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Single myofiber isolation

Notes:

- Fibers will stick to tissue culture plastic, so all clean-up should be done using *bacterial* 10 cm plates.

Preparation

- Prepare digest media and RV media:
    - Digest media (in 15 mL tube): 9 mL high-glucose DMEM, 100 U/mL penicillin G and 100 ug/mL streptomycin (p/s).
        - Add HEPES buffer (25 mM final) if digest cannot be performed in an incubator with 5% CO_2
        - 1 mL collagenase will be added at time of digest for a final digest volume of ≈10 mL
    - RV media: High glucose DMEM + 10% FBS + p/s.
- Polish the tips of Pasteur pipets in a flame until opening at end is very small
- Incubate several 10 cm bacterial plates (usually 2-3 per gastroc plus “clean” plates) containing 12-15 mL each of RV media for at least 30 min before adding fibers.

- Coat 24-well plates with 10% Matrigel (I do this once I have the muscle digesting. I have better luck with fibers sticking if I let them coat for a few hours rather than overnight):
    - Thaw Matrigel aliquot on ice (particulate debris may be spun out – do not filter!)
    - Using a repeat pipettor, dispense 50 uL Matrigel into each well
    - Ensure that wells are fully coated by shaking plate, then tilt and remove excess Matrigel with a p1000 (avoid bubbles). Matrigel may be re-used for multiple plates, but do not re-freeze aliquot.
- Incubate plate at 37°C until time of plating fibers.

Procedure:

Muscle excision and digest

1. Pre-warm digest media in water bath (37°C) for at least 10 min before beginning procedure. Thaw collagenase aliquot at RT. Just before excising muscle, add collagenase to digest media invert several times to mix.
2. Dissect gastrocnemius muscle from right and left hindlimbs.
    - Rip back skin over muscle and around ankle to expose Achilles tendon
    - Make small incision behind knee and use forceps and scissors to cut upper layer of fascia/connective tissue down length of the muscle. Peel away as much as possible down either side using scalpel.
    - Cut Achilles tendon and pull up gastroc while trimming connective tissue on either side.
    - Rinse muscle in PBS, and then slice down center of muscle with scalpel while holding tendon with forceps. Do NOT stretch the muscle.
    - Immediately transfer excised muscles to 15 mL tube containing digest mix.
3. Incubate tube in 37C water bath for 1 hour (have tube (with cap parafilmed) lie on its side in the water)
4. Rock for 30 minutes at 37C in the envirogenie (set speed to 15:30).
5. After digest, allow fibers to settle, then gently remove digest media (down to about 1.5-2 mL mark) with a pipet. Add ~10 mL fresh (pre-warmed) RV media. Invert tube gently, and allow fibers to settle.
6. Repeat 2-3 times.
7. Transfer 3-4 mL of digest to a series (usually do 2) of 10 cm plates containing warm RV media.
    - The more debris, the more plates the digest should be separated into

“Cleaning up” fibers

8. “Clean up” fibers away from surrounding dead (hypercontracted) fibers and debris by transferring to a clean plate using the fire-polished Pasteur pipet (live fibers will appear translucent and shiny, length and thickness will vary).

9. Keep plates out of incubator only for 10-15 min at a time, then warm in incubator 10 min before continuing.
10. Once all your fibers are picked into a clean plate, carefully, using a 10 ml pipet, transfer fibers to a 15 ml tube. Can rinse plate with a few ml of RV media.
11. Let fibers settle, aspirate media, and add 10ml RV media. Gently invert the tube to wash. Let fibers settle again. Repeat 2-3 times to ensure fibers are free of any contaminating cells. Resuspend in final volume of 10 ml and pour into a fresh 10cm plate. Can rinse tube with a few ml of RV media.
12. When ready to plate, add 1 mL warm RV media to each well of a Matrigel-coated 24 well plate. Allow to equilibrate in incubator 10-15 min before plating.

Plating fibers

13. Using a glass fired Pasteur pipet, transfer 1-3 fibers to each well. Try and pull up as little media as possible with each fiber.
14. After all fibers have been plated, let sit in incubator for at least 15 min.
15. (Optional) Centrifuge plate @ 1100g, 37° C, for 30-40 minutes to increase adherence of the fiber (this is important if immunostaining time 0 fibers!)
16. Maintain fibers @ 37° C in incubator (do not refeed!!) and fix at desired time.
    17. If you plan on culturing fibers for more than 3 days, add FGF to the RV media to prevent the cells from fusing.

Fixing fibers:

1. Carefully remove about 75% of the media. DO NOT remove all the media or the fibers will hypercontract.
2. Carefully add 1 mL 2% formaldehyde to each well, fix for 10-12 min
3. Aspirate about 75% of formaldehyde and fill wells with max amount of PBS per well.
4. Repeat several times by aspirating about 75% of the PBS and then diluting again, being careful not to dislodge fibers. Once fibers are fixed it's OK to aspirate all the liquid.

5. Stain fibers immediately or seal plate with parafilm and store at 4°C in PBS + 0.02% sodium azide to prevent contamination.

Reagents:

GFR Matrigel (GFR = growth factor reduced)

- BD Biosciences cat #354230 (10 mL) – store at –20°C
- To prepare 10% working solution: thaw stock ON ICE (Matrigel "gels" at room temp, so it must be kept on ice at all times) and dilute 1:10 in DMEM. Store aliquots at –20°C
  - Avoid repeated freeze/thawing of Matrigel stock

Collagenase Type I

- Worthington, cat# MIE4816
- Prepare 10X stock (= 4000 U/mL):
  - Each lot has a different activity (U/mg), so be sure to check!
  - Dissolve appropriate amount of powder in sterile H_2O
- Store 1 mL aliquots at –20°C, thaw at room temp at time of use