

Supplementary Methods

Materials

Biological materials (complete list)

- C41 competent cells (Sigma-Aldrich, cat. no. CMC0017)
- C43 competent cells (Sigma-Aldrich, cat. no. CMC0019)
- DH10Bac cells (Thermo Fisher, cat. no. 10361012)
- DH5 α competent cells (New England Biolabs, cat. no. C2987H)
- HEK293S GnTI⁻ cells (ATCC, cat. no. CRL-3022)
- HEK293T cells (Sigma-Aldrich, cat. no. 12022001)
- pBud4.1 vector (Thermo Fisher, cat. no. V53220)
- pEG_Bacmam vector (Addgene, cat. no. 160451)
- pET28b vector (Sigma-Aldrich, cat. no. 69865)
- pPICZ vectors (Thermo Fisher, cat. no. V19020)
- SMD1163 yeast strain (Invitrogen, Genbank accession no. XM_002489831)

Reagents (complete list)

- Agar (Fisher, cat. no BP1423500)
- Ammonium sulfate (Sigma-Aldrich, cat. no A5132)
- Anti-Mouse IgG peroxidase antibody (Sigma-Aldrich, cat. no. A4416)
- Biotin (Sigma-Aldrich, cat. no. B4501)
- 5-Bromo-4-chloro-3-indolyl- β D-galactopyranoside (X-Gal; Promega, cat. no. V3941)
- Can Get Signal immunoreaction enhancer solution (Cosmo Bio, cat. no. TYB-NKB-101)
- Dithiothreitol (DTT; Sigma-Aldrich, cat. no. D9779)
- Dulbecco's modified eagle medium (DMEM; high glucose; Thermo Fisher, cat. no. 11965084)
- n-dodecyl- β -D-maltopyranoside (DDM; Anatrace, cat. no. D310S)
- DpnI restriction enzyme (New England Biolabs, cat. no. R0176L)
- ECL western blotting substrate (Thermo Fisher, cat. no. 32109)
- ER-Tracker blue-white DPX (Invitrogen, cat. no E12353)
- Ethanol (Sigma-Aldrich, cat. no. E7023)

- Ethylenediaminetetraacetic acid (EDTA; disodium salt; Sigma-Aldrich, cat. no. E5134)
- Fetal bovine serum (FBS; Thermo Fisher, cat. no. 26140079)
- FM 4-64 (Invitrogen, cat. no. T13320)
- Gel extraction kit (Qiagen, cat. no. 28706)
- Gentamicin (Sigma-Aldrich, cat. no. G1914)
- Glycerol (Sigma-Aldrich, cat. no. G7893)
- HEPES (Sigma-Aldrich, cat. no. RDD002)
- His-tag antibody (mouse monoclonal; GenScript, cat. no. A00186)
- Imidazole (Sigma-Aldrich, cat. no. 56750)
- Isopropyl β -D-1-thiogalactopyranoside (IPTG; Anatrace, cat. no. I1003)
- Luria Broth (LB; Fisher, cat. no. 214906)
- Kanamycin (Sigma-Aldrich, cat. no. K1377)
- MemMeso crystallization screen kit (Molecular Dimensions, cat. no. MD1-87)
- Methanol (Sigma-Aldrich, cat. no. 179337)
- Miniprep Kit (Qiagen, cat. no. 27104)
- Monoolein (Nu Chek, cat. no. M-239)
- MitoTracker red FM (Invitrogen, cat. no. M22425)
- N-Z-case plus (Sigma-Aldrich, cat. no. N4642)
- Opti-MEM (Thermo Fisher, cat. no. 31985070)
- 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC; 25 mg/mL in chloroform; Avanti, cat. no. 850457C),
- 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE; 25 mg/mL in chloroform; Avanti, cat. no. 850757C)
- 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG; 25 mg/mL in chloroform; Avanti, cat. no. 840457C)
- PEG 400 (Sigma-Aldrich, cat. no. 91893)
- PEI MAX (Polysciences, cat. no. 24765-100)
- Penicillin-streptomycin (Pen Strep; 100 \times ; Corning, cat. no. 30002CI)
- Phusion® high-fidelity DNA polymerase (New England Biolabs, cat. no. M0530L)
- PmeI restriction enzyme (New England Biolabs, cat. no. R0560L)

- Phosphate-buffered saline (PBS; Sigma-Aldrich, cat. no. D8537)
- Potassium hydroxide (KOH; Sigma-Aldrich, cat. no. P1767)
- Potassium phosphate dibasic (K_2HPO_4 ; Sigma-Aldrich, cat. no. P3786)
- Potassium phosphate monobasic (KH_2PO_4 ; Sigma-Aldrich, cat. no. P0662)
- Protease inhibitor cocktail (cOmplete™ EDTA-free tablets; Roche, cat. no. 11873580001)
- Sodium butyrate (Sigma-Aldrich, cat. no. 30341)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S3014)
- Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L3771)
- D-sorbitol (Sigma-Aldrich, cat. no. S6021)
- Super optimal broth with glucose (SOC; Thermo Fisher, cat. no. 15544034)
- Tetracycline (Sigma-Aldrich, cat. no. T7660)
- Tris base (Sigma-Aldrich, cat. no. T1503)
- Trypsin-EDTA (0.05%; Thermo Fisher, cat. no. 25300054)
- Tween 20 (Sigma-Aldrich, cat. no. S6501)
- Triton X-100 (Sigma-Aldrich, cat. no. X100)
- Urea (Sigma-Aldrich, cat. no. U5378)
- Yeast nitrogen base (Sigma-Aldrich, cat. no. Y1251)
- Yeast extract (Sigma-Aldrich, cat. no. 92144)
- Zeocin (100 mg/mL; Thermo Fisher, cat. no. R25005)

Equipment (complete list)

- Thermal cycler
- Water bath
- Heat block
- DNA electrophoresis system
- SDS-PAGE electrophoresis system
- Western blotting transfer system
- Shakers
- CO₂ incubators
- Tube rotator (Thermo Scientific, cat. no. 56264-306)

- Micropulser electroporator (Biorad, cat. no. 1652100)
- Electroporation cuvette (Fisher, cat. no. FB102)
- Refrigerated incubator shaker with 12 X 2 L platform (New Brunswick, Excella E-25R model)
- Cell density meter (VWR, Ultrospec 10 model)
- Automatic cell counter (Logos Biosystems, Luna model)
- Mixer mill (Retsch, MM 400 model)
- Ball mill (Retsch, PM 100 model)
- Glass beads (0.5mm; Fisher, cat. no. NC0386496)
- Sonicator (550 watts; Emerson, Branson sonifier SFX 550 model)
- Thermomixer (Eppendorf, cat. no. 535031771)
- High capacity centrifuge (Thermo Scientific, RC-12BP model)
- Tabletop centrifuge
- Tabletop ultracentrifuge (Beckman Coulter, Optima Max-TL model)
- Microfuge tube (Beckman Coulter, cat. no. 357448)
- Glass bottom culture dishes (Thermo Scientific, cat. no 150680)
- Ultracentrifuge (Beckman Coulter, Optima XPN-100 model)
- 45 Ti ultracentrifuge rotor (Beckman Coulter, cat. no. 339160)
- 45 Ti ultracentrifuge bottle (Beckman Coulter, cat. no. 355622)
- Gravity flow chromatography column (Kimble, Flex-column models)
- FSEC system (Shimadzu, LC-2030C model with RF-20AXS fluorescence detector and autosampler)
- Protein chromatography system (Cytiva, AKTA pure model)
- Superdex 200 increase column (Cytiva, cat. no. 28990944)
- Superose 6 increase column (Cytiva, cat. no. 29091596)
- Ultrafiltration protein concentrator tubes (MilliporeSigma, Amicon Ultra filters)
- NanoDrop spectrophotometer (Thermo Scientific, ND-1000 model)
- LCP equipment
 - LCP robot (Art Robbins, Gryphon model)
 - Support glasses (Soda lime rectangle 128 mm x 86mm x 1.1mm; Specialty Glass Products, custom made).

- Cover glasses (D263 rectangle 115mm x 80mm x 0.175mm; Specialty Glass Products, custom made)
- Double side tapes with 96-well internal holes (Saunders, custom made)
- LCP syringe with needle (100 μ L; Art Robbins, cat. no. 620-1005-03)
- Mixing ferrule and coupling (Art Robbins, cat. no. 620-1200-01)
- Gel loading pipet tips (200 μ L; VWR, cat. no. 37001-150)
- Confocal microscope (Zeiss, LSM 880 II Airyscan model)
- Synchrotron radiation beamline (e.g., 24-ID-C beamline, Advanced Photon Source, Argonne National Laboratory)

Reagent setup (complete list)

Biotin (500 \times)

Dissolve 50 mg biotin in 250 mL ddH₂O. Filter to sterilize. This solution can be stored at 4 °C for several weeks.

Buffer minimal glycerol (BMG) medium

Add 25 mL 50% glycerol and 50 mL 2 M potassium phosphate pH 6.0 to 825 mL dH₂O. Autoclave and cool down to room temperature (22 °C). Add 100 mL 10 \times YNB and 2 mL 500 \times biotin before use. This medium should be freshly prepared.

Buffer minimal methanol (BMM) medium

Add 100 mL 2 M potassium phosphate pH 6.0 to 800 mL dH₂O. Autoclave and cool down to room temperature. Add 100 mL 10 \times YNB, 2 mL 500 \times biotin, and 7 mL methanol before use. This medium should be freshly prepared.

! CAUTION Add methanol in chemical fume hood.

DDM (16%)

Dissolve 163 mg DDM to 900 μ L FSEC lysis buffer to make 1 mL 16% stock. Prepare fresh solution before use.

DMEM growth media with FBS

Add 50 mL FBS and 10 mL 100× Pen Strep to 1 L DMEM. This medium can be stored at 4 °C for several weeks.

DTT (1 M)

Dissolve 1.54 g DTT in ddH₂O to a final volume of 10 mL. Filter to sterilize. This solution can be stored at -20 °C for several weeks.

EDTA (500 mM)

Add 186.1 g of disodium EDTA to ddH₂O, adjust the pH to 8.0 with NaOH and filter. This solution can be stored at room temperature for several months.

FSEC lysis buffer

Add 2.5 mL 1 M Tris-HCl stock pH 8.0, 1.5 mL 5 M NaCl, and 100 µL 0.5 M EDTA to ddH₂O. Adjust the final volume to 50 mL. Add 1× protease inhibitor cocktail. Prepare fresh solution before use.

FSEC running buffer

Add 30 mL 5 M NaCl and 20 mL 1 M Tris-HCl pH 8.0 to 950 mL ddH₂O. Add 500 mg DDM, dissolve and filter. Prepare fresh solution before use.

Glucose (20% w/v)

Dissolve 100 g glucose in ddH₂O to a final volume of 500 mL. Autoclave to sterilize. This solution can be stored at 4 °C for several months.

Glycerol (50% v/v)

Weigh 588 g glycerol into a 1 L bottle. Add 400 mL dH₂O to 800 mL final volume. Autoclave to sterilize. This solution can be stored at room temperature for several months.

HEPES-NaOH (1 M)

Dissolve 238.3 g HEPES in 800 mL ddH₂O, and adjust to pH 8.0 with NaOH. Bring final volume to 1 L with dH₂O and filter. This solution can be stored at room temperature for several months.

LB with kanamycin, gentamicin, tetracycline (LB KGT) medium

Dissolve 2.5 g LB to 100 mL dH₂O. Autoclave and cool down to 55 °C. Add 100 µL 50 mg/mL kanamycin, 15 µL 47 mg/mL gentamicin and 100 µL 10 mg/mL tetracycline. Prepare fresh medium before use.

LB KGT with X-Gal and IPTG plates

Dissolve 2.5 g LB and 1.5 g agar to 100 mL dH₂O. Autoclave and cool down to 55 °C. Add 100 µL 50 mg/mL kanamycin, 15 µL 47 mg/mL gentamicin and 100 µL 10 mg/mL tetracycline. Mix and pour plates. The plates can be stored at 4 °C for several weeks.

Lipid mixture (20 mg/mL, 200×)

Mix 960 µL POPC, 320 µL POPE and 320 µL POPG (3:1:1 w/w) in glass test tube. Dry lipids under argon stream. Put in freeze dryer to remove trace amount of chloroform. Add 2 mL ddH₂O and sonicate at top power in bath sonicator until the solution turns clear. Prepare fresh solution before use.

Membrane solubilization buffer

Dissolve 1.1 g DDM to 50 mL Tris-NaCl buffer. Prepare fresh solution before use.

NaCl stock (5 M)

Dissolve 292.5 g NaCl in ddH₂O, adjust to 1 L final volume and filter. This solution can be stored at room temperature for several months.

PEI-Max transfection solution

Dilute 4 mg PEI-Max stock to 1 mL ddH₂O to make stock solution. Dilute further in dH₂O to 200 ng/mL, and sterilize the solution with syringe filter. This solution can be stored at 4 °C for several weeks.

Pichia lysis buffer (1.5×)

Add 30 mL 1 M Tris-HCl pH 8.0 and 45 mL 5 M NaCl to ddH₂O, and adjust final volume to 1 L. This solution can be stored at room temperature for several weeks.

Potassium phosphate (2 M, pH 6.0)

Dissolve 91.96 g K₂HPO₄, 472.52g KH₂PO₄ and 36 g KOH in dH₂O to a final volume of 1 L. This solution can be stored at room temperature for several months.

Sodium butyrate (500 mM)

Dissolve 2.75 g sodium butyrate in 50 mL ddH₂O. Filter and aliquot. This solution can be stored at -20°C for several months.

Sorbitol (1 M)

Dissolve 182.2 g D-sorbitol in ddH₂O. Adjust to 1 L final volume and autoclave. This solution can be stored at 4°C for several months.

Tris buffered saline (TBS, 10×)

Dissolve 24 g Tris base and 88 g NaCl to 900 mL ddH₂O. Adjust to pH 7.6 and 1 L final volume. This solution can be stored at room temperature for several months.

Tris buffered saline with 0.1% Tween 20 (TBST, 1×)

Mix 100 mL 10× TBS and 1 mL Tween 20 with 900 mL ddH₂O. This solution can be stored at room temperature for several weeks.

Tris-HCl stock (1 M)

Dissolve 121.1 g Tris base in ddH₂O, adjust to desired pH (7.5 or 8.0) with HCl and to 1 L final volume with dH₂O. After filtering (0.22 μm), this solution can be stored at room temperature for several months.

Tris-NaCl buffer

Add 30 mL 5 M NaCl and 20 mL 1 M Tris-HCl pH 7.5 or 8.0 to ddH₂O, and adjust final volume to 1 L. This solution can be stored at room temperature for several weeks.

Yeast extract peptone dextrose (YPD) medium

Add 10 g yeast extract and 20 g N-Z case plus to 900 mL dH₂O. Autoclave and store at 4 °C. Add 100 mL 20% glucose and before use. This medium should be prepared fresh.

Yeast extract peptone dextrose medium with sorbitol (YPDS) plate

Add 10 g yeast extract, 20 g N-Z case plus, 15 g agar and 182.5 g sorbitol to 900 mL dH₂O. Autoclave and cool down to approximately 55 °C. Add 100 mL 20% glucose and 4 mL 100 mg/mL zeocin. Pour 20-25 mL to each 10 cm petri dish. Allow plates to cool at room temperature overnight. Plate can be store at 4 °C for several weeks.

Yeast nitrogen base (YNB; 10×)

Dissolve 34 g yeast nitrogen base (without amino acid and ammonium sulfate) and 100 g ammonium sulfate in ddH₂O to a final volume of 1 L. Filter to sterilize. This solution can be stored at 4 °C for several weeks.

Software (complete list)

- CCP4 Program Suite:
 - PHASER (https://www.phaser.cimr.cam.ac.uk/index.php/Phaser_Crystallographic_Software)
 - REFMAC (<http://www.ytbl.york.ac.uk/refmac/docs/usage/examples.html>)
 - PARROT (<http://www.ytbl.york.ac.uk/~cowtan/parrot/cparrot.html>)
 - DM (<http://www.ytbl.york.ac.uk/~cowtan/ccp4wiki/wiki66.html>)
 - DMMULTI (<http://legacy.ccp4.ac.uk/html/dmmulti.html>)
 - NCSMASK (<http://legacy.ccp4.ac.uk/html/ncsmask.html>)
 - MAPMASK (https://saf.bio.caltech.edu/hhmi_manuals/ccp4/mapmask.html)
 - SUPERPOSE (https://www.ccp4.ac.uk/MG/ccp4mg_help/superpose.html)
 - BUCCANEER (<http://legacy.ccp4.ac.uk/html/cbuccaneer.html>)
- COOT (<https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/>)
- Graphpad Prism (<https://www.graphpad.com/scientific-software/prism/>)
- HKL2000 (<https://www.hkl-xray.com/>)
- Notepad++ (<https://notepad-plus-plus.org/>)

- PHENIX software suite (<https://www.phenix-online.org/>)
- PeakFit (<https://systatsoftware.com/products/peakfit/>)
- PolyPhobius (<https://phobius.sbc.su.se/poly.html>)
- PSIPRED and MEMSAT (<http://bioinf.cs.ucl.ac.uk/psipred/>)
- SnapGene viewer (<https://www.snapgene.com/>)
- TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>)
- XDS (<https://xds.mr.mpg.de/>)

DNA transformation in Pichia cells • TIMING 2-3 d

- 1 To generate linearized DNA, mix 20-30 µg plasmid containing coding sequence of the coupler-restrained membrane protein with 20 µL 10× smart cut buffer, 3 µL PmeI, and dH₂O to 200 µL final volume. Incubate the mixture at 37 °C for 3 h.
- 2 Add 600 µL solubilization buffer QG (provided in gel extraction kit) and 200 µL isopropanol to the mixture. Purify the linearized DNA using spin column from the kit following manufacturer's instructions. Elute DNA with 25 µL dH₂O.
- 3 To prepare Pichia competent cells, prewarm 25 mL YPD medium at 30 °C in a 250 mL flask, and inoculate 500 µL glycerol stock of SMD1163 Pichia strain. Shake at 220 rpm and 30 °C overnight.
! CAUTION Pichia cells grow slowly in YPD medium not being prewarmed.
- 4 Measure OD₆₀₀ of the culture. Aliquot cells in the amount equivalent to 10 mL of OD₆₀₀ = 10 to a new 50 mL Falcon tube. Centrifuge at 1,200g and room temperature for 5 min.
- 5 Resuspend cell pellet in 30 mL YPD medium pre-warmed at 30 °C. Add 6 mL 1 M HEPES pH 8.0 and 1 mL 1 M DTT. Mix and incubate at 30 °C for 15 min without shaking.
- 6 Centrifuge at 1,200g and 4 °C for 5 min. Remove supernatant and resuspend cell pellet with 25 mL ice-cold 1 M sorbitol. Repeat once.
- 7 Resuspend cell pellet in 900 µL ice-cold 1 M sorbitol.
! CAUTION Used fresh competent cells and prepare cells on the same day for DNA transformation.
- 8 Add 10 µL linearized plasmid (Steps 1-2) to 50 µL aliquot of Pichia competent cells (Step 7) in an Eppendorf tube placed on ice. Mix gently and incubate on ice for 5 min.

- 9 Transfer the mixture to a sterile electroporation cuvette prechilled on ice. Flick the cuvette for the liquid to go down. Select Pichia setting of Micropulser to electroporate cells.
- 10 Add 600 μ L ice-cold 1 M sorbitol. Transfer the mixture to an Eppendorf tube. Incubate at 30 °C for 1 h without shaking.
- 11 Add 500 μ L YPD media. Shake at 220 rpm and 30 °C for 1-3 h.
- 12 Dilute the cells by 100 \times with YPD. Spread 200 μ L diluted cells onto YPDS plates with 400 μ g/mL Zeocin. Incubate plates at 30 °C for 48-60 h.

Large-scale Pichia culture • TIMING 3-6 d

- 13 Inoculate 50 μ L glycerol stock to 2.5 mL YPD medium. Shake at 220 rpm and 30 °C overnight.
- 14 Add the 2.5 mL culture to 500 mL BMG medium in 2 L flask. Grow 24-36 h until OD₆₀₀ > 20.
- 15 Centrifuge the cells at 3,000g and 4 °C for 10 min.
- 16 Resuspend cell pellet in 500 mL BMM medium to induce protein expression. Shake at 220 rpm and 25 °C for 1-3 d. Supplement with 2.5 mL methanol every 24 h.
- 17 Save 1 mL culture for live cell imaging.
- 18 Centrifuge rest of the cells at 3,000g and 4 °C for 15 min. Flash freeze cell pellets in liquid nitrogen. Store cell pellet at -80 °C.

Protein purification from Pichia cells • TIMING 2 d

- 19 Resuspend 20 g of cell powder with 40 mL 1.5 \times Pichia lysis buffer (60 mL final volume). Add 1.2 g DDM (2% final concentration) to the cell resuspension. Incubate for 2-3 h with stirring. Conduct all protein purification steps at 4 °C or on ice.

! CAUTION Before weighing the detergent, prewarm the bottle (stored in -20 °C) in room temperature because most detergents are hygroscopic.

▲ CRITICAL STEP Test different detergents for optimal membrane protein purification (DDM is a common choice). To stabilize some membrane proteins, supplement 0.1 mg/mL (final concentration) lipid mixture (3:1:1 w/w POPC:POPE:POPG) in buffers used for metal affinity chromatography and size exclusion chromatography.

- 20 Transfer the cell resuspension to 70 mL Ti45 centrifuge tubes. Centrifuge in Ti45 rotor at 200,000g for 30 min in a floor ultracentrifuge.
! CAUTION Avoid using cracked or damaged centrifuge tubes. To avoid tube rupture under the high centrifugal force, fill more than 2/3 volume of polycarbonate centrifuge tubes and close lids tightly. Precisely balance the tubes before ultracentrifugation.
- 21 Pre-equilibrate Talon metal affinity resin in Tris-NaCl buffer with 0.2% DDM. Incubate the supernatant with 3 mL (bed volume) of Talon resin for 3 h with rotating.
- 22 Collect resin in gravity flow column. Wash resin with 20 mL Tris-NaCl buffer with 0.2% DDM and 5-10 mM imidazole. Repeat the wash twice.
▲ CRITICAL STEP For initial test, wash in steps with 0, 5, 10, 15, 20 mM imidazole and collect fractions. Run SDS-PAGE to identify the highest imidazole concentration that can be used for washing (i.e. no target protein eluted).
- 23 To elute target protein, add 3 mL Tris-NaCl buffer with 0.05% DDM and 250-300 mM imidazole. Repeat elution 3 times. Combine eluted fractions.
- 24 (Optional) Remove the C-terminal GFP and His tag from proteins used for crystallization. Add 1/20 (w/w) Prescission protease and incubate at 4 °C overnight. Alternatively, remove the tags by on-column digestion before elution (Step 22).
- 25 Transfer to Amicon Ultra with appropriate molecular weight cutoff. Centrifuge at 1,700g to concentrate the protein to 0.5-1 mL.
▲ CRITICAL STEP To reduce nonspecific protein binding, pass 5-10 mL of detergent-containing buffer through Amicon Ultra before use. Check and mix frequently to avoid overconcentrating the protein. Choose largest possible molecular weight cutoff (protein size + micelle size) to reduce accumulation of detergent micelles in concentrated protein.
- 26 Centrifuge filter concentrated protein (0.2 µm) and load onto 24 mL Superdex 200 column preequilibrated in Tris-NaCl buffer with 0.05% DDM. Collect 0.5 mL fractions.
- 27 Analyze the fractions on SDS-PAGE.
- 28 Combine peak fractions and concentrate to 40-45 OD₂₈₀ for LCP crystallization.

Small scale protein expression in HEK293 cells • TIMING 4 d

- 29 Grow HEK293T cells in DMEM (with FBS and Pen Strep) until cell confluency reaches 70%-80%. Use 24 well plate for live cell imaging and 10 cm plate for whole cell fractionation.

- 30 For transient transfection in 10 cm culture plates, mix 15 µg plasmid (pBud4.1 with membrane protein and coupler inserted) with 720 µL Opti-MEM. Mix 144 µL 200 ng/mL PEI-Max stock with 656 µL Opti-MEM. Combine the two mixtures and incubate 20 min at room temperature. For transfection in 24-well plate, use 0.6 µg plasmid and mix reagents at the same ratio.
- 31 Remove most DMEM medium from 10 cm plate and add 3 mL warm Opti-MEM (240 µL for 24-well plate). Transfer the plasmid-PEI mixture to plate. Incubate at 37 °C in CO₂ incubator for 4-6 h.
- 32 Remove all medium from 10 cm plate and add 10 mL DMEM with FBS and Pen Strep (1 mL for 24-well plate). Incubate 48 h in 37 °C CO₂ incubator for whole cell fractionation experiment.

Protein expression and purification with Bacmam system ● TIMING 4-6 weeks

- 33 Transfect pEG_Bacmam-derivatized vector containing coding sequence of coupler-restrained membrane protein to HEK293T cells on 6-well plate (as in Steps 30-32) for preliminary assessment of protein expression level and monodispersity by FSEC.
- 34 Generate bacmid for restrained membrane proteins with well-behaved FSEC elution profile.
 - (i) Transform 10 ng plasmid to 50 µL competent DH10Bac cells. Shake at 37°C and 220 rpm for 4-5 h.
 - (ii) Serially dilute the transformed cells by 10, 100, 1000× in SOC medium. Spread 100 µL of each dilution on LB KGT plate with X-Gal and IPTG. Incubate at 37 °C for 48 h.
 - (iii) Pick white colonies and inoculate into 5 mL LB KGT medium. Shake at 37 °C and 220 rpm overnight.
 - (iv) Isolate the bacmid by miniprep.
 - ! CAUTION Do not use the miniprep spin column. Use cold isopropanol to precipitate DNA and 70% ethanol to wash.
 - (v) PCR to verify the bacmid using a primer hybridizes with protein-coding sequence and M13-forward or reverse primer.
- 35 Grow sf9 cells.
 - (i) Prewarm Sf-900 II SFM medium in 27 °C water bath. Thaw a cryotube with 4×10⁷ sf9 cells and transfer to 40 mL medium in 50 mL Falcon tube.

- (ii) Centrifuge at 600g and room temperature for 10 min.
 - (iii) Resuspend cells in 40 mL medium and transfer to 250 mL baffled flask. Incubate at 27 °C and 120 rpm for 3-4 d.
 - (iv) Evaluate cell number and viability by automatic cell counter following manufacturer's instructions. To get an accurate measurement, shake flask vigorously by hand, immediately take a cell aliquot, and pipet mix thoroughly with trypan blue.
- ! CAUTION To maintain cell health, keep cells below 3×10^6 /mL and use prewarmed medium.
- (v) Dilute sf9 cells in fresh medium to 0.5×10^6 /mL, and grow in a new baffled flask. Repeat (generally once) to reach 90% cell viability.
 - (vi) Maintain the sf9 cells, and grow large cultures when needed.

36 Transfect bacmid to sf9 cells to produce virus.

- (i) Mix 4-8 µg bacmid with 100 µL Sf-900 II SFM medium. Mix 6 µL cellfectin II with 100 µL medium. Combine the two mixtures and pipet to mix. Incubate at room temperature for 45 min.
 - (ii) At the same time, dilute sf9 cells in fresh prewarmed medium to 9×10^5 /mL. Transfer 2 mL cells to one well of 6-well culture plate. Incubate at 27 °C for 30 min. Inspect cell attachment under microscope.
 - (iii) Remove medium in the well. Add 800 µL fresh medium to the final mixture in Step (i), and transfer to this well.
 - (iv) Incubate at 27 °C for 5 h. Replace with fresh medium and incubate at 27 °C for 5 d.
 - (v) Transfer medium to Falcon tube. Centrifuge at 600g and room temperature for 10 min to collect P1 virus. Transfer supernatant to a new tube and add FBS to 2% final concentration. Store P1 virus at 4 °C.
 - (vi) Add 500 µL P1 virus (1:100 v/v) to 50 mL sf9 cells at 2×10^6 /mL. Incubate at 27 °C for 48-72 h. Check cell morphology and viability with automatic cell counter. Swelled cells and decreasing viability indicate successful viral infection.
 - (vii) At 40-60% cell viability, centrifuge at 600g and room temperature for 10 min to collect P2 virus.
 - (viii) Repeat Steps (vi)–(vii) to generate P3 virus.
- PAUSE POINT. Baculoviruses can be stored at 4 °C for several months.

37 Grow HEK293S GnTI⁻ cells. (Optional) To save time, simultaneously grow sf9 cells and HEK293 cells.

- (i) Thaw 1 mL frozen cell stock (4×10^6 cells with 10% DMSO in 2 mL cryotube) in 37 °C water bath.
- (ii) Transfer cells to 10 mL DMEM medium with FBS in Falcon tube. Centrifuge at 170g and room temperature for 5 min.
- (iii) Resuspend cell pellet in 10 mL medium. Transfer to 10 cm culture plate and grow in 37 °C CO₂ incubator.
- (iv) Replace medium overnight and continue cell growth for 2-4 d.
- (v) At 70-80% cell confluency, pass 1/4 cells of a plate to new plate. Repeat every 2-3 d to maintain cells on culture plate.
- (vi) After at least two passages on plate, detach cells from 10 cm plate by EDTA-trypsin. Resuspend cells to 10 mL Freestyle medium with 2% FBS. Centrifuge at 170g and room temperature to collect cells.
- (vii) Resuspend cells in fresh medium and transfer to 50 mL Freestyle medium with FBS in 250 mL flask. Shake at 120 rpm in 37 °C CO₂ incubator.
! CAUTION To prevent aggregation of suspension cells, use baffled flask and keep volume of the growth medium below 1/4 of the flask size.
- (viii) Check cell number and viability with automatic cell counter. When cells reach 2×10^6 /mL, inoculate 5-10 mL cells to 50 mL medium in fresh 250 mL flask.
- (ix) Repeat Step (viii) and maintain cells below 2×10^6 /mL. Allow 1-2 weeks for cell viability to reach >90% and adaptation to suspension growth.

38 Protein expression

- (i) Add 1 mL P3 virus (Step 36) to 50 mL HEK293S GnTI⁻ cells. Shake at 120 rpm in 37 °C CO₂ incubator for 8-24 h.
- (ii) Add 1 mL 500 mM sodium butyrate. Move cells to 30 °C CO₂ incubator and shake at 120 rpm for 48-90 h.
- (iii) Centrifuge 1 mL aliquot of cells. Lyse cell pellet in Tris-NaCl buffer with 2% DDM. Determine protein expression level by FSEC.
▲ CRITICAL STEP Before large-scale cell growth, optimize protein expression level by varying following parameters: ratio of virus to cells (1:100 – 1:10), concentration of

sodium butyrate (5-20 mM), expression temperature (30 °C or 37 °C), and expression time (48-90h).

(iv) Repeat Step (i)-(iii) for large scale expression at optimal conditions in 1-10 L media.

(v) Centrifuge at 4,000g and 4 °C for 15 min to collect cells.

39 Lyse cell pellet in 2% DDM and purify protein by metal affinity chromatography and size-exclusion chromatography as in Steps 21-28.

Protein expression in *E. coli* • TIMING 3 d

40 Transform 10 ng plasmid with restrained membrane protein to 50 µL C41(DE3) or C43(DE3) competent cells.

41 Spread transformed cells onto LB plate with appropriate antibiotic.

42 After overnight growth, add 10 mL LB medium to the plate. Scrape all colonies on the plate and collect into a 50 mL Falcon tube. Vortex to resuspend cells and inoculate 6× 1 L LB medium in 2 L flasks.

▲ **CRITICAL STEP** Large volume of culture is generally required for membrane protein expression. Colonies on plate are directly transferred to the large culture, minimizing cell generations after fresh transformation and increasing protein expression level. This avoids growing a small culture in between and saves time.

43 Shake at 220 rpm and 37 °C for 3-4 h until OD₆₀₀ reaches 0.6-1. Adjust the temperature to 25 °C. Add 400 µL 1 M IPTG to each flask (final concentration 0.4 mM) to induce protein expression. Continue the cell growth overnight. Alternatively, induce at 37 °C for 6 h.

▲ **CRITICAL STEP** Optimize expression temperature, time, IPTG concentration and starting OD₆₀₀ to increase protein expression level.

44 Centrifuge at 5,000g and 4 °C for 15 min to collect the cells.

■ **PAUSE POINT** Freeze cell pellet in liquid nitrogen and store at -80°C for future use.

Protein purification from *E. coli* cells • TIMING 2 d

45 Resuspend the cell pellet in 180 mL Tris-NaCl buffer. Use a hand blender to facilitate the process.

46 Transfer the cell suspension to 250 mL beaker in ice-water bath. Sonicate at 50% power with cycles of 1 s on and 1.5 s off for 20 min total.

- 47 Centrifuge at 4,700g and 4 °C for 15 min.
- 48 Transfer supernatant to 70 mL Ti45 centrifuge tubes. Ultracentrifuge in pre-chilled Ti45 rotor at 200,000g and 4 °C for 30 min.
- 49 Use douncer to homogenize membrane pellet in 50 mL ice-cold membrane solubilization buffer. Use overhead stirrer to facilitate homogenization.
- 50 Incubate at 4 °C for 1-2 h with rotation or gentle stirring.
- 51 Ultracentrifuge in pre-chilled Ti45 rotor at 200,000g and 4 °C for 30 min.
- 52 Follow Steps 21-28 for protein purification by metal affinity chromatography and size-exclusion chromatography.

Crystallization in lipidic cubic phase • TIMING 1-2 weeks

- 53 Thaw monoolein at 40 °C in a heat block.
- 54 Use ethanol to wash two syringes, two plungers, one needle, one mixing ferrule and one coupling. Dry completely.
- 55 To setup 75 μ L LCP mixture, insert plunger into one syringe and adjust to 40 μ L. Use a long gel-loading tip to pipet 40 μ L protein.
 - ▲ **CRITICAL STEP** We generally use 2:3 v/v ratio of protein to monoolein. The 75 μ L LCP mixture is sufficient for crystallization screens with 7 \times 96-well plates. Extra volumes of protein and monoolein are pipetted to avoid air bubble formation.
- 56 Transfer protein from gel-loading tip to syringe. To avoid bubble formation, pipet out 1-2 μ L protein first to generate a small air section at the tip end. Insert gel-loading tip from open end of the syringe until close to the plunger. Gently dispense the protein solution. With more solution entering the syringe, slowly pull back the gel-loading tip while keeping the tip end in solution. Assemble ferrule on syringe and push the plunger to 30 μ L; protein solution should appear at the ferrule end.
- 57 Use a regular 200 μ L tip to pipet 60 μ L monoolein to another syringe. Slightly tilt down the front end of syringe, and dispense monoolein slowly into the rear end. After pipetting, insert a plunger into the syringe from the rear end. Hold syringe vertically, quickly push and slowly pull the plunger several times to remove air bubble. Adjust plunger to 45 μ L position.
- 58 Connect the two syringes with coupling. Mix protein and monoolein by slowly pushing the two plungers back and forth. After thorough mixing, the protein-monoolein mixture should

become homogenous and transparent, indicating successful protein reconstitution into mesophase (LCP).

- 59 Push the LCP-reconstituted protein to one syringe. Remove the other syringe, mixing ferrule and coupling. Attach and fasten needle to the syringe with reconstituted protein.
- 60 Wash 96-tip head of Gryphon LCP robot following manufacturer's instructions. Alternatively, use hand tool to set up LCP crystallization if a robot is not available.
- 61 Assemble the syringe to robot dispensing arm.
- 62 Peel off one side of sticky 96-well spacer and attach to support glass plate. Put water drops on metal support and adhere the glass plate. Peel off other side of the sticky 96-well spacer. Place the metal support with plate on designated deck of robot.
- 63 Place 96-well block containing crystallization screening solutions on designated deck of robot. We recommend commercial screens (e.g., MemMeso) or homemade screens that focus on PEG ion conditions. We generally start with PEG 400 (20%, 30% and 40%) with various salts (e.g., NaCl, $(\text{NH}_4)_2\text{SO}_4$, Li_2SO_4 , MgCl_2 and CaCl_2) at 0.1-0.4 M concentrations.
- 64 Dispense the LCP mixture and crystallization solutions using robot with the following programmed steps: load 15 μL crystallization solutions to the 96-tip head of the robot; sequentially dispense 100 nL LCP mixture onto the 96 wells of LCP plate; dispense 800 nL crystallization solutions on top of the LCP mixture drops.
- 65 Remove LCP plate from decks and seal with cover glass. Ensure all 96 wells are covered and sealed tightly. Label the plate.
- 66 Remove 96-well block of crystallization solutions from deck. Seal for storage.
- 67 Wash 96-tip head. Repeat Steps 62-66 to screen more crystallization conditions.
- 68 Remove the syringe from robot dispensing arm. Dismantle syringes, plungers, needle, ferrules and coupling. Wash with ethanol and air dry.
- 69 Wash Gryphon LCP robot following manufacturer's instructions.
- 70 Place all LCP plates in 22°C incubator and allow crystals to grow.
- 71 Inspect LCP plates every 2 days in the first week, and every week thereafter. Identify initial crystallization hits of sfGFP-restrained proteins by yellow color under light microscope and green color under fluorescence microscope.
- 72 Optimize crystallization buffer conditions. For instance, use finer PEG and ion concentration ranges and change pH.

Harvest LCP crystals • TIMING 1 d

- 73 Inspect LCP plate under light microscope. Select appropriate cryoloops according to crystal size.
- ▲ **CRITICAL STEP** Although most crystals can be harvested at room temperature, some show better diffraction with 4 °C harvesting. Harvest crystals as quickly as possible because the mesophase bolus turns turbid at 4 °C and visualization of crystals becomes difficult. Finding crystals of sfGFP-restrained membrane proteins is easier owing to their yellow color.
- 74 Use glass-cutting tool to draw circles around the well on cover glass. Remove dust of broken glass with moistened Kimwipes. Break glass to release the circular piece. Use fine point forceps to flip the piece. Locate the mesophase bolus, which is either on this cut piece and/or remains on the support glass.
- 75 Adjust microscope zoom to view LCP crystals. Harvest crystal with selected cryoloop from freshly exposed bolus. Immediately plunge the crystals into liquid nitrogen.
- ▲ **CRITICAL STEP** To avoid deterioration of crystals, harvest as quickly as possible after cover glass is broken (Steps 74-75).
- 76 Transfer the frozen crystal to cryo puck used for data collection.

Crystallographic data collection and processing • TIMING 1 d

- 77 Identify suitable synchrotron beamline designed for macromolecular crystallography. Choose beamline equipped with insertion device and microfocus beam to handle LCP crystals.
- 78 Load a crystal from cryo puck following beamline instructions. Center the cryo loop. Zoom in to center crystal (crystal of sfGFP-restrained membrane proteins show yellow or green color).
- ▲ **CRITICAL STEP** LCP crystals that are small or buried in lipid bolus are difficult to visualize and center. Use loop scan and vertical scan mode to locate the crystal (Steps 79-81).
- 79 Perform loop scan to identify a position with best diffraction.
- 80 Rotate crystal by 90° and perform vertical scan to center the diffraction position.

- 81 Take two orthogonal snapshots to confirm good diffraction in both directions.
! CAUTION Small LCP crystals require relatively stronger beam for diffraction and therefore are particularly sensitive to radiation damage. Minimize test shots to lower radiation damage.
- 82 Collection dataset. We generally collect 180-360° with 0.2-0.3° oscillation per frame.
▲ CRITICAL STEP Carefully choose beam attenuation to reduce radiation damage and maximize crystal diffraction. Use one crystal for test: collect and process data (Step 94) to determine the appropriate attenuation for other similar crystals. Judge radiation damage by significantly decreased diffraction intensity and increased B factor.
- 83 Process the diffraction data using HKL2000 or XDS.
▲ CRITICAL STEP Examine radiation damage by frames and exclude later frames with severe decay.
- 84 Assess data quality using Xtrriage program installed in PHENIX.

Crystal structure determination (complete protocol) • TIMING 2-7 d

- 85 Under the program list of CCP4 Graphical User Interface (CCP4i; version 7.1 is used here), select Scalepack2mtz. Convert the .sca file from HKL2000 to .mtz file. Enter 'Estimated number of residues in the asymmetric unit' (Step 87). Select 'Ensure unique data & add Free R column for 0.05 fraction of data'.
- ! CAUTION Change to 0.1 fraction for low resolution (3.5 Å or worse) or high symmetry data to ensure use of sufficient number of reflections (500-1500) for R_{free} calculation.
- 86 (OPTIONAL) Use 'Pointless' and 'Aimless' in CCP4i to convert XDS generated data to .mtz data format.
- 87 Under CCP4i program list, select Matthews_coef. Input the mtz file generated in Steps 85-86 and calculate solvent content. Enter the 'molecular weight in Daltons' of the coupler-restrained membrane protein. Use the value of the solvent content, which is generally between 40-80% (usually <70% for well-diffracted crystals), to estimate the number of molecules exist in the asymmetric unit.
- 88 Under CCP4i program list, select Phaser MR for molecular replacement.
- (i) Input the mtz file generated in Steps 85-86. In 'Define Ensembles', 'Ensemble #1', and PDB #1, input the .pdb file of known structure of the coupler protein (a monomer should

be used). In ‘Define composition of the asymmetry unit’, enter the molecular weight or residue number of the coupler-membrane protein fusion protein, and ‘Number in asymmetric unit’ (estimated in Step 87). In ‘Search Parameters’, perform search using [ensemble 1] and enter 1 for ‘Number of copies to search’.

▲ **CRITICAL STEP** If there are multiple copies in the asymmetric unit, we recommend searching for 1 molecule of the coupler first. Although Phaser has options to search for multiple copies, a close inspection of the molecular replacement solutions, one molecule at a time, is generally required to obtain the correct solution.

(ii) After running the Phaser MR¹, inspect the .sol file. There should be an outstanding top solution with TFZ > 10 (after Phaser refinement). We often observe TFZ of 20-30 if there is only one copy of the molecule in the asymmetric unit, but this value is expected to be lower when there are multiple copies. In addition, inspect the crystal packing under Coot because lack of significant steric clashes is a strong indicator of correct molecular replacement solution.

(iii) (Optional) If multiple copies of the molecule exist in the asymmetric unit, perform a second round of Phaser MR. The search parameters are the same as in Step (i), except that the known solution of the first copy (from Steps i-ii) need to be used. Under ‘Additional Search parameters’ and ‘Define any known partial structure’, select [Solution file] and input the .sol file from the previous search (Steps i-ii). View the .sol file and click off (start with a #) all other solutions except the top one. After running the Phaser MR, inspect the Z scores and crystal packing of the second solution (as in Step ii).

(iv) (Optional) Repeat Step iii if more molecules exist in the asymmetry unit (estimated from Step 87). This step may not be necessary when sfGFP is used as the coupler because we observe only 1 or 2 fused molecules in the asymmetry unit in all previous cases.

89 Under CCP4i program list, select Refmac5² to perform rigid body refinement of the molecular replacement solution. Select [rigid body refinement] using [no prior phase information]. Input the .mtz data file and the .pdb output from Phaser MR (Step 88), and perform 20 cycles of refinement.

90 Under CCP4i program list, select ‘Parrot’ program³ for density modification of the partial model phases. Input the .mtz file from Refmac, and select the data labels for FP, SIGFP. Click ‘Use PHI/FOM instead of HL coefficients’, and select PHWT and FOM from the

Refmac output. Change the 'Number of cycles of phase improvement to run' to 20. Input accurate value of solvent content (from Step 87) with the knowledge of exact number of molecules from Step 88. If there are multiple molecules in the asymmetric unit, select 'Get NCS from MR/partial model'.

! CAUTION If there are multiple molecules, test both with and without NCS (i.e., solvent flattening only), and compare density maps from these outputs.

- 91 In Coot, open the coordinates of the rigid body refined model (.pdb output from Step 89). Open the .mtz file from Parrot output (Step 90), and select the data labels of parrot.F_phi.F and parrot.F_phi.phi to view the density modified map from Parrot. Inspect near the fused point of the coupler, and electron densities representing helices from unknown transmembrane region should be clearly visible.
- 92 Under CCP4i program list, select BUCCANEER-autobuild/refine³ for auto model building using the PARROT map (from Step 90).
 - (i) Select 'Perform model building/refinement starting from [molecular replacement] phases, and 'Use MR model to place and name chains and' [provide initial model]. Use the rigid body refined model (Step 89) for 'the MR model PDB'. For 'Work PDB', also input this model.
 - (ii) Input the sequence of the coupler fused to the membrane protein as 'Work SEQ'.
 - (iii) Use the Parrot output for 'Work MTZ' (Step 90), and select data labels for FP and SIGFP. Use the Hendrickson Lattman coefficients (parrot_ABCD.A, parrot_ABCD.B, parrot_ABCD.C and parrot_ABCD.D) from the Parrot output .mtz.
 - (iv) Click 'Use Free-R flag' and 'Apply anisotropy correction to input data'.
 - (v) Use multiple CPUs to expedite the calculation.
- 93 Confirm and rearrange protein chains.
 - (i) Inspect the output pdb from BUCCANEER (Step 92) in Coot. Judge whether the built segments fit properly into the PARROT density map (Step 90). Trace the segments to the coupler.
 - (ii) In a text editor (we recommend Notepad++ because it allows column selection), rename the chain ID of well fitted segments to the same chain ID as the coupler molecule that they connect to. Rearrange the order of all the segments in the .pdb file according to the fused protein sequence.

- (iii) In Coot, renumber residues according to the sequence. The pdb should now be organized to best represent the knowledge of the fused molecules, i.e., which segments of the membrane protein are connected and how they connect to each of the coupler molecule (gaps allowed).
- 94 Reiterate the model autobuilding process.
- (i) In BUCCANEER, change 'Work PDB' to the pdb generated from Step 93. Rerun BUCCANEER.
- (ii) Inspect the output pdb as in Step 93.
- (iii) Repeat the BUCCANEER autobuilding until there is no further improvement toward a more complete model (2-3 more rounds is generally sufficient). Combine all the segments as in Step 93. For a reasonably good dataset (2-3.5 Å without notable defect such as severe anisotropy), the autobuilding generally can generate 80-95% of correctly modeled membrane protein structure⁴.
- 95 Use the model from Step 94 for additional density modification to improve the overall phases and the density map (map from Step 90 may have missing or poor densities). Run rigid body refinement of the new model and perform density modification by PARROT (as in Steps 89-90).
- ▲ **CRITICAL STEP** The density maps (2FoFc and FoFc) from the rigid body refinement should be inspected and compared with the density modified maps. The rigid body maps are 'original'. With the nearly complete model, the partial model phases from rigid body refinement are often sufficient for resolving certain ambiguities.
- 96 Generate averaging masks for further density modification.
- (i) If there is more than one molecule in the asymmetric unit, use Coot to read in the model from Step 95 and select out one of the molecules for averaging mask generation. Alternatively, the coupler and membrane regions in this molecule can be used separately to generate two averaging masks that allow multi-domain averaging in DM (Step 99).
- (ii) Under CCP4i program list, select [create mask from coordinates] and input one of the models. Set map grid x, y, z to numbers (multiplies of 4 are recommended) close to the unit cell dimensions. Select 'Cleanup mask to have 1 continuous region(s)', 'Remove features less than 10 grid points', and 'Trim mask to minimum box'.
- 97 (Optional) Generate solvent masks for further density modification.

- (i) Use all molecules in the asymmetric unit and use both the coupler and membrane protein regions, and follow Step 96 to generate an NCS mask first.
 - (ii) Under CCP4i program list, select the 'Mapmask' program. Select [Extend a map/mask file] for a [mask] file, and input the NCS mask. Select 'Extend map to [cover all atoms in molecule]', and input the pdb of all molecules in the asymmetric unit. Select 'Extend map by [overlapping copy and symmetry] and pad with [0.0] where density unknown'.
 - (iii)(Optional) To increase the solvent mask size to cover a certain region, create a 'dummy' pdb with residues roughly placed in this region. Generate mask as in Steps (i)-(ii).
- 98 If there are more than one molecule in the asymmetric unit, generate NCS matrix for each additional molecule.
- (i) Under CCP4i program list, select the 'Superpose' program. Input the molecule with averaging mask as 'Moving' and another molecule in the asymmetric unit as 'Fixed'. Select the residue range (generally the entire molecule is used) and chain ID of each molecule to superpose. Run the Superpose program for each additional molecule.
 - ▲ **CRITICAL STEP** By definition of NCS averaging, the molecule for which the averaging mask is created is rotated onto each of other molecules in the asymmetric unit.
 - (ii) Inspect the Superpose logfile. The 'RMS XYZ DISPLACEMENT' should be low (generally $< 2 \text{ \AA}$). Use 'TRANSLATION VECTOR' (in \AA ; not fractional) and 'CROWTHER ALPHA BETA GAMMA' (Euler angles) input parameters for DM (Step 99) and DMMULT (Step 102).
- 99 Use DM⁵ for fine tuning of density modification. Under program list of CCP4i, select DM.
- (i) Under 'Select density modification modes', select 'Solvent' and 'Histogram'. Select 'Averaging' if multiple copies are present in the asymmetric unit.
 - (ii) Select 'Phase combination scheme [perturbation] using [automatic phase extension]'. Alternatively, select [all reflections] or [phase extension in resolution steps]. For phase extension in resolution steps, set 'Fixed number of cycles of phase extension' to 100 cycles, and set 'Resolution in starting set in phase extension' to $3.5 - 5 \text{ \AA}$ ($\sim 1-2 \text{ \AA}$ below the actual resolution).
 - (iii) Input the 'fraction solvent content' (from Step 87).
 - (iv) Input the .mtz from rigid body refinement of the new model (Step 95), and choose data labels FP, SIGFP, PHWT, and FOM.

- (v) In 'Define NCS Symmetry', select 'Define NCS operators in terms of [ruler angles]. Input the averaging mask file from Step 96. Input the rotational and translational matrix for each molecule; enter numbers of 0 for the molecule with averaging mask, and enter the numbers from Step 97 for additional molecules.
 - (vi)(Optional) Multidomain averaging can be applied with the coupler as one domain and the fused membrane protein as the other domain, addressing their relative movement in each NCS molecule. Use the preliminary model of the membrane protein molecules (from Step 95) to generate the NCS matrix for each molecule (as in 98).
 - (vii)(Optional) Input a solvent mask from Step 97; create a manually built solvent mask can be helpful if the automatic mask generated by density modification programs (DM, DMMULTI, and PARROT) results in artificial removal of the electron density at a certain region.
 - ▲ **CRITICAL STEP** Change solvent content to a lower value often can recover artificially pruned densities. Compare the DM maps calculated with different solvent contents specified (5% steps are generally sufficient for initial evaluation).
 - (viii) Compare DM maps generated with different phase extension schemes and parameters.
! **CAUTION** In the DM log file, inspect the correlation of NCS averaging during the first cycle. Low correlation (<0.1) indicates mistake that is often due to the use of a wrong NCS matrix.
- 100 Inspect the DM map in Coot and compare with Parrot maps from Step 95 and 90. If the map is improved, repeat the BUCCANEER autobuild (Step 94) with the PHIDM and FOMDM phases. Alternatively, missing part the model can be manually built to fit the densities of DM map.
- 101 Repeat Steps 96-100 until no further improvement in model building or density map.
- 102 (Optional) For low-resolution or low-quality data, improve density maps by multi crystal averaging.
- (i) Download the existing model and diffraction data of the coupler protein from PDB.
 - (ii) Create an averaging mask on one molecule of the coupler (as in Step 96).
 - (iii)(Optional) Create an averaging mask on one molecule of the preliminary model of the membrane protein.

(iv) With one molecule of the coupler as the ‘moving’ one (and that of the membrane protein), generate NCS matrix (as in Step 98) for each molecule in the two structural models: coupler alone and coupler fused with the membrane protein.

(v) Generate the model phases for the coupler alone crystal using REFMAC rigid body refinement (as in Step 89), with the downloaded model and data of the coupler as inputs.

(vi) Under CCP4i program list, select the top tab ‘Density Improvement’ and ‘Run DmMulti’.

(vii) Select a phase extension scheme (as in Step 99-ii).

(viii) In ‘Crystal number 1’, input the .mtz generated from rigid body refinement of the coupler crystal, using data labels FP, SIGFP, PHWT and FOM. In ‘Crystal number 2’, input the .mtz generated from rigid body refinement of the molecular replacement model of the coupler against the data of coupler fused with membrane protein (from Step 89), or the .mtz generated from the rigid body refinement of the model with membrane protein portion partially built (Step 95).

(ix) In ‘Describe domains’, enter the averaging masks.

(x) In ‘Describe Crystals’ and ‘Describe Crystal 1’ enter the solvent content (calculated as in Step 87) and the NCS matrix of the coupler alone crystal (enter 0 numbers if there is only one molecule). In ‘Describe Crystal 2’, enter those for the crystal of the coupler fused with membrane protein.

!CAUTION Inspect the NCS correlation (as Step 99) for each domain in each crystal.

(xi) Compare DMMULTI maps generated with different phase extension schemes and parameters.

103 Manually build the model to completeness using COOT.

104 Refine the model by the phenix.refine program in the PHENIX software suite⁶.

105 Use the 2FoFc and FoFc maps generated from PHENIX to correct the model or build missing part. Repeat Steps 103-105 until R_{free} , Ramachandran, and other validation requirements are satisfactorily met.

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Supplementary Table 1. List of couplers, insertion sites and membrane proteins, whose termini restrained constructs were successfully expressed. In several cases, the crystal structures were also determined.

Name	Coupler			Membrane protein	Expression system	Crystal structure
	PDB	Resolution	Insertion site			
sfGFP	2B3P	1.4 Å	N144-N146	VKOR	<i>Pichia</i>	2.0 Å
				VKORL	<i>Pichia</i>	2.4 Å
				DsbB	<i>E. coli</i>	2.9 Å
				JAGN1	<i>Pichia</i>	2.25 Å
				CD53	<i>Pichia</i>	2.9 Å
				SPCS1	<i>Pichia</i>	2.45 Å
				Mfrn1	<i>Pichia</i>	N/A
				Cldn4	<i>Pichia</i>	N/A
β-galactosidase	4CU6	2.7 Å	H326-G327	CybB	<i>E. coli</i>	N/A
				LspA	<i>E. coli</i>	N/A
				JAGN1	<i>Pichia</i>	N/A
			G570-D571	CybB	<i>E. coli</i>	N/A
			G608-G609	CybB	<i>E. coli</i>	N/A
Vinculin	1TR2	2.9 Å	P99-Y100	CybB	<i>E. coli</i>	N/A
				LspA	<i>E. coli</i>	N/A
			K219-N220	CybB	<i>E. coli</i>	N/A
				LspA	<i>E. coli</i>	N/A
Thioredoxin	4OO4	0.97 Å	D20-K21	JAGN1	<i>Pichia</i>	N/A
				apVKOR	<i>E. coli</i>	N/A
GatE	1ZQ1	3.0 Å	K271	DsbB	<i>E. coli</i>	N/A
			R275	DsbB	<i>E. coli</i>	N/A
			L371	DsbB	<i>E. coli</i>	N/A
minichromosome maintenance protein N-terminal domain	5IY0	3.0 Å	L98-E100	VKORL	<i>Pichia</i>	N/A
Maltodextrin binding protein	1ANF	1.67	R316-A319	JAGN1	<i>Pichia</i>	N/A