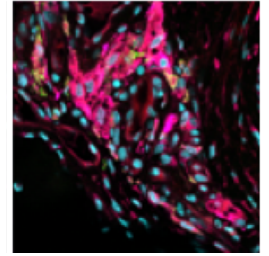


Mar 03, 2020

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

DOI

[dx.doi.org/10.17504/protocols.io.9sth6en](https://dx.doi.org/10.17504/protocols.io.9sth6en)



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Tendon Seed Network



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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:** November 26, 2019

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**Protocol Integer ID:** 30259

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



## 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.

## 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 4°C 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

### MATERIALS

- ⊗ Molecular Biology Grade Water **Fisher Scientific Catalog #10154604**
- ⊗ 15 mL Falcon tubes
- ⊗ RNasin(R) Plus RNase Inhibitor, 10,000u **Promega Catalog #N2615**
- ⊗ Dounce homogenizers **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8938-1SET**
- ⊗ Nuclei EZ Lysis Buffer **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N-3408**
- ⊗ cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack **Roche Catalog #05 892 791 001**
- ⊗ pluriStrainer 200 µm **pluriSelect Catalog #43-50200**
- ⊗ pluriStrainer 40 µm **pluriSelect Catalog #43-50040**
- ⊗ Falcon tube (50 mL)
- ⊗ Propidium iodide staining solution **Becton Dickinson (BD) Catalog #556463**
- ⊗ PBS **Invitrogen - Thermo Fisher**
- ⊗ SUPERase• In™ RNase Inhibitor (20 U/µL) **Thermo Fisher Scientific Catalog #cat# AM2694**
- ⊗ Dissection kit
- ⊗ DAPI **Thermo Fisher Scientific Catalog #62248**
- ⊗ Dissection Tray
- ⊗ Safety Scalpels, Scalpel No. 20 **Thermo Fisher Catalog #22079720**

### Safety warnings

- ⚠ Watch your hands when using the scalpel/razor blade



## Before start

### Buffers and Solutions

#### *Nuclear Isolation Buffer (NIB)*

- 10mM Tris-HCl
- 10mM NaCl
- 3mM MgCl<sub>2</sub>
- 0.1% NP40

For 40mL mix:

- 400μL 1M Tris-HCl
- 200μL 2M NaCl
- 120μL 1M MgCl<sub>2</sub>
- 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.



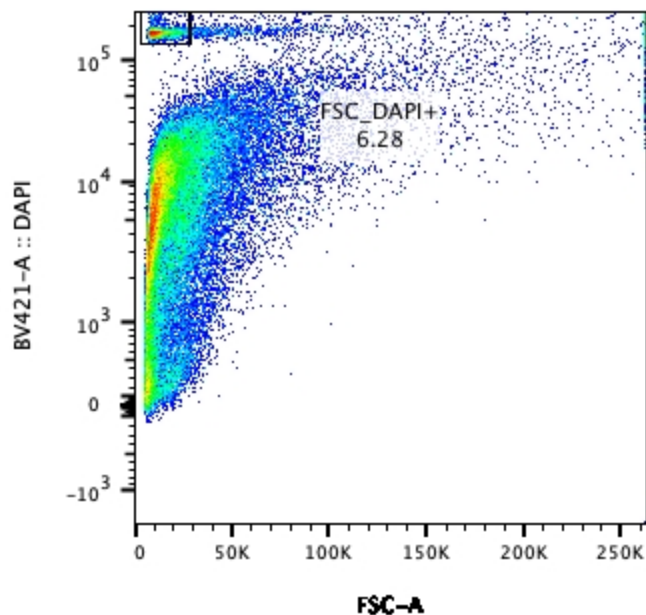
00:05:00

- 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 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.

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