Refrined approach for quantification of in vivo ischemia-reperfusion injury in the mouse heart

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Bohl S, Medway DJ, Schulz-Menger J, Schneider JE, Neubauer S, Lygate CA. Refined approach for quantification of in vivo ischemia-reperfusion injury in the mouse heart. Am J Physiol Heart Circ Physiol 297: H2054–H2058, 2009. First published October 9, 2009; doi:10.1152/ajpheart.00836.2009.—Cardiac ischemia-reperfusion experiments in the mouse are important in vivo models of human disease. Infarct size is a particularly important scientific readout as virtually all cardiocirculatory pathways are affected by it. Therefore, such measurements must be exact and valid. The histological analysis, however, remains technically challenging, and the resulting quality is often unsatisfactory. For this report we have scrutinized each step involved in standard double-staining histology. We have tested published approaches and challenged their practicality. As a result, we propose an improved and streamlined protocol, which consistently yields high-quality histology, thereby minimizing experimental noise and group sizes.

Methods

ANIMAL MODELS OF MYOCARDIAL ischemia-reperfusion (I/R) mimic the clinical scenario where a period of ischemia is followed by the restoration of myocardial blood flow (e.g., angioplasty or thrombolysis for treatment of acute myocardial infarction) and are important tools for testing novel strategies aimed at reducing ischemic damage and attenuating reperfusion injury. The mouse is increasingly the species of choice for in vivo I/R experiments, because of its genetic malleability, rapid breeding cycle, and economic husbandry. However, the small size of the mouse anatomy imposes challenges not only for surgery itself but also for subsequent histology, with the measurement of infarct size as the prime scientific readout. Unlike after permanent occlusion of a coronary artery where there is necrosis of the entire coronary territory, infarction after I/R is variable, nontransmural, and usually incomplete. Therefore, the principal requirement of I/R histology is the clear demarcation of three myocardial subsets: 1) the unaffected, remote myocardium; 2) the area at risk (AAR), i.e., the myocardium distal to the site of coronary occlusion that was initially subjected to ischemia; and 3) the area of necrosis (AON), which is the fraction of nonviable tissue within the AAR. The final infarct size is expressed as the ratio of AON to AAR, and to this end, gross histological double-staining techniques have served as a gold standard in many studies (e.g., see Refs. 2, 5, 8, 12).

The standard methodology is to reocclude the coronary artery before perfusing the heart with a vital dye (typically Evans blue) or microspheres. This stains the remote myocardium but leaves the AAR unstained. In a second step, nonviable myocardium within the AAR is identified using triphenyltetrazolium chloride (TTC) staining. This colorless dye is reduced to a deep-red precipitate by dehydrogenases in the presence of NADH (4, 7). Since lethally damaged cells do not retain these reactants, nonviable areas remain unstained and appear pale, whereas viable cells stain red. Some practical information on common protocols can be found in the methods sections of research papers or online, e.g., at http://www.southalabama.edu/ishr/help/ttc/.

Although relatively straightforward, the histological approach is strongly affected by the precise sequence and conditions in which the individual steps are carried out. As a result, the quality of histological images varies widely and is often poor in terms of contrast and border delineation. This introduces considerable subjectivity to AON/AAR measurements, compromising accuracy and, potentially, the validity of the conclusions.

This article describes an optimized protocol implemented in our laboratory, which reliably generates high-quality histological images. The key advantages include an entirely ex vivo staining method, greater color intensity in the remote myocardium (without smearing), and improved contrast and border delineation. Each step of the protocol is described in detail to assist other laboratories in implementing histology for in vivo I/R experiments.

METHODS

This investigation was approved by the Institutional Ethical Review Committee and conforms with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996), and with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act, 1986 (HMSO).

Considerations Before Histology

Surgery to induce ischemia with subsequent reperfusion is performed as described elsewhere (10). During this procedure, a 6-0 polypropylene suture is used to ensnare the coronary artery and tied against a short section of polyethylene tubing (PE-10; outer diameter, 0.61 mm). This allows coronary blood flow to be re-established by simply removing the tubing (see supplemental video file 1; note: supplemental material may be found posted with the online version of this article). However, the suture material itself

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should be left in situ with sufficiently long ends to later permit the reoclusion of the coronary in the identical location during histology. It should also be noted that proper infarct delineation by TTC staining requires a complete washout of NADH from the infarcted territory. For this reason, staining after \( t > 3 \) h of reperfusion arguably underestimates the true extent of ischemic necrosis (1, 6), and our experiments are routinely performed 20–24 h after reperfusion.

**Histology**

The AAR is demarcated by perfusing the heart with a dye after coronary reocclusion. In principle, this can be done in vivo using intravenous, intracavitary, or retrograde aortic injections. However, there are a number of disadvantages to this approach. First, it requires time-consuming intubation and thoracotomy. Second, such injections are associated with a risk of technical failure. Third, strong and homogeneous staining of remote myocardium is rarely achieved. We have therefore employed a simple Langendorff apparatus to perfuse the heart under ex vivo conditions.

The individual steps of the histological preparation are outlined and discussed below. The technique should be applicable, with some modifications, to larger species.

**Harvesting the heart.** The animal is euthanized by cervical dislocation. The beating heart is rapidly excised together with the diaphanous aorta. Once cannulated and secured using double knots, the heart is gently perfused with saline to wash out remaining blood. Subsequently, after reoclusion of the coronary artery, the heart is perfused with \( \sim 250 \mu l \) 5% Phthalocyanine blue, thereby coloring remote myocardium deeply blue. B: lateral view of cannulated heart. The area at risk (AAR) remains unstained after reoclusion of the left coronary artery using the original suture and a small piece of polyethylene (PE) tubing (asterisk). Remote myocardium is clearly demarcated (deep blue) after perfusion with \( \sim 250 \mu l \) 5% Phthalocyanine blue solution.

![Figure 1](image1.png)

**Fig. 1.** A: a simple clamp stand holds a saline-filled 2.5-ml syringe fitted with a blunted 20-gauge needle for cannulation of the aorta. The tip of the cannula (asterisk) must end proximally of the aortic valve to ensure proper coronary perfusion. The correct position can be checked visually by carefully bending the diaphanous aorta. Once cannulated and secured using double knots, the heart is gently perfused with saline to wash out remaining blood. Subsequently, after reoclusion of the coronary artery, the heart is perfused with \( \sim 250 \mu l \) 5% Phthalocyanine blue, thereby coloring remote myocardium deeply blue. B: lateral view of cannulated heart. The area at risk (AAR) remains unstained after reoclusion of the left coronary artery using the original suture and a small piece of polyethylene (PE) tubing (asterisk). Remote myocardium is clearly demarcated (deep blue) after perfusion with \( \sim 250 \mu l \) 5% Phthalocyanine blue solution.

![Figure 2](image2.png)

**Fig. 2.** The images exemplify the superior quality of the outlined dual-dye staining protocol. Top: set of photographs of both sides of each slice covering the entire left ventricle (from apex to base and from left to right). The 3 myocardial subsets are clearly identified with unambiguous border definition and strong tissue contrast. Bottom: schematic of manual infarct planimetry at greater magnification in a midventricular slice. AAR (non-blue area; 38% of entire slice area) and area of necrosis (AON, white area; 56% of AAR) are manually contoured. Relative AAR and AON sizes per slice are normalized to individual slice weight; slice results are added to yield overall infarct size. Note that the right ventricle was not removed.
lungs and connecting tissue as a precaution against damaging the aorta and then immersed in heparinized saline to remove the remaining intracavitary blood. The heart is then transferred to a petri dish covered with moist tissue paper. By lifting the thymus, the origins of the great vessels are exposed and the aortic arch identified. Extracardiac tissue is then trimmed off and the aorta clipped just proximal to the arch. The right ventricle must not be removed at this stage, as this would interfere with proper staining at the right ventricular/left ventricular insertions.

**Cannulation of aorta.** A 2.5-ml syringe with a 20-gauge blunted needle is filled with saline solution. Great care must be taken to avoid any air bubbles within the syringe and needle. The syringe is then secured vertically in a clamp stand with the tip 2 to 3 cm above the petri dish. The aortic stump is then picked with fine tweezers, put over the blunted needle, and secured in position with a 3-0 double knot so that the needle tip is safely distal of the aortic valve (Fig. 1A). The heart is very gently perfused with 1 to 2 ml saline until the eluate drains clear from the coronary sinus. This step markedly enhances the red/white contrast on TTC staining by rinsing out trapped blood/residual heme products, which would otherwise appear a brownish color (9). If perfusion pressure is too high, coronaries may rupture and, thereby, impair subsequent dye perfusion. This may also occur with an unintentional injection of air bubbles. With training, the procedure should be completed in <3 min.

**Religation of the coronary artery.** Since the suture material is left in situ, this is simply a case of retying a knot onto the tubing to obtain a perfect anatomical match to the initial occlusion. This is

Fig. 3. The images illustrate flaws and pitfalls of various dual-dye techniques in selected midventricular slices. A: Evans blue dye perfusion with subsequent triphenyltetrazolium chloride (TTC) immersion. The red/blue border definition is not clear-cut and may lead to inaccurate measurements. B: TTC perfusion with subsequent Evans blue dye perfusion. The TTC-induced tissue contraction obviates homogeneous blue staining of remote myocardium. C: Phthalocyanine blue perfusion with subsequent TTC immersion and before formalin treatment. The glossy surface impedes visual analysis. D: Phthalocyanine blue perfusion with subsequent TTC immersion and formalin treatment. Ruptured septal arteries due to injected air bubbles/excessive perfusion pressure prevent proper tissue staining. E and F: Phthalocyanine blue perfusion with subsequent TTC immersion and formalin treatment. The pronounced bleaching effect of formalin on TTC-stained tissue is shown. Excessive exposure (360 min; F) may lead to overestimation of nonviable myocardium compared with the usual 90 min (E).
most easily performed under a binocular microscope (e.g., OPMI 1-FR, Carl Zeiss, Oberkochen, Germany) using fine forceps.

Staining remote myocardium: dye perfusion. A water-soluble vital dye such as Evans blue is most commonly used to stain the remote myocardium. However, in our experience, this is not ideal, since such dyes can precipitate out of solution on a subsequent addition of TTC. They are also prone to smearing during slicing, resulting in an ambiguous border definition. Phthalo cyanine blue is a non-soluble pigment, which has been used as an alternative to Evans blue (11) and, with some modifications, also works well in our hands. A 5% (wt/vol) stock solution is made up of 0.9% NaCl and pigment powder (e.g., PB 15:3; D50 particle size, 0.2 μm; Heubach, Langelsheim, Germany). A small amount of household detergent is added to facilitate suspension. Aliquots are filtered (GD/X, 2.7 μm; Whatman International, Maidstone, United Kingdom) immediately before use. Again, care must be taken to eliminate air bubbles; otherwise, inhomogeneous staining will result. The cannula and syringe are carefully filled with dye, and the heart is gently perfused with ~250 μl. Ideally, this is done while the heart is still beating, thereby supporting an even distribution of the dye. Alternatively, the heart may be arrested in diastole to aid perfusion. Remote myocardium is clearly delineated against the unstained AAR (see Fig. 1B).

TTC staining for AAR. The intact heart is then wrapped in cling film, placed in an Eppendorf vial, and stored at −20°C for no longer than 15 min. After this period the heart is semifrozen and easy to slice in 6–8 parallel short-axis sections by hand using a razor blade on a cold surface: this approach circumvents more laborious preparations, such as embedding the heart in agarose to longer than 15 min. After this period the heart is semifrozen and ready for slicing (3), and we have found that freezing at −20°C for up to 120 min has no effect on the intensity of TTC staining. The slices are allowed to thaw. TTC perfusion induces severe tissue contraction, which strongly affects the geometry of the small-sized mouse heart and may prevent a successful subsequent dye perfusion. To minimize this, we therefore immerse the heart sections in freshly prepared TTC 1% (vol/vol) in 0.9% NaCl; General Purpose Grade 2, 3,5-TTC Fisher Scientific UK, Loughborough, United Kingdom] at room temperature, rather than perfusing hearts with TTC. After 2 to 3 min, the vial is transferred to a water bath at 37°C for 15 min. The vial is continuously agitated since tissue touching the wall will not be stained. The sections are then removed from the vial, and excess moisture is blotted; a possible distortion can be mended by gently flattening the sections between the index finger and the thumb. The samples are next placed in 10% neutral-buffered formalin (Shandon Formal Fixx, Thermo Scientific, Waltham, MA) for a maximum of 90 min. This step enhances the red/pale contrast between viable AAR and AON (see Fig. 2, top). In addition, the amphotrophic formalin eliminates the fatty surface gloss, which otherwise reduces tissue contrast on photographs. If exposed to formalin for too long, however, TTC red may bleach out and cause an overestimation of nonviable regions.

Figure 3 illustrates some of the flaws and pitfalls of the various dual-dye approaches.

Photography. A dissection microscope (e.g., SMZ 1000, Nikon, Tokyo, Japan) with adjustable fiber optic light sources (e.g., KL 1500, Schott, Mainz, Germany) and attached digital camera are used. The heart sections are blotted on tissue paper and photographed from both sides. The tissue contrast is strongly affected by the lighting conditions and must be carefully optimized.

Infarct quantification. Infarct quantification is performed on digital photographs by manually contouring the differentially colored left ventricle subsets (remote myocardium = blue, AAR = nonblue, and AON = white) using an image analysis software such as ImageJ (NIH, Bethesda, MD). The relative areas of these subsets are obtained using a pixel count tool, whereas absolute values may be obtained after scaling to an object of known length. AON and AAR can be determined as the average percent area per slice from the two sides of each section (see Fig. 2, bottom). They are then corrected for slice weight: Weightotal AAR = (WeightSlice 1 × %AARSlice 1) + (WeightSlice 2 × %AARSlice 2) + (WeightSlice 3 × %AARSlice 3). AON weight is calculated in the same manner. Finally, absolute infarct size is calculated as the ratio of Weightotal AON to Weightotal AAR.

Summary

Cardiac I/R surgery in the mouse is an increasingly important model of human disease but remains technically challenging in terms of both surgery and histological analysis. To minimize experimental noise and reduce group sizes, infarct measurements should be performed on high-quality tissue samples. For this report we have summarized each step involved in double staining histology. We have tested published approaches and challenged their practicability, validity, and reproducibility. As a result, we propose the described streamlined protocol, which consistently yields high-quality histology and should be helpful for other laboratories planning in vivo I/R experiments.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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