Demonstrated Protocol: Nuclei Isolation from FFPE Tissue

Overview

Single nuclei RNA sequencing (snRNA-Seq) is a powerful technique that allows for the analysis of gene expression and genetic variation amongst individual cellular nuclei within a tissue sample. One application of particular interest to researchers is the use of snRNA-Seq on formalin-fixed, paraffin-embedded (FFPE) tissue samples, which are commonly generated in clinical settings as a means of preserving patient samples for histological examination.

FFPE tissue samples have traditionally been difficult to study with molecular techniques due to the chemical modifications caused by the formalin fixation, which can lead to degradation of RNA and DNA. However, recent advances in snRNA-Seq technology have made it possible to overcome these challenges and generate high-quality genomic data from FFPE samples at the single nucleus level.

In this technical note, we discuss the use of the Singulator™ 100 and 200 systems in conjunction with manual pre-preparation of samples to isolate nuclei suitable for single nuclei RNA sequencing from FFPE samples. Human brain FFPE samples were used for this demonstration, and the isolated nuclei are suitable for subsequent generation of single nuclei sequencing libraries with the 10x Genomics Cell Flex™ chemistry. Single nuclei RNA sequencing (snRNA-Seq) is a powerful technique that allows for the analysis of gene expression and genetic variation amongst individual cellular nuclei within a tissue sample. One application of particular interest to researchers is the use of snRNA-Seq on formalin-fixed, paraffin-embedded (FFPE) tissue samples, which are commonly generated in clinical settings as a means of preserving patient samples for histological examination.

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Notes and Tips

1. FFPE Block Rehydration

   Rehydrate blocks at least 10 minutes in sterile water to decrease cracking and shattering of FFPE curls. Water can be warmed to 37°C if cracking of tissue still occurs after rehydration.

2. FFPE Block Quality

   RNA and nuclei quality will vary from block to block, tissue to tissue, age of tissue, fixation method, and tissue quality. This protocol does not guarantee successful generation of nuclei with high quality RNA for hybridization and library generation from poor quality blocks. DV200 can be determined from bulk RNA isolation from FFPE blocks, with higher DV200 preferable for downstream RNA assays.

Reagents and Consumables

Vendor	Item	Part Number
S2 Genomics	NIC+ Nuclei Isolation Cartridge	100-215-389
	Nuclei Isolation Reagent, 25 (NIR)	100-063-396
	Nuclei Storage Reagent, 25 (NSR)	100-063-405
	RNase Inhibitor	100-247-435
Millipore Sigma	Ethanol 200 Proof	E7023-1L
	Glycerol for Molecular Biology <99.0%	G5516-100ML
Eppendorf	DNA LoBind Tubes 1.5 mL	0030122275
VWR	15 mL High Performance Centrifuge Tubes	21008-089
	Pipette Tips RT LTS 1000 µL – Low Retention	30389219
	Pipette Tips RT LTS 250 µL – Low Retention	30389250
	Pipette Tips RT LTS 20 µL – Low Retention	30389226
	Bovine Serum Albumin – Lyophilized Powder	97061-420
	CitriSolv Hybrid – Xylene Substitute	89426-268
Revvity	Cellometer K2 Fluorescent Cell Counter	-
	SD025 Counting Chambers	CHT4-SD025
	ViaStain AO/PI Staining buffer	CS2-0106
Sysmex	CellTrics 30 µm, Sterile	04-004-2326
ThermoFisher	Nuclease-Free Water	430791
	UltraPure Bovine Serum Albumin	126615
Corning	PBS 1X (Without Calcium and Magnesium)	21-040-CM
10x Genomics	Conc. Quenching Buffer	2000516
	Conc. Fixation and Perm Buffer	2000517
	Enhancer	2000482

Required Buffers

Fix and Perm Buffer	Per Sample
Conc. Fixation and Perm Buffer	50 µL
Nuclease-Free Water	450 µL
Total	500 µL

Quenching Buffer	Per Sample
Conc. Quench Buffer	50 µL
Nuclease-Free Water	450 µL
Total	500 µL

50% Glycerol Solution	
Equal volume water and Glycerol 0.2 µm filter and store at room temperature	

Nuclei Wash Buffer 1 – Prepare 2 mL	Per 1 Samples
Nuclei Isolation Buffer	1.95 mL
S2 Genomics RNase Inhibitor	50 µL
Optional but recommended: BSA	20 mg
Total	2 mL

Nuclei Wash Buffer 2 – Prepare 2 mL	Per 1 Samples
PBS	1 mL
Nuclease Free Water	1 mL
S2 Genomics RNase Inhibitor	50 µL
Total	2.05 mL

Tips and Best Practices

1. The protocol was validated using three 50 µm thick FFPE human brain tissue curls.
2. FFPE slices must be intact and free of cracking and shattering. Incubating in a sterile 37°C water bath for 10 min and removing outer water contact layers in 5 µm slices before sectioning can be beneficial for creating intact 50 µm curls.
3. Place all tubes on ice for handling steps.
4. Minimize time as much as possible between steps to maintain nuclei integrity.
5. Use a swinging bucket centrifuge to pellet nuclei to stop nuclei from smearing against the sides of centrifuge tubes. This will help maintain nuclei membrane integrity.
6. Use wide bore pipette tips and resuspend pellet gently to avoid shearing nuclei. Do not vortex.

Nuclei Isolation from FFPE Tissue

A. Singulator and Buffer Preparation

1. Prepare buffers as described in **Required Buffers** section.
2. Turn on the Singulator by pressing the power button on the top of the tablet interface.
3. Pre-cool the Singulator by sliding the toggle in the upper right of the **User Home Screen** to **Cool** and tap the icon to initiate pre-cooling. The bar will turn orange indicating pre-cooling is in progress and will turn green and display **On √** upon completion.
4. Place NIC+ Nuclei Isolation Cartridges in a -20°C freezer.

B. Deparaffinization and Rehydration

> **!** Perform deparaffinization steps utilizing solvent and ethanol in a fume hood.
> Follow established safety guidelines for handling solvents.

1. Prepare 2 mL aliquots of 100%, 70%, 50%, and 30% ethanol.
2. Place tissue curls into a 1.5 mL tube.
3. Add 1 mL CitriSolv Hybrid and incubate for 15 minutes.

4. Remove CitriSolv Hybrid using a pipette, taking care not to break or remove tissue from the tube.
5. Add 1 mL CitriSolv Hybrid and incubate for 7 minutes.
6. Remove CitriSolv Hybrid, taking care not to break or remove tissue from the tube.
7. Add 1 mL CitriSolv Hybrid and incubate for 7 minutes.
8. Remove CitriSolv Hybrid, taking care not to break or remove tissue from the tube.
9. Add 1 mL of 100% ethanol to the curls and incubate for 1 minute.
10. Remove 100% ethanol, taking care not to break or remove tissue from the tube.
11. Repeat steps 8 and 9 for ethanol concentrations of 70%, 50%, and 30%, sequentially.
12. Rinse rehydrated tissue in 1 mL of PBS (-Ca/Mg) 3x.
13. Centrifuge sample at 1,000 g for 5 minutes to pellet the rehydrated tissue.

C. Nuclei Isolation

1. Once the Singulator is pre-cooled, select the desired protocol.
    a. Select **Run a Protocol** from the **User Home Screen**.
    b. Select the **Nuclei** button to toggle to nuclei protocols.
    c. Select the **FFPE Nuclei Isolation** protocol.
    d. Select **Next**.
    e. On the **Run Notes Screen**, add notes if desired to be saved in the internal log files, then select **Next**.
2. Resuspend the rehydrated tissue in 450 µL of NIR.
3. Remove the chilled cartridge from the -20°C freezer, remove the cap from the cartridge, place the 450 µL of rehydrated tissue and NIR inside the Dissociation Chamber, add 62.5 µL of RNase Inhibitor and replace the cap.
4. Lift the door of the Singulator and slide out the Cartridge Tray by lifting the red knob.
5. Place the cartridge on the Cartridge Tray and turn the white cartridge lock counterclockwise to lock cartridge into place.
6. Slide in the Cartridge Tray by pushing the back of the tray until the red knob fully clicks and drops into place. DO NOT USE THE RED KNOB TO PUSH THE CARTRIDGE TRAY.
7. Close the door of the Singulator.
8. Select **Run** – the nuclei isolation takes approximately 17 minutes.
9. After completion of the run, the instrument will display a **Run Complete Screen**. Raise the door and then, lifting the red knob, slide the Cartridge Tray and cartridge out of the instrument. Close the door. Rotate the cartridge lock clockwise and remove the cartridge from the Cartridge Tray.
10. Pierce the foil seal of the Output Chamber with a 1 mL widebore pipette and retrieve the sample (~2.2 mL) and place into a cold 15 mL conical tube. Make sure to tap the cartridge on the benchtop to get any remaining sample out of the filter unit.
11. Centrifuge the sample at 850 g for 5 minutes at 4° C in a swinging bucket rotor.

D. Nuclei Preparation

1. Remove the supernatant after centrifugation and gently resuspend pellet in 1 mL of Nuclei Wash Buffer 1.
2. Strain through a 30 µm CellTrics strainer, rinsing with 1 mL of Nuclei Wash Buffer 1.
3. Centrifuge sample at 850 g for 5 minutes and remove supernatant.
4. Resuspend in 500 µL of 1X Fix and Perm Buffer and incubate at room temperature (~20° C) for 1 hour.
    a. As 10x Genomics recommends, if temperature of room varies, use a controlled heat block to keep samples fixing at same temperature throughout the experiment.
5. Centrifuge sample at 850 g for 5 minutes and remove supernatant.
6. Resuspend in 500 µL 1X Concentration Quench Buffer by mixing 5 times.

E. Counting and Dilution

Follow manufacturers' instructions to obtain nuclei count using a fluorescence method, briefly described below.

1. Remove coverslip from a Nexcelom counting slide.
2. Mix 30 µL of the sample with 30 µL of ViaStain AO/PI dye, and place 20 µL in each side of counting slide.
3. Insert counting slide into the Cellometer K2 cell counter.
4. Open the matrix software on the K2 laptop. Select **K2_AOPI_Primary Cells** assay and enter dilution factor of 2.
5. Select **Preview**.
6. Using knob on right side of the instrument adjust focus until nuclei appear in **"Good Focus"** according to the **Cellometer Focus Guide**.
7. Select **Count**.
8. Proceed immediately to appropriate Cell Flex Kit protocol – Probe Hybridization step 1D.

F. Sample Storage

Follow 10x Genomics appendix Fixed Sample Storage Guidance (Fixation of Cells and Nuclei for Chromium Fixed RNA Profiling – pg. 6)

1. Thaw Enhancer at 65°C for 10 minutes.
2. Add 0.1 volume of Enhancer to the sample (*i.e.,* 50 µL Enhancer to 500 µL of sample).
3. Add 50% glycerol for final concentration of 10%.
4. Store at -80°C.

G. Post-Storage Processing

Follow 10x Genomics appendix Fixed Sample Storage Guidance (Fixation of Cells and Nuclei for Chromium Fixed RNA Profiling – pg. 6)

1. Thaw the sample at room temperature until no ice is present.
2. Centrifuge the sample at 850 g for 5 minutes at room temperature.
3. Remove the supernatant.
4. Resuspend the pellet in 0.5X PBS + 0.02% BSA (optionally supplemented with 0.2u/µL RNase inhibitor) or Quenching Buffer.
5. Determine cell concentration of the fixed sample using an Automated Cell Counter or hemocytometer. (See Section E above)
6. Proceed immediately to appropriate Chromium Fixed RNA profiling protocols.

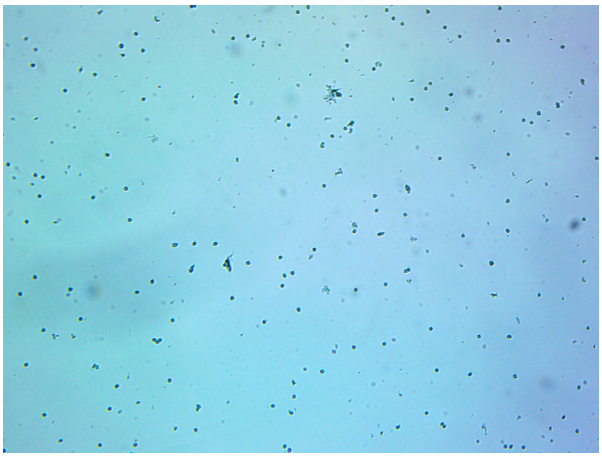
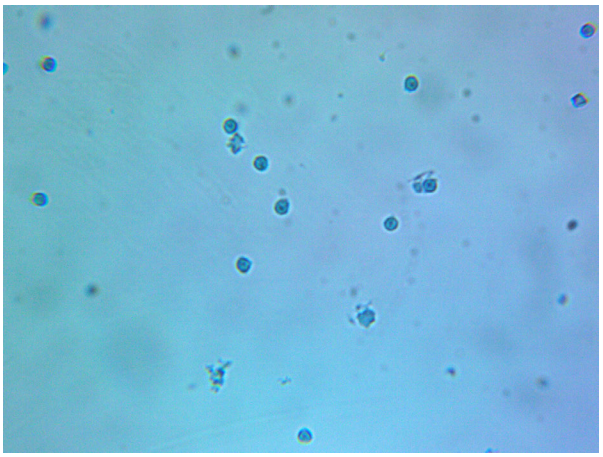
H. Chromium Fixed RNA Profiling

Follow 10x Genomics' recommended instructions in Chromium Fixed RNA Profiling Reagent Kits (CG000477 Rev D)

1. Probe Hybridization.
2. GEM Generation and Barcoding.
3. GEM Recovery and Pre-Amplification.
4. Fixed RNA – Gene Expression Library Construction.
5. Fixed RNA – Gene Expression Library Construction.
6. Sequencing.

Results

Nuclei Yield and Images

FFPE Brain Nuclei (10x Resolution)	FFPE Brain Nuclei (40x Resolution)
	
Nuclei Count (1:4 dilution) of Human Brain Tissue, 3x 50 µm curls	6,470,000 total nuclei

The images above show the nuclei isolated from three 50 µm curls, Trypan Blue stained, at 10x and 40x magnification. Automated cell counting indicated over six million nuclei were isolated. The nuclei have good morphology and the post-dissociation clean-up procedure was effective at removing the majority of the cellular debris.