Cell Counting and Viability Assessment of 2D and 3D Cell Cultures: Expected Reliability of the Trypan Blue Assay

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Abstract

Background: Whatever the target of an experiment in cell biology, cell counting and viability assessment are always computed. The Trypan Blue (TB) assay was proposed about a century ago and is still the most widely used method to perform cell viability analysis. Furthermore, the combined use of TB with a haemocytometer is also considered the standard approach to estimate cell population density. There are numerous research articles reporting the use of TB assays to compute cell number and viability of 2D and 3D cultures. However, the literature still lacks studies regarding the reliability of the TB assay in terms of assessment of its repeatability and reproducibility.

Methods: We compared the TB assay’s measurements obtained by two biologists who analysed 105 different samples in double-blind for a total of 210 counts performed. We measured: (a) the repeatability of the count performed by the same operator; (b) the reproducibility of counts performed by the two operators.

Results: There were no significant differences in the results obtained with 2D and 3D cell cultures: we estimated an approximate variability of 5% when the TB assay was used to assess the viability of the culture, and a variability of around 20% when it was used to determine the cell population density.

Conclusions: The main aim of this study was to make researchers aware of potential measurement errors when TB is used with a haemocytometer for counting and viability measurements in 2D and 3D cultures. We believe that these results can help researchers to determine whether the expected reliability of the TB assay is compliant with their applications.

Keywords: Microscopy, Oncology, Cell viability, Haemocytometer, Statistical analysis

Background

The evaluation of cell population density (i.e. the total number of living cells in the culture) and cell viability (i.e. the percentage of living cells in the sample) is fundamental during biology studies [1]. The majority of laboratories engaged in cell biology routinely perform cell viability and counting analysis for different purposes, ranging from ecosystem investigation [2] to proliferation studies [3], in both 2D (two-dimensional) [4] and 3D (three-dimensional) cell cultures [5].

Among the various typologies of 3D cell cultures, multicellular tumour spheroids are those typically used for testing drugs and radiation treatments [6]. The measurement of viability and the reduction of cancer culture population are fundamental parameters for evaluating the efficacy of the treatments under investigation [7]. Accordingly, the reliability of the method used to estimate these parameters plays a key role in this analysis [8]. In addition, cell counting and viability assessment often need to be performed for other 3D cell cultures, such as stem cell spheroids generated for regenerative medicine purposes [9], and organoids used to study (some) organ characteristics [10].

Many different methods (e.g. AlamarBlue® and MTT assay) and systems (e.g. Bio-Rad TC20™ Automated Cell Counter, ChemoMetec NucleoCounter®, Beckman Coulter...
Vi-CELL™ XR Cell Viability Analyzer [11]) can be used to
analyse cell viability [12]. Most of these share the same
approach: the cells are stained using a light (or a fluores-
cent) dye to highlight dead cells (or living cells), and a
detection system counts the number of cells highlighted,
in addition to the total number of cells. Finally, cell viabil-
ity is computed as the percentage of healthy cells in the
sample [13]. However, the Trypan Blue (TB) dye exclusion
assay [14], the first method proposed in the literature, is
considered the standard cell viability measurement method
[15] and is still the most widely used approach [16]. Fur-
thermore, TB paired with a haemocytometer grid (Fig. 1) is
regarded as the standard approach for estimating the cell
population density [17], i.e. the total number of living cells
in the culture [18].

TB was synthesised for the first time in 1904 by Paul
Ehrlich (Nobel prize in medicine, 1908) and was first
used for clinical analysis before becoming a standard
probe in biology. Today it is still widely used for several
medical purposes such as the visualization of the lymph-
associated primo vascular system [19] and of the anterior
capsule during cataract surgery [20]. Chemically, TB is
defined as toluidine-derived dye characterized by a
molecular weight of 960 Da [15]. Its chemical construc-
tion is \( C_{34}H_{28}N_{6}O_{14}S_{4} \). Azidine Blue, Benzamine Blue,
Chlorazol Blue, Diamine Blue, and Niagara Blue are syno-
yms for TB. TB is a cell membrane-impermeable mol-
ecule and therefore only enters cells having compromised
membrane. From a practical point of view, with TB the
cell viability is determined indirectly by detecting cell
membrane integrity [21]. Upon entry into the cell, TB
binds to intracellular proteins and in brightfield the dead
cells appear blue (apoptotic and necrotic cells are not
distinguished [1]), whereas the colour of living cells
remains unchanged (Fig. 1c).

Over the past two decades a number of studies com-
paring TB with other assays have been published [15]
and several methods have proven more efficient than TB
[22], especially those using fluorescent dyes [23]. The
use of TB has, in fact, several drawbacks [24]: (a) TB exerts a toxic effect on cells after a short exposure
period, thus limiting cell counting to only a brief period
after staining [25]; (b) As TB binds to cellular proteins,
there is a potential for binding to non-specific cellular
artifacts, especially in primary cells from clinical sam-
ple; (c) There is a large number of false positives, i.e.
“dead cells” resulting from irreversible damage to their
membrane, and false negatives from cells that have
already initiated the apoptotic pathway but still have
intact membranes; (d) There is no standardized TB
concentration for the measurement of cell viability; (e)
Manual counting using a haemocytometer and a light
microscope is time-consuming and operator-dependent.
Although the TB assay requires the use of a fluorescence
microscope, it has long been known that several fluores-
cent dyes are more reliable indicators of cell viability
than the more traditional coloured dyes [26]. For ex-
ample, Acridine Orange (AO) and Propidium Iodide (PI)
stainings have been shown to be more accurate in
detecting live and dead cells than TB [27]. AO is a
membrane-permeable cationic dye that binds to nucleic
acids of viable cells. At low concentrations it causes a
green fluorescence. PI is impermeable to intact mem-
branes but readily penetrates the membranes of nonvia-
ble cells and binds to DNA or RNA, causing orange
fluorescence. When AO and PI are used simultaneously,
viable cells fluoresce green and nonviable cells fluoresce
orange under fluorescence microscopy. Notwithstanding,
TB is still the most commonly used dye for cell viability
analysis because it is inexpensive, easy to use, it reacts
quickly, and can be visualized with a standard brightfield
microscope available in all biological laboratories [2]. TB
is also used in several automatic counters [28] and as
the reference method for comparing customized cell-
counting algorithms [29]. However, in-depth validation
studies of the TB assay used in combination with a
haemocytometer in viability and counting measurements
are lacking. Several articles have provided statistical

![Fig. 1 Haemocytometer grid containing cells stained with TB. a Picture of a Kova glasstic slide with grids (Hycor Biomedical Inc.). Each slide contains 10 counting chambers. b Schematic representation of the grid of a counting chamber. c Cells in brightfield are characterized by very low contrast. This magnified real-world detail shows some living and dead cells. In particular, a and b show the typical appearance of a living and a dead cell (stained with TB), respectively.](image-url)
analyses on its reliability. In 1964, Tennant [30] and Hathaway et al. [31] performed preliminary studies comparing TB, eosin Y and AO for the determination of the viability of in vitro and in vivo cultures. Twenty years later, Jones and Senft [26] also considered fluorescein diacetase (FDA) and PI. In 1999, Leite et al. [32] extended the research into this area, comparing the reliability of TB, AO and six other methods (i.e. Giemsa staining, ethidium bromide, PI, Annexin V, TUNEL assay and DNA ladder). In 2000, Mascotti et al. [27] published an in-depth comparison between AO/PI and TB assays in which the viability of 7 aliquots of hematopoietic progenitor cells (HPC) and the percentage of viable cells was calculated as the average of 5 viability measurements performed by two operators. However, as the raw counting data was not reported, it was not possible to quantitatively infer the repeatability (intra-rater reliability) and reproducibility (inter-rater reliability) of the counts. The first study on the repeatability and reproducibility of the TB assay appeared in 2011 when Sanfilippo et al. [33] assessed the reliability of TB and calcein AM/ethidium homodimer-1 (CaAM/EthD-1) staining in fresh and thawed human ovarian follicles. Measurements were performed by two independent operators. Reliability was evaluated by the intraclass correlation coefficient (ICC) and the differences between paired measurements were tested by the Wilcoxon signed-rank test. TB proved to be the more reliable staining method to evaluate follicle viability. However, the operators only evaluated 10 samples simultaneously. Finally, in 2015 Cadena-Herrera et al. [34] validated a manual, semi-automated, and fully automated TB exclusion-based methods. A single operator counted several samples in triplicate and the results obtained did not reveal a significant difference between the automated methods and the manual assay. However, 3D cell cultures were not taken into account and no considerations about measurement errors between different operators were made.

In this work we studied repeatability and reproducibility with the specific aim of assessing measurement errors occurring when TB is used in counting and viability applications in 2D and 3D cell cultures. Repeatability is the closeness of the agreement among subsequent measurements of the same object carried out under the same measurement conditions. Reproducibility is defined as the closeness of the agreement among measurements of the same object carried out under different measurement conditions [35]. In particular, the viability and total number of living cells of the culture were the "objects" being measured in our experiments. Thus, the operators performing the measurements represented the changing "condition" when assessing reproducibility. In practical terms, each operator generated and analysed 5 different samples from the same 13 2D cell cultures and 8 3D cell cultures (i.e. multicellular spheroids), making a total of 10 samples considered for each culture. Repeatability for each culture was evaluated by calculating the variability of the measurements obtained by the single operator. Conversely, reproducibility for each culture was estimated by comparing the measurements obtained by two operators. Overall, 210 samples were analysed (Table 1).

The main aim of this work was to make researchers aware of the measurement errors that can occur when the TB assay is used to evaluate population and viability of 2D and 3D cell cultures. Given that this is a preliminary study, global accurate overall accuracy values of assay reliability used in different contexts and with different cell lines cannot be provided. However, we believe that our findings can help researchers to evaluate whether the expected repeatability and reproducibility of the TB assay are compliant with those required by their own application.

**Methods**

**2D Cell Cultures**

To assess the TB reliability we prepared 8 25-cm² flasks (called A₁, i = 1, ..., 8) containing A549 cells (cells at the 36th passage) and 5 25-cm² flasks (called P₁, k = 1, ..., 5) containing PANC-1 cells (cells at the 116th passage). A549 and PANC-1 are well known and widely used commercial cancer cell lines (American Type Culture Collection - ATCC, Rockville, MD, USA). A549, a lung adenocarcinoma cell line of regular-shaped cells, was adhesion-cultured in Kaighn’s modification of Ham’s F-12 medium (F12 K, ATCC) and supplemented with 10% fetal bovine serum (FBS, EuroClone, Milan, Italy), 1% penicillin/streptomycin (GE Healthcare, Milan, Italy) and 2% amphotericin B (Euroclone). PANC-1, an epithelial cell line derived from a human pancreatic carcinoma of ductal cell origin, was grown in medium composed of DMEM/Ham’s F12 (1:1) (Euroclone) supplemented with 10% fetal calf serum (FCS, Euroclone), 2 mM glutamine (Euroclone) and 10 mg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA). All the cells were maintained in an incubator at 5% CO₂ humidified atmosphere at 37 °C and checked periodically for mycoplasma contamination using the MycoAlertTM Mycoplasma Detection Kit (Lonza, Basel, Switzerland). Once detached from the surface of the flask, cells started losing their morphology and gradually became round.

All flasks A₁ were prepared simultaneously in the morning and kept in the incubator for 24 h. Then, as previously done by Cadena-Herrera et al. [34], each flask A₁ was subjected to a different thermal shock to differentiate the cell viability between flasks. A₁ and A₂ were simply moved from the incubator to a sterile laminar flow hood at room temperature. A₃ and A₄ underwent a freeze-thaw cycle (incubator at 37 °C, freezer at -80 °C) and were then returned once to the incubator at 37 °C.
| Table 1 | Original measurements for all $S_k$ analysed by $O_1$ and $O_2$ |
|---------|----------------------------------------------------------------|
|         | $O_1$                             | $O_2$                             |
|         | Live cells | Dead cells | Viability [%] | Live cells | Dead cells | Viability [%] |
| A₁      | S₁         | 271        | 39           | 87.42      | 306        | 33          | 90.27       |
|         | S₂         | 330        | 51           | 86.61      | 339        | 41          | 89.21       |
|         | S₃         | 327        | 37           | 89.84      | 297        | 28          | 91.38       |
|         | S₄         | 363        | 24           | 93.80      | 345        | 23          | 93.75       |
|         | S₅         | 336        | 40           | 89.36      | 394        | 30          | 92.92       |
| A₂      | S₁         | 234        | 92           | 71.78      | 325        | 77          | 80.85       |
|         | S₂         | 178        | 57           | 75.74      | 320        | 71          | 81.84       |
|         | S₃         | 176        | 48           | 78.57      | 274        | 53          | 83.79       |
|         | S₄         | 250        | 67           | 78.86      | 204        | 55          | 78.76       |
|         | S₅         | 442        | 102          | 81.25      | 244        | 50          | 82.99       |
| A₃      | S₁         | 277        | 114          | 70.84      | 218        | 79          | 73.40       |
|         | S₂         | 259        | 108          | 70.57      | 241        | 87          | 73.48       |
|         | S₃         | 297        | 111          | 72.79      | 309        | 101         | 75.37       |
|         | S₄         | 253        | 76           | 76.90      | 220        | 182         | 54.73       |
|         | S₅         | 247        | 86           | 74.17      | 178        | 64          | 73.55       |
| A₄      | S₁         | 248        | 84           | 74.70      | 364        | 137         | 72.65       |
|         | S₂         | 326        | 121          | 72.93      | 390        | 136         | 74.14       |
|         | S₃         | 173        | 53           | 76.55      | 407        | 133         | 75.37       |
|         | S₄         | 303        | 105          | 74.26      | 343        | 119         | 74.24       |
|         | S₅         | 301        | 106          | 73.96      | 364        | 122         | 74.90       |
| A₅      | S₁         | 131        | 119          | 52.40      | 202        | 145         | 58.21       |
|         | S₂         | 130        | 113          | 53.50      | 218        | 227         | 48.99       |
|         | S₃         | 143        | 64           | 69.08      | 110        | 24          | 82.09       |
|         | S₄         | 166        | 64           | 72.17      | 172        | 49          | 77.83       |
|         | S₅         | 166        | 83           | 66.67      | 259        | 68          | 79.20       |
| A₆      | S₁         | 91         | 12           | 88.35      | 162        | 88          | 64.80       |
|         | S₂         | 46         | 35           | 56.79      | 116        | 76          | 60.42       |
|         | S₃         | 81         | 33           | 71.05      | 83         | 40          | 67.48       |
|         | S₄         | 93         | 49           | 65.49      | 100        | 48          | 67.57       |
|         | S₅         | 101        | 50           | 66.89      | 128        | 60          | 68.09       |
| A₇      | S₁         | 198        | 206          | 49.01      | 108        | 103         | 51.18       |
|         | S₂         | 244        | 267          | 47.75      | 165        | 126         | 56.70       |
|         | S₃         | 208        | 163          | 56.06      | 249        | 190         | 56.72       |
|         | S₄         | 207        | 130          | 61.42      | 177        | 146         | 54.80       |
|         | S₅         | 146        | 120          | 54.89      | 201        | 174         | 53.60       |
| A₈      | S₁         | 111        | 181          | 38.01      | 142        | 200         | 41.52       |
|         | S₂         | 147        | 294          | 33.33      | 121        | 220         | 35.48       |
|         | S₃         | 178        | 179          | 49.86      | 199        | 220         | 47.49       |
|         | S₄         | 169        | 137          | 55.23      | 129        | 142         | 47.60       |
|         | S₅         | 147        | 118          | 55.47      | 106        | 128         | 45.30       |
| P₁      | S₁         | 107        | 11           | 95.24      | 100        | 5           | 90.68       |
|         | S₂         | 80         | 8            | 96.25      | 77         | 3           | 90.91       |
|         | S₃         | 101        | 9            | 95.18      | 79         | 4           | 91.82       |
Table 1 Original measurements for all $S_k$ analysed by $O_1$ and $O_2$ (Continued)

| $S_k$ | $P_1$ | $S_1$ | 100 | 69 | 59.17 | 133 | 82 | 61.86 |
|-------|-------|-------|-----|----|-------|-----|----|-------|
| $S_2$ | 116 | 106 | 52.25 | 94 | 56.63 |
| $S_3$ | 136 | 88 | 60.71 | 72 | 64.86 |
| $S_4$ | 116 | 87 | 57.14 | 100 | 71.43 |
| $S_5$ | 163 | 96 | 62.93 | 80 | 64.00 |
| $P_2$ | $S_1$ | 55 | 48 | 54.17 | 39 | 53.40 |
|       | $S_2$ | 57 | 44 | 43.48 | 30 | 56.44 |
|       | $S_3$ | 49 | 44 | 51.04 | 49 | 52.69 |
|       | $S_4$ | 40 | 30 | 55.65 | 69 | 57.14 |
|       | $S_5$ | 38 | 42 | 57.43 | 85 | 47.50 |
| $P_3$ | $S_1$ | 14 | 116 | 11.59 | 8 | 10.77 |
|       | $S_2$ | 13 | 91 | 9.26 | 5 | 12.50 |
|       | $S_3$ | 15 | 127 | 16.22 | 12 | 10.56 |
|       | $S_4$ | 18 | 138 | 10.26 | 8 | 11.54 |
|       | $S_5$ | 11 | 71 | 13.33 | 10 | 13.41 |
| $P_4$ | $S_1$ | 55 | 48 | 54.17 | 39 | 53.40 |
|       | $S_2$ | 57 | 44 | 43.48 | 30 | 56.44 |
|       | $S_3$ | 49 | 44 | 51.04 | 49 | 52.69 |
|       | $S_4$ | 40 | 30 | 55.65 | 69 | 57.14 |
|       | $S_5$ | 38 | 42 | 57.43 | 85 | 47.50 |
| $S_2$ | $S_1$ | 155 | 120 | 56.36 | 66 | 47.48 |
|       | $S_2$ | 125 | 94 | 57.08 | 125 | 63.78 |
|       | $S_3$ | 158 | 87 | 64.49 | 103 | 58.19 |
|       | $S_4$ | 154 | 75 | 67.25 | 85 | 55.56 |
|       | $S_5$ | 156 | 81 | 65.82 | 219 | 55.30 |
| $S_3$ | $S_1$ | 167 | 42 | 79.90 | 117 | 86.67 |
|       | $S_1$ | 191 | 40 | 82.68 | 97 | 88.18 |
|       | $S_2$ | 128 | 41 | 75.74 | 180 | 88.67 |
|       | $S_3$ | 109 | 39 | 73.65 | 113 | 84.33 |
|       | $S_4$ | 146 | 34 | 81.11 | 130 | 85.53 |
| $S_4$ | $S_1$ | 101 | 71 | 58.72 | 58 | 63.74 |
|       | $S_2$ | 114 | 65 | 63.69 | 163 | 72.77 |
|       | $S_3$ | 92 | 60 | 60.53 | 141 | 75.81 |
|       | $S_4$ | 92 | 53 | 63.45 | 124 | 67.39 |
|       | $S_5$ | 179 | 77 | 69.92 | 121 | 68.36 |
| $S_5$ | $S_1$ | 260 | 96 | 73.03 | 140 | 71.07 |
|       | $S_2$ | 207 | 88 | 70.17 | 282 | 86.24 |
|       | $S_3$ | 232 | 64 | 78.38 | 173 | 76.55 |
A5 and A6 underwent the same procedure twice, and A7 and A8, three times. For each freeze-thaw cycle, A3, A5 and A7 were kept in the freezer for 15 min, and A4, A6 and A8 for 30 min. Of note, the thermal shocks were carried out sequentially in the morning and the counting measurements were performed for all the flasks in the afternoon of the same day.

We used gemcitabine, a well-known chemotherapeutic agent used to treat several tumours, including pancreatic cancer [36], to modulate the viability of the cells contained in the different Pk. All Pk were prepared simultaneously on the same morning and gemcitabine was tested at scalar concentrations of 5 μM (flask P2), 50 μM (P3), 500 μM (P4), and 1000 μM (P5). P1 contained untreated cells. An exposure time of 1 h followed by a 72-h washout was chosen on the basis of peak plasma levels defined in recent pharmacokinetic studies [37].

3D Cell Cultures
The A549 cells described in Section 2.1 were also used to produce the multicellular spheroids. Several systems and methods are available to generate in vitro multicellular spheroids of different dimensions [38]. We used a rotatory cell culture system, the RCCS-8DQ bioreactor (Synthecon Inc., Houston, TX, USA), which is capable of controlling up to 4 rotating chambers, even at different speeds. The rotator bases were placed inside a humidified, 37 °C, 5% CO2 incubator and connected to power supplies on the external side of the incubator. All activities were performed in sterile conditions under a laminar flow hood, as previously described [7]. Briefly: a single cell suspension of about 1 × 10⁶ cells/ml was placed in a single 50-ml rotating chamber at an initial speed of 12 rpm (rpm), increasing as the size of the spheroids increased to avoid aggregate sedimentation within the culture vessels. The culture medium was changed every 4 days. After 15 days the spheroids had reached a diameter of 0.5–1 mm and were transferred (one spheroid/well) under a sterile laminar flow hood to 96-well low-attachment culture plates (Corning Inc., Corning, NY, USA), each well previously filled with 100 μl of fresh culture medium. After the spheroidization time (i.e. 1 week [7]), each spheroid was imaged in brightfield using an inverted Olympus IX51 widefield microscope equipped with an Olympus UPlanFl 4×/0.13na as a standard objective lens and endowed with a Nikon Digital SightDS-Vi1 camera (CCD vision sensor, square pixels of 4.4 μm side length, 1600 × 1200 pixel resolution, 3-channel images, 8-bit grey level). For spheroids with partially out-of-focus borders, we acquired a z-stack of brightfield images and reconstructed a single 2D image fully in-focus by using the open-source tool previously described [39]. We then vignetting corrected the images with CIDRE [40], segmented the spheroids using AnaSP [41], and computed their volume by ReViSP [42, 43]. To assess TB reliability, eight compact spheroids with regular shape but a different volume (called SPi, i = 1, ..., 8, Fig. 2) were transferred to a different plate and digested into single cells using a Trypsin/EDTA 1× solution (Euroclone, Milan, Italy) [44].

Sample Preparation
We used a haemocytometer (Kova glass slide with grids, Hycor Biomedical Inc., Fig. 1b) and a commercially
available TB preparation (TB solution 0.4%, SIGMA-ALDRICH, Buchs, Switzerland) to perform the counts. A detailed description of the protocol adopted with TB is reported in [11, 21] and [45]. In brief, for each Ai we:

1) detached the cells from the flask by trypsinization;
2) centrifuged the cell suspension for 5 min at 1200 rpm;
3) resuspended the pellet in 1 ml of culture media using a pipette to obtain a single-cell suspension;
4) removed an aliquot of 100 μl;
5) added 100 μl of TB solution 0.4% to obtain a final 1:2 dilution;
6) waited for 5 min to allow the TB to stain the dead cells;
7) counted the cells using a haemocytometer and a light microscope;
8) calculated the percentage of viability and number of cells in the culture by considering the final dilution factor.

We followed the same protocol for the different Pk but used a 1:6 dilution. For the different SPi we used the same protocol as that used for Ai but with the pellet resuspended in 200 μl of culture media (not 1 ml, as described in point 3).

Two expert operators (hereafter O1 and O2) performed a double-blind evaluation of the viability and population of a set of 5 single-cell suspensions (S_{k}, k = 1, ..., 5) for each A_i, P_k and SP_i; making a total of 210 samples analysed. Of note, both O1 and O2 prepared their own suspensions for each A_i/P_k/SP_i. Using a Falcon 2 ml serological pipet for each S_k they gently pipetted up and down 30 times in about 15 s to disaggregate all the possible cell clumps before loading a drop into a counting chamber. Differences in viability due to different cultivation/waiting times were avoided by simultaneously counting the samples of the same flask/spheroid in double blind. In particular, the operators used two widefield microscopes with similar optics, located in the same room and used daily for counting applications. The first was an inverted Olympus IX51 widefield microscope equipped with an Olympus UPlanFl 10×/0.30na Ph1 objective infinity corrected, while the second was an inverted Zeiss Axiovert 200 widefield microscope equipped with a Zeiss Achromplan 10×/0.25na Ph1 objective infinity corrected. Both microscopes were used in brightfield, and the Köhler illumination alignment [46] was performed in advance.

Sources of Error for Counting Measurements
Several sources of error contributed to the variability in the counts performed with the TB assay and can be summarized as follows (https://chemometec.com/manual-cell-counting/):

1) Subjective definition of a “cell”: There are guidelines but no well defined rules to help an operator define a cell. From a practical point of view, distinguishing a cell from cell debris or other particles is often challenging, even for an expert biologist.
2) Subjective perception of a “dead cell”: With TB there is no official colour threshold for discriminating between a dead cell and a living one. Individual operators performing the manual count has a certain specific set of criteria to define the threshold of brightness of the stain in order to count a cell as being viable or not. Such interpersonal differences in the manual identification of dead cells are crucial for defining the percentage of viability of the cell culture.
3) Dilution and pipetting errors: The final sample of cells to be counted is the result of several dilutions
of the original cell culture. Small pipetting errors substantially influence the final estimation of the cell population density because they concatenate and contribute to the end result as multiplicative factors.

4) Time per sample: Counting cells at the microscope is tedious and time-consuming. In addition, and cells die due to the cytotoxic effect of TB and so, all the samples should be analysed at exactly the same time. However, standardization of the counting time is not possible because it is based on the number of cells in the sample.

5) Samples with a “right” number of cells: Even a few mismatches of dead cells can strongly influence the final evaluation of culture viability if the sample analysed with the haemocytometer contains a low number of cells. On the other hand, samples containing too high a number of cells can can lead to an incorrect estimation of cell population density because it is difficult to remember the cells that have been counted when using a haemocytometer with a grid that has only a few reference lines.

Statistical Analysis
The reproducibility and repeatability of the TB assay was measured by analysing the 210 counts performed by $O_1$ and $O_2$. In particular, for cell viability we computed the mean and standard deviation (i.e., $\mu$ and $\sigma$ values of the different $S_k$) of the percentage of living cells estimated by $O_1$ and $O_2$ for each $A_i$ (results reported in Table 2), $P_k$ (Table 5) and $SP_i$ (Table 8). As for the cell population density assessment, we estimated the mean and coefficient of variation (i.e., $\mu$ and CV of the different $S_k$) of the total number of living cells for each $A_i$ (Table 3), $P_k$ (Table 6) and $SP_i$ (Table 9). Specifically, we first computed $\mu$ and $\sigma$ of the 5 $S_k$ analysed by each operator for each $A_i/P_k/SP_i$, and then computed the CV values.

Table 2 Cell viability ($\mu$ and $\sigma$) estimated by $O_1$ and $O_2$ for the different $A_i$

| $A_i$ | Percentage of living cells [%] | $\mu$ | $\sigma$ | $O_1$ | $\mu$ | $\sigma$ | $p$-value |
|------|--------------------------------|------|--------|------|------|--------|----------|
|      |                                |      |        |      |      |        |          |
| $A_1$| 89.41                          | 2.79 | 91.51  | 1.86 | 0.31 |
| $A_2$| 77.24                          | 3.62 | 81.65  | 1.96 | 0.06 |
| $A_3$| 73.06                          | 2.61 | 70.10  | 8.64 | 1.00 |
| $A_4$| 74.48                          | 1.33 | 74.26  | 1.03 | 1.00 |
| $A_5$| 62.76                          | 9.18 | 69.26  | 14.75| 0.42 |
| $A_6$| 69.71                          | 11.64| 65.67  | 3.20 | 0.84 |
| $A_7$| 53.83                          | 5.57 | 54.60  | 2.32 | 0.84 |
| $A_8$| 46.38                          | 10.16| 43.48  | 5.10 | 0.55 |
| Average|                                | 5.86 |        | 4.86 |        |

$\mu$ mean, $\sigma$ standard deviation

Finally, we calculated the absolute percentage error ($E\%$) of the values obtained by the two operators, defined according to Eq. 1:

$$E\% = \left| \frac{v_1 - v_2}{v_1} \right| \times 100.$$  

For cell viability and total number of living cells, $v_1$ and $v_2$ are the mean values estimated by $O_1$ and $O_2$, respectively, while $v_{12}$ is the mean value estimated considering all 10 samples for each $A_i/P_k/SP_i$ analysed by the two operators. Finally, a two-sided Wilcoxon rank-sum test was used to compare the values obtained by the different operators for both cell viability and total number of living cells. MATLAB (©, The MathWorks, Inc., Natick, Massachusetts, USA) was used for statistical analysis. $p$-values < 0.05 were considered significant. The results obtained from the $A_i$ analysis are reported in Tables 2, 3, and 4. Tables 5, 6, and 7 report the results for $P_k$, and Tables 8, 9, and 10 show the results for $SP_i$.

Results
Analysis of the 2D Cell Cultures
We used the $\sigma$ values obtained for $A_i$ and $P_k$ to estimate the intra-rater reliability of cell viability (Tables 2 and 5, respectively). Given that cell viability is computed as a percentage, the standard deviation can be considered a direct estimation of the error that may occur when TB is used to estimate cell viability. All $\sigma$ values were lower than 15% for both $O_1$ and $O_2$. Furthermore, the average $\sigma$ values were approximately 5% for $A_i$ and 3% for $P_k$ (last row of Table 2 and Table 5, respectively), indicating the high reliability of the TB assay when used for this purpose. With regard to the inter-rater reliability of cell viability we considered the $E\%$ values reported in the
second column of Tables 4 and 7. It is worthy of note that the mean cell viability values estimated by \( O_1 \) and \( O_2 \) for each \( A_i/P_k \) were fairly similar (from left, the second and the forth column of Table 2 and Table 5). Accordingly, \( E\% \) values reported in Table 4 and Table 7 were very low, i.e. <10\%, and their average was <5\% (last row, second column of Table 4 and Table 7).

Conversely, both the intra- and inter-rater variability values obtained for the total amount of living cells were particularly high. Being the total amount of cells computed as the absolute value, we estimated the intra-rater variability by analysing the CV values for all \( A_i/P_k \), considering the different \( S_k \) counted by the operators. The majority of CVs reported in Table 3 and Table 6 were >15\%, which is fairly surprising. In particular, \( O_1 \) obtained a CV <10\% twice (i.e. for \( A_3 \) and \( P_2 \)) and \( O_2 \) only once (i.e. for \( A_4 \)). Furthermore, the average CV values (bottom row of Table 3 and Table 6) were particularly high (around 20\%) for both operators. Similarly, as the amount of living cells estimated by \( O_1 \) and \( O_2 \) for each \( A_i/P_k \) differed substantially (second and forth column of Table 3 and Table 6), the majority of \( E\% \) values reported in the third column of Table 4 and Table 7 were especially high. In particular, the average \( E\% \) (bottom row, right-hand column of Table 4 and Table 7) was >15\% for both \( A_1 \) and \( P_k \). These results, paired with the previously described high intra-rater variability, unexpectedly revealed a poor ability of the TB assay to estimate cell population density.

However, many of the \( p\)-values computed for both viability and total number of living cells were >0.05, this proving that the sets of counts obtained by \( O_1 \) and \( O_2 \) for the same \( A_i/P_k \) did not differ significantly from each other. In actual fact they differed in one only case for \( A_1 \) (Table 3, row \( A_4 \)), and in three cases for \( P_k \) (Table 5, row \( P_1 \) and Table 6, rows \( P_2 \) and \( P_3 \)). The differences obtained by the two operators in these cases were probably caused by a pipetting/resuspending error. For example, the data in Table 1 clearly show that the number of cells counted by \( O_1 \) for \( A_4 \) was significantly lower and more variable than those counted by \( O_2 \). However, a \( p\)-value <0.05 in 4 out of 26 cases simply means that, despite the high intra-rater reliability of the TB assay, especially when used for cell population density assessment, the sets of counts performed by different operators did not, in general, differ statistically.

### Analysis of the 3D Cell Cultures

The results obtained from the analysis of the 3D cell cultures were similar to those obtained for the 2D cultures.

### Table 4

| \( A_i \) | \% of living cells | Total number of living cells |
|----------|-------------------|----------------------------|
| \( A_1 \) | 2.32              | 3.26                       |
| \( A_2 \) | 5.55              | 6.57                       |
| \( A_3 \) | 4.13              | 13.37                      |
| \( A_4 \) | 0.29              | 32.12                      |
| \( A_5 \) | 9.85              | 26.52                      |
| \( A_6 \) | 5.98              | 35.36                      |
| \( A_7 \) | 1.43              | 10.83                      |
| \( A_8 \) | 6.46              | 7.59                       |
| Average  | 4.50              | 16.95                      |

| \( E\% \) absolute percentage error |

### Table 5

| \( O_i \) | \( \mu \) | \( \sigma \) | \( O_2 \) | \( \mu \) | \( \sigma \) | \( p\)-value |
|----------|----------|----------|----------|----------|----------|-------------|
| \( P_1 \) | 91.55    | 0.71     | 95.58    | 0.43     | 0.01     |             |
| \( P_2 \) | 87.93    | 2.23     | 84.60    | 5.04     | 0.55     |             |
| \( P_3 \) | 81.28    | 2.74     | 76.41    | 3.48     | 0.06     |             |
| \( P_4 \) | 53.43    | 3.83     | 52.35    | 5.49     | 1.00     |             |
| \( P_5 \) | 11.75    | 1.20     | 12.13    | 2.74     | 1.00     |             |
| Average  |         | 2.14     |         | 3.44     |           |             |

| \( \mu \) mean, \( \sigma \) standard deviation |

### Table 6

| \( P_k \) | \( \mu \) | \( CV \) [%] | \( O_1 \) | \( \mu \) | \( CV \) [%] | \( p\)-value |
|----------|----------|--------------|----------|----------|--------------|-------------|
| \( P_1 \) | 88.20    | 17.41        | 81.80    | 15.97    | 0.42         |
| \( P_2 \) | 109.80   | 7.77         | 91.00    | 12.09    | 0.04         |
| \( P_3 \) | 57.60    | 19.97        | 50.60    | 13.52    | 0.42         |
| \( P_4 \) | 47.08    | 17.96        | 55.40    | 41.22    | 0.88         |
| \( P_5 \) | 14.20    | 18.23        | 8.60     | 30.32    | 0.02         |
| Average  |         | 2.14         |         | 3.44     |             |             |

| \( E\% \) absolute percentage error | Total number of living cells | \( p\)-value |

### Table 7

| \( P_k \) | \% of living cells | Total number of living cells |
|----------|-------------------|----------------------------|
| \( P_1 \) | 4.31              | 7.53                       |
| \( P_2 \) | 2.04              | 18.73                      |
| \( P_3 \) | 6.18              | 12.94                      |
| \( P_4 \) | 3.18              | 16.28                      |
| \( P_5 \) | 5.48              | 49.12                      |
| Average  | 3.91              | 20.91                      |

| \( E\% \) absolute percentage error |
Only one p-value (Table 8 row SP3) was <0.05, which again indicates that the measurements obtained by O1 and O2 did not differ significantly.

All σ values reported in Table 8 were <15%, and the average σ were 4.84% and 4.23% for O1 and O2 respectively, once more confirming the high repeatability of the TB assay when used to estimate the viability of 2D and 3D cell cultures. The E% values reported in the second column of Table 10 were >15%, with an average E% of 17.23%. Notably, the CV value obtained by O2 for SP6, SP5, SP6, SP7 was triple that obtained by O1 because the total number of living cells counted by O2 for these SPi was much more variable than that of the counts performed by O1. Specifically, the σ of the counts performed by O2 was more than twice that of the counts performed by O1.

With regard to the analysis of cell population density, both intra- and inter-rater variability were once again exceptionally high. The majority of CVs reported in Table 9 were >20%, O2 never obtaining a CV <20%, and O1 only twice obtaining a value <10% (i.e. for SP2, SP6). Similarly to what happened for the 2D A549 cell cultures, the amount of living cells estimated by O1 for SPi differed substantially from that obtained by O2 (second column vs forth column, Table 9). Consequently, most of the E% values reported in the third column of Table 10 were >15%, with an average E% of 17.23%. Notably, the CV value obtained by O2 for SP6, SP5, SP6, SP7 was triple that obtained by O1 because the total number of living cells counted by O2 for these SPi was much more variable than that of the counts performed by O1.

Furthermore, O1 counted a lower number of cells than O2 for all but SP4, probably because there were more cell clusters in the samples prepared by O2 that must not be considered when counting with a haemocytometer (here, we remark that each operator prepared her/his own 5 SP). This resulted in a lower μ of the number of living cells counted by O2 which negatively contributed to the estimation of the CV values. Although both operators are biologists with more than 10 years’ experience in counting cells, the results are suggestive of a greater ability of O1 to resuspend the samples generated from 3D spheroids, effectively disgregating the cell clusters. This is indicative of the high subjectivity of the TB assay and of its poor reliability when used to estimate the total number of cells in a culture. However, as happened for the 2D cell cultures, almost all p-values computed for viability and total number of living cells were >0.05, once more proving that the sets of counts obtained by the different operators did not significantly differ from each other.

**Discussion**

In this work we studied repeatability and reproducibility of cell population and viability measurements obtained with the TB assay. We asked two experienced biologists to count the live and dead cells of 105 different samples of 2D and 3D cell cultures in a double blind manner.
(total 210 counts). Our aim being to measure: (a) the repeatability of the count performed by the same operator; (b) the reproducibility of counts performed by the two operators.

We estimated an approximate variability of 5% for both 2D and 3D cell cultures when the TB assay is used to assess the viability of the culture, and a variability of around 20% when it was used to determine the cell population density, i.e. total number of living cells in the culture. Our results show that, whilst the method is quite precise when used to assess viability, it is fairly unreliable at estimating the population of a cell culture, whether 2D or 3D. In practice, our findings serve to alert researchers evaluating cell culture populations that they should expect to find an appreciable difference between measurements (up to 20%) when performed by different operators.

Conclusions
The TB assay was introduced about a century ago and is still the most widely used method to perform viability and population assessments of cell cultures. However, no study has been published so far with regard to deep validation of the TB assay, especially for viability and counting measurements of 3D cell cultures.

The main aim of the statistical analyses performed in this work was to provide researchers with novel information on TB reliability and to make them aware of expected measurement errors when the assay is used to evaluate population and viability of 2D and 3D cell cultures. The results obtained prove that (a) there is no significant difference between 2D and 3D cell cultures as far as TB reliability is concerned; (b) the TB method is precise when used for viability assessments of a cell culture; (c) the method is fairly inaccurate at estimating cell population density, despite it is routinely used for this purpose in numerous laboratories.

For the sake of clarity we repeat that as mentioned before, the purpose of our work was not to provide overall accuracy of the reliability of an assay used in different contexts and with different cell lines. Nevertheless, once these performances are known and acknowledged, it will be up to researchers to determine when the TB assay can be used and whether the expected reliability of its measurements is compliant with their own experiments.

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Authors’ Contributions
FP, AT and AB conceived the study. AT and CA performed the experiments. FP prepared the figs. FP and AB performed the statistical analysis. FP and AT discussed the results and prepared the manuscript. CA and AB helped with the manuscript revision. All authors read and approved the final manuscript.

Competing Interests
The authors declare that they have no competing interests.

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