**TITLE:**

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

**AUTHORS AND AFFILIATIONS:**

Zhentao Zhang1, 2, 3, and Young-Jae Nam1, 2, 3

1Department of Medicine, Division of Cardiovascular Medicine, Vanderbilt University Medical Center, Nashville, TN, USA

2Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN, USA

3Vanderbilt Center for Stem Cell Biology, Vanderbilt University, Nashville, TN, USA

Corresponding author:

Young-Jae Nam

[Young-jae.nam@vanderbilt.edu](mailto:Young-jae.nam@vanderbilt.edu)

Email address of co-authors:

Zhentao Zhang (zhentao.zhang@vanderbilt.edu)

**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 stages1,2. 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 sequentially11. 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 mice12. 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: Rosa26EYFP13, Isl1Cre: Rosa26EYFP13, Hcn4H2BGFP14, Hcn4Cre: Rosa mT/mG14, Nkx2-5Cre: Rosa mT/mG14, Hcn4-eGFP15, Isl1Cre: Rosa mT/mG14, Nkx2.5Cre: Rosa26tdTomato15, and TgMef2c-AHF-GFP16 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 CO2 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 specification17. 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 E8.012 (**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 stage12. The various reporter mouse lines labeling earlier cardiac markers (e.g. Mesp1Cre: Rosa26EYFP13, Isl1Cre: Rosa26EYFP13, Hcn4H2BGFP14, Hcn4Cre: Rosa mT/mG14, Nkx2-5Cre: Rosa mT/mG14, Hcn4-eGFP15, Isl1Cre: Rosa mT/mG14, and Nkx2.5Cre: Rosa26tdTomato15, TgMef2c-AHF-GFP16 mice) can be used to examine earlier stages of chamber development using the method described in this article.

**ACKNOWLEDGEMENT**

This Work was supported by NIH R03 HL140264 (Y.-J. N) and Gilead Sciences Research Scholar Program (Y.-J. N).

**DISCLOSURES**

The authors have nothing to disclose.

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