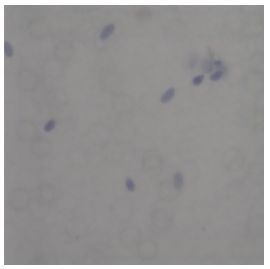
Sep 22, 2020

Nuclei Isolation for Single-Nuclei RNA sequencing



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

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Keywords: single-cell, single-nuclei, nuclei isolation, fatty tissue, adipose tissue, liposarcoma, sarcoma, PDX, nuclei from frozen liposarcoma tissue, nuclei isolation kit, nuclei isolation for single, nuclei rna, nuclei isolation, frozen liposarcoma tissue, nuclei ez prep, rna, nuclei, cultured adipocyte, sequencing, adipose tissue, fattier tissue,

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**.

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

MATERIALS

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

STEP MATERIALS

- pluriStrainer 40 µm **pluriSelect Life Science** **Catalog #**43-50040
- Nuclei EZ lysis buffer **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**EZ PREP NUC-101
- Minute Nuclei and Cytosol Isolation Kit for Adipose Tissues/Cultured Adipocytes **Fisher Scientific** **Catalog #**AN-029
- BioVortexer Mixer **Fisher Scientific** **Catalog #**50-153-2378
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- pluriStrainer Mini 70 UM **Fisher Scientific** **Catalog #**NC1444112

Consumables:

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

Protocol materials

- RNase-Free Disposable Pellet Pestles, With tube **Thermo Fisher Catalog #**12141368
- Nuclei Isolation Kit: Nuclei EZ Prep **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**NUC-101
- BioVortexer Mixer **Fisher Scientific Catalog #**50-153-2378
- pluriStrainer Mini 70 UM **Fisher Scientific Catalog #**NC1444112
- Protein LoBind Tubes, 1.5 mL **Eppendorf Catalog #**0030108116
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Before start

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.

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

Nuclei EZ lysis buffer **Merck MilliporeSigma (Sigma-Aldrich) 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 **Fisher Scientific Catalog #**50-153-2378

RNase-Free Disposable Pellet Pestles, With tube **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 **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 .

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 **pluriSelect Life Science Catalog** #43-50040

Visual Inspection and Sorting

1h

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.

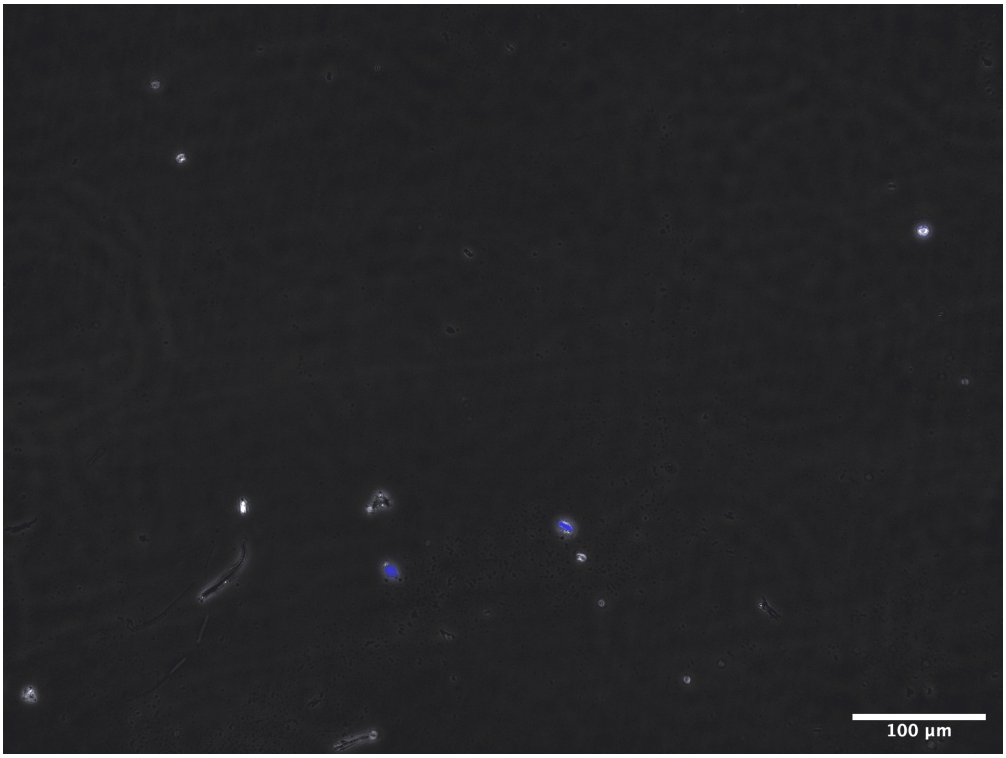
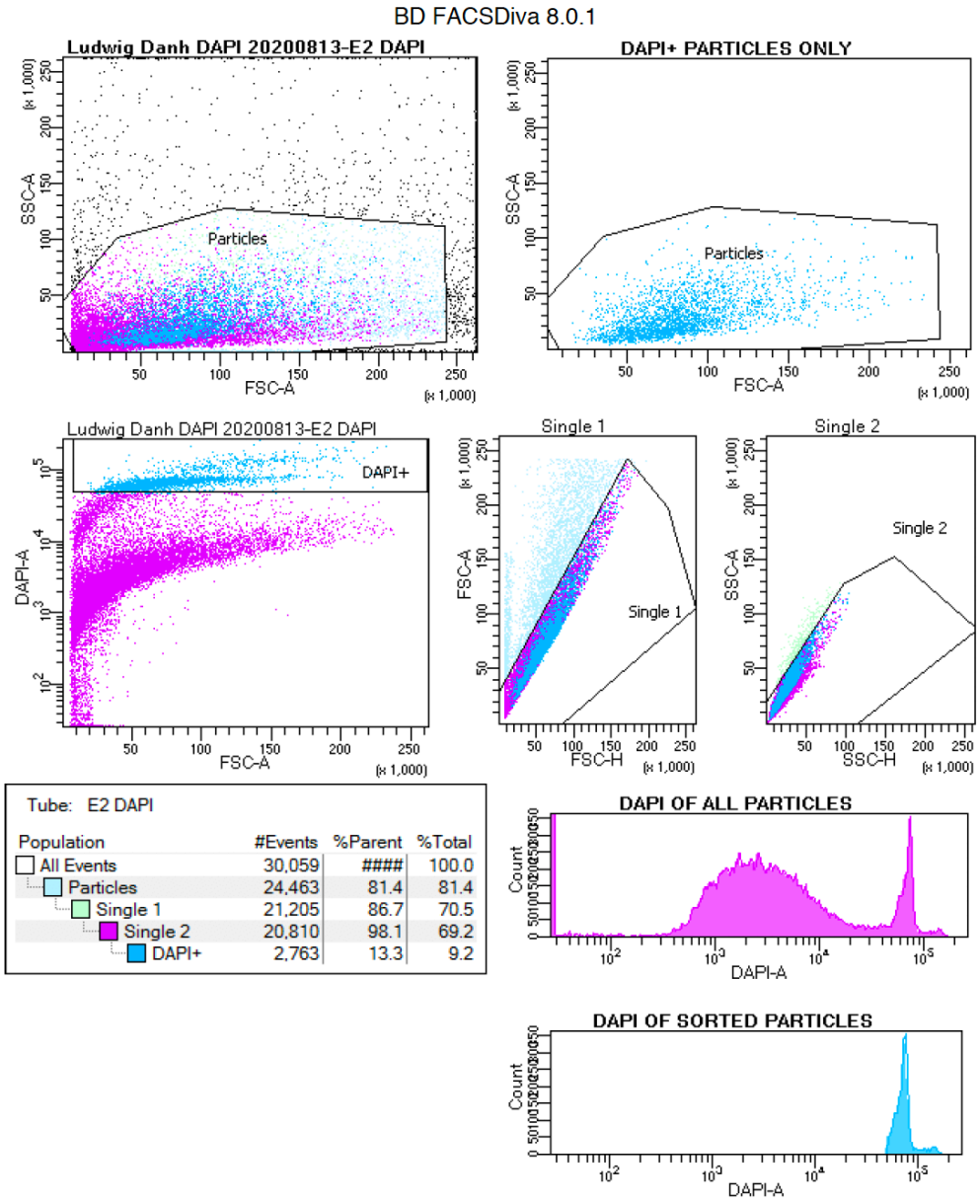


Image of isolated nuclei. There is significant debris prior to sorting.

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

45m



Report of DAPI sorted particles

21 Nuclei can be visualized after sort to confirm success.

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 **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 **Fisher Scientific Catalog #**50-153-2378

RNase-Free Disposable Pellet Pestles, With tube **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.

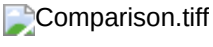
31 The supernatant can be saved if needed. It is the cytosolic fraction. I did not have a visible nuclei pellet but I marked where the pellet would be. Carefully aspirate the liquid and leave some behind if needed. Resuspend nuclei in 50 µL **Nuclei Wash and Resuspension Buffer.**

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.

Isolated nuclei from fatty tissue.

34 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.



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 01:00:00 then transfer to -80 °C

1h

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