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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 MMT assay) and systems (e.g. Bio-Rad TC20™ Automated Cell Counter, ChemoMetec NucleoCounter®, Beckman Coulter

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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 fluorescent) 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 viability 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]. Furthermore, 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 construction is $C_{34}H_{28}N_6O_{14}S_4$. Azidine Blue, Benzamine Blue, Chlorazol Blue, Diamine Blue, and Niagara Blue are synonyms for TB. TB is a cell membrane-impermeable molecule 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 comparing 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 samples; (*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 fluorescent dyes are more reliable indicators of cell viability than the more traditional coloured dyes [26]. For example, 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 membranes but readily penetrates the membranes of nonviable 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

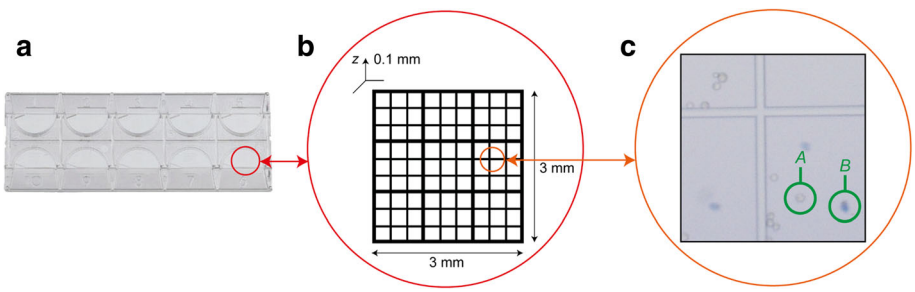

Fig. 1 Haemocytometer grid containing cells stained with TB. **a** Picture of a Kova glasstic slide with grids (Hycor Biomedical Inc.). Each slide contains 10 counting chambers. **b** Schematic representation of the grid of a counting chamber. **c** Cells in brightfield are characterized by very low 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

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-cm^2 flasks (called A_i, i = 1, …, 8) containing A549 cells (cells at the 36th passage) and 5 25-cm^2 flasks (called P_k, 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 epithelioid 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% CO_2 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 *Ai* 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 A_i was subjected to a different thermal shock to differentiate the cell viability between flasks. A_1 and A_2 were simply moved from the incubator to a sterile laminar flow hood at room temperature. A_3 and A_4 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 [%]
A_1	S_1	271	39	87.42	306	33	90.27
	S_2	330	51	86.61	339	41	89.21
	S_3	327	37	89.84	297	28	91.38
	S_4	363	24	93.80	345	23	93.75
	S_5	336	40	89.36	394	30	92.92
A_2	S_1	234	92	71.78	325	77	80.85
	S_2	178	57	75.74	320	71	81.84
	S_3	176	48	78.57	274	53	83.79
	S_4	250	67	78.86	204	55	78.76
	S_5	442	102	81.25	244	50	82.99
A_3	S_1	277	114	70.84	218	79	73.40
	S_2	259	108	70.57	241	87	73.48
	S_3	297	111	72.79	309	101	75.37
	S_4	253	76	76.90	220	182	54.73
	S_5	247	86	74.17	178	64	73.55
A_4	S_1	248	84	74.70	364	137	72.65
	S_2	326	121	72.93	390	136	74.14
	S_3	173	53	76.55	407	133	75.37
	S_4	303	105	74.26	343	119	74.24
	S_5	301	106	73.96	364	122	74.90
A_5	S_1	131	119	52.40	202	145	58.21
	S_2	130	113	53.50	218	227	48.99
	S_3	143	64	69.08	110	24	82.09
	S_4	166	64	72.17	172	49	77.83
	S_5	166	83	66.67	259	68	79.20
A_6	S_1	91	12	88.35	162	88	64.80
	S_2	46	35	56.79	116	76	60.42
	S_3	81	33	71.05	83	40	67.48
	S_4	93	49	65.49	100	48	67.57
	S_5	101	50	66.89	128	60	68.09
A_7	S_1	198	206	49.01	108	103	51.18
	S_2	244	267	47.75	165	126	56.70
	S_3	208	163	56.06	249	190	56.72
	S_4	207	130	61.42	177	146	54.80
	S_5	146	120	54.89	201	174	53.60
A_8	S_1	111	181	38.01	142	200	41.52
	S_2	147	294	33.33	121	220	35.48
	S_3	178	179	49.86	199	220	47.49
	S_4	169	137	55.23	129	142	47.60
	S_5	147	118	55.47	106	128	45.30
P_1	S_1	107	11	95.24	100	5	90.68
	S_2	80	8	96.25	77	3	90.91
	S_3	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_4	83	7	95.59	65	3	92.22
	S_5	70	6	95.65	88	4	92.11
P_2	S_1	106	17	86.87	86	13	86.18
	S_2	118	21	90.00	99	11	84.89
	S_3	99	12	87.60	106	15	89.19
	S_4	107	12	80.00	80	20	89.92
	S_5	119	14	78.50	84	23	89.47
P_3	S_1	63	14	77.61	52	15	81.82
	S_2	46	14	74.14	43	15	76.67
	S_3	52	10	81.69	58	13	83.87
	S_4	75	17	72.73	56	21	81.52
	S_5	52	11	75.86	44	14	82.53
P_4	S_1	55	48	54.17	39	33	53.40
	S_2	57	44	43.48	30	39	56.44
	S_3	49	44	51.04	49	47	52.69
	S_4	40	30	55.65	69	55	57.14
	S_5	38	42	57.43	85	63	47.50
P_5	S_1	14	116	11.59	8	61	10.77
	S_2	13	91	9.26	5	49	12.50
	S_3	15	127	16.22	12	62	10.56
	S_4	18	138	10.26	8	70	11.54
	S_5	11	71	13.33	10	65	13.41
SP_1	S_1	100	69	59.17	133	82	61.86
	S_2	116	106	52.25	94	72	56.63
	S_3	136	88	60.71	72	39	64.86
	S_4	116	87	57.14	100	40	71.43
	S_5	163	96	62.93	80	45	64.00
SP_2	S_1	155	120	56.36	66	73	47.48
	S_2	125	94	57.08	125	71	63.78
	S_3	158	87	64.49	103	74	58.19
	S_4	154	75	67.25	85	68	55.56
	S_5	156	81	65.82	219	177	55.30
SP_3	S_1	167	42	79.90	117	18	86.67
	S_2	191	40	82.68	97	13	88.18
	S_3	128	41	75.74	180	23	88.67
	S_4	109	39	73.65	113	21	84.33
	S_5	146	34	81.11	130	22	85.53
SP_4	S_1	101	71	58.72	58	33	63.74
	S_2	114	65	63.69	163	61	72.77
	S_3	92	60	60.53	141	45	75.81
	S_4	92	53	63.45	124	60	67.39
	S_5	179	77	69.92	121	56	68.36
SP_5	S_1	260	96	73.03	140	57	71.07
	S_2	207	88	70.17	282	45	86.24
	S_3	232	64	78.38	173	53	76.55

Table 1 Original measurements for all S_k analysed by O_1 and O_2 *(Continued)*

	S_4	192	56	77.42	209	53	79.77
	S_5	263	75	77.81	69	24	74.19
SP_6	S_1	222	65	77.35	175	41	81.02
	S_2	226	66	77.40	229	59	79.51
	S_3	216	53	80.30	108	29	78.83
	S_4	218	54	80.15	135	37	78.49
	S_5	205	44	82.33	254	43	85.52
SP_7	S_1	134	101	57.02	159	93	63.10
	S_2	161	128	55.71	235	124	65.46
	S_3	151	134	52.98	83	70	54.25
	S_4	180	106	62.94	134	97	58.01
	S_5	190	119	61.49	91	78	53.85
SP_8	S_1	146	197	42.57	67	105	38.95
	S_2	178	221	44.61	110	144	43.31
	S_3	110	159	40.89	188	241	43.82
	S_4	68	120	36.17	124	171	42.03
	S_5	157	214	42.32	127	154	45.20

A_5 and A_6 underwent the same procedure twice, and A_7 and A_8, three times. For each freeze-thaw cycle, A_3, A_5 and A_7 were kept in the freezer for 15 min, and A_4, A_6 and A_8 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 P_k. All P_k 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 wash out 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% CO_2 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 1×10^6 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 SP_i, $i = 1, \ldots, 8$, 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 glasstic slide with grids, Hycor Biomedical Inc., Fig. 1b) and a commercially

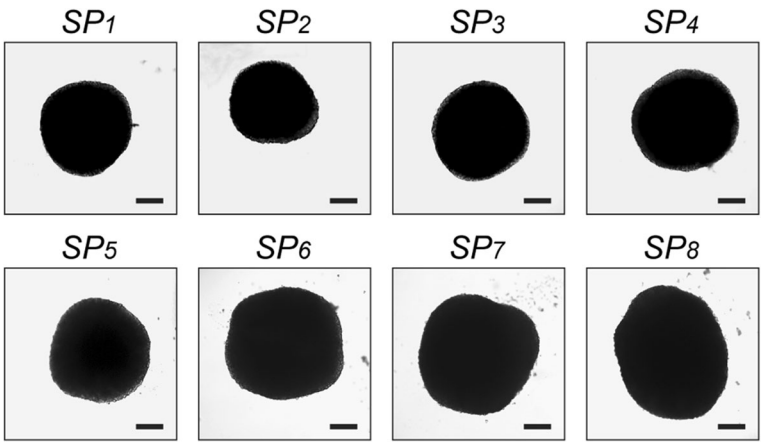

Fig. 2 Multicellular cancer spheroids obtained from lung cancer cells (line A549), built using a RCCS-8DQ bioreactor (Synthecon Inc., Houston, TX, USA). Scale bar 200 μm

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 P_k 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 O_1 and O_2) performed a double-blind evaluation of the viability and population of a set of 5 single-cell suspensions (S_k, $k = 1, \ldots, 5$) for each A_i, P_k and SP_i; making a total of 210 samples analysed. Of note, both O_1 and O_2 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 Achroplan 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., μ and σ 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., μ 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 μ and σ 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 (μ and σ) estimated by O_1 and O_2 for the different A_i

	Percentage of living cells [%]				*p*-value
	O_1		O_2		
	μ	σ	μ	σ	
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	

μ mean, σ standard deviation

Table 3 Cell population density (μ and CV) estimated by O_1 and O_2 for the different A_i

	Total number of living cells				*p*-value
	O_1		O_2		
	μ	CV [%]	μ	CV [%]	
A_1	325	10.32	336	11.40	0.69
A_2	256	42.61	273	18.75	0.42
A_3	267	7.64	233	20.63	0.15
A_4	270	22.72	373	6.70	0.01
A_5	147	12.17	192	28.96	0.13
A_6	82	26.16	118	25.43	0.10
A_7	201	17.57	180	28.63	0.55
A_8	150	17.22	139	25.67	0.38
Average	//	19.55	//	20.77	

μ mean, *CV* coefficient of variation

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

$$E\% = \left|\frac{\nu_1 - \nu_2}{\nu_{12}}\right| \cdot 100. \quad (1)$$

For cell viability and total number of living cells, ν_1 and ν_2 are the mean values estimated by O_1 and O_2, respectively, while ν_{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 *Ai* 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 *SPi*.

Results

Analysis of the 2D Cell Cultures

We used the σ 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 σ values were lower than 15% for both O_1 and O_2. Furthermore, the average σ 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

Table 4 *E*% computed between the μ value estimated by O_1 and O_2 for the different A_i

	E%	
	Percentage 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

second column of Tables 4 and Table 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

Table 5 Cell viability (μ and σ) estimated by O_1 and O_2 for the different P_k

	Percentage of living cells [%]				*p*-value
	O_1		O_2		
	μ	σ	μ	σ	
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	

μ mean, σ standard deviation

Table 6 Cell population density (μ and CV) estimated by O_1 and O_2 for the different P_k

	Total number of living cells				*p*-value
	O_1		O_2		
	μ	CV [%]	μ	CV [%]	
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	//	16.27	//	22.62	

μ mean, *CV coefficient of variation*

especially high. In particular, the average *E*% (*bottom row, right-hand column* of Table 4 and Table 7) was >15% for both A_i 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_i (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_5). 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 7 *E*% computed between the μ value estimated by O_1 and O_2 for the different P_k

	E%	
	Percentage of living cells [%]	Total number of living cells
P_1	4.31	7.53
P_2	3.86	18.73
P_3	6.18	12.94
P_4	2.04	16.28
P_5	3.18	49.12
Average	3.91	20.91

E% absolute percentage error

Table 8 Cell viability (μ and σ) estimated by O_1 and O_2 for the different SP_i

	Percentage of living cells [%]				*p*-value
	O_1		O_2		
	μ	σ	μ	σ	
SP_1	58.44	4.06	63.76	5.35	0.15
SP_2	62.20	5.10	56.06	5.88	0.10
SP_3	78.62	3.79	86.67	1.81	0.01
SP_4	63.26	4.26	69.61	4.73	0.06
SP_5	75.36	3.60	77.56	5.80	0.69
SP_6	79.50	2.13	80.68	2.88	0.69
SP_7	58.02	4.12	58.93	5.21	0.69
SP_8	41.31	13.05	42.66	2.36	0.54
Average	//	5.01	//	4.25	

μ mean, σ standard deviation

Only one *p*-value (Table 8 row SP_3) was <0.05, which again indicates that the measurements obtained by O_1 and O_2 did not differ significantly.

All σ values reported in Table 8 were <15%, and the average σ were 4.84% and 4.23% for O_1 and O_2, *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 slightly higher than those of Table 4 and Table 7, suggesting poorer reproducibility of cell viability values for 3D cultures (but still around 5%).

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%, O_2 never obtaining a CV <20%, and O_1 only twice obtaining a value <10% (i.e. for SP_2 and SP_6). Similarly to what happened for the 2D A549 cell cultures, the amount of living cells estimated by O_1 for SP_i differed substantially from that obtained by O_2 (*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 O_2 for SP_2, SP_5, SP_6, SP_7 was triple that obtained by O_1 because the total number of living cells counted by O_2 for these SP_i was much more variable than that of the counts performed by O_1. Specifically, the σ of the counts performed by O_2 was more than twice that of the counts performed by O_1. Furthermore, O_2 counted a lower number of cells than O_1 for all but SP_4, probably because there were more cell clusters in the samples prepared by O_2 that must not be considered when counting with a haemocytometer (here, we remark that each operator prepared her/his own 5 S_k). This resulted in a lower μ of the number of living cells counted by O_2 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 O_1 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 it 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.

Table 9 Cell population density (μ and CV) estimated by O_1 and O_2 for the different SP_i

	Total number of living cells				*p*-value
	O_1		O_2		
	μ	CV [%]	μ	CV [%]	
SP_1	126	19.19	96	24.60	0.07
SP_2	150	9.25	120	49.91	0.17
SP_3	148	21.69	127	24.86	0.42
SP_4	116	31.63	121	32.28	0.50
SP_5	231	13.64	175	45.34	0.31
SP_6	217	3.65	180	34.10	0.69
SP_7	163	13.72	140	43.72	0.33
SP_8	132	32.89	123	35.26	0.74
Average	//	18.21	//	36.26	

μ mean, *CV* coefficient of variation

Table 10 *E*% computed between the μ value estimated by O_1 and O_2 for the different SP_i

	E%	
	Percentage of living cells [%]	Total number of living cells
SP_1	8.70	27.38
SP_2	10.38	22.29
SP_3	9.75	15.09
SP_4	9.56	4.89
SP_5	2.88	27.73
SP_6	1.46	18.71
SP_7	1.55	15.01
SP_8	3.22	6.74
Average	5.94	17.23

E% absolute percentage error

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.

Abbreviations

TB: Trypan blue; 2D: Two-dimensional; 3D: Three-dimensional; Da: Dalton; AO: Acridine orange; PI: Propidium iodide; FDA: Fluorescein diacetase; HPC: Hematopoietic progenitor cells; CaAM: Calcein AM; EthD-1: Ethidium homodimer-1; ICC: Intraclass correlation coefficient; ATCC: American type culture collection; F12 K: Ham's F-12 medium; FBS: Fetal bovine serum; °C: Degree celsius; RCCS : Rotatory cell culture system; rpm: Revolutions per minute; mm: Millimetre; µl: Microlitre; CCD: Charge-coupled device; SP_i: Spheroid *i*; *O*: Operator; na: Numerical aperture; µ: Mean; σ: Standard deviation; CV: Coefficient of variation; *E%*: Absolute percentage error; v_i: Mean value estimated by O_i; ANOVA: One-way analysis of variance

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Availability of Data and Materials

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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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Ethics Approval and Consent to Participate

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