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OCT embedding (for spatial transcriptomics) and flash freezing (for single nucleus RNA-seq) of human myometrial and decidual tissues

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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 storing human myometrial and decidual tissues (obtained at Caesarean section) to later analyse using spatial transcriptomics (10x Genomics Visium platform) and single nucleus RNA-seq (10x Genomics Chromium platform).

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.

Materials

- Quantities of items listed in Materials lists are the recommended minimum to collect and store all three types of tissue samples (lower segment myometrium, upper segment (sub-parietalis region) myometrium-decidua, and decidua parietalis) per study participant. **It is highly recommended that spare items are made readily available**, especially for items that are part of pack P1.
- Items that can be pre-packed together are marked '**P1**' (for Part A; OCT embedding) and '**P2**' (for Part B; flash freezing in liquid nitrogen) - these packs should be topped up in advance of sample collections.
- Items that are single-use consumables to be UV-irradiated and sealed in ziplock bags can be stored (for topping-up packs P1 and P2) in packs (storage boxes) marked '**B2**' and '**B3**', along with pre-labelled (barcoded) cryovials.

	Item	Pack #	Quantity	Supplier	Product number	Note
	Insulated (Thermos) flask, 0.47 L capacity	P1	1	n/a	n/a	Fits at least 2 × 15 mL DPBS aliquots (one for each type of myometrial biopsy - i.e. upper and lower segment); to be taken into operating theatre
	Cooling gel pack	P1	1	n/a	n/a	Fits the insulated flask alongside 2 × 15 mL (in Universal tubes; 30 mL capacity each) DPBS aliquots; store in fridge
	Watchmaker forceps	P2 & P1	3	World Precision Instruments	501979-6	2x in P1, 1x in P2
	Blunt end 'filter' forceps	P1	1	Sigma-Aldrich	XX6200006P	
	Tissue dissection scissors	P2 & P1	2	World Precision Instruments	14393	1x in P1, 1x in P2
	Long reach blunt end forceps	P1	1	Fisher Scientific	12750916	
	Pocket microscope	P1	1	Carson	MM-450	
	Safety glasses	P1	1	n/a	n/a	For handling of isopentane; follow local safety procedures
	Plastic beaker, 100 mL capacity	P1	1	Scientific Laboratory Supplies	BEA5026	

	Item	Pack #	Quantity	Supplier	Product number	Note
	Stainless steel mortar and pestle set	P1	1	n/a	n/a	150-200 mL capacity mortar
	Stainless steel medicine cup, 30 mL capacity	P1	1	Fisher Scientific	15809721	Fits inside mortar whilst surrounded by powdered dry ice
	Plastic ice bucket, square and 1 L capacity	P1	1	VWR	11324085	To store dry ice with mortar and pestle for use at designated work area for OCT embedding
	Plastic ice bucket, rectangular and 4 L capacity	n/a	1	Fisher Scientific	11334085	To store wet ice for use at designated work area for OCT embedding
	Polystyrene box, 4 L capacity	n/a	1	n/a	n/a	To store wet ice for use in laboratory
	Dilvac dewar flask, (minimum) 1 L capacity	n/a	1	Scientific Laboratory Supplies	DEW1010	To store liquid nitrogen for use in laboratory
	Sponge-holding forceps; Rampley	n/a	1	Medisave	7969	

List of equipment. Pack P1 to be used in designated work area near operating theatre of labour ward (for collection of all tissues and OCT embedding of upper segment myometrial-decidual biopsies; Part A of protocol) and P2 to be used in laboratory (for flash freezing tissues using liquid nitrogen; Part B of protocol).

	Item	Pack #	Quantity	Supplier	Product number	Note
	Sterile-packed hand towel	P1	1 (+1 spare)	Bastos Viegas	4561-500	Scrub nurse will need to handle in order to prepare saline-soaked paper towel for placement of upper segment biopsies to be transferred from surgeon to researcher
	6 mm biopsy punch; Stiefel	P1	1 (+1 spare)	Medisave	BI2000	To be given to surgeon by scrub nurse for acquisition of upper segment myometrial-decidual biopsies - they will also need fine surgical scissors and forceps for retrieval of biopsies after each

	Item	Pack #	Quantity	Supplier	Product number	Note
						use of the biopsy punch
	Peel-A-Way 'truncated' OCT embedding moulds, 8 x 8 × 20 mm dimension	P1 & B2	2 (+2 spare)	VWR	15160-099	Each mould needs to be cleaned with RNAseZap, labelled (see note below), and UV-irradiated alongside the ziplock bag in which they are to be sealed in pairs prior to storing in packs P1 and B2
	Tissue cryovials, 5 mL capacity	P1 & B2	2 (+2 spare) pre-labelled, along with ~1 bag of unlabelled (as spares)	Fisher Scientific	15173109	Label (each with barcode) prior to sealing them in pairs, each pair in a ziplock bag prior to storing in packs P1 & B2
	Sterile-packed scalpels, #22 blade	P2 & P1	3 (+2 spare)	Appleton Woods	MP015	1x in P1, 2x in P2; also 1x spare in each pack
	Cell scraper	P1	1 (+1 spare)	Sarstedt	83.3590	
	Filter paper	P2 & P1	1 (+1 spare) pack	Fisher Scientific	11724183	
	Petri dishes, 10 cm diameter	P1 & B3	3 (+3 spare)	Greiner	632181	Keep sterile and UV irradiate alongside ziplock bags in which they are to be sealed 3 per bag prior to storing in packs P2 and B3
	Cryovials with external thread caps, 2 mL capacity	P2 & B2	8	Starlab	E3090-6222	Label (each with barcode) prior to storing in packs P1 & B2
	Placenta clinical disposal bags	n/a	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. Pack P1 to be used in designated work area near operating theatre of labour ward (for collection of all tissues and OCT embedding of upper segment myometrial-decidual biopsies; Part A of protocol) and P2 to be used in laboratory (for flash freezing tissues using liquid nitrogen; Part B of protocol). Packs B2 and B3 to be used in laboratory to top-up P1.

Note

For storing frozen OCT tissue blocks from 8 x 8 x 20 mm cryomoulds into 5 mL capacity cryovials, a fill limit of 0.5 cm height from the base (blunt apex) of each cryomould will need to be indicated by labelling this position using a permanent marker after cleaning them with RNAseZap and before UV-irradiation alongside their ziplock bag. Additionally, it is **important to also label each cryomould with 'M' (myometrium) and 'D' (decidua) on opposite walls** to indicate orientation of the upper segment biopsy to be OCT-embedded in them.

	Item	Pack #	Quantity	Supplier	Product number	Note
	Clinical grade saline (injection packs)	n/a	1 (+1 spare) x 5-10 mL packs			Scrub nurse will need to handle in order to prepare saline-soaked paper towel for placement of upper segment biopsies to be transferred from surgeon to researcher
	Optimal cutting temperature (OCT) compound	P1	1	VWR	SIFAAGR1180	*Alternative supplier: Labtech, product number 16-004004*
	Isopentane	n/a	~100 mL	Sigma-Aldrich	M32631	Store in 100 mL Duran bottle (refrigerated / chill on wet ice prior to use); follow local safety procedures
	Dulbecco's phosphate-buffered saline (DPBS; with CaCl2 and MgCl2)	n/a	4 (+1 spare) x 15 mL aliquots, 1 (+1 spare) x 50 mL aliquot	Sigma-Aldrich	D8662	15 mL aliquots in Universal tubes (30 mL capacity; Greiner 201151), 50 mL aliquots in skirted centrifuge tubes (50 mL capacity; Greiner 210261)
	Wet ice	n/a				See 'List of equipment' for storage container
	Dry ice	n/a				See 'List of equipment' for storage container; follow local safety procedures
	Liquid nitrogen	n/a				See 'List of equipment' for storage container; confirm availability of

	Item	Pack #	Quantity	Supplier	Product number	Note
						personal protective equipment prior to use, and follow local safety procedures
	Ethanol (absolute)	P1	~25 mL	VWR	20821.330	To use for cleaning metal instruments (along with Clinell disinfectant wipes; Medisave, product number CW200) prior to use for tissue handling
	Virkon disinfectant	n/a	variable	VWR	115-0021	To use as part of disposal of human tissue-containing waste in laboratory; follow local safety procedures
	RNaseZap	n/a	variable	Sigma-Aldrich	R2020	For cleaning OCT-embedding moulds prior to UV-irradiation and sealing into ziplock bags

List of chemicals. Pack P1 to be used in designated work area near operating theatre of labour ward (for collection of all tissues and OCT embedding of upper segment myometrial-decidual biopsies) and items can be stored in P1 in advance.

Other items (in addition to personal protective equipment)

- permanent marker pens (for labelling e.g. DPBS aliquot tubes)
- 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
- 1x foldable crate trolley (Office Depot, product number 4076007; to transport items from laboratory to designated work area near operating theatre of labour ward)
- Disinfectant wipes (e.g. Clinell)
- paper towels
- plastic clinical waste sharps bins

Safety warnings

In addition to assessing risks (**biohazards**) for the handling of human-derived tissues and biofluids, please seek local health and safety guidance for use of the following **hazardous chemicals**: liquid nitrogen, isopentane, dry ice.

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 responsible for hosting the study with regards to regulations to abide to 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 C-section (on day of sample collection) - confirm the following:

1. There is space available in -80^{O}C cryoboxes to store the upcoming set of tissue samples.
2. Cryovials for OCT-embedded (5.0 mL capacity; 2x for upper segment) and flash frozen (2.0 mL capacity; 2x for upper segment, 3x for lower segment, 3x for decidua parietalis) tissues have been pre-labelled (with barcodes) for sample tracking.
3. Tissue dissection bench has been disinfected (by wiping with e.g. Distel) and the items listed in **Table I** (for flash freezing tissues in liquid nitrogen) are present.
4. Fill the Dilvac flask (at least to half capacity) with liquid nitrogen and the polystryrene box with wet ice (to full capacity) ideally ~1-2 hours before C-section.
5. Confirm the items listed in **Table II** (for OCT-embedding of upper segment biopsies and collection of all three tissue types into DPBS to transport to laboratory) are present in pack P1 to take to designated work area (using the foldable crate trolley - line with a plastic bag to capture accidental spillage) as close as permissible to the C-section operating theatre.
6. 1 (+1 spare) x 50 mL DPBS aliquot has been set aside in fridge for washing tissues (to be flash frozen in liquid nitrogen) in laboratory.

	Item on tissue dissection bench
	1x Dilvac flask (for liquid nitrogen) and personal protective equipment (face shield and cryogen safety gloves)
	1x sponge-holding forceps (Rampley)
	1x Watchmaker forceps
	1x blunt end (filter) forceps
	1x tissue dissection scissors
	2 (+1 spare) x sterile-packed #22 blade scalpels
	1x polystyrene box (4 L capacity; for wet ice)

Table I. Items to make available (before C-section commences) at tissue dissection bench in laboratory for tissues to be flash frozen in liquid nitrogen prior to -80^{O}C storage.

	Item on work area for OCT-embedding and collection of tissues to transport to laboratory
	1x insulated flask with cooled gel pack and 2 × 15 mL DPBS aliquots
	# 1x plastic ice bucket (4 L capacity) with wet ice
	# On wet ice (in plastic bag): 1 × 100 mL Duran bottle of isopentane

	Item on work area for OCT-embedding and collection of tissues to transport to laboratory
	# On wet ice: 2 (+1 spare) x 15 mL DPBS aliquots
	# 1x plastic ice bucket (1 L capacity; loosely wrapped in a plastic bag to capture accidental spillage) - dry ice pellets outside of mortar, powdered dry ice (prepared using mortar / 100 mL plastic beaker and pestle) inside mortar and around medicine cup
	1 (+1 spare to also take into operating theatre) x sterile-packed tissue hand towels*
	1 (+1 spare to also take into operating theatre) x sterile-packed 6 mm biopsy punch*
	1 (+1 spare to also take into operating theatre) sterile (injection pack) saline*
	# ~25 mL ethanol (in 30 mL Universal tube)
	# 1x pocket microscope
	# 2x Watchmaker forceps
	# 1x tissue dissection scissors
	# 1 (+1 spare) cell scraper
	# 1 (+1 spare) pack of 3x UV-irradiated 10 cm diameter Petri dishes
	# 1 (+1 spare) pack of 2x RNAseZap-cleaned and UV-irradiated OCT-embedding moulds
	# 1 pack of 2x pre-labelled (barcoded) 5.0 mL capacity cryovials
	~1 bag of unlabelled 5.0 mL capacity cryovials
	# 1x pack of filter paper (+ 1 spare pack to keep in P1)
	# 1x long reach blunt end forceps
	# 1 (+1 spare) x sterile-packed #22 blade scalpel
	# OCT compound (1 bottle)
	2x permanent marker pens
	1x ballpoint pen
	Sample collection proforma (for noting down timings indicated in protocol)
	1x digital watch (confirm correct time shown in hh:mm)

Table II. Items to make available (before C-section commences) at designated work area as close as permissible to operating theatre; listed in Materials as part of pack P1 unless not stored at room temperature. Note that the dry ice bucket with dry ice, mortar and medicine cup can be prepared up to 2 days in advance of the C-section and stored within a polystyrene box (whilst wrapped loosely in a plastic bag) in a -80^{O}C freezer; dry ice will need to be refreshed every 2 days if not used and also after use. One of each item marked with '*' need to be given to scrub nurse prior to commencement of C-section. Items marked with '#' are to be placed on the work surface designated for OCT embedding of upper segment biopsies and isolation of decidua parietalis tissue.

Preparation at labour ward and closest permissable work area before commencement of C-section (on day of sample collection):

1. Confirm written consent to study participation has been obtained from participant, and the surgeon is aware that they have consented to upper and lower segment myometrial biopsies.
2. Provide the attending scrub nurse with sterile-packed 6 mm biopsy punch, sterile-packed paper towel (do NOT use gauze instead - this material is more likely to damage the decidual layer on the upper segment biopsies), and (injection pack) saline; the researcher is to hold onto the spare for each of these three items while in the operating theatre. Confirm they are aware that they need to (i) provide the surgeon with the biopsy punch after fetal delivery, and (ii) soak the paper towel with saline prior to placing the punch biopsies on it.
3. 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 lab after research samples have been acquired.
4. In the designated work area, where tissues will be OCT embedded, cover a clean (disinfected) dry work surface with paper towels to lay down the items listed in **Table II** that are marked with '#'.
5. Wipe all forceps and dissection scissors with a clinical disinfectant (e.g. Clinell) wipe.
6. Pre-chill both pre-labelled (barcoded) 5.0 mL capacity cryovials on the dry ice pellets (beside the mortar) in the 1 L plastic ice bucket.
7. Wrap one of the UV-irradiated Petri dishes in either a clean ziplock bag of large nitrile glove to take into theatre, along with the insulated flask still containing the cooling gel pack and 2 × 15 mL DPBS aliquots (one labelled for each type of myometrial biopsy to be obtained in theatre); confirm the items handed to the scrub nurse at step 2 are ready on the theatre instruments trolley.
8. Researcher is to hold on to the spares of a 6 mm biopsy punch tool, sterile-packed paper towel and (injection pack) saline in theatre before and during the C-section.

Acquisition of upper and lower uterine segment biopsies at C-section.

1 Excise **upper uterine segment (sub-parietalis region) biopsies** immediately after fetal and placental delivery:

- ⊕ Record times of excising first and last upper segment (myometrial-decidual) biopsies from the uterus.
- Surgeon will need to use a 6 mm biopsy punch, along with fine surgical scissors (to detach each biopsy from the uterus after inserting the punch) and forceps (to hold the biopsy at the myometrial 'stalk' while detaching from the uterus).
- A total of six upper segment punch biopsies per study participant is advised, where at least two of these biopsies are 'T-shaped' (the blunt end being the decidual end, the 'stalk' being the myometrial end) for OCT embedding.
- Biopsies needs to be retrieved from a site of the uterus that is as close to the opposite side of where the placenta was implanted as possible; researcher needs to confirm with surgeon the site of placenta implantation at the uterus at time of C-section.
- Swabbing the area of the uterus where biopsies are to be retrieved ideally should be kept to a minimum to ensure decidua is captured along with myometrium at each punch biopsy.

2 Place upper segment biopsies onto the **sterile saline-soaked paper towel** for transfer from surgeon to researcher *via* scrub nurse.

- Upon retrieval of the paper towel with punch biopsies, the researcher needs to gently fold the paper towel (to cover all the biopsies) and insert into the Petri dish, which is to be taken out of the operating theatre once the lower segment myometrial biopsy has also been acquired.

3 Excise **lower uterine segment biopsy** from the uterus immediately after retrieval of all upper segment biopsies.

- ⊕ Record time of excising the lower segment (myometrial) biopsy from the uterus.
- Surgeon will need to use surgical scissors to cut a ~2 × 1 cm biopsy from the upper edge of the C-section incision made to the lower uterine segment.

4 Transfer lower segment biopsy into **1 × 15 mL cold (pre-chilled in insulated flask with gel pack) sterile DPBS aliquot**.

- Surgeon will need to pass the biopsy to the scrub nurse for them to transfer into the DPBS aliquot (tube held by the researcher) using forceps; tube to be securely capped, put back into the insulated flask, and immediately taken out of the operating theatre with the upper segment biopsies to be chilled on wet ice in the designated work area for Part A of this protocol.

Part A: OCT embed upper uterine segment biopsies using isopentane-dry ice bath for -80°C storage.

5 **Chill, orientate and wash in DPBS.**

5.1 ⊕ Immediately chill the Petri dish base containing the upper segment punch biopsies on wet ice; be careful to ensure all of the saline-soaked paper towel is inside the Petri dish and not touching the ice. Place the Petri dish lid upside down onto wet ice (to be used from step 5.7).

5.2 Promptly transfer the 15 mL DPBS aliquot tube containing the lower segment myometrium from the insulated flask onto wet ice.

5.3 Carefully hold the paper towel with punch biopsies outside of the Petri dish. Use a pair of Watchmaker forceps (wiped clean with ethanol to remove residual disinfectant) to transfer the punch biopsies from the paper towel into the Petri dish base chilled on wet ice; dispose the paper towel after all punch biopsies are in the Petri dish.

5.4 Immediately add the 15 mL DPBS aliquot remaining in the insulated flask to the Petri dish to immerse the punch biopsies.

5.5 Fill the 30 mL medicine cup (on powered dry ice in mortar) with ~20 mL pre-chilled (on wet ice) isopentane.

> Note
>
> Remove any large amounts of (powdered) dry ice inside the medicine cup before filling with isopentane. This can help to minimise the isopentane seeping from the cup into the surrounding dry ice in the mortar and lowering the volume available for OCT freezing.

5.6 Wipe clean the dissection scissors and long reach (blunt end) forceps with ethanol to remove residual disinfectant.

5.7 Unpack 2 x OCT embedding moulds from their ziplock bag and check they are intact (i.e. the sides have not split open and thus remain leakproof). Orientate them on the Petri dish lid (resting on wet ice from step 5.1) so that their 'D' (for decidua) labelled side is facing left and 'M' (for myometrium) labelled side is facing right; this orientation will be referred to as **D-M orientation** from hereon.

5.8 Push air out of the nozel of the OCT compound bottle and add a droplet to the Petri dish lid before immediately (while not allowing air to go back into the nozel) also adding OCT

compound to the inside of both moulds up to approximately one-third below the pen-marked 0.5 cm from apex maximum fill line. **Avoid adding air bubbles to the OCT in both moulds** - use Watchmaker forceps to sweep any air bubbles onto the inner walls of the mould, away from where the punch biopsies will be placed for embedding.

5.9 Orientate two punch biopsies in the ice-cold DPBS so that their decidual and myometrial ends are identically aligned (to confirm these ends of the each punch biopsy are easy to identify).

5.10 Use the flat side of a scalpel blade, which will also be used at step 7.4 (so keep the plastic packaging to rest the scalpel in after the current step), to gently press onto each punch biopsy to squeeze out any loosely bound blood; this can help to improve visibility of the myometrial-decidual interface. Also use the scalpel to trim off any frayed tissue and use the Watchmaker forceps to remove any blood clots.

6 Dab off excess DPBS and immerse in OCT compound.

6.1 Place two sheets of filter paper onto dry paper towels next to the wet ice bucket.

6.2 Use the Watchmaker forceps to transfer one of the two punch biopsies (selected for OCT embedding at step 5.9) onto the double layer of filter paper to **gently dab off excess DPBS**. Do not lose track of its D-M orientation - if unsure, use the pocket microscope (wiped clean with disinfectant wipe and dried with paper towel) to check; confirm whether the view is inverted by looking for the position of the Watchmaker forceps when used to hold (ideally the myometrial end of) the biopsy while on the filter paper.

6.3 Use the Watchmaker forceps to promptly transfer the dabbed punch biopsy from the filter paper onto the droplet of OCT compound on the Petri dish lid (from step 5.8), whilst maintaining the D-M orientation, to coat one side of the biopsy.

6.4 Use the Watchmaker forceps to gently transfer the punch biopsy from the OCT compound droplet (on the Petri dish lid) into the first OCT-filled mould, with the OCT compound-coated side of the punch biopsy facing down and into direct contact with the OCT compound within the mould - ensure that the **D-M orientation of the punch biopsy matches the 'D' and 'M' labels on the mould** and point the decidual end of the punch biopsy towards the lower corner of the 'D' labelled side.

6.5 Push air out of the nozel of the OCT compound bottle and immediately (to minimise the punch biopsy sinking towards the bottom of the mould) add OCT compound to the top of the punch biopsy in the mould up to the pen-marked maximum fill level.

6.6 Ensure the punch **biopsy is completely covered with OCT compound** and there are **no air bubbles** close to it - gently push any air bubbles towards to the inner walls of the

mould using the Watchmaker forceps (whilst still not allowing the punch biopsy to sink too close towards the bottom of the mould).

7 **Freeze OCT compound.**

7.1 Use the long reach (blunt end) forceps to hold the mould containing the punch biopsy in the medicine cup on powdered dry ice so that it is half-immersed in isopentane (chilled from step 5.5) until the OCT compound has frozen solid (i.e. changed colour from clear to white), which should take approximately 2 minutes. **Do not release the forceps** from the mould and avoid allowing isopentane to enter the mould.

7.2 ⊕ Once the OCT compound at the punch biopsy has completely frozen solid, continue to hold the mould with the long reach forceps to transfer it onto the dry ice pellets surrounding the outside of the mortar. **Do not allow the OCT to thaw** from here onwards.

7.3 **Repeat from step 6.2 to 7.2** with the remaining one of two punch biopsies (still in DPBS on wet ice) selected for OCT embedding using the second mould filled with OCT compound.

7.4 Once both punch biopsies have been frozen in OCT, use the scalpel (from step 5.10; wipe clean with ethanol) to **chip off the upper corner of frozen OCT at the 'D' labelled side** of each mould to help indicate which side of each frozen OCT tissue block has the decidual end of their biopsy pointing towards the it (and under the chipped corner when positioned to the left); doing this will aid cryosectioning. It may be necessary to use the Watchmaker forceps to hold down the frozen OCT tissue block whilst chipping off the upper 'D' corner in case the tissue block slips out of the mould.

8 **De-mould the frozen OCT tissue block and keep on dry ice.**

8.1 Carefully transfer the isopentane in the medicine cup back into its Duran bottle on wet ice and secure the cap. Rest the empty medicine cup on wet ice.

8.2 Uncap the first pre-labelled 5.0 mL capacity cryovial (after checking label matches that identified on the sample collection proforma) and place into the powdered dry ice where the medicine cup was previously positioned. Rest the cap upside down on the dry ice pellets.

8.3 Release the first frozen OCT tissue block into its uncapped cryovial by gently pulling apart the walls of the mould to loosen their grip on the OCT; the mould walls do not necessarily have to snap away from each other.

8.4 Re-attach the cap of the cryovial, which now contains the frozen OCT tissue block, and move onto the dry ice pellets surrounding the outside of the mortar.

8.5 Repeat from step 8.2 to 8.4 for the second frozen OCT tissue block using the remaining pre-labelled 5.0 mL capacity cryovial.

8.6 Once both frozen OCT tissue blocks have been stored into their cryovials, use the Watchmaker forceps (wiped clean with ethanol to remove residual OCT compound) to transfer the remaining punch biopsies into a new 15 mL DPBS aliquot, securely cap, and keep chilled on wet ice alongside the DPBS aliquot tube containing the lower segment biopsy.

8.7 Use the handle of the long reach forceps to loosen the powdered dry ice so that space is made to store both cryovials containing frozen OCT tissue blocks in the mortar.

8.8 Dispose both DPBS in the Petri dish, along with the Petri dish (both base and lid chilled on wet ice) and empty moulds; the scalpel should be disposed into a plastic sharps bin too.

8.9 Cover the dry ice bucket with its lid and put aside ready for transport to the laboratory (once decidua parietalis tissue has been isolated from the chorion and stored into ice-cold DPBS to transport to the laboratory too).

Part B: Flash freeze all tissues in liquid nitrogen for -80°C storage.

9 **Isolate decidua parietalis tissue from chorionic membrane.**

9.1 Collect the placenta with fetal membranes attached (still in kidney dish wrapped with inco pad) once confirmed with the midwife that they have completed their clinical tasks with it.

9.2 ⊕ Separate a ~7 × 7 cm region of choriodecidua from the amnion, then use the tissue dissection scissors to cut the former off and lie it flat in the kidney dish with decidua side (i.e. side that was not in direct contact with the amnion) facing up.

9.3 ***For single cells isolation (see separate 'Single cells isolation from human decidua parietalis tissue to use for Chromium-based scRNA-seq and cryopreserve for single cell proteomics' protocol):*** *transfer the placenta with the rest of the fetal membranes into a clinical (disposal) bag, insert this bag into a second clinical (disposal) bag, and store in a specimen bucket; this needs to be transported to the laboratory after decidua parietalis tissue has been isolated from the chorion and stored into ice-cold DPBS to transport to the laboratory too.*

9.4 Carefully drain away the excess blood from the kidney dish (whilst holding the piece of choriodecidua securely to one side of the dish with the decidua side still facing up) into the clinical bag now containing the placenta.

9.5 Use the cell scraper to scrape off the decidua parietalis from the chorion in the kidney dish. The chorionic membrane will appear translucent (similar in appearance to the amnion) once the decidua parietalis (relatively more greyish-cream (and pink if residual blood is present) in colour than the chorion) has been scraped off it; take care not to tear the chorion and gather the decidua parietalis into a separate area of the dish once sure no chorion is attached.

9.6 Use a pair of Watchmaker forceps (wiped clean with ethanol) to transfer ~1-2 g decidua parietalis (scraped off the chorion) into its designated (labelled with marker pen) fresh 15 mL DPBS aliquot tube, securely cap, and keep chilled on wet ice.

10 **Clear designated (OCT embedding and decidua parietalis sampling) area and transport samples to laboratory.**

10.1 Clean all forceps, dissection scissors, and pocket microscope with disinfectant wipe; dry instruments with clean paper towels before returning to pack P1 along with the rest of its room temperature items that remain on the work surface.

10.2 Wipe dry the cleared work surface with the paper towels that lined it and clean with a disinfectant wipe.

10.3 ⊕ Transport pack P1, along with the dry ice and wet ice buckets (with their contents of tissue samples still chilled and all tubes securely capped), back to the laboratory using the crate trolley; *also transport the* ***placenta with fetal membranes*** *(from step 9.3) if decidua parietalis tissue is also needed for isolation of single cells*. Consider draining water from the wet ice bucket (while contents secured in place with bucket lid) if necessary; secure lids on both ice buckets. Loosely wrap all items together with the plastic bag lining the trolley to capture any accidental spillage while in transit.

10.4 ⊕ Upon arrival at the laboratory, place both ice buckets on the tissue dissection bench and put pack P1 to one side for topping up after completion of the protocol.

10.5 Empty the contents of the mortar into the dry ice bucket still containing the dry ice pellets - ensure both cryovials of frozen OCT tissue blocks are still resting on dry ice in the bucket. Put the empty mortar and medicine cup to one side to clean up (and refill with dry ice) after completion of the protocol.

11 **Prepare tissue dissection bench items before handling tissues for (dissection and) flash freezing in liquid nitrogen.**

11.1 Line the bench with dry paper towels next to the polystyrene box of wet ice together with both plastic ice buckets (from step 10.4).

11.2 Wipe clean the Watchmaker forceps, blunt end (filter) forceps, and dissection scissors (listed at Table I) with ethanol and air dry on paper towels lining the bench.

11.3 Place three pairs of filter paper onto the paper towels lining the bench; one pair for each type of tissue to be flash frozen in liquid nitrogen.

11.4 Confirm that all pre-labelled (barcoded) 2.0 mL capacity cryovials are present and identified on sample collection proforma, then chill on wet ice in polystyrene box alongside a fresh 50 mL DPBS aliquot.

12 **Flash freeze upper uterine segment biopsies in liquid nitrogen.**

12.1 Chill a new Petri dish base (from the UV-irradiated pack opened for collection of punch biopsies and OCT embedding) on wet ice in the polystyrene box and transfer the punch biopsies with its DPBS into the dish.

12.2 Use the Watchmaker forceps to gently agitate each punch biopsy in the DPBS to wash off as much loose blood as possible. If necessary, use a new scalpel to dissect out any fraying tissue and embedded blood clots without disturbing the myometrial-decidual interface at each punch biopsy.

12.3 Use the Watchmaker forceps to transfer the punch biopsies onto one double layer (i.e. pair) of filter paper sheets (from step 11.3) to briefly dab off excess DPBS.

12.4 Promptly transfer the dabbed punch biopsies from the filter paper into their designated (pre-labelled and pre-chilled) 2.0 mL capacity cryovials, ensuring they are each attached to the inner wall of the cryovials and well-separated from each other (to prevent freezing in clumps) within each cryovial.

12.5 ⊕ Securely cap the cryovials containing the punch biopsies and use the sponge-holding forceps to hold the cryovials horizontally into liquid nitrogen for flash freezing. **Do not drop throw the cryovials into the liquid nitrogen dewar flask** because this increases the chance of the tissue pieces clumping together at time of freezing.

12.6 Leave the cryovials in the liquid nitrogen dewar flask to promptly proceed to dissecting and flash freezing the lower uterine segment biopsy; wipe clean the Watchmaker forceps with ethanol and air dry.

13 **Dissect the lower uterine segment biopsy and flash freeze in liquid nitrogen.**

13.1 Chill a new Petri dish base (from the same UV-irradiated pack used at step 12.1) on wet ice in the polystyrene box and fill with ~20 mL fresh DPBS from the 50 mL aliquot (pre-chilled on wet ice from step 11.4).

13.2 Use the Watchmaker forceps to transfer the lower uterine segment biopsy from its DPBS aliquot tube into the Petri dish of fresh ~20 mL ice-cold DPBS.

13.3 ⊕ Use a new scalpel to cut the biopsy to reveal its core, where there is the least blood and most longitudinal smooth muscle, and dissect out 9-12 tissue pieces each of ~3-5mm^3 size.

13.4 **Undertake the same actions stated from step 12.3 to 12.5** with these dissected pieces of the lower uterine segment biopsy using a new double layer of filter paper and their separately designated (pre-labelled and pre-chilled) 2.0 mL capacity cryovials.

13.5 Leave the cryovials in the liquid nitrogen dewar flask to promptly proceed to dissecting and flash freezing the decidua parietalis; wipe clean the Watchmaker forceps with ethanol and air dry.

14 **Dissect the decidua parietalis tissue and flash freeze in liquid nitrogen.**

14.1 Chill a new Petri dish lid (separated from the base used at step 12.1 or 13.1) upside down on wet ice and fill with ~10 mL fresh DPBS from the 50 mL aliquot (pre-chilled on wet ice from step 11.4).

14.2 Use the Watchmaker forceps to transfer the decidua parietalis tissue from its DPBS aliquot tube into the Petri dish of fresh ~10 mL ice-cold DPBS.

14.3 ⊕ Use the blunt end (filter) forceps with tissue dissection scissors to dissect out 9-12 tissue pieces each of ~3-5mm^3 size.

14.4 **Undertake the same actions stated from step 12.3 to 12.5** with these dissected pieces of the decidua parietalis tissue using a new double layer of filter paper and their separately designated (pre-labelled and pre-chilled) 2.0 mL capacity cryovials.

15 ⊕ **Transfer flash frozen cryovials of all three types of tissues from liquid nitrogen onto dry ice using the sponge-holding forceps, and promptly store them and the**

frozen OCT tissue blocks (from step 10.5) into their designated (pre-labelled and frozen) cryoboxes in the -80^{o}C freezer.

Protocol references

Part A: The following 10x Genomics Demonstrated Protocols and User Guides (kit manuals) were used for guidance with regards to best approach to store samples for **spatial transcriptomics using the Visium platform**:
- CG000240 (Rev C): Visium Spatial Protocols – Tissue Preparation Guide
- CG000160 (Rev C): Methanol Fixation, H&E Staining & Imaging for Visium Spatial Protocols
- CG000238 (Rev D): Visium Spatial Gene Expression Reagent Kits – Tissue Optimization
- CG000239 (Ref F): Visium Spatial Gene Expression Reagent Kits

Part B: The following 10x Genomics User Guides (kit manuals) were used for guidance with regards to best approach to store samples for **single nucleus RNA-seq using the Chromium platform**:
- CG000505 (Rev A): Sample Prep User Guide - Chromium Nuclei Isolation Kit
- CG000315 (Rev C): User Guide - Chromium Next GEM Single Cell 3′ Reagent Kits v3.1 (Dual Index)