Three-dimensional islet graft histology: panoramic imaging of neural plasticity in sympathetic reinnervation of transplanted islets under the kidney capsule

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Juang J, Peng S, Kuo C, Tang S. Three-dimensional islet graft histology: panoramic imaging of neural plasticity in sympathetic reinnervation of transplanted islets under the kidney capsule. Am J Physiol Endocrinol Metab 306: E559–E570, 2014. First published January 14, 2014; doi:10.1152/ajpendo.00515.2013.—Microscopic examination of transplanted islets in an ectopic environment provides information to evaluate islet engraftment, including revascularization and reinnervation. However, because of the dispersed nature of blood vessels and nerves, global visualization of the graft neurovascular network has been difficult. In this research we revealed the neurovascular network by preparing transparent mouse islet grafts under the kidney capsule with optical clearing to investigate the sympathetic reinnervation via three-dimensional confocal microscopy. Normoglycemic and streptozotocin-induced diabetic mice were used in syngeneic islet transplantation, with both groups maintaining euglycemia after transplantation. Triple staining of insulin/glucagon, blood vessels, and tyrosine hydroxylase (sympathetic marker) was used to reveal the graft microstructure, vasculature, and sympathetic innervation. Three weeks after transplantation, we observed perigraft sympathetic innervation similar to the peri-islet sympathetic innervation in the pancreas. Six weeks after transplantation, prominent intragraft, perivascular sympathetic innervation was achieved, resembling the pancreatic intrasit, perivascular sympathetic innervation in situ. Meanwhile, in diabetic recipients, a higher graft sympathetic nerve density was found compared with grafts in normoglycemic recipients, indicating the graft neural plasticity in response to the physiological difference of the recipients and the resolving power of this imaging approach. Overall, this new graft imaging method provides a useful tool to identify the islet neurovascular complex in an ectopic environment to study islet engraftment.

islet transplantation; graft innervation; neural plasticity; sympathetic nerves; three dimensional

ISLET TRANSPLANTATION HAS BEEN PROPOSED AS AN EFFECTIVE CURE FOR PATIENTS WITH UNSTABLE TYPE 1 DIABETES IN WHOM THE DIABETES CARE ALONE IS INADEQUATE TO AVOID SERIOUS COMPLICATIONS (2, 8, 15, 25). TO ACHIEVE LONG-TERM GRAFT SURVIVAL, REVASCULARIZATION AND REINNERRATION OF ISLETS IN THE NEW MICROENVIRONMENT ARE ESSENTIAL FOR ISLET ENGRAFTMENT (5, 16, 27, 30, 34, 38). THE NEUROVASCULAR INTEGRATION IS PARTICULARLY IMPORTANT FOR GRANTS TO RECEIVE SIGNALS FROM THE CIRCULATION AND NERVES IN RESPONSE TO PHYSIOLOGICAL CUES TO MAINTAIN GLUCOSE HOMEOSTASIS AND AVOID HYPOGLYCEMIA. HOWEVER, BECAUSE OF THE DISPERSED NATURE OF BLOOD VESSELS AND NERVES, GLOBAL AND INTEGRATED VISUALIZATION OF THE GRAFT MICROSTRUCTURE, VASCULATURE, AND INNERRATION HAS BEEN DIFFICULT, EVEN IN ANIMALS. THE DIFFICULTY OF EXAMINING THE NEUROVASCULAR COMPLEX IN A THREE-DIMENSIONAL (3D) CONTINUUM HAS LIMITED OUR UNDERSTANDING OF ISLET ENGRAFTMENT AFTER TRANSPLANTATION TO HELP EVALUATE AND IMPROVE THE PROCESS.

BECAUSE OF THE INTRINSIC TISSUE OPACITY, THE STANDARD HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES OF ISLET GRAFTS IN AN ECTOPIQUE ENVIRONMENT (SUCH AS THE LIVER OR KIDNEY) ARE PERFORMED ON MICROTOME-SECTIONED TISSUE SLICES (7, 26), ~5 µM IN THICKNESS, TO AVOID LIGHT SCATTERING FOR OPTICAL MICROSCOPY. HOWEVER, THE THICKNESS IS ONE ORDER OF MAGNITUDE SMALLER THAN THE SIZE OF AN ISLET AND TWO ORDERS OF MAGNITUDE SMALLER THAN THE AGGREGATE(S) OF ISLETS UNDER THE KIDNEY CAPSULE. AS A RESULT, THE MICROTOME-BASED TISSUE ANALYSIS OFFERS ONLY A LIMITED VIEW OF THE GRAFT AND THE SURROUNDING TISSUES OF THE HOST. FOR EXAMPLE, IN ANIMAL MODELS OF ISLET TRANSPLANTATION, ALTHOUGH INVESTIGATORS WERE ABLE TO LOCALLY IDENTIFY GRAFT REINNERRATION (14, 20, 28–30, 32, 34, 35), GLOBAL VISUALIZATION AND COMPARISON OF THE INNERRATION PATTERNS BETWEEN THE PANCREATIC ISLETS IN SITU AND THE ENGRAFTED ISLETS IN ECTOPIQUE LOCATIONS HAVE NOT BEEN SYSTEMATICALLY PERFORMED TO SPECIFY THEIR DIFFERENCE IN NEUROANATOMY TO UNDERSTAND THE NEURAL NETWORK REINNERRATION AND REMODELING AFTER TRANSPLANTATION.

TO VISUALIZE THE ISLET NEUROVASCULAR COMPLEX IN SITU, WE PREVIOUSLY DEVELOPED A PENETRATIVE CONFOCAL IMAGING METHOD, BASED ON PREPARATION OF TRANSPARENT SPECIMENS BY OPTICAL CLEARING (9, 11, 12, 21–23, 37, 41), TO PERFORM 3D ISLET HISTOLOGY IN MICE (1, 6, 10, 39). THROUGH IN-DEPTH PROJECTION OF BLOOD VESSELS AND NERVES, WE REVEALED THE INTRASIT, PERIVASCULAR SYMPATHETIC INNERRATION, IN ADDITION TO PERI-ISLET CONTACTS OF SYMPATHETIC NERVES WITH α-Cells (6). THE NEUROVASCULAR COMPLEX IN THE ISLET CORE AND SYMPATHETIC NERVE-α-CELL CONTACTS IN THE MANTLE SUGGEST THAT SYMPATHETIC NERVES MODULATE ISLET HORMONE SECRETION THROUGH BLOOD VESSELS, IN ADDITION TO ACTING DIRECTLY ON α-Cells (1, 6).

HERE, IN 3D HISTOLOGY OF MOUSE ISLET GRAFTS UNDER THE KIDNEY CAPSULE, WE EMPLOYED THE SAME PENETRATIVE IMAGING APPROACH TO PROVIDE A GLOBAL AND IN-DEPTH VIEW OF THE GRAFT MICROSTRUCTURE, VASCULATURE, AND SYMPATHETIC INNERRATION. EXAMPLES ARE GIVEN TO ILLUSTRATE: 1) THE MORPHOLOGICAL FEATURES OF THE ISLET SYMPATHETIC REINNERRATION AFTER TRANSPLANTATION, 2) THE SIMILARITY IN THE SYMPATHETIC INNERRATION PATTERNS OF THE PANCREATIC ISLET IN SITU AND THE ISLET GRAFT IN KIDNEY, AND 3) A MARKED AND SURPRISING INCREASE IN GRANT SYMPATHETIC NERVE DENSITY OF DIAG...
abetic recipients over that in normoglycemic recipients, with both groups maintaining euglycemia after transplantation. Panoramas of the islet graft sympathetic neurovascular network under the kidney capsule and the implications of the network arrangement are presented and discussed in this report.

**MATERIALS AND METHODS**

*Animals and islet transplantation.* Male inbred C57BL/6 mice, age 8–12 wk, were used as donors and recipients for transplantation (17–19, 24). The recipients were diabetic or normoglycemic. The diabetic mice were induced by a single intraperitoneal injection of streptozotocin (STZ, 200 mg/kg body wt; Sigma, St. Louis, MO). Before transplantation, diabetic recipients were confirmed by hyperglycemia, weight loss, and polyuria; only the mice with blood glucose levels >350 mg/dl 2 wk after the STZ injection were transplanted. Blood glucose concentration was measured from the tail tip with a portable glucose analyzer (One Touch II; Lifescan, Milpitas, CA).

Islet isolation was performed under sodium amobarbital-induced anesthesia with the donor pancreases distended with 2.5 ml of digestion solution [RPMI-1640 medium supplemented with 1.5 mg/ml of collagenase; RPMI (Invitrogen, Carlsbad, CA); collagenase, from Clostridium histolyticum, type XI (Sigma)], excised, and incubated in a water bath at 37°C. Afterward, the islets were purified by a density gradient (Histopaque-1077; Sigma) and then handpicked under a stereomicroscope. Islets with a diameter between 75 and 250 μm were collected for transplantation. Three hundred islets were syngeneically transplanted under the left kidney capsule on the same day of isolation, weight loss, and polyuria; only the mice with blood glucose levels >350 mg/dl 2 wk after the STZ injection were transplanted. Blood glucose concentration was measured from the tail tip with a portable glucose analyzer (One Touch II; Lifescan, Milpitas, CA).

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![Fig. 1. Optical clearing increases light transmission of transplanted islets under the kidney capsule. A: transmitted light micrograph of the opaque islet graft in saline. Note that the dense connective tissue between the kidney capsule and islet graft is more opaque than the graft domain close to the kidney. B: transmitted light micrograph of the optically cleared islet graft. The graft-kidney interface can be clearly seen. In A and B, two images were stitched (the gray line) to generate a wide view of the graft.](http://ajpendo.physiology.org/)

![Fig. 2. Sympathetic nerves in mouse pancreatic islet and kidney before transplantation. A and B: remnants of sympathetic nerves in isolated mouse islet. B shows an in-depth projection of the islet microstructure in which glucagon staining was used to reveal the α-cells in the mantle. The oval and arrows indicate the remnants of sympathetic nerves, revealed by tyrosine hydroxylase (TH) staining. A and B were taken under the same view. *TH* endocrine cells. C–H: pancreatic peri- and intraislet sympathetic innervation in situ. In C–E, the transmitted light microscopy and paired staining and projection of glucagon and TH reveal the peri-islet sympathetic axons/varicosities and their association with α-cells. In F–H, the paired staining of insulin, blood vessels, and TH reveals the intraislet perivascular sympathetic innervation (circle in G) and the sympathetic nerves in the mantle (arrows in H, which follow the curvature of the islet). Additional examples can be found in Ref. 6. I and J: gross view of kidney sympathetic innervation. The sympathetic nerves were seen to associate with glomeruli (“G”) and extend from the renal organ domain toward the capsule area (arrows). The images in I and J were taken under the same view and then stitched to create a wide view of the tissue structures.](http://ajpendo.physiology.org/)
A Transmitted light image, isolated mouse islet

B \(\alpha\)-cells (green), sympathetic marker TH (white) & nuclei (red); projection depth: 120 \(\mu\)m

C Transmitted light image, islet in situ

D \(\alpha\)-cells (green) & TH (white), depth: 36 \(\mu\)m

E Sympathetic marker TH

F Insulin (blue), blood vessels (red) & TH (white)

G Blood vessels & TH, depth: 105 \(\mu\)m

H Sympathetic marker TH

I Gross view of mouse kidney

J Sympathetic marker TH (white) & nuclei (magenta); Projection depth: 105 \(\mu\)m

Kidney capsule
In diabetic recipients, mice with reversal of diabetes to normoglycemia 2 wk posttransplantation were included in the study. Islet transplantation in normoglycemic recipients followed the same procedure with euglycemia maintained after transplantation. The Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital approved all procedures with mice.

**Tissue labeling.** Blood vessels of the kidney and islet graft were labeled by vessel painting (13, 33) via cardiac perfusion of the lectin-Alexa Fluor 488 conjugate (30 μg/g of body wt, catalog no. W11261; Invitrogen) followed by 4% paraformaldehyde perfusion fixation. Afterward, grafts under the kidney capsule were harvested, and the vibratome sections of the tissue (~400 μm) were postfixed in 4% paraformaldehyde solution for 1 h at 25°C. The fixed tissues were then immersed in 2% Triton X-100 solution for 2 h at 25°C for permeabilization.

Three different primary antibodies were used to immunolabel the tissues following the protocol outlined below. The antibodies used were as follows: polyclonal rabbit anti-tyrosine hydroxylase (TH) (Millipore, Billerica, MA), polyclonal guinea pig anti-glucagon (Acris Antibodies, Herford, Germany), and polyclonal guinea pig anti-insulin (Gene Tex, Irvine, CA) antibodies. Before applying the antibody, the tissue was rinsed in PBS. This was followed by a blocking step, incubating the tissue with the blocking buffer (2% Triton X-100, 10% normal goat serum, and 0.02% sodium azide in PBS). The primary antibody was then diluted in the dilution buffer (1:50, 0.25% Triton X-100, 10% normal goat serum, and 0.02% sodium azide in PBS) to replace the blocking buffer and incubated for 1 day at 15°C.

Alexa Fluor 647-conjugated goat anti-rabbit secondary antibody and Alexa Fluor 546-conjugated goat anti-guinea pig secondary antibody (1:200; Invitrogen) were used to reveal the immunostained signals, including the transmitted light signals. The Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody was also used to stain TH-positive cells. Nuclear staining by propidium iodide (50 μg/ml; Invitrogen) or SYTO 16 (5 μM; Invitrogen) was performed at room temperature for 1 h to reveal the nuclei, if necessary. The labeled specimens were then immersed in the optical-clearing solution FocusClear (CelExplorer, Hsinchu, Taiwan) before being imaged via confocal microscopy (4).

**Confocal microscopy.** Imaging of the tissue structure was performed with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany) equipped with an objective of ×40 LD “C-Apochromat” water immersion lenses (working distance: 620 μm) (optical section: 3 μm; z-axis increment: 1.5 μm) under a regular or tile-scan mode with automatic image stitching. The laser-scanning process was operated under the multitrack scanning mode to sequentially acquire signals, including the transmitted light signals. The Alexa Fluor
647-labeled structures were excited at 633 nm, and the fluorescence was collected by the 650- to 710-nm bandpass filter. The propidium iodide-labeled nuclei and the Alexa Fluor 546-labeled structures were excited at 543 nm, and the signals were collected by the 560- to 615-nm bandpass filter. The SYTO 16-labeled nuclei and the lectin-Alexa Fluor 488-labeled blood vessels were excited at 488 nm, and the fluorescence was collected by the 500- to 550-nm bandpass filter.

**Image projection and analysis.** The LSM 510 software (Carl Zeiss) and the Avizo 6.2 image reconstruction software (VSG, Burlington, MA) were used for projection, signal segmentation, and analysis of the confocal images. Figures 2B, D–H, and J; 3, B–D and F–H; 4, B–D; 5, B–D; 6, B–D; 7, B–E, G–H, and J–K were derived from the projection module of the LSM 510 software. The 3D image in Fig. 8A was derived from the Voltex module of Avizo 6.2. In Supplemental Videos S1–S3 (Supplemental data for this article can be found on the American Journal of Physiology: Endocrinology and Metabolism website.), image stacks were recorded using the “Movie Maker” function of Avizo with the increase in display time in association with the depth of the optical section.

In density analysis, both the 3- and 6-wk grafts were derived from four normoglycemic and four diabetic recipients. Four image stacks were taken from each animal to assess their graft sympathetic nerve

**Normoglycemic recipient, 3 weeks post transplantation**

![Image of perigraft sympathetic nerves](image-url)

Fig. 4. Zoom-in examination of perigraft sympathetic nerves (3-wk graft in normoglycemic recipient). Transmitted light micrograph (A) and projections of insulin (blue), blood vessel (red), and TH (white) signals (B–D) identify the formation of TH⁺ sympathetic axons and varicosities surrounding the islet graft. In the meantime, the lack of the intragraft TH⁺ fibers (asterisk in C) was also noticeable at this early stage of graft remodeling.
and blood vessel densities. The sympathetic nerve and blood vessel densities of pancreatic islets in situ (22 islets from four normal C57BL/6 mice) were used as the control.

Quantitation of the graft neurovascular tissue density was shown in Fig. 8. The same tissue labeling, imaging, and quantitation processes were conducted on the transplanted islets under the kidney capsule and pancreatic islets in situ to compare their sympathetic nerve and blood vessel densities on the same basis. In estimation of the density, feature extraction and image segmentation were first performed by the “Label Field” function of Avizo to collect the voxels of the grafts and those of the sympathetic nerves (TH signals, excluding the globular TH⁺ endocrine cells) and blood vessels (signals from vessel painting). Afterward, voxels of the nerves (or blood vessels) in the acquired image stack were divided by those of the grafts ×100%. Values derived from the four image stacks of the same recipient were first averaged to estimate the density of one animal. Values derived from the four animals of the same recipient group were then averaged to estimate the nerve (or blood vessel) density of the group.

The quantitative values are presented as means ± SD. Statistical differences were determined by the unpaired Student’s t-test. Differences between groups were considered statistically significant when \( P < 0.05 \).

**RESULTS**

Optical clearing increases light transmission of islet graft under the kidney capsule. The vascular and neural integration between the islet graft and the host microenvironment has been...
widely suggested to influence the graft survival and its long-term function. However, because the islet graft strongly scatters light (particularly at the graft-capsule boundary; Fig. 1A), the intrinsic tissue opacity hinders in-depth imaging of the graft microstructure and its neurovascular connections to the host tissue networks. To visualize the graft-host integration, we immersed the engrafted kidney specimens in the optical-clearing solution with high refractive index to promote photon penetration by reducing scattering (9, 12, 41) (Fig. 1B). As can be seen, the optically cleared specimen allowed a direct visualization of the islet graft and its boundaries against the kidney capsule and the renal organ domain via transmitted light microscopy.

Taking advantage of the transparent specimens, in the next four sections we show the results derived from both the transmitted light and confocal microscopy to generate a connected view of the graft-kidney integration and to verify the signal fidelity by comparing the sources of tissue information with each other.

**Sympathetic nerves in islet and kidney before transplantation.** Figure 2, A and B, shows a typical isolated islet and the remnants of sympathetic nerves before transplantation. The nerve fiber remnants are a sharp contrast to the rich islet sympathetic innervation in situ (additional examples can be found in Ref. 6; Fig. 2, C–E and F–H). The result indicates the islet neural network was destroyed while harvesting the islets from pancreas, losing the peri- and intraislet sympathetic

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**Normoglycemic recipient, 6 weeks post transplantation**

![Transmitted light + fluorescence image of sympathetic marker TH & α-cells; depth: 105 μm](image)

![Sympathetic marker TH & blood vessels; depth: 42 μm](image)

![Sympathetic marker TH & α-cells, depth: 42 μm](image)

![Sympathetic marker TH & α-cells; depth: 185 μm](image)

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Fig. 6. Close proximity of sympathetic nerves to the perigraft α-cells (6-wk graft in normoglycemic recipient). The transmitted light image (A) and projections of the islet graft with different depths and signals (B–D) identify the graft domains (nos. 1–3) and the perigraft association of sympathetic axons with α-cells. The same innervation pattern was shown in Fig. 2D of the pancreatic islet in situ. Red, blood vessels; white, TH staining; green, glucagon staining.
Fig. 7. Intragraft perivascular sympathetic innervation (6-wk graft in STZ-induced diabetic recipient). A–D: zoom-in examination of the richly innervated islet graft. The progressive increase in projection depth from A to C reveals the abundant sympathetic nerves and their association with the graft microvessels (A and B). Blue, insulin staining; red, blood vessels; white, TH staining. An in-depth recording of the perivascular sympathetic innervation is presented in Supplemental Video S3. E: gross view of the islet graft. Box indicates the location of the enlarged graft in A–D. Projection depth: 105 μm. F–K: panoramic projection of islet graft sympathetic innervation. The panoramic view of the islet graft was derived by stitching and projection of high-resolution confocal image stacks. Viewers can zoom in to specify the peri- and intragraft sympathetic axons and varicosities.
innervation. Note that while using TH as the marker to reveal the sympathetic nerves, we also detected the TH⁺ endocrine cells (31, 40), which carry the enzyme of the catecholamine biosynthetic pathway but do not belong to the sympathetic nervous system. Their large globular patterns can be easily recognized and distinguished from the nerve fibers.

The gross views of the kidney microstructure and sympathetic innervation under the kidney capsule are shown in Fig. 2, I and J. Specifically, glomeruli and their association with sympathetic nerves can be visualized and identified through in-depth microscopy and projection. The renal sympathetic nerves were also seen to extend from the parenchymal domain toward the capsule area.

**Formation of perigraft sympathetic innervation at early stage of islet engraftment.** Three weeks after islet transplantation in STZ-induced diabetic recipients, we used deep-tissue confocal microscopy with optical clearing to detect the fluorescence signals of the islet cells (insulin/glucagon staining), blood vessels, and sympathetic nerves (Fig. 3). Figure 3, A and B, shows the gross view of an islet graft and its sympathetic innervation under the kidney capsule. Projection of the TH⁺ signals reveals the perigraft sympathetic nerves, which extend from the renal parenchyma and along the capsule into the graft boundary (Fig. 3, B–D). Supplemental Video S1 shows a 360-degree panoramic projection of the islet graft.

Figure 3, E–H, shows another example of the perigraft sympathetic innervation of a 3-wk graft. In this example, glucagon staining of α-cells shows four aggregates of islets at the center and two more aggregates at the far left and right sides of the gross image. Projection of the TH and glucagon signals reveals the perigraft association between the

![Fig. 8. Illustration of the graft neurovascular tissue quantitation. A: stereo projection of the graft image stack shown in Fig. 7, A–D. The image stack consists of 71 optical sections. Each section was 3 μm in depth and overlapped 50% with the adjacent sections. B: signal components of an optical section. We used section 35 of the graft as an example. The graft boundary (yellow drawing) was defined by the transmitted light and insulin fluorescence signals (panel iii). Within the boundary, the voxels occupied by the sympathetic nerves (panel i; excluding the TH⁺ endocrine cells, asterisks) and blood vessels (panel ii) were digitally extracted and divided by the graft voxels. C: nerve density profile of the 71 graft optical sections. The cyan bar indicates the sympathetic nerve density derived from section 35 (B). The nerve density of the image stack (A) is 1.79%, which was derived from the average of the 71 data points. However, it should be noted that, because the polyclonal primary and secondary antibodies used in immunostaining of TH allow one antigen molecule to bind with multiple antibody molecules to amplify the antigen signals for fluorescence detection, the real nerve density of the image stack was expected to be less than the apparent density shown here (1.79%).](http://ajpendo.physiology.org/)

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TH⁺ sympathetic nerves and the α-cells (Fig. 3, G and H, and Supplemental Video S2). This morphology resembles the mouse pancreatic peri-islet sympathetic innervation of α-cells in situ (Fig. 2, C–E).

Similar to the diabetic recipients, the perigraft sympathetic nerves were also found in the normoglycemic recipients 3 wk after transplantation. Figure 4 shows the zoom-in examination of the perigraft TH⁺ sympathetic axons and varicosities with high definition. Through in-depth projections, we also identified the lack of sympathetic nerves in the interior domain of the graft (Fig. 4C), suggesting that the islets were still in the remodeling phase after transplantation. The lack of intragraft sympathetic nerves was also found in diabetic recipients (Fig. 3, B and G).

Formation of peri- and intragraft sympathetic innervation in normoglycemic recipients. Six weeks after islet transplantation in normoglycemic recipients, both peri- and intragraft sympathetic innervation were formed, with the latter found primarily associated with the microvessels (Fig. 5). The intragraft TH⁺ sympathetic nerves consisted of the perivascular varicosities with the axons to bridge their paths across the nearby capillaries. In addition, prominent TH⁺ axons were found at the graft boundaries, similar to the perigraft TH⁺ sympathetic nerves observed in the 3-wk graft (Fig. 4). Also, using paired TH and glucagon staining, we revealed the close proximity of the sympathetic axons to the perigraft α-cells with high definition (Fig. 6).

Rich sympathetic innervation of islet grafts in diabetic recipients. Surprisingly, in diabetic recipients, we observed a marked increase in the graft sympathetic nerve density (also a 6-wk graft; Fig. 7, A–E) compared with that in normoglycemic recipients, with both groups maintaining euglycemia after transplantation. Specifically, the abundant intragraft sympathetic nerves followed the blood vessels (Fig. 7, A and B, and Supplemental Video S3), resembling the pancreatic intraislet perivascular sympathetic innervation in situ (Fig. 2, F–H, and Supplemental Video S3: the second part). Figure 7, F–K, shows another example of the intragraft sympathetic innervation in diabetic recipients. The rich peri- and intragraft sympathetic innervation underlines the graft neural plasticity in the islet sympathetic reinnervation after transplantation.

Quantitative assessment of islet graft reinnervation and revascularization. We have demonstrated the different islet graft sympathetic network morphologies under the kidney capsule. Figure 8 show how to acquire the sympathetic nerve and blood vessel densities in the islet graft via the 3D image stack. Using this approach, we next sought to quantify the sympathetic nerve density under different host conditions to show the different levels of neural plasticity of the islet graft under the kidney capsule.

The first level of plasticity was featured by recruiting the islet nerves after transplantation. Figure 9A shows that, in
normoglycemic and diabetic recipients, the sympathetic nerve density of islet grafts reached 29 and 25% of that of the pancreatic islets in situ, respectively, 3 wk after transplantation and reached 37 and 62% 6 wk after transplantation. The morphological and quantitative changes of the sympathetic nerves, from the nerve fiber remnants in isolated islets (Fig. 2B) to the network coupled with the renal sympathetic nervous system (Figs. 3–7), indicate the intrinsic ability of the islet graft to recruit sympathetic nerves for reinnervation after transplantation.

Also, in Fig. 9A, although there is no statistical difference in the sympathetic nerve density between the normoglycemic and diabetic recipients in the 3-wk grafts, in the 6-wk grafts we observed a significant 68% increase in the sympathetic nerve density in diabetic recipients over that in normoglycemic recipients. The difference indicated a second level of graft neural plasticity in response to the lack of active pancreatic β-cells in the STZ-induced diabetic recipients compared with their presence in the normoglycemic recipients.

Regarding the islet revascularization, Fig. 9B shows that the vascular density of the 3-wk grafts in normoglycemic and diabetic recipients reached 55 and 47% of that of the pancreatic islets in situ, respectively, and reached 82 and 76% in the 6-wk grafts without significant difference between the two recipient groups at both time points. Figure 9C summarizes the blood glucose levels of the normoglycemic and diabetic recipients over the course of the experiment.

Importantly, between the normoglycemic and diabetic recipients, the similar islet revascularization but different nerve densities after 6 wk of engraftment highlights the sensitivity of the neural network in response to the physiological difference of the recipients and the resolving power of this imaging approach.

**DISCUSSION**

Because of depth limitation, the spatial features of islet graft reinnervation in an ectopic environment cannot be easily portrayed by the standard microtome-based histology. In this study, we overcame this limitation by preparing transparent mouse islet grafts under the kidney capsule for panoramic visualization of the graft microstructure, vasculature, and sympathetic innervation in an integrated fashion. Taking advantage of the global and in-depth image data, we applied qualitative and quantitative analyses to reveal: 1) the peri- and intragraft sympathetic reinnervation, with the morphology resembling the pancreatic islet sympathetic innervation but at a larger scale (comparison between Figs. 3–7 and 2, C–H) and 2) the higher graft sympathetic nerve density in diabetic recipients over that in normoglycemic recipients (Figs. 5, 7, and 9A), reflecting the dynamic feature of islet graft reinnervation.

The panoramic visualization of the islet graft under the kidney capsule represents a technical advance of using tile scanning and deep-tissue confocal microscopy to illustrate the global features of the graft neurovascular network. This new imaging approach allowed us to differentiate the graft sympathetic innervation between the normoglycemic and diabetic recipients, which revealed the neural plasticity in response to the recipients’ physiological/metabolic states. This level of neural plasticity has not been demonstrated in the literature, although investigators have applied the standard microtome-based histology to investigate the graft reinnervation over time after transplantation (14, 20, 28–30, 32, 34). Here, our result underlines the importance of examining the islet graft as a union to investigate its innervation.

In both normoglycemic and diabetic recipients, the peri- and intragraft sympathetic reinnervation indicates the intrinsic ability of islets to recruit the sympathetic nerves, likely through the release of neurotrophic factors from β-cells (3, 36, 43), to initiate the graft-host integration. Similar development has been seen in the ontogenesis of the islet sympathetic nerves, in which the nerve growth factor guides the microvessels and sympathetic nerves toward the islets in the fetal and neonatal stages (3). Because the release of the nerve growth factor has been shown to couple with insulin secretion in response to the secretagogues applied to the β-cells in culture (3, 36), we suspect that the rich graft innervation in diabetic recipients (Fig. 7) was the result of the same mechanism with higher insulin secretion from the graft β-cells to compensate for the lack of active β-cells in the pancreatic islets to maintain euglycemia.

Regarding the progression of islet engraftment, we observed that the graft revascularization preceded reinnervation, particularly in the interior domain of the islet graft (from Figs. 3 and 4 to Figs. 5–7). This sequence of engraftment could be triggered by the release of angiogenic factors, such as the vascular endothelial growth factor, from the transplanted islets under hypoxia to initiate angiogenesis (16, 42), and then followed by the perivascular innervation to establish the graft-host neurovascular integration. However, it remains to be seen whether a similar process can be achieved and visualized after the portal vein infusion of islets or islet transplantation in other alternative sites.

In summary, we have developed a new islet graft imaging approach, based on preparation of transparent graft specimens, to reveal the peri- and intragraft sympathetic innervation under the mouse kidney capsule. The intragraft sympathetic nerves follow the microvessels, resembling the intraislet, perivascular sympathetic innervation in the pancreas. Our new graft imaging approach possesses the resolving power to qualitatively and quantitatively differentiate the sympathetic nerve density of grafts between normoglycemic and diabetic recipients in response to the different physiological conditions of the two model systems. Future studies on the reinnervation of human and animal islets in the liver and alternative transplantation sites will further benefit our understanding of the engraftment process and regulation of islet functions in an ectopic environment.

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**DISCLOSURES**

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**AUTHOR CONTRIBUTIONS**


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