Comparing Fluorescence-Based Viability Detection Method using the Cellometer Vision

WHITE PAPER

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Introduction

In this work, Cellometer Vision was employed to demonstrate rapid fluorescence-based viability measurements and the comparison of various fluorescent staining methods. First, fluorescent nucleic acid stains that examine membrane integrity were tested and validated by comparing against the standard trypan blue exclusion method. Similarly, fluorescent enzymatic stains that examine metabolic activities were tested and validated against the standard trypan blue exclusion method. Third, nucleic acid and enzymatic stains were compared by measuring viabilities of Jurkat cells incubated at different temperatures of water bath. Finally, to show the advantages of dual-staining method for "messy" primary cell samples Hoechst 33342, acridine orange, carboxyfluorescein diacetate, and Calcein AM in combination with propidium iodide were utilized [1-3] for isolated primary splenocytes and peripheral blood mononuclear cells with high level of debris and RBCs.

Materials and Methods

Image-based Cytometry Instrumentation and Disposable Counting Chamber

Cellometer Vision instrumentation has been described previously [4] and utilizes both brightfield (BR) and dual-fluorescent imaging modes. The software analyzed three image channels (BR, FL1, and FL2) and then the integrated proprietary algorithms converted the cell count to concentration and viability. For cell line samples, BR and FL1 images are analyzed for total and fluorescent positive cell enumeration, respectively. For primary cell samples, FL1 and FL2 images are analyzed for viable and nonviable cell enumeration, respectively. The concentration dynamic range of the Cellometer Vision was 1×10^5–7×10^7cells/ml.

Cell Line and Primary Cells Preparation

The Jurkat cell line was cultured in RPMI medium. The cell culture was maintained in an incubator at 37 °C and 5 % CO_2. Splenocytes and peripheral blood mononuclear cells (PBMCs) were given by Professor Xuemei Zhong (Boston University Medical Center, Boston, MA), which were prepared from the spleens and whole blood of BALB/c mice. Although most of the PBMCs were isolated from the sample, a noticeable percentage of

erythrocytes remained in the sample. Both splenocytes and PBMCs were resuspended in PBS before fluorescence-based viability measurement.

Viability Detection Method Using Nucleic Acid Stains

To test the nucleic acid staining detection method, nonviable Jurkat cells were stained with six fluorescent stains, which included 4′,6-diamidino-2-phenylindole (DAPI), Sytox Green, ethidium bromide (EB), propidium iodide (PI), 7- aminoactinomycin D (7AAD), and Sytox Red [5-9]. Two milliliters of Jurkat cells were heat-killed by incubation in a boiling water bath for 20 min. The heat-killed cells were then mixed with Jurkat cells directly from the cell culture at different ratios to produce five theoretical viability percentages at 100, 75, 50, 25, and 0 %. Each stain was mixed 1:1 with each of the five Jurkat samples. Sytox Green, EB, and PI stained samples were immediately analyzed after staining. DAPI and 7AAD were incubated for 5 min, while Sytox Red was incubated for 15 min at room temperature before image-based cytometric analysis. Each sample was measured in quadruplicate.

Automated viability measurements were compared to manual counting using a hemacytometer with trypan blue. Each of the five Jurkat samples was stained with trypan blue staining solution where the viable and nonviable cells were manually counted under a standard light microscope.

Viability Detection Method Using Enzymatic Stains

To test the enzymatic staining detection method, viable Jurkat cells were stained with two enzymatic stains, carboxyfluorescein diacetate (CFDA) and Calcein AM [7, 10]. Five theoretical viability percentages were prepared similar to that described above. Jurkat cells were incubated 1:1 with Calcein AM or CFDA for 15 min at 37 °C before image-based cytometric analysis. Each sample was measured in quadruplicate. The results were also compared to manual counting using hemacytometer and trypan blue, as described previously.

Comparison of Nucleic Acid and Enzymatic Stains Using Cell Line

In order to compare nucleic acid and enzymatic stain detection, we selected the combinations of acridine orange (AO)/PI and CFDA/PI to measure viable and nonviable cells simultaneously (dual-staining of acridine orange [11]. AO/PI staining solution was used as is, and CFDA/PI staining solution was mixed to a working concentration in PBS. Jurkat cells obtained directly from culture were incubated in four water baths of varying temperatures at 37, 45, 55, and 65 °C for a period of 20 min. Following the incubation, the Jurkat cells at each temperature were mixed 1:1 with AO/PI or CFDA/PI. AO/ PI was immediately analyzed with image-based cytometry after staining and CFDA/PI was allowed to incubate for 15 min before analysis. Each sample was measured in quadruplicate.

Dual-staining Method for Primary Cells

Due to the complexity of analyzing unpurified primary cells, the dual-staining method was performed to measure the viability of primary samples with high debris content. We selected

the combinations of AO/PI, CFDA/PI, Calcein AM/PI, and Hoechst 33342/PI to measure viable and non-viable primary cells simultaneously. AO/PI staining solution was used as is. CFDA/PI and Hoechst/PI staining solutions were mixed to a working concentration in PBS. Calcein AM/PI staining solution was mixed to a working concentration in cell culture H_2O. Splenocytes and PBMC samples were diluted. Following the dilution, CFDA/PI, Calcein AM/PI, or Hoechst/PI were added at a ratio of 1:1 to each primary cell sample and incubated for 15 min at 37 °C. Each primary cell sample was also stained similarly with AO/PI for comparison. Each sample was analyzed in quadruplicate.

Image-based Cytometry Viability Detection Method

To measure the viability of each cell sample, the appropriate fluorescence optics module is used to detect specific fluorescence emission wavelength. For nucleic acid viability stains, VB-450-302, VB-535-402, VB-595-502, VB-660-502, and VB-695-602 are used to detect DAPI, Sytox Green, EB/PI, 7AAD, and Sytox Red, respectively. For enzymatic viability stains, VB-535-402 is used to detect both CFDA and Calcein AM. For dual-staining methods, VB-450-302, VB-535-402, and VB-660-502 are used to detect Hoechst, AO, and PI, respectively. The Cellometer Vision software contained three equations for viability calculations. For nucleic acid and enzymatic staining method, the viability is calculated using Eqs. 1 and 2, respectively (shown below), where BR and FL represent the total number of cells counted in brightfield and fluorescence, respectively. It is important to note that the FL in Eqs. 1 and 2 represent nonviable and viable cells, respectively. For the dual-staining method, Eq. 3 (shown below) was used to calculate the viability of the sample, where FL1 and FL2 represent the total number of viable and nonviable cells, respectively.

Eq 1) Viability = (BR-FL)/BR x 100%

Eq 2) Viability = FL/BR x 100%

Eq 3) Viability = FL1/(FL1+FL2) x 100%

Results

Figure 1. Validation of Nucleic Acid Staining Viability Detection Method

- The nonviable Jurkat cells stained with DAPI, Sytox Green, EB, PI, 7AAD, or Sytox Red were counted under fluorescence detection, and total cells were counted through BR imaging. Since all the nucleic acid stains tested membrane integrity of the cells, the merged images were highly comparable. Trypan blue-stained Jurkat cell images at each percentage are also shown. By using the nucleic acid viability Eq. 1 in the software, the viability measurements for each nucleic acid stain were obtained.

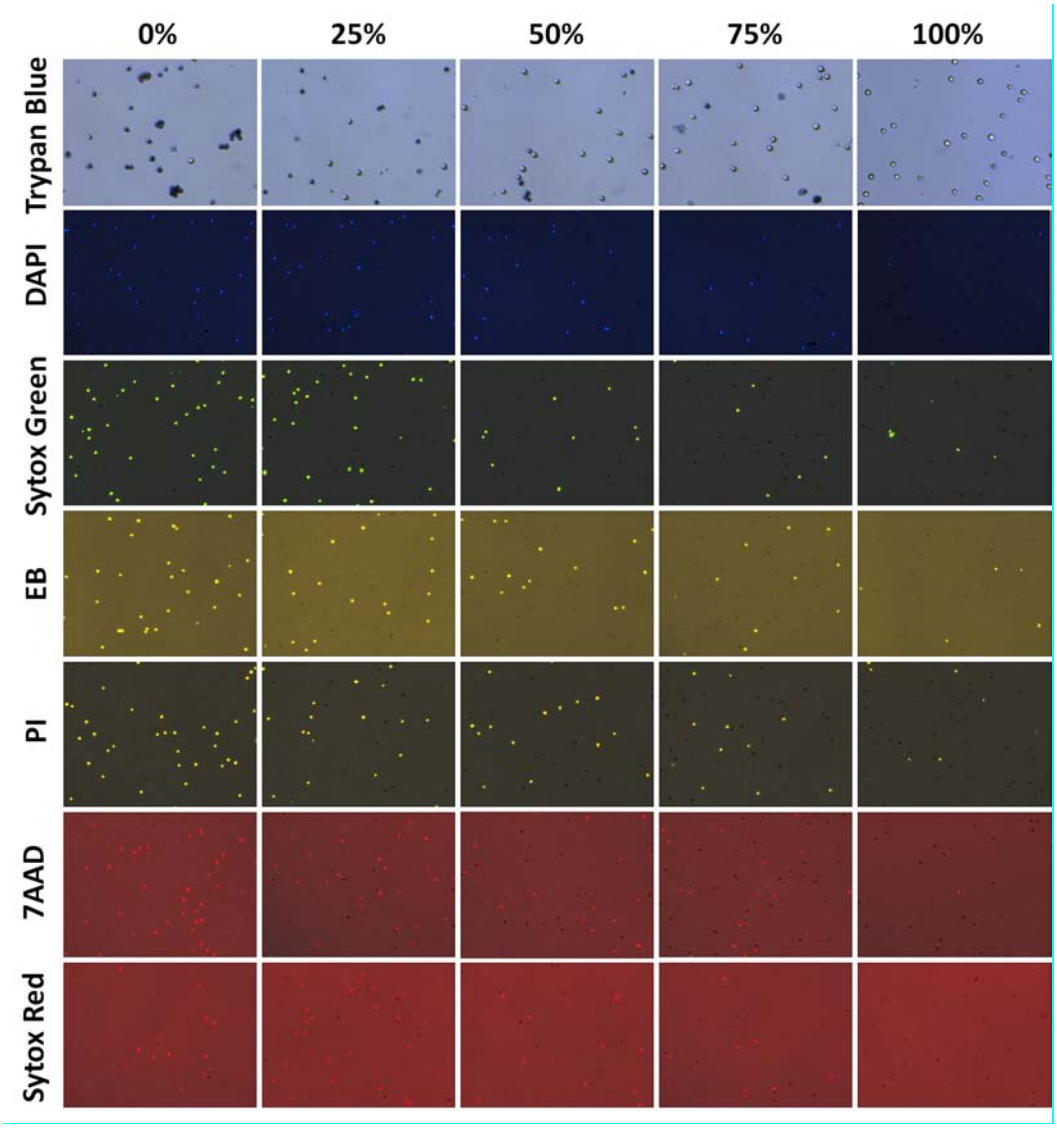


Figure 2. Validation of Enzymatic Staining Viability Detection Method

- The viable Jurkat cells stained with Calcein AM or CFDA were counted under fluorescence detection, and total cells were counted through BR imaging. Since all of the enzymatic stains tested the metabolic activity of cells, the merged images were highly comparable, where the increase in the number of fluorescent cells could be observed as the viability increased. Trypan blue-stained Jurkat cells images at each percentage are also shown. By using the enzymatic viability Eq. 2 in the software, the viability measurements for each enzymatic stain were obtained.

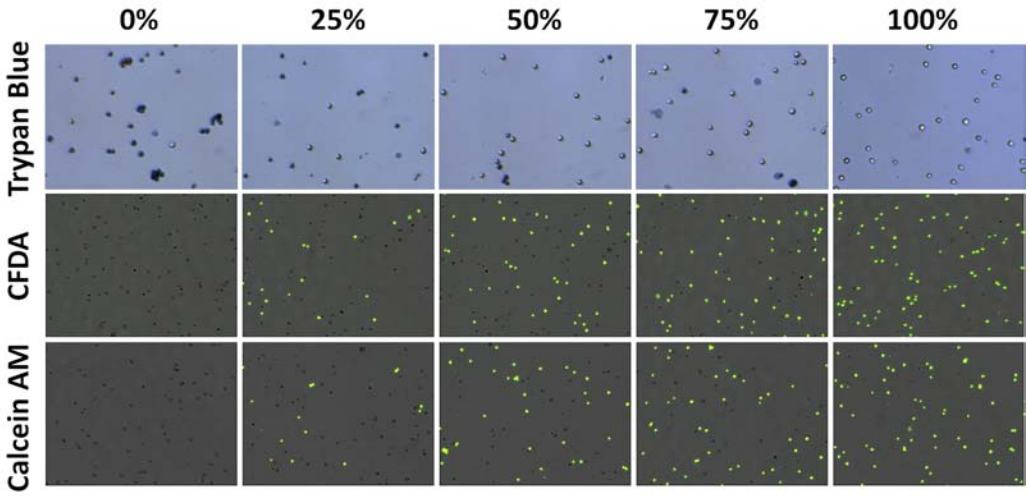


Figure 3. Comparison of Nucleic Acid and Enzymatic Dual-staining Method Using Jurkat Cell Line

- The combined fluorescent and brigthfield images are shown to compare AO/PI (nucleic acid) and CFDA/PI (enzymatic) staining methods. The number of nonviable cells is correlated positively to the temperature as expected. The increase and decrease in the number of red and green cells could be observed as the incubation temperature increased. By using the dual-staining viability Eq. 3, the viability results were obtained.

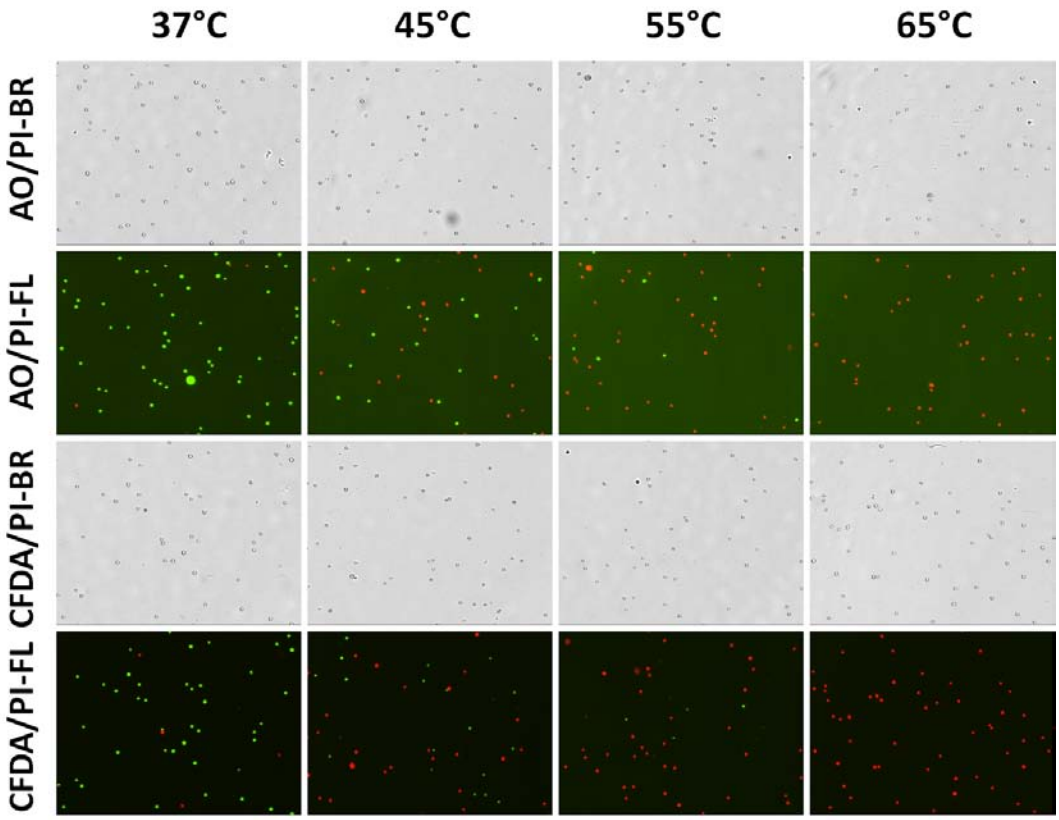


Figure 4. Dual-staining Method for Primary Splenocytes and PBMCs

- Viabilities of primary splenocytes and PBMCs were analyzed using: AO/PI, Hoechst/PI, Calcein AM/PI, and CFDA/PI. The dual-staining method induced large fluorescence signals for viable and nonviable nucleated cells, which aided the computer software in cell enumeration without counting the RBCs. Note that AO, Calcein AM, and CFDA exhibited some RBCs background fluorescence, but did not interfere with automated counting.

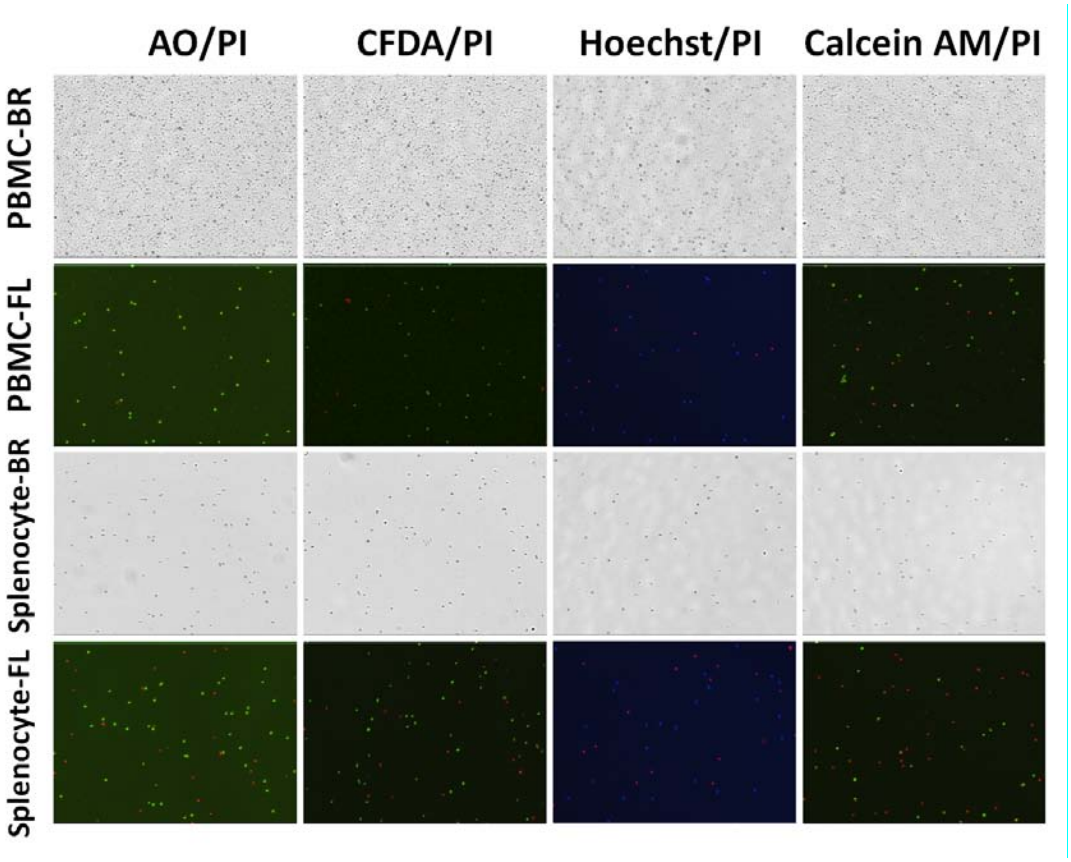


Figure 5. Histogram of image based viability measurements versus manual trypan blue counts.

- (5a) All tested nucleic acid stains produced comparable results to the trypan blue exclusion method via hemacytometer. The experimental viability measurements correlated closely with the theoretical values.
- (5b) The enzymatic stains also compared well to the trypan blue exclusion method via hemacytometer. The experimental viability measurement correlated closely with the theoretical percentages as well. Taken together, it has been demonstrated that the tested enzymatic stains using image-based cytometry could generate reliable viability measurements.

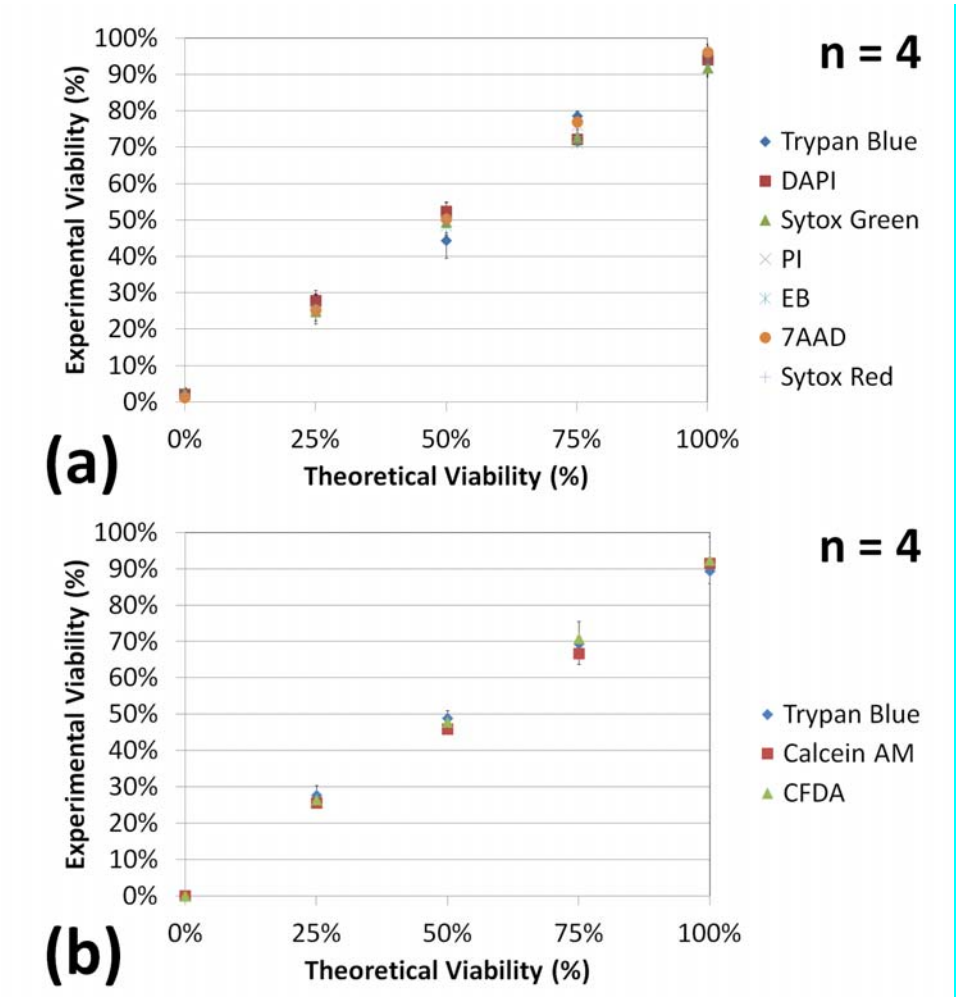
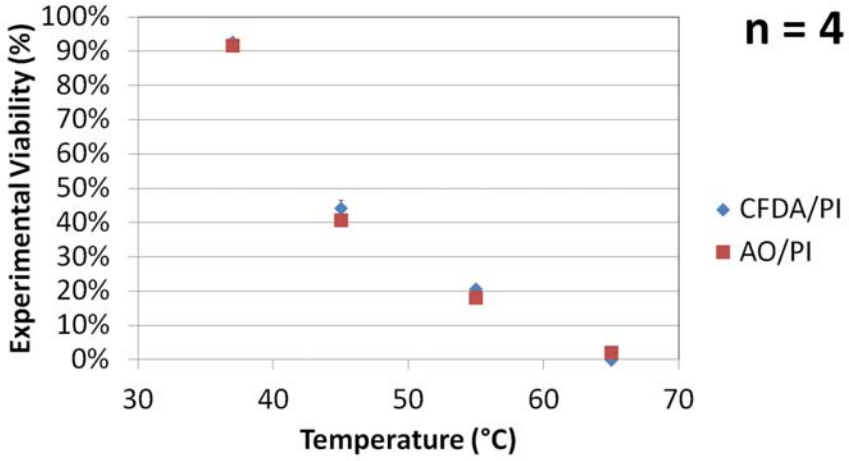
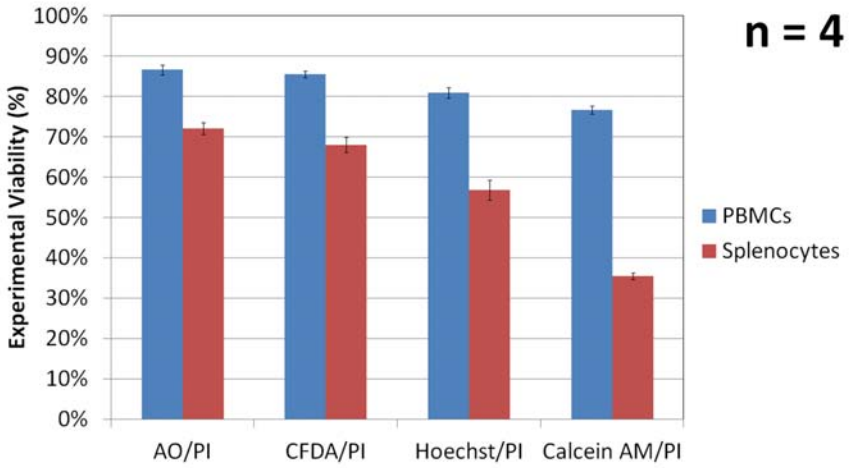


Figure 6. Histogram of viability measurements in Jurkat and primary cells with dual-staining method

- (6a) Comparable measurements between AO/PI and CFDA/PI are shown in Jurkat cels at each temperature. For both stain combinations, the viability reduced by ~45% when the temperature increased from 37 to 45 °C. The viability reduced at a lower rate of ~20% from 45 to 55° C and from 55 to 65° C.
- (6b) The PBMCs showed a viability of 86.6, 85.5, 80.9, 76.6%, and the splenocytes showed a viability of 72.0, 68.0, 56.8, and 35.4% for AO/PI, CFDA/PI, Hoechst/PI, and Calcein AM/PI, respectively. The measured viabilities for dual-staining AO/PI and CFDA/PI were highly comparable and were similar to the Jurkat cell line. However, the results obtained using Hoechst/ PI and Calcein AM/PI showed noticeable differences in viability (~10–30 %).





Conclusion

- The combination of brightfield and fluorescence imaging modes allows for the analysis of cell lines or purified primary cell samples using a single fluorescent viability stain. Fluorescence imaging mode using a dual-staining method allows the system to accurately measure viability of different primary cell samples without purification and processing [12].
- Image-based cytometry was able to validate each nucleic acid staining method against the traditional trypan blue method, which showed that each staining method was as accurate as trypan blue exclusion, as expected.
- Fluorescent enzymatic stains such as CFDA and Calcein AM were tested and validated against the traditional trypan blue exclusion, which gave comparable values. The viability results obtained from enzymatic stains showed slightly lower averages than the nucleic acid stains, which may be due to the fact that enzymatic stains are specific to metabolically active cells.
- For the dual-staining methods of nucleic acid and enzymatic stains in heat-killed Jurkat cells, AO/PI and CFDA/PI were highly comparable with differences of 0.9, 3.4, 2.5, and 2.2 % at temperatures 37, 45, 55, and 65 °C.
- In contrast, primary cells showed noticeable differences in viability measurements when comparing AO/PI, Hoechst/PI, CFDA/PI, and Calcein AM/PI. The measured viabilities showed a consistently decreasing trend from AO/PI, CFDA/PI, Hoechst/PI, to Calcein AM/PI, where the maximum reduction was 10.0 % for PBMCs and 36.6 % for splenocytes. The differences in viability measurement may be attributed to differences in stain molecular structure and functionality. Another possibility is that AO and CFDA may have higher nonspecific staining of debris.
- Of the four dual-staining methods, CFDA and Calcein AM induced a low amount of nonspecific fluorescence in the RBCs, but may require higher fluorescent threshold for more accurate counting.
- If the sample condition is clean, such as cell lines or purified primary cells, then the brightfield or single staining method can be employed. If the sample condition is complicated, such as unpurified primary cells, whole blood, or cell lines in killing assays, then a dual-staining method can be employed. These two methods have the potential to be integrated into cell-based research, which can improve the efficiency of the viability measurement step and allow more time for researchers to perform higher complexity cell-based analysis.

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