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| Janssen Vaccines & Prevention B.V. Archimedesweg 6  2333CN Leiden  The Netherlands |
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July 12th, 2018

Dear Dr. Nguyen,

Please find our revised manuscript entitled **“**A cell-based assay to study antibody-mediated tau clearance by microglia” [JoVE58576] that we hope is now acceptable for publication in Jove. We have taken into account the reviewers’ comments and have added the details requested for the protocol and provided clarifications in the text where requested.

A point-by-point response to the specific editorial comments and comments of the reviewers follows.

Sincerely Yours,

Donata de Marco, PhD

Adrian Apetri, PhD

Janssen Prevention Center, Janssen Pharmaceutical Companies of Johnson & Johnson

**Response to comments**

**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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

We proofread the manuscript.

2*. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*

We removed all trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

3. *Please add more details to your protocol steps. 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.*

We improved the protocol by including more experimental details.

4. *3.1.1: How much is used to wash?*

We added the volume (10 ml) used to wash as example since it depends on the size of the cell flask used.

5. *3.1.2/5.1.2: What volume of trypsin is used? Reaction conditions?*

We added details on the volume of trypsin used (given as example, since it depends on the size of the cell flask used) and the exact reaction conditions.

6. *Please provide RRIDs or lot numbers for all antibodies.*

All antibodies used are Janssen proprietary antibodies that are not commercially available. Therefore, lot numbers are not relevant here.

7. *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 highlighted the essential steps of the protocol in the revised manuscript.

*8. 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.*

We highlighted the essential steps of the protocol in the revised manuscript.

9. *Please do not abbreviate journal titles.*

We used the JoVE Endnote template to format the bibliography.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
*Manuscript Summary:  
This manuscript provided a very detailed protocol about a cell-based assay to study antibody-mediated tau clearance by microglia. It can be very helpful for therapeutic development of tau antibody based immunotherapy for AD treatment.*  
  
*Major Concerns:  
-None*

We thank the reviewer for his/her enthusiasm for our assay.

*Minor Concerns:*

*-line 56, what does "non-productive" mean here for neuroinflammation?*

During a productive inflammation, the proliferative phase predominates causing an infiltrate to appear in the tissue. Such an infiltrate doesn’t predominate in course of AD. This is common knowledge in the field.

-*It will be helpful to include the procedures for preparation of tau aggregation, a key experimental reagent in this assay, instead of Citation of the previous publication.*

We respectfully disagree with the reviewer. This protocol describes an assay in which tau aggregates are a reagent. Preparation of this reagent is described in detail in the referenced publication.

*-line 100, spread out ThT*

We spelled out Thioflavin T in the revised protocol

*-line 108, concentration of (~20?M)*

We added the right unit of measurement.

*-line 111, please specify the power in addition to 65%*

We have specified the sonicator power.

*-line120, please specify the column as PD10?*

JoVe guidelines indicate to avoid using commercial names.

*-line 197, explain why using a different sanitation protocol*

We assumed that the reviewer meant “sonication protocol”. We explained that a mild sonication improves microscopy results.

*-Figure 3, it will greatly beneficial if there will be imaging quantification method or figure provided.*

Microscopy is not used as a quantitative assay, but merely as a means to support observations from FACS analysis.

*-In discussion, it will be very helpful for others if the potential influencing factors that are key for the success of this assay. Also discuss the most important step(s) that controls the variation of the data. For example, how much will the semi-attachment of BV2 impact on the results; is any concern associated with the sickness of tau aggregates to pipets or tubes?*

We added extra discussion on role of cell density in assay reproducibility together with the importance of using stable and well characterized tau aggregates.

**Reviewer #2:**  
*Manuscript Summary:  
Dear Authors you paper is very interesting, some small clarification are necessary before final full acceptation.*

*Mayor/Minor concerns:*

*-You should add "TM" or "(R)" when the name of the product is a commercial one*

The editorial policy of JoVE actually prevents use of trademark (™) and registered (®) symbols.

*-You should add full name of the products then put their acronyms between parenthesis and after that you can use the acronyms. For example "EDTA", "PBS" etc etc*

We spelled out the names the first time used in the text and then replaced with acronyms.

*-In the sentence "2.1. Resuspend tau aggregates in 0.1 M sodium bicarbonate buffer (NaHCO3), pH 8.5 to a 108 concentration of 1 mg/mL as assessed by initial absorption of tau monomers at 280 109 nm using an extinction coefficient of 0.31 mL mg-1cm-1" is not clear. Is there a temperature that should be maintained during this procedure? If yes, could you please explain how you preserve your cold temperature.*

We re-wrote this sentence to me make it clearer. Concerning the temperature, we don’t control it, but we avoid over-heating during sonication keeping the aggregates on ice.

*-Line 119:"RT", you mean room temperature? Follow my suggestion below. Furthermore later in your paper you write "room temperature". Please try to be consistent with you nomenclature, it is important for well understanding your paper.*

We replaced “RT” with “room temperature”.

*-Line 125: remove "then", please*

We have removed the word “then”.

*-Line 131: "Store the labeled protein at -20°C" Please say clearly were you store your product, frigo? refrigerator?*

We specified that both -20 and -80 are freezers.

*-Line 145: please add between parenthesis "SFM", I think that this is the acronymous of serum-free medium*

We put “SFM” in parenthesis here and used it as acronym later on.

*-Line 162: "rcf": You should write it in extend and then cite by using the acronymous ref*

We spelled out “rcf” the first time used in the text and then used it as acronym.

*-Line 217:"For high quality images, use a 63x water immersion objective and acquire 0.5 μm 217 planes (20 per Z-stack) per imaged field" Which type microscope do you use? I think that you use confocal but it better to clarify.*

Point 5.3.6 specifies that we used a confocal microscopy. The materials table gives also further info on vendor name and catalog number of the microscopy used.

*-Lines 224 to 226:"In particular, we used a chimeric version (mouse IgG1 Fc region) of CBTAU-28.1. This human antibody binds to the N-terminal insert region of tau and is able to bind in vitro generated tau fibrils" Sorry but i did not find this antibody cited in your experiment...Why you are talking about something that you did not use ?*

The protocol refers in a general way to the incubation of antibody and tau aggregates. In the representative results, we give an example of the kind of results obtainable using the described assay and in particular we described results for one of our ant-tau antibodies: CBTAU-28.1.

**Reviewer #3:**

*Manuscript Summary:*

*The manuscript by De Marco et al., presents a detailed description of an in vitro based assay to examine the BV2 clearance of tau through antibody dependent mechanisms. They provide good descriptions of their assay with two different readouts (FACS vs. microscopy). While the majority of methodology employed is appropriate there are some methodological concerns. Finally, the use of BV2 cell line alone is inappropriate to achieve the goals stated in the abstract and discussion sections. If concerns are addressed I believe this manuscript will significantly add research and be of great interest to the Alzheimer's field.*

*Major Concerns:*

1. *-The use of BV2 cells as the only cells in the screening is significantly problematic. BV2 cells represented a significant advance in creating an easy to use microglia cell line resource when they were created in 1990. However in the nearly 3 decades since substantial technical and methodological innovations have made the isolation and culture of primary mouse cells significantly easier and new innovations have created protocols for human iPSC derived microglia like cells. Two recent studies (PMID: 29788964, PMID: 27400875) have shown what many in the field have known for years that BV2 baseline transcriptional signature and activation signature is dramatically different from primary microglia in vitro. No in vitro assay will be analogous to in vivo function but the microglial field in particular has realized the need to move towards better practices in order to get as close as possible to in vivo signatures. BV2 cells are sufficient for first pass analyses before proceeding to primary cells but not as sole analysis. The authors need to perform their assays in primary cells as well to recapitulate the results. If the results do not recapitulate in primary cells then the usefulness of this assay and the results obtained for development of future therapies is questionable. Finally validation of results is not sufficient to justify use of BV2 cells. The field needs to move beyond models simply because of ease of use when they do not significantly resemble their in vivo counterparts.*

*Particularly concerning for this paper and the focus on Fc receptor dependent functions is that quick glance at the supplemental table in PMID 29788964 reveals large differences in the expression of Fc receptors between BV2 and primary cells.*

1. *The authors claims of learning from BV2 mouse cells about development of human therapies is also problematic because of the significant difference between human and mouse Fc receptors and their function. Several protocols have emerged in the past year for differentiation of human iPSC to microglia-like cells. Of these protocols the manuscript from Albud et al., 2017 (PMID 28426964) produces large numbers of cells from minimal starting cells. The protocols are quick and highly reproducible and avoid the cross-species comparison and allow for use of patient specific lines to test differential therapeutic efficacies.*

We thank the reviewer for these insightful comments. The reviewer’s request to use alternatives for BV-2 cells is essentially a request to develop a different assay. Like any in vitro assay, it is a model of a more complex in vivo situation that will always require further study (ultimately in humans). As with any model, there is a balance between representativeness and practical applicability. While the relevance of any model system to answer a specific scientific question can be debated, the robustness of the current assay cannot be questioned. Nevertheless, in recognition of the limitations of the BV2 cells, we have revised the intro and discussion to narrow our claims.

1. *The FACS analysis either needs better description of controls and added controls.*

* *Creating histogram gate directly off the singlet gate is problematic. It means that even cells that failed to uptake tau are included and fails to gate out autofluorescence prior to analyzing fluorescent intensity.*

In our analysis we don’t create gates on the fluorescence histogram plot, but we show results as MFI of the singlet gate. This because we don’t observe a two picks/populations histogram for partially positive samples, but rather a shift of the pick which varies based on level of uptake. Comparison to BV-2 cells alone allows exclusion of cell autofluorescence.

* *Analysis of percentage of fluorescent cells is important characteristic that should also be examined.*

We added an example of analysis showing the percentage of fluorescent cells. We updated figure 1and 2 and corresponding legends to describe this analysis.

* *Following controls should be shown in methods and used to accurate quantify the internalize pHRodo signal:*
* *BV2 cells without any immune complexes (elim autoflurescence)*

We added this control in the revised figure 2.

* *BV2 cells fed unlabeled tau immune complexes (elim autofluorescence)*

While this is a useful suggestion, we don’t consider this a crucial control given our assay’s good experimental window that allows a strong separation between positive and negative samples.

* *Figure 1 also fails to state what the difference is between the two sets of panels (different antibodies, different replicates of same thing, etc)*

We amended legend to figure 1 to provide the requested information.

* *Singlet gate looks little strange (should be pure rectangular and narrow gate along the diagonal and not odd shape it currently is).*

A rectangular gate rather than a freely shaped gate doesn’t change the analysis as long as it excludes cells which follow outside the main population give their increased FSC area.

1. *The microscopy based assay has several issues. First and foremost, there is no quantification of the microscopy. This needs to be presented as comparison to the FACS based assay. Second, there is clearly significant autofluorescence in the microscopy assay (tau-Hoechst double labels with different pattern than tau that colocalizes with lysosomes).*

We don’t use the microscopy in a quantitative way, but merely to support observations from FACS analysis. Using the microscopy for quantitative analyses would require development of a separate assay altogether.

*Additionally, the authors do not present the same crucial controls on Fab antibody fragments that they use with the FACS assay. These are important controls and need to be included.*

We added the requested additional controls to the revised version of our manuscript.

*Minor Concerns:*

1. *Cells should ideally be serum starved overnight or 24 hours before incubation with tau-immune complexes to avoid problems with serum presence in phagocytosis assays and aid cells normalization to serum-free conditions.*

Based on our experience, an overnight/24hrs serum starvation drastically affect BV-2 adherence to the plate, which is crucial for assay success especially in the microscopy read-out.

1. *Use of word proved should be avoided in scientific publications. Please replace with more appropriate word in abstract.*

We replaced the word “proved” with “shown”