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Analysis of cardiac chamber development during mouse embryogenesis using whole mount epifluorescence

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Cover Letter

VANDERBILT UNIVERSITY MEDICAL CENTER

Department of Medicine
Division of Cardiovascular Medicine

Young-Jae Nam M.D., Ph.D.
Assistant Professor of Cardiovascular Medicine
Vanderbilt University Medical Center

January 2, 2019

Dear Dr. DSouza,

Enclosed please find our manuscript entitled “Analysis of cardiac chamber development during mouse embryogenesis using whole mount epifluorescence” that we wish to submit for publication in *JoVE.* We believe that this article will provide a useful and simple method for studying heart development.

In this manuscript, we describe a method for visualizing each stage of heart development during mouse embryogenesis using whole mount epifluorescent imaging of MLC-2v-tdTomato mice as an example. This simple method can be widely used with other fluorescent reporter mouse lines without performing tedious histochemical methods or using advanced new technologies which require special equipment.

We tried to address every editorial comments and reviewers’ concerns. However, we were not able to provide scale bars in Figure 2 (adopted from the previous publication), because each picture was manually zoomed using an epifluorescent dissecting microscope. It was impossible to provide scale bars in those manually zoomed pictures retrospectively. All the changes that we made were tracked in red.

We hope you and the reviewers will agree that this paper warrants publication in *JoVE*. Thank you for your consideration.

Sincerely,

Young-Jae Nam, M.D., Ph.D.

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TITLE:

Analysis of Cardiac Chamber Development During Mouse Embryogenesis Using Whole Mount Epifluorescence

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KEYWORDS:

Heart, Embryo, Ventricle, Chamber, Whole mount, and Epifluorescence

SUMMARY:

We present the protocols to examine mouse heart development using whole mount epifluorescent microscopy on mouse embryos dissected from ventricular specific MLC-2v-tdTomato reporter knock-in mice. This method allows us to directly visualize each stage of the ventricular formation during mouse heart development without labor-intensive histochemical methods.

ABSTRACT:

The goal of this protocol is to describe a method for the dissection of mouse embryos and visualization of embryonic mouse ventricular chambers during heart development using ventricular specific fluorescent reporter knock-in mice (MLC-2v-tdTomato mice). Heart development involves a linear heart tube formation, the heart tube looping, and four chamber septation. These complex processes are highly conserved in all vertebrates. The mouse embryonic heart has been widely used for heart developmental studies. However, due to their extremely small size, dissecting mouse embryonic hearts is technically challenging. In addition, visualization of cardiac chamber formation often needs in situ hybridization, beta-galactosidase staining using LacZ reporter mice, or immunostaining of sectioned embryonic hearts. Here, we describe how to dissect mouse embryonic hearts and directly visualize ventricular chamber formation of MLC-2v-tdTomato mice using whole mount epifluorescent microscopy. With this method, it is possible to directly examine heart tube formation and looping, and four chamber formation without further experimental manipulation of mouse embryos. Although the MLC-2v-

tdTomato reporter knock-in mouse line is used in this protocol as an example, this protocol can be applied to other heart-specific fluorescent reporter transgenic mouse lines.

INTRODUCTION:

Chamber formation during heart development is a complex process transitioning through several morphologically distinct embryonic stages[1,2]. The crescent shape of cardiac progenitor population cells forms a linear heart tube and then undergoes elongation and looping to form the spiral shape of the developing heart. After its septation process, the developing heart is transformed into the four-chambered heart. Interruption of any of these processes results in developmental heart defects. Thus, it is important to understand the molecular mechanisms underlying chamber formation during heart development. Despite numerous previous studies on heart development, our understanding of this complex process remains limited.

In situ hybridization, immunohistochemistry, and beta-galactosidase staining using LacZ reporter mice have been widely used to study chamber formation during mouse heart development by labeling cardiac specific or chamber specific structural genes or proteins (e.g., Nppa, Coup-TFII, Irx4, MLC-2a and MLC-2v)[3-10]. However, these experiments using mouse embryos require significant time and expertise, because several different experimental steps have to be performed sequentially[11]. Here, we describe a simple whole mount epifluorescent microscopy method to visualize the developing ventricles using embryos dissected from MLC-2v-tdTomato reporter knock-in mice[12]. The advantage of this method compared to previously used methods is to avoid complex experimental steps which may often create experimental variations. The main purpose of this protocol is to describe how to dissect mouse embryos and developing hearts and to examine each stage of mouse cardiac chamber development without tedious histochemical experiments. This method can be easily applied to assess heart development using various other transgenic mouse lines labeling early cardiac markers (e.g., Mesp1Cre: Rosa26EYFP[13], Isl1Cre: Rosa26EYFP[13], Hcn4H2BGFP[14], Hcn4Cre: Rosa mT/mG[14], Nkx2-5Cre: Rosa mT/mG[14], Hcn4-eGFP[15], Isl1Cre: Rosa mT/mG[14], Nkx2.5Cre: Rosa26tdTomato[15], and TgMef2c-AHF-GFP[16] mice).

PROTOCOL:

All animal procedures were performed with the approval of the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

1. Mouse embryo collection and dissection

1.1) Mate 8-10 week old female MLC-2v-tdTomato$^{+/-}$ mice with 8-10 week old male MLC-2v-tdTomato$^{+/-}$ mice to obtain MLC-2v-tdTomato$^{+/+}$, MLC-2v-tdTomato$^{+/-}$ and MLC-2v-tdTomato$^{-/-}$ embryos.

1.2) Check the dams for vaginal plugs every morning. Noon on the day of vaginal plug detection is considered as E0.5.

NOTE: Vaginal examination for detecting a vaginal plug should be performed in the morning (within 8-24 h after sexual activity), since a vaginal plug can be lost throughout the day.

1.3) Euthanize the pregnant dams at different days post coitum (e.g., E8.5, E10.5, and E12.5) using CO_2 inhalation followed by cervical dislocation.

1.4) Lay the mice supine and spray 70% ethanol on the abdomen of mice to avoid mouse hair contamination during dissection.

1.5) Open the abdominal cavity by incision of both the skin and abdominal wall using sharp surgical scissors and a forcep.

1.6) Locate bilateral uterine horns in the dorsal part of the abdominal cavity.

1.7) Separate the entire uterus by carefully cutting above the oviducts on both sides using sharp surgical scissors and a forcep.

1.8) Place the entire dissected uterus in a 10 cm Petri dish with ice-cold PBS and carefully separate each amniotic sac along the uterine horn using sharp surgical scissors and a forcep.

1.9) Transfer each embryo into individual wells of 6 well plate filled with ice-cold PBS using a transfer pipette.

1.10) Under a dissecting microscope, open up an amniotic sac and expose each embryo by cutting off the umbilical cord using sharp surgical scissors and a forcep in an individual well of a 6 well plate with ice-cold PBS.

1.11) Trim out extra-embryonic tissues as much as possible without damaging the embryo using sharp surgical scissors and a forcep.

NOTE: Whole mount epifluorescent imaging of whole mouse embryo is usually performed before dissecting the developing heart as described below.

1.12) Cut the embryo head using sharp surgical scissors and a forcep and transfer to a 1.5 mL tube with 100 µL of buffer A (25 mM NaOH and 0.2 mM EDTA) for genotyping to correlate with the results of epifluorescent imaging.

1.13) Open the chest of the embryo using fine forceps, remove the heart away from the lungs and vasculature using sharp surgical scissors and forceps, and transfer the dissected embryonic heart into a well of a 12 well plate with PBS using a transfer pipette. All dissection procedures are completed under a dissecting microscope with a fiber optic microscope illuminator.

NOTE: It was technically difficult to dissect out mouse embryonic hearts at E8.0 or E8.5, because of their extremely small size and fragile structure. Early embryonic hearts (i.e. E8.0 and E8.5) can be examined within the whole mount embryo without dissection.

2. Whole-mount epifluorescence imaging

2.1) Place the 12 well plate with mouse embryonic hearts under an epifluorescent dissecting microscope.

2.2) Under an epifluorescent dissecting microscope using fine forceps, position the embryonic heart such that developing ventricles are located close to the examiner.

2.3) Adjust focusing of the image using a 0.63x objective (zoom range between 3.15x and 18.9x) in bright field mode.

2.4) Take bright field exposures and capture multiple images. The images were usually obtained by one second exposure. However, exposure times may vary depending on illumination and camera specificationss and need to be optimized for each setup.

2.5) Turn off a fiber optic microscope illuminator, and set the filter for red fluorescence (Ex545 nm/Em 605 nm) to visualize tdTomato expression.

2.6) Re-adjust focusing of the image if necessary.

2.7) Adjust brightness and contrast, take red fluorescent exposures, and capture multiple images.

NOTE: The following image setting was usually used: 1 s exposure time, 2x gain, 1.0 saturation, and 1.0 gamma correction. The optimal setting needs to be optimized for each experiment. Once the optimal setting is determined, the same setting needs to be used for an entire experiment.

3. Genotyping

3.1) Boil the samples from step 1.12 for 1 h at 100 °C.

3.2) Centrifuge for 2 min at 11,360 x *g*, transfer 20 µL of supernatant into a new 1.5 mL tube with 20 µL of buffer B (40 mM Tris HCl, pH 5.5) and mix them.

3.3) Take 4.5 µL of the mixed supernatant from step 3.2 as a DNA template, combine it with 0.5 µL of each of the specific forward and reverse primers (10 µM), 10 µL of pre-mixed polymerase and reaction buffer (2x) (see **Table of Materials**), and then add water to a total volume of 20 µL. Primer sequences are as below.
F1: 5’-TACCCACGGAGAAGAGAAGGACT-3’
R1: 5’-TGGACTTCTTGGAACTGACTCTGT-3’
F2: 5’-ACGGCACGCTGATCTACAAGGT-3’

R2: 5'-TTTGCGCACAGCCCTGGGAT-3'

3.4) Run a polymerase chain reaction (PCR) with the following PCR program (**Table 1** and **Table 2**).

3.5) Run PCR samples and DNA ladder on a 1% agarose gel at 140 V in 1x TAE (Tris-acetate-EDTA) buffer (40 mM Tris-acetate and 1 mM EDTA) for 25 min. Use a 100 bp DNA ladder to estimate the size of the PCR bands.

3.6) Place the DNA gel on a UV transilluminator to identify the DNA bands and turn on the UV light.

REPRESENTATIVE RESULTS:

During heart development, MLC-2v is considered to be the earliest marker for ventricular chamber specification[17]. As depicted in **Figure 1**, we dissected out mouse whole embryos and embryonic hearts from MLC-2v-tdTomato reporter knock-in mice and examined MLC-2v-tdTomato reporter expression during heart development. In MLC-2v-tdTomato reporter knock-in mice, constitutive tdTomato expression in the developing heart is visualized via epifluorescence whole mount imaging as early as at E8.0[12] (**Figure 2**). Relatively weak expression of tdTomato in the linear heart tube at E8.0 becomes stronger at E8.5. At E10.5, MLC-2v-tdTomato reporter expression was demonstrated in the ventricular portion of the dissected looped heart from a whole mouse embryo, while it was not shown in the inflow tract, the outflow tract or the future atria. At E12.5-E13.5, whole mount epifluorescent imaging of the dissected heart of MLC-2v-tdTomato knock-in reporter mouse embryo showed that the tdTomato reporter is exclusively expressed in the ventricles of the four-chambered heart. The similar ventricular specific expression pattern of MLC-2v-tdTomato reporter was shown in the dissected mouse embryo at E16.5. Using this method, we can easily track down ventricular chamber formation during mouse heart development.

After whole mount epifluorescent imaging of mouse embryos or dissected developing hearts, we retrospectively confirmed the genotype of the embryo using the head of mouse embryos. Using two sets of primers as illustrated in **Figure 3A**, we performed PCR genotyping. The embryos carrying the wild type allele showed a 383 bp PCR product using the F1 and R1 primer set. The embryos carrying the tdTomato knock-in allele showed the 497 bp PCR product using the F2 and R2 primer set (**Figure 3B**). Heterozygous embryos were defined by demonstrating both the 383 bp and the 487 bp bands, while the wild type or homozygous genotype was determined by demonstrating a single 383 bp or 497 bp band, respectively.

FIGURE LEGENDS

Figure 1. Outline of stepwise experimental procedure for whole mount epifluorescent imaging of the embryonic MLC-2v-tdTomato reporter mouse hearts.

Figure 2. Representative epifluorescent images of whole embryos and developing hearts dissected from MLC-2v-tdTomato reporter knock-in mice. Epifluorescent imaging of whole mount embryos and dissected hearts at different embryonic stages demonstrates specific expression of tdTomato in the ventricles of developing hearts. (**A**) Whole embryo at E8.0, (**B**) Whole embryo at E8.5, (**C**) Whole embryo at E9.0, (**D**) Whole embryo at E10.5, (**E**) Whole embryo at E13.5, (**F**) Whole embryo at E16.5, **(G**) Embryonic heart at E9.0, (**H**) Embryonic heart at E10.5, (**I**) Embryonic heart at E13.5, and (J) Embryonic heart at E16.5. A, atrium; V, ventricle; IFT, inflow tract; OFT, outflow tract. Scale bar (**A–F**) = 1 mm; Scale bar (**G-J**) = 500 µm. This figure has been modified from reference #12 with permission.

Figure 3. Genotyping of MLC-2v-tdTomato reporter knock-in embryos. (**A**) Illustration of genotyping primer design. (**B**) Representative genotyping results using F1 and R1 primers (left) and F2 and R2 primers (right). +/+: homozygous, +/-: heterozygous, -/-: wild type. Exon: a segment of a gene that contains information required for protein synthesis; IRES: Internal ribosome entry site; FRT: flippase recombinase-recombination target.

Table 1: PCR program using F1 and R1 primers

Table 2: PCR program using F2 and R2 primers

DISCUSSION

The method described here is relatively simple to examine ventricular chamber development, without performing labor-intensive experiments to label ventricular or cardiac-specific structural genes or proteins. Thus, this method minimizes technical variabilities that were often found in immunostained heart sections.

There are two critical steps for successfully performing this method including precise estimation of the embryonic age of mice and dissection of embryonic hearts. We practically estimate the embryonic age of the mice by identifying vaginal plugs in female mice. However, the presence of a vaginal plug does not necessarily indicate pregnancy. It only indicates that sexual intercourse occurred within an approximate 8 to 24 h period. Even if vaginal plugs are found, it is often difficult to determine whether mice are indeed pregnant or not until 10-11 days post coitum by examining the abdomen of the female mice. Breeding multiple pairs of male and female mice is a safe breeding strategy to obtain the expected age of the mouse embryos. Isolating developing mouse hearts without significant damages to their structures is critical to accurately examine cardiac chamber development during mouse embryogenesis as illustrated in **Figure 1**. Careful dissection under a dissecting microscope and understanding developmental cardiac anatomy at each embryonic stage before dissection are necessary for successfully performing this protocol.

Since MLC-2v-tdTomato reporter expression is first observed at E8.0, it is not possible to examine the earlier embryonic heart tube or cardiac crescent stage[12]. The various reporter mouse lines labeling earlier cardiac markers (e.g. Mesp1Cre: Rosa26EYFP[13], Isl1Cre: Rosa26EYFP[13], Hcn4H2BGFP[14], Hcn4Cre: Rosa mT/mG[14], Nkx2-5Cre: Rosa mT/mG[14], Hcn4-eGFP[15], Isl1Cre: Rosa

mT/mG[14], and Nkx2.5Cre: Rosa26tdTomato[15], TgMef2c-AHF-GFP[16] mice) can be used to examine earlier stages of chamber development using the method described in this article.

ACKNOWLEDGEMENT

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DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1 Evans, S. M., Yelon, D., Conlon, F. L., Kirby, M. L. Myocardial lineage development. *Circulation Research.* **107** (12), 1428-1444 (2010).

2 Paige, S. L., Plonowska, K., Xu, A., Wu, S. M. Molecular regulation of cardiomyocyte differentiation. *Circulation Research.* **116** (2), 341-353 (2015).

3 Lee, K. J. et al. Myosin light chain-2 luciferase transgenic mice reveal distinct regulatory programs for cardiac and skeletal muscle-specific expression of a single contractile protein gene. *Journal of Biological Chemistry.* **267** (22), 15875-15885 (1992).

4 Chen, J. et al. Selective requirement of myosin light chain 2v in embryonic heart function. *Journal of Biological Chemistry.* **273** (2), 1252-1256 (1998).

5 Ross, R. S., Navankasattusas, S., Harvey, R. P., Chien, K. R. An HF-1a/HF-1b/MEF-2 combinatorial element confers cardiac ventricular specificity and established an anterior-posterior gradient of expression. *Development.* **122** (6), 1799-1809 (1996).

6 Christoffels, V. M. et al. Chamber formation and morphogenesis in the developing mammalian heart. *Dev Biol.* **223** (2), 266-278 (2000).

7 Wu, S. P. et al. Atrial identity is determined by a COUP-TFII regulatory network. *Developmental Cell.* **25** (4), 417-426 (2013).

8 Nelson, D. O., Jin, D. X., Downs, K. M., Kamp, T. J., Lyons, G. E. Irx4 identifies a chamber-specific cell population that contributes to ventricular myocardium development. *Developmental Dynamics.* **243** (3), 381-392 (2014).

9 Bao, Z. Z., Bruneau, B. G., Seidman, J. G., Seidman, C. E., Cepko, C. L. Regulation of chamber-specific gene expression in the developing heart by Irx4. *Science.* **283** (5405), 1161-1164 (1999).

10 Kubalak, S. W., Miller-Hance, W. C., O'Brien, T. X., Dyson, E., Chien, K. R. Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis. *Journal of Biological Chemistry.* **269** (24), 16961-16970 (1994).

11 Bardot, P. et al. The TAF10-containing TFIID and SAGA transcriptional complexes are dispensable for early somitogenesis in the mouse embryo. *Development.* **144** (20), 3808-3818 (2017).

12 Zhang, Z., Nam, Y. J. Generation of MLC-2v-tdTomato knock-in reporter mouse line. *Genesis.* 10.1002/dvg.23256 (2018).

13 Ieda, M. et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell.* **142** (3), 375-386 (2010).

14 Liang, X. et al. HCN4 dynamically marks the first heart field and conduction system precursors. *Circulation Research.* **113** (4), 399-407 (2013).
15 Nam, Y. J. et al. Induction of diverse cardiac cell types by reprogramming fibroblasts with cardiac transcription factors. *Development.* **141** (22), 4267-4278 (2014).
16 Spater, D. et al. A HCN4+ cardiomyogenic progenitor derived from the first heart field and human pluripotent stem cells. *Nature Cell Biology.* **15** (9), 1098-1106 (2013).
17 O'Brien, T. X., Lee, K. J., Chien, K. R. Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proceedings of the National Academy of Sciences of the United States of America.* **90** (11), 5157-5161 (1993).

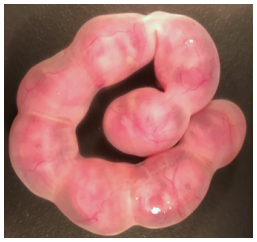
Figure 1

Time mate 8-10 week old male and female mice

Check vaginal plugs

Euthanize time pregnant female mice

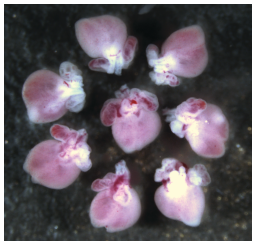
Dissect mouse uterus



Dissect mouse embryos

Epifluorescent whole mount imaging of mouse embryos

Dissect mouse embryonic hearts



Epifluorescent whole mount imaging of dissected embryonic hearts

Genotyping

Figure 2

Click here to access/download;Figure;Figure 2_rev.ai

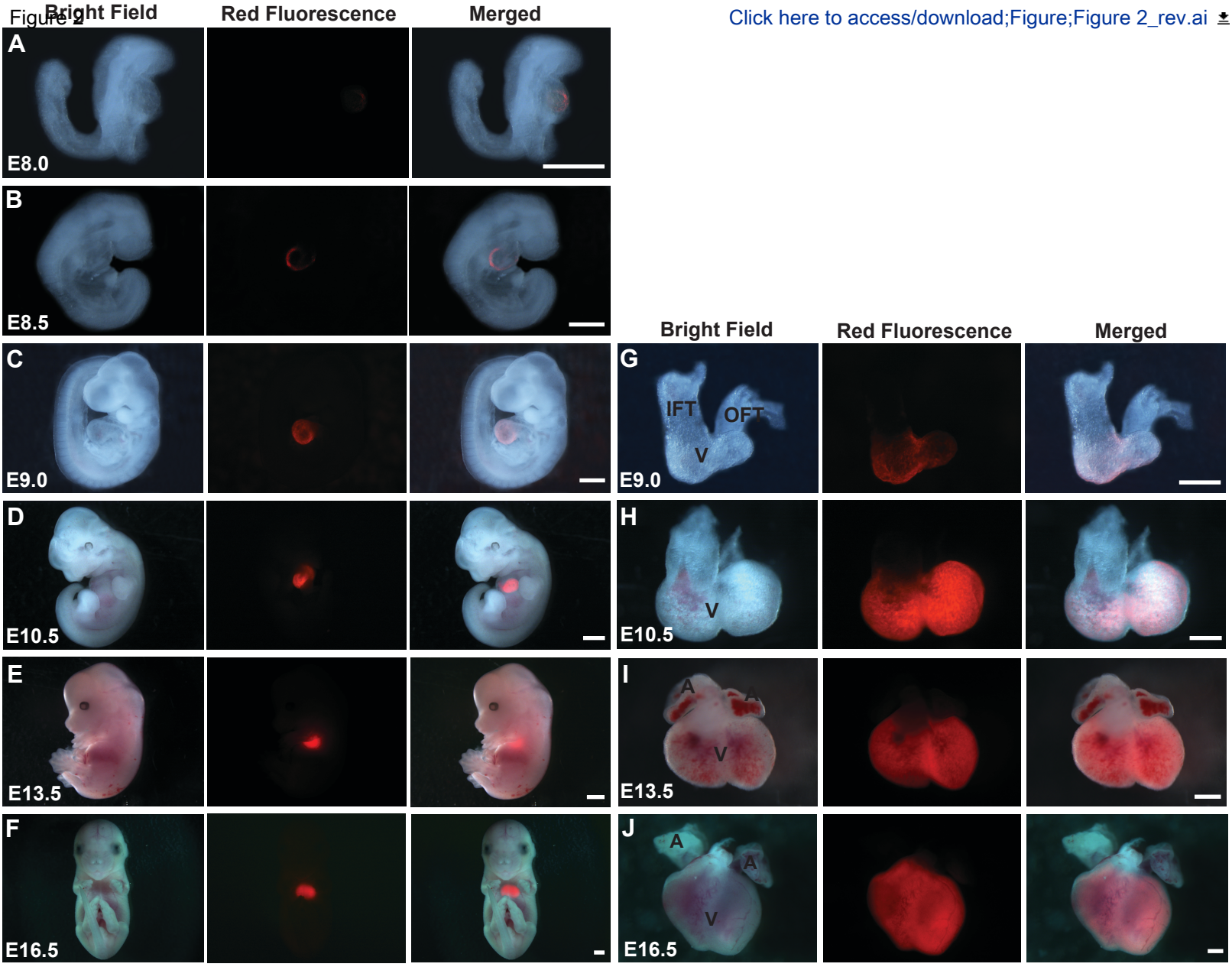


Figure 3

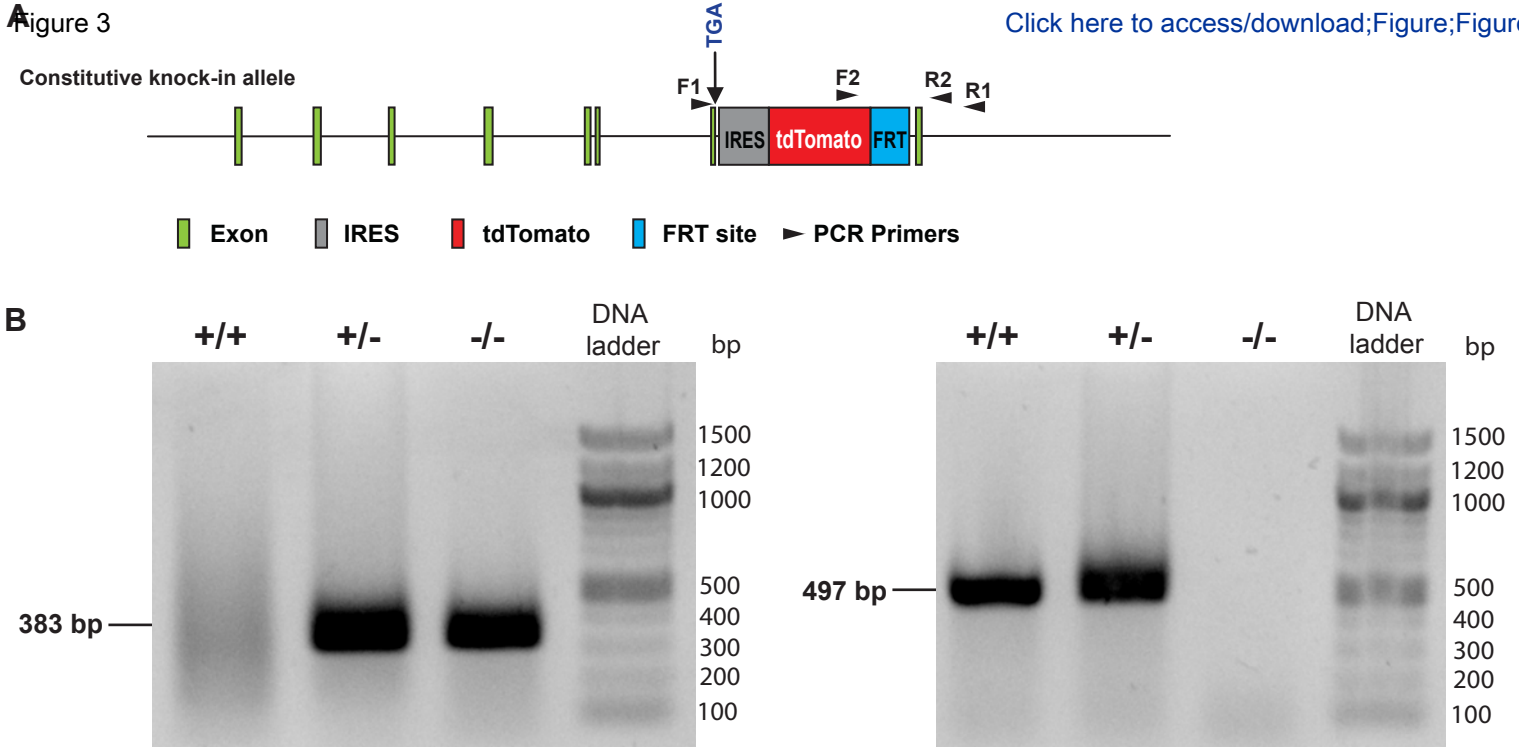


Table 1

Step	Temp ℃	Time	Note
1	94	3 min	
2	94	30 s	
3	60	35 s	
4	72	35 s	
5			repeat steps 2-4 for 38 cycles
6	72	5 min	
7	10	Hold	

Table 2

Step	Temp ℃	Time	Note
1	94	3 min	
2	94	30 s	
3	61.7	35 s	
4	72	35 s	
5			repeat steps 2-4 for 38 cycles
6	72	5 min	
7	10	Hold	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
dissecting microscope	Leica	MZ125	
DNA ladder (100 bp)	Promega	G2101	
epifluorescence dissecting microscope	Leica	M165 FC	
GoTaq Green master Mix	Promega	M712	
PCR machine (master cycler)	Eppendorf	6336000023	

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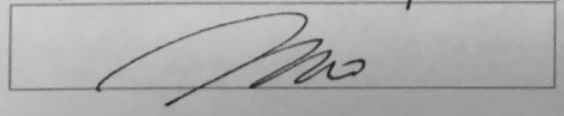
Signature:  Date: 11.19.2018

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We appreciate the constructive comments provided by the Reviewers, and we have attempted to address them on a point-by-point basis below. The changes that we made were tracked in red in the main text.

Reviewer #1

1) The title of the paper suggests that it will focus on the use of whole-mount epifluorescence microscope which might be an area with more potential than dissection. Yet, this section was not very specific (a lot of it may vary).

We described both embryonic heart dissection and whole-mount epifluorescence microscope to examine chamber specification during mouse embryogenesis using fluorescent reporter mice. Because successful dissection of embryonic hearts is a key to success of this protocol, we believe that it is important to describe the dissection method, in addition to epifluorescent microscope use.

2) Figure 2 showed the ventricular specific expression of tdTomato. However, ventricles are easy to identify morphologically without using epifluorescence images. It is not clear how epifluorescence microscope images of ventricles are useful for analysis of embryonic cardiac formation.

Although it is easy to identify ventricles in the post-natal heart, it is not straightforward to identify ventricles or future ventricles during early heart development. Our method will be helpful to study ventricular development or chamber specification during embryogenesis.

3) The steps shown in Figure 1 are routine in laboratories handling mouse embryos. Therefore, general impact of this paper may not be high.
We agree that the protocol described in this manuscript is not completely novel. However, it may fit into one of the aims in *JoVE*, "providing gold standard protocols that can serve trainees and other researchers trying to learn techniques".

4) Generation of the knock-in mouse model, such as vector construction, won't be a helpful content for a video. Perhaps those who want to perform electroporation, selection of ES cells, generation of mice from ES cells would be interested in seeing those procedures in a video.
Generation of a knock-in reporter mouse line is not a topic that we focus on in this manuscript. However, we added an illustration of knock-in allele to describe genotyping primer designs (Figure 3A).

5) All the important steps of the protocol were clearly stated and they lead to the outcomes presented in the paper.
Thanks for your support.

6) The main equipment and materials were listed with the company's name and the catalog number.
Thanks again for your support.

7) The genotyping section was clearly organized and had all the details like the sequences of the primers. However, genotyping is already a well-established procedure, and it won't be a helpful content for a video.
It may fit into one of the aims in *JoVE*, "providing gold standard protocols that can serve trainees and other researchers trying to learn techniques".

8) In the results, it was stated that tails were used for genotyping, but in the protocol it was stated that embryo's heads were used.
Thank you for pointing out the mistake. The embryo head was used for genotyping and the text has been changed accordingly.

Reviewer #2
1) Epifluorescent imaging is a very useful tool, but it is limited to examine inside cardiac chambers. Since early mouse embryos are relatively transparent that allows confocal imaging without a clearing step, providing a wholemount cardiac chamber imaging protocol with a confocal microscope in addition to the current epifluorescent microscopy protocol would be additional benefits for this type of paper.
We agree that confocal imaging technique is a very useful tool to examine embryonic hearts. However, the main advantage of this protocol is to provide a simple tool for studying heart chamber specification in a regular lab setting without a need of expensive equipment.

Reviewer #3
Although novelty is not required for publication, there are many such protocols available in the literature. The protocol is not a live imaging for embryonic hearts, nor is a three D imaging protocol.

We agree that the protocol described in this manuscript is not completely novel. However, this article may fit into one of the aims in *JoVE*, "providing gold standard protocols that can serve trainees and other researchers trying to learn techniques".

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