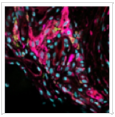


Mar 03, 2020

Single Nuclei RNA Sequencing of Tendon Tissue - small tissue biopsies

Adam Cribbs[1], Jolet Mimpen[1]

[1]University of Oxford

In Development dx.doi.org/10.17504/protocols.io.9sth6en

Tendon Seed Network

Adam Cribbs

ABSTRACT

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

EXTERNAL LINK

https://chanzuckerberg.com/science/programs-resources/humancellatlas/seednetworks/the-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

NAME	CATALOG #	VENDOR
Molecular Biology Grade Water	10154604	Fisher Scientific
15 mL Falcon tubes		
RNasin(R) Plus RNase Inhibitor, 10,000u	N2615	Promega
Dounce homogenizers	D8938-1SET	Sigma
Nuclei EZ Lysis Buffer	N-3408	Sigma
cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack	05 892 791 001	Roche
pluriStrainer 200 µm	43-50200	pluriSelect
pluriStrainer 40 µm	43-50040	pluriSelect
Falcon tube (50 mL)		

NAME	CATALOG #	VENDOR
Propidium iodide staining solution	556463	BD Biosciences
PBS		Invitrogen - Thermo Fisher
SUPERase• In™ RNase Inhibitor (20 U/µL)	cat# AM2694	Thermo Fisher Scientific
Dissection kit		
DAPI	62248	Thermo Fisher Scientific
Dissection Tray		
Safety Scalpels, Scalpel No. 20	22079720	Thermo Fisher

SAFETY WARNINGS

Watch your hands when using the scalpel/razor blade

BEFORE STARTING

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^{O}C), homogeniser (+4^{O}C), dissection Tray (-20^{O}C), scissors (-20^{O}C) and scalpel/razor blade (-20^{O}C)`).

All steps in the protocol should be performed on ice or in a cold room (4^{O}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 -20oC. 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.

00:05:00

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.

00:05:00

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

500 x g, 4°C 00:05:00

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

14 Incubate on ice for 5 mins.

00:05:00

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

16 Centrifuge at 500g for 5 mins at 4oC.
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 4oC.
500 x g, 4°C 00:05:00

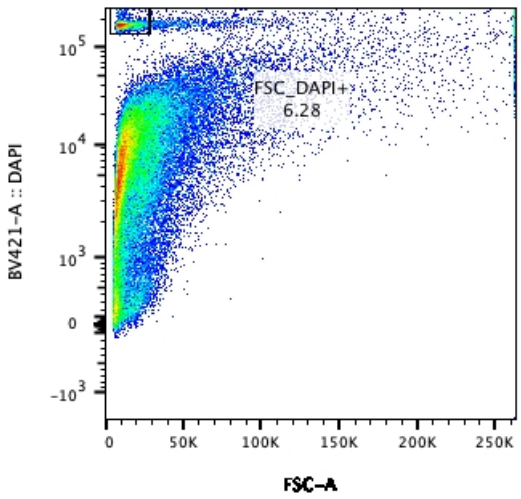
20 Discard the supernatant.

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 debri:



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.

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.

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