Pancreas Optical Clearing and 3-D Microscopy in Health and Diabetes

Martha Campbell-Thompson1* and Shiue-Cheng Tang2*

1 Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, FL, United States, 2 Department of Medical Science and Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan

Although first described over a hundred years ago, tissue optical clearing is undergoing renewed interest due to numerous advances in optical clearing methods, microscopy systems, and three-dimensional (3-D) image analysis programs. These advances are advantageous for intact mouse tissues or pieces of human tissues because samples sized several millimeters can be studied. Optical clearing methods are particularly useful for studies of the neuroanatomy of the central and peripheral nervous systems and tissue vasculature or lymphatic system. Using examples from solvent- and aqueous-based optical clearing methods, the mouse and human pancreatic structures and networks will be reviewed in 3-D for neuro-insular complexes, parasympathetic ganglia, and adipocyte infiltration as well as lymphatics in diabetes. Optical clearing with multiplex immunofluorescence microscopy provides new opportunities to examine the role of the nervous and circulatory systems in pancreatic and islet functions by defining their neurovascular anatomy in health and diabetes.

Keywords: islet, autonomic (vegetative) nervous system, lightsheet microscopy, CLARITY, adipocyte, Schwann cell, confocal 3-D microscopy, organoid

INTRODUCTION

Goals
Heterogeneity of the human pancreas is well accepted in terms of islet endocrine cell proportions and mass in healthy people and for lobularity in islet beta-cell losses and frequency of infiltrated islets in patients in type 1 diabetes (T1D) (1–7). Patients with type 2 diabetes (T2D) show similar heterogeneity in islet amyloidosis, fatty infiltration, fibrosis and inflammatory infiltrates (4, 8–10). Morphology-based studies of the human pancreas have been key to understanding regional heterogeneity yet examinations of the pancreas in its natural three-dimensional (3-D) configuration have been limited to laborious serial sectioning with subsequent reconstruction. Islets occupy only ~2% of the entire pancreas volume and sampling of multiple blocks is recommended to maximize islet analyzes by 2-D microscopy (11). Recent applications of optical clearing methods to the human and mouse pancreas provide new details for structure-function relationships in health and subsequent abnormalities in diabetes (12–15). This review provides an overview of recent optical clearing methods used in human and mouse pancreas studies and examples of pancreas optical clearing to define several components of the pancreas endocrine and exocrine compartments.
TECHNIQUES

Although basic optical clearing to render tissues transparent was first described by the German anatomist Walter Spalteholz over 100 years ago, the recent decade has seen a rapid growth in advanced clearing methods applicable for whole body or organ imaging to single cell resolution (16, 17). Optical clearing is readily accomplished in a standard laboratory and new procedures can be found nearly weekly in the literature for different organs and species (Table 1). Optical clearing methods are based on obtaining a high degree of tissue transparency and matching of the sample refractive index (RI) to that of the imaging media to remove light scattering (54). Most tissues are comprised of ~80% water (RI=1.33), 10% proteins (RI=1.44), and 10% lipids (RI=1.45) (55). Methods are broadly based on physical or chemical strategies with organic solvents or aqueous solutions used for the latter. Several excellent reviews are available that detail each optical clearing method advantages and disadvantages (45, 56-59).

Early optical clearing methods used organic chemicals [e.g., benzyl alcohol–methyl salicylate, benzyl alcohol–benzyl benzoate (BABB), and solvents used in 3-D imaging of solvent-cleared organs (3DISCO)] (27). Generally these methods achieve high transparency within a few days by removing lipids and homogenizing refractive indices (RIs) of the samples, and they are compatible with whole-mount immunolabeling (48, 60). Solvent-based clearing methods may use toxic chemicals and steps should be performed using a fume hood and suitable personal protective equipment. Recent methods have been named with acronyms such as immunolabeling-enabled imaging of solvent-cleared organs (iDISCO) (60), clear, unobstructed brain/body imaging cocktails and computational analysis (CUBIC) (17, 27, 61, 62), clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue hydrogel (CLARITY), and passive CLARITY technique (PACT) (38, 63-65).

The original CLARITY manuscript by Chung et al. (65) described four key steps: (1) hydrogel tissue embedding using a ratio of 4% acrylamide monomer to 0.05% bis-acrylamide followed by polymerization; (2) clearing secondary to lipid removal using 4% sodium dodecyl sulfate (SDS) detergent buffer within a custom built electrophoretic tissue clearing system (ETC); (3) immunostaining; and (4) tissue RI matching and imaging. Several modifications were subsequently published in favor of "passive clearing" without the use of ETC to avoid oxidative tissue artifacts with several variations in the amount of paraformaldehyde (0-4%), acrylamide monomer (1-4%) and bis-acrylamide in the hydrogel mixture depending on target organ (63, 66, 67).

Despite their differences in chemical and/or optical properties, the organic solvent, aqueous reagent, and electrophoresis-assisted clearing methods all extend our view of neurovascular networks (>100 μm) with 3-D microscopy compared with the images acquired from classic IHC and H&E histology (3-5 μm in thickness). To apply tissue clearing, the key question is whether a specific clearing technique changes the sample chemical and/or cellular environment that causes artifacts in signal detection. For example, in CLARITY, the use of sodium dodecyl sulfate (SDS) treatment and electrophoresis to remove cellular membranes is likely to disturb membrane receptor proteins. Thus, studies of the nerve-receptor association in space will be better accepted if a passive aqueous-based clearing method (e.g., sugar reagent) is employed compared with CLARITY. Likewise, leukocytes and their vascular receptor association in space will be better examined in an aqueous environment, because disturbing the membranes for 3-D imaging may create false negative results in signal detection. However, we need to stress that the false negative result may also come from scattering in deep-tissue imaging. Thus, adding a positive control in the specimen (e.g., nuclear staining) can help investigators monitor the resolving power (e.g., resolving two adjacent nuclei in an islet) across the optical depth in deep-tissue pancreatic and islet imaging.

Volumetric microscopy has also paralleled optical clearing methods with advancements in confocal, multiphoton and lightsheet microscopes. Advantages of the lightsheet microscopes are faster scanning speeds, reduced photobleaching, and good resolution at high tissue penetration depths. When access to lightsheet microscopes is not feasible, confocal microscopy provides an excellent alternative with single-cell resolution. While any type of fluorescent microscope can be used to image optically-cleared tissue, the microscope imaging chamber must be able to accommodate the size of the sample and the stage configuration and imaging depth are dependent on the working distance of the objective. Most images can be obtained with regular air objectives between ×2 and ×20 magnifications. Specialized objectives for optically cleared samples over larger distances are available from multiple vendors such as Olympus and Zeiss. Users of optical clearing methods and advanced microscopy need also consider data storage requirements for both image acquisition and analysis. Additional storage space for the imaging microscope is required as well as additional memory and processing speed for image analysis workstations. The open-source software Fiji/ImageJ can be used for 2-D stitching and basic 3-D adjustments (68). Zeiss Zen software will also provide stitching and maximum intensity projections. Commercial software packages are available for advanced 3-D volume rendering and reconstruction such as Neurolucida360/Vesselucida (MBF), Arivis (FEI), and Imaris (Bitplane, Concord MA). Consideration of sample size and existing microscopes and image analysis software may thus dictate which optical clearing method is most suitable for a given laboratory. For those without sufficient local resources, the growing popularity of optical clearing methods generated several commercial services for clearing, microscopy, and analysis services (Visikol, ClearLight, LifeCanvas).

CHALLENGES

Challenges related to optical clearing are relatively few. Acquisition of high-quality samples is important as for any down-stream application based on fixed samples. Fixation with 4% paraformaldehyde or 10% formalin is commonly employed in many laboratories. Cardiac perfusion is recommended for rodent studies in most part to remove red blood cells and their...
| Species | First Author | Year | Journal | Title                                                                 | Method         | Reference |
|---------|--------------|------|---------|----------------------------------------------------------------------|----------------|-----------|
| Mouse   | Kim          | 2010 | JoVE    | In situ quantification of pancreatic beta-cell mass in mice          | Sucrose        | (18)      |
| Mouse   | Fu           | 2010 | Gastroenterology | At the movies: 3-dimensional technology and gastrointestinal histology | FocusClear     | (19)      |
| Mouse   | Fu           | 2010 | Journal of Biomedical Optics | Three-dimensional optical method for integrated visualization of mouse islet microstructure and vascular network with subcellular-level resolution | FocusClear     | (20)      |
| Rat     | Li           | 2010 | J Cell Science | Activation of pancreatic-duct-derived progenitor cells during pancreas regeneration in adult rats | BABB           | (21)      |
| Mouse   | Chiu         | 2012 | Diabetologia | 3-D imaging and illustration of the perfusive mouse islet sympathetic innervation and its remodeling in injury | FocusClear     | (22)      |
| Mouse   | Tang         | 2013 | Diabetologia | Plasticity of Schwann cells and pericytes in response to islet injury in mice | FocusClear     | (23)      |
| Mouse   | Jiang        | 2014 | AJP     | Three-dimensional islet graft histology: panoramic imaging of neural plasticity in sympathetic reinnervation of transplanted islets under the kidney capsule | FocusClear     | (24)      |
| Mouse   | Tang         | 2014 | Diabetes, Obesity and Metabolism | Imaging of the islet neural network | FocusClear     | (25)      |
| Mouse   | Lee          | 2014 | BMC Developmental Biology | Improved application of the electrophotonic tissue clearing technology, CLARITY, to intact solid organs including brain, pancreas, liver, kidney, lung, and intestine | CLARITY        | (26)      |
| Mouse   | Susako       | 2015 | Nature Protocols | Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging 3-D imaging reveals participation of donor islet Schwann cells and pericytes in islet transplantation and graft neurovascular regeneration | CUBIC/FocusClear/RapiClear | (27)      |
| Mouse   | Jiang        | 2015 | EBioMedicine | Whole-body tissue stabilization and selective extractions via tissue-hydrogel hybrids for high-resolution intact circuit mapping and phenotyping | PACT           | (28)      |
| Human   | Treweek      | 2015 | Nature Protocols | Whole-body tissue stabilization and selective extractions via tissue-hydrogel hybrids for high-resolution intact circuit mapping and phenotyping | FocusClear     | (29)      |
| Mouse   | Chien        | 2016 | International Journal of Obesity | 3-D imaging of islets in obesity: formation of the islet-duct complex and neurovascular remodeling in young hyperphagic mice | RapiClear      | (30)      |
| Mouse   | Lin          | 2016 | AJP     | PannIN-associated pericyte, glial, and islet remodeling in mice revealed by 3-D pancreatic duct lesion histology | RapiClear      | (31)      |
| Mouse   | Simon        | 2017 | J Autoimmunity | Inhibition of effector antigen-specific T cells by intradermal administration of heme oxygenase-1 inducers | 3DISCO         | (32)      |
| Mouse   | Vlahos       | 2017 | PNAS    | Modular tissue engineering for the vascularization of subcutaneously transplanted pancreatic islets | RapiClear      | (33)      |
| Mouse   | Wong         | 2017 | Current Protocols Cell Biology | Simple and Rapid Tissue Clearing Method for Three-Dimensional Histology of the Pancreas | CLARITY        | (34)      |
| Mouse   | Yamamoto     | 2017 | Nat Comm | Neuronal signals regulate obesity induced β-cell proliferation by FoxM1 dependent mechanism | CUBIC          | (35)      |
| Mouse   | Pauerstein   | 2017 | Development | A radial axis defined by semaphorin-to-neuropilin signaling controls pancreatic islet morphogenesis | CLARITY        | (36)      |
| Mouse   | Chen         | 2017 | Scientific Reports | UbasM: An effective balanced optical clearing method for intact biomedical imaging | UbasM          | (37)      |
| Mouse, Human | Chen        | 2017 | Nature Protocols | Pathways to clinical CLARITY | CLARITY        | (38)      |
| Mouse   | Tang         | 2018 | Diabetologia | Pancreatic neuro-insular network in young mice revealed by 3-D pancreatic histology | RapiClear      | (39)      |
| Mouse   | Nishimura    | 2018 | Islets | Optical clearing of the pancreas for visualization of mature b-cells and vessels in mice | Sca/eS         | (40)      |
| Human   | Nold         | 2018 | American Journal of Pathology | Immunolabeling of Cleared Human Pancreata Provides Insights into Three-Dimensional Pancreatic Anatomy and Pathology | IDISCO        | (41)      |
| Human   | Tang         | 2018 | Diabetologia | Human pancreatic neuro-insular network in health and fatty infiltration | RapiClear      | (42)      |
| Human   | Tang         | 2018 | Current Diabetes | The role of accessory cells in islet homeostasis | RapiClear, PACT | (43)      |
| Human   | Fowler       | 2018 | Endocrinology | Three-Dimensional Analysis of the Human Pancreas | T3             | (44)      |
| Human   | Butterworth  | 2018 | JoVE    | High resolution 3D imaging of the Human Pancreas Neuro-insular network | PACT           | (45)      |
| Human   | Shen         | 2019 | EBioMedicine | Lymphatic vessel remodeling and invasion in pancreatic cancer progression | RapiClear      | (46)      |
| Mouse   | Chien        | 2019 | AJP     | Human pancreatic afferent and efferent nerves: mapping and 3-D illustration of exocrine, endocrine, and adipose innervation | RapiClear      | (47)      |
| Human   | Dybala       | 2019 | Diabetes | Heterogeneity Human Pancreatic Islet | T3             | (48)      |
| Human   | Hong         | 2019 | Advances in Anatomic Pathology | A “Clearer” View of Pancreatic Pathology: A Review of Tissue Clearing and Advanced Microscopy Techniques | IDISCO        | (49)      |
| Mouse   | Tokumoto     | 2020 | Diabetes | Generation and Characterization of a Novel Mouse Model That Allows Spatiotemporal Quantification of Pancreatic ß-Cell Proliferation | CUBIC          | (50)      |
| Mouse   | Hahn         | 2020 | Communications Biology | Topologically selective islet vulnerability and self-sustained downregulation of markers for ß-cell maturity in streptozotocin-induced diabetes | BABB           | (51)      |
| Mouse   | Maldonado    | 2020 | Stem Cells Tissue Repair | Painting the Pancreas in Three Dimensions: Whole-Mount Immunofluorescence | BABB           | (52)      |

(Continued)
inherent high autofluorescence. However, immersion fixation is a reasonable alternative for rodents and the only method available for human biosamples. As for traditional immunolocalization, the duration of fixation is ideally kept to a minimum to avoid over-fixation of tissue antigens. Many primary antibodies utilized for formalin-fixed paraffin embedded samples work well for optical clearing and those tested in our laboratory are provided as a reference (Table 2). A pre-testing step is advised for new primary antibodies and can be accomplished with fixed frozen thick sections (40µm) utilizing similar conditions as those for

**TABLE 2 | Primary antibodies for optical clearing.**

| Antigen | Cell type | Host | Vendor | Cat. # | Dilution | Comments |
|---------|-----------|------|--------|--------|----------|----------|
| **Endocrine Markers** | | | | | | |
| Glucagon | Alpha-cells | Mouse | BD Biosciences | 565891 | 1:50 | Worked |
| Glucagon | Alpha-cells | Rabbit | Cell Signaling | 2760S | 1:200 | Did not work |
| Glucagon | Alpha-cells | Mouse | Abcam | ab10988 | 1:200 | Worked |
| Insulin | Beta-cells | Guinea Pig | DAKO | A0564 | 1:200 | Worked |
| Secretogranin 3 | Neuroendocrine cells | Rabbit | Sigma | HP008880 | 1:200 | Worked |
| Somatostatin | Delta-cells | Goat | Santa Cruz | sc-7819 | 1:500 | Worked |
| **Neural Markers** | | | | | | |
| GFAP | Glial cells | Rabbit | DAKO | M730429-2 (also FITC-conjugate) | 1:50 | Did not work (both) |
| NCAM (CD56) | Pan-neural | Mouse | DAKO | | 1:50 | Did not work (both) |
| Peripherin | Pan-neural | Rabbit | EnCor | PCA-Peri | 1:200 | Worked |
| POPO9.5/UCHL1 | Pan-neural | Rabbit | DAKO | Z5116 | 1:50 | Did not work |
| POPO9.5/UCHL1 | Pan-neural | Chicken | EnCor | CPC0-UCHL1 | 1:100 | Worked |
| POPO9.5/UCHL1 | Pan-neural | Rabbit | Abcam | Ab103986 | 1:200 | Worked |
| b-Tubulin | Pan-neural | Mouse | EnCor | MCA-4E4 | 1:100 | Worked |
| Substance P | Sensory nerves | Rat | BioRad | 8450-0505 | 1:200 | Worked |
| Tyrosine Hydroxylase | Sympathetic neurons | Rabbit | Millipore | AB152 | 1:200 | Worked |
| Tyrosine Hydroxylase | Sympathetic neurons | Chicken | Abcam | Ab76442 | 1:50 | Worked, weak staining |
| Vasoactive Intestinal Peptide | Autonomic neurons | Rabbit | Immunostar | 20077 | 1:200 | Worked |
| Vascular Markers | | | | | | |
| CD31 (PECAM) | Endothelial cells | Rabbit | Abcam | Ab28384 | 1:30 | Worked |
| CD31 (PECAM) | Endothelial cells | Mouse | ThermoFisher | MS-353-S1 | 1:50 | Worked, weak staining |
| CD34 | Endothelial cells | Mouse | ThermoFisher | MA1-10202 | 1:50 | Did not work |
| Collagen IV | Basal lamina (blood vessels) | Mouse | Abcam | ab6311 | 1:200 | Did not work |
| Collagen IV | Basal lamina (blood vessels) | Rabbit | Abcam | ab6586 | 1:200 | Worked |
| Smooth muscle actin | Smooth muscle (arteries) | Mouse | Sigma | A5228; C6198 (Cy5) | 1:200; 1:200 | Worked; Conjugated better |

Primary antibodies tested for immunolocalization in human pancreas samples cleared by passive CLARITY (PACT) are shown by major headings for endocrine markers, neural markers, and vasculature. Comments include whether successful immunostaining was achieved. These antibodies are expected to work using similar clearing methods and may also work in other species as indicated by the vendor or literature.
permeabilization before immunostaining and optical clearing. Wide applicability of primary antibodies remains an issue, particularly for immune markers. While some methods have successfully employed preconjugated primary antibodies (43), the majority of optical clearing methods employ standard rounds of primary antibodies followed by secondary antibodies to promote antibody penetration. A reported benefit of CLARITY was the ability to reiteratively strip antibodies and reprobe a sample several times (65). In practice, we have been unsuccessful in fully stripping samples from human pancreas cleared using CLARITY and also found limited antigenicity and/or diffusion of subsequent primary antibodies (Campbell-Thompson, unpublished results). As such, our more recent studies employ single clearing methods with multiplex immunostaining on 500µm sections rather than several mm sized pieces to extend use of a given sample, particularly those from rare organ donors with diabetes.

Equipment utilized in optical clearing is generally found in any modern molecular pathology laboratory and include access to fume hoods, refrigeration or ovens, rocker plates, and other ancillary small equipment for immunostaining and clearing steps. Microscope and image analysis software are two aspects to be considered before conducting clearing studies as this will influence sample size and numbers of channels for multiplex staining and analysis. As costs for multiphoton and lightsheet microscopes decrease, access to these microscopes will increase whether through institutional shared resources, subcontract, or collaboration. Image analysis expertise is also limited and laboratory staff become proficient in the software available to them. Expense of commercial 3-D software analysis programs is high. Finally, the greatest hurdle may be the large image file sizes achieved by these methods. Here too, limiting the number of antigens needs to be balanced with the rarity of the sample since reiterative staining is difficult.

**OPTICAL CLEARING EXAMPLES**

This introduction on optical clearing and imaging provides a basic starting point for studies on human and mouse pancreas in health and diabetes. We will now show examples of application of different clearing methods to demonstrate the versatility of optical clearing in human and mouse pancreas for determination of normal states and changes found with diabetes.

**SAMPLE PROCESSING**

Human pancreata not suitable for clinical purposes were collected from nondiabetic, brain-dead organ donors after written informed consent from legal representative or next of kin and were processed by the Network for Pancreatic Organ donors with Diabetes (nPOD) program at the University of Florida (UF) Diabetes Institute using methods previously reported (69). The nPOD samples used in this specific study were approved as nonhuman by the UF IRB (IRB201902530). Collection and use of human pancreatectomy specimens were approved by the Institutional Review Board of National Taiwan University Hospital (201703131RIND). All UF animal studies were conducted using published guidelines and regulations of the National Institutes of Health for the care and use of laboratory animals. The protocol was approved by the UF Institutional Animal Care and Use Committee (IACUC 202009976). All animal studies conducted at the National Tsing Hua University were reviewed and approved by the institutional animal use review board.

**Pancreatic Islet Schwann Cells**

Passive CLARITY (PACT) provides for good tissue transparency and multiplex immunolabeling is quite feasible with image analysis such as using the open software Neurite tracer program in ImageJ (Figure 1) (15). Schwann cells are the peripheral counterpart to central nervous system oligodendrocytes and provide support to both myelinated and unmyelinated axons of motor and sensory neurons (70, 71). In addition to providing physical support, nonmyelinating Schwann cells are essential for maintenance and regeneration of damaged axons by production of neurotrophins and acting as "first responders" to injury (72, 73). Unlike the dense mesh-like network formed by islet Schwann cells in mouse islets, Schwann cells provide support for autonomic

![Figure 1](image-url) | Optical clearing of human pancreas by PACT and iDISCO. (A) Fixed pancreas sample from a control donor before and after clearing using passive CLARITY (PACT) showing the degree of sample transparency achieved with this method. (B) Representative example of 2-photon imaging for a 0.8 mm x 0.8 mm x 0.25 mm region (X, Y, Z axes) containing an islet immunostained for glucagon (red) and glial fibrillary acidic protein (GFAP, green). (C) GFAP-stained Schwann cells overlay an islet and cell projects were analyzed using the ImageJ neurite tracer program. (D) The traced Schwann cells are shown by Neurite tracer skeleton diagram.
nerves in human islets in a loose formation (Figure 2) (70). Schwann cells are also of particular interest in T1D as they have a role in antigen presentation, interact with the complement system, and secrete factors involved in immune interactions (74, 75) and animal studies report reactive Schwann cells in diabetes and islet injury (23, 76–78). Furthermore, glial fibrillary acidic protein (GFAP) is expressed in peri-islet Schwann cells and is reported to be an autoantigen for T1D with potential use as a biomarker (79).

**Pancreatic Ganglia and Neuroinsular Complexes**

Intrapancreatic ganglia represent the post-ganglionic neurons of the parasympathetic efferent network (80). They are widely distributed and in relatively low density throughout the human and rodent pancreas and thus optical clearing and 3-D imaging provides a greater opportunity to detect these ganglia (13). At low magnifications, interconnections of intrapancreatic ganglia and to islets are visualized in a human pancreas cleared using

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**FIGURE 2 | Mouse and human islet Schwann cells.** (A) A fixed frozen section (40µm) from a C57BL/6 mouse pancreas was stained for GFAP (green) using whole mount staining. A single 2-D slice (7th of 13 slices) and the 3-D maximum intensity projection (MIP, 12 µm stack) are shown to demonstrate the increase in cellular information obtained with a z-stack. (B) A human control pancreas sample (~1 mm3) was cleared by iDISCO and immunolabeling with glucagon (red) and GFAP (green) before confocal 3-D imaging (maximum intensity projection 50 µm). (C) The region identified by white box in (A) shows Schwann cells at the periphery of the islet that extended along nerves to islet interiors traveling along afferent vessels. (D) A single Schwann cell shows a clear nuclear region (white arrow) and numerous extensions with a termination at an alpha-cell (asterisk). See also Supplementary Video 1 for (A).
PACT (15) (Figures 3A, B). Such interconnections likely contribute to the synchronization of islet hormone secretions, particularly during the cephalic phase of digestion (81). The intrapancreatic ganglia vary in numbers of neurons and also observed are small clusters of neurons (Figure 3C) or small clusters of neurons with islet β-cells and α-cells can also be observed, so-called neuroinsular complex type II (Figure 3D) (82). These type II structures have not been previously reported in adult human pancreas and were found only in fetal pancreas. The lack of detection in adults could be due to limitations of 2-D microscopy in finding small structures compared to 3-D microscopy as demonstrated here (83).

Pancreatic Vasculature
The pancreatic vasculature in health and diabetes has been studied with newer studies showing detection of the Sars-CoV-2 receptor, ACE2, in pancreatic microvasculature, rather than islet endocrine cells, adding additional importance to understanding factors regulating islet blood flow in health and diabetes (84–87). The use of optical clearing provides an unprecedented opportunity to better examine structural-functional relationships of the islet microvasculature in the context of islet heterogeneity and inter-relationship to the surrounding acinar cells. Studies performed in rodents can be achieved by perfusion with fluorescent compounds including lectins or conjugated acinar cells. Studies performed in human samples, the vasculature can be readily labeled using CD31 or CD34 followed by multiplex immunofluorescence with islet endocrine cell markers and the high vascular density can be appreciated throughout 3-D microscopy (Figure 4) (53).

Pancreatic Acinar Ductal Metaplasia
Pancreatic cancer is one of the deadliest tumors and seminal studies showed that early lesions likely arise from acinar-ductal metaplasia forming so-called pancreatic intraductal neoplasia (PanIN) (88). Optical clearing studies in human and mice have shown characteristics of ductal lesions through 3-D imaging (31, 50). Cell culture models of human pancreatic cancer can also benefit from use of optical clearing and 3-D microscopy. Single cell details are apparent in an in vitro human primary acinar culture model showing duct formation following optical clearing using HISTO-M, a commercial product similar to DISCO clearing (Figure 5) (VisiKol) (T. Schmittgen, personal communication) (89).

Pancreatic Fatty Infiltration
Unlike the fatty liver, in which lipid droplets accumulate in the cytoplasm in the hepatocytes, pancreatic fatty infiltration involves the fat cells (adipocytes) ectopically developing and accumulating in and around the pancreatic lobules alongside the exocrine and endocrine tissues. The fat content in the pancreas increases with age (90) and is detectable as early as in adolescence (91), and the degree is linked with obesity (92–94). In the progression from obesity to type 2 diabetes, the state of hyperinsulinemia is likely to accelerate the pancreatic fat accumulation due to the organ’s high insulin concentration. Insulin is a potent factor to induce adipogenesis, in which preadipocytes (e.g., fibroblasts and myofibroblasts in the pancreatic stroma) differentiate into adipocytes (95, 96), and stimulate the proliferation of adipocytes (97). Thus, it is not surprising that multiple studies documented the correlation between type 2 diabetes and pancreatic fats and implicated the negative influence of these fats on the islet microenvironment and function (98–100).

Clinically, magnetic resonance imaging (MRI) and computed tomography (CT) are the preferred imaging modalities to detect...
and quantify the pancreatic fats (90–92, 94, 98–102). While these two methods provide valuable in vivo information for cross-sectional or longitudinal studies, they cannot resolve the cellular structures of fats and the pancreatic exocrine and endocrine tissues. At the cellular level, the classic microtome-based histology with H&E staining can identify the adipocytes and their association with blood vessels, acini, ducts, and islets with µm-level resolution. However, due to the hydrophobicity of fats and their weak mechanical connection with the pancreatic lobules, both microtome slicing and dewaxing (xylene wash) of the paraffin-embedded pancreas may create artifacts on the locations of adipocytes, affecting the analysis of the peri- and/or intra-lobular adipocyte association.

Modern 3-D histology with aqueous-based optical clearing alleviates the abovementioned technical concern by maintaining the native hydrophilicity of the tissue and avoiding the microtome slicing in sample preparation (42). This is particularly important in examination of the type 2 diabetic pancreas and the surgical
biopsy of pancreatic cancer [patient may have developed type 3c diabetes (103, 104)]. In both situations, investigators will likely encounter moderate-to-severe fatty infiltration, in which adipocytes generally or locally become a component of the pancreas (Figure 6). To investigate the pancreas in this condition, we advise careful comparison between the modern 3-D and the classic 2-D tissue images to confirm the adipocytes in and around the remodeled pancreatic lobules to avoid misrepresentation of the disease condition.

**Pancreatic Lymphatic Network**

The lymphatic drainage of pancreas is achieved by an intricate network of lymphatic vessels and nodes, in which the immune cells reside. The open-ended lymphatic network collects the

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**FIGURE 6** | Human pancreatic fatty infiltration. Images were derived from tile scanning of optically cleared pancreatic specimens. (A, B) Normal lobule of human pancreas. Adipocytes are clearly seen around the blood vessel and inside the lobule (magnified, arrows). Green, nuclear staining; red, CD31. (C, D) Acinar atrophy of diseased lobule. This view was acquired 2-cm distal to the pancreatic ductal adenocarcinoma. Overlay of transmitted light and fluorescence signals identifies the fatty infiltration.
interstitial fluids for water and lipid absorption and recycling (which balances the tissue osmotic pressure) and for immune surveillance (105). When pancreatic injury or disease occurs, the lymphatic system plays a central role in reaction to the exocrine [pancreatitis (44, 106)] and endocrine tissue inflammation. For example, in the nonobese diabetic (NOD) mice, the progression of type 1 diabetes features the migration of T lymphocytes from the circulatory system to the islet, attacking the β-cells (107, 108). In the process, the microtome-based 2-D histology has been used to evaluate the degree of islet inflammation, in which early,
moderate, and severe insulitis are assigned to evaluate the progression of the disease (109). However, due to the dispersed nature of lymphatic vessels, the classic 2-D histology cannot provide a global assessment of lymphatic endothelial remodeling in response to insulitis. To understand the associated lymphatic and immune response to insulitis, panoramic and in-depth imaging of the pancreas is needed to characterize the lymphatic network and T-cell migration in a global and integrated fashion.

As can be seen in Figure 7, the optically cleared NOD mouse pancreas provides an experimental setting to investigate the lymphatic and T-lymphocyte association in insulitis. Using the panoramic image (Figure 7A), we can detect the CD3+ T lymphocytes in the lymph node (positive control) and around the islets. The latter provides a clear target for examination of the islet under immune attack, featuring vasodilation and the packing of T lymphocytes in the lymphatic vessels (Figures 7B, C). Overall, the quadruple signals of tissue microstructure (nuclear staining), vasculature (blood and lymphatic vessels), and CD3+ T lymphocytes in the transparent pancreas provide an optimized condition to visualize the islet vascular remodeling and immune attack in insulitis. They also demonstrate the different scales of tissue information (from interlobular to subcellular features of islets under immune attack) that can be acquired from optically cleared pancreas samples to investigate experimental insulitis.

CONCLUSIONS

Both standard and modified optical clearing methods are well suited for studies of 3-D structure-function relationships for human and mouse pancreas and use readily available chemicals and imaging equipment. These methods are particularly advantageous for studies of diabetes due to known islet heterogeneity requiring examination of numerous islets and pancreas regions. Optical clearing methods can also be used in investigations of pancreatic cancer using patient or rodent samples or in vitro experiments examining acinar-ductal metaplasia. Advances in understanding failure of islet beta-cells in diabetes requires a wholistic examination of islets in the native environment as afforded by optical clearing and new findings are anticipated related to the role of the nervous, immune, and vascular systems in beta-cell biology from such studies.

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AUTHOR CONTRIBUTIONS

MC-T and S-CT designed the studies, performed experiments, prepared figures, and edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.644826/full#supplementary-material
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