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Dear Editor,

Please find enclosed a revised version of the manuscript entitled "**Labelling of extracellular vesicles for migration and uptake monitoring in cartilage explants.**" by Maria Antònia Forteza-Genestra, Miquel Antich-Rosselló, Francisco Gabriel Ortega, Guillem Ramis-Munar, Javier Calvo, Antoni Gayà, Marta Monjo and Joana Maria Ramis, Manuscript ID: **JoVE62780**, which we hereby submit to be considered for publication in *Journal of Visualized Experiments.*

In preparing this new version, we have taken into account all the editor and reviewer's suggestions. We also enclose an explanation of how the points have been dealt with.

We believe the manuscript has gained after taking into account the editor and reviewer's points. and we believe that the revised version of the manuscript will be of interest to the readers of *Journal of Visualized Experiments.*

Yours sincerely,

Joana M. Ramis

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. Please define all abbreviations at first use.

Manuscript have been proofread and abbreviations have been defined after first use.

2. Please adjust the word count of the abstract to be 150-300 words.

Abstract has been adjusted to 150-300 words.

3. Please revise the following lines to avoid overlap with previously published work: 47-48, 49-50, 52-54, 283-284, 288-289, 290-291, 293-294,

According to the editor's suggestion, these lines have been revised to avoid overlap with previously published work.

4. 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.

For example, Sepharose etc

In the new version of the manuscript, all commercial language has been removed and properly referenced in the Table of Materials.

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

In the new version of the manuscript all sentences including personal pronouns have been rewritten.

6. Please ensure 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 include all safety procedures and use of hoods, etc.

Imperative tense has been used and safety procedures have been added in the new version of the manuscript.

7. Instead of the heading "Previous steps", number it as 1 and called it something like column preparation. Then, refer to this as section 1 later.

According to editor's suggestion, the "Previous steps" section has been modified.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Thank you for the advice. We have reviewed that everything in the protocol that we would like to show in the video is contained in the manuscript.

9. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

We have highlighted the 3 pages of the protocol text for inclusion in the protocol section of the video.

10. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Scale bars have been included for all microscope images and defined at it figure legend.

11. Please upload Table 1 as an .xls or .xlsx file.

Table 1 uploaded as specified.

12. Please sort the Materials Table alphabetically by the name of the material.

Materials Table has been sorted alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This protocol describes the process of labelling platelet-derived EVs, isolating the labelled EVs, quantifying EVs, and then applying them to cartilage explants to see their uptake into chondrocytic cells over a 5 hour period, using confocal microscopy.

Major Concerns:

Introduction:

- The introduction should provide a more complete overview of the current landscape of using EVs for treating OA in the field of regenerative medicine, and better justify the rationale for the methods presented. Contrary to what appears to be suggested in the introduction (2nd

paragraph), EVs are an intercellular signalling mechanism and EVs secreted by the majority of cell types in the body would not be considered useful in the context of treating OA. The main cell types that have been investigated for producing therapeutic EVs for OA are mesenchymal stem cells (MSCs), as well as some other joint-related cells e.g. chondroprogenitors, immune cells. Progress in this area has been captured by several recent reviews: PMID 33809632, 31689923. These reviews need to be cited in the introduction, together with an expansion on the current preclinical landscape of using EVs to treat OA.

According to the reviewer's comment, we have added the recent reviews to the introduction in order to complete the landscape of using EVs for treating OA in the field of regenerative medicine.

- The rationale for the protocol needs to be better explained. Platelets are not a common source of EVs considered useful for OA treatment, although platelet-related products have been trialled for clinical OA treatment with somewhat conflicting results. These points need to be noted and justified in the introduction with regard to the focus of the protocol.

We agree that compared to other cell types, platelets are not the main source of EVs studied. However, as far as we know, there are some *in vitro* and *in vivo* studies using platelet-derived EVs (PL-EVs) obtaining great results. Some studies with chondrocyte cell culture studies have shown an increase on their proliferation and cell migration due to the activation of Wnt/β-catenin pathway by PL-EVs[1]. Other chondrocyte cell culture studies shown a decrease of the inflammatory conditions after treatment with PL-EV[1, 2] and promoted chondrogenic markers on chondrocytes derived from osteoarthritic patients[2], having shown a dose-dependent effect[1]. In *in vivo* studies using a rabbit model, animals treated with PL-EVs showed higher levels of chondrogenic proteins and less tissular abnormalities[1]. And finally, PL-EVs used with stem cells into articular injured tissue promote their engraftment and cartilage regeneration in intra-articular defects[3]. We have added this information in the introduction section of the new version of the manuscript.

Protocol/Discussion:

- Does this method have a requirement for the initial volume of EV suspension, concentration of EVs, or quality/purity of EVs (e.g. freeze-thaw cycles, isolation through other methods such as commercial kits) to produce the same results (for labelling, isolation, cell uptake, and imaging).

This method does not require a specific initial volume, but one should be aware of which final number of particles are needed in order to use an extra initial volume of EV suspension to concentrate. EV samples are concentrated to reassure that, when resuspended with diluent C and PKH26, all EVs contained in the suspension are labelled whatever the initial EV suspension volume is. A note has been added at the first step of "EV labelling" section.

- In the section "EVs USED AS OSTEOARTHRITIS TREATMENT", is the method for inducing the in vitro OA model verified? It appears that the cartilage explants were subjected to some inflammatory molecules to create an inhibitory environment, but whether this can induce OA-like changes particularly over the short term is debatable. Unless there is evidence provided in this respect, the manuscript should refer to the cartilage explants as being conditioned in an inflammatory environment, or similar, rather than specifically referring to them as a model for OA.

According to the reviewer's suggestion, we have named the model as "inflammation-driven OA", adopting the nomenclature used in the study by Kjelgaard-Petersen et al.[4], that we used as the basis to set up our model.

- It would have been highly relevant to see control group(s) for this protocol and better characterisation of the cells involved in EV uptake. From the microscopy images at 4-5 hours it is somewhat more apparent that the cells had a chondrocytic appearance, although this is not sufficient to define them as chondrocytes. It is also unclear whether these cells will unselectively uptake anything in the medium, e.g. blank lipid nanoparticles, EVs produced by other cell sources (e.g. MSCs), or only preferentially the platelet-derived EVs. Following on from this, it would be useful to verify that this labelling and preparation method can be applied to lipid nanoparticles/EVs from other origins rather than just platelets. This would greatly improve the value of the protocol to other scientists.

Yes, we have already proved that it is possible to label EVs from other sources with this method, but these results are presented in another manuscript which is under review. Please note that in the "EV LABELLING" section we did specify that this method can be used with EV samples from other sources. In fact, we have observed that MSC derived EVs are also uptaken by chondrocytes, but we did not test it with liposomes. We have discussed about it in the discussion section.

Finally, chondrocytes are the only cell type present in cartilage, which at the same time is one of the most avascular tissues. Therefore, since there is not any co-culture nor treatment with other cells and cartilage came directly from the donor, it seems obvious that the cells detected by DAPI are chondrocytes.

- The protocol described is very specific - specific source of EVs and applied to a very specific cell model. Only EV uptake is analysed and only through one imaging method. The usefulness to other researchers appears limited due to lack of descriptions or evidence for the generalisability of this protocol. Additional justifications would be useful.

The main aim of this paper is to set up a protocol to label EVs on one hand, and a method for monitoring its uptake, in this case with cartilage explants. Other sources of EVs can be used using the same labelling protocol, as stated previously. In addition, in another series of experiments performed in our lab we have been able to monitor *in vivo* MSC-EVs distribution labelled following the same protocol in mice.

We have added some comments to justify the generalisability of this protocol in the discussion section of the new version of the manuscript.

Minor Concerns:

- Some of the references in the discussion are very old (<2010s or even <2000s). It is suggested to replace or add to these with more updated references that better reflect the current technologies.

Following the reviewer's suggestion, in the new version of the manuscript references have been updated.

References:

1. Liu, X., Wang, L., Ma, C., Wang, G., Zhang, Y., Sun, S. Exosomes derived from platelet-rich plasma present a novel potential in alleviating knee osteoarthritis by promoting proliferation and inhibiting apoptosis of chondrocyte via Wnt/β-catenin signaling pathway. *Journal of orthopaedic surgery and research*. **14** (1), 470, doi: 10.1186/s13018-019-1529-7 (2019).
2. Otahal, A. *et al.* Characterization and Chondroprotective Effects of Extracellular Vesicles From Plasma- and Serum-Based Autologous Blood-Derived Products for Osteoarthritis Therapy. *Frontiers in bioengineering and biotechnology*. **8** (1), 584050, doi: 10.3389/fbioe.2020.584050 (2020).
3. Liang, C. *et al.* Platelet-Derived Microparticles Mediate the Intra-Articular Homing of Mesenchymal Stem Cells in Early-Stage Cartilage Lesions. *Stem cells and development*. **29** (7), 414–424, doi: 10.1089/scd.2019.0137 (2020).
4. Kjelgaard-Petersen, C.F. *et al.* Tofacitinib and TPCA-1 exert chondroprotective effects on extracellular matrix turnover in bovine articular cartilage ex vivo. *Biochemical pharmacology*. **165** (June), 91–98, doi: 10.1016/j.bcp.2018.07.034 (2019).

Reviewer #2:

Manuscript Summary:

The goal of this protocol is to label extracellular vesicles (EVs) for the purpose of monitoring their migration into cartilage explants and uptake by resident articular chondrocytes. Because there is still a poor understanding of the conditions under which EVs can efficiently reach chondrocytes within the cartilage extracellular matrix to deliver pro-regenerative signals, such a protocol should be of great interest to the field of osteoarthritis (OA) therapeutics. While the described methods can be applied to multiple EV populations, the authors focus on those derived from platelet lysate, which has translational potential for cell-free OA therapy. For EV tracking, the authors utilize a red fluorescent, lipophilic dye, PKH26, that is well established in

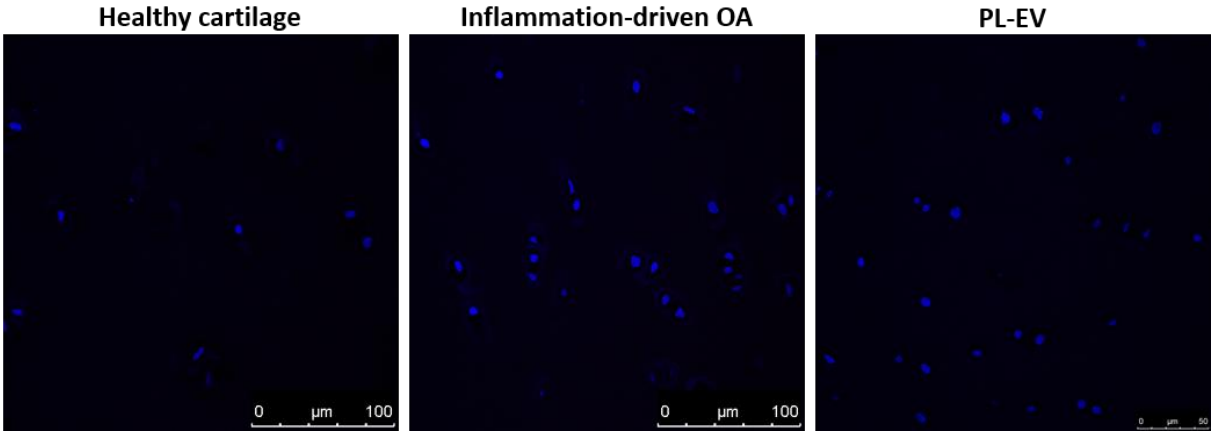
this field. They provide a protocol for labeling EVs with this dye that pays particular attention to post-labeling wash steps, in order to help avoid nonspecific dye uptake into target cells. Nanoparticle Tracking Analysis (NTA) is used to evaluate particle yield and size distribution before and after processing. Of translational relevance, the authors prepare disks of human cartilage obtained from biobanked tissue; to induce matrix degradation modeling OA, the authors challenge these disks with a cytokine cocktail prior to EV introduction. Using this model, the author report a time-dependent increase in red fluorescence within resident chondrocytes, as detected by confocal microscopy.

The general methodology presented here should be instructive to other investigators aiming to label their test EVs with PKH26 and similar lipophilic dyes for evaluation with cartilage disk cultures. The schematic overview of the protocol is well done. However, based on the protocol details and the representative results, there is some concern about the sensitivity of the protocol, as detailed below. Also, the manuscript could use some editing for English grammar and word choice.

Major Concerns:

1. While Figure 2 appears to show red fluorescence within chondrocytes during the 5 hr timecourse after PKH26-labeled EV delivery, as the authors point out, there is also apparent background fluorescence in the "OA Control" group (e.g., 1 hr, 4 hr panels). The authors attribute this background fluorescence to the potential presence of "remnant dye" within the control treatment, which incorporated dye but no EVs. To better clarify the source of background, however, it would have been useful to include a no-dye control (e.g., NTA-PL-EVs) to determine the contribution of autofluorescence at the chosen exposure time(s). For example, there appears to be varying levels of background fluorescence in the control images that is not dependent on time following treatment. This calls into question the overall sensitivity for the approach.

In preliminary studies we have confirmed that no red fluorescence is detected from tissue sections obtained after treating cartilage explants with non-labelled EVs or without EVs (control) under inflammatory and non-inflammatory conditions. Thus, these groups were not included in the series of experiments presented in this manuscript. We include you representative images of our previous results.



2. Related to comment #1, it is not clear why the authors choose to paraffin embed the fixed cartilage disks prior to sectioning for confocal microscopy, as opposed to using frozen sectioning. The alcohol/xylene steps associated with paraffin embedding, subsequent paraffin removal and rehydration are likely to have reduced overall dye fluroescence, thereby reducing the overall sensitivity of the assay. Indeed, this limitation is mentioned in the datasheet for PKH26, based on prior studies cited in that document. This choice should be discussed as a potential limitation to the approach, with frozen sectioning offered as an alternative to improve sensitivity.

Following the reviewer's suggestion, using paraffinized tissue sections instead of frozen tissue sections has been added as a limitation and frozen sectioning has been offered as an alternative to improve sensitivity.

3. Did the authors look at PKH-PL-EV uptake within disks not subjected to cytokine challenge to assess signaling within "healthy" cartilage? There is little information in the literature regarding the extent to which EVs can penetrate normal cartilage, so the result would be of interest to the field by helping define the limits of EV use with regards to the state of cartilage degeneration. On a related note, this deficiency in the literature would be a useful addition to the Discussion section.

We agree with the reviewer comment, EV uptake on "healthy" cartilage would be of interest since little information exist in the literature. However, the main aim of this manuscript is to set up a protocol to label EVs for monitoring their uptake on OA cartilage in order to study EVs as possible cell-free treatment for OA. Therefore, we did not include "healthy" cartilage in our experiments in order to avoid magnification of the experiment up to a scale difficult to be manipulated.

Minor Concerns:

4. Was the efficiency of EV labeling assessed in terms of percentage red fluorescent particles, for example using the NTA instrument? If the instrument available to the authors could not be

used to assess loading efficiency of a red fluorophore, might there be alternative instruments that could accomplish this task?

As far as we know, NTA could be used to assess loading efficiency of a red fluorophore if it disposes of the appropriate laser. Unfortunately, we do not have such a laser in our NTA instrument.

5. Protocol, lines 73-171: These methods describe using "Sepharose" columns, but the schematic (Figure 1) and Table of Materials/Equipment describes using "Exo-spin mini-HD" columns. It is not clear from the Exo-spin product page if they contain Sepharose (this seems unlikely). Please clarify whether the two different terms are referring to the same columns and, if not, please amend the protocol to describe only one product.

We agree with the reviewer comment. Since nomenclature is confusing, it has been corrected in the new version of the manuscript referring in the protocol to Exo-spin™ mini-HD columns merely as columns.

6. Protocol, EVs used as osteoarthritis treatment, lines 197-198: When the authors say they treated the cartilage disks with "oncostatin S", do they mean oncostatin M?

Thank you for pointing this out, we have amended this sentence in the new version of the manuscript.