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ORIGINAL PAPER

Rapid Image-based Cytometry for Comparison of Fluorescent Viability Staining Methods

Leo L. Chan · Alisha R. Wilkinson · Benjamin D. Paradis · Ning Lai

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Abstract The ability to accurately measure cell viability is important for any cell-based research. Traditionally, viability measurements have been performed using trypan blue exclusion method on hemacytometer, which allowed researchers to visually distinguish viable from nonviable cells. However, the trypan blue method is often limited to only cell lines or primary cells that have been rigorously purified. In the recent years, small desktop image-based cell counters have been developed for rapid cell concentration and viability measurement due to advances in imaging and optics technologies as well as novel fluorescent stains. In this work, we employed the Cellometer image-based cytometer to demonstrate the ability to simplify viability detection compared to the current methods. We compared various fluorescence viability detection methods using single- or dual-staining technique. Single-staining method using nucleic acid stains including ethidium bromide, propidium iodide, 7AAD, DAPI, Sytox Green and Sytox Red, and enzymatic stains including CFDA and Calcein AM were performed. All stains produced comparable results to trypan blue exclusion method for cell line samples. Dual-staining method using AO/PI, CFDA/PI, Calcein AM/PI and Hoechst 33342/PI that enumerates viable and non-viable cells was tested on primary cell samples with high debris contents. This method allowed exclusion of cellular debris and non-nucleated cells from analysis, which can eliminate the need to perform purification step during sample preparation, and improves the efficiency of viability detection method. Overall, these image-based fluorescent cell counters can simplify assay procedures as well as capture images for visual confirmation.

Keywords Image-based cytometry (IBC) · Viability · Enzymatic viability stain · Nucleic acid viability stain · Dual-staining viability method · Trypan blue exclusion method · Cellometer vision

Alisha R. Wilkinson and Benjamin D. Paradis contributed equally in this manuscript.

L. L. Chan (✉) · A. R. Wilkinson · B. D. Paradis
Department of Technology R&D, Nexcelom Bioscience LLC,
360 Merrimack St. Building 9,
Lawrence, MA 01843, USA
e-mail: lchan@nexcelom.com

N. Lai
Department of Applications, Nexcelom Bioscience LLC,
Lawrence, MA 01843, USA

A. R. Wilkinson
Department of Biology, Merrimack College,
North Andover, MA 01845, USA

L. L. Chan · A. R. Wilkinson · B. D. Paradis
Center for Biotechnology and Biomedical Sciences,
Merrimack College,
North Andover, MA 01845, USA

Introduction

The ability to accurately measure cell viability is important for any cell-based research, such as in oncology, immunology, stem cell, and toxicology research fields [1, 2]. Viability measurement has been performed primarily by selectively staining membrane-compromised nonviable cells with trypan blue, which allows the researcher to visually distinguish viable from nonviable cells [3]. Optimal viability measurements using the trypan blue method can be achieved for cell lines with high viabilities or primary cells that have been rigorously purified [4]. Cell viability measurements with trypan blue has been performed using hemacytometer on simple light microscopy for over a century [5, 6]. Over the years, various types of

hemacytometers have been developed to improve the accuracy of the device, but it does not resolve the underlying issues of manual cell counting method. First, there is inconsistency from user-to-user. Second, there is an inherent statistical error of $\frac{1}{\sqrt{n}}$, where n is the number of cells counted [7–10]. Typically 100–300 total cells are counted following standard procedures, which translate to approximately 5.8–10 % statistical error. In order to improve the accuracy of manual counting method, more than 400 cells are required to reduce the statistical error to below 5 %. However, it can increase the counting time significantly and makes the method more tedious.

In the recent years, advancements in imaging detector, high power light-emitting diode (LED), and optical lens technologies have allowed the development of a new generation of affordable automated image-based cytometry (IBC) systems, such as Cellometer AutoT4 (Nexcelom Bioscience), Countess (Invitrogen), and TC10 (BioRad), to address the known issues of manual counting method. These automated cell counters employ a combination of standard light microscopy, complementary metal-oxide-semiconductor (CMO) or charge-coupled device (CCD) detector, and innovative imaging analysis algorithm to enumerate viable and trypan blue stained non-viable cells in the bright-field images [11–14]. However, for more complex cell samples that contain cellular debris and contaminating non-nucleated cells, accuracy of viability measurements can be significantly affected.

Due to the increase in availability of fluorescent stains, new viability detection methods have been developed to more definitively identify viable and nonviable cells for any sample conditions. The commonly used fluorescent viability stains consist of membrane integrity and enzymatic stains. Fluorescent staining method can offer higher accuracy by specific staining only nucleated cells. Therefore, this method can be applied to cell line samples with high or low viability that contain high debris contents. By combining two fluorescent stains, one for viable population and the other for nonviable population, dual-staining method can be used to analyze primary cells with high red blood cells (RBCs) contamination, or white blood cells (WBCs) directly from whole blood. Therefore, the time-consuming purification or lysing procedures can be eliminated, which drastically improves the efficiency of viability measurement.

Fluorescence-based cell viability detection has been demonstrated using fluorescence microscopy and fluorescent stains for visual confirmation of viable and nonviable cells. However, the lack of automation and quantification of cells still render the fluorescence-based cell viability detection user-unfriendly. The common automated method for fluorescence analysis of cells requires the use of conventional flow cytometry, which has the ability to quickly enumerate millions of cells and considerably reduce the statistical error [15–17]. However, the systems remain relatively expensive, require considerable amount of maintenance (clogging issues), and highly skilled technicians. In addition, the lack of imaging capability in traditional flow cytometry systems may generate uncertainties in the results [18, 19]. To address the issues raised by the current fluorescent-based cell viability detection methods, image-based fluorescent cell counters were developed, such as Cellometer Vision (Nexcelom Bioscience), Tali (Invitrogen), and NucleoCounter (Chemometec), for rapid cell concentration and viability measurements. These image-based fluorescent cell counters can simplify assay procedures as well as capture images for visual confirmation.

In this work, Cellometer image-based cytometer was employed to demonstrate the ability to perform rapid fluorescence-based viability measurement and to compare various fluorescent staining methods. First, fluorescent nucleic acid stains that examine membrane integrity were tested and validated on image-based cytometry by comparing against the standard trypan blue exclusion method. Ethidium bromide (EX: 522 nm, EM: 606 nm), propidium iodide (EX: 538 nm, EM: 617 nm), 7-aminoactinomycin D (EX: 543 nm, EM: 647 nm), 4′,6-diamidino-2-phenylindole (EX: 358 nm, EM: 461 nm), Sytox Green (EX: 504 nm, EM: 523 nm), and Sytox Red (EX: 640 nm, EM: 658 nm) were used to stain membrane compromised Jurkat cells at different viabilities [20–24]. Similarly, fluorescent enzymatic stains that examine metabolic activities were tested and validated on IBC against the standard trypan blue exclusion method. Carboxyfluorescein diacetate (EX: 492 nm, EM: 517 nm) and Calcein AM (EX: 496 nm, EM: 516 nm) were used to stain metabolically active viable Jurkat cells at different viabilities [22, 25]. Third, nucleic acid and enzymatic stains were compared by measuring viabilities of Jurkat cells incubated at different temperatures of water bath. Dual-staining of acridine orange (EX: 501 nm, EM: 527 nm) or carboxyfluorescein diacetate in combination with propidium iodide was utilized for fluorescence-based enumeration of viable and nonviable cells [26]. Finally, to show the advantages of dual-staining method for messy primary cell samples, Hoechst 33342 (EX: 352 nm, EM: 455 nm), acridine orange, carboxyfluorescein diacetate, and Calcein AM in combination with propidium iodide were utilized [27–29] 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 [30], which utilizes bright-field (BR) and dual-fluorescent (FL1 and FL2) imaging modes to quantitatively

analyze and measure the concentration and viability of target cells (Fig. 1). Bright-field imaging used a white light-emitting diode (LED) and fluorescent imaging used four different monochromatic LEDs (375, 470, 525, and 630 nm) as the excitation light source. The monochromatic LEDs were combined with five specific fluorescence optics modules (excitation/emission), VB-450-302 (300/450), VB-535-402 (475/535), VB-595-502 (525/595), VB-660-502 (540/660), and VB-695-602 (630/695). The cell sample was pipetted into Nexcelom disposable counting chambers, which held precisely 20 μl and a fixed height of less than 100 μm. The counting chamber was held in position by a stage, which carefully moved to 4 locations on the chamber for cell analysis by the Cellometer software. 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 1×10^5–7×10^7 cells/ml. Acquisition of the images and cell analysis was less than 2 min depending on the exposure time of the two fluorescent channels.

Cell Line and Primary Cells Preparation

The Jurkat cell line (TIB-152, American Type Culture Collection (ATCC)) was cultured in RPMI medium supplemented with 10 % fetal bovine serum (FBS, ATCC) and 1 % pen/strep antibiotics (Sigma-Aldrich, St. Louis, MO). The cell culture was maintained in an incubator at 37 °C and 5 % CO_2 and medium was replenished every the other day.

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. All animal work was performed according to institutional guidelines and approved by the IACUC at Boston University Medical Center. Spleens were rinsed with phosphate buffered saline (PBS) and cut into two pieces. Next, single cell suspension was prepared by gently dissociating using the rubber end of syringe petrol, which was then passed

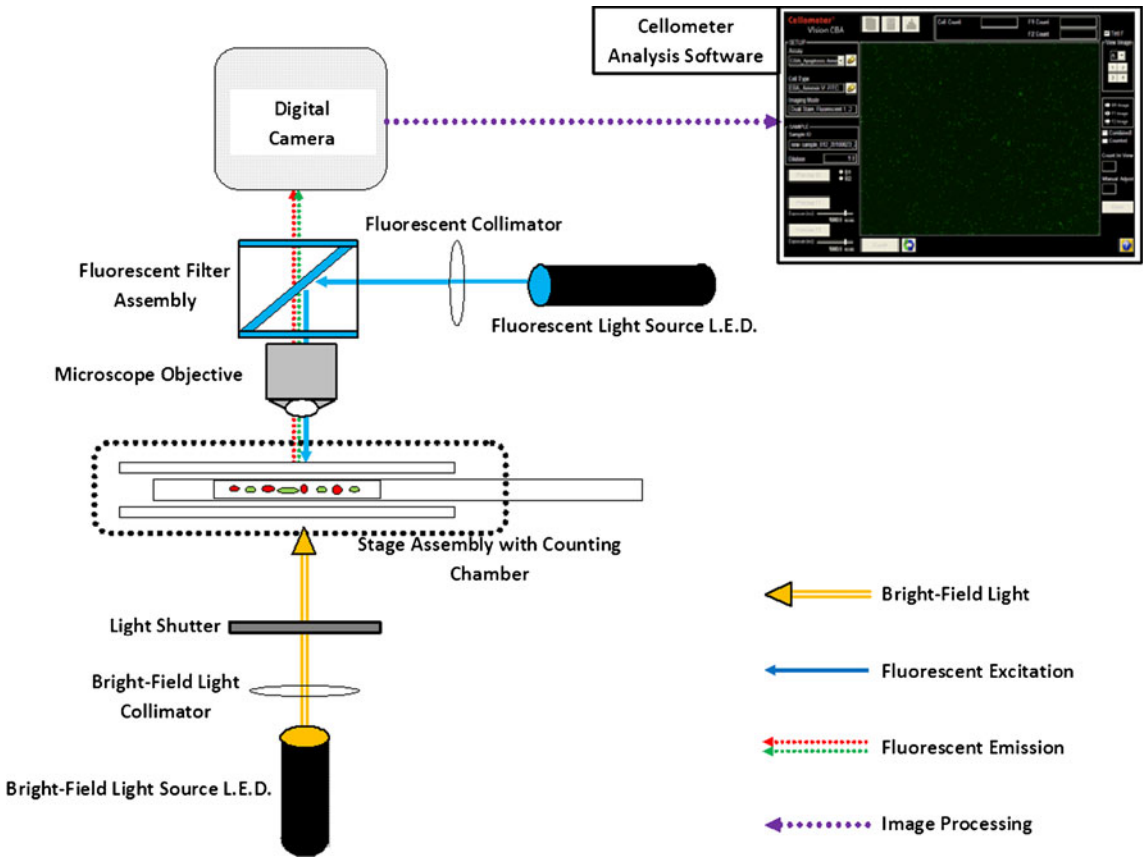

Fig. 1 Optical block diagram of Cellometer Vision. Cellometer Vision contains 3 signal pathways: (*1*) Bright-field light source allows transmission light microscopy imaging analysis. (*2*) Fluorescence excitation light source in combination with excitation, dichroic, and emission filter set allows epi-fluorescence imaging analysis. (*3*) Cellometer software allows imaging analysis for cell concentration, size, and fluorescence intensity measurement

through a cell strainer with 70 μm nylon mesh (BD Biosciences, San Diego, CA). The erythrocytes were not lysed to show the capability of fluorescence-based viability detection method of a primary sample. PBMCs were extracted by centrifugation of the blood sample to separate the nucleated cells from red blood cells. Although most of the PBMCs were isolated from the sample, a noticeable percentage of erythrocytes remain in the sample, which makes manual counting extremely difficult. 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, IBC was used to count the nonviable Jurkat cells stained with six fluorescent stains at various emission wavelengths, which included 4′,6-diamidino-2-phenylindole (DAPI), Sytox Green, ethidium bromide (EB), propidium iodide (PI), 7-aminoactinomycin D (7AAD), and Sytox Red. All of the stains were purchased from Invitrogen (Carlsbad, CA), except EB and PI, which were from Sigma-Aldrich and Nexcelom Bioscience, respectively. Sytox Green, EB, 7AAD, and Sytox Red were diluted in PBS to a working concentration of 1 μM, 100 μg/ml, 200 μg/ml, and 500 nM, respectively. DAPI was diluted in cell culture H_2O to a working concentration of 20 μg/ml and PI was used directly. Two milliliters of Jurkat cells were heat-killed by incubating 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 5 theoretical viability percentages at 100, 75, 50, 25, and 0 %.

Twenty microliters of each stain were mixed 1:1 with each of the 5 Jurkat samples. Sytox Green, EB, and PI stained samples were immediately analyzed with IBC 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 measurement using image-based cytometry was compared to manual counting using a hemacytometer with trypan blue to validate the fluorescence-based viability detection method. Each of the 5 Jurkat samples were stained with 0.2 % trypan blue staining solution (Invitrogen), 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, IBC was used to count the viable Jurkat cells stained with two enzymatic stains, carboxyfluorescein diacetate (CFDA) and Calcein AM, which were purchased from Invitrogen. Metabolically active cells will hydrolyze non-fluorescencent CFDA and Calcein AM to highly fluorescent carboxyfluorescein and Calcein using intracellular esterases. CFDA and Calcein AM were diluted in PBS and cell culture H_2O, respectively, to a working concentration of 10 μM. Five theoretical viability percentages were prepared similar to the procedure described for nucleic acid stains. Twenty microliters of 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 described previously.

Comparison of Nucleic Acid and Enzymatic Stains Using Cell Line

In order to compare nucleic acid and enzymatic stain detection using cell line on IBC, we selected the combinations of acridine orange (AO)/PI and CFDA/PI to measure viable and nonviable cells simultaneously. AO/PI staining solution was obtained from Nexcelom Bioscience and used as is. CFDA/PI staining solution was mixed to a working concentration of 20 μM/100 μg/ml in PBS. Jurkat cells obtained directly from culture were incubated in 4 different temperatures of water bath 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 unpurified primary cells, where large amount of debris and RBCs contamination existed, dual-staining method was performed with IBC 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 nonviable primary cells simultaneously. AO/PI staining solution was obtained from Nexcelom Bioscience and used as is. Hoechst 33342 (Hoechst) was purchased from Sigma-Aldrich. CFDA/PI and Hoechst/PI staining solutions were mixed to a working concentration of 20 μM/100 μg/ml in PBS. Calcein AM/PI staining solution was mixed to a working concentration of 20 μM/100 μg/ml in cell culture H_2O. Splenocytes and PBMC samples were diluted to approximately 1×10^6 cells/ml. Following the dilution, twenty microliters of CFDA/PI, Calcein AM/PI, or Hoechst/PI was 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 using image-based cytometry 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 imaging exposure times for DAPI, Sytox Green, EB, PI, 7AAD, Sytox Red, CFDA, Calcein AM, AO, and Hoechst were 500, 50, 500, 2000, 1000, 100, 100, 100, and 1000 ms, respectively. In dual-staining detection, PI exposure time was 2000 ms. The fluorescent threshold was set to 10 % for all experiments. The Cellometer Vision software contained 3 equations for viability calculations. For nucleic acid and enzymatic staining method, the viability is calculated using Eqs. 1 and 2, respectively, where BR and FL represent the total number of cells counted in bright-field and fluorescence, respectively. It is important to note that the FL in Eqs. 1 and 2 represent nonviable and viable cells, respectively. For dual-staining method, Eq. 3 is used to calculate the viability of the sample, where FL1 and FL2 represent the total number of viable and nonviable cells, respectively.

$$Viability = \frac{BR - FL}{BR} \times 100\% \quad (1)$$

$$Viability = \frac{FL}{BR} \times 100\% \quad (2)$$

$$Viability = \frac{FL1}{FL1 + FL2} \times 100\% \quad (3)$$

Results

Validation of Nucleic Acid Staining Viability Detection Method

To validate the nucleic acid staining viability detection method using IBC, the viabilities of Jurkat cells with theoretical viabilities of 0, 25, 50, 75, and 100 % were measured. 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 (Fig. 2). As displayed in Fig. 2, IBC allowed the acquisition of fluorescent images at a wide range of excitation and emission wavelengths. By using the nucleic acid viability Eq. 1 in the software, the viability measurements for each nucleic acid stain were obtained via IBC. All tested nucleic acid stains produced comparable results to the trypan blue exclusion method via hemacytometer (Fig. 6a). The experimental viability measurements correlated closely with the theoretical values. The results indicated that image-based cytometry can accurately measure viabilities ranging from 0 to 100 %.

Validation of Enzymatic Staining Viability Detection Method

To validate the enzymatic staining viability detection method using IBC, the viabilities of Jurkat cells with theoretical viabilities of 0, 25, 50, 75, 100 % were measured. The viable Jurkat cells stained with Calcein AM or CFDA were counted under fluorescence detection, and total cells were counted through BR imaging (Fig. 3). As displayed in Fig. 3, IBC allowed the acquisition of fluorescent images for each enzymatic stain, where viable and nonviable cells could be visually confirmed. By using the enzymatic viability Eq. 2 in the software, the viability measurements for each enzymatic stain was obtained via IBC, which both compares well to the trypan blue exclusion method via hemacytometer (Fig. 6b). The experimental viability measurement correlated closely with the theoretical percentages as well. Take together, it has been demonstrated that the tested enzymatic stains using image-based cytometry could generate reliable viability measurements.

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

To compare AO/PI (nucleic acid) and CFDA/PI (enzymatic) staining method, we employed IBC to measure viabilities of Jurkat cells incubated at 4 different temperatures (37, 45, 55, and 65 °C). The combined fluorescent and bright-field images are shown in Fig. 4, where the number of nonviable cells is correlated positively to the temperature as expected, which could be visually confirmed. By using the dual-staining viability Eq. 3, the viability results are obtained and shown in Fig. 7a, which showed comparable measurements between AO/PI and CFDA/PI 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.

Dual-staining Method for Primary Splenocytes and PBMCs

To demonstrate the advantage of dual-staining viability detection method, viabilities of primary splenocytes and PBMCs

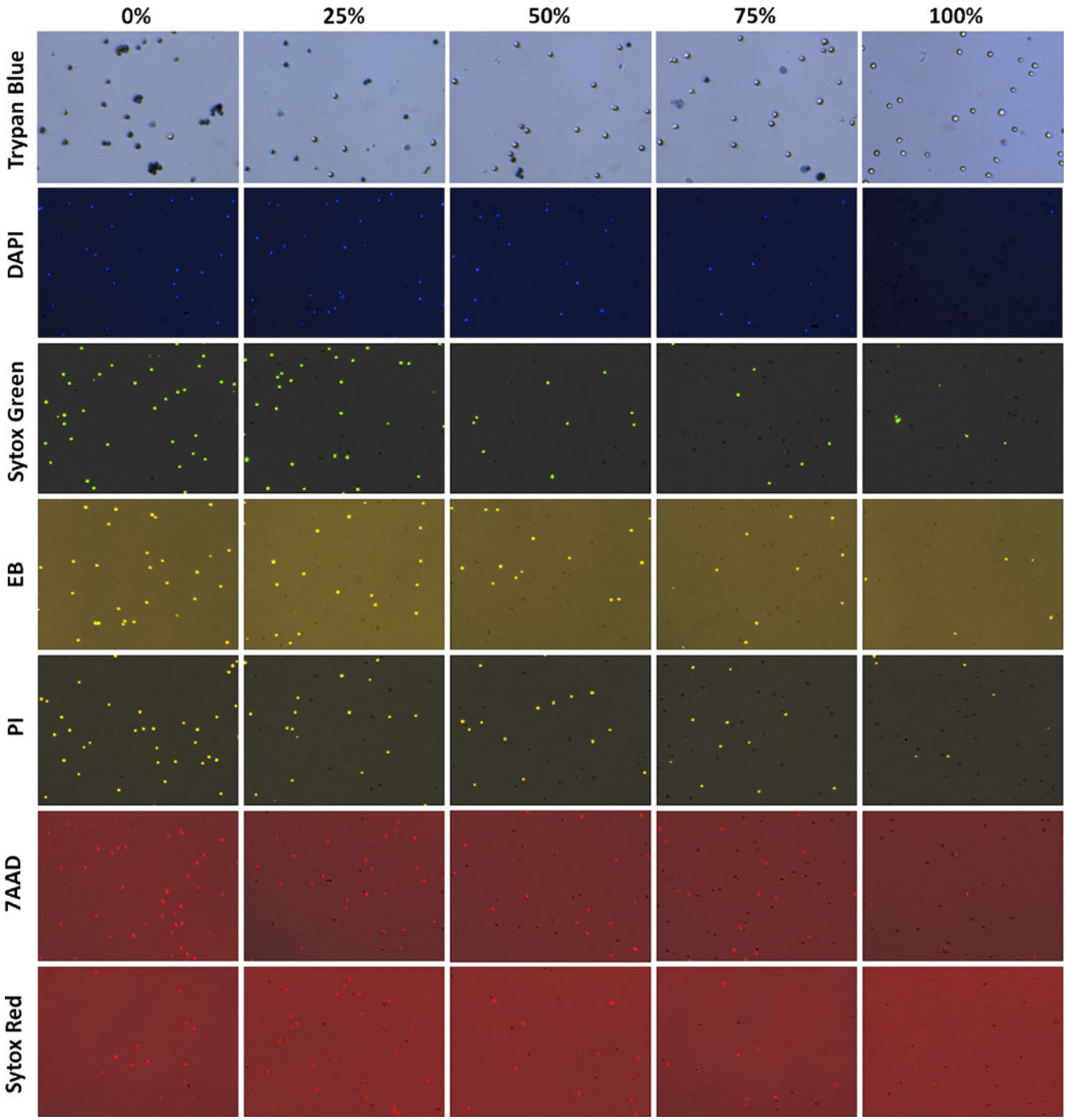

Fig. 2 Merged bright-field and fluorescent images of nucleic acid stained Jurkat cells. Five mixed Jurkat sample at 0, 25, 50, 75, and 100 % viability (column) were stained with DAPI, Sytox Green, EB, PI, 7AAD, and Sytox Red. Each stain was assigned a pseudo-color according to their emission wavelength of blue, green, orange, orange, red, and red, respectively. Since all of the nucleic acid stains tested membrane integrity of the cells, the merged images were highly comparable, where the decrease 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

were analyzed using 4 different fluorescent stain combinations, AO/PI, Hoechst/PI, Calcein AM/PI, and CFDA/PI. The combined fluorescent and bright-field images are shown in Fig. 5, where viable and nonviable were clearly identified by fluorescence emission above the background. Note that AO, Calcein AM, and CFDA exhibited some RBCs background fluorescence, but did not interfere with automated counting. The viability results are shown in Fig. 7b. 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

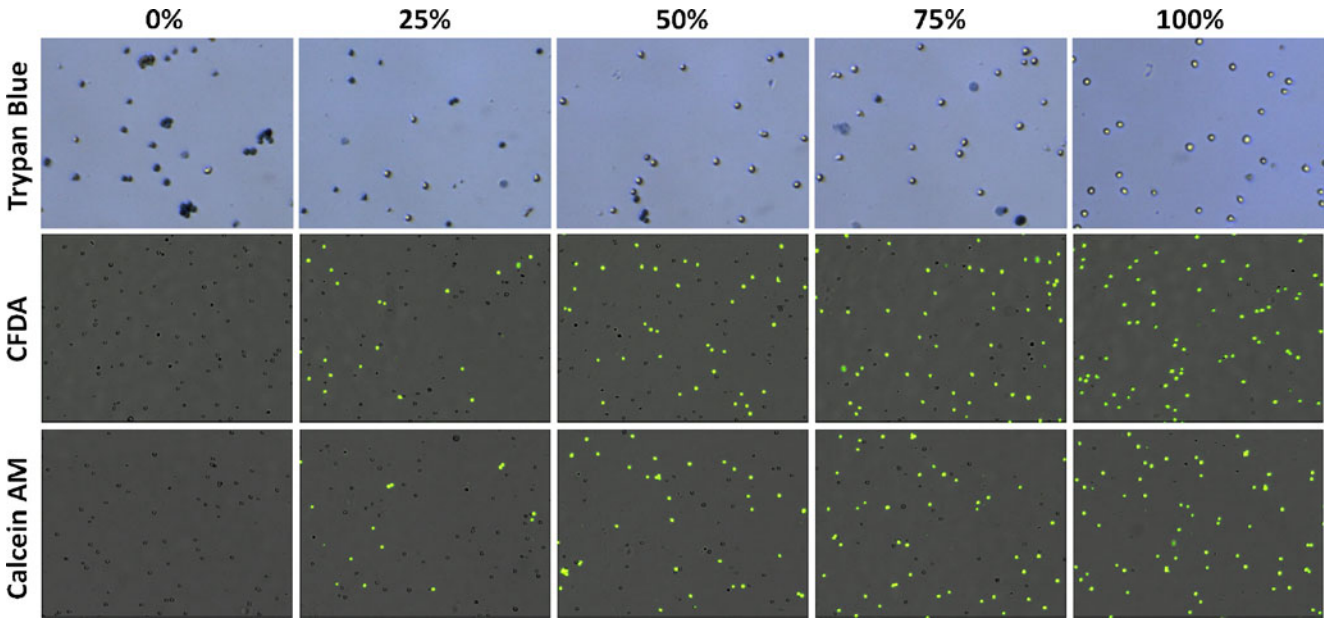

Fig. 3 Merged bright-field and fluorescent images of enzymatically stained Jurkat cells. Five mixed Jurkat sample at 0, 25, 50, 75, and 100 % viability (column) were stained with CFDA and Calcein AM. Both stains were assigned pseudo-color of green. Since all of the enzymatic stains tested the metabolic 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

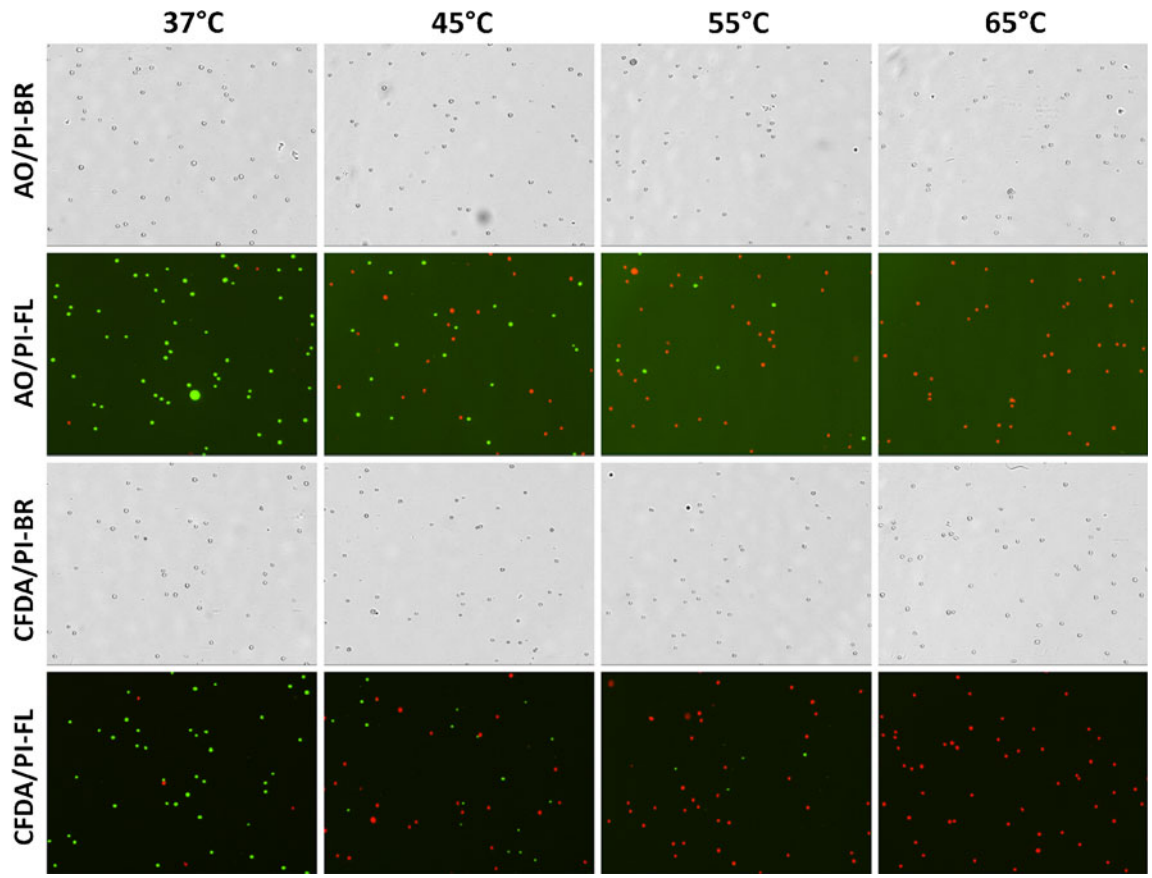

Fig. 4 Bright-field and fluorescent images of dual-staining AO/PI and CFDA/PI stained Jurkat cells. The cells were incubated at 37, 45, 55, and 65 °C and stained AO/PI or CFDA/PI, where viable and nonviable cells were colored green and red, respectively. The increase and decrease in the number of red and green cells could be observed as the incubation temperature increased. The background fluorescence of CFDA/PI was noticeably lower than AO/PI stains

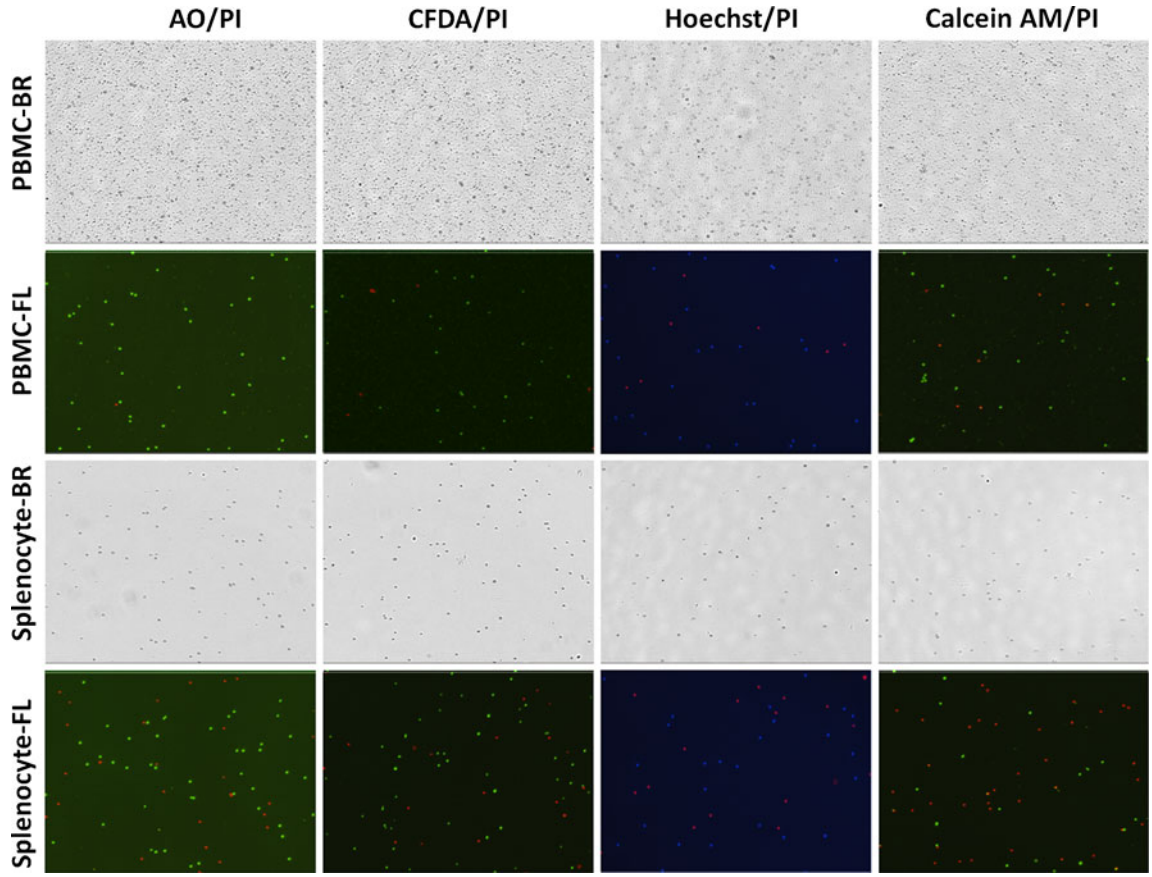

Fig. 5 Bright-field and fluorescent images of dual-stained primary cells. Primary PBMCs and splenocytes were isolated and made into single cell suspension for viability measurements using AO/PI, CFDA/PI, Hoechst/PI, and Calcein AM/PI. The dual-staining method induced large fluorescence signals for viable and nonviable nucleated cells, which aided the computer software in cell enumeration without counting the RBCs. Note that the RBCs showed small amounts of autofluorescence in the PBMC samples for CFDA and Calcein AM, but did not affect the counting algorithm

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, which 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 %).

Discussion

The integration of image-based fluorescent cell counters can address issues raised by manual cell counting as well as flow cytometry. Bright-field imaging mode allows the system to measure viability of highly viable cell lines or purified primary cell samples using trypan blue. The combination of bright-field and fluorescence imaging modes allows analysis of cell lines or purified primary cell samples with high viability or low viability using a single fluorescent viability stain. Fluorescence imaging mode using dual-staining method allows the system to accurately measure viability of different primary cell samples without purification and processing, such as bone marrow samples, isolated tumor samples, bronchoalveolar lavage and whole blood [31]. In addition, ability to easily change fluorescence optics module that enables various fluorescent excitation (EX: 375–630 nm) and emission (EM: 450–695 nm) wavelengths, could be beneficial to researchers experimenting with a variety of viability stains.

In this work, we were able to compare a variety of fluorescent viability staining methods for cell line and primary cells using image-based cytometry. The tested fluorescent nucleic acid stains such as DAPI, Sytox Green, EB, PI, 7AAD, and Sytox Red, are membrane integrity dyes that can enter membrane compromised cells and bind to the deoxyribonucleic acid (DNA). Image-based cytometry was able to validate each nucleic acid staining method against the traditional trypan blue method by comparing premixed Jurkat samples at different viabilities. The maximum measured differences between the stains were approximately 1.1, 3.1, 8.0, 7.4, and 4.4 % for 0, 25, 50, 75, and 100 % viability (Fig. 6a), which showed that each staining method was as accurate as trypan blue exclusion, as expected.

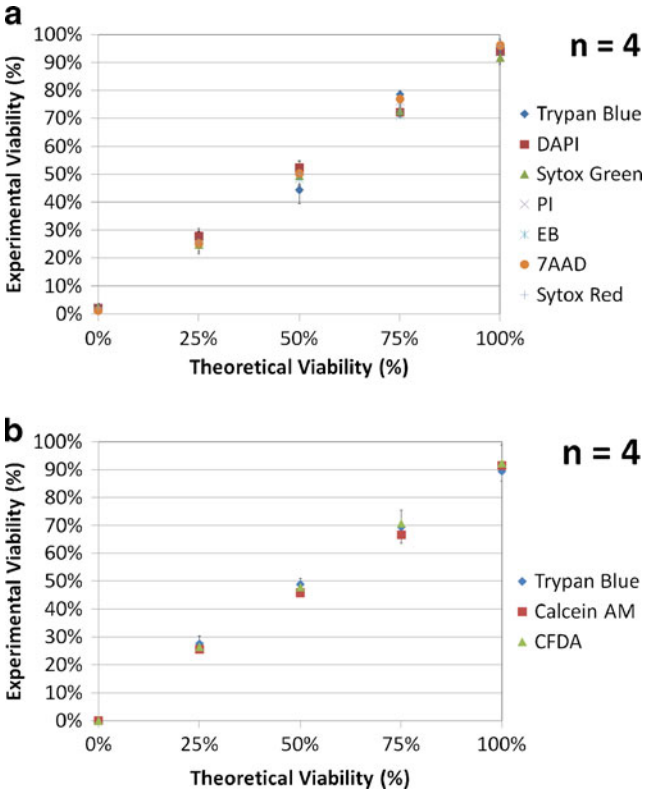

Fig. 6 Experimental viability measurements of Jurkat cells using (**a**) nucleic acid and (**b**) enzymatic stains compared to trypan blue. The viability results were comparable to a standard hemacytometer using trypan blue, which validated both nucleic acid and enzymatic staining method

The tested fluorescent enzymatic stains such as CFDA and Calcein AM, are membrane permeable fluorescin derivatives that can be cleaved by intracellular esterase enzyme to emit fluorescence and identify the viable cells. The method was again validated against the traditional trypan blue exclusion that showed maximum measured differences of 0.0, 2.1, 3.0, 2.7, and 2.9 % for 0, 25, 50, 75, and 100 % viability (Fig. 6b), which also confirmed the viability detection capability of enzymatic stains. The viability results obtained from enzymatic stains showed slightly lower average than the nucleic acid stains, which may be due to the fact that enzymatic stains are highly specific to metabolically active cells, thus "viable" cells that are not metabolically active may not get counted. It may also be due to the fact that bright-field total cell count could count debris particle appears similar to a cell. Therefore, in order to obtain a more accurate viability measurement under high debris content conditions, dual-staining method must be utilized.

The standard AO/PI dual-staining method employs an optical phenomenon called fluorescence resonance energy transfer (FRET), where AO can fluorescently label the nucleus of all cells and emit green fluorescence, and PI can only enter the membrane compromised cells and emit orange fluorescence [32, 33]. Since the AO and PI molecules are in close proximity when both are bound to the DNA in membrane compromised cells, the green fluorescent energy of AO is transferred to excite the PI molecules, thus eliminating the AO fluorescence of nonviable cells. In addition, the excitation and emission spectra of AO and PI have high percentage of overlap, which can enhance the efficiency of FRET [28, 34]. Hoechst behaves similar as AO, but induces less efficient FRET when used in conjunction with PI because their spectra are farther apart. On the other hand, CFDA and Calcein AM fluoresce only viable cells separately from PI, thus FRET does not occur.

In order to compare the dual-staining capability of nucleic acid and enzymatic stains, the method was tested on Jurkat cell line and primary mouse PBMCs and splenocytes. For 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 (Fig. 7a). Theoretically, the viability measured using CFDA/PI should be lower than AO/PI due to the possibility that not all the cells will be metabolically active, thus reducing the number of "viable" cells counted. However, it seems that Jurkat cells are highly susceptible to both AO/PI and CFDA/PI stains, generated comparable results. It was interesting to observe that the fluorescent images showed noticeable decrease in background

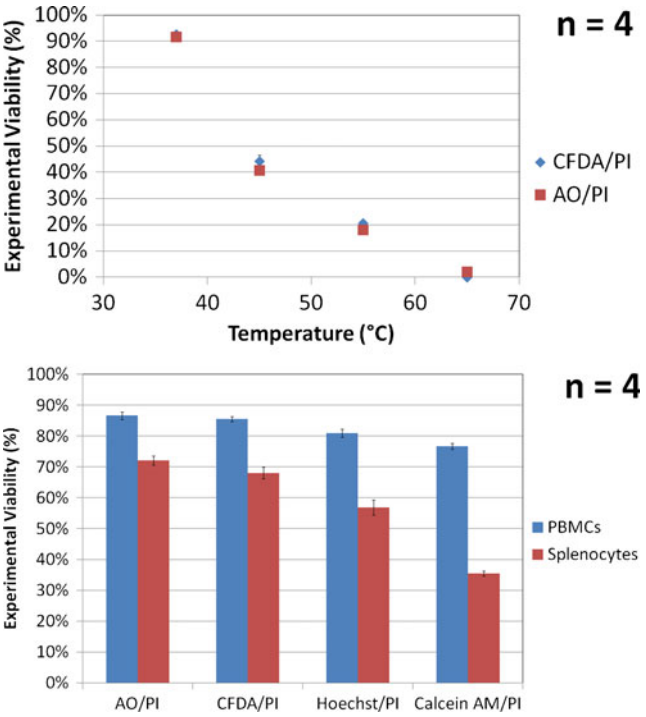

Fig. 7 Experimental viability measurements by dual-staining method with (**a**) Jurkat cell line and (**b**) primary PBMCs and splenocytes. AO/PI and CFDA/PI dual-stained Jurkat cells showed comparable viability results at each incubation temperature. AO/PI, CFDA/PI, Hoechst/PI and Calcein AM/PI dual-stained PBMCs and splenocytes showed a consistent reduction in viability, where the latter was more prominent

fluorescence of CFDA, which could be due to the specificity of CFDA in fluorescing only metabolic active cells.

In contrast, primary cells showed noticeable differences in viability measurements when comparing AO/PI, Hoechst/PI, CFDA/PI, and Calcein AM/PI (Fig. 7b). The measured viabilities showed a consistent 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, which may affect the staining ability of the tested dyes with different type of cells. Another possibility is that AO and CFDA may have higher nonspecific staining of debris, which could increase the viability percentages. It is important to note that viabilities of cell samples with high debris content or RBCs can be easily determined using the dual-staining method without further purification step, which can reduce assay time significantly. Of the 4 dual-staining methods, CFDA and Calcein AM induced a low amount of nonspecific fluorescence in the RBCs, which may require higher fluorescent threshold for more accurate counting. It can be stated from this experiment, the selection of fluorescent staining method requires the appropriate evaluation pertaining to cell types and sample conditions [35]. It is important to point out that in previous publications that as viability of primary cells decrease below an inflection percentage (dependent on cell types or sample conditions), the differences in measurement can increase significantly [28].

Among the different viability staining methods for image-based cytometry, the results showed that one should select the appropriate technique depending on the cell sample conditions. If the sample condition is clean, such as cell lines or purified primary cells, then the bright-field 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 (generally with high debris content), then dual-staining method can be employed. These two methods have the potential to be integrated into cell-based researches, which can improve the efficiency of the viability measurement step, and allow more time for researchers to perform higher complexity cell-based analysis. Besides the ability to determine viability using commercially available stains, image-based cytometry can also be used for rapid fluorescence-based cell death analysis such as apoptosis and autophagy using various fluorescent kits (Biolegend, Enzo Lifesciences, Invitrogen) [30, 36, 37]. These advance cell-based assays can further aid the analysis of the mechanism of cell survival, cell killing, and cytotoxicity, which can improve the understanding of researches in cancer, immunology, stem cell, and toxicology.

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Conflict of Interest The authors, LLC, BDP, and NL declare competing financial interests, and the work performed in this manuscript is for reporting on product performance of Nexcelom Bioscience, LLC. The performance of the instrumentation has been compared to standard approaches currently used in the biomedical research institutions.

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