

Trypan blue exclusion assay by flow cytometry

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Abstract

Dye exclusion tests are used to determine the number of live and dead cells. These assays are based on the principle that intact plasma membranes in live cells exclude specific dyes, whereas dead cells do not. Although widely used, the trypan blue (TB) exclusion assay has limitations. The dye can be incorporated by live cells after a short exposure time, and personal reliability, related to the expertise of the analyst, can affect the results. We propose an alternative assay for evaluating cell viability that combines the TB exclusion test and the high sensitivity of the flow cytometry technique. Previous studies have demonstrated the ability of TB to emit fluorescence when complexed with proteins. According to our results, TB/bovine serum albumin and TB/cytoplasmic protein complexes emit fluorescence at 660 nm, which is detectable by flow cytometry using a 650-nm low-pass band filter. TB at 0.002% (w/v) was defined as the optimum concentration for distinguishing unstained living cells from fluorescent dead cells, and fluorescence emission was stable for 30 min after cell treatment. Although previous studies have shown that TB promotes green fluorescence quenching, TB at 0.002% did not interfere with green fluorescence in human live T-cells stained with anti-CD3/fluorescein isothiocyanate (FITC) monoclonal antibody. We observed a high correlation between the percentage of propidium iodide + CD3/FITC⁺ and TB + CD3/FITC⁺ cells, as well as similar double-stained cell profiles in flow cytometry dot-plot graphs. Taken together, the results indicate that a TB exclusion assay by flow cytometry can be employed as an alternative tool for quick and reliable cell viability analysis.

Key words: Trypan blue; Fluorescence; Flow cytometry; Cytotoxicity

Introduction

Cell viability analysis is a useful tool in various experimental procedures, including those for tumor susceptibility, microbiological resistance, and spontaneous cell death after submission to different experimental conditions (1-4). It has been established that cell membrane integrity is a basic criterion for distinguishing dead from live cells (5). Thus, dyes capable of selectively penetrating the cytoplasm of dead cells have been widely used as vital dyes. The trypan blue (TB) method is a very common assay for evaluating cytotoxicity in experimental investigations (6-8) where dead cells absorb TB into the cytoplasm because of loss of membrane selectivity, whereas live cells remain unstained (6). Thus, the relative number of dead and live cells is obtained by optical microscopy by counting the

number of stained (dead) and unstained (live) cells using a Neubauer chamber. This conventional TB exclusion assay, when used for a large number of samples, can provide low-precision results because of the lengthy run time and intensive microscopic examination needed (9).

Previous studies have shown that TB-protein complexes emit fluorescence. As demonstrated by Harrison et al. (10), TB injected subcutaneously into Japanese quails was adsorbed on exogenous yolk, and the binding sites in oocytes were visualized by fluorescence microscopy. Furthermore, Kumar et al. (11) have suggested that TB is an appropriate dye for detection of arbuscular mycorrhiza spores by confocal fluorescence microscopy. This protocol used an excitation wavelength of 488 nm,

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with emission starting at 585 nm. Although TB has the ability to emit fluorescence when complexed with proteins, few studies have been conducted to improve the TB exclusion test by making the necessary adaptations for flow cytometry analysis. To date, propidium iodide (PI) staining followed by flow cytometry analysis is one of the most widely established assays for viability evaluation (12,13). Similar to TB, PI has the ability to penetrate into dead cells and to complex with DNA (14-16). We propose a TB exclusion test using flow cytometry as an alternative, cheap, and reliable technique to be used together with phenotype analysis, with no interference with cell viability and no quenching of green fluorescence, as has been previously reported (17).

Material and Methods

Peripheral blood mononuclear cells (PBMCs)

A venous blood sample (approximately 10 mL) from 7 healthy donors (22 ± 2.0 years of age) was collected in heparinized tubes 6 h before the experiment. Blood collection was performed between 7:30 and 8:30 am following these stringent blood-drawing criteria: no reported infection or symptoms of infection for 7 days prior to taking the sample, subject reported adequate sleep (6-9 h), no exercise or alcohol use for 24 h prior to withdrawal of the blood sample, no topical corticosteroid or aspirin use for the previous 48 h, no systemic antihistamines or corticosteroid use for 1 week prior to obtaining the sample, and no immunizations during the previous 3 weeks. All subjects included in this study gave their informed consent, and the local Ethics Committee approved the study (#CEP/UFVJM-146/10).

The blood and all the reagents used for PBMC isolation were kept at room temperature throughout the purification. PBMCs were isolated by centrifugation using Histopaque[®] 1077 (Sigma-Aldrich, USA), as described by Gazzinelli et al. (18) and adapted as follows. Briefly, the blood was gently added over Histopaque and centrifuged at 520 *g*. PBMCs were collected from the interphase after Ficoll separation and washed three times with PBS (0.015 M, pH 7.4). The cell suspensions were then adjusted to 1.0×10^7 cells/mL.

Determination of the fluorescence emitted by the TB-protein complex

Evaluation of the fluorescence emitted by the TB-protein complex was performed using a Shimadzu RF-5301PC spectrofluorophotometer (USA). Briefly, control solutions containing 0.02% TB (Sigma-Aldrich) or 10% bovine serum albumin (BSA; INLAB, Brazil), as well as the test solution (0.02% TB and 10% BSA) were prepared using distilled and deionized water, followed by spectrofluorophotometric analysis. We performed emission and excitation scans using the spectrum measurement mode setting to obtain both excitation and emission spectra. The

excitation slit was adjusted to 5 and the emission slit to 20; the spectral analysis was accomplished between 220 and 770 nm. Each analysis was performed in triplicate and collected using the RF-5301PC software. The spectral curve was generated and analyzed by the GraphPad Prism 5.0 software (USA). In a complementary experiment, BSA solutions with concentrations ranging from 1.8 to 10.0 mg/mL were added to 0.02% TB (w/v) solutions and analyzed by spectrofluorophotometry using fixed excitation and emission wavelengths of 488 and 660 nm, respectively. To confirm the fluorescence emitted by the interaction between TB and cytoplasmic proteins, PBMCs were submitted to simultaneous staining with 4',6-diamidino-2-phenylindole (DAPI) and 0.002% TB (w/v) and analyzed by fluorescence microscopy (AxioImager.M2, Germany).

TB exclusion test by counting in a Neubauer chamber

PBMCs (5.0×10^5 cells) were preincubated in a humidified air environment containing 5% CO₂ at 37°C or in a water bath at 50°C for 30 min. After preincubation, 1.0×10^5 PBMCs were placed in 5-mL round-bottom tubes and incubated with 190 μ L 0.4% TB solution diluted in PBS to perform the TB exclusion test with counting in a Neubauer chamber.

TB exclusion test using flow cytometry assay

To evaluate the fluorescence emitted by dead cells stained with TB, 10 μ L PBMC suspension (1.0×10^5 cells) was submitted to heat (50°C) pretreatment for 30 min, followed by staining with 190 μ L TB solutions at 0.002, 0.004, 0.04, 0.08, and 0.4% (w/v) in PBS. The cell suspensions were incubated in an air-humidified environment containing 5% CO₂ at 37°C for 5, 10, 20, and 30 min. The dead cell suspensions were treated with TB and were evaluated immediately after the addition of dye to be used as the zero time control. The same protocol was performed without the heat pretreatment step to evaluate the fluorescence emitted by live PBMCs. Cells stained with PI were evaluated by flow cytometry for comparison in all the experiments.

To evaluate whether TB promotes quenching of the green fluorescence as previously reported (17), 100 μ L heparinized blood samples was incubated with 2 μ L undiluted FITC-conjugated monoclonal anti-human CD3 antibody (Cat. No. 11-0038-73, Clone UCHT1, eBioscience, USA) in the dark for 30 min at room temperature. Following the incubation, erythrocytes were lysed with 100 μ L lysing solution (Optlyse-B, Immunotech, USA) for 5 min, followed by the addition of distilled water for 10 min. The cells were washed twice with 1 mL PBS containing 0.01% sodium azide and were then distributed into four new tubes. The cells were incubated with 190 μ L of either a 0.002 or 0.4% (w/v) TB solution in two of the tubes, or with 100 μ L PBS and 1 μ L of a 50 mg/mL PI stock solution (Cat. No. 51-66211E, BD Pharmingen, USA) in the third tube. The leukocytes were added to 190 μ L PBS in the fourth tube. After 15 min,

15,000 cells were removed from each tube and analyzed by flow cytometry.

Flow cytometry

The FACScan [Becton & Dickinson (BD), USA] flow cytometer used in this study was equipped with a blue argon laser (488 nm) and the following filters: 530/30 nm (FL1=green fluorescence) and 586/42 (FL2=orange fluorescence) band-pass filters, as well as the 650/LP (FL3=red fluorescence) long-pass filter. The size and granularity parameters were evaluated with the 488-nm detectors (blue) based on forward and side-scattered light, respectively. For both PI- and TB-treated cells, 15,000 events were acquired into the lymphocyte gate based on size vs granularity (FSC × SSC) dot plots. The fluorescence was evaluated in FL3 histograms or FL1 × FL3 dot plots. Unstained and stained cells were reported as a percentage of live and dead cells, respectively. Calibrite™ beads (BD, Cat. No. 340486) were used for the three-color flow cytometer compensation setup. The instrument settings for compensation were FL1: 1.3% FL2; FL2: 20.0% FL1; FL2: 0.0% FL3; and FL3: 12.7% FL2.

Statistical analysis

GraphPad Prism (version 5.00 for Windows; GraphPad Software, USA, www.graphpad.com) was used for statistical analysis. Data are reported as means ± SD. The Shapiro-Wilk test was used to assess the normality of the data. The two-way repeated-measures ANOVA was used to compare the viability assays, followed by the Tukey *post hoc* test when a significant *F* value was observed. Correlations between the viability assays were assessed by the Pearson correlation test. A significance level of $P \leq 0.05$ was used.

Results

TB-protein complex emits fluorescence

Previous studies have shown that TB emits fluorescence

when complexed to proteins (10). We analyzed the excitation and emission spectra using a TB solution at 0.02% or PBS containing 10% BSA, as well as a solution containing the TB-BSA complex to determine the optimum wavelengths for excitation and emission to be used in flow cytometry assays. The TB-BSA excitation spectrum presented strong maxima at 296, 485, and 648 nm (Figure 1A). Maximum emission was observed at 483 and 660 nm (Figure 1B), the latter being detected by the 650 nm/LP (FL3) long-pass filter of the FACScan cytometer. As demonstrated, there were no peaks in the emission spectrum curves corresponding to TB alone and not in the form of a complex with proteins. On the other hand, BSA solution presented an emission maximum at 463 nm in a region of the spectrum not detectable by the FL3 long-pass filter of the FACScan cytometer. Since the FACScan uses a laser light source at 488 nm, we evaluated the fluorescence emitted by the TB-BSA complex, fixing the excitation and emission wavelengths at 488 and 660 nm, respectively. By maintaining the TB concentration at 0.02% and by adding BSA solutions ranging from 1.8 to 10 mg/mL, the fluorescence emitted by the TB-BSA complex was observed to be dose-dependent (Figure 2A). Because the TB-BSA complex emits fluorescence, we evaluated whether TB inside cells in the form of complexes with cytoplasmic proteins could present a similar behavior. PBMCs were treated with TB and DAPI, simultaneously. According to the results, the cells containing TB-cytoplasmic protein complexes emitted fluorescence detectable by fluorescence microscopy at the excitation wavelength of 488 nm and the emission filter of 650 nm (Figure 2B).

Standardization of the TB exclusion assay using flow cytometry

Because TB can form complexes with proteins located in the cytoplasm and in the plasma membrane, different TB concentrations, as well as incubation times, were used to determine the optimum experimental condition for

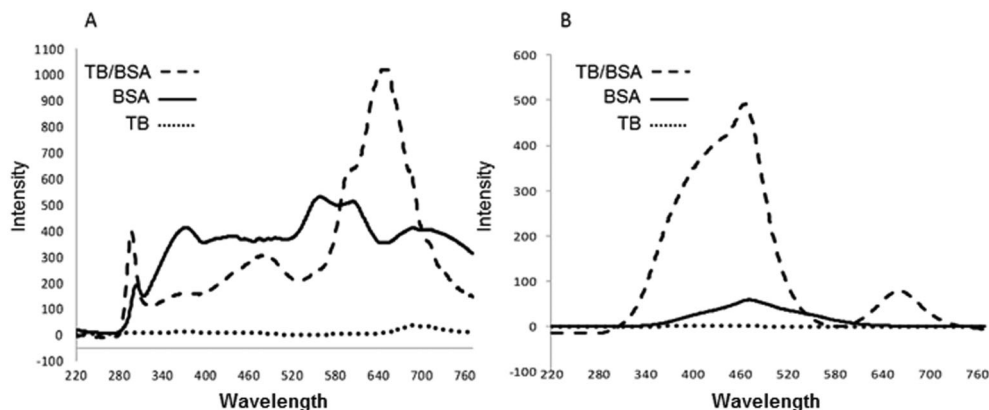


Figure 1. Spectrofluorophotometry analysis of the excitation (A) and emission (B) spectra of the solutions containing 0.02% (w/v) trypan blue (TB, dotted line), bovine serum albumin (BSA, solid line) at 10% (w/v), and TB-BSA (dashed line) interaction.

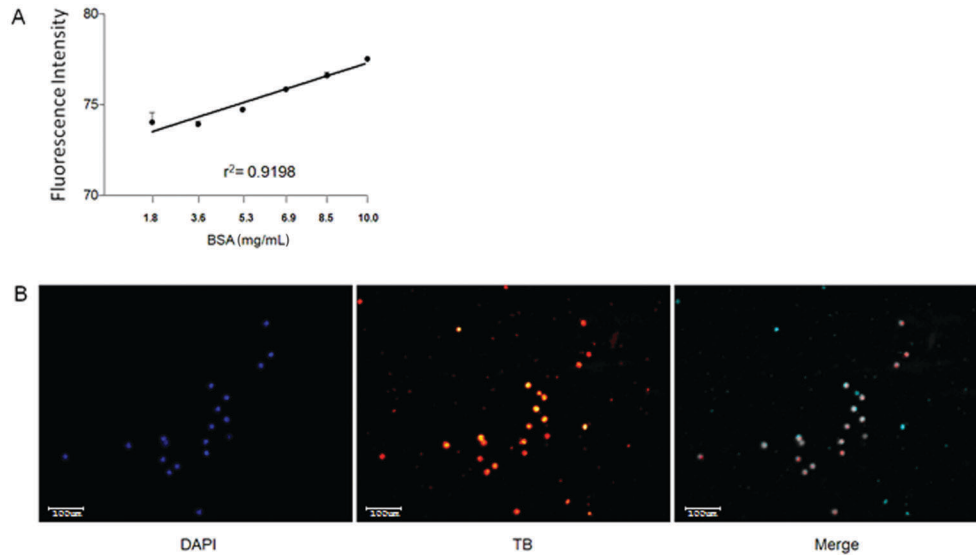


Figure 2. Fluorescence analysis of the trypan blue (TB)-bovine serum albumin (BSA) interaction. *A*, Increasing BSA concentrations were added to 0.002% TB solution. The fluorimeter was configured to use fixed excitation and emission wavelengths at 488 and 660 nm, respectively. *B*, Human PBMC co-stained with DAPI and TB. DAPI excitation at 365 nm and the emission band-pass filter DAPI-445/50, TB excitation at 488 nm and fluorescence emission detected by LP/515.

distinguishing live cells, whose TB-protein interactions exist only on the cell surface, and dead cells, whose TB-protein complex exists both in the cytoplasm and on the cell surface. A previous study (9) showed that live cells in contact with 0.4% TB for more than 5 min may incorporate the dye, leading to overestimation of the number of dead cells. We therefore evaluated whether the optimum TB concentration for use with flow cytometry had a similar imprecision. As shown in Figure 3, 0.002% (w/v) TB distinguished live and dead cells with the optimal fluorescence emission at 660 nm (FL3), and was able to distinguish dead (stained) cells, keeping live (unstained) cells in a negative region usually located between 10^0 and 10^1 on FL3-based histograms. Additionally, 0.002% (w/v) TB did not change the fluorescence intensity emitted by live cells that were incubated for 30 min after addition of the dye (Figure 4). All the results were comparable to those of the PI assay.

Comparison between the TB exclusion test by flow cytometry, PI staining, and the conventional TB exclusion test by cell counting in the Neubauer chamber

A comparison between the TB exclusion assay by flow cytometry [0.002% (w/v) TB], the PI exclusion test by flow cytometry, and the percentage of cells stained with 0.4% TB counted in a Neubauer chamber was performed to verify whether the results obtained with the standardized cytometry-based TB assay were similar to those obtained by conventional methods. The cell counting in a Neubauer chamber was performed no later than 3 min after cell staining. The three methods presented no differences in the

percentage of dead or live cells in both protocols (with or without heat pretreatment; Figure 5A). There was a very high correlation between the TB exclusion assay by flow cytometry, the PI ($r^2=0.9758$) assay, and the Neubauer chamber ($r^2=0.8986$) assay (Figure 5B). Interestingly, the assays using PI and TB analyzed on the long-pass filter at FL3=650 nm/LP had very similar profiles in the FL3-fluorescence histogram analysis (Figure 5C), as well as the dot-plot graphs (data not shown). Furthermore, according to data shown in Figure 1B, fluorescence emitted by TB was detected in the FL3 channel (650 nm/LP) on the FACScan cytometer and, contrary to PI, TB-protein fluorescence was not detected in the FL2 channel (Figure 5D).

TB at 0.002% (w/v) does not promote quenching in FL1

TB at 0.2 and 0.4% promoted a decrease in FL1 fluorescence (quenching) under acidic conditions. To determine whether cell treatment with 0.002% TB at pH 7.2 promoted FL1 quenching, we stained 100 μ L peripheral blood with specific monoclonal antibody anti-human CD3-FITC followed by TB or PI staining. The results showed that 0.002% (w/v) TB, at physiological pH, did not interfere with FL1-530/30 nm fluorescence (Figure 6) and produced results similar to those obtained by the cell cultures treated with PI or PBS (control). After cell death was induced using heat (50°C) pretreatment, the flow cytometry exclusion assays using TB or PI presented a similar percentage of live and dead cells, confirmed by the high correlation between these methods ($r^2=0.9805$), as well as very similar dot-plot profiles in flow cytometry analysis (Figure 7).

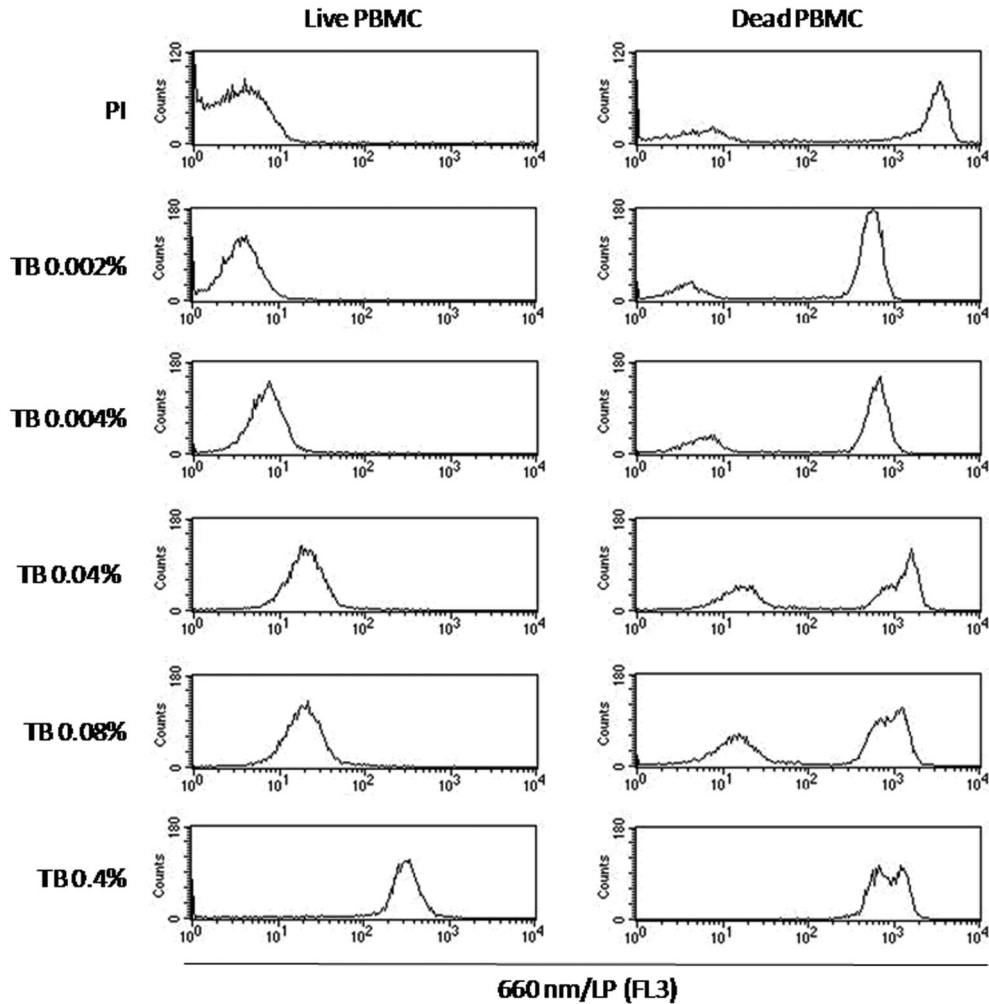


Figure 3. Evaluation of the optimum concentration for trypan blue (TB) solution ranging from 0.002 to 0.4% to discriminate between live and dead peripheral blood mononuclear cells (PBMC). The analysis was performed on a FACScan cytometer (Becton & Dickinson, USA) using the long-pass filter 650/LP (FL3 detector). Staining with propidium iodide (PI) was used as a reference.

Discussion

The number of live and dead cells in an experiment can be estimated by the use of several markers including dyes that intercalate into DNA (e.g., ethidium monoazide, PI, 7-aminoactinomycin D, DAPI), reagents that bind to phosphatidylserine (Annexin V), dyes that stain cells that lose membrane selectivity, and amine reactive dyes (UViD, ViViD, GrViD, OrViD: UV, violet, green, and orange fluorescence, respectively). Among the tests using dyes that penetrate into cells that lose membrane selectivity or that intercalate with DNA, the TB exclusion assay, using optical microscopy, and the labeling of cells with PI, followed by flow cytometry analysis, are widely used by the scientific community (14,19).

Studies have demonstrated the fluorescence emitted by TB-protein complexes (10,11,20). Davis and Sauter

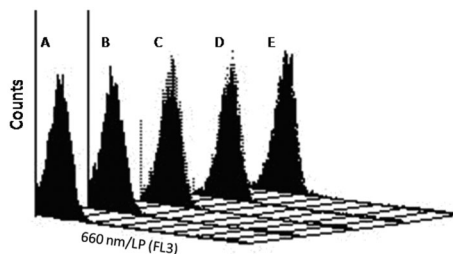


Figure 4. Histogram analysis of the trypan blue (TB) exclusion assay by flow cytometry to evaluate TB (at 0.002%, w/v) incorporation by live cells 0 (A), 5 (B), 10 (C), 20 (D), and 30 (E) min after dye addition.

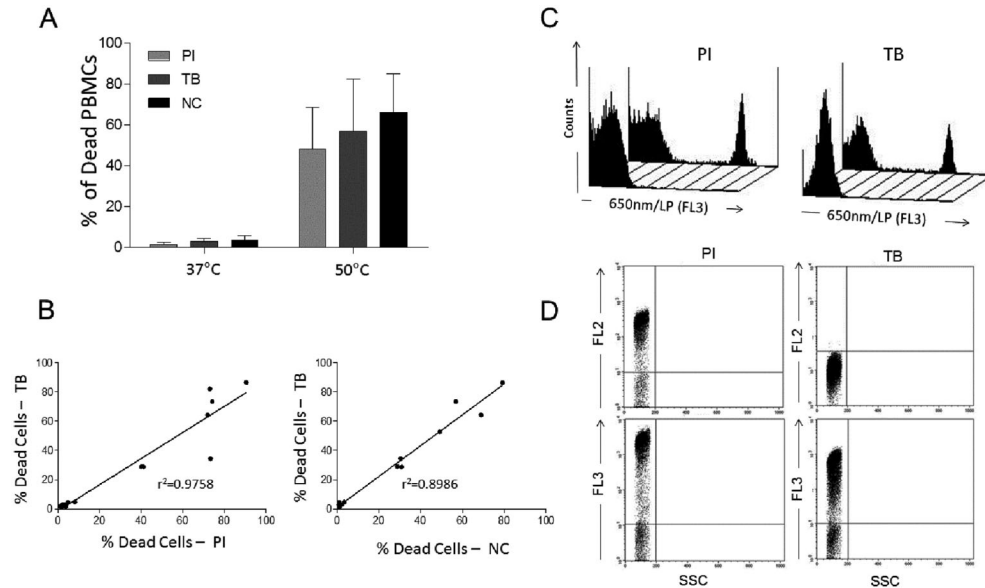


Figure 5. A, Comparison of the trypan blue (TB) exclusion test using flow cytometry, propidium iodide (PI) staining and the conventional trypan blue exclusion test employing cell counting in a Neubauer chamber (NC). B, Pearson's correlation test between PI and TB or NC and TB. C, Fluorescence intensity histogram profiles of the PI and TB flow cytometry analysis. D, Fluorescence emitted by cells stained with PI and TB dyes and analyzed on FL2 (585/42) and FL3 (650 nm/LP) detectors.

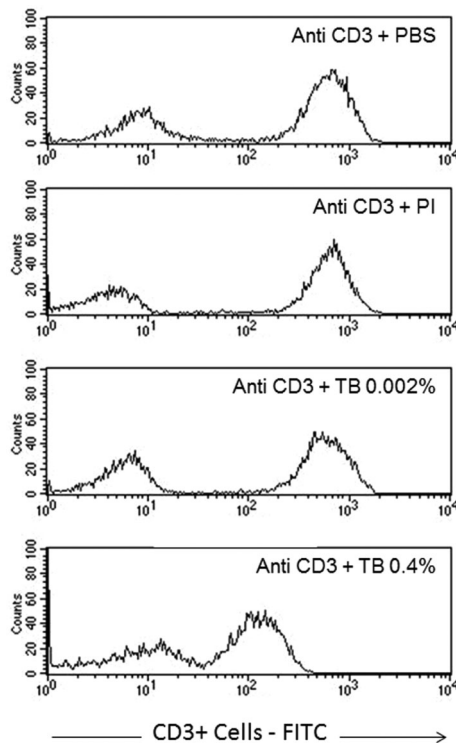


Figure 6. Profile of T-lymphocytes stained with monoclonal antibody anti-CD3-FITC followed by treatment with propidium iodide (PI) and trypan blue (TB) at 0.002 and 0.4% (w/v) or PBS (untreated control).

(21) developed a method for detection and characterization of intraembryonic dyes. Using this method, the fluorescence produced by protein-bound TB can be distinguished from non-protein-bound dye. Busetto et al. (22) reported a TB fluorescence-based assay to evaluate the phagocytic action of neutrophils *in vitro*. In this assay, fixed *Candida albicans* stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) were added to neutrophil cultures together with TB labeling. Since TB did not penetrate live cells, ingested yeasts retained their green fluorescence, while membrane-bound *C. albicans* displayed a double-positive red and green fluorescence. Because these and other previous studies showed the ability of the TB-protein complex to emit fluorescence, we questioned whether the cells assayed by TB exclusion might also be evaluated by flow cytometry, an issue that had not yet been reported. The main objective of this study was to present an adaptation of a technique already known and widely used in scientific experimentation to be employed as an additional and alternative tool for providing reliable results.

We demonstrated that the TB-BSA interaction emitted fluorescence at 490 and 660 nm. Moreover, human PBMC fluorescence was observed by the overlapping of blue and red fluorescence after simultaneous labeling with DAPI and TB (Figure 2B). This result, along with flow cytometry analysis for cell size (FSC) and granularity (SSC), demonstrated that the red fluorescence evaluated by flow cytometry was not produced by possible technical artifacts. The fluorescence emitted by interactions between TB and

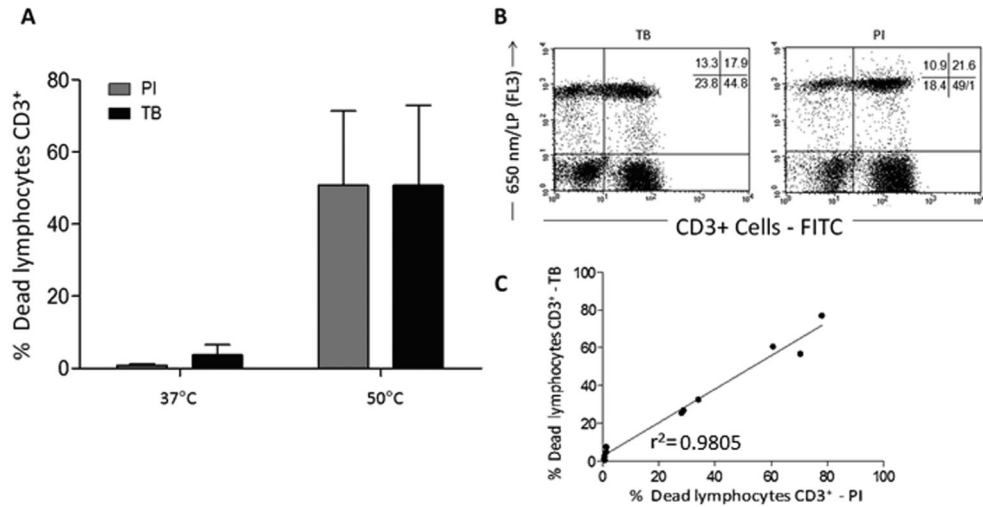


Figure 7. A, Percentage of dead human T-lymphocytes (CD3-FITC⁺ cells) submitted to cell culture at temperatures (T) of 37°C (physiologic temperature) or 50°C (high-stress temperature) followed by staining with trypan blue (TB) or propidium iodide (PI). B, Dot-plot graph profile between human lymphocytes submitted to pretreatment with high-stress temperature (50°C) followed by staining with TB and PI and monoclonal antibody anti-CD3-FITC⁺. C, Pearson's correlation test between dead CD3⁺ lymphocytes using PI and TB flow cytometry assays.

cytoplasmic proteins may, however, be influenced by the diversity and quantity of proteins within specific cell populations. Certain cells, such as monocytes and neutrophils, possess high proteogenic activity to support the effector functions in the phagocytosis process. Large numbers of protein granules inside the cells may lead to the phenomenon known as autofluorescence, a common effect observed in phenotypic analysis by flow cytometry (23). Therefore, it is recommended that, for each cell type, an optimum TB concentration adjustment is required.

Concerning the affinity of TB for proteins, the presence of red fluorescent cells after treatment with TB dye could indicate the presence of the TB-protein complex both inside and outside the cells. Flow cytometry is a highly sensitive technique. Therefore, it was necessary to determine the optimum concentration of TB necessary to distinguish live cells, which have a basal level of fluorescence due to the TB-protein complex on the cell surface from that of dead cells, where the TB-protein complex occurs both in the cytoplasm and outside the cell membrane. Thus, we treated a suspension of live and dead cells using different concentrations of TB to confine the population of live cells, which do not incorporate the dye, into a log-based scale region between 10⁰ and 10¹ (negative red-fluorescent region) as well as to distinguish dead intracellularly stained cells in FL3-based histograms. Heat pretreatment to induce cell death was chosen to avoid possible chemical interference of the cytotoxic agent with the formation of the TB-protein complex. According to our data, 0.002% TB promoted results similar to those of PI staining and was defined as the optimal concentration for further analysis (Figure 3). It is important to consider that, in flow cytometry,

it is common to use washing solutions supplemented with BSA or other Fc blockers, such as normal serum, to saturate Fc receptors on the cell surface. Because TB interacts nonspecifically with proteins, the formation of TB/Fc blocker complexes in the extracellular medium is possible. In the assay proposed herein, 0.002% TB is sufficient to form complexes with soluble proteins, as well as proteins located on the membrane and in the cytoplasm of live and dead cells, respectively. In the data obtained by flow cytometry, all those TB-protein complexes were considered, and the total fluorescence represents the sum of the TB-protein complexes both inside and outside the cells. TB-protein complexes not associated with cells are discarded in the cell population selection step (gated cells). Therefore, the presence of Fc blockers such as BSA in the medium does not interfere with the analysis of live and dead cells. Moreover, our data showed that the use of the washing solution (PBS) containing 0.5% BSA in the TB exclusion assay by flow cytometry did not cause a change in the cell viability analysis when compared to the protocol where BSA-free PBS was used (data not shown). In addition, the cell surface staining using anti-CD3-FITC monoclonal antibodies was also not affected, showing that 0.002% TB did not interfere in the quenching pattern presented in this study, even in the presence of Fc blockers. However, in experiments where cell cultures are treated with specific cytotoxic agents, it is recommended that a preliminary experiment be conducted to evaluate the possible chemical interference of the cytotoxic agent with TB-protein complex formation, as well as to evaluate the ability of the compound to emit fluorescence, such as doxorubicin, a widely used drug in cytotoxic assays that

emits FL3-detectable fluorescence by itself (24).

Previous studies have shown that live cells, when treated with 0.4% (w/v) TB, incorporate the dye after 5 min of TB treatment (25,26). We observed that live human leukocytes treated with 0.002% TB for 30 min after staining presented no differences in the fluorescence profile (Figure 4). This stable interaction between TB and cytoplasmic proteins could be useful for experiments that require the analysis of a large number of samples. The TB exclusion test by flow cytometry presented a strong positive correlation with the PI assay and the counting of stained cells in a Neubauer chamber by optical microscopy and confirmed the reliability of the proposed test (Figure 5B). The FL3 histograms obtained from PI and TB fluorescence analysis also presented a very similar pattern (Figure 5C). Considering that the FL2 detector is not sensitive to the fluorescence emitted by the TB-BSA complex, the TB exclusion assay by flow cytometry can be performed together with phycoerythrin-conjugated antibody staining. This issue represents a technical advantage over the use of PI, since this DNA marker is read simultaneously by FL2 and FL3 detectors (Figure 5D).

According to Van Amersfoort and Van Strijp (27), the use of TB at concentrations higher than 0.1% under acidic conditions can cause quenching of green fluorescence. The quenching effect promoted by TB on green fluorescence is an undesired event in cell phenotype analysis because this effect could be a limitation to double-staining with FITC-conjugated monoclonal antibodies. Thus, another option was to determine the possibility of simultaneous evaluation of cell viability by TB and cell surface staining using FITC by flow cytometry. PBS was used with the pH close to 7.2, and the levels of fluorescence emitted by CD3-FITC⁺ cells were maintained even at high TB

concentrations (0.4%). The FL1 fluorescence peak of the cells treated with anti-CD3-FITC and PBS was identical to the fluorescence peak of the cells treated with 0.002% TB. In comparison with PI and TB assays after FITC staining, a high correlation was observed, as well as a very similar profile in the dot plot obtained by the two techniques (Figure 7C).

In conclusion, the results are quite consistent and comparable to those obtained by the PI staining method. The large number of cells evaluated (30,000 cells), as well as the short time required by the cytometry assay, provide reliable results regarding the real amount of dead and live cells on the samples. Furthermore, the TB exclusion assay by flow cytometry means that simultaneous cell staining with monoclonal antibodies conjugated with fluorochromes detectable on the FL2 (585/42 nm) or FL1 (530/30 nm) channel is possible. This possibility represents an advantage over the PI assay. Flow cytometry is an expensive technique, but it has been widely employed. Thus, for the laboratories that make use of such equipment in their investigations, the TB exclusion assay by flow cytometry can be a reliable tool for viability analysis and has advantages over traditionally used methods.

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