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TITLE:

A Simple High Efficiency Protocol for Pancreatic Islet Isolation from Mice

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KEYWORDS:

Islets, pancreas, collagenase, density gradient, insulin, diabetes, mice

SUMMARY:

This islet isolation protocol described a novel route of collagenase injection to digest the exocrine tissue and a simplified gradient procedure to purify the islets from mice. It involves enzymatic digestion, gradient separation/purification, and islet hand-picking. Successful isolation can yield 250–350 high quality and fully functional islets per mouse.

ABSTRACT:

Pancreatic islets, also called the Islets of Langerhans, are a cluster of endocrine cells which produces hormones for glucose regulation and other important biological functions. The islets primarily consist of five types of hormone-secreting cells: α cells secrete glucagon, β cells secrete insulin, δ cells secrete somatostatin, ε cells secrete ghrelin, and PP cells secrete pancreatic polypeptide. Sixty to 80% of the cells in the islets are β cells, which are the most important cell population to study insulin secretion. Pancreatic islets are a crucial model system to study ex vivo insulin secretion. Acquiring high quality islets is of great importance for diabetes research. Most islet isolation procedures require technically difficult to access site of collagenase injection, harsh and complex digestion procedures, and multiple density gradient purification steps. This paper features a simple high yield mouse islet isolation method with detailed descriptions and realistic demonstrations, showing the following specific steps: 1) injection of collagenase P at the ampulla of Vater, a small area joining the pancreatic duct and the common bile duct, 2) enzymatic digestion and mechanical separation of the exocrine pancreas, and 3) a single gradient purification step. The advantages of this method are the injection of digestive enzyme using the

more accessible ampulla of Vater, more complete digestion using combination of enzymatic and mechanical approaches, and a simpler single gradient purification step. This protocol produces approximately 250—350 islets per mouse; and islets are suitable for various ex vivo studies. Possible caveats of this procedure are potentially damaged islets due to enzymatic digestion and/or prolonged gradient incubation, all of which can be largely avoided by careful ad justification of incubation time.

INTRODUCTION:

There are two common methods in the literature for pancreatic islet isolation. One requires excising the pancreas and dicing it into small pieces using surgical scissors, and then digesting it in a collagenase solution[1-3]. Another more precise method is to use the network of ducts present in the pancreas to introduce digestive enzyme. The following sites have been used for digestive enzyme injection: the junction of the bile and cystic duct, the gallbladder into the common bile duct, or the common bile duct itself[1,4,5]. It is known that islets are not evenly distributed in the pancreas; the splenic region contains the most islets[6]. While the second method using anatomical routes to deliver digestive enzymes allows for a more complete perfusion of the pancreas, including the splenic region, this procedure often requires clamping or suturing of the ampulla of Vater that is technically challenging. In terms of islet purification, multiple density gradients, as well as cell strainers and magnetic retraction have been used to purify the islets[3,7]. The utilization of these gradients can be time consuming and the Ficoll gradients can result in toxic damage of islets[8].

The current protocol is built on the method described by Li et al.[7], with additional modifications added based on the experience of ourselves and others[1,4]. The most critical steps of our protocol are clamping of the common bile duct near the liver end, injecting collagenase P via the ampulla of Vater to digest the exocrine tissue, and then using a shaking water bath to expedite the digestion mechanically[1,4,7]. Subsequently, a 'STOP' solution is applied to inhibit further digestion of the islets; HBSS is used to wash off the remaining collagenase P and STOP solution. When the Ficoll method was used to purify human islets, yield was reported to be twice the islets with greater functional capability (e.g., insulin secretion) as compared to the use of Percoll gradients[9]. However, studies have questioned the use of Ficoll gradient due to its toxic effect on the islets [1,10]. It has been reported that the Histopaque gradient provides optimal purification kinetics for mouse islet isolation, which produces good yield of high quality islets with simpler steps and lower cost[1]. In our protocol, Histopaque-1077 is used to purify islets from other residual tissue[8,11]. The harvested islets can be cultured in complete RPMI-1640 media, or directly utilized in RNA/protein quantitation.

Our protocol, using a combination of collagenase P digestion and a single gradient purification step, is simpler than other published protocols. Our method does not require demanding surgical procedures and has just a few simple steps. More importantly, this protocol consistently produces a good yield of high quality functional islets (250-350/mouse) as we reported [12].

PROTOCOL:

All methods described here have been approved by the Animal Care and Use Committee (ACUC) of Texas A&M University. The surgical tools need is shown in **Figure 1** and the schematic diagram of the procedure is shown in **Figure 2**.

1. Solutions

1.1. Prepare Hank's Balanced Salt Solution (HBSS) by adding 100 mL of 10X HBSS (from stock) to 900 mL of distilled water to make 1 L HBSS (1X).

1.1. Prepare STOP solution (must be made fresh and should be used within 1 h) by adding 50 mL of 100X fetal bovine serum (FBS) to 450 mL of ice cold 1X HBSS; this makes 500 mL STOP solution. Keep the STOP solution at 4 °C.

1.2. Prepare collagenase P solution (must be made fresh within 1 h of being used) by adding 1 mg/mL of collagenase P to ice cold 1X HBSS; use 6 mL/mouse.

1.2.1. Add 0.05% (w/v) bovine serum albumin (BSA) to collagenase P solution (this provides nutrients to the isolated islets). For example, use 3 mg BSA in 6 mL collagenase P solution. **Keep on ice**.

1.2.2. Calculate and prepare the amount of collagenase P needed for all mice in one 50 mL tube.

1.3. Prepare complete RPMI 1640 medium by adding 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, INS-1 cell supplement (10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol) to 500 mL RPMI 1640 media containing 11.1 mM glucose to be used for overnight culture and incubation.

2. **Preparation**

2.1. Fill three 50 mL tubes of 25 mL RNase inhibiting solution, 70% ethanol or distilled H_2O. These solutions will be used for cleaning surgical tools prior and during the procedure.

2.2. Soak the tips of the tools in RNase inhibiting solution for 30 min before starting the protocol; this eliminates any potential RNase on the tools.

2.3. Pre-cut absorbent pads to 6 in x 6 in size for use during surgery.

2.4. Prepare 1X HBSS solution in advance and store at 4 °C. Prepare STOP solution prior to surgery. Store at 4 °C.

2.5. Prepare collagenase P solution immediately prior to surgery and store on ice.

NOTE: This should be used within 2 h of preparation.

2.6. Label 50 mL tubes for digestion and purification; prepare 2 tubes for each mouse, one for digestion and the other for islet purification. Ensure that the animal ID is on both tubes.

2.7. Add 3 mL of collagenase P solution into the first 50 mL tube. The remaining 3 mL of collagenase P will be injected.

2.8. Draw the remaining 3 mL of collagenase solution into a 3 mL syringe mounted with 30 G ½ in needle. Place the syringe on ice.

3. Procedure

3.1. Remove all the tools from RNase inhibiting solution, then dip them first in the tube with 70% ethanol, then in the tube containing distilled H_2O, then air-dry on clean paper towel.

3.2. Place the mouse in a chamber containing 0.5 mL of isoflurane until mouse is deeply anaesthetized.

3.3. Remove mouse from the chamber and check the state of anesthesia by pinching a foot pad with forceps. Deep anesthetization is based on the observation that breathing become steadily slow and mouse is unreactive to foot pinching. After confirming that the mouse is deeply anaesthetized, euthanize the mouse with cervical dislocation, and then place the mouse on the absorbent pad.

3.3.1. Place anaesthetized mouse on its stomach, applying pressure to the neck and dislocating the spinal column from the brain by pulling tail.

3.4. Tape the limbs of the mouse in supine position to the absorbent pad, spray the body with 70% ethanol, and wipe excess off the excess ethanol.

3.5. Use cover glass forceps and curved surgical scissors to make incisions. First make a horizontal incision on the skin of the abdominal area (~3 cm), pull the skin wide open to expose the abdominal wall. Then make a vertical incision (~3–4cm) on the abdominal peritoneum to fully expose the pancreas in abdominal cavity (**Figure 3**).

3.6. Push the lobes of the liver superiorly to expose the bile duct, it will appear as a pale pink tube (**Figure 3**).

3.7. Carefully move the intestines from the right lumbar/iliac region of the abdominal cavity to the right, exposing the bile duct and hepatic artery (**Figure 3**).

3.8. Carefully clamp the common bile duct using the Schwartz micro serrefines (**Figure 1**) as close to the liver as possible.

3.9. Identify the ampulla of Vater, which is located at the duodenal papilla, formed by the union

of the pancreatic duct and the common bile duct. The ampulla of Vater appears swollen when viewed under a dissection microscope which is the entry point to the common bile duct (**Figure 4**).

NOTE: Adjusting intensity/angle of light of the dissection microscope can make it easier to locate.

3.10. Insert the syringe with 3 mL of the collagenase P solution into the ampulla of Vater. Push the needle into the duct for about 1/4 of the length of the common bile duct (ampulla leads into duct) as shown in **Figure 5.**

3.11. Once the needle is in the ampulla, ensure that the orientation of the needle is such that it is parallel with the duct.

3.12. Stabilize the needle by clamping with micro Adson forceps to prevent it from puncturing the duct (**Figure 5**).

3.13. Slowly and steadily inject 3 mL of the collagenase P solution from the syringe into the common bile duct (enough to feel resistance) as shown in **Figure 6**. The goal is to create backflow pressure to force the collagenase to enter the pancreatic duct. Injection is considered successful if the head, neck, body and tail region of the pancreas are all fully inflated.

NOTE: Islet yield will be low if either the pancreas is not fully inflated, or the splenic area is not fully inflated. The splenic area contains the highest number of islets[6]. Inflation can be confirmed by the appearance of open spaces between pancreatic tissue that are filled with solution.

3.14. Carefully dissect out the inflated pancreas and place it in a 50 mL digestion tube containing 3 mL of ice-cold collagenase P solution.

3.14.1. Remove the pancreas using 2 forceps (curved and cover glass; See **Figure 1**): (starting from the spleen, pull the pancreas away from spleen and continue removing from the stomach and along the duodenum.

NOTE: No incisions required.

3.14.2. Chop pancreas for 3–5 s in the digestion tube with 3 mL of ice-cold collagenase P solution using fine surgical scissors (**Figure 7A**).

3.15. Secure the tube to a rack in 37 °C water bath and shake at 100–120 rpm for approximately 12–13 min.

3.16. After incubation, gently invert the tubes by hand to disrupt the tissue until the collagenase P digestion solution becomes homogenous (**Figure 7B**). Homogeneity is confirmed by a sand-like appearance of fine particles of pancreas. About 30 s of gentle hand-shaking is usually enough. Hold the tube up to light to examine if the tissue is well homogenized; shake for another ~15 s if

needed.

3.17. Once digested, immediately place the tubes on ice and add 40 mL of ice-cold STOP solution to terminate the enzymatic digestion.

NOTE: At this point digestion tubes can be left on ice up to 2 h, if working on multiple mice.

3.18. Centrifuge the tube in a swinging-bucket centrifuge at 300 x *g* for 30 s (temperature of centrifuge is flexible).

NOTE: Use a swinging-bucket centrifuge so that the tissue pellet is formed at the bottom of the 50 mL tube and not on the wall of the tube; this is critical for better islet yield.

3.19. Decant and repeat the centrifugation with STOP solution 2 more times, decanting the solution after each spin. Use 20 mL of STOP solution for each subsequent wash.

3.19.1. Before each spin, disrupt the pellet by gently shaking the tissue pellet in the 50 mL tube in 20 mL STOP solution.

3.20. Re-suspend the tissue pellet with 40 mL of ice cold HBSS and centrifuge at 300 x *g* for 30 s.

3.21. Decant and repeat the centrifugation using a swinging-bucket centrifuge with HBSS solution two more times, decanting the solution after each spin. Use 20 mL of HBSS for each wash.

3.21.1. Before each spin, disrupt the pellet, to detach it from the bottom of the tube by gently shaking the tube containing 20 mL HBSS solution.

3.22. After the last centrifugation remove all HBSS.

3.23. Then add 5 mL of room temperature density gradient to the 50 mL tube containing the pellet. Vortex briefly at low speed until homogenized.

3.24. Add another 5 mL of the room temperature density gradient to the 50 mL tube. **Do not vortex/mix.**

NOTE: It is **critical** to **remain steady and still** so as to allow better gradient to form without disruption.

3.25. Pipette 10 mL of room temperature HBSS buffer into the tube (containing the density gradient), **drop-by-drop gently** and **slowly** to allow a gradient to form. Use a pipette gun with a 10 mL pipette to add HBSS dropwise.

3.26. Using a swinging-bucket centrifuge, spin tubes at 1700 x *g* for 15 min. Make sure to change the speed of both acceleration/deceleration to the lowest setting to maintain the gradient

(**Figure 7C**).

3.27. After the spin, carefully remove the tubes without disturbing the gradient. Using a pipette gun pre-wetted with cold HBSS (pipette HBSS up and down), pipette out the layer of islets (5–10 mL) formed between the density gradient and HBSS into the new 50 mL islet collection tube.

NOTE: It is helpful to wet the pipette with cold HBSS prior to pipetting the islets to prevent the islets from sticking to the inner walls of the pipette.

3.27.1. In case the separation is incomplete, islets would be visible in the density gradient layer, pipette out the entire 10 mL of the density gradient (bottom layer) along with the islet layer formed at the interface (only to leave about 8–10 mL of the HBSS top layer behind).

NOTE: Collecting both the islet layer and the density gradient layer (bottom layer) can result in the collection of debris which may lengthen the islet picking time, but no other steps will be altered. Collecting both these layers is not necessary when islet layer is well formed.

3.28. Add 20 mL of ice cold HBSS to the new 50 mL tube containing islets, then centrifuge at 350 x *g* for 3 min in a swinging-bucket centrifuge.

3.29. After the spin, carefully pipette (**do not decant**) out the supernatant (leave ~3 mL at the bottom) without disturbing the pellet containing the islets at the bottom. Discard the supernatant.

3.30. Repeat washing and centrifuging at least 3 times. Add 20 mL of HBSS each time, and be sure to suspend the pellet before each spin.

3.31. Warm the RPMI-1640 complete media bottle in a water bath at 37 °C prior to use. After the last centrifugation, remove all of the HBSS and add 4 mL of previously prepared complete RPMI-1640 media (containing FBS, INS-1 cell supplement and penicillin/streptomycin) to the pellet.

3.32. Dislodge the pellet by gently swirling the tube and immediately pour the RPMI-1640 media into a 100 mm Petri dish. Add another 5 mL of RPMI-1640 media to the tube and gently swirl it to wash off any remaining islets, then also pour the media into the same Petri dish.

3.32.1. Under a dissection microscope, pick healthy islets from the Petri dish using a 20 µL pipette, and put them in a new Petri dish containing 10 mL of complete RPMI 1640 media. When viewed under a dissection microscope, islets should appear spherical/oblong and golden-brown color with a smooth surface compared to the relatively transparent, wispy exocrine tissue.

NOTE: Magnification of dissection microscope is usually set at 12.5–16x. Islet yield will vary depending on various factors including strain, age and sex of mouse. This protocol usually yields 250–350 healthy islets from healthy C57BL/6J mouse age 4–10 months old.

3.33. Incubate the islets in a sterile incubator at 37 °C with 5% CO_2 infusion and 95% humidified air overnight for experiments the next day, or freeze the islets for desired analysis at a later time.

3.33.1. Once islets are frozen, they cannot be used for secretion assays, only RNA or protein quantification. To collect cells to freeze, place them in HBSS buffer, collect all islets in 200–500 µL of buffer, transfer to 1.5 mL tube, centrifuge 350 x *g* for 1–2 min, remove the supernatant leaving no more than 30–40 µL solution, place in -20 °C for short term storage (several days) or -80 °C for long term storage.

REPRESENTATIVE RESULTS:

Proper completion of this procedure requires some understanding of mouse anatomy in the abdominal cavity. This allows for proper identification of the ampulla of Vater and clamping of the common bile duct. The entire procedure normally takes 1–2 h. It is more efficient to isolate islets from 4–6 mice at the same time, so several samples can be centrifuged together. The time for islet-picking varies, depending on the number of islets and the efficiency of digestion; it may take roughly an hour to pick 250–350 islets from 1 mouse.

In this paper, a number of very realistic images are included: **Figure 3** shows the abdominal cavity of the mouse, exposing the common bile duct and hepatic artery. **Figure 4** shows the entire length of common bile duct, which appears to be a lighter color, as well as the ampulla of Vater, which is bigger and shinier near the juncture (where the needle will be inserted) between the pancreas and duodenum. A clamp is placed at the common bile duct and hepatic artery bundle close to the liver to block off the flow of collagenase P into liver. Failure to clamp the bile duct correctly or tight enough will result in leakage and incomplete perfusion of the pancreas. **Figure 5** shows the needle inserted at the ampulla of Vater into the common bile duct. Once the needle is in the common duct, forceps are used to stabilize the needle to prevent it from puncturing the duct while injecting. Once the injection begins, the pancreas will begin to swell from proximal end to distal end; the splenic region should begin to inflate after about 1 mL of collagenase injection. Backflow into the intestines can lead to an undesirable inflation of the duodenum; this can be remedied by readjusting the placement of the needle (pull it out a little, or reinsert slightly deeper) and by properly stabilizing the needle using forceps. The injection is considered a success if all regions of the pancreas are inflated (duodenal, gastric, and splenic lobes) as shown in **Figure 6**. Removal of the pancreas should begin from the splenic region. The inflated pancreas is chopped into chunks using fine surgical scissors (**Figure 7A**). After the 12–13 min digestion and mixing by hand-shaking, the tissue-containing suspension appears more homogeneous (**Figure 7B**). Subsequently, the islets are purified by the density gradient after centrifugation. **Figure 7C** shows that an islet suspension layer is formed between HBSS and the density gradient after centrifugation. Good/healthy islets appear as smooth round-shaped; bad/damaged islets show rough edges, and undigested exocrine tissue shows irregular shape and appears more translucent as shown in **Figure 8**.

FIGURE AND TABLE LEGENDS:

Figure 1: Surgical tools. Curved surgical scissors, cover glass forceps, micro Adson forceps, curved forceps, small surgical scissors, and Schwartz micro serrefines (microvascular clamp) are shown.

Figure 2: Schematic illustration of the protocol. The most critical steps of this procedure are the clamping of the common bile duct near the liver, and injecting collagenase P via the ampulla of Vater into common bile duct to digest the pancreas.

Figure 3: Location of common bile duct. Forceps hold up the common bile duct and hepatic artery bundle.

Figure 4: Illustration of common bile duct and ampulla of Vater. A clamp is placed on the common bile duct and hepatic artery bundle near the liver. The black arrows point to the ampulla of Vater where the needle will be inserted.

Figure 5: Cannulation of common bile duct. After proper cannulation of the common bile duct, the ampulla of Vater is injected with trypan blue (for demonstration purpose only) to better emphasize the placement of the common bile duct.

Figure 6: Fully inflated pancreas. The dotted line shows the boundary of the fully perfused pancreas. Forceps hold up the splenic region where islets are most concentrated.

Figure 7: Islet isolation and purification steps. (A) Mechanically chopped tissue pieces of pancreas before digestion. **(B)** Digested pancreas—homogeneous tissue suspension of collagenase-perfused pancreas after 13 min of incubation in a shaking water bath at 37 °C, followed by 30 s of mixing by hand. **(C)** Islet suspension layer is formed between the HBSS and the density gradient after centrifugation.

Figure 8: Representative islet images from Fluorescence Cell-Imager. (A) Good islets, bad islets and exocrine tissue are seen. **(B)** A cluster of good islets is shown. **(C)** Panel shows undigested exocrine tissue attached to a good islet. Good/healthy islets show smooth round edges, indicated by the red letter “G”; bad/damaged islets show irregular shape and rough edges, and are indicated by blue letter “B”. Undigested exocrine tissues appear translucent, often attached to islets, and are indicated by green letter “E”.

DISCUSSION:

This protocol includes collagenase perfusion and digestion, followed by purification of islets. The most critical steps of this protocol are effective injection and complete perfusion of the pancreas[1,4,7]. The delivery method of this protocol allows the enzyme to traverse the anatomical routes to better digest the exocrine tissue surrounding the islets[1]. In addition, this technique is well suited for complete digestion of the splenic region, which has the highest concentration of islets[4,6]. This protocol, with well-controlled digestion time and carefully executed purification steps, can produce 250–350 healthy islets. The islets isolated from this protocol have been used successfully to study glucose-stimulated insulin secretion ex vivo[12]. From our experience, insulin secretion increased 4-fold upon high glucose stimulation (22.2 mM) compared to baseline (3.3

mM) after overnight incubation in 5.5 mM glucose-containing RPMI-1640 complete media. The survivability/functionality of the islets under prolonged incubation (2–7 days) has not been tested.

Even though the presented protocol includes detailed notes and visual demonstrations, some adjustments are needed to achieve the optimal conditions for high yield and high-quality islets. Two most common problems that may impede the success are improper cannulation of the ampulla of Vater or accidental puncture of the duct. To avoid these, one should ensure that the site of penetration is precisely where the ampulla meets with the duodenum. This area is bigger and relatively easy to identify, which allows for multiple punctures without seriously compromising the integrity of the duct. Once within the ampulla, the orientation of the needle should be parallel with the duct rather than angled. At this point, push the needle into the duct for about 1/4 of the duct's length, and then stabilize the needle with forceps while slowly injecting collagenase; this will help to prevent the needle from bending under the pressure and accidentally puncturing the duct. Other problems may include over- or under-digestion of the pancreas; this may require modification based on age, strain and sex of the mice. Damaged islets due to over-digestion (enzymatic and mechanical) or prolonged exposure to the density gradient may occur. These are common problems that exist for similar methods; some minor adjustments should yield significant improvements. A suggestion when dealing with these types of issues is to choose one variable to modify at a time (e.g., time of digestion or concentration of collagenase).

The main advantage of this protocol is the route of enzyme delivery: having collagenase P to directly digest the exocrine pancreas using an easier anatomical route which increases digestion efficiency[1]. This method has been reported to yield a 50% increase in number of islets compared to the method of excising the pancreas, chopping it, and exposing it to collagenase[13]. This protocol only requires the use of a single density gradient, making it significantly less labor-intensive and more cost-effective, as compared to other methods which require the preparation of multiple gradients at different densities, or the complex Percoll method that requires additional time[1,8,11]. The gradient method employed in this protocol has also be used in islet isolation by others[4]. This method of islet isolation provides the scientist with an improved tool for studying pancreatic islets. Future applications of this protocol include alteration to enhance the efficacy when dealing with diabetic mice. As Do et al. have observed, diabetic mice, depending on glucose levels, yield fewer islets (less than 100), with reduced size and appearance of islets[4]. We have observed this phenomenon and believe that diabetic islets are more vulnerable to enzymatic and mechanical digestion which require special care. Additionally, islet density may alter its appearance in the density gradient, further optimization of the protocol would help to increase the yield of diabetic islets.

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DISCLOSURES:

The authors have nothing to disclose.

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Fig. 1

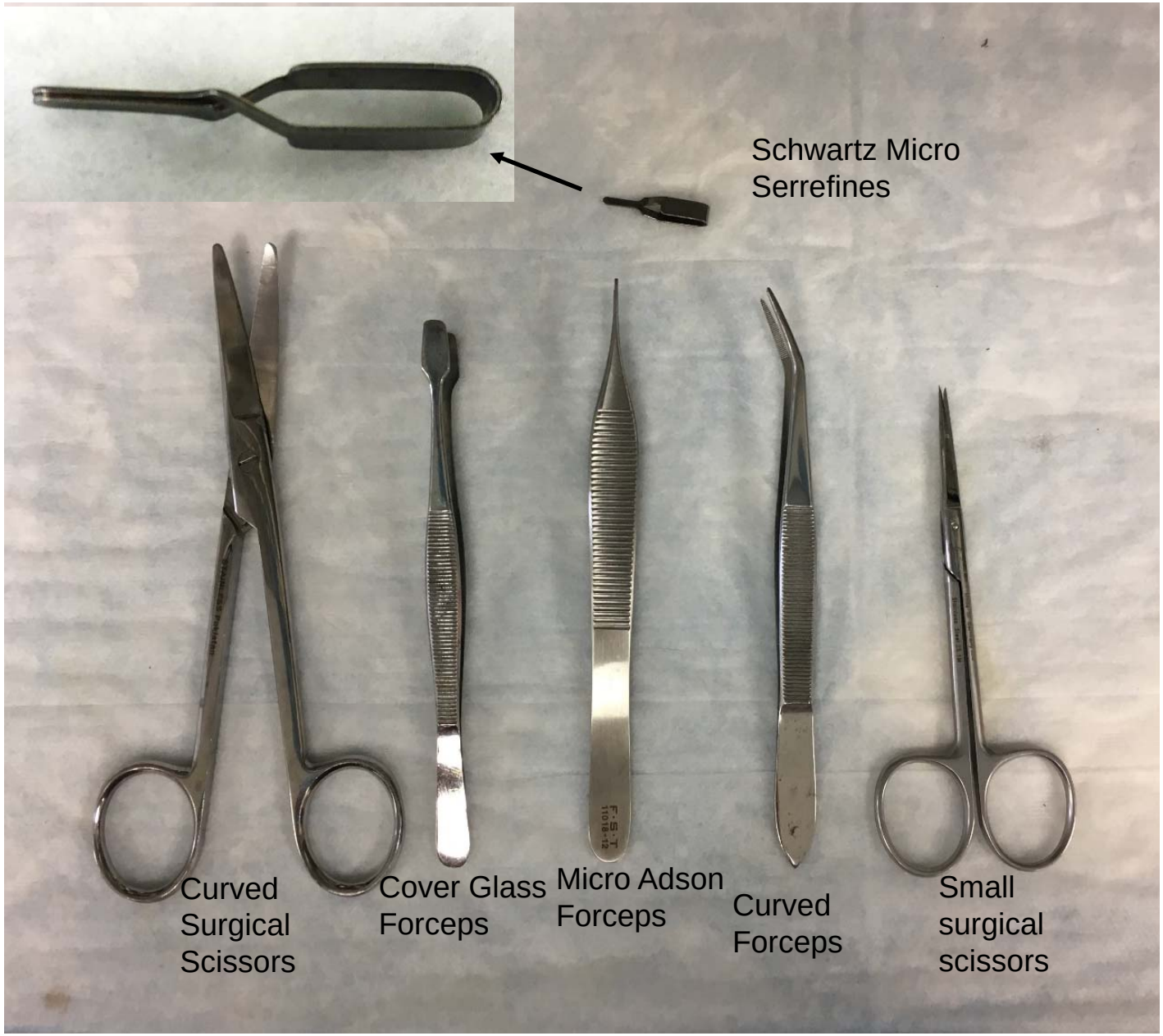


Fig. 2

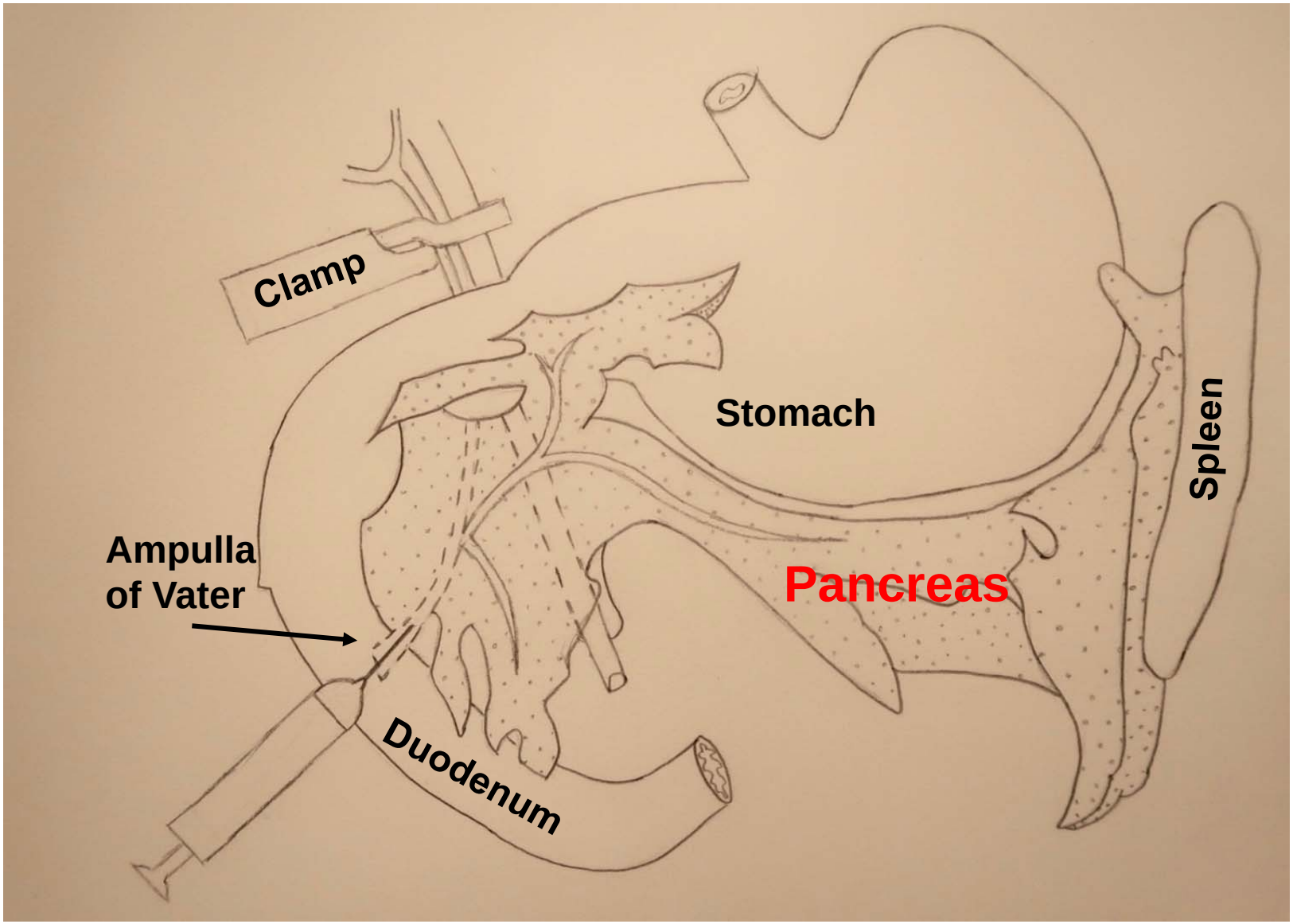


Fig. 3

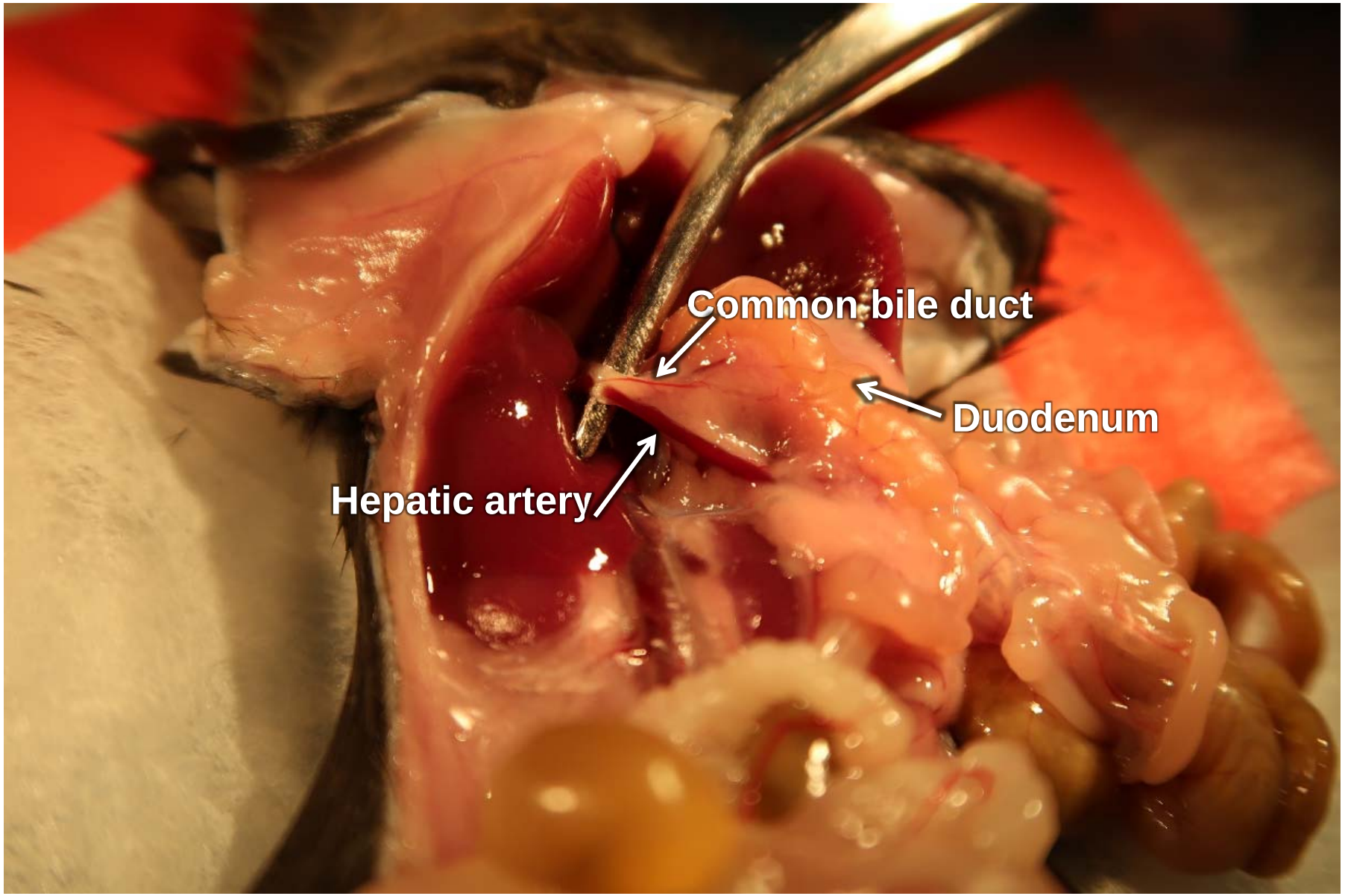


Fig. 4

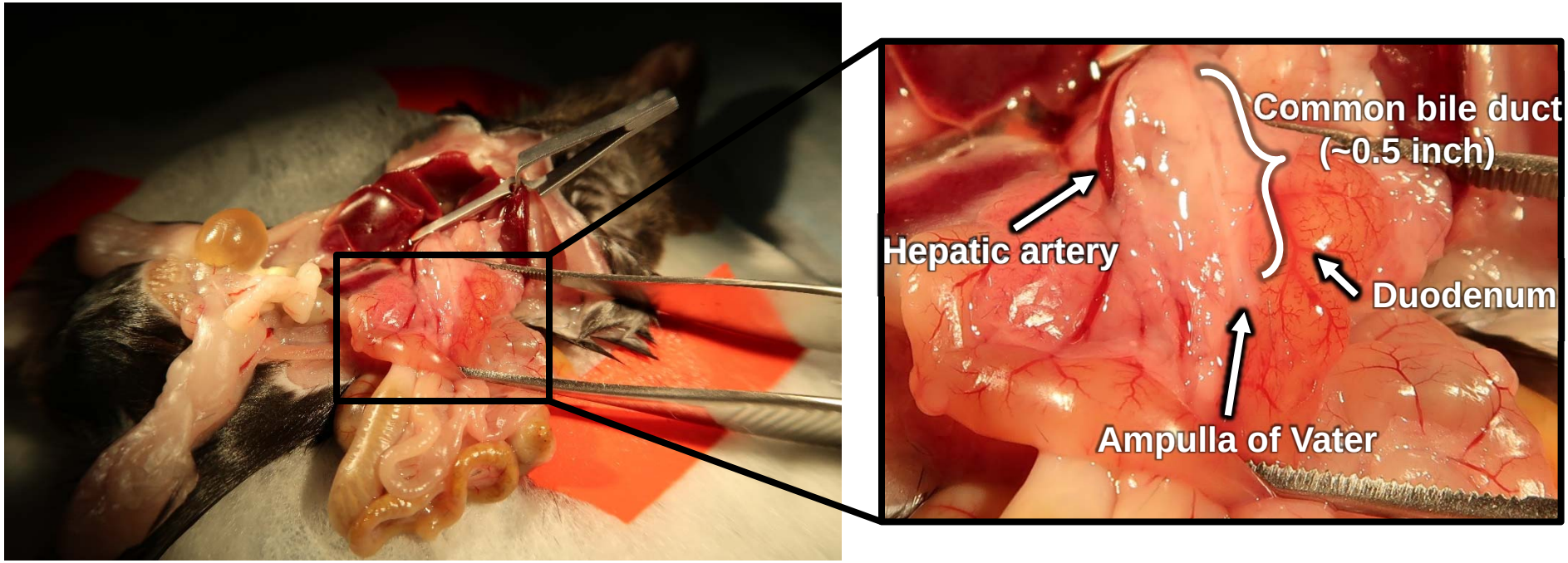


Fig. 5

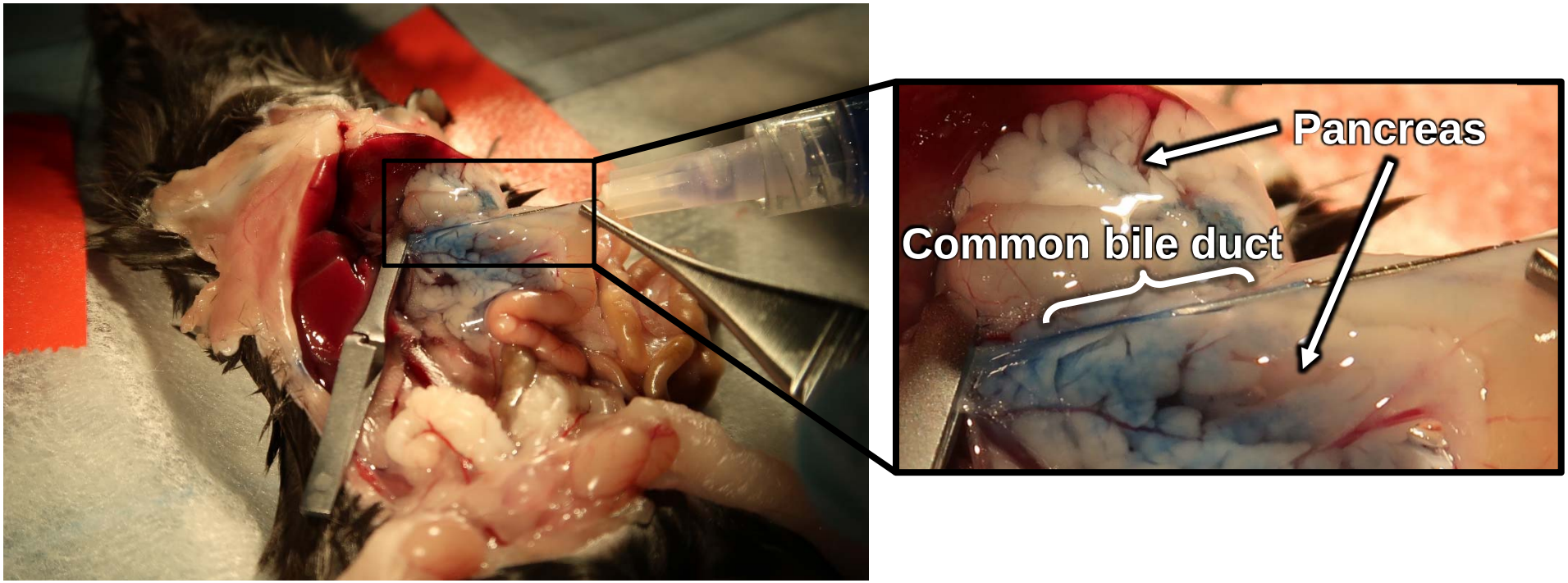


Fig. 6

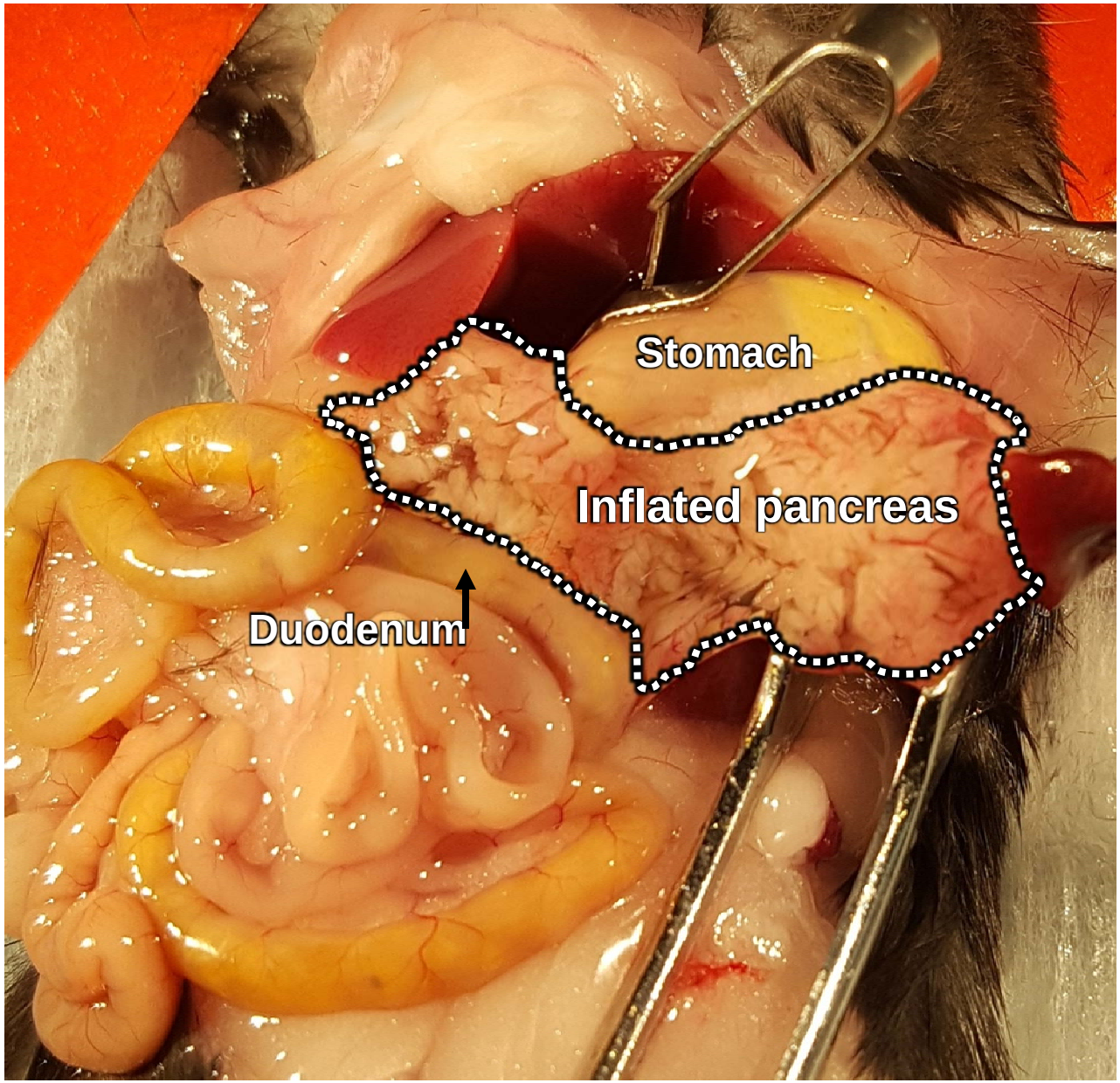
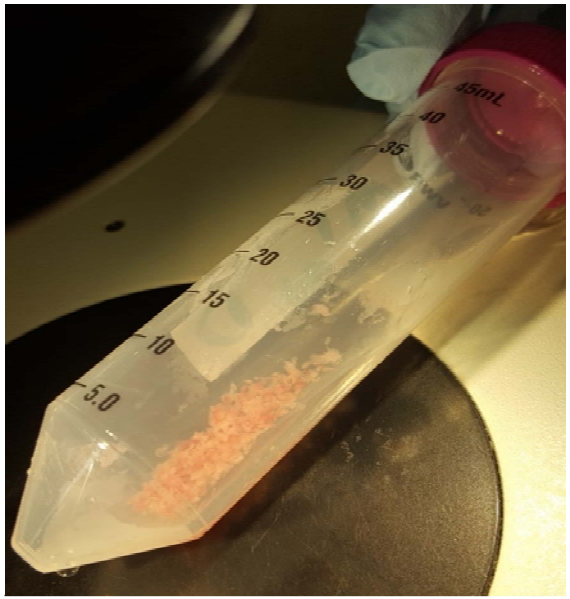
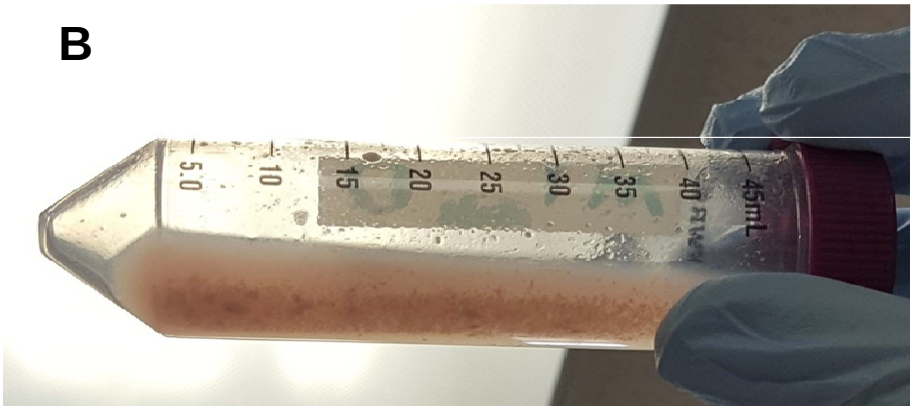


Fig. 7

A



B



C

HBSS Layer

Islet suspension layer

Density gradient layer

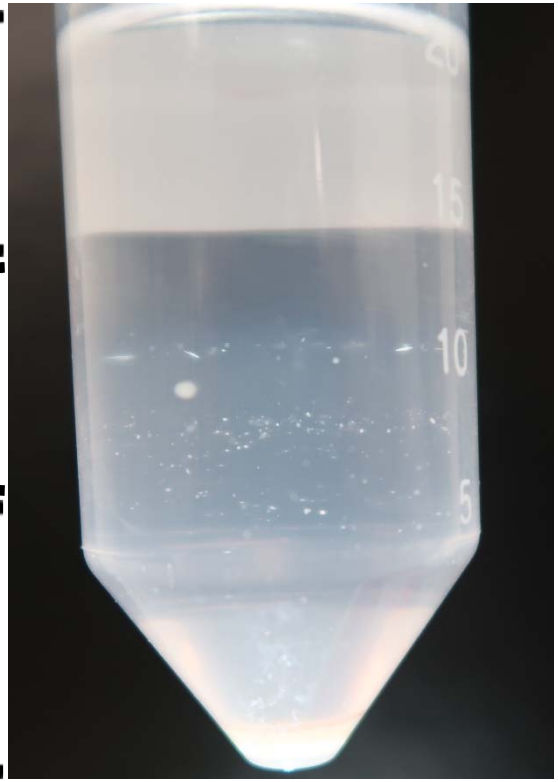
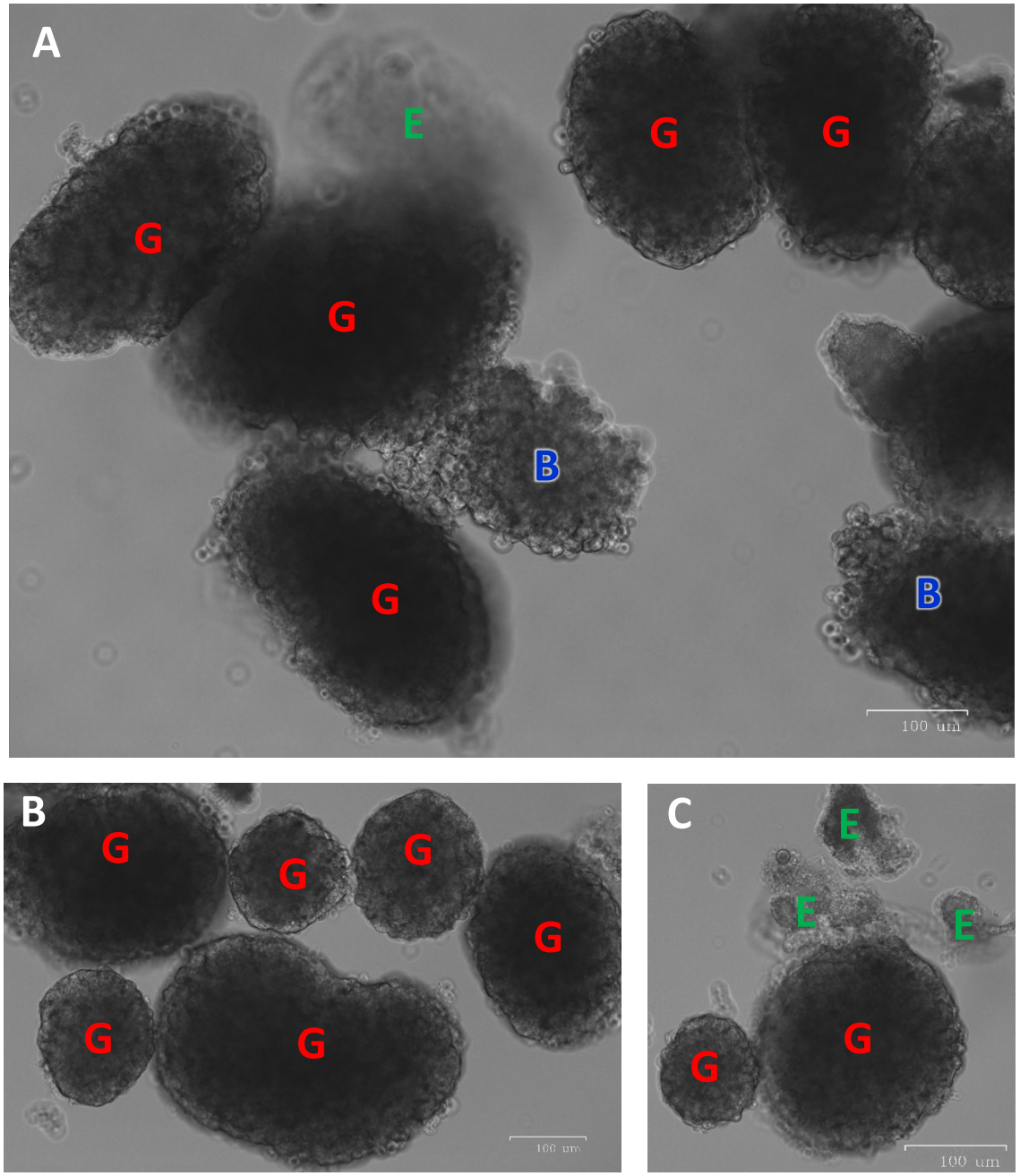


Fig. 8



G: Good islets

B: Bad islets

E: Exocrine tissue

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company
3 mL syringe	BD
Coverglass forceps	VWR
Curved forceps	Sigma-Aldrich
Isoflurane	Piramal
100 mm petri dishes	VWR
30 G. ½ inch needle	BD
50ml tube	VWR
Absorbent pads with waterproof moisture barrier	VWR
Centrifuge 5810R with swing bucket and deceleration capability	Eppendorf
Collagenase P- 1g	Roche Diagnostics
Curved surgical scissors	Fisher-Scientific
Dissection microscope	Olympus
Hank's Balanced Salt Solution 10x	Corning
Histopaque-1077	Sigma
Light source	Leeds
RNaseZap	Fisher-Scientific
RPMI-1640 Media w/o L-Glutamine	Corning
Schwartz micro serrefines (Microvascular clamp)	Fine Science Tools
Shaking waterbath	Boekel/Grant
Small surgical scissors	VWR

Catalog Number

309657

82027-396

Z168696

B13B16A

30-2041

305106

89039-658

82020-845

5811FJ478114

11249002001

13-804-21

SZX16

20-023-CV

RNBF5100

LR92240

AM9780

15-040-CV

18052-01

8R0534008

82027-578

Comments/Description

Hoding collagenase P

Holding skin of mouse to aid incision procedure

Holding tissues during pancreas removal

To anaesthetize mice prior surgery

Used for islet culture
For penetration of Ampulla of vater to deliver Collagenase P - this guage is used as it fits well in most CBDs

Holding digested pancreatic tissue, collagenase P, and purified islets

To absorb blood from syurgical procesdudes
Use for pelleting tissues, pellet is formed at bottom of conical tube - swing bucket centrifuge is needed. Also the decelaration feature is important to form the gradient

For digestion of exocrine pancreas

For cutting open mouse abdomen
Used for identification of key anatomical structures to accurately deliver collagenase into pancreas

Washing cells

For gradient formation

Enhancing visibility of microscope

For removing RNase

Culturing Islets

Clamping common bile duct and hepatic artery

Important for mechanical digestion of exocrine tissue

Cuttitng tissue that atached to pancreas

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Author(s): Daniel Villarreal, Geetali Pradhan, Chia-Shan Wu, Clinton D Allred, Shaodong Guo, YuxiangSun

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Article Title:	A simplified high-efficacy protocol for isolation of pancreatic islets from mice
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Dear JoVE editor & reviewers,

We greatly appreciate your insightful critiques, thank you for the opportunity to revise. We have carefully revised our manuscript; below please see our point-by-point responses to the reviewers' comments (in blue text). To note, some the procedure numbers may be shifted due to the revision.

Thank you do much!

Yuxiang & team

Changes recommended by the JoVE Scientific Review Editor:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Formatting:**
1. Please keep the current formatting, unless otherwise noted. For additional information please follow the link.

Reply: Yes.

2. Please define all abbreviations at first use.

Reply: Abbreviations are defined at first use.

3. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc., and h, min, s for time units. Please use a single space between numerical values and their units.

Reply: It has been corrected.

4. Please use 'Figure #' instead of 'Fig. #.'

Reply: It has been corrected.

5. For centrifugation force, please use 'x g' instead of 'g', e.g. '300 x g'

Reply: It has been corrected.

• **Authors and Affiliations:**
1. Please provide all authors' emails.

Reply: Emails of all authors have been added.

• **Keywords:**
1. Please remove commercial language (Histopaque)

Reply: The use of the term Histopaque is necessary for this paper. As its use is an important distinction from other protocols using other type of gradients, eg ficoll gradient. We believe in the context of our procedure, Histopaque gradient produces better result than other type of gradient. Also to note the Histopaque has previously used in J. Vis. Exp. 67: e4137 (2012).

• **Abstracts:** Please re-word the Short Abstract to more clearly state the goal of the protocol. For example, "This

protocol/manuscript describes…"
1. Please reword your Short Abstract because of the word limit (50 words).

Reply: It has been improved.

2. Please remove commercial language (Histopaque)

Reply: The use of the term Histopaque is necessary for this paper. As its use is an important distinction from other protocols using other type of gradients, eg ficoll gradient. We believe in the context of our procedure, Histopaque gradient produces better result than other type of gradient. Also to note the Histopaque has previously used in J. Vis. Exp. 67: e4137 (2012).

• **Introduction:** Please ensure the following points are covered:
1. advantages over alternative techniques with applicable references to previous studies

Reply: Advantages over other techniques has been included within this section. Particularly, the cost effectiveness and reduction of gradient steps.

2. the context of the technique in the wider body of literature

Reply: This technique is an amalgam of several other techniques, where certain integral points were utilized and adapted in our own protocol, these specific techniques were discussed at length. These included the anatomical choice of enzyme delivery, duct clamping, enzyme used, choice of gradient, detailed notation and visial demonstration.

3. information to help readers determine if the protocol is appropriate for their application.

Reply: Information that would help readers determine if the protocol is appropriate for them were included, such as age and sex of mice, whether the mice are diabetic, cost, etc.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video and must contain everything that you would like filmed. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. **Please add more details to the following protocol steps:**
1. Please add a blank line between every step and Note in the protocol section.

Reply: Concern has been addressed

2. Please ensure you answer the "how" question, i.e., how is the step performed? (Has been addressed) All steps should be explicitly stated to allow readers to replicate protocol. Alternatively, for steps that will not be filmed, add references to published material specifying how to perform the protocol action.

Reply: Concern has been addressed

3. 1 – Please move all materials listed to Materials Table.

Reply: Concern has been addressed

4. 2 – Please write in imperative tense and add all reagents to Materials Table. Please define 'fresh' (e.g. make day of use, good for a few days, etc.) If solutions need to be made during protocol, please integrate where appropriate. The protocol numbering order should reflect the order in which steps are done.

Reply: Written in imperative tense, "fresh" is defined; numbering order follows how steps are carried out.

5. 3.1 – When were tubes marked? How volume was added to each tube?

Reply: Tubes are marked during preparation, content can be added either by pipetting or pouring in directly form stock solution container.

6. 3.2 – Soak entire surface of tools? Partial? Please explain.

Reply: Tips of tools are soaked in the RNaseZap, areas that will be exposed to direct contact with pancreatic tissue.

7. 3.3 – Please specify size of material cut.

Reply: Size of material is 6" by 6".

8. 3.7 – Presumably 2 tubes ('digestion' and 'purification') are labelled per mouse? Please clarify.

Reply: Yes, it has been clarified.
"Label 50 mL tubes for digestion and purification – prepare 2 tubes for each mouse: one for digestion and the other for islet purification."

9. 4 Note – Please move this directly below the Protocol section heading on line 88.

Reply: This has been addressed.

10. 4.1 – 'rinse' – how? Are they left in the ethanol or water solutions for given amount of time? Please reference Figure 1.

Reply: They are rinsed by dipping and swirling tips in EtOH tube then ddH20. They are not left to soak, rinse only.

11. 4.2 – How was isoflurane added to chamber? How is 'deep' anesthetization confirmed? Move on to step 4.3 when mouse stops moving or after a certain amount of time?

Reply: 2 mL of Isofluorane is added to a covered beaker used to anaesthetize mice under ventilation hood.
"Deep anesthetization is based on the observation that breathing become steadily slow and mouse is unreactive to foot pinching."

12. 4.3 – Please note we cannot show euthanization in our videos. Please un-highlight steps 4.2-4.3. Please provide details how 'cervical dislocation' is accomplished. What equipment was used.

Reply: Once the mouse is deeply anaesthetized, the mouse will be sacrificed with cervical dislocation. "Deeply anaesthetized mouse will be placed on stomach, then one hand holds skull and one hand holds shoulder to apply firm pressure to the neck to dislocate the spinal cord from the brain by pulling the tail".

13. 4.3.1 – This should be re-numbered as 4.4. Was ethanol wiped away? Allowed to evaporate?

Reply: Yes, it has been renumbered, and excess ethanol is wiped away using paper towel.

14. 4.4 – What equipment was used? Location of incision? Length? Was peritoneum also cut at this step?

Reply: Spray 70% alcohol on the entire abdominal area to wet the fur to prevent hair flying under microscope. "Cover glass forceps and curved surgical scissors are used to make incisions. First a horizontal incision is made on the skin (~3cm), pull the skin wide open to expose the abdominal wall. Then a vertical incision (~3-4cm) on the abdominal peritoneum is made to fully expose the pancreas in abdominal cavity."

15. 4.5 – How is bile duct identified? Directions in up/down/left/right are all relative. Presumably liver is pushed superiorly?
Reply: The concern has been addressed as: Push the superior lobes of the liver to expose the bile duct, which appears as a pale pink color tube. Carefully push the intestines away from the lumbar/iliac region of the abdominal cavity to expose the bile duct and hepatic artery.

16. 4.6 – Please refer to Figure 3 for clarity.

Reply: It has been corrected ad requested.

17. 4.7 – 'clamp' – how? With what equipment?

Reply: Appropriate equipment has been noted.

18. 4.8.1/4.12.1/4.16.1/4.26.2/4.33.2 – Please change this to note.

Reply: Concern has been addressed.

19. 4.9 – How is common bile duct identified? It is difficult to identify even in Figure 5. Please refer here to Figure 5 for clarity, although a clearer image is required.

Reply: a clearer picture depicting common bile duct/Ampulla of Vater has been added along with following instructions "Identify the Ampulla of Vater, which is located at the duodenal papilla, formed by the union of the pancreatic duct and the common bile duct. The ampulla of Vater appears swollen when viewed under a dissection microscope and is the entry point to the common bile duct."

20. 4.11 – Please define slow and steady. How is proper flow rate confirmed?

Reply: Slow and steadily is defined as "enough to feel resistance". Proper flow rate is confirmed by full inflation of splenic area of pancreas.

21. 4.12 – Please combine with 4.11. How is 'fully inflated' confirmed?

Reply: The following note and reference to Figure 6 has been added "NOTE: Inflation can be confirmed by the appearance of open spaces between pancreatic tissue that is filled with solution".

22. 4.13.1 – 'remove' – how? Was incision made with scalpel? Please add equipment used.

Reply: The explanation depicting process of removal of pancreas has been added. "Remove the pancreas using curved and cover glass forceps (see Figure 1): starting from the spleen, pull the pancreas away from spleen and stomach, and then from the duodenum"

23. 4.13.2 – 'chop' – what size pieces should be made?

Reply: To increase the surface area of the tissue exposed to the collagenase, pancreases is chopped to small pieces (the size of peas), usually need to chop with scissors for 2-3 minutes.

24. 4.14/4.15/4.15.1 – How is tube rotated in water back? How is 'homogenously digested' confirmed?

Reply: Tubes are rotated in shaking water bath by placing them in a rack attached to the shaker. "Homogeneity of digested pancreas is defined as a sand like appearance. ~30 seconds of gentle hand-shaking is usually enough; hold the tube to light to examine if the tissue is well homogenized. If not, shake for another ~15 seconds if needed." This statement has been added to protocol.

25. 4.15.1 – Please integrate with 4.15.

Reply: Concern has been addressed.

26. 4.16.1 – How long can digestion tubes be left on ice for?

Reply: Preferably not longer than 2 hours. Longer than 2 hours the islets may not be healthy.

27. 4.17/4.19//4.25/4.27 – Centrifugation temperature?

Reply: Temperature is not a factor for this step, 22C room temperature is fine.

28. 4.17.1/4.26.1 – Please do not number notes.

Reply: Concern has been addressed.

29. 4.18 – How much STOP solution was added? Please add 'decanting' step prior to spinning with STOP solution, since this is the order in which the steps are conducted.

Reply: 40 mL of STOP solution is poured into the tubes containing digested islets and then 20 mL after each spin. Decanting step is added prior to spinning with solution. "Once the islets are digested, immediately place the tubes on ice and add 40 mL of STOP solution to terminate the enzymatic digestion. Repeat the washing and centrifugation with 20 mL of STOP solution 2 more times (Decanting the solution after each spin)."

30. 4.20 – What determined 2-3 times?

Reply: Just to ensure that all the collagenase/histopaque/undigested exocrine tissue are removed efficiently.

31. 4.20.1/4.20.2 – Please integrate with 4.20.

Reply: Concern has been addressed.

32. 4.22 – Presumably vortex till homogenous?

Reply: Yes, solution is vortexed at low speed until it becomes homogenous. "Then add 5 mL of room temperature Histopaque-1077 to the 50 mL tube containing the pellet. Vortex briefly at low speed until homogenized."

33. 4.23 – Was solution mixed?

Reply: Solution is not mixed after initial addition of 5 mL Histopaque. Clarifications have been added "NOTE: The following steps 4.24-4.25 require the user to remain steady to endure the gradient to form without disruption. Next, add another 5 mL of Histopaque -1077 to the 50 mL tube. Do not vortex/mix."

34. 4.24. – How was HBSS added drop by drop?

Reply: Clarification added: "Pipette 10 mL of room temp HBSS buffer into the tube (containing Histopaque), drop by drop, to allow a gradient to form."

35. 4.26 – 'pre-wet' – how? Presumably pre-wet not just the outside of the pipette? Is islet layer completely removed? How much HBSS remains to prevent uptake of other layers?

Reply: Following clarification has been added: "After the spin, carefully remove the tubes without disturbing the gradient. Using a pipette gun pre-wetted with cold HBSS (pipette HBSS up and down), pipette out the layer of islets (5-10 mL) formed between Histopaque-1077 and HBSS into the new 50 mL islet collection tube. NOTE: It is

necessary to wet the pipette with cold HBSS prior to pipetting the islets to prevent the islets from sticking to the inner walls of the pipette.

How much HBSS remains to prevent uptake of other layers?

In case the islets are visible in the Histopaque layer, pipette out the entire 10 mL of the Histopaque (bottom layer) along with the islet layer formed at the interface into a 50 mL islet collection tube. This will leave about 8-10 mL of top layer HBSS"

36. 4.26.2 – Do any subsequent steps change if bottom layer is collected? If not, is leaving this layer behind important?

Reply: The following has been added to clarify the reviewer's concern, "NOTE: Collecting both the islet layer and Histopaque layer (bottom layer) can result in the debris contamination, which will make the islet picking more challenging. Collecting both these layers is not necessary if islet layer is well formed."

37. 4.29.1 – Please integrate with 4.29

Reply: Concern has been addressed.

38. 4.31 – Presumably FBS, INS-1, and penicillin/streptomycin were already added and not further supplemented? Please clarify.

Reply: Yes, the formulation of this media is described which should be prepared beforehand, clarification and reference to this step has been added.

39. 4.32 – Please define 'gently shaking'. Please specify media on line 229 for washing tube.

Reply: "Gently shake" has been changed to "gently swirl" as it is more accurate description of the procedure used.

40. 4.33.1 – Please integrate with 4.33. Please provide settings used for dissection microscope.

Reply: Magnification of dissection microscope is 12.5X – 16.0X.

41. 4.34 – Please provide reference for freezing protocol.

Reply: Once islets are frozen they cannot be used for in vitro experiments (secretion assays), can only be used for RNA and protein quantification. To collect islets to freeze, collect all islets in 200-500 uL of HBSS buffer, then transfer to 1.5 mL tube, then centrifuge 350g for 1-2 minutes to remove supernatant. Place islet pellet in -20 C for short term storage (several days) or -80 C for long term storage.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. 'Do this', 'Measure that', etc.). Any text that cannot be written in the imperative tense may be added as a 'Note', however, notes should be used sparingly and supplement the protocol step.

Reply: Concern has been addressed.

• **Protocol Highlight:** After you have made all the recommended changes to your protocol, please re-evaluate the length of your Protocol section. There is a 10-page limit for the Protocol section excluding figures and tables. Please highlight **up to 2.75 pages** of text in yellow (including headings and spaces), to identify which steps should be visualized/filmed to tell the most cohesive narrative of the protocol. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the readers.

1. You may highlight portions of a protocol step, but must highlight complete sentences.
2. Notes cannot be filmed and should be excluded from highlighting.
3. Please revise title to reflect highlighted content only.

• **Figures/Legends:**
1. Figure 3-6 – It may be useful for readers to also label pancreas and duodenum for clarity.

Reply: Figures where it is applicable have had pancreas/duodenum labels added.

2. Figure 3 – Please label the bile duct and hepatic artery bundle separately for clarity.

Reply: Figures where it is applicable have had bile duct/hepatic artery labels added.

3. Figure 4 – Please provide new image as Ampulla of Vater is difficult to identify.

Reply: A better image of the ampulla has been added.

4. Figure 5 – Please provide a different image where the common bile duct is easily identifiable. This may require changing the angle of the light, the angle of the camera, or lower light intensity.

Reply: A better image where the common bile duct is easily identifiable has been added.

5. Figure 8 – Please make scale bars easy to read. Does panel C use the same scale bar?

Reply: Scale bar is the same for all images, scale bar has been added.

• **Discussion:** JoVE articles are focused on scientific protocols. Thus, the discussion should be similarly focused. Please ensure that the discussion covers:
1. modifications and troubleshooting
2. critical steps within the protocol
3. limitations of the technique
4. significance with respect to existing protocols
5. future applications
6. Please do not use bullets in this section.

• **References:** Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]
1. Please remove '&' when listing authors for a reference.
2. Please abbreviate all journal titles using ISO standard.
3. Please include volume, and issue numbers for all references.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial language, including trademarks (™), registered trademark symbols (®), and company brand names. Please replace all commercial language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the Materials Table. You may add the generic name(s) under the comments section of the Materials Table for clarification. Examples of commercial language in your manuscript include Histopaque, RNaseZap, etc.

• **Materials Table:** Please revise the table to include supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in an .xls/.xlsx file. It should include a comments column to clarify generic names in manuscript. Please include items such as surgical tools, all reagents and solutions in steps (1) and (2), 50 mL tubes, RNaseZap, adsorbent pad, isoflurane, dissection microscope,

surgical equipment, syringe, needles, 100 mm Petri dish, etc.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [AUTHOR] *et al.*[REFERENCE]"

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
This manuscript provides a simplified mouse islet isolation and purification method that will be of great interest to islet biology field. The protocol described a novel route of injecting collagenase P via the Ampulla of Vater to digest the exocrine tissue, thus greatly reducing the complexity of islet isolation techniques. The protocol is described in details so that it can be easily followed.

Minor Concerns:
In Discussion section, it would be interesting to know how long the isolated islets can be cultured in vitro and how many folds of insulin can be secreted by the isolated islets in response to high glucose stimulation.
Line 40, due combination, missing "to".
Line 42, in This protocol high produces high yield islets, first "high" should be deleted.,
Line 65, et al, missing name

Reply: We appreciate the reviewer's insightful critiques and comments. Typo errors associated have been corrected. We have only cultured them overnight for insulin secretion experiments.

We noted in revision: The islets isolated from this protocol has been used successfully to study glucose stimulated insulin secretion *ex vivo* in our previous publication ((Pradhan, G. *et al. Sci Rep.* **7**:, 979, 2017). 22.2mM glucose induced insulin secretion can be 4-fold higher than that of baseline (3.3mM). These results were achieved after overnight incubation in 5.5mM glucose RPMI-1640 complete media.

The current protocol has not been tested for prolonged incubation (2-7 days). Others have cultured islets for 2-7 days (Do, O. et all. *Lepr(db) mouse model of type 2 diabetes: pancreatic islet isolation and live-cell 2-photon imaging of intact islets*. J Vis Exp. 99, e52632, 2015; Phelps, E. A. et al. *Advances in Pancreatic Islet Monolayer Culture on Glass Surfaces Enable Super-Resolution Microscopy and Insights into Beta Cell Ciliogenesis and Proliferation*. Scientific Reports 7: 45961. 2017).

Reviewer #2:
Manuscript Summary:
It is a well written and easy to follow protocol for isolation of pancreatic islets from mice. The protocol is better than existing ones.

Major Concerns:
None

Minor Concerns:
Pay attention to some typos and grammatical in the Abstracts.

Reply: We appreciate the reviewer's kind comments, we have carefully corrected the typos and grammatical errors we find.

Reviewer #3:
Manuscript Summary:
I have read with great interest the manuscript entitled, "A simplified high-efficacy protocol for isolation of pancreatic islets from mice", by Villarreal and colleagues. The manuscript is well written and easy to read. The authors should

address the following one major point and one minor issue.
I am so surprised and disappointed with Figure 2 which shows incorrect pancreas anatomy in mice. The pancreas shown in Figure 2 appears to be human pancreas, not mouse pancreas. The authors should learn the differences in pancreas anatomy between human and mouse. Please read the following 2 published papers.
Changes in the mouse exocrine pancreas after pancreatic duct ligation: a qualitative and quantitative histological study. Arch Histol Cytol. 1995 Aug;58(3):365-74
Structural similarities and differences between the human and the mouse pancreas. Islets. 2015;7(1):e1024405
The authors should definitely replace Figure 2 with the correct one.

Reply: We apologize for the oversight, we greatly appreciate the reviewer's critique. We have redrawn the schematic diagram based on the papers kindly shared by the reviewer.

My another concern is about warm ischemia. The authors sacrificed the mice with cervical dislocation prior to surgery. This approach is unusual. The most researches sacrifice the mouse after intraductal distension of the pancreas (and just prior to pancreatectomy) by means of cardiac puncture so that the pancreas is subjected to minimum warm ischemia time. The authors should describe why they took the unusual approach.

Reply: The reviewer raises a very good question. In our initial studies, we perfused the pancreas while the mouse was under anesthesia (isofluorane), followed by cutting the hepatic artery and pancreatectomy. In this approach, excision of the perfused pancreas was challenging due to the excessive bleeding from the hepatic artery. Also it was difficult to inject the collagenase into the Ampula of Vater due to the heavy breathing. So, in this protocol we have choose our current approach.

Similar to our protocol, Do *et al.* sacrificed the mice by either cervical dislocation or CO_2 asphyxiation prior to exposing the abdominal cavity for islet perfusion. (Ref: J Vis Exp. 2015; (99): 52632). Further in the paper by Neuman *et al.*, the mouse was completely euthanized by opening the thoracic activity and cutting the aorta before perfusion of the pancreas to isolate islets (Ref: J Vis Exp. 2014; (88): 50374). In the book titled 'Pancreatic islet isolation: From the mouse to the clinic' by Miriam Ramírez-Domínguez, the author recommends euthanization of the mouse by cervical dislocation or CO_2 before proceeding with the islet isolation.

Reviewer #4:
Manuscript Summary:
This manuscript provides a detailed protocol for isolating pancreatic islets from mice, which is useful to the islet/diabetes research community. Isolating good quality islets is critical and impacts significantly on the quality of the research conducted. While there are several published protocols on islet isolation, this detailed protocol provides an excellent guide for islet isolation. Publication is recommended after addressing the below comments.

Major Concerns:
- A clear time estimate for completing the "procedure" (i.e., steps 4.2 - 4.34) with a specific number of mice (pancreata) should be provided.

Reply: We normally collect islets and a wide range of other central and peripheral tissues from 4-6 mice at a time. Following is the estimated time for collecting pancreatic islets from 6 mice by an experienced technician: 1. Excise and digestion of pancreas can be done in 30 minutes (~5 minutes/mouse) if all materials are prepared beforehand (eg, absorbent pads, syringes filled, 50 ml tubes filled with collagenase etc). 2. Pancreatic excision and digestion can be completed in 45-50 minutes. The centrifugation/washing steps may take 1 hour. 3. The gradient step requires slow, steady application of Histopaque and HBSS, which can take 1-1.5 hours. 4. The last step of islet picking time is highly variable depend on the amount and quality of islets. If digestion and separation are optimal, it normally takes 1-1.5 hours. (If digestion and separation are poor it will take more time to pick islets because one has to remove exocrine debris from islets, it can take 2-2.5 hrs). Thus normally it takes 3-4 hours in total for an experienced technician to collect islets from 6 mice. Comparing to other protocols involving multiple gradients, our sigle gradient purification procedure reduces the time and labor.

- The authors should comment in the manuscript on the time that would be needed to isolate islets with their method from larger numbers of mice (e.g., >15) as would be commonly needed for transplantation applications.

Reply: For research purpose, we normally not collect islets from mice greater than 8 mice per day to ensure the quality of the islets. However, if an additional person is available to assist, and there are additional centrifuge and dissecting scope, the group should be able to handle bigger number of mice, the time needed per mouse can be significantly reduced.

- The authors should comment why they combine the hand-picking step with the gradient step? This seems redundant as hand-picking is commonly done to bypass the gradient step...

Reply: In our protocol, we collect the entire volume of the HBSS (8-10ml) and the gradient layer (8-10ml) after carefully pipetting the formed islet layer. This leads to collection of islets that are dispersed in the gradient layer (due to incomplete separation) along with the islet layer, which helps to increase the overall islet yield. As a tradeoff, some debris and undigested exocrine tissue may be pipetted in as well. So, we use an additional step of hand-picking after the gradient step to increase the islet yield, and to obtain high quality islets (devoid of debris or exocrine tissue).

- Data on islet viability after Histopaque should be provided.

Reply: Islet viability using current protocol has been demonstrated in our previous publication (Pradhan, G. *et al. Sci Rep.* **7**:, 979, 2017), we showed that the islets are fully functional in glucose stimulated insulin secretion, as well as RNA and protein expression studies.

- The authors should comment whether islets are found in the pellet during step 4.26.

Reply: Islets can be found on the surface of the pellet but not within the pellet itself.

- In step 4.26.2, the sentence "In case the islets are visible in the Histopaque layer" should changed to the following "In case the separation is incomplete and islets are visible in the Histopaque layer" to indicate clearly that this is a contingency step to recover the islets after failure to separate in the 1st gradient.

Reply: The point is well taken, the text has been revised per reviewer's suggestion.

- The authors should reduce the emphasis on "high yields" in in the manuscript, as 300-350 islets/mouse is consistent with other isolation methods. Also, claims of simplicity/time-efficiency should also be dampened as the final step of islet hand-picking adds significant time requirements to this method; according to the authors, "it may take roughly an hour to pick 300-350 islets from 1 mouse", thus, the hand-picking step might add a few more hours with the recommended 4-6 mice per isolation, let alone in larger isolations (>10 mice).

Reply: We have turned down our statement accordingly.

Minor Concerns:
- Correct typographic errors in the manuscript to ensure correct execution of the protocol (e.g., line #162).
- Author name is missing in line #65.

Reply: Minor concerns have been addressed.

We greatly appreciate the reviewer's critical inputs, which has helped us to improve the manuscript dramatically.