Journal of Visualized Experiments

Observing Islet Function and Islet-immune Cell Interactions in Live Pancreatic Tissue Slices

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62207R1
Full Title:	Observing Islet Function and Islet-immune Cell Interactions in Live Pancreatic Tissue Slices
Corresponding Author:	Edward A Phelps, Ph.D. University of Florida Gainesville, FL UNITED STATES
Corresponding Author's Institution:	University of Florida
Corresponding Author E-Mail:	EPhelps@bme.ufl.edu
Order of Authors:	Mollie Huber
	Denise Drotar
	Helmut Hiller
	Maria Beery
	Paul Joseph
	Irina Kusmartseva
	Stephan Speier
	Mark Atkinson
	Clayton Mathews
	Edward Phelps
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Immunology and Infection
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Gainesville, Florida, United States of America
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	The manuscript is invited for special issues.

TITLE:

Observing Islet Function and Islet-immune Cell Interactions in Live Pancreatic Tissue Slices

AUTHORS AND AFFILIATIONS:

Mollie K. Huber[1], Denise M. Drotar[2,3,4], Helmut Hiller[1], Maria L. Beery[1], Paul Joseph[1], Irina Kusmartseva[1], Stephan Speier[2,3,4], Mark A. Atkinson[1], Clayton E. Mathews[1], Edward A. Phelps[5]

[1]Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL 32611, USA
[2]Paul Langerhans Institute Dresden (PLID) of the Helmholtz Zentrum München at the University Clinic Carl Gustav Carus of Technische Universität Dresden, Helmholtz Zentrum München, Neuherberg, Germany
[3]Institute of Physiology, Faculty of Medicine, Technische Universität Dresden, Germany
[4]German Center for Diabetes Research (DZD), München-Neuherberg, Germany
[5]J. Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL 32611, USA

Email addresses of co-authors:

Mollie K. Huber	mhuber@ufl.edu
Denise M. Drotar	denise-minerva.drotar@tu-dresden.de
Helmut Hiller	hhiller@ufl.edu
Maria L. Beery	marialpeterson@pathology.ufl.edu
Paul Joseph	pjoseph14@ufl.edu
Irina Kusmartseva	inkusmartseva@ufl.edu
Stephan Speier	stephan.speier@helmholtz-muenchen.de
Mark A. Atkinson	atkinson@pathology.ufl.edu
Clayton E. Mathews	Clayton.Mathews@pathology.ufl.edu

Corresponding author:

Edward A. Phelps ephelps@bme.ufl.edu

KEYWORDS:

Pancreas, tissue slices, immune cells, Ca^{2+} flux, type 1 diabetes, type 2 diabetes, physiology

SUMMARY:

This study presents the application of live pancreatic tissue slices to the study of islet physiology and islet-immune cell interactions.

ABSTRACT:

Live pancreatic tissue slices allow for the study of islet physiology and function in the context of an intact islet microenvironment. Slices are prepared from live human and mouse pancreatic tissue embedded in agarose and cut using a vibratome. This method allows for the tissue to maintain viability and function in addition to preserving underlying pathologies such as type 1 (T1D) and type 2 diabetes (T2D). The slice method enables new directions in the study of the

pancreas through the maintenance of the complex structures and various intercellular interactions that comprise the endocrine and exocrine tissues of the pancreas. This protocol demonstrates how to perform staining and time-lapse microscopy of live endogenous immune cells within pancreatic slices along with assessments of islet physiology. Further, this approach can be refined to discern immune cell populations specific for islet cell antigens using major histocompatibility complex-multimer reagents.

INTRODUCTION:

Involvement of the pancreas is pathognomonic to diseases such as pancreatitis, T1D, and T2D[1-3]. The study of function in isolated islets usually involves removal of the islets from their surrounding environment[4]. The live pancreatic tissue slice method was developed to allow for the study of pancreatic tissue while maintaining intact islet microenvironments and avoiding the use of stressful islet isolation procedures[5-7]. Pancreatic tissue slices from human donor tissue have been successfully used to study T1D and have demonstrated processes of beta cell loss and dysfunction in addition to immune cell infiltration[8-13]. The live pancreatic tissue slice method can be applied to both mouse and human pancreatic tissue[5,6,8]. Human pancreatic tissue slices from organ donor tissues are obtained through a collaboration with the Network for Pancreatic Organ Donors with Diabetes (nPOD). Mouse slices can be generated from a variety of different mouse strains.

This protocol will focus on non-obese diabetic-recombination activating gene-1-null (NOD.*Rag1*$^{-/-}$) and T cell receptor transgenic (AI4) (NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$) mouse strains. NOD.*Rag1*$^{-/-}$ mice are unable to develop T and B cells due to a disruption in the recombination-activating gene 1 (*Rag1*)[14]. NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mice are used as a model for accelerated type 1 diabetes because they produce a single T cell clone that targets an epitope of insulin, resulting in consistent islet infiltration and rapid disease development[15]. The protocol featured here describes procedures for functional and immunological studies using live human and mouse pancreatic slices through the application of confocal microscopy approaches. The techniques described herein include viability assessments, islet identification and location, cytosolic Ca^{2+} recordings, as well as staining and identification of immune cell populations.

PROTOCOL:

NOTES: All experimental protocols using mice were approved by the University of Florida Animal Care and Use Committee (201808642). Human pancreatic sections from tissue donors of both sexes were obtained via the Network for Pancreatic Organ Donors with Diabetes (nPOD) tissue bank, University of Florida. Human pancreata were harvested from cadaveric organ donors by certified organ procurement organizations partnering with nPOD in accordance with organ donation laws and regulations and classified as “Non-Human Subjects” by the University of Florida Institutional Review Board (IRB) (IRB no. 392-2008), waiving the need for consent. nPOD tissues specifically used for this project were approved as nonhuman by the University of Florida IRB (IRB20140093). The objectives of sections 1–3 of this protocol are to explain how to successfully dissect a mouse, prepare and process the pancreas, and generate live pancreatic tissue slices. Solutions should be prepared ahead of time, and the recipes can be found in

Supplemental Table 1. Time is the most critical factor during these protocol steps. Once the mouse has been sacrificed, tissue viability will begin to decline. All three parts of this protocol need to be completed as quickly as possible until all the necessary slices are generated.

1. **Preparation for generation of mouse pancreas slices**

1.1. Clamp the blade onto the vibratome blade holder, but do not attach it to the vibratome yet.

1.2. Melt 100 mL of 1.25% w/v low melting-point agarose in a microwave. Operate the microwave in 1 min intervals, and stop the microwave for 10 s if the agarose solution begins to boil. Repeat this process until the agarose is melted, and a homogeneous solution is produced. Place the bottle in a 37 °C water bath.

NOTE: The low agarose concentration is to account for the lower density of the mouse pancreas.

1.3. Fill a 10 mL Luer lock syringe with 3 mL of warm agarose. Fit a 27 G 25 mm needle onto the syringe. Keep the syringe with the capped attached needle in a 37 °C water bath until the solution is to be injected.

NOTE: A 27 G needle is preferred as it fits securely into the common bile duct of mice between 10–25 g in body weight and allows for the flow of the highly viscous agarose solution. While larger bore needles may be selected for use, smaller (larger gauge) needles are not recommended as these may be more easily clogged with the agarose solution.

1.4. Add 20 mL of chilled extracellular solution (ECS) to a 10 cm Petri dish.

NOTE: The ECS does not need to be bubbled at any point.

1.5. Fill two 10 cm untreated Petri dishes with 15 mL of Krebs-Ringer bicarbonate buffer (KRBH) containing 3 mM D-glucose and soybean trypsin inhibitor at a concentration of 0.1 mg/mL per dish.

NOTE: Throughout this protocol, it is essential that all solutions used for maintaining slices contain soybean trypsin inhibitor to prevent tissue damage caused by pancreatic digestive proteases.

2. Mouse pancreas excision and tissue processing

NOTE: The protocol for excising the pancreas, processing the tissue, and generating slices is modified from Marciniak et al[5]. To ensure tissue viability, minimize the amount of time between pancreas removal and slice generation. All necessary equipment should be prepared in advance and oriented in a manner to allow for rapid progression through the steps below. Bile duct canulation and injection as well as pancreas excision are best performed under a stereoscope.

2.1. The mouse is deeply anesthetized with isoflurane and sacrificed by cervical dislocation.

NOTE: Isoflurane is the preferred anesthetization method. A concentration of 5% isoflurane should be used. For example, 0.26 mL should be used with a 1 L chamber[16]. A decrease in pancreatic tissue viability was observed when CO_2 was used.

2.2. Spray the mouse with 70% v/v ethanol liberally to reduce tissue contamination with fur during the dissection and excision. Place the mouse in a dorsal down, ventral up orientation with the anterior side to the left.

2.3. Using scissors, open the peritoneum and remove the rib cage, taking care not to puncture the heart or adjacent vessels. Use forceps to flip the liver into the chest cavity, and to move the intestines out of the body cavity to expose the common bile duct. Use a Johns Hopkins bulldog clamp to occlude the ampulla of Vater.

2.4. Retrieve a 10 mL Luer lock syringe preloaded with 3 mL of warm agarose solution from the 37 °C water bath.

NOTE: Once the syringe with the agarose is removed from the water bath, the pancreas injection needs to be performed quickly before the agarose cools and sets in the syringe.

2.5. Holding the forceps in the left hand, use them to gently support and stabilize the bile duct for the injection.

2.6. Hold the syringe in the right hand, insert the needle bevel-side up into the bile duct. Slowly and steadily inject the pancreas. Once the injection starts, the flow cannot be stopped without the agarose hardening in the syringe and in the pancreas.

NOTE: The volume used will depend on the weight of the mouse. Based on experience, it is recommended that 1 mL of agarose solution be used per 10 g of mouse body weight with a maximum volume of 2 mL. The pancreas should look slightly inflated with a more definitive structure, but not overextended. Over-injection results in islets that become separated from the exocrine tissue and that have a “blown-out” appearance in the slices.

2.7. Excise the agarose-filled pancreas from the mouse. Using forceps and scissors, cut the pancreas away, starting at the stomach, moving to the intestines, and ending at the spleen. Once cut away, gently remove the injected pancreas with forceps, and place in a 10 cm Petri dish filled with chilled ECS.

2.8. Use scissors to remove the adipose, connective tissue, and fibrotic tissue, and parts of the pancreas that are not injected with agarose.

NOTE: Parts of the tissue that should be removed will not have strongly established structures and will appear somewhat gelatinous.

2.9. After trimming the tissue, use scissors to cut it into smaller sections that are approximately 5 mm in diameter while leaving it submerged under ECS. Cut the tissue carefully, taking care to not push the agarose out of the tissue.

2.10. Remove the pieces of tissue from the ECS, and place them on a two-ply wiper (see the **Table of Materials**). Gently roll them on the wiper using forceps to remove excess liquid.

2.11. Using forceps, carefully place the pieces of tissue in a 35 mm Petri dish with no more than 4 pieces per plate. Place the flattest side of the tissue block facing downward. Gently press down on the tissue using forceps.

NOTE: Make sure there is space, at least a few millimeters, between the pieces of tissue, and that they are not touching the edge of the plate.

2.12. Slowly pour the 37 °C agarose into the dish, taking care not to pour it directly on to the tissue. Pour enough so that the tissue pieces are completely covered. Make sure there is a layer of agarose above the tissue pieces as this part will be glued to the specimen holder.

2.13. Carefully transfer the dish with the pieces of tissue to a refrigerator to allow the agarose to set. Ensure the tissue pieces do not shift or start floating. If they do, quickly readjust them using forceps.

NOTE: Setting of the agarose should only take a few minutes.

2.14. Once the agarose has set, use a scalpel to cut around the tissue in straight lines to make agarose blocks as if making a grid between the pieces of tissue. Be sure to leave a few millimeters of agarose surrounding all sides of the tissue.

NOTE: There should not be any tissue protruding from the agarose. Each block should be a cube of approximately 5 mm x 5 mm x 5 mm volume.

2.15. Use the scalpel to flip out the empty sections of agarose that were cut around the edges of the plate. Remove the blocks with the tissue from the dish by lifting them carefully with forceps.

3. Mouse pancreatic slice generation

3.1. Using forceps, arrange the blocks on the specimen holder; place them sideways, keeping in mind that they will be flipped onto the super glue. Arrange the blocks so that they do not extend further than the blade width. As the vibratome moves slowly, arrange the blocks so that the blade has to move forward the least possible distance.

NOTE: Two rows of three or four blocks each with a few millimeters between the rows works well. The blocks within the same row can touch each other, but when both rows touch, it can be

difficult to retrieve slices as they come off the blocks.

3.2. Apply a line of super glue on the specimen holder, and use the end of the glue dispenser to spread the glue out into a thin layer. Flip the tissue blocks onto the glue so that the side closest to the tissue faces upward. Gently push down on the blocks, and let the glue dry for three minutes.

3.3. Attach the plate to the vibratome, typically with either a screw or magnet, depending on the vibratome model. Adjust the blade height and distance travelled so that the blade moves over the length of the blocks and just barely above them.

NOTE: Forceps can be helpful while adjusting the blade height. They can be placed on top of the tissue block to help place the blade as close to the top of the block as possible without touching the block.

3.4. Ensure that the glue has dried by gently nudging the blocks with forceps, and fill the vibratome tray with chilled ECS until the blade is covered. Set the vibratome to make 120 µm thickness slices at a speed of 0.175 mm/s, a frequency of 70 Hz, and an amplitude of 1 mm.

NOTE: The vibratome speed can be adjusted depending on the ease of cutting the tissue.

3.5. Start the vibratome, and watch for when slices start coming off of the tissue blocks. Use 10 cm Graefe forceps or a small No. 4 paintbrush to carefully remove the slices once they float off the block, and place them in 10 cm plates with KRBH containing 3 mM D-glucose and trypsin inhibitor. Pick up the slices by placing a paintbrush or forceps below them and gently lifting the slices. Do not incubate more than 15 slices together in a single plate.

NOTE: It is normal for the vibratome to have a few passes over the blocks where no slices are made, but these should be minimized for time. Have scissors ready in case the slices do not fully separate from the tissue blocks. If this happens, a corner or edge of the slice will be stuck to the block after the vibratome blade passes. Do not pull off the slice or block when removing the stuck slice.

3.6. Place the plates with the slices on a rocker at room temperature and at 25 rpm. Let the slices rest at room temperature for an hour. If they are going to be left for longer, place the slices in 15 mL of slice culture medium (see **Supplemental Table 1**) in an incubator. Incubate slices prepared for same-day studies at 37 °C, and culture slices that are cultured overnight at 24 °C, transferring them to 37 °C at least 1 h before experiments.

NOTE: Over the long term, the slices have better viability when cultured at 24 °C, although 37 °C is closer to their native physiological environment, probably due to the lower activity of the secreted protease enzymes at lower temperature. Mouse and human pancreatic tissue slices are both cultured at the same temperature and with a maximum of 15 slices per dish. However, the media recipes differ for human and mouse slices. Both formulations are listed in **Supplemental**

Table 1. Additionally, the procedure is the same for generating mouse and human slices with the exception of the mouse pancreas requiring injection with agarose for stabilization. Human slices are acquired through the nPOD Pancreas Slice Program. Both mouse and human slices are 120 µm thick. A variety of experiments can be performed on the slices; choose staining panels that work best for planned experiments.

4. Slice preparation for staining procedures

4.1. Culture the slices at 37 °C for at least 1 h ahead of the planned experiments. Warm KRBH containing 3 mM D-glucose in a 37 °C water bath. Transfer 2 mL of KRBH containing 3mM D-glucose in a 35 mm dish, and use a paintbrush to gently place the slice in it.

4.2. If the slice is being transferred from medium, wash it twice with KRBH containing 3 mM D-glucose. Carefully aspirate the KRBH with 3 mM D-glucose using a transfer pipette or Pasteur pipette, taking care not to disturb the slice. Keep the slice in the plate with KRBH containing 3 mM D-glucose while the staining panels are prepared.

5. Dithizone staining

NOTE: Although dithizone can be used to stain the islets red, it will kill the slice as it has been found to be cytotoxic to islets[17].

5.1. Measure 12.5 mg of dithizone, add it to 1.25 mL of dimethylsulfoxide, and take this mixture up in a 50 mL syringe. Fill the syringe to a volume of 25 mL using Hanks Balanced Salt Solution, and attach a filter to the end of the syringe. Aliquot 2 mL of KRBH with 3 mM D-glucose, and add 2 drops of the filtered dithizone solution from the 50 mL syringe into a 35 mm dish.

5.2. Using a paintbrush, carefully place a slice in a 35 mm petri dish. Image the slice with the islets indicated by red dithizone staining using a stereomicroscope.

6. Viability staining

NOTE: This section of the protocol describes how to assess slice viability using calcein-AM and blue-fluorescent nucleic acid stain (see the **Table of Materials**). Calcein-AM should be used at a concentration of 4 µM and blue-fluorescent nucleic acid stain at 1 µM.

6.1. Aliquot 2 mL of KRBH containing 3 mM D-glucose, and add 2 µL of calcein-AM dye and the nucleic acid stain to separate aliquots. Vortex the mixtures for 5 s.

6.2. Add 200 µL of KRBH containing 3 mM D-glucose and the dyes to each well of an 8-well chambered coverglass.

NOTE: Alternative plates and/or imaging chambers other than an 8-well chambered coverglass can be used.

6.3. Using a paintbrush, carefully place a slice in each well of the plate, and transfer the plate with the slices to a 37 °C incubator for 20 min. Wash the slices twice with KRBH containing 3 mM D-glucose. Carefully aspirate the KRBH using a transfer or Pasteur pipette, taking care not to disturb the slice.

6.4. Place the slice in a 35 mm coverglass-bottom Petri dish containing 2 mL of KRBH with 3 mM D-glucose and 2 µL of the nucleic acid stain at a concentration of 1 µL per 1 mL of solution. Cover the slice with a slice anchor, ensuring that the side with the harp faces downward. Take images of the slice.

NOTE: If the slice anchor keeps floating, wet it on both sides with KRBH containing 3 mM D-glucose to submerge it in the solution. It is critical to always maintain the slices in solutions containing protease inhibitor, even during dye loading. Viability stains used can be adapted for the specific experiment or microscope setup.

7. Slice Ca^{2+} indicator staining

NOTE: This section of the protocol describes how to stain slices for Ca^{2+} recordings using the cell-permeable Ca^{2+} indicator and nucleic acid stain in mouse slices (see the **Table of Materials**). The cell-permeable Ca^{2+} indicator should be used at a concentration of 5.6 µM and the nucleic acid stain at 1 µM. In human slices, Fluo-4-AM should be used at a concentration of 6.4 µM.

7.1. Aliquot 2 mL of KRBH containing 3 mM D-glucose, add 7 µL of the cell-permeable Ca^{2+} indicator, and vortex the mixture for 5 s.

NOTE: For human tissue slices, use Fluo-4-AM instead of the cell-permeable Ca^{2+} indicator. The Fluo-4-AM is preferable because it is brighter when the intracellular Ca^{2+} concentration increases; however, it does not load well in mouse pancreatic tissue. The protocol is the same as described above for Fluo-4-AM with the exception that Fluo-4-AM only needs to be incubated for 30 min.

7.2. Add 200 µL of KRBH containing 3mM D-glucose and the dye to each well of an 8-well chambered coverglass. Using a paintbrush, carefully place a slice in each well of the chambered coverglass. Transfer the chambered coverglass with the slices to a 37 °C incubator for 45 min.

7.3. Wash the slices twice with KRBH containing 3 mM D-glucose. Carefully aspirate the KRBH with 3 mM D-glucose using a transfer or Pasteur pipette, taking care to not disturb the slice.

7.4. Place the slice in an imaging plate or chamber with KRBH containing 3 mM D-glucose and the nucleic acid stain at a concentration of 1 µL per 1 mL, and cover with a slice anchor, ensuring that the harp faces downward. Take images of the slice.

NOTE: In this protocol, a 35 mm dish filled with 2 mL of KRBH containing 3 mM D-glucose and 2 µL of nucleic acid stain was used. If the slice anchor keeps floating, wet it on both sides with KRBH

containing 3 mM D-glucose to submerge it in solution.

8. Mouse slice Ca^{2+} recordings

NOTES: The following section describes how to perform Ca^{2+} recordings on mouse pancreatic tissue slices using the cell-permeable Ca^{2+} indicator and nucleic acid stain. Imaging was performed on a confocal laser-scanning microscope (see the **Table of Materials** for details). The lasers used were 405 nm for the nucleic acid stain, 488 nm for the cell-permeable Ca^{2+} indicator, and 638 nm for reflectance. A HyD detector was used for the cell-permeable Ca^{2+} indicator. Photomultiplier tube (PMT) detectors were used for reflectance and the nucleic acid stain. The Ca^{2+} imaging protocol is the same for human pancreatic tissue slices except that Fluo-4-AM was used as the indicator. Laser power levels, gain, and pinhole size should be adjusted based on the sample and particular islet imaged. Typically, a pinhole of 1.5 airy units and a laser power of 1% are good starting points.

8.1. At least 1 h before recording, switch on the microscope and equilibrate the stage-top or cage-style incubator to 37 °C. Place the 35 mm coverglass-bottom Petri dish containing the slice on the stage after removing the lid. Focus by setting the microscope to the 10x objective and brightfield mode. Locate islets using brightfield by looking for orange-brown ovals within the slice.

8.2. Once a probable islet is located, switch the microscope to the confocal imaging mode. To confirm islets by reflectance, switch on the 638 nm laser, set the laser power between 1% and 2%, and switch off the 638 nm notch filter that would normally remove backscattered light. Set the detection limits on the PMT detector to a bandwidth of approximately 20 nm centered around 638 nm.

NOTE: Exercise caution as operating the microscope in reflectance mode could damage the detector. Due to the high granularity of the endocrine tissue, reflected light can now be used to locate islets. The islets will appear as groups of brightly backscattering granular cells on this reflectance channel.

8.3. To view the nucleic acid stain, switch on the 405 nm laser and PMT detector, and set the laser power between 1% and 2%. Center the islet of interest in the field of view using the X and Y knobs of the stage controller. Once an islet of interest is located and confirmed by backscatter, switch to the 20x objective, and zoom in so the islet takes up most of the frame.

8.4. Take a z-stack of the islet with a z-step size of 1.5 µm. Find the best optical section of the islet where most of the cells are alive (negative for the nucleic acid stain) and well-loaded with the cell-permeable Ca^{2+} indicator or Fluo-4-AM.

NOTE: It is not unusual to see cells that are overloaded with a large amount of dye and that are very bright. These may be dying pancreatic cells in which Ca^{2+} storage in the endoplasmic reticulum may be released, resulting in high levels of loading; these are not ideal cells to record. Look for cells that have clearly loaded the dye, but are not oversaturating the detector so that an

increase in brightness that occurs when cytosolic Ca^{2+} levels fluctuate is visible. Dye loading of islets within slices is variable; however, the dye typically loads well through ~10–15 µm of the islet. However, dye loading can be difficult to visualize if the cells are deep in the tissue.

8.5. To prevent dye fading during the recording, ensure that the laser power on the 488 nm channel does not exceed 2%. Increase the pinhole to 2 airy units to collect more signal with lower excitation power.

8.6. Set the microscope to record in XYZT mode. Optimize the settings to reduce the frame rate to 2 s or less per frame.

NOTE: Settings adjustments that can be made to help decrease the frame rate include turning on bidirectional scanning, decreasing or turning off the line averaging, and increasing scanning speed.

8.7. Once the settings have been optimized, record several minutes of basal activity.

NOTE: Another good indicator of tissue viability is if the cells appear active and are visibly flashing during this recording of basal activity.

8.8. Add 100 µL of 20x concentrated KCl and glucose in KRBH to the plate using a 200 µL micropipette at the given time points to achieve a final concentration of 16.7 mM glucose or 30 mM KCl.

NOTES: Add the solutions carefully, taking care not to disturb the slice during the recording. Be sure not to bump the plate with the micropipette. It is typical to see the tissue contract in response to these stimulations. The Ca^{2+} flux recordings were processed and quantified in ImageJ[18]. Using ImageJ software, the staining intensity of the cell-permeable Ca^{2+} indicator was measured in the cells by manually selecting regions of interest (ROIs). The fluorescence intensity from these ROIs was calculated by dividing the fluorescence values at later timepoints by the initial fluorescence values of the cells (F/F_0). A perfusion system can be used along with a specialized imaging chamber to administer the solutions to slices dynamically as opposed to adding them manually. Perfusion system and imaging chamber recommendations can be found in the **Table of Materials**.

9. Staining of mouse T cells in live pancreatic slices

NOTE: This section of the protocol describes how to stain immune cells within mouse slices. The mouse strain used is the NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ as this model consistently develops disease with significant insulitis. The $CD8^+$ T cells in this mouse all target an epitope of insulin, allowing the use of a phycoerythrin (PE)-labelled insulin-D^b tetramer[15]. The CD8 antibody should be used at a concentration of 1:20 and the insulin tetramer at 1:50.

9.1. Aliquot 100 µL of KRBH containing 3 mM D-glucose, add 2 µL of PE insulin tetramer and 5 µL

of allophycocyanin (APC) CD8 antibody, and vortex the mixture for 5 s.

9.2. Add 100 µL of KRBH containing 3 mM D-glucose and the tetramer and antibody to a well of an 8-well chambered coverglass. Using a paintbrush, carefully place a slice in the well of the chambered coverglass. Transfer the chambered coverglass with the slice to a 37 °C incubator for 30 min.

9.3. Wash the slices twice with 2 mL KRBH containing 3 mM D-glucose. Carefully aspirate the KRBH with 3 mM D-glucose using a transfer or Pasteur pipette, taking care not to disturb the slice.

9.4. Place the slice in a 35 mm coverglass-bottom Petri dish containing KRBH with 3 mM D-glucose and the nucleic acid stain at a concentration of 1 µL per 1 mL, and cover with a slice anchor, placing the side with the harp facing downward. Take images of the slice.

NOTE: If the slice anchor keeps floating, wet it on both sides with KRBH containing 3 mM D-glucose to submerge it in the solution. The diluted antibody and tetramer can be reused once. After staining two slices, a fresh antibody mixture should be made.

10. Recording of mouse immune cells

NOTE: The following section describes how to perform immune cell recordings on mouse pancreatic tissue slices using CD8 antibody, PE insulin tetramer, and the nucleic acid stain. The imaging setup is as described in section 9. Recordings were made at 800 × 800 pixel resolution. The lasers used were 405 nm for the nucleic acid stain, 488 nm for the insulin tetramer, and 638 nm for CD8 antibody and reflectance. HyD detectors were used for CD8 antibody and PE insulin tetramer. PMT detectors were used for reflectance and the nucleic acid stain. The immune cell imaging protocol is the same for human pancreatic tissue slices except for the use of different antibodies and antigen-complexed HLA-multimers for human tissue. For both insulin tetramer staining in mouse tissue and HLA-multimer staining in human tissue, an immune cell co-stain should be used to verify the presence of the specific antigen-reactive T cells. Here, an anti-CD8 antibody was used. Antibodies, such as anti-CD3 or anti-CD4, can also be used depending on the target cell population.

10.1. At least 1 h before recording, switch on the microscope, and equilibrate the stage-top incubator to 37 °C. Secure the 35 mm coverglass-bottom Petri dish containing the slice on the stage. Focus the microscope by setting the 10x objective in the brightfield mode. Locate the islets using the brightfield mode by looking for orange-brown colored ovals within the slice.

10.2. Switch the microscope to confocal imaging by pressing the CS button on the microscope's touch screen controller. To view islets by reflectance, turn on the 638 laser and PMT detector, set the laser power between 1% and 2%, and turn off the notch filters.

NOTE: Due to the increased granularity of the endocrine tissue, reflected light can now be used

to locate islets. The islets will appear as groups of bright granular cells on this channel.

10.3. To view the nucleic acid stain, CD8 antibody, and insulin tetramer, check that the laser power is between 1% and 2%. Use the following settings to view each of the three: for the nucleic acid stain, turn on the 405 nm laser and PMT detector; for the CD8 antibody, turn on the HyD detector; and to view the insulin tetramer, turn on the 488 nm laser and HyD detector.

10.4. Center the islet of interest in the field of view using the X and Y knobs of the stage controller. Once an islet of interest is located, switch to the 20x objective, and zoom in so the islet takes up most of the frame. Take a z-stack of the islet with a z-step size of 1.5 µm. Find the best optical sections (a series of between 5 to 10 sections) of the islet where most of the cells are alive (negative for the nucleic acid stain) and any surrounding immune cells are in focus.

NOTE: Try to find frames where there are multiple CD8-positive and insulin tetramer-positive cells surrounding or infiltrating the islet.

10.5. Set the microscope to record in XYZT mode. Optimize the settings to record a Z-stack of the selected steps every 20 min over a period of several hours.

NOTE: If possible, it is best to do these recordings in an imaging chamber where temperature and CO_2 levels can be controlled, particularly when recording for over four hours. In the case of overnight recording, excess antibody can be added to the media to compensate for T cell receptor cycling and dye fading. Additionally, different fluorophores can be used for the T cell antibodies. Based on experience, antibodies in the far-red range work best for T cells.

REPRESENTATIVE RESULTS:

This protocol will yield live pancreatic tissue slices suitable for both functionality studies and immune cell recordings. Slice appearance in both brightfield and under reflected light are shown in **Figure 1A,B**. As discussed, islets can be found in slices using reflected light due to their increased granularity that occurs because of their insulin content (**Figure 1C**) and are clearly observed compared to the background tissue when reflected light is used. Viability should be assessed following slice generation, and islets should not be recorded if more than 20% of the islet is not viable. An islet with high viability is shown in **Figure 1D,** whereas an example of a poorly processed slice is shown in **Supplemental Figure 1**. Islets with low viability will have heavy nucleic acid staining, and the tissue will be covered with the stained nuclei of dead cells. Additionally, calcein-AM and Ca^{2+} indicators such as the cell permeable indicator used here and Fluo-4-AM will not load well in dead cells. Islets should be selected for Ca^{2+} recordings if they are viable and if the indicator is loaded throughout the islet. Ca^{2+} indicator loading is indicative of cell viability as both Ca^{2+} indicators discussed in this protocol (the cell permeable Ca^{2+} indicator and Fluo-4-AM) are loaded in cells through the same mechanism as the viability dye, calcein-AM.

The stain is loaded into cells, the acetoxymethyl ester is hydrolyzed within the cell, and the molecule becomes membrane impermeable[19]. Another positive indicator for viability is observable basal activity throughout the islet with cells flashing on and off. Basal activity should

also be observable in the exocrine tissue to a lesser degree. Although mouse tissue tends to have less visible basal activity than human tissue, it is still present. An islet from a slice made from a NOD.*Rag1*$^{-/-}$ mouse pancreas is shown in **Figure 2A**. As mentioned above, the cell-permeable Ca^{2+} indicator used here has a lower fluorescence intensity increase upon binding Ca^{2+} (~14-fold) than Fluo-4 (~100-fold). However, the cell-permeable Ca^{2+} indicator has the advantage of a lower calcium dissociation constant (K_d = 170 nM) than Fluo-4 (K_d = 335 nM), resulting in the cell-permeable Ca^{2+} indicator being more sensitive to lower concentrations of cytosolic Ca^{2+}. However, responses are still quantifiable, as shown by **Figure 2B**. Examples of an islet within a control human pancreatic tissue slice at rest and of one exhibiting a strong high glucose response are shown in **Figure 2C** and **Supplemental Video 1**. Fluo-4-AM dye is loaded well and is visible throughout the islet at low glucose concentrations. As discussed above, a typical occurrence is for a percentage of cells to load large amounts of dye and appear very bright. Moreover, the image parameters have been set for this recording so that most of the cells within the islet do not appear too bright at low glucose concentrations. This enables the detector to pick up on the increases in brightness that occur during changes in intracellular Ca^{2+} concentrations in response to high glucose levels. The quantification of the fluorescence of individual cells during this response is shown in **Figure 2D**, with the expected peak following the high glucose stimulation. ImageJ software was used to calculate the staining intensity of Fluo-4-AM and the cell-permeable Ca^{2+} indicator by manually selecting ROIs. The fold-increase in fluorescence intensity for each ROI was calculated by normalizing the fluorescence values at later timepoints using the initial fluorescence values of the cells (F/F_0).

Dithizone stains the islets red and is visible under a brightfield stereomicroscope. Intact islets and islets that are beginning to fall apart because of T1D onset can both be observed using this dye (**Figure 3A,B**). Islets can be found using reflected light (**Figure 3C**) and may begin to lose granularity due to immune cell infiltration and cell death (**Figure 3D**). Multiple CD3-positive cells can be seen infiltrating the islet in **Figure 3D**. Immune cell populations can be identified more specifically using CD8 antibody and insulin-tetramer staining. Imaging can then be applied to identify cells that co-stain for both markers (**Figure 3E**). The co-staining of the immune cells infiltrating the islet in **Figure 3D** indicates that the cells are effector T cells that are specifically targeting the insulin antigen. The CD8 co-stain is essential to distinguish that the areas that stain positive for tetramer are immune cells. The tetramer should not be used alone without an immune cell co-stain. A staining comparison of the mouse CD8 antibody and the isotype control Rat IgG2a, κ can be found in **Supplemental Figure 2**. An additional comparison of a control tetramer for lymphocytic choriomeningitis virus (LCMV) tetramer and the insulin tetramer can be found in **Supplemental Figure 3**. Some T cells remain stationary throughout the recording, many move slightly within a small area of the islet, and others are very mobile and can be seen moving throughout the islet and exocrine tissue. It is not unusual to see T cells exhibiting multiple mobility types within the same recording.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of slices and individual islets. (**A**) Darkfield stereomicroscopy image of a live human pancreatic tissue slice with islets indicated by red arrows. (**B**) Reflected light image of a live human pancreatic tissue slice with islets indicated by white arrows. (**C**) Reflected light image

of an islet (outlined in magenta) within a live human pancreatic tissue slice. (**D**) Viability staining of a high-viability islet (outlined in magenta) within a live human pancreatic tissue slice. Live cells are indicated in green and dead cells in blue. Scale bars (**A, B**) = 1 mm; scale bars (**C, D**) = 50 µm. Abbreviation: AM = acetoxymethyl ester.

Figure 2: Recordings of changes in intracellular Ca^{2+} concentrations and responses to high glucose concentration of a live NOD.*Rag1*$^{-/-}$ mouse pancreatic tissue slice and human pancreatic tissue slice from a donor without diabetes. (**A**) Images of an islet within a live NOD.*Rag1*$^{-/-}$ mouse pancreatic slice loaded with a cell-permeable Ca^{2+} indicator (see the **Table of Materials**) undergoing glucose stimulation. From left to right, a reflected light image of the islet, the islet in low glucose, and the islet in high glucose. (**B**) Fluorescence traces of the Ca^{2+} response of an islet within a live NOD.*Rag1*$^{-/-}$ tissue slice with the expected response to high glucose concentration [KRBH with 16.7 mM D-glucose (16.7G)] and KCl [KRBH with 30 mM KCl and 3 mM D-glucose]. (**C**) Images of an islet within a live human pancreatic slice loaded with Fluo-4-AM undergoing glucose stimulation. From left to right, a reflected light image of the islet, the islet in low glucose, and the islet in high glucose. (**D**) Fluorescence traces of the Ca^{2+} response of an islet within a live human pancreas tissue slice with the expected response to KRBH with 16.7 mM D-glucose (16.7G). Scale bars (**A**) = 100 µm; scale bars (**C**) = 50 µm. Abbreviations: KRBH = Krebs-Ringer bicarbonate buffer; KCl = potassium chloride; NOD.*Rag1*$^{-/-}$ = non-obese diabetic-recombination activating gene-1-null; NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ = T cell receptor transgenic (AI4) mouse strain.

Figure 3: Identification of islets and immune cell populations in NOD.*Rag1*$^{-/-}$ and NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse slices. (**A**) Dithizone staining of islets in a NOD.*Rag1*$^{-/-}$ mouse slice with the islets indicated in red. (**B**) Dithizone staining of islets in a NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse slice with the islets indicated in red. Islets are losing their shape due to disease onset. (**C**) Reflected light image of an islet in a NOD.*Rag1*$^{-/-}$ mouse slice. (**D**) Reflected light image of an islet in a NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse slice with CD3 antibody staining (green). (**E**) Viability staining of dead cells (blue) and immune cell staining (CD8 in green and insulin tetramer in red) in a NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse slice. Scale bars (**A**) = 500 µm; scale bars (**B**) = 50 µm; scale bars (**C**) = 100 µm. Abbreviations: NOD.*Rag1*$^{-/-}$ = non-obese diabetic-recombination activating gene-1-null; NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ = T cell receptor transgenic (AI4) mouse strain; CD = cluster of differentiation; insulin-tet = insulin tetramer.

Supplemental Figure 1: NOD.*Rag1*$^{-/-}$ mouse pancreatic slice following improper preparation without trypsin inhibitor and an overnight incubation at 37 °C. (**A**) Darkfield stereomicroscopy image of a live NOD.*Rag1*$^{-/-}$ mouse pancreatic tissue slice; scale bar = 1 mm. (**B**) Reflected light image of a live mouse pancreatic tissue slice; scale bar = 50 µm. (**C**) Viability staining of low-viability tissue. Dead cells are indicated in blue; scale bar = 50 µm. Abbreviation: NOD.*Rag1*$^{-/-}$ = non-obese diabetic-recombination activating gene-1-null.

Supplemental Figure 2: Rat IgG2a, κ isotype control antibody (left) and rat anti-mouse CD8 antibody (right) staining comparison in NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse slices. (**A**) Reflected light images of live NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse pancreatic tissue slices showing an islet (left) and blood

vessel (right). (**B**) Antibody staining of live NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse pancreatic tissue slices. (**C**) Overlay of the reflected light and antibody channels. Scale bars for control antibody (left panels) = 20 µm; scale bars for CD8 antibody (right panels) = 50 µm. Abbreviations: NOD.*Rag1*$^{-/-}$ = non-obese diabetic-recombination activating gene-1-null; NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ = T cell receptor transgenic (AI4) mouse strain; CD = cluster of differentiation; IgG = immunoglobulin G.

Supplemental Figure 3: Lymphocytic choriomeningitis virus tetramer (left) and insulin tetramer (right) staining comparison in NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse slices. (**A**) Reflected light images of live NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse pancreas tissue slices showing a blood vessel in exocrine tissue (left) and islets (right). (**B**) Tetramer staining of a live NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse tissue slices. (**C**) Overlay of the reflected light and tetramer channels. Abbreviations: NOD.*Rag1*$^{-/-}$ = non-obese diabetic-recombination activating gene-1-null; NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ = T cell receptor transgenic (AI4) mouse strain; LCMV = lymphocytic choriomeningitis virus; insulin-tet = insulin tetramer.

Supplemental Video 1: Recording of cytosolic Ca^{2+} detected with Fluo-4 in response to high glucose stimulation in a human pancreatic tissue slice from a control donor without diabetes. Cells within the tissue can be observed to exhibit basal Fluo-4 activity in a low glucose solution (3.0 mM), followed by an increase in Fluo-4 fluorescence intensity in response to a stimulation with high glucose (16.7 mM). The video corresponds to the still images and traces shown in **Figure 2C,D**.

DISCUSSION:

The objective of this protocol is to explicate the generation of pancreas slices and the procedures needed to employ the slices in functional and immunological studies. There are many benefits to using live pancreatic slices. However, there are several critical steps that are essential for the tissue to remain viable and useful during the described experiment protocols. It is imperative to work quickly. The length of time between injecting the pancreas and generating the slices on the vibratome should be minimized to maintain tissue viability. Viability is also improved by keeping the pancreas in cold ECS before slicing as opposed to room temperature ECS. Importantly, slices should never be in medium without protease inhibitor. When slices are incubated without the protease inhibitor, there are large decreases in viability.

When the slices were briefly left without inhibitor during dye loading, Ca^{2+} fluxes in response to high glucose and KCl could no longer be recorded despite basal activity still being visible in the slice. All solutions used for incubation of the slices—KRBH with 3 mM D-glucose solution the slices rest in, the solutions the slices are incubated in for staining, and any media the slices are cultured in—must all contain protease inhibitor at a concentration of 0.1 mg per mL. The indicator panels used for slice imaging can be modified depending on the objective of the experiment and the availability of microscope lasers. There are numerous cell viability dyes in different colors that can be used instead of the nucleic acid stain used here (see the **Table of Materials**). For Ca^{2+} experiments, Fluo-4-AM works well in human tissue. Some researchers have success using the cell-permeable Ca^{2+} indicator used here (see the **Table of Materials**) for mouse slices, whereas others obtain good results with Fluo-4-AM[20-22].

Additionally, mice engineered to express the genetically encoded Ca^{2+} indicator, GCaMP, in their islets could be used to circumvent the need to load the slices with a Ca^{2+} indicator dye. Although the cell-permeable Ca^{2+} indicator used here is not as bright as Fluo-4-AM, the Ca^{2+} responses are still observable and quantifiable. This is evidenced by the decrease in fluorescent peaks shown in the NOD.*Rag1*$^{-/-}$ slice recordings following high glucose and KCl stimulation. Other substances, such as sulfonylureas and arginine, can be used as positive controls at the end of the Ca^{2+} protocol, but they have not yet been used with slices[23-25]. While there are many benefits to the live pancreatic tissue slice method, there are also some limitations. Although the slices can remain viable for several days, there are steep declines in viability and functionality if they are cultured for longer, unless special culture conditions are employed[11,26]. Additionally, as the slices contain live pancreatic exocrine tissue, acinar cells in the slices will continue to produce and release digestive enzymes that need to be inhibited using protease inhibitor. Therefore, when using this protocol for human or mouse studies, always maintain slices in solutions with protease inhibitor.

The live pancreatic tissue slice method avoids placing the pancreatic tissue under chemical stress by only exposing the tissue to mechanical force during slice generation as opposed to chemicals used during islet isolation procedures[5]. Furthermore, intact pancreatic tissue is maintained, allowing for a more wholistic view of the pathologies and physiology that occur naturally within the organ[5]. Using the live pancreatic tissue slice method, immune cell activity can be observed *in situ* and real-time alongside tissue function. Additional *in vitro* imaging techniques, such as two-photon microscopy, have already been applied to tissue slices derived from thymus and could be applied to live pancreatic tissue slices[27]. Identification of immune cell populations that are present in the tissue along with their activities and impacts will allow for new knowledge to be gained on the pathogenesis of diseases such as T1D and T2D.

ACKNOWLEDGMENTS:

This work was funded by NIH grants R01 DK123292, T32 DK108736, UC4 DK104194, UG3 DK122638, and P01 AI042288. This research was performed with the support of the Network for Pancreatic Organ donors with Diabetes (nPOD; RRID:SCR_014641), a collaborative type 1 diabetes research project sponsored by JDRF (nPOD: 5-SRA-2018-557-Q-R), and The Leona M. & Harry B. Helmsley Charitable Trust (Grant #2018PG-T1D053). The content and views expressed are the responsibility of the authors and do not necessarily reflect the official view of nPOD. Organ Procurement Organizations (OPO) partnering with nPOD to provide research resources are listed at http://www.jdrfnpod.org/for-partners/npod-partners/. Thank you to Dr. Kevin Otto, University of Florida, for providing the vibratome used to generate mouse slices.

DISCLOSURES:

The authors declare no competing interests.

REFERENCES:

1 Uc, A., Fishman, D. S. Pancreatic disorders. *Pediatric Clinics of North America.* **64** (3), 685–706 (2017).

2 Bluestone, J. A., Herold, K., Eisenbarth, G. Genetics, pathogenesis and clinical

705 interventions in type 1 diabetes. *Nature.* **464** (7293), 1293–1300 (2010).
706 3 Taylor, R. Type 2 diabetes: etiology and reversibility. *Diabetes Care.* **36** (4), 1047–1055
707 (2013).
708 4 Meier, R. P. et al.. Islet of Langerhans isolation from pediatric and juvenile donor
709 pancreases. *Transplant International.* **27** (9), 949–955 (2014).
710 5 Marciniak, A. et al.. Using pancreas tissue slices for in situ studies of islet of Langerhans
711 and acinar cell biology. *Nature Protocols.* **9** (12), 2809–2822 (2014).
712 6 Panzer, J. K., Cohrs, C. M., Speier, S. Using pancreas tissue slices for the study of islet
713 physiology. *Methods in Molecular Biology.* **2128**, 301–312 (2020).
714 7 Speier, S., Rupnik, M. A novel approach to in situ characterization of pancreatic beta-cells.
715 *Pflugers Archive.* **446** (5), 553–558 (2003).
716 8 Panzer, J. K. et al. Pancreas tissue slices from organ donors enable in situ analysis of type
717 1 diabetes pathogenesis. *JCI Insight.* **5** (8), e134525 (2020).
718 9 Dolai, S. et al. Pancreatitis-induced depletion of syntaxin 2 promotes autophagy and
719 increases basolateral exocytosis. *Gastroenterology.* **154** (6), 1805–1821 e1805 (2018).
720 10 Dolai, S. et al. Pancreas-specific SNAP23 depletion prevents pancreatitis by attenuating
721 pathological basolateral exocytosis and formation of trypsin-activating autolysosomes.
722 *Autophagy.* 1–14, doi: 10.1080/15548627.2020.1852725 1-14 (2020).
723 11 Qadir, M. M. F. et al. Long-term culture of human pancreatic slices as a model to study
724 real-time islet regeneration. *Nature Communications.* **11** (1), 3265 (2020).
725 12 Cohrs, C. M. et al. Dysfunction of persisting β cells is a key feature of early type 2 diabetes
726 pathogenesis. *Cell Reports.* **31** (1), 107469 (2020).
727 13 Liang, T. et al. Ex vivo human pancreatic slice preparations offer a valuable model for
728 studying pancreatic exocrine biology. *Journal of Biological Chemistry.* **292** (14), 5957–5969
729 (2017).
730 14 Shultz, L. D., Ishikawa, F., Greiner, D. L. Humanized mice in translational biomedical
731 research. *Nat Reviews. Immunology.* **7** (2), 118–130 (2007).
732 15 Lamont, D. et al. Compensatory mechanisms allow undersized anchor-deficient class I
733 MHC ligands to mediate pathogenic autoreactive T cell responses. *Journal of Immunology.* **193**
734 (5), 2135–2146 (2014).
735 16 Fish, R., Danneman, P. J., Brown, M., Karas, A. (Eds) *Anesthesia and analgesia in*
736 *laboratory animals*. Academic Press (2011).
737 17 Clark, S. A., Borland, K. M., Sherman, S. D., Rusack, T. C., Chick, W. L. Staining and in vitro
738 toxicity of dithizone with canine, porcine, and bovine islets. *Cell Transplantation.* **3** (4), 299–306
739 (1994).
740 18 Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nature*
741 *Methods.* **9** (7), 676–682 (2012).
742 19 Monette, R., Small, D. L., Mealing, G., Morley, P. A fluorescence confocal assay to assess
743 neuronal viability in brain slices. *Brain Research Protocols.* **2** (2), 99–108 (1998).
744 20 Gál, E. et al. A novel in situ approach to studying pancreatic ducts in mice. *Frontiers in*
745 *Physiology.* **10**, 938 (2019).
746 21 Stožer, A., Dolenšek, J., Rupnik, M. S. Glucose-stimulated calcium dynamics in islets of
747 Langerhans in acute mouse pancreas tissue slices. *PloS One.* **8** (1), e54638 (2013).
748 22 Stožer, A. et al. Functional connectivity in islets of Langerhans from mouse pancreas tissue

slices. *PLoS Computational Biology.* **9** (2), e1002923 (2013).
23 Früh, E., Elgert, C., Eggert, F., Scherneck, S., Rustenbeck, I. Glucagonotropic and glucagonostatic effects of KATP channel closure and potassium depolarization. *Endocrinology.* **162** (1), bqaa136 (2021).
24 Satin, L. S. New mechanisms for sulfonylurea control of insulin secretion. *Endocrine.* **4** (3), 191–198 (1996).
25 Ren, J. et al.Slow oscillations of KATP conductance in mouse pancreatic islets provide support for electrical bursting driven by metabolic oscillations. *American Journal of Physiology-Endocrinology and Metabolism.* **305** (7), E805–E817 (2013).
26 Marciniak, A., Selck, C., Friedrich, B., Speier, S. Mouse pancreas tissue slice culture facilitates long-term studies of exocrine and endocrine cell physiology in situ. *PLoS One.* **8** (11), e78706 (2013).
27 Dzhagalov, I. L., Melichar, H. J., Ross, J. O., Herzmark, P., Robey, E. A. Two-photon imaging of the immune system. *Current Protocols in Cytometry.* **Chapter 12**, Unit12.26 (2012).

Figure 1

Click here to access/download;Figure;Manuscript Final_Jove Figure 1.pdf

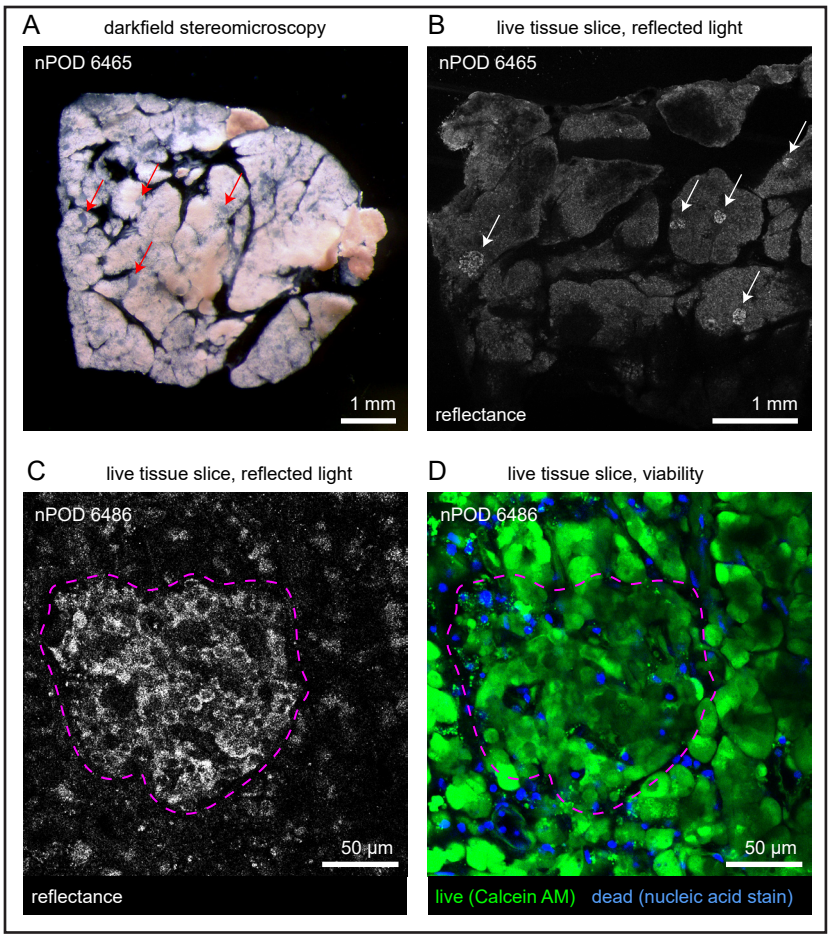


Figure 2

Click here to access/download;Figure;Manuscript Final_Jove Figure 2.pdf

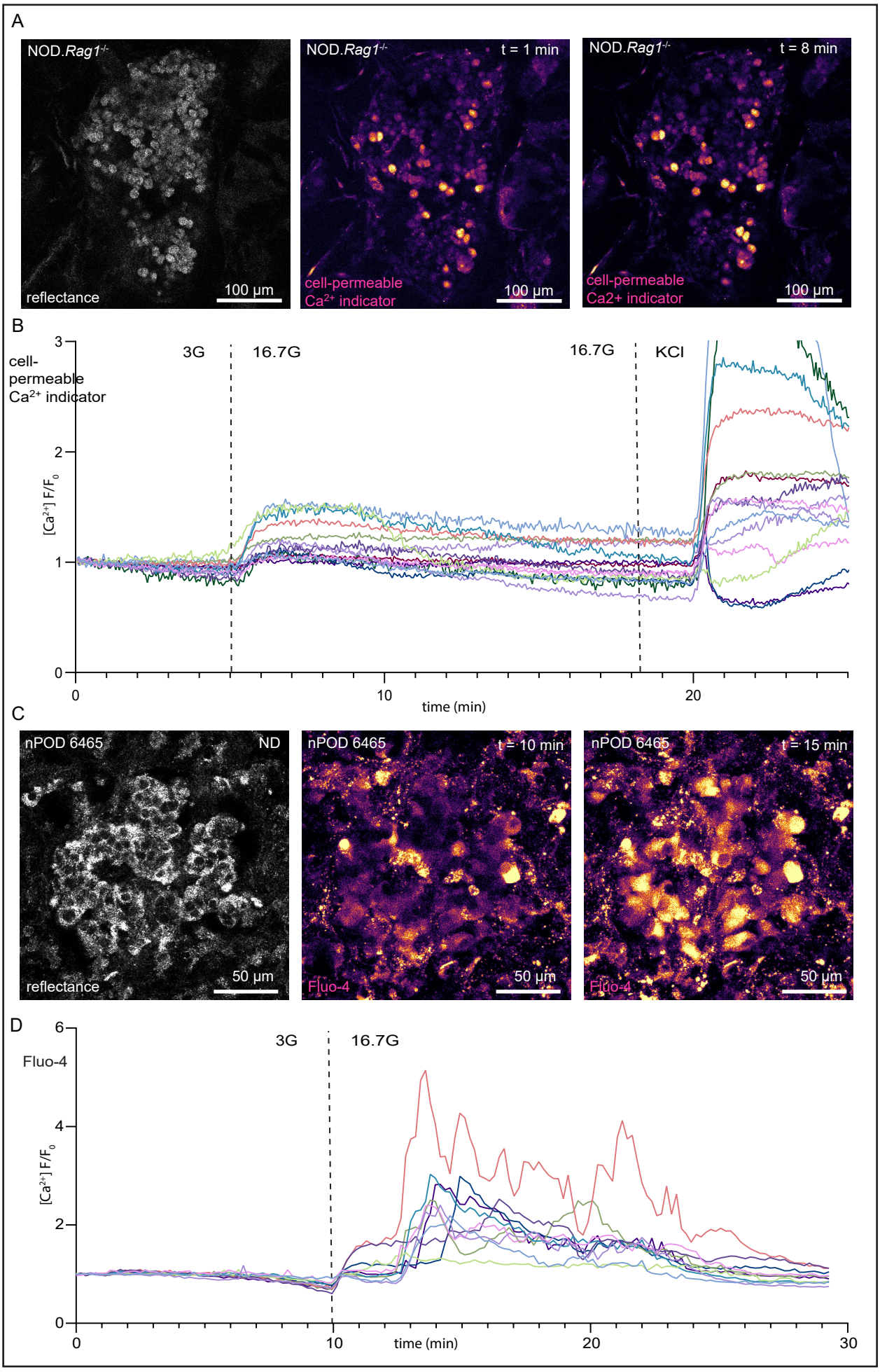
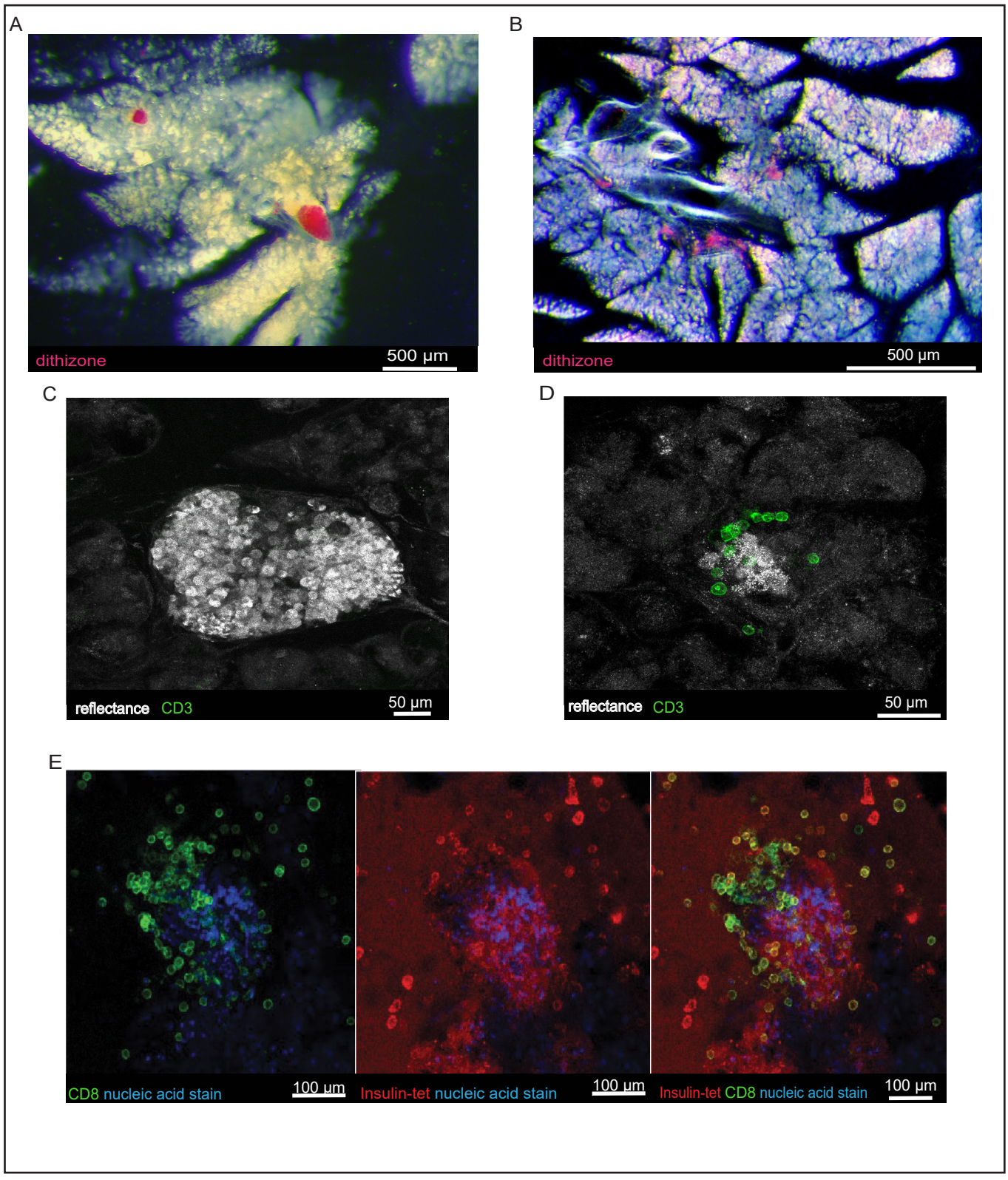


Figure 3

Click here to access/download;Figure;Manuscript Final_Jove Figure 3.pdf



Name of Material/ Equipment	Company	Catalog Number
#3 Style Scalpel Handle	Fisherbrand	12-000-163
1 M HEPES	Fisher Scientific	BP299-100
10 cm Untreated Culture Dish	Corning	430591
10 mL Luer-Lok Syringe	BD	301029
27 G Needle	BD	BD 305109
35 mm coverglass-bottom Petri dish	Ibidi	81156
50 mL syringe	BD	309653
8-well chambered coverglass	Ibidi	80826
APC anti-mouse CD8a antibody	Biolegend	100712
BSA	Fisher Scientific	199898
Calcium chloride	Sigma	C5670
Calcium chloride dihydrate	Sigma	C7902
Compact Digital Rocker	Thermo Fisher Scientific	88880020
Confocal laser-scanning microscope	Leica	SP8
D-(+)-Glucose	Sigma	G7021
ddiH2O		
Dithizone	Sigma-Aldrich	D5130-10G
DMSO	Invitrogen	D12345
Ethanol	Decon Laboratories	2805
Falcon 35 mm tissue culture dish	Corning	353001
FBS	Gibco	10082147
Feather No. 10 Surgical Blade	Electron Microscopy Sciences	7204410
fluo-4-AM	Invitrogen	F14201
Gel Control Super Glue	Loctite	45198
Graefe Forceps	Fine Science Tools	11049-10
Hardened Fine Scissors	Fine Science Tools	14090-09
HBSS	Gibco	14025092

HEPES	Sigma	H4034	
Ice bucket	Fisherbrand	03-395-150	
Isoflurane	Patterson Veterinary	NDC 14043-704-05	
Johns Hopkins Bulldog Clamp	Roboz Surgical Store	RS-7440	
Kimwipes	Kimberly-Clark Professional		34705
LIVE/DEAD Viability/Cytotoxicity Kit	Invitrogen	L3224	
Low glucose DMEM	Corning	10-014-CV	
Magnesium chloride hexahydrate	Sigma	M9272	
Magnesium sulfate heptahydrate	Sigma	M2773	
Magnetic Heated Platform	Warner Instruments	PM-1	
Microwave	GE	JES1460DSWW	
Nalgene Syringe Filter	Thermo Fisher Scientific	726-2520	
No.4 Paintbrush	Michaels		10269140
Open Diamond Bath Imaging Chamber	Warner Instruments	RC-26	
Oregon Green 488 BAPTA-1-AM	Invitrogen	O6807	
Overnight imaging chamber	Okolab	H201-LG	
PBS	Thermo Fisher Scientific		20012050
PE-labeled insulin tetramer	Emory Tetramer Research Core	sequence YAIENYLEL	
Penicillin Streptomycin	Gibco		15140122
Potassium chloride	Sigma	P5405	
Potassium phosphate monobasic	Sigma	P5655	
Razor Blades	Electron Microscopy Sciences		71998
RPMI 1640	Gibco		11875093
SeaPlaque low melting-point agarose	Lonza		50101
Slice anchor	Warner Instruments	64-1421	
Slice anchor (dynamic imaging)	Warner Instruments		640253

Sodium bicarbonate	Sigma	S5761	
Sodium chloride	Sigma	S5886	
Sodium phosphate monohydrate	Sigma	S9638	
Soybean Trypsin Inhibitor	Sigma	T6522-1G	
Stage Adapter	Warner Instruments	SA-20MW-AL	
Stage-top incubator	Okolab	H201	
Stereoscope	Leica	IC90 E MSV266	
SYTOX Blue Dead Cell Stain	Invitrogen	S34857	
Transfer Pipet	Falcon		357575
Valve Control System	Warner Instruments	VCS-8	
Vibratome VT1000 S	Leica	VT1000 S	
Water bath	Fisher Scientific	FSGPD02	

Comments/Description

HEPES Buffer, 1M Solution

BD Syringe with Luer-Lok Tips
BD General Use and PrecisionGlide Hypodermic Needles

µ-Dish 35 mm, high

µ-Slide 8 Well

$CaCl_2$
$CaCl_2$ (dihydrate)

Pinhole = 1.5-2 airy units; acquired with 10x/0.40 numerical aperture HC PL APO CS2 dry and 20x/0.75 numerical aperture HC PL APO CS2 dry objectives at 512 × 512 pixel resolution
$C_6H_{12}O_6$

Dimethyl sulfoxide

Falcon Easy-Grip Tissue Culture Dishes

cell-permeable Ca^{2+} indicator

Hanks Balanced Salt Solution

$C_8H_{18}N_2O_4S$

Straight; 500-900 Grams Pressure; 1.5" Length

Kimtech Science™ Kimwipes™ Delicate Task Wipers, 2-Ply

This kit contains the calcein-AM live cell dye.

$MgCl_2$ (hexahydrate)
$MgSO_4$ (heptahydrate)
Platform for imaging chamber for dynamic stimulation recordings

Imaging chamber for dynamic stimulation recordings
cell-permeable Ca^{2+} indicator

To make agarose for slice generation

KCl
KH_2PO_4

For Vibratome; Double Edge Stainless Steel, uncoated

To make agarose for slice generation

Slice anchor for dynamic imaging chamber

$NaHCO_3$

NaCl

NaH_2PO_4 (monohydrate)

Trypsin inhibitor from Glycine max (soybean)

To fit imaging chamber for dynamic stimulation recordings on the microscope stage

blue-fluorescent nucleic acid stain

Falcon™ Plastic Disposable Transfer Pipets

System for dynamic stimulation recordings

Fisherbrand Isotemp General Purpose Deluxe Water Bath GPD 02

Point-by-point Response to Editor and Reviewers' Critiques

We thank the Editor and the Reviewers for their time in evaluating our manuscript and for their insightful comments. The suggested changes substantially improved the manuscript. Please find below our point-by-point responses to the specific reviewer comments.

RESPONSES TO EDITOR:

SPECIFIC COMMENTS:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We read through the manuscript to check for and correct spelling and grammar issues.

2. Please provide an institutional email address for each author.

We added institutional email addresses for each author to the manuscript.

3. Please ensure that the corresponding reference numbers appear as numbered superscripts after the appropriate statement(s) before any punctuations.

We read through the manuscript to ensure the reference numbers appeared as superscripts after statements and before punctuations.

4. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

We added the ethics statement, "Human pancreatic sections from tissue donors of both sexes were obtained via the Network for Pancreatic Organ Donors with Diabetes (nPOD) tissue bank, University of Florida. Human pancreata were harvested from cadaveric organ donors by certified organ procurement organizations partnering with nPOD in accordance with organ donation laws and regulations and classified as 'Non-Human Subjects' by the University of Florida Institutional Review Board (IRB) (IRB no. 392-2008), waiving the need for consent. nPOD tissues specifically used for this project were approved as nonhuman by the University of Florida IRB (IRB20140093)."

5. Line 48: Please define the term "MHC" before using the abbreviated form.

We defined the term MHC as major histocompatibility complex.

6. Line 84: Please mention the volume and time required for melting in the microwave.

We added in the volume (100 mL) and that the microwave should be run in 1 minute intervals with 10 second stops if the agarose solution begins to boil.

7. Line 101: Please mention the required concentration of isoflurane.

We added in that the isoflurane concentration should be 5%.

8. Line 179: Please mention the units of amplitude, if any.

The units of amplitude are mm and were added to the manuscript.

9. Line 183: Please define the size of the forceps/ paint brush.

We added in that the forceps are 10cm Graefe forceps and the paintbrush size is No. 4.

10. Line 192: Please mention the volume of slice culture medium required to place the slices.

We added that the slices should be cultured in 15 mL of slice culture medium.

11. Line 284: Define the term "PMT" before using the abbreviated form.

We defined the term as photomultiplier tube.

12. Line 294: Please use lowercase x throughout the protocol to represent magnification ("10X" becomes "10x")

We went through the protocol and changed magnification to the lowercase x.

13. Line 352: Please specify the volume of KRBH used for washing.

We added that 2 mL of KRBH should be used for washes.

14. Please include a one line space between all protocol steps and substeps.

We added line spaces between all protocol steps and substeps.

15. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We selected the slice generation and recording of mouse immune cells to highlight in the protocol.

16. Do not use the &-sign or the word “and” when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. For more than 6 authors, list only the first author then et al. Example: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]limidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

We updated the reference formatting.

17. Figure 2B,2D: Please define the usage of terms 3G, 16.7G, KCl in the respective figure legends.

We edited the figure 2 legend and changed 3G, 16.7G, and KCl as KRBH with 3 mM D-glucose, KRBH with 16.7 mM D-glucose, and KRBH with 30 mM KCl and 3 mM D-glucose, respectively.

RESPONSES TO REVIEWER #1:

GENERAL COMMENTS:

This is a nicely written, highly detailed manuscript explaining the methodology for producing and imaging pancreas slices to observe interactions between immune cells and islets. The authors have done an excellent job of providing very clear instructions for the complex procedure and have explained their reasoning for why certain details must be attended to.

We thank Reviewer 1 for their positive comments.

RESPONSES TO REVIEWER #2:

GENERAL COMMENTS:

This a very good protocol presentation by a group who have pioneered major parts of the described protocol. The structure and language are both very clear, and the information presented should suffice for someone to be able to perform the experiments.

We thank Reviewer 2 for their positive comments and their time and effort in helping to improve the manuscript.

SPECIFIC COMMENTS:

Introduction and Protocol:

1. Some additional studies besides the mentioned one by Panzer et al have successfully used human slices, I suggest they are mentioned for great reference, e.g.: Dolai Gastroenterology 2018 & Autophagy 2020, Liang JBC 2017, Qadir Nat Commun 2020, and Cohrs Cell Rep 2020.
A suitable place would for instance be in the sentence in lines 55-57: Pancreas tissue slices from

human donor tissue have been successfully used to study type 1 diabetes and have demonstrated processes of beta cell loss and dysfunction in addition to immune cell infiltration.

These studies all discussed various applications of the live pancreas tissue slice model and citations were added to the manuscript following the suggested sentence.

2. Using a 27G needle might turn out to be practically challenging, especially with smaller strains and younger animals. Do you think smaller diameter needles, such as 30G could be used as well?

We have found that a 27 G needle is preferred since it fits securely into the common bile duct of mice between 10-25 g in body weight and allows for flow of the high viscosity agarose solution. A smaller gauge needle is not recommended as it may quickly clog with the agarose solution. We added this reasoning as a note in the manuscript.

3. Similarly, more concentrated agarose solutions have been used and are used by some groups. Could the authors briefly comment on or justify the practical value of using more dilute agarose?

The lower agarose concentration is to account for the lower density of the mouse pancreas. We added this as a note in the manuscript.

4. Throughout cutting the tissue into smaller pieces, bicarbonate-based ECS is used. Does it need to be "bubbled" constantly or can some of it be taken from a bigger flask and used for a couple of minutes without being "bubbled"? What about the solution in the vibratome bath chamber used for cutting?

We do not bubble the ECS with carbogen for our protocol and we added this clarification to the manuscript.

5. Does dithizone really universally "kill" the slices, does it affect the exocrine part? Does it really reduce viability of islets independently of concentration? This is a practically important issue, since DTZ staining may really help with islet recognition, especially during learning of human slice preparation. See for instance Latif Transplantation 1988, Conget Pancreas 1994, and Hansen Diab Res 1989.

We added a reference discussing the negative impact of dithizone on islet viability to the manuscript. In our experience, DTZ staining substantially reduces the overall viability, granularity, and glucose responsiveness of the slice. However, DTZ can be used (and we have used it) for training purposes to identify islets.

6. In point 8.4, for a better learning curve, switching to epifluorescence can sometimes really help identify islets before switching to confocal.

We experimented with using epifluorescence with the ocular port to manually find islets and found it suitable for locating islets in some slices by autofluorescence. We do not routinely employ this approach due to the high background, and usually prefer to scan for backscatter

using the confocal which we find to be higher contrast and routinely reliable. We do not have an epifluorescence camera on our scope so we cannot document the epifluorescence approach and thus have elected not to expand the manuscript to include it.

Representative Results and Discussion:

1. In 3mM glucose, would you typically expect a lot of basal activity in islets? In which parts of the islet? Isn´t responsiveness to KCl and high glucose a better indicator of viability than high basal activity, especially if beta cells are the main point of interest? Regarding their differential sensitivity to glucose, Is the situation the same for human and mouse slices?

In 3 mM glucose we typically see a moderate amount of basal activity in the islet with less being observed in the exocrine tissue. The activity is present throughout the islet and we have not found it to be localized to particular regions of the islet. While high glucose and KCl would be better indicators of viability, the goal at this stage is to distinguish viable from non-viable islets before beta cell stimulations occur to increase the odds of getting a good recording. The Ca^{2+} indicator also works to indicate cell viability because both Ca^{2+} indicators discussed in this protocol (Oregon Green BAPTA-1, AM and Fluo-4, AM) are loaded in cells through a similar mechanism as the viability dye Calcein, AM. The dye is permeable to cell membranes and is taken up. In live cells, the acetoxymethyl ester is hydrolyzed within the cell, and the molecule becomes membrane impermeable and trapped within the cell. Dead cells with permeable membranes do not retain the hydrolyzed dye. Lack of labeling with Sytox Blue is a second indicator for viability. We added a clarification about how the Ca^{2+} indicator dye works as a viability indicator to the manuscript.

2. It would be quite nice to cite some of the pioneering work by the group who performed first successful recording of calcium in mouse slices, e.g., Stozer PLoS One 2013 or Stozer PLoS Comput Biol 2013. More importantly, this can also be used as a reference for more details regarding imaging.
A suitable place would for instance be in the sentence in lines 502-503: Some labs have success using Oregon Green BAPTA-1, AM in mouse slices, while others obtain good results with Fluo-4, AM

These papers fit well with the techniques discussed in the manuscript and the references have been added at the suggested sentence.

Figures:

1. Is the slight decrease in Ca right upon stimulation a true transient phenomenon of Ca sequestration or an artifact due to drift? Please comment briefly in the text.

There is occasionally a small amount of tissue motion at the moment of glucose addition. The decrease indicated by the reviewer was due to such a motion artifact. We carefully re-analyzed the video and were able to stabilize the footage and remote the artifact.

2. Can substances other than KCl be used as positive controls at the end of a protocol? For instance, SUs for beta cells?

Sulfonylureas and arginine can both be used as alternative positive controls. We have not tried them in the slices yet but plan to. We added that sulfonylureas and arginine can be used to the discussion section of the manuscript.

RESPONSES TO REVIEWER #3:

GENERAL COMMENTS:

Huber and coworkers presented detailed protocols for preparing live pancreatic tissue slices of mouse and human. The manuscript also includes cell dye loading and imaging procedures for monitoring Ca2+ activity of islet cells in situ, and for imaging islet-immune cell interaction. This is a timely publication as the pancreas slice has been increasingly utilized by the islet biology community in recent years. This report and its companion video will serve as a very useful reference for those new to the technique and who like to adopt the method. The manuscript is well written by a group of people with extensive experience in this area.

We thank Reviewer 3 for their overall positive comments and excellent suggestions to improve the manuscript.

SPECIFIC COMMENTS:

1. Page 4, Section 2 describes Mouse pancreas Excision and Tissue Processing; Page 5, Section 3 describes Slice Generation (presumably for mouse pancreas). It is not clear if the procedure is the same for mouse and human, and whether there is a difference in processing human samples vs mouse samples.

We clarified that the protocol is for mouse slice generation and added a note that the mouse pancreas needs to be injected for stabilization.

2. P9, line 309: In isolated islets, small Ca2+ sensors can only be loaded to outer cell layers. In pancreas slice, what is the typical depth of dye penetration or labeling? Such information should help users to assess the level or the success of labeling. In addition, engineered mice expressing GCaMPs in islet cells are now routinely used. Do pancreas slices from these Tg mice provide a complementary system for Ca2+ imaging? The authors may consider discussing this possibility.

Dye loading in the slices can vary between donors, but a typical slice preparation visualizes Fluo-4 well up to a depth of 15 microns into the slice. If the optical plane of interest is located deeper in the tissue it can be more difficult to visualize due to light scattering. We clarified these points with a note in the manuscript. Additionally, we have not worked with GCaMP mice but theoretically the system should be complementary. We added this possibility to the discussion section of the manuscript.

3. It is commented that "It is not unusual to see cells that are overloaded with a large amount of dye and that are very bright". Could authors elaborate a bit on the identity of these bright cells and why they appear so bright? Are their cell membranes compromised?

We speculate that the very bright cells are likely undergoing apoptosis but do not yet have compromised plasma membranes, in which Ca2+ storage in the ER may be released causing these cells to fluoresce very brightly when loaded with fluo-4. We have expanded the note in the protocol to include this. The cells are pancreatic tissue cells but we have not studied their identity more specifically.

4. Figure 2, the latency in Ca2+ response is <20 sec in 2B; and the latency is ~ 3 min in 2D. Why was there such a sizable difference? Which response is more representative?

We often observe different latencies until glucose responsiveness between slice donors. Typically, Ca2+ begins to rise within 1-5 minutes after addition of glucose so both traces are representative. Several factors may affect the latency including the metabolic health of the slice, temperature control, positioning of the slice in the chamber, dead volume within perfusion tubing, or diffusion of glucose to reach islets at different depths in the slice. Reliability of timing of Ca2+ responses may be one limitation where isolated islets outperform slices.

RESPONSES TO REVIEWER #4:

GENERAL COMMENTS:

Huber et. al have developed a new approach to view the complex world of islet biology during different stages of immune destruction (type 1 diabetes) or dysfunction (type 2 diabetes). Complex imaging of islets from mouse has been accomplished in the past, but this is the first time that viable tissues can be imaged from human tissues. Adapting the ability to slice live tissue from thymus tissues for multiphoton imaging as E. Robey has eloquently performed in the past to a complex tissue like the pancreas is quite impressive. In addition to developing an exciting and revolutionary imaging approach, the addition of immunological markers opens this technique for vast scientific inquiry. This is a very exciting approach and I cannot wait to employ this method in my own research program. I strongly believe this approach should be published with only minor comments addressed as listed below.

We thank Reviewer 4 for their positive comments and for their constructive recommendations to improve the manuscript.

SPECIFIC COMMENTS:

1. Can you include some videos with this submission? I don't mean a video of the protocol, but I would really enjoy to see the video of the cellular interactions within the tissue slices!

We are also excited about the possibility to obtain time-lapse acquisitions of live endogenous immune cells interacting with islets in pancreas slices. We are currently conducting a study of these interactions hope to share such data in a future publication.

2. Does the gauge size of the needle for pancreas inflation need to be 27G for the agarose to flow? We typically use a 30 or 32 G needle for inflation, but this isn't agarose. Does the needle G change for age of recipient (larger common bile duct in older mice)?

27G works well to not tear the bile duct but smaller needles would likely cause issues with the agarose setting in them very quickly. For larger mice, a larger needle could probably be used. We have had success using the 27G with 10g and 25g mice. We added this elaboration to the note in the protocol.

3. A suggestion rather than comment - The addition of PBS to the dish will accelerate the "cure" time for the super glue. If you wait too long the super glue can penetrate into the agarose tissue plug and kill the tissue, much like capillary action. Setting the plug into the coated dish and quickly adding PBS cures the slice in place. In fact, we wipe away most of the glue with the tip of a kim-wipe but the remaining glue is more than enough to secure the plug to the plate. (Just a suggestions).

Thank you for the suggestion, we are going to try this with the mouse slices soon.

4. Can you add a slice that has not been properly processed to the supplemental data to illustrate what really bad islets and dead tissue looks like, maybe one on the way out and one past the point of no return. It might be helpful for readers to see so that they can compare with their own tissues to make sure the prep is performed fast enough and viable tissue. Death by laser looks very different than just plain dead tissue.

We processed a slice improperly by not putting it in medium with trypsin inhibitor and cultured the slice at 37°C overnight. We then took a darkfield stereomicroscopy image of the slice in addition to reflected light and viability staining with SYTOX Blue on the confocal microscope. We added these images to the supplemental data as Supplemental Figure 1.

5. Can you include a control slide with isotype control for CD3 or CD8 and an irrelevant tetramer for the insulin-tet? It would be helpful to see what the background stain looks like for these reagents. MHCII tetramers are inherently sticky to tissue and despite many labs efforts and years of modifications have been difficult due to extreme high background. MHCI is not as troublesome, but would be good to show. In addition, Can you please add a higher res image of the triple stain to visualize the yellow CD8 ins-Tet+ T cells? I would have envisioned these cells to be clustered near the dead islets with Sytox stain, but they appear to be near the exterior of the islet in Fig 3E.

We stained NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse slices with an isotype control for the mouse CD8 antibody (Rat IgG2a, κ) and also took images of slices stained with the rat anti-mouse CD8 antibody. The comparison can be found in Supplemental Figure 2. Additionally, we stained NOD.*Rag1*$^{-/-}$.AI4$^{\alpha/\beta}$ mouse slices with a tetramer control for lymphocytic choriomeningitis virus (LCMV) and collected images of slices stained with the insulin tetramer. The images can be found in Supplemental Figure 3. Our original image files are in high resolution but we think that the

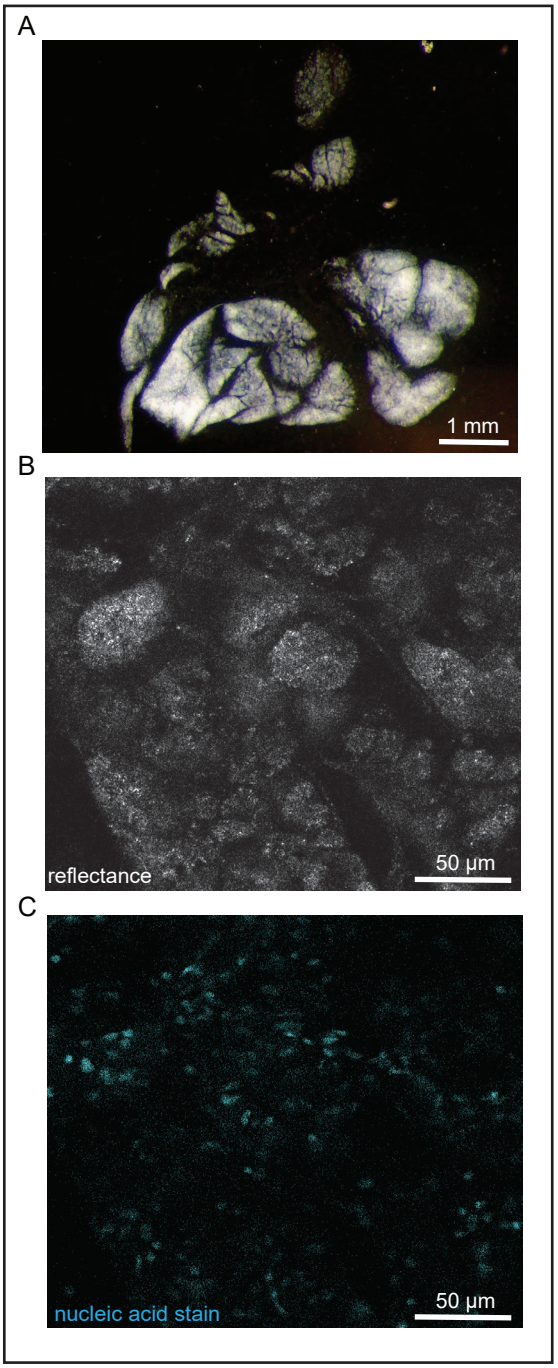
figure file didn’t render with the submission in high enough resolution. We edited the figure to make it a full page to help resolve this.

6. If possible a brief discussion of other approaches and adding citations for in vitro imaging such as PMCID: PMC3662370 and in vivo islet imaging from transplantation such as PMCID: PMC3538807, and PMCID: PMC2778301.

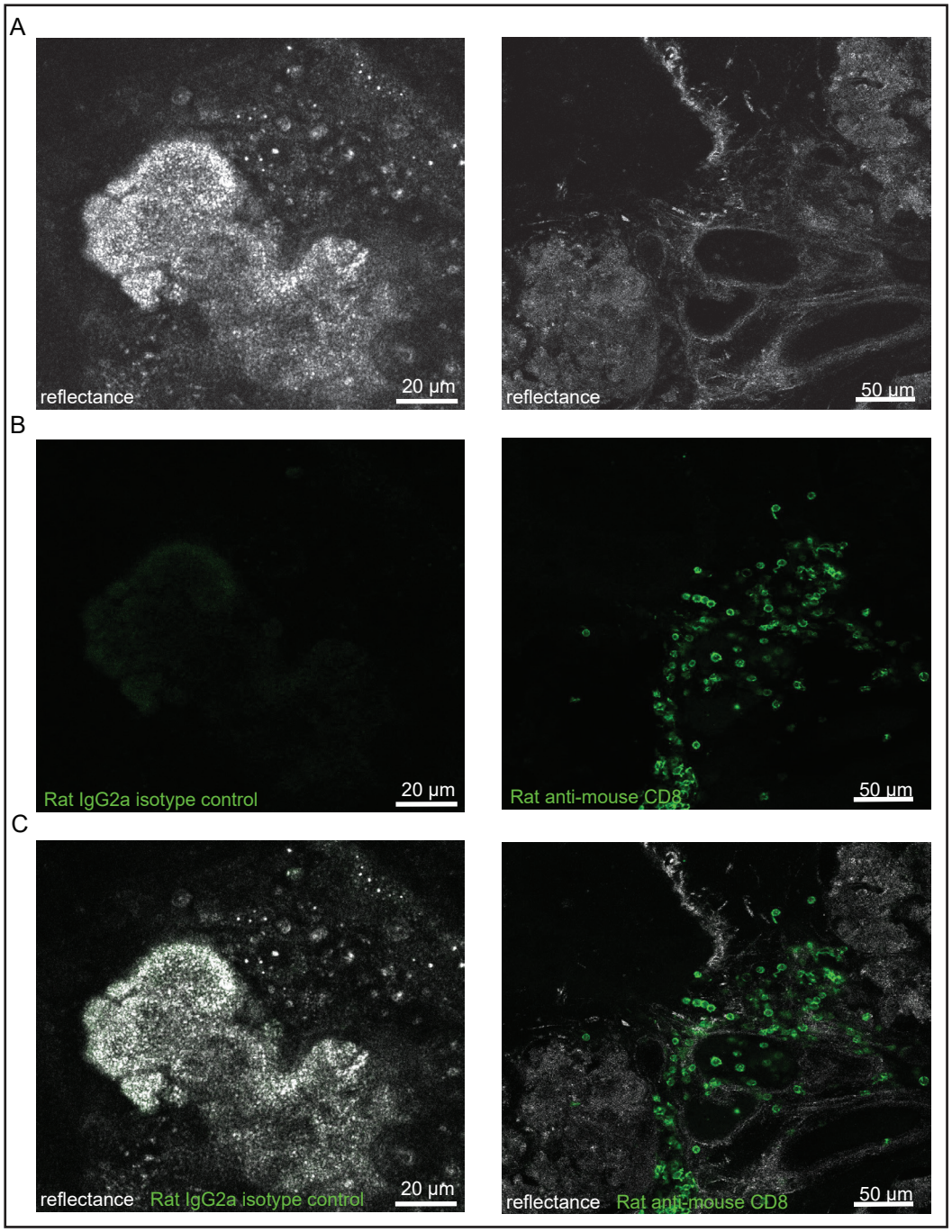
We added discussion of the different in vitro imaging techniques mentioned in the papers above to the manuscript. However, we felt that the in vivo islet imaging was beyond the scope of this paper.

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Manuscript Final_Supplemental Figure 1.pdf

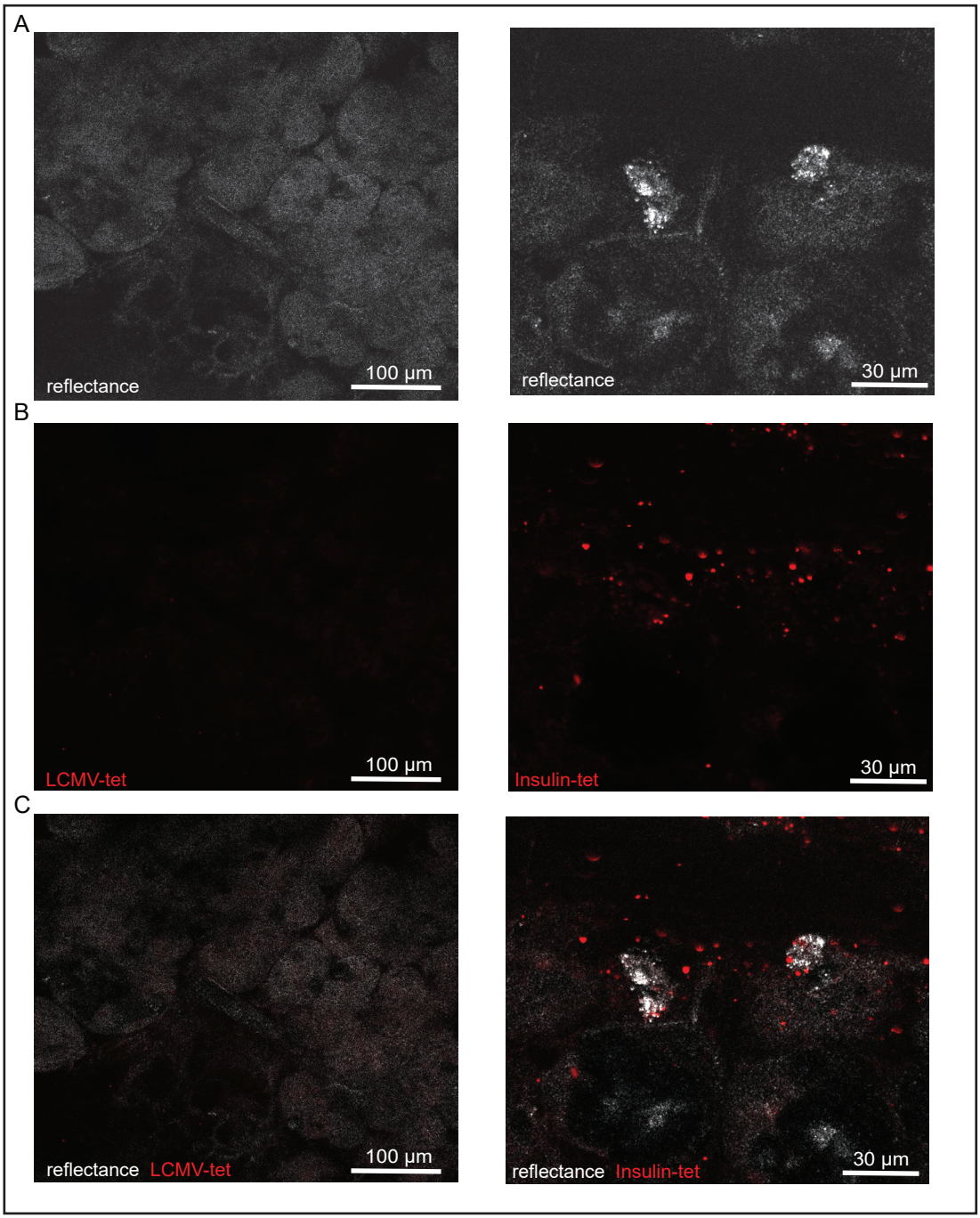


Supplemental Figure 2



Supplemental Figure 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Manuscript Final_Supplemental Figure 3.pdf



Solution	Description	Amount Needed
Agarose (100 mL)	Lonza SeaPlaque Agarose	1.25 g
	PBS	98.75 mL
Extracellular solution (ECS) (1 L)	NaCl	7.305 g
	KCl	0.186 g
	$NaHCO_3$	2.184 g
	NaH_2PO_4 (monohydrate)	0.172 g
	1M $MgCl_2$ (hexahydrate)	1 mL
	$CaCl_2$	0.222 g
	HEPES	2.383 g
	D-(+)-Glucose	0.540 g
Kreb's Buffer 10x Stock (KRBH) (1 L)	NaCl	67.2 g
	KCl	3.52 g
	$CaCl_2$ (dihydrate)	3.67 g
	KH_2PO_4	1.63 g
	$MgSO_4$ (heptahydrate)	2.96 g
	ddiH2O	1000 mL
KRBH with 3mM glucose (3G) (1 L)	10X KRBH	97.5 mL
	$ddiH_2O$	877.5 mL
	$NaHCO_3$	2.184 g
	BSA	2 g
	1 M HEPES	25 mL
	D-(+)-Glucose	552.468 mg
Mouse Slice Culture Medium (500 mL)	RPMI 1640	445 mL
	FBS	50 mL
	Penicillin Streptomycin	5 mL
	Soybean Trypsin Inhibitor	0.1 mg per 1 mL plate
Human Slice Culture Medium (500 mL)	Low glucose DMEM	445 mL
	FBS	50 mL
	Penicillin Streptomycin	5 mL
	Soybean Trypsin Inhibitor	0.1 mg per 1 mL plate

Supplemental video 1

Click here to access/download

Supplemental Coding Files

nPOD 6465 slice fluo4.avi