On-Chip Sort, 10x library preparation, and RNA sequencing of *Pet1* neurons in the mouse dorsal raphe- Detailed protocol

Preparing single-cell suspension

1. Prepare 500mL of artificial cerebral spinal fluid (ACSF) containing neuronal activity blockers (APV, DNQX, TTX) on ice (126 mM NaCl, 20 mM $NaHCO_3$, 20 mM dextrose, 3 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 50 μM APV, 20 μM DNQX, 100 nM TTX. Bubble thoroughly with 95% O_2/5% CO_2).
2. Deeply anesthetize the animal in 2% isoflurane and decapitate.
3. Dissect out brain and immediately submerge in cold ACSF.
4. Cut the brain coronally to create a flat base.
5. Using a vibratome (e.g. Leica Vibratome VT1000s or similar), glue down a 4% agarose block followed by the brain flat base down, laying vertically against the block. For more fragile tissue, the tissue can be embedded in 2% low melting point agarose/ACSF.
6. Fill the vibratome base with cold ACSF and cut 400um sections, carefully moving sections to room temperature bubbled ACSF.
7. Repeat until all necessary sections are in room temperature ACSF.
8. Repeat steps 2-7 until all tissue has been collected. Ensure all sections are in bubbled room temperature ACSF for at least 5min.
9. Transfer sections to bubbled ACSF containing 1mg/ml protease (Sigma P5147). Incubate for 15min + animals age up to 75min (e.g. sections from p50 animal would be incubated for 65min).
10. After incubation, return slices to protease-free ACSF for 15min.
11. Micro-dissect region of interest under an upright dissection microscope with fluorescence optics. Specifically, we use a Leica M165fc Fluorescence stereomicroscope (61.5 mm working distance with Plan APO 1x, M-Series Objective) with GFP2 Ultra filter set and EL 6000 external light source.
12. Combine tissue in 500-750μl of filtered ACSF/1%FBS depending on amount of tissue. According to the On-Chip user guide, cell density should not exceed 10^6 cells/mL. This can be determined using a hemocytometer (see below).
13. Gently triturate tissue using glass micropipettes of decreasing diameter until a mostly homogenous single cell suspension is achieved.
14. Move cell suspension to ice.
15. Add one drop of NucBlue to the cell suspension and allowed to sit for 20min.
16. In order to estimate overall cell density and relative abundance of fluorescently marked cells of interest prior to sorting, pipette 15ul of dissociated cells onto a hemocytometer and quantify both NucBlue and fluorescent cells. This also allows assessment of general cell health. Well preserved perisomatic morphology including intact proximal neurites indicates healthy cells, whereas the appearance of more uniformly round and bloated cells is indicative of poor cell health.

On-chip Sort

1. Turn on On-chip Sort and PC, boot OnChipFlow software and click “Online”.
2. Create a new sample file.
3. Place a new chip (#1002004 80 um chip) inside of the chip holder and load On-Chip T buffer (or On-chip Sheath Fluid) to Sheath Reservoir (4 mL), Sorting reservoir (1.5 mL), Sample reservoir (300 uL), and Collection reservoir (300 uL).
4. Insert chip holder, ensure correct chip alignment, and click “Prime” button.

5. Remove chip holder from sorter and remove buffer from all chambers.
6. Repeat steps 3 and 4 with cold ACSF/1%FBS instead of T buffer.
7. Remove all buffer from Sample reservoir, and ~280µl from Collection reservoir leaving ~20µl.
8. Add 400-500µl of single cell suspension to Sample reservoir
9. Open plots of interest based on fluorescence being sorted for. Plots used for sorts in Okaty, Sturrock, et al. 2020:
    - FSC (forward scatter) vs SSC (side scatter)
    - FSC vs FL-1 (445/20nm, to detect NucBlue)
    - FSC vs FL-2 (543/22nm, to detect GFP)
10. Push "Run" button to flow a small amount of sample.
11. Press stop once enough sample has flowed through to determine where gates should be set. Adjust gain and numerical factors to optimize separation of target population.
    - As a reference the gain used in this paper are described below. Numerical factors are all maintained at 1.00.
        i. FSC: 10
        ii. SSC: 30
        iii. FL1: 30
        iv. FL2: 30
12. Create an initial gate based on NucBlue population to exclude cell and tissue debris.
13. Change FSC vs FL-2 graph to be based on NucBlue gate (rather than "All (through)").
14. Create a gate around separated population of cells with higher FL-2 expression. Right click the gate and choose "Select population".
15. Press "Sort" to begin sorting. Continue sorting until event rate begins to slow down.
16. As event rate slows either:
    - The Sample reservoir is approaching empty
    - There is a clog
17. Remove chip holder
18. Remove all buffer within the Collection reservoir and add to a 1.5mL Eppendorf tube on ice.
19. Remove any remaining sample from the Sample reservoir and add back to tube containing unsorted single cell suspension.
20. If all unsorted single cell suspension has been sorted, proceed to next section. If there is remaining unsorted single cell suspension, add 300µl of cold ACSF/1%FBS to both the Sample reservoir and Collection reservoir.
21. Insert chip holder, ensure correct chip alignment, and click "Prime" button, to remove any clogs.
22. Remove chip holder, and remove all buffer from Sample reservoir, and ~280µl from Collection reservoir leaving ~20µl.
23. Add remaining single cell suspension and repeat sorting process until all suspension has been sorted, or the desired number of cells have been collected.

Note: If there is low purity of sorted cells, resort the collected cells. If there is low efficiency of collection, ensure that push and pull pressure is optimized. The parameters used in this paper are:

- Main Push 1: 0.9kPa, 0 second delay time
- Main Push 2: 3.4kPa, 6 second delay time
- Main Pull : -0.8kPa, 3 second delay time

<u>Determining final cell numbers and purity for 10x library preparation</u>

1. Prepare reagents for single cell master mix following the preparation and handling info described in the Chromium Single Cell 3' V3 user guide.
2. Pipette 15µl of sorted cell sample onto a hemocytometer.
3. Calculate the total number of cells (# NucBlue positive) and total number of target population (# GFP positive) within the sorted sample. At this stage you can either add the sorted cells back into the On-Chip sort to re-sort if greater purity is desired, or move on to 10x (note: residual non-GFP cells can often be filtered out post-hoc based on gene expression criteria).
4. Based on calculated total number of cells, use the Cell concentration table and Cell Suspension Volume Calculator table to determine the required volume of cell suspension stock per reaction.
5. Spin down the cell suspension for 6min at 100G to concentrate cells.
6. Remove ACSF carefully to achieve required concentrated volume of cells.
7. Prepare Master Mix and proceed through running the chromium controller and GEM-RT incubation as described.
8. Post GEM-RT cleanup and cDNA Amplification is completed as described in the Chromium Single Cell 3' V3 user guide.