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Corresponding Author:	Seena Ajit UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	ska52@drexel.edu
Order of Authors:	Richa Gupta
	Xuan Luo
	Zhucheng Lin
	Yuzhen Tian
	Seena Ajit
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TITLE:

Uptake of Fluorescent Labeled Small Extracellular Vesicles *In Vitro* and In Spinal Cord

AUTHORS AND AFFILIATIONS:

Richa Gupta, Xuan Luo, Zhucheng Lin, Yuzhen Tian, Seena K. Ajit

Department of Pharmacology & Physiology, Drexel University College of Medicine, 245 North 15th Street, MS 488, Philadelphia, USA

Email addresses of co-authors:

Richa Gupta (rg872@drexel.edu)
Xuan Luo (xl432@drexel.edu)
Zhucheng Lin (zl454@drexel.edu)
Yuzhen Tian (yt95@drexel.edu)

Corresponding author:

Seena K. Ajit (ska52@drexel.edu)

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Extracellular vesicles, exosomes, PKH dyes, spinal cord, macrophage-derived exosomes, astrocytes, neurons, microglia

SUMMARY:

We describe a protocol to label macrophage-derived small extracellular vesicles with PKH dyes and observe their uptake *in vitro* and in the spinal cord after intrathecal delivery.

ABSTRACT:

Small extracellular vesicles (sEVs) are 50–150 nm vesicles secreted by all cells and present in bodily fluids. sEVs transfer biomolecules such as RNA, proteins, and lipids from donor to acceptor cells, making them key signaling mediators between cells. In the central nervous system (CNS), sEVs can mediate intercellular signaling, including neuroimmune interactions. sEV functions can be studied by tracking the uptake of labeled sEVs in recipient cells both *in vitro* and *in vivo*. This paper describes the labeling of sEVs from the conditioned media of RAW 264.7 macrophage cells using a PKH membrane dye. It shows the uptake of different concentrations of labeled sEVs at multiple time points by Neuro-2a cells and primary astrocytes *in vitro*. Also shown is the uptake of sEVs delivered intrathecally in mouse spinal cord neurons, astrocytes, and microglia visualized by confocal microscopy. The representative results demonstrate time-dependent variation in the uptake of sEVs by different cells, which can help confirm successful sEVs delivery into the spinal cord.

INTRODUCTION:

Small extracellular vesicles (sEVs) are nanosized, membrane-derived vesicles with a size range of 50–150 nm. They originate from multi-vesicular bodies (MVBs) and are released from cells upon fusion of the MVBs with the plasma membrane. sEVs contain miRNAs, mRNAs, proteins, and

bioactive lipids, and these molecules are transferred between cells in the form of cell-to-cell communication. sEVs can be internalized by recipient cells by a variety of endocytic pathways, and this capture of sEVs by recipient cells is mediated by the recognition of surface molecules on both EVs and the target cells[1].

sEVs have gained interest due to their capacity to trigger molecular and phenotypic changes in acceptor cells, their utility as a therapeutic agent, and their potential as carriers for cargo molecules or pharmacological agents. Due to their small size, the imaging and tracking of sEVs can be challenging, especially for *in vivo* studies and clinical settings. Therefore, many methods have been developed to label and image sEVs to assist their biodistribution and tracking *in vitro* and *in vivo*[2].

The most common technique to study sEV biodistribution and target cell interactions involves labeling them with fluorescent dye molecules[3-7]. EVs were initially labeled with cell membrane dyes that were commonly used to image cells. These fluorescent dyes generally stain the lipid bilayer or proteins of interest on sEVs. Several lipophilic dyes display a strong fluorescent signal when incorporated into the cytosol, including DiR (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide), DiL (1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethyl indocarbocyanine perchlorate), and DiD (1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethyl indocarbocyanine 4-chlorobenzenesulfonate salt)[8-11].

Other lipophilic dyes, such as PKH67 and PKH26, have a highly fluorescent polar head group and a long aliphatic hydrocarbon tail that readily intercalates into any lipid structure and leads to long-term dye retention and stable fluorescence[12]. PKH dyes can also label EVs, which allows the study of EV properties *in vivo*[13]. Many other dyes have been used to observe exosomes using fluorescence microscopy and flow cytometry, including lipid-labeling dyes[14] and cell-permeable dyes such as carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)[15,16] and calcein acetoxymethyl (AM) ester[17].

Studies of sEV-mediated crosstalk between different cells in the CNS have provided important insights on the pathogenesis of neuroinflammatory and neurodegenerative diseases[18]. For example, sEVs from neurons can spread beta-amyloid peptides and phosphorylated tau proteins and aid in the pathogenesis of Alzheimer's disease[19]. Additionally, EVs derived from erythrocytes contain large amounts of alpha-synuclein and can cross the blood–brain barrier and contribute to Parkinson's pathology[20]. The ability of sEVs to cross physiological barriers[21] and transfer their biomolecules to target cells makes them convenient tools to deliver therapeutic drugs to the CNS[22].

Visualizing sEV uptake by myriad CNS cells in the spinal cord will enable both mechanistic studies and the evaluation of the therapeutic benefits of exogenously administered sEVs from various cellular sources. This paper describes the methodology to label sEVs derived from macrophages and image their uptake *in vitro* and *in vivo* in the lumbar spinal cord by neurons, microglia, and astrocytes to qualitatively confirm sEV delivery by visualization.

PROTOCOL:

NOTE: All procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care & Use Committee of Drexel University College of Medicine. Timed-pregnant CD-1 mice were used for astrocytic culture, and all dams were received 15 days after impregnation. Ten–twelve weeks old C57BL/6 mice were used for *in vivo* uptake experiments.

1. Isolation of sEVs from RAW 264.7 macrophage cells

1.1. Culture RAW 264.7 cells in 75 cm^2 flasks in DMEM exosome-depleted medium containing 10% exosome-depleted fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep) for 24–48 h.

1.2. Collect 300 mL of conditioned medium and centrifuge at 300 × *g* for 10 min at 4 °C.

1.3. Collect the supernatant and centrifuge at 2,000 × *g* for 20 min at 4 °C.

1.4. Transfer the supernatant to centrifuge tubes, centrifuge for 35 min at 12,000 × *g* at 4 °C.

1.5. Collect the supernatant and filter through a 0.22 µm syringe filter.

1.6. Transfer to ultracentrifuge tubes and centrifuge for 80 min at 110,000 × *g* at 4 °C.

1.7. Store the supernatant (exosome-depleted medium), resuspend the pellet in 100 µL of 1x phosphate-buffered saline (PBS), and centrifuge for 1 h at 110,000 × *g* at 4 °C.

1.8. Resuspend the pellet in PBS for further characterization using nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) or in radioimmunoprecipitation assay (RIPA) buffer for western blotting.

2. Characterization of sEVs

2.1. Nanoparticle tracking analysis (NTA)

NOTE: The size distribution and particle number/concentration of the purified sEVs from RAW 264.7 cells were measured by NTA.

2.1.1. Dilute the sEVs in filtered PBS to obtain 20–60 vesicles per field of view for optimal tracking.

2.1.2. Introduce the diluted sample into a flow cell using a syringe pump with a constant flow rate.

2.1.3. Take 3–5 videos of 30 s each. Set the shutter speed and gain, and manually focus the camera settings for the maximum number of vesicles to be visible and capable of being tracked and analyzed.

2.1.4. Advance the samples between each recording to perform replicate measurements. Optimize the NTA post-acquisition settings and keep the settings constant between the samples.

2.1.5. Analyze each video using the NTA software to obtain the average size and concentration of the vesicles.

2.1.6. Carry out all NTA measurements with identical system settings for consistency.

2.2. Western blot

2.2.1. Quantify the total protein amounts in sEVs, cell lysates, and exosome-depleted media using a protein assay kit following the manufacturer's instructions.

2.2.2. For cell lysate preparation, culture the RAW 264.7 cells in 75 cm^2 flasks until 80–90% confluent. Detach the cells with 0.25% trypsin, neutralize the trypsin with culture media, and pellet the cells by spinning at 400 × *g* for 5 min. Resuspend the cells in fresh growth medium.

2.2.3. Count the cells using a hemocytometer and transfer 1×10^6 cells to another tube. Wash the cells with PBS twice using the same centrifugation conditions as above and add 50 µL of lysis buffer (RIPA buffer with protease inhibitor cocktail added) to the cell pellet from the final spin.

2.2.4. Vortex the cells and keep them on ice for 20 min. Subject the mixture to centrifugation at 10,000 × *g* for 30 min at 4 °C, collect the supernatant (i.e., the lysate) in fresh microcentrifuge tubes, and keep at −80 °C until use.

2.2.5. Concentrate 2 mL of exosome-depleted media to 100 µL using 3 kDa-cutoff centrifugal filters before quantifying the amount of protein. Mix the sEVs with lysis buffer in a 1:1 ratio, vortex for 30 s, and incubate on ice for 15 min to quantify the amount of protein.

2.2.6. Mix equal amounts of protein (2 µg) of the sEVs, RAW 264.7 cell lysate, and exosome-depleted media with reducing sample buffer.

2.2.7. Denature the samples at 95 °C for 5 min, keep them on ice for 5 min, and spin for 2 min at 10,000 × *g*. Load the samples on a 12% sodium dodecylsulfate-polyacrylamide gel and run the gel at 125 V for 45 min.

2.2.8. Transfer the protein onto a polyvinylidene difluoride (PVDF) membrane at 25 V for 2 h.

2.2.9. Following the transfer, block the PVDF membranes with blocking buffer (see the **Table of Materials**) for 1 h at room temperature.

2.2.10. Incubate the blot with primary antibodies on a shaker overnight at 4 °C.

NOTE: Primary antibodies used were anti-CD81 (1:1,000), anti-alpha-1,3/1,6-mannosyltransferase (ALG-2)-interacting protein X (Alix) (1:1,000, anti-Calnexin (1:1,000), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000).

2.2.11. Wash the blots 3 x 15 min with 1x Tris-buffered saline, 0.1% Tween 20 (TBST), and incubate at room temperature with goat anti-mouse IgG-horseradish peroxidase (HRP)- or donkey anti-rabbit IgG-HRP-conjugated secondary antibodies (1:10,000) for 1 h on the shaker.

2.2.12. Wash the blots 3 x 15 min with 1x TBST, and detect the proteins using an HRP substrate.

2.2.13. Analyze the blots by enhanced chemiluminescence using a western blot imager.

2.3. Transmission electron microscopy (TEM)

2.3.1. Fix sEVs by resuspending them in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB); vortex for 2 x 15 s.

2.3.2. Place a drop of 10 µL of sEV suspension on clean parafilm. Float the carbon-coated formvar grid on the drop with their coated side facing the suspension. Let the membranes absorb for 20 min in a dry environment.

2.3.3. Place the grids (membrane side down) on a drop of PB to wash for 3 x 2 min.

2.3.4. Transfer the grids to 50 µL of 1% glutaraldehyde for 5 min.

2.3.5. Wash the grids with 100 µL of distilled water for 8 x 2 min.

2.3.6. Contrast the sample by placing the grids on a drop of 1% uranyl acetate for 2 min.

2.3.7. Embed the sample with 50 µL of 0.2% uranyl acetate with 2% methylcellulose solution for 10 min on a parafilm-covered ice dish.

2.3.8. Use stainless steel loops to hold the grids and remove excess fluid with filter paper.

2.3.9. Air-dry the grid for 10 min while still on the loop.

2.3.10. Observe under a transmission electron microscope at 80 kV.

3. Labeling of sEVs

3.1. Dilute 20 µg of sEVs in 1 mL of diluent buffer or the same volume of PBS in 1 mL of diluent buffer for dye control.

3.2. Dilute 3 µL of PKH67 or PKH26 dye in 1 mL of diluent buffer and mix by pipetting.

3.3. Add diluted PKH dye to the diluted sEVs and mix by pipetting. Incubate for 5 min in the dark at room temperature. For a dye control, mix the diluted dye with diluted PBS from step 3.1.

3.4. Add 2 mL of 1% bovine serum albumin (BSA) in PBS to the tube with the dye and the sEV mix and to the dye control tube to absorb excess dye.

3.5. Centrifuge for 1 h at 110,000 × *g* at 4 °C. Discard the supernatant, resuspend the pellet in 2 mL of PBS, and centrifuge for 1 h at 110,000 × *g* at 4 °C. Repeat the wash with PBS and resuspend the labeled sEVs or the dye control in an equal volume of PBS.

3.6. Quantify the amount of total protein by the Bradford method.

4. Uptake of sEVs by Neuro-2a cells

4.1. Place 18-mm coverslips in a 12-well plate and plate 10×10^4 Neuro-2a cells in each well in a total of 1 mL of complete DMEM medium containing 10% FBS and 1% pen-strep.

4.2. Change the medium to DMEM exosome-depleted medium when the cell confluency is 80–90%. Add 1, 5, or 10 µg of labeled sEVs in each well for 1, 4, and 24 h for dose- and time-dependent uptake, or add an equal volume of dye control.

5. Primary astrocytic cultures

5.1. Anesthetize 4 postnatal pups 4 days after birth by inducing hypothermia.

5.2. Collect the brains in a 60-mm Petri dish containing ice-cold Hank's Balanced Salt Solution (HBSS) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

5.3. Dissect both cortical lobes and remove the meninges. Mince the tissues with a sterilized blade.

5.4. Transfer the tissues to a 15 mL conical tube containing papain/deoxyribonuclease I dissociation buffer and incubate for 20 min at 37 °C. Swirl every 5 min.

NOTE: For 4 mouse cortices, 9 mL of 7.5 U/mL papain in HBSS is activated at 37 °C for at least 30 min, filtered through a 0.22 µm syringe filter, and mixed with deoxyribonuclease I to a final concentration of 0.1 mg/mL.

5.5. Aspirate the supernatant and add 5 mL of complete DMEM to inactivate the enzyme activity. Carefully triturate to dissociate the tissues with a 5 mL glass serological pipette and a flame-polished Pasteur pipette.

5.6. Pass the cell suspension through a 40 µm cell strainer and centrifuge the cells at 250 × *g* for 5 min at 4 °C. Aspirate the medium and seed the cells in 10 mL of complete DMEM in a 75 cm^2 flask. Replace the supernatant medium with 15 mL of fresh DMEM medium 4 h after plating.

5.7. After 14 days *in vitro*, transfer the flask to an orbital shaker to detach the microglia and oligodendrocytes at 320 rpm for 6 h.

5.8. Trypsinize the remaining astrocytes using 5 mL of the cell dissociation enzyme (**Table of Materials**) for 10 min at 37 °C. Add 5 mL of complete DMEM to inactivate the enzymatic action and pellet the cells at 250 × *g* for 5 min at 4 °C.

5.9. Resuspend the cells in complete DMEM. Seed 5 × 10^4 cells on 12 mm #1.5 coverslips in a 24-well plate.

6. Uptake of sEVs by astrocytes

6.1. When astrocytes reach 80–90% confluency, change the medium to DMEM exosome-depleted medium.

6.2. Add 1 µg of unlabeled, labeled sEVs, an equal volume of dye control, or PBS to the cells. Use the cells for staining 1 h and 24 h after sEV treatment.

7. Immunofluorescence

7.1. Rinse the cells with PBS 3x and fix them with 4% PFA in PB for 10 min at room temperature.

7.2. Wash the fixed cells 3 x 5 min with PB and permeabilize them using 0.1% Triton X-100 in PB for 10–15 min and wash with PB 3 x 5 min.

7.3. Block the cells with 5% normal goat serum (NGS) in PB for 1 h at room temperature.

7.4. Incubate the cells with primary antibodies: microtubule-associated protein 2 (MAP2A, 1:500) for Neuro-2a cells or glial fibrillary acidic protein (GFAP, 1:500) for primary astrocytes in fresh 5% NGS/PB overnight at 4 °C with gentle shaking.

7.5. Wash 3 x 10 min with PB and add fluorophore-conjugated secondary antibodies (Goat Anti-Mouse IgG1, Alexa Fluor 594; or Goat Anti-Mouse IgG H&L, Alexa Fluor 488) in 5% NGS and incubate for 2 h at room temperature on a rocker.

7.6. Wash 3 x 10 min with PB and incubate with 1 µg/mL of nuclear stain 4',6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Wash the cells again 3x with PB.

7.7. Mount the coverslips on #1 slides using an antifade mounting medium. Let them dry overnight in the dark, and store the prepared glass slides at 4 °C until imaging on a confocal microscope.

8. *In vivo* uptake of sEVs

8.1. Perform intrathecal injection of 5 µg of unlabeled or labeled sEVs resuspended in 10 µL of PBS, or equal volume (10 µL) of dye control (as prepared in section 3) into C57BL/6 mice.

8.2. After 6 and 18 h post-injection of sEVs, deeply anesthetize mice by intraperitoneal injection of 100 mg/kg body weight of ketamine and 10 mg/kg body weight of xylazine.

8.3. Perform intracardial perfusion of mice with 0.9% saline to flush out blood, followed by freshly made ice-cold 4% PFA/PB.

8.4. Dissect the spinal cord and fix in 4% PFA/PB at 4 °C for 24 h. Cryoprotect the tissues in 30% sucrose in PB at 4 °C for 24 h or until the tissues sink. Store the tissues at 4 °C until immunohistochemistry.

9. Immunohistochemistry

9.1. Embed L4-L5 spinal cord in O.C.T compound. Freeze on dry ice until completely solidified.

9.2. Section the tissues at 30 µm (cross-sectionally for the spinal cord) using a cryostat, and collect the sections in a 24-well plate containing PB. Wash the sections 3 x 5 min with 0.3% Triton in PB.

9.3. Block non-specific binding sites with 5% NGS in 0.3% Triton/PB for 2 h at room temperature.

9.4. Dilute primary antibodies: Anti-MAP2A (1:500), GFAP (1:1,000), Iba1 for microglia (1:2,000) with 5% NGS in 0.3% Triton/PB, and incubate the sections overnight at 4 °C on a shaker.

9.5. Wash the sections 3 x 5 min with 0.3% Triton/PB, and add secondary antibodies (Donkey Anti-Rabbit IgG Alexa Fluor 488, 1:500, or Goat Anti-Mouse IgG Alexa Fluor 488, 1:500) in 5% NGS/PB for 2 h at room temperature.

9.6. Wash 3 x 5 min with PB, and incubate the sections in 1 µg/mL of DAPI for 10 min at room temperature. Wash the sections 3 x 5 min with PB.

9.7. Mount the sections on a clean adhesive slide (**Table of Materials**) with a fine paintbrush under a light microscope.

9.8. Wet the coverslip with mounting medium. Cure overnight in the dark at room temperature.

9.9. Image under a confocal microscope with the respective lasers.

REPRESENTATIVE RESULTS:

After the isolation of sEVs from RAW 264.7 conditioned media via centrifugation, NTA was used to determine the concentration and size distribution of the purified sEVs. The average mean size of RAW 264.7-derived sEVs was 140 nm, and the peak particle size was 121.8 nm, confirming that most detectable particles in the light scattering measurement fell within the size range of exosomes or sEVs at 50–150 nm (**Figure 1A**). As suggested in the minimal information for studies of extracellular vesicles 2018 (MISEV2018)[23], we analyzed a set of proteins that should be present or excluded from distinct EV populations. Western blotting of sEVs, cell lysate, and exo-depleted media demonstrated that sEV-derived protein samples contained the sEV marker proteins Alix, CD81, and GAPDH. The cell lysate fraction was enriched with the endoplasmic reticulum resident protein, calnexin, which was absent in the sEVs. Thus, calnexin served as a negative marker for cellular contamination (**Figure 1B**).

We next performed dose-response and time-course experiments for sEV uptake *in vitro*. Neuro-2a cells were incubated with a single 1 µg dose of PKH67-labeled sEVs for 1, 4, and 24 h, following which the uptake of different concentrations of sEVs (1, 5, and 10 µg) was examined at 1 h. The results of the NTA indicated that 1 µg of protein on average was equal to ~1 x 10^9 particles. In parallel, PBS, unlabeled sEVs, and dye-alone controls were also tested. We observed that uptake of sEVs occurred at 1 h (**Figure 2A**) and for the 1, 5, and 10 µg sEVs (**Figure 2B**). Fluorescence could be detected at 4 h for 5 and 10 µg of sEVs (**Figure 2C**) post incubation. Next, uptake of PKH26-labeled sEVs by primary astrocytes was examined (**Figure 3**). Maximal fluorescence from sEV uptake in primary cortical astrocytes occurred at 24 h. Unlabeled sEVs did not show fluorescence, demonstrating that sEV autofluorescence does not significantly contribute to false positives (**Supplemental Figure S1A**).

Next, labeled sEVs were intrathecally injected into mice to assess the delivery and uptake of sEVs by different cells in the spinal cord using immunohistochemistry and confocal microscopy. We stained for MAP2 as a neuronal marker, GFAP as an astrocytic marker, and IBA1 as a microglial marker. Neurons **(Figure 4**), astrocytes (**Figure 5**), and microglial cells (**Figure 6**) all took up PKH26-labeled sEVs, and maximal sEV fluorescence was observed at 6 h post-injection. While the sEVs did not always colocalize with the cellular markers, we did not observe any differential uptake by CNS cells. Intrathecal injection with 5 µg of unlabeled RAW 264.7 sEVs or dye control did not show significant fluorescence (**Supplemental Figure S1B**). Fluorescent signals were observed in the meninges, both 6 h and 18 h after the injection of sEVs (**Supplemental Figure S1C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Characterization of purified RAW 264.7 sEVs. (**A**) Size and concentration of sEVs were determined using NanoSight NS300. The particles were tracked and sized based on Brownian motion and diffusion coefficient. The size distribution of sEVs is shown in nm. The concentration of sEVs was expressed as particles/mL. (**B**) Western blot of proteins derived from purified sEVs, cell lysate, and exosome-depleted media using sEV markers ALIX, GAPDH, and CD81. The endoplasmic reticulum protein marker, calnexin, serves as a control to monitor cellular contamination in sEV preparations. (**C**) Transmission electron microscopy demonstrated the size and morphology of sEVs. Scale bar = 100 nm. Abbreviations: sEVs = small extracellular vesicles; ALIX = Alpha-1,3/1,6-Mannosyltransferase (ALG-2)-interacting protein X; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; CD81 = cluster of differentiation 81.

Figure 2: Uptake of labeled RAW 264.7 sEVs by Neuro-2a cells. (**A**) PKH67-labeled sEVs (1 µg) were added to the cultured Neuro-2a cells for 1, 4, or 24 h. sEV uptake was observed at all time points with confocal microscopy. (**B**) PKH67-labeled sEVs (1, 5, or 10 µg) were added to Neuro-2a cells for 1 h. (**C**) PKH67-labeled sEVs (5 or 10 µg) were added to Neuro-2a cells for 4 h. sEV uptake was observed in all dosage groups with confocal microscopy. Negative control groups treated with PKH dye alone did not show sEV staining (**Supplemental Figure S1**). Neuro-2a cells were immunostained with MAP2A (probed with Alexa Fluor 594, shown in red), while cell nuclei were stained with DAPI (shown in blue) and sEVs with PKH67 (shown in green). Scale bar = 50 µm. Abbreviations: sEVs = small extracellular vesicles; MAP2A = microtubule-associated protein 2A; DAPI = 4’,6-diamidino-2-phenylindole.

Figure 3: Uptake of PKH26-labeled RAW 264.7 sEVs by primary mouse cortical astrocytes. One µg of sEVs was labeled with PKH26 dye and added to the primary astrocyte culture medium. Uptake of sEVs was observed at 1 and 24 h post-addition using a confocal laser scanning microscope. Astrocytes were stained with GFAP (probed with Alexa Fluor 488, shown in red), while cell nuclei were counterstained with DAPI (shown in blue), and sEVs were previously stained with PKH26 (shown in green). Scale bar = 20 µm. PKH26 dye alone served as a negative control for sEV staining. Abbreviations: sEVs = small extracellular vesicles; GFAP = glial fibrillary acidic protein; DAPI = 4’,6-diamidino-2-phenylindole.

Figure 4: Uptake of RAW 264.7 sEVs in neurons. PKH26-labeled sEVs were injected intrathecally in mice; 6 and 18 h later, the mice were perfused with 4% PFA, and the spinal cord was isolated and sectioned at 30 µm. Spinal cord sections were immunostained with a cell marker (probed with Alexa Fluor 488, shown in red) and DAPI nuclear counterstain (shown in blue), while sEVs were previously labeled with PKH26 (shown in green). Spinal cord sections were immunostained for MAP2A to visualize the neurons (red). Confocal microscopy shows sEVs in MAP2A-positive neurons at different time points. The negative control, PKH26 dye-alone group, did not show sEV staining. Scale bar = 20 µm. Abbreviations: sEVs = small extracellular vesicles; PFA = paraformaldehyde; MAP2A = microtubule-associated protein 2A; DAPI = 4’,6-diamidino-2-phenylindole.

Figure 5: Uptake of RAW 264.7 sEVs in astrocytes. PKH26-labeled sEVs were injected intrathecally in mice; 6 and 18 h later, the mice were perfused with 4% PFA, and the spinal cord was isolated and sectioned at 30 µm. Spinal cord sections were immunostained with a cell marker (probed with Alexa Fluor 488, shown in red) and DAPI nuclear counterstain (shown in blue), while sEVs were previously labeled with PKH26 (shown in green). Spinal cord sections were immunostained for GFAP to visualize the astrocytes (red). Confocal microscopy shows sEVs in GFAP-positive astrocytes at different time points. The negative control, PKH26 dye-alone group, did not show sEV staining. Scale bar = 20 µm. Abbreviations: sEVs = small extracellular vesicles; PFA = paraformaldehyde; GFAP = glial fibrillary acidic protein; DAPI = 4′,6-diamidino-2-phenylindole.

Figure 6: Uptake of RAW 264.7 sEVs in microglia. PKH26-labeled sEVs were injected intrathecally in mice; 6 and 18 h later, the mice were perfused with 4% PFA, and the spinal cord was isolated and sectioned at 30 µm. Spinal cord sections were immunostained with a cell marker (probed with Alexa Fluor 488, shown in red) and DAPI nuclear counterstain (shown in blue), while sEVs were previously labeled with PKH26 (shown in green). Spinal cord sections were immunostained for IBA1 to visualize the microglia (red). Confocal microscopy shows sEVs in IBA1-positive microglia at different time points. The negative control, PKH26 dye-alone group, did not show sEV staining. Scale bar = 20 µm. Abbreviations: sEVs = small extracellular vesicles; PFA = paraformaldehyde; IBA1 = ionized calcium-binding adaptor molecule 1; DAPI = 4′,6-diamidino-2-phenylindole.

Supplemental Figure S1: **Uptake of labeled RAW 264.7 sEVs by primary mouse cortical astrocytes and in spinal cord.** (**A**) Controls for the uptake of PKH26-labeled RAW 264.7 sEVs by primary mouse cortical astrocytes. One µg of unlabeled sEVs resuspended in PBS or an equal volume of PBS was added in parallel to the culture medium of astrocytes. No fluorescence was observed at 1 h for PBS and the unlabeled control using a confocal laser scanning microscope. Astrocytes were stained with GFAP (probed with Alexa Fluor 488, shown in red), while the nuclei were counterstained with DAPI (blue), and unlabeled sEVs were visualized under the same Alexa Fluor 546 channel as PKH26-labeled sEVs. Scale bar = 50 µm. (**B**) Controls for the uptake of PKH26-labeled RAW 264.7 sEVs by mouse spinal cord *in vivo*. Five µg of unlabeled sEVs or dye control were injected intrathecally in mice. Again, fluorescent signals were not observed for unlabeled sEVs or dye-alone control using a confocal laser scanning microscope. Astrocytes were stained with GFAP (probed with Alexa Fluor 488, shown in red), while nuclei were counterstained with DAPI (blue), and unlabeled sEVs were visualized under the same Alexa Fluor 546 channel as PKH26-labeled sEVs. Scale bar = 50 µm. (**C**) Representative images reveal the presence of RAW 264.7 sEVs in mouse spinal meninges 6 h and 18 h after intrathecal delivery. Five µg of sEVs were labeled with PKH26 dye (shown in green), and the nuclei were counterstained with DAPI (shown in blue). Asterisks indicate the anterior spinal artery. Scale bar = 50 µm. Abbreviations: sEVs = small extracellular vesicles; PBS = phosphate-buffered saline; GFAP = glial fibrillary acidic protein; DAPI = 4′,6-diamidino-2-phenylindole.

DISCUSSION:

In this protocol, we showed the labeling of sEVs with PKH dyes and the visualization of their uptake in the spinal cord. PKH lipophilic fluorescent dyes are widely used for labeling cells by flow

cytometry and fluorescent microscopy[3,5,6,12,24,25]. Due to their relatively long half-life and low cytotoxicity, PKH dyes can be used for a wide range of *in vivo* and *in vitro* cell-tracking studies[26,27]. Although excellent membrane retention and biochemical stability are advantageous, the intercalation of fluorescent probes with lipoprotein contaminants purified with sEVs can compromise the interpretation of sEV internalization and functional studies. Thus, the purification and labeling of sEVs are critical steps in the protocol because the persistence of the dyes with contaminants can lead to misinterpretation of the *in vivo* distribution[28]. The inclusion of controls is critical to avoid false-positive fluorescence signals due to non-specific labeling of particles and the long half-life of these dyes.

Aggregation and micelle formation of lipophilic dyes may also yield false signals. We addressed the problem of free or unbound dye by including a dye-alone control and visualizing the EV uptake at earlier time points. An important limitation reported for PKH labeling is that numerous PKH26 nanoparticles are formed during PKH26 dye labeling of sEVs. Although not included in this protocol, it is reported that PKH26 nanoparticles can be removed by a sucrose gradient[29]. Another study evaluated the effect of PKH labeling on the size of sEVs by NTA and reported an increase in size following PKH labeling[30]. Nevertheless, PKH dyes serve as a pragmatic and valuable tracer to show where the sEVs have traversed. Another limitation of this study is that we did not quantify sEVs as this protocol focuses on the confirmation of cellular uptake after intrathecal delivery. Novel cyanine-based membrane probes have been developed recently for highly sensitive fluorescence imaging of sEVs without altering the size or generating artifacts, such as the formation of PKH nanoparticles[31], and will undoubtedly improve future labeling studies.

Although macrophages play important roles in neuroinflammation, they also exert neuroprotective functions by delivering their cargo via exosomes[32]. Our studies show that labeled macrophage-derived sEVs are taken up by Neuro-2a cells, primary astrocytes, and in the lumbar spinal cord after intrathecal administration. The results indicate that a longer incubation time can lead to lower sEV signal intensity, which could be attributed to the degradation of sEVs or cell division by Neuro-2a cells in culture[33,34]. Although low-throughput, this protocol for visualizing labeled sEVs in the spinal cord can be used for initial validation studies that confirm sEV uptake before investigating the functional impact of sEVs delivered intrathecally. As we observed generally similar sEV uptake in several CNS cell types, the uptake process appears to be non-selective. If autofluorescence is an issue in imaging, unlabeled sEVs can be used as an additional control to negate sEV autofluorescence during imaging of tissues and cultures. Although the dose and the route of administration of sEVs can influence the pattern of biodistribution[11], this protocol is not optimized for the quantitative analysis of sEV uptake. Several different approaches and various imaging strategies are being employed to investigate sEVs, and these are being continually refined and optimized for *in vivo* tracking of sEVs[2].

This protocol is meant to be just one approach to confirm sEV uptake. As with all protocols, cross-validation using multimodal approaches can be beneficial. Specifically, the uptake of sEVs can be confirmed by investigating biomolecular cargo transfer to recipient cells and tissues. If the investigator knows the miRNA composition of delivered sEVs, an alternate approach to confirm

sEV transfer would be to check for miRNA changes in the recipient cells or determine the changes in expression levels of target genes for the miRNAs transferred. PBS-treated samples can be used as a control for this approach. Overall, these results support the concept that macrophage-derived sEVs are taken up by CNS cells *in vitro* and *in vivo*. This protocol can be used to investigate the role of sEVs in spinal disorders, pain, and inflammation and to determine whether sEVs can be developed as cellular vehicles for the delivery of therapeutic small molecules, RNA, and proteins.

ACKNOWLEDGMENTS:

This study was supported by grants from NIH NINDS R01NS102836 and the Pennsylvania Department of Health Commonwealth Universal Research Enhancement (CURE) awarded to Seena K. Ajit. We thank Dr. Bradley Nash for critical reading of the manuscript.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1 042921.pdf

Figure 1

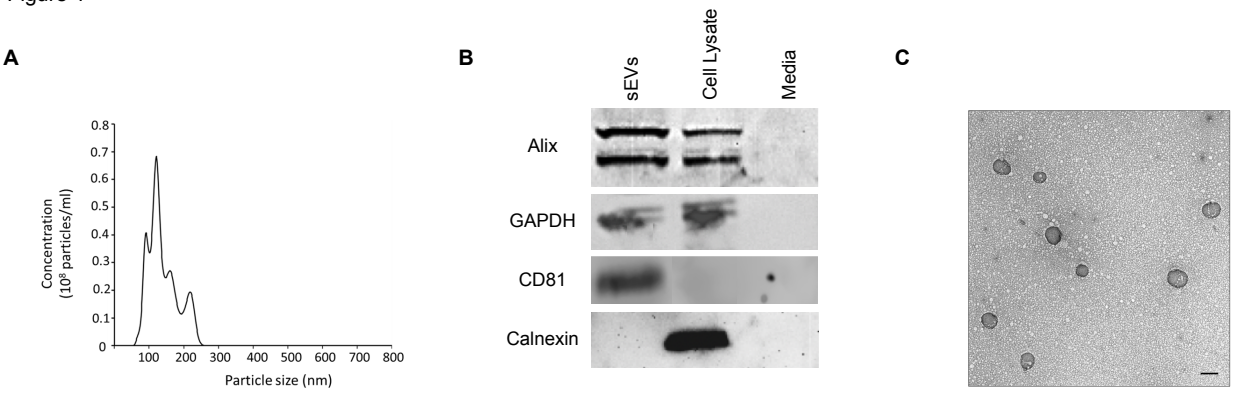


Figure 2

Figure 2

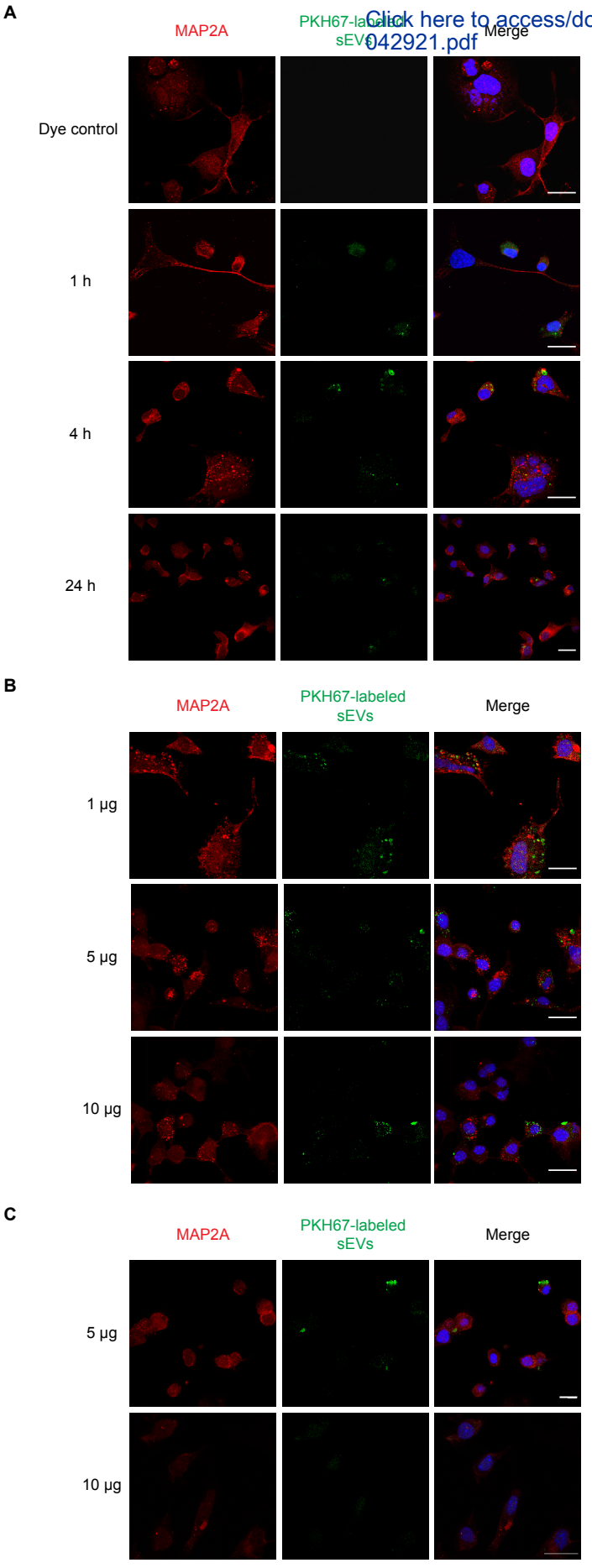


Figure 3

Figure 3

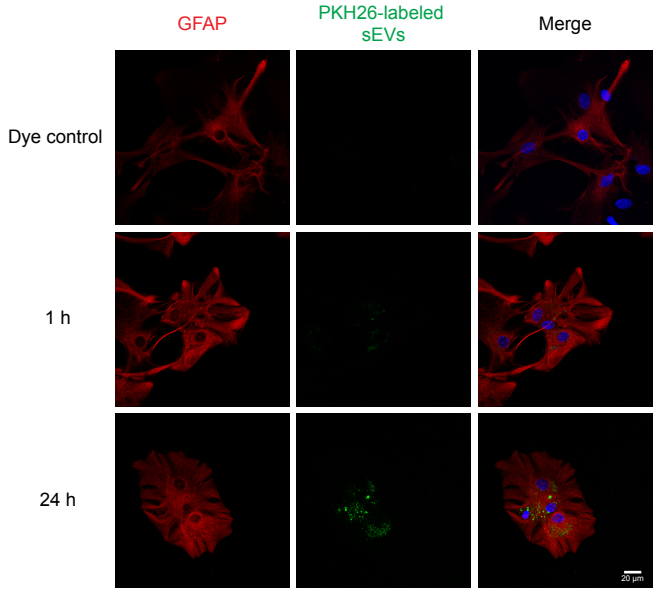


Figure 4

Figure 4

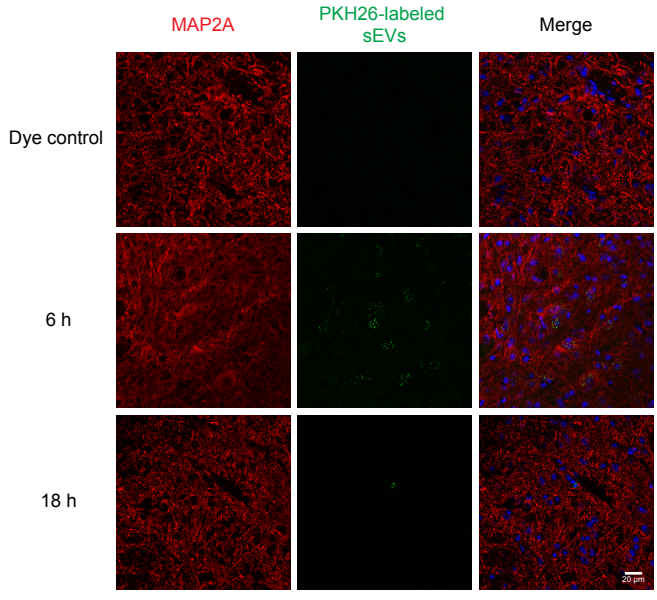


Figure 5

Figure 5

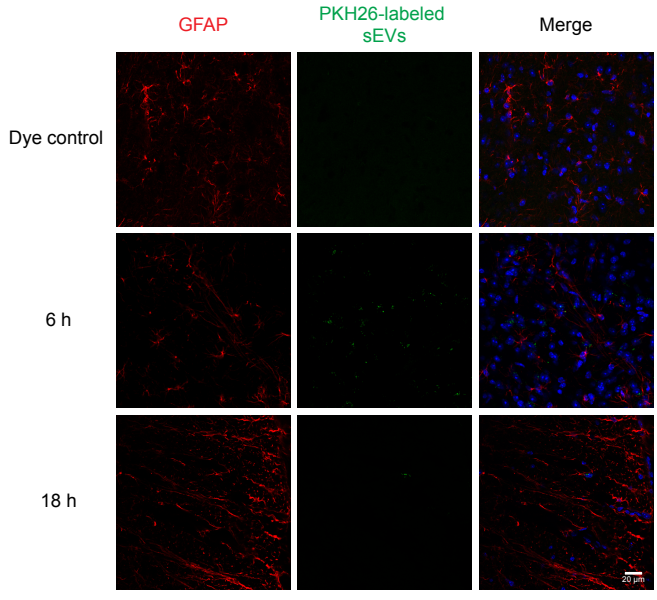
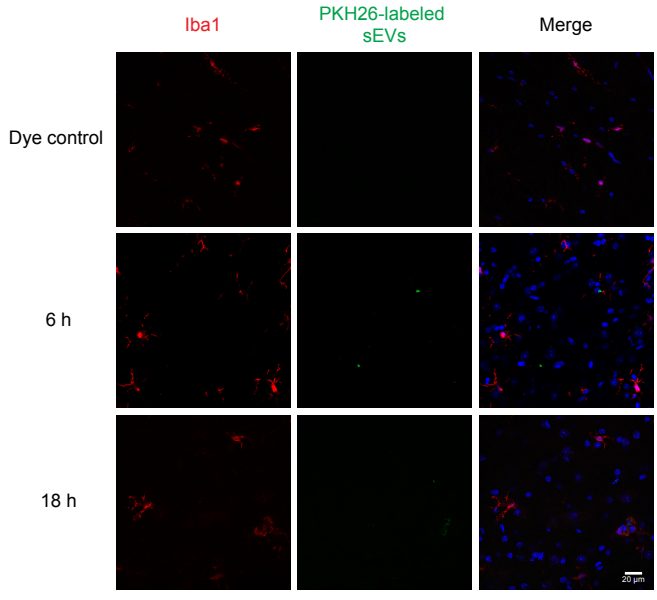


Figure 6

Figure 6



Name of Material	Company
Amicon Ultra 0.5 mL centrifugal filters	MilliporeSigma
Anti-Alix Antibody	Abcam
Anti-Calnexin Antibody	Abcam
Anti-CD81 Antibody	Santa Cruz Biotechnology
Anti-GAPDH Monoclonal Antibody (14C10)	Cell Signaling Technology
Anti-Glial Fibrillary Acidic Protein Antibody	Sigma-Aldrich
Anti-Iba1 Antibody	Wako
Anti-MAP2A Antibody	Sigma-Aldrich
Bovine Serum Albumin (BSA)	VWR
Cell Strainer, 40 µm	VWR
Centrifuge Tubes	Thermo Scientific
Coverslip, 12-mm, #1.5	Electron Microscopy Sciences
Coverslip, 18-mm, #1.5	Electron Microscopy Sciences
DAPI	Sigma-Aldrich
DC Protein Assay	Bio-Rad
Deoxyribonuclease I (DNAse I)	MilliporeSigma
Donkey Anti-Rabbit IgG H&L (HRP)	Abcam
Donkey Anti-Rabbit IgG H&L, Alexa Fluor 488	Invitrogen
Double Frosted Microscope Slides, #1	Thermo Scientific
DPBS without Calcium and Magnesium	Corning
Dulbecco's Modified Eagle Medium (DMEM)	Corning
Exosome-Depleted Fetal Bovine Serum	Gibco
Fetal Bovine Serum (FBS)	Corning
FluorChem M imaging system	ProteinSimple
FV3000 Confocal Microscope	Olympus
Goat Anti-Mouse IgG H&L (HRP)	Abcam
Goat Anti-Mouse IgG H&L, Alexa Fluor 488	Invitrogen
Goat Anti-Mouse IgG1, Alexa Fluor 594	Invitrogen
Hank's Balanced Salt Solution (HBSS)	VWR
HEPES	Gibco
HRP Substrate	Thermo Scientific
Intercept blocking buffer, TBS	LI-COR Biosciences
Laemmli SDS Sample Buffer	Alfa Aesar
Micro Cover Glass, #1	VWR
Microm HM550	Thermo Scientific
NanoSight NS300 system	Malvern Panalytical
NanoSight NTA 3.2 software	Malvern Panalytical
Neuro-2a Cell Line	ATCC
Normal Goat Serum	Vector Laboratories
O.C.T Compound	Sakura Finetek
Papain	Worthington Biochemical Corporat
Paraformaldehyde	Electron Microscopy Sciences
Penicillin-Streptomycin	Gibco
PKH26	Sigma-Aldrich
PKH67	Sigma-Aldrich
Protease Inhibitor Cocktail	Thermo Scientific
PVDF Transfer Membrane	MDI

RAW 267.4 Cell Line	ATCC
RIPA Buffer	Sigma-Aldrich
Sodium Chloride	AMRESCO
Superfrost Plus Gold Slides	Thermo Scientific
T-75 Flasks	Corning
Tecnai 12 Digital Transmission Electron Microscope	FEI Company
TEM Grids	Electron Microscopy Sciences
Tris-Glycine Protein Gel, 12%	Invitrogen
Tris-Glycine SDS Running Buffer	Invitrogen
Tris-Glycine Transfer Buffer	Invitrogen
TrypLE Express	
Triton X-100	Acros Organics
Trypsin, 0.25%	Corning
Tween 20	
Ultracentrifuge Tubes	Beckman

Catalog Number	Comments / Description
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Ab10286	1:1000
sc-166029	1:1000
2118	1:1000
MAB360	1:500 for IF; 1:1000 for IHC
019-19741	1:2000
MAB378	1:500
0332	
15-1040-1	
3118-0050	12,000 x g
72230-01	
72222-01	
D9542-1MG	1 µg/mL
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D4513-1VL	
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21-031-CV	
10-013-CV	
A27208-01	
35-011-CV	
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15-188-48 adhesive slides
431464U

FSF300-cu
XP00120BOX
LC26755
LC3675
cell dissociation enzyme
327371000
25-053-CL

344058 110,000 x g

April 30, 2021

Dear Editor:

We would like to thank the reviewers for their comments and suggestions on our manuscript titled “Uptake of fluorescent labeled small extracellular vesicles in vitro and in spinal cord”.

The attached manuscript has been revised to incorporate the reviewers’ recommendations. We have addressed the concerns raised by the reviewers, and we have provided answers to the questions raised (please see below) and how they have been addressed in the manuscript.

We have carefully and completely addressed the reviewers’ concerns and hope you will agree the manuscript is now suitable for publication in your journal.

Thank you very much.

Sincerely yours,

Seena Ajit

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.

Done

2. Please expand all abbreviations during the first-time use.

Done

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Done

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We have now moved the ethics statement immediately before the steps using mice.

5. We cannot have paragraph of texts in the protocol section. Please either make numbered action step or convert to a note.

Done

6. 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 and Reagents.

For example: Sorvall RC-5C Plus centrifuge (Sorvall), Sorvall SA-600, Optima XPN-80 ultracentrifuge, Malvern Instruments, NanoSight NTA 3.2 software, Santa Cruz, Abcam, Cell signaling, FluorChem M, ProteinSimple, Whatman NO. 1, Invitrogen, Wako Chemicals, etc.

We have deleted trademark symbols etc.
7. Please revise the following lines to avoid overlap with previously published work: 85-87, 90-92, 143-146, 152-155, 159-163, 167-177, 407-411
We have modified the lines mentioned above.
8. Please do not use personal pronouns in the protocol text.
Checked.
9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? This can be done by including mechanical actions, button clicks in the software, etc.
We have modified the text.
10. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 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.
Done
11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
We do not have any figures from previous publications.
12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We have tried to cover these as appropriate for this protocol.
13. Please combine all panels of one figure into a single image file. Please upload high-resolution images.
Since we are unable to combine multipanel figures, we have now included them as separate figures (Fig 4a, 4b and4c are now 4, 5 and 6 in high resolution).
14. Please sort the materials table in alphabetical order.
Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors present an interesting work, most of the paper is clear and well-presented, however, I have some criticisms as detailed below.

We thank the Reviewer for taking the time to evaluate our manuscript.

Major Concerns:

- In the summary section, the authors claim that "we describe a protocol to label macrophage-derived small extracellular vesicles (sEVs) with PKH dyes....", however, the same protocol is reported in several papers, even if it is applied to other cells type. Moreover, the authors should improve the description of the above -mentioned protocol; they claim that vesicles were labelled and then washed twice in PBS, it is not clear how this wash was performed.

We agree with the reviewer that this protocol is described in other papers. Here, we tried to optimize the dose and time required for the visualization of the labelled vesicles in spinal cord along with some of the appropriate controls.
We have now modified the labelling protocol to clarify the PBS wash.
"3.5. Centrifuge 1 h at 110,000 x g at 4 °C. Discard the supernatant, resuspend the pellet in 2 mL PBS and centrifuge 1 h at 110,000 x g at 4 °C. Repeat the wash with PBS and resuspend the labeled sEVs or dye control in PBS."

Minor Concerns:
- The authors used two types of dyes: PKH26 (red) and/or PKH67 (green), and two types of secondary antibodies: alexa fluor 488 (green) and alexa fluor 594 (red). However, in the figures, they showed only green staining for sEVs and red staining for cells. Further justification/details of this part are needed.
We used two types of dyes: PKH26 (red) and/or PKH67 (green). Alexa Fluor 488 was used when sEVs were labeled with PKH26, while Alexa Fluor 594 was used along with PKH67-labeled sEVs. However, for ease of visualization, we used pseudo-colors and showed sEVs in green and cells in red consistently throughout the manuscript. We have specified the Alexa Fluor conjugated secondary antibodies we used in all figure legends.

- In the western blot paragraph, it should be mentioned the cells used for this experiment (RAW 246.7). Additionally, how many cells were lysed? At point 2.2.4, it is stated that "Mix the same amount of sEVs, cell lysate, and exosome depleted media…". This concept is ambiguous. How many ug of protein were used? How did the authors quantify exosome depleted media? Please, described this in more detail.
We have now added additional details in the protocol describing sample preparation and protein quantification (point 2.2.2, 2.2.3, 2.2.4, 2.2.5, 2.2.6).

2.2.2. For cell lysate preparation, culture the RAW 264.7 cells in 75 cm2 flasks until 80–90% confluent. Detached the cells with 0.25% trypsin, neutralize with culture media and pellet by spinning at 400 x g for 5 min. Resuspend the cells in fresh growth medium.
2.2.3. Count the cells using hemocytometer and take 1 x 106 cells. Wash the cells with PBS twice using the same centrifugation conditions as above and add 50 µl lysis buffer (RIPA buffer with protease inhibitor cocktail added) to the cell pellet from the final spin.
2.2.4. Vortex the cells and keep on ice for 20 min. Subject the mixture to centrifugation at 10,000 x g for 30 min at 4°C, collect the supernatant (i.e. the lysate) in fresh microcentrifuge tubes and keep at −80°C until use.
2.2.5. Concentrate 2 mL exosome depleted media to 100 µL using 3 kDa cutoff centrifugal filters before quantifying the protein amount. Mix sEVs with lysis buffer in 1:1 ratio, vortex for 30 s and incubate on ice for 15 min to quantify the protein quantity.
2.2.6. Mix the same protein amounts (2 µg) of sEVs, RAW 264.7 cell lysate and exosome-depleted media with reducing sample buffer.

- In the section "in vivo uptake of sEVs", how many mice were used? What is "an equal volume of dye control?". Further justification/details of this part are needed.
We have modified the protocol. Though we had 5-6 mice in each group, we show images from one mouse for each condition based on image resolution and clarity from multiple sections in each mouse. We have now added details of dye control preparation in step 3. Dye control is used as a control for non-specific dye uptake along with labelled sEVs.

- Lines 342-343: the dose-dependent increase of uptake is from 1 to 10 µg, not from 1 to 5 µg...

We have now corrected this.

- Why the 1µg concentration was not inserted in figure 2C?
This is because we have already included the 1 µg time dependent uptake in figure 2A.

Reviewer #2:
Manuscript Summary:
In this work, the PKH dye labeling of macrophage small extracellular vesicles (sEVs) to study their uptake by different CNS cell types is examined. These types of studies have been published extensively, most frequently as a first part of papers showing the functional effect of sEV uptake in target cells. No novel protocols per se are shown, although a detailed workflow to fulfill the aim of the work may be useful.
We thank the Reviewer for the time and effort in reviewing our manuscript.

We agree with the Reviewer that labeling of sEVs and uptake have been reported in literature. We believe our protocol will be beneficial for neuroscientists pursuing sEV studies in spinal cord, specifically in the context of pain, spinal cord injury etc. We are showing representative images after intrathecal injection of different concentrations of sEVs at multiple time intervals.

We were also concerned about the novelty of the methods other than what we mentioned above and had confirmed with the editor this is of interest to the journal when we submitted a preliminary abstract before working on this manuscript.

Major Concerns:
* These types of studies have been published extensively, most frequently as a first part of papers showing the functional effect of sEV uptake in target cells. Please cite these works.
We have now included additional citations shown below.

3 Dehghani, M. & Gaborski, T. R. Fluorescent labeling of extracellular vesicles. Methods Enzymol. 645 15-42, (2020).

4 González, M. I. et al. Covalently Labeled Fluorescent Exosomes for In Vitro and In Vivo Applications. Biomedicines. 9 (1), (2021).

5 Chuo, S. T.-Y., Chien, J. C.-Y. & Lai, C. P.-K. Imaging extracellular vesicles: current and emerging methods. Journal of Biomedical Science. 25 (1), 91, (2018).

6 van der Vlist, E. J., Nolte-'t Hoen, E. N. M., Stoorvogel, W., Arkesteijn, G. J. A. & Wauben, M. H. M. Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry. Nat Protoc. 7 (7), 1311-1326, (2012).

7 Hoshino, A. et al. Tumour exosome integrins determine organotropic metastasis. Nature. 527 (7578), 329-335, (2015).

11 Wiklander, O. P. B. et al. Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. J Extracell Vesicles. 4 26316-26316, (2015).

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25 Gangadaran, P., Hong, C. M. & Ahn, B.-C. An Update on in Vivo Imaging of Extracellular Vesicles as Drug Delivery Vehicles. Frontiers in Pharmacology. 9 (169), (2018).

29 Pužar Dominkuš, P. et al. PKH26 labeling of extracellular vesicles: Characterization and cellular internalization of contaminating PKH26 nanoparticles. Biochim Biophys Acta Biomembr. 1860 (6), 1350-1361, (2018).

30 Dehghani, M., Gulvin, S. M., Flax, J. & Gaborski, T. R. Systematic Evaluation of PKH Labelling on Extracellular Vesicle Size by Nanoparticle Tracking Analysis. Sci Rep. 10 (1), 9533, (2020).

31 Shimomura, T. et al. New Lipophilic Fluorescent Dyes for Labeling Extracellular Vesicles: Characterization and Monitoring of Cellular Uptake. Bioconjugate Chemistry. 32 (4), 680-684, (2021).

* It is uncertain whether sEVs or contaminant labelled membranes/lipophilic aggregates/vesicles were taken up by cells. This should be addressed, e.g. by performing a density gradient to separate small EVs according to their density and using these EVs. The distribution of the dye along the gradient could also be a useful indicator (E.G. Wiklander et al 2015). This is the main concern regarding the present work.
We agree that density gradient separation and dye distribution would be a more rigorous approach. We have previously reported functional changes in pain behavior in mice injected with 0.5 and 1 µg of these sEVs (McDonald et al., 2014, Jean-Toussaint et al., 2021) and in this protocol we focused on labeling of all small EVs using PKH and visualization for their time and dose dependent uptake in spinal cord using sEVs isolated by the same method.
We have now acknowledged limitations of our method by including the following in the discussion: "An important limitation reported for PKH labeling is that numerous PKH26 nanoparticles are formed during PKH26 dye labeling of sEVs. Though not included in our protocol, it is reported that PKH26 nanoparticles can be removed by sucrose gradient[1]. Another study evaluated the effect of PKH labelling on the size of sEVs by NTA and reported an increase in size following PKH labelling[2]."

* It needs to be reported how many vesicles are used in the in vitro assays, e.g. number vesicles/cell in each case
We have determined that 1µg protein on average equals to ~ 1 x 10^9 particles. For Neuro-2A, we used 1, 5 and 10 µg vesicles per 10x10^4 cells in 12 wells. We performed the experiment in 12 well. We have now included this information in the manuscript.
Please see Page 9

* How did you quantify sEV uptake? Or why didn´t the authors quantify sEV uptake, at least in cells? How many independent experiments were performed in each case?
Our goal was not to quantify but to use this an approach to confirm uptake by visualization. We observed that in spinal cord the distribution can differ between different sections in the same mouse and thus accurate quantification is difficult.

We realize how the word “time and dose dependent” can be misleading. So, we have modified the abstract and deleted “time and dose dependent” from the manuscript to accurately reflect our intention in this manuscript is just for visualizing uptake.

Minor Concerns:
* The concept of exosomes vs small extracellular vesicles is sometimes confused. Small EVs can originate in MVBs but also at the plasma membrane. Instead, exosomes are defined by their exclusive biogenesis in MVB (e.g. Line 77-78, line 331-332). In consequence, both names cannot be interchanged: the sEV pellet after ultracentrifugation contains exosomes but also other small vesicle types
We agree with the Reviewer. However, when we cite published work, we tried to follow the terminology originally used by the authors in their publications. We didn’t want to change the term used in publications.

* How is the control used in sEV labeling (PBS-dye?)? Because after centrifugation, no pellet will remain. Specify the procedure. Please refer to supplementary figures for controls in figure legends or omit this result in the figure legend of individual figures (e.g. Negative control groups treated with PKH dye alone did not show sEV staining)
Yes, dye control is PBS-dye and procedure details are added in the labeling protocol. This control helps to see if there are any dye precipitates that can show false positive fluorescence signal.

* What means 10% ketamine/xylazine? How much ketamine and how much xylazine?
We have modified it and now provide detailed explanation for clarity. We now say “After 6 and 18 h post injection of sEVs, deeply anesthetize mice by intraperitoneal injection of 100 mg/kg body weight for ketamine and 10 mg/kg body weight for xylazine..”

Reviewer #3:
Manuscript Summary:
The authors first sought to characterise the size and expression profiles of the EVs that they isolated from RAW 264.7 cells. They utilised nanoparticle tracking analysis (NTA) to determine the concentration and size distribution of the isolated particles. Western blotting was used to confirm the protein expression of sEV markers and transmission electron microscopy was used to visualise and measure the size of isolated vesicles. (Figure 1)
The author next sought to assess the effect of time and dose on the uptake of labelled sEVs in vitro (Neuro-2a cells and primary astrocytes).
In Neuro-2a cells they incubated cells with 1 ug of labelled EVs and measured uptake at 1, 4 and 24 hours respectively using immunofluorescence and confocal microscopy. Maximum uptake of EVs occurred at 1hr. (Figure 2A)
Additionally in Neuro-2a cells, they examined the effect of dose at the following concentrations 1ug, 5ug and 10ug of EVs for 1 hour. The authors report a dose dependent uptake from 1 to 5 ug. (Figure 2B)
Primary cortical astrocytes were incubated with 1 ug of labelled EVs at two time points, 1hr and 24 hrs, they observed maximal uptake EV uptake at 24 hrs. (Figure 3)
Finally, the authors examined the in vivo uptake of labelled EVs. Mice were intrathecally injected and uptake was assessed in neurons (MAP2 used as a marker), astrocytes (GFAP used as a marker) and microglia (IBA1 used as a marker) at 6 and 18 hrs post injection via immunohistochemistry and immunofluorescence. The authors report that all cells took up labelled EVs and maximal uptake was at 6hrs. (Figure 4)
We thank the Reviewer for the careful evaluation of our manuscript.

Major Concerns:
The study lacks context. Although the authors have mentioned the availability of other dyes, they have failed to highlight what are the advantages and disadvantages of this protocol compared to others? Where is the novelty/improvement? Although the study has some merit in portraying a new protocol for EV labelling and visualisation, the study is mostly qualitative rather than quantitative, thus leaving a doubt on the quality and the robustness of this protocol over others already available.
Our goal was to confirm sEV uptake in spinal cord using different concentrations and at different time points by visualization and not to quantify. We acknowledge the technique of sEV labeling itself is not novel, but this is meant to be a method for confirmation of sEV delivery and ability to visualize its presence in spinal cord after 6 and 18 hours.
We have previously shown functional differences (attenuation of pain hypersensitivity using 0.5 µg (McDonald et al. 2014) and 1µg sEVs (Jean Toussaint et al. 2021) depending on the route on administration (intraplantar vs intrathecal). Thus, we are interested in visualizing sEVs at different time points in various cells. Though quantifying the uptake and correlating them to specific cell types will be interesting, it is beyond the scope of this manuscript.

Figure 1B. The CD81 western blot image is not convincing, a faint band is present in both EVs and cell lysate and the image is of poor quality. I have no doubt the authors have isolated EVs, but the image of that blot should be replaced with a better example or a different EV marker with higher expression or a stronger antibody signal
We have now replaced the CD81 western with a new figure.

Figures 2 and 4 need a higher magnification to be convincing. Also the panels in figure 2 have different dimensions.
We have now made the changes recommended by the Reviewer.

Figure 2B The authors claim a dose-dependent increase in EV uptake/fluorescence, but nothing has been quantified in this study. How can a dose-dependent increase be claimed or a maximum uptake without quantification? How many cells have the authors examined? What is the n number of the experiments? In the in vivo study through intrathecal delivery, one would imagine that most EVs would remain trapped in the meninges, as viruses and cells do. Have the authors investigated this point? has any precaution been taken to avoid this pitfall?
We have now modified the abstract to say “We show uptake of different concentrations of labeled sEVs at multiple time points by Neuro-2a cells and primary astrocytes in vitro. We also show uptake of sEVs delivered intrathecally in mouse spinal cord neurons, astrocytes, and microglia visualized by confocal microscopy”. As mentioned earlier, we had 5-6 mice in each group, but chose to show images from one mouse for each condition based on image resolution and clarity from multiple sections in each mouse.

We did observe some trapping in meninges. We have now included the new figure in the supplementary figure 1C.

Minor Concerns:
Figure 3. Line 344. The authors indicate that a similar pattern of uptake is observed in the astrocytes compared to Neuro-2a cells.
The sentence beginning on Line 346 contradicts the notion that a similar pattern is observed. The pattern observed in N2a cells being maximal uptake at 1hr, whilst maximal uptake within astrocytes

occurred at 24 hrs.
I would advise the removal of the words 'similar uptake pattern in a time dependent manner' (Line 345).
We have removed this information as recommended by the reviewer.

Part I of the protocol is missing indications of volumes, i.e. how much conditioned medium should be collected? how much PBS should be used to resuspend the pellet? Working with minute amounts of vesicles and proteins, one has to be precise in the dilutions performed.
We have now added details in section 1 as recommended by the reviewer.