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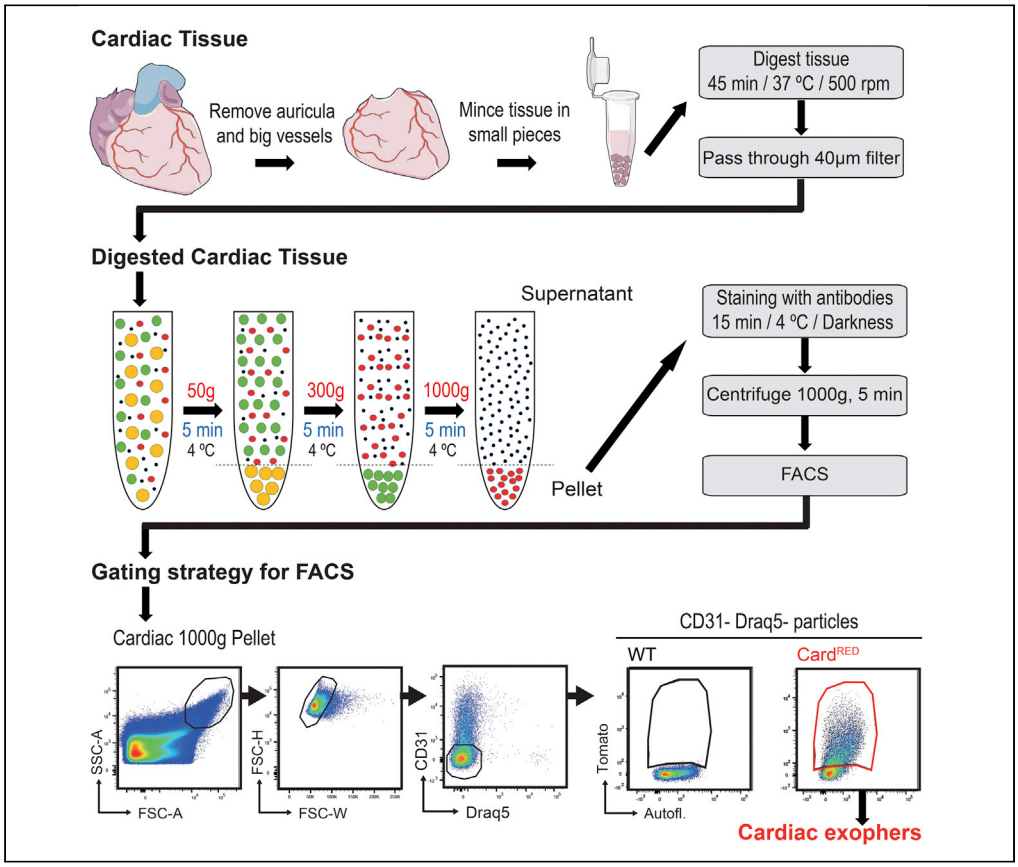
Protocol

Isolation of exophers from cardiomyocyte-reporter mouse strains by fluorescence-activated cell sorting



Cardiac exophers are membrane-bound extracellular vesicles released by cardiomyocytes with varied content and an average diameter of 3.5 µm. Here, we provide a detailed protocol to enable the identification and purification of cardiomyocyte-derived exophers by using fluorescence-activated cell sorting for downstream cellular and molecular analysis. This protocol requires the use of mouse strains expressing fluorescent proteins in cardiomyocytes.

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HIGHLIGHTS

Optimized protocol for exopher isolation from cardiomyocytes using FACS

Isolated exophers are suitable for downstream cellular and molecular analysis

Includes sample preparation and gating strategy for exopher identification

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Isolation of exophers from cardiomyocyte-reporter mouse strains by fluorescence-activated cell sorting

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SUMMARY

Cardiac exophers are membrane-bound extracellular vesicles released by cardiomyocytes with varied content and an average diameter of 3.5 μm. Here, we provide a detailed protocol to enable the identification and purification of cardiomyocyte-derived exophers by using fluorescence-activated cell sorting for downstream cellular and molecular analysis. This protocol requires the use of mouse strains expressing fluorescent proteins in cardiomyocytes.
For complete details on the use and execution of this protocol, please refer to Nicolás-Ávila et al. (2020).

BEFORE YOU BEGIN

To ensure success, it is critical to maintain everything on ice or at 4°C unless otherwise indicated. Have all essential reagents, equipment, and workstations prepared before starting the protocol.

Prepare the following

1. Prepare phosphate-buffered saline (PBS).
2. Prepare sorting buffer.
3. Set cell incubator to 37°C.
4. Cool a centrifuge for 15 mL tubes to 4°C.
5. Dissection tools: scissors and forceps.

⚠ CRITICAL: Note that this protocol may only work for mice with fluorescence protein expression restricted to cardiomyocytes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD31-Bv605 (clone 390)	BioLegend	CAT# 102427
Chemicals, peptides, and recombinant proteins		
Draq5	BioLegend	CAT# 424101
DAPI	Thermo Fisher	CAT# D3571
HBSS	Thermo Fisher	CAT# 14175-053
DNase I	Sigma	CAT# D4527-10KU
FBS	Gibco	CAT# 1027-106

(*Continued on next page*)

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
BSA	Sigma	CAT# A0976
Liberase	Roche	CAT# 5401127001
Experimental models: organisms/strains		
Mice: C57BL/6J	Jackson Laboratory	CAT# 000664
Mice: Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$	Miguel Torres	(Madisen et al., 2010)
Mice: B6.FVB(129)-A1cf$^{Tg(Myh6-cre/Esr1*)1Jmk}$/J	Juan Miguel Redondo	(Oka et al., 2006)

MATERIALS AND EQUIPMENT

PBS buffer

This solution can be prepared beforehand and stored at 4°C until use. PBS (phosphate-buffered saline) is a blend of phosphate buffers and saline solutions which contains:

- 137 mM NaCl
- 2.7 mM KCl
- 10 mM Na_2HPO_4
- 1.8 mM KH_2PO_4
- Adjust pH to 7.4

Alternatives: Commercial ready PBS can be bought from different companies such as Sigma (CAT# P5368-10PAK) or Thermo Fisher (CAT# 14190144).

Sorting buffer

This solution can be prepared beforehand and stored at 4°C until use. You will need to prepare 10–15 mL for each heart. Sorting Buffer contains:

- PBS buffer pH 7.4 (See above)
- 0.5% heat-inactivated FBS (fetal bovine serum).
- 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
- 1 mM EDTA (ethylenediaminetetraacetic acid)

Alternatives: Heat-inactivated FBS can be exchanged by bovine serum albumin (BSA) at a similar concentration. Other formats of FBS such as regular- or exosome depleted-FBS may work as well but this should be tested by each researcher.

Digestion solution

You will need 1 mL for each processed heart. This solution should be freshly prepared right before use and contains:

- Hank's balanced salts solution (HBSS)
- Liberase (0.2 mg/mL)
- DNase I (1 U/mL)

Alternatives: an alternative to Liberase in Digestion buffer is a protease-collagenase mix. Although we have not tested this for cardiac exopher preparation, other labs have successfully used it to obtain single cell suspensions form primary cardiac tissue (Aronoff et al., 2018; Pinto et al., 2013).

Collection buffer

You will need to prepare 0.1 mL for each processed heart. This solution can be prepared beforehand and stored at 4°C until use. Collection buffer contains:

- PBS buffer pH 7.4 (see above)
- 10% inactivated FBS (fetal bovine serum).

Alternatives: Heat-inactivated FBS can be exchanged by bovine serum albumin (BSA) at a similar concentration. Other formats of FBS such as regular- or exosome depleted-FBS may work as well but this should be tested by each researcher.

Centrifuge

You will need a centrifuge with adaptors for 15 mL (we use the Eppendorf 5810 R model) and 1.5 mL tubes (we use the both the Eppendorf 5810 R and 5415 R).

Heat pad

We use the Thermomixer comfort model from Eppendorf that allows temperature and mixing speed control.

Alternatives: If a heat block with agitation is not available, you can increase the incubation time with digestion buffer from 45 to 60 min and use a 37°C water bath instead.

STEP-BY-STEP METHOD DETAILS

Obtain a cell suspension enriched in cardiac exophers from primary cardiac tissue

Timing: 1 h

The primary method described here was adapted from other methods developed to isolate non-cardiomyocyte cell populations with the use of standard laboratory equipment and reagents for downstream cellular and molecular analyses (Aronoff et al., 2018; Pinto et al., 2013). The protocol is based on collagenase-mediated digestion of the extracellular matrix to release cells and, in this case, its subcellular particles. An explanatory video with steps 1–18 is provided (Methods Video S1).

1. Prepare the procedure space (Figure 1A). You will need
   a. Cold PBS for harvesting the heart and perfusion.
   b. Surgery scissors and forceps for the dissection
   c. Dissection surface.
   d. 10 mL syringe with a 21G needle loaded with fresh PBS.
   e. 70% ethanol.
2. Euthanize mouse using CO_2 asphyxiation or by any other institutionally accepted euthanasia procedure following your animal facility guidelines.
3. Place mouse in a supine position on the dissection stage and immobilize it by pinning its paws and feet to the stage, e.g., by using syringe needles (Figure 1B).
4. Spray the mouse torso with 70% ethanol to minimize fur dispersal and make an incision of superficial abdominal tissue below the lower costal bone and diaphragm by using forceps and scissors (Figure 1C). Then, cut upwards toward the head removing skin to reveal the peritoneal cavity (Figure 1D).
5. Access the peritoneal cavity by removing the peritoneum (Figure 1E).
6. Access the thoracic cavity and the heart by gently cutting the diaphragm laterally while holding the sternum with forceps (Figure 1F).
7. Cut the two lateral margins of the anterior thorax toward the head and flip it upwards. Be careful to avoid puncturing the lungs and heart (Figures 1F–1G).
8. You can now immobilize the anterior thorax by pinning it with a syringe needle to the dissection surface (Figure 1H).

9. Perfuse the heart (and the entire mouse), by nicking the right atrium using surgical scissors (Figure 1I) and hold the heart with the apex pointing upwards using forceps. Carefully insert the

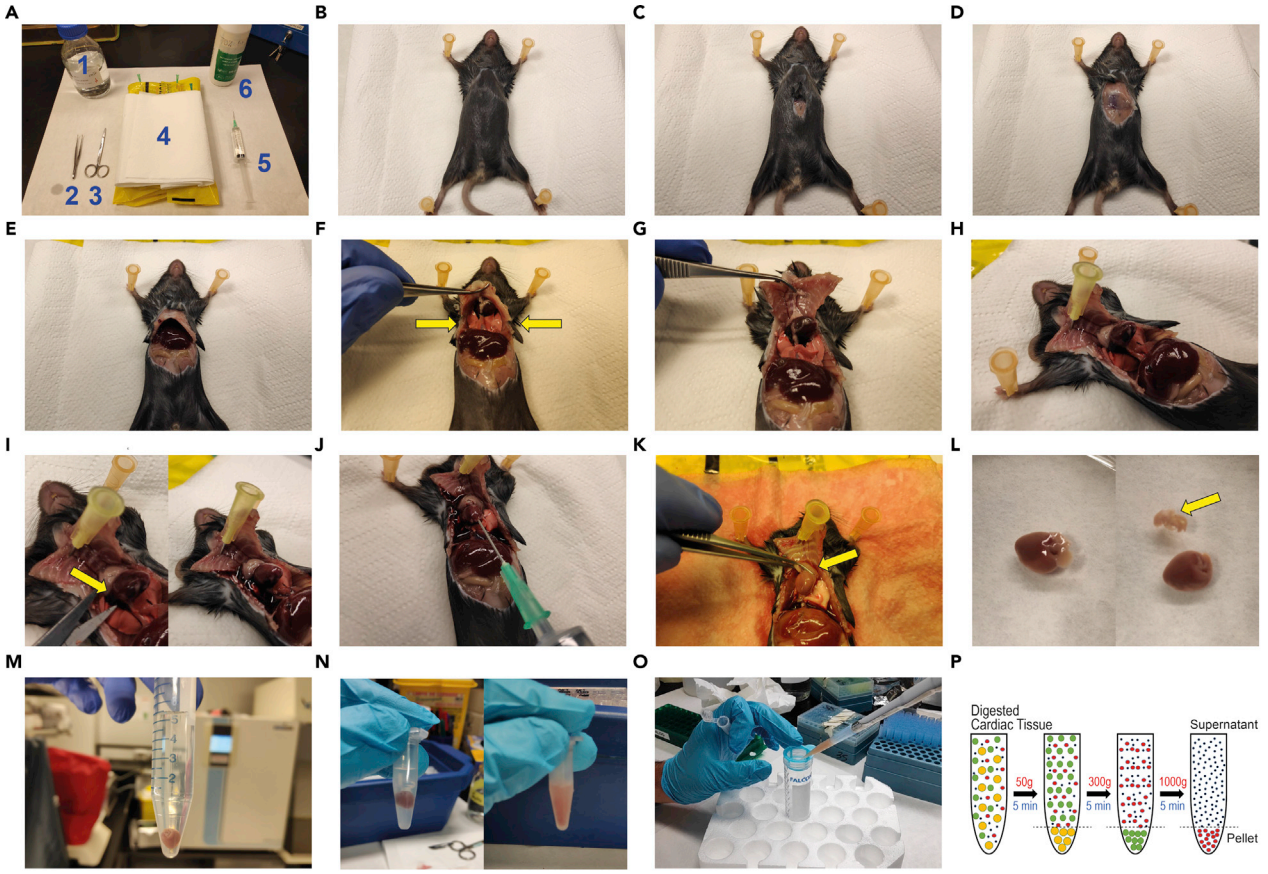

Figure 1. Perfusion and heart isolation for exopher preparation

(A) Material needed: (1) cold PBS, (2) forceps, (3) surgery scissors, (4) dissection table, (5) 10 mL syringe with 21G needle loaded with cold PBS and (6) 70% ethanol.

(B) Immobilization of the mouse.

(C) First incision.

(D) Skin removal.

(E) Access to peritoneal cavity.

(F) Access to thoracic cavity. Yellow arrows indicate the point where you should cut to separate the thorax and expose the heart.

(G) Heart exposure.

(H) Thorax immobilization.

(I) Right atrium puncturing. (Left) Yellow arrow points to the right atrium. (Right) Once punctured, blood will start flowing out fast.

(J) Needle insertion in left ventricle for perfusion.

(K) Heart collection. You should cut below the forceps, as indicated by the yellow arrow.

(L) (Left) Place the heart in a Petri dish with PBS. (Right) Remove the atria and big vessels (indicated by the yellow arrow).

(M) Place the ventricular myocardium into a Falcon tube with fresh PBS.

(N) Cardiac tissue before (left) and after (right) mincing with scissors in digestion buffer.

(O) Transference of digested tissue through a 40 μm strainer placed in a 50 mL Falcon tube.

(P) Summary of serial centrifugation steps.

syringe needle in the apex of the heart into the left ventricle and inject 10 mL of cold PBS at a flow rate of approximately 5 mL/min for 2 min (Figure 1J).

Note: Here you can follow different options:

a. Use a syringe with a 21G needle manually. This requires more skill and experience (Figures 1J).

b. Use a pump.

Note: Place needle gently in the left ventricle and be careful not to puncture the interventricular septum, pulmonary valves, or atrial valves. Effective perfusion is indicated by gradual

change of liver color from a deep red-brown to light yellow-brown, and of the lungs from pink to white (Figure 1J versus Figure 1K). Perfusion should be performed within less than 5 min after euthanasia in order to avoid blood coagulation, which would difficult the process and contaminate the organs of interest with circulating cells.

10. Remove the heart and trim away left the atria to ensure only the intact myocardium is isolated.

Note: This step is easier if you first remove the whole heart by cutting the major vessels below atria (aorta and major pulmonary arteries and veins; Figure 1K). Then you can carefully remove the vessels and the atria in a petri dish with cold PBS (Figure 1L).

11. Place the heart in a 15 mL Falcon tube filled with 3–5 mL of ice-cold PBS (Figure 1M).
12. Place the tube on ice, isolate any other desired tissues if needed and repeat steps 1–11 for any additional mice.

Note: Ideally, the time elapsed between harvesting of the tissues and processing should not be more than 1 h.

13. Once in the bench, prepare a 1.5 mL Eppendorf tube with 1 mL of digestion buffer, one tube for each heart.
14. Put the isolated hearts in the tubes with digestion buffer and mince them with surgical scissors into small pieces (Figure 1N).

Note: Hold the Eppendorf tube with one hand and mince using surgical scissors on the other hand. Clean your scissors between samples with PBS in a petri dish and paper. It is not necessary to pre-warm the Digestion buffer before this step.

15. Place the tubes in a 37°C heat block for 45 min with 100 rpm agitation.
16. After incubation, mix by pipetting up and down gently using a 1 mL pipette and a tip with the end trimmed (2 mm diameter). This is important to reduce the applied shear force and avoid rupture of non-disaggregated pieces of tissue.
17. Pre-wet a 40 μm strainer placed in a 50 mL Falcon tube with 1 mL sorting buffer. Then, transfer the digestion mixture to the strainer (Figure 1O).
18. Add 4–5 mL of cold sorting buffer through the strainer gently in order to stop the enzymatic reaction and help cells go through.
19. Remove the filter and discard the non-disaggregated pieces of tissue that had not gone through.
20. Transfer the cell suspension into a 15 mL Falcon tube.
21. Centrifuge samples at 50 × *g* for 5 min at 4°C and transfer the supernatant into a new 15 mL Falcon tube. Discard the pellet that contains mostly cardiomyocytes and cell aggregates (Figure 1P).
22. Centrifuge samples at 300 × *g* for 5 min at 4°C and transfer the supernatant into a new 15 mL Falcon tube. Discard the pellet that contains mostly small cells such as fibroblasts, leukocytes, and endothelial cells.
23. Centrifuge samples 1,000 × *g* for 5 min at 4°C, discard supernatant, and keep the pellet, which is enriched in cardiac exophers (Nicolás-Ávila et al., 2020).

Note: this serial centrifugation strategy is an adaptation from other protocols used to enrich the fraction containing apoptotic bodies (Atkin-Smith et al., 2017), similar in size (1–5 μm in diameter) to cardiac exophers (Nicolás-Ávila et al., 2020).

24. Re-suspend the pellet in 100 μL of sorting buffer containing antibodies at the desired concentration and incubate for 15 min at 4°C in the dark.

Note: in our case, we used CD31 (1:200 dilution) for the exclusion of endothelial cell derived particles, which are major components of the heart (Pinto et al., 2016), thereby cleaning our gating strategy. If assessment of other surface proteins in cardiac exophers, you should add the relevant probes/antibodies in this step.

25. Add 1–2 mL of sorting buffer to wash off the excess of antibody.
26. Centrifuge 1,000 × *g* for 5 min at 4°C, discard supernatant, and keep the pellet.
27. Re-suspend the pellet with 1 mL of sorting buffer containing Draq5 (1:5,000 dilution), a DNA probe that allows discrimination of nucleated versus non-nucleated structures. Keep samples at 4°C in the dark.
28. Samples are now ready to perform flow cytometry analysis. This analysis should be performed within the next 4 h.
29. If the goal is to sort exophers, prepare 1.5 mL Falcon tubes with 100 μL of collection buffer.

⚠ CRITICAL: Except for the enzymatic digestion, use always ice-cold solutions. In order to minimize the generation of cardiomyocyte-derived debris, avoid mechanically forced dissociation of the tissue by smashing or pipetting. Use only the cells/cardiac exophers disaggregated by enzymatic dissociation. The volumes indicated here are calculated for processing a whole heart. However, in order to obtain enough cardiac exophers for flow cytometry/FACS, 1/4 of the organ is sufficient so that the rest can be used for other purposes. If this is the case, you can modify the volumes accordingly.

Cardiac exopher identification by flow cytometry

Timing: 1–4 h

The purpose of this part of the protocol is to identify cardiac exophers in a flow cytometer, in combination with FACS or not, in order to perform downstream analysis on the exophers. The reduced size of these particles demands to increase laser potency and work at slower flow velocities than usual. For FACS it is likely that samples need to be diluted 1:10 or more to increase efficiency.

30. Gating strategy is provided in Figure 2.
31. Cardiac exophers will be among the particles with highest FSC-A and SSC-A signal in the 1,000 × *g* pellet obtained in the previous part of the protocol (Figure 2A).

Note: a logarithmic view for FSC-A and SSC-A instead of Linear will be useful to discriminate cardiac exophers in this plot. Also, you can use clean sorting buffer to identify the "noise" threshold and avoid contamination from buffer. However, exophers are much larger than any particle coming from sorting buffer (Not shown).

32. Discard doublets by using FSC-H and FSC-W (Figure 2A).
33. Discard particles containing DNA (Draq5+) or endothelial markers (CD31+). This will help "cleaning" the final gate (Figure 2A).
34. Use the fluorescence signal from cardiomyocytes in your fluorescent reporter model to identify cardiac derived exophers from the rest of particles of similar size. Use wild-type or Cre-negative mice as a control to establish the gate (Figure 2B). In our case, we induced Tdtomato fluorescent protein expression specifically in cardiomyocytes by crossing mice with a Cre recombinase under the control of the mouse Myh6 promoter (myosin, heavy polypeptide 6, alpha isoform; αMHC^{CRE}; Oka et al., 2006) with $Rosa26^{Tdtom}$ (Madisen et al., 2010). These mice were referred as $Card^{RED}$ in our original publication (Nicolás-Ávila et al., 2020), although similar results can be obtained with other reporter fluorescent proteins, such as GFP (Not

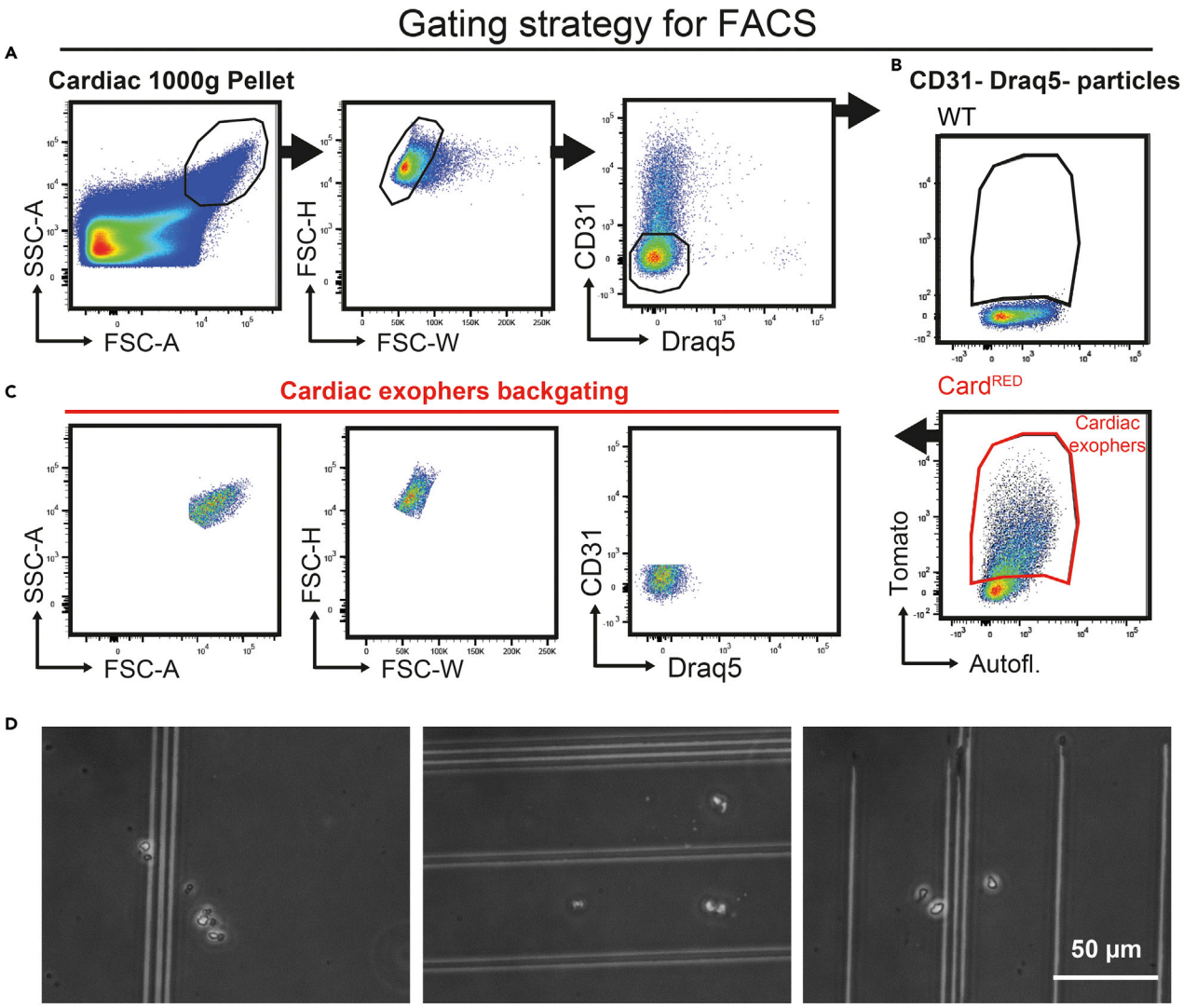

Figure 2. Identification of cardiac exophers by flow cytometry

(A and B) Gating strategy used to identify cardiac exophers based on (A) size, markers and (B) fluorescence in a cardiomyocyte-reporter mouse model (αMHC^{CRE} Rosa26^{TdTom}, referred as Card^{RED}) (Nicolás-Ávila et al., 2020).

(C) Back-gating of cardiac exophers, as identified in (A) and (B).

(D) Bright-field images of sorted exophers.

shown). We have used animals from 1 to 50 weeks of age and from both sexes with similar results.

35. After detection of the cardiac exophers by flow cytometry, you can back-gate those to better define your gates (Figure 2C).

EXPECTED OUTCOMES

A successful extraction process should routinely yield 1 × 10^6 or more cardiac exophers from one heart. Good quality cardiac exophers appear roundish and bright under the microscope (Figure 2D), and tdTomato signal that can be detected by fluorescent microscopy (Nicolás-Ávila et al., 2020). These exophers are suitable for flow cytometry, FACS, microscopy, proteomics or metabolic assays (Nicolás-Ávila et al., 2020). Note: we always use exophers 1–4 h after their isolation. For longer times each researcher must test the integrity of exophers at the needed time.

LIMITATIONS

Due to the lack of knowledge in surface markers specifically expressed on Exophers or Cardiomyocytes by flow cytometry, we rely on mouse strains expressing fluorescent proteins (i.e., tdTomato or EGFP) under cardiomyocyte-restricted Cre lines, such as αMHC-Cre, as this allows identification of those particles after previous enrichment with differential centrifugation steps. By using this protocol in combination with other molecular probes we have described the presence of Mitochondria inside cardiac exophers and phosphatidylserine on their membrane (Nicolás-Ávila et al., 2020); however, these properties are shared with other extracellular vesicles and cannot be used as unique criteria for their definition. An important consideration is that we use regular FBS in the preparation of our buffers and this may contain exosomes that can influence cultured cell behavior and downstream analysis (Ochieng et al., 2009; Shelke et al., 2014). If this is important for the user, there are protocols available to deplete exosomes from FBS (Shelke et al., 2014) and specific commercial formats.

TROUBLESHOOTING

Problem 1

Cardiac exophers are less than 1% of the events found in the 1,000 × *g* pellet obtained in step 23. High SSC/FSC laser voltage, necessary to properly visualize this structures by FACS (steps 30 and 31), may heavily increase the number of events detected by the cytometer (event overflow) causing problems with data acquisition and sorting efficiency.

Potential solution

To overcome this problem, there are two possibilities:

- If you are using exopher preparations only for cytometric analysis, an option is to use the sorting buffer to stablish a "noise" threshold in the SSC/FSC channels, so the number of events that the cytometer has to analyze by unit of time is strongly reduced. This helps significantly in terms of acquisition times and posterior data analysis.
- In the case of exopher sorting, you may want to avoid debris contamination of your sorted samples. Thus, increase SSC/FSC threshold is not a good option since it will reduce the sorter ability to discard drops containing exophers in combination with undesired elements. In this case, the best option is to dilute samples in a large sorting buffer volume to increase sorting efficiency, in detriment of acquisition time.

Problem 2

Rosa26^{Tdtom} mice may not be available for some researchers or are not compatible with other fluorophores in the range of tdTomato fluorescence, i.e., phycoerythrin (PE), Keima, etc.

Potential solution

We have successfully used Rosa26^{EGFP} in combination with αMHC^{CRE} with the same results that those presented for αMHC^{CRE} Rosa26^{Tdtom}. Any other combination between cardiomyocyte-specific Cre and inducible fluorescent protein expression may work to detect cardiac exophers. If this is your case, you will have to modify the gating strategy and laser used accordingly.

Problem 3

Lack of criteria to distinguish between cardiac exophers and other subcellular particles (such as apoptotic bodies) of cardiomyocyte origin in our setup.

Potential solution

To overcome this problem, we have followed a series of steps to minimize the generation of artifacts caused by mechanical rupture of cardiomyocytes. Although these are specified in each respective part of the step-by-step protocol, we summarize them here:

- After incubation with digestion buffer (step 15), trim the tip to create a larger opening (2 mm is enough) to pass the digested tissue through the strainer. This is important to reduce the applied shear force and to avoid massive rupture of cardiomyocytes.
- After transferring the digestion mixture to the strainer (steps 17 and 18), do not force the non-disaggregated pieces of tissue through, just discard them with the filter.
- The 50 × *g* centrifugation in step 21 before the 300 × *g* centrifugation in step 22 is meant to sediment cardiomyocytes smoothly without causing rupture. Do not skip this step since centrifuging cardiomyocytes at 300 × *g* will generate a lot of unspecific cardiomyocyte-derived debris.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by the Lead Contact, Andres Hidalgo (ahidalgo@cnic.es).

Materials availability

This study did not generate new unique resources, mice, or reagents. Further information on materials, datasets, and protocols should be directed to and will be fulfilled by the Lead Contact, Andres Hidalgo (ahidalgo@cnic.es).

Data and code availability

This study did not generate new unique Data or Code resources.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2020.100286.

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AUTHOR CONTRIBUTIONS

J.A.N.-A. and M.S.-D. performed experiments. J.A.N.-A. and A.H. designed and supervised the protocol. A.H. designed and supervised the study. J.A.N.-A. wrote the manuscript, which was edited by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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