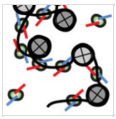

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snATAC-Seq on 10x Chromium™ platform for fresh, frozen and cryopreserved material: my notes from the lab (UPDATED VERSION)

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bda8i2hw

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ABSTRACT

-Here, I will **only** include tips and notes related to snATAC workflow. Thus, I assume you are familiar with Frankenstein protocol below:

'Frankenstein' protocol for nuclei isolation from fresh and frozen tissue for snRNAseq
by Luciano Martelotto,
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PREVIEW RUN

- These are ONLY a collection of my lab notes. This is not a protocol.

-These notes are derived from trial and error in order to get successful runs applied to fresh, snap/flash and cryopreserved frozen cell lines as well as to tissue derived from solid tumours and other tissues using Chromium Platform (10x Genomics).

-Be mindful these are just my notes and by no means are ground truth of how snATAC-Seq needs to be performed. I WELCOME to comments, suggestions and amendments.

GUIDELINES

Protocol Overview

You will be require acces to a cell sorter and familiarity with sorting cells/nuclei into 96-well plates.

SUCCESS of this tips and tricks heavily rely on **SAMPLE QUALITY**. Below are some of the steps I follow:

-For new sample types, and when possible (with minute samples is hard to do it) I evaluate the lysis efficacy by assessing under light or fluorescent microscope after **00:03:00** - **00:05:00** for single cell suspensions (cell lines) and **00:05:00** - **00:10:00** for tissues. This will ensure you avoid over- or under-lysis the cells.

-The cell lysis and washes are always carried out on wet-ice (i.e. **4 °C**) and in the presence of RNAse inhibitor 0.2-0.5 U/uL.

-Centrifugation, in my hands, works well at **500 x g, 4°C 00:05:00** and this is enough for most of the tissues types I worked on. However, some optimization might be needed for specific tissues.

-I always use LoBind nucleases free tubes.

-I always inspect nuclei under microscope using Trypan Blue to give more contrast and also to count.

-When debris and clump are an issue and I solved this issue I use sorting as explained in the protocols.

-Nuclei sizes and shapes under microscope varies from sample to sample so shape or roundness is not the only feature to check. Also look for signs of disorganisation of chromatin, this is usually quite visible as if burst nucleus.

-In good quality nuclei nucleoli may be visible.

-I use Flowmi 40 µm filters before FACS sorting or before loading onto chip.

-For additional tips on sample prep please check https://support.10xgenomics.com/single-cell-gene-expression/sample-prep.

1. Use a plastic pestle to mechanically homogenize tissue and release nuclei
2. Separate the nuclei from debris using a cell sorter (if not, then see note at the end)
3. Collect a specific number of nuclei in a 96-well plate containing 10x RT Buffer* or Wash Buffer**
4. Immediately load the sample into a Single Cell Chip for processing according the Single Cell 3' v3 Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide.
5. In our hands the use of DAPI, 7-AAD and DRAQ-7 dyes show very little or no effect on ATAC data metrics.

*Consider the event overestimation of some sorters (Check step 19). Also, assume that nuclei recovery is ~ 57 %; use this to determine the number of nuclei to collect for each of your samples. [This value is derived from the Cell Suspension Volume Calculator Table in the Single Cell 3' and v3 - Reagents User Guide or Single Cell V(D)J 5' v1 Reagents User Guide]
**Assume that nuclei loss can be up to ~ 50 — 60 % due to loss during washing and counting; use this to determine the number of nuclei to collect for each of your samples.

IMPORTANT NOTE:

If you are NOT sorting nuclei that is in Nuclei Wash and Resuspension Buffer directly into ATAC Wash Buffer-Dig, avoid resuspending pelleted nuclei that was in Nuclei Wash and Resuspension Buffer (PBS-based) directly in Diluted Nuclei Buffer or ATAC Wash Buffer-Dig as nuclei tend to clump.

Instead, you need to do a *buffer exchange* (from PBS to Tris) by adding at least 2x the volume of ATAC Wash Buffer-Dig [Tris-HCl 10 mM (pH 7.4), NaCl 10 mM, $MgCl_2$ 3 mM, BSA 1%, Tween-20 0.1 %, Digitonin 0.01%] to the nuclei that is **in suspension** in the Nuclei Wash and Resuspension Buffer (PBS-based).

Let equilibrate for **00:05:00** (buffer exchange). After this, pellet and do all washes in ATAC Wash Buffer-DIg before resuspending nuclei in Diluted Nuclei Buffer.

Below are examples of nuclei before and after sorting, bioanalyzer traces of 5' nuclei cDNA and snATAC library as well as representative metrics obtained with Cell Ranger for this example.

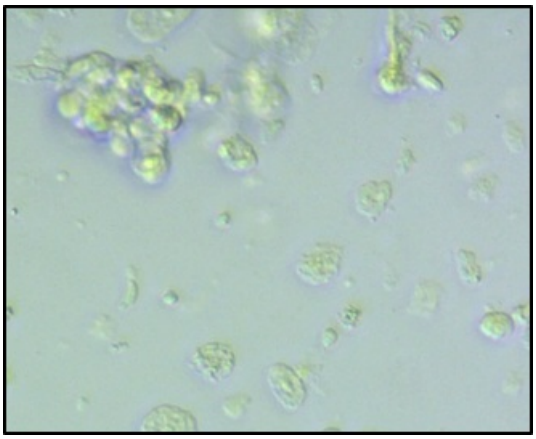

Fig. 1: Nuclei before sorting

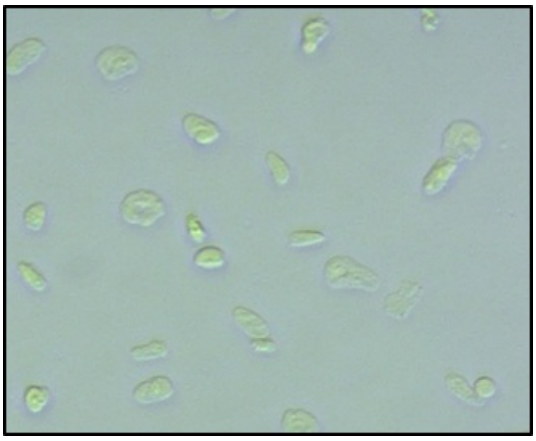

Fig. 2: Nuclei after sorting

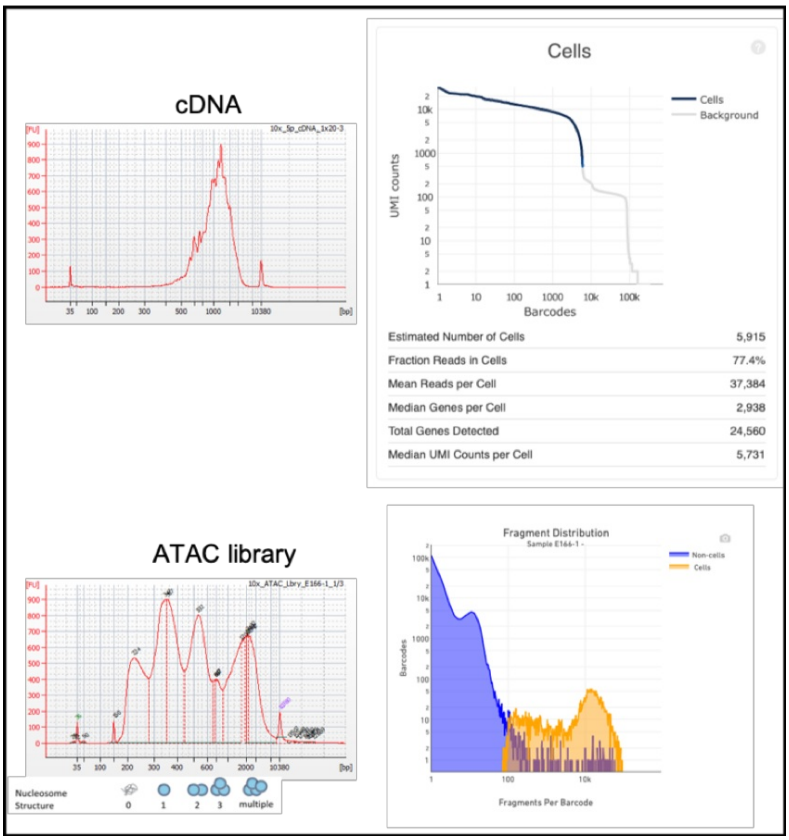

Fig. 3: Bioanalyzer traces, metrics obtained with Cell Ranger

MATERIALS TEXT

Required Buffers and Reagents

1. *Nuclei EZ Lysis Buffer* (Millipore Sigma) (chilled, **4 °C**). This is the same buffer used in Frankentein protocol, so nuclei prep and washes are done in the same way.

2. *Nuclei Wash and Resuspension Buffer* (prepare chilled, **4 °C**)

   1x PBS (No Ca^{2+}/Mg^{2+})

   1.0 % volume BSA

   0.2 U/µl – **0.5 U/µl** RNase Inhibitor

3. *Nuclei Wash and Resuspension Buffer with DNA binding dye* (prepare chilled, **4 °C**)

   1x PBS (No Ca^{2+}/Mg^{2+})

   1.0 % volume BSA

   0.2 U/µl – **0.5 U/µl** RNase Inhibitor

   10 µg/ml DAPI or **1 µg/ml** 7-AAD or **3 Micromolar (µM)** DRAQ-7 (see notes)

4. *Diluted Nuclei Buffer* (chilled, **4 °C**)

   20x Nuclei Buffer (ATAC kit, part 2000153): **50 µl**

   H_2O: **950 µl**

5. *ATAC Wash Buffer-Dig* (chilled, **4 °C**)

   Tris-HCl (pH **7.4**): **10 Milimolar (mM)**

NaCl: [M]**10 Milimolar (mM)**

$MgCl_2$: [M]**3 Milimolar (mM)**

BSA: [M]**1.0 %**

Tween-20: [M]**0.1 %**

Digitonin: [M]**0.01 %**

*** Buffer Notes**

- Follow Frankenstein's notes for the snRNA part.
- It is crucial to **work as fast as possible**. Do not leave nuclei sitting on ice for too long (e.g. 30' is too long).
- **Reduce as much as possible the time from sorting-to-controller run**, ideally keep it under 40'. The longer the time the higher the background will be.
- The sorting and/or resuspension of nuclei in Diluted Nuclei Buffer is critical for optimal snATAC-Seq assay performance.
- Use DNA LoBind tubes for all steps (when possible)!
- Since this protocol does not use nor need Digitonin during the initial cell lysis, it is important to **include Tween-20 and Digitonin 0.01% in the ATAC Wash Buffer-Dig** as it helps in nucleus envelope permeabilisation. Note, Digitonin is optional but recommended.

Additional equipment required for snRNA- and snATAC-Seq paths

- Protector RNAse Inhibitor (Cat. Number: RNAINH-RO, Merck/Roche)
- Nuclei EZ Lysis Buffer (sold as *Nuclei Isolation Kit: Nuclei EZ Prep* by Merck/Sigma, Cat. Number: NUC101)
- 1.5 ml DNA LoBind Eppendorf tubes
- 0.2 µl PCR-tubes/strips (LoBind)
- 15 ml Falcon tubes
- 70 µm-strainer mesh to fit a 15 mL Falcon tube (e.g. pluriStrainer Mini 70 um, Cell Strainer or Flowmi® Cell Strainer)
- 40-µm cell strainer (e.g. Falcon® RoundBottom Tubes with Cell Strainer or Flowmi® Cell Strainer)
- Round-bottom 96-well plate
- Light or fluorescent microscope
- FACS instrument (i.e. BD FACSAria™ Fusion, SONY SH800S), ideally with 70 µm nozzle.
- Thermocycler
- TapeStation or Bioanalyzer plus consumables
- Cell counter/hematocytometer
- Douncer/pestle
- Refrigerated centrifuge
- Razor blades

SAFETY WARNINGS

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

BEFORE STARTING

Prepare required Buffers and Reagents.

All samples and reagents are kept **On ice** or at **4 °C** .

snATAC-Seq

1

Ideally, **perform the following steps WHILE the RT reaction is RUNNING.** See Frankenstein protocol for nuclei prep. The steps below assumes the nuclei input for snATAC is relatively low.

Sort as many nuclei as possible into a round-bottom 96-well plate well containing **100 µl** of ice-cold ATAC Wash Buffer-Dig.

DO NOT, I repeat DO NOT sort into Diluted Nuclei Buffer.

Avoid excessive number of steps to reduce nuclei loss. Work as quicky as you can. Always leave some volume (10-15 uL) of supernatant behind when washing.

Note there will be significant nuclei loss during washes and nuclei counting, so you may want to make sure the washing steps are done carefully. Take into account this loss when deciding aimed nuclei. To reduce loss, follow the tips below (**bold**).

2 Transfer the entire volume to 0.2 ml PCR tube (LoBind!).

3 Add **50 µl** of ATAC Wash Buffer-Dig to the well.

4 Transfer any remanent nuclei to the 0.2 ml PCR tube (~ **150 µl**).

5 Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

6 Remove supernatant **leaving behind ~ 10 µl** .

7 Gently add **100 µl** ice-cold Diluted Nuclei Buffer.

DO NOT resuspend nuclei. This is just a buffer exchange step.

8 Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

9 Remove **100 µl** of the supernatant **in two steps**, namely, remove **90 µl** first and then **10 µl** (pellet may not be visible!).

10 Gently add **100 µl** ice-cold Diluted Nuclei Buffer.

DO NOT resuspend nuclei. This is just a buffer exchange step.

11 Centrifuge the nuclei at **500 x g** for **00:05:00** at **4 °C** .

12 Remove the supernatant (~ **100 µl**) **in two steps**, namely, remove **90 µl** first and then as much volume as possible to leave **leaving behind ~ 7 µl — 10 µl (avoid disturbing pellet)**.

If you manage to remove the whole volume without disturbing the pellet, then add **7 µl** of Diluted Nuclei Buffer and proceed to step 13.

13 Resuspend nuclei in the ~ **7 µl — 10 µl** of ice-cold Diluted Nuclei Buffer, **carefully washing walls of the tube** to ensure all nuclei are in solution.

14 Take **1 µl — 2 µl** and dilute 1:5 with Diluted Nuclei Buffer.

15 Mix 1:1 with Trypan Blue and count the number of nuclei with a cell counter (Countess II FL Automated Cell Counter) or hematocytometer (the counting is to have an idea of how many nuclei to expect based on the recovery factor).

Note that Trypan Blue (TB) tend to precipitate, so centrifuge vial of TP at top speed and use supernatant for staining.

16 Inspect under the microscope. Nuclei should look clean and intact. See attached photo:

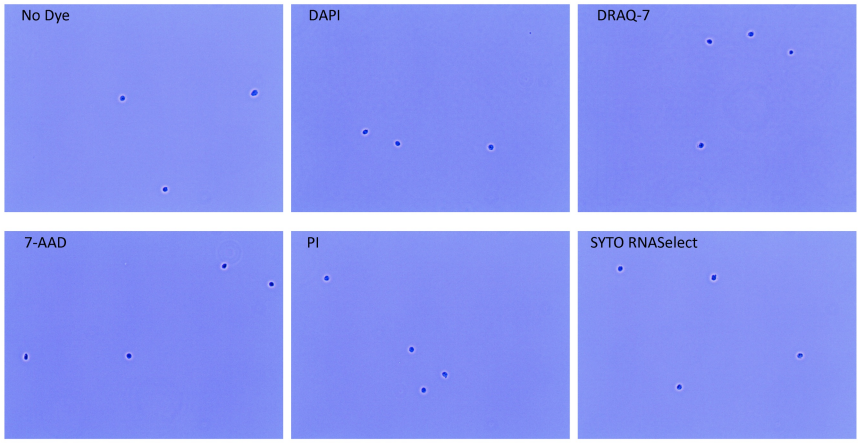

Fig. 4: Representative photographs of nuclei post sorting and clean up, right before entering 10x ATAC workflow.

17 Take **5 µl** of nuclei in Diluted Nuclei Buffer and proceed directly to Chromium Single Cell ATAC Reagent Kits protocol (CG000168 Rev A). The volume added to the Transposition reaction will vary; **for low input samples we usually use all 5 µl of nuclei prep.**

Alternatively, follow recommendations of the User Guide to estimate volume of nuclei to add to recover a determined targeted nuclei recovery (Page 20, CG000168 Rev A).

To *estimate* the Number of Recovered Nuclei, do the following calculation:
[Nuclei Concentration (from step 35) x Volume of Nuclei (up to 5 µl)] / 1.53 (recovery efficiency factor)