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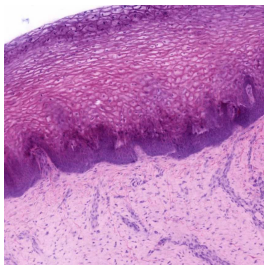
BIDMC-TMC Nuclei Isolation from Frozen Cervix for Single Cell RNA-Seq



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We use this protocol and it's working

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Abstract

As a fibromuscular organ, human cervix samples are naturally resilient to withstand strains and deformation during pregnancy and birth. Due to its consistency and composition, dissociation and disrupting the cervix or other parts of uterus to extract nuclei is a challenging step for single cell applications.

Here, we present a comparably simple and fast protocol for single nucleus RNA sequencing that has been developed at BIDMC TMC, Spatial Technologies Unit of the Beth Israel Deaconess Medical Center of HuBMAP Consortium in collaboration with Luciano Martelotto from the University of Adelaide.

We optimized this protocol for human cervix and other uterine parts, but also tested it successfully for other tissue types very rich (90%) in fibrous connective tissues. Our protocol combines liquid nitrogen facilitated tissue disruption with the fixed RNA (flex) kit from 10x Genomics and generating high quality libraries for HuBMAP.

Materials

- Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit [PN-1000414]
- Chromium Fixed RNA Kit, Human Transcriptome, 4 rxns x 1 BC [PN-1000474]
- Chromium Next GEM Chip Q [PN-1000418 or PN-1000422]
- Dual Index Kit TS Set A 96 rxns [PN-1000251]

- Protector RNase Inhibitor (40U/µL) [RNAINH-RO]
- Bovine Serum Albumin 10% that is sterile filtered and cell-culture tested [Sigma Aldrich A1595 or 126615]
- Paraformaldehyde, use fresh ampule from Paraformaldehyde Aqueous Solution, EM Grade (EMS)
- Nuclease-free Water [Invitrogen/Ambion AM9939]
- PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free [Invitrogen/Ambion AM9624]
- 50% Glycerol - In case of storage only
- EZ Lysis Buffer [Sigma Aldrich EZ PREP NUC-101]

- Sterile and RNAse-free Tissue Culture Dish, 6cm or 10cm from Corning, Falcon or similar.
- Forceps
- Razor Blades
- Wide-Bore Pipette Tips
- Miltenyi Biotec Pre-Separation Filters (30 µm) [130-041-407]
- pluriStrainer Mini 70 µm (Cell Strainer) [SEK560.00]
- DNA LoBind Tubes 1.5 mL [022431021]
- DNA LoBind Tubes 2.0 mL [022431048]

- Tabletop centrifuge with swing rotor adapter, for example Eppendorf 5810R.
- Eppendorf ThermoMixer C with heated lid. Regular thermal blocks do not suffice.
- Liquid Nitrogen and tools stable at cryogenic temperature: Mortar and pestles (porcelain or agate), ladle and dewar for adding liquid nitrogen, stainless steel spatula for transferring pulverized tissue.
- Bel-Art Disposable Pestles [BAF199230001]
- Ice bucket/blocks
- Tissue-Tek Cold Plate, Sakura Finetek [4650]
- Thermal cycler, for example BioRad C1000
- Automated Cell counter, for example Luna-FX7

Protocol materials

Nuclei EZ lysis buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EZ PREP NUC-101 Step 14

Safety warnings

- Take precautions when using alternatives, such as thermal blocks instead of ThermoMixer with heated lid. Temperature variation during hybridization and wash steps will strongly impact assay outcome.
- All buffers and reagents must be freshly prepared.
- Correct cell counting is crucial for a successful assay. Buffers used in flex protocol have lower buoyancy so make sure the cells are well-resuspended at counting and chip loading.

Before start

- Get liquid nitrogen. 1 liter suffices for preparation of 2-4 sample. Industrial dry ice is very dirty and should not be used as substitute.
- Pre-cool centrifuge and other utensils.
- Take precautions when using alternatives, such as thermal blocks instead of ThermoMixer with heated lid, wrong filters.

Buffer Preparation

1

> **Note**
>
> The protocol will indicate the best times in which to prepare the buffers for optimal time usage. Still, all buffers can be prepared ahead of time and kept at their designated temperatures, if desired.
>
> All buffer volumes are indicated for **one sample**. Adjust volumes accordingly.

2 Prepare fresh buffers based on CG000478, page 2, including Fixation Buffer, Quenching Buffer and Wash-Resuspension buffer. 1 ml Fixation Buffer will be used per sample. Prepare the Sample Fixation Buffer as described above and aliquot 1mL into a 1.5mL Eppendorf Tube;

Fixation Buffer – 1mL per Sample – Room Temp.

	A	Stock Concentration	Final Concentration	Per 25 mg Sample (uL)
	Conc. Fix & Perm Buffer* (10x Genomics PN 2000517)	10x	1x	100
	Formaldehyde	32%	4%	125
	Nuclease-free Water	-	-	775

Quenching Buffer – 1 mL per Sample – 4°C

	Reagent	Stock Concentration	Final Concentration	Per 25 mg Sample (uL)
	Nuclease-free Water	-	-	875
	Conc. Quench Buffer* (10x Genomics PN 2000516)	8x	1x	125

Tissue Wash Buffer – 4mL per Sample – 4°C

	Reagent	Stock Concentration	Final Concentration	Per 25 mg Sample (uL)
	Nuclease-free Water	-	-	3200
	BSA	10%	1%	400
	PBS	10X	1X	400

Tissue Resuspension Buffer – 1 mL per Sample – 4°C

	Reagent	Stock Concentration	Final Concentration	Per 25 mg Sample (uL)
	Nuclease-free Water	-	-	948
	BSA	10%	0.02%	2

	Reagent	Stock Concentration	Final Concentration	Per 25 mg Sample (uL)
	PBS	10X	0.5X	50
	RNase Inhibitor (U/uL)	40	0.2	5

Sample Preparation

3 Ensure the samples are free of OCT if they were previously embedded. Coarsely trim off the peripheral OCT and rinse the sample core with RNase-free chilled PBS thoroughly before processing.

4 Pre-cool a mortar and pestle by filling with sufficient liquid nitrogen;

5 Place the tissue on a pre-chilled 6cm or 10cm dish maintained on a cold plate, and mince tissue finely with a sterile razor blade to facilitate the fixative penetration. Do not over-mince. Transfer the minced tissue into the mortar;

6 Add enough liquid nitrogen to submerge the whole sample and wait until the tissue is fully frozen, then grind the tissue carefully to generate a granular fine power.

6.1 Repeat this step until the tissue becomes a fine powder. **Ensure the tissue never thaws throughout this process.**

7 Using a sterile disposable spatula, transfer the suspension of tissue powder carefully into a pre-cooled 1.5 or 2ml low-bind and safe-lock Eppendorf tube and allow liquid nitrogen to evaporate without thawing the sample powder. The exact process needs to be optimized depending on the tissue composition.

For immediate processing, transfer the powder to the pre-filled 1.5 mL tube containing the Fixation Buffer. Pipette mix with a wide-bore pipette tip.

Alternatively, the powder can be stored at -80°C in an Eppendorf Tube or a cryovial for extended period.

Note

The powder will most likely begin to thaw during the transfer and may stick to the spatula. Transferring the powder directly to a pre-filled tube allows you to "clean" the spatula and not waste any tissue. Alternatively, if not immediately processing the sample, pre-cool an empty tube by dipping it into liquid nitrogen immediately before transferring the powder.

Sample Fixation

1h

8 Incubate at Room temperature for at least 01:00:00, or at 4 °C Overnight. 1h

8.1 Gently invert the sample tube to ensure contact between Fixation Buffer and powered samples.

Sample Dissociation

20m

9 Centrifuge the samples at 850 rcf, Room temperature, 00:05:00. 5m

10 Prepare the Sample Quenching Buffer as described above;

11 Cool the centrifuge to 4°C;

12 Remove the supernatant and re-suspend the pellet in Quenching Buffer;
From this moment forward the sample should be kept on ice;

13 Centrifuge the samples again at 850 rpm, 4°C, 00:05:00. 5m

14 Remove the supernatant, and add 200 µL (or enough to cover the tissue) of

Nuclei EZ lysis buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EZ PREP NUC-101

.

15 Homogenize the tissue using a small plastic pellet pestle to ensure complete dissociation;

16 Add more EZ Lysis buffer for a total of 1 mL, incubate for 00:10:00 on ice; 10m

> **Note**
>
> Depending on how fibrous your tissue is and how many samples are being processed at once, dessociation might take up to 10 minutes. We suggest to add the initial lysis buffer to all samples and start a timer. Avoid exceeding 20 minutes between the moment the sample first gets in contact with the lysis buffer and the next step.

Sample Cleanup 10m

17 Prepare the Sample Wash Buffer and the Sample Resuspension Buffer as described above;

18 Centrifugate the samples at 850 rpm, 4°C, 00:05:00 5m

19 Remove the supernatant and re-suspend in 800 µL of Wash Buffer;

20 Filter the resuspension through a 70µm sterile filter and into a **2 mL** Eppendorf LoBind tube. Do not discard original tube.

21 Add 800 µL more of wash buffer to the original tube and wash the walls. Pass this through the same filter to increase nuclei capture;

22 Centrifugate the samples at 850 rpm, 4°C, 00:05:00 5m

23 Remove the supernatant and re-suspend in 1 mL of Wash Buffer;

24 Repeat steps 21 and 22 at least one more time for a total of 3 washes.

> **Note**
>
> If desired, check the sample under a microscope to check for cleanliness; Repeat washes as needed depending on amount of debris present.

25 After the final centrifugation, resuspend pellet in 1 mL of Resuspension Buffer;

> **Note**
>
> For maximal nuclei recovery, consider rinsing the filter with extra Resuspension Buffer;

26 Filter final nuclei suspension through a 30µm sterile filter and into a 1.5 mL LoBind Eppendorf tube.

27 Count the nuclei, and proceed to immediately to 10X protocol CG000691 (see Ref.);

> **Note**
>
> Alternatively, the sample can be stored as follows:
>
> - For storage of up to 1 week at 4 °C : add 0.1 volume of Enhancer;
> - For storage of up to 6 months at -80 °C : add 0.1 volume of Enhancer and 50% Glycerol so that it is at a final concentration of 10%; [e.g. for 1mL of sample, add 100µL of Enhancer and 275µL of 50% Glycerol]

Sample Storage

28 Thaw Enhancer (10x Genomics PN-2000482) for 10 minutes at 65°C;

29 Samples can be kept for up to 1 week at 4°C with 0.1 volume of Enhancer;

30 Samples can be kept for up to 6 months at -80°C with 0.1 volume of Enhancer, and 10% volume of 50% Glycerol.

Protocol references

Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling
10x Genomics Sample Preparation Products, CG000478
https://www.10xgenomics.com/support/single-cell-gene-expression-flex/documentation/steps/sample-prep/fixation-of-cells-and-nuclei-for-chromium-single-cell-gene-expression-flex

Chromium Fixed RNA Profiling Reagent Kits for Singleplexed Samples
10x Genomics User Guide, CG000691
https://www.10xgenomics.com/support/single-cell-gene-expression-flex/documentation/steps/library-prep/chromium-fixed-rna-profiling-reagent-kits-for-singleplexed-samples