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Nuclei isolation from frozen gonad tissue

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We use this protocol and it's working

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Abstract

This protocol describes how to perform nuclei isolation from frozen gonad tissues using Dounce homogenizers. It can be used as well for ovary strips frozen in OCT

Guidelines

Human tissue and cells from primary samples may contain uncharacterized adventitious agents, including blood-borne viruses. No attempt will be made to culture these agents deliberately. Correct use of PPE will drastically reduce the risks.
Tissue collection for this protocol requires prior approval by the users' Institutional Ethics Board or equivalent ethics committee

Materials

- Sucrose **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S0389
- KCl 2M **Catalog #**AM9640G
- 1M MgCl2 **Invitrogen - Thermo Fisher** **Catalog #**AM9530G
- 1M Tris buffer pH8.0 **Invitrogen** **Catalog #**AM9855G
- Triton X-100, 10% solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**93443
- 1xPBS (RNase free) pH7.4 **Gibco - Thermo Fisher Scientific** **Catalog #**10010-023
- 1M DL-Dithiothreitol solution (DTT) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**646563
- cOmplete™ Protease Inhibitor Cocktail **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**11697498001
- SUPERase• In™ RNase Inhibitor (20 U/µL) **Thermo Fisher Scientific** **Catalog #**cat# AM2694
- UltraPure™ BSA (50 mg/mL) **Thermo Fisher** **Catalog #**AM2618
- RNasin(R) Plus RNase Inhibitor, 10,000u **Promega** **Catalog #**N2615
- KIMBLE Dounce tissue grinder set 7 mL complete **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D9063
- Flowmi Cell Strainer **Merck Millipore (EMD Millipore)** **Catalog #**BAH136800040-50EA

Troubleshooting

Buffer preparation

1 **Nuclei Isolation Buffer 1 (NIM1)**

Can be made, filtered and stored at 4°C for up to 6 months

	A	B	C	D
		Start M/MW	Final M	Volume/amount
	Sucrose	342.3 g/mol	0.25	4.279 g
	KCL	2 mol/l	0.025	625 µl
	MgCl2	1 mol/l	0.005	250 µl
	Tris buffer pH8	1 mol/l	0.01	500 µl
	Nuclease free water	-	-	48.625 ml
	Total			**50 ml**

Nuclei Isolation Buffer 2 (NIM2)

To be made and used on the day of experiment, keep on ice.

- Pre-dilute 1M DTT: 5 ml 1 M DTT in 4.995 ml water.
- For 100x Protease inhibitor stock, dissolve 1 tablet in 500 ml nuclease free water. Stock solution can be stored at 2 to 8 °C for 1 to 2 weeks, or at least 12 weeks at -15 to -25 °C.

	A	B	C	D
		Start M/X	Final M	Volume/amount
	DTT pre-made stock	1 mol/l	1	10 µl
	100x protease inh.	100x	1	100 µl
	NIM1	-	-	9.89 ml
	Total			**10 ml**

Homogenization Buffer

To be made and used on the day of experiment, keep on ice. Do NOT vortex.

	A	B	C	D
		Start conc/%	Final conc/%	Volume/amount
	RNaseIn Plus	40 U/ µl	0.4	100 µl
	Superasin	20 U/ µl	0.2	100 µl
	Triton X-100	10%	0.1	100 µl
	NIM2			9.7 ml
	Total			**10 ml**

Wash Buffer

	A	B	C	D
		Start conc	Final	Volume/amount
	BSA		1%	0.1 g
	RNasin	40 U/ µl	0.2	50 µl
	1xPBS	10%		9.9496 ml
	Total			**10 ml**

Diluted Nuclei Buffer

Prepare this buffer while centrifuging nuclei in step 11

	A	B
	20x Nuclei buffer	25 µl
	DTT	0.5 µl
	RNase Inh	12.5 µl

	A	B
	Nuclease free water	460 µl

90% Percoll solution

Prepare fresh and keep on ice

	A	B
	Percoll	4.5 mL
	10x PBS	0.5 mL

2 Clean Dounce homogenizers and pestle B with 70 % ethanol, then Milli-Q water. Place on ice with 50 ml Falcon tube to pre-chill

3 Collect tissue on dry ice from -80 °C freezer and proceed immediately

4 Transfer tissue biopsy to homogenizer using sterile, disposable tweezers. If the piece of tissue is in OCT, cutted slides should be directly transferred to the dounce homogenizer already seating on dry ice

5 Mix **Homogenization buffer** and transfer 3 mL to the homogenizer making sure to wash the tissue to the bottom of the tube. Wait until buffer is liquid again

6 Gently homogenize the tissue using the tight pestle (B). Up and down but do not grind. Try to avoid bubbles. 10-20 strokes depending on how the tissue behaves. Rinse the pestle with 0.5 mL of **Homogenization buffer**. Place the pestle in Virkon for cleaning.

7 Pour homogenate through a 40 µm cell strainer into 50 ml Falcon tube on ice. Rinse homogenizer with additional 0.5 mL **Homogenization buffer** and pour it through the strainer. Make sure to transfer droplets of the filtrate from the under side of the strainer using a pipette.

Glass homogenizers should be left for 24 hours in Virkon, then cleaned with 70 % ethanol and Milli-Q water.

8 Centrifuge the 50 ml Falcon tube @ 500 g, 6 mins, 4 °C with **acceleration set at 0 and deacceleration set to 3** in Eppendorf centrifuge.

9 Carefully remove most of the supernatant with 1 ml pipette tip. Leave a small amount and gently resuspend the nuclei using a wide bore tip (optional). Add 0.5 mL of **Wash buffer** and carefully resuspend nuclei and transfer to an 1.5 ml eppendorf (NOT low bind).

10 Check how is the nuclei quality using a C-Chip (5 µl filtered Trypan blue, 5 µl nuclei suspension)

10.1 If the nuclei solution is clean enough and not many debris present, add 0.5 mL more of **Wash buffer** and go to **step 12**

10.2 If many debris is present, start Percoll clean-up:

- Prepare **90% Percoll solution**
- Prepare 2 epp with 475 µL of Wash Buffer
- Add 225 µL of the nuclei solution to each one of the epp
- Add 300 µL of 90% Percoll solution to each one of the epp
- Mix by inverting eppendorfs 5 times
- Centrifuge @ 20,000 g, 15 min, 4 °C
- Take supernatant without touching pellet that would be "floating" in the middle-lower part of the tube
- Add 500-900 µl Wash Buffer
- Centrifuge @ 500 g, 3 min, 4 °C
- Take as much supernatant as possible and add 500 µl Wash Buffer in each epp and combine them in one
- Centrifuge @ 500 g, 3 min, 4 °C
-Take most of the supernatant leaving 50-100 µl for resuspension.
- Count nuclei using a C-Chip (5 µl filtered Trypan blue, 5 µl nuclei suspension)
- Concentrate down if necessary, centrifuging at 500 g, 3 mins, 4 °C and taking out extra supernatant volume according to desirable nuclei stock concentration versus targeted nuclei recovery.
- Exemple if the nuclei stock is in100 µl:
    add 5 µl 20x Nuclei buffer
    add 1 µl DTT(diluted 1:10 in water)
    add 2.5 µl **RNase Inhibitor????**

11 Centrifuge at 500 g, 3 mins, 4 °C. During centrifuge time, prepare **Diluted Nuclei Buffer**

Remove all supernatant and resuspend nuclei in the remaining (~100 µl) wash buffer. Check how is the nuclei quality using a C-Chip (5 µl filtered Trypan blue, 5 µl nuclei suspension)

12

Skip filtering with Flowmi filter if there is not a lot of debris.

Adjust here depending on the size and appearance of the nuclei. If there is enough nuclei density, resuspend in 100 µL of **Diluted Nuclei Buffer**

13 Check the nuclei in the new buffer using a C-Chip (5 µl filtered Trypan blue, 5 µl nuclei suspension). Normally will appear clumpy in this buffer!!!!

14 Filter with a 40 µm Flowmi cell strainer and check nuclei suspension again (a little bit less volume due to filtering)

15 Count nuclei using a C-Chip (5 µl filtered Trypan blue, 5 µl nuclei suspension). Concentrate down if necessary, centrifuging at 500 g, 3 mins, 4 °C and taking out extra supernatant volume according to desirable nuclei stock concentration versus targeted nuclei recovery.

16 Follow 10X Single Cell Multiome ATAC + Gene Expression kit protocol (isothermal incubation + GEM generation and barcoding + GEM incubation + quenching reaction) Keep GEMs at -80 °C