Video Article

Methods for the Isolation, Culture, and Functional Characterization of Sinoatrial Node Myocytes from Adult Mice

Emily J. Sharpe*[1], Joshua R. St. Clair*[1,2], Catherine Proenza[1,3]

[1]Department of Physiology and Biophysics, University of Colorado Anschutz Medical Campus

[2]Department of Bioengineering, University of Colorado Anschutz Medical Campus

[3]Department of Medicine, Division of Cardiology, University of Colorado Anschutz Medical Campus

*These authors contributed equally

Correspondence to: Catherine Proenza at Catherine.Proenza@ucdenver.edu

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Abstract

Sinoatrial node myocytes (SAMs) act as the natural pacemakers of the heart, initiating each heart beat by generating spontaneous action potentials (APs). These pacemaker APs reflect the coordinated activity of numerous membrane currents and intracellular calcium cycling. However the precise mechanisms that drive spontaneous pacemaker activity in SAMs remain elusive. Acutely isolated SAMs are an essential preparation for experiments to dissect the molecular basis of cardiac pacemaking. However, the indistinct anatomy, complex microdissection, and finicky enzymatic digestion conditions have prevented widespread use of acutely isolated SAMs. In addition, methods were not available until recently to permit longer-term culture of SAMs for protein expression studies. Here we provide a step-by-step protocol and video demonstration for the isolation of SAMs from adult mice. A method is also demonstrated for maintaining adult mouse SAMs *in vitro* and for expression of exogenous proteins via adenoviral infection. Acutely isolated and cultured SAMs prepared via these methods are suitable for a variety of electrophysiological and imaging studies.

Video Link

The video component of this article can be found at https://www.jove.com/video/54555/

Introduction

Pacemaker myocytes in the sinoatrial node of the heart (sinoatrial myocytes, "SAMs") generate spontaneous, rhythmic action potentials (APs) that propagate through the myocardium to initiate each heartbeat. Experiments using acutely isolated SAMs from many species have been essential for elucidation of mechanisms that contribute to the generation of pacemaker activity. SAMs are highly specialized cardiomyocytes that differ substantially from their counterparts in the atrial and ventricular myocardium in terms of morphology, function, and protein expression. The hallmark of spontaneous APs in SAMs is a spontaneous depolarization during diastole that drives the membrane potential to threshold to trigger the next AP[1,2]. This "pacemaker potential" depends on the coordinated activity of many different membrane currents including the "funny current" (I_f), T- and L-type calcium currents, and the sodium-calcium exchanger current (I_{NCX}), which is driven by Ca^{2+} release from the sarcoplasmic reticulum[3,4].

While acutely isolated mouse SAMs are an essential experimental preparation for the study of pacemaking, the isolation of SAMs from mice can be a challenging method to adopt because the indistinct anatomy and small size of the mouse SAN requires a nuanced microdissection and the combined enzymatic and mechanical dissociation of the cells requires careful optimization.

We provide here a detailed video demonstration of a protocol that has been successfully used to isolate SAMs from adult mice for patch clamp recordings[5-8]. To our knowledge, there is no such visual demonstration available from any other source. In addition, a new method is demonstrated in which isolated SAMs from adult mice can be maintained *in vitro* for several days, thereby permitting the introduction of proteins, genetically encoded reporter molecules or RNAi via adenoviral infection[9].

Protocol

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus. The standard protocol below has been optimized using male C57BL/6J mice of 2-3 months of age.

1. Prepare Solution Stocks and Supplies in Advance of Experiments

NOTE: Refer to **Materials Table** for necessary equipment and supplies.

1. Prepare 1 L each of the following solutions as indicated in **Table 1**: Complete Tyrode's, Low Ca^{2+}/Mg^{2+} Tyrode's, Modified Kraft-Brühe (KB) Solution, and Bovine Serum Albumin (BSA) Solution. Use ultrapure filtered deionized water for all solutions. Divide each solution into 50 ml aliquots and store at 20 °C for up to six months. Thaw individual aliquots immediately before experiments, and store for up to one week at 4 °C.
2. Prepare 50 ml of 10 mM NaCl and 1.8 mM $CaCl_2$ Adaptation Solution by dissolving NaCl and $CaCl_2$ in ultrapure filtered deionized water (**Table 1**). Store at room temperature for up to six months.
3. Prepare 4.75 enzyme activity unit (U) aliquots of elastase by pipetting into microfuge tubes. Store at 4 °C for up to three months.
4. Prepare 375 µg aliquots (in ultrapure H_2O) of collagenase-protease enzyme blend by pipetting into microfuge tubes. Store at -20 °C for up to three months.
5. Prepare two fire-polished Pasteur pipettes, one for tissue transfer (~1.5 mm final opening diameter; **Figure 1Aiv** and **Figure 1C**) and one for dissociation (~2 mm diameter; **Figure 1Aiii** and **Figure 1C**).
    1. Score Pasteur pipettes with a glass cutter and snap along the score to produce an opening slightly larger than the desired size. Fire-polish the cut end of each pipette for ~30-60 sec over a low flame on a Bunsen burner to produce a thick polished wall and an opening of the desired diameter. Ensure the fire-polished opening is free of any cracks or rough edges.
6. Prepare two dissection dishes by adding ~25 ml silicone elastomer mixed according to manufacturer's directions to each 100 mm Petri dish (**Figure 1Ai** and **Figure 1B**). Allow to cure at room temperature for 48 hr.
7. For culture only: prepare 25-50 ml each Plating Medium and Culture Medium as per **Table 2**. Store for up to two weeks at 4 °C.

2. Prepare Solutions to Be Used on Day of Cell Isolation

NOTE: The following amounts are for isolation of sinoatrial myocytes from one mouse.

1. Add 2.5 ml of Low Ca^{2+}/Mg^{2+} Tyrode's (pH 6.9) to each of three small, round-bottomed culture tubes. Place tubes in 35 ± 1 °C water bath.
2. Add 2.5 ml of Low Ca^{2+}/Mg^{2+} Tyrode's to one large round-bottomed culture tube. To this tube, add 1 aliquot (4.75 U) elastase and one aliquot (375 µg) collagenase-protease enzyme blend. Swirl to mix. Place tube in 35 ± 1 °C water bath.
3. Add 2.5 ml of KB Medium to three additional small, round-bottomed culture tubes and one additional large, round-bottomed culture tube. Place tubes in 35 ± 1 °C water bath.
4. Add ~7 ml of BSA solution to one large bottomed culture tube. Keep this tube at room temperature.
5. Place 20-40 ml of Complete Tyrode's solution in a 50 ml beaker (**Figure 1Aii**). Add 10 USP/ml heparin, swirl to mix, and place the beaker in the 35 ± 1 °C water bath.

3. Prepare Additional Solutions and Materials for Cultured Cells (Skip These Steps for Acutely Isolated Cells)

1. In a sterile tissue culture hood, place two 12 mm round glass coverslips per mouse into individual wells of a 24-well plate.
   NOTE: The 24 well plate is used because the wells are a convenient size, which serves to limit the volume for subsequent viral infections.
2. Pipette approximately 200 µl of a 100 ng/ml solution of mouse laminin diluted in sterile phosphate buffered saline (PBS) onto each coverslip.
3. Incubate coverslips with laminin for the duration of the isolation (at least 1 hr) in an incubator at 37 °C.
4. Pre-warm the Plating Medium and Culture Medium (from Step 1.7 and **Table 3**) to 37 °C.

4. Sinoatrial Node Isolation

1. In a chemical fume hood, place a mouse in one chamber of a two-chamber box and anesthetize with ~200 µl liquid isoflurane introduced via a cotton swab into the other chamber. Confirm anesthesia (usually within ~30-60 sec) with a toe pinch. Euthanize mouse by cervical dislocation.
   NOTE: An empty 1 ml pipette tip box can be used to form the two-chamber box; turn the rack upside down in the box to create the separate compartment for the isoflurane to prevent the mouse from contacting it directly.
2. Remove fur from chest with scissors and transect rib cage to expose the chest cavity using external tissue forceps (**Figure 1Aviii**) and dissection scissors (**Figure 1Avix**). Bathe the chest cavity with ~2 ml warmed Complete Tyrode's with Heparin using a transfer pipette.
   NOTE: Continue bathing chest cavity when necessary, do not allow the preparation to dry out.
3. Under a dissecting microscope, carefully remove the lungs and thymus using internal scissors (**Figure 1Avi**) and dissection forceps (**Figure 1Avii**).
4. While gently holding the apex of the heart with the internal dissection forceps, carefully cut the inferior vena cava and the aorta with the internal scissors to remove the heart from the chest cavity. Transfer the heart to one of the silicone dissection dishes and bathe with ~4 ml warmed heparinized Complete Tyrode's using the transfer pipette.
5. Orient the heart such that the posterior vessels are visible and facing up, with the animal's right atrium on the experimenter's right and left atrium on the experimenter's left. Once oriented, immobilize the heart by pinning through the apex into the silicone dissection dish.
6. Locate the groove between the ventricles and the atria (clear ring above the ventricles).
7. Using the internal dissection scissors, make an incision at the groove, keeping closer to the ventricles than the atria. Flush the groove and incision with additional warmed heparinized Complete Tyrode's as needed to allow a clear view of the atria and the valves. Continue to cut along the groove to separate the atria from the ventricles.
8. Transfer the atrial tissue to the second silicone dissection dish and bathe with ~3 ml warmed heparinized Complete Tyrode's.

9. Orient the tissue so that the animal's right atrium is now on the experimenter's left, and the left atrium is on the right.
   NOTE: The right atrium is more transparent, while the left atrium has more of a dark red tone.
10. Pin the tissue through the inferior and superior vena cavae and the right and left atrial appendages, stretching the preparation gently. Remove any remaining fat or other tissue to allow a clear view of the preparation (be careful to not cut into the atrial wall, as the sinoatrial node is quite delicate and can be easily damaged).
11. Open the anterior wall of the atria by cutting through the venae cavae. Re-position the pins as necessary to visualize the interatrial septum.
12. Cut along the interatrial septum to remove the left atrium. Re-pin the preparation, stretching gently.
13. Remove the right atrial appendage and free the sinoatrial node by cutting along the cristae terminalis, which appears as a dark orange streak bordering the atrial appendage.
14. Re-pin the nodal tissue and cut it laterally (perpendicular to the crista terminalis) to produce three equally sized strips.

5. Sinoatrial Node Digestion

1. Using the narrow fire-polished pipette (**Figure 1Aiv**), transfer the three strips of sinoatrial node tissue into the first of three small, round-bottomed tube containing 2.5 ml of low Ca^{2+}/Mg^{2+} Tyrode's in the 35 ± 1 °C water bath. Incubate for 5 min.
2. Transfer tissue strips to the second small, round bottom tube containing 2.5 ml low Ca^{2+}/Mg^{2+} Tyrode's in the 35 ± 1 °C water bath, using the same narrow pipette. Wash the tissue strips by gentle swirling the tube or by gently pipetting with the narrow pipette. Do not invert the tube.
3. Transfer tissue strips to the third small, round bottom tube containing 2.5 ml low Ca^{2+}/Mg^{2+} Tyrode's, and repeat the washing step described in step 5.2.
4. Transfer strips into the large round-bottomed tube containing 2.5 ml of low Ca^{2+}/Mg^{2+} Tyrode's with enzymes (elastase plus collagenase-protease blend) in the 35 °C water bath. Ensure that all three tissue strips are present. Incubate for 10-15 min at 35 ± 1 °C. Mix every 5 min by gently swirling the tube. Do not invert the tube.

6. Sinoatrial Node Myocyte Dissociation

1. Following the enzyme digestion, use the narrow fire-polished pipette to gently transfer the tissue strips to the first small, round-bottomed tube containing 2.5 ml KB solution at 35 ± 1 °C. Wash tissue by gently swirling the tube.
   Note: Tissue strips will appear somewhat translucent and may clump together at this point. Handle very gently after enzymatic digestion to avoid losing cells.
2. Transfer the tissue to the second small round-bottomed tube containing 2.5 ml KB at 35 ± 1 °C. Swirl gently to wash.
3. Transfer the tissue to the third small round-bottomed tube containing 2.5 ml KB at 35 ± 1 °C. Swirl gently to wash.
4. Transfer the tissue to the large, round-bottomed tube containing 2.5 ml KB at 35 ± 1 °C.
5. Using the larger fire-polished pipette (**Figure 1Aiii** and **Figure 1C**), dissociate the cells in the large round-bottomed tube by constant trituration at approximately 0.5-1 Hz for 5-10 min, taking care to keep the dissociation tube submerged in the 35 ± 1 °C water bath and to avoid introducing bubbles into the solution.
   Note: Trituration time varies with diameter of the dissociation pipette and force of pipetting. Time should be adjusted so that the tissue pieces remaining at the end of the dissociation appear thin, transparent and wispy. If tissue retains any color, the dissociation is likely to be incomplete. Frequency (0.5-1 Hz) is determined by hand.
6. Remove round-bottomed tube containing dissociated SAMs from the water bath and equilibrate at room temperature for 5 min.

7. Sinoatrial Node Calcium Re-adaptation (Performed at Room Temperature)

NOTE: For SAMs destined for culture experiments, the procedures in the following section should be performed in a sterile tissue culture hood. If SAMs are to be used for acute experiments, there is no need to perform these steps in a sterile environment.

1. Add 75 µl of NaCl/$CaCl_2$ adaptation solution (**Table 2**). Swirl gently to mix and incubate for 5 min.
2. Add 160 µl of NaCl/$CaCl_2$ adaptation solution. Swirl gently to mix and incubate for 5 min.
3. Add 390 µl of BSA solution (**Table 2**). Swirl gently to mix and incubate for 4 min.
4. Add 1.25 ml of BSA solution. Swirl gently to mix and incubate for 4 min.
5. Add 4.37 ml of BSA solution. Swirl gently to mix and incubate for 4 min.
   Note: The final concentration of calcium will be 1.8 mM.
6. Following calcium re-adaptation, collect SAMs by allowing to settle by gravity for ~10 min or by centrifugation at ~2,000 x g for 3 min.
   1. For acutely isolated cells, gently remove and discard about 5 ml of the supernatant using a glass Pasteur pipette, leaving cells in a volume of ~2 ml. Store these cells at room temperature for up to ~8 hr for patch clamp recordings.
   2. For cultured cells, remove as much of the supernatant as possible using a sterile glass Pasteur pipette. Resuspend cell pellet in 1 ml pre-warmed (37 °C) Plating Medium (**Table 2**).

8. Plating and Culture of Sinoatrial Myocytes (Skip for Acutely Isolated Cells)

1. Remove laminin solution from coverslips from Step 3.3 with a Pasteur pipette. Immediately seed 500 µl (~50-100 cells) onto each laminin-coated coverslip (from step 3).
   NOTE: The contractile inhibitor 2,3-butanedione monoxime (BDM) is included in the plating and culture media to prevent contraction, which causes attrition of cells[9].
2. Return the 24-well plate containing newly seeded SAMs to the incubator and maintain at 37 °C in an atmosphere of 95% air/5% CO_2. Allow cells to adhere to coverslips for 4-6 hr in plating media (**Table 2**).
3. Gently remove Plating Medium using a sterile Pasteur pipette. Replace with 500 µl per well of pre-warmed (37 °C) Culture Medium (**Table 2**).

9. Adenoviral Transduction of Adult Sinoatrial Myocyte Cultures (Skip for Acutely Isolated Cells)

1. Estimate number of cells per coverslip immediately before applying adenovirus by counting the cells in a field of view under a microscope, adjusting for magnification. Count all cells, not just SAMs.
2. Dilute adenovirus in Medium 199 and adjust the dilution for the viral titer so that application of 1-10 µl is required to achieve a final multiplicity of infection (MOI) of 100 viral units per cell. Add adenoviral solution in a dropwise fashion directly onto plated SAMs.
3. Incubate cells with the virus-containing medium overnight (~12-14 hr). Then exchange with fresh culture medium. Maintain cells in incubator, changing culture medium every 48 hr, until desired protein expression is obtained.

10. Functional Evaluation of Acutely Isolated or Cultured SAMs

NOTE: The following protocol is an example of functional assessments of isolated SAMs using the amphotericin perforated-patch technique to record both spontaneous APs and I_f from the same cell (see reference[9]).

1. Prepare recording solutions as described in **Table 3**.
2. Prepare a stock solution of 20 mg/ml amphotericin-B in DMSO fresh on the day of recording. Keep stock at room temperature and protect from light. Dilute amphotericin-B stock in intracellular solution to a final concentration of 200 ug/ml just before use. Maintain the final pipette solution on ice and protect from light.
   NOTE: The amphotericin-containing pipette solution for experiments should be prepared fresh hourly by diluting an aliquot of the stock solution into the intracellular solution and vortexing for at least 1 min.
3. Transfer an aliquot of the acutely dissociated SAM cell suspension or a fragment of glass coverslip bearing cultured SAMs to a recording chamber containing Tyrode's solution at 35 ± 1 °C. Perfuse cells with Tyrode's solution for at least 2 min prior to electrophysiological recordings to remove any residual BDM remaining from culture medium.
   NOTE: Spontaneous contractions should be evident immediately upon transfer to the Tyrode's solution. SAMs for recording can be identified by a combination of features including spontaneous contractile activity, characteristic morphology (*e.g.*, **Figure 2**), lack of striations, expression of HCN4 protein, presence of I_f current, membrane capacitance <50 pF, and spontaneous APs with waveforms that include a diastolic depolarization phase and a slow upstroke.
4. Using borosilicate glass pipettes with resistances of 1.5-3.0 MΩ, fill the tip with intracellular solution lacking amphotericin by dipping for 10-30 sec. Then back-fill the pipette with the amphotericin-containing solution. A GΩ cell-attached seal should be obtained as quickly as possible. If seal formation is difficult, increase the tip-fill time.
   NOTE: The access resistance should be continuously monitored following formation of the cell-attached seal, and recordings should only be commenced after obtaining a stable access resistance of <10 MΩ.
5. To record spontaneous APs, switch the amplifier to fast current-clamp mode with no current injection.
   NOTE: 1 nM isoproterenol is included in the extracellular Tyrode's solution when recording APs to stabilize the firing rate, as previously reported[10].

Representative Results

The protocols described here have been previously employed to isolate spontaneously active SAMs from adult mice that are suitable for a variety of different patch clamp studies[5-8]. In addition, the protocols allow for isolated SAMs that can be maintained in culture for up to one week. Gene transfer into the cultured cells can be accomplished via adenoviral infection[9]. The results presented in this section derive from our previous work and are shown here as examples of the characteristics of acutely isolated and cultured SAMs.

As shown in **Figure 2**, spontaneously active SAMs can be maintained in culture for up to 6 days. The cultured cells retain an overall morphology that is very similar to that of acutely isolated SAMs, with no significant changes in the average length, width, cross-section area, or membrane capacitance of cultured cells compared to acutely isolated cells (**Figure 2B**-**E**). There was however attrition of the number of viable SAMs over time in culture. Hence, it is recommended that cultures be prepared from pooled cells from multiple animals if the experimental design requires extensive datasets.

A major goal in developing the protocol for culture of sinoatrial myocytes from adult mice was to create a system that would allow the expression of exogenous proteins in the native cellular context of adult SAMs. An example of this type of protein expression is shown in **Figure 3** for the case of cells co-infected with viruses expressing the fluorescent marker proteins GFP and mCherry. We found that protein expression was clearly evident within 24 hr of adenoviral infection, and was maximal for the test proteins within 48 hr (**Figure 3**). A viral MOI of 100 resulted in nearly 100% infection efficiency with no evidence of cellular toxicity. In contrast, transfection using lipid-based reagents failed to produce in any detectable gene transfer into adult SAMs, even after 72 hr[9].

Functional characterization via patch-clamp electrophysiology is critical for evaluation of both acutely isolated and cultured SAMs. **Figure 4A** shows typical current-clamp recordings of spontaneous action potentials recorded from acutely isolated or cultured SAMs using the amphotericin perforated-patch recording configuration. Action potential waveforms were similar in acutely isolated and cultured SAMs and there was no difference in the average AP firing rate (**Figure 4B**).

I_f is a hallmark of SAMs and the HCN4 channels that produce I_f are used as immunocytochemical markers of the sinoatrial node. Hence the presence of I_f in patch clamp recordings can be used to support the idea that the cells are in fact SAMs. All of the spontaneously active acute and cultured SAMs described herein also exhibited I_f >100 pA in response to a 1 sec voltage step to -120 mV. For the representative results shown here, cells were perfused with Tyrode's solution containing 1 mM $BaCl_2$ to block K^+ currents (**Table 3**) and the voltage dependence of activation of I_f was assayed by 3 sec hyperpolarizing voltage steps from -60 to -160 mV from a holding potential of -50 mV, as we have previously described[5-8]. The current density was evaluated in response to 3 sec hyperpolarizing test pulses to -150 mV from a holding potential of -50 mV. There were no significant differences in either the voltage-dependence of activation of I_f or the I_f current density between acutely isolated and cultured SAMs (**Figure 4C**-**D**). These co-recordings of spontaneous APs and I_f from individual SAMs require approximately 9 min in total (including a 2 min initial wash, 30-60 sec of spontaneous APs in current clamp mode, a 2 min solution change, and approximately 5 min to collect sufficient voltage clamp data to describe the voltage-dependence of activation of I_f). Hence the data shown in **Figure 4C** also illustrate the robustness of the cell preparation.

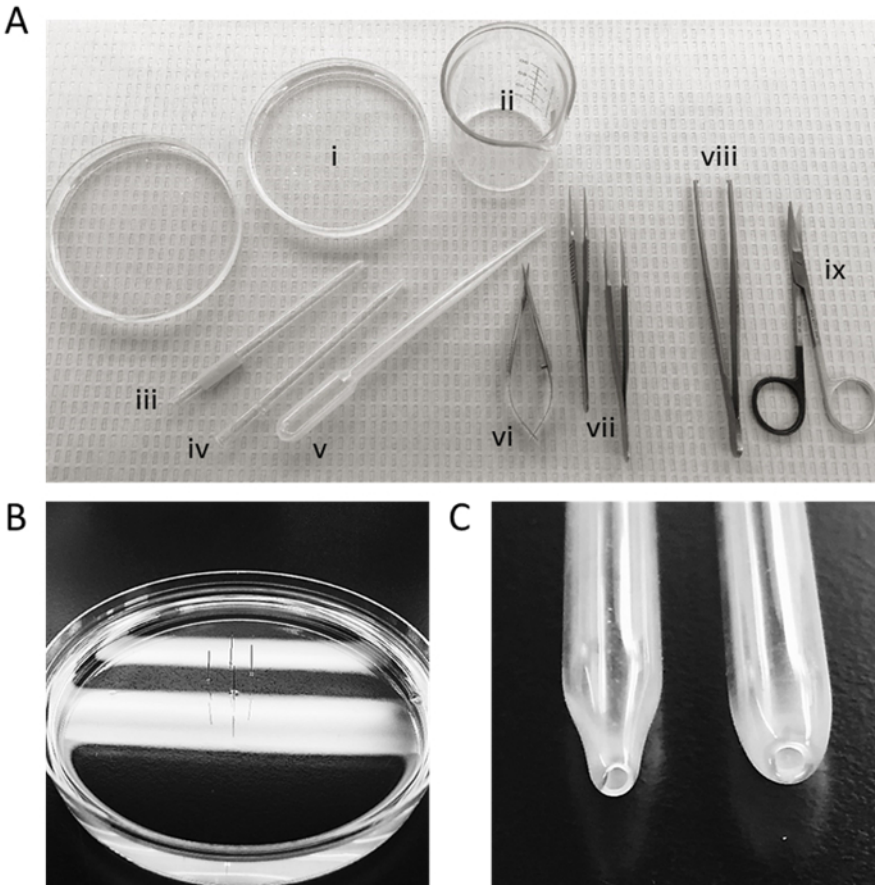

Figure 1: Dissection instruments for the isolation and dissociation of mouse SAMs. (**A**) *i.* Two 10 cm Petri dishes containing ~25 ml cured silicone elastomer. Each dish is preloaded with 6-10 small dissection pins for immobilizing tissue. *ii.* 100 ml beaker for holding Complete Tyrode's in the 35 °C water bath. *iii.* Wide (~2 mm diameter), fire-polished dissociation pipette. *iv.* Narrow (~1.5 mm diameter) fire-polished transfer pipette. *v.* Plastic transfer pipette for bathing preparation with Complete Tyrode's with heparin. *vi.* Self-opening micro scissors for internal cutting procedures. *vii.* Fine forceps (tip size 0.06 x 0.02 mm) for internal tissue manipulation. *viii.* Tissue forceps, 5.5 in, 1 x 2 teeth for external tissue manipulation. *ix.* Curved iris scissors 4.3 in for external cutting procedures. (**B**) Close-up photo highlighting small dissection pins placed in dissection dish. (**C**) Close-up photo highlighting fire-polished narrow transfer pipette (*left*) and fire-polished wide-mouthed pipette for mechanical trituration (*right*). Please click here to view a larger version of this figure.

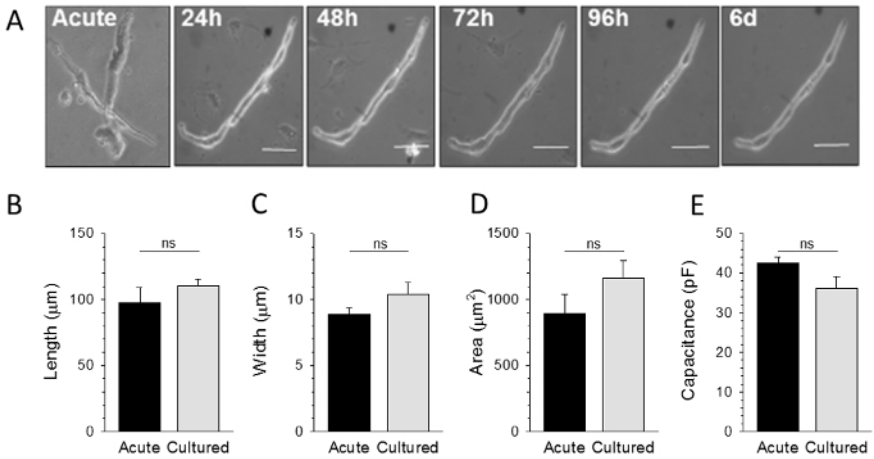

Figure 2: Acutely isolated and cultured SAMs are morphologically indistinguishable. A: Bright field images of representative SAMs either immediately after isolation (acute) or after 24 hr, 48 hr, 72 hr, 96 hr, or 6 days *in vitro*. **B**-**D**: average (± SEM) maximum length, width and cross-sectional area for acutely isolated versus 48 hr cultured SAMs. **E**: Average (± SEM) membrane capacitance from voltage-clamp recordings from acutely isolated or cultured SAMs. All comparisons are not significant: $p > 0.05$ vs. acute. Adapted from reference[9]. Please click here to view a larger version of this figure.

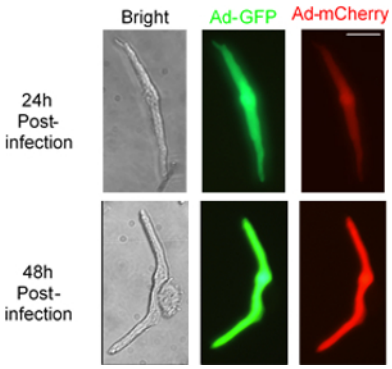

Figure 3: Adenoviral expression of exogenous proteins in cultured SAMs. Bright field and epifluorescent images of representative cultured SAMs that have been infected at an MOI of 100 for adenoviruses expressing eGFP (green) and mCherry (red), either at 24 or 48 hr after double infection. Scale bar = 20 µm. Reproduced from reference[9].

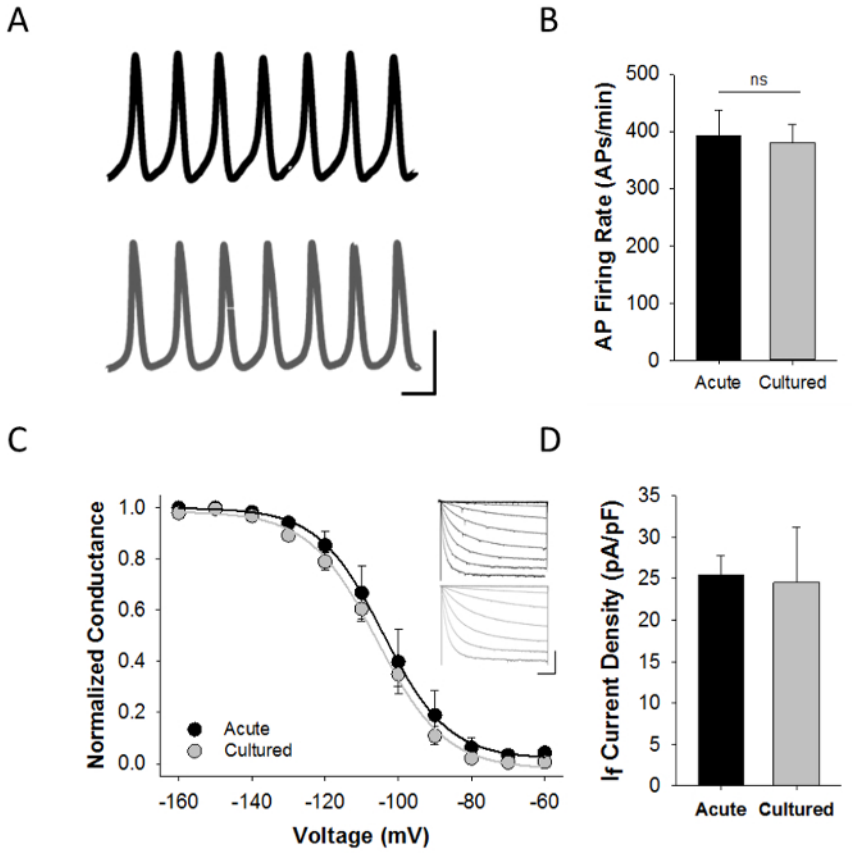

Figure 4: Electrophysiological characterization of cultured SAMs. A: Representative current clamp recordings from acutely isolated (*black*) or cultured (*grey*) SAMs in Tyrode's solution containing 1 nM isoproterenol. Scale bars: 40 mV, 100 msec. **B**: Average (± SEM) instantaneous firing frequency in acutely isolated (*black*; n = 6) or 48 hr cultured (*gray*; n=14) SAMs. **C**: Normalized average (± SEM) conductance-voltage relationships for I_f in acutely isolated (*black*; n = 8) or cultured (*grey*; n = 14) SAMs. *Insets*: representative current families from acute (*black*) or cultured (*grey*) SAMs elicited by 3 sec hyperpolarizing test pulses from -60 to -160 mV in 10 mV increments. **D**: Average (± SEM) I_f current density at -150 mV in acutely isolated (*black*; n = 7) and cultured (*grey*; n = 8) SAMs. Adapted from reference[9]. Please click here to view a larger version of this figure.

	Complete Tyrode's	low Ca^{2+}/Mg^{2+} Tyrode's	KB Medium	BSA Solution	NaCl/$CaCl_2$ Adaptation Solution
NaCl	140	140		140	10
KCl	5.4	5.4	25	5.4	
KH_2PO_4	1.2	1.2	10	1.2	
HEPES	5	5	5	5	
glucose	5.55	18.5	20	5.55	
$MgCl_2$	1			1	
$CaCl_2$	1.8	0.066		1.8	1.8
taurine		50	20		
BSA		1 mg/ml	1 mg/ml	1 mg/ml	
K-glutamate			100		
K-aspartate			10		
$MgSO_4$			2		
creatine			5		
EGTA			0.5		
pH adjusted to	7.4 with NaOH	6.9 with NaOH	7.2 with KOH	7.4 with NaOH	

Table 1: Dissociation solutions. Compositions of solutions used in the dissociation of sinoatrial node myocytes. Concentrations are given in mM except where noted.

	Plating Medium	Culture Medium
Media199	base	base
2,3-butanedione monoxime (BDM)	10 mM	10 mM
Fetal Bovine Serum (FBS)	5%	--
Bovine Serum Albumin (BSA)		0.1 mg/ml
Insulin		10 µg/ml
Transferrin		5.5 µg/ml
Selenium		5 ng/ml
Penicillin	100 U/ml	100 U/ml
Streptomycin	100 mg/ml	100 mg/ml

Table 2: Plating and culture solutions. Compositions of solutions used for plating and culture of sinoatrial node myocytes.

	Tyrode's	PP Intracellular
NaCl	140	5
KCl	5.4	135
Kh_2PO_4	1.2	
HEPES	5	10
glucose	5.55	
$MgCl_2$	1	1
$CaCl_2$	1.8	0.1
EGTA		10
Mg-ATP		4
Amphotericin-B		200 µg/ml as needed
Isoproterenol	1 nM as needed	
pH adjusted to	7.4 with NaOH	7.2 with KOH

Table 3: Electrophysiology recording solutions. Compositions of extracellular (Tyrode's) and intracellular (PP) solutions used for amphotericin perforated-patch recordings from sinoatrial node myocytes.

Discussion

This paper presents detailed protocols for the isolation and culture of fully differentiated sinoatrial node myocytes from adult mice. The isolation protocol reliably produces spontaneously active mouse SAMs suitable for either immediate electrophysiological analysis or subsequent culture. Similar protocols have been reported by many other groups (for example, see references[11,12,10,13-17]). However, our protocol for maintaining adult mouse SAMs *in vitro* preserves the characteristic morphology, spontaneous activity, and electrophysiological properties of the cells and allows for adenoviral delivery of proteins[9].

For modification and troubleshooting of the protocols, a few critical steps should be noted. The first critical factor is a significant lot-to-lot variability in the activity of the digesting enzymes (Step 1.4), which can dramatically alter the number and quality of SAMs isolated in a given preparation. This variability is particularly true for the elastase, which typically requires lot-specific optimization, in which the concentration and exposure times should be bracketing over the course of several preparations (often done in parallel) to maximize the cell number and health. Some protocols for the isolation of SAMs from various species use individual collagenase and protease enzymes instead of the collagenase-protease enzyme blend in the present protocol. However, the lot-to-lot variability for each of these enzymes is fairly high (similar to that of elastase), necessitating repeated rounds of co-optimization. The enzyme blend provides more consistency across lots and requires fewer optimization steps.

A second critical, and unobvious, factor for troubleshooting the SAM isolation is the integrity of the fire-polished glass dissociation pipette (**Figure 1C**). If the rim of the pipette contains any sharp edges, cracks or accumulated cellular debris, the mechanical trituration step can damage the cells, resulting in calcium toxicity. The fire-polished dissociation pipette should be replaced at least every 2 months of regular use, or at any time that the cell yield or quality declines markedly over the course of several preparations.

The timing and the force of the mechanical dissociation is a third critical step for troubleshooting the isolation protocol. The trituration requires slow (approximately 0.5-1 Hz) but forceful turbulence for 5-10 min. Faster rates and reduced times could also be used, but it is important to avoid introducing bubbles or generating froth in the solution. When the sinoatrial node samples have been fully dissociated, the remaining tissue strips should appear wispy and white in color; subtle pink-ish coloring indicates incomplete trituration. When examined at 200-400X magnification, cells from optimally prepared samples have smooth cell membranes, whereas cell membranes from over-digested or over-triturated samples have a cratered and striated appearance. In healthy cells, contractions are observed primarily as subtle twitching of the ends of the cells. Waves of contraction are a sign of damaged cells, which can be observed to contract vigorously for a short time, before balling-up. A cracked dissociation pipette is the most common cause of such cell damage. In samples that have been under-digested or under-triturated, cells are present as clumps or aggregates instead of single cells, and significant numbers of contracting SAMs can be observed in the remaining tissue chunks.

Each user will need to individually optimize the dissociation. Although the enzymatic digestion and mechanical dissociation times should both be adjusted, it is recommended to initially keep the enzymatic digestion time constant while modifying the dissociation time. Additional fine tuning relies mainly on the user's ability to recognize the appearance of the tissue pieces remaining at the completion of trituration — when the pieces are wispy and whitish in color, then the trituration should be stopped. Optimization is also necessary to accommodate variations in the sinoatrial node that accompany differences in age, gender, or strain of mice. For example, dissociation of cells from older animals[7] requires reduction in both enzymatic digestion and mechanical dissociation times. As a general guideline, a typical preparation from a 2-3 month old male C57BL/6 mouse yields roughly 50-200 viable SAMs, of which perhaps 5-10 can be successfully patch-clamped in a good day's session, depending on the experiments. The yield is considerably (~30%) lower when older mice are used[7].

The success of the culture protocol also relies on a few key steps. Foremost, the success of a primary cell culture requires a high-quality dissociation, as discussed above. It is recommended that the acutely isolated cell preparation be optimized before attempting to maintain the cells in culture. A second critical factor for cultured cells lies in the selection of cells for electrophysiological recordings. For best results, cultured

SAMs selected for recordings should exhibit spontaneous contractions immediately upon washout of the BDM-containing culture medium. A slow onset of spontaneous contractions upon wash-out of BDM is a sign of unhealthy cells.

The SAM culture system does have some limitations, most notably the very small size of the mouse SAN. While the methods demonstrated here allow for sufficient cell numbers for electrophysiological and imaging studies, the limited amount of tissue precludes biochemical analyses of cultured SAMs. Another limitation of the technique is that SAMs must be cultured in the presence of the reversible myosin ATPase inhibitor, BDM. This is a potential concern because BDM has other cellular effects, including non-specific phosphatase activity, inhibition of cardiac transcription factors[19], and inhibition of sodium channels, calcium channels and ryanodine receptors[20]. However, the data show that BDM has minimal effects on the morphological and electrophysiological properties of SAMs assayed here.

In future applications, it seems likely that the methods outlined here could be adapted to prepare SAM cultures from larger mammals. In combination with adenoviral gene transfer, such cultures could allow genetic manipulations of pacemaking in large animal models. The ability to introduce proteins of interest also provides for future applications in which genetically encoded reporter molecules can be used to probe intracellular signaling pathways in SAMs.

Disclosures

None.

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