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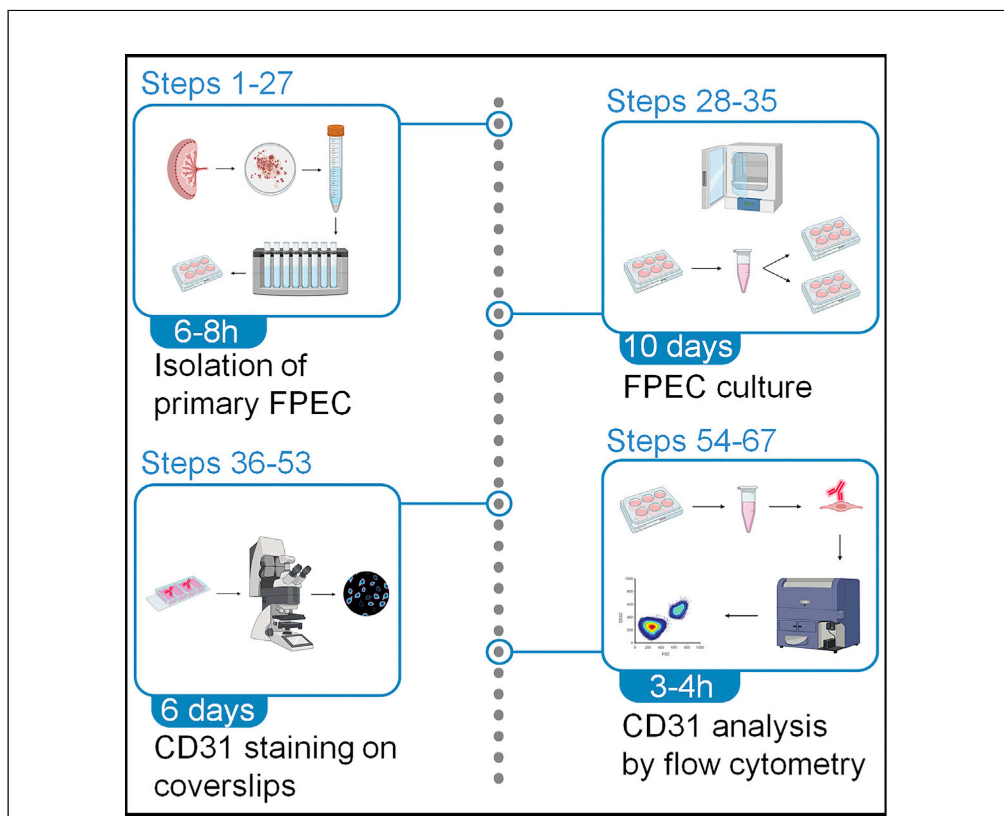
Protocol to isolate and culture primary mouse feto-placental endothelial cells



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Highlights

Procedures for isolating primary mouse feto-placental endothelial cells (FPEC)

Conditions for optimal maintenance and expansion of primary FPEC *ex vivo*

Protocol for assessing purity of primary FPEC by immunofluorescence staining

Protocol for assessing purity of primary FPEC by flow cytometry analysis

In the mouse, feto-placental endothelial cells (FPEC) line the inner surface of the feto-placental blood vessels located within placental labyrinthine zone and play critical roles in placental development and function. Here, we present a detailed protocol for isolation and culture of primary mouse FPEC, as well as two complementary methods (immunohistochemistry staining and flow cytometry analysis) to assess their purity. These cells are suitable for downstream *ex vivo* studies to investigate their functional properties, both in normal and pathological contexts.

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

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Protocol to isolate and culture primary mouse feto-placental endothelial cells

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SUMMARY

In the mouse, feto-placental endothelial cells (FPEC) line the inner surface of the feto-placental blood vessels located within placental labyrinthine zone and play critical roles in placental development and function. Here, we present a detailed protocol for isolation and culture of primary mouse FPEC, as well as two complementary methods (immunohistochemistry staining and flow cytometry analysis) to assess their purity. These cells are suitable for downstream *ex vivo* studies to investigate their functional properties, both in normal and pathological contexts.
For complete details on the use and execution of this protocol, please refer to Sandovici et al. (2022).

BEFORE YOU BEGIN

Endothelial cells of the feto-placental vasculature originate from cells of the extra-embryonic mesoderm that form the allantois and the umbilical cord, and ultimately have their cell lineage origin in the inner cell mass and epiblast (Rossant and Cross, 2001). The allantois grows out and attaches to the chorion, at around embryonic day E8.5, in a process called chorio-allantoic fusion (Rossant and Cross, 2001), which is critical for developmental progress. Following the chorio-allantoic fusion, the feto-placental vasculature undergoes extensive branching angiogenesis, which contributes to the expansion of the placental labyrinthine zone, in order to meet the increasing nutritional needs of the fast growing fetus (Woods et al., 2018). The maternal blood spaces in the placental labyrinthine zone of mouse placenta are not lined by maternal endothelial cells, but by fetal cells of the trophoblast lineage (Rai and Cross, 2014).

Here we describe a magnetic beads-based protocol for FPEC isolation from E15.5 mouse placentas, as well as the conditions used for culture, which allows *ex vivo* expansion for downstream applications, such as angiogenesis assays or molecular analyses. By isolating placental labyrinthine zone, we remove any endothelial cell of maternal origin that line maternal vessels located in the decidua basalis. We also report here on two methods to ascertain the purity of cell isolation, one based on immunofluorescence staining and another on flow cytometry analysis.

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FPEC have been isolated before by fluorescence-activated cell sorting (FACS) from conceptuses of *Rosa26*$^{mT/mG}$ mice mated to *Tie2*-Cre transgenic mice, driving the expression of GFP in the endothelium (Chi and Delgado-Olguin, 2018). However, a high level of cell loss is observed during isolation by FACS (Sutermaster and Darling, 2019), hindering further expansion for subsequent adequate experimentation *ex vivo*, which is overcome by using the protocol describe herein. Moreover, for functional studies, adequate preservation of these primary cells requires a gentler method for isolation as well as physiologically relevant culture conditions. Finally, this protocol allows the study of FPEC isolated from wild type or mutant mice, without the additional need to cross them with reporter mice.

Institutional permissions

The mouse experiments described below were performed in accordance with the University of Cambridge Animal Welfare and Ethical Review Body and the United Kingdom Home Office Regulations.

Setup mouse mating

Timing: 12–19 days

1. In the morning, move one adult male (age 8–12 weeks) into a clean breeding cage (prepare as many as needed, making sure males are single housed).
2. In the afternoon add one female (age 8–12 weeks) to each breeding cage.
3. Regularly check for a vaginal plug starting the morning after, and record as embryonic day 0.5 (E0.5) as soon as plug is detected.
4. Keep pregnant females separate from the males until FPEC isolation, here described for placentas removed at E15.5. Additional optimization may be required for FPEC isolation at other developmental time points.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BD Pharmingen purified rat anti-mouse CD31 (dilution 1.2:10)	BD Biosciences	RRID: AB_396660
Rabbit anti-CD31 (dilution 1:50)	Abcam	RRID: AB_726362
Rabbit IgG, polyclonal - isotype control (dilution 1:50)	Abcam	RRID: AB_2631996
Donkey anti-rabbit-AF594 (dilution 1:250)	Jackson ImmunoResearch	RRID: AB_2340619
Rat anti-CD16/32 (dilution 1:100)	BioLegend	RRID: AB_1574975
Rat anti-mouse CD31-AF647 (dilution 1:400)	BioLegend	RRID: AB_2161029
Chemicals, peptides, and recombinant proteins		
Calcium chloride solution	Merck	Cat#21115
Magnesium sulfate solution	Merck	Cat#M3409
Collagenase type I from *Clostridium histolyticum*	Merck	Cat#SCR103
HBSS, no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	Cat#14175095
Ham's F-12 nutrient mix	Thermo Fisher Scientific	Cat#11765054
DMEM, low glucose	Thermo Fisher Scientific	Cat#31885023
MEM non-essential amino acids solution (100×)	Thermo Fisher Scientific	Cat#11140035
Sodium pyruvate solution	Thermo Fisher Scientific	Cat#11360039
HEPES, 1 M buffer solution	Thermo Fisher Scientific	Cat#15630056
Heparin sodium salt from porcine intestinal mucosa	Merck	Cat#H3149
Fetal bovine serum	Thermo Fisher Scientific	Cat#26140087
Endothelial cell growth supplement	Merck	Cat#E2759
Trypsin-EDTA solution (0.25% trypsin)	Merck	Cat#59428C
Trypsin-EDTA solution (0.05% trypsin)	Merck	Cat#59417C

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RPMI 1640 medium	Thermo Fisher Scientific	Cat#21875034
Penicillin-streptomycin	Merck	Cat#P0781
PBS, no calcium, no magnesium	Thermo Fisher Scientific	Cat#10010023
Bovine serum albumin solution	Merck	Cat#A7979
Normal donkey serum, sterile	Merck	Cat#566460
Triton X-100	Merck	Cat#T8787
DAPI	Merck	Cat#D9542
Staining buffer	BioLegend	Cat#420201
7-aminoactinomycin (7-AAD)	Thermo Fisher Scientific	Cat#A1310
Propidium iodide solution	Merck	Cat#P4864
Trypan blue solution	Merck	Cat#T8154
Accutase	Merck	Cat#A6964
Gelatin solution	Merck	Cat#G1393
Formaldehyde solution	Merck	Cat#F1635
TWEEN 20	Merck	Cat#P9416
ProLong gold antifade mountant	Thermo Fisher Scientific	Cat#P10144
Critical commercial assays		
Dynabeads sheep anti-rat IgG	Thermo Fisher Scientific	Cat#11035
Experimental models: Organisms/strains		
Wild-type C57BL/6J (JAX Mice Strain), 8–12 weeks old, males and females	Charles River	Strain Code:632
Other		
Dumont tweezers #5, 11 cm, straight, 0.1 × 0.06 mm tips, titanium	World Precision Instruments	Cat#14096
Dumont tweezers #7, 12 cm, curved, 0.17 × 0.1 mm tips	World Precision Instruments	Cat#14097
Fisherbrand sterile cell strainers	Fisher Scientific	Cat#22-363-548
Conical tubes (50 mL)	Thermo Fisher Scientific	Cat#AM12501
Conical tubes (15 mL)	Thermo Fisher Scientific	Cat#AM12500
RotoFlex plus bench top tube rotator	Merck	Cat#Z740290
2.0 mL round bottom microcentrifuge tubes	Eppendorf	Cat#0030108132
DynaMag-2 magnet	Thermo Fisher Scientific	Cat#12321D
Serological pipettes (5, 10 and 25 mL)	Thermo Fisher Scientific	Cat#170355T
10 μL graduated TipOne filter tip, natural, racks (sterile)	Starlab	Cat#S1121-3810
200 μL graduated TipOne filter tip, natural (sterile), racks	Starlab	Cat#S1120-8810
1,000 μL XL graduated TipOne filter tip, natural, racks (sterile)	Starlab	Cat#S1122-1830
Round bottom polystyrene test tube, with cell strainer snap cap	Fisher Scientific	Cat#352235
Corning Costar ultra-low attachment 6 well plate	Merck	Cat#CLS3471-24EA
Cover slips, round, 16 mm	VWR	Cat#631-0152P
SuperFrost plus, cellophane wrapped, box in cellophane	VWR	Cat#631-9483
ADAM-MC automatic cell counter	NanoEntek	Cat#ADAM-MC II
ADAM AccuChip Kit	NanoEntek	Cat#AD4K-200

MATERIALS AND EQUIPMENT

All media and solutions listed below are prepared using sterilized consumables and instruments (pipet tips, microcentrifuge tubes and flasks), using strict aseptic and endotoxin-free environment to avoid introduction of contaminants that may alter the behavior and viability of the purified cells. We recommend sterilizing all media by filtration using a 0.22 μm syringe filter prior to use.

Note: All media and solutions should be kept at 4°C.

⚠ CRITICAL: Reagents stocks kept at −20°C, such as enzymes, heparin and growth factors, are prepared in aliquots of volumes required for single use, to avoid frequent freeze-thaw cycles.

Dissection media		
Reagent	Final concentration	Amount
RPMI 1640	1 ×	49.5 mL
Penicillin-streptomycin	1 ×	0.5 mL
Total	N/A	**50 mL**
Store at 4°C for up to 1 month.		

⚠ CRITICAL: Do not discard dissection media until starting digestion steps.

Alternatives: RPMI 1640 can be substituted with HBSS. The penicillin-streptomycin stock solution (100 ×) contains 10,000 units penicillin and 10 mg streptomycin per mL. It can be replaced with an antibiotic-antimycotic solution, which contains 25 mg/mL of Amphotericin B in addition to penicillin-streptomycin.

HBSS++ solution		
Reagent	Final concentration	Amount
1 M calcium chloride solution	2 mM	0.5 mL
1 M magnesium sulfate solution	2 mM	0.5 mL
1 M HEPES, buffer solution	20 mM	5 mL
HBSS, no calcium, no magnesium, no phenol red	1 ×	244 mL
Total	n/a	**250 mL**
Sterilize using 0.22 μm vacuum filter and store at 4°C for up to 1 month.		

Note: This solution has several uses in this protocol: washing cell pellets and reconstitution of collagenase type I stock solution (see below). HBSS without calcium and magnesium are important to avoid inhibition of collagenase type I activity. HBSS without phenol red allows better visual inspection of the cell pellet during washing steps.

Collagenase type I stock solution		
Reagent	Final concentration	Amount
Collagenase type I from *Clostridium histolyticum*	10%	250 mg
Sterile PBS, no calcium, no magnesium	1 ×	2.5 mL
Total	N/A	**2.5 mL**
Aliquot in sterile microcentrifuge tubes (0.4 mL/tube) and store at −20°C for up to 6 months.		

Note: PBS without calcium and magnesium is required to avoid inhibition of collagenase type I activity.

⚠ CRITICAL: To avoid exposure of collagenase to contaminants, prepare the stock solution in sterile conditions directly within the original flask. Collagenase is difficult to dissolve. We recommend replacing the rubber stopper and shaking the vial vigorously at regular intervals for about 20 min (while keeping the content cold), before aliquoting.

Digestion solution		
Reagent	Final concentration	Amount
Collagenase type I stock solution	0.2%	0.4 mL
HBSS++ solution	1 ×	19.6 mL
Total	N/A	**20 mL**
Prepare fresh and pre-warm at 37°C right before use.		

Note: Digestion volume optimized for 6–8 placentas (approximately 0.8 g total weight); adjust and prepare aliquots in adequate required volumes depending on amount of tissue of origin.

Alternatives: Purified versions of bacterial collagenase A that allow efficient harvesting of endothelial cells, while removing non-specific components, such as proteases, phospholipases and bacterial wall debris, may be beneficial in certain applications, for example, when isolated FPEC are immediately used for in vivo applications.

Magnetic bead washing and antibody binding solution (Beads Buffer)

Reagent	Final concentration	Amount
Bovine serum albumin solution (BSA, 35%)	0.1%	0.1 mL
Sterile PBS, no calcium, no magnesium	1 ×	34.9 mL
Total	**N/A**	**35 mL**

Prepare fresh and keep at room temperature (beads washing) or on ice until use.

Note: The addition of BSA to this solution reduces cell and beads stickiness to the plastic, therefore, improving their recovery during washing steps. BSA also acts as a blocking agent to reduce non-specific antibody binding.

FPEC basal growth medium

Reagent	Final concentration	Amount
Ham's F-12 nutrient mix	0.5 ×	200 mL
DMEM, low glucose	0.5 ×	200 mL
MEM non-essential amino acids solution (100×)	1 ×	5 mL
100 mM sodium pyruvate solution	2 mM	10 mL
1 M HEPES, buffer solution	20 mM	10 mL
Total	**N/A**	**425 mL**

Filter sterilize and store at 4°C for up to 1 month.

Endothelial cell growth supplement stock solution

Reagent	Final concentration	Amount
Endothelial cell growth supplement (ECGS)	7.5 mg/mL	15 mg
Sterile water	N/A	2 mL
Total	**N/A**	**2 mL**

Aliquot in sterile Eppendorf tubes (0.5 mL/tube) and store at −20°C for up to 3 months.

Note: Lyophilized ECGS is stable for 2 years at −20°C.

Heparin stock solution

Reagent	Final concentration	Amount
Heparin sodium salt from porcine intestinal mucosa	10 mg/mL	55.55 mg
Sterile water	N/A	5.5 mL
Total	**N/A**	**5.5 mL**

Aliquot in sterile Eppendorf tubes (0.5 mL/tube) and store at −20°C for up to 3 months.

Note: Heparin grade I-A, suitable for cell culture, has ≥180 USP (United States Pharmacopeia) units/mg powder.

FPEC growth medium without heparin		
Reagent	Final concentration	Amount
Fetal bovine serum	20%	10 mL
Endothelial cell growth supplement stock solution	0.75 mg/mL	0.5 mL
FPEC basal growth medium	N/A	39.5 mL
Total	**N/A**	**50 mL**
Prepare fresh or store at 4°C for up to 3 days.		

Note: FPEC growth medium without heparin is recommended in the initial stages of the FPEC growth. However, heparin potentiates the growth stimulatory effects of the endothelial cell growth supplement (Thornton et al., 1983), which is the reason for it to be added in the media once the cells attach.

FPEC growth medium with heparin		
Reagent	Final concentration	Amount
Fetal bovine serum	20%	10 mL
Endothelial cell growth supplement stock solution	0.75 mg/mL	0.5 mL
Heparin stock solution	1 mg/mL	0.5 mL
FPEC basal growth medium	N/A	39 mL
Total	**N/A**	**50 mL**
Prepare fresh or store at 4°C for up to 3 days.		

STEP-BY-STEP METHOD DETAILS

Section 1: Dissection of mouse placental labyrinthine zone

Timing: 1 h per litter

This part describes the procedures required to isolate the placental labyrinthine zone (Lz) that contains FPEC (Figure 1). Isolation of Lz eliminates the risk of contamination with endothelial cells of maternal origin. One standard litter of 6–8 embryos is required to isolate enough FPEC for n=1 biological replicate for downstream applications. The steps are performed on ice, under sterile conditions.

1. In the morning, euthanize the E15.5 pregnant female(s) by cervical dislocation.

   CRITICAL: Avoid using inhaled CO_2 or anesthetics as a method for humane euthanasia of the pregnant mouse. These methods will impair the recovery of the FPEC.

2. Open immediately the abdomen and remove the uterus using scissors, and place it into a 12 cm Petri dish containing ice-cold dissection media. Quickly place the Petri dish on ice (Figure 1A).
3. Open the uterus and peel off all conceptuses using a curved forceps (Figures 1B and 1C).
4. Isolate each placenta by removing the yolk sac and sectioning the umbilical cord (Figures 1D and 1E).
5. Transfer placentas to another Petri dish filled with ice-cold dissection media, and isolate the Lz from each under a dissecting microscope, by removing the junctional zone and decidua basalis using one pair of Dumont tweezers #5 (straight) and one pair of Dumont tweezers #7 (curved) (Figures 1F and 1G).

Section 2: Preparation of placental Lz single-cell suspension

Timing: 2 h

This part of the protocol describes the steps required to dissociate the isolated placental Lz into a single-cell suspension. The conditions used for the collagenase digestion (concentration, temperature, time) were optimized to maximize the isolation of single cells, while avoiding over-digestion and damaging cell membranes.

6. Place all placental Lz in a new Petri dish and wash twice with ice-cold HBSS++ solution to remove the excess blood.
7. Using scalpels or small, sharp scissors mince finely the Lz tissue (Figure 1H).
8. Place the entire volume of minced placental Lz tissue into a 50 mL Falcon tube filled with 20 mL pre-warmed collagenase-containing digestion solution, and incubate for 1.5 h at 37°C, with gentle rotation (35 RPM).

   Note: While cells are undergoing the 1.5 h collagenase digestion, perform the antibody coating of the beads as described in Section 3: Coating the magnetic beads with rat anti-mouse CD31 antibody, under steps 13–20. Alternatively, the coating can be done the day before (see below).

9. Filter the digested tissue through a sterile 70-μm nylon mesh to remove clumps of cells or fragments of undigested tissue; press tissue through the filter with a cell lifter to ensure maximal cell recovery.
10. Pellet the cells by gentle centrifugation for 5 min at 260 × *g* in a centrifuge pre-chilled at 4°C, and then gently aspirate the supernatant (the pellet may be loose).
11. Wash once with 20 mL ice-cold HBSS++ solution by gently re-suspending the cell pellet in 1 mL solution, followed by mixing with the remaining 19 mL. Repeat step 10.
12. Re-suspend the pellet in 3 mL ice-cold Beads Buffer and transfer to a 15 mL Falcon tube.

   ⚠ CRITICAL: It is important to perform one wash only (cells are fragile) and to carefully aspirate the supernatant to avoid disturbing or accidentally aspirating the cell pellet.

   Note: The volume of 3 mL Beads Buffer is recommended when working with Lz tissue isolated from one standard litter of 6–8 conceptuses. This volume should be adjusted when working with different amounts of placental tissue (e.g., 1 mL extra for a litter of 10).

Section 3: Coating the magnetic beads with rat anti-mouse CD31 antibody

Timing: 1.5 h

The procedures described in this part (Figure 2A) are performed during the 1.5 h collagenase digestion (Section 2: Preparation of placental Lz single-cell suspension, step 8).

⚠ CRITICAL: All the steps described in this part are performed at room temperature.

13. Resuspend the sheep anti-rat IgG magnetic beads suspension by gentle shaking, to avoid bubbles.
14. For a typical litter of eight embryos, transfer 8–10 μL of sheep anti-rat IgG Dynabeads into a sterile 2.0 mL round bottom microcentrifuge tube.

   Note: The use of 2.0 mL round bottom microcentrifuge tubes for this step is recommended in order to avoid the beads becoming stuck to the bottom of 1.5 mL conical-shaped tubes.

15. Add 1 mL of Beads Buffer and gently mix by pipetting. Place the Eppendorf tube on the DynaMag magnetic stand and allow the separation of beads from solution (approximately 1 min). Aspirate the solution without disturbing the beads. Repeat twice (total three washes).

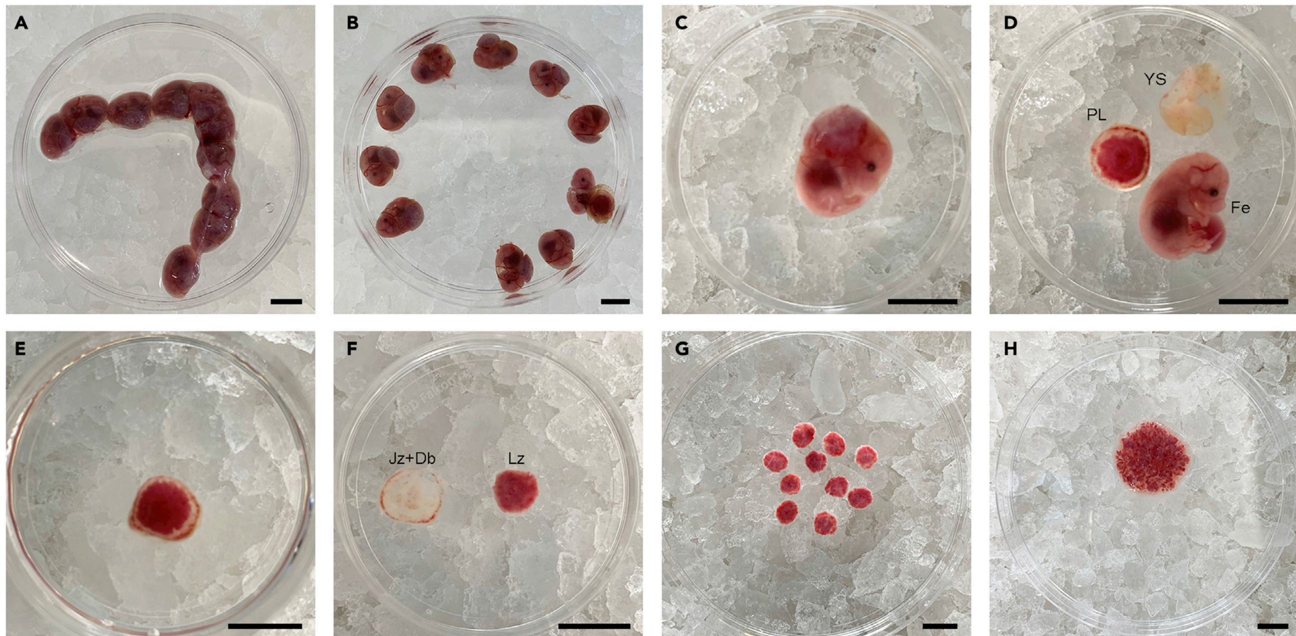

Figure 1. Isolation of labyrinthine zone (Lz) from E15.5 mouse placenta

(A) Isolated gravid uterus at E15.5 mouse pregnancy.
(B) Isolated E15.5 mouse conceptuses.
(C) Individual E15.5 conceptus prior to opening the yolk sack.
(D) Separated fetus (Fe), placenta (PL) and yolk sack (YS).
(E) Fetal side view of a E15.5 placenta, with a darker, intense vascularized central area corresponding to the labyrinthine zone.
(F) Micro-dissected labyrinthine zone (Lz), which is separated from the junctional zone (Jz) and decidua basalis (Db).
(G) Lz collected from all E15.5 placentae in the litter.
(H) The pooled Lz tissue after mincing into small fragments. For all panels, scale bars are 1 cm.

16. Re-suspend the washed beads in 1 mL of Beads Buffer by gentle pipetting.
17. Add 1.2 μL rat anti-mouse CD31 antibody to 10 μL Dynabeads and mix by gentle pipetting.

Note: The Dynabeads (sheep anti-rat IgG) are supplied at 4×10^8 per mL, and the concentration of the rat anti-mouse CD31 antibody is 0.5 mg/mL. 10 μL Dynabeads incubated with 1.2 μL antibody was determined as optimal for isolation of endothelial cells from one litter of eight; adjust volume if preparing for different litter size or litter number.

18. Allow antibody to bind to the beads for 1 h at room temperature with gentle rotation (35 RPM), using a rotator.

Alternatives: Steps 13–18 can be performed the day before, using an overnight incubation of the beads with the antibody (step 18) at 4°C.

19. Wash the beads three times, using the magnetic stand (as described in step 15) to remove the unbound antibody.
20. After the three washes, re-suspend the beads in the Beads Buffer, using the original volume (8–10 μL) and immediately add to the digested placental tissue (see step 21) or keep on ice until needed.

Section 4: Isolation of pure FPEC

Timing: 1.5 h

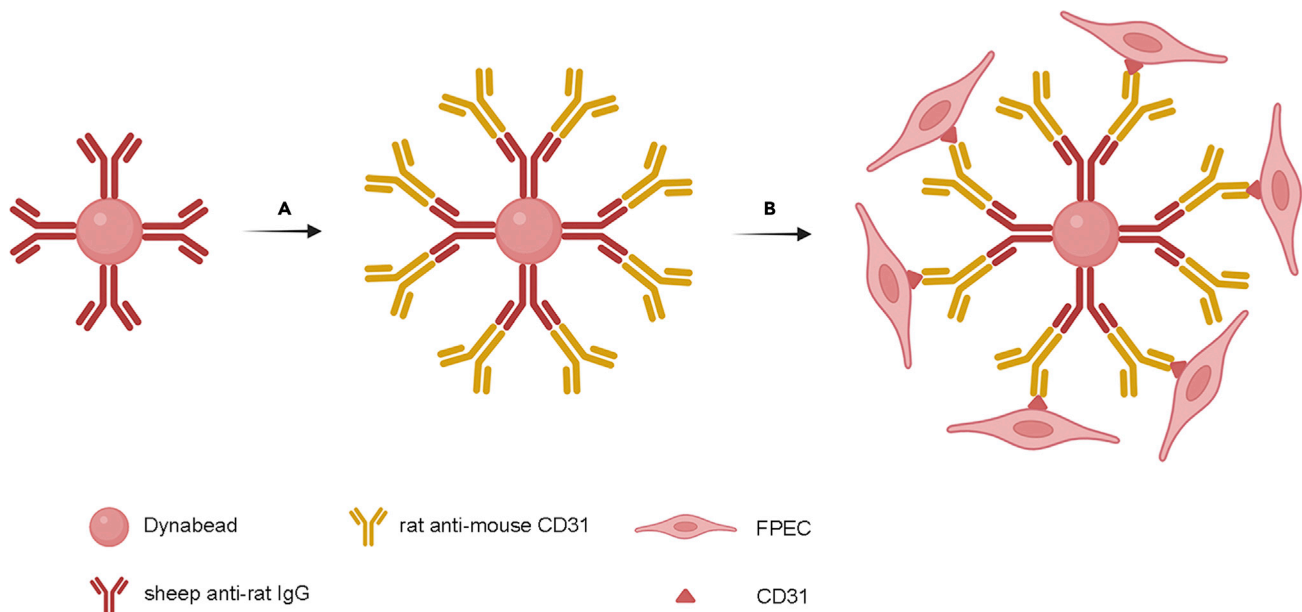

Figure 2. FPEC isolation using antibody-coated magnetic beads
(A) Incubation of Dynabeads pre-coated with sheep anti-rat IgG with rat anti-mouse CD31 (steps 13–20).
(B) FPEC pulldown from the single-cell Lz suspension (steps 21–27).

This part of the protocol describes the steps to isolate FPEC from the single-cell suspension prepared in steps 6–12 (Figure 2B).

21. Add the coated beads (step 20) to the 15 mL Falcon tube containing the single-cell placental Lz suspension (step 12).
22. Incubate the Falcon tube for 1 h at 4°C with slow rotation (35 RPM) to allow the binding of FPEC to the CD31-coated beads.
23. After incubation, transfer the beads + cells suspension into three 2 mL microfuge tubes with round bottom, placed on the DynaMag magnetic rack. Beads and bound cells are collected by the magnet, and after 1 min the bead-free suspension is removed.
24. Wash beads in each tube in 1 mL room temperature Beads Buffer by removing from the magnet, gently pipetting to avoid foam or bubbles. Place the tubes back on the magnetic rack for 1 min and carefully aspirate the bead-free Beads Buffer.

Note: The presence of BSA in the Beads Buffer makes it foamy. Bubbles can lead to loss of beads and cells.

25. To pool all beads into one single tube for the remaining washes, remove tubes from the magnet and resuspend beads from one tube in 1 mL Beads Buffer, transfer to the second one, gently mix the two aliquots of beads, and finally transfer again to the last tube – all beads are now suspended in 1 mL of Beads Buffer.
26. Wash three more times as in step 24, with 1 mL room temperature Beads Buffer each time.
27. Re-suspend the bead-bound cells in 1 mL FPEC growth medium without heparin, pre-warmed at 37°C.

Section 5: Culture and passage of FPEC

Timing: 10–14 days

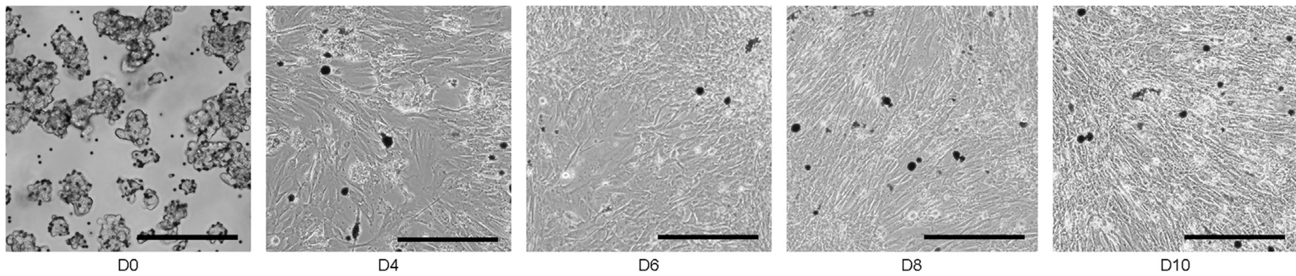

Figure 3. ***Ex vivo*** **culture of E15.5 FPEC**
Each panel shows representative images of primary FPEC at various stages of growth post-isolation: D0 – day zero – shows clusters of FPEC with antibody-coated Dynabeads attached to their surface; D4 – day 4 – attached FPEC after the removal of dead cells; D6 – D10 FPEC at various levels of cell confluence (full confluence at D10) with beads visible as dark clusters. Scale bars are 100 μm.

The procedures described in this part allow for the optimal culture and expansion of FPEC isolated as per steps 21–27 (Figure 3).

28. Transfer bead-bound cells to two wells of a 6-well tissue culture plate and add 1.5 mL FPEC growth medium without heparin for a total of 2 mL media/well.
29. Incubate the plate in a tissue culture incubator at 37°C, under 5% CO_2 and 5% O_2.

Note: 5% O_2 mimic the average physiological oxygen tension found in the placenta, and this was found to significantly improve the yield of FPEC, and prevents metabolic and functional adaptations to hyperoxia.

⚠ CRITICAL: Primary endothelial cells may take time to adhere, and to ensure cell adherence and reduce cell loss, growth medium should not be changed for at least four days.

30. After the cells attach, gently aspirate the medium, and perform an additional wash, with 1 mL FPEC growth medium without heparin, to remove the dead cells.
31. Feed the cells with 2 mL FPEC growth medium without heparin per well every other day until the cells reach 70%–80% confluence.

Note: FPEC are ready for first passage around 10 days after seeding.

32. To passage the cells, remove the media and add 0.2 mL Accutase, pre-warmed at 37°C. Incubate for 5 min at 37°C, until the cells detach.

Note: Accutase is efficient in detaching endothelial cells and is suitable for downstream experiments, such as flow cytometry-based analyses.

Alternatives: Instead of Accutase, FPEC can be detached using a trypsin-EDTA solution. Incubation times depend on trypsin concentration (2–3 min for a 0.25% trypsin solution; 5–6 min for a 0.05% trypsin solution).

33. Add 1.8 mL media into the well and transfer the cells into a microcentrifuge tube.
34. Pellet the cells by centrifugation at 260 × *g* for 3 min. Carefully aspirate the supernatant and resuspend cell pellet evenly in 1 mL of FPEC growth medium with heparin.
35. Add 0.5 mL cell suspension (obtained at the end of step 34) into two wells of a new 6-well tissue culture plate and place back in the incubator.

Note: Some beads remain present after the first or even the second passage, because the Accutase treatment is not 100% efficient in detaching them from FPEC. Usually, persistent beads do not interfere with cell growth or with downstream experiments.

⚠ CRITICAL: Ideal split ratio for FPEC is 1:2. Do not split FPEC at low density, as this will compromise their ability to survive and expand.

Section 6: CD31 staining of FPEC on coverslips

Timing: 6–7 days

This part describes the procedures that are required to assess purity of the endothelial cell preparations by immunofluorescence staining.

36. Place sterilized coverslips into the desired number of wells of a 24-well tissue culture plate.
37. Add 400 μL of the gelatin-coating solution. Incubate the coverslips for 10 min at room temperature inside a laminar flow hood.
38. Remove the gelatin-coating solution by gentle aspiration. Air-dry the coverslips for 15 min.
39. Seed approximately 5,000 cells in 500 μL of FPEC growth medium with heparin in each well containing gelatin-coated coverslips.
40. Incubate the plate in a tissue culture incubator at 37°C, under 5% CO2 and 5% O_2 for approximately 4–5 days, until the cells reach the desired density (80%). Feed the cells on alternate days.
41. Remove the culture media from each well by gentle aspiration and wash twice with 500 μL PBS.
42. Add 500 μL of 2% formaldehyde solution (in PBS) to each well. Incubate for 20 min at room temperature.

Note: To avoid detachment of endothelial cells due to the change in surface tension during step 42, an alternative method is to pre-fix the cells by adding 500 μL of 4% formaldehyde solution directly into the culture medium. After 2 min, replace the pre-fixation culture medium with 500 μL of 2% formaldehyde solution and incubate for 20 min at room temperature. This alternative fixation method replaces steps 41 and 42.

43. Wash the wells twice with 500 PBS μL for 5 min at room temperature.
44. Block non-specific staining by adding 400 μL of blocking buffer (10% normal donkey serum, 0.3% Triton X-100 in PBS). Incubate for 45 min at room temperature.
45. Remove the blocking buffer. No rinsing is necessary.
46. Dilute the unconjugated primary antibody (rabbit polyclonal anti-CD31) 1:50 in staining buffer, supplemented with 1% normal donkey serum. Add 400 μL per well and incubate overnight at 2°C–8°C.

Note: Each staining experiment should include at least one negative control well, using the polyclonal rabbit IgG antibody (isotype control) diluted 1:50 in staining buffer, supplemented with 1% normal donkey serum, to identify non-specific staining caused by non-specific secondary antibody binding.

47. Next morning, wash twice with 400 μL of PBS supplemented with 0.05% Tween 20 (PBS-T) for 5 min per wash, at room temperature.
48. Dilute the secondary antibody (donkey anti-rabbit-AF594) 1:250 in staining buffer, supplemented with 1% normal donkey serum. Add 400 μL to each well. Incubate at room temperature for 1 h in the dark.

Note: From this step forward, all incubations should be performed in the dark.

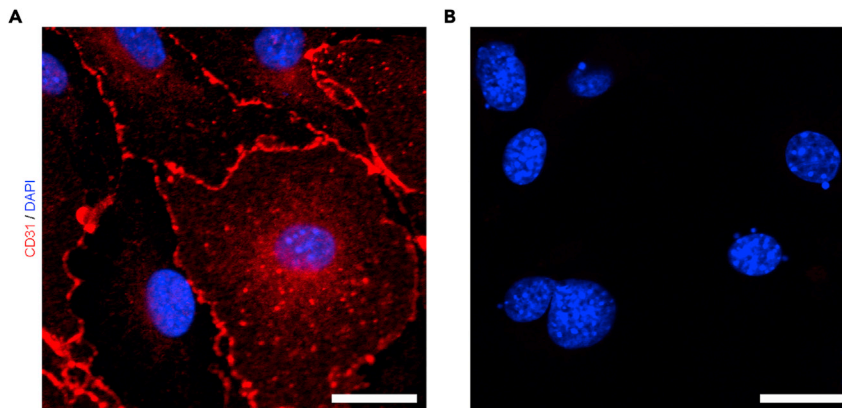

Figure 4. Immunofluorescent staining of FPEC grown on coverslips
(A) Staining for the endothelial cell marker CD31 (also known as PECAM1).
(B) Negative control – FPEC incubated with polyclonal rabbit IgG. For both panels, DAPI stains the DNA in the nucleus; scale bars are 20 μm.

49. Wash two times in 400 μL PBS-T (5 min each).
50. Add 300 μL of 5 μM DAPI solution in PBS to each well. Incubate 2–5 min at room temperature.
51. Rinse once with PBS and once with water (5 min each).
52. Carefully remove the coverslips from the wells and blot to remove any excess water. Dispense 1 drop of antifade mountant onto the microscope slide per coverslip. Mount the coverslip with the cells facing towards the microscope slide.
53. Visualize using a confocal microscope (Figure 4). On a Leica SP8 confocal microscope, use 405 nm excitation and 425–465 nm emission to detect DAPI, and 580 nm excitation and 600–615 nm emission to detect AF594.

Note: The slides can also be stored in a slide box at 4°C, protected against light, for later examination. If the coverslips are sealed with nail varnish, the fluorophores are stable for at least two months.

Section 7: Flow cytometry analysis of FPEC for CD31 protein expression

Timing: 3–4 h

This part describes the procedures required to assess the purity of the endothelial cell preparations by flow cytometry.

54. When FPEC reach confluence in one well of a 6-well plate, remove the culture media and wash each well twice with 1 mL PBS at room temperature.

Note: When confluent, expect around 1–1.5 million FPEC/well.

55. Add 0.2 mL Accutase per well (pre-warmed) and then incubate for 5 min at 37°C.

Note: We recommend Accutase over Trypsin/EDTA digestion as method to detach FPEC prior to flow cytometry analyses, as Accutase does not remove cell-surface antigens such as CD31. Trypsin solutions require careful monitoring of the incubation time, to avoid loss of membrane-bound CD31.

56. Add 1.8 mL media into the well and transfer the cells into a microcentrifuge tube.

57. Pellet the cells by centrifugation (400 × *g* for 5 min at 4°C), and discard supernatant.
58. Wash cells once with 1 mL of 0.1% BSA in PBS, then pellet the cells as above.
59. Re-suspend the cells in a small volume of PBS (e.g., 100 μL) and use a small aliquot (10 μL) to count the cells.

Note: We recommend counting FPEC with the aid of an automated cell counting system based on the propidium iodide exclusion method (e.g., using the ADAM-MC system).

60. Re-suspend the cells at 1 × 10^7 cells/mL in staining buffer. For each experimental sample prepare one aliquot of 1 million cells (100 μL volume) and place it in 1.5 mL microcentrifuge tubes. Additionally, each experiment includes three control tubes (negative control, 7-AAD control, CD31 control) with same amount of 1 million cells each. Add another 100 μL staining buffer to all tubes, for a final volume of 200 μL to each tube.
61. To block Fc receptors, add 1 μg anti-CD16/32 antibody per 1 million cells and incubate on ice for 20 min.
62. At the end of the blocking incubation, add 0.25 μg of rat anti-mouse CD31-AF647 antibody in the CD31 control and experimental samples. Incubate all tubes on ice in the dark for 1 h.
63. After the 1-h incubation ends, add 800 μL staining buffer to all tubes and centrifuge at 400 × *g* for 5 min.
64. Remove the supernatant and wash the cells one more time with 1 mL staining buffer.
65. Re-suspend the cells in 0.5 mL PBS and filter the cells through 70-μm cell strainers into 5 mL polystyrene test tubes.
66. Add 5 μL 7-AAD solution per tube in all sample tubes and in the 7-AAD control tube and incubate on ice for 5 min.
67. Perform the flow cytometry analysis (Figure 5).

EXPECTED OUTCOMES

Using this protocol, the researcher should be able to isolate, and culture E15.5 mouse FPEC. The expected population doubling time is about 5 days. Once the cells are passaged from the initial isolation plate, >95% of cells in the culture should be CD31+ positive, as assessed by immunostaining or flow cytometry analysis. Although cells isolated using this protocol are able to proliferate for at least 7 passages, we recommend their use for downstream protocols within the first 2–3 passages, as cells with higher passage number may exhibit undesirable changes in specific cellular characteristics, such as migration, proliferation rates etc. due to the inevitable adaptation to artificial culture environments (Liao et al., 2014).

LIMITATIONS

This protocol was optimized for the isolation and culture of primary FPEC obtained from E15.5 mouse placentae. Additional optimization may be required for isolation of FPEC at other developmental stages. An important aspect to consider is the change in the proliferation capacity of FPEC as pregnancy progresses. Our *in vivo* observations, based on EdU incorporation in FPEC over a period of 16 h, showed a progressive decline in proliferation rates from approximately 30% at E13.5, to 15% at E15.5, and to almost undetectable levels at E18.5 (Sandovici et al., 2022). Therefore, *ex vivo* expansion of FPEC isolated from placentae may be unsuccessful near the end of gestation.

Another limitation of this protocol is the requirement to use an entire E15.5 litter for a single biological replicate. This requirement precludes the possibility to apply this protocol in litters with mixed genotypes to study the impact of specific genetic mutations, or to study differences between FPEC isolated from male and female mouse placentae (in the absence of rapid genotyping tests of individual conceptuses, to allow correct pooling of placentae).

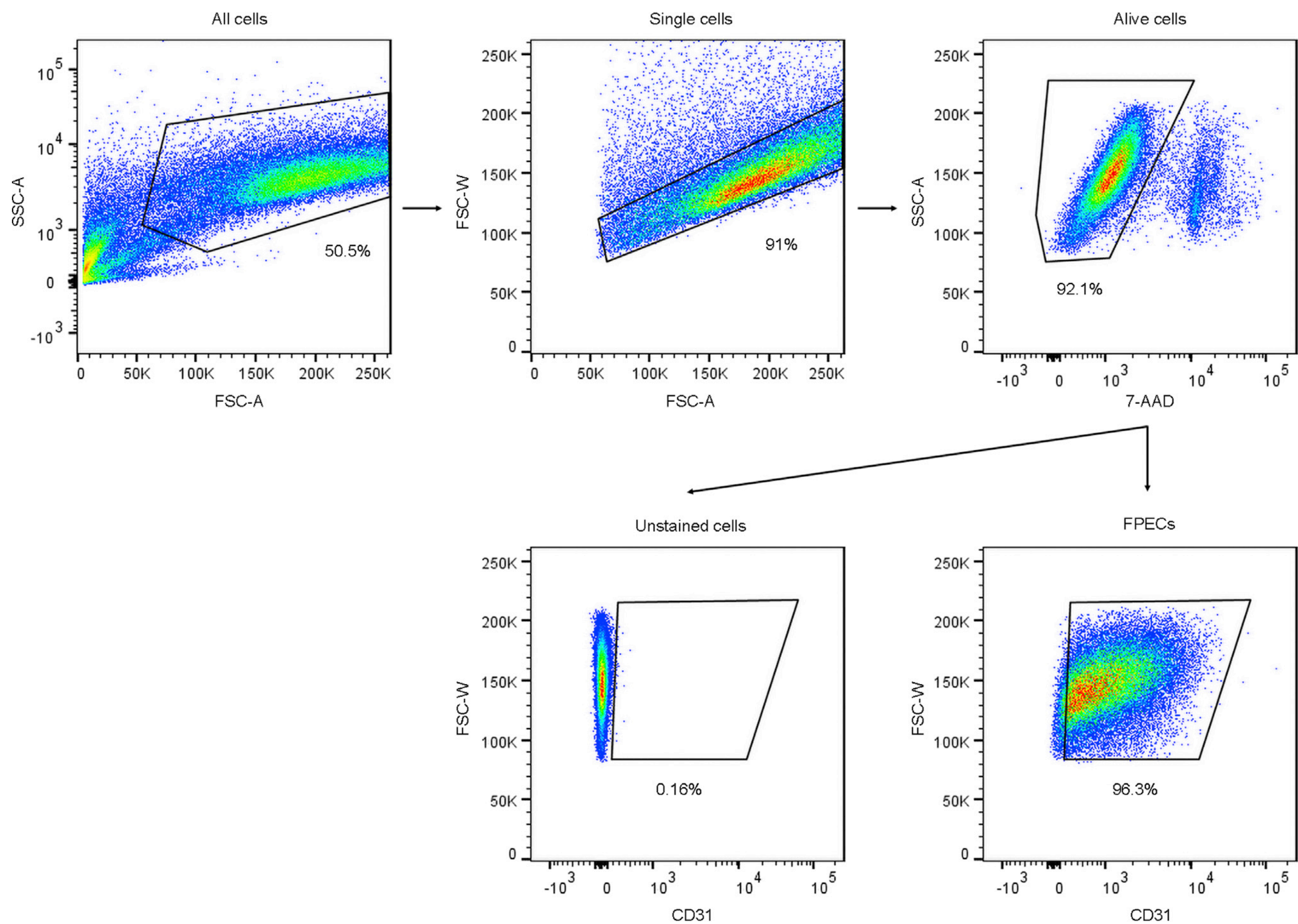

Figure 5. Gating strategy used for flow cytometry analysis of CD31 expression on passage one FPEC
After flow cytometry gating, more than 95% of the stained cells are CD31+, demonstrating the high purity of isolated FPEC (polygonal gate in the bottom right panel). For each panel, the polygonal shapes represent the defined populations (the percentage values shown under each polygonal gate relate to the parent gate). SSC-A – side scatter area; FSC-A – forward scatter area; FSC-W forward scatter width; 7-AAD – 7-aminoactinomycin (marker of cell death).

Furthermore, as with any *ex vivo* model, FPEC isolated and cultured using this protocol are likely to only partially recapitulate their functional *in vivo* characteristics. Co-culture of endothelial cells with other cells that exist in placenta may better preserve and recapitulate their functions. Co-culture of endothelial cells with pericytes has been used to model *ex vivo* human placental microvasculature and vascular conditions such as pre-eclampsia (Haase et al., 2019). Trophoblast and blood vessel organoid cultures, recently achieved using explants of early human abortions (Zhao et al., 2022) bring the promise of a more physiological model to study FPEC *ex vivo*.

TROUBLESHOOTING

Problem 1

Collagenase under- or over-digestion (step 8).

Potential solution

Collagenase type I from *Clostridium histolyticum* purchased from other suppliers may require initial testing and optimization. Some collagenase preparations may contain other proteases that could digest cell surface proteins, including CD31, which slightly damages cells and reduces FPEC pull-down efficiency.

Problem 2

Low number of cells have attached to culture plate (step 30).

Potential solution

Check that all reagents have been stored and applied correctly. If the problem persists, following FPEC isolation, plate all cells within one well of a 6-well plate.

Problem 3

FPEC have detached during the immunocytochemistry procedure (steps 43–52).

Potential solution

Do not agitate plate during incubations. During the washing steps, use a pipette instead of an aspirator to remove the media or other solutions, which will reduce the risk of disturbing the cells.

Problem 4

Patchy staining for CD31 during immunocytochemistry (step 53).

Potential solution

Avoid complete confluence of FPEC grown on coverslips, which may limit the access of the antibody to the CD31 epitope. Avoid over-fixing the cells with formaldehyde.

Problem 5

Percentage of cells positive for CD31 during flow cytometry analysis is low (step 67).

Potential solution

This may be due to contaminating non-endothelial cells (e.g., pericytes) that are tightly bound to FPEC. A potential solution is to optimize the collagenase digestion (step 8) and to filter the single-cell suspension after the collagenase digestion using 40-μm instead of 70-μm cell strainers (step 9), to reduce the risk of pericyte-FPEC doublets being bound by the coated Dynabeads (step 22). Another potential cause is the loss of the CD31 epitope when cell detachment was performed using Trypsin instead of Accutase (step 55).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Cristina Branco: c.branco@qub.ac.uk.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique data sets or code.

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AUTHOR CONTRIBUTIONS

I.S., M.C., and C.M.B. designed the study. I.S. and M.R. performed the experiments, with guidance from C.M.B. I.S. wrote the manuscript, with contributions from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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