Evaluation of Conventional Adherent Cell Enumeration Methodologies alongside Image-Enhanced Flow Cytometry

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Background incl. aims
Enumeration and characterisation of cell populations are vital processes in both clinical and industrial laboratories. Accurate, reproducible counts of viable and apoptotic cells are crucial to many biological assays and require the development of robust experimental protocols. Currently, interlaboratory comparability between cell measurements is limited due to the lack of alignment in cell analysis techniques and methodologies. In general, the enumeration of adherent cells poses a greater metrological challenge than suspension cells, due to morphological complexity and diversity these cells can present. The impact of cellular morphology and growth characteristics on cell counting is yet to be fully investigated for many conventional enumeration techniques. The use of DNA and membrane stains allowed for quantification of cell confluency as a function of cell count using microscopy, demonstrating the impact of cell type on limits of quantification and linearity. Overall, this study evaluated the efficacy of three common enumeration techniques: haemocytometry, microscopy and flow cytometry using five distinct adherent cell types. Finally, a novel counting technique, which uses combined brightfield imaging flow cytometry, was investigated as a means of overcoming the current limitations associated with conventional flow cytometry.

Methods
Five adherent cell types; MCF-7, HeLa, MRC-5, HUVEC, and CHO were selected to cover a range of morphologies and disease states. Cells were seeded at commonly used concentrations, recommended by the ATCC. Cells were analysed forty-eight hours after seeding, using six separate instruments across three different counting techniques: haemocytometry (automated Countess and manual), microscopy (confocal and widefield), and flow cytometry (Attune CytPix and Beckmann Coulter CytoFlex). Cells for haemocytometry and flow cytometry were detached for counting using TrypLE. Cells for microscopy were fixed using 4 % paraformaldehyde, then DNA and membranes stained using Hoechst 33342 (2 µg/ml) and Wheat Germ Agglutin 594 (2.5 µg/ml) respectively. Imaging was performed using a Zeiss LSM 880 confocal or a EVOS FL 2 Auto widefield microscope. Automated image analysis of nuclei counts, and membrane area was performed using CellProfiler 4.2.1.

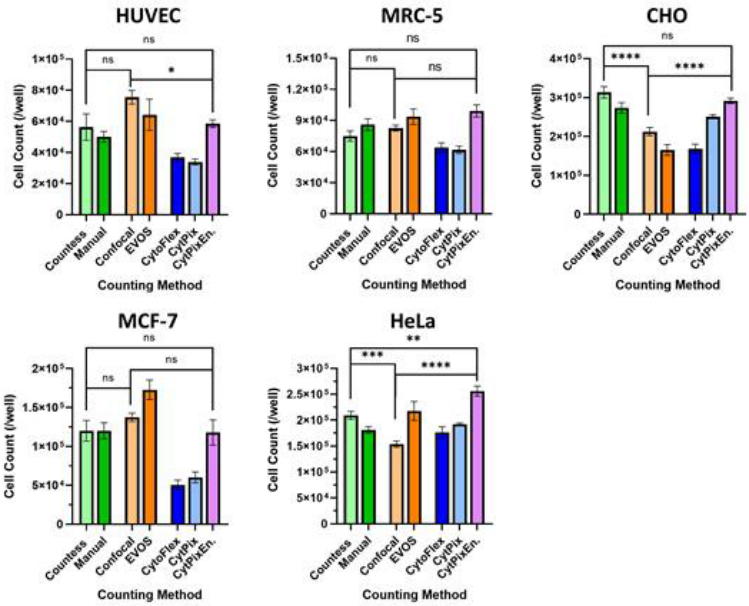
Results
Cell counts measured using hemocytometry demonstrated that this technique is least impacted by cellular morphology and growth characteristics. Low sampling volumes and user bias are the primary sources of counting variations which result in a relatively high limit of detection when compared to other techniques. The efficacy of microscopy-based counting was the most influenced by cellular morphology. Cells which displayed densely populated growth characteristics, such as CHO and HeLa, proved challenging in the identification and segmentation of single cell nuclei. These limitations resulted in a loss of linearity in cell count v confluency plots, which showed cell counts continuing to rise despite having a 100 % confluency. Both conventional flow cytometers consistently underestimated cell counts when compared to the other four techniques. This was primarily due to the presence of cell aggregates in the sample which were incorrectly counted as single cell events and was not resolved following single gating approach of scatter height against width. In particular, cells displaying clustered growth morphologies, such as MCF-7, were found to produce the lowest

cell counts in flow cytometry. Using the Attune CytPix brightfield imaging and analysis capabilities, cell aggregates could be identified and the number of cells in each aggregate determined. Image enhanced cell counts (CytPix En.) displayed values significantly more in-line with alternative techniques, demonstrating the efficacy of this novel counting methodology (graphic).

Conclusion

Results throughout this cell counting study demonstrated the impact of cell morphology and growth characteristics on countability. Several key factors have been highlighted which must be considered when aiming to develop robust cell counting methodologies and standards. Microscopy has been shown to be a useful tool for cell counting, however, this study notes that its limit of linearity and quantification is dictated by cell type. The combined use of flow cytometry and imaging for cell counting presents a quantitative method of determining cell count without the use of reference materials. This method overcomes the limitations of both microscopy and flow cytometry, in particular, removing the uncertainties associated with user-based single-cell gating strategies.

Graphic:



Keywords:

Cell-counting, Imaging Flow Cytometry, Microscopy

Reference:

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