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Rodent Heart and Brain Tissue Preparation for Digital Macro Photography after Ischemia-reperfusion

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TITLE:

Rodent Heart and Brain Tissue Preparation for Digital Macro Photography after Ischemia-reperfusion

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SUMMARY:

Presented here is a protocol for the standardized methodology of rodent tissue preparation after the ischemia-reperfusion experiment and guidelines for establishing lighting and camera setups for high-resolution image acquisition. This method is applicable to all experimental small-animal organ photography.

ABSTRACT:

Macro photography is applicable for imaging various tissue samples at high magnification to perform qualitative and quantitative analyses. Tissue preparation and subsequent image capture are steps performed immediately after the ischemia-reperfusion (IR) experiment and must be performed in a timely manner and with appropriate care. For the evaluation of IR-induced damage in the heart and brain, this paper describes 2,3,5-triphenyl-2H-tetrazolium chloride (TTC)-based staining followed by macro photography. Scientific macro photography requires controlled lighting and an appropriate imaging setup. The standardized methodology ensures high-quality, detailed digital images even if a combination of an inexpensive up-to-date digital camera and macro lens is used. Proper techniques and potential mistakes in sample preparation and image acquisition are discussed, and examples of the influence of correct and incorrect setups on image quality are provided. Specific tips are provided on how to avoid common mistakes, such as overstaining, improper sample storage, and suboptimal lighting conditions. This paper shows the appropriate methodology for rat heart and brain tissue slicing and staining and provides guidelines for establishing lighting and camera setups and photography techniques for high-resolution image acquisition.

INTRODUCTION:

For decades, photography and analysis of heart and brain tissue specimens have been an important part of life science experiments. Science and innovation progress drives the development of expensive microscopes capable of superresolution. Photomicrographs are

obtained in a well-controlled light environment following detailed instructions. In contrast, macro photography (at 1:2 or greater magnification) is frequently performed in an uncontrolled light environment using inappropriate imaging setups. Often, the techniques of sample preparation and camera setup need to be substantially optimized. As a result, macro photographs of limited quality have been widely published in scientific journals. Insufficient image resolution and contrast limit the possibilities of precise image quantification in IR studies.

Experimental procedures of myocardial[1,2] and brain[3,4] infarctions have been described in detail. The purpose of this study is to provide a step-by-step guide on how to set up a system for photography and standardized analysis of rodent heart and brain tissue specimens after infarction experiments. This includes tissue slicing, staining, and macro photography of heart and brain samples. The preparation of tissue specimens is an essential part of the experiment, and the planimetric image results depend highly on the quality of the obtained images[5].

These methods are particularly useful for performing measurements and image planimetric analysis in rodent tissues and could be of value for general scientific macro photography. In addition, the high quality and consistency of images allow automated analysis of digital photographs to be performed, which helps to save time, avoid user input, and minimize the risk of errors or bias during image analysis. This will result in the generation of robust and reliable data and increase the translation of preclinical discoveries into novel antiischemic treatments in clinics.

PROTOCOL:

The experimental procedures were performed in accordance with the guidelines of the European Community and local laws and policies (Directive 2010/63/EU), and all the procedures were approved by the Food and Veterinary Service, Riga, Latvia.

1. Heart staining and slicing

NOTE: Techniques described in this protocol can be used after both Langendorff-perfused isolated rat or mouse heart[6,7] and *in vivo* rat heart IR injury assays[8-11]. For staining after an *in vivo* IR injury assay, it is assumed that the heart is excised, mounted on a cannula, and briefly perfused in the Langendorff perfusion mode.

1.1. Detach the heart cannula from the syringe filled with Krebs-Henseleit solution and connect it to a syringe filled with a warm (37 °C) solution of 0.1% methylene blue in Krebs-Henseleit solution. Use a 5 mL syringe for rat hearts and a 1–2 mL syringe for mouse hearts.

NOTE: An alternative is to fill the pressure- or flow-controlled (e.g., Langendorff) apparatus with a blue dye-containing solution. During the detachment and mounting procedure, it is essential not to leave any air bubbles in the cannula and not loosen the suture used for coronary artery reocclusion.

1.2. Further perfuse rat hearts with 4 mL of the methylene blue solution at a rate of ~4 mL/min and perfuse mouse hearts with 1 mL of methylene blue solution at a rate of ~0.5–1 mL/min.

NOTE: Based on experience, both techniques are safe and provide adequate staining; however, using a pressure-controlled pump/hydrostatic pressure system is a more time-consuming but safer option against overstaining for novice scientists.

1.3. Disconnect the cannula from the syringe and remove the heart from the cannula.

1.4. Remove excess methylene blue by gentle rolling of the heart on tissue paper. Loosen the ligature around the coronary artery by opening the hemostatic forceps and removing the plastic tubing from the surgical suture only after removing excess methylene blue.

NOTE: At this stage, it is possible to place the mouse heart in a small plastic bag or a 5 mL centrifuge microtube in the freezer (-20 °C) for up to 5–10 min. Maximal freezing time should be determined experimentally in each laboratory. Short-term freezing of a mouse heart can help a novice experimenter cut it into 1-mm-thick slices. The freezing of rat hearts is not recommended. Overfreezing for more than 10 min at -20 °C must be avoided.

1.5. Place the stained rat heart in a stainless-steel matrix (see the **Table of Materials**) for heart slicing (**Figure 1A**). Then, cut the ventricles of the heart into 2-mm-thick slices (aim for 6–7 slices of an adult rat heart). For mouse hearts, cut the ventricles of the heart into 1.5-mm-thick slices (aim for at least 4 slices of an adult mouse heart).

NOTE: Slicing matrix-compatible razor blades must be used. In general, compatible single-edge razor blades (e.g., thickness of up to 0.01 inch (0.254 mm)) can be used for slicing rat hearts. Double-edge razor blades are generally used for mouse hearts and are usually up to 0.004 inches (0.1 mm) in thickness.

[Place **Figure 1** here].

1.6. After cutting, transfer the slices to a 15 mL plastic tube. Add 5 mL of 1% triphenyltetrazolium chloride (TTC) dissolved in phosphate-buffered saline (PBS) to the tube with the heart slices, and incubate for 10 min in a water bath at 37 °C.

1.7. After incubation in TTC solution, wash the heart slices at least 2–3 times with PBS and prepare for image capture.

2. Brain staining and slicing

2.1. After the middle cerebral artery occlusion experiment[3,12], remove the brain, including the brainstem, from the skull, and wash it in ice-cold PBS.

2.2. Choose the correct size of the brain stainless-steel matrix (see the **Table of Materials**) depending on the weight of the animals (**Figure 1B**). Place the brain with its ventral side up in the brain matrix.

NOTE: When seated in the matrix, the brain's ventral surface must be parallel to the top surface of the mold.

2.3. Using blades, restrict the frontal and caudal parts (2 blades from both sides) of the brain.

NOTE: Slicing matrix-compatible razor blades must be used. In general, a compatible, single-edge razor blade (thickness of up to 0.01 inch (0.254 mm)) can be used for rat brain slicing.

2.4. Put the blades partially (not fully cutting the brain) into the channels between the first and the last blades. When all the blades are inserted and arranged in parallel, press all the blades down with the palm at the same time to cut the brain into 2 mm coronal slices.

2.5. Grasp the blades firmly along the sides with two fingers and remove them together with the sliced brain from the matrix.

2.6. Arrange the brain slices one by one in a tray (70 mL, 72 x 72 mm). When arranging the slices, ensure that the anterior surface of each slice is always facing up.

2.7. Pour warm (+37 °C) 1% TTC solution in PBS onto the brain slices, and incubate them for 8 min at 37 °C in the dark.

NOTE: The brain slices must be fully immersed in TTC solution during the incubation.

2.8. After incubation in 1% TTC solution, transfer the brain slices to the blue plastic tray to capture images. Arrange the brain slices in sequential order from the frontal to the caudal part, and use a scalpel to separate the hemispheres in the sagittal plane.

NOTE: The surface of the tray should be washable, matte, and of a color that contrasts brain slices (i.e., not red, white, or pale pink).

3. Macro photography

3.1. Photograph the tissue slices immediately after staining.

NOTE: Heart slices can be stored in cold PBS (at +4 °C) or formalin solution for up to 30 min. Brain slices can be stored in formalin for a prolonged period (1–2 weeks).

3.2. Set up the camera of choice with a charged battery, memory card, and attached lens on a stand (**Figure 2**)

NOTE: Turn the lights on at least 5–10 min before image acquisition to warm up the equipment. LED lights reach full brightness in microseconds.

[Place **Figure 2** here].

3.3. Depending on the available light sources, select the appropriate white balance settings or perform color temperature calibration according to the instructions in the camera manual.

NOTE: White LED light (color temperature 6,500 K) is the preferred light source to avoid light flickering by fluorescent light bulbs.

3.4. Switch the camera to fully manual mode, set the ISO 100 and aperture to f/10, and adjust the shutter speed for optimal image exposure. Ensure that the camera focal plane is parallel to the surface where the sample will be placed.

NOTE: Histogram function is useful to ensure that tissue slices are not overexposed.

3.5. Attach or enable a wired or wireless remote trigger to prevent camera shake when the shutter is released.

NOTE: An alternative is to enable a delayed shutter function, which will delay the trigger for 2 or 10 s after pressing the trigger button.

3.6. Immerse the heart slices completely in a container with PBS.

NOTE: Immersed slides tend to float away from their position. To minimize floating of the slices, use the smallest possible tray into which all slices can fit and the minimal amount of immersion solution, ensuring that the specimen is fully immersed. Alternative methods include placing the slices between glass slides or using a polarizing filter on the lens. A circular polarizing filter is attached to the lens and rotated until reflections in a live-view display of the camera disappear.

3.7. Arrange the brain slices in a dry tray without PBS or other liquids.

NOTE: A polarizing filter is very convenient to capture images of brain slices.

3.8. Place the container with slices under the camera with the macro lens and ensure that all slices fully fit in the field of view. Ensure that the slices are on the same plane, i.e., not curved or rolled.

3.9. Check the exposure and adjust the camera settings if needed.

NOTE: Once set, do not change the exposure and other settings during the entire experiment.

3.10. Capture the number (or other identification) of the sample and image the tissue slice using a remote trigger.

NOTE: A size marker, such as a mm ruler, should be included in the field of view when absolute quantification of specimen size is necessary.

3.11. Rotate the slices and capture their images from the other side.

REPRESENTATIVE RESULTS:

Figure 3A is a photograph of a methylene blue- and TTC-stained heart slice after myocardial infarction, which contains enough detail and color information for further planimetric analysis of infarct size (**Figure 3B**). We tested how freezing of the heart for 24 h affects the integrity of heart tissues (**Figure 3C**). Freezing for a prolonged period (>1 h, **Figure 3C**) reduces mitochondrial function; thus, TTC staining of the heart is not red but pale pink, and the border between necrotic and viable tissues is blurred (**Figure 3C**).

Further, two methods were compared for the reduction of reflections in the specimens. Immersion is the most efficient method and produces detailed images with good contrast (**Figure 4A**). The second method is the use of a polarizing filter attached to the lens. The polarizing filter also is effective; however, the filter slightly reduces the resolution and microcontrast of the image (**Figure 4B**). An example image of a heart slice without immersion or filter (**Figure 4C**) contains many reflections and is not suitable for further analysis.

Brain slices are not immersed because of slice management (floating) problems. In the planimetric analysis, it is important to compare the unaffected (healthy) side of the brain (**Figure 5A**) with the stroke-affected side (**Figure 5B**). Brain slices are easier to manage on a dry plate or tray, and a polarizing filter is used to remove reflections. A tray with blue background is used for brain slice photography (background selection described previously[5]).

Manual camera settings were used to ensure full control of exposure and white balance. Camera settings should be adjusted before or at the beginning of the experiment according to the available light source. This ensures optimal exposure and white balance of all images to allow uniform analysis (**Figure 6A**). The automatic settings of the camera are not perfect and can result in varying camera parameters, causing inappropriate results and the introduction of image-to-image variability.

Figure 6 shows examples of overexposed (**Figure 6B**) and underexposed images (**Figure 6C**) of heart slices. Sufficient attention should be paid to the correct white balance settings of the camera to match a particular light source used in the camera–light setup. Incorrect white balance settings may result in a shift to blue or yellow (**Figure 6D**) and magenta or green (**Figure 6E**) cast in the image.

FIGURE AND TABLE LEGENDS:

Figure 1: Matrices for the rat heart and brain slicing. (**A**) Rat heart, (**B**) rat brain.

Figure 2: Camera and lights set up for macro photography. Camera is perpendicular to the imaging surface to ensure that the focal plane of the camera is parallel to the samples. Abbreviation: LED = light-emitting diode.

Figure 3: Images of rat cardiac slices. (**A**) Fresh heart slice was analyzed in ImageProPlus 6.3 software using color segmentation (**B**). (**C**) TTC staining poorly discriminates between viable

and necrotic tissue in the frozen heart slice (frozen for 24 h). Abbreviation: TTC = 2,3,5-triphenyl-2H-tetrazolium chloride.

Figure 4: Techniques for the reduction of reflections. Rat heart slice image captured immersed in PBS (**A**) and using polarizing filter (**B**). (**C**) Heart slice with reflections when neither immersion nor filter is used. Abbreviation = PBS = phosphate-buffered saline.

Figure 5: Images of rat brain slices. Rat brain was cut into seven slices and stained with TTC after ischemia-reperfusion. Using polarizing filter results in acquisition of reflection-free image. (**A**) Slices from the undamaged hemisphere ; (**B**)Slices from the stroke-affected hemisphere. Abbreviation: TTC = 2,3,5-triphenyl-2H-tetrazolium chloride.

Figure 6: Rat heart slice images. Correctly (**A**) and incorrectly (**B–E**) captured heart slice images. Incorrect exposure settings result in overexposed (**B**) and underexposed images (**C**). Incorrect white balance settings result in yellow (**D**) or green cast in the image (**E**).

DISCUSSION

Preparation of the heart after IR starts with the reocclusion of blood cardiac arteries and the perfusion of blue dye for the discrimination of at-risk areas from non-risk areas. Methylene blue or Evans blue dyes are most frequently used for this purpose[2]. As an excessively high pressure might damage heart valves and, thus, partially or completely stain at-risk areas, it is better to perfuse the heart with a pressure-controlled system, such as the Langendorff apparatus or a simplified version of a hydrostatic pressure system-equipped syringe or pump. Controlled perfusion will ensure physiological pressure, and the dye will usually not enter the occluded region of the heart. Both flow speed- and pressure-controlled techniques are safeguards against overstaining.

One of the most serious mistakes in viable tissue processing is keeping tissues in a freezer for a prolonged time before staining. Freezing is mainly used because researchers want to perform heart staining the day after the IR experiment or later. Moreover, freezing is used to make the cutting of the heart easier. We found that short-term freezing of the heart for up to 5–10 min negligibly affects the integrity of heart tissues and facilitates cutting the tissues (particularly for mouse hearts) into thin slices. However, freezing for prolonged periods damages membranes and decreases cell viability and mitochondrial function[13]. As a result, TTC staining of functioning mitochondria is affected, and the border between necrotic and viable tissues is poorly delineated (blurry). Overall, freezing of rat harts should be avoided, and only short-term freezing of mouse hearts can be used for easier cutting.

The next step is tissue staining in 1% TTC solution at 37 °C[14]. The staining solution should be prewarmed—particularly important for staining of brain slices. When using the prewarmed solution, the optimal staining time for heart slices is 10 min. A longer incubation or a temperature higher than 37 °C results in brown colorization of the heart tissues. Proper staining of specimens and consistent red color intensity are important for further image analysis. In the final steps before photography, the tissue slices are rinsed 2–3 times with cold PBS or a similar buffer to remove TTC and excess methylene blue from the solution to avoid blue casting in the photograph. Heart slices should be photographed shortly after staining to obtain the best image quality. Heart staining remains of good quality if stored for

up to 60 min in the cold (+4 °C) PBS. Stained brain slices and aortic tissues are usually stored in a 4% neutral formaldehyde solution and retain good quality for a week. Overnight storage of brain tissues in formalin (+4 °C) does not impair the color intensity of normal tissue and is acceptable for image acquisition. However, formalin induces swelling and destaining of heart slices. Therefore, the storage of heart tissues in formalin is not recommended.

The next step is image acquisition. Many laboratories use flatbed scanners as an image acquisition tool that is expected to replace a digital camera and lighting setup. We determined that the scanning of slices does not provide sufficient image resolution and color separation and therefore is not suitable for imaging heart slices. In particular, scanner resolution is insufficient for mouse hearts, and we noticed poor rendering of methylene blue. In contrast, a scanner might be an alternative to a photo camera for imaging brain slices stained only with TTC or other single dyes. For the scanning of tissue slices, scanning software that ensures constant exposure settings is essential. Overall, a flatbed scanner is less capable and cannot replace a digital camera for most imaging applications.

The background behind specimens is also important. Ideally, the bottom of the tray should be of a color not present in the stained specimen. For example, to quantify the area of methylene blue and TTC (red) staining in an automated or semiautomated manner, white, red, blue, yellow, and brown backgrounds should be avoided. Thus, a green background would be preferable. Nevertheless, color selection depends on the preferences of the operator, who postprocesses the image. Many scientists prefer a white background because a white background can be deleted in image postprocessing and converted into completely white (RGB white code 255,255,255). Then, one should completely exclude white from the list of selected colors used for semiautomated analysis and count only pale necrotic areas, which are not completely white if not overexposed. Blue and green backgrounds are suitable for the photography of brain slices and aortas.

The optimal imaging tool for tissue photography is a single-lens reflex or mirrorless interchangeable lens digital camera with a compatible macro lens. Capturing very small objects might require a combination of a camera and a microscope; nevertheless, a macro lens usually has sufficient (at least 1:2) magnification to obtain detailed images of a mouse heart. Many manufacturers offer affordable digital cameras and macro lenses to obtain high-resolution and high-magnification photographs. All up-to-date digital cameras have characteristics and functions necessary for macro photography, including the possibility to mount on a stand, a high number of pixels (usually >20 Mpx), live view, mirror lock-up, time-lapse features, remote shutter, and the ability to manually set camera parameters, thus ensuring a constant shutter speed, aperture, white balance, and ISO setting. Compact cameras with the above-mentioned features and lens magnification of at least 1:2 can also be used for macro photography. Because of lens characteristics, some compact cameras should be placed in close proximity to the object, and the experimenter must ensure that the camera body does not affect the illumination of the specimen.

For macro photography with any type of interchangeable lens camera, a high magnification (1:1–1:2) macro lens is required. We suggest using macro lenses with a focal length ranging from 50 mm to 100 (120) mm or equivalent to the full-frame (24 mm x 36 mm) sensor. Smaller sensor cameras have different sensor sizes, and magnification should be

recalculated accordingly. For the photography of heart slices, an ergonomic distance of the 100 mm macro lens front element to the subject is approximately 150 mm. This setting allows operators to keep all the equipment on a table, with easy access to the camera controls. A 50 mm macro lens might be considered for photography of larger objects, such as brain slices, because a wider field of view is necessary to obtain all slices in a single photograph.

To obtain sharp images with high resolution, a camera should be mounted onto a sturdy stand, which, together with a light setup, is called a photography copy stand. Mounting the camera on a stand and a remote (wired or wireless) trigger eliminates camera shake and ensures a constant distance from the target. A camera–lighting setup with two constant-light sources from both sides, angled approximately 30–60° relative to the subject plane, ensures sufficient illumination of specimens and helps avoid reflections at the same time. The camera should be mounted precisely so that the sensor is parallel to the subject plane. To evenly illuminate the image field, both lamps should be equally oriented and placed at the same distance from the subject. Light sources placed at various distances from the subject cause uneven illumination. Additionally, blinking light sources are a reason for variations in image exposure. Overall, it is important to accurately place the camera and light sources to precisely acquire images of well-illuminated specimens.

Tissue samples reflect light (glisten), which appear as white spots in the images. These light reflection spots do not contain useful color information, and accordingly, these parts of the images cannot be used for accurate quantitative analysis of images. Light reflections from tissue slices can be removed by various methods. The most efficient is the full immersion of tissue samples in a container with a saline or PBS solution. A similar approach is the insertion of tissue slices below (or between) glass plates. This method is efficient against reflections; however, the image resolution can be lower than that of photographs of immersed tissues.

One can also use a polarizing filter mounted on a lens to eliminate light reflections. Circular polarizing filters are widely available but vary considerably in quality depending on price, and cheap filters can significantly reduce image resolution. Reflected light can be filtered off by turning the moving part of the polarizing filter at an angle. The efficacy of the polarizing filter might be affected by some light sources (e.g., strong LED light). Overall, after removal of extra liquid, a polarizing filter can eliminate all reflections from the brain slices; however, sample immersion in buffer solution is the easiest and most cost-efficient approach for heart slices.

Manual settings of the shutter speed, aperture, ISO, and white balance are important to maintain full control of the imaging process. The sample, background, and characteristics of the light source influence the camera exposure metering system in automatic settings; therefore, manual settings are necessary to maintain constant exposure and white balance between multiple photographs during the experiment. For macro photography, the suggested aperture setting is between f/8 and f/16. By decreasing the aperture, the depth of field increases, which is helpful if the object is not in a single plane. However, diffraction limits the total resolution of photography in the case of smaller apertures. The optimal aperture for most lenses is usually f/10 because in this setting, resolution drop is negligible, and depth of field is sufficient. ISO values that range from 50 to 400 (lower is better) are

usually optimal to minimize image artifacts (noise). The shutter speed then remains to be changed to obtain correct exposure using the mentioned aperture and ISO settings in existing light conditions. Manual settings are important for consistent image analysis. Standardized imaging ensures the use of the same color thresholding settings throughout any study, which requires segmentation analysis. For example, semiautomated analysis by ImagePro software based on a segmentation file with predefined colors of blue, red, and white (+pale pink) can be used over the years if specimen images have consistent colors, WB, and exposure.

The white balance setting should be adjusted depending on the color temperature of the light source that is used to illuminate a sample. White balance can be selected from camera built-in presets or using the manual calibration of a grey target. The benefit of image capturing in RAW format is that white balance can be adjusted during software postprocessing of the image. As RAW files contain much more information than JPEG files, RAW file postprocessing provides an excellent opportunity for the correction of color balance and exposure, as well as to obtain better image resolution. Because most cameras can capture JPEG and RAW files simultaneously, we suggest capturing the RAW file and saving it as a backup. Overall, this protocol describes a methodology for rat heart and brain tissue slicing and staining and provides guidelines for establishing lighting and camera setups and photography techniques for high-resolution image acquisition for further analysis. This method is applicable to all experimental small-animal organ photography.

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DISCLOSURES:

The authors declare that they have no conflicts of interest.

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Figure 1

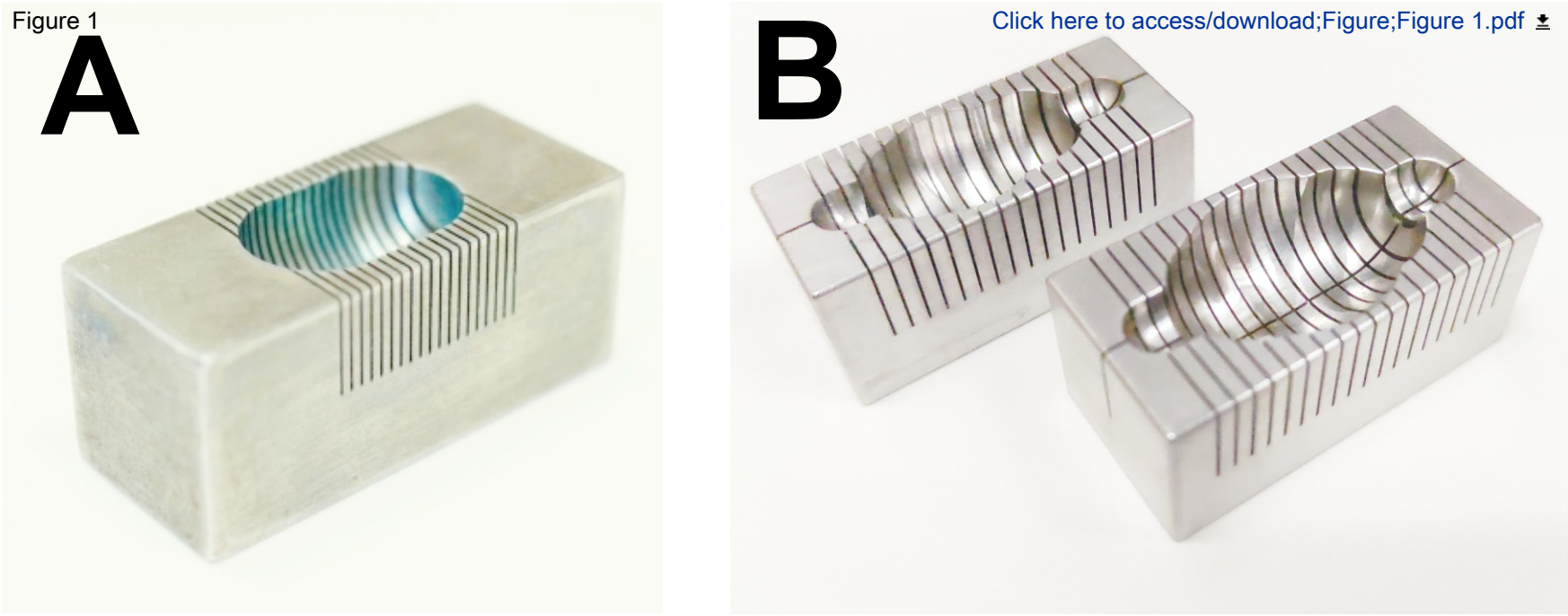

Figure 2

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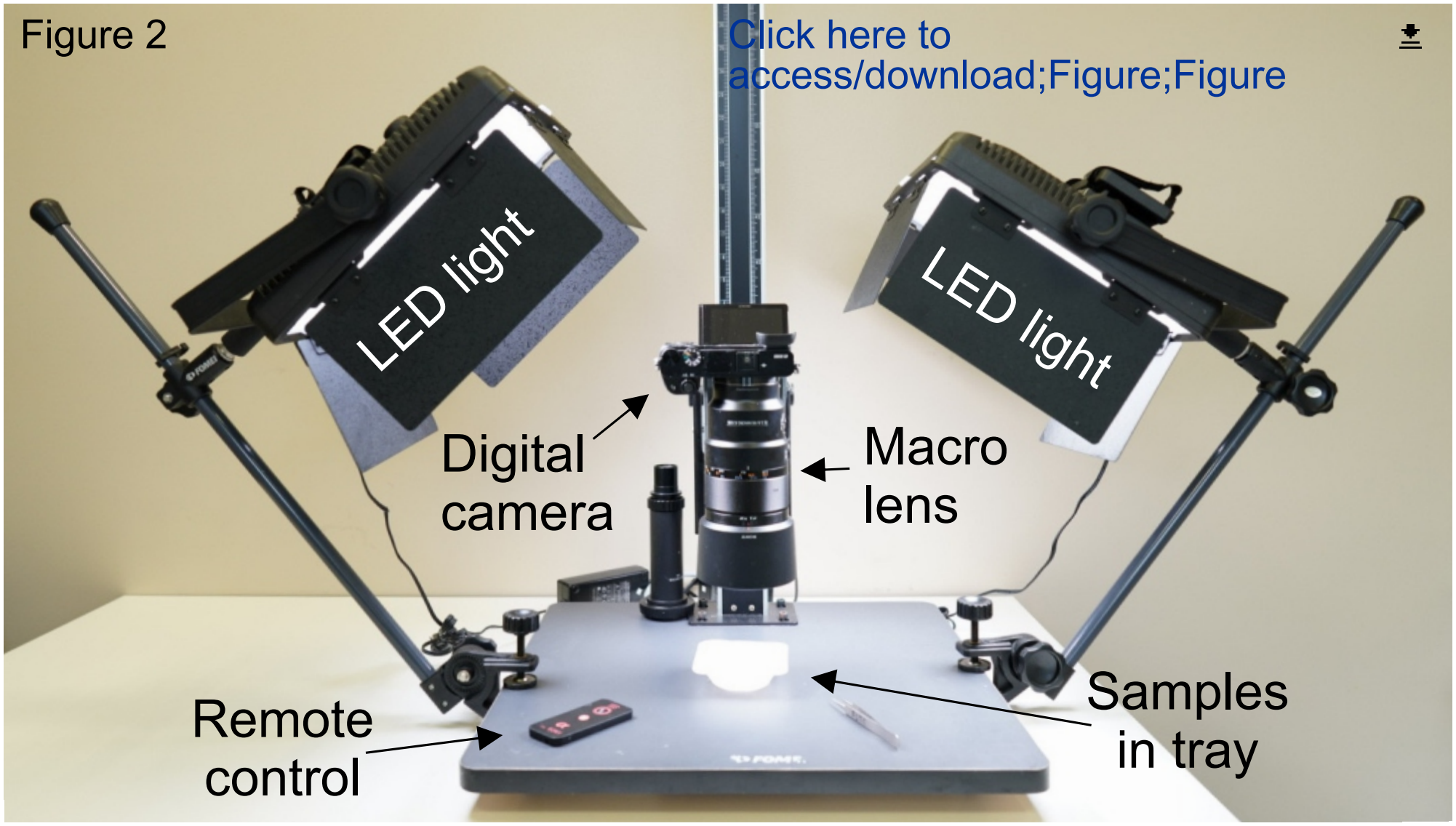

Figure 3

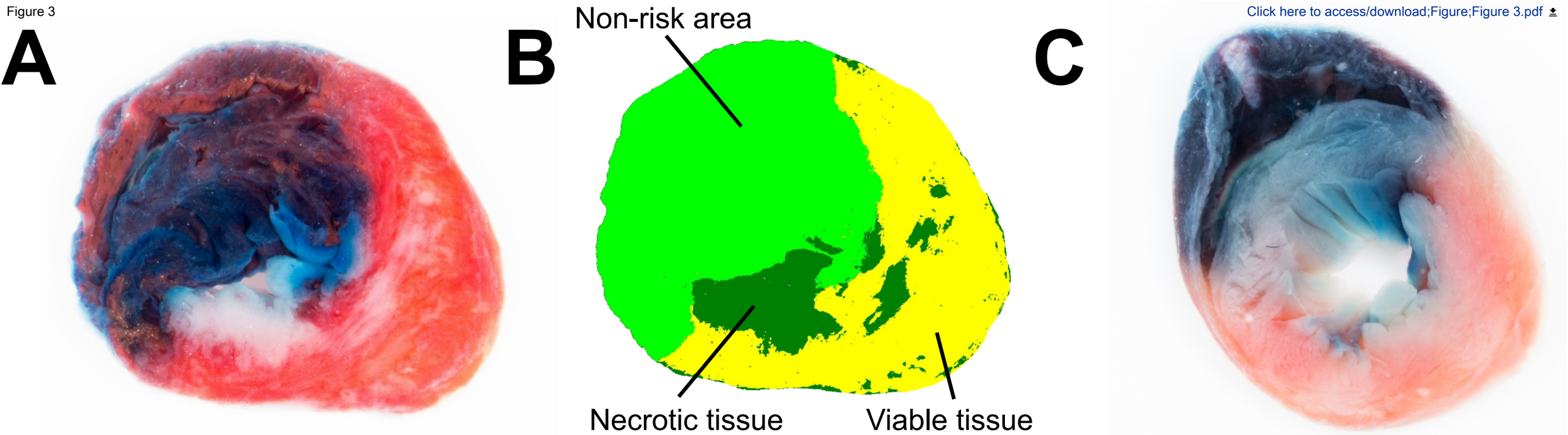

Figure 4

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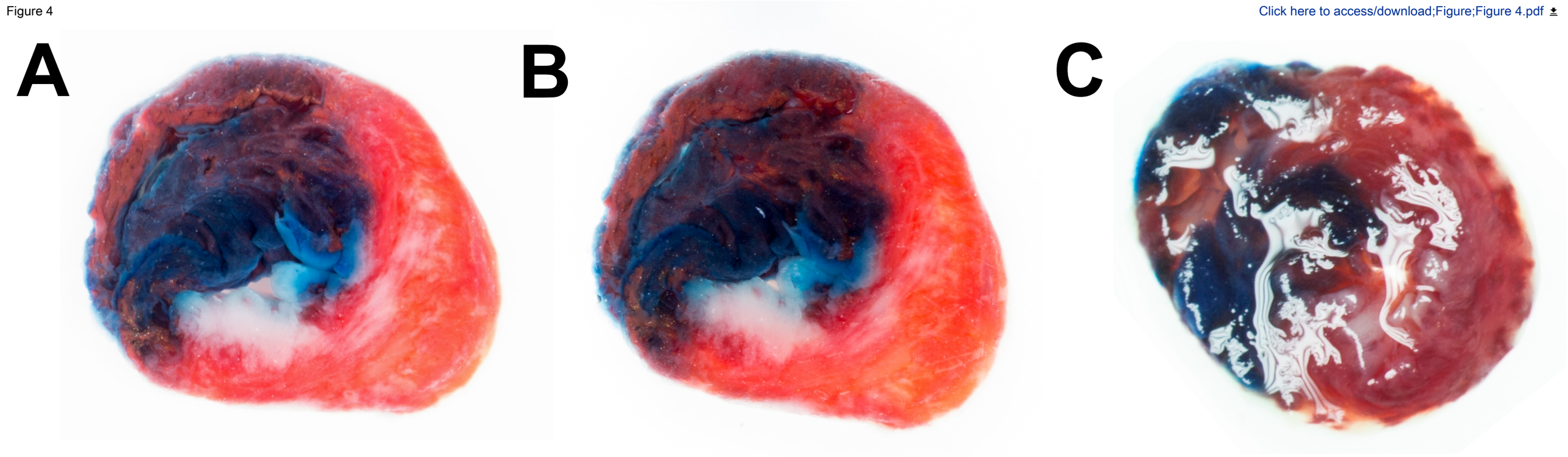

Figure 5

Figure 6

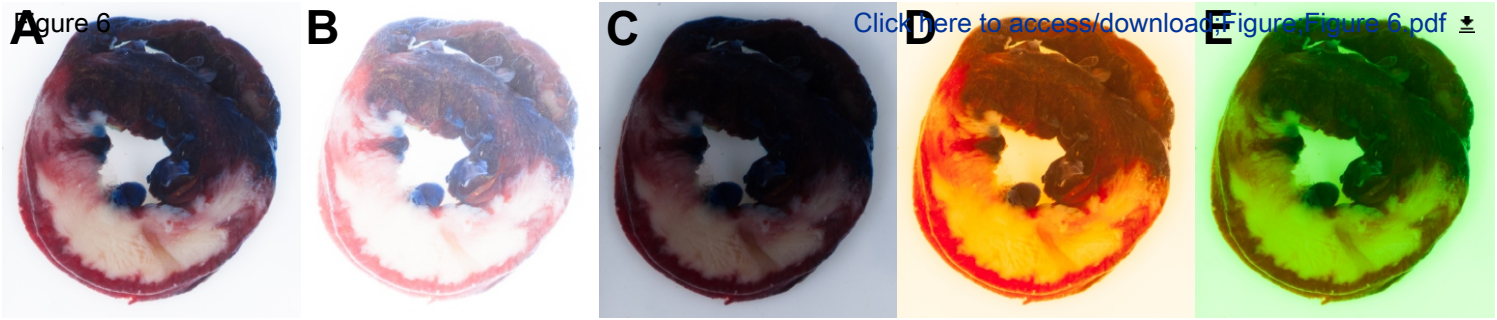

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Table of Materials

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December 2, 2021

To Whom It May Concern:

Please find, attached, our revised manuscript (JoVE submission JoVE62942). We want to thank reviewers for their helpful insights and comments. Our responses and the corresponding revisions are described, in detail, below.

Editorial and production comments:
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. Please use American English throughout.

The text was proofread by the AJE experts. We have now used American English throughout the manuscript.

AJE

Editing Certificate

This document certifies that the manuscript

Heart and brain tissue preparation for digital macro photography after ischaemia-reperfusion

prepared by the authors

Edgars Liepinsh, Janis Kuka, Liga Zvejniece, Reinis Vilskersts, Maija Dambrova

was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at AJE.

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2. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without brackets'

The reference format has been changed as suggested.

3. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
We believe that all the requested information is present in the Introduction text. We

have described the rationale behind the use of improved and up-to-date techniques to get high-quality images for the quantification of infarcted tissues. This is expected to reduce the variability of preclinical data sets and inconclusive results/outcomes. The very detailed previous studies and techniques for heart and brain infarction experiments have been cited, allowing us to concentrate on macro photography and image analysis steps. To help readers to determine whether the method is appropriate for their application, we state that these methods are particularly useful for performing image planimetric analysis in rodent tissues and general scientific macro photography to obtain high-quality images for automated analysis of digital photographs. We also believe that these techniques will help readers to save time, avoid user input and minimize the risk of errors or bias during image analysis. To highlight the information regarding the overall goal of this method, we modified the respective sentence in the Introduction and rewrote the Summary:

The purpose of this study is to provide a step-by-step guide on how to set up a system for photography and standardized analysis of rodent heart and brain tissue specimens after infarction experiments.

Presented here is a protocol for standardized methodology of rodent tissue preparation after the ischemia-reperfusion experiment and guidelines for establishing lighting and camera setups for high-resolution image acquisition. This method is applicable to all experimental small animal organ photography.

4. 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."

The texts are rearranged and corrected as suggested.

5. Please ensure you answer the "how" question, i.e., how is the step performed?

The texts are corrected as suggested.

6. Age, sex, strain of the rat used in the experiment.

The age, sex, and strain of animals are not relevant for the photography and image analysis techniques. Our contribution is related to the heart and brain tissue preparation for digital macro photography after ischemia-reperfusion, i.e., tissue preparation and subsequent image capture are steps performed immediately after the ischemia-reperfusion experiment. The readers are referred to previous publications that describe all details for the heart infarction and stroke experiments (Botker et al., 2018, Basic Research in Cardiology; Zvejniece et al., 2012, J Neurosci Methods; Bell et al., 2011, J Mol Cell Cardiol; Liu et al., 2011, J Biomed Biotechnol; Kolwicz et al., 2010, J Vis Exp; Herr et al., 2015, J Vis Exp; Liepinsh et al., 2015, Br J Pharmacol.; Nakamura et al., 2005, J Card Surg.; Li et al., 2010, Lab Anim (NY); Wu et al., 2011, J Vis Exp.; Vavers, E. et al. 2016, Pharmacol Res).

7. 1.2: Do you check the depth of anesthesia?

Yes, we check the depth of anesthesia when we do in vivo infarction experiments. All experimental procedures are performed in accordance with the guidelines of the European Community and local laws and policies (Directive 2010/63/EU), and approved by Food and Veterinary Service, Riga, Latvia. However, our present manuscript describes tissue preparation and subsequent image capture techniques performed immediately after the ischemia-reperfusion experiment, and this does not include the anesthesia step.

8. 1. Please describe how big is the incision in each step.

Our contribution is related to the heart and brain tissue preparation for digital macro photography after ischemia-reperfusion, i.e., tissue preparation and subsequent image capture are steps performed immediately after the ischemia-reperfusion experiment. The readers are referred to previous publications that describe all details for the heart infarction and stroke experiments (Botker et al., 2018, Basic Research in Cardiology; Zvejniece et al., 2012, J Neurosci Methods; Bell et al., 2011, J Mol Cell Cardiol; Liu et al., 2011, J Biomed Biotechnol; Kolwicz et al., 2010, J Vis Exp; Herr et al., 2015, J Vis Exp; Liepinsh et al., 2015, Br J Pharmacol.; Nakamura et al., 2005, J Card Surg.; Li et al., 2010, Lab Anim (NY); Wu et al., 2011, J Vis Exp.; Vavers, E. et al. 2016, Pharmacol Res).

9. 3: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.

The description of protocols is simplified and now contains only 2-3 actions per step.

10. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

The Representative Results section now describes the obtained example images in Figures in the context of used techniques.

11. Please ensure the results are described in the context of the presented technique.

The results are now described in the context of the presented techniques.

12. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Please combine all panels into one image file.

All panels are now combined into one image file and uploaded separately to the Editorial Manager account.

13. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

The manuscript file isre- organized as requested.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation].

All figures are prepared for the current publication, no re-used from any previous one.

15. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have revised the Discussion text and hope that it now includes all important discussion points related to the applicability of the suggested techniques.

16. Please number the references in the order of citation. Please ensure at least 10 references are cited.

References are now numbered in the order of citation.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This text gives some useful guidance on the analysis of infarct size in small animal's hearts such as rats and rodents. These hearts are usually too small to trace directly and require macro photography to get an accurate analysis. They propose that an inexpensive digital camera along with a macro lens to be used.

Concerns:
I agree with this premise but, depending on the camera, an accessory macro lens may not be needed. My old Pentax pocket camera has a super macro setting and I can get a sharp 1000 pixel wide picture of a mouse heart easily without a macro lens. The authors should give a guideline as to what resolution in pixels needs to be achieved so the investigator can determine if his camera is suitable. I shoot for an absolute minimum width of 800. Odds are many of today's cameras would work without a macro lens.

Thank you for pointing this out. We agree that any camera can be used if it fulfills the following criteria: can be fixed on a stand, has remote shutter/shutter delay function;

it has live view function; manual mode of focus, exposure and white balance settings; lens can reach magnification of at least 1:2 and has sufficient distance to the object (to not affect the illumination of the object) at high magnification; camera has sufficient dynamic range (to make it possible to capture dark viable tissue and necrotic bright tissue with sufficient detail and color information).

Cheapest setups of a new interchangeable lens camera and macro lens cost around 1000EUR/USD. It is generally the same price as for 1-2 ELISA kits. In contrast to ELISA kits, the camera-lens combo will last at least 10 years in indoor conditions on the stand. Most of the up-to-date interchangeable lens cameras available on the market today have 20+Mpix sensors (see camera selection from B&H store below as an example). Nevertheless, pixels count is not the only or main criteria of a camera. For instance, a modern interchangeable lens camera with a larger and thus "higher quality" pixel size but "only" 20Mpix will capture much more color information and higher dynamic range than any latest smartphone with 50-100Mpix or pocket cameras. Lens quality (resolution across the field, low distortion) often is at least equally important than is the senor of the camera.

To answer the reviewer's concerns, we have added more details on camera and lens characteristics to the Protocol texts and Discussion.

Compact cameras, which have the above-mentioned features and lens magnification of at least 1:2 also can be used for macrophotography..

The section describing the ischemic insult is inadequate (Lines 98-102). Line 101 says "reocclude the artery" but the original occlusion is not mentioned. You should describe a recovery period of at least 20 minutes normal perfusion, then an occlusion time (the insult) followed by a reperfusion time of at least an hour to allow TTC-reactive enzymes to wash out. Also, temperature must be maintained at 37 degrees, especially during ischemia. Immersion in a temperature controlled bath is best during ischemia when warm buffer is not circulating through the heart. Also, you should warn that the heart is ischemic between the time it is excised from the animal to the start of perfusion. The transfer must be very quick as an ischemic time of more than a couple minutes can precondition the heart (even with cooling) and cause an artifactualy small infarct size.

Our contribution is related to the heart and brain tissue preparation for digital macro photography after ischemia-reperfusion, i.e., tissue preparation and subsequent image capture are steps performed immediately after the ischemia-reperfusion experiment. In the Introduction part the readers are referred to previous publications that describe all details for the heart infarction and stroke experiments (Botker et al., 2018, Basic Research in Cardiology; Zvejniece et al., 2012, J Neurosci Methods; Bell

et al., 2011, J Mol Cell Cardiol; Liu et al., 2011, J Biomed Biotechnol; Kolwicz et al., 2010, J Vis Exp; Herr et al., 2015, J Vis Exp; Liepinsh et al., 2015, Br J Pharmacol.; Nakamura et al., 2005, J Card Surg.; Li et al., 2010, Lab Anim (NY); Wu et al., 2011, J Vis Exp.; Vavers, E. et al. 2016, Pharmacol Res).

The text only discusses regional ischemia in a Langendorf perfused heart. Regional ischemia is well suited to open chest models. On the other hand regional ischemia is hardly the optimal choice for an isolated heart. There global ischemia is the best. The risk zone simply becomes the whole heart so the blue dye is not needed. Also, if a balloon is placed in the heart's lumen you can measure the recovery of mechanical function at the end of reperfusion.

Our contribution is related to the heart and brain tissue preparation for digital macro photography after ischemia-reperfusion, i.e., tissue preparation and subsequent image capture are steps performed immediately after the ischemia-reperfusion experiment. We believe that the regional ischemia experiments are relevant for translational research of emerging cardioprotective therapies. We hope that our guide on how to set up a system for photography and standardized analysis of heart and brain tissue specimens after infarction experiments will help to save time, avoid user input and minimize the risk of errors or bias during image analysis to increase the translation of preclinical discoveries into novel antiischemic treatments in clinics.

On line 301 you mention eliminating glare by sandwiching the tissue slices between glass plates. Actually this should be done with accurately measured shims in the 4 corners of the glass plates When the tissue is slightly squashed to ensure good contact with the glass the slice will be distorted to slightly increase its diameter. However, however the shims will allow you to calculate an accurate volume of each slice (the surface area times the thickness of the shims). The glass sandwich can be flipped to photograph the other side. You should also mention that a size standard should be included in the picture such as a mm ruler or a piece of paper of known size so areas and volumes can be calculated in mm.

Thank you for pointing this out. In our laboratory, we are using immersion for the heart slices and aortas mounted on a silicone block, while a polarizing filter is used for the brain without immersion or glass slides. Since we know that many scientists are placing specimens between glass plates, we tried and found that this method of choice may present some problems e.g. challenges with bubbles, specimen unequal thickness, etc. Immersion is therefore much easier without compromising image quality. To answer the reviewer's comment, we have now included a Note about the alternative methods and placing slices between glass slides in the method description.

Alternative methods include placing slices between glass slides or using a polarizing filter on the lens.

In our laboratory, we do not use a ruler when absolute quantification is not necessary. The infarct size is quantified as % to the area of the left ventricle or area at risk. Stroke also is quantified relatively to the unaffected side of the brain. To answer the reviewer's comment, we added a sentence to the manuscript:

Size marker such as a mm ruler should be included in the picture when absolute quantification of specimen size is necessary.

The background is also important. A royal blue background is recommended which will allow selection of the infarct and living tissue by color thresholding using an image analysis program such as ImageJ. Blue is not included in colors generated by TTC staining.

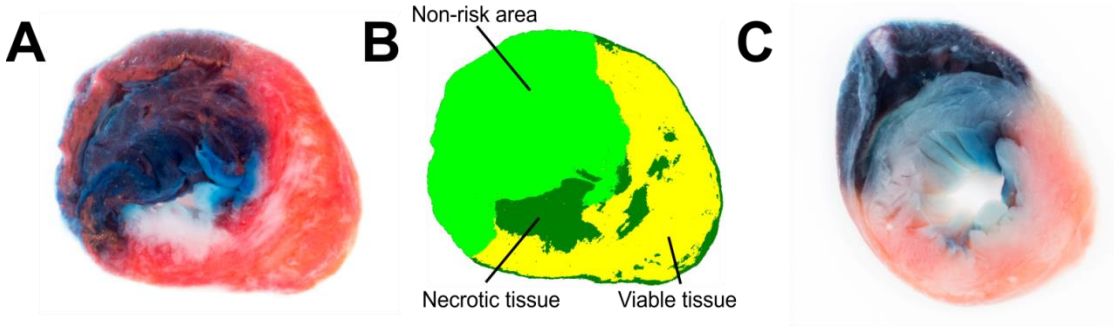
Thank you for pointing this out. Ideally, the tray should be in a color not present in the stained specimen. For example, to quantify the area of methylene blue and TTC (red) staining in an automated or semi-automated manner, white, red, blue, yellow, brown, colors of background should be avoided. So green would be preferable. However, color selection depends on the preferences of the operator, who is doing image postprocessing. We like the white background because in image postprocessing you can delete the background and thus make it completely white (RGB white code 255,255,255). Then exclude completely white color from the list of selected colors used for semiautomated analysis and count only pale necrotic area, which is not completely white if not overexposed. For the photography of brain slices and aortas, blue and green is suitable.

We have now referred to our previous publication (Liepinsh et al, 2013) in the Representative Results section.

A tray with blue background is used for brain slice photography (background selection described in details previously[5]).

You should discuss image analysis in sizing the infarcts. For many years infarct size studies have suffered from inaccurate subjective analyses by supposedly blinded observers. Excellent discrimination can be achieved with image analysis programs. Color thresholding settings should be established and then rigorously adhered to throughout any study. That way anybody can analyze the same picture and get the same exact infarct size result. Blinding is no longer required.

Thank you for noting this. We agree with the reviewer that low-quality images and subjective analysis are issues in experimental infarct studies. In our manuscript we describe techniques how to get images usable for image analysis – this protocol works very well and we have shared it with several international collaborators who found it very useful. Our analysis workflow is based on software ImagePro 6.3 that is not available any more (we are not familiar with ImageJ). We are using macro-driven semiautomated analysis by ImagePro software based on a segmentation file with predefined colors of blue, red, and white (+pale pink) for about 15 years. Over the years we also kept the photography setup at a constant output. We changed cameras and lightings but always adjusted settings to get the same colors, WB, and exposure in the image. Still, we cannot avoid manual postprocessing because of 2 issues. First, methylene blue is sometimes not perfectly staining the whole area of non-risk (see example below). Second, since the heart is not cylindrical but the round shape, some slices are conical. These issues and image analysis were discussed in our previous article (Liepinsh et al 2013).



To answer the reviewer's concerns, we have added the Representative results section and an example of planimetric image analysis in Fig. 3B.

I suggest you look at the H.E.L.P files in the International Society for Heart Research's web site (https://www.ishrworrld.org). They have an excellent page discussing these issues (https://www.southalabama.edu/ishr/help/ttc/). I am not sure if you can cite a web page in JOVE, but if it is allowed it would be useful.

Thank you for the information. We have added references to the previously published infarction protocols both to Introduction and Protocol part. We hope that also our manuscript if accepted, will help readers to save time, avoid user input and minimize the risk of errors or bias during image analysis.

Experimental procedures of myocardial[1,2] and brain[3,4] infarctions have been described in detail before.

Techniques described in this protocol can be used after both Langendorff-perfused isolated rat or mouse heart [6,7] and in vivo rat heart ischemia-reperfusion (IR) injury assays[8-11].

2.1 After the middle cerebral artery occlusion experiment[3,12] remove the brain, including the brainstem, from the skull, and wash in ice-cold PBS.

Reviewer #2:
Manuscript Summary:
The manuscript introduced a method of macro photography in ischemia-reperfusion-induced damage in the heart and brain with a high-resolution image, which has a standardized methodology and is easy to implement.

We thank the reviewer for the comment!

Reviewer #3:
Manuscript Summary:
Heart and brain tissue preparation for digital macro photography

The authors describe photography using a digital camera with macro lens for qual and quant analysis. Pre photography methods include Tissue prep, Dissection, Slicing, staining. The Standardised photography Set up includes hardware (Lighting, copy stand, camera specifications) and camera settings (ISO, white balance, aperture). This article appears to be based upon the authors previous article (Liepinsh E, Kuka J,

Dambrova M. Troubleshooting digital macro photography for image acquisition and the analysis of biological samples. Journal of Pharmacological and Toxicological Methods. 2013;67:98-106.) with some updates to equipment. The methods and rationale for method choices are discussed in more detail in the 2013 article. I would suggest the authors refer more frequently to their 2013 article for in depth discussion of method choices that are not so detailed in this 2021 article (lighting specifications, background selection, avoiding reflection,) or increase the detail in this 2021 article.

Thank you for a suggestion. We have added more technical details to the Protocol texts, Representative results section, and Discussion.

JoVE is a video journal, and Discussion is limited to 3-6 paragraphs. We have already exceeded this limit (10 paragraphs). While participating in the establishment of an international network that will evaluate the effects of cardioprotective compounds and treatment strategies, we noticed that our written instructions (article Liepinsh et al., 2013) are not enough illustrative, and therefore decided to film a video to demonstrate this very efficient process.

Major Concerns:
Nil

Minor Concerns:
Minor Grammar 'ischaemic/ schaemia' British English -JOVE required American English. Overuse of the word 'proper' in most cases this word can be deleted.
Thank you for noting this. We have now used American English throughout the manuscript. We have now corrected the use of "proper".

Line 2. Consider adding Rodent to the title to be specific to the specimen type.
Thank you for noting this. We have added "Rodent" to the title of this manuscript.

Line 48. Macro photography should be defined. Does the macro refer to macroscopic eg naked eye scale image (compared to microscopic) or methods using a macro lens on the camera?
Macro photography refers to photographing at high magnifications. Macro photography can be achieved when the lens can ensure at least 1:2 magnification, and ideally 1:1 or even greater magnification. Some operation microscopes can be used instead of a macro lens. The magnification ratio indicated the relation of the projection size of the subject on the image sensor of its life-size.

We have now defined macro photography in the Abstract, Introduction, and suggested magnification of the lens in Discussion, in Paragraphs 5 and 6.

In contrast, macro photography (photographing at 1:2 or greater magnification) is frequently performed in an uncontrolled light environment using inappropriate imaging setups.

Line 56; later steps

Thank you for noting this. We have revised the Introduction text, and this part is now deleted.

Line 78: left or right or both axillae?

We have now deleted in vivo part of tissue preparation (references to protocols and techniques provided in the Introduction) to concentrate this manuscript on imaging techniques of various tissue samples. We make two incisions: one is done to the direction to the left axilla and the second to the direction of the right axilla.

Line 79: do you mean muscles of the abdominal wall superficial (not above) to the xiphoid process? Above could mean superior ie thoracic muscles. Using standardised anatomical terms is recommended.

Thank you for pointing this out. The abdominal muscles were grabbed superficially to the xiphoid process with the forceps. This part is deleted from the manuscript because we have now deleted in vivo part of tissue preparation (references to previously published protocols and techniques provided in the Introduction) to concentrate this manuscript on imaging techniques of various tissue samples.

Line 84. Describe the location of the line of rib cutting, is it para sternal, mid clavicular, anterior axillary or mid axillary? Is the clavicle transected as well?

The line of rib cutting corresponds to the anterior axillary line and the clavicle is not transected. This part is now deleted from the manuscript because we have now deleted in vivo part of tissue preparation (references to previously published protocols and techniques provided in the Introduction) to concentrate this manuscript on imaging techniques of various tissue samples.

Line 86. Do you really recommend using fingers? Fingers are large and imprecise and is opposite to your thesis of promoting quality and precision in methods. Describe the level at which superior and inferior thoracic structures are transected. Eg transect the thoracic aorta at the level of the diaphragm and the great vessels of the heart (describe the level, eg is the aortic arch and pulmonary trunk bifurcation included?) what level is the trachea transected?

Is the oesophagus removed with the heart/lungs. Do you use blunt dissection to separate the heart and lungs from the oesophagus?

This part is now deleted from the manuscript because we have now deleted in vivo part of tissue preparation (references to previously published protocols and techniques provided in the Introduction) to concentrate this manuscript on imaging techniques of various tissue samples.

To cut out the heart, we use two partly similar techniques. In the first method after the opening of the thorax, we grab the lungs and the esophagus with the forceps distally from the heart. Then we cut the esophagus just above the diaphragm and lift everything up. Further, we cut the aorta at the level of 5-6 thoracic vertebrae and proceed with the cutting of the aorta from the posterior wall of the thorax. When the thoracic aorta is separated from the thorax, we cut brachiocephalic, left common carotid, and left subclavian arteries as well as trachea and esophagus proximally to the thymus. Heart together with lungs, thymus and esophagus is put in the ice-cold

Krebs-Henseleit buffer solution. Later the heart is separated from other tissues, stained, and cut into slices.

In the case of the second method, after the opening of the thorax, lungs and heart are gently grasped with fingers. The esophagus is cut just above the diaphragm. Aorta is cut at the same level and separated from the posterior wall of the thorax similarly as in the first method. Brachiocephalic, left common carotid, and left subclavian arteries, as well as trachea and esophagus, are cut proximally to the thymus. Afterward, the heart together with the lungs, thymus, and esophagus is put in the ice-cold Krebs-Henseleit buffer solution. In the first version of the manuscript we included the second method as it can be performed much faster and if done properly heart is not squeezed during dissection and gradually stops beating after immersion in ice-cold Krebs-Henseleit solution.

The thoracic part of the esophagus is cut out together with the heart and lungs and later separated from the heart.

Line 90; separate/dissect it (not clean). The non cardiac organs are not 'dirty'.

Thank you for pointing this out. In future we will use correct verbs.

Line 111 rate not pace

Thank you for pointing this out. It is now corrected.

Line 120. What temperature freezer -20 or -80 degrees. Caution against over freezing

Thank you for pointing this out. It is now indicated in the text, that the freezer temperature should not be below -20ºC, and a warning against over-freezing is added. Nevertheless, optimal time in each laboratory should be determined experimentally.

NOTE: At this stage, it is possible to place the mouse heart in a small plastic bag or a 5-ml microtube in the freezer (-20ºC) for up to 5-10 min. Maximal freezing time should be determined experimentally in each laboratory. The short-term freezing of mouse heart can help a novice experimenter to cut it in 1-mm-thick slices. The freezing of rat heart is not recommended. Over-freezing must be avoided.

Line 125 - is cooling (4 degrees ?) of the stainless steel matrix recommended to keep the semifrozen tissue solid?

Since "freezing" of the tissues is optional, we would not recommend cooling the steel matrix as a go-to option for the cutting procedure. Stainless steel matrix can be at room temperature. We would avoid cooling down the matrix because the heart can stick to very cool metal.

Line 126- specify the type of blade used for cutting

Blades from Ziwic instruments or any compatible single edge razor blade (e.g. thickness up to 0.01 inch (0.254 mm)) can be used for slicing rat hearts. For mouse

hearts, double edge razor blades are generally used and are usually up to 0.004 inches (0.1 mm) in thickness. This is indicated now in the text.
NOTE: Slicing matrix-compatible razor blades must be used. In general, compatible single edge razor blades (e.g. thickness up to 0.01 inch (0.254 mm)) can be used for slicing of rat hearts. For mouse hearts, double edge razor blades are generally used and are usually up to 0.004 inches (0.1 mm) in thickness.

Line 126 slicing is what you do with hand and blade/knife/scalpel. Sectioning is microtomy. You are slicing. Cut ventricles of the heart transversely into….

Thank you for noting this; corrected.

Line 134 spell out MACO
MCAO now is spelled out in the text: Middle cerebral artery occlusion.

Line 148. Describe razor blades
Blades from Ziwic instruments or any compatible single edge razor blade (e.g. thickness up to 0.01 inch (0.254 mm)) can be used for slicing rat brain. This is now indicated in the text.
NOTE: Slicing matrix-compatible razor blades must be used. In general, compatible single edge razor blade (thickness up to 0.01 inch (0.254 mm)) can be used for rat brain slicing.

Line 156. Slices not slides. Top surface is not an anatomical term, do you mean superior or anterior?
This part has now been described in a bit more detail.

2.6 Arrange the brain slices one by one in a tray (70 ml, 72 x 72 mm). When arranging the slices make sure that the anterior surface of each slice is always facing up.

Line 160 . specify the blue surface which you are using as a background. Is it an acrylic sheet? Cutting mat? Plastic tray?, matt or glossy, washable or disposable?

The blue surface now is specified in the text: 2.8 After incubation in 1% TTC solution, transfer the brain slices to the blue plastic tray to capture images. The brain slices are arranged in sequence order from the frontal to the caudal part, and then, using a scalpel, hemispheres are separated in the sagittal plane. NOTE: The surface should be washable, matt, and at a color that contrasts brain slices (i.e. not red, white, pale pink).

Line 161; in sequence not ascending order
Thank you for noting this. Error in line 161 is now corrected.

The brain slices are arranged in sequence order from the frontal to the caudal part, and then, using a scalpel, hemispheres are separated in the sagittal plane.

Line 163. How do you keep the brain slides from floating off the blue background when immersed in formalin?
Thank you for noting this. Brain slices are not immersed, but reflections are removed

by a polarizing filter. The misleading sentence is removed and the reduction of reflections part is now explained in the Representative Results section.

Line 174; LED lights emit hardly any heat, what equipment needs to warm up?

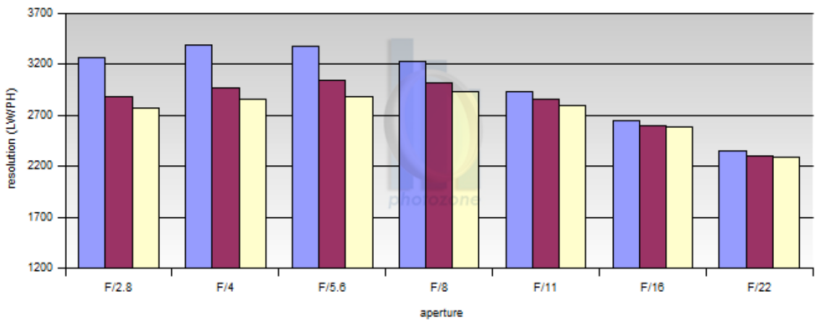
"Warm-up" time is a general term (probably slang) to characterize time when lights reach constant brightness and color temperature. It is suggested that LED lights reach working condition instantly, still, it is recommended by manufacturers of at least a few minutes of "warm-up" time even for the most sophisticated LED lasers used in confocal microscopes. Probably this is an outdated tradition. Warm-up time is necessary if lighting other than LEDs is used. We have explained this in the text now:

NOTE. LED lights reach full brightness in microseconds.

Line 177. Aperture is usually expressed as a f stop fraction eg f/8 or f/11 or F16. It may be better to specify a range, and explain the aperture number is a fraction, the larger the number the smaller the aperture. A small aperture is desirable for this method.
By decreasing aperture, the depth of field increases, which is helpful if the object is not in a single plane, however, aperture decrease has limited benefit, because of diffraction. Diffraction is an optical effect that limits the total resolution of photography. The light begins to disperse or "diffract" when passing through a small opening (such as the camera's aperture). This effect is normally negligible since smaller apertures often improve sharpness by minimizing lens aberrations. However, for sufficiently small apertures, this strategy becomes counterproductive — at which point the camera becomes diffraction-limited. Below are examples of how lens resolution changes in response to aperture. The optimal aperture usually is around f/8, we are suggesting f/10 because resolution drop, in this case, is ~10-15% and it is possible to achieve sufficient depth of field.

Canon EF 100mm f/2.8 USM L macro

100mm	F/2.8	F/4	F/5.6	F/8	F/11	F/16	F/22
Center	3270,5	3395	3373	3229	2937	2643	2347
Border	2885	2970	3041	3016	2861	2598	2299
Extreme	2767	2853	2889	2937	2800	2580	2295



Sony FE 90mm f/2.8 OSS macro

90mm	F/2.8	F/4	F/5.6	F/8	F/11	F/16	F/22
Center	4734	5002	4884	4434	3881	3423	2389
Near Center	4486	4856	4716	4299	3875	3373	2219
Border	3964	4167	4218	4011	3695	3266	1998
Extreme	3772	3930	4124	4007	3657	3241	1992

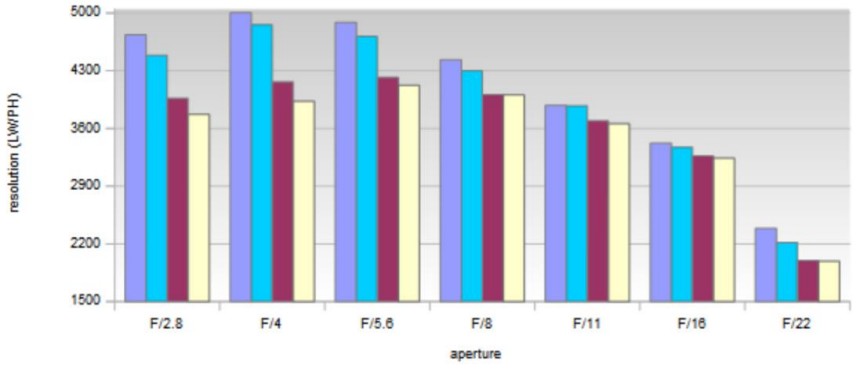


Figure Lens resolution depending on aperture. Graphs are from the website https://www.opticallimits.com/all-tests

Aperture f/10 is suggested in 5.4. step of the Protocol.

Line 182. Describe the background for the specimens, is it blue, or white or another colour? The background is better explained in your previous 2013 article.

The color of the tray should be in a color which is not present in the stained specimen. For example, to quantify the area of methylene blue and TTC (red) staining in an automated or semi-automated manner, white, red, blue, yellow, brown, colors of background should be avoided. So green would be preferable. However, color selection depends on operator preferences, who is doing image postprocessing. We like the white background and because in image postprocessing you can delete the background and thus make it completely white (RGB white code 255,255,255). Then exclude completely white color from the list of selected colors used for semiautomated analysis and cont only pale necrotic area, which is not completely white if not overexposed. For the brain and aortas, blue and green background is suitable.

We have now referred to our previous publication (Liepinsh et al, 2013) in the Representative Results section.

A tray with blue background is used for brain slice photography (background selection described in details previously[5]).

Line 183 'with objective' do you mean macro lens?

Thank you for noting this. Error is now corrected.

Line 193 turn the slices over. Around means rotate.

Thank you for noting this. Error is now corrected.

Line 203. Is 3B the frozen slice an example of suboptimal tissue prep for photography? Say so. Fig 3A, label and explain the different areas. Blue is the non ischaemic are, red is the TTC positive area at risk, White it's the TTC negative area of infarction or similar. Here is an example article with a labelled diagram that explains the different areas https://www.hindawi.com/journals/bmri/2011/195483/fig3/

We have now added an example of color segmentation in Fig 3B, representative results section:

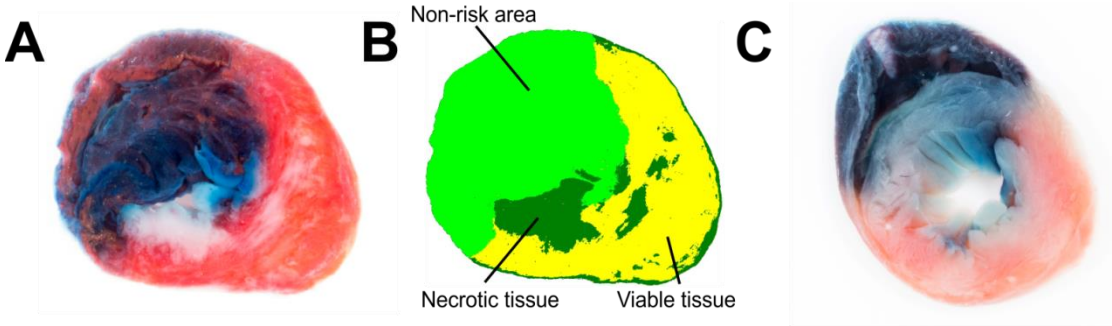


Figure 3 Images of fresh (A), fresh segmented (B), and 24h frozen (C) cardiac slices. Heart slice (A) was analyzed using color segmentation (B) in ImageProPlus 6.3 software. TTC staining poorly discriminates viable and necrotic tissue in the frozen heart slice (C).

You want your article is to be understandable to a wider audience beyond those just doing TTC ischaemia experiments.

Line 220/1: re-occlusion of coronary arteries

Line 221: elaborate specifically the blue dye stains non ischaemic areas

Line 229. Fresh tissue, not live.

Thank you for noting this. The respective part of the manuscript is corrected/modified.

Line 232 freezins (sp)

Line 236 functioning mitochondria, not live. They are an organelle and thus cannot be 'live'

Thank you for noting this. The respective sentences of the manuscript are now corrected/modified.

Line 338. Good you say that the frozen example here is suboptimal, but you also need to say this in the figure legend. ?

Figure 3 legend is modified as follows:

Images of fresh (A) and 24h frozen (B) cardiac slices. Note how TTC staining poorly discriminates between viable and necrotic tissue in the frozen heart slice.

Line 248/9. Not clear about the lyophilization or the temperature of freezing, its it -20 or -80 degrees. What is your experience and recommendation about freezing, if no experience, then quote a reference. ?

Thank you for noting this. We have deleted this sentence as it was misleading.

Line 251: incubation means storing at warm temperature eg 37 degrees for cells, does this apply to the slices in formalin. Do you mean brain slice storage in formalin at ambient temperature? The fixed tissue will be of good microscopic quality forever, but do you mean the quality of the TTC and methylene blue staining regions? Be specific. Line 252: same, are you meaning 37 degrees formalin? Line 254. Formalin causes decolourisation of normal tissues, do you mean destaining of the methylene blue, or decolourisation of unstained tissues. In my experience, the colour of methylene blue is not changed by formalin. In fact we use methylene blue to stain formalin fixed tissues for microscopy. Do you mean diffusion of stain? ?

Storage in formalin is now decribed in a more detail in the Discussion:

Overnight storage of brain tissues in formalin (+4□C) does not impair the color intensity of normal tissue and is acceptable for image acquisition. However, formalin induces swelling and destaining of heart slices. Therefore the storage of heart tissues in formalin is not recommended..

Line 265, what about compact cameras with a macro function? They can do the job without the expense and complexity of interchangeable lenses/special macro lens.

Thank you for pointing this out. We agree that any camera can be used if it fulfills the following criteria: it can be fixed on a stand, it has remote shutter/shutter delay function; it has live view function; manual mode of focus, exposure and white balance settings; lens can reach magnification of at least 1:2 and has sufficient distance to the object (to not affect the illumination of the object) at high magnification; camera has sufficient dynamic range (to make it possible to capture dark viable tissue and necrotic bright tissue with sufficient detail and color information). Cheapest setups of a new interchangeable lens camera and macro lens cost around 1000EUR/USD. It is generally the same price as for 1-2 ELISA kits. In contrast to ELISA kits, the camera-lens combo will last at least 10 years in indoor conditions on the stand.

To answer the reviewer's concerns, we have added more details on camera and lens characteristics to the Protocol texts and Discussion.

Compact cameras, which have the above-mentioned features and lens magnification of at least 1:2 also can be used for macrophotography.

Line 273 ..and manual white balance setting.

The white balance setting is now added to the respective sentence in the Discussion.

All up-to-date digital cameras have characteristics and functions necessary for macro photography, including the possibility to mount on a stand, a high number of pixels (usually >20 Mpx), live view, mirror lock-up, time-lapse features, remote shutter, and the ability to manually set camera parameters, thus ensuring a constant shutter speed, aperture, white balance, and ISO setting.

Line 285 not sure the quotes are needed around photography copy stand. This is a standard term, it has been around since the 19th century for film photography.

Corrected.

Line 289: elaborate on the recommended light source. You label Fig 2 as LED light. What colour temperature is recommended (eg 6500K, same as white of a LED computer screen), advantages of LED (not heat generating, will not heat up specimen), high CRI . Be explicit, eg do not use fluorescent light sources which flicker with AC power supply.

We don't want to be definitive in our suggestions. If someone is buying new lights, LED is preferable. A good (up-to-date) camera can tolerate color temperatures from 6500K down to 4500K. It is possible to adapt to almost any light source if it is placed correctly and camera settings are adjusted according to the parameters of the light source. We found that for brain slices more convenient it is to use a polarizing filter, not an immersion method. Then we noticed that a circular polarizing filter cannot filter all reflections if the LED light is used. Therefore we are still using 20+ years old stand with fluorescent light sources for brain slices (it is present in our video guide). We adjusted camera settings accordingly: WB setting 5700K, exposure time 0.5s to remove any flickering.

We have now added a Note about the light sources, Protocol point 3.3:

NOTE: White LED light (color temperature 6500K) is the preferred light source to avoid light flickering by fluorescent light bulbs.

Line 293. Not blinking. Flickering light sources such as fluorescent.

Corrected.

Lines 299-302. The methods of reflection removal are an important contribution of this methods article. The method needs to be illustrated (not just the result). A photo or video including how exactly the tissue is placed between glass plates, the size of the glass, thickness, how to ensure even contact, how to deal with different thickness tissue slices causing irregular contact between slices , air bubbles etc. These are this details required for a methods article. Similarly, the article will be improved with visual elaboration of photos and or video of the immersion method. What type of container is used to immerse the specimen in - glass, acrylic? What size? How deep should the immersion fluid be? Specify the background below the container, or is it integrated into the floor of the container, how to deal with bubbles, diffusion of blue dye into the immersion solution, how to fix the tissue down so it does not float off. Describe what you have found to work well and what does not.

We are using immersion for heart slices and a polarizing filter for the brain. Since we know that many scientists are placing specimens between glass plates. We tried and found that this method of choice, however with some problems. We agree with the reviewer that using glass plates is a lot of challenges with bubbles, specimen thickness, etc. Immersion is much easier.

No specific requirements for containers except the color. Probably for easy manipulation size and depth might matter. We are using a usual weighing tray for hearts, a pet drinking tray for the brain (both are in the video), and a cover from the tips box for the aorta. The weighing tray is selected because of the appropriate size and white color, pet drinking try is selected because of blue color. We would suggest minimum immersion depth and overall size of tray in order to avoid a large buffer volume, which moves in the tray and causes floating of specimens. In a small tray you don't need to fix specimens. At the same time, we have seen (in publications) that specimens are not fully immersed and then reflections and other artifacts appear in the images.

The color of the tray should be in a color which is not present in the stained specimen. For example, to quantify the area of methylene blue and TTC (red) staining in an automated or semi-automated manner, white, red, blue, yellow, brown, colors of background should be avoided. So green would be preferable. However, color selection depends on operator preferences, who is doing image postprocessing. We like the white background and because in image postprocessing you can delete the background and thus make it completely white (RGB white code 255,255,255). Then exclude completely white color from the list of selected colors used for semiautomated analysis and count only pale necrotic area, which is not completely white if not overexposed. For the brain and aortas, blue and green background is suitable.

If the (heart) specimen is not overstained (too much volume/concentration of methylene blue used for perfusion) and/or rinsed enough, then no problems with bubbles and diffusion of methylene blue. Therefore in the manuscript, we are stressing the point about sufficient washing of specimens. In the case of Evans blue stain diffusion is not a problem at all, but there is a higher risk of overstaining.

We have now referred to our previous publication (Liepinsh et al, 2013) in the Representative Results section.

A tray with blue background is used for brain slice photography (background selection described in details previously[5]).

We added a Note about the use of glass slides, Protocol point 3.6:

Alternative methods include placing slices between glass slides or using a polarizing filter on the lens. A circular polarizing filter is attached to the lens and is rotated until reflections in a live-view display of the camera disappear.

Line 309 water or PBS- contradicts with line 300

Corrected to buffer solution.

Line 316 is a better discussion of aperture. Refer section 5.5 where a aperture of 10 is stated without the f which does not make sense.

Protocol text corrected, f/10 added.

Discussion,
Are there other potential applications for the methods - other animal TTC ischaemia models besides rodents? could this be used in humans in the autopsy setting?

We agree that it can be useful for the photography of any specimen. Since we are mainly concerned with the poor quality of images in the experimental cardiology field, we narrowed it down to this topic.

The macro photography set up is generalisable to all laboratory experimental small animal organ photography, perhaps this may be stated as a conclusion. The article ends abruptly with RAW files without a concluding paragraph.

Thank you for pointing this out. We have now added a concluding paragraph:

Overall, this protocol describes a methodology for rat heart and brain tissue slicing and staining and provides guidelines for establishing lighting and camera setups and photography techniques for high-resolution image acquisition for further analysis. This method is applicable to all experimental small animal organ photography.

Summary is also modified:

Presented here is a protocol for standardized methodology of rodent tissue preparation after the ischemia-reperfusion experiment and guidelines for establishing lighting and camera setups for high-resolution image acquisition. This method is applicable to all experimental small animal organ photography.

Reviewer #4:
Manuscript Summary:
The method of heart and brain tissue slicing and staining are clearly described. The guidelines for establishing lighting and camera setups and photograph techniques for high-resolution image acquisition are well stated.
We thank the reviewer for the comment!

Major Concerns:
none.

Minor Concerns:
1.3: "above" the xiphoid process. Should it be "beneath?"

This part is now deleted from the manuscript because we have now deleted in vivo part of tissue preparation (references to previously published protocols and techniques provided in the Introduction) to concentrate this manuscript on imaging techniques of various tissue samples. We thank the reviewer for pointing to the terminological error. The abdominal muscles were grabbed superficially to xiphoid process with the forceps.

2.3: "buffer solution" what buffer? Can be more specific

The heart was washed with oxygenated Krebs-Henseleit solution. This part is now deleted and readers referred to previous publications:

Techniques described in this protocol can be used after both Langendorff-perfused isolated rat or mouse heart [6,7] and in vivo rat heart ischemia-reperfusion (IR) injury assays[8-11].

2.4, 4.8 and 4.9: a picture may be needed to better illustrate the steps.

Point 2.4. is now deleted, 2.4 and 2.5(former 4.8 un 4.9) are captured in the video.

3.1, 3.2 and 5.8: the last sentence need to be moved to "discussion."

The format of the Protocol section is now changed according to the Editor's suggestions. All important explanations are now presented as NOTEs.

3.3: sentences after "At this stage" need to be moved to "discussion."

This part of the Protocol is re-written now.

NOTE: At this stage, it is possible to place the mouse heart in a small plastic bag or a 5-ml microtube in the freezer (-20ºC) for up to 5-10 min. Maximal freezing time should be determined experimentally in each laboratory. The short-term freezing of mouse heart can help a novice experimenter to cut it in 1-mm-thick slices. The freezing of rat heart is not recommended. Over-freezing must be avoided.

In addition to the edits requested by the referees, we have also made a number of minor spelling and grammatical corrections throughout the text. Overall, we believe we have adequately addressed all the concerns raised by the referees. Certainly, their suggestions have greatly improved the quality of the manuscript and we are most grateful. We are hopeful that the revised manuscript is now acceptable for publication.

Sincerely,

Prof. Maija Dambrova, on behalf of the LIOS team

Video Produced by Author: Less than 50 MB. If your video is greater than 50 MB, click "offline" as the delivery method and our

This piece of the submission is being sent via mail.