Adult Neuron Isolation Protocol (R. Kaletsky 9/2014)

Notes before starting:

- Begin protocol ~45min before sort time to minimize time between cell harvesting and sorting
- Prepare pronase solution immediately before isolation
- Lysis buffer is made prior to isolation and is frozen in 1mL aliquots
- Prepare solution of 2% FBS in PBS-/-. Or 2% FBS in L15 media. Keep on ice
- Start with at least 3 plates of synchronized, well-fed worms on HG plates
- Always include age-matched N2-negative control to set FACS gates

Protocol:

- **Wash worms into 1.5ml clear epi tube using M9**
  - Be careful not to collect chunks of bacteria with worms
  - If worm pellet exceeds 250ul, split sample between 2 tubes and process separately
  - Rapid washes are performed using MiniSpin centrifuge to pellet worms

- **Wash worms 5x in M9**
  - Wash until all of food is removed

- **Wash 1x in room temp lysis buffer ~500ul. Remove supe**

- **Add 750ul lysis buffer**

- **Incubate at RT for exactly 6.5 min for Day 1 adults**
  - We have found that dpy and roller strains are more sensitive to lysis conditions. Incubate for exactly 6 min
  - Monitor cuticle disruption by examining 1ul of sample under a microscope without a coverslip. Near the completion of this incubation, worms should be dead and the head should appear slightly blunt as a result of the weakened cuticle. However, worms should not rupture at this step. Overexposure to SDS-DTT will diminish cell integrity and GFP signal.

- **Wash rapidly 5x in M9**
  - Tubes should no longer smell like DTT
  - Carryover of SDS-GFP will damage cells

- **Add 500ul of freshly made, room temp, 20 mg/ml Pronase (resuspended in water or osmolarity adjusted L15 buffer) to worm pellet**
  - Pronase and/or salts will precipitate if resuspended in PBS or M9. H20 works well for us, but other buffers (ie, egg buffer) can be tried.

- **Incubate up to 20 min at RT.**
    - Dissociation can take between 12-20 min depending on lot of Pronase.

- **Pipette vigorously with P200 tip for 100 times every 2-3 min, rotating between samples.**
    - Monitor dissociation after each round of pipetting by checking 1ul of pronase suspension under microscope. Large worm chunks will gradually disappear, and the suspension will become particulate and cloudy.
    - Move on to next step when the majority of worm chunks (especially intact heads for neuron isolation) are no longer visible

- **Use ice cold PBS solution to pre-wet a 5uM syringe filter (for neuron isolation) using a 1mL syringe**
    - Do this between pipetting steps or at beginning of protocol
    - Store filters on ice

- **Examine 2ul of worms on dissecting scope after every pipetting stage to assess level of dissociation**
    - Different batches of pronase exhibit variable activity and dissociation rates. Incubation times vary anywhere from 15-25min. Proceed to next step immediately when intact worms are no longer visible. Large worm fragments are acceptable. Avoid overdigestion.

- **Add 250ul of PBS/FBS to stop cell digestion**

- **(Optional: Transfer 50ul aliquot of dissociated, pre-filtered worm sample to tube containing Trizol LS . This sample can serve as a 'whole worm' reference upon sequencing.)**

- **Transfer dissociated cells to 1mL syringe using 27 gauge needle.**
    - Can also pipette into syringe/filter setup

- **Swap out needle for filter and gently pass cells through the syringe filter directly into FACS tube on ice**

- **Wash filter with 0.5-1 mL PBS/FBS solution, depending on cell dilution requirements for cell sorter**
    - Filtered cell suspension should appear cloudy

- **Keep on ice and in dark until sort**

- **Sort GFP+ cells directly into Trizol LS**
    - Prepare 850ul aliquots of Trizol LS in collection tubes
    - Can add sorted cell volume of 150ul max per tube to maintain Trizol effectiveness
        - ~250,000 cells max per tube

- **Freeze cells in Trizol at -80C until RNA isolation.**

Notes:

It is critical to work rapidly through this protocol. Cells and cell fragments are highly fragile. Optimal sorting conditions are achieved when cells are harvested and on ice within 30-35 min of starting isolation.

Cell isolation and sorting can successfully be performed in the presence of Actinomycin D (*see note below*) to prevent new transcription that occurs during cell preparation. Act D is added to all solutions (Lysis buffer, M9, Pronase, and PBS-FBS) immediately before isolation. Solutions are kept in the dark. Act D can diminish GFP intensity and/or increase autofluorescence. Use Act D-treated controls to establish sorting gates.

We have successfully isolated RNA from aged animal tissues (testing up to Day 10 adults) using the same protocol. Incubation times in pronase may be slightly reduced and should be monitored closely.

Protocol modifications for hypodermis, muscle, and intestine: Other cell types and fragments require modified filtering steps. Following pronase incubation, muscle and hypodermis are filtered through a pre-wet 20 micron Nitex Nylon (Sefar Filtration) mesh attached to a 50mL Falcon tube. Samples are filtered using gravity flow. Intestinal cells are filtered using a 35 micron mesh. Eggs from dissociated adult animals will pass through a 35 micron pore. To avoid sorting embryonic tissues, a marker must be used that does not appear in eggs, or alternatively sort intestine from a sterile strain (ie, *fem-1;fer-15*).

Recipes

Lysis buffer
200 mM DTT
0.25% SDS
20 mM HEPES pH 8.0
3% sucrose
Prepare 50mL. Freeze in 1.5ml aliquots at -20C.

Pronase (Protease from Streptomyces griseus Sigma-Aldrich Cat No: P6911-1G)
20 mg/mL in ddH20
Prepare fresh each time

PBS-FBS solution
Dilute 10x PBS-/- stock in ddH20
Thaw heat inactivated FBS (Fetal Bovine Serum, Certified, Heat-Inactivated Invitrogen Cat No: 10082-139)
Add to PBS to make 2% final concentration

L15-PBS solution
Adjust osmolarity of L15 to 340mM. (Check lot for current osmolarity. Add 1mM sucrose for every 1mM osm to adjust. For example, if currently 312mM, require 28mM osm adjustment. Calculate how much solid sucrose to get 28mM sucrose added. Sterile filterize.)
Add FBS to get 2% final conc in L15.

Optional: Prepare all solutions in the presence of 100 ug/mL Actinomycin D.

RNA isolation from sorted cells for RNA-seq

- Thaw worm/trizol mixture at RT. Incubate at 65C for 5 min with occasional vortexing
- Spin in centrifuge for 10 min at 14k at 4C
    - ALL steps are performed in RNAse free conditions
- Move supernatant to new 1.7mL eppendorf tube. Debris pellet may not be visible
- Add 0.2mL $CHCl_3$ to tube. Invert 15 sec. Incubate RT for 2 min
- Spin 10 min at 14K and 4C to separate phases
- Fill a new 1.7mL tube with 0.55mL isopropanol
- Move upper aqueous layer to isopropanol and mix. Collect as much of aqueous phase as possible without Trizol carryover
- Incubate at -20C overnight
- Pellet RNA at 14K for 10min at 4C
- Dispose of supernatant in Trizol waste

- Wash pellet (may not be visible) with 0.1mL of 75% EtOH
- Spin 7.5K for 5min at 4C
- Dispose of supernatant without disrupting pellet
- Air dry pellet (~10min until no liquid visible)
- Resuspend in 87.5uL RNAse-free H_2O
- DNAse treat sample in solution using RNase-Free DNase kit (Qiagen)
- Clean RNA using RNeasy MinElute Cleanup Kit (Qiagen)
- Bioanalyze RNA (Agilent RNA 6000 Pico Kit)
- Use maximum volume permitted (<100ng) as input for RNA amplification using Ovation® RNA-Seq System V2 (NuGEN)

RNA amplification

Use Ovation® RNA-Seq System V2 (NuGEN) according to manufacturer's instructions. Minimum input is 500pg RNA. We have demonstrate successful neuron profiling using at least 300pg.

Assess amplification using DNA1000 Bioanalyzer chip

cDNA Shearing

Use Covaris to shear cDNA to 200bp average size. Use manufacturer's protocols. Larger fragments can be obtained if performing 144-nt paired-end sequencing, but 200bp is sufficient for most single-end sequencing applications

Library preparation

500ng – 1ug of sheared cDNA is used as input for Illumina TruSeq DNA library preparation.