Good Lab FACS Protocol

Advanced preparation:

1. Preparation to do days or weeks ahead of time
2. **Consult and schedule with flow cytometry core**
3. Hoechst 33324: suspend in DMSO
    a. 10mg/mL, flash freeze, thaw overnight
    b. Must be Invitrogen Hoechst (the kind that comes as a solid, not liquid)
    c. After suspending in DMSO, should be very bright green and clumpy NOT dark green and liquid
4. Propidium Iodide (PI): suspend in molecular grade water
    a. 1mg/mL
    b. Keep in dark!
5. DNase I: comes lyophilized and needs to be suspended in molecular grade water
    a. For Qiagen DNase I: peel back plastic on lid, use a 1mL syringe and needle to add 550uL molecular-grade water (DNase and RNase free)
    b. invert a few times to mix and make sure all enzyme is dissolved, then use syringe to pull liquid out of container it comes in and transfer to clean, labeled epitube (color recommended); aliquots of ~150uL recommended
    c. Qiagen DNase kit comes with instructions for this as well
    d. Try to avoid refreezing

Preparation (morning of sort):

1. **Plan for about 1 hour of preparation on the morning of the sort before you start the mouse dissection and testis disassociation**
2. Prep enzymes (best to do this the morning of the sort, plan for 20-30min)
    a. Collagenase: 10mg collagenase dissolved in 200 uL Gey's balanced salt solution (GBSS)
        i. Notes: stock (dry) collagenase and GBSS should be stored in fridge
        ii. Pipette most of 200uL of liquid into small weigh boat and put the rest of it into a clean, labeled, colored epitube (colors easier to see on ice, label cap or use lab tape if colored tubes not available)
        iii. Place weight boat with liquid in balance, tare, and then use clean (wipe with EtOH) spatula to add enzyme
        iv. Mix with pipette until all enzyme is dissolved; **pipette very slowly without letting air in to prevent the solution from getting bubbly**; if needed, add some of the liquid that was put in the epitube to the weigh boat to help dissolve
        v. Pipette as much of the dissolved enzyme as possible into the colored epitube from step i
        vi. Store on ice in ice bucket
    b. Trypsin: 10mg Trypsin dissolved in 200 uL**1mM** HCl

        i. Notes: stock (dry) trypsin should be stored in fridge, stock HCl should be stored in inorganic acids cabinet at room temperature, molecular grade water for diluting HCl should be stored at room temperature
        ii. **HCl may need to be diluted!** Check concentration of stock solution
        iii. Dissolve enzyme using same protocol as collagenase (steps i-iv above)
        iv. Store on ice in ice bucket
    c. DNase I – take out of freezer to thaw
3. Turn on incubators – do this early so that they have time to heat up
    a. SciGene oven with rotator for testes disassociation: 33°C
    b. Incubator in dissection room: 37°C
    c. If doing sperm counts, bench top incubator in dissection room: 60°C
4. Set up for testes disassociation (on clean lab bench)
    a. Large styrofoam ice bucket with lid, full of ice
    b. Incubator set to 33°C with rotator inside (or shaker that can gently rotate)
    c. Gather these items and put on ice: GBSS, Collagenase solution, Trypsin solution, DNase I
    d. Gather these items and put in drawer (need to thaw at room temp in dark): fetal calf serum (FCS) and Hoechst 33342 solution. Put the Hoechst aliquot in an epitube holder so it doesn't roll around. Put DNAse I in the same holder if it isn't thawed yet but **move to ice bucket as soon as it is**
    e. Gather these items and place on bench (room temp and light okay): holder for 15mL tubes, several disposable transfer pipettes, waste beaker, two 50mL conical tubes and holder, four disposable cell strainers, 5mL syringe, 18 gauge needle, 22 gauge needle, paper towel, sharp forceps, flat forceps
    f. Gather these items and place on metal tray (these are to take to sorter): colored epitube (1mL or larger), glass vials with and without filter caps, p10 and p1000 pipettes, p10 and p1000 pipette tips (no filter okay)
    g. Fill two 15mL conical tubes with 3mL GBSS and 30uL collagenase solution
        i. Store on ice
5. Set-up for mouse dissection
    a. Make sure incubator set to 37°C and hot plate to 60°C
    b. Pipette ~1mL Dulbecco's solution into epi tube and place in 37C dissection room incubator
    c. Place two watch glasses in 37°C dissection room incubator
    d. Gather paper towels, two forceps, scissors, razor, ruler, balance, mouse phenotyping binder, two pre-weighed epitubes, square of parafilm, Makler chamber, paintbrush, labeled side, counter, lens paper/kim wipes, tape, pens
    e. Make sure microscope and CO2 chamber are ready to go
6. Set-up for clean room – do this AFTER sort
    a. Make sure everything is sterile and RNase free!
    b. Clean area with RNase away

c. Gather RTL buffer from Qiagen RNA extraction kits, β-mercaptoethanol (BME), 16 sterile screw-cap tubes with lids, sterile rack for holding these tubes, sharpie, Qiagen RNA extraction kits, pipettes and tips

Mouse dissection and phenotyping:

1. Bring a Sherman trap and small box to LAR facility and put mouse in Sherman trap to bring up to lab; don't forget cage card too!
2. **Plan 3-4 hours for mouse dissection and testis disassociation**
3. Euthanize mouse in CO2 chamber, followed by cervical dislocation
4. Tape cage card to phenotyping sheet – fill this out as you take measurements/count sperm
5. Weigh mouse
6. Make incision across abdomen, remove testes with cauda epididymis
7. Pour warm Dulbecco's into watch glass and place cauda epididymides in watch glass, tear apart and mix with forceps and razor blade, cover with parafilm and place other watch glass on top, return to incubator for **10 minutes**
8. Place testes in preweighed epitube, weigh, record
9. GO TO TESTIS DISASSOCIATION PROCEDURE (step 1)
10. After testes are in incubator on shaker, dissect out seminal vesicles, place in preweighed epitube, weigh, record
11. IF COLLECTING TISSUE: dissect out liver (and whatever other tissues you want), place in preweighed, labeled epitubes and place in -80 freezer
12. Record mouse measurements: nose to tail length, tail length, foot length, ear length (opposite sides for foot and ear)
13. Place mouse in Ziploc bag and place in freezer
14. Make sure sperm have incubated for 10 min in watch glass
15. Remove large chunks of debris, mix gently w/ wide-bore pipette tip, transfer at least 200 uL to epitube and place on bench top incubator (60°C) to heat shock
16. GO BACK TO TESTIS DISASSOCIATON PROCEDURE (step 7)
17. Remove epitube from heat block and mix gently with wide-bore pipette
18. Spread 25 uL of heat-shocked sperm solution evenly on labeled slide without touching slide with pipette tip
19. Pipette 5 uL of heat-shocked sperm solution on Makler chamber and count 5 rows of sperm (every other row); record
20. If there are sperm clumps, redo
21. Clean up
22. GO BACK TO TESTIS DISASSOCIATON PROCEDURE (step 13)

Testis disassociation:

1. Move testes to ice bucket on lab bench
2. Make a small tear in testis with sharp forceps, use flat forceps to squeeze tubules out into 15mL conical tube with GBSS and collagenase (one tube per testis)
3. Add **15 uL** DNase I to each tube

4. SHAKE GENTLY – this is important to have enough disassociation, but do not shake vigorously because cells can lyse
5. Place **horizontally** on rotator in incubator for **15 minutes,** tape tubes down
    a. Incubator should be at **33C** and rotator in SciGene oven should be around 3-4
    b. If using a shaker, should be at **120rpm**
6. GO BACK TO MOUSE PROCESSING PROCEDURE (step 10)
7. Move tubes to ice bucket and let settle for ~1min
    a. **If testes don't look like they're breaking up or are still in compact clumps, agitate gently by hand again and/or incubate for a few more minutes**
    b. **Leave tubes on ice while adding reagents, instead of moving to holder!!!**
8. Use disposable pipette to transfer liquid from tubes to waste beaker
    a. Don't get too close to testis to avoid sucking them into pipette!
9. Add: **3mL** GBSS, **30 uL** collagenase solution, **15 uL** DNase 1
10. Agitate gently by hand
11. Incubate for **15 minutes** at **120rpm** and **33C**
12. GO BACK TO MOUSE PHENOTYPING PROCEDURE (step 17)
13. Move tubes to ice bucket and let settle for ~1min – tubules should look like long strings!
    a. **Agitate more or incubate longer if tubules don't look like long strings**
    b. **Leave tubes on ice**
14. Use disposable pipette to transfer liquid from tubes to waste beaker (see step 8 details)
    a. This is the last wash step, so better to get a bit closer to the tubules and remove more debris this time
15. Add: **2.5mL** of GBSS (less than before!), **30 uL** collagenase solution, **50 uL trypsin** solution (new this time!), **15 uL** DNase I
16. Agitate gently by hand
17. Incubate for **15 minutes** at **120rpm** and **33C**
18. **During the above incubation**, move to clean room and mix lysis buffers
    a. **New gloves!**
    b. These are the tubes/buffers cells will be sorted into on FACS machine
    c. Label 16 sterile screw cap tubes AND caps with cell type and number
        i. Sorter will take 7 rounds or sorting to get through a whole sample
        ii. One screw cap tube can hold 2 rounds worth of sorting volume
        iii. So each cell type requires four screw-cap tubes (see table for labeling)
    d. Add RTL buffer from Qiagen kit to each tube, amount depends on type of mouse and which tube (for tubes that will hold 2 rounds of sorting: 600 for less fertile mouse, 650 for more fertile mouse; for tubes that will hold 1 round of sorting: 300 for less fertile mouse, 350 for more fertile mouse) (see table)
    e. Add 15uL BME per mL lysis buffer
    f. Place tubes in ice bucket

Labeling and volume example for a more fertile mouse and 4 spermatogenesis cell types (SP = spermatogonia, LZ = leptotene-zygotene, DIP = diplotene, RS = round spermatids):

SP1,2 (600uL RLT, 9ul BME)	SP3,4 (600uL RLT, 9uL BME)	SP5,6 (600uL RLT, 9uL BME)	SP7 (300uL RLT, 4.5uL BME)
LZ1,2 (600uL RLT, 9uL BME)	LZ3,4 (600uL RLT, 9uL BME)	LZ5,6 (600uL RLT, 9uL BME)	LZ7 (300uL RLT, 4.5uL BME)
DIP1,2 (600uL RLT, 9uL BME)	DIP3,4 (600uL RLT, 9uL BME)	DIP5,6 (600uL RLT, 9uL BME)	DIP7 (300uL RLT, 4.5uL BME)
RS1,2 (600uL RLT, 9uL BME)	RS3,4 (600uL RLT, 9uL BME)	RS5,6 (600uL RLT, 9uL BME)	RS7 (300uL RLT, 4.5uL BME)

19. Move tubes to **ice bucket** on bench– tubules should look like short segments!
    a. **Agitate or incubate longer if not in short segments**
20. Use disposable pipette to gently mix each tube until no clumps visible (~1 min)
21. Add: **30 uL** trypsin solution, **15 uL** DNase I
22. Remove Hoescht 33342 from drawer and mix gently by pipetting up and down
23. Add **60 uL** Hoechst 33342 to each 15mL conical tube
24. Agitate gently by hand
25. Incubate for **15 minutes** at **120rpm** and **33C**
26. **During above incubation,** finish setting up tubes with buffer/BME, clean up dissection room, clean up testis disassociation stuff no longer needed (ex: forceps, enzymes)
27. Move tubes to **ice bucket** on bench
28. Add **400 uL** fetal calf serum, **70 uL** Hoechst, **15 uL** DNase I
29. Agitate gently by hand
30. Incubate for **15 minutes** at **120rpm** and **33C**
31. **During above incubation** prep stuff to go to sorter
    a. Aliquot PI – surround with foil to keep in dark and place in ice bucket!
    b. During last ~5 mins of incubation, start cell strainer prep (steps 33-35)
32. Move tubes to **ice bucket** on bench
33. Remove the lid from a 50mL conical tube and place in tube holder
34. Place a disposable cell strainer on 50mL conical tube (should sit nicely in the opening) and place a second strainer on top of the first (they don't fit into each other so you need to hold the top one in place)
35. Wet both strainers with GBSS and be sure to get the sides
36. Pipette extra GBSS out of the conical tube
37. Use a disposable pipette to slowly drop liquid from the 15mL conical vials (disassociated testis sample) over the top cell strainer
    a. If set-up is correct, liquid should go from disposable pipette through the first strainer, then the second, then into 50mL conical vial
    b. Do this SLOWLY so that the strainers don't clog
    c. May need to rotate top strainer so that liquid goes through the sides if bottom gets clogged
    d. May need to lift up bottom strainer occasionally so that suction doesn't prevent liquid from flowing into 50mL conical tube

38. **Repeat** steps 33-37 in a new 50mL conical tube with two new cell strainers; repeat again for a total of three times if you have time
39. Add DNase to 50mL conical tube (~15uL for low sperm count mouse and ~30uL for higher sperm count mouse)
    a. If not enough DNase, loose DNA will clog the sorter, so higher end of estimates is best
40. Attach 18-gauge needle to 5mL syringe and use to pull as much of sample as possible from 50mL conical tube into syringe
41. Remove 18-gauge needle and dispose of in sharps container
42. Attach 22-gauge needle to 5mL syringe (which is now full of sample); leave cap on!
43. Place syringe with sample, needle, and needle cap in ice bucket
44. Go down to sorter; before you go make sure you have these things, and RNase-away them if possible:
    a. In ice bucket: 16 labeled tubes with lysis buffer and BME, 5mL syringe with sample in it and capped 22-gauge needle attached, PI aliquot (wrapped in foil), DNase I, lid for ice bucket
    b. On tray: empty epitube (colorful!), vials with and without filter caps, p10 and p1000 pipettes, p10 and p1000 pipette tips (no filter needed), copy of cell sort procedure
    c. If time, clean up, but if running late or cutting it close for cell sort appointment just deal with essential things and take care of rest later
        i. Essential things: needles and razors disposed of in sharps container, FCS back in freezer, all reagents either in ice bucket or back in proper storage

Sorting Procedure:

1. May very depending on machine used for FACS and different flow cytometry core protocols
2. **Plan for 4-5 hours**
3. Place four screw-cap tubes (one for each cell type) into FACS machine; make sure tube label is correct for the round of sorting you are on (ex: you should start with SP1,2; LZ1,2; DIP1,2; RS1;2)
4. FOR FIRST ROUND OF SORTING:
    a. Use the syringe to gently place **500 uL** of sample into colored epitube by slowly ejecting sample along the side of the tube
    b. Add **1.5 uL PI** and **15 uL DNase I** to epitube and mix by pipetting gently
    c. Use a pipette to move sample from epitube into vial **with filter cap** (should pipette slowly onto filter cap)
    d. Remove filter cap from vial and **save filter cap** by placing on to clean glass vial and putting new vial with old cap on ice; place vial on sorter
    e. Close ice bucket lid (keep dark and cold!)
5. For other rounds of sorting (rounds 2-6 or 7 depending on how much sample you have)
    f. Use syringe to gently place **700 uL** of sample into colored epitube by slowly ejecting sample along the side of the tube

   g. Add **1.8 uL PI** and **15-20uL DNase I** (volume depending on if sample is clogging or not) to epitube and mix by pipetting gently
   h. Add **15-20 uL** DNase I and mix by pipetting gently
   i. Use pipette to move sample from epitube into vial; **reuse filter cap from first vial**
   j. Remove filter cap and **save**; give vial to Pam for sorting
6. Between rounds of sorting, vortex screw cap tubes being used to store sorted cells
7. After two rounds of sorting, remove screw cap tube and place in ice bucket; move next set of labeled screw cap tubes to sorter

RNA extraction:

1. BE VERY CAREFUL OF RNases!!!
   a. Best done in a clean room, if possible
   b. **Spray everything with RNase away** before moving it into clean room or starting the next steps
2. For each cell type, combine the four screw cap tubes of sorted cells into one 5mL screw cap tube
3. Add an equal volume of 80% EtOH to each 5mL screw cap tube
   a. Make this fresh! Use only molecular grate EtOH and H2O that are specific to the clean room
   b. Mix samples well after adding EtOH
4. Step 1 of the RNeasey protocol is to pipette <=700uL of the sample+EtOH solution into a spin column and centrifuge – THIS NEEDS TO BE REPEATED UNTIL THE WHOLE SAMPLE HAS BEEN ADDED TO THE COLUMN
   a. RNeasy tubes can only hold 700uL but the output from sorts+EtOH will be ~5mL for each cell type, so:
      i. Add 700uL of sample to a spin column tube, one tube per sample
      ii. Centrifuge as directed by the RNeasy protocol
      iii. Discard flow-through
      iv. Repeat i-iii with the same spin column until all 5mL of sample have been run through the same column, such that all RNA from one cell type should be in the membrane of one column
5. Follow the rest of the RNeasy protocol from Qiagen RNA extraction kit
   a. Include DNA digestion and optional dry step described in the RNeasy protocol
   b. Do NOT need to do RNA cleanup procedure in part II
   c. Elute into 55 uL molecular grade H2O that is specific to clean room
   d. Use non-stick epitubes or tubes provided in kit
6. Check extractions with Agilent tapestation
   a. See tapestation protocol for RNA
   b. RINs should be >8 to continue with library preps
7. Move RNA to -80 ASAP for storage