APPLICATION NOTE

Importance of accurate cell counting for single cell sequencing platforms using the Cellometer K2 fluorescent viability cell counter.

Authors

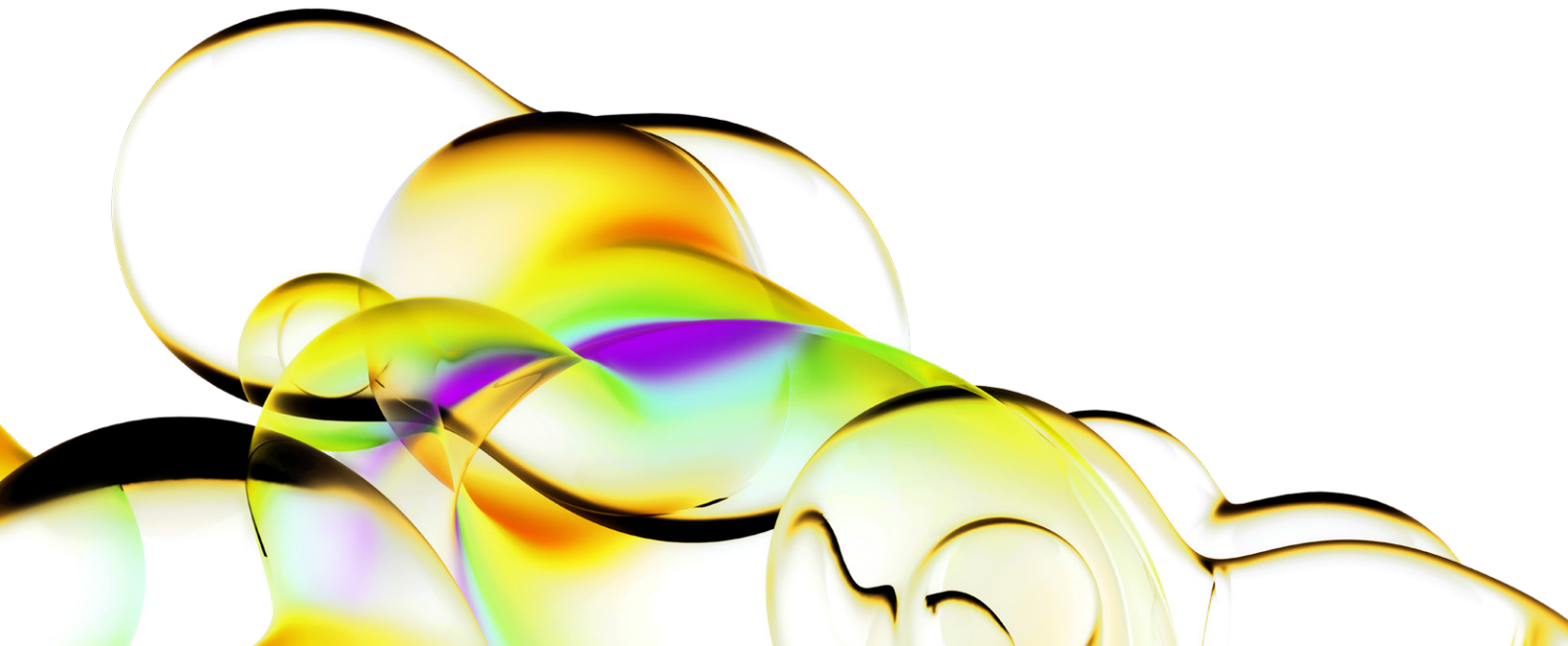
Leo Li-Ying Chan,
Belen Belete-Gilbert,
Krishna Vallabhaneni

Introduction

The discovery of "nuclein" (now known as DNA) in 1871 by Friedrich Miescher has set scientists on a quest to identify & characterize the building blocks of life. From the discovery of DNA double helix structure by James Watson and Francis Crick in 1953 to the launch of the Human Genome Project in 1990, significant technological advancements have been made. After the publication of initial human genome in 2001 and the completion in 2003, new and novel next-generation sequencing (NGS) platforms have allowed significant reduction in assay time as well as sequencing cost [1].

Standard genome sequencing characterizes the genome through analysis of DNA content extracted from thousands to millions of cells using microarray or RT-qPCR. This type of bulk sequencing measures average gene expression levels across a population of cells, which is useful for quantifying gene expression in homogeneous populations. It is cheaper, easier, and more straightforward to analyze [2, 3].

In the recent years, the development of single-cell sequencing (SCS) technique has shifted genomic research towards more comprehensive analyses of individual cells for immunology, oncology, neuro and developmental biology, as well as stem cell research. The SCS method can be used to identify rare cell subtypes, trace lineage and developmental relationships between cell states, track gene expression changes between normal and diseased state, and measure stochasticity of gene expression [4].



Single-cell sequencing technique

Single-cell sequencing requires the isolation of a single cell and the amplification of extracted DNA to ensure enough content is sequenced. SCS measures the distribution of gene expression levels across a population of cells, which is useful for studying cell-specific transcriptomic differences in heterogeneous populations. However, it is more difficult to collect and analyze, as well as introducing error to the final sequence [5, 6].

Single cells can be isolated by using FACS, limiting dilution, microfluidic chip (Fluidigm), laser capture microdissection (LCM), cell-printing technique, or droplet-based isolation. Companies such as Illumina, BioRad, BD Biosciences, and 10x Genomics have introduced their single cell sequencing technologies to the market [7-12]. One major challenge that cuts across many fields is the integration of massive data sets generated by NGS technology.

No matter what isolation or sequencing techniques are used, it is important to ensure cell conditions are characterized and appropriate for the selected workflow. Single cell sequencing results can be affected by poor cell viability, high apoptosis rate, clumpy and doublet cells, thus many of the single cell sequencing techniques have stringent cell condition and preparation guidelines [13, 14].

Cell counting for single cell sequencing

One of the most important cell preparation step when performing single cell sequencing is cell counting and analysis for cell characterization. Traditionally, counting is performed by manually by counting trypan blue (TB) stained cells in a hemacytometer using standard light microscopy. However, it is known this method is time-consuming and high operator variation, which can drastically affect the performance of single cell sequencing. Therefore, it is recommended to use automated cell counters to reduce time and improve accuracy of cell counting [15].

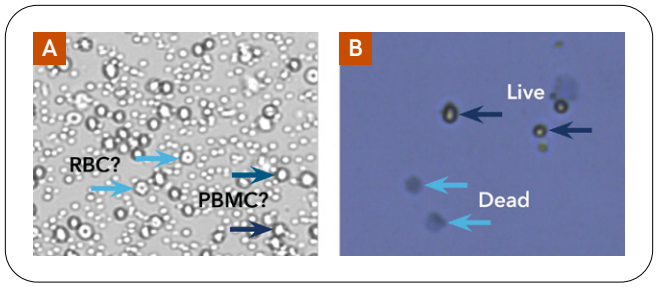


Figure 1: Issues with brightfield manual cell counting with the use of trypan blue staining. (left) Numerous nonspecific particles in the sample, making it difficult to distinguish PBMCs from RBCs and platelets. (right) Trypan blue affects the morphology of dead cells creating a dark and diffused profile.

A majority of automated cell counters on the market utilize brightfield imaging and trypan blue staining for cell count and viability measurement. However, after extensive study we have discovered two important issues with the use of trypan blue. First, primary cell sample contains RBCs, platelets, and debris, which without tedious purification process, can over-estimate live cell count and viability using only brightfield imaging (Figure 1A). Second, trypan blue staining can affect the morphology of unhealthy and dead cells, thus under-estimate dead cell count and viability (Figure 1B) [16].

In order to eliminate issues raised with trypan blue, fluorescence-based automated cell counters have been introduced to specifically count nucleated cells in the sample. By using dual-fluorescent stain mixture, acridine orange (AO) and propidium iodide (PI), live and dead nucleated cells are effectively stained, respectively, and automatically counted [17].

Cellometer fluorescence cell counting workflow

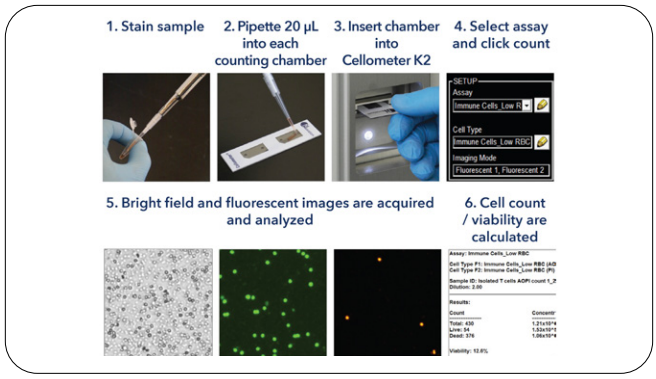


Figure 2: Cell counting workflow for Cellometer. (1) Stain the cell sample 1:1 with AO/PI. (2) Pipette 20 µL into the Nexcelom Counting Chamber. (3) Insert the Counting Chamber into the Cellometer. (4) Select the Assay Type and click count. (5) Brightfield and fluorescent images are captured. (6) Counting results are automatically generated.

Figure 3: Cellometer results showing cell count, concentration, mean diameter, as well as viability. In addition, cell sizes are plotted in a histogram showing size distribution.

The Cellometer® K2 fluorescent cell counter has been used to ensure consistent cell count and viability measurement prior to single cell sequencing. Using the AO/PI stain, primary and fragile cells can be accurately counted such as PBMCs and hepatocytes. In addition, automated size measurement can indicate percentages of clumpy and doublet cells to ensure cell samples are appropriate and not clog microfluidics.

The Cellometer K2 workflow is shown in Figure 2, which showed simple and quick procedure to determine cell count and viability, as well as cell size information. The counting takes approximately 30 seconds per count, and the entire protocol from staining to results requires only less than 2 minutes per sample. After automated counting, cell count, concentration, cell diameter, as well as viability results are generated. Cell size histogram is also produced to determine clumpiness of cell sample (Figure 3).

Discussion

Cellometer K2 improves accuracy in cell count and viability measurement

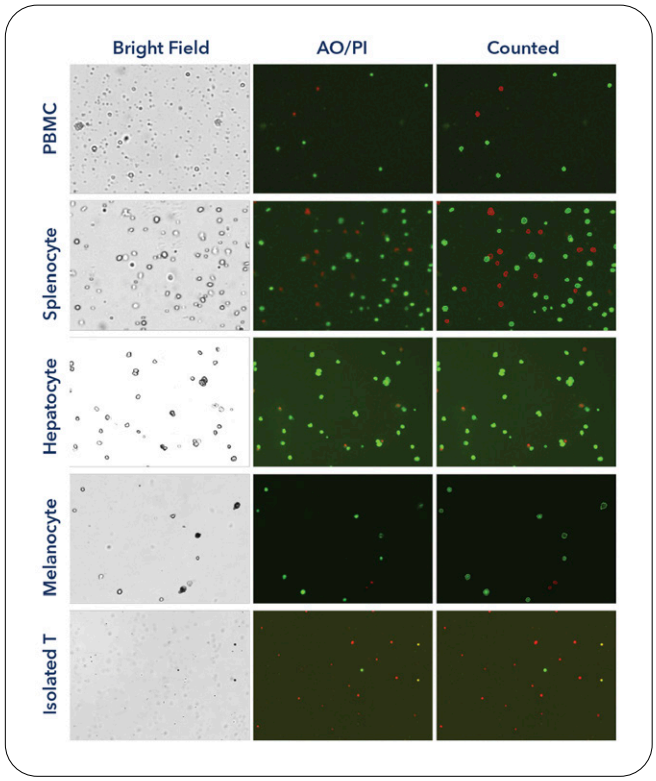


Figure 4: Brightfield and AO/PI fluorescent images for PBMC, splenocyte, hepatocyte, melanocyte, and isolated T cells. The panel on the far right shows the counted image where the live and dead cells are outlined with green and red, respectively.

Live/Dead AO/PI fluorescent dye specifically stains nucleated cells

Stained nucleated cells in primary and fragile cell samples such as whole blood, PBMCs, hepatocytes, and mouse splenocytes are accurately identified and counted, where non-nucleated objects such as RBCs, platelets, or debris are excluded (Figure 4). The AO/PI staining also allowed proper declustering of crowded cells to improve accuracy in cell counting (Figure 5). In addition, Cellometer K2 can quantify RBC and platelet contamination percentages from the same sample (Figure 6).

Cellometer software segments a wide range of cell morphology

Cells come in all shape and sizes, thus it is important for the cell counting technology to be able to identify and segment different cell types and morphologies (Figure 7). The Cellometer software can identify and segment cell sizes ranging from 3 to 300 µm in diameter. For single cell sequencing, it is critical the cell sample is composed mostly of single cells instead of cell clumps to prevent clogging of microfluidic devices. Cellometer K2 can also determine the amount of cell clumps and doublets to ensure the cell sample is appropriate for single cell sequencing (Figure 8).

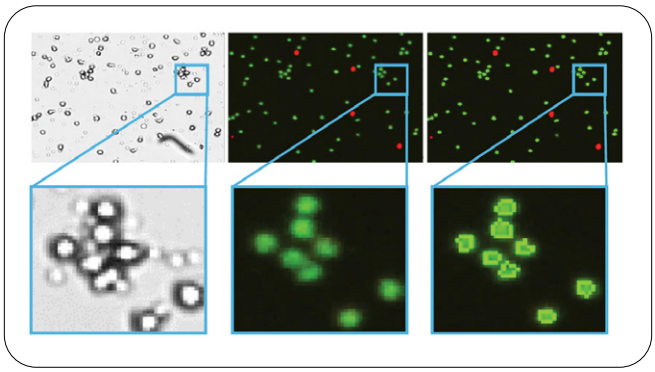


Figure 5: Brightfield and AO/PI images of cells clustered together, which can be accurately counted using the declustering software function in Cellometer.

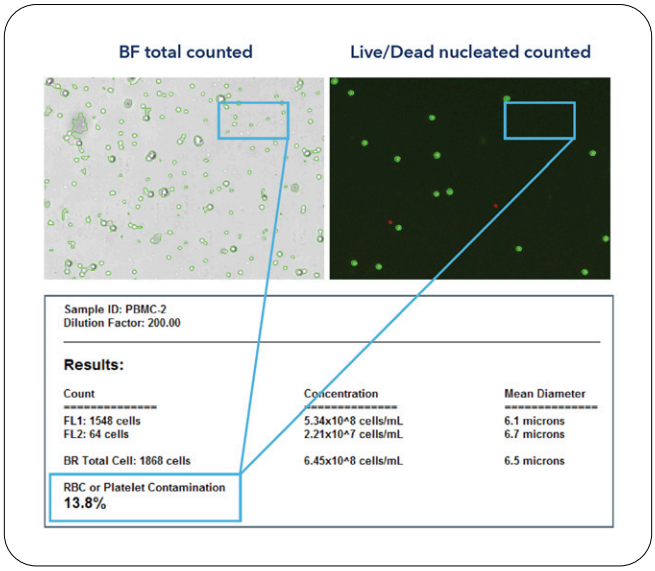


Figure 6: Captured brightfield and fluorescent images can be analyzed to determine RBC contaminations. By counting total cell count in brightfield and compare to nucleated cell count in fluorescence, the RBC contamination % can be calculated.

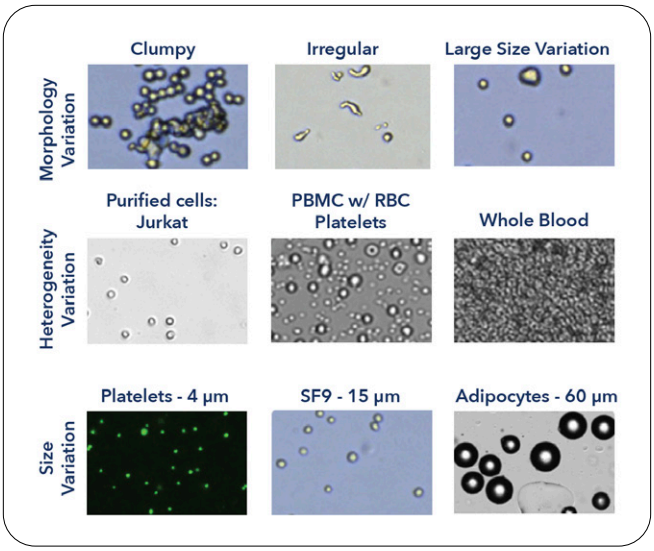


Figure 7: Cells can be different by morphology, heterogeneity, as well as cell size. These differences should be considered when selecting a cell counter to be flexible with different cell types.

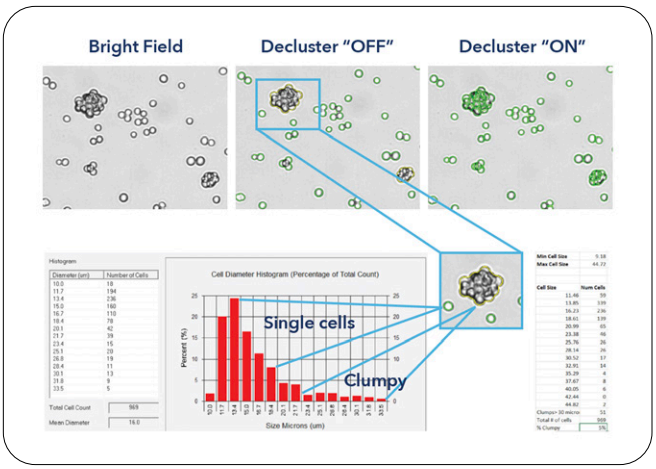


Figure 8: Brightfield counting using Cellometer showing the declustering function as well as analysis of clumpy cells in the sample using cell size histogram.

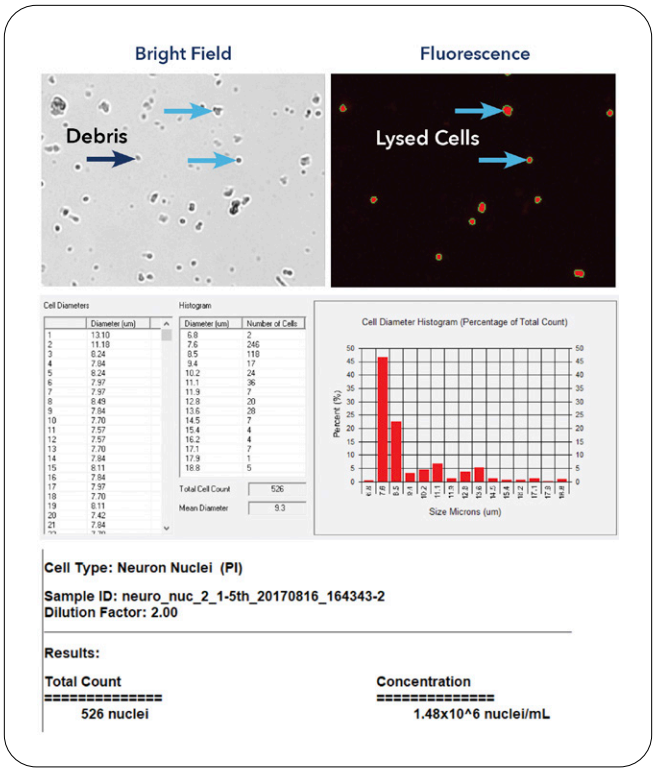


Figure 9: Brightfield and PI fluorescent images showing direct counting of nuclei.

Ability to optimize counting using multiple parameters

The Cellometer software can easily customize specific cell counting programs for different assays and cell types. There are 15 parameters that can be adjusted to optimize cell counting segmentation and locked for future use. An example of specific counting program is to adjust cell size range and threshold to count total nuclei (Figure 9). In this example, cells are lysed and stained with propidium iodide, then counted using Cellometer K2.

Cellometer K2 can accommodate cell samples at low concentration or sample volume

Increase in captured images improves accuracy for low concentration sample

The cell concentration detection ranges from 105 to 107 cells/mL, which covers the majority of cell samples used for sequencing. For low cell concentration samples, instead of physical manipulation such as centrifuging and concentrating the cell sample, the number of images captured can be increased from the default of 4 to 8 images, thus doubling the number of counted cells to improve statistical analysis.

Utilize different counting chambers that can handle different volumes

For precious samples such as circulating tumor cells (CTC), infiltrating T cells, or stem cells, often times the final collected volume is very low, thus causing a problem for cell counting that usually requires volume of 10 µL or higher. By using low volume slide (SD025), only 2 – 4 µL of cell sample is required, thus providing an alternative method to count precious samples (Figure 10). The regular counting chamber (SD100) holds 20 µL for any standard cell types. Furthermore, for low concentration samples, higher volume can be used in larger counting chamber at 55 µL (PD300) to increase the number of counted cells, thus improving statistical accuracy.

Cellometer K2 has built-in QA/QC program for digital record keeping and cell type management

Built-in GMP/GLP ready modules for audit trails

For any GMP/GLP laboratories performing single cell sequencing, it is critical to have proper record and documentations for the cell samples tested. By utilizing the access control and automated data and image saving/export, digital records are properly preserved for any future auditing.

In conclusion, NGS platforms provide a gold mine of data based on systems approach to illuminate genotype–phenotype relationships. Eliminating missteps on sample preparation would be one simple guarantee for quality assurance and quality control.

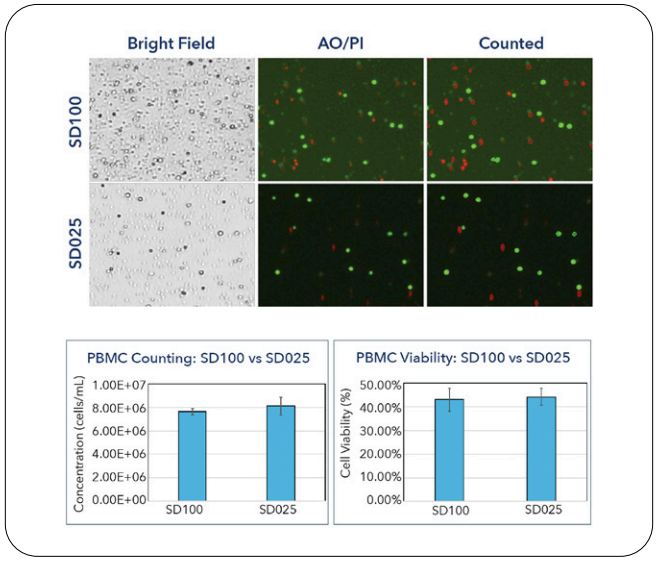


Figure 10: Cell samples such as circulating tumor cells or stem cells can be very precious, thus it is critical to be able to use only a small volume for testing cell concentration. By using the SD025 counting chamber, only 4 µL is needed per sample, thus minimizing the use of the precious cell sample.

References

1. https://www.yourgenome.org/facts/timeline-history-of-genomics
2. Jeffrey M. Perkel. (2017) Single-cell sequencing made simple, Nature, 541(7661): 125-126
3. Yong Wang and Nichloas E. Navin (2015) Advances and Applications of Single Cell Sequencing Technologies, Molecular Cell, 58(4): 598-609
4. Lander et al. (2001) Initial sequencing and analysis of the human genome, Nature, 409(6822): 860-921
5. Rickard Sandberg. (2013) Entering the era of single-cell transcriptomics in biology and medicine, Nature Methods, 11(1): 22-24
6. Gross et al. (2015) Technologies for Single-Cell Isolation, International Journal of Molecular Science, 16(8): 16897-16919
7. Tang et al. (2009) mRNA-Seq whole-transcriptome analysis of a single cell, Nature Methods, 6: 377-382
8. Hashimshony et al. (2012) CEL-Seq: Single-Cell RNA-Seq by Multiplexed Linear Amplification, Cell Reports, 2(3): 666-673
9. Jaitin et al. (2014) Massively parallel single cell RNA-Seq for marker-free decomposition of tissues into cell types, 343(6172): 776-779
10. Klein et al. (2015) Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic Stem Cells, Cell, 161(5): P1187-1201
11. Macosko et al. (2015) Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets, Cell, 161(5): P1202-1214
12. Cao et al. (2017) Comprehensive single-cell transcriptional profiling of a multicellular organism, Science, 357(6352): 661-667
13. Svensson et al. (2017). Exponential scaling of single-cell RNA-seq in the last decade. arXiv:1704.01379v2
14. Stegle et al. (2015). Computational and analytical challenges in single cell transcriptomics. Nat. Rev. Genetics 16, 133-45.
15. Chan et al. (2012) Rapid Image-based Cytometry for Comparison of Fluorescent Viability Staining Methods, Journal of Fluorescence, 22(5): 1301-1311
16. Chan et al. (2015) Morphological observation and analysis using automated image cytometry for the comparison of trypan blue and fluorescence-based viability detection method, Cytotechnology, 67(3): 461-473
17. Chan et al. (2013) Accurate measurement of peripheral blood mononuclear cell concentration using image cytometry to eliminate RBC-induced counting error, Journal of Immunological Methods, 388: 25-32.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com