

Harvard Medical School P20 Center for Interdisciplinary Research in Benign Urology

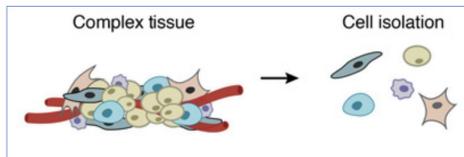
Single cell transcriptome profiling to define cell types in brain nuclei controlling bladder function (DK119789)

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Rationale:

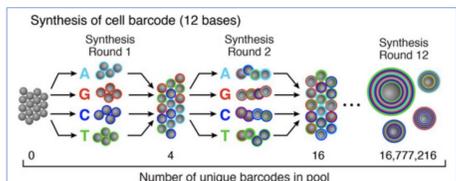
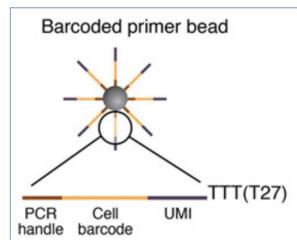
- Millions of people suffer from urinary incontinence, urgency, frequency and bladder pain. Little is known about the mechanisms that cause these symptoms, but disruptions to brain function as seen in dementia, stroke and brain injury often result in LUTS. Neural circuit defects may therefore contribute to, or cause, many benign urological disorders.
- It is now possible to manipulate and map specific neuron populations (e.g. glutamatergic neurons) in specific brain regions (e.g. the pontine micturition center (PMC) or the periaqueductal gray (PAG)). However, subsets of neurons which utilize a common neurotransmitter still likely consist of many different subpopulations, each of which has a different set of inputs, a distinct set of projections, and different functions.
- Single cell transcriptome profiling via the use of microfluidic device; **Drop-seq** is a particularly powerful solution because information from rare cell types is not lost among the many. Unlike approaches that analyze DNA from a population of cells at once, single-cell analysis captures the heterogeneity of cells in a tissue.
- Members of our Center have successfully created a neuron atlas in mice, of the hypothalamic arcuate–median eminence complex (Arc-ME) which controls energy balance, fertility and growth. They profiled gene expression in 20,921 individual cells in and around the adult mouse Arc-ME using Drop-seq (Campbell JN *et al.* (2017) *Nature Neuroscience*, 20:484). This approach identified 50 transcriptionally distinct Arc-ME cell populations (Figure 1).

Methods and Principles: (1) Generate cells and barcoded primer beads

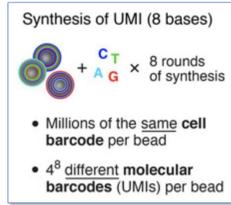


1. Proteolytic digestion of tissue into live single cells

2. Each microparticle contains more than 10⁸ individual primers that share the same “PCR handle” and “cell barcode”, but have different unique molecular identifiers (UMIs). A 30-bp oligodT sequence is present at the end of all primer sequences for capturing mRNAs and priming reverse transcription.

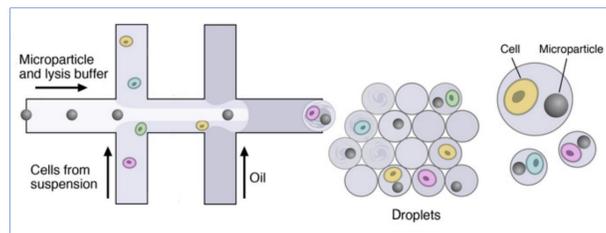


3. To generate the cell barcode, the pool of microparticles is repeatedly split into four oligonucleotide synthesis reactions, to which one of the four DNA bases is added. The result is a pool of beads, each possessing one of 4¹² (16,777,216) possible sequences.

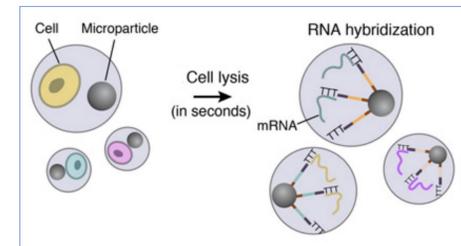


4. The synthesis of UMIs (Unique Molecular Identifiers) takes place after the completion of the “split-and-pool” synthesis cycles. Each individual primer receives one of 4⁸ (65,536) possible sequences (UMIs).

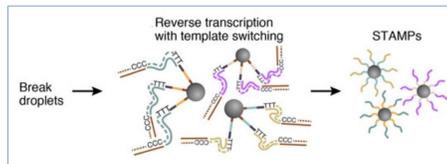
Methods and Principles: (2) Microfluidic separation of cells, mixing with beads, cell lysis, hybridization and PCR amplification



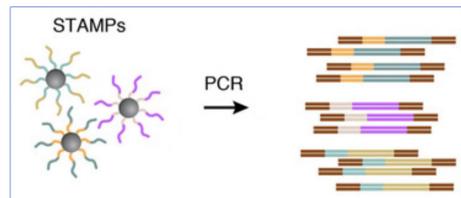
5. Individual cells are encapsulated in droplets together with microparticles, using a microfluidic device.



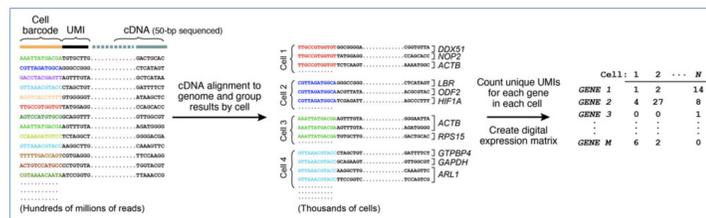
6. Immediately following droplet formation, each cell is lysed and its mRNAs are released and hybridize to the primers on the microparticle.



7. The mRNAs are then reverse-transcribed into cDNAs together in one reaction, forming a set of beads called “single-cell transcriptomes attached to microparticles” (STAMPs)



8. The barcoded STAMPs can then be amplified in pools by PCR reaction for high-throughput mRNA-sequencing.



9. Resulting molecules are sequenced from each end using high-capacity parallel sequencing. The reads are first aligned to a reference genome to identify the gene-of-origin of the cDNA. Next, reads are organized by their cell barcodes, and the number of mRNA transcripts of each gene ascertained in each cell is digitally counted. A matrix of digital gene-expression measurements can then be established for further analysis.

Acknowledgment: Drop-seq images were originally published in Macosko *et al.* (2017) Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. Cell 161:1202.

Prior Data:

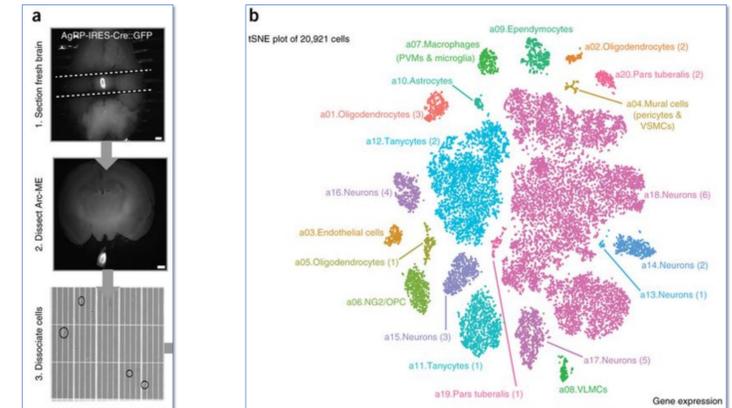


Figure 1. (a) Fresh brain section showing GFP labelled arcuate nucleus, excised in middle panel and dissociated into cells in bottom panel. (b) Following transcriptomics the data are presented as a spectral t-distributed stochastic neighbor embedded (tSNE) plot of 20,921 cells, colored per density clustering and annotated according to known cell types (Campbell JN *et al.* (2017) *Nature Neuroscience*, 20:484)

Specific Aims: Research Plan

Aim 1: Use single cell (sc) RNA-Seq to create an atlas of the neuron subpopulations of the pontine micturition center-locus coeruleus (PMC-LC) and the ventrolateral periaqueductal gray (vlPAG) regions in the mouse brain. *We hypothesized that neurons in PMC and vlPAG regions are transcriptionally diverse and can be distinguished based on transcriptional markers*

Aim 2: Use sc-RNA-Seq with anterograde and retrograde tracing to identify the PMC-LC and vlPAG neurons in the bladder controlling circuit. *We hypothesized that neurons that form this bladder-controlling circuit can be profiled based on their connectivity, then be identified based on their transcriptional markers.*

Aim 3: Identify functionally relevant PAG_{VL} and PMC-LC neuron subpopulations based on their response to a physiological challenge (chronic polyuria). *We hypothesize that PMC and vlPAG neurons that control bladder function can be identified based on their transcriptional response to a relevant physiological stimulus.*

Progress:

- We have modified our approach by moving away from fresh tissue digestion into single cells, to direct isolation of single nuclei (Drop-seq with nuclei; DroNc-Seq). There are several major advantages; i) enzymatic digestion and cell dispersion is avoided; ii) tissue punches can be frozen for later nuclei and mRNA isolation; iii) the injection site can be validated while tissue is stored.
- We have identified ten subpopulations from among PMC (and adjacent) glutamatergic neurons
- Further characterization of these ten has revealed five which appear to be PMC-specific, spinally projecting and influence voiding as registered by chemogenetics and conscious cystometry.