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Extracellular Vesicles Tracking and Quantification Using CT and Optical Imaging in Rats

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[Abstract] Exosomes, a subtype of extracellular vesicles, are nanovesicles of endocytic origin. Exosomes contain a plethora of proteins, lipids, and genetic materials of parent cells to facilitate intercellular communications. Tracking exosomes *in vivo* is fundamentally important to understand their biodistribution pattern and the mechanism of biological actions in experimental models. Until now, a number of tracking protocols have been developed, including fluorescence labeling, bioluminescence imaging, magnetic resonance imaging, and computed tomography (CT) tracking of exosomes. Recently, we have shown the tracking and quantification of exosomes in a spinal cord injury model, by using two tracking approaches. More specifically, following intranasal administration of gold nanoparticle-encapsulated exosomes to rats bearing complete spinal cord injury, exosomes in the whole central nervous system were tracked by using microCT, and quantified by using inductively coupled plasma and flame atomic absorption spectroscopy. In addition, optical imaging of fluorescently labeled exosomes was performed to understand the abundance of migrating exosomes in the spinal cord lesion, as compared to the healthy controls, and to further examine their affinity to different cell types in the lesion. Thus, the protocol presented here aids in the study of exosome biodistribution at both cellular and organ levels, in the context of spinal cord injury. This protocol will also enable researchers to better elucidate the fate of administered exosomes in other models of interest.

Keywords: Exosomes, Extracellular vesicles, Tracking, Biodistribution, *In vivo* imaging, Spinal cord injury

[Background] Exosomes are natural nanovesicles (30-150 nm in diameter) of endosomal origin, secreted by a variety of cells including mesenchymal stem cells (MSCs) (Yang *et al.*, 2018). Exosomes carry proteins, lipids and genetic materials of parent cells, and are capable of inducing a multitude of biological effects to adjacent or distal cells and tissues (Valadi *et al.*, 2007; van Niel *et al.*, 2018). Due to their innate advantages, such as low immunogenicity, potential of crossing blood brain barrier, and amenability to the loading of exogenous cargos, exosomes have emerged as an alternative to cell-based therapeutics in the treatment of cancer, neurodegenerative and many other diseases (Alvarez-Erviti *et al.*, 2011; Kamerkar *et al.*, 2017; Guo *et al.*, 2019; Yong *et al.*, 2019). While exosomes have gained

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increasing interest as diagnostic and therapeutic vehicles, the knowledge of their *in vivo* behavior is limited, which restricts the clinical translation of exosome therapy. Thus, advanced imaging approaches for *in vivo* tracking of exosomes are highly anticipated.

To elucidate the *in vivo* behavior of exosomes, several strategies have been developed. Among them, optical imaging represents the most commonly used exosome tracking method. Up until now, organic dyes, immunofluorescent reporters, genetically encoded fluorescent proteins, or fluorescent nanomaterials have been used to label exosomes (Pegtel *et al.*, 2010; Alvarez-Erviti *et al.*, 2011; Suetsugu *et al.*, 2013; Lai *et al.*, 2015; Jiang *et al.*, 2018; Shen *et al.*, 2018; Betzer *et al.*, 2019). Optical imaging allows high-throughput efficiency, facilitating the observation of the interplay between exosomes and recipient cells. However, this technique has several drawbacks, such as artifacts due to lipophilic dye aggregation (Grange *et al.*, 2014) or presence of autofluorescence background (Faruqu *et al.*, 2019), as well as limited tissue penetration depth (Shen *et al.*, 2018). Magnetic resonance imaging (MRI) constitutes another promising tracking methodology, featured by high spatial resolution with anatomical details and radiation-free operation (Busato *et al.*, 2017). Exosomes can be loaded with superparamagnetic iron oxide nanoparticles (SPIONs), and tracked in animal models using MRI (Hu *et al.*, 2015; Busato *et al.*, 2016; Jia *et al.*, 2018). MRI, however, has inherent disadvantages, including susceptibility to movement artifacts, long image acquisition, and high cost. Computed tomography (CT) is a powerful, and widely used imaging modality which offers high spatial and temporal resolution. Recently, Betzer *et al* established a protocol for labeling exosomes with gold nanoparticles (GNPs) for tracking exosome migration *in vivo* using CT (Betzer *et al.*, 2018). This technology has been successfully utilized to track the biodistribution of intranasally delivered exosomes derived from mesenchymal stem cell (MSC), in rodents with focal brain ischemia (Betzer *et al.*, 2017), various brain pathologies (namely, stroke, Parkinson's disease, Alzheimer's disease, or autism) (Perets *et al.*, 2019), or spinal cord injury (SCI) (Guo *et al.*, 2019). Guo *et al* combined optical imaging and CT neuroimaging modalities, to examine the biodistribution of intranasally administered MSC-derived exosomes in rats with complete SCI (Guo *et al.*, 2019). Coupled with quantitative methodologies, *i.e.*, inductively coupled plasma (ICP), flame atomic absorption spectroscopy (FAAS), and analysis of immunofluorescently stained tissues, a more comprehensive exosome tracking was demonstrated, unraveling the interactions between exosomes and different cell populations in the spinal cord, and the localization of exosomes in several major organs, in the context of SCI.

Multimodal imaging modalities are more anticipated than single imaging model with regard to elucidating the behavior of exosomes *in vivo* through complementary advantages. The protocol presented here can be expanded to investigate how exosomes behave in other diseased models.

Materials and Reagents

1. 0.22 μm filter
2. 70 ml polycarbonate tubes
3. Permanent marker

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4. Coverslips
5. Single-neck round-bottom flask (Sigma-Aldrich, catalog number: Z414514)
6. 0.45 µm syringe filters (CSI Analytical Innovations, catalog number: 30103)
7. Spinal cord hook (Fine Science Tools, catalog number: 1016212)
8. #10 blade (Albion, catalog number: 15478491)
9. 5-0 Absorbable sutures (Assut, catalog number: 704031)
10. 4-0 sutures (Assut, catalog number: 6543)
11. Female Sprague Dawley rats, 200-250 gram, 8-10 weeks old
12. Bone marrow mesenchymal stem cells (Lonza, catalog number: 10HU-217)
13. DMEM medium (Biological Industries, catalog number: 01-056-1A)
14. L-glutamine (Biological Industries, catalog number: 03-022-1B)
15. Penicillin-streptomycin-neomycin (Biological Industries, catalog number: 03-034-1B)
16. MEM-eagle non-essential amino acid (Biological Industries, catalog number: 01-340-1B)
17. Heparin (Sigma, catalog number: H9267-1MG)
18. Platelets lysate (Rabin Medical Center, Israel)
19. PKH26 dye (Sigma, catalog number: PKH26GL-1KT)
20. Deuterium-depleted water (DDW) (Thermo Scientific)
21. Sodium Hydroxide (NaOH) (Sigma, catalog number: 1310-73-2)
22. Oleic acid (Sigma, catalog number: 112-80-1)
23. Palmitic acid (Chem-Implex Int'l, catalog number: 14036)
24. $HAuCl_4$ (Sigma, catalog number: 27988-77-8)
25. Ascorbic acid (Sigma, catalog number: 50-81-7)
26. O-(2-Carboxyethyl)-O′-(2-mercaptoethyl)heptaethylene glycol (PEG7) solution (Sigma, catalog number: 866889-02)
27. n-hexane (Bio-Lab, catalog number: 110-54-3)
28. HCl solution (Deajung, catalog number: 764701-0)
29. N-Hydroxysulfosuccinimide sodium salt (NHS) (Sigma, catalog number: 106627-54-7)
30. N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) (Sigma-Aldrich, catalog number: 25952-53-8)
31. Glucose-2 (2GF) (D-(+)-glucosamine hydrochloride (Sigma-Aldrich, catalog number: 66-84-2)
32. Acetone (Romical, catalog number: 19-009001-72)
33. Ketamine (Vetoquinol, catalog number: 8c0903c)
34. Xylazine (Eurovet, catalog number: 084697)
35. Isoflurane (Abbott Laboratories, catalog number: 6041795)
36. Eye ointment (Alcon, catalog number: 16D12E)
37. Buprenorphine (Medi-market, catalog number: V82806J)
38. 0.9% saline (Baxter, catalog number: 18K29E0B)
39. Polydine-iodine (Dr. Fisher, catalog number: B.N.D0410PT20.2)
40. Phosphate buffered saline (PBS) (Gibco, catalog number: 14200067)

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41. 4% Paraformaldehyde (ChemCruz, catalog number: sc281692)
42. Sucrose (Baker Analyzed, catalog number: 57501)
43. Triton X-100 (Biolab, catalog number: 20180501)
44. Tween-20 solution (Sigma, catalog number: P2287)
45. Primary antibodies
    a. NeuN antibody (Abcam, catalog number: ab104225)
    b. GFAP antibody (Millipore, catalog number: AB5804)
    c. TMEM119 antibody (NovuBio, catalog number: NBP23055)
46. Secondary antibodies
    Goat anti rabbit 647 (LifeTechnologies, catalog number: A21244)
47. DAPI (Sigma, catalog number: D9592)
48. Bovine serum albumin (BSA) (Millipore, catalog number: 820451)
49. Sucrose solution (see Recipes)
50. Blocking solution (see Recipes)
51. Tween solution (see Recipes)

Equipment

1. Tunstem Carbide Noyes microscissor (Fine Science Tools, catalog number: 1551412)
2. Friedman-Pearson Rongeur (Fine Science Tools, catalog number: 1602114)
3. Heating pad (Gaymar Industries, model: TP702)
4. Electric shaver (Oster Golden A5 Two-speed, model: 07800550002)
5. Inhalation set-up (Matrx™ VIP 3000 Calibrated Vaporizer, catalog number: V1419556)
6. Funnel (Separatory funnel 250 ML/glass stopcock) (Sigma, catalog number: 64803-U)
7. Vacuum pump (Millivac-Mini Vacuum Pump) (Merck, catalog number: XF5423050)
8. Inductively-Coupled-Plasma Optical-Emission-Spectrometry (ICP-OES) (Santa-Clara, USA, Agilent, model: 5100VDV)
9. Flame atomic absorption spectroscopy (Agilent Technologies, model: SpectrAA 140)
10. Micro-CT scanner (Skyscan High Resolution, model: 1176)
11. Cryostat (Germany, Leica, model: CM1850)
12. Confocal microscope (ZEISS, model: LSM700)
13. Ultracentrifuge (Beckman, model: Optima XE-90 Ultracentrifuge)

Software

1. Fiji: ImageJ
2. GraphPad Prism 7
3. Matlab
4. SkyScan CT-Voxel (“CTVox”)

5. SkyScan CT-Volume (“CTVol”)

Procedure

A. Exosome production and purification

1. Culture human bone marrow mesenchymal stem cells in the DMEM medium, enriched with 1% L-glutamine, 1% penicillin-streptomycin-neomycin, 1% MEM-eagle non-essential amino acid, 0.04% heparin, and 10% exosome-free platelets lysate, for 3 days.
2. Collect 10 ml medium per T75 flask when cells are at 80-90% confluency (3 x 10^6 cells/flask). Perform exosome isolation for a total of 110 ml medium using a standard differential centrifugation protocol, at 4 °C. In brief, centrifuge medium at 300 *x g* for 10 min. Centrifuge the supernatant at 2,000 *x g* for 10 min, re-centrifuge at 10,000 *x g* for 30 min, and then vacuum through a 0.22 μm filter. Centrifuge the collected supernatant at 100,000 *x g* for 70 min (rotor: TI-45 Beckman; 70 ml polycarbonate tubes). Wash the pellet in 70 ml PBS and centrifuge again at 100,000 *x g* for 70 min. Resuspend the final pellet in 200 μl sterile PBS.

B. Exosome labeling with GNPs or PKH26

1. Synthesize GNPs
   a. Into a single-neck round-bottom flask with stirring magnet, add 100 ml deuterium-depleted water (DDW) with a mixture of oleic acid (2.437 ml), palmitic acid (600 mg) and ethanol (15 ml, 99%).
   b. In a separate tube stir NaOH (200 mg) and DDW (30 ml) until the solution is dissolved and add into the mixture above.
   c. While stirring the solution add $HAuCl_4$ (100 μl, 50 mg/ml), then add ascorbic acid (5 ml, 0.05 M) drop by drop, very slowly, wait additional 5 min while stirring.
   d. To the GNP solution add 15.O-(2-Carboxyethyl)-O′-(2-mercaptoethyl)heptaethylene glycol (PEG7, 2.26 x 10^{-3} g), followed by 1 h stirring.
   e. Achieve pH = 9 of the mixture by NaOH, and then stir for additional 1 h.
   f. Add n-hexane (80 ml), then stir the solution for 1 h.
   g. Adjust the pH to 7, using HCl solution (100 μl, 37%).
   h. In a funnel (Separatory funnel 250 ml/glass stopcock, Sigma), perform phase separation (aqueous and organic) of the mixture at room temperature (Figure 1). Repeat the phase separation 4 times, until only aqueous solution is left.

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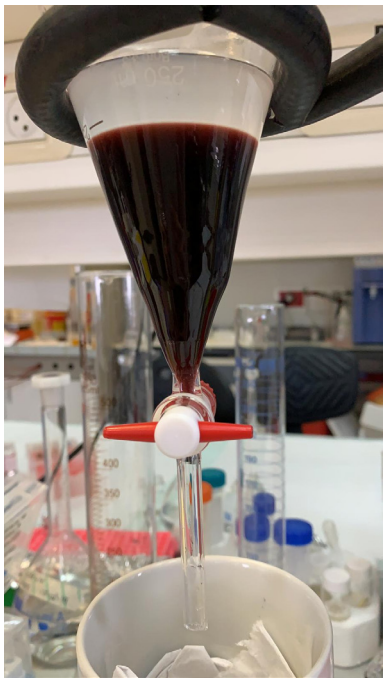

Figure 1. Phase separation. Following 5 nm GNP formation, a funnel is used in order to separate between the organic phase (top, white) and aqueous phase (bottom, purple). This step is repeated until a pure aqueous solution that contains the GNPs is left.

i. Add the solution with N-Hydroxysulfosuccinimide sodium salt (2.12 x 10^{-3} g), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (1.87 x 10^{-3} g), and glucose amine (D-(+)-glucosamine hydrochloride) (1.75 x 10^{-3} g), and stir for 3 h.
j. Add acetone to the GNP solution (GNP:Aceton 1:4 ratio), and centrifuge for 30 min at 24 °C, 20,000 *x g*, gently pump out the particles that have sunk into the bottom of the tube with a pipette.
k. Attach the particle's veil to a vacuum pump (Millivac-Mini Vacuum Pump, Merck), to evaporate all the acetone left in the liquid.
l. Measure the particle's concentration using ICP-OES, gold concentration should be 30-35 mg/ml.

2. Incubate a total of 2.8 x 10^{10} MSC-Exo (200 µl exosomes in 1 ml saline) with GNPs (35 mg/ml, 100 µl) coated with glucose, at 37 °C for 3 h.
3. Centrifuge the MSC-Exo at 100,000 *x g* for 2 h, at 4 °C. Resuspend GNP-labeled MSC-Exo in 200 µl PBS.
4. For PKH26 labeling. Label 2.8 x 10^{10} MSC-Exo with PKH26 dye for 5 min at 37 °C. Wash with 70 ml PBS using ultracentrifugation for 2 h at 100,000 *x g*, at 4 °C. Resuspend PKH26-labeled MSC-Exo in 200 µl PBS.

C. Spinal cord injury model

Note: All animal experimentations should be reviewed and approved by local ethical committee.

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1. Keep female Sprague Dawley rats (200-250 g) on a 12 h light/dark cycle with ad libitum access to food and water.
2. Ensure an acclimation period of about one week before surgery.
3. Ensure the surgery station and tools are clean and sterile during the whole procedures to minimize the risk of infections.
4. Anesthetize rats by intraperitoneal injections of ketamine (60-90 mg/kg) and xylazine (10-15 mg/kg) mixture, with inhalation of 1-2% isoflurane at a flow rate of 1 L/min.
5. Place the anesthetized rats on a supine position, on a heating pad to maintain core body temperate at 37 °C.
6. Ensure adequate anesthesia, as indicated by the absence of paw withdrawal upon pinch or corneal reflex.
7. Apply eye ointment on both eyes to protect the eyes.
8. Shave the fur on the back (about 5 cm x 6 cm) using an electric shaver.
9. Palpate the prominence when the trunk of the rats is curved up, and mark the point using a permanent marker.
10. Sterilize the area using polydine solution, followed by twice 70% ethanol solution.
11. Inject buprenorphine (0.01-0.05 mg/kg) subcutaneously to minimize post-operative pain.
12. Perform a 2 cm sagittal midline incision using a #10 scalpel, centering at the point identified earlier.
13. Separate the paravertebral muscles to expose the vertebral processes.
14. Remove the T10 vertebral body using a Rongeur to expose the spinal cord.
15. Lift up the spinal cord using a spinal cord hook.
16. Perform the full transection using a microscissor.
17. Irrigate the injured field using 0.9% saline.
18. Clean the bloods to make the surgical view clear.
19. Verity lesion completeness by lifting up the severed spinal cord stumps and passing the spinal cord hook inside the gap to ensure no residual fibers on the bottom and lateral side of the spinal cord canal.
20. Suture the muscle and skin layer by layer using 4.0 sutures.
21. Gently place the rats in a temperate controlled recovery chamber, supplemented with oxygen, to allow full recovery.
22. Three-hour post-injury, for microCT, FAAS, and ICP studies, use a micropipetter to inject 10 µl GNP-labeled exosomes to one side of the nostrils, wait for 2 min before injecting another 10 µl dose to other side. This step repeats once again 2 min later, for a final volume of 20 µl exosomes injected to each side. For immunofluorescence studies, use a micropipetter to inject 10 µl PKH26-lableled exosomes to one side of the nostrils, wait for 2 min before injecting another 10 µl dose to other side. This step repeats once again 2 min later.
23. Gently massage the bladder and inject buprenorphine (0.01-0.05 mg/kg) subcutaneously every 8 h.

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D. Tissue processing and microCT imaging acquisitions
   1. Twenty-four hours after GNP-labeled exosome treatment, euthanize the rats using CO_2 inside a CO_2 euthanasia chamber, via a building supplied CO_2 setup.
   2. Harvest the olfactory bulbs, whole brain and spinal cord tissues gently, after removing their surrounding bone structures using rongeurs and scissor.
   3. Immerse the dissected tissues in 4% paraformaldehyde (PFA) overnight at 4 °C.
   4. Wash the tissues using 1x PBS for 5 min.
   5. Scan the tissues using microCT scanner (Skyscan High Resolution Model 1176). Imaging parameters are as follow: 45 kV tube voltage, 0.2 mm aluminum filter, and 35 µm nominal resolution. Use a modified algorithm (Feldkamp) in the GPU-accelerated SkyScanNRecon software for reconstruction. Use 25% beam hardening correction, 3% Gaussian smoothing, and ring artifact reduction. Use RGBA transfer function in the SkyScan CT-Voxel ("CTVox") and SkyScan CT-Volume ("CTVol") software to generate volume-rendered three-dimensional images.

E. Tissue processing for FAAS, and ICP studies
   1. Twenty-four hours after GNP-labeled exosome treatment, euthanize the rats using CO_2.
   2. Harvest and weigh the olfactory bulbs, whole brain, spinal cord tissues, lungs, liver, kidneys, heart and spleen (use rongeurs and scissor to remove bone structures surrounding the olfactory bulbs, whole brain and spinal cord tissues; collect whole lungs and heart in the thoracic cavity and liver, kidneys, and spleen in the abdominal cavity).
   3. For FAAS study, digest the lungs, liver, kidneys, heart, and spleen with 1 ml aqua regia acid (a mixture of nitric acid (BioLab Ltd) and hydrochloric acid (Daejung, CAS: 7647010) in a volume ratio of 1:3). Allow the solution to evaporate at room temperature, within the chemical hood for 24 h, followed by half an hour of heating to 60 °C, and dilute with DDW to 4 ml total solution. Filter smaples via 0.45 µm syringe filters. Measure gold concentrations using a linear calibration curve prepared using a solution with known gold (Au) concentrations of 0.1, 1, 2, and 5 mg/ml.
   4. For ICP study, combust the olfactory bulbs and brain, and spinal cord tissues at 550 °C for 5 h. Add 5 ml 1% HNO_3 to the digested tissues and filter via 0.45 µm syringe filters. Measure gold concentrations in the samples with correlation to the gold standard curve.

F. Tissue processing for immunofluorescence studies
   1. Twenty-four hours after PKH26-labeled exosome treatment, euthanize the rats using CO_2.
   2. Harvest the spinal cord tissues after removing the surrounding vertebral column using a rongeur and immerse the tissues in 5 ml 4% PFA at 4 °C overnight.
   3. Rinse the tissues with 1x PBS for 2 min, and immerse the tissues in 10 ml 30% sucrose solution at 4 °C overnight.
   4. Embed the tissues in optimal cutting temperature solution.
   5. Section the tissues longitudinally to 20 µm thickness.

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6. Permeabilize the tissue slices in 100 µl 0.5% Tween solution.
7. Rinse the tissue slices thrice with 1x PBS, 5 min each wash.
8. Block the tissues with 100 µl 5% BSA for 1 h, at room temperature.
9. Incubate the tissues with 50 µl primary antibodies diluted in 5% BSA, at 4 °C overnight.
10. Rinse the tissue slices thrice with 1x PBS, 5 min each wash.
11. Incubate the tissues with 50 µl secondary antibodies diluted in 1x PBS, at room temperature, for 1 h.
12. Rinse the tissue slices thrice with 1x PBS, 5 min each wash.
13. Mount the tissue slices with coverslips.
14. Scan the tissue slices using confocal microscope (ZEISS).

Data analysis

A. GNP-exosome quantification
   1. Measure the weight of olfactory bulbs and whole brain, spinal cord tissues, lungs, liver, kidneys, heart and spleen.
   2. Measure the GNP concentration in the olfactory bulbs and whole brain, and spinal cord tissues, using the ICP test, and lungs, liver, kidneys, heart and spleen, using the FAAS test.
   3. Compare the GNP concentration per mg tissue in olfactory bulbs and whole brain, spinal cord tissues, lungs, liver, kidneys, heart and spleen, between the injured and health rats (Table 1).

Table 1. ICP test result of spinal cord tissue

Samples	mg Au per mg tissue (n = 3)
Healthy spinal cord	6.44E-08 ± 1.54E-08
Injured spinal cord	1.20E-07 ± 1.30E-08

B. PKH26-labeled-exosome quantification
   1. Stain cryosectioned injured spinal cord tissue treated with intranasal PKH26-Exosomes, using antibodies against NeuN, GFAP, or TMEM119 respectively.
   2. Take immunofluorescent images of those labeled tissues (Figure 2).

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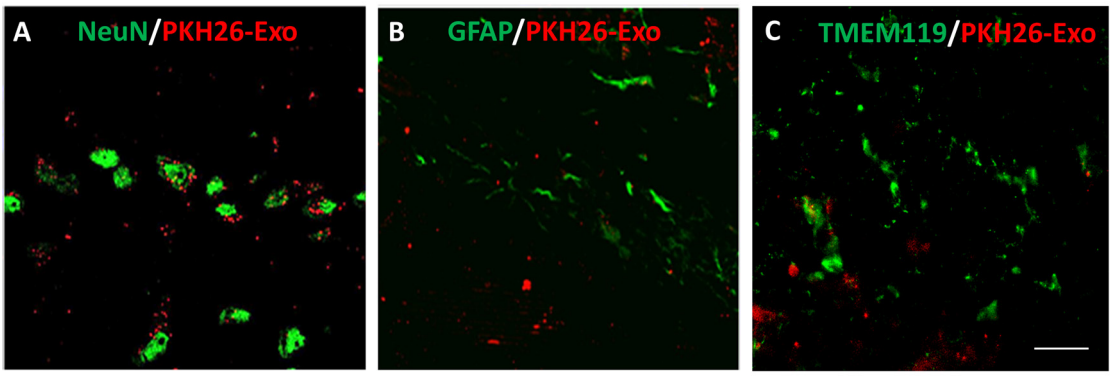

Figure 2. Immunofluorescent staining of spinal cord rostral to the lesion. A. Neurons. B. Astrocytes, and C. Microglia were in green, and exosomes in red. Scale bar = 25 μm.

3. In each group of immunofluorescent images, calculate the signals of PKH26 colocalized with NeuN, or GFAP, or TMEM119, using Matlab code as follows (Figure 3).

```
clear all;
close all;
[filename,pathname] = uigetfile('*.*','Select Image');
im = imread(strcat(pathname,filename),'tif');
imgsize = size(im);
imgpixX = imgsize(2);
ROI = roipoly(im);
imR=double(im(:,:,1)).*ROI/255;
imG=double(im(:,:,2)).*ROI/255;
% imG=double(im(:,:,2)).*ROI/255;
%imR=double(im).*ROI/255;
%imG=double(im).*ROI/255;
imBWR = im2bw(imR,0.2);
imBWG = im2bw(imG,0.1);
figure(1)
imagesc(imBWR);
figure(2)
imagesc(imBWG);
greenAndred = imBWR.*imBWG;
figure(3)
imagesc(greenAndred);
Total_red=sum(sum(imBWR));
redINgreen = sum(sum(greenAndred));
percentage = 100*redINgreen/Total_red;
fid = fopen('exosome.csv', 'a+');%name of excel file
```

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```
fprintf(fid,
'%s, %f, %f, %f\n',filename,Total_red,redINgreen,precentage);
fclose(fid);
```

4. Compare the colocalizations amongst NeuN, GFAP, or TMEM119-stained tissues.

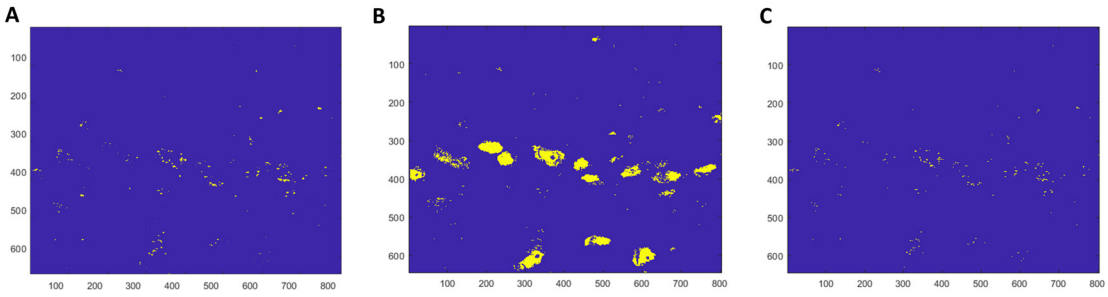

Figure 3. Matlab analysis of exosome colocalization with neurons. A. Exosome signal. B. neuron signal, and C. colocalized signal between exosomes and neurons.

C. To analyze data between two groups, use Student's *t*-test or one-way ANOVA with *post-hoc* Turkey's multiple comparison test for more than two groups. Data are presented with means ± SEM. Significance levels: *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ****$P < 0.0001$.

Recipes

1. Sucrose solution
   30 g Sucrose
   Add 100 ml PBS
2. Blocking solution
   2.5 g BSA
   Add 50 ml PBS
3. Tween solution
   200 µl Tween-20
   Add 40 ml PBS

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Competing interests

The authors declare no conflict of interest.

Ethics

All experimental procedures have been reviewed and approved by the ethical committee of the Technion-Israel Institute of Technology (IL1130817, validity period: 01.01.2018 to 01.01.2022).

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