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FACS Nuclei Isolation

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

This protocol provides a meticulous approach to extracting and processing nuclei from frozen tissues, ensuring high-quality samples suitable for droplet-based single-nuclei sequencing.

Abstract

This protocol details the isolation of nuclei from frozen tissue for subsequent single-nuclei sequencing using a droplet-based platform, such as 10X Genomics. The procedure emphasizes maintaining the integrity of nuclei and minimizing RNA degradation, using a series of specialized buffers and careful handling techniques.

Nuclei Extraction protocol, optimized for small tissue pieces

1 Prepare Reagents and Buffers

Note

ALL buffers to be cooled to 4 °C ***before use***

1.1 **Dissociation Buffer (DB) Preparation**: Dissolve these components in ultrapure, nuclease-free water to a final volume of 500 mL

- 5.83 g Na2so4
- 2.615 g K2SO4
- 0.905 g Glucose
- 1.2 g HEPES
- 2.5 mL MgCl2

1.2 **Extraction Buffer (ExB):**

- 15 mL DB (Dissociation Buffer)
- 150 mg Kollidon VA64
- 150 µL TX-100 (final concentration 1%)
- 15 µL 10% BSA (final concentration 0.01%)
- RNase inhibitor: 1 tube (Lucigen, 10,000 units)

1.3 **Wash Buffer (WB):** Prepare 30 mL per sample

- 30 mL DB (Dissociation Buffer)
- 30 µL 10%BSA
- 50 µL of RNase inhibitor (Lucigen, 10,000 units)

1.4 **FACS Capture Buffer** (**CDB):**

- 20 mL Dissociation Buffer (DB)
- 20 µL 10% BSA
- RNase inhibitor: 1 tube (Lucigen, 10,000 units)

1.5 Prepare: **5% BSA–DB for FACS (Prepare** 200 µL per sample **)**

2 Procedure

2.1 Pre-Cooling:

- Ensure all buffers are cooled to 4 °C before use
- Pre-cool centrifuge, tubes, well plates, 26-gauge needle, 40 µm cell strainer, and syringe at 4 °C for at least 20 minutes.

2.2 Tissue Dissociation:

- Transfer the frozen tissue sample to the prepared 6-well plate using 150 µL of ExB buffer.
- Triturate by pipetting the 1 mL volume slowly up and down using a 1 mL Rainin pipette tip (#30389212) without creating froth or bubbles. Perform 25-30 strokes, pause for 2 minutes, and repeat 4-5 times. Monitor progress; add a fifth pass only if dissociation appears incomplete.
- Pass the entire volume through a 26-gauge needle twice into the same well.

2.3 Sample Processing:

- Transfer the extracted sample (~1 mL) into a 50 mL Falcon tube. Add 30 mL of wash buffer to dissociate the nuclei, then split the volume between two pre-coated Falcon tubes (15+ mL each)
- Centrifuge at 600xg for 10 minutes at 4°C.

2.4 Supernatant Removal:

- Aspirate the supernatant, leaving 500 µL in each tube. Pool the two samples to obtain 1 mL of nuclei suspension.
- Filter the sample through a pre-cooled 40 µm cell strainer using gravity only (no pressure) into a new, clean, pre-cooled Falcon tube.

2.5 Staining:

- Transfer and measure the volume of the filtrate into a pre-cooled 1.5 mL Eppendorf tube.
- Stain the nuclei by adding DAPI (Thermo, #62248) at a 1:1000 dilution.

2.6 FACS Preparation:

- Coat a 0.2 mL PCR tube with 5% BSA. Use a chilled 96-well FACS plate (Sony M800 FACSorter) to capture nuclei in the PCR tube. Pre-fill the PCR tube with 20 µL of CDB to cushion the FACS stream/nuclei.

2.7 FACS Sorting:

- Perform FACS enrichment for singlets using the singlet DAPI peak. Sort at speed 6 with a forward scatter gain of 1% on the DAPI gate. Use the "purity" mode. Avoid spinning down after FACS unless using the gentle spin protocol.

Nuclei FACS enrichment for singlets on singlet DAPI peak

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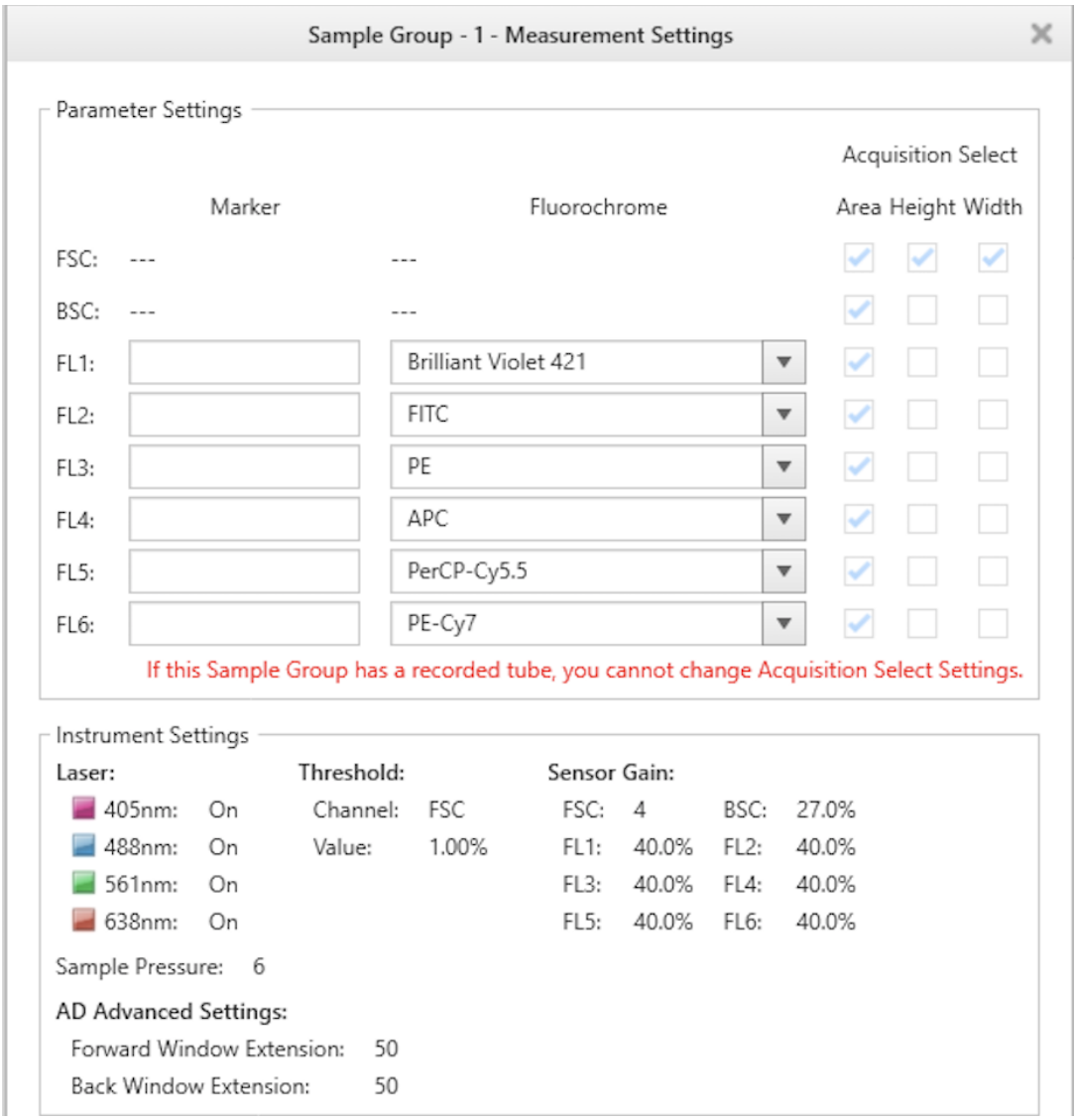
Note

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

FACS Settings:

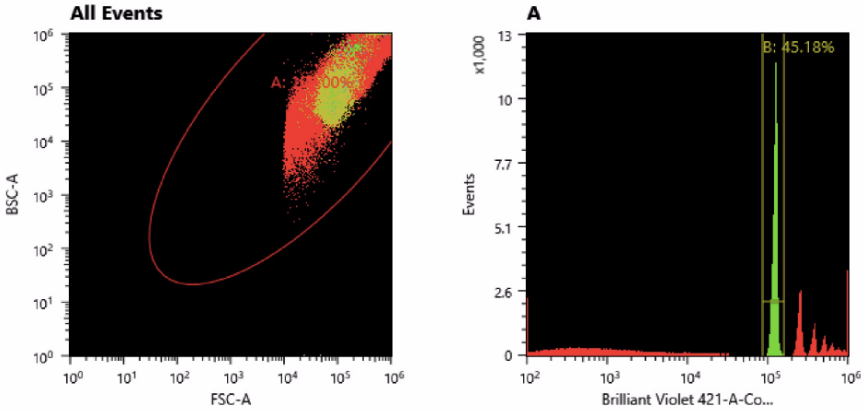
- **Instrument:** Sony SH800/SH900 cell sorter
- **Chip:** 70 µm chip
- **Gating:** Focus on the singlet DAPI peak to enrich for singlet nuclei.

- DAPI peak: y axis = events, x axis = brilliant violet 421-A
- DAPI gate on **purity mode**
- Sample pressure of **3-6**
- Cell size on **regular**
- Sort rate at **4eps**



Note

We gate on the singlet DAPI peak:



4 **Collection of FACS-Sorted Samples:**
To ensure the integrity and minimal collection volume of FACS-sorted nuclei, samples are collected in PCR tubes positioned within a chilled (-20°C) 96-well cold block. This setup is essential to maintain nuclei quality and prevent degradation. A suitable cold block can be found here: Eppendorf PCR Coolers.

Determination of Nuclei Concentration Post-FACS:

5 **1. Volume Measurement:**

- Use a pipette to accurately determine the volume of the collected nuclei suspension in each PCR tube post-FACS.

2. Dilution Preparation:

- Prepare a 1:10 dilution by combining 18 µL of chilled Dissociation Buffer (DB) with 2 µL of the nuclei suspension in a PCR tube. Mix thoroughly.

3. Counting Nuclei:

- Load the diluted sample onto a Fuchs-Rosenthal hemocytometer (16 chambers).

- Visualize using a fluorescent microscope, capturing images under both brightfield and DAPI excitation to ensure debris removal and accurate nuclei identification.
- Count the nuclei across all 16 large squares for precise concentration determination.
- Calculate the average nuclei count per square, multiply by 10 (to account for the 1:10 dilution), and then multiply by 5 (the hemocytometer factor) to determine the final nuclei concentration in nuclei/µL.

Proceeding to the 10X Genomics Protocol:

6 - For the 10X Genomics v3 workflow, the input volume is 46.6 µL. To maximize data output, the optimal nuclei concentration should be 364 nuclei/µL, translating to an input of approximately 17,000 nuclei per 46.6 µL next gem kit.

This protocol provides a meticulous approach to extracting and processing nuclei from frozen tissues, ensuring high-quality samples suitable for droplet-based single-nuclei sequencing.