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Labeling of Extracellular Vesicles for Monitoring Migration and Uptake in Cartilage Explants

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Corresponding Author:	Joana Ramis Universitat de les Illes Balears SPAIN
Corresponding Author's Institution:	Universitat de les Illes Balears
Corresponding Author E-Mail:	joana.ramis@uib.es
Order of Authors:	Maria Antònia Forteza-Genestra
	Miquel Antich-Rosselló
	Francisco Gabriel Ortega
	Guillem Ramis-Munar
	Javier Calvo
	Antoni Gayà
	Marta Monjo
	Joana Ramis
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TITLE:

Labeling of Extracellular Vesicles for Monitoring Migration and Uptake in Cartilage Explants

AUTHORS AND AFFILIATIONS:

Maria Antònia Forteza-Genestra[1,2], Miquel Antich-Rosselló[1,2], Francisco Gabriel Ortega[1,2], Guillem Ramis-Munar[3], Javier Calvo[1,2,4], Antoni Gayà[1,2,4], Marta Monjo[1,2], Joana Maria Ramis[1,2]

[1]Cell Therapy and Tissue Engineering Group, Research Institute on Health Sciences (IUNICS), University of the Balearic Islands, Crta. Valldemossa km 7.5, 07122 Palma, Spain
[2]Health Research Institute of the Balearic Islands (IdISBa), 07120, Palma, Spain
[3]Cellomics Unit, Institut Universitari d'Investigació en Ciències de la Salut (IUNICS), Universitat de les Illes Balears, Palma, Illes Balears, Spain
[4]Fundació Banc de Sang i Teixits de les Illes Balears (FBSTIB), 07004, Palma, Spain

Email addresses of co-authors:

Maria Antònia Forteza-Genestra	(maria.forteza@ssib.es)
Miquel Antich-Rosselló	(miquel.antich1@estudiant.uib.es)
Francisco Gabriel Ortega	(f.ortega@uib.cat)
Guillem Ramis-Munar	(guillem.ramis@uib.es)
Javier Calvo	(jcalvo@fbstib.org)
Antoni Gayà	(agaya@fbstib.org)

Corresponding authors:

Marta Monjo	(marta.monjo@uib.es)
Joana Maria Ramis	(joana.ramis@uib.es)

SUMMARY:

Here, we present a protocol to label platelet lysate-derived extracellular vesicles to monitor their migration and uptake in cartilage explants used as a model for osteoarthritis.

ABSTRACT:

Extracellular vesicles (EVs) are used in different studies to prove their potential as a cell-free treatment due to their cargo derived from their cellular source, such as platelet lysate (PL). When used as treatment, EVs are expected to enter the target cells and effect a response from these. In this research, PL-derived EVs have been studied as a cell-free treatment for osteoarthritis (OA). Thus, a method was set up to label EVs and test their uptake on cartilage explants. PL-derived EVs are labeled with the lipophilic dye PKH26, washed twice through a column, and then tested in an *in vitro* inflammation-driven OA model for 5 h after particle quantification by nanoparticle tracking analysis (NTA). Hourly, cartilage explants are fixed, paraffined, cut into 6 µm sections to mount on slides, and observed under a confocal microscope. This allows verification of whether EVs enter the target cells (chondrocytes) during this period and analyze their direct effect.

INTRODUCTION:

Osteoarthritis (OA) is an articular degenerative disease that implies a progressive and irreversible

inflammation and destruction of the extracellular matrix of the articular cartilage[1]. Although various forms of arthritis have numerous treatments[2–4], these are restricted by their side effects and limited efficacy. Tissue engineering techniques using autologous chondrocyte implantation are routinely applied for the regenerative treatment of injured cartilage in early OA cartilage lesions[4]. Cell-based therapies are restricted mainly due to the limited number of phenotypically stable chondrocytes or chondroprogenitors capable of effectively repairing the cartilage[3]. Therefore, the development of new therapeutic strategies to prevent disease progression and regenerate large cartilage lesions is of paramount importance.

Extracellular vesicles (EVs) have been suggested as a treatment for OA by different authors[5,6]. EVs are membranous bodies secreted by the majority of cell types, are involved in intercellular signaling, and have been shown to exert stem cells' therapeutic effects[7–9], due to which they have recently elicited interest in regenerative medicine[10]. EVs derived from mesenchymal stromal cells (MSCs) are the main therapeutic EVs investigated for OA, although other joint-related cells have been used as EV sources, e.g., chondroprogenitors or immune cells[11,12].

Platelet concentrates, such as platelet lysates (PLs), are used to enhance wound healing in different injuries, such as corneal ulcers[13–15] or in tendon tissue regeneration[16], because of the hypothesis that the EV component of platelet concentrates may be responsible for these effects[17]. Some studies related to joint-related diseases use platelet-derived EVs (PL-EVs) as a treatment to ameliorate osteoarthritic conditions. PL-EVs improve chondrocyte proliferation and cell migration by activating the Wnt/β-catenin pathway[18], promoting the expression of chondrogenic markers in osteoarthritic chondrocytes[19], or showing higher levels of chondrogenic proteins and fewer tissular abnormalities in osteoarthritic rabbits treated with PL-EVs[18].

EVs contain proteins, lipids, and nucleic acids that are liberated to the target cell, establishing cell-to-cell communication, which is the main feature related to their therapeutic applications[20]. The effects of EVs depend on their reaching cells and subsequent cargo release. This effect can be confirmed indirectly by changes caused in cells, such as metabolic activity or gene expression modification. However, these methods do not allow the visualization of how EVs reach cells to exert their function. Thus, this paper presents a method to label these PL-derived EVs to be used as a treatment for inflammation-driven OA cartilage explants. Confocal microscopy was used to monitor EV uptake and progression to the chondrocytes present in the explants in a time-lapse of 5 h.

PROTOCOL:

NOTE: Cartilage explants were obtained from the IdISBa Biobank (IB 1995/12 BIO) in compliance with institutional guidelines after ethical approval of the project by the CEI-IB (IB 3656118 PI).

1. Column preparation

1.1. Equilibrate columns following the manufacturer's instructions or as follows:

1.1.1. Remove the column cap and equilibrate the column. Remove the storage buffer by elution.

1.1.2. Wash the column 3 times with 2.5 mL of phosphate-buffered saline (PBS). During each wash, wait for the column to absorb the whole volume.

NOTE: Do not let the column dry.

1.1.3. Cover the column with the cap after the last wash and until sample preparation.

2. EV labeling

NOTE: This EV labeling protocol uses a PL-EV sample previously isolated by size exclusion chromatography (SEC) with previously described conditions[21,22]. However, any EV sample from any source may be used with this protocol.

2.1. Concentrate the EV sample and the control (PBS) using a concentrating tube.

2.1.1. Place the samples in a 15 mL or 500 µL concentrating tube, depending on the starting volume of the EV sample starting. Centrifuge the tubes according to the manufacturer's instructions until an almost-dry sample is obtained.

NOTE: The control sample is necessary to check for any dye background. Although this method does not require any specific initial volume, it is necessary to know the final number of particles to concentrate an extra initial volume of EV suspension.

2.2. Resuspend the concentrated samples with diluent C. Resuspend EV samples with 200 µL and the control group with 100 µL and transfer them to new 1.5 mL centrifuge tubes.

2.3. Separate the EV sample into two aliquots of 100 µL. Mark one with dye and use it as treatment (PKH-PL-EV); leave the other unmarked but process it (NTA-PL-EV) like the EV sample and use it to quantify the EV concentration by NTA.

2.4. Prepare 2x dye solution, resulting in 8 µM PKH26 solution in diluent C.

2.5. Mix 1 µL of 1 mM PKH26 linker per 125 µL of diluent C in the sample. Prepare a volume required to add to the samples in a 1:1 ratio.

2.6. Add 2x dye solution to PKH-PL-EV and control samples in a 1:1 ratio to achieve 1x dye concentration and 4 µM PKH26 concentration. Add the same volume of PBS to the NTA-PL-EV sample. Incubate for 5 min at room temperature.

2.7. Add 5% bovine serum albumin-PBS solution to the samples in a 1:1 ratio and ensure that the final volume is ~400 µL.

NOTE: This step allows the removal of nonspecific dye interactions or unbound dye.

2.8. Proceed to separate the labeled EVs from the unbound dye and nonspecific interactions of the dye with the column.

3. Labeled-EV isolation

3.1. Remove the cap from the column, add 400 µL of the sample (PKH-PL-EV, NTA-PL-EV, or control), and discard all eluted liquid.

3.2. Wait for the sample to enter the column completely before proceeding to the next step. Add 600 µL of PBS and discard all eluted liquid.

3.3. Wait for the PBS to enter the column completely before proceeding to the next step. Add 600 µL of PBS and collect a fraction of 600 µL in a 1.5. mL centrifuge tube (EVs or control).

NOTE: These steps are needed to remove the excess dye from the samples. Another separation by column is needed to obtain purer EVs. Thus, the following steps should be performed in a new equilibrated column (step 4.1.) or the same column after an initial washing step (step 4.2).

3.4. Prepare the column for a new EV separation step to obtain purer EVs. If it is a new column, repeat steps 2.1. and 2.2. If it is the same column, wash the column with 2.5 mL of 20% isopropanol and then repeat steps 2.1 and 2.2.

3.5. Add 600 µL of previously eluted EVs obtained in step 2.5 to the column and discard the eluted volume. Wait for the liquid to enter the column completely before proceeding to the next step.

3.6. Add 400 µL of PBS and discard all eluted volume. Wait for the liquid to enter the column completely before proceeding to the next step.

3.7. Add 600 µL of PBS and collect a fraction of 600 µL in a 1.5 mL centrifuge tube. Use the EVs and control samples for further analyses or store them overnight at 4 °C.

3.8. Store the used columns for future use.

3.8.1. Wash the column with 25 mL of 20% isopropanol and discard the eluted volume. Wash the column 3 times with 2.5 mL of PBS.

3.8.2. Add the storage buffer removed in step 1.1.1 and wait for the buffer to enter the column. Cover the column with the cap and store at 4 °C until subsequent use.

4. EV quantification

4.1. Prepare 1:1,000 dilutions of the NTA-PL-EV sample and the initial PL-EV sample as described by the following two steps.

4.1.1. Prepare 1 mL of 1:10 diluted NTA-PL-EV and 1 mL of 1:10 diluted initial PL-EV with PBS filtered through a 0.2 µm filter.

4.1.2. Prepare 1 mL of a 1:100 dilution of the previous diluted samples with PBS filtered through a 0.2 µm filter.

4.2. Inject the 1:1,000 diluted NTA-PL-EV sample or the initial PL-EV sample using a sterile syringe into the NTA pump. Follow the manufacturer's instructions and recommendations for particle concentration and size distribution determination.

NOTE: As EV concentration depends on the sample starter volume, it may be necessary to read intermediate dilutions and make adjustments to obtain a correct NTA determination.

5. EVs used as a treatment for inflammation-driven OA

5.1. Wash the cartilage twice with PBS and excise it using a 3 mm diameter biopsy punch under sterile conditions.

NOTE: Perform the procedure from steps 5.1 to 5.6 in a cell culture hood.

5.2. Place the explants in 96-well culture plates with DMEM-F12 medium supplemented with 1% penicillin-streptomycin at 37 °C, 5% CO_2, and 80% humidity.

5.2.1. To establish an inflammation-driven OA model, supplement the cell culture medium with 10 ng/mL oncostatin M and 2 ng/mL tumor necrosis factor-alpha (TNFα).

5.3. Treat the explants with 1×10^9 particles/well of labeled EVs (PKH-PL-EV) or control in cell culture medium supplemented with oncostatin M and TNFα.

NOTE: Measure the volume of the sample containing 1×10^9 particles/well and use the same volume for the control.

5.4. Remove the medium from the 96-well cell culture plates containing cartilage explants. Add 200 µL of the cell culture medium described in step 5.3 to each well.

NOTE: If the 96-well plates have been in contact with fetal bovine serum (FBS), wash each well three times with 200 µL of PBS to remove any EVs from the FBS.

5.5. Stop the *in vitro* assay at different times: 0, 1, 2, 3, 4, and 5 h.

5.6. Wash the cell culture wells containing the cartilage explants twice with 200 µL of PBS.

5.7. Add 100 µL of 4% paraformaldehyde (PFA) to the tissue to fix it for 3 h at 4 °C.

NOTE: Steps involving PFA should be performed in a fume hood following the Safety Data Sheet recommendations.

5.8. Remove the PFA, add 100 µL of PBS, store the fixed tissue at 4 °C, and process the samples within 48 h.

6. Microscopy preparation and visualization

NOTE: This histological procedure consists of dehydration, paraffin embedding, and rehydration steps. These steps may reduce overall dye fluorescence (a limitation mentioned in the datasheet for PKH26). Therefore, other procedures, such as frozen sectioning, may be more suitable for EV visualization by confocal microscopy.

6.1. Embed the fixed tissues in paraffin blocks. Cut the tissue into 6 µm-thick sections.

6.2. Deparaffinize the tissue sections.

NOTE: All steps using xylene should be performed in a fume hood.

6.2.1. Immerse the tissue sections in xylene for 30 min, 100% ethanol for 2 min, 96% ethanol for 2 min, 75% ethanol for 1 min, and finally in distilled water for 30 s.

6.3. Permeabilize the tissue sections.

6.3.1. Prepare a 0.1% Triton-0.1% sodium citrate solution to permeabilize the tissue. Add a 20 µL drop to each tissue section and incubate for 10 min at room temperature. Wash each section twice with 20 µL of PBS.

6.4. On a microscopic slide, add a drop of mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) with an aqueous mounting medium for preserving fluorescence. Cover the slide containing 3 tissue sections from step 6.3.1.

6.5. Incubate the slides overnight at room temperature, protected from light.

6.6. Store at 4 °C, protected from light until confocal microscopy.

REPRESENTATIVE RESULTS:

A schematic overview for EV labeling and uptake monitoring is displayed in **Figure 1**. The particle concentration and EV size detected by NTA in **Table 1** show that the EV concentration decreases during the process due to the purification step performed twice after labeling with the column.

However, the amount obtained is in the optimal range of the number of particles to use for treatment. This particle concentration is used to calculate the volume of PKH-PL-EV and control that are used to treat osteoarthritic cartilage explants.

Once the cartilage explants are treated with EVs or the control group, they are fixed for different periods: 0, 1, 2, 3, 4, and 5 h. Each group is then paraffinized, sliced, and prepared for confocal microscopy with a mounting medium containing DAPI. Representative images for each group at each time point are presented in **Figure 2**, showing how EVs enter the tissue until they reach the chondrocytes and enter them over time.

As seen in **Figure 2**, labeled EVs are already localized around chondrocytes (shown in blue with DAPI staining) after 1 h of incubation (shown in red with PKH26 dye). Some background due to remnant dye can be observed for the control group, which does not have EVs but is processed following the same protocol as the EV sample. These results confirm the success of the protocol to label EVs, which can be used to monitor their migration through tissue in *in vitro* assays, as shown here, and in *in vivo* experiments.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic overview for EV labeling and uptake monitoring protocol. Abbreviations: PBS = phosphate-buffered saline; EV = extracellular vesicle; RT = room temperature; BSA = bovine serum albumin; NTA = nanoparticle tracking analysis; OA = osteoarthritis; TNFα = tumor necrosis factor-alpha; PL = platelet lysate; PFA = paraformaldehyde; DAPI = 4',6-diamidino-2-phenylindole.

Figure 2: Representative images of EV uptake at different times. Confocal representative images taken after 0, 1, 2, 3, 4, and 5 h of osteoarthritic cartilage explants incubated with PKH-labeled EVs or with a control group. Images were taken at 400x. Scale bars = 50 µm. Abbreviations: OA = osteoarthritis; PL = platelet lysate; EV = extracellular vesicle.

Table 1: Characterization by nanoparticle tracking analysis. Abbreviations: OA = osteoarthritis; PL = platelet lysate; EV = extracellular vesicle; NTA = nanoparticle tracking analysis.

DISCUSSION:

EV imaging helps to understand EV properties, such as their release and uptake mechanisms. Their imaging allows the monitoring of their biodistribution and the characterization of their pharmacokinetic properties as drug vehicles. However, EV imaging and tracking may be difficult due to their small sizes, although many imaging devices and labeling techniques have been developed to help researchers monitor EVs under *in vitro* and *in vivo* conditions[23–25].

Two possibilities exist when tracking EVs by optical microscopy: bioluminescence and fluorescence imaging. Both are used to detect EVs within the visible light spectrum (390–700 nm). Bioluminescence is a type of chemiluminescence produced after a luciferase catalyzes the oxidation of its substrate. Although this signal requires an ultrasensitive charge-coupled device (CCD) camera for detection, it has a high signal-to-noise ratio as the signal does not require an

external light source[26].

Fluorescence imaging uses proteins or organic dyes that emit signals after excitation from an external light source. Compared to bioluminescence, fluorescence is easier to detect by a CCD camera. Moreover, in bioluminescence, substrate toxicity and half-life of the substrate's bioluminescence should be considered for real-time EV tracking[27,28].

In contrast, fluorescent protein- and organic dye-based labeling have been used with excellent resolution in optical microscopy. Although the fluorescence intensity depends on EV protein expression levels, the efficiency of EV labeling at membrane domains, and the excitation light source, fluorescence dyes provide stable and strong signals for EV imaging[25]. Most organic fluorescent dyes were initially used for cell membrane imaging. These dyes generally combine fluorophores that label the lipid bilayer or proteins of interest on EVs via different functional groups[29].

One organic fluorescent dye family is the lipophilic PKH dye family consisting of fluorophores with a lipophilic carbocyanine that anchors into the lipid bilayer for fluorescence imaging[30,31]. PKH dyes have been used for *in vitro* and *in vivo* studies as their *in vivo* half-life ranges from 5 to >100 days. Thus, the persistence of the dye *in vivo* may lead to misleading results in studies of shorter duration than the half-life of the dye. However, PKH dyes are useful as tracers to show EV migration[32].

PKH26 is a member of this PKH lipophilic fluorophore family, found in the red spectrum with a peak of excitation at 551 nm and emission at 567 nm. This makes it compatible with other detection channels, such as rhodamine, phycoerythrin, or DAPI[33], allowing the detection of, in this case, the migration of EVs marked with PKH26 toward chondrocytes marked with DAPI. It is important to note that although PL was used as an EV source here, this protocol can be used with EVs from other sources and for other purposes, for example, to track labeled the *in vivo* distribution of EVs.

This protocol has some limitations; for instance, there are some concerns that PKH26 increases EV size, which may affect their biodistribution and cellular uptake. However, in such cases where EV size was increased by PKH26, the labeling procedure was different from that described in this protocol[34]. These researchers did not include the washing and purification steps, thus leading to higher levels of free dye, which could cause the larger EV size. Moreover, the present protocol overcomes this problem by performing a parallel EV purification with and without PKH26. This allows the characterization of an unlabeled EV sample, which was processed identically as the labeled one. Thus, a misleading quantification due to confounding nonspecifically labeled particles (lipoprotein or protein sample contaminants) or by the presence of non-EV particles within the labeling mixture can be avoided, as demonstrated previously[35,36].

In this paper, two cycles of purification were performed by size exclusion chromatography using columns. The first one may be substituted by sucrose gradient centrifugation. However, all EV populations were collected in the same eluted aliquot with this column and not subjected to high

g forces encountered in high-speed centrifugation. However, avoiding centrifugation may lead to a slower process, especially if column washes are needed between separation cycles. Another limitation of this protocol is the need for sample concentration before starting the labeling process due to the limited volume of the column. This handicap may be overcome by using columns with higher loading capacity.

Other EV-labeling protocols describe the use of different dyes; however, their use of ultracentrifugation steps may damage EV integrity[37]. The protocol described here has allowed the monitoring of EV migration and uptake in cartilage explants easily with a confocal microscope without any particular function. Furthermore, this may be extrapolated to other tissues and lipidic samples or conditions, such as an *in vivo* assay.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

1. Column preparation

x3 washes with PBS

Store with cap till use

2. EV labeling

1. EV concentration

Pool of previous isolated PL-EV

Concentrate sample

2-7. PKH26 labeling

Add **diluent C**

Add 2x Dye solution (**PKH26**)

Incubate 5 min at RT

Add **5% BSA-PBS**

Labeled-EV isolation

3. Labeled EV isolation

1-3. Removing excess dye

Load Labeled sample

Load PBS

Load PBS

Equilibrated column

Discard eluate

Discard eluate

4-8. Purifying labeled-EVs

Load

Collect eluate (**EV or control fraction**)

New equilibrated or washed column

Load PBS

Load PBS

Discard eluate

Discard eluate

Collect eluate (**PKH-PL-EV, NTA-PLE-EV or control**)

4. EV quantification

1-2. NTA quantification

Dilute 1:1000 with PBS

Proceed to **NTA**

EVs used as OA treatment

5. EVs used as OA treatment

1-2. Obtaining cartilage explants

3-5. Cartilage explant treatment

DMEM-F12 + **inflammatory condition**:
10 ng/µL Oncostatin S
2 ng/µL TNFα

+ **control**

+ **PKH-PL-EV treatment**

6. Cartilage explant fixation

Finish experiment after: **0, 1, 2, 3, 4 and 5 hours**

x2 PBS washes

PFA 4% 3 hours at 4 °C

Store in PBS at 4 °C

Microscopy preparation and visualization

6. Microscopy preparation and visualization

1-2. Paraffin blocks and cutting sections

3-5. Deparaffinize and permeabilize tissue sections

6-9. Confocal microscopy visualization

Cut 6 µm sections from paraffin-embedded tissues

1. Xylene
2. 100% Ethanol
3. 96% Ethanol
4. 75% Ethanol
5. Distilled water

0.1% Triton-0.1% sodium citrate solution

Incubate 10 min at RT

+ x2 PBS washes

Mounting medium with **DAPI** + coverslip

Incubate at RT overnight protected from light

Take photos or store at 4 °C protected from light until visualization

Figure 2

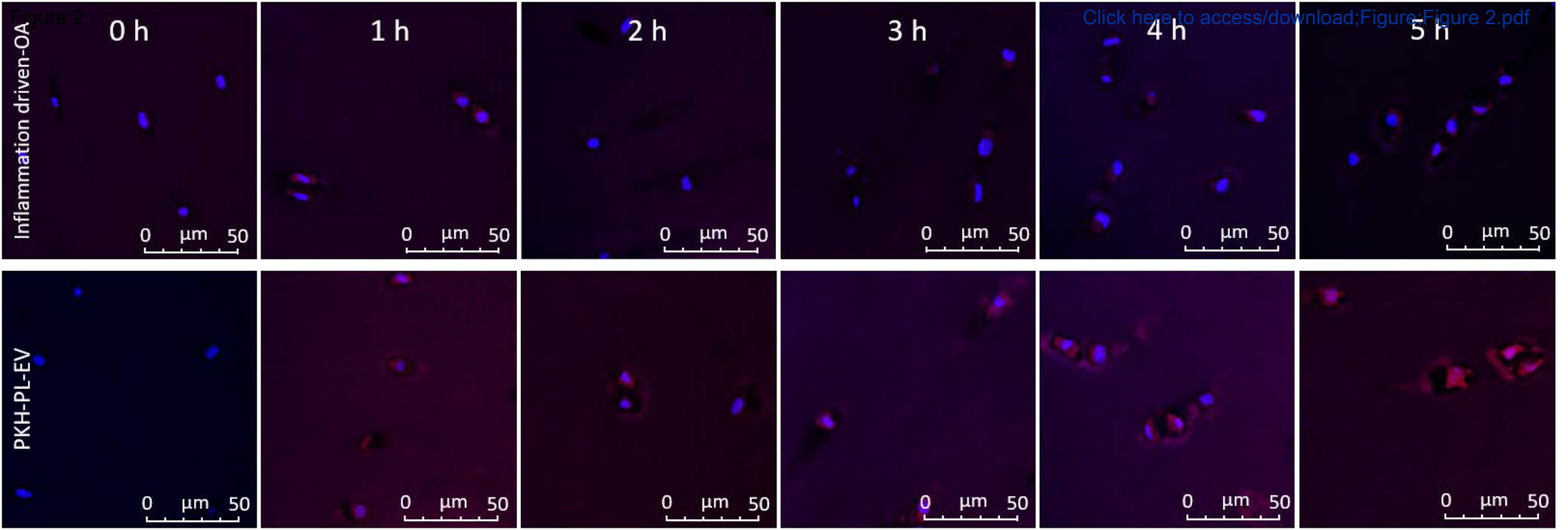


Table 1.

	Concentration (particles/mL)	Particle size (nm)
PL-EVs (initial)	3.03×10^{11}	134.0
NTA-PL-EVs without PKH26 (after the protocol)	8.30×10^{10}	132.0

Universitat de les Illes Balears

Joana M. Ramis, Ph.D.
Research Institute on Health Sciences (IUNICS)
Group of Cell Therapy and Tissue Engineering
University of Balearic Islands
Ctra. Valldemossa km 7.5, 07122 Palma de Mallorca, Spain

e-mail: joana.ramis@uib.es
Phone: +34 971259607
Fax: +34 971173184

Palma de Mallorca, 29th of June 2021

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

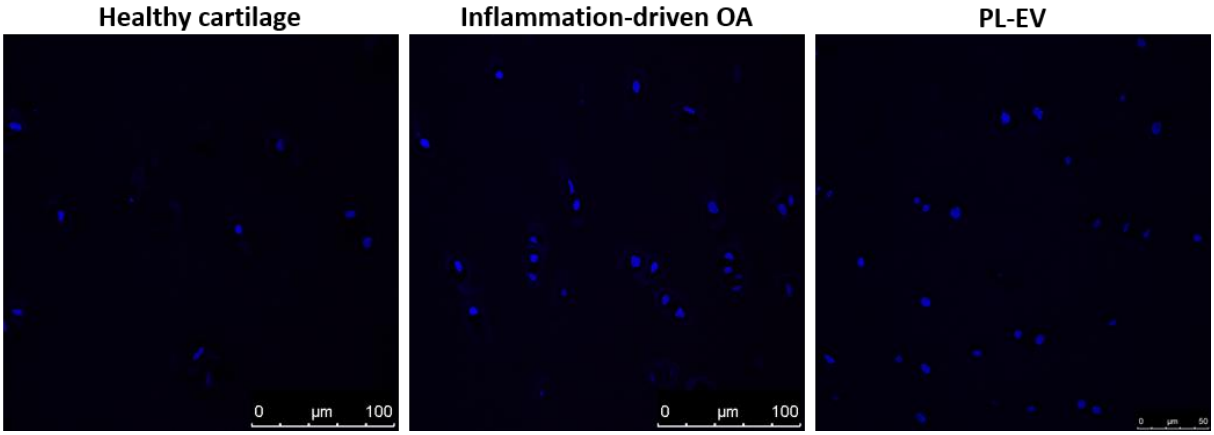
this field. They provide a protocol for labeling EVs with this dye that pays particular attention to post-labeling wash steps, in order to help avoid nonspecific dye uptake into target cells. Nanoparticle Tracking Analysis (NTA) is used to evaluate particle yield and size distribution before and after 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.