Image-Based High-Throughput Cell Viability Assay Using Erythrosin B Instead of Trypan Blue

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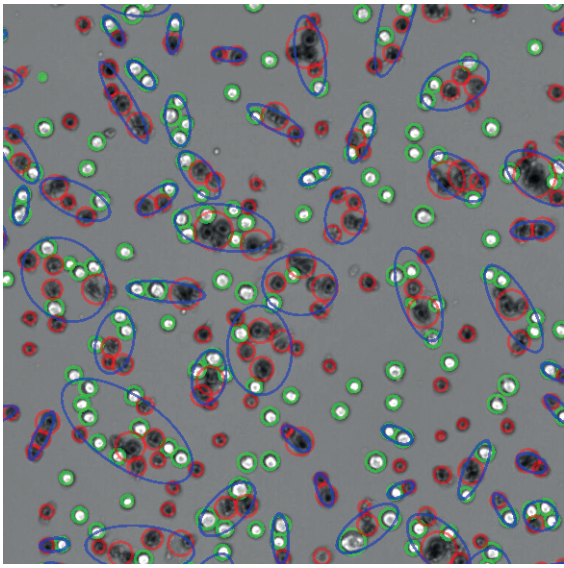
ABSTRACT

Viable cell counting is essential in biological research and biomanufacturing to assess cell health and proliferation. Traditionally, cell viability is most often assessed using trypan blue staining. However, despite its widespread use, trypan blue poses considerable health and environmental risks, which complicate its handling and disposal, especially in high-throughput applications and settings. Consequently, there is increasing interest in alternative methods that offer similarly reliable results without the associated hazards. One such alternative is erythrosin B, a red colorant, noted for its biosafety and comparable staining properties to trypan blue.

In this study, we explored erythrosin B as a potential substitute for trypan blue staining using SYNENTEC's high-throughput imagers, CELLAVISTA® 4K and NYONE® Scientific. We tested various concentrations of erythrosine B under different filter settings to determine the optimal conditions for cell viability assessment. Based on these optimized parameters, we evaluated the viability of two widely used cell lines, CHO-K1 and HEK293T, and compared the results to those obtained with the standard trypan blue assay. Both staining methods showed comparable results regarding cell viability and cell counts.

Our findings enabled us to establish a robust staining protocol and image analysis algorithm for viable cell counting with erythrosin B, making it a suitable, economic and safer alternative for high-throughput applications.



KEYWORDS: CELL VIABILITY, CELL COUNTING, ERYTHROSIN B, TRYPAN BLUE, STAINING, CELL CULTURE, BRIGHTFIELD, AUTOMATION, HIGH-THROUGHPUT, BIOSAFETY, ENVIRONMENTALLY FRIENDLY, SUSTAINABILITY, GREEN LAB

Benefits of the Erythrosin B application:

- With our optical flexibility, easily switch from your existing trypan blue protocol.
- Minimize risks and increases safety during handling and disposal of your reagents.
- Reduce costs as no expensive consumables are required.
- Analyse 96 samples from any cell culture system in less than five minutes.
- Use any SBS plate - no need of proprietary sample carriers.
- Stick to existing liquid handling methods - no in-depth re-coding necessary.

INTRODUCTION

The assessment of viable cell counting represents a basic but fundamental step in *in vitro* cell culture in biological research and the biomanufacturing industry to regularly evaluate the health and functionality of cells. For this purpose, viability tests are used to distinguish living from dead cells. Among the various dyes used in these assays, trypan blue is one of the most commonly employed. Many automated cell counters without fluorescence detection are specifically optimized for trypan blue-stained suspension cells. The function of trypan blue is based on the principle of membrane permeability: viable cells exclude the dye due to their intact membranes, while non-viable cells, having compromised membranes, take up the dye and appear blue in brightfield microscopy.

Despite its widespread use, trypan blue has its drawbacks. According to the European Chemicals Agency (ECHA), trypan blue is associated with significant health and environmental concerns, including potential carcinogenicity and cytotoxicity [1-4]. These hazards complicate the proper disposal and handling of the dye. Especially, the combination of trypan blue with genetically modified organisms (GMOs) is challenging. These cells need to be inactivated, which is usually done by autoclaving. However, autoclaving trypan blue is not recommended as toxic aerosols might be formed. Therefore, the stained GMOs need to be inactivated by other methods like incineration, resulting in significant costs.

Additionally, trypan blue has a high affinity for proteins in solution, which may interfere with the serum proteins present in the medium. To avoid this, samples should be prepared in phosphate-buffered saline (PBS), adding an extra preparation step [5, 6]. Another limitation is the stability of the dye. Trypan blue solutions tend to precipitate under suboptimal storage conditions, such as temperature fluctuation or extended light exposure. This can compromise the staining efficacy and reproducibility of cell viability assessment.

These concerns necessitate the development of biosafe and cost-effective alternatives for cell viability assays. One such alternative is the red dye erythrosin B, which is a vital exclusion stain like trypan blue, selectively staining cells that have lost membrane integrity. Erythrosin B is also known as FD&C Red No. 3 (US) or E127 (EU) food coloring dye and dental plaque disclosing agent [7-9]. Although the Food and Drug Administration (FDA) has recently banned erythrosin B in food and ingested drugs, due to its potential cancer risk in male rats, it remains authorized in the EU for use in cocktail and candied cherries, as well as Bigarreaux cherries [9-10]. The European Food Safety Authority (EFSA) considers an acceptable daily intake (ADI) of up to 0.1 mg/kg body weight per day to pose no safety concern [8].

The aim of this work was to determine whether cell viability assessments routinely performed using trypan blue in combination with our imagers and image analysis software could be replaced by erythrosin B. To do this, we tested various concentrations of

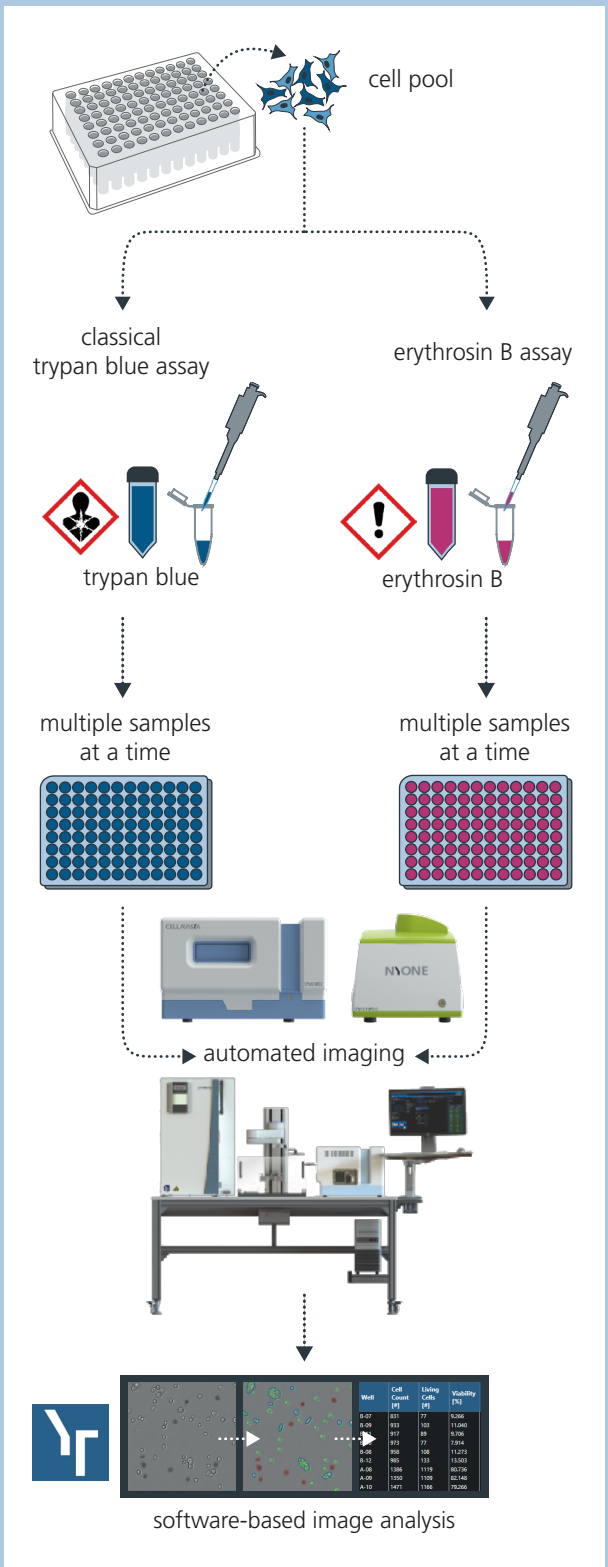


FIG. 1. EXPERIMENTAL WORKFLOW

Adherent cells were detached, diluted in PBS, and stained with either trypan blue or erythrosin B. The cell suspension was transferred to a 96-well plate, centrifuged, and imaged using a CELLAVISTA® or NYONE® Scientific imaging system. Images were automatically analyzed using YT-SOFTWARE®.

erythrosin B with different filter settings and established the best conditions. Using these, we analyzed the viability of the widely used cell lines CHO-K1 and HEK293T and compared the results with our standard trypan blue assay. Lastly, we analyzed the cytotoxic effects of both dyes on the cells after several hours of exposure.

MATERIAL

Cell culture

- adherent HEK293T (human embryonic kidney (HEK) 293 T (SV40 large T antigen) cells
- adherent CHO-K1 (Chinese hamster ovary (CHO) K1 (subclone K1) cells
- DMEM (HEK293T) or DMEM F12 (CHO-K1) medium supplemented with 10 % (v/v) FCS, 1 % (v/v) L-Glutamine, 1 % (v/v) Sodium Pyruvate
- Trypsin 0.05 %/EDTA 0.02 % in PBS
- Trypan blue 0.02 % (v/v) in PBS (Gibco™, catalog number: 15250061)
- Erythrosin B 0.01 % (w/v) in PBS or H_2O (made from Thermo Fisher Erythrosin B powder, catalog number: A14180.14)
- Phosphate-buffered saline (PBS) w/o Ca^{2+} w/o Mg^{2+}

Viability assay

- 96-well plate (e.g. CytoOne®, starlab #CC7682-7596)
- Trypan blue 0.02 % in PBS (made from Gibco™, catalog number: 15250061)
- Erythrosin B 0.01 % w/v in PBS or H_2O (made from Thermo Fisher Erythrosin B powder, catalog number: A14180.14)
- Phosphate-buffered saline (PBS) w/o Ca^{2+} w/o Mg^{2+}

Imaging and analysis

- SYNENTEC's imaging device (here CELLAVISTA® 4K and NYONE® Scientific)
- SYNENTEC's YT-SOFTWARE®

METHODS

Cell culture and cell counting

We routinely cultured CHO-K1 cells in DMEM F12 medium and HEK293T cells in DMEM medium under standardized cell culture conditions (37 °C, 5 % CO_2, humidified atmosphere). Both media were supplemented with 10 % (v/v) FCS, 1 % (v/v) Sodium Pyruvate, and 1 % (v/v) L-Glutamine.

Cells were routinely passaged at a subconfluent state between 70-80 % confluence as determined by visual inspection. For passaging, cells were detached with trypsin, diluted in PBS/0.01 % trypan blue and counted using SYNENTEC's **Trypan Blue** application [11]. The appropriate cell number for reseeding was calculated based on the viable cell density.

Viability assay

Dead cells were generated by incubating cells in a falcon tube with a limited amount of medium overnight or for several days. Viable cells were obtained from exponentially growing standard cell culture. Cell suspensions with varying viabilities were prepared by mixing the dead and viable cells. The cell suspensions were diluted in PBS to achieve different cell densities of viable or serum-starved cells. 50 µL of cell suspension were pipetted into each well of a 96-well plate. An equal volume (50 µL) of staining solution containing either 0.02 % (w/v) trypan blue or 0.01 % (w/v) erythrosin B was then added to each well, resulting in a 1:2 dilution and a total volume of 100 µL per well. The plate was centrifuged at 30 x g for 1 min in a swing-out rotor. Samples were analyzed with NYONE® Scientific or CELLAVISTA® 4K using the **Erythrosin B** or **Trypan Blue** image analysis application, respectively (Fig. 1).

In general, it is important to ensure that the cell suspension is free of clumps and that the cells are evenly distributed within each well. Additionally, to achieve precise results, a final cell concentration of approximately 3×10^5 cells/mL and the preparation of at least three technical replicates per cell suspension are recommended. For detailed information see technical note [12].

Imaging and image analysis

Imaging was performed using the 10x objective of CELLAVISTA® 4K (2x2 High Sensitivity option) or NYONE® Scientific. Depending on the staining solution, the application wizard **Trypan Blue** or **Erythrosin B** of YT-SOFTWARE® was used for image analysis.

RESULTS & DISCUSSION

1. Erythrosin B staining

To determine the compatibility of erythrosin B staining with our imaging platforms and image analysis software, we pipetted HEK293T cells into 96-well plates and stained them with a range of erythrosin B concentrations (0.00125 % (w/v) to 0.4 % (w/v)). Staining intensity and imaging results were compared to those obtained using our established trypan blue protocol (0.01 % (w/v) trypan blue) [11].

The appearance of the cell suspensions varied with erythrosin B concentration, ranging from dark red at higher concentrations to light red at lower concentrations (Fig. 2A). Using appropriate filter settings (see technical note for details [12]), erythrosin B-stained cells were readily visualized as darker objects in brightfield imaging at final staining concentrations between 0.015 % (w/v) and 0.005 % (w/v). At concentrations above 0.015 % (w/v), the staining solution was opaque, affecting light transmission and

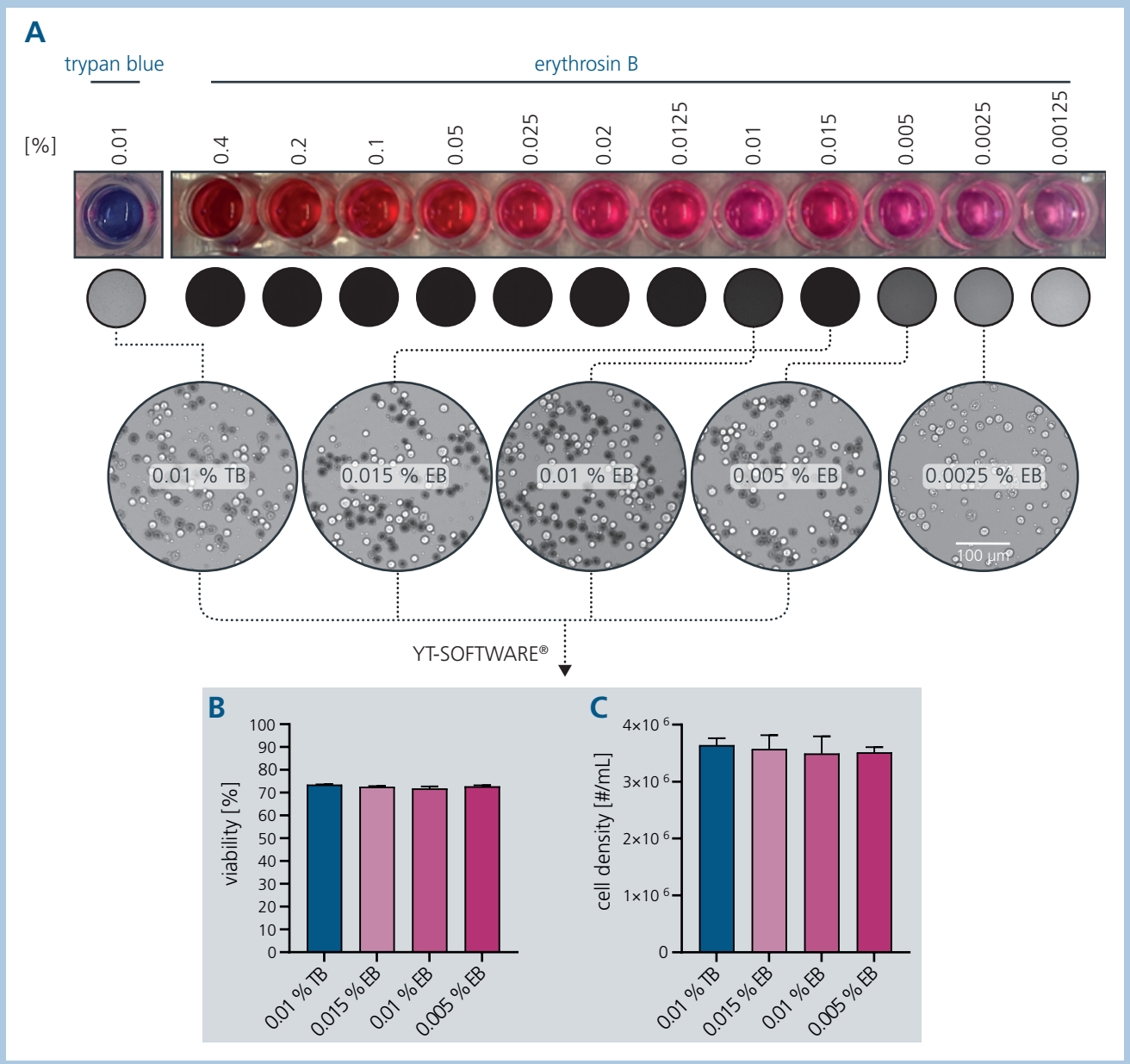


FIG. 2. FIG. 2: CELL VIABILITY ASSESSMENT USING ERYTHROSIN B AND CELLAVISTA® 4K

HEK293T cells were serum-starved and transferred into a 96-well plate. (A) Cells were stained with various erythrosin B (EB) concentrations or 0.01 % (w/v) trypan blue (TB) and imaged directly using CELLAVISTA® 4K. (B, C) Images were analyzed with the **Trypan Blue** or **Erythrosin B** image analysis application of YT-SOFTWARE® and viability (B) and cell density (C) were plotted. Shown are representative results from one experiment, performed in four technical replicates. EB: erythrosin B; TB: trypan blue.

image quality. Conversely, erythrosin B concentrations below 0.005 % (w/v) resulted in insufficient staining of dead cells, making it difficult to reliably distinguish them from viable cells (Fig. 2A).

Following optimization of image analysis parameters, our YT-SOFTWARE® platform was able to automatically and accurately detect both stained (non-viable) and unstained (viable) cells for quantification. Comparative analyses demonstrated that erythrosin B at concentrations of 0.015 % (w/v), 0.01 % (w/v), and 0.005 % (w/v) yielded results for cell viability and cell density that were consistent with those obtained using 0.01 % (w/v) trypan blue (Fig. 2B, C).

Based on optimal staining intensity, exposure time, image clarity, and quantification accuracy, we selected 0.005 % (w/v) erythrosin B as the preferred concentration for viability assessment with our imaging systems. Notably, this concentration is lower than those recommended in the literature (0.02-0.2 % (w/v erythrosin B)), thereby minimizing potential cytotoxic effects and reducing reagent consumption [13, 14]. Furthermore, the total measurement time for 0.005 % (w/v) erythrosin B staining was comparable to that of the established trypan blue protocol, requiring approximately 4 minutes to process a full 96-well plate.

2. Trypan blue versus erythrosin B cell viability assay

To evaluate the accuracy of erythrosin B in staining dead cells, we compared our standard trypan blue protocol with a 0.005 % (w/v) erythrosin B staining method. To generate samples with varying viable cell concentrations, we serum-starved HEK293T and CHO-K1 cells by incubating them overnight or for extended periods in a small volume of medium within a tube. These serum-starved dead cells were then mixed in various ratios with living HEK293T or CHO-K1 cells and pipetted into 96-well plates. After the addition of either erythrosin B or trypan blue staining solutions, the cells were imaged using the CELLAVISTA® 4K system.

Both trypan blue and erythrosin B staining produced comparable results in terms of cell viability, viable cell density, and average cell size (Fig. 3). However, erythrosin B appeared to be more sensitive in detecting dead cells, particularly in HEK293T samples, as indicated by a lower measured viability compared to trypan blue (Fig. 3A). This difference was most pronounced in samples with low viability. Both, trypan blue and erythrosin B stainings produced results with a high correlation to the theoretical viabilities (HEK293T: trypan blue R^2 = 0.9940, erythrosin B R^2 = 0.9970; CHO-K1: typan blue R^2 = 0.9994, erythrosin B R^2 = 0.9988). In line with the viability results, the viable cell density was slightly higher in trypan blue-

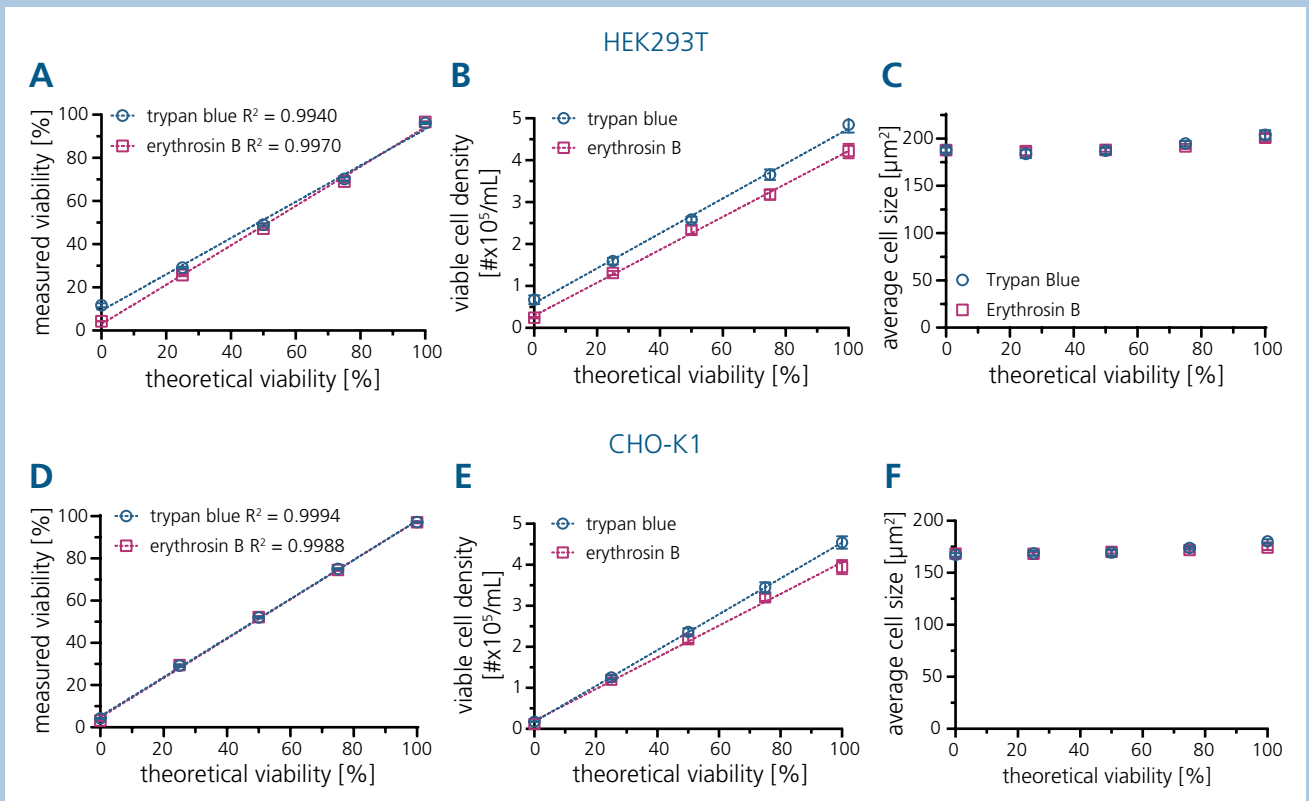


FIG. 3. TRYPAN BLUE VERSUS ERYTHROSIN B

Various ratios of live and dead HEK293T or CHO-K1 cells were transferred into a 96-well plate and stained with 0.005 % (w/v) erythrosin B or 0.01 % (w/v) trypan blue. Cells were imaged directly using CELLAVISTA® 4K. Images were analyzed with the **Trypan Blue** or **Erythrosin B** image analysis application of YT-SOFTWARE® and cell viability (A, D), viable cell density (B, E) and average cell size (C, F) were plotted. Representative results from one experiment are shown, performed in five technical replicates.

stained samples, especially in HEK293T cells (Fig. 3B, E).
These results are in line with previous studies showing that erythrosin B stains all non-viable cells immediately after lethal treatment, while trypan blue optimally stains only about 60 % of dead cells under similar conditions [15]. Additionally, Chan et al. showed that trypan blue can rupture cells and change the morphology of dead cells, resulting in dim and diffuse shapes that are difficult to detect and may lead to an over-estimation of cell viability [16, 17].

3. Cytotoxic effect of erythrosin B and trypan blue

The cytotoxicity of trypan blue at elevated concentrations is well documented, whereas erythrosin B is presumed to exhibit lower cytotoxicity towards mammalian cells [14]. This is a well-known challenge as sometimes samples cannot be immediately measured but remain some time in a plate. To find out, if our routinely used trypan blue and erythrosin B concentrations induce cytotoxicity and find the safe time frame for measurement, we exposed CHO-K1 and HEK293T cells to 0.01 % (w/v) trypan blue or 0.005 % (w/v) erythrosin B for varying durations at room temperature. Cell viability was monitored using the respective **Trypan Blue** and **Erythrosin B** image analysis applications within the YT-SOFTWARE® platform. Parallel control samples were incubated at room temperature in either standard cell culture medium, PBS (control trypan blue) or PBS/H_2O (control erythrosin B).
Our results indicate that neither 0.01 % (w/v) trypan blue nor 0.005 % (w/v) erythrosin B significantly affected cell viability over a 6-hour incubation period when compared to controls (Fig. 4). These findings indicate that, at the tested concentrations, both dyes are suitable for short-term viability assays in CHO-K1 and HEK293T cells without inducing significant cytotoxic effects. This highlights the advantages of using our imaging-based cell counters over alternative methods, especially regarding trypan blue. Trypan blue is widely used at a staining concentration of 0.4 % (w/v), which has been shown to reduce mammalian cell viability within 10 min of exposure. In contrast, 0.2 % (w/v) erythrosin B showed no toxic effect on the cells [14,15].

4. Optimizing the assay for speed and data storage

After establishing the experimental setup for the erythrosin B viability assay, we aimed to optimize the imaging parameters for both acquisition time and data storage – two critical factors in high-throughput analysis in automated environments. To this end, we imaged the same plate of erythrosin B-stained cells sequentially using either CELLAVISTA® 4K or NYONE® Scientific, applying different subwell settings, and subsequently compared the results using the **Erythrosin B** image analysis application.
The results were comparable across both imaging devices and subwell settings (Fig. 5). Based on these findings, we recommend measuring a well area of the inner 4 subwells to significantly reduce measurement time and data storage requirements. This configuration corresponds to a processed area of 21.1 % with NYONE Scientific and 34.9 % with CELLAVISTA 4K. Please note that an even cell distribution within each well is essential to achieve consistent and reproducible data accuracy across samples and devices.

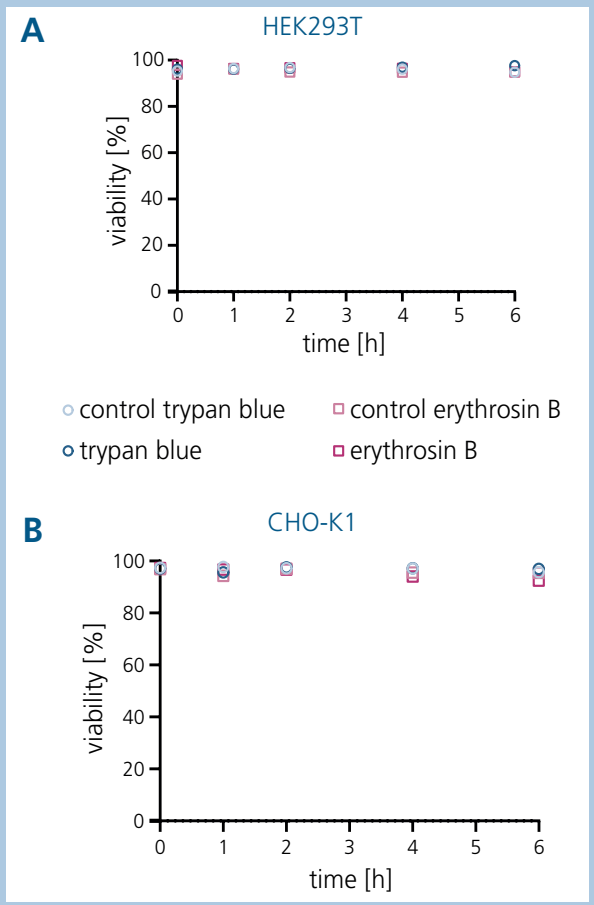


FIG. 4. NO CYTOTOXIC EFFECT OF ERYTHROSIN B AND TRYPAN BLUE

HEK293T (A) or CHO-K1 (B) cells were exposed to 0.005 % (w/v) erythrosin B, 0.01 % (w/v) trypan blue, PBS (control trypan blue) or PBS/H_2O (control erythrosin B) over different time periods. Cells were measured using CELLAVISTA® 4K and images were analyzed with the **Trypan Blue** or **Erythrosin B** image analysis application of YT-SOFTWARE®. Representative results from one experiment are shown, performed in three technical replicates.

CONCLUSION

In line with previous publications, our findings underscore the potential of using erythrosin B as a reliable, safer and cost-effective alternative to trypan blue for cell viability assays [13, 14, 15]. It minimizes the risks associated with the traditional trypan blue method while maintaining accuracy and efficiency in cell counting protocols, using the same hardware and similar device settings. Notably, erythrosin B is compatible with automated environments, making it well-suited for high throughput applications and settings.

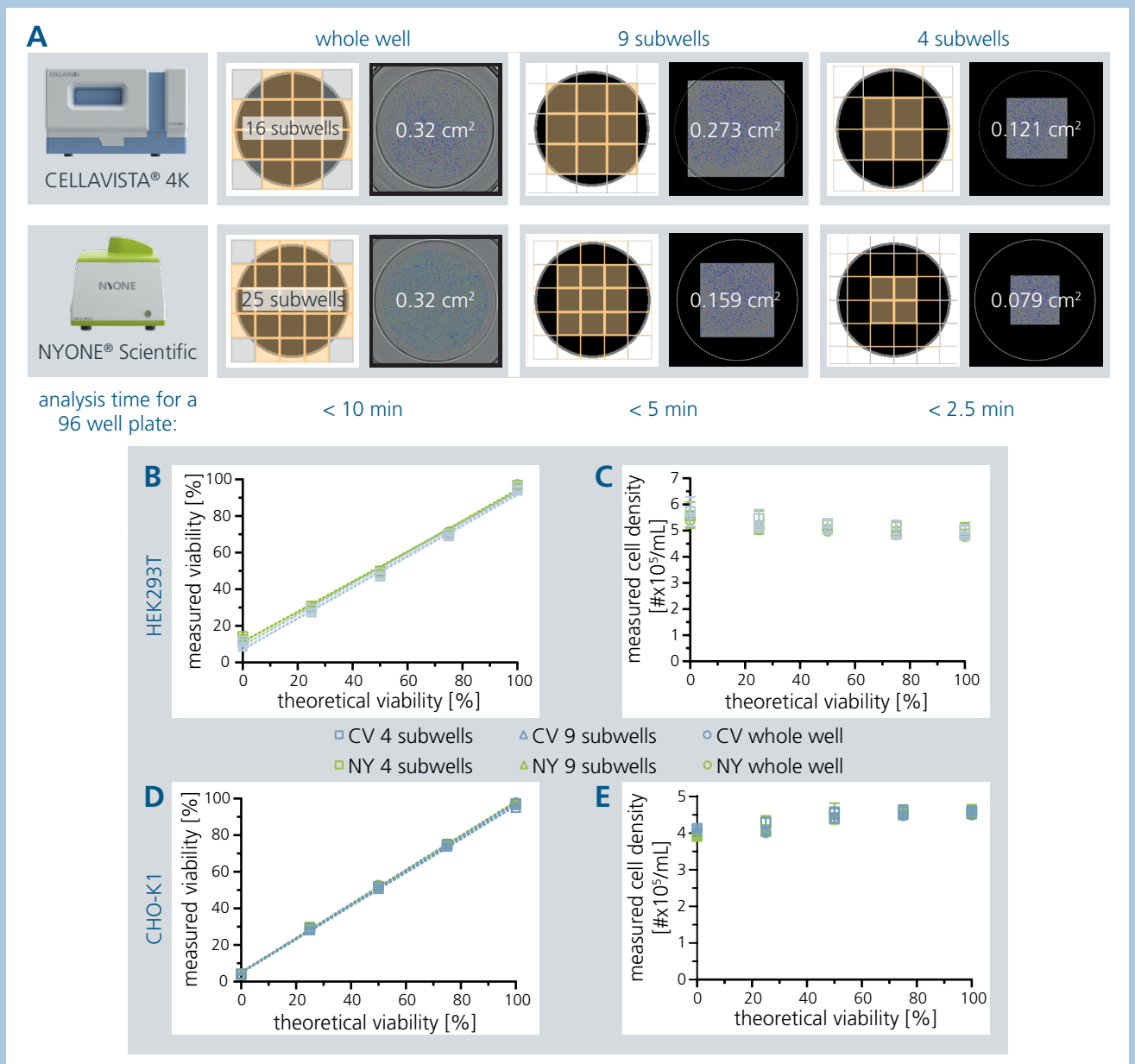


FIG. 5. OPTIMIZING THE IMAGING TIME FOR THE ERYTHROSIN B VIABILITY ASSAY

(A) Our YT-SOFTWARE® allows the user to selectively image specific well areas. (B, C) Various ratios of live and dead HEK293T (B, C) or CHO-K1 (D, E) cells were pipetted into a 96-well plate and stained with 0.005 % (w/v) erythrosin B. Cells were imaged with CELLAVISTA® 4K (CV) or NYONE® Scientific (NY) using the subwell setting whole well, 9 subwells or 4 subwells. Images were analyzed with the **Erythrosin B** image analysis application of YT-SOFTWARE®, and cell viability (B, D) and cell density (C, E) were plotted. Representative results from one experiment are shown, performed in five technical replicates. CV: CELLAVISTA 4K; NY: NYONE Scientific.

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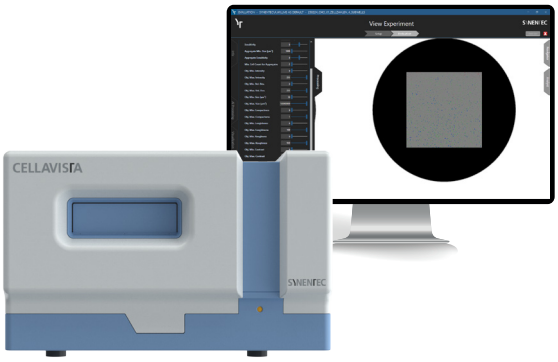
Imaging Devices



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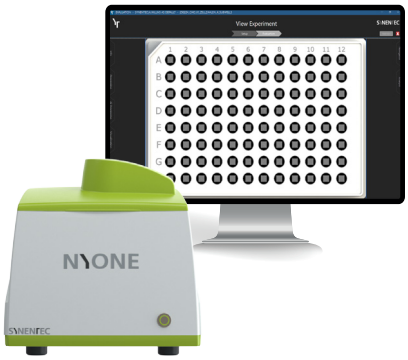

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