Customer Developed Protocol

‘Frankenstein’ protocol for nuclei isolation from fresh and frozen tissue

Contributed by:
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Contributor Research Profile

Dr. Luciano Martelotto leads the single-cell innovation team at the University of Melbourne, Centre for Cancer Research, Victorian Comprehensive Cancer Centre. His team, in collaboration with Dr. Richard Tothill (Rare Diseases Oncogenomic Lab) and Prof. Sean Grimmond (Centre Director), implements new single-cell technologies and develops new techniques and protocols.

Learn more about their research: https://mdhs.unimelb.edu.au/our-organisation/institutes-centres-departments/umccr/research/our-research

The University of Melbourne, Centre for Cancer Research is part of an alliance whose members are The University of Melbourne, Peter MacCallum Cancer Centre, The Royal Melbourne Hospital, The Walter and Eliza Hall Institute of Medical Research, The Royal Women's Hospital, The Royal Children's Hospital, Western Health, St Vincent's Hospital Melbourne, Austin Health and the Murdoch Children's Research Institute.

Read more here: https://www.viccompcancerctr.org/

References

This protocol is the result of the combination of various nuclei isolation protocols for single cell RNA-seq experiments using droplet-based methods, hence the name *Frankenstein*. Developed to prepare nuclei isolates from small sample sizes (as little as grain of rice), this protocol uses FACS to identify cell subpopulations based on ploidy (e.g. tumor versus stroma), to ensure that nuclei suspensions are not clumped, and to remove any debris, especially ambient RNA, to help reduce background. The reference protocols can be found in the following papers: Hu, *et al*., *Habib, et al. (2016)*, *Habib, et al. (2017)*, Lake, *et al*., and Lacar, *et al*.

This protocol is routinely used in the single-cell innovation lab for single nuclei experiments using 10x Genomics technologies.

The protocol has been demonstrated to work successfully with fresh, snap/flash frozen, cryopreserved cells, and cell lines, as well as various solid cancers: pancreas, pheochromocytomas, paragangliomas, breast cancer, lymphoma, xenografts and tumors.

NOTE: The protocol did not work with cardiomyocytes due to the high level of myosin heavy chain (MHC).

10x Genomics Products

Chromium Single Cell Immune Profiling Solution - https://www.10xgenomics.com/solutions/vdj/
Chromium Single Cell Gene Expression Solution - https://www.10xgenomics.com/single-cell/

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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
3. Collect a specific number of nuclei in a 96-well plate containing 10x RT Buffer*
4. Immediately load the sample into a Single Cell Chip for processing according the Single Cell 3' Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide.

*Assume that nuclei recovery is 57%, and 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' Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide)

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 U/µl RNase Inhibitor

3. Nuclei wash and resuspension buffer with DAPI (prepare chilled, 4°C)
   - 1x PBS
   - 1.0% BSA
   - 0.2 U/µl RNase Inhibitor
   - 10 ug/mL DAPI

4. 10x RT Buffer[1] for Single Cell Gene Expression 3' reagents (DO NOT add RT enzyme)
   - RT Reagent Mix: 50 uL
   - RT primer: 3.8 uL
   - Additive A: 2.4 uL
   - H_2O: (33.8 – X – Y) uL

5. 10x RT Buffer[1] for Single Cell Immune Profiling 5' reagents (DO NOT add RT enzyme)
   - RT Reagent Mix: 50 uL
   - RT primer: 5.9 uL
   - Additive A: 2.4 uL
   - H_2O: (31.7 – X – Y) uL

[1]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 will 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 minutes. The longer the time the higher the background will be.

Protocol

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 a grain of rice.
2. Add 500 uL chilled Nuclei EZ Lysis Buffer to the tissue in 1.5 mL tube. Homogenize the sample using a douncer (stroking ~10-20 times). For mincing the tissue, you may take the tube out of ice, however, be quick and return to ice.
3. Transfer the homogenate (~500 uL) into a 2 mL tube and add 1 mL of chilled Nuclei EZ Lysis Buffer, mix gently and incubate on ice for 5 min. Gently mix with a wide bore tip and repeat 1-2 times during the incubation.
4. Filter homogenate using a 70 µm-strainer mesh. Collect flow through in a Polystyrene round-bottom FACS tube and transfer volume back into a new 2 mL tube.
5. Centrifuge the nuclei at 500g for 5 minutes at 4°C and remove supernatant leaving behind ~50 uL. Gently resuspend nuclei in another 1.5 mL of EZ Lysis buffer, incubate for 5 minutes on ice.
6. Centrifuge the nuclei at 500g for 5 min at 4°C, remove supernatant as much as possible without disturbing pellet (if pellet looks loose leave ~50 uL behind) and add 500 uL Nuclei Wash and Resuspension Buffer and incubate 5 minutes **without resuspending** to allow buffer interchange. After incubation, add 1 mL of Nuclei Wash and Resuspension Buffer and resuspend the nuclei.
7. Centrifuge the nuclei at 500g for 5 min at 4°C, remove supernatant leaving behind ~50 uL and gently resuspend nuclei in 1.4 mL Nuclei Wash and Resuspension Buffer and transfer to a 1.5 mL tube (easier to see small pellets).
8. Repeat 7 and resuspend in 500 uL **Nuclei Wash and Resuspension Buffer supplemented with DAPI**. Collect all nuclei by washing off nuclei from the wall of centrifuge tube.

IMPORTANT*:* Protect from light from here forward.

9. Filter nuclei (at least once) with a 35-µm cell strainer. Visually inspect nuclei integrity under a microscope and count the number of nuclei with a cell counter or hematocytometer if required.
10. Perform cytometric analysis. Identify single nuclei and sub-populations based on DNA content, gate and sort directly into 10x RT Buffer prepared **without the RT Enzyme Mix**.
11. Proceed immediately with the 10x Genomics Single Cell Protocol and minimize the time between nuclei preparation/sorting and chip loading. Add 10 uL RT Enzyme Mix to the sorted nuclei in RT buffer, mix well but gently and load chip as per the Single Cell 3' Reagents User Guide or Single Cell V(D)J 5' Reagents User Guide.

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