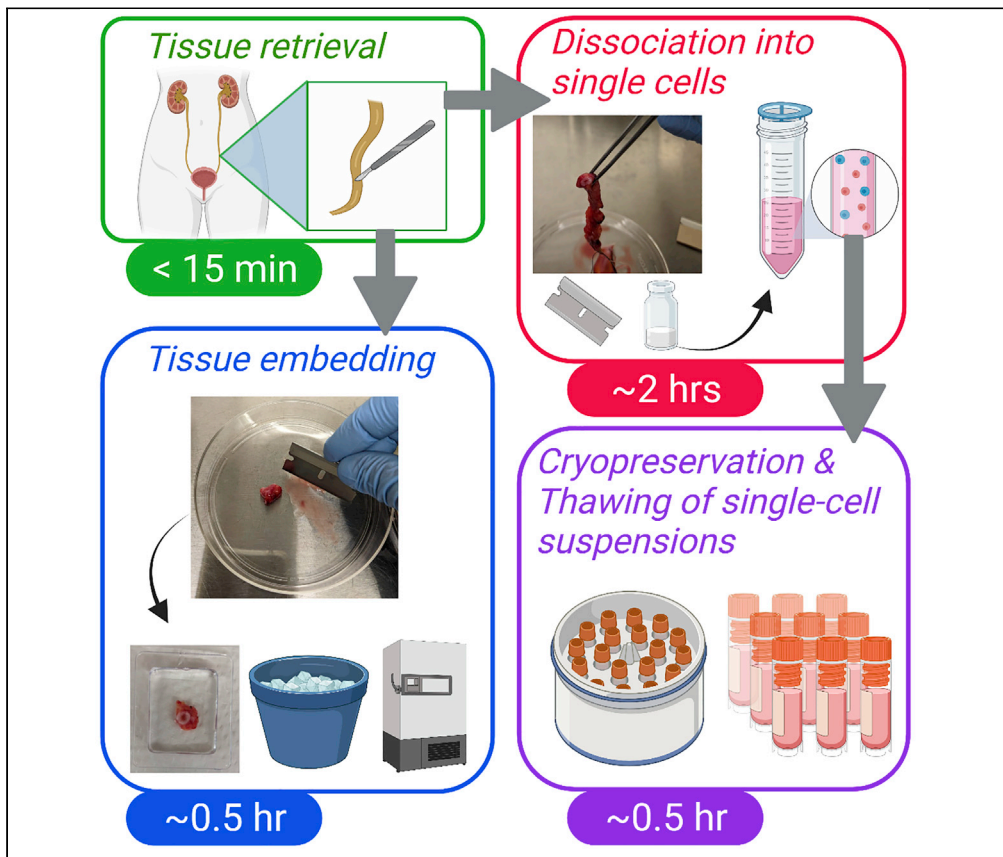


Protocol

Processing and cryopreservation of human ureter tissues for single-cell and spatial transcriptomics assays



Emily E. Fink, Surbhi Sona, Byron H. Lee, Angela H. Ting

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Highlights

Generation of high-viability single-cell suspensions from human ureter tissues

Steps to cryopreserve single-cell suspensions

Counting and quality control of single-cell suspensions

O.C.T. embedding of human ureter tissue for spatial transcriptomics

Characterizing the cellular heterogeneity of human ureter tissues using single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics provides a detailed atlas of cell types, signaling networks, and potential cell-cell cross talk underlying developmental and regenerative pathways. We describe an optimized protocol for generating, cryopreserving, and thawing single-cell suspensions from ureter tissues isolated post-cystectomy for scRNA-seq. In addition, we describe an optimized protocol for cryopreserving human ureter tissues for 10x Genomics Visium spatial gene expression platform.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Processing and cryopreservation of human ureter tissues for single-cell and spatial transcriptomics assays

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<https://doi.org/10.1016/j.xpro.2022.101854>

SUMMARY

Characterizing the cellular heterogeneity of human ureter tissues using single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics provides a detailed atlas of cell types, signaling networks, and potential cell-cell cross talk underlying developmental and regenerative pathways. We describe an optimized protocol for generating, cryopreserving, and thawing single-cell suspensions from ureter tissues isolated post-cystectomy for scRNA-seq. In addition, we describe an optimized protocol for cryopreserving human ureter tissues for 10x Genomics Visium spatial gene expression platform.

For complete details on the use and execution of this protocol, please refer to Fink et al. (2022).¹

BEFORE YOU BEGIN

The protocol herein describes the detailed steps for (1) generating a single-cell suspension of ureter cells with > 90% viability from human ureter tissues ranging in length of approximately 0.5–5 cm for downstream 10x Genomics single-cell RNA sequencing (scRNA-seq), (2) how to embed and preserve human ureter tissues for downstream 10x Genomics Visium spatial gene expression analysis, and (3) how to cryopreserve and thaw the human ureter single cell suspensions for downstream scRNA-seq using the 10x Genomics platform. We have also used this protocol on tissues from whole mouse bladders, normal human bladder, bladder tumor, and human prostate. In addition, we have used this dissociation protocol for the isolation of single cells used in the generation of ureter and bladder organoids.

Before retrieving the tissue from the operating room, it is necessary that all reagents are in place for tissue processing in order to move through the stages efficiently, so that high-viability cell suspensions are reproducibly generated. Record any variations in tissue retrieval and processing times.

Institutional permissions (if applicable)

Human ureter tissues should only be obtained under an institutional review board (IRB)-approved protocol with patient informed consent. All experiments included in this protocol involved the voluntary participation of patients with written informed consent prior to sample acquisition. All users should abide by their institutional policies with regards to working with human subjects.



Preparation for tissue dissociation

⌚ Timing: Days to hours before experiment

1. Set up a clean working station in a tissue culture hood for tissue dissociation, including ample pipettes/aids, a 10 cm dish for cutting up tissue, 50 mL tubes, 15 mL tubes, 1.5 mL tubes, ACK lysis buffer, trypan blue, PBS, 70 μ m filters, Flowmi 40 μ m cell strainers, razor blades, and tissue forceps.
 - a. Ensure all razor blades and tissue forceps have been autoclaved beforehand.
2. Resuspend lyophilized papain in 5 mL Earl's Balanced Salt Solution (EBSS) and warm to 37°C in a water bath about 10 min prior to tissue dissociation.
3. Resuspend lyophilized DNase in 0.5 mL EBSS and place on ice until ready to use. The DNase is a component of the Papain Dissociation System, each single use vial containing 1,000 units of DNase.
4. Resuspend the lyophilized ovoid inhibitor in 32 mL EBSS and keep at 20°C–25°C until ready to use. The ovoid inhibitor is a component of the Papain Dissociation System.

Note: The EBSS, lyophilized papain, lyophilized DNase, and lyophilized ovoid inhibitor are components of the Papain Dissociation system.

Preparation for tissue embedding

⌚ Timing: 30 min before experiment

5. Set up a station and gather the supplies for tissue embedding, including O.C.T. compound, labeled cryomolds, dry ice or liquid nitrogen, isopentane, a container suitable for making a dry ice/isopentane bath, forceps, and aluminum foil.
 - a. At least 10 min prior to freezing the tissue in O.C.T., the isopentane needs to be pre-chilled on dry ice or liquid nitrogen.
 - b. Have a pre-chilled box in the –80°C freezer for keeping the samples once they are frozen.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human ureter tissues from patients undergoing cystectomy	Fink et al. ¹	N/A
Chemicals, peptides, and recombinant proteins		
ACK lysis buffer	Thermo Fisher Scientific	Cat#A1049201
DMEM/F-12, GlutaMAX supplement	Thermo Fisher Scientific	Cat#10565018
Fetal bovine serum (FBS)	Corning	Cat#35010CV
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D2650
Trypan blue	Thermo Fisher Scientific	Cat#15250-061
PBS	Thermo Fisher Scientific	Cat#10010-023
Tissue-Tek® O.C.T. Compound	Sakura	Cat#4583
Isopentane (2-methylbutane)	Sigma-Aldrich	Cat#M32631
Critical commercial assays		
Papain Dissociation System	Worthington	Cat#LK003150
Other		
Flowmi 40 μ m Cell Strainers	Bel-Art	Cat#136800040
70 μ m cell strainer	Falcon	Cat#352350
Tissue-Tek® Cryomolds	Sakura	Cat#4557
Razor blades	Fisherbrand	Cat#12-640
Tissue forceps	Fisherbrand	Cat#13-812-38
Disposable hemocytometers	Thermo Fisher Scientific	Cat#C10283

MATERIALS AND EQUIPMENT

Freezing medium for single cell suspensions		
Reagent	Final concentration	Amount
Dimethyl sulfoxide (DMSO)	10%	1 mL
Fetal bovine serum (FBS)	50%	1 mL
DMEM/F-12, GlutaMAX supplement	40%	5 mL
ddH ₂ O	N/A	42 mL
Total	N/A	50 mL

Sterile filter and store at 4°C for one month.

Alternatives: You can also use a 90%FBS/10%DMSO freezing media to improve viability after thawing.

STEP-BY-STEP METHOD DETAILS

Ureter tissue retrieval and preparation for digestion

⌚ Timing: 20 min

This protocol begins with the retrieval of ureter tissues from the operating room, which vary in size. Use of surgical energy devices such as electrocautery should be avoided during tissue collection. The tissue should be brought back to the lab immediately, keeping it in a container of PBS and on ice the whole time. It is best to minimize the time between collection and processing, and record any potential variations. While this protocol has only been used for downstream 10X Genomics scRNA-seq platform, it is hypothesized to be universally applicable to additional scRNA-seq platforms requiring an input of high-viability single-cell suspensions. Papain has proven to be a less-damaging proteolytic enzyme in a variety of tissue types.^{2–4}

- Record any necessary details and observations about the tissue in your lab notebook.
 - Include information such as the date, time of retrieval, variations in elapsed time from transporting the tissue, sample identifiers, and length of the ureters.
- Wash tissue with enough PBS to cover in 50 mL conical (Figure 1), centrifuge at 1,000 × g for 5 min at 20°C–25°C, and aspirate PBS.
- Transfer tissue to a sterile 10 cm tissue culture dish using tissue forceps.
- If the tissue is large, cut a piece that is approximately 1 cm in length for each separate aliquot of papain.
- Thoroughly mince the tissue at 20°C–25°C using a sterile blade.
 - First start by cutting it with razor blades into as small of pieces as possible.
 - Once the pieces are too small to slice, mince the tissue until a paste-like consistency is achieved (Figure 2).
- Transfer minced tissue to a 50 mL conical containing 5 mL papain and 500 μL DNase (make sure there is enough volume to submerge the tissue).

⚠ **CRITICAL:** To prevent tissue from drying while mincing it, you can add a few drops of the papain/EBSS solution on to the dish.

Ureter tissue enzymatic digestion and cell clean-up

⌚ Timing: 1.5–2 h

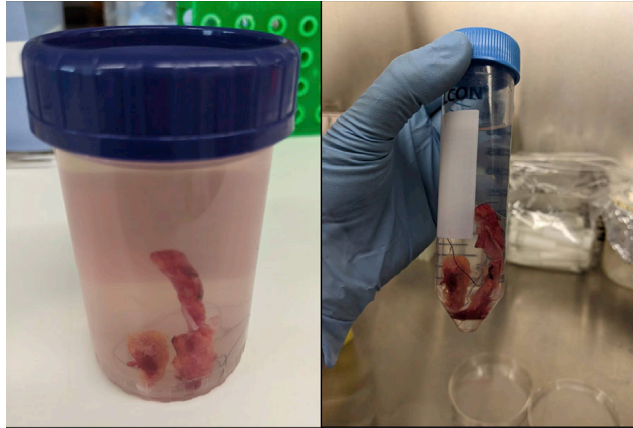


Figure 1. Physical appearance of ureter specimen

Example of a ureter tissue sample post-cystectomy (left) and after being transferred to a new 50 mL conical in PBS.

During this step, the ureter tissue is enzymatically digested using papain, DNase, and gentle shaking to release single cells. The aliquot of DNase should be kept on ice until this step.

7. Wrap cap with parafilm and incubate horizontally with gentle shaking (100 rpm) at 37°C for 1 h.

Note: Keeping the tube horizontal is important for maintaining a homogenous mixture and minimizing the settling of tissue onto the bottom of the conical tube, contributing to the overall efficiency of the digestion.

Optional: Shaking can be achieved with a standard bacterial shaker or by placing a rotating shaker inside of a 37°C incubator.

8. Gently triturate using P1000 pipette to check if tissue is digested (can cut the tip if there are chunks).

Optional: The incubation time can be prolonged to 90 min in order to increase the cell yield. Additionally, you can take the tube out and gently vortex every 15 min.

9. After incubation, add 5.5 mL of ovoid inhibitor to the tissue suspension to stop the digestion.

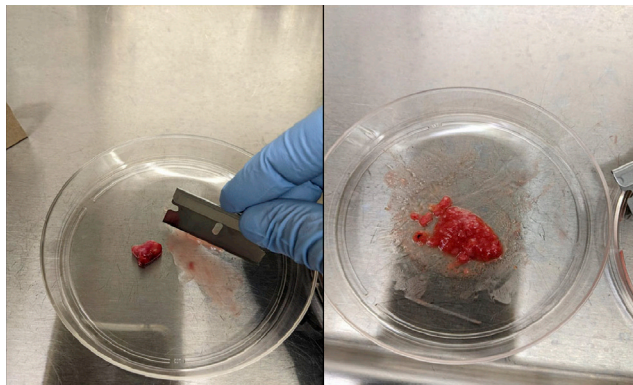


Figure 2. Initial mincing of ureter specimen

Before (left) and after (right) thoroughly mincing a 1 cm long section of human ureter.

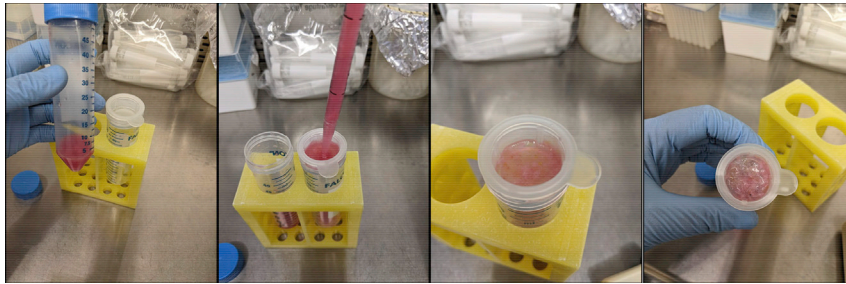


Figure 3. Removal of large debris

Post-digestion filtering through a 70 µm cell strainer (panels 1–3) and an example of residual tissue left in the filter afterwards (far-right panel).

10. Filter the mixture by passing the entire volume (~11 mL) through a 70 µm cell strainer to remove large debris (Figure 3).
 - a. Place a new 70 µm filter onto the top of an open 50 mL conical tube.
 - b. Carefully and slowly pipette up the cell suspension and expel it through the middle of the filter, being careful not to overflow.
 - c. Once the cell suspension has passed through the filter, discard the filter and cap the 50 mL conical.

Note: There will be clumps of indigestible tissue left in the filter. If you started with a large piece of tissue, there may be a propensity to clog the filter. Use a sterile pipette tip to move the extra tissue around the filter so there is a clear path for the liquid to flow through.

11. Centrifuge cell suspension at 1,000 × g for 5 min at 20°C–25°C in a rotating bucket centrifuge to pellet cells and carefully discard supernatant.

Note: The pellet may be small, loose, and hard to see so be careful when aspirating the supernatant to avoid accidentally sucking up any of the pellet. It helps to leave a few hundred microliters of supernatant in the bottom to avoid removing any of the pellets by accident. The safest way to minimize cell loss is to gently aspirate and avoid decanting.

12. Resuspend cell pellet in 3 mL ACK lysis buffer and incubate at 20°C–25°C for 3 min, centrifuge at 1,000 × g for 5 min at 20°C–25°C, and carefully discard supernatant.

Optional: Transfer the ACK/cell suspension to a new 15 mL conical. A smaller conical will help in visualizing the pellet.

Note: The pellet should go from a red color to nearly white/colorless after the incubation with ACK lysis buffer (Figure 4). Maintain the same careful aspiration of the supernatant as in the previous step.

13. Resuspend the cell pellet with 5 mL PBS at 20°C–25°C, centrifuge at 1,000 × g for 5 min at 20°C–25°C, and carefully discard supernatant.
14. Resuspend the pellet in 200 µL of PBS at 20°C–25°C and transfer to a 1.5 mL Eppendorf tube.

Note: Larger pellets may need to be resuspended in 500 µL–1 mL of PBS in order to obtain a countable cell density. On the other hand, smaller pellets may require a smaller volume. For 10x Genomics scRNA-seq sequencing, the optimum cell density is 1,000 cells/µL, and the pellet depicted in Figure 4 (right side) was resuspended in 250 µL PBS.

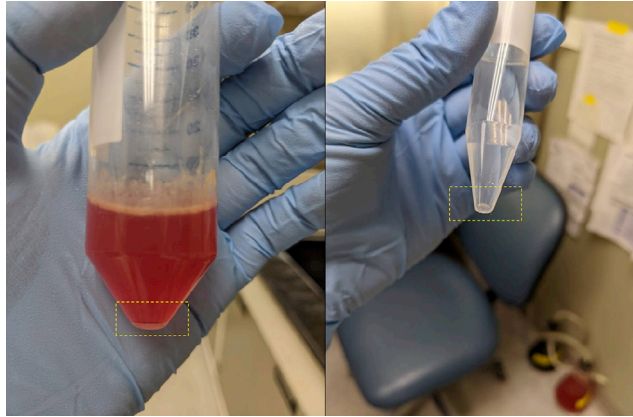


Figure 4. Elimination of red blood cells

An example of the cell pellet color and size before (left) and after (right) ACK lysis buffer treatment and centrifugation.

15. Pass the PBS/cell suspension through a 40 μm Flowmi tip (Figure 5).
 - a. First, pull the PBS/cell suspension into the tip of a p1000 pipette.
 - b. Once the suspension is inside the tip, attach a 40 μm Flowmi tip to the tip of the pipette tip.
 - c. Once firmly in place, slowly expel the cell mixture into a new 1.5 mL Eppendorf tube. Do not go past the first stop on the pipette when expelling the liquid.
16. Take an aliquot of cells (10 μL) and mix 1:1 with Trypan blue to determine cell viability, concentration, and extent of cellular aggregation. (Figure 6).

Note: Counts can be performed manually using a hemocytometer or an automated cell counter such as the Countess (Figure 6). It is generally advisable to do a manual count regardless.

Note: For 10x Genomics scRNA-seq, there should be minimal cell “clumps” (defined as two or more cells aggregated together). If there are substantial clumps, the sample can be passed through a new Flowmi tip.

Optional: BSA can be added to the PBS at a concentration of 0.04%–2% to decrease aggregation and minimize cell loss.

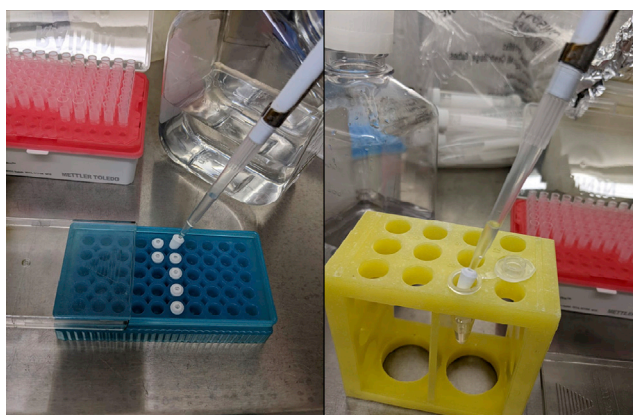


Figure 5. Passage of cells through Flowmi tip

The proper attachment of a 40 μm Flowmi tip (left panel) and expelling the sample through the Flowmi tip (right panel).

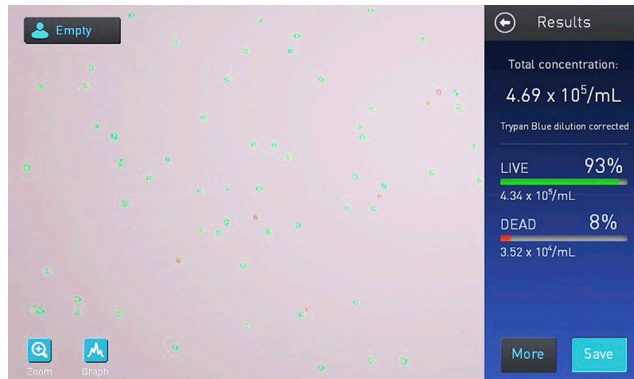


Figure 6. Cell suspension QC

An example of the cell viability, count, and relative lack of cell aggregation, optimal for downstream 10x Genomics scRNA-seq sequencing.

17. At this stage, the cells in PBS should be kept on ice for up to 30 min until they are ready to be processed for scRNA-seq or other downstream applications. If cryopreserving the cell suspensions, move on to step 18.

Cryopreservation and thawing of ureter single cell suspensions

⌚ Timing: 30 min

This portion of the protocol is performed in order to store single cell suspensions in the -80°C freezer for long-term (several months) storage, that, when thawed, retain similar cell viability, heterogeneity, and can be used for downstream scRNA-seq and other applications. This method has also been used for preserving and thawing cells for successful organoid generation.

18. Determine the total number of cells to cryopreserve in order to approximate the best volume of freezing media to use.
 - a. From the example cell count in [Figure 6](#), with the given resuspension volume of $250\ \mu\text{L}$ and factoring in the volume of Trypan blue, the total number of cells from 1 cm piece of ureter is roughly 250,000.
 - b. For cell numbers less than 1 million total cells, resuspend the pellet in 1 mL of freezing media, enough for one cryovial.
 - c. For higher cell yields, keep the cells at a concentration of 1 million/mL of freezing media.

Note: Multiple simultaneous dissociations can be performed if the section of ureter is long enough. It is most efficient to break it into 1 cm long sections for each separate dissociation. This will maximize the cell yield from each ureter sample.

19. Prepare the correct number of labeled cryovials.
20. Resuspend the cell pellet in the appropriate volume of freezing media and aliquot into the pre-determined number of cryovials for freezing.
21. Quickly transfer the vials of cells into a Styrofoam box or an isopropanol cooler and place in the -80°C freezer.
22. To thaw the frozen cells, remove the desired number of vials from the -80°C freezer and quickly transfer the vial to a 37°C water bath.
23. Once the majority ($>75\%$) of the sample is thawed and only a small chunk of ice remains, resuspend the cells in 10 mL of pre-warmed DMEM/F-12 media that has been supplemented with 10% FBS and transfer the liquid to a 15 mL conical.
24. Centrifuge at $1,000 \times g$ for 5 min at 20°C – 25°C and gently remove the supernatant.



Figure 7. Removal of excess liquid from tissue

An example of a ureter cross section approximately 5 mm in length on a paper towel to remove excess moisture.

25. To wash, resuspend the cell pellet in 10 mL of PBS and centrifuge at $1,000 \times g$ for 5 min at 20°C – 25°C .
26. Depending on the downstream application, resuspend the cell pellet in 250 μL –1 mL of PBS and keep on ice.
 - a. Take a 10 μL aliquot of cell suspension and mix 1:1 with Trypan blue for a viability assessment.

Note: If the cells have become aggregated during the cryopreservation, the user can pass the suspension through a 40 μm Flowmi tip if the downstream application requires a homogenous mixture of single cells.

Note: Handling time during dissociation, length of time in the freezer, and general batch effects are all factors that dictate cell viability after thawing. Long-term storage (> 8 months) results in a significant drop in cell viability (< 70% viable) following this protocol, whereas short-term storage of 1 week to several months maintains similar viability as the cells prior to freezing.

O.C.T. embedding of ureter tissue sections

⌚ Timing: 30 min

This tangential portion of the ureter processing protocol is performed in order to cryopreserve whole pieces of tissue for downstream sectioning and spatial analysis using Visium or other methodologies such as immunofluorescence and immunohistochemistry. This protocol can be performed in parallel with the dissociation if there are multiple people processing the tissue. If there is only one person processing, this portion of the protocol can be performed during the one hour incubation period (step 7), as long as the piece of ureter for embedding has been kept on ice in PBS. From each 5 mm thick imbedded ureter tissue, approximately $500 \times 10 \mu\text{m}$ sections, the optimum thickness for Visium, can be reached, with >75% of the sections usable for staining and Visium.

27. Begin by taking a horizontal cross section of the ureter approximately 5 mm in length and typically < 0.5 cm in width (Figure 7) and rinse in PBS.
28. Using non-toothed forceps, gently pick up the tissue and wick off excess moisture using a paper towel (Figure 7).



Figure 8. Freezing tissue in O.C.T.

An example of ureter embedding and freezing in O.C.T., followed by wrapping in aluminum foil and transferring to a bucket of dry ice.

29. Obtain a pre-labeled cryomold and fill halfway with O.C.T., being careful not to introduce any bubbles.
30. Gently place the ureter tissue in the middle of the O.C.T./cryomold (Figure 8).
 - a. Layer more O.C.T. on top of the ureter tissue to fill the mold and completely cover the tissue.
31. Transfer the filled cryomold to the pre-chilled isopentane bath for freezing (Figure 8).

Note: the isopentane is cold enough when you add a small piece of dry ice and see no bubbles/boiling.

- a. The O.C.T. will quickly start to turn opaque white around the edges until the whole sample is opaque white. This happens < 5 min for smaller tissues as described in this protocol.
- b. Once the whole sample is frozen, wrap the sample in aluminum foil and place it in dry ice until it is ready to be transferred to the -80°C freezer.
- c. Seal the foil-wrapped samples in a plastic bag and place in a pre-chilled box in the -80°C freezer.

△ CRITICAL: The vapors from the isopentane are harmful when inhaled in large quantities, always make sure to have the isopentane covered when it is not being used and store the isopentane in a proper cabinet for flammable liquids.

EXPECTED OUTCOMES

This single-cell dissociation protocol has been optimized to obtain cells from different cellular compartments within the adult human ureter; including the basic cell types of the stroma, epithelial, and immune compartments.¹ From a 1 cm length of human ureter, the expected cell yield is typically $\sim 250,000$ cells with a viability of > 90%, which is sustainable after cryopreserving and thawing. Furthermore, the basic cell types of the stroma, epithelial, and immune compartments are nearly identical before and after cryopreservation.

LIMITATIONS

As with any tissue dissociation performed using enzymatic digestion, there will be a bias towards cell types that are more amenable and easier to enzymatically digest, such as the urothelial and immune cells. Cells that are harder to isolate due to their rarity and/or difficulty extracting from a complex matrix (such as neuronal cells with long processes) will be under-represented.^{5,6} Therefore, it is imperative not to make strong conclusions relating to proportional differences between cellular compartments. To validate the relative abundance of a particular cell population with respect to the other compartments, complementary methods such as immunofluorescence can be used if there is a known cell-type specific marker protein.

TROUBLESHOOTING

Problem 1

A high number of dead cells after the final washing stage of the single cell suspension with PBS.

Potential solution

Some potential reasons for a high percentage of dead cells (> 30% dead cells) could be the following; 1) The processing time is too long, 2) The tissue was sitting on ice for too long prior to processing, 3) during the tissue mincing in step 5, the tissue became dry, or 4) Some unknown underlying pathology within the tissue.

To ensure maximum viability, it is imperative to move through each step as quickly as possible.

Problem 2

Low cell yield.

Potential solution

To ensure maximum cell yield, it is first and foremost important to make sure that the tissue has been minced as thoroughly as possible. This may take several minutes. If the piece is small, it helps to use one razor blade to hold the tissue in place and a second razor blade to slice the tissue apart. The tissue should be the consistency of grits or polenta when it is minced to completion. The enzyme must also be fresh.

Additionally, the cell pellets tend to be loosely attached to the conical wall. Therefore, it can be very easy to accidentally suck up some of the cells when aspirating any liquid from a small pellet. To avoid this, it is best to leave a few hundred microliters of liquid surrounding the pellet to avoid disturbing it.

Problem 3

Poor viability after thawing the cryopreserved cells.

Potential solution

It is not uncommon to see a small drop in viability between initial freezing and thawing. The length of time in the freezer can greatly impact the survivability of cryopreserved cells so it is advisable to empirically determine the optimum "window of opportunity" for each individual user and tissue type. Transferring the samples to liquid nitrogen has not yet been tested within the scope of this protocol, but it might be an additional viability-boosting option.

Single cell suspensions are thawed following conventional freezing and thawing techniques of cryopreserved cells. Following removal from the freezer, cryovials are placed in a 37°C water bath until the ice is ~80% melted, followed by the addition of 10 mL pre-warmed DMEM/F-12 media supplemented with 10% FBS. Cells are centrifuged at 1,000 × g for 5 min at 20°C–25°C, washed 1 × with 10 mL of PBS, and viability is assessed using Trypan blue stain.

Problem 4

Excessive red blood cells in the final cell suspension.

Potential solution

In some instances, the tissue may be larger and/or have more blood on it, and one 3-min incubation with ACK lysis buffer may not be sufficient for lysing all of the red blood cells. To ensure the proper lysis and removal of all red blood cells, the volume of ACK lysis buffer can be increased to 10 mL and the lysis repeated.

Problem 5

The presence of aggregates of cells (>20%).

Potential solution

Cellular aggregation is not uncommon and can be remedied by passing the suspension through a Flowmi tip a second time. Additionally, BSA (bovine serum albumin) can be added to the PBS to a final concentration of 0.04–2% without any adverse effects on the workflow or data.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Angela Ting (tinga@ccf.org).

Materials availability

This protocol did not generate new unique materials or reagents.

Data and code availability

This protocol did not generate any new data or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.E.F. and A.H.T.; Methodology, E.E.F., B.H.L., and A.H.T.; Investigation, E.E.F. and S.S.; Writing – Original Draft, E.E.F.; Writing – Review & Editing, S.S., B.H.L., and A.H.T.; Funding Acquisition, B.H.L. and A.H.T.; Supervision, A.H.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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