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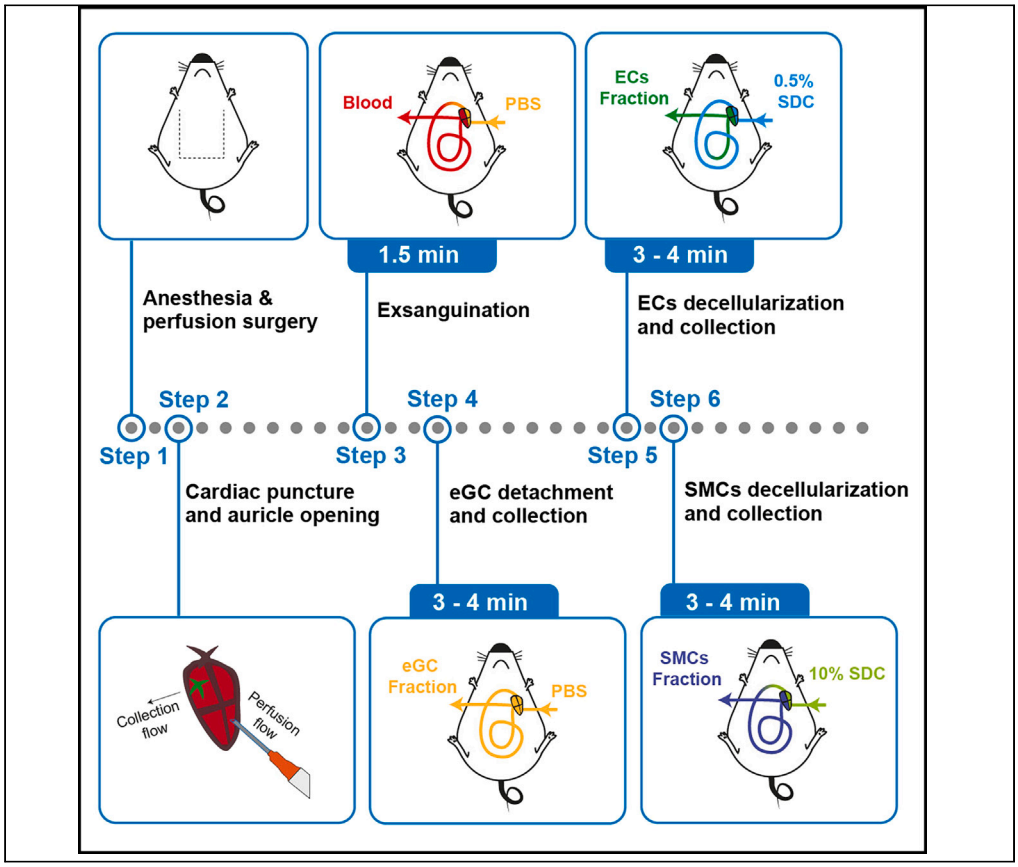
Differential systemic decellularization *in vivo* to study molecular changes in each vasculature layer in murine models of disease



Vascular dysfunction underlies the onset and progression of many life-threatening diseases, highlighting the need for improved understanding of its molecular basis. Here, we present differential systemic decellularization *in vivo* (DISDIVO), a protocol that enables systemic and independent study of the molecular changes in each vasculature layer in murine models of disease. We describe steps for anesthesia, perfusion surgery, and exsanguination. We then detail detachment and collection of glycocalyx and decellularization and collection of both endothelial and smooth muscle cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

DISDIVO allows biochemical characterization of whole-body vascular beds

Vascular beds' cell layers can be sequentially lysed and collected

DISDIVO protocol is based on intracardiac whole-body perfusion

DISDIVO can be adapted and applied to multiple animal models of disease

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Differential systemic decellularization *in vivo* to study molecular changes in each vasculature layer in murine models of disease

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SUMMARY

Vascular dysfunction underlies the onset and progression of many life-threatening diseases, highlighting the need for improved understanding of its molecular basis. Here, we present differential systemic decellularization *in vivo* (DISDIVO), a protocol that enables systemic and independent study of the molecular changes in each vasculature layer in murine models of disease. We describe steps for anesthesia, perfusion surgery, and exsanguination. We then detail detachment and collection of glycocalyx and decellularization and collection of both endothelial and smooth muscle cells.

For complete details on the use and execution of this protocol, please refer to Serra et al., Gallart-Palau et al., and Vinaiphat et al.[1–3]

BEFORE YOU BEGIN

In vivo models of disease offer new insight on the molecular basis of vascular dysfunction linked to the onset of major human diseases (i.e., stroke, heart attack, sepsis, dementia, and cancer, to mention just a few). The exploitation of these models, though, to understand the systemic affectation of the different vascular beds, with special emphasis on capillaries, in specific organism systems or in whole organism, cannot be achieved by traditional workflows.[1]

In this protocol article, we present a system-wide approach for the molecular profiling of vascular beds. In DISDIVO by employing whole body perfusion in mice with increasing concentrations of detergent buffers, the different vascular layers (glycocalyx (eGC), endothelial cells (ECs) and smooth muscle cells (SMCs)) become sequentially decellularized and can be collected for further biochemical analysis.

> ***Note:*** This protocol besides being suitable for mice models can be adapted to other animal models that allow whole-body perfusion. Larger animals (i.e., rat and pig) might allow an easier implementation of the DISDIVO procedures detailed.

Institutional permissions

All animal procedures were approved by the Nanyang Technological University Institutional Animal Care and Use Committee and were performed in strict accordance with the International Guiding Principles for Animal Research in a humane manner.

Animal-related procedures must be done in compliance with the specific institutional regulations. All animal procedures have to be performed according to the 3Rs principle in animal experimentation.[4]

Preparation of materials and buffers for sequential whole-body perfusion

Timing: 0.5–1.5 h

Before starting the DISDIVO procedure different buffers as well as apparatus must be prepared.

1. Preparation of buffers for sequential whole-body perfusion. Approximate required volume of each buffer is indicated per animal. These volumes must be adapted depending on the dead volume of the perfusion system used for the procedure.
   a. Prepare 1× PBS. Ideally use commercial 1× PBS, or dilute 10× PBS with deionized water. At least 25 mL per animal are required.
   b. Prepare 0.5% sodium deoxycholate (SDC) in 100 mM ammonium acetate (AA) buffer. At least 10 mL per animal are required. More details about preparation of this solution are included in 'materials and equipment' section.
   c. Prepare 10% SDC in 100 mM AA. At least 10 mL per animal are required. More details about preparation of this solution are included in 'materials and equipment' section.

   Note: Concentration of ammonium acetate can range from 25 mM to 150 mM.

   Note: Make sure there is enough remaining of all buffers before starting the animal procedure.

   Note: SDC-containing buffers can be stored at room temperature and do not need to be prepared fresh every day. Make sure to use SDC-containing buffers < 2 months old.

   Note: Buffers containing SDC can also be prepared in 50 mM Tris HCl buffer (pH ranging from 7 to 9) or 1× PBS if incompatibilities with AA and analytical strategies used for the characterization of the decellularized vascular beds are expected.

2. Apparatus preparation. A multichannel syringe pump was used in this study. Nonetheless, multiple single-channel pumps (working in parallel) can also be used (see picture of this pump system in Figure 1A). The detailed instructions refer to the preparation of the two-channel syringe pump or two single-channel syringe pumps (working in parallel) perfusion system:
   a. Attach a 30-gauge needle (perfusion inlet needle) to a perfusion line (Figure 1B).
   b. Fill three 50-mL syringes with 1× PBS, 0.5% SDC buffer and 10% SDC buffer.
   c. Connect the perfusion line from step *a.* to the 50-mL syringe filled with 1× PBS.
   d. Manually clear the line to remove air bubbles.
   e. Place the 1× PBS syringe connected to the perfusion line into the syringe pump holder.
   f. Place the 0.5% SDC buffer filled syringe (not connected to any perfusion line) into a second peristaltic syringe holder.
   g. Set the flow rate of the two channels at 1.5 mL/min. Do not turn on the pump yet.

   Note: Multichannel syringe pump or multiple single-channel pumps (working in parallel) are preferred over gravitational perfusion systems. Both systems are detailed in the key resources table.

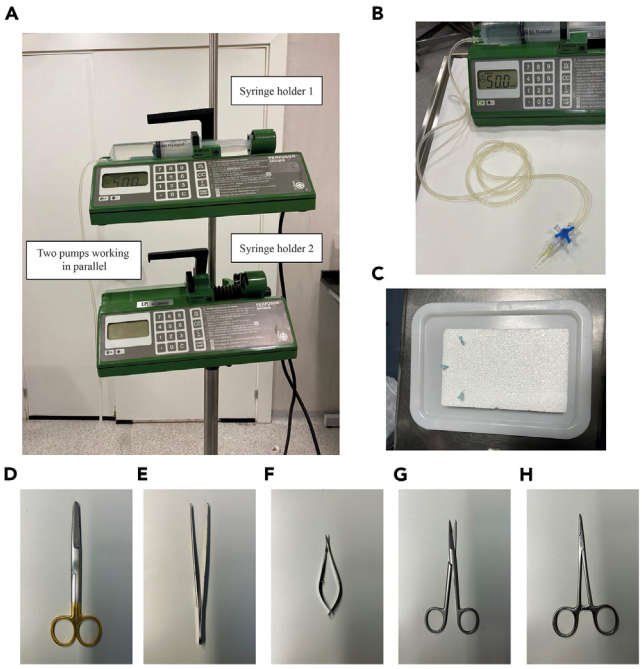

Figure 1. Materials used in the DISDIVO protocol

(A–E) Surgical tools: (A) Operating scissors, (B) Forceps, (C) Castroviejo spring scissors, (D) Fine scissors, and (E) Halsted-mosquito hemostatic forceps.

(F) Picture of two single-channel pumps working in parallel. Syringe holders are indicated. A 50 mL syringe connected to the perfusion line and filled with PBS is located in syringe holder 1.

(G) Close picture of the 50 mL syringe filled with PBS and connected to the perfusion line. A 30-gauge syringe is connected at the end of the perfusion line.

Note: the use of politetrafluoroetileno (PTFE) tape to seal the connection between the syringe and the perfusion line is recommended to prevent leakage due to the high working pressure generated by the pump.

Note: The perfusion line will be disconnected and reconnected to the three syringes filled with i) 1× PBS, ii) 0.5% SDC buffer and iii) 10% SDC buffer, in a sequential manner alongside the DISDIVO protocol as shown in steps 4 to 6 of the graphical abstract. Only one perfusion inlet needle will be used alongside the whole procedure.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Mouse: C57BL/6NT (wild type, male, 8–10 weeks)	The Jackson Laboratory	RRID: IMSR_JAX:000664
Chemicals, peptides, and recombinant proteins		
1× PBS pH 7.4	Thermo Fisher Scientific	10010031
Sodium deoxycholate ≥ 97%	Merck	D6570
Ammonium acetate	Merck	A7262
70% Ethanol	Sigma	109-56-8
Antifade mounting medium with 4′, 6-diamidino-2-phenylindole (DAPI)	Vector Laboratories	H-1200-10
Isoflurane	IsoFlo	26675-46-7
Ketamine hydrochloride/xylazine hydrochloride solution	Merck	K113

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-PECAM1 (10 μg/mL)	Abcam	ab28364
Alexa Fluor anti-rabbit 546-conjugated secondary antibody (2 μg/mL)	Jackson ImmunoResearch	A-11035
Alexa Fluor 594-conjugated wheat germ agglutinin (2 μg/mL)	Thermo Scientific Pierce	W11262
Deposited data		
Repository PRIDE (identifiers PXD008794, PXD031228, PXD018274)	Pride	http://www.ebi.ac.uk/pride
Software and algorithms		
GraphPad Prism 9	GraphPad software	https://www.graphpad.com/scientific-software/prism
Other		
Needle (25G)	Sterican	9186158
Needle (30G)	Sterican	4656300
50 mL centrifuge tubes	Corning	CLS430828
1 mL syringe	Terumo	050001
50 mL syringe	Biosigma	BSS131
Dissecting styrofoam tray	Nebraska Scientific	D 4025A
Dual-rate syringe pump	WPI	SPLG-G88PLUS
Perfusor Secura FT	B Braun	WA11578
VetFlo vaporizer single channel anesthesia system	VetFlo	VetFlo-1205S
Low-profile anesthesia mask for traditional vaporizer for animals under 100 g	VetFlo	VetFlo-0801
Operating scissors	HEBU	HB2261-18
Castroviejo spring scissors	Fine Science Tools	15017–10
Forceps	Iberomed	PIDI001
Fine scissors	Fine Science Tools	9910834
Student Halsted-mosquito hemostatic forceps	Hartmann	9910413

MATERIALS AND EQUIPMENT

0.5% sodium deoxycholate in 50 mM ammonium acetate		
Reagent	Final concentration	Amount
Sodium deoxycholate	0.5%	5 g
Ammonium acetate	100 mM	7.71 g
ddH_2O	-	1 L
Total	**N/A**	**1 L**
Store at room temperature for up to 2 months.		

Alternatives: 50 mM ammonium acetate can be substituted by 50 mM Tris HCl or 1× PBS.

10% sodium deoxycholate in 50 mM ammonium acetate		
Reagent	Final concentration	Amount
Sodium deoxycholate	10%	100 g
Ammonium acetate	100 mM	7.71 g
ddH_2O	-	1 L
Total	**N/A**	**1 L**
Store at room temperature for up to 2 months.		

Alternatives: 100 mM ammonium acetate can be substituted by 50 mM Tris HCl pH 7–9 or 1 × PBS.

STEP-BY-STEP METHOD DETAILS

Anesthesia

Timing: 30 min (for step 1)

Timing: 45 min

Animals will be anesthetized using ketamine/xylazine. General anesthesia will be maintained alongside the DISDIVO procedure using isoflurane.

1. Anesthesia of animals.
   a. Measure and record body weight of the animal and calculate amount of ketamine/xylazine at a dose of 90:10 mg/kg required.
   b. Administer the ketamine/xylazine through intraperitoneal injection using a 1 mL syringe with a 25-gauge needle. Intraperitoneal administration is injected at the lower left or right quadra of the mice abdomen.[5]
   c. Wait until the animal has reached a surgical plane and no tail pinch response is observed.
   d. Place the mouse on its back on a Styrofoam dissecting tray.
   e. Pin the four paws of the mouse using pins or needles to accomplish proper immobilization of the anesthetized animal.
   f. Connect the anesthesia mask for mice (nose cone) of the isoflurane infuser system to the nose of the animal.
   g. Turn on the isoflurane gas to 1.5% v/v isoflurane level at a flow rate of 0.4–0.8 L/min.

Note: The use of a Styrofoam board positioned in a large stainless steel or autoclavable tray is highly recommended for collection of any possible fluid overflow (see Figure 1C).

Note: animal surgical plane is generally accomplished in less than 5 min.

Note: for animal immobilization, the use of tape for immobilization of paws is not recommended as tape is detached easily if it gets in contact with liquids.

Once the animal is fully anesthetized the DISDIVO procedure will start with the perfusion surgery. For the DISDIVO the animal is transcardially perfused with different buffers: i) PBS, for the detachment and collection of eGC;, ii) 0.5% SDC, for the ECs decellularization, lysis and collection; and iii) 10% SDC, for the SMCs decellularization, lysis and collection.

DISDIVO perfusion

2. Perfusion surgery (see Figures 2A–2F).
   a. Prepare the autoclaved surgery tools (Figures 1D–1H).
   b. Sterilize the mouse by wetting the ventral fur surface with 70% ethanol.
   c. Use forceps and a scissor cut a patch of skin to uncover the abdominal wall.
   d. Make a 2 cm lateral incision into the abdominal wall beneath the rib cage using forceps and scissors.
   e. Locate the diaphragm. If necessary, carefully separate the liver from the diaphragm.
   f. Cut the diaphragm laterally using curved blunt scissors exposing the pleural cavity.
   g. Locate the xiphoid process, and gently lift it with forceps.
   h. Cut in vertical along both sides of the ribs up to the collarbone creating a flap. During this step carefully displace the lungs if necessary to avoid damaging them.

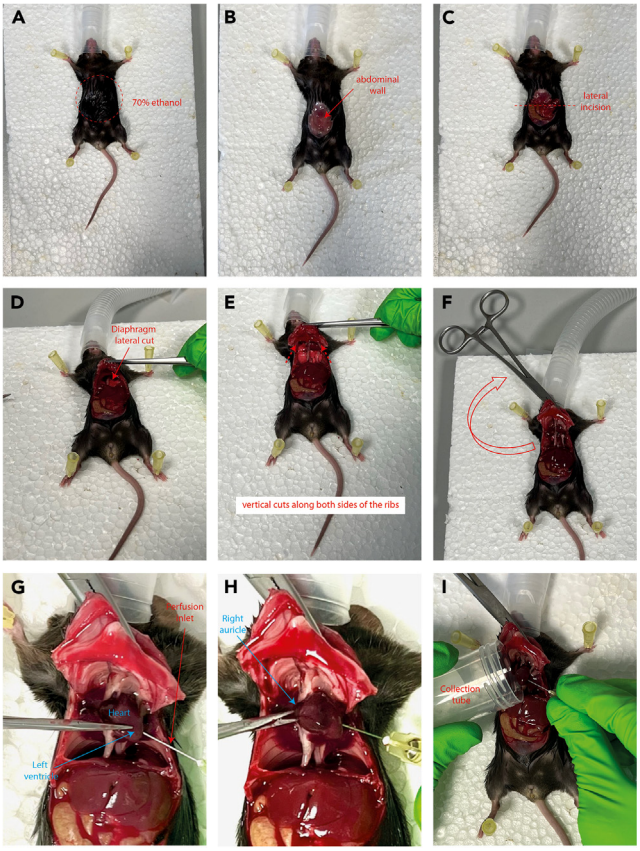

Figure 2. Surgery and initiation of the DISDIVO cardiac perfusion

(A) Sterilization of the ventral fur surface with 70% ethanol.

(B) Cut of a patch of skin to uncover the abdominal wall.

(C) Abdominal lateral incision to open the abdominal cavity.

(D) Cut a lateral incision at the diaphragm to expose the pleural cavity.

(E) Opening the pleural cavity by cutting vertically along both sides of the ribs.

(F) Flipping the ribs flap to uncover the heart and immobilizing the flap using halsted-mosquito hemostatic forceps.

(G) Insertion of the 30-gauge needle connected to the 1× PBS perfusion line into the left ventricle. A blue line has been used to draw the heart for better interpretation, the left ventricle is shaded in blue.

(H) Cut a small incision into the right auricle to allow drainage of perfusates.

(I) Placement of a 50 mL tube for outflow collection.

i. Fold the created flap over the head immobilizing it using halsted-mosquito hemostatic forceps. If needed cut any tissue connecting the ribs flap to the heart.

3. Exsanguination (see Figures 2G–2I).
   a. Insert the 30-gauge needle connected to the 1× PBS perfusion line into the left ventricle.
   b. Turn on the syringe pump to run 1× PBS at a flow rate of 1.5 mL/min.
   c. Once perfusion has begun, rapidly create a small incision into the right auricle using fine scissors to allow drainage of perfusates. Alternatively, the collection of perfusates can be assisted by the injection of a 25-gauge needle into the right auricle.
   d. Place a 50 mL tube at the side of the open right auricle to collect blood.
   e. Change the 50 mL tube after ~1.5 min of perfusion (almost no traces of blood are visible), maintain the tube containing the collected blood on ice and place a new 50 mL tube for the collection of the eGC in the next step.

Note: After injecting the needle of the perfusion line into the ventricle, it is important to not move the needle to ensure a proper perfusion.

Note: During exsanguination whole blood can be collected. Transferring the collected blood into tubes with the interior wall coated with heparin or EDTA anticoagulants is recommended, at the end of the procedure, if plasma will be used for further analysis.

Note: To reduce residual presence of blood components in subsequent DISDIVO fractions the perfusion with PBS in the exsanguination step performed at 1.5 mL/min can be prolonged, up to 3 min. A higher risk of eGC degradation may be observed when using long exsanguination times.

Note: blood samples obtained in this step can be stored during the DISDIVO procedure on ice, it is therefore recommended to have a box with ice during the execution of the procedure.

4. eGC detachment and collection (DISDIVO-PBS).
   a. increase the flow rate to 2 mL/min to detach the eGC from vessel wall.
   b. Perfuse the animal with 1× PBS for additional 3–4 min to completely collect the eGC.
   c. Momentarily stop the pump.
   d. Place the tube with the collected eGC outflow on ice.

Note: Characterization of tissues can also be performed by dissecting the different organs or tissues of interest post DISDIVO procedure. For example, characterization of the main protein components of the eGC remaining in the aorta tissue after DISDIVO is included in Figure 3A and was performed by liquid chromatography-based proteomics as described.[1] Images of kidney sections obtained after DISDIVO indicating disruption of eGC with intact ECs are also shown in Figure 3A. eGC determination by immunostaining was labeled with Alexa Fluor 594-conjugated wheat germ agglutinin as detailed.[3]

Note: In this step a higher flow rate of 2 mL/min is used for proper eGC detachment and collection.

Note: the eGC outflow can be frozen at −80°C for further analysis but as it is not possible to stop the DISDIVO procedure to store samples properly, it is recommended to have a box with ice to keep store samples during the execution of the procedure.

5. ECs decellularization, lysis and collection (DISDIVO-0.5% SDC).
   a. Rapidly disconnect the tubing from the 50 mL syringe filled with 1× PBS and connect it to the 0.5% SDC buffer filled syringe.
   b. Turn on the pump at 1.5 mL/min and collect the outflow containing the decellularized EC for 3–4 min.
   c. Momentarily stop the pump.
   d. Maintain the collected EC outflow on ice until the finalization of the DISDIVO procedure.

Immunohistochemical characterization of the EC-marker platelet and endothelial cell adhesion molecule 1 (PECAM1) is included in Figures 3B and 3C and was performed as described in [1].

Note: due to the use of detergent EC are lysed during the DISDIVO procedure.

Note: the EC outflow can be frozen at −80°C for further analysis but as it is not possible to stop the DISDIVO procedure to store samples properly, it is recommended to have a box with ice to keep store samples during the execution of the procedure.

6. SMCs decellularization, lysis and collection (DISDIVO-10% SDC).
   a. Rapidly disconnect the perfusion line from the 50 mL syringe filled with 0.5% SDC buffer.
   b. Place the syringe filled with 10% SDC buffer into the pump holder.

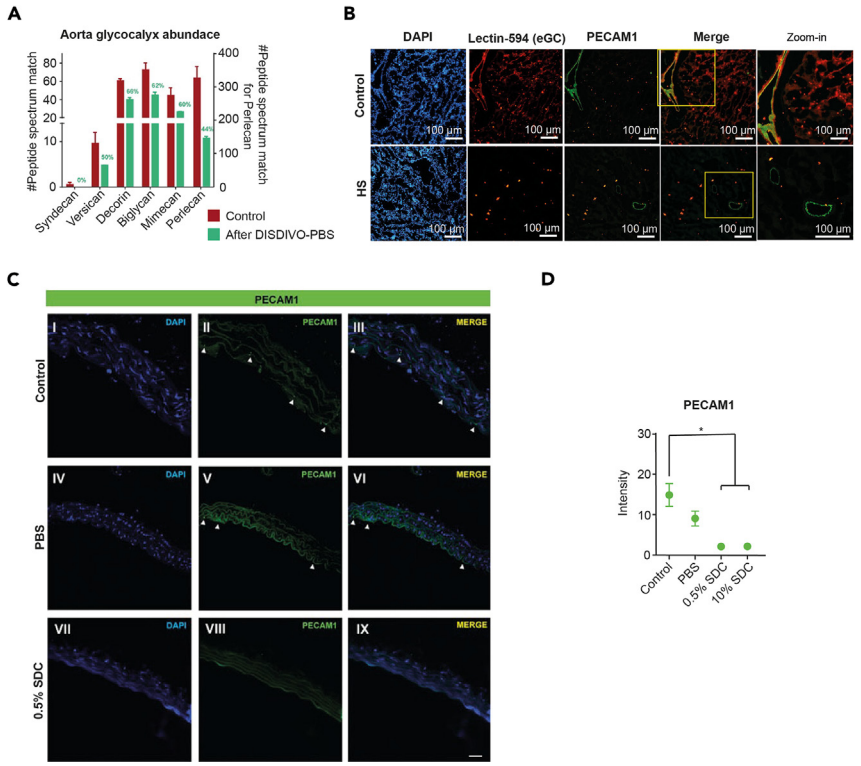

Figure 3. Characterization of murine vascular beds treated by differential systemic decellularization *in vivo* (DISDIVO)

(A) Label-free proteomic quantification of glycocalyx (eGC) markers present in aorta tissues before (control) and after eGC collection with 1× PBS (After DISDIVO-PBS). Numbers in green above green columns indicate the percentage of each respective remaining marker in thoracic aorta tissues after eGC collection by DISDIVO-PBS. Perlecan quantification is indicated in the right *y* axis.

(B and C) (B) Micrographs of kidney sections from control and high salt (HT) treated mice for platelet and endothelial cell adhesion molecule 1 (PECAM1) and (C) Immunostaining of PECAM1 in thoracic aorta from untreated control mice (I, II and III), after eGC collection (IV, V and VI) [DISDIVO-PBS] and after ECs decellularization and collection (VII, VIII and IX) [DISDIVO-0.5% SDC]. White arrows point PECAM1 signal. In section D scale bar = 30 µm.

(D) Integrated density measurement of PECAM1 in micrographs quantified using imageJ software. Nuclei were stained using DAPI and eGC was stained using 594-conjugated wheat germ agglutinin. Immunostaining relative quantitation of PECAM1 in thoracic aorta demonstrates the efficiency of low concentrations of sodium deoxycholate (SDC) to efficiently disrupt endothelial cells depleting PECAM1 from thoracic aorta. All experiments were performed per triplicate. *Statistical significance between conditions was assessed by ANOVA with Bonferroni correction for multiple comparisons ($P < 0.05$). A, B and C sections were reprinted with permission from Serra et al. 2018.[1] Section D was reprinted with permission from Vinaiphat, et al. 2023.[3] Immunostaining was performed as detailed in[1] for sections B and C, and in[3] for section D.

c. Connect the perfusion line to the syringe filled with 10% SDC buffer.
d. Turn on the pump at 1.5 mL/min and collect the outflow for 3–4 min. This collected outflow contains the decellularized SMCs.
e. Maintain the collected SMCs outflow on ice.

Note: due to the use of detergent SMCs are lysed during DISDIVO procedure.

7. Store all fractions at −80°C until analysis.

⚠ CRITICAL: After completion of the procedure, gently flush all the tubing and rinse the tools with water to completely remove salts and prevent SDC deposits.

Note: the SMC outflow can be frozen at −80°C for further analysis but as it is not possible to stop the DISDIVO procedure to store samples properly, it is recommended to have a box with ice to keep store samples during the execution of the procedure.

EXPECTED OUTCOMES

Identification of proteoglycan eGC markers is expected in the eGC fraction, with an extraction of near the 50% of the total proteoglycan eGC markers in the aorta can be achieved as shown in Figure 3A. The expected proteins to be found in this fraction include, but are not limited to: syndecan, versican, decorin, biglycan, mimecan and perlecan (Figure 3A). Lumican a protein involved in the homeostasis of the endothelial barrier can also be preferentially identified in this eGC fraction.

Enrichment of the endothelial marker PECAM1 can be expected in the EC collected fraction after eGC fraction decellularization due to lysis of the endothelial bed as shown for aorta in Figures 3B and 3C. Similarly, identification of the SMC markers such as smooth muscle actin alpha 2 (ACTA2) can be performed in SMC fraction collected as indicated.[1] If the DISDIVO collected sample are analyzed by systems biology,[6] proteins of the different systemic vascular beds can be identified and characterized, allowing the characterization of specific vascular beds such as the capillary vascular beds, brain vascular beds or liver vascular beds. Characterization of the obtained proteins may provide crucial information on the molecular compositions of the endothelium mantle within the capillary beds of these specific organs and systems.

Finally, the DISDIVO procedure may be applied to organ isolated tissues by adapting an *ex vivo* organ perfusion procedure[7] with the DISDIVO perfusion strategy.

LIMITATIONS

DISDIVO lyses all the luminal vascular components and cells in a sequential and structured manner to obtain the different mantles that compose the vasculature beds. However, this protocol does not allow independent study of vascular cell organelles. The specific location of biomarkers detected from the whole body DISDIVO by the systems biology method can be further confirmed using histological approaches.

It is also important to note that the proper accomplishment of the whole organism perfusion is essential to obtain the best results out of this protocol, thus it is important to execute carefully all the steps of the intracardial perfusion as described to avoid trespassing of the heart ventricle and penetration into the pulmonary artery.

Finally, it is also important to attentively respect the established times linked to the detergent concentrations and flow rate detailed in the step-by-step procedures of this protocol, to avoid overlapping contamination of blood and specific vasculature cells throughout the collected fractions.

TROUBLESHOOTING

Problem 1

Incorrect perfusion of mice in steps 3–6 due to: i) puncturing of the aorta during perfusion or ii) trespassing the septum and perfusing through the right ventricle.

Potential solution

- If the aorta has been punctured during the perfusion or the needle has crossed to the right ventricle damaging the septum animal will not be properly perfused. The problem cannot be solved at this point, but to reduce the risk of puncturing the aorta or crossing the septum during perfusion, the tip of the 25-gauge needle used as perfusion inlet needle can be trimmed and polished with sandpaper, as detailed earlier[8] before starting the procedure.

Problem 2

Unstable flow rate, during steps from 3 to 6, alongside the procedure because the volume of solvent in the 50 mL syringe is too low for the proper function of the pump.

Potential solution

- Refill the three syringes used for the multistage perfusion with 1× PBS, 0.5% SDC and 10% SDC buffers, before starting the procedure and refill all the syringes before starting the procedure especially when more than one animal is perfused in the same experimental session.

Problem 3

Unstable flow rate alongside the procedure, during steps from 3 to 6, due to presence of air bubble trapped in the syringe or perfusion line.

Potential solution

- Refill the three syringes used for the multistage perfusion with 1× PBS, 0.5% SDC and 10% SDC buffers, and flush a small amount of liquid to remove any air bubble before starting the procedure. If more than one animal is perfused in the same experimental session; thus, repeat this step before every animal.

Problem 4

Isoflurane level is not enough, in step 1.

Potential solution

- Revise the isoflurane level before starting the DISDIVO procedure and refill it when needed. This is very important when more than one animal is perfused in the same experimental session; thus, revise the isoflurane level before every animal.

Problem 5

Detection of presence of precipitated SDC in the tools and tubing during preparation of materials.

Potential solution

- SDC is used at high concentration in DISDIVO. If materials are not cleaned and rinsed properly potential precipitation of SDC in the tools and tubs is possible. If SDC salts precipitates are observed, clean the materials with lukewarm water and rinse extensively with distilled water.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xavier Gallart-Palau, PhD (xgallart@irblleida.cat).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets generated from the proteomics characterization of the vascular beds obtained by DISDIVO from three different studies: i) WT mice,[1] ii) mice with endothelial dysfunction in Gram-negative sepsis[2] and iii) mice with endothelial damage induced by high salt hypertension[3] are publicly available at the PRIDE repository (http://www.ebi.ac.uk/pride) and can be accessed by project accession PXD008794, PXD018274 and PXD031228, respectively.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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