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Video Article

A Novel *Ex vivo* Culture Method for the Embryonic Mouse Heart

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Abstract

Developmental studies in the mouse are hampered by the inaccessibility of the embryo during gestation. Thus, protocols to isolate and culture individual organs of interest are essential to provide a method of both visualizing changes in development and allowing novel treatment strategies. To promote the long-term culture of the embryonic heart at late stages of gestation, we developed a protocol in which the excised heart is cultured in a semi-solid, dilute Matrigel. This substrate provides enough support to maintain the three-dimensional structure but is flexible enough to allow continued contraction. In brief, hearts are excised from the embryo and placed in a mixture of cold Matrigel diluted 1:1 with growth medium. After the diluted Matrigel solidifies, growth medium is added to the culture dish. Hearts excised as late as embryonic day 16.5 were viable for four days post-dissection. Analysis of the coronary plexus shows that this method does not disrupt coronary vascular development. Thus, we present a novel method for long-term culture of embryonic hearts.

Video Link

The video component of this article can be found at http://www.jove.com/video/50359/

Introduction

Over recent years, the transgenic mouse has been the predominant model system for studying development heart defects. However, other model organisms, such as the zebrafish, have proven to have significant advantages over the mouse. Three major advantages of the zebrafish are the external laying of eggs, for ease of access to the embryos; the optical transparency of the embryos, which allows easy visualization of cardiac development; and the ease of applying small molecular treatments to modulate development of the embryo[1]. Thus, the development of a culture technique that allowed ex utero growth of an embryonic organ would bypass, at least in part, the limitations currently experienced by researchers studying developmental processes in transgenic mice.

Ex vivo cardiac culture systems have been developed in both the chick and mouse embryo that allow treatment with small molecules and analysis of how different regions of the heart communicate[2-6]. For whole mouse heart culture, hearts taken from embryos up to embryonic (E) 12.5 of age can be placed in culture medium with or without rocking[2,3,5]. Using this technique, embryonic hearts have been successfully incubated to the equivalency of E13.5, and hearts cultured with rocking have been maintained as long as three days (starting at E10.5)[3]. However, no studies have reported the successful culture of hearts from older embryos. Likewise, rescue experiments have been limited to applying the therapeutic agent globally to the culture medium[2].

A slice culture system, in which hearts are excised, embedded, and sectioned using a vibratome, has also been utilized for both younger hearts, such as E12.5 mouse hearts and Hamburger-Hamilton stage 36 (approximately E16 in the mouse) chick hearts[2,4,6], and older hearts, such as post-natal and adult mouse hearts and adult human hearts[7,8]. While the embryonic analyses have typically utilized 150-µm thick sections[2,4], section thickness can be a great as 500 µm without evidence of oxygen deprivation[8]. These slice cultures have been maintained as long as two months in culture, with most slices maintaining contractility throughout this period[9]. Compared to studies in isolated cardiomyocytes, these slice cultures allow the co-culture of cardiomyocytes with their neighboring cell types and provide a useful method for *ex vivo* analysis. However, these cultures require more elaborate set-up than simply placing a heart in culture medium (*e.g.* embedding the live heart for sectioning on a vibratome), and any analysis is obviously limited to the portion of the heart within the section.

Given the limitations described above for culturing embryonic mouse hearts and the wealth of transgenic mice available for study, we developed an *ex vivo* mouse heart culture system similar to an ex vivo lung culture system developed by Weaver, *et al.*[10] Our culture system permits long-term culture and visualization of the remodeling coronary circulation within whole embryonic mouse hearts. In addition, the use of Matrigel allows beads to be held in place near the heart, thus providing a localized treatment with therapeutic agents. These experiments can be performed at different developmental time points to compare the effect of a given treatment on a process such as coronary artery formation. Because small molecules can diffuse through Matrigel, this culture system can also be used to culture dissected regions of the heart near each other to

determine whether specific cell-cell contacts are necessary for certain developmental processes or whether paracrine signaling from one region to the other is necessary.

This culture system is relatively simple and, unlike the slice culture system, makes use of basic culture reagents and set-ups that are readily available in most laboratories. In brief, excised embryonic hearts are cultured in dilute Matrigel, which provides a semi-solid support. This support is sufficient to maintain the three-dimensional morphology of the heart while also allowing the heart to contract. Using this system, whole hearts from older mouse embryos (E14.5-E16.5) can be maintained in culture for up to four days. The entire coronary plexus is maintained, unlike in the slice cultures, so any signaling cues that occur from different regions of the heart remain present. Furthermore, live-cell fluorescent dyes can permeate the Matrigel to allow visualization of the live heart, and protein-conjugated beads can be placed near the heart to provide a localized signaling source. Together, these benefits make this technique an ideal method for studying developmental processes in the embryonic mouse heart.

Protocol

1. Excising the Embryonic Hearts

1. Euthanize a timed-pregnant mouse at the desired embryonic day using an approved euthanasia technique. All experiments were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill.
2. Liberally spray the female with 70% ethanol prior to dissection. Open the female's abdominal cavity to retrieve and excise the uterine horn.
3. Place the uterine horn in a Petri dish containing cold 1x phosphate-buffered saline (PBS) and rinse as needed. Cut open the uterine horn via the midline to expose the attached embryonic sacs.
4. Cut open an embryonic sac and retrieve an embryo. Place the embryo in a second Petri dish with cold PBS. Return the Petri dish with the uterine horn to ice.
5. Decapitate the embryo to improve access to the chest wall. If genotyping is necessary, remove and save the tail in an Eppendorf tube placed on ice.
6. With the embryo on its back, cut open the chest wall to visualize the heart and lungs. Depending on the stage, the chest wall may be transparent.
7. Carefully lift the heart using forceps and cut the vessels below. Then, cut above the great vessels to free the heart. If necessary, remove extraneous tissues.
8. Place the heart in one well of a 24-well dish filled with cold PBS and place the dish on ice.
9. Repeat the previous steps until hearts have been excised from all embryos.

2. Culture Set-up

1. In a laminar hood, combine cold Matrigel and the desired culture medium (*e.g.* 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM)) at a 1:1 ratio. Do not pre-warm the culture medium.
2. Keeping the dilute Matrigel on ice, pipette 500-1,000 µl of the diluted solution into a well of a 24-well culture dish that is also maintained on ice.
3. In the hood, carefully pick up and place the excised heart in a well of the Matrigel-containing culture dish while keeping the culture dish on ice.
4. If orientation of the embedded heart is crucial: Liberally spray an inverted microscope and the surrounding space with 70% ethanol. In the hood, place the excised heart in a well of the Matrigel-containing culture plate while still on ice. Then, place the plate on a microscope and quickly orient the excised heart as the Matrigel begins to solidify.
5. Place the culture dish in a humidified 37 °C incubator (5% CO_2) and allow the Matrigel to solidify for approximately 30 min.
6. Add 1 ml pre-warmed culture medium to each well containing a heart.
7. Hearts can remain in culture for at least four days. Changing the culture medium every two days is recommended.

3. Fixation and Visualization

1. If desired, add a fluorescent live-cell dye (*e.g.* Syto-16) to visualize the live heart. Photography will be limited to the side of the heart that is visible based on the position in which it was embedded.
2. If post-culturing analyses (*e.g.* whole mount imaging or sectioning) will be performed, remove the culture medium and replace with cold 4% formaldehyde. Place the dish on ice for 30 min.
3. After the cold formaldehyde and ice promote dissolution of the Matrigel, replace the fixative (and Matrigel) with fresh formaldehyde and fix hearts overnight at 4 °C.
4. Wash the hearts with buffer (*e.g.* PBS or Tris) and store at 4 °C until further analyses.

Representative Results

Using this technique, the heart maintains its three-dimensional morphology and remains viable, as indicated by continued contractions (**Movie 1**). These contractions are consistently more prominent in the atria than in the ventricles. Following culture, hearts can be fixed and processed for either immunohistochemistry or histology to examine specific marker expression or structures. **Figure 1A** shows the base of the ventricles and great arteries of an embryonic mouse heart that was cultured for 24 hr, fixed, and processed for immunohistochemistry to label the endothelium. These confocal micrographs demonstrate that the coronary vasculature appears similar to a heart excised from a comparably aged embryo that was maintained *in utero* (**Figure 1B**). However, the coronary vessels in the heart cultured *ex vivo* do appear smaller than in

the heart that was maintained in utero. In both hearts, the apices of the ventricles were removed to allow better access of the antibodies to the tissue.

Hematoxylin and eosin (H&E) analysis of hearts excised at E16.5 and cultured *ex vivo* for 4 d demonstrates that the overall morphology, including the walls of the great arteries (**Figure 2A**) and the ventricular and interventricular septal myocardium (**Figure 2B**) of the heart remains intact. Despite the lack of circulation, the coronary ostia are apparent (**Figure 2A**). However, DNA fragmentation, observed both with H&E (**Figures 2A, 2B)** and DAPI (data not shown), is apparent in the compact myocardium and around the great vessels. Further, the myocardium is less dense as compared with a heart from an E18.5 embryo that developed in utero (**Figures 2C, 2D**), even though the *ex vivo* cultured heart was still beating. Thus, there are limitations to the length of time for or the stage at which these hearts can be maintained.

This culture technique can also be used to fluorescently label hearts while in culture. In this case, hearts are cultured for 48 hr after being incubated with an endothelial-specific fluorescent dye. In this instance, visualization is limited based on the orientation of the heart and the opacity of the tissue (**Figures 3A, 3B**). However, subsequent histological sections through the ventricular myocardium confirm that this fluorescent molecule can penetrate the outer cell layers to label sub-epicardial vessels within the ventricles (**Figures 3C, 3D**), and this fluorescent dye colocalizes with iso-lectin (**Figure 3D**).

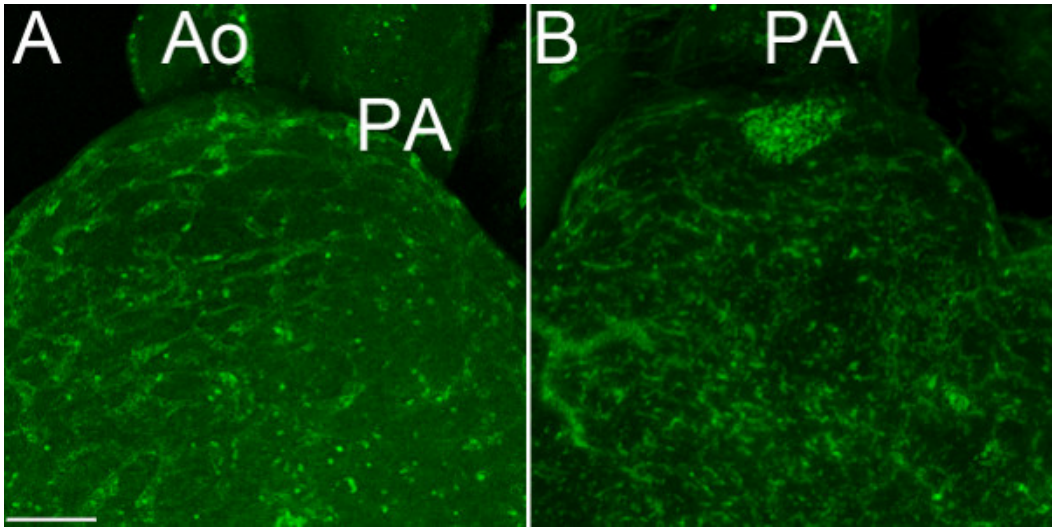

Figure 1. Development appears normal in *ex vivo* conditions. (A) After 24 hr in culture, *ex vivo* hearts from E14.5 embryos were fixed, labeled with an endothelial marker (green), cleared, and imaged with confocal microscopy. The pulmonary artery (PA), aorta (Ao), and base of the heart are shown. **(B)** Hearts excised from E15.5 embryos that developed *in utero* were similarly processed. Scale bar, 100 μm.

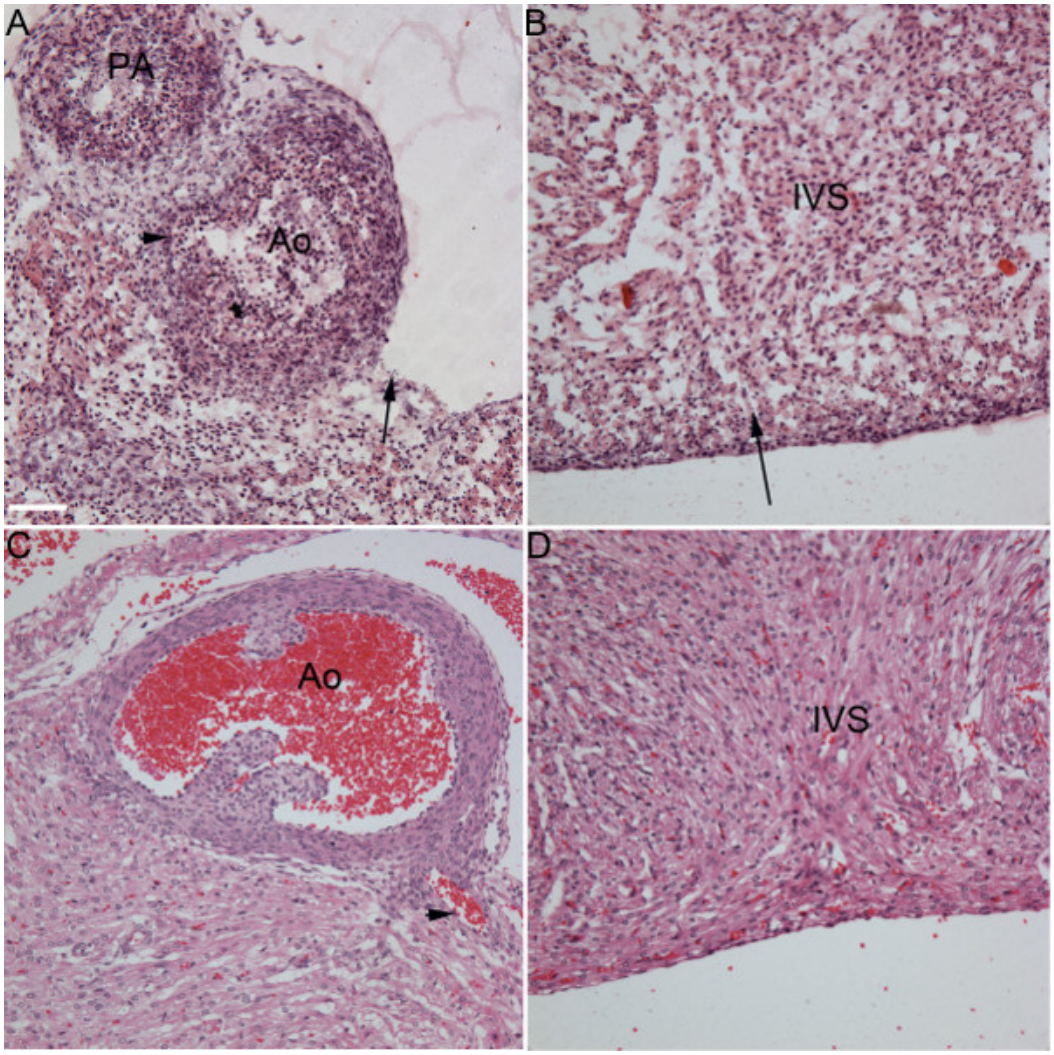

Figure 2. Morphology is grossly maintained during *ex vivo* culture. (A, B) Hearts were excised from E16.5 embryos and cultured *ex vivo* for 4 days. Hearts were then sectioned and stained with hematoxylin and eosin (H&E). **(A)** A transverse section showing the alignment of the aorta (Ao) and pulmonary artery (PA). One of the coronary ostia (arrowhead) is visible. The myocardium surrounding the aorta is less dense than in the control **(C)**, and some nuclear fragmentation (arrow) is also present. **(B)** The overall morphology of the compact myocardium of the ventricles and the interventricular septum (IVS) appears normal, but the myocardium is less dense than in the control **(D)**, and some nuclear fragmentation (arrow) is apparent in the IVS. (**C, D**) For comparison, frontal H&E-stained sections from an E18.5 heart are shown. **(C)** A coronary ostium is observed adjacent to the Ao (arrowhead). **(D)** The interventricular septum is well defined. Scale bar, 70 μm.

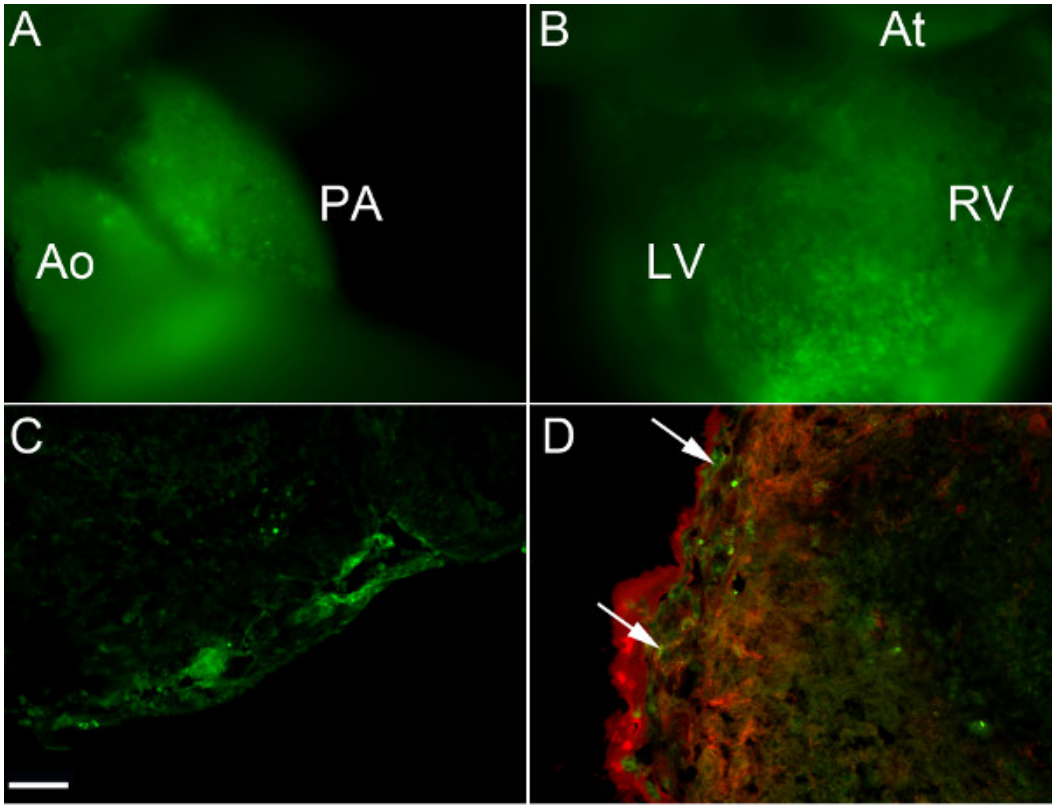

Figure 3. *Ex vivo* hearts can be fluorescently labeled during culture. (A, B) After 48 hr in culture, *ex vivo* hearts from E16.5 embryos were incubated with Syto 16 (green) for 1 hr, and fluorescence could be observed as late as 48 hr after incubation. **(C, D)** This fluorescence was maintained through frozen sectioning and could be observed in the subepicardial epithelial cells within the ventricular myocardium. In **D**, the section within the ventricular myocardium was co-labeled with iso-lectin (red). The iso-lectin co-labels the green Syto 16-positive cells (arrows) and also labels vessels within the myocardium that were not stained with Syto 16. A-B, 4x magnification; scale bar in **C-D**, 120 µm.

Movie 1. After *ex vivo* culturing for 30 hr, a heart excised from an E14.5 embryo shows consistent contractility and pacing between the atria and ventricles. Click here to view movie.

Discussion

The current culture system poses significant advantages for embryonic mouse heart studies. This culture system preserves myocardial contractility and the coronary plexus, with limited signs of necrosis, even after four days in culture. Further, the semi-solid matrix provides enough support to maintain the three-dimensional morphology of the developing heart while allowing flexibility to contract and also to hold coated beads in place during culture. Despite this support, this matrix is permeable to fluorescent dyes such as Syto 16, allowing fluorescent analysis of the live heart.

Slice culture methods place the heart section, containing its portion of the coronary plexus, on a porous membrane at an air-liquid interface to maintain oxygenation through capillary action[7]. In contrast, previous whole heart culture methods rely on rocking and restricted studies to embryonic ages before a functional coronary vasculature is necessary[2,3,5]. Using the current technique, however, hearts can easily be cultured after the coronary plexus has formed without the additional complexity of an air-liquid interface.

Despite our concerns that late embryonic hearts (*e.g.* E16.5) would become necrotic after an extended period of time in culture, only limited necrosis was observed after four days of culture (*i.e.* the equivalent of a post-natal day 1 heart). The original protocol, as performed using embryonic lung buds,[10] reported no necrosis but also utilized lungs from earlier staged embryos. Thus, necrosis may be avoided by restricting studies to earlier embryonic time points.

This *ex vivo* culture system does have limitations. After two days in culture, the epicardium begins spreading to the matrix, and within four days, the epicardium attaches to the culture dish. Because the epicardial outgrowth does not affect visualization, however, this issue may be of primary importance to epicardial studies. At the stages analyzed herein (E14.5-E16.5), the epicardium has long since encompassed the heart[11] and provided signals to the developing plexus[12]; thus, its outgrowth does not appear to have negative effects on the coronary plexus. Further, the hearts in culture appear to stop growing larger like their in utero counterparts. Another limitation is that the embryonic mouse heart is not transparent like the zebrafish embryo. Any live visualization is thus limited to what can be observed on the outer layers of the heart. However, a live endothelial marker can sufficiently permeate the heart to label some of the coronary plexus and is visible in culture.

Despite these limitations, we anticipate that this *ex vivo* culture method will be useful for a number of applications. The wide variety of transgenic mice lines that carry fluorescently labeled cell types could be easily used to assess different aspects of development using time-lapse imaging. Further, treatment of embryonic hearts *ex vivo* with small molecular compounds, whether novel or already in clinical use, will be easy to accomplish and mimics how drug therapies are applied in humans.

In conclusion, we have adapted an *ex vivo* culture method for the embryonic mouse heart. This technique will allow easy access to otherwise inaccessible organs, such as the liver or kidney, during development and will allow for the manipulation and live analysis of these organs.

Disclosures

The authors declare that they have no competing financial interests.

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