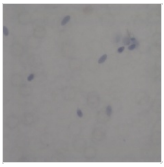

Sep 22, 2020

Nuclei Isolation for Single-Nuclei RNA sequencing

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1 Works for me dx.doi.org/10.17504/protocols.io.bkacksaw

Human Cell Atlas Method Development Community | Ludwig Lab

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ABSTRACT

We developed this protocol while trying to isolate single-nuclei from frozen liposarcoma tissue. We tested this protocol on a variety of PDX's. For fattier tissues, we use a modified protocol based on a different kit. Thanks to Dr. Luciano Martelotto for his assistance and tips on the first section.

This protocol is based on prior work: 'Frankenstein' protocol, Nuclei Isolation Kit: Nuclei EZ Prep, and Minute™ Total Protein Extraction Kit for Adipose Tissues/Cultured Adipocytes.

DOI

dx.doi.org/10.17504/protocols.io.bkacksaw

PROTOCOL CITATION

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KEYWORDS

single-cell, single-nuclei, nuclei isolation, fatty tissue, adipose tissue, liposarcoma, sarcoma, PDX

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GUIDELINES

Refer to Isolation of Nuclei for Single Cell RNA Sequencing from 10x genomics for additional information.

For filtering the nuclei, we don't use use Flowmi filters as we find that some of the tips do not fit properly into the filters and I lost some samples due to this. I prefer to use the pluriStrainer Mini.

For nuclei sequencing, since nuclei have lower RNA content compared to whole cell, protocols could increase the number of cDNA amplification cycles by 1 or 2 cycles, as suggested by 10x genomics.

MATERIALS

NAME	CATALOG #	VENDOR
RNAlater	AM7020	Thermo Fisher Scientific
DAPI	D3571	Invitrogen - Thermo Fisher
Protein LoBind Tubes, 1.5 mL	0030108116	Eppendorf
RNase-Free Disposable Pellet Pestles, With tube	12141368	Thermo Fisher
RNase Inhibitor	N8080119	Thermo Fisher
Nuclei Isolation Kit: Nuclei EZ Prep	NUC-101	Sigma Aldrich
PluriStrainer Mini 40µm	43-10040-60	pluriSelect
Minute Nuclei and Cytosol Isolation Kit for Adipose Tissues/Cultured Adipocytes	AN-029	Fisher Scientific
BioVortexer Mixer	50-153-2378	Fisher Scientific
pluriStrainer Mini 70 UM	NC1444112	Fisher Scientific

STEPS MATERIALS

NAME	CATALOG #	VENDOR
pluriStrainer 40 µm	43-50040	pluriSelect
Nuclei EZ lysis buffer	EZ PREP NUC-101	Sigma
Minute Nuclei and Cytosol Isolation Kit for Adipose Tissues/Cultured Adipocytes	AN-029	Fisher Scientific
BioVortexer Mixer	50-153-2378	Fisher Scientific
RNase-Free Disposable Pellet Pestles, With tube	12141368	Thermo Fisher
pluriStrainer Mini 70 UM	NC1444112	Fisher Scientific

MATERIALS TEXT

Consumables:
- Scalpels
- 1.5 mL tubes
- 15 mL centrifuge tubes
- 50 mL centrifuge tubes
- PBS, sterile

BEFORE STARTING

All solutions should be kept at **4 °C** or on **On ice** prior to use.

Turn on benchtop centrifuge and keep at **4 °C**

Prepare Nuclei Wash and Resuspension Buffer (**4 °C**)
- 1X PBS with 1.0% BSA and 0.2U/µl RNase Inhibitor

Nuclei Isolation for Fatty tissues
- use a thermometer to make sure freezer is about **-20 °C**
- Chill plastic consumables in ice before use

Nuclei Isolation (most tissues) 25m

1 ⚠

This protocol should work for most tissues. If the tissue that you are working is fatty or contains fat, including adipose or brain, **skip to Nuclei Isolation for Fatty tissues section.**

2 Use a scalpel and mince tissue on ice. Mincing tissue will improve nuclei isolation efficiency.

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3 Add **500 µl** of EZ Lysis Buffer to tissue in a 1.5 mL microcentrifuge tube.

Nuclei EZ lysis buffer
by Sigma
Catalog #: EZ PREP NUC-101

4 Homogenize tissue using plastic microcentrifuge pestles. A Chemglass Life SciencesSupplier BioVortexer Mixer [Fisher Scientific: 50-153-2378] can be used. This should be done in ice but you can take it out for visual inspection. The timing will be based on tissue type and size. 45s

BioVortexer Mixer
by Fisher Scientific
Catalog #: 50-153-2378

RNase-Free Disposable Pellet Pestles, With tube
by Thermo Fisher
Catalog #: 12141368

5 Add **1 mL** of EZ Lysis Buffer to the 1.5 mL microcentrifuge tube and incubate **On ice** for **00:05:00** . This time can be modified depending on your tissue type and size. 5m

6 Filter the solution using pluriStrainer Mini 70 µm [Fisher Scientific: NC1444112] into a new 1.5 mL microcentrifuge tube.

pluriStrainer Mini 70 UM
by Fisher Scientific
Catalog #: NC1444112

7 Centrifuge the solution at **500 x g, 4°C, 00:05:00** . 5m

8 Add **1 mL** of EZ Lysis Buffer to the 1.5 mL microcentrifuge tube and incubate **On ice** for **00:05:00** .

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9 Centrifuge the solution at **500 x g, 4°C, 00:05:00** .

10 ⚠

Remove the supernatant while being careful not to disturb the pellet. If you can not see the pellet, it is advisable to leave behind **50 µl** .

Washing 15m

11 Carefully add **0.5 mL** of the **Nuclei Wash and Resuspension Buffer** and incubate for **00:05:00** without resuspending.

12 Add **0.5 mL** of the **Nuclei Wash and Resuspension Buffer** and resuspend the nuclei.

13 Centrifuge the solution at **500 x g, 4°C, 00:05:00** . 5m

14 Aspirate the supernatant while being careful not to disturb the pellet and leave behind **50 µl** .

15 Add **1 mL** of **Nuclei Wash and Resuspension Buffer**.

16 Centrifuge the solution at **500 x g, 4°C, 00:05:00** . 5m

17 Aspirate the supernatant and resuspend the nuclei in **500 µl** of **Nuclei Wash and Resuspension Buffer** supplemented with [M]**10 µg/µl** DAPI.

18 Filter nuclei with a pluriStrainer Mini 40 µm [Fisher Scientific: NC1469671].

pluriStrainer 40 µm
by pluriSelect
Catalog #: 43-50040

Visual Inspection and Sorting 1h

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19

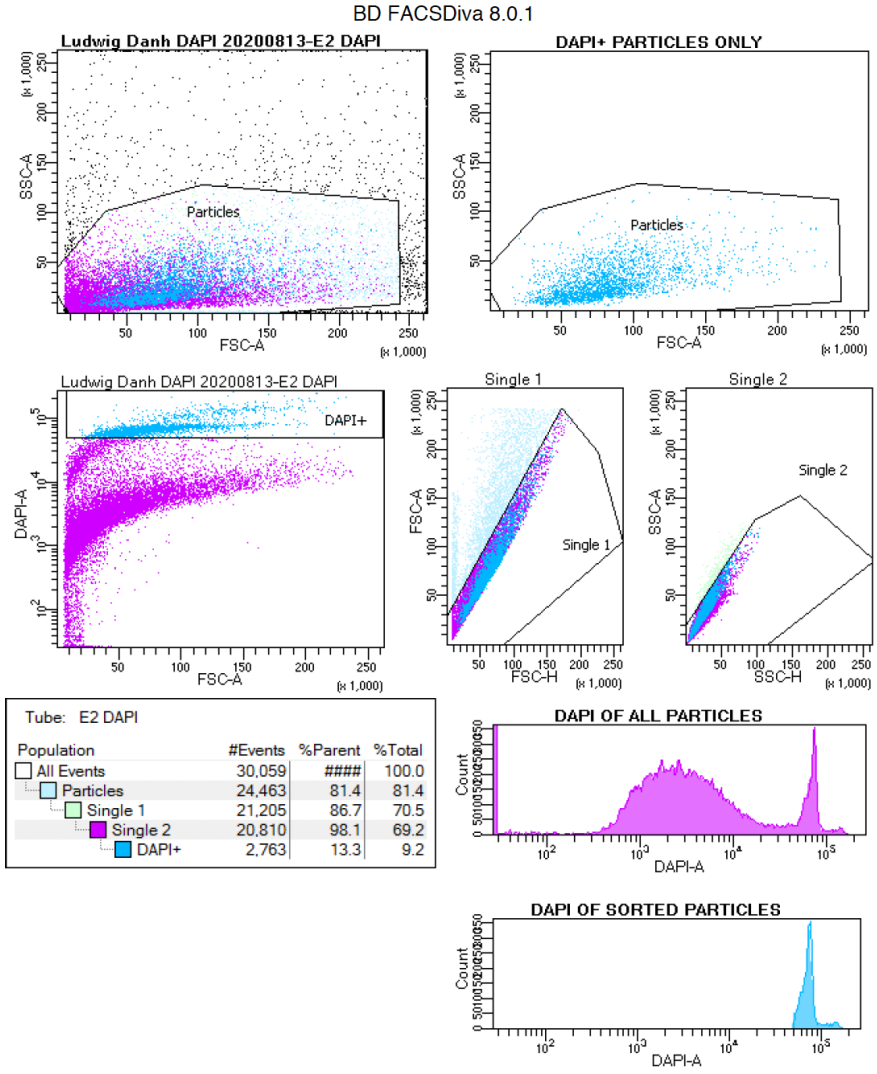
Visually inspect nuclei integrity under a microscope and count the number of nuclei with a cell counter. This is important to check before continuing to see if the protocol was successful.

Image_Overlay-1.tif

Image of isolated nuclei. There is significant debris prior to sorting.

20 45m

Nuclei should be sorted as there will be significant debris after dissociation.



Report of DAPI sorted particles

21

Nuclei can be visualized after sort to confirm success.

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Image_Overlay-1.tif

Image of isolated nuclei after sorting.

22 After sorting, adjust concentration of nuclei to 700 nuclei/µl if needed. Continue with 10x Genomics Single Cell Protocol.

Nuclei Isolation for Fatty tissues 10m

23 ⚠

For fatty tissues, including brain and adipose, the lipids can interfere with the collection. This is a different protocol that can assist in enhancing nuclei isolation efficiency. We will use a modified protocol from the Minute Nuclei and Cytosol Isolation Kit for Adipose Tissues/Cultured Adipocytes kit.

Minute Nuclei and Cytosol Isolation Kit for Adipose Tissues/Cultured Adipocytes
by Fisher Scientific
Catalog #: AN-029

24 Tissue weight should range from **120 mg** to **150 mg** . Mince tissue using a scalpel and place inside a 1.5 mL microcentrifuge tube.

25 Add **600 µl** of **N/C buffer** to tissue in a 1.5 mL microcentrifuge tube.

26 Homogenize tissue using plastic microcentrifuge pestles. A Chemglass Life SciencesSupplier BioVortexer Mixer [Fisher Scientific: 50-153-2378] can be used. This should be done in ice but you can take it out for visual inspection. The timing will be based on tissue type and size.

BioVortexer Mixer
by Fisher Scientific
Catalog #: 50-153-2378

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RNase-Free Disposable Pellet Pestles, With tube
by Thermo Fisher
Catalog #: 12141368

Filter Nuclei and Lipids 30m

27 Place the filter catridge that comes with the kit onto the collection tube. Pour the homognized tissue and liquid into the filter. Pipette any remaining liquid into the filter.

28 Incubate the filter cartridge with the cap open in a freezer at **-20 °C** for **00:20:00** 20m

28.1 The timing can be optimized if you do not have a thermometer. Add **0.5 mL** of ddH2O in a 1.5 mL microcentrifuge tube and incubate in the freezer until the liquid freezes. This is the time that can be used.

29 Place the tube with cap open in a microcentrifuge. Centrifuge at **500 x g, 4°C, 00:03:00** . Increase to **1000 x g, 4°C, 00:01:00** if there is some liquid retention. You will see fat stuck on top of filter. The supernatant below will contain the nuclei.

30 Discard the filter and close the microcentrifuge tube. Vortex briefly to mix up the nuclei. Make sure you know where the pellet will end up based on the placement inside the centrifuge. The nuclei pellet will be white or almost invisibible. Centrifuge at **1000 x g, 4°C, 00:04:00** .

31 The supernatant can be saved if needed. It is the cytosolic fraction. I did not have a visible nuclei pellet but I marked where the pellet would be. Carefully aspirate the liquid and leave some behind if needed. Resuspend nuclei in **50 µl Nuclei Wash and Resuspension Buffer.**

32 Transfer nuclei to a clean microcentrifuge tube to avoid lipid contamination leftover on the tube walls.

33

The nuclei can visualized using trypan blue. You will notice lipid contamination still, which can be removed using FACS.

LipoAsp_Fresh_20x_1.tif

Isolated nuclei from fatty tissue.

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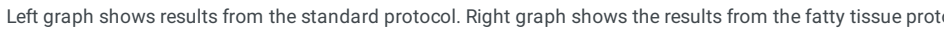
While both protocols will result in many lipid droplets in addition to the nuclei, the fatty tissue protocol will have a higher nuclei count overall.

Comparison.tiff



Left graph shows results from the standard protocol. Right graph shows the results from the fatty tissue protocol.

35 **go to step #22** for sorting prior to 10x Genomics Single Cell protocol.

Cryopreservation 1h 30m

36 Add **900 µl** RNALater to **100 µl** nuclei in **Nuclei Wash and Resuspension Buffer**, incubate at **4 °C** for 1h **01:00:00** then transfer to **-80 °C**

37 To remove RNALater, centrifuge nuclei at **4000 x g, 4°C, 00:10:00** . Aspirate RNALater and resuspend in **Nuclei Wash and Resuspension Buffer** 10m

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