Video Article

Laser Doppler: A Tool for Measuring Pancreatic Islet Microvascular Vasomotion *In Vivo*

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Abstract

As a functional status of microcirculation, microvascular vasomotion is important for the delivery of oxygen and nutrients and the removal of carbon dioxide and waste products. The impairment of microvascular vasomotion might be a crucial step in the development of microcirculation-related diseases. In addition, the highly vascularized pancreatic islet is adapted to support endocrine function. In this respect, it seems possible to infer that the functional status of pancreatic islet microvascular vasomotion might affect pancreatic islet function. Analyzing the pathological changes of the functional status of pancreatic islet microvascular vasomotion may be a feasible strategy to determine contributions that pancreatic islet microcirculation makes to related diseases, such as diabetes mellitus, pancreatitis, *etc*. Therefore, this protocol describes using a laser Doppler blood flow monitor to determine the functional status of pancreatic islet microvascular vasomotion, and to establish parameters (including average blood perfusion, amplitude, frequency, and relative velocity of pancreatic islet microvascular vasomotion) for evaluation of the microcirculatory functional status. In a streptozotocin-induced diabetic mouse model, we observed an impaired functional status of pancreatic islet microvascular vasomotion. In conclusion, this approach for assessing pancreatic islet microvascular vasomotion *in vivo* may reveal mechanisms relating to pancreatic islet diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/56028/

Introduction

As a parameter of the functional status of microcirculation, microvascular vasomotion takes responsibility for the delivery and exchange of oxygen, nutrients, and hormones and is crucial to the removal of metabolic products, such as carbon dioxide and cell waste[1]. Microvascular vasomotion also regulates blood flow distribution and tissue perfusion, thereby affecting local microcirculatory blood pressure and responses to inflammation, which can induce edema in many diseases. Therefore, microvascular vasomotion is extremely important to maintain the physiological function of organs[2,3,4], tissues, and component cells. The impairment of microvascular vasomotion might be one of the key steps in the development of microcirculation-related diseases[5].

Laser Doppler was initially developed for observation and quantification in the field of microcirculation research[6]. This technique, together with other technical approaches (*e.g.,* laser speckle[7], transcutaneous oxygen, *etc.*), has been regarded as the golden standard for assessing blood flow in microcirculation. The rationale that the blood perfusion of local microcirculation (*i.e.,* capillaries, arterioles, venules, *etc.*) can be determined by apparatus equipped with laser Doppler, is based on the Doppler shift principle. The wavelength and frequency of stimulated emission light change when light particles encounter moving blood cells in microvessels, or they remain unchanged. Therefore, in microcirculation, the number and the velocity of blood cells are the key factors relating to the magnitude and frequency distribution of the Doppler-shifted light, while the direction of microvascular blood flow is irrelevant. Using different methods, a variety of tissues have been used for microcirculatory studies, including the mesenteries and dorsal skinfold chambers of mice, rats, hamsters, and even humans[8]. However, in the current protocol, we focus on the functional status of pancreatic islet microvascular vasomotion, which is evaluated using laser Doppler and a homemade assessment parameter system.

Pancreatic islet microcirculation is mainly composed of pancreatic islet microvessels and exhibits distinctive features. A pancreatic islet capillary network shows a five-times-higher density than the capillary network of its exocrine counterpart[9]. Providing a conduit for the delivery of input glucose and disseminating insulin, islet endothelial cells deliver oxygen to metabolically active cells in islet β cells. Furthermore, emerging evidence also demonstrates that islet microvessels are involved not only in regulating insulin gene expression and β-cell survival, but also in affecting β-cell function; promoting β-cell proliferation; and producing a number of vasoactive, angiogenic substances and growth factors[10]. Therefore, in this respect, we infer that the functional status of pancreatic islet microvascular vasomotion may affect islet β-cell function and get involved in the pathogenesis of diseases such as acute/chronic pancreatitis, diabetes, and other pancreas-related diseases.

Analyzing the pathological changes of the functional status of pancreatic islet microvascular vasomotion might be a feasible strategy to determine the contributions of the pancreatic islet microcirculation to the diseases mentioned above. A detailed step-by-step procedure

describing the approach to determine pancreatic islet microvascular vasomotion *in vivo* provide here. Typical measurements are then shown in the **Representative Results**. Finally, the benefits and limitations of the method are highlighted in the **Discussion**, along with further applications.

Protocol

All animal experiments were executed in compliance with all relevant guidelines, regulations, and regulatory agencies. The present protocol being demonstrated was performed under the guidance and approval of the Institute of Microcirculation Animal Ethics Committee (IMAEC) at the Peking Union Medical College (PUMC).

1. Animals

1. Before the start of the experiment, keep three BALB/c mice per cage, with controlled temperature (24 ± 1 °C) and humidity (55 ± 5%), under a 12-h light-dark cycle. Allow the mice free access to regular food and water.
2. Randomly divide the mice into a non-diabetic control group and a diabetic group. Accurately weigh each individual mouse and calculate the injection volume using the body mass of each mouse.
3. Fast the mice for 4 h before streptozotocin (STZ) injection and provide regular water as normal on experimental day 1.
4. Prepare 0.1 M sodium citrate buffer at pH 4.3. Put 1 mL of the solution into a 1.5-mL microcentrifuge tube and wrap the microcentrifuge tube in aluminum foil to avoid light exposure.
5. Dissolve the STZ in sodium citrate buffer (pH 4.3) to a final working concentration of 5 mg/mL before use.
6. Give the mice of the diabetic group intraperitoneal injections of STZ at a dose of 40 mg/kg using a 1-mL syringe and a 25-G needle. Inject the mice of the non-diabetic control with the same volume of sodium citrate buffer (pH 4.3).
7. Put the mice back into the cages and supply them with regular food and 10% sucrose water.
8. Repeat steps 1.3-1.7 on experimental days 2 to 5 (*i.e.,* the next 4 consecutive days).
9. Replace the 10% sucrose water with regular water after the last STZ injection.
10. Fast the mice for 6 h, but give them free access to water, and measure their blood glucose levels nine days later (experimental day 14). Collect a blood sample from the tail vein to confirm hyperglycemia using a blood glucose monitoring system.
    NOTE: Mice with blood glucose levels > 200 mg/dL are considered diabetic.

2. Preparation of the Instrument

1. Clean the optical surfaces of the probe tip and probe connector of the laser Doppler apparatus with a soft, non-abrasive cloth to remove any dust or particles. Plug the cable into the port of the instrument (**Figure 1A**).
2. Assemble the calibration stand by allowing the flux standard to be in thermal equilibrium with experimental surroundings (room temperature, usually for 30 min). Shake the flux standard gently for 10 s and let it rest for 2 min.
3. Position the flux standard container in the middle of the calibration base. Adjust the clamp to the maximum height and secure the probe in the clamp such that it points downwards to the container. Make sure the flux standard is correctly positioned underneath the probe.
4. Slowly move the probe down until the tip is correctly submerged in the flux standard. Select and press "calibration" on the laser Doppler apparatus and choose the working channel that the probe is connected to. Run the calibration program until a "Calibration successful" notice is displayed on the screen of laser Doppler apparatus.
5. Secure the probe using probe holders. Manually secure the probe to avoid movement.
6. Maintain the experimental room at constant temperature (24 ± 1 °C) and humidity (~50-60%).
7. Turn off any external light (such as fluorescent and spot lamps) before performing the experiment to avoid external light-induced change.

3. Preparation of the Animals

1. Autoclave the surgical instruments and allow them to cool to room temperature before use.
2. Give the mice 10 min to acclimatize to the experimental environment before detecting pancreatic islet microvascular vasomotion by laser Doppler.
3. Fill a 1-mL syringe with 1 mL of 3 % pentobarbital sodium. Inject the pentobarbital sodium solution (75 mg/kg i.p.) to anesthetize the mice.
4. Cover the eyes of the mouse with pre-moistened medical gauze to prevent dryness.
5. Ensure that the mouse completely loses consciousness and no longer responds to tail or hindfoot pinches with forceps. Monitor the anesthesia throughout the anesthetic and intra-operative event every 15 min. Maintain the anesthesia by supplementing with 10 % of the initial injection volume of the pentobarbital solution when necessary.
6. Place a heating pad with a semi-insulating layer below the animal and place the animal in supine position and transfer it to the working station of the laser Doppler apparatus. Fix the mouse to the working platform with surgical tape.
7. Swab the abdominal skin of the mouse with betadine, and then 75% ethanol is used to swab the abdominal area clean.
8. Inject 2% lidocaine/0.5% bupivacaine (50/50) mixture subcutaneously. Cut a ~3 cm-diameter hole in the center of a gauze sponge. Cover the abdominal region with the gauze sponge.
9. Lift the abdominal skin with forceps and make an initial vertical incision along the midline of abdomen using a scalpel or skin scissors.
10. Grasp the underlying muscle with forceps and incise to enter the abdominal cavity. Do not injure any organs. Fold the skin and underlying muscle over the chest to reveal the abdominal cavity. Gently expose the pancreatic body and the spleen using a pair of blunt-nosed forceps.

4. Data Acquisition for Analysis

1. Run the software of the laser Doppler apparatus by clicking on "File" → "New" to create a new measurement file. To configure the connected monitors, under the "General" tab, set up the monitoring duration to "Free run." Use the factory default for the "LDF Monitor" tab. Click "Next."

2. Set up the graph display in the "display setup dialog box." Select the "Flux, Conc, Speed" channels by checking the respective boxes. Select the following parameters: "Data source for the channel" and "Label, Units and Color." Click "Next."
3. Enter user information about the subject and measurement (*i.e.,* name and subject number, operator, monitoring time, comments, *etc.*) in the "file information dialog box" and click "Next" to finish the measurement configuration.
   NOTE: A measurement window is automatically created by the software (**Figure 1B**).
4. Manually advance the electrode to the pancreas. Make sure the distance between the probe and pancreas tissues is within 1 mm. An inappropriate distance gives an artificially increased or decreased blood flow reading.
5. Click the "Start" toolbar icon to start recording the microvascular blood perfusion units (PU) data. Collect the PU data continuously for 1 min every run. Click "Stop" to stop the measurement. Select "File" → "Save as" to name and save the finished measurement file.
6. Manually reposition the probe after each run to avoid additive effects and the localized exhaustion of contractile and relaxation capacity. Repeat steps 4.1-4.4 to harvest multi-point (*i.e.,* three randomly chosen points from pancreatic tissue) microvascular PU data for each mouse. Measure the PU data of a non-reflective plate as a baseline control.
7. Close the abdominal muscle layer and the skin layer with a suture. Place the animals in clean cages after the experiments.
8. Keep the animal warm by placing the recovery cage half-on the heating pad.
   NOTE: Pay attention to warmth, hygiene, fluid and food intake, and infection. Administrate mice with 2 mg/kg Carprofen for 48 h as postoperative pain management. Perform euthanasia by injecting 150 mg/kg pentobarbital sodium i.p. when mice are observed to be in a state of severe pain or distress that cannot be alleviated.

5. Calculating the Parameters of Microvascular Vasomotion

1. Use the "Export" command of the laser Doppler software to export the time and PU raw data as a *.xlsx file and open the file in a spreadsheet.
2. Calculate the average baseline perfusion unit (PU_b) (see step 4.6).
3. Calculate the average blood perfusion (PU_a) for 1 min of a measurement as follows: Average blood perfusion (PU_a) = PU - PU_b (Equation 1).
4. Calculate the frequency (cycles/min) for each 1 min of measurement.
   NOTE: The frequency of microvascular vasomotion is defined as the number of peaks that occurred in a microvascular vasomotion wave per minute.
5. Calculate the amplitude (ΔPU) for each 1 min of measurement.
   1. Calculate the amplitude of microvascular vasomotion as the difference between the maximum (PU_{max}) and minimum (PU_{min}): Amplitude (ΔPU) = PU_{max} - PU_{min} (Equation 2).
6. Calculate the relative velocity (PU) for each 1 min of measurement.

Representative Results

A photograph of the microvascular vasomotion measurement laser Doppler apparatus equipped with a semi-conductor laser diode is shown in **Figure 1A**. User interface software is presented in **Figure 1B**. Using the method mentioned above, the hemodynamic parameters of pancreatic islet microvascular vasomotion were detected for both non-diabetic control and diabetic mice. A variety of techniques, including laser Doppler flowmetry, reflected and scattered light, infrared spectroscopy, and imaging techniques, have been used to study microvascular vasomotion since it was first defined. **Table 1** summarizes research groups and published articles that use laser Doppler technology to determine the role of microcirculation in diabetes and related diseases.

In general, the microcirculatory conditions of the pancreatic islet are represented by the functional status of pancreatic islet microvascular vasomotion using the microvascular parameters, including average blood perfusion, amplitude, frequency, relative velocity (**Figure 2**). The representative microvascular vasomotion schematic diagram is mainly composed of periodic contraction and relaxation phases (**Figure 2A**). The hemodynamic phenomena present a pattern of blood flow perfusion in microvascular networks. PU data collected by laser Doppler apparatus were employed to chart scatter diagrams and to show the distribution pattern of microvascular blood perfusion. In the current protocol, the distribution patterns of pancreatic islet microvascular blood perfusion in non-diabetic and diabetic mice were totally different (**Figure 2B**).
A lower scale of blood perfusion of pancreatic islet microvascular vasomotion was observed in diabetic mice compared to the non-diabetic control. The rhythm of contractions and relaxations of pancreatic islet microvascular vasomotion was chaotic and irregular in STZ-induced diabetic mice, whereas non-diabetic controls had rhythmic oscillations (**Figure 2C** and **Figure 2D**). We extracted the 5-s data of pancreatic islet microvascular blood perfusion within the dashed lines in **Figure 2C** and **Figure 2D** and demonstrated that the chaotic fluctuations of pancreatic islet microvascular blood perfusion in diabetic mice lost the ability to regulate the functional status of pancreatic islet microvascular vasomotion, which should occur in response to blood glucose fluctuation (**Figure 2E**).

Furthermore, to respond to hyperglycemia, pancreatic islets need sufficient and biorhythmic blood flow perfusion to transport insulin. The pancreatic islet microvascular vasomotion parameters (including average blood perfusion, amplitude, frequency, and relative velocity) were then calculated and quantitatively analyzed based on the PU profiles. As shown in **Figure 3**, compared with non-diabetic controls, the average blood perfusion of the pancreatic islet microcirculation was decreased in STZ-induced diabetic mice (**Figure 3A**). Meanwhile, there were significant decreases noted in the amplitude (**Figure 3B**) and frequency (**Figure 3C**) of pancreatic islet microvascular vasomotion in STZ-induced diabetic mice. The relative velocity of pancreatic islet blood perfusion significantly decreased in the STZ-induced diabetic group compared to the non-diabetic control (**Figure 3D**). As mentioned above, the functional status of pancreatic islet microvascular vasomotion was impaired in diabetic mice. We speculate that rhythm abnormalities, together with a decreased frequency, amplitude, and relative velocity of pancreatic islet microvascular vasomotion, might result in a deficiency of microvascular blood perfusion, which can damage islet β cells and reduce insulin secretion.

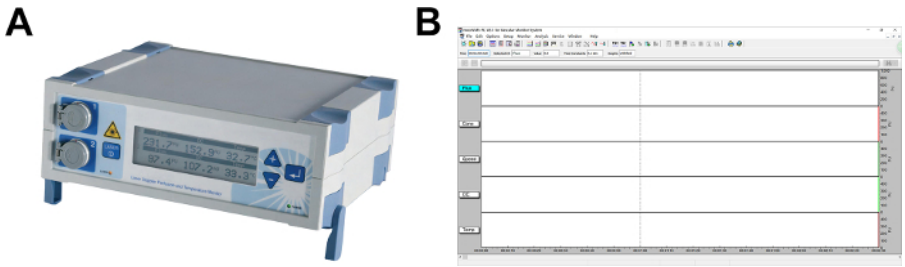

Figure 1. Apparatus used to determine pancreatic islet microvascular vasomotion *in vivo*. A. Photograph of the measurement apparatus used to determine the pancreatic islet microvascular vasomotion of mice. The probe sockets and laser switch button are in the left panel. The liquid crystal display is in the middle panel. Menu buttons (*i.e.,* the up, down, and enter buttons) and the power light-emitting diode are in the right panel. The peripheral devices (*i.e.,* computers and cables) are not shown. **B.** Screenshot illustrating the typical elements and graph channels of the laser Doppler apparatus software. "Flux," "Conc," "DC," and "Speed" measurement readings are displayed in the graph channels. "Flux" represents tissue microvascular blood perfusion, "Conc" represents the tissue microvascular blood cell concentration, "DC" represents the mean intensity of reflective light, and "Speed" represents the relative velocity of microvascular blood flow. Please click here to view a larger version of this figure.

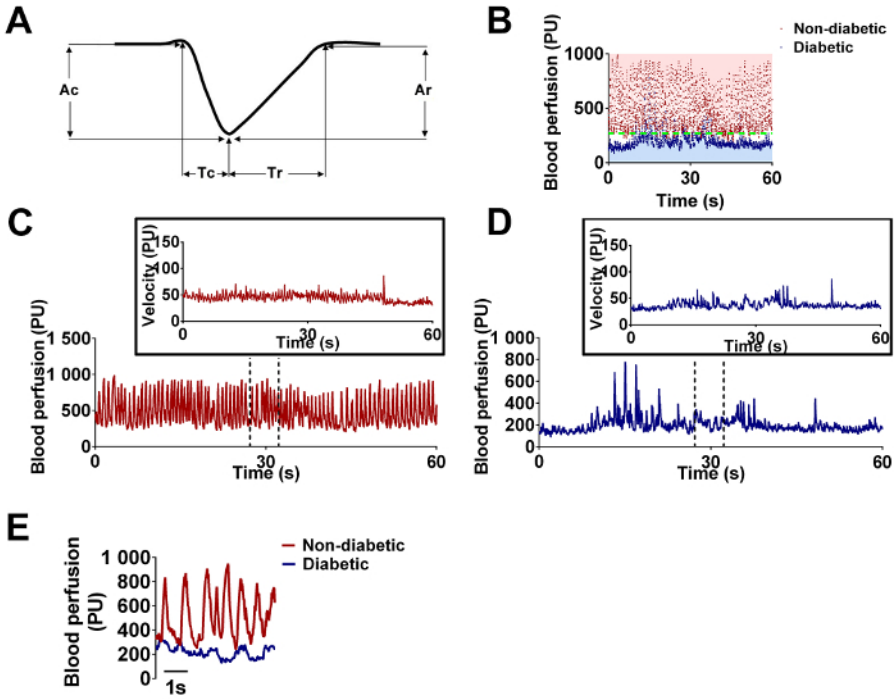

Figure 2. Functional status of pancreatic islet microvascular vasomotion in mice. The blood perfusion of pancreatic islet microvascular vasomotion was assessed by a laser Doppler apparatus, and the functional status was analyzed. **A.** Schematic of parameters relating to microvascular vasomotion. Ac represents the amplitude of a microvascular vasomotion contraction, Ar represents the amplitude of a microvascular vasomotion relaxation, Tc represents the length of the time of a microvascular vasomotion contraction, and Tr represents the length of the time of a microvascular vasomotion relaxation. **B.** Distribution pattern of pancreatic islet microvascular blood perfusion in non-diabetic and diabetic mice. Red dots: non-diabetic mice. Blue dots: diabetic mice. The dashed green line shows the demarcation between the non-diabetic and diabetic microvascular blood perfusion pattern. **C.** Pancreatic islet microvascular vasomotion in the control group was assessed on the basis of the dynamic microvascular perfusion of the blood flow. **D.** Pancreatic islet microvascular vasomotion in the diabetic mice was assessed on the basis of the dynamic microvascular perfusion of the blood flow. **E.** Diagram of representative (5-s range) pancreatic islet microvascular vasomotion between the non-diabetic control and diabetic mice. Red line: non-diabetic control. Blue line: diabetic mice. PU: perfusion units. Please click here to view a larger version of this figure.

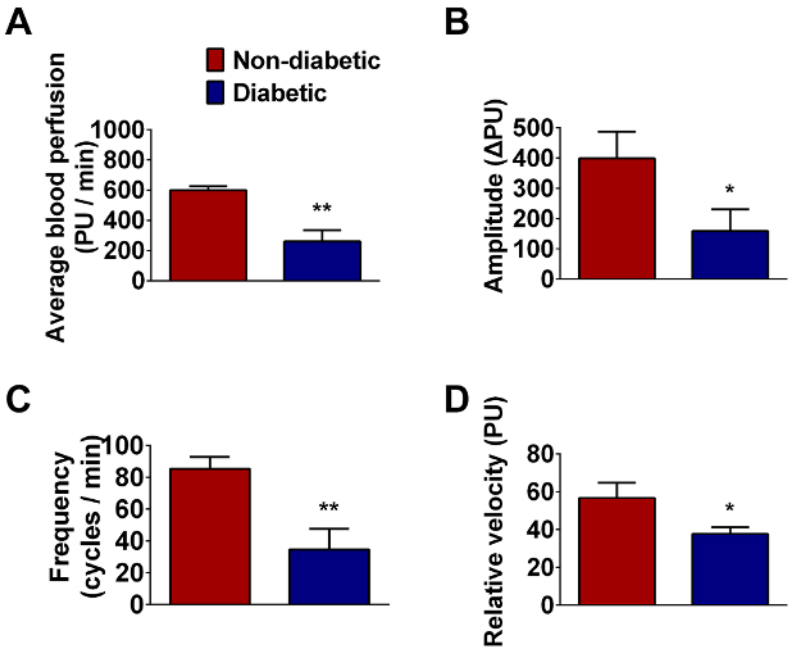

Figure 3. Quantification of the parameters of pancreatic islet microvascular vasomotion. Pancreatic islet microvascular vasomotion parameters, including average blood perfusion, amplitude, frequency, and relative velocity were analyzed and compared between non-diabetic control and diabetic mice. **A.** Quantification of the average blood perfusion (PU/min) of pancreatic islet microvascular vasomotion in non-diabetic and diabetic mice. The **B.** amplitude (ΔPU), **C.** frequency (cycles/min), and **D.** relative velocity (PU) of pancreatic islet microvascular vasomotion were lower in diabetic mice than in non-diabetic control mice. The amplitude of microvascular vasomotion was calculated as the difference between the maximum (PU_{max}) and minimum (PU_{min}). The frequency of microvascular vasomotion was defined as the number of peaks or valleys that occurred in a microvascular vasomotion wave per minute. The data were presented as the mean ± SD (n = 6 in each group). *P < 0.05, **P < 0.01. Please click here to view a larger version of this figure.

Diseases	Object	Apparatus	Refs No.
Endothelial function	H	LDF, LSCI	11, 12, *etc.*
DN	H, R	LDF	13, 14, 15, *etc.*
DR	H	LDF	16, 17, 18, *etc.*
Skin/cutaneous microcirculation	H	LDF	11, 19, 20, *etc.*
Cardiac microcirculation	R	LDF	21
Hearing impairment	M	LDF	22
DN, diabetic neuropathy. DR, diabetic retinopathy. LDF, laser Doppler flowmetry.			
LSCI, laser speckle contrast imaging. R, rat. H, human, M, mouse.			

Table 1. The role of microcirculation in diabetes and its complications. Research groups have used laser Doppler to determine the role of microcirculation in diabetes and its complications for decades. Related articles in recent years are listed here. These published articles mainly focus on endothelial dysfunction, diabetic neuropathy (DN), diabetic retinopathy (DR), skin and cutaneous microvascular impairment, and relatively rare complications such as cardiac microcirculation dysfunction and hearing impairment. DN: diabetic neuropathy. DR: diabetic retinopathy. LDF: laser Doppler flowmetry. LSCI: laser speckle contrast imaging. R: rat. H: human. M: mouse.

Discussion

In the cases that involve microvascular dysfunction (*e.g.,* diabetes, acute pancreatitis, peripheral microvascular diseases, *etc.*), some diseases lead to reduced blood flow. Other than changes in blood flow, there are important indicators, such as microvascular vasomotion, that mirror the functional status of microcirculation. The specific indicator, microvascular vasomotion, is generally defined as the oscillation of the microvascular tone in microvascular beds. In the current protocol, a microvascular blood perfusion monitoring system allows for the direct visualization and quantitative analysis of the functional status of microvascular vasomotion. Our microcirculatory evaluation approach can be applied selectively to the targeted tissues and organs by identifying dynamic changes in blood perfusion. Reports published by other groups about using laser Doppler to determine the role of microvascular blood perfusion in diabetes and its complications were summarized in **Table 1**. In the current study, to demonstrate our approach, the functional status of the pancreatic islet microvascular vasomotion of diabetic mice was evaluated.

Microvascular vasomotion is recognized as a parameter of the functional status of microcirculation and is capable of regulating blood flow perfusion by adjusting the distribution in local tissue[23]. The microvasculature of the pancreas, which can be divided into islets, acini, and ducts, has been studied for decades. Basically, this separation of the pancreas into different parts is for convenience only because the microvasculature

is actually interconnected and homogeneous as an organic entity[24]. This microvasculature network supports the regulation of pancreatic islet blood flow. Hence, we used parameters of the functional status, determined by laser Doppler, to represent pancreatic islet microvasculature vasomotion. However, because of the characteristics of pancreatic architecture, we still failed to make a judgment after applying the current method to ascertain whether the blood perfusion is derived from the endocrine part or from the exocrine part of pancreas. Using islet-specific labeling dyes, such as dithizone and neutral red, may become one of the possible ways to understand this issue, at least to some extent.

An important aspect of the measurement step is the distance between the probe and the pancreas tissue. An inappropriate distance gives an artificially increased blood flow reading. The physical force applied to the tissue and organ by a probe tip will reduce microvascular blood flow. Hence, minimal pressure should be applied when taking measurements. Another point to note is the power of lasers. High-power lasers generally easily injure microvessels in the pancreatic islet, so the frequency of the laser beam needs to be controlled, within limitations. For general and temporal measurements, a frequency of 1 Hz or less is recommended. To avoid the localized exhaustion of microvascular vasomotion capacity (including contractile and relaxation) and the additive effect, multipoint determination and repositioning site after each measurement are suggested in any experiments.

In the current method, the PU data are used to represent the blood flux of microvascular blood flow. Because of the characteristics of microvascular blood flow in microcirculation, it is not feasible to determine the absolute flow units (*e.g.,* mL/min/100 g of specific organs or tissues). Therefore, the assessment parameter system used here is based on the relative blood flow perfusion units. Wavelet analysis, fast Fourier transform, and other spectral analysis algorithms are common methods that conduct laser Doppler signals. In the present protocol, we established an approach that uses hemodynamic parameters (*i.e.,* blood perfusion, amplitude, frequency, and relative velocity) to show the functional status of microvascular vasomotion. Moreover, the accuracy of the measurement is related to the depth of the target and the probe design, which is generally around 1 mm. Thus, thicker or compact organs and tissues may not be appropriate for the application of laser Doppler and for the current method. Additionally, because the data derived from blood flow perfusion could be affected by other conditions that causes noticeable changes, including temperature, humidity, external light, and alterations in the position of the mice, some rules should be obeyed during the experimental proceedings. The laboratory needs to maintain constant temperature and humidity, and external lighting needs to be shielded. It is recommended to fix the mice to avoid changes in position. It is believed that these strategies can overcome the limitations mentioned above and will increase the accuracy of blood flow perfusion data.

The advantage of the present protocol comparing with others reported in the literatures is that it is sensitive and responsive to the local microvascular vasomotion of tissues and organs. This will facilitate the broader application of the method to the assessment or investigation of microcirculation, especially the functional status of microvascular vasomotion, in both clinical and laboratorial research. The applications include but are not limited to: ischemia visualization, blood perfusion assessment, and the evaluation of the functional status of microvascular vasomotion. In conclusion, our method can be used to investigate and evaluate the functional status of pancreatic islet microvascular vasomotion in mice *in vivo* and may be able to meet the clinical need to assess microcirculation function.

Disclosures

The authors have nothing to disclose.

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