**July 9th, 2018**

**Rebuttal Letter for JOVE Manuscript “Whole Body and Regional Quantification of Active Human Brown Adipose Tissue Using 18F-FDG PET/CT”**

**EDITORIAL COMMENTS**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.  
   ***Thank you for the suggestion. We have made careful edits throughout the manuscript.***
2. Please provide an email address for each author.  
   ***All emails have now been added.***
3. Please rephrase the Introduction to include a clear statement of the overall goal of this method.  
   ***Thank you for the suggestion. We have now revised this section to enhance focus of the introduction.***
4. 3.1 (3.1.1-3.1.3), 3.2 (3.2.1-3.2.3), 3.3, 3.4.1-.4.2, 3.6.1, 3.7, 3.7.1, 3.7.2, 3.8.1.2, 3.9.1, 4.1, 4.2, etc: Please revise the protocol so that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please move the discussion about the protocol to the Discussion.  
   ***The protocol has been adjusted accordingly. The paragraph originally in the introduction that discussed limitations of other methods has also been moved to the Discussion.***
5. For critical computational steps, it would be helpful if software screenshots are provided as supplementary files to match each step.  
   ***We believe that the most critical steps have been highlighted in the current set of figures.***
6. There is a 2.75 page limit for filmable content. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.  
   ***We have reduced the highlighted text from just over 3.0 pages to about 2.75 pages.***
7. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. 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. Please do not highlight any steps describing euthanasia.  
   ***Revised as recommended.***  
     
   8. 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.  
   ***Revised as recommended with detail in multiple steps.***  
   9. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.  
   ***Revised as recommended. We chose to keep some shorter steps to keep the protocol succinct. We believe these steps will make more sense for the video.***  
   10. Discussion: Please also discuss critical steps within the protocol.  
   ***We have added more information about several critical steps in the protocol as suggested.***

**REVIEWER 1 COMMENTS**

Major Concerns:  
  
INTRODUCTION:  
In general terms the introduction lacks of key references and the authors do some assumptions that need to be cited.  
***We now added some key references to address these concerns.***

PROTOCOL  
  
1. It will be helpful to prepare a tutorial/video recording of the screen analysing BAT of one participant. This video could be really helpful for the scientific community.  
***We thank the reviewer for this comment, and we agree that a video version will complement the written instructions of this manuscript, which is the unique approach of this journal. If the paper is accepted, the JoVE staff will provide professional assistance to produce such a video.***  
  
2. In the point that stablished in the protocol section, it is mentioned that you used the option of "Any". However, if you read the instruction of the authors you will find the following sentences:  
  
"Even though in most cases the CT has many more points than the corresponding PET point, only nearest neighbours are used. The "best" choice would be average since that would be expected to be fairly close to the central point. The all choice would be more stringent in that all nearest neighbours would have to be in the fat window. Any is the least stringent as even a single CT point in the fat window would accept the PET point."  
  
Based on that argument, the creator of the software stablished (also co-author of this paper), stablished that the best option will be to use "average" instead of "any" or "all". Justify, why you have selected "any".  
-Do you have any sensitivity analysis of that?  
-How can affect the use of "any" or "all" to the analyses?  
  
If not, I will suggest to replace and re-do it the set of the results based on the average option instead of use any.

***We agree that the option to select Any/Average/All will lead to different results of BAT volume and activity. However, the main objectives of this manuscript are primarily to instruct the readers how to construct the axial ROI’s and navigate through the image analysis software. While we include the description of each of these options, we prefer to leave this choice to the user depending on their specific research goals. We have modified the text as follows: “Select one of the three voxel inclusion criteria (“Any”, “Average”, or “All”). NOTE: “Any” was used in Leitner et al. 2017. For a detailed explanation of other options, refer to petctviewer.org”***  
  
3. Why do you select the range of -300 to -10, when recently a panel of experts (some of the co-authors of this work) proposed -190 to -10?

***We thank the reviewer for this detailed observation. We selected the range of -300 to -10 to be consistent with our last publication using this method (Leitner et al 2017). We have added a clarification to suggest that the user can use the HU range recommended by BARCIST. The text now reads: “NOTE: a -300 HU lower limit and -10 HU upper limit were used in Leitner et al. 2017 and a range from -190 to -10 was also previously recommended (Chen et al 2016)”***  
  
4. I will add a section to "Load" the ROIs that were previously drawn.  
***Revised as recommended in step 2.2. Thank you.***  
5. Based on the BARCIST 1.0 paper, I will add a section named "Reference tissues" and I will show what is (in your opinion) the best way to draw the ROI in cerebellum, liver and descending aorta as reference tissues.

***Thank you for this comment. BARCIST 1.0 is only a recommendation guideline, and there is ongoing debate as to which tissue should be used as reference tissue. The focus of this manuscript is on quantifying human BAT, and thus we do not include the steps to quantifying other tissues. However, we have added the following statement to the discussion to inform readers about analysis of reference tissues: “The PETCT viewer software can also be used to quantify the activity of tissues other than BAT, for instance shivering skeletal muscle which also major plays a role in cold induced thermogenesis (Leitner et al 2017), or various areas of the brain or liver which have been suggested as reference tissues for PET/CT analysis (Chen et al 2016). However, these tissues will have densities and anatomical distributions that differ from BAT and are outside the focus of our current protocol. We direct readers to the consensus document for greater detail on these subjects (Chen et al 2016).”***  
6. Why the cervical region is stablished from C3 to C7 instead of atlas vertebrae to C7? C1 and C2 you can also find cervical BAT.  
***It is possible to find cervical BAT near C1 and C2 vertebrae, however these regions are close to the base of the skull and the jaw, where spillover from brain FDG uptake and skeletal muscle uptake may be problematic. We believe that starting at C3 as the vertebral landmark may be the easiest way to standardize BAT quantification across subjects and across researchers. We have clarified this in the revision. The note for this section now reads: “NOTE: C1-C2 regions may also contain BAT, but BAT detection there is likely to be confounded by high uptake of FDG in brain and skeletal muscle.”***  
7. Which are the problematic zones that should be excluded? Please clarify.  
***Thank you, we believe this is quite an important concept to be made clear through the use of the video which will include these problematic zones in each of the BAT regions. We believe that the screenshots captured in our figures highlight the most common problematic zones as examples.***  
8. In the paper of Leitner et al. 2017 you also measured skeletal muscle activity. Which skeletal muscles have you measured? Maybe, add this section to this protocol?  
***Thank you again for this comment. As stated previously, because the focus of this manuscript is on how to quantify active BAT, we have not included a section on how to measure skeletal muscle activity. However, we have added the following statement to the discussion section to inform readers that it is possible to do this using the software: “The PETCT viewer software can also be used to quantify the activity of tissues other than BAT, for instance shivering skeletal muscle which also major plays a role cold induced thermogenesis (Leitner et al 2017), or various areas of the brain or liver which have been suggested as reference tissues for PET/CT analysis (Chen et al 2016). However, these tissues will have densities and anatomical distributions that differ from BAT and are outside the focus of our current protocol. We direct readers to the consensus document for greater detail on these subjects (Chen et al 2016).”***

9. During the description of the protocol, I will add a short intro before starting every point. For instance 6. Segmenting BAT into individuals Depots. I will describe shortly which is the aim to do that and why is better to analyse BAT from the mask instead of analysed BAT directly from the original data.  
***Thank you for the suggestion. However, due to the strict limitation of space and the format dictated by the journal (see editors comments) we are unable to put a short description before every point in the protocol section. However, for the section that you have mentioned, we added a clarifying note to the beginning of the section and we added further discussion of the segmentation to the discussion. The note now reads: “NOTE: The following section is focused only on quantifying regional depots of BAT17. The steps are not necessary to obtain whole body BAT volume and activity.”***

***The portion added to the discussion is also in reference to your next question (#10) and the new text can be found there.***

10. Which is the main aim to perform a mask? Is it really needed for human BAT quantification?  
***We apologize to the reviewer that this part of the manuscript was not clearer. The mask is not required to obtain whole-body BAT volume and activity, it is only required to quantify BAT in regional depots, as noted in our response to your previous question (#9). BAT from multiple regional depots can occur in each axial slice. The PETCT Viewer software only allows the user to draw ROIs in one plane at a time. We chose to perform the axial slice-by-slice analysis first to identify all active BAT throughout the body. The mask is a re-generated PET image where only voxels identified as BAT retain their SUV value. The SUV value for all other voxels is set to 0. Once generated, the user can create ROIs on the mask in the sagittal plane, rather than the axial plane, which is much easier to separate the seven specific depots that we have previously defined, as seen in Figure 3 (new figure numbering).***

***To address this, we have added several clarifying passages to the text. To section 6 in the protocol, we have added the following: “ NOTE: The following section is focused only on quantifying regional depots of BAT17. The steps are not necessary to obtain whole body BAT volume and activity. Generate a BAT mask in the “brown fat, ROI” editor (Figure 1). NOTE: The mask is a PET image containing SUV values only for voxels identified as BAT with the ROIs created during the previous steps of this protocol. The SUV value for all other voxels is set to 0.”***

***And to the discussion we have added: “As noted in the protocol, BAT is distributed in several distinct anatomical regions, including the cervical, dorsocervical, supraclavicular, axillary, mediastinal, paraspinal, and abdominal depots. These depots are distributed such that one axial slice may contain more than BAT from multiple depots. For instance, an axial slice in the thoracic region can contain BAT from the mediastinal depot (proximal and anterior), paraspinal depot (proximal and posterior, along the spine), and axillary depot (lateral and near the mid-antero-posterior line). Knowledge of these depots can help users create ROIs in the various regions of the body since they occur in pre-described locations are largely contiguous, as described in our protocol. However, because we encourage users to draw only one ROI per slice to avoid ROI overlap, the additional steps of generating a BAT mask and drawing sagittal ROIs is required to separate the previously-identified BAT voxels into the distinct regional depots if information of BAT distribution is desired, i.e. separating mediastinal, paraspinal, and axillary BAT detected in the same axial ROI into depots based on sagital location as shown in Figure 3.”***

***We also hope that the “chapters” available in the video format of this article will also make this section more clear for readers and users.***  
11. I have followed all the protocol and I did not have any problem to do every step. However, from the 6.2 to 6.10 I have not been able to do it and I do not understand the logic behind. Please explain these steps a little bit better.  
***As mentioned in the previous comment from the reviewer, we hope that our revisions help clarify the steps in mapping BAT in different depots.***

12. Although the proposed method is able to reduce the number false positive cases, it should be considered that it might increase the false negatives, since some specific containing-BAT depots might be avoided (e.g. see Figure 1C, in which paravertebral and pericardial BAT depots seem not to be included within the ROIs).  
***This is an excellent point.***

***We have included a note in the discussion to state: “It is also possible that the stringent ROI selection may introduce false negatives since some BAT-containing depots may not be included.”***  
  
RESULTS  
  
1. Several studies (see PMID: 25384777 and 23362317) have addressed that there are several skeletal muscles such as longuis colli, scalene or sternocleidomastoid which can be confused with cervical BAT in a cold exposure. Nowadays, there is a huge gap in literature about how skeletal muscle glucose uptake is quantified (e.g. anatomical delimitation, slice height, applied SUV and HU range), it might be useful for future studies adding to this extensive protocol a section related to this issue.

***We agree that skeletal muscle can play a role in thermogenesis, thus our BAT ROI’s try to stay away from skeletal muscles as mentioned by the reviewer. But as stated previously, the purpose of this article focuses on BAT quantification so we chose not to divert our focus to other tissues. However, as stated in our response to question #8 in the protocol section, we have added some text to the discussion section for readers with possible interest in muscle activity.***

o Please, explain how to exclude these muscles

***In our figures, we show ROIs that try to avoid including muscle groups that tend to experience shivering. We hope to demonstrate these approaches in the video version.***

o Which skeletal muscles would you measure or have you measured in Leitner et al. 2017? I will report how to draw a ROI for these skeletal muscles, because in Leitner et al. 2017 you published glucose uptake of this tissue.

***The deltoid muscle was measured using a spherical ROI in Leitner et al 2017. As we have described above, the purpose of skeletal muscle quantification in that paper was beyond of the scope of this manuscript.***   
  
2. An important result of this study is to add some information about which is the average time to analyse 1 participant. ***We added a sentence in discussion: “The time it takes to complete analysis of a single scan can range from three to eight hours, which gets shorter with practice and experience.”***  
  
3. Other interesting results are how to calculate the total BAT parameters after the delimitation of ROIs.  
***While we agree with this comment in principle, we believe that, due to the different needs of potential users, it is hard to answer this question with any specificity. We therefore did not include a step on post-processing data analysis because it is out of the scope of this manuscript.***   
  
DISCUSSION:  
1. It should be mentioned as a limitation that this protocol is time consuming, specially for large intervention studies.  
***Addressed. See concern #2 in RESULTS section above.***  
2. I agree that 18F-FDG is the most used method to quantify BAT, however this protocol should be replicated in studies using other tracers, for instance 18F-FTHA.  
***We agree with the reviewer that such a replication would be extremely important for the field. However, our group does not perform BAT studies with any other tracer than 18F-FDG. For this reason we have clarified throughout the text (beginning with the title) that the quantification demonstrated here has been done only with 18F-FDG.***   
  
  
Minor Concerns: Abstract:  
- Lines 31-32: It is stated that "BAT´s ability to expend energy highlight a potential target for novel therapies to ameliorate obesity and associated metabolic disorders in humans"… However this affirmation should be toned down ("might highlight a potential target"), and it should be noted that not only the BAT´s capacity to expend energy is a potential target for novel therapies, but also its endocrine role in human metabolism.  
***Thank you, we have reworded the sentence and eliminated the word “highlight” to tone down the sentence. We agree that the endocrine roles of BAT are likely to have substantial influences on human metabolism as well, but for brevity and clarity, we have not included descriptions regarding such roles.***

Introduction:  
- What about the dose use for PET and CT? It is of interest to mention in the introduction that this issue could reduce the overlapping of the PET over the CT.  
***This method has been used by our group on multiple doses of both PET and CT scans. Because these factors are likely to vary across all groups that use this method, we have opted not to introduce any confusion that may lead readers to believe that this analysis is limited by the PET and CT dosages of the scans obtained.***  
- Line 50: It is mentioned that "obesity is attributed to an excess of white adipose tissue (WAT), which stores energy in the form of triglycerides". This sentence might be too simplistic.  
***We agree, there are many factors that contribute to obesity and we have adjusted the text to reflect this. The text now reads: “Obesity is due in part to excess energy stored in the white adipose tissue (WAT) in the form of triglycerides2”***  
- Line 52: BAT also presents a larger innervation…  
- Line 56: "…ATP", long  
***Thank you for these suggestions, we have adjusted the first sentence and shortened the section as suggested. The sentences now read: “Brown adipose tissue (BAT) differs from WAT most notably due to its higher mitochondrial content, smaller, multilocular lipid droplets, distinct anatomical distribution, greater sympathetic innervation, and heat generating ability”***  
- Lines 61-63. These correlations have been reported. However, not consistently (e.g. Blondin et al. 2015 J Phys, Bakker et al. 2014 Lancet End Metab; Lee et al Diabetes 2014). Please state it.  
***Agreed. We thank the reviewer; the appropriate citations have been added.***  
- Line 68: It is stated that "the precise measurements are challenging due to BAT´s unique anatomic location in humans". This sentence should be modified since it could lead to the equivocal believe that BAT is located in a unique anatomical location in humans. In the following sentence (lines 69-70), you properly mention how BAT is distributed through the neck, thorax and abdomen.

***Agreed. As suggested, we changed the word “location” to “distribution”.***  
- Line 74: "This heterogeneity also makes automatic quantification of BAT more difficult than homogenous structures such as the liver" this sentence needs reference.  
***The reference by Chauvie et al, 2018 in Computer Methods and Programs in Biomedicine has been added to support this claim.***  
- Line 78: "The radiolabeled glucose analog 18F-Fluourodeoxyglucose (18F-FDG) is the most widely used tracer to study metabolic activity in BAT". This sentence needs reference (e.g. PMID: 29119565)  
***Thank you for the suggestion. We used the study provided by the reviewer as the reference.***  
- Lines 88-89. You explain how patient motion can affect to the overlapping of both PET and CT images. It would be desirable to mention in the limitations paragraph that the method that you are proposing still have to face with this limitation.  
***The reviewer is correct. We have moved this paragraph to the discussion as suggested by the editor and we have also included that this is a limitation to our method in a separate limitation paragraph of the discussion.***   
  
Protocol  
- Figure 3, should be figure 1 because is the first figure that has been mentioned in the text.  
***We agree and have changed the order of the figures.***  
- Please, explained what is a DICOM file when is the first time that is mentioned.  
***We have added a note in the text that clarifies what is meant by “Dicom image” and “DICOM Path”. The NOTE for this section now reads: “a DICOM (Digital Imaging and Communications in Medicine) is a file format commonly used for medical images and the “DICOM path” refers to the set of folders that contains all raw DICOM images.”***  
- 3.1.2. The order of images that will appear in FIJI is firs PET, CT and Fusion and I will show a screenshot of this.  
***This will be shown in the video. Excess screenshots have not been added to the figures because the video will provide much richer information than many screenshots.***   
  
- Line 180: change "Brown fat ROIs" by "Brown fat, ROIs".  
***Addressed.***  
  
- Line 189: I will show to readers a screenshot of the "Brown fat, ROIs" option.  
***This will be shown in the video. Excess screenshots have not been added to the figures because the video will provide much richer information than many screenshots.***  
- Line 211: Please refer to PMID 29867076 and discuss.  
***Thank you for the suggestion, a line has been provided to refer users to PMID 29867076, and the citation has been added.***   
  
- In the section: [3.9.2.1](http://3.9.2.1/" \t "_blank): slice limits: Please, explain how to do that.  
***Thank you for the suggestion. We modified the text to clarify this step, but we hope that the video will provide additional clarity: “Set the starting and ending “slice limit” to the same slice, so that the ROI will only apply to the current axial slice (e.g. starting slice = 90 and ending slice = 90).”***  
- Line 300: etc and … is the same. Please only use one, I would recommend etc.  
***It is not the same. We have attempted to make the distinction between the two types of deleting clearer.***  
- Line 331: "Thyroid and BAT have the same density". Please reference this assumption. To my knowledge, there is no paper published concluding a definitive radiodensity of thyroid.  
***We agree with the reviewer. In order to refrain from certainty regarding the densities of BAT and thyroid, we have changed that sentence to: “Exclude the thyroid, which may have similar density and activity level as BAT”***  
  
- It is important to stablish that Fiji software is in continuous development and the actualization of this software are automatic, however the researcher needs to update the software every time that they observe the message of update.  
***To address this point, we have added to our sentence that directs the user to petctviewer.org, where all updates affecting the PET CT Viewer can be found. (Section 1.2)***

**REVIEWER #2 COMMENTS:**

Major Concerns:  
  
L92: The authors should clarify what they mean by "user specified regions".  
***We now clarified it to read: “one should apply PET and CT criteria only within the regions of interest (ROIs) that users construct”* Note that this paragraph has been moved to the second paragraph of the discussion section per the editor’s instructions.**  
  
L96: Would the ROIs be drawn on every slice based on experience, the literature, or some other source?  
***We believe that the ROIs should be drawn on every axial slice where active BAT is found based on experience and literature. Although our revised paragraph does not include this statement based on the instructions by the Editor, we hope that this point is made clear in other sections and in the final video.***

L197: The authors refer to "more lenient voxel inclusion". Doesn't this broader selection of voxels create more false positives?  
***Similar to our previous response to Reviewer 1, we clarified that this option should be made by the user. The revised statement is now in Section 5.ii.***  
  
L198: The new/innovative component of this method is not clear. Please, add some more information.  
***Thank you for the comment. We revised the introduction and discussions to clarify our aim for this work.***

L210-212: Don't we have BARCIST for that?  
***The reviewer is correct. We have revised this section to simplify it.***  
  
L214: Shouldn't the authors provide a threshold here? They should also define how this threshold is determined.  
***The reviewer is correct and we have reorganized the sentence to make the upper threshold recommendation based on our previous publication.***  
  
L237: It would be helpful for the reader if the authors explain why that would be the case.  
***We revised this section to address this point by adding “NOTE: It is easiest to have a maximum of one ROI per axial slice. Including more than one ROI per slice may lead to inadvertent overlap. Voxels identified as BAT in overlapping regions would then be counted more than once toward total BAT volume”. It is now in section 3.10.i.***   
  
L240: What does "commonly" mean? Is it not always the case?  
***Thank you for the comment. This has been revised and removed.***  
  
L288-290: Does this refer to identification that the user has done on their own?  
***Yes, this is to visualize the confirmed BAT. We now clarify this in the revision to read “Check the checkbox located beneath “Vol\*mean” so that all voxels deemed to be BAT will be highlighted in blue while the “brown fat, ROI” window is open. NOTE: The SUVmax will appear in red and the adjustable number next to this checkbox dictates the thickness of the highlight.” This is now moved to section 3.8.***  
  
L438: Why is this change to the lower limit needed?  
***We have restated this to read “Uncheck the density (HU) threshold and change the lower limit of the PET (SUV) threshold to 0.01 SUV to exclude any non-BAT voxels which now have an SUV value of 0.” This is now in section 6.2.iii.***  
  
L510-511: Does this mean that everything is done according to the BARCIST?  
***We believe that only users who are interested in performing regional BAT analysis will be knowledgeable in this area. Thus, we removed this statement.***  
  
L519: Does this mean that there is also shivering thermogenesis involved?  
***It is certainly possible that when there is 18F-FDG uptake in skeletal muscle that shivering thermogenesis is occurring. The purpose of this protocol is to focus on quantifying active human BAT, but we now added a paragraph in the discussion addressing this.***   
  
L520: The authors refer to "highlighted several common regions… supraclavicular BAT vs. shivering muscle near borders of air and solid tissue". Does this mean that this was not done thus far?  
***We apologize for the lack of clarity in this part of the text. We did not mean highlight in the way that this protocol was the first to show common false positives. We revised this section to bring more clarity.***   
  
L606-612: The authors should consider briefly indicating the innovative/different component as compared to existing analyses.  
***Thank you for the comment. It is our view that, while this method is not necessarily innovative as we and others have been using it, the step-by-step instructions on how to use the software for BAT quantification do not exist. Furthermore, the JOVE video provides the unique ability to visually demonstrate how to accomplish this, which cannot be fully explained in written instructions.***