Overview

Additional resources

Need more help?

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Main content

**Part 1: Solution Preparation**

Glassware:

600 mL beaker

3 small falcon tubes- labeled BDM, 100mM CaCl2, 10mM CaCl2

2 large falcon tubes- labeled Stop 1, Stop 2

250 mL beaker- labeled digestion buffer

100 mL beaker- labeled warm perfusion

500 mL glass cylinder

Solutions/Compounds needed

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Compound/Solution | Source | Purity | Amount | Location |
| [Perfusion Stock](https://sites.google.com/a/campbellmusclelab.org/wiki/protocols/stock-solution-recepies/perfusion-buffer) | Lab |  | 500 mL | Fridge B |
| [Tyrode's Stock](https://sites.google.com/a/campbellmusclelab.org/wiki/protocols/stock-solution-recepies/tyrode-s-solution) | Lab |  | 250 mL | Fridge B |
| 2-3, Butanedione monoxime (BDM) | Sigma | 98.0% | 0.506 g | Shelf 11M |
| Glucose | Sigma | 99.5% | 0.500 g + 0.450 g | Shelf 11M |
| 100 mM Calcium Chloride Stock (CaCl2) |  | 100 mM | 5 mL | Shelf 11L |
| 10 mM Calcium Chloride Stock (CaCl2) |  | 10 mM | 10 mL (1 mL 100 mM CaCl2 + 9 mL     DI H2O) | Shelf 11L |
| Fetal Bovine Serum | Bioquest |  | 2 mL (Stop 1) + 1.26 mL (Stop 2) | Freezer A (in bag labeled FBS) |
| [Reconstituted Liberase TH Solution\*](https://sites.google.com/a/campbellmusclelab.org/wiki/protocols/rat-cardiomyocyte-isolation/liberase-and-collagenase) | Roche |  | 1.50 mL | Freezer A (in white box labeled liberase) |
| OR if using [collagenase instead of Liberase:](https://sites.google.com/a/campbellmusclelab.org/wiki/protocols/rat-cardiomyocyte-isolation/liberase-and-collagenase) | Worthington (Type 2) |  | 100 mg | Freezer A (in white box labeled collagenase) |
| Pluronic Acid |  |  |  |  |
| Fura-2 |  |  |  |  |

1. Add 0.500 g glucose to 500 mL of the Perfusion stock in a 600 mL beaker and bubble with 95% O2 5% CO2for 18 seconds.  Check the pH and O2 level of the solution, make sure the setup has been calibrated. The pH should be around 7.3 and O2 between 10-15.
2. After the perfusion buffer has been bubbled, add 0.506 g of 2,3-butanedione monoxime to 8 mL of DI water, dissolve, and then add DI water until total volume of the solution is 10 mL.  This is the **BDM Solution**.
3. Add 5 mL of 100 mM CaCl2 to a pre-washed falcon tube.  This is the **100 mM CaCl2 Solution**.
4. Add 1 mL of 100 mM CaCl2 to 9 mL of DI water in a pre-washed falcon tube.  This is the **10 mM CaCl2 Solution**.
5. After the Perfusion stock with glucose has been bubbled, add 18 mL of the perfusion solution to 2 mL FBS and 0.025 mL of 10 mM CaCl2  together in a large falcon tube.  This is the **Stop 1 Buffer**.
6. Add 23.74 mL of bubbled Perfusion to 1.26 mL FBS and 0.032 mL of 10 mM CaCl2 together in a large falcon tube.  This is the **Stop 2 Buffer**.
7. Add 9.4 mL BDM solution to the remaining 458 mL of Perfusion stock with glucose.  This is the **Perfusion Buffer**.
8. Add 0.031 mL of 100 mM CaCl2 to 150 mL of Perfusion Buffer in a 250 mL beaker and heat to 37 degrees Celsius.\*  This is the **Digestion Buffer**.
9. Separate 60 mL of the remaining Perfusion Buffer into a 100 mL beaker and heat to 37 degrees Celsius.\*  This is the **Warm Perfusion**.  Store the remaining perfusion buffer on ice.

**Part 2: Cannulation, Perfusion, And Digestion**

Pre-Part 2

1. \*Put Digestion Buffer & Warm Perfusion in water bath
2. Put 6, 10mL beakers in water bath
3. Add water to bath if needed (enough but beakers not floating)
4. Check amount of water in bottom water heater (add to the shadow area if needed)
5. Set up area (go by photos)
6. Set up 6 sets of 3 little centrifuge tubes in blue carrier
7. Fill out DEA inventory

Part 2

1. Anesthetize the rat with beuthaniasa ( NEW DOSAGE ~.064-.055 mL FOR 150 g RAT).
2. Inject the rat with 0.07 mL of 10000 U Heparin.
3. Add 1.5 mL of Liberase TH Solution to the 150 mL of Digestion Buffer.  Stir or shake gently to mix.
4. Add 100mL of the digestion buffer to the top set up and fill the lower set up with warm perfusion.
5. Set aside two weight boats filled with the cold perfusion buffer before you anesthetize the rat.  Prepare a dissection board with all of the required surgical instruments.  It is also important to setup a syringe with a canula attached full of cold perfusion.  An image of this setup is shown below.
6. Place the animal on the dissection board and check to see if it is properly anesthetized.  If not, inject with more Pentobarb.
7. Open up its chest just below the xiphoid process.  It might be helpful to tape down the rats arms, legs, and tail. Warm water can be spread over the place you will cut to put the hair down and out of your way. Remove the heart, dip it in one of the weigh boats filled with the cold perfusion buffer and then place in the other weigh boat.  While the heart is in the weigh boat, tie the aorta around a canula while pushing perfusion buffer through the canula to clear the coronaries of the heart.
8. Start flow before attaching cannula to remove bubbles.  Attach the cannula to the perfusion system and run 37 degree perfusion buffer through the heart until it drips clear.
9. After this switch to 37 degree digestion buffer and digest the heart on the perfusion system for 12 minutes.  It is important to make sure you do not run out of fluid in the top beaker, if level is low add more.  Also, do not allow the level in the bottom (where the heart is located) to overflow.  This level should stay to the top of the heart.  Some hearts may only need about 10 minutes to digest.  Pay attention to the heart squishyness and color.
10. After the initial digestion is complete, remove the cannula from the perfusion system and cut the LV free-wall out of the heart. When looking at the heart you should see a smile and an eye.  The smile is the RV and the eye is the LV.  Then separate the LV free-wall into three sections based on the layer of the myocardium removed (endo, mid, epi).  Place each layer into a separate beaker with 1 mL of digestion buffer at 37 degrees Celsius.
11. Pull apart the tissues until you see yellow liquid in bottom of the beakers.  Use the small, black scissors to pull cells apart. Gently gyrate each beaker while allowing the layers to digest.  Pull 6 uL of the digest out of the beakers while the tissue is dissecting and inspect under a microscope.  After 3 minutes you should see cells in the solution (under the microscope).
12. Aggravate the myocardium in each beaker and then transfer the myocardial pieces to a second set of beakers holding 1 mL of fresh digestion buffer.
13. Use a wide-tipped pipette (cut the end of a normal tip with a razor blade) and pipette ~750 ul of cells into a matching set of eppendorf tubes. Place nylon mesh (~150 microns) on top of the tubes when you pipette to act as a course filter and catch chunks of tissue.
14. Then top off each eppendorf tube to the top with stop 1 buffer. These tubes give you the **first batch of cells**.
15. Repeat steps 11 to 14, 3-5 times more, as long as the cells are viable.  After time is up you will have 4-6 batches of cells, with 3 myocardial layers for each batch.
16. After digestion is finished, let the cells settle out for 5-10 minutes, until a pellet is visible.  Then, pipette out the liquid from the top of each eppendorf tube until less 0.1 mL of cells/stop 1 solution is left (leave the pellet intact).  Then add 0.5 mL of stop 2 buffer to each eppendorf.

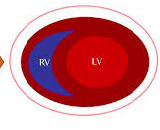




Image of the setup of the syringe and cannula with cold perfusion in boar and in syringe



Arial view of the setup for the dissections. The rat will be placed on the covered dissection board along with the instruments.

**Part 3: Calcium Ladder**

After the addition to each eppendorf tube it is important that each tube is inverted twice to ensure mixing.

If certain tubes have been selected for preservation then the following procedure should be stopped after addition number 4; the 0.0025 mL of 100mM CaCl2 should not be added to the tubes selected for preservation.

1. Add 0.0025 mL of 10 mM CaCl2 to each eppendorf tube and let stand for 4 minutes.
2. Add another 0.0025 mL of 10 mM CaCl2 to each eppendorf tube and let stand for 4 minutes.
3. Add 0.005 mL of 10 mM CaCl2 to each eppendorf tube and let stand for 4 minutes.
4. Add 0.0015 mL of 100 mM CaCl2 to each eppendorf tube and let stand for 4 minutes.
   * do not continue to step five for the tubes you plan on storing.
5. Add 0.0025 mL of 100 mM CaCl2 to each eppendorf tube and let stand for 1 hour.

**Part 4: Cell Loading**

*If doing a pharmalogical experiment consult specific protocols. OM and Blebb protocols can be found here:*[*Blebbistatin and OM experiments*](https://sites.google.com/a/campbellmusclelab.org/wiki/protocols/stock-solution-recepies/blebbistatin-and-om-experiments)

1. Add 0.450 g glucose to 250 mL of the Tyrode's stock in a beaker, stir, and bubble with 95% O2 5% CO2 for  20 seconds.
2. Adjust the pH of the bubbled Tyrode's solution until it is 7.25-7.35.  Check the O2 level, it should be between 10-15 ppm.
3. Combine 3.680 mL of Tyrode's with 0.004 mL of 20% pluronic acid in DMSO and 0.008 fura-2 am in DMSO (50 uL DMSO/fura-2 container), without letting the fura-2 or pluronic to be exposed to light. This is the **Loading Solution.**
4. While still in the dark, separate 0.230 mL of loading solution in 9 eppendorf tubes and add 0.040 mL of cells taken from the bottom of the 9 cell tubes.  Allow the cells to load for 15 minutes. Load only 1-2sets of cells at a time, consider how large of a pellet you are loading, consider light triteration.
5. In 9 separate eppendorf tubes add 0.150 mL of Tyrode's and then add 0.030 mL of cells taken from the bottom of the loaded cell eppendorfs. Cut off bottom 1/3 of the pipet tip before collecting the cells. Allow the cells to stand for 15 minutes.
6. The cells should now be loaded by slowly loading the cells back and forth across the cell bath and may be viewed on the slide in 0.030 mL aliquots taken from the bottom of each Tyrode's/loaded cell eppendorf.

**Part 5: Cleaning Chamber**

1. Pipet Tyrode's solution into the chamber with high velocity and agitate the liquid inside.
2. Unscrew vacuum/ suction line and directly suction up the remaining liquid in the chamber.
3. If a drug was being tested before cleaning out chamber, it is important to run control solution through the lines and into the chamber.
4. It might be helpful to take a look in the eye piece to be sure all cells were suctioned up. If cells are still present repeat the steps listed.

**\*\*\*WHEN FINISHED WITH ISOLATION BE SURE TO CHANGE TEMPERATURE SET POINT BACK TO 15 C\*\*\***

The Day After an Isolation

1. Prepare a solution of 25 mL of Perfusion stock, 0.005 g glucose, 1.25 mL 10 mM Ca (made yesterday) in a large falcon tube.

2. Oxygenate for 1-2 seconds.

3. Remove the supernatant from the cells you wish to measure today and add .5 mL of the solution made it step 1.

4. Let the cells sit in this solution for 20 minutes, then remove the solution, and re-add 0.5 mL to the cells.

5. Repeat this step so that the cells have been in the new solution for a total of three rounds. This is done in order to clean out the myosin ATPase inhibitor.

6. Continue onto step five of the calcium loading...Add 0.0025 mL of 100 mM CaCl2 to each eppendorf tube and let stand for 1 hour.