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Expression and Purification of the Human Lipid-sensitive Cation Channel TRPC3 for Structural Determination by Single-particle Cryo-electron Microscopy

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Dear Editors,

I am writing to resubmit the edited manuscript entitled “Expression and Purification of the Human Lipid-sensitive Cation Channel TRPC3 for Structural Determination by Single-particle Cryo-electron Microscopy” by Emery Haley, Wooyoung Choi, Chen Fan, Weinan Sun, Juan Du and Wei Lü for publication at *JoVE*.

We have revised the manuscript in accord with reviewers’ comments and improved the quality of the manuscript significantly.

Please contact me if you have any questions,

Thank you for your time and assistance with this manuscript and we look forward to working with you on the video production.

Sincerely,

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TITLE:

Expression and Purification of the Human Lipid-sensitive Cation Channel TRPC3 for Structural Determination by Single-particle Cryo-electron Microscopy

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KEYWORDS:

Ion channel, membrane protein, protein purification, structure determination, cryo-electron microscopy, cryo-EM, baculovirus

SUMMARY:

This protocol describes techniques used to determine ion channel structures by cryo-electron microscopy, including a baculovirus system used to efficiently express genes in mammalian cells with minimum effort and toxicity, protein extraction, purification, and quality checking, sample grid preparation and screening, as well as data collection and processing.

ABSTRACT:

Transient receptor potential channels (TRPCs) of the canonical TRP subfamily are nonselective cation channels that play an essential role in calcium homeostasis, particularly store-operated calcium entry, which is critical to maintaining proper function of synaptic vesicle release and intracellular signaling pathways. Accordingly, TRPC channels have been implicated in a variety of human diseases including cardiovascular disorders such as cardiac hypertrophy, neurodegenerative disorders such as Parkinson's disease, and neurologic disorders such as spinocerebellar ataxia. Therefore, TRPC channels represent a potential pharmacologic target in human diseases. However, the molecular mechanisms of gating in these channels are still unclear. The difficulty in obtaining large quantities of stable, homogeneous, and purified protein has been a limiting factor in structure determination studies, particularly for mammalian

membrane proteins such as the TRPC ion channels. Here, we present a protocol for the large-scale expression of mammalian ion channel membrane proteins using a modified baculovirus gene transfer system and the purification of these proteins by affinity and size-exclusion chromatography. We further present a protocol to collect single-particle cryo-electron microscopy images from purified protein and to use these images to determine the protein structure. Structure determination is a powerful method for understanding the mechanisms of gating and function in ion channels.

INTRODUCTION:

Calcium is involved in most cellular processes including signaling cascades, transcription control, neurotransmitter release, and hormone molecule synthesis[1-3]. The homeostatic maintenance of cytosolic free calcium is crucial to the health and function of cells. One of the major mechanisms of intracellular calcium homeostasis is store-operated calcium entry (SOCE), a process in which depletion of calcium stored in the endoplasmic reticulum (ER) triggers the opening of ion channels on the plasma membrane to facilitate the replenishment of ER calcium, which can then be used in further signaling[4-6]. Transient receptor potential channels (TRPCs), which are calcium-permeable channels belonging to the TRP superfamily, have been identified as a major participant in SOCE[7-9] .

Among the seven members in the TRPC family, TRPC3, TRPC6, and TRPC7 form a homologue subgroup, and they are unique in the ability to be activated by the lipid secondary messenger diacylglycerol (DAG), a degradation product of the signaling lipid phosphatidylinositol 4,5-bisphosphate (PIP2)[10,11]. TRPC3 is highly expressed in smooth muscle and in the cerebral and cerebellar regions of the brain, where it plays essential roles in calcium signaling that impacts neurotransmission and neurogenesis[12,13]. Dysfunction of TRPC3 has been linked to central nervous system disorders, cardiovascular disorders, and certain cancers such as ovarian adenocarcinoma[14-16]. Therefore, TRPC3 holds promise as a pharmaceutical target for treatment of these diseases. The development of specifically targeted drugs acting on TRPC3 has been limited by a lack of understanding of its molecular activation mechanisms, including lipid binding sites[17,18]. We have reported the first atomic-resolution structure of the human TRPC3 channel (hTRPC3) and its two lipid binding sites in a closed state, providing important insights into these mechanisms[19].

The key factor for determining the structure of a membrane protein at high resolution is to obtain protein of high quality. The corresponding screening of expression and purification conditions necessary to obtain high quality protein can be a time-consuming and costly endeavor. Here we present a protocol describing in detail how we identify the optimal conditions for the expression and purification of hTRPC3, which behaved poorly in our initial screening. We present several key points on how to troubleshoot and optimize the protein behavior, which lay a solid foundation for our cryo-electron microscopy (cryo-EM) studies. We use a modified baculoviral generating vector (pEG), developed by Gouaux and colleagues, which is optimized for screening assays and efficient generation of baculovirus in mammalian cells[20]. This expression method is appropriate for rapid and cost-effective overexpression of proteins in the mammalian cell membrane. We combine the use of this vector with a fluorescence-detection size-exclusion chromatography-based (FSEC) prescreening method[21]. This method uses a green fluorescent protein (GFP) tag fused to the construct of interest and improves visualization of the target protein in small, whole-cell solubilized samples. This allows for screening of protein stability in the presence of different detergents and additives and, with thermostabilizing mutations, allows the use of a small number of cells from small-scale transient transfection. In this way, a multitude of conditions can be rapidly screened before moving to a large-scale protein purification. Following expression, screening, and purification, we present a protocol for obtaining and processing images from cryo-EM to generate a *de novo* structural determination of the protein. We believe that the approaches described here will serve as a generalizable protocol for structural studies of TRP channel receptors and other membrane proteins.

PROTOCOL:

1. Transformation of DH10α Competent Cells to Produce Bacmid DNA

1.1 Synthesize the gene of interest and subclone it into a modified version of the pEG vector containing a twin strep-tag, a His8-tag, and GFP with a thrombin cleavage site at the N terminus (pFastBacI)[20].

1.2 Transform competent cells by adding 5 ng of plasmid containing a desired gene in pFastBacI to 50 µL of DH10α cells in a 1.5 mL tube and incubate for 10 min on ice. Heat shock the cells for 45 s at 42 °C. Add 200 µL of super optimal broth with catabolic repressor (SOC) medium to the tube and incubate for 4 - 8 h at 37 °C in an orbital shaker at 225 rpm.

1.3 Plate 5 µL of cells on a bacmid LB agar plate (50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL isopropyl β-D-1-thiogalactopyranoside [IPTG], agar).

1.4 Incubate the plate for 48 h at 37 °C.

NOTE: The Bluo-gal indicator stains colonies that are still expressing lacZ (vector insertion unsuccessful), allowing for selection of white (successfully transformed) colonies.

1.5 Carefully select an isolated white colony, avoiding any white colonies that are in contact with blue colonies, and grow cells overnight in 6 mL of acmid LB medium (50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline) at 37 °C in an orbital shaker at 225 rpm.

2. Bacterial Preparation for Isolation of Bacmid DNA

2.1 To isolate bacmid DNA, spin down *Escherichia coli* cells for 10 min at 2880 x *g*.

2.2 Discard the supernatant and resuspend the pellet in 200 µL of cell resuspension solution from the miniprep kit (see **Table of Materials**) by pipetting. Be sure the pellet is fully and homogenously suspended. Then, transfer the cell suspension into 1.5 mL tubes.

2.3 Add 200 µL of the cell lysis solution from the miniprep kit and mix by inverting the tube a few times. Incubate for up to 5 min at room temperature (RT) to lyse the cells. Add 200 µL of neutralization solution from the miniprep kit and mix by inverting tube a few times to stop the lysis reaction.

2.4 Spin down for 10 min at 21,130 x *g* in a table-top centrifuge. Collect 600 µL of supernatant in a 2 mL tube. Add 600 µL phenol:chloroform:isoamyl alcohol solution (see **Table of Materials**) and mix thoroughly to extract the DNA from the remainder of the cell lysis products.

CAUTION: Phenol:chloroform:isoamyl alcohol solution is toxic by inhalation, in contact with skin, and if swallowed. It can cause chemical burns and may be carcinogenic. Wear gloves and a buttoned lab coat. Work in a fume hood. Dispose of this hazardous waste appropriately.

2.5 Spin the tube for 10 min at 21130 x *g* in a table-top centrifuge. Two separate liquid phases will be visible. Carefully transfer 300 µL of the upper aqueous phase to a new tube. Add 600 µL of 100% ethanol to wash the DNA. Gently invert the tube to mix.

NOTE: Do not vortex, as this can shear bacmid DNA.

2.6 Cool tubes by placing them in a -20 °C freezer for 10 min. Spin down for 10 min at 21,130 x *g* in a table-top centrifuge. Discard the supernatant and preserve the DNA pellet.

2.7 Add 1 mL of 70% ethanol to wash the pellet. Gently invert the tube to mix. Spin for 10 min at 21,130 x *g* in a table top centrifuge. Discard the supernatant and allow the pellet to air dry for approximately 5 min or until no liquid is visible in the tube and the DNA pellet becomes translucent.

2.8 Resuspend the dry pellet in 50 µL of sterile, DNase-free, deionized water. Measure the DNA concentration.

NOTE: Do not freeze bacmid DNA. Store at 4 °C for up to several days.

3. Transfection of Sf9 Insect Cells with Bacmid to Produce P1 Baculovirus

3.1 Seed 0.9 x 10^6 Sf9 cells/well in 2 mL of appropriate medium (see **Table of Materials**) in each well of a 6-well tissue culture plate. Incubate cells at 27 °C for 20 min to promote attachment to the plate.

CAUTION: Cell cultures are a potential biohazard. Work in an approved laminar flow hood using aseptic techniques and check institutional and governmental guidelines for recommended protective clothing and proper disposal of waste prior to performing experiments.

3.2 After attachment, add 8 µL of transfection reagent (see **Table of Materials**) to 100 µL of media for each well of the 6 well plate being transfected in a sterile tube. Add 6 µg of bacmid DNA to 100 µL of medium in a separate sterile tube. Incubate 5 min at RT. Combine the two solutions and incubate for 45 min at RT.

3.3 Replace the medium in the 6 wells with 2 mL of fresh medium. Add the mixture from previous step to each well dropwise (200 µL per well). Gently rock the plate to ensure mixing of the transfection solution into the medium.

NOTE: Do not swirl or shake the plate because this will cause cells to detach.

3.4 Incubate cells for 5 d (120 h) in a 27 °C humidified incubator. Check GFP fluorescence before harvesting to verify that virus is being produced in a large percentage of cells; if the percentage is low, extend the incubation time as necessary (see **Figure 1C**).

3.5 Collect the supernatant containing P1 virus (about 2 mL from each well). Filter the medium containing P1 virus into 2-mL tubes using a 3 mL syringe and small 0.2 µm filter. Add sterile fetal bovine serum (FBS) to a final concentration of 1%.

NOTE: This stock of P1 virus should be stored at 4 °C and be protected from light.

4. Infection of Sf9 Insect Cells with P1 Baculovirus to Produce P2 Baculovirus

4.1 Prepare 200 mL (or desired volume) of Sf9 cells at a concentration of 0.8 - 0.9 x 10^6 cells/mL in appropriate medium (see **Table of Materials**) in a flat bottom Erlenmeyer culture flask of sufficient size.

NOTE: For suspension culture, the volume used should not exceed 40% of the total capacity of the flask.

4.2 Add 1:2500 ratio (v/v) of P1 virus stock from 3.5 to the Sf9 cell suspension culture. Incubate for the time of optimal virus expression (usually 48 - 120 h depending on the protein construct) at 27 °C in an orbital shaker at 115 rpm.

NOTE: The relative virus expression can be determined by viewing the GFP fluorescence of the virus in a sample of the culture.

4.3 Centrifuge the cell suspension for 40 min at 11,520 x *g* and collect the supernatant containing P2 virus. Filter the supernatant using disposable 0.2 µm filters. Add FBS to a final concentration of 0.5%.

NOTE: This stock of P2 virus should be stored at 4 °C and be protected from light.

4.4 Obtain a titer for the P2 virus using Sf9easy cells or a virus counter.

5. Infection of HEK293 Mammalian Cells with P2 Baculovirus for Large-scale Protein Expression

5.1 Prepare a desirable volume of HEK293 mammalian cell suspension culture (4 - 6 L is recommended for preparation of frozen grids) at a concentration of 3.5 - 3.8 x 10^6 cells/mL in the expression medium (see **Table of Materials**) supplemented with 1% (v/v) sterile FBS in baffled-bottom Erlenmeyer culture flasks of sufficient size.

NOTE: For suspension culture, the volume used should not exceed 40% of the total volume of the flask.

5.2 Add 8% (v/v) of P2 virus stock solution from step 4.3 to the HEK293 cell suspension culture. Incubate at 37 °C in an orbital shaker at 135 rpm.

5.3 Add 10 mM sodium butyrate at 12 - 18 h post-infection. Incubate for the time of optimal protein expression (usually 36 - 72 h) at 30 °C.

5.4 Harvest cells by centrifuging for 20 min at 2,880 x *g*. Wash cells by resuspending in approximately 100 mL tris-buffered saline (TBS) per liter of cells harvested. Centrifuge again for 20 min at 2,880 x *g* and collect the cell pellet.

NOTE: The protocol may be paused here. Cell pellets can be snap-frozen in liquid nitrogen and stored at -80 °C until purification.

CAUTION: Liquid nitrogen may cause cryogenic burns or injury. It may cause frostbite. It may displace oxygen and cause rapid suffocation. Wear cold insulating gloves and face shield.

5.5 Collect small 1 mL harvests at varying time points and solubilize for 2 h at 4 °C with rocking or stirring in the presence of different detergents and/or additives. These small whole-cell solubilized samples can be clarified by ultracentrifugation at 235,000 × *g* for 10 min at 4 °C and run as a 30 µL sample on a size-exclusion chromatography (SEC) column (see **Table of Materials**) to determine the best time for expression and the best solubilization conditions.

NOTE: In the case of hTRPC3, this screening included different buffers with pH values from 4.0 - 9.5 and salt concentrations of 50 - 500 mM; different ionic compositions (such as $MgCl_2$ or NaCl); different detergents with critical micelle concentration (CMC) values of 0.1 - 20 mM; reducing additives such as dithiothreitol, tris(2-carboxyethyl)phosphine, and β-mercaptoethanol; and the calcium-chelating additive ethylenediaminetetraacetic acid (EDTA).

6. Purification of Htrpc3 Protein from the Frozen Cell Pellet

6.1 Thaw the pellet in buffer containing 20 mM Tris (pH 8.0), 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.8 µM aprotinin, 2 µg/mL leupeptin, 2 mM pepstatin A, and 1% digitonin, using 100 mL of buffer per liter of cells harvested. Once thawed, ensure homogeneity of the solution by pipetting or stirring. Allow to solubilize for 2 h at 4 °C in a beaker immersed in ice with a stir bar rotating.

6.2 Remove cell debris by ultracentrifugation at 235,000 × *g* for 1 h at 4 °C. Verify protein quantity by running a 30 µL sample on an SEC column (see **Table of Materials**) by high performance liquid chromatography (HPLC) and visualize the target protein by the GFP signal output.

6.3 Incubate the solubilized protein (supernatant) with cobalt affinity resin for 1 - 2 h at 4 °C. Verify protein binding to the resin by running a 30 µL sample on an SEC column.

NOTE: If protein binding has occurred, the GFP tagged protein target will be retained on the column, not found in the flow-through. Therefore, no GFP signal will be present at the position corresponding to the target protein size when the flow-through is run on HPLC.

6.4 Wash the resin with 10 column volumes of buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 15 mM imidazole, and 0.1% digitonin). Check for protein loss by running a 30 µL sample on an SEC column.

NOTE: If protein loss from the column has occurred, the GFP tagged protein target will be found in the wash buffer that has passed over the column. Therefore, GFP signal will be present at the position corresponding to the target protein size when the wash buffer is run on HPLC. If protein loss has occurred, the imidazole concentration of the wash buffer may need to be lowered to prevent disrupting His tag binding to the affinity column.

6.5 Elute the resin-bound hTRPC3 with buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 250 mM imidazole, and 0.1% digitonin). Add thrombin at a 1:20 molar ratio (to cleave the GFP tag) and add 10 mM EDTA (an hTRPC3 stabilizing agent) to the eluted sample and incubate for 3 h at 4 °C. Check that protein has been eluted by running 90 µL of a sample diluted 1:100 on an SEC column and verifying the presence of a GFP signal at the position corresponding to the target protein size.

NOTE: At this point, the tryptophan signal from total protein in the elution can also be viewed. Only the target affinity-purified protein will remain in the elution and the GFP and tryptophan signals will be near identical in profile. If the target protein is not seen in large quantity in the elution but was not lost in the flow-through or wash, the protein has likely remained bound to the column and can be eluted using a higher concentration of imidazole in the buffer.

6.6 Concentrate the eluate to 500 µL or less in a 15 mL 100K centrifugal filter tube (see **Table of Materials**) by spinning at 2,880 x *g* at 4 °C in 5 min increments. Resuspend the protein by pipetting the solution up and down between spins to avoid overconcentrating.

NOTE: Centrifuge time may be shortened as the volume approaches the desired final volume.

6.7 Load the concentrate onto an SEC column in buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA, and 0.1% digitonin). Run fast protein liquid chromatography (FPLC) and collect 300 µL fractions.

6.8 Combine peak fractions containing the intact TRPC3 tetramer, as visualized by UV absorbance signal, and concentrate again to a final concentration of at least 5 mg/mL.

7. Screening of Protein by Negative-stain Electron Microscopy

7.1 Turn on the glow-discharge machine. Set the program for discharging a carbon-coated grid using argon and oxygen for 30 s. Run the program to make the carbon-coating on copper 400-mesh grids hydrophilic prior to addition of the protein solution.

7.2 Set up five 40 µL drops of sterile water and two 40 µL drops of 1% uranyl formate solution (about 40 µL each on lab film, wax paper, or a similar surface, see **Table of Materials**). Take the grid from step 7.1 and add 2.5 µL of protein sample 5 mg/mL (50 - 200 µM) onto the dark side and let it sit for 1 min.

7.3 After 1 min, dry the grid using filter paper. Do not touch the filter paper directly to the grid surface; instead, bring the paper to the edge of the liquid droplet and allow capillary action to pull the liquid from the grid into the filter paper.

7.4 Dip the grid into first drop of water. Dry with filter paper and repeat with the remaining drops of water and the first drop of uranyl formate. Allow the second drop of uranyl formate sit for 1 min and then dry with filter paper. Allow the grid to fully air dry (about 1 min) before storing.

NOTE: This staining protocol may not be ideal for all protein-detergent combinations. Different concentrations of uranyl formate stain and different lengths of time for stain exposure should be tested if the steps above do not provide a stain with good contrast.

7.5 Image the grids on an electron microscope (see **Table of Materials**) to check the protein particle quality. Ensure that the micrographs show numerous particles that are homogenous in general appearance and distribution, display good contrast, and match the predicted size of the target protein.

7.6 Generate preliminary, low-resolution, two-dimensional (2D) classifications using 50 - 100 micrographs (see data processing – step 10) to check that the particles represent different views of a single consistent structure.

NOTE: Micrographs and preliminary 2D classes of sufficient quality, as described above, are a strong indicator that the protocol has been sufficiently optimized for protein purification. Preparation and screening of cryo-EM grids is warranted at this point.

8. EM Sample Preparation

8.1 Glow-discharge a gold holey carbon grid (see **Table of Materials**) as described in step 7.1.

8.2 Apply 2.5 µL of the concentrated hTRPC3 protein sample (5 mg/mL) onto the grid. Blot the grid for 1.5 s using a blot force of 1 and a wait time of 5 s at 100% humidity and 4 °C, then plunge the grid into liquid ethane cooled by liquid nitrogen using a vitrification machine.

NOTE: The humidity, temperature, blot-force, blot time, and wait time listed here were used for the authors' hTRPC3 study[19]. They may need to be changed to produce optimal vitreous ice for other proteins and detergents.

8.3 Screen frozen grids for optimal ice conditions using a cryo-EM microscope (see **Table of Materials**) and manually view regions of thick ice (grid squares that appear smaller and darker), thin ice (grid squares that appear larger and brighter), and medium ice.

NOTE: Thicker ice often holds more particles, while thinner ice often yields better contrast and resolution. Use manual screening of images to determine which ice conditions results in a large number of monodispersed particles with good contrast and resolution. Once good conditions are verified, move to image collection on a 300 kV cryo-EM microscope.

9. EM Data Collection

9.1 Using an automated acquisition program, record image stacks in super-resolution counting mode with a binned pixel size of 1.074 Å on an electron microscope operated at 300 kV with a nominal magnification of 130,000X direct electron detector.

9.2 Dose-fractionate every image to 40 frames with a total exposure time of 8 s, with 0.2 s per frame and a dose rate of 6.76 e^{-} $Å^{-2}$ s^{-1} (nominal defocus values varied from 1.0 to 2.5 µm in the authors' experiment).

10. EM Data Processing

10.1 Implement motion correction of summed movie stacks[22] and estimate defocus values[23] using the data processing software (see **Table of Materials**)[24].

10.2 Pick particles from the micrographs. Use these picked particles to construct an initial reference-free 2D classification using the software[24]. Select ideal 2D class averages to use as templates for automated particle picking for the entire data set.

10.3 Manually check the quality of the auto-picked particles and remove bad particles. Use multiple rounds of 2D classification to clean up picked particles.

10.4 Generate an initial model[25]. Subject 2D picked particles to three-dimensional (3D) classification (about 5 classes) using C1 symmetry and an initial reconstruction low-pass filter of

60 Å as a reference model. Determine which classes have high-resolution features and combine particles within such a class.

10.5 Further refine particles using the local refinement with C4 symmetry (in the case of hTRPC3) applied and a high-resolution limit for particle alignment set to 4.5 Å[26].

11. Model Building

11.1 Build a model (see **Table of Materials** for the software used). For hTRPC3, use the transmembrane domain (TMD) of the transient receptor potential melastatin 4 (TRPM4) structure protein data bank (PDB) 5wp6 as a guide[27]. Use bulky residues and secondary structure prediction to guide *de novo* building.

11.2 Subject the initial model to real space refinement with secondary structure restraints[28]. Manually examine the refined model and remodify as needed (see **Table of Materials** for the software used).

11.3 Apply Fourier shell correlation (FSC) curves to calculate the difference between the final model and the EM map for validation of the refined structure. Evaluate the geometries of the atomic models (see **Table of Materials** for the software used)[29,30].

REPRESENTATIVE RESULTS:

A schematic overview of the protocol for expression and purification of hTRPC3 is shown in **Figure 1A**. An image of the hTRPC3 bacmid plate with ideal white colonies, similar to the one selected for bacmid DNA purification, is shown in **Figure 1B**. We found that 48 h is ideal for clear Bluo-gal staining while maintaining the presence of isolated colonies. Peak production of P2 virus for hTRPC3, as visualized by GFP fluorescence, was seen after 4 d of infection in Sf9 insect cells (**Figure 1C**).

P2 baulovirus was harvested from the media, supplemented with 1% FBS, and used to infect a suspension of HEK293 mammalian cells. Sodium butyrate was added to the infected cells 12 - 18 h after the virus to boost protein expression[20]. Cells were then incubated for an additional 36 h at 30 °C. The cells were harvested and subjected to solubility and stability screening by FSEC (**Figure 2A**)[21]. The cells were solubilized using seven different detergents with CMC values of 0.01 - 2 mM at a detergent concentration approximately 10 times the CMC value. After whole-cell solubilization, the cell lysis debris was removed by ultracentrifugation and the supernatant containing solubilized protein was loaded on an SEC column and run on HPLC in n-dodecyl-β-D-maltopyranoside/cholesteryl hemisuccinate (DDM/CHS) detergent-containing buffer to compare absolute solubility and peak volume position of hTRPC3 under different conditions relative to a TRPM4 control (**Figure 2B**). We chose to use DDM/CHS as the initial running buffer for the samples solubilized in different detergents because DDM/CHS is the most commonly used detergent when solving the structures of membrane proteins. All of the different detergent-solubilized TRPC3 samples showed peak positions at about 11.9 mL, which is likely too large, because the tetrameric form of hTRPC3 has a smaller molecular weight than the positive control human TRPM4 (**Figure 2A and Figure 2B**).

Nevertheless, because there was no structure of any TRPC channel receptor to compare our results to and because the large molecular weight might be caused by the architecture of TRPC3 or other factors, we carried out a small-scale purification of hTRPC3 using 25 mL of cells using DDM/CHS throughout the experiment as DDM/CHS gave the best solubility of hTRPC3. The S profile of hTRPC3 showed a monodisperse peak but was still in a peak position representing a higher molecular weight than TRPM4 (data not shown). We checked the protein in the peak fraction of S profile by negative-stain EM, because it is a rapid method of verifying the protein quality and requires a very small amount of protein. In the micrograph, particles were observed with two transmembrane domains present in the same particle. It appeared that two hTRPC3 particles dimerized in a head-to-head manner through the interaction between two cytosolic domains (**Figure 3D)**. We then included the reducing reagent dithiothreitol (DTT) in the purification buffer for the second small-scale purification, hoping to disrupt the dimerization of hTRPC3. Indeed, a second peak with lower molecular weight appeared by S fractionation. However, the particles appeared too small to be an intact tetramer and showed no features of membrane proteins such as a transmembrane domain. It turned out that DDM/CHS was not a suitable detergent for purifying hTRPC3, despite giving the best solubility for hTRPC3.

Next, we tried a milder detergent, digitonin, to solubilize hTRPC3, and compared it with the protein solubilized in DDM/CHS. Here we used two different running buffers containing DDM/CHS and digitonin, respectively. This was important given that the detergent in the running buffer often contributes to protein stability and that the membrane protein may become instable when changing the detergents from solubilization to FS. The protein run in buffer containing digitonin showed a promising peak shift toward a lower molecular weight when solubilized in either DDM/CHS or digitonin. The protein solubilized by and run in digitonin yielded the highest peak in a reasonable position relative to the position of the positive control, human TRPM4 (**Figure 3A**). Then we moved forward by performing a small-scale purification using 25 mL of cells. Although multiple and broad peaks were observed by S (**Figure 3B**), the protein showed features of a single tetrameric hTRPC3 channel in 2D classification by negative-stain EM (**Figure 3C** and **Figure 3D**). We conclude that digitonin is an ideal detergent for purifying hTRPC3.

We scaled up the expression of hTRPC3 and performed a medium-scale purification using 400 mL of cells in digitonin. By doing so, we hoped to obtain sufficient protein for a few frozen grids without wasting medium and detergent before we figured out the final conditions for purifying hTRPC3 on a large scale. It happens often that membrane proteins show instability when highly concentrated for the preparation of frozen grids. The purified and concentrated protein not only shifted toward a higher molecular weight by FSEC, but also showed noisy background in cryo-EM grids imaged using an electron microscope equipped with an EMCCD camera. Even though we were able to observe single, intact tetrameric receptors, hTRPC3 purified in digitonin was not ideal for high-resolution cryo-EM studies.

To further improve the protein stability, we screened a great number of conditions described in **Protocol** step 5. We chose to screen additives based on the physiological character of hTRPC3; *e.g.*, EDTA was selected because hTRPC3 is permeable to calcium and removing the calcium may stabilize the protein. Of all the samples tested by FSEC running in the digitonin-containing buffer,10 mM EDTA showed a remarkable effect on stabilizing hTRPC3 in an intact tetrameric peak position (**Figure 4A**). It also significantly increased the number of particles in the cryo-EM micrograph and decreased the noise in the background, as shown in **Figure 5**.

Having identified the ideal conditions for expression and purification, we performed a large-scale expression (2 L) of hTRPC3. The cells containing hTRPC3 were harvested and then solubilized. Ultracentrifuge-clarified lysate was subjected to metal-affinity column purification by batch-binding with a cobalt resin followed by purification in a gravity column. Retention of solubilized protein within the column and elution of affinity-purified protein from the column was verified by FPLC (**Figure 4B**). We found that for hTRPC3, an imidazole concentration of 15 mM in the wash buffer was sufficient to remove contaminating proteins with nonspecific resin binding, and 250 mM imidazole in the elution buffer was sufficient to elute the majority of solubilized hTRPC3 protein bound to the resin. All the samples were checked by FSEC, and the eluted protein showed a sharp, monodisperse peak at the correct peak position. As intrinsic flexibility between the GFP tag and the hTRPC3 protein may make the alignment of protein particles during image processing more challenging, and because a thrombin cleavage site is located between GFP and hTRPC3, we tested a small amount of purified protein at different cleavage times using thrombin protease. Given that the eluted protein incubated with thrombin at a 1:20 molar ratio showed reasonable stability and complete cleavage after 2 h at 4 °C, we cleaved off the GFP from all the purified protein using the same conditions and checked by HPLC to confirm that GFP has been completely removed (**Figure 4C**). The protein was further purified by S, and the cleavage of GFP can again be visualized by the peak position shift toward a smaller molecular weight (**Figure 4D**). A small unconcentrated sample from the main peak fraction was used to make grids for negative-stain EM. All fractions containing the tetrameric hTRPC3 were then combined and reconcentrated to a final concentration of 5 mg/mL, which was used to prepare grids for cryo-EM imaging. As we have observed that part of the protein still gradually shifted toward a larger molecular weight, we collected only the fractions at the correct molecular weight and froze the grids immediately after protein purification. We usually completed the sequence of experiments from purification of protein to preparation of frozen grids within a single day.

A 2.5 µL sample of purified hTRPC3 protein (concentration 5 mg/mL) was applied onto a glow-discharged holey carbon grid. A vitrification machine was used to prepare the grid, using a 1.5 s blotting time under 100% humidity followed by a plunge into liquid ethane cooled by liquid nitrogen. This step freezes the sample into vitreous ice for imaging. Images were captured at 130,000X nominal magnification by a cryo electron microscope operated at 300 kV. Image stacks were recorded in super-resolution counting mode with a binned pixel size of 1.074 Å and a nominal defocus value ranging from 1.0 to 2.5 µm using a direct electron detector and automated acquisition software. Each image was dose-fractionated to 40 frames with a total exposure time of 8 s (0.2 s per frame) and a dose rate of 6.76 e^{-} $Å^{-2}$ s^{-1}. A representative micrograph from this data set is shown in **Figure 5**.

Motion correction and estimation of defocus values of the summed movie stacks was performed. Approximately 200 resulting micrographs were used as the source for manual particle picking and initial reference-free 2D classification[31]. Nine representative 2D class averages from this initial classification were selected and used as templates for automated particle picking for the entire data set. A manual check of autopicked particles was performed to remove obviously bad particles, then three rounds of 2D classification were applied to refine the selection of autopicked particles (**Figure 5**). These 2D-class selected particles were classified into five 3D classes using a low-pass-filter of 60 Å as an initial reference model. Of these five classes, only one displayed high-resolution features (**Figure 5**). Particles from this class were further subjected to local refinement with a high-resolution limit of 4.5 Å and C4 symmetry applied (**Figure 5**)[32]. The refined model was manually examined and remodified. The refined structure was validated by calculation of FSC curves to determine the difference between the final model and EM map and by evaluation of the geometries of the atomic models[33].

Initial model construction was performed using the TMD domain of the TRPM4 structure (PDB 5wp6) as a guide[34]. *De novo* building of the model of hTRPC3 was mainly guided by bulky residues and secondary structure prediction, with the many α helices in the structure greatly facilitating register assignment. In the initial *de novo*-built model, the order, length, and position of secondary structural features and bulky residues are in close agreement with the prediction. During refinement, the resolution was held to a lower limit than the resolution estimated for the final reconstruction. Three-dimensional FSC was used to measure the normalized cross-correlation coefficient between two independently generated 3D maps (each using half of the data set) over corresponding shells in Fourier space (as a function of spatial frequency). We employed a soft mask of 4.3 Å from the reconstruction and an additional 4.3 Å cosine soft edge along with a low-pass filter of 10 Å, then used the gold standard FSC 0.143 cutoff threshold. This was used for final resolution reporting.

FIGURE LEGENDS:

Figure 1: Expression and purification of hTRPC3 using the BacMam system. (**A**) Schematic procedure for hTRPC3 expression and purification. (**B**) Bacmid colony selection on blue-white indicator plate. Choose an isolated white colony (black arrow), not a white colony with an imbedded blue colony or a white colony in contact with a blue colony (red arrows). (**C**) GFP fluorescence of P2 baculovirus in Sf9 cells under 20X magnification. Fluorescence should be bright and present on the cell membrane and/or the interior of the majority of cells for a potent virus.

Figure 2: Stability screening of hTRPC3 by FS. (**A**) Detergent screening of hTRPC3. The hTRPC3 was whole-cell solubilized in a variety of detergents with a critical micelle concentration of 0.01 - 20 mM and was run in DDM/CHS-containing buffer. Although DDM/CHS shows the highest solubility of hTRPC3, the peak appears at the wrong position, too large to be the tetrameric hTRPC3 (blue trace). (**B**) TRPM4 control, with a tetrameric molecular weight of approximately 540 kDa, solubilized and run in DDM/CHS, had an elution volume of approximately 12.9 mL, while TRPC3, with a tetrameric molecular weight of approximately 400 kDa, solubilized and run in

DDM/CHS, had an elution volume of approximately 11.9 mL. With a smaller molecular weight, hTRPC3 would be expected to have an elution volume slightly larger than TRPM4, not slightly smaller, suggesting that DDM/CHS may not be an ideal detergent for hTRPC3 solubilization and purification.

Figure 3: Detergent buffer screening of hTRPC3 by FS. (**A**) Solubility and stability test of hTRPC3 using digitonin. Digitonin was tested both for solubilization and in running buffer. Note the peak position of hTRPC3 protein solubilized in digitonin shifted toward a larger elution volume in comparison to the protein solubilized in DDM/CHS (yellow trace *vs.* blue and red traces). Only the combination using digitonin in both solubilization and running buffers shows the correct size of hTRPC3 by FSEC (yellow trace, asterisk). (**B**) Similar to the FSEC results of whole-cell solubilized hTRPC3, the peak position of hTRPC3 protein affinity purified in digitonin (red trace, 14 mL) shifted toward a larger elution volume in comparison to the protein affinity purified in DDM/CHS (blue trace, 12 mL) as measured by S-fractionation. (**C**) Negative-stain EM was used to verify the quality of purified protein prior to cryo-EM data collection. An ideal stain should present particles (red circles) negatively stained (appearing white) and surrounded by a detergent ring (appearing black). A representative, ideal micrograph in which these particles are abundant, monodispersed, and present in multiple orientations is shown. (**D**) Quality data from negative-stain EM should provide 2D classes with a clear and consistent general architecture and should encompass multiple orientations of the protein, with averages contained and centered within the mask (black circle). 2D classes from negative-stain EM micrographs of hTRPC3 solubilized in DDM/CHS present particles that appear to be a head-to-head dimerization of hTRPC3 monomers. In contrast, a rough (but clear and consistent) overall acorn-like tetrameric structure is apparent in 2D classes from negative-stain EM micrographs of hTRPC3 solubilized in digitonin.

Figure 4: Purification of hTRPC3. (**A**) Stability test of hTRPC3 in the presence of EDTA. hTRPC3 was solubilized in digitonin in the presence (red trace) or absence (blue trace) of 10 mM EDTA. The former showed a single and narrower peak at the tetramer protein position (red trace, asterisk), indicating that EDTA stabilized hTRPC3 in its tetrameric conformation. (**B**) GFP fluorescence signal FSEC for hTRPC3 solubilized in digitonin and run in digitonin buffer. The tetramer peak is observed in the solubilized material before metal-affinity column purification (blue trace, asterisk). The absence of this peak in the flow-through fraction indicates a complete binding of hTRPC3 protein in the affinity column (red trace). After elution by imidazole, the fractions in the elution peak were checked by FSEC (green trace). All the fractions showing intact tetramer peak were collected for further purification. (**C**) Test of GFP cleavage of hTRPC3 using thrombin. Digestion time was tested using a small amount of protein at 4 °C, and the completeness of digestion was checked by FS. In each trace, the peak on the left corresponds to the hTRPC3 tetramer peak (asterisk) and the peak on the right is the free GFP peak. As the length of the digestion increased, the ratio of tetrameric protein to free GFP decreased, indicating that GFP was being cleaved from the protein. The 2 h digestion shows a complete cleavage of GFP. (**D**) S profile of hTRPC3 before or after thrombin digestion in digitonin containing running buffer with 10 mM EDTA added. As expected, the peak position is shifted toward the smaller elution volume after thrombin digestion (red trace). The main peak (asterisk) represents the tetrameric hTRPC3. The fractions between red lines were collected for cryo-EM experiments.

Figure 5: Schematic of cryo-EM data collection and processing for hTRPC3. The collection of 3,660 movie stacks of purified hTRPC3 was done using an electron microscope with a direct electron detector. Motion correction and manual selection were applied resulting in 3,580 micrographs. Particles were autopicked and subjected to 2D classification. 2D classes were further refined by 3D classification. Only one out of five 3D classes displayed high-resolution features. This class was selected for 3D refinement and Frealign local refinement, resulting in a structure for hTRPC3 at 3.3 Å resolution.

DISCUSSION:

Structural determination of proteins by cryo-EM has revolutionized the field of structural biology in the past few years, thanks to the development of new cameras and algorithms that significantly speeds up the structure determination of proteins that do not readily crystalize, particularly membrane proteins. Despite all of the recent advances in the cryo-EM technique, the preparation of purified proteins sufficient in quality and quantity to facilitate high-quality imaging often remains time-consuming, costly, and challenging. The ability to rapidly express and screen multiple gene constructs under a variety of purification conditions, as described in the protocol above, improves the efficiency of this process by allowing the rapid and cost-effective production of high-quality purified protein for use in cryo-EM studies.

While the method described here provides a way to purify large quantities of mammalian membrane proteins at less cost than by direct transfection and more quickly than by generation of a stably transfected cell line, it has many steps, each of which must be optimized to provide a high-quality protein yield. Within the biochemical techniques used to produce and purify hTRPC3, there are a number of critical steps and checkpoints. The first critical checkpoint is the production of bacmid DNA. As described in the results, ideal colony selection is the first step in generation of a quality virus for protein expression. Additionally, if the concentration of bacmid DNA purified from the selected colony is less than 1 µg/µL, the resulting virus will not be sufficient for robust protein expression. The second critical checkpoint is the production of P1 and P2 baculovirus. Virus titer can be estimated by GFP fluorescence, as shown in **Figure 1C** or can be measured as described by Coleman *et al.*[35]. In addition to viral titer, a time course of infection time should be undertaken for each new construct to determine the optimal time of infection for maximal protein expression. Critical checkpoint three is the solubility and stability screening shown in **Figure 2**. Detergents with a high CMC, such as DDM, may provide high solubility, but they are not ideal for cryo-EM imaging because they can result in an abundance of contaminating micelles. A milder detergent, such as digitonin, can provide sufficient solubility while preserving the protein stability. The screening of additives, such as EDTA in the case of hTRPC3, is important for discovering a purification condition that results in intact and homogenous protein, probably by removing the calcium from hTRPC3, which is permeable to calcium. Critical checkpoint four is the verification of specific and efficient protein capture during the affinity column purification and the observation of an ideal protein peak during S-fractionation (**Figure 4**). Avoidance of the inclusion of contaminating protein particles while retaining all of the target protein is crucial to obtaining a high enough yield of protein for cryo-EM imaging. Alteration of the batch-binding time and ratio of resin to solubilized protein can help improve protein retention by the resin column. The overall quality of the S-fractionation peak is in our experience the best indicator, prior to imaging, of the quality of purified protein particles. A low or a broad peak during S-fractionation indicates possible problems of too few protein particles per image or the presence of contaminating protein aggregation/degradation products, respectively. Additionally, changes to the affinity tag within the construct itself has proven necessary in some cases to ensure sufficient affinity-tag binding. The final checkpoint prior to collection of cryo-EM data sets is the verification of protein particle quality by negative-stain EM. While not strictly necessary, we find that this provides an excellent opportunity to spot and correct protein quality issues before investing in the time- and cost-intensive process of collecting data by cryo-EM.

One alternative method of producing protein for cryo-EM structural studies is the direct purification of protein from the native tissue source. While this method often encounters issues with protein yield, it is an excellent alternative for proteins that are highly abundant in cells or that exist as part of a large multi-protein complex, which can be difficult to produce using a recombinant overexpression system[36]. However, for single proteins expressed in moderate or low amounts under physiologic conditions, the expression and purification system presented here is an efficient, relatively low-cost and high-yield method for producing purified mammalian membrane protein for cryo-EM structural studies. One method that could strongly complement that presented here is the reconstitution of purified protein into lipid nanodiscs prior to cryo-EM data collection[37]. Under this method, the proteins are imbedded into a small disk of lipid bilayer, probably providing a native-like microenvironment in comparison to detergent micelles, although there is no evidence so far that the structures dissolved in detergent and nanodisc are different[38-40]. Another approach is to extract the protein directly using amphipathic polymers like styrene maleic anhydride (SMA), which has been successfully used for determination of membrane protein structures[41,42].

These approaches described in this manuscript have proven to be readily adaptable in our lab to a variety of other mammalian membrane proteins beyond ion channels. Therefore, we believe this protocol will be a valuable tool in the structural and functional analyses that underlie high-specificity targeted drug design. This will be particularly useful in neurodegenerative disease, cardiovascular disease, and cancer, in which ion channels are difficult but high-potential drug targets.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

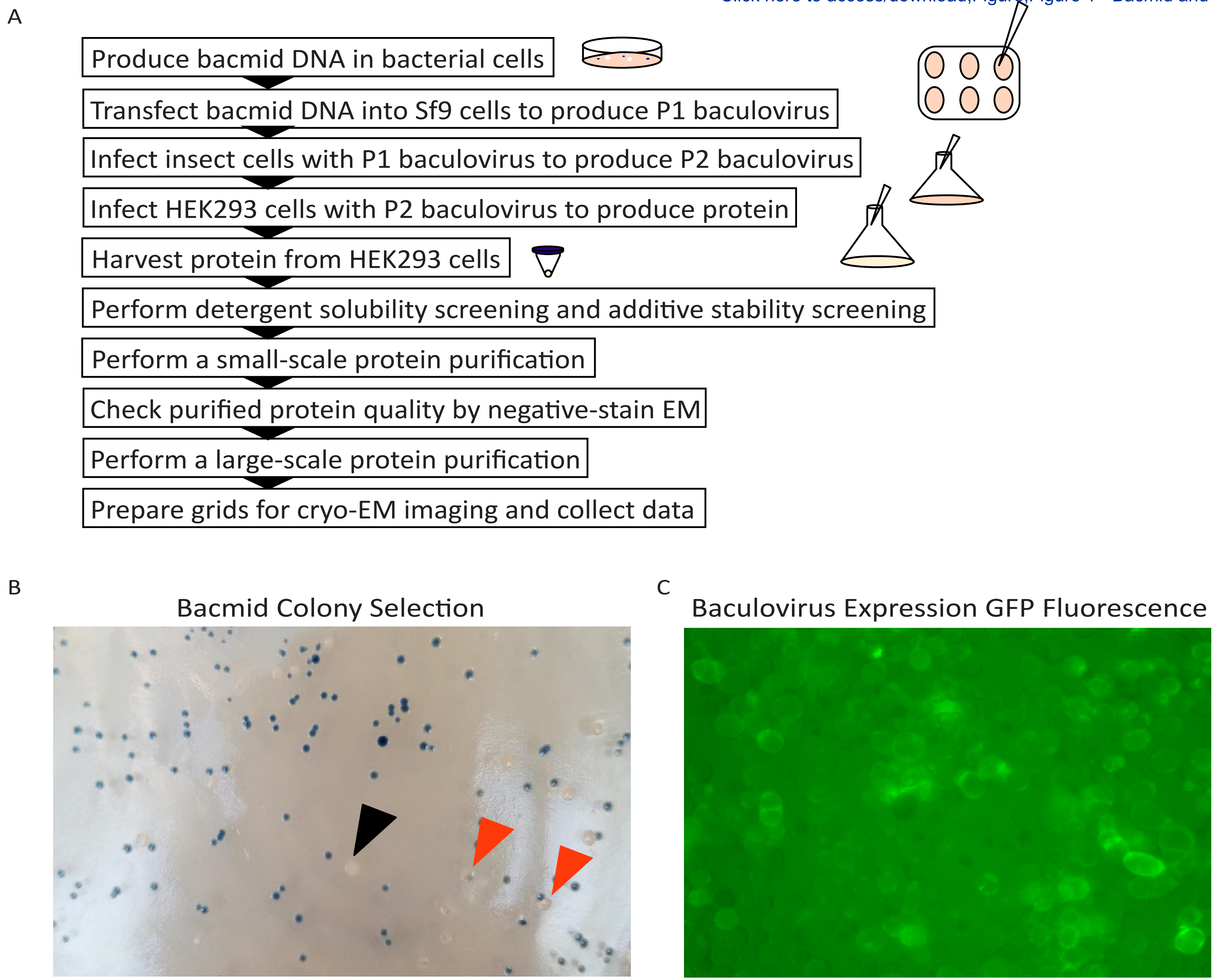

A
Produce bacmid DNA in bacterial cells
Transfect bacmid DNA into Sf9 cells to produce P1 baculovirus
Infect insect cells with P1 baculovirus to produce P2 baculovirus
Infect HEK293 cells with P2 baculovirus to produce protein
Harvest protein from HEK293 cells
Perform detergent solubility screening and additive stability screening
Perform a small-scale protein purification
Check purified protein quality by negative-stain EM
Perform a large-scale protein purification
Prepare grids for cryo-EM imaging and collect data
B
Bacmid Colony Selection
C
Baculovirus Expression GFP Fluorescence

Figure 2

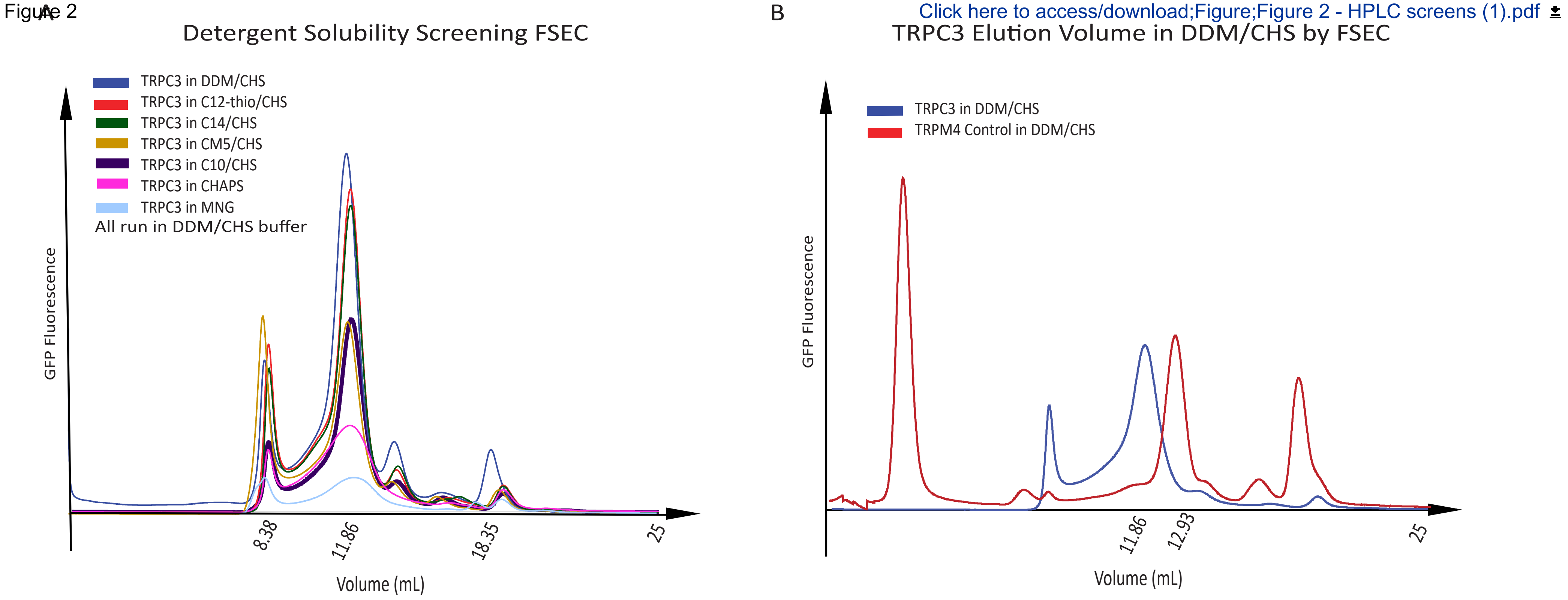

A
Detergent Solubility Screening FSEC
TRPC3 in DDM/CHS
TRPC3 in C12-thio/CHS
TRPC3 in C14/CHS
TRPC3 in CM5/CHS
TRPC3 in C10/CHS
TRPC3 in CHAPS
TRPC3 in MNG
All run in DDM/CHS buffer
GFP Fluorescence
8.38
11.86
18.35
25
Volume (mL)
B
TRPC3 Elution Volume in DDM/CHS by FSEC
TRPC3 in DDM/CHS
TRPM4 Control in DDM/CHS
GFP Fluorescence
11.86
12.93
25
Volume (mL)

Figure 3

Click here to access/download;Figure;Figure 3 - Detergent Buffer Screens.ai

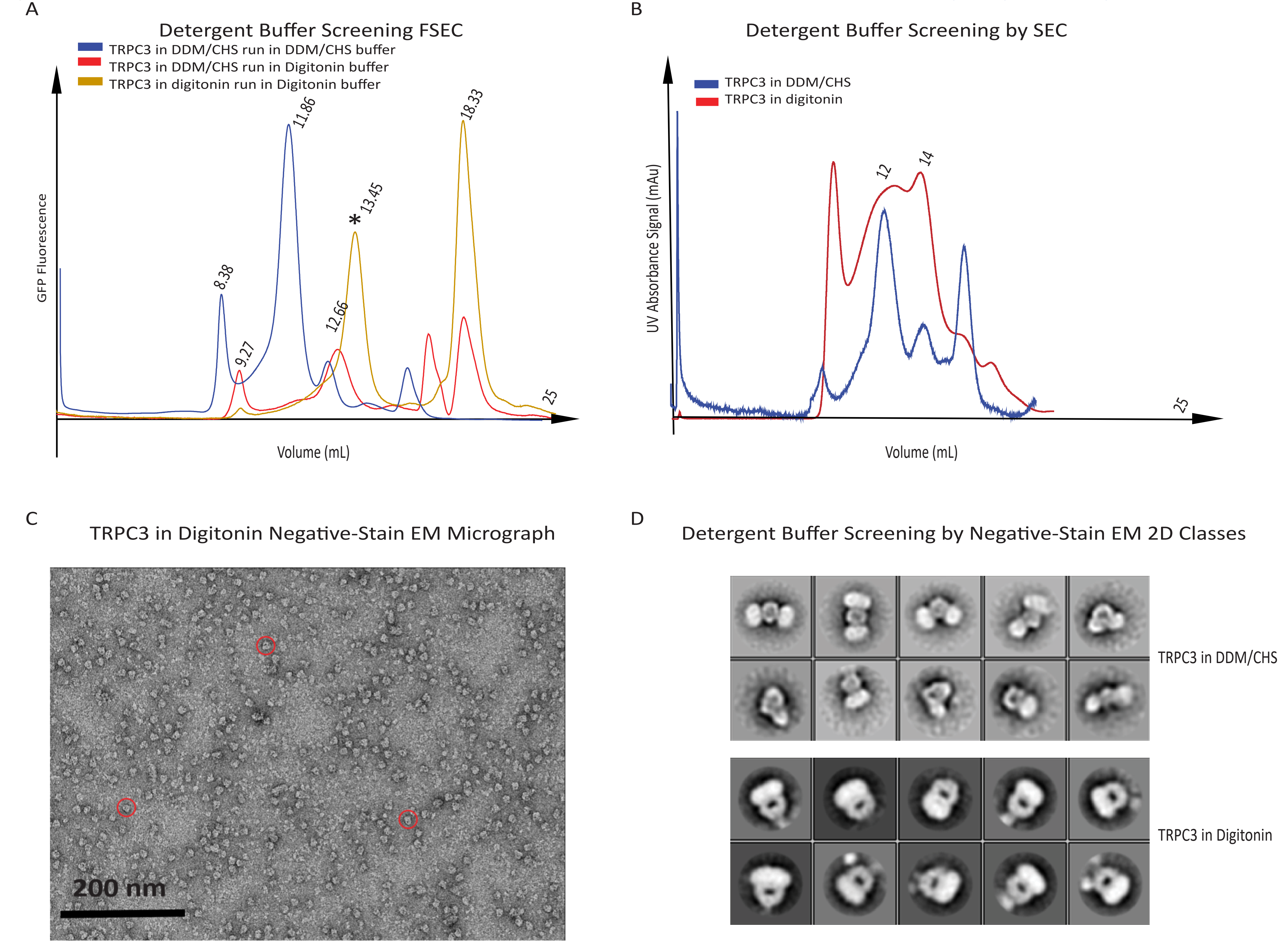


Figure 4

Click here to access/download;Figure;Figure 4 - Purification (1).ai

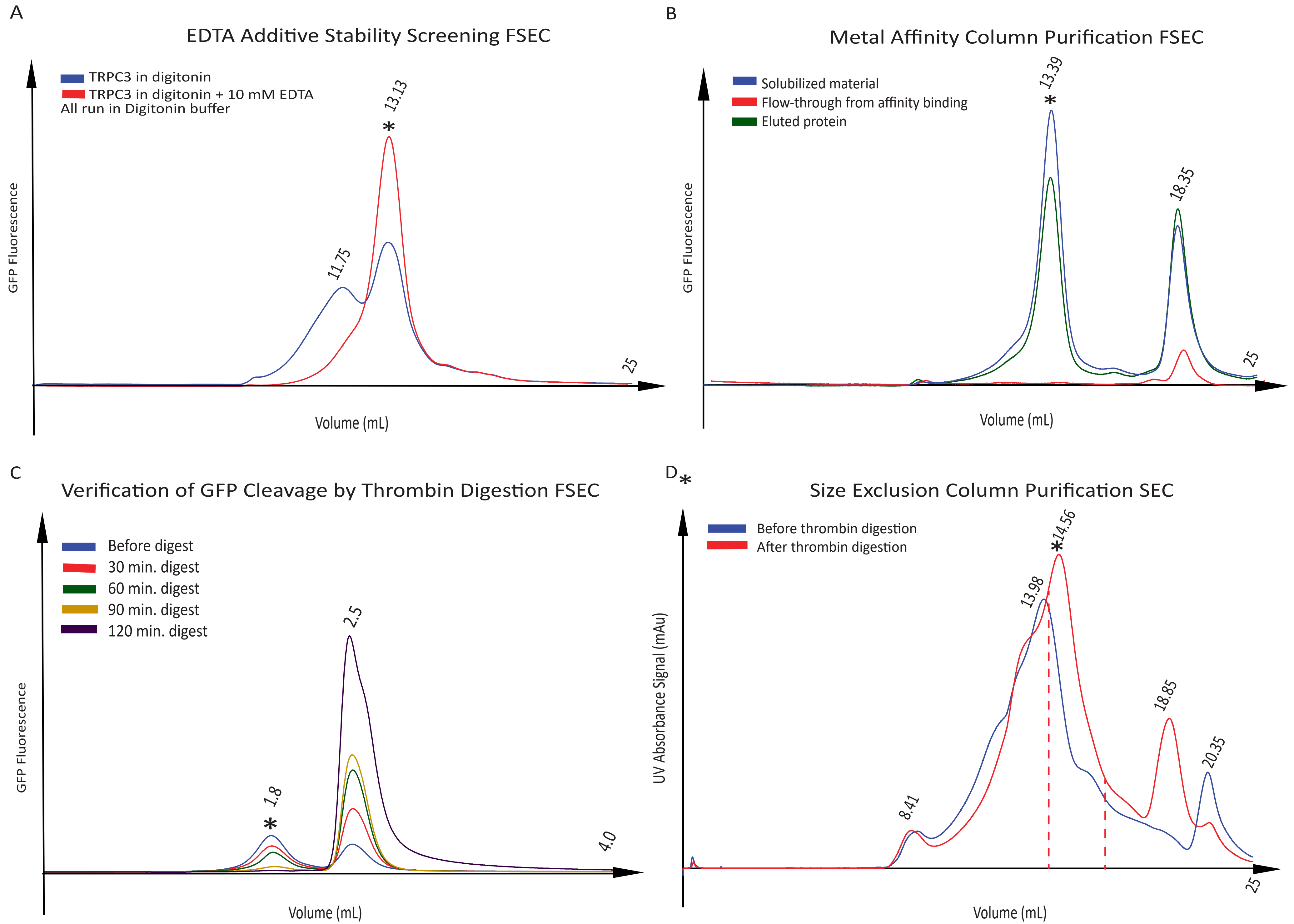
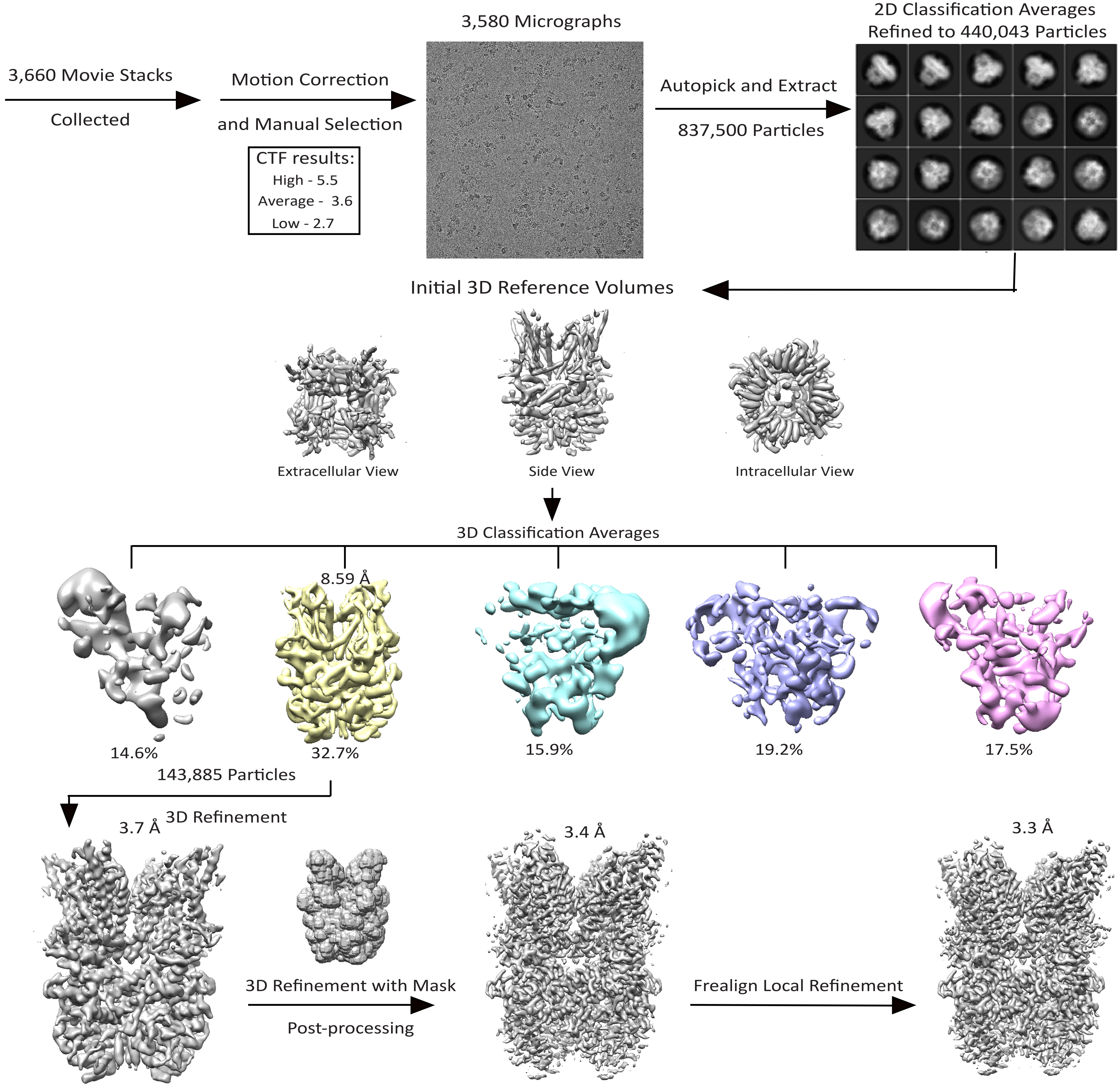


Figure 5



3,660 Movie Stacks
Collected
Motion Correction
and Manual Selection
CTF results:
High - 5.5
Average - 3.6
Low - 2.7
3,580 Micrographs
Autopick and Extract
837,500 Particles
2D Classification Averages
Refined to 440,043 Particles
Initial 3D Reference Volumes
Extracellular View
Side View
Intracellular View
3D Classification Averages
8.59 Å
14.6%
32.7%
15.9%
19.2%
17.5%
143,885 Particles
3D Refinement
3.7 Å
3D Refinement with Mask
Post-processing
3.4 Å
Frealign Local Refinement
3.3 Å

Name of Material/ Equipment
pEG BacMam vector (pFastBacI)
DH10α cells
S.O.C. media
Bacmam culture plates
Incubation shaker for bacterial cells
Incubated orbital shaker for insect cells
Reach-in CO_2 incubator for mammalian cells
Table-top orbital shaker
Incubator
QIAprep Spin Miniprep Kit
Phenol:Chloroform:Isoamyl alcohol
Sf9 cells
Sf-900 media
FBS
Cellfectin II
lipofectamine 2000
0.2 µm syringe filter
0.2 µm filter flasks 500ml resevoir
erlenmeyer culture flask (flat bottom 2L)
erlenmeyer culture flask (baffled 2L)
nanodrop 2000 spectrophotometer
HEK293
Freestyle 293 expression Medium
Butyric Acid Sodium Salt
PMSF
Aprotinin from bovine lung
Leupeptin hydrochloride
pepstatin A
digitonin
imidazole
TALON resin

superose6 incease columns
Prominence Modular HPLC System
Controller Module
Solvent Delivery System
Fluorescence Detector
Autosampler with Cooling
Pure FPLC System with Fractionator
thrombin (alpha)
Amicon Ultra 15 mL 100K centrifugal filter tube
EDTA
400 mesh carbon-coated copper grids
Quantifoil holey carbon grid (gold, 1.2/1.3 µm size/hole space, 300 mesh)
Vitrobot Mark III
liquid nitrogen
ethane gas
Solarus Plasma System
Tecnai Spirit electron microscope
Talos Arctica electron microsocope
Titan Krios electron microscope
Software
Gautomatch software
Relion 2.1 software
CryoSPARC software
Frealign software
Coot software
MolProbity software
SerialEM software
MortionCor2 software
GCTF software

Phenix.real_space_refine software

Company	Catalog Number
addgene	31488
Life Technologies	10361-012
Corning	46003CR
Teknova	L5919
Infors HT	Multitron standard
Thermo-Fisher	SHKE8000
Thermo-Fisher	3951
Thermo-Fisher	SHKE416HP
VWR	1535
Qiagen	27106
Invitrogen	15593031
Life Technologies	12659017
Gibco	12658-027
Atlanta Biologicals	S11550
Gibco	10362100
Invitrogen	11668-027
VWR	28145-501
Corning	430758
Gene Mate	F-5909-2000
Gene Mate	F-5909-2000B
Thermo-Fisher	ND-2000
ATCC	CRL-3022
Gibco	1238-018
Acros	263195000
Acros	215740500
Sigma-Aldrich	A1153-100MG
Sigma-Aldrich	24125-16-4
Fisher Scientific	BP2671-250
EMD Millipore	300410
Sigma	792527
Clonetech	635504

GE	29091596; 29091597
Shimadzu	See Below
"	CBM20A
"	LC30AD
"	RF20AXS
"	SIL20ACHT
Akta	
Haematologic Technologies Incorporated	HCT-0020 Human alpha
Millipore	UFC910008
Fisher	E478500
Ted Pella Inc.	01754-F
Electron Microscopy Sciences	Q3100AR1.3
FEI	
Dura-Cyl	UN1977
Airgas	UN1035
Gatan	Model 950
FEI	
FEI	
FEI	

http://www.mrc-lmb.cam.ac.uk/kzhang/Gautomatch/
https://github.com/3dem/relion
https://cryosparc.com/
http://grigoriefflab.janelia.org/frealign
https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/
http://molprobity.biochem.duke.edu/
http://bio3d.colorado.edu/SerialEM/
http://msg.ucsf.edu/em/software/motioncor2.html
https://www.mrc-lmb.cam.ac.uk/kzhang/Gctf/
https://www.phenix-online.org/documentation/reference/real_space_refine.html

Comments/Description

for transformation of DH10α cells for Bacmid
for culture of transformed DH10α cells

used in Reach-in CO2 incubator for mammalian cells
for bacterial plates
for plasmid extraction and purification
for DNA extraction
insect cells for producing virus
insect cell media

for transfecting insect cells
for transfecting mamalian cells
for filtering P1 virus
for filtering P2 virus
for culturing insect cells
for culturing mammalian cells
for determining DNA and protein concentrations
mammalian cells for producing protein
mammalian cell media for protein expression
to amplify protein expression
protease inhibitor
protease inhibitor
protease inhibitor
protease inhibitor
detergent - to solubilize protein from membrane
to elute protein from resin column
for affinity purification by His-tag

for HPLC and FPLC

for cleaving GFP tag
for concentrating protein
for stabilizing protein
grids for negative stain
grids for Cryo-EM
for preparing sample grids by liquid ethane freezing

for cleaning grids before sample freezing
for negative stain EM imaging
for screening and low resolution imaging of Cryo-EM grids
for high-resolution Cryo-EM imaging

to pick particles from micrographs
to construct 2D and 3D classification
to generate an initial structure model
to refine particles
to build a model
to evaluate the geometries of the atomic model
for automated serial image stack acquisition
for motion correction of summed movie stacks
for measuring defocus values in movie stacks

for real space refinement of the initial 3D model

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Title of Article:	Expression and Purification of the Human Lipid-sensitive Cation Channel TRPC3 for Structural Determination by Single-particle Cryo-electron Microscopy
Author(s):	Emery Haley, Wooyoung Choi, Chen Fan, Weinan Sun, Juan Du, Wei Lü

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Department: Cancer and Cell

Institution: Van Andel Research Institute

Title: Assistant Professor

Signature: Juan Du Date: 07-04-2018

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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Reply: We appreciate the editors' observation that the manuscript was in need of a thorough revision to improve the English grammar and spelling and to check for typing errors. We have reviewed the document ourselves for such errors. Additionally, we have sent the edited document for review by the Van Andel Research Institute Science Editor and we have addressed all grammar, spelling, and typing errors discovered.

2. Please revise lines 83-85 and 406-410 to avoid previously published text.

Reply: We recognize the need to avoid copyright infringement and appreciate the editors delineating selected regions of text in need of rephrasing. We have edited the referenced passages to avoid similarity to our previously published work.

Lines 78-801: **"**We have reported the first atomic-resolution structure of the human TRPC3 channel (hTRPC3) and its two lipid binding sites in a closed state, providing important insights into these mechanisms19."

Lines 565-571: "During refinement, the resolution was held to a lower limit than the resolution estimated for the final reconstruction. Three-dimensional Fourier shell correlation (FSC) was used to measure the normalised cross-correlation coefficient between two independently generated 3D maps (each using half of the data set) over corresponding shells in Fourier space (as a function of spatial frequency). We employed a soft mask of 4.3 Å from the reconstruction and an additional 4.3 Å cosine soft edge along with a low-pass filter of 10 Å, then used the gold standard Fourier shell correlation (FSC) 0.143 cutoff threshold. This was used for final resolution reporting."

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Reply: Because we will focus on the expression and purification of human TRPC3 in this manuscript, and because we have determined that a full description of the electrophysiology methods for functional validation of an ion channel structure is beyond the scope of this article, we have excluded the part of electrophysiology, negating the need to acquire reprint permission.

4. Please spell out each abbreviation the first time it is used.

Reply: We have taken great care to locate each abbreviation used and to spell out each ahead of the abbreviation.

Line 39: **"**transient receptor potential channels of the canonical subfamily (TRPC)"
Lines 52-53: "cryo-electron microscopy (cryo-EM)"
Line 62: "store-operated calcium entry (SOCE)"
Line 63: "endoplasmic reticulum (ER)"
Line 70: "diacylglycerol (DAG)"
Lines 70-71: "phosphatidylinositol 4,5-bisphosphate (PIP2)"
Line 92: "fluorescence-detection size-exclusion chromatography-based (FSEC)"
Line 93: "green fluorescent protein (GFP)"
Lines 115-116: "super optimal broth with catabolic repressor (S.O.C. media)"
Lines 120-121: "isopropyl β-D-1-thiogalactopyranoside (IPTG)"
Line 208: "fetal bovine serum (FBS)"
Line 253**:** "tris-buffered saline (TBS)"

Lines 271: “critical micelle concentration (CMC)”
Line 273: “ethylenediaminetetraacetic acid (EDTA)”
Line 302-303: “high-pressure liquid chromatography (HPLC)”

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Reply: We have taken great care to locate each instance of commercial language and replace the term with a neutral term and reference to the materials list.
Lines 137-138: “cell resuspension solution from the miniprep kit (see materials list)”
Line 141: “cell lysis solution from the miniprep kit”
Line 143: “neutralization solution from the miniprep kit”
Line 182**:** “appropriate medium (see materials list)”
Line 190: “transfection reagent (see materials list)”
Line 215: “appropriate medium (see materials list)”
Line 240: “expression medium (see materials list)”
Lines 265-266: “size-exclusion chromatography column (see materials list)**”**
Lines 320-321: **“**15 mL 100K centrifugal filter tube (see materials list)”
Line 343: “lab film, wax paper, or a similar surface (see materials list)”
Line 357: “electron microscope (see materials list)”
Line 370: “gold holey carbon grid (see materials list)”
Lines 381-382: “cryo-EM microscope (see materials list)”
Line 388: “300-kV cryo-EM microscope (see materials list)”
Line 403: “using software in materials list”

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Reply: We have revised the protocol to ensure each sentence starts with a verb, and rewritten all other indispensable descriptions, etc., as notes within the protocol. For example Lines 123-131:
“1.1.4 Incubate the plate for 48 h at 37 °C.

Note: The Bluo-gal indicator stains colonies still expressing lacZ (vector insertion unsuccessful), allowing for selection of white (successfully transformed) colonies.

1.1.5 Carefully select an isolated white colony, avoiding any white colonies that are in contact with blue colonies, and grow cells overnight in 6 mL of acmid LB medium (50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline) at 37 °C in an orbital shaker at 225 rpm.”

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how”

question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
2.1.2, 2.1.3: What do P1, P2 and N3 refer to? Please cite the Table of Materials for composition of these solutions.
4.1.4: What is tittered?
5.1.1: Listing an approximate volume to prepare would be helpful.
5.1.3: What is the incubation temperature?
5.1.4: Please spell out TBS. What volume of TBS is used to wash?
5.1.5: Please specify the detergents and/or additives in this step. Such details are needed for filming.
6.1.1: What container is used? What volume of buffer is used?
6.1.2: How to visualize protein by GFP signal? What instrument is used?
6.1.3: How to confirm protein binding? What results would indicate protein binding? Please specify.

Reply:
We made additions, based on the input of new lab members who are novices to the methods described, and who read through the protocol for clarity. Additionally, all of the changes and clarifications specifically requested by the editors have been made.
Lines 137-138: "cell resuspension solution from the miniprep kit (see materials list)"
Line 141: "cell lysis solution from the miniprep kit"
Line 143: "neutralization solution from the miniprep kit"
Line 234: **"**Obtain a titer for the P2 virus using Sf9easy cells or Virus counter."
Lines 238-239: "Prepare a desirable volume of HEK293 mammalian cell suspension culture (4–6 L is recommended for preparation of frozen grids)**"**
Line 250: **"**at 30 °C."
Lines 252-253: "Wash cells by resuspending in approximately 100 mL tris-buffered saline (TBS) per liter of cells harvested."
Lines 269-273: "Note: In the case of hTRPC3, this screening included different buffers with pH values from 4.0–9.5, salt concentrations of 50-500 mM; different ionic compositions (such as MgCl2 or NaCl); different detergents with critical micelle concentration (CMC) values of 0.1 mM to 20 mM; reducing additives such as dithiothreitol, tris(2-carboxyethyl)phosphine, and β-mercaptoethanol; and the calcium-chelating additive ethylenediaminetetraacetic acid (EDTA). ."
Lines 277-281: "Thaw the pellet in buffer containing 20 mM Tris, pH 8.0,500 mM NaCl, 1 mM PMSF, 0.8 μM aprotinin, 2 μg/ml leupeptin, 2 mM pepstatin A, and 1% digitonin, using 100 mL of buffer per liter of cells harvested. Once thawed, ensure homogeneity of the solution by pipetting or stirring. Allow to solubilize for 2 h at 4 °C in a beaker immersed in ice with a stir bar rotating."
Lines 283-285: "Remove cell debris by ultracentrifugation at 235 000 × g for 1 h at 4 °C. Verify protein quantity by running a 30-μL sample on a size-exclusion chromatography column (see materials list) by HPLC and visualize the target protein by the GFP signal output."
Line 291-294: "Note: If protein binding has occurred, the GFP tagged protein target will be retained on the column, not found in the flow-through. Therefore, no GFP signal will be present at the position corresponding to the target protein size when the flow-through is run on HPLC."

8. 6.1.6: Please break up into two steps.

Reply:
We have split the protocol step into two as requested.
Lines 320-329: "6.1.6 Concentrate the eluate to 500 μL or less in a 15-mL 100K centrifugal filter tube (see materials list) by spinning at 2880 x g at 4 °C in 5-min increments. Resuspend the protein by pipetting the solution up and down between spins to avoid overconcentrating.

Note: Centrifuge time may be shortened as the volume approaches the desired final volume.

6.1.7 Load the concentrate onto a size-exclusion chromatography column in buffer containing 20 mM Tris, pH 8.0, 500 mM NaCl, 1 mM EDTA, and 0.1% digitonin and run fast protein liquid chromatography (FPLC), collecting 300-µL fractions. ”

9. Please include single-line spaces between all paragraphs, headings, steps, etc.

Reply: We have checked the formatting to ensure that a single blank space was included between all paragraphs, headings, steps, notes, and other relevant text breaks.

10. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Reply: We have chosen a protocol section to highlight.

11. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Reply: The highlighted section includes only complete sentences and each included step begins with a verb.

12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Reply: We were careful to include all steps and sub-steps that contain details for actions chosen for inclusion in the highlighted section.

13. Figures 2 and 3: Please change “ml” to “mL”. Please include a space between numbers and their units (10 mM).

Reply: We have made the format changes to the units and spacing in figures 2 and 3.

14. Discussion: Please also discuss any limitation of the technique.

Reply: We strongly agree the importance of truly addressing the limitations of the technique, not only the complimentary strategies that can be used to address some of the limitations.
Line 653-657: “While the method described here provides a way to purify large quantities of mammalian membrane proteins at less cost than by direct transfection and more quickly than by generation of a stably transfected cell line, it has many steps, each of which must be optimized to provide a high-quality protein yield. Within the biochemical techniques used to produce and purify hTRPC3, there are a number of critical steps and checkpoints. ”

15. References: Please do not abbreviate journal titles.

Reply: We have removed all abbreviations and included the full name of journal titles in all references.

Reviewers' comments:
Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.

Reviewer #1:
The manuscript described in detail the procedure for constructing the viruses for expressing hTRPC3 in mammalian cells, testing proper conditions for extracting the recombinant proteins and for maintaining better homogeneity and stability for protein purification, examining the protein specimens by negative-stain EM before making cryoEM grids, and lastly cryo-EM imaging, 3D reconstruction and refinement, and model building and interpretation. The physiological test of the channels expressed in cells was excerpted from a published paper showing the effects of an agonist and an antagonist. The description of the protocol has sufficient details and is suitable for publication in JoVE after modifications listed in the below.

Reply: We appreciate the reviewer's positive comments and have addressed each concern below.

1) line 48, "human diseases" instead of " …disease"

Reply: We have corrected this grammatical error.
Line 46: **"**human diseases"

2) line 50, structure determination is better than "structural …"

Reply: We have corrected this grammatical error.
Line 54: "structure determination**"**

3) line 106, should read as 'synthesize the gene of interest and subclone it into …'. Similar errors in the use of articles and the proper sense for verbs are found in many places. It is recommended to request professional editorial service to improve the English of the manuscript.

Reply: We appreciate the editors' observation that the manuscript was in need of a thorough revision to improve the English grammar and spelling and to check for typing errors. We have reviewed the document among ourselves for such errors. Additionally, we have sent the edited document for review by the Van Andel Research Institute Science Editor and we have addressed all grammar, spelling, and typing errors discovered.
Lines 109-111: "Synthesize the gene of interest and subclone it into a modified version of the pEG vector containing a twin strep-tag, a His8-tag, and GFP with a thrombin cleavage site at the N terminus (pFastBacI)20"

4) Lines 173 and 174, it is better to use "protein expression" than using "virus expression".

Reply: We recognize that the imprecise description coupled with prior grammatical errors in the manuscript led the reviewer to believe that the protein expression was being measured in this step. We have rewritten the protocol step to clarify that the baculovirusis being expressed at this stage and viral expression can be visualized by GFP fluorescence.

Lines 201-204 “Incubate cells for 5 d (120 h) in 27 ?C humidified incubator. Check GFP fluorescence before harvesting to verify that virus is being produced in a large percentage of cells; if the percentage is low, extend the incubation time as necessary (see Figure 1C).”

5) Lines 202, "Superose 6 column", not "superpose6". Similar mistakes in multiple places.

Reply: We have removed all commercial language per the editors request and replaced all instances of “superpose 6 column” with “size-exclusion chromatography column (see materials list)”.

6) Line 237, "dark side" not "dark site"

Reply: We have corrected this typing error.
Line 345: “dark side**”**

7) Line 238, "once time is up,", not "…minute it up".

Reply: We have corrected this grammatical error.
Line 345: “After 1 minute”

8) Line 248 "blot the grid for …", not "blot for".

Reply: We have corrected this grammatical error.
Line 373: “Blot the grid for”

9) Line 263, "defocus", not "Defocus"

Reply: We have corrected this typing error.
Line 397: “defocus”

10) In section 10, please specify if the initial 3D classification was imposed of C4 symmetry. Please show the initial 3D reference map generated from the 2D class in Fig. 5.

Reply: We recognize that the point at which C4 symmetry is added to the reconstruction was unclear. We have added the clarification that initial 3D modeling is performed under C1 symmetry. We have also included the initial 3D reference map (intracellular, extracellular, and side views) generated from 2D classes (figure 5) as requested.
Lines 412-415: **“**Generate an initial model25. Subject 2D picked particles to 3D classification (about 5 classes) using C1 symmetry and an initial reconstruction low-pass filter of 60 Å as a reference model. Determine which classes have high-resolution features and combine particles within such a class.”

11) In Fig. 5, please add the following details: a) ~3 typical CTF-finding results, and resolution limits in the CTF fitting process; b) the micrograph showed has poor contrast, not easy to see the particles; better contrast and a smaller area might be shown to highlight the individual particles; c) for three rounds of 2D classifications, how many classes were generated in each, and what fractions of data were retained during each round? These would be helpful to serve as benchmarks; d) for the 3D classification, the initial reference volume was generated from what types of 2D classes. Please show the reference map before filtering to 60 Å. E) FOR 3D CLASSIFICATION INTO FIVE CLASSES, what is the resolution for the selected class?

Reply: To help clarify the intermediate results during data processing and model building, we have added the requested results to Figure 5. Specifically, we have added a box specifying the highest, lowest, and average CTF results from our data processing; a new micrograph displaying better contrast of the particles; intracellular, extracellular, and side views of the initial 3D reference volume constructed from all 2D classes shown prior to filtering; and a label for the 8.59 Å resolution of the selected 3D class.

12) If EDTA is really an agonist, why is the structure in the closed-state instead of an open-state? Please provide an alternative explanation.

Reply: While we often use agonists to stabilize proteins during purification, we appreciate the reviewer noting that EDTA is not a TRPC3 agonist. We have corrected the manuscript to indicate that EDTA was chosen as a stabilizing additive, due to its calcium chelating properties, during purification of the TRPC3 cation channel.
Line 273: "calcium chelating additive ethylenediaminetetraacetic acid (EDTA)"
Lines 501-503: **"**We chose to screen additives based on the physiological character of hTRPC3; e.g, EDTA was selected because hTRPC3 is permeable to calcium and removing the calcium may stabilize the protein.**"**
Lines 669-671: **"**The screening of additives, such as EDTA in the case of hTRPC3, is important for discovering a purification condition that results in intact and homogenous protein, probably by removing the calcium from hTRPC3, which is permeable to calcium***."***

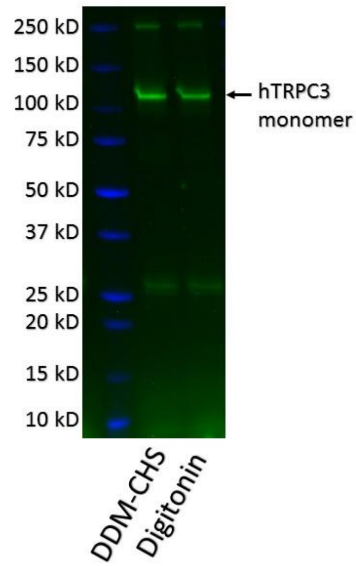
13) Line 299, "using a micro…" is better.

Reply: We have determined that a full description of the electrophysiology methods for functional validation of an ion channel structure is beyond the scope of this article. As such, the method description has been removed.

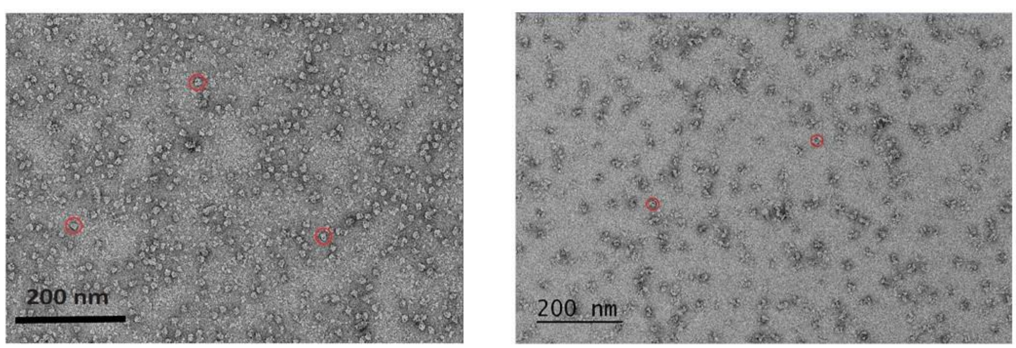
14) Figs 2 & 3, the retention volume in the X-axes must be given. The peak positions (retention volumes) should be marked for the main extraction peak and the stable tetramer peaks (*) in Fig. 2B. There seems a monomer peak or a detergent micelle peak in Fig. 2B. SDS-PAGE gels should be given to show the distribution of proteins among those different peaks, serving good benchmarks.

Reply: We have added the retention volumes to the X-axes of all graphs in Figures 2 and 3 to clarify the position of tetrameric protein and other peaks. An SDS-PAGE gel was run for hTRPC3 samples solubilized in either DDM-CHS or Digitonin (as in Figures 2 and 3). However, as an SDS-PAGE gel results in denaturing of proteins, the tetrameric quaternary structure is lost, and the resulting GFP signal only indicates the monomeric protein. We believe the peak shift shown in Figure 3A indicates a change in the quaternary structure of the protein, and therefore, a concurrent shift in band position on an SDS-PAGE gel would not be expected. To illustrate this we have included a side by side comparison of negative stain micrographs from hTRPC3 solubilized in either digitonin or DDM-CHS below. The images from hTRPC3 solubilized in DDM-CHS do not show tetrameric protein particles and instead predominantly display dimeric protein particles. In addition to the data included in this letter (not to be published), we have modified Figures 2-4 to include data which further explain these observations. We have included FSEC comparisons of the TRPC3 peak in DDM/CHS, to the peak of a known standard control, TRPM4, in DDM/CHS. We have included SEC comparisons of TRPC3 purified in DDM/CHS compared to digitonin to compliment the FSEC results previously shown in Figure 2B (now Figure 3A), and we have included 2D classes from negative stain EM data of TRPC3 purified in DDM/CHS compared to digitonin to show the dimerized particles versus the complete tetrameric channel particles.

GFP Signal from hTRPC3 on SDS-Page



Negative Stain Images of hTRPC3



Solubilized in Digitonin (from Figure 4)

Solubilized in DDM-CHS

15) Line 384, "direct electron detector" not "direct elector …"

Reply: We have corrected this typing error.
Line 394: **"**direct electron detector"

16) Line 382, "operated" not "operating". The instrument does not operate itself.

Reply: We have corrected this grammatical error.
Lines 393-394: **"**electron microscope operated at 300 kV**"**

17) Lines 371 and 462, "a ring of positively-stained lipids" appears not well supported by what was done. In negative stain EM, it is generally assumed that the local contrast comes from the stain microcrystals formed next to the protein particles during drying of the stain solution while the proteins maintain their 3D architectures. The stain particles may form in crevices of proteins, giving out the structural details. The surrounding area of the protein is darker due to the accumulated stain, not due to positive staining, which refers to the reaction of the stain molecules with the particle and would make certain parts of the particles merged with the background and disappear from the reconstruction. That means that "the positive staining of lipids" must be demonstrated by showing that the stain molecules react with the lipids. Further, because lipids were not added during the protein purification, it is necessary to provide direct evidence that there are still lipids next to the purified TRPC3 proteins, which are expected not to be purely detergent micelles. Lipid analysis must be performed for the purified proteins.

Reply: We recognize that "positively-stained lipids" was an inaccurate description and was merely intended to emphasize the contrasting appearance of the black ring surrounding a white negatively stained protein particle. For this purpose, it is not necessary to determine the composition of the ring, nor determine whether the uranyl formate stain reacts with the components of the black ring. We have amended the text to clarify the visual description of an ideal grid, while avoiding any unproven claims.
Lines 603-605: **"**An ideal stain should present with particles negatively stained (appearing white) and surrounded by a detergent ring (appearing black)."

18) Line 485, it is too bold to claim that the purified membrane proteins are in "native state" when detergents are used to disrupt the membranes.

Reply: We agree that the use of detergents to extract the protein from membrane results in the protein being suspended in a non-native environment, and we have therefore changing the wording to reflect this distinction.
Line 643: **"**Structural determination of membrane proteins by Cryo-EM"

19) Because of the need for functional evidence of the purified proteins, it is important to show that the purified TRPC3, when reconstituted into lipid vesicles, remains functional. It is not sufficient to show that the starting state of the protein is functional (Fig. 6).

Reply: We agree that to truly demonstrate that purified hTRPC3 is functional, we must demonstrate channel activity of the purified protein reconstituted in lipid vesicles. While these experiments are planned in our ongoing work, we have determined that a full description of the electrophysiology and other methods for accurate functional validation of an ion channel structure is beyond the scope of this article. As such, the method description and accompanying figure has been removed.

20) Line 508, delete "and" for homogeneous protein.

Reply: We have corrected this grammatical error.

21) There are many other typos and grammatical errors that need to be corrected.

Reply: We have reviewed the document among ourselves for such errors. Additionally, we have sent the edited document for review by the Van Andel Research Institute Science Editor and we have addressed all grammar, spelling, and typing errors discovered.

Reviewer #2:

Manuscript Summary:

This manuscript describes a potentially very useful set of procedures involved in expressing a recombinant ion channel in mammalian cells, using the Bacmam system, screening for well-behaved variants using fluorescent protein tags and gel filtration, purifying the protein, imaging it by cryo-EM, and determining its structure by single particle analysis. This is an important set of procedures in which many groups are interested, and the authors' published work demonstrates that the procedures they use are effective.

Major Concerns:

1) There are, however, serious problems with the protocol as written. The authors seem to have given little thought to what constitutes a useful protocol for helping others in different laboratories carry out similar procedures without having to do excessive literature searching or trouble-shooting. Rather, they seem to have selected without good criteria, portions of the protocols followed, which in some places leave out information essential for reproducing the procedures, and in others simply repeating material that has been provided in another section.

Examples (there are too many to list all):

"Discard the supernatant and resuspend the pellet in 200 L of cell resuspension solution

125 (P1 of miniprep kit) by pipetting." Which miniprep kit is this? There has been no mention of such a kit up to this point, no manufacturer is indicated, and there is no reference. Moreover, if this is simply following a kit protocol, why not just say, "...use miniprep kit ** from ** Co., and follow manufacturer's instructions," or "follow kit instructions with the following modifications?"

Later, we find this bonus information: "CAUTION: Phenol:Chloroform:Isoamyl Alcohol solution is toxic by inhalation, in contact with skin 147 and if swallowed. It..." etc., but we are never told the recipe (or the reference for the recipe) for this reagent. Although it is a common reagent, some kit-dependent labs don't use it these days.

Reply: We appreciate that the details regarding some reagents and materials may be unclear when referencing only the text provided. According to the editors' restrictions on commercial language, the name of the kits, reagents, and manufacturers cannot be explicitly listed in the text of the protocol. Where relevant, we have inserted the note "(see materials list)" beside the generic description of certain kit components, reagents, and materials to in order to direct the readers to these relevant details that will provide clarification for the protocol step.

In the example provided, the reagents from a miniprep kit are used, but the protocol steps have very minimal overlap with the manufacturers' directions. Therefore, we have chosen to explicitly list our protocol steps with no mention of the directions provided with the kit in order to avoid confusion. Additionally, the Phenol:Chloroform:Isoamyl Alcohol solution reagent is purchased directly from a supplier (found in the materials list), so no recipe is needed.

2) The next section refers to reagents such as Sf-900 and Cellfectin II, with no information on where these come from or what is in them. How about at least a supplier's name?

Reply: The supplier name, full product name, and catalog numbers can be found in the materials list that was submitted along with the original manuscript. We have added the note "(see materials list)" beside the generic descriptions in order to direct the reader to these details.

3) In the next section, we are told to use "culture flask of sufficient size." How about some guidance on what "sufficient size" is? Of course someone who cultures Sf9 cells all the time will know, but they will not need this detailed protocol anyway.

Reply: We recognize that not all readers will be familiar with the relevant cell culture techniques. We have therefore added recommended volumes of cell culture suspension to prepare for each protocol step, as well as a note on choosing the appropriate flask size, while continuing to indicate that these volumes may not be the same for all experiments.

Lines 214-219: "Prepare 200 mL (or desired volume) of Sf9 cells at a concentration of 0.8-0.9 □□106 cells /mL in appropriate medium (see materials list) in a flat bottom erlenmeyer culture flask of sufficient size.

Note: For suspension culture, the volume used should not exceed 40% of the total capacity of the flask."

Lines 238-244: "Prepare a desirable volume of HEK293 mammalian cell suspension culture (4–6 L is recommended for preparation of frozen grids) at a concentration of 3.5-3.8 □□106 cells/mL in expression medium (see materials list) supplemented with 1% (V/V) sterile FBS in baffled-bottom erlenmeyer culture flasks of sufficient size.

Note: For suspension culture, the volume used should not exceed 40% of the total volume of the flask."

4) "Turn on the plasma machine. Set program, carbon argon O2 Ar 0:30. Run to clean grids." Either refer the reader to a reference on how to plasma clean grids, or provide sufficient information here. Presumably the settings mentioned are specific for a particular instrument. "Run to clean grids," is not necessary for an expert, but too vague for a novice.

Reply: The settings described were for a specific instrument (found in the materials list), however, they are widely applicable to any instrument used for this purpose. We have clarified the description of the settings in consideration of those unfamiliar with preparing grids in this manner and have removed unnecessary or redundant language. In addition, we have provided a description of the purpose of this step.

Lines 338-340: **"**Turn on the glow-discharge machine. Set the program for discharging a carbon-coated grid using argon and oxygen for 30 s. Run the program to make the carbon-coating on copper 400-mesh grids hydrophilic prior to addition of the protein solution.""

5) It is odd that the protocol provides details on how to screen different constructs for expression and stability, but makes no mention of screening freezing conditions or checking sample with negative stain or lower voltage instrument before securing time for a long acquisition time on a 300 keV instrument with direct electron detector.

Reply: We agree that our protocol skipped a crucial step of screening grid conditions on a lower power cryo-EM microscope. We have added steps and notes to the protocol to describe this process of screening and troubleshooting.
Lines 335-398:

"7. Screen protein by negative-stain electron microscopy

7.1.1 Turn on the glow-discharge machine. Set the program for discharging a carbon-coated grid using argon and oxygen for 30 s. Run the program to make the carbon-coating on copper 400-mesh grids hydrophilic prior to addition of the protein solution.

7.1.2 Set up 5 40 µl drops of sterile water and 2 40 µl drops of 1% uranyl formate solution (about 40 µl each on lab film, wax paper, or a similar surface (see materials list). Take the grid from step 7.1.1 and add 2.5 µl of protein sample 5 mg/mL (50-200 µM) onto the dark side and let it sit for 1 min. After 1 min, dry the grid using filter paper. Do not touch the filter paper directly to the grid surface; instead, bring the paper to the edge of the liquid droplet and allow capillary action to pull the liquid from the grid into the filter paper. Dip the grid into first drop of water. Dry with filter paper and repeat with the remaining drops of water and the first drop of uranyl formate. Allow the second drop of uranyl formate sit for 1 min and then dry with filter paper. Allow the grid to fully air dry (about 1 min) before storing.

Note: This staining protocol may not be ideal for all protein–detergent combinations. Different concentrations of uranyl formate stain and different lengths of time for stain exposure should be tested if the steps above do not provide a stain with good contrast.

7.1.3 Image the grids on an electron microscope (see materials list) to check the protein particle quality. Ensure that the micrographs show numerous particles that are homogenous is general appearance and distribution, display good contrast, and match the predicted size of the target protein. Generate preliminary, low-resolution, 2D classifications using 50–100 micrographs (see data processing – section 10) to check that the particles represent different views of a single consistent structure.

Note: Micrographs and preliminary 2D classes of sufficient quality, as described above, are a strong indicator that the protocol has been sufficiently optimized for protein purification. Preparation and screening of cryo-EM grids is warranted at this point.

8. EM sample preparation

8.1.1 Glow-discharge a gold holey carbon grid (see materials list) as described in step

8.1.2 Apply 2.5 µL of the concentrated hTRPC3 protein sample (5 mg/mL) onto the grid. Blot the grid for 1.5 s using a blot force of 1 and a wait time of 5 s at 100% humidity and 4 °C, then plunge the grid into liquid ethane cooled by liquid nitrogen using a vitrification machine.

Note: The humidity, temperature, blot-force, blot time, and wait time listed here were used for our hTRPC3 study. They may need to be changed to produce optimal vitreous ice for other proteins and detergents.

8.2 Screen frozen grids for optimal ice conditions using a cryo-EM microscope (see materials list) and manually view regions of thick ice (grid squares that appear smaller and darker), thin ice (grid squares that appear larger and brighter), and medium ice.

Note: Thicker ice often holds more particles, while thinner ice often yields better contrast and resolution. Use manual screening of images to determine which ice conditions results in a large number of monodispersed particles with good contrast and resolution. Once good conditions are verified, move to image collection on a 300-kV cryo-EM microscope (see materials list).

9. EM data collection

9.1.1 Using an automated acquisition program, record image stacks in super-resolution counting mode with a binned pixel size of 1.074 Å on an electron microscope operated at 300 kV with a nominal magnification of 130,000× direct electron detector.

9.1.2 Dose-fractionate every image to 40 frames with a total exposure time of 8 s, with 0.2 s per frame and a dose rate of 6.76 e^{-} Å^{-2} s^{-1} (nominal defocus values varied from 1.0 to 2.5 µm in our experiment)."

6) The section on EM data processing consists of 12 lines. This is a very involved and protracted process relying on multiple software components and numerous decisions on the part of the investigator, based on intermediate results obtained. The level of detail provided is, again, probably not necessary for the expert, and not very useful for a novice. Likewise, "Build a model." Really? How? Using what software?

Reply: We recognize that the data processing section in our protocol provides only an overview of how to convert raw micrograph data into a high-resolution model. We agree that the many software components involved and the need to make numerous intermediate decisions within the process will require considerable explanation in order to be properly executed by a novice. To provide such extensive and nuanced detail is beyond the scope of this 10 page protocol. We have included several references (22-30) within our protocol step descriptions in order to aid novices in finding the necessary resources to learn the proper use of each software component.

7) "Apply FSC curves to calculate the difference between the final model and EM map for validation of the refined structure. Evaluate the geometries of the atomic models" The procedure described refines the model, not the structure (map). There is nothing about the procedure used to estimate the resolution by the FSC of halves of data set. There is mention of the "gold standard" in results, but no indication of what is correlated with what.

Reply: We have rewritten the protocol and results sections pertaining to FSC curves in order to clarify how and why FSC curves and the "gold-standard" cutoff are used to determine the resolution of the final structure.
Lines 565-571: "During refinement, the resolution was held to a lower limit than the resolution estimated for the final reconstruction. Three-dimensional Fourier shell correlation (FSC) was used to measure the normalised cross-correlation coefficient between two independently generated 3D maps (each using half of the data set) over corresponding shells in Fourier space (as a function of spatial frequency). We employed a soft mask of 4.3 Å from

the reconstruction and an additional 4.3 Å cosine soft edge along with a low-pass filter of 10 Å, then used the gold standard Fourier shell correlation (FSC) 0.143 cutoff threshold. This was used for final resolution reporting."

8) Finally, much of what is given in "results" has already been given in the protocol or should be. Put all the needed information to repeat the procedure in one place and confine Results to results.

Reply: We have reviewed the results section and moved any descriptions of protocol action, not explicitly necessary to understanding the results shown into the Protocol section. Example:
Results Lines 437-442: "A schematic overview of the protocol for expression and purification of hTRPC3 is shown in Figure 1A. An image of the hTRPC3 bacmid plate with ideal white colonies, similar to the one selected for bacmid DNA purification, is shown in Figure 1B. We found that 48 h is ideal for clear Bluo-gal staining while maintaining the presence of isolated colonies. Peak production of P2 virus for hTRPC3, as visualized by GFP fluorescence, was seen after 4 d of infection in sf9 insect cells (Figure 1C). "
Protocol Lines 123-131:
"1.1.4 Incubate the plate for 48 h at 37 °C.

Note: The Bluo-gal indicator stains colonies still expressing lacZ (vector insertion unsuccessful), allowing for selection of white (successfully transformed) colonies.

1.1.5 Carefully select an isolated white colony, avoiding any white colonies that are in contact with blue colonies, and grow cells overnight in 6 mL of acmid LB medium (50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline) at 37 °C in an orbital shaker at 225 rpm."