Additional Flex information and resources

This document collects some general useful information for 10X Flex projects, as well as links to some of the available protocols and resources. Please note that NGI does not guarantee that it contains the most up-to-date information. The latest protocols and recommendations can always be found on the 10X Genomics webpage

For any 10X Flex project at NGI, please always read and adhere to NGI's **10X Genomics FLEX (Fixed RNA) GEM-X User guidelines.**

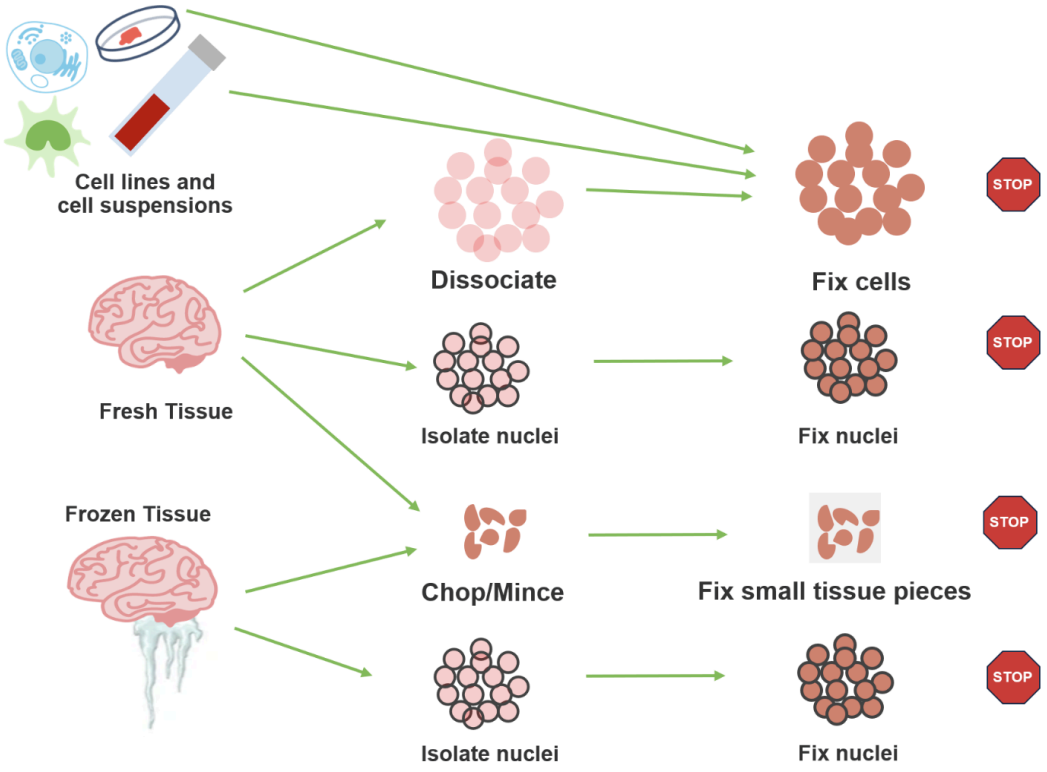
Starting material for Flex

For an overview of the merits of using different starting materials for a Flex project see this 10X blog entry. *NB! This is written for the older Next-GEM version, so cell numbers are not up to date, but the discussion is useful.*

It is possible to use either cells/nuclei in suspension, tissues or FFPE scrolls as starting material for the Flex sample preparation. For fresh tissues, it is possible to fix before or after cell dissociation. Fixing before dissociation is often considered preferred, in order to preserve RNA quality and sensitive cell types, and is recommended if possible. For snap-frozen tissues, tissue must either be fixed directly when thawing, or alternatively nuclei can be isolated prior to fixation.

Take into account that fixation, dissociation and freezing/thawing is associated with some sample loss. Please plan accordingly when deciding on the amount of starting material.

Figure 1. Overview of sample preparation options for Flex
Figure displaying fixation and dissociation options for fresh starting material. FFPE material can also be used for Flex, but is not included in this overview.



Adapted from:
https://www.10xgenomics.com/blog/planning-your-single-cell-experiment-with-flex-faqs-for-fresh-frozen-and-ffpe-samples

Demonstrated protocols from 10X Genomics

Always prepare samples for long-term storage. This includes following the protocol with overnight fixation. Refer to the appendix of the following Demonstrated Protocols when preparing fixed sample suspension for storage. Please ensure that you are always following the most up-to-date protocol from 10X Genomics (check 10X Genomics website).

1. **Cells/Nuclei**.
    a. Protocol for tubes: Fixation of Cells & Nuclei for GEM-X FLEX gene expression
    b. Protocol for plates with V2: Plate-based Sample Preparation for GEM-X Flex v2
2. **Tissue samples**. Protocol: Tissue Fixation & Dissociation for GEM-X FLEX gene expression. This protocol is preferred if you do not have an optimised tissue dissociation protocol for single cell suspensions.
3. **FFPE blocks**. Protocol: Isolation of Cells from FFPE Tissue Sections for GEM-X FLEX gene expression

4. **Blood**. Protocol: Blood fixation and cell isolation for GEM-X FLEX gene expression

For general advice on sample preparation for single-cell experiments please see: Cell Preparation for Single Cell Protocols

Importantly, previously established tissue dissociation protocols for fresh tissue that yielded good results for 3' GEX are also expected to perform similarly in Fixed RNA Profiling, and can be used with this assay.

The fixation of whole tissues for use in the Fixed RNA Profiling assay is not supported. Tissues must first be chopped into smaller pieces before fixation for optimal assay performance. This step is required for uniform fixation and permeabilization. Please also see: Which tissue dissociation protocols are supported for use with the Fixed RNA Profiling assay?

List of tissues tested by 10X Genomics

For a list of tested tissues (fresh or frozen) with the 10X Genomics FLEX assay see here.
For a list of tested FFPE tissues, see here.

Multiplet rates

For each multiplexed sample, assuming 1 unique Probe Barcode is used per sample, the undetected (i.e. with same Probe Barcode) cell multiplet rate for the GEM-X FX chip is approximately 0.4% multiplets per 1,000 cells recovered. Up to 320,000 cells with V1 and 1 million cells with V2 Apex can be recovered with a low multiplet rate since GEMs with multiplets derived from different Probe Barcodes can be demultiplexed.

Undetectable Multiplet Rate (%)	Cells Loaded/ Sample Barcode	Cells Recovered/ Sample Barcode	Number of Sample Barcodes (cells equally distributed; cells/well)									
			4		16		24		96		384	
			Loaded	Recovered	Loaded	Recovered	Loaded	Recovered	Loaded	Recovered	Loaded	Recovered
~0.2	725	500	2,900	2,000	11,600	8,000	17,400	12,000	69,600	48,000	278,400	192,000
~0.32	1,160	800	4,640	3,200	18,560	12,800	27,840	19,200	111,360	76,800	445,440	307,200
~0.4	1,450	1,000	5,800	4,000	23,200	16,000	34,800	24,000	139,200	96,000	556,800	384,000
~0.72	2,610	1,800	10,440	7,200	41,760	28,800	62,640	43,200	250,560	172,800	1,002,240	691,200
~0.8	2,900	2,000	11,600	8,000	46,400	32,000	69,600	48,000	278,400	192,000	1,113,600	768,000
~1.04	3,770	2,600	15,080	10,400	60,320	41,600	90,480	62,400	361,920	249,600	1,447,680	998,400
~1.6	5,800	4,000	23,200	16,000	92,800	64,000	139,200	96,000	556,800	384,000		
~2.4	8,700	6,000	34,800	24,000	139,200	96,000	208,800	144,000	835,200	576,000		
~3.2	11,600	8,000	46,400	32,000	185,600	128,000	278,400	192,000	1,113,600	768,000		
~4.0	14,500	10,000	58,000	40,000	232,000	160,000	348,000	240,000	1,392,000	960,000		
~4.2	15,225	10,500	60,900	42,000	243,600	168,000	365,400	252,000	1,461,600	1,008,000		
~5.0	18,125	12,500	72,500	50,000	290,000	200,000	435,000	300,000				
~5.32	19,285	13,300	77,140	53,200	308,560	212,800	462,840	319,200				
~6.0	21,750	15,000	87,000	60,000	348,000	240,000	522,000	360,000				
~7.0	25,375	17,500	101,500	70,000	406,000	280,000	609,000	420,000				
~8.0	29,000	20,000	116,000	80,000	464,000	320,000	696,000	480,000				

Compatibility with protein labeling

GEM-X Flex is compatible with cell-surface and intracellular protein labeling using antibody-oligonucleotide conjugates, for example with TotalSeq-C antibodies and Proteintech Multipro antibodies. Antibody staining must be performed before cell fixation. For intracellular proteins, a brief initial fixation is performed prior to cell permeabilization, staining and final fixation. For guidelines on staining, and also antibody-oligonucleotide conjugation, best practise advice etc., see Cell Surface & Intracellular Protein Labeling for GEM-X Flex Gene Expression

Staining and any necessary optimisation is performed by the researcher, prior to delivering the samples to NGI.

Compatibility of cell types with high RNase levels

10X Genomics has performed limited testing in-house on RNase-rich tissues (e.g. spleen). These samples performed similarly to other non-RNase-rich tissues in the Fixed RNA Profiling Assay. Fixation of these samples limits the degradation after collection that is often observed due to high levels of RNases and other inhibitory compounds, but it is recommended to limit the cell number for the hybridisation step to 100,000.

Can fixed samples be FACS sorted?

Post-fixation samples can be sorted using FACS for advanced sample clean-up, as well as for the enrichment of specific populations in the Fixed RNA Profiling Assay. For more details, see Can post-fixation samples be sorted in the Flex Gene Expression assay?. For FFPE derived samples, see Can dissociated FFPE samples be flow sorted for use with the Flex Gene Expression

Multiplexing strategies

The Flex barcodes can be used either for multiplexing different samples or for increasing the number of cells analysed by using multiple barcodes per sample (subpooling).

This illustration provides an overview of multiplexing configurations, using the Chromium Flex V2 kit, with 4-plex barcodes, but the sample principle applies for the V1 kit and for higher levels of multiplexing. The same principle can be applied when using up to 16 barcodes; Max # of cells per Probe Barcode is 20,000 cells.

Pooling Samples with Different RNA Content

Due to the nature of a multiplexing pool, the sequencing reads for the pool will be distributed to different samples in proportion to their inherent RNA content and all the samples will have the same sequencing saturation. As a result, a sample with cells high in RNA will receive more reads per cell, whereas a sample with cells low in RNA will have proportionally fewer reads per cell, and sequencing saturation will be approximately the same for each sample. Because the distribution of reads across samples is determined by the composition of the pool, it is not possible to add reads to specific samples in the pool.

The recommendation therefore is to:

1. Pool samples when comfortable with sequencing to the same percent saturation for each sample.
2. Keep samples separate if wishing to sequence one closer to saturation than the others is preferred

Custom Probe Design

To detect expression of exogenous genes or other transcripts, not included in the probe set, it is possible to design custom probes. While this is not supported by 10X, and performance of custom probes cannot be guaranteed, there is still guidance on probe design available here for GEM-X Flex V1 and here for GEM-X V2