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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.

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

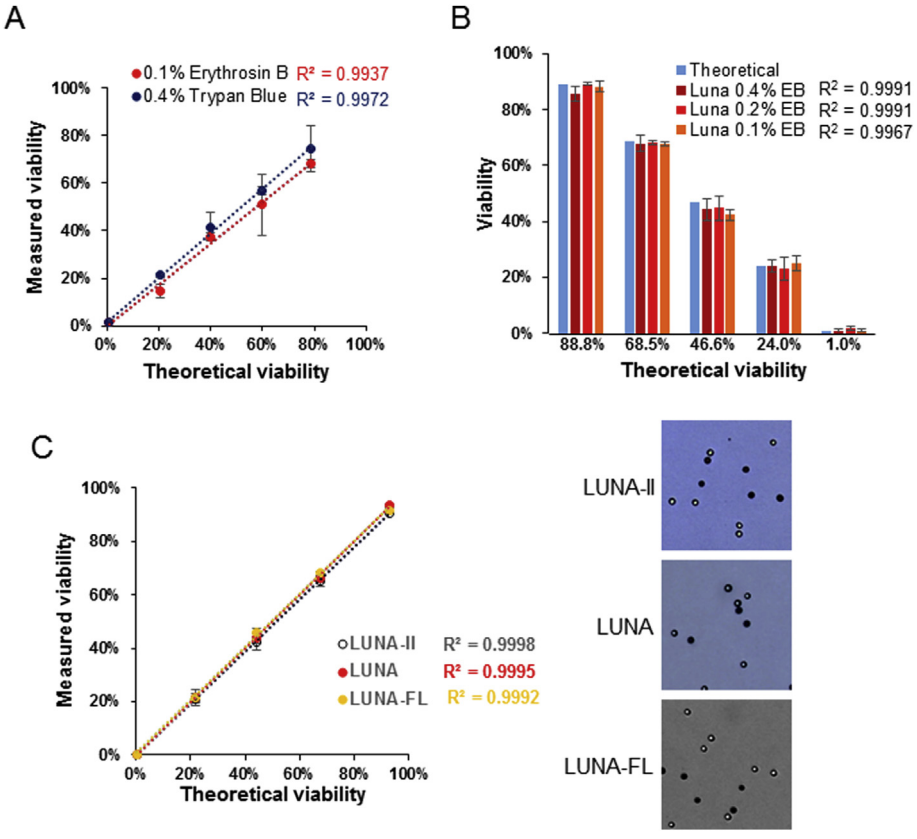

Fig.1. Application of EB for cell counting with automated cell counters. (A) Comparison of TB and EB. Cells with differential viabilities were counted with the LUNA after staining with either 0.4% TB or 0.1% EB. The measured viabilities were plotted against theoretical viabilities. Statistical analysis 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.

serum-free medium, HL-60 cells was mixed with an equal volume of 0.4% TB or 0.2% EB and incubated at room temperature. The cell number and viability were further determined over a period of time after vigorous vortexing to ensure the homogeneous suspension of cells. For live cell imaging, cells were resuspended in RPMI-1640 containing 20% glycerol to prevent rapid evaporation of liquid during live cell imaging. After mixing the cell suspension with an equal volume of 0.4% TB, live cell images were captured with the iRiS Digital Cell Imaging System (Logos Biosystems) equipped with a TC PlanAchro 4× Ph objective lens.

Statistical analysis

For simple comparisons, a two-tailed Student's *t*-test was applied for statistical analysis. For multiple comparisons, one-way analysis of variance (ANOVA) was performed.

Results and discussion

We first examined whether EB staining can be applied to cell counting with automated cell counters. As a starting point, we used 0.1% EB dissolved in DPBS [19]. The accuracy of cell counting was determined with the LUNA automated cell counter by counting HL-60 cells with various viabilities (the theoretical viabilities are ~ 0, 20, 40, 60, and 80%) after staining with EB. As a control, 0.4% TB was also used to measure the viabilities. As shown in Fig. 1A, EB and TB staining provided comparable results. To further verify linearity, different concentrations of EB were further tested. Increased concentrations of EB (0.1, 0.2, and 0.4%) produced results with a high correlation ($R^2 > 0.999$) to the theoretical viabilities (Fig. 1B). To extend the application of EB, we further tested 0.2% EB with different automated cell counters. HL-60 cells with different viabilities were stained with EB and counted with three different automated cell counters. As shown in Fig. 1C, there was no significant difference in the results from cell counters tested. EB staining successfully differentiated live cells from dead cells; only dead cells were stained with EB (Fig. 1C, right). Vital staining by EB was also well detected by the LUNA-II autofocusing liquid lens. All of these data indicate that EB is a vital dye as effective as TB for cell counting with automated cell counters.

The dynamic working range of EB was further tested by counting HL-60 with different concentrations. HL-60 cells were serially diluted with concentrations ranging from approximately ~5×10^4 to ~1×10^7 cells/ml. The range of cell concentrations was decided according to the recommended cell concentration range of the LUNA-II for TB staining. After staining with 0.2% EB, the cells were counted with the LUNA-II. Cell concentrations (either total or live cells) determined by EB staining well matched theoretical cell concentrations (Fig. 2A).

Cell counting with EB staining was also determined with distinct cell lines. Comparative cell counting with both TB and EB was performed for HeLa and 293 cells with the LUNA-II. As shown in Fig. 2B, there was no significant difference in cell size, viability, or cell concentration regardless of cell type or staining method. These data indicate that EB staining is comparable to TB staining in counting adherent and suspension cells with automated cell counters.

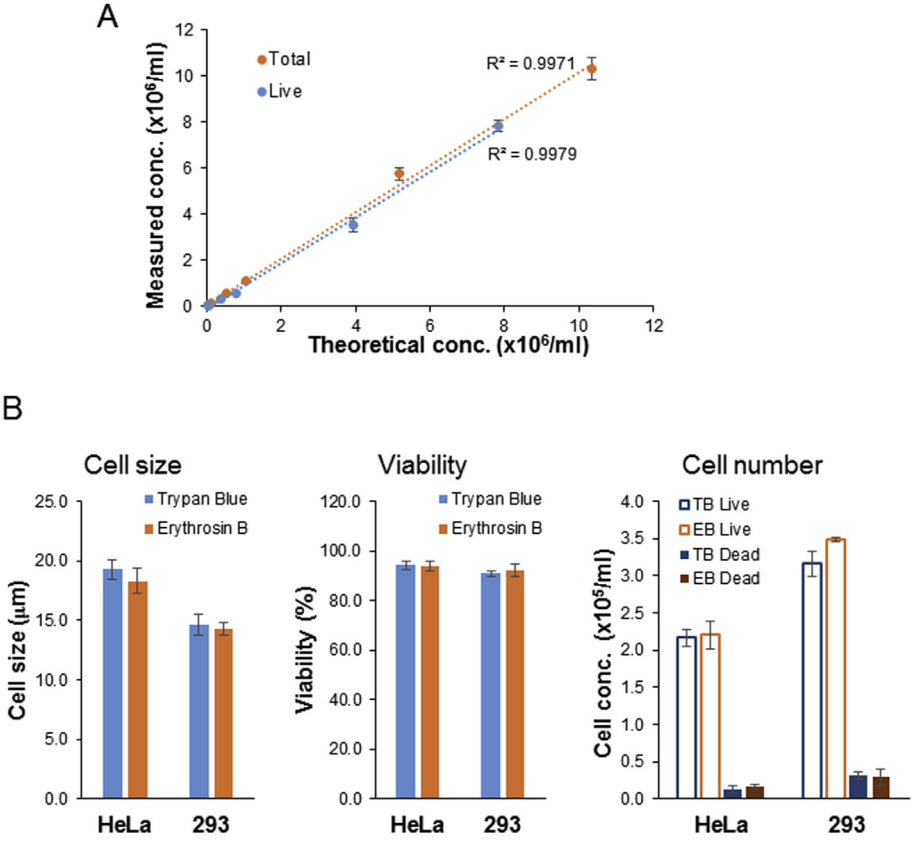

Fig.2. Application of EB to count cultured mammalian cells. (A) Range of cell concentrations with EB staining. Serially diluted HL-60 cells were counted with EB staining with the LUNA-II. Data from a representative experiment performed in triplicate are shown as means ± standard deviations. (B) Application of EB to count distinct cell lines. HeLa or 293 cells were trypsinized and resuspended in appropriate medium. The number of cells was determined by either TB or EB staining with the LUNA-II. The size, viability, and the number of live and dead cells are represented as means ± standard deviations from triplicates. No significant difference was observed between TB and EB staining.

Biosafety is one major concern in modern biomedical and bio-industry fields. As mentioned earlier, TB is a potential teratogenic agent [8–12]. In addition, it has been reported that TB has a toxic effect on cultured human cells [20,21]. The toxic effect of TB is less potent on cells suspended in serum-containing medium than on cells suspended in DPBS [20]. However, the standard protocol suggests the use of cells suspended in a serum-free solution for TB staining because TB, but not EB, also stains serum proteins that may cause misleading results [4]. To compare the toxic effect of TB and EB, we performed a series of experiments. First, HL-60 cells were washed twice with DPBS to remove FBS and were resuspended in DPBS. The initial viability of HL-60 cells was determined by either 0.4% TB or 0.2% EB staining with the LUNA-II cell counter. The cells were mixed with an equal volume of either TB or EB and incubated at room temperature. The viability was determined over a period of time. As shown in Fig. 3A, the viability of HL-60 cells decreased when the cells were incubated with 0.4% TB within 10 min. In addition, the concentration of total cells was also reduced when the cells were incubated with TB. On the contrary, no significant change in cell viability or total cell concentration was observed when the cells were incubated with EB. Second, DPBS-washed HL-60 cells were resuspended in serum-free medium and mixed with an equal volume of the vital dyes. Again the viability of cells was determined over the time. The toxic effect of TB was delayed when the cells were suspended in serum-free medium. The toxic effect of TB was observed as early as 60 min after incubation with TB (Fig. 3B). Both

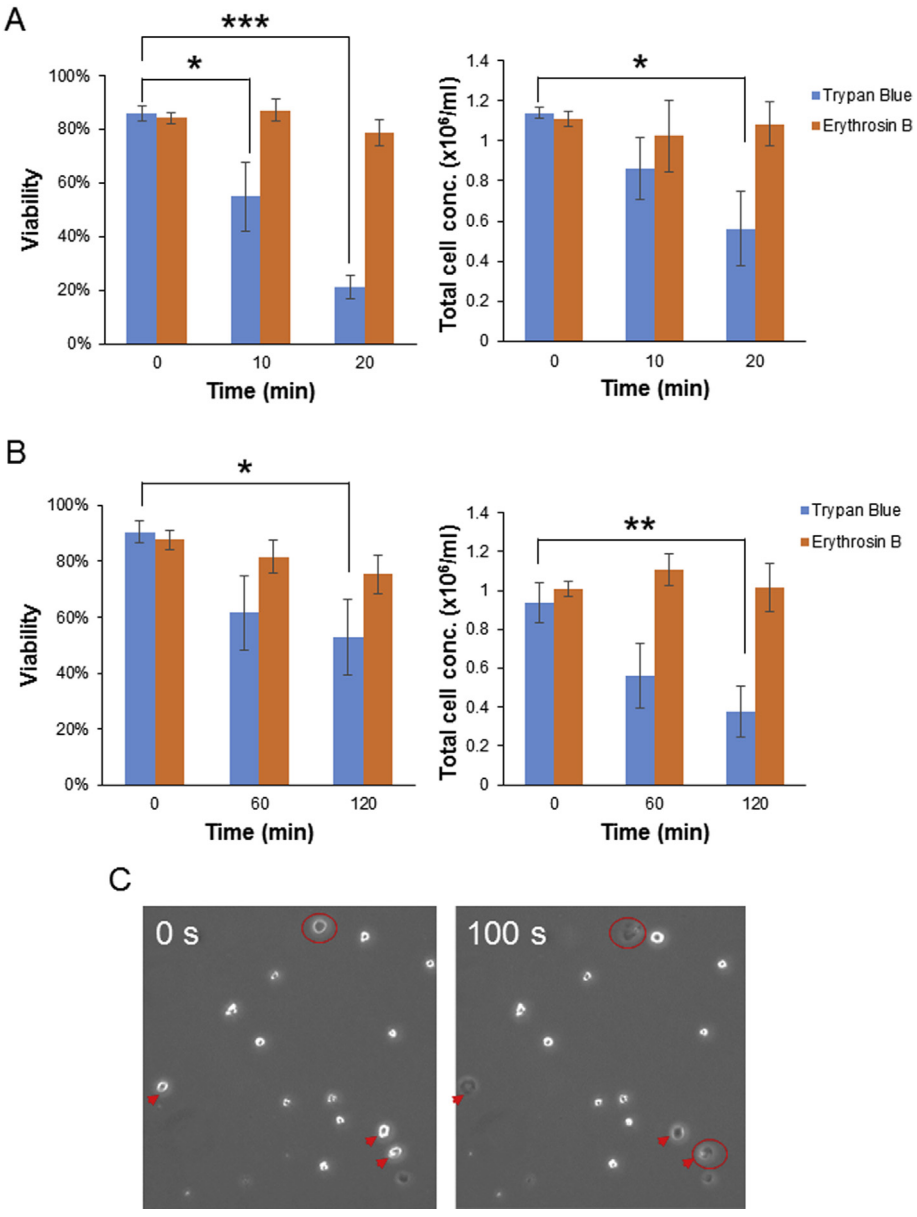

Fig.3. Toxic effect of TB on cultured mammalian cells. (A) Toxicity of TB and EB on HL-60 cells in DPBS. (B) Toxicity of TB and EB on HL-60 cells in RPMI-1640. (A,B) The cells were washed twice with DPBS and resuspended in DPBS (A) or RPMI-1640 (B) without FBS. The number and viability of cells were determined by LUNA-II after incubating with TB or EB. Data from a representative experiment performed in triplicate are shown as means ± standard deviations. Significant differences are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (C) Live cell images from cells stained with 0.4% TB. HL-60 cells were washed twice with DPBS and resuspended in RPMI-1640 without FBS. After mixing with 0.4% TB, live cell images were captured by the iRiS Digital Cell Imaging System. Arrowheads indicate cells that died after TB incubation. Red circles indicate ruptured cells. (For interpretation of the references to color in this figure legend and in the description of this figure in the text, the reader is referred to the Web version of this article.)

viability and the concentration of cells were markedly decreased after 120 min of TB incubation, whereas there was a marginal decrease of cell viability when the cells were incubated with EB for the same amount of time. To understand the decrease of total cell concentration, live cell imaging was conducted in the presence of TB. HL-60 cells, suspended in serum-free medium, were mixed with an equal volume of 0.4% TB and loaded into a disposable counting slide, and then live cell images were captured with a digital microscope. As shown in Fig. 3C, some viable cells became stained with TB within 100 s after imaging (red arrowheads). In addition, nonviable TB-stained cells eventually ruptured and disintegrated (red circles). The ruptured cells completely disintegrated to debris and resulted in a loss of total cell concentration after prolonged incubation with TB (data not shown). Taken together, our data indicate that EB is an alternative biosafe vital dye for automated cell counters without significant toxic effects on cultured cells.

Conclusions

In this study, we determined that EB is an alternative vital dye for counting mammalian cells with automated cell counters. The importance of cell counting (cell enumeration) has increased during recent years because the inaccuracy of cell counts may affect the potency and efficacy of cell therapy treatment, disease diagnosis, rate of growth of regenerated tissue in a biomaterial scaffold, and bioassays that are normalized by cellular activity [22]. As an alternative biosafe vital dye, EB is as accurate as TB in counting and measuring the viability of cells with automated cell counters. In addition, EB is less toxic than TB on cultured mammalian cells suspended in serum-free solutions. The European Union (EU) banned the use of TB in textiles and leather due to its potential hazardous effect on human skin [23]. Introducing EB for use with automated cell counters will significantly reduce the hazardous concern caused by TB.

Competing interests

The all authors are employees of Logos Biosystems. D.H. and H.L. are stockholders of Logos Biosystems. K.C. and N.J. are founders and stockholders of Logos Biosystems.

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References

[1] G. Johnston, Automated handled instrument improves counting precision across multiple cell lines, BioTechniques 48 (2010) 325–327.
[2] D. Cadena-Herrera, J.E. Esparza-De Lara, N.D. Ramírez-Ibañez, C.A. López-Morales, N.O. Pérez, L.F. Flores-Ortiz, E. Medina-Rivero, Validation of three viable-cell counting methods: manual, semi-automated, and automated, Biotechnol. Rep. 7 (2015) 9–16, http://dx.doi.org/10.1016/j.btre.2015.04.004.
[3] A. Tholudur, L. Giron, K. Alam, T. Thomas, E. Garr, G. Weatherly, K. Kulowiec, M. Quick, S. Shepard, Comparing automated and manual cell counts for cell culture applications, BioProcess Int. (October 2006) 28–34, in: http://www.bioprocessintl.com/wp-content/uploads/bpi-content/06049ar04_76381a.pdf.
[4] W. Strober, Trypan blue exclusion test of cell viability, Curr. Protoc. Immunol. (1997). A3.B.1–A3.B.2.
[5] K.G. Tucker, S. Chalder, M. . al-Rubeai, C.R. Thomas, Measurement of hybridoma cell number, viability, and morphology using fully automated image analysis, Enzyme Microb. Technol. 16 (1994) 29–35.
[6] H. Lecoeur, Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases, Exp. Cell Res. 277 (2002) 1–14.
[7] N.J. Philpott, A.J. Turner, J. Scopes, M. Westby, J.C. Marsh, E.C. Gordon-Smith, A.G. Dalgleish, F.M. Gibson, The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques, Blood 87 (1996) 2244–2251.
[8] A.N. Barber, J.C. Geer, Studies on the teratogenic properties of trypan blue and its components in mice, J. Embryol. Exp. Morphol. 12 (1964) 1–14.
[9] F. Beck, J.B. Lloyd, Dose–response curves for the teratogenic activity of trypan blue, Nature 201 (1964) 1136–1137.
[10] M.M. Turbow, Teratogenic effect of trypan blue on rat embryos cultivated in vitro, Nature 206 (1965) 637.
[11] F.E. Field, G. Roberts, R.C. Hallowes, A.K. Palmer, K.E. Williams, J.B. Lloyd, Trypan blue: identification and teratogenic and oncogenic activities of its coloured constituents, Chem. Biol. Interact. 16 (1977) 69–88.
[12] J.G. Wilson, A.R. Beaudoin, H.J. Free, Studies on the mechanism of teratogenic action of trypan blue, Anat. Rec. 133 (1959) 115–128.
[13] D.M. Marmion, Handbook of U.S. Colorants for Foods, Drugs, and Cosmetics, Wiley Interscience, New York, 1979.
[14] A.W. Krause, W.W. Carley, W.W. Webb, Fluorescent erythrosin B is preferable to trypan blue as a vital exclusion dye for mammalian cells in monolayer culture, J. Histochem. Cytochem 35 (1984) 1084–1090.
[15] H.J. Philips, J.E. Terryberry, Counting actively metabolizing tissue cultured cells, Exp. Cell Res. 13 (1957) 341–347.
[16] Logos Biosystems, Automated fluorescence cell counting [application note], Logos Biosystems, Gyunggi-do, Korea. http://www.logosbio.com/download/pdf/Review-fluorescence_cell_counting.pdf (accessed 20.05.15).
[17] D. Hong, G. Lee, N.C. Jung, M. Jeon, Fast automated yeast cell counting algorithm using bright-field and fluorescence microscopic images, Biol. Proced. Online 15 (1) (2013), http://dx.doi.org/10.1186/1480-9222-15-13.
[18] H.-C. Lin, M.-S. Chen, Y.-H. Lin, A review of electrically tunable focusing liquid crystal lenses, Trans. Electr. Electron. Mater 12 (2011) 234–240.
[19] A. Sahaphong, B.F. Trump, Studies of cellular injury in isolated kidney tubules of the flounder: V. Effects of inhibiting sulfhydryl groups of plasma membrane with the organic mercurial PCMB (parachloromercuribenzoate) and PCMB (parachloromercuribenzenesulfonate), Am. J. Pathol. 63 (1971) 277–298.
[20] B.T. van Dooren, W.H. Beekhuis, E. Pels, Biocompatibility of trypan blue with human corneal cells, Arch. Ophthalmol. 122 (2004) 736–742.
[21] K.T. Tsaousis, N. Kapsachilis, I.T. Tsinopoulos, S.A. Dimitrakos, F.E. Kruse, U. Welge-Luessen, Time-dependent morphological alterations and viability of cultured human trabecular cells after exposure to trypan blue, Clin. Exp. Ophthalmol. 41 (2013) 484–490.
[22] S. Sarkar, ISCT Webinar: Measurement Assurance for Cell Enumeration, ISCT North American Legal and Regulatory Affairs (LRA) Committee, 2015. https://isct-beacon.app.box.com/isctwebinar29042015 (accessed 9.06.15).
[23] U.S. Environmental Protection Agency, Dyes Derived from Benzidine and its Congeners, 2010. http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/DCB%20Action%20Plan_06232010.noheader.pdf (accessed 9.06.15).