CELL COUNTING: HOW RELIABLE (ARE THESE METHODS?)

Franca Nneka Alaribe (PhD)

Biomedical Consulting Agency
Via Pontisette 3, 44124 Ferrara, Italy
Phone; +393292229258, Email; management@bcafra.com

Received for Publication: July 24, 2014 Accepted: August 18, 2014

Abstract
Cell counting is an inevitable step involved in experiments of many cell–based research studies. The technique allows standardization of cell concentration between samples with minimized error and variation in downstream results. Manual cell counting technique has been the most popular in use. Recently, technological improvement in cell imaging has lead to the manufacture of automated cell counters. The advancements in imaging technology, enabled by automated cell counters such as Countess™ and LUNA-FLTM, are improving experimental accuracy and reliability, with much less time and effort. This review explores different types of cell counting methods with more emphasis on the most recent counting method—the automated cell counters. The methodology, accuracy, precision, benefits and drawbacks of these methods are also highlighted.

Keywords: counting techniques, cell counting, reliability, mammalian cell, white blood cells.

INTRODUCTION
Cell counting is an inevitable step involved in experiments of many cell–based research studies. Scientists are faced with the problem of knowing the exact cell density in many common procedures and assays. Cell counting enhances their work in the determination of viability in microbial samples, error reduction, variation in downstream results and standardization of cell concentration between samples. The procedures range from cell splitting in cell culture maintenance, to proliferation assays and quantitative experiments such as quantitative polymerase chain reaction (qPCR) [1].

Furthermore, cell counting enhances microbiological methods in environmental samples. Despite limitations, account of viable bacteria provide information, on soil and water quality, environmental contamination, and bioremediation [2].

To date, the haemocytometer is the most commonly used cell counting method. Recently, advancements in imaging technology has facilitated the invention of the automated cell counting techniques, which are capable of providing less time and effort with improved experimental accuracy and reliability [3]. For example, previously, the original Coulter Counter counted and measured only red blood cells, however, with the enhancement in technology and evolving instrumentation, it enabled clinicians to also count and measure white blood cells.

Recent studies have observed that modern cell analyzers are multi-channeled and perform automated haematology measurements simultaneously using different techniques. The Cell-Dyn.RTM 3000 instrument, uses "impedance" measurements to count and size red blood cells (RBCs), plateletcrit and platelets (PLTs), "absorption" measurements to for mean corpuscular haemoglobin (MCH). Furthermore, "optical scatter" measurements to count and classify white blood cells (WBCs) and the five part differential are used [3,4].

Cells can be counted using methods such as haemocytometers, spectrophotometers, plate counting, flow cytometers, and automated cell counters. The article reviews the types of cell counting methods with more emphasis, on the most recent counting method namely, the automated cell counters. The methodology, accuracy, precision, benefits and drawbacks of these methods are highlighted.
HAEMOCYTOMETERS/COUNTING CHAMBERS METHODS

Haemocytometers, were the first method developed specifically for obtaining accurate cell counts. The haemocytometer cell counting method uses a specially crafted gridded-glass slide consisting of two chambers, each divided into nine 1 x 1 mm squares. A glass cover is supported by the grid, and the cell suspension is loaded via capillary action into the chamber. The microscope is then focused on an area of the counting chamber and the cells are counted using a tally counter. It is always preferable to use a clean haemocytometer (70% ethanol) and lens. To determine the viability of the cells, trypan blue is added to the cell suspension, in the ratio of 1:1, before loading it to the counting chamber (Fig.1) [5].

![Haemocytometer with counting chambers](image)

Table 1: Merits and Demerits of Haemocytometer Method

<table>
<thead>
<tr>
<th>Merits of Haemocytometers Method</th>
<th>Demerits of Haemocytometers Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the haemocytometer technique, results can be obtained in minutes rather than days as required for plate counts, thereby creating room for immediate judgments and decisions about the sample analysis.</td>
<td>Where there are many samples to process, the microscopy is labour intensive and can lead to operator fatigue. To date, new technology is gradually replaced with image analysis and flow cytometers.</td>
</tr>
<tr>
<td>It is a good method for samples full of debris and contains multiple object shapes and sizes.</td>
<td>Only capable of analyzing small number of cells (100-250 cells can be counted) and statistically unreliable.</td>
</tr>
<tr>
<td>The instrument does not require as such trained operators/professionals as in the case of flow cytometers.</td>
<td>Sometimes, there is no uniform distribution of cells which gives imprecise results. Coefficients of Variation (CV) ranging from 30 – 110% .</td>
</tr>
<tr>
<td>The glass haemocytometers, are subject to over-filling and with high error in cell counting. This leads to unreliable cell counts with a high standard deviation. At times, monocytes tend to be underestimated and the lymphocytes tend to be overestimated.</td>
<td>The procedure is tedious and requires careful cleaning and handling of the haemocytometer. Validity of the practice has been questioned because of low specificity, accuracy, and precision of counts.</td>
</tr>
</tbody>
</table>

Generally, cells are counted manually and are usually placed over the grid occupying a volume of 0.1 μl for each 1 x 1 mm square. This process is usually repeated using four different 1 mm² areas with an average result. To determine the original concentration in cells/ml, the number of cells counted in a specific number of squares and the dilution factors are used. To determine cell viability, the number of dead and live cells, are separately determined. The dead cells are permeable with trypan blue due to their damaged membranes hence the cells always appear blue when viewed under the microscope [3, 6, 9, 10].
SPECTROPHOTOMETER METHOD
A cell counting technique seldom used for obtaining relative estimates of cell density. With the method, increase in cell density leads to passage of less light through the cuvette. The action is possible due to the turbid nature of cells. With the spectrometer, the optical density (OD) of a culture is proportional to the biomass in the cell suspension. The process generates an immediate result. Using spectrophotometry for measuring the turbidity of cultures (turbidometry) has its advantages and disadvantages [7].

Advantages: Some spectrophotometers are characterized with many cuvette spaces in which several cuvettes can be inserted, thereby further reducing work time. Additionally, there are spectrophotometers that require extremely small volumes of culture, such as 1 u/l thereby reducing waste.

Disadvantage: The spectrophotometer’s drawback is its limited accuracy. It is the only method in which the cells are not directly counted. The machine measures the amount of light rather than cells. Combined with the stochastic nature of liquid cultures, the methodology enables only an estimation of cell numbers. Thus spectrophotometer seems to be unreliable in cell density estimation because absorbance is liable to be affected by other variable components of cell suspensions [8].

PLATE COUNTING METHOD
Cell plating is another common method of counting cells in for example bacterial colonies. A sample to be counted is diluted in a solution rendering it harmless to the microbe. The most used methods of plate counting are viable plate count and pour plate count techniques. This enables the cells to be counted by plating the cells and streaking the diluted cells onto a plate. After sufficient time is allowed for growth, colonies are counted. Based on the dilution and the known volume of suspension, the density of the original suspension can be determined. Using a Pour plate technique involves mixing a portion of the dilution with molten agar, after which the mixture is poured into a petri plate. According to the sample dilution, individual cells are dropped on the agar to form colonies. To determine the colony forming units (CFUs) on the plate, the total number of colony is counted (Fig 2). To find the total number of CFUs in the original sample, the total number of CFUs is multiplied by the total dilution of the solution [9].

Figure 2: Illustration of series of pour plates showing the appearance of viable plate count. The four plates show a $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ of a natural sample and the number of colony forming units which decreases 10 folds between the plates. No of bacterial/ml = number of colonies X dilution of sample.
FLOW CYTOMETER METHOD

Flow cytometry is another technology adopted to count fluorescently labeled cells. The technique uses a fluorescence detection system. Flow cytometry technology is basically made for passing cells singly through a sensing region of a flow cell. The cell counting and sizing is carried out by calculating the changes in the optical technique, Optical scattering of light (laser beam) by each cell is measured [3].

Flow cytometry has its origins in the analysis of microorganisms. It has developed over the last 30 years as a technique primarily optimized for, and associated with, the analysis of clinical samples [10, 11, 12].

Currently, specialized type of flow cytometry techniques such as the Fluorescence-activated cell sorting, are available. These are based upon integrating certain substances known as “fluorophores” as labels into the cells. Immunological analysis for cell antigens (e.g. lymphocytes) is now possible by fluorescence flow cytometry, using monoclonal antibodies [3, 9].

Further studies have shown that only volumetric flow cytometry can count the absolute number of cells in unit volumes of liquid to provide highly accurate cell numbers proved comparable to those obtained with haemocytometers [13].

Benefits/advantages: Despite the fact that Flow cytometers are most frequently used for more detailed cellular analysis, it is also capable of providing highly accurate cell counts, and can discriminate cells based on factors, such as protein expression, by using fluorescently labeled antibodies. Flow cytometers are very easy to use and depend more on the experimental set-up. There is room for measurement of higher cell concentrations than the manual method. Flow cytometry analyzes individual cells, which means that their viability determination is assured. The viability measurements include but not limited to measurement of metabolic activity, membrane energizing, RNA and/or DNA content, membrane permeability [1, 3, 9].

Disadvantages: Notwithstanding the benefits of flow cytometric cell counting, the devices are expensive and are rarely used for general cell counting applications. The instrument requires professionals due to its complex and complicated mechanical configuration. Additionally, for acquisition of reliable data, constant maintenance is required [1, 3, 9].

AUTOMATED CELL COUNTERS

Previously, manual use of haemocytometers and counting chambers in blood counts were very common. Today technological evolution and technological advancement has made it possible for the development of more sophisticated automated blood cell counters/analyzers in research analysis. These automated counters has come a long way in enhancing throughput, due to their multiple functions and high speed performance.

Almost two decades ago, a series of image-based automated cell counters has been introduced to provide accurate cell number and viability data. Basically, these cell counters use light microscopy and computer-based image analysis software. Instead of manual counting, magnified cell images are automatically captured by a digital camera and transported to computer software. Algorithms are used to accurately find cells and discriminate against non cellular objects such as cell debris [13].

Recently, these advancements in imaging technology has enabled inventions like the Countess™ Automated Cell Counter with the best promise for improving experimental accuracy, reliability, and time-to-results, with much less time and effort[14].

Unlike the haemocytometer, Countess™ Automated Cell Counter is a bench top instrument that does not involve
The use of the Countess™ Automated Cell Counter involves three steps:

1. Prepared cells are suspended to a concentration of approximately $1 \times 10^6$ cells/ml, aliquots are mixed with 0.4% trypan blue and 10 μl is suspended using a pipette into Countess™ chamber slide.

2. The slide is inserted into the instrument.

3. Press the “Count cells” button and results are displayed. The image that appears on the screen represents the same area as four 1 x 1 mm squares on a haemocytometer. Adjusting zoom and focusing knob produces the desired image with total readout of cells/ml, live cells/ml, dead cells/ml and % viability of cells [3, 6, 13, 14].

Advantages of Countess™ Automated Cell Counter:

Due to the use of digital image capture and computer analysis for counting, Countess™ Automated Cell Counter always show significantly higher effective concentration range than the haemocytometer. As an important technological advance in automated cell counting, Countess™ Automated Cell Counter readily provides the researcher with high-quality cell count data in less time and effort. It also eliminates the tediousness and subjectivity of manual cell counting. With the method of instant image generation of samples, the Countess™ instrument generates an image of the actual cells, increasing the researcher’s confidence in the results. In automated cell counter, there is no slide distribution error. Many studies have suggested that laboratory efficiency could be improved by relying more on instruments such as the Countess™ Automated Cell Counter. Due to its capability to perform accurate count on leukocyte and monocyte cells used in diagnosis of infection with less reliance on manual differential counts [3, 6, 15, 16].

Finally, the techniques are more affordable, easier to learn than the flow-based system and essentially maintenance free.

More sensitive and accurate cell counting techniques have been introduced to overcome the shortcomings of bright field image based cell counting. Cell counters such as LUNA-FLTM and others, which are equipped with a fluorescence microscope module, and an image analyzing/counting algorithm, has been developed. The LUNA-FLTM is a cell counter based on sensitive and accurate fluorescence staining methods to provide cost-effective and reliable cell counting solutions.

The latest development in the instrument is that, unlike the cytometry, the LUNA-FLTM captures and analyzes three different images: bright field, green fluorescence, and red fluorescence. Within 30 seconds, total cell numbers, viability, cell size and histogram data are displayed on a touch screen LCD monitor. In summary, it is designed in a small, all in one configuration, that it can be applied in space limited environments and are used to count cells without the limitation of the cell types [12, 13].

CONCLUSION

Despite the successful use of mammalian cells with popular dyes such as trypan blue and methylene for cell viability and bright field imaging techniques, most mammalian cell counting still remain challenging. Cells like the peripheral blood mononuclear cells (PBMCs), yeast cells, sperm cells and primary cells are not easily counted with the bright field image analysis [14]. Additionally, the accuracy of counting chambers seem to fall short and subjective means of counting are poor in
discriminating between multiple types of cells in a suspension. The technique is accomplished when the size or shape differences between the various cell types are structurally distinguishable.

Whilst on the other hand, flow cytometry has many advantages and exciting opportunities for research, it has not yet become as widely used as cell counting potential deserves. Instruments seems to be extremely expensive and sometimes challenging in cell counting. Commercial instruments lack the sensitivity required for the analysis of certain cells such as microbial cells.

With the proliferation of automated cell counters like Countess™ and LUNA-FLTM these shortcomings are addressed. Currently, these alternatives has demonstrated success for all the mammalian cell types. The methodology has a high ability of producing or generating an image of the actual cells rather than relying on representation on a dot plot. The outcome generally tends to increase the researcher’s confidence in results obtained. However, despite the efficiency of automated cell counting analyzers the instrument is still not efficient in detecting abnormal WBCs. Therefore, the manual cell counting techniques and automated cell counters still attend brighter prospects especially when haematological malignancy or rare hereditary disease is suspected.

REFERENCES