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Ovarian Hilum Tissue Digestion and Magnetic-Activated Cell Sorting (MACS) of CD31+ Endothelial Cell Isolation

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We use this protocol and it's working.

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Disclaimer

Use recommended biosafety protocols and precautions for human tissue for your institution.

Abstract

The ovarian hilum serves as a conduit of nerves, lymphatic vessels and blood vessels for the ovary.[1] Digestion of cryopreserved human hilar tissue allows for further primary cell isolation, subsequent experiments and downstream analysis. For example, magnetic-activated cell sorting (MACS) can be performed on cells harvested from digested hilar tissue to isolate desired cell types with cell surface markers. This protocol describes hilar tissue digestion and subsequent MACS isolation for primary endothelial cells using anti-CD31-conjugated magnetic beads.[2] Isolated cells and the negative population can then be used in primary cell culture and further downstream analyses.

Hilar tissue was obtained from pediatric and adolescent patients undergoing ovarian tissue cryopreservation (OTC) at Ann & Robert H. Lurie Children's Hospital of Chicago who consented to donate tissue for research under approved IRBs. The hilum is surgically removed from the ovary, processed into 2mm x 5mm pieces and cryopreserved using the previously published slow freezing method: **dx.doi.org/10.17504/protocols.io.5jyl8jzr6g2w/v1**.

Immunofluorescence staining of MACS-isolated cells with Ulex europaeus agglutinin I vascular dye, which binds to endothelial cell glycoconjugates, verified the presence of CD31-expressing cells within our positive fraction when compared to CD31-negative cells collected in the MACS flowthrough. Other interstitial stromal cells were also present in the culture as indicated by positive HMGB1 staining.

Materials

Hilar Tissue Digestion

- α-MEM with Glutamax (1X): R&D Systems M34750
- Human Serum Albumin: Sigma Aldrich A9511-5G
- Insulin-Transferrin-Selenium (100X): Corning 25-800-CR
- Liberase DH (preferred over Liberase TL): Sigma Aldrich 05401089001
- Collagenase IV: Sigma Aldrich C5138-100MG
- DNase I: Roche 10104159001
- Antibiotic-Antimycotic (100X): R&D Systems B22110
- Bovine Serum Albumin: Sigma-Aldrich A9418-50G
- Phosphate Buffered Saline: Cytiva SH30258.02
- Fetal Bovine Serum: Cytiva SH30088.03
- DMEM/F12: Cytiva SH30261.01
- 6-well tissue culture treated plate: Celltreat 229106 or equivalent
- 15mL polypropylene centrifuge tube: Celltreat 667115R or equivalent
- 50 mL polypropylene centrifuge tube: Celltreat 229420 or equivalent
- 100mm x 15mm Petri dish: Celltreat 229695 or equivalent
- 0.22µm filter: Celltreat 229747
- 10mL syringe: Air-Tite 5100.X00V0
- 70µm cell strainer: Celltreat 229483
- Eppendorf LoBind 1.5mL polypropylene microcentrifuge tubes: Fisher-Scientific 13-698-794 or equivalent
- McIlwain Tissue Chopper, Petri Dish Table, 120V (optional): Ted Pella 10184-220

MACS

- DMEM-F12 + Glutamax + FBS 10%
- PromoCell Endothelial Cell Growth Medium (+ Supp): PromoCell Inc. C-22022
- Phosphate Buffered Saline
- Trypsin 0.25%: Cassion Labs TRL01-100ML
- Miltenyi Biotec CD31 MicroBead Kit: Miltenyi BioTec 130-091-935
- Miltenyi MACS MultiStand
- Miltenyi MACs MS Columns: Miltenyi BioTec 130-042-201
- 15mL conical vial
- 50mL conical vial
- CryoStor CS10: STEMCELL Technologies 07940
- Ulex Europaeus Agglutinin I (UEA I), DyLight 594 Vascular Dye: Vector Laboratories DL-1067-1

Digestion Media Preparation

1 Combine the following components in a sterile conical vial, adding the enzymes (Liberase DH, Collagenase IV, DNase I) last.

	Component	Stock concentration	Working concentration	Amount (for 10mL)
	α-MEM with Glutamax	1X		To 10mL
	Human Serum Albumin		1mg/mL	10mg
	Insulin-Transferrin-Selenium	100X	1X	100ul
	Liberase DH		40 µg/mL	80ul
	Collagenase IV		0.4 mg/mL	40ul
	DNase I		0.2 mg/mL	200ul
	Anti-anti (optional)	100X	1X	100ul

2 Filter the digestion media using a 0.22µm filter and 10mL syringe. Transfer to a 10cm dish and place in an incubator (37C, 5% CO2) to equilibrate.

Quench Media Preparation *This is used to prevent overdigestion of cells and improve viability.*

3 Add 10% FBS in DMEM/F12 for a total of 50mL quench media.

4 Transfer to a conical vial and leave in hood at room temperature.

Tissue Preparation

5 Thaw tissue (2×2×5 mm pieces) using a bead bath or water bath set at 37ºC for approximately 5 minutes, depending on tissue size, or until there is a small piece of ice

left in the tube.

6 After thawing, transfer contents into a 15mL tube, either by pouring or using a transfer pipet. Wet the transfer pipet in media to prevent cells from sticking to a dry pipet.

7 Working **dropwise**, add about 5mL of quench media (RT) into the 15mL tube. Be sure to add quench media slowly to avoid a sudden change in osmotic pressure. Roll the tube against hands until the cryoprotectant interface is no longer visible.

8 Spin at 300 x g for 5 mins.

9 Aspirate supernatant and resuspend in 1mL quench media.

10 Cut tissue using scissors or tissue chopper (optional) into roughly 1 mm x 1 mm pieces. This can be done in PBS or L15 since it is in open air. *This will improve the rate of digestion.*

11 Transfer chopped tissue to a 15mL tube using a sterile wet transfer pipet. Add about 5mL quench media and spin 15mL tube at 300 x g for 5 mins.

Tissue Digestion

12 Aspirate supernatant and resuspend in 2mL of digestion media from the 10cm plate to cover the tissue.

13 Pull up resuspended tissue using a wet transfer pipet and transfer to a 6-well tissue culture plate. Try to add an equal amount of tissue/cells in each well. Then add a maximum of 2mL of digestion media into each well with tissue.

14 Shake 6-well plate on orbital shaker in incubator (37ºC, 5% CO_2) at 100 rpm x 15 mins.

15 At 15 mins, remove plate from incubator and mechanically digest by pipetting up and down with a sterile wet transfer pipette. *If the tissue pieces are too large to pipette, use a sterile transfer pipette to break up the tissue mechanically.*

16 Return to shake in the incubator at 100 rpm for 15 mins.

17 Set up a 70µm cell strainer in a 50mL conical tube and prime with 1mL quench media.

18 After 15 mins, remove the plate from the incubator and mechanically digest with a sterile wet transfer pipet.

19 Use a transfer pipet to move well contents into the filter and into the 50mL conical, then add n mL quench media, where n = amount of digestion media (1:1 ratio).

19.1 If large chunks remain, leave them in the 6-well plate, add some more digestion media and incubate and shake again. Only transfer the media with properly digested tissue into the filter.

20 Plate or freeze the collected stromal cells as desired.

Plating Stromal Cells

21 Following digestion, spin at 300 x g for 5 min to pellet the cell suspension.

22 Aspirate the supernatant and resuspend in 1mL DMEM/F12.

23 Count cells and resuspend in an appropriate amount of DMEM/F12 to have a final concentration of ~500,000 cells per mL.

24 Plate 2mL of cell suspension per well of a 6-well plate. Replace spent media with fresh media after the first day and every two days thereafter until 80% confluent or until media is yellow.

Post expansion: Magnetic-Activated Cell Separation of CD31+ Cells from Digested Hilar Tissue

25 Bring DMEM-F12 and trypsin to room temperature.

26 Aspirate media from 6-well plates and wash with PBS.

27 Add 1mL trypsin to each well and incubate for 2-5 minutes.

28 Gently knock plate and check cells under microscope to ensure that they have lifted.

29 Neutralize with DMEM-F12 + 10% FBS (2x the amount of trypsin). Transfer cell suspension to a conical vial and centrifuge at 300 x g for 5 minutes.

30 Aspirate supernatant and count cells. Resuspend in a total of 5mL DMEM-F12.

31 Centrifuge vial at 300 x g for 5 min.



32 For cell count < 1×10^7: Resuspend in 60ul media (DMEM-F12) and transfer the suspension to a sterile low-bind Eppendorf tube. Add 20ul Fc block. Vortex for 5 seconds.

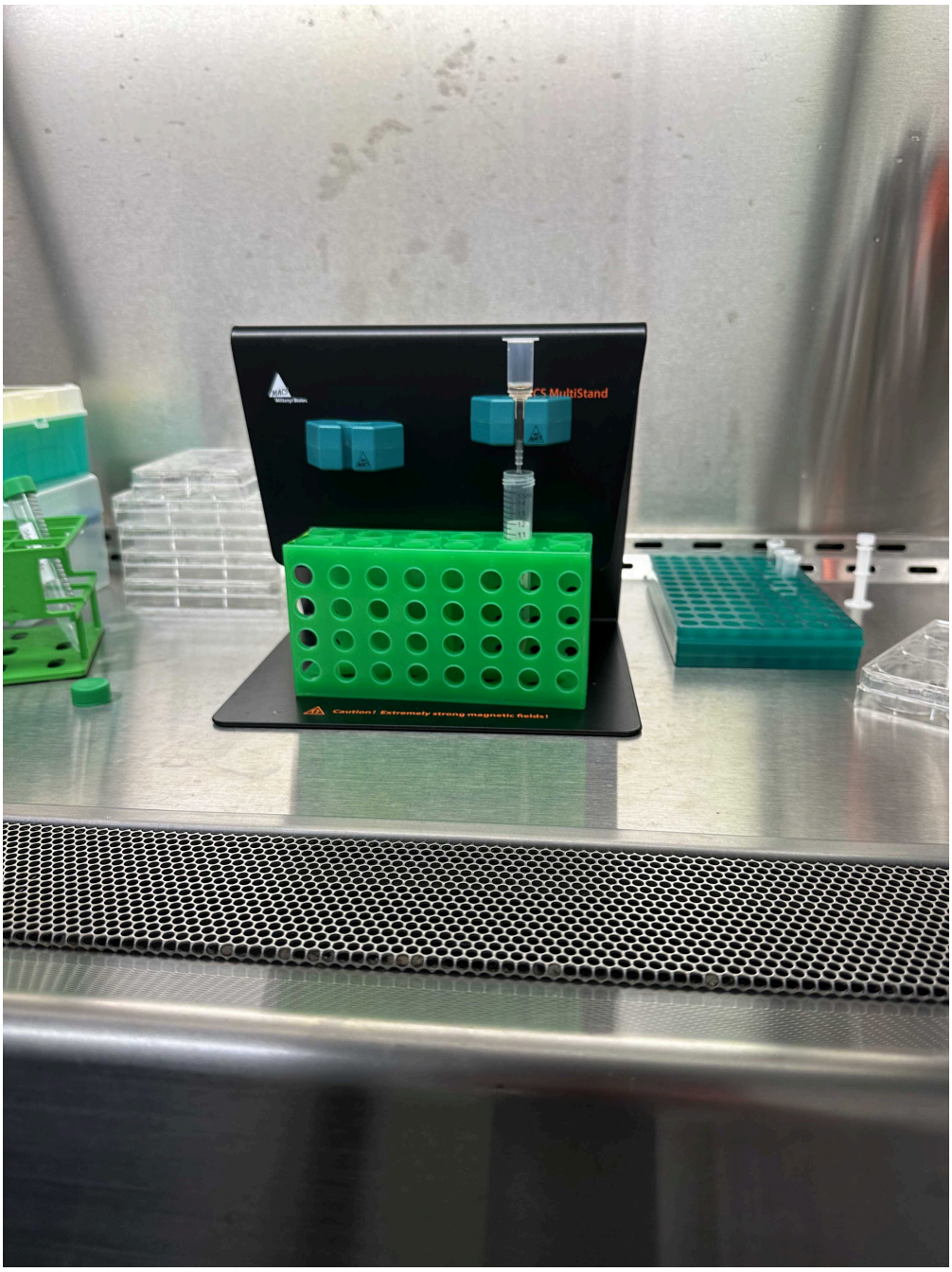
32.1 For cell count > 1×10^7: Split cell suspension into aliquots of < 1 × 107 each and proceed.

33 For cell count < 1×10^7: Add 20ul anti-CD31 magnetic beads (shake the bottle before adding).

34 Incubate at 4ºC for 15 mins.



35 While incubating, set up the MACS column. Remove the plunger and place it aside so that the portion that will come in contact with the cell suspension is facing up. Set a conical vial underneath each column stand, one for the stromal cells (CD31-negative) and one for the endothelial (CD31+) cells, and add 1-2mLs of the appropriate media in each (DMEM-F12 for stromal cells, endothelial growth media for the CD31+ cells).



MACS Multistand set-up. Only one column is pictured here.

36 Prime each column with 1mL media (DMEM for the first flow-through, which will be stromal cells).

37 After the 15 minutes have elapsed, add 1mL DMEM-F12 to the Eppendorf tube containing the cell and magnetic bead suspension and centrifuge at 300 x g for 5 minutes.

38 Aspirate supernatant and resuspend in 1 mL DMEM.

39 Add cell suspension to the column.

40 The first flowthrough should be stromal cells (CD31-negative, non-target cells). **Do NOT use the plunger** for this first flowthrough as this will disrupt the magnetic beads and result in a heterogenous population.

41 Add another 1mL of DMEM/F12 to the top of the magnetic column and allow for flowthrough. This will ensure that all remaining cells are pushed through the column.

42 Plate stromal cells as desired OR freeze down for later use in Cryostor CS10.

43 To isolate the endothelial cells, remove the column from the magnet and move it above the second conical vial primed with the endothelial cell growth media.

44 Add 1mL endothelial cell growth media to the column. Apply the plunger and push.

45 Plate the isolated CD31-expressing cells (endothelial cell enrichment) in endothelial cell growth media.

Immunofluorescence staining of MACS-isolated cells

46 Immunofluorescence staining of MACS-isolated cells with Ulex europaeus agglutinin I vascular dye, which binds to endothelial cell glycoconjugates, verified the presence of endothelial cells, especially when compared to CD31-negative sort cells collected in the MACS flowthrough. An analysis of the percentage of area covered by vascular dye over the percentage of phalloidin, 25.75% of cells expressed human-specific endothelial vascular dye. Other interstitial stromal cells were also present in the culture as indicated by positive HMGB1 staining. Images were taken with BZX710 Keyence Microscope.

Fig. 1

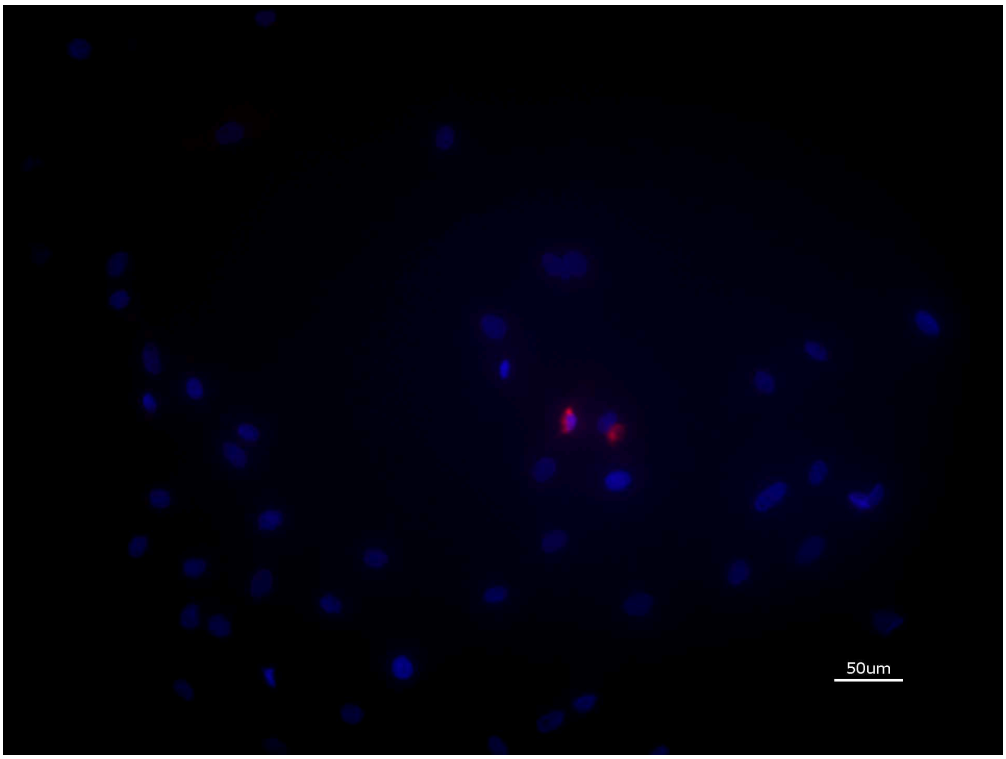


Fig. 1. **CD31-negative MACS flowthrough.** Vascular dye (red), DAPI (blue). 20X magnification.

Fig. 2

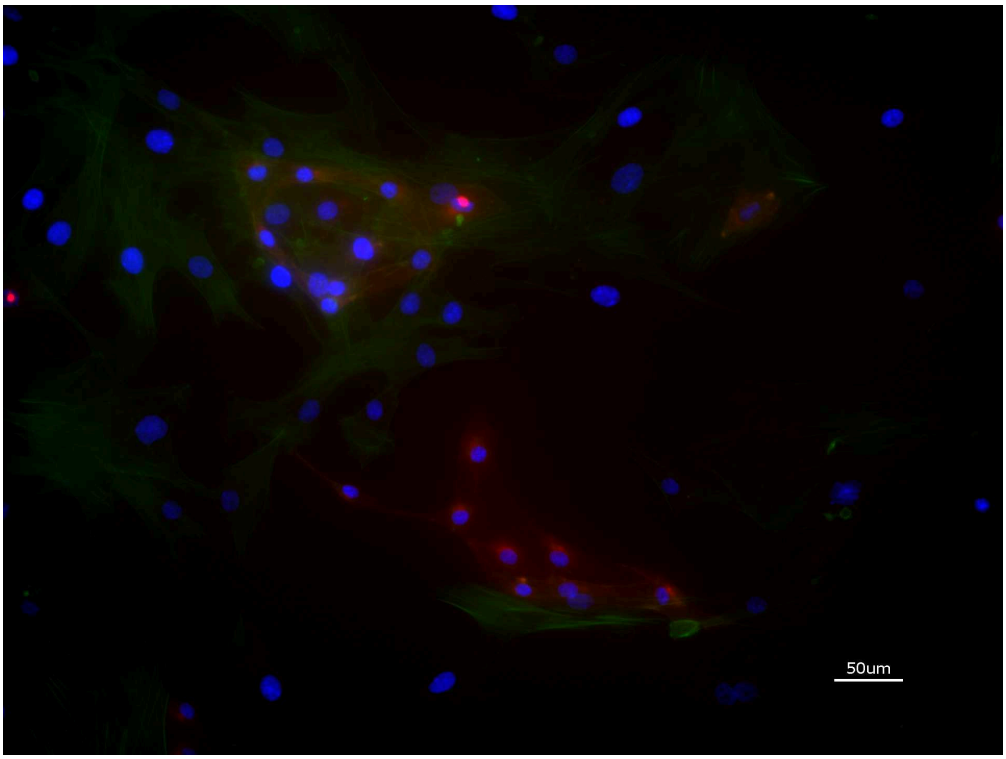


Fig. 2. **CD31-enrichment cells.** Vascular dye (red), phalloidin (green), DAPI (blue). 20X magnification.

Fig. 3

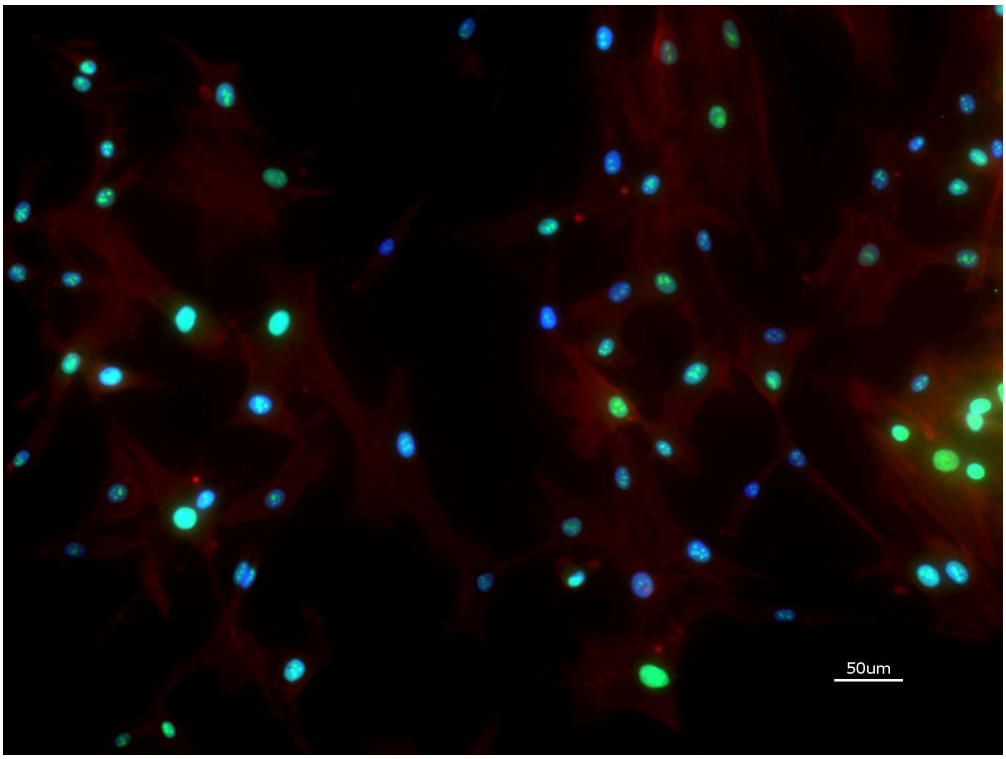


Fig. 3. **CD31-enrichment cells.** HMGB1 (green), phalloidin (red), DAPI (blue). 20X magnification.

Protocol references

1. Tsui, E., O'Neill, K., LeDuc, R., Shikanov, A., Gomez-Lobo, V., Laronda, M. Creating a common language for the subanatomy of the ovary. *Biol Reprod* **108**, 1 (2023). https://doi.org/10.1093/biolre/ioac199

2. Wagner, M., Yoshihara, M., Douagi, I. *et al.* Single-cell analysis of human ovarian cortex identifies distinct cell populations but no oogonial stem cells. *Nat Commun* **11**, 1147 (2020). https://doi.org/10.1038/s41467-020-14936-3

CD31+ MACs protocol from Miltenyi Biotec: https://static.miltenyibiotec.com/asset/150655405641/document_87215qami11ah2nd7a6agrim07?content-disposition=inline