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Application of a non-hazardous vital dye for cell counting with automated cell counters

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ABSTRACT

Recent advances in automated cell counters enable us to count cells more easily with consistency. However, the wide use of the traditional vital dye trypan blue (TB) raises environmental and health concerns due to its potential teratogenic effects. To avoid this chemical hazard, it is of importance to introduce an alternative non-hazardous vital dye that is compatible with automated cell counters. Erythrosin B (EB) is a vital dye that is impermeable to biological membranes and is used as a food additive. Similarly to TB, EB stains only nonviable cells with disintegrated membranes. However, EB is less popular than TB and is seldom used with automated cell counters. We found that cell counting accuracy with EB was comparable to that with TB. EB was found to be an effective dye for accurate counting of cells with different viabilities across three different automated cell counters. In contrast to TB, EB was less toxic to cultured HL-60 cells during the cell counting process. These results indicate that replacing TB with EB for use with automated cell counters will significantly reduce the hazardous risk while producing comparable results.

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Viable cell counting of eukaryotic cells is the first step in maintaining and expanding in vitro cell cultures in modern biomedical research and the biomanufacturing industry. Cell counting, or cell enumeration, is important for the routine monitoring of cell health and proliferation rate, and for scheduling and seeding cells for use in subsequent experiments, including transfection or infection and various cell-based assays [1,2]. The most common traditional way of determining cell viability is to use a hemocytometer to manually count cells stained with a vital dye under microscopic observation [1–4]. Although manual counting has benefits such as low cost and versatility [1], its procedure is time-consuming and labor-intensive. Potential disadvantages include contamination of the reusable hemocytometer, variations of hemocytometer filling rates, and inter-user variations as well [1,2,5]. Although one survey demonstrated that more than 70% of researchers still use a hemocytometer to count their cells [1], the recent automation of cell counting instruments has provided more consistent results with easy-to-use instrumentation [1–3].

The vital dye exclusion assay is used to determine the fraction of viable cells in suspension [4]. Because viable cells have intact cell membranes that prevent penetration of polar molecules, staining of cells with vital dyes differentiates nonviable cells with disintegrated cell membranes from viable intact cells [4]. Because vital staining indirectly determines cells' viability from cell membrane integrity, the results are limited; a cell might not grow or proliferate even though its membrane integrity is maintained, a cell may repair the membrane integrity and become fully viable, and small amounts of dye uptake may be unnoticed [4]. However, the simple and rapid vital dye exclusion assay is widely used.

Various vital dyes, including trypan blue (TB), methylene blue, erythrosin B (EB), nigrosine, eosin, safranin, propidium iodide, and 7-aminoactinomycin D, have been introduced to count viable cells [2,4,6,7]. Among these, TB is widely used for viable cell counting with bright-field optics. In addition, most of automated cell counters without fluorescence optics are optimized for viable cell counting based on TB dye exclusion [1–3]. Although TB has been widely used as a vital dye, a potential teratogenic effect of TB has been reported [8–12].

EB, also known as erythrosine or Red No. 3, is primarily used for food coloring [13]. Although EB has already been introduced as a vital dye [14,15], it is not widely used to count viable cells manually or with automated instruments. Because biosafety is a growing

Abbreviations: TB, trypan blue; EB, erythrosin B; FBS, fetal bovine serum; DPBS, Dulbecco's phosphate-buffered saline.

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concern, the use of EB with automated instruments is an option to lessen consumption of TB. Here we report, for the first time, the use of EB for vital staining of three cell lines for automatic counting and demonstrate that the use of safer alternative EB avoids the toxic side effect of trypan blue exposure on mammalian cells.

Materials and methods

Automated cell counters

All of the automated cell counters used in this study were manufactured by Logos Biosystems. The LUNA family of cell counters produces data about cell size, concentration, and viability. The LUNA™ is optimized for bright-field imaging with double-folded optics. The LUNA-FL™ is a dual fluorescence cell counter with bright-field optics included [16,17]. The LUNA-II™ is optimized for bright-field imaging and had an integrated liquid lens [18] that supports rapid autofocusing. All cell count and viability measurements in this study were performed on the aforementioned automated cell counters.

Cell culture and reagents

HL-60, a human promyelocytic leukemia cell line, was maintained in RPMI-1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies). A human cervical adenocarcinoma cell line, HeLa, and a human embryonic kidney cell line, 293, were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% FBS and 100 U/ml penicillin/streptomycin (Life Technologies). TB (Sigma, St. Louis, MO, USA) and EB (MP Biomedicals, Solon, OH, USA) were dissolved in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies) and sterilized by filtration.

Measurement of cell viability

To prepare dead cells, an appropriate number of cells were incubated at 70 °C for 30 min. Live cells were prepared from exponentially growing cells. A series of cells with different viabilities was prepared by mixing the dead and live cells. The dead and live cell suspensions were stained with 0.4% TB, and the viabilities of each were measured with the LUNA. A series of cell suspensions with different viabilities was prepared by mixing the dead and live cells. The measured viabilities of these cells were determined by either 0.4% TB or different concentrations of EB. For optimal cell counting, the instruments were recalibrated to the appropriate concentration of vital dye prior to each count.

Toxicity assay

HL-60 cells were washed twice with DPBS and resuspended in DPBS or RPMI-1640 without FBS. The initial viability of the HL-60 cell suspension was determined with the LUNA-II by either 0.4% TB or 0.2% EB. To compare the toxicity of TB and EB in DPBS or

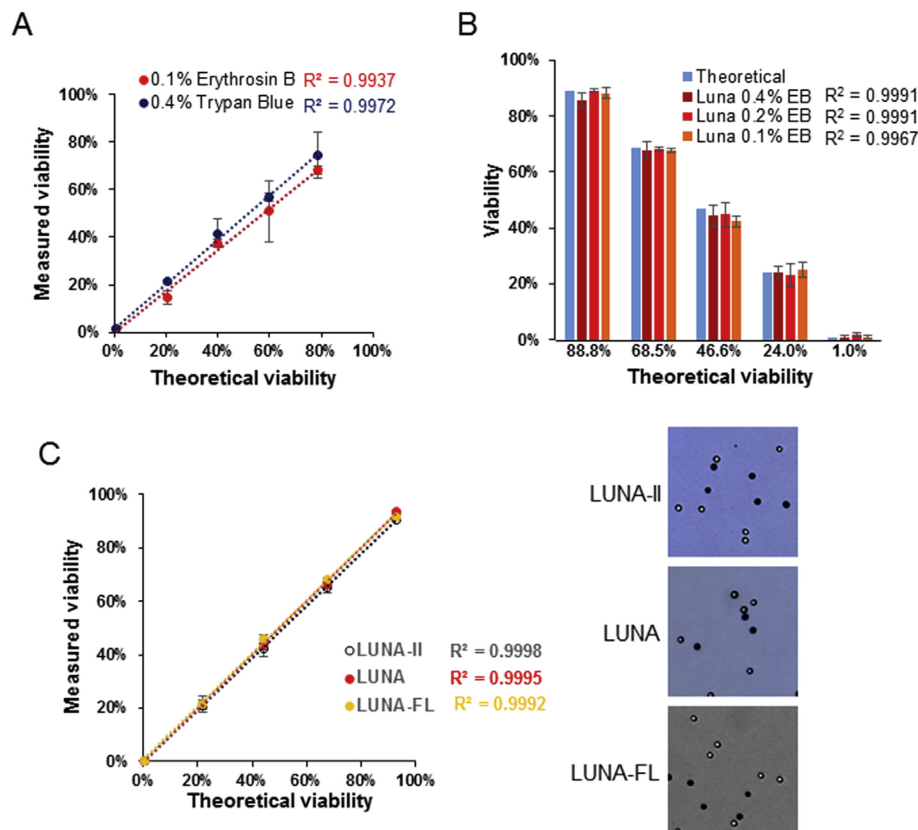

Fig.1. Application of EB for cell counting with automated cell counters. (A) Comparison of TB and EB. Cells with differential viabilities were counted with the LUNA after staining with either 0.4% TB or 0.1% EB. The measured viabilities were plotted against theoretical viabilities. Statistical analysis revealed that there is no significant difference between TB and EB. (B) Comparison of different concentrations of EB. Cells with differential viabilities were counted with the LUNA after staining with 0.1, 0.2, or 0.4% EB. No significant difference was observed. (C) Cell counting with EB with three different automated cell counters. Cells were counted with the LUNA-II, LUNA, and LUNA-FL after staining with 0.2% EB. The measured viabilities were plotted against theoretical viabilities. No significant statistical difference across the instruments was observed. Representative images are shown at the right. (A–C) Representative data from three independent experiments performed in triplicate are shown as means ± standard deviations.

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