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Frozen Tissue Nuclei Extraction (v2)

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Human Cell Atlas Metho...

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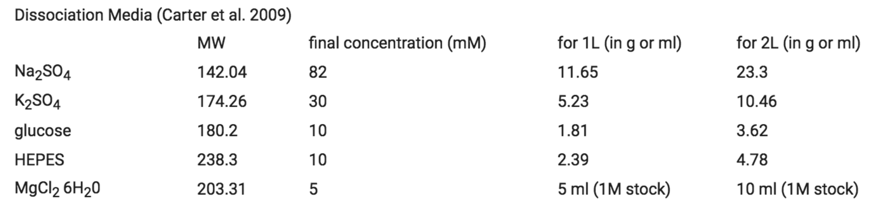
Abstract

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 **Make dissociation buffer**, 50 mLs per sample:



Dissociation Media (Carter et al. 2009)

	MW	final concentration (mM)	for 1L (in g or ml)	for 2L (in g or ml)
Na_2SO_4	142.04	82	11.65	23.3
K_2SO_4	174.26	30	5.23	10.46
glucose	180.2	10	1.81	3.62
HEPES	238.3	10	2.39	4.78
$MgCl_2\ 6H_20$	203.31	5	5 ml (1M stock)	10 ml (1M stock)

2 **Make extraction buffer**, 3 mLs per sample:

Dissociation Buffer + 1% Kollidon VA64 + 1% Triton X100 + 1:40 RNase-inhibitor

3 **Chill all buffers to 4C**, and keep all buffers on ice when in use.

4 **Prepare equipment:**

Set out and chill the following equipment/supplies. The instructions below are for one sample.

- Cold block dissecting tray, kept at -20C
- Chill clean razor blades and glass slides on the cold dissecting tray
- Chill 3 mL syringe with 26 ½ gauge needle, at 4C, one syringe and needle per sample
- Place a 12-well tissue-culture plate, well-bottoms colored with marker, on ice

* Color the underside of the culture plate with dark marker so you can see your sample dissociate

- Place 2 mL chilled extraction buffer in the first well of 12-well plate

- Place ~50 mL chilled DB in a falcon tube on ice

- Locate DAPI (1:1000) (https://www.thermofisher.com/order/catalog/product/D1306)

- Locate 50 µm eppendorf tube filter, place in 4C (https://us.sysmex-flowcytometry.com/consumables/celltrics-filters/sterile-single-pack-celltrics filters/1445/sterile-single-pack-celltrics-filters-50/box?number=04-00 4-2327)

- Place 2 50 mL tubes on ice

- Locate a c-Chip FR hemocytometer

5 **Locate supplies for FACS:**

- 96 well cold block, chilled to -20C: (https://www.daigger.com/eppendorf-pcr-coolers-14616-group?gclid=EAIaIQobChMI2rKM7aDN4AIVDRgMCh2jkwRMEAQYBSABEgKeA_D_BwE)

- 200uL 5% BSA-DB

- 20uL collection buffer: DB with 1:40 RNAse-inhibitor
  * Prepare the collection buffer right before beginning FACS session

6 **Begin extraction. Perform all steps with cold buffers on ice. Nuclei should never leave the ice or 4C environment.**

Chop up tissue on the chilled dissection tray. If your sample is a very small piece (less than 3mm in any dimension) you can forgo this step and place sample immediately into the extraction buffer

Place the frozen sample on the glass slide and shave the frosty/freezer-burned tissue face you want to sample from off the sample and discard. This is damaged and not worth extracting. Then, moving rapidly but carefully, shave off “enough” sample to work with.

Rapidly mince the shaved sample with the 2 chilled razor blades that were used to shave the tissue block and scrape into the first well of a 6-well plate that contains 2 mL of cold extraction buffer. Pipette up and down 20 times.

7

Incubate in detergent with mechanical trituration:

Commence 10-min incubation, pipetting up and down 20x every ~2.5 min with a 1000 uL pipette set to 1000uL.

8 **Perform mechanical dissociation:**

Pull the sample into the chilled needle/syringe and express into the same well for a final mechanical dissociation.
If large chunks remain, repeat this step, pulling the sample into the same syringe and expressing it into the same well.

9 **Large volume wash:**

Extract the nuclei into empty 30ml tube. Add chilled DB to the extracted filtered nuclei to a total volume of just under 30 mL.

10 **Split sample:**

Divide these diluted nuclei among 2 50 mL conical tubes (~15 mL per tube).

11 **Spin tubes** at 600 rcf in a swinging bucket centrifuge for 10 minutes at 4C.

12 **Remove supernatant:**

Carefully aspirate ~13 mLs wash buffer with a serological pipette. To keep from disturbing pellet, gently aspirate the rest of the supernatant with a P1000 pipette. Leave about 500-300uL of sample in the bottom of the tube. Pipette sample to resuspend nuclei, being careful not to introduce bubbles. Pool the appropriate sample pairs back together.

13 **Filter nuclei clumps:**

Wet the 40um eppendorf tube filter with cold DB, pass the ~0.5-1mL of washed sample through the filter into an epi tube.

14 **Stain DNA:**

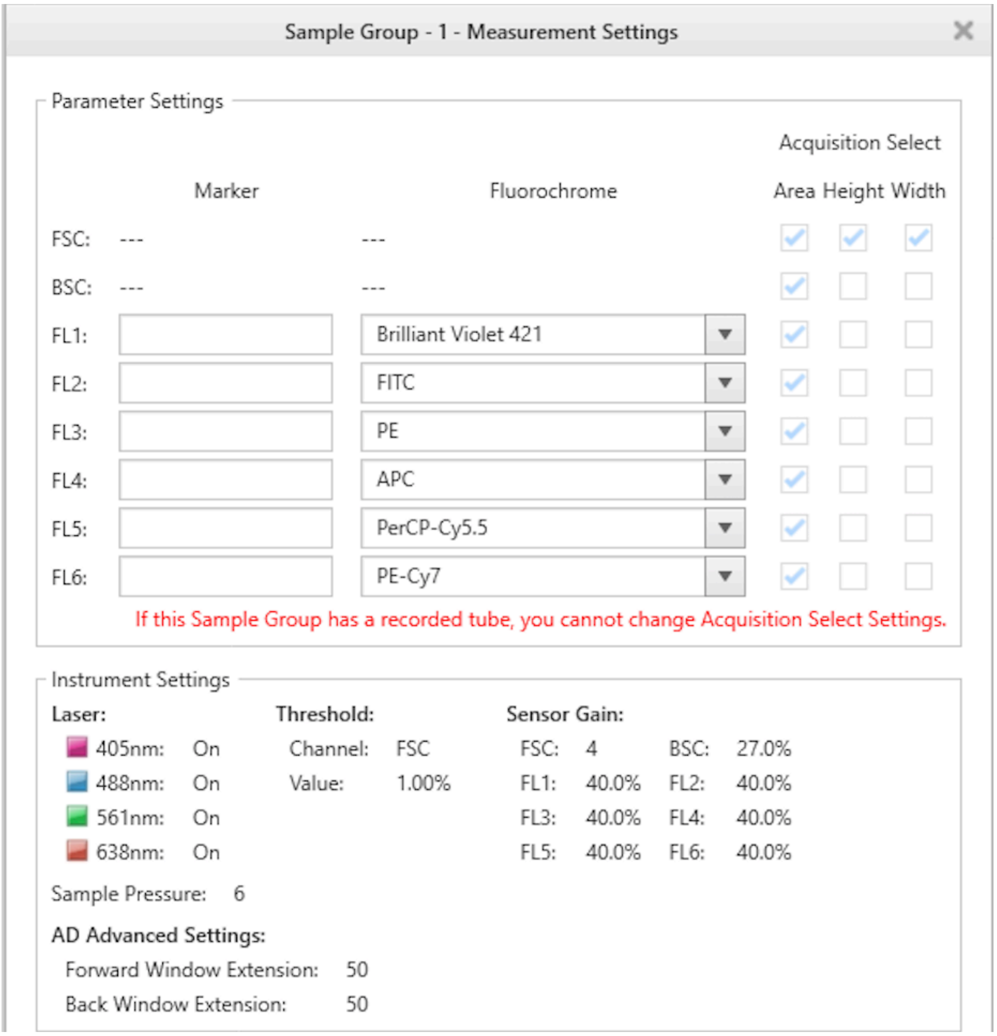
Add 1ul of DAPI to 1ml of nuclei. Remove 20ul and place into FR hemocytometer. Check your nuclei concentration and nuclei quality. Some debris is ok.

15 **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:



Sample Group - 1 - Measurement Settings

Parameter Settings

	Marker	Fluorochrome	Acquisition Select Area	Height	Width
FSC:	---	---	☑	☑	☑
BSC:	---	---	☑	☐	☐
FL1:		Brilliant Violet 421	☑	☐	☐
FL2:		FITC	☑	☐	☐
FL3:		PE	☑	☐	☐
FL4:		APC	☑	☐	☐
FL5:		PerCP-Cy5.5	☑	☐	☐
FL6:		PE-Cy7	☑	☐	☐

If this Sample Group has a recorded tube, you cannot change Acquisition Select Settings.

Instrument Settings

Laser:

- 🟪 405nm: On
- 🟦 488nm: On
- 🟩 561nm: On
- 🟥 638nm: On

Threshold:

Channel: FSC
Value: 1.00%

Sensor Gain:

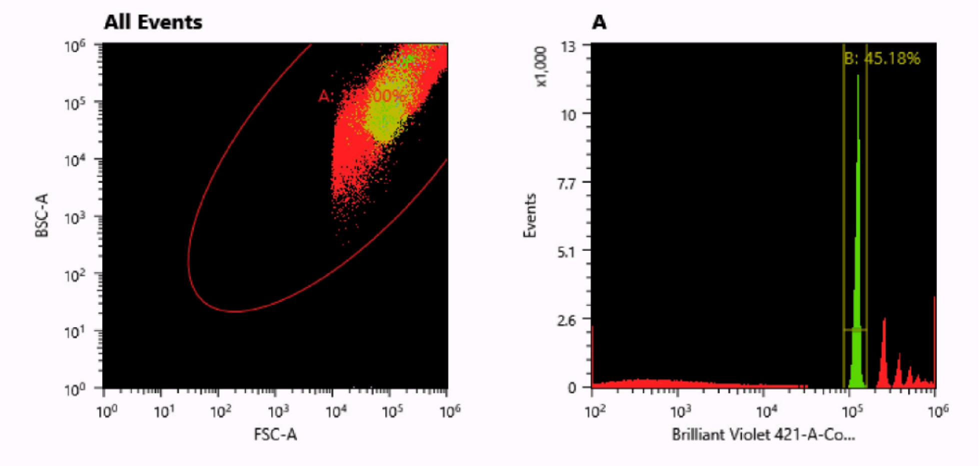
FSC:	4	BSC:	27.0%
FL1:	40.0%	FL2:	40.0%
FL3:	40.0%	FL4:	40.0%
FL5:	40.0%	FL6:	40.0%

Sample Pressure: 6

AD Advanced Settings:

Forward Window Extension: 50
Back Window Extension: 50

We gate on the singlet DAPI peak:



16 **Prepare the collection PCR tubes:**

○ Fill the PCR tube with 5% BSA-DB, vortex, and remove BSA. *This coats the sides of the tube so that if nuclei hit the side of the tube they will fall to the bottom and not dry out.*
○ Wash tube with 200 uL DB
○ Make collection buffer, add 20uL collection buffer (DB with 1:40 RNase-inhibitor) to the PCR tube.

FACS into the tube using a 70um chip with 1%FSC threshold on the DAPI peak.

17 **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.

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.

18 **Prep for 10X sequencing:**

The maximum nuclei you can input is 17000 (which comes out to 10000 after loss). For 10x v3 the input nuclei volume is 46.6, which means the maximum concentration you can input is 364 n/uL.

19