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TITLE:
Multiplexed analysis of retinal gene expression and chromatin accessibility using scRNA-Seq and scATAC-Seq

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KEYWORDS:
Development; Disease; scRNA-Seq; scATAC-Seq; MULTI-Seq; multiplex; Chromatin

SUMMARY:
Here, the authors showcase the utility of MULTI-seq for phenotyping and subsequent paired scRNA-seq and scATAC-seq in characterizing the transcriptomic and chromatin accessibility profiles in retina.

ABSTRACT:
Powerful next generation sequencing techniques offer robust and comprehensive analysis to investigate how retinal gene regulatory networks function during development and in disease states. Single-cell RNA sequencing allows us to comprehensively profile gene expression changes observed in retinal development and disease at a cellular level, while single-cell ATAC-Seq allows analysis of chromatin accessibility and transcription factor binding to be profiled at similar resolution. Here the use of these techniques in the developing retina is described, and MULTI-Seq is demonstrated, where individual samples are labeled with a modified oligonucleotide-lipid complex, enabling researchers to both increase the scope of individual experiments and substantially reduce costs.

INTRODUCTION:
Understanding how genes can influence cell fate plays a key role in interrogating processes such as disease and embryonic progression. The complex relationships between transcription factors and their target genes can be grouped in gene regulatory

networks. Mounting evidence places these gene regulatory networks at the center of both disease and development across evolutionary lineages[1]. While previous techniques such as qRT-PCR focused on a single gene or set of genes, the application of high-throughput sequencing technology allows for the profiling of complete cellular transcriptomes.

RNA-seq offers a glimpse into large scale transcriptomics[2,3]. Single-cell RNA sequencing (scRNA-seq) gives investigators the ability to not only profile transcriptomes but link specific cell types with gene expression profiles[4]. This is achieved bioinformatically by feeding individual cell profiles into sorting algorithms using known gene markers[5]. Multiplexing using lipid-tagged indices sequencing (MULTI-seq) offers unprecedented diversity in the number of scRNA-Seq profiles that can be collected[6]. This lipid based technique differs from other sample indexing techniques such as cell-hashing that rely on the presence of surface antigens and high affinity antibodies instead of plasma membrane integration[7]. Not only is it now possible to profile gene expression profiles into cell types but different experiments can be combined into a single sequencing library, dramatically lowering the cost of an individual scRNA-seq experiment[6]. The cost of scRNA-seq may seem prohibitive for use in phenotyping experiments where many different genotypes, conditions or or patient samples are analyzed, but multiplexing allows the combination of up to 96 samples in a single library[6].

Profiling gene expression via scRNA-seq has not been the only high-throughput sequencing-based technique to revolutionize the current understanding of how molecular mechanisms dictate cell fate. While understanding which gene transcripts are present in a cell enables the identification of cell type, equally important is understanding how genomic organization regulates development and disease progression. Early studies relied on detecting DNase-mediated cleavage of sequences not bound to histones, followed by sequencing of the resulting DNA fragments to identify regions of open chromatin. In contrast, single cell assay for transposon accessible chromatin sequencing (scATAC-seq) allows researchers to probe DNA with a domesticated transposon to readily profile open chromatin at the single nucleotide level[8]. This has gone through a similar scaling to scRNA-seq and now investigators can identify individual cell types and profile phenotypes across thousands of individual genomes[8].

The pairing of scRNA-seq and scATAC-seq has allowed researchers the ability to profile thousands of cells to determine cell populations, genomic organization, and gene regulatory networks in disease models and developmental processes[9–12]. Here the authors outline how to first utilize MULTI-seq to condense phenotyping of a myriad of animal models and employ paired scRNA-seq and scATAC-seq to gain a better understanding of the chromatin landscape and regulatory networks in these animal models.

PROTOCOL:

The use of animals for these studies was conducted using protocols approved by the Johns Hopkins Animal Care and Use Committee, in compliance with ARRIVE guidelines, and were performed in accordance with relevant guidelines and regulations.

1. MULTI-seq

1.1. Media preparation

1.1.1. Prepare and equilibrate ovomucoid inhibitor, 10 mg of ovomucoid inhibitor and 10 mg of albumin per mL of Earle's Balanced Salt Solution (EBSS), for 30 min prior to use[13]. Equilibrate with 95% O_2:5% CO_2 in an incubator.

1.1.1.1. Prepare papain dissociation solution, 20 units/mL papain and 0.005% DNase in EBSS, during this incubation step[13].

NOTE: One vial of DNase solution will be sufficient for 5 samples. Additional vials may need to be reconstituted if performing MULTI-seq on more than 5 samples.

1.1.2. Prepare lipid modified oligo barcoding solutions.

1.1.2.1. Make a unique barcode dilution for each sample by combining 0.5 µL of 100 µM barcode stock solution with 4.5 µL of Phosphate Buffered Saline (PBS).

1.1.2.2. To prepare anchor:barcode solution at 1:1 molar ratio, combine 4.4 µL of 10 µM barcode dilution, 0.9 µL of 50 µM anchor solution, and 16.7 µL of PBS and place on ice. The final concentration for both the barcode strands and anchor strands should be 2 µM.

1.1.2.3. To prepare co-anchor solution, combine 0.88 µL of 50 µM co-anchor solution and 21.12 µL of PBS and place on ice. The final concentration of the co-anchor should be 2 µM. Multiply the volumes in this step by 1.1x the number of samples.

1.1.3. To make 1% BSA in PBS, dissolve 0.1 g of bovine serum albumin (BSA) in 10 mL of PBS and place on ice.

1.1.4. To make PBS with RNase inhibitor, combine 2.5 µL of 40 U/µL RNase inhibitor with 197.5 µL of PBS and store on ice. Multiply the volumes in this step by 1.1x the number of samples.

1.2. Sample dissociation

1.2.1. Euthanize animals through cervical dislocation and/or CO_2 asphyxia in accordance with institutional IACUC requirements. Method for euthanasia will depend on institutional requirements and model organism used.

1.2.2. Dissect retina from both eyes in cold PBS under a dissection microscope and transfer to a sterile 1.5 mL microcentrifuge tube using a transfer pipette.

1.2.3. Transfer 500 µL of the papain solution to a 1.5 mL tube for each sample.

1.2.4. Place samples in the papain solution and immediately cap the tubes.

1.2.5. Incubate the tubes containing the samples at 37 °C, 5% CO_2 for 30 min. Invert the vials 3 times every 10 minutes. The samples should progressively dissociate with each round of inversions.

1.2.6. Return the vials to room temperature and triturate each sample 3-4 times with a 1 mL pipette set to 300 µL.

1.2.7. Allow any pieces of undissociated tissue remaining after trituration to settle.

1.2.8. Remove the cloudy cell suspension while avoiding any pieces of undissociated tissue and place each sample's cell suspension in a new sterile 15 mL screw-capped tube.

1.2.9. Centrifuge the cell suspensions at 300 x g for 5 min at room temperature.

1.2.10. During the centrifugation step, prepare the cell resuspension media.

1.2.10.1. Mix 857 µL of EBSS (Vial 1) with 95 µL of reconstituted albumin-ovomucoid inhibitor solution in a sterile 1.5 mL screw-capped tube. Add 48 µL of DNase solution saved from 1.1.1. Multiply the volumes in this step by 1.1x the number of samples.

1.2.11. Discard the supernatant, careful to retain the cell pellets, and immediately resuspend the cell pellets in 1 mL cell resuspension solution prepared in step 1.2.10.

1.2.12. Prepare discontinuous density gradient.

1.2.12.1. For each sample, add 1.6 mL of ovomucoid inhibitor solution to a new 15 mL sterile screw-capped tube and carefully layer the cell suspension on top. The interface between the two layers of the gradient should be visible but some mixing does not affect results.

1.2.13. Centrifuge the discontinuous density gradients at 70 x g for 6 min at room temperature. The dissociated cells should pellet at the bottom of the tubes while membrane fragments remain at the interface.

1.2.14. Discard the supernatant and add 200 µL of PBS to wash the pelleted cells. Centrifuge at 300 x *g* for 5 min at room temperature.

1.2.15. Discard the supernatant and resuspend the cells in 180 µL of PBS with RNase inhibitor. Pass the cells through a 40 µm cell strainer into a sterile 1.5 mL microcentrifuge tube.

1.3. **Cell barcoding**

1.3.1. Add 20 µL of anchor:barcode solution to each sample and pipette gently to mix. Ensure that each sample receives a unique anchor:barcode solution and incubate on ice for 5 min.

1.3.1.1. Prepare the materials required for gel bead-in-emulsion (GEM) generation and barcoding during this incubation[14].

1.3.2. Add 20 µL of co-anchor solution to each sample and pipette gently to mix. Incubate on ice for 5 min.

1.3.3. Add 1 mL of ice-cold 1% BSA in PBS to each sample and centrifuge the cells at 300 x g for 5 min at 4 °C.

1.3.4. Remove the supernatant without disturbing the cell pellet and add 400 µL ice cold 1% BSA in PBS. Centrifuge at 300 x g for 5 min at 4 °C.

1.3.4.1. Repeat step 1.3.4 for a total of 2 washes. Remove the supernatant and resuspend in 400 µL ice cold 1% BSA in PBS.

1.3.5. Determine the concentration of cells for each sample using a hemocytometer.

1.3.5.1. Determine the total number of cells to load for each sample. Typically, this is the total number of cells desired divided by the number of samples. However, if a biological replicate for one condition was lost, its share can be split amongst the other biological replicates from that condition.

1.3.5.2. Divide the number of cells desired for each sample by the cell concentration of that sample calculated in step 3.5 to determine the volume required to load the desired number of cells.

1.3.6. Combine the volumes calculated in step 3.5.2 from each sample in a new sterile 1.5 mL centrifuge tube. It is prudent to double the volume taken from each sample in case of failure of GEM generation.

1.3.6.1. Determine “final” cell concentration of the combined samples using a hemocytometer.

1.4. GEM generation and barcoding

1.4.1. Use combined samples to generate and barcode GEMs[14].

1.5. Post GEM-RT Cleanup and cDNA Amplification

1.5.1. Prepare materials for post GEM reverse transcriptase cleanup and cDNA amplification according to manufacturer's instructions with the following modifications[14]. Perform post GEM-RT cleanup by size selection[14].

1.5.1.1. Increase volume of 80% ethanol prepared by 2 mL.

1.5.1.2. Prepare cDNA Amplification Mix on ice[14]. Add 1 µL of 2.5 µM MULTI-seq primer to the cDNA Amplification mix. Vortex for 5 s and centrifuge for 5 s in a benchtop mini centrifuge.

1.5.2. Perform cDNA amplification[14]. The cDNA can be stored at 4 °C for up to 72 h at this point.

1.5.3. Perform cDNA cleanup by size selection[14]. The endogenous transcript cDNA can be stored at 4 °C for up to 72 h or at –20 °C for up to 4 weeks at this point.

1.5.3.1. **Do not discard the supernatant from the first round of size selection. This fraction contains the sample barcodes.** Transfer the supernatant to a new sterile 1.5 mL microcentrifuge tube.

1.5.3.2. Store the supernatant on ice.

1.5.4. Vortex to resuspend paramagnetic bead-based size selection reagent and add 260 µL paramagnetic bead-based size selection reagent (final 3.2x) and 180 µL of 100% isopropanol (1.8x) to the supernatant. Pipette the mix 10 times and incubate at room temperature for 5 min.

1.5.5. Place the tube on a magnetic rack and wait for the solution to clear. Then remove and discard the supernatant.

1.5.6. Add 500 µL of 80% ethanol to the beads and let stand for 30 s. Remove and discard the supernatant.

1.5.6.1. Repeat for a total of 2 washes.

1.5.7. Briefly centrifuge the beads and return to the magnetic rack. Start a timer for 2 min.

1.5.8. Remove the remaining ethanol with a P10 pipette and air-dry the beads on the magnetic rack for the remainder of the 2 min. Do not exceed 2 min.

1.5.9. Remove the beads from the magnetic rack and resuspend in 100 µL elution buffer (EB). Pipette thoroughly to resuspend.

1.5.10. Incubate at room temperature for 2 min.

1.5.11. Return to the magnetic rack and wait for the solution to clear.

1.5.12. Transfer the supernatant to a fresh 1.5 mL microcentrifuge tube.

1.5.13. Repeat steps 1.5.4 to 1.5.12. Halve the volume of buffer EB used in step 1.5.9.

1.5.14. Determine barcode DNA concentration and size distribution using a dsDNA high sensitivity assay kit and fluorometer and fragment analyzer[15,16]. The barcode cDNA can be stored at 4 °C for up to 72 h or at –20 °C for up to 4 weeks at this point.

1.6. Barcode library construction

1.6.1. Prepare 1 mL of 80% ethanol.

1.6.2. Prepare barcode library PCR master mix in a sterile PCR strip tube: combine 26.25 µL of 2x hot start PCR ready mix, 2.5 µL of 10 µM Universal i5 primer, 2.5 µL of 10 µM RPI primer, 3.5 ng of barcode cDNA (volume determined by concentration from Step 5.14), and enough nuclease-free water to bring the final volume to 50 µL.

1.6.2.1. Use a unique RPI primer for each barcode library if multiple MULTI-seq libraries are sequenced together.

1.6.3. Subject the barcode library master mix to a library preparation PCR: 95 °C for 5 min; 10 cycles of 98 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s; 72 °C for 1 min; and then hold at 4 °C.

1.6.4. Vortex to resuspend the paramagnetic bead-based size selection reagent.

1.6.5. Remove the PCR product from the thermocycler and add 80 µL (1.6x) of paramagnetic bead-based size selection reagent. Pipette thoroughly.

1.6.6. Incubate at room temperature for 5 min. Place on magnet separator in the high position and wait for the solution to clear.

1.6.7. Remove and discard the supernatant.

1.6.8. Add 200 µL of 80% ethanol to the beads and allow to stand for 30 s. Remove and discard the supernatant.

1.6.8.1. Repeat step 1.6.8 for a total of 2 washes.

1.6.9. Briefly centrifuge the tube and place on the magnetic separator in the low position. Start a timer for 2 min.

1.6.10. Remove the remaining ethanol with a P20 pipette and air-dry the beads on the magnetic separator for the remainder of the 2 min. Do not exceed 2 min.

1.6.11. Remove the tube from the magnetic separator and resuspend the beads in 25 µL of buffer EB. Pipette mix thoroughly to resuspend the beads.

1.6.12. Incubate for 2 min at room temperature.

1.6.13. Return to the magnetic separator in the low position and wait for the solution to clear. Transfer the supernatant to a new PCR strip tube.

1.6.14. Quantify expression library and barcode library concentration and fragment size distribution using a dsDNA high sensitivity assay kit and fluorometer and fragment analyzer.

1.7. 3' Gene Expression Library Construction

1.7.1. Perform 3' gene expression library construction according to manufacturer's instructions[14].

1.8. Sequencing

1.8.1. In addition to the endogenous cDNA library amplified in step 5, submit the barcode library amplified in step 6. For simplicity, submit these libraries separately, or as a cost-effective measure include an aliquot of the sample barcoding material with the endogenous cDNA library. Target between 20,000-50,000 cDNA and 3,000-5,000 barcode reads per cell.

1.8.2. Convert the resulting sequencing bcl files to fastq files by cellranger mkfastq and count matrices generated from these by cellranger count.

1.8.3. Deconvolute samples post Cellranger analysis using the deMULTIplex r package available at the MULTI-seq GitHub repository (https://github.com/chris-mcginnis-ucsf/MULTI-seq).

2. Paired scRNA-seq and scATAC-seq

2.1. Flash freeze tissue for scATAC-seq and methanol fixation of retinal cells for scRNA-seq

2.1.1. Media preparation

2.1.1.1. To prepare the solutions for retinal dissociation, repeat steps 1.1.1 and 1.1.4.

2.1.1.2. Prepare an ethanol dry ice bath in a small ice bucket.

2.1.1.3. Prepare 15 mL of 70% ethanol by adding 10.5 mL of ethanol to 4.5 mL of nuclease-free water.

2.1.1.4. Add enough dry ice to the ice bucket (preferably in pellet form) that 15 mL of liquid will rise to just cover the ice. Add the 15 mL of ethanol.

2.1.1.5. Place PBS on ice and 1 mL aliquots of 100% methanol in a convenient –20 °C freezer.

2.1.2. Sample preparation

2.1.2.1. Follow steps 1.2.1 and 1.2.2 to isolate retinas; however, this time place each retina into a separate microcentrifuge tube.

2.1.2.2. For the samples destined for scATAC-seq, remove any liquid from the microcentrifuge tube using a P200 pipette and immediately cap and place the tube in ethanol-dry ice bath to flash freeze the sample.

2.1.2.3. For the samples destined for scRNA-seq, to dissociate the cells, repeat steps 1.2.3 through 1.2.15 on the dissected retina.

2.1.2.4. Centrifuge the cells at 300 x g for 3 min at room temperature.

2.1.2.5. Remove the supernatant without disrupting the cell pellet.

2.1.2.6. Add 1 mL of chilled PBS and gently pipette mix 10 times or until the cells are completely suspended.

2.1.2.7. Centrifuge at 300 x g for 5 min at 4 °C.

2.1.2.7.1. Repeat steps 2.1.2.5 to 2.1.2.7 for a total of 2 washes.

2.1.2.8. Remove the supernatant without disrupting the cell pellet.

2.1.2.9. Add 100 µL of chilled PBS and gently mix 10x or until cells are completely suspended.

2.1.2.10. Start vortexing the tube at the lowest speed setting. Add 900 µL chilled methanol (–20 °C) drop by drop while continuing to gently vortex the tube to prevent the cells from clumping.

2.1.2.11. Incubate the cells on ice for 15 min.

2.1.2.12. Determine the concentration of the fixed cells using a hemocytometer.

2.1.2.13. Assess the efficacy of the fixation step using trypan blue. A high fraction of viable cells indicates that more fixation time is required.

2.1.2.14. Store the frozen tissue and fixed cells at –80 °C.

2.2. Nuclei Isolation from flash frozen tissue for scATAC-seq

2.2.1. Media preparation

2.2.1.1. Prepare the lysis dilution buffer by combining 9.77 mL of nuclease-free water, 100 µL of 1 M Tris-HCl (pH 7.4), 100 µL of 1 M NaCl, 30 µL of 1 M $MgCl_2$, and 0.1 g of BSA.

2.2.1.2. Prepare the lysis dilution buffer ahead, such as the day before, and store at 4 °C. On the day of the protocol, place on ice.

2.2.1.3. Equilibrate 20x nuclei buffer provided by the manufacturer of the scATAC kit from -20 °C to room temperature. Vortex and centrifuge briefly.

2.2.1.4. Prepare the 1x lysis buffer by incubating 5% Digitonin solution in a 65 °C water bath until the precipitate is dissolved. Then combine 2 mL of lysis dilution buffer, 20 µL of 10% Tween-20, 20 µL of Nonidet P40 Substitute (may alternatively be labeled IGEPAL), and 4 µL of Digitonin.

2.2.1.5. Store the 1x lysis buffer on ice.

2.2.1.6. To prepare the 0.1x lysis buffer, combine 1.8 mL of lysis dilution buffer with 200 µL of the 1x lysis buffer and store on ice.

2.2.1.7. To prepare the wash buffer, combine 2 mL of lysis dilution buffer and 20 µL of 10% Tween-20 and store on ice.

2.2.1.8. To prepare the diluted nuclei buffer, combine 950 µL of nuclease-free water with 50 µL of nuclei buffer (20x) and store on ice.

2.2.2. Nuclei Isolation

2.2.2.1. **Do not thaw the sample prior to lysis.** Add 500 µL of chilled 0.1x lysis buffer to a 1.5 mL microcentrifuge tube containing the frozen sample. Immediately homogenize 15x using a pellet pestle.

2.2.2.2. Incubate for 5 min on ice. Prepare the materials required for scATAC transposition during this incubation step[17].

2.2.2.3. Pipette mix 10x with a 1 mL pipette and incubate for 10 min on ice. Complete preparation of the materials required for scATAC transposition during this incubation step if not already completed. It is unnecessary to prepare more Diluted Nuclei Buffer.

2.2.2.4. Add 500 µL of chilled wash buffer to the lysed cells. Pipette mix 5x.

2.2.2.5. Pass the suspension through a 70 µm cell strainer into a new 1.5 mL microcentrifuge tube. Filter ~300 µL at a time using a new 70 µm cell strainer each time.

2.2.2.6. Pass the collected flowthrough through a 40 µm cell strainer into a new 1.5 mL microcentrifuge tube and store on ice.

2.2.2.7. Determine the nuclei concentration using a hemocytometer. Based on the nuclei concentration and the total volume of the cell suspension, calculate the total number of dissociated nuclei.

2.2.2.8. Centrifuge at 500 x g for 5 min at 4 °C and remove the supernatant without disrupting the nuclei pellet.

2.2.2.9. To create the nuclei stock based on the total nuclei count determined in step 2.2.2.7., resuspend the nuclei in enough diluted nuclei buffer to reach a desired nuclei concentration.

NOTE: Desired nuclei concentration is based on the targeted nuclei recovery for the scATAC-seq experiment and is determined from the Nuclei Concentration Guidelines found in the manufacturer's protocol[17]. For instance, if 10,000 nuclei are ultimately desired, the desired nuclei concentration for the Nuclei Stock is between 3,080 and 7,700 nuclei/µL. It might be best to aim for the middle of the range in concentration.

2.2.2.10. Determine the nuclei concentration using a hemocytometer.

2.2.2.11. Immediately proceed to scATAC transposition using the prepared nuclei stock[17].

2.3. Rehydration of Methanol Fixed Cells for use in scRNA-Seq

2.3.1. Media Preparation

2.3.1.1. To prepare the wash/dilution buffer, dissolve 0.01 g of BSA in 1 mL of PBS. Then add 12.5 µL of 40 U/µL RNase Inhibitor and mix gently. Store on ice.

2.3.2. Cell Rehydration

2.3.2.1. Centrifuge methanol fixed cells in a 1.5 mL microcentrifuge tube at 3000 x g for 10 min at 4 °C. Then remove the supernatant.

2.3.2.2. Add 200 µL chilled wash/dilution buffer to the microcentrifuge tube. Centrifuge at 300 x g for 10 min at 4 °C and remove the supernatant. Repeat for a total of 2 washes.

NOTE: Prepare the materials required for GEM generation and barcoding during the previous two steps[14].

2.3.2.3. To prepare the cell stock, based on the total cell number calculated in 2.1.2.12., resuspend the cells in enough wash/dilution buffer to achieve the desired cell concentration. Preferred cell concentrations are between 700 and 1200 cells/µL.

2.3.2.4. Determine the cell concentration of the cell stock using a hemocytometer.

2.3.2.5. Immediately proceed to GEM generation and barcoding using the cell stock[14].

2.3.3. Sequencing

2.3.3.1. When performing time course analysis (as in development or disease), sequence multiple samples in a multiplexed run (i.e., multiple libraries on a single flow cell) to cut down on technical variation.

REPRESENTATIVE RESULTS:

This workflow lays out a strategy for investigation of developmental phenotypes and regulatory processes using single cell sequencing. MULTI-seq sample multiplexing enables an initial low-cost phenotyping assay while paired collection and fixation of samples for scRNA-seq and scATAC-seq allows for more in-depth investigation (**Figure 1**).

MULTI-seq barcoding enables the combined sequencing of multiple samples and their subsequent computational deconvolution. The sample of origin can be determined for each cell based on their barcode expression (**Figure 2A**). These combined samples can be analyzed as a single dataset for the purposes of cell clustering and cell type identification (**Figure 2B**). Because each cell is barcoded before GEM generation, cell doublets will have a high probability of showing expression for multiple MULTI-seq barcodes and a majority of doublets can therefore be identified and removed prior to clustering and cell type identification (**Figure 2C**). Increasing the number of cells used in the GEM generation step will increase the proportion of doublets found. scATAC-seq can be used to generate a dataset with cell types to match those found by scRNA-seq (**Figure 2D**). The pairing of scRNA-seq gene expression and scATAC-seq DNA accessibility information enables the reconstruction of gene regulatory networks.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic demonstrating the use of MULTI-Seq in initial analysis, followed by separate scRNA-Seq and scATAC-Seq analysis in in-depth characterization of phenotypes, treatments, or disease states of interest.

Figure 2: UMAP dimensional reduction representations of MULTI-seq data for an allelic series of P0 *Sstr2* knockout mice demonstrating (a) the deconvolution of genotype for each cell in the dataset and (b) the identification of cell types in the dataset. Overloading cells during the GEM generation and barcoding step will result in an increase in cell doublets like those as seen in (c), which shows the data from (a) and (b) before doublet removal and reclustering. In (d), scATAC-Seq data from GFP-positive cells obtained at E16 from retinal explants electroporated with a GFP-expressing control plasmid at E14. Cell types are annotated based on accessibility of cell type-specific genes. This figure has been modified from Weir, K., Kim, D. W., Blackshaw, S. Regulation of retinal neurogenesis by somatostatin signaling. *bioRxiv* 2020.09.26.314104 (2020) doi:10.1101/2020.09.26.314104[18] and original, unpublished data.

DISCUSSION:

The power of MULTI-seq stems from seamless integration of data from multiple experimental conditions or models and the enormous benefit in terms of cost and limiting batch effects. Utilizing MULTI-seq offers a laboratory unprecedented phenotyping depth. Non-genetic multiplexing methods such as cell hashing or nuclei hashing opened the door to multiplexed samples through the use of barcoded antibodies[7,19,20]. However, this relies on the availability of high affinity antibodies that recognize surface proteins expressed on cells or their nuclei, which will not be possible if these antibodies are unavailable or the cells do not express appropriate cell surface or nuclear antigens[7]. Because MULTI-seq utilizes lipid-modified oligo barcodes to stably incorporate into the cells or their nuclei, it allows researchers to gather transcriptomic data from up to 96 fresh or fixed samples in a cheaper and more broadly applicable manner[6].

Following up on the initial MULTI-Seq phenotyping with paired scRNA-Seq and scATAC-Seq is suggested and showcased to gain an understanding of the genomic organization that coincides with the transcriptomic data[1]. This not only gives an idea of the heterochromatin and euchromatin regions but also valuable understanding of the transcription factor networks driving gene expression. A multi-omic approach can be used to reveal the dynamic chromatin changes that take place at key points of cell fate decisions and determine cellular trajectories in development and disease[1,21,22]. This can be accomplished through bioinformatically subjecting the data to pseudotime, *cis*-regulatory interactions and footprinting analysis[5,23–25]. Fixing the samples and sequencing multiple in a multiplexed run reduces sources of batch effect, enabling comparison across samples such as through a time course experiment. The number of tissue samples required depends on the scope of the experiment. When examining the phenotypes associated with embryonic time points, single retinas are often sufficient and can provide hundreds of thousands of cells. A single retina may not provide enough cells in more complex experimental schemes: *ex vivo* electroporations of retinal explants, probing for rare cell populations, or genetic models with insufficient CRE activation. While a single retina may provide a few hundred cells, these will not capture the full tissue complexity. For such experiments, optimization will be required based on a researcher's needs. Utilizing these techniques, one can gain insight into how the genes analyzed in MULTI-seq are regulated during dynamic processes such as development and disease.

Regulation of gene regulatory networks lies at the heart of understanding cellular processes and how they contribute to development and disease[1,26,27]. The workflow presented here can be used to identify these gene regulatory networks in specific cell types. However, this protocol has been optimized for use with mouse retinal tissue. Optimization of various steps, such as lysis or dissociation time, centrifugation speeds/times, and number of filtration steps may be required to maximize the number of cells or nuclei and minimize the cell debris in cell suspensions from other tissue types, *ex vivo* samples, or species, whether samples are fresh, frozen, or fixed in methanol. Methanol fixation time may need to be increased if samples show high levels of viable cells with trypan blue staining. The MULTI-seq technique introduces many additional wash steps over traditional scRNA-seq. To avoid the accidental disposal of valuable cells or DNA, it is prudent to optimize centrifugation speeds and maintain supernatants that would normally be discarded in the cell barcoding, post-GEM RT cleanup, and barcode library construction steps on ice until that step has been verified to be successful. One limitation to MULTI-seq is the limited number of cells, and therefore samples, that can be sequenced from a single well in the GEM generation step. It is recommended to not try to excessively overload cells during this step to avoid a substantial increase in doublet cell capture. Avoid loading a GEM well with more than 20,000 cells. Rather, multiple wells can be prepared from a single combined suspension and multiplexed during sequencing. This will require preparing a large enough volume of cell suspension for GEM generation and the addition of replicates to steps 5, 6, and 7 and will increase the cost of sequencing as more total reads are needed for more cells. With proper optimization, this workflow will enable cost and time efficient identification of cell type-specific phenotypes and gene regulatory networks.

ACKNOWLEDGMENTS:

We thank Linda Orzolek from the Johns Hopkins Transcriptomics and Deep Sequencing Core for help in sequencing the produced libraries and Lizhi Jiang for performing the *ex vivo* retinal explants.

DISCLOSURES:

Nothing to disclose.

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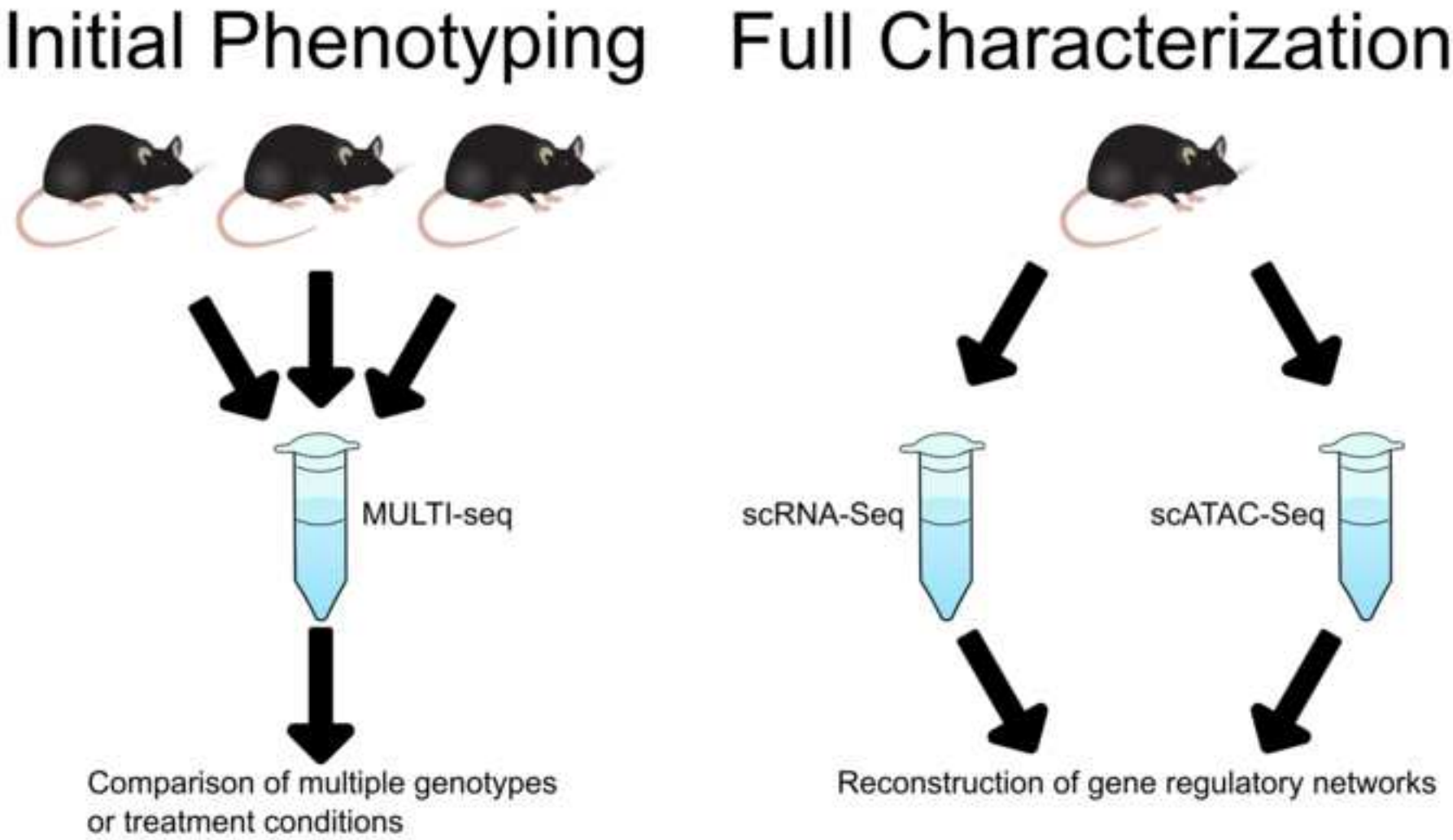
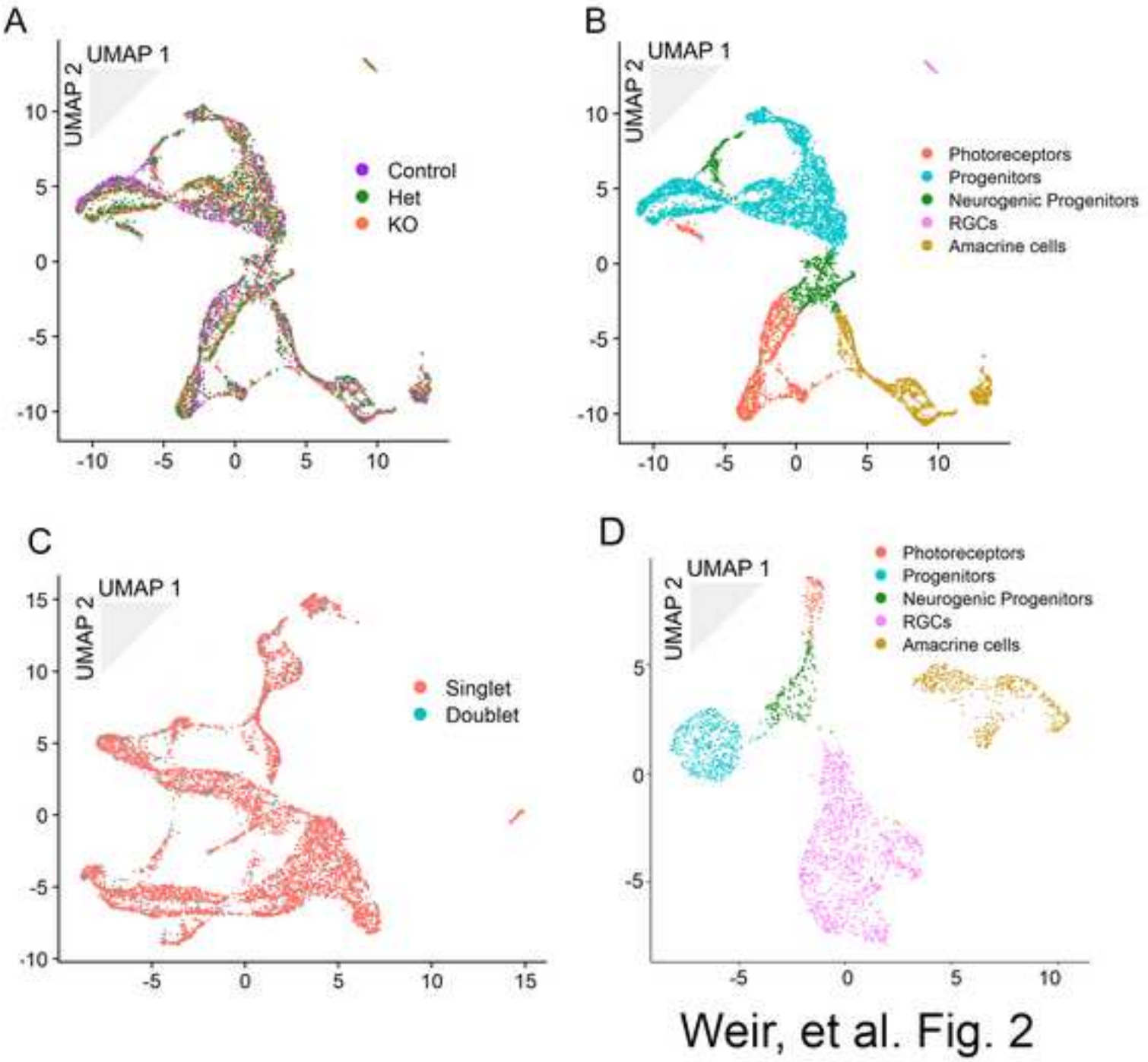


Figure 2



Weir, et al. Fig. 2

Table of Materials

Click here to access/download;Table of Materials;Weir et al_JoVE_Materials_Blackshaw.xls

Name of Material/ Equipment	Company
10 µL, 200 µL, 1000 µL pipette filter tips	
10% Tween 20	Bio-Rad
100 µM Barcode Solution	Request from Gartner lab
100% Ethanol	Millipore Sigma
100% Methanol	Millipore Sigma
10x Chip Holder	10x Genomics
10x Chromium controller & Accessory Kit	10x Genomics
15mL Centrifuge Tube	Quality Biological
40 µm FlowMi Cell Strainer	Bel-Art
50 µM Anchor Solution	Sigma or request from Gartner lab
50 µM Co-Anchor Solution	Sigma or request from Gartner lab
5200 Fragment Analyzer system	Agilent
70 um FlowMi cell strainer	Bel-Art
Allegra X-12R Centrifuge	VWR
Bovine Serum Albumin	Sigma-Aldrich
Chromium Next GEM Chip G	10x Genomics
Chromium Next GEM Chip H	10x Genomics
Chromium Next Gem Single Cell ATAC Reagent Kit v1.1	10x Genomics
Chromium Single Cell 3' GEM, Library & Gel Bead Kit v3.1	10x Genomics
Digitonin	Fisher Scientific
Dissection microscope	Leica
DNA LoBind Tubes, 1.5 mL	Eppendorf
Dry Ice	
EVA Foam Ice Pan	Tequipment
FA 12-Capillary Array Short, 33 cm	Agilent
Fisherbrand Isotemp Water Bath	Fisher Scientific
Forma CO2 Water Jacketed Incubator	ThermoFisher Scientific
Glycerol 50% Aqueous solution	Ricca Chemical Company
Hausser Scientific Bright-Line Counting Chamber	Fisher Scientific
Illumina NextSeq or NovaSeq	Illumina
Kapa Hifi Hotstart ReadyMix	HiFi
Low TE Buffer	Quality Biological

Magnesium Chloride Solution 1 M	Sigma-Aldrich
Magnetic Separator Rack for 1.5 mL tubes	Millipore Sigma
Magnetic Separator Rack for 200 µL tubes	10x Genomics
MULTI-seq Primer	Sigma or IDT
MyFuge Mini Centrifuge	Benchmark Scientific
Nonidet P40 Substitute	Sigma-Aldrich
Nuclease-free water	Fisher Scientific
P2, P10, P20, P200, P1000 micropipettes	Eppendorf
Papain Dissociation System	Worthington Biochemical Corporation
PBS pH 7.4 (1X)	Fisher Scientific
Qiagen Buffer EB	Qiagen
Refridgerated Centrifuge 5424 R	Eppendorf
RNase-free Disposable Pellet Pestles	Fisher Scientific
RNasin Plus RNase Inhibitor	Promega
RPI primer	Sigma or IDT
Single Index Kit N, Set A	10x Genomics
Single Index Kit T Set A	10x Genomics
Sodium Chloride Solution 5 M	Sigma-Aldrich
SPRIselect Reagent Kit	Beckman Coulter
Standard Disposable Transfer Pipettes	Fisher Scientific
TempAssure PCR 8-tube strip	USA Scientific
Trizma Hydrochloride Solution, pH 7.4	Sigma-Aldrich
Trypan Blue Solution, 0.4% (w/v)	Corning
Universal I5 primer	Sigma or IDT
Veriti Thermal Cycler	Applied Biosystems
Vortex Mixer	VWR

Catalog Number

1662404
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B23318
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1402-4700
T2194
25-900-CI
See sequence list
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10153-838

Comments/Description

Primer Name

Anchor LMO
Co-Anchor LMO
Universal I5 primer
MULTI-seq Primer
TruSeq RPI1
TruSeq RPI2
TruSeq RPI3
TruSeq RPI4
TruSeq RPI5
TruSeq RPI6
TruSeq RPI7
TruSeq RPI8
TruSeq RPI9
TruSeq RPI10
TruSeq RPI11
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TruSeq RPI40
TruSeq RPI41

TruSeq RPI42
TruSeq RPI43
TruSeq RPI44
TruSeq RPI45
TruSeq RPI46
TruSeq RPI47
TruSeq RPI48

Sequence

5’-{Lipid}-TGGAATTCTCGGGTGCCAAGGgtaacgatccagctgtcact-3'

5’-AGTGACAGCTGGATCGTTAC-{Lipid}-3‘

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5’-CTTGGCACCCGAGAATTCC-3’

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We thank the editors for their constructive comments. We have made the following edits, as requested:

1) We have verified the step references in the protocol to reflect the addition of numbering for previously unnumbered sections.
2) We have removed the highlighting on the section concerning amplification of the barcode library. This brings our highlighted section below 3 pages in length.
3) A paragraph describing the figures in detail has been added to the representative results section as requested.
4) At the beginning of the protocol steps, we have included an ethics statement concerning the animals used in this paper.

We hope that these address any remaining concerns you may have, and look forward to hearing from you soon.