

Morphology-Dependent Bias in the Enumeration of Adherent Cells

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Abstract

Enumeration and characterisation of cell populations are critical in fundamental research, clinical diagnostics, and biotechnology. Accurate cell enumeration is central to many biological assays which require the development of complex experimental protocols. Currently, interlaboratory comparability between cell measurements is challenging due to poor traceability in cell analysis which is further compounded by diverse morphologies, growth behaviour, and inherent heterogeneity of adherent cell populations. This study assessed five adherent cell types with distinct morphological characteristics using common cell enumeration methods including haemocytometry, bio-imaging, conventional and image-enhanced flow cytometry. Results revealed that enumeration accuracy between methods was highly dependent on cell type and demonstrated the method-dependent bias that variations in cell morphology introduce. Cell clustering and morphological characteristics were identified as a major source of discrepancy in suspension-based counting approaches, which could be mitigated by employing image-enhanced flow cytometry to quantify the number of cells in cluster events. These findings demonstrate that cell morphology is a key determinant of cell countability and should be considered when selecting enumeration methodologies, assay development and establishment of standardised adherent cell enumeration workflows.

1. Introduction

Accurate cell enumeration is fundamental to cell culture research, biotechnology, biomanufacturing and cell based therapeutic development. Cell number concentration measurements frequently underpin subsequent analyses, including the quantification of proliferating, quiescent, or apoptotic cells in critical quality attribute (CQA) assessment, and the enumeration of marker expression in clinical characterisation assays (4, 5). Furthermore, many cell-based assays produce outputs which must be normalised with respect to the cell number concentration to allow for data comparability (6). Therefore, inaccuracies in cell counting can influence downstream analyses resulting in variability in assay performance, and reduced measurement reliability in bioprocessing workflows. The ability to produce accurate measurements of cell number concentration between laboratories is one of the most prominent challenges currently facing the field of cell measurement science due to diverse morphological, physiological and growth characteristics exhibited across different cell types. Development of robust, validated cell counting methodologies would benefit life science communities spanning clinical and industrial sectors, including in cell-based biomanufacturing and cell therapeutics (1–3). Accuracy in number concentration estimation is therefore an important consideration not only for improving interlaboratory comparability and reproducibility but also for fundamental analytical setup and application (7).

It is well recognised that there is a need for improved accuracy and better standardisation of methods related to cell counting, cell identification, and characterisation of biological activity (8–12). The metrological and standards communities are working to address this challenge, including through the publication of several ISO guidelines (ISO 20391-1, ISO 20391-2) and subsequent studies have been

conducted to determine their efficacy (13, 14). However, early activities have generally focused on the quantification of suspension cells and do not address the challenges associated with the quantification of adherent cells. The diverse tissue and pathological origins of adherent cells result in additional complexities such as variable growth characteristics and morphological features. Nutrient availability, cell density, cell-cell interactions, and cell aging are additional factors which are known to influence cell morphology and can further impact their ability to be effectively counted. This presents challenges in accurate number concentration estimation due to the limitations of individual counting techniques (15). Many established cell counting methodologies must measure adherent cells after detachment to enable their analysis in suspension. However, these suspended adherent cells can contain cell clusters, cell debris, and fragments which introduce a counting bias that is inherently linked to the morphological properties of the cells being analysed.

The choice of cell counting method is typically dictated by the wider assay requirements, with flow cytometry, bioimaging, and haemocytometry being commonly employed techniques. In clinical practice, flow cytometry is widely used to identify and quantify specific cell populations using volumetric measurements (10, 16, 17); however, counting uncertainties can arise from coincidence events, thresholding, gating strategies, and the inability to account for clustered detached cells (5, 18). For drug discovery and bioactivity studies, bioimaging is a popular alternative offering the ability to analyse adherent cells directly, without detachment. Determination of cell number concentrations, often expressed as cells/cm², is typically performed through the counting of cell nuclei stained with DAPI or Hoechst 33342. The incorporation of additional organelle staining also enables further assessment of cell morphological changes (19–22). Despite this, bioimaging-based cell counting is subject to user bias, segmentation errors, and significant variability due to limited standardisation. Ongoing efforts by groups such as QUAREP-LIMI and metrological organisations including ISO Biotechnology and CCQM CAWG (P197) aim to improve data quality, comparability, and traceability (13, 23–29). Haemocytometry, which also requires detachment, remains the gold standard for routine cell culture where cells are counted (manually or automated) in a defined area of known volume (30, 31). This technique is limited by low sampling volumes, preparation uncertainties, and a high limit of detection although recent advances incorporating fluorescent nuclear and viability stains alongside performance beads have improved precision and traceability (32–34).

Although previous studies have evaluated the performance of individual cell counting methods, little attention has been given to understanding how cell morphology influences enumeration accuracy across different counting platforms. This knowledge gap is particularly important as adherent cell types frequently display distinct morphological and growth characteristics that may affect measurement performance in different ways. Five diverse adherent cell types were selected for this study to cover a range of biotechnological applications, disease states, growth characteristics, and morphologies: HeLa, CHO, MCF-7, MRC-5, and HUVEC. HeLa cells are a widely used immortalised cervical carcinoma cell line used for applications such as viral infection and replication studies, and telomere research (35, 36). Chinese Hamster Ovary (CHO) cells are commonly used for recombinant protein and antibody production due to their resilience to culture variations (37). MCF-7 cells are an immortalised breast

adenocarcinoma cell line often used in cancer models due to their hormone dependence and oestrogen responsiveness (38). MRC-5 cells are a human diploid lung fibroblast cell line commonly used as a substrate for viral vaccine manufacturing, drug screening, and tissue co-culture systems (39, 40). Human Umbilical Vein Endothelial Cells (HUVECs) are a model cell line used for study vascular related processes such as tumour angiogenesis, inflammation, and response to hypoxia (41, 42).

This study aimed to investigate how cellular morphology influences countability, assess agreement between commonly used enumeration methods, and determine factors that contribute to variability in adherent cell measurements. Haemocytometry, bioimaging, and volumetric flow cytometry were each evaluated along with an emerging technique employing the use of imaging flow cytometry. This comparison provides practical guidance for method selection and generates insights relevant to the development of robust and standardised approaches for adherent cell enumeration. Overall, this work has quantified the impact of cell morphology on counting performance and highlighted key factors which must be considered for the development of robust, standardised cell enumeration workflows.

2. Materials and Methods

Comparison of all counting techniques was first performed using cells taken at a single seeding concentration which was determined using the ATCC recommended growth conditions. The Attune CytPix, Countess, and Confocal counting methods were then further compared in a series of experiments which investigate the impact of variable cell seeding densities on cell counting. A diagram depicting the experimental counting comparison workflow for each technique is shown in Fig. 1.

2.1 Cell Culture

HeLa (ATCC-CCL-2), MCF-7 (ATCC-HTB-22), MRC-5 (CCL-171), and CHO-K1 (CCL-61) cells were all purchased from ATCC and maintained in DMEM (Gibco, high glucose, 41965-039) or F-12K (CHO, Gibco, 21127-002) complete with 10% Foetal Bovine Serum (FBS, Gibco, 10500-064), 1% Penicillin-Streptomycin (Gibco, 15140-122), and 1% Glutamax (Gibco, 35050-038). HUVECs (PromoCell, C-12203) were purchased from PromoCell and grown using Endothelial Growth Medium (PromoCell, C-22110) complete with the growth supplement pack and 2% FBS. In preparation for well-plate seeding, cells were washed with PBS (Gibco, 12190-144), detached using 1X TrypLE (Gibco, 12563-011), then neutralised with complete media. The Countess 2 haemocytometer was then used to determine seeding concentration. Cells were seeded in flat bottomed 24-well plates (ThermoFisher, 142475) for flow cytometry and haemocytometry, and flat bottomed #1.5 thickness 24-well plates for bioimaging (CellVis, P24-1.5P) to enable imaging using short working distance objectives. Cell concentrations for the single-density, multi-instrument counting comparison study (section 3.1) were HeLa (8×10^4 cells/mL), MCF-7 (1.2×10^5 cells/mL), MRC-5 (4×10^4 cells/mL), CHO (4×10^4 cells/mL), and HUVEC (4×10^4 cells/mL). For the variable-density, single-instrument comparison study (section 3.2), the four seeding densities selected for each cell type were: HeLa ($2, 4, 8, 12 \times 10^4$ cells/mL), MCF-7 ($4, 8, 12, 16 \times 10^4$ cells/mL), MRC-5 ($0.5, 1, 2, 4 \times 10^4$ cells/mL), CHO ($1, 2, 4, 8 \times 10^4$ cells/mL), and HUVEC ($1, 2, 4, 8 \times 10^4$ cells/mL). Whilst

uncertainties in the cell counts will be present from the use of an automated hemocytometer, the use of serial dilutions allowed for consistent ratios of cells to be seeded. As results were being normalised within each repeat, it was not necessary to consider the limitations of this counting technique at this stage.

2.2 Haemocytometry Counting

Both manual and automatic haemocytometer analysis was conducted. Media was removed from each well then cells were washed briefly with PBS. 300 μL of 1X TrypLE was then added to each well to detach the cells. Once fully detached, 700 μL of DMEM was added to neutralise the TrypLE, giving a total volume of 1 mL per well. 100 μL of cell suspension was removed for Haemocytometry-based counting. Each of the 4, 4 x 4 grids were counted and the number of cells per 4x4 grid averaged. This was repeated, giving a total of four averaged manual measurements of cell count. A Countess II automatic cell counter (Invitrogen) was used to perform cell counting. Serially diluted Countess Test Beads (Invitrogen, C10284) were measured at $10^4 - 10^6$ /mL and values plotted to verify the linear working range of the instrument. Each chamber of a disposable Countess counting chamber slide (Invitrogen C10228) was filled with 10 μL of cell suspension. Slides were input into the Countess which automatically determined the optimal exposure and focus settings. Cell counts were then recorded and analysed.

2.3 Flow Cytometry

The remaining 900 μL of cell suspension prepared in section 2.2 was used for flow cytometry-based cell counting. When performing the 7-method counting comparison study, the 900 μL solution was split equally between the 3-laser BRV Attune CytPix and 2-laser BR Beckman Coulter CytoFlex flow cytometers. Instrument validation performed prior to each experiment following manufacturing instructions: Attune Performance Tracking Beads (Thermo Fisher, 4449754) and Cytoflex Daily QC Fluorophores (Beckmann, C65719). Each 450 μL solution was split into three wells of a 96-well plate, each containing 150 μL . 50 μL of dead volume was required per well, therefore the number of cells in 100 μL was recorded. CytoFlex and CytPix measurements were ran at rates of 60 $\mu\text{L}/\text{min}$ and 200 $\mu\text{L}/\text{min}$, respectively. To ensure that cell debris did not impact cell count, a threshold of 70,000 was applied to FSC-A and SSC-A plots in both instruments. No further gates were imposed on recorded events. For image-enhanced flow cytometry (CytPix En.), the CytPix was set to capture images of cells at 6000 images/s. Image analysis was performed using the Attune Image Processing tool built-in to the Attune Cytomic software 6.0. The Particle Analysis tool was capable of identifying the number of cells present in each recorded event. Gates were applied to ParticleCount plots from singlet (1) up to sextet (6) which then allowed for calculation of the total number of cells passing through the flow cell. When analysing dense cell suspensions, the events/s rate often exceeded the maximum images/s acquisition rate. To account for this, the cell cluster fractions determined from the images acquired were applied to the population of cells which were not imaged.

2.4 Bioimage Acquisition

On the day of cell counting, media was removed from each well then cells were briefly washed with 1 mL of PBS (Gibco, 14190-144). Cells were then fixed using 1 mL of 4% paraformaldehyde in PBS (PFA, Thermo Scientific, AAJ19943K2) for 15 minutes on a thermoshaker (Grant-Bio PCMT) at 21 °C. Cell nuclei and membranes were then stained using a 500 µL, 2 µg/mL Hoechst 33342 (Invitrogen, H3570) and 2.5 µg/mL Wheat Germ Agglutinin 594 (WGA, Biotium 29023-1) – PBS solution for 15 minutes at 21 °C. A 3 X PBS wash was then performed, then cells stored in PBS prior to image acquisition. Confocal imaging was performed using a Zeiss LSM880 microscope and a Zeiss Plan-Apochromat 20X/0.8 M27 objective lens. Microscope quality controls were performed using the ImageJ MetroloJ-QC plugin, in accordance with QUAREP-Limi guidelines. Field uniformity was measured using 1 µM NIST-traceable fluorescein (Invitrogen, F36915) to assess objective performance. A laser power meter (PM400, S170C Thorlabs) was used to measure laser power at a range of settings, and 5 µm multicolour PS beads were used to determine fluorescence co-localisation. Measurements were performed on a monthly basis to ensure stable microscope performance across all experimental repeats. Hoechst 33342 was excited using a 405 nm diode laser (4%) and emission detected using a PMT ($\lambda = 410\text{--}556$ nm) with a gain of 580. WGA was excited using a 561 nm DPSS laser (6%) and emission detected using a PMT ($\lambda = 585\text{--}733$ nm) with a gain of 570. To ensure bioimaging sufficiently sampled the cell culture population, three separate sets of images were acquired which were set equidistant across the centre of each well. Each set of images consisted of a 'cell ruler'-type configuration, where a vertical set of images 1 x 10 were captured – producing a 0.425 x 3.87 mm (1024 x 9318 px) image. Overall, this resulted in 2.6% of each well being sampled. Widefield imaging was performed using an EVOS Auto2 microscope using an Olympus SApo 20X/0.75 objective. Stains were excited with an LED light source using DAPI (Ex 357/44 nm, Em 447/60 nm) and RFP (Ex 531/40, Em 593/40) filter cubes. Automated tilescan image acquisition was performed which captured three separate 'cell ruler' image stacks each with 1 x 20 images – producing a 0.26 x 3.88 mm (2080 x 31010 px) image. Overall, this resulted in 6.4% of each well being sampled.

2.5 Bioimage Analysis

CellProfiler (4.2.1) was used to analyse images acquired from confocal and widefield imaging. The 'IdentifyPrimaryObjects' module was used to first identify nuclei in the Hoechst 33342 channel. An adaptive thresholding strategy combined with a Robust Background thresholding method was used to account for variations in fluorescent intensity. Nuclei objects were then used as seed points to identify individual cell membrane boundaries in the WGA channel using 'IdentifySecondaryObjects' again with adaptive, robust background thresholding. Finally, object intensity and shape measurements were performed to output values for the number of nuclei and area covered by cell membranes. Image analysis pipeline segmentation performance was assessed by overlaying nuclei and membrane outlines onto the original image. Cell confluency was calculated by determining the percentage of the imaged area covered by cell membrane areas. For the analysis of the relationship between confluency and cell area, an exponential plateau curve was fitted to each cell type to allow for quantification of this relationship through determination of the exponent rate constant, k_H

2.6 Statistical Analysis

Data from each instrument, time point, and seeding density was compiled into excel files. Columns included count, well, plate, type (instrument type), replicate, seeding density, and timepoint. R (4.2.1) was used to perform statistical analysis of the data. As the dataset consisted of both fixed effects (counting methodology) and random effects (plate, well nested within plate, and replicate), a linear mixed effect model was used to estimate the means by replicate. A natural log transformation Then the residual variation was stratified by method, which was found to be necessary for a well-fitting model. To compare absolute cell-count values, it was necessary to back-transform the log-transformed estimates and standard errors using the following equation:

$$s = \bar{x} \sqrt{\exp s_{log}^2 - 1}$$

Where s_{log} is the standard deviation on the log scale, s is the corresponding standard deviation on the linear scale and $\bar{x}$ is the estimated mean in counts per well. Values were then normalised with respect to the confocal cell counts per well, which allowed values for each repeat to be combined. ANOVA and Tukey testing was then performed to determine p-values and statistically significant variations in mean count values.

3. Results

3.1 Impact of Cell Type on Counting Performance

Performance testing of the Countess FLII was performed using Countess test beads serially diluted between $10^4 - 10^6$ beads/mL. Number concentration estimations showed a linear trend (R-square = 0.94) and closely agreed with expected counts within a $5 \times 10^4 - 1 \times 10^6$ bead/mL range (figure S1). Values acquired at 10^4 /mL diverged from linearity and were identified as outliers when subject to robust regression and outlier removal (ROUT), with a Q value of 1 (43). This indicated that the limit of linearity and quantification using the Countess was $> 10^4$ /mL. The initial multi-instrument counting comparison investigated the cell counts acquired from each technique from a single cell seeding concentration (for each cell type), and a single growth time point. 48 hours after seeding, cells were counted following the methods described throughout sections 2.2–2.4. As the dataset contained both fixed effects (counting methodology) and random effects (plate, well nested within plate, replicate), standard errors therefore included plate-to-plate, well-to-well, and random error variability. The error variation was found to be visibly different for each method, with a different residual standard deviation for each. A mixed effects model was used to estimate the means by replicate and a natural log transformation was applied to ensure the distribution was more symmetrical. To ensure a well-fitting model, residual variation was stratified by method due to the variability across methods. The relative standard deviations were calculated for each cell type and method (Fig. 2b). Plotting the normalised residual distributions for each

cell type and method revealed that values were acceptably homogeneous and normally distributed (supplementary figure S2).

Observation of the standard deviation components from random and fixed effects revealed that the variation in well-to-well and plate-to-plate was insignificant when compared with the variation arising from biological replicates and counting methodologies. Cell counts from each method were therefore compared separately for each replicate. $\log_{10}$ counts/well for each method, repeat, and cell type were determined (Fig. 2a) and median values (dotted lines) calculated using the combined data from all methods. Interaction plots are used to visualise how different measurement techniques affect the cell count values across all three repeats (Fig. 3). Parallel, non-intersecting lines in interaction plots indicate no interaction between counting techniques and each repeat, suggesting that counting techniques give consistent counting performance regardless of variations in the true cell count value. Non-parallel intersecting lines suggest the opposite and indicate intrinsic variability in the counts produced by a specific technique or techniques. Analysis of the degree of interaction for each cell type revealed that CHO and MRC-5 data displayed the most parallelism, followed by HUVEC, HeLa, and MCF-7.

Comparison of the combined, confocal-normalised cell counts for all repeats, cell types, and techniques allowed for visualisation of the variance and counting performance for each cell type (Fig. 4). p-values and statistical significance was determined using ANOVA and Tukey tests. In general, the most significant statistical differences were observed between adhered (bioimaging) and suspended (FC and hemocytometry) counting techniques. Calculation of the median count, $\bar{x}$ and expanded ($k = 2$) uncertainty values, SD from confocal-normalised values ($\bar{x} \pm \text{SD}$) revealed that MRC-5 showed the best agreement between techniques (1.01 ± 0.19), followed by HUVEC (0.74 ± 0.37), HeLa (1.32 ± 0.46), CHO (1.29 ± 0.56), and MCF-7 (1.27 ± 0.57).

3.2 Impact of Cell Density on Counting Performance

To further investigate the impact of adherent cellular morphology and growth characteristics on cell countability, a follow-up counting study was conducted which assessed cell counts at four different seeding densities. Whilst previous cell counting studies have evaluated the quality of a cell counting measurement process using serial dilutions, this process cannot be directly applied to cell counting methods in which cells remain adhered to a surface (44). Exploration of a range of seeding densities, centred around the ATCC recommended guidelines for each cell type (25–150%), aimed to use a similar approach to evaluate the efficacy of each counting method in this study. To improve study feasibility, one instrument was taken from each of the three counting techniques: Countess, Confocal, and CytPix - in addition to image-enhanced CytPix measurements. Cells were counted using the same methodologies in the previous section as outlined throughout the methods sections 2.3–2.5. The $\log_{10}$ cell count/well for each cell type, technique, concentration was again determined and mean and median values calculated (Fig. 5a). The separation of the horizontal lines allows for observation of the separation in final, day 3, cell count values as a result of different seeding concentrations. Examination of normalised residual

values (Fig. 5b) revealed that values obtained using flow cytometry were normally distributed across all seeding densities, suggesting that sparse or dense cell cultures did not impact counting precision. Whereas, for haemocytometry and microscopy, several measurements taken from the lowest or highest seeding densities were found to contain residuals which exceeded the generally accepted range of two standard deviations.

The average counts, and associated standard deviations, for each technique, cell type, and seeding densities were determined (Fig. 5c). p-values and statistical significance was again determined using ANOVA and Tukey tests (supplementary Fig. 3). Examination of CHO cell counts at lower seeding densities than previously considered (1 and 2×10^4 cells/cm²), found that all techniques showed close agreement, with no statistically significant differences calculated. Consideration of counts obtained from the highest seeding density (8×10^4 cells/cm²) found increased overall disagreement between values from each technique. Haemocytometry results showed significantly increased variance (1.9 ± 1.2), suggesting that the high cell counts obtained at this seeding density exceeded the optimal, linear working range of the Countess. This is supported by consideration of the absolute values obtained, which ranged between $0.8\text{--}1.6 \times 10^6$ cells/mL. CytPix En. values were found to be significantly lower than confocal values (0.56 ± 0.35 and 1.00 ± 0.16 , respectively), which may be attributed to an overestimation by microscopy or an underestimation from flow cytometry.

Observation of the impact of variable seeding densities on HUVEC counts revealed a similar trend in counts from samples seeded at 2 and 4×10^4 cells/mL, which primarily show an underestimation of total cell count when using conventional flow cytometry, but a significant improvement in agreement with comparative techniques when image-enhanced cluster analysis is performed. Interestingly, the use of cluster analysis resulted in an overestimation of cell counts at the lowest seeding density, but an underestimation of cell counts at the highest seeding density, when compared to other techniques. Overall, HUVEC counts values obtained across all seeding densities displayed high variance or poor agreement between techniques.

MRC-5 cells were previously found to produce the most precise cell counts between techniques, however observation of lower seeding densities found significant variations in values. Values acquired from the most sparsely seeded cell cultures found no statistically significant differences, however this was primarily due to the high variance associated with each set of values, rather than good agreement between the values. HeLa cell counts displayed a relatively consistent trend across many seeding densities, with microscopy values being lower than those obtained by haemocytometry or flow cytometry. Results showed either no (ns) or minor (*) statistically significant differences however, this was often accompanied by high variances in values from each technique. MCF-7 cell counts displayed high variances at low seeding densities which decreased at higher seeding densities. This, however, was not accompanied by increased agreeability between counting techniques.

3.3 Bioimaging-based Cell Counting

To investigate how morphology changed with confluency, and ultimately impact enumeration accuracy, we assessed the average cell area (μm^2) against confluency (%) (Fig. 6a). In general, cells displayed increased cell area at low confluency (< 20%) which decreased as cultures reached high confluency (> 80%). The decrease in cell area corresponded to a decrease in the associated variance, showing that trend towards a more consistent cell size when cell packing, with increased confluency, becomes a factor. MCF and HUVEC cells displayed the most consistent cell areas across a range of confluencies, with a $22 \pm 9\%$ and $28 \pm 9\%$ decrease in area in low and high confluency cultures, respectively. Whereas MRC-5 and HeLa cells displayed the greatest decrease in cell area, with a $50 \pm 30\%$ and $42 \pm 14\%$ decrease, respectively. The overall size and growth characteristics for each cell type is reflected in the trend of count v confluency (Fig. 6c), along with the rate constants, k_H determined from exponential plateau fitting (Fig. 6d). HUVECs were found to reach 100% confluency at the lowest total cell count (approximately 2×10^5 cells/well), conversely, MCF-7 cells failed to reach 100% confluency at cell counts exceeding 6×10^5 cells/well. Images used to determine cell counts (Fig. 6b) show the nuclei (blue) and WGA (red) staining alongside the CellProfiler image analysis outlines of nuclei (pink) and cell membranes (green).

Overlapping cell growth alongside the variability between cell morphologies imposes differing limits of linearity on bioimaging-based cell quantification. The limit of linearity for each cell type was determined by considering root mean square error (RMSE) values taken from linear fits of cell count v confluency (supplementary Figure S4).

3.4 Cell Counting using Conventional and Image Enhanced Flow Cytometry

To further assess the impact of cellular morphology on flow cytometry-derived cell counts and to demonstrate how image-enhanced flow cytometry may mitigate uncertainties associated with manual gating, comparative analyses between conventional and image-enhanced measurements were conducted to support the observations detailed in earlier sections. Side scatter-area (SSC-A) v forward scatter area (FSC-A) and forward scatter height (FSC-H) v FSC-A plots, from CHO (Fig. 7a) and MCF-7 (Fig. 3b) cell acquisitions with the Attune CytPix, demonstrate the heterogeneity found between cell types. The single cell gate shows a typical user-defined gate used to identify the events associated with single-cells based on the linear relationship between signal area and signal height. CHO cells were found to be easily

disaggregated into single cell populations, as confirmed by the high percentage (94.5%) of single cells. Whereas MCF-7 cells were found to remain as clusters, which is shown by the low percentage of single cells (41.6%) and large population deviating from the linear FSC-H v FSC-A trend. Particle Count histograms output from Attune Cytometric image analysis (Fig. 7c) of each cell type, quantify the percentages of events containing cell clusters (Figs. 7a and 7b). FSC-H v FSC-A graphs now showing the distribution of cell clusters, colour coded by particle count gates, display how intermixed single cell and cell cluster populations are (Figs. 7a and 7b).

Image analysis was performed on all cell samples acquired throughout this study and the average cell cluster fractions calculated (Fig. 7d). CHO cells demonstrated the highest fraction of single cells ($82.3 \pm 0.4\%$) whereas MCF-7 and HUVEC cells demonstrated the lowest fractions at $59.0 \pm 0.6\%$ and $54.5 \pm 0.4\%$, respectively (Fig. 7e). The cell cluster fractions were used to calculate the magnitude difference between image-enhanced cell counts when compared to considering all events as single cells. Values were found to be highest for HUVEC (1.75 ± 0.01) and MCF-7 (1.78 ± 0.02), followed by HeLa (1.58 ± 0.02), MRC-5 (1.52 ± 0.01), and CHO (1.15 ± 0.01).

4. Discussion

This study evaluated the enumeration performance of three commonly used techniques across five cell types with distinct morphological and growth characteristics (Fig. 1), and assessed their quantitative performance in relation to a range of cell types and their associated morphological characteristics. Consideration of cell lines with distinct morphologies enabled the assessment of how intrinsic biological variation influences the accuracy and precision of cell counting measurements. Variations in cell size, clustering propensity, and adherent growth characteristics were found to influence counting performance across haemocytometry, bioimaging, and flow cytometry-based approaches. It was also found that differences were not consistent across cell types, indicating that the performance of an enumeration method is highly dependent on biological characteristics of the cell types being measured. These findings demonstrate that cell morphology is an important determinant of countability and suggest that no single enumeration strategy is universally optimal for all adherent cell types - further highlighting the importance of selecting counting methodologies that are appropriate for morphology and culture characteristics of the cell type of interest.

Enumeration performance was initially assessed at a single seeding density for each cell type using log10-transformed cell counts and associated residual variation (Fig. 2). Differences in median cell counts between experimental repeats reflected the inherent biological variability exhibited by the different cell types (Fig. 2a). Residual standard deviations were found to vary both between instrument/technique, as well as between each cell type (Fig. 2b). This indicated that the precision of each cell counting technique was not consistent with each cell type, suggesting that intrinsic cell morphological and growth characteristics directly impacted counting performance. This effect could be further observed in interaction plots which visualized the variation in cell count values for each technique and cell type (Fig. 3). The absence of consistent variation in parallel and intersecting lines between cell types further suggested that counting performance is variable depending on cell type. Together, this data suggests cell type is key factor influencing cell counting performance and that the level of associated error varies for the different methods with cell type. This is more clearly demonstrated when the confocal-normalised median cell counts are plotted (Fig. 4) with varying degrees of statistical significance positive or negative values depending on cell types being measured. Observations made throughout bioimaging (Fig. 6) and flow cytometry-based counting (Fig. 7) aid in explaining results observed when assessing cell counting performance and overall count comparisons. Cells which show proclivity to grow in dense clusters, such as MCF-7, present challenges when microscopy-based

counting is used, due to limitations associated with effective nuclei staining and segmentation analysis. Such growth characteristics were also shown to be independent of overall cell confluency (Fig. 7c), indicating that counting performance is affected across all seeding densities. The presence of cell clusters also negatively impacts conventional flow cytometry-based counting, where user-based gating either discounts a significant population of cell clusters, or inaccurately identifies clusters as single cells (Fig. 7a and 7b). Furthermore, cells which do not show closely associated 2D growth, such as HUVECs, produced significant clusters once detached, due to their ability to form strong cell-to-cell connections. The range over which cell clustering can impact flow cytometry-based counting is illustrated in the evaluation of cell cluster fractions (Figs. 7d and 7e) highlighting the variation due to cellular morphology. Together, these findings indicate that cellular morphology and growth behaviour are major determinants of enumeration performance.

To assess the impact of culture density on cell enumeration, each cell type was seeded at various concentrations spanning 25–150% of the ATCC-recommended seeding density (45–49). Analysis of confocal-normalised residuals (Fig. 5b) and cell count values (Fig. 5c) revealed that counting performance was dependent on both the biological characteristics of the cell type and the density-dependent changes in cell growth and organisation within the culture. Normalised residual values outside of the generally accepted range of two standard deviations were observed for both Confocal and Countess techniques, most prominently cultures seeded with the highest and lowest initial cell concentrations (Fig. 5b). Overall, the density at which cells were seeded did not positively or negatively impact the agreement in cell counting techniques in a uniform manner. The use of image enhanced flow cytometry (CytPix En.) consistently increased the measured cell count to values close to that of the Countess. Whereas, Confocal and conventional flow measurements (CytPix), generally reported values lower than each of the aforementioned techniques. Together, this suggests that the combination of imaging and flow cytometry that image-enhanced flow cytometry offers, presents a means of overcoming the limitations imaging and flow cytometry encounter when used separately. As described previously, conventional flow cytometry is unable to accurately count cells which display a proclivity for cell clustering upon detachment (Fig. 7). Image-enhanced flow cytometry (CytPix En.) presented a means by which the limitations associated with conventional flow cytometry could be overcome, removing the bias and uncertainties introduced through manual gating strategies (54, 55). Cell clustering fractions, a metric which described the adjusted cell concentration values when image-enhanced cytometry was employed, were found to align well with the morphological characteristics of each cell type (Fig. 7). This was further reflected in results throughout the study, which found that manually gated values obtained from CytPix and CytoFlex showed an underestimation of cell counts for cell types susceptible to clustering, whereas CytPix En. cell concentration values showed improved agreement with comparative counting techniques – suggesting an improvement in enumeration efficacy. However, it should be noted that, whilst biological and technical repeats were included, this study was performed in a single laboratory. It is expected that the inclusion of additional metrological institutes would expand the uncertainties associated with each technique due to instrument, analyst, and analysis variabilities. In the case of image-enhanced flow cytometry, this may further highlight the practicality of using

automated flow cytometry analysis tools to overcome user-based reproducibility. Such tools have previously been of interest across the community; however widespread adoption of these tools remains a challenge (56, 57).

As with conventional flow cytometry, bioimaging-based counting was confronted with similar challenges associated with the identification of individual cells in densely growing, overlapping cell cultures (Fig. 6). The impact of this was evident in the single-density study (Fig. 4), where both confocal and widefield-based imaging significantly underestimated cell counting in dense (CHO) or clustered (MCF-7) cultures, when compared to image-enhanced flow cytometry. It is evident that cell morphology and subsequent image analysis challenges are key contributing factors in the limitations observed with this technique. Accurate quantification of the number of cell nuclei is dictated by stain efficacy, image quality, and the choice of segmentation algorithm – presenting a multi-faceted challenge in terms of standardisation. CellProfiler, the tool used throughout this study, is a powerful, customisable software which allows the user to fine tune many of the segmentation algorithm properties. However, as it is user-based, it is also prone to bias which introduces further uncertainties into microscopy-based counting. Several sophisticated image analysis algorithms employing the use of machine learning have been developed which employ the use of thresholding and segmentation techniques, presenting a means of reducing the uncertainties associated with this technique (50–53). AI driven analysis presents its own metrological challenges, due to lack of algorithm traceability, training data bias, and lack of transparency and verifiability. Therefore, fully evaluating the effectiveness of these cell-counting pipelines will require additional coordinated efforts across industry and metrological institutions. Challenges were also presented when quantifying sparsely populated cell cultures, where several cell types, such as MCF-7, display a propensity to proliferate in colonies, leaving large areas devoid of cells. The area required for imaging is therefore dependent on culture homogeneity, making it challenging to suggest a minimum recommended sampling area to ensure accurate total cell populations are calculated. Plots of cell count v confluency (Fig. 6c) reveal the variable limit of linearity for each cell type due to their growth characteristics and cell size. This variability highlights the challenges associated with the production of universal microscopy-based cell counting standards and standardised methodologies, indicating that standards may need to be developed for specific cell types across a set cell density or confluency.

Overall, results highlighted the importance of method selection and cell type selection for practical applications. Accurate cell enumeration is a critical quality attribute (CQA) in many medical and biotechnological applications, influencing downstream processes such as dose accuracy, batch consistency, potency, or toxicity assays (14, 58). It is therefore essential to consider the accuracy and precision of each cell enumeration methodology in relation to its downstream application. Whilst ISO standards, ICH Q8 (R2) Pharmaceutical Development guidelines, and tools like COMET provide frameworks for evaluating cell counting systems, including instrumentation, sample preparation, and data analysis, this study highlights the additional uncertainties introduced from morphological and biological variations (13, 14, 59, 60). Findings throughout the variable density study (section 3.2) show that deviations from ATCC-recommended seeding densities can affect cell count accuracy, particularly when assessed against the linear working ranges of various counting methods. Although cell counting is

not explicitly covered in ICH Q5D: Cell Substrates, this work supports its emphasis on morphological characterisation by demonstrating the importance of optimal seeding densities in monitoring cell growth dynamics. Notably, the study also identifies unique measurement challenges associated with specific cell types, highlighting the importance of selecting appropriate starting materials for research and cell-based therapeutic biomanufacturing. It is evident that any counting standard developed to serve as a reference material must take into consideration the morphology of the cell type in question along with the density at which they are being analysed. Building on the findings of this research, further work can support the refinement of standardised methodologies and reference materials to align with ISO guidelines, ensuring inter-laboratory reproducibility.

5. Conclusions

This study has highlighted how the morphology and inherent biological characteristics of cells can impact their ability to be accurately counted across a range of enumeration techniques. The application of image-enhanced flow cytometry has been shown to overcome the morphology-associated limitations of conventional flow cytometry and thus offers an attractive method of improving the counting performance of cytometry for both adherent and suspension cells. It is evident from the study, that cell morphology and cell growth characteristics must be carefully considered when aiming to develop an adherent cell counting reference method and associated reference material. Our findings demonstrate that cell and method selection should be key considerations when designing experiments where accurate cell counting is critical.

Declarations

Author Contribution

MB, LD, and JR performed the experiments and wrote the main manuscript text MB analysed the data and prepared all of the figures SC performed the statistical analysis JB, SC, and JH reviewed the manuscript

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Data Availability

The datasets supporting the findings of this study, including raw and processed cell enumeration data, are publicly available in the Zenodo repository at [10.5281/zenodo.22912507](https://zenodo.org/record/22912507).

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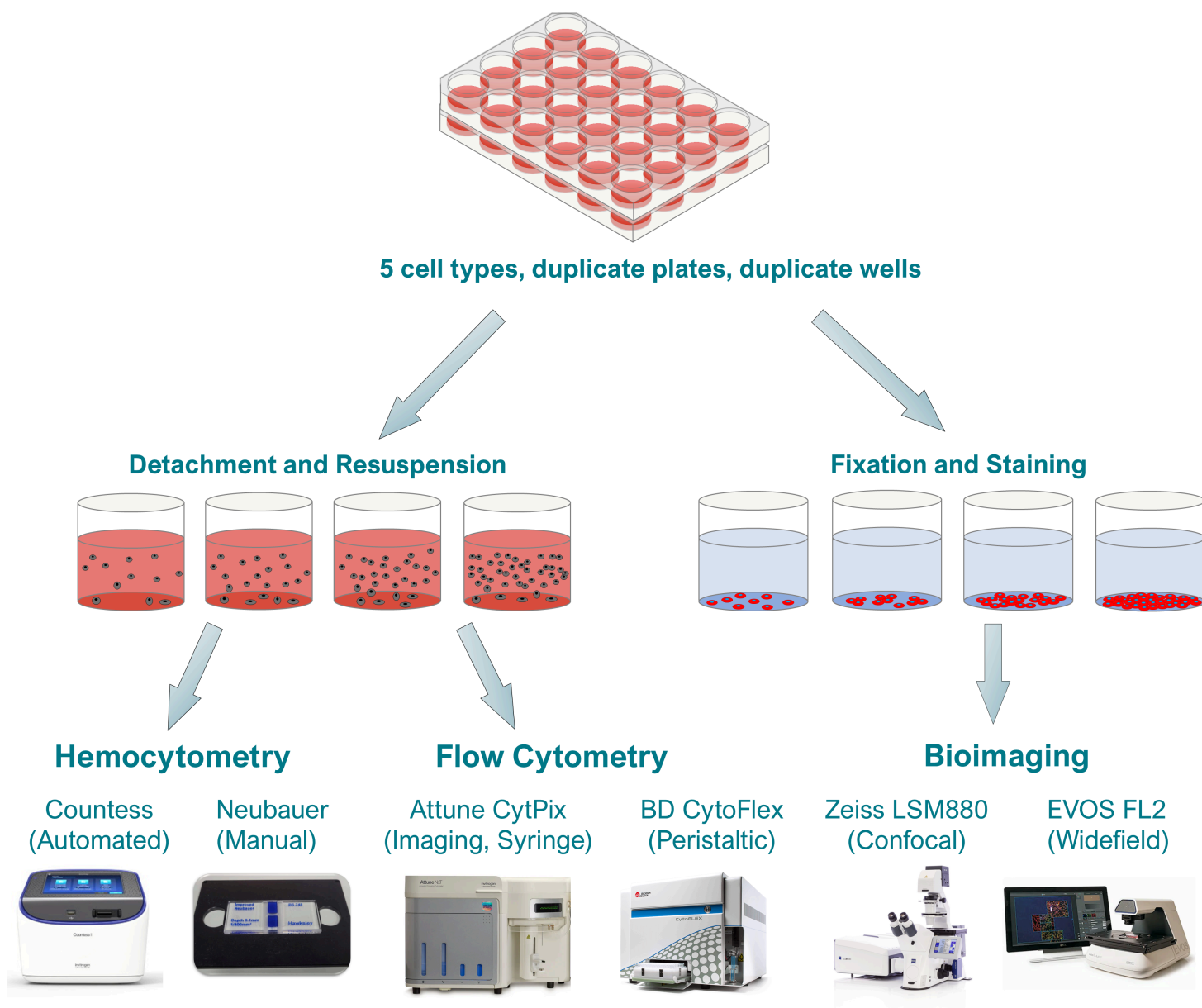
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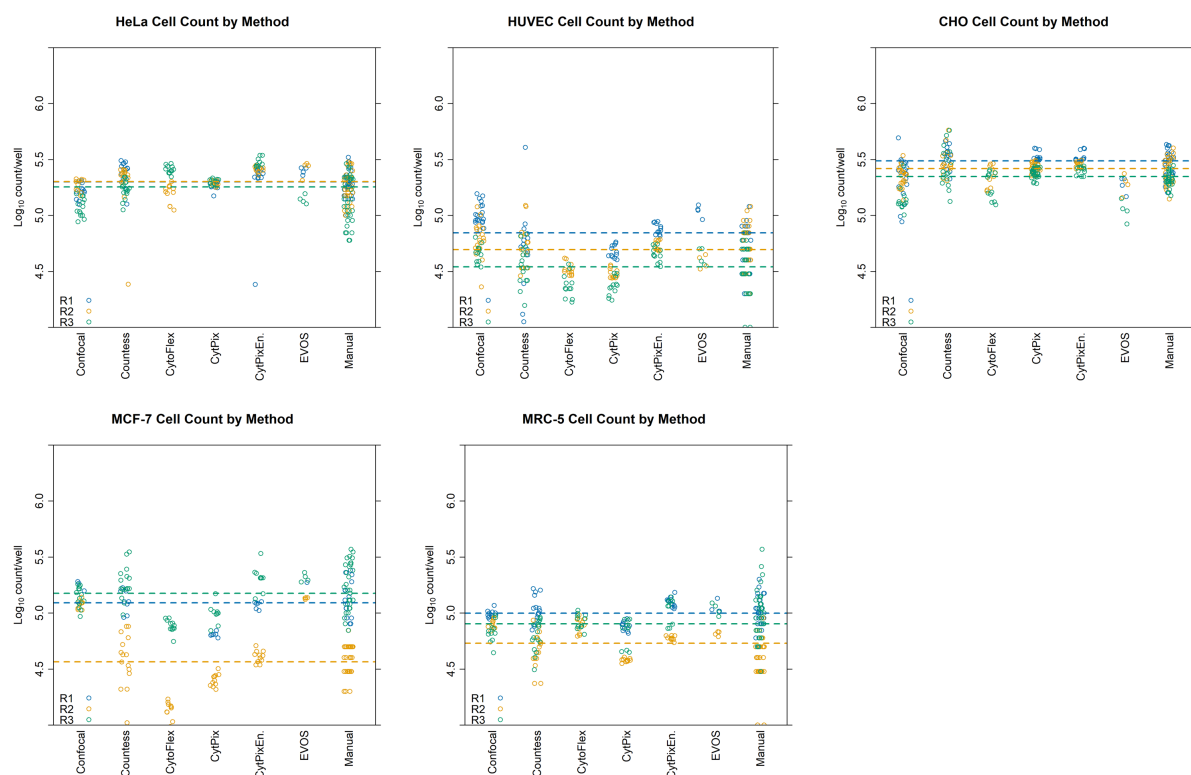
Figures



Study	Countess	Neubauer	CytPix (En.)	CytoFlex	LSM880	EVOS
3.1 Single-density	✓	✓	✓	✓	✓	✓
3.2 Variable-density	✓		✓		✓	

Figure 1

A diagram depicting the experimental counting comparison workflow for each technique. For each seeding concentration, well plates are seeded with five cell types in doublet wells across two well plates. Cells from one plate are detached and resuspended in preparation for haemocytometry or flow cytometry counting. Cells from the other plate are fixed and stained with Hoechst 33342 (nuclei) and WGA (membrane) in preparation for bioimaging counting. Table 1 outlines the counting techniques implemented in single seeding density (section 3.1), and variable seeding density (section 3.2) studies.



	Confocal	EVOS	Countess	Manual	CytoFlex	CytPix	CytPix En.
MRC-5	12.9 %	10.1 %	30.5 %	44.9 %	14.6 %	13.0 %	12.8 %
MCF-7	12.7 %	5.3 %	36.7 %	37.9 %	18.6 %	13.3 %	15.8 %
HUVEC	25.2 %	14.2 %	72.4 %	40.5 %	19.1 %	13.9 %	11.8 %
HELA	15.5 %	10.4 %	21.6 %	33.7 %	12.9 %	5.8 %	14.9 %
CHO	30.5 %	19.1 %	31.7 %	22.7 %	23.0 %	11.2 %	8.1 %

Figure 2

(a) Log_{10} cell counts/well for each cell type and method. Each point indicates an individual measurement, which are coloured according to correspond to different days. Dotted lines show median values for each repeat, depicting the variance in cell count values due to intrinsic biological variability. (b) Table showing relative standard deviations of cell count for each method and cell type.

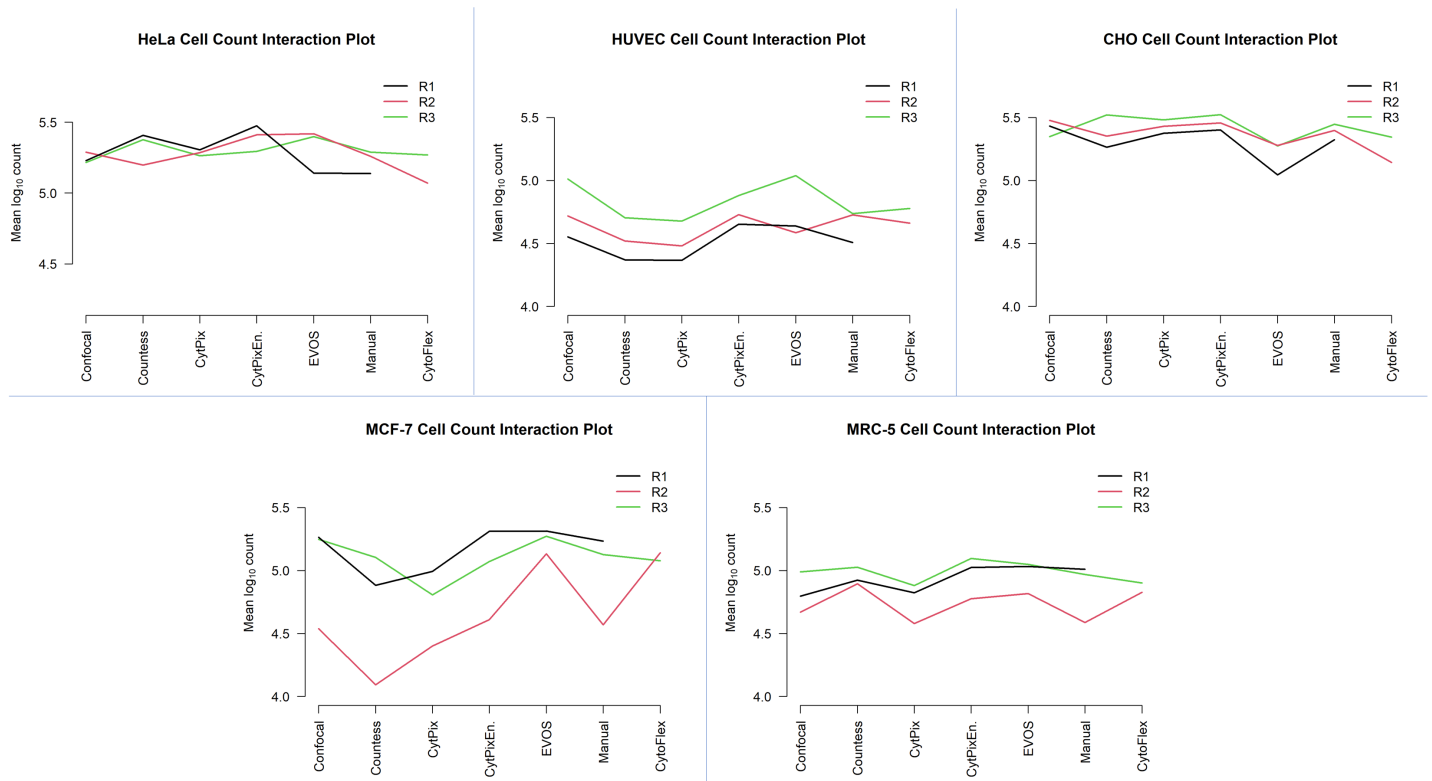


Figure 3

Mean $\log_{10}$ cell count interaction plots for each cell type, method, and repeat. The plot summary shows the combined interaction plot for all repeats. The degree of line parallelism indicates the interactions between each counting technique, with greater parallelism indicating consistent counting performance, regardless of overall cell count. Overall, MRC-5 and CHO demonstrated the most parallel plots, whereas HeLa and MCF-7 gave the most intersecting lines.

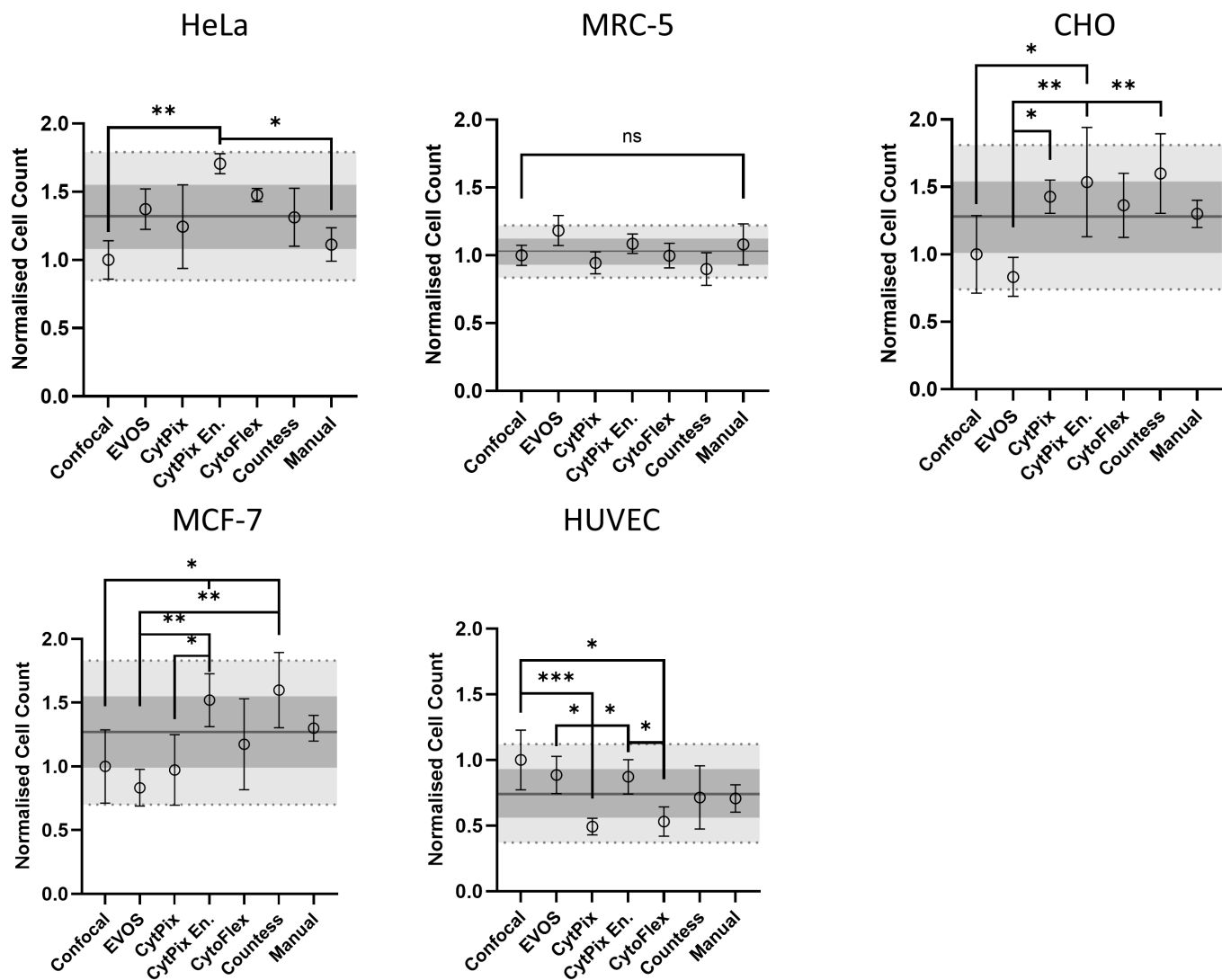


Figure 4

Summary graphs showing the combined confocal-normalised cell count values for all techniques and cell types. Error bars show standard error for each data point. The solid grey lines depict the median count value, the dark grey shaded region shows $k=1$ uncertainty from all values, and light grey shows $k=2$ expanded uncertainty from all values. ANOVA and Tukey statistical testing was performed to determine p-value significance values between each data point. Key significance values are shown - all values are non-significant unless stated otherwise.

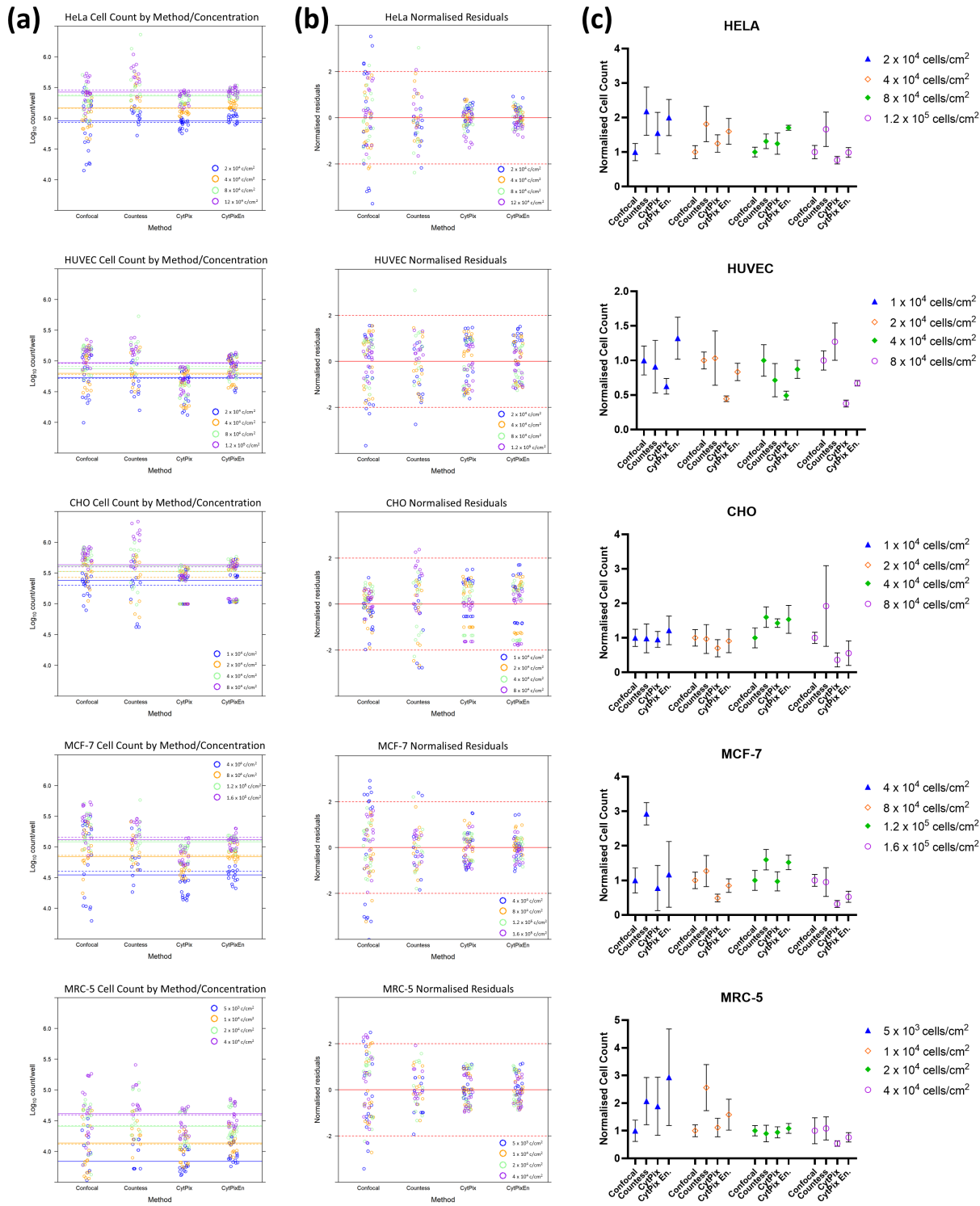


Figure 5

(a) Log₁₀ cell counts/well for each cell type, method, and initial seeding concentration. Each point indicates an individual measurement, which are coloured according to each seeding concentration. Solid and dashed lines show mean and median values for each repeat, respectively. (b) Confocal normalised residuals for each cell type, counting method, and seeding density. (c) Summary graphs showing the combined confocal-normalised cell count values for all techniques, cell types, and initial seeding concentrations.

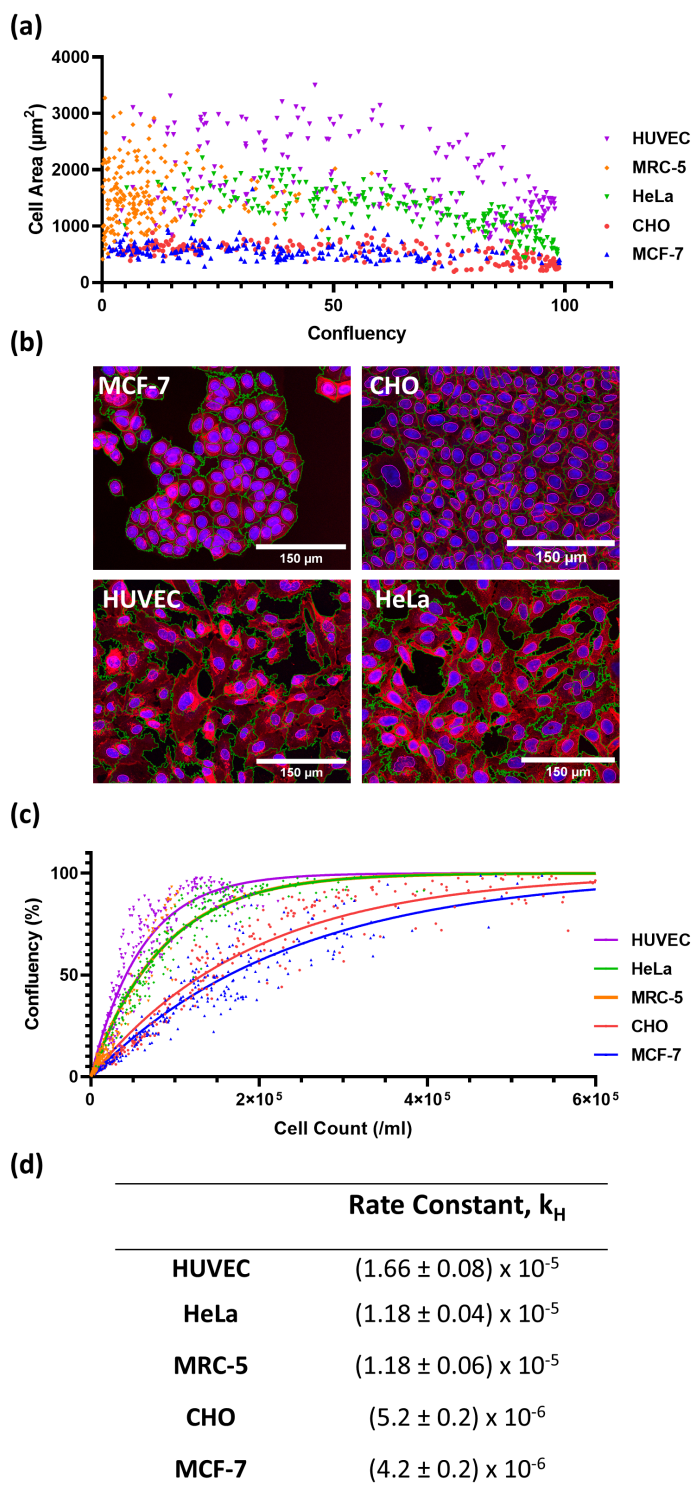


Figure 6

(a) Graph showing the measured cell area vs cell culture confluency for all cell types compiled from all cultures used throughout this study. (b) Confocal fluorescent images showing nuclei (Hoechst, blue) and membrane (WGA, red) channels overlayed with CellProfiler image analysis outlines of nuclei (pink) and membranes (green). (c) A graph of confluency vs cell counts for each cell type. Exponential plateau curves

fitted to each cell type quantifies the growth characteristics for each cell type. (d) A table showing the exponent rate constants, k_{μ} for each curve fitted in (c).

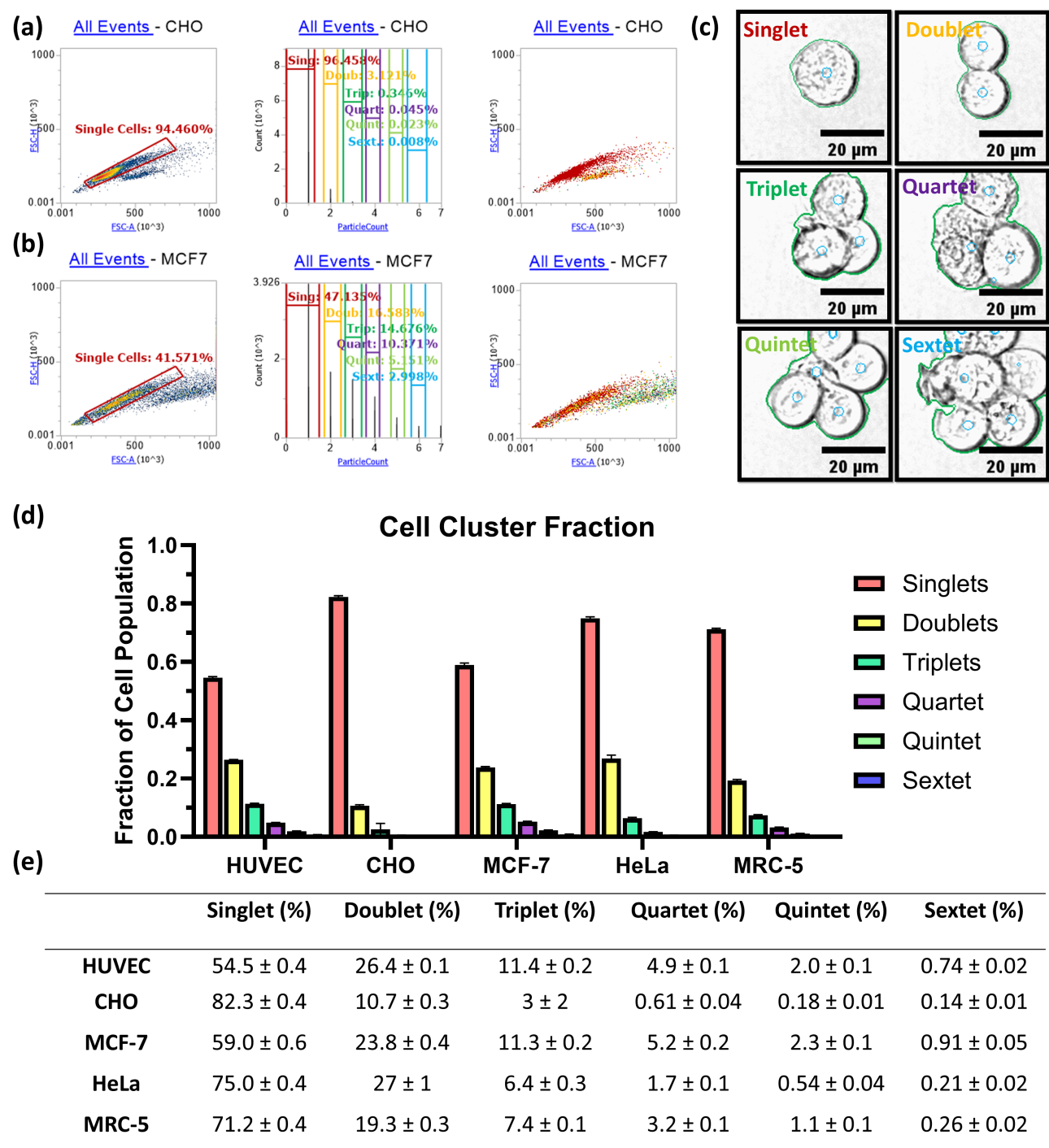


Figure 7

A FSC-H v FSC-A plot of conventionally-gated single cell population alongside an image-enhanced Particle Count histogram plot, and an FSC-H v FSC-A plot showing particle count distribution by colour. This is shown for (a) CHO cells and (b) MCF-7 cells. (c) CytPix brightfield images for singlet (1) to sextet (6)

clusters for MCF-7 cells. Green and blue outlines indicate cell edge and centroid analysis, respectively. (d) Graph showing the average cluster fractions with standard error of the mean, for each cell type considered throughout this study (e) Table showing the cluster percentages ($\pm$ SEM) for each cell type. Data was compiled using all time points and seeding densities.

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