DEMONSTRATED PROTOCOL

Nuclei Isolation from FFPE Tissues for Single-Nuclei Sequencing Applications

Overview

This protocol describes how to isolate, count, and prepare single nuclei from FFPE (formalin-fixed, paraffin-embedded) tissues for single-nuclei sequencing assays using the Singulator Platform. Deparaffinization and rehydration of the tissue using solvent and various concentrations of ethanol are conducted prior to automated nuclei isolation on the Singulator Platform.

Some optimizations of Singulator protocol parameters and post-isolation cleanup steps may be needed based on the species, tissue type, storage time, and condition of the FFPE tissue.

Compatible Downstream Applications

Be sure to read associated user guides for applicable assay below before proceeding with nuclei isolation for sequencing runs, see references section.

Platform	Assay	Part Number
10x Genomics – Single Cell Gene Expression Flex Assay	Chromium Fixed RNA Kit, Human Transcriptome	1000474 (4rxns x 1 BC) 1000475 (4rxns x 4 BC) 1000476 (4rxns x 16 BC) 1000547 (16rxns x 16 BC)
10x Genomics – Single Cell Gene Expression Flex Assay	Chromium Fixed RNA Kit, Mouse Transcriptome	1000495 (4rxns x 1 BC) 1000496 (4rxns x 4 BC) 1000497 (4rxns x 16 BC) 1000568 (16rxns x 16 BC)

Reagents and Consumables

Vendor	Item	Part Number
S2 Genomics	NIC+ Isolation Bundle with RNase Inhibitor V2 (8 Samples)	100-291-531
	NIC+ Isolation Bundle with RNase Inhibitor V2 (24 Samples)	100-288-807
	RNase Inhibitor V2	100-288-916
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
	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 (BSA) (50mg/mL)	AM2616
Corning	PBS 1X (Without Calcium and Magnesium)	21-040-CM
10x Genomics	Concentrated Quench Buffer*	PN-2000516

*Included in the 10x Genomics Chromium Next GEM Single Cell Fixed RNA Sample Preparation Kit, 16 rxns (PN-1000414)

Getting Started

Prepare Buffers

Wash Buffer 1 – Prepare 1 mL (Place on Ice)	**Per 1 Samples**
PBS	485.5 µL
Nuclease Free Water	485.5 µL
RNase Inhibitor V2	25 µL
Bovine Serum Albumin (BSA) (50mg/mL)	4 µL
Total	1.0 mL

Prepare 500uL of Quenching Buffer or Resuspension Buffer for final pellet resuspension prior to moving into appropriate downstream Chromium Fixed RNA Assay.

Quenching Buffer (Place on Ice)	**Per Sample**
Nuclease Free Water	473.5 µL
Conc. Quench Buffer* (Thaw at room temperature. Vortex and centrifuge briefly)	62.5 µL
Total	500 µL
Or	
Resuspension Buffer (Place on Ice)	**Per Sample**
PBS	248 µL
Tris Buffer (pH 8.0; 1000mM)	25 µL
BSA (UltraPure, 50mg/mL)	2 µL
RNase inhibitor V2	3 µL
Nuclease-free Water	222 µL
Total	500 µL

50% Glycerol Solution (If storing samples for processing at a later date)
Mix equal volumes of water and glycerol. Use 0.2 µm filter and store at room temperature.

Tips and Best Practices

1. Rehydration: Rehydrate FFPE blocks in 37°C sterile water for at least 10 minutes to minimize cracking and shattering of FFPE curls. If excessive tissue cracking still occurs after rehydration, proceed with caution as this may result in sample loss during deparffinization and rehydration steps, see Appendix 1 for recommendations.
2. FFPE Block RNA Quality: RNA and nuclei quality may vary between blocks due to factors like tissue type, age, fixation method, and initial tissue quality. This protocol does not guarantee high-quality RNA or intact nuclei for library generation from poor-quality FFPE blocks. DV200 values, which can be determined from bulk RNA isolations, should ideally be above 30% (50% is optimal) for downstream RNA assays.
3. Temperature Control: Keep all tubes and buffers on ice during isolation steps to preserve RNA integrity.
4. Centrifugation: Use a swinging bucket centrifuge to pellet nuclei, which prevents nuclei from smearing against sides of centrifuge tubes and helps maintain nuclei integrity.
5. Resuspension: Gently resuspend pellet by pipetting to avoid shearing nuclei. Do not vortex.
6. Safety: Perform deparaffinization steps in a chemical fume hood and follow established safety guidelines for handling solvents.

Nuclei Isolation from FFPE Tissue

A. FFPE Tissue Preparation and Sectioning

1. Trim excess paraffin from around the tissue in the block.
2. Using a microtome expose or “face” the tissue block by removing the outer layers of paraffin in 5 µm increment slices to reveal the tissue.
3. Rehydrate the FFPE tissue block by incubating in 37°C sterile water for 10 minutes, to prevent shattering or cracking of tissue during slicing. Using a kim wipe, dry off FFPE block.
4. Using a microtome remove rehydrated white layer in 5 µm increment slices. Usually 1-2 slices.
5. Slice a 50 µm section of tissue and transfer it in sterile 1.5mL tube.

B. Singulator Setup

1. Place the NIC+ Nuclei Isolation Cartridge(s) in a -20°C freezer overnight or for at least 20 minutes before the run(s).
2. Prepare buffers as described in **Getting Started** section.
3. Turn on the Singulator by pressing the power button on the top of the tablet interface.
4. 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.

   Tip: The Singulator will turn off Pre-Cooling function after 15 minutes of inactivity. Select Continue Cooling after 15 minutes of inactivity or proceed with Pre-Cooling after step C.6 below.
5. Ensure 3 mL of Nuclei Isolation Reagent (NIR) and 3 mL of Nuclei Storage Reagent (NSR) are present in Chiller unit.

C. Deparaffinization and Rehydration of FFPE Tissue

Tip: Perform deparaffinization steps using solvent and ethanol in a fume hood, following established safety guidelines for handling solvents.

1. Prepare 2 mL aliquots (per sample) of 100%, 70%, 50%, and 30% ethanol.
2. Place one 50 µm FFPE tissue curls into a 1.5 mL tube.
3. Add 1 mL CitriSolv Hybrid and incubate for 15 minutes.
4. Carefully remove CitriSolv Hybrid using a pipette, ensuring the tissue remains intact in the tube.

   Tip: Solvent and ethanol incubation supernatant may be removed with a syringe and needle to help prevent loss of sample. Slant tube and gently remove supernatant from meniscus using needle.
5. Add 1 mL CitriSolv Hybrid and incubate for 7.5 minutes.
6. Carefully remove CitriSolv Hybrid using a pipette, ensuring the tissue remains intact in the tube.
7. Add 1 mL CitriSolv Hybrid and incubate for 7.5 minutes.
8. Carefully remove CitriSolv Hybrid using a pipette, ensuring the tissue remains intact in the tube.
9. Add 1 mL of 100% ethanol to the tissue curl and incubate for 1 minute.
10. Carefully remove 100% ethanol using a pipette, ensuring the tissue remains intact in the tube.
11. Repeat steps 8 and 9 sequentially with ethanol concentrations of 70%, 50%, and 30%.
12. Remove the final ethanol wash and rinse rehydrated tissue in 1 mL of PBS (-Ca/Mg) three times.

D. Nuclei Isolation

1. 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 **FFPE Nuclei Isolation**.
   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. Remove the chilled NIC+ Nuclei Isolation Cartridge from the -20°C freezer. Remove the cartridge cap, transfer the tissue using forceps, add 450 µL NIR, and add 62.5 µL RNase Inhibitor V2 inside the Dissociation Chamber. Replace the cap.

   Tip: If the tissue pieces are too small to transfer with forceps, centrifuge at 1000g for 3 minutes to pellet the tissue. Remove the supernatant, resuspend in 450 µL of NIR, and add to Dissociation Chamber using a wide-bore pipette tip, along with 62.5 µL of RNase Inhibitor V2.Mix 30 µL of sample with 30 µL of ViaStain AO/PI dye, and place 20 µL in each side of counting slide.
3. Lift the door open of the Singulator and slide out the Cartridge Tray while lifting the red knob.
4. Place the cartridge on the Cartridge Tray and turn the white cartridge lock counterclockwise to lock the cartridge into place.

5. Slide in the Cartridge Tray by pushing on the back of the tray until the red knob fully drops into place. **DO NOT USE THE RED KNOB TO PUSH THE CARTRIDGE TRAY.**
6. Close the door shut of the Singulator.
7. Select **Run** – the nuclei isolation takes approximately 17 minutes.
8. After completion of the run, the instrument will display a **Run Complete Screen**. Raise the door, lift the red knob, and 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.
9. Pierce the foil seal of the Output Chamber with a 1 mL pipette, retrieve the sample and place into a cold 15 mL conical tube. Tap the cartridge on the benchtop to ensure any remaining sample is collected from the filter unit.
10. Centrifuge sample at 850g for 5 minutes at 4°C in a swinging bucket rotor.

E. Nuclei Preparation

1. After centrifugation, carefully remove the supernatant and gently resuspend pellet in 1 mL of Nuclei Wash Buffer 1.
2. Strain the sample through a 30 µm CellTrics strainer by gently pressing the pipette tip against the nylon mesh and slowly pipette the sample through the mesh.
3. Centrifuge the sample at 850 g for 5 minutes and remove the supernatant.
4. After centrifugation, carefully remove the supernatant and gently resuspend the pellet in 500 µL of Quenching Buffer.

F. Counting

Follow manufacturer's instructions (see references below) to obtain a nuclei count using a fluorescence method, briefly described below. A fluorescent based counting method is required for accurate determination of nuclei yield due to decreased fluoresce of nuclei prepared from FFPE samples. See **Counting Using PI Staining Solution** section of the Appendix in "Sample Preparation from FFPE Tissue Sections for Chromium Fixed RNA Profiling" (10x Genomics CG000632) for more information.

1. Remove the top and bottom coverslip from a Nexcelom counting slide.
2. Mix 20 µL of the sample with 20 µL of ViaStain AO/PI dye, and place 20 µL on each side of counting slide.
3. Insert the 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 a dilution factor of 2.
5. Select **Preview**.
6. Using the knob on the right side of the instrument, adjust the focus until nuclei appear in "**Good Focus**" according to the **Cellometer Focus Guide**.
7. Adjust the fluorescent exposure (FL Exposure (ms)) to ensure dimly fluorescing FFPE nuclei are visible in the preview. Set Channel 2 (Red Channel) FL exposure (ms) setting to 9000.
8. Select **Count**.

Note: If high viability readings (above 5%) are seen recount sample and adjust Channel 1 (Green Channel) FL Exposure (ms) to below 600.

Note: Nuclei samples may appear dirty based on sample type. Removal of debris will occur during downstream probe hybridization steps and hybridization washes of Chromium Fixed RNA Profiling Kits. See representative images below in Results section.

G. Chromium Fixed RNA Profiling – Single Cell Gene Expression Flex Assay

Follow the recommended instructions provided by 10x Genomics for the appropriate Chromium Fixed RNA Profiling Reagent Kits. See References section for compatible user guides.

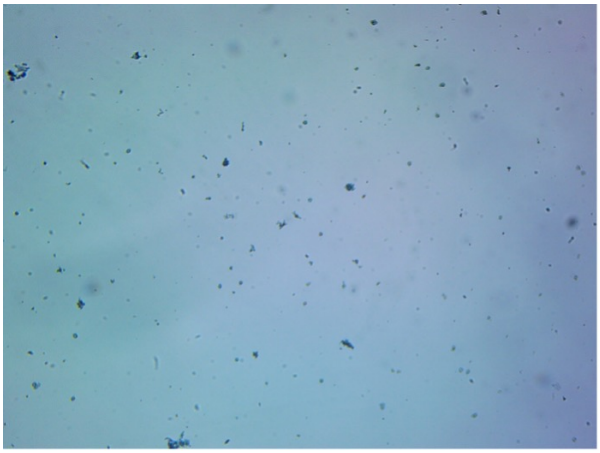
1. Proceed immediately with desired number of nuclei to the appropriate Chromium Fixed RNA Profiling protocols – Probe Hybridization step 1.1d. See references for compatible user guides.
2. If samples will not be processed immediately for Fixed RNA profiling, store the samples as described in **Fixed Sample Storage Guidance** section of the Appendix in "Sample Preparation from FFPE Tissue Sections for Chromium Fixed RNA Profiling" (10x Genomics CG000632).
3. When ready to proceed with nuclei processing follow the steps in **Post-Storage Processing** section of the Appendix in "Sample Preparation from FFPE Tissue Sections for Chromium Fixed RNA Profiling" (10x Genomics CG000632) to thaw, prepare, and count nuclei before immediately proceeding to the appropriate Chromium Fixed RNA Profiling protocols – Probe Hybridization step 1.1d. See references for compatible user guides.

Results

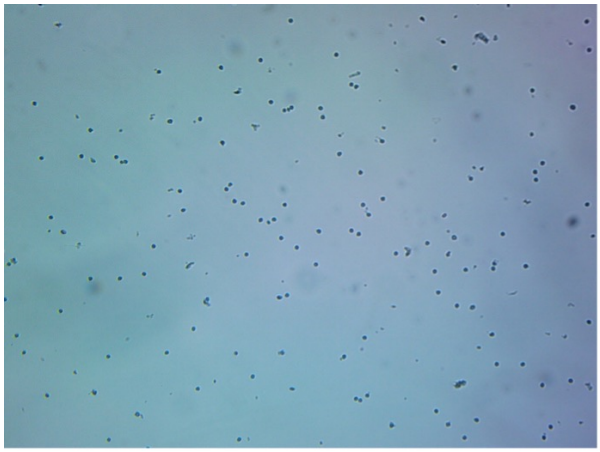
Representative Images:

Human Brain FFPE Sample

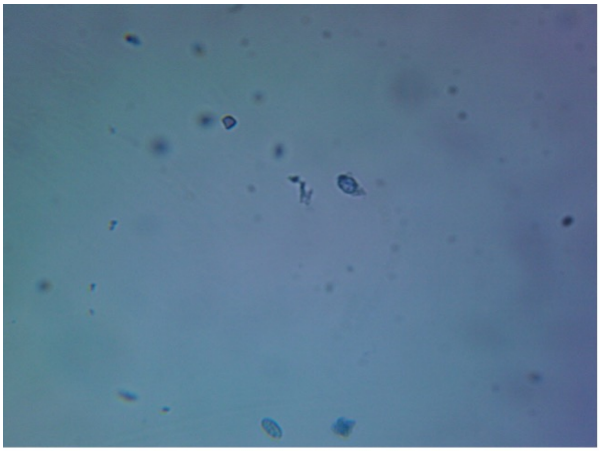
Post Nuclei Isolation
(10x Resolution)



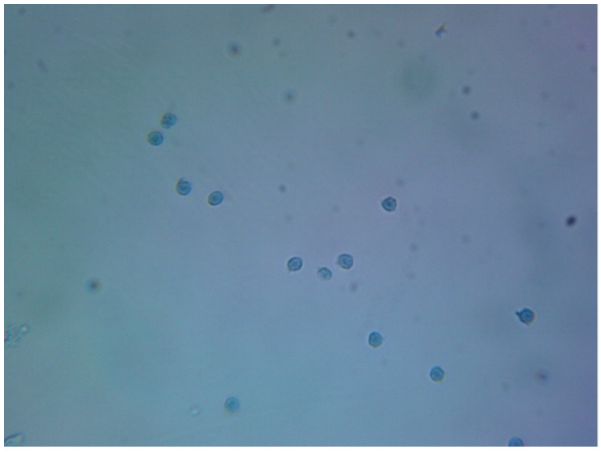
Post Hybridization and Hybridization Washes
(10x Resolution)



Post Nuclei Isolation
(40x Resolution)

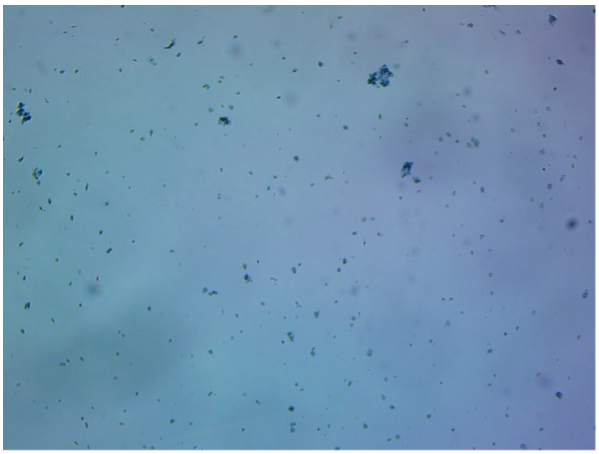


Post Hybridization and Hybridization Washes
(40x Resolution)

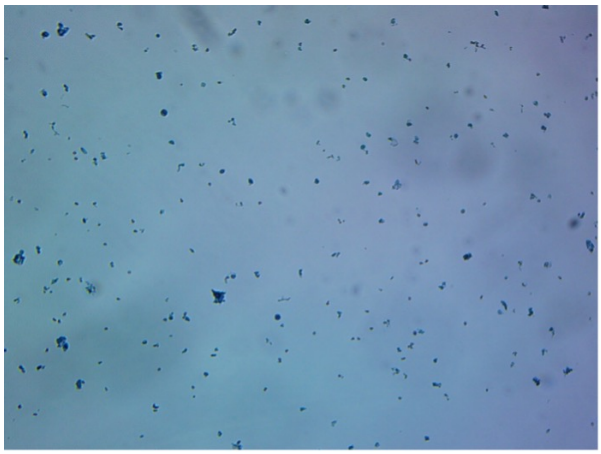


Human Lung FPPE Sample

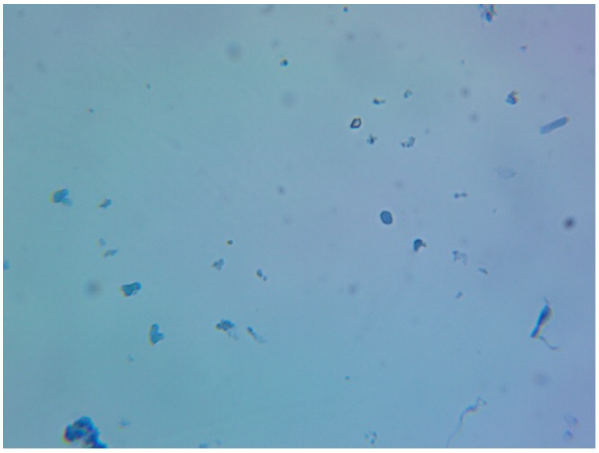
Post Nuclei Isolation
(10x Resolution)



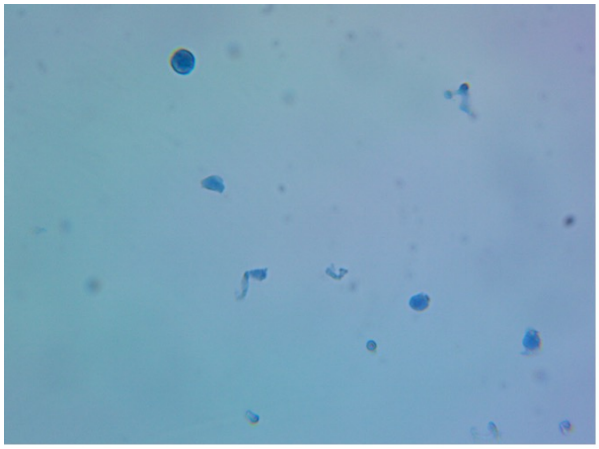
Post Hybridization and Hybridization Washes
(10x Resolution)



Post Nuclei Isolation
(40x Resolution)

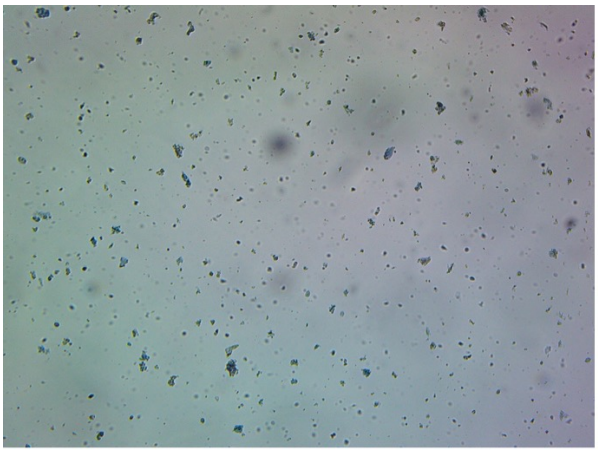


Post Hybridization and Hybridization Washes
(40x Resolution)

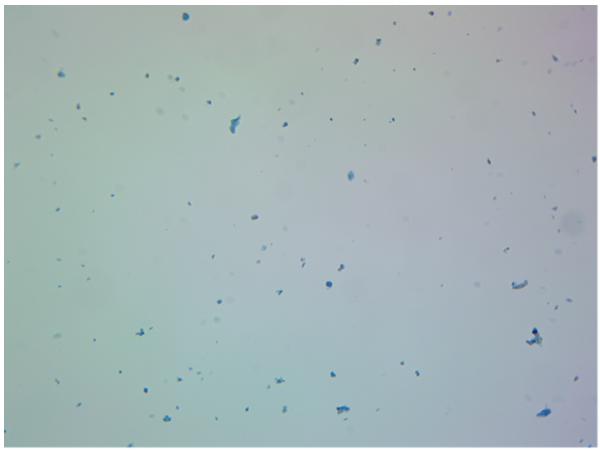


Mouse Pancreatic Ductaladenocarcinoma

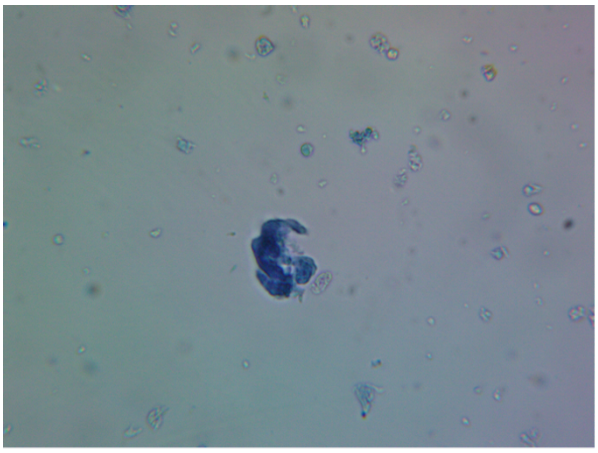
FFPE Sample Post Nuclei Isolation
(10x Resolution)



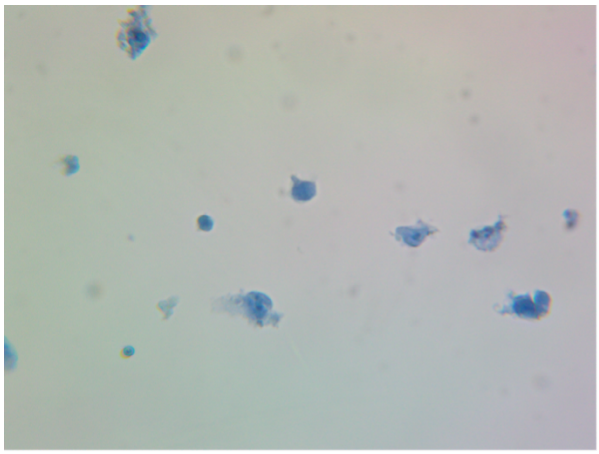
FFPE Sample Post Hybridization and Hybridization Washes (10x Resolution)



Sample Post Nuclei Isolation
(40x Resolution)



FFPE Sample Post Hybridization and Hybridization Washes (40x Resolution)



Appendix 1 – Issues and Recommendations

Issue	Recommendation
Cracking or shattering of FFPE curls	• Extend hydration time in step A.3. • Proceed with caution during deparaffinization and rehydration steps to minimize sample loss. Samples may be centrifuged at 850 g for 1 minute to pellet before reagent exchange in steps C.3 through C.12.
Low yield	• Process up to three 50 µm sections through protocol. • Run Increased Disruption Protocol: 1. Select "3x Disrupt Nuclei Isolation" in step D.1.c. 2. Select modify and change NIR source to manual, NIR volume to 0.5mL, and incubation time to 8 minutes. 3. Rename modified protocol to desired name and proceed to step D.1.d. • Test "Demonstrated Protocol – Cell Isolation from FFPE and Fixed Tissues for Single-Cell Sequencing Applications"
High Debris post downstream hybridization and hybridization washes performed in Chromium Fixed RNA Profiling Kit	• FACs sort hybridized nuclei with 7-AAD dye to isolate nuclei from debris. • Test "Demonstrated Protocol – Cell Isolation from FFPE and Fixed Tissues for Single-Cell Sequencing Applications"

References

1. Cellometer K2 Matrix User Manual (8003393)
2. Demonstrated Protocol Sample Preparation from FFPE Tissue Section for Chromium Fixed RNA Profiling (CG000632)
3. Chromium Fixed RNA Profiling Reagent Kit for Multiplexed Samples User Guide (CG000527)
4. Chromium Fixed RNA Profiling Reagent Kit for Singleplexed Samples User Guide (CG000527)