10x GENOMICS®

# TECHNICAL NOTE

# Removal of Dead Cells from Single Cell Suspensions Improves Performance for 10x Genomics® Single Cell Applications

## INTRODUCTION

10x Genomics® Single Cell Protocols require suspensions of viable, single cells as input (*Single Cell Protocols – Cell Preparation Guide* - CG00053). The removal of dead cells and other contaminants from single cell suspensions is critical to obtaining high quality data. Depending on the sample type and sample preparation method, the fraction of dead cells in a single cell suspension can vary significantly. Dead cells easily lyse, resulting in the release of ambient RNA. This cell-free RNA can contribute to the background noise of the assay and will compromise the quality of single cell data. This Technical Note discusses the impact of non-viable cells in single cell suspensions on single cell data genomics quality. In this Technical Note we compare datasets generated from single cell suspensions containing varying amounts of non-viable cells to highlight the importance of removing dead cells from single cell suspensions prior to use with the Single Cell 3' v2 Protocol.

## METHOD

We prepared single cell suspensions from peripheral blood mononuclear cells (PBMCs) from a healthy donor by following the Demonstrated Protocol *Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing* – CG00039. The single cell suspension was then split into four groups for further processing. In order to elevate the fraction of dead cells we treated each cell suspension as follows:

| | |
|---|---|
| PBMC suspension #1 (Control): | Cells loaded immediately for partitioning after sample preparation |
| PBMC suspension #2 (24 h at RT): | Cells left at room temperature (RT) in PBS for 24 h |
| PBMC suspension #3 (Digitonin Low): | Cells treated with 5 ng/ml Digitonin. Cell suspension resulted in < 5% viable cells and was mixed with >90% viable PBMCs at a 1:1 ratio |
| PBMC suspension #4 (Digitonin High): | Cells treated with 5 ng/ml Digitonin. Cell suspension resulted in < 5% viable cells and was mixed with >90% viable PBMCs at a 5:1 ratio |

Prolonged incubation of PBMCs at room temperature will result in increased cell death. Digitonin permeabilizes the cell membrane which mimics a similar phenotype of non-viable cells that prematurely lyse as they undergo apoptosis.

To examine cell viability for each cell group, we stained cells with Trypan Blue and assessed cells with the Countess® II FL Automated Cell Counter (Table 1). In an effort to increase the fraction of viable cells in all 4 PBMC suspensions we used a Dead Cell Removal kit (Miltenyi Biotec) and followed the Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* – CG000093. Cell viability for each PBMC group was assessed again after removal of dead cells (Table 1).

We next prepared Chromium™ Single Cell 3' v2 libraries for each PBMC group (Pre and Post Dead Cell Removal treatment – Pre-DCR and Post-DCR, respectively) according to the *Chromium Single Cell 3' Reagent Kits v2 User Guide* – CG00052. All eight libraries were sequenced using paired-end sequencing (26bp Read 1 and 98bp Read 2) with a single sample index (8bp) on an Illumina® HiSeq 2500 in Rapid Run mode with approximately ~90,000 reads per cell. Raw sequencing data were processed with Cell Ranger™ 2.0.

## RESULTS

To assess single cell data quality and compare application performance between all eight PBMC samples we evaluated the following metrics that are reported by Cell Ranger in the *web_summary.html* file:

- Barcode Rank Plot
- *Estimated Number of Cells*
- *Fraction Reads in Cells*
- *Total Genes Detected*
- *Median Genes per Cell*
- Fraction of mitochondrial transcripts

Fig. 1 shows the Barcode Rank Plots for all eight libraries. The Barcode Rank Plot presents a distribution of cell barcodes ranked according to the number of unique molecular identifiers (UMIs) that are associated with a given barcode. Note that each cell or partition is represented by a unique barcode. The y-axis is the number of UMI counts mapped to each barcode and the x-axis is the number of barcodes below that value. Each barcode is ranked from left to right based on the decreasing number of UMI counts associated with a given barcode.

| | Control | 24 h at RT | Digitonin Low | Digitonin High |
|---|---|---|---|---|
| Pre Dead Cell Removal | 85% | 50% | 50% | 20% |
| Post Dead Cell Removal | 85% | 86% | 85% | 82% |

Table 1. Cell viability for each PBMC suspension assessed with Trypan Blue and the Countess II Automated Cell Counter after sample treatment and removal of dead cells with the Dead Cell Removal Protocol.

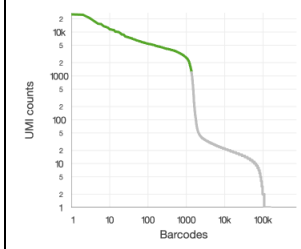
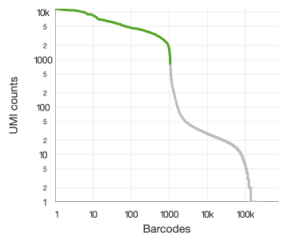
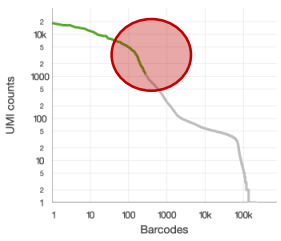
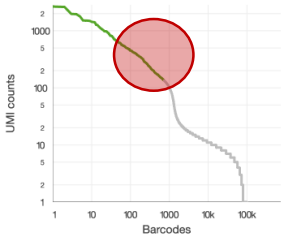
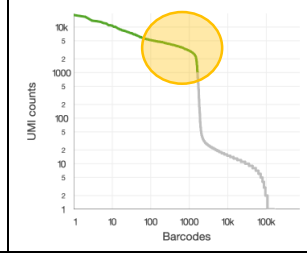
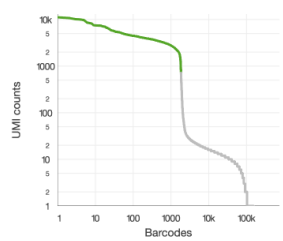
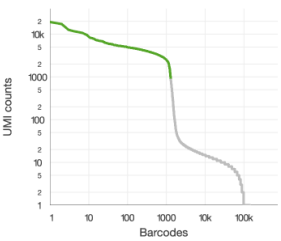
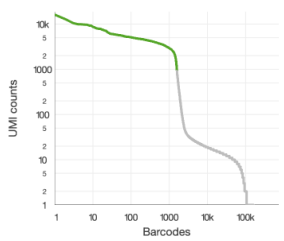
| Treatment | Control | 24 h at RT | Digitonin Low | Digitonin High |
|---|---|---|---|---|
| Pre-Dead Cell Removal |  |  |  |  |
| Post-Dead Cell Removal |  |  |  |  |

Fig.1. Barcode Rank Plot for 8 samples pre and post Dead Cell Removal treatment. Plotted is the distribution of barcode counts (x-axis) and the corresponding total UMI (Unique Molecular Identifier) counts (y-axis). Cell-containing partitions (barcodes on x-axis) are shown in green. Background partitions are shown in grey. Red circle indicates lack of steep drop-off. Yellow circle indicates one example of clear steep drop-off.

A steep drop-off is indicative of good separation between the cell-associated partitions in green and all background partitions lacking a cell in grey. In addition, good separation indicates the presence of low amounts of ambient RNA in the sample. A high fraction of ambient RNA is typically the result of non-viable cells that prematurely lyse before or during single cell partitioning. As a result, UMI counts that originate from background partitions increase, which consequently reduces the separation between cell-associated and empty partitions. Digitonin Low and Digitonin High samples that had not been processed with the Dead Cell Removal kit (50% and 20% viable cells, respectively) lack a good separation, suggesting the presence of many background partitions.

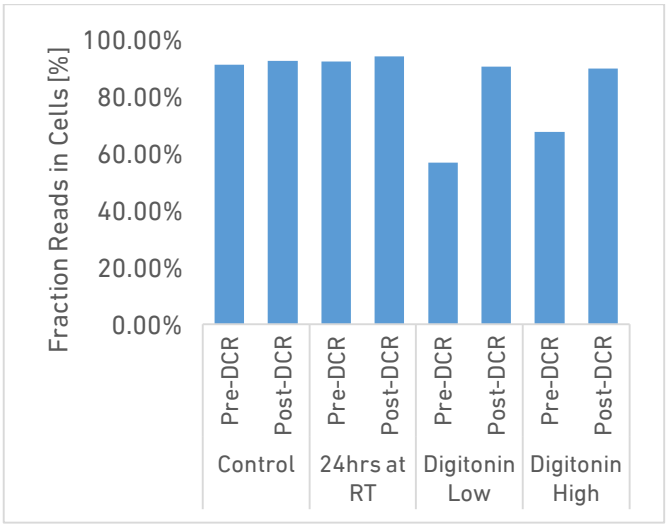
### Increased Sample Cleanliness after Removal of Dead Cells

We next compared application performance metrics reported by Cell Ranger between each PBMC sample that was processed with or without the Dead Cell Removal kit (Fig. 2 and 3). Fig. 2a and 2b show *the Fraction Reads in Cells* and *Estimated Number of Cells* for each sample, respectively. The *Fraction Reads in Cells* is the percentage of confidently mapped reads that are associated with a cell-associated barcode (partition). The samples treated with Digitonin showed the lowest *Fraction of Reads in Cells* before removal of dead cells (~56-67%), suggesting that a higher percentage of reads was associated with empty partitions (barcodes not associated with a cell). Note that each partition containing ambient RNA will contribute to background and lower the *Fraction Reads in Cells*. The *Fraction Reads in Cells* increased to > 80% for each sample after removal of dead cells.

### Increased Accuracy in Target Cell Count after Removal of Dead Cells

For cell recovery we targeted 1,500 cells for each samples. The *Estimated Number of Cells* metric reports the total number of partitions that contained a barcode associated with a cell (green line in Fig. 1). The calculated *Estimated Number of Cells* closely matched the target of 1,500 cells for both control samples and samples processed for Dead Cell Removal. In contrast, we recovered lower than expected numbers of cells for samples left at room temperature for 24 h or treated with Digitonin (24 h RT: 1,066 cells, Digitonin Low: 278 cells, Digitonin High: 730 cells).

a)




b)

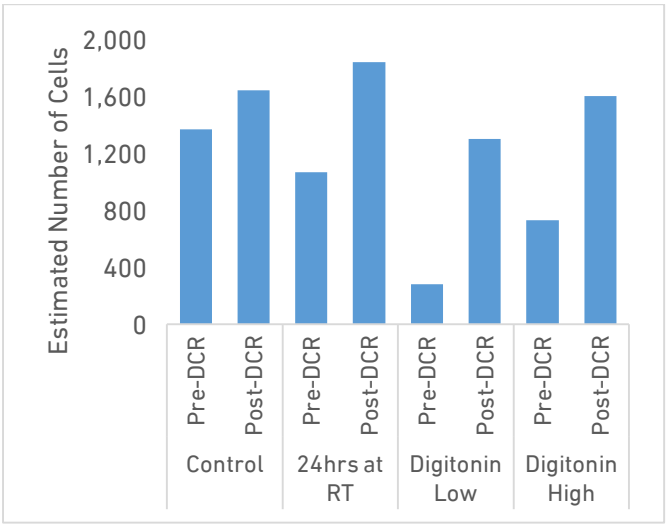



Fig.2. Application performance metrics for 8 samples pre and post Dead Cell Removal treatment (Pre-DCR and Post-DCR, respectively). (a) *Fraction of Reads in Cells* and (b) *Estimated Number of Cells* increase for each sample after treatment with the Dead Cell Removal kit.

## Increased Library Complexity after Removal of Dead Cells

We next evaluated the impact of cell viability on library complexity (*i.e.* number of genes expressed in each library) for all 8 samples. Fig. 3 shows the *Median Genes per Cell* (a) and the *Total Genes Detected* (b) for each sample downsampled to ~50,000 reads per cell to allow for a direct comparison. *Median Genes per Cell* remained relatively consistent (~ 1,000) across all samples with the exception of the Digitonin High samples. Only 27 *Median Genes per Cell* were detected for the Digitonin High sample Pre-DCR which contained 20% viable cells. The library complexity significantly improved with the removal of dead cells and reached a median of > 1,000 genes per cell. Interestingly, the sample that was left at room temperature for 24 hours showed a decrease in library complexity which could not be recovered when processed with the Dead Cell Removal kit.

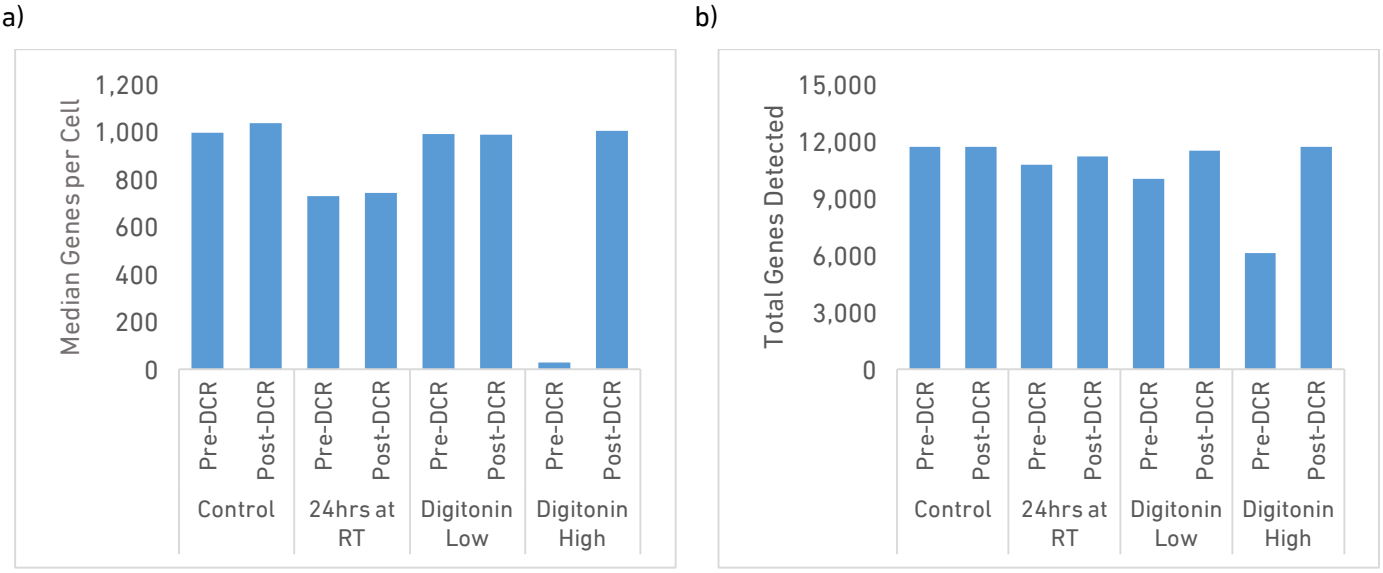



Fig.3. Application performance metrics for 8 samples pre and post Dead Cell Removal treatment (Pre-DCR and Post-DCR, respectively). (a) *Median Genes per Cell* and (b) *Total Genes Detected* increase for each sample after treatment with the Dead Cell Removal kit

*Total Genes Detected* significantly improved with the removal of dead cells and doubled from ~6,000 genes to ~12,000 genes in the Digitonin High sample. We detected ~11,500 total genes for all remaining samples that were treated with the Dead Cell Removal kit and contained >80% viable cells which is a modest increase in library complexity compared to samples Pre-DCR.

## Decreased Number of Mitochondrial Genes after Removal of Dead Cells

Mitochondria are one of the cells' primary organelles involved in the initiation and execution of apoptosis. The process of apoptosis is dependent on a cascade of signaling events that includes the increased expression of mitochondrial genes and the activation of caspases[1,2]. We thus assessed the fraction of unique transcripts in the calculation of the number of UMIs that originated from mitochondrial gene expression for each sample. We detected only a low fraction of mitochondrial UMIs (~3%) in samples that were of high quality with > 80% viable cells. As expected, the number of mitochondrial transcripts was highest in the Digitonin High sample that contained only 20% viable cells (74%). In contrast, samples that contained 50% viable cells only showed a slight increase in mitochondrial gene expression (5-7%), indicating that viability does correlate with the number of mitochondrial transcripts observed.

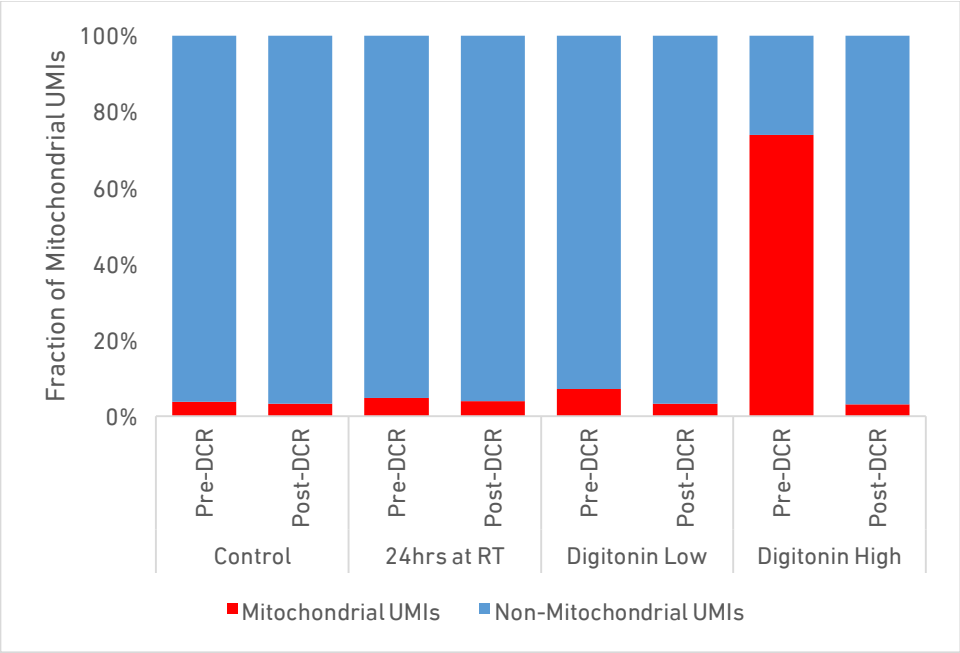



Fig.4. Fraction of mitochondrial UMIs in each PBMC sample.

### Cluster Analysis using Loupe Cell Browser™

We then used the subsampled datasets for a more direct comparison and for downstream analysis in Loupe Cell Browser™. Specifically, we used the *cellranger aggr* command and subsampled reads from each library (stored in the molecule_info.h5 file produced by *cellranger count*) until all libraries had the same mean reads per cell, to control for sequencing batch effects. The normalized libraries were aggregated into a single gene-barcode matrix followed by secondary analysis (PCA, clustering, differential expression and t-SNE).

We performed cluster analysis and used the *AMLBloodCell.csv* gene list to identify each of the major cell subtypes found in human PBMCs:

- Lymphocytes: T cells, B cells
- Myeloid cells: Monocytes

The gene list can be downloaded on the Support website at https://support.10xgenomics.com/single-cell-gene-expression/software/visualization/latest/tutorial-celltypes.

We found that the majority of the samples clustered similarly and a total of 14 sub-clusters were identified via Graph based clustering (Fig. 5 – 8). With the exception of the samples left at room temperature for 24 hours we clearly identified all major cell subtypes in PBMC samples that have been processed with the Dead Cell Removal kit and contained cells that were >80% viable. The monocyte subpopulation was virtually absent in PBMC samples that were left at room temperature for 24 hours, suggesting that the majority of cells have lost CD14 marker expression (Fig. 6). Moreover, despite the identification of all other major PBMC subtypes in this sample Post-DCR, subtypes clustered differently via Graph based clustering compared to other samples. In contrast, subpopulations were either absent or represented by significantly fewer cells in samples that were of low quality (< 50% viable) based on marker gene expression (Fig. 7 and 8). We failed to identify any major PBMC subtypes in the sample with the lowest cell viability (Digitonin High Pre-DCR, 20% viable cells).

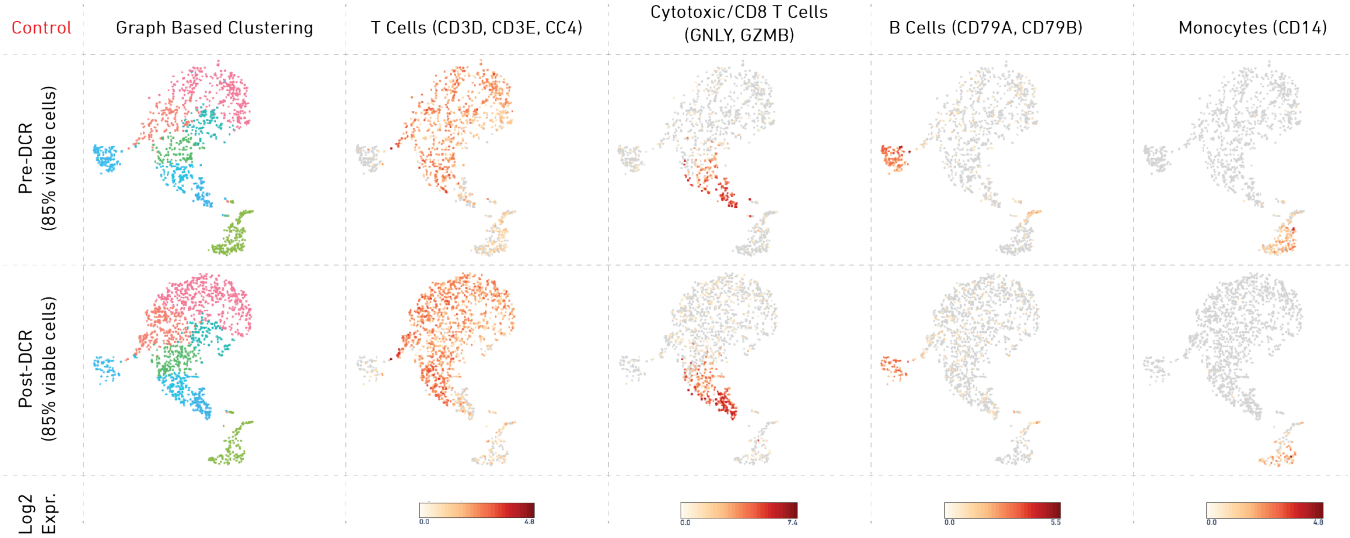



Fig.5. Cluster analysis of Control samples with Loupe Cell Browser.

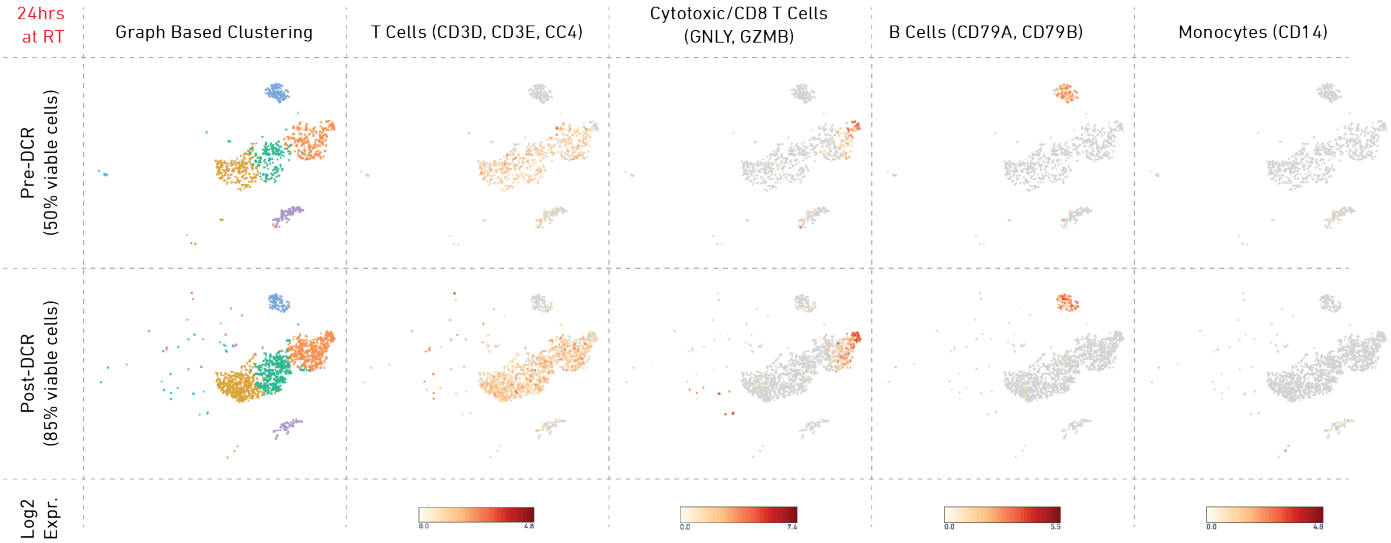



Fig.6. Cluster analysis of 24 h at RT samples with Loupe Cell Browser.

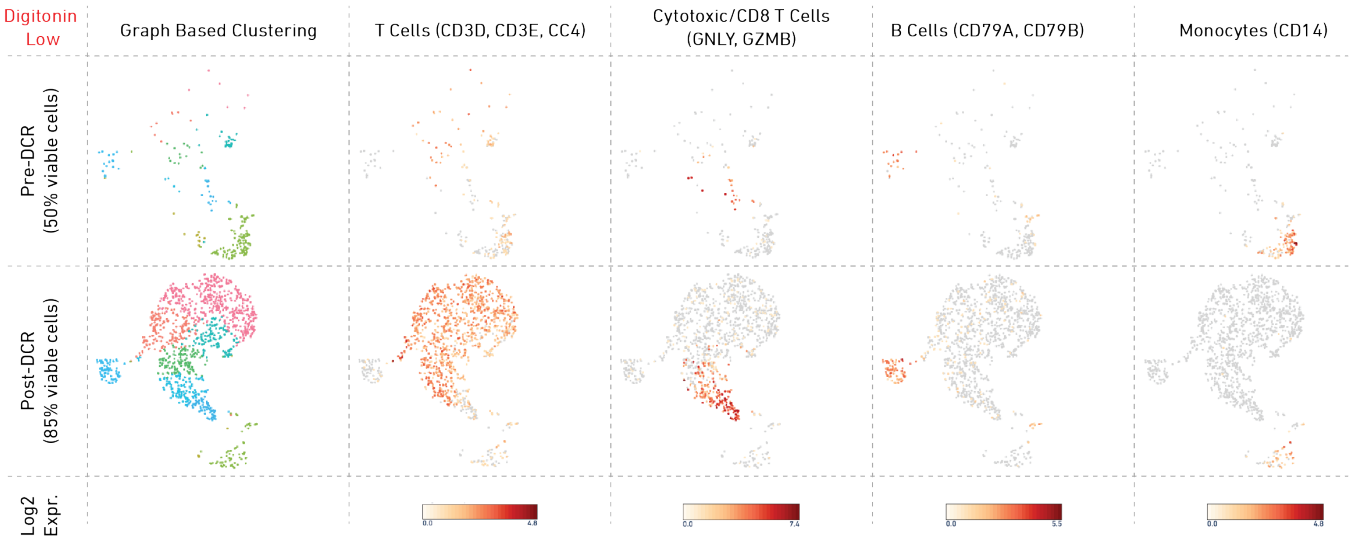



Fig.7. Cluster analysis of Digitonin Low samples with Loupe Cell Browser.

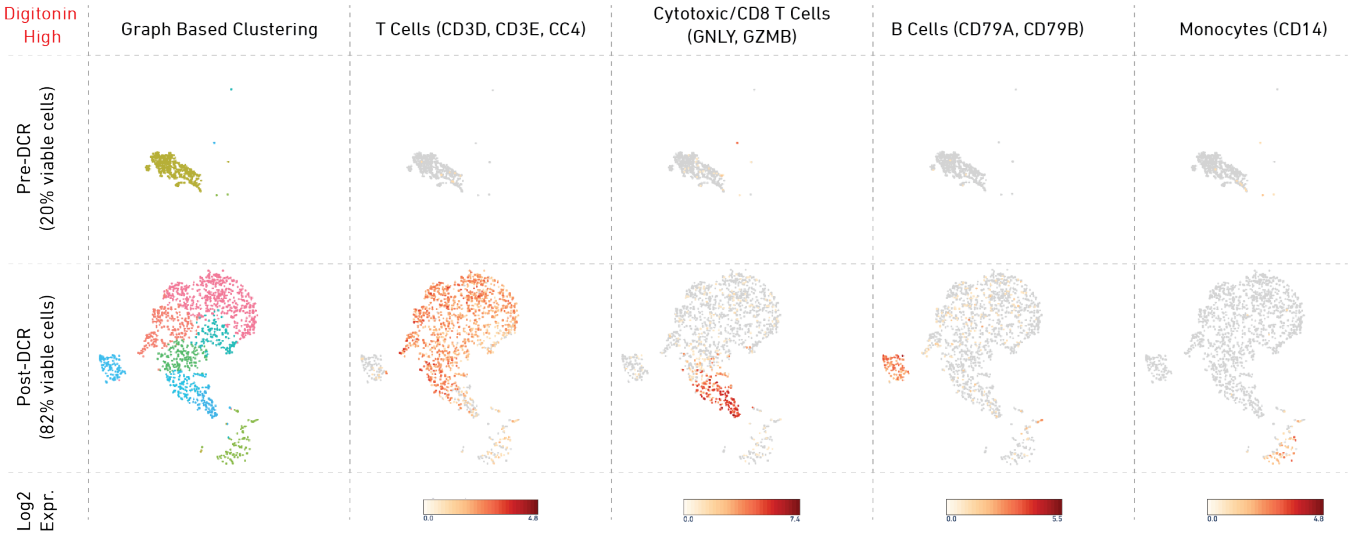



Fig.8. Cluster analysis of Digitonin High samples with Loupe Cell Browser.

## DISCUSSION

Our studies highlight the variability in application performance for Single Cell 3' v2 libraries prepared with human PBMCs that varied significantly in cell viability and cell handling. The data underscores the importance of starting with high quality single cell suspensions in order to retrieve high quality data. Overall, the presence of non-viable cells decreased application performance including library complexity and targeting accurate cell recovery numbers. Non-viable cells are included when calculating the total cell count and can thus result in inaccurate counts of recovered cells. Notably, the sample that contained only 20% viable cells performed worse in the majority of metrics (*i.e.* library complexity, *Fraction of Reads in Cells*, accuracy in target cell count) that we have assessed. While the number of mitochondrial transcripts increased, the total number of genes detected per cells dropped significantly (from ~1,000 to 37 genes per cell). As a result, cluster analysis failed to reveal major cell subtypes found in human PBMCs (*i.e.* T Cells, B Cells, Monocytes).

Importantly, low application performance for low quality samples was overcome by processing the sample with the Dead Cell Removal kit. Removal of dead cells and ambient RNA during the column-based clean up and wash steps are primary reasons for the improvement in data quality. Additional guidance for use of the Dead Cell Removal Kit includes:

- Human PBMCs form cell aggregates that cannot be resuspended back into a single cell suspension when kept in PBS for a prolonged period of time (> 2 hours). This lowers the effective concentration of suspended single cells which can result in less accurate target cell counts during data analysis.
- Cells should be loaded immediately after sample preparation and cell suspensions should always be kept on ice.
- We also encourage Users to carefully assess cell viability before starting the Single Cell 3' v2 Protocol. Note that our analysis was performed on human PBMCs and that other cell types and/or experimental designs may perform differently with respect to application performance.

## CONCLUSION

We have discussed the impact of non-viable cells present in single cell suspensions on single cell data quality. The use of the Dead Cell Removal kit to remove non-viable cells is recommended for single cell suspensions with <70 % viable cells. Application performance metrics reported in this Technical Note serve as a guideline to assess single cell data quality of Chromium™ Single Cell 3' v2 libraries generated from human PBMCs.

## REFERENCES


- *Chromium™ Single Cell Reagent Kits v2 User Guide* (CG00052)
- *Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing* (CG00039)
- *Single Cell Protocols – Cell Preparation Guide* (CG00053)
- *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (CG000093)
- Wang. The expanding role of mitochondria in apoptosis (2001), *Genes & Dev*
- Ferguson-Miller *et al.* Mitochodnria – Releasing Power for Life Unleashing the Machineries of Deat (2003), *Cell*

# Notices

## Document Number

CG000130 Rev A *Technical Note*

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