Three-dimensional optical method for integrated visualization of mouse islet microstructure and vascular network with subcellular-level resolution

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Abstract. Microscopic visualization of islets of Langerhans under normal and diabetic conditions is essential for understanding the pathophysiology of the disease. The intrinsic opacity of pancreata, however, limits optical accessibility for high-resolution light microscopy of islets in situ. Because the standard microtome-based, 2-D tissue analysis confines visualization of the islet architecture at a specific cut plane, 3-D representation of image data is preferable for islet assessment. We applied optical clearing to minimize the random light scattering in the mouse pancreatic tissue. The optical-cleared pancreas allowed penetrative, 3-D microscopic imaging of the islet microstructure and vasculature. Specifically, the islet vasculature was revealed by vessel painting—lipophilic dye labeling of blood vessels—for confocal microscopy. The voxel-based confocal micrographs were digitally processed with projection algorithms for 3-D visualization. Unlike the microtome-based tissue imaging, this optical method for penetrative imaging of mouse islets yielded clear, continuous optical sections for an integrated visualization of the islet microstructure and vasculature with subcellular-level resolution. We thus provide a useful imaging approach to change our conventional planar view of the islet structure into a 3-D panorama for better understanding of the islet physiology. © 2010 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3470241]

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1 Introduction

The islets of Langerhans are small endocrine organs scattered in the pancreas that are crucial for maintaining the glucose homeostasis. The spherical- or ovoid-shaped islet consists of four major secretory cells of insulin, glucagon, somatostatin, and pancreatic polypeptide, i.e., β, α, δ, and F cells, respectively. The balance of the four cell types and the release of the metabolic peptides are tightly regulated by a committee of paracrine, endocrine, and neuronal cues, which poses a challenging task of analyzing the islet physiology as well as its critical disorder—diabetes mellitus.1,2 Anatomically, the endocrine islets are surrounded by the exocrine acini and constantly receive paracrine signals from the neighboring acinar cells. In addition, islets are densely vascularized, receiving 5 to 15% of the pancreatic blood supply, despite contributing only approximately 1% to the pancreatic volume.3,4 The structural and functional connections between the islet endocrine cells and vascular endothelial cells are important to provide oxygen and nutrients to the endocrine cells and allow rapid transport of endocrine hor-

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mes such as insulin to the circulation for regulation of blood glucose levels.

Although there is an intensive need to characterize the islet microstructure and vasculature in an integrated fashion, visualization of the pancreatic islet and its vascular network is limited by the spatial resolution of the imaging tools. In routine laboratory practice, the microtome-based imaging method allows μm-level resolution but confines the view at a specific cut plane. In addition, the disconnection between sectioned tissues as well as the distortions and artifacts caused by microtome slicing prevent an integral, 3-D visualization of the islet in situ.

For transparent tissues such as the retina, where light can pass through with minimal scattering, optical imaging such as confocal microscopy provides a useful tool to study the 3-D tissue structure with appropriate fluorescent labeling. Unfortunately, pancreatic tissues are nontransparent [Fig. 1(a)], which seriously limits their optical accessibility. Recent progress in optical clearing of tissues and cells,5–11 however, sheds light on deep-tissue microscopy by improving the tissue match between the tissue constituents immersed in optical-clearing solution to reduce refractive mismatch between the tissue constituents (refractive index at ~1.46) and fluids (water has a refractive index at 1.33). This avoids random scattering when light moves between media, therefore facilitating laser penetration and fluorescence detection in confocal microscopy.17 In this research, we integrated optical clearing with confocal microscopy to achieve in situ, 3-D imaging of mouse pancreatic islets with subcellular-level resolution. Using this imaging approach, for the first time we were able to apply vessel painting (lipophilic dye labeling of blood vessels)16,17 to visualize the islet/pancreatic vasculature network with high definition, which is critically missing in previous studies of optical imaging of islets.20,21 Panoramas of the mouse islet and its tissue network and our approach to acquire the 3-D images are presented and discussed.

2 Materials and Methods

2.1 Animals

Pancreata from the female wild-type BALB/c mice (Bio-LASCO, Taipei, Taiwan) were used in insulin staining and tests of optical clearing. The tissues were also used in membrane and nuclear staining to examine the islet/pancreatic microstructure. Pancreata from the nestin–green fluorescent protein (GFP) transgenic mice were used in vessel painting to examine the islet microvasculature. In these transgenic animals, the GFP expression is under the control of the 5.8-kb protomer and the 1.8-kb second intron of the nestin gene, which encodes a type VI intermediate filament protein.22 Particularly, in the pancreas, this mouse line shows strong GFP

Fig. 1 (a) and (b) Optical clearing improves the transparency of the mouse pancreas. The specimens were immersed in phosphate-buffered saline (PBS) (a) or FocusClear solution (b) prior to being imaged by the transmitted light channel of confocal microscopy. (c) and (d) Gross images of islets of Langerhans (revealed by insulin immunostaining) and vasculature (revealed by vessel painting), respectively, in the optical-cleared pancreas. (e) to (j) Reversibility test of the optical clearing effect. The intensity of transmitted light is presented in gray scale. In (e) to (g) and (h) to (j), the immersion solution was changed from FocusClear to PBS and from PBS to FocusClear, respectively, FC: FocusClear. The dotted circle indicates the location of the islet. (k) Kinetics of the optical clearing effect and the reverse of optical clearing. The intensity of transmitted light was measured over time. A similar trend was seen in all three rounds of optical clearing. The letters e to j in the upper panel of (k) correspond to the grayscale micrographs of (e) to (j). The volume ratio of tissue/FocusClear is ~1/50. In (a) to (e), bar=100 μm.
expression in the exocrine acinar cells and, minimal, if any, GFP expression in the endocrine islet cells. Overall, 15 and 7 wild-type BALB/c (36 islets) and nestin-GFP (32 islets) mice were used, respectively, to generate representative images. The care of the animals was consistent with Guidelines for Animal Experiments, National Tsing Hua University, Taiwan.

2.2 Sample Preparation

Mouse pancreata were fixed by paraformaldehyde (4%) perfusion alone (used in BALB/c mice) or vessel painting of lipophilic dye DiD (4-chlorobenze sulfonate salt, purchased from Invitrogen, Carlsbad, California, used in the nestin-GFP mice)\textsuperscript{18,19} followed by paraformaldehyde perfusion prior to being harvested for examination. Once harvested, the tissues were fixed in paraformaldehyde (4%) for an additional 1 h. Mouse insulin staining was achieved by incubating the specimen with the primary rabbit-anti-mouse insulin antibody (1:50, Cell Signaling, Danvers, Massachusetts) and then revealing with Alexa Fluor 488-conjugated goat-anti-rabbit IgG (1:200, Invitrogen). Propidiod iodide staining (PI staining, 25 µg/ml, Invitrogen) was performed at room temperature for 90 min to label the nuclei. DiD staining of membranes (2 µg/ml) was performed at room temperature overnight. Specimens from the DiD-perfused nestin-GFP mice (vessel painting) were stained with PI only. The labeled specimens were immersed in FocusClear solution (Cell Explorer, Hsinchu, Taiwan) for optical clearing before being imaged via confocal microscopy.

2.3 Imaging Settings

Confocal imaging of the optical-cleared specimens was performed with a Zeiss LSM 510 confocal microscope equipped with a 25× objective LD Plan-Apochromat glycerine immersion lens or 40× objective LD C-Apochromat water immersion lens. A 10× objective Fluor was used to acquire gross images. The PI- and DiD-labeled samples were excited with helium/neon lasers at 543 nm and 633 nm, respectively. A 488-nm argon laser and a bandpass 505- to 530-nm filter were used to collect signals from PI and DiD, respectively. A 560-to 615-nm filter and a long-pass 650-nm filter were used to collect signals from PI and DiD, respectively. Two islets were imaged in the scanned volume at depths of ~180 µm and ~270 µm (MPEG 4.9 MB). (Color online only.)

Video 1 Complete serial optical sections of the mouse pancreas shown in Figs. 2(a)–2(e) (depth=330 µm). The specimen was stained by DiD and propidium iodide to reveal the cellular membranes (green) and nuclei (red), respectively. Two islets were imaged in the scanned volume at depths of ~180 µm and ~270 µm (MPEG 4.9 MB). (Color online only.)

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 scans, and the value ranged from 0 to 4095 (12-bit data) for the transmitted light imaging and from 0 to 255 (8-bit data) for the rest of the confocal imaging.

2.4 Post-Recording Image Processing and Projection

The voxel-based confocal micrographs were processed with the use of the LSM 510 software (Version 3.2, Carl Zeiss) and the Amira 4.1.2 (Mercury, Chelmsford, Massachusetts) for projection and analysis. Typically, for a three-channel confocal microscopy (such as to collect signals from GFP, PI, and DiD), the size of the image data was ~630 megabytes (1024×1024 pixels×200 slices×3 channels of signals) for an imaging depth of 300 µm. The LSM image data were uploaded into Amira, which was operated under a Dell Precision T5400 workstation. We applied the Gaussian Filter function of Amira for noise reduction of the micrographs. In Videos 1, 2, and 5 the image stacks were displayed using the Ortho Slice function, and the video was made via the Movie Maker function with an increase in display time in association with the depth of the optical section. In Videos 2 to 4, 6, and 7, the Voltex module of Amira was used to project the image stacks. In addition, the Volume Editing function was used to subtract a cuboid(s) from the scanned volume to expose the interior domain of the specimen for visualization. In video preparation, the Demo Maker function was used to arrange the sequence of the image objects at different time intervals. The Camera Path function was used to adjust the projection angles and zoom-in and zoom-out movements of the 3-D images.
3 Results

3.1 Optical Clearing of the Mouse Pancreas

We applied an optical-clearing solution FocusClear (U.S. Patent No. 6472216) to facilitate photon penetration across the mouse pancreatic tissue. The optical-clearing effect allowed the opaque specimen [Fig. 1(a), in saline] to become transparent when it was immersed in the FocusClear solution [Fig. 1(b)]. In the transparent pancreas, we were able to visualize islets [Fig. 1(c), insulin immunostaining] and the vascular structure [Fig. 1(d), details described later] in situ. Traditionally, pancreata are sectioned into thin slices (e.g., 10 μm in thickness) to expose their interior domain for microscopic examination. Here, the image area can be as large as 1000 μm (x) × 1000 μm (y) × 400 μm (z, depth). The thickness of the specimen can be in the 800-μm range, given that separate visuals can be made from both sides of the specimen.

A significant feature of the FocusClear-mediated optical clearing is its reversibility. Figure 1(e) shows an islet residing in the transparent pancreas, which was acquired using the transmitted light channel of confocal microscopy. When we replaced FocusClear with saline [Figs. 1(e)–1(g)] and then repeated the optical-clearing immersion [Figs. 1(h)–1(j)], the optical-clearing effect was reproduced [Figs. 1(e)–1(j)]. We measured the kinetics of this effect: Figure 1(k) shows a similar trend in all three tests, confirming the reversibility of the FocusClear-mediated optical clearing. Note that in sample preparation, paraformaldehyde was used to fix the specimens prior to the FocusClear immersion. Importantly, the size of the islet in the paraformaldehyde-fixed specimen remained the same, while the tissue optics changed during the test [Figs. 1(e)–1(j)]—this is essential with the use of a new optical method for tissue examination. However, we would like to stress the finding by Goldstein et al. that upon comparison of the freshly excised and the formalin-treated specimens, there was shrinkage after fixation. Thus, there are still disparate observations in the size of tissue structures between in vivo and in vitro measurements.

3.2 Penetrative Confocal Imaging of Islets of Langerhans in the Mouse Pancreas

Taking advantage of the transparent specimen, we next applied deep-tissue confocal microscopy to examine the fluorescent-dye-labeled structure. Figures 2(a)–2(e) are examples of the confocal micrographs at different depths in the specimen, where the nuclei and membranes were revealed by propidium iodide (PI) and DiD (4-chlorobenzene sulfonate salt) staining, respectively. The complete serial optical sections, from the surface to depth=330 μm, are shown in Video 1, which reveals two islets in the imaged region. Because the two islets are embedded in the exocrine acini, we used the Amira image software to digitally subtract two cuboids from the scanned volume to expose their locations [Figs. 2(f)–2(h)].

In a second example, Figs. 2(i)–2(k), we used a higher magnification to examine the pancreatic islet structure. Video 2 uses stereo projection to display continuous orthogonal views of the imaged islet. Because there were no physical sections involved in sample preparation, we can digitally quantify the size of the islet by gathering the islet signals based on their locations in the micrograph and calculating the occupied voxels. For example, the size of the scanned volume shown in Figs. 2(i)–2(k) is $175 \times 175 \times 122$ μm$^3$, and the size of each voxel is $0.17 \times 0.17 \times 1.5$ μm$^3$. By calculating the voxels occupied by the islet [Fig. 2(k)], we estimate that the volume of the islet is $7.1 \times 10^7$ μm$^3$ in the specimen. A
its vascular network. In high resolution, 3-D projections of an embedded islet in connection with animals. Previously, this method has been used to visualize of lipophilic dye to label blood vessels in small experimental Vessel painting is a direct staining method that uses perfusion

3.3 High-Resolution, 3-D Imaging of Islet Vasculature in the Mouse Pancreas

Vessel painting is a direct staining method that uses perfusion of lipophilic dye to label blood vessels in small experimental animals. Previously, this method has been used to visualize the vasculature of the retina, skin, lung, heart, and brain in mice, but with limited imaging depth.18 19 Here, we tested the compatibility of vessel painting with our optical method to visualize the mouse islet/pancreatic vasculature in a 3-D fashion. Figures 3(a)–3(c) show that the mouse tissues, including the pancreas, turned blue after perfusion with the lipophilic dye DiD. The labeled pancreatic tissue was then optically cleared by the FocusClear solution and underwent confocal microscopy for deep-tissue imaging. This approach led to a clear visualization of the vascular network, including the capillaries in the islet. Figures 3(d)–3(f) are the gross views of the labeled pancreatic tissue, where there are three embedded islets, residing at 50 to 200 μm under the tissue surface. In Fig. 3(e), we used the Amira software to digitally subtract a cuboid of signals from acini to reveal the vasculature inside. In Fig. 3(f), we subtracted the vasculature signals on top of the cuboid (from 0 to 80 μm in depth) to reveal the locations of the three islets. Stereo projections of Figs. 3(d)–3(f) are shown in Video 4.

We next zoomed in to visualize the islet vasculature with high resolution. The optical sections at different depths of a typical islet are shown in Figs. 4(a)–4(e). Images from the transmitted light and fluorescence channels of confocal microscopy are presented on the left and right sides of the figure, respectively. Video 5 displays the serial optical sections of the scanned region. Specifically, on the left side of Fig. 4(e), large blood vessels can be seen using transmitted light imaging. On the right side, these vessels are also revealed by fluorescence imaging, confirming the success of vessel painting. Notably, the microvessels with a diameter of ~5 μm are clearly visualized in the islet and acini.

360-deg panoramic presentation of Fig. 2(k) is shown in Video 3. This approach allows an integral view and assessment of the islet structure, which cannot be done by the standard microtome-based 2-D analysis.
Figures 4(f) and 4(g) are stereo projections of the blood vessels and an orthogonal view of the islet shown in Figs. 4(a)–4(e). The 3-D top view and bottom view of the islet vasculature, shown in Figs. 4(h) and 4(i), are created by using the Amira software to merge the signals shown in Figs. 4(f) and 4(g) and rotate the viewing angle to project the top and bottom halves of the imaged region. Figures 4(j) and 4(k) are two additional results of the high-resolution, 3-D vasculature imaging of the mouse pancreatic islets. A fly-through presentation of Fig. 4(k) is shown in Video 6. Furthermore, in Video 7, we applied the Image Segmentation function of the Amira software to highlight the vasculature in the interior and exterior domains of the islet. These results explore the feasibility of an in situ 3-D image comparison between the endocrine and exocrine parts of the pancreas, which could be used to examine the remodeling of microvessels during the progression of autoimmune diabetes.

4 Discussion
The worldwide prevalence of diabetes mellitus underlines the complexity of the disease and an urgent need to better understand the islet physiology and developing diagnostic and monitoring tools for the disease. In this research, we develop a new imaging approach to extend our conventional planar view of the microscopic islet/pancreatic architecture into a 3-D panorama for characterization of islets in situ. In our approach, we explored the efficiency of an optical-clearing reagent, FocusClear, in improving photon penetration in the mouse pancreatic tissue. We engineered the tissue optics to
reveal the embedded islets in the pancreas, where random light scattering was suppressed and the size of the islet in the paraformaldehyde-fixed specimen remained the same. Using fluorescent labeling, the islet/pancreatic microstructure and vasculature were revealed by 3-D confocal microscopy with efficient fluorescence excitation and emission in the optical-cleared specimen. The 3-D figures and videos presented in this report make possible viewing the spatial relationship of the endocrine islet and the exocrine acini. Significantly, because microtome sectioning is not involved in sample preparation, our approach provides a useful tool for an integral, 3-D presentation of the intricate islet microvasculature.

Recently, significant progress has been made in developing optical methods for in vivo imaging of islet cell mass and vasculature.26–29 For example, Nyman et al.28 used confocal microscopy to examine the blood flow in the exteriorized pancreatic islets. Speier et al.29 transplanted islets into the anterior chamber of the mouse eye for a transparent environment to study islet cell biology via two-photon microscopy. However, the extension of confocal and two-photon microscopy to in vivo imaging still leaves unresolved the problem of light scattering as photons encounter the opaque tissue. In two-photon microscopy, for instance, although the infrared lasers excite fluorophores twice as deep in comparison to confocal microscopy, the resolution is not significantly improved because the emitted fluorescence still needs to travel through the turbid tissue for detection. Evidently, in both Nyman’s and Speier’s studies, images were limited at cellular-level resolution, in addition to lacking a clear view of the vascular structure in the interior domain of the islet. Results of these studies confirm that the process of optical clearing is essential in using optical microscopy to acquire high-definition images of the microvascular network, as shown in Figs. 3 and 4. However, it should be noted that the optical-clearing-enhanced microscopy can be done only on a deceased animal, so the ability to perform longitudinal measurements is restricted to a single time point. Although in vivo microscopy is limited due to optical scattering, a key advantage remains that it can be done in a longitudinal fashion. Therefore, both approaches carry pros and cons.

In this study, we applied the FocusClear solution (U.S. Patent No. 6472216)24 for optical clearing to improve photon penetration in the pancreatic tissue. FocusClear is an aqueous mixture of chemicals consisting of sugars and their derivatives with high refractive indices (∼1.46) similar to those of the tissue constitutes. Immersion of pancreatic tissues in the solution reduces the amount of refractive mismatch between tissue constitutes and the surrounding fluids, thereby avoiding scattering and making the pancreatic tissue transparent for optical imaging.

In addition to optical microscopy, advances have been made in using noninvasive imaging methods, such as magnetic resonance imaging (MRI) and positron emission tomography (PET), in animal models to detect the altered β-cell mass and the microvascular remodeling during the progression of type 1 (autoimmune) diabetes.27–30,32 MRI, for example, can image deep into the pancreas in vivo and provide 3-D information such as the angiogram.27–30 Continued advances in noninvasive monitoring of the pathophysiological islet changes would provide crucial help in early diagnosis and therapeutic intervention of the disease. However, to date, the spatial resolution of MRI and PET is too low to observe...
either the islet cellular structure or the microvasculature. Thus, additional imaging approaches, such as the 3-D optical method developed in this study, will need to fill the gap between the routine planar microscopy and the noninvasive imaging methods to provide insights into tissue’s microstructure and networks.

5 Conclusions

In this report, we present an optical method for imaging the islet microstructure and vasculature in the pancreas. The integration of fluorescent labeling, optical clearing, confocal microscopy, and 3-D image rendering provides a unique approach to visualize the islet and its vascular network in situ. Unlike the microscope-based tissue imaging, this optical method for penetrative imaging of the pancreas yields clear, continuous optical sections for an integrated visualization of the islet microstructure and vascular network with subcellular-level resolution. This new optical approach will change our conventional planar view of the islet structure into a 3-D panorama for better understanding the islet physiology and diabetes mellitus.

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