

Cell counting technology on a global scale: navigating automated, accurate and safe techniques

Cell therapies have huge potential for the treatment of a range of human diseases including cancer, metabolic disorders, tissue degradation and immune deficiencies.

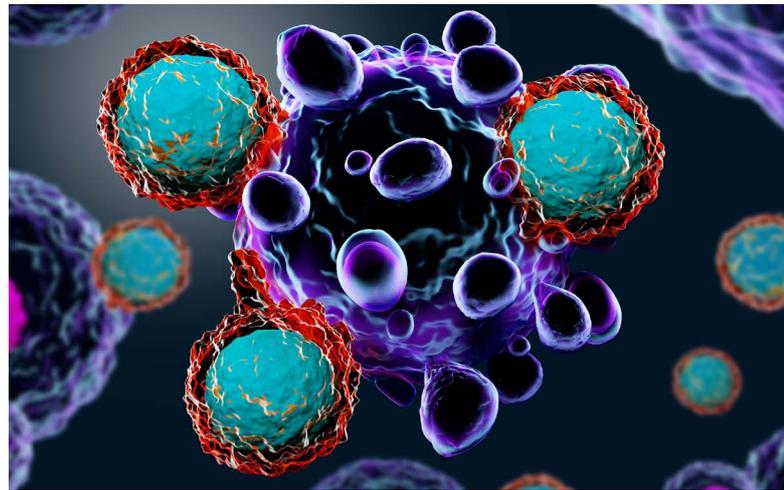
However, before these therapies can be effectively commercialized for widespread clinical use, there is a need to find robust, repeatable, cost-effective and scalable ways to generate and test large volumes of cells to ensure they constitute a safe and effective therapy.

Currently, there is no consensus on a single technology for counting cells during expansion accurately and safely. In this whitepaper Nick Collier, Steven Deane, and Thore Bücking assess the strengths and weaknesses of the current technologies available for cell counting and identify the requirements for developing an optimal system.

The importance of automation

Many of the therapies in clinical trials are autologous. Using the patient's own cells as the starting material requires production of one clinical batch for each individual patient. Such personalized processes are currently costly and highly labour intensive. For example, manufacturing a re-engineered cell therapy product such as a CAR-T cell product is particularly complex.

Firstly, a patient's own immune cells must be harvested in sufficient quantities, then re-engineered to provide the ability to target specific cancer cells. These engineered cells are then expanded ex-vivo, characterized and injected back into the patient.



Although only a relatively small number of cells may be required for each dose, it may be necessary to set up thousands of small bioreactor systems, each operating independently and with subtly different operating parameters. The more operators involved, the greater the cost and the greater the chance of process contamination, hence the drive towards achieving closed and fully automated systems.

Given the large variation in the number of patient cells and the cells response to the to the expansion process, measurement and feedback is essential to ensure both high-quality and a fast-turnaround in automated systems. Whilst there are many options to measure process variables such as temperature, pH, dissolved gases etc, there are very few cost-effective options to continuously measure the progress of viable cell production at frequent stages in the process. Such sensors would have two purposes, one to warn an operator that the process is not going to achieve the desired purity and yield ("early fail"), giving time to start again with a fresh sample; the other to allow correction to expansion conditions to be made, in a quality control (QC) mode.

Further benefits of integrated sensors lie in assuring safety for example, by detecting contamination (endotoxin, mycoplasma, retro or lentovirus), if the cell detection method lends itself to this degree of discrimination.

The challenges to cell counting

The objective of the manufacturing process is to produce transduced cells in sufficient numbers and at the correct concentration to form one or more patient doses. A large number of factors affect the process including the number of starting cells, the transduction efficiency, and the ability of the cells to proliferate.

The process usually starts with apheresis and further purification to obtain the desired starting cells. The number of cells at this stage can be low, depending on the transduction target and the patient, particularly those that have already undergone conventional treatment. For example, one study¹ found the lymphocyte count in 15 patients varied over a 5:1 range whilst the CD3+ count varied over a 13:1 range and the CD8+ count varied over a 13:1 range. Typically CAR-T cell therapy requires 0.6×10^9 CD3+ cells to be confident of adequate expansion² and preferably >2

$\times 10^9$, however the number actually collected by apheresis can be hugely variable.

The target patient dose of transduced cells in CAR-T cell therapy trials is in the range of 1×10^7 to 1×10^9 cells.³ This usually depends on the patient weight and dose escalation strategy. However the total cell count can be higher, as not all of the cells will be CAR+, potentially as low as 20%.

Additionally, cell therapy requires viable cells and its important when performing cell counts to distinguish viable and non-viable cells. It is important to also count the non-viable cells as infusing a large number of dead cells into a patient raises safety concerns.

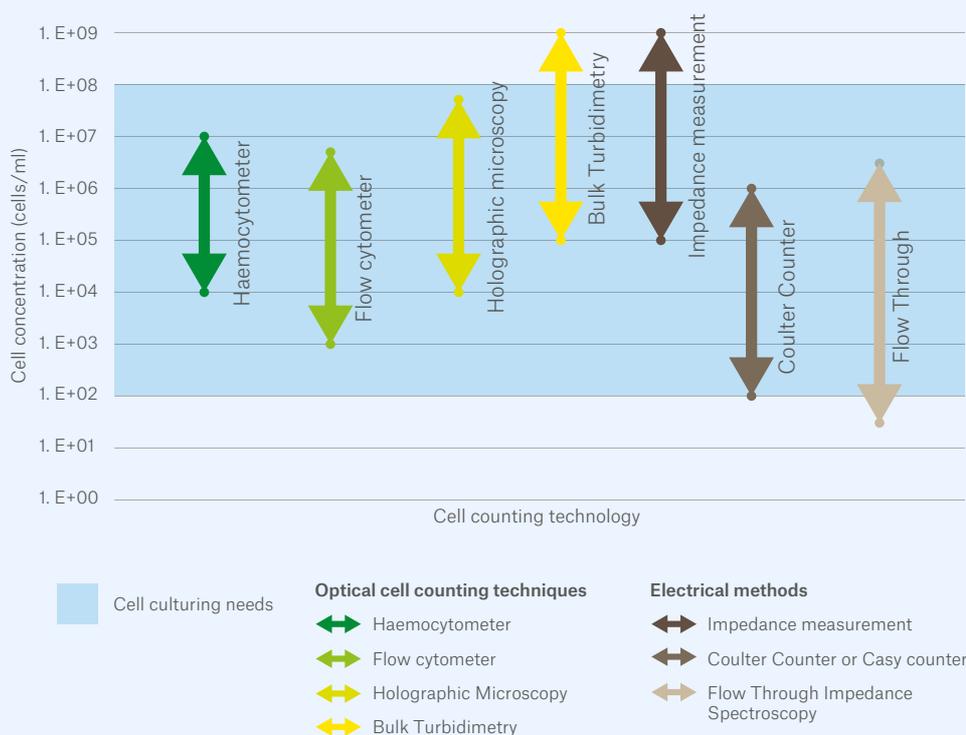
Assessing existing measurement technologies

A number of cell counting technologies exist and they fall into two approaches: optical or electrical cytometry. In figure 1 we compare the ranges they can count reliably to the desired range in cell culturing.

Optical cell counting techniques

Optical cell counting is used in a number of technologies:

Figure 1: the range of cell densities in cell culturing compared to the range available by main cell counting technologies. No single technique covers the full range.



¹DOI 10.1182/bloodadvances.2017011254

²Transfusion. 2017 May ; 57(5): 1133-1141. doi:10.1111/trf.14003

³EMBO Mol Med (2017) 9: 1183-1197 DOI 10.15252/emmm.201607485

Haemocytometer

The most commonplace technology is optical cell counting with a haemocytometer, either manually or by an automated instrument. This can be combined with a simple viability stain such as trypan blue to distinguish between viable and non-viable cells.

However, this counting method only works well over a limited range, which typically requires the sample to be diluted to the best range for counting, and the resulting concentrations corrected for the dilution. This requires either manual or automated dilutions steps, both of which add cost, introduce potential for errors, and increase the risk of contamination of the culture.

Flow cytometer

A second common optical cell counting technology is the flow cytometer. Here a sample is drawn through a narrow channel, typically a quartz capillary. Often, a sheath fluid surrounds the sample, and assists in ensuring the cells to be counted are approximately centred in the channel, and pass through in 'single file' to avoid co-incident events. The channel is then probed by a laser. The forward and side scatter can be measured label free, and give measurements of the cell volume and information on scattering properties or granularity. Thus, some differentiation of cells is possible at the same volume. Enhanced separation is typically achieved with fluorescent labelled antibodies, which can be used to give highly specific measurement of many surface markers simultaneously by using different wavelengths for the fluorescence. Typically the sample must be below 10^7 cells / ml to avoid too many coincident events.

Issues with flow cytometry are commonly the cost of the instrument, typically >\$10k, the need for careful control of sample preparation and analysis, precise alignment between the optics and flow channel, and gating for reproducibility makes use in a cartridge based instrument problematic unless the sample is removed from the cartridge, which introduces contamination risks. The ability to differentiate live / dead cells label free is limited; often stains like propidium iodide are used. The ability to give accurate absolute concentrations is also poor; if absolute counts are needed, a known bead concentration is typically mixed into the sample, to reference the counted cells

to. The need for these labels to give live / dead differentiation and absolute counts means samples would need to be taken from a cell culture and not returned, requiring additional complexity in a culturing system, and greater risk of culture contamination.

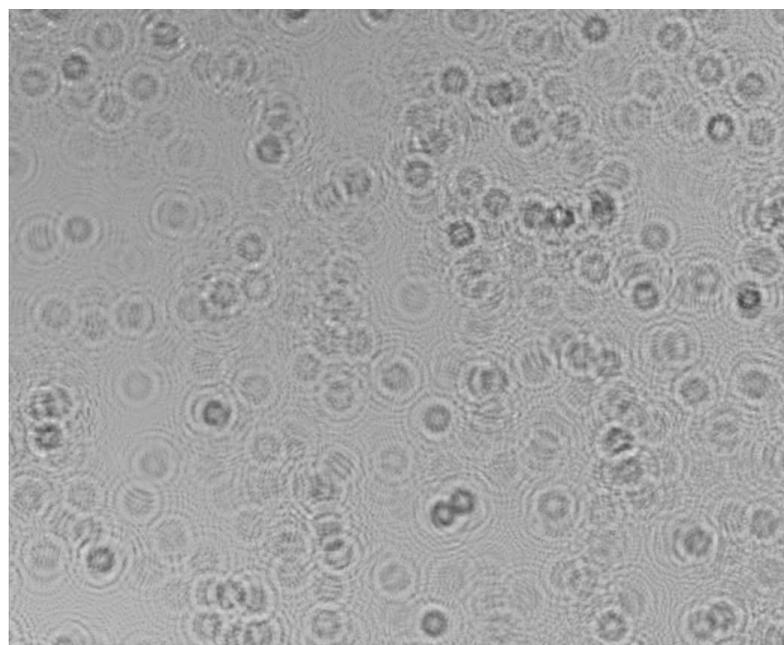
Turbidimetry

A further optical method is spectrophotometry or turbidimetry. Here, the bulk culture light scattering is measured, either by measuring the attenuation of transmitted light, or by measuring the scattered light. This has difficulty in measuring low cell concentrations and great difficulty in differentiating live and dead cells. Better differentiation is possible with cell viability staining; though again, this means a sample needs to be taken from the culture and not returned, increasing system complexity and contamination risk.

Holographic microscopy

This approach is based on encoding the phase information of a light field distorted by the varying refractive indices of the cells. Being a purely optical technique, holographic microscopy can gain information about the microstructure of cells without the need for chemical labels or staining and thus viability can be assessed without interfering with a cell culture.

A holographic microscopy technique of particular interest is lens-free microscopy, which has recently emerged as a solution for low-cost and high-throughput viability assays.⁴ Here, the sample is



brought into close proximity (~1mm) with a CMOS sensor and illuminated with a coherent light source. Provided the sample is sufficiently transparent, light scattered by the sample (the object beam) interferes with the background (the reference beam) to form an in-line hologram on the sensor. A mathematical construction of the image in 3D is possible by making valid assumptions about the nature of the reference beam. The result is a 3D image stack of reconstructed object planes, visualising in-focus cells which were originally in those planes.

Different viability measures have been reported using this technique, the underlying principle being the observation that dead cells appear to scatter the light more diffusely. Hence, by analysing the contrast within regions of interest of the hologram, individual cells can be characterised.

Lens-free microscopy enables sampling a large field of view (20-40mm²) at micron scale resolution without the use of expensive optical components. It's possible to assemble components for a complete imaging setup at a parts cost below that of conventional high resolution imaging equipment. The use of this approach for monitoring cell viability is relatively young and few commercial products exist. A notable example is the Norma series by Iprasense which claim functionality for concentrations ranging from 10⁴ to 5x10⁷ cells/ml.

Sagentia has experience in this type of system and its algorithms and understands what is involved in establishing an in-line derivative for cell counting and viability analysis. For example, we may look for a single-sided approach monitoring a relevant depth of the bulk medium. A significant advantage offered is an enhanced depth of field compared to conventional reflection microscopic imaging from the vessel wall.

Electrical methods

Additionally there are various technologies which use electrical methods. These can be divided into bulk and pore / channel-based measurements.

Bulk measurements

A bulk measurement works by measuring the frequency dependent impedance of the mixture of

cells and culture medium. Viable cells with low frequencies and intact membranes act as insulators, while at higher frequencies the membrane capacitance allows conduction inside the cell. This allows the volume fraction of viable cells to be directly measured. Importantly, this differentiation is label-free, so there is no impact on the ongoing culture. However, because it is measuring the change in capacitance in the bulk medium due to the suspended cells, it can only work for high cell concentrations. This limits its ability to measure the potentially crucial early stages of cell culturing.

Pore based measurements

- The original pore based electrical cell counting technology is the **Coulter counter**⁵. This consists of a small pore through which cells suspended in a medium are drawn. Electrodes on either side measure the resistance of the pore and when a cell enters the pore the resistance rises due to the insulating nature of the cell at low frequencies. These resistance pulses are then counted, allowing the volume and number of cells to be determined. A limitation of this technology is that the effective sensing region surrounds the pore at both sides, and the typical number of cells in this sensing volume must be <0.1 to avoid multiple cells giving overlapping pulses, appearing as single larger cells. This limits the technology to lower cell concentrations, and so is often used with a manual or automated dilution step. There is a trade-off between using a short pore to allow higher cell concentrations to be measured, or a longer pore which allows more accurate size measurements. A further challenge for continuous monitoring is that the pore can be blocked by debris, either stopping counting completely or resulting in an error in cell numbers and size. Using a larger pore is limited by the need for the cell signal to be distinguished from the noise and further reduces the maximum cell concentration which can be measured. As an example, making the pore 10x wider will reduce the maximum measurable cell density by a factor of 1000. Cell viability is only weakly distinguished, as non-viable cells appear electrically slightly smaller due to their porous membranes.
- A variation on the Coulter counter is the **Casy counter**, which is similar, but uses a higher

frequency electrical signal to give improved differentiation of viable and non-viable cells.

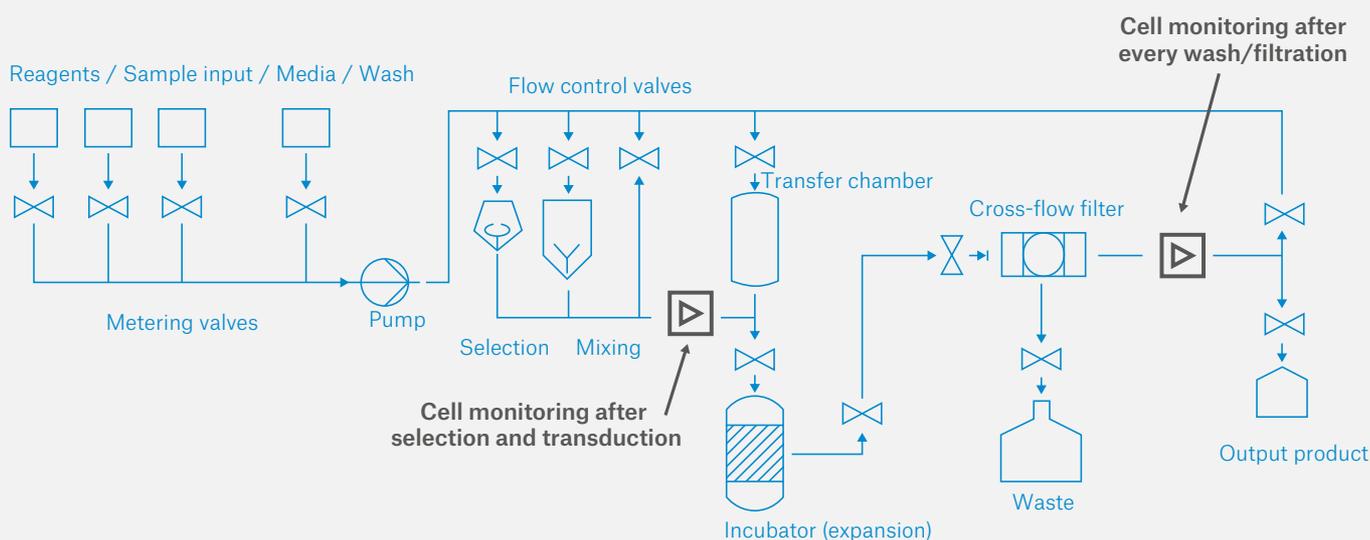
- **Flow through impedance spectroscopy⁶** is related to Coulter counting, but instead of the electrodes being positioned either side of a pore, they are positioned on either side of a microfluidic channel. In addition, multiple electrodes and multiple frequencies may be used. This has advantages over the Coulter counter in that the detection volume is smaller for a given channel size, allowing slightly higher cell concentrations to be measured. The lower detection volume combined with the option to use multiple electrodes allows a better signal to noise, enabling smaller particles to be counted in a given pore / channel diameter. If multiple frequencies are used, these can be used to differentiate differently shaped cells of the same volume, or viable and non-viable cells. However, a downside of this technology is increased complexity of manufacture, making its use in a disposable cartridge more challenging.

An evolutionary need for sensors

Any development of CBMP (Cell Based Medicinal Products) relies on a manufacturing system which is designed and proved to meet cGMP (cell-based Good Manufacturing Practice). As CMNP evolve, initial systems will require sampling or will benefit from automated sensors to validate the process and fall in with cGMP requirements. Data from manual analysis of samples and release tests performed on the subsequent batch yield will ratify in-line tests. Analysis of accumulated data on repeat growth cycles should also show that some in-line tests become unnecessary as protocols become validated.

As a minimum, we would expect QC (quality control) cell monitoring to be performed in the two critical locations indicated in the following diagrammatic representation of a cell growth system, with additional QA (quality assurance) tests performed on the output product.

Figure 2: Cell monitoring locations in a typical cell expansion process



⁵<https://media.beckman.com/-/media/pdf-assets/application-notes/particle-counting-multisizer4-application-note-characterized-by-ingenuity.pdf>
⁶<https://www.ncbi.nlm.nih.gov/pubmed/21331413>

The desirability of sampling/ label-free measurements

Measurements that need labelling of the cells, dilution to reach a measurable concentration range, or a measurement technique that cannot be performed inside the cartridge, will require removal of a sample from the culture, for example to avoid the labelling or dilution agent contaminating the cell culture. This can either involve breaching the sterile confinement of the culture, or integrating a sampling and non-return system into the cartridge. The former risks contaminating the culture, while the latter increases the complexity and therefore cost of the cartridge.

In addition, if non-return sampling is performed, careful attention to sample volume and the number of measurements is needed to avoid depletion of the culture, when low cell densities are being measured. The cell sampling volume needed for an accurate count may change with cell density, being larger at low concentrations to keep Poisson noise acceptable.

These challenges mean that when aiming to achieve a low cost of disposable cartridges with minimised risks of contamination, a label-free measurement technique which can be cheaply integrated inside the cartridge, and where the sample can be returned to the culture is preferable

The requirements of an optimal system

In order for fully-automated closed-system cell manufacturing solutions to come to market, technologies that allow the continuous monitoring of the viable and non-viable cell count at all stages of the cell expansion process from sample input to final formulation are required.

Key requirements of such a system will be:

Wide measurement range to cope with sample variation, low growth rates, fault conditions and many different process protocols

Ability to count viable and non-viable cells

Accuracy of better than +/- 10%

Fully automated, requiring no manual intervention

Cell preserving – the cells are precious and losses from measurement must be minimal

Maintains system sterility

Introduces no reagents into the growth chamber and does not dilute or concentrate cells

Works with different cell types

Is easily cleaned and sterilized for reuse or has low-cost disposable elements

Overall costs not prohibitive

There are clearly challenges to be overcome in developing a technique which can deliver against these requirements but for cell & gene therapy to be delivered – at scale to large patient populations – technology solutions must be found.

Cell counting development areas

There are numerous areas of development which would enhance basic capability and improve measurement accuracy and cell identification, starting with counting and viability:

Counting – impedance, image analysis and morphological processing; include distinguishing beads to check bead removal

Viability – live vs dead – microscopic image analysis, impedance, CASY/Coulter counter

Debris measurement – for quality control eg. aggressive filtration produces debris, measured from pass-through filters

Size distribution – assist identity, viability and cell population determination

Volume – calculates average cell diameter

Shape – assist ID of target or unwanted cells, image analysis or multi-angle diffraction

Aggregation – a measure of which may help to improve cell count accuracy for certain counting techniques

Identity – can use surface protein affinity to molecular labels or sensitive biosensor surfaces, can use shape and size.

Summary

As we have discussed, there are several challenges to consider in the development of cell counting technology, there are a number of technologies and techniques in play, and the benefit to developing a more automated approach is clear. Our medical team at Sagentia are continuously future gazing to find the major growth potential areas that will be core to the evolution of the healthcare industry; with the fantastic progression of cell therapy treatment the technology to make this treatment scalable is going to be on high demand and will need to be ready to gain market share fast when this major development in the healthcare world hits. A real opportunity exists for companies in this industry to build upon their current technologies, or even to diversify into this technology area before the major market disruption occurs.

Sagentia is able to support the development of a manufacturing system to include components, sensors or the whole package including the control system. We are familiar with both cartridge and tube-based aseptic systems, pump choices, filter choices and incubator formats. Our expertise in optical systems and high-resolution electrical measurement allows us to pick the best technique for purpose, with no specific bias. We are confident that there are answers to the challenges and that technological research and development will make automated, safe and accurate cell counting a reality.

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