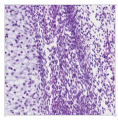

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Embedding and freezing fresh human tissue in OCT using isopentane V.3

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1 Works for me dx.doi.org/10.17504/protocols.io.95mh846

Human Cell Atlas Method Development Community

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ABSTRACT

For many cutting-edge spatial transcriptomic analysis methods, it is essential to prepare and store tissue in a manner that preserves RNA integrity, tissue morphology, and spatial orientation.
This protocol describes the process of freezing fresh or fixed and cryoprotected tissues in optimal cutting temperature compound (OCT) using an isopentane-dry ice slurry. Rapid controlled freezing preserves RNA and mimises ice damage, while embedding in OCT prepares tissues for future cryosectioning.

GUIDELINES

This is a general protocol that may be used for many fresh or fixed adult or foetal human tissues including adrenal gland, kidney, gut, brain, endometrium, and foetal limb.

Fresh and/or fixed frozen OCT-embedded tissues are suitable for many spatial analyses, including RNAscope, Spatial Transcriptomics (fresh only), in situ sequencing, and immunohistochemistry.

MATERIALS

NAME	CATALOG #	VENDOR
Optimal Cutting Temperature Compound (OCT)	361603E	VWR international Ltd
DPBS no calcium no magnesium	14190144	Thermo Fisher Scientific
CellStor™ Pot - 60ml - 10% neutral buffered formalin	BAF-6000-08A	
Sucrose	S7903	Sigma Aldrich
Corning® non-treated culture dishes 60 mm × 15 mm	CLS430589	Sigma Aldrich
Corning® non-treated culture dishes 150 mm × 25 mm	CLS430597	Sigma Aldrich
HypoThermosol® FRS Preservation Solution	H4416	Sigma Aldrich
Isopentane	10468030	Fisher Scientific
Tissue-Tek Cryomold Moulds	View	
Moria perforated spoon	View	Fine Science Tools

MATERIALS TEXT

CellStor™ Pots and sucrose are required only for fixed-frozen tissue preparation.
Different sizes of cyromould are available; 15 x 15 mm will suit most foetal tissues, but adult tissues may require larger moulds (e.g. 20 x 25 mm).

SAFETY WARNINGS

Unscreened human tissues may be host to aventitious biological agents including blood-bourne pathogens, such as HIV, hepatitis B, hepatitis C, and malaria. These tissues should be processed in a laboratory with appropriate biosafety containment level infrastructure. Contaminated waste should also be isolated. Liquid waste should be treated with Virkon; solid waste should be collected for incineration.

Surfaces and equipment should be cleaned using appropriate biocidal cleaning agent, such as Chemgene High Level Disinfectant.

Dry ice is ~78°C, and so has the potential to cause severe cold burns and frostbite: wear thermally insulating gloves when handling. Sublimation yields carbon dioxide, which displaces air and may cause suffocation: store and use in a well-ventilated area.

Isopentane is extremely volatile and extremely flammable, and is a respiratory hazard: it should be stored in a ventilated safety cabinet and used in a ventilated fume cabinet or on a ventilated downflow table. Finally, it is also very toxic to aquatic life, and must be disposed of carefully following local guidelines.

Sharp tools, including scalpels, may be required in this protocol. Use with care (including disposal), and only if necessary

BEFORE STARTING

Handling, use, storage, and disposal of human tissues must be conducted in a respectful manner in line with Human Tissue Authority (HTA) guidelines, reflecting the sensitive nature and origin of the material.
Ensure prior to start that collection, carriage, and receipt of tissues is compliant with all HTA guidelines, including Research Ethics Service/Committee approval, and thorough labelling and tracking of all human materials.

When preparing for spatial analyses, it is advantageous to obtain and preserve maximal anatomical context. It is advisable to communicate with the source of tissue - be it a tissue bank, pathologist, or a fellow researcher who conducted dissection - to obtain as much information as possible about anatomical boundaries and orientation. While this protocol includes opportunity for microscopic examination of tissues prior to freezing, this is no substitute for thorough prior details. Contextualising and re-orienting tissues after freezing in OCT is very challenging.
Different sizes of cryomould are available to suit different tissue sizes, but downstream applications should also be taken into account. For example, if downstream staining or processing requires tissue sections to fit onto a region of a slide, or to be covered by a hybridisation chamber, then the tissue may require trimming prior to embedding.

Prior to processing, it is recommended that tissues are shipped and stored at 4°C in HypoThermosol, which has been shown to preserve molecular integrity for >24 hours (Maddssoon et al. bioRxiv https://doi.org/10.1101/741405).

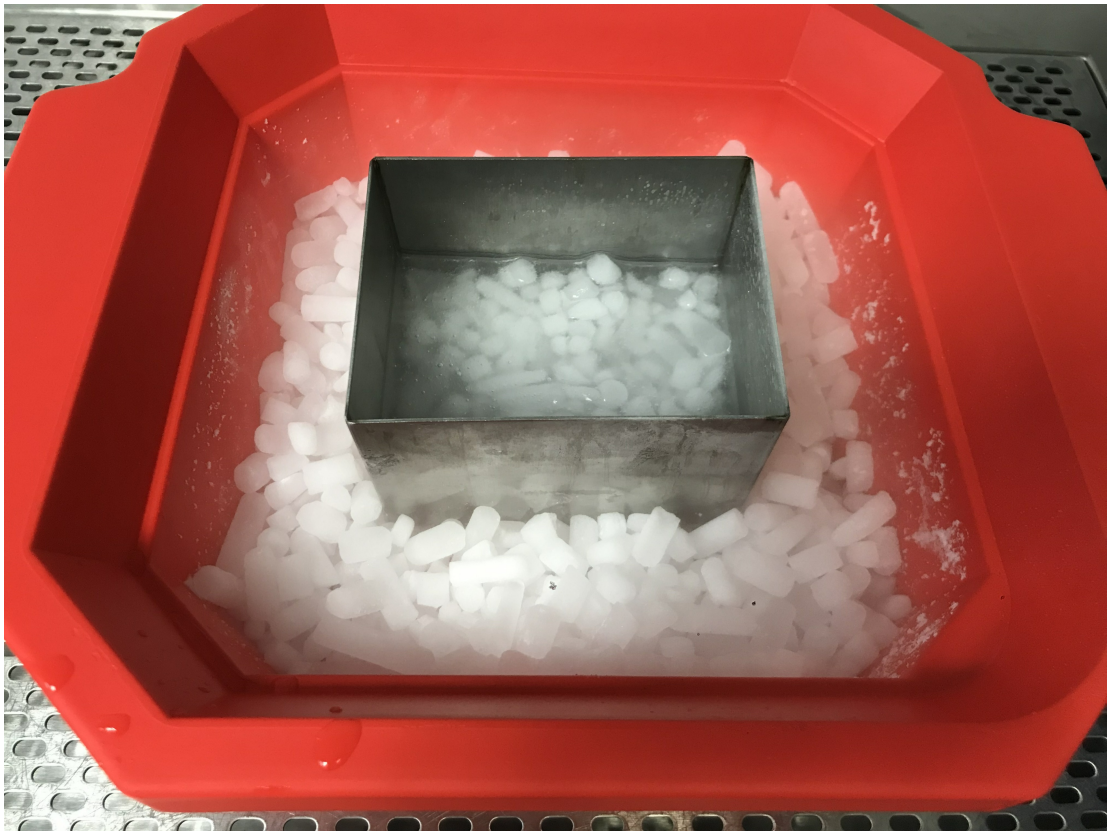
Pre-embedding and freezing (fixed frozen only)

1 For tissues that are to be fixed prior to freezing, transfer tissues to 10% neutral-buffered formalin (such as a CellStor Pot) and fix at 4°C or room temperature for between 2 and 36 hours, depending upon the tissue and application.

2 Fixed tissues may be cryoprotected prior to embedding and freezing. Prepare a 30% (w/v) sucrose solution using sterile deionised water. Transfer the tissue directly from formalin into 30% sucrose, and incubate at 4°C overnight. *Most* tissues will initially float in 30% sucrose, but sink once permeation is complete; at this point, cryoprotection may be considered complete. Cryoprotection, while desirable, is not recommended for non-fixed tissues due to the possibility of molecular degradation.

Embedding

3 Prepare an isopentane-dry ice slurry. Place a small metal box onto dry ice, and fill with isopentane to a depth of several centimetres. Slowly (to minimise violent boiling) add dry ice to the isopentane. When sufficient dry ice is added, the mixture will reach approximately -78°C and adding further pieces of dry ice will not induce strong bubbling (the temperature may be monitored with an appropriate thermometer).



This assembly is ready for freezing. The metal box is chilled on dry ice, in an appropriate insulated container, and isopentane-dry ice slurry has equilibrated, now bubbling little. The entire aparatus is situated on a downflow table to minimise operator exposure to carbon dioxide, isopentane, and tissue-derived aerosols.

4 Label cryomoulds with both sample and orientation information; it is recommended that at least one axis, e.g. dorsoventral, anteroposterior, is noted on the cryomould. Fill cryomoulds with OCT then leave for at least several minutes; this will minimise bubbles.

5 Pre-chill ~20 ml of sterile PBS (or HBSS) on ice. Fill a medium-sized disposable Petri dish (~150 mm) with ice, and place onto this a smaller dish (~30-60 mm) to be filled with the pre-chilled sterile PBS.

6 Process tissues one at a time. First, rinse tissues in pre-chilled PBS on ice, as prepared above.
Prior to embedding and freezing, it is recommended that tissues are examined using a dissection microscope. This will yield maximum spatial anatomical context that may be important in later sample preparation and experiments.

7 If necessary, use a disposable scalpel to trim or dissect the tissue to yield multiple pieces or a specific anatomical region, or to separate tissues that were collected and shipped together.

8 Following all necessary examination and dissection, use a perforated Moria spoon to pick up the tissue. Quickly and *gently* wick away any excess buffer using blotting filter paper. **For most tissues, the sample should not come into direct contact with the paper - this may cause damage.** Instead, place the tissue sample on the spoon, and either bring a corner of the filter paper to any surrounding liquid droplets, or place the perforated spoon onto filter paper and allow the liquid to seep through the holes. Take care, especially with delicate foetal tissues, not to damage the tissue sample by catching it on the filter paper.

9 Using a spoon or spatula, transfer the tissue into a cryomould pre-filled with OCT. If necessary, use forceps or a small spoon to gently 'stir' the OCT to remove streaks of any residual buffer (these should be visible by eye). Then carefully manipulate the orientation of the material to suit future cryosectioning. If using fine forceps, take care not to puncture or rip the sample. Some samples will initially float, and should be gently pushed down to the bottom of the mould.
It is recommended that a photo or sketch is taken of the tissue in the cryomould in its final orientation. Amend or complete any orientation markings on the cryomould prior to freezing.

Freezing

10 Use a pair of forceps to place the cryomould into the isopentane-dry ice slurry, keeping it level during movement to prevent loss of OCT and sample movement.
Ideally, rest or hold the cryomould immersed in the top of the isopentane liquid until the OCT has started to freeze; if isopentane floods the top of the OCT early during freezing, it may promote the presence of bubbles in the block.
The duration of freezing will depend upon the size of the tissue and the cryomould, but most tissues smaller than ~500 mm^3 in an appropriately sized cryomould will be frozen sufficiently within 5 minutes.

This sample is mid-freezing. The mould is labelled with the sample ID and the date - this is essential for sample tracking, especially in the case of human tissues - as well as orientation information, in this case the anteroposterior (A-P) axis.

If the sample is slightly deeper than the mould, additional OCT may be added to cover it once the bottom of the mould has frozen, but take care not to let any isopentane flood the top of the frozen OCT prior to further OCT addition - this may cause the two pieces of frozen OCT to separate later.

11 Once completely frozen, lift the cryomould out of the isopentane-dry ice slurry, and onto fresh dry ice, to allow excess isopentane to drain and evaporate. The sample may be paused on dry ice while others are processed.

12 Wrap each sample, frozen in individual cryomoulds, in aluminium foil. This keeps samples clean, and separate, and minimises dehydration in the -80°C freezer. Minimise the time each sample spends away from the dry ice while wrapping to minimise temperature changes. Carefully label the outside of the foil prior to wrapping around each sample.

13 Transfer samples to a pre-cooled cryobox and store at -80°C.