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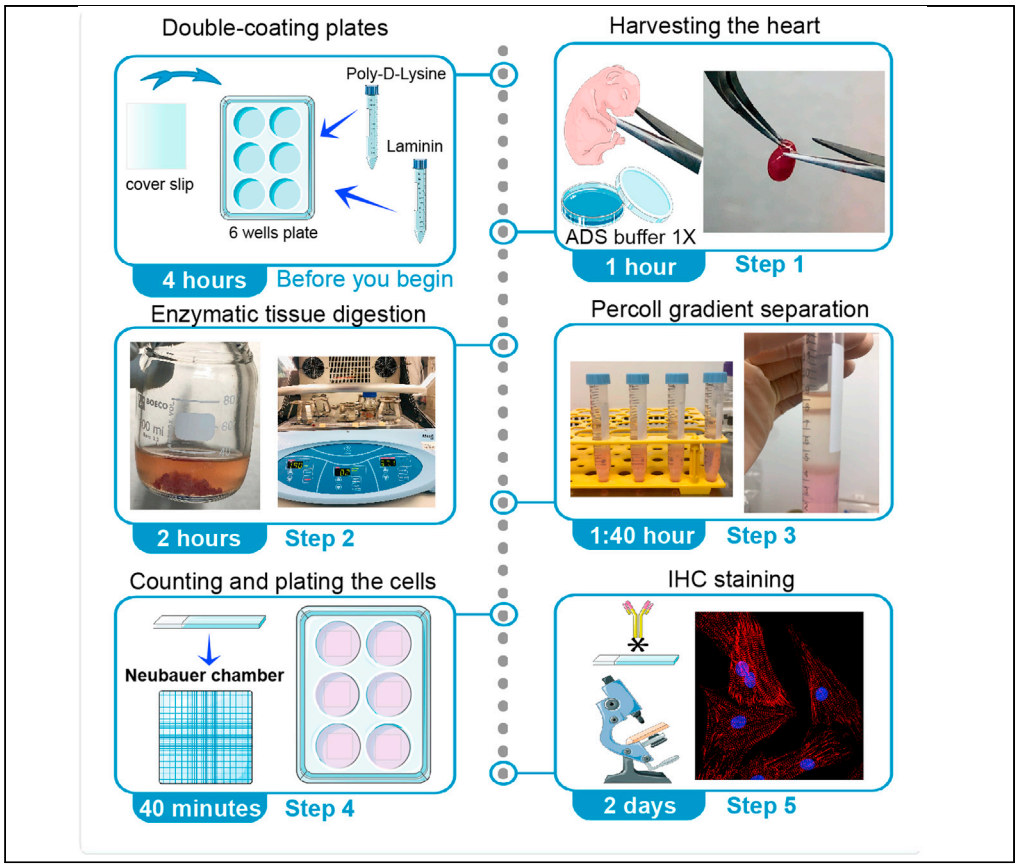
Isolation, culture, and immunostaining of neonatal rat ventricular myocytes



Isolation and culture of ventricular cardiomyocytes from neonatal rats (NRVMs) is a powerful model to study neonatal cardiac development, cell cycle regulation, and cardiac physiology and pathology *in vitro*. Here, we present our modified enzymatic digestion protocol followed by two-step discontinuous Percoll gradient centrifugation to isolate a high yield of viable ventricular cardiomyocytes from neonatal rats. Finally, here we describe an immunostaining protocol for cytosolic and nuclear staining of NRVMs.

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Highlights

Isolate a high yield of viable ventricular cardiomyocytes from neonatal rats (NRVMs)

NRVMs can be maintained in culture for several days

Enzymatic digestion of cardiac tissue and Percoll gradient separation

Detailed subsection of immunofluorescence for nuclear and cytosolic staining

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Isolation, culture, and immunostaining of neonatal rat ventricular myocytes

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SUMMARY

Isolation and culture of ventricular cardiomyocytes from neonatal rats (NRVMs) is a powerful model to study neonatal cardiac development, cell cycle regulation, and cardiac physiology and pathology *in vitro*. Here, we present our modified enzymatic digestion protocol followed by two-step discontinuous Percoll gradient centrifugation to isolate a high yield of viable ventricular cardiomyocytes from neonatal rats. Finally, here we describe an immunostaining protocol for cytosolic and nuclear staining of NRVMs.

For complete details on the use and execution of this protocol, please refer to Pereira et al. (2020).

BEFORE YOU BEGIN

Prepare the solutions before you start the neonatal cardiomyocytes isolation. Refer to the key resources table and materials and equipment sections for a complete list of materials and equipment.

All solutions are sterile or sterile filtered, and tools are surface sterilization with 70% ethanol. All steps are performed in a sterile laminar flow cell culture hood. This protocol is intended to isolate neonatal rat hearts from two-three litters (approximately 30 pups). For work with neonatal rodents, please refer to your local institutional guidelines and rules set forth by the legislature and animal care programs, and adhere to your institutionally approved animal protocol. All methods described in this protocol have been approved by the CEUA CNPEM (protocol number 68).

Prepare poly-D-lysine and laminin double-coated plates

Timing: 4 h

1. Rinse the coverslips with distilled water.
2. Autoclave the coverslips in 100 mL glass bottle Schott containing 50 mL of distilled water.
3. Prepare a 100 μg/mL Poly-D-Lysine working solution in sterile, distilled water.
4. Using sterile forceps, transfer the coverslips to each well of a 6-well plate.
5. Add 1 mL of the 100 μg/mL Poly-D-Lysine working solution to each well of a 6-well plate.
6. Incubate the coated plates at room temperature for 1–2 h.
7. Remove the Poly-D-Lysine solution and rinse it three times with distilled water.
8. Prepare a 15 μg/mL working solution of laminin in sterile distilled water.
9. Add 1 mL of the 15 μg/mL laminin working solution to each well of a 6-well plate.
10. Incubate the coated plates at 37°C for 2 h or overnight at 4°C.

11. Before use, pre-warm the culture vessel to room temperature for at least 1 h before aspirating and discarding the laminin solution.

Note: You can use the coated culture plate immediately or store it at 4°C wrapped in laboratory film for up to 1 week.

⚠ CRITICAL: All steps should be performed in a sterile laminar flow cell culture hood. In addition, during the coating procedure, do not allow the plate to dry for extended periods at any point.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rhodamine Phalloidin	Invitrogen™	Cat# R415
α-Actinin antibody	Sigma-Aldrich	Cat#A7811
MEF2 antibody	Santa Cruz Biotechnology, Inc.	Cat#sc313
AlexaFluor-568-conjugated goat anti-mouse	Thermo Fisher Scientific	Cat# A-11004
AlexaFluor-488-conjugated goat anti-rabbit	Thermo Fisher Scientific	Cat# A-27034
AlexaFluor-488-Phalloidin	Thermo Fisher Scientific	Cat# A-12379
Chemicals, peptides, and recombinant proteins		
Collagenase, Type 2	Worthington Biochemical Corporation	Cat# LS004176
Percoll^{R}	Sigma-Aldrich	Cat# GE17-0891-01
Laminin Mouse Protein, Natural	Gibco™	Cat# 23017015
Poly-D-Lysine	Sigma-Aldrich	Cat# P7280
Horse Serum, New Zealand origin	Gibco™	Cat# 16-050-122
Fetal bovine serum	Vitrocell Embriolife	Cat# 50011
Penicillin-Streptomycin (10,000 U/mL)	Gibco™	Cat#15140122
Pancreatin from porcine pancreas	Sigma-Aldrich	Cat# P1750
Hepes sodium salt	Sigma-Aldrich	Cat# H7006-100G
NaCl	Merck	Cat# 1.06404-1000
NaH_2PO_4	Merck	Cat# 1.06346.1000
D-glucose	Sigma-Aldrich	Cat# G5400
KCl	Sigma-Aldrich	Cat# P-9333
$MgSO_4.7H_2O$	Merck	Cat# 1.05886.1000
Sodium bicarbonate	Sigma-Aldrich	Cat# 55761
DMEM, powder, high glucose, pyruvate	Gibco™	Cat# 12800017
Phenol Red	Sigma-Aldrich	Cat# P3532-5g
DAPI	Sigma-Aldrich	Cat# D9542
Glycine	Sigma-Aldrich	Cat# G7126
Bovine Serum Albumin lyophilized powder (BSA)	Sigma-Aldrich	Cat# A3733
VECTASHIELD® Mounting Medium	Vector Laboratories	Cat# H-1000-100
Experimental models: Organisms/strains		
Neonatal Wistar HanUnib Rats (1–3 days old)	CEMIB/Unicamp, Campinas, SP	Protocol CEUA #68
Other		
50 mL/100 mL beakers	Pyrex®	CLS100050
Laboratory bottle (Schott) with cap	Boeco®	Z305200
0.22μm filter	Millex Millipore	SLGV033RB
15 mL and 50 mL Centrifuge tube	Falcon A Corning Brand	Ref 352070
20 mL Syringe	BD Plastipak	Ref 990173
6-wells cell culture plate standard	SARSTEDT	Ref 833920
5, 10mL Serological pipette	SARSTEDT	Ref 861253001
Refrigerated centrifuge	Eppendorf	5810R
Inverted microscope	Nikon	TMS-F

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Confocal microscope	Leica Microsystems	TCS SP8
Large Incubated and Refrigerated Benchtop Orbital Shaker	Thermo Scientific	MaxQ 4000 SHKE4000 8CE
CO_2 incubator cell	Thermo Electron Corporation	HEPA Class 100
Biological Safety cabinet	Forma Scientific	Class II A/B3 Model 1184

MATERIALS AND EQUIPMENT

Solution preparation

Note: All procedures are performed with standard aseptic practices, such as a sterile laminar flow cell culture hood in all steps. Additionally, all solutions should be prepared using double-distilled purified water.

ADS buffer 10×, 100 mL

Reagent	Final concentration (mM)	Molecular weight	Amount (g)
NaCl	11.64	58.44	6.8
KCl	0.54	74.55	0.4
HEPES sodium salt	1.83	260.29	4.76
NaH2PO4	0.08	141.96	0.12
Glucose	0.56	180.16	1.0
MgSO4 x 7H2O	0.04	246.48	0.1
ddH2O	n/a	n/a	100 mL
Total	n/a	n/a	100 mL

Note: pH 7.35 ± 0.05
The solution needs to be sterilized through 0.22 μm filter.

ADS buffer 1×, 500 mL

Reagent	Final concentration	Amount
ADS buffer 10×	1×	50 mL
ddH_2O	n/a	450 mL
Total	**n/a**	**500 mL**

Note: pH 7.35 ± 0.05
The solution needs to be sterilized through 0.22 μm filter.

Collagenase buffer, 60 mL

Reagent	Final concentration	Amount
ADS buffer 1×	1×	60 mL
Pancreatin	0.2 mg/mL	12 mg
Collagenase II	207 U/mL	12,420 U
Total	n/a	60 mL

Note: The solution needs to be sterilized through the filter 0.22μm and should be used immediately; do not store it.

Percoll stock solution, 20 mL

Reagent	Final concentration	Amount
Percoll	90%	18 mL
ADS 10×	n/a	2 mL
Total	n/a	20 mL

Note: Should be used immediately, do not store.

Low-density Percoll separation solution (density = 1.059), 20 mL		
Reagent	Final concentration	Amount
Percoll stock solution	40.5%	9 mL
ADS 1×	n/a	11 mL
Total	n/a	20 mL
Note: Should be used immediately, do not store.		

High-density Percoll separation solution (density = 1.082), 16.8 mL		
Reagent	Final concentration	Amount
Percoll stock solution	58.9%	11 mL
ADS 1×	n/a	5.8 mL
Phenol red	0.02mM	7 mg
Total	n/a	~16.8 mL
Note: Should be used immediately, do not store.		

Plating culture medium (pH 7.4), 200 mL		
Reagent	Final concentration	Amount
Horse serum	10%	20 mL
Fetal bovine serum	5%	10 mL
Pen/Strep	1%	2 mL
DMEM	n/a	200 mL
Total	n/a	200 mL

Maintaining culture medium (pH 7.4), 200 mL		
Reagent	Final concentration	Amount
Fetal bovine serum	2%	4 mL
Pen/Strep	1%	2 mL
DMEM	n/a	200 mL
Total	n/a	200 mL
Note: Warm the medium to 37°C before use.		

PBS buffer 1×, 1 L			
Reagent	Final concentration (mM)	Molecular weight (g/mol)	Amount (g)
NaCl	137	58.44	8.01
KCl	2.7	74.55	0.20
Na_2HPO_4	10	141.96	1.42
KH_2PO_4	1.8	136.08	0.24
ddH2O	n/a	n/a	Up to 1L
Total	n/a	n/a	1L
Note: pH 7.4 ± 0.05			

STEP-BY-STEP METHOD DETAILS

Harvesting heart ventricles from neonatal rat

Timing: 1 h

⚠ CRITICAL: Sterilize scissors (one curved; one straight) and forceps.

Note: All following steps are performed in the sterile cell-culture hood.

1. Transfer 20 mL of 1× ADS buffer into each of the two sterile 100 mm dishes placed on ice.
2. Prepare 60 mL of Collagenase Buffer in 100 mL sterile Beaker.
3. Filter 15 mL of Collagenase Buffer with a 0.22 μm filter using a syringe in a 100 mL sterile laboratory bottle (Schott) with cap.

Note: The Collagenase Buffer volume for the first digestion is 15 mL. For the following digestions, the volume is reduced to 10 mL.

4. 1–3 days old neonatal rats are rinsed quickly in 75% ethanol solution for surface sterilization. The ethanol was spread using a wash bottle before decapitation focusing on the pup's chest. Pups are decapitated using sterile scissors (straight), and the chest is opened along the sternum to allow access to the chest cavity and the heart.
5. Hearts are extracted from the body with curved scissors and transferred immediately into the 100 mm dish containing ~20 mL of 1× ADS buffer.
6. Remove lung tissue, large vessels and atria. Rinse the heart in the 1× ADS solution to remove blood.
7. Transfer the heart to the second 100 mm dish containing 1× ADS to remove any additional blood.
8. Using a scissor, cut the hearts in 4–5 pieces directly into the 100 mL Schott containing 10 mL of filtered Collagenase Buffer (Figures 1A and 1B).
9. Repeat the procedure (steps 4–8) with the remaining pups
10. After the last heart has been collected and cut into small pieces, cap the Schott and incubate the heart tissue with the collagenase solution under continuous agitation,180 rpm, at 37°C for 10 min (Figure 1C).

Enzymatic tissue digestion

Timing: 2 h

⚠ CRITICAL: The digestion temperature (37°C) is a crucial point for the complete digestion of the hearts.

11. After 10 min, use a sterile 10 mL serological pipette to pipette up and down 10 times to homogenize the sample and improve the tissue dissociation.
12. Allow the tissues to settle by gravity for 1 min, collect the supernatant (approximately 11 mL) and transfer to 50 mL conical tubes.
13. Add 10 mL 0.22 μm filtered Collagenase Buffer solution to the remaining cardiac tissue and incubate under 180 rpm agitation at 37°C for 10 min.

Note: The shaker utilized here display an orbital motion rage of 1.9 cm (0.75 in).

14. Stop enzymatic degradation by removing the Schott from the 37°C shaker and by adding 1 mL of fetal bovine serum to the 50 mL conical tube containing the 11 mL of the supernatant (step 12).
15. Centrifuge at 2,000×*g* for 5 min at 25°C.
16. Carefully discard the supernatant and add 2 mL of the fetal bovine serum to the cell pellet and homogenize pipetting up and down using a serological pipette.

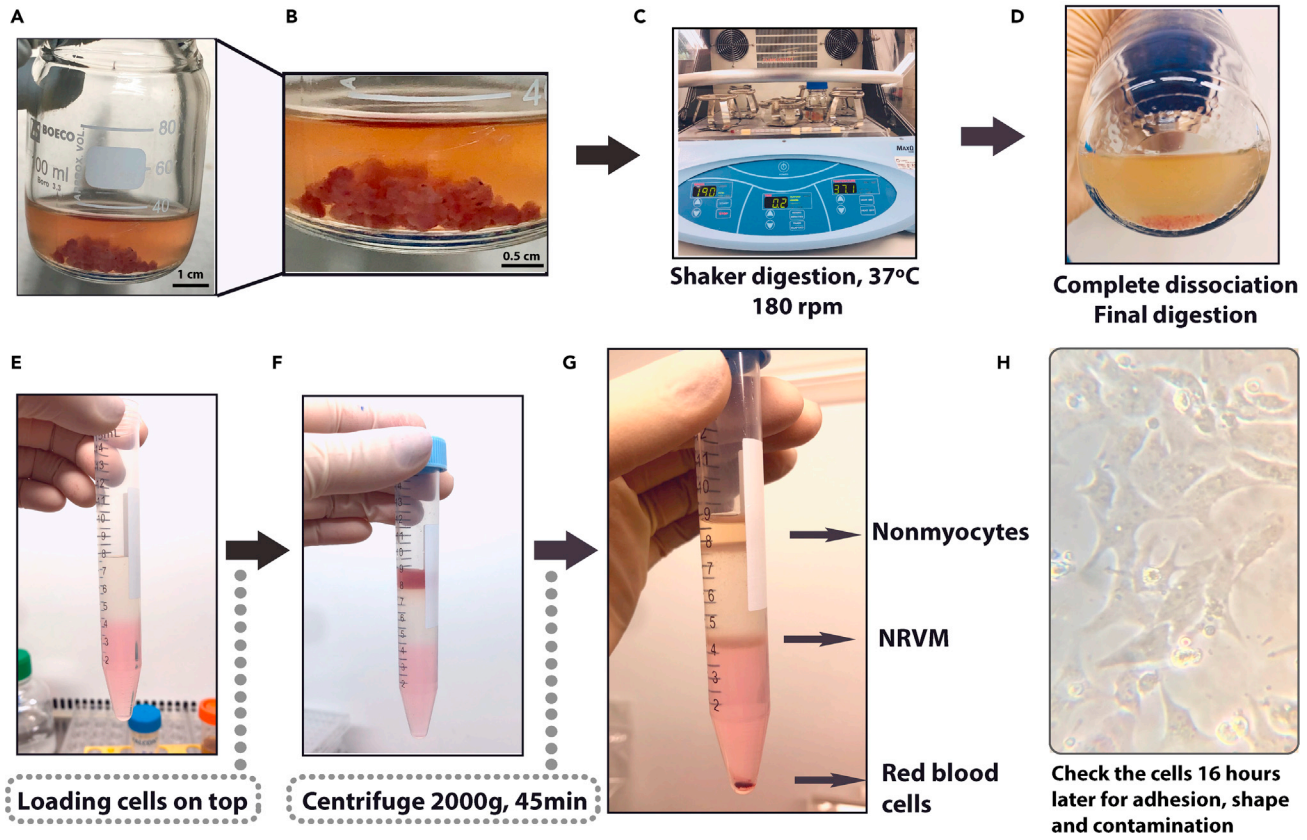

Figure 1. Enzymatic digestion of cardiac tissue and Percoll gradient separation

(A and B) The heart ventricles were minced in 4–6 pieces and transfer to a bottle containing 10 mL of Collagenase Buffer.

(C) For optimal digestion, the enzymatic solution with the cardiac tissue was placed under agitation at 37°C, 180 rpm.

(D) After ~60 min, the cardiac tissue is almost complete digested.

(E–G) Cardiomyocyte enrichment step by discontinuous Percoll gradient centrifugation.

(H) After 16 h in culture, check the cells under microscopy for shape, adhesion and spontaneous contractions (see Methods video S2) (40× magnification).

17. Transfer the cell suspension to a sterilized 100 mm glass petri dish (non-treated) to allow the fibroblasts to adhere. Incubate at 37°C, 5% CO_2 for at least 1 h, the time it takes to finish all digestions.
18. Repeat the digestion, centrifugation and the pre-plating process (steps 11–17) 4–5 times with the remaining tissue fragments.
19. After finishing the last digestion (Figure 1D) and pre-plating cycle, transfer the supernatant of all five Petri dishes into a 50 mL conical tube.

Note: Pre plating is a process that consists in seeding the cells on an uncoated glass petri dish to allow fibroblasts and endothelial cells to adhere, improving the purity of the culture. In addition to increasing the purity of the cardiomyocyte isolation, this procedure enables isolated and cultivate high yields of cardiac fibroblasts and endothelial cells from neonatal rats.

Note: The number of Petri dishes used in this step is variable accordantly to the number of digestion cycles performed in step 18.

20. Gently wash the Petri dishes with 6 mL fetal bovine serum and transfer the supernatant to one conical tube.

21. Centrifuge at 2,000×*g* for 10 min at 25°C.
22. Resuspend the cells in 3 mL of 1× ADS buffer.

Note: The Petri dishes have a high yield of viable primary fibroblasts attached and can be maintained in Culture Medium for further experiments. Cardiac fibroblasts in culture proliferate to form a confluent monolayer. Please refer to Golden et al. (2012).

Percoll gradient separation

Timing: 1 h 40 min

23. In this step, a high yield of viable cardiomyocytes will be separated from non-cardiomyocytes using two-step discontinuous Percoll gradient centrifugation.
24. Prepare the two-layers Percoll gradient in a 15 mL conical centrifuge tube. For 30–40 hearts, prepare four 15 mL centrifuge tubes.
25. To each of the four 15 mL centrifuge tubes, the two-layers Percoll density gradient is created by carefully loading 4 mL of the low-density Percoll solution on top of the 4 mL of the high-density Percoll solution (Figure 1E). Tilt the tube during this procedure to avoid mixing the Percoll layers.
26. Slowly load up to 1 mL of cell suspension (obtained in step 22) on the top layer of the Percoll gradient prepared above. During cell loading, the tube should be tilted (Figure 1F and Methods video S1).
27. Centrifuge the tubes at 1,800×*g* for 45 min at 25°C.

⚠ CRITICAL: This step requires that acceleration and deceleration be switched off completely to prevent the layers' remix. Therefore, the centrifuge accelerates and stop slowly, avoiding disturbing the Percoll gradient.

28. After centrifugation, the NRVMs are located at the interface of the low and high-density gradients (Figure 1G).
29. Using a sterile 5 mL pipette, start carefully discarding the layers corresponding to the high-density Percoll layer or non-cardiomyocytes.
30. Repeat this step with the remaining centrifuged tubes.
31. Using a new sterile 5 mL pipette, collect the band corresponding to the NRVMs and transfer it to a further 50 mL conical centrifuge tube.
32. Repeat this procedure with the remaining centrifuged tubes and combine them into the same 50 mL conical tube.
33. Add 30 mL of 1× ADS buffer.
34. Centrifuge the 50 mL tube at 1,800×g for 10 min at 25°C. Discard the supernatant in a waste container.
35. Gently resuspend the cell pellet by pipetting 30 mL of 1× ADS buffer and repeat the previous wash step (step 34).
36. Resuspend the pellet with 5 mL of warm Plating Culture Medium by gently pipetting to ensure a homogeneous solution of single cells.

Counting and plating the cells

Timing: 40 min

The NRVMs yield and viability are determined by a Neubauer chamber and trypan blue staining. Trypan blue staining is used to indicate the ratio of live to dead cells as the stain turns dead cells that have compromised membranes blue, while live cells will remain colorless.

37. Take 10 μL of resuspended cells (step 36) into a new 1.5 mL tube and add 10 μL of 0.4% trypan blue dye solution. Mix gently.
38. Load 10 μL of the mixture above into the well of the Neubauer counting chamber.
39. Using the microscope to focus on the grid of the Neubauer counting chamber determine the total number of viable cells, following the standard procedure for counting cells and the calculation for determining total cell number.
40. Add 3 mL of fresh Plating Culture Medium per 35 mm dish or per well in 6-wells plate.
41. Plate the cells on the double coated surface. For immunostaining, we recommend 500,000 cells/35 mm dish or per well in a 6-wells plate containing coverslip on the bottom to achieve 70–80% of confluence.

Note: If desired, before plating, add Bromodeoxyuridine (BrdU), a thymidine analog (100 μM final concentration), to avoid the proliferation of non-myocytes.

42. Incubate at 37°C, 5% CO_2.
43. Forty-eight hours after plating, gently change the medium with 3 mL of Maintaining culture medium. Using the microscope confirm that NRVMs are attached and exhibit spontaneous beating (Methods video S2). Any non-attached cells should be removed during the medium exchange procedure. At this point, any treatment, transfection or virus transduction may be performed according to the experimental design.

Immunofluorescence staining

Timing: 2 days

44. Remove the medium and rinse briefly in 1× phosphate-buffered saline (PBS).
45. Fix cells with 4% paraformaldehyde in PBS pH 7.4 for 30 min on ice (Figure 2A).
46. Wash three times for 5 min with 1× ice-cold PBS.
47. Next, permeabilize and block any unspecific binding of the antibodies by incubating the cell with 1% BSA, 0.1% Triton-X100, 50 mM glycine in PBS for 30 min (Figure 2B).
48. Wash three times for 5 min with 1× PBS.
49. Incubate the cells with primary antibodies (1:200 dilution) in 1% BSA in PBS overnight at 4°C (Figure 2C).
50. Wash three times for 5 min with 1× PBS.
51. Incubate the cells with Alexa Fluor-conjugated secondary antibody (1:200) for 2 h at room temperature. All subsequent steps should be carried out in the dark as fluorescent probes are light sensitive (Figure 2D).
52. Wash three times for 5 min with 1× PBS.
53. If applied, perform the cytosolic staining with phalloidin (1:50) for 1 h at room temperature (Figure 2E).

Note: Please use fluorophores with different emission spectra in multi-color immunofluorescence experiments to avoid spectral overlap.

54. Wash for 5 min with 1× PBS.
55. Incubate with the nuclear stain DAPI (1:500) at room temperature for 5 min (Figure 2E).
56. Wash for 5 min with 1× PBS.
57. Place the coverslip onto a drop of mounting media (Vectashield) with the cells facing the slide (Figure 2F).
58. Seal the coverslip with nail polish by gently outlining the perimeter of the slide.
59. Samples can then be examined using a fluorescence microscope (Figures 2G–2I) or stored in the dark at −20°C.

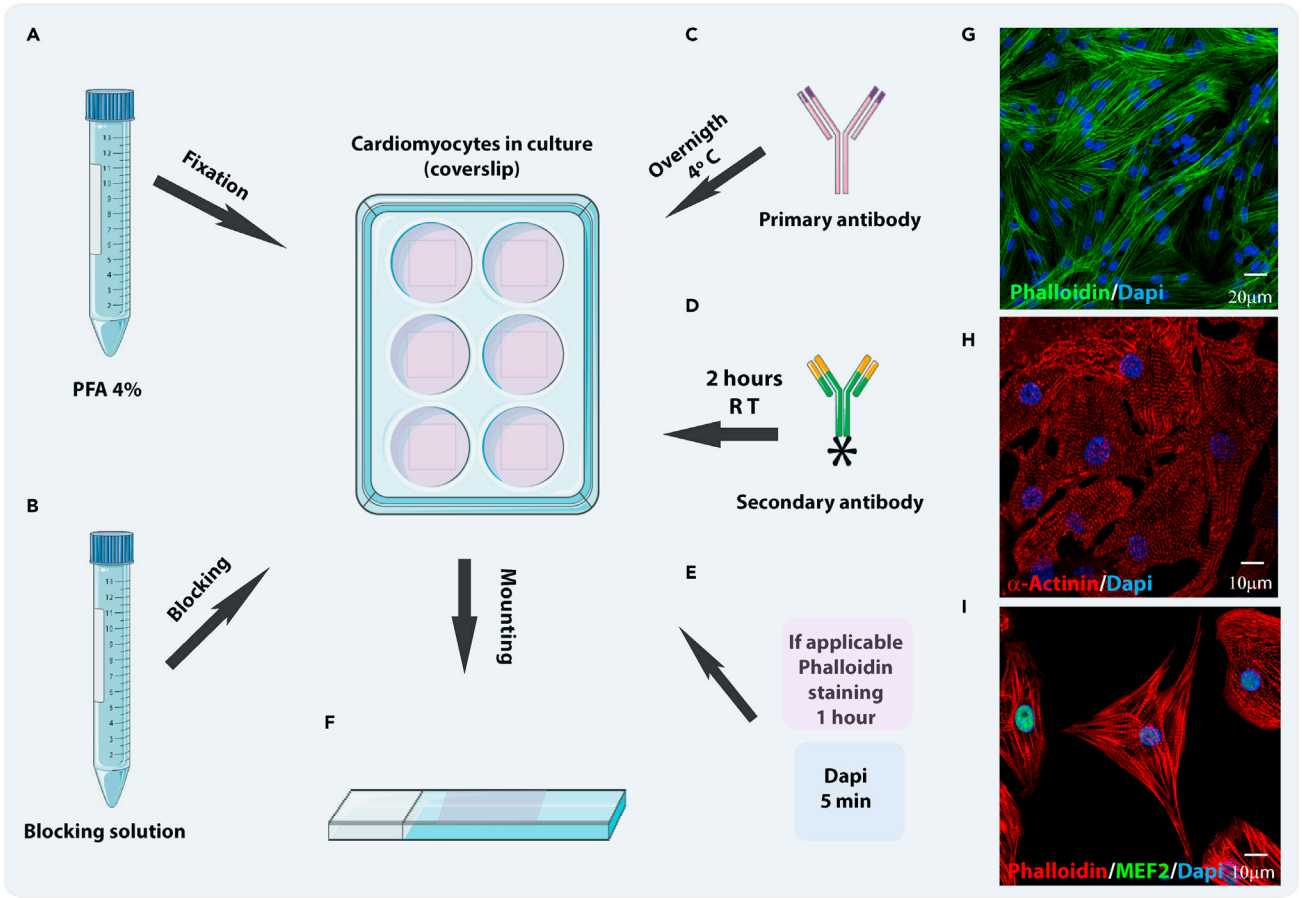

Figure 2. Immunofluorescence staining and imaging

(A) Fix the cells with 4% paraformaldehyde in PBS.

(B) Blocking any unspecific antibodies binding by incubating the cell with 1% BSA, 50 mM glycine in PBS.

(C) Incubate with primary antibody.

(D) Incubate the cells with a secondary antibody.

(E) Alternatively, incubate the cells with phalloidin-rodhamin and DAPI.

(F) Mounting the coverslip using mounting medium Vectashield.

(G) Representative images of cardiomyocytes staining with phalloidin in green and Dapi in blue.

(H) Representative pictures of cardiomyocytes staining with α-actinin antibody in red and Dapi in blue.

(I) Representative images of cardiomyocytes stained with MEF2 antibody in green, phalloidin in red and Dapi in blue. Scale bar, 10 µm.

EXPECTED OUTCOMES

A high yield of viable cardiomyocytes was isolated from 25 neonatal rat hearts through our protocol. The average result of purified cells was (1.27 ± 0.11) x 10^6 cells per neonatal heart, and the average viability accessed by Trypan blue staining was approximately 80 ± 3.3% (n=3). The cardiomyocyte purity calculated by the ratio of myocyte specific antibody (sarcomeric α-actinin) positive cells to total cells determined by DAPI nuclear staining was 87 ± 2.08% (n=3). Our results are similar to previous studies that reported approximately 1 × 10^6 NRVM per neonate rat heart with 70–90% viability (GOLDEN, H. B et al., 2012). The cardiomyocytes can be maintained in culture for 6–7 days with a medium change every two days. Sixteen hours after plating, the cardiomyocytes can be observed under the microscopy for attachment to the laminin-coated cell culture dishes (with approximately 70–80% confluency) (Figure 1H) and display spontaneous contractility (Methods video S2).

LIMITATIONS

Addition of proliferation inhibitors

Even with the pre-plating step followed by the Percoll gradient separation to improve cardiomyocyte purity, the remaining population of nonmyocytes, such as cardiac fibroblasts and endothelial cells, will undergo cell proliferation at high rate, and significantly affect the cell-population over time. Therefore, the addition of proliferation inhibitors, such as Bromodeoxyuridine (BrdU) to the Maintaining Culture Medium is recommended to prevent the proliferation of nonmyocytes. However, the addition of proliferation inhibitors may affect the experimental design and results interpretation(Ehler et al., 2013). Therefore, proliferation inhibitors must be used with caution depending on experimental purposes.

Non-viral transfection of NRVMs

The efficiency of transfection of NRVMs with liposomal reagents or electroporation is limited, typically reported lower than 10% (Ehler et al., 2013). Alternatively, gene transfer using viral vectors (such as adenovirus, adeno-associated virus, and retrovirus) have been reported to achieve higher efficiency rates (Djurovic et al., 2004), with minimal impact in cell viability and cellular morphology (Consonni et al., 2021). For more details about gene transfer in culture cardiomyocytes, please refer to (Djurovic et al., 2004).

Passaging and cryopreservation

Subculturing the primary cardiomyocytes is challenging and not recommendable (Ehler et al., 2013). The cardiomyocyte viability and beating capacity were significantly reduced during this procedure.

TROUBLESHOOTING

Problem 1

Cardiac tissue does not digest appropriately; the amount of the tissue does not decrease after 5–6 rounds of digestion. (Step 17)

Potential solution

Check the temperature of the shaker.

Increase the enzyme concentration.

Add 0.2mg/mL of DNaseI to Collagenase Buffer

Problem 2

Low cell viability (many trypan blue positive cells). (Step 39)

Potential solution

Reduce the digestion time. It is not recommended more than 5–6 rounds of digestion due to loss of cell viability

Reduce the pre-plating time. Pre-plating time longer than 2–3 h may reduce the cell viability

Check the centrifuge speed and temperature

Check the pH of the solutions

Problem 3

No viable or adherent NRVMs 16–18 h after plating. (Step 43)

Potential solution

Try different substrate coatings like collagen or gelatin.

Check cell culture incubator (temperature, humidity and CO_2)

Check for contamination

Problem 4
The percoll layers are not observed, or no layer forms on the Percoll gradient. (Step 28)

Potential solution
Check the brake and acceleration of centrifugation.

The ADS 1× solution should be done from the same ADS 10× stock solution.

Problem 5
Immunofluorescence staining does not work, or unspecific staining. (Step 59)

Potential solution
Increase antibody concentration.

Increase blocking time.

The secondary antibody control should be performed following the same staining protocol without the addition of the primary antibody

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the lead contact Kleber Gomes Franchini (kleber.franchini@lnbio.cnpem.br)

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100950.

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AUTHOR CONTRIBUTIONS
A.H.M.P. conducted most of the experiments and data analysis. A.C.C. performed partial experiments and data analysis. A.H.M.P. and A.C.C. wrote the manuscript. K.G.F. supervised the project and revised the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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