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

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Jyuhn-Huarng Juang, Shib-Jung Peng, Chien-Hung Kuo, and Shiue-Cheng Tang. 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, perivasular sympathetic innervation was achieved, resembling the pancreatic intraislet and perivasular sympathetic innervation in situ. Meanwhile, in diabetic recipients, a higher graft sympathetic nerve density was found compared with grafts in normoglycemic recipients, Meanwhile, in diabetic recipients, a higher graft sympathetic nerve density was found compared with grafts in normoglycemic recipients, although investigators were able to locally identify graft reinnervation via three-dimensional confocal microscopy. In this study, 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, 3D islet histology in vivo has been systematically performed to specify their difference in neuroanatomy to understand the neural network reinnervation 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 to perform 3D islet histology in mice (1, 6, 10, 39). Through in-depth projection of blood vessels and nerves, we revealed the intragraft vasculature and sympathetic innervation, in addition to peri-islet contacts of sympathetic nerves with a-cells (6). The neurovascular complex in the islet core and sympathetic nerve-a-cell contacts in the mantle suggest that sympathetic nerves modulate islet hormone secretion through blood vessels, in addition to acting directly on a-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 innervation. Examples are given to illustrate: 1) the morphological features of the islet sympathetic reinnervation after transplantation, and 2) the similarity in the sympathetic innervation patterns of the pancreatic islet in situ and the islet graft in kidney.
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 isola-
A Transmitted light image, isolated mouse islet

B α-cells (green), sympathetic marker TH (white) & nuclei (red); projection depth: 120 μm

C Transmitted light image, islet in situ

D α-cells (green) & TH (white), depth: 36 μm

E Sympathetic marker TH

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

G Blood vessels & TH, depth: 105 μm

H Sympathetic marker TH

I Gross view of mouse kidney

J Sympathetic marker TH (white) & nuclei (magenta); Projection depth: 105 μm
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, 1% 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 647-conjugated goat anti-rabbit secondary antibody was used to reveal the nuclei, if necessary. The labeled specimens were then immersed in the optical-clearing solution FocusClear (CellExplorer, 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 density.

**Normoglycemic recipient, 3 weeks post transplantation**

![Transmitted light image](A)

**Insulin staining**

![Sympathetic marker TH, blood vessel & insulin signals](D)

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 innervation.
Diabetic recipient, 6 weeks post transplantation

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
TH\(^+\) sympathetic nerves and the \(\alpha\)-cells (Fig. 3, G and H, and Supplemental Video S2). This morphology resembles the mouse pancreatic peri-islet sympathetic innervation of \(\alpha\)-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 \(\alpha\)-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 recipi-
ents, the similar islet revascularization but different nerve densi-
ties after 6 wk of engraftment highlights the sensitivity of the
nerve 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 por-
trayed by the standard microtome-based histology. In this
study, we overcame the 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 sympa-
thetic innervation between the normoglycemic and diabetic
recipients, which revealed the neural plasticity in response to
the recipients’ physiological/metabolic states. This level of
neral 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 abil-
ity 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, partic-
ularly 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 perivas-
cular innervation to establish the graft-host neurovascular integra-
tion. 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 quan-
titatively 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

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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