# Journal of Visualized Experiments

## Stabilized longitudinal in vivo cellular-level visualization of the pancreas in a murine model with a pancreatic intravital imaging window

--Manuscript Draft--

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|---|---|
| **Article Type:** | Invited Methods Collection - JoVE Produced Video |
| **Manuscript Number:** | JoVE62538R2 |
| **Full Title:** | Stabilized longitudinal in vivo cellular-level visualization of the pancreas in a murine model with a pancreatic intravital imaging window |
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| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
| Please specify the section of the submitted manuscript. | Medicine |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea |
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**TITLE:**

# Stabilized Longitudinal *In Vivo* Cellular-Level Visualization of the Pancreas in a Murine Model with a Pancreatic Intravital Imaging Window

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**SUMMARY:**

*In vivo* high-resolution imaging of the pancreas was facilitated with the pancreatic intravital imaging window.

**ABSTRACT:**

Direct *in vivo* cellular-resolution imaging of the pancreas in a live small animal model has been technically challenging. A recent intravital imaging study, with an abdominal imaging window, enabled visualization of the cellular dynamics in abdominal organs *in vivo*. However, due to the soft sheet-like architecture of the mouse pancreas that can be easily influenced by physiologic movement (e.g., peristalsis and respiration), it was difficult to perform stabilized longitudinal *in vivo* imaging over several weeks at the cellular level to identify, track, and quantify islets or cancer cells in the mouse pancreas. Herein, we describe a method for implanting a novel supporting base, an integrated pancreatic intravital imaging window, that can spatially separate the pancreas from the bowel for longitudinal time-lapse intravital imaging of the pancreas microstructure. Longitudinal *in vivo* imaging with the imaging window enables stable visualization, allowing for the tracking of islets over a period of 3 weeks and high-resolution three-dimensional imaging of the microstructure, as evidenced here in an orthotopic pancreatic cancer model. With our method, further intravital imaging studies can elucidate the pathophysiology of various diseases involving the pancreas at the cellular level.

**INTRODUCTION:**

The pancreas is an abdominal organ with an exocrine function in the digestive tract and an endocrine function of secreting hormones into the bloodstream. High-resolution cellular imaging

of the pancreas could reveal the pathophysiology of various diseases involving the pancreas, including pancreatitis, pancreatic cancer, and diabetes mellitus[1]. Conventional diagnostic imaging tools such as computed tomography, magnetic resolution imaging, and ultrasonography are widely available in the clinical field[1,2]. However, these imaging modalities are restricted to visualizing only structural or anatomical changes, while alterations at the cellular or molecular level cannot be determined. Given that molecular changes in diabetes mellitus or pancreatic cancer can initiate more than 10 years prior to the diagnosis[3,4], the detection of pancreatic diseases from their molecular transition during the latent period has the potential to provide an early diagnosis and a timely intervention. Thus, imaging that will overcome the limitations of resolution and provide valuable insights into the function will remarkably gain attention by providing early diagnosis of pancreatic cancer or advanced identification of the alteration of the islets during the progression of diabetes mellitus[5].

In particular with the islets, nuclear imaging, bioluminescence imaging, and optical coherence tomography have been suggested as non-invasive islet imaging techniques[6]. However, the resolution of these methods is substantially low, with typical values ranging from several tens to hundreds of micrometers, offering a limited capability to detect changes at the cellular level in the islets. On the other hand, previous high-resolution studies of islets were performed under *ex vivo*[7,8] (e.g., slicing or digestion of the pancreas), non-physiologic[9] (e.g., exteriorization of the pancreas), and heterotopic conditions[10–12] (e.g., implantation under the kidney capsule, inside the liver, and in the anterior chamber of the eye), which restricts their interpretation and clinical implications. If *in vivo*, physiologic, and orthotopic model of high-resolution imaging can be established, it will be a critical platform for the investigation of pancreatic islets.

Intravital imaging, which reveals the pathophysiology at a microscopic resolution level in a live animal, has recently received great attention[13]. Of the *in vivo* imaging methods, the development of an abdominal imaging window[14], which implants a window into the abdomen of a mouse, has allowed the discovery of novel findings (i.e., a pre-micrometastasis stage of early liver metastasis[15] and mechanism of stem cell maintenance in the intestinal epithelium[16]). Although the abdominal imaging window provides valuable results, the applications of this window for the pancreas and the resulting intravital imaging research based on diseases involving pancreas, have not been extensively investigated.

Unlike the well-defined solid organ characteristics of the human pancreas, the pancreas of a mouse is a diffusely distributed soft tissue-like structure[17]. Therefore, it is incessantly affected by physiological movements including peristalsis and respiration. A previous study on the application of an abdominal imaging window for the pancreas demonstrated that wandering occurred due to motion-artifacts induced by bowel movements[18]. Severe blurring was observed in the resulting averaged image, which impeded the visualization and identification of the microscale structures.

Herein, we describe the use of a novel supporting base integrated pancreatic intravital imaging window combined with intravital microscopy[19,20] to investigate the longitudinal cellular level events in diseases involving the pancreas. In addition to a detailed description of the

methodology in the previous study[18], the extended application of pancreatic imaging window for various diseases involving the pancreas will be addressed in this paper. In this protocol, a custom-built video-rate laser-scanning confocal microscopy system was utilized as an intravital microscopy system. Four laser modules (wavelengths at 405, 488, 561, and 640 nm) were utilized as an excitation source, and four channels of emission signals were detected by photomultiplier tubes (PMT) through bandpass filters (BPF1: FF01-442/46; BPF2: FF02-525/50; BPF3: FF01-600/37; BPF4: FF01-685/40). Laser scanning consisted of a rotating polygonal mirror (X-axis) and a galvanometer scanning mirror (Y-axis) that enabled the video-rate scanning (30 frames per second). Detailed information about intravital microscopy has been described in the previous studies[10,18–23].

In our previous islet study[18], we successfully and stably imaged the islets in live mice using a transgenic mouse model (MIP-GFP)[24] in which the islets were tagged with GFP. The method enabled high-resolution visualization of the changes in the islets over a period of 1 week. It also facilitated imaging of the same islets for up to 3 weeks, which suggests the feasibility of long-term studies of the pancreatic islets for the functional tracking or monitoring during the pathogenesis of diabetes mellitus[18]. Furthermore, we developed an orthotopic pancreatic cancer model in which fluorescent pancreatic cancer cells (PANC-1 NucLight Red)[25] were directly implanted into the pancreas of the mouse. With the application of the pancreatic intravital imaging window, this model could be utilized as a platform for investigating the cellular and molecular pathophysiology in the tumor microenvironment of pancreatic cancer and for the therapeutic monitoring of novel drug candidates.

**PROTOCOL:**

All procedures described in this paper were conducted in accordance with the 8th edition of the Guide for the Care and Use of Laboratory Animals (2011)[26] and approved by the Institutional Animal Care and Use Committee at the Korea Advanced Institute of Science and Technology (KAIST) and Seoul National University Bundang Hospital (SNUBH).

## 1. Preparation of the window and other materials

1.1. Custom design the pancreatic intravital imaging window to seclude the pancreas from the bowel in the abdominal cavity[18] (**Figure 1A,B**). A detailed blueprint of the window is described in a supplementary figure of a previous study[18].

1.2. Use C57BL/6N mice, 8–12-week-old males, for intravital pancreatic imaging. Inject anti-CD31 antibody conjugated with an Alexa 647 fluorophore, 2 h prior to the imaging, for the purpose of vessel labeling[18].

1.3. For the islets study, prepare a transgenic mouse model in which the islets are tagged with a fluorescent reporter protein. Here, we utilized MIP-GFP, where green fluorescent protein was expressed under the control of the mouse insulin 1 gene promoter, which is active in the beta cells of all islets in the mouse[24].

1.4. For the pancreatic cancer study, prepare transgenic cancer cells tagged with a fluorescent reporter protein and BALB/C nude mice. In this study, the PANC-1 NucLight Red cells were used. PANC-1 cancer cells[25] were labeled with the NucLight Red fluorescent probe.

1.5. Sterilize all surgical tools and imaging windows using an autoclave.

1.6. Apply PEG coating to the cover glass to prevent inflammatory response and increase biocompatibility, which is suitable for long-term imaging.

## 2. Surgery

2.1. Prepare a sterile surgical platform and sterilize the surfaces with 70% ethanol.

NOTE: For longitudinal imaging sessions, consider using aseptic techniques.

2.2. Anesthetize mice with a mixture of tiletamine/zolazepam (30 mg/kg) and xylazine (10 mg/kg).

NOTE: Use of tiletamine/zolazepam is recommended instead of ketamine because of its adverse effect of hyperglycemia. Optimal anesthesia should be selected for the purpose of the experiment[9,27].

2.3. Monitor the body temperature using a rectal probe with a homeothermic controlled heating pad.

2.4. Shave the left flank of the mouse and apply three rounds of alternating alcohol and iodine-based scrub.

2.5. Make a 1.5 cm incision on the left flank of the mouse and dissect the skin and muscle.

2.6. Perform a purse-string suture with a black or nylon 4-0 suture in the incision margin.

2.7. Use a micro retractor on the incision and gently expose the spleen.

2.8. Carefully pool the spleen with ring forceps and identify the pancreas.

2.9. Place the window at the flank of the mouse and pass the spleen and pancreas through the open space of the window.

2.10. Gently place the pancreas on the plate of the imaging window; the spleen will be placed on the open space of the window.

2.11. For the cancer cell study, inject PANC-1 NucLight Red ($1.0 \times 10^6$ cells) directly into the pancreas.

NOTE: For imaging the orthotopic human pancreatic cancer xenografts, direct implantation of cancer cell such as PANC-1 or other human pancreatic cancer cell could be facilitated[28]. To visualize with intravital fluorescence microscopy, PANC-1 cells were transduced with red fluorescent protein using the NucLight Red lentiviral reagent that labels the nucleus[29].

2.12. Apply drops of N-butyl cyanoacrylate glue on the margin of the imaging window.

2.12.1. To minimize the amount of the applied glue, use a 31 G catheter needle for the application. If the amount of the drop is large, then the tissue will unintentionally adhere to the window or cover glass.

2.13. Gently apply a 12 mm round cover glass to the margin of the imaging window.

2.14. Pull the suture loop to fit into the lateral groove of the window and tie it three times.

2.15. Cut the maximal proximal site of the tie to prevent the interruption of tight stitches when these mice are awake.

2.16. Let mice recover from anesthesia and inject ketoprofen (5 mg/kg, intramuscular) for pain relief.

NOTE: Analgesia influences insulin secretion in response to glucose[9]. The choice and timing of analgesia must be individualized for the experimental purpose.

## 3. Intravital imaging

3.1. Turn on the intravital microscope including the laser power.

3.2. Turn on the heating pad and set the homeothermic regulation to 37 °C.

NOTE: Alternatively, use a passive heating pad or lamp with frequent control if there is no homeothermic regulation.

3.3. Perform intramuscular anesthesia with a mixture of tiletamine/zolazepam (30 mg/kg) and xylazine (10 mg/kg).

NOTE: Use of tiletamine/zolazepam is recommended instead of ketamine because of its adverse effect of hyperglycemia. Optimal anesthesia should be selected for the purpose of the experiments[9,27].

3.4. Insert a vascular catheter for the injection.

3.4.1. Apply pressure on the proximal side of the tail with the index and third finger as an

alternative to a tourniquet application. Heat the tail with a lamp if needed.

3.4.2. Sterilize the tail vein with a 70% ethanol spray.

3.4.3. Insert a 30 G catheter into the lateral tail vein. Regurgitation of blood will be visualized in the PE10 tube.

3.4.4. Apply a silk tape on the catheter to stabilize it.

3.4.5. Inject FITC/TMR dextran or other fluorescent probes (25 µg of anti-CD31 conjugated with Alexa 647), as appropriate, according to the combination of fluorescent probes[18].

NOTE: For fluorescent conjugated antibody probes, inject 2 h before the imaging session.

3.5. Transfer the mouse from the surgical platform to the imaging stage.

3.6. Insert a rectal probe to automatically control the body temperature with the homeothermic heating pad system.

3.7. Insert the pancreatic imaging window into the window holder prepared during the intravital microscopy setup (**Figure 2**). For an inverted microscope, a window holder might not be required.

3.8. Perform intravital imaging.

3.8.1. For imaging the pancreas, start with a low magnification objective lens (e.g., 4x) for scanning the whole view of the pancreas in the pancreatic imaging window (recommended field of view: 2500 x 2500 µm).

3.8.2. After determination of the region of interest, switch to higher magnification objective lens (20x or 40x) to perform the cellular level imaging (recommended field of view: 500 x 500 µm or 250 x 250 µm). In this experiment, the lateral and axial resolution was approximately 0.5 µm and 3 µm, respectively.

3.8.3. Perform z-stack or time-lapse imaging to observe the 3D structure or cellular-level dynamics, such as cell migration.

NOTE: For imaging the fluorescent protein expressing cells of transgenic animals (MIP-GFP), 30 s of intermittent 488 nm laser exposure with power up to 0.43 mW was tolerable without noticeable photobleaching or tissue damage. For imaging the fluorescent proteins labeled with Alexa 647, the 640 nm laser power up to 0.17 mW was tolerable without noticeable photobleaching or tissue damage. Prolonged excitation laser exposure with a power above this setting may lead to photobleaching or tissue damage by phototoxicity. Adjust the adequate gain and power to appropriately image the region of interest. Detailed setting of parameters in

intravital microscopy must be individualized for each intravital microscopy prepared in the institute.

**REPRESENTATIVE RESULTS:**

Intravital microscopy combined with the supporting base integrated pancreatic intravital imaging window enables longitudinal cellular level imaging of the pancreas in a mouse. This protocol with the pancreatic intravital imaging window provides long-term tissue stability that enables the acquisition of high-resolution imaging to track individual islets for up to 3 weeks. As a result, mosaic imaging for an extended field of view, three-dimensional (3D) reconstruction of z-stack imaging, and longitudinal tracking of the same position can be achieved. In addition, our intravital microscopy provides four channels (405, 488, 561, and 647 nm) of acquisition, which enables simultaneous multiple cell visualization with their interactions.

For the preliminary imaging, the window was implanted in a C57BL/6N mouse with intravenously injected anti-CD31 antibody conjugated with an Alexa 647 fluorophore. Wide-area imaging (**Figure 3A**) and magnified 3D imaging (**Figure 3B-D, Supplementary Video 1**) of the pancreas were facilitated with this system. Pancreatic tissue was visualized with autofluorescence, and the adjacent vasculature labeled with the anti-CD31 antibody was identified. Oscillation due to either peristalsis or respiration was not identified, resulting in averaged imaging with a high signal-to-noise ratio (**Figure 4**). Acinar cells, which require visualization at a microscale resolution in the pancreas, were clearly visualized in the averaged images.

For imaging of the islets, a MIP-GFP mouse was utilized. Using the mosaic imaging method, a wide-field view with high-resolution imaging enabled the visualization of the islets with the adjacent vasculature (**Figure 5**). Approximately 40–50 islets were identified in the wide-field view. This stable imaging method could further facilitate the tracking of the islets for up to 3 weeks, as shown in a previous study (**Figure 6**)[18].

For the cancer cell imaging, PANC-1 NucLight Red cells were directly implanted into the mouse pancreas during surgery (**Figure 7**). A dual-labeling strategy was used, consisting of PANC-1 NucLight Red cells and nearby vessels stained with anti-CD31 conjugated with Alexa 647. With our protocol, wide-field imaging of pancreatic cancer (**Figure 7A**), which delineates the margin of the tumor, and high-resolution 3D imaging at the single-cell level, was achieved (**Figure 7B–D, Supplementary Video 2**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Design and photograph of the pancreatic intravital imaging window**. (**A**) A 3D and cross-sectional view of the pancreatic intravital imaging window. A detailed blueprint of the size and diameter is described in the previous paper[18]. (**B**) Anterior and posterior photograph of the pancreatic imaging window. Copyright 2020 Korean Diabetes Association from Diabetes Metab J. 2020 44:1:193-198. Reprinted with permission from The Korean Diabetes Association.

**Figure 2: Photograph of the implementation of the pancreatic intravital imaging window**. A pancreatic intravital imaging window is implanted in the mouse in the XYZ translational stage and

the imaging chamber holder attached to the tilting mount is connected to the pancreatic imaging window. Copyright 2020 Korean Diabetes Association from Diabetes Metab J. 2020 44:1:193-198. Reprinted with permission from The Korean Diabetes Association.

**Figure 3: Representative intravital pancreatic imaging in C57BL/6N mouse**. (**A**) Wide-area image and (**B**) magnified 3D image of the pancreas (green) and its microvasculature (red) in the C57BL/6N mouse. Vessels are labeled with an anti-CD31 antibody conjugated with the Alexa 647 fluorophore. (**C**) 3D reconstructed image and (**D**) surface-rendering image of the mouse pancreas. Scale bar: 200 µm (A) and 50 µm (B–D). Also see **Supplementary Video 1**.

**Figure 4: Intravital imaging of acinar cell and adjacent vasculature**. Acinar cells (green) and adjacent vasculature (red) in the C57BL/6N mouse. Vessels are labeled with Anti-CD31 antibody conjugated with the Alexa 647 fluorophore. Tissue stability accomplished with the pancreatic imaging window provides a high signal-to-noise ratio image. Scale bar: 50 µm.

**Figure 5: Representative intravital imaging of pancreatic islets in the MIP-GFP mouse.** Wide-area mosaic and magnified image of the islets (green) and adjacent vasculature (red) processed with maximum intensity projection method in the pancreas of the MIP-GFP mouse. Vessels are labeled with an anti-CD31 antibody conjugated with the Alexa 647 fluorophore. Scale bar: 500 µm (wide area) and 50 µm (magnified).

**Figure 6: Longitudinal intravital imaging of islets in the pancreas of the MIP-GFP mouse.** Longitudinal image of the islets for up to 3 weeks in the pancreas in the MIP-GFP mouse. Each arrowhead with different colors indicates the same islets. Scale bar: 100 µm. Copyright 2020 Korean Diabetes Association from Diabetes Metab J. 2020 44:1:193-198. Reprinted with permission from The Korean Diabetes Association.

**Figure 7: Representative intravital imaging of the pancreatic cancer model.** (**A**) Wide-area image and (**B**) magnified 3D image of the implanted PANC-1 NucLight Red cells (red) in the BALB/c Nude mouse. Vessels (blue) are labeled with an anti-CD31 antibody conjugated with the Alexa 647 fluorophore. (**C**) 3D reconstructed image and (**D**) surface-rendering image of pancreatic cancer in the mouse model. Scale bar: 500 µm (A) and 50 µm (B–D). Also see **Supplementary Video 2**.

**Supplementary video 1: *In vivo* 3D pancreatic imaging in C57BL/6N mouse.** *In vivo* 3D imaging of pancreas (green) of C57BL/6N mouse intravenously injected with an anti-CD31 antibody conjugated with the Alexa 647 fluorophore (red). Scale bar is depicted in the video. This video corresponds to **Figure 3C,D**.

**Supplementary video 2: *In vivo* 3D pancreatic cancer (PANC-1 NucLight Red) imaging in BALB/C Nude mouse.** *In vivo* 3D imaging of pancreatic cancer (red) implanted in BALB/C Nude mouse intravenously injected with an anti-CD31 antibody conjugated with the Alexa 647 fluorophore (blue). Scale bar is depicted in the video. This video corresponds to **Figure 7C,D**.

**DISCUSSION:**

The protocol described here consists of intravital imaging of the pancreas using a novel supporting base integrated pancreatic intravital imaging window modified from an abdominal imaging window. Among the protocols described above, the first critical step is the implantation of the intravital pancreatic imaging window in the mouse. For the application of the glue in the window, it is important to apply the glue between the margin of the window and the cover glass, but not on the pancreatic tissue, as it may significantly interrupt intravital imaging. Not only the glue itself between the glass and tissue but also adjunct dust particles may induce light scattering during imaging if the glue is directly applied to the tissue. In addition, the application of the adhesive may have toxic and non-physiological effects on the pancreas.

The second step is the amount of pancreatic tissue placed on the metal-supporting base plate. Because the pancreatic tissue is a sheet-like structure, the volume of pancreatic tissue on the plate needs to be controlled. If too large a volume is placed on the plate, the glue applied to the margin of the ring might adhere to the tissue, and the mass effect might hamper the perfusion of the pancreas. On the other hand, if too small a volume is placed on there, the field of view that can be visualized might be limited. This protocol of surgery on the window may require several trials to meet a consistent standard.

For long-term imaging over a period of 3 weeks, the most concerning issue was potential damage to the pancreatic imaging window. Unintended destruction of the cover glass in the pancreatic imaging window could occur during the long-term observation period. To prevent this, the mouse with the window must be housed separately, and hard objects with sharp edges should be removed from the cage. The euthanasia of mice should be considered should the cover glass break, if there are the severe signs of inflammation near the window, or if the animal appears to be in distress. In our experience, mice with the pancreatic imaging window were able to eat and exercise normally when the recovery after the surgery was appropriate and no other complication was developed.

In our previous experience with the abdominal imaging window, we failed to acquire high-quality cellular level imaging as well as longitudinal tracking of the same spots over multiple days. Compared to the abdominal imaging window, which provides a diverse platform for various abdominal organs, the pancreatic imaging window is further specified for imaging the pancreas as well as other organs that are soft and easily influenced by movements such as mesentery, spleen, and small bowel. However, the liver and kidney might be unfeasible in the pancreatic imaging window because of the limited space.

While the combination of a fluorescent mouse, cells, and antibody probes enables the visualization of the dynamic interactions between endothelial cells and either the islets or cancer cells, the protocol described here could be reproduced with other compositions of fluorescent-labeled cells or molecular probes suitable for each respective condition. Furthermore, expansive applications integrated with our method are expected, such as the CpepSfGFP reporter mouse with insulin secretion[9,30], AAV8-mediated gene delivery targeting reactive oxygen species (ROS)[31], or orthotopic tumor model[32–34] in which the tumor *in situ* can fully stimulate the tumor microenvironment, including tumorigenesis, development, and metastasis[35]. Furthermore,

patient-derived xenograft models can also be studied using our platform[36].

There are a few limitations to be addressed in this study. First, even when we utilized the metal base for stabilization, we were unable to determine the mechanical stress induced on the tissue by the base and cover glass, which could affect blood flow. However, as depicted in the above figures, intravenous injection of a fluorescence-conjugated antibody (CD31) or dextran adequately labeled the vessel with no distinguishable non-perfused area, suggesting a minimal impact of mechanical stress on the normal blood flow inside the pancreatic tissue. Second, adverse reactions due to the adhesive could not be assessed in the pancreatic tissue. Nevertheless, we attempted to avoid touching pancreas with adhesives as carefully as possible to avoid any additional effects. Third, as discussed above, the unintended impact of anesthetic agents might affect insulin sensitivity and secretion, as described in the previous study[9,27]. In our experience, a mixture of ketamine and xylazine induced hyperglycemia compared to the mixture of tiletamine, zolazepam, and xylazine. A further study investigating the effect of anesthesia on insulin secretion should be performed and proper anesthesia with minimal adverse effects should be selected according to each experiment. Fourth, imaging of the pancreas is focused on the tail portion, and imaging of the head portion of the pancreas could be limited with our window.

In summary, a stabilized longitudinal imaging of the pancreas at the cellular level for up to several weeks was facilitated by our imaging system integrated with the pancreatic intravital imaging window optimized for *in vivo* pancreas imaging. Because intravital imaging provides dynamic insights into cell biology, immunology, and tumor biology, this protocol could be a useful method for investigating the pathophysiology of various diseases involving the pancreas.

**ACKNOWLEDGMENTS:**
This study was supported by grant No. 14-2020-002 from the SNUBH Research Fund and by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NRF-2020R1F1A1058381, NRF-2020R1A2C3005694).

**DISCLOSURES:**
The authors have nothing to disclose.

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Figure 1



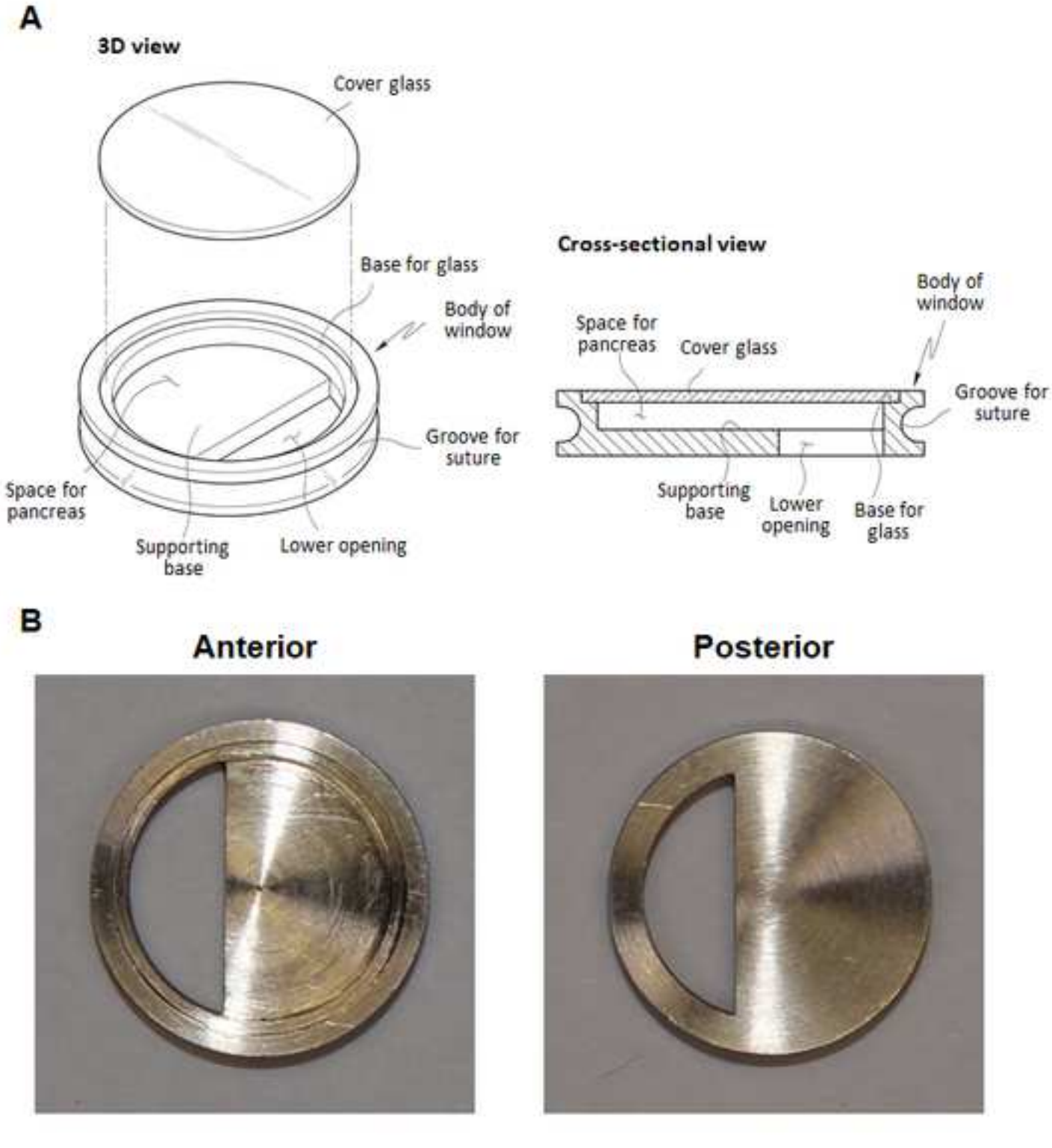

Figure 2

Click here to access/download;Figure;Figure2.psd

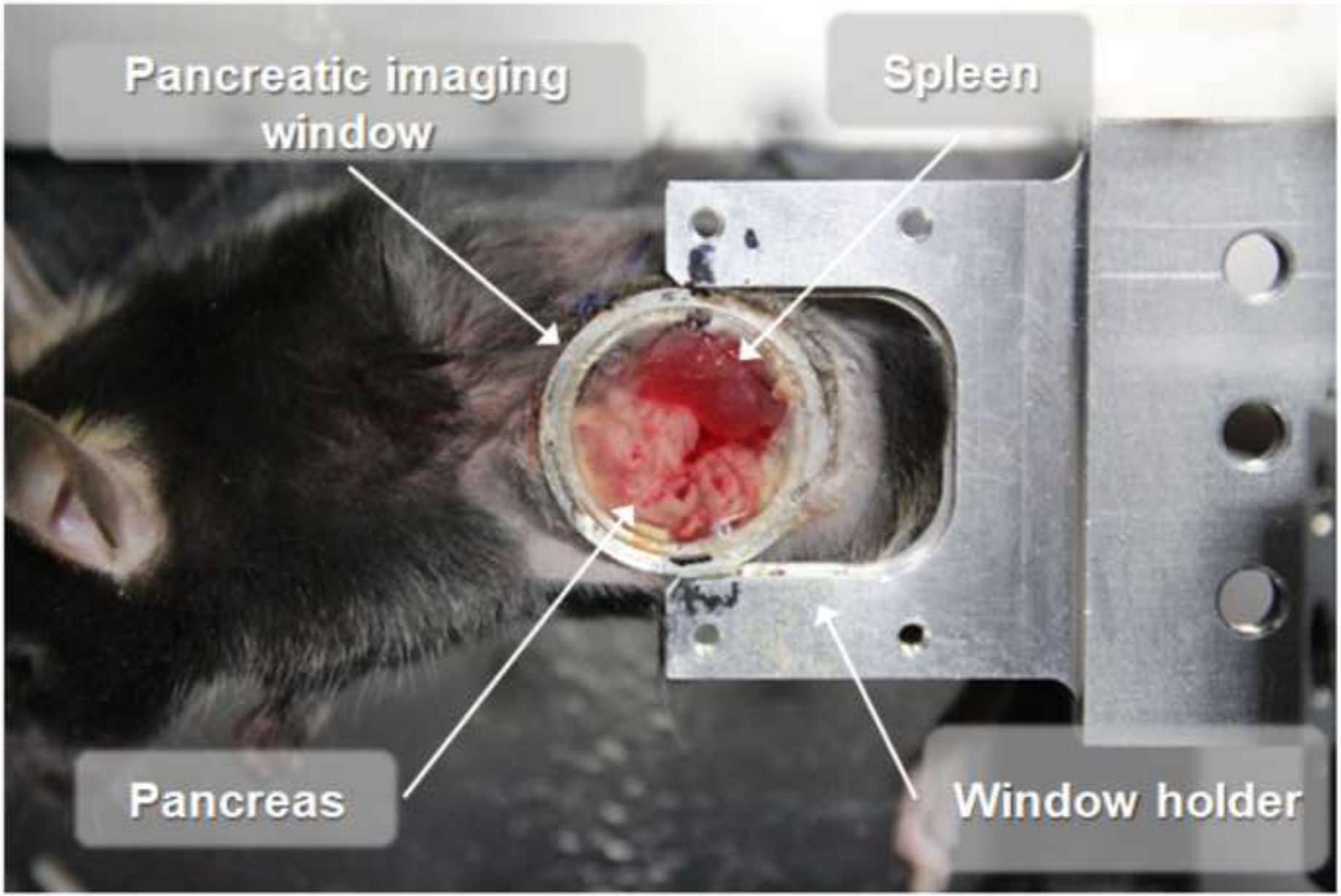

Figure 3

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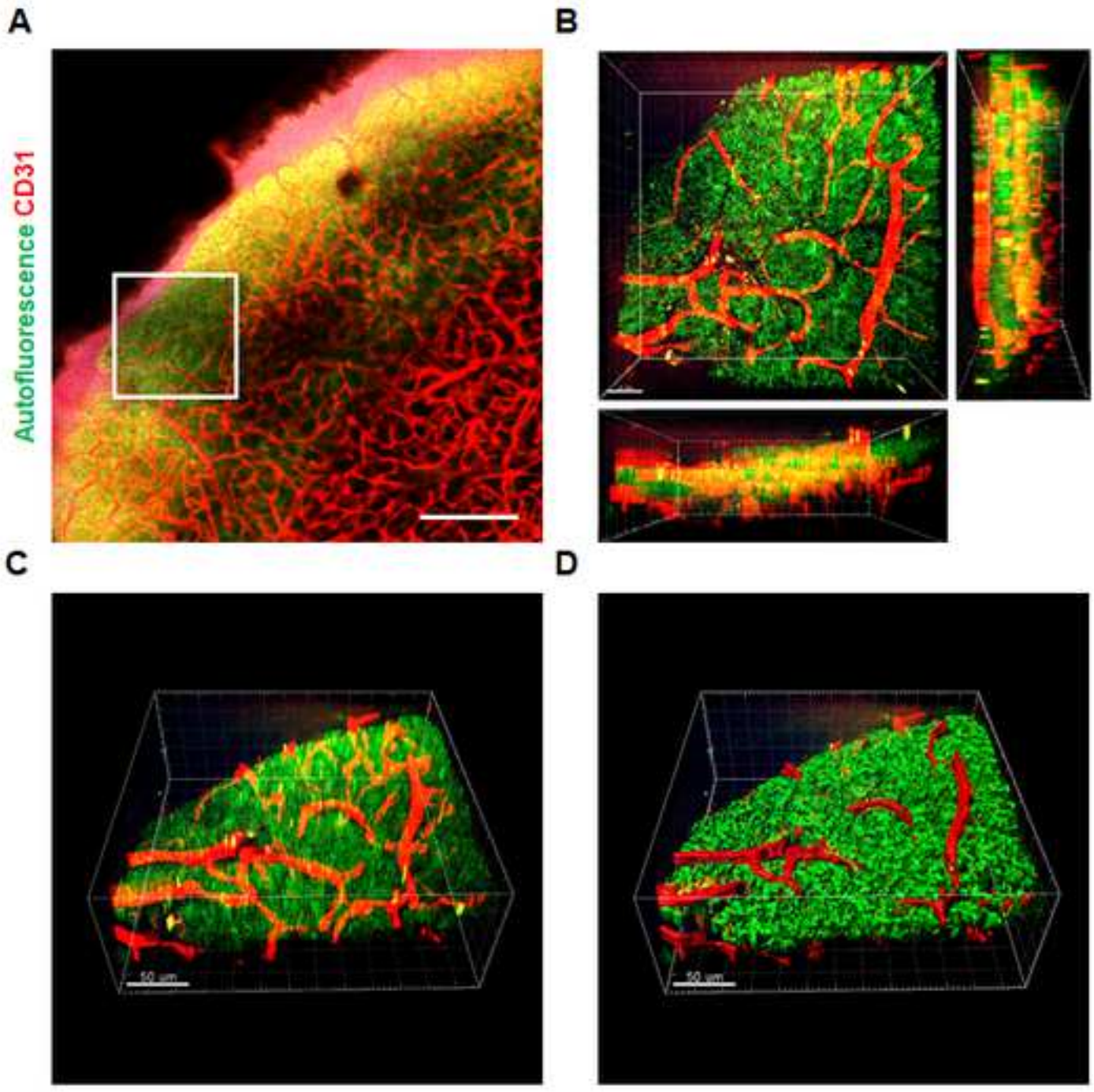

Figure 4

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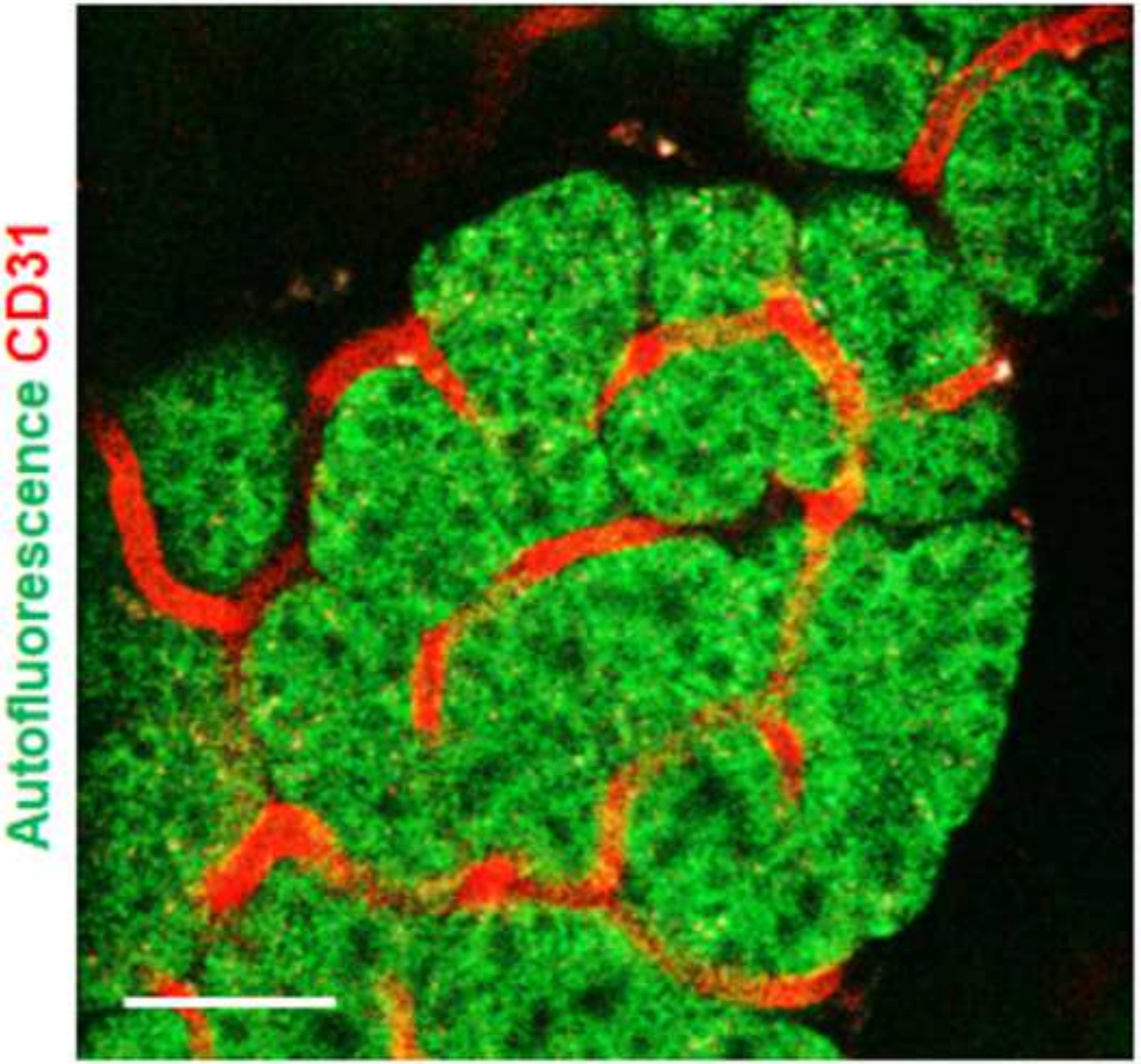

Figure 5

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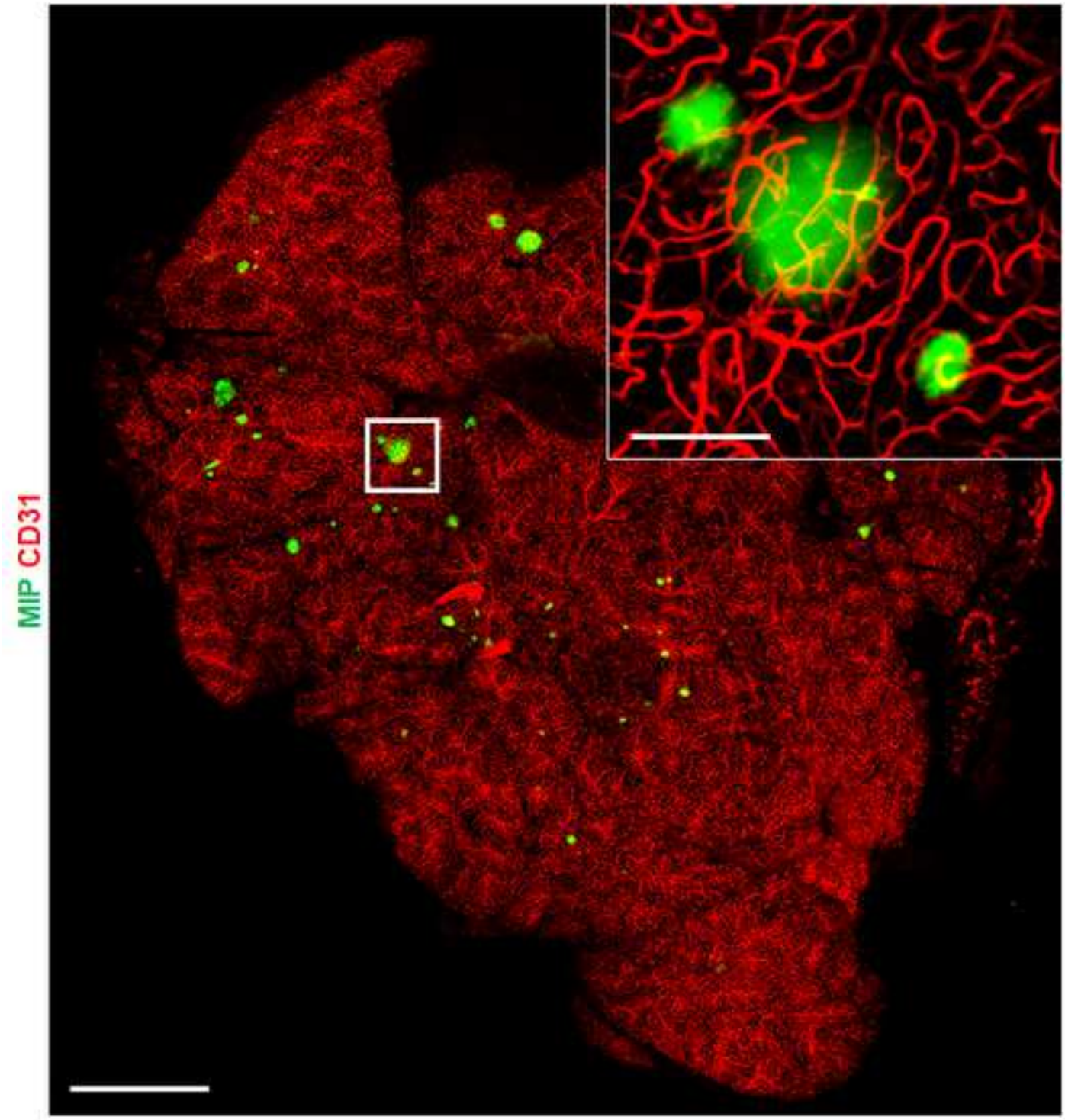

Figure 6

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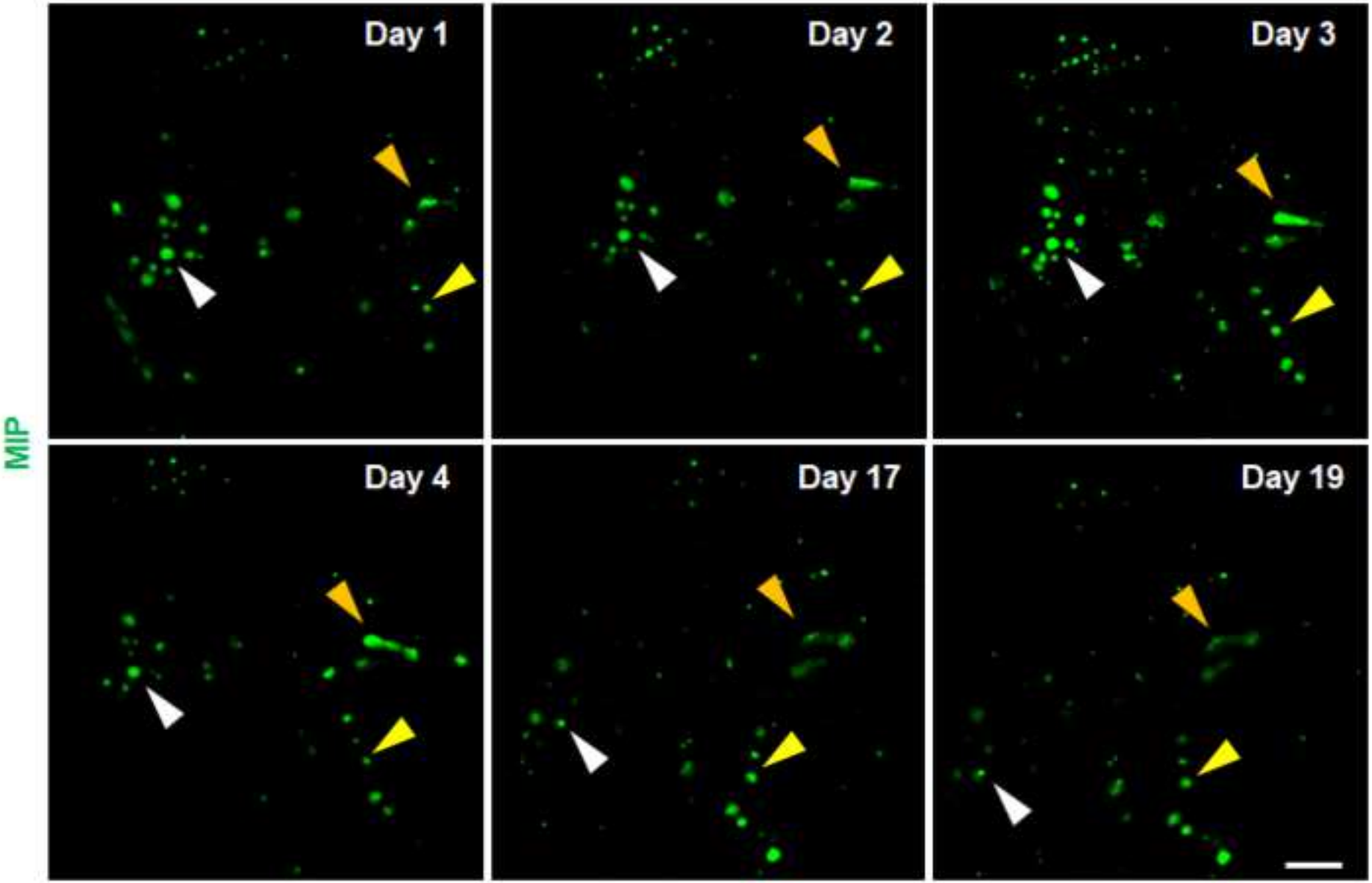

Figure 7

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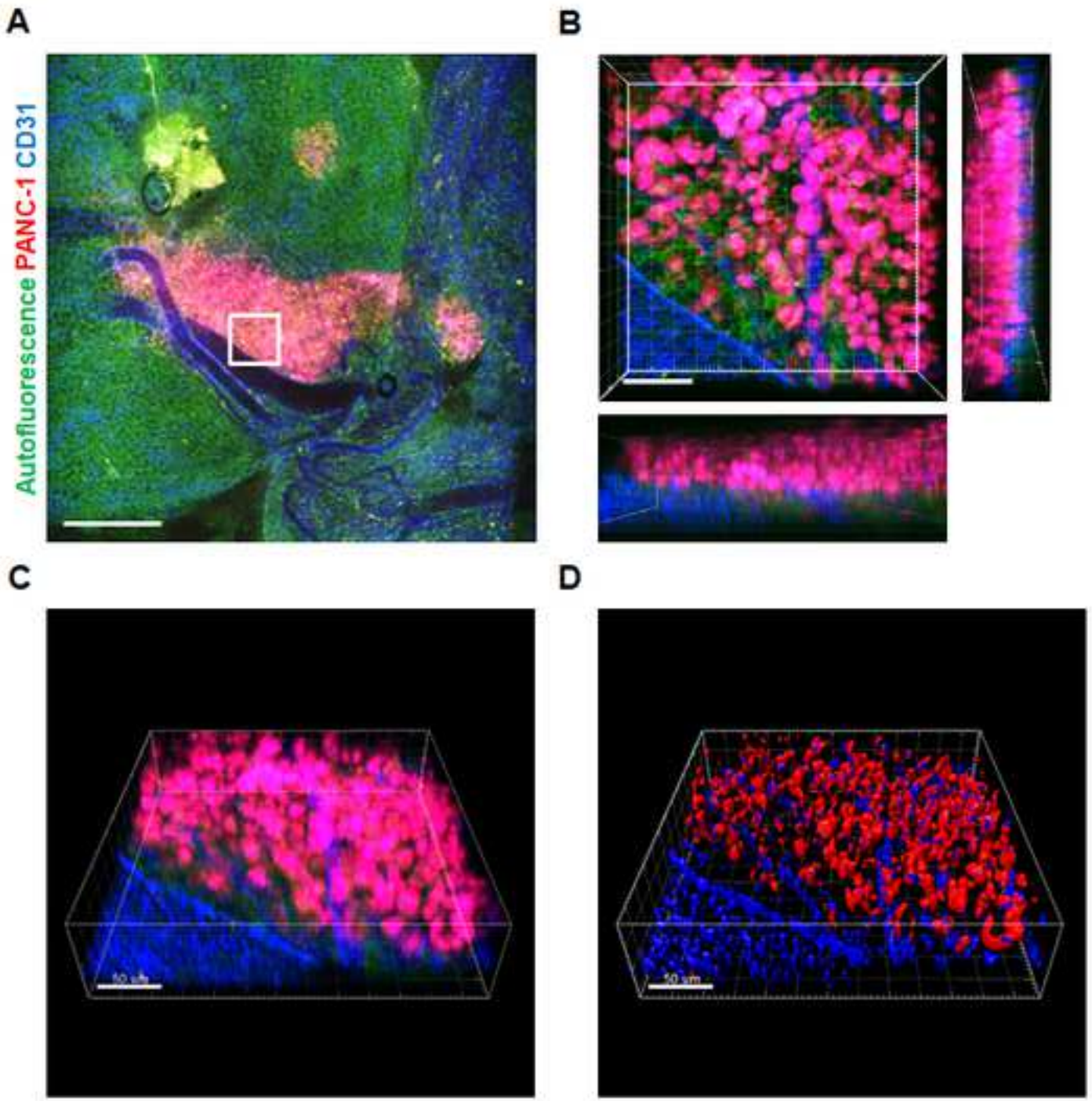
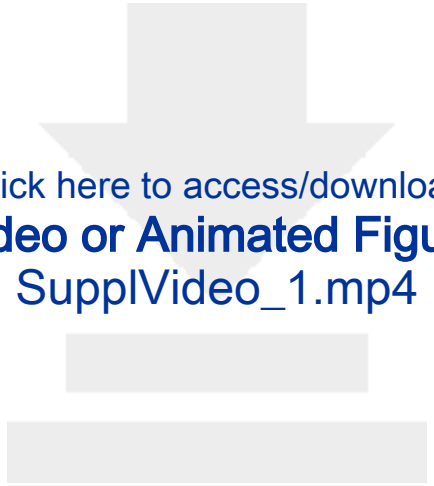


Supplementary Video 1



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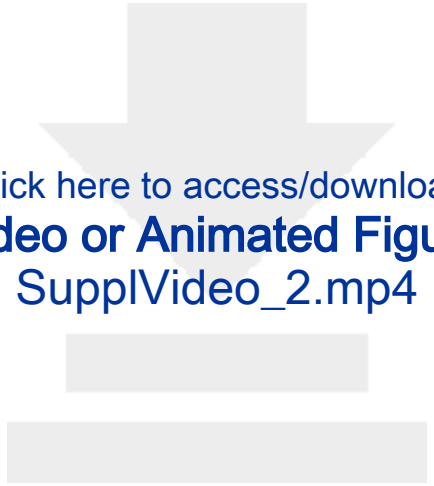
**Video or Animated Figure**

SupplVideo_1.mp4

Supplementary Video 2



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**Video or Animated Figure**

SupplVideo_2.mp4

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| Alexa Fluor 647 Succinimidyl Esters (NHS esters) | Invitrogen | A20006 |
| BALB/C Nude | OrientBio | BALB/C Nude |
| BD Intramedic polyethylene tubing | BD Biosciences | 427401 |
| C57BL/6N | OrientBio | C57BL/6N |
| Cover glasses circular | Marienfeld | 0111520 |
| FITC Dextran 2MDa | Merck (Former Sigma Aldrich) | FD200S |
| IMARIS 8.1 | Bitplane | IMARIS |
| Intravital Microscopy | IVIM tech | IVM-C |
| IRIS Scissor | JEUNGDO BIO & PLANT CO, LTD | S-1107-10 |
| Loctite 401 | Henkel | 401 |
| Micro Needle holder | JEUNGDO BIO & PLANT CO, LTD | H-1126-10 |
| Micro rectractor | JEUNGDO BIO & PLANT CO, LTD | 17004-03 |
| Microforceps | JEUNGDO BIO & PLANT CO, LTD | F-1034 |
| MIP-GFP | The Jackson Laboratory | 006864 |
| Nylon 4-0 | AILEE | NB434 |
| Omnican N 100 30G | B BRAUN | FT9172220S |
| PANC-1 NucLightRed | Custom-made | Custom-made |
| Pancreatic imaging window | Geumto Engineering | Custom order |
| Physiosuite | Kent Scientific | PS-02 |
| Purified NA/LE Rat Anti-Mouse CD31 | BD Biosciences | 553708 |
| Ring Forceps | JEUNGDO BIO & PLANT CO, LTD | F-1090-3 |
| Rompun | Bayer | Rompun |
| TMR Dextran 65-85kDa | Merck (Former Sigma Aldrich) | T1162 |
| Window holder | Geumto Engineering | Custom order |
| Zoletil | Virbac | Zoletil 100 |

| Comments/Description |
| --- |
| Fluorescent probe for conjugate with antibody |
| BALB/C Nude |
| PE10 catheter for connection with needle |
| C57BL/6N |
| Cover glass for pancreatic imaging window |
| For vessel identification |
| Image processing |
| Intravital Microscopy |
| This product can be replaced with the product from other company |
| N-butyl cyanoacrylate glue |
| This product can be replaced with the product from other company |
| This product can be replaced with the product from other company |
| This product can be replaced with the product from other company |
| B6.Cg-Tg(Ins1-EGFP)1Hara/J |
| Non-Absorbable Suture |
| For Vascular Catheter, Use only Needle part |
| Made in laboratory |
| Pancreatic imaging window - custom order |
| Homeothermic temperature controller |
| Antibody for in vivo vessel labeling |
| This product can be replaced with the product from other company |
| Anesthetic agent |
| For vessel identification |
| Window holder - custom order |
| Anesthetic agent |

## Answers to reviewers' comments

We deeply appreciate the editor and reviewers for their thoughtful, critical, and constructive comments, which have undoubtedly provided us with valuable opportunities to improve our work. We meticulously reviewed comments and revised our paper accordingly to address the issues raised by the editor and reviewers.

**Editorial comments:**
Editorial Changes
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We appreciate the editor for the helpful and constructive comments. We have proofread the manuscript once more to assure the spelling and grammar issues.

2. Please remove the "(Instructions)" links in the section headers.

We removed every "(Instructions)" link in the section headers.

3. Please include the author list first, followed by their affiliations.

We realigned the author list first, followed by their affiliations.

4. Line 107: Please provide details about the age and sex of the mice used.

We provided details about the age and sex of the mice used.

5. Use "1 X $10^6$" instead of "1 * 10^6".

We corrected as described above.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We revised the phrases written as "could be", "should be", and "would be" and wrote in the imperative tense.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from

your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Zoletil, Rompun, etc.

We removed the registered symbols and company names.

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We highlighted with yellow background color where it should be visualized

9. Please add a scale bar to Figure 3A.

We added a scale bar to Figure 3A and also changed the 3D view of previous images in Figure 3C&D and Figure 7C&D.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names. Do not use “&/and” in the author list of the references. Please include volume and issue numbers for all references.

We revised the reference as described above.

11. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

We have revised the names of the material as described above. Once again, we would like to appreciate the editor for the meticulous review.

_____

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

The manuscript by Park and coworkers describes a surgical procedure for creating a pancreatic imaging window (PIW) in mice. There have been growing interests in developing intravital imaging platforms for studying exocrine or endocrine cells of pancreas. Both abdominal imaging window (AIW) and exteriorized pancreas have been used in recent years. PIW is similar to AIW, yet providing potentially useful enhancements for stabilizing the pancreas by using a customized supporting base. The protocol described herein together with the accompanying video should help readers to adopt PIW for studying pancreas in vivo by intravital imaging.

Major Concerns:
As it currently stands, AIW and likely PIW as well, is limited by the short survival time post-surgery. Park and co-workerer were able to track pancreas and islets over a period of 3 weeks. It would be helpful to elaborate key issues or experimental details that are important for maintaining animal health and survival, both during the surgery and in the subsequent recovery period. What are the common causes(s) for the mouse death in this procedure? For people who are trying to adopt this procedure, what things they should watch out for to keep mice alive, and how should they trouble shoot when animals die?

We appreciate the reviewer for the critical comment. We revised our manuscript in the protocol and discussion section to address issues that reviewers have raised. In the discussion section, we have added key issues in the discussion part as below.

For long-term imaging over a period of three weeks, the most concerning issue was potential damage to the pancreatic imaging window. Unintended destruction of the cover glass in the pancreatic imaging window could occur during the long-term observation period. To prevent this, the mouse with the window must be housed separately, and hard objects with sharp edges should be removed from the cage. The euthanasia of mice should be considered should the cover glass break, if there are the severe signs of inflammation near the window, or if the animal appears to be in distress. In our experience, mice with the pancreatic imaging window were able to eat and exercise normally when the recovery after the surgery was appropriate and no other complication was developed.

Minor Concerns:
Some discussions on the pros and cons of PIW vs AIW would enhance the paper.

We appreciate the reviewer for the valuable comment. We have added our comments on the pros and cons of PIW and AIW in the discussion section below.

In our previous experience with the abdominal imaging window, we failed to acquire high-quality cellular level imaging as well as longitudinal tracking of the same spots over multiple days. Compared to the abdominal imaging window which provides a diverse platform for various abdominal organs, the pancreatic imaging window is further specified for imaging the pancreas as well as other organs that are soft and easily influenced by movements such as mesentery, spleen, and small bowel. However, the liver and kidney might be unfeasible in the pancreatic imaging window because of the limited space.

**Reviewer #2:**
Manuscript Summary:

In this manuscript titled "Stabilized longitudinal in vivo cellular-level visualization of the pancreas in a murine model with a pancreatic intravital imaging window", Park and Kim describe a novel method to image pancreatic tissue in vivo and longitudinally (over a period of up to three weeks). Their method uses an abdominal window where the tissue is immobilized in a chamber using adhesives. The manuscript is clear and well presented, but would benefit from some clarifications and additional details.

Major Concerns:
* It's not clear whether this manuscript describes a novel technique altogether or whether it presents details from Reference 11 (by the same authors). This point should be clarified.

We appreciate the reviewer for the valuable comments.
In the Introduction section, we have added the comments in the introduction part as below

…involving the pancreas. In addition to a detailed description of the methodology in the previous study, the extended application of pancreatic imaging window for various diseases involving the pancreas will be addressed in this paper. In this protocol…

* The authors don't seem to have taken in consideration several parameters of their platform over the physiological response of the islets placed under the chamber. For instance, they need to clarify whether the mechanical stress on the tissue could affect the blood flow, whether the adhesive used here is inert… Anesthetics have also been well known to affect insulin secretion and insulin sensitivity (e.g. Golstein et al. doi:10.1371/journal.pone, Windelov, et al. doi:10.14814/phy2.12824 (2016) or Frikke-Schmidt, et al. doi.org/10.1038/s41598-020-79727-8). The impact of their anesthesia protocol should be mentioned and discussed in the manuscript.

We appreciate the reviewer for the critical comments. We have added the limitation paragraph in the discussion part as below.

There are a few limitations to be addressed in this study. First, even when we utilized the metal base for stabilization, we were unable to determine the mechanical stress induced on the tissue by the base and cover glass, which could affect blood flow. However, as depicted in the above figures, intravenous injection of a fluorescence-conjugated antibody (CD31) or dextran adequately labeled the vessel with no distinguishable non-perfused area, suggesting a minimal impact of mechanical stress on the normal blood flow inside the pancreatic tissue. Second, adverse reactions due to the adhesive could not be assessed in the pancreatic tissue. Nevertheless, we attempted to avoid touching pancreas with adhesives as carefully as possible to avoid any additional effects. Third, as discussed above, the unintended impact of anesthetic agents might affect insulin sensitivity and secretion, as described in

the previous study. In our experience, a mixture of ketamine and xylazine induced hyperglycemia compared to the mixture of tiletamine, zolazepam, and xylazine. A further study investigating the effect of anesthesia on insulin secretion should be performed and proper anesthesia with minimal adverse effects should be selected according to each experiment. Fourth, imaging…

* There are also very few details on the microscopy parameters (magnification, resolution of the imaging, exposure time). In addition to additional details in point 3.8 of the protocol, it would also be important to discuss whether the imaging parameters used in this experimental setup could lead to photobleaching or tissue damage.

We appreciated the reviewer for the comments. We have added method parts (3.8) as below.

3.8.1. For imaging of the pancreas, start with a low magnification objective lens (e.g. 4X) for scanning the whole view of the pancreas in the pancreatic imaging window (recommended field of view: 2500 X 2500 μm).
3.8.2. After determination of the region of interest, switch to higher magnification objective lens (20X or 40X) to perform the cellular level imaging (recommended field of view: 500 X 500 μm or 250 X 250 μm). In our experiments, the lateral and axial resolution was approximately 0.5 and 3 micrometers, respectively.
3.8.3. Upon the purpose of imaging, perform z-stack or time-lapse imaging to observe the 3D structure or cellular-level dynamics, such as cell migration.
3.8.4. For imaging the fluorescent protein expressing cells of transgenic animals (MIP-GFP), 30 seconds of intermittent 488nm laser exposure with power up to 0.43mW was tolerable without noticeable photobleaching or tissue damage. For imaging the fluorescent proteins labeled with Alexa 647, the 640nm laser power up to 0.17mW was tolerable without noticeable photobleaching or tissue damage. Prolonged excitation laser exposure with a power above this setting may lead to photobleaching or tissue damage by phototoxicity. Adjust the adequate gain and power to appropriately image the region of interest.
NOTE: Detailed setting of parameters in intravital microscopy must be individualized for each intravital microscopy prepared in the institute.

* For longitudinal studies, the animals keep the imaging window affixed on their abdomen. The authors need to assess the impact it has on the animals, on stress, eating patterns and other factors that might contribute to an altered physiological response of the mice.

We appreciated the reviewer for the comments. We have added the additional comments of impact on the animal in the discussion part as below.

For long-term imaging over a period of three weeks, the most concerning issue was potential damage to the pancreatic imaging window. Unintended

destruction of the cover glass in the pancreatic imaging window could occur during the long-term observation period. To prevent this, the mouse with the window must be housed separately, and hard objects with sharp edges should be removed from the cage. The euthanasia of mice should be considered should the cover glass break, if there are the severe signs of inflammation near the window, or if the animal appears to be in distress. In our experience, mice with the pancreatic imaging window were able to eat and exercise normally when the recovery after the surgery was appropriate and no other complication was developed.

Minor Concerns:
* The introduction would benefit from mentioning other approaches that have been used to study islets in vivo (anterior chamber of the eye, pancreatic slices, erization of the pancreas) and emphasize the advantages of the presented approach in comparison.

* It could be of value also to mention the different imaging methods used by other investigators a bit more in detail (see Holmberg et al. doi:10.1007/s00125-008-1140-7) in order to justify the method suggested here.

We appreciated the reviewer for the two beneficial comments. We have added the additional paragraph mentioning other approaches in vivo and different imaging methods in the introduction part as below.

In particular with the islets, nuclear imaging, bioluminescence imaging, and optical coherence tomography have been suggested as non-invasive islet imaging techniques. However, the resolution of these methods is substantially low, with typical values ranging from several tens to hundreds of micrometers, offering a limited capability to detect changes at the cellular level in the islets. On the other hand, previous high-resolution studies of islets were performed under ex vivo (slicing or digestion of the pancreas), non-physiologic (exteriorization of the pancreas), and heterotopic conditions (implantation under the kidney capsule, inside the liver, and in the anterior chamber of the eye) which restricts their interpretation and clinical implications. If in vivo, physiologic, and orthotopic model of high-resolution imaging could be established, it would be a critical platform for the investigation of pancreatic islets.

* The authors mention the use of analgesics (which can also have a physiological impact as emphasize for a related platform in Frikke-Schmidt, et al. doi.org/10.1038/s41598-020-79727-8), but didn't explain why it was administered at the end of the experiments instead of before the first incision.

We appreciated the reviewer for the critical comments. We have added the NOTE comments in 2.16 as below.

NOTE: Analgesia influences insulin secretion in response to glucose. The choice and timing of analgesia must be individualized for the experimental purpose.

* The imaging method presented here is restricted to a limited portion of the tail of the pancreas. It would be important to mention how much of it can be imaged and to add a caveat emphasizing that this method cannot be used to image the head of the pancreas.

We appreciate the reviewer for the comment. We have added the description in the limitation paragraph in the discussion part as below.

… proper anesthesia of minimal adverse effect should be selected according to each experiment. Fourth, imaging of the pancreas is focused on the tail portion, and imaging of the head portion of the pancreas could be limited with our window.

* Similarly, it would be important to mention how many islets can be imaged at the time.

We appreciate the reviewer for the important comment. We have added the description in the limitation paragraph in the discussion part as below.

For imaging of the islets, a MIP-GFP mouse was utilized. Using the mosaic imaging method, a wide-field view with high-resolution imaging enabled the visualization of the islets with the adjacent vasculature (Figure 5). Approximately 40 – 50 islets were identified in the wide-field view. This stable imaging method could further facilitate the tracking of the islets for up to three weeks, as shown in a previous study (Figure 6).

* In the protocol, the point should be rephrased. The reporter is expressed under the control of the insulin promoter, not in the promoter.

We appreciate the reviewer for the delicate comment. We have revised the manuscript as the reviewer has suggested.

Here, we utilized MIP-GFP, where green fluorescent protein was expressed under the control of the mouse insulin 1 gene promoter present in the beta cells of all islets in the mouse.

Once again, we would like to appreciate the critical and helpful comments.

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April 23, 2021

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- Publication: Diabetes & Metabolism Journal
- Title: A Novel Pancreatic Imaging Window for Stabilized Longitudinal In Vivo Observation of Pancreatic Islets in Murine Model
- Author: Inwon Park, Sujung Hong, Yoonha Hwang, Pilhan Kim
- Diabetes Metab J 2020;44:193-198 (DOI: https://doi.org/10.4093/dmj.2018.0268)
- Portion: Figure 1, 2, 6

**New Article Information**

- Publication: JoVE (Journal of Visualized Experiments)
- Title: Stabilized longitudinal in vivo cellular-level visualization of the pancreas in a murine model with a pancreatic intravital imaging window
- Author: Inwon Park[1], Pilhan Kim[2]
- Affiliation: [1]Department of Emergency Medicine, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seongnam, Korea
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- Publication date: under review

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