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## Processing Adult Human Kidney Tissues for Single Cell Studies

**Abstract:** Critical to generating robust single cell or tissue gene expression data is ensuring that the source samples are procured and processed in a way that minimizes artifacts. This is especially critical for generating single cell/nucleus gene expression data from adult human tissue samples that are difficult to dissociate and might require prolonged manipulations that will introduce stress response signatures. We describe methods to: minimize these unwanted processing effects to enable interrogation of limited tissues, such as biopsies; allow histological validation and quality assurance and control of the samples; ensure compatibility with both single cell assays and orthogonal technologies. This involves preparing fresh frozen O.C.T. embedded tissue cryoblocks to collect thick sections for gene expression studies and adjacent sections for QA/QC, bulk RNAseq, DNA preparation or multiplex *in situ* RNA hybridization.

***Applications: Histology, snRNA-Seq, in situ RNA imaging (DART-FISH)***

***Materials:***

1. Two Cryomold (cassettes): Tissue-Tek Cryomold Intermediate (#4566); for larger tissue Standard size can be used. One is used for bathing the tissue and the other for embedding.
2. O.C.T. compound: Tissue-Tek (#4583)
3. RNaseZAP: Sigma (R2020-250ml)
4. Cryostat: set at -20°C to -25°C
5. Powdered dry ice
6. Small ziplock bags
7. 2 ml cryovials (bigger tubes if you plan to harvest large chunks of tissue)
8. Pipet tips with aerosol barrier
9. Sterile PBS (phosphate-buffered saline)
10. Forceps
11. Scalpel blade
12. RNAlater® (AM7021). If the solution appears cloudy, warm it at 37°C for 30 min or refer to the Ambion protocol. Specimen labels to be provided by the Central Hub or printed locally from a centrally managed specimen software if available.

****General Note****: Perform procedures in a RNase-free working area (e.g. clean and sterile environment)*

***Procedure:***

***Tissue embedding and OCT Block Preparation (Figure 1)***

1. Label one cryomold with specimen number and place it on powdered dry ice. This will be the cassette in which tissue will be embedded
2. Take the second cryomold and fill it with O.C.T. Tissue will be bathed in it before transferring to the cryomold in step 1
3. Rapidly dissect the tissue, dab away excess blood using a pad or gauze, cut desired size section that will fit easily in the cryomold chosen, and briefly bathe the tissue in O.C.T. in the cassette in step 2, gently swishing around with a clean instrument (~2min) (Figure 1A)

*Note: Keep separate forceps to handle tissue during dissection from the one used for bathing as the O.C.T. will stick to the forceps and can make dissection difficult. Handle the tissue gently, for a long biopsy one could also use a pipette tip held in hand to bathe the tissue and lift to transfer without squeezing it*

**QA/QC**: Using a smartphone camera, an image of the tissue can be captured while the tissue is bathing that can be used for gross identification of cortex and documentation.

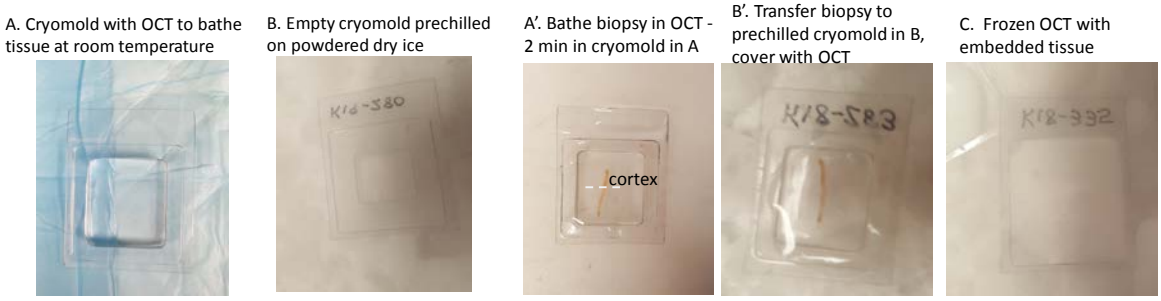


**Figure 1. Preparation of cryomolds for fresh frozen OCT embedding of research core.**

4. Transfer the tissue into the pre-labeled and chilled cryomold (step 1), orient the tissue as desired and pour O.C.T. till it completely covers the tissue (Figure 1B, C)

   *Note: avoid bubbles; can move the bubbles out of the way with forceps or pipette tip*

5. Keep the cassette on powdered dry ice for freezing. Keep the dry ice bucket covered to keep a cold environment. Avoid flash freezing as that could result in tissue fracturing and thus loss of integrity.

6. Transfer the frozen block into a pre-chilled ziplock (on dry ice), seal it taking as much air out as possible and keeping the block chilled, and store it in a freezer box at -80°C until ready to section or ship overnight packed in dry ice shipping container. To avoid damaging the block during transit, place it securely in a pre-chilled freezer box and pack in a Styrofoam container with ample dry ice.

***Sectioning***

1. Acclimate O.C.T. blocks in cryostat for about 10 minutes (check cryostat temperature, usually kept at -20 to -25°C)
2. Prepare tubes, slides, containers before cutting and as needed. Pre-chill the tubes for RNAlater in wet ice. Slides on which sections will be mounted for histology can be at room temperature
3. At room temperature spray all instruments (forceps/paintbrushes) with RNaseZAP
4. Add O.C.T. to a pre-chilled metal chuck kept in the cryostat and while freezing place the O.C.T. block with the tissue (tissue facing up) on the chuck. In a few minutes the O.C.T. block with the tissue will be attached to the metal chuck
5. Mount block with the tissue on the cryostat stage (tissue facing the user)

   *Caution: Be careful when inserting or changing or cutting with cryostat blade and use gloves while cutting. All tissue should be handled keeping universal precautions in mind and consider them biohazardous. In the event of injury contact your local safety office.*

6. Sections are collected for various processing conditions (see table as an example)

| | | | | | |
|---|---|---|---|---|---|
| Slides 1-5 (for histology -5-10µm) | Thick sections RNALater/Dry Ice (for snRNA-seq; RNAseq) | Slides 6-10 (for histology-5-10µm) | Thick sections RNALater/Dry Ice (for snRNA-seq; RNAseq) | Slides 11-15 (for histology-5-10µm) | Sections on Coverslips (For DART-FISH- 10-20µm) |

a) Sections **on slides** are collected in 3-5 slide intervals at 5-10µm, 1 section per slide, for histology/immunofluorescence to **validate** the tissue being used. Slowly bring slide to the cut section and as sections transfer to the slides, briefly touch the back surface of the slide (opposite to the section) with your finger to facilitate adherence of the tissue section.

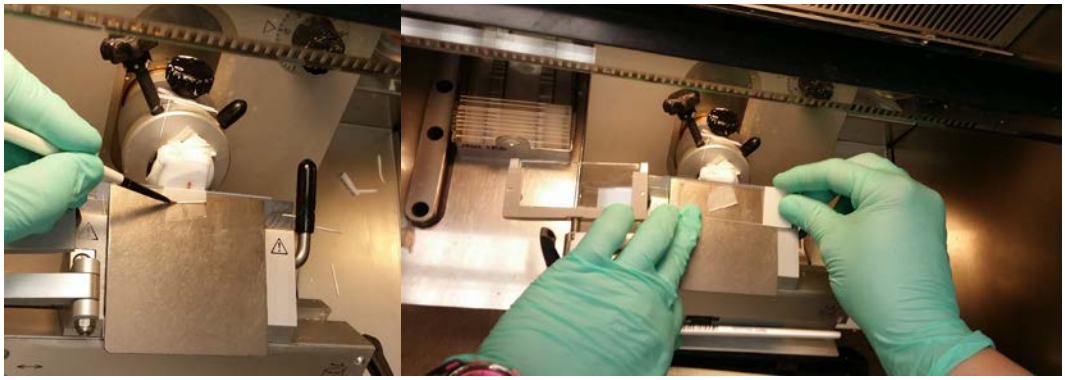

**Figure 2. Collecting cryosections on a slide.**

One slide from each interval will be H&E stained for adequacy, quality, composition and integrity (**QA/QC)**. Keep the remainder in the cryostat and transfer to -80°C.

b) **Thick section rolls** (35-40 µm) (use for **RNA** or **protein** studies) (**series of tubes with tissue curls allows for reproducibility testing and comparing different processing methods – QA/QC)** are collected in microfuge tubes (**Figure 3**).

Examples:

- Dry Ice, no solution: 7 rolled sections (total 280µm) collected in chilled 1.7ml centrifuge tube. Keep chilled at all times so OCT doesn't melt. Process immediately. Used for: **RNA or protein** assays - e.g. snRNA-seq or single cell chromatin accessibility
- RNALater: 7 rolled sections are collected in centrifuge tube containing 1mL RNAlater and kept chilled on ice (**RNA** - snRNA-seq or total RNA). At this stage, the RNAlater containing tubes can be shipped overnight (packed with ice packs) to another site or stored at -20°C for a few weeks (RNAlater will not freeze). Used for: **RNA** assays – e.g. snRNA-seq or total RNA isolation.

**Figure 3.** Cryosection rolls

- Collected in Other media: as needed

*Note: Be careful when handling the tubes, avoid touching the surface with the tissue as it could cause local thawing, better to hold at the lip of the tube or anywhere that is away from the tissue if need be.*

c) Sections for **coverslips** are collected as follows (for **DART-FISH):**
- Keep the prepared coverslips at room temperature
- Section tissue at desired thickness (we cut at 10 µM and are in the process of testing 20 µM) and place the coverslip so tissue is in the center. Use forceps to pick up and flip the coverslip so the tissue is now facing upwards. Keep chilled.

*Note: Be gentle and avoid pressing too firmly as this will smash the tissue. Gently tilting the coverslip (or slides in case of histology) usually will cause the tissue to lift and adhere due to static generated.*

*Note: Adjacent tissue sections allows for reproducibility testing and comparing different processing conditions – **QA/QC***

- Place a slide in the cryostat and, once chilled, put a drop of water (~25uL) towards the center of the slide and place coverslip (tissue facing upwards) over the water. Keep the slide flat until the water freezes, attaching the coverslip to the slide. This makes it easier to ship the coverslips.
- Place slides with the attached coverslip in pre-chilled slide container for shipment or store at – 80°C

***Specimen preservation in RNAlater (modified from USER GUIDE from Ambion)***

***Things to do before starting:*** Plan how many tubes and what type of sections or tissues you want to preserve. Use clean equipment/instruments and sterile technique as much possible to ensure best quality of RNA is obtained.

1. Obtain desired tissue (preferably < 0.5cm at least in one of the dimensions), you can rinse in sterile PBS to remove excess blood if needed, wipe excess solution

***Tip:*** *If you do not have this tissue available for histology, you may want to slice an adjacent section for histology and then use the other half for RNAlater*

2. Immerse in RNAlater (5-10x volume) overnight at 4°C. You may cut the tissue in small pieces to fit in the vial to ensure faster stabilization of the RNA. Alternatively, you can ship the vial with tissue in RNAlater packed with icepacks overnight

   ***Tip:*** *For small biopsies, 2ml cryovial with prefilled RNAlater can be provided to the collector*

   ***Tip****: For tissue pieces, depending on size, the tissue can be collected in 5ml or 15 ml sterile tube*

   *Note: RNAlater is an RNA stabilizing solution that needs to penetrate the tissue to preserve the RNA. While the tissue may be OK in RNAlater at room temperature, DO NOT leave tissue in it for days. If you do anything different make a note in the comment section in the tissue repository software. The specimen can be kept at 4°C for several days with minimal effect on RNA quality, but remember this will change the texture of the tissue that may need more vigorous homogenization in RNA preparation steps later*

3. Remove supernatant, store at -80°C (removing excess RNAlater helps reduce salt formation in frozen tissue) indefinitely. Alternatively, tissue can be stored at -20°C in RNAlater solution (it will not freeze); you may see some crystals but those are of no concern and will go away upon warming when ready to make RNA

   ***Tip:*** *If you keep the tissue at -20°C, then make sure it is a reliable freezer with alarm system since heavily used freezers may be warmer than -20°C*

4. When ready to use, thaw at room temperature. You can use an absorbent pad to wipe excess RNAlater. Proceed with RNA isolation method (e.g. TriZol)

   ***Tip:*** *It may help to cut the tissue in small pieces to aid in homogenization*

   ***Tip:*** *Removing excess RNAlater before lysing the tissue improves the quality of RNA obtained by several RIN units. The TriZol can be added directly to the 2ml cryovial and homogenized or the tissue can be transferred into a larger tube with clean forceps (autoclaved or RNAzap treated)*