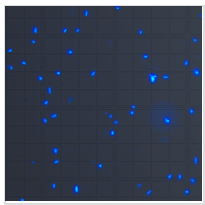

Mar 23, 2022

Adaption for small samples - Nuclei isolation from snap-frozen tissue for single nucleus RNA Sequencing

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Tendon Seed Network

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Previously, we described a robust protocol for the isolation of nuclei from snap frozen fibrous tissues, including tendon and ligament, for use in single nucleus RNA Sequencing. This use of snap-frozen tissue enables tissues that are collected from different centres or/and over a period of time to be processed together. Therefore, this is not only convenient for prevention of a stress response and for collecting and processing tissue (especially tissues that are not very accessible), but it might also reduce batch effects by preventing the need to process tissues fresh on different days.

Jolet Y Mimpen, Claudia Paul, Tendon Seed Network, Adam Cribbs, Sarah Snelling. Nuclei isolation from snap-frozen tendon tissue for single nucleus RNA Sequencing.
http://dx.doi.org/10.17504/protocols.io.bc6xizfn

The collection of (healthy) human tissues is difficult, meaning that there are often only small pieces available for research. Therefore, we present an adapted protocol for the isolation of nuclei for single nucleus RNA sequencing from small pieces of tissue (biopsies and tissues up to ~100mg). This protocol makes use of smaller tubes and small volumes of buffers, thereby reducing nuclei loss through transfer steps, leading to great nuclei yields per tissue weight.

This protocol was adapted from Slyper *et al.* (2020) Nature Medicine.

Citation: Jolet Y Mimpen, Claudia Paul, Tendon Seed Network, Adam Cribbs, Sarah Snelling Adaption for small samples - Nuclei isolation from snap-frozen tissue for single nucleus RNA Sequencing https://dx.doi.org/10.17504/protocols.io.rm7vzyx75lx1/v1

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Nuclei isolation, tissue digestion, single nucleus RNA Sequencing, tendon, fibrous tissue, single nuclei RNA Sequencing

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Due to the small surface area of the recommended strainer, we do not recommend to use this protocol with more than ~100mg of tissue. However, the maximum volume/weight of tissue used may need to be optimised for each tissue type. If more than 100mg of tissue needs to be used, we recommend using our original protocol:

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Materials:

- 5.0 mL Eppendorf tubes
- Petri dish
- Forceps
- Scalpels
- 40 µm mini cell strainer (pluriStrainer Mini 40 µm, cat no. 43-10040-40)
- Neubauer Improved Haemocytometer (NanoEntek DHC-N01)
- 1.5 mL eppendorf tubes
- 0.5 mL eppendorf tubes
- Weighing boat (optional)
- P10, P200, and P1000 (low retention) filter tips
- Wide bore P1000 filter tips (or P1000 filter tips with the tip cut off)

NOTE: All used materials need to be sterile

Reagents:

- BSA (Sigma, cat no. A7030)
- D-PBS (Sigma, cat no. D8537)
- Water, RNase and DNase free (Invitrogen, cat no. 10977-035)
- 1M Tris-HCl pH 7.5
- 2M NaCl
- 1M CaCl2
- 1M MgCl2
- CHAPS hydrate (Sigma, cat no. C3023)
- RNaseIn Plus (Promega, cat no. N2615)
- SUPERase In (Invitrogen, cat no. AM2696)
- cOmplete tablet (Roche, cat no. 05892791001)
- DAPI (Thermo Fisher Scientific, cat no. 62248)

Equipment:

- Rotor
- Swinging bucket centrifuge (for 15 mL tubes)

Other:

- Dry ice
- Ice

Scalpel

- Do not reuse or resterilize. Discard after one procedure. Structural integrity and/or function may be impaired through reuse or resterilization.
- Do not use device that has been damaged in any way. Damaged device may cause complications.
- Device blade is sharp. Handle with care. Do not touch cutting edge.
- Scalpels must be disposed of in a yellow sharps box.

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Dry ice

- Avoid contact with skin and eyes. Wear eye protection or safety glasses
- Use and store only in well-ventilated areas
- Do not store dry ice in airtight containers
- Contact with water releases carbon dioxide (CO_2)
- Do not discard in sinks
- Do not save or reuse dry ice leftovers in ice buckets

Preparation

1 **Prepare and pre-cool all reagents, materials, and equipment:**

- Get a box of ice to cool down buffers and tubes
- Get a box of dry ice. Put a Petri dish on the dry ice to allow it to cool. Stick a scalpel and a pair of forceps in the dry ice to allow them to cool.
- Let swinging bucket centrifuge with 15mL tube inserts cool down to 4ºC.

All steps need to be carried out on ice or in a cold room (4ºC) to minimise RNA degradation.

2 If running the experiment beyond step 5, prepare the buffers:
We recommend making all the buffers fresh on the day.

Prepare 2x Salts and Tris (ST) buffer. For 5 mL, mix:

- 0.73 mL of 2M NaCl
- 0.10 mL of 1M Tris-HCl pH 7.5
- 0.01 mL of 1M CaCl2
- 0.21 mL of 1M MgCl2
- 3.95 mL sterile H2O

Mix well and store on ice until use.

Prepare 1% CHAPS. For 10 mL, mix:

- 10 ml sterile H2O
- 0.1g CHAPS hydrate

Mix well and store on ice until use.

Prepare 1X CST

- 5.0 mL 2X ST
- 4.9 mL 1% CHAPS
- 100 µL 1% BSA
- 40 µL SUPERase IN
- 40 µL RNasein Plus
- 1 cOmplete tablet

Mix well and store on ice until use.

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Prepare PBS + 1% BSA. For 20 mL, mix:

- 20 mL PBS
- 0.2g BSA

Mix well and store on ice until use.

Tissue preparation

3 Take snap-frozen tendon tissue out of -80ºC or LN2 and put on dry ice to keep frozen

3.1 *Optional:* weigh tissue. This should be done at this point and not after the tissue is cut into small pieces as the tissue pieces will thaw straight away.

IMPORTANT NOTE: for this protocol, we do not recommend using more than ~100mg of tissue (especially fibrous tissues such as tendon). If large pieces of tissue are to be used, please use our original protocol:

> Jolet Y Mimpen, Claudia Paul, Tendon Seed Network, Adam Cribbs, Sarah Snelling. Nuclei isolation from snap-frozen tendon tissue for single nucleus RNA Sequencing.
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4 Use pre-cooled forceps to hold tissue and use scalpel to cut very thin slices of tissue and then cut these slices into small pieces (approx. 1-2mm in diameter). The thinner the pieces, the more efficient the lysis step will be.

5 Add tissue to a pre-labelled 5.0mL Eppendorf tube that has been pre-cooled on dry ice. Store on dry ice.

Now there are two options:

Store samples
Store at -80°C until use. On day of use, make sure that you thaw the tube with the warmth of your hands before continuing to step 6. You know that the pieces of tissue start to defrost when the pieces of tissue start sticking to the sides of the tubes.

Continue
If sample is to be digested and lysed immediately, continue to step 6.

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6 Add 1.5 mL of 1X CST buffer. Put the tube on a rotor in the coldroom to ensure it stays cold. Leave for 10 minutes to incubate.

Straining sample

7 ***! Make sure that all these steps are done on ice to keep the samples cold !***

Strain the sample into a clean 5.0 mL Eppendorf tube as follows:

7.1 Put a 40 µm mini cell strainer into a clean pre-labelled 5.0 mL Eppendorf tube. Slightly re-suspend the lysed tissue using a wide bore P1000 tip and transfer the tissue/buffer-mixture to the strainer.

7.2 Add 1 mL of PBS + 1% BSA to the tube used for tissue digestion to wash out any remaining tissue and nuclei; transfer to strainer. Repeat this step once for a total of 2 washes.

Please note: If it takes a long time to filter the liquid through the strainer, this wash step can also be reduced to 1 wash of 1 mL of PBS + 1% BSA. If this is the case, an additional 1 mL of PBS + 1% BSA should be added to the final mixture in step 7.5 (i.e. add a total of 2 mL of PBS + 1% BSA in step 7.5).

7.3 If a lot of liquid remains in the strainer, either carefully move the tissue around using a pipette tip, resuspend the tissue/buffer-mixture in the strainer using a wide bore pipette tip, and/or use the end of a sterile 1.5 mL Eppendorf tube to gently press the liquid through the strainer.

7.4 Take off the strainer and use a pipette to transfer any remaining liquid on the bottom of the strainer into the 5.0 mL Eppendorf tube. Dispose of strainer.

7.5 Add another 1 mL of PBS + 1% BSA to the tube to further dilute the lysis buffer.

Centrifugation and resuspending 5m

8 Put 5.0 mL Eppendorf tube in a pre-cooled centrifuge: **500 x g, 4°C, 00:05:00** 5m

9 During centrifugation, make up a DAPI solution in PBS+1%BSA. We recommend to start with a 500x dilution of DAPI (1 µL of DAPI + 499 µL of PBS+1%BSA), so that the nuclei solution can be

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diluted 2x for counting. However, other dilutions can be chosen depending on the expected yield/concentration:

For high yield/concentration:
If expected yield is high, we recommend to dilute the solution at least 4x. We would recommend to add 9mL of 750x DAPI to 3mL of nuclei (4x dilution)

10 After centrifugation, pour off supernatant and carefully rest the rim of the tube on a paper towel to remove any liquid drops around the top of the tube.

11 Centrifuge the tubes again for ±20 seconds at 500xg at 4°C to get all remaining liquid to the bottom. This will help to accurately determine the remaining nuclei in the next steps.

Counting nuclei 5m

12 Resuspend the nuclei and use a pipet to determine how much volume is left in the tube.

!** Make sure to keep samples on ice when not being handled **!

13 Add 6 µL of the 500x diluted DAPI solution to a 0.5 mL Eppendorf tube. Take off 6 µL of the resuspended nuclei solution and add to 0.5mL Eppendorf tube (to get to a final DAPI concentration of 1:1000). Leave to incubate for 5 minutes.

***Important**: change the relative volumes if another dilution of DAPI and nuclei is used!*

14 Add 10 µL of the nuclei/DAPI solution to one well of a Neubauer Improved haemocytometer.

15 Count nuclei in three 1x1mm squares. Calculate the concentration:

(total number of counted nuclei / 3 squares) x dilution factor (2) x 10^4 = nuclei / mL

Optional: Determine total nuclei yield:

total volume in mL (as determined in step 12) x concentration (as determined above) = total number of nuclei

For more information about Neubauer Improved haemocytometer and an example of the

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counting-grid, please visit:
http://nanoentek.com/theme/nanont2_en/shop/02/product01_view.php?it_id=1547539282#detail_down

Dilution for library prep 5m

16 Proceed to library preparation immediately.

If necessary, further dilute the nuclei solution for the 10X library prep with PBS + 1% BSA.

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