I would like to thank the reviewers for the valuable feedback that will help improve the manuscript. Please find below the point-by-point responses to the comments of the reviewers.

Reviewers' comments:

Reviewer #1:
Manuscript Summary: The author precisely explained methods to measure H+ currents through the mitochondrial inner membrane. Two different examples were demonstrated: one is H+ current through UCP1 in bran fat mitochondria and the other is the one mediated via AD/ATP carrier in heart mitochondria. This is an excellent method paper and very much helpful for mitochondria researchers.

I thank the reviewer for the compliments. The technique described here was optimized by the Kirichok laboratory, which I learned during my postdoctoral training.

Major Concerns: I have no major concerns
Minor Concerns:
1. With the TMA-based pipette solution with 2-4 mM Cl, I am afraid that adjusting zero-current level might be difficult. Did the author use a 3 M KCl-agar salt bridge for the pipette electrode or use conventional Ag/AgCl wire?

This is correct. We use an agar bridge inserted into a microelectrode holder with a 2 mm Ag/AgCl pellet as a reference electrode. The Ag/AgCl pellet molded into the body of the holder provides a more stable low-noise baseline than that obtained with a silver wire. I now indicate in the text the type of pipette holder holding the agar bridge and have referenced the catalogue number in the Excel file of the material.

2. Please explain how the experimental temperature was controlled.

We do not precisely control the temperature of the perfusion chamber with a heating plate. We patch in a room where the temperature is fairly stable and only fluctuates between 22 and 24°C.

3. In my experience, it may be difficult to transfer a thin coverslip (0.1 mm thickness) to the recording chamber. Please explain more how the author performed the transfer.

We use a standard thin microdissection forceps with a curved tip. The coverslip can be broken, especially when learning, but after several tries it is really easy to insert it gently into the bottom of the chamber. I have added a line in the protocol explaining the transfer of the coverslip into the chamber.

Reviewer #2:
Review according manuscript: "Use of the Patch-clamp Technique to Study the Thermogenic Capacity of Mitochondria" by Ambre Bertholet.

Manuscript Number: JoVE62618

In these manuscript, author has been described patch-clamp method with main steps in measuring H+ leak across the inner mitochondrial membrane as a new approach to study the thermogenic capacity of mitochondria.
In seems that patch-clamp methodology applied to mitochondria enabled the opportunity to direct study of proton leak through the inner mitochondrial membrane. It allows describe biophysical and pharmacological properties of mitochondrial transporters like uncoupling protein specific of brown and beige fats. This unique approach will provide new insights into the study of mitochondrial thermogenesis mechanisms
The topic is interesting and important, and the findings have a high impact to broad ranged readers. The experiments are carefully designed and well performed. I believe the scientific merit is worthy for publication in a prestigious journal such as Journal of Visualized Experiments. Generally speaking, the paper is sound; however there are some problems. Main body of the manuscript
Introduction:
In my opinion the introduction lacks a paragraph on the patch-clamp technique, especially in the context of experiments performed on the inner mitochondrial membrane. Such a historical outline would be helpful in enhancing the application of the patch-clamp method in the proposed studies H+ leak across the inner mitochondrial membrane. I suggest adding a paragraph regarding patch-clamp technique and possibilities of its use for potassium or chloride measurements through inner mitochondrial membranes.

Thank you for the constructive comments of the reviewer. As suggested, I have added a paragraph in the introduction about the whole IMM patch-clamp technique established by Yuriy Kirichok in a reproducible way during his postdoctoral training in David Clapham's laboratory. The whole IMM patch-clamp can also be used to measure other conductances across the IMM, such as K^+ and Cl^- currents, but the study of these two conductances with the whole IMM patch-clamp has not yet been published. I have chosen to cite in this review only the bibliography of the whole IMM experiment to support the protocol described here. Several seminal works of the group of Ildiko Szabo, one of the world leaders in K^+ conductances in mitochondria using the electrophysiology approach, are not referenced here, as these studies are mainly based on recordings of single K^+ channels from IMM patches and added channels in lipid bilayers.

Protocol
This part is well prepared. Only, photos are missing here. If possible, I recommend show, for example, a patch-clamp setup, French press and the like. Question: is it possible to use the proposed protocol for H+ transport studies in the inner mitochondrial membrane derived from cell lines? Please extend these issue and add adequate comments to the manuscript, not only in the Discussion section.

I have added a picture of a standard electrophysiological setup used for patch-clamp applied to mitochondria (Faraday cage, microscope, air table) and a picture of a French press.
This protocol can also be applied to cell lines. Historically, the first paper reporting IMM recordings was from COS7 cell lines. I referred in the introductory section when I added a paragraph on the whole IMM patch-clamp. I also mentioned it in the discussion section.

Representative Results
Presented results shows possibilities of measurements of H+ current through inner mitochondrial membrane. How author exclude the possibility of measuring other transporters or channel activities

present in the inner mitochondrial membrane? It is also unclear whether the protons pumped out through the respiratory chain are not a disturbance in the proposed protocol? The above issues requires in-depth discussion in part of the discussion.

Pharmacological studies and the use of KO mice were essential for the characterization and identification of the proteins responsible for the H^+ current through the IMM of various tissues. These studies established that UCP1 is the main UCP of brown and beige fat, and AAC in non-adipose tissues. We cannot completely exclude the possibility of the existence of other H^+ currents not mediated by UCP1 and AAC. However, if other H^+ currents exist, their amplitude was beyond the resolution of our electrophysiological setup. We do not measure H^+ pumping of the ETC under the conditions described in this paper. Once we have reached the full IMM configuration, the mitochondrial matrix is washed out by perfusion of the intra-pipette solution. The intermembrane space no longer exists since the step of breaking the OMM with the French Press. No substrates of the respiratory complexes crucial for H^+ pumping through the ETC have been added. It is therefore unlikely to develop active H^+ pumping under our electrophysiological conditions.
I have added a paragraph to the discussion regarding this issue.

Figures
Red/Green figures are challenging for those with color vision deficiencies. I highly recommend not using the red and green. Here are some helpful items to aid in recoloring: Optimizing color maps with consideration for color vision deficiency to enable accurate interpretation of scientific data (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0199239) Coloring for colorblindness (https://davidmathlogic.com/colorblind/#%23D81B60-%231E88E5-%23FFC107 %23004D40)

This is modified according to the visible color for persons with color vision deficiencies.

Tables
The fullness of the protocol would be reinforced by the insertion of the catalog numbers of the used proposed chemicals in the tables. It has been prepared brilliantly in table "Name of Material/Equipment". In case "Potter-Elvehjem homogenizer" I suggest add recommended type and volume.

This is done.

Reviewer #3:
This is a proposal to present very important and challenging technique that deals with measuring ionic currents in mitochondrial inner membrane (IMM) with the patch-clamp in whole-mitoplast configuration. This work is extremely important, and author is one of very few researchers world-wide who mastered this approach. Overall methods described in sufficient details and this referee will be looking forward for the video to be published.

Thank you for the reviewer's encouragement.

I have several points that I suggest need to be addressed in the presentation:

1. Pipettes used for mitoplast patch-clamp are much smaller than regular (whole-cell) pipettes. If will be nice if author gave more details regarding the specifics of these pipettes. I hope that video will include images of the pipette tip shape and fire-polishing process. Also, it might be useful if author could give some suggestions regarding strategies on how to adjust the puller settings to achieve proper shape (perhaps initial puller setting and which parameters can be adjusted to achieve desirable shape).

This is done. The video will show pipette tip shape and the fire-polishing process.

2. It does not appear that Axon amplifier is essential. If this is correct may be author could mention that other amplifiers could be used.

The electrophysiological setup we use requires a standard amplifier allowing low noise recordings. Axopatch is used here but Sutter IPA is cited as great by Polina Lishko's recent article in JOVE. I have never experienced it.

3. In the discussion author could reference the original work by Kirichok and Clapham on MCU in which many of the "tricks" to achieve the successful whole-mitoplast configuration were developed for the first time.

I am very grateful to the reviewer for noticing this missing citation. I am very happy to add the pioneering paper by Yuriy Kirichok which, for the first time, shows reproducible recordings of the whole IMM. Being too focused on protons, I forgot to mention this nice article about calcium conductance through the IMM via the MCU.
I added a paragraph telling the story of the mitochondrial patch-clamp as suggested by the reviewer. I have now described and cited Yuriy Kirichok's first paper on mitochondria and other important papers on calcium currents through the IMM in the introduction.

Reviewer #4:
Manuscript Summary: The methods manuscript is excellent. I would recommend a few small changes: Please make one figure at least with a traditional scale bar with current instead of current divided by capacitance. Even though the latter is correct, the former is more familiar to many investigators and would serve as a nice comparison to other published reports.
Can you show a micrograph of the patch electrode on the mitoplast? How is this imaging done during seal formation? Is it performed by looking through the microscope eye pieces or is it performed using a camera and imaging on a monitor (so it can be further enlarged)? Can a micrograph be shown of the field (showing the usual debris and the mitoplast identified amongst the debris)?
This is not important for this manuscript, but I am curious about the difference in the outward current in the heart recordings and why is it not sensitive to carboxyatractyloside?

I thank the reviewer for these great words.
I have added in Figure 4B the same trace not normalized below that represents the UCP1-dependent H^+ current titrated with different ATP concentrations. The brown fat mitoplast in this figure had a membrane capacitance of 0.624 pF.

I have added two photographs in Figure 2 to show what is seen with the microscope equipped with DIC optics. Figure 2B on the left panel shows a schematic of the formation of the gigaohm seal between the glass patch pipette and the IMM (mitoplast-attached configuration). Figure 2B on the right panel shows a photograph of a mitoplast attached to the glass patch-pipette. The pipette is attached to the lobe of the IMM. We use 60X magnification to visualize the IMM lobe which is less optically dense as compared to the lobe that contains both, IMM and the remnants of the OMM. Once the mitoplast of interest is selected, we approach the glass pipette to the mitoplast using a micromanipulator. The whole procedure (the selection of the mitoplast, glass pipette sealing) are performed under the microscope optics. Figure 2C shows the whole-IMM configuration after breaking the IMM with voltage pulses.

The outward current noticed by the reviewer is a very interesting current. The article published in 2019 demonstrated that the outward current CATR-insensitive is not a H^+ current. Both AAC and UCP1 require free fatty acids to induce a H^+ current. Our data suggest that this current is certainly due to FA anion currents at positive voltage (anion inward). We do not know which protein or proteins are responsible for this transport. We have been able to dissociate the outward current (FA anion) from the inward H^+ current by using Mersalyl, a cysteine modifier. We think there is still an outward H^+ current at positive voltages but the amplitude is below pA. Further research will be needed to characterize and identify this FA anion transporter.