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Single cells isolation from human decidua parietalis tissue to use for Chromium-based scRNA-seq and cryopreserve for single cell proteomics

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Abstract

This protocol was developed as part of the Borne Uterine Mapping Project (BUMP) feasibility study; specifically for the goal of isolating suspensions of single cells from decidua parietalis tissue to be used for single cell (sc)RNA-seq and stored for single cell proteomics.

Guidelines

- Where ⊕ symbol is present at a protocol step, record the time that the associated step has been reached or what has been stated in the step to be noted for timing.
- Follow aseptic technique for all steps to be undertaken in the Class II biological safety ('biosafety') cabinet.

Materials

- Quantities of items listed in Materials lists are the recommended minimum to isolate decidua parietalis tissue from one study participant at a sufficient quantity to isolate cells for both (i) use with the 10x Genomics Chromium Next GEM Single Cell 3′ Reagents Kits v3.1 (Dual Index) components for scRNA-seq library preparation, and (ii) cryopreservation using CryoStor CS10 reagent for single cell proteomics. **It is highly recommended that spare items are made readily available**.
- Items marked '*' need to made ready for use inside a decontaminated **Class II biological safety cabinet** (ideally before collection of placenta with fetal membranes).
- Items marked '~' are components of the **10x Genomics Chromium Next GEM Single Cell 3′ Reagents Kits v3.1 (Dual Index)** package for scRNA-seq library preparation.
- Items marked '#' are specifically for **preparing suspension of single cells for scRNA-seq** but are not components of the 10x Genomics Chromium Next GEM Single Cell 3′ Reagents Kits v3.1 (Dual Index) package.

	Item	Quantity	Supplier	Product number	Note
	~ Chromium controller	1	10x Genomics	PN-120270	
	~ Accessory kit for Chromium controller	1	10x Genomics	PN-1000142	Specific components of kit required: secondary holder (PN-3000332), vortex adaptor (PN-330002), and magnetic separator (PN-230003)
	# Thermal cycler	1	site-dependent		e.g. Eppendorf Mastercycler Nexus X2, Bio-Rad C1000
	Electronic balance	1	site-dependent		2 decimal place accuracy is sufficient
	Shaking water bath	1	site-dependent		e.g. Julabo SW21, Clifton WA0261
	Wire tube rack	1	Scientific Laboratory Supplies	STA20020	
	Centrifuge with swing bucket rotor; fits 15 and 50 mL centrifuge tubes, adjustable temperature settings	2; 1 of which needs to have adjustable acceleration and brake speed settings	site-dependent		e.g. Eppendorf 5804R (adjustable brake speed), Eppendorf 5910R (fixed brake speed); both suitable for 400 g centrifugation
	Light microscope	1	site-dependent		e.g. Leica DM IL LED; suitable for Trypan Blue manual cell counting
	Blunt end (filter) forceps	1	Sigma-Aldrich	XX6200006P	
	Tissue dissection scissors	1	World Precision Instruments	14393	
	Polystyrene box, 4 L capacity	1	n/a	n/a	To store wet ice
	Cell freezing container; Nalgene (Mr Frosty)	1	Thermo Fisher Scientific	5100-0001	
	Plastic beaker, 100 mL capacity	1	Scientific Laboratory Supplies	BEA5026	
	* Serological pipette controller; adjustable speed	1	site-dependent		e.g. HTL SwiftPet Pro

	Item	Quantity	Supplier	Product number	Note
	* 50 mL centrifuge tube racks	3	Starlab	E2345-1099	
	* Haemocytometer	1	site-dependent		For Trypan Blue-based manual cell counting
	gentleMACS Dissociator	1	Miltenyi Biotec	130-093-235	
	*# MidiMACS Separator	1	Miltenyi Biotec	130-042-302	
	*# MACS MultiStand	1	Miltenyi Biotec	130-042-303	
	*[#] Micropipettes; P10, P20, P200 and P1000 capacity	2 per type (1x in biosafety cabinet, [1x] on Chromium controller bench) and 1x P1000 for tissue dissection bench	site-dependent		1x P1000 micropipette on tissue dissection bench to be used for resuspending cells in CryoStor CS10 reagent

List of equipment. Items marked '[#]' indicate only a partial quantity is specifically for preparing suspension of single cells for scRNA-seq (and are not components of the 10x Genomics Chromium Next GEM Single Cell 3' Reagents Kits v3.1 (Dual Index) package); the quantity allocated to scRNA-seq is noted (within square brackets) in the 'Quantity' column.

	Item	Quantity	Supplier	Product number	Note
	Cell scraper	1 (minimium)	Sarstedt	83.3590	
	*[#] 10 cm diameter Petri dishes; sterile-packed	3 (minimum)	Greiner	632181	1 [+1]x within Class II biosafety cabinet, 1x on bench
	Absorbent 'inco' pads	2	Medisave	55525	
	*[#] gentleMACS C-tubes	2	Miltenyi Biotec	130-096-334	1 [+1]x within Class II biosafety cabinet
	*# MACS LS column	1	Miltenyi Biotec	130-042-401	
	*[#] 70 µm (pore diameter) cell strainers	6	Fisher Scientific	11597522	3 [+3]x within Class II biosafety cabinet
	*[#] 40 µm (pore diameter) cell strainers	4	Greiner	542040	2 [+2]x within Class II biosafety cabinet
	*[#] 10 mL syringes	2	VWR	5100-000V0	1 [+1]x within Class II biosafety cabinet
	*[#] Pasteur pipettes, 1 mL capacity	7	Starlab	E1414-0111	4 [+3]x within Class II biosafety cabinet
	*[#] Non-skirted (canonical) centrifuge tubes, 50 mL capacity and fits cell strainers	8	Greiner	227261	4 [+4]x within Class II biosafety cabinet
	*[#] Centrifuge tubes, 15 mL capacity	4	Costar	430791	2 [+2]x within Class II biosafety cabinet
	*# Bijou container, 7 mL capacity	1	Greiner	189175	1 x within Class II biosafety cabinet

	Item	Quantity	Supplier	Product number	Note
	Cryovials with external thread caps, 2 mL capacity	3	Greiner	126280	Pre-label (with barcodes for tracking) prior to sample collection
	Serological pipettes, individually sterile-wrapped; 5, 10 and 25 mL capacity	~1 pack of each size	site-dependent		e.g. Costar Stripettes
	*[#] Sterile and nuclease-free filter micropipette tips; to fit P10, P20, P200 and P1000 capacity micropipettes	2 boxes minimum per tip size (1x in biosafety cabinet, [1x] on Chromium Controller bench, and 1× 1 mL tips for tissue dissection bench)	site-dependent		Use low retention tips for Chromium workflow; see 10x Genomics manual CG000315, Rev C); 1 mL micropipette tips on tissue dissection bench to be used for resuspending cells in CryoStor CS10 reagent
	Standard laboratory film (i.e. Parafilm)	~1 roll			
	Masking tape	~1 roll			
	*# Universal tube, 30 mL capacity	1	Greiner	201151	
	# PCR tubes	~1 pack	VWR	732-0548	Alternative (if >1 reaction per cell suspension): ~1 pack of 8-strip (non-flex) PCR tubes; Starlab I1402-3700
	# DNA LoBind Eppendorf tubes, 1.5 mL capacity	~1 pack	VWR	525-0130	
	# 0.2 µm (pore size) syringe filter	1	Fisher Scientific	15392388	Only needed for preparing 50% v/v glycerol stock (for use at Chromium Controller)
	Placenta clinical disposal bags	2	n/a	n/a	Follow waste disposal procedure instructed by labour ward; also use a specimen bucket to transport placenta with fetal membranes to laboratory if to be used for isolation of single cells

List of single-use consumables. Items marked '[#]' indicate only a partial quantity is specifically for preparing suspension of single cells for scRNA-seq (and are not components of the 10x Genomics Chromium Next GEM Single Cell 3' Reagents Kits v3.1 (Dual Index) package); the quantity allocated to scRNA-seq is noted (within square brackets) in the 'Note' column. **Quantities of tubes do not include those for aliquots listed in the 'chemicals and reagents' table**.

	Item	Quantity	Supplier	Product number	Note
	[#] Accutase	1[+1]x 12 mL aliquot, each stored in 15 mL (centrifuge) tubes; sterile	Thermo Fisher Scientific	00-4555-56	
	Dulbecco's phosphate buffered saline (DPBS;	2× 50 mL aliquot,	Sigma-Aldrich	D8662	

	Item	Quantity	Supplier	Product number	Note
	CaCl2 and MgCl2 included)	each stored in 50 mL (centrifuge) tubes; sterile			
	DPBS (no CaCl2, no MgCl2)	1x ~500 mL bottle, and 1[+1]x 20 mL aliquots stored in 50 mL conical (non-skirted) centrifuge tubes; sterile	Thermo Fisher Scientific (Gibco)	14190169	Need to weigh these tubes before and after adding decidua parietalis (to calculate tissue weight present for adding appropriate working volume of accutase); write the weights down on the tube label
	* Histopaque-1077	1× 20 mL aliquot stored in 50 mL conical (non-skirted) centrifuge tube, sterile	Sigma-Aldrich	10771	
	CryoStor CS10	1× 3.3 mL aliquot stored in 7 mL Bijou tube, sterile	Sigma-Aldrich	C2874	
	Trypan blue	~1 mL	Thermo Fisher Scientific (Gibco)	15250-061	
	*# ACK Lysing Buffer	1× 3.3 mL aliquot stored in 7 mL Bijou tube, sterile	Thermo Fisher Scientific (Gibco)	A1049201	
	2-propanol (HPLC grade)	check volume required to fill cell freezing container	Sigma-Aldrich	34863	
	*# Distilled water, sterile and nuclease-free	1 bottle (~500 mL)	Thermo Fisher	10977035	
	# Nuclease-free water	1 bottle (~50 mL)	Thermo Fisher Scientific	AM9937	
	Wet ice	n/a			To fill the (4 L capacity) polystyrene box
	Ethanol (absolute)	variable	VWR	20821.330	Use to prepare 70% v/v ethanol for cleaning equipment and work surfaces, especially for items to be used within the Class II biological safety cabinet
	Virkon disinfectant (tablets)	variable	VWR	115-0021	To use as part of disposal of human tissue-containing waste in laboratory - solution in spray bottle for

	Item	Quantity	Supplier	Product number	Note
					tissue dissection bench, tablet in 500 mL waste container in Class II biological safety cabinet; follow local safety procedures
	# RNaseZap	variable	Sigma-Aldrich	R2020	

List of chemicals and reagents. Items marked '[#]' indicate only a partial quantity is specifically for preparing suspension of single cells for scRNA-seq (and are not components of the 10x Genomics Chromium Next GEM Single Cell 3' Reagents Kits v3.1 (Dual Index) package); the quantity allocated to scRNA-seq is noted (within square brackets) in the 'Quantity' column.

	Item	Quantity	Supplier	Product number	Note
	# 7.5% w/v bovine serum albumin (BSA)	0.27 mL, sterile	Sigma-Aldrich	A8412	To prepare Chromium resuspension buffer, mix with 50 mL DPBS (no CaCl2, no MgCl2) and prepare 10 × 5 mL aliquots of this mixture (i.e. 0.04% w/v BSA in DPBS) in 15 mL centrifuge tubes; see 10x Genomics CG000053 (Rev C – page 3) Demonstrated Protocol.
	# DPBS (no CaCl2, no MgCl2)	50 mL, sterile	Thermo Fisher Scientific (Gibco)	14190169	
	# Glycerol (molecular biology grade)	20 mL, nuclease-free	Sigma-Aldrich	G5516	Mix 1:1 to prepare 50% v/v glycerol, filter through a 0.2 µm (pore size) syringe filter, and aliquot 20 × 1.0 mL in DNA LoBind Eppendorf tubes; see 10x Genomics CG000315 manual, Rev C – page 19.
	# Nuclease-free water	20 mL	Thermo Fisher Scientific	AM9937	

List of chemicals for preparing Chromium resuspension buffer and 50% v/v glycerol solution for use of live cells enriched suspension with Chromium Controller for GEM generation. Use each directly from their stock bottle, which need to be allocated specifically to the Chromium (scRNA-seq) workflow.

	Item	Quantity	Supplier	Product number	Note
	# MACS Dead Cell Removal kit	1x kit	Miltenyi Biotec	130-090-101	
	~ Chromium Next GEM Single Cell 3' GEM kit v3.1	1x vial for each component	10x Genomics	PN-1000130	Only equilibrate and use RT Reagent B, RT Enzyme C, Template Switch Oligo, and Reducing Agent B.
	~ Chromium Next GEM Single Cell 3' Gel Bead kit v3.1	1x vial of gel beads per sample	10x Genomics	PN-1000129	
	~ Chromium Next GEM Chip G Single Cell kit	1x of each component	10x Genomics	PN-1000127	Only equilibrate and use Partitioning Oil, Chip G, and Chip Gasket.

List of kits. For use of the Chromium Controller on the same day as isolation of cell suspension from decidua parietalis tissue for scRNA-seq, when only the 10x Genomics Chromium kit components required for 'GEM Generation & Barcoding' ('Step 1' of 10x Genomics CG000315 manual, Rev C;

pages 24-31) will need to be used; all other components remain in storage at temperatures recommended by the manufacturer. PN numbers refer to the 4-reaction Chromium 'v3.1' kit (PN-1000269) or 16-reaction 'Chip G' kit (PN-1000127).

Other items (in addition to personal protective equipment)

- permanent marker pens (for labelling e.g. DPBS aliquot tubes; both on bench and in Class II biological safety cabinet)
- ballpoint pens (for documenting timings of tissue collection, dissection, freezing, and storage)
- printouts of sample collection proforma (for documenting timings and number of cryovials stored)
- 1x digital watch
- Disinfectant wipes (e.g. Clinell)
- paper towels
- plastic clinical waste sharps bins

Safety warnings

Seek local health and safety guidance for use of liquid nitrogen (**hazardous chemical**) and a Class II biological safety (biosafety) cabinet; in addition to assessing risks (**biohazards**) for the handling of human-derived tissues and biofluids.

Ethics statement

Ethical approval must be obtained from the Local Research Ethics Committee (LREC), Institutional Review Board, or equivalent body to recruit pregnant study participants for the purpose of acquisition, use, and storage of human tissue and biofluid samples specified in the protocol. Researchers must confirm that written consent has been obtained (by Good Clinical Practice (GCP) certified personnel; or equivalent for outside of the UK) via a LREC approved study consent form before proceeding with the use of this protocol. Seek further guidance from the institution(s) responsible for hosting the study with regards to regulations to abide by for the use of human-derived samples.

This protocol does not involve the use of (non-human) animal tissues.

Before start

Preparation in the laboratory before commencement of fetal/placental delivery (on day of sample collection) - confirm the following:

1. There is space available in the **liquid nitrogen sample storage dewar** to store the upcoming set of CryoStor-preserved cells (for single cell proteomics). The GEM-RT incubation products (for scRNA-seq) will need to be stored at either 4^{o}C for up to 72 h or -20^{o}C for up to a week; see 10x Genomics manual CG00315, Rev C - page 31.
2. **Thermal cycler** is switched on and GEM-RT reaction program (see 10x Genomics manual CG000315 Rev C - page 31 for program steps) is set for use.
3. **Shaking water bath** has been set to 37^{o}C and shaking function (100 rpm) is operating.
4. **Centrifuges** have been set to 20^{o}C and swing buckets for both 15 mL and 50 mL centrifuge tubes are ready to use. Also check settings for acceleration and brake speeds; *it is advisable that the centrifuge to be used for Histopaque-1077 layering, which needs low acceleration and brake speeds (see step 14.2 in protocol), is positioned as close to the Class II biological safety cabinet as possible*.
5. Volume of isopropanol in **cell freezing container** is sufficient (top-up with fresh isopropanol if needed); pre-chill the container in the fridge.
6. **Tissue dissection bench** has been disinfected (by wiping with e.g. Distel) and the items listed in **Table I** (below) are present; wipe clean dissection scissors and forceps with 70% v/v ethanol.
7. Reagents at **Table II** (below) are present in the fridge and ready (e.g. weighed) for use.
8. **Class II biological safety cabinet** has been switched on and airflow has stabilised. Its work surfaces have been wiped clean with 70% v/v ethanol and the items listed in **Table III** (below) are present (and also decontaminated with 70% v/v ethanol if possible; sterile-packed items remain in packaging when placed into Class II biological safety cabinet).
9. **Chromium Controller bench** has been wiped clean with disintectant (e.g. Distel) followed by 70% v/v ethanol. Also, the equipment itself switches on with no errors, items for GEM Generation & Barcoding (Step 1; listed in 10x Genomics manual CG000315 Rev C - page 25) are all available for use, and items listed in **Table IV** (below) are on the bench; reagents marked as 'equilibrate to room temperature' can be chilled on wet ice until it has been determined that enough live cells are available to proceed with use of the Chromium Controller.

	Item on tissue dissection bench
	* 1x blunt end (filter) forceps
	* 1x tissue dissection scissors
	* 1x polystyrene box (for wet ice)
	2x absorbent 'inco' pads - absorbent sides facing up
	1 (+1 spare) sterile-packed cell scraper
	1× 10 cm diameter Petri dish
	1× 50 mL centrifuge tube rack
	1x P1000 micropipette + 1 mL sterile filter tips
	1× 3.3 mL CryoStor CS10 aliquot - chill on wet ice
	3× 2.0 mL capacity cryovials (pre-labelled to store cells in CryoStor CS10 reagent; 2x Histopaque-1077 interface layer, 1x Histopaque-1077 lower layer) - chill on wet ice
	70% v/v ethanol (in spray bottle)
	Virkon disinfectant solution (in spray bottle)
	1x permanent marker pen

Table I. Items to make available (before C-section commences) at tissue dissection bench in laboratory for isolation of decidua parietalis tissue (from chorionic membrane) to use for isolation of single cells. *If decidua parietalis tissue is also to be flash frozen in liquid nitrogen from the same study participant (using a separate protocol) by the same researcher, undertake this first before proceeding with isolation of single cells using the current protocol; the items marked '*' are the same as those in Table I of the 'OCT embedding (for spatial transcriptomics) and flash freezing (for single nucleus RNA-seq) of human myometrial and decidual tissues' protocol.*

	Item to reserve in fridge for isolation of single cells
	2× 50 mL DPBS (CaCl2 and MgCl2 included) aliquots
	2× 20 mL DPBS (no CaCl2, no MgCl2) aliquot - record the WEIGHT of both tubes (with DPBS stored); will need to weigh both tubes once they are filled with decidua parietalis (scraped off the the chorion) to calculate tissue weight
	1× 5 mL Chromium resuspension buffer aliquot
	1x MACS Dead Cell Removal kit

Table II. Items to make available (before C-section commences) for isolation of single cells from decidua parietalis tissue but keep refrigerated until placenta with fetal membranes has been transported to the laboratory.

	Item in Class II biological safety cabinet
	1x serological pipette controller
	Serological pipettes: 5, 10 and 25 mL (maximum) volume capacity
	2× 50 mL centrifuge tube racks
	# 1x MidiMACS Separator
	# 1x MACS MultiStand
	A set of micropipettes: 1 each of P10, P20, P200 and P1000 capacity
	A set of sterile and nuclease-free filter tips for micropipettes: 1 box for each size (minimum)
	1x haemocytometer
	[#] 2× 10 cm diameter Petri dishes (1x CryoStor cells, 1x Chromium Controller cells)
	[#] 2x gentleMACS C-tubes (1x CryoStor cells, 1x Chromium Controller cells)
	# 1x MACS LS column
	[#] 6× 70 µm (pore diameter) cell strainers (3x CryoStor cells, 3x Chromium Controller cells; minimum)
	[#] 4× 40 µm (pore diameter) cell strainers (2x CryoStor cells, 2x Chromium Controller cells; minimum)
	[#] 2× 10 mL syringes (1x CryoStor cells, 1x Chromium Controller cells; minimum)
	[#] 7× 1 mL Pasteur pipettes (4x CryoStor cells, 3x Chromium Controller cells; minimum)
	[#] 8× 50 mL non-skirted (canonical) centrifuge tubes (4x CryoStor cells, 4x Chromium Controller cells)
	[#] 4× 15 mL centrifuge tubes (2x CryoStor cells, 2x Chromium Controller cells)
	# 1× 7 mL Bijou container
	# 1× 30 mL Universal tube
	1x ~500 mL DPBS (stock bottle; no CaCl2, no MgCl2)
	1× 20 mL Histopaque-1077 aliquot - equilibrate to room temperature
	1 × 3.3 mL ACK Lysing Buffer aliquot
	Distilled water; 1x ~500 mL (stock) bottle

	Item in Class II biological safety cabinet
	1× 500 mL container (e.g. empty DPBS stock bottle) containing a Virkon disinfectant tablet
	1x permanent marker pen

Table III. Items to make available (before C-section commences) in Class II biological safety cabinet, where '#' indicates the item is specifically for isolation of single cells for scRNA-seq, and '[#]' indicates a partial quantity of the item is also for isolation of cells to cryopreserve in CryoStor CS10 reagent for single cell proteomics.

	Item (not supplied by 10x Genomics) on Chromium Controller bench
	A set of micropipettes: 1 each of P10, P20, P200 and P1000 capacity
	A set of low retention nuclease-free filter tips for micropipettes: 1 box for each size (minimum)
	3 (+ spares) x PCR tubes
	3 (+ spares) x DNA LoBind Eppendorf tubes
	RNAseZap (1x ~250 mL bottle)
	Nuclease-free water; 1x ~50 mL (stock) bottle

Table IV. Nuclease-free items to make available (before C-section commences) at Chromium Controller bench.

Preparation at labour ward before commencement of fetal/placental delivery (on day of sample collection) - confirm the following; *same as at 'OCT embedding (for spatial transcriptomics) and flash freezing (for single nucleus RNA-seq) of human myometrial and decidual tissues' protocol if also obtaining decidua parietalis tissue to flash freeze in liquid nitrogen*:

1. Confirm written consent to study participation has been obtained from participant.
2. Inform the attending midwife that the placenta with fetal membranes will need to be given to the researcher immediately after their routine clinical checks; they are to be left in the kidney dish wrapped with the inco pad at room temperature. Make appropriate arrangements if the placenta needs to be sent to a clinical pathology laboratory after research samples have been acquired.

Tissue dissection to isolate decidua parietalis tissue

1 Transport placenta with attached fetal membranes (double-bagged in clinical disposal bags and stored in specimen bucket) at room temperature to laboratory immediately after all its routine clinical checks have been completed (confirm with the attending midwife). ***If tissues are to also be flash frozen in liquid nitrogen*** *(using the separate 'OCT embedding (for spatial transcriptomics) and flash freezing (for single nucleus RNA-seq) of human myometrial and decidual tissues' protocol), the decidua parietalis tissue sample stored in 1 × 15 mL DPBS aliquot on wet ice will also need to be transported to the laboratory too.*

> Note
>
> If decidua parietalis tissue is to be flash frozen in liquid nitrogen from the same study participant (to store at -80^{o}C), complete this first before proceeding to isolate the remaining decidua parietalis tissue still attached to the chorionic membrane in the laboratory using the current protocol.

2 ⊕ As soon as placenta with fetal membranes arrives in the laboratory, **defrost 2× 12 mL accutase aliquots** (1x for CryoStor cells, 1x for Chromium Controller cells) by placing the frozen tubes in a 100 mL beaker filled with room temperature (deionised) water - do NOT thaw in a heated water bath or equivalent.

3 Take the **pre-weighed 2× 20 mL DPBS (no $CaCl_2$, no $MgCl_2$) aliquots** out of the fridge to equilibrate to room temperature.

4 Take the placenta with fetal membranes out of the bag and lay onto the top of the double-layered 'inco' pads (absorbent sides facing up) on the tissue dissection bench with fetal side (i.e. amnionic membrane) facing down.

5 **Isolate 2x ~6 g decidua parietalis tissue.**

5.1 ⊕ Manually separate the choriodecidua from the amnion whilst keeping the decidua parietalis side facing up.

5.2 Use the dissection scissors (wiped clean with 70% v/v ethanol) to cut **multiple ~7×7 cm pieces of choriodecidua**, where ~4 pieces (from a term gestation pregnancy) is often sufficient per ~6 g decidua parietalis tissue (so need ~8 pieces for ~12 g; although ~5 g decidua parietalis should be sufficient for isolation of single cells to use with the MACS Dead Cell Removal kit), and lay these on the top 'inco' pad with decidua parietalis side facing up.

5.3 Add a layer of chilled DPBS ($CaCl_2$ and $MgCl_2$ included; from the 2 × 50 mL aliquots stored in the fridge) onto the decidua layer of all the ~7×7 cm choriodecidua pieces to keep them hydrated while waiting to be scraped off.

5.4 Put a Petri dish (base or lid) upside down onto the top 'inco' pad and place a ~7×7 cm piece of choriodecidua onto it with decidua layer facing up. Use the blunt end (filter) forceps to pick off any blood clots on the decidua layer.

5.5 Use a newly unwrapped cell scraper to gently scrape off the decidua layer from the chorion without tearing the latter. The chorionic membrane should become visibly translucent (from opaque), while the texture of the decidua can vary from soft to rough and is often a greyish-cream (and pink if residual blood is present) colour.

5.6 Promptly transfer the decidual tissue equally into the 2x pre-weighed 20 mL DPBS (no $CaCl_2$, no $MgCl_2$) aliquot tubes.

5.7 Repeat from step 5.4 to 5.6 with the remaining ~7×7 cm pieces of choriodecidua, using the same Petri dish (base or lid) and cell scraper to isolate the decidua parietalis and transfer into the DPBS tubes until they each contain ~6 g tissue.

5.8 Once 2 x ~6 g decidua parietalis tissues have been stored into both DPBS tubes, clean dissection scissors and forceps with a disinfectant (e.g. Clinell) wipe to thoroughly remove blood and tissue, then wipe dry with clean paper towels. Put the remaining fetal membranes and placenta back into the waste bags for clinical disposal, along with the 'inco' pads, or storage for histology.

Tissue dissociation (mechanical disruption and enzymatic digestion)

6 ⊕ Securely cap both tubes of decidua parietalis tissue in DPBS (before taking out of the biosafety cabinet) and centrifuge at **400 g for 5 minutes at 20°C**.

400 x g, 20°C, 00:05:00 , maximum acceleration and brake speeds; tissue dissociation

7 Aspirate the supernatant (without disturbing the tissue pellet) at both tubes and dispose into Virkon (i.e. disinfectant) waste container within the biosafety cabinet.

8 ⊕ Resuspend each tissue pellet in fresh 20 mL DPBS (no $CaCl_2$, no $MgCl_2$) and repeat steps 6 and 7.

9 Resuspend each tissue pellet in **accutase** at 1:2 volume (e.g. 6 g tissue = 12 mL accutase).

10 Transfer the each tissue-accutase mixture into its own labelled **gentleMACS C-tube**, cap securely, and invert both C-tubes several times to loosen the tissue pellets before attaching to the **gentleMACS dissociator**. Keep both 50 mL centrifuge tubes in the biosafety cabinet.

10.1 ⊕ Set the gentleMACS dissociator to run the preset 'Spleen' program (**m_spleen_01.01; 56 seconds/cycle**) for both C-tubes.

10.2 Carefully detach the C-tubes from the dissociator (without twisting the tube to avoid uncapping) and, in the biosafety cabinet, use a 25 mL serological pipette to transfer each tissue-accutase mixture back into their 50 mL centrifuge tube (emptied at step 10.0). **Repeat steps 10.0 and 10.1 (ideally no more than once)** if the tissue-accutase mixtures are unable to pass through the 25 mL serological pipette smoothly, and re-attempt transfer of each mixture into their 50 mL centrifuge tubes using a 25 mL serological pipette.

11 Securely cap both tubes of tissue-accutase mixtures (before taking out of biosafety cabinet) and wrap laboratory film (i.e. Parafilm) securely around each cap before

attaching them horizontally to the wire tube rack (using masking tape).

11.1 ⊕ Insert the wire rack with the securely attached 50 mL tubes of tissue-accutase mixtures positioned horizontally into the **37°C water bath** to incubate for 45 minutes with (100 rpm) shaking function on for constant agitation. Confirm that both tubes are no more than half-immersed in 37°C water of the shaking bath.

11.2 Take the rack with attached tubes out of the 37°C water bath after completing the 45-minute incubation, remove the masking tape to detach the tubes, and wipe dry the tubes with clean paper towels. Remove the laboratory film at each lid and wipe both tubes with 70% v/v ethanol thoroughly before putting back into the biosafety cabinet.

11.3 Use a 25 mL serological pipette to combine the two tissue-accutase mixtures into one of their 50 mL tubes.

12 Uncap two new 50 mL centrifuge tubes (standing in a tube rack) and insert a **70 µm cell strainer** into each of them.

12.1 Use a 25 mL serological pipette to fill each cell strainer equally (two-thirds full at a time to minimise blockages) with the tissue-accutase mixture. Triturate the mixture at the top of each cell strainer in-between fills using a **1 mL Pasteur pipette** to encourage the supernatant to pass through into the connected 50 mL tubes.

12.2 If the 70 µm cell strainer becomes blocked, change to a new 70 µm cell strainer and rest the blocked cell strainer in a **Petri dish** to capture residual supernatant.

> Note
>
> Do not fill each cell strainer more than two-thirds its maximum capacity. Each cell strainer should be able to process 7-10 mL tissue-accutase mixture.

12.3 Gently press the remaining supernatant out of the solid debris for ~1 minute using the **plunger of a 10 mL syringe**. After which, rest the cell strainer in the Petri dish (of blocked cell strainers) to capture residual supernatant.

12.4 Use a new **1 mL Pasteur pipette** to draw up all of the residual supernatant that has drained into the Petri dish (at steps 12.2 and 12.3) from the cell strainers and transfer into the 50 mL tubes they were connected to.

12.5 Return each cell strainer (still containing tissue debris) to their connected 50 mL tube and use a 10 mL serological pipette to add fresh DPBS (no $CaCl_2$, no $MgCl_2$). Allow to wash over the tissue debris and drain through the cell strainers; distribute the DPBS volume so that a total of 20 mL supernatant is captured at each 50 mL tube.

13 Uncap two new 50 mL centrifuge tubes (inserted into a tube rack) and insert a **40 µm cell strainer** into each of them. Transfer 20 mL supernatant (from step 12.5) into each of these cell strainers.

13.1 Use a **1 mL Pasteur pipette** to gently (<10 times) triturate any suspension trapped at the top of each 40 µm cell strainer (and not passing through the strainer by gently agitating back and forth). Typically, <1 mL may remain trapped in the cell strainer; if >1 mL remains trapped, use the Pasteur pipette to transfer into a new 40 µm cell strainer.

13.2 Keep the 2 x ~20 mL supernatants (i.e. suspensions of single cells) separate; 1 x ~20 mL for treatment with ACK lysing buffer (scRNA-seq), 1 x ~20 mL for Histopaque-1077 layering (single cell proteomics).

Preparation of single cells for use with Chromium Controller and preservation in CryoStor reagent.

14 Gently swirl the securely capped 20 mL **Histopaque-1077** aliquot (equilibrated to room temperature in biosafety cabinet) to coat the inside wall of its tube with the reagent.

14.1 Use a 10 mL serological pipette, with low speed setting at the pipette controller, to gently layer 1 × 20 mL cell suspension (from step 13.2) on top of the 20 mL Histopaque-1077 reagent (i.e. 1:1 volume). To layer, hold the tube of 20 mL Histopaque-1077 at a ~45^{o} angle (resting the base of the tube on the work surface), rest the serological pipette tip on the inner wall of the tube (at the side in closest distance to the surface of the Histopaque-1077) and maintain a slow steady flow of the cell suspension onto the reagent, whilst always keeping the pipette tip above the cell suspension layered onto the reagent, until all 20 mL of the cell suspension has been dispensed.

14.2 ⊕ Gently tilt the 50 mL tube back into a vertical position without disturbing the layers of cell suspension and Histopaque-1077, cap securely, and centrifuge at **400 g for 20 minutes at 20^{o}C with acceleration and brake speeds both at LOW settings**, which will complete in >20 minutes.

400 x g, 20°C, 00:20:00 , setting '3' acceleration and '0' brake speeds for the Eppendorf 5804R centrifuge; single cell proteomics

15 *After starting centrifugation of Histopaque-layered cells - prepare cells allocated to scRNA-seq:* securely cap and centrifuge the remaining 20 mL cell suspension (in the second centrifuge) at **400 g for 10 minutes at 20^{o}C**.

400 x g, 20°C, 00:10:00 , maximum acceleration and brake speeds; scRNA-seq

15.1 ⊕ *During both centrifugations at step 15:* transfer Chromium kit items from -20^{o}C (reagents) and -80^{o}C (gel beads) storage onto wet ice; see 10x Genomics CG000315 (Rev C) manual, page 25.

15.2 *Immediately after centrifugation at the cell suspension allocated to scRNA-seq has ended (and while the centrifugation of Histopaque-layered cells is still in progress):* aspirate the supernatant (without disturbing the pellet) in the tube of cells allocated to scRNA-seq within the biosafety cabinet, dispose this supernatant into Virkon waste container, and gently resuspend the pellet in **1 mL ACK lysing buffer per ~2g tissue** (that was used to isolate the cells) to incubate in the biosafety cabinet for **5 minutes at room temperature**.

15.3 *While Histopaque-layered cells are still in the centrifuge:* securely cap and start centrifugation of the cells resuspended in ACK lysing buffer at **400 g for 10 minutes at 20^{o}C**.

400 x g, 20°C, 00:10:00 , maximum acceleration and brake speeds; scRNA-seq

16 *After starting centrifugation of cells in ACK lysing buffer - isolate cells from Histopaque layering:* use a new 1 mL Pasteur pipette to carefully transfer **~12 mL interface layer**, which appears as a ~3 mm thin cloudy-white layer between the upper (blood-stained DPBS) and lower (clear Histopaque-1077) layers, into a new 50 mL centrifuge tube standing in the tube rack.

Note

Position the tip of the Pasteur pipette at the base of the DPBS layer (and top of the interface layer) when drawing up the interface layer into the pipette. As the interface layer is retrieved, the boundary between the DPBS and Histopaque-1077 layers becomes visibly sharper (i.e. better defined).

16.1 Use a new 1 mL Pasteur pipette to transfer the **~8 mL lower (Histopaque-1077) layer**, which also will contain cells (albeit different proportions of each cell type when compared to the interface layer), into a separate 50 mL centrifuge tube.

Note

The interface layer of cells is used for single cell proteomics. The lower (clear Histopaque-1077) layer is collected to create a balance tube for the interface layer of cells and worth storing for future analysis because it also contains cells that may be of interest.

16.2 *While cells in ACK lysing buffer are still in the centrifuge:* Add fresh DPBS (no $CaCl_2$, no $MgCl_2$) to the 50 mL centrifuge tubes of retrieved interface and lower layers of cells to make up to a final suspension volume of 35 mL, securely cap, and centrifuge both tubes at **400 g for 10 minutes at 20°C**.

400 x g, 20°C, 00:10:00 , maximum acceleration and brake speeds; single cell proteomics

17 *After starting centrifugation of interface and lower (Histopaque-1077) layer cells - isolate cells following ACK lysis buffer treatment:* aspirate the supernatant (without disturbing the pellet) in the ACK lysis buffer-treated cells tube within the biosafety cabinet; dispose this supernatant into the Virkon waste container.

17.1 Gently resuspend the pellet in 0.5-1 mL fresh DPBS (no $CaCl_2$, no $MgCl_2$) and briefly agitate the cell suspension to dissociate clumps.

17.2 Use a micropipette to retrieve 10 µL of the resuspended cells for manual **Trypan blue cell counting** using the haemocytometer - calculate % viability and concentration of cells in the suspension.

18 After centrifugation at step 16.2 has ended, aspirate the supernatant (without disturbing the pellet) in both the interface and lower (Histopaque-1077) layer cells tubes within the biosafety cabinet; dispose this supernatant into Virkon waste container.

18.1 Resuspend the interface and lower layer cell pellets in fresh DPBS (no $CaCl_2$, no $MgCl_2$) at 5 mL and 1 mL volume, respectively; leave to rest in the biosafety cabinet (tubes capped) until step 20.

19 Use manual Trypan blue cell counting values from step 17 to calculate the volume of MACS Dead Cell Removal Kit MicroBeads needed to obtain a suspension of **100 µL MicroBeads per 1×10^7 all (i.e. live and dead combined) cells - i.e. 1×10^5 cells/µL concentration**; see section 2.4 of MACS Dead Cell Removal kit manual.

19.1 ⊕ Add 3 mL fresh DPBS (no $CaCl_2$, no $MgCl_2$) to the 0.5-1 mL cell suspension (from step 17), securely cap, and centrifuge at **300 g for 10 minutes at 20°C**.

300 x g, 20°C, 00:10:00 , maximum acceleration and brake speeds; scRNA-seq

20 *After starting centrifugation of the cells to be used with the MACS Dead Cell Removal kit*: briefly agitate the cells isolated by Histopaque-1077 layering and resuspended in DPBS (from step 18) to dissociate clumps, and use a micropipette to retrieve 10 µL (per cell suspension) for manual **Trypan blue manual cell counting** using the haemocytometer - calculate % viability and concentration of cells in each suspension.

21 After centrifugation at step 19.1 has ended, aspirate the supernatant (without disturbing the pellet) in the tube of cells in DPBS (after ACK lysis buffer treatment) within the biosafety cabinet; dispose this supernatant into Virkon waste container.

21.1 Turn the light off in the biosafety cabinet, transfer the MACS Dead Cell Removal kit vial of **MicroBeads (light-sensitive)** from 2-4^{o}C storage into the biosafety cabinet, resuspend the cell pellet with the volume of MicroBeads calculated at step 19.0.

21.2 ⊕ Incubate the cell suspension + MicroBeads mixture in the dark biosafety cabinet for **15 minutes at room temperature**.

21.3 Transfer the MACS Dead Cell Removal kit vial of **20x Binding Buffer** from 2-4^{o}C storage into the biosafety cabinet. Prepare **25 mL of 1x Binding Buffer** in a Universal tube (30 mL capacity) using sterile and nuclease-free water; see section 2.3 of MACS Dead Cell Removal kit manual.

22 *During 15-minute incubation of scRNA-seq cells in MicroBeads*: add DPBS to the lower (clear Histopaque-1077) layer of cells to obtain a cell suspension volume that is equal to that of the interface layer of cells; for balance in the centrifuge. Securely cap and centrifuge both tubes at **400 g for 10 minutes at 20^{o}C**.

400 x g, 20°C, 00:10:00 , maximum acceleration and brake speeds; single cell proteomics

23 *After starting centrifugation of interface and lower (clear Histopaque-1077) layers of cells - setup the LS column for enrichment of live cells for scRNA-seq (as guided by the MACS Dead Cell Removal kit manual)*: Attach an **LS column** to the MidiMACS Separator on the MACS MultiStand (magnetic stand). Place a Bijou container under the column and add 3 mL of 1x Binding Buffer (prepared at step 21.3) to the column for its first wash.

23.1 Add **1x Binding Buffer** to the mixture of cell suspension + MicroBeads (from step 21.1) to make up to a total volume of 1 mL (if not already 1 mL).

23.2 Label **2 × 15 mL centrifuge tubes** to be used to capture the LIVE (1 tube) and DEAD (1 tube) fractions from the LS column, and fill both tubes with 1 mL of 1x Binding Buffer each.

23.3 Load all of the 1 mL cell suspension + MicroBeads mixture into the LS column and confirm the flow-through is being captured by the 'LIVE' labelled 15 mL centrifuge tube.

23.4 Proceed to the 1x Binding Buffer wash steps, as instructed in the kit manual, during the next steps of the current protocol; flow rate should be ~3 minutes per 3 mL wash.

24 *After centrifugation at step 22 has ended and while the cell suspension allocated to scRNA-seq continues to be processed through the LS column*: aspirate the supernatant (without disturbing the pellet) in both tubes derived from Histopaque-1077 layering **until only ~50-100 µL is left at each pellet** in the biosafety cabinet; dispose the supernatant in the serological pipette into Virkon waste container.

24.1 Use a 1 mL micropipette tip to carefully remove the remaining ~50-100 µL supernatant at each pellet before placing the 15 mL centrifuge tubes on wet ice.

Note

This step can be undertaken on a laboratory bench outside of the biosafety cabinet, along with the rest of step 24's sub-steps.

24.2 Use a new 1 mL micropipette tip to resuspend each pellet in pre-chilled (on wet ice) **CryoStor CS10** reagent at a volume that will equate to **≥1 million cells per cryovial**; use the manual Trypan blue cell counting values from step 20.

24.3 ⊕ Once resuspended in CryoStor CS10 reagent, transfer the cell suspensions into their pre-labelled and pre-chilled (on wet ice) cryovials, and **incubate on wet ice for 10 minutes**.

25 *During 10-minute incubation of CryoStor cell suspensions on wet ice*: After the last wash step at the LS column, move the 15 mL centrifuge tube containing the flow-through (i.e. **LIVE cells fraction**) from under the column and securely cap the tube; leave in the tube rack.

25.1 Uncap the 'DEAD' labelled 15 mL centrifuge tube, insert the LS column detached from the MidiMACS Separator, add 5 mL of 1x Binding Buffer to the LS column, and use its plunger to expel the **DEAD cells fraction** into the connected 15 mL centrifuge tube. Cap the 15 mL tube to leave in the tube rack and dispose the LS column afterwards.

25.2 Securely cap and centrifuge both 15 mL centrifuge tubes of cell suspensions (from the LS column) at **400 g for 10 minutes at 20°C**.

400 x g, 20°C, 00:10:00 , maximum acceleration and brake speeds; single cell proteomics

26 ⊕ *After starting centrifugation of LIVE and DEAD cells fractions*: Once 10-minute incubation of CryoStor cell suspensions has completed, transfer their cryovials into the **cell freezing container** and store in a -80°C freezer overnight before proceeding to step 34.

27 ⊕ Transfer Chromium kit reagents that have been on wet ice (since step 15.1) into a tube rack on the bench to equilibrate to room temperature for 30 minutes; see 10x Genomics manual CG000315 (Rev C) manual, page 25. **Confirm the Template Switch Oligo has been resuspended in Low TE buffer**.

28 After centrifugation at step 25.2 has ended, aspirate supernatant (without disturbing the pellet) in both the LIVE and DEAD cells fraction tubes in the biosafety cabinet; dispose the supernatant in the serological pipette into Virkon waste container.

28.1 Resuspend each cell pellet with ~300 µL Chromium Resuspension Buffer.

28.2 Use a micropipette to retrieve 10 µL (per cell suspension) for manual **Trypan blue manual cell counting** of both the LIVE (and DEAD) cells fractions, now in Chromium Resuspension Buffer, using the haemocytometer - calculate % viability and concentration of cells in each suspension.

Note

Do not proceed with use of the Chromium Controller if <80% viable cells present in the LIVE fraction. Instead, it is advisable to undertake Trypan blue cell counting of the DEAD cells fraction to determine (for future reference) whether the low % viability was due to inefficiencies at the column-based live cell enrichment part of this protocol.

Use Chromium Controller with LIVE fraction cells in Chromium Resuspension Buffer for GEM generation & barcoding.

29 Decontaminate micropipettes, tubes and other wipeable equipment/plasticware with **RNaseZap**.

30 Using the Trypan blue manual cell count for the LIVE cells fraction (from step 28.2), calculate the volume of additional Chromium Resuspension Buffer needed to adjust the concentration of cells to **1000 cells/µL** (or any concentration within a range of 700-1200 cells/µL; see 10x Genomics CG000315 (Rev C) manual, page 27. Make the calculated volume adjustment in the biosafety cabinet.

31 ⊕ Securely cap the LIVE cells fraction tube and promptly transfer it to wet ice on the Chromium Controller bench.

32 ⊕ Promptly proceed to following the protocol for **Chromium Step 1 (GEM Generation & Barcoding)** at pages 26-31 (confirm all items at page 25 are ready) of the 10x Genomics CG000315 (Rev C) manual.

33 ⊕ Store the PCR tube of **GEM-RT incubation product** in the -20^{o}C freezer for ≤1 week.

Note

After which, proceed with the remaining steps described in the 10x Genomics CG000315 (Rev C) manual for construction of cDNA sequencing libraries.

34 ⊕ **Transfer cryovials of CryoStor preserved cells from the cell freezing container (after overnight incubation in a -80^{o}C freezer) into a liquid nitrogen storage vessel for long term storage.**

Note

It is advised that CryoStor preserved cells are stored in liquid nitrogen for up to 4-6 weeks prior to processing with a fluorescence-activated cell sorter for optimal quality of recovered cells.

Protocol references

Photographs for isolation of decidua parietalis from the chorion can be found in the following publication:

Alexander T H Cocker, Emily M Whettlock, Brendan Browne, Pei F Lai, Jonathan K H Li, Sivatharjini P Sivarajasingam, Nesrina Imami, Mark R Johnson, Victoria Male, Isolation of single cells from human uterus in the third trimester of pregnancy: myometrium, decidua, amnion and chorion, *Oxford Open Immunology*, Volume 3, Issue 1, 2022, iqac010, https://doi.org/10.1093/oxfimm/iqac010