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Nuclei Isolation from Flash Frozen Ovarian Tissue using Sucrose Gradient

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We use this protocol and it's working

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Abstract

High-throughput single-nuclei RNA sequencing (snRNAseq) can profile nuclear gene expression of various cell types within the ovary. In contrast to single-cell RNA sequencing, which requires fresh tissue and immediate tissue processing and sequencing, snRNAseq with flash-frozen tissue may be a more practical and nuclear-focused method of RNA sequencing. Difficult-to-dissociate cell types may be left out during the single-cell isolation process and artificial transcriptional stress responses could occur. snRNAseq may more accurately represent the gene expression levels in all cell types and exclude RNA from the cytoplasm. Additionally, tissue flash freezing can be performed easily at the time of ovarian tissue removal in the operating room.

This protocol outlines how to isolate nuclei from flash-frozen ovarian tissue. The protocol was modified from the 10X Genomics Chromium Nuclei Isolation Kit protocol as well as previous protocols used for nuclei isolation from adult mouse brain. Flash-frozen pieces of the ovary containing both medulla and cortex obtained from an adult donor were used to optimize this protocol prior to downstream applications of FACS sorting and single nuclei RNA sequencing.

Guidelines

This protocol has been validated for single nuclei RNA sequencing from flash frozen ovarian tissue that includes ovarian cortex and medulla. Validation methods included Live/Dead staining and FACS.

Materials

- 1 small forceps,
- 1 sharp tip fine scissors,
- 1 flat edge blade,
- One metal block stored at -20C,
- 1 of 4-7 ml Glass Grinder,
- 1 of 70um and 1 of 40 um cell strainers (Greiner EAZY strainer Small, cat# 542120)
- UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml ThermoFisher cat# AM2616)
- 2 of 3 ml Falcon Plastic Transfer pipet (wide-bore, individually wrapped andSterile, BD Catalog 35-7575)
- 1 of 5 mL DNA LoBind Tube, PCR Clean (Eppendrof, # 0030108310)
- 3 of 2 ml DNA LoBind Tubes (Eppendrof, # 022431048)
- 1 of 1.5ml microcentrifuge tube,
- 2 of 15 ml and
- 2 × 50 mL conical tubes,
- Refrigerated Microcentrifuge for 1.5-2 ml microtubes, pre-cool at 4C.
- Cell counter and AO/PI fluorescent dye for cell availability evaluation (Avoid light)
- One Dry-ice container
- One ice container

Troubleshooting

Before start

Best practices for handling cell line/tissue samples include using sterile techniques, nuclease-free reagents and consumables, minimizing pipetting steps, and using wide-bore pipette tips when possible, to minimize cell damage.

It is critical to keep all steps at 4^0C to prevent RNA degradation and minimize cell and nuclei handling time because the viability may significantly decrease when cells/nuclear are kept in suspension for a prolonged period.

Ovarian Tissue Preparation

1 In our current protocol, immediately after unilateral oophorectomy in the operating room, a 1-2 mm transverse section of the ovary (salami cut) is performed in order to include both cortex and medulla.

Recommended weight of ovarian tissue per sample: 30-50 mg
Goal number of nuclei per sample for 10X Genomics: 1 X 10^6 nuclei/ml

1.1 Sample is immediately placed in a cassette and buried in dry ice (flash frozen). Sample is stored in liquid nitrogen freezer (LNF) until needed.

Pre-Procedure Setup

2 Prepare all buffers:

1. Low Sucrose buffer: (0.32 M sucrose, 10 mM HEPES [pH 8.0], 5 mM CaCl2, 3 mM Mg-acetate, 0.1 mM EDTA, 1 mM DTT) (Add fresh DTT)
2. Detergent Lysis Buffer: Low Sucrose buffer with 0.1% NP-40 (10ul 10% solution in 1ml)
3. Nuclei suspension buffer: 1x PBS with 1% BSA (from 10% stock), and add fresh RNase Inhibitor (0.2u/ul).
4. Sucrose Cushion Buffer I: 2.7 ml Nuclei PURE 2M Sucrose Cushion Solution with 0.3 ml Nuclei PURE Sucrose Cushion Buffer. At room temperature!
5. 1X PBS: 10 ml on ice

2.1 Add 1.0 ml lysis buffer to a 5 ml LoBind tube (Tube #1) and place on ice for tissue cutting and digestion.

2.2 Place 3 × 2 mL LoBind tube (Tube #2 and #3) on ice.

2.3 Place a 40 mm strainer #1 over the 2 mL tubes (#2 and #3) and pre-wet the filter with 750 uL of low sucrose buffer.

2.4 Add 3 mL of low sucrose buffer in one 15ml centrifuge tube and place on ice without cap on.

Mechanical-Detergent Cell Lysis

3 Take out the frozen tissue from the LNF, place the tissue in dry ice before processing.

4 Cut a portion of the tissue (approximately 5 × 5 mm) with a flat edge blade over a cold block, weighing out 40mg -50mg of tissue on a scale.

5 Wrap the rest of the tissue in the original foil and place back in dry ice to snap freeze.

6 Place the tissue sample in Tube #1 (5 ml tube containing 1.0 ml pre-chilled lysis buffer on ice).

7 Use sharp tip fine scissors to cut tissue into small pieces (2–3 mm) in the tube containing the lysis buffer.

8 Transfer the tissue fragments to the pre-chilled glass homogenizer with a 3 ml transfer pipet.

9 Grind tissue with approximately 5 strokes using a twisting motion (Avoid lifting the homogenizer out of the lysis solution in between strokes to prevent bubbles).

10 Take out the pestle from the homogenizer and place it into the 5 ml Tube #1 on ice.

11 Incubate the tissue fragments in the glass homogenizer for 5 min on ice for lysis (total time from cutting and grinding is 10 mins).

12 Gently mix the tissue lysis 7-10 times with the transfer pipette.

13 Transfer the homogenate on to the pre-wet 70 mm strainer in the 2 mL Tube #2 and Tube #3 on ice.

14 (Optional) Transfer the 70 mm strainer #1 onto the 2 mL Tube #3 for additional grinding if there are large pieces of tissue sample remaining in the grinder.

15 Second grinding: Add 1 mL of low sucrose buffer (from the 15 ml tube) to the homogenizer with the remaining tissue if large pieces are visible, then proceed to grind for 5 strokes.

16 Remove the pestle and place it into the 5 ml Tube #1 on ice.

17 Gently mix the homogenate for 5 times with the 3 ml transfer pipet.

18 Transfer the homogenate into the 70 mm strainer over the 2 mL Tube #3.

19 Rinse the pestle with 1 mL low sucrose buffer over the 40 mm strainer#1. The final volume is about 4 mL (2×2 ml).

20 Centrifuge the homogenate in Tube #2 and Tube #3 at 350 x g for 5 min at 4 °C in a fixed angle rotor (1.7 rpm).

21 Remove the supernatant carefully not to disturb the pellet, leaving a small amount of buffer just to cover the pellet.

22 Add 1.9 ml of low sucrose buffer into the tube, set on ice for 2 min to loosen the pellet, then mix gently to wash the pellet with a clean 3 ml transfer pipet.

23 Centrifuge the sample at 350 x g for 5 min at 4 °C in a fixed-angle microcentrifuge.

24 Remove the supernatant carefully and use a small-bore pipet to remove the residual buffer.

25 Add 1.9 ml of low sucrose buffer (combine 2 tubes in one) into the tube, set on ice for 2 min to loosen the pellet, then mix gently to wash the pellet with clean 3 ml transfer pipet,

26 Centrifuge the sample at 350 x g for 5 min at 4 °C in a fixed-angle microcentrifuge.

27 Add 0.5 mL Nuclei suspension buffer to the tube, set on ice for 2 min, then gently swirl to move the pellet from the wall to facilitate the resuspension, and pipet gently to suspend the nuclear pellet.

28 If both nuclear pellets are very small or barely visible, the contents in the 2 tubes can be combined into one tube with 500 ul of Nuclei suspension buffer. This crude nuclear suspension will proceed with the Sucrose Density Gradient purification steps below.

Sucrose Density Gradient

29 Pre-load 500 uL of Sucrose Cushing Buffer I into the bottom of the 2 ml Tube #3.

30 Add 900 ul of Sucrose Cushing Buffer I to the crude nuclear suspension with 0.5 mL of Nuclei Suspension Buffer from step 27. Mix gently 3 times.

31 Slowly layer the 1.4 mL of nuclei suspension on top of Sucrose Cushing Buffer I in the 2 ml Tube #3, taking care not to create a bubble or disrupt the density layers.

32 Centrifuge the gradients at 13,000 x g for 45 min at 4 °C in a fixed-angel rotor (11,000 RPM).

33 Once the centrifugation is complete, immediately decant the supernatant with a pipet. NOTE: A residual volume of sucrose buffer can be left in the tube if there is a small amount of nucleus (little or no pellet visible in the wall of the tube).

34 Add 200 -500 uL of Nuclei suspension buffer to the pellet, using an appropriate volume according to the size of the nuclei pellet to achieve the target nuclei concentration of 1000 nuclei/µl (1×10^6 nuclei/ml).

35 Set the tube on ice for 2-4 min, then gently pipette 8–10 times or until nuclei are completely suspended. Note: Nuclei suspensions should also be checked visually for debris or nuclei aggregates as these can clog microfluidic channels. If observed, additional pipetting may be required to obtain optimal performance.

36 During the centrifugation, place a 40 µm Cell Strainer #2 over a fresh 2ml Tube #4 on ice. Load 0.5 ml cold nuclei suspension buffer to pre-wet the filter. Then remove the buffer completely.

37 Filter the nuclei suspension through the 40 µm Cell Strainer #2 and nuclei will be collected in Tube #4 (to remove aggregates and debris). Do not tap the filter to force the liquid flow through but slowly rock the filter from side-to-side gently. (This last filtering step also can be performed before sorting).

Determining Nuclei Concentration

38 Turn on the CellDrop, an automated cell counter, and select the AO/PI default program.

39 Clean the cell counter surface with 10 ul of 75% Ethanol and wipe off. Check the screen display for cleanness.

40 Add 10 ul of AO/PI dye into a microcentrifuge tube.

41 Resuspended the nuclei well, pipette 10 ul of nuclei suspension, and mix it with 10 ul of AO/PI dye to visualize nuclei viability.

42 Load 10ul of nuclei/Dye mix into the slot of the CellDrop counter.

43 When the cell/nuclei stopped moving in the screen display, start the count and record the result.

44 If the concentration is less than 500 nuclei/µl (5×10^5 nuclei/ml), adjust the volume accordingly by centrifuging and resuspending the pellet with a lower volume of the buffer. The target nuclei concentration is 1000 nuclei/µl (1×10^6 nuclei/ml).