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January 24, 2020

Ronald Myers, PhD
Senior Science Editor
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Dear Dr. Myers,

We submit a revised original research manuscript entitled" 'Delivery of Modified mRNA in a Myocardial Infarction Mouse Model' for consideration for publication in The Journal of Visualized Experiments (*Jove*). The data are unpublished and are not under consideration by any other journal. All authors have approved the manuscript for submission.

Myocardial infarction (MI) remains a major cause of morbidity and mortality and is associated with significant societal and economic burden. In order to develop better therapeutics and improve outcomes for patients, researchers are focusing to evaluate the role of gene therapy in the mitigating the heart disease.

Till date, various traditional genes delivery methods have been employed in the clinics in attempt to increase the expression of a specific protein in the heart. Despite the initial success with these integrating vectors, their use in therapeutic medicine has declined due to complications like poor and uncontrolled expression of the delivered genes. In contrast, modified RNA (modRNA) offers gene transfer both dividing and non-dividing mammalian cells that mediate fast, robust, transient expression of proteins in the targeted cells or tissue and thus has become a promising avenue in ischemic heart disease research.

Thus, in this protocol we present a standardized and optimized method of preparation and delivery of modRNA intracardiac injection in mouse MI model to maximize modRNA delivery efficiency. With this paper, we attempt to make modRNA-based cardiac delivery accessible and consistent for *in vivo* applications.

The initial reviews were positive but pointed out few shortcomings in the manuscript. In the revision, we have comprehensively addressed all these issues that substantially strengthen the manuscript.

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We hope you will find our study acceptable for review and publication in *Jove*.

Please do not hesitate to contact me with any questions you might have.

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1   **TITLE:**
2   **Delivery of Modified mRNA in a Myocardial Infarction Mouse Model**
3

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24   **KEYWORDS:**
25   modified RNA, gene therapy, myocardial infarction, cardiovascular regeneration, cardiac
26   protection, cardiac regeneration
27

28   **SUMMARY:**
29   This protocol presents a simple and coherent way to transiently upregulate a gene of interest
30   using modRNA after myocardial infarction in mice.
31
32   **ABSTRACT:**
33   Myocardial infarction (MI) is a leading cause of morbidity and mortality in the Western world. In
34   the past decade, gene therapy has become a promising treatment option for heart disease, owing
35   to its efficiency and exceptional therapeutic effects. In an effort to repair the damaged tissue
36   post-MI, various studies have employed DNA-based or viral gene therapy but have
37   faced considerable hurdles due to the poor and uncontrolled expression of the delivered genes,
38   edema, arrhythmia, and cardiac hypertrophy. Synthetic modified mRNA (modRNA) presents a
39   novel gene therapy approach that offers high, transient, safe, nonimmunogenic, and controlled
40   mRNA delivery to the heart tissue without any risk of genomic integration. Due to these
41   remarkable characteristics combined with its bell-shaped pharmacokinetics in the
42   heart, modRNA has become an attractive approach for the treatment of heart disease.
43   However, to increase its effectiveness in vivo, a consistent and reliable delivery method needs to
44   be followed.   Hence,   to   maximize modRNA delivery   efficiency   and   yield   consistency

45 in modRNA use for in vivo applications, an optimized method of preparation and delivery
46 of modRNA intracardiac injection in a mouse MI model is presented. This protocol will
47 make modRNA delivery more accessible for basic and translational research.
48
49 **INTRODUCTION:**
50 Gene therapy is a powerful tool involving delivery of nucleic acids for the treatment, cure, or
51 prevention of human diseases. Despite the progress in the diagnostic and therapeutic
52 approaches for heart disease, there has been limited success in the delivery of genes in
53 myocardial infarction (MI) and heart failure (HF). As straightforward as the process of gene
54 therapy seems, it is a markedly complex approach considering the many factors that need to be
55 optimized before employing a particular delivery vehicle. The correct delivery vector should be
56 non-immunogenic, efficient, and stable inside the human body. Efforts in this field have
57 generated two types of delivery systems: viral or non-viral. The widely used viral systems,
58 including gene transfer by adenovirus, retrovirus, lentivirus, or adeno-associated virus, have
59 shown exceptional transduction capacity. However, their use in clinics is limited due to the strong
60 immune response induced[1], risk of tumorigenesis[2], or the presence of neutralizing antibodies[3],
61 all of which remain a major obstacle to broad and effective application of viral vectors in human
62 gene therapy. On the other hand, despite their impressive expression pattern, the delivery of
63 naked plasmid DNA displays a low transfection efficiency, while mRNA transfer presents high
64 immunogenicity and susceptibility to degradation by RNase[4].
65
66 With the extensive research in the field of mRNA, modRNA has become an attractive tool for
67 delivery of genes to the heart and various other organs due to its numerous advantages over
68 traditional vectors[5]. Complete replacement of uridine with naturally occurring pseudouridine
69 results in more robust and transient protein expression, with minimal induction of innate
70 immune response and risk of genomic integration[6]. Recently established protocols use an
71 optimized amount of anti-reverse cap analog (ARCA) that further enhances the protein
72 translation by increasing the stability and transability of the synthetic mRNA[7].
73
74 Previous reports have shown the expression of various reporter or functional genes delivered by
75 modRNA in the rodent myocardium after MI. With modRNA applications, significant areas of the
76 myocardium, including both cardiomyocytes and noncardiomyocytes, have been successfully
77 transfected post-cardiac injury[8] to induce angiogenesis[9,10], cardiac cell survival[11], and
78 cardiomyocyte proliferation[12]. A single administration of modRNA encoded for mutated human
79 follistatin-like 1 induces the proliferation of mouse adult CMs and significantly increases cardiac
80 function, decreases scar size, and increases capillary density 4 weeks post-MI[12]. A more recent
81 study reported improved cardiac function after MI with application of VEGFA modRNA in a swine
82 model[10].
83
84 Thus, with the increased popularity of modRNA in the cardiac field, it is essential to develop and
85 optimize a protocol for the delivery of modRNA to the heart post-MI. Herein is a protocol
86 describing the preparation and delivery of purified and optimized modRNA in a biocompatible
87 citrate-saline formulation that provides robust, stable protein expression without stimulating any
88 immune response. The method shown in this protocol and video demonstrates the standard

89   surgical procedure of a mouse MI by permanent ligation of the left anterior descending artery
90   (LAD), followed by three site intracardiac injections of modRNA. The aim for this paper is to
91   clearly define a highly accurate and reproducible method of modRNA delivery to the murine
92   myocardium to make modRNA application widely accessible for cardiac gene therapy.
93
94   **PROTOCOL:**
95
96   All animal procedures outlined here have been approved by Icahn School of Medicine at Mount
97   Sinai Institutional Care and Use Committee.
98
99   **1. Synthesis of modRNA**
100
101   NOTE: The details of modRNA synthesis can be found in Kondrat et al.[13].
102
103   1.1. Order the plasmid templates (**Table of Materials**) and generate a clean PCR product to use
104   as the mRNA template.
105
106   1.2. Prepare the modRNAs by transcription in vitro with a customized ribonucleoside blend of
107   the following (**Table of Materials**): T7 transcription kit, 6 mM anti-reverse cap analog (ARCA) 30-
108   O-Me-m7G(50) ppp(50)G, 75 mM guanosine triphosphate, 75 mM adenosine triphosphate, 75
109   mM cytidine triphosphate, 100 mM pseudouridine-5-triphosphate, and T7 DNase enzyme.
110
111   1.3. Purify the modRNA made in step 1.2 with a transcription cleanup kit (**Table of Materials**) and
112   treat with antarctic phosphatase. Next, repurify the modRNA with the transcription cleanup kit
113   and quantify it using a spectrophotometer.
114
115   1.4. Precipitate the modRNA with ethanol and ammonium acetate and resuspend in 10 mM Tris-
116   HCl and 1 mM EDTA.
117
118   1.5. Concentrate the modRNA for in vivo delivery by centrifugal filters and measure the quality
119   using an automated electrophoresis platform (**Table of Materials**).
120
121   **2. Preparation of modRNA injection for in vivo delivery**
122
123   2.1. Calculate the volume needed for 100 µg of luciferase (Luc)/Cre modRNA injection based on
124   the modRNA concentration.
125
126   2.2. Mix the calculated volume of modRNA with equal parts of sucrose solution prepared in
127   nuclease free water (0.3 g/mL) and 0.1 M citrate solution (pH = 7) (20 µL each recommended).
128
129   2.3. Bring the final volume of the injection to 60 µL by adding saline solution.
130
131   **3. Myocardial infarction surgery**
132

3.1. Anesthesia and preparation of the animal

NOTE: This study uses male and female CFW mice 8–12 weeks old.

3.1.1. Anesthetize the mouse with 2% isoflurane using an induction chamber and place the animal on its back in a dorsal position with a facemask over its nose and mouth to keep up the anesthesia. Maintain the anesthesia by mechanical ventilation using a respirator equipped with a 7–8 mm airway tube and a filter.

3.1.2. To immobilize the mouse on the surgery platform, secure its limbs with surgical tape. Shave the neck area and the left side of the ribcage and disinfect using 70% ethanol and dry the area with wipes. Check its reflexes, pinching the tail and hind feet to assess the depth of anesthesia.

3.1.3. Continuously monitor and maintain the core body temperature at normothermia (37.5–38 °C).

3.1.4. Maintain an open airway by performing intratracheal intubation. To do this, place the mouse in a ventral position with its mouth facing towards the experimenter and gently pull out the tongue. Adjust the angle of the neck and straighten the intubation path and push in the intubation cannula.

3.1.5. In case the animal is unable to breathe through it, tracheostomy can be performed. Perform a cervical incision along the midline isolating the skin, muscle, and tissue outlining the trachea using a microscope. When the trachea is clearly visible, insert the endotracheal tube into the tissue between two cartridge rings below the glottis by making a small hole and holding the cranial part of the trachea using microsurgical forceps.

3.1.6. Observe the thoracic movement of the mouse to verify that both lungs are receiving oxygen. The respiratory rate (RR) should be approximately 120 breaths per minute, with an inspiratory pressure of 17–18 cm H_2O.

3.2. Left anterior descending (LAD) artery ligation

3.2.1. To perform the LAD ligation, turn the mouse carefully so it is lying on its right side. Lift the skin and perform a left sided thoracotomy between the 3rd and the 4th rib and dissect the tissue and muscle carefully. Use a cautery to prevent bleeding.

3.2.2. Open the thorax carefully and once it is open, find the heart, without touching the lung with any sharp object. Place the retractors into the incision to keep the thoracic cavity open and have a clear view of the heart. Now move the lungs to the edge of the incision and remove the part of the pericardial sac that is covering the heart to access the anterior surface of the heart.

3.2.3. Carefully identify the LAD, which can be seen as a deep light red vessel located between the pulmonary artery and the left auricle. Ligate the LAD proximal with one single suture of a 7-0 silk suture. Place a chest tube (28 G, venal catheter), between the 4th and the 5th rib.

3.2.4. Close the thoracic incision in layers. Use the 6-0 silk running sutures to adapt the ribs and use 5-0 silk running sutures to close the skin. Make sure to leave a proper window for future echocardiographic measurement.

3.2.5. Carefully drain the thorax with warm isotonic solution (9 g of sodium chloride in 1 L of water) using a 2 mL syringe. Place the mouse on its back. If performing tracheostomy, take the endotracheal tube out and using 7-0 silk sutures adapt the tracheal cartridge rings with one single stitch. Place the face mask on the mouse and close the skin using 5-0 silk running sutures.

3.2.6. For the recovery phase, disconnect the intubation cannula from the ventilator and allow spontaneous breathing. Place the animal under a heat lamp until it attains consciousness. Do not leave the animal unattended after surgery until it is fully awake.

3.2.7. Manage pain therapy with buprenophine at 0.1 mg/kg body weight, injected subcutaneously for the next 3 days at 12 h intervals.

4. Cardiac delivery of modRNA

4.1. Deliver a total of 60 µL of the modRNA prepared in step 2.3 intramuscularly (IM) using an insulin syringe (31 G) following an MI.

4.1.1. Inject 20 µL of the modRNA at three different sites of the heart muscle surrounding the infarct area (two on either side of ligation and one in the apex). Perform the injections immediately after the LAD, before closing the chest.

NOTE: Representative images of modRNA injection sites can be seen in the **Figure 1**.

5. Protein expression validation in heart post MI

5.1. Luciferase modRNA expression using a bioluminescence imaging system

NOTE: A total of 100 µg of Luc mRNA prepared in 60 µL of the sucrose citrate buffer is directly injected into the heart of CFW mice after the MI.

5.1.1. At 24 h post MI and modRNA injection, anesthetize the mice with 2% isoflurane using an induction chamber and intraperitoneally inject luciferin (150 mg/g body weight) to validate the Luc signal in vivo.

5.1.2. Image the mice using a bioluminescence imaging system every 2 min until the Luc signal reaches saturation.

219

220 5.1.3. Quantify the collected imaging data with imaging analysis software. Use the mice injected
221 with saline only as a baseline reading for Luc expression and subtract the background signal
222 collected from the saline-injected mice.

223

224 NOTE: Luc signal is expressed in $p/s/cm^2/sr \times 10^6$.

225

226 5.2. Immunostaining for Cre transfection validation

227

228 5.2.1. To validate the transfection with Cre, sacrifice the animals 24 h postinjection using an
229 intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine followed by cervical
230 dislocation. Before making an incision, disinfect the chest and abdomen using a 70% alcohol
231 swab.

232

233 5.2.2. Open the thoracic cavity by making a transverse incision ~1 cm lower than the sternum
234 and move the scissors towards the head, cutting through the rib cage.

235

236 5.2.3. Open the chest and inject 1 mL of sterile PBS in the right ventricular chamber to remove
237 excess blood. Excise the heart immediately and place it in the sterile PBS to wash away the
238 remaining blood.

239

240 5.2.4. Fix the heart in 4% paraformaldehyde (PFA) for 24 h, followed by overnight incubation in
241 30% sucrose solution at 4 °C. The following day embed the hearts in optimal cutting temperature
242 medium and section them at a thickness of 10 μm using a cryostat.

243

244 5.2.5. Stain the sections with primary antibody against cardiac troponin I (cTNI) and label them
245 with a fluorescent secondary antibody. To identify the nucleus, stain with 4',6-diamidino-2-
246 phenylindole (DAPI) for 5 min. Image the slides using a fluorescent microscope.

247

248 **6. Statistical analysis**

249

250 6.1. Plot a bar graph based on the Luc expression in saline and Luc-injected mice and report the
251 values as mean ± SEM. Compare the two groups using an unpaired t-test (****$p < 0.0001$).

252

253 **REPRESENTATIVE RESULTS:**
254 Eight to ten-week-old mice were anesthetized with isoflurane and intubated. After the animal
255 was under anesthesia, the left thoracic region was shaved and sterilized with ethanol, and the
256 heart was exposed for LAD ligation. The left coronary artery was occluded by firmly knotting the
257 suture under the artery (diagram representation **Figure 1A**). After a successful infarction
258 (indicated by the paling of the left ventricular free wall), a direct injection of 100 μg of Luc or Cre
259 modRNA dissolved in sucrose citrate buffer was delivered directly into the myocardium at three
260 different sites (**Figure 1B**) surrounding the injury area using an insulin syringe. The MI procedure
261 with modRNA injections lasted for 30–45 min per animal. The animals showed approximately
262 90% survival rate postprocedure. After the procedure, the chest and the skin were firmly sutured

263 in layers and the animal was removed from ventilation as soon as it started breathing normally.
264

265 After conducting the LAD ligation and subsequent delivery of Luc modRNA injection, we validated
266 the Luc modRNA transfection by checking for Luc protein expression 24 h postinjection using a
267 bioluminescence imaging system (**Figure 2A**). We established in previous publications that
268 although the protein expression can be seen until day 6 posttransfection, the highest transfection
269 efficiency of modRNA is observed at 24 h[8]. Similarly, we successfully detected the Luc signal in
270 the heart treated with Luc modRNA injection (1.76×10^8) compared to the mice injected with
271 sucrose citrate buffer after MI (3.0×10^5) (**Figure 2B**).
272

273 Further, we sought to validate the modRNA expression by checking its translation and
274 biodistribution in a transgenic Rosa26^{mTmG} mouse. This mouse model system expresses the cell
275 membrane-localized tdTomato (mT) fluorescence expression in all body cells/tissues and changes
276 to cell membrane-localized EGFP (mG) fluorescence expression upon Cre recombination. Thus,
277 to observe the expression of the Cre modRNA, 100 mg Cre modRNA was injected directly into the
278 myocardium post-MI in Rosa26^{mTmG} male and female mice, and the animals were sacrificed 24 h
279 postsurgery. Hearts were fixed and processed for immunostaining with cardiomyocyte marker
280 cTNI and nuclear marker DAPI (**Figure 3A**). Successful Cre expression was evident due to the
281 appearance of green colored cells (**Figure 3B**), which were a result of recombination with the Cre
282 modRNA delivered to the mouse, represented by the change of the tdTomato color to EGFP
283 around the Cre injection site (**Figure 3C**).
284

285 **FIGURE LEGENDS:**
286 **Figure 1: LAD ligation and cardiac delivery of modRNA.** (**A**) Schematic diagram showing the area
287 of LAD ligation and three sites of modRNA injection. (**B**) Representative images of the whole
288 mouse heart post a successful MI induced by permanent ligation (**a**) and sites of modRNA
289 injection following the MI. The 100 µg of modRNA dissolved in 60 µL of sucrose citrate buffer was
290 delivered in the border zone area surrounding the infarction, two on either side of the ligation
291 (**b**,**c**) and one in the apex (**d**). Scale bar = 1 cm.
292

293 **Figure 2: Luc expression analysis post modRNA injection.** Sucrose citrate buffer containing 100
294 µg of Luc modRNA was injected directly into myocardium of CFW mice in an open-chest surgery.
295 The bioluminescence imaging system was used to calculate Luc protein expression at 24 hours
296 after injection. (**A**) Comparative bioluminescent images of control mice (transfected with buffer
297 only) vs. mice injected with Luc modRNA. (**B**) Quantification of Luc signal compared with the
298 control mice measured after 24 hours using bioluminescence imager. Error bar represents SEM
299 with p < 0.0001.
300

301 **Figure 3: Validation of Cre expression in vivo.** Representative images of transfected heart cross
302 sections (short-axis view) validating the expression of Cre modRNA in Rosa26^{mTmG} mouse 24
303 hours postinjection. (**A**) The cardiomyocytes immunostained with cTNI (red). (**B**) Green colored
304 cells represent the Cre transfected cells. (**C**) Merged image showing Cre activated cells around
305 the two injections. Blue is the nuclear stain DAPI. Scale bar = 1 cm.
306

DISCUSSION:

Gene therapy has shown tremendous potential to significantly advance the treatment of cardiac disease. However, traditional tools employed in the initial clinical trials for treatment of HF have shown limited success and are associated with severe side effects. Modified RNA presents a nonviral gene delivery that is continuously gaining popularity as a gene transfer tool in the heart. ModRNA requires no nuclear localization of genes for translation, and thus offers an efficient and fast expression of the protein. Further, as the mRNA does not integrate into the genome host, gene delivery by modRNA skips the risk of mutagenesis. Consequently, owing to its advantages over conventional gene delivery vehicles, modRNA has become one of the most attractive platforms for delivery of single gene or gene combinations to the heart. However, to date there is no standardized protocol for the preparation and transfer of the modRNA into the heart postinjury. Thus, the aim here was to establish a standard and optimized protocol for intracardiac delivery of modRNA in rodent heart to improve the use of modRNA as a gene therapy tool and to make it suitable for therapeutic purposes.

In this protocol, modRNA is prepared by replacing uridine with pseudouridine, followed by capping with ARCA at the 5' end. These changes in the secondary structure of mRNA have been shown to render higher protein translation compared to various other nucleotide modifications[8]. Moreover, this altered mRNA structure escapes the immunogenicity after IM injections in mice by limiting its recognition by toll-like receptors and nucleases[6]. Here, we showed that naked mRNA delivery with sucrose citrate buffer produces strong protein translation in the heart using a mouse MI model. In our previous studies, we established the superiority of modRNA delivered with sucrose citrate buffer in the heart in comparison to encapsulation of modRNA in nanoparticles, such as in vivo fectamine or in vivo jetPEI, which may hinder modRNA translation into protein[8]. The high preservation of the RNA by citrate and extra energy provided by the sucrose for endocytosis in addition of rescuing the single-stranded modRNA clumping could be the reason for the marked increase in translation of modRNA delivered in sucrose citrate buffer.

Although the use of modRNA has escalated in preclinical and clinical studies[10,14] in the past decade, certain areas need to be investigated for improved success of modRNA in the clinic. First, synthesizing modRNA in the high quantities required for therapeutic investigations can be cost-prohibitive. Thus, it is essential to develop clinical-grade and cost-effective modRNA to move the field toward a clinical RNA therapy phase in humans. Second, a clinically applicable delivery method for modRNA needs to be identified. Considering the damage caused by the cardiac IM injections, less invasive catheter-based delivery methods might be more plausible in transferring the large amount of modRNA necessary to transfect large hearts.

In conclusion, this work demonstrated the successful delivery of RNA containing pseudouridine modifications in the mouse heart post MI. The delivery of modRNA in sucrose citrate buffer yielded a strong protein expression 24 hours after the injection. This protocol enables the researchers to follow a standardized delivery and protein evaluation in the heart post injury and thus provide more accessibility in the preparation and delivery of modRNA for their research.
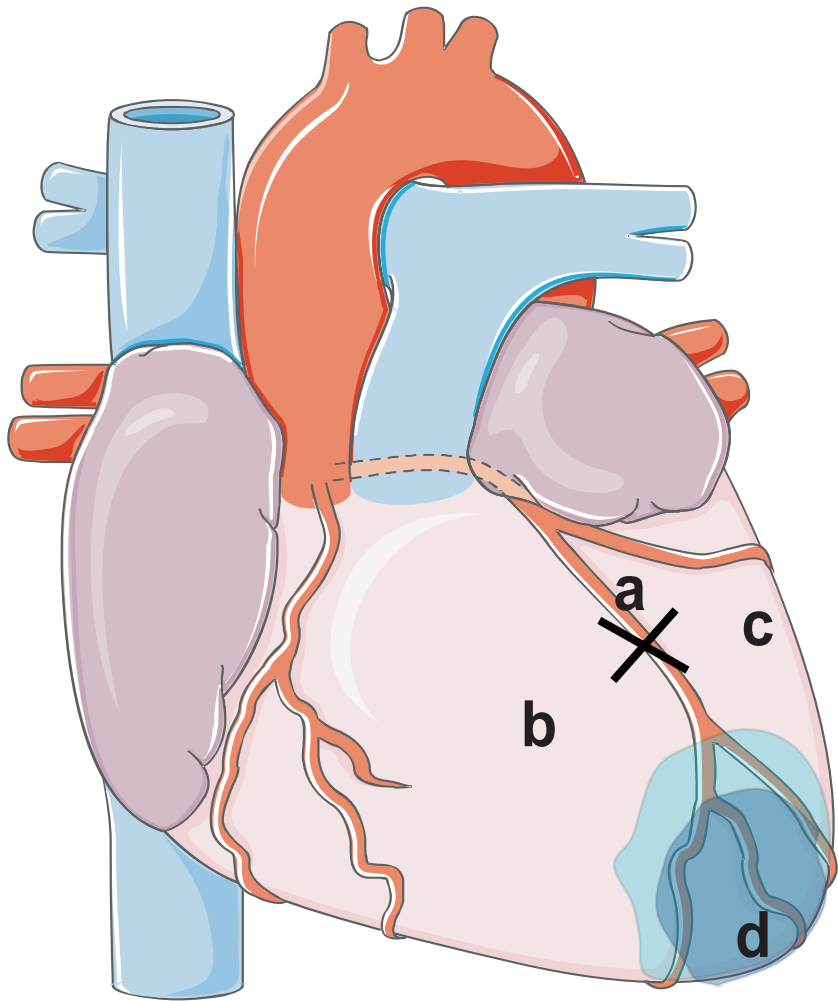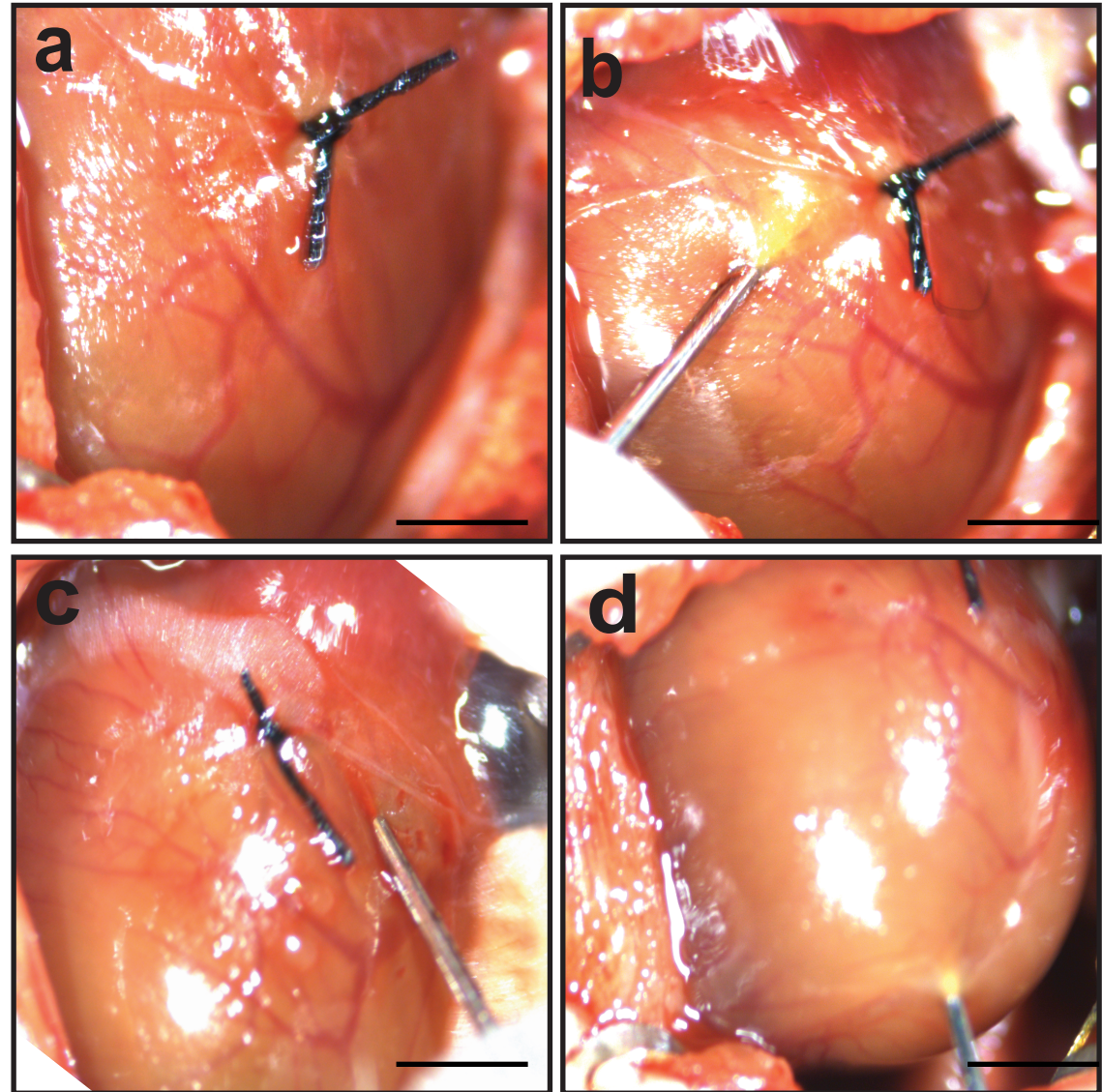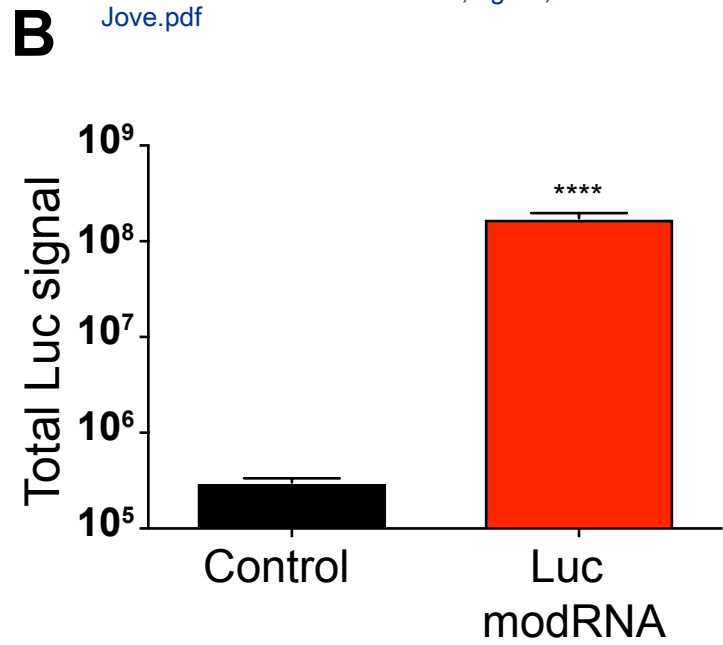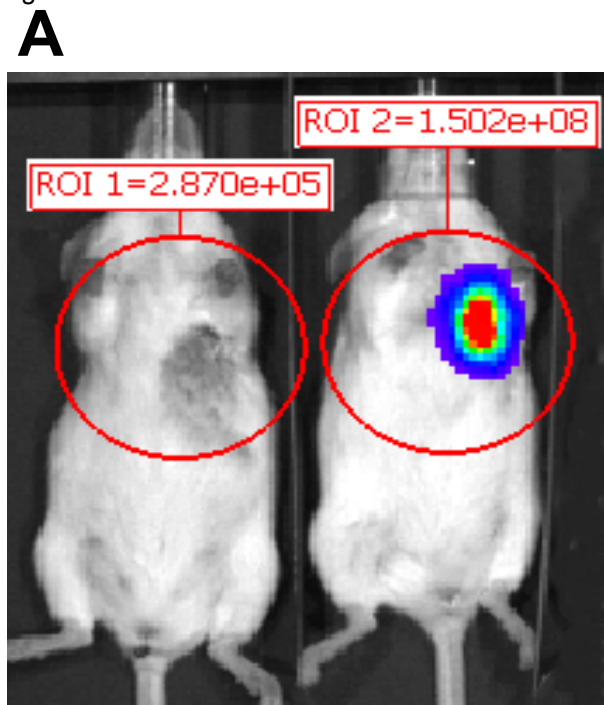
DISCLOSURES:

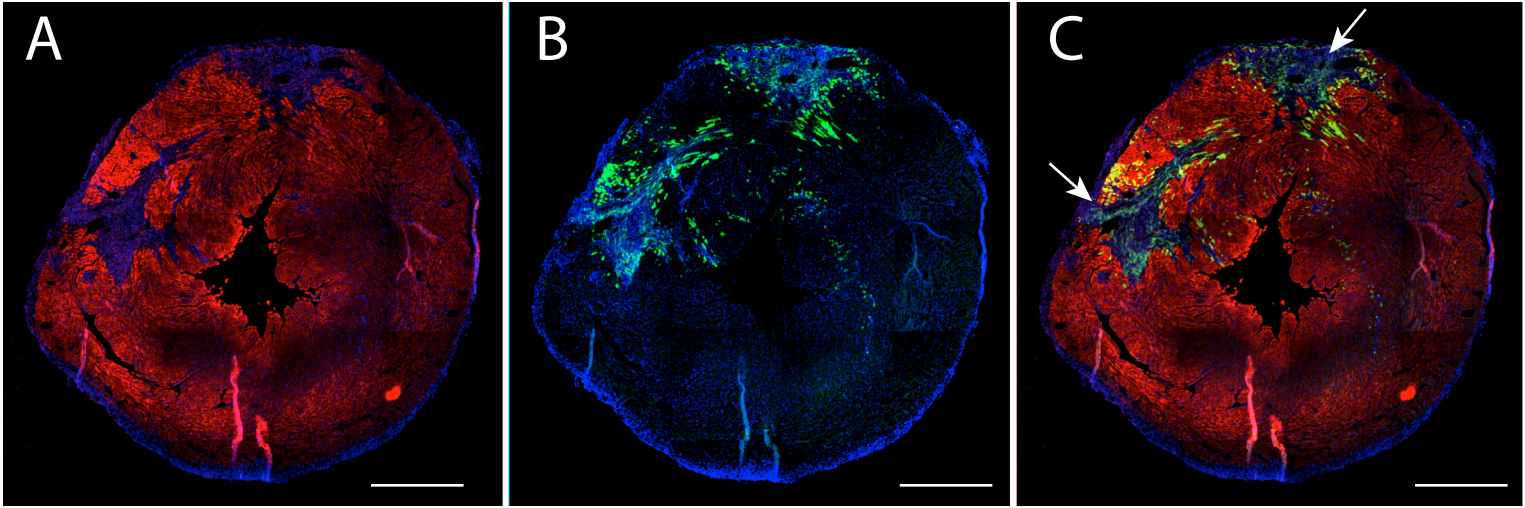The authors have nothing to disclose.

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Figure 1

Figure 2

A

B

ROI 2=1.502e+08

ROI 1=2.870e+05

****

Total Luc signal

10^9
10^8
10^7
10^6
10^5

Control

Luc modRNA

Figure 3

DAPI / **Troponin I** / **Cre activated cells**

Name of Material/Equipment	Company	Catalog Number
Adenosine triphosphate	Invitrogen	AMB13345
Antarctic Phosphatase	New England Biolabs	M0289L
Anti-reverse cap analog, 30-O-Mem7G(50) ppp(50)G	TriLink Biotechnologies	N-7003
Bioluminescense imaging system	Perkin Elmer	124262
Blunt retractors	FST	18200-09
Cardiac tropnin I	Abcam	47003
Cytidine triphosphate	Invitrogen	AMB13345
Dual Anesthesia System	Harvard Apparatus	75-2001
Forceps- Adson	FST	91106-12
Forceps- Dumont #7	FST	91197-00
Guanosine triphosphate	Invitrogen	AMB13345
In vitro transcription kit	Invitrogen	AMB13345
Intubation cannula	Harvard Apparatus	
Megaclear kit	Life Technologies	
Mouse ventilator	Harvard Apparatus	73-4279
N1-methylpseudouridine-5-triphosphate	TriLink Biotechnologies	N-1081
NanoDrop Spectrometer	Thermo Scientific	
Olsen hegar needle holder with suture scissors	FST	12002-12
Plasmid templates	GeneArt, Thermo Fisher Scientific	
Sharp-Pointed Dissecting Scissors	FST	14200-12
Stereomicroscope	Zeiss	
Sutures	Ethicon	Y433H
Sutures	Ethicon	Y432H
Sutures	Ethicon	7733G
T7 DNase enzyme	Invitrogen	AMB13345
Tape station	Aligent	4200
Transcription clean up kit	Invitrogen	AM1908
Ultra-4 centrifugal filters 10k	Amicon	UFC801096

Comments/Description

Included in Megascript kit

IVIS100 charge-coupled device imaging system

Included in Megascript kit

Included in Megascript kit
5X MEGAscript T7 Kit

5.00
6.00
7.00
Included in Megascript kit

Megaclear

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested. Please also take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done

2. Please rephrase lines 157-160 and 162-163 as much as you can to avoid previously published text.

Done

3. Please address specific comments marked in the attached manuscript.

Done

4. Please include all commercial products (listed in Table 1 and Table 2) in the Table of Materials using the template attached. Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

Done