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We use this protocol and it's working

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Preparation of Nuclei Suspension from Human Musculoskeletal Tissues

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ABSTRACT

An adapted version of the 'Frankenstein' protocol (Luciano G Martelotto 2020. ‘Frankenstein’ protocol for nuclei isolation from fresh and frozen tissue for snRNAseq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bqxymxpw) to prepare single nuclei suspensions from freshly frozen human musculoskeletal tissues before nuclei sorting by flow cytometry.

Protocol described for one sample.

This protocol has been used in the following available studies:
PREPRINT: **Distinct myofibre domains of the human myotendinous junction revealed by single nucleus RNA-seq.** Anders Karlsen et al. https://doi.org/10.1101/2022.12.16.519020

GUIDELINES

- Avoid freeze-thawing of tissues
- Keep materials and reagents chilled
- Keep work space RNAse free
- All pipetting steps are performed on ice

MATERIALS

Reagents

- PBS, sterile
- Penicillin-Streptomycin (5,000 U/mL) (15070063, Thermo Fisher Scientific)
- Nuclei Isolation Kit: Nuclei EZ Prep (NUC101-1KT, Sigma-Aldrich)
- PBS without magnesium
- Phosphate Buffered Saline with 10% Bovine Albumin (SRE0036-250ML, Sigma-Aldrich)
- 10x PBS (AM9625, ThermoFisher Scientific)
- 1 M $MgCl_2$ (M1028-10X1ML, Sigma)
- Protector RNase Inhibitor (03335399001, Roche)
- Trypan Blue Solution, 0.4% (15250061, Thermo Fisher Scientific)

Materials

- Scalpel
- 50 ml tube
- 15 ml tube
- Cryotube
- 2 ml screw cap tubes
- 2 ml microcentrifuge tubes
- 1.5 ml microcentrifuge tube
- 2.3 mm diameter stainless steel (11079123ss, BioSpec Products)
- 1.0 mm silicon carbide beads (11079110sc, BioSpec Products)
- pluriStrainer Mini 70 µm (Cell Strainer) (43-10070-40, PluriSelect)
- pluriStrainer Mini 40 µm (Cell Strainer) (43-10040-40, PluriSelect)

Equipment

- Liquid nitrogen
- Freezer cabinet or dry ice
- MP Biomedicals FastPrep-24
- Microcentrifuge with a cooling function
- Centrifuge that can hold 15 ml tubes and has a cooling function
- Neubauer Improved haemocytometer

BEFORE START INSTRUCTIONS

- Pre-cool centrifuges
- Obtain ice
- Label and pre-chill tubes on ice

Tissue Preparation

1 Collect fresh tissue in a 50-ml tube containing 25 mL ice-cold 1X phosphate-buffered saline containing 50 U/ml penicillin and 50 µg/ml streptomycin and keep On ice for transport.

2 Using a scalpel, dissect the tissue as desired On ice.

Note

E.g. For hamstring tendons, use a scalpel to scrape off any muscle fibres. For muscle tissues, cut away tendon tissue.

3 Cut up tissue into small pieces of around 100 μm^3, remove excess PBS from tissue pieces and snap freeze in cryotubes in liquid nitrogen. Store samples at -80 °C.

Note

Each tissue sample may be divided into multiple small pieces, each frozen separately.

4 In a freezer cabinet or on dry ice, cut each piece of frozen tissue into smaller pieces using a scalpel. Transfer ~ 50 mg tissue into a 2 ml screw cap tube containing homogenising beads.

Note

E.g. For tendon tissue use five stainless steel balls of 2.3 mm. For muscle tissue use five stainless steel balls of 2.3 mm and an additional 1.0 mm silicon carbide bead.

5 Return samples to storage at -80 °C until ready for homogenisation.

Nuclei Wash and Suspension Buffer

6 Freshly prepare Nuclei Wash and Suspension Buffer (2% bovine serum albumin in PBS without magnesium, 2 mM $MgCl_2$, 0.2 U/l RNA inhibitor). Keep On ice.

Note

For each sample prepare 5 ml Nuclei Wash and Suspension Buffer.

Tissue Homogenisation

7 Pipette 1 mL chilled Nuclei EZ lysis buffer **Sigma Catalog #EZ PREP NUC-101** to each 2 ml screw cap tube containing ~50 mg cut up frozen tissue and homogenising beads.

8 Homogenise the tissues at 4.0 M/S for 00:00:20 in a FastPrep 24. Immediately after, transfer the tubes and incubate On ice for 5 min . 20s

9 Repeat homogenisation step. Immediately after, transfer the tubes and incubate On ice for 5 to 15 min for the bubbles to settle.

Note

The tissue may not be completely homogenised. Extra homogenisation steps could lead to the sample overheating. This step will need to be optimised for other tissue types.

10 Transfer the homogenate from each tube to a pre-chilled 2 ml microcentrifuge tube and add 500 µL chilled Nuclei EZ lysis buffer **Sigma Catalog #EZ PREP NUC-101** to each tube. Mix gently using a wide bore 1000 µl pipette. Incubate the tubes On ice and gently mix two more times using a wide bore 1000 µl pipette to help release more nuclei from the remaining tissue.

11 Place a 70 µm cell strainer over a pre-chilled 15 ml tube. Filter all the homogenate from the same sample. After, wash the cell strainer with 1.5 mL chilled Nuclei EZ lysis buffer **Sigma Catalog #EZ PREP NUC-101** . Centrifuge at 500 x g, 4°C, 00:05:00 . 5m

12 Remove as much supernatant as possible without disturbing the pellet. Pipette 1.5 mL chilled Nuclei EZ lysis buffer **Sigma Catalog #EZ PREP NUC-101** to the tube. Using a wide bore 1000 µl pipette gently resuspend the pellet by pipetting up and down 10 times. Transfer 5m

the suspension to a pre-chilled 2 ml microcentrifuge tube. Centrifuge at 500 x g, 4°C, 00:05:00.

13 Remove as much supernatant as possible without disturbing the pellet. Pipette 500 µl chilled Nuclei Wash and Suspension Buffer **without disturbing the pellet**. Incubate On ice for 5 min.

14 Add 1 mL Nuclei Wash and Suspension Buffer. Resuspend the pellet using an uncut 1000 µl pipette tip, gently pipetting up and down 20 times.

15 Place a 40 µm cell strainer over a pre-chilled 2 ml microcentrifuge tube. Filter the nuclei suspension. After, wash the cell strainer with 0.5 mL chilled Nuclei Wash and Suspension Buffer. Centrifuge 500 x g, 4°C, 00:05:00 and resuspend the pellet in the desired volume of Nuclei Wash and Suspension Buffer. Keep samples on ice. 5m

Nuclei Visualisation and Counting

16 Mix a small volume nuclei suspension with Trypan blue. E.g. 10 µl of each. Visualise on a Neubauer Improved Haemocytometer.

Note

Nuclei will be stained blue. They can be distinguished from debris by their clear outlines and bean shape.

Nuclei purification

17 To purify the nuclei suspension from tissue debris, e.g., collagen extracellular matrix in tendon samples, samples may be further purified by fluorescence-activated nuclei sorting or by ultracentrifugation using an iodixanol gradient (DOI: 10.1126/sciadv.abn836).