

Histology of Type 1 Diabetes Pancreas

Abby Willcox and Kathleen M. Gillespie

Abstract

The islets of Langerhans play a critical role in glucose homeostasis. Islets are predominantly composed of insulin-secreting beta cells and glucagon-secreting alpha cells. In type 1 diabetes, the beta cells are destroyed by autoimmune destruction of insulin producing beta cells resulting in hyperglycemia. This is a gradual process, taking from several months to decades. Much of the beta cell destruction takes place during a silent, asymptomatic phase. Type 1 diabetes becomes clinically evident upon destruction of approximately 70–80 % of beta cell mass. Studying the decline in beta cell mass and the cells which are responsible for their demise is difficult as pancreatic biopsies are not feasible in patients with type 1 diabetes. The relative size of islets and their dispersed location throughout the pancreas means in vivo imaging of human islets is currently not manageable. At present, there are no validated biomarkers which accurately track the decline in beta cell mass in individuals who are at risk of developing, or have already developed, type 1 diabetes. Therefore, studies of pancreatic tissue retrieved at autopsy from donors with type 1 diabetes, or donors with high risk markers of type 1 diabetes such as circulating islet-associated autoantibodies, is currently the best method for studying beta cells and the associated inflammatory milieu in situ. In recent years, concerted efforts have been made to source such tissues for histological studies, enabling great insights to be made into the relationship between islets and the inflammatory insult to which they are subjected. This article describes in detail, a robust immunohistochemical method which can be utilized to study both recent, and archival human pancreatic tissue, in order to examine islet endocrine cells and the surrounding immune cells.

Keywords: Type 1 diabetes, Beta cell, Alpha cell, Islet, Pancreas, Histology, Immunohistochemistry, Insulinitis, DAB, Antibody

1 Introduction

The human pancreas is a multifunctional organ. The exocrine compartment comprises acinar cells which produce and secrete digestive enzymes into the pancreatic ductal system which are then drained into the gut. The endocrine compartment synthesizes and secretes glucose homeostasis hormones which are secreted into the circulation in response to blood glucose. The exocrine compartment comprises approximately 98 % of pancreatic mass, with the endocrine comprising just 2 %. The endocrine cells of the pancreas are clustered into discrete “islands” known as islets of Langerhan, which are scattered throughout the pancreas. Islets are composed of several different endocrine cell types; alpha cells

which secrete glucagon, beta cells which secrete insulin, delta cells which secrete somatostatin, PP cells which secrete pancreatic polypeptide, and a minor fraction of epsilon cells which secrete ghrelin. Islets are highly vascularized by capillaries and are also innervated by the sympathetic, parasympathetic, and sensory nervous system [1]. Islets are approximately 150–300 μm in diameter and contain approximately 1500 cells each [2].

The autoimmune destruction of beta cells results in the development of type 1 diabetes. The inflammatory infiltrate which targets the islets is known as “insulinitis.” The wide distribution of islets throughout the organ makes the study of the endocrine compartment of the pancreas very difficult as the islets are too small to resolve by *in vivo* imaging in humans. Advances are being made in the field of *in vivo* imaging of pancreatic beta cells and insulinitis [3] but information on beta cell function can currently only be gained indirectly by measurement of islet hormones in the circulation, or by measuring the 31-amino-acid C-peptide, produced in equimolar quantities when proinsulin is cleaved into insulin, in the circulation or the urine. Blood glucose levels following a meal can also be measured in order to gain information on pancreatic function but this does not correlate with beta cell mass as glucose homeostasis can be maintained with as little as 30 % of normal beta cell mass [4, 5]. These issues are further complicated by the heterogeneous preclinical phase of the disease during which beta cell destruction can take place over months up to several years and is unique to each individual. Circulating islet associated autoantibodies are useful biomarkers which indicate that beta cell autoimmunity is taking place and aid in determining risk of developing type 1 diabetes [6]. They offer no insight however into the extent of beta cell destruction or the cells which are responsible for it.

Study of the human pancreas using histological methods is therefore extremely important as it provides direct insights into the autoimmune process in the type 1 diabetic pancreas. Such tissue is generally only available at autopsy as taking pancreatic biopsies is a complicated procedure and there are many issues, both ethical and practical, with taking pancreatic samples from individuals with type 1 diabetes [7]. Immunohistochemical staining of human pancreas tissue sections for various endocrine, immune cell and proliferative markers provides valuable information on the state of the islets in terms of beta cell number remaining at various stages of disease, which cells are involved in beta cell death, and importantly, whether there are any indications that beta cell mass may be able to recover or regenerate following the autoimmune attack.

This chapter aims to describe a robust immunohistochemistry method which can be applied to new and archival pancreatic tissues in order to reveal important features of the pancreatic islets which can be examined and quantified in order to gauge the degree of

beta cell loss and the severity of any ongoing autoimmune insult in the pancreas of donors with new onset type 1 diabetes.

Pancreatic tissue from individuals with type 1 diabetes, in particular new onset type 1 diabetes, is an extremely rare resource. Availability of tissue sections is limited and therefore, it is very important that all staining procedures are optimized on control tissues which are available in greater abundance. Nondiabetic pancreatic tissue is recommended for islet endocrine cell antibodies, and human spleen or tonsil tissue for antibodies raised against immune cell or proliferation markers. This article assumes the use of formalin-fixed, paraffin-embedded (FFPE) tissue sections which have been thinly sectioned (4–8 μm) with a microtome onto standard glass microscope slides, however, the adaptation of the method for the use of snap-frozen sections will be discussed in Sect. 4. Suggestions are also made as to which antibodies can be used for a general examination of the type 1 diabetic pancreas, however any primary antibody of interest can be switched into this protocol and therefore, the selection of secondary antibodies outside of the scope of those described below, and all relevant control reagents, should be identified and sourced by the end user.

2 Materials

1. Xylene.
2. Absolute ethanol (use distilled water (dH_2O) to make dilutions of 90 %, 70 %, and 50 % ethanol).
3. Antigen retrieval buffers: Tris-EDTA pH 9.0: 10 mM Tris-base, 1 mM EDTA in dH_2O , or, citrate buffer pH 6.0: 100 mM citric acid in dH_2O .
4. Tris-buffered saline (TBS) pH 7.4: 50 mM Tris, 150 mM NaCl in dH_2O .
5. A microwave suitable for laboratory use.
6. A microwavable pot with a vented lid, large enough to hold 1 L of antigen retrieval buffer and completely immerse a rack of microscope slides (standard slide dimensions 26 mm \times 76 mm \times 1 mm).
7. Glass Coplin jars (standard 10-slide Coplin jars hold approximately 50 ml of solution).
8. An incubation chamber with a lid in which glass microscope slides can be laid flat and slightly raised from the bottom of the chamber. Place a dampened paper towel along one edge of the chamber in order to maintain humidity and prevent drying out of the tissue sections. A large, shallow Tupperware pot with 5 ml serological pipettes firmly attached to the bottom in rows will suffice.

9. Blocking reagent: TBS supplemented with 5 % normal serum (Vector Laboratories).
10. Endogenous peroxidase 3 % blocking solution: H₂O₂ stock reagent (30 %) diluted 1/10 with dH₂O.
11. Primary antibodies to detect islet beta and alpha cells respectively: guinea-pig anti-insulin (Dako; A0564), rabbit anti-glucagon (Abcam; ab18461). Dilute in either TBS + 0.5 % normal serum or commercial diluents can be purchased, e.g., Dako REAL™ Antibody Diluent.
12. Primary antibodies to detect immune cell infiltrates, including CD45 (pan-lymphocyte), CD3 (pan-T cell), CD4 (helper T cells), CD8 (cytotoxic T cells), CD20 (B cells), and CD68 (macrophages), have been described in detail previously [8].
13. Isotype control antibodies: immunoglobulin (Ig) of the same isoform and species of the primary antibody, e.g., mouse IgG1 or, IgG2a (available from multiple sources).
14. Dako Envision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse secondary detection kit: contains a ready-to-use cocktail of goat anti-rabbit IgG and goat anti-mouse IgG polymer-horseradish peroxidase (HRP) conjugated secondary antibodies, and DAB+ chromogen and DAB+ substrate buffer. The secondary antibody cocktail is also able to detect guinea-pig IgG so can be used as a universal secondary antibody for all three species of primary antibody.
15. Mayer's hematoxylin. Filter the hematoxylin through Whatman filter paper before use.
16. Blueing solution; Scots Tap Water Substitute (STWS): 3.5 g sodium bicarbonate, 20 g magnesium sulfate, 1 L dH₂O.
17. DPX mounting medium.
18. Glass coverslips, 25 × 60 mm.

3 Method

3.1 *Setting Up Antibody Controls*

As with any method which involves antibody labeling, appropriate controls must be employed to confirm that both primary and secondary antibodies are binding specifically. These are very important when staining human pancreatic tissue as islets can be “sticky” and are prone to staining with a wash of light nonspecific background when using immuno-peroxidase techniques. This needs to be distinguishable from true immunopositive signal.

To control for primary antibody specificity, an antibody pre- quenching step using the relevant immunizing peptide or protein, should be employed. Immunizing peptides and proteins can be purchased from the manufacturer of which the antibodies were

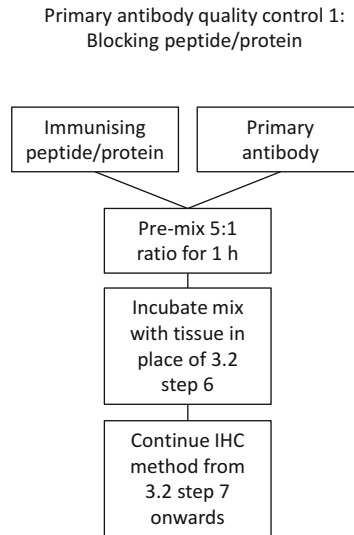


Fig. 1 The flowchart demonstrates how to adapt the method to include a control condition under which the primary antibody would be quenched by immunizing peptide or protein prior to incubation with the tissue. If positive labeling persists under these conditions, it suggests that the primary antibody exhibits off-target binding properties

sourced. Premix the primary antibody with its corresponding peptide/protein in TBS at a ratio of at least 1:5 for 1 h with frequent agitation, followed by a brief centrifugation. Apply the premixed solution to the section in the same way that primary antibody alone would be incubated (*see* Sect. 3.2, **step 6**) (Fig. 1). If the primary antibody is specific for the immunizing protein, it should be quenched during the premixing step and unable to bind to the tissue section. This should result in the absence of positive staining. A second quality control which can be employed in certain circumstances in human tissue is the use of alternative tissues which are known not to express the protein of interest. This is easily accomplished for insulin staining as the protein is only expressed in the pancreas. However, this control step may not be feasible if the protein of interest is widely expressed in many cell types, or if its expression profile is ambiguous.

There are two quality control steps which should be employed to confirm the specificity of secondary antibodies. The first requires replacing the primary antibodies with isotype control antibodies. These are irrelevant Ig of the same isotype and derived from the same host species of the primary antibody, e.g., mouse IgG1 or IgG2a (Fig. 2a). Replacing the primary antibody with isotype control Ig should result in a lack of positive staining as the control Ig should target any epitopes in the human tissue and therefore, should not bind. In this case, the secondary antibody should have no primary antibody to bind to. If, under these control conditions

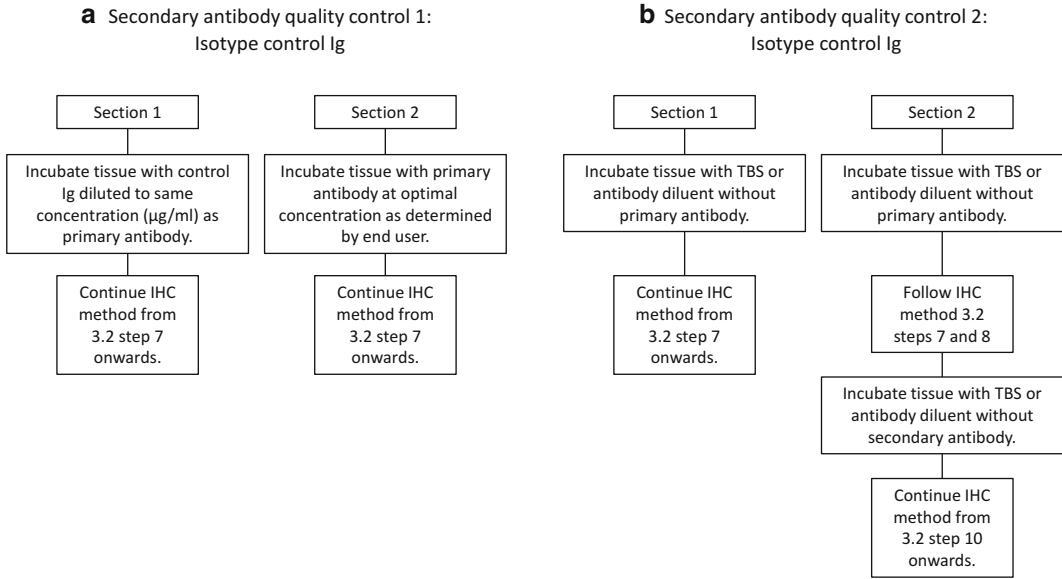


Fig. 2 (a) The flowchart demonstrates how to include the quality control condition in which primary antibody is replaced by Ig of the same isotype and host species. If positive labeling persists under these conditions, it suggests that the secondary antibody exhibits off-target binding properties, or, in some cases the Ig itself may have bound to the tissue. **(b)** demonstrates an additional control measure which should always be employed. It will also determine the cause of any nonspecific staining which presents under the control conditions described in Figs. 1a and 2a

there is still staining then it suggests that the secondary antibody is binding nonspecifically to the tissue. The second quality control step for secondary antibody specificity requires (a) the incubation of one tissue section with only TBS or antibody diluent without addition of primary antibody (Sect. 3.2, step 6) and (b) an adjacent tissue section incubated with only TBS or antibody diluent in place of both the primary and secondary antibodies (Sect. 3.2, steps 6 and 9, respectively) (Fig. 2b). Should positive staining develop under condition “a” but not condition “b” then it points to a nonspecificity issue with the secondary antibody. However, if positive staining develops under conditions “a” and “b,” it suggests that endogenous peroxidase activity in the tissue was not completely quenched by the endogenous peroxidase 3 % blocking solution and has reacted with the DAB reagent. To overcome this issue a higher concentration of H₂O₂ or a slightly increased incubation time will be required (e.g., increase from 5 min to 10 min).

3.2 Immunohistochemistry Staining Method

Steps 1–2 and 17–20 must be carried out in a fume cupboard.

1. Place the slides in a Coplin jar filled with Xylene to fully immerse the tissue sections, for 5 min. Repeat with a second Coplin jar of Xylene. This step will dissolve the wax and remove it from the tissue.

2. Bring the slides through a series of Coplin jars containing decreasing concentrations of ethanol (100 %, 90 %, 70 % and 50 %), and finally into a Coplin jar of dH₂O. This step will rehydrate the tissue, enabling the binding of antibodies in subsequent steps.
3. Gaining access to antigen binding epitopes within FFPE tissue can be problematic. Cross-links formed during formalin fixation can physically block antibodies from accessing their cognate epitope. The process of antigen retrieval, also known as heat-mediated epitope retrieval (HIER), is often required to break down the cross-links. If this method is modified for the use of fresh-frozen tissues then HIER (**step 3a–d**) should be omitted (*see Note 1*).
 - (a) Fill a microwaveable pot with 1 L of antigen retrieval buffer. The choice of antigen retrieval buffer is specific to each primary antibody, and must be determined by the end user following optimization experiments. There is not one buffer which optimally unmasks epitopes for all antibodies (*see Note 2*).
 - (b) Place the slides into a microwavable plastic slide rack and immerse fully in the antigen retrieval buffer.
 - (c) Secure the lid onto the pot, while allowing steam to escape through a vent or small hole in the lid.
 - (d) Place the pot containing the slides into a microwave and set to full power (“high” on an 800 W microwave) for 20 min, then remove the pot from the microwave, loosen the lid and leave the sections in the buffer to cool for 20 min.
4. Remove the slides from the antigen retrieval buffer one at a time, being careful not to allow the tissue sections to dry out as the slides will still be warm. Lay the slide flat, tissue side up, in the incubation chamber and immerse the tissue section in 100–500 μ l (depending on the size of the section, use the same volume for all incubation chamber steps) of blocking reagent, ensuring it is fully covered with solution. When choosing serum for the blocking reagent it is optimal to use normal serum from the host species in which the secondary antibodies are raised, or a serum which is irrelevant to any of your primary antibody species (e.g., if you are using a primary antibody which was raised in rabbit then you should never use normal rabbit serum for blocking as this will generate a blanket of nonspecific staining). Place the lid on the chamber and incubate for 30 min at room temperature.
5. Pour the blocking reagent off of the sections into the bottom of the tray and lay the slides down flat.

6. Incubate the sections with primary antibodies diluted appropriately (as suggested by the antibody data sheet combined with end user optimization experiments) in antibody diluent, in the same manner as described for the blocking reagent. Sections can be incubated for 1–2 h at room temperature or, overnight at 4 °C, depending on the individual requirements of each primary antibody (e.g., if the antigen is low in abundance, the tissue autolysed, or the primary antibody only available at low concentration, then a longer incubation time is often required (*see Note 3*)).
7. Pour the primary antibody solution off of the sections and place the slides in a Coplin jar filled with TBS for 5 min. Repeat twice more to wash all unbound primary antibody from the sections.
8. Lay the slides flat in the incubation chamber and incubate with endogenous peroxide blocking solution for 5 min. Pour off the solution and wash once more in a Coplin jar of TBS for 5 min to stop the reaction. If this method is being employed for immunofluorescence staining, this step should be omitted and the modified method described in **Note 4** should be followed from this stage onwards.
9. Lay the slides back in the incubation chamber and immerse with secondary antibody for 1 h. The Envision™ secondary antibody described in **step 13** of Sect. 2 is ready-to-use. Approximately 3–5 drops will be suitable to immerse most tissue sections. If using a concentrated stock HRP-conjugated secondary antibody, ensure that it targets the relevant primary antibody host species and dilute in the same manner as described for primary antibodies (Sect. 2, **step 11**).
10. Pour the secondary antibody solution off of the sections and place the slides in a Coplin jar of TBS for 5 min. Repeat twice more to wash all unbound antibody from the sections.
11. Using the Envision™ Detection Systems Peroxidase/DAB kit (described in Sect. 2, **step 14**), dilute the DAB+ chromogen in the DAB+ substrate buffer at a ratio of 1:50 and mix well.
12. To develop the chromogen, enabling visualization of the location of the protein of interest, lay each of the slide flat in the incubation chamber and incubate with the mixed DAB+ solution for 10 min (*see Note 5*). When labeling antigens which are expressed in abundance, it is possible to visualize a brown precipitate forming by eye as the antibody-bound peroxidase oxidases the 3,3'-diaminobenzidine. If staining for insulin or glucagon on nondiabetic pancreas sections, brown “spots” should appear throughout the section, highlighting the location of the islets (Fig. 3a, b). Antigens which are expressed in low abundance may not be visible without the aid of a microscope. If the entire section turns a shade of light–dark brown it

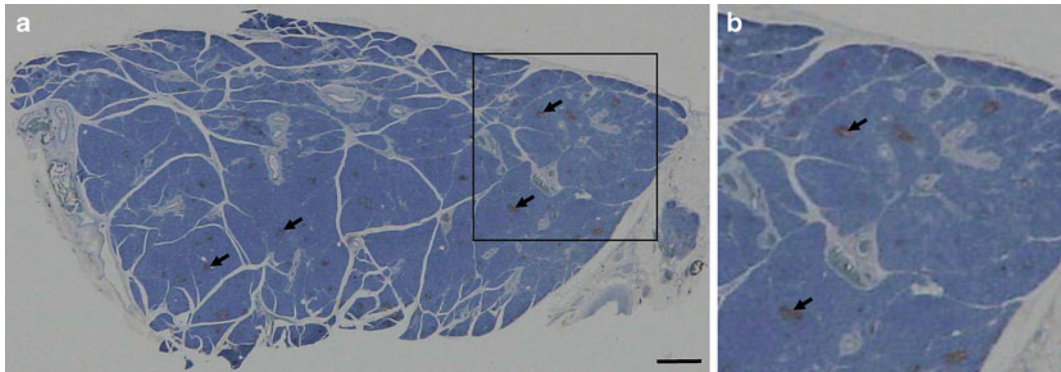


Fig. 3 (a) A section of human pancreas stained with rabbit anti-glucagon primary antibody and counterstained with hematoxylin (*blue areas*) using the method described in this chapter. Focal areas of brown staining which represent glucagon-positive alpha cells, and therefore islets, are visible by eye. A selection of such islets is highlighted by the *black arrows*. The region highlighted by the *black box* is shown at higher magnification in image **(b)**. Scale bar, 1000 μm

suggests that too high a concentration of primary antibody was used or that the blocking steps were not completely effective.

13. Following the DAB incubation, dispose of the DAB solution as per the material safety data sheet suggests. Place the slides in a Coplin jar of dH_2O for 5 min to inhibit any further oxidization reaction.
14. To counterstain the tissue a purple/blue color, place the slides in a Coplin jar of filtered Mayer's hematoxylin for 1–2 min.
15. Place the slides into a Coplin jar of tap water and leave under a gently running tap for 5 min. The color of the sections will change when immersed in tap water to a slightly darker shade of blue/purple due to the slightly acidic pH of tap water.
16. The counterstained sections can optionally be placed in a Coplin jar of STWS blueing solution for 10 s. This step will induce further blueing of the sections and increase the sharpness of the counterstain.
17. To complete the procedure, dehydrate the sections by placing the slides in 50 %, 70 %, 90 % and 2×100 % ethanol for 5 min each. It is crucial that all water in the tissue is displaced by ethanol to enable effective mounting and preservation of the sections.
18. Place the slides in xylene for 5 min, and repeat. The xylene will expel the ethanol from the tissue, and is also suitable for mixing with the DPX mounting medium, while ethanol is not.
19. Lay the slides flat on a paper towel. Using a plastic Pasteur pipette, apply approximately 600 μl of DPX mounting medium to the section. Gently lower a glass coverslip at a diagonal angle over the slide, using slow movements to prevent air bubbles

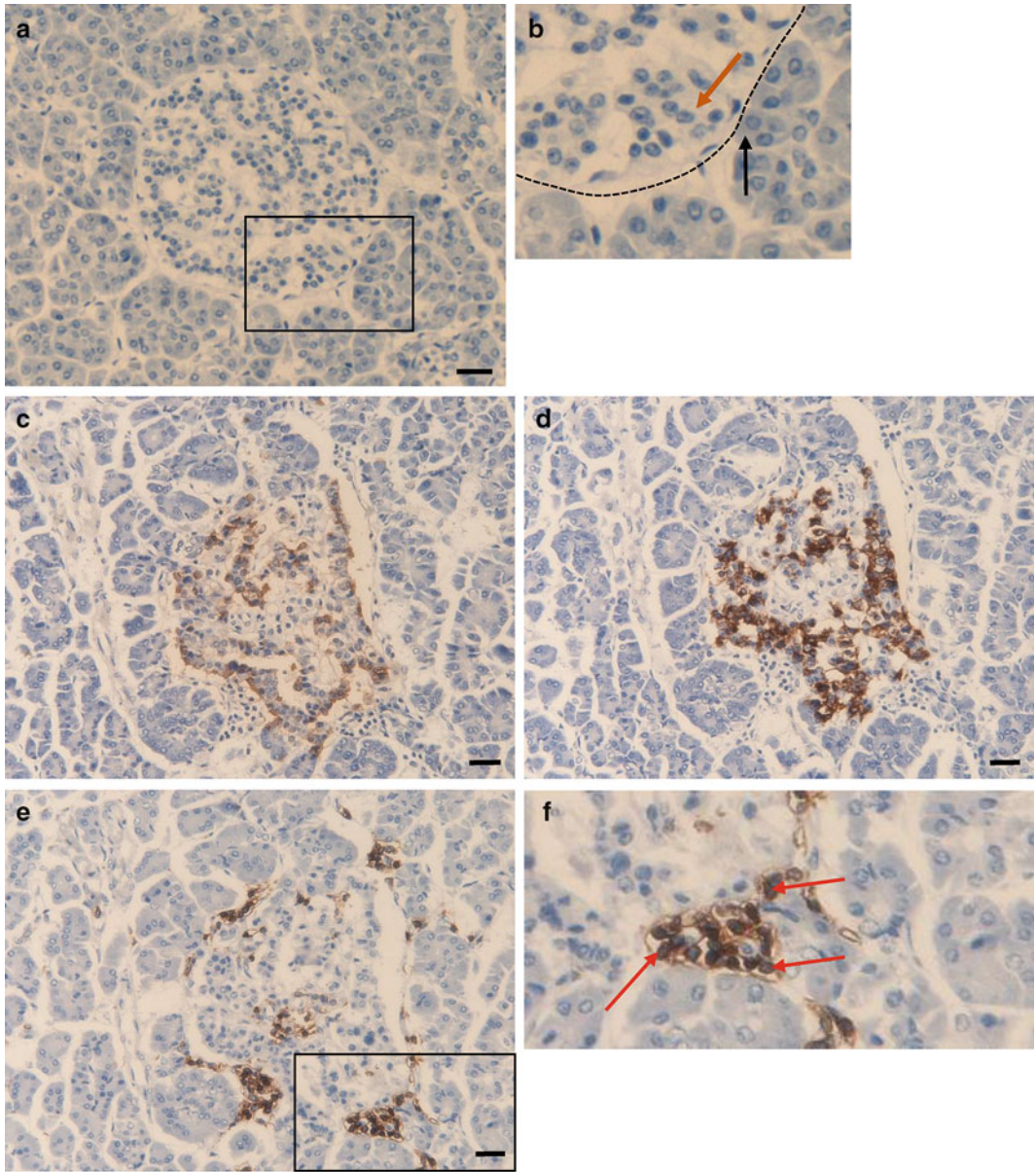


Fig. 4 Images of human pancreatic islets in situ. (a) shows an islet stained with hematoxylin (*blue*), surrounded by acinar cells. (b) highlights the area outlined by the *black box* in (a). It shows the distinction between the lightly stained cytoplasm of the islet cells (*orange arrow*) and the darker stained cytoplasm of the acinar cells (*black arrow*). The boundary between the islet and exocrine compartments is marked by the *dashed black line*. Images (c), (d) and (e) show islets stained for glucagon, insulin, and the pan-lymphocyte marker CD45, respectively. Image (f) shows a highlighted area represented by the *black box* in image (e). CD45-immunopositive lymphocytes are visible as dark nuclei with minimal visible cytoplasm. Scale bars, 20 μm

forming and being trapped on the section. Turn the slide over and apply gentle pressure to spread out the mounting medium evenly and remove any excess. Turn the slide back upright and square up the coverslip with the slide.

20. Lay the slides down flat to dry overnight.

3.3 Imaging

The tissue sections can be visualized using any microscope with bright-field capability. If image capture is required then a bright-field microscope with a mounted color camera linked to appropriate image capture software is necessary.

Islets can be identified histologically as discrete structures which are embedded within the exocrine tissue and are surrounded by a basement membrane [9]. As described above, islets stain a lighter shade with hematoxylin than the surrounding acinar cells (Fig. 4a, b). While the whole islet can be readily identified based on hematoxylin staining alone, in human pancreas immunohistochemical labeling of the islet hormones is crucial in order to confidently identify individual cell types as this cannot be done based on location alone. In humans, the alpha and beta cells do not display clear differential localization as seen in mouse islets (i.e., an alpha cell mantle with a beta cell core) [10]. Therefore, to fully establish the beta cell content of the islets or the alpha cell–beta cell ratio, cell-type specific staining must be employed (Fig. 4c, d). Insulinitis can be identified histologically as small mononuclear cells, the nuclei of which stain intensely with hematoxylin, with very little or no visible cytoplasm (Fig. 4e, f). These cells represent the lymphocyte population, however there is no way of distinguishing between lymphocytes sub-types histologically so subtype specific antibodies must be employed to reveal the composition of the infiltrate. This is also important for detection of the non-lymphocyte component of the infiltrate which is not so readily identifiable in the pancreas using histology alone.

4 Notes

1. The described method can be easily modified for either fixed-frozen or fresh-frozen tissue sections. For fixed sections, wash the sections in TBS 3×5 min to remove any OCT cutting medium and then proceed from **step 4** of the method (incubating with blocking reagent). If using fresh frozen sections, bring them to room temperature and dry any condensation from the slides using tissue paper. Lay the slides down flat and incubate with 1 ml of 10 % formalin per slide for 15 min (in a fume cupboard). Wash the sections three times 5 min with TBS and then proceed from **step 4**.

2. Antigen retrieval is a step which often requires careful optimization before application to your test samples. There are many variations on the buffers and method described in this article. However, the two buffers described have consistently produced robust results and are very commonly employed for this purpose. The exact mechanisms which underlie antigen retrieval are not fully understood, however pH has been shown to be very important and extremes of pH are more effective at un-masking epitopes [11]. Each epitope is optimally un-masked under different conditions, therefore conditions for each primary antibody need to be ascertained as they are unlikely to be the same for each primary antibody being used. There are also a wide range of methods used. This article describes the use of a microwave to heat the buffer and sections, however, the use of a pressure cooker has also been found to be very effective. It is also possible to unmask epitopes enzymatically using commercially available kits. Antigen retrieval is not required for frozen sections as they are not subject to the same intense cross-linking which affects FFPE tissue. Also, some antibodies, including the Dako anti-insulin and anti-glucagon antibodies described above, do not require antigen retrieval, therefore this step can be omitted as it is always preferable to expose your tissue sections to as few processing steps as possible to better preserve their integrity.
3. Human pancreatic tissue retrieved at autopsy can be particularly difficult to work with. This is in part due to the digestive nature of the pancreas which makes it susceptible to degradation by autolysis. Given that it is not possible to retrieve the organ immediately following death, even short ischemia times of a few hours can result in some autolysis. Very pronounced autolysis can lead to reduced labeling of some antigens, although fortunately the islets and also the immune infiltrate are reasonably resistant to autolysis in the short term, likely due to protection provided by the double basement membrane and the fact that the pancreatic ducts do not pass through the islets. Labeling issues caused by mild autolysis can often be resolved by an increased incubation time with primary antibody from 1 h to overnight at 4 °C. However, there is no way to improve the disturbed morphology of any affected cells.
4. The method can also easily be adapted for immunofluorescence staining. Firstly, omit the endogenous peroxidase blocking step. Secondly, replace the HRP-conjugated secondary antibody with a relevant fluorescent probe-conjugated secondary antibody, and finally terminate the procedure at **step 10** after washing off the secondary antibody. Mount the sections using a mounting medium appropriate for immunofluorescent staining, such as VECTASHIELD® (Vector Laboratories) or

ProLong® Gold antifade reagent (Life Technologies) and keep them protected from light. If required, mounting medium can be purchased premixed with a nuclear dye such as DAPI or Hoechst to stain all of the cell nuclei. Both of these stains emit fluorescence in the blue spectrum.

5. While 10 min incubation with Dako DAB reagent is the recommended time for optimal development of most reactions, this time can be modified to suit individual end users. Some reactions develop precipitate rapidly and may only require a 2–3 min incubation time, whereas others may take longer. Too long an incubation in DAB reagent will eventually result in nonspecific background staining of the tissue which might mask any specific immunopositive staining. It is important to determine equilibrium between developing a good visual precipitate and minimizing the degree of background staining. The most important aspect of the DAB incubation time is to keep it consistent between experiments. For example, if an incubation time of 5 min is determined for one sample, then this time point must be used consistently for all other samples so as not to gain false information regarding different staining intensity between specimens.

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