Video Article

Pancreatic Islet Embedding for Paraffin Sections

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URL: https://www.jove.com/video/57931
DOI: doi:10.3791/57931

Keywords: Biology, Issue 136, Islets, morphology, microscope slides, paraffin embedding, paraffin sections, hematoxylin and eosin staining, immunofluorescence staining, alpha cells, beta cells

Date Published: 6/29/2018

Citation: Kong, Y., Ebrahimpour, P., Liu, Y., Yang, C., Alonso, L.C. Pancreatic Islet Embedding for Paraffin Sections. *J. Vis. Exp.* (136), e57931, doi:10.3791/57931 (2018).

Abstract

Experiments using isolated pancreatic islets are important for diabetes research, but islets are expensive and of limited abundance. Islets contain a mixed cell population in a structured architecture that impacts function, and human islets are widely variable in cell type composition. Current frequently used methods to study cultured islets include molecular studies performed on whole islets, lumping disparate islet cell types together, or microscopy or molecular studies on dispersed islet cells, disrupting islet architecture. For *in vivo* islet studies, paraffin-embedded pancreas sectioning is a powerful technique to assess cell-specific outcomes in the native pancreatic environment. Studying post-culture islets by paraffin sectioning would offer several advantages: detection of multiple outcomes on the same islets (potentially even the exact-same islets, using serial sections), cell-type-specific measurements, and maintaining native islet cell-cell and cell-substratum interactions both during experimental exposure and for analysis. However, existing techniques for embedding isolated islets post-culture are inefficient, time consuming, prone to loss of material, and generally produce sections with inadequate islet numbers to be useful for quantifying outcomes. Clinical pathology laboratory cell block preparation facilities are inaccessible and impractical for basic research laboratories. We have developed an improved, simplified bench-top method that generates sections with robust yield and distribution of islets. Fixed islets are resuspended in warm histological agarose gel and pipetted into a flat disc on a standard glass slide, such that the islets are distributed in a plane. After standard dehydration and embedding, multiple (10+) 4 - 5 µm sections can be cut from the same islet block. Using this method, histological and immunofluorescent analyses can be performed on mouse, rat, and human islets. This is an effective, inexpensive, time-saving approach to assess cell-type-specific, intact-architecture outcomes from cultured islets.

Video Link

The video component of this article can be found at https://www.jove.com/video/57931/

Introduction

Pancreatic islets of Langerhans, the sole source of circulating insulin, are a critical tissue for investigators studying diabetes mellitus. From any given organism, islets have variable size, cell type frequency, and architecture[1,2,3]. The conventional strategy to study *in vivo* structure and endocrine cell composition of pancreatic islets is by sectioning pancreas tissue[4,5]. Since islets comprise only a small fraction of total pancreatic cellular content, molecular studies are performed on isolated islets. *Ex vivo* islet culture experiments testing response to nutrients, gene modulation (transfection, transduction), or experimental treatments provide important insight into mechanisms modulating endocrine cell survival, proliferation, and function[5,6].

Ex vivo islet experiments often are analyzed using molecular studies of whole islets or histological or molecular studies of dispersed islet cells grown in monolayer[5,6]. Molecular analysis of whole islets introduces the serious caveat of intermixing cell types, which may produce false-negative or false-positive results when extrapolated to any individual cell type. Cell dispersion onto coverslips for post-culture microscopy allows cell-type-specific outcome measurement, but disrupts islet architecture, which may alter response to intervention and precludes identification of architecture-related outcomes. In addition, generally only a single outcome can be measured; for example, to measure beta cell proliferation and beta cell death under the same conditions, two separate experiments need to be performed. These approaches are also blind to inter-islet variability, an area of increasing interest in the field. Sorting islet cells by flow cytometry for cell-type-specific molecular studies, or single-cell RNA studies are elegant but expensive, time consuming, limited by tissue abundance, architecture-eradicating, and not well suited to routine cell culture analyses[5,7]. Confocal imaging of whole-mount immunostained islets provides high-quality intact-architecture data, but is labor intensive, and data obtained from each sample is limited to outcomes identifiable in a single immunostaining[8].

The ability to generate high-quality paraffin sections of post-culture whole islets would address many of these concerns. High-cost, low-abundance islet tissue from unique genetic models or from human organ donors, or islets status post *in vivo* or *in vitro* experimental manipulation, are precious. Obtaining multiple paraffin sections from the same islets would allow multiple cell-type-specific, intact-architecture analyses from the same experiment.

Existing techniques to generate islet pellets for sectioning are imperfect. Histology-optimized agarose is an aqueous low melting point gel that is widely used in processing histological and cytological specimens including small or fragmented tissue samples that are difficult to process[9]. One islet embedding approach is to suspend the islets in agarose in a microcentrifuge tube, centrifuge to pellet the material, retrieve the agar plug, then process and embed for sectioning[10,11]. Extracting the solidified sample from the bottom of the tube is time consuming and difficult, leading to occasional fragmentation of the sample and risk of personal injury. The islets are concentrated in the tip of the plug, leading to inadequate islet distribution in sections obtained from this method. The round bottom of the plug complicates embedding such that an islet-poor region may be presented for sectioning. Overall, this method leads to low yield and clumped islet distribution in the resulting sections.

This new method is a simplified and improved approach for the preparation of islet sections. Islets are concentrated in a small volume and then placed on the smooth surface of a microscope slide to form a small disc, with the islets in a single plane. The Histogel-islet disc is subsequently processed for paraffin embedding in a shortened dehydration and xylene infiltration protocol. The previous approach, which concentrates the islets in the bottom of a microfuge tube, is also carried out as a comparison. This new technique improves the yield of islets per section, the distribution of islets in each section, and takes less time to transfer the islet blocks to cassettes. This technique is useful for islet biologists or other scientists studying small pieces of tissue wishing to maximize productive use of a low-abundance tissue by measuring multiple outcomes on a single sample in its native tissue architecture.

Protocol

All procedures involving animals were approved by the UMass Medical School Institutional Animal Care and Use Committee. Human islet studies were determined by the UMass Institutional Review Board to not qualify for IRB review or exemption because they do not involve the use of human subjects.

1. Islet Isolation and Culture

1. Isolate islets and separate from contaminating exocrine and ductal tissue using the method of your choice.
   NOTE: This method was optimized using islets isolated by collagenase ductal insufflation and Ficoll separation[12] (rodent) or post-shipment islets from the Integrated Islet Distribution Program (IIDP[13]; human). Islets were handpicked with a P200 micropipette.
2. To optimize islet morphology, allow islets to recover overnight in 10 mL complete islet medium (RPMI with 10% FBS, penicillin/streptomycin, and 5.5 mmol/L glucose) in a humidified chamber containing 5% CO_2 at 37 °C. Although this step is not required to obtain sections, the islets are more intact after a recovery period (see **Figure 2**).

2. Islet Fixation

1. Using a low-binding P200 tip, handpick approximately 250 islet equivalents (IEQ)[13] using a calibrated grid under a stereomicroscope into a 1.5 mL low binding microfuge tube. Low-binding tips and tubes reduce islet loss.
2. Allow islets to settle to the bottom of the microfuge tube. Remove most supernatant with a P200 pipet tip, taking care not to remove any islets.
3. Add 1 mL of PBS. Centrifuge tube in a swinging bucket centrifuge until the speed reaches 200 x g and then halt the spin. Remove the supernatant. Repeat PBS wash, for a total of two washes.
4. Add 500 µL of 10% formalin solution or 4% freshly made paraformaldehyde. Fix at room temperature for 30 minutes. For the outcomes tested in this method, these fixation methods were indistinguishable. Shorter fixation may also be possible; it is recommended to optimize fixation duration for desired outcome.
5. Remove the fixative with a P200 tip.
6. Add 1 mL of PBS. Centrifuge tube until the speed reaches 200 x g and then halt the spin. Remove the supernatant. Repeat PBS wash, for a total of two washes.
7. Proceed immediately to the next step.

3. Preparation of Islet Disc (Figure 1)

1. On first use, melt the gel (*e.g.,* Histogel) at 70 °C and make aliquots in 1.5 mL microfuge tubes for long term storage. Aliquot volume is not critical, since aliquots can be re-used.
2. Warm the gel (approximately 100 µL per sample) at 70 °C by placing microfuge tube containing the gel aliquot in a heat block set to 70 °C.
3. Transfer agarose blue beads (10 µL for each sample) into a 1.5 mL clean microfuge tube. Thoroughly resuspend the beads before pipetting.
   NOTE: Blue beads are mixed with the islets to assist in visual identification of the embedded material in the agarose button and the paraffin block. The number of blue beads used is not critical to the outcome, but avoid excessive beads (>5x the number of islets) to allow optimal distribution of islets in sections.
4. Wash the blue beads with 1mL PBS, twice. For each wash, spin the beads 1 min at 800 x g. After the second wash, resuspend the beads in (n+2) x 10 µL PBS, where n is the number of sample tubes; for example, for 2 sample tubes, the PBS volume to add to the beads would be 40 µL.
5. Centrifuge the microfuge tube containing islets (brief spin to 200 x g) and remove most of the supernatant. Cut off the tip of a 10 µL micropipette tip with scissors or a blade and add 10 µL of beads to each islet tube, using a clean tip for each sample. Do not mix (to avoid losing islets).
6. Centrifuge islets and beads (brief spin to 200 x g). Remove as much PBS as possible with an uncut 10 µL micropipette tip.
7. Label the microscope slides for sample identification. Two to three samples can be prepared on each slide. Place the slide on the bench near the heat block with warmed gel. Cut the tips of three to five 20 µL low binding micropipette tips per sample with a razor blade or scissors. Load two P20 micropipettes with cut tips to allow rapid switching from one to the other.

8. Using the first micropipette, add 15 - 20 µL of warm gel to the islets/beads microfuge tube; immediately mix, avoiding bubbles; and apply the liquid agar/islets/beads mixture to the slide to form a <1 cm diameter disc. Place the disc near the edge of the slide, leaving space for the outer gel ring (next step). Tap the slide gently a few times to settle the islets and beads.
9. Using a second micropipette, add 20 µL of warm gel to the sample tube. Using the first micropipette, mix new gel with any remaining islets/beads, re-warming in the heat block if it starts to solidify, then apply this mixture to the slide, surrounding the original disc. Repeat as necessary, using additional pre-cut tips, until the disc is the desired size and thickness.
   NOTE: Handling the gelled disc is easier if the outside ring is slightly thicker. Usually, 3 x 20 µL of gel is sufficient. This step minimizes loss of islets by tube washes and adds islet-poor agarose to the outside of the disc to facilitate disc handling.
10. Prepare each disc individually and keep careful track of sample order if placing multiple discs on each slide.
11. Place the slides on a flat surface of wet ice, with a cover, for 10 minutes or until the gel solidifies. It is more difficult to slide the disc off the glass if it dries out.
12. Label biopsy processing/embedding tissue cassettes with pencil, one per sample. Dry the back of the slide to avoid wetting the blue paper.
13. Using the blunt edge of a razor blade, gently push the disc in each direction to free it from the glass. When it slides easily, slowly push the disc from the microscope slide onto the blue tissue paper. Place the disc, flat side down, directly on the paper.
14. Keeping the disc flat, fold the paper around the disc to prevent movement, place the disc in folded paper in the cassette, and close the cassette. If the disc does not slide cleanly off the glass, add more gel and/or return the slide to ice for a few more minutes.
15. Submerge the cassette in PBS in a beaker. Process for paraffin embedding the same day for optimal morphology.

4. Paraffin Embedding

NOTE: Process the islet gel discs to paraffin blocks using an abbreviated dehydration series as described below. This technique was optimized using an automated processor, but manual processing should produce similar results.

1. Immerse cassettes in 85% ethanol for 15 minutes.
2. Immerse cassettes in 95% ethanol for 15 minutes.
3. Immerse cassettes in 100% ethanol for 15 minutes. Repeat twice for a total of three washes.
4. Immerse cassettes in xylene for 15 minutes. Repeat twice for a total of three washes.
5. Immerse cassettes in molten paraffin for 10 minutes.
6. Transfer cassettes to fresh molten paraffin for 10 - 30 minutes.
7. Carefully open the cassette and unwrap the blue paper. Remove the gel disc, keeping track of the flat surface that was opposed to the paper. Embed the disc in a small mold, with the flat (islet-containing) surface down, parallel to the cutting surface. For the plug, carefully remove it from the cassette and embed it in a small mold with the tip pointing towards the cutting surface.

5. Paraffin Sectioning and Staining

1. Label slides sequentially if serial sections are required.
2. Have 5 µm paraffin sections cut by an experienced histotechnologist. To maximize yield, save all sections containing material. Generally, collecting 20 sections allows capture of most of the material.
3. Store sections at room temperature. Sections are amenable to routine histological stains (*e.g.,* H&E) and immunofluorescence (*e.g.,* insulin, glucagon and DAPI). Optimize fixation for other antigens, if necessary.

Representative Results

An illustrated schematic of the steps to prepare the gel disc is shown in **Figure 1**. This gel disc method results in paraffin sections that contain a sufficient number of islets distributed in a single plane to allow meaningful quantification of outcomes. **Figure 2** shows low-magnification images of the resulting sections to illustrate the number of islets captured per section. In general, >35 islets were visible in each section when 250 IEQ were used for the procedure, and >10 sections (4 - 5 µm) containing islets were obtained from each sample. Increasing the number of islets used for the procedure increased the number of islets in the sections. Islets in sections were structurally diverse and of varying sizes, supporting the concept that new and interesting data may be obtained using sections rather than techniques blind to inter-islet differences. The method worked equally well for rodent islets isolated in the laboratory (rat, mouse) and for human islets received after shipment by the IIDP. Rodent islets had noticeably more intact morphology after post-isolation recovery overnight in islet medium, with a smooth rounded surface and compact spherical shape (**Figure 2A**). Human islet morphology was similar when embedded on the day of arrival or when embedded after post-shipment recovery overnight, perhaps because the islets had already recovered from isolation stress prior to shipment. The microtube method yielded a smaller tissue cross-sectional area, with fewer islets per section, and densely packed islets and beads (**Figure 2B**). The microtube images shown in **Figure 2B** were the best we were able to obtain with this method; one of the samples had to be sent back to cut more sections because no islets were found in the first set of sections.

This technique generates high quality islet sections for histological and immunofluorescence staining (**Figure 3**). Insulin and glucagon staining showed the diverse composition and heterogeneity of islet architecture. The sections clearly recapitulated known architecture differences between human, mouse and rat islets, with human islets comprised of abundant α-cells intermingled with beta cells, whereas mouse and rat islets showed similar architecture with a beta cell core and alpha cells in the periphery. Serial sections were obtainable; the panels in **Figure 3** show serial sections of the same islet stained for H&E (left) and immunofluorescence (right).

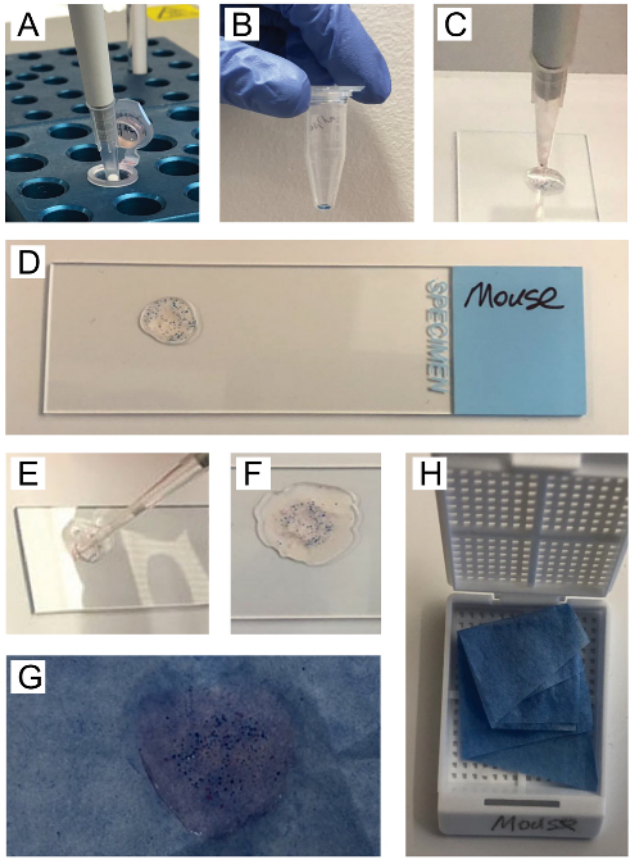

Figure 1. Illustration of the critical steps for making the islet gel disc. Gel warmed to 70 °C in a heat block **(A)** is transferred to the low binding microfuge tube containing the islet/bead pellet **(B)**. The islets are resuspended in warm gel and transferred to a glass slide, spreading the material in a disc shape **(C-D)**. Additional clean gel is transferred to the microfuge tube, then all remaining material is removed and spread circumferentially around the initial islet/bead-containing disc on the glass slide **(E-F)**. After gelling on ice, the disc is transferred, flat side down, to histology paper **(G),** which is folded and placed in a cassette **(H)** for processing. Please click here to view a larger version of this figure.

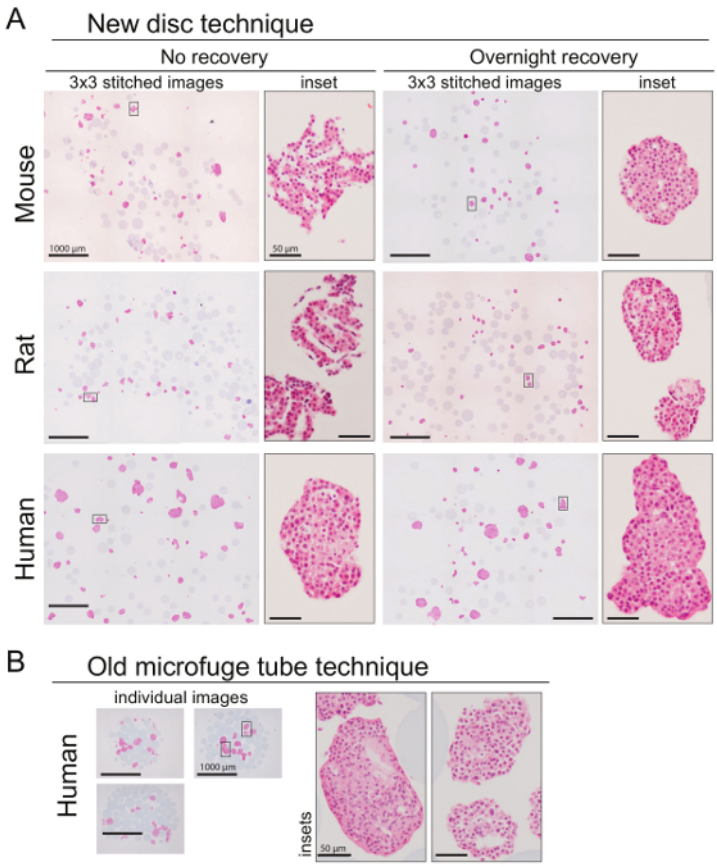

Figure 2. **Paraffin sections of gel discs contain a large number of well-distributed islets**. **(A)** Low magnification panels (40X, 3 x 3 images stitched together, with 15% overlap) of mouse, rat or human islet sections stained with H&E show that many islets are visible in each section. High magnification insets (200X) show representative islets. Left panels show sections of islets embedded immediately after isolation (mouse, rat) or shipment (human); right panels show islets embedded after overnight recovery in culture. Pale blue circles seen in 40X images are the beads included for visualization during embedding and sectioning. These particular samples are from a C57BL/6N mouse (1 year old) and BBDR rat (4 weeks old), islets isolated by ductal collagenase injection and Ficoll separation, and human T2D islets received from the IIDP. **(B)** 250 IEQ of human islets embedded per the old microfuge tube technique, for comparison. Low magnification panels (40X; a single unstitched image captured all material) and high mag insets (200X) show representative sections. Culture medium was RPMI with 10% FBS, penicillin/ streptomycin, and 5.5 mmol/L glucose. Scale bars for low magnification panels are 1000 µm; scale bars for high magnification panels are 50 µm. Please click here to view a larger version of this figure.

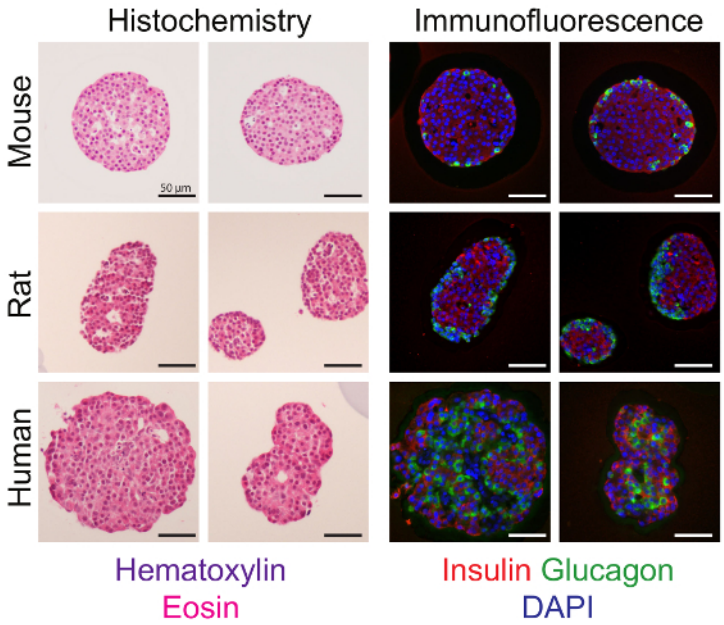

Figure 3. Representative histochemical and immunofluorescence staining on serial islet sections. Sections of mouse, rat, and human islets embedded after overnight culture were stained for H&E and imaged by brightfield microscopy (200X; left). An adjacent section was stained for insulin (red), glucagon (green), mounted in DAPI-containing media (blue) and imaged using fluorescent microscopy (200X; right). Scale bars: 50 µm. Please click here to view a larger version of this figure.

Discussion

This modified gel-disc-based embedding method provides a simple, inexpensive, and efficient way to generate a high yield of islets per section. Constructing the gel disc on a flat glass surface facilitates spreading islets in an even distribution over a well-defined area. Spreading the islets in a flat disc offers the advantage of placing many islets in the plane of section, optimizing yield and allowing fewer islets to be used. The disc thickness can be adjusted to meet investigators' needs. Since multiple sections can be obtained, serial sections of the same islets can be analyzed, increasing the number of distinct outcomes that can be measured on the same experimental sample. Although optimized here for pancreatic islets, the technique could be applied to other low-abundance material, including those with small pieces, friable nature, or viscous samples that would otherwise be difficult to process. This method is optimized for fresh-fixed islets and has not been tested for other types of material, such as previously frozen islets. These sections may also be useful for *in situ* hybridization assays to detect DNA or RNA. The addition of extra gel serves two purposes: to maximize islet collection and to protect the islet-rich disc center from disruption during handling/transferring. The thin gel disc solidifies quickly and allows shortened dehydration and xylene infiltration steps for paraffin embedding. Forming the disc on a flat surface rather than in a secondary container (microfuge tube or culture plate well) makes it easier to physically transfer the disc to the cassette for processing. When compared with the disc technique results, the microtube technique had reduced islet yield and clumped islet distribution, although the non-planar tissue arrangement could generate a higher number of islet-containing sections.

The critical steps of the protocol include tissue fixation, formation of the gel disc, paraffin embedding, and sectioning. With respect to fixation, as tested by H&E and immunofluorescence for insulin and glucagon, no difference was detected between PFA and formalin, or fixation duration ranging from 10 - 30 minutes (not shown). However, fixation intensity and duration should be optimized for end-user needs. The principal innovation contained in this protocol is the preparation of the gel islet disc; critical steps are summarized in **Figure 1**. Key steps include using a small volume of gel to concentrate tissue in a small area and forming the disc on a flat surface to arrange the tissue in a single plane for sectioning. Use of low-binding microfuge tubes and tips and a swinging bucket centrifuge for all spins improves tissue yield; islets stick to plastic after fixation. Addition of easily visible beads assists with disc formation, embedding and sectioning. Removing as much PBS as possible before adding the gel is critical to avoid diluting the agarose, which leads to poor solidification and difficulty transferring the disc to the paper. Keeping the thin disc intact while sliding it from the glass slide to the paper can be challenging. If a wrinkle occurs in the disc, allow it to return to the original position, add more gel and re-chill the slide. With respect to paraffin embedding, it is important that the disc be embedded flat-side down and parallel to the plane of cutting. A histotechnologist experienced with cutting paraffin sections is essential to the success of this procedure, since the material is concentrated at the leading edge of the block. Excessive facing off of the block may lead to loss of the sample. It is recommended to start collecting sections as soon as the blue beads are visible.

Fixation intensity and duration should be optimized for the end user's specific tissue and needs. The number of islets used per sample can be modified if necessary; however, reducing starting material generates sections with fewer islets. Thicker or larger discs can be generated using this technique; dehydration and xylene infiltration steps may need to be lengthened and should be optimized. If resulting sections contain only a partial disc shape, consider the possibility that the disc was not embedded parallel to the cutting surface. If too few sections are obtained, the technologist may have lost material during initial block preparation.

This method generates sections containing fixed islet tissue and, as such, can only be used for histological outcomes. To obtain a large number of islets per section, 200 - 250 IEQ starting material was used, which is challenging to generate for some experiment types. High quality sections are dependent on having an experienced histotechnologist.

The ability to generate 10+ sections containing many islets from a single experimental condition will allow quantification of multiple outcomes on the same material, improving experimental efficiency. Routine analysis of intact islets may lead to advances in understanding in tissue heterogeneity, not only on a cellular level, but also at the islet level, about which little is currently understood. Applying this technique to other limited-abundance tissue samples may offer benefits to other fields as well.

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgements

We gratefully acknowledge the Beta Cell Biology Group at the UMass Diabetes Center of Excellence for helpful advice and discussions. Human pancreatic islets were provided by the NIDDK-funded Integrated Islet Distribution Program (IIDP) at City of Hope. This work was funded by NIH/NIDDK: R01-DK114686 (LCA), R01-DK105837 (CY), 2UC4DK098085 (IIDP) and by the American Diabetes Association grant #1-15-BS-003 (LCA) in collaboration with the Order of the Amaranth.

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