

2.4. Force assessment in multicellular cardiac preparations in mice

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A. OBJECTIVE

This protocol describes the assessment of contractile strength in isolated multicellular cardiac preparations.

B. CAUTIONS

- Myocardial multicellular preparations are very easily damaged. It will typically take several months of daily practice before publication-quality results can be obtained. It takes fine-tuned micro-dissection skills that not everyone can master.
- Good quality preparations can never be obtained by cutting larger muscles. Any cutting in multicellular tissue will cause damage that typically travels 200-250 μm into the preparation.¹⁶ Only free running trabeculae or small and whole papillary muscles will render unambiguous results.
- Some hearts, especially some murine strains, simply will not have a suitably sized muscle. C57 mice typically have less suitable trabeculae than FVBN or SV129 mice.¹⁴
- The attachment of the muscle to the force-transducer and length displacement device is critical to a quality outcome. Intact muscles should never be sutured or glued to attach.
- Only with a binocular microscope and suitable micro-dissection tools can sufficiently quality muscle be obtained.
- Even a poorly dissected muscle, provided a few cells are still alive, will show some force development. So it is important to use a nicely dissected muscle.
- Force development must always be related to cross-sectional area as a quality measure.

C. MATERIALS

- Binocular microscope: It is critical to view the to-be-dissected muscles in 3-dimensional space. Optimally, a microscope that can zoom out is preferred so it can aid in the macro-dissection and opening of the ventricle. A minimal resolution of 10 μm or less is required for accurate assessment of the muscles' dimensions.
- Dissection dish: The dissection dish size is highly dependent on the species used. For mice, rats, small rodents, and up to rabbits a 4-6 inch diameter dish is fine, for larger species (dog, sheep, human), a larger dish is needed. The bottom of the dish should be very soft as to accommodate pins or syringes to pin-open the ventricle. A circular dish filled with (black) silicone is ideal. The black color aids in creating a sharp contrast between tissue and background, and prevents too much light scattering. A simple way to make a dissection dish is to use the bottom 2 inches of a plastic

large graduated 1000 or 2000 ml cylinder. Fill with 1 inch of regular bathroom silicone caulk (black), and let harden. Then, peel out the silicone, apply a small amount of fresh silicone into the cylinder, and flip over the hardened silicone disc smooth side now facing up, and push back in cylinder. A cannula (can be made from a syringe, with tip cut off, and edges filed smooth), should protrude through the side, a few mm above the bottom.

- Micro scissors, and sharp-pointed micro tweezers: Sharpen (or have sharpened by the manufacturer) regularly, and replace when worn.
- Krebs Henseleit (KH) Solution for experiments⁶: Make fresh daily, or make from 10X stock. In 10X stock, do not add bicarbonate nor glucose or 2,3-butanedione monoxime (BDM), these must always be added fresh on the day of use. 1X KH solution contains:
 - 137 mM NaCl
 - 5 mM KCl
 - 0.25 CaCl₂
 - 2.0 mM Mg₂SO₄
 - 1.2 mM NaH₂PO₄
 - 10 mM Glucose
 - 20 mM NaHCO₃ (This amount may vary depending on the altitude, adjust amount so that when bubbled with the gas mixture, the pH results in 7.4)
 - use 95% O₂- 5% CO₂ gas mixture
 - After addition of ~ 20 mM BDM, the solution is called KH-BDM.
- Tissue Bath. The tissue bath should ideally be small enough so that a flow of ~5 ml of solution per minute refreshes the volume of the entire bath every ~5-10 seconds. This will provide proper oxygenation and prevents a build-up of a gradient of ions and waste products around the muscle. On the other hand, a bath should be big enough to allow for mounting the muscle without damaging it. A typical muscle bath dimension would be 30-40 mm long, and 3-4 mm wide, and 2-3 mm deep (volume ~300-400 microliter).
- Force Transducer. The force transducer should be sensitive enough to pick up the small forces, and fast enough to record accurate kinetics of a twitch contraction. Typically, the absolute baseline twitch force of a small muscle (0.1 x 0.1 x 2 mm) is only ~0.5 mN, a minimum sensitivity of 0.01 mg would be required for accurate force assessment. A mouse twitch contraction lasts <100 ms, so the resonance frequency of the force-transducer should be >500 Hz in order to accurately capture such fast kinetics.
- Stimulation. Most typically field stimulation is required. This requires the placement of two electrodes (platinum ideally), on either side of the muscle, spanning the full length and width of the muscle. 0.2 mm wire or so should suffice, making sure they are close enough apart for a strong field (~1-2 mm). Most physiological stimulators will provide enough

voltage and current for a full muscle contraction; make sure to test by starting at low voltage, and making sure at one point a threshold is reached after which the muscle no longer contracts more forcefully. Throughout the protocol, ideally ~120-140% of threshold voltage should be used to ensure full stimulation. Pulse duration needs to be only 1-2 ms, with a bi-polar stimulation profile needed in order to prevent electrolysis (and thus gas formation) in the muscle bath.

- Length displacement device (servo motor or micro positioner): It is critical that the length of the muscle can be accurately set. For this purpose, you can use either a manual displacement device (microscrew, or micropositioner), or a small motor. Total travel should be 10-25 mm, while accuracy of length control should be better than 10 μm . A micro-screw with a position change of ~100 μm per full rotation will suffice. For most servo motors, note that the lever arm rotates on an axis, and thus only small ranges can result in the muscle staying in the same z-plane. Linear servo motors can have a longer range while keeping the muscle in the exact same Z-plane.
- Heparin

D. METHODS

- 1) Removal of the heart. This is highly dependent on the species used. For small rodents, after opening the thorax, to inject the heart (via the apex) with 500-1000 U of heparin while the heart is still beating. This will make it much easier to flush out the blood, and remove negative impacts of remaining blood and clots in the tissue. After removal of the heart, place the heart immediately in room temperature KH-BDM solution. In case the heart is not immediately further processed (> 5 minutes), use ice-cold KH solution to store the heart in a refrigerator.
- 2) Placement in dissection dish: Transport the heart to dissection dish, and connect the aorta to a cannula (put aorta 1-2 mm over the cannula, and use a clip, suture, or a pin to keep in place) in order to flush the heart with the KH-BDM solution (at room temperature), make sure the solution is continuously bubbled with the gas mixture to maintain constant pH. Suck up and discard the bloody solution. Once the heart is completely flushed and clear, proceed to the next step.
- 3) Opening of the ventricle: Orient the heart on its side with the ventricular septum running longitudinally and the apex pointing towards you. The right ventricle should be facing upwards. Using a small pin (insect pins with diameter under 1mm work well), pin the aorta to the base of the dish so that the heart is in the center of the dissection dish. Place another pin through the tip of the left ventricular apex so that the heart is held in position. Using micro tweezers, gently grasp the right ventricular epicardium (without puncturing the ventricle) near the RV apex and make a small longitudinal cut immediately adjacent to the ventricular septum into the RV with micro scissors. Next, with the scissors pointing towards the base of the heart, insert one blade of the

micro scissors into the newly opened slit, immediately beneath the RV free wall and with a careful cutting motion, continue to the RV base. Next, make two horizontal incisions; one at the RV base and another at the RV apex, extending away from the long incision previously made along the septum and into the RV. You should now be able to lay open the RV free wall and pin its two free corners to the dish. A similar technique is used for LV muscles, however, in most murine and rat hearts, the ideally sized muscles are hardly ever found: the papillary muscles are too big, and the trabeculae in the LV are typically more branched and less suitable for mechanical experiments. If the use of LV muscles is a must, plan on using a much larger initial sample size.

- 4) Selection of muscles: With the RV lying flat on the dissection dish, scan the tissue for appropriately sized trabeculae or papillary muscle. There will typically be 3 papillary muscles in the RV, with the base of each attached to the septum or free wall and the tip attached via chordae tendineae to the tricuspid valve. Typically, papillary muscles are used for live perfusion studies only from mouse hearts, due to the large size of papillary muscle in larger species. Often, papillary muscles are located very close together, so you may need to decide to isolate one or two muscles at the cost of another. Trabeculae that are unbranched and no more than $\sim 250 \mu\text{m}$ in diameter may also be used and are preferred in larger species where the papillary muscle exceeds these size limitations. In muscle that exceed $\sim 250 \mu\text{m}$ in diameter, the core of the muscle will likely be or become hypoxic during the experiment. A large number of studies have empirically shown that using muscles larger than $250 \mu\text{m}$ in diameter will result in sub-optimal contractions, impaired relaxation, and results are therefore rather ambiguous.^{12, 13, 15, 16} Under very demanding conditions, i.e. high frequency and under inotropic stimulation (high calcium concentration, or with beta-stimulation), even muscles of $250 \mu\text{m}$ may not be thin enough, and if such demanding conditions are needed in the protocol, only muscles with a diameter of $\sim 100\text{-}125 \mu\text{m}$ should be used.¹⁵
- 5) Dissection of the muscle: To dissect papillary muscle, grasp tissue near the base of the muscle, but be very careful to avoid touching the body of the muscle or stretching the muscle. At no time during the dissection should the muscle itself be touched, nor any tissue closer than $250 \mu\text{m}$. Using micro scissors, cut a small chunk of RV tissue at the base of the muscle to free it from the ventricle. The muscle should now only be attached to the heart via the chordae tendineae at the tip. Next, by carefully holding only the RV chunk that you just freed (and not the muscle itself), cut the muscle free by severing the chordae tendineae from the valve. There should be enough chordae tendineae left to pierce the tissue over a small hook in the muscle bath and enough RV tissue at the base to hold the muscle within a small "basket". Dissect trabeculae similarly, but with two chunks of RV at each end. Again, do not touch the muscle, nor anything within $250 \mu\text{m}$

of its ends. Once the muscle is dissected, measure the width of the muscle, and the thickness. To measure thickness, place the muscle on its side.

- 6) Mounting of the muscle: The muscle will need to be mounted horizontally in a muscle bath that contains circulating K-H solution warmed to 37°C and bubbled constantly with 95% O₂- 5% CO₂ gas. Attached to the force transducer should be a “basket”, made using a minuten pin bent into a circular shape and inserted into a needle shaft that is connected to the transducer. At the other end of the bath should be another minuten pin bent into a hook shape that is attached to a micromanipulator. Transfer the muscle to the muscle bath by gently drawing KH-BDM (along with the muscle) into a ~ 2 cm long piece of flexible tubing attached to a syringe. With muscle inside the tubing, position the syringe over the muscle bath so that the tip of the tubing breaks the surface tension of the KH in the bath. Do not push KH-BDM from the syringe into the bath, but allow gravity to draw the muscle out of the tube and into the muscle bath. Using micro tweezers and touching only the chunks/tendon on either end of the muscle at the part furthest from the actual muscle, pull one chunk (or tendon) through the basket towards the other end of the bath. Make sure the hook and basket are closer together than the resting length of the muscle. Pull the muscle through the basket without dragging the muscle against the basket so that the chunk of RV catches in the basket and hold the muscle in place. Pierce the chunk (or tendon) at the other end of the muscle over the hook without stretching the muscle beyond its resting length.
- 7) Assessment of optimal length: Be sure KH solution is circulating in the muscle bath and turn the stimulation on, set to baseline frequency (typically below resting frequency), to avoid initial calcium overload injury. Raise the calcium concentration stepwise from the current 0.25 mM to 1.5 mM. For mouse, start at 4 Hz, for rat, at 2 Hz, for rabbit, start at 1 Hz, for larger species including human, start at either 0.5 or 1 Hz.⁸ With the muscle hanging at slack length (no tension on muscle), zero the force transducer readout and measure width of the muscle. Stretch the muscle in small increments (~5-10 μm at a time) until optimal length is reached. Optimal length can be defined as the point just before which there is a disproportionate increase in resting tension, relative to the increase in developed tension.
- 8) Contraction assays: Project specific assays can now be performed on the contracting muscle. Basic assays include evaluation of length-force, frequency-force, and isoproterenol-force relationships.^{3, 4} For length-force assessment, measure contraction at optimal length, then at 85%, 90%, 95% and 100% of optimal length, allowing the muscle to stabilize between each length change (~2-3 minutes). These 4 points will roughly account for end systolic length (85%), end diastolic length (95%), a value in between (90%), and optimal length (high volume,

100%). For frequency-force assessment, measure contraction at optimal length at baseline frequency, followed by frequency alterations, with stabilization between each frequency (0.5-3 minutes). Set the frequencies such that they at least encompass the species entire working range. Mouse: 8-14 Hz, Rat: 4-9 Hz, Rabbit: 1-4 Hz, Dog, 1-4 Hz, Human, 0.5-3 Hz. For isoproterenol-force assessment, record all data at baseline frequency with increasing concentrations of isoproterenol (typically semi-log increments from pre-prepared stock solutions of 10^{-5} and 10^{-2} M), starting at 10^{-9} M up to 10^{-6} M, allowing the muscle to stabilize between each isoproterenol addition (3-5 minutes).

E. EVALUATION AND INTERPRETATION OF RESULTS

For wild-type murine muscles, forces obtained at optimal length, at a baseline of 4 Hz should be in the 10-30 mN/mm² range¹⁵. Forces well below that range typically indicate a damaged or compromised muscle. At maximal isoproterenol concentration, force should typically increase to the 30-70 mN/mm² range. Kinetics of the muscle should typically be about 40-45 ms for time to peak tension (TTP), and about 20-25 ms from time from peak tension to 50% relaxation (RT50%). The response to increases in length should be an increase in tension, and a small decrease in kinetics (slower time to peak and slower time to 50% relaxation). The increase in frequency, when assessed, should be a small increase from 4 to ~10 Hz, followed by a plateau and/or a decrease, whereas the kinetics should slightly accelerate (shorter TTP and RT50%) as frequency increases over the entire range. The response to isoproterenol should be a robust dose-dependent increase in tension (EC50 ~30 nM), and an acceleration of kinetics. In other species, force levels are remarkably similar; rat, rabbit, dog, and human all should produce (if free from disease), a similar baseline force level. Kinetics however are increasingly slower as the size of the species increases. For examples of values see: rat^{9, 10}, rabbit^{10, 11}, dog¹, human^{5, 7}.

Clearly, muscles from a genetic model, or a disease model, can vary from these values. However, there is a bottom limit below which values are likely to be not reflecting true function. If force is below 5 mN/mm² during the entire protocol, the ejection fraction of this heart would be so poor that the mouse would unlikely live. Given that the muscle was taken from a recently living animal, it is more likely damaged than it is a result of the genetic manipulation or disease. For most, but perhaps not all (i.e. in severe cases of cardiac hypertrophy the large muscle mass may compensate for a very low specific force and allow the animal to survive), studies, muscles not generating at least 5 mN/mm² during any time in the protocol should be discarded from analysis. If kinetics are much slower than indicated above, first make sure the temperature of the muscle bath is correctly set for 37 degrees, even a few degrees less causes much slower kinetics.²

F. REFERENCES

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