Sample Preparation from FFPE Tissue Sections for GEM-X Flex Gene Expression

Introduction

This protocol outlines how to isolate nuclei from formaldehyde fixed & paraffin embedded (FFPE) Tissue Sections for use with GEM-X Flex Gene Expression workflow. The isolated population primarily consists of nuclei suspension (intact nuclear membrane with attached ribosomes), along with a minor fraction of whole cells and some dissociated cells/cellular debris (as described by Chung et al. 2022, Vallejo et al. 2022; see References). For simplicity, the document refers to this population collectively as "nuclei", which are used as the input for the GEM-X Flex Gene Expression assay.

The protocol includes instructions for deparaffinizing and rehydrating the tissue sections, followed by preparation and addition of a Dissociation Enzyme Mix to the sections. The tissue sections can then be dissociated using either a pellet pestle or a gentleMACS Octo Dissociator. Once dissociated, nuclei are resuspended in the Tissue Resuspension Buffer or Quenching Buffer B prior to counting. Refer to the Nuclei Input recommendations for GEM-X Flex Gene Expression hybridization.

This protocol was demonstrated using FFPE tissue blocks ranging 1-20 years in age. Each block may yield different amounts of material and data quality, depending on age, tissue type, pre-fixation tissue quality, tissue density, size/area of tissue in the scrolls, and other factors. See Appendix for nuclei yields derived from indicated tissue types. In most instances, a single 25 µm or 50 µm section will yield adequate nuclei.

Additional Guidance

This protocol was demonstrated using one or more 25 µm or 50 µm tissue sections from a diverse spectrum of tissue types. For human and mouse tissue, it is recommended to use at least one 25 µm section. Depending on tissue type and cross sectional area, some tissue types require more than two sections to yield enough nuclei. See Nuclei Isolation from FFPE Tissue Sections table.

Nuclei yield and recovery from FFPE blocks may be often lower or variable compared to freshly isolated and fixed samples. Lower than expected targeted nuclei recovery in the GEM-X Flex Gene Expression Web Summary File (HTML output of CellRanger) may be observed. This can be attributed to lower complexity, cell calling algorithm of the CellRanger pipeline, and/or other factors such as nuclei counting.

Users should expect to recover ~50-60% of the nuclei they are targeting during chip loading, and therefore, it is recommended that users target a minimum of 10,000 nuclei and follow the counting and handling Tips & Best Practices.

FFPE block* properties will also impact yields & data quality. Note that poor quality blocks will likely yield data that cannot be interpreted accurately or salvaged.

**Previously used high-performing FFPE blocks for Visium CytAssist Spatial Gene Expression assay will likely perform well if used for GEM-X Flex Gene Expression. Similar to the recommendation for Visium CytAssist assay, bulk RNA extraction for assessing DV200 value may be performed prior to GEM-X Flex Gene Expression.*

Consult the Visium CytAssist Spatial Gene Expression for FFPE Tissue Preparation Guide (CG000518) for DV200-related guidance.

Tissue and cells may carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

Nuclei Input

Recommended FFPE dissociated nuclei inputs for GEM-X Flex Gene Expression hybridization following this protocol are specified in the table below.

Performing a pilot multiplex assay run (4 rxns with 4 Probe Barcodes) is recommended prior to committing to larger studies.

For optimal assay results, targeting a minimum of 10,000 nuclei per Probe Barcode is recommended when performing the GEM-X Flex assay.

Recommended Tissue Section Input
1–2 25 µm tissue sections
Recommended FFPE Dissociated Nuclei Input # per Hybridization
25,000–500,000 nuclei *(if sufficient nuclei are available, it is recommended to default to 300,000 nuclei)*
Important Considerations It may be possible to use ≤25,000 nuclei, but it may lead to: • Loss of pellet • Not enough nuclei for storage • Difficulty in pooling samples in equal number when multiplexing • Not enough nuclei left after washing to target maximum cell load (20,000 cells/Probe Barcode) • Difficulty in counting samples; may require concentrating the sample • Observance of an atypical library trace and increase in wasted data due to excess supernatant left behind during post hybridization washes
Mitigation Strategies • Follow better sample preparation practices including use of a swinging bucket rotor • During probe hybridization, up to 15 µl supernatant can be left behind to avoid losing the pellet • During post-hybridization wash, up to 30 µl supernatant can be left behind to avoid losing the pellet • Follow pooled wash workflow during post-hybridization wash

Table 1. Recommended FFPE dissociated nuclei input for GEM-X Flex Gene Expression assay.

Tips & Best Practices

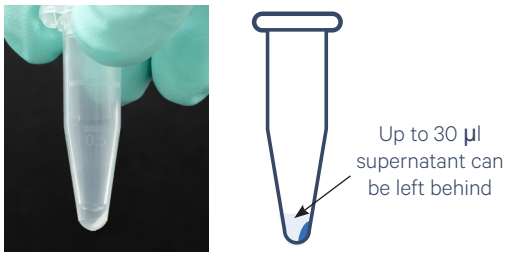
The following recommendations are critical for optimal performance of the GEM-X Flex Gene Expression assay.

Sample Handling

- Before starting, ensure that the tissue block is well hydrated. Remove and discard the first few sections from the block and place the block on an ice water bath (tissue facing the water surface) for sufficient rehydration. Rehydration times may vary; start with 10 min for freshly prepared blocks and extend the time for older blocks (≥60 min).
- Gently roll the sectioned tissue ribbons into compact scrolls.
- Use a paint brush (instead of forceps) for gentler handling and transferring of the tissue scrolls.
- Always pipette reagents to the side of the tubes and not directly on to the scrolls in the tube.

Centrifugation & Pellet Resuspension

- Use low binding micro-centrifuge tubes and a swinging-bucket rotor for centrifugation for higher cell recovery.
- When isolating nuclei from samples with low nuclei numbers (i.e. <300,000), complete removal of the supernatant is not required. Up to 30 µl supernatant may be left behind to optimize cell recovery without significantly impacting assay performance.



- When adding buffer to resuspend a pellet, pipette mix without introducing bubbles.

Nuclei Counting

- Accurate sample counting is critical for achieving desired cell recovery.

Consult the Cell Preparation for Single Cell Protocols Handbook (CG00053) for general cell counting guidelines.

- Fluorescent staining enables accurate counting even in the presence of sub-cellular debris and hence, is strongly recommended when counting nuclei isolated from FFPE tissue sections.
- Ensure that the cell counter laser/filter is compatible with the fluorescent dye used.
- Focus nuclei under the brightfield before switching to the fluorescent channel.
- Increase exposure time to help adjust signal to noise during counting.
- Do a final visual inspection to confirm the counting number is accurate. After obtaining the counting number, switch between brightfield and fluorescent channel to ensure that the counts include minimal to no debris.
- Including debris in the count will result in lower chip loading numbers, which may contribute to lower cell recovery.
- See Appendix for details on fixed nuclei counting, cell counters used, and dyes tested.

Nuclei Yield & Block Quality

- Nuclei yield can vary from different FFPE tissue types and blocks. If tissue of interest is likely to yield lower nuclei (based on past data or on yields table in Appendix), double the scrolls input to 2 x 25 µm or perform multiple rounds of FFPE tissue dissociation.
- For certain tissue types and for larger tissue section areas, one 10 µm scroll may be adequate.
- Final cell recovery after sequencing can be affected by counting accuracy, as well as the FFPE block quality. Over counting debris is likely to cause a lower loading number of cells into the chip, which might contribute to lower cell recovery.
- Poor quality of FFPE blocks are likely to generate nuclei with lower complexity. Note that the cell calling algorithm has a complexity threshold (only barcodes >500 UMIs can be called as cells) that may cause lower complexity samples to be under-called and can be interpreted as lower cell recovery.

Sample Storage & Transportation

- Fixed cell or nuclei suspensions can be stored at 4°C for up to 1 week or at -80°C for up to 12 months after resuspending in appropriate reagents.
- Sample storage and post-storage guidelines are provided in the Appendix.
- If shipping tissue scrolls (instead of FFPE blocks), scroll breakage impacting sample quality is possible. To minimize the risk of breakage, place an FFPE tissue scroll in individual 1.5-ml Eppendorf tubes. Cap the tube and secure the tube in the shipping container to minimize shaking. If significant temperature change is expected, ship the tube with ice packs.

Specific Reagents & Consumables

Vendor	Item	Part Number
For Tissue Section Transfer & Deparaffinization		
Millipore Sigma	Xylene, Reagent Grade	214736
	See alternate options at the end of this table	
	Ethyl Alcohol, 200 Proof, anhydrous	E7023
VWR	Ethanol absolute ≥99.5%, TechniSolv, pure (for Europe)	83813.360DP
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV
	15 ml PP Centrifuge Tubes	430791
Thermo Fisher	Nuclease-free Water (not DEPC-Treated)	AM9937
If using gentleMACS Octo Dissociator		
Miltenyi Biotec	gentleMACS C Tubes	130-093-237
Xylene Alternatives *Tested as viable alternatives for Tissue Deparaffinization*		
Millipore Sigma	Neo-Clear	1098435000
VWR	CitriSolv	89426-270
For Tissue Section Dissociation		
Millipore Sigma	Liberase TH	5401151001
Corning	RPMI	10-040-CV
If using pellet pestle:		
Fisher Scientific	RNase-Free Disposable Pellet Pestles	12-141-364
Fisher Scientific	BD Luer-Lok PrecisionGlide Disposable Syringes with Detachable Needles *OPTIONAL*	14-823-37
If using gentleMACS Octo Dissociator:		
Miltenyi Biotec	gentleMACS Octo Dissociator with Heaters	130-096-427
For Sample Filtration		
Sysmex	Sterile Single-Pack CellTrics Filters*(use 30 µm)	04-004-2326
Miltenyi Biotec	MACS SmartStrainers* (30 µm) *OR*	130-098-458
	Pre-Separation Filters*(30 µm)	130-041-407
**Choose either Sysmex or Miltenyi Biotec filter.*		
For Counting		
Nexcelom Biosciences	ViaStain PI Staining Solution	CS2-0109-5mL
	ViaStain AOPI Staining Solution *Alternative to PI Staining Solution*	CS2-0106-5mL
	Cellaca MX High-throughput† Automated Cell Counter	MX-112-0127
	Cellometer K2 Fluorescent Cell Counter	CMT-K2-MX-150
	PD100 Counting Chambers 1 case	CHT4-PD100-003
Biotium	NucSpot 470 *Alternative to PI Staining Solution.*	40083
	Dilute the stock to 1:100 and mix 1:1 with the sample. For example, add 10 µl diluted dye to 10 µl sample.	

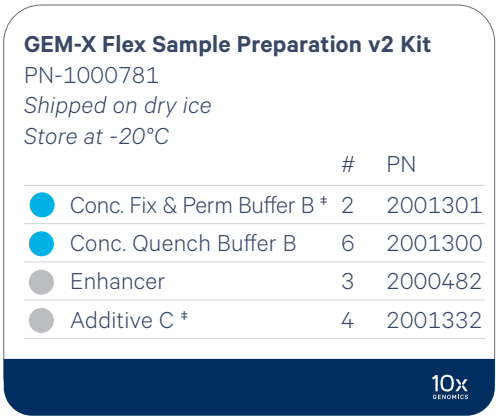
Thermo Fisher Scientific	†Countess II FL Automated Cell Counter *Discontinued*	AMAQAF1000
	Countess Automated Cell Counting Chamber Slides	C10228
	†Countess 3 FL Automated Cell Counter	AMQAF2000
	Trypan Blue Stain (0.4%)	T10282
†Choose Countess II/3, Cellaca, or equivalent fluorescent counter. See Appendix for more details on cell counters and dyes tested.		
For Storage & Post-Storage Processing		
10x Genomics	Enhancer*	2000482
**Included in the 10x Genomics GEM-X Flex Sample Preparation v2 Kit (PN-1000781).*		
Acros Organics	Glycerol**, 99.5%, for molecular biology, DNAse, RNAse and Protease free	327255000
Millipore Sigma	Glycerol for molecular biology**, ≥99.0%	G5516-100ML
***Choose either Millipore or Acros Organics glycerol*		
If using Tissue Resuspension Buffer:		
VWR	Tris Buffer, 1M sterile sol., pH 8.0	E199-100ML
Corning	Phosphate-Buffered Saline, 1X without Calcium and Magnesium	21-040-CV
Millipore Sigma	Protector RNase Inhibitor	3335399001
	Albumin, Bovine Serum***, 10% Aqueous Solution, Nuclease-Free	126615
Thermo Fisher Scientific	UltraPure Bovine Serum Albumin*** (BSA, 50 mg/ml) *This is a 5% BSA solution*	AM2616
****Choose either Millipore or Thermo Fisher Scientific BSA*		
If using Quenching Buffer B:		
10x Genomics	Conc. Quench Buffer B*	2001300

Additional Materials
Disposable plastic Pasteur pipette
Nuclease-free Water
DNA LoBind Tubes 2.0 ml (Eppendorf 022431048)
Water bath
Microtome
Centrifuge

This list may not include some standard laboratory equipment.

Refer to SDS and follow local and institutional guidelines for proper handling and disposal of all chemicals.

GEM-X Flex Sample Preparation v2 Kit, PN-1000781



‡ *Not used for this protocol*

The sample preparation kit provides sufficient reagents to process 96 FFPE samples.

Preparation - Buffers

Prepare all buffers fresh. Prepare Liberase TH stock solution (5 mg/ml) by adding 1 ml nuclease-free water to 5 mg Liberase TH. Mix at 2-8°C until completely resuspended. Store in single-use aliquots at -20°C.

Prepare Dissociation Enzyme Mix, incubate at **37°C** for **10 min** before proceeding with dissociation.

For pestle-based protocol

Dissociation Enzyme Mix	Stock	Final	1 rxn (µl)	4 rxn + 10% (µl)
Liberase TH (mg/ml)	5	1	210	924
RPMI	-	-	840	3696
Total Volume (µl)			**1050**	**4620**

For gentleMACS Octo Dissociator protocol

Dissociation Enzyme Mix	Stock	Final	1 rxn (µl)	4 rxn + 10% (µl)
Liberase TH (mg/ml)	5	1	420	1848
RPMI	-	-	1680	7392
Total Volume (µl)			**2100**	**9240**

*Prepare **either** Tissue Resuspension Buffer or Quenching Buffer B. Quenching Buffer B has been reformulated to boost cell recovery. Using Tissue Resuspension Buffer instead of Quenching Buffer B will result in 10-20% additional cell loss.*

Tissue Resuspension Buffer *(Maintain at 4°C)*	Stock	Final	1 rxn (µl)	4 rxn + 10% (µl)
PBS	1X	0.496X	248	1091.2
Tris buffer (pH 8.0; mM)	1000	50	25	110
BSA (RNase free)	10%	0.02%	1	4.4
RNase Inhibitor (U/µl)	40	0.24	3	13.2
Nuclease-free Water	-	-	223	981.2
Total Volume (µl)			**500**	**2200**

OR

Quenching Buffer B *(Maintain at 4°C)*	Stock	Final	1 rxn (µl)	4 rxn + 10% (µl)
Nuclease-free Water	-	-	437.5	1925
Conc. Quench Buffer B* (10x Genomics PN 2001300) *Thaw at room temperature. Vortex and centrifuge briefly. Maintain at 4°C*	8X	1X	62.5	275
Total Volume (µl)			**500**	**2200**

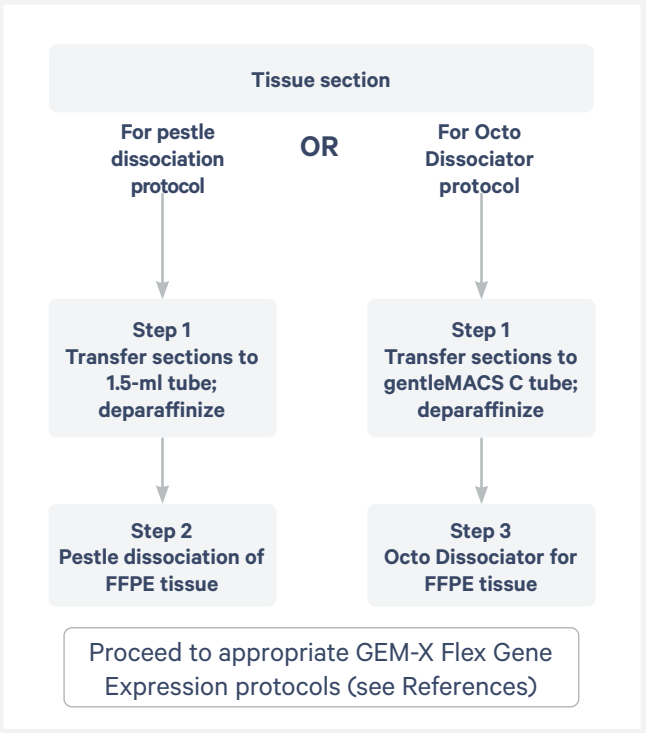
**Included in the 10x Genomics GEM-X Flex Sample Preparation v2 Kit (PN-1000781).*

Buffers for Storage of Fixed Samples
50% glycerol solution
• Mix an equal volume of water and 99% Glycerol. • Filter through a 0.2-µm filter. • Store at –20°C in 2-ml LoBind tubes.

Ethanol: Prepare fresh 70% and 50% Ethanol (1 ml each/sample).

Refer to SDS and follow local and institutional guidelines for proper handling and disposal of all chemicals.

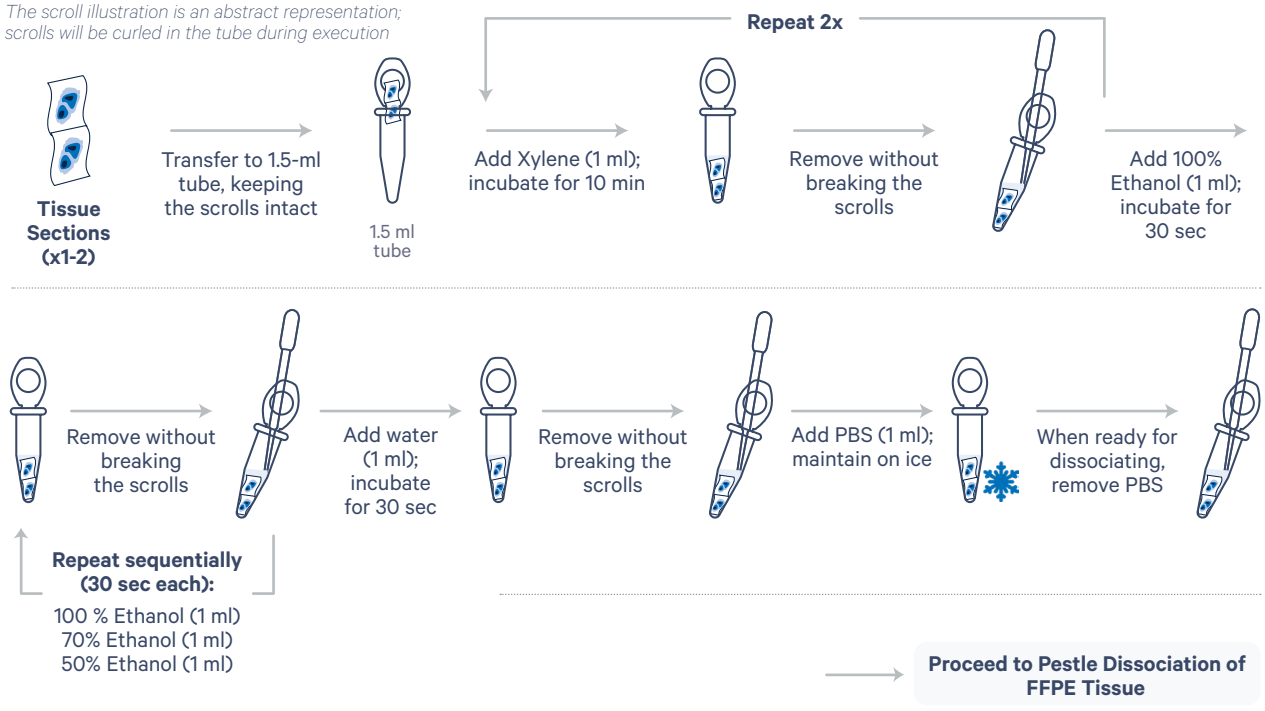
Step Selection Overview



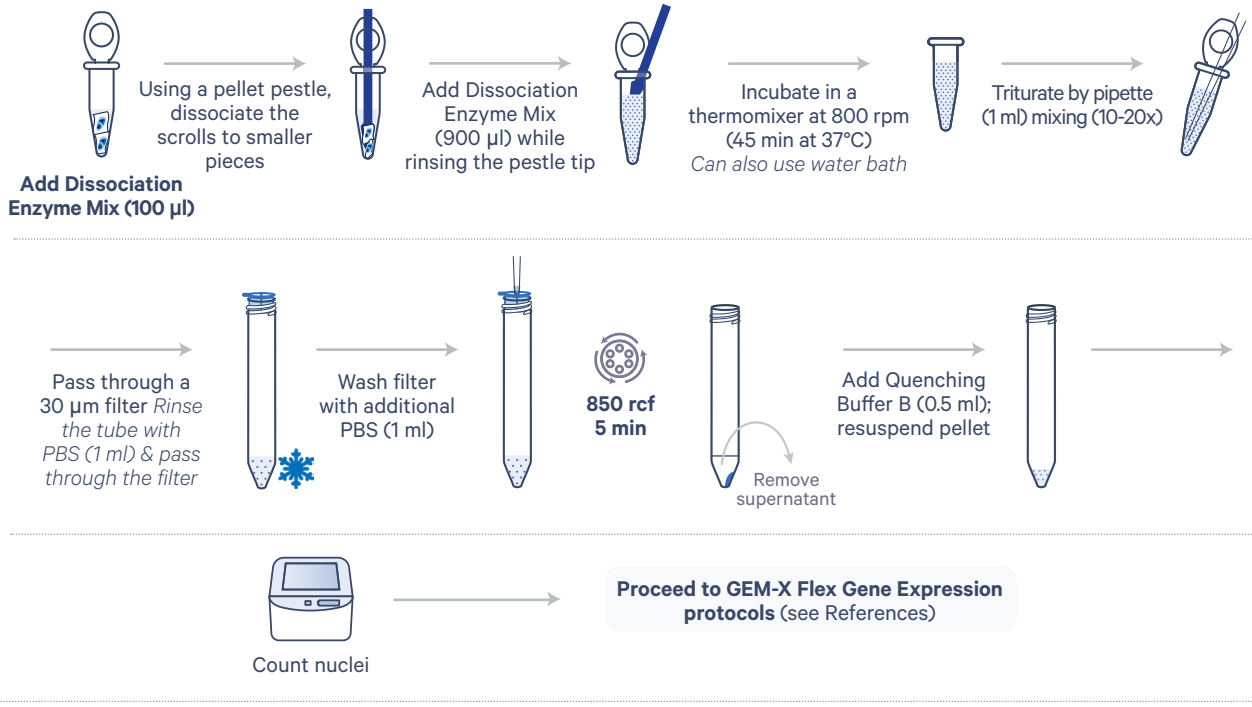
Protocol Overview: Pestle for Isolation of Nuclei from FFPE Tissue Sections

1. Tissue Section Transfer & Deparaffinization

The scroll illustration is an abstract representation; scrolls will be curled in the tube during execution

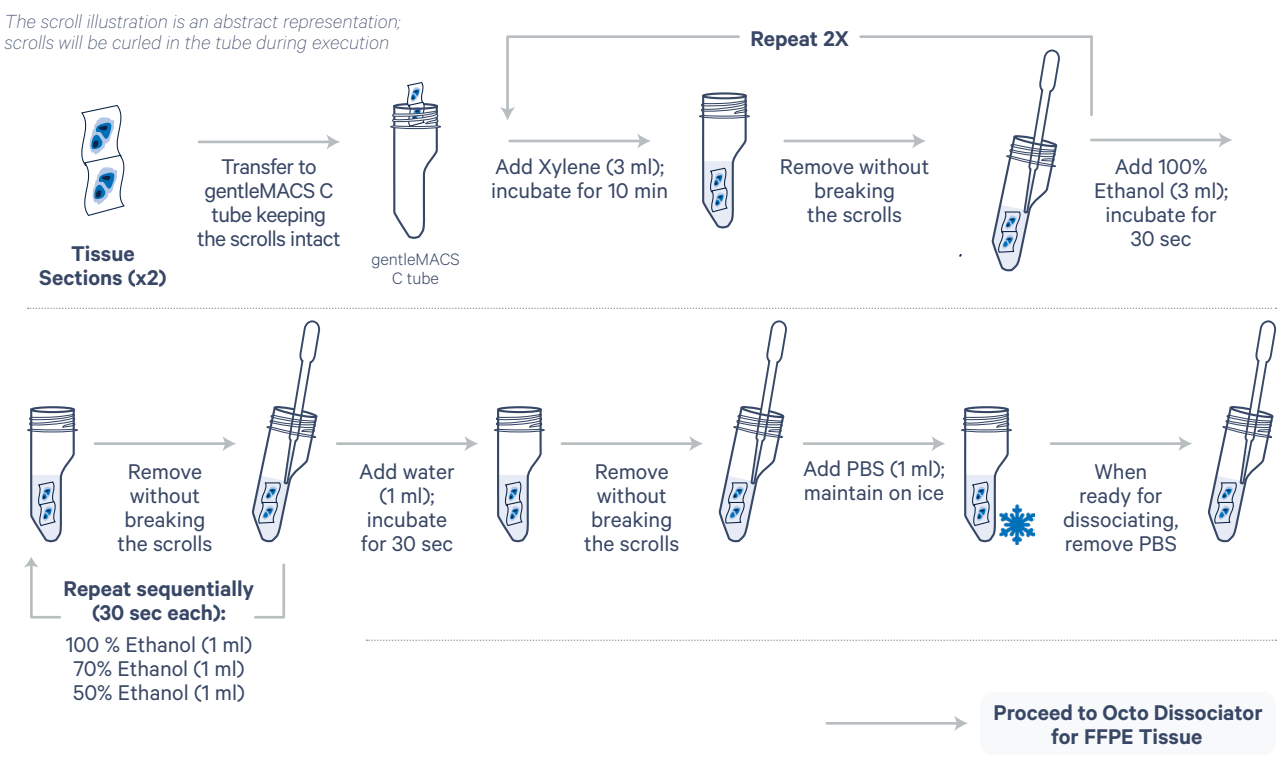


2. Pestle Dissociation of FFPE Tissue

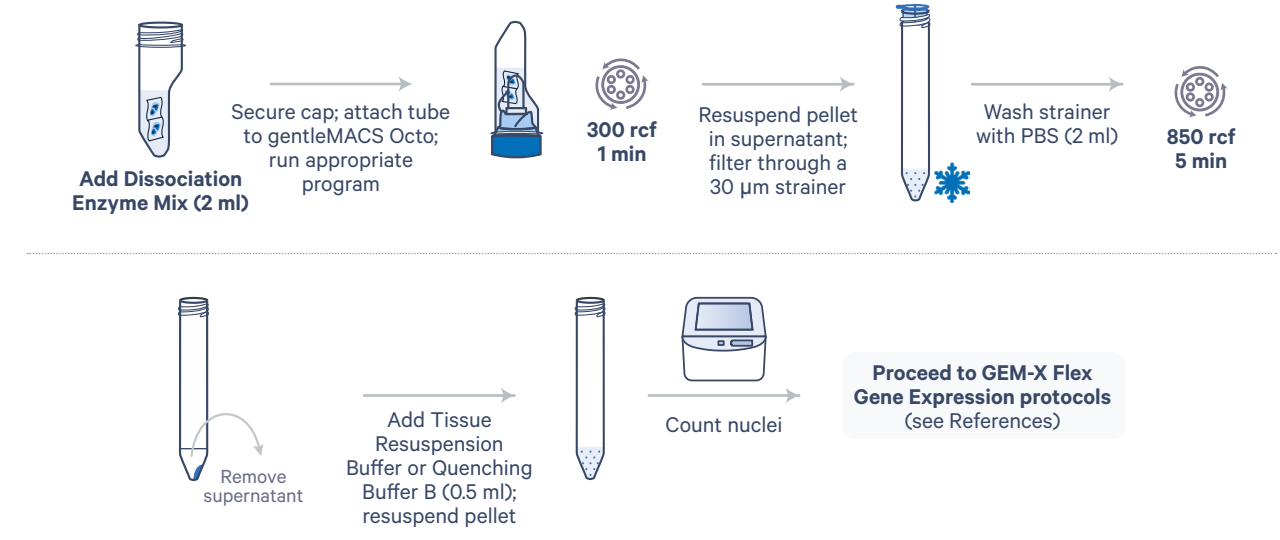


Protocol Overview: gentleMACS Octo Dissociator for Isolation of Nuclei from FFPE Tissue Sections

1. Tissue Section Transfer & Deparaffinization



3. gentleMACS Octo Dissociator for FFPE Tissue



Isolating Nuclei from FFPE Tissue Sections

This protocol was demonstrated using 25 µm or 50 µm FFPE tissue sections of various tissue types (maximum of six 50 µm sections were dissociated). The nuclei derived from the FFPE sections are compatible with the GEM-X Flex Gene Expression. Nuclei can also be isolated after scraping FFPE sections off from slides. See Appendix for details.

1. Tissue Section Transfer & Deparaffinization

Before starting, carefully inspect the tissue block to assess dehydration. Remove and discard the first few sections from the block and place the block on an ice water bath (tissue facing the water surface) for sufficient rehydration. Rehydration times may vary; start with 10 min for freshly prepared blocks and extend the time for older blocks (≥60 min).

See representative images of scrolls derived from well rehydrated and less rehydrated blocks in the Troubleshooting section.

Prepare the first section from the rehydrated block by setting the microtome to 5 µm. Discard the first section; set the microtome for 25 µm or 50 µm sections.

Volumes added in steps 1b, 1e, and 1g are based on the type of tube being used for the protocol.

a. After discarding the first few sections, start with preparing up to two 25 or two 50 µm sections* from the rehydrated tissue block and transfer into a 1.5 ml tube (or to a gentleMACS C Tube) while keeping the scrolls intact. The scrolls in the tube can be stored at **4°C** for up to **1 year**.

**One section may be sufficient. See Appendix for yields from up to 6 sections.*

Ensure that the scrolls stay intact during transfer and through steps 1a-1n. Add reagents along the side of the tube and not directly onto the scrolls. Intact sections enable easier liquid aspiration and minimize tissue loss. If scrolls break, see guidance in Troubleshooting section. Proceed with the protocol and based on nuclei yields process more sections if needed.

TIPS *Always use freshly prepared reagents for the deparaffinization steps. Ensure the scrolls are fully submerged in reagents in the following steps.*

Maximum 6x 25 µm or 3x 50 µm scrolls should be used per tube; if using more, additional tubes are required.

b. Add **1 ml** xylene to the 1.5-ml tube (**3 ml** to gentleMACS C Tube) and incubate for **10 min** at **room temperature**.

TIPS *Xylene alternatives specified in Specific Reagents & Consumables may be used.*

c. Using a Pasteur pipette, remove the liquid from the tube without breaking the scrolls.

d. Repeat **steps 1b-1c** twice (for a total of three xylene incubations).

e. Add **1 ml** 100% ethanol to the 1.5 ml tube (**3 ml** to gentleMACS C Tube) and incubate for **30 sec** at **room temperature.**

f. Using a Pasteur pipette remove the liquid from the tube without breaking the scrolls.

g. Add **1 ml** 100% ethanol (to both tube types) and incubate for **30 sec** at **room temperature.**

h. Using a Pasteur pipette remove the liquid from the tube without breaking the scrolls.

i. Add **1 ml** 70% ethanol (to both tube types) and incubate for **30 sec** at **room temperature.**

j. Using a Pasteur pipette remove the liquid from the tube without breaking the scrolls.

k. Add **1 ml** 50% ethanol (to both tube types) and incubate for **30 sec** at **room temperature.**

l. Using a Pasteur pipette remove the liquid from the tube without breaking the scrolls.

m. Add **1 ml** nuclease-free water to the tube and incubate for **30 sec** at **room temperature**.

n. Using a Pasteur pipette remove the liquid from the tube without breaking the scrolls.

o. Add **1 ml** PBS and maintain on ice.

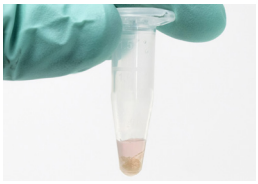
p. Proceed either to **Step 2 Pestle Dissociation of FFPE Tissue** (sections are in 1.5-ml tube) or to **Step 3 gentleMACS Octo Dissociator for FFPE Tissue** (sections are in gentleMACS C Tube).

2. Pestle Dissociation of FFPE Tissue

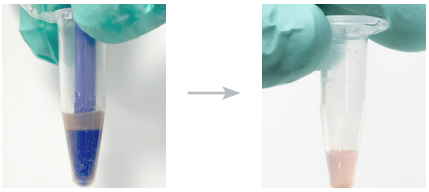
Refer to Preparation - Buffers section to prepare Dissociation Enzyme Mix. Incubate at **37°C** for **10 min** before proceeding with dissociation.

a. Remove PBS from the tube without breaking the scrolls.

b. Add **100 µl** Dissociation Enzyme Mix to the tube.



c. Using a 1.5 ml pellet pestle, dissociate the tissue scrolls breaking them to smaller pieces.



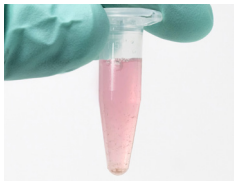
TIPS *Grasp the pestle between the thumb and fingers and rotate the pestle (clockwise and counterclockwise 10-20X) inside the tube and go up and down with the scrolls trapped between the wall of the tube and the pestle for dissociation or until the scrolls are broken into similarly sized smaller pieces. The pellet pestle can also be attached to a compatible cordless motor and operated inside the tube until the scrolls are broken into similarly sized smaller pieces.*

d. Add **900 µl** Dissociation Enzyme Mix to the tube while rinsing the pestle tip into the tube to collect any additional tissue pieces sticking to the pestle. Pipette mix.

e. Incubate for **45 min** at **37°C** in a thermomixer at **800 rpm**. Alternatively, incubate in a **37°C** water bath, mixing by inversion every **15 min** during the incubation.

TIPS *Ensure no tissue pieces are stuck to the tube cap. Centrifuge at 300 rcf for ~20 sec to remove pieces stuck on the cap.*

f. Using a 1,000 µl pipette, triturate the tissue pieces in the tube by pipetting 10-20x.



TIPS *The progress of the dissociation can be monitored by taking a 10 µl aliquot and counting. If the concentration is lower than recommended for hybridization, additional dissociation maybe required; recommend performing step g.*

g. **OPTIONAL:** Aspirate and push the tissue pieces and solution through a 23G needle 5x to improve cell recovery.

h. Pass the suspension through a Pre-Separation Filter (30 µm) placed on a 5- or 15-ml tube placed on ice.

i. Rinse the original tube (step 2f) with **1 ml** chilled PBS and rinse the 30 µm filter with additional **1 ml** chilled PBS to minimize sample loss. Collect the filtrate in the same tube as step 2h.

j. Centrifuge the suspension at **850 rcf** at **4°C** for **5 min**.

TIPS *Use of swinging-bucket rotor is recommended for higher cell recovery.*

k. Remove supernatant without disturbing the pellet.

l. Resuspend the pellet in **0.5 ml** chilled Tissue Resuspension Buffer or Quenching Buffer B, pipette mix 5x, and maintain on ice.

m. Determine concentration of the fixed sample using an Automated Cell Counter or hemocytometer. See Appendix for Fixed Cell Counting along with details on cell counters and dyes tested.

TIPS *For accurate counting, it is strongly recommended that the nuclei suspension be stained with a fluorescent nucleic acid dye such as PI Staining Solution and counted using an automated fluorescent cell counter.*

n. Proceed **immediately** to appropriate GEM-X Flex protocols – Probe Hybridization step 1 (see References) or store (**Fixed Sample** Storage Guidance in Appendix).

3. gentleMACS Octo Dissociator for FFPE Tissue

Refer to Preparation - Buffers section to prepare Dissociation Enzyme Mix. Incubate at **37°C** for **10 min** before proceeding with dissociation.

a. Remove the PBS from the gentleMACS C Tube without breaking the scrolls.

b. Add **2 ml** Dissociation Enzyme Mix to the gentleMACS C Tube and close securely.

TIPS *Ensure the scrolls are fully submerged in reagents in the following steps.*

c. Place the gentleMACS C Tube on the gentleMACS Octo Dissociator , apply Heating units and run the gentleMACS Program 37C_FFPE_1. Run time **~48 min**. Ensure that the Octo Dissociator blades are moving before walking away.

gentleMACS Program 37C_FFPE_1

1	temp ON
2	spin -20 rpm, 5 min *(counterclock)*
3	loop 3X
4	spin -20 rpm, 14 min *(counterclock)*
5	spin 1700 rpm, 7 sec
6	spin -1700 rpm, 1 sec *(counterclock)*
7	spin 1700 rpm, 2 sec
8	spin -1700 rpm, 1 sec *(counterclock)*
9	spin 1700 rpm, 4 sec
10	end loop
11	end

d. At the end of the run, detach the tube from the gentleMACS Octo Dissociator and visually inspect to ensure the scrolls have been dissociated. If scrolls are not dissociated, see guidance in Troubleshooting section.

e. Centrifuge at **~300 rcf** for **1 min** and resuspend the pellet in the supernatant.

f. Pass the suspension through a Pre-Separation Filter (30 µm) placed on a 15-ml tube on ice.

g. Rinse the original gentleMACs tube (step 3d) with **2 ml** chilled PBS and use that rinse for an additional wash of the **30 µm** filter to minimize sample loss. Collect the filtrate in the same tube as step 3f.

TIPS *Use of swinging-bucket rotor is recommended for higher cell recovery.*

h. Centrifuge the suspension at **850 rcf** at **4°C** for **5 min**.

i. Remove the supernatant without disturbing the pellet.

j. Resuspend the pellet in **0.5 ml** chilled Tissue Resuspension Buffer or Quenching Buffer B, pipette mix 5x, and maintain on ice.

k. Determine concentration of the fixed sample using an Automated Cell Counter or hemocytometer. See Appendix for Fixed Cell Counting, along with details on cell counters and dyes tested.

TIPS *For accurate counting, it is strongly recommended that the suspension be stained with a fluorescent nucleic acid dye (such as PI Staining Solution) and counted using an automated fluorescent cell counter.*

l. Proceed **immediately** to appropriate GEM-X Flex Gene Expression protocols or store according to Fixed Sample Storage Guidance provided in Appendix.

Appendix

Fluorescent staining is strongly recommended when counting dissociated FFPE nuclei. PI stained nuclei can be counted using an automated cell counter of hemocytometer.

The use of fluorescent dye during counting enables accurate quantification even in the presence of sub-cellular debris.

Counting using PI Staining Solution

This protocol provides instructions for counting sample using PI staining solution and the Cellaca Counter to enable accurate quantification even in the presence of sub-cellular debris. The optimal concentration for the Cellaca Counter is 100-10,000 cells/µl (10^5–10^7cells/ml). Refer to manufacturer's instructions for details on operations.

- Add **25 µl** PI Staining Solution into Mixing Row of Cellaca plate
- Gently mix the sample. If the sample is too concentrated, a 1:1 dilution in PBS can also be prepared. For example, add 15 µl fixed sample suspension to 15 µl PBS.
- Add **25 µl** sample to Mixing Row of plate containing PI Staining Solution. Gently pipette mix 8X.
- Transfer stained sample to Loading Row of Cellaca plate.
- For counting fixed samples, only use the PI (Propidium Iodide) channel. Refer to manufacturer's instructions for details.

When counting after dissociation, the suspension may include some debris. However, during the GEM-X Flex Gene Expression workflow, the post-hybridization wash step will reduce the debris.

Samples stained with PI staining solution can also be counted using Countess II FL or 3 FL Automated Cell Counter or Cellometer K2 Fluorescent Cell Counter. See manufacturer's instructions for details. Additional counters listed in the Cell Preparation for Single Cell Protocols Handbook (CG00053) have not been tested with FFPE tissues specifically. Pilot counting experiment is strongly recommended to determine the optimal counter and fluorescent dye combination.

Table 2 shows the combination of counters and dyes tested for counting nuclei post-dissociation and post-hybridization wash. Representative images post-dissociation and post-hybridization wash (ready for loading on to the chip for GEM generation) are shown in Figures 1-2.

Counter Type	Fluorescent Dye	Counting Comparison
Cellaca *Range: 1 x 10^5–1 x 10^7cells/ml* *Automated exclusion of debris from cell count*	• Propidium Iodide • NucSpot 470 • DAPI	Comparable counting results at both counting steps for all three dyes
Countess II FL/Countess 3 FL *Range: 1 x 10^4–1 x 10^7cells/ml (optimal 1 x 10^5–4 x 10^6)* *Manual debris exclusion from cell count post-image capture, using gates on the instrument program*	• Propidium Iodide • NucSpot 470 • DAPI	Comparable counting results at both counting steps for the three dyes
Cellometer K2 *Range: 1 x 10^5–1 x 10^7cells/ml* *Debris exclusion from cell count by adjusting instrument program settings before image capture*	• Propidium Iodide • NucSpot 470	Comparable counting results at both counting steps for the two dyes Propidium Iodide stained nuclei require longer exposure compared to NucSpot 470 but can still be relatively dimmer

Table 2. Cell counters and dyes used for counting nuclei isolated from FFPE sections of four different human tissue types (glioblastoma, tonsil, liver, and kidney). Counting performed post-dissociation and post-hybridization wash.

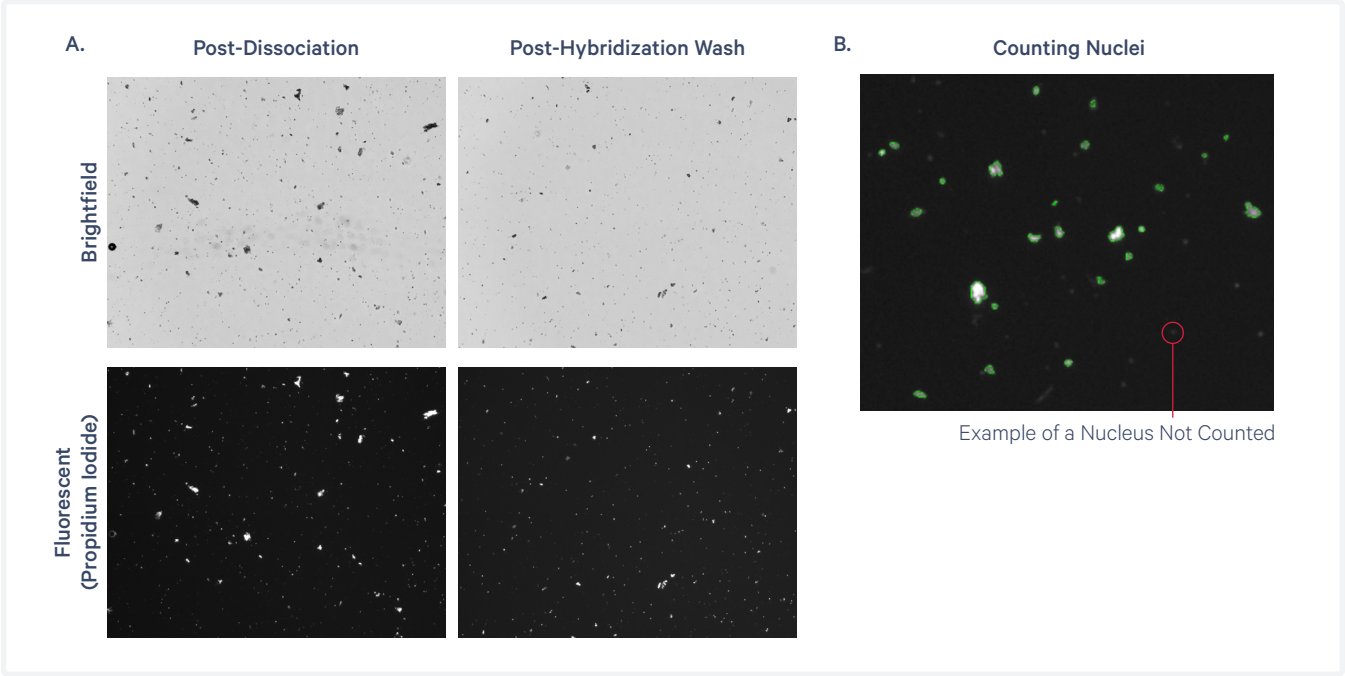


Figure 1. Nuclei derived from human glioblastoma FFPE tissue sections post-dissociation and post-hybridization wash (A), shown in brightfield and fluorescent (Propidium Iodide stained) formats. Representative image (B) shows examples of nuclei included and excluded from the count. Nuclei counted using Cellaca counter.

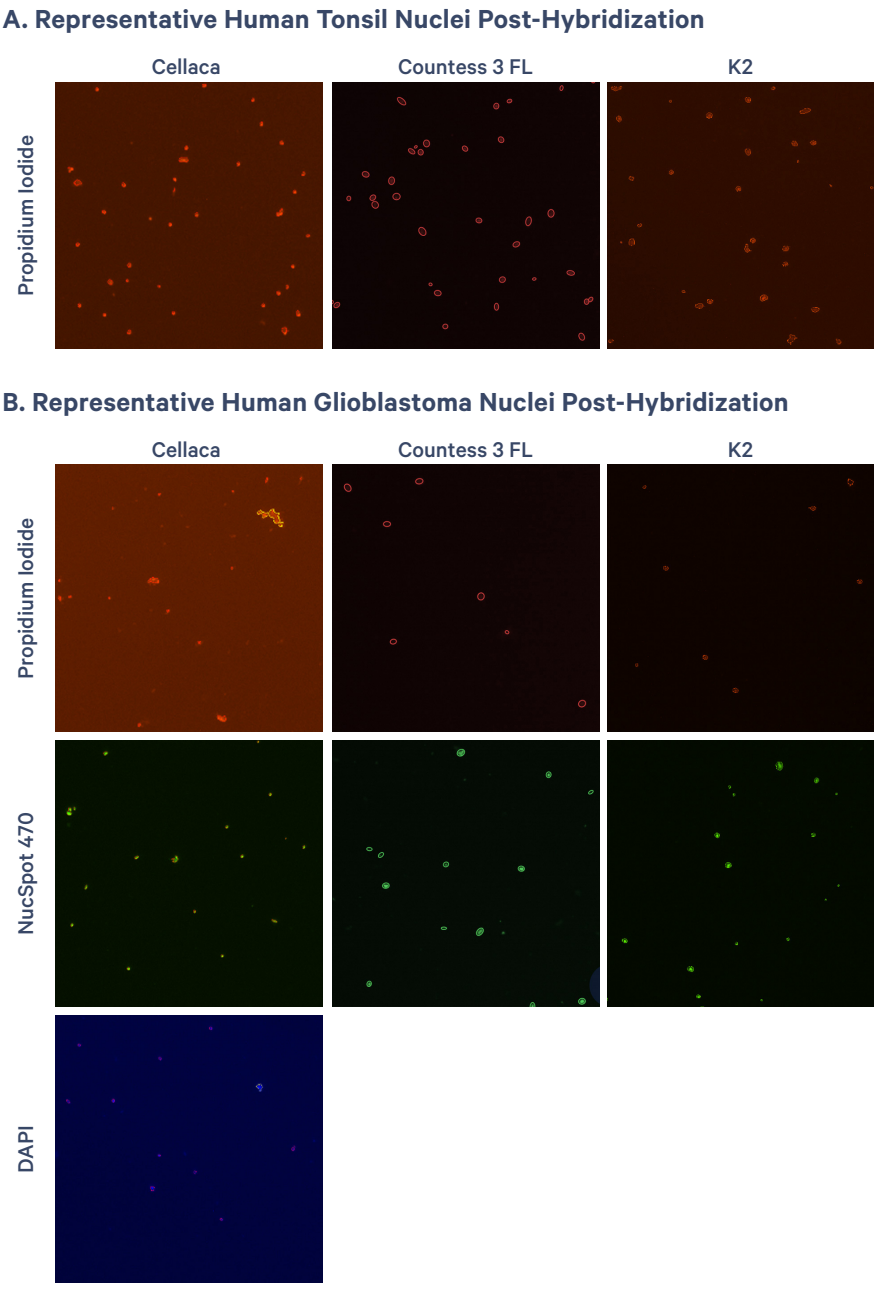


Figure 2. Representative images showing nuclei derived from two different tissue types post-hybridization, along with the corresponding cell counter and staining dye used.

Fixed Sample Storage Guidance

Fixed samples (dissociated nuclei resuspended in Quenching Buffer B or in Tissue Resuspension Buffer) can be stored for short or long-term as described below. Choosing only one storage condition per sample (either short or long term) is recommended.

Short-term Storage at 4°C

a. Thaw Enhancer (10x Genomics PN-2000482) for **10 min** at **65°C**. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min. If not used within 10 min and precipitation is observed, reheat at 65°C to ensure enhancer is fully dissolved again before use.

b. Add **0.1 volume** pre-warmed Enhancer to fixed sample in Quenching Buffer B or in Tissue Resuspension Buffer. For example, add **50 µl** Enhancer to **500 µl** fixed sample in Quenching Buffer B or in Tissue Resuspension Buffer. Pipette mix.

c. Store sample at **4°C** for up to **1 week**.

Long-term Storage at −80°C

a. Thaw Enhancer (10x Genomics PN-2000482) for **10 min** at **65°C**. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min. If not used within 10 min and precipitation is observed, reheat at 65°C to ensure enhancer is fully dissolved again before use.

b. Add **0.1 volume** pre-warmed Enhancer to fixed sample in Quenching Buffer B or in Tissue Resuspension Buffer. For example, add **50 µl** Enhancer to **500 µl** fixed sample in Quenching Buffer B or in Tissue Resuspension Buffer. Pipette mix.

c. Add 50% glycerol for a final concentration of 10%. For example: add **137.5 µl** 50% glycerol to **550 µl** fixed sample in Quenching Buffer B or in Tissue Resuspension Buffer with Enhancer. Pipette mix.

d. Store at **−80°C** for up to **12 months**.

Post-Storage Processing

Samples may undergo a color change during storage (e.g. black, light gray, or green), however this will not impact assay performance.

If samples were stored at −80°C, thaw at room temperature until no ice is present.

a. Centrifuge sample at **850 rcf** for **5 min** at **room temperature**.

b. Remove the supernatant without disturbing the pellet.

c. Resuspend the pellet in **0.5 ml** Quenching Buffer B or Tissue Resuspension Buffer and maintain on ice.

d. Determine concentration of the fixed sample using an Automated Cell Counter or hemocytometer. See Counting guidelines. See Appendix for additional cell counters and dyes tested.

e. Proceed **immediately** to appropriate GEM-X Flex Gene Expression protocols (see References).

Isolating Nuclei by Scraping FFPE Tissue Sections on Slides

In limited testing, FFPE tissue sections placed on slides were used to isolate nuclei as input for the assay.

Using one human endocervical cancer and one healthy human lymph node block, tissue sections placed on 20 slides/tissue type (5 µm FFPE tissue sections on glass slides; ~100 µm of total tissue) were used to isolate nuclei as per the following protocol steps.

Deparaffinization on Slides & Tissue Transfer by Scraping

a. Two Xylene washes for **10 min**.

b. Two 100% Ethanol washes for **3 min**.

c. Two 95% Ethanol washes for **3 min**.

d. One 70% Ethanol wash for **3 min**.

e. One Nuclease-free Water wash for **20 sec**.

f. One 1X PBS wash for **20 sec**.

g. Using a razor blade (Stanley High Carbon Steel Single Edge Razor Blade 1-½"), scrape off the tissue sections from the slide while holding it at a 45° angle and transfer the scrapings to a 1.5 ml Eppendorf tube.

h. To isolate nuclei, follow the Pestle Dissociation of FFPE Tissue Protocol starting at Step 2a.

It is likely that, depending on the tissues, less than 20 sections (for example, one 5 µm FFPE section) could yield sufficient nuclei (>25 K) for hybridization. For example, using the information in Table 3, the amount of nuclei from a single 5 µm endocervical would yield ~146,500 nuclei. See comparison of nuclei yields and data derived from scraping FFPE tissue sections from slides versus tissue scrolls in the table below.

Refer to the Nuclei Yields from FFPE Tissue Sections table when optimizing a new sample type. If scraping FFPE tissue sections from slides, double the amount of tissue sections listed in the table may be required to ensure the minimum sample input requirements are met.

	Tissue Type	Nuclei Isolated From	Nuclei Count	Estimated Nuclei Number	Median UMI/ Cell	MedianGenes/ Cell	Fraction Reads in Cells
HUMAN	Endocervical Cancer	Tissue scrolls	2.34×10^6	5,200	950	600	72%
		Slide scrapings	2.93×10^6	4,200	1,100	630	72%
	Healthy Lymph Node	Tissue scrolls	4.68×10^6	7,100	1,400	1,050	82%
		Slide scrapings	5.41×10^6	6,200	1,100	950	78%

Table 3. Yield of nuclei isolated from FFPE tissue sections scraped off of slides.

Nuclei Isolation from FFPE Tissue Sections

Listed below are the nuclei yields from FFPE tissue sections processed as described in this protocol. Based on this information, approximate nuclei yield from a specific tissue type/section may be estimated. For example, based on the information in this table, a 1 x 25 µm lung cancer section would yield ~460,000 nuclei using the manual protocol. Note that the tissue density in combination with tissue cross section area (tissue volume) will impact final yields.

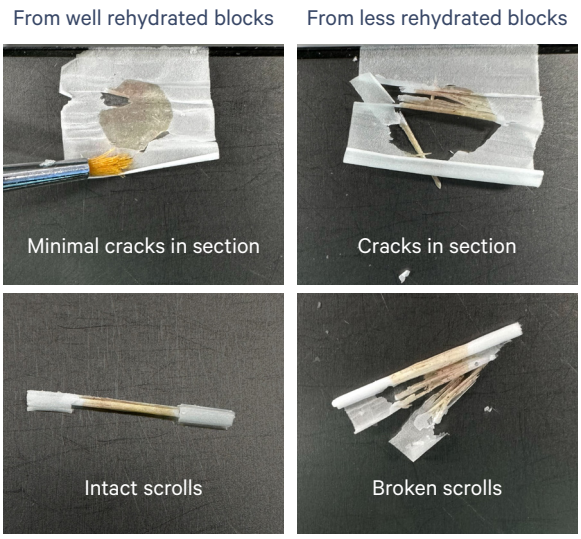
	Tissue Type	Tissue State *(Healthy or Diseased)*	No. of Sections x Thickness *(µm)*	Tissue Cross Section *(x*y mm)*	Cubic Volume *(# of sections* x*y mm^3)*	Nuclei Yields	
						Manual Protocol	gentleMACS Octo Dissociator
MOUSE	Brain (Cerebellum)	Healthy	6 x 50 µm	2 x 2 mm	1.2	1.88 x 10^6	1.24 x 10^6
	Brain (Forebrain)	Healthy	6 x 50 µm	4 x 5 mm	6.0	0.545 x 10^6	0.2 x 10^6
	Kidney	Healthy	3 x 50 µm	5 x 9 mm	6.8	4.73 x 10^6	3.89 x 10^6
	Liver	Healthy	3 x 50 µm	6 x 9 mm	8.1	1.7 x 10^6	1.6 x 10^6
	Spleen	Healthy	3 x 50 µm	1 x 9 mm	1.4	5.15 x 10^6	4.865 x 10^6
HUMAN	Brain	Healthy	2 x 25 µm	5 x 10 mm	2.5	0.4 x 10^6	0.36 x 10^6
	Brain	Glioblastoma	2 x 25 µm	10 x 13 mm	6.5	0.77 x 10^6	0.76 x 10^6
	Breast	Healthy	2 x 25 µm	10 x 6 mm	3.0	0.04 x 10^6	0.02 x 10^6
	Breast	Invasive Ductal Carcinoma	2 x 25 µm	13 x 7 mm	4.6	1.47 x 10^6	1.87 x 10^6
	Cervix	Endocervical Adenocarcinoma	2 x 25 µm	10 x 7 mm	3.5	1.2 x 10^6	1.7 x 10^6
	Colon	Cancer, Colorectal	2 x 25 µm	10 x 9 mm	4.5	0.6 x 10^6	0.76 x 10^6
	Kidney	Healthy	2 x 25 µm	7 x 6 mm	2.1	0.36 x 10^6	0.53 x 10^6
	Liver	Healthy	2 x 25 µm	5 x 6 mm	1.5	0.38 x 10^6	0.61 x 10^6
	Liver	Hepatocellular Carcinoma	2 x 25 µm	5 x 9 mm	2.3	0.56 x 10^6	0.67 x 10^6
	Lung	Healthy	2 x 25 µm	9 x 7 mm	3.2	0.44 x 10^6	0.79 x 10^6
	Lung	Cancer	2 x 25 µm	9 x 8 mm	3.6	0.92 x 10^6	0.76 x 10^6
	Lymph Node	Healthy	2 x 25 µm	7 x 5 mm	1.8	2.34 x 10^6	2.65 x 10^6
	Lymph Node	Diseased, Reactive	2 x 25 µm	10 x 12 mm	6.0	6.75 x 10^6	7.45 x 10^6
	Ovary	Cancer	2 x 25 µm	10 x 18 mm	9.0	2.2 x 10^6	1.92 x 10^6
	Pancreas	Healthy	2 x 25 µm	11 x 9 mm	5.0	0.38 x 10^6	0.58 x 10^6
	Prostate	Cancer	2 x 25 µm	9 x 9 mm	4.1	0.8 x 10^6	1.6 x 10^6
	Skin	Malignant Melanoma	2 x 25 µm	4 x 4 mm	0.8	0.22 x 10^6	0.23 x 10^6

Table 4. Yield of nuclei isolated from FFPE tissue sections derived from various mouse and human tissue types.

Troubleshooting

Problem	Solution
Tissue block not adequately rehydrated	Soak in water longer, until rehydrated
Tissue section scroll breaks during deparaffinization	Ensure tissue blocks is adequately rehydrated *(see adjacent representative tissue section images from well rehydrated and less rehydrated tissue blocks)* DO NOT pipette directly on top of the scrolls; pipette against the tube wall
Tissue section scroll breaks during rehydration	Centrifuge at 850 rcf for 1 min prior to reagent exchange to pellet tissue pieces at the bottom of the tubes
Lower than expected nuclei yields	Refer to Nuclei Yields from FFPE Tissue Sections table when planning the experiment If possible, repeat sample preparation protocol with more sections to increase yield (not more than six 25 µm scrolls or three 50 µm scrolls should be placed per tube; if dissociating more scrolls, additional tubes should be used) If additional sections are not available and fewer than 25,000 nuclei are used, the following risks apply: -Loss of sample during Post-Hyb Washes -Observance of an atypical library trace and increase in wasted data due to excess supernatant left behind during Post-Hyb Washes -Difficult to pool samples in equal number for Multiplexing and dropouts may be more likely. Refer to the Fixed RNA Profiling for Multiplexed Samples Pooling Workbook (CG000565) for guidance. Consider pooling nuclei derived from tissues of similar complexity

Representative Tissue Section Images



	If executing probe hybridization step with lower input, ensure that no nuclei are removed during post-hybridization washes by leaving behind slightly more buffer in the tube during the washes
Debris/large chunks during cell counting	Pass sample through a 30 µm filter followed by rinsing the filter with PBS to minimize loss Remaining debris (that passes through the 30 µm filter) will be further reduced during the post-hybridization wash step in the downstream workflow (see Figure 1B)
Using gentleMACS Octo Dissociator	
Intact scroll at the end of run or run fails midway	Run a "spin only" program on the Octo Dissociator with steps 5-9 (from the protocol in step 3C)
Octo Dissociator without heated lids	Use a water bath for 14 min, followed by completing steps 5-9 as written (from the protocol in step 3C). Repeat two more times for a total of three times (total time ~45-50 min)

References

1. H Chung et al, SnFFPE-Seq: towards scalable single nucleus RNA-Seq of formalin-fixed paraffin-embedded (FFPE) tissue. BioRxiv (2022) https://doi.org/10.1101/2022.08.25.505257
2. A Vallejo et al, snPATHO-seq: unlocking the FFPE archives for single nucleus RNA profiling (2022) https://doi.org/10.1101/2022.08.23.505054

Compatible User Guides:

3. GEM-X Flex Gene Expression Reagent Kit for Multiplexed Samples (CG000787)
4. GEM-X Flex Gene Expression Reagent Kits For Singleplexed Samples (CG000786)

Take 1 minute to evaluate this protocol. Scan this code or click here.

Document Revision Summary

Document Number	CG000784
Title	Sample preparation from FFPE Tissue Sections for GEM-X Flex Gene Expression
Revision	Rev A to Rev B
Revision Date	March 2025
Summary of Changes	Centrifugation & Pellet Resuspension section includes updated nuclei numbers (page 3)
	Additional guidance regarding tissue block rehydration time (pages 3 & 9)
	Updated GEM-X Flex Sample Preparation v2 Kit information (pages 5-6)
	Updated to include a merged table with Additional Materials information (page 5)
	Minor updates in guidance, language, and format throughout

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