Title

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ISBN

9780323853767

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Publication Date

2021

DOI

10.1016/bs.mie.2020.12.020

Peer reviewed

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CHAPTER TEN

SARS-CoV-2 3a expression, purification, and reconstitution into lipid nanodiscs

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Contents

1. Introduction 208
2. Before you begin 210
3. Key resources table 211
4. Materials and equipment 212
5. Step-by-step method details 214
   - 5.1 Preparation of Bacmid 214
   - 5.2 Making 3a protein-expressing Sf9 cell pellets 215
   - 5.3 Protein-preparation-day eve 218
   - 5.4 3a protein purification 219
   - 5.5 3a nanodisc incorporation 227
6. Expected outcomes 230
7. Advantages 230
8. Limitations 231
9. Safety considerations and standards 231
10. Alternative methods/procedures 231
    - 10.1 Making MSP1E3D1 stocks 231
    - 10.2 Bacterial preparation of human rhinovirus 3C protease 232
    - 10.3 Bacterial preparation of anti-GFP nanobody 232
    - 10.4 Preparation of anti-GFP nanobody resin 232
    - 10.5 Storage and regeneration of anti-GFP nanobody resin 233

Acknowledgments 233
References 234

Abstract

The SARS-CoV-2 3a protein is a putative ion channel implicated in virus life cycle and pathogenesis. We recently expressed, purified, and reconstituted 3a into lipid nanodiscs to solve its structure by cryo-EM to 2.1 Å resolution. In this chapter, we describe methods

Methods in Enzymology, Volume 653
ISSN 0076-6879
https://doi.org/10.1016/bs.mie.2020.12.020

we developed in order to facilitate the study of this protein in other laboratories. We emphasize factors that enabled rapid progression from gene sequence to reconstituted protein (3 weeks in the case of 3a) and provide general observations and tips for adapting these protocols to other membrane proteins of interest.

1. Introduction

The global pandemic of Coronavirus disease 2019 (Covid-19) has focused many biomedical research efforts on the properties and pathogenesis of the causative virus, SARS-CoV-2. We were particularly interested in understanding the structure and function of the accessory protein orf3a (3a) as studies on the related SARS-CoV-1 virus implicated 3a as a putative ion channel with roles in inflammasome activation and cell death (Castaño-Rodriguez et al., 2018; Chan et al., 2009; Cui, Li, & Shi, 2019; Lu et al., 2006; Ren et al., 2020; Siu et al., 2019; Yue et al., 2018). We therefore expressed, purified, and reconstituted 3a into proteoliposomes for electrophysiological recordings (using the protocol described in del Mármol, Rietmeijer, & Brohawn, 2018; see also Martinac et al., 2010) and into lipid nanodiscs for structural characterization by cryo-EM. We found that purified 3a assembles into dimers and tetramers (as a dimer-of-dimers) with a novel structural fold and displays non-selective cation channel activity (Kern et al., 2020). However, many questions remain unanswered including the basis of 3a channel gating, the mechanism of inhibition by putative small molecule therapeutics, and the structural and functional impact of proteins that interact with 3a N- and C-terminal regions. Answering these and other questions will necessitate further biochemical and structural experiments. To facilitate this work, we detail here our methods for the rapid production and reconstitution of 3a protein.

We intend that following this chapter will allow you to produce 3a for your own experiments. We also note that these methods have been readily adapted to study other membrane proteins in our lab. Therefore, we emphasize general observations and tips that should facilitate application of the procedure to additional targets. The first major hurdle for this procedure is whether a membrane protein of interest can be successfully expressed and purified from insect cells. We prefer using insect cells when possible as it tends to yield high protein expression and purity in a short time frame. However, proteins that cannot be expressed and purified from insect cells can often be made in mammalian cells in suspension in a similar manner (Goehring et al., 2014). The second major hurdle for this procedure is to

optimize biochemical parameters of the purification and reconstitution. While the parameters we detail for 3a are typical in our hands and good starting points, in other cases the use of different detergents (e.g., some proteins require cholesterol hemisuccinate supplementation during extraction) and optimization of the ratios for nanodisc reconstitution (e.g., adjusting for protein oligomeric state) have been necessary for optimal results (Kern, Oh, Hite, & Brohawn, 2019; Li, Rietmeijer, & Brohawn, 2020; Reid, Kern, & Brohawn, 2020).

The procedure here is straightforward to adapt to different purification approaches and laboratory settings, but we note three features below that we find particularly useful and encourage using to your advantage, if possible. We refer readers to prior work for more detail (Goehring et al., 2014; Kawate & Gouaux, 2006; Ritchie et al., 2009).

First, we tag 3a with a variant of GFP (sfGFP) as a visualization and purification tag (Pédelacq, Cabantous, Tran, Terwilliger, & Waldo, 2006). The visible green color of the GFP-tagged protein allows tracking of the protein of interest throughout expression and purification steps using fluorescence microscopy, illumination of samples by typical blue LED light used in common DNA gel lightboxes, and simple direct visualization when GFP-tagged proteins are at high concentrations. FSEC allows small-scale rapid screening of detergent and other buffer conditions to quickly optimize biochemical parameters for protein purification (Kawate & Gouaux, 2006). This is useful both for teaching researchers who are new to membrane protein purification and for fast troubleshooting of purifications. Affinity purification using an anti-GFP nanobody resin followed by protease cleavage to release the protein of interest typically leads to a very clean purification even before size exclusion (Rothbauer et al., 2008).

Second, instead of directly extracting insect cells in a detergent buffer, we first perform a purification of the membrane fraction by ultracentrifugation and pellet washing. This step removes soluble, cytosolic contaminants including free GFP and cleavage products often made as a consequence of overexpressing tagged protein. It also allows for the removal of nucleic acids which can interact with the anti-GFP nanobody resin through electrostatic interactions and negatively impact both the speed and quality of downstream steps.

Third, we and others have seen many benefits of cryo-EM structure determination of membrane proteins in lipid nanodiscs compared to in detergent micelles (Ritchie et al., 2009). First, although a lipid nanodisc does not share all properties of a lipid bilayer, the presence of lipid can promote

proper membrane protein function (TRPV1), stabilize domains (NOMPC), or promote alternate conformations (LRRC8A) compared to detergent (Gao, Cao, Julius, & Cheng, 2016; Jin et al., 2017; Kern et al., 2019). We have seen improved particle distribution and freezing behavior of proteins in nanodiscs relative to detergent. Interactions with the membrane scaffold proteins and lipids can also serve to add stable mass to small membrane proteins that can aid in their reconstruction (Cianfrocco & Kellogg, 2020; Li et al., 2020; Reid et al., 2020). This can help with both the initial steps of particle selection and pruning and final refinements. In the case of 3a, we see full nanodisc density in 2D class averages and lower resolution reconstructions and specific interactions between 3a, lipids, and the scaffold protein MSP1E3D1 in high resolution refinements (Kern et al., 2020).

2. Before you begin

Timing: ~1 week to prepare healthy cells for baculovirus steps and competent cells for transformations, ~1 week during baculovirus steps to prepare reagents for protein preparation, ~1 day before protein preparation for setup.

1. The first part of the protocol consists of the molecular biology, tissue culture, and baculoviral techniques to generate frozen pellets of cells expressing 3a protein
   - **a)** You will require competent MultiBAC cells or equivalent for generating bacmid
   - **b)** For this portion of the protocol you need robustly growing Sf9 cells in shaking culture
   - **c)** Passage cells several times post-thawing until they display high viability (≥95%) and typical morphology (round and of a similar size)
   - **d)** Generating 3a protein pellets will take ~2 weeks
2. The second part of the protocol takes this 3a cell pellet and goes through all protein purification and nanodisc incorporation steps in ~1.5 days
   - **a)** You will need reagents and equipment for these steps ready in advance including anti-GFP nanobody coupled resin for protein purification, human rhinovirus 3C protease for cleaving the GFP tag from 3a, and MSP1E3D1 scaffold protein for forming nanodiscs. These can be generated in house in various ways and/or purchased. See Section 10: Alternative methods/procedures
   - **b)** On the day before the protein purification and nanodisc incorporation you will need to prepare reagents for each step

c) For protein purification, it is advisable to make the detergent stock solution in advance

d) For nanodisc incorporation, you will need to dry down lipids and prepare hydrated biobeads a day in advance

3. Key resources table

Reagent or resource	Source	Identifier
Bacterial and Virus Strains		
DH10MultiBAC *E. coli* strain competent cells	Geneva Biotech	DH10MultiBac
Chemicals, Peptides, and Recombinant Proteins		
Large construct miniprep kit (e.g., Purelink HiPure)	Thermo Fisher	K210002
ESF 921 Sf9 culture medium	Expression Systems	96–001-01
Antibiotic-antimycotic	Thermo Fisher (Gibco)	15240062
Escort IV transfection reagent	Millipore Sigma	L3287
E64	Gold Biotechnology	E-064-5
Pepstatin A	Gold Biotechnology	P-020-100
Soy Trypsin Inhibitor	Millipore Sigma	T9128
Benzamidine	Gold Biotechnology	B-050-100
Aprotinin	Gold Biotechnology	A-655-100
Leupeptin	Gold Biotechnology	L-010-5
AEBSF	Gold Biotechnology	A-540-500
PMSF	Gold Biotechnology	P-470-10
Benzonase	Millipore Sigma	E1014

PreScission (Human rhinovirus 3C) protease	Cytiva (Or prepare)	27084301
Benzonase nuclease	Millipore Sigma	70746
n-Dodecyl-β-D-Maltopyranoside (DDM)	Anatrace	D310S
1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)	Avanti	850725C
1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS)	Avanti	840034C
1-Palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC)	Avanti	850457C
Pentane		
Anti-GFP nanobody coupled resin	Prepare in advance	See Section 10
MSP1E3D1 for nanodisc frozen at 5 mg/mL	Prepare in advance	See Section 10
Biobeads SM2	Bio-Rad	1523920
Experimental Models: Cell Lines		
Spodoptera frugiperda (Sf9) cell line	Expression Systems	94-001F
Recombinant DNA		
3a-sfGFP expression plasmid (pFASTBAC1 derivative)	Addgene	#156423
MSP1E3D1 expression plasmid	Addgene	#20066
Anti-GFP nanobody expression plasmid	Addgene	#49172
Human rhinovirus 3C protease plasmid	Addgene	#78571

4. Materials and equipment

- Electroporator (e.g., Biorad MicroPulser) and cuvettes
- Lysogeny Broth (LB) media
- 100 mm LB-Agar plates with antibiotics and chemicals (Kanamycin, Tetracycline, Gentamicin, IPTG, BluoGal, as indicated)

- 37 °C/250 rpm shaking incubator for bacterial growth (e.g., Infors Multitron)
- Flasks for culturing Sf9 cells (e.g., glass flat bottom 300 mL and 2800 mL flasks from Corning, with foam stoppers covered with foil as caps)
- 27 °C incubator for Sf9 cells (e.g., Binder BD line)
- 27 °C/130 rpm shaking incubator for Sf9 growth (e.g., Infors Multitron)
- Tissue culture hood for Sf9 work
- Hemocytometer for counting cells
- Microscope capable of GFP excitation/detection for tissue culture work
- −80 °C freezer
- Argon tank with regulator and spray nozzle
- Glass vials with screw caps for lipid and detergent preparation (e.g., Fisherbrand 1495925B)
- Pentane (HPLC grade)
- Chloroform (ACS grade)
- Ultrasonic bath for solubilizing detergent and lipids (e.g., Branson CPX-952-219R) with stand and clamps to hold vials
- Chemical fume hood
- Vacuum desiccator
- Tris Buffer (titrate TRIS base to final pH with HCl)
- HEPES buffer (titrate HEPES free acid to final pH with KOH)
- KCl
- EDTA (titrate to final pH with KOH)
- 4 °C cold room
- Plastic or glass beakers (250 and 600 mL) and graduated cylinders (100 and 250 mL)
- 15- and 50-mL polypropylene tubes
- 1.5 mL plastic tubes
- Parafilm
- Aluminum foil
- Magnetic stir bar
- Magnetic stir plate
- Refrigerated floor centrifuge (e.g., Sorvall Lynx 6000), rotors (e.g., Fiberlite F9–6 × 1000 LEX and Fiberlite F14-14X50CY), and corresponding 1 L bottles and 35 mL tubes and adapters for spins at ~3000 × *g* (for ~1 L volumes) and ~35,000 × *g* (for ~35 mL volumes)
- Refrigerated tabletop centrifuge (e.g., Sorvall Legend), rotor (e.g., BIOFlex HC Swinging-Bucket), and conical tube adapters capable of spins at 1000–5000 × *g*
- Ultracentrifuge (e.g., Beckman Optima), rotor (e.g., Ti45), and corresponding tubes capable of spins at ~150,000 × *g*

- Refrigerated desktop centrifuge (e.g., Eppendorf 5430R), microcentrifuge tube rotor, and tubes capable of spins at $\sim 20{,}000 \times g$
- Dounce homogenizer (100 mL recommended, e.g., Kimble)
- Tip Sonificator for cell lysis (Branson Cell Disruptor 450)
- Column for accumulating, washing, and eluting resin (e.g., Econo-Column from Bio-Rad)
- Spin concentration devices with 10 kDa cutoff (15 and 4 mL)
- FPLC for gel filtration chromatography
- Superdex 200 Increase 10/300 GL column (Cytiva)
- Nanodrop with detection at 280 nm for determining protein concentration
- 1.5 mL tube rotator
- Equipment for running Coomassie gel

5. Step-by-step method details

5.1 Preparation of Bacmid

5.1.1 Timing: 3 days (prepare miniprepped 3a vector, MultiBAC competent cells, and plates in advance)

1. Electroporate MultiBAC competent cells with 3a plasmid
   - **a)** Thaw 100 μL of competent cells in a 1.5 mL tube on ice.
   - **b)** Dilute plasmid in water and then into cells to achieve a final DNA concentration of ~10 ng in 100 μL.
   - **c)** Mix by vortexing or by tapping tube and then let the mixture sit on ice for 15 min.
   - **d)** Electroporate cells in a cuvette according to manufacturer instructions.
   - **e)** Quickly add 400 μL Lysogeny broth (LB) and transfer mix back to an 1.5 mL tube.
   - **f)** Recover cells by shaking at 250 rpm at 37 °C for 4–16 h (**Note**: choose incubation time as convenient, everything in this range should be successful).
   - **g)** During this time prepare 100 mm plates. Plates should be standard LB-agar with Kanamycin (50 μg/mL), Tetracycline (10 μg/mL), and Gentamycin (15 μg/mL).
   - **h)** Top spread on IPTG (20 μL of a 40 mM stock solution) and Bluo-gal (30 μL of a 20 mg/mL stock solution) with your favorite plating method and let the plate dry at room temperature or 37 °C

depending on timing. (**Note:** Make sure chemicals are absorbed and plate is dry before plating bacteria).

i) After cell incubation, plate 150 μL of recovered cells and incubate at 37 °C for 24 h.

j) At 24 h, check plate and make sure colonies are clearly blue or white. If unclear, plate can be left at room temperature or 4 °C to develop color.

k) Pick 1 clearly white colony (white paper as a background to the plate can be helpful for visualization) into 10 mL of LB with Kanamycin, Tetracycline, and Gentamycin at the same concentrations as plating media and incubate for ~16 h to generate a saturated culture.

2. Miniprep the 3a bacmid

a) Make a glycerol stock of the bacmid containing cells as a backup and for future bacmid preparation.

b) We recommend using a large construct miniprep kit such as Purelink HiPure that uses gravity flow instead of a spin column.

c) Following manufacturer's instructions for this type of kit should work well. However, a few steps should be performed with extra attention:

d) **Critical:** For the spindown after precipitation, make sure that you only transfer cleared supernatant to the purification column. If there is visible debris or a cloudy supernatant, spin the sample again until it is clear and pipet carefully.

e) **Note:** After isopropanol precipitation and ethanol washes, there should be a small white DNA pellet at the bottom of the 1.5 mL tube.

f) **Note:** For drying down the pellet, remove the majority of the ethanol carefully. Then briefly spin the remainder of the liquid down and carefully remove it with a P200, avoiding the pellet. Let the tube sit open for 10 min to fully dry and then resuspend the pellet in 50 μL TE buffer.

5.2 Making 3a protein-expressing Sf9 cell pellets

5.2.1 Timing: 9–12 days

Note: Sf9 cell manipulations should be performed in a tissue culture hood using sterile technique.

Note: For full Sf9 and baculoviral techniques and troubleshooting, you can consult manuals such as the Invitrogen BEVS manual. This is an abbreviated protocol with short cuts to generate protein quickly.

3. Sf9 culture

a) We culture cells shaking at 27 °C at 130 rpm in non-baffled glass Erlenmeyer flasks with a foam cap and foil covering. These flasks and caps are sterilized by washing, autoclaving, and drying. You can alternatively use many varieties of sterile plastic flasks. Common volumes we use are 100 mL of cell suspension in a 300 mL flask for passage and 500 mL to 1 L of cell suspension in a 2.8 L flask for protein expression.

b) We use ESF921 media from Expression Systems supplemented with 1 × Antibiotic-antimycotic (anti-anti) from Thermo Fisher for culturing cells.

c) We keep the cell density between 750,000 and 12 million cells/mL, assessed by hemocytometer. As healthy cells should double daily, this makes for a passage every 4 days.

4. Bacmid transfection of Sf9 cells to generate P1 virus

a) Seed 1.5 million cells per well in a 6-well plate (use 1 well per construct and 1 control well) in ESF921 with anti-anti and put in the 27 °C incubator.

b) For each construct, add 5 μL of bacmid miniprep DNA to 100 μL of ESF921 without antibiotic in an Eppendorf tube. Tap the tube to mix.

c) For each construct, add 5 μL of Escort IV transfection reagent to 100 μL of ESF921 without antibiotic in an Eppendorf tube. Tap the tube to mix.

d) Wait 5 min, then add the transfection mix to the DNA mix. Tap the tube to mix.

e) Wait 45 min for transfection complexes to form.

f) Take out the plate with settled cells, aspirate the media, rinse with 2 mL of ESF921 without anti-anti, and then add the transfection complexes to the wells in 1 mL of ESF921 without anti-anti.

g) Place the cells into the 27 °C incubator. This mix can sit on the cells for anywhere from 5 h to overnight depending on what is convenient.

h) After incubation, replace the transfection complex media with 2 mL of ESF 921 with anti-anti and place back into the incubator.

i) Wait **3 days** after addition of the transfection mix. **Note:** If you fear or observe significant evaporation in your incubator, you can make a crude humidity chamber for the plate by placing a wet Kimwipe on top of the closed plate and wrapping both in aluminum foil.

j) During this time, expand and pass Sf9 cells such that you will have enough cells for a P2 generation day 3 post transfection and P3 generation and/or protein production on day 6 post-transfection. We generally use 100 mL at 2 million cells/mL for P2 generation, 500 mL at 2 million cells/mL for P3 generation, and 1 L at 4 million cells/mL for protein production.

k) On day 3 post-transfection, inspect transfected cells for signs of baculovirus and protein production. Using a tissue culture microscope setup for GFP detection, inspect cells for the presence of 3a-GFP. Transfection rates are variable, but we generally see somewhere between 10 and 50% GFP^+ cells at this timepoint.

l) Assuming fluorescence looks good, you can either proceed immediately to the next step or 0.2 μm filter (**Note:** make sure filter is low-protein-binding) and store the P1 virus at 4 °C.

5. Generation of P2 and P3 baculovirus

a) **Note:** For viral amplification, you ideally want to infect cells with a multiplicity of infection (MOI) of between 0.01 and 0.1. Being precise about MOI requires measuring the viral titer and applying the formula: Volume virus required = MOI × total cells/viral titer. However, this takes additional time and effort at each step.

b) In lieu of titering virus, we have found that if fluorescence of bacmid-transfected cells looked good (10–50% GFP^+ cells), adding 250 μL of the P1 virus to 100 mL of Sf9 cells at 2 million cells/mL results in an appropriate MOI for amplification.

c) Wait 3 days. During this time, prepare flasks to be ready on the third day for P3 or protein production. You should also carefully inspect aliquots of the P2 producing cells for GFP^+ cells. You should begin to see GFP^+ cells on the second day post infection and a majority (~90%) of GFP^+ cells on day 3 immediately prior to harvest.

d) Harvest your P2 virus by spinning down cells in a 50 mL tube at 3000 × g and then filtering the supernatant with a 0.2 μm low-protein-binding filter. If you plan to store the P2 for an extended period of time, you can also add up to 2% FBS and keep at 4 °C.

e) If you want to make a P3 viral stock (in order to generate large viral volumes for multiple infections and protein preps) continue to part (f), otherwise skip to step 6.

f) For P3 generation, add 500 μL P2 virus to 500 mL Sf9 cells at 2 million cells/mL.

g) Wait 3 days and harvest and store as with the P2 virus, using 1 L bottles for centrifugation.

6. 3a protein production in Sf9 cells

a) For protein production, you ideally would want to infect cells with a MOI of 2 or greater by titering your virus. However, similarly to virus production, we have found that if fluorescence of cells used to generate virus is high (~90–100%), you can use the volumes indicated here without titering with good results.

b) Add 50 mL of P2 or P3 stock to 1 L of Sf9 cells at 4 million cells/mL.

c) Harvest cells on the third day post-infection. Approximately 60 h post-infection is ideal in our hands.

d) Spin cells down in 1 L bottles at 3000 × *g*. Dispose all but 20 mL of the supernatant and use that remaining solution to resuspend and transfer pellets to a 50 mL tube.

e) Spindown again at 3000 × *g* and dispose of supernatant.

f) Place tube at −80 °C and store until needed for protein preparation.

5.3 Protein-preparation-day eve

5.3.1 Timing: 2 h

7. Getting ready for the big day

a) Prepare DDM detergent for protein purification. We make stocks of 10% detergent in 200 mM Tris pH 8 in glass vials. For purification of 3a from 1 L of cells, make up 12 mL of stock DDM solution. Weigh detergent and put into a tube (e.g., 0.6 g for 6 mL in two tubes). Add buffer on top of the detergent and mix with a gentle vortex. Add a layer of argon on top of the mixture, cap, and then sonicate in a bath sonicator until clear. See Fig. 1 for sonication setup and an example of cleared detergent. Store detergent overnight wrapped in foil at room temperature.

b) Prepare lipids for nanodisc reconstitution. We use a mixture of DOPE:POPS:POPC at a 2:1:1 weight ratio. This mixture can be made and aliquoted in advance. Add 10 mg of the lipid mixture in chloroform to a glass tube in a chemical fume hood. Place the glass tube in a small beaker with water such that the bottom of the tube with lipid will be in water (do not submerge the entire tube). Establish a gentle stream of argon through a glass Pasteur pipet at the end of tubing connected to the gas tank and slowly evaporate the chloroform while spreading the lipid out over the lower portion of the tube. When the lipid appears to be dry, turn off the stream and

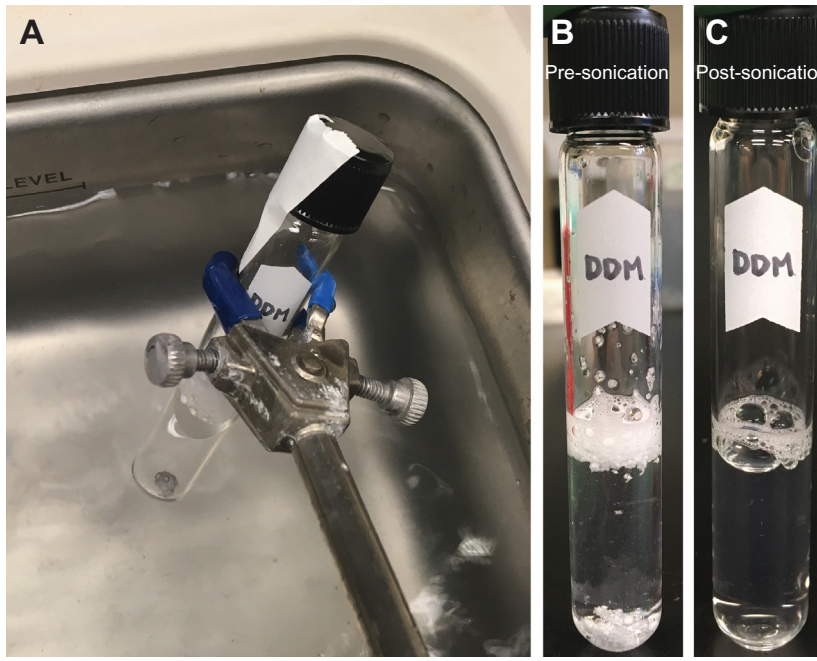

Fig. 1 (A) Set up for bath sonication. (B) Detergent solution pre- and (C) post-sonication.

gently prod the lipid to make sure there are no pockets with trapped chloroform. Next add ~1–2 mL of pentane to the dried lipid and swirl to resuspend. Gently evaporate the pentane with the same technique to generate a coating of lipid as even as possible at the bottom of the tube. See Fig. 2 for technique and dried lipid example. Store the tube, blocked from light with aluminum foil, in a vacuum desiccator overnight to fully dry the lipid of solvent.

c) Prepare biobeads for the nanodisc reconstitution. Weigh out 1–2 g of biobeads in a 50 mL tube and add 20 mL methanol. Rock for ≥15 min, dispose of methanol and then repeat this wash step two additional times. Perform the same washing steps with water and then buffer (20 mM HEPES, 150 mM KCl, 1 mM EDTA, pH 7.4). Store the washed and equilibrated beads at 4 °C in buffer.

5.4 3a protein purification

5.4.1 Timing: 1 day (~12 h)

8. Lyse cells

Fig. 2 (A) Set up for removing solvent from lipids. (B) Properly dried lipid is distributed in an even thin film at the base of the glass tube.

a) Make 100 mL lysis buffer: 50 mM Tris, 150 mM KCl, 1 mM EDTA, pH 8 with protease inhibitors at the final concentrations listed: E64 (1 μM), Pepstatin A (1 μg/mL), Soy Trypsin Inhibitor (10 μg/mL), Benzamidine (1 mM), Aprotinin (1 μg/mL), Leupeptin (1 μg/mL), AEBSF (1 mM), and PMSF (1 mM). Stock solutions of protease inhibitors can be prepared in advance and stored at −20 °C. Place buffer in a 250 mL beaker on ice with a magnetic stir bar.

d) Obtain 50 mL tube containing the frozen 3a pellet, pour a portion of the lysis buffer on top of the pellet, and place the closed tube in a 600 mL beaker containing room temperature (or slightly colder) water to thaw.

e) Every couple of minutes, rock the tube back and forth to gradually thaw cell pellet. You can also exchange buffer solution back and forth between the beaker and 50 mL tube.

f) When mostly thawed (only small frozen pieces left), pour the contents of the tube into the beaker with all of the lysis buffer and thawed cell pellet.

g) Add 4 μL Benzonase and stir to fully dissolve pellet and mix. Wait 10 min on ice or in a 4 °C room to make sure the mix is cold before proceeding to sonication.

h) Remove the stir bar with a magnet and set aside. Prepare a larger beaker with ice (you can reuse the beaker you thawed the pellet in) and place the smaller beaker containing the cell mixture in the beaker containing ice.

i) We sonicate with a Branson Sonifier 450 located in a 4 °C room. As the power of sonication can vary between models, sonifier tips, and over time, we recommend using a microscope to test for and validate complete cell lysis. Ideally, sonication will result in rupture of all cells leaving only pieces of GFP^+ membrane visible. However, you also do not want to warm the mixture too much. On our model we use the lowest power setting (~10% output) and sonicate for 1-min total with a program of 15 s on, 59 s off for 4 rounds. We recommend following a similar sonication scheme and adjusting power or total time as necessary.

9. Membrane preparation and extraction

a) Aliquot the lysis mixture evenly between ultracentrifuge tubes. If you have a Ti45 rotor, you can use two tubes for the spin. Wash the beaker and magnetic stir bar and set aside to use for extraction later.

b) Spin down the tubes at 4 °C in an ultracentrifuge at $\sim 150{,}000 \times g$ (~36,000 rpm in the Ti45 rotor) for at least 45 min. If pellets appear loosely packed against the tube bottom, you should increase centrifugation time to an hour or longer to achieve a compact pellet.

c) During the spin, prepare 100 mL of your extraction buffer: 30 mM Tris, 150 mM KCl, 1 mM EDTA, and 1% DDM (1/10 v/v from stock solution). Final Tris concentration will be 50 mM with the addition of the DDM mix. Store the buffer on ice and then add all protease inhibitors as in step (8a) just before use. Set aside the remaining DDM stock for making your nanodisc mixture later in the day.

d) Discard the supernatant containing non-membrane components and inspect the cell pellet. The pellet should have a lower layer that looks green and a thin upper layer that looks cloudy or white. The upper layer contains nucleotide that you want to remove now. Using a serological pipet and buffer (we use DPBS), rinse the pellet to remove the nucleotide layer. If the pellet is well compacted, you should be able to pipet with enough force to dislodge the upper layer without disturbing the lower membrane-containing pellet. You should see the buffer get cloudy as the nucleotide-containing contaminant layer dissolves. When you are satisfied that only the green membrane pellet is remaining, discard the buffer and proceed to the next step. **Note:** We find that the composition and ratio of the pellet changes somewhat prep-to-prep and, if you are prepping another protein out of insect cells, protein-to-protein. Even if there is not a visible nucleotide layer it is worth performing this rinse step to remove trace remaining nucleotide that would interfere with the prep. See Fig. 3 for representative images of the membrane pellet clean-up.

e) Pour a quantity of extraction buffer into a dounce homogenizer. Then scoop the membrane pellet into the dounce with a metal spatula. Use the extraction buffer and a serological pipet to transfer

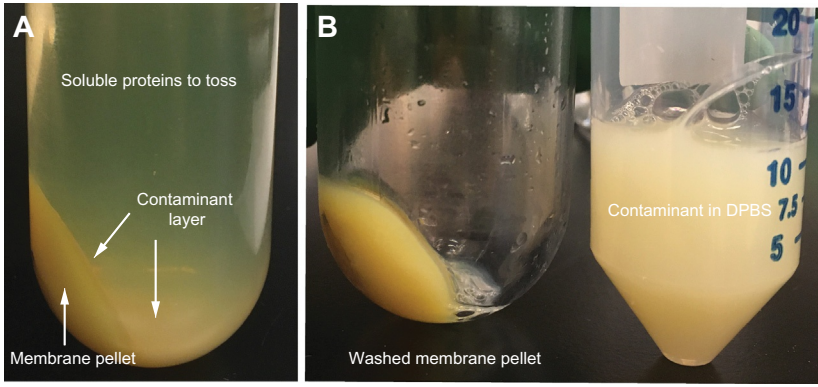

Fig. 3 (A) After ultracentrifugation, the compact membrane-containing fraction in the lower layer of the pellet can be distinguished from a loose nucleotide-containing upper layer and soluble proteins, which should be discarded. (B) An image of the washed membrane-containing fraction to be extracted with detergent (left) and the solubilized nucleotide-containing upper layer in buffer to be discarded (right).

any remaining pelleted material to the dounce homogenizer. See Fig. 4 for images of the dounce homogenization process.

f) First use a plunger with a loose fitting to break up membrane pellet portions and make an evenly dissolved mixture. Try to trap membrane pieces between the sides of the plunger and glass to accelerate the process.

g) Next use a plunger with a tight fitting and perform 10 plunges (up and down) to fully homogenize the pellet.

h) Pour the extraction mixture back into the washed beaker with the stir bar that you used during preparation of the lysate.

i) Stir at ~200 rpm for 1 h at 4 °C to extract 3a. This step can be extended, if needed, but substantial additional time (hours) may lead to more truncation products.

10. Resin binding, washing, and elution

a) Distribute the extraction mix evenly into centrifuge tubes and spin down at 33,000 × *g* for 45 min.

b) During this time, prepare your wash buffer (50 mL) and column buffer (250 mL). Column buffer: 19.5 mM HEPES, 150 mM KCl, 1 mM EDTA, 0.025% DDM (1/400 from stock solution),

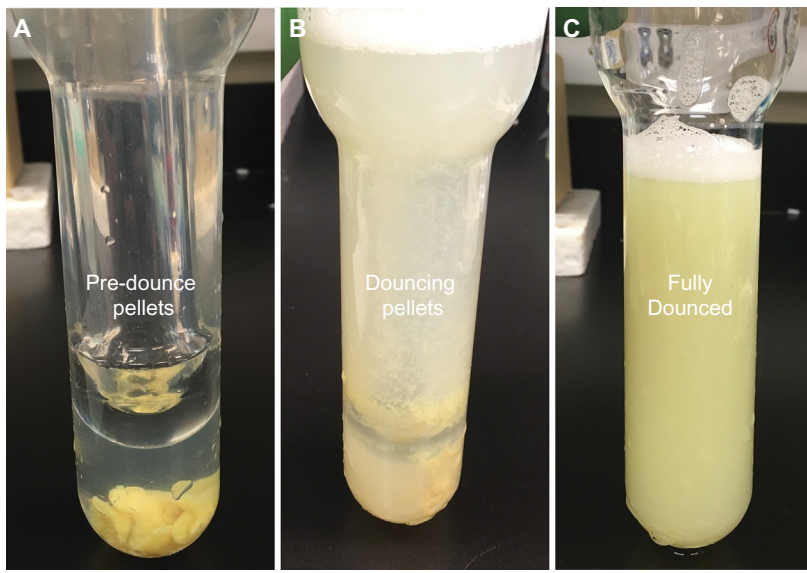

Fig. 4 (A) Pieces of the membrane pellet at the bottom of a Dounce homogenizer in detergent-containing buffer. (B) Partially homogenized membrane pellet. (C) Fully homogenized membrane pellet.

pH 7.4. Wash buffer: Same as column buffer, but with 500 mM KCl. Final HEPES concentration will be 20 mM.

c) Equilibrate your anti-GFP nanobody resin (we generally use ~4–6 mL of resin, but this can be increased as needed) in column buffer.

d) When the spin is done, transfer the supernatant together with the washed GFP nanobody resin into a beaker with a stir bar. See Fig. 5 for an example of a post-spin tube with extracted 3a-GFP protein in the supernatant.

e) Stir at ~200 rpm for 1 h at 4 °C to bind resin. This step can be extended as needed, but substantial extended time (hours) may lead to more truncation products.

f) When complete, transfer the resin to a gravity column and let the unbound solution flow through at 4 °C. You can use a small amount of column buffer to rinse the beaker and collect remaining resin. See Fig. 6A for an example of accumulated resin in our gravity flow column.

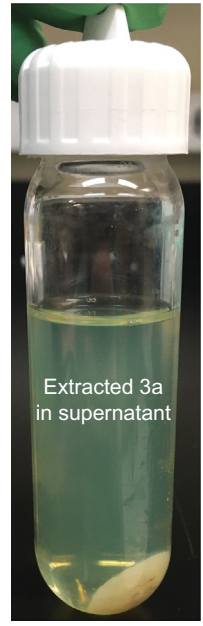

Fig. 5 Solubilized 3a-GFP in the supernatant separated from insoluble material after centrifugation.

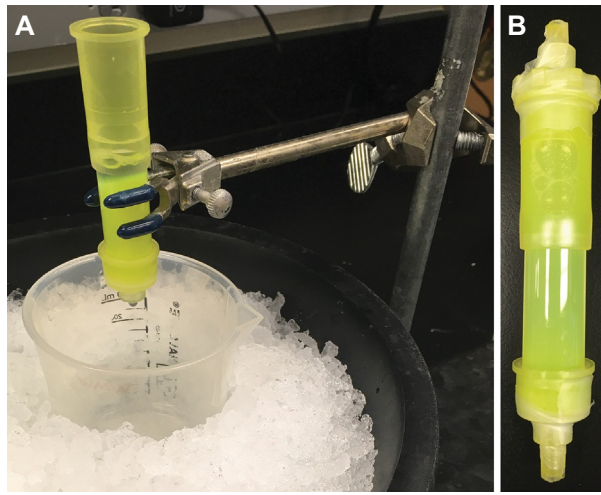

Fig. 6 (A) Set up for washing 3a-GFP bound to anti-GFP nanobody resin in a gravity flow column over an ice bath. (B) Sealed column after washing and addition of protease-containing buffer to cleave 3a from GFP.

g) When the liquid level is just above the resin, add the wash buffer one portion at a time until you have used it all. **Note:** You can test washes for the presence of protein by monitoring absorbance at 260–280 nm with a nanodrop to see if contaminant protein or nucleotide is still coming off. ~40–50 mL of wash buffer should be sufficient.

h) Rinse the resin with 2 bed volumes of column buffer.

i) Cap the column on the bottom, add ~6 mL of column buffer (or volume needed to almost fill the column), then add the HRV 3C protease (~0.5 mg or as needed). Cap the column, seal with parafilm, wrap in foil, and incubate with rocking at 4 °C for at least **2 h**. See Fig. 6B for an example of a column capped and wrapped in parafilm.

j) During this time, first set aside ~20 mL of column buffer for equilibrating your spin concentrator and for eluting protein. Then use your remaining column buffer to equilibrate your size exclusion column.

k) When the cleavage step is complete, let the elution drip into an equilibrated (with column buffer) 15 mL, 10 kDa cutoff, spin-concentrator on ice. Use the column buffer to rinse off all tube walls and collect all eluted protein into the concentrator (up to ~14 mL).

l) Spin concentrate sample in 5-min increments at 3000 × *g*. Pipet or rock the tube gently to mix and equilibrate the sample in between spins. We spin concentrate to ~450 μL in order to load into a 500 μL loop on our FPLC. Modify as necessary for your FPLC set-up.

m) When the sample is sufficiently concentrated, spindown at 21,000 × *g* at 4 °C. Move the cleared supernatant to a new tube, collect a small amount for a Coomassie gel, and proceed to load sample into an FPLC.

11. Gel filtration of 3a into detergent

a) Load 3a sample into FPLC for purification by gel filtration in a column equilibrated with column buffer (20 mM HEPES, 150 mM KCl, 0.025% DDM, 1 mM EDTA, pH 7.4) as per your setup and manufacturer's instructions. We use a Superdex 200 Increase 10/300 GL column from Cytiva, but other similar gel filtration columns would also work. **Note:** You can also begin preparing nanodisc components now, see step 11.

b) 3a preparations, in our experience, have minimal protein in the column void volume, a peak for the tetrameric component (~10–11 mL), and a peak for the dimeric component (~12–13 mL). See Fig. 7 for an example of a gel filtration trace and Coomassie-stained gel of 3a in DDM detergent.

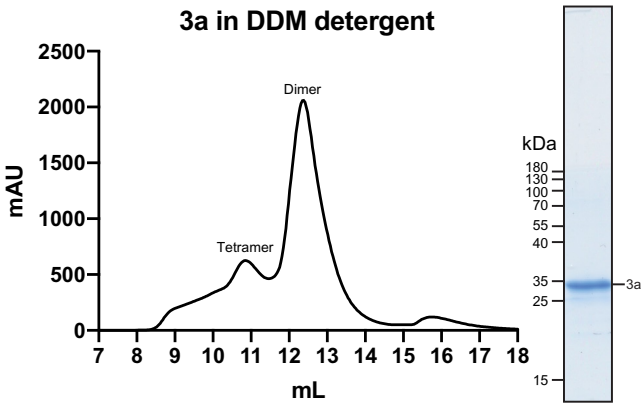

Fig. 7 Representative chromatogram from gel filtration chromatography on an S200 column of 3a in DDM detergent-containing buffer (left) and Coomassie-stained SDS-PAGE lane of the peak dimeric fraction. The position of 3a is indicated.

c) Collect the fractions corresponding to the dimer peak, and, if possible, avoid the right peak shoulder that may contain truncation products. Check protein concentration by nanodrop and, if necessary to make your nanodisc mixture, spin concentrate (10 kDa cutoff) to the appropriate concentration.

5.5 3a nanodisc incorporation

5.5.1 Timing: 2 h day 1, overnight, 2 h day 2

12. Preparation of nanodisc materials

a) Take the fully dried lipid cake in a glass tube out of the vacuum desiccator and resuspend in buffer (20 mM HEPES, 150 mM KCl, 1 mM EDTA, pH 7.4) to ~20 mM lipid (~650 μL for 10 mg lipid).

b) Layer argon on top of the lipid solution and set up your bath sonicator to maximize power. To do this, set the water level to the point with the most visible water disturbance and highest pitch and loudest sound. Then set the tube in the bath sonicator such that you can see the lipid mix moving with energy transferred from the sonicator.

c) Alternate bouts of sonication with gentle rocking or vortexing of the tube to resuspend and fully clarify the lipid mix. This typically takes 30 min to an hour in our hands. See Fig. 8 for an example of lipid mix that is resuspended (~1 min of sonication) and cleared after ~45 min of sonication.

d) Try to time clearing the lipid to the end of the gel filtration run if possible.

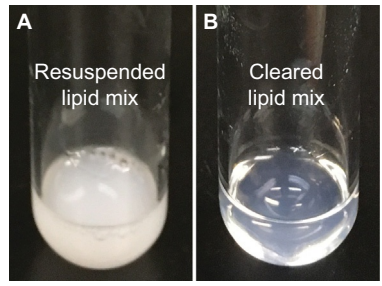

Fig. 8 (A) Resuspended lipid mixture in buffer after briefly sonicating and vortexing. (B) Fully clarified lipid mixture in buffer after sonication is complete.

e) If you prepared fully equilibrated biobeads in advance you can just set these aside, otherwise complete the final washes and equilibration now.

13. Making the nanodisc mixture

a) To make the nanodisc mixture you need purified 3a protein, clarified lipid mixture, purified MSP1E3D1 scaffold protein, buffer (20 mM HEPES, 150 mM KCl, 1 mM EDTA, pH 7.4) and some remaining 10% DDM from your stock solution. We use a nanodrop with absorbance measurement at 280 nm to determine protein concentration (Note: For 3a prepared as described, the molecular weight is 32,141 Da and the extinction coefficient is 58,330 M^{-1} cm^{-1}).

b) First, calculate all concentrations and volumes necessary for the final mix and make sure you have everything prepared.

c) For 3a (and similar dimeric proteins) and the scaffold protein MSP1E3D1 we use a final molar ratio of approximately 1 3a:4 MSP1E3D1: 400 lipid. We also add DDM at a molar ratio of ~5 detergent: 3 lipid to form the initial mixed-micelles. In this example, we will use 15 μM 3a monomer, 60 μM MSP1E3D1 monomer, 6 mM Lipid mix, and 10 mM DDM in 500 uL total volume. You may need to scale concentrations based on your yield of 3a. After disc formation, for this standard 500 μL mix with 10 mM DDM, we use 400 mg of Biobeads SM2 (damp weight) to remove detergent.

d) Mix together the lipid, DDM, 3a protein, and buffer. Incubate this mixture for 30 min (or longer) at 4 °C with gentle rocking or rotation.

e) During this incubation, thaw frozen aliquots of MSP1E3D1 for your mix on ice. We purify His-tagged MSP1E3D1 (see (Ritchie et al., 2009) and Alternative methods) and freeze it at 5 mg/mL.

f) Add MSP1E3D1 to your mixture, mix by gentle inversion or vortexing, and then incubate at 4 °C for 10 min.

g) After this incubation, add half of your total biobeads and rock or rotate in the cold room for 30 min. We weigh biobeads by putting them on a small dish and removing buffer with a P1000. We then weigh the damp beads and add them to the tube with a small metal spatula. For this example, we would add 200 mg of beads for the first addition.

h) After the first biobead addition, add the second half of biobeads. Layer a small amount of argon on top of the mix, wrap the tube in parafilm, and **incubate overnight** at 4 °C in the dark. We generally incubate **8–12 h** for this step. For some proteins, longer incubations have led to a reduction in yield.

14. Harvesting 3a in nanodiscs

a) Spin the tube down gently at 1 min for 1000 × *g* at 4 °C and recover the nanodisc solution.

b) Use a P200 to transfer the supernatant to a new tube while avoiding all biobeads. It can be helpful to first pipet out all liquid above the biobeads and then place the pipet tip at the bottom of the tube to remove the rest. It may take a few tube transfers or an additional spindown to separate out all the beads.

c) Spin the recovered nanodisc solution for 10 min at 21,000 × *g* at 4 °C to pellet insoluble material. Transfer the supernatant to a new tube and take a small sample for a gel.

15. Gel filtration of 3a in nanodiscs

a) Load 3a in MSP1E3D1 sample into the FPLC as per your setup and manufacturer's instructions. We run into 20 mM HEPES and 150 mM KCl, pH 7.4.

b) On our setup on the S200 column, 3a in MSP1E3D1 nanodiscs elutes at ~12–12.5 mL. See Fig. 9 for an example gel filtration

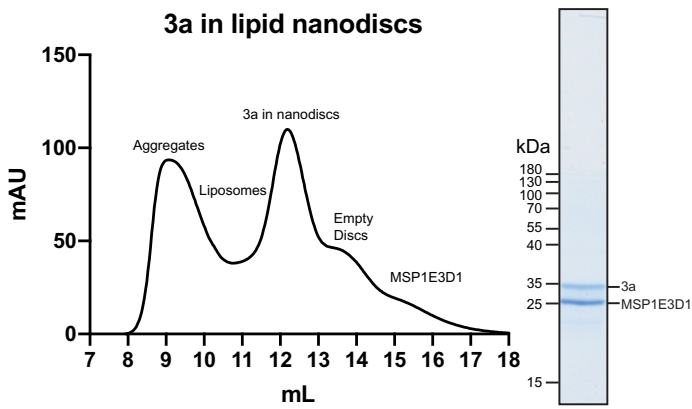

Fig. 9 Representative chromatogram from gel filtration chromatography on an S200 column of 3a in MSP1E3D1-lipid nanodiscs (left) and Coomassie-stained SDS-PAGE lane of the peak dimeric fraction. The positions of high molecular weight aggregates, liposomes, 3a-reconstitued lipid nanodiscs, lipid nanodiscs lacking 3a, and free MSP1E3D1 are indicated on the chromatogram. The positions of 3a and MSP1E3D1 are indicated on the gel.

run with labeled peak components and a Coomassie-stained gel of 3a in MSP1E3D1 nanodiscs.

c) Collect the peak fractions, collect a sample for gel, and concentrate 3a in MSP1E3D1 nanodiscs for cryo-EM structure determination or other experiments.

d) Run a gel to assess sample purity.

6. Expected outcomes

You should be able to obtain ~1–2 mg of purified 3a protein in detergent. If you use all of this protein for nanodisc incorporation you should get ~0.2–0.5 mg of pure 3a in lipid nanodiscs for cryo-EM or another usage. These methods may also be adapted for other membrane proteins.

7. Advantages

We have found that Sf9 cell expression is useful for multiple membrane proteins. If the protein expresses and folds properly in insect cells, we often see higher yield and a cleaner preparation from Sf9 than a comparable HEK293 cell system. This expression method is a fast version of a baculoviral protocol. If experiments proceed as expected you can generate substantial quantity of protein in as little as ~2–3 weeks after preparing entry vector.

The membrane prep described here allows removal of many contaminants (including cytosolic components and nucleotide) using one spin and wash step after cell lysis.

The use of a GFP-tag and anti-GFP nanobody resin allows for tracking the progress of the protein-of-interest and for excellent affinity purification, respectively.

In our hands, incorporation of 3a (or other targets) into lipid nanodiscs is advantageous for cryo-EM analysis compared to the same protein in a detergent micelle for several reasons. First, the protein may adopt conformations in a lipid environment that more closely resemble native cellular membranes. Second, the combination of removing detergent from the buffer and nanodisc incorporation can improve freezing behavior and particle distribution. Third, especially for small membrane proteins like 3a (with a dimeric molecular mass of 62 kDa), the extra mass and relatively uniform size of the nanodisc can increase the likelihood of accurate particle alignment and high-resolution reconstructions.

8. Limitations

If using this protocol for another protein, you may not be able to use Sf9 cells for successful expression. This protocol may serve as a starting point for purification and nanodisc incorporation, but it is likely that you would need to optimize the protocol (e.g., extraction buffer and reconstitution ratios) for your protein of interest.

9. Safety considerations and standards

Perform steps with chloroform and pentane carefully inside a working fume hood.

10. Alternative methods/procedures

10.1 Making MSP1E3D1 stocks

Note: We have had success following the methods chapter from the Sligar lab (Ritchie et al., 2009). This protocol can be followed verbatim or with modifications with successful results. For completeness, here we list our observations and recommendations from our experience with this preparation.

1. Expression of MSP1E3D1
   - **a)** We use LOBSTR-BL21 cells (Kerafast) (Andersen, Leksa, & Schwartz, 2013) and always pick from a freshly transformed, single colony.
   - **b)** We use shaking culture with baffled flasks for growth of bacteria and protein expression and otherwise follow the OD and timing recommendations from the Sligar Lab.
   - **c)** Purification from a 4 L growth (in Terrific Broth) typically yields ~8–15 mg of pure protein.
2. Purification of MSP1E3D1
   - **a)** We use Talon cobalt resin (Takara) and with modified wash buffers containing 5 , 10, and 30 mM imidazole respectively. Our elution buffer contains 200 mM imidazole.
   - **b)** After elution, we spin concentrate the MSP1E3D1 and run on a gel filtration column (16/60 Sephacryl S-100 HR). The majority of the protein will be MSP1E3D1, but often there will be multiple peaks and the appearance of a large right shoulder on the main peak.

c) We then run a Coomassie gel across the peak fractions and select only fractions without small-sized truncation products (i.e., smaller than the main band at ~25 kDa). Often this means discarding much of the right shoulder of the main peak.

d) We then spin concentrate the protein to 5 mg/mL, aliquot, snap-freeze in liquid nitrogen, and store at −80 °C until use.

10.2 Bacterial preparation of human rhinovirus 3C protease

Note: You can obtain a plasmid encoding this protease from Addgene and add a tag for purification. We use a standard His-tag purification, add 15% glycerol and 10 mM BME to the purified protease, snap-freeze aliquots (1–2 mg/mL) in liquid nitrogen, and store at −80 °C until use. Alternatively, PreScission protease can be purchased commercially.

10.3 Bacterial preparation of anti-GFP nanobody

Note: You can obtain a plasmid of this protein from Addgene and modify with an affinity tag of your choosing.

1. We use a standard His-tag purification with two caveats to safeguard against protein precipitation:
   a) Keep the protein in a buffer with at least 500 mM salt throughout
   b) Do not let the protein concentration exceed 10 mg/mL
2. Protein storage
   a) After purification we dialyze (10 kDa cutoff) the nanobody into Coupling Buffer: 50 mM $NaHCO_3$ pH 8.3, 500 mM NaCl
   b) We then snap-freeze aliquots in liquid nitrogen at 1 mg/mL and store at −80 °C until ready for resin coupling

10.4 Preparation of anti-GFP nanobody resin

Note: Anti-GFP nanobody resin can be obtained multiple ways. First, you can buy it pre-made from a company. Second, you can buy CNBr-activated Sepharose and couple it (following manufacturer's instructions) to prepared anti-GFP nanobody. Finally, you can buy Sepharose resin, activate it with CNBr in your laboratory, and couple it to prepared anti-GFP nanobody. This is the cheapest option, but you will need to have appropriate practices in place to handle CNBr, which is very toxic.

Note: We use ~1–1.5 mg anti-GFP nanobody per 1 mL of hydrated resin for coupling.

10.5 Storage and regeneration of anti-GFP nanobody resin

Note: We have found that resin can be washed and regenerated many times without a noticeable loss in effectiveness. Resin will, however, accumulate green color (from bound GFP protein that is not removed by low pH stripping) overtime.

1. Storage of resin
   a) After the resin is used for elution, we recommend storing used resin at 4 °C in a non-TRIS containing storage buffer (we use DPBS) with high salt (add up to 500 mM or more).
   b) If resin is to be stored for a long time, you can also add Sodium Azide to 10 mM final concentration as preservative.
2. Regenerating the resin
   a) First, accumulate used resin in a column. Use DPBS or other buffer to make sure that all resin is transferred.
   b) Add resin wash buffer: 100 mM Glycine, 1 M NaCl, **pH 2.4**. Upon contact with this buffer, the used resin will turn from green (folded GFP) to white (eluting/unfolded GFP). We use ~10 × bed volumes of wash buffer or more.
   c) Add neutralization buffer: 100 mM Tris pH 8. Run ~4 × bed volumes of this buffer. You will notice that some of the GFP that is now attached to the resin will refold and the resin will become slightly green again (it should be some amount less green than your initial used resin).
   d) You can repeat steps (b) or (c) as needed. You can check the Glycine eluate for GFP by neutralizing it with Tris. In our experience, one pass should be enough.
   e) Store resin as in step 1 for future use.

Acknowledgments

We would like to thank all the members of the Brohawn lab for help, suggestions, and troubleshooting during the development of these protocols. For help in optimizing our Dounce homogenization protocol we thank Robert Rietmeijer. For insect cell methods and troubleshooting the webpages of the Institute of Cancer, London (http://strubiol.icr.ac.uk/extra/baculovirus/) and UCB Cell Culture Facility (https://bds.berkeley.edu/facilities/cell-culture#protocols/) were helpful to us and we recommend them to others. We thank Juliane Meyer for recommending the Escort IV reagent. We would like to thank Daniel Asarnow and the lab of Yifan Cheng for early suggestions on development of nanodisc protocols. SGB is a New York Stem Cell Foundation-Robertson Neuroscience Investigator. This work was funded by the New York Stem Cell Foundation, NIGMS grant GM123496, a McKnight Foundation Scholar Award, a Sloan Research Fellowship (to SGB), a Fast Grants Award from Emergent Ventures at the Mercatus Center, George Mason University (to SGB, Hillel Adesnik, and Diana Bautista), and NIGMS grant GM128263 (to DMK).

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