DEMONSTRATED PROTOCOL

# Cell Isolation from FFPE and Paraformaldehyde Fixed Tissues for Single-Cell Sequencing Applications

## Overview

This protocol outlines the steps for isolating, counting, and preparing single cells from FFPE (formalin-fixed, paraffin-embedded) or paraformaldehyde (PFA) fixed whole tissues for single-cell sequencing assays using Singulator™ Platform. The process includes deparaffinization and rehydration of the tissue using specific solvents and various concentrations of ethanol prior to automated cell isolation on the Singulator™ Platform.

Optimizations to the Singulator protocol parameters and post-isolation cleanup steps may be needed depending on factors such as the species, tissue type, storage duration, and condition of the FFPE/PFA tissue.

## Compatible Downstream Applications

Before beginning cell isolations for sequencing runs, be sure to have read associated user guides for applicable assays listed below.

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

## Reagents and Consumables

| Vendor | Item | Part Number |
|---|---|---|
| S2 Genomics | Cell Isolation Cartridge Pack (8 Cartridges) | 100-290-414 |
| | Cell Isolation Cartridge Pack (24 Cartridges) | 100-290-313 |
| | RNase Inhibitor V2 | 100-288-916 |
| Millipore Sigma | Ethanol 200 Proof | E7023-1L |
| | Glycerol for Molecular Biology <99.0% | G5516-100ML |
| | Liberase TH Research Grade | 5401135001 |
| 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 (BSA) (50mg/mL) | AM2616 |
| Corning | PBS 1X (Without Calcium and Magnesium) | 21-040-CM |
| | RPMI 1640 Medium | 10-040-CV |
| 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

| **Digestion Enzyme Buffer**<br>Prepare 3 mL by fully dissolving Liberase Enzyme in RPMI at room temperature by shaking for 20 minutes in 15 mL conical tube. *Do not heat Digestion Enzyme Buffer.* | **Per 1 Sample** |
|---|---|
| RPMI | 3 mL |
| Liberase TH (Research Grade) | 3 mg |
| Total | 3.0 mL |

| **Cell Wash Buffer 1**<br>Prepare 2 mL (place on ice) | **Per 1 Sample** |
|---|---|
| PBS | 971 µL |
| Nuclease-Free Water | 971 µL |
| RNase Inhibitor V2 | 50 µL |
| BSA | 8 µL |
| Total | 2 mL |

Prepare 500 µL 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 1 Sample** |
|---|---|
| Nuclease Free Water | 473.5 µL |
| Conc. Quench Buffer*<br>(Thaw at room temperature. Vortex and centrifuge briefly) | 62.5 µL |
| Total | 500 µL |
| **Or** | |
| **Resuspension Buffer** (Place on Ice) | **Per 1 Sample** |
| PBS | 248 µL |
| Tris Buffer (pH 8.0; 1000 mM) | 25 µL |
| BSA (UltraPure, 50 mg/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 sterile water at 37°C for at least 10 minutes to minimize cracking and shattering of FFPE curls. If excessive tissue cracking still occurs, proceed with caution, as this may result in sample loss during deparaffinization and rehydration steps. See Appendix 1 for recommendations.
2. RNA Quality of FFPE Block: RNA quality may vary between FFPE blocks based on factors like tissue type, storage duration, fixation method, and initial tissue quality. This protocol does not guarantee high-quality RNA or intact cells 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 on ice during isolation steps to preserve RNA integrity.
4. Centrifugation: Use a swinging bucket centrifuge to pellet cells, which prevents cells from smearing against sides of centrifuge tubes and helps maintain RNA integrity.
5. Resuspension: Gently resuspend pellet by pipetting to avoid shearing cells. Do not vortex.
6. Safety: Perform deparaffinization steps in a chemical fume hood and follow established safety guidelines for handling solvents

## Cell Isolation from FFPE Tissue

### A. FFPE Tissue Preparation and Sectioning

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

### B. Singulator Setup

1. Obtain a Cell Isolation Cartridge and equilibrate to room temperature.
2. Prepare buffers as described in **Getting Started** section.
3. Turn on the Singulator by pressing the power button located on the top of the tablet interface.
4. Pre-heat the Singulator by sliding the toggle in the upper right of the **User Home Screen** to **Heat** and tap the icon to initiate pre-heating. The bar will turn orange indicating pre-heating is in progress and will turn green and display **On √** upon completion.

   **Tip:** The Singulator will turn off Pre-Heating function after 15 minutes of inactivity. Select **Continue** after 15 minutes of inactivity or proceed with Pre-Heating after step C.6 below.

**C. Deparaffinization and Rehydration of FFPE Tissue**

If preparing cells from whole PFA-fixed tissue, mince tissue into 1mm$^2$ pieces and proceed to Section D.

**Tip:** 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 for each sample.
2. Place one 50 µm FFPE tissue curl into a 1.5 mL tube.
3. Add 1 mL CitriSolv Hybrid and incubate for 15 minutes.
4. Carefully remove the CitriSolv Hybrid using a pipette, ensuring the tissue remains intact in the tube.

   **Tip:** To prevent sample loss, a syringe and needle may be used to remove the solvent and ethanol supernatant. Tilt the tube and gently remove supernatant from meniscus with the needle.
5. Add 1 mL of CitriSolv Hybrid and incubate for 7.5 minutes.
6. Carefully remove the CitriSolv Hybrid using a pipette, ensuring the tissue remains intact in the tube.
7. Add 1 mL of CitriSolv Hybrid and incubate for 7.5 minutes.
8. Carefully remove the 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. Sequentially repeat steps 8 and 9 sequentially with ethanol concentrations of 70%, 50%, and 30%.
12. After the final ethanol wash, rinse the rehydrated tissue in 1 mL of PBS (-Ca/Mg) three times.

**D. Cell Isolation**

1. Once the Singulator is pre-heated, select the desired protocol.
   a. Select **Run a Protocol** from the **User Home Screen**.
   b. Select the **Cells** button to toggle to cell protocols.
   c. Select **Tumor Cells V2**.
   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. Using the red knob, pull and slide down the tube holder of the Single Shot Mechanism next to the designated processing bay. Remove the cap and place a 15 mL conical tube containing 3 mL of Digestion Enzyme Buffer into the right side of the Single Shot Mechanism, in the slot labeled "Enzyme".
3. Add 6 mL of RPMI to a 15 mL conical and place in the left side of the Single Shot Mechanism labeled "Buffer".
4. Remove the cap from a Cell Isolation Cartridge, add minced PFA-fixed tissue or deparaffinized and rehydrated FFPE tissue using forceps and add 50 µL RNase Inhibitor V2 inside the Dissociation Chamber, and replace the cap.

5. Lift the door open of the Singulator and slide out the Cartridge Tray while lifting the red knob.
6. Place the cartridge on the Cartridge Tray and turn the white cartridge lock counterclockwise to lock the cartridge into place.
7. 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**.
8. Close the door shut of the Singulator.
9. Select Run – the cell isolation takes approximately 55 minutes.
10. 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 containing the cartridge out of the instrument. Close the door, rotate the cartridge lock clockwise, and remove the cartridge from the Cartridge Tray.
11. 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.
12. Centrifuge sample at 850 g for 5 minutes at 4°C in a swinging bucket rotor.
13. Remove used 15 mL conical tubes from Single Shot Mechanism and discard.

**E. Cell Preparation**

1. After centrifugation, carefully remove the supernatant and gently resuspend pellet in 1 mL of Cell 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 or Resuspension Buffer

**F. Counting**

Follow manufacturer's instructions (see references below) to obtain a cell count using a fluorescence method, briefly described below. A fluorescent based counting method is required for accurate determination of cell yield due to decreased fluoresce of cell prepared from PFA-fixed or 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. Place 20 µL of the mixture 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 cells appear in "**Good Focus**" according to the **Cellometer Focus Guide**.
7. Adjust the fluorescent exposure (FL Exposure (ms)) to ensure dimly fluorescing cells 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:** Cell samples may appear dirty based on sample type. Removal of debris will occur during downstream during probe hybridization steps and hybridization washes of Chromium Fixed RNA Profiling Kits.

**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.

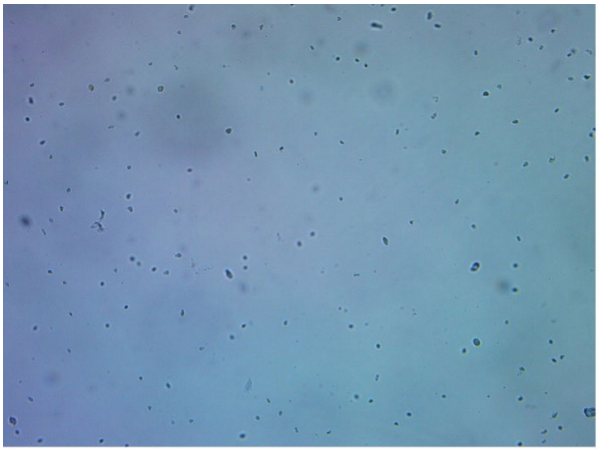
1. Proceed immediately with desired number of cells to the appropriate Chromium Fixed RNA Profiling protocols – Probe Hybridization step 1.1d. See references for compatible user guides.
2. If samples will not be processed immediately for Fixed RNA profiling, store the samples as described in **Fixed 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 cells 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 cells before immediately preceding to the appropriate Chromium Fixed RNA Profiling protocols – Probe Hybridization step 1.1d. See references for compatible user guides.

## Results

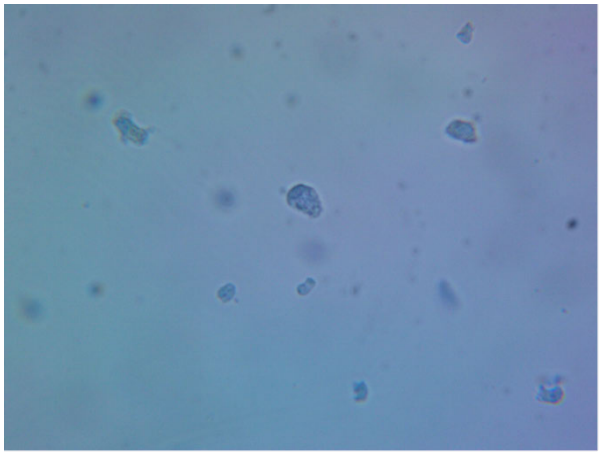
### Representative Images:

**PFA-Fixed Mouse Lung Sample**

Post-Cell Isolation
(10x Magnification)

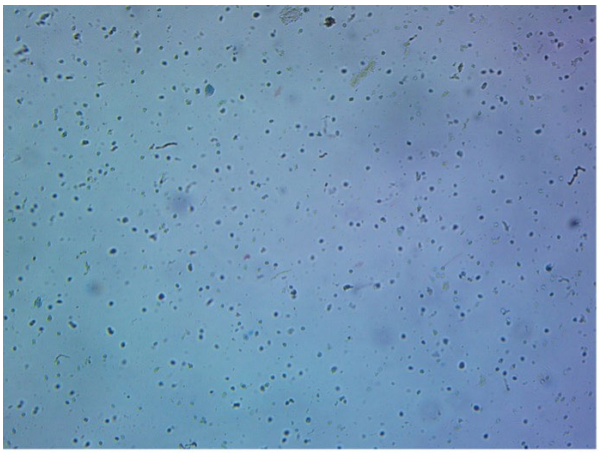


Post-Cell Isolation
(40x Magnification)

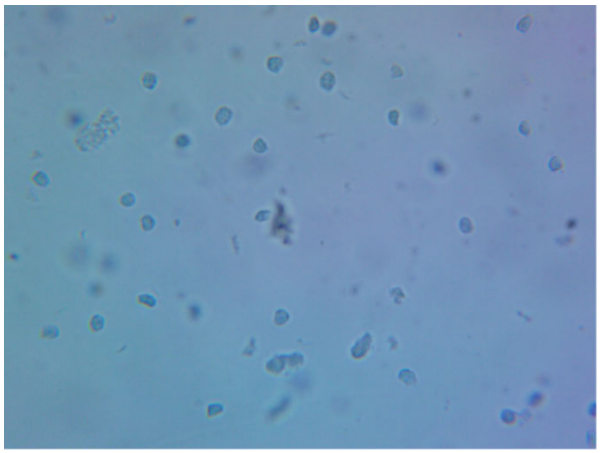


**PFA-Fixed Mouse Brain Sample**

Post-Cell Isolation
(10x Magnification)

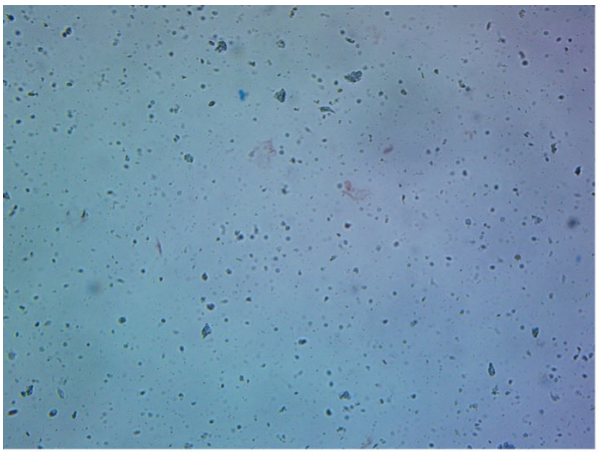


Post-Cell Isolation
(40x Magnification)

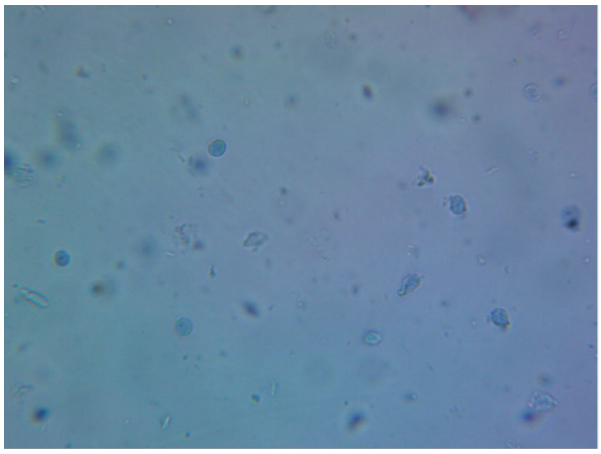


**PFA-Fixed Mouse Kidney Sample**

Post-Cell Isolation
(10x Magnification)

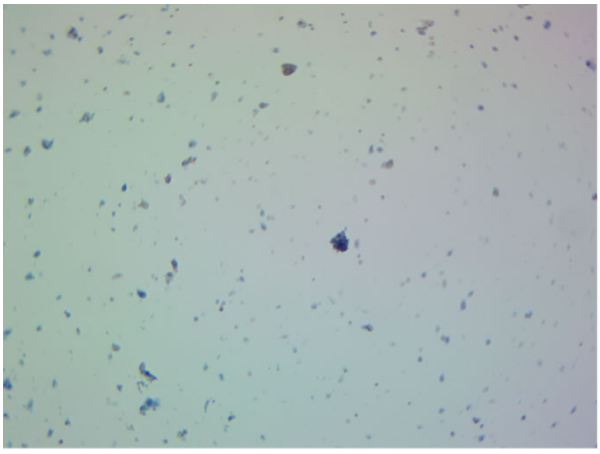


Post-Cell Isolation
(10x Magnification)

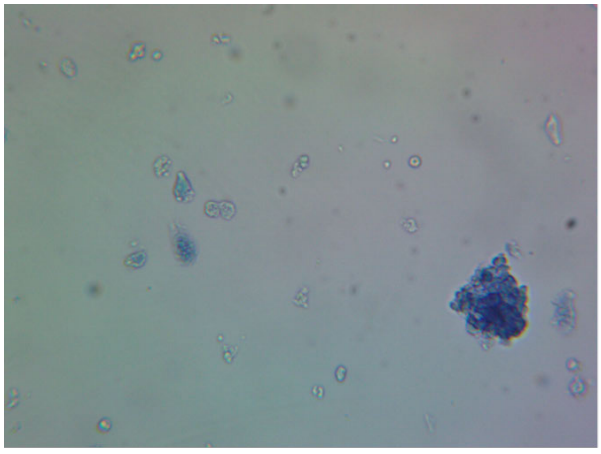


**Mouse Pancreatic Ductaladenocarcinoma FFPE Sample**

Post-Cell Isolation
(10x Magnification)



Post-Cell Isolation
(40x Magnification)



## Appendix 1 – Issues and Recommendations

| Issue | Recommendation |
|---|---|
| Cracking or shattering of FFPE curls | • Extend hydration time in step A.3.<br>• Proceed with caution during deparaffinization and rehydration steps in Section C, 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.<br>• Test "Demonstrated Protocol – Nuclei Isolation from FFPE 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. |

### 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)