CELL & GENE THERAPY INSIGHTS

SEPARATION AND EXPANSION TECHNOLOGIES

SPOTLIGHT

EXPERT INSIGHT

Technological developments in dielectrophoresis and its path to commercialization

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One of the bottlenecks for cell therapy development is the need to isolate specific cells, be it stem cells with specific differentiation fates, or specific white cells from a blood cell sort. However, the nature of the application means that the separation method should ideally be label-free and GMP-compliant, as well as achieving appropriate levels of throughput and cell recovery. One emergent field in cell separation is dielectrophoresis, an electrostatic method that has the potential to meet this growing need. Recent commercial developments mean that for the first time, this technique will be more widely available to the cell therapy sector.

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THE NEED FOR LABEL-FREE CELL SEPARATION

As limitations grow on developing pharmaceutical therapies to intervene in diseases of cancer and aging, such as neurodegenerative disease, medicine is turning to alternative approaches to intervention. One of the growth areas of greatest impact in clinical research is in the use of cell therapies; the use of living cells to repair, replace and augment the existing body tissues [1]. This can take the form of neural stem cells selected to replace missing or damaged tissue in Alzheimer's or Parkinson's disease [2]; or it can be used to treat disease by modifying specific cells, such as the use of lentivirus-augmented T lymphocytes for CAR-T therapies, where altered immune cells are used to combat cancer by reprogramming them [3]. As the interest in cellular therapeutics grow, so does the need for isolating cells of therapeutic importance. Stem cells can differentiate into multiple cell types, requiring a selection method to isolate the ones of interest; white blood cells are easily extracted using common clinical apheresis [4], but the T lymphocytes required for CAR-T must then be extracted from the other nucleated cells.

Cell separation has been achieved by many methods since its first

demonstration in the early 1960s [5], but it is currently overwhelmingly achieved by three methods, each of which occupies slightly over 30% of the cell separation market globally [6]. The first of these is the density gradient method. Cells are placed in a centrifuge tube [7], density of which varies from top to bottom. Cells move in this gradient through the spin process, coming to rest at the level where the density of cell and medium are equal. This method is commonly used where there are significant physical differences between the cells in question; for example, its primary application is in the sorting of red and white blood cells. However, as such changes correspond to significant differences between cells, it is less useful when separating stem cells from a common progenitor but with different differentiation fates, nor is it sufficiently sensitive to discriminate between subpopulations. However, it is the simplest and cheapest cell separation method, as well as offering the highest throughput; tens of billions of blood cells can be processed in as little as 30 minutes.

The next two common cell separation methods both employ the use of chemical labels to identify cells of interest; Fluorescent-Activated Cell Sorting (FACS) [8] and Magnetic-Activated Cell Sorting (MACS) [9]. FACS uses fluorescent labels to identify target cells, giving the technique its name. These labels can interact with antigens on the cell surface or within the cell interior; they may identify the presence of a particular large molecule, or could depend on a different physiological effect such as membrane potential (with fluorescence intensity dependent on transmembrane voltage). Cells are then passed through a machine containing one or more lasers used to interrogate the cells; fired in droplets containing a single cell, the cells pass through the beam before being electrostatically sorted into appropriate outlet streams. The final common method, MACS relies on discriminating between cells according to the presence or absence of specific antigens on the surface of the cells. Magnetic microbeads coated with antibodies raised against the surface marker are incubated, causing the beads to attach to the cells of interest (or the other, non-interesting population). A magnet is then used to extract the cells containing the beads, allowing separation.

These two methods share common characteristics; the most significant is that they identify cells by attaching a label (fluorescent or magnetic) to a protein of interest, as a basis for separation. This is significant, for a number of reasons. Firstly, the label will persist in the separated population, potentially limiting the uses (particularly clinical) to which the separated population can be put. Secondly, the cost of labels can be significant. Thirdly, incomplete labeling of the population can lead to losses of target cells of up to 50% of the population [10,11]. Fourthly, there is a risk that the label will interfere chemically with the cells. Finally, the use of labels adds a sample preparation step that adds time and complexity to the workflow. However, the methods also have advantages; whilst not as fast as centrifugation, MACS can process up to one billion cells in an hour, whilst FACS is limited to a few tens of millions of cells over 2–3 hours including sample preparation time and setup, whilst FACS in particular is also good for pinpointing

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very rare cells through the use of multiple dyes; MACS is ultimately limited to identifying cells by surface markers alone. Consequently, these methods are of questionable application for cell therapeutic applications [12].

In order to address these limitations, researchers have spent many years attempting to develop non-label-based (more commonly, 'label-free') separation methods that offer the benefits of centrifugation techniques with the added benefit of a broader range of selectivities. In order to achieve this, separation strategies must be identified that exploit physical differences between the cells, since only the properties inherent in the cells (such as density) are available in the absence of additional labels [13]. However, to be meaningful, such an approach must also offer the advantages of MACS and FACS, such as high throughput. Label-free strategies also potentially lack the flexibility of high-throughput systems such as FACS, where cells are sorted regardless of density; label-free, different approaches are commonly taken for circumstances where large numbers of cells are required, or where a very small number are to be sorted from a much larger population.

DEP FOR LABEL-FREE SEPARATION

Fundamentally, any separation method – particularly something that performs binary separation, rather than multi-population fractionation – performs some form of physical interrogation on the cells; the response of the cells to that intervention determines whether they are in the 'in' or 'out' groups – a form of shibboleth. In label-free sorting, this typically involves applying an external force field, and the response of the cell to that force field determines the direction in which it moves. To consider the example of gradient centrifugation, a force (the centrifugal force) is applied to the cells, which then respond differently according to where they lie in the gradient field. These different responses to the force then allow the cells to be moved to different locations within the tube, and subsequently separated. For label-free separation, it is necessary to identify ways in which the cells might physically be different between populations; examples typically include (but are not limited to) differences in cell size, mechanical compliance, and electromagnetic properties [13]. We can also classify separation methods according to whether they sort on a continuous or batch process; that is, whether solution containing cells to be separated is constantly introduced to the separation chamber and the separated components depart the process without interruption, or whether a defined volume is processed in a single process, then removed and repeated. FACS is an example of the former (separation can occur for as long as there is flow to the device), whilst centrifugation is an example of the latter (a batch of cells is processed at one time in a centrifuge). This being the case, what is the most suitable approach for cell separation for therapeutic purposes? The key figure is the total number of cells to be separated. Where a separation technique is continuous, there is no upper limit before the device becomes saturated; instead, the limit is one of time – how long does the separation of a

usable number of cells take? Taking T-cell therapy as an example, we can consider separation on the 'input' and 'output' ends of the process. If we were to extract all of the white blood cells from a healthy patient by apheresis, we would be looking at a starting solution of 10^9-10^{10} cells. From this, we need to extract perhaps 10% of the cells [14]. Cell numbers are smaller when grown in culture – a T175 flask at confluence contains perhaps $2x10^7$ cells. This then defines the way forward; a viable label-free separation method needs to discriminate on the basis of physical differences within the cells at a rate suitable for the application at hand.

Other than density gradient methods, several strategies have been put forward for label-free separation at volume; few strategies have developed beyond laboratory proof-of-concept stage, and none is in widespread use. One of the most advanced is separation on the basis of different electrical properties, using a phenomenon called dielectrophoresis (DEP) [15]. Related to electrophoresis, a much more commonly understood technique in biological sciences, DEP does not use the intrinsic charge of the moieties to be separated (which can include proteins but which are more commonly cells), but instead interacts with the dipole (with positive and negative components) induced when suspended in an electric field. The induced electrical dipole has an orientation and magnitude that is dependent on the electrical properties of the cell at the frequency of the field. By using a non-uniform field – that is, one with a gradient – cells are be attracted toward, or repelled from, the electrodes generating the field depending on the orientation of the dipole with respect to the field. A mixture of cells with different properties may experience frequency windows where the cell types respond by moving in opposite directions, causing physical separation that can then be exploited.

Whilst earlier observations of particle movement had been made, it was Herbert A Pohl of Oklahoma State University who first analyzed the phenomenon in detail, giving it both its definition and name. After studying the effect on a range of particles including bacteria, cell separation by DEP was first demonstrated in 1966 [16], with the separation of live and dead yeast cells by Pohl and his student, Ira Hawk. Since then, a range of technologies have been developed to make the technology suitable for industrial and biological applications. These have focused on improving throughput, specificity or providing features such as multiple separation outputs. Early systems used machined electrodes to generate the inhomogeneous electric fields required for DEP to work; however, limitations on electrode design and the relatively low field gradients precluded these devices from high throughput and selectivity. Technology moved forward significantly in the late 1980s when the group of Ronald Pethig at the University College of North Wales at Bangor developed the first microengineered electrodes [17]. Using principles from the fabrication of microelectronics, these electrodes featured sharply defined contours that allowed much larger gradients in electric field strength. Using their pioneering 'interdigitated, castellated' electrodes etched into a thin film of gold across the bottom of a chamber and then flowing cells across these, the group

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demonstrated separations of the order of those achieved with FACS and MACS. The group demonstrated the efficacy of the system in multiple applications, beginning with live and dead yeast [18], and progressing to cells of therapeutic use in 1994 by demonstrating the enrichment of $CD34^+$ cells from bone marrow [19,20]. Others using the same technology subsequently showed the separation of circulating tumor cells from general leukocyte populations [21].

THE PATH TO COMMERCIALIZATION

Whilst this technology showed great potential, it was never developed commercially. In the following years, many variants of this separation technology were published, presenting multiple separation outputs [22], vertical electrodes [23] and more, and several cell types used; several excellent review can be found describing both technology and applications elsewhere (e.g., [24]). However, this period did not see the advancement of DEP separation outside of engineering departments; it could be argued that there was a disconnection between the technology providers and the ultimate end users. Nevertheless, a number of scientists in the biomedical field used DEP separators to show that the technology could be used to separate stem cells with different differentiation fates on the basis of differences in membrane capacitance, including stem cells of neural, adipose, stromal and skeletal origin [25–30]. Another work by the Gascoyne group at the MD Anderson Cancer Center on the characterization of cells showed how differences in cells could be exploited for separation, showing that T-cell properties differ sufficiently from other leukocytes to be potentially separable by this method [31].

Whilst the development of DEP technology and applications has largely remained within academia, three platforms have emerged to bring DEP separation devices to reach market. Two offer high throughput, but are developed for very different applications; the third eschews throughput for precision. The first of these [32] was developed by American company Apocell, based on technology originally developed in the laboratory of Peter Gascoyne at the MD Anderson Cancer Center in Houston, TX, and has thus far been developed specifically for the isolation of circulating tumor cells from white blood cells. This is a continuous process whereby a mixture of cells passes along the edge of a chamber. The mixture passes over DEP electrodes that induce a repulsive force in normal white cells, but not the desired tumor cells. Those remain next to the channel wall, where they are extracted through a thin slit; the remained flow with the bulk medium towards a waste outlet. The system is still highly specialized in its application but offers very high rates of selectivity whilst processing cells at rates of up to 5000 per second. However, the platform is still expensive to purchase, on par with high-throughput flow cytometry.

The second DEP platform [33], only reaching market now, was developed at the University of Surrey, UK. Based on technology developed previously for high-speed cell analysis [34] and called the DEParator, the technology takes a different approach to that taken by Apocell.

Rather than aiming for very high selectivity (the 'one in a million' separations), the device uses high throughput and low cell loss to enrich populations rapidly, the intention being that multiple passes through the separator allow the user to achieve high degrees of separation with minimal outlay. The device operates by pushing a mixture (typically 10–15 ml) of cells through a chip containing 4-500 'wells', each striped along the side with 12 electrodes. These generate fields that trap one population of cells whilst the other falls through and is collected; once this is finished, the field is removed and the trapped portion can be recovered. The whole process takes approximately 10–15 minutes, and both the 'passed through' and 'trapped' populations can then be re-separated to further enrich the desired population. Cell losses are sufficiently small (typically 2% per pass) for several passes to be used without losing the target cells. On the other hand, the cell numbers that can be processed by the device are significantly higher than can be performed by any process other than centrifugation – separation speeds in excel of 150,000 per second have been demonstrated, whilst total cell numbers approach one billion. Furthermore, the cells spend the entire process contained within a disposable container containing the chip and pumping mechanism, meaning that there is no risk of cross-contamination, and hence that the system can be engineered to meet Good Manufacturing Practice standards for cell separation for therapeutic purposes. It is anticipated that the consumable would retail for under $25, whilst the base station containing the support electronics is likely to be comparable to a MACS device. It is anticipated that this system will reach market in 2018.

The third commercial system is the DEParray form Silicon Biosystems [35]. This differs from the other two systems in that separation does not occur on the basis of electrical properties, but instead by optical detection of physical characteristics (which may include markers, but could also use cell morphology). Instead, the technology locks tens of thousands of cells into DEP-actuated 'cages', and then unlocks adjacent cages to allow the target cells to be 'corralled' into areas where they can be collected. Aimed much more at identifying rare cells than at processing large numbers of cells, it nevertheless offers a different approach to DEP-actuated separation.

CONCLUSION

As technologies for cell therapies progress towards general use, there is a need to address potential bottlenecks to production. There is a need to isolate patient cells in a manner that is faster, cheaper and more compatible with good manufacturing practice than are currently available. Whilst there is development work yet to do, it is possible that DEP may step forward to fill this gap in the coming years.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The author is Director of Deparator, which manufactures a DEP-based cell separation system. No writing assistance was utilized in the production of this manuscript.

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