*e.g.* HisTrap Excel 1 mL, GE Healthcare, ref. 17371205)

Automated FPLC purification system (*e.g.* ÄKTA Purifier, GE Healthcare, or equivalent)

Size exclusion chromatography (SEC) column (*e.g.* HiLoad 16/600 Superdex 200 Increase PG, GE Healthcare, ref. 28989335)

Additional reagents and equipment for SDS-PAGE (Gallagher, 2012), electrotransfer (Goldman et al. 2015), and immunoblot analyses (Olson and Markwell, 2007)^{[A37][A38]}

1. Resuspend the cell pellet in 40 mL of milli-Q water.

2. Centrifuge for 5 min at 5,000 × *g*, 4 °C.

3. Discard the supernatant and wash the pellet with 40 mL of SED buffer.
4. Centrifuge for 5 min at $5,000 \times g$, 4 °C.
5. Discard the supernatant and wash the pellet with 40 mL of 1 M sorbitol.
6. Centrifuge for 5 min at $5,000 \times g$, 4 °C.
7. Discard the supernatant and resuspend the cell pellet in 40 mL of the solubilization S3 buffer.
8. Place the suspension in a standard bottle and gently agitate with a magnetic stirrer.
9. Dropwise add 10 U of Zymolyase[®] per gram of initial cell paste.

As previously mentioned in Alternative Protocol 2, the amount and/or the duration of Zymolyase digestion have to be adjusted to the IMP of interest.

10. Dropwise add the DDM solution to reach a 1 % final concentration.
11. Incubate for 30 min at room temperature under gentle agitation.
12. Ultracentrifuge for 30 min at $100,000 \times g$, 4 °C.
13. Recover the supernatant containing the solubilized fraction.
14. Proceed to IMAC and SEC purifications as described in Basic Protocol 4, starting from step 4, with the following adjustments for IMAC:

- Use a 1 mL HisTrap Excel column pre-equilibrated in buffer A3 supplemented with 4 % buffer B3 (20 mM imidazole).
- Successively wash with 10 CV of 4% buffer B3 (20 mM imidazole), 10 CV of 10% buffer B3 (50% imidazole) and 10 CV of 20% buffer B3 (100 mM imidazole).
- Elute with 10 CV of 100% buffer B3 (500 mM imidazole).

Figure 5 presents the classical IMAC, SEC and SDS-PAGE profiles obtained for a human lipase enzyme purified with the present procedure. Of importance, only a very homogeneous peak of monomeric lipase is eluted from the SEC, whereas a significant additional population

of multimeric / aggregated forms of the protein can be observed when starting from membrane preparations as described in Basic Protocol 4 (data not shown).

BASIC PROTOCOL 5: RECONSTITUTION OF DETERGENT-PURIFIED MEMBRANE PROTEINS IN LIPID NANOPARTICLES

A common challenge regarding IMPs isolated in detergent is to maintain them stable in solution for a reasonable period of time. Indeed, the hydrophobic environment engendered by detergents significantly differs from the one of lipids in native membranes. In general, the weaker hydrophobicity of detergents leads to more dynamic molecular interactions with the IMPs' hydrophobic domains. This may result in a looser packing of the protein that in turn facilitates the insertion of detergent within its hydrocarbon core. Overall, this combination of events contributes to the structural destabilization of IMPs and often lead to their denaturation (Chipot et al. 2018). In addition, during the manipulation of membrane proteins, detergents may co-concentrate with the protein target and then amplify these phenomena. A number of strategies aiming at minimizing these instability issues are regularly reported, including the development of more adapted detergents (refs in Breyton et al. 2019) and the engineering of more stable IMPs (deletion of unstructured and flexible regions of the protein, introduction of stabilizing point mutations or fusion sequences) (Chun et al. 2012).

Alternatively, because the presence of free detergents and/or detergent micelles may also interfere with a number of techniques and assay formats, trapping purified IMPs in detergent-free lipid particles may prove highly beneficial for their further analysis. Here too, a number of strategies have been developed for the isolation of IMPs in lipid-based membrane mimetics, including liposomes (Jorgensen et al. 2017), disc-shaped structures such as bicelles (Dürr et al. 2012), lipid nanoparticles stabilized with styrene maleic acid co-polymers

(Overduin and Klumperman 2019) or with lipoproteins (Bayburt and Sligar 2010; Frauenfeld et al. 2016).

With different strengths and limitations, these approaches have proven successful for the stabilization of various IMPs in aqueous solutions for a period of time compatible with a number of biochemical and biophysical investigations.

We here describe a protocol allowing to recover lipoprotein nanoparticles containing the adenosine A2A receptor (AA2A), a prototypic class A GPCR. These so-called nanodiscs are obtained from a detergent-purified AA2A that, upon detergent removal, self-assembles within a lipid bilayer encircled by two amphipathic membrane scaffold proteins (MSPs).

Material

Detergent-purified AA2A (AA2A is produced as described in Basic Protocol 1; membranes are prepared as described in Basic Protocol 2; AA2A is purified following Basic Protocol 3 in a 50 mM HEPES pH 7.4, 150 mM NaCl, 0.02 % β -DDM (w/v), 0.002 % CHS (w/v), 1 μ M DPCPX buffer)

Membrane scaffold protein MSP1E3D1(-) (MSP) produced and purified as described in Denisov et al. 2004 in a 20 mM HEPES pH 7.4, 100 mM NaCl, 0.5 mM EDTA buffer
25 mg/mL stock solution of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) in chloroform (Avanti Polar, ref 850457C)

25 mg/mL stock solution of 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) in chloroform^[A39]^[A40] (Avanti Polar, ref 840457C)

Argon (or nitrogen) gas bottle

Lipid resuspension buffer (see recipe in Reagents and Solutions)

5 mL glass tube

Polystyrene Biobeads SM-2 (BioRad, ref. 1523920)

Vacuum dessicator

Fritted glass support

Benchtop tube rotation device (*e.g.*, RotoFlex tube rotator)

Benchtop centrifuge

IMAC column (*e.g.*, HisTrapHP 1 mL, GE Healthcare)

SEC column (*e.g.*, Superdex 200 10-300 Increase, GE Healthcare)

Automated protein purification system (*e.g.*, ÄKTA Purifier, GE Healthcare)

1. Mix POPC and POPG at a 3:2 molar ratio in a 5 mL (or adapted) glass tube.
2. Evaporate the chloroform under an argon (or nitrogen) stream to form a lipid film at the bottom of the tube.
3. Let the lipid film thoroughly dry overnight in a vacuum dessicator.
4. Suspend the lipid mixture at a 24 mM final concentration in the lipid resuspension buffer.

Allow the mixture to hydrate above the transition temperature of the lipids (typically here at RT) for 30-60 min and with regular vortexing.

5. Add the purified MSP to the lipid suspension at a 1:70 molar ratio and incubate on ice for 15 min.
6. Add the purified AA2A receptor to the MSP:lipid mixture at a 1:10 AA2A:MSP molar ratio and incubate on ice for an additional 60 min.
7. Add the BioBeads SM-2 (0.25 g of dry beads per mL of reconstitution mixture) to initiate the nanodiscs self-assembly towards detergent removal.

Batches of dry BioBeads should be prepared in advance by three successive washes with 20 mL methanol, then deionized-water, followed by a vacuum filtration step on a fritted glass support^{[A41][A42]}.

8. Incubate overnight at 4°C on a tube rotator.
9. Decant the Biobeads by a 3,000 × g short spin centrifugation at room temperature^{[A43][A44]}.

10. Carefully recover the supernatant with a syringe mounted with a 0.8 mm gauge needle.
11. Proceed to IMAC and SEC purification as described in Basic Protocol 4.

Figure 6 illustrates the IMAC, SEC and SDS-PAGE profiles that we typically obtain for the isolation of AA2A-containing nanodiscs. Whereas the flow through (FT) fraction observed during the IMAC purification step mainly contains free MSP or empty nanodiscs, the eluted ones correspond to homogeneous populations of AA2A-nanodiscs as confirmed by the SDS-PAGE and by negative staining EM analyses (not shown). The two bands observed for AA2A correspond to wild-type (upper band) and C-terminally truncated (lower band) populations of the receptor as determined by mass spectrometry analyses.

REAGENTS AND SOLUTIONS^[A45]

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

YPD agar plate

70 g YPD agar (Formedium, UK, ref. CCM0110)

Bring to a final volume of 1 liter with water.

Heat sterilize in autoclave. Let the solution cool to about 50 °C and sterilely pour into petri dishes. Store up to 1 month at 4 °C

BMGY medium

For 1 liter of BMGY solution, prepare 700 mL of YEP (10 g yeast extract (Formedium, UK, ref. YEA03), 20 g peptone (Formedium, UK, ref. PEP03) and complete with deionized water) and sterilize by autoclaving.

Just before use, sterilely add the following volumes of filter-sterilized stock solutions:

- 100 mL of 13.4 % (w/v) yeast nitrogen base w/o amino acid (10X stock solution) (Formedium, UK, ref. CYN0410);
- 100 mL of 10 % (v/v) glycerol (10X stock solution) (Euromedex, FR, ref. EU3550);
- 100 mL of 1 M phosphate buffer pH6 (10X stock solution);

YEP and other 10X stock solutions can be stored up to 1-2 months at 4 °C.

BMMY medium

For 1 liter of BMMY, proceed the same as for BMGY with 100 mL of 5 % methanol (10X stock solution) (Sigma Aldrich, ref. 179957) instead of glycerol.

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

TNGE buffer

50 mM Tris (Euromedex, FR, ref. 26-128-30-94-B) pH 7.4

0.5 M NaCl (Euromedex, FR, ref. 1112-A)

1 mM EDTA (Euromedex, FR, ref. EU0007)

1 mM PMSF (Sigma Aldrich, ref. P7626)

10 % (v/v) glycerol

Prepare fresh

TNG buffer

50 mM Tris pH 7.4

0.5 M NaCl

1 mM PMSF

10 % (v/v) glycerol

Prepare fresh

SED buffer

1 M sorbitol (Sigma, ref. S6021)

25 mM EDTA

50 mM DTT (Euromedex, FR, ref. EU00006-D) (add fresh before use)

CG buffer

20 mM trisodium citrate (Euromedex, FR, ref. 1126) pH 5.8

10 % (v/v) glycerol

1 mM PMSF (add fresh before use)

Solubilization buffer S0

50 mM Tris pH 7.4

500 mM NaCl

1 mM EDTA

Solubilization buffer S1 (TRPV4)

50 mM HEPES (Euromedex, FR, ref. 10-110), pH 7.4

500 mM NaCl

0.5 % (w/v) / 0.05 % (w/v) LMNG/CHS (LMNG: Anatrace, ref. NG310; CHS: Anatrace, ref. CH210)

0.3 mM EDTA

Protease inhibitor cocktail (Complete ultra tablets, Roche, ref. 06538282001)

Buffer A1 (TRPV4)

50 mM HEPES, pH 7.4

500 mM NaCl

0.05 % (w/v) / 0.005 % (w/v) LMNG/CHS

25 mM imidazole (Sigma Aldrich, ref. I0250)

Buffer B1 (TRPV4)

50 mM HEPES pH 7.4

500 mM NaCl

0.05 % (w/v) / 0.005 (w/v) % LMNG/CHS

500 mM imidazole

SEC running buffer GF1 (TRPV4)

50 mM HEPES pH 7.4

150 mM NaCl

0.05 % (w/v) / 0.005 % (w/v) LMNG/CHS

Solubilization buffer S2 (TREK1)

50 mM Tris-HCl pH 7,4

500 mM KCl (Euromedex, FR, ref. P017-A)

10 % (v/v) glycerol

1 % (w/v) DDM (Anatrace, ref. D310S)

20 mM imidazole

Protease inhibitor cocktail

Buffer A2 (TREK1)

50 mM Tris pH 7.4

150 mM KCl

0.01 % (w/v) DDM

Buffer B2 (TREK1)

50 mM Tris HCl pH 7.4

150 mM KCl

0.01 % (w/v) DDM

500 mM imidazole

SEC running buffer GF2 (TREK1)

50 mM Tris pH 7.4

150 mM KCl

0.1 % (w/v) DDM

1 mM EDTA

Solubilization buffer S3 (Lipase)

50 mM Tris pH 7.4

300 mM NaCl

10 % (v/v) glycerol

1 % (w/v) DDM

2 mM EDTA

Buffer A3 (Lipase)

50 mM Tris pH 7.4

300 mM NaCl

0.1 % (w/v) DDM

Buffer B3 (Lipase)

50 mM Tris pH 7.4

300 mM NaCl

500 mM imidazole

0.1 % (w/v) DDM

SEC running buffer GF3 (Lipase)

50 mM Tris pH 7.4

300 mM NaCl

0.1 % (w/v) DDM

Lipid resuspension buffer

50 mM HEPES pH 7.4

150 mM NaCl

48 mM sodium cholate (Sigma Aldrich, ref. C6445)

COMMENTARY

Background information

IMPs comprise about one third of all proteomes, where they are in charge of vital functions for cell life, including fluxes of solutes and information, energy conversion, enzymatic activities, cell shape and integrity, intra- and intercellular contacts and communication. As key players in these essential physiological processes, they are also involved in a large number of associated disorders, and they consistently represent the largest class of targets – more than 60% of the commercialized drugs – for the pharmaceutical industry (Santos et al. 2017). In this context, obtaining a detailed understanding on how these proteins function at the molecular level is crucial for both fundamental knowledge and biomedical applications.

Such investigations are however complicated by the low abundance of IMPs in native membranes and thus necessitate effective recombinant expression systems to generate the amounts of biological material required for these studies. Over years, *P. pastoris* has proven one of these best performing systems, not only for the wide number of eukaryotic IMPs that have been successfully produced with this host, but also because these IMPs are representative of a large panel of membrane functions from different organisms, of various membrane spanning topologies and protein sizes, and of diverse molecular assemblies including hetero-multimeric protein complexes (references in Alkhalfioui et al. 2011). Applying preparative procedures similar to those described in this article, a large number of these IMPs could then be extracted from *P. pastoris* membranes and isolated to quantity and quality levels compatible with their thorough characterization at the molecular level. A rapid, far from exhaustive, survey conducted on the last decade actually retrieved an abundance of information on a variety of IMPs produced in these conditions, covering screening and functional characterization of compounds (Wöhri et al. 2013; Logez et al. 2014; Scalise et al. 2014; Dekki-Shalaly et al. 2015; Zehnpfenning et al. 2015; Zollmann et al. 2015; Westh Hansen et al. 2016; Igonet et al. 2018), interactions with and regulation by proteins (Bornert et al. 2014; Rosell et al. 2014; Doshi et al. 2017; Damian et al. 2018) and lipids (Schölz et al. 2011; Brohawn et al. 2014), functional impact of critical mutations (Kapri-Pardes et al. 2011; Ampah-Korsah et al. 2017; Yang et al. 2017; Christenson et al. 2018), as well as structural insights and mechanistic details observed at the atomic level (Hino et al. 2012; Kodan et al. 2014; Fan et al. 2011; Vinothkumar et al. 2016; Wang et al. 2016; Lolicato et al. 2017; Deng et al. 2018; Eddy et al. 2018; Ye et al. 2018; Garaeva et al. 2019; Wang et al. 2020).

Nonetheless, the wealth of data described in these papers was obtained from investigations involving a large panel of biochemical and biophysical techniques and analyses. These include radiometric and fluorescent assays in various formats, thermal shift assays, circular

dichroism spectroscopy, isothermal titration calorimetry and differential scanning calorimetry, electrophysiology, surface plasmon resonance, frontal affinity chromatography, mass spectrometry in native or denaturing conditions, transmission electron microscopy, cryo-electron microscopy, X-ray crystallography, solid and solution state NMR with various probes and formats... [A46][A47] Overall, this wide diversity of studies and their major achievements highlight the strong potential of IMP samples generated from *Pichia pastoris* membranes, as well as the flexibility of the preparative procedures to comply with various demanding applications and their specific technical requirements.

Critical parameters and troubleshooting

A number of technical parameters and hints are specifically presented within each of the Basic Protocols sections, together with some anticipated results. Following are additional general guidelines and recommendations that may be helpful for the readers.

As indicated in the Basic Protocol headers and their illustrative figure examples, each step leading from overexpression to extraction and purification still remains a protein-dependent exercise. In addition, the formulation and the quality grade requirements for each generated sample also vary upon the type of biochemical and/or biophysical analyses that are further planned. Accordingly, the parameters that reveal critical for the preparation of one given IMP in a given study are rarely transposable to another IMP and have thus to be experienced on a case-by-case basis.

Whatever the IMP, however, the main issue relies on the ability to properly assess the quality of samples at every step of the production and preparation process: while yield is often a serious concern for a number of biochemical or biophysical investigations, proper folding of functional IMPs is obviously a requisite. Whenever possible, this characterization should be achieved with a specific activity assay that inform on both IMP's yield and functionality in

the analyzed sample. Such tests are highly beneficial, not only to monitor the optimal production of functional IMPs (André et al. 2006; Singh et al. 2012; Logez et al. 2014; Vasseur et al. 2019), but also to help setting the subsequent extraction and purification procedures. As previously mentioned and exemplified in Basic Protocol 3, this may be particularly valuable when screening for solubilizing detergents and conditions. In an illustrative study, a ligand binding assay was notably key to the identification of a particular Fos14/CHAPS detergent mixture that allowed extracting and maintaining the human melatonin MT1 receptor in an active form (Logez et al. 2014). Similarly, specific activity assays are a mean of choice to evaluate the stability of IMP in solution. As an example, a time-resolved scintillation proximity assay was developed in a comparative analysis of the human kappa opioid receptor solubilized in various environment, showing that receptor stability was dramatically improved in nanodiscs compared to detergent (Westh Hansen et al. 2016).

For a number of IMPs, however, no activity assays are readily available, or at least not at every step of the process. This is typically the^{[A48][A49]} situation for many IMPs, such as transporters or ion channels, that necessitate a structured compartmentation to assess their functionality. In this case, activity is evaluated only after the reconstitution of IMPs in lipid vesicles or planar bilayers (Brohawn et al. 2014; Zollmann et al. 2015; Wang et al. 2016; Christenson et al. 2018) and other quality control criteria are performed during the preceding extraction and purification steps. These criteria, that should also be investigated for every recombinant IMPs, include the evaluation of purity, integrity and homogeneity of the samples that are measured by any suitable techniques such as denaturing and native electrophoresis, size exclusion chromatography, UV-visible spectroscopy, mass spectrometry, dynamic light scattering, circular dichroism, transmission electron microscopy, etc.

Time considerations

The development of a complete procedure leading to the production and isolation of a functional membrane protein in solution is usually not a straightforward process. As already stated, it is always protein-dependent and may vary from few weeks to several months, with often no guarantee of success.

However, once the different parameters for either production, solubilization and purification are set and validated, obtaining pure protein in solution is not very time consuming. In a typical scheme, the whole procedure may be planned as follows:

- IMP production: starting from a freshly streaked colony, preculture is launched at the end of the **Day 1** and methanol induction is carried out on **Day 2**. Yeasts are then collected on **Day 3** and cells pellets can be directly processed or stored at -80°C for several months before membrane preparation step.
- Membrane preparation: the equivalent of 1 to 5 L of culture can be easily processed in and protein concentration determination can be performed in 1 day (**Day 4**).
Membrane preparations can also be stored at -80°C for several months and don't need to be prepared freshly prior to protein extraction and purification.
- IMP extraction and purification: once the proteins are extracted from the membrane, all the following steps should be performed in the continuity and as fast as possible. Either starting from membrane preparations (Basic Protocol 4) or from yeast cells (Alternative Protocols 4A and 4B), the time required to carry out the extraction and purification of IMPs in the condition described necessitates 1 day (**Day 5**). Analyses are then performed on the following day (**Day 6**).
- IMP reconstitution in lipid nanodiscs: nanodiscs self-assembly is usually performed overnight in the continuity of the extraction / purification step. They are purified and analyzed the day after (**Day 6**).

Overall, the minimal amount of time required to produce, prepare and isolate recombinant IMPs from a yeast clone is approximately 2 weeks.

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LITERATURE CITED

- Ahmad, M., Hirz, M., Pichler, H., Schwab, H. 2014. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Appl. Microbiol. Biotechnol.* 98:5301-17.
- Alkhalfioui, F., Logez, C., Bornert, O., Wagner, R. 2011. Expression systems: *Pichia pastoris*. In: Production of Membrane Proteins – Strategies for Expression and Isolation (Robinson A.S., ed.), Wiley-VCH, pp. 75-108.
- Ampah-Korsah, H., Sonntag, Y., Engfors, A., Kirscht, A., Kjellbom, P., Johanson, U. 2017. Single amino acid substitutions in the selectivity filter render NbXIP1;1 α aquaporin water permeable. *BMC Plant Biol.* 17:61.
- André, N., Cherouati, N., Prual, C., Steffan, T., Zeder-Lutz, G., Magnin, T., Pattus, F., Michel, H., Wagner, R., Reinhart, C. 2006. Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen. *Protein Sci.* 15:1115-1126.

- Bada Juarez, J. F., Harper, A. J., Judge, P. J., Tonge, S. R., Watts, A. 2019. From polymer chemistry to structural biology: The development of SMA and related amphipathic polymers for membrane protein extraction and solubilisation. *Chem. Phys. Lipids* 221:167-175.
- Bayburt, T.H., Sligar, S.G. 2010. Membrane protein assembly into Nanodiscs. *FEBS Lett.* 584:1721-1727.
- Bornert, O., Alkhalfioui, F., Logez, C., Wagner, R. 2012. Overexpression of membrane proteins using *Pichia pastoris*. *Curr Protoc Protein Sci.* Chapter 29:Unit 29.2.
- Bornert, O., Møller, T. C., Boeuf, J., Candusso, M. P., Wagner, R., Martinez, K. L., Simonin, F. 2013. Identification of a novel protein-protein interaction motif mediating interaction of GPCR-associated sorting proteins with G protein-coupled receptors. *PLoS One* 8:e56336.
- Breyton, C., Javed, W., Vermot, A., Arnaud, C. A., Hajjar, C., Dupuy, J., Petit-Hartlein, I., Le Roy, A., Martel, A., Thépaut, M., Orelle, C., Jault, J. M., Fieschi, F., Porcar, L., Ebel, C. 2019. Assemblies of lauryl maltose neopentyl glycol (LMNG) and LMNG-solubilized membrane proteins. *Biochim. Biophys. Acta Biomembr.* 1861:939-957.
- Brohawn, S.G., Su, Z., MacKinnon, R. 2014. Mechanosensitivity is mediated directly by the lipid membrane in TRAAK and TREK1 K⁺ channels. *Proc. Natl. Acad. Sci. U. S. A.* 111:3614-9.
- Buck, T. M., Jordan, R., Lyons-Weiler, J., Adelman, J. L., Needham, P. G., Kleyman, T. R., Brodsky, J. L. 2015. Expression of three topologically distinct membrane proteins elicits unique stress response pathways in the yeast *Saccharomyces cerevisiae*. *Physiol. Genomics* 47:198-214.

- Cabib, E., Arroyo, J. 2013. How carbohydrates sculpt cells: Chemical control of morphogenesis in the yeast cell wall. *Nat. Rev. Microbiol.* 11:648-655.
- Champeil, P., Orlowski, S., Babin, S., Lund, S., le Maire, M., Møller, J., Lenoir, G., Montigny, C. 2016. A robust method to screen detergents for membrane protein stabilization, revisited. *Anal. Biochem.* 511:31-5.
- Chipot, C., Dehez, F., Schnell, J. R., Zitzmann, N., Pebay-Peyroula, E., Catoire, L. J., Miroux, B., Kunji, E. R. S., Veglia, G., Cross, T. A., Schanda, P. 2018. Perturbations of Native Membrane Protein Structure in Alkyl Phosphocholine Detergents: A Critical Assessment of NMR and Biophysical Studies. *Chem. Rev.* 118:3559-3607.
- Christenson, E. T., Gallegos, A. S., Banerjee, A. 2018. *In vitro* reconstitution, functional dissection, and mutational analysis of metal ion transport by mitoferrin-1. *J. Biol. Chem.* 293:3819-3828.
- Chun, E., Thompson, A. A., Liu W., Roth, C. B., Griffith, M. T., Katritch, V., Kunken, J., Xu, F., Cherezov, V., Hanson, M. A., Stevens, R. C. 2012. Fusion partner toolchest for the stabilization and crystallization of G protein-coupled receptors. *Structure* 20:967-976.
- Damian, M., Pons, V., Renault, P., M'Kadmi, C., Delort, B., Hartmann, L., Kaya, A. I., Louet, M., Gagne, D., Ben Haj Salah, K., Denoyelle, S., Ferry, G., Boutin, J. A., Wagner, R., Fehrentz, J. A., Martinez, J., Marie, J., Floquet, N., Galès, C., Mary, S., Hamm, H. E., Banères, J. L. 2018. GHSR-D2R heteromerization modulates dopamine signaling through an effect on G protein conformation. *Proc. Natl. Acad. Sci. U. S. A.* 115:4501-4506.
- Dekki-Shalaly, N. D., Aneiros, E., Blank, M., Mueller, J., Nyman, E., Blind, M., Dabrowski, M. A., Andersson, C. V., Sandberg, K. 2015. Positive Modulation of the Glycine

- Receptor by Means of Glycine Receptor-Binding Aptamers. *J. Biomol. Screen.* 20:1112-23.
- Deng, Z., Paknejad, N., Maksaev, G., Sala-Rabanal, M., Nichols, C. G., Hite, R. K., Yuan, P. 2018. Cryo-EM and X-ray structures of TRPV4 reveal insight into ion permeation and gating mechanisms. *Nat. Struct. Mol. Biol.* 25:252-260.
- Denisov, I. G., Grinkova, Y. V., Lazarides, A. A., Sligar, S. G. 2004. Directed Self-Assembly of Monodisperse Phospholipid Bilayer Nanodiscs with Controlled Size. *J. Am. Chem. Soc.* 126:3477-348.
- Doshi, R., McGrath, A. P., Piñeros, M., Szewczyk, P., Garza, D. M., Kochian, L. V., Chang, G. 2017. Functional characterization and discovery of modulators of SbMATE, the agronomically important aluminium tolerance transporter from *Sorghum bicolor*. *Sci. Rep.* 7:17996.
- Duquesne, K., Prima, V., Sturgis, J. N. 2016. Membrane Protein Solubilization and Composition of Protein Detergent Complexes. *Methods Mol. Biol.* 1432:243-60.
- Dürr, U. H. N., Gildenberg, M., Ramamoorthy, A. 2012. The magic of bicelles lights up membrane protein structure. *Chem. Rev.* 112:6054-6074.
- Eddy, M. T., Gao, Z. G., Mannes, P., Patel, N., Jacobson, K. A., Katritch, V., Stevens, R. C., Wüthrich, K. 2018. Extrinsic Tryptophans as NMR Probes of Allosteric Coupling in Membrane Proteins: Application to the A2A Adenosine Receptor. *J. Am. Chem. Soc.* 140:8228-8235.
- Fan, Y., Shi, L., Ladizhansky, V., Brown, L. S. 2011. Uniform isotope labeling of a eukaryotic seven-transmembrane helical protein in yeast enables high-resolution solid-state NMR studies in the lipid environment. *J. Biomol. NMR* 49:151-61.

- Fischer, J. E., Glieder, A. 2019. Current advances in engineering tools for *Pichia pastoris*. *Curr. Opin. Biotechnol.* 59:175-181.
- Frauenfeld, J., Löving, R., Armache, J.-P., Sonnen, A.F.-P., Guettou, F., Moberg, P., Zhu, L., Jegerschöld, C., Flayhan, A., Briggs, J. A., Garoff, H., Löw, C., Cheng, Y., Nordlund, P. 2016. A saposin-lipoprotein nanoparticle system for membrane proteins. *Nat. Methods* 13:345-351.
- Gallagher, S. R. 2012. One-dimensional SDS gel electrophoresis of proteins. *Curr Protoc Protein Sci.* Chapter 10:Unit 10.1.
- Garaeva, A. A., Guskov, A., Slotboom, D. J., Paulino, C. 2019. A one-gate elevator mechanism for the human neutral amino acid transporter ASCT2. *Nat. Commun.* Jul 10:3427.
- Gasser, B., Mattanovich, D. 2018. A yeast for all seasons - Is *Pichia pastoris* a suitable chassis organism for future bioproduction? *FEMS Microbiol. Lett.* 365.
- Goldman, A., Ursitti, J. A., Mozdzanowski, J., Speicher, D. W. 2015. Electroblothing from Polyacrylamide Gels. *Curr Protoc Protein Sci.* 2;82:10.7.
- Hartmann, L., Kugler, V., Wagner, R. 2016. Expression of Eukaryotic Membrane Proteins in *Pichia pastoris*. *Methods Mol. Biol.* 1432:143-62.
- Hino, T., Arakawa, T., Iwanari, H., Yurugi-Kobayashi, T., Ikeda-Suno, C., Nakada-Nakura, Y., Kusano-Arai, O., Weyand, S., Shimamura, T., Nomura, N., Cameron, A. D., Kobayashi, T., Hamakubo, T., Iwata, S., Murata, T. 2012. G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. *Nature.* 482:237-40.
- Igonet, S., Raingeval, C., Cecon, E., Pučić-Baković, M., Lauc, G., Cala, O., Baranowski, M., Perez, J., Jockers, R., Krimm, I., Jawhari, A. 2018. Enabling STD-NMR fragment

screening using stabilized native GPCR: A case study of adenosine receptor. *Sci. Rep.* 8:8142.

Jørgensen, I.L., Kemmer, G. C., Pomorski, T. G. 2017. Membrane protein reconstitution into giant unilamellar vesicles: a review on current techniques. *Eur. Biophys. J.* 46:103-119.

Kapri-Pardes, E., Katz, A., Haviv, H., Mahmmoud, Y., Ilan, M., Khalfin-Penigel, I., Carmeli, S., Yarden, O., Karlish, S. J. 2011. Stabilization of the $\alpha 2$ isoform of Na,K-ATPase by mutations in a phospholipid binding pocket. *J. Biol. Chem.* 286:42888-99.

Kodan, A., Yamaguchi, T., Nakatsu, T., Sakiyama, K., Hipolito, C. J., Fujioka, A., Hirokane, R., Ikeguchi, K., Watanabe, B., Hiratake, J., Kimura, Y., Suga, H., Ueda, K., Kato, H. 2014. Structural basis for gating mechanisms of a eukaryotic P-glycoprotein homolog. *Proc. Natl. Acad. Sci. U. S. A.* 111:4049-54.

Kotov, V., Bartels, K., Veith, K., Josts, I., Subhramanyam, U. K. T., Günther, C., Labahn, J., Marlovits, T. C., Moraes, I., Tidow, H., Löw, C., Garcia-Alai, M. M. 2019. High-throughput stability screening for detergent-solubilized membrane proteins. *Sci. Rep.* 9:10379.

Liu, W. C., Inwood, S., Gong, T., Sharma, A., Yu, L. Y., Zhu, P. 2019. Fed-batch high-cell-density fermentation strategies for *Pichia pastoris* growth and production. *Crit. Rev. Biotechnol.* 39:258-271.

Logez, C., Alkhalfioui, F., Byrne, B., Wagner, R. 2012. Preparation of *Pichia pastoris* expression plasmids. *Methods Mol. Biol.* 866:25-40.

Logez, C., Berger, S., Legros, C., Banères, J.-L., Cohen, W., Delagrange, P., Nosjean, O., Boutin, J. A., Ferry, G., Simonin, F., Wagner, R. 2014. Recombinant human melatonin

- receptor MT1 isolated in mixed detergents shows pharmacology similar to that in mammalian cell membranes. *PLoS One* 9:e100616.
- Lolicato, M., Arrigoni, C., Mori, T., Sekioka, Y., Bryant, C., Clark, K. A., Minor, D. L. Jr. 2017. K₂P2.1 (TREK-1)-activator complexes reveal a cryptic selectivity filter binding site. *Nature* 547:364-368.
- Lórenz-Fonfría, V., Perálvarez-Marín, A., Padrós, E., Lazarova, T. 2011. Solubilization, Purification, and Characterization of Integral Membrane Proteins. In: Production of Membrane Proteins – Strategies for Expression and Isolation (Robinson A.S., ed.), Wiley-VCH, pp. 317-360.
- Love, K. R., Dalvie, N. C., Love, J. C. 2018. The yeast stands alone: the future of protein biologic production. *Curr. Opin. Biotechnol.* 53:50-58.
- Macauley-Patrick, S., Fazenda, M. L., McNeil, B., Harvey, L. M. 2005. Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* 22:249-70.
- Mattanovich, D., Sauer, M., Gasser, B. 2014. Yeast biotechnology: teaching the old dog new tricks. *Microb. Cell Fact.* 13:34.
- Olson, B. J., Markwell, J. 2007. Assays for determination of protein concentration. *Curr Protoc Protein Sci.* Chapter 3:Unit 3.4.
- Overduin, M., Klumperman, B. 2019. Advancing membrane biology with poly(styrene-co-maleic acid)-based native nanodiscs. *Eur. Polym. J.* 110:63-68.
- Pandey, A., Shin, K., Patterson, R. E., Liu, X. Q., Rainey, J. K. 2016. Current strategies for protein production and purification enabling membrane protein structural biology. *Biochem. Cell. Biol.* 94:507-527.

- Rosell, A., Meury, M., Álvarez-Marimon, E., Costa, M., Pérez-Cano, L., Zorzano, A., Fernández-Recio, J., Palacín, M., Fotiadis, D. 2014. Structural bases for the interaction and stabilization of the human amino acid transporter LAT2 with its ancillary protein 4F2hc. *Proc. Natl. Acad. Sci. U. S. A.* 111:2966-71.
- Santos, R., Ursu, O., Gaulton, A., Bento, A. P., Donadi, R. S., Bologa, C. G., Karlsson, A., Al-Lazikani, B., Hersey, A., Oprea, T. I., Overington, J. P. 2017. A comprehensive map of molecular drug targets. *Nat. Rev. Drug Discov.* 16:19-34.
- Scalise, M., Pochini, L., Panni, S., Pingitore, P., Hedfalk, K., Indiveri, C. 2014. Transport mechanism and regulatory properties of the human amino acid transporter ASCT2 (SLC1A5). *Amino Acids* 46:2463-75.
- Schlebach, J. P., Sanders, C. R. 2014. The safety dance: Biophysics of membrane protein folding and misfolding in a cellular context. *Q. Rev. Biophys.* 44:1-34.
- Schölz, C., Parcej, D., Ejsing, C. S., Robenek, H., Urbatsch, I. L., Tampé, R. 2011. Specific lipids modulate the transporter associated with antigen processing (TAP). *J. Biol. Chem.* 286:13346-56.
- Seddon, A. M., Curnow, P., Booth, P. J. 2004. Membrane proteins, lipids and detergents: not just a soap opera. *Biochim. Biophys. Acta* 1666:105-17.
- Singh, S., Gras, A., Fiez-Vandal, C., Martinez, M., Wagner, R., Byrne, B. 2012a. Screening for high-yielding *Pichia pastoris* clones: the production of G protein-coupled receptors as a case study. *Methods Mol. Biol.* 866:65-73.
- Singh, S., Gras, A., Fiez-Vandal, C., Martinez, M., Wagner, R., Byrne, B. 2012b. Large-scale production of membrane proteins in *Pichia pastoris*: the production of G protein-coupled receptors as a case study. *Methods Mol. Biol.* 866:197-207.

- Smith, S. M. 2017. Strategies for the Purification of Membrane Proteins. *Methods Mol. Biol.* 1485:389-400.
- Spohner, S. C., Müller, H., Quitmann, H., Czermak, P. 2015. Expression of enzymes for the usage in food and feed industry with *Pichia pastoris*. *J. Biotechnol.* 202:118-34.
- Tate, C. G. 2010. Practical considerations of membrane protein instability during purification and crystallisation. *Methods Mol. Biol.* 601:187-203.
- Vasseur, L., Cens, T., Wagner, R., Saint, N., Kugler, V., Chavanieu, A., Ouvry, C., Dupré, C., Ferry, G., Boutin, J. A. 2019. Importance of the Choice of a Recombinant System to Produce Large Amounts of Functional Membrane Protein hERG. *Int. J. Mol. Sci.* 20 pii: E3181.
- Vinothkumar, K. R., Montgomery, M. G., Liu, S., Walker, J. E. 2016. Structure of the mitochondrial ATP synthase from *Pichia angusta* determined by electron cryo-microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 113:12709-12714.
- Vogl, T., Thallinger, G. G., Zellnig, G., Drew, D., Cregg, J. M., Glieder, A., Freigassner, M. 2014. Towards improved membrane protein production in *Pichia pastoris*: general and specific transcriptional response to membrane protein overexpression. *N Biotechnol.* 31:538-52.
- von Hagen, J., Michelsen, U. 2013. Cellular fractionation--yeast cells. *Methods Enzymol.* 533:31-9.
- Wang, W., Touhara, K. K., Weir, K., Bean, B. P., MacKinnon, R. 2016. Cooperative regulation by G proteins and Na(+) of neuronal GIRK2 K(+) channels. *Elife* 5 pii: e15751.

- Wang, H., Schoebel, S., Schmitz, F., Dong, H., Hedfalk, K. 2020. Characterization of aquaporin-driven hydrogen peroxide transport. *Biochim. Biophys. Acta Biomembr.* 1862:183065.
- Westh Hansen, R., Wang, X., Golab, A., Bornert, O., Oswald, C., Wagner, R., Martinez, K. L. 2016. Functional Stability of the Human Kappa Opioid Receptor Reconstituted in Nanodiscs Revealed by a Time-Resolved Scintillation Proximity Assay. *PLoS One* 11:e0150658.
- Wöhri, A. B., Hillertz, P., Eriksson, P.-O., Meuller, J., Dekker, N., Snijder, A. 2013. Thermodynamic studies of ligand binding to the human homopentameric glycine receptor using isothermal titration calorimetry. *Mol. Membr. Biol.* 30:169-183.
- Yang, Z., Zhou, Q., Mok, L., Singh, A., Swartz, D. J., Urbatsch, I. L., Brouillette, C. G. 2017. Interactions and cooperativity between P-glycoprotein structural domains determined by thermal unfolding provides insights into its solution structure and function. *Biochim. Biophys. Acta Biomembr.* 1859:48-60.
- Ye, L., Neale, C., Sljoka, A., Lyda, B., Pichugin, D., Tsuchimura, N., Larda, S. T., Pomès, R., García, A. E., Ernst, O. P., Sunahara, R. K., Prosser, R. S. 2018. Mechanistic insights into allosteric regulation of the A_{2A} adenosine G protein-coupled receptor by physiological cations. *Nat. Commun.* 9(1):1372.
- Zehnpfenning, B., Wiriyasermkul, P., Carlson, D. A., Quick, M. 2015. Interaction of α -Lipoic Acid with the Human Na⁺/Multivitamin Transporter (hSMVT). *J. Biol. Chem.* 290:16372-82.
- Zhu, T., Sun, H., Wang, M., Li, Y. 2019. *Pichia pastoris* as a Versatile Cell Factory for the Production of Industrial Enzymes and Chemicals: Current Status and Future Perspectives. *Biotechnol. J.* 2019 14:e1800694.

Zollmann, T., Moiset, G., Tumulka, F., Tampé, R., Poolman, B., Abele, R. 2015. Single liposome analysis of peptide translocation by the ABC transporter TAPL. *Proc. Natl. Acad. Sci. U. S. A.* 112:2046-51.

FIGURE LEGENDS

Figure 1: Immunoblot analysis of a human 4TM cell surface receptor extracted with seven different detergents. Proteins were extracted with 1 % (w/v) of each detergent (2 % (w/v) for OG). Equal volumes of samples (*i.e.* supernatant – S – and resuspended pellet – P –) were loaded in each lane (15 μ L). Proteins were separated by SDS-PAGE on a 10 % (w/v) polyacrylamide gel and immunoblotted with a mouse penta-his antibody (1/1000 v/v) (Qiagen, ref. 34660). The secondary antibody is a donkey anti-mouse DyLight 800 (1/10000 v/v) (Euromedex, FR, ref A90-337D8). Molecular masses (kDa) are indicated on the left. The band corresponding to the immunodetected protein of interest is indicated by the arrow on the right.

MW: Molecular weight; Mb: membrane; No det.: no detergent ; OG: n-octyl β -D-glucopyranoside; OGNG: 2,2-dihexylpropane-1,3-bis- β -D-glucopyranoside; DM: n-decyl- β -D-maltopyranoside; DDM: n-dodecyl- β -D-maltopyranoside; LMNG: 2,2-didecylpropane-1,3-bis- β -D-maltopyranoside; Fos12: Fos-choline 12; Fos14: Fos-choline 14.

Figure 2: Detergent screening for the human ADRA2B receptor extraction. A) Immunoblot analysis of membrane protein extracted with 1 % (w/v) of various detergents. Equal volumes of the solubilized fractions were loaded in each lane (15 μ L). Proteins were separated by SDS-PAGE on a 10 % polyacrylamide gel and immunoblotted with an M2 anti-flag antibody (1/8000 v/v) (Sigma, F3165). The secondary antibody is a donkey anti-mouse DyLight 800 (1/10000 v/v). Molecular masses (kDa) are indicated on the left. The band corresponding to the immunodetected protein of interest is indicated by the arrow on the right. Bands of higher molecular weight most likely correspond to SDS-resistant oligomeric forms of the receptor. B) Specific ligand binding activity determined by a radioligand binding assay on 10 μ g of each solubilized fraction with [3 H]Rauwolscine (André et al. 2006).

MW: Molecular weight; Mb: membrane (control); 1: n-dodecyl- β -D-maltopyranoside (DDM); 2: n-octyl β -D-glucopyranoside (OG); 3: n-undecyl- β -D-maltopyranoside (UM); 4: n-decyl- β -D-maltopyranoside (DM); 5: digitonin; 6: lauryl-sucrose; 7: Fos-choline 12 (Fos12); 8: Fos-choline 14 (Fos14); 9: Fos-choline 16 (Fos16); 10: cyclohexyl-hexyl- β -D-maltoside (CYMAL-6); 11: zwittergent 3-12; 12: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS).

Figure 3: Two-step purification of a TRPV4 ion channel. A) IMAC profile obtained with a HisTrap HP column. The dashed trace represents the percentage of B buffer applied onto the column. W1, wash 1: 30 % B (150 mM imidazole); E, elution: 100 % B (500 mM imidazole). Fraction indicated with a red arrow was injected on a size exclusion column. B) SEC profile, obtained on a Superdex 200 10-300 column. C) Coomassie Blue stained SDS PAGE. Equal volumes of the analyzed fractions were loaded in each lane (10 μ L). Molecular masses (kDa) are indicated on the left. MW: molecular weight (kDa), H_{pool}: elution fraction from HisTrapHP; S₂₀₀: elution fraction from Superdex 200.

Figure 4: Purification of a human TREK1 channel from *P. pastoris* protoplasts. A) IMAC profile obtained with a HisTrapHP column. The dashed trace represents the percentage of B buffer applied onto the column. W1, wash 1: 20 mM imidazole; W2: 50 mM imidazole; W3: 100 mM imidazole; E, elution: 500 mM imidazole. Fractions in between the red lines were pooled (H_{pool}), concentrated up to 500 μ L and injected on a size exclusion column. B) SEC profile of H_{pool}, obtained on a Superdex 200 10-300 Increase column. Arrows point to the indicated fractions that were further analyzed by SDS-PAGE. C) Coomassie Blue stained SDS PAGE (10 % (w/v) polyacrylamide gel) of the representative fractions issued from the IMAC and SEC. SEC fractions from were also immunoblotted with an M2 anti-flag antibody (1/8000 v/v). The secondary antibody is a IRDye 800CW goat anti-mouse (1/10000 v/v). Molecular masses (kDa) are indicated on the left. MW: molecular weight.

Figure 5: Purification profiles of a human lipase extracted from *P. pastoris* protoplasts. A) IMAC profile obtained with a HisTrap Excel column with absorbance measured at 280 nm. The dashed trace represents the percentage of B buffer applied onto the column. W1, wash 1: 20 mM imidazole; W2: 50 mM imidazole; E, elution: 500 mM imidazole. Fractions in between the red lines were pooled and concentrated up to 2 mL prior injection on a size exclusion

column. B) SEC profile, obtained with a Superdex 200 HiLoad PG column. C) Coomassie Blue stained SDS-PAGE. Equal volume of membrane proteins were loaded in each lane (10 μ L). Molecular masses (kDa) are indicated on the left. MW: molecular weight; H_{pool}: pool of the elution fractions from HisTrapHP; S₂₀₀: elution fraction from Superdex 200.

Figure 6: Purification of the nanodisc-reconstituted human AA2A receptor. A) IMAC elution profile (HisTrap HP 1mL column) of the self-assembled nanodisc mixture (ND mix). Elution is achieved with 500 mM imidazole (100 % B buffer, dashed line). The eluted fractions were pooled (H_{pool}), concentrated to 500 μ L and injected on a size exclusion column. B) SEC profile obtained on a Superdex 200 10-300 Increase column. The indicated fractions 21 to 25 were further analyzed by SDS-PAGE. C) Coomassie Blue stained SDS PAGE of the purification representative fractions. Molecular masses (kDa) are indicated on the left. MW: molecular weight; FT: IMAC flow through.