Protocol for nuclei isolation from fresh and frozen tissues for snRNA-Seq and snATAC-Seq on Chromium™ platform using the same nuclei preparation.

Contributed by:

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The *'Van Helsing'* protocol is an adaptation and extension of the 'Frankenstein' protocol – originally developed for nuclei isolation from fresh and frozen tissue for snRNA-Seq – in order to perform snATAC-Seq on the **same** nuclei prep.

This protocol currently used in the Single Cell Innovation Lab (SCIL) for single nuclei experiments using 10x Genomics technologies. It has been successfully applied to fresh, snap/flash and cryopreserved frozen cell lines as well as to tissue derived from solid tumours such as pancreas and breast cancer, pheochromocytomas and paragangliomas using Chromium Platform (10x Genomics).

Protocol Overview

NOTE: This protocol requires access to a cell sorter and familiarity with sorting cells/nuclei into 96-well plates.

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 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' v1 Reagents User Guide.

*Consider the event overestimation of some sorters (see below). 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' 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.

Required Buffers and Reagents

1. Nuclei EZ Lysis Buffer (Millipore Sigma) (chilled, 4°C)
2. Nuclei Wash and Resuspension Buffer (prepare chilled, 4°C)
   1x PBS
   1.0% BSA
   0.2-0.5 U/µl RNase Inhibitor
3. Nuclei wash and resuspension buffer with DAPI (prepare chilled, 4°C)
   1x PBS
   1.0% BSA
   0.2-0.5 U/µl RNase Inhibitor
   10 ug/mL DAPI
4. RT Buffer for Single Cell Gene Expression 3' v3 reagents (DO NOT add RT Enzyme C)
   RT Reagent: 20 uL

Template Switch Oligo: 3.1 uL
Reducing Agent B: 2.0 uL
H_2O: (33.4 – X – Y) uL

5. RT Buffer[1] for Single Cell Immune Profiling 5' reagents (DO NOT add RT Enzyme Mix B)
   RT Reagent Mix: 50 uL
   RT primer: 5.9 uL
   Additive A: 2.4 uL
   H_2O: (31.7 – X – Y) uL
6. Diluted Nuclei Buffer (chilled, 4°C)
   10x Nuclei Buffer: 50 uL
   H_2O: 950 uL
7. ATAC Wash Buffer-Dig (chilled, 4°C)
   Tris-HCl (pH 7.4): 10 mM
   NaCl: 10 mM
   $MgCl_2$: 3 mM
   BSA: 1%
   Tween-20: 0.1% **(CRITICAL)**
   Digitonin: 0.01% **(CRITICAL)**

RT Buffer Notes

- X ('sorting volume'): In the cytometric analysis setup described in this protocol, each droplet is 1 nL. Example: 10,000 nuclei = 10,000 nL = 10 uL 'sorting volume'.
- Y ('additional volume'): This accounts for any additional volume deposited by the flow cytometer nozzle. In the cytometric analysis setup described in this protocol (i.e. 75 um nozzle) there is no additional volume deposited by the nozzle, so Y = 0. If in doubt, or to be on the safe side, just make Y= 5-10 uL.
- The 1 nuclei/nL assumption was corroborated empirically by sorting 10,000 nuclei in ten wells containing 70 uL PBS and then measuring the final volume post sorting. It is highly recommended to determine X empirically as value may vary depending on different sorters/nozzle combinations. It is recommended to determine it at least once.
- **Always measure the volume after sorting** and top up to 90 uL with PBS or H_2O if required.
- After adding the RT Enzyme Mix the final volume should be ~100 uL.
- 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 it is important to include Tween-20 and Digitonin in the ATAC Wash Buffer-Dig as it helps in nucleus envelope permeabilization.

Step-by-step Protocol

Nuclei prep and snRNA-Seq

NOTE: All samples and reagents are kept on ice or at 4 °C (wet ice).
1. Mince/chop tissue with a razor blade to small pieces. The tissue may be as small as 2-3 grains of (cooked) rice to accommodate the sorting of sufficient nuclei for both snRNA-Seq and snATAC-Seq (larger fragments are better). Note that this largely depends on tissue cellularity.
2. Add 300-500 uL chilled Nuclei EZ Lysis Buffer (+RNAse inhibitor) to the tissue in 1.5 mL DNA LoBind tube. Homogenise the sample using a douncer/pestle (gently stroking ~10-15 times; for small pieces use 300 uL). For mincing the tissue, you may take the DNA LoBind tube out of the ice, however, be quick and return to ice. Add more lysis buffer to 1 mL, mix gently (bore tips preferred) and incubate on ice for at least 5'. Note: lysis time of 5' has been enough for most tissues we tested, but you may need some optimisation.
3. Filter homogenate using a 70-um strainer mesh to fit a 15 mL Falcon tube (e.g. pluriStrainer Mini 70-um, Cell Strainer. My preferred one is 70-um Flowmi® Cell Strainer, in which case you would collect directly in 1.5 mL DNA LoBind tube). Collect flow through in a 15 Falcon tube and transfer volume back into a new 1.5 mL DNA LoBind tube.
4. Centrifuge the nuclei at 500xg for 5' at 4°C and remove supernatant leaving behind ~50 uL, add 1 mL Nuclei Wash and Resuspension Buffer and incubate 5' without resuspending the pellet.
5. Centrifuge the nuclei at 500xg for 5' at 4°C, remove supernatant leaving behind ~50 uL and gently resuspend nuclei in 500 uL Nuclei Wash and Resuspension Buffer.
6. Repeat step 5 but resuspend in 400 uL Nuclei Wash and Resuspension Buffer supplemented with DAPI (resuspension volume can be less if you have low nuclei number). Collect all nuclei by washing off nuclei from the wall of centrifuge DNA LoBind tube. IMPORTANT: Protect from light from here forward.
7. Filter nuclei (at least once) with a 40-um cell strainer (e.g. Falcon® Round-Bottom Tubes with Cell Strainer or 40-um Flowmi® Cell Strainer) before sorting. Visually inspect nuclei integrity under a microscope and (optionally) count the number of nuclei with a cell counter (Countess II FL Automated Cell Counter) or hematocytometer. Prior to sorting, you may want to dilute sample to have ~150-200 events/second to get better defined peaks in cytometric analysis.
8. Perform cytometric analysis. Identify single nuclei and sub-populations based on DNA content, gate and sort directly into a round-bottom 96-well plate well containing the respective RT Buffer prepared without the RT Enzyme. IMPORTANT: for snRNA-Seq, we have seen that FACS sorters tend to overestimate the number of nuclei sorted in about >/= 40% depending on instrument, so we usually sort 35-40% more nuclei than aimed (e.g. for ~5000 nuclei recovery, you would need ~8,700 but we sort ~12,000 nuclei).
9. Proceed immediately with the 10x Genomics Single Cell 3' v3 or 5' protocol (Standard or NextGEM), minimising the time between nuclei preparation/sorting and chip loading. Add the corresponding volume of RT Enzyme (depending on the kit, 10 ul for 5' v1, and 8.3 uL for 3' v3) to the sorted nuclei in RT buffer, mix well but gently and load chip as per the Single Cell 3' v3 Reagents User Guide or Single Cell V(D)J 5' v1 Reagents User Guide.

snATAC-Seq

Ideally, perform the following steps while the RT reaction is running. The protocol below assumes the nuclei input is low.

10. Sort as many nuclei as possible into a round-bottom 96-well plate well containing 100 uL of ice-cold Wash Buffer (DO NOT, I repeat DO NOT sort into Diluted Nuclei Buffer). We have successfully sorted as little as ~7000 nuclei and recovered ~3500+ profiled nuclei (~50% recovery as expected, 1.53

recovery efficiency factor). 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 and underlined**).
11. Transfer to 0.2 mL PCR tube (**LoBind!**), add 50 uL of ATAC Wash Buffer-Dig to the well and transfer any remanent nuclei to the 0.2 uL PCR tube (~150 uL).
12. Centrifuge the nuclei at 500xg for 5' at 4°C, remove supernatant **leaving behind ~10 uL** and gently add 100 uL ice-cold Diluted Nuclei Buffer. **DO NOT resuspend.**
13. Centrifuge the nuclei at 500xg for 5' at 4°C, remove 100 uL of the supernatant **in two steps**, namely, remove 90 uL first and then 10 uL (pellet may not be visible!) and gently add 100 uL ice-cold Diluted Nuclei Buffer. **DO NOT resuspend.**
14. Centrifuge the nuclei at 500xg for 5' at 4°C, remove the supernatant (~100 uL) **in two steps**, namely, remove 90 uL first and then as much volume as possible to leave **leaving behind ~7-10 uL (avoid disturbing pellet)**. If you manage to remove the whole volume without disturbing the pellet, then add 7 uL of Diluted Nuclei Buffer and proceed to step 15.
15. Resuspend nuclei in the ~7-10 uL of ice-cold Diluted Nuclei Buffer, **carefully washing walls of the tube** to ensure all nuclei are in solution.
16. Take 1-2 uL and dilute 1:5 with Diluted Nuclei Buffer, 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). Inspect under the microscope.
17. Take 5 uL 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 uL of nuclei prep**. Alternative, 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).

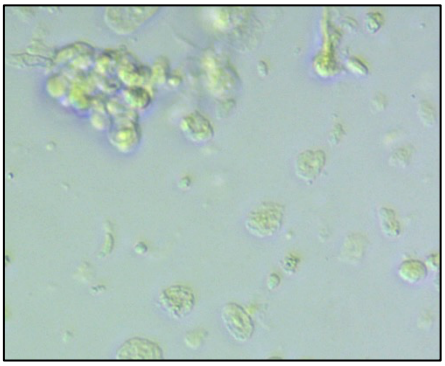
To **estimate** the Number of Recovered Nuclei, do the following calculation:

[Nuclei Concentration (from 15.) x Volume of Nuclei (up to 5 uL)] / 1.53 (recovery efficiency factor)

IMPORTANT NOTE: If you are NOT sorting nuclei that is in Nuclei Wash and Resuspension Buffer directly into Wash Buffer (e.g. you are doing single **cell** RNAseq and then also single nuclei ATAC-seq, instead of single nuclei RNA seq and single nuclei ATAC-seq), avoid trying to resuspend pelleted nuclei that was in Nuclei Wash and Resuspension Buffer (PBS-based) directly in Diluted Nuclei Buffer or ATAC Wash buffer-Dig as they 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 to the nuclei that is **in suspension** in the Nuclei Wash and Resuspension Buffer (PBS-based), and let equilibrate for 5 minutes (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' v1 nuclei cDNA and snATAC library as well representative metrics obtained with Cell Ranger for this example.

Before sorting



After sorting