**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.”

***3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”***

**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)”

***5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Cellfectin, Freestyle, Superpose6, Parafilm, etc.***

**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.**”**

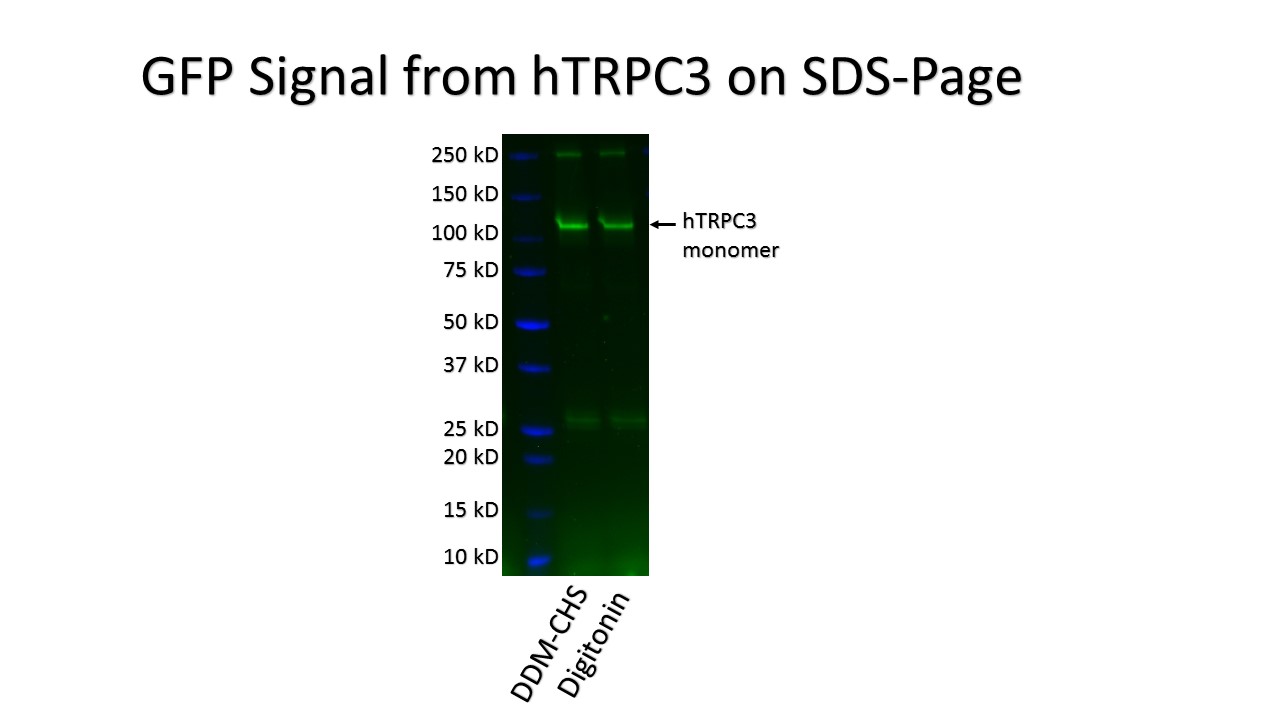
Lines 669-671: **“**The screening of additives, such as EDTA in the case of hTRPC3, is important for discovering a purification condition that results in intact and homogenous protein, probably by removing the calcium from hTRPC3, which is permeable to calcium**.*”***

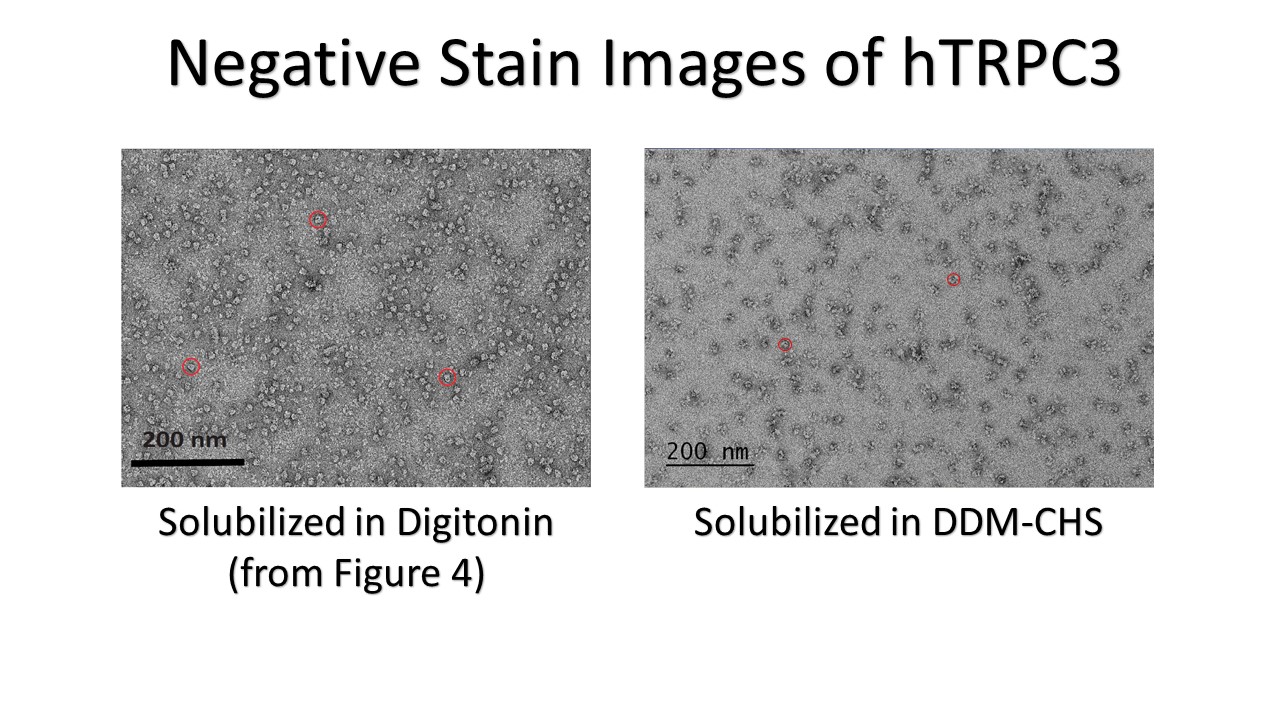
***13) Line 299, "using a micro…" is better.***

**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.

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***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.”