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Automatic Electrorotation-based Cell-Physiometry Platform

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Abstract

Electrokinetic techniques have gained growing attention as label-free and non-invasive tools for investigating the dielectric properties of biological cells. These approaches enable cell characterization and manipulation by exploiting the interaction between external electric fields and the induced dipole movement of the cells. Among them, Electrorotation (ROT) is one of the most accurate and informative methods for analyzing the dielectric response of individual cells. This technique allows to extract information about the cell membrane in the low-frequency regime and the cytoplasm in the high-frequency regime, which together form the cell's dielectric signature. Conventional techniques for cellular characterization, such as flow cytometry, rely on chemical labels, which limit their scalability, invasiveness, and real-time applicability. These constraints highlight the need for automated, low-cost, and portable systems capable of performing dielectric analysis under fully controlled and reproducible conditions.

This research thesis presents the design, development, and validation of a fully integrated ROT-based platform for high-throughput and automated dielectric cell characterization. The work spans from hardware realization to software automation and biological validation, progressively advancing the state of the art in electrokinetic analysis. First, an automated low-frequency ROT platform was developed to analyze membrane-dominated electrorotation measurements. This initial platform comprises a Quadrature Signal Generator (QSG) electronic system and a first-generation angular-velocity extraction algorithm, the PiXel Intensity Algorithm (PxI). This setup allows for accurate and repeatable measurement of the angular velocity of multiple cells, enabling the derivation of their dielectric signature. The platform was experimentally validated with distinct cell lines—CaCo-2, CCD-841, and OPM2—demonstrating reliable discrimination capabilities and quantitative robustness in determining the membrane capacitance characteristics. The platform was then validated using Multiple Myeloma (MM) plasma cells exhibiting different metabolic and drug-resistance profiles. The results show that ROT analysis can distinguish tumor subpopulations based on intrinsic dielectric signatures, supporting its potential role as a complementary tool to conventional minimal residual disease (MRD) detection techniques. Following

the development of the initial low-frequency ROT platform and its biological validation, the need emerged to further enhance the system in terms of experimental throughput and spectral completeness. In particular, clinically relevant investigations required both scalable data processing and the extension of dielectric characterization across the full ROT frequency spectrum through the integration of high-frequency operation. To address these, the Electro-Cell Physiometry (ECP) Framework and the High-Frequency Quadrature Signal Generator (HF-QSG) have been developed. The ECP-Framework is a comprehensive and fully automated software environment governing the entire analytical pipeline, from image segmentation and cell tracking to angular velocity estimation and dielectric parameter extraction. At its core, the framework integrates POLARIS, a dedicated rotational analysis algorithm that significantly enhances estimation precision, robustness to noise, and reproducibility. Validation was conducted using both simulated datasets, generated through a dedicated experimental ROT digital-twin developed—*Electrorotation Game*—, and experimental measurements. Finally, the HF-QSG extended the previous QSG version toward higher frequencies, enabling full ROT spectral acquisition and a more comprehensive dielectric characterization of intracellular structures.

Overall, this thesis provides a unified technological and methodological foundation for automated ROT-based cell analysis. By implementing a fully automated and standardized workflow, the developed ROT platform enables reproducible characterization that does not require expert intervention, allowing non-engineering operators to perform high-throughput dielectric cell analysis reliably. This highlights the critical role of systematic automation and precise process control in ensuring quantitative, scalable, and reproducible measurements. Moreover, by integrating hardware design, advanced computational pipelines, and biological validation, it demonstrates how ROT can evolve from a laboratory technique to a robust, operator-independent, and reproducible tool, suitable for high-throughput diagnostics and monitoring for next-generation bioelectronic and clinical applications.

Introduction

The characterization of living cells is a cornerstone of modern biomedical research, providing crucial information for understanding physiological processes, disease mechanisms, and therapeutic responses. Conventional methods for cell analysis—including flow cytometry, fluorescence microscopy, and molecular assays—have been instrumental in advancing knowledge across the life sciences. These label-based techniques, however, rely on chemical or fluorescent markers to identify cellular targets or processes, often altering the cell's native state, requiring complex preparation protocols, and providing only endpoint information. Furthermore, their dependence on expensive and bulky instrumentation limits portability, throughput, and accessibility.

In recent years, label-free approaches have emerged as powerful alternatives, offering the possibility to probe intrinsic biophysical and biochemical properties of cells without perturbing their physiological conditions. Among these, techniques exploiting electrical and electrokinetic phenomena, such as impedance spectroscopy, Dielectrophoresis (DEP), and Electrorotation (ROT), enable the quantification of cellular dielectric parameters, including membrane capacitance, cytoplasmic conductivity, and permittivity. These parameters are intimately related to cell morphology, composition, and metabolic state, making them valuable physical biomarkers for disease detection, cell sorting, and monitoring of treatment efficacy. Importantly, electrokinetic techniques are contact-free, non-invasive, and compatible with microfluidic integration, offering a route toward automated and high-throughput analysis within compact lab-on-chip devices.

Despite their promise, several limitations have hindered the full deployment of electrokinetic methods in biological and clinical settings. Conventional impedance-based systems require complex instrumentation and limited scalability. DEP-based techniques, while suitable for manipulation and sorting, often suffer from heterogeneous electric field distribution that complicates accurate dielectric modeling and characterization. In contrast, the ROT-based approach using a uniform rotating electric field provides a well-defined electrical environment that enables direct and quantitative assessment of dielectric properties. By measuring the rotational motion of a cell suspended in a rotating electric field, ROT allows the extraction of key electrical parameters across a broad frequency spectrum, linking them to the cell's physiological

and pathological state. However, traditional ROT systems are typically bulky, costly, and operator-dependent, relying on commercial multi-phase generators and manual image-based analysis of cell rotation. These factors have limited its use primarily to specialized laboratories and small-scale experiments, preventing broader adoption in applied biomedical contexts. Addressing these challenges requires innovation across both hardware and software domains, aimed at achieving compactness, automation, high throughput, and integration with microfluidic and computational tools.

The motivation of this research lies in bridging the existing technological gap by developing a fully automated, scalable, and quantitatively reliable ROT-based platform for label-free dielectric cell characterization. The objective is to transform ROT from a laboratory-level experimental technique into a robust, automated analytical system for single-cell analysis, suitable for applications ranging from cancer diagnostics to therapy monitoring.

The scientific and technological contributions of this thesis include advances in both methodological and technological aspects, and follow a developmental trajectory, moving from the first system implementation to full workflow validation and automation:

The scientific and technological contributions of this thesis converge in the conception, realization, and validation of the ***Automatic Electrorotation-based Cell-Physiometry Platform***, an integrated platform for quantitative, fully automated dielectric characterization at the single-cell level. The platform combines wideband hardware architecture, digital simulation tools, and advanced computational analysis into a unified and controlled electro-rotation workflow. The main contributions of this work can be articulated across the following complementary components:

- **Wideband hardware architecture for quantitative electrorotation.** Design and implementation of a broadband electrorotation system based on a custom microfabricated ROT-chip and a High-Frequency Quadrature Signal Generator (HF-QSG). The system enables accurate generation of controlled rotating electric fields over an extended frequency range, allowing full electrorotation spectrum acquisition and broadband dielectric spectroscopy at the single-cell level.
- **Electrorotation Game: a digital twin for controlled benchmarking.** Development of the Electrorotation Game simulation environment as a digital twin of real ROT experiments. The simulator generates synthetic image sequences with known ground-truth angular velocities and dielectric parameters, providing a rigorous benchmarking framework for algorithm validation and enabling the decoupling of signal-processing performance from experimental variability.
- **Electro-Cell Physiometry (ECP) Framework.** Development and validation of a fully automated and integrated software environment governing the complete ROT analytical workflow. The ECP Framework unifies image segmentation, cell tracking, rotational velocity estimation, spectrum reconstruction, and dielectric parameter extraction within a coherent and operator-independent pipeline, constituting the first end-to-end automated software architecture specifically designed for quantitative electrorotation analysis.

- **Experimental and biological validation.** Comprehensive validation of the platform across multiple cells, including various established cell lines and clinically relevant Multiple Myeloma (MM) cells characterized by distinct metabolic and drug-resistance profiles. The experimental campaign demonstrates the capability of ROT-based dielectric signatures to discriminate heterogeneous cellular subpopulations and to resolve both membrane- and cytoplasm-related dielectric contributions across the investigated frequency range. Comparative analyses against standard biological assays further support the potential relevance of the proposed approach in functional phenotyping and minimal residual disease (MRD) assessment.
- **Standardization and automation of the complete ROT workflow.** Definition of a reproducible and controlled experimental protocol enabling reliable, fully automated ROT measurements. From an automation perspective, the platform evolves into a closed-loop analytical system, where controlled excitation, image processing, and dielectric modeling are coherently integrated. This step paves the way for broader adoption of electrorotation in biological and clinical laboratories.

This thesis is organized into five chapters, following a progressive development from theoretical foundations to a fully automated and wideband electrorotation platform:

Chapter 1 establishes the theoretical framework of the work. It introduces the fundamental principles of cell characterization, with particular emphasis on electrical and electrokinetic techniques, and motivates electrorotation as a quantitative, label-free approach for dielectric analysis. This chapter defines the physical and modeling basis upon which the entire platform is developed.

Chapter 2 describes the first operative implementation of the ROT platform. It presents the design and realization of the complete low-frequency system architecture, including a first-generation Quadrature Signal Generator (QSG) specifically designed for membrane-dominated electrorotation measurements and a first-generation angular velocity extraction algorithm—the *PiXel Intensity Algorithm (PxI)*—, developed to automatically estimate the cell angular velocity from image sequences. The system is experimentally validated through membrane capacitance estimation in three distinct cell lines—CaCo-2, CCD-841, and OPM2—thereby demonstrating feasibility, quantitative consistency, and the foundational capabilities of the approach.

Chapter 3 builds upon this validated platform and shifts the focus toward biological relevance. Electrorotation is applied to Multiple Myeloma plasma cells to investigate whether dielectric signatures reflect metabolic states and resistance mechanisms. This chapter represents the first demonstration of the platform’s diagnostic potential, revealing its capability to discriminate tumor subtypes and suggesting applications in minimal residual disease (MRD) evaluation.

Chapter 4 addresses the methodological limitations emerging from the first-generation implementation and introduces the Electro-Cell Physiometry (ECP) Framework as the second major evolution of the platform. This chapter marks a structural transition—from an isolated, task-specific algorithmic implementation to a fully automated and systematically integrated framework governing the entire analytical pipeline. A key advancement is the replacement of the first-generation PxI angular velocity estimator with *POLARIS*, a significantly more robust and accurate rotational analysis algorithm, representing a substantial improvement in estimation reliability, noise resilience, and quantitative precision. Beyond algorithmic refinement, this chapter marks the shift from a partially automated processing strategy to a fully integrated computational framework. The ECP Framework automatically manages the entire ROT workflow, including cell segmentation, tracking, angular velocity estimation, spectral reconstruction, and dielectric model fitting, thereby eliminating operator-dependent steps and ensuring reproducibility. The framework is validated through a two-stage process: first under controlled simulation conditions using the developed *Electrorotation Game simulator environment*, and subsequently on experimental datasets. This combined verification strategy consolidates the analytical robustness and automation level of the platform, establishing it as a complete and operator-independent ROT analysis environment.

Chapter 5 completes the technological evolution of the system by extending its operational bandwidth. It presents the High-Frequency enhanced ROT-Platform and the development of the High-Frequency Quadrature Signal Generator (HF-QSG), a wideband electronic system designed for accurate full-spectrum dielectric characterization at the single-cell level. Broadband validation on HL60 and HeLa cells demonstrates the transition from low-frequency characterization to extended ROT spectrum, thereby achieving the definitive hardware configuration of the platform and resolving both membrane- and cytoplasm-related dielectric characteristics of the investigated cell lines.

In summary, this thesis contributes to the advancement of label-free electrokinetic technologies by providing a complete, integrated, and validated Platform for automated dielectric cell characterization. Through the convergence of experimental engineering, computational analysis, and biological validation, it lays the foundation for the next generation of bioelectronic tools capable of transforming cellular diagnostics and research.

Chapter 1

Understanding Cellular Properties: Methods and Applications

This chapter provides an overview of the main principles and experimental methods used to investigate cellular properties, with a particular focus on electrokinetic techniques. It begins by presenting standard laboratory and on-chip strategies for cell characterization, including optical, mechanical, hydrodynamic, and dielectric approaches, with specific emphasis on label-based and label-free methodologies, highlighting their differences, advantages, and limitations. The second section discusses electrokinetic phenomena that influence the interaction between biological particles and electric fields. It covers methods such as impedance spectroscopy, Dielectrophoresis (DEP), and Electrorotation (ROT), which offer complementary approaches to non-invasively probe the dielectric properties of cells. The third section focuses on applications of electrokinetic techniques, including membrane characterization, cell integrity assessment, cell sorting, and integration with lab-on-chip systems, demonstrating their versatility in both research and biomedical contexts, emphasizing their advantages in terms of quantitative, real-time, and non-invasive analysis, and motivating the use of electrokinetic approaches as a powerful tool for detailed cellular characterization.

1.1 Cell Characterization

Studying the characteristics of cells is essential to understanding their function and the mechanisms underlying disease, forming one of the cornerstones of life sciences. The analysis of cellular biophysical properties provides critical insights for diagnostics, clinical research, and pharmaceutical development. These properties often serve as early indicators of pathological or abnormal

physiological conditions, making them valuable markers for identifying cancers [1–3], bacterial infections [4, 5], toxin exposure [6], and tissue health status [7]. These biophysical signatures arise primarily from the structural and functional organization of the cell, where both the membrane and the cytoplasm play distinct yet interrelated roles in determining overall cellular behavior. In particular, the interplay between membrane properties and intracellular organization governs how cells interact with their environment and respond to external physical or biochemical stimuli. The cell membrane’s composition and transport processes regulate nutrient uptake and signal transduction, while the cell cytoplasm’s organelles and cytoskeletal structures maintain metabolic activity and mechanical integrity [8]. Insights derived from these studies have driven advances in biomedical research, drug discovery, and the treatment of disorders such as cancer and neurodegenerative diseases [9].

In recent decades, single-cell analysis has emerged as a major research focus, driving the development of experimental tools and technologies capable of probing individual cell behavior with high precision. Continuous technological and methodological progress has led to a wide range of strategies for single-cell characterization. These can be broadly categorized into standard laboratory techniques, which rely on well-established biochemical or optical assays [10], and On-Chip methods, which exploit micro- and nanoscale platforms to enable more precise, real-time, and label-free cellular analysis [11]. Building on decades of experimental development, standard laboratory techniques remain the cornerstone of cell characterization. These methods—ranging from optical microscopy [12] and flow cytometry [13] to biochemical and spectroscopic assays [14]—enable the analysis of cellular morphology, viability, and molecular composition with high reliability. In particular, assay-based techniques, which rely on chemical or fluorescent labeling to quantify cell activity or detect specific biomolecules, have long served as fundamental tools for studying cell behavior in culture. However, despite their robustness and reproducibility, these standard approaches often require extensive sample preparation, labeling steps, and destructive protocols, which can limit their ability to capture real-time cellular dynamics. To overcome these limitations, recent advances have shifted attention toward On-Chip and label-free technologies, which enable dynamic and non-invasive analysis at the single-cell level.

1.1.1 Standard Laboratory Approaches

Technological progress and methodological innovation have introduced a broad range of strategies for cell characterization. These approaches can be broadly categorized into standard laboratories and On-Chip methods.

Standard laboratory techniques have long formed the backbone of cellular characterization in biological research. These standard methods span several complementary categories, including optical imaging [15], cytometric analysis [16], biochemical assays [14], and mechanical or spectroscopic probes [17], each targeting distinct cellular attributes such as morphology, phenotypic markers, metabolic activity, and biomechanical properties. Together, they provide a well-established toolkit for both population-level and single-cell studies, balancing sensitivity, specificity, and throughput according to experimental needs.

Among standard techniques, optical microscopy [18] has long been a cornerstone of cellular characterization, providing an immediate and intuitive

understanding of their morphology. Indeed, unlike indirect analytical methods, microscopy provides direct visualization of biological processes, revealing cell morphology, and organization at the single-cell level. Continuous advances in optical microscopy have expanded its capabilities, allowing detailed investigation of cellular and molecular features [19]. In recent years, quantitative imaging has further enhanced the role of microscopy. The combination of high-sensitivity cameras, improved optics, and computational reconstruction now allows the extraction of biophysical parameters directly from image data. Moreover, the integration of Artificial Intelligence (AI) has accelerated image analysis, enabling automated segmentation and feature classification with higher precision and reproducibility [20].

In addition to optical microscopy, Flow Cytometry (FCM) represents a cornerstone technique for quantitative cellular analysis, allowing the characterization of thousands of cells per second [21]. By passing single cells through a focused laser beam and measuring their light scatter and fluorescence properties, FCM enables rapid, multiparametric assessment of large cell populations. This approach is widely used to evaluate cell size, granularity, viability, and expression of specific surface or intracellular markers. Its high-throughput nature makes it particularly suitable for immunophenotyping, cell sorting, and monitoring heterogeneous samples in both research and clinical settings [22]. While flow cytometry offers robust quantitative data, it generally relies on fluorescent labeling and does not provide direct morphological information at the subcellular level.

Complementing optical microscopy and cytometric analysis, spectrophotometric [23], and fluorometric assays [24] are routinely employed to assess cell viability, proliferation, and metabolic activity. Assays such as MTT, WST-1, or resazurin-based tests provide sensitive, reproducible measurements that inform on cellular responses to drugs, toxins, or environmental conditions [25, 26]. These methods are straightforward and widely accessible in standard laboratory settings, but they typically offer only bulk measurements and may require cell fixation or lysis, limiting real-time or single-cell resolution. Together with optical microscopy and cytometric analysis, these three classes of conventional techniques comprehensively cover the main aspects of cell characterization: morphology, phenotype, and functionality. They form the well-established foundation upon which more advanced on-chip and microfluidic approaches have been developed, enabling higher throughput, real-time monitoring, and integration of multiple analytical modalities within compact platforms.

1.1.2 On-Chip Approaches

The development of Micro-Electro-Mechanical Systems (MEMS) paved the way for microfluidics, a multidisciplinary field enabling the precise manipulation of fluids in micrometer-scale channels [27]. Their ability to operate with minimal sample volumes, shorten solvents or solutes diffusion times, and enable parallel processing has made microfluidic systems ideal for applications requiring high throughput and precise control. Building on these advantages, the concept of the Lab-on-a-Chip (LOC) — also known as the micro Total Analysis System (μ TAS) — emerged [28]. LOC platforms integrate multiple laboratory operations, such as sample preparation, detection, and separation,

onto a single chip, effectively transforming complex laboratory workflows into compact, automated, and portable analytical systems.

Over the past two decades, Lab-on-a-Chip and microfluidic technologies have become key enablers of modern bioanalysis, diagnostics, and cell characterization [29]. Their ability to replicate conventional assays within microscale environments not only reduces reagent consumption and analysis time but also enables seamless integration with optical, electrical, or mechanical sensing modalities, thereby extending analytical capabilities beyond those of standard laboratory methods. These on-chip systems offer several key advantages over traditional approaches:

- **Reduced sample and reagent consumption**, due to the microscale operating volumes;
- **High-throughput single-cell analysis**, allowing the study of heterogeneous populations;
- **Integration of multiple analytical modalities**, such as optical imaging, impedance spectroscopy, and dielectrophoretic manipulation within a single platform;
- **Enhanced control of the cellular microenvironment**, including gradients, shear stress, and chemical stimulation, which improves reproducibility and physiological relevance.

These capabilities have made on-chip platforms increasingly important in drug screening, diagnostics, and fundamental cell biology research [30]. A major focus of current microfluidic and On-chip research lies in single-cell diagnostics, where the integration of fluidic control, sensing, and analysis within compact chips enables the detection of rare or disease-related cells with high precision. Microfluidic diagnostic platforms have been developed for a wide range of biomedical targets, including circulating tumor cells (CTCs) [31–33], blood-borne parasites [34–36], and fertility-related cells [37, 38], infection biomarkers [39, 40]. Their capability to operate with microscale sample volumes and to leverage well-established lithographic fabrication techniques makes these systems highly suitable for mass production, resulting in low-cost, portable diagnostic tools with potential for point-of-care applications, even in resource-limited settings [41].

Different on-chip detection strategies have been explored, each relying on distinct physical or biochemical transduction mechanisms—such as optical, electrical, acoustic, or mechanical sensing. The versatility of microfluidic technology allows seamless integration with advanced analytical tools, including light microscopy, automated image processing, optical and acoustic trapping, chemical gradient generation, and on-chip cell culture. This high degree of adaptability enables not only the study of single cells but also the investigation of cell–cell interactions and subcellular processes within controlled microenvironments [11].

Owing to these capabilities, microfluidic diagnostic systems are increasingly regarded as a transformative approach in biomedical analysis. They combine the precision and automation of microengineering with the complexity of biological assays, paving the way for next-generation lab-on-chip platforms capable of addressing a wide variety of diagnostic and research challenges. Moreover, they provide the foundation for both label-based and label-free strategies — including electrokinetic techniques such as dielectrophoresis

(DEP) and electrorotation (ROT) — which will be discussed in the following sections.

1.1.3 Label-Based and Label-Free Strategies

In cell analysis, experimental approaches are generally classified into *label-based* and *label-free* strategies, depending on whether external markers are required to detect or quantify specific cellular features [42]. This distinction reflects two complementary philosophies of investigation: one focused on molecular specificity, the other on intrinsic biophysical characterization [43].

Label-based methods rely on the use of fluorescent dyes [44], or genetic tags [45] to visualize or quantify selected biomolecules or structures. Techniques such as fluorescence microscopy [46], flow cytometry, or immunoassays [47] exemplify this category, providing high sensitivity and molecular selectivity. However, they often require complex sample preparation, chemical or genetic modification, and may interfere with the natural physiological state of the cell. Moreover, labeling procedures can be time-consuming and costly, making them less suitable for continuous, real-time, or point-of-care analysis [48].

Conversely, label-free approaches extract information from the intrinsic physical, chemical, or electrical properties of cells, such as refractive index, mass, or dielectric response [49]. These techniques eliminate the need for exogenous markers, allowing for non-invasive, reproducible, and often high-throughput analysis while preserving cell viability and functionality. Label-free methods are therefore particularly appealing for long-term monitoring, single-cell characterization, and integration within microfluidic or on-chip systems, where minimal perturbation and automation are key [50].

In recent years, label-free strategies have gained increasing attention as they enable the exploration of cellular properties that are not easily accessible through conventional labeling—such as membrane capacitance, cytoplasmic conductivity, or mechanical deformability. Among these, electrokinetic techniques, including dielectrophoresis (DEP) and electrorotation (ROT), have proven especially powerful for characterizing the dielectric properties of biological cells in a quantitative and non-destructive manner.

Overall, while label-based techniques remain indispensable for molecular-level identification, label-free approaches offer a complementary and increasingly advantageous alternative for biophysical analysis, particularly when preserving cell integrity and minimizing assay complexity are priorities.

1.2 AC Electrokinetic Methods

Electrokinetic methods exploit the interaction between suspended cells and externally applied electric fields to extract information about their physical and dielectric properties. These techniques rely on the fact that biological cells behave as polarizable particles whose response to electric stimulation depends on their dielectric properties, i.e., membrane capacitance, cytoplasmic conductivity, and overall geometry.

Electrokinetic techniques investigate the intrinsic electrical properties of cells and are intrinsically label-free, offering a powerful means for non-invasive and quantitative analysis at the single-cell level. By observing how a cell translates, rotates, or alters its impedance under controlled electric excitation,

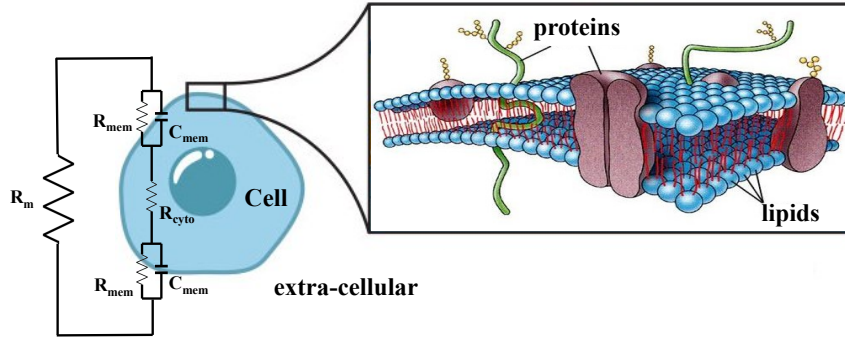


FIGURE 1.1: Electrical Model of Bioparticles in Electrokinetic Effects

it is possible to analyze its internal composition, physiological state, and even detect pathological alterations.

Electrokinetic approaches encompass a family of related phenomena, including impedance spectroscopy, dielectrophoresis (DEP), and electrorotation (ROT). Each of these effects originates from distinct interactions between the applied field and the induced dipole within the cell, providing complementary insights into its electrical and structural characteristics.

1.2.1 Electrokinetic Effects on Biological Cell

The interaction between biological cells and externally applied AC electric fields lies at the core of all electrokinetic techniques. When a cell is suspended in a conductive medium and subjected to an electric field, the distribution of free and bound charges within and around the cell becomes perturbed, leading to the formation of an induced dipole moment [51]. This process, known as *cellular polarization*, provides insight into the dielectric composition and structural organization of the cell.

Biological cells can be modeled as heterogeneous particles composed of a conductive cytoplasm surrounded by a thin dielectric membrane and immersed in a conductive suspending medium [52]. The cell membrane, primarily composed of phospholipid bilayers, behaves as an electrical insulator that separates two conductive domains—the intracellular and extracellular regions [53]. This configuration is commonly represented as a single-shell spherical model consisting of a resistive–capacitive (RC) membrane enclosing a conductive core, see Fig. 1.1. The main electrical parameters of interest are the membrane capacitance and conductivity, and the cytoplasm permittivity and conductivity. These quantities determine how the cell interacts with an external field, and thus, how its polarization develops over time and frequency [54].

At the microscopic level, two main mechanisms govern the cell–field interaction: *conduction*, where free charges move through conductive regions, and *polarization*, where charges accumulate at interfaces due to restrictions in mobility. In biological particles, interfacial (Maxwell–Wagner) polarization dominates in the frequency range typically from tens of kHz to tens of MHz , as ions become trapped at the membrane interface, forming space-charge regions that manifest as dipoles aligned with the electric field [55]. As the

frequency increases, the capacitive effect of the membrane decreases, allowing current to penetrate the cytoplasm and rendering the internal structure accessible to electrical probing.

For a spherical bioparticle with complex permittivity ε_p^* suspended in a medium of complex permittivity ε_m^* , the induced dipole moment $\mathbf{p}$ is given by

$$\mathbf{p} = 4\pi\varepsilon_m r^3 CM^*(\omega) \mathbf{E}, \quad (1.1)$$

where r is the cell radius, $\mathbf{E}$ the applied electric field, and $CM^*(\omega)$ is the complex *Clausius–Mossotti factor*, defined as

$$CM^*(\omega) = \frac{\varepsilon_{cell}^* - \varepsilon_m^*}{\varepsilon_{cell}^* + 2\varepsilon_m^*}, \quad (1.2)$$

with $\varepsilon^* = \varepsilon - j\sigma/\omega$, where ε is the permittivity, σ the conductivity, $\omega = 2\pi f$ the angular frequency of the rotating electric field, and $j = \sqrt{-1}$.

The Clausius–Mossotti factor describes both the magnitude and phase of the polarization response, linking the dielectric properties between the cell ε_{cell}^* and its environment ε_m^* to measurable electrokinetic behaviors. Its real and imaginary components govern different physical effects: the real part $\text{Re}[CM^*(\omega)]$ determines the direction and strength of translational forces in DEP, while the imaginary part $\text{Im}[CM^*(\omega)]$ is responsible for the torque observed in ROT [56]. Moreover, the frequency dependence of $CM^*(\omega)$ explains the dispersive nature of cell impedance and the capability of impedance spectroscopy to probe distinct subcellular features.

Overall, the polarization of biological cells under electric fields represents the fundamental mechanism underlying electrokinetic phenomena. By quantifying how cells polarize and interact with AC electric fields across frequencies, one can extract label-free information on their dielectric properties, morphology, and physiological state—forming the physical basis for modern electrokinetic diagnostic and analytical platforms [57]. Several electrokinetic techniques have been developed for cellular characterization. Among them, the most established and widely adopted—owing to their versatility and strong diagnostic relevance—are Electrical Impedance Spectroscopy (EIS), Dielectrophoresis (DEP), and Electrorotation (ROT).

1.2.2 Electrical Impedance Spectroscopy (EIS)

Electrical Impedance Spectroscopy (EIS) is one of the most established noninvasive bioparticle characterization techniques for probing the dielectric properties of biological cells [57]. In EIS, cells are exposed to a low-amplitude alternating electric field, commonly sinusoidal, applied across two or more electrodes generated by a voltage signal $V(j\omega)$. The cells interact with the field, and their resistive and capacitive effects are reflected in the measured response current $I(j\omega)$. By varying the frequency of the applied field and recording the corresponding response, an impedance spectrum is generated, i.e., impedance as a function of frequency. This spectrum allows the extraction of key dielectric properties such as membrane capacitance and cytoplasmic conductivity.

EIS can be implemented using different electrode configurations, including two-, three-, or four-electrode setups. In the simplest two-electrode configuration, the same electrodes act as the working electrode (WE) and the counter electrode (CE). While increasing the number of electrodes can improve precision, it also increases experimental complexity; the optimal configuration depends on the specific application constraints [58].

The electrical behavior of the cells is formally described by the complex impedance $Z(j\omega)$, which relates the applied voltage $V(j\omega)$ to the measured response current $I(j\omega)$:

$$Z(j\omega) = \frac{V(j\omega)}{I(j\omega)} = |Z|e^{j\text{Arg}(Z)} = \text{Re}(Z) + j\text{Im}(Z) \quad (1.3)$$

where $\text{Re}(Z)$ and $\text{Im}(Z)$ represent the resistive and reactive components, respectively. Pure (Ohmic) resistance does not vary with frequency; however, in biological cells, the effective resistance is frequency-dependent due to the capacitive effect of the cell membrane. The corresponding complex permittivity $\varepsilon^*(j\omega)$ captures both conductive and polarization effects, linking electrical behavior to the physical properties of the cell.

At low frequencies, the insulating cell membrane dominates the response, acting as a capacitive barrier that limits current flow into the cytoplasm. As the frequency increases, the membrane is progressively bypassed, allowing current to penetrate the cell interior. This transition produces a characteristic dielectric dispersion, known as the β -dispersion, reflecting interfacial (Maxwell–Wagner) polarization effects arising from charge accumulation at the membrane–medium interface [59].

Through proper modeling (e.g., single-shell or multi-shell approximations), parameters such as membrane capacitance, cytoplasmic conductivity, and cell size can be extracted [60]. These quantities serve as quantitative markers of cell health, differentiation state, or pathological alterations.

EIS offers several advantages: it is label-free, non-destructive, and compatible with microfluidic integration, enabling high-throughput single-cell analysis [57]. However, since impedance measurements typically reflect ensemble averages, complementary methods such as dielectrophoresis (DEP) and electrorotation (ROT) are often employed to provide localized or dynamic dielectric information at the single-cell level. Moreover, this method is accurate, but requires specialized hardware, whereas DEP and ROT utilize simpler, cost-effective systems to induce cell movement using electric fields—non-uniform in DEP, and uniform in ROT.

1.2.3 Dielectrophoresis (DEP)

Dielectrophoresis (DEP) refers to the translational motion of a polarizable particle when subjected to a *non-uniform* electric field [61]. When a biological cell is suspended in a medium and exposed to such a field, an induced dipole moment arises due to the mismatch between the dielectric properties of the cell and its surrounding medium. The resulting dielectrophoretic force acting on the cell $\langle \mathbf{F}_{DEP} \rangle$ depends on the degree and direction of polarization and can move the cell either toward regions of higher or lower electric field intensity [56].

The time-averaged DEP force acting on a spherical particle of radius r in a medium of permittivity ε_m is expressed as:

$$\langle \mathbf{F}_{DEP} \rangle = 2\pi r^3 \varepsilon_m \operatorname{Re}[CM^*(\omega)] \nabla |\mathbf{E}|^2 \quad (1.4)$$

where $\mathbf{E}$ is the electric field and $CM^*(\omega)$ is the *complex Clausius–Mossotti factor*, representing the frequency-dependent polarizability contrast between the particle and the medium.

If $\operatorname{Re}[CM^*(\omega)] > 0$, the particle experiences *positive DEP* (attraction toward high-field regions), whereas $\operatorname{Re}[CM^*(\omega)] < 0$ leads to *negative DEP* (repulsion from high-field regions). By analyzing the DEP response over a range of frequencies, it is possible to infer key electrical parameters such as membrane capacitance and cytoplasmic conductivity, enabling label-free discrimination of cells based on their dielectric signature [54].

DEP has been extensively applied for cell trapping [62], manipulation [63], separation [64], and classification [65], and represents one of the most widely used electrokinetic methods in both conventional and microfluidic (on-chip) settings.

1.2.4 Electrorotation (ROT)

Electrorotation (ROT) is a complementary electrokinetic phenomenon with respect to DEP in which a polarizable particle experiences a *rotational torque* when exposed to a *uniform rotating* electric field. This torque arises from the phase shift between the induced dipole moment and the applied rotating electric field and provides access to the *imaginary part* of the Clausius–Mossotti factor, which is not accessible via DEP measurements [54].

The average torque $\langle \Gamma_{ROT}(\omega) \rangle$ acting on a spherical particle is given by:

$$\langle \Gamma_{ROT}(\omega) \rangle = -4\pi \varepsilon_m r^3 \operatorname{Im}[CM^*] |E|^2 \quad (1.5)$$

where the direction and magnitude depend on the sign and amplitude of $\operatorname{Im}[CM^*(\omega)]$. At frequencies where $\operatorname{Im}[CM^*(\omega)] = 0$, no torque is generated and the cell remains stationary. Conversely, when the imaginary part is nonzero, the dipole either lags or leads the field, producing *co-field* or *counter-field* rotation, respectively.

By monitoring the rotation rate as a function of frequency, ROT provides a powerful means to extract dielectric parameters related to both membrane and cytoplasmic properties [66]. In combination with DEP, it enables a comprehensive dielectric characterization—capturing both the magnitude and phase of the cell’s polarizability. Together, DEP and ROT offer complementary insights into the dielectric behavior of biological cells, forming the foundation for modern label-free electrokinetic characterization techniques [67].

1.3 On-Chip Electrokinetic Applications

In recent years, electrokinetic techniques have evolved from fundamental physical principles into powerful analytical and manipulation tools for biological systems. Their ability to probe the intrinsic dielectric properties of single cells in a label-free and non-invasive manner has paved the way for numerous applications in biotechnology, diagnostics, and life sciences. These methods have

found particular relevance when integrated within microfluidic and lab-on-chip platforms, where precise control of the electric field and fluid environment enables high-throughput, low-volume, and automated cellular analysis. The following sections highlight key application domains of electrokinetic methods, focusing on their technological integration, diagnostic capabilities, and their role in cell sorting and manipulation.

1.3.1 Advantages of Electrokinetic Approaches

Electrokinetic methods leverage the intrinsic electrical properties of cells to perform both characterization and manipulation in a label-free, non-invasive manner. Unlike traditional assays—which often require chemical markers, complex preparation, or fixed cells—techniques such as impedance spectroscopy, dielectrophoresis (DEP), and electrorotation (ROT) can probe live, suspended cells with minimal perturbation.

Measuring the electrical parameters offers a simple and scalable form of assessing a broad range of information. In fact, one of the key advantages lies in the ability to access dielectric parameters such as membrane capacitance, cytoplasmic conductivity, and cell polarization dynamics [68]. These parameters reflect cellular morphology, membrane integrity, and physiological state, enabling early detection of disease, differentiation, or treatment response without relying on antibodies or dyes. Moreover, electrokinetic platforms are readily integrable with microfluidic architectures, facilitating low-volume, high-throughput, and automated workflows. In addition, the combination of microfluidics with AC electric fields allows for compact, scalable devices that consume fewer sample quantities, reduce analysis times, and support real-time monitoring of single cells or populations. These features make electrokinetic approaches particularly attractive for point-of-care devices, personalized diagnostics, and automated screening platforms [69]. Another benefit is the dual functionality offered by many electrokinetic systems: they can both characterize and manipulate cells [70]. For example, DEP can discriminate cells based on dielectric contrasts and then direct their movement or trapping; ROT can extract detailed dielectric spectra, reveal subtle biophysical changes, and precisely control the cell's orientation through the induced torques. This “characterize then act” paradigm provides a seamless path from measurement to sorting or manipulation, all in a single and compact device.

Finally, while challenges still remain—such as standardization of protocols or device fabrication complexity—the trajectory of recent research and commercialization [71,72] indicates that electrokinetic approaches are increasingly complementing, and in some cases replacing, standard laboratory methods in cellular characterization and manipulation.

1.3.2 Microfluidic integration and On-Chip Systems

The integration of electrokinetic methods within microfluidic and lab-on-chip platforms has enabled the realization of miniaturized systems capable of performing complex biological analyses with high precision and automation. These devices allow the application and control of electric fields in confined fluidic environments, enabling functions such as cell trapping, manipulation, and dielectric characterization directly on-chip.

Recent advances in materials science and microfabrication have made substrates and components suitable for nearly all biological applications readily available [73]. Continuous development in sample handling and preparation techniques has also contributed to making bioassays more compact, automated, and customizable. These advances significantly enhance the throughput, reproducibility, and versatility of on-chip electrokinetic platforms.

Multicomponent fabrication and device architecture. Electrokinetic devices typically rely on a multicomponent architecture in which conductive, dielectric, and structural layers are integrated to achieve specific electrical, mechanical, and optical functionalities. A typical configuration includes a rigid substrate—such as glass or silicon—on which metallic microelectrodes are patterned [74]. These electrodes generate controlled electric fields within a microfluidic channel, allowing the manipulation or characterization of suspended cells. Depending on the intended operation (e.g., impedance measurement, dielectrophoretic trapping, or electrorotation), different electrode geometries are adopted, such as interdigitated [75], castellated [76], or quadrupolar designs [77]. The microchannel network, which guides the fluid and positions the cells in the active region, is generally fabricated from a transparent polymer and bonded to the electrode substrate, forming a hybrid electrofluidic device [78].

Materials and fabrication processes. The choice of materials for electrokinetic microdevices must balance electrical, optical, and chemical requirements with ease of fabrication and biocompatibility [41]. The most common substrates are glass and silicon. Glass offers excellent optical transparency, chemical inertness, and thermal stability—making it particularly suited for imaging applications, including fluorescence or phase-contrast microscopy [79]. Silicon, conversely, is preferred when high electrical performance or integration with existing microelectronic technologies is required; however, its opacity limits optical access and requires reflective or infrared imaging setups [80]. Gold (Au) is widely used as the electrode material due to its chemical stability, high conductivity, and biocompatibility. Because gold does not adhere strongly to glass or silicon, a thin adhesion layer—typically chromium (Cr) or titanium (Ti)—is deposited prior to gold deposition. The metallic layers are patterned using standard microfabrication techniques such as photolithography combined with thermal evaporation or sputtering. These processes ensure precise control over electrode geometry and thickness, which are critical for achieving the desired field distributions. The microfluidic components, responsible for fluid handling and sample positioning, are most often fabricated from polydimethylsiloxane (PDMS). PDMS offers key advantages such as optical transparency, elasticity, and ease of prototyping through replica molding against a master template [81]. Once cured, the PDMS layer containing the channel network is aligned and irreversibly bonded to the electrode-bearing substrate, forming a sealed microfluidic device capable of operating under controlled electrical and hydrodynamic conditions.

Microfluidic manipulation and electrokinetic actuation form the foundation of modern electrokinetic lab-on-chip technologies. By combining conductive, dielectric, and structural materials into compact architectures, it is

possible to create multifunctional platforms capable of performing cell analysis, sorting, and diagnostics with minimal reagent consumption and high spatial precision.

1.3.3 Cell Characterization and Integrity Assessment

Electrokinetic characterization methods have been successfully applied to a broad spectrum of biological systems, offering powerful means for quantifying dielectric signatures associated with cell health, differentiation, or infection. These approaches, including impedance spectroscopy, dielectrophoresis (DEP), and electrorotation (ROT), provide complementary perspectives on membrane capacitance and cytoplasmic conductivity, enabling label-free monitoring of cell function and pathology [82].

Several studies have focused on red blood cells (RBCs) owing to their well-defined geometry and physiological importance. Gawad et al. demonstrated the capability of impedance flow cytometry to distinguish erythrocytes and ghost cells through frequency-dependent impedance profiles [83], while Cheung et al. extended this approach to assess fixed versus native RBCs [84]. Electrorotation has been particularly effective for probing RBC membrane and cytoplasmic properties, as shown in early works by Becker et al. [85] and Gascoyne et al., who analyzed both healthy and *Plasmodium falciparum*-infected RBCs, revealing marked changes in dielectric parameters during infection [86,87]. ROT, DEP and EIS have been extensively applied to cancer cell analysis, enabling the discrimination of malignant phenotypes based on dielectric contrast. Zhang et al. used a constriction-channel EIS platform to differentiate between primary and metastatic salivary gland carcinoma cells through variations in membrane capacitance [88], while Zhao et al. applied multifrequency impedance to distinguish between lung and cervical cancer cell lines [89]. Using ROT, Wang et al. observed reduced membrane capacitance in erythroleukemia cells following induced differentiation [90], and Keim et al. achieved multiplexed single-cell characterization of cancer and neuronal lines [91]. DEP has also been exploited to discriminate cells according to malignancy or differentiation state. Salmanzadeh et al. [92] investigated ovarian cancer MOSE cells, revealing distinct dielectric signatures associated with cancer stage. In addition, DEP field-flow fractionation (DEP-FFF) enabled the discrimination of malignant MDA-435 cancer cells from stem cells based on differences in levitation height, yielding 92% purity [93]. ROT-based studies have also extended to immune cell profiling. Becker et al. characterized HL-60 leukemic cells and T-lymphocytes [94], and further developments enabled dielectric mapping of multiple leukocyte subtypes in integrated microchip platforms [95], underscoring the potential of electrorotation for immunophenotyping. Beyond mammalian systems, electrokinetic methods have been applied to other bioparticles. For example, DEP characterization of the alga *Chlamydomonas reinhardtii* revealed shifts in crossover frequencies linked to cytoplasmic conductivity changes under nitrogen depletion [96], demonstrating how electrokinetics can serve as a proxy for metabolic state and stress responses.

Overall, these applications confirm that electrokinetic techniques constitute a powerful toolkit for cell characterization, offering label-free, quantitative, and highly sensitive analyses across a wide range of biological contexts [97].

1.3.4 Cell Sorting, Trapping and Manipulation

Beyond dielectric characterization, electrokinetic techniques play a crucial role in the active manipulation of biological particles. By exploiting the forces generated through applied electric fields, it is possible to translate, trap, or selectively separate cells according to their intrinsic electrical and physical properties [98]. Among these methods, dielectrophoresis (DEP) and its derivatives represent the most established approaches for electrokinetic manipulation. Depending on the frequency and field configuration, cells can experience positive or negative DEP, leading to motion toward or away from high-field regions [99]. Owing to its versatility and label-free operation, DEP has been extensively explored for applications ranging from single-cell trapping to large-scale separation. Its capability to discriminate cells based on dielectric contrasts has enabled selective enrichment of viable, malignant, or rare cell types in heterogeneous samples [100]. The robustness and maturity of DEP-based methods have also facilitated their transition toward commercial implementations; a notable example is the *ApoStream* platform [72].

Recent studies have demonstrated a broad range of DEP-assisted operations, including cancer cell sorting, selective trapping, and viability-based separation, using both electrode-based (eDEP) and contactless (cDEP) architectures. DEP-based separation has been extensively applied to cancer cell discrimination. Alshareef et al. [101] employed an eDEP platform to separate breast cancer cells (MCF-7) from colorectal cancer cells (HCT-116), later optimizing electrode geometry and materials to enhance the recovery rate. Similarly, Alazzam et al. [102] demonstrated efficient isolation of metastatic breast cancer cells (MDA-MB-231) from blood cells using interdigitated comb electrodes, achieving a separation efficiency of 96%. Further advancements were proposed by Marchalot et al. [103], who implemented three-dimensional carbon-PDMS electrodes for positive DEP trapping, obtaining up to 97% efficiency. Other studies have focused on heterogeneous or mixed samples. Chen et al. [104] used an eDEP device to separate HeLa cells from a mixture containing MCF-7 and red blood cells (RBCs), achieving viability and efficiency values near 80%. Further improvements in design have enabled size-based discrimination, such as the four-zone device described in [105], which separated PC-3 prostate cancer cells from leukocytes with 85.6% efficiency and 54.7% recovery. Finally, contactless DEP (cDEP) architectures have emerged as an alternative to conventional electrode systems. Shaffie et al. [106] employed cDEP to manipulate THP-1 leukemia cells while quantifying translational and rotational dynamics under actuation. Similarly, the use of vertical eDEP configurations [107] and coil-based cDEP concentrators [108] demonstrated high selectivity (up to 95%) in guiding or trapping target cell populations such as K562 and Jurkat T-lymphoblasts.

Overall, DEP-based manipulation demonstrates remarkable versatility for both analytical and preparative purposes. By tuning the field frequency, amplitude, and device geometry, electrokinetic platforms can be engineered to perform selective trapping, enrichment, or separation across a wide range of cell types and physiological states. Collectively, these advances illustrate how electrokinetic methods are evolving from diagnostic characterization toward active and precise cellular manipulation. A summary of representative applications, encompassing both dielectric characterization and sorting operations, is reported in Table 1.1.

TABLE 1.1: Summary of electrokinetic techniques application for cellular characterization and manipulation.

Technique	Application	Cell Type(s)	Description	Advantages / Limitations	Ref.
EIS	Characterization	RBCs and ghost cells	Impedance flow cytometry distinguishes intact from lysed RBCs based on impedance spectra	Label-free; real-time monitoring. Requires accurate fluidic control	[83]
EIS	Characterization	SACC-83, SACC-LM carcinoma cells	Crossing-constriction channel device differentiates carcinoma subtypes via dielectric parameters (C_m , σ_{cyto})	Sensitive to subtle dielectric contrasts; single-cell resolution	[88]
EIS	Characterization	H1299, HeLa, A549	Frequency-dependent impedance profiling (100–250 kHz) for cancer cell discrimination	High-throughput; minimal labeling. Limited selectivity at low frequencies	[89]
ROT	Characterization	HL-60, T-lymphocytes, RBCs	ROT spectra used to determine crossover frequencies and derive C_m	Quantitative; precise dielectric mapping. Sensitive to medium conductivity	[85]
ROT	Characterization	MEL (treated vs. untreated)	Membrane capacitance decreased after differentiation-inducing treatment	Enables monitoring of differentiation; requires careful calibration	[90]
ROT	Characterization	HeLa, C3H10, B lymphocytes, HepaRG	3D ROT combined with imaging for electrophysiological and morphological measurements	Integrates optical and electrical data; complex fabrication	[109]

Continued on next page

Table 1.1 – continued from previous page

Technique	Application	Cell Type(s)	Description	Advantages / Limitations	Ref.
ROT	Characterization	Healthy vs. P. falciparum-infected RBCs	Detection of dielectric alterations (increased σ_{cyto} , C_m) due to infection progression	High sensitivity to infection state; limited throughput	[87]
DEP	Characterization	Chlamydomonas reinhardtii (algae)	Shift in 2nd crossover frequency under nitrogen stress indicates lipid accumulation	Non-destructive; correlates dielectric response with metabolic state	[96]
DEP	Sorting	MCF-7, HCT-116	eDEP device separates breast from colorectal cancer cells; optimization of electrode geometry improved efficiency	High separation selectivity; dependent on geometry/materials	[101]
DEP	Sorting	MDA-MB-231, blood cells	Interdigitated electrodes separate metastatic cells from blood with 96% efficiency	High recovery; label-free. Performance decreases in high-conductivity media	[102]
DEP	Trapping	HeLa cells	3D Carbon-PDMS electrodes enable efficient single-cell trapping (97% efficiency)	3D field enhances trapping; fabrication complexity	[103]
DEP-FFF	Sorting	Stem cells, MDA-435	Field-flow fractionation separates malignant and stem cells by levitation height (92% purity)	Continuous flow; high throughput. Requires uniform field gradients	[93]
DEP	Differentiation analysis	MOSE ovarian cancer cells	DEP response used to differentiate cancer stage through dielectric properties.	Stage-sensitive; simple setup. May require calibration media	[92]

Continued on next page

Table 1.1 – continued from previous page

Technique	Application	Cell Type(s)	Description	Advantages / Limitations	Ref.
DEP	Sorting	HeLa, MCF-7, RBCs	eDEP separation with viability and efficiency in 76~80% range	Moderate selectivity; compatible with flow operation	[104]
DEP	Sorting	PC-3, leukocytes	Four-zone device separates prostate cancer cells by size (85.6% efficiency)	Size-selective; tunable geometry. Partial recovery (55%)	[105]
cDEP	Manipulation	THP-1 leukemia cells	cDEP platform measures translational/rotational motion under electric actuation	Electrode-free; reduced fouling. Limited force control	[106]
eDEP	Sorting	K562 cells	Vertical Cr–Au electrodes direct trajectories toward outlet (94.7% separation)	Compact design; scalable. Reduced recovery efficiency (49%)	[107]
cDEP	Concentration	Jurkat T-lymphoblasts	Coil-driven cDEP microdevice concentrates cells with 95% efficiency	High selectivity; avoids electrode degradation	[108]

1.3.5 Electrorotation for Dielectric Cell Characterization

As discussed in the previous sections, several electrokinetic techniques are currently used for cell characterization [110–112], in which cells are modeled as electrical networks to extract their dielectric properties. In particular, impedance-based techniques [113], such as impedance flow cytometry (IFC) [114] and electric impedance spectroscopy (EIS) [58], correlate the dielectric properties of a cell population with the amplitude and phase response of the induced current when an alternating voltage is applied at fixed or variable frequencies [115]. Although highly accurate, these approaches typically require complex instrumentation and precise cell–electrode coupling, which can hinder measurement reproducibility and scalability.

Conversely, dielectrophoresis (DEP) and electrorotation (ROT) rely on electric-field-induced motion—translational in DEP [56] and rotational in ROT [116]—to derive the dielectric parameters of individual cells. By analyzing the frequency-dependent motion of suspended cells, these methods yield the so-called DEP (translation–frequency) and ROT (rotation–frequency) spectra. Typically, the frequency range explored spans from tens of kHz up to a hundred of MHz , encompassing both the low-frequency region dominated by the capacitive response of the cell membrane and the higher-frequency region where the electric field penetrates into the cytoplasm [52,68,117]. This broadband probing capability allows a comprehensive assessment of both membrane and intracellular properties. In addition to their use in dielectric spectroscopy, DEP and ROT enable dynamic operations such as trapping [62,118], manipulation [63,119], and separation of cells [64]. While DEP is particularly suited for manipulation and sorting, ROT provides a more stable platform for accurate dielectric characterization, as cells experience rotational torque while remaining localized near a fixed point—thereby minimizing the influence of positional variability and local field inhomogeneity.

Therefore, among the various electrokinetic methods, ROT stands out as a powerful technique for quantitative, label-free cell characterization. This method ensures non-contact measurements, reduces experimental variability, and simplifies device fabrication. Furthermore, the rotating field employed in ROT is inherently uniform than the non-uniform field required for DEP, leading to a more homogeneous torque distribution and, consequently, more accurate extraction of dielectric parameters. The uniformity of the electric field also facilitates the simultaneous analysis of multiple cells and enables longer observation times, yielding richer and more robust datasets.

Taken together, these features make electrorotation particularly advantageous for studying cellular biophysical properties, especially when high precision, minimal perturbation, and scalability are required. For these reasons, ROT represents the most suitable technique for the development of the present work, combining the analytical power of impedance spectroscopy with the flexibility and label-free nature of electrokinetic systems.

Chapter 2

Automated, High-Throughput and Label-Free ROT-Platform

This chapter presents the development and validation of the automated low-frequency ROT-platform for label-free dielectric characterization of biological cells. The system consists of three main components: (i) a custom electronic device, the *Quadrature Signal Generator (QSG)*; (ii) a specifically designed electrorotation chip, the *ROT-chip*; and (iii) a dedicated image processing algorithm, the *PiXel Intensity Algorithm (PxI)*. Its high level of automation enhances results consistency and lowers the operational barrier for non-specialist users, enabling routine, label-free dielectric characterization with minimal supervision. Designed to be compact, automated, and user-friendly, the platform is well-suited for biological environments, eliminating the need for bulky, expensive, and complex instrumentation. The ROT-Platform has been tested and validated through the determination of the specific membrane capacitance in three different cell lines: CaCo-2, CCD-841, and OPM2. The consistency of measurements both within and across these cell lines confirms the platform's functionality and highlights its potential as a label-free, high-throughput electrokinetic characterization of cell populations. The work related to the proposed platform and methodology has been published in [66].

2.1 Cell Characterization Through Rotating Electric Field

As discussed in Chap.1, ROT is a well-established electrokinetic technique that enables the label-free dielectric characterization of single cells suspended in a fluid medium. When exposed to a rotating electric field, a polarizable particle experiences an induced torque that causes it to rotate. The angular

velocity of this rotation depends on the frequency-dependent complex permittivity of both the particle and the surrounding medium.

By analyzing the cell's rotational response across a range of excitation frequencies, ROT provides detailed information on intrinsic dielectric parameters such as membrane capacitance, cytoplasmic conductivity, and membrane permittivity. These properties are directly related to cell morphology, composition, and physiological state, making electrorotation a powerful diagnostic and analytical tool for biological studies.

Compared to other electrokinetic methods, e.g., dielectrophoresis (DEP), ROT offers a simpler, direct, and quantitative means to assess the complex polarization behavior of cells under controlled electric stimuli. For this reason, it has become a fundamental technique for probing the electrical and structural properties of biological cells in a non-invasive, label-free, and highly sensitive manner.

2.1.1 Theory and Operating Principles

In the ROT phenomenon, a suspended biological cell rotates when exposed to a uniform rotating electric field. More specifically, the dipole induced in the cell by the Maxwell-Wagner polarization [120] interacting with the external rotating electric field with a magnitude $|E|$ and an angular frequency ω , generates a time-averaged torque $\langle \Gamma_{ROT} \rangle$, that causes the cell to rotate with a time-averaged angular velocity $\langle \Omega_{ROT} \rangle$ [121], defined as follows

$$\langle \Gamma_{ROT}(\omega) \rangle = -4\pi\epsilon_m r^3 \text{Im}[CM^*] |E|^2 \quad (2.1)$$

$$\langle \Omega_{ROT}(\omega) \rangle = -\frac{\epsilon_m \text{Im}[CM^*] |E|^2}{2\eta} \quad (2.2)$$

where ϵ_m and η are the absolute permittivity and the viscosity of the suspension medium, r is the radius of the cell, and $\text{Im}[CM^*]$ represents the imaginary part of the Clausius-Mossotti factor, which for the case of a spherical particle suspended in a medium, is given by

$$CM^* = \frac{\epsilon_{cell}^* - \epsilon_m^*}{\epsilon_{cell}^* + 2\epsilon_m^*} \quad \text{with} \quad \epsilon_m^* = \epsilon_m - j \frac{\sigma_m}{\omega} \quad (2.3)$$

where ϵ_{cell}^* and ϵ_m^* are the complex permittivity of the cell and suspension medium, respectively, and σ_m is the conductivity of the suspension medium. The Clausius-Mossotti factor represents the effective polarizability of the cell, relating the dielectric properties of the cell and the medium in which it is suspended. Specifically, in ROT, it determines the magnitude and phase of the induced dipole moment of the suspended particle and thus governs its rotational behavior under a rotating electric field. The complex nature of CM^* implies that it can assume both positive and negative real and imaginary parts depending on the excitation frequency and the contrast between the dielectric properties of the particle and the medium.

In ROT, a nonzero imaginary component of CM^* gives rise to a torque acting on the particle, causing it to rotate. When the imaginary part of CM^* is negative, the induced dipole moment lags the rotating electric field, resulting in rotation in the same direction as the field (co-field rotation). Conversely, when the imaginary part of CM^* is positive, the dipole leads

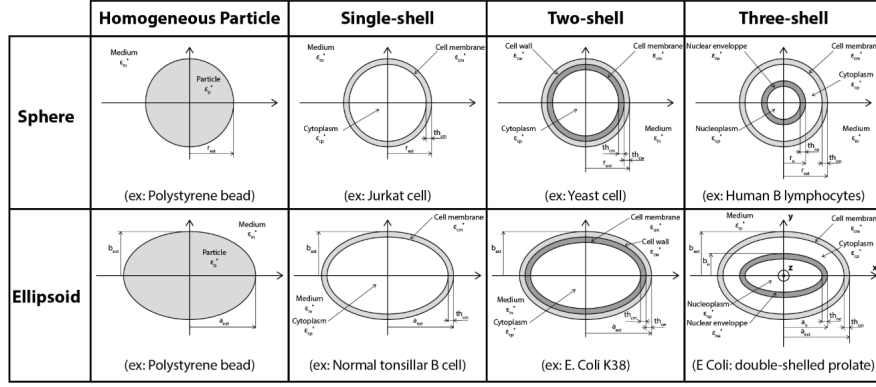


FIGURE 2.1: Illustration of the different spherical and ellipsoidal cell and particle models. *Cottet, Jonathan, et al. "MyDEP: a new computational tool for dielectric modeling of particles and cells." Biophysical journal 116.1 (2019): 12-18.*

the applied field, producing rotation in the opposite direction (counter-field rotation). At frequencies where CM^* is purely real, no torque is generated, and the particle remains stationary [122].

2.1.2 Electrokinetic Cell Modeling: Single and Multi-Shell

To accurately interpret ROT measurements, a suitable electrical model of the cell is required. Cells are inherently heterogeneous systems, consisting of distinct compartments such as the plasma membrane, cytoplasm, and, in some cases, a nucleus or internal organelles, each with different dielectric properties. These structural features strongly influence the cell's frequency-dependent polarization and therefore its electrokinetic response.

To capture this complexity, cells are commonly represented through equivalent dielectric models, such as the Single-Shell or Multi-Shell spherical or ellipsoidal models, as shown in Fig.2.1. In the Single-Shell model, the cell is represented as a conductive cytoplasmic core surrounded by a thin insulating membrane. This simplified description effectively accounts for the dominant interfacial polarization effects and is generally sufficient for most non-nucleated or homogeneous cells.

When higher accuracy is required—such as for nucleated mammalian cells or microorganisms with complex envelopes—Multi-Shell models (two- or three-layer) can be employed. These extend the Single-Shell framework by introducing additional dielectric layers (e.g., nucleus), allowing finer estimation of intracellular permittivities and conductivities. However, this increased complexity also makes parameter estimation more challenging and often unnecessary outside specialized studies. Despite these extensions, the Single-Shell model remains the most widely used approach in current electrokinetic literature, providing a good compromise between physical realism and analytical tractability.

Therefore, to model the complex permittivity of a biological cell, the *Single-Shell Model* is commonly adopted [54]. In this model, the cell is represented as a concentric spherical structure composed of an inner cytoplasm core with radius r_{in} and an outer membrane layer of thickness $d = r - r_{in}$, where r is the cell radius. The corresponding effective complex permittivity ϵ_{cell}^* is then given by:

$$\varepsilon_{\text{cell}}^* = \varepsilon_{\text{mem}}^* \frac{\left(\frac{r}{r_{\text{in}}}\right)^3 + 2 \left(\frac{\varepsilon_{\text{cyto}}^* - \varepsilon_{\text{mem}}^*}{\varepsilon_{\text{cyto}}^* + 2\varepsilon_{\text{mem}}^*}\right)}{\left(\frac{r}{r_{\text{in}}}\right)^3 - \left(\frac{\varepsilon_{\text{cyto}}^* - \varepsilon_{\text{mem}}^*}{\varepsilon_{\text{cyto}}^* + 2\varepsilon_{\text{mem}}^*}\right)} \quad (2.4)$$

were $\varepsilon_{\text{mem}}^*$ and $\varepsilon_{\text{cyto}}^*$ represent the complex permittivity of the membrane and cytoplasm, defined as follows

$$\varepsilon_{\text{mem}}^* = \varepsilon_{\text{mem}} - j \frac{\sigma_{\text{mem}}}{\omega} \quad \text{and} \quad \varepsilon_{\text{cyto}}^* = \varepsilon_{\text{cyto}} - j \frac{\sigma_{\text{cyto}}}{\omega} \quad (2.5)$$

The dielectric response of a cell to a known electrical stimulus (E , ω) depends on four intrinsic parameters: the membrane permittivity (ε_{mem}), membrane conductivity (σ_{mem}), cytoplasm permittivity ($\varepsilon_{\text{cyto}}$), and cytoplasm conductivity (σ_{cyto}). Indeed, it is not possible to analytically determine their values from a single frequency (ω_0) measurement; it is therefore necessary to acquire the electrorotation over a range of frequencies and derive the ROT-spectrum $\langle \Omega_{\text{ROT}_{\text{exp}}}(\omega_i) \rangle$. Finally, to evaluate these parameters, a least-squares fitting procedure minimizing the discrepancy between the experimentally measured spectra $\langle \Omega_{\text{ROT}_{\text{exp}}}(\omega_i) \rangle$ and the theoretical model predictions $\langle \Omega_{\text{ROT}_{\text{theory}}}(\omega_i) \rangle$ can then be used.

Among all, the estimated membrane permittivity ε_{mem} can be used to extract the area-specific membrane capacitance C_m as follows:

$$C_m = \frac{\varepsilon_0 \varepsilon_{\text{mem}}}{d} \quad (2.6)$$

where $d = r - r_{\text{in}}$ represents the membrane thickness. The membrane capacitance (C_m) is a key indicator of the biophysical characteristics of the cell membrane, particularly its complexity and surface roughness. Higher values of C_m are typically associated with increased membrane roughness, often linked to pathological conditions such as cancer.

2.1.3 Complementary Dielectric Insights: ROT and DEP

As discussed in Chap.1, Dielectrophoresis (DEP) and Electrorotation (ROT) represent two complementary electrokinetic techniques that probe the dielectric properties of suspended cells through their distinct responses to non-uniform and uniform rotating electric fields, respectively. While DEP measures translational motion resulting from induced dipole-field gradients, ROT detects the rotational torque arising from phase shifts between the applied field and the induced dipole. Together, they provide a comprehensive picture of cell polarizability, enabling the extraction of both the magnitude and phase of the Clausius–Mossotti factor across frequency.

In fact, DEP and ROT probe complementary aspects of the Clausius–Mossotti factor. The dielectrophoretic force is proportional to its real part, which governs the strength and direction of the induced dipole in a non-uniform field, whereas the electrorotational torque depends on its imaginary part, arising from the phase lag between the field and the dipole moment. Therefore, while DEP provides information on the magnitude of cell polarizability, ROT reveals its frequency-dependent phase behavior.

The two techniques are inherently interconnected, since both originate from the same polarization mechanism and share the same theoretical formulation of the Clausius–Mossotti factor. In principle, knowledge of one allows

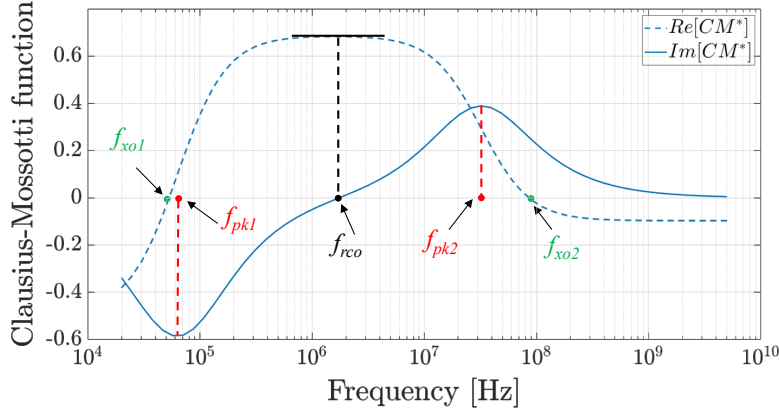


FIGURE 2.2: Dielectrophoresis (DEP) and electrorotation (ROT) responses modelled using the Single-Shell model. The low- and high-frequency DEP crossover points (f_{xo1} , f_{xo2}) are indicated, together with the contra-field and co-field peaks of rotation at f_{pk1} and f_{pk2} , respectively. A transition from contra- to cofield ROT occurs at f_{rco} .

the derivation of the other across the frequency spectrum [54]. Thus, invoking the Kramers–Kronig theorem, for a fixed frequency ω_o , a relation between the DEP force F_{DEP} and the ROT torque Γ could be derived as

$$\Gamma(\omega_o) = \frac{2\omega_o}{\pi k} \int_0^\infty \frac{F_{DEP}(\omega)}{\omega^2 - \omega_o^2} d\omega \quad (2.7)$$

The quantity k is a frequency-independent, positive factor related to the DEP and ROT applied electric fields E by:

$$k = \frac{|\nabla E_{DEP}^2|}{|E_{ROT}^2|} \quad (2.8)$$

This relationship indicates that, through the Kramers–Kronig theorem, we can obtain the DEP frequency response of a particle from its ROT spectrum and vice versa. From this interrelationship between DEP and ROT can be noticed that: (i) in a frequency range where the DEP force acting on a particle increases with frequency ($\partial F_{DEP}/\partial\omega > 0$) the rotational torque $\Gamma(\omega)$ is negative, to give a contrafield rotation; (ii) where the DEP force decreases with frequency ($\partial F_{DEP}/\partial\omega < 0$) the rotational torque $\Gamma(\omega)$ is positive, to give a co-field rotation; (iii) at frequencies where $\partial F_{DEP}/\partial\omega = 0$ the rotational torque is zero. These relationships can be seen in Fig.2.2 for the DEP and ROT response modelled for a generic particle.

More importantly, when both DEP and ROT data are available for the same sample, the combined analysis enables the direct determination of the four dielectric parameters of the Single-Shell model—namely, the permittivity and conductivity of the cytoplasm and the membrane—from four characteristic frequencies (two crossover frequencies from DEP and two rotational peaks from ROT). This provides a self-consistent solution without relying on iterative fitting procedures.

However, in practice, simultaneously obtaining accurate DEP and ROT responses from the same cell population is experimentally demanding due to differences in setup configuration, field distribution, and measurement conditions [123]. Consequently, most studies rely on one of the two techniques and

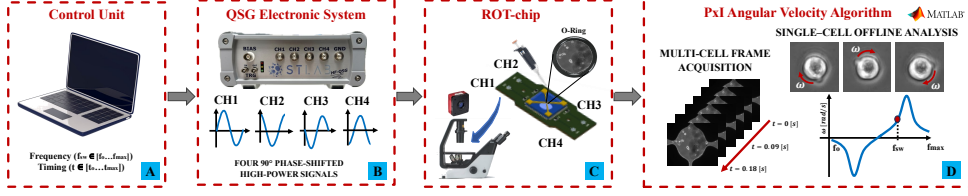


FIGURE 2.3: ROT-Platform architecture: (A) the PC unit for the automation and control of the experimental procedure, (B) the high-power and broadband Quadrature Signal Generation (QSG) for 90-degree phase-shifted signal, (C) the plug-and-play ROT-chip, (D) the image process acquisition and PiXel Intensity (PxI) Algorithm for the automatic extraction of the cells angular velocity vs frequency (ROT-spectrum).

employ model-based fitting to estimate the dielectric parameters with good accuracy. For further details about DEP and ROT theory, see [54].

2.2 ROT-Platform Design and Architecture

The developed ROT-Platform is composed of (i) a Quadrature Signal Generator (QSG), (ii) ROT-chip, and (iii) Pixel-Intensity Algorithm (PxI). The operating principle is illustrated in Fig.2.3. The QSG, controlled via PC (see Fig.2.3(A)), generates four high-power quadrature sinusoidal signals (see Fig.2.3(B)). These signals are connected to the ROT-chip via high-frequency compatible cables (see Fig. 2.3(C)), where a uniform rotating electric field is generated. At the center of the ROT-chip, a rubber O-ring is placed to form a fluidic chamber and filled with the cells. A slide cover is then placed on top to seal the chamber, minimizing cell translational movement. The ROT-chip is positioned in the microscope, and cell rotation is monitored using a digital camera. A custom script running on the PC was implemented to fully automate and control the experimental procedure and automatically perform the ROT-spectrum experiments.

Specifically, the script, initially, define the experimental parameters: the frequency vector $F = [f_1, f_2, \dots, f_k]$, the corresponding rotation durations $t_{\text{ROT}} = [t_{\text{ROT}_1}, t_{\text{ROT}_2}, \dots, t_{\text{ROT}_k}]$, the resting duration t_{resting} , and the amplitude of the applied signal A_0 . The script initializes the process by setting the first frequency and starting the video acquisition. The experiment proceeds through an iterative loop composed of two main phases for each frequency step:

- **rotation-phase**: the signal amplitude is set to $A = A_0$, and the selected frequency f_{count} is applied for a time duration $t_{\text{ROT}_{\text{count}}}$, inducing a rotation of the cells;
- **resting-phase**: the signal amplitude is set to zero $A = 0$, effectively turning off the electric field for a fixed interval t_{resting} . During this phase, the electrorotational torque is null ($\langle \Gamma_{\text{ROT}} \rangle = 0$), and the cell's rotation ceases. This interruption is crucial for detecting transitions between frequencies and simplifying and automating the post-processing step.

Once all frequencies in the vector F have been spanned, the video acquisition is stopped and saved. Finally, the recorded video is analyzed using an automated algorithm that calculates the angular velocity of the rotating cell as a function of frequency, enabling the extraction of the cell's ROT-spectrum

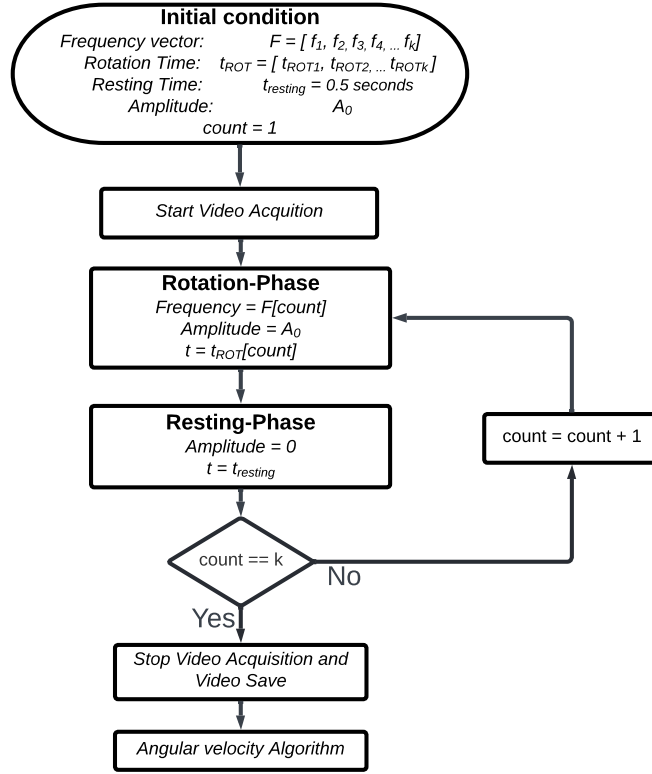


FIGURE 2.4: Flowchart of the experimental algorithm that fully automates and controls the experimental procedure to automatically perform the ROT-spectrum experiments.

(see also Fig. 2.3(D)). The complete workflow of the experimental control algorithm is summarized in the flowchart shown in Fig. 2.4. In the following, the in-depth description of the ROT-Platform component.

2.2.1 Quadrature Signal Generator

The QSG is a custom-designed quadrature sinusoidal signal generator, specifically engineered to meet the demanding requirements of electrokinetic experiments, see Fig.2.5A. The system is capable of delivering four signals with amplitudes up to $8 V_{pp}$ into a 50Ω load, maintaining a -3 dB bandwidth of 60 MHz , thus ensuring both high-power (200 mW) and wide frequency response (20 MHz at -0.1 dB bandwidth) suitable for ROT demanding. The QSG system architecture is modular and consists of three main subsystems, as described in Fig.2.5B: the Control Unit (highlighted in yellow), the signal generation module (highlighted in red), and the Quadrature and AM-Plication stage (QAMP) (highlighted in green). Below, each subsystem is described to provide a general understanding of its function and integration.

Control Unit

The Control Unit consists of a Raspberry Pi Model 4B, which acts as the central processor coordinating all functional aspects of the system. This embedded platform was chosen for its flexibility, low power consumption, and ease of integration with custom software environments. A dedicated control software has been developed to manage the device hardware. This software

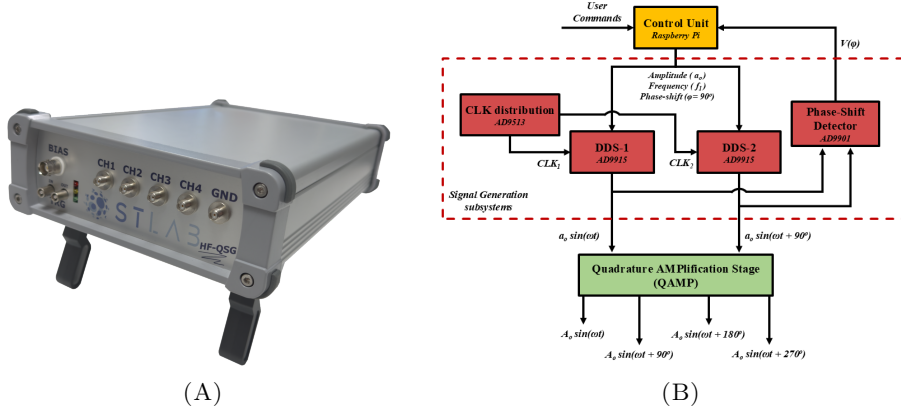


FIGURE 2.5: Quadrature Signal Generator electronic system (QSG) (A) picture and (B) functional block diagram.

provides a set of low-level commands, enabling configuration of output parameters—including frequency (f_1), amplitude (a_o), and phase (φ)—along with full automation of the generator’s operation during experimental protocols. Communication between the user and the QSG is established via a TCP/IP protocol over an Ethernet connection, ensuring fast and reliable data transmission.

Signal Generation

The signal generation module is responsible for producing two low-power sinusoidal signals with a 90-degree phase shift between them ($V_1 = a_o \sin(\omega t)$ and $V_2 = a_o \sin(\omega t + 90^\circ)$). At the core of this module are two Direct Digital Synthesis (DDS) units (AD9915, by Analog Devices), which enable high-resolution frequency and phase control across a wide operating range. The AD9915 is a DDS unit designed to operate with an internal clock frequency of up to 2.5 GHz, allowing it to synthesize output signals with frequencies of up to 1 GHz. A clock distribution circuit based on the AD9513 (by Analog Devices) is employed to deliver a common reference clock to both DDS units, enabling synchronization through the integrated multi-chip synchronization feature. This capability locks the DDS in frequency and maintains phase coherence across the units, ensuring the generation of the signals with the required frequency, while maintaining the exact phase relationship imposed (90-degree phase shift). Moreover, to monitor the phase relationship between the DDS outputs, a dedicated digital-to-analog phase feedback circuit based on AD9901 (by Analog Devices), named a phase-detector, is incorporated, providing an output voltage proportional to the phase-shift ($V(\varphi)$) of the two signals. This module allows for high-resolution phase comparison and continuous monitoring of synchronization, enabling fine-tuning during operation. The DDS units are managed by the Control Unit via the SPI (Serial Peripheral Interface) protocol, allowing for startup initialization and dynamic adjustment of output parameters. User-defined commands enable real-time changes to frequency, amplitude, and phase for each DDS output, providing flexibility for experimental applications.

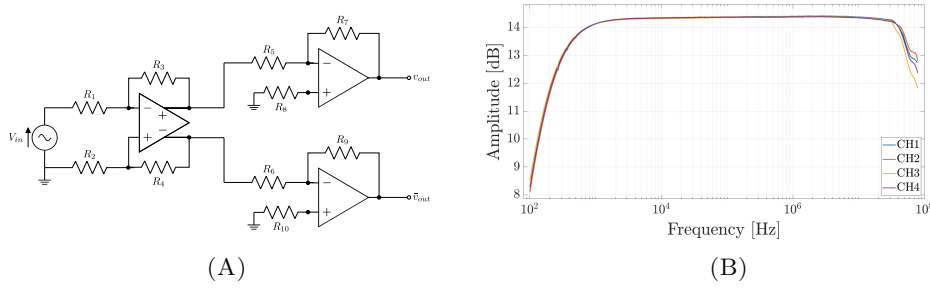


FIGURE 2.6: QSG Amplification Stage: (A) Simplified electrical schematic of a single channel; (B) Magnitude response of the four channel, showing a perfect overlap with a flat gain of approximately 14 dB in the 10 kHz-20 MHz range, and a -3 dB bandwidth extending up to 60 MHz.

Quadrature AMPlification (QAMP)

The Quadrature AMPlification stage (QAMP) has been designed as a double, low-power input system. Each input signal (tone) is elaborated by a conditioning chain named "channel", where the Fig.2.6A depicts the simplified schematic. The single channel is capable of generating two 180° out-of-phase signals from the input one, while amplifying both voltage and power. For this application context, two 90° out-of-phase signals are supplied by the signal generation module so that the QAMP will provide four amplified, power-backed, and in quadrature signals.

The QAMP is composed of two independent channels where the mutual phase is solid, and the dynamic input/output voltage range and bandwidth are almost equal. The system is composed of a low-noise and high linearity pre-amplification stage and a power amplifier. The single channel can be divided into four main blocks: the input tone source, the fully-differential pre-amplifier, and two identical power amplifiers. The beating heart of the pre-amplifying stage is made up of a dual-channel, fully-differential amplifier (ADA4927, by Analog Devices). This is powered with a dual voltage ± 5 V and provides initial amplification (6 dB) of the input tones. The choice of a dual-channel chip is thought out to minimize inter-channel mismatches and guarantee a low phase-shift discrepancy between the two elaborated tones. At the output of this circuit, there are two signals in phase opposition with amplitudes limited to ± 5 V. The power amplification stage is made up of an operational amplifier (THS3491, by Texas Instruments) which is powered with a dual voltage supply, ± 15 V. The voltage gain has been set at 14 dB using external resistors. Extensive electrical characterization of each signal conditioning chain has been carried out in the frequency and time domains. The magnitude diagram reported in Fig.2.6B shows a band-pass gain equal to about 14 dB, reduced by 6 dB for a 50 Ω standard measurement setup. A high gain flatness (gain variation < 0.2 dB in the band) is maintained in the frequency range between 10 kHz and 20 MHz, with an inter-channel gain error lesser than 1%, with a final -3 dB bandwidth of 60 MHz. All four output signals exhibit almost the same amplitude, with a maximum output-to-output amplitude difference equal to 40 mV, corresponding to 0.5% of the output signal, and the maximum phase error is 0.4°. The measured total harmonic distortions are equal to 0.1%, and 0.4% for an output voltage peak-to-peak value of 1 V, and 7.5 V, respectively.

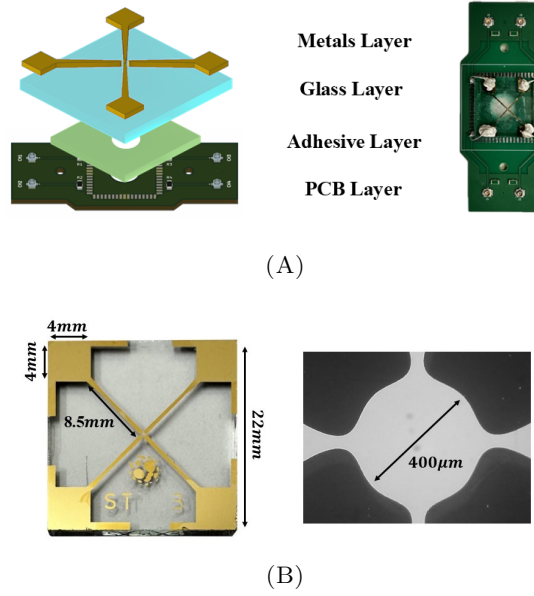


FIGURE 2.7: (A) The plug-and-play ROT-chip layer structure and (B) ROT-microchip dimensions and electrode geometry.

2.2.2 ROT-chip: Design and Fabrication

The ROT-chip consists of a custom PCB designed to minimize parasitic capacitances, enabling high-frequency operations, and a specially engineered, glass-based, ROT-microchip, as shown in Fig.2.7A. The PCB facilitates easy handling of the ROT-microchip as well as easy electrical connections for signal transmission through four RF coaxial connectors between the QSG and the ROT-microchip electrodes. Indeed, to ensure impedance matching between the QSG and the ROT-chip, each electrode is equipped with a $50\ \Omega$ termination resistor. This configuration maximizes power transfer efficiency and preserves signal integrity across the high-frequency operating range.

The ROT-microchip is composed by four metal electrodes on a glass substrate defining a central circular region of radius $200\ \mu\text{m}$, corresponding to the region where the rotating electric field is generated, see Fig.2.7B. A specific "Bone electrode" configuration, described in [124], was used in order to minimize field non-uniformities, thus minimizing dielectrophoretic (DEP) forces, which would cause the cells to drift away from the region of interest (ROI). The geometries of the electrodes and a picture of the ROT-microchip are shown in Fig.2.7B. The ROT-microchip was manufactured using standard photo-lithographic techniques [125]. The microelectrodes were realized with a $20\ \text{nm}$ chrome adhesion layer and a $100\ \text{nm}$ gold layer on top of a glass substrate, chosen for its biocompatibility and transparency. The ROT-microchip was mechanically attached to the PCB support through an adhesive layer, see Fig.2.7A. Finally, the electrodes were connected to the PCB using copper wires and silver paste to ensure a stable and robust connection during handling and transport.

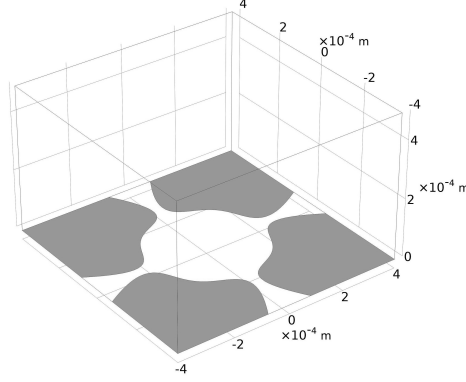
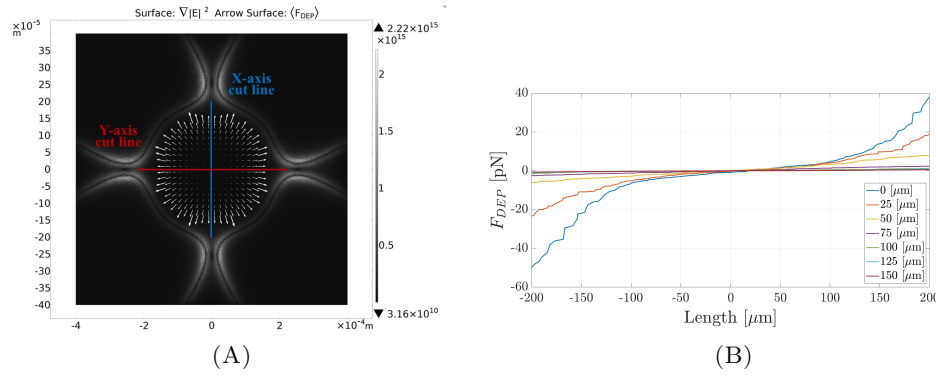


FIGURE 2.8: Geometry employed in the COMSOL simulation.

FIGURE 2.9: *COMSOL Multiphysics* simulations: (A) $\nabla|E|^2$ surface in the plane $10 \mu m$ above the electrode layer and (B) $\langle F_{DEP} \rangle$ acting along the x -axis for different z -planes. The particle was assumed to have a radius of $6.2 \mu m$ (consistent with the dimension of the CaCo-2 cells) and a $Re[CM^*] = 0.8$ for a frequency of $80 kHz$.

Comsol Simulations

A simulation was performed using *COMSOL Multiphysics*, a finite element simulation commercial software package, to assess the influence of the dielectrophoretic and ROT force on cells in different positions. The simulation focused on the ROT-microchip, made up of four electrodes, to study the dielectrophoretic effects induced inside the chamber. A 3D model of the chamber was used, with a volume of $800 \mu m \times 800 \mu m \times 500 \mu m$, beginning from the metal layers and up to the $500 \mu m$ of the o-ring thickness, as shown in Fig.2.8. The volume above the electrode layer was assumed composed of water having an electrical conductivity of $0.033 S/m$ and a relative permittivity of 80, representing the cells suspension medium (aqueous buffer). A non-uniform tetrahedral 3D mesh was used for space discretization. The mesh was narrower around the electrodes, and then decreased along the z -axis to best simulate the region of interest in the electron plane, while minimizing the simulation time. The study was a frequency-domain analysis, with four quadrature sinusoidal signals considered, having a frequency of $80 kHz$ and an amplitude of $10 V_{pp}$. The study allowed the analysis of the gradient of the electric field ∇E inside the chamber and the derivation of both the dielectrophoretic and electrorotation forces $\langle F_{DEP} \rangle$ in a tridimensional space.

The simulation results (Fig.2.9A) indicate that the resulting $\langle F_{DEP} \rangle$ is null at the center of the chamber. However, approaching the electrodes, the

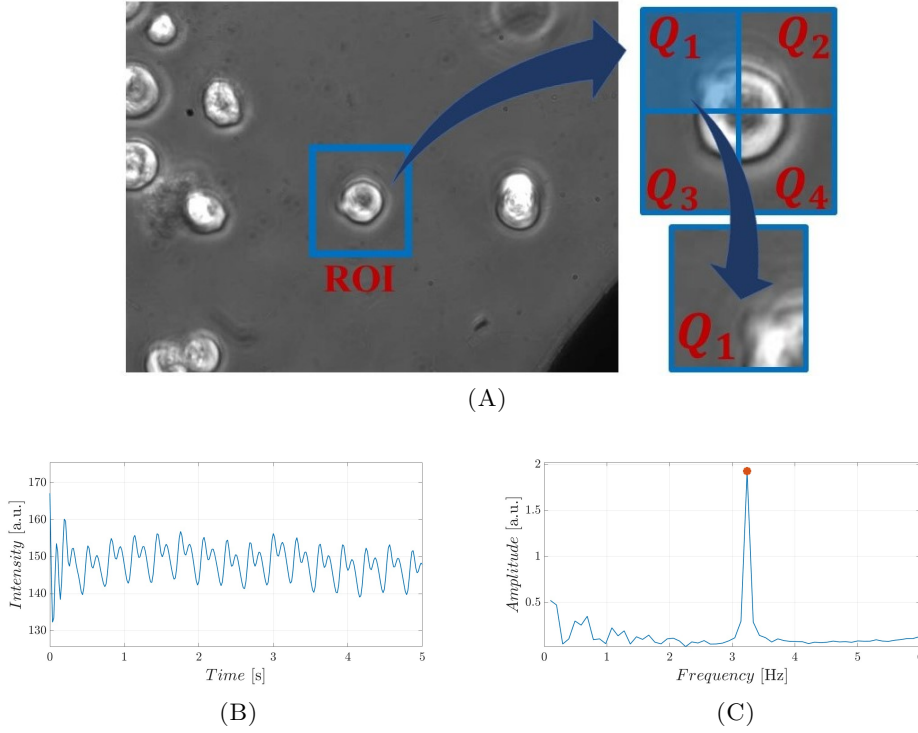


FIGURE 2.10: (a) Subdivision of the frames in the four quadrants, (b) $P_q(t)$ signal behavior in the first quadrant Q_1 , and (c) FFT analysis with peak extraction.

electric field becomes non-uniform, thereby generating a $\langle F_{DEP} \rangle \neq 0$, which leads to unwanted cells movements. This means that the optimal cell position is located inside a circular region with a radius of $150 \mu m$, smaller than the $200 \mu m$ of the electrodes, beginning from the center of the electrodes. Outside this region, i.e. close to the electrodes, the cells are subjected to an $\langle F_{DEP} \rangle \neq 0$, resulting in non-pure rotations. Fig.2.9B depicts the average DEP force $\langle F_{DEP} \rangle$ along the x -axis at various z -plane, with a $25 \mu m$ increment, ranging from the electrode plane up to $150 \mu m$. The plotted data illustrates the trend of the DEP force $\langle F_{DEP} \rangle$ to diminish as the distance from the electrode plane increases. This behavior is characterized by a rapid saturation in a quadratic pattern, particularly evident after approximately $100 \mu m$. The underlying cause of this phenomenon is the swift dispersion of the electric field along the z -plane. This poses a notable limitation on the ideal positioning of the cell along this plane.

2.2.3 PiXel Intensity Algorithm

The video acquired during the experiments is analyzed through the image processing *Pixel-Intensity Algorithm (PxI)* that allows the automatic extraction of the cells' angular velocity vs frequency ($\langle \Omega_{ROT}(\omega_i) \rangle$). The PxI algorithm estimates the cell's angular velocity over time by analyzing variations in grayscale pixel intensity patterns, enabling precise characterization of the electrorotational response.

The automated ROT analysis pipeline begins with a user-assisted cell selection step, performed via a graphical user interface. At this stage, cells located outside the optimal trapping region—defined as a radius of $150 \mu m$ from the geometric center of the electrodes—or exhibiting clustering or signs

of being biologically compromised (e.g., membrane rupture) are excluded to ensure biological and analytical reliability. Each selected cell is assigned a unique label, and its centroid is localized within the initial reference frame. Subsequently, a segmentation-based tracking routine is employed to monitor the position of each selected cell throughout the duration of the experiment. For this purpose, the Cellpose algorithm—a robust and widely used segmentation tool—is utilized to ensure robust and accurate centroid detection across all video frames. Once the centroid trajectory is established, a square Region of Interest (ROI) is dynamically extracted from each frame, centered on the instantaneous centroid position. This ROI is then passed to the angular velocity extraction module, implemented through the PxI.

Specifically, suppose the cell is rotating around the z-axis (optical axis) and its centroid has been determined using Cellpose. For a recorded sequence of frames F_i for $i = 1, 2, \dots, \ell$ in the xy-plane, acquired at uniform time intervals, the PxI algorithm operates as follows:

- ROI extraction as a $n \times n$ intensity matrix around the identified centroid of the cell, see Fig.2.10A;
- subdivision of the ROI into four equal regions, identified as quadrants, Q_1 , Q_2 , Q_3 , and Q_4 , each represented by an $\frac{n}{2} \times \frac{n}{2}$ matrix, see Fig.2.10A;
- Determination of the spatial average intensity value of the k -th frame $\bar{P}_k^q$ for each quadrant, independently, as

$$\bar{P}_k^q = \frac{1}{n} \sum_{i=1}^{\frac{n}{2}} \left(\sum_{j=1}^{\frac{n}{2}} p_k^q(i, j) \right) \quad (2.9)$$

for $q = 1, 2, \dots, 4$, where $p_k^q(i, j)$ is the intensity of the pixel in position (i, j) of the k -th frame of the q -th quadrant;

- calculation of the average intensity value $\bar{P}_q(t)$ over time for each independent quadrant, as shown in Fig.2.10B;
- Fast Fourier Transform (FFT) of the resulting signal $\bar{P}_q(t)$;
- frequency peak identification as shown in Fig.2.10C;
- cell's angular velocity ω_{PxI}^q determination as

$$\omega_{PxI}^q = 2\pi f_p \quad (2.10)$$

- cell's mean angular velocity ω_{PxI} determination as a average of the four quadrant ω_{PxI}^q .

As observed in the $\bar{P}_q(t)$ signal, the temporal behavior is periodic, with periodicity dependent on the cell's rotation. Therefore, in the frequency domain, the frequency peak corresponds to the number of revolutions per second that the cell performs. To reduce the impact of background noise in ROT experiments, the ROI around the cell is sized based on the cell radius and divided into four quadrants. This accounts for uneven noise sources—such as cell

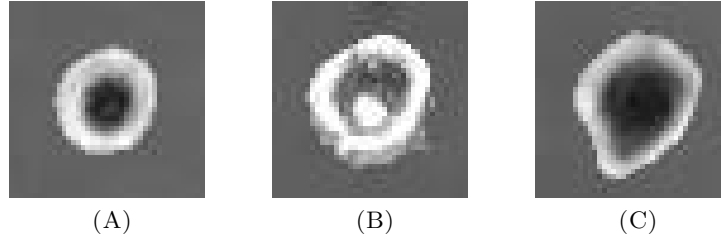


FIGURE 2.11: (a) Round-shaped cell, (b) elliptic-shaped cell, and (c) asymmetric-shaped cell.

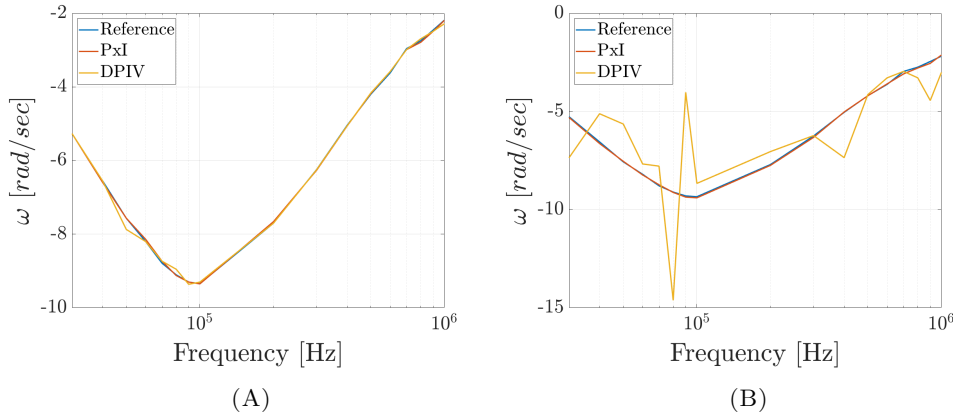


FIGURE 2.12: Electrorotation Spectrum for round-shaped cell with (a) no residual tracking error and (b) high residual tracking error.

movement, out-of-focus objects, and lighting variations—which may affect each quadrant differently. Averaging the results from all quadrants improves the reliability of the angular velocity estimation.

PxI Performances

In order to evaluate the accuracy and computational efficiency of the developed PxI rotation algorithm, a simulated electrorotation environment has been implemented. The simulator mimics the behavior of cells rotating under electrorotation conditions, generating a synthetic video of the experiment. The output video from the simulation is then processed by the PxI algorithm, which estimates the angular velocities, enabling a comparison between the estimated parameter and the original input value, providing a controlled environment to evaluate the algorithm's performance. To generate the simulated video, the simulator environment takes as input a cell image extracted from a pre-existing database composed of real cell images acquired during previous ROT experimental campaigns. Alongside the cell input, the simulator also receives an angular velocity vector, representing the expected ROT spectrum, and a corresponding time vector that defines the duration for which the cell rotates at each specified angular velocity. As output, the algorithm produces a simulated video replicating the ROT experiment.

Three different cell shapes were considered in four different video conditions. Fig.2.11 shows the three representative cell shapes considered for the simulator. Specifically, a round, an elliptical, and an asymmetric shape were

Tracking residuals	Cell Shape	PxI		DPIV	
		RMSE [rad/s]	t_{el} [s]	RMSE [rad/s]	t_{el} [s]
Null	round	0.031	1.82	0.090	210
	elliptical	0.032	1.47	2.704	241
	asymmetric	0.035	1.24	0.047	274
Low	round	0.041	1.28	3.178	453
	elliptical	0.045	1.58	2.044	669
	asymmetric	0.040	1.09	1.229	471
Medium	round	0.055	1.02	4.001	663
	elliptical	0.049	2.59	2.493	444
	asymmetric	0.063	2.06	2.049	569
High	round	0.058	1.85	2.182	489
	elliptical	0.083	1.27	3.526	793
	asymmetric	0.111	1.73	2.197	799

TABLE 2.1: Summary table reporting the accuracy and computational efficiency of both the PxI and DPIV algorithms across all combinations of tracking conditions and cell shapes considered.

selected as reference cases. These three morphologies were chosen as they collectively encompass the vast majority of cellular geometries typically observed in electrorotation experiments. Furthermore, the accuracy of the PxI algorithm is affected by the precision of the tracking process. Tracking errors can introduce residual offsets, leading to a random displacement of the cell within the region of interest (ROI) over time. To assess the robustness of the PxI algorithm under varying tracking conditions, we created four levels of tracking residuals: Null, Low, Medium, and High. These levels represent scenarios ranging from ideal tracking with no centroid drift to severe tracking errors with significant centroid drift for each cell shape. Finally, to benchmark the developed algorithm against standard methodologies, all simulated videos were analyzed using both the PxI algorithm and Digital Particle Image Velocimetry (DPIV), [126]. For each simulated video, the resulting rotation spectrum was estimated, and both the Root Mean Square Error (RMSE) and elapsed time (t_{el}) were evaluated across the entire electrorotation frequency range.

Fig.2.12 presents rotation spectra obtained with both analysis methods, PxI and DPIV, for the round-shaped cell under the two extreme tracking scenarios: no residual tracking error (Null condition, Fig.2.12A) and high residual tracking error (High condition, Fig.2.12B). It can be observed that, under ideal tracking conditions, where the tracking residual error is null, both algorithms are able to extract the full electrorotation spectrum. However, under the worst-case scenario, characterized by high tracking residuals, the DPIV algorithm fails to estimate the angular velocity, resulting in an inaccurate reconstruction of the rotation spectrum. Table 2.1 summarizes the results obtained across all tested conditions and cell shapes. The table highlights a clear trend observed in both algorithms: as tracking residuals increase, the RMSE rises, primarily due to the displacement of the cell within the ROI. However, while the PxI algorithm consistently estimates the angular velocity across all levels of tracking residuals considered, with an error ranging from just 3.5% under ideal conditions to 11% in the presence of high residuals, the DPIV algorithm is only able to reliably reconstruct the rotation spectrum when no tracking error is present. One of the primary reasons why Particle Tracking Velocimetry (PTV) algorithms, such as DPIV in this case, tend to

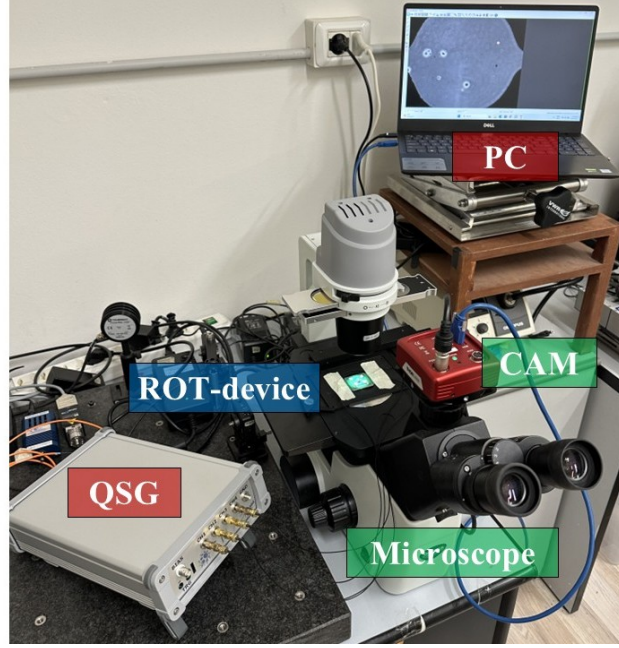


FIGURE 2.13: Picture of the experimental setup used.

fail in rotational velocity analysis is that they are inherently designed to estimate translational and tangential displacements, rather than angular motion. As a result, they struggle to accurately capture the rotational dynamics of cells, particularly when tracking residuals introduce centroid drift within the ROI. Moreover, there is a significant difference in computational time between the two methods: Pxl extracts angular velocity values across the entire spectrum more than 100 times faster than DPIV, making it vastly more efficient for high-throughput or real-time applications.

2.3 Experimental Setup and Campaign

A picture of the ROT-Platform used for the experimental validation is shown in Fig.2.13, as previously mentioned, the system is compact, easily transportable, and can be used with standard microscopes. The electrical connection between the QSG and the ROT-chip was made using SMA-UMC coaxial cables of equal length, in order to maintain the symmetry of the system to avoid a mismatch or a phase-shift between the signals. The ROT-chip is inserted under a phase contrast inverted microscope (OPTIKA IM-300 PHi) with a $\times 20$ magnification. A CCD camera (340M Fast Frame, Thorlabs) with a resolution of 640×480 px (pixel size of $7.4 \mu m$, square) coupled with the microscope, was connected through a USB cable to a PC for the video acquisition using its proprietary software (ThorCam™ v.3.7.0), using a frame rate of 60 FPS.

As described in Sec.2.2, a Matlab script was used to control the QSG and automate the experimental procedure. To automate the frequency spanning, we have set a variable t_{ROT} time and a fixed $t_{resting}$ time, equal to 0.5 seconds, sufficient for the algorithm to separate the analysis of each frequency. The choice of a variable t_{ROT} time depends on the algorithm's capability to estimate the rotation accurately. To ensure a valid result and minimize the error between the real and estimated rotation, it is essential for the cell to complete

multiple rotations around its axis. Although an infinite number of rotation periods would yield an average angular velocity nearly coincident with the real value and an error approaching zero, the objective is to analyze as many cells as possible within the shortest time possible. Consequently, we have identified four completed cell rotations as the optimal number. Moreover, the rotation of the cell is contingent upon the applied frequency, necessitating a t_{ROT} time dependent on the signal frequency. The higher the angular velocity of the cell, the shorter the acquisition time, and vice versa. Therefore, for each experiment, the variable t_{ROT} time spans between 4 to 10 seconds. For all the experiments, the frequency step used is the logarithmic scale, ranging between 30 kHz and 1 MHz . The duration of each experiment aimed at extracting a ROT-spectrum approximates 120 seconds. The measurable number of cells per unit time ($cells/h$) was evaluated as the system throughput. Based on the number of cells analyzed within the measurement time, defined as the sum of the time required to obtain the ROT spectra ($\sim 120\ s$) and the time required to replace and introduce cells ($\sim 60\ s$), the system achieves a throughput of up to $\sim 200\ cells/h$. Moreover, due to the compact and easily transportable nature of the system, the entire experimental campaign was conducted within the biology laboratories. This arrangement has provided an optimal environment for monitoring cell lines while also eliminating the need for travel and the associated delays that arise from changing laboratories.

2.3.1 Cell cultures and preparation

Three different cell lines were considered for the performance evaluation of the platform: Normal Colorectal cells (CCD-841), human colorectal adenocarcinoma cells (CaCo-2), and human myeloma cells (OPM2). Normal Colorectal cells (CCD-841 CoN CRL-1790, American Type Culture Collection, Manassas, VA, USA) and Human colorectal adenocarcinoma cells (CaCo-2 HTB-37TM, American Type Culture Collection, Manassas, VA, USA) were grown respectively in Dulbecco's Modified Eagle Medium (DMEM) and in Modified Eagle Medium (MEM) both with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-Alanyl-L-Glutamine, penicillin-streptomycin (50 units - 50 μg for mL) and incubated at $37^\circ C$ in a humidified atmosphere of 5% CO_2 , 95% air. Human myeloma cells (OPM2, from DSMZ) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin at $37^\circ C$ and 5% CO_2 . Cells were seeded at a concentration of 0.5×10^6 cells per mL and split every 48 hours. For the experiment, cells were collected in a centrifuge tube and centrifuged at 1500 rpm for 5 minutes. After discarding the supernatant medium, the cells were washed with the buffer, already reported in [127], after which the cells were incubated for about 5 minutes with the same buffer, which has a conductivity of 33 mS/m . Then this buffer was removed, the cells underwent trypsinization, and trypsin was inactivated with the buffer containing 10% FBS, the cells were centrifuged at 1300 rpm for 5 minutes and were counted. Finally, the cells were resuspended in the same buffer to obtain a working concentration of 500 $cells/\mu L$.

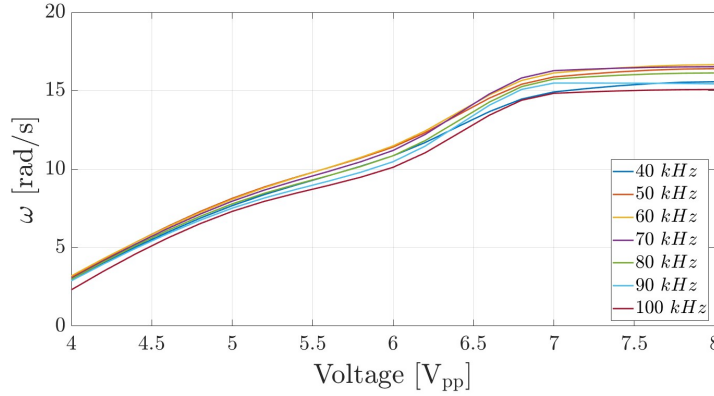


FIGURE 2.14: Amplitude modulated rotation spectrum for different frequencies of the applied electric field.

2.4 Experimental Validation

Before starting cell characterization, the optimal operating conditions were investigated. In particular, a series of ROT-spectra was performed on the same cell at different amplitudes of the applied signal. From the theory, see Sec.2.1, it was expected that as the intensity of the applied electric field increases, the angular velocity of the cell also increases. This is clearly illustrated in Fig.2.15A, where a single CCD-841 cell with an amplitude signal range from $4V_{pp}$ to $8V_{pp}$ was investigated. The relationship between the angular velocity of the cell and the electric field intensity follows as expected quadratic behaviors (see Eq.2.2). Indeed, to accurately extract the angular velocity through the algorithm and minimize the duration of the experiment, it is essential to maximize the intensity of the electric field. However, the cell rotates within a fluid of specified viscosity, which limits the maximum angular velocity it can achieve, see Fig.2.14. Furthermore, excessive electric field intensity may induce electrolysis. Consequently, the optimal working condition has been established with an amplitude voltage of $6V_{pp}$.

Initially, the ROT-Platform validation was performed by computing the ROT-spectra intra-cell line. In particular, in one of the CaCo-2 experiments, it was possible to extract four CaCo-2 cells, which are in the observation field simultaneously. Fig.2.15B shows their spectra. As can be seen, all the spectra share, as expected, the same shape and the same frequency of maximum rotation, which, for the CaCo-2, corresponds to around $40 - 55 \text{ kHz}$. Although the frequency of maximum rotation and the shape of the ROT-spectrum are consistent across cells, the rotational velocities are different from each other. The variability in angular velocity observed among cells primarily stems from slight spatial non-uniformities in the electric field and, more notably, from hydrodynamic interactions resulting from the cells' proximity to the wall substrate. Even without direct adhesion, close positioning to the surface increases the effective viscosity experienced by the cell, thereby reducing its rotational velocity. This near-wall effect has been previously studied and incorporated into ROT models, particularly in the context of metallic particles [128]. Additional minor factors may also contribute and will be explored in future work. Despite minor biological variability, the ROT spectra consistently exhibit the

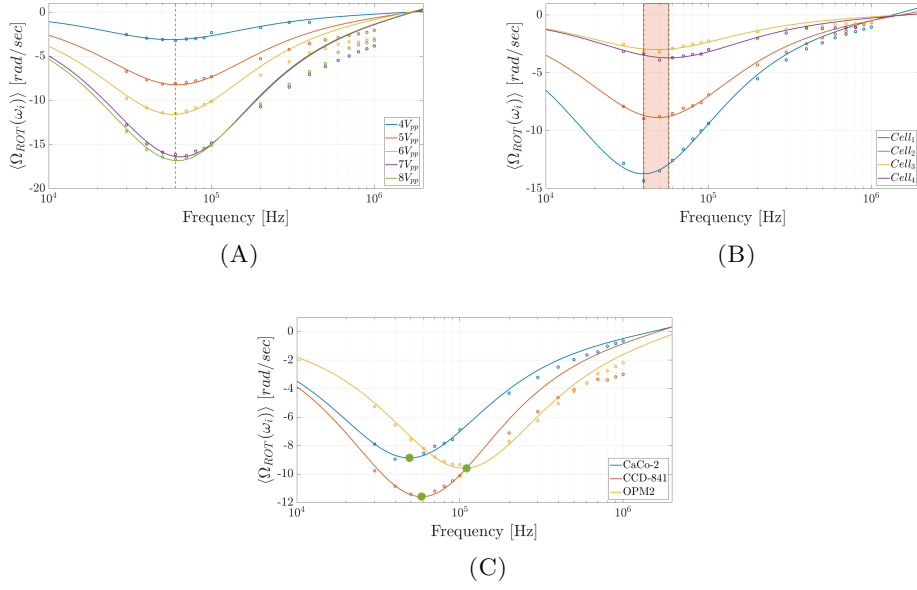


FIGURE 2.15: ROT-spectra ($\langle \Omega_{ROT}(\omega_i) \rangle$) of the experimental campaign. The cell angular velocity is plotted versus the frequency of the rotating electric field ($f_i = \omega_i/2\pi$). In particular: (a) ROT-spectra of CCD-841 cells at different voltage operating conditions, (b) ROT-spectra of four different CaCo-2 cells at 6 V_{pp} extracted during the same experiment, and (c) ROT-spectra of the three different cell lines considered in this study. One cell per cell line is reported. The data points were gathered from experiments and represent the measured ROT-spectra obtained through the *PxI* Algorithm. The solid lines show the fitting curve of data points with the Single-Shell Model.

same characteristic shape and peak frequency range, confirming the robustness of the dielectric property extraction.

In Fig.2.15C, the ROT spectra of three cells related to the considered cell lines are reported. Unlike the previous case, Fig.2.15B, where the maximum rotation coincides at the same frequency, in this case, the maximum rotation is different for each cell line, indicating, as expected, different dielectric characteristics. Specifically, CaCo-2 cells exhibit a maximum rotation point of around 40 – 55 kHz , while CCD-841 and OPM2 cells show maximum rotation points at frequencies around 60 kHz and 100 kHz , respectively. These findings confirm the distinct nature of the three cell lines and highlight their varying dielectric membrane characteristics.

2.4.1 Specific Membrane Capacitance

To estimate the dielectric properties of the cells, the Single-Shell Model (see Eq.2.4, combined with Eqs.2.2, 2.3, and 2.5) was employed, as it represents a well-established and widely validated model that enables reliable extraction of cell dielectric properties. A least-squares fitting procedure was applied to minimize the discrepancy between the experimentally measured spectra and the theoretical predictions. As reported in Sec.2.1, the dielectric characteristics of a biological cell is described by four parameters: membrane permittivity (ϵ_{mem}), membrane conductivity (σ_{mem}), cytoplasm permittivity (ϵ_{cyto}), and cytoplasm conductivity (σ_{cyto}). In the considered frequency range, 30 kHz – 1 MHz , the ROT phenomenon is dominated by the cell

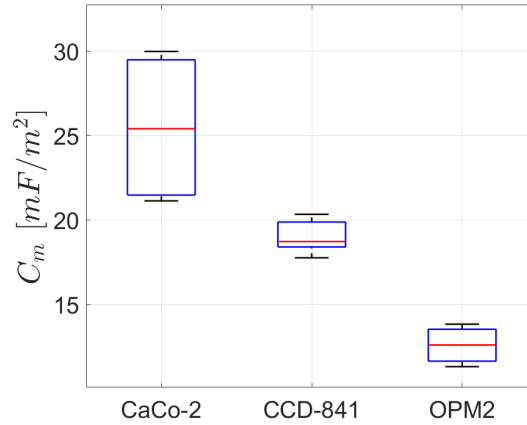


FIGURE 2.16: Membrane capacitance C_m of CaCo-2, CCD-841, and OPM2 cells estimated by fitting 30 ROT-spectra using the Single-Shell Model.

membrane permittivity ε_{mem} so that the cytoplasm permittivity (ε_{cyto}), the cytoplasm conductivity (σ_{cyto}) and the membrane conductivity (σ_{mem}) cannot be reliably extracted through fitting procedures with the ROT-spectrum [109], and typical fixed values of 80, 0.3 S/m and 10^{-6} S/m were, respectively assumed—typical values for physiological conditions.

The solid curves in Fig.2.15 show the results obtained through least-squares fitting with the Single-Shell model. The experimentally measured spectra show a strong agreement with the corresponding theoretical predictions. For each cell type analyzed in this study (CaCo-2, CCD-841, and OPM2), the relative permittivity of the membrane ε_{mem} was estimated. Finally, by considering a membrane thickness of $d = 8$ nm—typical values for physiological conditions—the area-specific membrane capacitance C_m for each cell line was subsequently calculated using Eq.2.6. Fig.2.16 shows the resulting values of membrane capacitance C_m estimated by the best fit over approximately ~ 10 experimental spectra per cell type.

2.4.2 Statistical Analysis

The comparative analysis of the three investigated cell types—CaCo-2, CCD-841, and OPM2—revealed distinct distributions of membrane capacitance values, reflecting differences in membrane morphologies and structural organization. An analysis of variance (ANOVA) was carried out to analyze the statistical significance of the obtained membrane capacitance C_m values, and are reported in Table 2.2. The performed analysis shows that differences in membrane capacitance between the three investigated cell lines were statistically significant (p -value < 0.001). Moreover, the analysis of variance shows that the between-groups variability ($SS = 332.14$) is much larger than the within-groups residual variability ($SS = 72.82$), confirming that the variance in C_m can be attributed to the cell type. In fact, post-hoc Tukey's multiple comparison test indicated that all pairwise comparisons were significant: CaCo-2 vs CCD-841 (p -value = 0.013), CaCo-2 vs OPM2 (p -value < 0.001), CCD-841 vs OPM2 (p -value = 0.013).

Among the colon-derived cell lines, CaCo-2, a colorectal adenocarcinoma line, exhibited the highest membrane capacitance ($C_m = 25.47 \pm 4.6$ mF/m²),

Source	Sum of Squares (SS)	df	Mean Square	F-value	p-value
Groups	332.14	2	166.07	22.80	0.000188
Error	72.82	27	7.28		
Total	404.96	29			

TABLE 2.2: ANOVA performed on membrane capacitance (C_m) values across the three considered cell lines: CaCo-2, CCD-841, and OPM2.

reflecting a complex and irregular membrane surface. In contrast, the CCD-841 line, derived from normal human colon epithelium, presented a significantly lower capacitance ($C_m = 19.02 \pm 1 \text{ mF/m}^2$), consistent with the relatively smoother and more organized membrane structure of healthy epithelial cells. This trend aligns well with findings in the literature, which report that cancer cells tend to exhibit increased membrane capacitance compared to their non-tumorigenic counterparts, due to enhanced microvilli density and membrane roughness [54, 65]. The OPM2 line, representing a hematopoietic cancer model (multiple myeloma), showed the lowest capacitance overall ($C_m = 12.58 \pm 1.1 \text{ mF/m}^2$), likely reflecting the intrinsically different morphology and membrane composition of non-adherent, spherical blood-derived cells compared to epithelial cells. This observation is consistent with findings reported in [129], where subpopulations of peripheral blood cells exhibited significantly higher crossover frequencies—corresponding to higher peak ROT rate—compared to all NCI-60 cell lines grown under adherent culture conditions. These results suggest that blood-derived cells tend to have lower membrane capacitance values, likely influenced by their smoother, more spherical morphology, which implies reduced membrane surface complexity or roughness.

Chapter 3

ROT-Platform Validation in Multiple Myeloma Cells

This chapter explored the dielectric properties of three Multiple Myeloma (MM) cell lines with distinct metabolic profiles and varying sensitivities to proteasome inhibitors. Using the ROT-Platform described before, we demonstrated the capability of ROT to differentiate MM cells with unique metabolic features and resistance mechanisms. The findings underscore the potential of electrokinetic approaches, such as ROT, to advance our understanding of MM heterogeneity and enhance MRD assessment. By providing new insights into cell behavior and dielectric response, these techniques open promising avenues for improving diagnostic precision and guiding therapeutic decision-making in MM. The related work, its validation, and the experimental results have been detailed in a recent submission [130] (accepted with revision).

3.1 Introduction to Multiple Myeloma

Multiple myeloma (MM) is an incurable hematological malignancy characterized by the clonal expansion of tumor plasma cells that produce non-functional monoclonal antibodies. The disease is marked by significant genetic and clinical heterogeneity, which complicates treatment strategies and leads to highly variable therapeutic outcomes. This is particularly evident in relapsed or refractory MM, where the efficacy of successive therapies diminishes due to the development of resistance and persistence mechanisms. A key challenge in managing MM is the accurate evaluation of minimal residual disease (MRD), a critical prognostic factor that has gained even greater importance with recent advancements in immunotherapy. However, current MRD detection methods face significant limitations, highlighting the need for innovative, label-free techniques capable of overcoming these challenges. In this context, the electrical properties of malignant cells, such as membrane capacitance and electrical impedance, have emerged as promising biomarker-independent

indicators for cell characterization. These properties form the basis of physical separation methods that exploit differences in cellular density, size, and dielectric characteristics.

3.1.1 Clinical Features and Therapeutic Challenges

Multiple myeloma (MM) is an incurable hematologic malignancy that accounts for approximately 1% of all cancers and about 10% of blood malignancies [131]. It is characterized by the clonal expansion of terminally differentiated plasma cells that produce non-functional monoclonal antibodies (M proteins), which accumulate in the serum and urine of most patients. Hallmark features include elevated clonal plasma cell count in the bone marrow and the presence of CRAB criteria (hypercalcemia, renal insufficiency, anemia, and osteolytic bone destruction) [132, 133]. MM exhibits substantial heterogeneity at both genetic and clinical levels, with its molecular profile undergoing notable changes during the transition from precursor conditions, such as monoclonal gammopathy of undetermined significance (MGUS) or smoldering multiple myeloma (SMM), to symptomatic disease [134–138]. This complexity drives the biological intricacy of the disease, characterized by clonal diversity and a wide range of clinical outcomes, which frequently result in varied therapeutic responses among patients [136, 137]. Despite significant advances in therapy, including the introduction of immunomodulators, proteasome inhibitors, monoclonal antibodies, CAR-T cells, and bispecific antibodies, MM remains challenging to treat, particularly in relapsed or refractory cases [131, 139–141]. Therapy resistance and disease persistence have been linked to numerous cellular alterations in tumor PCs, including metabolic and morphological changes, stress adaptation, and modifications in membrane antigens and transporters [142–147]. These changes also affect the electrical properties of malignant cells, including membrane capacitance and electrical impedance, which have been shown to differ between malignant cells and normal counterparts (e.g., leukemic cells vs. T lymphocytes and erythrocytes) [148–150].

3.1.2 Minimal Residual Disease

Minimal residual disease (MRD) represents one of the most powerful prognostic markers in MM, strongly correlating with progression-free and overall survival [151, 152]. Current methods for MRD assessment include multiparametric flow cytometry (MFC) [153], allele-specific oligonucleotide PCR (ASO-PCR) [154], and next-generation sequencing (NGS) [155], all capable of detecting one malignant cell among 10^4 – 10^6 normal cells. However, despite their sensitivity, these approaches are costly, time-consuming, and require specialized facilities and expertise. Moreover, MRD assays often rely on prior knowledge of patient-specific genetic markers or antibody panels, which limits their applicability and introduces variability due to clonal evolution and antigen loss during disease progression [156]. These challenges underscore the need for complementary, label-free analytical approaches capable of detecting phenotypic or biophysical differences in residual malignant cells without requiring specific molecular targets.

3.1.3 Electrical Properties as Label-Free Biomarkers

In this context, the dielectric properties of tumor plasma cells have emerged as promising, non-invasive indicators of cellular phenotype and physiological state [54]. Parameters such as membrane capacitance (C_m) and cytoplasmic conductivity (σ_{cyto}) are highly sensitive to variations in membrane composition, surface charge, and internal organization, which often reflect metabolic activity, differentiation state, or drug resistance mechanisms [111]. These characteristics make electrical measurements an appealing tool for label-free discrimination of malignant cells, offering insights into disease heterogeneity and treatment response. Furthermore, when combined with microfluidic and electrokinetic platforms—such as dielectrophoresis (DEP) and electrorotation (ROT)—these measurements can be obtained with high precision, minimal sample preparation, and no need for biochemical labeling. The integration of such physical characterization methods into MM research could thus provide a novel framework for studying tumor biology and for refining MRD evaluation beyond current molecular boundaries.

3.2 Materials and Method

3.2.1 Cell cultures

Experiments were performed using three immortalized HMCLs: NCI-H929, OPM2, and U266. Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, 88365) supplemented with 10% or 20% heat-inactivated fetal bovine serum (Gibco™, 10091148), 1% penicillin/streptomycin (Gibco™, 15140122), and 2 mM L-Glutamine (Gibco™, 25030081). Cells were cultured under standard growth conditions in an incubator at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Bortezomib (15 nM) from Sigma-Aldrich (St. Louis, MO) was used to treat cells for 24 hours.

3.2.2 Sample preparation for Metabolites Analysis

For metabolic analysis, 3×10^6 cells were harvested and immediately frozen in liquid nitrogen. The cells were then deproteinized using ice-cold HPLC-grade acetonitrile (CH₃CN) mixed with 10 mM KH₂PO₄ (pH 7.40) in a 3:1 (v/v) ratio, and vigorously agitated for 60 seconds. After deproteinization, the sample was centrifuged at 12400 rpm for 10 minutes at 4°C. The resulting clear supernatant was then combined with HPLC-grade chloroform, mixed vigorously, and centrifuged again at maximum speed for 5 minutes. The upper aqueous phase was collected for HPLC analysis.

This analysis included the simultaneous separation and quantification of high-energy phosphates, oxidized and reduced nicotinamide coenzymes, glycosylated UDP-derivatives, nitrite and nitrate, as well as purines and pyrimidines. Amino acids and amino group-containing compounds were separated and quantified using chromatographic conditions established in our laboratory. Both analyses were carried out with an HPLC system consisting of a Spectra System P4000 pump, a Hypersil C-18 column (250 × 4.6 mm, 5 μm particle size, with a guard column), and a UV6000 LP diode array detector with a 5 cm light path flow cell (Thermo Fisher Scientific, Rodano, Milano, Italy).

3.2.3 RNA extraction and gene expression Analysis

The mRNA expression levels were measured by quantitative real-time PCR (RT-qPCR). Total RNA was extracted from cells using Trizol[®] reagent according to the manufacturer's instructions (Invitrogen). The extracted RNA was then quantified, and its quality and purity were assessed using a NanoDrop spectrophotometer (ThermoScientific) before proceeding with cDNA synthesis (Thermo Fisher Scientific, Milan, Italy) as per the manufacturer's protocol. RT-qPCR was performed on the StepOne Fast Real-Time PCR System with SYBR Green PCR Master Mix (Life Technologies), and thermocycling reactions were completed using the 7900HT Fast Real-Time PCR System (Thermo Fisher). The expression levels of the target genes are the following

- *hspa5* - Fw: TAGCGTATGGTGCTGCTGTC; Rv: TGACACCTCCCACAGTTTCA;
- *atf4* - Fw: GCTAAGCGGGCTCCTCCGA; Rv: ACCCAACAGGGCATCCAAGTCG;
- *hmox1* - Fw: ACTGCGTTCCTGCTCAACAT; Rv: GGGGCAGAATCTTGCACTTT;
- *tlr4* - Fw: AAGCCGAAAGGTGATTGTTG; Rv: CTGAGCAGGGTCTTCTCCAC;
- *p62* - Fw: GGGGCGCCCTGAGGAACAGA; Rv: CCTGGTGAGCCAGCCGCCTT;
- *mnf1* - Fw: GGCATCTGTGGCCGAGTT; Rv: ATTATGCTAAGTCTCCGCTCCAA;
- *mnf2* - Fw: GCTCGGAGGCACATGAAAGT; Rv: ATCACGGTGCTCTTCCCAT;
- *opa1* - Fw: GTGCTGCCCGCCTAGAAA; Rv: TGACAGGCACCCGTACTCAGT;
- *drp1* - Fw: TGGGCGCCGACATCA; Rv: GCTCTGCGTTCCCACTACGA;
- *fis1* - Fw: TACGTCCGCGGGTTGCT; Rv: CCAGTTCCTTGGCCTGGTT;

were detected by SYBR Green fluorescence. For each sample, the expression levels of the target mRNAs were normalized to the housekeeping gene b2m (Fw: AGCAGCATCATGGAGGTTTG; Rv: AGCCCTCCTAGAGCTACCTG) using the $2^{-\Delta\Delta C_t}$ method.

Finally, mitochondrial mass was detected using the MitoTracker RED CMXRos probe (Thermo Fisher Scientific, Milan, Italy), according to the manufacturer's instructions. Cells were stained using 200 nM of the MitoTracker RED CMXRos for 30 minutes at 37°C. After incubation, cells were washed three times and analysed by using the flow cytometry MACSQuant Analyzer 10 (Miltenyi Biotec).

3.2.4 ROT Setup and Cell Viability Assessment

To perform electrorotation experiments, HMCLs were resuspended at a concentration of 500 cells/ μ L in ROT buffer (9.5% sucrose, 0.1 mg/mL dextrose, 0.1% BSA, 0.1 mM calcium acetate $[(CH_3COO)_2Ca]$), which was filtered using a Millex Millipore syringe filter with 0.22 μ m pores (Biosigma S.p.A., Cona, Italy). The conductivity was adjusted to a final value of 33 mS/m using a 0.1 mM potassium chloride solution, measured with an Electric Conductometer DDS Digital Lab (DDS-307, Changzhou W&J Instrument Co., Ltd., Changzhou, Jiangsu, China).

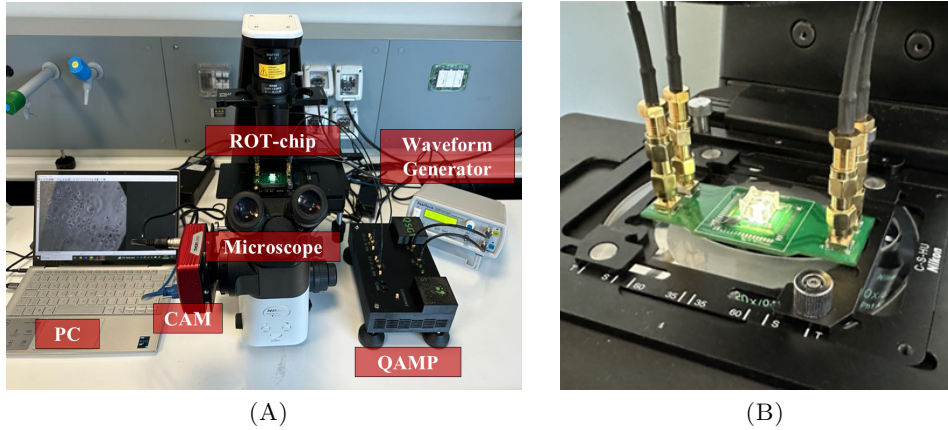


FIGURE 3.1: Quadrature Signal Generator electronic system (QSG) (A) picture and (B) functional block diagram.

A figure of the ROT-Platform employed during the experimental campaign is shown in Fig.3.1A¹. The ROT-chip, see Fig.3.1B was positioned on a phase-contrast inverted microscope equipped with a $\times 20$ objective. Video acquisition was performed using a CCD camera (340M Fast Frame, Thorlabs) with a native resolution of 640×480 pixels and a square pixel size of $7.4 \mu m$. The camera, interfaced to a PC via USB connection, was operated through its proprietary software (ThorCamTM v.3.7.0) at a frame rate of 60 FPS. Due to the current bandwidth limitations of the Quadrature AMplification (QAMP) unit, the electrorotation spectrum used in this work spans from 30 kHz to 1 MHz. This frequency interval is sufficient to capture the membrane-dominated dispersion region, enabling the estimation of the dielectric properties of the cellular membrane. The experimental setup and acquisition protocol strictly follow the configuration described in Sec.2.2, ensuring full consistency with previous work and enabling complete automation of the electrorotation measurement campaign.

The Cells were incubated with complete medium (Control) and electro-rotation buffer (9.5% Sucrose, 0.1 mg/mL Dextrose, 0.1% BSA, 0.1 mM $(CH_3COO)_2Ca$) [157] at room temperature for 1 and 3 hours. Then, cell viability was assessed using the Annexin V/7-AAD assay kit (BioLegend, Belgium) according to the manufacturer's instructions. Cells were washed with PBS and resuspended in 100 μL of cold binding buffer. Then, 5 μL of Annexin V-FITC and 5 μL of 7-AAD were added to the cell suspension and mixed gently. Cells were incubated for 15 minutes at room temperature in the dark. Finally, 400 μL of binding buffer was added to stop the staining, and the percentage of viable cells was determined by flow cytometry (MACSQuant Analyzer 10 (Miltenyi Biotech) and analyzed using FlowLogic software (Miltenyi Biotech).

Characterization using the ROT technique involves suspending cells in a high-conductivity buffer that allows rotation when an electric field is applied

¹It is worth noting that the electronic system shown in Fig.3.1A corresponds to an earlier, pre-QSG configuration of the system described in Sec.2.2.1. At this stage, the signal-generation module and the quadrature and power stage (QAMP) were not yet integrated into the main architecture. These subsystems were instead implemented as external units and interfaced with the platform during the experimental campaign. Their full integration was carried out only in the subsequent development phase that led to the final QSG design.

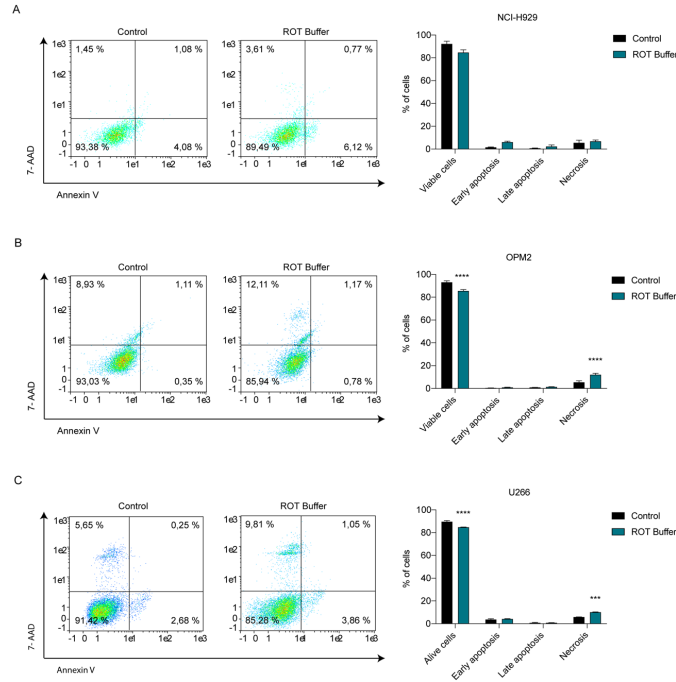


FIGURE 3.2: ROT buffer slightly affects cell viability in HMCLs after 1 hour of incubation. (A-C) Representative dot plots (left) and quantification (right) of cell viability after 1 hour of incubation with ROT buffer in NCI-H929, OPM2, and U266 cell lines, respectively. Data are shown as mean $\pm$ SEM, not significant (ns), * p -value < 0.05, ** p -value < 0.01, *** p -value < 0.0001

at room temperature. To assess the effect of the ROT buffer on cell viability, three immortalized myeloma cell lines—NCI-H929, OPM2, and U266—were incubated at room temperature for 1 and 3 hours. Cell viability was not affected in NCI-H929 cells resuspended in the ROT buffer compared to the control after 1 hour of incubation (Fig.3.2(A)). However, a slight reduction in viability was observed in OPM2 and U266 cells after 1 hour of incubation with the buffer compared to the control (mean reduction of 7.55 ± 0.12 SEM for the OPM2 cell line and 4.86 ± 0.79 SEM for the U266 cell line; Fig.3.2(B-C)). We observed similar results in NCI-H929 and OPM2 cells incubated for 3 hours with the ROT buffer, while the U266 cell line showed a significant reduction in cell viability compared to the control after 3 hours of incubation (mean reduction of 16.41 ± 4.60 SEM; Fig.3.3(C)). These results suggest that the ROT buffer can be used for electrorotation experiments for up to three hours.

3.3 Experimental Results

For statistical analyses, a two-tailed unpaired Student's t-test was used for the comparison of two groups ($n = 2$). For comparisons involving three or more groups ($n \geq 3$), a one-way analysis of variance (ANOVA) or repeated-measures ANOVA with Holm-Sidak post-hoc test for multiple comparisons was used where appropriate. Principal component analysis (PCA) was performed on metabolite data. Explained variances and the quality of variable representation on the factor map, expressed as squared cosines (Cos^2), are shown. PCA results are displayed as a biplot, with variables represented by

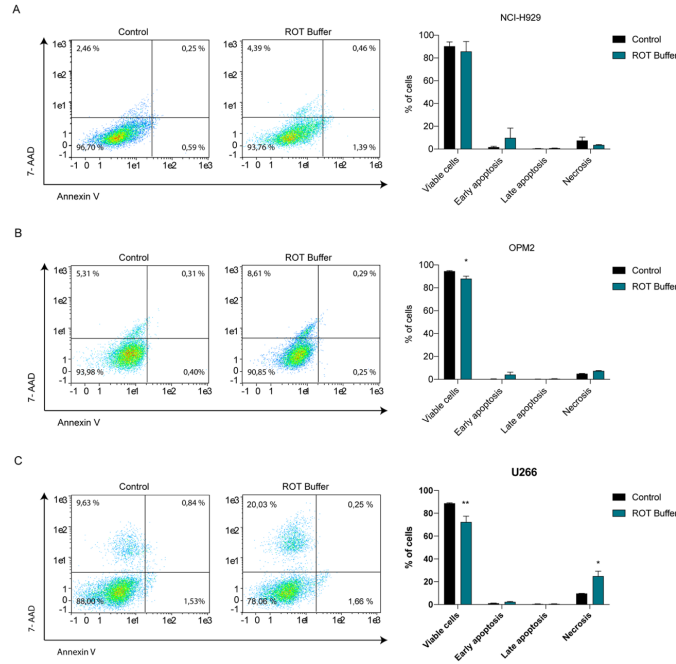


FIGURE 3.3: ROT buffer slightly affects cell viability in HMCLs after 3 hour of incubation. (A-C) Representative dot plots (left) and quantification (right) of cell viability after 3 hours of incubation with ROT buffer in NCI-H929, OPM2, and U266 cell lines, respectively. Data are shown as mean $\pm$ SEM, not significant (ns), * p - value < 0.05, ** p - value < 0.01, *** p - value < 0.0001

color-coded arrows. Analyses were conducted using RStudio software (Version 1.0.153 for Mac) with the *corrplot*, *FactoMineR*, and *Factoextra* packages for computing and visualizing PCA diagrams. Data are presented as mean $\pm$ standard deviation (SD) unless otherwise stated. Data analysis was performed using GraphPad Prism software version 5.00 or RStudio version 1.0.153. A p - value of < 0.05 was considered statistically significant, and symbols used to indicate statistical differences are described in the figure legends.

3.3.1 Metabolism and Stress-Response Pathways

The in vitro experiments were conducted on three different HMCLs: NCI-H929, OPM2, and U266. To assess their metabolic characteristics, individual metabolites were analyzed using HPLC followed by PCA. This analysis revealed that PC1 plays a crucial role in distinguishing OPM2 cells from both NCI-H929 and U266 cells (Fig.3.4(B)). Notably, the most significant contributors to PC1 were ADP, ATP, GTP, NAD⁺, NADH, and the NAD⁺/NADH ratio, highlighting the impact of energy metabolism on the distinctive metabolic profiles of these cells (Fig.3.4(A)). Additionally, the levels of energetic nucleotides (GMP, ADP, GDP, ATP, CTP, GTP, and UTP) were higher, and the oxidized forms of NAD and NADPH, compared to their reduced counterparts, were elevated in OPM2 cells relative to NCI-H929 and U266 cells. Furthermore, the higher mitochondrial redox potential in OPM2 cells, compared to the other two cell lines, confirmed an enhancement in energy production (Fig.3.4(C)). These findings were further validated

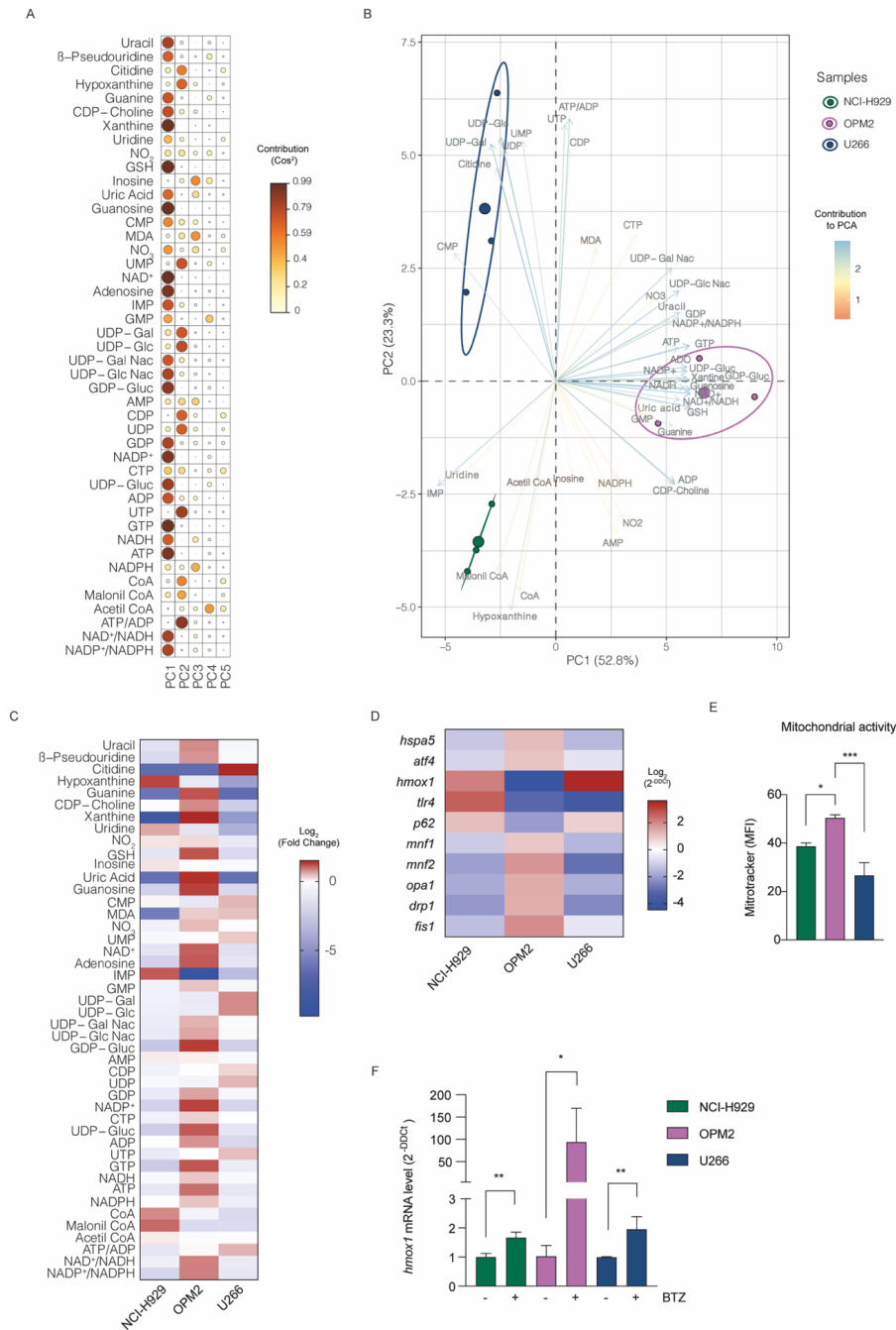


FIGURE 3.4: OPM2 cells differ from NCI-H929 and U266 cell lines in metabolism and antioxidant pathways. (A) Factor map representing the quality of variables on PC1-PC5, expressed as square cosine (Cos2). (B) PCA biplot of 46 metabolites for OPM2, NCI-H929, and U266 cell lines; key-colored arrows represent the contribution of each variable to the PCA; small circles represent single subjects and large circles represent the mean point of the confidence ellipses. (C) Heat maps of 46 metabolites in the OPM2, NCI-H929, and U266 cell lines showing the abundance of 46 metabolites. Data are expressed as $\text{Log}_2(\text{Fold Change})$. (D) Heat maps showing mRNA expression of genes *HSPA5*, *ATF4*, *HMOX1*, *TLR4*, *P62*, *MNF1*, *MNF2*, *OPA1*, *DRP1* and *FIS1* in the OPM2, NCI-H929 and U266 cell lines. Data are expressed as $\text{Log}_2(2^{-\Delta\Delta C_t})$. (E) Quantification of Mitotracker fluorescence (MFI) in OPM2, NCI-H929, and U266 cell lines. (F) mRNA expression of *HMOX1* genes after treatment with btz. Data are expressed as $2^{-\Delta\Delta C_t}$. Data are shown as mean $\pm$ SEM, * p -value < 0.05 , *** p -value < 0.001

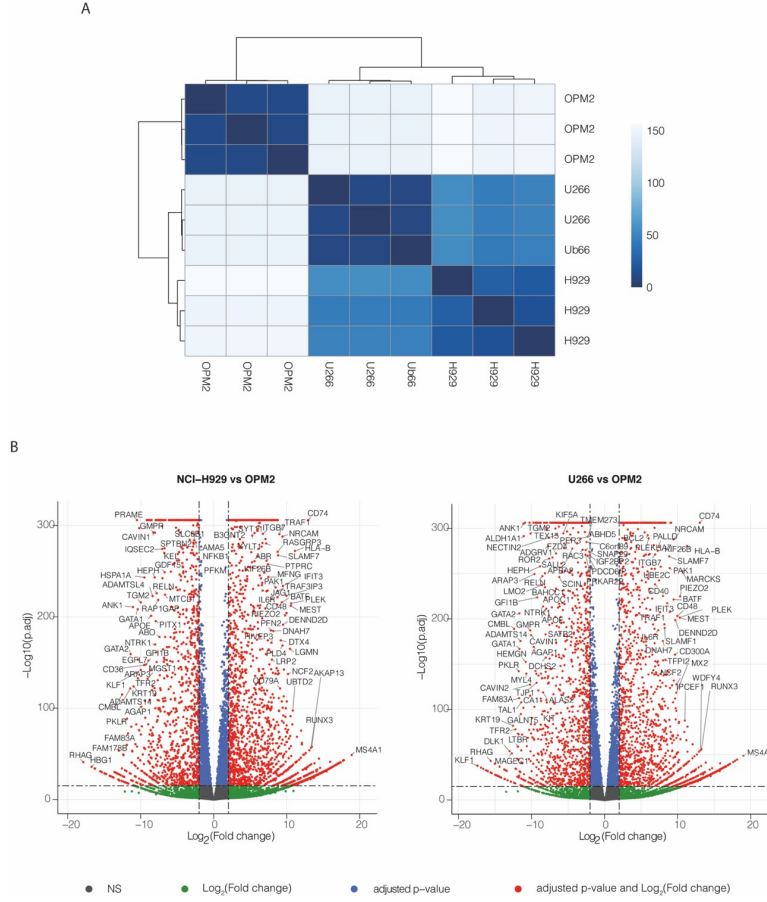


FIGURE 3.5: Transcriptomic analysis of HMCLs. (A) Correlation heat map analysis between samples. (B) Volcano plot showing the DEGs in NCI-H929 and U266 cell lines.

by gene expression analysis using RT-PCR, which showed elevated expression of genes involved in mitochondrial homeostasis, particularly those regulating mitochondrial fusion (MNF1 and MNF2), fission (DRP1 and FIS1), and biogenesis (OPA1) (Fig.3.4(D)). These results were corroborated by the higher Mitotracker MFI observed in OPM2 cells compared to the other two cell lines (Fig.3.4(E)). Additionally, we observed differences in the expression of genes related to autophagy (P62) and stress response (HSPA5, ATF4, HMOX1), which further distinguish OPM2 cells from NCI-H929 and U266 cells Fig.3.4(D)). Finally, we assessed *hmx1* mRNA levels after treatment with BTZ. BTZ significantly increased *hmx1* expression in all three cell lines, with the highest expression observed in OPM2 compared to the other two cell lines (Fig.3.4(F)). Overall, these results demonstrate that the OPM2 cell line differs significantly from NCIH929 and U266, particularly in mitochondrial function, cellular stress response pathways, and sensitivity to BTZ treatment.

3.3.2 Transcriptomic Profiles Analysis

To further investigate the dielectric properties of HMCLs, we analysed their transcriptomic profiles. Differentially expressed genes (DEGs) were identified using the *DESeq2* package in R. The analysis followed four main steps: construction of a gene expression matrix, creation of an experimental design

matrix, generation of a contrast matrix, and production of results. In this study, DEGs were defined as those with an adjusted p - value < 0.05 and an absolute $|\text{Log}_2(\text{Fold Change})| > 1$. The identified DEGs were then used to perform over-representation analysis for gene ontology (GO) terms. A q - value < 0.05 was considered the threshold for significance.

ONTOLOGY	GO Terms	GeneRatio	p-value	q-value	-Log ₁₀ (q-value)
BP	Lipid localization (GO:0010876)	225/6770	2.24E-05	1.89E-04	3.723
BP	Response to nutrient (GO:0007584)	82/6770	2.48E-05	2.05E-04	3.687
BP	Inorganic cation import across plasma membrane (GO:0098659)	72/6770	5.57E-05	4.08E-04	3.389
BP	Inorganic ion import across plasma membrane (GO:0099587)	72/6770	5.57E-05	4.08E-04	3.389
BP	Receptor clustering (GO:0043113)	32/6770	1.14E-04	7.34E-04	3.134
BP	Cell-cell junction organization (GO:0045216)	104/6770	1.54E-04	9.42E-04	3.026
BP	Positive regulation of cytoskeleton organization (GO:0051495)	93/6770	2.43E-04	1.37E-03	2.864
BP	Ruffle organization (GO:0031529)	32/6770	7.96E-04	3.48E-03	2.459
BP	Positive regulation of amine transport (GO:0051954)	26/6770	1.11E-03	4.63E-03	2.335
BP	Plasma membrane invagination (GO:0051954)	33/6770	1.13E-03	4.72E-03	2.326
BP	Potassium ion transport (GO:0051954)	111/6770	1.19E-03	4.91E-03	2.309
BP	Regulation of transporter activity (GO:0032409)	143/6770	2.24E-03	8.23E-03	2.085
BP	Membrane raft assembly (GO:0032409)	8/6770	6.32E-03	1.90E-02	1.722
BP	Plasma membrane organization (GO:0007009)	78/6770	6.58E-03	1.97E-02	1.705
CC	Cytoplasmic side of plasma membrane (GO:0009898)	83/7088	2.92E-05	2.42E-04	3.615
CC	Voltage-gated calcium channel complex (GO:0005891)	29/7088	9.31E-03	3.44E-02	1.464
MF	Symporter activity (GO:0015293)	77/7035	1.47E-04	2.39E-03	2.622
MF	MHC protein complex binding (GO:0023023)	25/7035	1.48E-04	2.39E-03	2.622
MF	Solute:sodium symporter activity (GO:0015370)	45/7035	5.74E-04	6.45E-03	2.191

TABLE 3.1: Enriched GO terms in NCI-H929 vs OPM2 cell line.

Correlation analysis revealed distinct clustering among the cell lines, with OPM2 displaying a unique transcriptomic profile compared to the NCI-H929 and U266 cell lines. Conversely, NCI-H929 and U266 cell lines exhibited similar transcriptomic profiles, suggesting that their dielectric properties differ from those of OPM2 (Fig.3.5(A)). To identify genes potentially associated with dielectric properties, we analysed DEGs between NCIH929 and OPM2, as well as between U266 and OPM2. Our results showed 7.648 DEGs in NCI-H929 and 7.099 DEGs in U266, based on an adjusted p - value cutoff of < 0.05 and a $\text{Log}_2(\text{Fold Change}) > |1|$ (Fig.3.5(B)).

DEGs were then analysed using traditional GO enrichment analysis, which includes three categories: biological process (BP), cellular component (CC), and molecular function (MF). Over-representation analysis (ORA) revealed enriched GO terms related to plasma membrane organization (GO:0007009) and structure, such as ruffle organization (GO:0031529) and plasma membrane invagination (GO:009924), as well as transport processes across the plasma membrane. These included terms like inorganic ion import across the plasma membrane (GO:0099587) and voltage-gated calcium channel complex (GO:0005891), which were identified in both cell lines (Fig.3.6(A-B),

ONTOLOGY	GO Terms	GeneRatio	p-value	q-value	-Log ₁₀ (q-value)
BP	Plasma membrane organization (GO:0007009)	81/6280	1.14E-04	7.85E-04	3.105
BP	Lipid transport (GO:0006869)	185/6280	3.84E-04	2.10E-03	2.678
BP	Protein localization to extracellular region (GO:0071692)	159/6280	7.04E-04	3.41E-03	2.467
BP	Peptide transport (GO:0015833)	112/6280	8.46E-04	3.99E-03	2.399
BP	Regulation of transporter activity (GO:0032409)	136/6280	1.11E-03	4.93E-03	2.308
BP	Membrane protein ectodomain proteolysis (GO:0006509)	25/6280	1.39E-03	5.09E-03	2.223
BP	Establishment of protein localization to extracellular region (GO:0035592)	154/6280	1.45E-03	6.22E-03	2.206
BP	Plasma membrane invagination (GO:0099024)	31/6280	1.54E-03	6.53E-03	2.185
BP	Positive regulation of transporter activity (GO:0032411)	58/6280	1.73E-03	7.15E-03	2.146
BP	Regulation of carbohydrate metabolic process (GO:0006109)	79/6280	4.76E-03	1.54E-02	1.811
BP	Regulation of membrane lipid distribution (GO:0097035)	31/6280	5.87E-03	1.84E-02	1.735
BP	Ruffle organization (GO:0031529)	28/6280	6.30E-03	1.96E-02	1.707
BP	Regulation of protein localization to cell surface (GO:2000008)	22/6280	9.69E-03	2.66E-02	1.576
CC	Cytoplasmic side of plasma membrane (GO:0009898)	80/6588	1.13E-05	1.10E-04	3.958
CC	Extrinsic component of membrane (GO:0019898)	132/6588	1.25E-05	1.19E-04	3.923
CC	Voltage-gated potassium channel complex (GO:0008076)	41/6588	4.69E-04	2.87E-03	2.542
MF	Inorganic anion transmembrane transporter activity (GO:0015103)	71/6540	2.21E-04	3.73E-03	2.428
MF	ABC-type transporter activity (GO:0140359)	29/6540	6.38E-04	8.13E-03	2.090
MF	Transmembrane receptor protein phosphatase activity (GO:0019198)	13/6540	7.08E-04	8.58E-03	2.066
MF	Lipid transporter activity (GO:0005319)	79/6540	1.79E-03	1.70E-02	1.770

TABLE 3.2: Enriched GO terms in U266 vs OPM2 cell line.

Tab.3.1 and 3.2). Additionally, we found GO terms significantly enriched for solute symporter activity (GO:0015370), regulation of transporter activity (GO:0032409), and peptide transport (GO:0015833; Fig.3.6(A-B); Tab.3.1 and 3.2). In summary, our results highlight that the tested HMCLs differ in their plasma membrane characteristics, which likely play a key role in determining their dielectric properties.

3.3.3 ROT Analysis

To derive the ROT characteristic spectra and assess the dielectric properties of HMCLs under a rotating electric field, the videos acquired during the ROT experiments were analysed using the Pixel Intensity Algorithm (PxI) described in Sec.2.2.3. The Single-Shell Model [54] was then employed to estimate the dielectric parameters of the cells. A least-squares fitting procedure was applied to minimize the discrepancy between the experimentally measured ROT spectra and the theoretical predictions. Fig.3.7 reports the ROT spectra of three representative cells from the analysed cell lines. The experimentally measured spectra show a strong agreement with the corresponding theoretical predictions.

Specifically, NCI-H929 cells show a maximum rotation frequency of approximately 250 *kHz*, whereas OPM2 and U266 cells exhibit peak rotation at around 100 *kHz* and 150 *kHz*, respectively. These results underscore the distinct dielectric responses of the three cell lines, confirming their differential electrical properties under rotating field conditions. For each

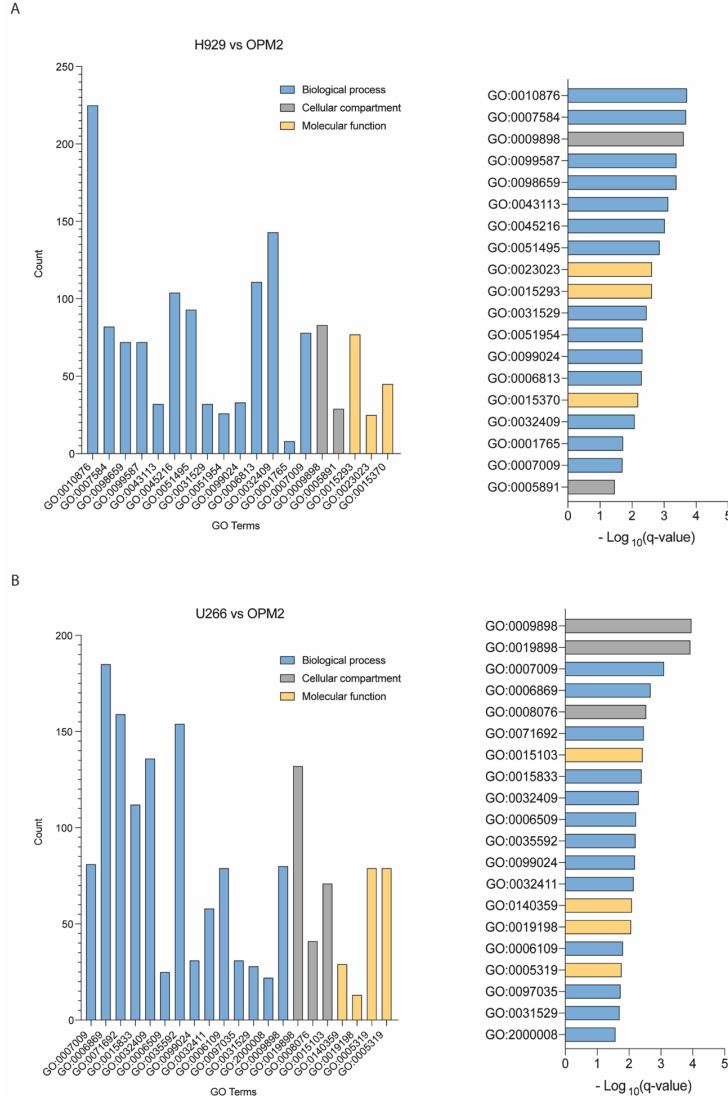


FIGURE 3.6: Significantly enriched Gene Ontology (GO) terms of common DEGs.

analysed cell line, the membrane permittivity (ϵ_{mem}) was extracted from the Single-Shell Model parameters obtained through the fitting of the experimental ROT spectra. Finally, by considering a membrane thickness of $d = 8 \text{ nm}$ (typical values for physiological conditions are reported in the range $5 - 10 \text{ nm}$ [57, 158]), the area-specific membrane capacitance C_m was subsequently calculated using Eq.2.6. Based on the best-fit of the experimental ROT spectra obtained over 30 cells, the corresponding membrane capacitance (C_m) values were derived, as illustrated in Fig.3.8. The comparison across the three analysed cell types —NCI-H929, OPM2, and U266— highlighted distinct distributions of C_m , indicative of differences in membrane morphology and structural organization. A summary of these results is provided in Tab.3.3. In particular, OPM2 exhibited the highest membrane capacitance ($C_m = 8.97 \pm 1.47 \text{ [mF/m}^2\text{]}$), compared to U266 ($C_m = 5.83 \pm 1.14 \text{ [mF/m}^2\text{]}$) and HCI-H929 ($C_m = 4.67 \pm 0.71 \text{ [mF/m}^2\text{]}$) showed markedly lower values.

Statistical analysis confirmed these differences: one-way ANOVA yielded a highly significant effect of the cell line on membrane capacitance ($p\text{-value} = 4.11 \times 10^{-8}$). Moreover, post-hoc multiple comparisons (Tukey–Kramer test)

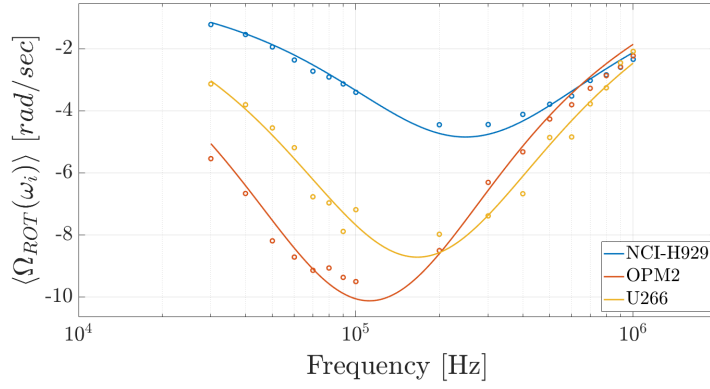


FIGURE 3.7: ROT spectra of the investigated cell lines. The cell angular velocity is plotted versus the frequency of the rotating electric field ($f_i = \omega_i/2\pi$). The data points were gathered from experiments and represent the measured ROT spectra obtained through the PxI Algorithm. The solid lines show the fitting curve of data points with the Single-Shell Model.

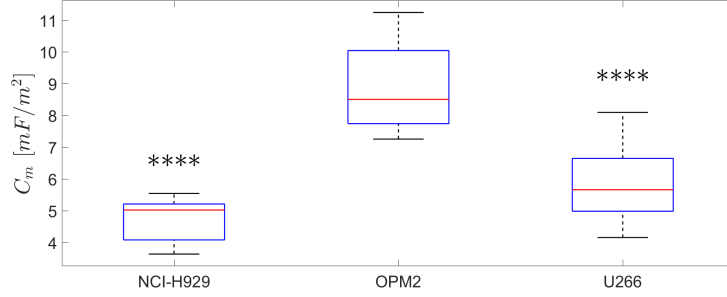


FIGURE 3.8: Estimated Membrane Capacitance C_m of HMCLs using the Single-Shell Model. Data are shown as mean $\pm$ SEM, **** p - value < 0.0001 .

revealed that OPM2 differed significantly from both HCI-H929 (p - value = 0.00000005) and U266 (p - value = 0.00000385), whereas no significant difference was detected between HCI-H929 and U266 (p - value = 0.07332).

Cell type	Sample Number	Radius [μm]	C_m [mF/m^2]
HCI-H929	9	9.90 ± 1.52	4.67 ± 0.71
OPM2	9	9.27 ± 2.33	8.97 ± 1.47
U266	12	10.70 ± 1.05	5.83 ± 1.14

TABLE 3.3: Dielectric properties of HMCLs cells using the ROT method

3.3.4 Dielectric Biomarkers as Transcriptomic Profiles

In this chapter, we employed three HMCLs to investigate, for the first time, the dielectric behavior using the ROT technique. To assess metabolic heterogeneity among HMCLs reflecting patients' variability, individual metabolites were analyzed using HPLC. PCA revealed distinct metabolic profiles among the three cell lines. Specifically, OPM2 cells exhibited the highest levels of energetic nucleotides (e.g., GMP, ADP, GDP, ATP, CTP, GTP, and UTP) and increased mitochondrial dynamics, indicating enhanced mitochondrial activity and energy production compared to NCI-H929 and U266

cells. Elevated levels of oxidized NAD⁺ and NADP⁺, together with upregulation of HSPA5, suggested heightened endoplasmic reticulum (ER) stress, likely associated with increased protein synthesis and glycosylation activity. These findings were supported by higher ATF4 expression and accumulation of glycosylated UDP derivatives, indicating that OPM2 represents a more aggressive, BTZ-resistant phenotype, consistent with previous studies linking BTZ response to mitochondrial function, antioxidant capacity, and glycosylation substrates [143]. BTZ treatment activates the TLR4/HO-1/CO axis, a stress-responsive pathway that enhances cell survival by protecting mitochondria from BTZ-induced damage, and ultimately promoting drug resistance [159]. Specifically, ER stress induced by BTZ directly drives HO-1 upregulation, which plays a critical role in maintaining mitochondrial homeostasis [152, 160]. Although OPM2 cells exhibited lower basal expression of HMOX1 compared to the other two cell lines, BTZ treatment significantly increased HMOX1 expression in all three cell lines, with the highest levels observed in OPM2, confirming their reduced sensitivity to BTZ. These results demonstrate that the three cell lines differ in their metabolism, antioxidant systems, and responses to proteasome inhibitors.

Consistent with these findings, transcriptomic analysis revealed clearly distinct expression signatures among the HMCLs, with OPM2 exhibiting a unique gene expression pattern, whereas NCI-H929 and U266 shared more similar profiles. GO enrichment analysis of DEGs between OPM2 and the other two cell lines identified enriched GO terms related to plasma membrane organization (e.g., ruffle organization and invagination) and transport processes, including inorganic ion import, solute symporter activity, and peptide transport, suggesting that plasma membrane architecture and transport capacity contribute to phenotypic diversity. Plasma membrane features, including ruffle organization and transport channels, are critical determinants of cellular dielectric properties [161]. Variations in plasma membrane composition and structural organization, which influence cellular biophysical characteristics, may reflect underlying metabolic differences that result in distinct dielectric signatures.

In accordance, using the ROT characterization, each cell line showed a distinct ROT spectrum, with a maximum rotation frequency around 250 kHz for NCI-H929 cells, 150 kHz for U266, and 100 kHz for OPM2 cells, allowing clear discrimination. Moreover, calculation of the area-specific C_m further confirmed differences in membrane morphology and structural organization, with NCI-H929 exhibiting the lowest C_m , in line with its smoother and less complex surface morphology compared with the other cell lines.

This study demonstrates that the ROT-Platform can effectively distinguish MM cell lines exhibiting variations in mitochondrial function, stress responses, and plasma membrane organization. The distinct metabolic and transcriptomic profiles associated with unique dielectric properties provide a multidimensional characterization of cellular heterogeneity in MM. This represents the first direct evidence linking dielectric membrane properties to the molecular determinants of drug resistance in MM. Overall, the proposed electrokinetic approach provides a powerful tool for identifying and characterizing subpopulations of myeloma PCs based on their dielectric membrane features.

Chapter 4

Integrated Image Analysis Framework for Automated ROT Analysis

This chapter marks a structural transition, from an isolated, task-specific algorithmic implementation (PxI) to a systematically integrated and fully automated framework governing the entire ROT analytical pipeline. It presents the development and validation of the Electro-Cell Physiometry (ECP) Framework, an integrated software environment designed for the end-to-end automation of electrorotation experiments. By combining advanced image processing, robust rotational velocity estimation, and dielectric model fitting within a unified architecture, the framework enables standardized, reproducible, and quantitatively controlled assessment of cellular dielectric properties. The ECP-Framework has been validated using a strategy focused on assessing the computational performance and reliability of the entire analysis workflow. A comprehensive comparative study was conducted using both simulated datasets—with known ground-truth parameters—and experimental ROT measurements to quantify accuracy, robustness, and consistency across different operating conditions. The new proposed angular velocity estimation module, POLARIS, was benchmarked against existing approaches and our previously developed PxI algorithm, demonstrating superior performance in terms of automation, accuracy, robustness, and noise resilience. Overall, the ECP-Framework establishes a robust and versatile foundation for quantitative electrokinetic analysis, providing a validated and modular toolset for future applications in single-cell dielectric characterization and electro-physiological research. The related work, its validation, and the experimental results have been detailed in a recent manuscript currently awaiting review [162].

4.1 Current Limitations in ROT Analysis

As discussed in Chap.1, over the past decades, electrorotation has been successfully employed to characterize a wide range of biological cells, including red blood cells (RBCs) [86, 163], circulating tumor cells (CTCs) [91, 164], leukocytes [95, 165], demonstrating its potential for label-free dielectric phenotyping at the single-cell level. Despite its proven capability to extract key electrical parameters with high accuracy, ROT has not yet achieved widespread adoption outside specialized research settings. Two main factors have limited its broader technological diffusion. First, the generation of a stable rotating electric field typically requires multi-phase signal generators and precise synchronization hardware, often relying on bulky and expensive benchtop instruments [166], which constrains portability and integration into compact microfluidic systems. Second, the ROT workflow still lacks automation: most experimental procedures, from field generation and cell trapping to image acquisition and angular velocity extraction, are manually performed and analyzed [167]. This high level of user intervention hampers reproducibility, throughput, and accessibility for non-specialist users.

4.1.1 The Critical Role of Angular Velocity Estimation

Within the overall ROT workflow, one of the most critical and least automated stages is the estimation of the cell's angular velocity, which directly influences the accuracy of dielectric parameter extraction. Traditionally, this has been obtained manually by inspecting video sequences of rotating cells frame by frame [68]. Although conceptually simple, this approach is highly time-consuming, operator-dependent, and unsuitable for large-scale or real-time analysis, severely limiting the practical throughput of ROT experiments. To overcome these limitations, several studies have proposed automated or semi-automated algorithms for angular velocity extraction [168, 169]. Most of these methods rely on image correlation [170–172], or feature-based rotation detection [173, 174], typically applied to two-dimensional video sequences of single cells. While they have demonstrated the feasibility of algorithmic analysis, their performance often lacks robustness against noise, cell shape irregularities, or variations in angular velocity, which restricts their general applicability in practical settings. These constraints highlight the need for more reliable, adaptable, and fully integrated computational approaches to ROT analysis.

4.1.2 A Comprehensive Solution for Automated ROT Analysis

Within this context, in Chap.2 we introduced the ROT-Platform, an initial platform toward the automation of the ROT characterization process. That study proposed a first-generation algorithm for extracting the angular velocity of single cells and outlined the conceptual integration between the experimental and analytical stages. In this chapter, we introduce the Electro-Cell Physiometry (ECP) Framework, a fully integrated software dedicated to the automated analysis of electrorotation experiments. Given raw video recordings of rotating cells, the ECP-Framework provides the complete processing pipeline—cell segmentation, tracking, angular velocity estimation,

and dielectric parameter extraction—within a single, user-friendly environment. The ECP-Framework provides real-time visualization, batch analysis capabilities, and structured data management, enabling reproducible and operator-independent ROT characterization. It complements existing tools for experimental automation, described in Chap.2, by focusing exclusively on the analytical and computational stages of the workflow. At the core of ECP-Framework lies POLARIS (*POLar Angular Rotation Intensity Signature*), the new proposed algorithm for angular velocity extraction. In this study, the proposed method is benchmarked against our previously described PxI algorithm, and the widely adopted image-correlation approach reported in [172], allowing for a quantitative evaluation of performance in terms of accuracy, stability, and computational efficiency.

In typical ROT experiments, the observed motion of a suspended cell results from a combination of rotation and translation. Although translation motion is theoretically absent, a small lateral drift can still occur due to local field asymmetries or hydrodynamic perturbations. When the cell remains sufficiently close to the central region, this drift is generally limited but nonetheless introduces an additional source of uncertainty in the estimation of the angular velocity. The main objective is to determine the cell’s angular velocity Ω as rapidly and accurately as possible across the range of applied field frequencies. Automated systems must therefore be capable of extracting reliable angular velocity values from sequences of microscope images, even when cells exhibit irregular morphologies, low optical contrast, or slight translational motion. To render the estimation problem tractable, three practical assumptions are adopted: (i) imaging is performed using a phase-contrast microscope equipped with an RGB or grayscale camera, ensuring a minimal optical contrast; (ii) the center of cell rotation coincides with the geometric centroid of the cell; and (iii) the rotational motion occurs around the optical z -axis, remaining confined within the focal plane of the imaging system. These assumptions preserve the physical realism of the observed motion while simplifying the analytical treatment required for angular velocity estimation under realistic imaging conditions.

4.2 ECP-Framework Design and Architecture

The developed ECP-Framework, implemented in Python, provides an integrated environment for the automated analysis of ROT experiments. Its architecture, see Fig.4.1, is designed to streamline the entire analytical workflow—from video import and preprocessing to cell tracking, angular velocity estimation, and dielectric parameter computation—within a single, user-friendly graphical user interface (GUI), providing real-time visualization of intermediate and final outputs, interactive parameter tuning, and batch-processing capabilities for high-throughput experimentation.

The ECP-Framework is organized into four main functional modules reflecting the stages of the analytical ROT workflow. In particular, a *Cell Centroid Selection* module enables users to identify and select target cells from raw microscopy recordings. The *Segmentation and Tracking* module performs frame-by-frame cell segmentation and trajectory reconstruction. The extracted cells are then processed by the *Angular Velocity Extraction* module,

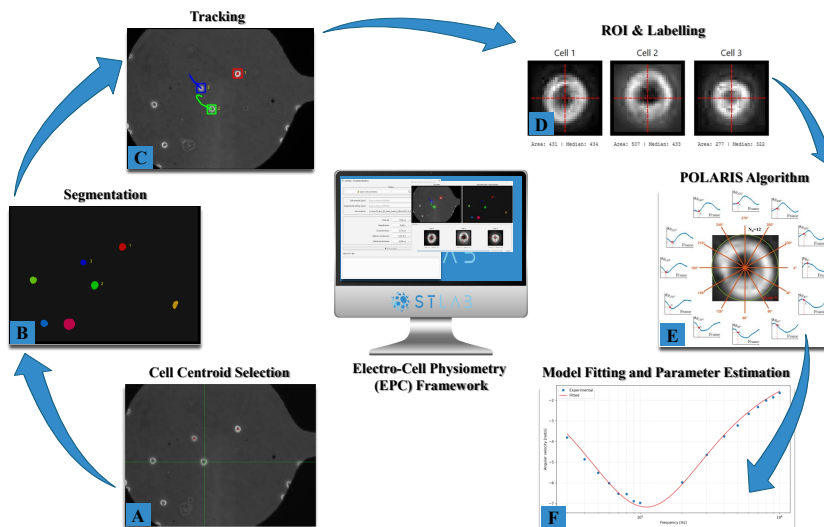


FIGURE 4.1: Overview of the Electro-Cell Physiometry (ECP) Framework workflow, integrating all key stages required for quantitative single-cell electrorotation analysis: (A) selection of cell centroids within the recorded experimental video, (B) frame-by-frame segmentation mask, with each selected cell assigned a unique label, (C) centroid-based tracking paths across frames for motion consistency assessment, (D) cropped and centered ROIs extracted for each selected tracked cell, (E) angular velocity estimation through the POLARIS algorithm, and (F) model fitting of the measured spectra for dielectric parameter estimation, enabling comprehensive single-cell physiometric characterization.

which integrates the proposed POLARIS algorithm for angular velocity estimation. Finally, the *ROT Spectrum Fitting Module* fits the experimental data to the theoretical ROT model to extract membrane capacitance, cytoplasmic conductivity, and other dielectric and morphological parameters.

4.2.1 Cell Centroid Selection

The *Cell Centroid Selection* module represents the only stage of the analytical pipeline that requires direct user intervention. Its purpose is to initialize the automated workflow by allowing users to identify the target cells to be analyzed. The user begins by importing one or multiple video recordings corresponding to independent ROT experiments. Each video is displayed sequentially, enabling manual specification of the initial frame marking the onset of cell rotation. Cell selection is performed interactively by simple mouse-clicking on the desired cells, see Fig.4.1(A). At this stage, cells outside the ROT region, forming aggregates, adhering to surfaces, or showing morphological damage (e.g., membrane rupture) are excluded to ensure biological and analytical consistency. Upon completion, the module compiles a configuration file in `.json` format containing all selection metadata and preprocessing parameters. This file serves as input for the subsequent *Segmentation and Tracking* module, thus ensuring reproducible and fully automated processing of the selected dataset.

4.2.2 Segmentation and Tracking

The videos and cell selected in the previous stage are passed to the *Segmentation and Tracking* module, which performs automatic frame-by-frame cell segmentation and trajectory reconstruction. Prior to segmentation, each frame undergoes optional intensity equalization to enhance contrast uniformity across the field of view, a step that can be enabled or disabled by the user from the GUI. This preprocessing operation mitigates illumination inhomogeneities and improves the subsequent mask consistency, particularly under low-contrast imaging conditions. Cell segmentation is performed using the *CellPose* deep-learning framework [175], which leverages a convolutional neural network trained on diverse microscopy modalities for generalizable cell boundary detection. In this work, the *cyto3* model (pretrained on bright-field and phase-contrast images) was employed. This configuration provided stable mask generation across a wide range of experimental conditions. The segmentation is executed independently on each frame, yielding a labeled mask in which each connected component corresponds to a detected cell region, see Fig.4.1(B). From the resulting masks, the geometric centroid (c_x, c_y) and the equivalent diameter d_{eq} are computed for all segmented regions. Only the cells previously selected by the user are retained for further analysis. Tracking across frames is then performed by centroid association: for each labeled cell in frame k , the corresponding cell in frame $k+1$ is identified by minimizing the euclidean distance between centroids, subject to a maximum displacement constraint proportional to d_{eq} . The resulting trajectories are visualized within the GUI, as illustrated in Fig.4.1(C), where centroid positions are connected frame-by-frame using color-coded paths. For each tracked cell, a region of interest (ROI) is then cropped around its centroid. The ROI size is dynamically adapted to the estimated cell diameter, with an additional margin of N pixels to fully capture the rotation dynamics. Each ROI sequence is stored as an independent image stack $\{I_k(x, y)\}_{k=1}^L$, which serves as the direct input for the subsequent *Angular Velocity Extraction* module, see Fig.4.1(D). This ensures spatial and temporal alignment of the tracked object across the entire video and provides a standardized input domain for the POLARIS algorithm.

4.2.3 Angular Velocity Extraction - POLARIS

The POLARIS (*POLar Angular Rotation Intensity Signature*) algorithm estimates the angular velocity of a rotating cell from a temporal sequence of microscopy frames. Let $\{I_k(x, y)\}_{k=1}^L$ denote the preprocessed image stack, where k indexes the frame within the sequence of length L . The algorithm employs a polar-coordinate transformation to capture angular intensity variations over time and quantifies rotational dynamics through temporal cross-correlation analysis.

In particular, each frame $I_k(x, y)$ of size $N \times N$ is first converted from cartesian to polar coordinates with respect to the central pixel (c_x, c_y) , which corresponds both to the ROI center and to the geometric centroid of the cell, as determined by the segmentation and tracking module. Using the standard polar coordinate transformation

$$r = \sqrt{(x - c_x)^2 + (y - c_y)^2} \quad \theta = \tan^{-1} \left(\frac{y - c_y}{x - c_x} \right) \quad (4.1)$$

each pixel intensity in $I_k(x, y)$ is mapped into the polar domain $I_k(r, \theta)$. Defining N_θ as the number of angular samples—i.e., the set of discrete angular positions partitioning the full 2π range around the cell into equally spaced sectors (see Fig.4.1(E))—the mean radial intensity profile is computed over the N_θ angular samples $\theta_i = 2\pi i/N_\theta$ for $i = 0, 1, \dots, N_\theta - 1$ as

$$s(\theta_i, k) = \frac{1}{R} \sum_{r=1}^R I_k(r, \theta_i) \quad (4.2)$$

where R denotes the maximum radial extent of the cell within the ROI. By evaluating Eq.(4.2) for each frame $k = 1, \dots, L$, we construct a discrete angular-temporal map $S(\theta_i, k) \in \mathbb{R}^{N_\theta \times L}$, where each row represents the temporal evolution of the mean radial intensity at the angular position θ_i . For a fixed angular position θ_i , the corresponding angular-temporal profile is defined as the vector

$$\mathbf{s}_{\theta_i} = [s(\theta_i, k)]_{k=1}^L \quad (4.3)$$

To estimate the angular velocity, we analyze the temporal behaviour of intensity variations at adjacent angular positions. In particular, during steady rotation, the angular-temporal profile $\mathbf{s}_{\theta_i}$ measured at two angular positions θ_i and $\theta_j = \theta_i + \Delta\theta$ exhibit similar waveforms that are mutually time-shifted: the waveform at θ_j can be approximated as a delayed version of the waveform at θ_i . Therefore, the angular velocity Ω can be estimated from the angular separation $\Delta\theta$ and the corresponding temporal delay τ estimated between the two successive angular-temporal profiles $\mathbf{s}_{\theta_i}$ and $\mathbf{s}_{\theta_j}$

$$\Omega \approx \frac{\Delta\theta}{\tau} \quad (4.4)$$

under the assumption of approximately constant angular velocity over the analysed interval. The time delay τ between two angular signals can be directly estimated simply by computing the cross-correlation between $\mathbf{s}_{\theta_i}$ and $\mathbf{s}_{\theta_j}$. Before computing the cross-correlation, each angular-temporal profile signal $\mathbf{s}_{\theta_i}$, is detrended by subtracting its sample mean μ_{θ_i} , and then low-pass filtered to suppress high-frequency noise. Denoting by $\mathcal{F}_{\text{LP}}\{\cdot\}$ the applied Butterworth low-pass operator, the preprocessed signal is

$$\tilde{\mathbf{s}}_{\theta_i} = [\mathcal{F}_{\text{LP}}(s(\theta_i, k) - \mu_{\theta_i})]_{k=1}^L \quad (4.5)$$

The discrete cross-correlation is computed as a function of integer lag n :

$$CC_{ij}[n] = \begin{cases} \sum_{k=1}^{L-n} \tilde{s}(\theta_i, k) \tilde{s}(\theta_j, k+n) & n \geq 0 \\ \sum_{k=1-n}^L \tilde{s}(\theta_i, k) \tilde{s}(\theta_j, k+n) & n < 0 \end{cases} \quad (4.6)$$

for $n = -(L-1), \dots, (L-1)$. The integer lag n_{ij}^* that maximizes $CC_{ij}[n]$ corresponds to the relative frame shift between the two angular-temporal profiles $\mathbf{s}_{\theta_i}$ and $\mathbf{s}_{\theta_j}$. Given the frame period $\Delta t = 1/\text{FPS}$, the corresponding time delay is $\tau_{ij}^* = n_{ij}^* \Delta t$. Using the geometric angular separation $\Delta\theta$ between

θ_i and θ_j , the angular velocity is given by

$$\Omega_{ij} = \frac{\Delta\theta}{\tau_{ij}^*} = \frac{\Delta\theta}{n_{ij}^* \Delta t} \quad (4.7)$$

To enhance robustness under varying image contrast and noise conditions, multiple angular-temporal profile pairs and a set of N_θ configurations are evaluated, yielding a collection $\{\Omega_m\}$ of candidate angular velocity values. Finally, the estimated angular velocity Ω_{est} is obtained using an α -trimmed mean estimator [176], with $\alpha = 30\%$.

4.2.4 ROT Spectrum Fitting Module

The last module performs the extraction of cell dielectric parameters by fitting the experimentally measured ROT spectra to the theoretical model. Specifically, for each analyzed cell, the experimental dataset $\{f_k, \langle\Omega_{ROT,\text{exp}}(f_k)\rangle\}$ obtained from the POLARIS algorithm is compared with the theoretical model $\{f_k, \langle\Omega_{ROT,\text{theory}}(f_k, \mathbf{p})\rangle\}$ derived from Eq.(2.2), (2.3), and (2.4). The fitting procedure aims to identify the parameter vector $\mathbf{p}$, containing the intrinsic dielectric quantities of the cell, that minimizes the discrepancy between theoretical and experimental angular velocities:

$$J(\mathbf{p}) = \sum_{k=1}^{N_f} [\langle\Omega_{ROT,\text{exp}}(f_k)\rangle - \langle\Omega_{ROT,\text{theory}}(f_k, \mathbf{p})\rangle]^2 \quad (4.8)$$

The parameter estimation is performed using a Differential Evolution (DE) algorithm, a global optimization method particularly suited for non-linear and multimodal problems. Parameter bounds are imposed to ensure physically consistent solutions. At present, the implemented theoretical model follows the Single-Shell model representation introduced in Sec.2.1.2. However, the module structure allows straightforward extension to more complex geometries (e.g., *Double-Shell* models accounting for the nucleus or internal organelles).

Upon convergence, the fitted parameters are used to compute secondary quantities such as the area-specific membrane capacitance C_m from Eq.(2.6), enabling a direct link between the electrical and morphological features of the analyzed cell. Finally, for each processed video and each identified cell, the module automatically saves:

- the optimized dielectric parameters $\mathbf{p}_{\text{opt}}$;
- the experimental spectrum $\langle\Omega_{ROT,\text{exp}}(f)\rangle$;
- the fitted theoretical curve $\langle\Omega_{ROT,\text{theory}}(f, \mathbf{p}_{\text{opt}})\rangle$;
- and a graphical output overlaying both experimental and fitted curves for visual validation, see Fig.4.1(F).

This structure ensures transparency, reproducibility, and ease of post-processing, allowing batch-level analysis across multiple cells or experimental conditions.

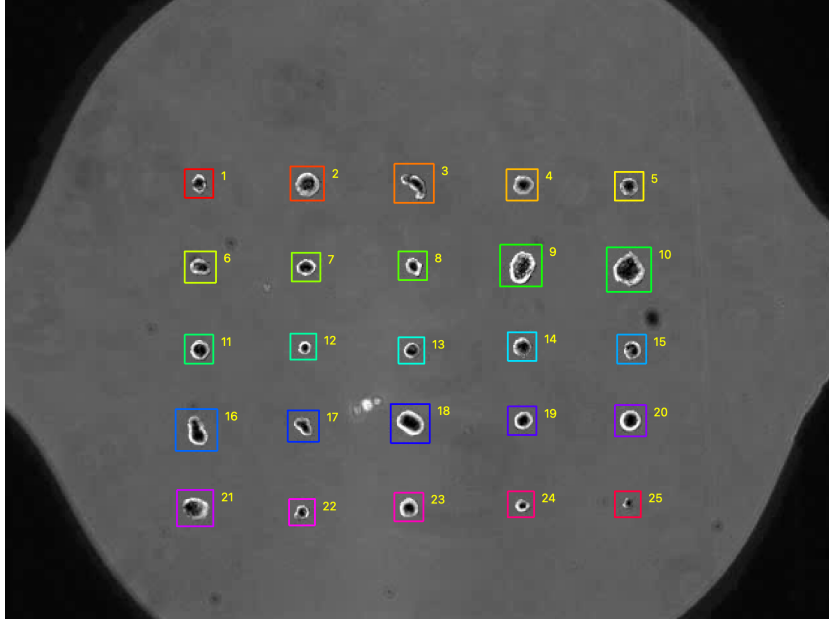


FIGURE 4.2: Representative frame from the simulated electroration dataset used to evaluate angular-velocity estimation performance across heterogeneous cell morphologies, generated by the simulator environment described in Appendix A. The image shows the five human cell lines considered in the study, arranged by rows: CaCo2 (first row), CCD-841 (second row), NCI-H929 (third row), OPM2 (fourth row), and U266 (fifth row). For each line, five individual cells are displayed, selected to span a broad spectrum of geometrical and morphological features.

4.3 Simulator-based Validation

To quantitatively evaluate the proposed angular velocity estimation algorithm, two simulation-based datasets were generated, each designed to assess distinct aspects of performance and robustness. This assessment was done using the electroration simulator developed and described in Appendix A, which represents an extension of the first prototype employed in Sec.2.2.3. Building upon that initial version, a complete graphical environment was designed and implemented, providing a more realistic and comprehensive representation of the electroration experiments compared with the original tool. See Appendix A for more information regarding the ROT-Game Simulator.

4.3.1 Dataset and Metrics

The first dataset aims to verify the algorithm’s capability to accurately estimate the angular velocity across the full ROT spectrum. Cell images used in the simulations were extracted from previous ROT experimental campaigns, ensuring realistic morphological and optical characteristics. The five previously analyzed cell lines were considered—CaCo2, CCD-841, OPM2, NCI-H929, and U266—each represented by five individual cells differing in size and morphology (circular, elliptical, irregular, symmetric, and asymmetric). A frame of the simulated video is shown in Fig.4.2. This variability allows the analysis of how geometrical and morphological heterogeneity affects the estimation accuracy. Moreover, although segmentation and tracking are considered established processes, they inevitably introduce a residual misalignment of the cell within the cropped ROI due to the image discretizations.

Therefore, to assess the robustness to such tracking residuals, each simulated video incorporates a random spatial displacement of the cell, referred to as the tracking residual. Three different levels of tracking residuals were considered: 0 pixel variation (cell perfectly centered within the ROI), and random displacements of $[1, 2]$ pixels around the ROI center position. Simulations were performed under conditions replicating our experimental setup reported Chap.2, i.e., 60 FPS acquisition rate, 640×480 pixel frame size, and $20\times$ optical magnification. Each simulated video reproduces the experimental protocol described always in Sec.2.2, alternating rotation and resting phases across the full frequency range, in order to automate the analysis.

The second dataset was designed to explore the operational limits of the algorithm, identifying the minimal experimental conditions required for reliable estimation. In this case, one representative cell (with a 1-pixel tracking residual) was analyzed while varying two parameters: (i) the acquisition frame rate, ranging from 60 FPS down to 20 FPS, and (ii) the number of full rotations per frequency interval, ranging from three to one-half rotation. This analysis provides quantitative insight into the trade-offs between temporal sampling, rotational completeness, and estimation accuracy, highlighting the minimal conditions under which the algorithm maintains stable performance in order to minimize the experimental time.

For each study and condition, the estimated angular velocity was compared to the imposed ground-truth, and three main metrics were computed: the Root Mean Square Error (RMSE), the Mean Absolute Percentage Error (MAPE), and the Coefficient of Determination (R^2) across the entire ROT spectrum.

4.3.2 Results

The quantitative evaluation performed on the first dataset highlights a clear performance hierarchy among the three tested algorithms, with POLARIS exhibiting higher accuracy, stability, and robustness across all cell lines and almost all noise conditions. A representative spectrum per tracking residual performed on the first simulated dataset is shown in Fig.4.3. Tab.A.1 in the Appendix A presents detailed results of the performance indices among all conditions, while Fig.4.4 illustrates the corresponding performance trends, calculated as the mean across all considered cells. It is important to note that NCC is reliable only for angular velocities above ~ 3 rad/s; slower rotations produce inter-frame displacements too small for detectable image correlation shifts. Consequently, angular velocities below 3 rad/s were excluded from NCC analysis. Furthermore, while POLARIS intrinsically provides the rotation direction, both PxI and NCC lack directional sensitivity; therefore, the sign of the angular velocity for these two methods was manually corrected to ensure consistency across algorithms and performance indexes.

Under ideal tracking conditions (0-pixel tracking residuals), POLARIS consistently achieves low RMSE values below 0.2 rad/s and MAPE values under 1.5 % across all cells considered. The coefficient of determination (R^2) is approximately 0.9997, indicating nearly perfect alignment between estimated and imposed angular velocities. In comparison, both PxI and NCC exhibit markedly higher RMSE and MAPE values, together with reduced R^2 scores, primarily because their performance deteriorates sharply for cells with highly symmetric geometries. While both methods operate reliably on

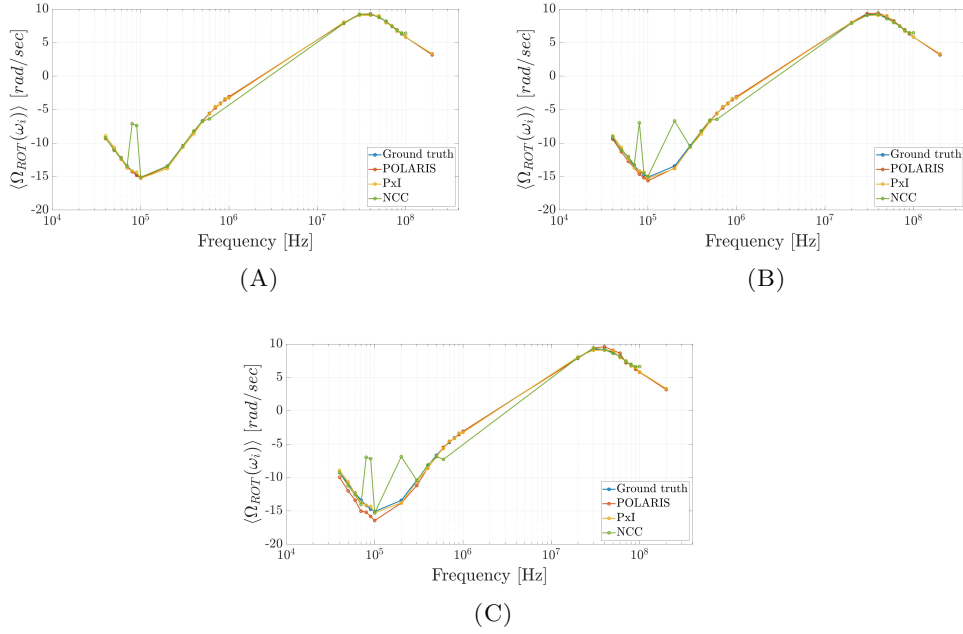


FIGURE 4.3: Representative ROT-spectra per tracking residual levels. (a) *0-pixel*: the center of the cell remains at the nominal coordinates (c_x, c_y) . (b) *1-pixel*: the center of the cell is randomly shifted in both coordinates (c_x, c_y) up to ± 1 pixel, using a uniform distribution. (c) *2-pixel*: the same random shift is applied with a range of ± 2 pixels. Data points show the angular velocity ground-truth imposed using the simulator and the angular velocity automatically estimated by POLARIS, Pxl, and NCC.

irregular shapes, circular or elliptical profiles introduce intrinsic ambiguities that lead to substantial estimation failures. Overall, these results demonstrate that POLARIS achieves high precision across all cell shapes, making it cell-shape independent. When a moderate tracking residual is introduced (1-pixel tracking residuals), POLARIS retains a high level of accuracy for the majority of cells, with RMSE values that remain below 0.3 rad/s in most cases and R^2 values still close to one. Only a limited subset of cells exhibits a noticeable degradation, reflected in increased RMSE and MAPE values. This behavior is expected, as POLARIS inherently assumes that the cell is perfectly centered within the ROI; any residual displacement violates this condition, consequently reducing performance in the estimation. Nonetheless, the degradation remains contained compared to Pxl and NCC. Finally, under the most challenging condition (2-pixel tracking residuals), while the RMSE for POLARIS increases as expected due to the larger spatial decentralization, it remains within a bounded range for most of the cells, and the R^2 values stay high (above 0.98 for the majority of cases). A subset of small cells represents the worst-case scenarios, exhibiting RMSE excursions and a partial reduction in R^2 . This arises because smaller cells undergo a proportionally larger spatial decentralization, which amplifies the misalignment-induced error during the estimation. By contrast, RMSE values for Pxl remain nearly constant across noise levels, reflecting its quadrant-based nature, whereas NCC demonstrates sensitivity to misalignment.

The results of the second simulated dataset are summarized in Tab. 4.1. In this case, reducing the number of available rotations progressively degrades

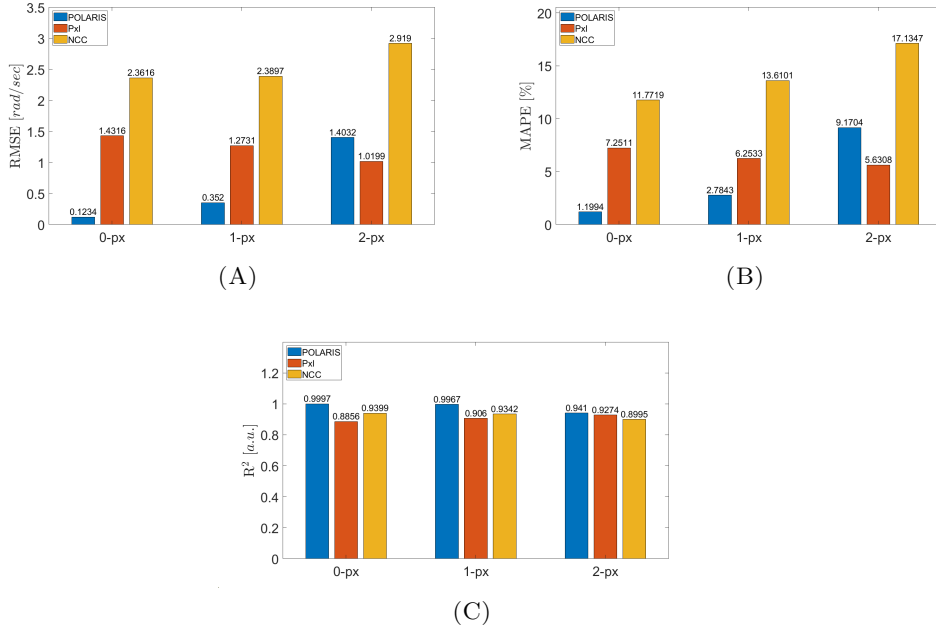


FIGURE 4.4: Simulation-based performance assessment computed on the first datasets generated with different levels of tracking residual. Each bar reports the mean value over all simulated cells. (a) Root Mean Square Error (RMSE), (b) Mean Absolute Percentage Error (MAPE), and (c) Coefficient of Determination (R^2), evaluated by comparing the estimated angular velocity spectra with the corresponding ground-truth profiles.

	POLARIS			PxI			NCC		
Rotations	RMSE	MAPE	R^2	RMSE	MAPE	R^2	RMSE	MAPE	R^2
3	0.268	1.713	0.999	0.758	5.876	0.993	2.238	11.034	0.947
2	0.263	2.156	0.999	1.494	9.983	0.971	2.781	15.704	0.917
1	0.089	1.798	1.000	1.282	17.985	0.914	NaN	NaN	NaN
1/2	0.063	2.951	0.999	2.815	59.021	-1.532	NaN	NaN	NaN
FPS									
60	0.064	0.640	0.999	0.211	2.377	0.999	2.143	10.103	0.963
50	0.079	0.383	0.999	0.229	2.466	0.999	3.352	3.352	0.880
40	1.905	7.675	0.953	0.188	1.992	0.999	4.258	4.258	0.806
30	3.660	33.248	0.827	0.184	2.071	0.999	4.532	4.532	0.781
20	3.103	34.274	0.875	0.238	2.556	0.999	6.268	6.268	0.580

TABLE 4.1: Simulation-based performance assessment computed on the second dataset generated with different full cell rotations and FPS. The Root Mean Square Error (RMSE), Mean Absolute Percentage Error (MAPE), and Coefficient of Determination (R^2), evaluated by comparing the estimated angular velocity spectra with the corresponding ground-truth profiles.

the performance of all methods, although in different ways. Since the POLARIS algorithm does not require the full rotational profile of the cell but relies on local angular information, it maintains accurate estimation even when fewer than two complete rotations are available. PxI, in contrast, loses reliability under incomplete rotations because the mean-intensity signal progressively loses its periodicity, causing the spectral peak to weaken or disappear and leading to inconsistent angular-velocity estimates. NCC also shows reduced reliability in this regime because it relies on detecting frame-to-frame

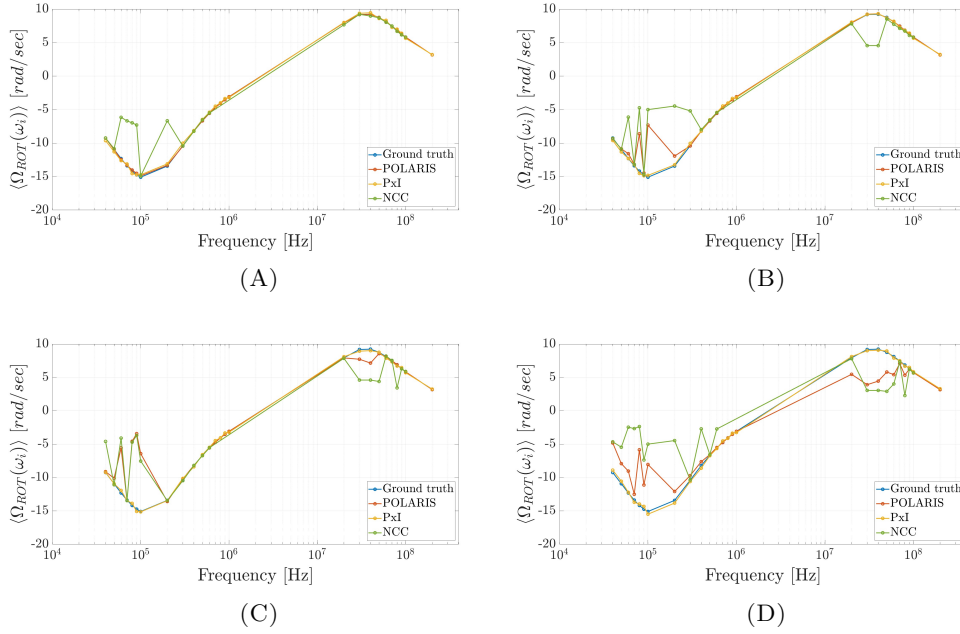


FIGURE 4.5: ROT-spectra assessment computed on the second datasets generated with different FPS levels. Each bar reports the mean value over all simulated cells: (a) 50 FPS, (b) 40 FPS, (c) 30 FPS, and (d) 20 FPS.

similarity that becomes intrinsically unobservable when fewer than one full rotation is present, and as a result, it fails to return meaningful angular-velocity estimates under one-rotation or half-rotation conditions.

Instead, decreasing FPS values highlights the temporal-sampling constraints inherent to correlation-based techniques. Both POLARIS and NCC rely on correlation-based measurements, and their resolution in angular velocity estimation is limited by the temporal sampling of the video. In POLARIS, reducing the FPS lowers the maximum angular velocity that can be reliably estimated, as can be seen in Fig.4.5. NCC is similarly affected because it depends on detecting frame-to-frame similarity, and a lower sampling rate increases the chance of missing overlapping or partially repeating patterns, which degrades the resulting estimates. Pxl, in contrast, remains largely insensitive to frame-rate variations, since its angular velocity estimation is not based on correlation methods but on spectral analysis. Even when undersampling (within certain limits), the main frequency component correlated to the cell rotation is preserved, allowing for the extraction of angular velocity.

Overall, the results of the first simulated dataset demonstrate that POLARIS can reliably estimate the angular velocity across heterogeneous cell morphologies and under realistic experimental perturbations. Moreover, the results of the second dataset confirm that POLARIS offers the best trade-off between accuracy, robustness, and experimental requirements. The capability of POLARIS to drastically reduce the duration of ROT experiments—since it is able to estimate angular velocity for incomplete rotations—thereby increasing throughput, while maintaining robustness and accuracy, is particularly notable. This is achieved within the constraints imposed by acquisition rates and tracking errors, which in our setup fall well within the operational range, ensuring reliable and efficient measurements. These findings justify its use as the core estimator within the ECP-Framework for the subsequent analysis of

real experimental ROT datasets.

4.4 ECP-Framework Experimental Validation

4.4.1 Experimental Setup

For the experimental validation, the ROT-Platform described in Sec.2.3 was employed. The ROT-chip was positioned on a phase-contrast inverted microscope (OPTIKA IM-300 PHi) equipped with a $\times 20$ objective. Video acquisition was performed using a CCD camera (340M Fast Frame, Thorlabs) with a native resolution of 640×480 pixels and a square pixel size of $7.4 \mu m$. The camera, interfaced to a PC via USB connection, was operated through its proprietary software (ThorCamTM v.3.7.0) at a frame rate of 60 FPS. Due to the current bandwidth limitations of the QSG unit, the ROT spectrum performed in this work spans from 20 kHz to 1 MHz. This frequency interval is sufficient to capture the membrane-dominated dispersion region, enabling the estimation of the dielectric properties of the cellular membrane. The experimental setup and acquisition protocol strictly follow the configuration described in Sec.2.2, ensuring full consistency with previous work and enabling complete automation of the ROT experimental validation.

4.4.2 Cell cultures and preparation

Two distinct cell lines were used to evaluate system performance: the human leukemia suspension cell line HL-60 and the adherent cervical carcinoma cell line HeLa. HeLa cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin ($100\times$). HL-60 cells were maintained in RPMI-1640 medium supplemented with 20% FBS and 1% penicillin–streptomycin ($100\times$). All cultures were incubated at $37^\circ C$ in a humidified atmosphere with 5% CO_2 and were processed when they reached approximately 80% confluence. Sample preparation followed a common workflow for both cell lines, with adjustments based on their growth characteristics. HL-60 cells were collected directly from suspension, whereas HeLa cells were detached from the culture surface by incubation with EDTA for 4 min and then neutralized by adding complete medium before centrifugation. Cell suspensions were transferred to centrifuge tubes and pelleted at 1500 rpm for 5 min. After removing the supernatant, pellets were washed 3 times and resuspended in 1 mL of buffer solution, already reported in [127], achieving a final concentration of approximately $8 \times 10^5 \text{ cells/mL}$.

4.4.3 Results

The validation of the ECP-Framework was conducted on the entire workflow of ROT characterization, automatically, without user intervention. This included cell segmentation, tracking, angular velocity estimation, model fitting, and determination of dielectric parameters. In Fig.4.6, two representative ROT spectra from the investigated cell lines are presented. The data points represent the estimated angular velocity obtained through the *POLARIS* Algorithm, while the solid lines illustrate the fitting curves corresponding to these data points, based on the Single-Shell Model. The experimentally measured spectra show a strong agreement with the corresponding theoretical predictions. As can be observed, the maximum anti-field rotation differs

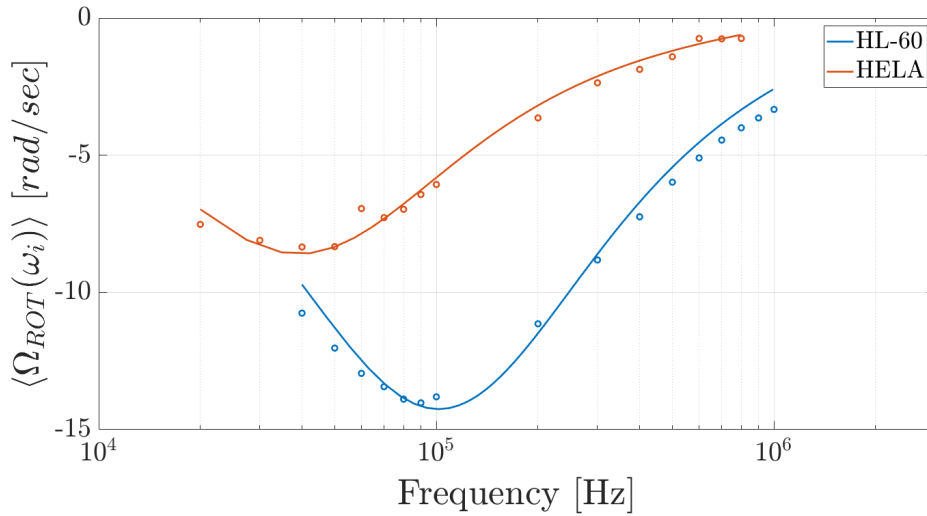


FIGURE 4.6: ROT-spectra ($\langle \Omega_{ROT}(\omega_i) \rangle$) of the investigated cell lines: HL-60 and HeLa. The data points were gathered from experiments and represent the estimated angular velocity obtained through the *POLARIS* Algorithm using the ECP-Framework. The solid lines show the fitting curve of data points with the Single-Shell Model.

Source	Sum of Squares (SS)	df	Mean Square	F-value	p-value
Groups	2040.47	1	2040.47	94.88	1.71×10^{-10}
Error	602.14	28	21.51		
Total	2642.61	29			

TABLE 4.2: ANOVA performed on membrane capacitance (C_m) values across the considered cell lines: HL-60 and HeLa.

for each cell line, indicating, as expected, distinct dielectric characteristics. Specifically, HL-60 cells exhibit a maximum rotation point of around 100 kHz , while HeLa cells show maximum rotation points at frequencies around 40 kHz . These findings confirm the distinct nature of the two cell lines and highlight their varying dielectric membrane characteristics.

From the ECP-Framework output, the membrane capacitance (C_m) and the equivalent cell radius (r) were extracted for each individual cell. These parameters, automatically estimated within the processing pipeline, were collected over the entire dataset to enable a quantitative comparison of the dielectric properties. Among the two examined cell lines, HeLa cells exhibited the highest membrane capacitance ($C_m = 30.5 \pm 6.43 \text{ mF/m}^2$), consistent with their highly structured and morphologically heterogeneous surface. HL-60 cells, in contrast, displayed a substantially lower capacitance ($C_m = 13.9 \pm 2.58 \text{ mF/m}^2$), reflecting the smoother and less articulated membrane typical of suspension-grown leukemic cells. The extracted equivalent radii were $r_{\text{HL-60}} = 5.76 \pm 0.44 \text{ }\mu\text{m}$ and $r_{\text{HeLa}} = 6.94 \pm 0.93 \text{ }\mu\text{m}$. To determine whether the membrane-capacitance distributions of the two cell lines originated from statistically distinct populations, an analysis of variance (ANOVA) was performed. As reported in Tab. 4.2, the test yielded an F -value of 94.88 with a corresponding p -value of 1.71×10^{-10} , confirming a statistically significant difference between the two groups.

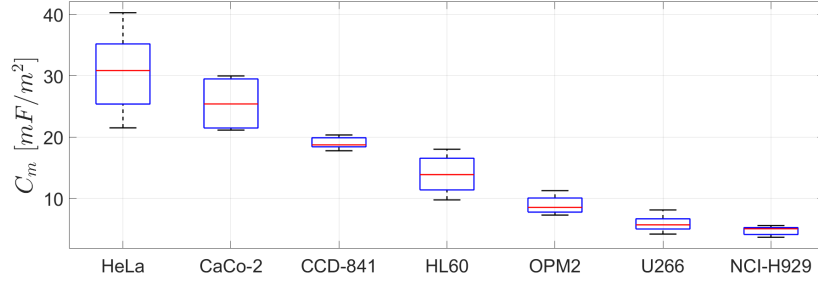


FIGURE 4.7: Membrane capacitance C_m values extracted for all analyzed cells. The figure reports the aggregated results obtained from the HeLa and HL-60 measurements acquired in the present study, together with additional datasets processed in previous experiments. For each cell line, the mean membrane capacitance and its standard deviation are shown, enabling a direct comparison across all available samples.

Beyond the two cell lines characterized in this study, all experimentally acquired membrane-capacitance datasets available from previous campaigns—including CaCo-2, CCD-841, OPM2, U266, and NCI-H929—were aggregated and represented in Fig.4.7. This comprehensive visualization enables a direct comparison across all cell types investigated so far through the ROT-Platform, allowing the newly acquired results to be contextualized with respect to pre-existing trends, highlighting the consistency of the capacitance distributions and reinforcing the reliability of the automated extraction procedure. The box-plot representation, ordered by decreasing membrane capacitance, provides a compact overview of the variability observed across the different cell lines, and highlights the capability of the ECP-Framework to systematically extract dielectric descriptors in a consistent and fully automated manner across diverse experimental datasets. Overall, the observed separation between the capacitance values of HeLa and HL-60 cells remains clearly preserved even when considered within the broader dataset. This confirms that the differences identified in the present study are not isolated outcomes but reflect stable and reproducible dielectric signatures intrinsic to the two cell lines.

Chapter 5

High-Frequency Enhanced ROT-Platform

This chapter presents the development and validation of the High-Frequency Quadrature Signal Generator (HF-QSG), a newly designed, wideband electronic system for single-cell dielectric characterization. The system integrates synchronized DDS-based generation, RF-optimized amplification, and embedded digital control into a compact architecture capable of delivering phase-coherent quadrature signals up to 300 MHz. Compared to previous implementations, the HF-QSG adopts a fully impedance-controlled, symmetry-preserving PCB design that ensures extended bandwidth, minimized inter-channel mismatch, and high stability under continuous operation. The system was validated through broadband ROT measurements on two biologically distinct cell lines, HL-60 and HeLa, previously characterized only in the low-frequency regime. Using the automated ECP-Framework workflow, we extracted both specific membrane capacitance and cytoplasmic conductivity, revealing a statistically significant separation in membrane properties and comparable cytoplasmic conductivities across the two considered populations. These results demonstrate the capability of the HF-QSG to enable reliable, broadband, single-cell dielectric profiling, opening the way to next-generation ROT and DEP assays with expanded diagnostic and biophysical relevance. The related work, its validation, and the experimental results have been detailed in a recent manuscript currently awaiting submission.

5.1 Current Limitations in ROT Instrumentation

As discussed in Chap.1 and 4, two main factors have limited ROT technological diffusion. First, the ROT workflow still lacks automation: most experimental procedures, from field generation and cell trapping to image acquisition and angular velocity extraction, are manually performed and analyzed [167]. This high level of user intervention hampers reproducibility, throughput, and accessibility for non-specialist users. In this context, the

previous chapter introduced the newly developed ECP-Framework, a unified and fully automated software environment specifically designed to bridge this gap.

The second main factor is the generation of a stable broadband rotating electric field, which typically requires multi-phase signal generators and precise synchronization hardware, often relying on bulky, expensive benchtop instruments [166], thereby constraining portability and integration into compact microfluidic systems.

5.1.1 Practical Constraints in Wideband AC Actuation

In most practical implementations, the required AC signals are generated using off-the-shelf waveform generators. For example, in [177], a laboratory waveform generator was employed to perform DEP-based characterization of *Mycobacterium smegmatis*, using AC signals between 100 kHz and 15 MHz. While suitable for analyses dominated by membrane polarization, this configuration inherently restricts access to higher-frequency cytoplasmic features due to the limited bandwidth of the driving electronics. In [91], similar constraints emerge in electrorotation platforms. In that study, two synchronized generators were used, one to produce the trapping field through negative DEP and a second to supply the four quadrature signals needed for ROT. Although functional, this multi-instrument architecture relies on benchtop equipment, increases system complexity, and remains bandwidth-limited, thereby reducing the accessible dielectric spectrum.

To overcome the intrinsic frequency limitations of low-frequency generators, some DEP studies have resorted to hybrid architectures. In [178], low-frequency actuation was produced by a standard waveform generator, which was then switched, via an external RF switching and attenuation module, to a high-frequency RF signal generator and dedicated power amplifier for UHF-DEP characterization. This approach successfully extended the operational range, revealing phenotypic differences in glioblastoma cell lines, but required complex switching circuitry, RF probes, and multiple high-end instruments. More recently [68], high-frequency ROT has been demonstrated by combining an RF waveform generator with cascaded broadband power amplifiers, where four synchronized AWG channels were amplified to approximately 18 V over a 50 Ω load, enabling ROT characterization from 50 kHz to 5 GHz. Although this setup achieved unprecedented bandwidth, it relied on two full amplifier blocks, extensive RF conditioning, and instrumentation whose cost and complexity are incompatible with portable or point-of-use systems.

Other dedicated platforms have been developed to overcome the intrinsic limitations of benchtop instrumentation. A notable example is the 3DEP system [71], which employs a disposable 3D electrode geometry and performs DEP characterization of large cell populations by optically monitoring the field-induced redistribution of cells. The system integrates twenty independently controlled DDS-based signal generators (Direct Digital Synthesis), each capable of delivering 20 V_{pp} up to 45 MHz. Despite its efficiency and throughput, the operational bandwidth remains confined to the low-mid MHz range, restricting access to the complete higher-frequency dielectric features. A second example is the ADEP platform [179], a portable system designed for DEP manipulation and ROT orientation. Its signal-generation module comprises six independent channels, each implemented with dual DDS sources followed

by amplification stages, providing frequency tunability from DC to 10 MHz. While compact and highly flexible, the system is intrinsically limited to the low-frequency DEP and ROT regime and does not address the challenges associated with high-frequency. Similarly, the platform presented in [180] integrates DEP and ROT functionalities using a microfluidic system integrated with an on-chip impedance readout, employing a DDS-based excitation unit, providing a programmable sinusoidal output over a sweep range from 10 kHz to 45 MHz. Despite the improved compactness and integration, the limited voltage swing and moderate upper frequency boundary still confine the analysis primarily to membrane-dominated regimes.

5.1.2 Advanced ROT and DEP Spectroscopy

Across the systems cited before, a common limitation emerges: although custom architectures can provide flexibility and integration, their operational bandwidth is generally confined to the classical DEP/ROT range below a few tens of MHz. Extending excitation toward the RF domain introduces significant challenges associated with wideband signal generation, power delivery, impedance matching, and signal integrity at the electrode interface. These constraints continue to hinder the development of compact and broadband system capable of spanning the full dielectric spectrum of biological cells.

Within this context, in Chap.2 we introduced the Quadrature Signal Generator (QSG), a custom electronic system tailored for electrokinetic experiments. The system was able to deliver four high-power sinusoidal signals with a -3 dB bandwidth of 60 MHz. Despite these capabilities, the finite bandwidth constrained its use to the conventional frequency range typically explored in electrokinetic studies. In this chapter, we overcome this limitation by introducing the High-Frequency Quadrature Signal Generator (HF-QSG), an extended version of the original architecture capable of operating up to 300 MHz. This advancement enables a broadband excitation platform, expanding the accessible spectral window far beyond classical DEP/ROT regimes and thereby supporting full-range dielectric interrogation within a single, compact system.

5.2 HF-QSG Electronic System Design

The HF-QSG is a high-performance quadrature sinusoidal signal generator developed to satisfy the stringent requirements of advanced electrokinetic measurements. The functional block diagram and a picture of the electronic device are shown in Fig.5.1A-5.1B, respectively. The system delivers four phase-controlled sinusoidal outputs with amplitudes up to $8 V_{pp}$ on a 50Ω load, while providing a wide operational bandwidth up to 300 MHz.

The HF-QSG is structured as a modular electronic system composed of four main hardware subsystems: (i) a distributed power supply network, (ii) a broadband signal generation unit based on dual Direct Digital Synthesis (DDS) devices, (iii) a quadrature amplification stage providing high-power sinusoidal outputs, and (iv) a phase detection module for real-time monitoring and calibration. These blocks operate in a tightly coordinated manner to ensure phase-coherent excitation and stable amplitude delivery up to 300 MHz. Fig.5.2 illustrates the overall system architecture, highlighting the signal flow from clock distribution and DDS synchronization, through the amplification

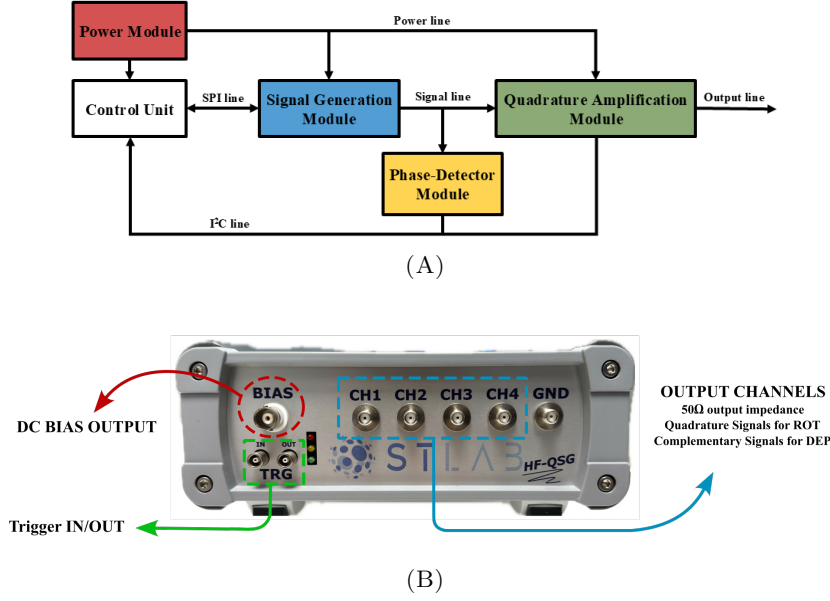


FIGURE 5.1: (a) Functional Block diagram, and (b) front view of the HF-QSG.

chain, to the phase sensing path and digital control unit. This modular organization facilitates electrical isolation between sensitive analog blocks and high-power stages, enabling robust operation across a broad frequency range. The following sections describe each module in detail.

5.2.1 Power Supply distribution

The HF-QSG adopts a distributed power delivery architecture in which each hardware block is supplied through a dedicated regulation stage, see Fig.5.2. (*Power-Line*). This approach enhances electrical isolation among modules and improves thermal dispersion across the Printed Circuit Board (PCB), preventing localized overheating during high-power operation. Particular attention is given to the signal generation subsystem, where each DDS unit is powered by an independent low-noise linear regulator. DDS architectures are intrinsically sensitive to supply fluctuations and ground coupling; therefore, isolating their power domains substantially reduces phase jitter and spurious spectral components. The use of multiple regulators also mitigates the high current demand associated with broadband and 50 Ω operation, reducing thermal gradients and ensuring stable performance across the full operating frequency range. Overall, the power distribution strategy ensures clean supply rails, minimized coupling between analog and digital sections, and improved thermal management—conditions essential for maintaining phase accuracy and signal integrity up to 300 MHz.

5.2.2 Signal Generation Module

The signal generation module is responsible for synthesizing the baseband sinusoidal waveforms from which the four high-frequency outputs of the HF-QSG are derived. It consists of two high-performance DDS devices (AD9915, Analog Devices), each capable of synthesizing broadband sinusoidal signals up

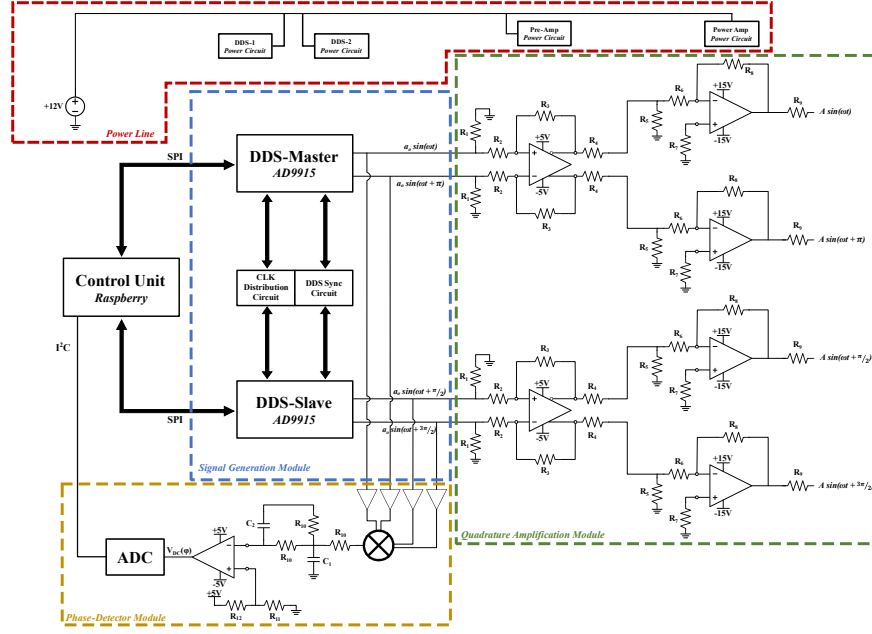


FIGURE 5.2: Block-level architecture of the HF-QSG electronic system. The diagram highlights the modular structure of the platform: the power-distribution network (red block) supplies each subsystem through dedicated regulation stages; the signal-generation module (blue block) hosts the two synchronized DDS devices and the associated clock- and sync-distribution circuitry; the quadrature amplification module (green block) provide high-power outputs for DEP and ROT excitation; and the phase-detection module (yellow block) monitors the relative phase between channels. All subsystems interface with the digital control unit, which manages configuration, synchronization, and signal management.

to the GHz range, see Fig.5.2.(*Signal-Generation-Module*). The AD9915 provides a differential output DAC stage, which is exploited in this design to generate two complementary waveforms. This configuration naturally produces signals in counterphase, at the expense of halving the single-ended available power, but it ensures excellent symmetry, minimizing distortion—an advantageous property for electrokinetic applications. Clocking and synchronization are handled by two dedicated AD9513 clock-management units. The first AD9513 distributes a low-jitter reference clock to both DDS devices using differential LVDS lines, ensuring aligned clock edges and minimizing phase noise contributions. The second AD9513 is employed to implement the multichip synchronization procedure between the two DDS units: it receives the synchronization signal from the DDS-Master and redistributes the regenerated sync signals toward both devices. This mechanism aligns the internal phase accumulators of the two AD9915 units, guaranteeing deterministic phase coherence across the entire operating band. Each DDS synthesizes a waveform of the form

$$v(t) = a_0 \sin(2\pi f_{\text{out}} t + \phi)$$

where the output frequency and phase are controlled respectively via the 32-bit frequency tuning word (FTW) and the 16-bit phase offset word (POW):

$$f_{\text{out}} = \frac{\text{FTW}}{2^{32}} f_{\text{clk}} \quad \phi = \frac{\text{POW}}{2^{16}} 2\pi$$

The HF-QSG generates the four signals required for ROT and DEP by appropriately programming the phase offset between the two DDS units. When the DDS phase offset is set to $\phi = \pi/2$, the combined outputs yield four waveforms in perfect quadrature ($0, \pi/2, \pi, 3\pi/2$). Conversely, when the DDSs are programmed with zero phase offset, the system produces two in-phase sinusoidal waveforms whose differential outputs correspond naturally to the required π pair used in DEP applications. This dual-mode configuration allows the HF-QSG to natively support both DEP and ROT without reconfiguring the hardware, requiring only a change in the programmed phase relationship between the DDS units. All DDS parameters—frequency, amplitude, and phase—are controlled by the digital control unit through an SPI interface, allowing deterministic reconfiguration during operation and enabling rapid switching between DEP and ROT modes as required by the experimental protocol.

5.2.3 Phase Detector Module

To ensure that the phase relationship imposed by the DDS units is preserved, the HF-QSG integrates a phase detector module based on a four-quadrant multiplier, see Fig.5.2.(*Phase-Detector-Module*). The two differential sinusoidal signals generated by the master and slave DDS paths are routed to the phase-detection stage through dedicated buffers, which preserve the controlled-impedance connection toward the amplification chain. The buffered signals are then applied to the inputs of the four-quadrant multiplier. Given the two sinusoidal waveforms

$$v_1(t) = a_0 \sin(\omega t) \quad v_2(t) = a_0 \sin(\omega t + \Delta\phi)$$

the multiplier produces an output signal

$$v_{\text{mul}}(t) = v_1(t) v_2(t) = a_0^2 \left[\frac{1}{2} \cos(\Delta\phi) - \frac{1}{2} \cos(2\omega t + \Delta\phi) \right]$$

that contains a high-frequency component at 2ω and a DC term proportional to $\cos(\Delta\phi)$, which encodes the phase difference between the two input waveforms. A second-order low-pass filter extracts the DC component of $v_{\text{mul}}(t)$, generating a voltage $V_{DC}(\phi)$ that is directly related to the phase offset $\Delta\phi$. The filter stage also introduces a fixed positive offset so that $V_{DC}(\phi)$ remains strictly within the input range of the subsequent analog-to-digital converter (ADC), ensuring compatibility with the unipolar sampling constraints. The filtered and level-shifted phase signal is therefore digitized by the ADC and transmitted to the control unit, where it is used for real-time monitoring of the system phase alignment. This feedback mechanism enables precise verification of quadrature conditions during ROT operation and ensures stable alignment when operating in DEP mode.

5.2.4 Quadrature Amplification Module

The quadrature amplification module receives the two low-power differential signals generated by the DDS stage and delivers the four high-power outputs required for DEP and ROT operations. Since the relative phase between the two signals is already imposed digitally at the signal-generation stage— $\Delta\phi = \pi/2$ for ROT or $\Delta\phi = 0$ for DEP—the primary objective of this module is to preserve that phase relationship while providing the voltage

and current levels needed at the electrode interface. This subsystem replaces and extends the design described in Sec.2.2.1, with a specific emphasis on bandwidth enhancement and RF-grade signal integrity.

The module is organized into two fully symmetric amplification channels, each processing the differential output of one DDS device, see Fig.5.2. (*Quadrature-Amplification-Module*). These signals are routed on controlled-impedance ($50\ \Omega$) differential transmission lines to the input of a fully differential pre-amplifier (ADA4927). This first stage performs a low-noise gain, maintains differential routing to suppress common-mode disturbances. Each single-ended output from the fully differential stage then drives a dedicated high-power branch based on a wideband current-feedback amplifier (THS3491), capable of supplying output currents up to $\pm 400\text{ mA}$. These power stages operate from a $\pm 15\text{ V}$ supply and incorporate input and output matching networks to maintain the $50\ \Omega$ environment along the entire path.

Compared with the earlier QSG implementation, the HF-QSG adopts a fully RF-oriented multilayer PCB design. Particular care was devoted to enforcing geometric symmetry between channels, equalizing the electrical length of all transmission paths from the DDS outputs to the final connectors, and maintaining continuous ground referencing along the entire signal route. The differential-to-single-ended transitions were optimized to minimize imbalance, while controlled-impedance ($50\ \Omega$) routing, matched interstage interfaces, and careful reduction of parasitic capacitances were applied throughout the layout. These RF-grade measures effectively suppress reflections, enhance high-frequency transmission, and preserve the phase coherence required to ensure that all four amplified signals remain closely aligned in both amplitude and phase over the extended operating bandwidth of the HF-QSG. Thermal management is then achieved through the internal temperature-monitoring features of the THS3491 devices. The control unit continuously reads these temperature indicators and can selectively enable or disable each power-amplifier branch to limit self-heating and avoid high-temperature conditions that may degrade high-frequency performance. This supervisory mechanism contributes to the long-term stability and reliability of the HF-QSG, especially during prolonged high-power operation.

5.2.5 Control System and Software Interface

The HF-QSG is managed by an embedded Control Unit based on a Raspberry Pi 4B, which coordinates all hardware modules and ensures deterministic operation across the entire signal-generation chain. This platform was selected for its computational capability, mature software ecosystem, low power consumption, and native support for high-speed digital communication interfaces, making it well-suited for integrated electronic instrumentation.

The internal software running on the Control Unit has been entirely developed in C/C++ to guarantee maximum stability, precise timing, and low-latency interaction with the hardware. This layer directly interfaces with the DDSs via SPI, with the ADCs for phase and diagnostic acquisition, with the digital lines controlling the power stages, and with additional auxiliary functions such as trigger in/out and programmable DC bias outputs. A set of low-level commands defines the core functionalities of the HF-QSG, e.g., frequency tuning, amplitude configuration, phase assignment, power-amplifier enable states, and acquisition of diagnostic parameters. These commands are

exposed to the user through a TCP/IP communication protocol, allowing external clients to send configuration messages or execute customized routines. This structure enables full programmability for users who require direct control of the hardware for specialized or automated experimental protocols.

Above the embedded layer, a high-level graphical user interface (GUI), implemented in Python, provides an intuitive control environment tailored for experimental workflows. Through this interface, the user can select the operating mode (DEP or ROT), set amplitude and frequency parameters, monitor system diagnostics, and execute predefined experimental routines. In particular, the GUI integrates automated spectral-sweep procedures—such as the experimental protocol described in Sec.2.2—where the HF-QSG scans the frequency range required for DEP or ROT characterization according to user-defined timing and step specifications. This abstraction layer allows complex experimental sequences to be executed reliably without requiring direct low-level programming, while still preserving the full flexibility of the underlying command-based architecture.

5.3 Experimental Validation

To assess the performance of the HF-QSG and verify its suitability for broadband electrokinetic characterization, we conducted a series of ROT experiments on two distinct biological cell lines—HL-60 and HeLa—previously analyzed in the low-frequency regime. This enables a direct extension of their dielectric characterization toward higher frequencies, allowing the expanded bandwidth of the HF-QSG to uncover spectral features that were not accessible in earlier measurements. Although the system is inherently compatible with both DEP and ROT methods, the present validation focuses on ROT-based analysis, leveraging the standardized ECP-Framework previously introduced in Chap.4. This choice enables a direct comparison with established reference data while exploiting the expanded bandwidth of the HF-QSG to probe frequency regimes beyond the range accessible in earlier implementations. The following subsections describe the cell preparation procedures, the experimental setup adopted for the ROT assays, and the related results.

5.3.1 Cell cultures and preparation

The two distinct cell lines used to evaluate HF-QSG electronic system performance were the human leukemia suspension cell line HL-60 and the adherent cervical carcinoma cell line HeLa. HeLa cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (100×). HL-60 cells were maintained in RPMI-1640 medium supplemented with 20% FBS and 1% penicillin–streptomycin (100×). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂ and were processed when they reached approximately 80% confluence. Sample preparation followed a common workflow for both cell lines, with adjustments based on their growth characteristics. HL-60 cells were collected directly from suspension, whereas HeLa cells were detached from the culture surface by incubation with EDTA for 4 min and then neutralized by adding complete medium before centrifugation. Cell suspensions were transferred to centrifuge tubes and pelleted at 1500 rpm for 5 min. After removing the supernatant, pellets were washed 3 times and resuspended in 1 mL of buffer

solution, already reported in [127], achieving a final concentration of approximately $8 \times 10^5 \text{ cells/mL}$.

5.3.2 Experimental setup and campaign

For the experimental validation, the ROT-Platform described in Sec.2.3 was employed, with the only difference being the replacement of the original signal-generation system by the newly developed HF-QSG. In particular, the ROT-chip was mounted on a phase-contrast inverted microscope (OPTIKA IM-300 PHi) equipped with a $20\times$ objective. Image acquisition was performed using a CCD camera (Thorlabs 340M Fast Frame) with a resolution of 640×480 pixels (square pixel size of $7.4 \mu\text{m}$). The camera was connected to a host PC via USB and operated at 60 fps using the ThorCamTM software (v.3.7.0). The ROT experiments conducted in this work range from 20 kHz to 200 MHz, thus covering both the membrane-dominated dispersion and the higher-frequency regime in which cytoplasmic conductivity becomes the primary contributor to the dielectric response. This extended spectral window allows the HF-QSG to probe cellular features not accessible with low-frequency generators.

The acquisition protocol generally follows the experimental configuration described in Sec.2.2, ensuring compatibility with our established ROT-platform and analysis pipeline. In the original workflow, the experiment proceeds through repeated cycles comprising: (i) a *rotation phase*, in which the sinusoidal signals are applied with non-zero amplitude to induce cell rotation for a time interval t_1 ; (ii) a *resting phase*, where the signal amplitude is set to zero for a fixed time t_2 , allowing the cell to stop and providing temporal markers for frequency transitions; and (iii) a *sweep phase*, during which the excitation frequency is updated to traverse the electrorotation spectrum. In this case, this protocol has been significantly optimized through the integration of the POLARIS algorithm and the fully automated control software described in the previous chapter. POLARIS enables robust angular-velocity estimation even in the presence of incomplete rotations or low angular velocity, effectively removing the need for frequency-dependent t_1 durations. As a consequence, each experimental cycle now consists of a fixed 1 second *rotation phase* followed by a 0.5 second *resting phase*. This reduction shortens the acquisition of a full ROT spectrum to less than 40 s, substantially increasing the throughput of the characterization pipeline while maintaining reliable extraction of the dielectric response over the full frequency range.

5.3.3 Experimental Results

Fig.5.3 shows the two representative ROT spectra acquired using the HF-QSG for HL-60 and HeLa cells. As said before, the angular velocities are obtained automatically by the POLARIS Algorithm within the ECP-Framework, which also performs the model fitting (using the Single-Shell Model) and the per-cell parameter extraction; all subsequent aggregation, statistical analysis, and plotting were carried out in MATLAB. The spectra exhibit differences in the low-frequency anti-field rotation peaks, with HL-60 centered around $\sim 100 \text{ kHz}$ and HeLa around $\sim 40 \text{ kHz}$, reflecting distinct membrane properties. At higher frequencies, the co-field rotation maxima occur at approximately $\sim 50\text{--}60 \text{ MHz}$ for HL-60 and $\sim 40 \text{ MHz}$ for HeLa; the proximity of the

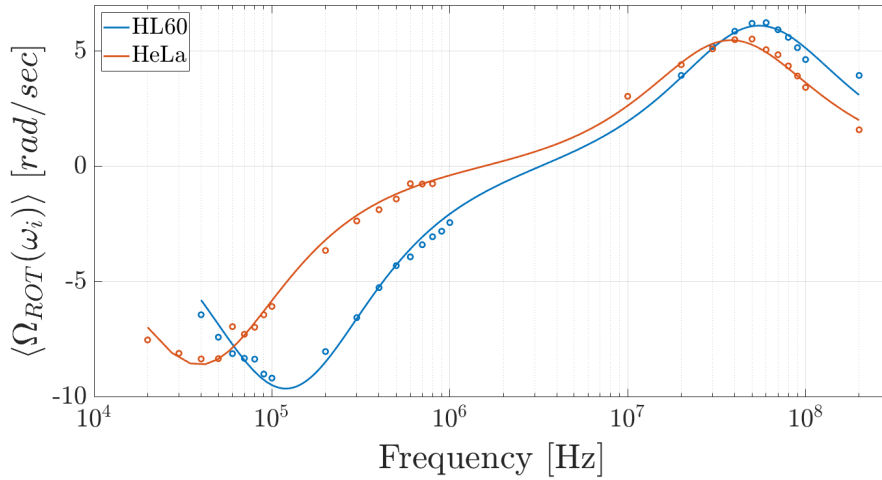


FIGURE 5.3: Representative full ROT-spectra ($\langle \Omega_{ROT}(\omega_i) \rangle$) of the two investigated cell lines: HL-60 and HeLa. The data points represent the measured ROT-spectra obtained through the *POLARIS* Algorithm, and the solid lines show the fitting curve using the Single-Shell Model.

Source	Sum of Squares (SS)	df	Mean Square	F-value	p-value
Groups	1283.99	1	1283.99	61.9	2.14×10^{-7}
Error	394.15	19	20.74		
Total	1678.13	20			

TABLE 5.1: ANOVA performed on membrane capacitance (C_m) values across the considered cell lines: HL-60 and HeLa.

Source	Sum of Squares (SS)	df	Mean Square	F-value	p-value
Groups	0.04	1	0.04	3.91	0.0627
Error	0.21	28	0.01		
Total	0.25	29			

TABLE 5.2: ANOVA performed on cytoplasm conductivity (σ_{cyto}) values across the considered cell lines: HL-60 and HeLa.

high-frequency peaks indicates a comparable level of cytoplasmic conductivity between the two lines.

To quantitatively assess whether the two cell populations exhibit statistically distinguishable dielectric behaviours, an ANOVA test was applied to both the specific membrane capacitance C_m and the cytoplasmic conductivity σ_{cyto} . The analysis reveals a clear distinction in specific membrane capacitance: the ANOVA reported in Tab.5.1 yields an F -value of 61.9 with a corresponding p -value of 2.14×10^{-7} , confirming that the two groups originate from statistically independent distributions. This result is fully consistent with the markedly different capacitance values extracted for each cell line, with HL-60 cells exhibiting $C_m = 14.8 \pm 2.68$ mF/m² and HeLa cells $C_m = 30.4 \pm 5.74$ mF/m². The corresponding radii, 5.86 ± 0.36 μ m for HL-60 and 6.83 ± 0.94 μ m for HeLa, fall within the expected morphological ranges and do not account for the observed difference in capacitance.

In contrast, the ANOVA test performed on the cytoplasmic conductivity (Tab.5.2) returns a p -value of 0.0627, indicating that the two conductivity distributions cannot be considered statistically distinct at the 5% level. This

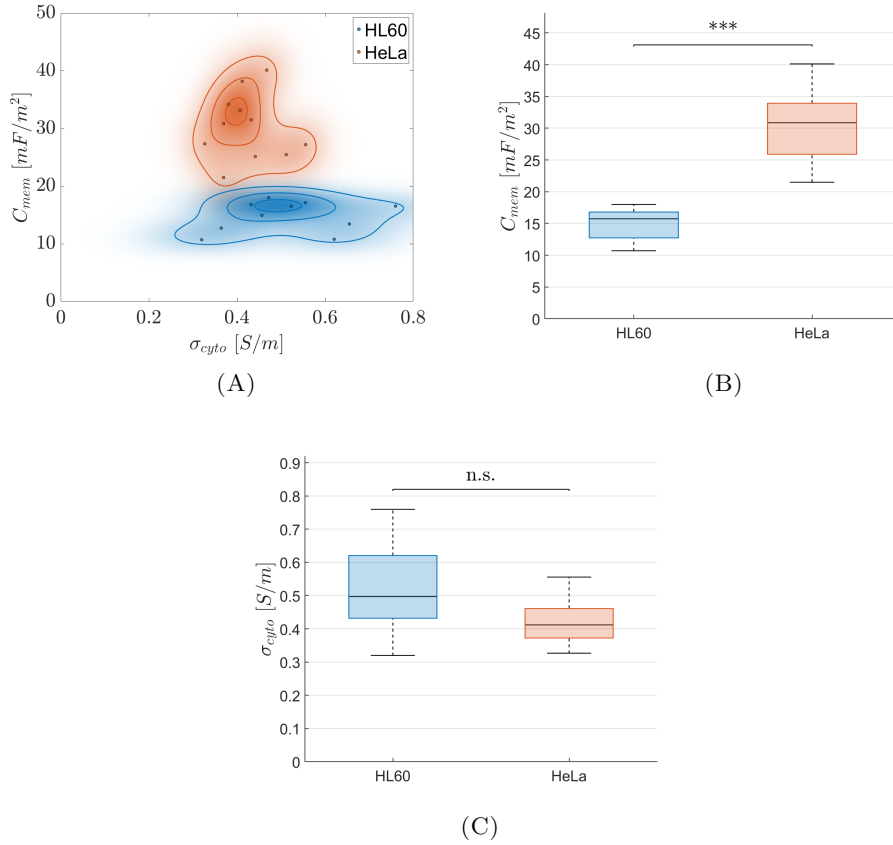


FIGURE 5.4: (A) Scatter plot and density map of specific membrane capacitance vs. cytoplasm conductivity of HL-60 and HeLa cell lines. The averages and standard deviations of (B) specific membrane capacitances, and (C) cytoplasm conductivities. Significant difference: *** $p < 0.00001$, and $n.s > 0.05$.

outcome aligns with the values extracted from the spectra, where HL-60 cells show $\sigma_{cyto} = 0.516 \pm 0.14$ S/m and HeLa cells 0.425 ± 0.07 S/m, a difference that is appreciable but not sufficiently large to achieve statistical significance. These trends are visually captured in Fig.5.4. The two-dimensional density map in Fig.5.4A. clearly shows the distinct separation between HL-60 and HeLa cells along the membrane-capacitance axis. However, the distributions along the cytoplasmic conductivity axis reveal similar values for both cell lines. The boxplots reinforce this interpretation, see Fig.5.4B-5.4C: the membrane-capacitance shows a clear distinction with strong statistical significance, whereas the conductivity exhibits overlapping interquartile ranges and no significance marker. Overall, the combined statistical and visual evidence confirms that the two cell lines are clearly distinguishable in terms of membrane dielectric properties, while their cytoplasmic conductivities remain comparatively similar.

Overall, the experimental findings confirm that the HF-QSG provides stable, broadband, and high-fidelity signal generation suitable for extracting both membrane-related and cytoplasm-related dielectric features from single-cell ROT spectra. The clear separation observed in membrane capacitance between the two cell lines, together with the reliable access to the high-frequency region that informs cytoplasmic conductivity, demonstrates that the proposed

system effectively extends the measurable dielectric domain while preserving the accuracy and consistency of the extracted parameters. These results validate the HF-QSG as a robust and versatile signal-generation system for comprehensive single-cell dielectric characterization.

Conclusions

This PhD thesis presented the design, development, validation, and optimization of an automatic electrorotation-based platform for single-cell dielectric analysis, addressing fundamental limitations of existing approaches and advancing electrorotation (ROT) from a predominantly research-oriented technique toward a practical and automated cell-physiometry tool. By integrating novel hardware architectures, advanced image processing techniques, and a unified software framework, this work establishes a comprehensive technological foundation for next-generation label-free cell characterization.

The first contribution of this thesis concerns the design and implementation of an automated low-frequency ROT-Platform capable of high-throughput dielectric characterization [66]. This platform combines a novel hardware architecture, the *Quadrature Signal Generator* (QSG), with a dedicated first-generation algorithm for the extraction of the electrorotation angular velocity of suspended cells, *PiXel Intensity* (PxI) algorithm. The platform was validated using three distinct cell lines, demonstrating reproducibility, stability, and suitability for population-level dielectric studies.

Building upon this foundation, the second major contribution focuses on the biological application of electrorotation for the dielectric profiling of tumor plasma cells in the clinical context of Multiple Myeloma [130]. This work demonstrated, for the first time, the feasibility of using electrorotation to extract biologically relevant dielectric biomarkers from clinically relevant samples, benchmarking the methodology against conventional bioanalytical techniques. These results highlight the potential of ROT as a complementary physical assay to support hematological diagnostics and deepen the understanding of biophysical alterations associated with malignancies.

A further step forward is represented by the development of the Electro-Cell Physiometry (ECP) Framework [162], a fully integrated software solution designed to automate the complete ROT workflow, from image acquisition to parameter extraction, marking a structural transition from the previous isolated, task-specific algorithmic implementation (*PxI*) to a fully automated and systematically integrated framework governing the entire analytical pipeline. The framework includes: (i) an advanced segmentation and tracking module; (ii) POLARIS, the second-generation angular velocity estimation algorithm that significantly outperforms the previous approach; and

(iii) an automated pipeline for multi-parameter dielectric reconstruction. The ECP-Framework was validated using both a custom simulation environment and experimental datasets, achieving high accuracy, robustness, and no user intervention. This marks an important transition from manual, operator-dependent ROT analysis to standardized and automated single-cell dielectric profiling.

The final advancement presented in this thesis is the High-Frequency QSG-based ROT system as a step forward to the previous low-frequency QSG, enabling full-spectrum electrorotation measurements. This system introduces a new class of sinusoidal generator for high-frequency field synthesis, extending ROT into the MHz regime and enhancing sensitivity to membrane and cytoplasmic dielectric features. Experimental validation confirmed its reliability and opened the way for full broadband dielectric spectroscopy on single cells.

Overall, the combined hardware and software innovations enabled the creation of the first unified, automatic, and high-throughput electrorotation platform, capable of providing reproducible dielectric characterizations with no-operator supervision. This constitutes a significant contribution to the bioengineering and lab-on-chip community, addressing long-standing barriers related to experimental variability, low throughput, and lack of standardization in ROT-based cellular analysis.

The *Automatic Electrorotation-based Cell-Physiometry Platform* has reached a significant level of technological maturity, corresponding to an advanced Technology Readiness Level (TRL). The system is fully operational, experimentally validated, and has already been transferred beyond the laboratory prototype stage, with multiple units deployed to external research institutions. This transition from proof-of-concept to reproducible instrumentation demonstrates the robustness, reliability, and applicability of the developed architecture.

Current developments are focused on further strengthening automation and biological integration. In particular, the integration of microfluidic infrastructures for controlled sample handling is actively underway. The incorporation of microchannels and electroosmotic flow control will enable automated injection, washing, and repositioning procedures, extending the platform toward continuous and long-duration experimental protocols. In parallel, ongoing research aims at deepening the biological interpretation of dielectric parameters, establishing quantitative correlations between ROT-derived signatures and membrane and cytoplasmic biomarkers, metabolic states, and functional phenotypes. This effort, carried out in collaboration with biologists and clinicians, is central to consolidating the translational potential of dielectric cell-physiometry.

Additional technological evolutions are envisioned to further enhance performance and usability. The implementation of AI-driven real-time electrorotation analysis represents an important opportunity to accelerate data processing and provide immediate experimental feedback. However, it should

be emphasized that the current POLARIS framework already ensures high-accuracy and robust post-processing analysis; real-time deep-learning integration would therefore constitute an optimization step rather than a functional requirement. Similarly, the transition toward a fully integrated compact instrument—featuring embedded optics, illumination, and on-chip sensing—represents a natural evolution of the platform toward portability and decentralized diagnostics. While the existing system already performs complete electrorotation experiments with validated workflows, further miniaturization would expand its accessibility and facilitate broader clinical adoption.

Overall, future developments will build upon an already consolidated and validated technological foundation, moving from advanced research instrumentation toward increasingly automated, compact, and clinically translatable dielectric cell-physiometry systems.

In conclusion, this thesis demonstrates that automatic electrorotation represents a powerful and versatile platform for label-free cell characterization, capable of bridging physics-based measurements with biological relevance. The technological advancements developed provide the basis for next-generation dielectric systems and open promising avenues toward compact, automated, and clinically meaningful tools for electro-cell physiometry. The contributions of this work mark an important step in transforming electrorotation from a specialized laboratory technique into an accessible and impactful technology for biomedical research, diagnostics, and single-cell biophysics.

Appendix A

Electrorotation Game: A Simulated Environment for Controlled ROT Experiment

In this appendix, we provide a general description of the *Electrorotation Game*, a compact simulation framework designed to generate microscopy-like sequences with fully prescribed translational and rotational dynamics. The tool offers a controlled environment for benchmarking translation-rotation-aware image-analysis algorithms, enabling systematic exploration of motion-estimation accuracy under reproducible and configurable conditions. In addition to detailing the simulation principles, rendering pipeline, and ground-truth generation, this appendix outlines the software architecture and its intended use as a companion resource for method validation. All source code, together with installation instructions and full documentation, is made openly available in *Electrorotation Game*. The related work has been detailed in a recent manuscript currently under submission.

A.1 Introduction

Automated video analysis is central to bioimage informatics, enabling segmentation, tracking, and motion quantification of individual cells. However, constructing reliable algorithms remains hampered by the scarcity of annotated benchmarks, particularly for phenomena beyond simple translation. Electrorotation (ROT) provides a paradigmatic example of this limitation. In ROT experiments, a rotating electric field induces a torque on polarizable particles or cells, causing them to rotate at an angular velocity dependent on their dielectric properties. These controlled rotation patterns are widely used to probe membrane conductivity, cytoplasmic permittivity, and other biophysical parameters. Yet, despite their relevance, quantitative analysis pipelines for ROT still rely on small, experiment-specific videos, and the field lacks

standardized datasets for evaluating how well algorithms recover rotational dynamics under variable imaging conditions. Indeed, existing methods for self-rotation—such as block matching [169] or area-change analysis [181]—are validated on bespoke electric-field-induced rotation videos and lack standardized test data. Although live-cell simulators such as *SimuCell* and *CellSium* generate realistic images with accurate segmentations [182, 183], they focus on heterogeneity, division, or growth and do not support explicit, user-defined rotation schedules. As a result, there is no widely adopted synthetic benchmark for evaluating rotation-rate extraction or assessing how segmentation and tracking methods cope with rotating cells. To address this gap, we present a lightweight simulator that generates microscopy-like videos in which each cell follows a prescribed translational trajectory and a signed angular-velocity schedule. The simulator is designed to provide controlled user-defined kinematics for individual cells in otherwise realistic microscopy-like scenes, enabling the systematic study of cell rotation in a reproducible and configurable environment.

A.2 Existing Synthetic Data Generators and Motion-Extraction Approaches

Several rule-based simulators underpin bio-image analysis research. *SimuCell* synthesizes fluorescence images by combining cell and organelle models with specified marker distributions and spatial dependencies, producing ground-truth segmentations for downstream analyses [182]. *CellSium* simulates bacterial microcolonies growing in monolayers with configurable growth models and noise, outputting time-lapse images and tracking files for segmentation and lineage analysis [183]. TRAgen generates 2D time-lapse sequences of moving and dividing cells, adding textures, blur and noise [184], while *MitoGen/CytoPacq* produce 3D fluorescence sequences with cell motion, division and photobleaching [185]. These simulators provide realistic cell appearance and translational ground truth but do not offer user control over rotation.

Generative pipelines offer a complementary approach. For example, *SynCellFactory* fine-tunes a spatial cells diffusion model to produce photorealistic cell videos with annotations for segmentation [186], whereas GAN-based frameworks synthesize cell images to augment training data [187]. Such models excel at realism but typically do not allow the user to prescribe motion or rotation at will. Our simulator differs in that it uses simple geometric transformations of real templates to yield interpretable, deterministic motion control.

Rotational–translational motion extraction has been studied in systems where electric fields induce cell movement, including dielectrophoresis and electrorotation [181, 188], in which cells experience a force in non-uniform electric fields and a torque in uniform rotating electric fields, respectively. Early approaches use block matching and template matching to track surface features in each frame [169, 171], and area-change methods infer angular velocity from oscillations in the projected cell area [181]. These methods operate on small, experiment-specific datasets and lack reproducible benchmarks. Comprehensive initiatives such as the *Cell Tracking Challenge* provide annotated movies for segmentation and tracking [189], but they do not include tasks

with controlled rotation. The simulator presented here complements existing resources by focusing on rotation-aware synthetic data with deterministic ground truth.

A.3 Simulation principle and data model

The simulator operates at the image and kinematic level, producing microscopy-like videos by overlaying real cellular appearances onto cell-free background recordings. Backgrounds are provided as short microscopy videos devoid of foreground cells, preserving realistic illumination, optical artifacts and noise. Cell templates are two-dimensional crops of individual cells extracted from experimental phase-contrast microscopy images; each template includes a binary mask defining the cell region and is organized by cell line in a catalogue. Templates retain genuine texture and morphology and are not synthetically generated shapes. Each instantiated template represents a simulated cell whose state at any time is defined by its translational position in the image plane and its in-plane orientation. Translation and rotation are fully prescribed by user-defined kinematic parameters. No physical interactions, dielectrophoresis forces, or biomechanical dynamics are modeled; the simulator is intentionally restricted to geometric and kinematic transformations only. At each frame, the positions and orientations of all cells are evaluated, the corresponding templates are geometrically transformed, and the transformed images are composited onto the background using their masks. In this way, the simulator generates deterministic, fully controlled motion patterns within visually realistic microscopy scenes.

A.3.1 Motion and rotation parameterization

Translational motion is specified by parametric trajectories in two dimensions. The simulator supports stationary, linear, and higher-order polynomial paths. Each trajectory is defined by start point and end points, with optional Bézier control points for curved motion. Rotational motion is prescribed independently of translation through a temporal schedule of signed angular velocities. The schedule consists of contiguous time intervals; within each interval, the angular velocity ω , expressed in radians per second, is fixed. Positive values of ω denote clockwise rotation, negative values denote counterclockwise rotation, and $\omega = 0$ indicates a stationary phase. Given a desired angular velocity ω in radians per second and a video with frame rate f_{fps} (frames per second), the cell's cumulative orientation at frame n is computed by converting the continuous angular velocity into a per-frame increment. The angular increment per frame is

$$\omega_{\text{frame}} = \frac{\omega}{f_{\text{fps}}}$$

The cumulative orientation θ_n at frame n is then given by

$$\theta_n = n \cdot \omega_{\text{frame}} = n \cdot \frac{\omega}{f_{\text{fps}}} \quad n = 0, 1, \dots, N$$

where θ_n represents the total rotation of the cell up to frame n . Each frame contributes a fixed angular increment, producing a piecewise-linear evolution of orientation that strictly follows the specified rotation schedule while remaining consistent with the discrete nature of the simulated video frames. In

the present implementation, the angular-velocity schedule is defined globally and applied identically to all cells, enabling synchronized rotational behavior if required.

A.3.2 Image composition and rendering

Each output frame is generated by overlaying the transformed cell templates onto the corresponding background frame using their associated masks. To approximate experimental image characteristics, Gaussian noise is applied within the masked cell regions, mimicking local camera read noise while preserving the original background intensity distribution. To prevent sharp intensity discontinuities between cells and background, an edge-feathering operation is applied along the mask boundary. This smoothing produces a gradual transition between foreground and background intensities, improving the overall realism of the composite image.

A.3.3 Ground-truth generation

In parallel with video synthesis, the simulator exports a structured ground-truth table that records the kinematic state of each cell at every frame. Each row corresponds to a unique {cell, frame} pair. For each entry, the centroid coordinates (x, y) in pixel units, the instantaneous angular velocity ω in radians per second, and the cumulative rotation angle θ in radians are recorded. These values provide an exact reference for quantitative evaluation of translation and rotation-rate extraction algorithms and can be used to benchmark segmentation and tracking methods via precise cell localization.

A.3.4 Software implementation

The simulator is implemented in Python and makes use of standard scientific and image-processing libraries, including NumPy for numerical operations and OpenCV for image manipulation. The graphical interface is built with PySide6 and PyQtGraph and supports interactive scenario definition and visual preview of simulated sequences. The codebase follows a modular design that separates background handling, template management, motion parameterization, rendering, and ground-truth export. Scenario configurations are stored in configuration files, enabling reproducible experiments and integration into automated evaluation workflows.

A.4 Discussion and conclusions

We introduced a simulator for synthetic microscopy videos that provides explicit control over the translational and rotational motion of cells while preserving a realistic appearance through the use of real backgrounds and cell templates. By combining simple and geometric transformations with local noise and feathering, the simulator yields annotated sequences that support rigorous benchmarking of rotation-rate extraction algorithms and can complement existing segmentation and tracking benchmarks. Our focus on controlled kinematics rather than physical modeling keeps the tool lightweight and accessible. Limitations include the restriction to two-dimensional motion and the absence of interactions or three-dimensional structures. Future extensions

could incorporate per-cell rotation schedules, more complex motion models, or three-dimensional volumes, as well as additional noise models to approximate different imaging modalities. We provide the simulator and associated assets as open source to encourage adoption and further development by the community.

A.5 Results

TABLE A.1: Simulation-based performance assessment (RMSE, MAPE, R^2) computed on the first datasets generated with different levels of tracking residual.

RMSE																													
	CaCo-2					CCD-841					0-pixel Noise					NCI-H929					OPM2					U266			
POLARIS	0.036	0.064	0.172	0.079	0.084	0.252	0.044	0.083	0.047	0.069	0.206	0.121	0.117	0.145	0.127	0.112	0.081	0.085	0.119	0.195	0.165	0.074	0.145	0.146	0.315				
PxI	9.207	0.211	0.211	0.210	0.230	0.210	7.378	2.608	0.435	0.211	0.212	0.210	0.212	0.210	0.219	5.686	0.196	0.213	0.210	0.211	0.210	6.456	0.211	0.212	0.212				
NCC	2.238	2.238	2.238	2.238	2.238	2.536	2.536	2.238	2.813	2.238	2.238	2.238	2.238	2.238	2.238	2.848	2.941	2.848	2.238	2.238	2.238	2.238	2.238	2.238	2.238				
1-pixel Noise																													
POLARIS	0.081	0.227	0.104	0.564	0.247	0.166	0.168	0.160	0.070	0.225	0.568	0.756	0.464	0.342	0.168	0.106	0.132	0.132	1.752	0.917	0.197	0.237	0.558	0.215	0.243				
PxI	9.205	0.210	0.209	0.213	0.233	0.212	7.139	2.632	0.210	0.207	0.214	0.209	0.213	0.213	0.220	5.080	0.229	0.203	0.212	0.211	0.211	3.510	0.210	0.214	0.209				
NCC	3.055	2.169	2.781	2.093	2.708	1.495	2.731	1.116	2.818	2.148	2.726	2.755	0.334	2.316	2.974	2.747	3.293	3.133	2.590	2.706	1.559	1.892	2.103	2.773	2.729				
2-pixel Noise																													
POLARIS	0.604	0.658	0.745	1.638	0.612	0.224	0.796	2.220	0.230	0.549	1.307	1.819	1.639	0.841	0.244	0.531	0.459	0.317	12.057	3.229	0.295	0.787	2.103	0.562	0.612				
PxI	9.193	0.215	0.206	0.210	0.210	6.311	2.361	0.217	0.205	0.213	0.202	0.215	0.210	0.217	3.050	0.219	0.216	0.217	0.207	3.229	0.211	0.351	0.208	0.207	0.217				
NCC	3.383	2.731	2.746	2.669	2.222	2.743	1.841	3.113	3.552	2.834	1.611	4.847	2.663	2.217	2.609	3.539	3.593	3.886	2.851	5.700	2.224	1.650	3.496	1.615	2.638				
MAPE																													
0-pixel Noise																													
POLARIS	0.390	0.640	1.632	0.769	0.827	2.083	0.426	0.846	0.535	0.520	2.043	1.537	1.179	1.327	1.228	0.999	0.776	0.917	1.259	1.779	1.460	0.716	1.247	1.861	2.990				
PxI	49.800	2.377	2.345	2.366	2.444	2.357	33.694	8.555	4.364	2.389	2.391	2.362	2.385	2.370	2.412	20.182	2.153	2.403	2.375	2.366	2.359	19.694	2.366	2.382	2.384				
NCC	11.034	11.034	11.034	11.034	11.051	12.791	12.791	11.034	14.700	11.034	11.034	11.034	11.034	11.034	11.051	14.773	14.786	14.773	11.034	11.034	11.034	11.034	11.043	11.034	11.034				
1-pixel Noise																													
POLARIS	0.706	1.431	1.091	2.939	1.805	1.436	1.148	1.179	0.605	1.382	4.438	3.959	3.198	2.790	1.593	1.021	1.294	1.134	19.424	5.790	1.793	1.631	3.483	1.913	2.424				
PxI	49.787	2.368	2.337	2.378	2.427	2.384	29.933	8.686	2.376	2.378	2.395	2.358	2.371	2.389	2.373	11.578	2.457	2.340	2.386	2.366	2.360	8.796	2.364	2.383	2.362				
NCC	20.584	11.790	15.704	11.723	16.140	6.734	15.004	3.433	14.875	11.293	17.010	16.896	2.772	11.639	18.087	14.854	19.782	19.900	13.574	17.533	6.422	8.872	12.101	16.716	16.817				
2-pixel Noise																													
POLARIS	3.195	4.027	3.276	9.214	4.347	1.950	5.938	14.962	1.078	3.054	9.142	11.707	11.442	5.884	2.184	2.145	2.889	2.309	85.107	17.177	2.481	4.404	11.659	4.407	5.282				
PxI	49.205	2.402	2.342	2.377	2.324	2.363	26.339	8.890	2.437	2.358	2.368	2.322	2.400	2.349	2.375	5.605	2.392	2.423	2.360	2.363	2.365	3.284	2.351	2.358	2.418				
NCC	23.118	17.576	16.055	17.377	12.242	16.276	8.386	19.931	24.390	16.837	8.574	13.794	16.849	11.963	14.635	24.574	24.380	29.386	20.257	22.673	11.649	6.069	26.458	7.815	17.101				
R ²																													
0-pixel Noise																													
POLARIS	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.999					
PxI	-0.097	0.999	0.999	0.999	0.999	0.999	0.999	0.295	0.912	0.998	0.999	0.999	0.999	0.999	0.999	0.582	0.999	0.999	0.999	0.999	0.999	0.461	0.999	0.999	0.999				
NCC	0.947	0.947	0.947	0.947	0.947	0.931	0.931	0.947	0.916	0.947	0.947	0.947	0.947	0.947	0.947	0.913	0.908	0.913	0.947	0.947	0.947	0.947	0.947	0.947	0.947				
1-pixel Noise																													
POLARIS	0.999	0.999	0.999	0.996	0.999	0.999	0.999	0.999	0.999	0.999	0.996	0.993	0.997	0.998	0.999	0.999	0.999	0.999	0.960	0.989	0.999	0.999	0.996	0.999	0.999				
PxI	-0.097	0.999	0.999	0.999	0.999	0.999	0.999	0.340	0.910	0.999	0.999	0.999	0.999	0.999	0.999	0.666	0.999	0.999	0.999	0.999	0.999	0.841	0.999	0.999	0.999				
NCC	0.900	0.950	0.917	0.953	0.922	0.976	0.920	0.987	0.915	0.951	0.921	0.919	0.999	0.943	0.906	0.919	0.884	0.895	0.928	0.922	0.974	0.962	0.953	0.918	0.920				
2-pixel Noise																													
POLARIS	0.995	0.994	0.993	0.965	0.995	0.999	0.992	0.936	0.999	0.996	0.978	0.957	0.965	0.991	0.999	0.996	0.997	0.999	-0.013	0.865	0.999	0.992	0.943	0.996	0.995				
PxI	-0.094	0.999	0.999	0.999	0.999	0.999	0.484	0.928	0.999	0.999	0.999	0.999	0.999	0.999	0.999	0.880	0.999	0.999	0.999	0.999	0.999	0.998	0.999	0.999	0.999				
NCC	0.878	0.920	0.919	0.924	0.947	0.920	0.964	0.896	0.865	0.914	0.972	0.749	0.924	0.948	0.927	0.866	0.862	0.839	0.913	0.653	0.947	0.971	0.869	0.972	0.926				

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