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Minimal invasive micro-surgical procedure of inducing myocardial infarction in mice

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Abstract:	Myocardial infarction still remains the main cause of death in western countries, despite considerable progress in the stent development area in the last decades, For clarification of the underlying mechanisms and the development of new therapeutic strategies, the availability of valide animal models are mandatory. Since we need new insights into pathomechanisms of cardiovascular diseases under in vivo conditions to combat myocardial infarction, the validity of the animal is a crucial aspect. However, protection of animals are highly relevant in this context. Therefore, we establish a minimally invasive and simple model of myocardial infarction in mice, which assures a high reproducibility and survival rate of animals. Thus, this models fulfils the requirements of the 3R principle (Replacement, Refinement and Reduction) for animal experimentation and assure the scientific information needed for further developing of therapeutical strategies for cardiovascular diseases.
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To Editorial Board of
Journal of Visualized Experiments

05. October 2014

Dear Sir/Madam,

please find enclosed the revised manuscript entitled 'Minimal invasive surgical procedure of inducing myocardial infarction in mice' by A. Curaj et al.
We have made now all the changes required by the referees and responde adequately to all remaining issues.

The current manuscript is not under consideration elsewhere. Moreover, all authors have read and approved the manuscript submission to *Journal of Visualized Experiments* and no any potential conflict of interest exists.

We hope that the manuscript is suitable now to be considered for publication in *Journal of Visualized Experiments* and we look forward to hearing from you.

Sincerely yours,

Elisa A. Liehn, MD, PhD

TITLE:
Minimal invasive surgical procedure of inducing myocardial infarction in mice

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KEYWORDS:
Heart, mouse model, myocardial infarction, myocardial ischemia, ventricular remodeling, scar formation, LAD ligature

SHORT ABSTRACT:
A highly reproducible model for myocardial infarction in mice with minimal invasive manipulations is described. The model can be easily performed, resulting in a high reproducibility and survival rate. Thus, the described model will reduce the number of required animals as requested by the 3R principle (Replacement, Refinement and Reduction).

LONG ABSTRACT:
Myocardial infarction still remains the main cause of death in western countries, despite considerable progress in the stent development area in the last decades, For clarification of the underlying mechanisms and the development of new therapeutic strategies, the availability of valid animal models are mandatory. Since we need new insights into pathomechanisms of cardiovascular diseases under *in vivo* conditions to combat myocardial infarction, the validity of the animal is a crucial aspect. However, protection of animals are highly relevant in this context. Therefore, we establish a minimally invasive and simple model of myocardial infarction in mice, which assures a high reproducibility and survival rate of animals. Thus, this models fulfils the requirements of the 3R principle (Replacement, Refinement and Reduction) for animal experimentation and assure the scientific information needed for further developing of therapeutical strategies for cardiovascular diseases.

INTRODUCTION:
Myocardial infarction is one of the main causes of death in industrialized countries. Despite undeniable progress of diagnostic and therapeutic approaches, cardiovascular diseases are still the major cause of mortality. Given the improved life expectancy and life-related risks, a continuous increase in the incidence of cardiovascular diseases is expected in the future. Therefore, there is a strong need to establish and validate novel approaches for the treatment of cardiovascular disease. The information of human studies suffer from its limitations, these studies generally are insufficient to explain and understand the mechanisms at the molecular level, being unable to provide solutions to these major health problems.

Moreover, basic research has been limited due to complexity and difficulty to reproduce the mechanisms of cardiovascular disease in the laboratory. Therefore, to increase our knowledge about the pathophysiology of cardiovascular diseases, it is essential to validate animal models [1,2]. However, to identify all cascades of molecular events involved in the healing after myocardial infarction, analysis at different time points is necessary, causing a large number of animals experiments.

Myocardial infarction experiments are often performed by using animal models. Inducing myocardial infarction in small animals[3-11] is the most suitable and efficient model employed to investigate cellular and molecular events than large animal models. Moreover, no other species presents the availability of transgenic or knockout strains as mice[12]. These mouse models are highly useful in other diseases, including cardiovascular pathologies (such as atherosclerosis, in stent restenosis)[13,14]. In addition, the low pregnancy period and the high number of progenies qualify mouse models as most attractive system to study molecular mechanisms of myocardial infarction[12].

Nevertheless, the size of the heart in mice expects high precision of manipulation during microsurgery. Teaching such qualified and skilled surgery personnel is a time-consuming and work-intensive process. Therefore, we herein present a detailed microsurgery procedure, including tips and tricks to guide collaborators even with average qualifications, such as students or technicians to perform the complex myocardial infarction model in mice.

Initially, intubation is performed by means of a short cannula without using the tracheotomy. The thoracic incision is located in the intercostal area, avoiding injury of ribs or/and surrounding tissue. This sub-step is highly relevant to assure fast recovery and healing[15]. The ligature is made differential for chronic ischemia and ischemia/reperfusion models, for a high survival rate while still maintaining a significant infarction size. Our experience shows that using silk suture assures a higher reproducibility compared to cryo-injuries[16].

In conclusion, the method described here is applicable in both chronic ischemia and ischemia/reperfusion models in small animals. The tips and tricks presented in this procedure are meant to enable personnel with even low or average qualification to apply it in small animal models.

PROTOCOL:

Experiments presented in this paper are performed accordingly to the German low and to the European animal care guidelines. The animals are bred in the Animal facility of Institute for Laboratory Animal Science, Universiy hospital Aachen, Germany, under supervision of Prof. Dr. R. Tolba and Dr. A. Teubner (animal welfare officer).

1) Animal care

1.1) Keep the mice in a specialized care unit, assuring proper access to food and specialized veterinary control and treatment. If the animals are moved or purchased from outside, please assure one-week accommodation before undergoing the procedure.

2) Intubation

2.1) Anesthetize 8-10 weeks old male C57Bl/6 wild type mice, 25-27g using intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. Monitor the level of anesthesia by toe pinch reflexes. Place vet ointment on the eyes to prevent dryness during the procedure.

2.2) Manage pain therapy with buprenophine 0.1 mg/kg body weight subcutaneously before starting the surgical procedure, following the animal care guidelines of your institution.

2.3) Assure the maintenance of sterile conditions to avoid infections during surgery by using sterile materials and instruments.

2.4) Place the anesthetized mouse in a supine position on a heated surgery table. Remove the hair from both ventral neck area and left half of the thorax using a small razor and disinfect with 70% alcohol prior to incision.

2.4.1) Perform a small median incision of 0.5 cm using surgery scissors in the center of the neck. Under the skin, go through the 2 fatty bodies with sterile curved forceps and visualize the trachea under stereomicroscope through the transparence of the covering muscle.

2.5) Introduce orally the intubation cannula into the trachea under view by using the stereomicroscope (Figure 1A). Distinguish the metal cannula through the transparent tissue. And check, the position and location during the operation at any moment (Figure 1B).

2.6) Connect the cannula to the small animal ventilator and adjust ventilation settings according manufactures guidelines (tidal volume between 100-150 µl and a respiration rate between 100-150/min).

3) Myocardial infarction induction

3.1) Perform a skin incision less than 0.5 cm in the middle of a line between xyphoid and left axila. Use forceps to separate the muscle layer from the underlying ribs.

3.2) Perform a small incision between ribs by using a small scissor until the thoracic cavity is opened[17]. For chronic infarction, perform the incision in the second inter costal space (Figure 1C) and/or for the ischemia/reperfusion model, in the third inter costal space, numbered from below (Figure 1D).

3.3) Place the retractors into the incision to open thoracic cavity and to visualize the heart.

3.4) Carefully remove the pericardium to prevent excessive fibrotic processes.

3.5) Visualize the left descending coronary artery (LAD) as a deep positioned light red vessel. If the LAD cannot be visualized, consider some reference points to increase the reproducibility.

3.5.1) For chronic infarction model, place the ligature in the middle of the ventral side of the heart (between the auricle and apex), having as reference the vein as shown in Figure 1C. Bind both branches of the artery using 0/7 silk suture to obtain a transmural anterior and posterior infarction. The gray color indicates the position of the ligature and can be repeated if needed (Figure 1C).

3.5.2) For ischemia/reperfusion model, place the ligature under the auricle, over the main body of LAD (Figure 1D). The ligature is located over a silicon tube to protect the integrity of the vessel. The gray color indicates the infarcted area and should appear in the entire heart (Figure 1D). Place temporal sutures on the ribs during the ischemia period and moisten using a compress to avoid tissue drying. After ischemia, remove the silicon tube and cut the suture with small scissors to visualize the reperfusion.

3.6) Narcotize the mouse during surgery with 0,5% isoflurane, following the animal care guidelines of your institution.

4) Suture and recovery

4.1) Eliminate the residual air from thorax by filling with warm isotonic salt solution.

4.2) Close the thorax with 3 sutures 0/6 (as shown in Figure 2A and 2B). Position the medial sutures at an angle of 90°, to assure a sealed closure of the ribs, as shown in Figure 2 (Figure 2A, B).

4.3) Close the muscle layer with 2 sutures (Figure 2C) and the skin with 3-4 sutures 0/6 (Figure 2D). Perform these sutures separately to obtain a proper window for further echocardiographic measurement.

4.4) Disconnect the intubation cannula from the ventilator and allow spontaneous breath. For later identification, mark the mouse using the local system (ask the animal welfare officer from your institution).

4.5) Lay down the mouse on the left side under the red lamp until it wakes up. Do not leave an animal unattended until it has regained sufficient consciousness. Do not allow an animal that has undergone surgery to be in the company of other animals until fully recovered.

4.6) Manage pain therapy with buprenophine 0.1 mg/kg body weight, subcutaneously for the next 3 days, following the animal care guidelines of your institution.

5) Analysis of the myocardial infarction

5.1) Regularly monitor the heart function by means of echocardiography (Figure 3A): the ejection fraction, fractional shortening, cardiac output and heart dimensions.

5.2) Anesthetize the animals using intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. Confirm proper anesthetization prior to surgery by the lack of reflexes.

5.3) Open the thoracic cavity and excise the heart, placing it in sterile PBS solution washing extensively the remaining blood.

5.4) If needed, collect the blood directly from the heart by avoiding the injury of the infracted regions, or after removal of the heart, from the thoracic cavity.

5.5) After washing, stop the heart in diastola in saturated KCl solution (steril filtered 3M KCl in PBS). For histological analysis fix the heart in 10% formalin and proceed with Step 5.7.

5.6) If necessary, measure the viability of the cardiac cells by Evans-Blue/ Triphenyl tetrazolium chloride (TTC) staining. After rebuilding the ligature at the initial place, perfuse the heart with 200µl 1% Evans Blue Solution using an aortic cannula and freeze the heart in a small plastic bag at -20°C, without washing.

5.6.1) After 2 hours, perform 5 transversally slides using a sharp scalpel and incubate them for 10-15 min in TTC solution at 37°C, as described by manufactured. Fix the slides for 10 min in 10% formalin and put them between the microscopic slides for further analysis.

5.6.2) Embed the heart tissue in paraffin, by positioning the heart on the tip, to perform transversal sectioning. Perform serial section of 5 µm. Collect the first 20 sections and discard the next 300 µm. Continue the section protocol until the mitral valve level has been reached (Figure 3A, B). Serial sections, 400 µm apart along the entire heart are collected and can be stained for the qualitative and quantitative analysis.

5.7) Measure the infarction size using Gomori's one-step staining[6-8].

5.8) Analyze angiogenesis, collagen content or inflammatory cells recruitment in serial section using usual immunohistological staining.

REPRESENTATIVE RESULTS

The myocardial infarction procedure occurs within 25-30 minutes and shows a mortality rate of 10%. After surgery, the mice recover from anesthesia within the next 15 minutes. No physical impairment was observed to the operated mouse. However, there is a higher risk of heart rupture one week after post-chronic myocardial infarction, if the repairing processes are disturbed during the inflammatory phase. Since heart is able to change significantly its dimensions during the pumping, it is important for all the collected hearts to be stopped in the same position, for example in diastola. This can be achieved by perfusing the heart with saturated KCl solution. Increased extracellular K+ concentration blocks the ionic pumps, decreases the membrane resting potential of cardiac cells, resulting in a diastolic arrest of cardiac activity.

The infarction area can be seen in ultrasound analysis (**Figure 3A**, lower panel). In comparison to the normal myocardium, ischemic regions appear thin and hypokinetic (**Figure 3A**, upper panel). Depending on the model used, the infarction size will differ. The chronic infarction model induces circular, transmural infarction of the apex (**Figure 3B**), while the ischemia/reperfusion induces a thin, middle-wall and throughout all heart (Figure 3C). There are many methods to determine infarction size. If the aim is to analyze the direct effect on cardiac viability, an Evans-Blue/TTC staining[18] is indicated to be performed at least 2 hours after reperfusion, to be able to see any changes in the myocardium., Sections can be analyzed immediately (**Figure 3B**, middle panel) after staining or can be kept between glass slides in formalin for 2-3 days (**Figure 3C**, middle panel). The blue area represents the healthy myocardium, not affected by ischemia. The red area represents the viable myocardium inside the ischemic area (risk myocardium), and the white area represents the dead tissue. Usually, the infarction size is expressed as percent from the risk area.

The mature scar resulting after remodeling processes can be easily measured by immunohistolgy using Gomori's one-step staining. Blue-stained infarcted and red-stained healthy ventricular areas (**Figure 3B andC**, right panels) are determined in the first section from each level until the mitral valve. To avoid the variation due to binding of LAD at different levels, the infarction from all section is considered and expressed as a percentage of total left ventricular volume. An infarction volume of 15-20% in chronic infarction model and of 10-15% after ischemia/reperfusion model can be achieved. Further, the chronic infarction model will induce an accentuated dilatation, not observed in the ischemic/reperfusion model (**Figure 3B and C** right panel).

Conventional staining procedures can be used, such as: CD31 staining used to reveal the angiogenesis (red, **Figure 4A**) or smooth muscle actin staining to determine myofibroblasts (green, **Figure 4B**). Double fluorescence staining can also be applied to identify different target molecules in the infarction area, since the absence of cardiomyocytes gives no auto-immunofluorescence (**Figure 4C**).

Figures:

Figure 1: Medial incision and insertion of the intubation's cannula

(**A**). The stereomicroscopic visualization of the metal cannula through the transparence of the tissue (B). The tracheal rings (blue arrows) and the cannula (black arrow) are pointed out. The intercostal incision for the chronic infarction model and the ligature of LAD (C). The ligature is located at middle of the heart (between the auricle and apex, black in lower panel), taking as reference the end of the vein (schematic in blue, lower panel). Both branches of the artery should be bound (red in lower panel). The gray color indicates infarcted area and it appears in the lower half part of the heart (right lower panel). The ligature for the ischemia/reperfusion model is made under the auricle, binding the main body of LAD (red in lower panel) over a silicon tube (right side) (D). The gray color indicates the infracted area, which is present on the entire heart (right lower panel).

Figure 2: The ribs suture seals the thoracic incision if the medial sutures are positioned at an angle of 90° in both chronic

(**A**) and ischemia/reperfusion model (B, left panel). *In vivo* imaging of ribs suture (**C**, left panel), muscle suture (**C**, middle panel) and skin suture (C, right panel).

Figure 3: **Echocardiographic images**.

Images of normal (A, upper panel) and infarcted areas (A, lower panel), are acquired in the long axis (longitudinal, left panels) or in the short axis (transversal, right panels).. Infarction induced by chronic ligature (B) and by one hour ischemia followed by reperfusion (C).

Evans Blue/TTC Staining allows identification of perfused (blue)/non-perfused areas as well as the viable (red)/dead (white) myocardium (B, C middle panels). Gomori's one-step staining allows the identification of infarcted areas (blue), and differentiates them from the normal regions (red) (B, C, right panels).

Figure 4: Different stainings can be performed in infracted area, such as CD31 to described neo-angiogenesis (A, red, simple arrows), or smooth muscle actin for myofibroblasts (B, green, simple arrows), as well as double staining (C, CD31-red/smooth muscle actin-green), counterstained with DAPI for nuclei (blue). Myofibroblasts can be differentiated easily from smooth muscle cells from small or big arteries, which are always accompanied by a endothelial layer (C, arrows). Double arrows point the erythrocytes autofluorescence. Scale bars 50 µm.

DISCUSSION:

During the procedure, there are some critical points to be noted: the intubation, the opening the thoracic cavity and the LAD ligature. The first critical is the intubation of the animal before experiements. Many groups are using a vertical support for fixing the mouse and a source of light to insert the cannula directly into the trachea. This method has uncertainty concerning the correct insertion of the cannula into the trachea and is the most prone to failure by the novices. Making a small incision, the position of the cannula can be controlled during the entire maneuver, thus decreasing the default rate. Moreover, the tracheotomy is surpassed, thus decreasing complications and reducing the time of operation.

The next critical step is the opening of the thoracic cavity. The median sternotomy represents a high-risk maneuver delaying the recovery of the animals. The lateral left incision implying the

cutting of 2-3 ribs[15], leads to deficient recovery and increased mortality. We used in the model small, discrete incision between the ribs offering minimal burden. The animals recover very quickly after the surgery and do not present defects or disturbed healing. The lower inter-costal space is taken as a reference point. Considering this, the proper and differentiated access to the ligature place for chronic and ischemia/reperfusion model, does not raise serious problems.

The ligature itself represents the most critical step. The left descending coronary artery is hard to be visualized, and often needs to be bound without view. Therefore, some anatomic reference points are pointed out to help the surgeon to perform the correct ligation. For the chronic infarction model, the ligature is placed in the middle of the ventral side of the heart, between the auricle and the apex, above the ending of the major anterior vein (**Figure 2B**). The efficiency can be controlled by visualizing the appearance of the grey color in the affected areas. If the infarcted area appears anterior and does not include the posterior wall, a new suture can be placed to the left of the first suture. The main root of LAD is always visible under the auricle[18], and therefore does not present serious problems in detecting this part. However, the auricle presents the major risk of bleeding and needs to be handled carefully.

The procedure is limited by existence of appropriate equipment. A ventilator and appropriate anesthesia system for the small animals are expensive and require connections to gas and ventilation system of the room. Further, a close supervision of the animals is necessary in the first week after procedure to detect the possible clinical. To examine the heart function during the experiment, high-resolution ultrasound, complex Langendorf perfusion-system, or small intraventricular catheter measurements are required, involving high costs and additional expertise.

Considering the myocardial infarction, there is no alternative methods available to reproduce the complexity of the events *in vitro*. Depending on the point of interest, *ex vivo* perfusion of an isolated heart in Langendorff system provides information about the contractility, heart function and myocardial viability in response to different stimuli or drugs. However, it excludes all interferences of blood components and immune system, and it is not indicated for long-studies of remodeling and healing after myocardial infarction.

After performing the myocardial infarction procedure, all other functional analysis can be carried out, like intraventricular pressure measurements, ultrasound (small animal ultrasound systems) or isolated heart Langendorff-perfusion. Moreover, all biological and molecular analysis can be performed to identify cells, proteins, mRNAs, microRNAs, genes or other biomarkers, which can be used as therapeutic targets to develop new treatment strategies for myocardial infarction.

DISCLOSURES

The authors have nothing to disclose.

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REFERENCES:

1 Liehn, E. A., Postea, O., Curaj, A. & Marx, N. Repair after myocardial infarction, between fantasy and reality: the role of chemokines. *J Am Coll Cardiol.* **58** (23), 2357-2362, doi: doi: 10.1016/j.jacc.2011.08.034 (2011).

2 Liehn, E. A., Radu, E. & Schuh, A. Chemokine contribution in stem cell engraftment into the infarcted myocardium. *Curr Stem Cell Res Ther.* **8** (4), 278-283, doi: 10.2174/1574888X11308040003 (2013).

3 Alexander, S. *et al.* Repetitive transplantation of different cell types sequentially improves heart function after infarction. *J Cell Mol Med.* **16** (7), 1640-1647, doi: 10.1111/j.1582-4934.2011.01477.x (2012).

4 Liehn, E. A. *et al.* Compartmentalized protective and detrimental effects of endogenous macrophage migration-inhibitory factor mediated by CXCR2 in a mouse model of myocardial ischemia/reperfusion. *Arterioscler Thromb Vasc Biol.* **33** (9), 2180-2186, doi: 10.1161/ATVBAHA.113.301633 (2013).

5 Liehn, E. A. *et al.* Ccr1 deficiency reduces inflammatory remodelling and preserves left ventricular function after myocardial infarction. *J Cell Mol Med.* **12** (2), 496-506, doi: 10.1111/j.1582-4934.2007.00194.x (2008).

6 Liehn, E. A. *et al.* A new monocyte chemotactic protein-1/chemokine CC motif ligand-2 competitor limiting neointima formation and myocardial ischemia/reperfusion injury in mice. *J Am Coll Cardiol.* **56** (22), 1847-1857, doi: 0.1016/j.jacc.2010.04.066 (2010).

7 Liehn, E. A. *et al.* Double-edged role of the CXCL12/CXCR4 axis in experimental myocardial infarction. *J Am Coll Cardiol.* **58** (23), 2415-2423, doi: 10.1016/j.jacc.2011.08.033 (2011).

8 Oral, H. *et al.* CXC chemokine KC fails to induce neutrophil infiltration and neoangiogenesis in a mouse model of myocardial infarction. *J Mol Cell Cardiol.* **60**, 1-7, doi: 10.1016/j.yjmcc.2013.04.006 (2013).

9 Projahn, D. *et al.* Controlled intramyocardial release of engineered chemokines by biodegradable hydrogels as a treatment approach of myocardial infarction. *J Cell Mol Med.* **18** (5), 790-800 doi: 10.1111/jcmm.12225 (2014).

10 Schuh, A. *et al.* Novel insights into the mechanism of cell-based therapy after chronic myocardial infarction. *Discoveries.* **1** (2), e9, doi: 10.15190/d.2014.1 (2014).

11 Schuh, A. *et al.* Effect of SDF-1 alpha on Endogenous Mobilized and Transplanted Stem Cells in Regeneration after Myocardial Infarction. *Curr Pharm Des.* **20** (12), 1964-70, doi: 10.2174/13816128113199990443 (2013).

12 Zaragoza, C. *et al.* Animal models of cardiovascular diseases. *J Biomed Biotechnol.* **2011**, 497841 doi:10.1155/2011/497841 (2011).

13 Kanzler, I., Liehn, E. A., Koenen, R. R. & Weber, C. Anti-inflammatory therapeutic approaches to reduce acute atherosclerotic complications. *Curr Pharm Biotechnol.* **13** (1), 37-45, doi: 10.2174/138920112798868557 (2012).

14 Liehn, E. A., Zernecke, A., Postea, O. & Weber, C. Chemokines: inflammatory mediators of atherosclerosis. *Arch Physiol Biochem.* **112** (4-5), 229-238, (2006).

15 Kolk, M. V. V. *et al.* LAD-Ligation: A Murine Model of Myocardial Infarction. *J. Vis. Exp.* (**32**). pii: 1438. doi: 10.3791/1438 (2009).

16 Ryu, J. H. *et al.* Implantation of bone marrow mononuclear cells using injectable fibrin matrix enhances neovascularization in infarcted myocardium. *Biomaterials.* **26** (3), 319-326, doi: 10.1016/j.biomaterials.2004.02.058 (2005).

17 Frobert, A., Valentin, J., Cook, S., Lopes-Vicente, J. & Giraud, M. N. Cell-based Therapy for Heart Failure in Rat: Double Thoracotomy for Myocardial Infarction and Epicardial Implantation of Cells and Biomatrix. *J. Vis. Exp.* **91** e51390, doi: 10.3791/51390 (2014).
18 Xu, Z., Alloush, J., Beck, E. & Weisleder, N. A Murine Model of Myocardial Ischemia-reperfusion Injury through Ligation of the Left Anterior Descending Artery. *J. Vis. Exp.* **86** e51329, doi: 10.3791/51329 (2014).

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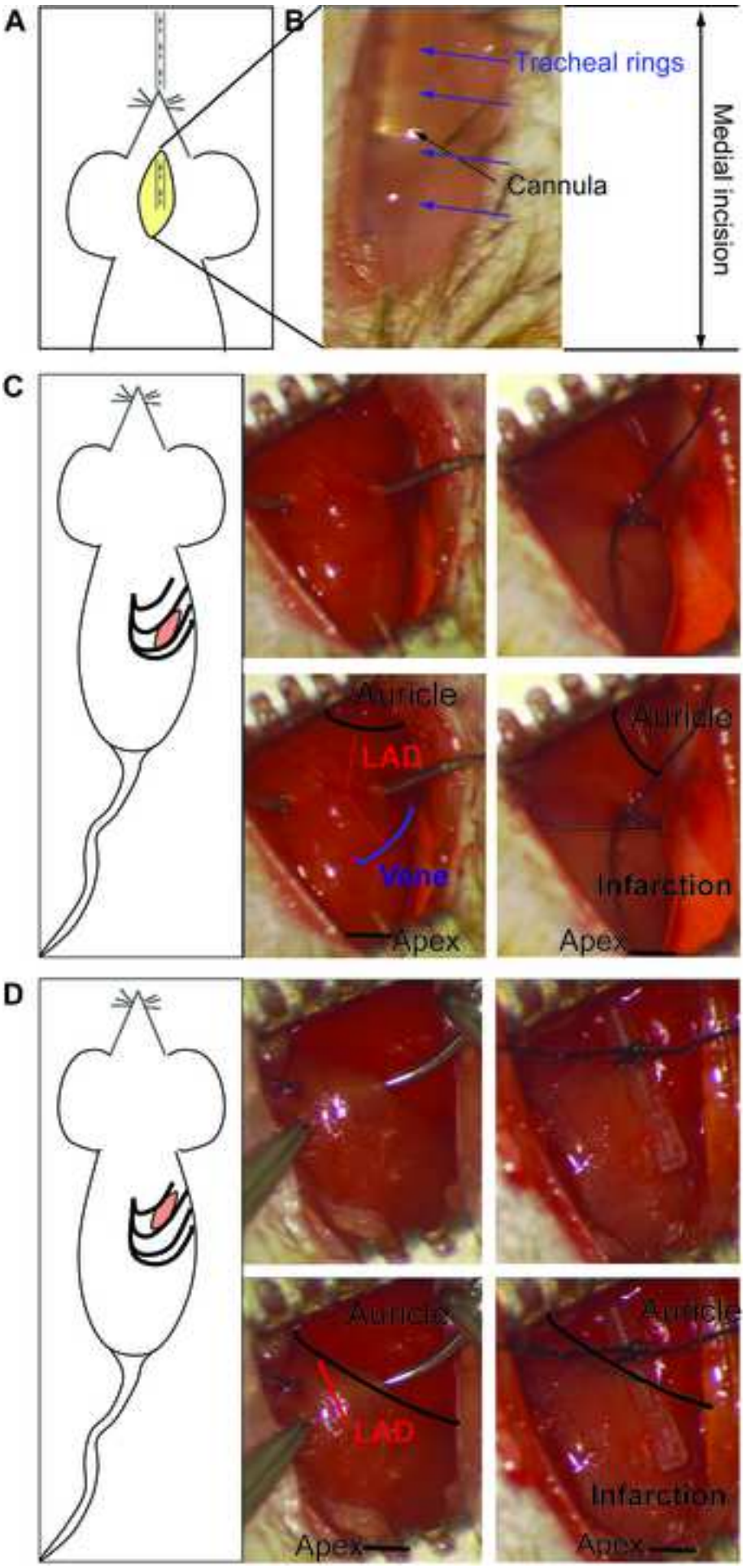

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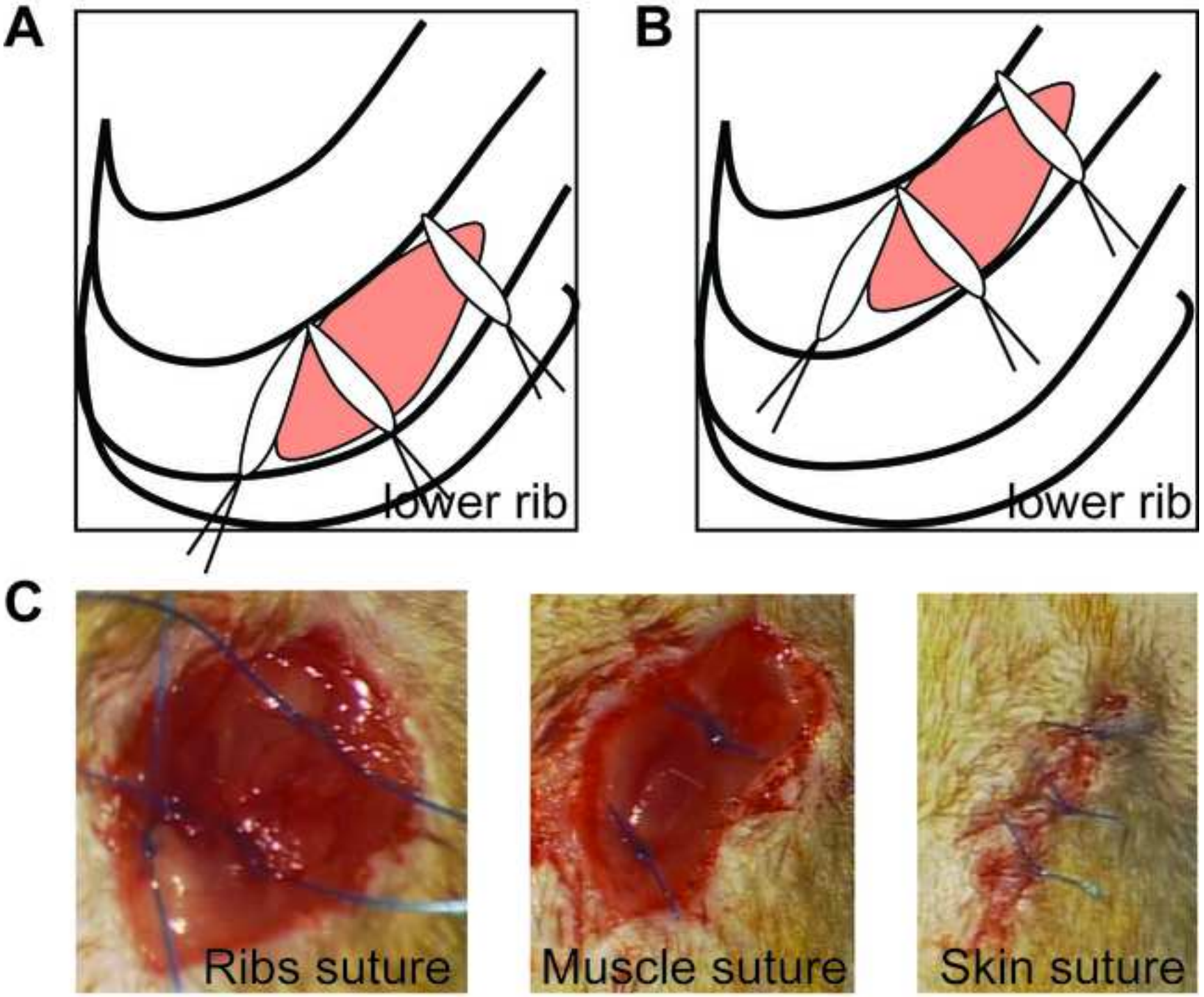

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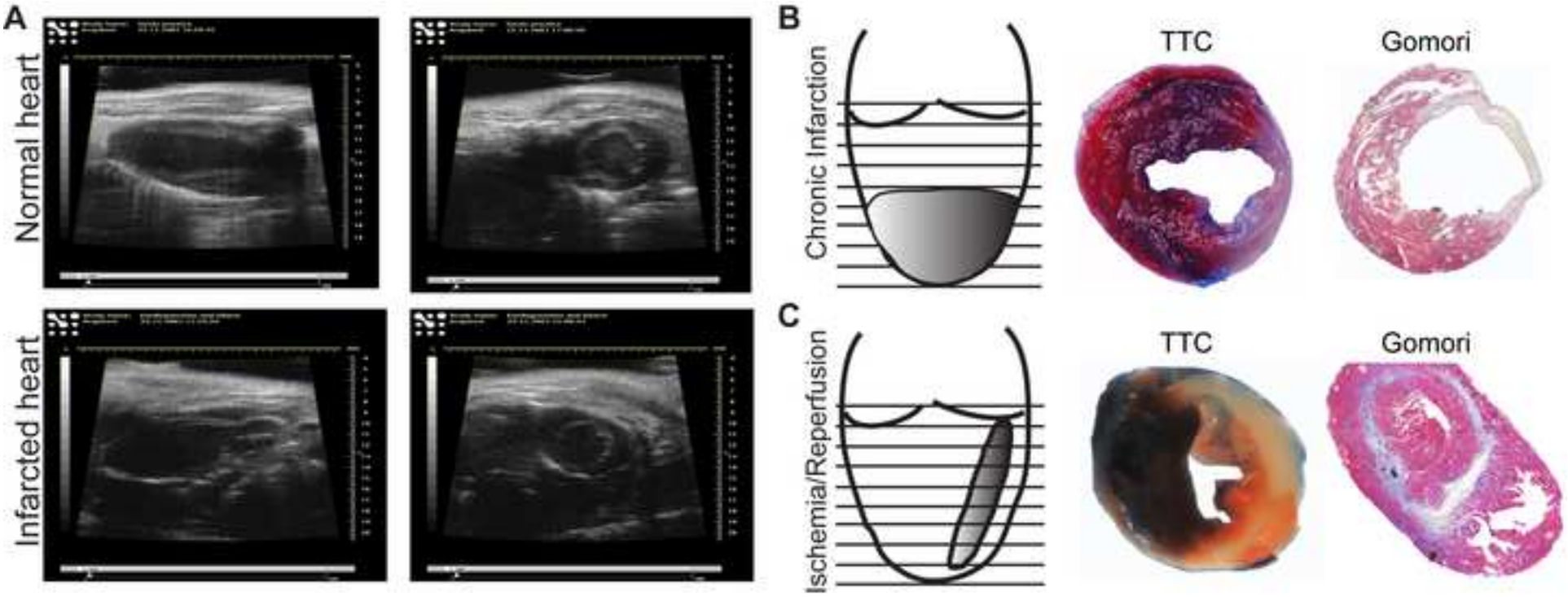

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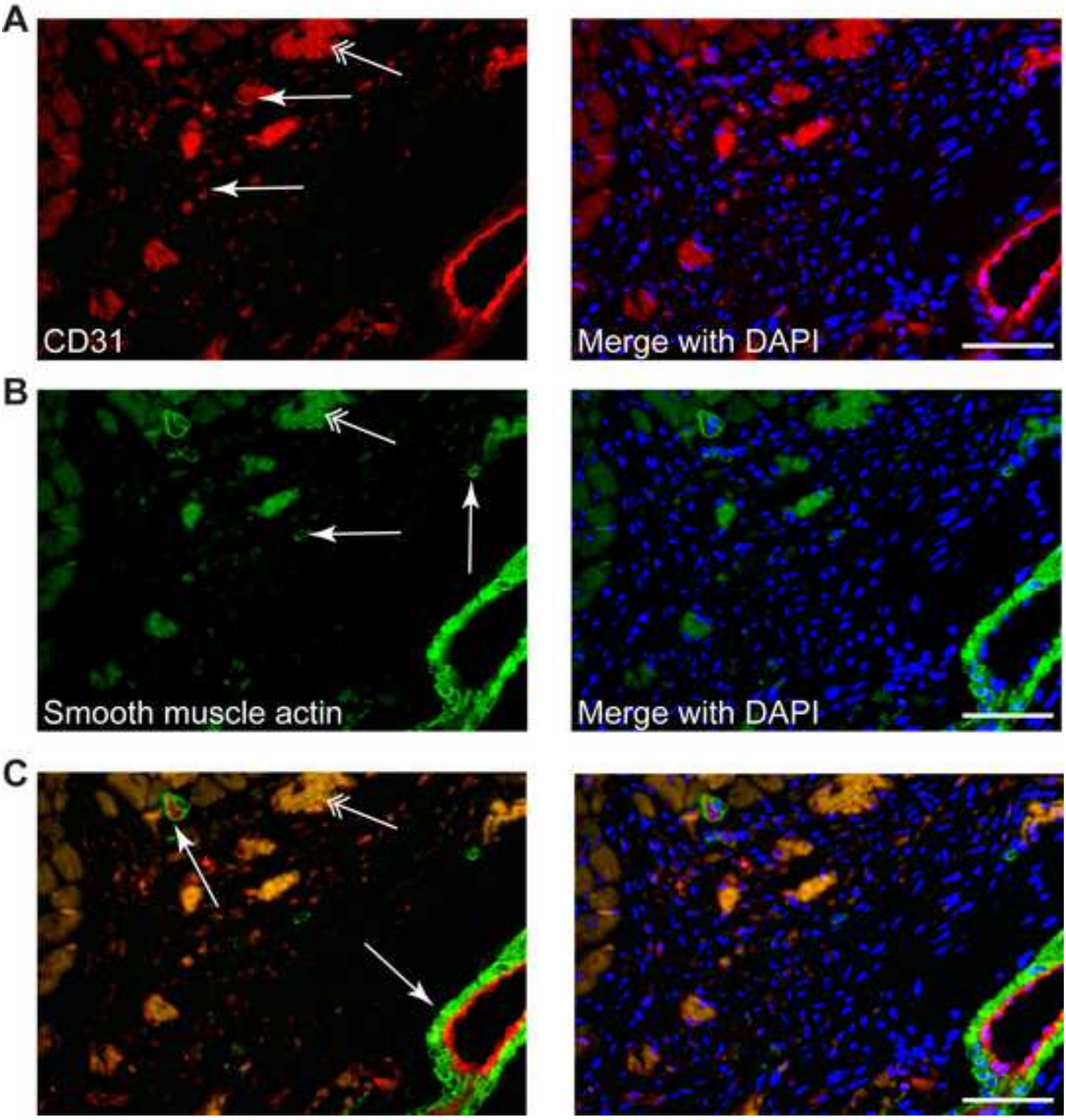

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Stereomicroscope	Olympus	SZ/X9	-
Mouse ventilator	Harvard Apparatus	730043	Model Minient 845
Dual Anesthesia System (Tabletop Version)	Harvard Apparatus	-	Self-contained isoflurane-based anesthesia unit for use on lab tables, with a compact 8" x 11" footprint.
Intubation cannula	Harvard Apparatus	732737	-
Forceps	FST, Germany	91197-00	standard tip curved 0.17 mm x 0.1 mm
Scissors	FST, Germany	91460-11	straight
Vannas scissor	Aesculap, Germany	OC 498 R	-
Retractors	FST, Germany	18200-10	2.5mm wide
Retractors	FST, Germany	18200-11	5mm wide
Wire handles	FST, Germany	18200-05	10cm
Wire handles	FST, Germany	18200-06	14cm
Ketamine 10%	CEVA, Germany	-	-
Xylazine 2%	Medistar, Germany	-	-

Bepanthene eye and nose cream	Bayer, Germany	-	-
Silicon tube	IFK Isofluor, Germany	custom-made product	diameter 500µm
			section thickness 100 µm
			polytetrafluorethylene catheter
PROLENE Suture 6/0	ETHICON	8707H	polypropylene monofilament suture, unresorbable, needle CC-1, 13mm, 3/8 Circle
7/0 Silk	Seraflex	IC 1005171Z	-
Ultrasound	Vevo, Canada	770 Vevo	-

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Author(s): Curaj A, Simsekyilmaz S, Staudt M, Liehn EA

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Article Title:	Minimal invasive method of inducing myocardial infarction in mice
Signature:	
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52197- R3 - 052814
Comments to referees:

Editorial comments:

1) Tense throughout short abstract is inconsistent. 3R should be defined. ("Compared to other methods, this procedure is accessible, assuring a high survival rate, minimal complications, higher reproducibility, shortening the learning curve and reducing the number of animals as demanded by the 3R principle.").
We have modified the abstract according to the Referee #3 and defined the principle of 3R.

2) The wording in the Long Abstract is a bit awkward and would benefit from revision.
We have reformulate the long abstract according to the Referee #3.

3) Step 5.1 should be expanded to mention what is measured by echocardiography.
We have mentioned the parameters measured by Echocardiography: "*5.1) Regularly monitor the heart function by means of echocardiography (Figure 3A): the ejection fraction, fractional shortening, cardiac output and heart dimensions"*.

4) In step 5.5, how do you ensure the heart is stopped in diastola?
Stopping the heart in diastola is an established procedure and is used for a long time. Increased extracellular K+ concentration blocks the ionic pumps, decreases the membrane resting potential of cardiac cells, resulting in a diastolic arrest of cardiac activity. We have now completed the 5.5 step by adding the procedure of preparing the saturated KCl solution and discussed this issue in the results.

5) How should the heart be positioned for sectioning? (Step 5.5).
We have now added an information about the positioning the heart: "*Embed the heart tissue in paraffin, by positioning the heart on the tip, to perform transversal sectioning"*.

6) The scale bar for figure 3E should be defined in the figure legend.
The scale bar is now defined in the Figure legend.

7) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
We have proved again the manuscript by a native English speaker.

8) If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.
Our Figures are original and were not published elsewhere.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The article could be significant to scientists and beginners who want to learn thoracotomy and coronary artery ligation particularly in mouse model. The introduction is lengthy with redundant statements. In the methodology sections, the authors fail to provide rationale and background of the procedure. Often the techniques are poorly explained and inadequately detailed especially considered this is a methodology paper. Nor do they discuss limitations and mention alternative approaches.

We apologize for the inconsistence of our manuscript and we thank for the improvement suggestions. We have now eliminated the redundant statements from the introduction and reorganized the discussion, introducing separate paragraphs for the limitations (forth paragraph) and alternative approaches (fifth paragraph).
The methodology sections were completed, as suggested by this and other referees.

The authors do provide some results which could be important to the study. Again, they fail to provide details of how the echo is performed which could be a great learning process and even a manuscript by itself.
As the referee mentioned, the procedure of performing echocardiography is very complex and we intentionally avoid to provide more details, since is not a part of the microsurgery protocol. However, we added information about the parameters measured during the ultrasound analysis (5.1). As result, we exemplified the visualization of the infarction by ultrasound (Figure 3A), and mentioned it in discussion part by the future applications after mastering this technique. We thank the referee for the suggestion to write a manuscript about the measuring the heart function by echocardiography.

They neglect to provide the detail how to fix the heart diastole.
We have provided details about the KCl solution (Step 5.5). Moreover, we have discussed shortly this issue in the results: "*Increased extracellular K+ concentration blocks the ionic pumps, decreases the membrane resting potential of cardiac cells, resulting in a diastolic arrest of cardiac activity.*"

Worst of all, they indicate euthanasia is performed "by opening the thoracic cavity and excising the heart" after the proper anesthesia!
We have now corrected this issue (Methods 5.3).

The photography could yield better resolution despite they can be downloaded. Yet, they still fail to provide detail structures, and none of the images have any labels.
We appreciate this constructive criticism. We have now increased the resolution of the pictures and labeled them, to improve the understanding of the method.

There are quite a few articles related to thoracotomy and LAD. The authors may compare those papers with their methodology.
We have introduced new references about related techniques of thoracotomy and LAD, which are mentioned compared with out method. (Kolk et al, Frobert et al, Xu et al)

Major Concerns:

Line 51. Avoid using colloquial 'don't'.
We have corrected this issue.

Line 103. Are there any province, city or local animal welfare regulations, guidelines, approval, etc. to regulate the animal welfare? Are the mice freshly purchased? Are the animals quarantined (# of days) prior to surgery. Who is the vendor? Please elaborate.
As we mentioned at the beggining of the protocol, the experiments presented in this paper are performed accordingly to the German low and to the european animal care guidelines. The animals are bred in the Animal facility of Institute for Laboratory Animal Science, Aachen, Germany, under supervision of Prof. Dr. R. Tolba and Dr. A. Teubner (animal welfare officer). Depending on the experiments, we use mice available in our institute, but also we can purchese them from outside (wild type, as well as knock-out animals). If they are

purchesed from outside, it is important to permit one week accomodation before undergo the surgical procedure. This aspect is now mentioned in Step 1.1

Line 116. Please specify details of eye cream, brand name, dose, etc.
We have specified that we have used Bepanthen cream from Bayer.

Line 125-129. What is "mouse upward"? Is the mouse positioned in head upward and tail downward position. Is the surgical table specially built to accommodate the mouse in such position, and how is the mouse held in such position? Where exactly is the incision, and what are the "two fatty bodies"?
We have now replace the word "*upward*" with "*on the back*". We have also localized the incision in the middle of the neck and we described the 2 fatty bodies in the neck area, under the skin.

Line 131-135. How is the cannula connected to the ventilator?
Cannula will be connected to the ventilator as described by the manufacturer. This explanation is now included in Step 2.6.

Line 143-152. How is the animal positioned, and which side is the surgery performed. Is disinfectant (povidone-iodine) been used prior to incision?
We have added all this information in the revised manuscript: "*Place the anesthetized mouse on the back on the surgery table. Shave the mice using a small razor and disinfect both ventral neck area and left half of the thorax with alcohol prior to incision*".

Line 170. The statements in both procedures are unclear. Please elaborate.
We have now modified the pictures and we added supplementary information (Figure 1,2,3), as well as in Figure legends.

Line 175. Is 0/7 same as 7-0? Are stitches used to tie the ribs together, and the residual air ever removed from the pleural space after the ribs are closed?
We thank very much for this observation. We added now in step 4.1: "*Eliminate the residual air from thorax by filling with warm isotonic salt solution*".

Line 182. Does the animal breath spontaneously once the extubation is completed? Does the mouse need to be identified or tagged?
If the procedure is perform without mistake, the mouse will start to breath spontaneously. The identification of the animal is important and we have now mentioned this in step 4.4: "*For later identification, mark the mouse using the local system (ask the animal welfare officer from your institution)*".

Line 195. What kind of instrument is used for the echo, and how is it done?
As mentioned before, we have added all these information. The instrument used is Vevo770, however, if the resolution is enough, any kind of apparatus can be used (technical details described in material tables).

Line 201. How exactly is the mouse euthanized? Is it "by opening the thoracic cavity and excising the heart" after the anesthesia?
We have now corrected this issue. However, for proper washing, as well as for further functional studies (Langendorff perfusion), the heart needs to be excised during beating. These aspects are mentioned now in step 5.3.

Line 204. Why is the blood collected. Would perfusion fixation a better way to fix the heart diastole?

The cannulation of the heart to perform perfusion fixation is very difficult and require additionally instruments. After excision, the heart will beat autonomic for other minutes, enough to wash the blood if immersed in PBS.

Line 215. There are no data in infarction size. collagen content, etc. though the authors do mention in the methodology section.

We have now extended the results with information about the infarction size, added figures with TTC staining and Gomori in both models, as well as immunofluorescence (Figure 4).

Reviewer #2:

This manuscript describes an improved method of inducing myocardial infarction in mice. Several points are mentioned in the method that constitute either an improvement in the effectiveness or the execution of the technique. The methods are described in sufficient detail to allow readers to perform the procedures using the improved techniques.

We thank this referee for the appreciations and for the suggestions to improve the manuscript. I hope we were able to address accordingly all remaining issues.

The major problem with the manuscript is that the English used has many errors or sound awkward in many places. While efforts were made initially to point out each of the error/awkward passages, it was clear that there were simply too many throughout the manuscript to make this effort feasible. Therefore, it is not only recommended but imperative that the authors copyedit the entire manuscript with the help of someone fluent in the English language.

We have now proved again the manuscript by a native English speaker.

Generally speaking, the introduction and protocol are contain all the necessary components and written with sufficient detail. The discussion, however, contains repetitive information and is not very well organized. It is recommended that the authors clearly lay out in the discussion the main advantages of their method compared to the conventional method and list the advantages of each of the techniques that is unique to their method.

We reorganized and modified the discussion, as requested. We pointed out the critical steps and main disadvantage (first 3 paragraphs), discussing the modifications and troubleshooting, as well as the limitations (forth paragraph), alternative approaches (fifth paragraph), and future applications (sixth paragraph).

Editors Note:
The "Discussion" section should covers the following points running between 3 – 6 paragraphs.
a.Critical steps within the protocol.
b.Modifications and troubleshooting.
c.Limitations of the technique.
d.Significance of the technique with respect to existing/alternative methods.
e.Future applications or directions after mastering this technique.

Reviewer #3:

General comments
Curaj et al described a minimal invasive and simple model of myocardial infarction in mice that is of interest for readers of JoVE. In the study, an in vivo procedure is introduced that is feasible and clinically important to be reproducible under laboratory conditions. Although the manuscript is well organized, the text is sometimes hard to read and to follow, due to the complicated style, numerous errors and over-statements throughout the manuscript. The conclusions are mainly supported by the experimental/descriptive, but are somewhat repetitive between results and introduction sections.

We thank particularly this referee for the recognition of our method and for the detailed suggestions to improve the manuscript. We have also edited the manuscript for the English grammar and style, as suggested.

Specific comments
1. Section 3.4. "For chronic infarction model …"
Section 5.7 "… Gomori's one-step staining…."
Section 3.5. "For ischemia/reperfusion model …" . There is no explanation about the time of reperfusion injury, but if it would be acute (Figure 3B) a description of TTC staining is required in the protocol. If it would be a chronic reperfusion (Figure 3C), one has to establish the optimal point to perform Gomori's staining.
We thank for the suggestions, we have made the mentioned corrections and we have added a step about the TTC staining as suggested (5.6). We also included and discussed this staining in the results.

2. The journal is focued on visualized experiments: here, it is necessary to show TTC staining after the acute myocardial infarction and Gomori´s staining after chronic myocardial infarction showing a control group (without infarction) compared to the ischemia/reperfusion injury in pictures, in addition to the schemes shown in the Figure 3 B,C,D.
We added now the information in the results and Figures (new Figure 3) in both cases: chronic infarction (Figure 3B) and ischemia/reperfusion (Figure 3C).

3. Figure 3E. The figure should be improved, including a separate panel for CD31+ DAPI; SMA+ DAPI and auto-florescence of erythrocytes + DAPI.
We have now included a separate Figure (Figure 4) for the indicated staining.

4. Recommended text changes for short abstract:
Text:
This report describes a pathogenesis model of myocardial infarction model in mice with minimal invasive manipulations. Compared to other methods, this procedure is easy to establish and to control, assuring a high reproducibility and survival rate for animals with minimal complications. Also, the procedure helps to reduce the number of required animals as requested by the 3R principle (1).
5. Recommended text changes for long abstract: Both long abstracts should be identical (front page and within the manuscript).
Text:
Despite the considerable progress made in the stent development in the last decades, myocardial infarction remains the main cause of death in western countries. Human studies offer only limited information and do not permit a deeper understanding of molecular mechanisms, impairing the development of future therapeutic strategies. Since the insights into pathomechanisms of cardiovascular diseases under in vivo conditions need to be increased to combat this worldwide health problem, unfortunately, there is no alternative way to establish and refine animal models that will reflect the pathology in humans as close as possible. The present report presents a minimally invasive and simple model of myocardial infarction in mice, which assures a high reproducibility and survival rate of animals with minimal complications. Also, it fulfils the requirements of the 3R principle (Replacement, Refinement and Reduction) for animal experimentation.
We very much appreciated the suggestions and we changed now the short and long abstracts accordingly.

6. The numbering of manuscript pages is missing.
We have now introduced the page numbering (left, bottom).

7. The English style throughout the text needs improvement and checking by a native English speaker.
We have now edited for the English grammar and style as requested.

Reviewer #4:

Manuscript Summary:

The authors describe a very important and complex technique that is essential to develop new therapies for ischemic heart disease, the single leading cause of death in the industrialized world.
We thank very much this referee for the appreciation of our manuscript. We have now addressed all remaining concerns.

Major Concerns:
None
Minor Concerns:
Line 207: "saturated KCl solution", please provide details how to prepare this solution
Line 242: please change "CD31can" to "CD31 can"
We provided now details about the preparation of saturated KCl solution in Step 5.5 and corrected the remaining issues.