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ABSTRACT

Regenerative medicine has the capability to revolutionise many aspects of medical care, but for it to make the step from small scale autologous treatments to larger scale allogeneic approaches, robust and scalable label free cell sorting technologies are needed as part of a cell therapy bioprocessing pipeline. In this proceedings we describe several strategies for addressing the requirements for high throughput without labeling via: dimensional scaling, rare species targeting and sorting from a stable state. These three approaches are demonstrated through a combination of optical and ultrasonic forces. By combining mostly conservative and non-conservative forces from two different modalities it is possible to reduce the influence of flow velocity on sorting efficiency, hence increasing robustness and scalability. One such approach can be termed "optically enhanced acoustophoresis" which combines the ability of acoustics to handle large volumes of analyte with the high specificity of optical sorting.

Keywords: Cell sorting, acoustophoresis, optical trapping, optical guiding, cell therapy, bioprocessing.

1. INTRODUCTION

Progress in stem cell research and regenerative medical treatments is leading towards the development of more complex cell-based therapeutic products that promise to revolutionise healthcare. Cell-based products create new challenges for bio-production and processing requiring a re-think of current manufacturing technology. A key consideration in the manufacturing of any product is its purity and freedom from contamination. In the case of stem cell derived therapeutics, this encompasses freedom from unknown or adventitious pathogens, through to the homogeneity of cell populations. This homogeneity is important throughout the manufacturing process, to ensure robustness and reproducibility of protocols through to the resulting end product regardless of whether it is used *in vitro*, such as in high-throughput screening platforms, or for transplantation *in vivo* to treat degenerative disease or damaged tissues. A critical concern for cell therapies, especially cells derived from embryonic stem cells, will be the absence of undifferentiated cell populations with a potential to form teratomas, benign tumours capable of interfering with tissue physiology, or worse, teratocarcinomas, with metastatic potential. Furthermore, realisation of the true potential of allogeneic cellular therapies demands the manufacture of cellular products on a larger and larger scale, far exceeding the throughput of currently available separation technologies. Consequently there exists a pressing manufacturing need for effective high throughput separation technologies, a solution which has still not been found by the bioprocessing research community or industry.

1.1 The challenge of allogeneic cell therapies

There are two main approaches to achieving a cell product for cell therapies. The first, and by far the simplest, is an autologous therapy. Autologous therapies are ones where the recipient of the therapy is also the donor. This vastly simplifies issues such as compatibility and regulatory approval. It tends to involve relatively low volumes of cells and is a small scale, non-industrial process by its very nature. However, it is a slow and expensive process and is not suited to the widespread adoption of cell therapies. In order to obtain large volumes of cells for cell therapies, one instead needs to use an allogeneic process, where there is one donor and many recipients. This allows for the scaling of a therapy, but

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introduces complex issues for ensuring the purity of the cell product and subsequently for obtaining regulatory approvals. Figure 1 shows graphically and in numbers the main differences between the two approaches. Importantly, the cell sorting technologies needed for autologous therapies already exist, but they are not directly transferable to the greater demands on throughput and purity required in an allogeneic cell therapy bioprocess.

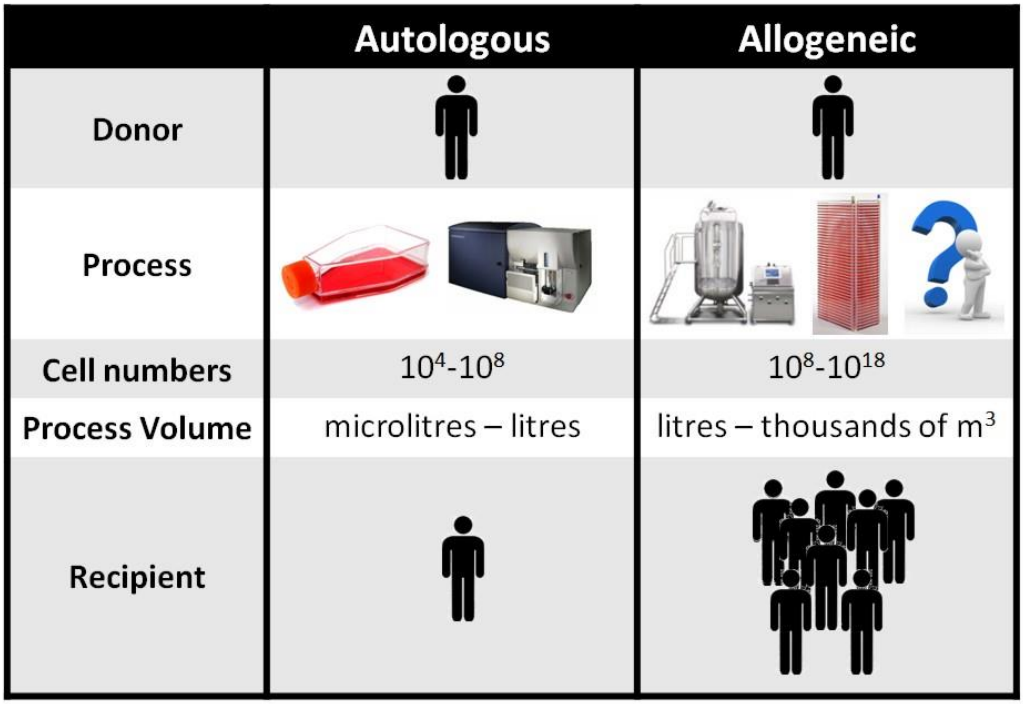

Figure 1. The differences between autologous and allogeneic bioprocesses lead to different challenges, which for allogeneic therapies have not yet been solved.

1.2 Current approaches

Cell sorting and cytometry are well developed and mature methods in the life sciences and medicine. However, none of the existing technologies is likely to be capable of meeting the future needs of a cell therapy bioprocessing industry. Approaches such as FACS and MACS rely on labelling of cells with antibodies, an approach which would be expensive for an industrial cell separation process and which introduces regulatory issues due to the difficulty in removing labels before the introduction of the cells into a patient. MACS is relatively scalable, but FACS is a single-file flow process which requires an active decision for every cell. More traditional approaches such as centrifugation and cross-flow filtration are likely to form part of the bio-processing pipeline, but they lack the specificity needed to ensure the removal of undifferentiated cells or may be too harsh in their handling of the cells (e.g. shear forces for in-line centrifugation).

1.3 Passive cell sorting as a solution

In this proceedings we present methods which are simultaneously label free, scalable, sensitive enough to distinguish cells with similar properties and compatible with in-line manufacturing processes. A key consideration in achieving these attributes is for the sorting mechanism to be passive, that is that it does not require any decision based on a measurement of a cell property to determine where it should be directed. Instead, a passive sorting technique relies on inherently selective separation of particles. Some studies have wrongly stated that a passive sorting technique is one where there is no externally applied force [1], however, passivity comes solely from whether or not a decision making step is required before an "intervention", such as electrostatic deflection in a FACS machine. Hence, passive sorting can be achieved using many different approaches including, but not restricted to, hydrodynamics (e.g. pinched flow fractionation), ultrasonics (e.g. acoustophoresis), magnetics, dielectrophoresis and optics[2-5]. Each of these approaches has its benefits and challenges. Many of them require a precisely calibrated flow, be it to achieve the right hydrodynamic conditions alone or to balance fluid drag against an applied force. Though it is not possible to entirely remove the role of the carrier fluid in a passive sorting technique, there are mechanisms with which its role in determining the sorting outcome can be

reduced. One such approach is to achieve a fractionation effect through the balance of two externally applied forces, rather than balancing the applied force purely against fluid drag. In the work presented here we use a combination of ultrasonic and optical forces to achieve sorting.

Ultrasonic manipulation of cells has been shown to be effective for handling large volumes of analyte and has even been incorporated within some FACS machines, such as the products from SonoSep and BioSep. However, it can be difficult to achieve the required levels of selectivity and specificity that many applications require using the selectivity of ultrasonic forces alone. Optical sorting of cells has been shown to be highly specific and can be easily customised to a specific sorting task, but is inherently best suited to handling low volumes of analyte and is difficult to scale. We show that a combination of ultrasonic cell handling with optical forcing, a combination of conservative and non-conservative forces, can lead to a robust and scalable cell sorting approach which may be suited to incorporation into a cell therapy bio-processing pipeline. Importantly our approach requires only the simplest of microfluidics, ultrasonics and optics, making the combination of technologies an affordable and adaptable solution as well a technically promising one.

2. SCALING STRATEGIES

There are many candidate technologies suited to addressing the challenges of cell sorting for allogeneic cell therapies. Here we present some different generic strategies for overcoming the scaling challenge and how they might be implemented using a combination of optical and acoustic sorting.

2.1 Dimensional scaling

One of the most logical ways in which to scale up a sorting process is to go from a 1D process, such as the single file flow of particles used in fluorescence activated cell sorting (FACS) to sorting in a single 2D plane, as seen in our previous fractionation publications [6], to full 3D volume sorting (see fig. 2). One way in which the latter can be achieved is through the use of radiation force-balanced, 3D optical lattices. Such an approach ensures that the gradient force on a particle dominates and particles are not pushed to the walls of a channel, allowing sorting to be performed throughout a volume flow. Another approach, and the one which we present in this proceedings, involves the parallel manipulation of particle flows using ultrasonic standing waves in a microcapillary, with simultaneous lateral guiding, similarly to the VALOR (Vertical Acoustic Levitation with Optical Routing) concept [7].

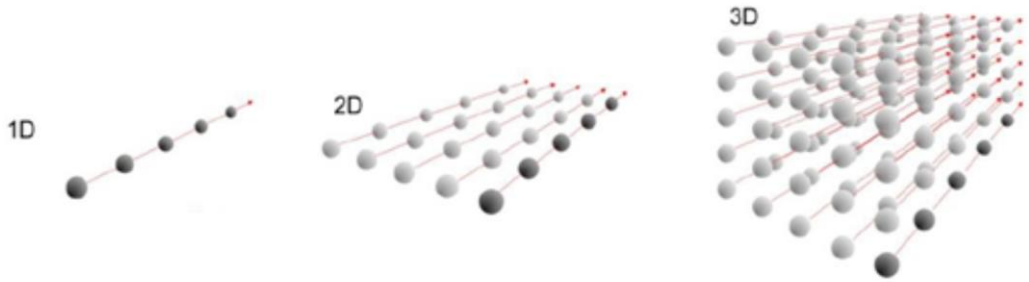

Figure 2. Dimensional scaling: by scaling from a single-file flow to planar then volume flow it is possible to inherently scale a sorting method, essentially moving from a 1-dimensional to a 3-dimensional approach.

2.2 Sorting from a stable state

An inherent challenge in label free sorting is finding the “sweet spot” where the spread of physical properties of an analyte leads to the desired particle deflections and separation. A key requirement for scaling of any process is that it is robust enough to be insensitive to the inevitable flow perturbations and heterogeneity of cell properties that will occur. Hence, a label free sorting method that requires a perfect balance between flow and applied field strength will prove impractical for high throughput sorting (blue line in figure 3). The ideal sorting method would be flow rate-independent (red line in figure 3), but the reality is that flow rate always plays a role. We propose two solutions which address this challenge: 1) sorting from an equilibrium, or stable, state where the sorting outcome is the same for a wide range of flow rates (green line in figure 3) and 2) sorting approaches which, though they produce a range of deflections for different flows, lead to the same final sorting outcome and which are hence robust to changes in flow rate, within a range of flow rates.

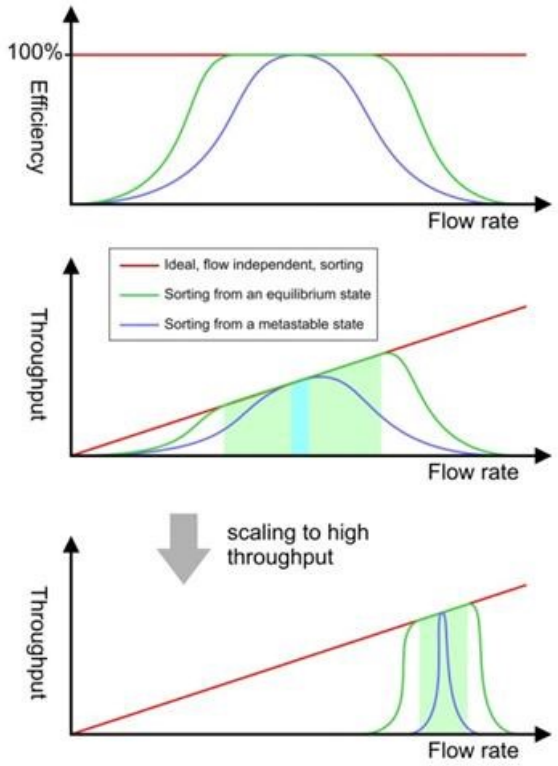

Figure 3. Graphical representation of sorting from a stable state. It is important to ensure that a sorting mechanism is robust enough to be scaled. If sorting from a metastable state it will become increasingly difficult to hit the sorting "sweet spot", but when sorting from an equilibrium the process should be robust to scaling.

One approach to achieving sorting from an equilibrium is to use a balance of applied forces, rather than to balance viscous drag against an applied force. This avoids issues with flow instabilities which will become increasingly problematic at higher throughputs. An exemplar of this strategy, and one which we present here, is to balance an acoustic gradient force against an optical radiation force, balancing a conservative force against a non-conservative force.

2.3 Sorting rare species

Sorting for cell therapy will require the ability to remove very rare species as the overwhelming majority of cells will be differentiated into the target state. Hence, the demands on a sorting method depend largely on whether it is designed to deflect the majority species or the minority species. The former requires almost all of the cells in a sample to be deflected whilst the latter requires just that tiny portion which needs to be identified/removed to be deflected, greatly reducing the burden on the system as we scale to ever greater throughput. An example of this approach is described in this proceedings, where small deflections in periodic ultrasonic standing waves can be used to amplify acoustophoresis through the addition of optical deflection within fractionation zones to achieve a highly specific form of acoustophoresis: optically enhance-acoustophoresis.

3. SCALING SOLUTIONS

In this section we describe the implementation of the strategies discussed in section 2. Each of them uses a combination of acoustic cell handling in an ultrasonic standing wave in combination with optical radiation forces and/or optical guiding.

3.1 Acoustic/Optical force balance sorting

In this subsection we discuss the balancing of optical and acoustic forces in an attempt to reduce the importance of the flow velocity in finding a stable sorting state. The forces on a particle due to acoustic trapping, scale with particle radius cubed [8,9], meaning that larger particles are more strongly held in the acoustic nodes than smaller particles. Although the optical radiation pressure also scales with particle radius (Equation 2), the force scales much more slowly as the particle size becomes larger than the beam diameter [10]. This presents an opportunity to exploit the contrast in the applied forces from optics and acoustics on different particle sizes to achieve separation.

To demonstrate the proposed separation method, a single beam optical setup was constructed, with a low NA (0.25) aspheric lens used to focus the beam in to the sample chamber. From below, the beam enters a glass capillary of inner dimensions (300x300) μm, which is bonded to a transparent LNO-ITO transducer [11]. The use of a transparent transducer allows for all planes of optical access. The capillary is viewed from the side with a simple microscope system using Köhler illumination, a 5X long working distance objective and a CMOS camera.

The transducer is driven at 4.34 MHz with an arbitrary waveform generator, which is empirically found to give the 2nd harmonic across the capillary walls. This results in four acoustic streams along the length of the capillary, illustrated by the black dashed lines in Figure 1. The beam is positioned to intersect with two of the acoustic streams, such that particles are deflected from the lower stream into the upper stream, as depicted in Figure 4.

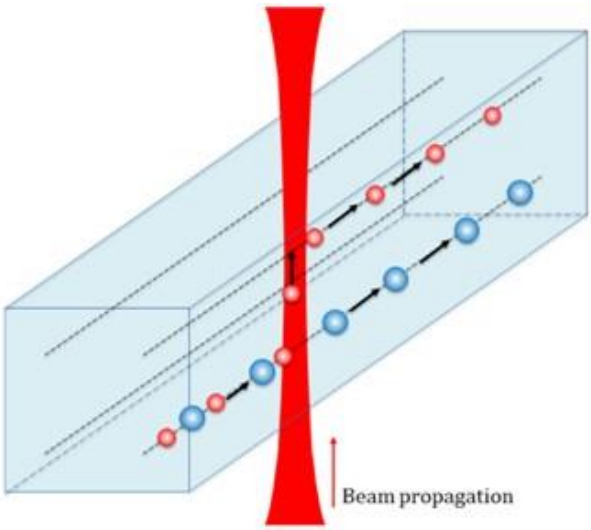

Figure 4. The loosely focused laser beam enters the glass capillary from below, and is positioned to intersect with two of the four acoustic streams (black dashed lines). The balance of forces from the laser beam and the acoustic trapping elicits a differing response from the different particle sizes, such that separation of the two particle species can occur. The smaller particles (red) are deflected in to the upper acoustic stream more readily than the larger particles (blue).

The main driver of deflection arises from the optical radiation force in the direction of beam propagation. This force is balanced against the forces generated from the acoustic trapping, providing a binary separation of the particles between the upper and lower streams. The optical gradient force of the beam will also play a role in keeping the particles confined in the optical beam for the duration of deflection, balancing with the force from the Stokes drag due to the fluid flow. However, the separation is still dominated by the balance of the optical radiation force and the acoustic gradient forces. It

is worth noting that there is an upper limit to the optical power which can be used, essentially limited to below a level where significant heating of the channel takes place, and leads to distortion of the acoustic standing wave.

A solution of 7 and 10 µm polymer microspheres in distilled water is flowed through the capillary at a constant average particle (flow) speed of 58 µm/s (0.3 µl/min) measured by video analysis. The particles need only be deflected just over half way between the two acoustic nodes to be drawn in to the upper acoustic stream. Particles which do not reach the half way point are drawn back down in to the lower acoustic stream. In this way, a small difference in optical separation is emphasised by the acoustic trapping. The laser is gradually increased in incident power from 93 mW, where there is enough force present to slightly displace the particles, but not enough to deflect either particle size in to the upper acoustic stream, to 184 mW, the upper limit found where all particles are readily deflected in to the upper acoustic stream, and no separation of particle sizes is observed. The acoustic power is kept constant at 12 Vpp throughout the experiment.

For quantification, particles are counted as sorted if they are deflected from the lower stream in to the upper stream. Particles that are not deflected to the upper stream are counted as unsorted. This selective deflection is illustrated in Figure 5.

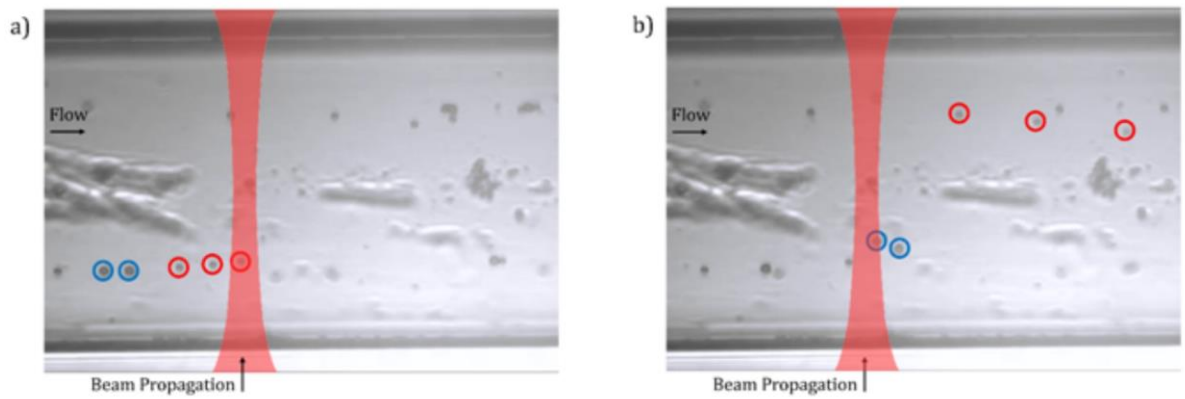

Figure 5. The 7 µm (red) and 10 µm (blue) particles are shown a) acoustically confined prior to entering the optical field, and b) separated by size due to their response to the balance of optical and acoustic forces.

The results of the experiment are displayed in Figure 6, for the percentage of each particle size deflected in to the upper acoustic stream as a function of increasing laser power. The results show a peak separation at a laser power of 112 mW. At this power, 62% and 1% of the 7 and 10 µm particles respectively are deflected from the lower to the upper acoustic stream. This leads to a 98.4% enrichment of the 7 µm particles in the upper acoustic stream for a single pass through the sorting region.

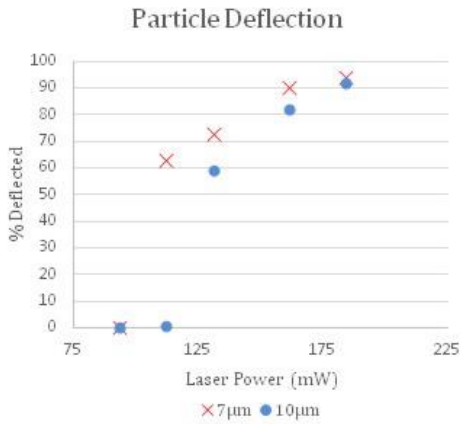

Figure 6. Results showing the particle deflection efficiencies as a function of increasing laser power. It can be seen that the smaller 7 µm particles are more readily deflected at lower laser powers, leading to a separation of the particles by size. The optimum laser power for sorting is observed at 112 mW.

If the optical radiation force is capable of deflecting a single 7 µm particle from the lower into the upper stream, it should reach a deflection efficiency near 100%. There are some practical reasons why in this case the deflection efficiencies are less than expected. In this experiment, the particles are not all confined to precisely the same lateral position in the capillary, and although slightly off-axis particles can be brought in to line by the optical gradient forces present, this does lead to some particles having less interaction time with the laser beam. Additionally, secondary radiation forces from the acoustic field can cause particles to form agglomerates [12], which causes some particles to enter the laser beam as a group of particles. These agglomerates are typically not deflected, leading to a reduction in the measured efficiency, though it could be argued that it is beneficial to keep agglomerates out of the enriched upper stream, in which case efficiencies could be deemed to be even higher than the 98.4% we report here.

A proposed solution to overcome these issues is to have the particles hydrodynamically or otherwise focussed in such a way that the spacing of the particles can be controlled [13], such that they enter the path of the laser beam one by one. This is not dissimilar to the manner in which particles are prepared for processing in FACS.

Where this technique can differentiate itself from other single-file techniques such as FACS is the possibility of using multiple beams to affect more than one pair of streams, or the same pair of streams multiple times, in an entirely passive nature. Affecting the same pair of streams multiple times could help increase the enrichment with each additional processing step. The demonstration in this paper details one pair of acoustic streams, however the addition of several independent optical beams acting on different pairs of streams can increase both the number of sorting sites and the range of parameters by which particles are deflected. This could be a possible way to scale up to selectively deflecting many more types of particles in the same solution. This can be further expanded upon by producing more acoustic streams by driving the system at higher harmonics, resulting in many more possible configurations of particle deflection, as discussed in the next subsection.

3.2 Acoustic/Optical sorting with dimensional scaling

Here we discuss how the use of acoustic standing waves within simple microcapillaries can lead to the simultaneous confinement of multiple particle streams throughout the capillary's whole volume. If combined with the force balance sorting shown in section 3.1 this gives dimensional scaling from an equilibrium state, hence combining two of the strategies identified in section 2.

The experimental set-up used in this section was essentially identical to that described in section 3.1. The main difference however came from the driving frequencies used to generate the ultrasonic standing wave in the microcapillary. In 3.1 the acoustic mode excited was the 2nd harmonic of the system, such that two trapping planes are created within the microcapillary. However, it is also possible to drive at the fundamental frequency or at higher harmonics. In fact, for sufficiently symmetrical square capillaries the trapping nodes are not restricted to vertical confinement, but are also produced laterally, such that multiple particle streams can be produced throughout the flow volume within the capillary, as illustrated in Fig. 7.

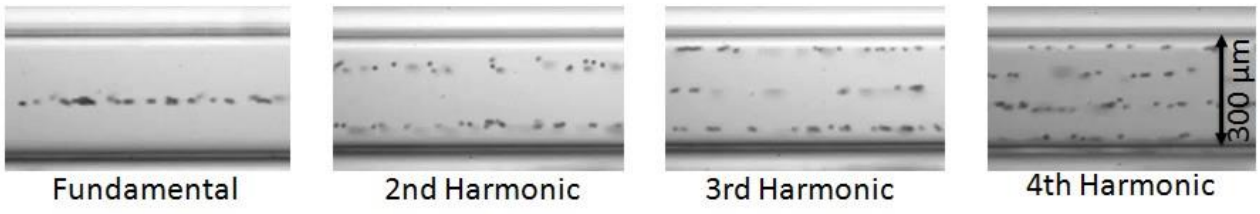

Figure 7. Multiple acoustic harmonics, and hence multiple flow confinement patterns, can be excited in a single microcapillary. For symmetric capillaries these patterns are in both lateral directions, resulting in 1, 3, 9 and 16 particle streams for the fundamental, 2nd harmonic, 3rd harmonic and 4th harmonic respectively. Note that the out of focus images of further particles streams at different depths within the capillary can be seen for the 2nd 3rd and 4th harmonics.

Using the multiple particle streams facilitates either direct dimensional scaling or, alternatively, the ability to deflect particles from one stream to the next, at multiple locations within a single microcapillary.

3.3 Optically enhance acoustophoresis

In subsection 3.2 it was seen that particles form long laterally confined streams within a microcapillary. However, because both the capillaries and the ultrasound transducers used to excite the standing waves are finite in length, end effects can lead to longitudinal inhomogeneities along the capillary. This in turn leads to longitudinally periodic "dispersion" regions, where particles are no longer laterally confined. This effect can be seen in Fig. 8. This mechanism can in fact facilitate a new form of sorting which allows the selection of rare species only and as such is an example of the third strategy described in section 2.

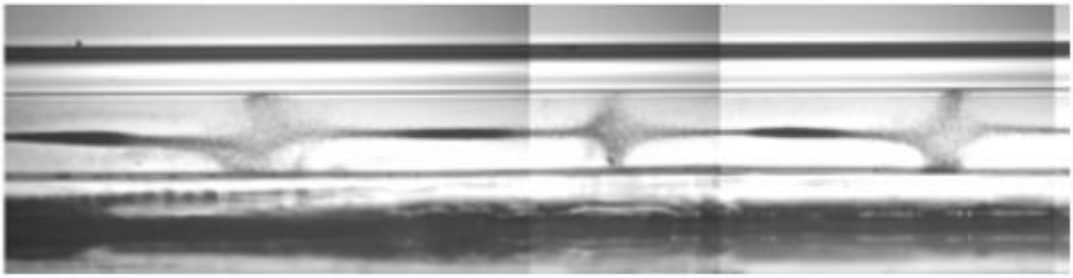

Figure 8. Cross section of 300 μm wide square capillary showing vertical confinement within the fundamental mode of the capillary. Due to end effects of the capillary and the finite length of the ultrasound transducer there are periodic zones with poor lateral/vertical confinement, as seen by the dispersion of 1 μm silica spheres.

In order to determine the mechanisms which lead to this phenomenon, a finite element model was built.

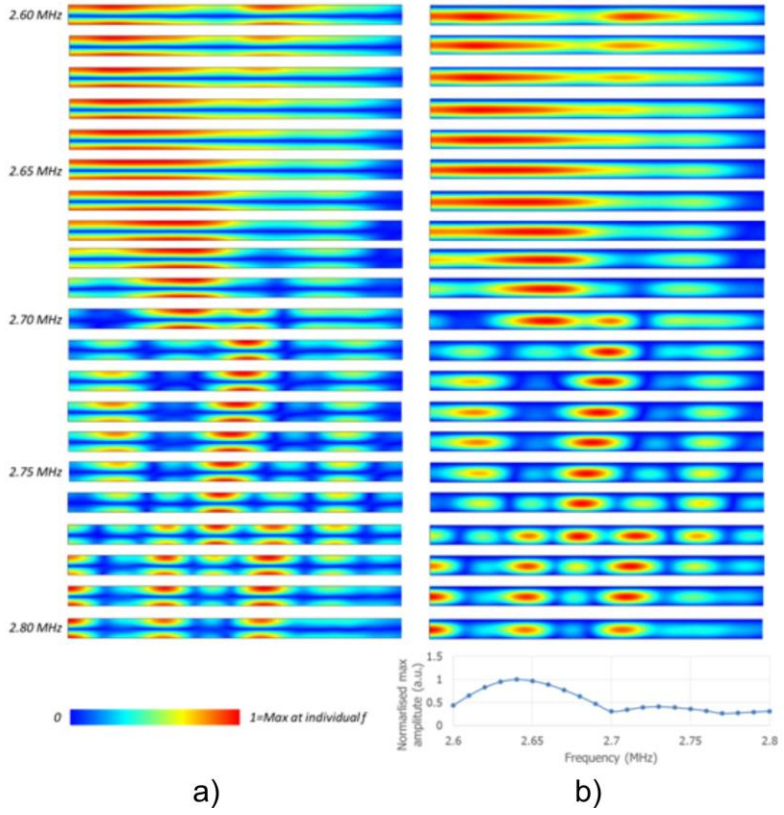

Figure 9. Normalised (a) pressure and (b) velocity magnitudes of the water channel between 2.60 to 2.80MHz with the increment of 10 kHz.

In figure 9 we see that depending on the precise excitation frequency used, many different periodic ultrasonic standing wave patterns can be excited within even a single simple microcapillary. Under static conditions (Fig. 8), tracer particles were used to demonstrate the intermittent nature of the acoustic confinement along the length of the capillary at a driving frequency of 2.724 MHz. The particles suspended in the fluid were observed to be disperse prior to activation of the transducer, but trapped at intervals along the capillary with the transducer active.

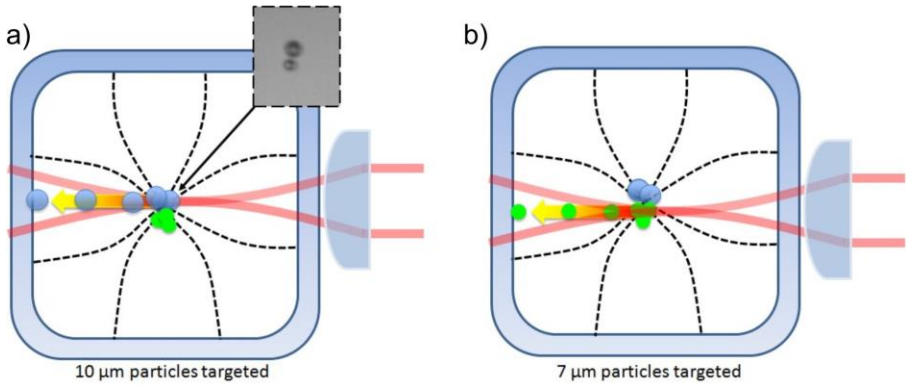

Figure 10. Schematic representation of how dispersive regions within an acoustic standing wave can be targetted with a laser to turn a small acoustophoretic fractionation into a large spatial separation. Particles can be targetted at different heights, as shown in a) and b). The inset in a) shows an image taken from the side of the capillary of 10 µm and 7 µm particles sitting at different heights in the flow. The dotted lines indicate the lateral acoustic pressure and the red lines the laser focussed into the capillary to achieve complete spatial separation of different fractions within the weakly acoustically confined regions.

Under flow conditions, particles in a polydisperse sample were observed to fractionate at locations where the acoustic confinement changed from a gradient trap at the capillary midline to an acoustic field "null". By locating the laser at the null in the acoustic field (both pressure and velocity) where the separation was greatest, it was possible to selectively deflect one species in the presence of another by selecting the particle of interest's unique trajectory, as illustrated in Fig. 10. Under the same flow conditions, particles will always follow the same trajectory predetermined by their physical properties and will be automatically selected by the laser. The same effect was observed repeatedly along the channel at the repeating locations of acoustic field nulls. Attempts to separate the particles in areas with no acoustic fractionation using optical forcing alone failed. The combination of the acoustic fractionation and optical forcing was absolutely necessary for particle separation in this experimental arrangement.

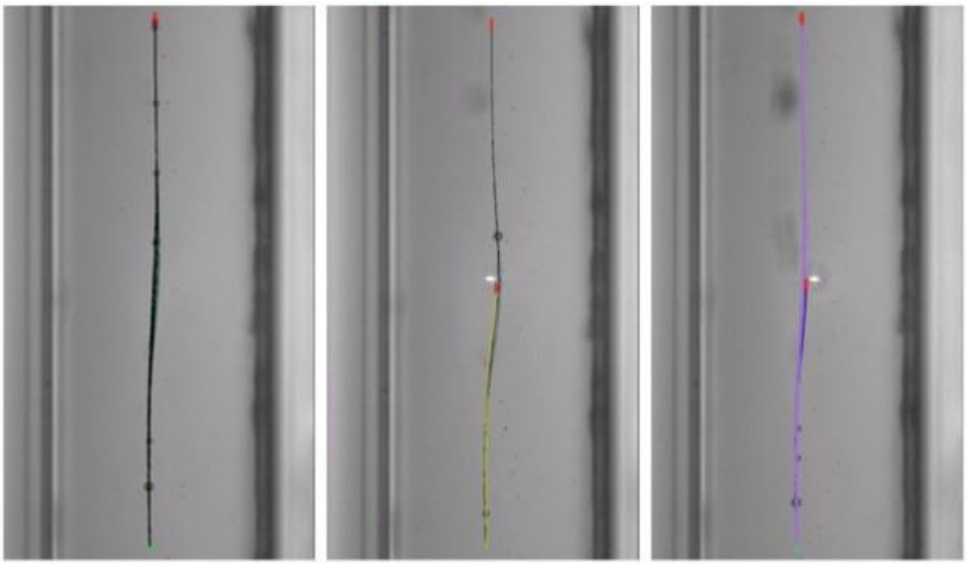

Figure 11. Optically enhanced acoustophoresis.. The left hand image shows the particle trajectories for a polydisperse flow of 7 and 10 mm particles. The centre image shows the targeting of the larger particles with a laser and the right hand image the targeting of the smaller particles for laser deflection.

A representative sorting location is illustrated in figure 10a (inset shows particles in a sample of different sized polystyrene particles). This difference in height between the two particle sizes is typically too small to spatially separate the two species with individual physical outputs as in acoustophoretic separation, but with the selectivity of optical forcing, selected particles may deflected orthogonally by a laser beam. Note that without the slight separation provided by the acoustic trapping nulls the optical forcing would not be able to distinguish between the particles, unlike in 3.1. Figure 11 shows the situation where the laser is used to target first the larger 10 μm particles (magenta trajectories) then smaller particles (yellow trajectory) in turn. The traced particle trajectories clearly show that one particle type may be rejected in the presence of another almost identical species.

4. CONCLUSIONS

There exists an applications need for future cell therapy bioprocesses. One example of such a process is the industrial production of red blood cells. However, though this application is potentially one of the simplest from a regulatory point of view (a full differentiated erythrocyte has ejected its genetic material) it presents a challenge in throughput never before seen for label free cell sorting (up to 10^{18} cells per year for the UK alone). It is unlikely that any single technology will be able to meet the throughput and selectivity/sensitivity demands in a single step, however, by combining more traditional techniques such as cross-flow filtration with new scalable label free techniques could find a solution to this challenge before it becomes a manufacturing bottleneck capable of halting the translation of cell therapy into wide clinical use.

In this proceedings we introduced and demonstrated three possible strategies for scaling label free cell sorting. By combining the ability of acoustics to handle large throughputs with the specificity of optical manipulation we were able to demonstrate approaches which do not rely heavily upon precise flow or which have the ability to be scaled into multiple dimensions, opening up the possibility for sorting mechanisms which can truly be scale up and not just out.

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