

## METHODS PAGE

# An isolated rat pancreas preparation for studying pancreatic spinal mechanosensitive and chemosensitive afferent activity

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## Introduction

Sensory information from the viscera to the brain is conducted by vagal and spinal afferent neurons (1). The pancreatobiliary system is innervated by both vagal and splanchnic afferents however there are few reports describing their functional electrophysiological properties. Under pathological conditions such as pancreatitis and pancreatic cancer, these sensory inputs are believed to be essential to the perception of the pain associated with these conditions and additional symptoms e.g. nausea. The pancreatic mechanosensitive afferents have been poorly investigated. Some information has been generated using anesthetized animal studies (2, 3). Due to the limited access to the pancreas in such preparations, the extent and distribution of mechanosensitive nerve endings has not been fully described.

Information regarding pancreatic afferent nerve chemosensitivity is also limited. A few studies have applied algescic agents such as substance P, bradykinin, and prostaglandins i.v. or on the surface of the pancreas via pledgets or wells (5, 6). Although these studies demonstrated the

action of these agents, the precise location of the receptive fields and dose-response characteristics of these afferent nerves were not defined.

Previous studies have highlighted the limitations of whole animal experiments (limited access to the entire pancreas and possible confounding effects of anaesthesia) and these shortcomings prompted us to develop the isolated rat pancreas preparation for extracellular recordings described below. Studies using this preparation have been reported by Schloithe *et al.*, (6).

## 1. Materials

### 1.1 Chemicals/Reagents

2. Dextran (MW, 64,000-76,000; Sigma-Aldrich, St Louis, MO, USA)
3. Soybean trypsin-chymotrypsin inhibitor, (T-9777, Sigma-Aldrich)
4. Protease inhibitor cocktail (P 8340; Sigma-Aldrich)
5. Nembutal (Rhone Merieux Australia, Pinkemba, QLD, Australia).

6. Silicone high vacuum grease (Dow Corning Australia, NSW, Australia)
7. Sylgard (Dow Corning Australia)
8. Paraffin oil (100%) obtained from several reputable suppliers Cover slips or poly-L-lysine coated glass bottom 35 mm culture dishes (MatTek Corp, Ashland, MA, USA).

### 1.2 Dissection Instruments

1. Olympus SZ stereo microscope; 0.5x objective 7.5 – 64 zoom
2. Scalpel and blade (size 10)
3. Standard scissors; straight 12 cm
4. Metzenbaum scissors; curved 13 cm
5. Iris scissors; curved 12 cm
6. Plain Adson forceps; straight 12 cm
7. Mosquito hemostats; curved 12 cm
8. LigaClip® applicator (for small LigaClips®)
9. Small LigaClips®; vessel ligating clips (Ethicon Endo-Surgery Inc, OH, USA)
10. Jewellers forceps; curved 11 cm
11. Jewellers forceps; straight 11 cm
12. Jewellers forceps 45 °; 0.10x0.06 mm tips 11 cm
13. Jewellers forceps; straight fine 0.10x0.06 mm tips 11 cm
14. Microscissors; spring 11 cm

### 1.3 Electrical Equipment

1. Standard electrophysiology recording equipment; Faraday cage, recording and reference platinum electrodes, headstage, micromanipulators, differential amplifier, band-pass filter, recording system (Power lab or equivalent), sound mixer with headphones and computer

2. Digital thermometer with a flexible probe (CIE, model 305 thermometer, Industrial; Equipment Corp, India)
3. Magnetic stirrer
4. Peristaltic pump for dextran-Krebs solution
5. Water heater and pump, water cooler and pump and heat exchangers

### 1.4 Other Materials (apart from standard laboratory items)

1. Organ bath (**Note 10**)
2. Silk suture 6/0; cut 4 x 10 cm long for each experiment
3. Cotton buds (Q-tips)
4. Gauze swabs
5. Disposable needles; 27 g
6. Entomology pins (Headless pins Product # E182 (0.193 x 12.5 mm) Australian Entomology Supplies Pty Ltd, NSW Australia)
7. Optiva® i.v. catheters (Smiths Medical, Carlsbad, Ca, USA)
  - 24 g i.v. catheter (without needle) with attached 1 ml syringe
  - 18 g i.v. catheter (without needle) with attached 10 ml syringe
8. Glass Petri dish; 116 mm OD x 20 mm deep (Duran Group GmbH, Mainz, Germany) (**Note 1**)
9. Shallow ice container (polystyrene or equivalent); to accommodate dissection dish and ice
10. Acrylic sheet; black 120 mm x 120 mm, 2-4 mm thick
11. 5 % CO<sub>2</sub>/ 95 % O<sub>2</sub> (Carbogen) gas cylinder and polyvinyl tubing to permit oxygenation of two 5L beakers (5 mm OD tubing), the

dissecting dish (1 mm OD tubing) and the organ bath (1 mm OD tubing)

## 2. Methods

### 2.1 One day prior to experiment (usually late afternoon)

Prepare the following two solutions, 5 L of each, at room temperature. Due to the time it can take to prepare these solutions, it is convenient to prepare them 15-18 hours in advance. One solution is modified Krebs buffer and the other is modified Krebs buffer supplemented with dextran and protease inhibitors (dextran-Krebs). The modified Krebs solution is used during gross dissection and the dextran-Krebs used during the remaining dissection and recordings.

The modified Krebs buffer consists of 133.4 mM NaCl, 4.7 mM KCl, 1.3 mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 16.3 mM  $\text{NaHCO}_3$ , 7.7 mM D-Glucose, 0.6 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.12 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . This solution is oxygenated with Carbogen, during the preparation to adjust the pH to 7.4 (**Note 2**). The supplements for the dextran-Krebs buffer are 1.5 % dextran (**Note 3**), protease inhibitor cocktail (0.2 ml/l) and trypsin-chymotrypsin inhibitor (2 mg/l); the latter two are added the next day (see below). Both solutions are stored in the refrigerator overnight.

Rat (approximately 200 g body weight) is fasted overnight with free access to water. Fasting rats overnight results in smaller stomachs which aids tissue harvest and dissection. Rat weighing in excess of 250 g tend to have more visceral fat which is not conducive to a good dissection.

### 2.2 Day of Experiment

Ensure instruments, other materials, organ bath (**Note 7**), dissection dish (**Note 1**) and all recording equipment is prepared, setup and in place.

Turn on electrical equipment except the Krebs peristaltic pump.

Place beakers containing each solution prepared as described above in separate ice buckets positioned on a magnetic stirrer and continuously oxygenate with Carbogen. Place a magnetic stirring bar in each beaker and commence mixing. Add the protease inhibitor cocktail and soybean trypsin-chymotrypsin inhibitor to the dextran-Krebs. To keep the tissue cool during dissection put ice in the shallow ice container, place the black acrylic sheet on the ice, then place the dissection dish on top of the black sheet and heap ice around the edge of the dissection dish. A black plastic or acrylic sheet is used to provide visual contrast with the tissue and this facilitates the dissection.

### 2.3 Tissue Harvest

Anesthetise the rat with approximately 300 ul Nembutal, i.p. (60 mg/kg). Once the rat is unconscious, place the animal in a supine position on a dissection board and secure its front legs and tail with adhesive tape.

Perform a laparotomy and to aid exposure, cut down each side of abdomen avoiding the epigastric vessels running within the muscle of the abdomen. Using cotton buds tease fat away from the vena cava and aorta below the renal vessels to isolate/clear. With curved forceps, free tissue under the combined vessels and then position 2 sutures underneath. Tie off carefully with a 2-3 mm gap between each suture (to allow for subsequent transection), leave the most proximal (i.e. cranial) suture long and clip with a hemostat. Locate the distal colon and cut. Identify each kidney and using Krebs soaked gauze gently tease away the fat/connective tissue attaching them to the back of the abdominal wall. In the same way, clear viscera away from the posterior abdominal wall on both sides up to the diaphragm, being careful to avoid the area around the adrenal glands. If the experimental design

involves the bile or pancreatic ducts, locate the bile duct close to the liver, tie with a silk suture and transect (keep the suture several cm long for future retraction). *Alternatively this can be done later when the tissues are in the dish.*

Working quickly, retract the viscera then cut through the diaphragm adjacent to the spine and cut around its periphery (i.e. near ribs) to the other side. Cut open the thoracic cavity and retract each side with hemostats. Locate the aorta in the thoracic cavity and using hemostats separate the aorta from the back abdominal wall attachments. Clip the aorta with 2 LigaClips®, 3-4 mm apart and transect between the clips using iris scissors. Near the transected aorta, cut the esophagus and vena cava vertically and deeply to the level of the spine with Metzenbaum scissors.

Using moist gauze swabs as a wrap, lift the intestines, other visceral organs and importantly the kidneys (the nerves of interest are close) to gain access beneath. Cut beneath the tied distal aorta/vena cava using Metzenbaum scissors. Cut deeply by moving the tips of the scissors closely along the spine whilst lifting out visceral organs. Cut along the spine up into the thoracic cavity above the level of the transected aorta and remove the tissue *en bloc* (alternately, the spine can be included in the *en bloc* dissection to avoid cutting the nerve trunks that lie along the aorta; the segment of spine can be removed by dissection subsequently when isolating the nerve trunks).

Place all organs in a beaker containing ice-cold, oxygenated modified Krebs solution. Euthanize the rat and clean up.

## 2.4 Dissection

Rinse visceral organs in oxygenated ice-cold modified Krebs 2-3 times to remove blood and hence improve visibility. Transfer the viscera to the dissecting dish containing modified Krebs which is positioned on the black acrylic sheet in the ice container (**Figure 1**). Continuously

oxygenate the solution in the dish during the dissection with Carbogen via the flexible tubing (**Note 4**). Importantly, during this procedure the pancreas should not be handled with forceps and its manipulation should be kept to a minimum.

Loosely pin the stomach and liver towards the top of dish, and the kidneys, and intestines towards the bottom of the dish (**Note 5**). Maintain this orientation for the remainder of the dissection (**Figure 1**).

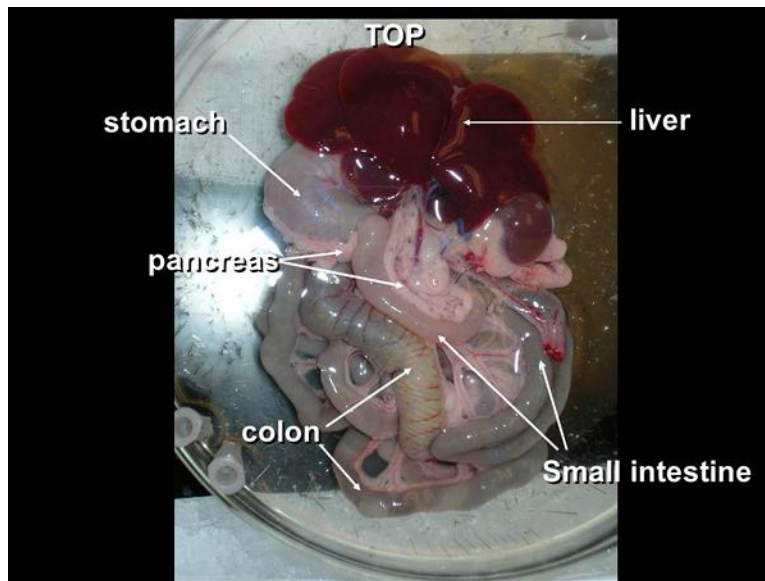
If not done previously, locate and tie bile duct close to the liver and transect (keep the suture several cm long for future retraction). Place a LigaClip® on the hepatic artery close to the liver. Lift the left lobe of the liver to locate the portal vein (a large vessel) and apply a LigaClip® allowing some space between clip and liver to cut the vein later (do not transect the portal vein at this time). In preparation for removal of the liver cut connective tissue between liver and stomach and any attachments to other organs and ensure all liver lobes are free. Then cut the portal vein and hepatic artery (liver side of clips). This should free the liver which can then be discarded. Reorientate the stomach, duodenum and remaining tissues as seen in **Figure 2**. Refresh the modified Krebs in the dissection dish.

Carefully dissect pancreas from colon by retracting the colon away from the pancreas (**Note 6**). Dissect pancreas from the distal small intestine. Find the segment of the duodenum where pancreatic attachment cease, and transect the duodenum distal to this point. The colon and distal small intestine should be free and can be discarded. Refresh the modified Krebs solution in the dissection dish.

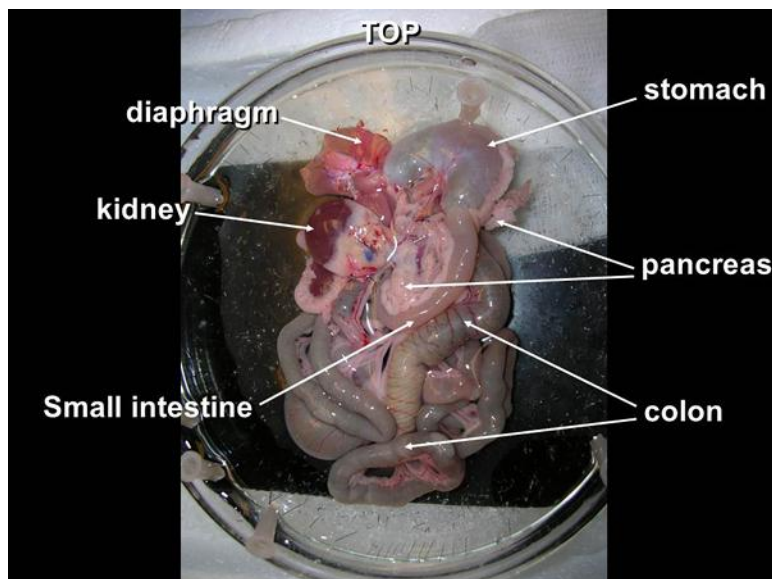
Re-arrange the tissue in the dish to expose the portal vein. If the orientation is correct, the portal vein will be visible on top. Mobilize the stomach by cutting the gastric artery, connective tissue and attached pancreas (cut close to the stomach), which will also mobilize the spleen and pancreas. Gently retract the stomach and spleen. Pin the kidneys to

the left and right and be aware of possible torsion (twist) in tissues. Then pin the top and bottom ends of the aorta/vena cava to lie approximately vertically

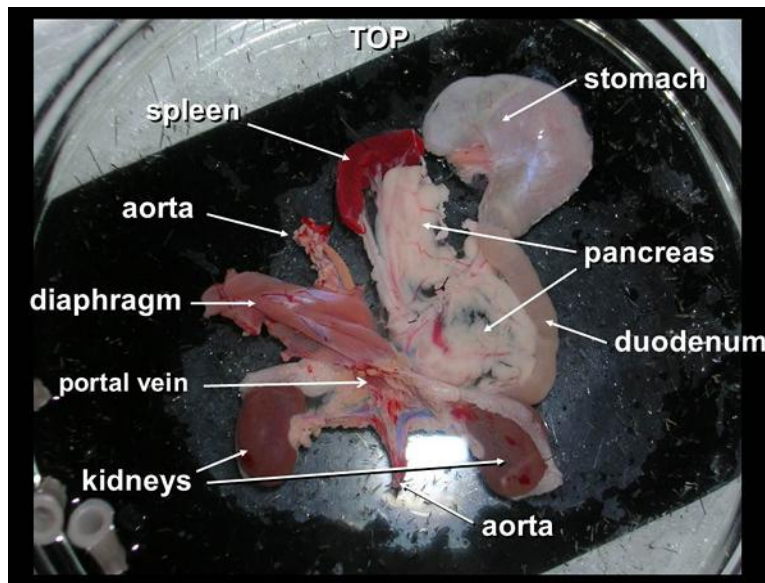
(i.e. north-south) between the kidneys (to produce a “cross configuration” as shown in **Figure 3**).



**Figure 1.** Image illustrating the viscera, immediately post-harvest, in the dissection dish. Note the pins located around the edge of the dish and the black acrylic sheet under the dish.



**Figure 2.** Another image illustrating the viscera shortly after harvest (with the liver removed) in the dissection dish.



**Figure 3.** Image of a preliminary dissection. Note that the aorta is oriented roughly north-south with the kidneys on either side. The stomach is positioned upper most, the duodenal segment on the right, the pancreas central and the spleen on the upper left.

Assemble fine dissection instruments. Position a dissection microscope over the inferior aorta/vena cava to aid with subsequent dissection. Using microscissors and fine forceps, remove by dissection the 'brown' fat associated with the portal vein, renal veins and inferior vena cava and aorta below the kidneys. Beginning from the transected distal ends of the vena cava and aorta, separate the two by blunt dissection using fine forceps and then clear the distal portion of the aorta using the microscissors. Dissect the renal veins, vena cava and portal vein from the aorta and discard these vessels. Refresh the modified Krebs solution in the dissection dish.

The nerve network (NN, the complex consisting of the celiac and superior mesenteric ganglia - **Figure 4**) is just above the renal veins but at the level of the aorta. Avoid any manipulation of the NN particularly during the following dissections. Identify the nerve trunk. The nerve trunk is bright white, shiny and appears to be striated (i.e. circumferential stripes) and is very often buried in fat and connective tissue.

In preparation to dissect the right nerve trunk, lift and pin the duodenal segment over to the left side. Find the top end of aorta, slide it under and to the right and then pin in position. After these manoeuvres, the view of the preparation is slightly different to that shown in **Figure 4**, see later. Identify the right and left adrenal glands located above each kidney and pin at  $90^\circ$  to the aorta (**Figure 4**). Care is required as the relevant nerve trunks are close to the adrenal glands. Locate the NN which should be at the level of the adrenal glands and over the aorta. Locate the right nerve trunk (animal's left nerve trunk); it runs from the NN towards the adrenal gland then deviates upwards, through and behind the muscle of the diaphragm, and along the superior aorta (it may be necessary to retract/lift/manoeuvre the diaphragm muscle which is located on the underside of the preparation, to visualize the nerve trunk). Dissect fat/connective tissue away from the nerve trunk as it becomes visible, working a few mm from the NN towards the superior aorta. Do not handle the nerve trunk with forceps, instead use fine forceps to hold and

retract fat or connective tissue around the nerve trunk to inspect before dissecting/cutting. (If the nerve trunk becomes difficult to visualise then it may be necessary to locate it and begin its dissection from the superior end of the aorta). Once the nerve trunk is free of attached tissues to its termination reduce retraction of the adrenal gland and allow the nerve to float; do not pin the nerve trunk. Refresh the modified Krebs solution in the dissection dish.

To dissect the left nerve trunk (animals right) move and pin the superior aorta end to the left side and move the duodenal segment to the right side to aid visualization, as shown in **Figure 4**. Repeat the nerve trunk dissection described above. Refresh the modified Krebs solution in the dissection dish.

Transect the renal arteries close to the aorta and remove together with the kidneys but retain the adrenal glands for retraction. Complete clearing the aorta by dissecting remaining 'brown' fat from the superior aorta, being very careful near the NN. Remove the diaphragm and cut out any lymph nodes. Remove the stomach. Remove fat and vessels to spleen that are not associated with pancreas. Orientate and pin the tissue gently so that the duodenum is on the left with the attached pancreas to its right, the aorta vertically on the right of the pancreas and the spleen to the right and above the aorta, similar to the layout in **Figure 5**.

Cut off each end of the aorta to remove the LigaClips®, trim to a length of 2 cm ensuring the NN is approximately central to its length. Flush the aorta using the 24 g i.v. catheter with attached syringe filled with ice-cold oxygenated dextran-Krebs. Flush the luminal contents of the duodenal segment using the 18 g i.v. catheter with attached syringe filled with ice-cold oxygenated dextran-Krebs. Replace the modified Krebs in the dissection dish with ice-cold oxygenated dextran-Krebs.

## 2.5 Bile duct cannulation

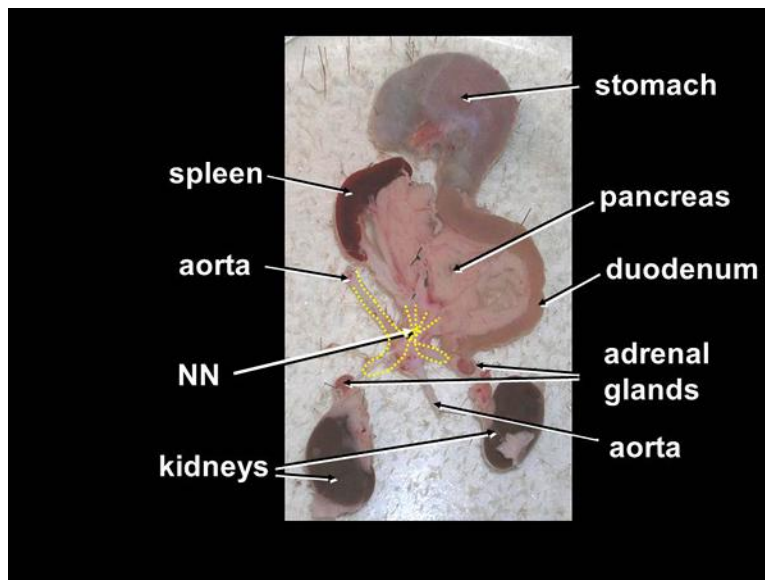
If the bile duct is required for the experiment, e.g. for pancreatic duct distension, proceed as follows.

Prepare a bile duct cannula using a 10 cm length of polyvinyl tubing (0.5 mm OD, 0.2 mm ID, 10 cm in length). To one end of the tubing attach a male luer, a 3 way tap and a syringe, filled with ice-cold oxygenated dextran-Krebs. Flush the tubing with the syringe solution and then close the tap to the tubing. To the other end of the tubing, cut at a 45 ° angle (cut off the sharp tip to avoid duct wall damage) to aid cannulation.

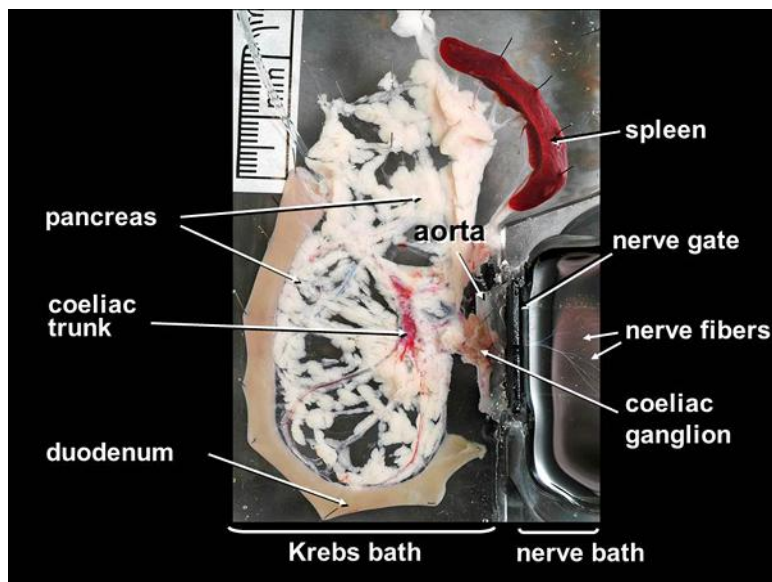
Locate the junction of the bile duct and the duodenum and secure this area by pinning the duodenum. With straight jewellers forceps use blunt dissection to separate pancreas from around the bile duct only at this junction. Loop a 6/0 silk suture underneath the exposed duct using curved forceps and ligate.

Locate the hepatic end of the bile duct which lies under the pancreas (using cotton buds gently fold the pancreas over to aid exposure of the duct) and use the previously attached suture to retract the duct slightly and pin in place. Loop a 6/0 silk suture underneath the duct using curved forceps and tie it very loosely a few mm from the end of the duct. Using microscissors, make a small cut (a slit) into the top of the duct between the 2 sutures, insert the prepared cannula through the cut and tighten the looped suture around the duct and cannula to secure them well and prevent subsequent leaks. Keep one end of the suture several cm long to restrain the duct and prevent twists once in the organ bath.

Refresh the dextran-Krebs solution in the dissection dish and ensure that it is well oxygenated with Carbogen and add more ice around the dissection dish if necessary. The tissue is ready to transfer to the organ bath.



**Figure 4. Image of an advanced stage of the dissection.** The nerve network (NN; the coeliac ganglion/mesenteric ganglion complex) is indicated. The broken yellow lines indicate the approximate location of the left and right nerve trunks.



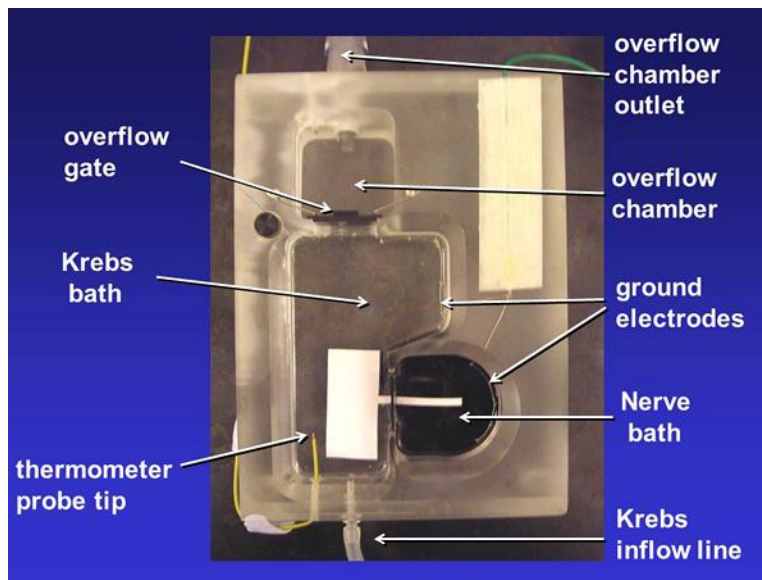
**Figure 5. Image of the final preparation (with duodenal segment retained for orientation) pinned in the Krebs-bath.** Note that the duodenal segment is normally removed. The spleen is usually retained as it provides a means of maintaining the pancreas in a stretched state without the direct insertion of pins in the pancreas. The nerve trunks with teased bundles and fibers are visible in the nerve-bath. A scale bar is included in the image (1 division = 1 mm).



## 2.6 Organ bath and tissue arrangement

Key features of the organ bath are shown in **Figure 6**. Position the organ bath in the Faraday cage so that the nerve-bath is on the right side, the Krebs-bath is left and the overflow-chamber toward the top (away from open side of the cage). To prepare the organ bath (**Note 7 and Figure 6**) to receive the tissue, fix the overflow-chamber gate in place, fill the Krebs-bath and nerve-bath (without the nerve-bath gate in place) with ice-cold oxygenated dextran-Krebs solution and

position a “gauze wick” over the overflow-chamber gate (**Note 7 and Figure 7**). Begin continuous perfusion of the bath with ice-cold oxygenated dextran-Krebs solution via a peristaltic pump (3-5 ml/min). Turn on the vacuum/suction to drain away the accumulating solution from the overflow-chamber via the outlet tube. Place a polyvinyl tube connected to the Carbogen source in the Krebs-bath to maintain solution oxygenation (**Figures 7 and 8**). Secure a temperature probe in the Krebs-bath to continuously monitor bath temperature.



**Figure 6. An image of the empty organ bath.** Key features are: 3 compartments (Krebs and nerve baths and overflow-chamber). The overflow-chamber and nerve-bath gates are in position. The Krebs and nerve baths have a ground electrode pinned to their respective base. A T-shape piece of paper is included to highlight the nerve gate. Also shown are the Krebs-bath inlet line, overflow-chamber outlet and the tip of a thermometer in the Krebs-bath.

Transfer the tissue to the Krebs-bath and loosely orientate the aorta to the right (near the nerve-bath Krebs-bath junction), the pancreas centrally, the duodenum on the left and the spleen on the right, above the aorta. If a bile duct catheter has been introduced, ensure it is not twisted and anchor it loosely in position with an inverted U-shaped pin. Align and firmly pin the aorta close to the nerve-bath entrance so the free nerve trunks,

emanating from the NN, are positioned  $90^\circ$  to the aorta, located centrally to and lie across the bottom of the nerve-bath entrance and into the nerve bath (see **Figures 5, 7 and 8**). The nerve position across the nerve-bath entrance is critical and the nerve-bath gate can be used as a guide to ensure that the nerve lies directly under the notch in the gate and so avoid damage due to crushing (**Note 7**).

Use tension along the duodenum length to progressively retract and gently flatten the pancreas. Pin the connective tissue between the pancreas and duodenum to secure the tensioned pancreas. Once the pancreas is well pinned out, separate (by sharp dissection) and discard the duodenal segment.

Retract the spleen gently to the right and pin to tension the attached blood vessels and pancreas. Adjust the position of the bile duct catheter, if used, to ensure it is lying untwisted and as straight as possible, then secure firmly using the inverted U-shaped pin. Tension the remaining pancreas, pinning the connective tissue where possible, otherwise pin the edges of pancreas, to flatten/stretch gently and secure firmly (**Figures 7 and 8**).

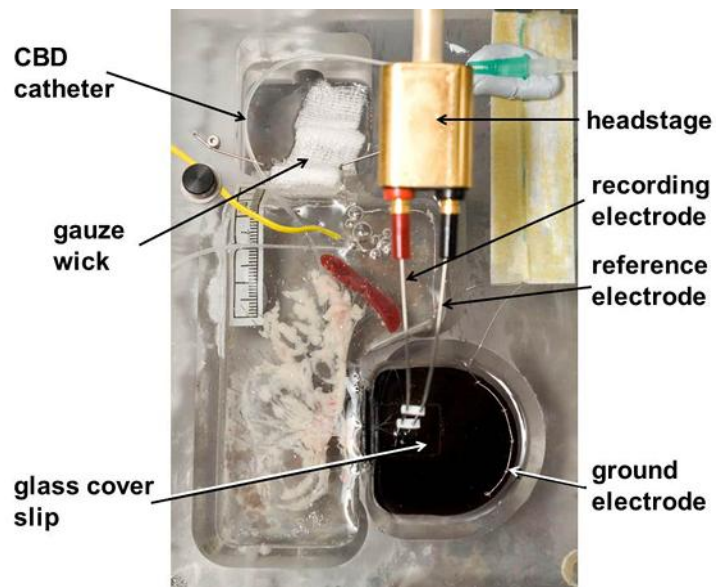
## 2.7 Nerve-bath and nerve arrangement

To position the nerve within the nerve-bath perform the following steps relatively quickly to prevent the nerve from drying out. Stop the Krebs pump, lift the outflow-bath gate and gently tilt the bath to drain the dextran-Krebs from the Krebs-bath and the nerve-bath. Alternatively, strong suction could be used with care, to rapidly remove the solution.

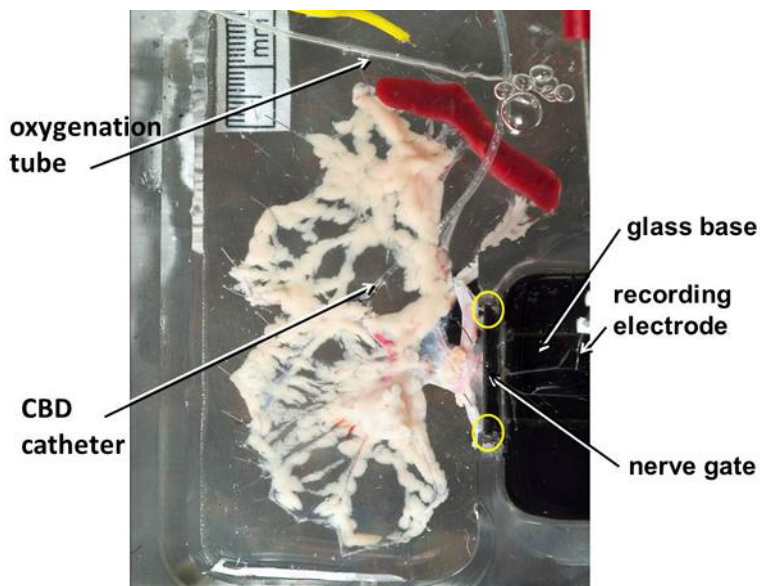
Rapidly blot the nerve-bath entrance with facial tissue paper being careful not to damage the nerve. Quickly syringe a continuous, generous long bead of silicon grease along both sides of the nerve-bath entrance (in the gate channels) and along the bottom. Re-position the unconstrained nerve trunks so that they lay centrally across the nerve-gate entrance, over the bead of silicone and into the nerve-bath along the glass base, then quickly syringe more silicone on top of the nerve trunks. Slide the nerve gate into the gate channels and position the tips of the 45 ° forceps under the gate notch (this helps to align the nerve under the notch) then slide the gate almost completely down, ensure that the nerve trunks are positioned correctly, remove the forceps and slide

the gate completely down to securely separate the 2 baths (**Figure 8**). With the aid of a cotton bud, smooth the grease along the gate joints to create a good seal between the Krebs-bath and the nerve-bath. Blot up residual dextran-Krebs from the nerve-bath then add sufficient paraffin to generously cover the nerve trunks (approximately 10 mm in depth). Syringe/pour dextran-Krebs into the Krebs-bath to generously cover the tissue then re-start the dextran-Krebs pump (12-15 ml/min).

The nerve may need gentle untwisting and tensioning to position it centrally along the length of glass bottom in the nerve-bath. The properties of the glass and paraffin ensure that the nerve adhere to the glass, keeping it in position. If the nerve starts floating in the paraffin then it is likely due to too much Krebs under the nerve. In this case, wick the Krebs away, using the corner of 1 ply tissue paper rolled/twisted finely. Search for the source of the Krebs which is under the nerve; it may be residual Krebs from the dissection or due to a leak in the gate which needs to be resealed with the silicone grease (use a damp cotton bud to apply gentle pressure along the silicone to help reseal the gate; additional silicone may be required). Cut off the associated adrenal glands (if still attached) and discard. Check the tension of the tissue in the Krebs-bath and pin out to retention (stretch) if necessary as shown in **Figures 5, 7 and 8**. Bath temperature should be 15-20 °C (**Note 8**).



**Figure 7.** This image shows a preparation pinned in the Krebs-bath. The gauze wick is visible in the top left side. Also shown are the headstage (with recording and reference electrodes) and the nerve trunks in the nerve bath lying on the glass base. The common bile duct (CBD) catheter and oxygenation tube are also visible.



**Figure 8.** Image of the final preparation pinned in the Krebs-bath. Note that the duodenal segment is removed. The common bile duct (CBD) catheter, oxygenation tube and temperature probe are shown. In the nerve bath, the nerve trunks, bundles and fibers are visible. The channels in which the nerve gate is positioned (sides of the Krebs-bath) are highlighted by yellow circles. The recording electrode is visible at the bottom of the image. A teased nerve fiber is on the recording electrode; however this is not visible at this magnification. (Scale bar; 1 division = 1 mm).

## 2.8 Nerve de-sheathing and division

Using fine forceps and with the aid of the dissecting microscope, tease the nerve trunk sheath apart at a point several mm from its end. To do this, grasp the opposite edges of the nerve trunk sheath with the fine forceps and then pull in opposite directions (i.e. at 90 ° to the nerve length). Once the sheath is cleared in the one point, around the circumference of the nerve trunk, it may be possible to roll the sheath towards the cut end and pull it off (like rolling down a sock). Be careful not to pull lengthwise too much otherwise the nerve may be damaged. Continue until the sheath is completely removed but avoid clearing the portion near the gate (do not disturb the nerve trunks near the gate otherwise it may cause the gate to leak).

Once the sheath is removed it will be evident that the trunk is composed of smaller bundles. Position one tip from each of 2 pairs of fine jeweller's forceps between these smaller bundles at one point several mm from the end of the trunk and pull the forceps in opposite directions to separate the bundles. Work along the length of the trunk to completely separate the bundle. Continue using this method until the trunks are separated into a number of bundles.

Use the same method to separate each bundle into finer fibers. As each bundle is divided more finely, arrange the fibers on the glass base so that they are separated from one another to prevent tangling, as seen in **Figure 8**.

## 2.9 Recording apparatus arrangement

Attach the headstage in a micromanipulator firmly and ensure the micromanipulators' horizontal and vertical adjustments are centralised for greatest manoeuvrability and flexibility, then attach the electrode to the headstage. Grossly manoeuvre the micromanipulator with attached headstage so that the electrodes are positioned over the nerve

bath and nerve (it may be necessary to move the organ bath to achieve the ideal position).

Using the micromanipulators' fine adjustments, position the recording electrode directly above the nerve fiber required for recording neural activity. The reference electrode should be set several mm away from the recording electrode, be in good contact with the glass base of the nerve-bath and be positioned several mm above the nerve fibers. Place a small piece of connective tissue cut from near the spleen over the reference electrode, to complete the circuit for recording. Using fine forceps gently drape the nerve fiber over the recording electrode (**Figure 8**).

Commence perfusion of the Krebs bath with pre-heated dextran-Krebs perfusion at a rate of 6-7 ml/min. This rate is varied to maintain the Krebs-bath temperature between 32 – 33 ° C for the recording period (**Note 8**). Turn on the differential amplifier to begin recording however a 30 min equilibration period is required to allow nerve activity to stabilise at the elevated temperature before commencing the protocol. Periodically monitor the bath temperature, fluid levels and the nerve-bath gate for leaks. Total preparation time is 4-5 hours.

This preparation is suitable for identifying pancreatic afferent nerve endings (receptive fields) using a fine stimulating electrode. Once located, the receptive fields can be tested for mechanical and chemical sensitivity. Standard extracellular recording equipment is used. The preparation should provide 4-6 hours of recordings (6).

## 3. Notes

1. Prepare the dissection dish several days prior to being required. Mix the Sylgard in a disposable container as specified in the instructions (Sylgard parts are very sticky and to aid handling during measuring and mixing use disposable syringes of the appropriate volume). Pour a 5-7 mm layer

of Sylgard on the bottom (to extract bubbles from the Sylgard place the dissection dish in a vacuum chamber, under vacuum, for approximately an hour) and leave to cure on a level surface for the specified period (see Sylgard instructions). Once cured, soak the dissection dish in distilled water for 24 hours to remove chemical residues. Once prepared, this dish can be washed and reused for numerous experiments. Insert several 27 g needles and entomology pins into the silicone layer around the edge of the dish in preparation for the dissection.

2. Modified Krebs solution. This solution is bubbled with Carbogen, during the preparation to adjust the pH. If this is not done, a precipitate of calcium phosphate may develop and the solution will appear whitish rather than clear. We use 5L plastic beakers and cover with food-wrap for overnight storage in the refrigerator.
3. Dextran is added in small amounts (5-10 g lots) as it is easier to dissolve; it takes time to dissolve and continuous stirring is essential. The solubility of the dextran is temperature dependent – room temperature is adequate, however warming the solution will aid solubility. The larger surface area of the 5 L beaker also decreases the time to dissolve the dextran.
4. We have found that a cold dissection results in a better preparation. Presumably the low temperature reduces cellular metabolism and protease activity. An oxygenation tube is placed in the dissection dish for the duration of the dissection to maintain the oxygenation of the Krebs solution. This tube is pinned to the edge of the dissection dish under the surface of the solution and the open end covered with a small piece of gauze to prevent the bursting gas bubbles from disturbing the surface of the solution (which would otherwise impede visibility, particularly when using the dissecting microscope).
5. Pins. To pin down the tissues, we use 27 g hypodermic needles to restrain the larger organs (e.g. stomach) and entomology pins for the remainder (**see Figures 3-5**).
6. Tissue handling. The rat pancreatic tissue is relatively diffuse compared with that of human or mouse and is attached to the stomach, small intestine, colon and spleen with connective tissue. Therefore to adequately isolate the pancreas, these attachments need to be cut. As the pancreas is a fragile tissue, it should be handled as little as possible. Consequently the attached organs and connective tissue should be used for manipulation. For example, to dissect the pancreas away from the colon, handle the colon with forceps and put the tissue under gentle tension to aid separation and cutting between the organs.
7. We use a custom made 3-compartment bath (**see Figures 5 - 8**). This is milled from solid plexiglass. The larger compartment (Krebs-bath) has a Sylgard base which allows the tissues to be pinned down. The nerve bath also had a Sylgard base (which is coloured black to improve colour contrast with the nerve trunk and nerve fibers; **Figures, 5 - 8**) and also a square of glass microscope slide (which was positioned onto the liquid Sylgard prior to hardening). The glass provides a surface to which the nerve trunks and individual bundle and fibers will adhere to under paraffin oil and facilitates bundle/fiber manipulation (**Figures 5 & 7**). Adjacent to the Krebs-bath is an overflow-chamber which is separated from the Krebs-bath by a removable plexiglass gate. Adjusting the height of this gate, in conjunction with the perfusion flow rate, regulates the volume in the Krebs-bath. Drainage into the overflow-chamber is

facilitated by placing a piece of gauze over the gate to act as a wick (see **Figure 7**). A vacuum pump is connected to the overflow-chamber outlet to provide continuous suction which prevents accumulation of fluid. The Krebs and nerve baths are separated by a removable acetal homopolymer resin (black) gate which fits into channels constructed into the sides of the organ bath at the nerve-bath entrance (**Figure 8**). This gate has an inverted U-shaped notch cut in the centre of the base to allow passage of the nerve bundles into the nerve bath without being crushed or compressed when the gate is in position. When the nerve gate and nerve trunks are in their final positions, silicone grease is generously applied to the joints of the gate to provide leak-proof seals.

8. Solution temperatures: the temperature of the dextran-Krebs in the Krebs-bath is kept at 15-20 °C until the preparation is ready for recording. The Krebs is perfused through a heat exchanger connected to a refrigeration pump unit to cool the Krebs. Subsequently, the Krebs-bath temperature is then raised to 32-33 °C by pumping the Krebs through another heat exchanger connected to a heat pump unit. Following a 15-30 min equilibration period recording is commenced. Because of the temperature change, gas bubbles will be generated in the Krebs lines, so a bubble trap needs to be included in the setup, just before the Krebs-bath inlet.

## 4. References

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