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# Frozen Tissue Nuclei Extraction (for 10xV3 snSEQ) V.1

Forked from Frozen Tissue Nuclei Extraction (v2)

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**Protocol status:** Working
**This is the most up-to-date version of the single nuclei preparation protocol from our group (Feb 2020) and it is working.**

**Created:** February 15, 2020

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**Keywords:** nuclei extraction, isolation, frozen, single-nuclei sequencing, frozen tissue nuclei extraction, nuclei from frozen tissue, similar extraction protocol from the mccarroll lab, protocol for extraction, extraction protocol, similar extraction protocol, snseq, frozen tissue, nuclei, extraction, mccarroll lab

## Abstract

Protocol for extraction of nuclei from frozen tissue in preparation for single-nuclei sequencing (droplet-based/10X).

**This protocol is based strongly on a similar extraction protocol from the McCarroll lab.**

## Troubleshooting

1 **Nuclei Extraction protocol, optimized for small tissue pieces**
**Macosko Lab, Stanley Center, Broad Institute**

2 **Dissociation Buffer: DB**
A stock of 500mls of Dissociation Buffer (DB) using ultrapure nuclease-free water and these reagents:
$Na_2SO_4$ - 5.83 g
$K_2SO_4$ - 2.615 g
Glucose - 0.905 g
HEPES - 1.2 g
$MgCl_2$- 2.5 ml

3 **Extraction Buffer: ExB**
DB - 15 ml
1% Kollidon - 0.150 mg
0.1% TX-100 - 150 ul
10% BSA - 15 ul
1 tube RNase inhibitor (Lucigen, 10,000 units)

4 **Wash Buffer: WB (30 ml per sample)**
DB - 30 ul
10%BSA - 30 ul
20ul of RNase inhibitor (Lucigen, 10,000 units)

5 **FACS Capture Buffer (20 ul per sample) CDB**
DB - 20 ml
10% BSA- 20 ul
1 tube RNase inhibitor (Lucigen, 10,000 units)

6 **5% BSA–DB for FACS (200 ul per sample)**

7 **Procedure:**
ALL buffers to be cooled to 4$^{o}$C before use
Pre-cool centrifuge to 4$^{o}$C
Chill all tubes and well plates on ice for 20 minutes before use.
Perform all steps on ice.

8 Chill a 26-gauge needle, 40um cell strainer and syringe at 4$^{o}$C at least 20min before use.

9 Coat 12 well culture plate with 1% BSA then wash with DB and fill well with 1ml ExB buffer.

10 Wash frozen tissue sample out of PCR tube with 150ul ExB buffer and deposit into well of well plate.

11 Triturate by pipetting 1ml volume slowly up and down with a 1ml Rainin (tip #30389212) without creating froth/bubbles 20x, then pause 2 mins, repeat 4-5 times (observe progress, add 5th pass only if dissociation appears incomplete).

12 Pass entire volume twice through 26-gauge needle into same well.

13 Transfer extracted sample (~1ml) into a 50 ml falcon tube, pre-coated with 1% BSA. Add 30 ml of wash buffer to dissociate, then split volume between two pre-coated falcon tubes (15+ ml each).

14 Spin down at 600xg for 10 mins 4$^{o}$C (centrifuge should be pre cooled to 4$^{o}$C)

15 Aspirate supernatant until 500ul remains in each tube. Pool these two half samples to 1ml.

16 Pass through a pre-cooled 40um cell strainer and filter (gravity only, no pressure) the sample into a new, clean, pre-cooled falcon tube.

17 Transfer (and measure volume of) filtrate to a 1.5ml Eppendorf tube (pre-cooled).

18 Stain the filtered nuclei by adding DAPI (Thermo, #62248) at 1:1000 dilution.

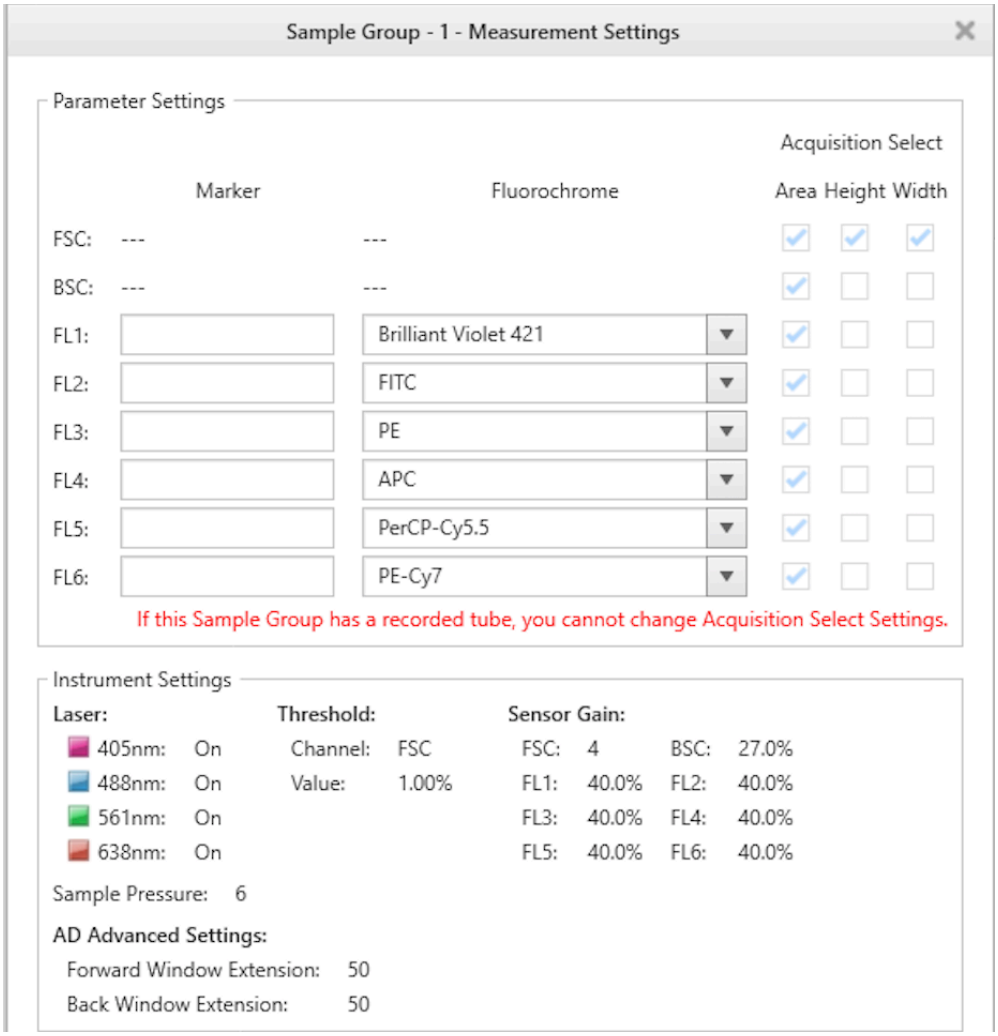
19 **Prepare the collection PCR tubes:**
Coat a 0.2ml PCR tube with 5% BSA. Use the chilled 96 well FACS plate (Sony M800 FACSorter) to capture nuclei into this PCR tube. Pre-fill the PCR tube with 20 ul of CDB to act as a cushion for FACS stream/nuclei.

20 **FACS enrichment for singlets on singlet DAPI peak:**
FACS at speed 6 with forward scatter gain of 1% on DAPI gate. Use “purity” mode. DO not spin down after FACS unless you use the gentle spin protocol (see below).

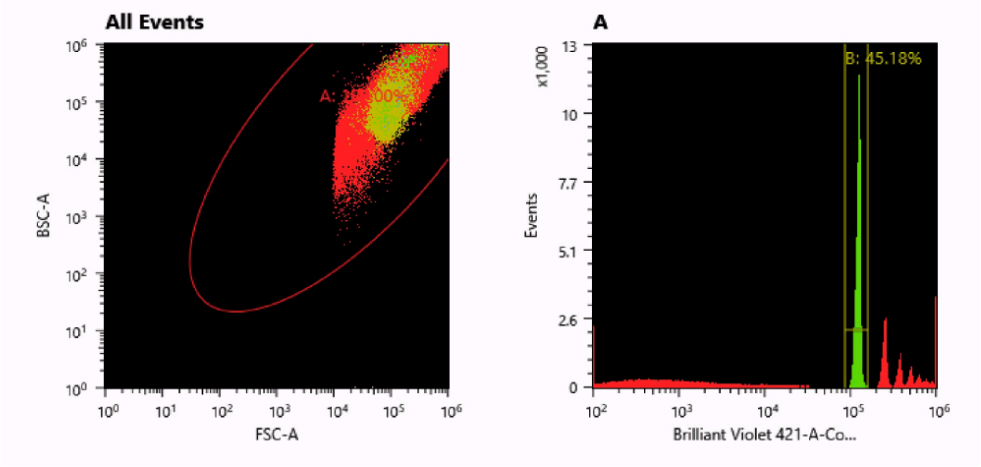
21 **FACS enrichment for singlets on singlet DAPI peak:**

We collect FACS'ed samples in PCR tubes held in a chilled (-20C) 96-well cold block to keep the collection volume as minimal as possible: ( https://www.daigger.com/eppendorf-pcr-coolers-14616-group?gclid=EAIaIQobChMI2rKM7aDN4AIVDRgMCh2jkwRMEAQYBSABEgKeA_D_BwE)

We FACS on a Sony SH800 sorter, using a 70um chip, with these settings:




We gate on the singlet DAPI peak:




22 **Calculate nuclei concentration:**

After FACS, use a pipette to determine the volume in each tube.

Make a 1:10 dilution of your sample: combine 18ul chilled DB with 2ul of nuclei a PCR tube to make a 1:10 dilution. Mix and put into FR hemocytometer.

**Count nuclei with Fuchs- Rosenthal Hemocytometer (16 chambers):**

Visualize with the fluorescent scope, getting images of brightfield and DAPI-excited nuclei. Notice that debris is gone.

Count all 16 large squares to get the most accurate concentration estimate.Calculate the average of the 16 squares. Multiply that number by 10 (accounts for 1:10 dilution) then multiply by 5 (FC hemocytometer factor). This number is your final concentration in nuclei/uL.

23 Proceed to 10X protocol with nuclei. For 10x v3 the input nuclei volume is 46.6, which means the maximum concentration you can input is 364 n/uL. You should input 17k nuclei into (46.6ul) 10X well to maximize data output. If you collect less concentrated

nuclei prep. after FACs than is required, then follow gentle spinning protocol to increase nuclei concentration.

24 **Gentle Spinning Protocol:**

25 Use a 0.5 ml Axygen 321-05-051 tube with 2.0 ml Axygen MCT-200-C tube as tube holder in centrifuge (Labnet PrismR). Coat 0.5 ml tube with BSA, drain very well (0 remaining volume) and cool on ice. Also, cool “holder” tube. This tube and centrifuge combo. are important because they impart a specific g-force to the system, something that was empirically determined to NOT damage the nuclei (as evidenced by actual 10x results).

26 Add your faxed nuclei to cooled tubes. Measure volume, 200 ul max volume.

27 Spin in mini-fuge at 4 degrees C. 1 minute @ 200 xg. Let stop. 1 minute @ 200 xg again. Let stop.

28 Carefully aspirate from top to remove the volume needed to leave desired concentration in 46.6ul 10X input volume. Check aspirate to be sure no nuclei are being lost before you discard (some are lost, but it should be a low number).

29 Gently resuspend nuclei “pellet” before adding to 10X via gentle up and down 2-3 times. Note - There is no actual pellet – that’s why there is minimal damage.