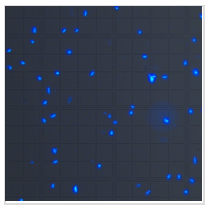

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Nuclei isolation from snap-frozen tendon tissue for single nucleus RNA Sequencing

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Next generation sequencing, especially single cell RNA Sequencing (scRNA-Seq) using enzymatic digestion, has revolutionised our ability to study tissues. However, several problems remain as these techniques have traditionally been used for tissues from which substantial cell numbers are easily liberated. Tissues with low-cellularity and a substantial extracellular matrix present a significant challenge for enzymatic scRNA-Seq approaches due to stress responses to digestion. Here, we describe 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.

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

DOI

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Materials:

- 50 mL Falcon tubes
- 15 mL Falcon tubes
- Petri dish
- Forceps
- Scalpels
- 40um cell strainer PET-mesh (pluriStrainer, cat no. 43-50040-01)
- Neubauer Improved Haemocytometer (NanoEntek DHC-N01)
- 1.5 mL eppendorf tubes
- 0.5 mL eppendorf tubes
- 10 mL syringe (optional)
- Weighing boat (optional)

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

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.
- Scalepls must be disposed of in a yellow sharps box.

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

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- 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 H_2O

Mix well and store on ice until use.

Prepare 1% CHAPS. For 10 mL, mix:
- 10 ml sterile H_2O
- 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.

Prepare PBS + 1% BSA. For 50 mL, mix:
- 50 mL PBS
- 0.5g BSA

Mix well and store on ice until use.

Tissue preparation

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3 Take snap-frozen tendon tissue out of -80ºC or LN_2 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 they will thaw straight away.

4 Use pre-cooled forceps to hold tissue and use scalpel to cut very thin slices of tissue and then cut these slices into smal pieces (approx. 1-2mm in diameter). The smaller the pieces, the more efficient the lysis step will be.

5 Add tissue to pre-cooled 50mL tube. Now there are two options:

Store samples
1. If sample is to be stored at this point, make sure the 50 mL tube was cooled on dry ice while cutting to make sure the tissue does not defrost during the transfer. 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
2. If sample is to be digested and lysed immediately, continue to step 6.

6 Add 4 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 Strain the sample into a clean 50 mL tube:

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

7.1 Put 40 µm strainer in pre-labelled clean 50mL tubes and pour digested/lysed tissue solution through the strainers.

7.2 Add 2x 2 mL of PBS + 1% BSA to the tubes/well to wash out any remaining nuclei and strain through strainer.

7.3 Use the end of a plunger of a 10 mL syringe to gently press on the tissue to make sure all the liquid/cells/nuclei come out of the tissue.

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7.4 Take off the strainer and pipette the remaining liquid on the bottom of the strainer into the 50mL tube. Dispose of strainer.

8 Transfer the ±8mL of nuclei suspension into a pre-labelled 15 mL tube. Add 4 mL of PBS + 1% BSA to the 50 mL tube to remove any remaining nuclei, transfer to 15 mL tube.

Centrifugation and resuspending

9 Put 15mL Falcon tube in a pre-cooled centrifuge **500 x g, 4°C, 00:05:00**

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

For low yield/concentration:
If the expected yield is low, try to dilute the solution as little as possible. We recommend adding 1mL of 100x DAPI to 9mL of nuclei (1.1111x dilution)

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)

11 Pour off supernatant and tap on a paper towel to remove any liquid drops around the rim of the tube.

12 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.

13 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 **!

Counting nuclei

14 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

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concentration of 1:1000). Leave to incubate for 5 minutes.

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

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

16 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 13) x concentration (as determined above) = total number of nuclei

For more information about Neubauer Improved haemocytometer and an example of the counting-grid, please visit:
http://nanoentek.com/theme/nanont2_en/shop/02/product01_view.php?it_id=1547539282#detail_down

Dilution for library prep

17 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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