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Quantitation of Acute Necrosis After Experimental Myocardial Infarction

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Abstract

Myocardial infarction (MI) is death and necrosis of myocardial tissue secondary to ischemia. MI is associated with adverse cardiac remodeling, progressive heart chamber dilation, ventricular wall thinning, and loss of cardiac function. Myocardial necrosis can be experimentally induced in rodents to simulate human MI by surgical occlusion of coronary arteries. When induced in knockout or transgenic mice, this model is useful for the identification of molecular modulators of cell death, cardiac remodeling, and preclinical therapeutic potential. Herein we outline in tandem, methods for microsurgical ligation of the left anterior descending artery followed by quantitation of myocardial necrosis. Necrosis is quantified after staining the heart with triphenyltetrazolium chloride.

Keywords

Necrosis; Myocardial infarction; Infarct size; Area at risk (AAR)

1 Introduction

Myocardial infarction (MI) is necrosis and death of heart muscle secondary to ischemia and acute coronary artery thrombosis. Thrombal occlusion of epicardial coronary arteries leads to cell death of the underlying subendocardium. Loss of non-regenerative and contractile cardiomyocytes results in reduced cardiac output and predisposes to heart failure [1]. The extent of necrosis after MI is linked to the duration of ischemia and also to the size of the area at risk (AAR), which is the region of tissue that relies on perfusion downstream of the arterial blockage. Location of blockage affects infarct size; in rodents, ligation of the left anterior descending (LAD) artery proximal to its origin can create an infarction consisting of >65 % of the left ventricle with a mortality rate of 100 % [2]. In humans, proximal LAD occlusions are associated with a greater extent of necrosis and a less favorable prognosis [3]. Necrosis is also a function of genetic and cellular factors that control cell-death susceptibility and in turn modulate the extent of infarct expansion within the AAR, as described below. Thus, molecular factors that causally promote or reduce susceptibility to necrosis after MI can be identified and characterized in experimental animals after surgically induced infarction.

In humans, MI is precipitated after atherosclerotic plaque rupture [4, 5]. Experimental mice are naturally less susceptible to both atherosclerosis and MI, and therefore genetic and surgical means have been devised to, in a controlled fashion, model the physiology, histology, and cell biology of human MI [2]. In patients, MI often manifests at the left ventricle and/or ventricular septum, with isolated right ventricle infarcts occurring in the minority of cases [6]. In mice, left ventricular infarction is induced by surgical occlusion of the LAD artery to generate the ischemic AAR (Fig. 1) [7]. Within the AAR, necrotic cells can initially compose ~10 % of the ischemic area after as little as 30 min [8]. Early functional consequences of cell injury are potentially reversible with restoration of blood flow (i.e., reperfusion) [9]. However, severe ischemia lasting at least 20 min causes irreversible myocyte death via necrosis and apoptosis [6, 8]. Over time, necrosis and cell death can expand; transmural necrosis can lead to the risk of myocardial rupture. Alternatively, the heart in coordination with the innate inflammatory response initiates cardiac repair and healing as necrotic and apoptotic cells are cleared and replaced with a provisional granulation tissue that leads to formation of a collagenous scar [10–12]. Therefore, a measurement of collagen content weeks after MI can be an indirect evaluation of necrosis.

Acute indications of myocardial necrosis

Within hours, infarcted tissue is identified under light microscopy by the buckling of non-contractile dead myofibers at the border of the infarct, giving a “wavy” appearance [6]. At this time, the primary form of myocyte death is coagulation necrosis; initial denaturation due to intracellular acidosis permits the outline of the cell to be maintained but internal proteins, including proteolytic enzymes, are degraded. These anucleated cells can remain for days following MI, until lysosomal enzymes provided by infiltrating leukocytes from the collateral circulation arrive in association with edema fluid that fills extracellular space [13]. In addition, increased binding of eosin to denatured intracytoplasmic proteins causes an eosinophilia of necrotic tissue during staining. At the ultrastructural level, the development of large myocardial vacuoles, dilated and misshapen mitochondria, nonspecific DNA breakdown, and nuclear shrinkage (pyknosis) and fragmentation are indications of cell death by ischemia [6, 13]. Cardiomyocytes may also undergo oncosis and appear swollen with ruptured sarcolemma [8]. In contrast, apoptotic cardiomyocytes exhibit condensed nuclei and formation of apoptotic bodies.

In addition to the aforementioned histological indications of necrosis, alternative and complementary experimental methods have been developed to quantify myocardial necrosis and normalize variances in ischemic AAR between experimental subjects. This is a key point as differences in AAR are directly correlated with infarct/necrosis area. Below we outline the measurement of infarct/necrotic area as a function of AAR after permanent occlusion of the mouse LAD. Post surgery, this method utilizes two separate dyes: Evans blue and triphenyltetrazolium chloride (TTC). Injection of Evans blue through experimental hearts after LAD-ligation stains perfused myocardium and leaves ischemic area unstained, revealing the AAR. Subsequent incubation in TTC reveals live tissue, which converts TTC to a red precipitate within the AAR. Infarcted/necrotic tissue remains white. Necrosis is calculated as a percentage of the AAR.

2 Materials

A clean and uncluttered work area will facilitate reproducible and shorter duration surgeries. Be sure to wear a mouth face mask, proper laboratory covering, eye goggles, and gloves. Work aseptically and follow all waste disposal regulations when disposing of biohazard materials. Prepare proper sham-surgery controls.

2.1 Surgical Coronary Occlusion

1. *Anesthesia*: This protocol describes MI after inducing anesthesia with Avertin (tribromoethanol from Sigma-Aldrich, St. Louis, MO), which produces hemodynamic stability in our hands. Avertin is prepared by mixing tribromoethanol and tert-amyl-alcohol to a final stock concentration of 15 mg/ml. This requires vigorous stirring for 2–3 h for dissolving. For intraperitoneal injection, the dose is 0.1 ml/10 g mouse. It is important to note that toxicity and mortality are associated with improper storage of this drug. Therefore, care must be taken to store Avertin properly at 4 °C and in the dark (*see*^{Note 1}) [14]. Post-op analgesics include buprenorphine or alternatively, the nonsteroidal anti-inflammatory meloxicam, both of which are obtained with permission and through your animal oversight committee and facility.
2. *Tools to remove chest hair*: Electric animal shaver and hair depilatory agent such as Nair tape is helpful to remove hair after shaving if the shaver does not have an attached vacuum source. Moist gauze for removal of Nair. Cotton-tipped applicators are useful to apply Nair and to absorb bleeding during surgery.
3. *Intubation*
 - a. We prefer an intubation stand from which the mouse is “hung” by a string and at an angle from its upper incisors, permitting the operator to look straight down into the larynx. Alternatively, the mouse may be laid in a supine position and the operator can bend down for visualization.
 - b. A blunt non-serrated forceps can be used to gently reposition the tongue prior to intubation.
 - c. Paper towels cut into small triangles are also useful to insert into the mouth to absorb saliva and air bubbles during the procedure.
 - d. A proper light source such as a gooseneck lamp is critical for transillumination of the neck and visualization through the oral cavity [15].
 - e. An intubation tube is fastened from a 20-gauge intravenous catheter cut at 25 mm in length with a fastener to attach to a Y-shaped ventilator connection.

¹Alternative anesthetics include pentobarbital sodium (87.5 mg/kg body weight for mouse) after intraperitoneal (IP) injection. Ketamine/xylazine can also be utilized for IP injection; however this combination is a cardio-depressant and may increase mortality. Isoflurane has the advantages of rapid recovery time. If using isoflurane, you will also need a vaporizer and oxygen source along with tubing, nose mask, and induction chamber. Gas must be properly scavenged with charcoal. Lidocaine (0.1 ml of 0.1 % stock injected subcutaneously) can be applied to prevent lethal arrhythmia; however, this may have effects on cardiomyocyte cell death.

Between surgeries the intubation tube is kept in alcohol for disinfection and washed with water prior to use (*see* ^{Note 2}).

4. *Ventilator*: We use the volume-controlled Harvard Apparatus Model 687 ventilator. For a 25 g mouse, the ventilation rate (breaths/min) is set to 140 and the tidal volume is 300 μ l. Variations will exist depending on the volume of dead space in the tubing that runs from the vent to the mouse and therefore optimal settings should be empirically validated, including periodically after heavy usage. A water column should be prepared for administration of positive end expiratory pressure (PEEP) [16]. Alternatively, an automatic PEEP can be programmed with a pressure/volume-controlled *Inspira* vent, also from Harvard Apparatus (*see* ^{Note 3}).
5. *Surgical platform with heating element*: Adjustable heating platforms can be purchased from *Kent Scientific*. Alternatively, a heating system can be custom made and placed underneath the surgery platform. For example, a reptilian tank warmer pad can be affixed to the bottom of plexiglass. The following is one such warmer, which can be purchased from a pet store: *Exo Terra Vivarium Heater Model PT-2030*. Alternatives include a cardboard platform wrapped in aluminum foil (which can be easily positioned for intubation). You will also need surgical tape to fasten the mouse to the platform (*see* ^{Note 4}).
6. Prior to surgery, 70 % ethanol and betadine (povidone-iodine from *Fisher HealthCare*) are necessary to apply to the surgical area for disinfection.
7. We recommend the following microsurgical instruments from *Fine Science Tools*:
 - a. A pair of curved micro-point forceps for tearing the pericardium.
 - b. Curved medium-point forceps with blunt ends and serrated edges are necessary for probing into the thoracic cavity and grabbing at the skin.
 - c. A pair of angled sharp forceps is useful for manipulation of sutures.
 - d. One locking needle holder for ligation of the coronary artery and another non-locking needle holder for wound closing.
 - e. Fine and spring scissors (one large and one micro-spring scissors) for dissection and separating skin from underlying fascia.
 - f. A micro-chest retractor is optional as sutures can be utilized for retraction (*see* ^{Note 5}).

²We find that a guidewire/stylet is useful in facilitating maneuvering of the intubation tube into the trachea, although experienced technicians may not need this. Premade and reusable stainless steel intubation cannulae may be purchased from *Harvard Apparatus*. A drop of 1 % lidocaine may also be applied on the tip of the catheter to numb the throat and reduce gag reflex.

³If using a water column to generate a PEEP in combination with a gaseous anesthetic such as isoflurane, the output gas from the vent that is immersed in water will need to be captured in a closed system, such as either a fume hood or alternatively a water-filled flask with an output that feeds into a gas-scavenging system.

⁴Mice can also be positioned on a monitoring pad such as from *Indus instruments* for monitoring of ECG, respiration, and temperature control. This pad uses noninvasive ECG electrodes. Alternatively electrodes can be inserted subcutaneously. Finally, a rectal probe may also be inserted to monitor body temperature. We recommend *ThermoWorks MicroTherma 2*.

⁵For surgical induction of MI, it is important to use sterilized instruments prior to and between surgeries. Instruments can initially be autoclaved for the first surgery and subsequently sterilized in a hot bead-sterilizer between individual animal subjects.

8. Cautery, such as the thermal cautery unit from Geiger Medical Technologies with “201” tips. Alternatively, a more affordable system is the battery-operated Gemini cautery system sold through Harvard apparatus. You will need to replace tips periodically.
9. Dissecting microscope with adjustable head and light source from gooseneck lamp. An attached digital camera with a high frame capture rate for video acquisition is helpful for teaching purposes and documentation. You will need a trinocular microscope if a camera is attached.
10. Sutures can be purchased from *eSutures.com* or *Ethicon*. We use 7 or 8-0 Surgipro or prolene sutures for ligation and to minimize bleeding when threading through the myocardium. For rib, muscle, and skin closure: 6-0 silk cutting sutures from Suture. 6-0 sutures can also be used to retract ribs.
11. Electric heating pad, circulating water blanket, or forced warm-air blanket for postsurgical recovery and to maintain core body temperature within normal physiologic range. Radiant heat is provided through the rodent cage. A heating lamp can also be used with monitoring (*see* ^{Note 6}).
12. Analgesic *Buprenex* (obtained with permission and through your local animal supervisory center) at 0.1 mg/kg mouse body weight for subcutaneous injection 4–6 h after surgery and then once a day for 3 days post surgery.

2.2 Infarct and AAR Measurement

1. Isoflurane (from your local/institutional animal care center) and cotton pads. Isoflurane-soaked cotton to be placed in induction or small chamber of known volume and secure-lid for anesthesia. Wire mesh for placement at the bottom of the container; cotton-soaked isoflurane is placed underneath so that solution does not come into contact with the mouse skin and cause irritation. Alternatively, a nose cone with isoflurane and gauze can be used. Fume hood to protect surgeon from inhalation of vapors.
2. Surgical Styrofoam board (lid from a Styrofoam cooler) wrapped in aluminum foil, pins for securing animal, and blunt dissecting scissors and forceps.
3. 20 gauge catheter for injection of Evans blue dye. A 5 ml syringe with Luer lock to fill with dye. Alternatively, the heart can be retrograde-perfused after cannulation of the aorta followed by slow perfusion of the dye as described below.
4. Evans Blue (Sigma) 2 % weight/volume in phosphate-buffered saline. Care must be taken to eliminate air bubbles; otherwise, nonhomogeneous staining will result (*see* ^{Note 7}).

⁶Electrical heating pads can cause burns to anesthetized or debilitated animals. To reduce the risk of burn, pads must only be used at low or medium settings and always positioned so that animals can move away from the heat source.

⁷Evans blue is the most commonly utilized dye for measurement of AAR; however this dye may slightly precipitate out of solution during subsequent incubations, thereby leading to less defined borders. Methylthiazolyl blue tetrazolium (MTT) tends to have less of this problem; however, the color is less blue. MTT is made into a 0.5 % solution in phosphate-buffered saline.

5. A 1 mm heart slicer for sectioning hearts: *Zivic Rodent Heart Slicer Matrix*. New razor blades. Saran wrap if placing hearts in freezer as described below.
6. TTC from *Sigma* is an enzyme substrate that stains viable, non-injured tissue. Necrotic cells lack intact lactate dehydrogenase enzyme activity and are therefore unable to convert tetrazolium to its red precipitate. TTC is 1 % in saline (*see* ^{Note 8}). Small petri dishes or tubes for incubation in TTC-stain. 37 °C incubator.
7. 10 % buffered formalin for fixation of tissue, histology grade. This is harmful if inhaled or absorbed through skin and can cause irritation to eyes and respiratory tract.
8. Plexiglass to position heart-slices for imaging. Imaging device and software for morphometry. Blue, red, and white areas after staining are measured with Adobe Photoshop, NIH Image, or Image J. From these measurements, infarct size is calculated as a percentage of the AAR.

3 Methods

All methods must be Institutional Animal Care and Use Committee (IACUC)-approved prior to beginning.

3.1 Permanent Occlusion of the Left Anterior Descending Artery

Below is outlined a procedure for permanent occlusion of the LAD artery [17]. We recommend 10–12-week-old C57BL/6 mice from Jackson or Charles River Laboratories. Younger mice can be utilized; however the survival rate may be diminished. One should have everything prepared prior to starting (tape, ligation suture on locking needle holder, retracting sutures, intubation catheter/stand). Average LAD ligation surgery should be about 15–20 min per animal with an experienced surgeon. Surgical consistency is critical for downstream quantitation of necrosis.

1. *Weigh mouse and calculate anesthetic dose.* This protocol describes surgery with Avertin. Non-pharmaceutical grade Avertin must be justified with IACUC. Avertin is an unstable compound. Hence stock solution is to be discarded after 4 months as this compound can degrade into toxic compounds. Discard solution if it turns purple or yellow. Pinch tail feet to confirm anesthetic depth after injection. Anesthetic depth is confirmed by lack of pedal reflex (*see* ^{Note 9}).
2. *Remove chest hair.* Once under anesthesia, shave hair from chest (left side of rib cage) with an electric razor. Also shave neck area over trachea in case tracheotomy is required.
3. *Intubate mouse.* Secure animal by passing a loop over its top incisor on an intubation stand. Intubation can be performed with mouse positioned at an angle or alternatively with mouse positioned flat on its back. Otherwise a flat cardboard

⁸Tetrazolium can be purchased as nitro blue or triphenyl. The nitro blue tetrazolium will not cross membranes and therefore can only be used with sliced tissue. The triphenyltetrazolium will cross the cell membrane and therefore be also included in the perfusate.

⁹When using Avertin keep stock and working solution away from light or wrapped in foil/amber bottle. Keep working solution for 2 weeks and stock solution for 4 months maximum.

stand wrapped in aluminum foil will suffice. Gently pull out the tongue and position it to the side. Gooseneck light is to be shined at the throat of the mouse to illuminate larynx. While securing the tongue with your thumb and index finger, apply slight pressure with middle or index finger near the throat. This will make the vocal cord more visible (Fig. 2). Insert endotracheal catheter approximately 5 mm into the trachea [18]. Once inserted, tape down intubation tube to secure and prevent extubation. Extubation will also be prevented by securing top incisors (teeth) of mouth with string. To verify a successful endotracheal intubation (as opposed to an esophageal intubation), one can turn on the ventilator and connect the intubation catheter to check for proper rhythmic breathing pattern. Another verification is to place a chilled dental mirror in front of intubation catheter and to examine for visual evidence of breathing on mirror (*see* ^{Note 10}).

4. *Position for surgery.* Proper positioning is critical to stretch the mouse chest and expose heart optimally for ligation. Position mouse supine (lying on its back) on surgical platform. Left arm should be secured toward head. Right paw down and toward body axis. Left foot free (Fig. 3).
5. *Remove surface hair.* Remove hair with depilatory agent such as *Nair*. Remove hair from left chest from bottom rib cage up to arm level, from just right of the midline to left arm. Remove hair with tape and clean with 70 % EtOH. Chest area is disinfected further with *betadine*. Disinfecting starts at the center of the surgical area with a circular motion outwards. Never go back to the middle with the same gauze pad.
6. *Initial incision.* Make initial parasternal/midline incision 2 mm left of sternal middle border on the left side of the rib cage below the left mouse elbow and armpit, near the fourth intercostal space. Cut through the skin and subcutaneous fascia. Extend an oblique incision left toward just below (1–2 mm) left armpit. Incision is about 1.5 cm long away from midline.
7. *Loosen skin.* Loosen/separate skin from connective tissue/fascia carefully and blunt dissect with curved forceps or scissors (by prodding scissors under skin and opening and closing).
8. *Dissect pectoralis major.* Dissect through the first muscle layer, i.e., the *pectoralis major*. Spare the rectus (abdominal) muscle. The pectoral muscles are dissected and loosened through the natural divisions of the muscle layer and retracted gently.
9. *Dissect pectoralis minor.* Loosen minor pectoral muscle and cut over the fourth intercostal space (second large rib space from lower margin of the ribs).
10. *Identify intercostals.* Identify the third and fourth intercostal space for thoracotomy (Fig. 4). Count to the third and fourth intercostal from the first (*see* ^{Note 11}).

¹⁰In case tracheotomy is required due to failed endotracheal intubation trim IV catheter (20 G 1–1/4) off of hub and remove pointed end. Replace on insert. Make a 1 cm incision on midline over trachea. Blunt dissect down to trachea. Pull tongue aside and insert trachea tube making sure to angle upward so as not to enter the esophagus. Watch for white of the tube in trachea to know that the tube is in the proper position. Quickly remove insert, and attach trachea tube to ventilator tubing and start ventilation.

¹¹To identify the desired intercostal, one can look for various landmarks. A noticeable vessel runs from bottom of the ribcage toward the head until reaching the fourth intercostal. You will cauterize the intercostal one space above if entering at the third intercostal. Also

11. *Cauterize intercostal.* Cauterize the intercostal that you will enter through (third or fourth) to prevent bleeding. Bleeding will obstruct the view of LAD. Also be careful during cauterization of intercostal so as not to penetrate the underlying heart/pericardium.
12. *Turn on ventilator.* Confirm proper breathing pattern and rate (~115 beats per minute). Ventilation is not necessary until puncture of thoracic cavity (*see* ^{Note 12}).
13. *Perform left-sided thoracotomy.* Penetrate w/small spring scissors, taking care not to damage the heart or the lung below. Extend incision up to margin of the lung, taking care not to touch lung. Pull up on the rib cage with forceps to create distance from lung. One can insert a moist sponge (gauze dipped in saline) to displace lungs if necessary (*see* ^{Note 13}).
14. *Retract ribs.* Retract ribs above incision and separately, below the incision, with retracting sutures. Alternatively, a micro-chest retractor can be inserted.
15. *Pericardium removal.* Turn up the light setting for best viewing. Avoid tearing pericardial vessels as the pericardium is vascularized and tearing vessels will lead to profuse bleeding. Pull up on the pericardium near the top of the heart near the atrium with precision sharp forceps and tear. Pulling away should generate a pericardial “cradle” that slightly lifts the heart for better viewing of the LAD. Overlay pericardium on left lung.
16. *Identification of the LAD coronary artery (see Fig. 5).* Visualization of the LAD is not trivial and requires experience so as not to confuse with other prominent coronary vessels. The LAD is best observed with a dissecting microscope and focused light that is not too bright; gooseneck lighting from two tangentially directed light sources is recommended. Early after thoracotomy and pericardial removal, the LAD should appear as an orange pulsating color. Prolonged time (greater than 5 min after pericardial removal) and reduced temperature from ambient air will make the LAD difficult to visualize (*see* ^{Note 14}).
17. *Ligation.* Ligate the proximal LAD with an 8-0 prolene suture. As evidence of successful occlusion, look for immediate blanching/paleness of the downstream ventricle along with altered myocardial movement. Ligate 2–3 mm below the atria to produce a 40–50 % infarct of the left ventricle. Pull suture through slowly. Double-loop knot and pull tight.

you can check for the curvature of the ribs. Intercostal space for entry is after first rib that is less curved than the rib just above. Check for the largest appearance of the beating heart to verify this position.

¹²Lung inflation by blocking air export from mouse should be done at the following time points: (1) before tearing off pericardium, (2) before suturing ribcage (after permanent occlusion of LAD), (3) before suturing the skin, and (4) before taking the animal off the ventilator. Note that a healthy lung is “bubblegum” pink in color. Blocking air export from mouse on ventilator will inflate lung and create PEEP.

¹³In case of convulsions be careful not to perforate the artery that runs parallel to the sternum as this will cause convulsions. If convulsions occur after retracting the ribs, it is most likely due to lack of ventilation, spoiled Avertin, or lack of anesthetic depth.

¹⁴Tips for good LAD visualization include utilizing a retracting suture on the top border of the incision and pulling first toward the head first followed by pulling 45° left of the operator’s viewpoint. Bottom ribcage retracting suture should be pulled 90° downward toward the mouse feet. Other tips include lifting the atria to visualize the pulsating LAD emanating from underneath as a pulsating bright red spike, running in the middle of the heart wall from underneath of the left atrium toward the apex. You may apply soft pressure to the apex with a warm sponge–armored blunt angled forceps. This maneuver induces slight paleness of the myocardium and increases the tissue contrast to the perfused and brighter LAD.

18. *Loosen retractors.* Remove retractors to alleviate stress on the mouse.
19. *Evacuation catheter.* Insert evacuation catheter through the skin and into thoracic cavity for aspiration of fluid as described below.
20. *Close openings.* Carefully suture ribs together so as not to pinch the lung. Pull tautly and secure knot. Reposition pectoralis major and suture together. Take care not to overtighten muscles when closing chest with sutures. Suture the subcutaneous layer and skin.
21. *Evacuation of pneumothoraces.* Remove tape. With evacuation tube, remove air and fluid while slightly squeezing chest. Take mouse off vent.
22. *Position mouse on heating pad.* Ensure that the animal has means to escape heat after waking. This can be accomplished by positioning the heating pad so that only half of the bottom surface area of the recovery cage is exposed to the pad. Wait for mouse to come out of anesthesia. If using isoflurane, stop isoflurane and wait until mouse is awake and begins to move before extubation. In this case, leave mouse attached to the ventilator while lying on the heating pad during recovery. Then, as mouse starts to move, disconnect ventilator, being careful not to remove trachea tube. Watch to make sure that mouse is breathing on its own. If not breathing well, reattach to the ventilator.
23. *Post-op documentation.* Make notes regarding surgery, body weight, and length of surgery. Note comments about movement, coat condition, eyes, eating, or drinking in the lab notebook with original mouse surgery record. If mouse appears to be in any form of distress, consult the animal veterinarian immediately.
24. *Analgesia.* For post-op pain, the opioid buprenorphine is administered. Alternatively, the nonsteroidal anti-inflammatory meloxicam can be used. Fluids may also be given (~1.0 ml of 5 % dextrose in water i.p.) and 100 % oxygen via nasal cone. Mouse must be monitored for distress two times a day for the next 3 days. After the first 5 days, mouse is to be checked three times per week.

3.2 Measurement of Infarct Necrosis Within the Ischemic Area at Risk

TTC staining, when used in conjunction with Evans blue, identifies the viable tissue within the AAR and can be used to determine infarct area as early as 20 min after occlusion [8]. Infarct size is expressed as a ratio of necrotic area to AAR [19]. After the heart is sliced into 1 mm pieces, the slices are incubated in TTC enzyme solution (a colorless dye). This enzyme is reduced to a deep-red precipitate by dehydrogenases in the presence of NADH. Hence, nonviable tissues will not turn red. Fixation with 10 % formalin brings out the contrast between colors.

1. *Prepare Evans blue dye for perfusion.* Prepare 0.4–0.5 ml of 2 % Evans blue solution in a 1 ml syringe.
2. *Anesthetize mouse.* Add isoflurane to gauze pad. Work in fume hood. Leave mouse in chamber and subsequently check anesthetic depth by pinching toe. Transfer mouse to a surgical board and work quickly. Cervical dislocation will serve a

secondary euthanasia. Careful not to be too forceful with dislocation as this can lead to tearing of internal organs. Tape or pin down the mouse to the surgical board.

3. *Dissect.* Cut open mouse. Make initial incision below diaphragm and extend incisions toward armpits. Pull up on sternum and position rib cage away from heart.
4. *Inject dye in situ* (see^{Note 15}). Displace abdominal organs to expose the left renal vein [20]. Do this under the dissecting microscope. Insert the catheter and attach syringe containing Evans blue solution. Make sure to avoid bubbles. Perfuse slowly. You should begin to see the heart turning blue except for the AAR (see Fig. 6 and Note 16).
5. *Remove the heart.*
6. *Section heart.* Place the heart in the heart slicer (Fig. 7). Insert razor blades into the matrix. Do not fully insert until all blades are in place. Once in place, apply downward pressure to all and cut (see^{Note 17}).
7. *Stain with TTC.* Incubate heart slices in 1 ml solution of 1 % TTC. Incubate the tube in a 37 °C incubator for 5–10 min until you see development of red stain.
8. *Fixation.* Stack heart slices on a needle; each slice is separated by a square western blot film to prevent curling during fixation with formalin (Fig. 8). Keep the needle-stacked hearts in 4–5 ml of 10 % formalin in a 15 ml tube at 4 °C for 20 min.
9. *Prepare heart slices for imaging.* Lay heart slices on plexiglass in saline. Place on top with cover glass/microscope slide. A 2 mm shim/spacer should separate bottom-surface from top to standardize area measurements. Spring clamp to press top-cover down to press slices to a uniform 2 mm (Fig. 9). This will make the diameter of the rings larger and prevent glistening of uneven heart surfaces.
10. *Acquire images.* Digital photomicrographs of infarcts should be blinded for analysis of all slices. Image should be taken of both sides of each slice.
11. *Quantify.* Infarct size is determined as a percentage of the AAR. Infarct area and AAR are traced in the digital images (Fig. 10). Infarct size, expressed as a percentage, is calculated by dividing the sum of infarct areas from all sections by the sum of LV areas from all sections (including those without infarct scar) and multiplying by 100. Infarct size can also be normalized to the weight of the corresponding heart slice(see^{Note 18}).

¹⁵For AAR quantification staining in situ, it is important for the heart to be beating so that the dye can circulate. It is critical that no bubbles are formed during injection of dye.

¹⁶As an alternative to perfusing the heart with Evans blue in situ, one may excise the heart for retrograde dye perfusion at the end of a cannula [19]. For this approach, mice are euthanized by cervical dislocation. The still beating heart is excised along with the thymus and connective tissue to prevent damage to the aorta. The organs are rinsed in heparinized saline to remove excess blood. On a moist tissue, the thymus is lifted to expose the aortic arch and the aorta. Extra-cardiac tissue is trimmed away. A 20 gauge blunted needle is inserted into the aorta, taking care to avoid air bubbles. The syringe is then secured in a clamp stand and heart is very gently perfused to prevent coronary rupture with 1–2 ml saline to remove brown-staining blood.

¹⁷After perfusion, heart is rinsed in 0.9 % saline or PBS, connective tissue removed, and stored in –20 °C freezer. To freeze the tissue, wrap it in clear food wrap and put it in a –20 °C freezer for 1–2 h (any longer and risk freeze-drying). Wrap is important to prevent freeze-drying (which will then artificially become tetrazolium negative).

¹⁸Acute necrosis can also be measured by Masson's trichrome stain, which will color necrotic cardiomyocytes blue and viable myocytes red. Unlike collagen at chronic time points post MI, acute necrotic myocytes will be picosirius red negative.

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Fig. 2. Positioning of mouse for intubation. Transilluminated light from a gooseneck lamp permits operator visualization from above in the larynx. While securing the tongue with your thumb and index finger, apply slight pressure with middle or index finger near the throat. This will make the vocal cord more visible

Fig. 3. Positioning of mouse for surgery. Position mouse supine on surgical platform. *Left arm* should be secured toward head. *Right paw* down and toward body axis. *Left foot* free and therefore not indicated in the figure

Fig. 4. Identify intercostals. To identify the desired intercostal, one can look for various landmarks. A noticeable vessel runs from bottom of the ribcage toward the head until reaching the fourth intercostal

Fig. 5. Identification of the LAD. The pulsating LAD emanates from underneath the *left atrium* as a pulsating bright red spike, running in the middle of the heart wall toward the apex. Ligate 2–3 mm below atrium

Fig. 6.
Visualization of the AAR. After perfusion with Evans blue, you should begin to see the heart turning dark except for the AAR

Fig. 7. Heart slicing. Place the heart in matrix as shown on *left*. Insert razor blades as shown on *right* and slice

Fig. 8.
Stacking heart slices. Stack heart slices on a needle; each slice is separated by a square western blot film to prevent curling during fixation with formalin

Fig. 9.

Prepare heart slices for imaging. Lay heart slices on plexiglass in saline. Place on top with cover glass/microscope slide. A 2 mm shim/spacer should separate bottom-surface from top to standardize area measurements. Spring clamp to press top-cover down to press slices to a uniform 2 mm

Fig. 10.

AAR and infarct size. Infarct size is determined as a percentage of the AAR. AAR is outlined in *dotted red lines*. Infarct area (*black line tracking*) and AAR are traced in the digital images. Infarct size, expressed as a percentage, is calculated by dividing the sum of infarct areas from all sections by the sum of LV areas from all sections (including those without infarct scar) and multiplying by 100