RESEARCH SNAPSHOT

Improve Sample and Data Quality by Avoiding RBC Lysis

Overview

Single-cell analysis is a powerful scientific approach that provides exceptional resolution in the study of complex biological systems, significantly advancing our ability to answer otherwise intractable scientific questions. Single-cell RNA sequencing (scRNA-seq) allows researchers to measure changes in gene expression in entire transcriptomes of potentially rare cell populations from complex biological samples[1]. However, those tissue samples often contain variable amounts of contaminants such as dead/dying cells, red blood cells (RBCs), and debris that impede single-cell sequencing results. RBCs take up precious space in the library preparation and sequencing reactions, driving down representation of other cell types of interest. Such contamination can obscure and bias data, making valid interpretations difficult at best[2]. Conventional methods to remove RBCs, such as lysis using ammonium chloride solutions[3], are incomplete and can have significant impacts on cells other than RBCs[4]. The LeviSelect™ Tissue RBC Depletion Kit coupled with Levitation Technology™ provides a novel approach to this problem by offering a reliable way to gently enrich viable, untouched cells of interest while simultaneously depleting RBCs in one streamlined workflow.

Efficient Removal of Contaminating Ambient RBC Related RNA

To compare the effectiveness of LeviSelect RBC depletion to conventional RBC lysis, two mouse spleens were dissociated and 7000 total cells were targeted for scRNA-seq from each sample using the 10x Genomics Chromium Next GEM Single Cell 3' Kit v3.1, and the Illumina NovaSeqX Plus sequencing platform.

KEY HIGHLIGHTS: Advantages of LeviSelect RBC Depletion Over RBC Lysis

- Efficient and complete removal of both erythroblasts and mature RBCs
- Simple multiplex step simultaneously improves sample viability and quality
- Increases the resolution of cell types of interest
- Provides higher-quality sequencing results and more usable data

The resulting sequencing data were analyzed using SoupX to subtract ambient RNA[5], Scrublet to remove doublets[6] and Seurat to perform normalization, dimensionality reduction, clustering and gene expression analysis[7]. Leaving ambient RNA in the data set reveals that RBC lysis results in a significant amount of RBC-related gene contamination in all cell clusters as compared to RBC-depleted samples (Figure 1A and 1B). Even after bioinformatic removal of ambient RNA, clusters of cells with high levels of hemoglobin (Hba-a1) expression remain following RBC lysis, but were eliminated in the RBC-depleted samples (Figure 1B and 1D).

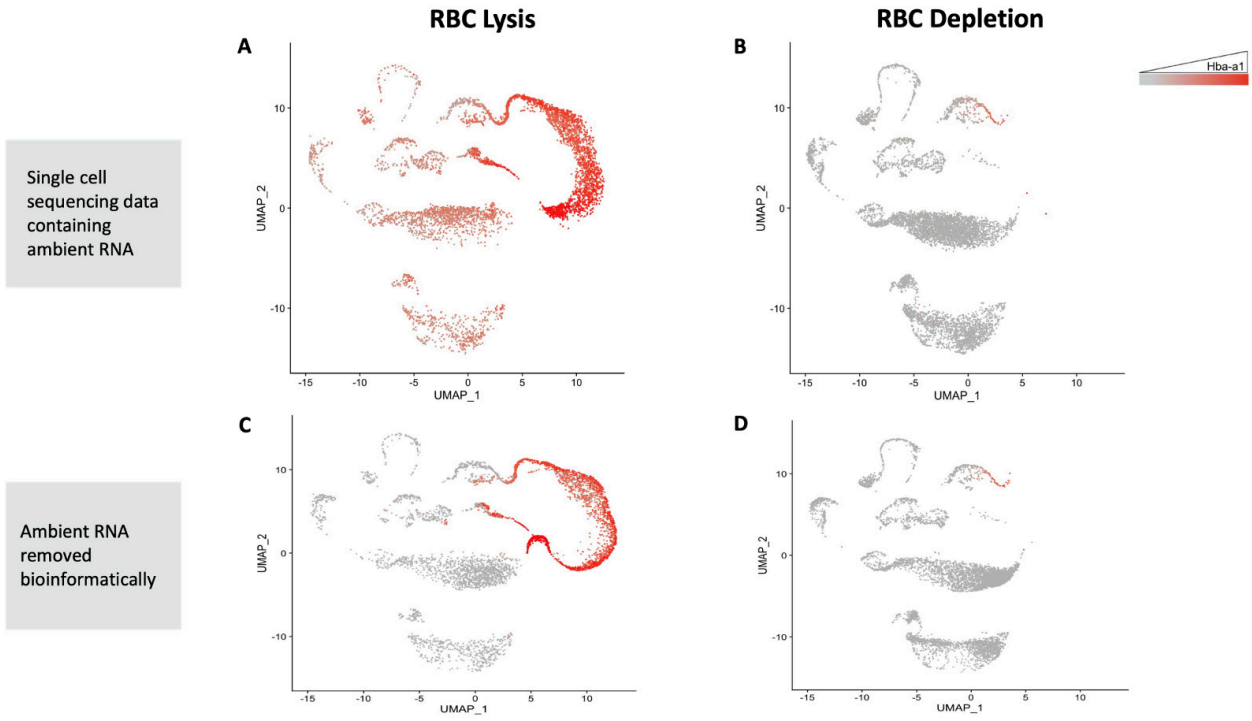

Figure 1.Eliminate RBC-related RNA contaminants to maximize biological insights. *Spleens from two separate C57BL/6 mice were dissociated and either treated with RBC depletion (B and D) or with RBC lysis solution (150mM Ammonium Chloride, 10mM Tris-HCl pH7.2) for 10 min at RT to lyse RBC (A and C). Representative UMAPs are shown above with the expression levels of the RBC gene Hba-a1 superimposed in red.*

Single-cell suspensions from at least 4 different mouse spleens were subjected to either RBC depletion or RBC lysis methods. The number of RBCs left behind were calculated as the difference between the number of total cells counted in brightfield and the number of nucleated cells stained with the nuclear dyes acridine orange (live nucleated cells) and propidium iodide (dead nucleated cells). Both methods very effectively removed mature non-nucleated RBCs; however, only RBC depletion enriched for viable cells (Table 1).

Readout	% RBC (stdev)	% Viability (stdev)
Before RBC Removal	30% (6%)	74% (8%)
After RBC Lysis	2% (2%)	64% (15%)
After RBC Depletion	Not Detected	90% (93%)

Table 1. RBC depletion improves sample viability as compared to RBC lysis.

RBC Depletion Results in Higher-Quality Sequencing Data

After removing ambient RNA and doublets, the scRNA-seq data set was organized into a Seurat object and the number of features per cell and percent of reads mapping to mitochondrial genes for each cell was visualized in violin plots to set quality control thresholds. RBC lysis correlated with more cells associated with low numbers of features and high mitochondrial reads (Figure 2), both indicative of dead/dying cells[2]. The inclusion of such dead/dying cells in downstream gene expression analysis can create noise and even skew data interpretation, thus these cells are generally removed before further analysis. If thresholds for removal are set at <200 features per cell or >10% mitochondrial reads per cell, RBC lysis resulted in an average of 21% loss of cells from the data set as compared to only 6% for RBC-depleted samples (Table 2). RBC depletion resulted in more usable data from the same number of input cells when compared to RBC lysis.

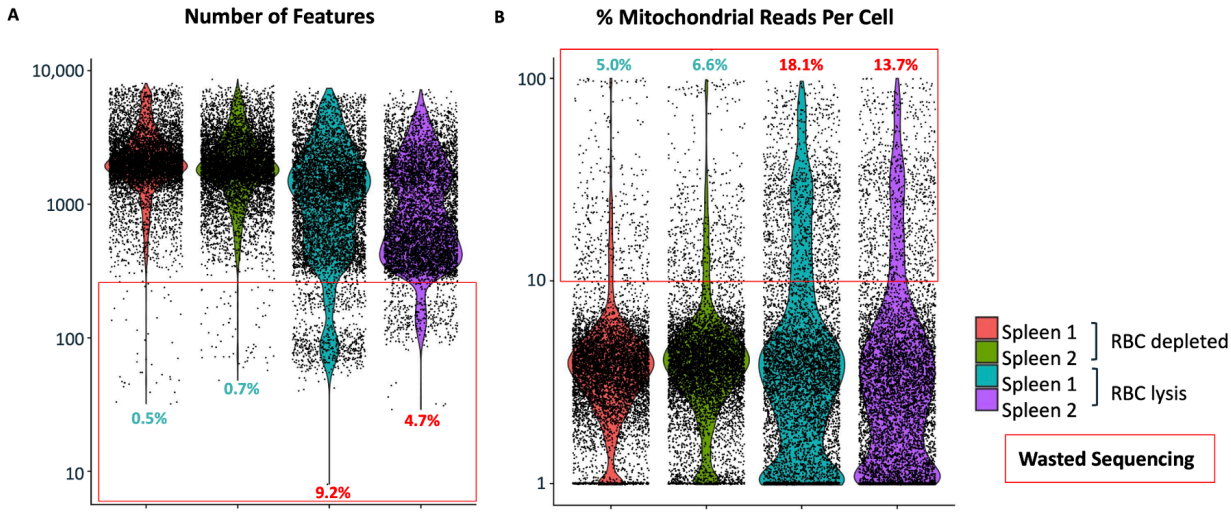

Figure 2. RBC depletion reduces the loss of usable data by decreasing percent of cells with low number of features and high mitochondrial reads. *The number of features (left) and the percent of reads mapped to mitochondrial genes (right) is shown for the splenocyte single cell RNA sequencing data sets described in figure 1 (after removing ambient RNA and doublets bioinformatically). Red highlighted boxes indicate cell barcodes associated with low numbers of features and/or a high percentage of reads mapped to mitochondrial genes and so must be removed bioinformatically.*

% of Total Cells:	RBC depleted		RBC lysis	
	Spleen 1	Spleen 2	Spleen1	Spleen 2
Cells with < 200 features:	0.5%	0.7%	9.2%	4.7%
Cells with >10% mitochondrial genes:	5.0%	6.6%	18.1%	13.7%
Cells associated with both:	5.1%	6.6%	25.9%	16.9%

Table 2. Gain better insight by reducing low-quality indicators.

Decrease Noise and Obtain Higher Resolution of Target Cells

After normalization, dimensionality reduction and clustering analysis, each cell cluster was identified using the ScType tool[8]. The cell clusters associated with high amounts of hemoglobin (Figure 1C) were identified as erythroblasts and erythroblastic island macrophages (EIM, Figure 3A and 3B). Erythroblasts are immature red blood cells that still have a nucleus and are less sensitive to RBC lysis. However, erythroblasts still express RBC surface markers targeted by the RBC depletion antibody cocktail, leading to their near complete depletion from the cell mixture (Figure 3B). The mouse spleen is a site of erythropoiesis and contains specific niches called erythroblastic islands where erythroblasts develop in close association with specialized macrophages (EIM) that aid with RBC development[9]. While EIM do not express RBC surface markers, these cells remain attached to developing erythroblasts after tissue dissociation and therefore also become targets of RBC depletion. By removing erythroblasts and these other RBC-related cell types, RBC depletion effectively increases the representation of other non-RBC-related cell types in the data set (Figure 4A), amplifying the potential for detecting rare cell types and strengthening the validity of the conclusions that may be drawn from the data overall.

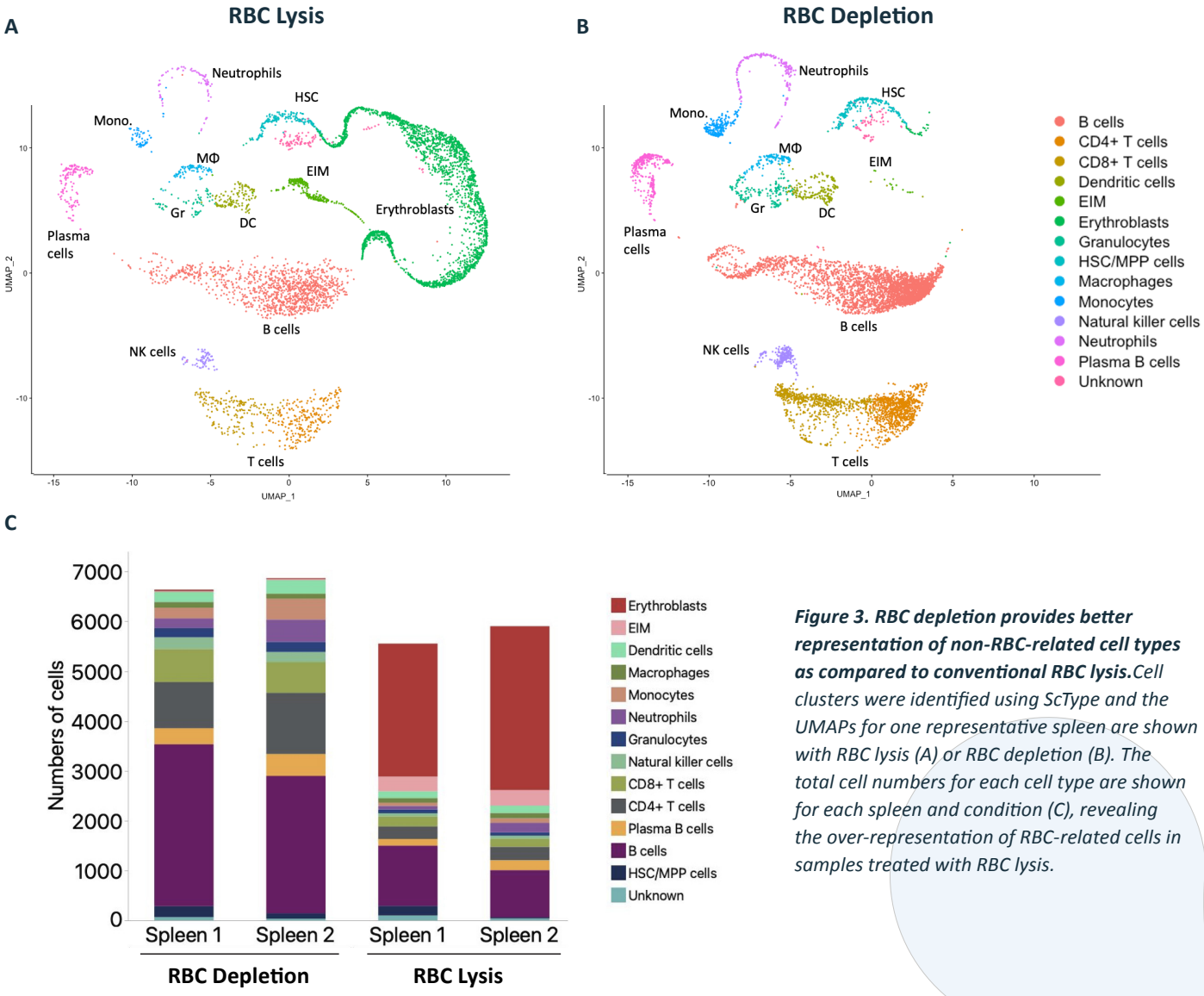

Figure 3. RBC depletion provides better representation of non-RBC-related cell types as compared to conventional RBC lysis. *Cell clusters were identified using ScType and the UMAPs for one representative spleen are shown with RBC lysis (A) or RBC depletion (B). The total cell numbers for each cell type are shown for each spleen and condition (C), revealing the over-representation of RBC-related cells in samples treated with RBC lysis.*

Conclusion

The LeviSelect Human and Mouse Tissue RBC Depletion Kits accelerate and improve sample processing to help uncover true biological cell profiles. This novel approach using Levitation Technology eliminates RBCs and improves sample quality while avoiding the costly trade-offs and limitations associated with RBC lysis. These trade-offs include, but are not limited to, cell stress and death, sample loss, excessive debris, and negative impacts on downstream applications such as single cell sequencing. By increasing the quality of cells that are submitted to downstream sequencing, RBC depletion allows researchers to maximize their sequencing funds and data potential by returning high-quality analyzable data from every cell and ultimately aiding scientific discovery.

References

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