Label-Free High-Throughput Cell Viability Analysis Using Advanced Machine Learning Algorithms

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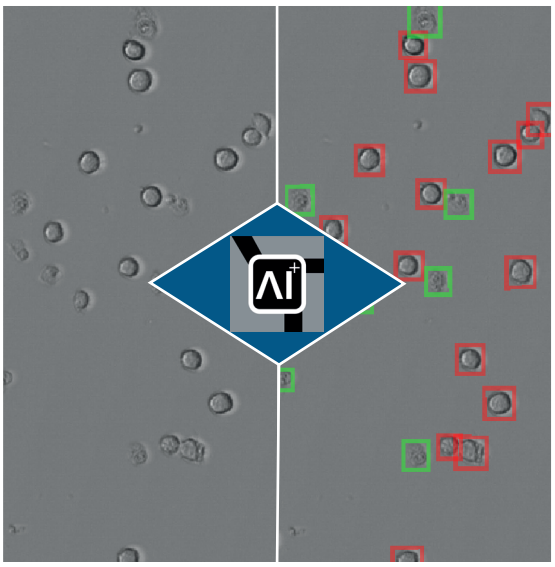
ABSTRACT

Cell viability assays are essential tools in various research fields, like drug discovery, cancer research, bioprocessing, or cell line development. Commonly, cells are stained with a dye to distinguish between living and dead cells. Dyes not only complicate these assays but also may introduce challenges like interference with test compounds, handling and disposing of toxic substances, and the generation of single-use plastic waste. Thus, we explored a solution for a label-free, high-throughput viability assay. To do so, we used algorithms of machine learning/artificial intelligence (AI). Initially, we trained a machine-learning model using only brightfield images. To differentiate between living and dead cells fluorescence images were employed as an auxiliary tool to classify the cells as viable or dead. Cells were labeled and assigned into the two classes using our AI-STUDIO+ software. Subsequently, we validated the accuracy of the AI model by comparing its results with those obtained from classical image processing of Hoechst 33342/Calcein-AM/Propidium Iodide and Trypan Blue stainings. The Trypan Blue viability assay is widely used during cell culture routine in many laboratories. Both assays showed similar results regarding Viability and Cell Count in comparison to AI analysis. Moreover, the standard deviation was lower for the AI-analyzed data than for the classical Trypan Blue assay. Thus, we developed an AI-driven solution to assess the cell viability of suspension cells using brightfield images of unlabeled cells suitable for high-throughput purposes.



KEYWORDS: CELL COUNT, CELL VIABILIY, LABEL-FREE, MACHINE LEARNING, ARTIFICIAL INTELLIGENCE, HIGH-TROUGHPUT

Benefits of SYNENTEC's AI-based viability assay:

- Imaging and image analysis in one software platform
- Label-free, non-invasive, no interference with test compounds
- Safe, rapid, reliable, efficient, and time-and cost-effective
- Suitable for high throughput

INTRODUCTION

Cell viability assays are pivotal in life sciences, enabling the evaluation of cell vitality to determine the toxicity of components or estimate cell health during the cell culture routine. Commonly used methods are dye exclusion assays, in which cells are labeled with either a brightfield or fluorescent dye to distinguish between live and dead cells, and a detection system quantifies the number of cells falling into each category, in addition to the total cell count. Ultimately, cell viability is calculated as the percentage of viable cells in the sample. The underlying principle of dye exclusion assays is that living cells possess intact cell membranes that prevent the penetration of the stain, whereas dead or dying cells lack such barriers and the dye can penetrate damaged membranes.

One of the most common standard techniques used in academic research and industry to access viability is Trypan Blue (TB) staining [1]. The cell suspension is mixed with the TB dye and is visually examined under a light microscope to determine whether the cells include or exclude the stain. A viable cell is characterized by a clear cytoplasm, whereas a nonviable cell exhibits a blue cytoplasm as TB has entered the dead cell. Although the assay is simple, well-established, and the gold standard for the assessment of cell viability in many laboratories, TB dye has limitations and drawbacks. On the one hand, TB becomes toxic to cells after a short exposure time. This presents the risk of miscounting viable cells because they die over time. On the other hand, TB staining was shown to overestimate cell viability in samples that are below 80% viable [2]–[4]. Moreover, since TB binds to proteins, it might also interfere with proteins in the serum of the medium, therefore the sample should be prepared in PBS, which adds a preparation step. In addition, the dye might also interfere with other test components, for example regarding drug screenings [4]. Concerning automation systems, stains add unwanted complexity to automated liquid handling processes. Despite that, the most challenging disadvantage might represent the toxicity of TB. According to the European Chemicals Agency (ECHA), TB may cause cancer, genetic defects, and may damage fertility or the unborn child [5]. This poses huge obstacles in terms of the proper disposal and handling of the toxic dye. Frequently, the cells stained with TB are categorized as genetically modified organisms (GMOs) that need to be autoclaved before disposal. Since it is not allowed to autoclave TB, the stained GMOs need to be incinerated at high temperatures. Thus, there is a need to explore an alternative method that overcomes these challenges, while being reliable, time- and cost-effective. Therefore, we aimed to develop a solution using brightfield images of unlabeled suspension cells to assess cell viability. To do so, we used algorithms of machine learning that were initially trained with brightfield images of labeled living and dead cells. The AI models learned from the input data to detect cells and assess their viability based on morphological differences.

MATERIAL

Cell Culture and Staining

- CHO-K1 (adherent Chinease hamster ovary cells); HEK293T (adherent human embryonic kidney cells)
- Ham's F12 medium or DMEM supplemented with 10% (v/v) FCS, 1% (v/v) L-Glutamine, 1% (v/v) Sodium Pyruvate
- Trypsin 0.05%/EDTA 0.02% in PBS, e.g. Gibco
- Trypan Blue (0.4%), Gibco # 15250061
- Calcein-AM, Biolegend # 425201
- Hoechst 33342, Invitrogen # H1399
- Propidium Iodide, Biolegend # 421301
- 96 well plate, Starlab, CytoOne® # CC76827596

Imaging and Analysis

- SYNENTEC's imaging device (here CELLAVISTA® 4K)
- SYNENTEC's YT-SOFTWARE®
- SYNENTEC's AI-SUITE®

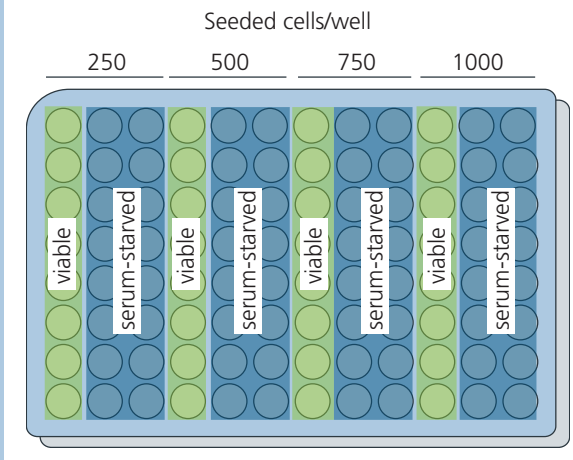


FIG. 1. PLATE LAYOUT

Different cell densities of viable and serum-starved HEK293T cells were seeded in a 96 well plate.

TAB. 1: LIVE/DEAD STAINING

Dye	Stock Concentration	Dilution	Final Concentration
Hoechst 33342	5 mg/mL	1:1,000	5 µg/mL
Propidium Iodide	0.5 mg/mL	1:1,000	5 µg/mL
Calcein-AM	1 mM	1:10,000	0.1 µM

MATERIAL

Cell Culture

We routinely cultured Chinese ovary hamster (CHO-K1) cells in Ham's F12 medium and human embryonic kidney (HEK293T) 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 generally passaged at 70-80% confluence. To do so, cells were detached with trypsin and counted using SYNENTEC's **Trypan Blue** application (see Trypan Blue application note for details). The appropriate cell number was calculated based on the viable cell density or depending on the assay, the cell density.

Generation of Training and Validation Data

For AI viability training, a population of viable and dead cells was obtained by subjecting cells to a period of starvation, achieved by storing them in a 15 mL falcon tube with a limited amount of medium overnight. The viable cells were obtained from the standard cell culture by trypsinization. Various cell densities of viable or serum-starved cells were pipetted in a 96 well plate each (200 µL/well, FIG. 1). A staining solution, comprising Hoechst 33342, Propidium Iodide, and Calcein-AM was prepared at a 20x concentration and added to the wells (10 µL, TAB. 1). The plates were then incubated for 15-30 min in a standard incubator (37 °C, 5% CO_2, humidified atmosphere) and immediately imaged thereafter.

Imaging and Image Analysis

Imaging was performed using the 10x objective of CELLAVISTA® 4K and the whole well was imaged. Regarding Live/Dead stainings the imaging settings are described in TAB. 2. Depending on the assay, image analysis was performed using the image analysis applications **Trypan Blue**, **Virtual Cytoplasm (2F)** or the AI-SUITE® of YT-SOFTWARE®.

Machine Learning

A state-of-the-art computer vision Deep Learning model, specifically an object detection model, was trained on the training data. Furthermore, the correct localization and classification of each detected cell was validated on the validation data, which was separated from the training data. This step ensured that the quality and accuracy of the model were assessed on unseen, high-quality data. Thus, making the model more robust to changes and fluctuations in the real-world application.

TAB. 2: IMAGE SETTINGS FOR LIVE/DEAD STAININGS

Dye	Excitation LED [nm]	Dichro	Emission [nm]
Hoechst 33342	UV (377/50)	UV	Blue (452/45)
Propidium Iodide	Lime (562/40)	Lime	Lime (593-LP)
Calcein-AM	Blue (475/28)	Blue	Green (530/43)

RESULTS & DISCUSSION

Data Generation for Machine Learning

The first step in the development process of an AI-driven solution for a viability assay was the generation of training data for machine learning. Therefore, we stained CHO-K1 or HEK293T cells having different viabilities with Hoechst 33342, Calcein-AM, and Propidium Iodide (PI) and imaged the cells with CELLAVISTA® 4K. Hoechst 33342 (not shown) stains the nuclei of each cell and was used for general cell identification. Calcein-AM is a non-fluorescent cell-permeant dye that is converted to a highly fluorescent form by intracellular esterases in live cells (FIG. 2A right, green cells). In contrast, PI can only penetrate cells with compromised membranes, such as dead or dying cells (FIG. 2A right, yellow cells). This Calcein-AM/PI dual-staining method is a common technique in cell biology and fluorescence microscopy to distinguish live, dead, and apoptotic

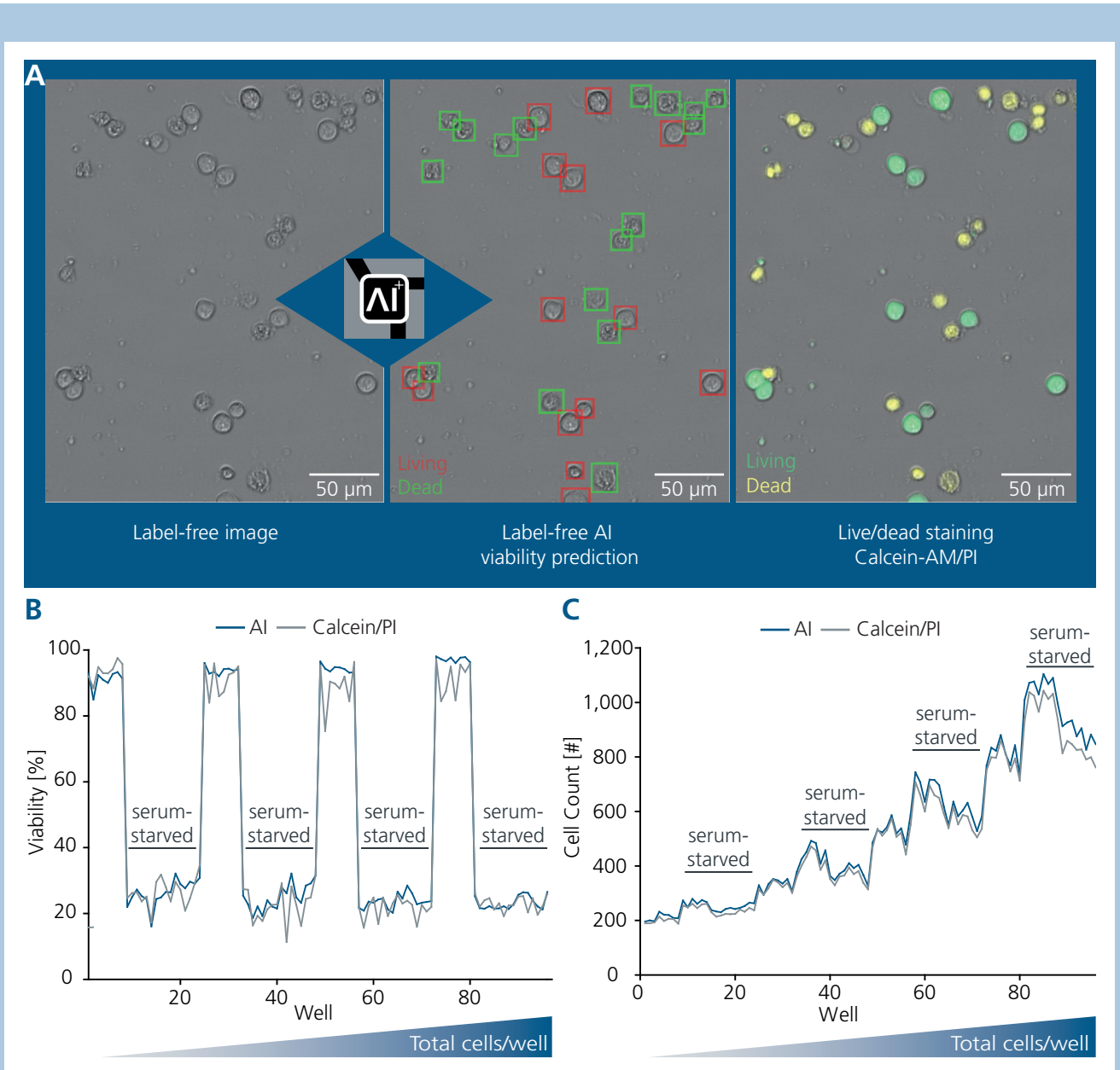


FIG. 2. COMPARISON OF AI VIABILITY AND LIVE/DEAD STAINING

(A) Viable or serum-starved HEK293T cells were seeded in a 96 well plate, stained with Hoechst 33342, Calcein-AM, and Propidium Iodide (PI), and were imaged directly with CELLAVISTA® 4K. Brightfield images were analyzed by AI and fluorescence images utilizing the image analysis application Virtual Cytoplasm (2F). (B) Viability and (C) Cell Count results were plotted to compare both assays. Data from one representative experiment are presented.

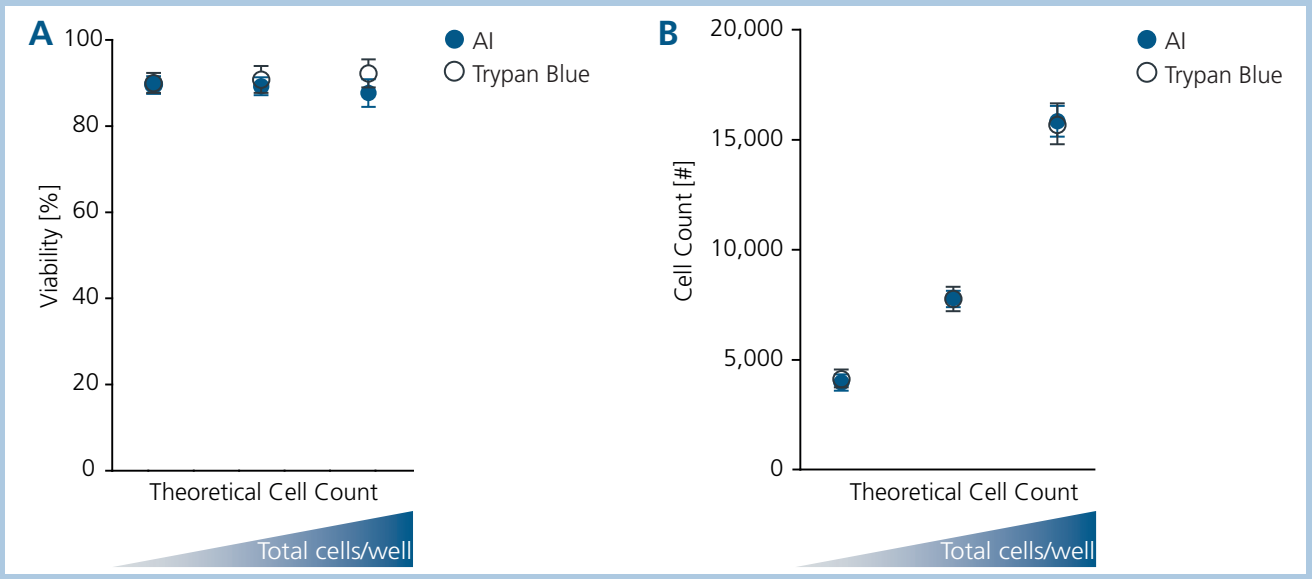


FIG. 3. COMPARISON OF AI VIABILITY AND TRYPAN BLUE ASSAY

(A) CHO-K1 cells were seeded in a 96 well plate and half of the cells were stained with Trypan Blue. Cells were imaged with CELLAVISTA® 4K. Images of unstained cells were analyzed by AI and stained cells using Trypan Blue application. (A) Viability and (B) Cell Count data are presented as mean and standard deviation from one representative experiment performed in 16 technical replicates.

cells within a cell population. Based on this staining, the cells were labeled and assigned to the class living or dead using our in-house software AI-Studio⁺. Utilizing these data, Machine Learning models were trained and subsequently validated.

Live/Dead Staining *versus* AI Viability

In addition, we analyzed the Hoechst 33342/Calcein AM/PI staining with our classical image analysis application **Virtual Cytoplasm (2F)** of YT-SOFTWARE® and compared the results with the data obtained by the AI models (FIG. 2). We compared the AI viability results with the percentage of Calcein-AM positive/PI negative cells (FIG. 2B). Although both data sets exhibited variances, overall, their outcome was similar. Analogous results were observed overlaying the AI *Cell Count* and *Nuclei Count* of the **Virtual Cytoplasm (2F)** application (FIG. 2C). Altogether, *Viability* and *Cell Count* results from both, AI and classical image processing, lead to comparable results, pointing out that machine learning was successful.

Classical Trypan Blue Staining *versus* AI Viability

Following machine learning, we evaluated AI performance in the field during our routine cell culture procedures and compared the results with our classical **Trypan Blue** assay. We seeded different numbers of CHO-K1 cells in a 96 well plate and stained half of the cells with TB afterward. Cells were imaged with CELLAVISTA® 4K and subsequently, the stained cells were analyzed using the image analysis application **Trypan Blue** of YT-SOFWARE®, while unstained cells were analyzed using the AI viability model. Notably, the data unveiled similar results in both *Viability* and *Cell Count* (FIG. 3, TAB. 3). Interestingly, the standard deviation was lower for the AI-analyzed data than for the classical TB assay (FIG. 3, TAB. 3).

TAB. 3: RESULTS FROM AI AND TB ASSAYS

Sample	Viability [%]		Cell Count [#]	
	Artificial Intelligence	Trypan Blue	Artificial Intelligence	Trypan Blue
1	89.94 ± 1.74	90.18 ± 2.33	3,974 ± 4.12%	4,131 ± 6.21%
2	89.48 ± 2.08	91.02 ± 3.13	7,809 ± 2.69%	7,769 ± 5.92%
3	87.87 ± 3.19	92.44 ± 3.25	15,830 ± 3.76%	15,664 ± 5.61%

CONCLUSION

It's elegantly simple: capture brightfield cell images with our automated imagers NYONE® or CELLAVISTA® and analyze them using our YT-SOFTWARE®, empowered by an integrated AI tool to predict cell viability. Making cell staining redundant, the assay is time- and cost-effective, while being safe, rapid, and reliable. Moreover, since our AI-based solution is non-invasive to the cells, the *Viability* and *Cell Count* of suspension cells can be monitored over time and the cells can be used in follow-up studies (FIG. 4). In times where the need to explore all possibilities for contributing to greater sustainability is inevitable, this assay has the potential to revolutionize cell culture, research, and clinical applications by avoiding the use of toxic substances and minimizing waste.

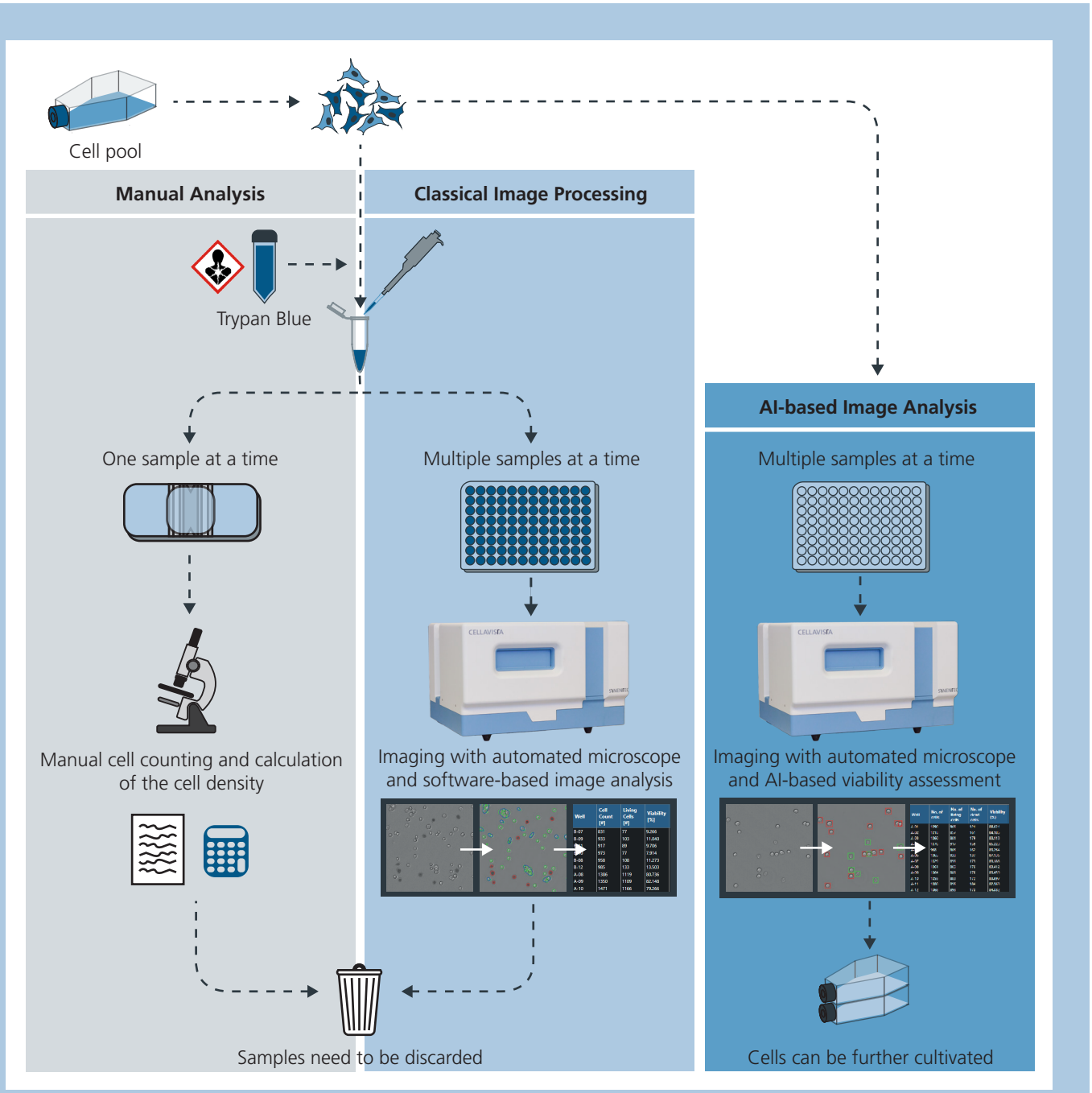


FIG. 4. OVERVIEW OF DIFFERENT VIABILITY ASSAY PROCEDURES

For classical cell counting and viability determination, cells are initially stained with Trypan Blue. In the traditional method, cells are placed in a hemocytometer and are manually counted under a conventional microscope (left). In our previous plate-based approach, cells are measured, and the cell count, and viability are calculated automatically (middle). With the use of AI, cell staining is not required, and samples are measured and evaluated automatically (right).

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Acknowledgement

We thank the Institute for Experimental Cancer Research, especially Prof. Susanne Sebens, for outstanding support, fruitful discussions and a great working atmosphere during this cooperation.

Institut für Experimentelle Tumorforschung

published 12/2023

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