Video Article

Inducing Ischemia-reperfusion Injury in the Mouse Ear Skin for Intravital Multiphoton Imaging of Immune Responses

Chi Ching Goh[1,2], Jackson LiangYao Li[1], David Becker[3], Wolfgang Weninger[4,5,6], Veronique Angeli[2,7], Lai Guan Ng[1,2,5,8]

[1]Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Biopolis

[2]Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore

[3]Lee Kong Chian School of Medicine, Nanyang Technological University

[4]Centenary Institute for Cancer Medicine and Cell Biology

[5]Discipline of Dermatology, University of Sydney

[6]Department of Dermatology, Royal Prince Alfred Hospital

[7]LSI Immunology Programme, National University of Singapore

[8]School of Biological Sciences, Nanyang Technological University

Correspondence to: Lai Guan Ng at Ng_Lai_Guan@immunol.a-star.edu.sg

URL: https://www.jove.com/video/54956
DOI: doi:10.3791/54956

Keywords: Medicine, Issue 118, skin, immunology, ischemia-reperfusion, inflammation, interstitial migration, multiphoton microscopy, intravital imaging

Date Published: 12/22/2016

Citation: Goh, C.C., Li, J.L., Becker, D., Weninger, W., Angeli, V., Ng, L.G. Inducing Ischemia-reperfusion Injury in the Mouse Ear Skin for Intravital Multiphoton Imaging of Immune Responses. *J. Vis. Exp.* (118), e54956, doi:10.3791/54956 (2016).

Abstract

Ischemia-reperfusion injury (IRI) occurs when there is transient hypoxia due to the obstruction of blood flow (ischemia) followed by a subsequent re-oxygenation of the tissues (reperfusion). In the skin, ischemia-reperfusion (IR) is the main contributing factor to the pathophysiology of pressure ulcers. While the cascade of events leading up to the inflammatory response has been well studied, the spatial and temporal responses of the different subsets of immune cells to an IR injury are not well understood. Existing models of IR using the clamping technique on the skin flank are highly invasive and unsuitable for studying immune responses to injury, while similar non-invasive magnet clamping studies in the skin flank are less-than-ideal for intravital imaging studies. In this protocol, we describe a robust model of non-invasive IR developed on mouse ear skin, where we aim to visualize in real-time the cellular response of immune cells after reperfusion via multiphoton intravital imaging (MP-IVM).

Video Link

The video component of this article can be found at https://www.jove.com/video/54956/

Introduction

Ischemia-reperfusion injury (IRI) occurs when there is a transient hypoxia due to the obstruction of blood flow (ischemia) followed by a subsequent re-oxygenation of the tissues (reperfusion). In the skin, ischemia-reperfusion (IR) is thought to be one of the contributing factors to the pathophysiology of pressure ulcers, where prolonged bed rest predisposes long-term hospital patients to injury. In these patients, both the skin and the underlying muscles are constantly exposed to weight pressure exerted over areas of bony prominence, resulting in localized injuries that, if left untreated, may become necrotic[1].

The damages involved in an IRI are twofold. During ischemia, the occlusion of blood vessels leads to a drastic drop of oxygen delivery to the tissues. This results in a decrease of ATP and pH, which inactivates ATPases involved in cellular metabolism. In turn, cellular calcium levels spike, and stressed or damaged cells undergo apoptosis or necrosis[2]. The release of intracellular contents or damage associated molecular patterns (DAMP), like HMGB1, contributes to the inflammatory response[3]. The second insult occurs during reperfusion. Although oxygen and pH levels are restored during reperfusion, this results in the generation of reactive oxygen species (ROS), which leads to the oxidation of intracellular lipids, DNA, and proteins. Consequently, pro-inflammatory mediators are activated, which sets off a secondary inflammatory response that involves the recruitment of immune cells to the inflammatory site[2]. While the cascade of biochemical events leading up to the inflammatory response has been well described, the spatial and temporal regulation of the immune cell activities are not well understood.

Here, we describe a robust IR model on mouse ear skin using simple magnet clamping. Coupled with multiphoton intravital imaging (MP-IVM), we established a model to study the *in vivo* inflammatory responses that occur after reperfusion takes place. The rationale behind the development and use of this technique is to try to understand how both interstitial and infiltrating cells respond to IR in real time.

Existing models of IR using the clamping technique on the skin flank are highly invasive, as they require the surgical implantation of steel plates in the skin flank, making them less-than-ideal for immunological studies[4]. A similar non-invasive clamping technique has been described in the mouse skin flank[5,6]. However, because of the incorporation of the intravital imaging component in this method, we instead chose the ear skin as the targeted IR site, as it circumvents movements due to breathing and offers stability during imaging[7,8]. Moreover, leukocyte subsets that span

the interstitium are identical between the ear skin and the skin flank, although the numbers and proportions may vary slightly[9]. Thus, the ear skin represents an ideal imaging site.

In addition, most data retrieved from these IRI models are limited to macroscopic evaluations (grading of ulcers) and microscopic analyses of endpoint inflammatory indicators[10]. Using this model, real-time visualization of the cellular response of neutrophils after reperfusion in the skin of a fluorescent reporter mouse is enabled. A previously published intravital ear imaging model is utilized[8] with additional modifications (**Figures 1, 2**).

Protocol

All experiments dealing with live animals were conducted in accordance to all relevant animal use and care guidelines and regulations.

1. Choice of Fluorescent Reporter Mice

1. Use 6- to 12-week-old LysM-eGFP[11] mice (no preference for either males or females).
   Note: The use of various cell-specific fluorescent reporter mice enables the visualization of different immune cells *in vivo*. In this strain, circulating neutrophils (GFP^{hi} cells), circulating monocytes (GFP^{lo} cells), and dermal macrophages (GFP^{lo} cells) can be visualized. With the imaging parameters used, only the bright signals from GFP-positive neutrophils will be detected.
   Note: A list of immune-cell-specific fluorescent reporter mouse strains suitable for this type of skin imaging study can be found in Reference 8.
   Note: It is highly recommended that albino mice be used for imaging, as pigmented mice are more prone to photodamage. This is because the pigmented ear skin is much more sensitive to laser-induced speckling (indicative of tissue burning). As a result, neutrophil recruitment and accumulation may be observed even during the steady state[8,12].
2. Keep the mice in specific pathogen-free (SPF) conditions with 12-h light-dark cycles.

2. Mouse Anesthesia

1. Anesthetize the mouse with an intra-peritoneal injection of ketamine-xylazine (8 µL g^{-1} bodyweight), composed of a mixture of 15 mg mL^{-1} ketamine and 1 mg mL^{-1} xylazine dissolved in sterile water.
2. Place the mouse on a heating pad to maintain its body temperature at 37 °C throughout the preparation procedure. Check for sufficient anesthesia by observing an absence of a toe pinch reflex.
   Note: After the first hour, subsequent quarter doses of anesthetic will need to be administered subcutaneously and will last for approximately 0.5 h each. The twitching of whiskers or the tail may also indicate that the anesthesia is wearing off and that a top-up is required.
3. Use ophthalmic lubricant on the eyes to prevent dryness while under anesthesia.

3. Depilation

1. Carefully apply depilatory cream to the upper two-thirds of the dorsal mouse ear using cotton-tip applicators.
2. Wait for 2 - 3 min before removing the cream using wet cotton-tip applicators in a thorough but gentle manner.
   Note: Do not allow hair removal cream to stay on the mouse ear for too long, as it may induce inflammation[13,14].

4. Induction of Ischemia and Reperfusion Injury

1. Use gold-plated, N42-grade neodymium magnets, 12 mm diameter x 2 mm thick, and with a Gauss rating of approximately 3,000 to induce ischemia in the mouse ear skin.
   Note: In this case, the dimpled face of the magnets denotes its north pole.
2. Slot the magnets into their individual plastic guides.
   Note: The plastic guide serves to ease the placement and separation of the high-strength magnets. Due to their strong magnetic force yet low resistance to breakage, do not place individual magnets in close proximity to each other or to other metals. Breakage and splintering may occur if they pull towards each other.
3. Position the first (dorsal) magnet such that only the edge is in contact with the second (ventral) magnet (**Figure 3a**)
   Note: This prevents the magnets from snapping together before they have been properly positioned.
4. Position both magnets such that the ventral magnet lies flat on the ear (**Figure 3a**).
   Note: Before ischemia is induced, ensure that the mouse is kept at 37 °C and that sufficient anesthesia is maintained (see step 2.2).
5. Once ready, carefully let the magnets come together (**Figure 3a**).
   Note: For imaging purposes, clamp only half of the ear so that an IR and non-IR region can be observed.
6. After 1.5 h of ischemia, remove the magnets by twisting the magnets away from each other using the plastic guides, allowing reperfusion to take place.
   Note: Care must be taken to prevent the ears from creasing when the magnets are placed. Incomplete ischemia is evident if the macroscopically visible major blood vessels re-perfuse immediately (*i.e.,* blood fills the vessels immediately) after the magnets are removed. Although reperfusion does not occur immediately after the removal of magnets, blood vessel occlusion is only transient. As such, it is imperative to prepare the mouse ear for imaging as swiftly as possible.

5. Injection of Blood Vessel Labeling Agents

1. Immediately after magnet removal, administer intravenously (via retro-orbital or tail vein injection) Evans blue (10 mg mL^{-1} in PBS or saline; 1 µL g^{-1} bodyweight) or another blood vessel labeling agent of choice.
   Note: Before injection, ensure that sufficient anesthesia is still maintained by performing gentle toe pinching.

6. Placement of the Ear on the Imaging Platform

1. Cut 2 pieces of masking tape 1.5 cm in length and 1.8 cm in width.
2. Allow the adhesive sides to stick together whilst leaving about 1 mm of adhesive along its width.
3. Cut the masking tape in two, lengthwise, to accommodate its placement within the slit on the ear platform.
4. Insert this masking tape about halfway through the slit, such that the adhesive side faces up.
5. Position the mouse on the heating pad, such that the ear to be imaged is next to the masking tape strip.
6. By using two PBS-moistened cotton-tip applicators, gently press the ear against the adhesive strip.
7. Using the strip as a guide, bring the mouse ear through the slit while simultaneously adjusting the mouse closer towards the stage.
8. To remove the sticky tape, first add a drop of PBS to reduce the adhesiveness of the tape.
9. Separate the mouse ear from the masking tape as gently as possible using a fine paintbrush.
10. Flatten the ear against the ear platform by gently rolling a moist cotton-tip applicator over the ear.
11. Put a drop of PBS underneath the coverslip (which is kept in position on the coverslip holder using grease; **Figure 2**) and gently place it over the ear. Top up with more PBS if necessary.
    Note: The coverslip holder increases the stability during imaging.
12. Insert the rectal temperature probe and connect the wires to the heating system according to manufacturer's instructions.
    Note: Set the temperature of the body heating pad to 37 °C and the ear stage platform to 35 °C.

7. Multiphoton Microscope Setup and Imaging Parameters

Note: This protocol uses a single beam, multiphoton microscope with a tunable (680 - 1,080 nm) Ti:Sa laser (3.3 W at 800 nm; pulse length of 140 fs; 80 MHz repetition rate) with a 20X water objective (NA = 1.0) for intravital imaging studies.

1. Open the imaging software.
2. Align the laser according to the manufacturer's instructions.
3. Adjust the excitation wavelength to 950 nm.
   Note: GFP and Evans blue can be simultaneously excited at 950 nm.
4. To preview, use the following settings: 500 $µm^2$ scanfield, 505 x 505 pixel resolution, and a scan frequency of 800 Hz in a single line scan. Click "Preview."
5. Toggle the attenuator (laser) power. Ensure that all essential signals are picked up without exposing the imaging field to excessive amounts of laser power, which may induce heat damage.
   Note: Start at a low attenuator power and increase if the signal is dim. As neutrophils will be absent from the interstitium at early time points post-reperfusion, macrophages may be used as a gauge to determine the minimal power required, as the former are dimmer than neutrophils.
   Note: If this step is to be done for the first time, adjust the settings in the non-ischemic zone, where intact vascular integrity is expected and will facilitate the setting of the attenuator power. In subsequent experiments, these settings do not require much modification unless the laser power is unstable.
6. Adjust the PMT settings.
   Note: Check with the manufacturer for the maximal optimal gain voltage for the PMT. Setting the PMT voltage beyond the recommended threshold will result in a higher signal-to-noise ratio. The general recommendation is to set the PMT gain voltage to the recommended threshold and to increase the attenuator power as necessary should the signal be too dim.
7. Select an imaging region that is in close proximity to the edge of the ischemia, characterized by massive Evans blue leakage.
8. Collect GFP and Evans blue signals using 525/50 bandpass (BP) and 655/40 BP filters, respectively. For second harmonic generation (SHG) signals of collagen fibers within the dermal compartment, use a 475/42 BP filter.
9. Create a folder to save the images in a format that is compatible for the available image analysis software.
10. To acquire, use the following settings: 500 $µm^2$ scanfield, 505 x 505 pixel resolution, and a scan frequency 400 Hz in a single line scan.
11. A 100-µm z-stack with a step size of 4 µm can be acquired repeatedly over time, at 1 min intervals, to monitor neutrophil infiltration.
    Note: Especially for leukocytes (*e.g.,* neutrophils) with a higher migratory velocity, an interval that is longer than 1 min may result in difficulties during cell tracking analysis. In this case, the user can either reduce the thickness of the acquisition stack or increase the scan frequency.
    Note: While the required acquisition stack of the ischemic zone may appear smaller as a result of the compression (alternatively characterized by a higher SHG intensity), subsequent reperfusion will result in massive inflammation that causes the ear to swell. Significant drifts in the Z-direction are expected. As such, the acquisition of a large z-stack is necessary to accommodate the drift.
12. Throughout the imaging, top up the PBS and water regularly to keep the ear moist and the objective lens immersed in water.
    Note: A typical experiment usually lasts for 2 - 4 h. Depending on the experimental design, and coupled with a well-controlled anesthesia regime, extending the duration of imaging is possible.

8. Terminating the Experiment

1. Euthanize the mouse by carbon dioxide asphyxiation according to the institution's Institutional Animal Care and Use Committee (IACUC) procedures.

Note: Always euthanize the mouse by approved methods as determined by the institution's rules, regulations, and guidelines. If repeated imaging is needed, keep the mouse on the heating pad until the anesthesia wears off. Once the mouse has regained sufficient consciousness and is mobile, return the mouse to its cage.

9. Image Analysis

Note: Data generated from the imaging experiment can be visualized by different software packages.

1. Open the imaging analysis software.
2. Under Surpass Mode, import the file (Open → select any file in the desired folder → Click "open").
3. Edit pseudo colors if default colors do not apply (Edit → Display adjustment → Under the channel tab, select the desired color from the color palette).
4. Adjust the brightness, contrast, and background (Edit → Display Adjustment → Toggle Max and Min values).
5. Ensure that the file dimensions are correct (Edit → Image properties → Under the geometry tab, check the voxel size. In this protocol, X = 0.99, Y = 0.99, and Z = 4).
   Note: Derive X and Y by dividing the scanfield dimension by the pixel resolution. Z is the thickness between each slice.
   Note: The output of these data sets can be presented as maximum projection movies.
   Note: Tracking analysis is required to fully characterize the cellular activities and interactions in order to understand their function *in vivo*. Detailed instructions of cell tracking using the spot function available in the software can be found in the protocol in Reference 15.
   Note: There are many ways of quantifying leukocyte migration, details of which can be found in the review article in Reference 16.

Representative Results

This protocol uses a custom-built ear skin imaging platform, as shown in **Figure 1**. Several features of this platform are specifically designed to facilitate imaging while maintaining physiological settings. Placing the ear on the heated brass platform not only maintains the ear at a physiological temperature of 35 °C, but it also isolates the ear from inevitable movements due to breathing. The addition of a metal clip on the brass platform creates a gap to prevent the coverslip holder from exerting weight on the ear, thereby maintaining uninterrupted blood flow. The stage platform is also designed to accommodate a heating pad that will maintain the mouse at 37 °C throughout the imaging procedure.

The coverslip holder (**Figure 2**) is designed to allow the coverslip to be attached onto the metal holder with the help of vacuum grease. The coverslip holder is connected to a stand that allows for flexible adjustment in the vertical and horizontal axes with the help of the adaptor. This way, the user can first accurately adjust the position of the coverslip over the ear and subsequently fix that position by tightening the screws on the adaptor.

In this protocol, we have described the use of magnets to simulate ischemia and reperfusion. **Figure 3b** displays representative views of the ears before and immediately after ischemia. Under physiological conditions, major blood vessels can be visualized macroscopically. The pinching effect of the magnets temporarily stems the blood flow, which can be observed by a transient blanching effect (black arrow) when the magnets are removed.

In this particular demonstration, imaging is focused on the edge of the ischemic zone (**Figure 3c**). This interface between the ischemic (clamped) and non-ischemic (clamp-free) zone is usually marked by massive Evans Blue leakage into the interstitium (white solid arrow), while the ischemic zone at early time points is devoid of any Evans Blue signal (white arrowhead) (**Figure 3d**). This creates important landmarks for intravital imaging studies and helps to maintain consistency between independent experiments.

The data shows that, in response to an IR insult, infiltrating neutrophils exit into the interstitium from intact blood vessels lining the edge of the ischemic zone and migrate towards the injury site (**Figure 3d** and **Movie 1**). We expect a delayed response of neutrophils migrating into the interstitium as compared to other inflammation models due to a lack of viable perfusing blood vessels at earlier time points post-reperfusion. To fully characterize these cellular activities, cell tracking analysis may be employed to understand their function *in vivo*. Speed, mean displacement, and chemotactic index are some of the potential read-outs that may be obtained from the cell tracking analysis.

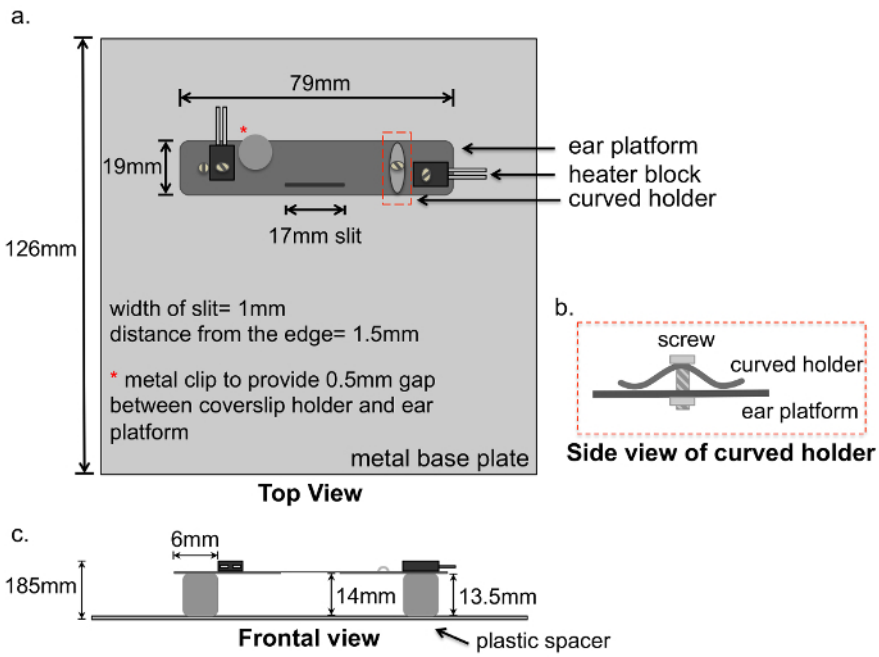


Figure 1. Schematic Diagram of a Custom-made Ear Skin Stage for Intravital Multiphoton Imaging. (a) Top view of the ear imaging stage with its dimensions. **(b)** Frontal view and dimensions. Note that the brass plate is thicker at the sides, in order to provide stability to the platform, and thinner in the middle, to minimize contact area with the skin, ensuring uninterrupted blood flow when the ear is folded over the edge of the slit and rested on the platform. **(c)** Side view of the curved holder that pins down the feedback probe. The heating pad can be fixed onto the platform using masking tape (not shown). Modified from Li *et al.*, Nat Protoc, 2012[8]. Please click here to view a larger version of this figure.

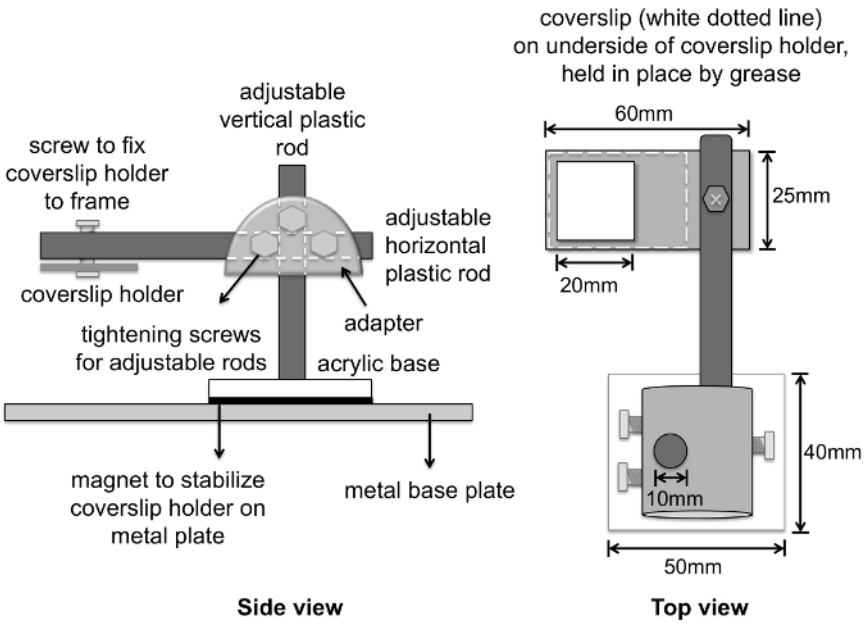


Figure 2. Design of the Coverslip Holder; Side and Top View. The coverslip holder holds the coverslip in a fixed position and thus reduces z-drift. Please click here to view a larger version of this figure.

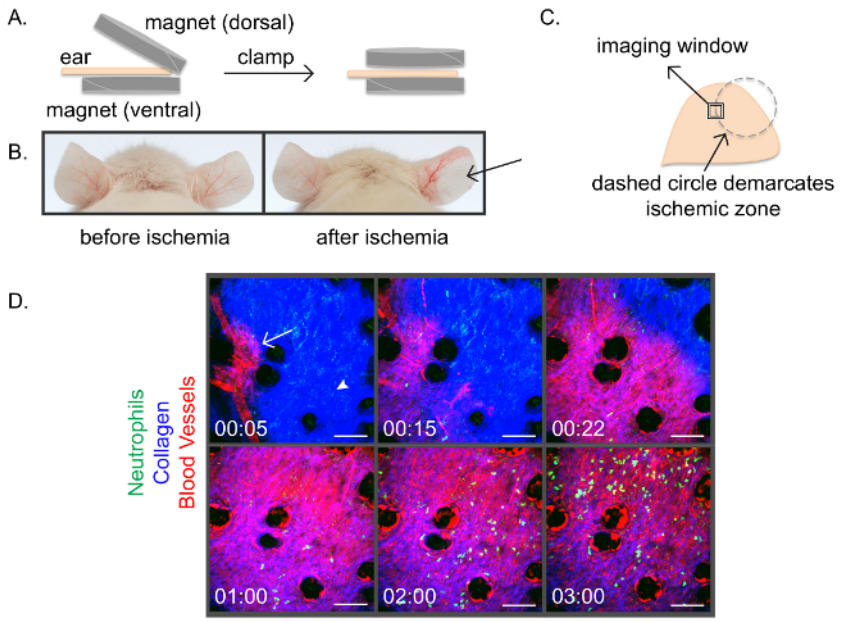
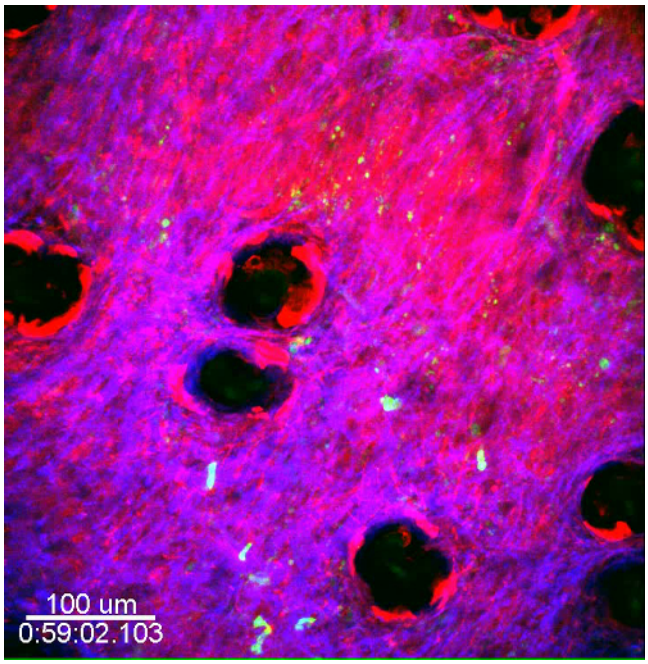


Figure 3. The IR Model. (A) Schematic diagram showing the positioning of magnets. **(B)** Photos showing a pre-ischemic (left) and a post-ischemic ear (right; right ear) after magnet clamping. **(C)** Schematic of the ear showing the region imaged with respect to where the magnet had been placed. **(D)** Maximum intensity z-projected snapshots from a time-lapse sequence showing neutrophil infiltration post-reperfusion in a LysM-eGFP mouse. Elapsed time shown in hh:mm format. Scale Bar: 100 µm. Please click here to view a larger version of this figure.



Movie 1. Neutrophil Recruitment in IR Injury. A time-lapse sequence of maximum projection showing neutrophil (green) infiltration in response to reperfusion in LysM-eGFP albino mouse ear skin after 1.5 h of ischemia. At earlier time points, the lack of Evans blue signal (red) indicates temporary vessel occlusion after ischemia. Collagen (blue, second harmonic generation). Please click here to view this video. (Right-click to download.)

Discussion

Significance

IR is one of the leading causes of skin pressure ulcers. The early stages (I and II) of pressure ulcers describe the condition of the human skin (as compared to the underlying subcutaneous tissues and muscles). However, an understanding of the immunological etiology is still lacking. Here, we present a simple and robust IR model on mouse ear skin in order to address this gap. We simulate ischemia by clamping the mouse ear between two magnets and subsequently study the downstream immune responses after magnet removal (reperfusion). By using magnets

to generate a constant pressure on each ear for a fixed period of time, inducing ischemia, we are able to ensure reproducibility and consistency between experiments.

The strength of this non-invasive IR model also lies in its ability to demonstrate, in real time, *in vivo* immune responses by utilizing a well-established model of intravital imaging of the ear skin, which offers more stability during imaging as compared to the skin flank procedure. Some of the current skin flank models involve surgical procedures that may alter the immune landscape of the skin[4]. In addition, imaging the skin flank itself presents a technical challenge, as respiratory movements may contribute to motion-related artifacts during imaging[5,6]. Our current model circumvents these problems.

In addition to studying the physiological responses of immune cells to IR, this model may also be applicable to pathophysiological settings like diabetes mellitus, where diabetes may worsen IR injury through increased oxidative stress. Understanding the immune responses will contribute to an understanding of wound healing.

Critical Steps

Once the mouse is under anesthesia, thermoregulation is impaired and the core body temperature drops drastically. To prevent hypothermia, external heating is essential to maintain the core body temperature at 37 °C. The ear, located distally to the body core, is physiologically cooler by 1 - 2 °C and must be kept at 35 °C. This consistency in heating will also ensure reproducible read-outs of cell dynamics. At the same time, the heating system must be checked to ensure that the mouse is not over-heating throughout the entire length of anesthesia.

The use of albino mice (BALB/c and C57BL/6-C^{2J}) for all skin imaging studies is highly recommended to avoid pigment-induced speckling injuries. If albino mice are not available, decreasing the laser power during image acquisition may alleviate the problem for pigmented mice. However, further optimization may be needed, as a reduced depth of tissue penetration may result in a poorer acquisition outcome.

The ear skin is very delicate; hence, care must be taken to avoid any circumstances that may cause inflammation. Examples of improper techniques include, but are not limited to, the following: 1) leaving the depilatory cream on for an excessive amount of time over what is recommended; 2) applying excessive friction on the ear when bringing the ear through the slit of the ear imaging platform or when detaching the ear from the masking tape; and 3) overheating the ear, either due to a faulty heating system or a misplaced temperature feedback probe that may be recording ambient temperature instead of the mouse core temperature.

Modifications and Troubleshooting

Most of the troubleshooting pertaining to the ear preparation procedure has been previously listed[8]. If the ear is imaged at a later time point post-reperfusion, the ear may swell beyond the 0.5-mm gap created by the metal clip to accommodate the thickness of the ear (**Figure 1**). In this scenario, the placement of the coverslip over the ear may hinder blood flow, which is evident when the macroscopically visible blood vessels disappear from view. This must be avoided by creating a larger gap between the coverslip holder and the brass platform. This can be done in two ways, either by manually adjusting the height of the coverslip holder (**Figure 2**) or by using a thicker metal clip.

Limitations of the Technique

In this protocol, we used Evans blue to determine the location of the ischemic zone, since leakage of the Evans blue in the interstitium marks the boundary of the ischemic zone at early time points post-ischemia. Evans Blue is commonly used as a blood vessel labeling agent. However, under inflammatory conditions, vascular integrity is severely compromised. This results in the leakage of Evans Blue into the interstitium. In this model, we observe a drastic loss of contrast between blood vessels and interstitium soon after reperfusion. As such, in studies where leukocyte-endothelial interactions are involved, we recommend experimenting with other blood vessel labeling agents of different sizes (*e.g.,* dextrans, *etc.*).

Future Applications

Besides utilizing the LysM-eGFP mouse to demonstrate how neutrophils respond to skin IR, this model can also be used for studying other leukocyte responses, both in infiltrating (monocytes) and in resident interstitial (macrophages) leukocytes. This model can also be extended to other studies examining blood and lymphatic integrity, as well as leukocyte-endothelial interactions after an IR injury. Coupled with the ability to visualize collagen as SHG signals through MP-IVM, it would also be interesting to monitor collagen integrity after IR or during wound healing. In summary, we have set up a non-invasive, robust model of IR for intravital imaging in order to study immune responses in the mouse ear skin.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Thomas Graf for providing us with the LysM-eGFP mice.

References

1. Black, J. *et al.* National Pressure Ulcer Advisory Panel's updated pressure ulcer staging system. *Adv Skin Wound Care.* **20**, 269-274 (2007).
2. Kalogeris, T., Baines, C. P., Krenz, M., & Korthuis, R. J. Cell biology of ischemia/reperfusion injury. *Int Rev Cell Mol Biol.* **298**, 229-317 (2012).
3. Huebener, P. *et al.* The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. *J Clin Invest.* **125**, 539-550 (2015).

4. Wassermann, E. *et al.* A chronic pressure ulcer model in the nude mouse. *Wound Repair Regen.* **17**, 480-484 (2009).
5. Stadler, I., Zhang, R. Y., Oskoui, P., Whittaker, M. S., & Lanzafame, R. J. Development of a simple, noninvasive, clinically relevant model of pressure ulcers in the mouse. *J Invest Surg.* **17**, 221-227 (2004).
6. Tsuji, S., Ichioka, S., Sekiya, N., & Nakatsuka, T. Analysis of ischemia-reperfusion injury in a microcirculatory model of pressure ulcers. *Wound Repair Regen.* **13**, 209-215 (2005).
7. Ng, L. G. *et al.* Visualizing the neutrophil response to sterile tissue injury in mouse dermis reveals a three-phase cascade of events. *J Invest Dermatol.* **131**, 2058-2068 (2011).
8. Li, J. L. *et al.* Intravital multiphoton imaging of immune responses in the mouse ear skin. *Nat Protoc.* **7**, 221-234 (2012).
9. Tong, P. L. *et al.* The skin immune atlas: three-dimensional analysis of cutaneous leukocyte subsets by multiphoton microscopy. *J Invest Dermatol.* **135**, 84-93 (2015).
10. Saito, Y. *et al.* The loss of MCP-1 attenuates cutaneous ischemia-reperfusion injury in a mouse model of pressure ulcer. *J Invest Dermatol.* **128**, 1838-1851 (2008).
11. Faust, N., Varas, F., Kelly, L. M., Heck, S., & Graf, T. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood.* **96**, 719-726 (2000).
12. Roediger, B., Ng, L. G., Smith, A. L., Fazekas de St Groth, B., & Weninger, W. Visualizing dendritic cell migration within the skin. *Histochem Cell Biol.* **130**, 1131-1146 (2008).
13. Kikushima, K., Kita, S., & Higuchi, H. A non-invasive imaging for the *in vivo* tracking of high-speed vesicle transport in mouse neutrophils. *Sci Rep.* **3**, 1913 (2013).
14. Ng, L. G. *et al.* Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. *PLoS Pathog.* **4**, e1000222 (2008).
15. Soohoo, A. L., Bowersox, S. L., & Puthenveedu, M. A. Visualizing clathrin-mediated endocytosis of G protein-coupled receptors at single-event resolution via TIRF microscopy. *J Vis Exp.*, e51805 (2014).
16. Beltman, J. B., Maree, A. F., & de Boer, R. J. Analysing immune cell migration. *Nat Rev Immunol.* **9**, 789-798 (2009).

