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# Intravital imaging of neutrophil recruitment in intestinal ischemia-reperfusion injury

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A B S T R A C T

*Background:* Neutrophils are known to be key players in innate immunity. Activated neutrophils induce local inflammation, which results in pathophysiologic changes during intestinal ischemia-reperfusion injury (IRI). However, most studies have been based on static assessments, and few have examined real-time intravital neutrophil recruitment. We herein report a method for imaging and evaluating dynamic changes in the neutrophil recruitment in intestinal IRI using two-photon laser scanning microscopy (TPLSM).
*Methods:* LysM-eGFP mice were subjected to 45 min of warm intestinal ischemia followed by reperfusion. Mice received an intravenous injection of tetramethylrhodamine isothiocyanate-labeled albumin to visualize the microvasculature. Using a time-lapse TPLSM technique, we directly observed the behavior of neutrophils in intestinal IRI.
*Results:* We were able to image all layers of the intestine without invasive surgical stress. At low-magnification, the number of neutrophils per field of view continued to increase for 4 h after reperfusion. High-magnification images revealed the presence or absence of blood circulation. At 0–2 h after reperfusion, rolling and adhesive neutrophils increased along the vasculature. At 2–4 h after reperfusion, the irregularity of crypt architecture and transmigration of neutrophils were observed in the lamina propria. Furthermore, TPLSM imaging revealed the villus height, the diameters of the crypt, and the number of infiltrating neutrophils in the crypt. In the IRI group, the villus height 4 h after reperfusion was significantly shorter than in the control group.
*Conclusions:* TPLSM imaging revealed the real-time neutrophil recruitment in intestinal IRI. Z-stack imaging was useful for evaluating pathophysiological changes in the intestinal wall.
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## 1. Introduction

Ischemia-reperfusion injury (IRI) of the intestine occurs in a variety of clinical conditions, including intestinal malrotation, strangulated ileus, superior mesenteric artery thrombosis, hemorrhagic shock, trauma, surgery, and transplantation [1]. The interruption of the blood supply results in ischemic injury that rapidly damages metabolically active tissues. Paradoxically, however, the restoration of the blood flow to the ischemic tissue initiates a cascade of events that can lead to additional cell injury, known as reperfusion injury [2,3].

Neutrophils, which are one of the earliest innate immune cells recruited to the sites of infection and inflammatory response, are crucial for the pathophysiology of IRI [4–8]. Neutrophils can cause tissue damage in several ways, including via the secretion of

*Abbreviations:* TPLSM, two-photon laser-scanning microscopy; IRI, ischemia-reperfusion injury; GFP, green fluorescent protein; ROS, reactive oxygen species; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; SEM, standard error of the mean.

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proteolytic enzymes from cytoplasmic granules, neutrophil extracellular traps formation, and physical impairment of the microcirculation. Many neutrophils promote uncontrolled inflammation, and the depletion of redundant neutrophils is also implied to ameliorate tissue injury in various IRI models [9,10]. In intestinal IRI, the emigration of neutrophils from the postcapillary venules to areas of inflammation is a complex and highly coordinated process [11].

Thus far, the analysis of the status of neutrophils in intestinal IRI has been mainly based on the static assessment of histologic sections or an alternative index, such as myeloperoxidase activity, and few studies have analyzed the *in vivo*, real-time neutrophil recruitment. Therefore, the complexity and the dynamic features of the immune responses induced by neutrophils have been difficult to capture. We previously demonstrated the *in vivo* imaging of neutrophil recruitment in hepatic IRI using two-photon laser-scanning microscopy (TPLSM) [12,13]. TPLSM is one of the most progressive new developments in the field of imaging technology. It has numerous advantages over other modalities, including high-resolution, deep-site imaging, less phototoxicity, and less photobleaching than conventional confocal laser-scanning microscopy. These advantages are particularly useful for imaging tissues and organs in living beings [14–16].

In the present study, we present a method for examining neutrophil recruitment in intestinal IRI using TPLSM. We established our methodology by combining 1) a mouse intestinal IRI model, 2) LysM-eGFP transgenic mice, 3) the extension of the intestinal wall by phosphate-buffered saline (PBS) introduction and a tie technique, 4) butylscopolamine, 5) a stereotaxic device, and 6) TPLSM. With this novel method, we were able to visualize all of the layers of the intestine and analyzed the real-time neutrophil recruitment in live mice.

## 2. Materials and methods

### 2.1. Animals

LysM-eGFP mice were a generous gift from Dr. T. Graf (Center for Genomic Regulation, Barcelona, Spain). These mice express eGFP under the lysozyme M promoter, and their neutrophils are visualized by the expression of eGFP$^{hi}$ [17]. Mice were maintained in a specific-pathogen-free environment with free access to standard food and water throughout the experimental period at the Animal Resource Facility at Kumamoto University. Male LysM-eGFP mice at 8–12 weeks of age weighing 22–26 g were used for all experiments. The temperature was maintained at $22 \pm 2$ °C. All experiments were performed according to the guidelines of the Institutional Animal Committee of Kumamoto University.

### 2.2. Intestinal I/R injury model

Mice were anesthetized with an intraperitoneal injection of xylazine (10 mg/kg; Tokyo Kasei Kogyo, Tokyo, Japan) and ketamine (100 mg/kg; Fujita, Tokyo, Japan). After midline laparotomy, mice underwent a sham or I/R operation. In the I/R group, a part of the ileum supplied by a single branch of the superior mesenteric artery was exteriorized. Ligatures using 6-0 non-absorbable sutures were tightly applied to the marginal arteries and veins to prevent blood flow. An atraumatic microvascular clip (0.29 N; BEAR Medic; Tokyo, Japan) was placed across the superior mesenteric artery and vein, and the abdomen was closed. After 45 min of ischemia, the abdomen was reopened, and the clip was removed. Evidence of ischemia and reperfusion was confirmed by the intestinal color changes. In the control group, a sham operation was performed using the same protocol but without vascular occlusion. The mice were placed on a heating pad to maintain their body temperature at 37 °C throughout the procedure.

### 2.3. Intravital imaging of the intestine using TPLSM

Mice were prepared for intravital imaging using a protocol modified from previous reports [15,18,19]. After exteriorization of the target ileum, 0.5 mL of PBS was introduced into the collapsed ileum using a 30-G needle (Terumo Corporation, Tokyo, Japan) to visualize vertically all layers of the ileum, from the serosa to the mucosa. The exteriorized ileum was tied to the muscle layer of the abdomen using 6-0 non-absorbable sutures, and a 20-mm-diameter cover-ring was attached with glue. The ring was fixed into the arms of a stereotaxic holder. The hole of the ring was filled with PBS, and a cover-glass was placed onto the ring. General anesthesia was maintained with subcutaneous injections of xylazine (5 mg/kg) and ketamine (50 mg/kg) at 1- to 2-h intervals, and hydration was maintained by subcutaneous injections of 0.5 mL warmed saline every hour. The mice received an intravenous injection of tetramethylrhodamine isothiocyanate (TRITC)-labeled albumin (500 μg; Sigma-Aldrich, Tokyo, Japan) just prior to imaging to visualize the blood vessels. At the same time, butylscopolamine (0.2 mg/kg; Boehringer Ingelheim, Tokyo, Japan) was administered intravenously to minimize peristalsis of the intestine. Fig. 1A shows the intestinal preparation and setup of TPLSM for intravital imaging. The imaging system comprised a BX61WI upright microscope and FV1000MPE (Olympus, Tokyo, Japan) laser-scanning microscope system. In TPLSM mode, a Mai Tai HP Deep See femtosecond-pulsed laser (Spectra Physics, Santa Clara, CA, USA) was turned to and mode-locked at 840 nm. The intestine was scanned and fluorescence emission captured by the external non-descanned detectors (570-nm mirror and bandpass emission filters at 495–540 nm for eGFP and at 575–630 nm for TRITC). Time-lapse images were taken using the FV10-ASW version 3.0 software program (Olympus). For imaging at low-magnification, x-y planes spanning $1270 \times 1270$ μm at a resolution of 2.485 μm/pixel were imaged continuously at 1 frame every 1.644 s for 5–10 min using an Olympus UMPLFLN x10 W (numerical aperture, 0.30; working distance, 3.5 mm) objective lens. The intestine images were recorded hourly for 4 h at 37 frames/sec in the same position throughout imaging. For imaging at high-magnification, images were recorded using an Olympus XLPLN 25X WMP (water immersion; numerical aperture, 1.05; working distance, 2.0 mm) objective lens. High-magnification images were recorded by zooming in suitably to focus on the neutrophil morphology at the single-cell level.

### 2.4. Z-stack imaging of the intestinal wall

Using TPLSM, optical tomographic slices on the z-plane were obtained by moving the tomographic images on the x-y plane in the z-direction. Image stacks were collected at a 2-μm vertical step size from the serosa to the mucosa of the ileum. We calculated the villus height in the control and the IRI groups using z-stack imaging.

### 2.5. Image analyses

For analysis of the number of neutrophils, the TPLSM images were evaluated using the 'analyze and measure' command in the Image J software program (National Institutes of Health, Bethesda, MD, USA). Neutrophils were identified based on characteristics of fluorescent intensity (threshold of >50 in low-magnification imaging and 150 in high-magnification imaging) and cell size (5–15 μm). Under these conditions, neutrophils (GFP$^{hi}$) and monocytes/macrophages (GFP$^{low}$ and larger than 20 μm) were differentiated based on their brightness levels and distinct

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