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🌐 Single Nuclei RNA Sequencing of Tendon Tissue - small tissue biopsies

Adam Cribbs¹, Jolet Y Mimpén¹

¹University of Oxford

Tendon Seed Network

Adam Cribbs

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External link:
<https://chanzuckerberg.com/science/programs-resources/humancellatlas/seednetworks/the-tendon-seed-network/>

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Protocol status: In development
We are still developing and optimizing this protocol

Created: Nov 26, 2019

Last Modified: Mar 03, 2020

ABSTRACT

This protocol is used the the Tendon Seed Network to extract nuclei from small tissue biopsies of Tendon tissue. For larger pieces of tissue we use the GentleMACS protocol.

This protocol is an iteration of various nuclei extraction protocols (Martelotto, Humphreys, Macosko) developed specifically for tendon single-cell RNA-seq experiments, using drop-seq based techniques. The protocol can be used to isolate nuclei from both small (less than 3mmx3mm) pieces of tissue. The protocol has an optional (but recommended) FACS based isolation step if the sample contains lots of debris that can make nuclei counting difficult and sample blockages in the microfluidics more likely. However, this leads to significantly increased loss of nuclei so you may not have enough to perform your single-nuclei experiments at the end of the protocol.

The protocol used has been developed iteratively using healthy snap-frozen hamstring tendon but should be amenable for any tendon.

The protocol is used routinely within the tendon seed network for single nuclei experiments using drop-seq methods.

PROTOCOL integer ID:
30259

Keywords: Tendon, single-nuclei, dronc-seq, nuclei, Tendon Seed Network

GUIDELINES

1. This tissue has only been optimised for tendon samples.
2. It is important to note that further optimisation is required for muscle tissues.
3. It is important that the whole sample should be kept at 4oC throughout the protocol, this includes during the centrifugation steps.
4. FACS purification of nuclei after tissue is important for maintaining good nuclei sequencing.
5. Bypassing the FACS purification leads to low levels of UMIs, false high cell numbers and poor UMAP clustering.
6. We do not recommend freezing the nuclei, as we have obtained our best results when the samples are directly loaded onto the drop-seq. However, we have not extensively evaluated this but we plan on evaluating this soon.

MATERIALS

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Molecular Biology Grade Water Fisher Scientific Catalog
#10154604

15 mL Falcon tubes Contributed by
users

RNasin(R) Plus RNase Inhibitor, 10,000u Promega Catalog
#N2615

Dounce homogenizers Sigma Catalog #D8938-1SET

Nuclei EZ Lysis Buffer Sigma Catalog #N-
3408

cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack Roche Catalog #05 892 79
001

pluriStrainer 200 µm pluriSelect Catalog #43-
50200

pluriStrainer 40 µm pluriSelect Catalog #43-
50040

Falcon tube (50 mL) Contributed by
users

Propidium iodide staining solution BD Biosciences Catalog
#556463

PBS Invitrogen - Thermo Fisher

SUPERase• In™ RNase Inhibitor (20 U/µL) Thermo Fisher Scientific Catalog #cat
AM2694

Dissection kit Contributed by
users

DAPI Thermo Fisher Scientific Catalog #62248

Dissection Tray Contributed by
users

Safety Scalpels, Scalpel No. 20 Thermo Fisher Catalog
#22079720

SAFETY WARNINGS

Watch your hands when using the scalpel/razor
blade

BEFORE START INSTRUCTIONS

Buffers and Solutions

Nuclear Isolation Buffer (NIB)

- 10mM Tris-HCl
- 10mM NaCl
- 3mM MgCl₂
- 0.1% NP40

For 40mL mix:

- 400µL 1M Tris-HCl
- 200µL 2M NaCl
- 120µL 1M MgCl₂
- 800µL 5% NP40
- 38.48 mL sterile water

Nuclear Lysis 1 (NL1) buffer

10mL of NIB buffer

1 tablet of cOmplete ULTRA

40 uL of RNasin Plus

40 uL of SUPERaseIN

Nuclear Lysis 2 (NL2) buffer

4mL of NIB Buffer

4 uL of RNasin Plus

4 uL of SUPERaseIN

Nuclei wash (NW) buffer

2mL of 1X PBS

2 uL of RNasin Plus

Cell sorting buffer

1mL of 1X PBS

1 uL of DAPI

Preparation

- 1 Pre-cool all solutions and equipment (including centrifuge (+4°C), homogeniser (+4°C), dissection Tray (-20°C), scissors (-20°C) and scalpel/razor blade (-20°C)).

Note

All steps in the protocol should be performed on ice or in a cold room (4°C) to minimise RNA degradation

Large tissue digestion

- 2 Chop up tissue into 4 pieces on a cold dissection tray. Use scissors and razor blades that have been chilled to -20°C. The best approach to begin with is to use a razor blade and shave off a small piece of tissue. Then cut this tissue into smaller pieces and set aside on the dissection tray. Sometimes tendon can be very “stringy” and this may require the use of dissection scissors.
- 3 Scrape the tissue into a Dounce homogeniser from the dissection tray.
- 4 Add 2-4 ml of NL1 buffer (We usually start with 2ml and then add more if required)
- 5 Pipette the tissue up and down 10 times using a wide bore P1000 tip (Slightly cut the top of the tip off a P1000).

Tissue homogenisation

- 6 Transfer the sample to a 7mL or smaller Dounce homogeniser. (If any sample remains in the dissection tray add NL1 buffer to this and add to Dounce)
- 7 Homogenise the sample using pestle A and stroke 20 times.

8 Place the homogeniser back onto ice and wait 5 mins.

9 Optional: Homogenise the sample using pestle B and stroke 20 times.

10 Filter the sample into a 50mL Falcon Tube passing through a 200uM strainer.

11 Incubate on ice for 5 mins.

12 Centrifuge the tube at 500g for 5mins at 4°C.

13 Remove the supernatant and resuspend in 4mL of NL2 buffer.

14 Incubate on ice for 5 mins.

15 Filter the sample into a 50mL Falcon Tube passing through a 40uM strainer.

16 Centrifuge at 500g for 5 mins at 4°C.

500 x g, 4°C,
00:05:00

17 Discard the supernatant.

18 Resuspend pellet in 2mL of NW buffer.

19 Centrifuge at 500g for 5 mins at 4°C.

500 x g, 4°C,
00:05:00

20 Discard the supernatant.

21 Resuspend pellet in 0.5-1mL of Cell sorting buffer.

22 Proceed to FACS sorting of nuclei.

FACS purification of nuclei

23 FACS purification was performed using a Sony SH800 cell sorter. With our current laser settings we are unable to sort DAPI so have relied on DAPI staining of nuclei and sorting based on FSC and FL3. An example gate is shown below of a sample that shows a high level of debris:

Sample showing high levels of debris

Post-FACS purification of nuclei

- 24 Sort nuclei into FACS tubes containing 1mL of NW buffer.
- 25 Centrifuge nuclei at 300g for 10 mins.
- 26 Resuspend in appropriate volume of NW buffer so that there is a ~1000 nuclei/uL.

Note

A reliable nuclei count can be difficult, but we suggest that this is performed using trypan blue and confirmed using an automatic cell counter if available.

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- 27** Nuclei that have been counted are now ready for loading into a 10X or drop-seq experiment, according to the manufacturers conditions.