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HTAPP_NST- Nuclei isolation from frozen tissue V.2

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Eugene Drokhlyansky[1,2], Nicholas Van Wittenberghe[1,2], Michal Slyper[1,2], Julia Waldman[1,2], Asa Segerstolpe[1,2], Orit Rozenblatt-Rosen[1,2], Aviv Regev[1,2,3,4]

[1]Broad Institute; [2]Human Tumor Atlas Pilot Project; [3]Massachusetts Institute of Technology; [4]Howard Hughes Medical Institute

NCIHTAN

Judit Jane-Valbuena
The Broad Institute

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol describes a nuclei isolation method for frozen tissues based on the previously published protocol by ***Gao et al.*** and modified by ***Drokhlyansky et al***. It can be used on both healthy and disease tissues and is compatible with droplet-based single-nucleus RNA-Seq technology ***Slyper et al***.

This method is part of a toolbox for processing frozen tissue samples for single-nucleus RNA-Seq, including the NST (this protocol), CST and TST protocols (all available in **protocols.io**). We recommend users to test all three protocols on their tissue type of interest and perform side-by-side comparison of the data generated.

A description of the complete toolbox and guidance for testing and selecting methods from the toolbox for processing other tumors can be found in ***Slyper et al***.

For the Human Tumor Atlas Pilot Project (HTAPP), this protocol was tested on metastatic breast cancer, ovarian cancer and pediatric neuroblastoma frozen samples. For these cancer types, however, TST was the protocol of choice. When tested on HTAPP samples, NST, in general, underperformed compared to TST and CST based on several QC parameters.

Guidelines

Work quickly on ice and reduce the time nuclei stand as much as possible.
Work gently when pipetting nuclei.
Optimization might be required for the tissue and cell type of interest.
Recommended sample size: range between ~10 mm x 1 mm to ~0.5 cm^3

Materials

MATERIALS

- Costar® 6-Well Flat-Bottom Plate, Tissue Culture-Treated 50 Plates **STEMCELL Technologies Inc. Catalog #**38015
- Magnesium chloride solution for molecular biology (1.00 M) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M1028
- Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap **Corning Catalog #**352235
- 5M NaCl **Thermo Fisher Scientific Catalog #**AM9760G
- CALCIUM CHLORIDE 1M STERILE **VWR International (Avantor) Catalog #**97062-820
- UltraPure 1M Tris-HCl Buffer pH 7.5 **Thermo Fisher Scientific Catalog #**15567027
- INCYTO C-Chip Neubauer Improved Disposable Hemacytometers **VWR International (Avantor) Catalog #**22-600-100
- Noyes Spring Scissors - Tungsten Carbide/Straight **Catalog #**15514-12
- Nonidet™ P40 Substitute Ultrapure Affymetrix/USB **Fisher Scientific Catalog #**AAJ19628AP
- UltraPure DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- BSA Molecular Biology Grade **New England Biolabs Catalog #**B9000S
- Falcon™ Cell Strainers - Mesh size: 40um; blue **Thermo Fisher Scientific Catalog #**08-771-1

Troubleshooting

Safety warnings

When working with human tissue, use a BL2 biosafety cabinet or fume hood to protect from splashing. Submerge used tools in 10% bleach immediately after use, followed by wash with DI water and 70% ethanol. Collect all liquid waste into 10% bleach and dispose down the drain carefully. All discarded tissue waste should be sealed inside a secondary waste container before being disposed into BL2 waste bins.

Before start

ST Buffer preparation:

	Reagent	Reagent stock concentration	Reagent volume for 50ml 2X stock	Concentration (2X)	Final concentraion (1X)
	NaCl	5 M	2.92 ml	292 mM	146 mM
	Tris	1 M	1 ml	20 mM	10 mM
	CaCl2	1 M	100 µl	2 mM	1 mM
	MgCl2	1 M	2.1 ml	42 mM	21 mM
	H2O	—	43.88 ml	—	—

2 ml NST (for 1 sample):
1 ml 2X ST
10 µl BSA
40 µl 10% NP40
950 µl H2O

1X ST (for 1 sample):
2 ml 2X ST
2 ml H2O

Set-up:

- Ice bucket
- 40 µm filter
- 50 ml conical tube
- 15 ml conical tube
- NST Buffer (2 ml per sample)
- 1X ST
- FACS tube (with 35 µm filter attached)
- Scissors, cleaned with 10% bleach and 70% ethanol
- Frozen tissue
- 6-well plate
- Centrifuge with swing bucket rotor pre-cooled to 4C

1 Place all buffers, tubes, and a 6-well plate on ice; pre-fill one well with 1 ml NST buffer. Keep tissue frozen (on dry ice) until begining of processing.

1 mL NST

Note: If the sample is frozen in OCT, before starting the nuclei isolation protocol cut and discard as much of the frozen OCT surrounding the tissue using a razor and remove the remaining OCT by washing the sample in cold PBS in a 10 cm tissue culture dish (on ice). If needed, using forceps gently assist the removal of the remaining OCT. This should be done on wet ice and as quickly as possible. Quickly after proceed to Step 2.

If the sample, however, is an OCT tissue scroll, place the scroll in cold PBS in a tissue culture dish (on ice) and allow for the OCT to dissolve (this should only take a few seconds). Quickly after proceed to Step 2.

Nuclei isolation

2 Using forceps, place tissue in the pre-filled well of the 6-well plate on ice (containing the 1 ml of NST; Step 1) and chop tissue for 10 minutes in the buffer using spring scissors.

00:10:00

4 °C On ice

3 With a 1 ml pipette transfer the suspension from the 6-well plate to a 40 µm filter, and filter the entire volume into a 50 ml conical tube.

4 °C on ice

Note: Some small pieces of tissue may be left after 10 minutes of chopping.

4 Wash the well with an additional 1 ml of NST buffer and then pass through the same filter.

1 mL NST

4 °C on ice

5 Wash filter with additional 3 ml of 1X ST buffer. Discard filter.

3 mL ST 1X

6 Transfer total volume (~5 ml) from the 50 ml tube into a clean 15 ml conical tube.

4 °C on ice

7 Centrifuge in a swinging bucket rotor for 5 minutes at 500 g at 4°C. Set stop break to 'soft'.

500 x g
00:05:00
4 °C

8 Carefully take the sample out of centrifuge, and place it on ice. Remove supernatant with a 1 ml pipette and discard.

9 Resuspend nuclei pellet in 1X ST buffer (determine the ST volume based on nuclei pellet size - usually around 100-150 µl 1X ST).

Note: The volume used for resuspension may vary between tissues and will depend on pellet size and nuclei concentration desired, usually 1000 nuclei / µl. It is better to resuspend initially in a small volume and dilute as needed to avoid an extra centrifugation step to concentrate the nuclei.

10 Using a P200 pipette, collect the nuclei suspension and place it on a 35 µm falcon cup filter. Filter the suspension into the 5 ml falcon tube.

Nuclei counting

11 Count nuclei using INCYTO C-Chip Neubauer Improved Disposable Hemacytometers and dilute if necessary. The recommended concentration is 1,000 nuclei/µl, but concentrations between 175 and 2,000 nuclei/µl are acceptable. 8,000-10,000 nuclei are typically loaded per channel of a 10x Genomics chip in a volume that should not exceed 43.2 µl for v3.1 chips.

Counting Instructions:

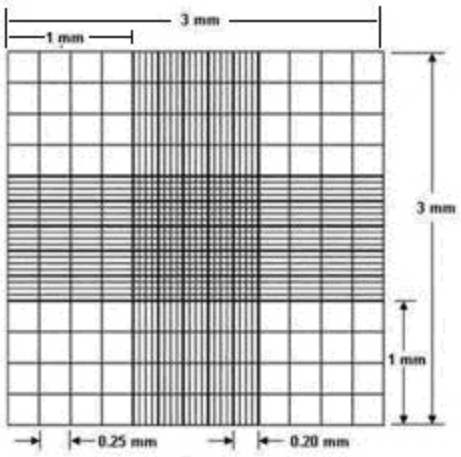
Load 10 µl of nuclei suspension onto a hemocytometer. Under a microscope, count nuclei that are within the hemocytometer grid (figure below). The full grid contains 9 squares, each of which is 1 mm^2. The thickness of liquid in the hemocytometer is 0.1 mm. Consequently, the volume above each of these squares is 0.1 µl and the concentration of nuclei can be calculated as:

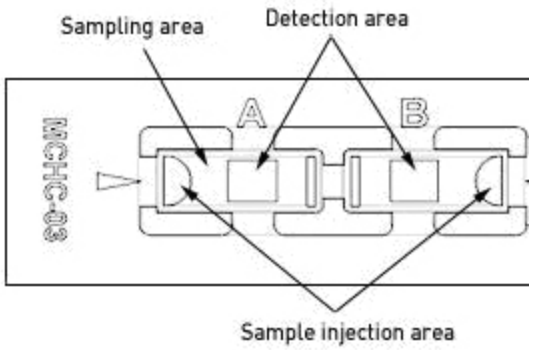
concentration in nuclei/µl = number of nuclei in 1 mm^2 square x 10

If the sample has been diluted before loading onto the hemocytometer, make sure to take this dilution into consideration when calculating the sample concentration. For a robust estimate of nuclei concentration, count at least 30 nuclei ideally located in different areas of the grid.

Alternatively, nuclei can be stained using DAPI and counted under a fluorescence microscope. In this case, mix 3.3 µl of nuclei suspension with 6.6 µl of DAPI (2.5 µg/µl stock in PBS), load on the hemocytometer, and count as described above. Taking into account the dilution with DAPI solution, the nuclei concentration can be calculated as:

concentration in nuclei/µl = number of nuclei in 1 mm^2 square x 10 × 3





10x loading

12 Load sample on 10x (recommended to load between 8,000-10,000 nuclei per 10x channel).