“CAROL DAVILA” UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST

DOCTORAL SCHOOL

MEDICINE

ABSTRACT OF DOCTORAL THESIS

USE OF OPTICAL TWEEZERS AND DIELECTROPHORESIS FOR RETINAL PIGMENT EPITHELIAL CELLS’ CHARACTERIZATION AND SEPARATION TOWARD CELL REPLACEMENT AND TRANSPLANTATION THERAPY

PhD Supervisor

Prof. Dr. Tudor Savopol

PhD Student

Dharm Singh Yadav

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Contents of thesis

Publication List

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   (*This article provides a critical review of the biomedical applications of optical tweezers, which served as the central focus of this thesis*)

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   (*This article is covered in chapter 3, page 43-75*)

General Overview

This thesis is dedicated to advancing the biophysical techniques, specifically optical tweezers (OT) and dielectrophoresis (DEP), for the characterization, manipulation, and separation of retinal pigment epithelial (RPE) cells, with a focus on contributing to improve therapeutic strategies for retinal disorders such as age-related macular degeneration (AMD). The research is divided into two main studies, each addressing critical challenges in the field of regenerative medicine and cell-based therapies on cell population and single-cell level. The dielectrophoretic characterization provides valuable insights into the electrical properties of RPE cells under oxidative stress, while the single-cell analysis using OT and DEP offers a deeper understanding of individual cell optical and electrical properties. The results highlight the usefulness of DEP and OT in the field of regenerative medicine, particularly for developing precise cell-based therapies for retinal diseases. This work highlights the potential of biophysical methods in advancing the characterization, separation, and analysis of therapeutic cells, ultimately contributing to improved outcomes in cell transplantation and optogenetic therapies aimed at restoring vision in patients suffering from retinal degenerative disorders.

The thesis is structured to first provide a general introduction and background in **Chapter 1** and **Chapter 2**, setting the stage for the specific studies presented in **Chapter 3** and **Chapter 4**.

Chapter 1 entitled “Introduction to Human Vision and Retinal Diseases” provides an overview of human vision, the structure of the retina, and common retinal diseases such as AMD, diabetic retinopathy, and retinitis pigmentosa. It discusses the role of RPE cells in maintaining retinal health and introduces emerging therapies like cell transplantation and optogenetics as potential treatments for vision disorders.

Chapter 2 entitled “Biophysical Methods for Label-Free Cell Characterization and Separation” introduces OT and DEP as key biophysical tools for manipulating and characterizing cells. It explains the principles of OT, including optical trapping and force calibration, and DEP, focusing on its use for cell separation based on dielectric properties. The chapter also highlights the importance of real-time cell analysis (RTCA) for monitoring cell behavior.

Personal contribution

Chapter 3: Dielectrophoretic characterization of peroxidized retinal pigment epithelial cells as a model of age-related macular degeneration

4.1. Age-Related Macular Degeneration (AMD)

AMD is one of the most common causes of vision loss in adults over the age of 50, particularly in developed countries [1]. As the global population ages, AMD is becoming an increasingly significant public health issue, with millions of individuals worldwide suffering from its debilitating effects. The disease primarily targets the macula, the central portion of the retina that enables us to perform tasks requiring sharp, central vision, such as reading, recognizing faces, and driving. The macula is a specialized area within the retina that contains a high concentration of photoreceptor cells, which convert light into visual signals. In AMD, the macula deteriorates, leading to a gradual loss of central vision. This vision loss can be devastating, as it significantly impacts daily activities and quality of life, although peripheral vision typically remains intact.

The pathophysiology of AMD is complex, involving genetic, environmental, and lifestyle factors. Oxidative stress plays a central role in AMD progression, particularly due to the retina's high metabolic activity, constant light exposure, and abundance of polyunsaturated fatty acids, which are prone to oxidation. Over time, oxidative stress damages RPE and photoreceptor cells, leading to drusen accumulation and macular degeneration. Environmental factors like smoking, poor diet, and sunlight exposure further exacerbate oxidative stress and inflammation, increasing AMD risk [2],[3], [4].

Currently, there is no cure for AMD, but treatments aim to manage the disease and slow its progression. Emerging regenerative therapies, particularly RPE cell transplantation, offer hope for restoring vision in AMD patients. Induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) can be differentiated into RPE-like cells and transplanted into the subretinal space, where they may integrate with existing retinal tissue and restore function. While challenges such as ensuring long-term cell survival and preventing immune rejection remain, advances in

regenerative medicine hold promise for developing effective treatments to halt or reverse AMD progression [5], [6].

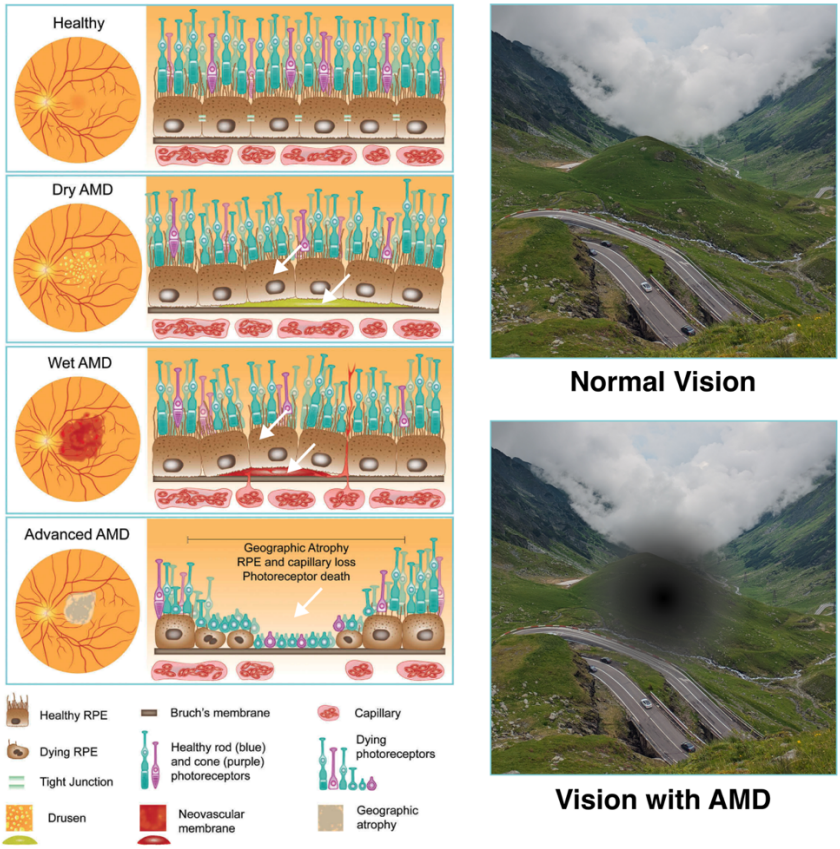

Fig. 3.1 Illustration of the stages of AMD. The progression of AMD begins with the formation of drusen, which are deposits that accumulate beneath the layer of RPE cells. This stage is known as dry AMD. In the advanced form, known as wet AMD, abnormal blood vessels from the choroid penetrate the RPE layer and retina, causing disruptions. These blood vessels can leak fluid or blood, leading to gradual vision loss. Figure adapted from [7] under permission to reuse for thesis.

4.2. RPE Cell-Based AMD Therapies

The RPE cells form a monolayer between the photoreceptor cells and the highly vascularized choroid layer. Each RPE cell has unique apical and basal surfaces. The basal side interacts with the choroidal blood supply, whereas the apical side faces the photoreceptor's outer segments [8]. Understanding RPE cells' importance in eye health and other vision-related diseases necessitates an appreciation for their role in human vision.

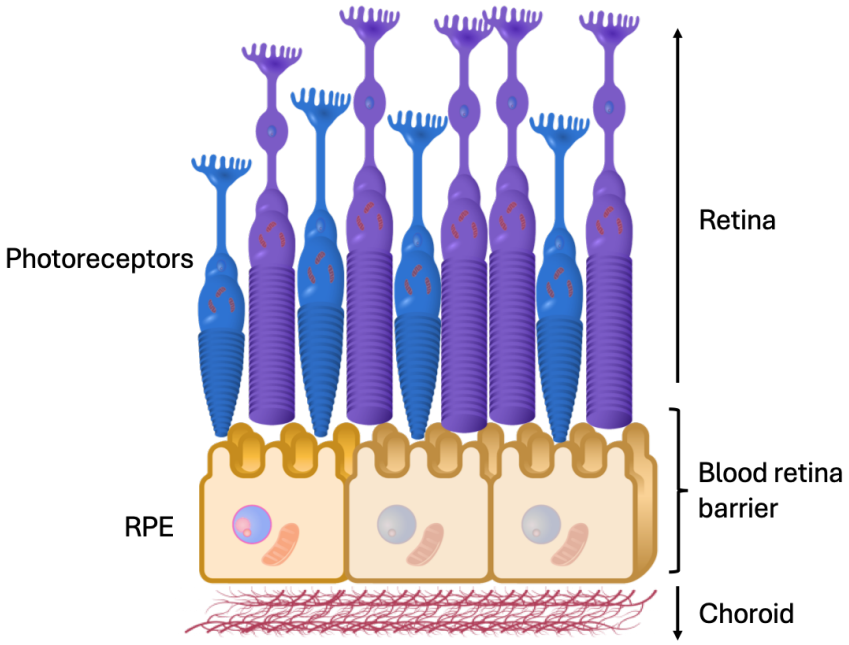

Fig. 1.1 Illustration showing RPE cells forming a blood retina barrier necessary for the transport of nutrients from blood to retina and removal of waste generated by photoreceptor cells. RPE is essential to maintain a healthy retinal environment. Figure created using Reactome icon library.

RPE cell transplantation has emerged as a promising therapeutic option for retinal disorders, including AMD. RPE cells, located between the retina and choroid, are essential for maintaining photoreceptor function and overall retinal health. Their loss or dysfunction is a hallmark of AMD and other retinal degenerative diseases. Unlike other retinal cells, RPE cells can be easily cultured in vitro and do not require synaptic connections, making them ideal candidates for transplantation. Recent advancements in RPE cell transplantation have demonstrated the potential to restore photoreceptor function in AMD patients [5],[9], [10], [11].

Recent advances in cell separation techniques, such as fluorescence-activated cell sorting and dielectrophoresis (DEP), have improved the efficiency and precision of isolating RPE and photoreceptor cells. DEP, in particular, offers a label-free, non-invasive method for purifying RPE cells based on their dielectric properties, minimizing contamination and improving transplantation outcomes.

Despite these advancements, challenges remain in RPE cell transplantation, including ensuring cell survival, integration, and preventing immune rejection [12], [13],[14]. Current purification methods, such as immunomagnetic sorting and density gradient centrifugation, face limitations like cell viability loss and heterogeneity [15], [16], [17]. DEP-based approaches provide a potential

solution by enabling high-throughput, precise isolation of healthy RPE cells [18],[19], addressing these challenges and enhancing the therapeutic potential of RPE cell transplantation. In summary, RPE cell transplantation holds significant promise for treating AMD, with ongoing research focused on overcoming challenges related to cell survival, integration, and purification. Advances in techniques like DEP are critical for improving the quality and efficacy of RPE-based therapies, offering hope for restoring vision in AMD patients.

4.3. Results

The study focused on characterization and separation of healthy and oxidatively stressed RPE cells using DEP as a model for AMD. Key findings include:

4.3.1. DEP Analysis of External Medium Conductivity

DEP spectra of BPEI-1, a rat RPE cell line, were recorded under various conductivity of the external medium to establish appropriate experiment conditions. A low-conductivity sucrose-based buffer (0.01 S/m) provided the strongest positive DEP (p-DEP) force and the most accurate measurements, minimizing Joule heating and unwanted electrophoretic effects. Higher conductivities (0.02 S/m and 0.04 S/m) led to increased membrane permittivity and cytoplasmic conductivity, complicating data interpretation, especially in oxidative stress experiments.

4.3.2. Cell Morphology and Viability

Microscopy and MTS assays revealed that H_2O_2 induced oxidative stress caused dose-dependent morphological changes and reduced cell viability in BPEI-1 cells. At 100 μM H_2O_2, cells exhibited rounding and detachment, indicative of apoptosis. However, the antioxidant N-Acetylcysteine (NAC) effectively impede these effects, preserving cell morphology and viability. NAC at 1.0 mM provided optimal protection, restoring cell viability to near-control levels.

4.3.3. Real-Time Cell Proliferation Monitoring

Real-Time Cell Analysis (RTCA) showed that H_2O_2 exposure significantly reduced cell proliferation, with 100 μM H_2O_2 causing rapid cell death. NAC treatment, particularly at 1.0 mM,

allowed cells to recover proliferation rates, demonstrating its protective role against oxidative damage. Higher NAC concentrations (2.5 mM) showed delayed recovery, suggesting potential toxicity at excessive doses.

4.3.4. Dielectrophoretic Characterization

DEP spectra of H_2O_2-treated RPE cells showed increased membrane permittivity, indicating oxidative damage to the lipid bilayer. NAC treatment reduced this effect, restoring membrane properties closer to those of healthy cells. The first crossover (CO) frequency shifted to lower values in H_2O_2-treated cells, reflecting changes in membrane and cytoplasmic conductivity. These dielectric differences enabled the separation of healthy and oxidatively stressed cells using DEP.

4.3.5. Simulations for Cell Separation

COMSOL simulations of a microfluidic DEP separation chip demonstrated the potential to separate healthy and H_2O_2-treated RPE cells with 100% efficiency. The simulations used experimentally derived dielectric properties and accounted for cell size variability, confirming the feasibility of DEP-based cell sorting for transplantation therapies.

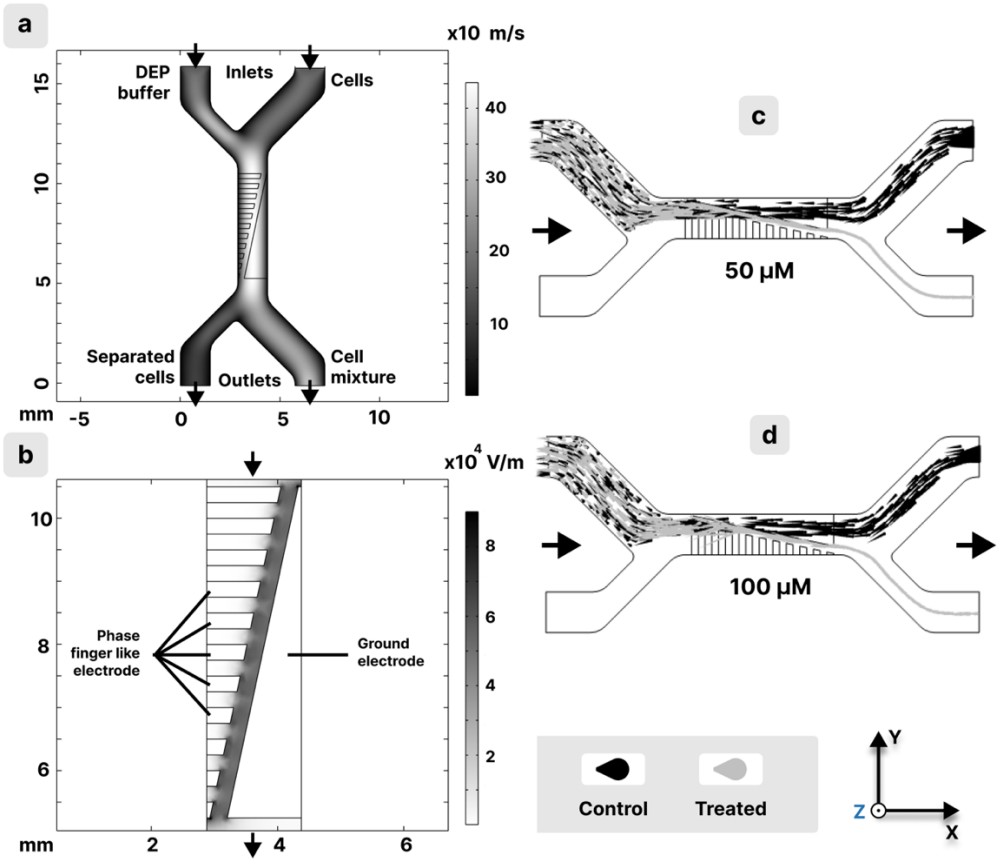

Fig. 3.2 Simulations in the DEP separation chip model of: (a) flow rate, (b) electric field intensity, (c), (d) separation of the Control (healthy) cells from mixtures of healthy and cells treated with 50 and 100 µM H_2O_2, respectively. The DEP field frequency was 28.941 kHz, which is the first CO frequency of the Control cells.

4.4. Conclusion

In this study, we developed an in-vitro AMD model and demonstrated how DEP can assess and separate healthy and oxidized BPEI-1 RPE cells. Increasing H_2O_2 concentrations induced membrane peroxidation, leading to a progressive rise in cell membrane permittivity. The reduction in these effects with NAC confirmed that oxidative damage caused the increased permittivity, consistent with previous studies on lipid peroxidation in cells [20], [21]. ROS generated during oxidative stress modified proteins and lipids, initiating chain reactions that altered membrane structure, fluidity, and ion permeability, ultimately disrupting cellular function and leading to cell death [22],[23], [24].

DEP proved valuable for evaluating the electrical properties of peroxidized cell membranes and cytosol. While the single-shell model effectively interpreted the data, it is a simplification, and more advanced models are needed to fully capture oxidative damage, including DNA fragmentation and metabolic dysfunction. The shift in the first crossover (CO) frequency to lower values in H_2O_2-treated cells suggested an "all or nothing" response, demanding further biochemical assays to quantify peroxidation levels.

In conclusion, DEP-based methods successfully characterized and separated healthy and oxidized RPE cells, validating their utility in AMD research. Simulations confirmed DEP's ability to isolate healthy cells from mixtures, offering a promising approach for cell sorting in transplantation therapies. This work advances regenerative medicine by enabling the purification of healthy RPE cells, a critical step in personalized cell replacement therapies [20],[25].

Chapter 4: Optical trapping force measurement and single cell characterization using combined dielectrophoresis

4.1. Introduction

In recent years, single-cell manipulation techniques have become essential tools in biological research [26]–[28]. Among these methods, OT stand out as a prominent technique, utilizing highly focused laser beams to trap microscopic objects without the need for labels or physical contact. This approach has gained widespread use for micromanipulation and precise force measurements on cells and organelles [29]. The principle behind OT was first uncovered by Arthur Ashkin in the 1970s, who identified a gradient force capable of trapping microparticles [30], which led him the development of optical tweezers in 1986. Since its discovery, OT is applied in various fields, particularly in manipulating single cells and microparticles in three-dimensional space [31]. In biological contexts, OT are employed to trap viruses, bacteria, and cells, as well as to facilitate processes like cell fusion, sorting, and even performing intracellular surgery [32]. This technique enables highly precise manipulation of individual particles and provides accurate force measurements, enabling in-depth observations of DNA characteristics, enzyme activities, molecular motor operations, and protein-DNA interactions [33],[34]. By calculating optical trapping forces with precision, researchers can explore cellular responses to mechanical stimuli, manipulate subcellular structures, and measure forces generated by molecular motors, such as

those involved in actin-myosin binding [35]–[37]. As a result, OT plays a crucial role in revealing fundamental biological processes like cell division, motility, and intracellular transport [38],[39].

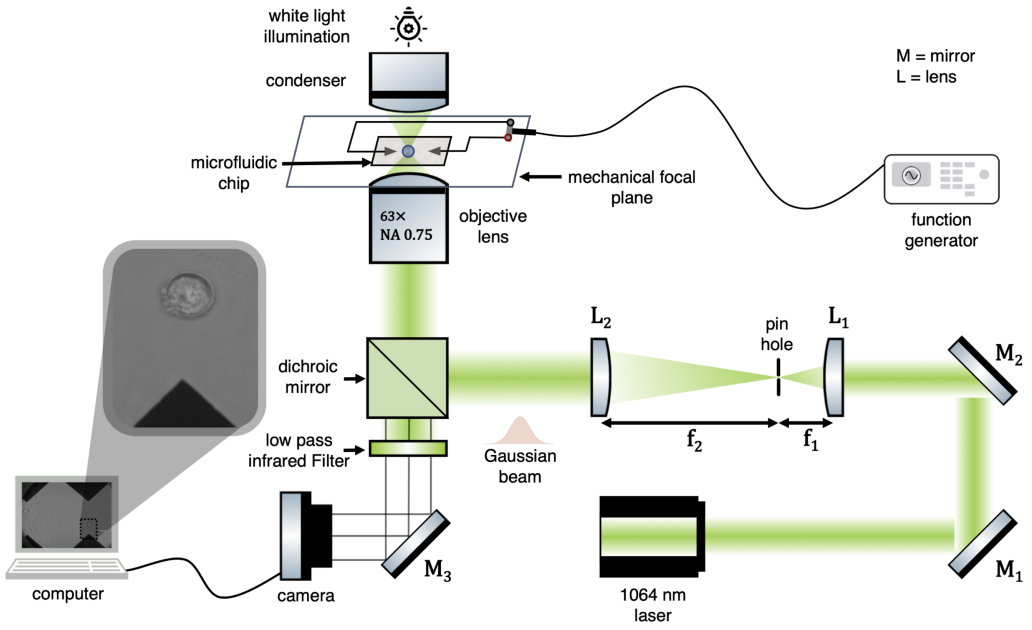

Fig. 4.1 A dual trapping setup designed on an inverted microscope by combining the single beam OT with DEP on the microscopy focal plane.

There are various methods available to measure forces using OT on small sized particles and simple cells such as yeast or red blood cells [40]–[46]. The positions of the trapped particle in these methods are usually measured using QPD. Theoretical force estimates are also available for a spherical geometry of a trapped homogenous particle [47]. Both the QPD and theoretical force estimation methods are reliable when dealing with spherical, homogenous particles. However, eukaryotic cells, being large, non-spherical, and inhomogeneous, pose challenges for precise OT force calibration using conventional techniques. To address this, we explored a calibration method based on combined OT with DEP, enabling simultaneous direct measurement of optical trapping forces and evaluation of optical and electrical properties of living cells. This approach integrates DEP with OT by balancing the forces and allow to evaluate OT force and cell properties in a label free manner.

DEP is an electro kinetic effect first discovered by Pohl in the 1950s, where dielectric particles move and align in response to an inhomogeneous electric field gradient [48]. Since its discovery,

numerous studies have further explored DEP theory and its biological applications [49]–[51]. Originally identified as a fundamental electro kinetic process, DEP has been shown to hold significant potential in biological applications, such as label-free, non-contact methods for cell sorting, purification, and trapping [52]. DEP leverages the interaction between electric fields and the intrinsic polarizability of particles [53]. In this work, we advanced the method to evaluate the maximum OT trapping force on living cells by applying a ramping DEP force (achieved by adjusting the voltage). As the DEP force increases, the trapped cell is gradually displaced while maintaining the balance between optical and electrical forces. The maximum trapping force is identified when the applied DEP force surpasses the OT force, referred to as the "escaping voltage." For cells subjected to equal DEP forces, the maximum optical trapping force depends solely on the cells' optical properties.

4.2. Experimental Method

The maximum optical trapping force on cells (BPEI-1, NIH3T3 and Caco-2) was accessed by the “escaping voltage” at which the cell makes an escape from OT. The DEP forces were applied in a ramping until the equilibrium between the two forces breaks. Since the OT and DEP forces are in equilibrium till the escaping point where the OT force reaches its maximum, the system allows to estimate the OT force by calculating the DEP force.

$$\vec{F}_{\mathrm{OT}} = \vec{F}_{\mathrm{DEP}} = 2\pi\varepsilon_0\varepsilon_m r^3 Re[K(f)]\vec{\nabla}|E^2|$$

To establish the frequency of the electric field to be applied during the escape experiments and to measure $Re[K(f)]$ for BPEI-1, NIH3T3 and Caco-2 cells, population DEP spectra were recorded and processed using OpenDEP [54].

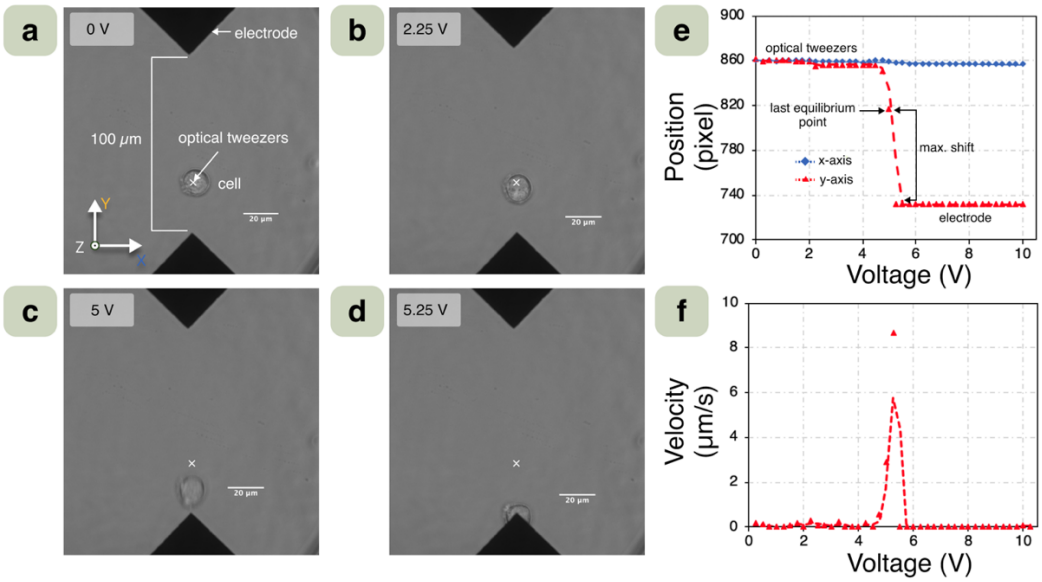

Fig. 4.2 Experimental steps to determine the escaping voltages: (a) A cell (radius 6.24 µm) is trapped by OT (indicated by a white '×') and positioned between two triangular electrodes, closer to one of them. (b) The cell's position is adjusted in response to a very low DEP voltage. (c) The cell escapes from the optical trap, as observed in the frame corresponding to 5 Vpp. (d) The cell becomes trapped on the electrode at voltages exceeding the equilibrium of DEP and OT forces. (e) and (f) depict the tracking of the cell's position and velocity.

The experimental values of $Re[K(f)]$ were calculated based on the average measured cell size in population DEP; however, in single-cell experiments, the cells exhibit a range of sizes, which is known to influence the value of $Re[K(f)]$. We simulated the DEP spectra for cell sizes (ranging from the maximum to the minimum) measured in our experiments, utilizing the electrical parameters of the cells derived from the population DEP data. Based on this data, the DEP frequency (2 MHz) was determined where $Re[K(f)]$ does not have an influence of cell sizes for all 3 cell types. Although, we have also performed the single cell experiments at 100 KHz frequency at which the system senses the electrical differences of the cells. The 100 kHz frequency allows to observe the variation of $Re[K(f)]$ which arises due to the electrical and size differences between cells whereas, at the 2 MHz frequency, the cells exhibit equal $Re[K(f)]$ which is also cell size independent. The 2 MHz choice enabled the generation of the maximum possible p-DEP force while mitigating any potential size-related effects on subsequent calculations which allowed to evaluate the optical differences (Refractive indices) between the cells.

4.3. Results

The study focused on developing a method to measure OT forces on living cells using DEP as a complementary technique. Key findings include:

4.3.1. Optimization and Preliminary Tests

Propidium iodide (PI) fluorescence assays and Real-Time Cell Analysis (RTCA) were used to assess cell viability under DEP field exposure. Results showed that DEP voltages above 15 V significantly damaged cells, while lower voltages (below 10 V) maintained high cell viability. This guided the optimization of OT laser power to 6.4 mW, ensuring minimal cell damage during experiments.

4.3.2. Electric Field Simulations

COMSOL simulations of the electric field gradient in the microfluidic chip revealed the highest gradient near the apexes of triangular electrodes. The simulations provided numerical values for the electric field intensity and gradient, which were used to calculate the DEP force acting on cells during experiments.

4.3.3. Escaping Voltage Estimation

The escaping voltage, where the DEP force overcomes the OT force, was determined by tracking cell positions under increasing DEP voltages. At 2 MHz, the DEP force was size-independent, allowing for precise measurements of OT forces. The average escaping voltage and corresponding OT forces were calculated for BPEI-1, NIH3T3, and Caco-2 cells, revealing differences in their optical properties, particularly refractive indices.

4.3.4. Single-Cell Characterization

The refractive indices of Caco-2 and BPEI-1 cells were estimated using NIH3T3 cells as a reference. BPEI-1 cells exhibited the highest refractive index, likely due to their high melanin content, while Caco-2 cells had a lower refractive index. These differences in optical properties

were reflected in the measured OT forces, validating the method's ability to distinguish cells based on their intrinsic characteristics.

4.4. Conclusion

OT have proven to be invaluable tools in biological research, enabling the manipulation and force measurement of microscopic particles through highly focused laser beams. However, traditional OT setups encounter challenges when applied to larger, inhomogeneous, and scattering particles like cells. Typically, these methods rely on measuring the Brownian motion of the trapped particle using a Quadrant Photodiode (QPD), which is less effective for the larger dimensions of cells. In such cases, the standard approach is viscous drag force calibration, requiring either a precisely calibrated microfluidic chip and pump or a highly accurate moving stage.

In this article, we propose a calibration method based on DEP that simplifies the process. This method requires only a slide with deposited electrodes and a function generator. The complexity of our approach primarily lies in acquiring the DEP spectra necessary to compute the real part of the Clausius-Mossotti factor, $Re[K(f)]$. Fortunately, $Re[K(f)]$ can be derived using known electric parameters for many cell types. Our experimental technique leverages the interplay between dielectrophoretic forces and the trapping forces of OT, facilitating direct force detection on individual cells. This design allows for the determination of the DEP voltage at which a cell escapes the OT, enabling the calculation of the escaping force under controlled conditions. When two cell types with similar sizes and electric polarizabilities are analyzed, our method reveals differences in optical trapping escaping forces, highlighting variations in their optical properties. Additionally, we established a positive correlation between escaping force and cell size, further validating the method.

One of the key advantages of this approach is its ability to calibrate OT forces on structurally complex eukaryotic nucleated cells, which exhibit significant optical inhomogeneity. The adaptability of our method enables OT force measurement for both negative and positive DEP, making it suitable for a diverse range of particles. Furthermore, the technique can be extended to non-spherical objects, such as rod-shaped bacteria, provided that the true Clausius-Mossotti factor can be determined. This method also opens avenues for investigating the effects of applied DEP voltage, as well as physical and chemical stresses on cells, by precisely measuring force responses. Our system demonstrated highly reliable results for complex cells without requiring detailed

knowledge of their local refractive indices and textural parameters, which can be challenging to quantify in living cells. Our findings suggest that variations in the intrinsic optical properties of the cells can be effectively detected by this system, potentially contributing to applications in single-cell characterization and separation.

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