Murine Ventricular Cardiomyocyte Isolation, Calcium Adaptation, and Fura-2/AM Dye Loading Protocol

Isolation Solutions:

1. Prepare stock solutions: 0-Ca^{2+} Physiologic Saline Solution (PSS), MEM Base, and 2mM-Ca^{2+} PSS (see solution supplement)
2. Prepare enzyme aliquot (see solution supplement)

Immediately Prior to Isolation:

3. Make isolation solutions (see solution supplement)
   a. Wait to add BSA to solution 3 until immediately prior to anesthetizing the rodent – pre-measure and store at 5° C.
4. Check oxygen tanks
5. Rinse DI water through isolation rig
   a. Cover top reservoir until use
6. Check vacuum flasks
7. Check water level in recirculation pump
   a. Check bath temp
8. Boil 1L Millipore water to clean rig after isolation
9. Turn on water bath, recirculation pump, and temperature controllers
10. Add solution 1 to top reservoir and turn on oxygen to outgas
11. Perfuse solution 1 through tubing, removing all bubbles
12. Tie double knot sutures onto cannula

Cannulation and Isolation Protocol:

13. After sufficiently anesthetizing rodent, excise the heart per lab protocol (varies by ACUC)
14. Place heart in cold 0-Ca^{2+} PSS, removing excess tissue as needed
15. Cannulate aorta and secure with pre-tied suture; begin solution 1 perfusion
16. Perfuse solution 1 ~2 minutes, ensuring heart fully submerged in solution
17. During this time, add enzyme to solution 2
18. Perfuse solution 2 and set enzymatic digestion timer to coincide with solution arrival at heart
19. Once solution 2 is in the heart, drain the bath of all excess solution 1 via transfer pipette to ensure heart is bathed only in solution 2
20. Solution 2 perfusion time is ~6-12 minutes (highly condition- and tissue-dependent). Barely visible cellular detritus should be apparent after sufficient digestion. Tissue should be orange in color and soft but not slimy.
21. Fill weigh boats with solution 3 (stop solution with BSA)
22. After sufficient digestion, carefully remove heart from cannula and submerge in solution 3
    a. Remove atria, excess fat, and right ventricle (with or without septum) via fine surgical scissors
    b. Move trimmed left ventricle to separate weigh boat and make 5-6 fine cuts to help dissociate cells

c. Gently agitate with fine forceps for ~5-10 minutes to further dissociate cells
d. Check cells under microscope for live/dead ratio
e. Remove heart by scraping gently up the side of the weigh boat to further remove cells

23. Cells are filtered into 15ml falcon tube through 100-200 µm mesh filter
    a. A few drops of solution 3 to pre-wet will break static seal on mesh
24. Use an angled surface or box to keep tube at ~45° angle during all subsequent procedures
    a. Failure to allow pelleting at an angle can result in cell clumping and sarcolemmal damage (followed by calcium overload)
25. Allow cells to pellet ~10 minutes, remove supernatant, and replace with fresh BSA/Stop solution to bring cells to desired density (solution 3)
26. Excess tissue can be discarded appropriately

Following Isolation (this can be done while plating/dye loading cells):

27. Once heart is removed from rig: oxygen, water bath, and recirculation pump can be turned off.
28. Wash rig (make sure vacuum dish is open):
    a. Flush with ~500ml of boiling Millipore water through rig
    b. Flush with ~500ml of 70% ethanol
    c. Flush again with ~500ml of boiling Millipore water
    d. Flush a final time with Millipore water to ensure all ethanol is removed
29. After all solution has been flushed, turn off hotplate and vacuum.

Assessing Cardiomyocyte Isolation Quality:

30. Assess newly isolated cardiomyocytes for live:dead ratio
    a. >60% represents experimentally viable cells
    b. This percentage is model- and protocol-dependent as isolations in disease models (e.g. aging, muscular dystrophy, western diet, etc.) may have significantly lower yields
31. Visually inspect isolated cardiomyocytes under bright field for:
    a. Distinct sarcomeric striations
    b. Lack of sarcolemmal “blebbing” (bubble-like visual indicators of compromised sarcolemma integrity)
    c. Lack of non-stimulated cardiomyocyte contraction (spontaneous contraction), indicative of compromised sarcolemmal integrity
    d. Crisply defined cellular borders (not “chewed up” or clumped together with other cells, indicative of over- and under-digestion, respectively)
    e. If time permits, resting sarcomere lengths >1.8 µm indicate experimentally viable cells
32. Critically, ensure visual markers of cellular integrity are intact following adaptation to physiologic calcium concentration and cells are not spontaneously contracting.
    a. Physiologic calcium adapted cardiomyocyte functional integrity is the *critical* hallmark of a good isolation

Adaptation and Plating

33. Re-suspend freshly isolated cardiomyocytes in fresh BSA/STOP solution after removing old BSA
34. Step-wise over 20 minutes, adapt cells from nominal to 250 µM Ca^{2+}
    a. Suggested interval: 5 minutes

b. Lightly re-suspend cells prior to additions using large-bore transfer pipette

35. Allow cells to pellet, remove supernatant, and re-suspend in 500 μM Ca^{2+} PSS
    a. *NOTE*: Cells can be further adapted and loaded either plated on laminin-coated coverslips or suspended in falcon tubes. Read further for plating instructions:
        i. For coverslips, coat with ~3-5 μL 1M laminin and smear evenly in appropriate area.
        ii. Once completely dry, transfer pipette sufficient suspended cardiomyocytes to cover laminin-coated area. To minimize dessication, place moistened pieces of kimwipe in petri dishes containing slides and cover with lid.
        iii. Allow 25-30 minutes for cardiomyocyte attachment.
        iv. Below, ignore pelleting instructions and simply pipette off excess solution between solution changes, replacing with indicated solution
36. After 5 minutes, adapt cells to 750 μM Ca^{2+}
37. Allow cells to pellet, remove supernatant, and re-suspend in 1 mM Ca^{2+} PSS

Fura-2/AM Dye Loading

38. Dye load isolated cardiomyocytes in 2-5 μM fura-2/AM in DMSO (empirically derived)
39. Allow cells to load for 20-25 minutes (empirically derived)
40. Wash (de-esterification of acetoxymethyl ester) with 2 mM Ca^{2+} for ~30 minutes
    a. Ensure near-complete removal of fura-2/AM solution prior to washing
41. Cells are ready for experimentation

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