

# Simultaneous single-cell profiling of the transcriptome and accessible chromatin using SHARE-seq

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## Abstract

The ability to analyze the transcriptomic and epigenomic states of individual single cells has in recent years transformed our ability to measure and understand biological processes. Recent advancements have focused on increasing sensitivity and throughput to provide richer and deeper biological insights at the cellular level. The next frontier is the development of multiomic methods capable of analyzing multiple features from the same cell, such as the simultaneous measurement of the transcriptome and the chromatin accessibility of candidate regulatory elements. In this chapter we discuss and describe SHARE-seq (**S**imultaneous **h**igh-throughput **A**TAC and **R**NA **e**xpression with **s**equencing) for carrying out simultaneous chromatin accessibility and transcriptome measurements in single cells, together with the experimental and analytical considerations for achieving optimal results.

**Key words:** scRNA-seq, scATAC-seq, multiomics, chromatin accessibility, transcriptomics, split-pool

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## 1 Introduction

The basic unit of biological organization is the individual cell. In combination with their surrounding cellular microenvironments within the context of a multicellular organism, each cell integrates across internal and external stimuli to maintain or alter its state for biological function. Understanding the cellular state at the single cell resolution, therefore, is critical to defining the regulatory processes driving health and disease. A key advancement toward understanding cellular states have been in the development of transcriptomic methods. With the advent of high-throughput sequencing methods in the late 2000s, RNA-seq was developed to profile transcriptomes at base-pair resolutions [1–4]. Subsequently, the molecular biology approaches that enabled ever improved RNA-seq sensitivity has led to the development of single-cell RNA-seq

(scRNA-seq) to measure transcriptomes at the single-cell level. The first scRNA-seq methods [5–8] were very low throughput, only able to measure a few cells at a time. Further technical advancements utilized microfluidics- and plate-based approaches to increase throughput to the  $10^2$ – $10^3$  range [9, 10], while droplet- and bead-based methods later boosted it to the  $10^4$ – $10^5$  range [11–14]. However, the approach that holds the most promise for ultra-high throughput single-cell measurements is combinatorial indexing. The core concept of these approaches is to dynamically assign barcodes through multiple rounds of splitting and pooling cells to create a combinatorial set of barcodes that can be used to uniquely identify each cell. Specifically, a set of cells can be split into a 96- or 384-well plates, each well given a specific barcode, then pooled back together to be randomly split into another set of plates. Iteratively performing these split-pool rounds with an optimal number of input cells, barcodes, and number of rounds of barcoding, one can create a sufficient diversity of barcodes to uniquely assign each cell to a combination of barcodes. In comparison to physical isolation of each cell in a droplet or a well, combinatorial indexing provides a scalable platform for single cell measurements. This is the basis of all “sci” (single-cell combinatorial indexing) methods, such as sci-RNA-seq [15] and SPLiT-seq [16].

While scRNA-seq measures the current amount of transcripts in a given cell, it does not provide insight into how that transcriptional state is achieved and maintained through regulation. Mapping active *cis*-regulatory elements (cREs) provides key insight to address this need. A common property of active cREs, originally recognized more than four decades ago [17–19], is that they are depleted of nucleosomes and exhibit an open, “accessible” conformation. This property has been the basis for the numerous methods that have been developed over the years to profile these elements [20], which rely on the preferential enzymatic cleavage or labeling of open chromatin regions. ATAC-seq [21, 22] (Assay for Transposase-Accessible Chromatin using sequencing) has emerged as the most versatile instance of such assays. ATAC-seq takes advantage of the preferential insertion of a hyperactive Tn5 [23] transposase, preloaded with sequencing adapters into open chromatin. Tn5 had been previously adapted and successfully used for the generation of high-throughput sequencing libraries from low-input DNA samples [24]. The realization that it can also be used to tag open chromatin regions with ready-for-amplification sequencing adapters in a single reaction allowed for chromatin accessibility profiling to be carried out in bulk on very low-input samples (typically 50,000 cells, but also down to just a few thousand [21]), and eventually in single-cells, in the form of scATAC-seq, in the mid-2010s[25]. As with scRNA-seq, the throughput of scATAC-seq has also been dramatically increased over the years, using combinatorial indexing (sciATAC-seq [26–28]), microwell plates ( $\mu$ ATAC-seq [29]), droplet-based methods [30], and combinations of combinatorial indexing and droplets (dsciATAC-seq [31]).

Techniques such as scRNA-seq and scATAC-seq has provided unprece-

dent ed insights into the diversity of cell types, their developmental dynamics, and cellular responses to external stimuli in a wide variety of context. However, the ideal measurements would provide information about all relevant aspects of the state from the same cell. To this end, a variety of single-cell multiomic methods, measuring multiple such modalities in the same individual cells, have been under active development in recent years. These include methods for sequencing the genomes and transcriptomes of single cells (G&T-seq [32], PRDD-seq [33], DNTR-seq [34], sci-L3-RNA/DNA [35], TARGET-seq [36], and others), for sequencing methylomes and transcriptomes (scTrio-seq [37], scMT-seq [38], and scM&T-seq [39]), for mapping accessible chromatin and methylomes (e.g. scNOMe-seq [40]), for measuring proteins and transcripts (REAP-seq [41], CITE-seq [42], QBC [43], inCITE-seq [44], iNS-seq [45], using methylation-based labeling of open chromatin to map accessible DNA and transcripts (COOL-seq [46], scNMT-seq [47], scNOMeRe-seq [48], snmC2T-seq [49]), mapping protein occupancy and transcriptomes (CoTECH [50], Paired-Tag [51], scDam&T-seq [52]), for quantifying proteins levels and mapping open chromatin (PHAGE-ATAC [53], ASAP-seq [54]), for quantifying proteins and transcriptome levels and mapping open chromatin (DOGMA-seq [54], TEA-seq [55]), and others [56].

As regulatory elements and RNA levels are the two perhaps most informative modalities, joint scATAC-seq + scRNA-seq methods are the most sought after multiomic assays. A number of these have been developed in recent years – sci-CAR-seq [57], Paired-seq [58], ASTAR-seq [59], SNARE-seq [60], SHARE-seq [61], and others. The ideal such assay should capture as many of the transcripts present in each cell as possible and also as many of the open chromatin regions in the nucleus, with high specificity and little noise. The SHARE-seq assay, which is based on the combinatorial indexing described above, provides high-quality and high throughput transcriptome and accessible chromatin measurements in the same single cells.

In this chapter, we describe in detail the SHARE-seq procedure and discuss the key optimization points and considerations for the generation of high-quality scATAC+scRNA-seq datasets.

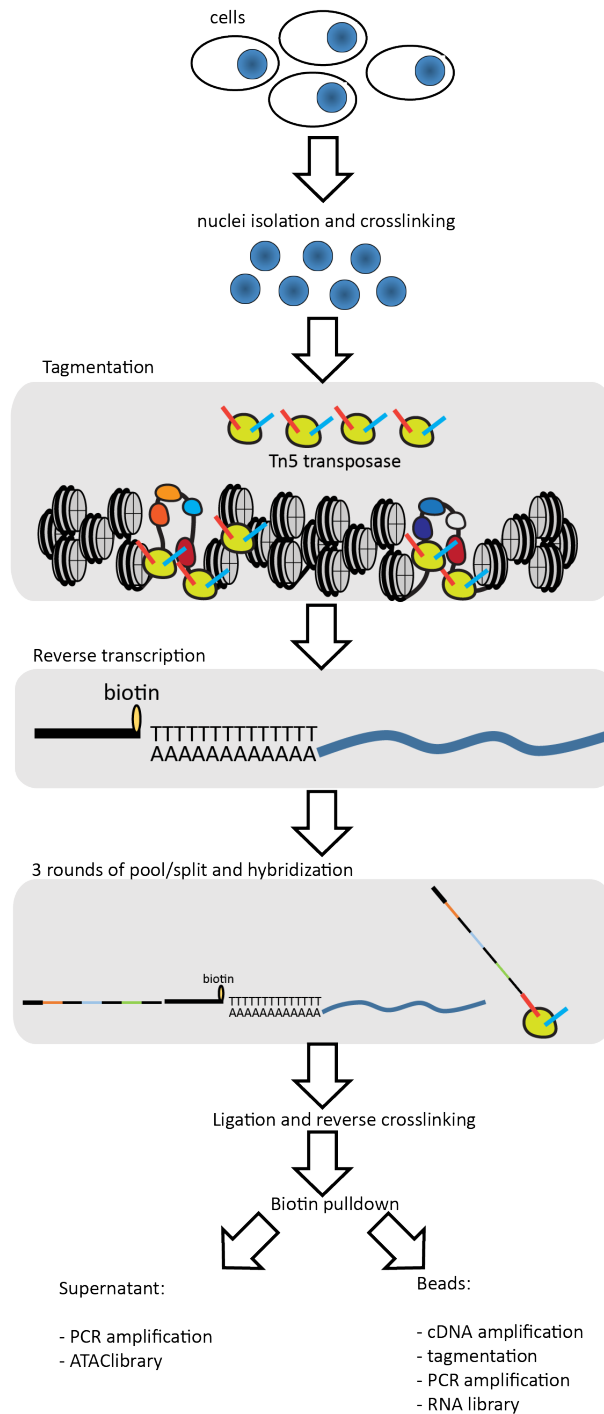
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## 2 Materials

### 2.1 DNA oligos and primers

All oligonucleotides can be obtained through IDT. The exact scale and purification methods are listed below.

1. Round 1 linker (1  $\mu$ mol scale, standard desalting):  
CCGAGCCCACGAGACTCGGACGATCATGGG
2. Round 2 linker (1  $\mu$ mol scale, standard desalting):  
CAAGTATGCAGCGCTCAAGCACGTGGAT
3. Round 3 linker (1  $\mu$ mol scale, standard desalting):  
AGTCGTACGCCGATGCGAAACATCGGCCAC



**Fig. 1: Outline of the SHARE-seq assay.** Nuclei are isolated from cells or tissues and crosslinked. Transposition is then carried out on chromatin, followed by reverse transcription with a biotinylated RT primer. Three pool/split rounds of hybridization of barcode oligos are then performed. Hybridized barcodes are then ligated, and crosslinks are reversed. The ATAC and RNA portions are separated by streptavidin pull-down. The ATAC is directly amplified, the RNA is subjected to cDNA amplification, tagmentation, and final library amplification.

4. Round 1 blocking (1  $\mu$ mol scale, standard desalting):  
CCCATGATCGTCCGAGTCTCGTGGGCTCGG
5. Round 2 blocking (1  $\mu$ mol scale, standard desalting):  
ATCCACGTGCTTGAGCGCGCTGCATACTTG
6. Round 3 blocking (1  $\mu$ mol scale, standard desalting):  
GTGGCCGATGTTTCGCATCGGCGTACGACT
7. Read 1 (100 nmol scale, HPLC purified):  
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
8. Template Switching Oligo (TSO) (100nmol scale, HPLC purified):  
AAGCAGTGGTATCAACGCAGAGTGAATrGrG+G
9. RNA PCR primer (100 nmol scale, standard desalting):  
AAGCAGTGGTATCAACGCAGAGT
10. P7 primer (100 nmol scale, standard desalting):  
CAAGCAGAAGACGGCATACGAGAT
11. Phosphorylated Read2 (100 nmol scale, HPLC purified):  
/5Phos/GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG—
12. Reverse transcription primer (RT primer) (100 nmol scale, HPLC purified)  
/5Phos/GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNNN  
/iBiodT/TTTTTTTTTTTTTTTVN
13. Blocked\_ME\_Comp (100 nmol scale, HPLC purified):  
/5Phos/C\*T\*G\* T\*C\*T\* C\*T\*T\* A\*T\*A\* C\*A\*/3ddC/
14. Pool-split ligation Plate R1 (*see Note 4*):  
/5Phos/CGCGCTGCATACTTG[8-bp-barcode]CCCATGATCGTCCGA
15. Pool-split ligation Plate R2 (*see Note 4*):  
/5Phos/CATCGGCGTACGACT[8-bp-barcode]ATCCACGTGCTTGAG
16. Pool-split ligation Plate R3 (*see Note 4*):  
CAAGCAGAAGACGGCATACGAGAT[8-bp-barcode]GTGGCCGATGTTTCG
17. PCR Library indexing primers plate:  
AATGATACGGCGACCACCGAGATCTACAC[8bp-index]  
TCGTCGGCAGCGTCAGATGTGTAT

An example set of 96 barcodes is listed below

```

AACGTGAT AAGGTACA CACTTCGA GATAGACA TGGAACAA ATCATTCC
AAACATCG ACACAGAA CAGCGTTA GCCACATA TGGCTTCA ATTGGCTC
ATGCCTAA ACAGCAGA CATACCAA GCGAGTAA TGGTGGTA CAAGGAGC
AGTGGTCA ACCTCCAA CCAGTTCA GCTAACGA TTCACGCA CACCTTAC
ACCACTGT ACGCTCGA CCGAAGTA GCTCGGTA AACTCACC CCATCCTC
ACATTGGC ACGTATCA CCGTGAGA GGAGAACA AAGAGATC CCGACAAC
CAGATCTG ACTATGCA CCTCCTGA GGTGCGAA AAGGACAC CCTAATCC
CATCAAGT AGAGTCAA CGAACTTA GTACGCAA AATCCGTC CCTCTATC

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CGCTGATC AGATCGCA CGACTGGA GTCGTAGA AATGTTGC CGACACAC  
 ACAAGCTA AGCAGGAA CGCATACA GTCTGTCA ACACGACC CGGATTGC  
 CTGTAGCC AGTCACTA CTCAATGA GTGTTCTA ACAGATTC CTAAGGTC  
 AGTACAAG ATCCTGTA CTGAGCCA TAGGATGA AGATGTAC GAACAGGC  
 AACAACCA ATTGAGGA CTGGCATA TATCAGCA AGCACCTC GACAGTGC  
 AACCGAGA CAACCACA GAATCTGA TCCGTCTA AGCCATGC GAGTTAGC  
 AACGCTTA GACTAGTA CAAGACTA TCTTCACA AGGCTAAC GATGAATC  
 AAGACGGA CAATGGAA GAGCTGAA TGAAGAGA ATAGCGAC GCCAAGAC

## 2.2 General Reagents Equipment

1. Eppendorf Thermomixer C (96 well plate adapter)
  2. Tabletop centrifuge
  3. Swing bucket centrifuge with temperature control
  4. Thermal cycler
  5. Cold room
  6. qPCR machine (QuantStudio 3)
  7. Qubit fluorometer or equivalent
  8. E-gel electrophoresis system (Thermo Fisher Scientific)
  9. TapeStation (Agilent) or equivalent, e.g. BioAnalyzer (Agilent).
  10. Multichannel pipettes or liquid handling instruments
  11. gentleMACS Dissociator (Miltenyi Biotec)
  12. Automated cell counter, e.g. Countess 3 (ThermoFisher Scientific) or equivalent.
1. 1× PBS buffer solution (Thermo Fisher Scientific, Cat #10010049)
  2. Bovine Albumin Fraction V (7.5% solution) (Thermo Fisher Scientific, Cat #15260037)
  3. Trypan Blue Stain (0.4%) (Thermo Fisher Scientific, Cat #T10282)
  4. Enzymatic RI (Qiagen, Cat #Y9240L)
  5. SUPERase RI (Thermo Fisher Scientific, Cat #AM2696)
  6. Lucigen RI (Lucigen Cat # 30281-2)
  7. Protector RI (Sigma Aldrich Cat # 3335399001)
  8. 16% FA (Thermo Fisher Scientific, Cat # 28906)
  9. Glycine (Sigma Aldrich, Cat #50049)
  10. 1 M Tris HCl pH 7.5 (Thermo Fisher Scientific, Cat #15567027)
  11. 1 M Tris HCl pH 8.0 (Thermo Fisher Scientific, Cat #15568025)
  12. 5 M NaCl (Thermo Fisher Scientific, Cat #AM9760G)
  13. 1 M MgCl<sub>2</sub> (Sigma Aldrich, Cat #63069)
  14. 1 M CaCl<sub>2</sub> (Sigma Aldrich, Cat #21115-100ML)
  15. DMF (Dimethyl Formamide) (Sigma, Cat #227056)

16. 0.2 M Tris-acetate pH 7.8 (Bioworld, Cat #40120265-2)
17. 5 M Potassium acetate (Sigma Aldrich, Cat #95843-100ML-F)
18. 1 M Magnesium acetate (Sigma Aldrich, Cat #63052-100ML)
19. 10% NP-40 (Thermo Fisher Scientific, Cat #28324)
20. Buffer EB (Qiagen, Cat #19086)
21. PEG 6000 (Sigma Aldrich, Cat #528877)
22. Maxima H Minus Reverse Transcriptase with buffer (Thermo Fisher Scientific, Cat #EP075)
23. 10 mM dNTPs (NEB, Cat #N0447L)
24. T4 DNA Ligase (NEB, Cat #M0202L)
25. Additional 10× T4 Ligase buffer (NEB, Cat #B0202S)
26. Proteinase K (20 mg/mL) (NEB, Cat #P8107S)
27. 20% SDS (VWR, Cat #97062+440)
28. 100 mM PMSF/IPA (Sigma Aldrich, Cat # P7626)
29. cOmplete Protease Inhibitor Cocktail (Sigma Aldrich, Cat # 11697498001)
30. 0.5 M EDTA (Sigma Aldrich, Cat #AM9260G)
31. Tween-20 (Sigma Aldrich, Cat #P9416-100ML)
32. Digitonin (Promega, Cat #G9441)
33. MyOne C1 Dynabeads (Thermo Fisher Scientific, Cat #65001)
34. Ficoll PM-400 (20%) (Sigma Aldrich, Cat #F5415-25ML)
35. Kapa HiFi 2× mix (Fisher Scientific, Cat #NC0295239)
36. SPRiselect beads (Beckman Coulter, Cat #B23318)
37. 100% EtOH
38. 100 mM DTT (Thermo Fisher Scientific, Cat #707265ML)
39. NEBnext 2× Mix (NEB, Cat #M0541L)
40. Glycerol (Thermo Fisher Scientific, Cat #15514011)
41. TD buffer from Nextera kit
42. SYBR Green I Nucleic Acid Gel Stain (Thermo Fisher Scientific, Cat #S7563)
43. EVAGreen Dye, 20x in water (Biotium, Cat #31000)
44. Nuclease-free H<sub>2</sub>O
45. 96-well plates (Eppendorf, Cat #0030129300) (preferably low protein and DNA binding; *see Note 5*)
46. 1.5-mL microcentrifuge tubes, preferably low protein and DNA binding (*see Note 5*)
47. 2-mL, 15-mL and 50-mL tubes
48. gentleMACS M Tubes (Miltenyi Biotec, Cat #130-093-236)
49. 30 μm Sterile single-pack CellTrics filters (Sysmex, Cat #04-004-2326)

50. 200- $\mu$ L PCR tubes
51. Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat # Q32851)
52. TapeStation D1000 and D5000 tape and reagents (Agilent)
53. Tn5 transposase (*see Note 1*)
54. MinElute PCR Purification Kit (Qiagen Cat# 28004/28006), Zymo DNA Clean and Concentrator Kit (Zymo Cat# D4013/D4014), or equivalent

## 2.4 Buffers and Reagents

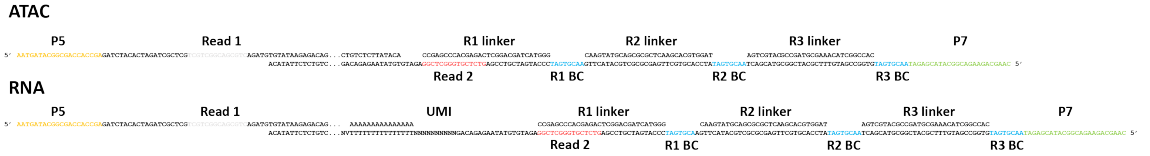
Make all buffers using ultrapure molecular biology-grade ddH<sub>2</sub>O.

1. 2.5M Glycine (50 mL)
  - 9.375 g Glycine (powder)
  - 1 $\times$  PBS up to 50 mL
 Filter through a 0.22  $\mu$ M filter. Store at room temperature.
2. Tissue Dissociation (MACS) buffer
  - 10 mM Tris-HCl pH 8.0
  - 5 mM CaCl<sub>2</sub>
  - 5 mM EDTA
  - 3 mM MgAc
  - 0.6 mM DTT
  - cOmplete protease inhibitor
 Make fresh every time.
3. Nuclei Isolation Buffer (NIB)
  - 10 mM Tris-HCl pH 7.4
  - 10 mM NaCl
  - 3 mM MgCl<sub>2</sub>
  - 0.1% IGEPAL CA-630
 Store at 4 °C.
4. 2 $\times$  TD buffer
  - 20 mM Tris-HCl pH 7.6
  - 10 mM MgCl<sub>2</sub>
  - 20% Dimethyl Formamide
 Store at -20 °C.
5. PEG 6000 50%
  - Mix equal mass of PEG6000 and H<sub>2</sub>O, heat to 65 °C) for 4 minutes, then cool down to room temperature.
6. 2 $\times$  RCB buffer
  - 100 mM Tris pH 8.0
  - 100 mM NaCl
  - 0.40% SDS
 Store at room temperature.
7. 2 $\times$  BW buffer
  - 10 mM Tris pH 8.0

- 2 M NaCl
- 1 mM EDTA
- Store at 4 °C.
- 8. 1× B&W-T Buffer
  - 5 mM Tris pH 8.0
  - 1 M NaCl
  - 0.5 mM EDTA
  - 0.05% Tween-20
 Store at 4 °C.
- 9. Oligo resuspension buffer (IDTE)
  - 10 mM Tris pH 8.0
  - 0.1 mM EDTA
 Store at room temperature.
- 10. Oligo annealing buffer (STE)
  - 10 mM Tris pH 8.0
  - 50 mM NaCl
  - 1 mM EDTA
 Store at room temperature.
- 11. Dilution Buffer
  - 50% glycerol
  - 50 mM Tris pH 7.5
  - 100 mM NaCl
  - 0.1 mM EDTA
  - 0.1% NP-40
 Store at -20 °C.

## 2.5 Software packages

1. Bowtie [62] (<http://bowtie-bio.sourceforge.net/index.shtml>).
2. samtools [63]: <http://www.htslib.org/>
3. PicardTools <https://broadinstitute.github.io/picard/>
4. UCSC Genome Browser [64, 65] utilities: <http://hgdownload.cse.ucsc.edu/admin/exe/>
5. STAR [66] <https://github.com/alexdobin/STAR>
6. R: <https://www.r-project.org/>
7. Python (version 2.7 or higher) <https://www.python.org/>
8. ArchR [67]: <https://www.archrproject.com/>
9. Seurat [68]: <https://satijalab.org/seurat/>
10. Additional scripts: <https://github.com/georgimarinov/GeorgiScripts>. Contains python scripts used in the examples shown below; some of the scripts depend on having pysam (<https://pysam.readthedocs.io/en/latest/index.html>) and pyBigWig (<https://github.com/deeptools/pyBigWig>) installed.



**Fig. 2: Structure of final SHARE-seq libraries.** ATAC (top) and RNA (bottom). Dots represent the actual library insert.

### 3 Methods

The general outline of the SHARE-seq assay is shown in Figure 1. The first of the two basic ideas behind SHARE-seq and other pool/split-based assays is to label molecules originating from each cells with a unique combination of barcodes that are added serially and randomly by pooling cells and then randomly redistributing them across subsequent sets of barcodes, thus ensuring that statistically each cell can be identified through a unique combination of barcodes. The second is the separation of chromatin and transcriptome molecules through the use of a biotinylated reverse transcription (RT) primer, which can then be used for a streptavidin pulldown of the transcriptome.

In brief, before the beginning of a SHARE-seq experiment, the needed barcode plates and transposases are prepared and stored. The experiment itself begins with the isolation of nuclei from cells in culture or from tissues (*see Note 2*. Nuclei are then cross-linked, usually lightly (*see Note 3*. Transposition is then carried out, followed by reverse transcription using a biotinylated RT primer containing a random unique molecular identifier (UMI). Three rounds of pool/split hybridization and blocking are then carried out, after which the hybridized oligos are ligated into single molecules to each other and to the transposed chromatin fragments and reverse transcribed mRNA. Crosslinks are then reversed, and streptavidin pulldown is used to separate the chromatin from the transcriptome. ATAC libraries are directly amplified from the supernatant. The transcriptome is first amplified on-beads into cDNAs, which are then tagged into sequenceable fragments and PCR-amplified into final libraries.

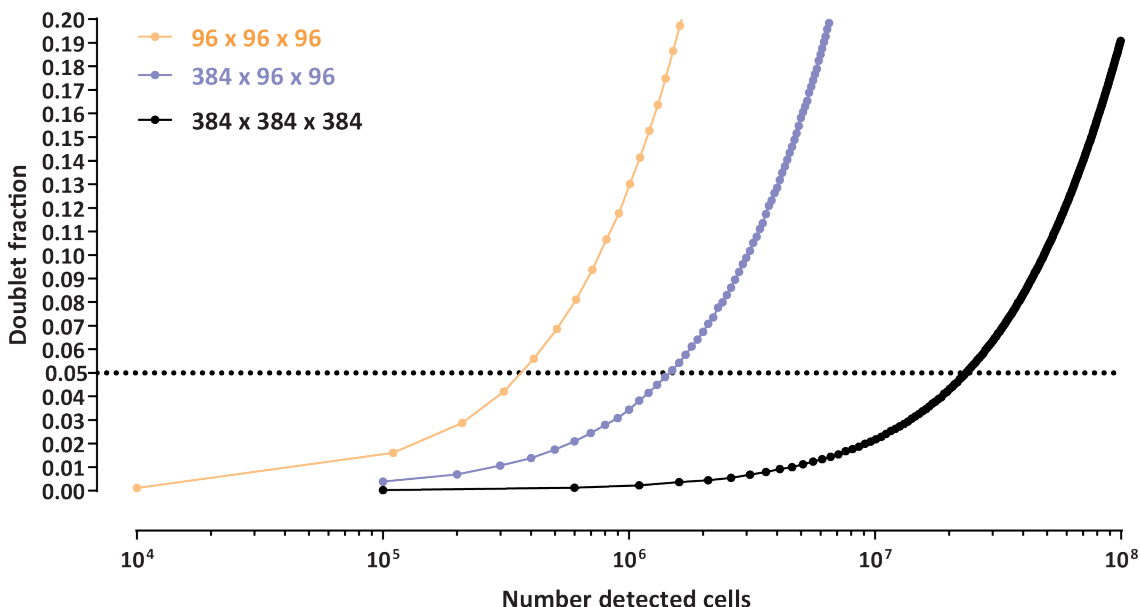
The resulting library structures for ATAC and RNA are shown in Figure 2. ATAC libraries contain three barcodes while RNA libraries also include the UMI. Note that with many Illumina-based sequencing read-outs the first barcode to be read is actually the third one added during the pool/split procedure.

#### 3.1 Determining the optimal cell number

It is important to carefully track the number of cells going into the SHARE-seq assays and being retained at each key step of the procedure. Pool/split assays rely on the statistical uniqueness of barcode combinations through which cells pass, which in turn means that having too many cells entering the pool/split procedure will lead to an unacceptably high rate of doublets (two or more cells with the same

barcode). In the same time some of the reactions have an efficiency-imposed limit on the number of cells that can enter them, and need to be distributed into parallel reactions for optimal results. This applies to the initial transposition and reverse transcription reactions, as well as to the final amplification, where the existing protocol is optimized for libraries of size  $\sim 20,000$  cells, which means that after the final pooling cells are split into separate subpools of that size and processed into individual sublibraries.

Figure 3 shows the theoretical number of detected cells and doublet rate for different pool/split setups with three rounds, accounting for a certain level of cell loss during repeated handling. Based on these calculations and empirical experience, we usually start the pool/split rounds with  $\sim 5 \times 10^5$  cells for a  $96 \times 96 \times 96$  pool/split experiment.



**Fig. 3: Combinatorial indexing and SHARE-seq’s throughput.** Shown is the number of cells that can be detected at a given doublet rate; the pool-split process was simulated as a random Poisson loading at a 50% loss of cells during each pool/split round.

### 3.2 Annealing of oligo plates

In this step, barcode containing oligonucleotides for each round of split pool are annealed and distributed into 96-well plates prior to the actual assay. These plates can be stored at  $-20^\circ\text{C}$  indefinitely. It is advisable for the purposes of time saving to prepare sufficiently many such plates in advance to support multiple experiments. It is critical to thaw these plates to room temperature prior to use.

(see Note 4)

1. Dilute Round 1 linker oligos ( $120\ \mu\text{L}$  at  $1\ \text{mM}$  concentration) with  $11,880\ \mu\text{L}$  STE buffer.

2. Mix 90  $\mu\text{L}$  diluted Round 1 linker oligo with 10  $\mu\text{L}$  Round 1 oligo (at 100  $\mu\text{M}$ ) in the wells of a multiwell plate
3. Dilute Round 2 linker oligos (120  $\mu\text{L}$  at 1 mM concentration) with 9,480  $\mu\text{L}$  STE buffer.
4. Mix 88  $\mu\text{L}$  diluted Round 2 linker oligo with 12  $\mu\text{L}$  Round 2 oligo (at 100  $\mu\text{M}$ ) in the wells of a multiwell plate
5. Dilute Round 3 linker oligos (144  $\mu\text{L}$  at 1 mM concentration) with 9,360  $\mu\text{L}$  STE buffer.
6. Mix 86  $\mu\text{L}$  diluted Round 3 linker oligo with 14  $\mu\text{L}$  Round 3 oligo (at 100  $\mu\text{M}$ ) in the wells of a multiwell plate
7. Anneal the Round 1, Round 2, and Round 3 plates as follows in a thermocycler:
  - 2 minutes at 95 °C
  - Slow ramp at -1 °C per minute to 20 °C
  - 2 minutes at 20 °C
  - Indefinitely at 4 °C
8. Check if there has been significant water evaporation for wells situated at the corners. If yes, add water to equalize volumes
9. Aliquot 10  $\mu\text{L}$  of the annealed oligos to new plates. This should be enough for 9 experiments. Store these plates at -20 °C.

### **3.3 Anneal adapter oligos**

In this step, Tn5 adapters are prepared for both transposition of chromatin and tagmentation during cDNA library preparation.

1. Dilute the Phosphorylated Read2, Read1, and Blocked ME Comp oligos to a 100  $\mu\text{M}$  concentration with the IDTE buffer.
2. Prepare the transposition adapter mix in a PCR tube as follows:
  - 6.5  $\mu\text{L}$  100  $\mu\text{M}$  Phosphorylated Read2 oligo
  - 6.5  $\mu\text{L}$  100  $\mu\text{M}$  Read1 oligo
  - 13  $\mu\text{L}$  100  $\mu\text{M}$  Blocked ME Comp oligo
  - 0.26  $\mu\text{L}$  1 M Tris pH 8.0
  - 0.26  $\mu\text{L}$  5 M NaCl
3. Prepare the tagmentation adapter mix in a PCR tube as follows:
  - 13  $\mu\text{L}$  100  $\mu\text{M}$  Read1 oligo
  - 13  $\mu\text{L}$  100  $\mu\text{M}$  Blocked ME Comp oligo
  - 0.26  $\mu\text{L}$  1 M Tris pH 8.0
  - 0.26  $\mu\text{L}$  5 M NaCl
4. Anneal oligos as follows in a thermocycler:
  - 2 minutes at 85 °C
  - Slow ramp at -1 °C per minute to 20 °C
  - 2 minutes at 20 °C
  - Indefinitely at 4 °C
5. Heat glycerol to 65 °C, then equilibrate to room temperature
6. Mix 25  $\mu\text{L}$  glycerol with 25  $\mu\text{L}$  of annealed oligo

The annealed adapters can be immediately used or stored at  $-20^{\circ}\text{C}$ .

### **3.4 Transposome assembly**

In this step, Tn5 transposomes are assembled together with the annealed adapter oligos.

1. Assemble Tn5 transposomes by mixing the following components.  
0.625×*N* 1× home-made Tn5  
0.625×*N* Dilution Buffer  
1.25×*N* annealed transposition adapter with glycerol  
Total volume: 2.5×*N*
2. Incubate at room temperature for 30 minute.  
The assembled transposome can be stored at  $-20^{\circ}\text{C}$  for up to 2 weeks.

### **3.5 Tissue dissociation**

Here, we describe an example tissue dissociation protocol that has worked successfully in our hands for several human embryonic tissues. However, users should be aware that generally each tissue requires separate optimization of dissociation conditions and it is likely that a different protocol will have to be adapted in most situations.

1. Set swing bucket centrifuge to  $4^{\circ}\text{C}$  Fast Temp and thaw 1M DTT.
2. Transfer tissue samples onto dry ice.
3. Prepare MACS buffer (2 mL for each sample) as described above. Make sure the buffer is cold on ice.
4. Add 10  $\mu\text{L}$  Protector RNase Inhibitor for each 1 mL in GentleMACS M-tubes. Add 1 mL of MACS buffer to each GentleMACS M-tube and chill on ice.
5. Transfer 30-50 mg of tissue into each GentleMACS M-tube containing 1 mL MACS buffer.
6. Allow the tissue to thaw in buffer. Transition to a cold room.
7. Homogenize using a `Protein_01_01` dissociation protocol on a GentleMACS Tissue Dissociator instrument.
8. Filter the homogenate through 30  $\mu\text{m}$  CellTrics filter into a 2mL DNA lo-bind tube by pipetting directly onto the top of the filter and gently tapping to allow flow.
9. Wash the GentleMACS M tube with 1 mL MACS buffer and filter the wash again through the 30  $\mu\text{m}$  CellTrics filter.
10. Spin down the homogenate in a swing bucket centrifuge at 500 *g* for 5 minutes at  $4^{\circ}\text{C}$  (ramp up and down both at 3/9).
11. Remove and discard supernatant.
12. Resuspend in 1mL PBS-2RI.
13. Count cells/nuclei and proceed with desired number of cells/nuclei.

### **3.6 Fixation of cells in culture and of dissociated nuclei from tissue**

The next step, if starting with a dissociated tissue, is to fix the nuclei. This is also the first step if starting with cells in culture. The procedure

used is generally the same, with the difference that with nuclei the first step is directly the fixation.

1. Prepare PBS-2RI Buffer (~4 mL) by mixing the following:
  - 4 mL 1× PBS
  - 21.4  $\mu$ L 7.5% BSA
  - 10  $\mu$ L Enzymatic RI
  - 5  $\mu$ L SUPERase RI

Keep on ice.

2. Prepare NIB-RI Buffer (~8 mL) by mixing the following:
  - 8 mL NIB
  - 20  $\mu$ L Enzymatic RI
  - 20  $\mu$ L SUPERase RI

Keep on ice.

3. Spin down cells at 500 *g*
4. Wash cells with 0.5 ml PBS-2RI
5. Count cells with Trypan blue
6. Resuspend cells with cold PBS-2RI at concentration of  $1 \times 10^6$  cells/mL.
7. For each 1 mL of cells in PBS-2RI, add 66.7  $\mu$ L of 1.6% FA (final concentration 0.1% FA) for cells or 66.7  $\mu$ L of 3.2% FA for tissues. Mix and incubate at room temperature for 5 minutes.
8. Quench the reaction by adding to each 1 mL of cells in PBS-2RI the following:
  - 56.1  $\mu$ L 2.5 M Glycine
  - 50  $\mu$ L 1M Tris pH 8.0
  - 13.3  $\mu$ L of 7.5% BSA

Mix well and incubate on ice for 5 minutes.

9. Spin down at 500 *g*. Remove supernatant, and add 0.5 mL PBS-2RI without disturbing the cell pellet.
10. Prepare RSB-RI by mixing the following:
  - 2.5  $\mu$ L 1 M Tris-HCl pH 7.5
  - 0.5  $\mu$ L 5 M NaCl
  - 0.75  $\mu$ L 1 M MgCl<sub>2</sub>
  - 2.5  $\mu$ L 10% Tween-20
  - 2.5  $\mu$ L 10% NP-40
  - 2.5  $\mu$ L 1% Digitonin
  - 33.3  $\mu$ L 7.5% BSA
  - 0.25  $\mu$ L 1 M DTT
  - 204  $\mu$ L Ultrapure water
  - 1.25  $\mu$ L Enzymatic RI
11. Spin down again at 500 *g*. Remove supernatant, and resuspend cells in 100  $\mu$ L RSB-RI and incubate on ice for 3 minutes.
12. Prepare RSB-T by mixing the following:
  - 25  $\mu$ L 1 M Tris-HCl pH 7.5
  - 5  $\mu$ L 5 M NaCl

7.5  $\mu\text{L}$  1 M  $\text{MgCl}_2$   
25  $\mu\text{L}$  10% Tween-20  
333.3  $\mu\text{L}$  7.5% BSA  
2.5  $\mu\text{L}$  1 M DTT  
2089.5  $\mu\text{L}$  Ultrapure water  
12.5  $\mu\text{L}$  Enzymatic RI

13. Pipette 1 mL of RSB-T to cells and mix. Spin down at 500  $g$  for 5 minutes.

### 3.7 ATAC reaction

In this step, transposition of the entire sample is performed by splitting it into 10,000-20,000 cells in 50- $\mu\text{L}$  reactions each in a 96-well plate. The smaller volume and the number of cells per reaction improves the quality of transposition.

The cell lysis conditions described here are adapted from the omniATAC bulk ATAC protocol [22] (see **Note 7**).

1. Prepare PBS-RI by mixing the following:  
800  $\mu\text{L}$  PBS  
2  $\mu\text{L}$  Enzymatic RI
2. After the last centrifugation, remove supernatant and resuspend the cells with PBS-RI to  $2 \times 10^6$  cells/mL.
3. Prepare 2 $\times$  TB buffer (sufficient for 96 reactions) by mixing the following:  
874.5  $\mu\text{L}$  0.2 M Tris-acetate  
70  $\mu\text{L}$  5 M Potassium acetate  
53  $\mu\text{L}$  1 M Magnesium acetate  
53  $\mu\text{L}$  10% Tween-20  
53  $\mu\text{L}$  1% Digitonin  
848  $\mu\text{L}$  100% DMF  
698.5  $\mu\text{L}$   $\text{H}_2\text{O}$
4. Prepare 1 $\times$  TB buffer according to the number of reactions  $N$  to be carried out.  $N = 1$  corresponds to  $10^4$  input cells.  
 $25 \times N$  2 $\times$  TB  
 $16.45 \times N$   $\text{H}_2\text{O}$   
 $0.2 \times N$  PIC  
 $0.85 \times N$  Enzymatic RI  
Total volume:  $42.5 \times N$ .
5. Aliquot  $5 \times N$   $\mu\text{L}$  of the diluted cells to a new tube. e.g. for  $10 \times 10^5$  cells,  $N = 10$ , so aliquot 50  $\mu\text{L}$  cells to a new tube.
6. Add  $42.5 \times N$   $\mu\text{L}$  1 $\times$  TB to sample.
7. Add  $2.5 \times N$   $\mu\text{L}$  of assembled Tn5 to sample. Mix well.
8. Aliquot 50  $\mu\text{L}$  of sample in the wells of a 96- or 384-well plate.
9. Seal the plate and incubate with shaking at 500 rpm for 30 minutes at 37  $^\circ\text{C}$ .
10. Pool the reactions and spin down at 500  $g$ .

11. Add 0.5 mL NIB-RI without disturbing the pellet and spin down again at 500 *g*
12. Resuspend the cells in 60  $\mu\text{L}$  EB.

### 3.8 Reverse transcription

In this step, reverse transcription is performed *in situ*. The conditions are optimized for  $1 \times 10^5$  cells entering each 50- $\mu\text{L}$  reaction.

1. Prepare the Reverse Transcription (RT) mix (sufficient for 6 reactions) as follows:
  - 70  $\mu\text{L}$  5 $\times$  RT buffer
  - 2.19  $\mu\text{L}$  Enzymatics RNase Inhibitor
  - 4.38  $\mu\text{L}$  SUPERase RI
  - 17.5  $\mu\text{L}$  dNTPs
  - 35  $\mu\text{L}$  RT Primer
  - 10.94  $\mu\text{L}$  H<sub>2</sub>O
  - 105  $\mu\text{L}$  50% PEG
  - 35  $\mu\text{L}$  Maxima H Minus Reverse Transcriptase (add right before RT reaction)
 Total volume: 280  $\mu\text{L}$ .
2. Add 240  $\mu\text{L}$  RT mix to 60  $\mu\text{L}$  cells in EB.
3. Aliquot 50  $\mu\text{L}$  to 6 PCR wells.
4. Start thawing the oligo plates while the RT is ongoing.
5. Run the reverse transcription reaction in a thermocycler as follows:
  - 50 °C for 10 minutes
  - 3 cycles of:
    - 8 °C for 12 seconds
    - 15 °C for 45 seconds
    - 20 °C for 45 seconds
    - 30 °C for 30 seconds
    - 42 °C for 2 minutes
    - 50 °C for 3 minutes
  - 50 °C for 5 minutes.
6. Pool samples and mix with 500  $\mu\text{L}$  NIB-RI.
7. Spin down at 500 *g*.
8. Wash with 1000  $\mu\text{L}$  NIB.
9. Spin down at 500 *g*.
10. Resuspend with 1,152  $\mu\text{L}$  NIB-RI.

### 3.9 Hybridization-ligation and pool-split

In this step, cells/nuclei are iteratively split into individual wells to dynamically create a combinatorial index statistically unique to each cell. All handling is performed at room temperature so make absolutely sure that oligo plates have been fully thawed before proceeding.

If different samples are multiplexed in a single run, they can be individually identified based on the first-round barcodes. If such a strategy is deployed, each sample needs to be processed through transposition and

reverse transcription separately, then loaded into specified positions in the first-round plate(s).

1. Prepare 3,456  $\mu\text{L}$  hybridization buffer as follows:
  - 2761.9  $\mu\text{L}$   $\text{H}_2\text{O}$
  - 576  $\mu\text{L}$  10 $\times$  T4 ligase buffer
  - 14.4  $\mu\text{L}$  SUPERase RI 20 U/ $\mu\text{L}$
  - 46.08  $\mu\text{L}$  Enzymatics RI 40 U/ $\mu\text{L}$
  - 57.60  $\mu\text{L}$  10% NP40
2. Mix 1,152  $\mu\text{L}$  of sample with 3,456  $\mu\text{L}$  hybridization buffer. Keep the sample at RT
3. Aliquot 40  $\mu\text{L}$  of mixture to a Round 1 plate.
4. Mix and shake at 300 rpm for 30 minutes at RT
5. Prepare 1,152  $\mu\text{L}$  Blocking Oligo 1 mix as follows:
  - 253.4  $\mu\text{L}$  100  $\mu\text{M}$  Round 1 blocking oligo
  - 211.2  $\mu\text{L}$  10 $\times$  T4 DNA Ligase buffer
  - 687.4  $\mu\text{L}$   $\text{H}_2\text{O}$
6. Add 10  $\mu\text{L}$  Blocking Oligo 1 mix to each well
7. Mix and shake at 300 rpm for 30 minutes at RT
8. Pool samples from all wells
9. Aliquot 50  $\mu\text{L}$  of mixture to a Round 2 plate.
10. Mix and shake at 300 rpm for 30 minutes at RT
11. Prepare 1,152  $\mu\text{L}$  Blocking Oligo 2 mix as follows:
  - 304.1  $\mu\text{L}$  100  $\mu\text{M}$  Round 2 blocking oligo
  - 211.2  $\mu\text{L}$  10 $\times$  T4 DNA Ligase buffer
  - 636.7  $\mu\text{L}$   $\text{H}_2\text{O}$
12. Add 10  $\mu\text{L}$  Blocking Oligo 2 mix to each well
13. Mix and shake at 300 rpm for 30 minutes at RT
14. Pool samples from all wells
15. Aliquot 60  $\mu\text{L}$  of mixture to a Round 2 plate.
16. Mix and shake at 300 rpm for 30 minutes at RT
17. Prepare 1,152  $\mu\text{L}$  Blocking Oligo 3 mix as follows:
  - 265.0  $\mu\text{L}$  100  $\mu\text{M}$  Round 3 blocking oligo
  - 11.5  $\mu\text{L}$  10% NP-40
  - 875.5  $\mu\text{L}$   $\text{H}_2\text{O}$
18. Add 10  $\mu\text{L}$  Blocking Oligo 1 mix to each well
19. Mix and shake at 300 rpm for 30 minutes at RT
20. Pool samples from all wells
21. Spin down at 500  $g$  5 minutes
22. Wash with 1 mL NIB-RI
23. Spin down at 500  $g$  5 minutes
24. Resuspend in 80  $\mu\text{L}$  NIB-RI

25. Prepare 320  $\mu\text{L}$  Ligation mix as follows:
  - 3.2  $\mu\text{L}$  Enzymatics RI
  - 1.00  $\mu\text{L}$  SUPERase RI
  - 40  $\mu\text{L}$  10 $\times$  T4 DNA Ligase Ligation buffer
  - 20  $\mu\text{L}$  T4 DNA Ligase 400 U/ $\mu\text{L}$
  - 251.8  $\mu\text{L}$  H<sub>2</sub>O
  - 4  $\mu\text{L}$  10% NP40
26. Mix sample with the 320  $\mu\text{L}$  Ligation mix
27. Aliquot 8 $\times$  50  $\mu\text{L}$  in PCR tubes
28. Shake at 300 rpm for 30 minutes at RT
29. Pool samples from all tubes
30. Spin down at 500  $g$  5 minutes
31. Wash with 1 mL NIB-RI
32. Spin down at 500  $g$  5 minutes
33. Resuspend in 400  $\mu\text{L}$  NIB-RI
34. Count the number of nuclei.

Note: If fewer cells are preferred per sub-library, count cells to desired concentration and add more NIB to make the volume up to 50  $\mu\text{L}$  per sub-library.

### 3.10 Reverse crosslinking

In this step, cells are reverse crosslinked to release DNA from the bound proteins so that the ATAC libraries can be amplified. As the crosslinking is relatively gentle (at 0.1 or 0.2%), a milder reverse crosslinking condition of 1 hour incubation at 55  $^{\circ}\text{C}$  is generally sufficient.

Further reverse crosslinking optimization might be needed if the crosslinking protocol has been modified.

1. For each  $N$  of 50- $\mu\text{L}$  sub-library, add the following:
  - 50  $\mu\text{L}$  2 $\times$  RCB
  - 2  $\mu\text{L}$  Proteinase K
  - 1  $\mu\text{L}$  SUPERase RI
2. Incubate at 55  $^{\circ}\text{C}$  for 1 hour.
3. Add 5  $\mu\text{L}$  100 mM PMSF/IPA
4. Incubate at room temperature for 10 minutes

Note: this is an optional stopping point. The reverse crosslinked product can be stored at  $-80^{\circ}\text{C}$  for a few days.

### 3.11 Pull down

In this step, the cDNA is separated from the transposition products by pulling down on the biotin that is part of the reverse transcription primer. The supernatant constitutes the transposition products, and is processed separately from the cDNA.

1. Prepare 1 $\times$  B&W-T/RI buffer by mixing the following:
  - 400 $\times$ ( $N + 1$ )  $\mu\text{L}$  1 $\times$  B&W-T buffer
  - 4 $\times$ ( $N + 1$ )  $\mu\text{L}$  SUPERase RI

2. Prepare  $1\times$  B&W/RI buffer by mixing the following:
  - $100\times(N + 1)$   $\mu\text{L}$   $1\times$  BW buffer
  - $2\times(N + 1)$   $\mu\text{L}$  SUPERase RI
3. Prepare  $1\times$  STE/RI buffer by mixing the following:
  - $200\times(N + 1)$   $\mu\text{L}$   $1\times$  STE buffer
  - $N + 1$   $\mu\text{L}$  SUPERase RI
4. In a fresh tube, mix  $10\times N$   $\mu\text{L}$  MyOne C1 Dynabeads with  $100\times N$   $\mu\text{L}$   $1\times$  B&W-T
5. Separate on a magnetic rack and remove supernatant
6. Wash twice with  $100\times N$   $\mu\text{L}$  B&W-T without RI.
7. Wash once with  $100\times N$   $\mu\text{L}$  B&W-T/RI
8. Resuspend beads in  $100\times N$   $\mu\text{L}$   $2\times$  B&W/RI.
9. Add 100  $\mu\text{L}$  beads to each sample
10. Incubate at room temperature on a rotator for 60 minutes
11. Place the tube on a magnetic rack
12. Transfer the supernatant (which contains chromatin fragments) to a new tube for ATAC library preparation. The ATAC fragments are stable for a few hours at room temperature and can be processed concurrently or after cDNA library construction is complete.
13. Wash cDNA/RNA-bound beads three times with 100  $\mu\text{L}$   $1\times$  B&W-T/RI.
14. Wash with 100  $\mu\text{L}$   $1\times$  STE/RI without resuspending beads.

### **3.12 ATAC library preparation**

In this step, ATAC fragments are purified and amplified into a final library ready for sequencing.

1. Clean up the ATAC part of the sample using Zymo DNA Clean & Concentrate. Elute in 11  $\mu\text{L}$  EB buffer, then elute again with additional 11  $\mu\text{L}$  EB buffer (a total of 22  $\mu\text{L}$  EB buffer).
2. Prepare ATAC PCR Master Mix by mixing the following:
  - 225  $\mu\text{L}$   $2\times$  NEBnext Master Mix
  - 9  $\mu\text{L}$  P7 primer 25  $\mu\text{M}$
  - 27  $\mu\text{L}$   $\text{H}_2\text{O}$
3. Mix the following:
  - $\sim 20$   $\mu\text{L}$  sample
  - 29  $\mu\text{L}$  ATAC PCR Master Mix
  - 1  $\mu\text{L}$  of 25  $\mu\text{M}$  Adapter 1 Primer (from the PCR Library indexing primers plate)
4. Run PCR for 5 cycles as follows:
  - 72  $^\circ\text{C}$  for 5 minutes
  - 98  $^\circ\text{C}$  for 30 seconds
  - 5 cycles of:
    - 98  $^\circ\text{C}$  for 10 seconds
    - 65  $^\circ\text{C}$  for 30 seconds
    - 72  $^\circ\text{C}$  for 30 seconds

- Determining additional cycles using qPCR. Add 5  $\mu\text{L}$  of the pre-amplified reaction to 10  $\mu\text{L}$  qPCR mastermix for a total qPCR reaction of 15  $\mu\text{L}$  as follows:

5  $\mu\text{L}$  NEBnext Master Mix  
0.2  $\mu\text{L}$  25  $\mu\text{M}$  Adapter 1.1  
0.2  $\mu\text{L}$  25  $\mu\text{M}$  P7  
0.9  $\mu\text{L}$  10x SYBR Green  
3.7  $\mu\text{L}$   $\text{H}_2\text{O}$

- Assess the amplification profiles and determine the required number of additional cycles to amplify. Please refer to Figure 2 in Buenrostro et al. (2015).
- Carry out final amplification by placing the remaining 45  $\mu\text{L}$  in a thermocycler and running the following program:  
 $N_{add}$  cycles of:  
98  $^\circ\text{C}$  for 10 seconds  
65  $^\circ\text{C}$  for 30 seconds  
72  $^\circ\text{C}$  for 30 seconds  
Where  $N_{add}$  is the number of additional cycles.
- Clean up the finally library using Zymo DNA Clean & Concentrate, eluting in 15  $\mu\text{L}$ .

### **3.13 RNA library preparation step 1. Template switching**

In this step, RNA library generation is initiated by carrying out template switching on the pulled down cDNA.

- Prepare the Template switch mix by mixing the following:  
11.25  $\mu\text{L}$   $\text{H}_2\text{O}$   
125  $\mu\text{L}$  50% PEG 6000  
90  $\mu\text{L}$  5 $\times$  Maxima RT buffer  
90  $\mu\text{L}$  Ficoll PM-400 (20%)  
45  $\mu\text{L}$  10 mM dNTPs  
45  $\mu\text{L}$  RNase inhibitor (Lucigen)  
11.25  $\mu\text{L}$  100  $\mu\text{M}$  TSO oligo  
22.5  $\mu\text{L}$  Maxima RT Rnase H Minus (add last right before reaction)
- Remove all supernatant. Be careful to avoid drying the beads
- Resuspend beads in 50  $\mu\text{L}$  Template switch mix
- Incubate samples for 30 minutes at room temperature with rotation
- Incubate samples for 90 minutes at 42  $^\circ\text{C}$  at 300 rpm. Resuspend every 30 minutes by pipetting up and down.

### **3.14 RNA library preparation step 2. Amplification of cDNA**

The next step is to amplify the individual cDNA molecules.

- Prepare cDNA PCR Mix by mixing the following:  
247.5  $\mu\text{L}$  Kapa Hifi 2 $\times$  mix

7.92  $\mu\text{L}$  25  $\mu\text{M}$  RNA PCR primer  
7.92  $\mu\text{L}$  25  $\mu\text{M}$  P7 primer  
231.7  $\mu\text{L}$   $\text{H}_2\text{O}$

- Mix samples with 100  $\mu\text{L}$   $\text{H}_2\text{O}$ .
- Separate beads on magnet. Wash with 200  $\mu\text{L}$  STE without resuspending the beads
- Mix beads with 55  $\mu\text{L}$  cDNA PCR Mix and transfer to PCR tubes/plates
- Run PCR as follows:  
95  $^\circ\text{C}$  for 3 minutes  
5 cycles of:  
98  $^\circ\text{C}$  for 20 seconds  
65  $^\circ\text{C}$  for 45 seconds  
72  $^\circ\text{C}$  for 3 minutes
- Determining additional cycles using qPCR. Add 2.5  $\mu\text{L}$  of the pre-amplified reaction to 7.5  $\mu\text{L}$  qPCR mastermix in a total qPCR reaction of 10  $\mu\text{L}$  as follows:

3.75  $\mu\text{L}$  Kapa Hifi 2 $\times$  mix  
0.12  $\mu\text{L}$  25  $\mu\text{M}$  RNA PCR primer  
0.12  $\mu\text{L}$  25  $\mu\text{M}$  P7 primer  
0.5  $\mu\text{L}$  20x EVAgreen  
3.01  $\mu\text{L}$   $\text{H}_2\text{O}$

- Determine additional cycles as described above for ATAC libraries.  
5 cycles of:  
98  $^\circ\text{C}$  for 20 seconds  
65  $^\circ\text{C}$  for 45 seconds  
72  $^\circ\text{C}$  for 3 minutes
- Purify using SPRI beads. Mix the reaction with 0.8 $\times$  volume of SPRI beads and incubate at room temperature for 10 minutes. Separate the beads on magnet and wash twice with 200  $\mu\text{L}$  freshly prepare 70% EtOH. Make sure to remove all liquid, and elute in 20  $\mu\text{L}$ .
- Optional: check size of the cDNA using the D5000 TapeStation.

### **3.15 RNA library preparation step 3. Tagmentation**

The next step is to tagment the amplified cDNA, which will prepare it for the final library amplification step.

- Quantify cDNA concentration using Qubit.
- Dilute cDNA to a concentration of 5 ng/ $\mu\text{L}$  for tagmentation  
Note: expect more than 50 ng cDNA. If cDNA amount is low, it can get away with tagmenting 20 ng cDNA; in this case, adjust the volume of  $\text{H}_2\text{O}$  and cDNA accordingly
- Prepare tagmentation transposome by mixing the following:  
11.25  $\mu\text{L}$  1 $\times$  Tn5  
11.25  $\mu\text{L}$  Dilution Buffer  
22.5  $\mu\text{L}$  annealed tagmentation adapter with glycerol

4. Mix the following:
  - 10  $\mu\text{L}$  5 ng/ $\mu\text{L}$  cDNA
  - 10  $\mu\text{L}$   $\text{H}_2\text{O}$
  - 25  $\mu\text{L}$  2 $\times$  TD buffer
  - 5  $\mu\text{L}$  assembled Tn5
5. Incubate for 5 minutes at 55  $^\circ\text{C}$ .
6. Purify tagged library using the Zymo kit (use 250  $\mu\text{L}$  binding buffer). Elute twice with 11  $\mu\text{L}$  EB (a total of 22  $\mu\text{L}$ )

### **3.16 RNA library preparation step 4. Final Amplification**

Final libraries are generated by PCR.

1. Prepare Post-tagmentation PCR mix by mixing the following:
  - 20  $\mu\text{L}$  sample
  - 25  $\mu\text{L}$  2 $\times$  NEB Next Master Mix
  - 1  $\mu\text{L}$  25  $\mu\text{M}$  P7 primer
  - 1  $\mu\text{L}$  25  $\mu\text{M}$  Adapter 1 Primer (from the PCR Library indexing primers plate)
  - 3  $\mu\text{L}$   $\text{H}_2\text{O}$
2. Run PCR as follows:
  - 72  $^\circ\text{C}$  for 5 minutes
  - 9 cycles of:
    - 98  $^\circ\text{C}$  for 10 seconds
    - 65  $^\circ\text{C}$  for 30 seconds
    - 72  $^\circ\text{C}$  for 60 seconds

### **3.17 Library quantification and evaluation of library quality**

Before libraries can be sequenced, they need to be properly quantified and be subjected to quality evaluation. This is done by first, evaluation of the insert distribution, and second, quantification.

1. Examination of library size distribution. This step can be carried out using several different instruments, such as a TapeStation or a BioAnalyzer. We prefer to use a TapeStation (with the D1000 or HS D1000 kits) due to flexibility, ease of use, and rapid turnaround time.
2. Quantification of library concentration. For most high-throughput sequencing applications, this step is standardly carried out using a Qubit fluorometer. While this works well for libraries with a unimodal fragment length distribution, ATAC libraries typically exhibit a multimodal fragment distribution and also often contain fragments of length higher than what can be sequenced on standard Illumina instruments. As a result effective library concentrations often differ from apparent library concentrations measured using Qubit, and the optimal way for estimating effective library concentration is qPCR
3. Estimation of effective library concentration using qPCR. Standard Illumina library quantification kits can be used to quantify the concentration of the library that will be able to be sequenced. Products from NEB or KAPA are appropriate for this use.

### 3.18 Sequencing

The protocol described here generates libraries designed to be sequenced on Illumina sequencers, the most widely available of which is the NextSeq. On a NextSeq, SHARE-seq libraries are to be run as follows using a 150-cycle kit:

For the RNA libraries, use a 50 bp × 10 bp × 99 bp × 8 bp configuration (Read 1 × Read 2 × Index1 × Index2, respectively).

For the ATAC libraries, use a 30 bp × 30 bp × 99 bp × 8 bp configuration (Read 1 × Read 2 × Index1 × Index2, respectively).

For RNA, the 10bp of Read 2 capture the UMI, the 50 bp capture the actual RNA sequence.

For ATAC, fragments are sequenced in a 2×30 bp format.

The 8 bp of Index 2 capture the library barcode (if more than one library is sequenced in a single run). The 99 bp of Index 1 capture the pool-split barcodes.

For other Illumina instruments, different configurations can be used. For example, using a 200-cycle kit on NovaSeq, run ATAC libraries in 55 bp × 55 bp × 99 bp × 8 bp configuration and RNA libraries in a 100 bp × 10 bp × 99 bp × 8 bp configuration.

An important consideration to take into account before sequencing is that the standard Illumina run recipes do not allow for the 99-bp index read configuration that is necessary for SHARE-seq libraries. This necessitates the creation of custom recipes in which the limits on the length of the index reads are increased accordingly. However, different methods for creating these custom recipes are necessary depending on the Illumina instrument used and the versions of the control software that the machine is equipped with; resolving this issues may on occasions require seeking help from Illumina’s customer support service.

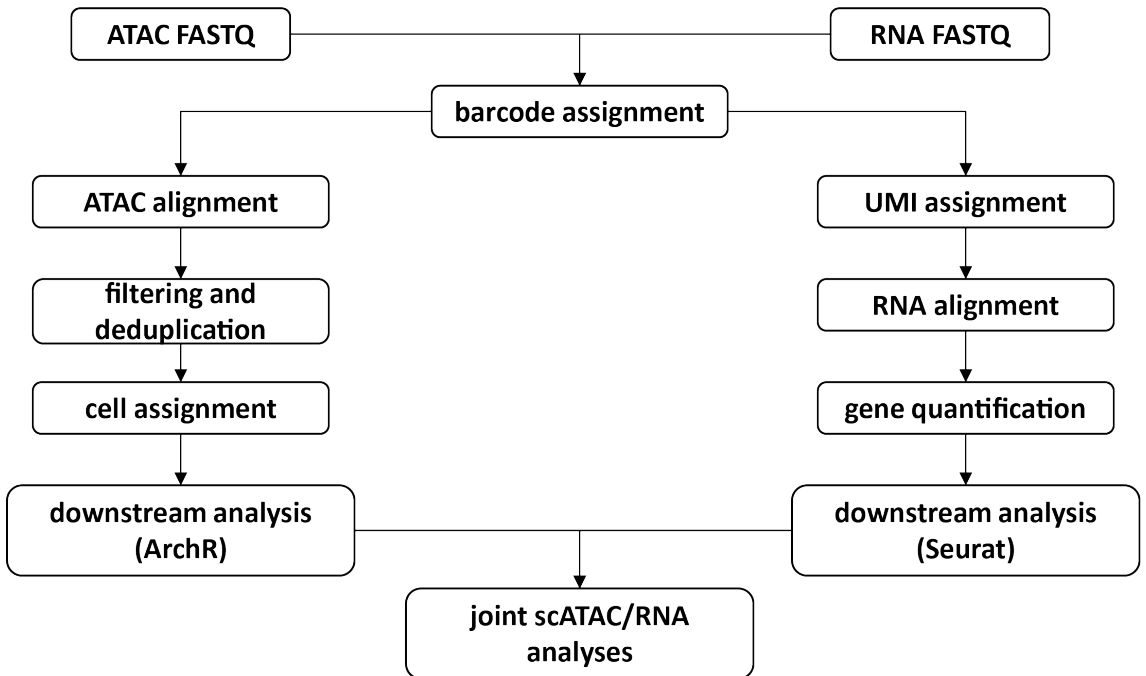
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## 4 Computational processing

At present there is no standard tool for analyzing pool-split-based multiomics datasets. The pipeline presented here is the one we have been using in our practice. It’s objective is to take the raw SHARE-seq reads and to produce object that can be used for further analysis with established tools for scRNA-seq/scATAC-seq processing such as **Seurat** and **ArchR** (e.g. sparse matrices and BAM files). The outline of the processing is show in Figure 4. For both ATAC and RNA, reads are first assigned their cellular barcodes. RNA reads are additionally annotated with the sequenced UMIs. RNA reads are aligned against the genome, a quantification is carried out for each gene in each cell, and a final sparse matrix is created. For ATAC, reads are mapped against the genome, then filtered and deduplicated within each cell, and a final BAM file with cellular barcodes appended to each alignment is created.

### 4.1 RNA

1. As a first step in the RNA processing, annotated barcodes for each read pair, using the `SHARE-seq-barcode-annotate.py` script.



**Fig. 4: Outline of the SHARE-seq computational processing procedures.** As a first step, cell barcodes are annotated for all reads in both ATAC and RNA FASTQ files. Subsequently, UMIs are consolidated and assigned to reads in the RNA set. RNA reads are then aligned against the genome, and gene expression is quantified in single cells, resulting in a final data matrix that can be analyzed in Seurat (or other scRNA-seq) tools. ATAC reads are aligned against the genome, filtered (removing mitochondria-mapping reads), and deduplicated within each barcode. Alignments are then annotated with their cell barcodes and can be used as input for further analysis in ArchR. Further joint analysis of the ATAC and RNA can be carried out downstream.

```

python SHARE-seq-barcode-annotate.py
    BC1file fieldID pos1 lenBC1 BC2file
    fieldID2 pos2 lenBC2 BC3file fieldID3
    pos3 lenBC3 [-BCedit N] [-revcompBC]
  
```

The script is flexible and can be used to assign barcodes to almost any kind of pool-split experiment in which the indexes are in Index Read 1. It takes as input files containing the barcodes for each round of pool-split and the column positions of the barcode sequences in each file (0-based), their position in Index Read 1 (0-based), their length, and their orientation (use the `[-revcompBC]` option if the sequences are reverse complement, depending on the exact format of the sequencing). Use the `[-BCedit]` option to increase/decrease the stringency of matching barcode sequences to the master list (the default value is 1). In this case the barcode files are in the following format:

```
#WellPosition Name Sequence
```

```
A1      Round1_01      AACGTGAT
B1      Round1_02      AAACATCG
C1      Round1_03      ATGCCTAA
[...]
```

And barcodes are assigned in a single step as follows:

```
python PEFastqToTabDelimited.py RNA.end1.fastq.gz
      RNA.end2.fastq.gz | python SHARE-seq-barcode-annotate.py
      Plate_R1.tsv 2 15 8 Plate_R2.tsv 2 53 8 Plate_R3.tsv
      2 91 8 -revcompBC
      | PEFastqToTabDelimited-reverse.py -
      RNA.barcodes_annotated
```

This will produce FASTQ files with headers looking as follows:

```
@[readID]:::[GTTAGCCT+TAGTCTTG+TACCGAGC] 1:N:0:
TGGGGNCACAGAGCCAAACCATATCAGCTG
+
AAAAA#EEEEEAEEEEEEEEEEEEEEEEEEEE
```

In which barcode combinations have been appended to the read headers, with `nan` if no matching barcode was found due to sequencing errors or other issues, e.g:

```
@[readID]:::[GACGGATT+GATAGAGG+nan] 1:N:0:
ACCAANCTGTGCACAAGCGTGAATCAACCT
+
6AAAA#E/EEEEEEEEEAEEEEEEEEEEEE
```

Note that it is considerably faster to split the FASTQ files into smaller pieces and process them in parallel.

## 2. Compress the output files:

```
gzip RNA.barcodes_annotated.barcodes_annotated.end1.fastq
gzip RNA.barcodes_annotated.barcodes_annotated.end1.fastq
```

## 3. Annotated UMIs using the `SHARE-seq-RNA-UMI-Add.py` script, which is also flexible and can read UMIs of different length in each read in the pair:

```
python SHARE-seq-RNA-UMI-Add.py UMIlen read1|read2
```

As follows:

```
python PEFastqToTabDelimited.py
      RNA.barcodes_annotated.end1.fastq.gz
      RNA.barcodes_annotated.end2.fastq.gz |
python SHARE-seq-RNA-UMI-Add.py 10 read2 |
python PEFastqToTabDelimited-reverse.py -
      RNA.barcodes_annotated.RNA_UMI
```

This step will append the UMI sequence to the cell barcodes in the read ID:

```
@[readID]:::[TGACCACT+GGTCGTGT+TGCTGATA+TTTATGATAG]
CCTCTNGCTCAGCCTATATACCGCCATCTTCAGCAAACCCTGATGAAGGC
+
AAAAA#EEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEEE/EEEEEEEEEEEE
```

4. Compress the output files:

```
gzip RNA.barcodes_annotated.RNA_UMI.end1.fastq
gzip RNA.barcodes_annotated.RNA_UMI.end2.fastq
```

5. Merge the individual files:

```
cat RNA_*.barcodes_annotated.RNA_UMI.end1.fastq >
  RNA.barcodes_annotated.RNA_UMI.end1.fastq.gz
cat RNA_*.barcodes_annotated.RNA_UMI.end2.fastq >
  RNA.barcodes_annotated.RNA_UMI.end2.fastq.gz
```

6. Align the Read 1 FASTQ file against the genome using STAR as follows (the commands given here use the standard ENCODE Project Consortium[69] STAR settings):

```
STAR --limitSjdbInsertNsj 10000000 --genomeDir genome/STAR
--outFileNamePrefix RNA.end1.STAR/
--readFilesIn RNA.barcodes_annotated.RNA_UMI.end1.fastq.gz
--runThreadN 20 --outSAMunmapped Within --outFilterType
BySJout --outSAMattributes NH HI AS NM MD
--outFilterMultimapNmax 50 --outSAMstrandField intronMotif
--outFilterMismatchNmax 999 --outFilterMismatchNoverReadLmax
0.04 --alignIntronMin 10 --alignIntronMax 1000000
--alignMatesGapMax 1000000 --alignSJoverhangMin 8
--alignSJBoverhangMin 1 --sjdbScore 1 --readFilesCommand
zcat --outSAMtype BAM SortedByCoordinate --outWigStrand
Stranded --twopassMode Basic --twopass1readsN -1
--limitBAMsortRAM 50000000000
```

7. Index the output BAM file:

```
samtools index
  RNA.end1.STAR/Aligned.sortedByCoord.out.bam
```

8. Calculate global mapping statistics:

```
python SAMstats.py
  RNA.end1.STAR/Aligned.sortedByCoord.out.bam
  SAMstats-RNA.end1.STAR.hg38
  -bam genome.chrom.sizes samtools
```

This script will output the number of mapped reads in various categories (uniquely mapping, spliced, etc.) as well as the molecular complexity of the alignment.

9. Calculate read distribution relative to the genome annotation:

```
python SAM_reads_in_genes3_BAM.py annotation.gtf
      RNA.end1.STAR/Aligned.sortedByCoord.out.bam
      genome.chrom.sizes
      sam_reads_genes-RNA.end1.STAR -nomulti
```

This script will output the fraction of exonic, intronic, and intergenic reads. This is important information for single-cell assays for evaluating to what extent the cytoplasm (which is enriched for exonic reads relative to the nucleus) is captured in the final libraries.

10. Make a RPM-normalized (Reads Per Million mapped reads) global coverage track:

```
python makewigglefromBAM-NH.py title
      RNA.end1.STAR/Aligned.sortedByCoord.out.bam
      genome.chrom.sizes
      RNA.end1.STAR/Aligned.sortedByCoord.out.wig -RPM
```

11. Evaluate read coverage along transcripts:

```
python gene_coverage_wig_gtf.py annotation.gtf
      RNA.end1.STAR/Aligned.sortedByCoord.out.wig
      1000 coverage-RNA -normalize -singlemodelgenes
```

This script run with these settings will output the average read profile over all genes with only a single transcript annotated (in order to avoid confounding by the presence of multiple isoforms) and  $\geq$  1,000 bp in length. Use a simple annotation with few isoforms, such as refSeq to get as many genes meeting these requirement as possible.

12. Calculate UMI counts per gene and per cell barcode combination using the `SHARE-seq_RNA_counts.py`. For faster processing, run this on each chromosome in parallel, as follows (shown is chr1)

```
python SHARE-seq_RNA_counts.py
      RNA.end1.STAR/Aligned.sortedByCoord.out.bam
      annotation.gtf.chr1 genome.chrom.sizes
      RNA.SHARE-seq_RNA_counts.chr1 -UMIedit 1
```

The `[-UMIedit]` option can be used to tweak the level of UMI collapsing (in this case UMIs within an edit distance of 1 from each other will be collapsed into a single UMI).

13. Calculate per-cell statistics by mering the individual outputs using the `SHARE-seq-RNA-BC-sum-across-files.py` script as follows:

```
python SHARE-seq-RNA-BC-sum-across-files.py
      list_of_per_chromosome_outputs
      RNA.SHARE-seq_RNA_counts.UMIs_per_cell
```

This will output a file in the following format:

#BC1+BC2+BC3	rank3	UMIs3	Aligned Positions	genes
GCCAATGT+CAGATCTG+TAACGCTG	1	64660	171969	8369
GTTGTCGG+TAAGCGTT+GATCAGCG	2	47079	123008	7864
TGACCACT+GGTCGTGT+TGCTGATA	3	45034	109960	7652

Which shows the number of UMIs and number of detected genes for each cell barcode combination.

14. Extract cell barcode combinations above a desired threshold, e.g.  $\geq 500$  UMIs into a separate file.
15. Create final sparse matrix format files that can be used as input to Seurat for further analysis with the `SHARE-seq-RNA-UMIs-sum-across-files.py` script:

```
python SHARE-seq-RNA-UMIs-sum-across-files.py
  list_of_per_chromosome_outputs
  RNA.SHARE-seq_RNA_counts.UMIs_per_cell.min500 0
  RNA.SHARE-seq_RNA_counts.UMIs_per_cell.min500.sparse
  -sparse
```

## 4.2 ATAC

The first steps of the ATAC processing are analogous to those of the RNA pipeline.

1. First, annotate cellular barcodes:

```
python PEFastqToTabDelimited.py
  ATAC.end1.fastq.gz ATAC.end2.fastq.gz |
  python SHARE-seq-barcode-annotate.py
  Plate_R1.tsv 2 15 8 Plate_R2.tsv 2 53 8 Plate_R3.tsv 2
  91 8 -revcompBC |
  PEFastqToTabDelimited-reverse.py -
  ATAC.barcodes_annotated
```

Note as before that it is considerably faster to split the FASTQ files into smaller pieces and process them in parallel.

2. Compress the output files:

```
gzip ATAC.barcodes_annotated.end1.fastq
gzip ATAC.barcodes_annotated.end2.fastq
```

3. Merge the individual files:

```
cat ATAC_*.barcodes_annotated.end1.fastq >
  ATAC.barcodes_annotated.end1.fastq.gz
cat ATAC_*.barcodes_annotated.end2.fastq >
  ATAC.barcodes_annotated.end2.fastq.gz
```

4. Align reads against the mitochondrial genome with Bowtie as follows:

```
python PEFastqToTabDelimited.py
  ATAC.barcodes_annotated.end1.fastq.gz
  ATAC.barcodes_annotated.end2.fastq.gz -trim 30 30 |
  bowtie bowtie-indexes/chrM -p 20 -v 2 -a -t --best
  --strata -q -X 1000 --sam --12 - |
  samtools view -F4 -bT genome.fa - |
  samtools sort - ATAC.2x30mers.chrM
```

This step is for the purpose of evaluating the extent of mitochondrial contamination in the overall library.

- Align reads against the full genome with Bowtie and filter out mitochondrial reads as follows:

```
python PEFastqToTabDelimited.py
  ATAC.barcodes_annotated.end1.fastq.gz
  ATAC.barcodes_annotated.end2.fastq.gz
  -trim 30 30 | bowtie bowtie-indexes/genome
  -p 20 -v 2 -k 2 -m 1 -t --best --strata -q
  -X 1000 --sam --12 - | egrep -v chrM |
  samtools view -F4 -bT genome.fa - | samtools sort -
  ATAC.2x30mers.unique.nochrM
```

Adjust accordingly if working a genome in which the mitochondrial chromosome/contigs are named differently or there are multiple contigs to be filtered out (e.g. in plants where there is also a plastid in addition to the mitochondrion).

- Index the resulting BAM files.

```
samtools index ATAC.2x30mers.unique.nochrM.bam
samtools index ATAC.2x30mers.chrM.bam
```

- Calculate mapping statistics for the two sets of alignments.

```
python SAMstats.py ATAC.2x30mers.chrM.bam
  SAMstats-ATAC.2x30mers.chrM
  -bam genome.chrom.sizes samtools
  -paired -noNHinfo
python SAMstats.py ATAC.2x30mers.unique.nochrM.bam
  SAMstats-ATAC.2x30mers.unique.nochrM
  -bam genome.chrom.sizes samtools
  -paired -uniqueBAM
```

- Calculate the mitochondrial reads fraction  $MRF$  as follows:

$$MRF = \frac{|R_M|}{|R_M| + |R_N|} \quad (1)$$

Where  $R_M$  is the total number of reads that map to the mitochondrial genome and  $R_N$  is the number of reads that map to the nuclear genome after filtering out mito-mapping reads.

- Evaluate the fragment size distribution over the nuclear genome:

```
python PEInsertDistFromBAM.py
    ATAC.2x30mers.unique.nochrM.bam
    genome.chrom.sizes
    ATAC.2x30mers.unique.nochrM.InsLen
    -uniqueBAM -normalize
```

10. Create a normalized genome coverage track:

```
python makewigglefromBAM-NH.py title
    ATAC.2x30mers.unique.nochrM.bam
    genome.chrom.sizes ATAC.2x30mers.unique.nochrM.wig
    -notitle -RPM -uniqueBAM
```

11. Create a bigWig file using the wigToBigWig program from the UCSC Genome Browser utilities suite.

```
wigToBigWig ATAC.2x30mers.unique.nochrM.wig
    genome.chrom.sizes
    ATAC.2x30mers.unique.nochrM.bigWig
```

12. Calculate the global TSS enrichment. The TSS enrichment  $TSS_E$  is the most informative ATAC-seq, and is based on generating an average read distribution profile around annotated transcription start sites for protein coding genes, then calculating the ratio between the number of reads in the immediate neighborhood of the TSS and the number of reads falling in the regions on the flanks of the TSS peak. The advantage of the  $TSS_E$  metric is that it is an internal to the dataset measure independent of peak calling. We use a TSS window of  $\pm 100$  bp and a TSS flank distances of 2,000 bp, i.e.  $TSS_E$  is calculated as follows:

$$TSS_E = \frac{|R \in [TSS \pm 100]|}{|R \in [TSS - 2050, TSS - 1950]| + |R \in [TSS + 1950, TSS + 2050]|} \quad (2)$$

First, generate the TSS metaprofile:

```
python signalAroundCoordinate-BW.py
    annotation.TSS-0bp.bed 0 1 3 4000
    ATAC.2x30mers.unique.nochrM.bigWig
    ATAC.2x30mers.unique.nochrM.TSS_profile -normalize
```

Note that you need a BED file containing the start positions and the strands of annotated TSSs in the genome, e.g.:

```
#chr TSS    TSS strand geneName
chr1 1000    1000  +      GENE1
```

Second, calculate the TSS score:

```
python ATACTSSscore.py
    ATAC.2x30mers.unique.nochrM.TSS_profile
    100 2000 >> ATACTSSscore.txt
```

13. Deduplicate the BAM file. Note that this step is different from the typical deduplication carried out in most high-throughput sequencing pipelines, based on tools such as `MarkDups` in `picard`. Here, we perform deduplication of fragments only within the same cell barcode, i.e. for two fragments to be collapsed, they need to have the same coordinates, orientation, and cell barcode.

```
python SHARE-seq_ATAC_dedup.py
    ATAC.2x30mers.unique.nochrM.bam
    genome.chrom.sizes
    ATAC.2x30mers.unique.nochrM.BC_dedup.bam
    -addBC
```

Use the `[-addBC]` to append the cell barcodes to each alignment as a BC tag, making these final files ready to use with `ArchR`.

14. Index the deduplicated BAM file:

```
samtools index ATAC.2x30mers.unique.nochrM.BC_dedup.bam
```

15. Calculate alignment stats for the deduplicated BAM file:

```
python SAMstats.py ATAC.2x30mers.unique.nochrM.BC_dedup.bam
    SAMstats-ATAC.2x30mers.unique.nochrM.BC_dedup
    -bam genome.chrom.sizes samtools -paired -uniqueBAM
```

16. Calculate fragment count and TSS enrichment statistics for each cell barcode.

```
python SHARE-seq_ATAC_stats_per_cell.py
    ATAC.2x30mers.unique.nochrM.BC_dedup.bam
    genome.chrom.sizes annotation.TSS-0bp.bed 0 1 2000 200
    ATAC.2x30mers.unique.nochrM.BC_dedup.per_cell_stats
```

This script will output a file containing information about the number of fragments and TSS enrichment for each barcode that can be used to filter barcodes for downstream analysis.

More sophisticated filtering, in addition to these simple metrics, i.e. of doublet cells, can be performed in `ArchR` [67].

---

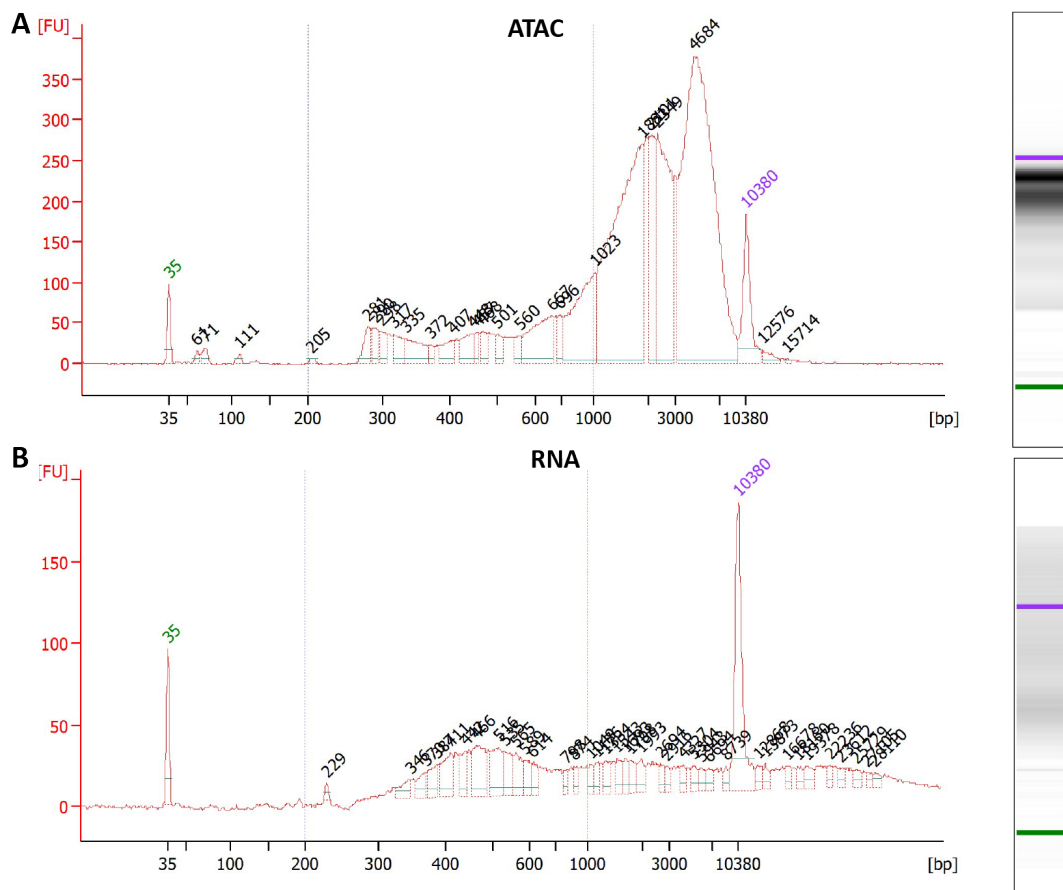
## 5 Expected results

### 5.1 Sequencing libraries

Figure 5 shows the typical fragment profiles for ATAC and RNA SHARE-seq libraries. ATAC libraries are expected to show a nucleosomal signature, with a prominent subnucleosomal, mononucleosomal and perhaps dinucleosomal peaks, shifted to the right by the length of the adapters and barcodes added to the original fragments. In contrast, RNA libraries are primarily unimodal in length.

### 5.2 Species mixing experiments

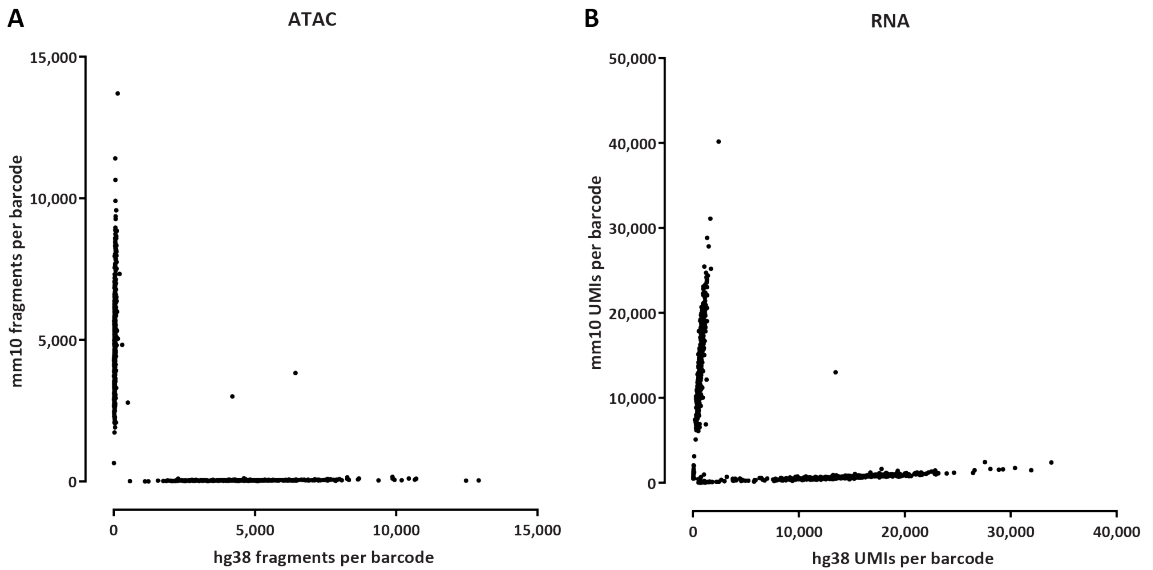
A customary experiment to be carried out when testing, adopting or developing any new single-cell protocol is the species mixing experiment, in which cells from two different species, usually mouse and human, are



**Fig. 5: Typical fragment-length profiles of SHARE-seq libraries.** (A) BioAnalyzer profile of a SHARE-seq ATAC library; (B) BioAnalyzer profile of a SHARE-seq RNA library.

mixed together and the extent of crosstalk/contamination of individual barcodes or of doublet formation (in which two cells are processed together with the same barcode) is assessed based on how many reads in each barcode map to each species. Ideally, all barcodes should feature reads coming from only one of the two species. Doublets arise from loading of multiple cells in the same droplets/wells (depending on the method used) or from physical clumping of cells early in the protocol that then are processed together throughout the rest of the procedure.

Figure 6 shows typical species mixing results for a SHARE-seq experiment. We note that in our hands ATAC experiments usually show virtually no crosstalk between barcodes and very few doublets. On the other hand, pool-split RNA experiments in general often exhibit a small fraction of reads resulting from “leakage”, likely because of some cells opening up during cell handling and releasing their content into the general reaction pool. This issue does not significantly affect most analyses, but it should be kept in mind in the cases in which it could be a



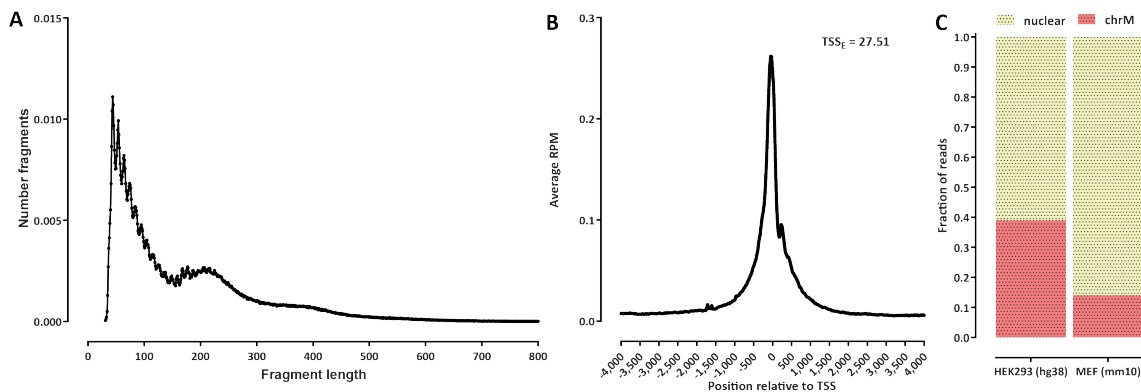
**Fig. 6:** Typical results from a species mixing SHARE-seq experiment. Human HEK293 and mouse embryonic fibroblast (MEF) cells were mixed in equal proportions and carried through the SHARE-seq workflow. (A) ATAC fragments per cell. (B) RNA UMIs per cell

confounding factor.

### 5.3 ATAC *post-sequencing quality evaluation*

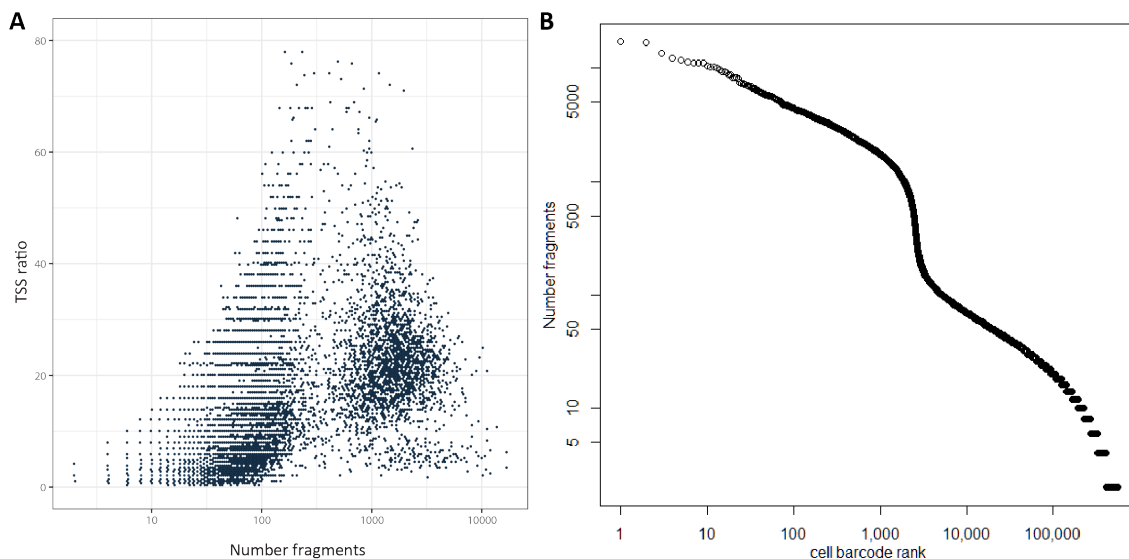
Figure 7 shows the key ATAC-seq bulk-level metrics. The fragment length distribution (Figure 7A) usually shows strong subnucleosomal and nucleosomal peaks as well as a weaker dinucleosomal one. High TSS enrichment is desirable; in this case (Figure 7B) it is very high ( $TSS_E \geq 25$ ). See Note 8 for more details. Figure 7C shows the fraction of mitochondrial reads in the human and mouse cells in the species mixing experiment. Note that the fraction can vary greatly depending on the properties of the cell type (cancer cell lines and highly metabolically active cells tend to have more mitochondria [70]) and not just on the experimental variation (which in this case is completely minimized as the cells were processed together).

Figure 8 shows the key scATAC metrics. One such metric is the relationship between the number of fragments per cell barcode and the TSS enrichment within each cell barcode (Figure 8A). Another is the curve of the number of fragments per cell barcode plotted against the rank (by the number of fragments per cell barcode) of the cell barcodes (Figure 8B). Ideally, there should be a clear inflection point between the cell barcodes with high fragment counts and the cell barcodes with low fragment counts, indicating that a set of high-quality cells has been captured and preserved intact through the full pool-split procedure. A flatter, diagonal-like shape of that curve can be indicative of loss of cell integrity during handling and is potentially concerning regarding the biological interpretability of the experiment if the lack of inflection is



**Fig. 7: Basic evaluation of bulk-level ATAC quality and enrichment.** (A) Fragment length distribution. (B) TSS enrichment. Shown are the same experiments as those featured in Figure 6. (C) Mitochondrial read fraction for each species in this experiment.

too extreme.



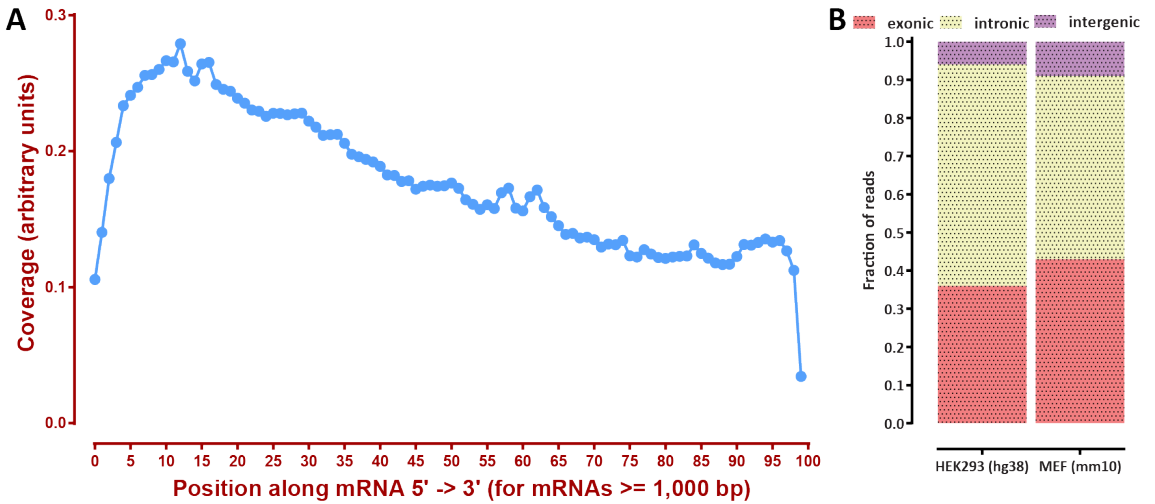
**Fig. 8: Basic evaluation of scATAC-seq-level quality and enrichment.** (A) Number fragments per cell barcode vs. TSS enrichment; (B) Cell barcode rank (by fragment counts) vs. fragment counts per cell barcode

### 5.4 RNA post-sequencing quality evaluation

Figure 9 shows the typical parameters to be evaluated for a bulk-level RNA-seq dataset. One is the distribution of reads along transcripts (Figure 9A). SHARE-seq is not a 3'-tagging experiment the way some scRNA-seq approaches are as it attaches UMIs to the 3' end of tran-

scripts but cDNAs are tagged at random after cDNA amplification, thus the first reads of the RNA part of a SHARE-seq dataset can be some distance away from the 3' end.

Another is the distribution of reads relative to the annotation (Figure 9B). As is often observed in scRNA-seq datasets, SHARE-seq RNA libraries contain a significant portion of reads originating from introns, presumably from unspliced transcripts present in the nucleus. This is likely due to the fact that the ATAC reaction has to happen first in the workflow, thus a substantial portion of the cytoplasm is lost and the final libraries are enriched for nuclear material.

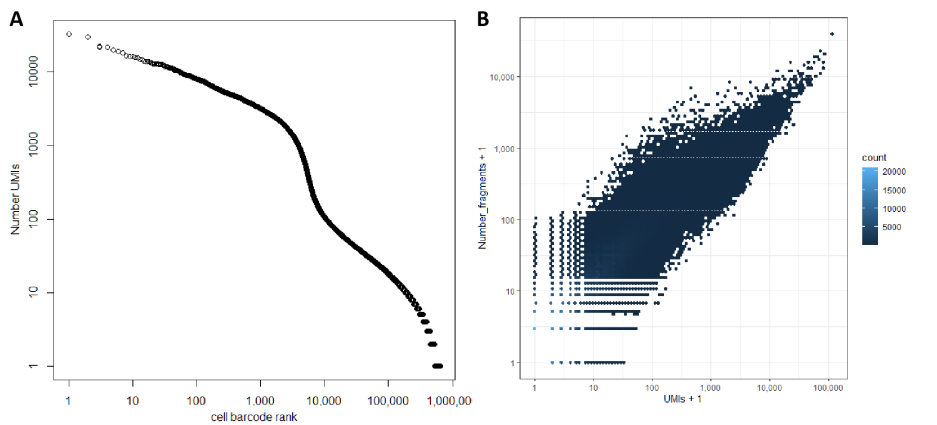


**Fig. 9: Basic evaluation of the bulk-level RNA-seq properties.** (A) Read distribution along transcript lengths; (B) Read distribution relative to the exonic, intronic and intergenic genomic spaces.

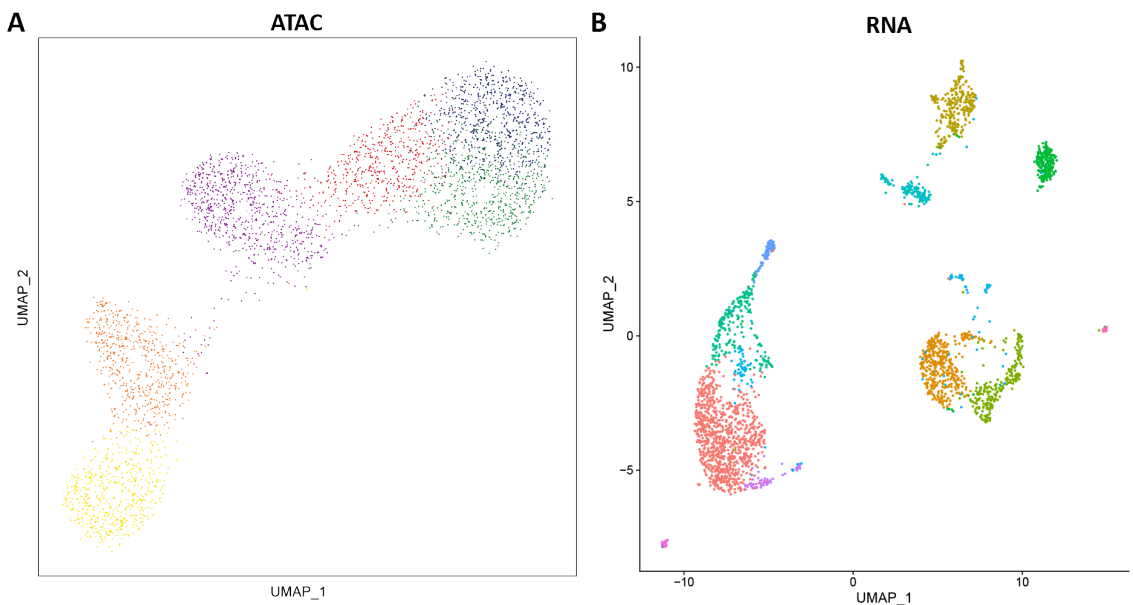
Figure 10 shows the key metric for evaluating the success of the RNA portion of a SHARE-seq experiment. As with ATAC above, the curve of the number of UMIs per cell barcode plotted against the rank (by the number of UMIs per cell barcode) of the cell barcodes should ideally feature a clear inflection point between the cell barcodes with high UMI counts and the cell barcodes with low UMI counts (Figure 10A). There should also be a concordance between the cell barcodes with high ATAC fragment counts and those with high UMI counts, i.e. the same cells are of high quality in both modalities, and are thus usable for joint analysis (Figure 10B)

### 5.5 Dimensional-reduction and cell type/cluster identification

Following initial data processing, clusters and cell types can be identified using standard tools for that purpose such as Seurat [68] and/or ArchR [67]. Figure 11 shows typical such output in UMAP space for both the ATAC and RNA sides of a SHARE-seq experiment from a human embryonic lung tissue sample.



**Fig. 10: Basic evaluation of SHARE-seq RNA single cell-level quality and enrichment.** (A) Cell barcode rank (by UMI counts) vs. UMI counts per cell barcode (B) UMI counts per barcode vs. ATAC fragment counts per barcode.



**Fig. 11: Example SHARE-seq output on human embryonic lung samples.** (A) ArchR iterative LSI UMAP on the ATAC-seq dataset; (B) Seurat UMAP on the RNA dataset. Individual ArchR- and Seurat-defined clusters are colored separately

## 6 Notes

1. The details of the production of hyperactive transposition are beyond the scope of this chapter. However, detailed instructions for how to carry it out can be found in Picelli et al. 2014 [71].
2. In this chapter we presented one of many available protocols for tis-

sue dissociation and nuclei isolation that has worked in our hands in some contexts. However, the variety of tissues and their properties that can be encountered in different organisms is vast, making it practically impossible to have one common such protocol for all situations. Thus novel optimal procedures for tissue dissociation often have to be empirically devised or adapted.

3. The protocol we described here used light 0.1% FA crosslinking. This does not mean that optimal results will be obtained in all contexts with the same conditions, and crosslinking may have to be optimized depending on the specifics of the experimental system being studied.
4. The protocol described here is for a  $96 \times 96 \times 96$  indexing. However, it can be expanded to more cycles and/or more barcodes, e.g. to a 3-round  $384 \times 384 \times 384$  indexing, or 4-round or 5-round  $96/384 \times 96/384 \times 96/384$ . Pick the optimal design based on the availability of robotic liquids handlers (it is generally not practical to carry out pipetting of 384-well plates by hand), the desired throughput, and other considerations. Note that additional barcodes and linker would have to be designed so that they are compatible with each other and with further rounds of barcoding. Aim for as much distance in sequence space between the 8-bp barcodes (or increase their length, if the sequencing format allows for it). The set of 8-bp barcodes can be identical throughout all rounds of indexing.
5. Low-binding tubes are preferable for all reactions in order to ensure maximum yields.
6. It is optimal in terms of effort to anneal a sufficient amount of oligos for multiple experiment on many separate plates. These can then be used immediately when cells/tissues become available, saving a considerable amount of experimental time.
7. The TB buffer described here is modified from the original omni-ATAC protocol with the addition of acetate. In our experience, this provides superior results compared to the traditional buffer formulation.
8. In our (and not only ours) experience, experiments in cell lines always produce much higher quality ATAC datasets than those obtained from tissues, especially frozen tissues. This is not limited to SHARE-seq but is what has been observed by numerous previous studies mapping chromatin accessibility in tissues samples in contexts such as cancer, development, adult tissues [27, 28, 72, 73]. This is likely due to the extensive handling and freezing and thawing of tissues leading to the breaking up of nuclei and the release of unprotected free DNA that is tagmented by Tn5, increasing the background fragments and decreasing the signal-to-noise. Whether future protocol optimizations can resolve these issues or they are fundamentally insurmountable is not known at present.

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