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Microinjection-based System for In Vivo Implantation of Embryonic Cardiomyocytes in the Avian Embryo

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Dear Editorial Review Board,

We are pleased to submit our manuscript entitled " Microinjection-based system for *in vivo* implantation of embryonic cardiomyocytes in the avian embryo" for your consideration. The manuscript outlines a technique that our research group has optimized for *in vivo* cellular implantation into the embryonic heart. Our protocol overcomes many of the physical limitations inherent in attempting to "graft" cells onto the moving embryonic heart via classical experimental embryology techniques and allows for precision deliver of donor cells into multiple locations within the cardiac muscle and surrounding tissue.

I had spoken with Nandita Sing regarding this submission earlier this summer. In consultation with Nandita, we had determined that the protocol described in this manuscript would be of high interest to the field, particularly if presented in a video format.

If there is any further information that I can provide, please do not hesitate to contact me. We look forward to hearing your response.

Sincerely

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TITLE:

Microinjection-based System for In Vivo Implantation of Embryonic Cardiomyocytes in the Avian Embryo

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KEYWORDS:

embryonic development, in vivo, implantation, cellular integration, cell tracing, cardiac lineage

SUMMARY:

In this method, embryonic cardiac tissues are surgically microdissected, dissociated, fluorescently labeled, and implanted into host embryonic tissues. This provides a platform for studying the individual or tissue level developmental organization under ectopic hemodynamic conditions, and/or altered paracrine/juxtacrine environments.

ABSTRACT:

Interpreting the relative impact of cell autonomous patterning versus extrinsic microenvironmental influence on cell lineage determination represents a general challenge in developmental biology research. In the embryonic heart, this can be particularly difficult as regional differences in the expression of transcriptional regulators, paracrine/juxtacrine signaling cues, and hemodynamic force are all known to influence cardiomyocyte maturation. A simplified method to alter a developing cardiomyocyte's molecular and biomechanical microenvironment would, therefore, serve as a powerful technique to examine how local conditions influence cell fate and function. To address this, we have optimized a method to physically transplant juvenile cardiomyocytes into ectopic locations in the heart or the surrounding embryonic tissue. This allows us to examine how microenvironmental conditions influence cardiomyocyte fate transitions at single cell resolution within the intact embryo. Here, we describe a protocol in which embryonic myocytes can be isolated from a variety of cardiac sub-domains, dissociated, fluorescently labeled, and microinjected into host embryos with high precision. Cells can then be directly analyzed in situ using a variety of imaging and histological techniques. This protocol is a powerful alternative to traditional grafting experiments that can be prohibitively difficult in a moving tissue such as the heart. The general outline of this method can also be adapted to a variety of donor tissues and host environments, and its ease of use, low cost, and speed make it

a potentially useful application for a variety of developmental studies.

INTRODUCTION:

Cardiac developmental research has benefitted enormously from the advent of germline transgenic model systems which have identified many of the gene regulatory networks that pattern different cell lineages and functional domains in the heart. However, identifying how these gene networks interact with and respond to microenvironmental conditions, including paracrine/juxtacrine signals and biophysical inputs (stretch, strain, hemodynamic flow), can be challenging. As such, it is not always easy to determine whether a cellular phenotype arises as a direct consequence of a genetic perturbation or as a secondary result of an adaption to changes in cardiac biomechanics or higher order tissue composition[1,2].

Grafting experiments, which have classically been used to address concepts of fate specification, commitment, induction, and competence[3,4], would represent an ideal approach to circumvent some of the challenges inherent in defining cell autonomous versus environmental influence in the heart. Unfortunately, heart contractions make standard grafting approaches difficult. Rapid movement of the tissue often prevents grafted cells from adhering to the heart and large tissue punctures (normally required for grafting) frequently lead to heart failure and embryonic lethality[5-7]. Therefore, we have developed a pressure-based, microinjection system for precision cellular implantation into the developing chick heart, circumventing the technical hurdles of tissue grafting described previously[8,9]. Using this technique, individual or small groups of cardiac cells isolated from a donor embryo can be microinjected into a variety of regions of a host embryonic heart eliminating the need for extensive host preparation and the large tissue insults that arise using standard grafting techniques. The microinjection needles used for these implantation studies have an outer diameter of ~30−40 µm, which means that the needle can be placed directly in the target tissue (i.e., can penetrate the embryonic myocardial wall) and cells can be focally delivered with minimal damage to the surrounding tissue. The protocol can be used to perform a variety of isotopic, heterotopic, isochoric, and heterochronic manipulations, providing a rapid, flexible, and low-cost approach to directly examine classical experimental embryological paradigms in the developing four-chambered heart.

In the protocol outlined below, we label donor cells with a cell permeant fluorescent dye, which allows for the success of a microinjection experiment to be monitored in real-time and the location of engrafted cells to be documented without the need for any additional staining. However, it should be noted that this approach is best suited for short term experiments (approximately 48 h) as the fluorescent dye can be lost through cell division. Alternative approaches can be used for longer term experiments.

While we are presenting this technique in the context of cardiac development, we have used it to great effect for cell implantation experiments into the mesoderm, head, limbs, and somites. As such the basic approach described below is highly tractable and can be used in a variety of organ systems.

PROTOCOL:

All methods described adhere to animal care guidelines of The University of North Carolina at Chapel Hill.

1. Preparation of micro-injection pipettes

1.1 Pull glass capillaries using a micropipette puller. For some injections, needle beveling is recommended as it provides an extremely sharp surface devoid of structural impurities. To do this, polish the end of a pulled needle on a beveling wheel at an angle of 45° for 15–20 min.

NOTE: Exact settings for pulling will vary based on the puller being used. The final inner diameter of the bevel should be between 20–40 µm.

1.2 Coat the inner and external surfaces of the glass capillary with silicone. First dip the needles in the siliconizing agent to coat the external surface of the needle and then backload the siliconizing agent solution into each micropipette to coat the inner surface.

NOTE: Coating of the glass capillaries should be done 24 h before the implantation experiment. Coating the glass capillaries with silicone provides a chemically inert surface to the glass. If the capillaries are left untreated, the cell suspension generated in later steps will adhere to the glass and plug the needle. Therefore, coating is necessary and vital to the success of the method.

CAUTION: The siliconizing agent is a ready-made commercially available mixture of heptane and 1,7-dichloro-1,1,3,3,5,5,7,7-octamethyltetrasiloxane (**Table of Materials**). It is extremely flammable and acutely toxic. Always handle with proper PPE inside a fume hood.

1.2.1 To backload the needle, load ~5–10 µL of the siliconizing agent into a micro-injection pipette tip (**Table of Materials**). Place the micro-injection tip in the wide end of the pulled glass capillary and position the tip as far down as possible (close to the glass needle tip). Eject the siliconizing agent while slowly removing the loading pipette in order to minimize air bubbles in the needle.

1.2.2 Leave the siliconizing agent in the glass needle for 10 min, remove by aspirating with a new loading pipette and allow needles dry overnight in a fume hood.

1.3 The morning of the experiment, rinse the glass capillaries with deionized water following the procedure in step 1.2 and allow to dry for 3–4 h.

2. Preparation of solutions

2.1 Prepare 5 mL of trypsin neutralizing solution by supplementing 4.2 mL of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (DMEM/F12) with 750 µL of Fetal Bovine Serum (FBS) and 50 µL of Penicillin/Streptomycin. Store at 37 °C until use.

2.2 Prepare 5 µM labeling dye solution by pipetting 5 µL of 1 mM stock labeling dye (in dimethyl sulfoxide [DMSO], **Table of Materials**) into 995 µL of Hank's balanced salt solution (HBSS). Vortex

for 1 min and store at 37 °C until use.

2.3 Prepare fresh paraformaldehyde (PFA) by combining 10 mL of 32% PFA stock solution with 62 mL of molecular biology grade water and 8 mL of 10x Dulbecco's phosphate-buffered saline (DPBS). The final concentration is 4% PFA in 1x DPBS.

3. Preparation of host embryos

3.1 Incubate fertile chicken eggs in a horizontal orientation in a humidified incubator at 38 °C until Hamburger and Hamilton (HH) Stage 19[10].

NOTE: The stage chosen for manipulation is flexible and entirely dependent upon the aims of each individual experiment.

3.2 Score the "flat" end of the egg shell along the egg equator using angled forceps to make a small puncture >1 mm in diameter. Insert an 18 G needle with attached 10 mL syringe through the puncture and remove ~5 mL of albumin.

NOTE: This anatomical location is external to the "air cell" inside the egg and keeps albumin from leaking once the puncture is made. This step is recommended as it "drops" the embryo away from the egg shell, preventing potential damage in the subsequent steps.

3.3 Apply transparent tape to the top of the egg shell. Score with angled forceps and cut a ~2.5 cm window using curved tenotomy scissors.

3.4 Inspect and stage the embryo based on criteria established by Hamburger and Hamilton[10] and seal the puncture from step 3.2 with transparent tape.

NOTE: Here, stage HH 19 embryos are used which have 37−40 somites extending into the tail bud. The puncture should not be sealed until after the window is opened along the top of the egg (step 3.3).

3.5 Inject ~200 µL of India Ink/HBSS mixture (1:5) beneath the embryo using a 32 G needle with attached 1 mL syringe.

NOTE: India ink provides visual contrast between the embryo and the yolk beneath. Alternative dyes such as neutral red or commercially available cyan fluorescent protein (CFP) or blue fluorescent protein (BFP) fluorescence filter sets can be used to improve contrast.

3.6 Add 1 mL of HBSS dropwise onto the embryonic disc and seal windowed shells with paraffin film. Place eggs back in the humidified incubator until ready for injection.

4. Isolation of donor tissue

4.1 Incubate donor fertile chicken eggs in a humidified incubator at 38 °C until Stage HH 19 (or desired stage).

4.2 Remove the embryo from the egg and place in a 100 mm x 15 mm petri dish containing sterile HBSS at room temperature (RT).

4.3 Surgically microdissect atrial donor tissue from each embryo by first isolating the entire embryonic heart from the embryo and then by isolating the atria from the heart using forceps, tenotomy scissors, and microspatula under a stereo dissecting microscope. Pool in a sterile 1.5 mL microcentrifuge tube containing 1 mL of HBSS on ice.

4.4 Once all donor tissue has been collected, pellet the tissue by centrifugation at 1000 x *g* for 5 min at 4 °C in a fixed-angle microcentrifuge.

5. Trypsin digestion of donor tissue

5.1 Resuspend cell pellets in 1 mL of prewarmed 0.05% trypsin-EDTA and incubate at 37 °C for 15 min in a shaking heat block at 300 rpm. Alternatively, use a water bath with periodical agitation of the sample.

5.2 Pipette the digestion solution up and down to break up any remaining tissue, and pellet as in step 4.4.

5.3 Resuspend the pellet in 1 mL of the trypsin neutralizing solution and centrifuge as in step 4.4.

5.4 Resuspend the cells in 400 µL of red fluorescent labeling dye solution and incubate at 37 °C for 20 min in a heat block. Alternatively, use a water bath.

5.5 Once the labeling reaction is finished, pellet the cells as in step 4.4 and wash with 1 mL of HBSS (number of wash steps can be varied between 1 and 3).

5.6 Resuspend the labeled, pelleted cells at a concentration of ~50,000 cells/µL, which generally results in a 5−10 µL working volume depending on the total cell yield.

NOTE: Cell concentrations below ~50,000 cells/µL can result in poor injection efficiency.

6. In-vivo injection

6.1 Backload the cell suspension into a silicone treated glass capillary pipette following the procedures in step 1.2. Mount the pipette into the pressure microinjector apparatus.

6.2 Remove host embryos from humidified incubator and place in an egg holder underneath the fluorescent stereo dissecting microscope.

6.2.1 Open the vitelline membrane using sterile fine forceps and make a small incision (~0.5−1.0 mm in length) in the pericardium. Additional manipulation/dissection may be needed depending on target region for injection.

6.3 Position the microinjector such that the tip of the microinjection needle penetrates the target tissue.

6.3.1 Pressure inject cells, and use the fluorescent label to determine that implanted cells are present in desired tissue. For typical injections, apply single pulses less than 0.5 s in duration ranging from 100–400 hectopascals in pressure.

NOTE: Pulse length and absolute pressure will vary depending on the number of cells to be injected and can be modified to suit individual needs.

6.3.2 Retract the microinjector apparatus and remove the egg from the holder after pressure injecting.

6.4 Add 1 mL of warm HBSS dropwise onto the embryo, seal eggs using transparent packing tape, and incubate in the humidified incubator at 38 °C for 24 h post implantation.

7. Isolation and analysis

7.1 Isolate host embryos in RT HBSS using forceps, tenotomy scissors, and microspatula similar to step 4.3, and fix in 4% PFA overnight at 4 °C with gentle rocking.

7.2 Wash embryos 3 x 5 min in HBSS at RT with gentle rocking, and store in HBSS at 4 °C for further downstream analysis (microscopy, immunohistochemistry, in-situ hybridization, etc.).

REPRESENTATIVE RESULTS:

After 24 h incubation, the heart and surround tissue of host embryos were isolated, photographed (**Figure 1A**), and processed for immunofluorescent analysis. In this example, donor atrial myocytes were microinjected into the proepicardium of a similar staged host embryo. The host embryo was then stained with the muscle marker (MF20 green) and 4',6-diamidino-2-phenylindole (DAPI; blue). Injected cells (red) are clearly visible (**Figure 1B**). Possible adjustments to consider if cells are not visible include: donor tissue was over digested (cells would be unable to attach), labeling dye solution was too dilute, cells were over-washed, or multiple cell divisions resulted in loss of the label.

To confirm that the injected cells in this example were myocardial, we optically sectioned this embryo using a confocal microscope (**Figure 1C–E**). The only MF20 positive cells within the proepicardium (PE) are the fluorescent red positive cells that were focally implanted.

FIGURE LEGEND:

Figure 1: Representative images of embryos isolated 24 hours post injection. (**A**) Low

magnification brightfield image of the trunk region of an E3.5 (HH Stage 19) chick embryo. (**B**) Merged image showing injected cells (red), cardiomyocytes (green), and DAPI. Cells were isolated from the atria and microinjected into the proepicardium. (**C**) High magnification confocal imaging showing labeled cells in the core of the proepicardium. (**D**) High magnification confocal imaging confirming CT Red labeled cells are cardiomyocytes. (**E**) Three-dimensional (3D) reconstruction of injected cells from panels **D** and **E**. At, atria; OFT, outflow tract; PE, proepicardium; Vt, ventricle; MF20, Myosin 4.

DISCUSSION:

The ability to define how microenvironmental conditions impact cardiac cell fate specification and lineage stabilization is fundamental to creating a compressive understanding of congenital heart disease as well as to developing efficient protocols for proper maturation of stem cell or somatic cell reprograming-based cardiomyocytes. The protocol outlined above gives investigators the ability to directly assay cardiac cell development under altered in vivo conditions, allowing for cell autonomous maturation processes to be separated from paracrine/juxtacrine and/or hemodynamic cues. When combined with high resolution imaging, genetic analysis, and physiological assays, this technique can serve as a powerful complement to existing transgenic models.

Form a technical stand point, the protocol presented here relies on efficient isolation, labeling, and precise implantation of donor heart cells into host embryonic tissues. The use of a microinjection system greatly aids in the targeting of the donor cells and allows for successful implantation without the need for creating a large engraftment site in the host tissue. Some operational skill is required to perform this technique however, as reduced viability can result if the injection needle is not carefully placed in the target tissue (causing rupture of the heart or local vasculature). Care and thought should also be given to the isolation and labeling steps. Over digestion of the donor tissue can lead to poor implantation efficiency, and transient labeling techniques can limit the time window over which donor cells can be tracked (as cell division can dilute the label).

This technique is highly modifiable and can be adapted for a variety of purposes. For example, donor cells from a large range of tissues and stages can be isolated (though optimization of the enzymatic digestion is required) and can similarly be injected into a variety of host tissues across different stages of development. Similarly, the labeling approach can be modified to track cells across different temporal windows, including the use of fluorescent inorganic semiconductor nanocrystals for longer transient labeling and implantation of quail cells or cells from green fluorescent protein transgenic (GFP+) donor embryos[11] for permeant labeling.

While we currently use this technique for avian implantation studies, we feel that it could be used for a large range of chimeric studies in the future. For example, genetically altered cardiac cells from transgenic organisms could be isolated and microinjected into the avian heart using a very similar protocol. Furthermore, cells differentiated into cardiomyocytes from stems cells or via somatic cell reprograming approaches could be microinjected into the embryonic heart to evaluate their integration into the tissue and/or maturation under in vivo biomechanical

conditions.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Guo, Y. X., Pu, W. T. Genetic Mosaics for Greater Precision in Cardiovascular Research. *Circulation Research.* **123**, 27-29, doi:10.1161/Circresaha.118.313386 (2018).
2. Guo, Y. X. et al. Analysis of Cardiac Myocyte Maturation Using CASAAV, a Platform for Rapid Dissection of Cardiac Myocyte Gene Function In Vivo. *Circulation Research.* **120** (12), 1874-1888, doi:10.1161/Circresaha.116.310283 (2017).
3. Rugh, R. *Experimental embryology; techniques and procedures*. 3rd edition, (Burgess Pub. Co., 1962).
4. Slack, J. M. W. *Essential developmental biology*. 3rd edition, (Wiley, 2013).
5. Reinecke, H., Zhang, M., Bartosek, T., Murray, C. E. Survival, integration, and differentation of cardiomyocyte grafts: a study in normal and injured rat hearts. *Circulation.* **100**, 193-202, doi:10.1161/circ.100.2.193 (1999).
6. Rojas, S. V. et al. Transplantation of purified iPSC-derived cardiomyocytes in myocardial infarction. *PLOS ONE.* **12**, e0173222, doi:10.1371/journal.pone.0173222 (2017).
7. Zhang, M. et al. Cardiomyocyte Grafting for Cardiac Repair: Graft Cell Death and Anti-Death Strategies. *Journal of Molecular and Cellular Cardiology*. **33** (5), 907-921, doi:10.1006/jmcc.2001.1367 (2001).
8. Bressan, M., Liu, G., Mikawa, T. Early mesodermal cues assign avian cardiac pacemaker fate potential in a tertiary heart field. *Science.* **340** (6133), 744-748, doi:10.1126/science.1232877 (2013).
9. Bressan, M. et al. Reciprocal myocardial-endocardial interactions pattern the delay in atrioventricular junction conduction. *Development.* **141** (21), 4149-4157, doi:10.1242/dev.110007 (2014).
10. Hamburger, V., Hamilton, H. L. A series of normal stages in the development of the chick embryo. *Journal of Morphology.* **88** (1), 49-92 (1951).

Figure

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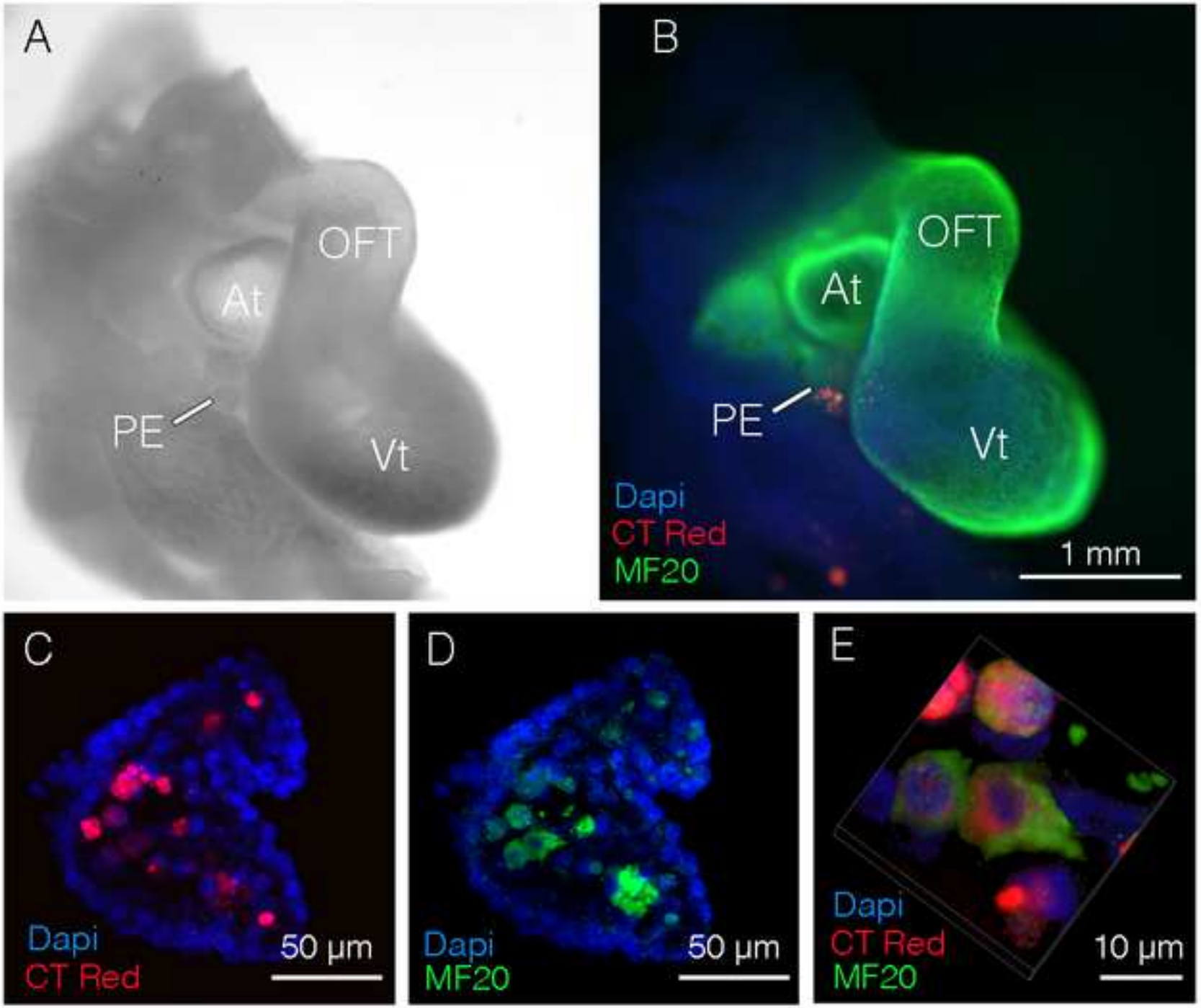

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 mL Insulin Syringe	BD	329654	
1.7 mL Microtubes, Clear	Genesee Scientific	24-282	
10 ml Syringe	BD	305482	
1000ul Reach Barrier Tip Racked, Sterile	Genesee Scientific	24-430	
15 mL Centriguge Tubes, Racked	Genesee Scientific	28-101	
1588 Genesis Hova-Bator Incubator	GQF	813927021221	
18G x 1 1/2 Needle	BD	305196	
200ul Barrier Tip Low Binding, Racked, Sterile	Genesee Scientific	24-412	
32G x 1/2" Needle	TSK Steriject Air-Tite	TSK3213	
Alchohol Wipes 70%	Thermo Fisher Scientific	19015744	
Angled Forceps	Fine Scientific Tools	11260-20	
Backloading Tips	Eppendorf	930001007	
Black India Ink	KOH-I-NOOR	3084-F	
CellTracker Green CMF	Thermo Fisher Scientific	C7025	1 mM in DMSO
CellTracker Red CMTPX	Thermo Fisher Scientific	C34552	1 mM in DMSO
Centrifuge	Eppendorf	5424R	
Commercial Grade Packing Tape	Staples	2619001	
Curved Tenotomy Scissors	Fine Scientific Tools	14067-11	
DMEM/F12	Thermo Fisher Scientific	11330-032	
DMSO, anhydrous	Thermo Fisher Scientific	D12345	
DPBS (10X), no calcium, no magnesium	Thermo Fisher Scientific	14025092	
Femtojet 4i	Eppendorf	5252000021	
Fetal Bovine Serum	Thermo Fisher Scientific	10437-028	
Hatching Eggs	Pilgrim's Hatchery	--	
HBSS, calcium, magnesium, no phenol red	Thermo Fisher Scientific	14025-092	
Injectman 4	Eppendorf	5192000027D	
Micromanipulator	Leica Microsystems	--	
Parafilm	SIGMA	P6543-1EA	
Paraformaldehyde 32% in aqueous solution, EM Grade	VWR	100496-496	
Penicillin/Streptomycin	Thermo Fisher Scientific	15140-022	
Petri Dish	Genesee Scientific	32-107	
Pipette Grinder	Narishige	EG-44	
Pipette Puller	HEKA	PIP 6	
Scotch Transparent Tape	Staples	487909	
Sigmacote	SIGMA	SL2-25ML	
Stereo Microscope	Leica	--	
ThermoMixer C	Eppendorf	5382000023	
Thin Wall Glass Capillaries	World Precision Instruments	TW100F-4	
Transfer Pipette	Thermo Fisher Scientific	273	
Trypsin-EDTA (0.05%), phenol red	Thermo Fisher Scientific	25300-054	

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We would like to thank the JoVE editorial team and the manuscript reviewers for their careful reading of our protocol. A number of issues were raised with the initial submission that we have corrected. In general, the editorial team and reviewers asked us to provide more detail on several components of the protocol, expand the introduction and discussion, and fix some grammatical errors. Please find our point-by-point response below.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have reviewed the manuscript and corrected a number of proofreading mistakes.

2. Please expand your Introduction to include the following the advantages over alternative techniques with applicable references to previous studies.

The Introduction has been expanded to include the following:

Using this technique, individual or small groups of cardiac cells isolated from a donor embryo can be microinjected into a variety of regions of a host embryonic heart eliminating the need for extensive host preparation and the large tissue insults that arise using standard grafting techniques. The microinjection needles used for these implantation studies have an outer diameter of ~30-40 µm, which means the needle can be placed directly in the target tissue (i.e. can penetrate the embryonic myocardial wall) and cells can be focally delivered with minimal damage to the surrounding tissue. The protocol can be used to perform a variety of isotopic, heterotopic, isochoric, and heterochronic manipulations, providing a rapid, flexible, and low-cost approach to directly examine classical experimental embryological paradigms in the developing four-chambered heart.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

We have corrected our scientific notation as requested.

4. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

We have corrected our spacing to meet the JoVE formatting requirements as requested.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

The following ethics statement has been included:

All methods described adhere to animal care guidelines of The University of North Carolina at Chapel Hill.

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

7. 1.3: Please provide the composition of siliconizing agent. Please describe how to backload the solution into micropipette.

The siliconizing agent is commercially available and is listed in the Table of Materials.

8. 1.4: How to dry the glass capillaries and for how long?

The timing for drying the needles has been included.

9. 3.1, 4.1: Please specify the desired stage in this protocol.

The stage for manipulation has been included.

10. 3.2.4: Please provide the criteria for staging the embryo.

A reference to staging criteria (proposed by Hamilton and Hamburger) is now included.

11. 3.3: Is a syringe used to inject the mixture?

Details regarding the syringe are now included.

12. 4.2: How large is the petri dish?

Petri dish size is now included.

13. 5.3, 5.5: Please specify centrifugation parameters. Note that there is no step 3.7.

All centrifugation sets are as described in procedure 4.4. We have now indicated this in the text.

14. 5.4: Please provide the composition of the labeling dye solution.

The composition of the dye labeling solution is now provided in procedure 2.2

15. 6.2.1: How small is the incision?

Length of the incision is now provided in procedure 6.2.1

16. 6.3.2: Please describe how.

We are not sure what is being requested in the comment. This procedure is just visual inspection of the tissue.

17. Representative Results: Please describe all panels of Figure 1 in the text.

All panels are now described.

18. Discussion: Please discuss the significance with respect to existing methods and any future applications of the technique.

We have modified the discussion substantially to emphasize the points requested.

19. Figure 1: Please use the micro symbol μ instead of u and include a space between the number and the units of the scale bar (1 mm, etc.). In the figure legend, please define all abbreviations in the figure.

The figure and figure legend have been modified as requested.

20. A minimum of 10 references should be cited in the manuscript. For instance, please include applicable references to previous studies when describing advantages over alternative techniques.

We have added additional references.

21. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Table of materials has been reformatted.

Reviewers' comments:

Reviewer #1:

Minor Concerns:

There are a few inadequacies that might improve the manuscript, and I highlight

those here along with some editorial suggestions:
1) Silicone coating of the pipettes is novel, and likely helpful in preventing the frustration of needle plugs. However, the siliconizing reagents don't seem to be listed in the materials section. An image of a prepared needled might also be helpful.

The Siliconizing reagent is listed in the Table of Materials. We agree that an image of the needle would be helpful, and expect that the video recording of the procedure will be adequate for viewers.

2) A more detailed description of "backloading" of the injection needle would be helpful, since this can be a difficult procedure.

We have modified the text to provide more details regarding the materials needed to backload the needle and the process of doing so.

3) In line 41, the word "pression" is used. Because of the context, I initially thought this was a misspelling of "precision." Pression is an uncommon term, and perhaps the standard "pressure" could be used to avoid confusion by the reader.

This is, in fact, a grammatical error and we have corrected it. We did intend to use the term "precision".

4) In line 115 and 138, the phrase White Leghorn Horn Chicken eggs is used. Take out Horn as it is incorrect, unless this is a new breed. Alternatively, since the breed of chicken is likely not important, fertile chicken eggs would be an acceptable phrase.

The reviewer is correct, "horn" in this context was a typo. We have taken the reviewer's advice and simply used the term "fertile chicken eggs".

5) Line 118 describes a location on an egg shell, but it is unclear. Removing albumin from an egg usually occurs near the more pointed end, so as to avoid the air pocket on the more blunt end of the egg. Perhaps the authors could clarify. Perhaps an image would be helpful?

We have expanded the description of the text in this area of the protocol. We did not specify that we typically incubate the eggs in a horizontal orientation and puncture the blunt end for removing the albumen. This will be clear in the video recording.

6) Lines 181-182 describe the pressure for injection. Can the pulse length also be variable and modified? Is it important? Please clarify.

We typically use a series of 0.5 s pulses, however, the pressure is far more important for effective cell delivery. We have given a clearer description of this in the text.

7) Line 197 describes using PFA, but it is not in the materials list. Also, HBSS is not listed, or the ingredients to make it. Perhaps a better description of these reagents is warranted, since 4% PFA could be in water or in a salt solution like PBS or HBSS.

Hanks Balanced Salt Solution (HBSS) is included in the Table of Materials and we have added our procedure for making 4% PFA (procedure 2.3)

8) The discussion section seems to have written hastily. Please consider editing for sentence structure and flow. For example: line 221-222 - "drops in viability" could be changed to "reduced viability." Line 224 - "easiest" to "most". Line 226 - "too short a half-life for the application other…" is confusing. Line 229 - "…viable by time of injection." Needs modification.

We have rewritten the discussion, we hope that this draft is more consistent with the format requested by the JoVE editors.

Reviewer #2:

Major Concerns:
The authors should make explicit why is necessary to coat the glass capillaries with silicone.

We have added the following text to the manuscript:

NOTE: Coating the glass capillaries with silicone provides a chemically inert surface to the glass. If the capillaries are left untreated the cell suspension generated in later steps will adhere to the glass and plug the needle; therefore, coating is **NECESSARY** and **VITAL** to the success of the method.

It should also be clearly explained whether the tip of the glass needle needs to be in contact to the targeted tissue (if not, at which distance should it be?).

The needle tip should penetrate the target tissue. We have stated this in the revised procedure 6.3

Finally, is there any optimal cell concentration for this experiment? (will always work independently from the cell concentration in the injected suspension?).

We have poor injection quality at lower than 50,00 cells per ul. We have now stated this in the text (procedure 5.6).

Minor Concerns:
The protocol should provide alternatives. The use of indian ink to give some contrast during the injection (3.3) can be substituted by proper illumination with transmitted light (normally the light beam incidence angle should be of 90º with respect to the eyesight) or by using vital dyes like neutral red.

As the reviewer points out, there are many methods to increase contrast in the embryo. We felt that India ink would provide the best contrast for the video recording of the procedure (and that is why we included it). We have, however, referenced some alternative approaches as requested (procedure 3.3).