Intracellular Dielectric Tagging for Improved Optical Manipulation of Mammalian Cells

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(Invited Paper)

***Abstract*—Optical micromanipulation of transparent microparticles such as cellular materials relies upon the application of optical forces that are crucially dependent on the refractive index contrast between the particle and the surrounding medium. We briefly review the application of optical forces for cell manipulation and sorting, highlighting some of the key experiments over the last twenty years. We then introduce a new technique for enhancing the dielectric contrast of mammalian cells, which is a result of cells naturally taking up microspheres from their environment. We explore how these intracellular dielectric tags can influence the scattering and gradient forces upon these cells from an externally applied optical field. We show that intracellular polymer microspheres can serve as highly directional optical scatterers and that scattering forces can enable sorting through axial guiding onto laminin-coated glass coverslips upon which the selected cells adhere. Such internal dielectric tagging presents a simple, inexpensive, sterile technique to enhance optical manipulation procedures for cellular material and may enable new sorting techniques within microfluidic systems.**

***Index Terms*—Intracellular dielectric tagging, optical cell sorting and axial and scattering forces.**

I. Introduction

THE ADVENT of optical tweezers in 1986 [1] presented a step change for single-molecule biophysics. Whilst optical forces, induced by the transfer of momentum between the incoming light field and a transparent microscopic object, have very successfully enabled a broad range of studies at the single-molecule level, there remain outstanding issues when these techniques are applied directly to move and manipulate cellular materials [2]. These problems are, in large part due to the low refractive index contrast between the cell and its surrounding medium.

Optical trapping has proved very successful in biophysics when applied as a calibration device to measure miniscule forces in a quantified manner [3], [4]. In this way, major studies of single molecules, particularly molecular motors, have been accomplished [5]. This includes studies of kinesin motion upon microtubules, the famous muscle contraction protein system of actin–myosin and deoxyribonucleic acid (DNA) [6]. Optical traps have also been applied to important studies at the cellular scale and cell biologists have used such techniques to explore cell–cell interactions and signaling, the viscoelastic properties of given cell types and the transfer of cells between different buffer media [2]. Additionally, related techniques have been applied to various topics, including fundamental physics [7], colloidal science [8], and microrheology [9].

An emergent area in cell biology and biomedical research is the identification and characterization of individual cells or cell subpopulations and their subsequent isolation or purification from a sample solution that contains a complex mixture of various different cell types [10]. For example, the ability to enrich easily and isolate both rare and not so rare populations of cancer and stem cells from large mixtures of contaminating cells is an urgent need in clinical oncology and related biomedical research fields [11]. Conventional means of fractionation and sorting from biological samples rely on rather laborious protocols, some involving chemical pretreatment or tagging of the sample and the use of equipment requiring bulk volumes of analyte. Although fluorescent-activated cell sorting (FACS) [12] provides fast, high-precision results for biomedical diagnosis and efficient active sorting, a portable microfluidic device would offer a disposable, sterile cell-separation platform for a wider usage across the biomedical sciences. Recently, cell sorting at the microfluidic scale has come to the fore particularly with the use of optical and dielectrophoretic sources [13]–[15]. The use of optical forces in this respect is attractive. However, their application directly to cells may be hampered by the weak dielectric contrast between the cells and the surrounding buffer solution [14], [16]. In our previous research, we have discussed the possibility of enhancing the transport of cells and the sorting process by tagging microspheres onto the surface of cells [17]–[19]. We have shown that cells with tagged microspheres offer an enhanced response to an optical potential energy landscape and have demonstrated a very simple sorting scheme using a "nondiffracting" Bessel beam [17]. Although reasonable results were achieved, this tagging approach requires the proper selection of particle-specimen bonding reagents, i.e., the use of specific ligand or antibody for coating the surface of the tagging particle, thereby promoting either receptor–ligand or antigen–antibody interactions. We note that such a form of

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dielectric sphere tagging is a well-established method in manipulation of macromolecules [20]. It would, therefore, be a significant step for sorting, and more general optical manipulation, to explore a simplified method for enhancing cell manipulation.

In many instances, the refractive index of the cell is very close to the refractive index of the cell culture medium, so cells are relatively difficult to manipulate optically, in contrast to silica or polymer microspheres. As an example, Chang *et al.* [21] showed that the optical forces on CHO cells can be modeled in a single multilayer sphere system. They showed that the optical forces from a simulated CHO cell (with average refractive index of 1.3) are around 3 times weaker than that on a polymer sphere (average refractive index of 1.45) of the same size. In practice this means that higher optical powers must be used to manipulate cellular materials than may be expected which places a restriction on the lasers that can be used and may cause damage to the cells being manipulated. In addition, cells are known to possess an inhomogeneous scattering volume due to their varying morphological features [22] which weakens the strength of optically induced forces acting upon them.

Internalization of a foreign body by a cell is a process that can be categorized as phagocytosis [23] or endocytosis. In higher organisms, "professional" phagocytes including macrophages, neutrophils, and dendritic cells facilitate elimination of senescent cells and invading pathogens through this process. Unicellular organisms can utilize phagocytosis for nutritional purposes [24]. An interesting application of such processes is to allow the cell to internalize an object of interest such as a dielectric microsphere, which can subsequently be used to perform a particular function on the cell. Recently, optical tweezers have been used in several studies investigating specific membrane binding mechanisms behind phagocytosis [25], [26]. In other studies, optical tweezers were employed to move internalized beads within cells to identify the anomalous diffusion scaling to the density of the network within which the bead was embedded [27]. In other applications, the internalization of functionalized beads has enabled intracellular studies, including calcium signaling detection [28], pH detection, force measurements, and cytoskeletal rearrangements [29].

In this report, we first briefly review the application of optical traps to cell biology highlighting some of the types of study that may be performed. We then explore a new technique for enhancing the intrinsic dielectric contrast of different mammalian cells by microsphere internalization, where cells naturally uptake polymer microspheres from their environment, and thus, respond more strongly to an applied optical field. Contrary to external handle attachments that require accurate consideration of the surface chemistry and often disengage the cells once exposed to the trapping beam, as a result of weak cell–sphere binding affinity, this intracellular tagging process provides stable, nontoxic handles. This eliminates the use of complex, costly reagents to facilitate cell tagging. We characterize the optical forces on these cells, and show that this simple, inexpensive and nontoxic internal tagging technique can be used to investigate enhanced optical manipulation and enable a simple new technique to sort cells.

II. Optical Manipulation and Sorting of Cells

In this section, we briefly review the application of optical forces particularly to cellular material giving a flavor of the types of studies performed. We note that other reviews cover the individual topics presented in more depth and refer the interested reader to [2]–[4] and [30].

A cell's response to an applied laser beam is largely dictated by the laser wavelength, the laser power, and indeed, the manner in which the light is distributed over the object. This latter point is exemplified by the fact that there are different power densities over a cell when we use, for example, a high numerical aperture (NA) objective lens (as for single-beam optical tweezers) versus a dual-beam optical trap (with two weakly focused light fields) [2]. A variety of experiments have been performed at the cellular size scale using optical forces. Following the advent of a single-beam optical trap, Ashkin's first studies showed the manipulation of both *Escherichia coli (E. coli)* and tobacco mosaic virus [31]. Both individual tobacco mosaic viruses and dense oriented arrays of viruses dispersed in an aqueous media were both trapped. Furthermore, using only a few milliwatts of laser power, trapping and manipulation of single live motile *E. coli* bacteria was observed. Subsequently, single-beam traps or counter-propagating beam traps have been used extensively for single-cell studies [2].

The dual-beam optical trap was first demonstrated by Ashkin [32]. A major advance was made when such a trap was made using optical fibers in 1993 [33]. Recently, this trap has been used in a wide range of studies due to a number of key features including the ease of holding and manipulation of large cells, and the compatibility with microfluidics thereby potentially enabling the development of an integrated device. The dual-beam trap was also central to the development of the optical stretcher [34]–[37] within which, perhaps counter intuitively, placing a deformable object of higher refractive index than its surroundings between two counter-propagating beams actually causes a bulging of the object outward. This is a consequence of the change in photon momentum that actually increases when transiting from low- to high-refractive-index media, resulting in an outward reaction force at the boundary between the trapped object and surrounding medium. Interestingly, neoplastic or cancerous cells have distinct changes in their actin cytoskeleton, which, in turn, means they are generally more deformable than healthy normal cells. By recording the cell's ellipticity with a stretcher, researchers were able to distinguish breast cancer cells from normal cells in a given population [34]–[37]. Separate studies have used the dual-beam trap in conjunction with Raman spectroscopy where Raman signals were acquired from different parts of the cell and then analyzed using multivariate statistical techniques [38]. Raman analysis of single cells in a variety of traps is now an important area of research [39]. Ashkin, in the first studies with tweezers for biological material, recognized that near-IR (NIR) laser trapping could reduce photo-induced damage in biological cells [40] when compared to traps made using visible light. To investigate laser damage Neuman *et al.*, later looked at the impact of trapping fields from 790 nm–1064 nm on *E. coli* bacteria [41]. They discovered that the

action spectra for photodamage exhibited minima at 830 nm and 970 nm and maxima at 870 nm and 930 nm. The study by Liang *et al.* found comparable results when the cloning efficiency of Chinese hamster ovary (CHO) cells after optical trapping was investigated [42]. A crucial point from such experiments was that though typically associated with pulsed laser sources with accompanying high peak powers, continuous-wave (CW) lasers could also induce two-photon effects that, in turn, could destroy cells [43]. Pulsed sources often introduce photodamage and cells irradiated with such beams for long time periods can receive irreparable damage. However, they have been used in tandem with CW lasers for trapping, where the pulsed source is used for precisely controlled laser ablation or microsurgery [44].

As an example of the practical application of optical trapping to cellular materials, we now turn to some of the research performed upon red blood cells (RBCs). Human RBCs (erythrocytes) can experience a shearing force ($\approx$100% elastic deformation) as they flow through narrow capillaries with an inner diameter smaller than 3 μm. In many cases, the structural integrity (elasticity) of the RBC under external stress can be used a measure of the "health" of such cell types. The diameter of a RBC is typically around 8 μm, which is within the trapping range of optical tweezers. Using optical forces and video microscopy, it is possible to selectively impose an external shearing force and simultaneously measure the overall elasticity of RBCs. There are a number of innovative ways to impose an optical shearing (stretching) force using the optical tweezers. A single-beam setup is used by a number of groups [45]–[47] to deform RBCs either by holding them against flow (which imposes a shearing force) [47] or with attached microspheres (to stretch opposing ends of the blood cells) [48]–[50]. In addition, static linearly polarized optical tweezers can initiate RBC folding [51] or rotation [52] upon trapping. The relaxation time (unfolding) of the RBC (760 ms) may be used to infer its elasticity while the comparison of the rotation speed of the healthy RBC with a malaria-parasite-infected RBC shows a marked difference. With a scanning optical tweezer, Liao *et al.* [53] showed that the RBC can be stably trapped and stretched with any adhered microspheres. More recently, a team from Australia [54] designed an evanescent wave near-field laser trapping technique that can stretch, rotate and fold RBCs at a much lower optical power. Alternatively, the use of two diverging beams, an optical cell stretcher, can also be applied to the stretching of RBC. Guck *et al.* [34], [55] showed that the forces from the beams (the output from optical fiber ends) can steadily measure the mechanical deformation on RBCs without the requirement of dielectric sphere tethering [56]. The optically induced surface stresses can be sufficient to measurably deform a cell while maintaining its trapping position. For a given force, the recorded deformation (shape) may be used to determine the shear modulus. As a stretched and deformed RBC relaxes back to its original state, the time taken is again indicative of the state of the cell. This type of single-cell diagnostic technique can be integrated into a microfluidic chip [37]. Hence, in many ways, the elastic and viscoelastic properties of RBCs that are directly interrogated with optical forces can be correlated with the cells' response due to structural and molecular alterations.

A further exciting area of current research is based on multiple optical traps that can be generated using diffractive and holographic optical elements. Time-sharing optical traps achieve rapid toggling between trap sites at a time scale shorter than the diffusion time for particles within a viscous medium. Acoustooptic elements, electrooptic devices or galvanometer mirrors may be used, with acoustooptic devices proving the most popular to date [57]. The use of holographic methods [58], [59] or those using phase contrast [60] may also create multiple trap sites: these are potentially more powerful due to the ability to generate simultaneously all traps, and create trap arrays in three dimensions as well as create more exotic beam shapes such as Laguerre–Gaussian or Bessel modes [57]. From a technology standpoint, spatial light modulators (SLMs) have proved very popular to generate holographic arrays of traps in two and three dimensions. The generalized phase contrast has also been used as a more direct imaging method using a counter-propagating technique [61]. Additionally, a variety of multitrap studies with cellular material, some incorporating microfluidics [62], are emerging in the literature. For example, Akselrod *et al*. [63] used a novel combination of both a SLM and an acousto optic deflector (AOD) to create living cell microarrays in a hydrogel where the AOD produced large arrays in 2D and the SLM was used to generate axial planes, thus combing the merits of both systems. In tandem this seems to be a powerful technology. With this arrangement, Akselrod *et al*. [63] created heterotypic microarrays of living cells using optical traps for exact positioning of hundreds of cells at the same time. Specifically, the team organized large numbers of Swiss 3T3 fibroblasts that were surrounded by a ring of *Pseudomonas aeruginosa* bacteria that were trapped and maneuvered simultaneously.

The studies showed cell viability was not compromised. Naturally, once the laser is removed, the cells are free to migrate at will so the researchers fixed the cell positions permanently using a hydrogel matrix. In other research studies, Mirsaidov *et al.* [64] used multiple laminar fluid flows in a microfluidic network to transport *E. coli* bacteria to an assembly area. Within this area, multiple time-shared optical traps were used to pattern the bacteria into a complex array. Encapsulation of the cells in a photopolymerizable hydrogel allowed the experimenters to mimic the presence of an extracellular matrix. To extend the patterned cell array without loss of viability, they then moved to an adjacent location while maintaining registration of their original (reference) array, and repeated this patterning method. In this way, a heterogeneous array of *E. coli* was formed. Notably, the arrayed bacteria were one of three different types that had been genetically engineered to express one of three different colored fluorescent proteins under the control of a sugar inducible promoter (a *lac* switch). Therefore, when the bacteria arrays were induced with the synthetic sugar (isopropyl β-D-thiogalactopyranoside) using a microfluidic network, the different colored fluorescence from the different bacteria, could be monitored. The different intensity of fluorescence was utilized as a basic measure of the metabolism/viability of each bacterium relative to the other bacteria within an array. It is important to note that though these experiments were performed with bacteria, they are significant, as it is possible that this approach

could be extended to eukaryotic cells. This advancement could have the potential to produce synthetic tissue with the ability to capture the three-dimensional (3-D) complexity of a multicellular organism.

Other work at the cellular size scale has explored the activation dynamics of membrane receptors using microparticles covered with ligands. Monnoret *et al.* [65] used arrays of holographic optical traps to move multiple beads to well-defined regions of individually targeted COS-7 cells within a microfluidic chamber. Using an alternative approach with an SLM, termed the "generalized phase contrast technique," Arneborg *et al.* [66] organized two species of yeast cells in order to explore their growth characteristics. The geometry was designed such that one species, the *Hanseniaspora uvarum* was surrounded by *Saccharomyces cerevisiae*. The *S. cerevisiae* cells indeed influenced the growth of *H. uvarum*. The average time of generation time of surrounded *H. uvarum* cells was shown to be 15% higher than that of nonsurrounded cells. Thus, the confinement imposed by viable *S. cerevisiae* cells on *H. uvarum* inhibits growth of the latter cell confirming that confinement is a determinant of growth in a microbial ecosystem. Combining optical traps with advanced imaging is a topic of major interest particularly for cell biology. In 2004, optical tweezers were incorporated into a multiphoton microscope to allow 3-D imaging of trapped cells [67]. Eriksson *et al.* [68] recently combined multipoint holographic optical tweezers with image analysis. The aim was to optimize the axial position of trapped cells in a given array to bring all of the nuclei into a single imaging plane. This eliminated the need for multiple stacks of images to ensure that each nucleus is imaged properly and notably this novel modality reduces photobleaching.

There are various methods of separating and sorting cells of interest and this is another area where optical forces may play a key role, though not necessarily in direct trapping but in deflecting or guiding selected cells to a reservoir. First, we can make a distinction between immunological and nonimmunological cell-separation methods. Immunological techniques are based on commercial cell-separation methods such as FACS and magnetic-activated cell sorting (MACS) [69]. Both of these macroscopic cell-sorting techniques possess high specificity and selectivity because they consist of extremely precise immunoreactions between the membrane marker proteins and labeling antibodies. Another advantage of FACS and MACS schemes is the achievement of high-throughput cell separation. This fact not only means that there is a requirement of large numbers of cells for efficient separation, but also means that immunologically isolated cells may often experience damage during follow-on processes such as the elution of cells from the capturing antibodies, and additionally, these cell-separation systems are also bulky and expensive.

Nonimmunological methods are relatively fast and simple techniques. In these methods, the type of cells are determined and separated according to their cell size, shape, and other physical properties, and the use of optical forces in a passive geometry falls into this category. Nonimmunological cell-sorting techniques demonstrate a low specificity for cell separation, as cells do not show remarkable differences between each cell type with the exception of their immunological properties. Examples of such technologies include dielectrophoresis (DEP) where dielectric particles, such as cells, have two different types of behavior, i.e., positive and negative DEP, depending on the direction of particle movement under the presence of a nonuniform electric field. The DEP cell separators have been developed using positive and negative DEP phenomena [70]. In a positive DEP-dependent separator, cells are deflected toward the electrodes resulting in adsorption onto the electrodes, which may cause reduced cell recovery. Therefore, they are not adequate for rare cell recovery after isolation because of the trapped cells on the electrodes. Contrarily, the strategy of negative DEP is to provide a repulsive force that acts on the cells, eliminating cell adsorption onto the electrodes. Although the negative DEP force is decreased as the cells are moving away from the electrodes, which gradually gives rise to diminished cell deflection velocity, the negative DEP is more adaptable to a continuous-flow cell separation because of its reliability in cell recovery. The use of optical forces in many ways mirrors the use of DEP when used in a passive regime, i.e., no immunological tags are involved.

Active optical sorting also exists where the combination of optical forces typically with microfluidics aims to replicate more bulky FACS machines or similar but in a more compact geometry and can be achieved by a variety of ways [13]. One such example is the study of Wang *et al.* [14] who developed a micro-FACS (μFACS) system that used a microfluidic cartridge and a laser at 488 nm to excite fluorescence, and a subsequent 1064 nm laser to extract the sorted cells in a microfluidic flow. They used optical forces for the rapid routing on the millisecond timescale on their chip. This therefore produced a fluorescence-activated microfluidic cell sorter. The researchers assessed the performance on live, stably transfected HeLa cells that expressed a fused histone-green fluorescent protein. Viability was measured by evaluation of the transcriptional expression of two genes, *HSPA6* and *FOS*, known indicators of cellular stress, and no detrimental effects on the cells from the optical sorting were observed.

Another example is shown in Fig. 1 [71], where the principle of active cell sorting via optical forces in such μFACS devices is illustrated. A second method utilized multiple optical traps, which can be utilized to form what are termed potential energy landscapes, in which the motion of microparticles and cells across such a landscape is dictated by the physical properties of the particles. This, in turn, can be used as a discriminative parameter and enable sorting. MacDonald *et al*. showed how particles could be separated by parameters such as size or their refractive index by flowing them over a multibeam interference pattern (an optical lattice) [72]. This system was then extended to study sorting of RBCs and white blood cells [73]. Various other sorting schemes using passive techniques are emerging and may hold promise for biological applications [74]. Using the potential landscape created by a Bessel light mode, Paterson *et al*. separated RBCs and white blood cells, in this instance, without flow [19]. In 2007 further studies, using this method, showed that how using dielectric tagging could enhance the sorting [17] and detailed data were acquired for human

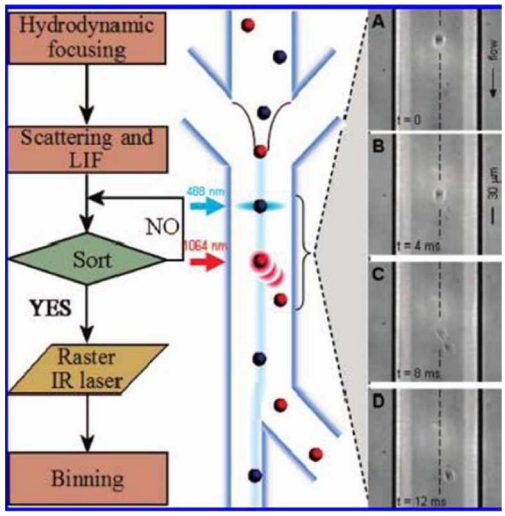

Fig. 1. Principle and illustration of μFACS based on optical forces. The hydrodynamically focused macrophage: (A) detected by forward scattering, (B) enters the IR laser spot, (C) deflected by optical gradient forces, (D) released in a different laminar flow stream (reprint by permission from *Analytical Chemistry* [71]).

promyelocytic leukemia (HL60) cells, murine bone marrow, and murine stem/progenitor cells.

In all of these studies, it is worth commenting on some aspects of cell manipulation: we typically have not yet seen many studies of cell mechanotransduction using quantified aspects of optical tweezers. In addition, as eluded in many studies, the poor refractive-index contrast between cells and their surrounding buffer media can lead to difficulties in basic manipulation and sorting. Therefore, we now describe a new experimental procedure that aims to show how incubation and phagocytosis of polymer microparticles by cells can increase this contrast and lead to enhanced lateral forces and longitudinal guiding velocities for cellular samples.

III. Incubating Cells With Microspheres: Enhanced Manipulation

In this technique, we rely on the internalization of polymer microspheres by cells through the natural cellular processes and aim to show that the subsequent increase in refractive index contrast, produced by the polymer microsphere, provides an enhancement of optical trapping and manipulation efficiency.

To quantify the cell-trapping process, we used the well-known Q-value method. This method works by dragging a known trapped particle through a sample medium and recording the terminal velocity at which the particle leaves the trap. This occurs when the viscous Stokes drag, given by $F = 6\pi\eta r v$, where η is the viscosity of the medium, r the particle diameter, and v the velocity of the particle with respect to the fluid, exceeds the applied trapping force. Previously, researchers have reported on both the axial (Q_{axial}) as well as the lateral (Q_{lateral}) trapping efficiencies of microparticles to quantify the degree to which these particles are effectively trapped [75]. The dimensionless parameter Q is related to the force on the particle F_{trap}, the power output of the laser P, and the refractive index of the surrounding medium n_m through the relationship: $F_{\mathrm{trap}} = (Qn_m P)/c$ is the speed of light. Data on Q-values for a variety of experimental optical trapping geometries consisting of differing laser beam shapes are available in the literature, though this work has been mostly performed upon microspheres [76], [77]. Due to the possibility that stable 3-D optical trapping of cells incubated with microspheres, might enhance the refractive index, we sought to measure the lateral trapping efficiency of a variety of cell types. We report the Q_{lateral} values of cells internalizing different numbers of polymer microspheres and compare how strongly they trap to those containing no microspheres.

A. Methodology

The procedures for cell preparation and the optical setup used for the data presented are outlined in the following section. In this study, we have used a range of cell types to reflect the cell types used in medical and biological research. The CHO cells are widely used as a cell line that grows rapidly as an attached cell population. The retinal pigment epithelial cells (RPE) are an example of a human epithelial cell line that has been immortalized by transduction with human telomerase and retain a near-diploid karyotype. The HL60 cells grow as a suspension and are commonly used for studies on drug sensitivity and for investigating factors that induce cell differentiation in leukemia. The FDCP-mix C2GM cell line is a murine cell line with stem-cell-like properties whose growth is dependent on the presence of growth factors.

1) Materials, Microsphere Preparation and Cell Culturing: For the incubation and sorting process, we made use of polymer microspheres with diameters of 2 and 3 μm.

These beads were purchased from Duke Scientific and had a green-fluorescing (GF) dye incorporated into the polymer matrix.

From a stock concentration of 6.7×10^8 spheres/mL, these were diluted 1:1000 in complete growth medium.

a) CHO cells: CHO cells were grown in minimum essential medium (MEM) with 2 mM L-glutamine, 1% penicillin–streptomycin, 0.5 mg/mL geneticin and supplemented with 10% fetal calf serum (FCS). These particular cells were used in previous work and had been phototransfected with a plasmid containing an antibiotic-resistant gene and a gene encoding a mitochondrially targeted red fluorescent protein (pDsRED-Mito gene), thereby providing an internal fluorescent red marker [78]. Since these are an adherent cell line, 0.25% (w/v) trypsin–ethylenediaminetetraacetic acid (EDTA) solution was used to harvest the cells for experiments.

b) RPE cells: These cells were grown in 10% FCS Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 μg/mL gentamicin, 0.348% sodium bicarbonate (w/v), 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, and 10 μg/mL hygromycin B in phosphate buffered saline (PBS). This adherent human epithelial cell line was trypsinized using a 0.25% (w/v) trypsin–EDTA solution for cell sample preparation prior to optical treatment.

c) HL60 cells: HL60 is a human promyelocytic leukemic cell line that grows in suspension. It was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented

with 10% FCS and penicillin (50 U/mL), streptomycin (50 μg/mL), and L-glutamine (2 mM).

d) Hematopoietic stem-cell like (FDCP-mix C2GM) cells: *FDCP-mix C2GM* (denoted as *C2GM*) is a growth-factor-dependent hematopoietic stem-cell-like cell line that grows in suspension. These cells are derived from long-term murine bone marrow cultures and have the ability to differentiate into different hematopoietic cells [79]. They were cultured in DMEM containing 2 mM L-glutamine, 1% penicillin–streptomycin, 10 ng/mL murine recombinant granulocyte macrophage colony stimulating factor (GM-CSF), and supplemented with 20% horse serum.

2) Cell–Microsphere Incubation: The cells were incubated with 2 or 3 μm GF polymer microspheres. Postincubation, fluorescence, and confocal microscopy were used to confirm microsphere internalization, and trypan blue exclusion tests indicated cell viability following microsphere uptake. Before their incubation with microspheres, the four different cell lines were grown at 37 °C with 5% CO_2 and 85% humidity (optimum growth condition) in 75 cm^2 flasks and subcultured twice weekly. Every experiment was performed in triplicate and repeated five times. For the adherent cell lines, i.e., CHO and RPE cells, approximately 10^5 cells in 2 mL complete medium were seeded from 75 cm^2 flasks into sterile 30-mm-diameter culture plates before microsphere treatment. These cells were grown to subconfluency overnight in optimum growth conditions. The 2 mL of culture supernatant left in the samples was aspirated and replaced with the same volume of media containing 2 or 3 μm diameter spheres. For HL60 and C2GM suspension cell lines, seeding was performed at a similar cell concentration as with the other two cell lines. However, these cell samples were grown in microsphere containing media immediately postseeding. To quantify cell–sphere uptake, the red fluorescent expressing CHO cells were incubated with the GF microspheres over five different time periods, namely, 3, 6, 24, 48 and 72 h. Following the microsphere incubations at the recorded times, non internalized spheres were removed by rinsing the cells twice using 2 mL complete medium, leaving approximately 200–250 μL of medium covering the sample surface to prevent the monolayer from desiccating.

Samples were then analyzed via fluorescence microscopy where a 10× microscope objective (MO) lens was utilized to take fluorescent images of cells containing spheres. These images were then imported onto a Labview particle tracking program that was written specifically to count sphere uptake per cell. In another experiment, the 24 h incubated samples were evaluated using confocal microscopy to confirm microsphere uptake by cells. Cell viability for all indicated time periods was also determined. This was measured by replacing the 200–250 μL of medium with 160 μL of 0.4% trypan blue per dish. The samples were thereafter allowed to incubate at room temperature for about five minutes before imaging via Köhler illumination created using a white light source.

B. Optical Trapping and Guiding Apparatus

Using a high numerical aperture MO, an inverted microscope system (Nikon TE2000U), and a motorized translation stage (Prior Scientific), we measured the lateral Q-values and the guiding velocities of cells both with and without internalized microspheres. The trapping beam and guiding beams were both generated using a 1070-nm, 5-W fiber laser (YLM-5, IPG Photonics). For the lateral trapping studies, the Gaussian beam was magnified to fill the back aperture of the oil immersion MO of NA 1.25, magnification 100× to form a diffraction-limited spot that acted as a 3-D optical trap. The transverse beam waist ($w_{x,y}$) at the full-width at half maximum [80], [81] is measured to be ≈380 nm. A beam steering system was formed using a conjugate lens pair where the image of the steering mirror was imaged onto the back focal plane of the MO. The optical trap was maintained at a power of 22 mW. The 3-D optical trap formed provided an optimized gradient force in both the axial and transverse plane. A low NA (0.25) objective was used for the cell-guiding experiment, where the power was kept at 250 mW. Here, both the axial distance and time taken for cell–sphere guiding were determined to calculate the optical guiding velocities. For sample imaging, a charge-coupled-device (CCD) camera was aligned to one of the side ports of the microscope and connected to a monitor or a computer for capturing videos (see Fig. 2). With the low NA objective, we were able to obtain a beam with a longer Rayleigh range, thus propelling the particle further along in axial direction (optical guiding) as opposed to a high NA objective with a shorter Rayleigh range.

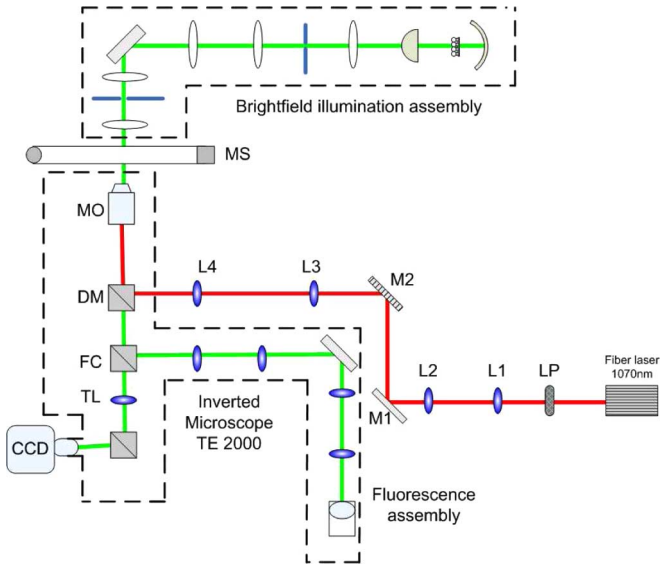

Fig. 2. Optical tweezing and guiding setup for our studies. A fiber laser (5 W, ytterbium-doped fiber laser, IPG) provides a Gaussian beam (M2 < 1.1, beam diameter 1.6 mm) that is expanded by lens L1 and L2 to 9.6 mm. A linear polarizer (LP) is used to rotate the polarization of the beam. The expanded Gaussian beam is relayed through a beam steering lens system (L3 and L4), via reflecting off M1 and M2 (sliver mirrors). The beam is then reflected by NIR dichroic mirror (DM) (z900dcsp, Chroma) onto the back aperture of the MO. The sample is mounted onto a motorized stage (MS). For brightfield and fluorescent illumination, we make use of the brightfield and fluorescence assembly supported by a commercial inverted microscopy (TE2000E, Nikon). FC is the fluorescence cube and TL is the tube lens. A color digital camera (CCD) (HAD, Pulnix) is used to record the microscopic images.

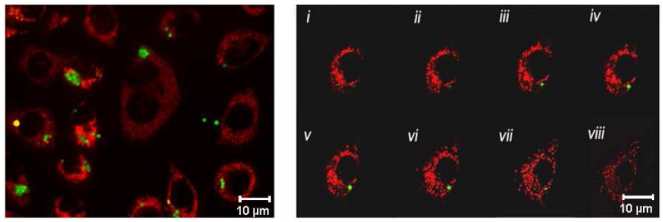

Fig. 3. (A) Fluorescent micrograph of red fluorescing CHO cells containing 2 μm GF ploymer microspheres. (B) Data obtained via confocal microscopy [(i)–(viii) z-scan of 1 μm] confirmed internalization of 2 μm GF microspheres.

C. Sorting of CHO Cells With Internalized Microspheres

A mixed population of CHO cells with a range of numbers of internalized 3 μm beads was trypsinized from a T25 culture flask and suspended in complete growth medium. These were strained through a 40 μm pore size filter to obtain a monodispersed cell sample. The concentration used for the sorting experiment was calculated to be 6.2×10^5 cells/mL. For the CHO cell-sorting experiments, we used 100 μL of this cell sample per sorting experiment. Sample chambers used in these experiments consisted of 30 mm-diameter type-zero glass bottom petri dishes (bottom) and 23 mm-diameter round glass type-one coverslips (top). To prevent cell adhesion to the 24 mm glass surface of the dishes, the dishes were coated with sigmacote (Sigma-Aldrich), a reagent that reacts with surface silanol groups on glass to produce a neutral, hydrophobic microscopic thin film. In contrast, the top coverslips were coated with 2 μg/cm^2 laminin solution made up in sterile water to promote cell adhesion upon close physical contact [82].

IV. Results

A. Quantification of Microsphere Uptake After Culturing

Initial experiments focused on the internalization parameters of the microspheres into CHO cells. These cells were also used for viability, fluorescent, and confocal microscopy measurements. Fluorescent microscopy (mercury lamp and confocal laser scanning) was used to measure the fluorescence from the internalized fluorescent microspheres within the CHO cells. In Fig. 3, we show the cultured CHO cell samples that were analyzed indicating the successful internalization of the microspheres. Specifically, Fig. 3(B) displays confocal microscopy image stacks (i–viii), where each image slice has a depth size of 1 μm along the axial plane through the cell to confirm sphere encapsulation 24 h postincubation within actively growing CHO cells. A 10$\times$ MO was used to capture images of the fluorescing cell sample with a larger field of view over different time periods. The fluorescence images were then imported onto a Labview © particle-tracking program that was written specifically to count sphere uptake per cell.

In Fig. 4, we show the quantification of microsphere ingestion by mammalian cells, which shows that the maximum internalization was achieved at 24 h postincubation.

After quantifying microsphere ingestion by mammalian cells and noting that the maximum engulfment occurred at 24 h postincubation, further experiments were performed over this timescale.

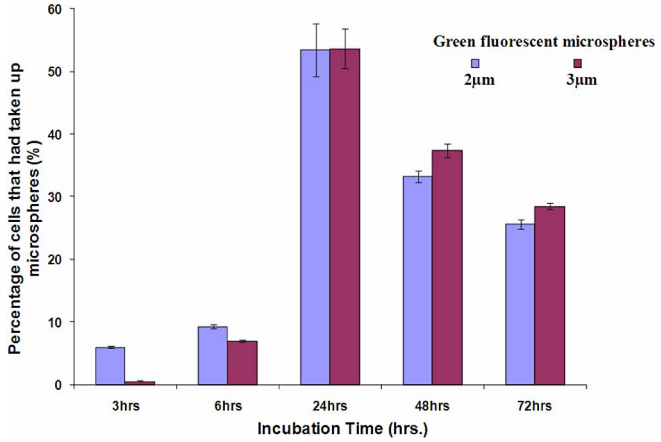

Fig. 4. Cellular uptake of 2 and 3 μm diameter GF microspheres by CHO cells. The maximum number of cells that internalized microspheres occurred at 24 h postincubation for both sphere sizes.

Trypan blue exclusion experiments were performed for all reported time periods, and confirmed no compromise to cell viability, prior to microsphere ingestion as well as postoptical treatment.

B. Measuring Q-Values and Guiding Velocities

Having established these initial internalization parameters, we next measured the lateral Q_{lateral}-values and the guiding velocities of different cell types both with and without internalized microspheres. As described earlier, we used the Q_{lateral}-value measurement technique to quantify the lateral trapping forces. Briefly, cells were held in the trap and traversing the sample stage at constant velocity until the cell fell out of the trap (upon attaining the critical drag force velocity), the amount of momentum transferred onto the cells with and without the engulfed microspheres from the trapping beam, was established. Fig. 5 shows the Q_{lateral}-values obtained for the different cell types but also dependent on the number of spheres that had been internalized. During the tweezing of the cells with internalized spheres, we noted that the cell would orientate such that the internalized microspheres are aligned to the tweezing beam. This then resulted in the trapping of the engulfed microspheres and not necessarily the whole cell.

For most cells, one to two internalized microspheres provided the highest Q-values for trapping in the lateral plane. The consistency of the Q-values was dependent on the quality of the trap and the trapped particle. Hence, the internalized microspheres provided an increase of the lateral gradient force. Notably, the HL60 cells did not appear to respond as well as the other cell lines. As these cells had successfully internalized the microspheres, this apparent difference could be due to their smaller size ($\sim$10 μm) and containing a larger nucleus to cytoplasm ratio, in comparison to the other cell types [83].

By using a weakly focused Gaussian beam, the axial gradient force is much reduced so that the scattering force dominates allowing cell guiding. Therefore, in the following experiment,

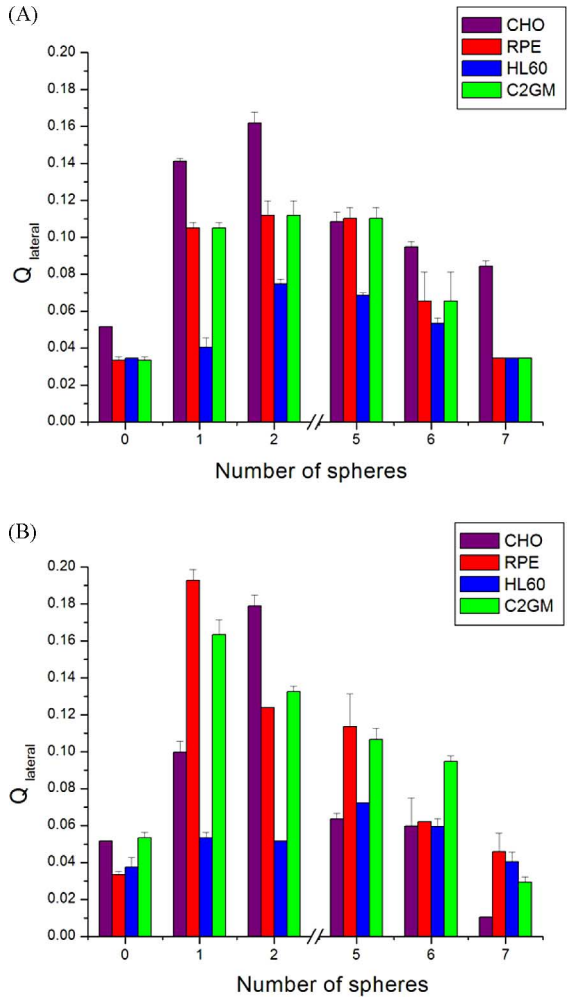

Fig. 5. Lateral Q-values of different cell types containing different amounts of (A) 2 μm and (B) 3 μm polymer microspheres. In general, cells containing one or two spheres per cell, trapped with the highest efficiency. The average values with standard deviation are shown.

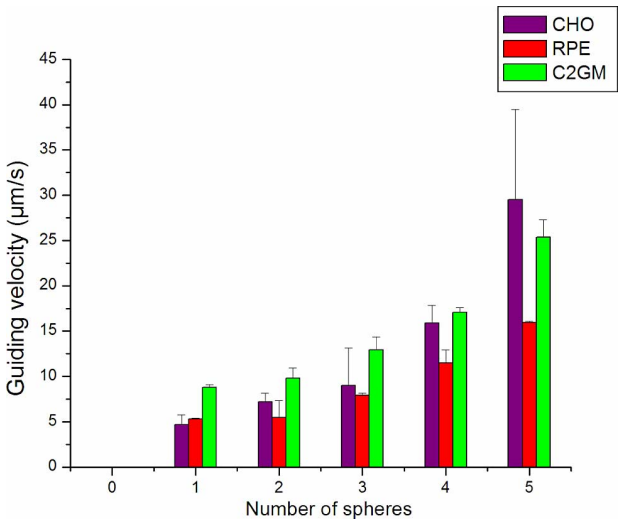

Fig. 6. Axial cell guiding data, displaying maximum guiding velocities for cells containing different numbers of microspheres per cell. Cells with no spheres displayed no axial guiding. Experiments were performed in triplicates and repeated five times. The average values with standard deviation are shown.

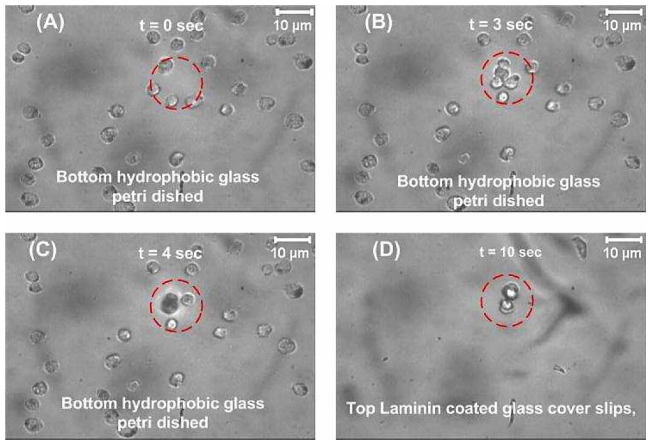

Fig. 7. Sample chamber containing 100 μL of a mixed population (with and without ingested beads) of CHO cells was suspended between a hydrophobic glass bottom petri dish and laminin-coated top coverslip, while a diffracting Gaussian beam (red dotted circle) emerging from a 10× objective with NA 0.28 was illuminated toward the laminin-coated coverslip. (A) and (B) CHO cells begin to migrate toward the centre of the beam. (C) CHO cells with ingested microspheres start to be axial propelled onto the top laminin-coated glass coverslip. (D) CHO cells with ingested spheres adhere to the top glass coverslip, and subsequently, can be cultured separately.

we positioned cells with and without ingested microspheres in the beam path of a Gaussian beam (using an objective of magnification 10×/NA 0.25). By observing the guiding velocity of the cells with and without ingested microspheres, we were able to infer that cells with polymer spheres guided an order of magnitude faster than cells without spheres. With larger numbers of ingested microspheres, we observed a direct increase in the guiding velocity of the cells. This increase of the guiding velocity of the cells was attributed to an increase in the axial scattering due to the presence of the ingested spheres. This effect, therefore, allowed us to confirm that the increase in the effective axial scattering exerted onto the cells was due to the ingested microspheres (see Fig. 6).

C. Selectively Separating CHO Cells With Internalized Microspheres

As mentioned earlier, we have shown that on exposing CHO cells to a diverging optical field obtained through use of a low numerical aperture objective, cells with ingested microspheres have enhanced axial guiding (see Fig. 6). Therefore, we sought to capitalize upon this feature to develop a novel optical cell-sorting methodology for the separation of cells from a mixed cell population. To do this, we implemented the sorting of the cells with microspheres on a self-built inverted optical trapping setup, using analyte volumes of 100 μL. A mixed population (with and without internalized microspheres) of CHO cells was suspended between a hydrophobic glass bottom petri dish and a laminin-coated top coverslip, while a diffracting Gaussian beam was directed onto a MO (Comar magnification 10×/NA 0.28) upward toward the laminin-coated coverslip. Only cells that had internalized microspheres were propelled axially to the top of the sample chamber and attached to the laminin-coated glass coverslips (see Fig. 7).

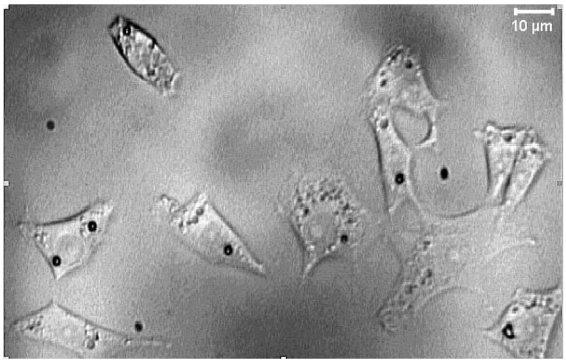

Fig. 8. Brightfield image of a sample chamber containing recultured CHO cells with ingested microspheres (dark spots) viewed with a 20× objective lens of NA 0.54.

An average of 50 cells with ingested spheres per experiment were optically sorted and separated from the rest of the sample. This was achieved due to an increased axial scattering force exerted onto those cells with internalized microspheres compared to those with no ingested spheres. The sorting occurred at a maximum rate of 11 cells/min. The separated cells that adhered to the laminin-coated coverslip were then allowed to grow further by placing the coverslips into 500 μL of filter-sterilized conditioning medium in 30 mm diameter plastic petri dishes at 37 °C with 5% CO_2, 85% humidity, and the medium changed every 48 h.

After four days of culturing, the optically sorted cells were trypsinized; their viability tested using the trypan blue exclusion dye method, and counted. A total number of 1.2×10^3 cells/mL were obtained with 100% viability. In Fig. 8, a brightfield image of the recultured CHO cells viewed with a 20×/NA 0.54 MO is shown.

V. Conclusion

Effective optical trapping, guiding, and sorting of cells remain a key topic for biophotonics. In this paper, we have briefly reviewed the application of optical forces for the manipulation and sorting of cells, and additionally presented a new technique for enhancing the dielectric contrast of the mammalian cells for enhanced manipulation. In this new technique, cells were allowed to naturally uptake nonfunctionalized polymer microspheres from their surroundings. By studying how these intracellular microspheres influenced the cell's response to an applied optical field, we were able to exploit both the lateral gradient as well as the axial scattering forces to successfully sort cells via a simple, inexpensive, and nontoxic optical process. We have shown that a range of different cell types (both adherent and suspension cell lines) are capable of taking up these microspheres without harm, but notably there are some cell types that do not respond to guidance despite taking up microspheres. Therefore, we have shown that it is possible to enhance the dielectric contrast of cells to promote effective optical micromanipulation of these cells by the uptake of nontoxic microspheres. In the future, it might be possible to apply this new method to studies within, for example, the immune system, to isolate different phagocytic and nonphagocytic cells. We can also develop this technique for the microfluidic environment with computer-based analysis system [84], [85] for automated high-throughput cell sorting.

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