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May 17, 2022

HuBMAP | VU TMC Eye/Pancreas | Human Pancreas Processing for Multiple Applications V.2

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VU Biomolecular Multimodal Imaging Center
Human BioMolecular Atlas Program (HuBMAP) Method Development Community

Carrie Romer

Developed in collaboration with Dr. Rita Bottino (Imagine Pharma) and Dr. Marcela Brissova (Vanderbilt University); adapted from this standard operating procedure established by the Network for Pancreatic Organ Donors with Diabetes (nPOD).

This protocol describes the workflow for collecting human pancreas samples for multimodal downstream assays, in use by the Vanderbilt University HuBMAP Tissue Mapping Center.

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Diane Saunders

protocol

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HuBMAP, Vanderbilt, Pancreas, Paraffin-embedding, cryosections, Flash frozen sections, RNA isolation, BIOMIC

protocol ,

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May 17, 2022

May 12, 2022 Carrie Romer

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In steps of
HuBMAP | VU TMC Eye/Pancreas | Pancreas Pipeline Overview

Materials and Consumables:

- ☒ Stainless steel sterilizable tray (12.25 x 7.75 x 2) **Cardinal Health Catalog #DYND051202Z**
- ☒ All Purpose Sponge sterile **Covidien Catalog #8044**
- ☒ 4.5 straight scissors **General Econopack (STERIS Life Sciences) Catalog #AH.50-1401**
- ☒ 5.5 straight scissors **General Econopack (STERIS Life Sciences) Catalog #AH.50-9591**
- ☒ 5” Adson Kocher tweezers **McKesson Corporation Catalog #43-1-775**
- ☒ 6.25” Debakey tissue forceps **General Econopack (STERIS Life Sciences) Catalog #30-3751**

  ☒ Tissue Marking Dyes **Cancer Diagnostics Catalog #MD2000** Step 7
- ☒ Fisherbrand™ Tissue Path™ II Cassettes **Fisher Scientific Catalog #15-120-400A** Step 16
- ☒ Tissue-Tek Cryomold Standard 25x20x5mm **VWR International Catalog #25608-916** Step 21
- ☒ Aluminum Weighing Dishes 68.2-mm diameter **Fisher Scientific Catalog #08-732-103** Step 26
- ☒ Epredia™ Peel-A-Way™ Disposable Embedding Molds **Fisher Scientific Catalog #18-41** Step 26.2

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- 50-mL and 15-mL conical tubes
- Large insulated, lidded container for dry ice
- Large plastic weigh boats
- iPad or camera to take images
- Metric ruler
- Disposable scalpels
- Kimwipes
- Balance
- Adjustable tilt rocker

Reagents (see step #2 for solutions):

- 10% Neutral Buffered Formalin **Electron Microscopy Sciences Catalog #15740-01** Step 12
- 16% Paraformaldehyde **Electron Microscopy Sciences Catalog #15710** Step 17
- 2-Methylbutane **Fisher Scientific Catalog #O3551-4** Step 25
- RNAlater™ **Thermo Scientific Catalog #AM7021** Step 28
- 32% Paraformaldehyde **Electron Microscopy Sciences Catalog #50-980-495** Step 30
- D-Sucrose **Fisher Scientific Catalog #BP220-1**
- Carboxymethyl Cellulose Sodium Salt **Fisher Scientific Catalog #C9481**

Equipment:

Excelsior™ AS
Tissue Processor
Epredia™ **A82300001ER**

Safety glasses, proper gloves, and a lab coat required. The area should be adequately vented.

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1 Prepare reagents, tubes, labels, etc. as outlined in the template linked below.

HuBMAP Pancreas Processing Template

2 Prepare solutions as follows.

- **100 mM PBS, 1L:** Add 12.07 g Na_2HPO_4 (dibasic), 2.04 g KH_2PO_4 (monobasic), 8.0 g NaCl, and 2.0 g KCl to 1L glass bottle, then add MilliQ water to volume. Place on stir plate until dissolved. Filter through a 0.22 µm filter and store at 4ºC for up to 1 month.
- **70% Ethanol, 1L:** Combine 700 mL of 190 Proof ethanol with 300 mL of dH_2O.
- **16% Paraformaldehyde (PFA), for FCMC:** Add 60 mL of 16% PFA stock into a 500 mL glass bottle, then add 180 mL of 100 mM PBS. Mix gently and transfer 40-mL aliquots into prelabeled 50-mL conical tubes for fixation.
  ⚠ *This solution should be made fresh within 12 hours of use.*
- **16% Paraformaldehyde (PFA), for CLR:** Add 20 mL of freshly-opened 32% PFA ampule into a 500 mL glass bottle, then add 140 mL of 100 mM PBS. Mix gently and transfer 30-mL aliquots into prelabeled 50-mL conical tubes for fixation.
  ⚠ *This solution must be made fresh immediately prior to use.*
- **30% Sucrose:** Add 72 g sucrose to approximately 100 mL of 10 mM PBS in a 500 mL glass bottle. Place on stir plate until dissolved, then bring to 240 mL total volume with 10 mM PBS. Filter through a 0.22 µm filter and store at 4ºC for up to 1 month.
- **2.6% Carboxymethylcellulose (CMC)**: Add 500 mL water to 500 mL glass bottle and microwave for 2 minutes. Place bottle on heated stir plate (~70ºC), and slowly add 13g CMC to warmed water. Stir on low hotplate overnight, occasionally tightening lid and shaking bottle. Store at 4ºC for up to 2 months. Before each use, pour solution into 125 mL bottle and sonicate for 10 minutes.
- **10% Neutral Buffered Formalin:** Pipet 18-mL aliquots into prelabeled 50-mL conical tubes.
- **RNA*later*™ Stabilization Solution:** Pipet 40-mL aliquots into prelabeled 50-mL conical tubes.

Organ Dissection and Cross-Sectioning

3 Upon arrival, remove the pancreas from transport solution (UW or HTK) and transfer into a large

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dissection tray placed on ice. Photograph the organ and note the following prior to dissection:

Gross morphology:

- **Peri-pancreatic fat:** normal or excessive?
- **Peri-pancreatic adhesions**: yes or no?
- **Texture:** soft, firm, or hard? Any palpable masses?
- **Outer surface:** is contour smooth or irregular? Note any localized bulge, hemorrhage, breakdown, chalk-white areas, or changes in color.
- **Other external features:** describe as needed.

4 Dissect the pancreas by carefully removing duodenum, mesentery, fat, and spleen (**Figure 1**).

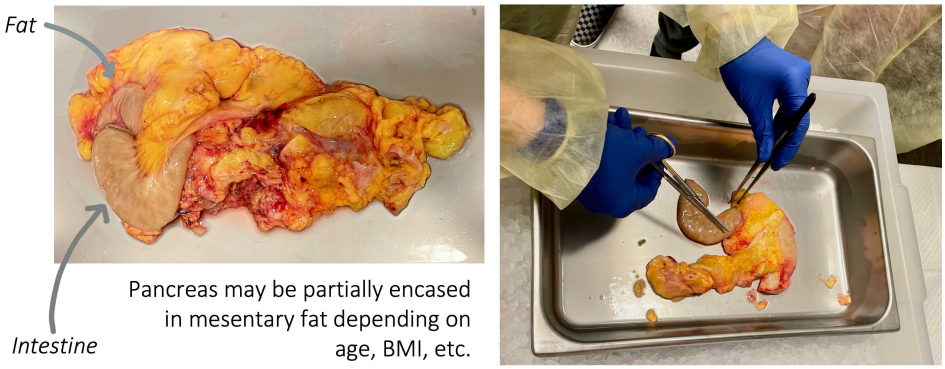

Figure 1. Pancreas may require removal of mesentary fat as well as portions of intestine or spleen.

⚠ *Note:* If time and personnel permit and there are pieces of duodenum and/or spleen, retain and freeze as tissue blocks (see **Flash Freezing** subheading below).

5 Once the pancreas is cleaned, blot with a sterile gauze square and quickly transfer to a clean plastic weigh boat. Place on a pre-tared balance and record **pancreas weight**.

6 Immediately transfer the pancreas on an ice-cooled dissecting platform and take a photograph with a ruler as shown in **Figure 2**. Record **pancreas size**.

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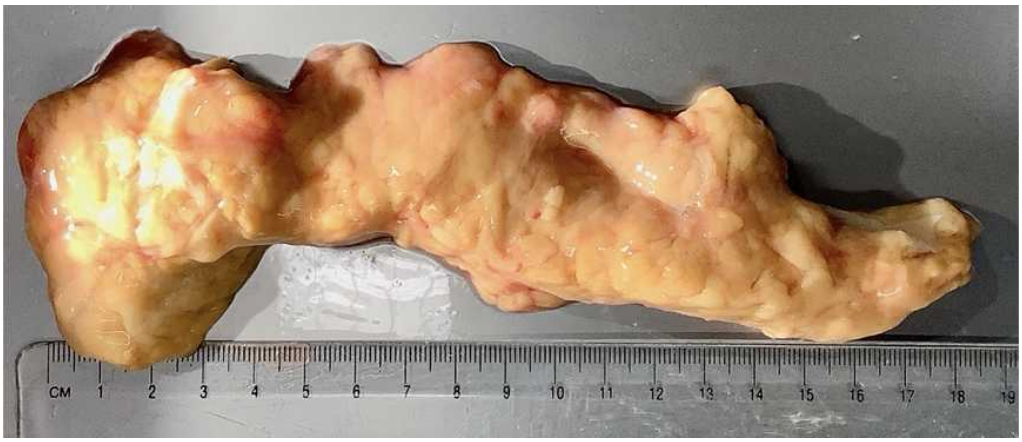

Figure 2. Example photograph of pancreas after dissecting away fat.

7 Tissue Marking Dyes **Cancer**

Apply **Diagnostics Catalog #MD2000** to length of organ as follows:

- **Anterior:** blue
- **Superior:** black
- **Posterior:** green
- **Inferior:** red

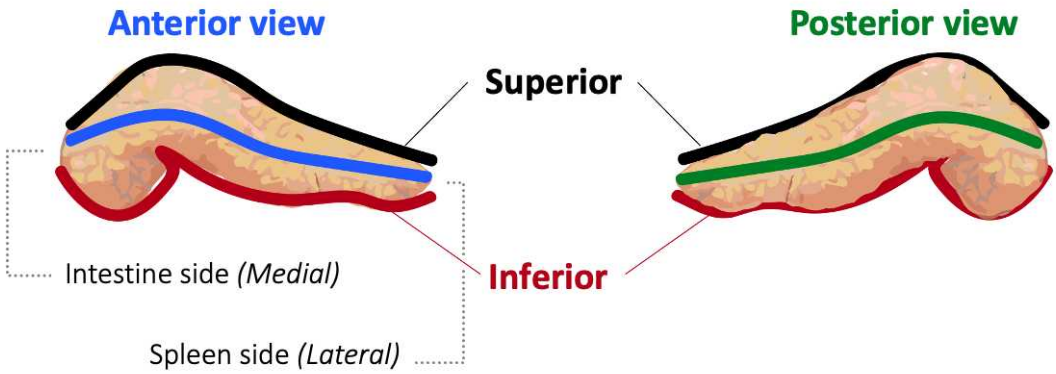

Figure 3. Schematic of marking dyes in relation to organ orientation in the body.

8 Once the pancreas has been marked, start collecting approximately **5 mm** cross-sectional slices ("discs") starting from the head (duodenum) and moving laterally towards the tail (spleen) as outlined in **Figure 4**. Each disc should contain dye markings on the exterior to provide spatial orientation.

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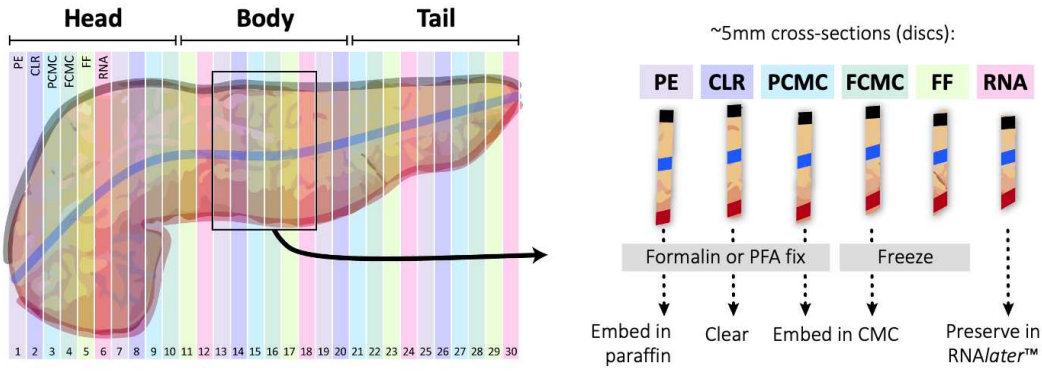

Figure 4. Map illustrating serial collection of cross-sections (discs) for different modalities/applications, moving from Head to Tail region. Processing types are alternated in sequence.

9 Photograph each disc, medial side down, using prelabeled cards with nomenclature developed for Vanderbilt LIMS (encodes positional information and processing type).

As discs are photoraphed, make notes on the experimental spreadsheet if any of the following **gross morphology** is observed:

- Dilated ducts, cystic change, or stones
- Hemorrhage
- Tissue breakdown (necrosis)
- Chalk-white areas (calcification)
- Visible neoplasms
- Other features

10 Divide each disc into four approximately equal-sized quadrants, attempting to bisect dye markings so that each tissue piece contains 2 dye colors (**Figure 5**). Photograph disc again after quadrants are cut.

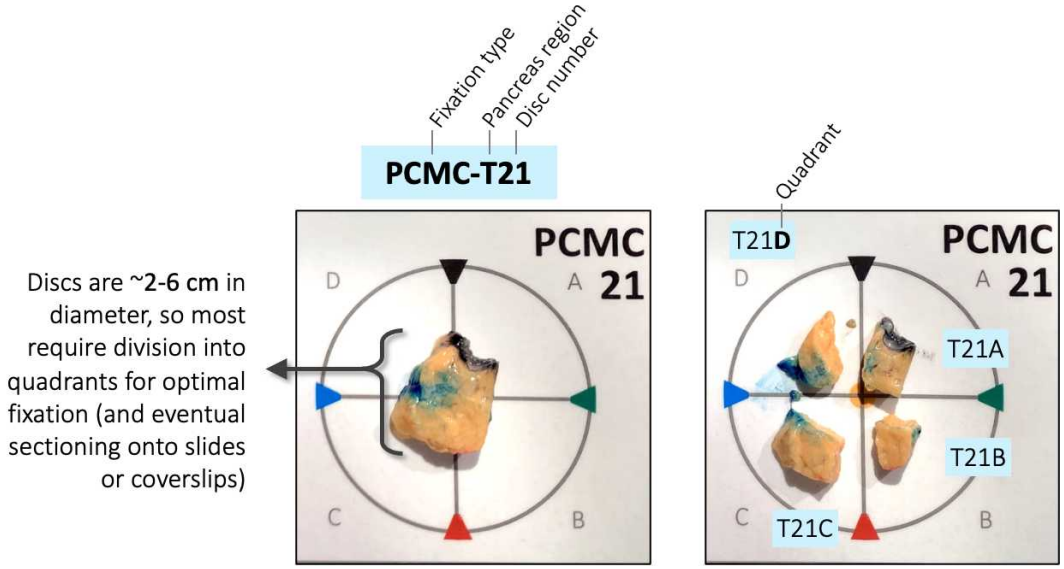

Figure 5. Example of photographing a disc before (left) and after (right) division into quadrants. This particular disc (#21) was assigned to PFA Fixation and CMC Embedding (PCMC) and falls within the Tail region; blue labels indicate nomenclature to record processing and spatial orientation.

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11 Transfer each set of quadrants to the appropriate processing protocol as outlined in **Figure 4**.

Formalin Fixation and Paraffin Embedding (PE)

12 Place all tissue quadrants from one disc into a 50-mL Falcon tube containing **40 mL** of 10% Neutral Buffered Formalin **Electron Microscopy Sciences Catalog #15740-01** .

13 Fix **Overnight** or at least **18:00:00** at **4 °C** under mild agitation using an adjustable tilt rocker (LabNet).

14 Wash tissue four times in **40 mL** of 100 mM PBS at **4 °C** for a period of **03:00:00** , rocking. Blot the tube with paper towel before adding the fresh washing solution.

15 After the last wash, add **40 mL** of 70% ethanol and store at **4 °C** .

16 To process for paraffin embedding, transfer fixed tissue samples from 50-mL tube into a 10-cm petri dish. Using the photographs described in step #10 and tissue marking dyes, identify quadrants A-D. Place each quadrant, medial side down, into prelabeled Fisherbrand™ Tissue Path™ II Cassettes **Fisher Scientific Catalog #15-120-400A** .

16.1 Follow schedule as follows using **Epredia™ Excelsior™ AS Tissue Processor** (total 16 hours):

- 70% Ethanol, one change - **00:45:00**
- 90% Ethanol, one change - **00:45:00**
- 95% Ethanol, one change - **00:45:00**
- 100% Ethanol, three changes - **00:35:00** each
- Xylene or xylene substitute (e.g., Clear Rite 3), three changes - **00:35:00** each
- Paraffin wax, **58 °C** to **60 °C** , three changes - **01:00:00** each

16.2 Trim blocks as necessary and store at **Room temperature** .

Light PFA Fixation and CMC Embedding (PCMC)

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17 Place all tissue quadrants from one disc into a 50-mL Falcon tube containing **40 mL** of freshly prepared fixative (4% PFA*/100 mM PBS).

16% Paraformaldehyde **Electron Microscopy Sciences Catalog #15710**

* is used; see step #2 for preparation details.

18 Fix for **03:00:00** **On ice** under mild agitation using an adjustable tilt rocker (LabNet).

19 Wash tissue four times in **40 mL** of 100 mM PBS **On ice** for a period of 2-3 hours (**00:30:00** per wash), rocking. Blot the tube with paper towel before adding the fresh washing solution.

20 After the last wash, equilibrate the tissue in **40 mL** of sucrose solution (30% sucrose/10 mM PBS) at **4 °C** **Overnight** . Tissue will settle to bottom of the tube.

21 Fill a prelabeled

Tissue-Tek Cryomold Standard 25x20x5mm **VWR International Catalog #25608-916**

with a thin layer of carboxymethylcellulose (CMC). Pour tissue samples and sucrose solution from 50-mL tube into a 10-cm petri dish. Using the photographs described in step #10 and tissue marking dyes, identify quadrants A-D.

22 Pick each quadrant up with a pair of fine forceps, blot on a Kimwipe to remove excess sucrose, and place into the appropriate CMC-prepared cryomold. Using the forceps, push the tissue lightly to bottom of the cryomold, medial side down.

23 Transfer the cryomold to a dry ice block to freeze, adding more CMC to completely fill the indentation. When CMC compound is completely frozen, wrap each tissue block tightly in a pre-labeled aluminum foil and place it a storage bag to prevent it from drying out.

24 Store samples at **-80 °C** .

▷ *If sample transfer is required, ship blocks on dry ice.*

Flash Freezing +/- CMC Embedding (FF, FCMC)

25 **Prepare isopentane slurry:**

Place a layer of dry ice into an insulated, lidded container and add enough

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2-Methylbutane **Fisher Scientific Catalog #O3551-4** (isopentane) to make a slurry.

26 Place each tissue quadrant into a prelabeled Aluminum Weighing Dishes 68.2-mm diameter **Fisher Scientific Catalog #08-732-103** and transfer to isopentane slurry. Cover box with lid and allow tissue to completely freeze.

26.1 For **FF** samples (no embedding), wrap frozen tissue samples tightly in prelabeled squares of aluminum foil.

26.2 For **FCMC**, apply a thin layer of CMC into prelabeled Epredia™ Peel-A-Way™ Disposable Embedding Molds **Fisher Scientific Catalog #18-41** on the isopentane slurry. Allow the CMC to begin freezing (noted by turning opaque) around the edges before placing tissue quadrant into the mold. Slowly add more CMC on top of the tissue until it is covered, keeping the lid closed over the slurry as much as possible. When completely frozen, wrap mold in prelabeled square of aluminum foil.

27 Place all wrapped samples in prelabeled storage bags and store at **-80 °C** .

▷ *If sample transfer is required, ship blocks on dry ice.*

Preservation for Spatial Transcriptomics (RNA)

28 Place all tissue quadrants from one disc into a 50-mL Falcon tube with approximately 5 volumes of RNAlater™ **Thermo Scientific Catalog #AM7021** (four quadrants ≈ 3.5 g or **18 mL**).

29 Store samples at **4 °C** .

▷ *If sample transfer is required, ship sealed tubes on ice packs.*

PFA Fixation for Tissue Clearing (CLR)

30 Place all tissue quadrants from one disc into a 50-mL Falcon tube containing **40 mL** of freshly prepared fixative (4% PFA*/0.1 M PBS).

32% Paraformaldehyde **Electron Microscopy** * **Sciences Catalog #50-980-495** stock is used; see step #2 for preparation details.

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31 Fix for **16:00:00** at **4 °C** under mild agitation using an adjustable tilt rocker (LabNet).

32 Wash tissue three times in **40 mL** of 100 mM PBS **On ice** under mild agitation.

33 Continue immediately to tissue clearing protocol, or store samples at **4 °C** in 100 mM PBS containing 0.1% sodium azide.

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