Evaluating Short-term Storage of Cells for Single Cell Sequencing

Introduction

Cryopreservation is a common method for preserving samples for long-term storage and transport. However, some cell types may be sensitive to freeze-thaw cycles or may need to be stored for only a few hours, highlighting a need for alternative methods of sample preservation. Protocols for short-term storage of cells without cryopreservation are limited, and the impact of these on single cell sequencing data has not been thoroughly evaluated. This Technical Note evaluates HypoThermosol FRS and RPMI + 10% FBS as short-term cell storage solutions in 10x Genomics Single Cell 3' Gene Expression Assays.

Madissoon et. al. (Genome Biology, 2020) evaluated the storage of lung, esophagus, and spleen tissue from human donors in HypoThermosol FRS (Stem Cell Technologies, PN-101373), an optimized hypothermic (2-8°C) preservation media designed to preserve cells and tissues, in single cell RNA sequencing experiments. The investigators found that storage of tissues in HypoThermosol FRS preserved cell yield, total number of reads per cell, and other quality metrics on the tissues within the first 24 hours of storage. However, broader application of HypoThermosol FRS in cells needs further investigation.

In this document, the impact of different short-term cell storage solutions on performance of single cell RNA sequencing assays was assessed (Figure 1).

Cells were stored either in RPMI + 10% FBS or HypoThermosol FRS for up to seven days at 4°C. Apoptotic cells were excluded using Annexin V-based dead cell removal. Samples were subsequently divided into two groups: with or without dead cell removal, and were then processed using Chromium Single Cell 3' Gene Expression assay followed by sequencing and data analysis to determine gene expression profiles, transcriptomic and mitochondrial read mapping, and cell type annotation.

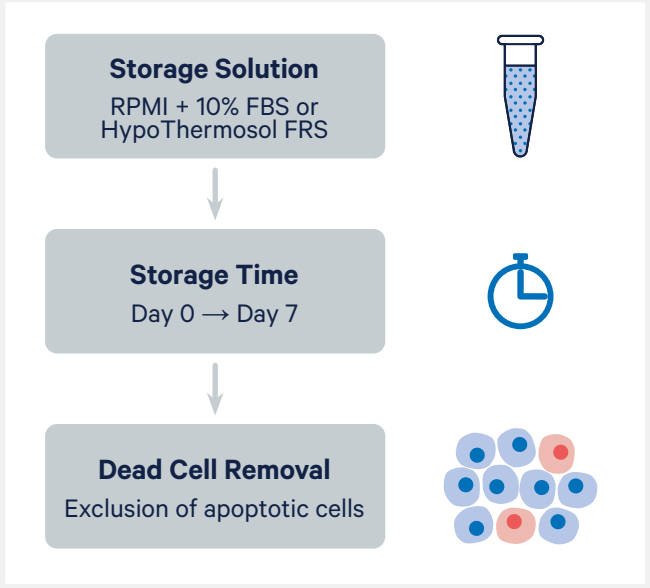


Figure 1. Key variables tested in this Technical Note were storage solution, storage time, and dead cell removal.

Methods

Sample types tested are summarized in Table 1. PBMCs were resuspended into HypoThermosol FRS or RPMI + 10% FBS storage solutions. Human and mouse cell lines were cultured separately in their respective culture media, mixed together in a 1:1 proportion, and then resuspended in HypoThermosol FRS or RPMI + 10% FBS storage solutions. Samples were stored at 4°C for up to seven days and cell viability was measured at each timepoint using trypan blue staining. The impact of dead cell removal using Annexin-V was also evaluated. All timepoints and conditions were then processed with the Single Cell 3' v3.1 (Dual Index) reagents, targeting 6,000 cells. Sequencing data were analyzed with Cell Ranger 6.1 and visualized with Loupe Browser 5.1. See Figure 2 for a detailed schematic of the experimental design.

Table 1. Summary of Sample Types

Cell Type	Cell Type Specifics	Vendor	Total Cells Profiled
Human Peripheral Mononuclear Blood Cells (PBMCs)	Donor 1	All Cells	395,245
	Donor 2	All Cells	
Adherent Cells	Human embryonic kidney epithelial cells (HEK293T)	ATCC	244,525
	Mouse embryonic fibroblasts (3T3)	ATCC	
Suspension Cells	Human immortalized T lymphocyte cells (Jurkat)	ATCC	244,906
	Mouse T lymphocytes (EL4)	ATCC	

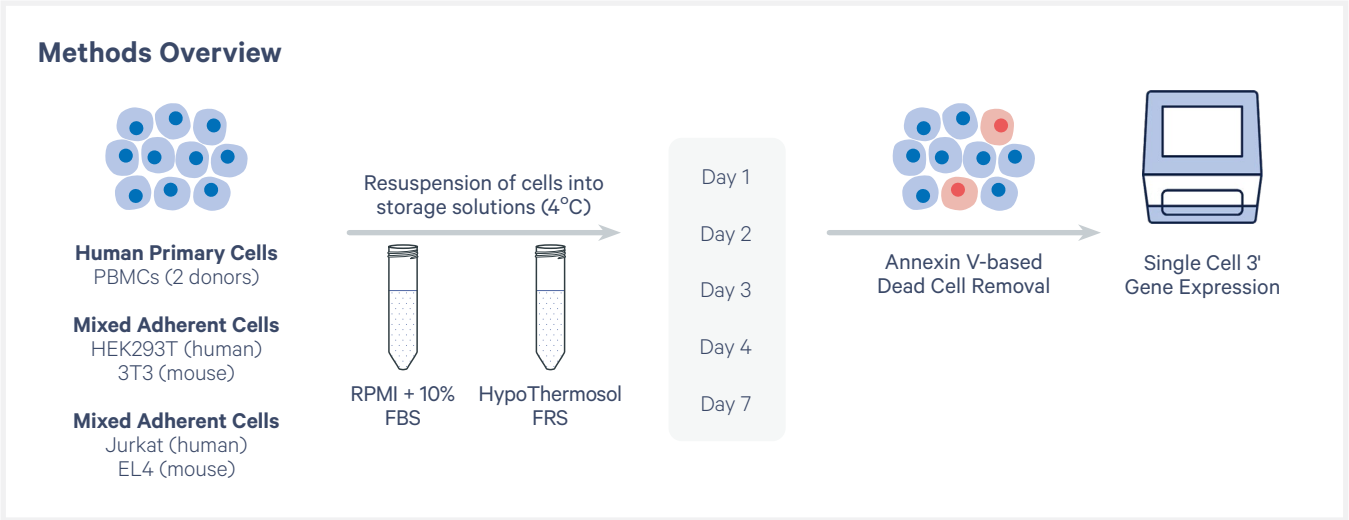


Figure 2. Schematic of overall experimental design.

Results

Cell Viability and Sensitivity

Trypan blue staining was used to assess cell viability (Figure 3A). Single cell gene expression profiles were generated for PBMCs and the two cell line mixtures, fresh (day 0) and at intermediate timepoints, until 7 days in short-term storage solution (Figures 3B-D). Median Genes and Median UMIs per cell were reduced in PBMCs, as well as human adherent cells, when stored in HypoThermosol FRS (with and without dead cell removal) as compared to mouse adherent cells, where increases were observed. Human and mouse suspension cells showed reduced Median Genes and Median UMIs per cell when stored in HypoThermosol FRS (with and without dead cell removal), whereas mouse suspension cells showed an increase in both metrics when stored in RPMI + 10% FBS until day 7. The differential response of cells to storage treatment emphasizes the importance for users to validate a storage protocol for cells and tissues of interest.

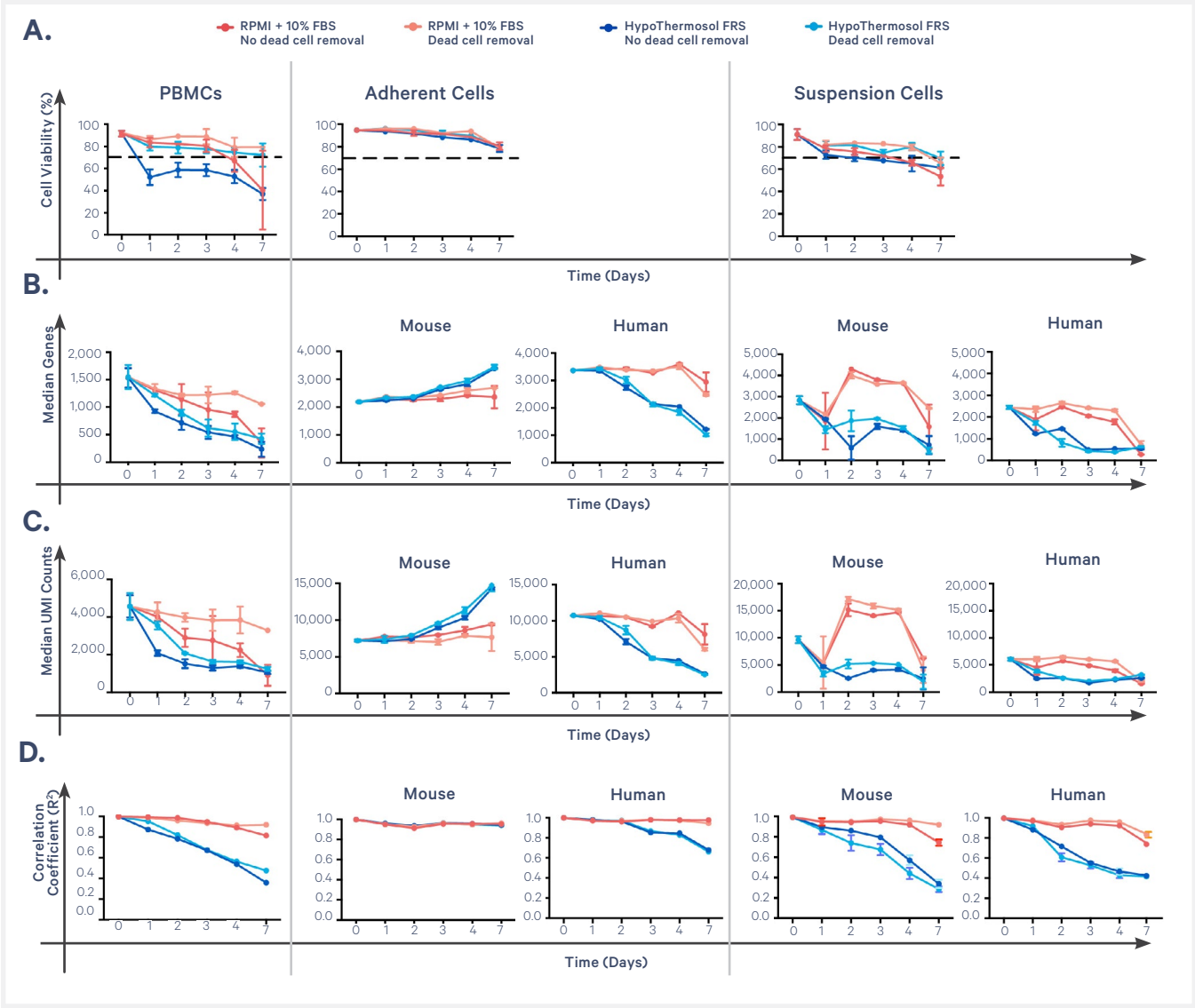


Figure 3. A. Cell viability for PBMCs, adherent, and suspension cells. PBMC data is derived from two independent donors. Adherent and suspension cells include 4 and 3 technical replicates, respectively. Mean +/- SD is plotted. **B.** Median genes per cell at a sequencing depth of 20K raw read pairs per cell. **C.** Median UMI counts per cell at a sequencing depth of 20K raw read pairs per cell. **D.** Correlation coefficient (R^2) of total cell-associated UMI counts (pseudo-bulk) per gene against day 0 with each indicated time point. Values of 2 technical replicates are depicted for each condition in Figures B-D and mean +/-SD is plotted.

Transcriptomic and Mitochondrial Read Mapping

The fraction of reads mapping to the transcriptome was relatively stable over time in RPMI + 10% FBS. In contrast, storage in HypoThermosol FRS showed a decreased transcriptomic mapping and a corresponding increase in mitochondrial RNA content, an indicator of decreased sample quality (Figure 4).

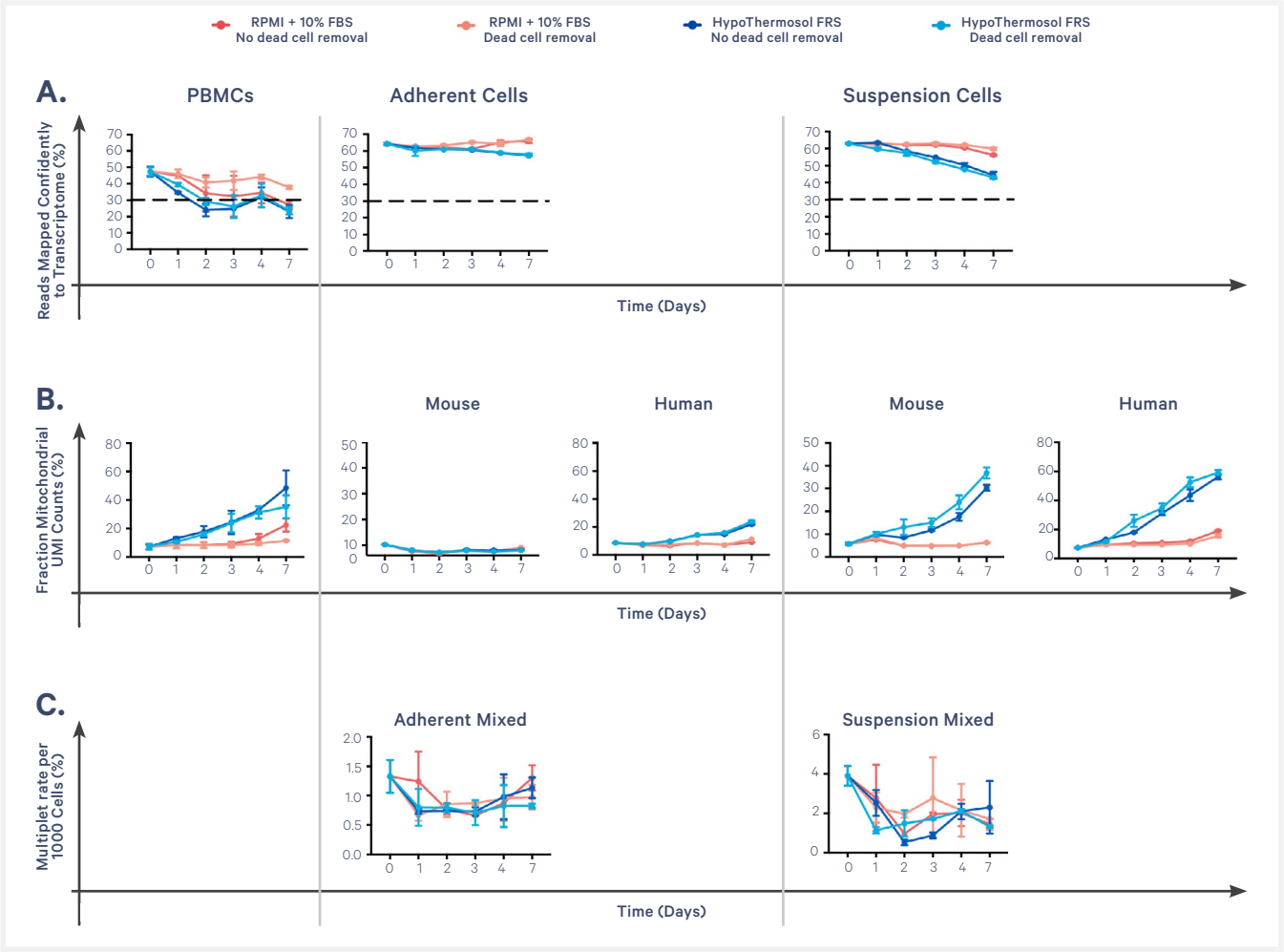


Figure 4. Change in quality metrics of single cell gene expression data over time, showing **(A)** reads confidently mapping to the transcriptome, **(B)** percentage of mitochondrial UMI counts, and **(C)** multiplet rate.

Human PBMCs: Cell Type Distribution and UMAP Representations

PBMCs were annotated using a custom annotation pipeline. PBMCs for fresh (day 0) and for intermediate time points until 7 days in either RPMI + 10% FBS or HypoThermosol FRS, with and without dead cell removal, were compared. PBMCs stored in HypoThermosol FRS had a higher fraction of undetermined cell types, with accompanying losses of T-cell subtypes (Figure 5A-B). Data from all conditions was combined and UMAP plots were generated. Cells stored in HypoThermosol FRS showed less overlap between time-points, thus indicating differences in gene expression that increase with storage-time (Figure 5C). This effect was significantly less pronounced for cells stored in RPMI + 10% FBS. These observations are consistent with the changes in cell type distribution observed in Figure 5A-B.

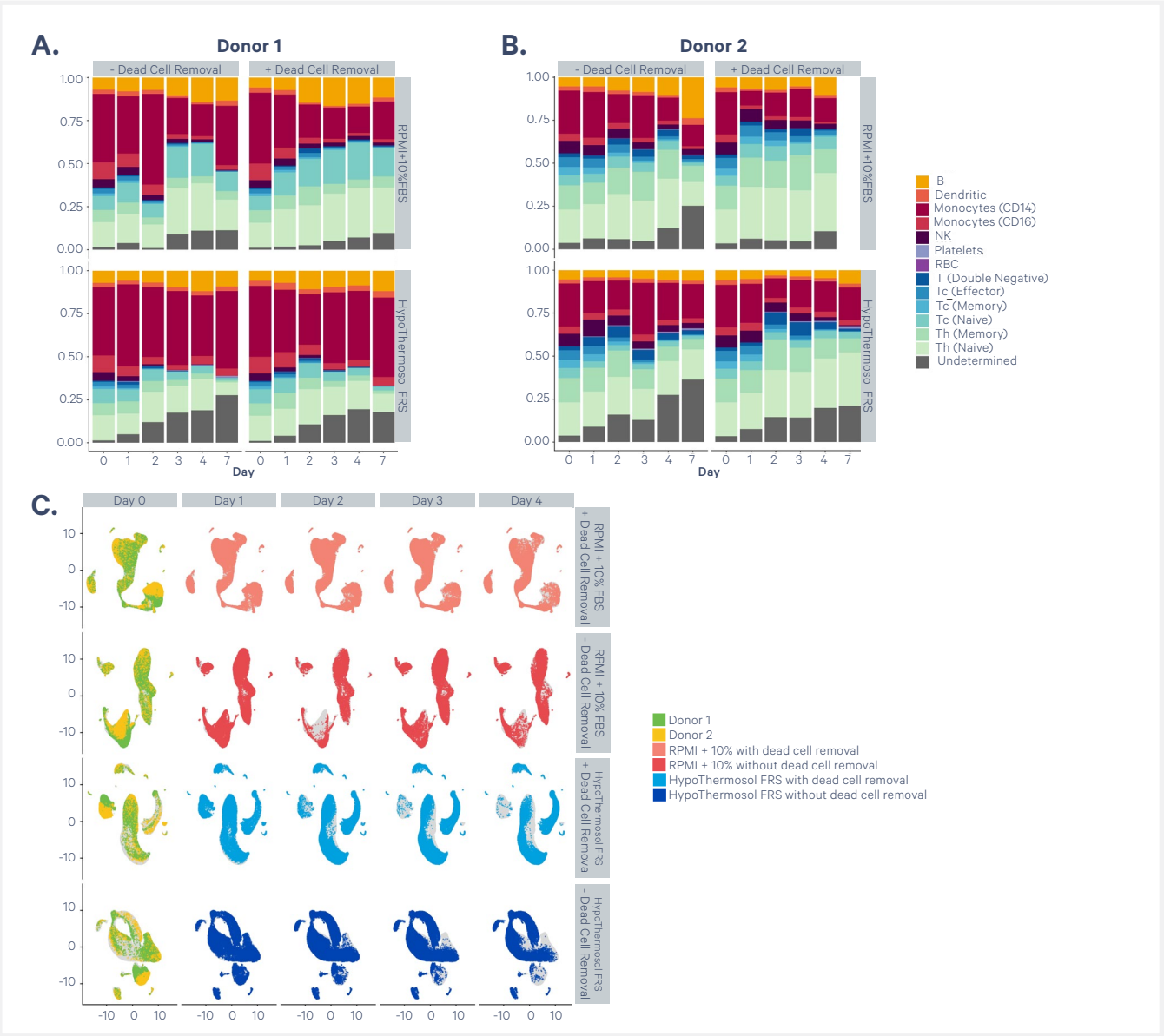


Figure 5. Cell type distributions of Donor 1 **(A)** and Donor 2 **(B)** human PBMCs. **C.** UMAPs are shown for day 0 controls (yellow/green) and indicated timepoints. UMAPs for cells stored in RPMI + 10% FBS and HypoThermosol FRS are colored in red and dark blue, respectively. UMAPs for cells stored in RPMI + 10% FBS or HypoThermosol FRS undergoing additional dead cell removal are colored light red and light blue, respectively. Changes in the 2D distribution of cells across timepoints for HypoThermosol FRS indicates a batch effect that is significantly less pronounced for RPMI + 10% FBS. Grey cells indicate cells from other timepoints.

Cell Line Mixtures: UMAP Representations

UMAP plots were generated for cell line mixtures across the different conditions. Cell line mixtures cluster by species as seen by the non-overlapping green and yellow clusters on day 0. Cells stored in HypoThermosol FRS showed less overlap across timepoints as compared to cells stored in RPMI + 10% FBS. This effect is less pronounced in RPMI + 10% FBS. These observations are consistent with what was observed in human PBMCs.

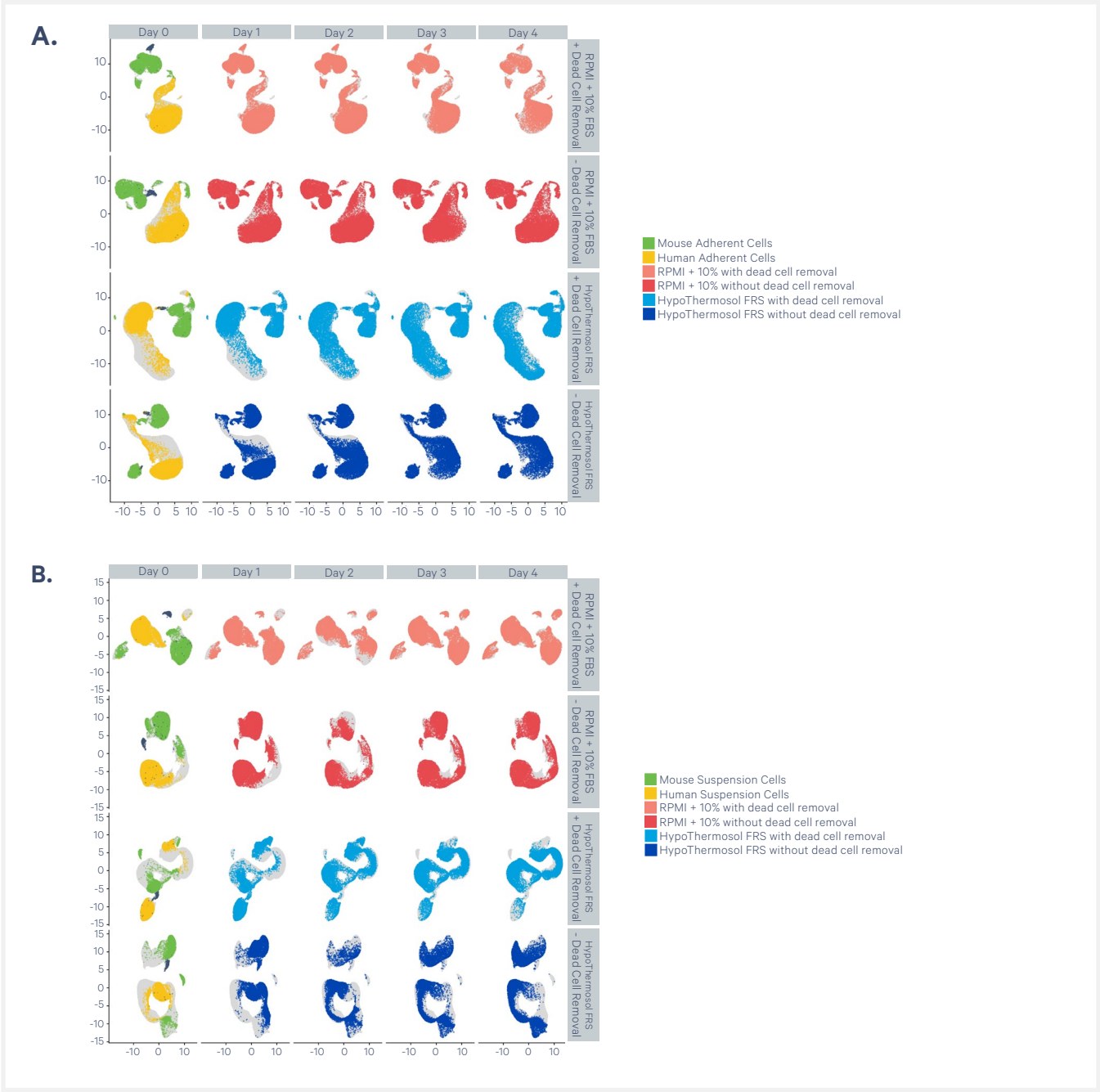
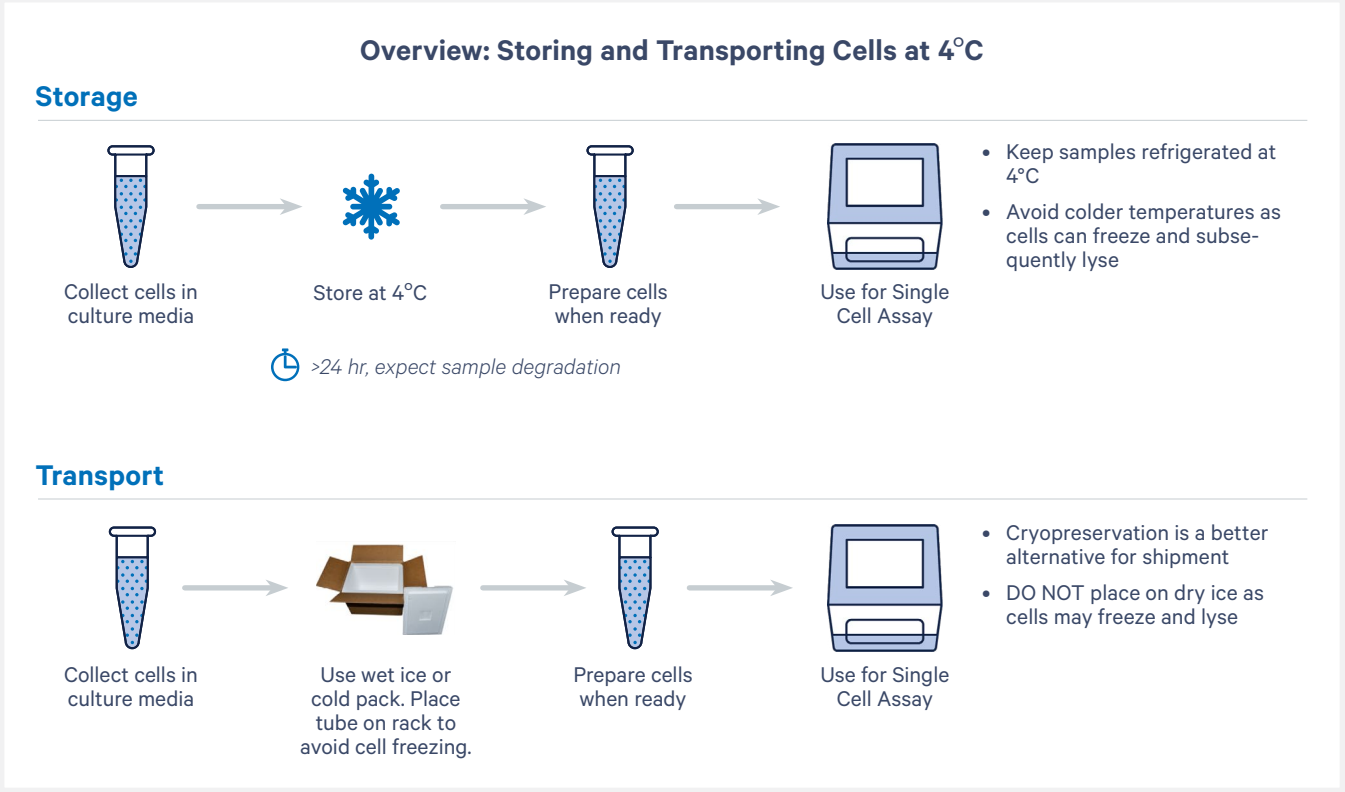


Figure 6. Single Cell 3' Gene Expression clustering data of adherent **(A)** and suspension **(B)** human/mouse mixed cells. UMAPs are shown for day 0 controls (yellow/green) and indicated timepoints. UMAPs for cells stored in RPMI + 10% FBS and HypoThermosol FRS are colored in red and dark blue, respectively. UMAPs for cells stored in RPMI + 10% FBS/HypoThermosol FRS undergoing additional dead cell removal are colored orange and light blue, respectively.

Conclusions

Short-term storage solutions are a useful option for transporting cell lines from one lab to another. Human PBMCs, adherent, and suspension human/mouse mixed cell lines tested in this series of experiments were best preserved in RPMI + 10% FBS as a short-term storage solution. Cells stored in RPMI + 10% FBS had improved cell viability, sensitivity, and correlation to day 0 gene expression as compared to cells stored in HypoThermosol FRS. Dead cell removal improved cell viability and overall sample metrics and did not negatively impact gene expression though it did incur significant cell loss. One day timepoints (24 hours) for both RPMI + 10% FBS and HypoThermosol FRS (with dead cell removal) were closest to day 0 timepoint metrics. After 24 hours, cell viability, changes to cell type distribution, and reduced mapping metrics were observed for both storage solutions. Taken together, RPMI + 10% FBS can be used as a short-term transport and storage solution, ideally less than 24 hours, for cells in single cell gene expression sequencing experiments. For long-term storage needs, cryopreservation of cell suspensions may be a better option to preserve gene expression.



References

1. E Madissoon et. al., scRNA-Seq Assessment of the Human Lung, Spleen, and Esophagus Tissue Stability after Cold Preservation. *Genome Biology*. 21(1):1 (2019)
2. Chromium Single Cell 3' v3.1 (Dual Index) Reagent Kits User Guide (CG000315)

Document Revision Summary

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