

FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO



Biophotonics Signal Analysis for Biomarker Discovery in Bio-Fluids

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Resumo

O desenvolvimento de dispositivos analíticos com seletividade e sensibilidade, capazes de detectar e diferenciar diferentes classes de partículas, presentes em baixas concentrações, é amplamente exigido por sistemas farmacêuticos e de saúde. Uma das abordagens mais promissoras para diagnóstico e prognóstico de doenças consiste na detecção de biomarcadores em fluidos circulantes humanos (sangue, sêrum, plasma), uma vez que a medição e avaliação dessas moléculas permite detectar mudanças na composição e concentração que podem ser indicadoras de processos patogênicos ou resposta a intervenções terapêuticas. Biossensores baseados em plataformas de fibra ótica são particularmente promissores para responder aos desafios associados com a análise *in-vivo* de moléculas individuais. Recentemente, a ampla variedade de características benéficas apresentadas por fibras óticas levaram ao desenvolvimento de um novo conceito de ferramentas, as *Optical Fiber Tweezers*. O INESC TEC obteve um grande avanço neste tipo de ferramentas ao publicar e patentear um novo método designado *intelligent Lab on Fiber (iLoF)*, capaz de simultaneamente imobilizar pequenas partículas e categorizar a classe, ao analisar o sinal refletido, através de técnicas de Inteligência Artificial. Esta diferenciação é baseada nas assinaturas presentes na luz back-scattered pelas partículas, resultantes da interação do feixe de luz com materiais e meios não homogêneos. De forma a analisar as manifestações dessas interações, e deduzir propriedades de uma amostra, diferentes parâmetros de um sinal ótico podem ser investigados. No método iLoF, como na maioria dos sistemas de processamento de sinal, a informação é analisada através de parâmetros derivados do domínio do tempo e magnitude do espectro de frequências. Nesta dissertação, a fase dos sinais bio-fotônicos adquiridos durante o desenvolvimento da iLoF foi estudada, ao realizar uma análise estatística exploratória a 8 parâmetros derivados do espectro da fase do sinal de *back-scattering* e à porção da fase dos primeiros 40 coeficientes da transformada de Fourier correspondente. Este conjunto de parâmetros baseados na fase mostrou ser sensível e robusto para detectar e discriminar desde simples micropartículas sintéticas a células tumorais mamíferas e nano-vesículas extracelulares com elevado grau de similaridade em suspensões de meio biológico líquido, a concentrações semelhantes às que são encontradas no sangue e derivados. O espectro da fase da luz *back-scattering* adquirida mostrou reter padrões relacionados com propriedades intrínsecas de cada partícula, que foram evidenciados com o aumento de grau de heterogeneidade e complexidade estrutural. Considerando a crescente procura por parâmetros eficazes e discriminativos, capazes de providenciar uma boa performance de técnicas de classificação, os resultados obtidos demonstram que a incorporação de atributos derivados da fase parecem ter uma maior poder discriminativo que os de magnitude, podendo estender os horizontes para sistemas de processamento de sinal bio-fotônico. Assim, a fase é introduzida como uma nova dimensão para extrair parâmetros de padrões óticos discriminativos, com potencial para futuras aplicações em diagnóstico como detecção e identificação de biomarcadores, circulantes em sangue e seus derivados.

Abstract

The development of selective, sensitive, and reliable analytical devices, capable of detecting and discriminating different classes of particles at low concentrations, is highly demanded by health-care and pharmaceutical systems. One of the most promising approaches to disease diagnosis and prognosis is the detection of biomarkers in human circulating fluids (blood, serum, plasma) since the measurement and evaluation of such molecules allows detecting changes in composition and concentration that can be indicators of pathogenic processes or responses to therapeutic interventions. Biosensing probes based on optical fiber platforms are particularly promising to meet the challenges associated with *in-vivo* analysis of single-molecules. Recently, the wide range of beneficial characteristics presented by optical fibers led to the development of a novel concept of “opto-tools”, the *Optical Fiber Tweezers*. INESC TEC has obtained a breakthrough in this type of tools by publishing and patenting a new method called *intelligent Lab on Fiber (iLoF)*, an optical-fiber based approach capable of simultaneously trap small particles and analyze the originating back-scattered signal through AI techniques, that allow automatic particle characterization. This differentiation is based on the light scattering signatures encountered in the signal reflected by the trapped particles, a result of the interactions of light with non-homogeneous materials and media. To analyze the manifestations of such interactions and deduce properties of the samples, different parameters of an optical signal can be investigated. In the iLoF technique and the majority of signal processing systems, information is analyzed through features extracted from the time domain and magnitude of the frequency spectrum. In this dissertation, the phase component of the biophotonic signals acquired during iLoF development was studied, by conducting an exploratory statistical analysis to 8 parameters derived from the phase spectrum of back-scattering signals, and phase portion of the first 40 coefficients of Fourier Transform. This set of attributes proved to be sensitive and robust to detect and discriminate from simple synthetic microparticles to complex cancer-derived mammalian cells and highly similar extracellular nano-vesicles in suspensions of biological liquid media, at “naturally” occurring levels in the blood or derivatives. The phase spectrum of back-scattering signals showed to retain patterns related to the intrinsic properties of each particle, which were enhanced with higher heterogeneity degree and structural complexity. Considering the increasing demand for effective, discriminative features that provide a good performance of classification techniques, the results obtained demonstrate that phase-based features have better discriminative potential than their spectral magnitude counterparts and can extend the horizons of the above-mentioned biophotonic signal processing systems. Furthermore, these findings introduce phase as a potential new domain to obtain discriminative light pattern features, with great potential for future applications in point-of-care diagnosis such as detection and identification of cells or other sub-cell bio-particles with important clinical outcomes, circulating in the blood or its derivatives.

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‘Would you tell me, please, which way I ought to go from here?’
‘That depends a good deal on where you want to get to’, said the Cat.
‘I don’t much care where...’ said Alice.
‘Then it doesn’t matter which way you go’, said the Cat.
‘... so long as I get somewhere’, Alice added as an explanation.
‘Oh, you’re sure to do that’, said the Cat, ‘if you only walk long enough.’

Alice’s Adventures in Wonderland - Lewis Carroll

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Abbreviations and Symbols

1D	1 Dimension
2D	2 Dimensions
3D	3 Dimensions
AI	Artificial Intelligence
CAP	Center of Applied Photonics
C-BER	Center for Biomedical Engineering Research
COT	Conventional Optical Tweezers
DAQ	Data Acquisition Board
DCT	Discrete Cosine Transform
DFT	Discrete Fourier Transform
EVs	Extracellular Vesicles
FBS	Fetal Bovine Serum
FFT	Fast-Fourier Transform
iLoF	<i>intelligent</i> Lab on Fiber
IQR	Interquartile Range
LEDs	Light Emitting Diodes
LOD	Limit of Detection
NA	Numerical aperture
NTA	Nanoparticle tracking analysis
OFTs	Optical Fiber Tweezers
OTs	Optical Tweezers
PBS	Phosphate Buffered Saline
PMMA	Poly(methyl methacrylate)
PS	Polystyrene
RI	Refractive Index
RMS	Root-Mean-Square
SNR	Signal-to-Noise Ratio
STD	Standard Deviation
TEM	Transmission Electron Microscopy

Chapter 1

Introduction

This chapter describes the context and motivation of the research line that will be followed in the present work. Additionally, the goals, main contributions and outline of the document are presented.

1.1 Context

The development of selective, sensitive, and reliable analytical devices, capable of detecting and discriminating different classes of particles at low concentrations, and cope with the challenges associated with *in-vivo* measurements, is highly demanded by healthcare and pharmaceutical systems [1, 2, 3]. This is prompt by the number of biological structures encountered in the human body, capable of not only provide useful insights about human physiology and eminent diseases but also be efficiently used for early diagnosis, drug delivery, and cell targeting. [4, 5]

One of the most promising approaches to disease diagnosis and prognosis is the detection of biomarkers such as circulating tumor cells, RNA, proteins, extracellular vesicles (EVs), among others, in human circulating fluids (blood, serum, plasma) [6]. The measurement and evaluation of such molecules allow to detection of changes in composition and concentration that can be indicators of pathogenic processes or pharmacologic responses to therapeutic interventions [7, 8, 6]. Despite the major potential of biomarker-identification strategies, the detection of structures of such reduced size is a challenging task for conventional single-cell techniques, limited by their bulky systems and inherent diffraction barrier of light [9, 10] and challenged by the required scalability to smaller dimensions [11, 12]. Therefore, a suitable detection method must be selected. In this regard, optical-based detection techniques have been highlighted as very efficient methods, in comparison with chemical, electrical, or even electrochemical ones, by expanding the limits of sensitivity, portability, and spatial resolution [13, 12]. In particular, optical fibers are considered promising tools due to a wide range of beneficial characteristics, namely the minimal size, biocompatibility, flexibility, and non-toxicity [14, 3], which prompts a great interest in Lab-on-Fiber technologies, where new features and functionalities are integrated on the optical-fiber with

the vision of an advanced and miniaturized “all-in-fiber” probe [14, 1, 3]. This increasing popularity led to the development of a novel concept of “opto-tools” - the Optical Fiber Tweezers (OFTs) [14, 15, 16, 17], where the extremity of the optical fiber is patterned or modified to form a microlens that tightly focuses a propagating laser beam, allowing to optically trap particles. [14, 17, 18]

In a recent joint effort from the Center of Biomedical Engineering Research (C-BER) and the Center for Applied Photonics (CAP), INESC TEC has obtained a breakthrough in this type of tools by publishing [19] and patenting [20] a new method called intelligent Lab on Fiber (iLoF), an optical-fiber based approach capable of simultaneously trap small particles and analyze the originating back-scattered signal through Artificial Intelligence (AI) techniques, based on a set of time and magnitude spectral features. On contrary to the common fiber sensors, that are designed to measure one single attribute for sensing purposes, the application of advanced data analysis methods in the iLoF technique allowed to extract 53 parameters from the sensor output signal, that was robust to the detection and discrimination of a wide range of micro and nanoparticles including synthetic microspheres - Poly(methyl methacrylate) (PMMA), Polystyrene (PS) - simple yeast cells, two complex cancer-derived cells, and associated extracellular nano-vesicles, only differing in surface glycosylation profiles. This differentiation is based on the light scattering signatures encountered in the back-scattered signal. The interactions of light with a biological material are a great source of information that can provide valuable insights in life sciences research, to understand the basic functions of molecules and genesis of diseases [21]. By analyzing the different manifestations of such interactions - absorption, scattering, reflection - through changes in the control variables of the light field such as amplitude, frequency, polarization, phase among others, several properties of the samples can be investigated [22]. For instance, analysis of the light scattered by a particle is a widely used method to the identification and characterization of micro and nanoparticles, since it has been widely reported that important properties can be deduced including morphological characteristics (size, shape, composition, orientation, and granularity), subcellular organization [23, 24] or refractive index changes, that can reflect differential protein expression and intercellular cargo among cells, and even reveal infectious status [24, 25, 26, 27]. Considering that scattered signal processing methods are associated with a lower computational cost, allow the detection of objects with dimensions below the light diffraction limit and require less expensive and complex equipment, the combination of such methods with the promising inherent properties of optical fibers and, more specifically, OFTs, proved to be a very efficient, robust and sensitive technique to analyze and differentiate small particles in complex media, which is very promising to “discover novel “biological signatures” of each main disease and contributing for disease subtyping”. [19]

1.2 Motivation

Although fiber-based optical devices present the potential to provide valuable diagnostic properties, a great margin for optimization still exists for application in point-of-care diagnosis, which

requires more efficient measurement capabilities and functionality degree [2, 1]. One major limitation lies in the standard practice of measuring a single attribute, while the sensor output signal can contain much more information that could be used to perform a more complete analysis [28]. Considering this, the application of advanced data analytics methods is pointed to as a promising approach to the development of multifunctional, flexible tools capable to analyze several different parameters. Very few fiber optic sensor configurations involving such kinds of techniques have been proposed in the literature, probably because such techniques require mathematically complex data processing algorithms, associated with a substantial degree of abstraction, and difficult visual interpretation [29, 30]. However, the application of machine learning techniques allows the analysis of a large amount of input data and provides an efficient way to make generalizations, through biostatistics and layered mathematical models [31]. This way is possible to detect patterns and analytical relations that would be difficult to decipher by visual inspection, or by simply analyzing one single attribute [30]. In this process, algorithms characterize each object based on a set of features – a key component in advanced data analytics techniques. Features must be carefully selected since important requirements must be fulfilled so that the differentiation ability of the system is not compromised [32]. Examples of such conditions include robustness to noise, sensibility to the properties of the target and minimal redundancy [33, 32]. Therefore, the increasing demand for sensitive and accurate classification techniques gives rise to the need for effective, discriminative features that assure good performance of the learning techniques. [34]

In the iLoF technique, similar to the majority of signal processing systems, information is captured from temporal-related and magnitude spectral features. In contrast, the phase spectrum of the signals is generally discarded mainly due to the computational challenges associated [35, 34, 36, 37]. However, it has been concluded for different contexts and applications that phase retains important information of the original object for one-dimensional, two-dimensional, and three-dimensional signals. The past results revealed that back-scattered signals acquired through the iLoF OFT setup contain scattering patterns, that allow particle discrimination [38, 19, 9]. It is widely observed and successfully explored - for instance, in radar and sonar systems - that the occurrence of oscillations in time series, due to the interaction of light with the inherent properties of each structure, can provide important object signatures. Considering that phase retains information on the location of events, discriminative patterns can be retained in the phase spectrum, related to the light interactions with structural parameters such as the nature and number of cell layers. If so, the phase-based features can constitute a robust set of information for micro and nanoparticle detection and differentiation, able to be adapted for more complex differentiation problems and applied in bio-sensing platforms, which would represent an important contribution towards the implementation of effective classification systems for real-time diagnostic tools, to be deployed in multi-functional single-molecule analytic tools. Besides, considering the informative content of the phase reported in the literature, it is possible that phase-based features perform better than the commonly derived magnitude parameters in discriminating micro and nano-particles. All these hypotheses, that will be studied during the present dissertation, can be summarized in the following research questions:

1. Can phase information of back-scattered signals be correlated with the structural properties and heterogeneity degree that characterize different synthetic and biological micro and nanoparticles?
2. If so, do phase-derived features have discriminative potential to detect and differentiate micro and nanoparticles, suspended in bio-fluids, based on OFT laser the back-scattering signals?
3. Is the discriminative potential enhanced with an increase in structural complexity of the particles and conditions evaluated?
4. Can phase-based features perform better than the commonly derived magnitude parameters in OFT back-scattering signal differentiation problems?

1.3 Goals

Considering the research questions presented, the main goals of this dissertation are:

1. Create a pipeline for phase computation and processing of biophotonic signals.
2. Create a set of features derived from phase spectrum.
3. Evaluate the potential of phase-based features for micro and nanoparticles detection and discrimination, through multi-class statistical analysis.
4. Compare the results with the previously applied set of features extracted in iLoF approach, based on time and magnitude spectral information.
5. Study and derive conclusions on the relation between the phase component of back-scattered signals and structural properties of biological cells, based on state-of-the-art concepts for light-matter interactions and light scattering measurements for particle characterization.

1.4 Contributions

The main contributions of this dissertation are:

- Pipeline for phase computation, processing and feature extraction.
- New insights on the information content of phase spectrum derived from back-scattering signals, and the relation with structural properties of biological particles.
- New evidences on the potential of phase information to derive features for classification problems, through a set of parameters statistically robust to identify and discriminate microscopic biological particles under optical trapping and extracellular nano-vesicles suspended in bio-fluids, at “naturally” occurring levels in the blood or derivatives (as serum or plasma).

The presented results are reported in a scientific article, entitled "Micron-Sized Bioparticles Detection through Phase Analysis of Back-scattering Signal from Optical Fiber Tweezers: An Exploratory Study", prepared to be submitted on the international peer-reviewed scientific journal *IEEE Transactions on Biomedical Engineering*.

1.5 Outline

Besides Introduction, this document contains 6 more chapters.

Chapter 2 introduces the concepts of single-cell sensing and cell characterization by light scattering and presents the iLoF technology.

Chapter 3 focuses on the exploitation of phase-based features and approaches the mathematical description of phase spectrum, main applications and computational challenges to an efficient extraction and interpretation, where the phase unwrapping problem is explained.

Chapter 4 describes all the methodologies transversal to the experiments conducted in the framework of the present work, including back-scattered signal acquisition and processing procedures, phase spectral representations computation, phase-based features extraction and statistical tests applied.

Chapter 5 describes and discusses the statistical results of the two experiments conducted for Microparticles detection and discrimination.

Chapter 6 describes and discusses the statistical results obtained considering the detection and discrimination of non-static extracellular nano-vesicles in biological fluids and presents general conclusions from the three experiments performed.

Chapter 7 describes the main conclusions, contributions of the present dissertation and gives suggestions on posterior future work.

Chapter 2

Single-cell sensing technologies

This chapter introduces the concept of single-cell analysis to assess cell-to-cell variability among isogenic populations. The principles of light scattering are explained, as well as the applications of such phenomenon for particle characterization. A new fiber-based method called iLoF is presented and described regarding the optical setup and Advanced Data Analytic methods applied. A brief explanation of optical trapping is also provided.

2.1 Cell-to-cell variability among populations

The development of microscopy revolutionized science by revealing morphological and structural information, on a scale and detail that was never been achieved before. This cellular analysis has lead to a shift in the categorization of different cells from macro to microscopic features [39] and the study of molecular characteristics originated classification schemes that provided a better understanding and fundamental knowledge of biological systems [5]. Over the past years, the increasing resolving power of analytical tools revealed new phenotypical characteristics and inter-relationships that have challenged the concept of uniformity among populations [40, 5]. Nowadays, a plethora of evidence exists for heterogeneity in isogenic or clonal cell populations, in both mammalian and bacterial cells [39, 5, 40, 41, 42]. It is reported that individual cells can present several morphological, behavioral, and biochemical differences from each other, as well as shifts in genetic composition and patterns of molecular expression [5]. Such heterogeneity has a strong influence in cell-fate mechanisms like apoptosis and division [43, 44], that are translated into variations in a wide range of important processes including division, gene expression, age, or drug response [40, 11]. Therefore, single-particle characterization has become an indispensable approach to study cells and sub-cell vesicle behavior and understand the mechanisms behind different disease models. For instance, in the development of targeted cancer therapies, molecular heterogeneity is a major barrier since it appears to have a strong link with tumor metastasis, drug resistance, and clinical diagnosis. [43, 45]

For these reasons, there is an increasing interest in characterizing micro and nanoparticles, from different sources – both synthetic and biologic. Besides the exploitation of synthetically produced micro/nanoparticles for cosmetic and food fields, organic particles such as cells and sub-cell vesicles have been highlighted for Biomedical Applications, due to the potential to provide useful information about human physiology [46, 47]. Many examples are reported in the literature of micro and nanostructures that are not only able to provide useful insights about human physiology and eminent diseases but also be efficiently used for tissue engineering, drug delivery, cell targeting, and early diagnosis [4, 5]. For instance, the refractive index (RI) values of cells and cellular organelles can present modifications when infected by parasites, as reported for Red Blood Cells infected by Malaria [27]. The RI distribution can also be used to distinguish different cells, due to differential patterns of protein expression [26]. Other important biological particles include lipoproteins and extracellular vesicles, which have been considered suitable biomarkers for the early diagnosis of certain chronic diseases (e.g., cancer, autoimmune, cardiovascular, infectious, and metabolic diseases) [4]. The precise and tight measurement of such properties and possible changes over time can provide diagnostic insights about an eminent disease.

Considering this, the conventional techniques for cell characterization present a major limitation by performing measurements on population-level data [5, 42, 11]. For instance, Microscopy and Affinity-based techniques, Spectroscopy and Mass Spectrometry analyze thousands of cells per sample [11], yielding information of the average responses of the population [5]. As explained by Di Carlo and Lee [48], the results obtained from bulk-scale measurements “can lead to hidden and misleading biological information”, since cell-to-cell differences are not considered in the mean behavior of the population. Therefore, an examination of cells at an individual level is the ideal approach to understand the cellular plasticity and molecular mechanisms of a cell and its role in a complex environment. [41, 44]

2.2 Single-cell characterization by light scattering

The amount of light scattered by a particle is a widely used parameter to the identification and characterization of micro and nanoparticles, once it retrieves important information related to the sample characteristics. A variety of light scattering-based techniques have been developed over the years, that are now considered gold-standard tools for single-cell detection and characterization [11, 49, 50]. Such techniques allow a rapid, nondestructive, and noninvasive analysis of single particles, thus being suitable for several applications from food industries to chemical and pharmaceutical fields [51]. Once this dissertation is focused on the extraction of relevant parameters from scattered light, it is important to briefly understand the theory behind this type of signal.

Absorption and scattering are the two major interactions that occur when a light beam irradiates a fraction of matter. Scattering is a change in the direction of photons, that is observed when the following conditions are encountered: (1) the irradiated material presents heterogeneity or optical RI variations or (2) when exists optical heterogeneity of the material relative to the surrounding media [50]. In a biological medium, the light beam encounters heterogeneities caused

by the particles suspended in the fluid. The different refractive index between organelles within the cells and particles compared to the fluid medium generates a scattering signal, resultant from the changes in dipole oscillation in the particle electron cloud, induced by the electromagnetic radiation of the incident photons [24, 52]. Considering the interaction of light with a single particle, the presence of multiple layers and diverse internal and external components cause the photons in a light beam to diverge from their original path and propagate in different directions. These phenomena are schematically represented in Figure 2.1.

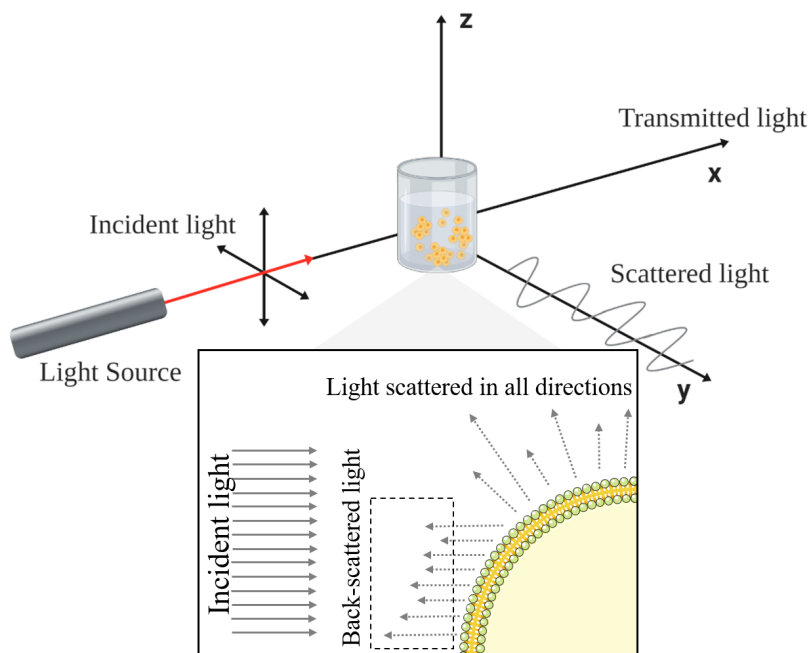


Figure 2.1: Principles of light scattering. The electric field of the incident light hits the molecule, that turns into an oscillating dipole, scattering radiation in all the directions. Heterogeneity and refractive index variations between particles and/or relatively to the surrounding media, lead to changes in the direction of photons, translated in light patterns that allow particle characterization. Back-scattered light is highlighted since it is the type of signal acquired and processed during this work. Based on [53]

Typically, in light scattering experiments, a monochromatic beam is directed to the sample and then a detector records the scattered light at a certain angle. Then, quantitative measurements of laser light scatter characteristics allow the study of crucial features of cells since the amount of light scattered is affected by several important factors including the morphological characteristic of the particle (size, shape, molecular weight, composition, orientation, and granularity), the refractive index and type (biological, synthetic). [23, 24]

Considering the analysis of nanoparticles, current identification and detection techniques have several limitations, mainly related to the size of such particles, that are far beyond the limit of light diffraction [9]. Consequently, suitable equipment and measurement strategies are not yet available. Usually, nanoparticles are analyzed in dispersions, where the light is scattered by a

number of small particles within a solution. In this case, the scattering intensity is the composite of the scattering from all the particles within the scattering volume [53, 50]. Since the particles are in proximity within the volume, effects such as multiple scattering, mutual polarization, and interference between particles can occur, which will be reflected in the signal collected from the detector of a light scattering reading system [53, 54]. Besides, when in a suspension, particles are found in constant movement, mainly due to random thermal translational or rotational motions (Brownian motion). These movements lead to intensity changes in the scattered light, such as fluctuations or jumps, that can retrieve important information of both the motion of the particles and about the particles themselves. [53, 55]

2.3 iLoF: Simultaneous cell trapping and sensing

Optical-based detection techniques have been highlighted as very efficient methods, in comparison with chemical, electrical, or even electrochemical ones, by overcoming the limited sensitivity, portability, and spatial resolution that characterize these techniques [13, 12]. Due to the promising characteristics of light, several optical components such as laser diodes, Light Emitting Diodes (LEDs), or optical fibers have been used to develop miniaturized and portable micro-sensing devices, applied in a wide variety of areas since Biochemistry and Bioanalytics to Optogenetics and Biology [11, 13, 12, 56]. Considering the wide range of beneficial characteristics, since the minimal size, biocompatibility, flexibility, and non-toxicity, optical fiber tools are revealing a great potential [14]. The increasing popularity gave rise to the emerging field of “Lab-on-Fiber” technology, a new concept that envisages an innovating platform where different materials and devices are integrated onto a single optical fiber, enabling the development of sophisticated devices [57, 2]. More specifically, optical fiber tips are being increasingly used to explore micromanipulation, due to their ability to exert piconewton forces on particles and conduct strongly focused optical beams [58]. This led to the origin of Optical Fiber Tweezers, where the optical fiber tip is modified to deliver light to a remote location and simultaneously extract accurate environmental information, creating an effective tool not only to trap and handle particles with translational and rotational precision [59] but also to function as multifunctional, miniaturized sensors for “extracellular, intracellular and intrabody applications”. [14]

Considering all of the above potentialities of optical fiber tools, this research line was followed in INESC TEC, with a joint effort from the C-BER and the CAP. As a result, a breakthrough in this type of tools was obtained with a new published [19] and patented [20] method called intelligent Lab on Fiber (iLoF), capable of simultaneously trap microparticles and analyze short-term back-scattered signals [38, 9, 19]. This approach consists of an optical fiber with a polymeric lens on top, that exerts optical trapping forces that immobilize the particle under test, in a touchless manner. Simultaneously, the back-scattered signal arising from the trapped cell is acquired and analyzed through an Artificial Intelligence algorithm. With this method, optical fiber tools are converted into “intelligent” devices with flexibility, portability, and high resolution, capable of isolation and identification of single cells. [9, 19]

2.3.1 Optical Trapping and Back-Scattered Signal Acquisition Setup

Optical trapping was first described by Arthur Ashkin in 1970 through a series of seminal papers that demonstrated the possibility to stably trap micron-sized dielectric particles through the application of optical forces, which became known as Optical Tweezers (OTs) [60]. In the Optical trapping technique, fluctuating dipoles are generated in the dielectric particle near the focus through a tightly focused laser beam combined with a high numerical aperture (NA) objective lens. In the momentum transference, the particle experiences optical forces during the reflection and refraction of the incident photons, that lead to a trapping phenomenon [60, 59]. Since the particles under analysis during the developed work have dimensions much smaller than the wavelength of light used, the Rayleigh scattering regime is assumed. According to this theory, the trapping forces that act on a particle can be decomposed into two components: scattering and gradient forces, represented in Figure 2.2. Scattering force is generated in the momentum transfer from the radiation field to the particle, pushing the particle away from the beam in the direction of light propagation. On the other hand, the gradient force, proportionally related to the gradient of the intensity of the electric field, is generated in the direction of the spatial light gradient and leads the particle to change the direction into the highest intensity region. [61, 59]. In Figure 2.2, the Conventional Optical Tweezers configuration and optical trapping representation are presented.

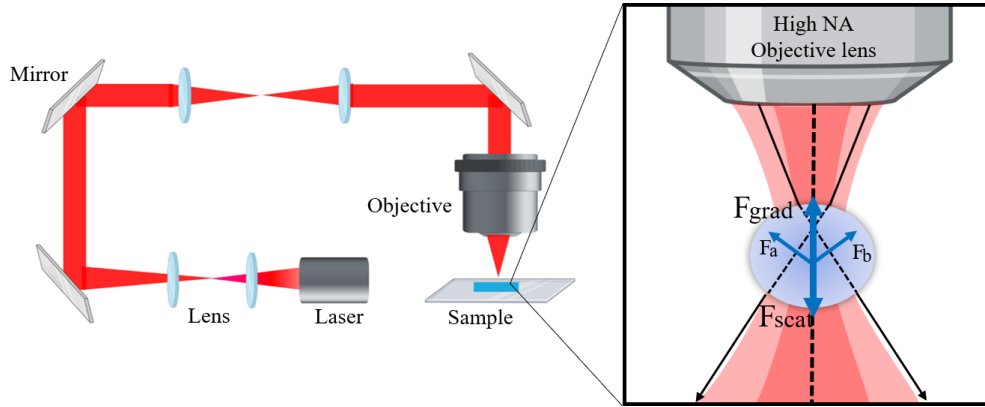


Figure 2.2: (A) Scheme illustrating a Conventional Optical Tweezers configuration [18] (B) Representation of optical trapping. Forces resultant from the interaction of the light beam (black arrows) with the particle. F_{grad} represents the gradient forces and F_{scat} the scattering forces, that allow particle immobilization. F_a and F_b are the reactionary force vectors of each ray on the particle. Based on [14]

In the iLoF method, the trapping of cells is performed through a simple lens-like microstructure, fabricated on the top of a single-mode optical fiber. This is the key element ensuring, in the experiments involving microstructures, stable trapping of particles. A detailed description of the fabrication procedure is provided in several previous works [19, 17]. It consists of a low-cost photopolymerization method, based on assembling, on top of a single-mode fiber, cross-linked polymeric structures through monomers linking, triggered by light of a specific wavelength [18, 62].

The fabrication process and a schematic representation of the setup used for the visualization, manipulation, and acquisition of the back-scattered signal are presented in Figure 2.3. The optical configuration is composed of an inverted microscope connected to three different components: a digital camera, to allow image acquisition of the particles trapped, a motorized micromanipulator to hold a capillary with the optical fiber tip, and a signal acquisition module that acquires the back-scattered signal for the trapped cell through a photodetector connected to a Data Acquisition Board (DAQ). Each sample is irradiated by a 980 nm laser, connected to the optical tip and to the photodetector through an optical fiber coupler. For each solution, the optical fiber tip is inserted into a sample drop, and the laser is then turned on, with the immersed lensed tip carefully positioned in front of an isolated particle. Once the particle is stably trapped, the image acquisition system, connected to a laptop, allows its visualization and the back-scattered signal is acquired [9, 19]. A more detailed description of the back-scattered signal acquisition setup and procedure will be provided in Section 4.2.

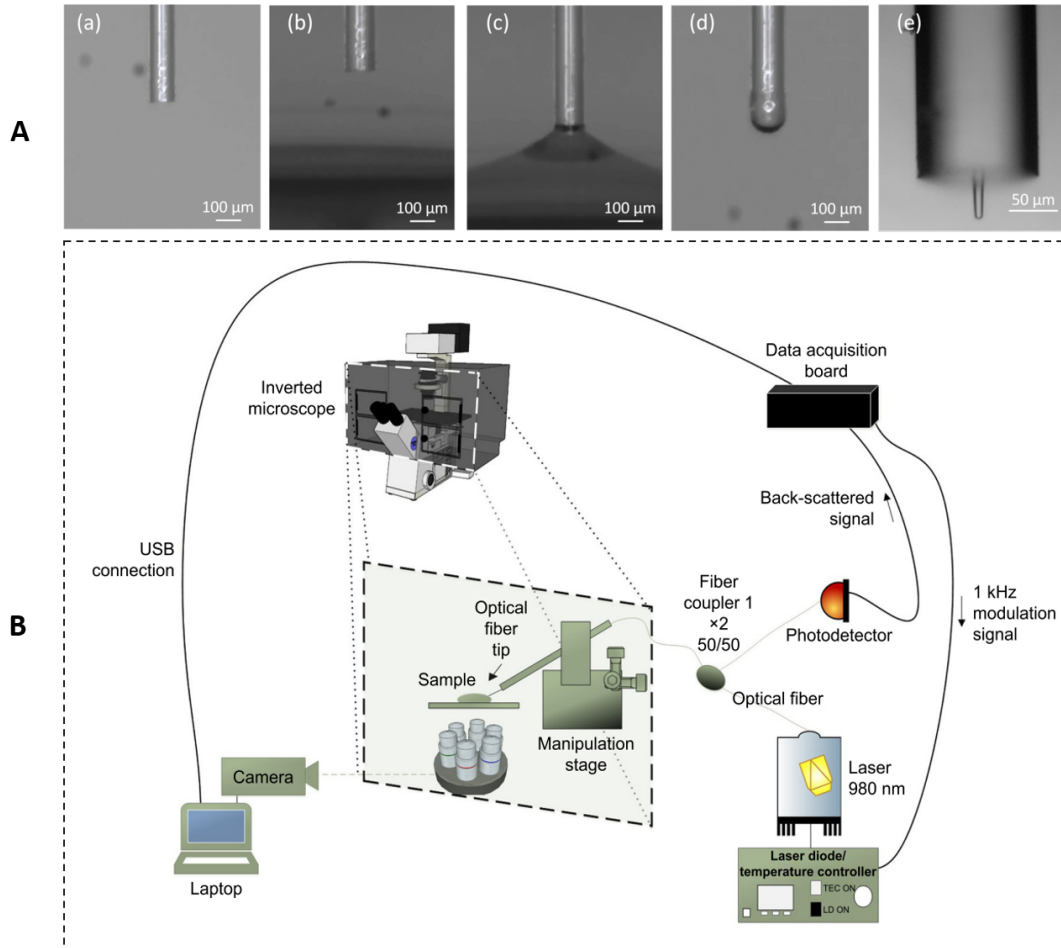


Figure 2.3: (A) Fabrication of the polymeric spherical tips on top of optical fiber, through a photo-polymerization technique [38, 63] (B) Optical manipulation setup for particles trapping and sensing, retrieved from [14, 38]

2.3.2 Advanced Data Analytics Methods

The use of back-scattered signal for target identification is reported in several studies, mainly for fish species recognition in underwater conditions [64, 65, 66, 67, 68] or objects identification in the surrounding environment (in air, water) [69, 70]. It is reported that back-scattering signals retain highly relevant information, that can be used as “signatures” of the target to develop object classification systems. That was the analytical approach in the iLoF technique through which optical fiber tools were converted into “intelligent” devices, capable of characterizing and/or measuring specific properties of the trapped cells and classify them into different types (synthetic vs biological, tumorigenic vs non-tumorigenic) [38, 19]. To do so, a set of 55 features was extracted from the backscatter signals obtained for each particle, based on time-domain and frequency-domain magnitude information [38, 9]. Time-domain features were divided into two subsets: (1) time-domain statistics, including common parameters such Mean, Root Mean Square, Entropy, Standard Deviation, Kurtosis and Skewness, and (2) time-domain histogram-derived features, including two parameters from Nakagami distribution, that is widely and successfully applied in back-scattered signal analysis. The frequency-domain magnitude features extracted from the back-scattered signal also contain two subsets – one based on Discrete Cosine Transform and other derived from Wavelet analysis [9, 19]. The extracted features were then evaluated through statistical analysis and applied into two categories of machine learning algorithms: Supervised Learning (Linear Discriminant Analysis and Artificial Intelligence algorithms) and Unsupervised Learning (Principal Component Analysis and K-means Clustering). [38, 9, 19]

2.3.3 Main Results

Until now, the polymeric fiber tip was able to stably trap and manipulate several particles, from a different source (synthetic, biological), size, complexity degree, and shape [71, 38, 19]. The backscattered signal of trapped particles was then analyzed to study its potential to differentiate distinct types of trapped particles [38]. In an exploratory statistical analysis, the most relevant backscattered signal attributes for particles differentiation were assessed for a dataset involving synthetic particles (PS, PMMA), and biological (Living yeast cell). The results revealed that frequency-domain parameters and Discrete Cosine Transform-based attributes can provide important information about the particle type thus contributing to the development of an OT technique to differentiate particles through its immobilization and back-scattered signal analysis [38]. Linear Discriminant Analysis was also applied in a study with nanoparticles of polystyrene, where a 55 set of features was tested to detect the 100 μm particles in different concentration values. Accuracy values above 90% were achieved, demonstrating that the information provided from the back-scattered signal portions can be used to detect the presence of nanoparticles in a solution. In further works, AI-based algorithms were introduced to automatically identify the type of optically trapped particles [9, 19]. The previous developments lead to the validation of the iLoF method in a study using a human gastric carcinoma cell line [19]. The two types of cancer cell models were successfully immobilized by the fabricated microlens and distinguished by the iLoF

method, which revealed a high distinction power of this approach for two highly similar cancer cells, which represents major progress in the development of detection methodologies for cancer and other diseases based on single-cell fast screening. [19]

2.4 Conclusions

This chapter highlights the importance of single-particle characterization tools to study cells and sub-cell vesicle behaviors and understand the mechanisms behind different disease models. Besides, several examples are presented of micro and nano-structures able to provide insights about human physiology and eminent diseases. The relation between the light scattered by a particle and its morphological properties is presented, and the theory behind the generation and measurement of this type of signal is explained. A fiber-based method developed at INESC TEC, named iLoF, is described regarding the particle trapping and sensing capabilities, optical setup composition, and advanced data analysis methods applied, that allowed the conversion of optical fiber tools in "intelligent" devices able to detect and classify different particles.

Chapter 3

Exploitation of phase-based features

This chapter provides an in-depth description of the mathematical principles of phase spectral information, followed by an explanation of the challenges in its computation and processing. The phase unwrapping technique is discussed regarding the different methods proposed in the past years and the most conventional algorithm - applied in this work - is explained and mathematically described. Besides, the main applications of phase-based information are presented and the differential information content of Fourier magnitude and phase is discussed through the reproduction of well-known experiments of phase intelligibility in reconstructed images.

3.1 Feature Selection

As explained in Chapter 2, back-scattering has been observed and studied for many years, being nowadays an important process for a variety of applications including radar systems (military, atmospheric), astronomy, photography, and medical imaging (ultrasonography, back-scattering x-ray). Besides, as discussed in section 2.2, many different scattering-based techniques have emerged as suitable tools to detect and identify different pathologies in a minimal, non-invasive manner [72, 73]. These developments were driven by the recognition that rich information about the target is retained in back-scattered signals, with great potential to classification/identification tasks [74, 75]. Therefore, light scattering signatures have been exploited for automatic target identification systems in water [64, 65, 66, 67, 68] and air [69, 70] environments. More recently, these are also analyzed for biomedical applications, namely diagnosis algorithms based on the detection of solid breast lesions [76], circulating tumor cell clusters [77], changes in sub-epithelial tissue texture [78] and discrimination between leukemic cells and normal red and white blood cells [79].

In the majority of signal processing systems, information is captured from temporal-related and magnitude spectral features. In contrast, the phase spectrum of a signal is generally discarded [80, 34, 36, 37]. Different reasons are pointed to explain why phase-related information is not commonly used to derive features. In 1843, Ohm [81] stated that only magnitude spectrum information is used by the human auditory system for speech perception, which was confirmed by Helmholtz in 1875 [82], in a series of experiments involving audio signals with equal magnitude

but differential phase spectra. Although the study concluded that no audible differences existed between the different stimulus, many different authors since then have demonstrated the opposite – two signals having the same magnitude spectrum are clearly distinguishable if they have different phase spectral information [83, 84, 85, 86]. Additionally, a considerable problem associated with phase is the complexity in its computation [87, 88]. While the magnitude spectrum is simple to analyze and parametrize into features, the phase spectrum is an abstract representation, and its calculation results in a wrapped, noise-like signal, that requires additional processing to extract meaningful information [34, 37, 89]. Besides, it has been shown, for short frames (20-40 ms), the phase spectrum does not retain a significant amount of information [90, 91, 92, 80], despite opposite conclusions are also found in the literature. [93, 37, 35]

Although these considerations have been supported by different researchers over the years, this is not a consensual topic in the literature and a plethora of evidence exists to support the high informative content of phase for one-dimensional (1D), two-dimensional (2D), and three-dimensional signals (3D). [93, 36, 92]

3.2 Phase calculation and processing

Before exploring the applications of phase-related information, it is important to fully understand the mathematical description of phase spectrum and computational challenges to an efficient calculation and interpretation. The most common method to analyze a phase spectrum is through the Fourier transform function, described in the polar form by [94, 92]:

$$X(\omega) = |X(\omega)|e^{j\phi(\omega)} \quad (3.1)$$

where $|X(\omega)|$ and $\phi(\omega)$ are the magnitude and phase, respectively. Phase angles are calculated from the inverse tangent function [94, 92]:

$$\phi(\omega) = \arctan \left[\frac{\text{Im}(X(\omega))}{\text{Re}(X(\omega))} \right] \quad (3.2)$$

with Im and Re being the imaginary and real components of the complex Fourier spectrum of the data. Due to this calculation, the retrieved phase distribution is forced to the principal value range [94, 92]:

$$-\pi < \phi(\omega) < \pi \quad (3.3)$$

When a true phase value exceeds that interval, it is wrapped around zero, resulting in a discontinuity. Consequently, the signal generated is characterized by rapid phase transitions known as 2π “jumps” [94, 37, 95]. Such discontinuities need to be removed/compensated from the principal values to a continuous phase map, to apply feature extraction methods and extract useful information [37, 89, 96]. The process of determining discontinuities on the wrapped phase, resolving them, and achieving a continuous phase spectrum is called phase unwrapping. [89]

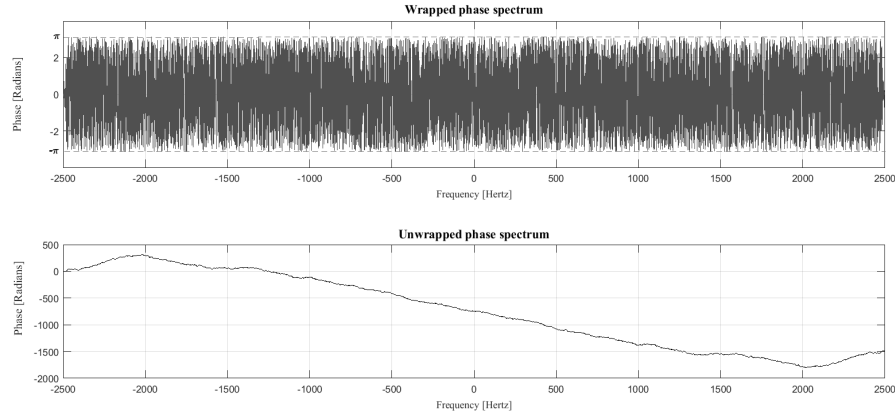


Figure 3.1: Example of Fourier phase spectrum extracted from the a back-scattered signal acquired from a trapped PS particle. Top: Wrapped phase, characterized by discontinuities in the interval $[-\pi, \pi]$. Down: Unwrapped phase map, where discontinuities are removed/compensated through numerical integration phase unwrapping algorithm [97]

3.3 Phase unwrapping

The phase unwrapping process is addressed by a number of authors and has been adapted to a variety of applications such as synthetic aperture radar (SAR), acoustic imaging, magnetic resonance imaging (MRI), among others [98, 96]. Over the years, different methods were presented including Quatieri and Oppenheim's iterative phase unwrapping [99], Steiglitz and Dickinson polynomial factorization technique [100], discontinuities detection method presented by Oppenheim and Schaffer [101] and a more recent algorithm based unwrapping at Nyquist frequency [102]. Despite the variety of methods, these are not commonly used or tested and several difficulties are reported by the authors. The most widely used and accepted phase unwrapping algorithm is the numerical integration method developed by Tribolet [97]. This is considered the conventional phase unwrapping algorithm, which consists of detecting phase wraps, followed by compensation through a 2π addition or subtraction to ensure the continuity of the spectrum. [103, 34]

Mathematically, this phase unwrapping operation is described as [97, 104]:

$$\varphi(x) = \phi(x) + 2\pi k(x) \quad (3.4)$$

where $\varphi(x)$ is the estimated unwrapped phase, $\phi(x)$ the wrapped phase obtained from Eq.(2), and $k(x)$ is the integer value that specifies the corrective offset required [97, 104]. Therefore, the procedure is based on transversing, in the x direction, through the wrapped phase vector to detect the presence of discontinuities between adjacent samples, that are compensated with a 2π addition or 2π subtraction when a difference larger than $+\pi$ or smaller than $-\pi$, respectively, is found [97]. This procedure allows removing the discontinuities to obtain a continuous phase signal [97, 37, 89]. A more detailed description of the phase calculation and unwrapping procedures applied will be provided in Chapter 4, where a validation step was performed using synthesized signals to fully illustrate the operations involved in phase computation and processing.

3.4 Applications of phase information

Despite the challenges of processing the discontinuities in the phase spectrum, it has been concluded for different contexts and applications that phase retains more of the important information of the original object [92]. One common approach used to illustrate this consists on comparing two types of representations: (1) phase-only representations, by discarding the magnitude spectrum information, that is replaced by a unitary value and (2) magnitude-only representations, where the phase is assumed to be zero [90, 93, 92, 105]. Another common experiment involves the reconstruction of two different images, using the inverse Fourier transform, maintaining the magnitude of the original image, and switching the phase vector between the two images. Reproductions of such representations, performed by the Oppenheim method [92] to compare intelligibility of images reconstructed from phase and magnitude content, are presented in Figure 3.2. In general, comparing with the original object, the reconstruction obtained from magnitude-only representations does not present much informative value whereas in phase-only representations many important features in common with the original object are found. In phase-swapping experiments, both reconstructed images present more similarity to the image the phase was taken from, thus confirming that the phase of the Fourier transform is the one that has the most information about the original imaging. [105, 92]

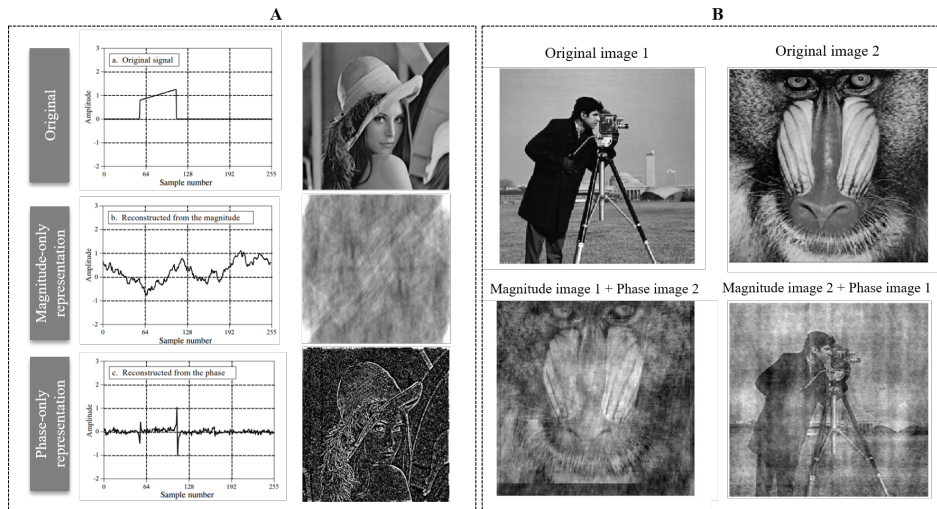


Figure 3.2: Evidence of phase intelligibility. (A) The original object is reconstructed via Inverse Fourier Transform considering magnitude-only (phase is assumed to be zero) and phase-only information (magnitude is replaced by a unitary value). In phase-only representation, the 1D signal retained the location of the edges (From [106]) and in 2D, the main characteristics of the original object are clearly identifiable, while magnitude values do not present much representative value (From [107]). (B) Phase swapping experiment, where the magnitude of the original image is maintained and the Fourier transform phase is switched between the two images, enlightening the presence of important characteristics of the object in phase representation (Based on [92]).

The main fields and applications where phase spectral information is used are presented in Table 3.1. Phase-based features have been successfully applied mostly in audio processing applica-

tions such as speech recognition, speech enhancement, and speaker recognition. For such fields, a large body of previous studies has shown that after compensating the discontinuities and obtaining a continuous phase distribution, the phase-related information significantly assists the classification process, increasing the system performance and may possibly serve to improve recognition accuracy [36, 89]. However, a very limited range of areas explores information extracted from the phase. For instance, the role and potential of phase-based measures for biomedical applications are insufficiently studied and explored, which is exactly the goal of the present dissertation work.

Table 3.1: Table summarizing the main fields and applications where phase spectral information is used and the corresponding contributions

Area	Application	Authors	Main results
Audio processing	Speech synthesis	[108, 109, 110]	Phase spectrum contributes to the quality and naturalness of the synthesized speech
	Automatic Speech Recognition	[80, 111, 112]	Significant improvement in speech intelligibility is gained by the inclusion of phase information
	Speech enhancement	[113, 93]	Utilization of phase information leads to improvements in speech quality
	Speaker verification	[114, 115]	Phase information captures the formant and pitch related vocal differences among speakers
	Voice pathology detection	[116, 117, 118]	Phase-based features are particularly suited for capturing phonation irregularities in the speech signal
	Spoofing signals detection	[119, 120, 121, 122]	Phase-based features present higher sensitivity in detecting synthetic speech
Image processing	Image Coding	[123, 124]	Assigning more bits to coding the phase than the magnitude leads to more intelligibility
	Infrared small target detection	[125, 126]	Image reconstruction from phase spectrum of Fourier Transform is helpful to enhance infrared small target
	Medical image analysis	[127, 128, 123]	Phase-based methods are more robust to noise and attenuation artifacts in ultrasound images Phase image contains structural information that can be used to distinguishing different structures

3.5 Conclusions

In this chapter, the reasons why phase-spectral information is usually not exploited were presented and the theoretical principles behind its calculation were described. The computational challenges to an efficient extraction and interpretation were explained and the phase unwrapping process was discussed. The most conventional method was described regarding its calculation steps and disadvantages. Lastly, the main applications where phase-based features have been successfully applied were presented and examples of phase-intelligibility experiments were provided to discuss the high degree of intelligibility of phase for different contexts and signals.

Chapter 4

Methodologies overview

This chapter provides a description of all the methodologies involved in the framework of the present dissertation. The datasets obtained in the scope of iLoF technique development, and the procedures followed for back-scattered signal acquisition and processing, will be described. Furthermore, the pipeline applied in this work to extract the phase spectral representation of each class will be addressed, as well as the challenges and tests performed to assess its validity to provide a unwrapped phase map. Additionally, the set of phase-based features extracted will be characterized and an explanation of the statistical tests applied will be provided.

4.1 Dataset

In order to study the potential of phase-derived information for microparticles/cells categorization, three sets of experiments were conducted in this dissertation, using the back-scattering signals acquired in the scope of iLoF technique development and validation, where the complexity of targets was sequentially increased. All the information regarding particle characterization and sample preparation protocols, signal acquisition, and processing was explored and provided from such previous works, namely, [38] for simple microparticles, [19] considering cancer-derived cells, and [129] for extracellular nano-vesicles discrimination. The particles involved in each experimental stage and general representation of the work-plan are represented in the scheme of Figure 4.1.

Firstly, phase-derived features were tested to differentiate three simple microspheres and cells trapped by the fiber tip - PMMA, Polystyrene, and living yeast – in distilled water. In the second experiment, the set of features was applied to complex human cells as targets, suspended in PBS. As well-reported in literature, cancer presents a wide variety of subtypes, with a high degree of heterogeneity, which constitutes a major barrier to the effective diagnosis and treatment of this pathology. Recent reports highlight cancer-associated glycoforms at the surface of circulation cells as potential contributors to increase the specificity of cancer biomarker assays and therapeutic approaches [130, 131]. One example is the link between the presence of shorter truncated O-glycans and poor cancer diagnosis [130, 132]. However, the selective detection of such structures requires complex and time-consuming methods [131]. Since previous studies conducted through the iLoF

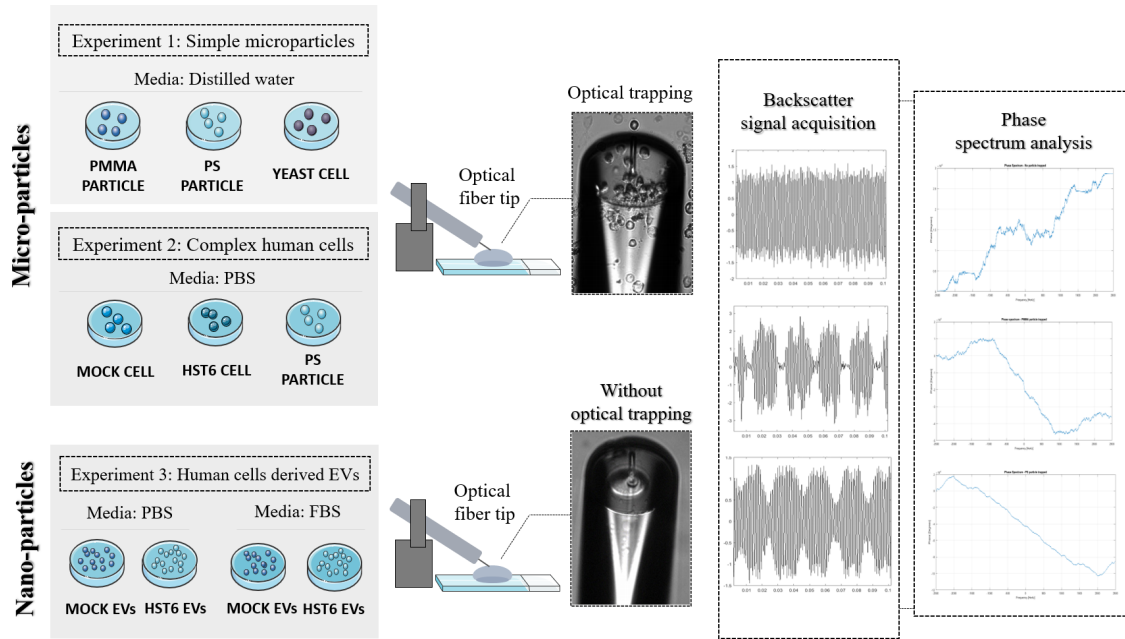


Figure 4.1: Work-plan of the present dissertation, using the dataset acquired in the scope of iLoF technique development and validation. Experiment 1 involves both simple synthetic Poly-methyl methacrylate (PMMA) and Polystyrene (PS) microspheres and Living yeast cells in distilled water [38]; In Experiment 2, the back-scattered signal is acquired from two identical cancer-derived cells, Mock and HST6, and PS particles in Phosphate Buffered Saline (PBS) dispersion [19]. The third experiment involves the analysis of back-scattering signals arising from PBS and Fetal Bovine Serum solutions (FBS) containing extracellular nano-vesicles (EVs) derived from the human cancer cells [129]. After signal processing, the phase spectrum of the back-scattered signals is extracted and analyzed to derive a set of phase-based features for particle detection and differentiation.

method presented a great performance for detection and classification of cancer cell alterations, with high inter-cell similarity, the same dataset was used to test the proposed phase-derived features [19]. It is based on a human gastric carcinoma cell line, where two different cells were used: Mock and HST6 cancer cells [19]. These present the same genetic composition and morphology, only differing in the surface glycosylation since HST6 cells are transfected with a vector over-expressing the ST6GalNAc1 glycosyltransferase, an enzyme leading to the biosynthesis of the well-established tumor-derived carbohydrate STn antigen (Neu5Ac2-6GalNAc-O-Ser/Thr), associated with metastasis and poor prognosis of cancer patients [133]. The third class with PS particles was added, functioning as a control target. [19]

In the third experiment, a smaller target dimension was tested with the use of extracellular vesicles derived from the human-cancer cell line of experiment 2 (MKN45 gastric cell line). Tumor-derived EVs have been considered a suitable source of biomarkers for specific cancer types due to reported changes in protein expression, cargo, and glycoconjugates presence, thus being able to provide a straight categorization of tumor subtypes [134] and contribute for an early cancer diagnosis [47, 135]. Considering its small diameter (about 100 nm), a stable trapping of particles is

Table 4.1: Description of the particles involved in each experiment, corresponding optical and morphological properties and signal acquisition conditions. PMMA - Poly-methyl methacrylate. EVs - Extracellular Vesicles. PBS - Phosphate Buffured Saline. FBS - Fetal Bovine Serum

Experiment	Particles type	Particles source	Particles diameter	Particles RI	Number of particles	Liquid phase	Trapping conditions	
1	Polystyrene spheres	Synthetic	8 (μm)	1.580	18	Distilled water	Yes	
	PMMA spheres	Synthetic	8 (μm)	1.480	16			
	Living yeast cells	Biological	6-7 (μm)	1.500	16			
2	Mock cells	Biological	15.6 $\pm$ 2.9 (μm)	1.360 - 1.370	15	PBS	Yes	
	HST6 cells	Biological	16.2 $\pm$ 3.1 (μm)	1.360 - 1.370	15			
	Polystyrene spheres	Synthetic	8.0 $\pm$ 1.6 (μm)	1.570	10			
3	Mock EVs HST6 EVs	Biological	129.6 $\pm$ 2.7 (nm) 114.9 $\pm$ 1.1 (nm)	1.3345	13	PBS	No	
				1.3345	13			
				1.3362	15	Culture medium + FBS		
				1.3361	15			

not expected [136], contrary to the previous microparticles. The decreased particle diameter leads to a substantial reduction of scattering and gradient forces acting on the particles, consequently reducing the trapping forces profile, which precludes a stable immobilization of particles [136]. However, taking into account the nanoparticle’s geometry and structural properties, there is previous evidence that the reflected signal is able to capture significant information for the detection of nanostructures [9]. Therefore, the EVs dataset was used to analyze the potential of phase-based features, considering two classes of particles - Mock EVs and ST6 EVs – suspended in two types of biological-derived media: Phosphate Buffered Saline (PBS) and culture medium supplemented with Fetal Bovine Serum (FBS). The two different EVs are produced by the cancer cell models of experiment 2, with the same genetic background, only differing in their surface glycosylation. As reported in several studies, EVs acquire the biochemical and structural key features of their “mother” cells, carrying their molecular identity [137, 138]. In this case, EVs present similar size and RI, differing only on the length of EVs surface glycans, although this difference is not statistically significant [129]. More details on EVs characterization will be provided on Chapter 6. For each experiment, a “No particle” class was created by acquiring the signal with the polymeric tip into an empty area, with no particle trapped, in order to evaluate the ability to detect the presence of micro and nanostructures [38, 9]. The information about the different experiments, samples used and signal acquisition conditions are provided in Table 4.1, which describes the complete dataset used in the present study.

4.1.1 Samples preparation protocols

The cancer-derived cells were cultured in RPMI 1640 GlutaMAX, HEPES culture cell medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biowest, Riverside, MO, USA) and maintained at 37n an atmosphere of 5 % CO₂. Both Mock and HST6 cultured cells were routinely tested for mycoplasma contamination and STR profiling was used to confirm cell line identity. Prior to cell trapping and analysis, cells were detached from the flasks by gentle non-enzymatic cell dissociation (Versene, Gibco),

resuspended in PBS, and plated into 35 mm μ -dishes (Ibidi, Germany) [19]. Considering the isolation of EVs from cultured tumor cells, differential centrifugation was firstly conducted. When a confluence of 70% was reached, cells were washed with PBS, and culture medium (non-FBS supplemented RPMI medium) was added. After 48 hours, the supernatant was collected and cell debris was spun at 1200 rpm for 5 minutes followed by another centrifugation at 4000g for 10 minutes and 4 in open-top Ultra-Clear tubes (Beckman Coulter, Fullerton, CA, USA), followed by a washing step with saline solution (0.9% of NaCl) at 100000g and 4°C for 2 hours. Pelleted EVs (for Mock and HST6 types) were then resuspended in saline solution for further analysis, where 3 ml of each dispersion were placed over 35 mm separate μ -dishes. In the supplementation with FBS, a previous filtration step was performed to remove “naturally” occurring EVs providing from bovine-derived cells (for ensuring that the detected exosomes would be only from Mock or ST6 types added to the solution during preparation). The dispersions present an EV concentration of $3.45\text{E}+07$ EVs/ml for the Mock EVs class and $4.38\text{E}+07$ EVs/ml for the HST6 EVs class. [129]

4.2 Optical Trapping and Particles Sensing

As introduced in Chapter 2, the visualization, manipulation, and acquisition of the back-scattered signal is performed through an optical setup composed by an inverted microscope connected to a digital camera, a motorized micromanipulator, and a signal acquisition module, acquiring the back-scattered signal arising from the trapped cell through a photodetector connected to a DAQ, that modulates the light with a 1 kHz sinusoidal signal to translate the frequency analysis of the signal features from the low to the high-frequency range, and reduce the influence of the low-frequency noise, such the 50 Hz electrical grid. Each sample is irradiated by a 980 nm laser, connected to the optical tip and to the photodetector through an optical fiber coupler, that establishes a bidirectional connection between the optical fiber lensed tip, the output signal acquisition system, and the light source, ensuring the simultaneous particle laser light irradiation and trapping, and the collection of the back-scattered light arising from the trapped particle. [38, 9, 19]

4.2.1 Back-Scattered Signal Acquisition

For each solution considered, the optical fiber with the lensed tip on its extremity is inserted into a sample drop containing the particles, placed over a glass coverslip over the inverted microscope setup. The laser is then turned on (@980 nm; optical fiber tip output power of 10 ± 2 mW), with the immersed lensed tip carefully positioned in front of an isolated particle, with the help of the mounted imaging system. Once the particles are stably trapped, the image acquisition system, connected to a laptop, allows its visualization and the backscattered signal is acquired through the photodetector, at a sampling rate of 5 kHz. 120 seconds are acquired in experiment 1 and 80 seconds in experiment 2. Then, in order to create the “No particle trapped” class, signal acquisitions are conducted with no particle in front of the tip [19, 14, 9]. In experiment 3, despite particles are not trapped, the same setup and polymeric lens were used, with a similar acquisition protocol: the optical fiber tip is dipped into a dispersion of EVs and, once completely immersed,

the laser is turned on and the back-scattered signal is continuously acquired for 10 different fiber tip positions determined randomly within the sample, during 60 seconds with a sampling rate of 5 kHz [9].

4.2.2 Back-Scattered Signal Processing Steps

For all the experiments conducted, the same signal processing pipeline was applied to the back-scattered signals, represented in Figure 4.2. After the acquisition, each signal was analyzed and processed through a MATLAB 2019b® custom-built script. Since the input radiation laser was modulated with a 1 kHz sinusoidal signal, low-frequency components, such as the 50 Hz electrical grid component, were removed through a second-order 500 Hz Butterworth high-pass filter. Then, artifacts were eliminated by computing the z-score, followed by the segmentation of each signal into signal portions of 2 seconds. A threshold of $|z\text{-score}| = 5$ was defined to remove noisy signal epochs whose values did exceed the condition, in order to improve the Signal-to-Noise Ratio (SNR). Through these steps, 2-second normalized back-scattered signal portions were obtained for each particle and nanoparticle dispersion was analyzed through the sensing setup [38, 9, 19]. Sketches of processed back-scattered signals for each class and media tested are presented in Figure 4.3. The different classes for each experiment were then created (4-class problem for experiments 1 and 2, two 3-class problem for experiment 3). The following steps involve the computation of fast-Fourier Transform to obtain the frequency-domain representation signals, in order to extract the phase spectrum that is processed through a phase unwrapping procedure. More details on these steps will be further provided. After continuous phase spectral representations were obtained, the set of phase-derived features was extracted and each feature was individually evaluated in the statistical analysis stage, between the selected classes for each problem and all possible pairwise combinations. The architecture of the code employed to perform each step of the signal processing and analysis pipeline is described in Appendix, Section A.1.

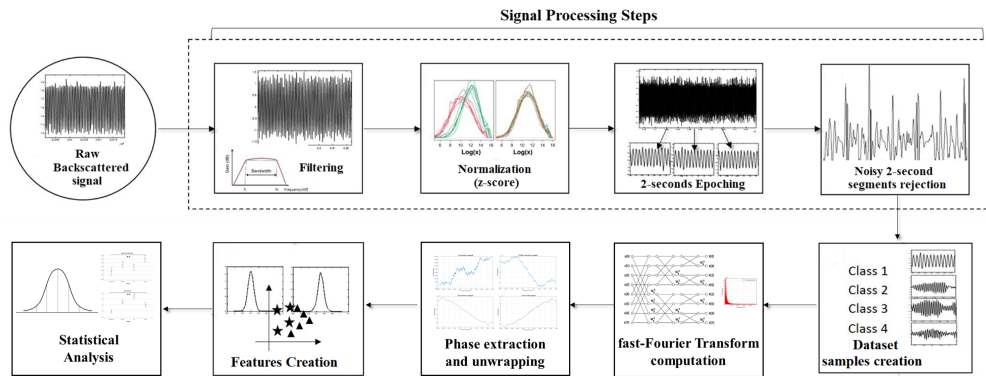


Figure 4.2: Representation of the signal processing and analysis pipeline applied to the acquired back-scattered signals. Adapted from [38]

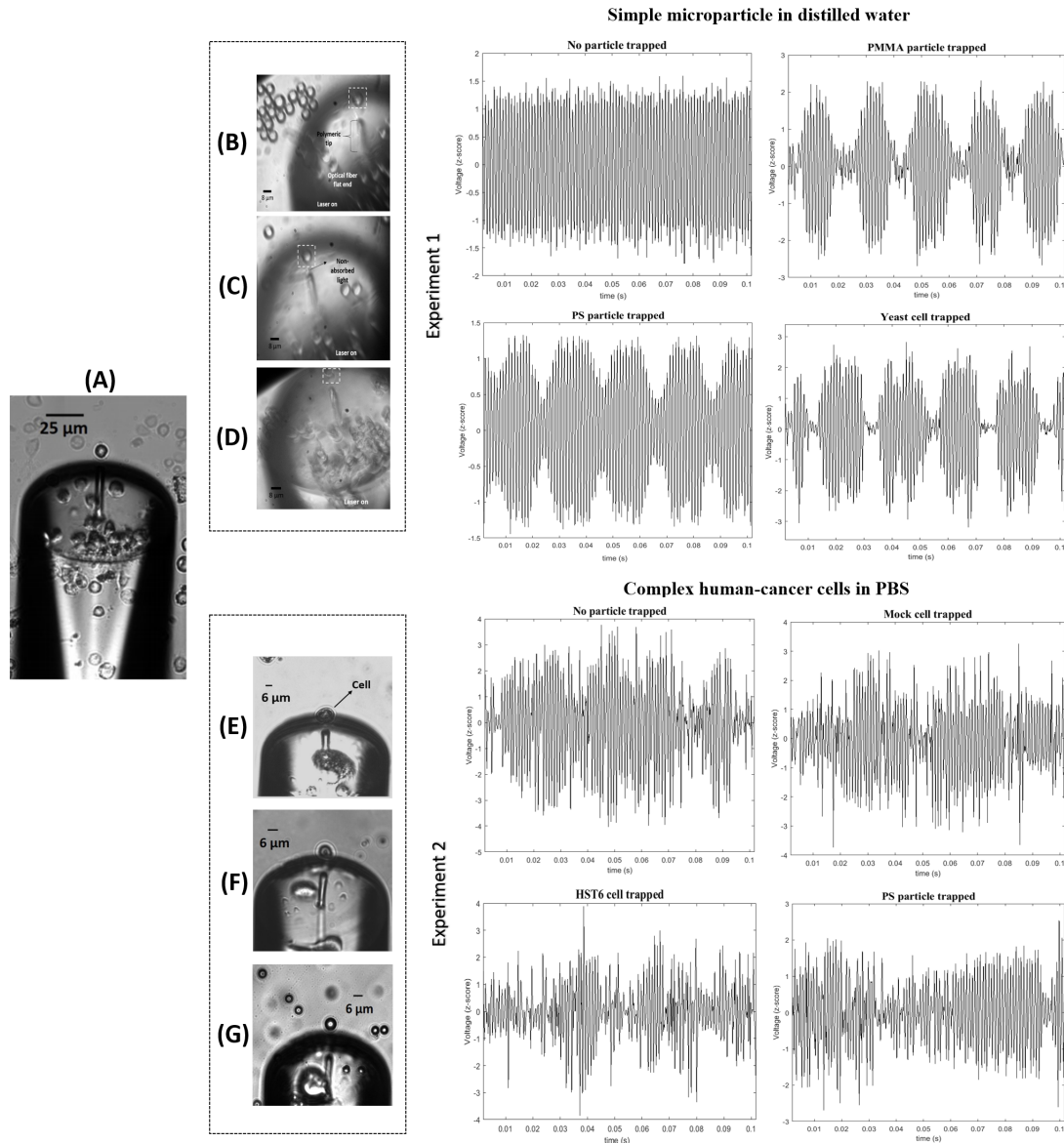


Figure 4.3: (A) Biological particle stably trapped in front of a polymeric lensed optical fiber tip. Bright-field microscopic images acquired for the different particles trapped: (B) PMMA, (C) Polystyrene and (D) Living yeast cell in distilled water (From [38]); (E) Mock cell, (F) HST6 cell, (G) PS particle in Phosphate Buffered Saline (PBS) (From [19]). Particles are trapped while the back-scattered signal is acquired through the same micro-lens on the top of the fiber. Filtered portions of back-scattered signals obtained for each class of particles under analysis after processing steps, for Experiment 1 (top) and Experiment 2 (bottom).

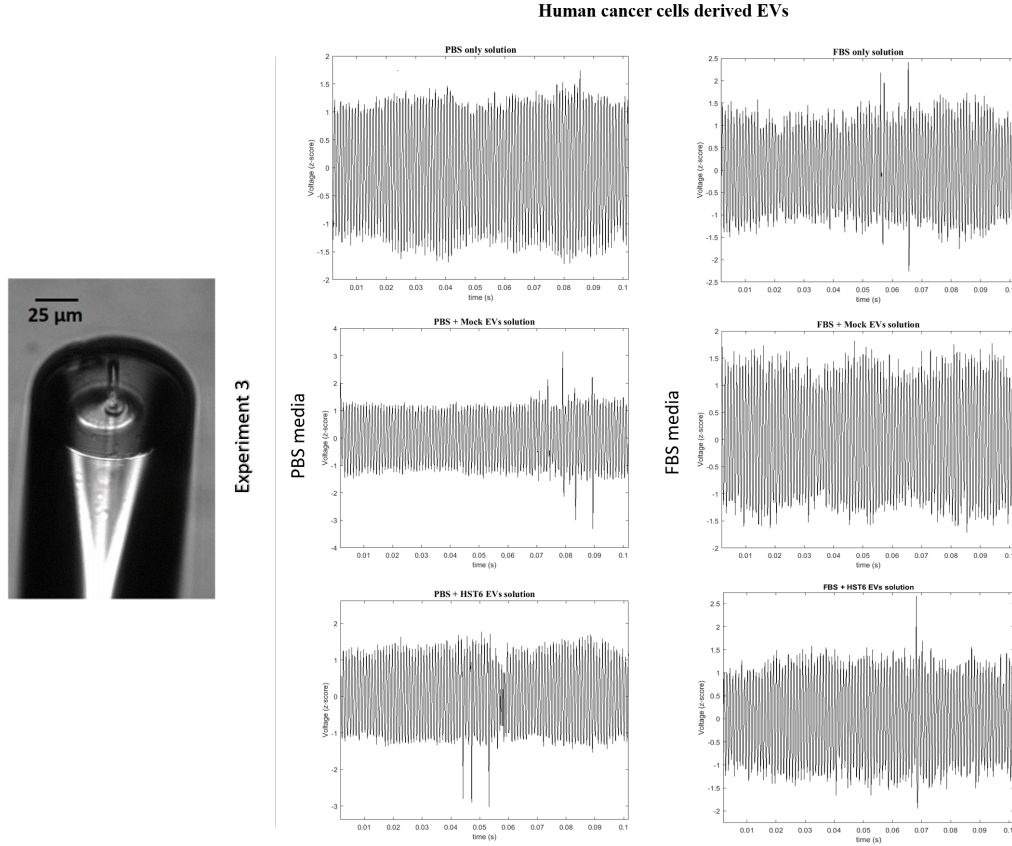


Figure 4.4: Microscopic image of the polymeric lensed tip immersed in a colloidal dispersion of Phosphate Buffered Saline (PBS) containing human cancer-derived extracellular vesicles (EVs) (From [129]). Filtered portions of back-scattered signals obtained for each class of particles under analysis after processing steps. FBS - Fetal Bovine Serum.

4.3 Back-scattered signal-based features

After signal processing, a set of features based on the back-scattered signal phase information, extracted from frequency-domain analysis, was created in order to distinguish particles belonging to different classes or to identify the class of particle, for all the experiments conducted. In order to extract the encoded phase information, the frequency-domain spectral representation of the signal was obtained through the widely known Discrete Fourier Transform (DFT) [106]. Considering a discrete-time signal with N samples,

$$x(n), \quad n = 0, 1, \dots, N-1. \quad (4.1)$$

DFT can be described by [139]:

$$X(k) = \sum_{n=0}^{N-1} x(n) e^{-j \frac{2\pi n k}{N}}, \quad k = 0, 1, \dots, N-1 \quad (4.2)$$

where $x(n)$ is the signal value at sample n and $X(k)$ is a sequence of N complex coefficients

constituting the frequency-domain representations of the finite-time signal sequence, that allow an examination of the information encoded in the frequency, phase, and amplitude. [139, 106, 140]

To implement DFT, the efficient fast-Fourier Transform (FFT) algorithm was applied through the built-in MATLAB function *fft* that, by exploiting the computational redundancy in the calculation of DFT, reduces the number of calculations which results in much faster and precise implementation, with fewer round-off errors [141, 139, 106]. This algorithm is the standard, most commonly used method to extract and study phase encoded information of signals. [106]

4.3.1 Phase calculation

With the signal in frequency-domain after FFT computation, each signal portion was processed through three main procedures. Firstly, the computation of the Fourier phase spectrum was performed according to the previous mathematical description provided in section 3.2. This was followed by small-magnitude values removal, in order to decrease round-off errors. As discussed in Chapter 3, to apply feature extraction methods and obtain useful information, the discontinuities present in the wrapped phase need to be removed/compensated from the principal value range $([-\pi, +\pi])$ to a continuous phase map through phase unwrapping. This constitutes the third procedure, which was performed through the built-in MATLAB function *unwrap*, that applied the conventional Tribolet unwrapping algorithm previously described (section 3.3).

Considering the reduced amount of literature on phase unwrapping and well-known challenges associated, the aforementioned pipeline was tested through a series of validation experiments involving synthetic signals with a linear phase, based on [106]. To do so, a signal with left-right symmetry was generated through the *sinc* function [142]:

$$\text{sinc}(t) = \frac{\sin(\pi t)}{\pi t}. \quad (4.3)$$

When the signal is symmetric with respect to the origin, it is expected to be zero phase. By shifting the signal in time, a change in the slope of the linear phase must be observed. To test this, two variations of the first signal were created. Given a signal $x(t)$:

$$x(t) = A \sin(2\pi f t) \quad (4.4)$$

a time delay and advance were introduced, respectively, by [142]:

$$x_2(t) = A \sin(2\pi f (t - \tau)) \quad (4.5)$$

$$x_3(t) = A \sin(2\pi f (t + \tau)) \quad (4.6)$$

where τ represents the time shift in seconds. This results in the original signal $x(t)$ shifted to the right in $x_2(t)$ and to the left in $x_3(t)$ [142, 106]. This operation is represented in Figure 4.5, for $\tau = 0.5$ seconds.

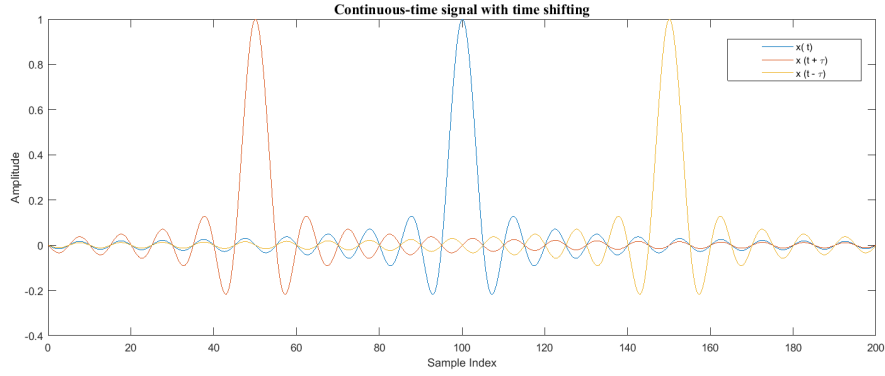


Figure 4.5: Continuous-time signal and its advanced and delayed versions. Based on [106]

The spectral representations obtained after applying FFT, small magnitude values removal, phase calculation and unwrapping are presented on Figure 4.6. After phase calculation, the π discontinuities present were compensated through the phase unwrapping algorithm. As it was expected, a linear phase response is obtained from the symmetrical time domain waveform. When the peak is centered on sample $N/2$, it presents zero phase [106]. By shifting in the time domain, a changing in the slope of the linear phase is observed [106]. Therefore, it confirms that the algorithm applied provides a correct phase representation and can then be used to extract phase spectrum from the back-scattered signals.

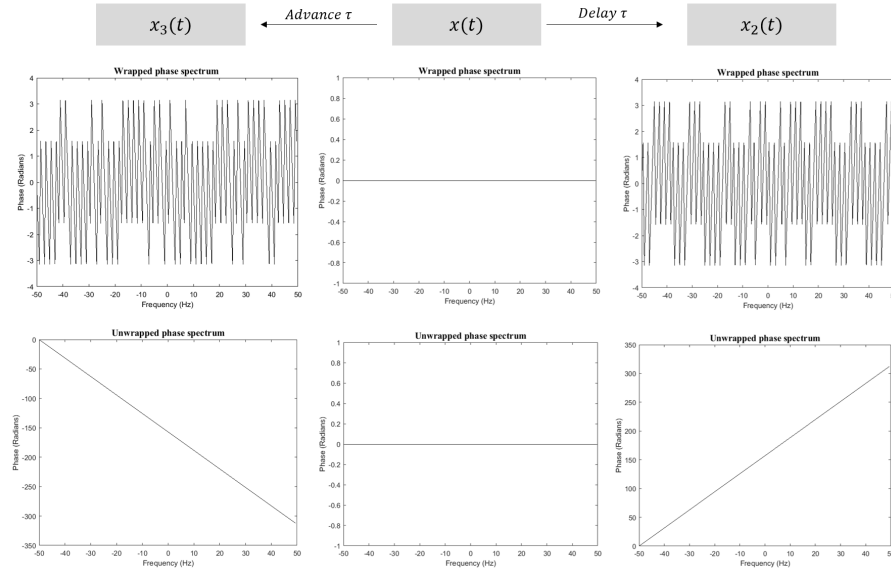


Figure 4.6: Phase changes observed with a time domain shift. After phase calculation and unwrapping, a linear phase is obtained as expected for the symmetrical signals use, which confirms that the algorithm applied provides a correct phase representation.

It is also important to consider that synthetic signals are very simple to unwrap since errors caused by under-sampling and noise can be avoided. However, in practical real-world applications, these problems can be present. One disadvantage of the conventional unwrapping method is

that it cannot discriminate whether the phase "jumps" is due to rapidly changing angles or due to the wrapping ambiguities that can occur when the phase map is disturbed by error sources such as low signal-to-noise ratio or under-sampling [143]. During each back-scattered signal acquisition session, the Nyquist sampling was always considered so under sampling problems were not expected during signal processing. However, low SNR conditions are present [9]. The effect of noise in phase unwrapping is presented in Figure 4.7. The presence of fake discontinuities in the signal is a major concern with phase unwrapping algorithms since an error in the determination of only a single-phase wrap affects all the samples to the right of the signal. This is one of the reasons phase unwrapping is considered a challenging task in digital signal processing [37, 89]. Despite that, as it will be presented, the phase spectrum obtained for each class of particles does not present major discontinuities, thus demonstrating that the back-scattered phase spectrum analysis is performing properly in this type of signal.

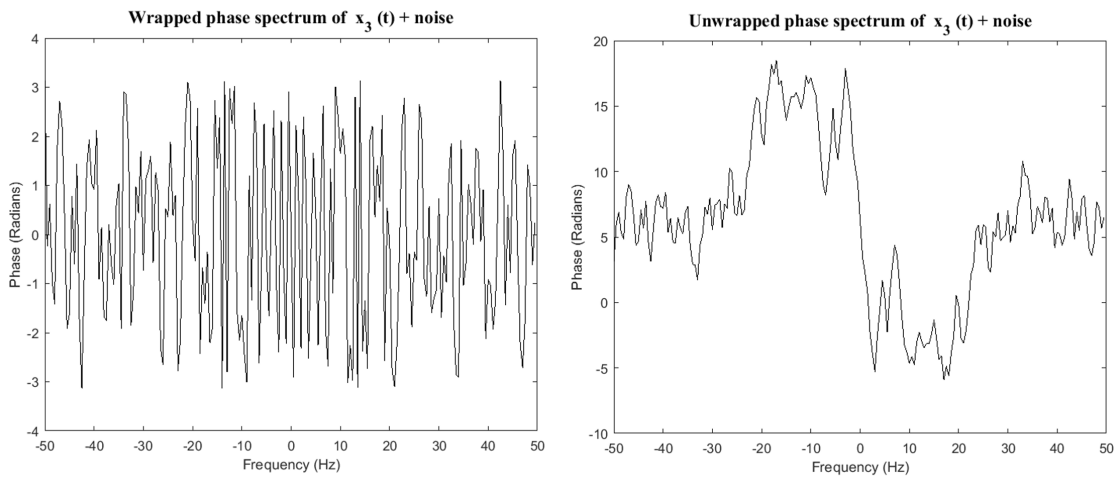


Figure 4.7: Phase unwrapping algorithm applied to a synthetic signal with added noise, that affects the spectrum calculation by inducing fake discontinuities.

A last test was conducted to observe the information content of phase in a 1D signal, also based on [106]. DFT was applied to two different pulse-like waveforms and signal was reconstructed by calculating the phase, replacing the magnitude with random numbers, and taking the Inverse DFT. The results are presented in Figure 4.8. It is observed that the location of the edges is retained, which reveals that information of the location of events in the time domain signal is contained in phase. Therefore, it is suitable to the present problem where it is intended to analyze patterns of back-scattering signal through phase spectrum.

A detailed description of the code used to generate the experiments conducted in this section is provided in Appendix, Section A.2.

4.3.2 Phase-derived features

The high discriminative ability of frequency-domain derived features are well-documented in the literature, with different authors reporting a better performance comparing with groups of fea-

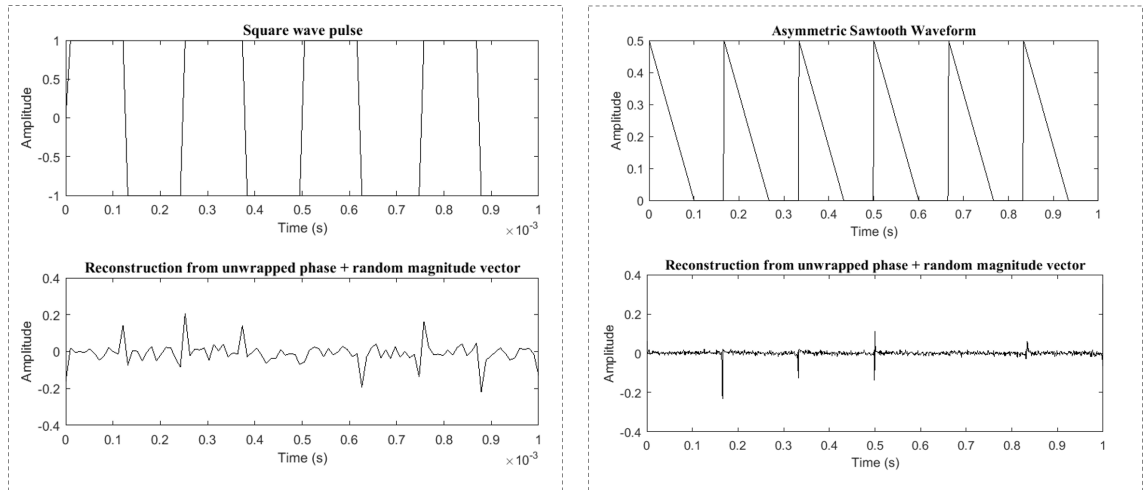


Figure 4.8: Pulse-like waveforms reconstructed to observe the informative content of phase. By replacing the magnitude for random values, the reconstructed signal is based solely on the information contained in the phase. The presence of the edges presented in the original signal reveals the potential of phase to capture patterns related with the location of events in the time domain.

tures such as simple time-domain features [144], wavelet coefficients [145] or spectral energy and entropy derived features [144]. Therefore, there is strong evidence of the suitability of frequency-domain information to characterize mainly short-duration signals [145, 106]. Besides the discriminative potential, the use of FFT is also computationally advantageous, since it is acknowledged that only a small number of coefficients is required to obtain a good representation of the original object [141, 146, 147, 148, 149, 150]. In the mid-'70s, this was explored with great enthusiasm in the information processing area [147]. One of the first examples is the McClure works with NIR spectra in 1984 [141], where it was shown that a limited number of FFT coefficients (~ 50) was sufficient to capture the predominant information contained in the original spectral data and obtain a reliable representation [141]. Since then, FFT-derived information has been used successfully in different areas since face recognition [146] and chemical composition models [148, 149] to gait activity characterization [150, 144, 145] and speech detection experiments [112, 151, 152, 35]. Thus, by retaining important spectral information and providing a simple and direct implementation, with conservation of computer time and space, FFT-derived features show to be suitable to the particle differentiation goal of the present work.

Therefore, after applying the phase extraction procedure presented previously and obtaining the unwrapped phase, a set of frequency-domain derived features was extracted, composed of two different subsets. The first subset, based on descriptive statistics, contains 8 statistical measurements extracted from the phase spectrum (phase as a function of frequency), that include Standard Deviation (STD), Root Mean square (RMS), Interquartile Range (IQR), Skewness, Kurtosis, Range, Variance and Entropy. Scale statistics such as STD, Variance, and Range provide information about the dispersion of the distribution i.e., the amount of scattering. Kurtosis and Skewness are shapes and distribution statistical parameters, reported to efficiently encapsulate distinctive features of the distribution [64, 89]. Kurtosis (4th order moment) indicates the flatness degree of

the distribution, and Skewness (3rd order moment) is related with the degree of asymmetry [153]. RMS and IQR are commonly used as location and quartiles measurements [89]. Such parameters were already applied in time-domain information in previous works [38, 19] and are reported to be efficient in differentiating periodic signals from different origins and sources (synthetic, biological) [154, 155, 156], discriminate pathological from healthy electrocardiograms, identify tumor cell clusters in cell lines [77] and identify different objects also through the back-scattered signal in underwater conditions. [154, 65, 157]

The second subset of features is based on FFT coefficients. It is found in the literature that the majority of feature extraction methods are heavily based on the magnitude of the FFT coefficients, despite being reported that the phase of the coefficients can also play a useful role in various applications [158, 159, 92]. Thus, in this work, the unwrapped phase of the first 40 complex-valued FFT coefficients, given by Equation 4.2, was calculated and used as 40 individual features. This number was already applied in previous works and is supported by different reports that present higher accuracy values for a similar number of coefficients [19, 149, 160]. The complete set of features extracted is presented in Table 4.2.

4.4 Statistical Analysis

After obtaining the complete set of phase-derived features, statistical tests were conducted to evaluate the discrimination ability between the different classes of particles in each experiment.

To choose the correct statistical approach to analyze data, the underlying assumptions of each statistical technique must be verified to avoid unreliable results. In order to check the data variables (in this case, each feature) for any violation of assumptions, data needs to be characterized by obtaining descriptive statistics on the variables. Two important requirements to assess are Normality and Homogeneity. A normal distribution of the variable (symmetrical, bell-shaped curve) and equal variance between populations (in this case, classes of particles) is assumed by many of the parametric statistical techniques [161]. Thereupon, after obtaining the standardized residuals of each variable, the Shapiro-Wilk test was used to evaluate normality, and the Levene test was applied to assess the equality of variances [161]. For all the variables evaluated, the p-value obtained from both tests was below the significance threshold of 0.05, thus indicating a violation of both Normality and Homogeneity assumptions. As a consequence, Non-Parametric tests were selected, since these present less stringent requirements and do not make assumptions about the underlying population distribution [161].

Firstly, a comparison between all the conditions of each experiment was conducted (4-class comparison problem for experiments 1 and 2 and 3-class comparison for each media considered in experiment 3). This was followed by a post-hoc analysis to compare groups in a pairwise manner (2-classes). Post-hoc comparisons are designed to guard against the possibility of an increased Type 1 error (rejection of a true null hypothesis) due to the large number of different comparisons being made [161]. Such tests set more stringent criteria for significance so it is often harder to achieve. In order to evaluate differences between all the conditions, the Kruskal-Wallis test was

Table 4.2: Table summarizing the back-scattered signal phase-derived features evaluated in this study.

Subset	Type	Parameters
1	Unwrapped Phase spectrum statistics	Standard Deviation Root mean square Kurtosis Interquartile Range Skewness Entropy Variance Range
2	FFT Unwrapped Phase Coefficients	1st Coefficient [$\angle X(1)$] 2nd Coefficient [$\angle X(2)$] 3rd Coefficient [$\angle X(3)$] 4th Coefficient [$\angle X(4)$] 5th Coefficient [$\angle X(5)$] 6th Coefficient [$\angle X(6)$] 7th Coefficient [$\angle X(7)$] 8th Coefficient [$\angle X(8)$] 9th Coefficient [$\angle X(9)$] 10th Coefficient [$\angle X(10)$] 11th Coefficient [$\angle X(11)$] 12th Coefficient [$\angle X(12)$] 13th Coefficient [$\angle X(13)$] 14th Coefficient [$\angle X(14)$] 15th Coefficient [$\angle X(15)$] 16th Coefficient [$\angle X(16)$] 17th Coefficient [$\angle X(17)$] 18th Coefficient [$\angle X(18)$] 19th Coefficient [$\angle X(19)$] 20th Coefficient [$\angle X(20)$] 21th Coefficient [$\angle X(21)$] 22th Coefficient [$\angle X(22)$] 23th Coefficient [$\angle X(23)$] 24th Coefficient [$\angle X(24)$] 25th Coefficient [$\angle X(25)$] 26th Coefficient [$\angle X(26)$] 27th Coefficient [$\angle X(27)$] 28th Coefficient [$\angle X(28)$] 29th Coefficient [$\angle X(29)$] 30th Coefficient [$\angle X(30)$] 31th Coefficient [$\angle X(31)$] 32th Coefficient [$\angle X(32)$] 33th Coefficient [$\angle X(33)$] 34th Coefficient [$\angle X(34)$] 35th Coefficient [$\angle X(35)$] 36th Coefficient [$\angle X(36)$] 37th Coefficient [$\angle X(37)$] 38th Coefficient [$\angle X(38)$] 39th Coefficient [$\angle X(39)$] 40th Coefficient [$\angle X(40)$]

selected. This represents the non-parametric alternative to a one-way between-groups analysis of variance, that allows comparing scores for three or more groups, as it was intended [161]. To analyze differences between pairs of conditions, the Mann-Whitney test was selected, the non-parametric alternative to the t-test for independent samples. This is commonly used to test for differences between two independent groups on a continuous measure. For all the statistical tests performed, a significance level of $p - value = 0.05$ was considered, as applied in the previous statistical analysis performed in iLoF [38]. The comparisons evaluated for each experiment are presented in Figure 4.9.

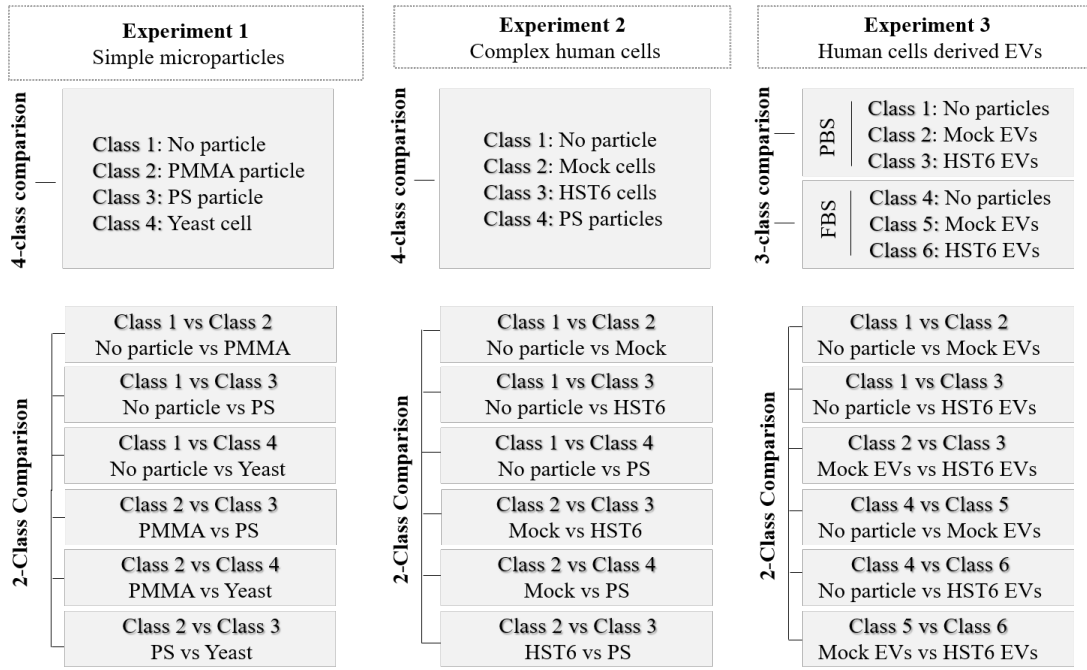


Figure 4.9: Comparisons evaluated by statistical analysis.

Once concluding all the steps of the pipeline for back-scattering signal processing and analysis presented in this chapter (Figure 4.2), the results obtained from both statistical tests described above will be used to analyse the discriminative capacity of the features derived from the unwrapped phase spectrum. Statistical results considering micro-particles trapped by the OFT setup are discussed in Chapter 5, while results regarding detection of nanoparticles in suspension, with no optical trapping, are presented in Chapter 6.

4.5 Conclusion

In this chapter, a detailed description of the methodologies applied during the present work was provided. The particles under analysis for each experiment were characterized and the procedure for acquisition and processing of back-scattered signals was explained. Furthermore, the pipeline used for phase extraction and analysis was presented, regarding its calculation and unwrapping,

followed by a description of the phase-based features selected to characterize each class of particles and statistical tests applied to evaluate the differentiation ability of each feature for micro and nanoparticles.

Chapter 5

Microparticles detection and type distinction

This chapter describes the results regarding the two experiments conducted to evaluate the potential of phase-derived features for trapped microparticles detection and discrimination. More details on the data analysis conducted are provided, the phase spectrum representations obtained for each particle are presented and the statistical results are graphically depicted and discussed considering the discrimination ability of each individual feature.

5.1 Introduction

As explained in section 4.1, the discrimination ability of the unwrapped phase-based features was evaluated for three experiments involving different particles and conditions. The first two experiments involve the analysis of back-scattered signals collected from both synthetic and biological microparticles under trapping conditions, through the optical fiber tip of the OFT setup previously presented. The results obtained in such experiments will be further described and discussed.

5.2 Simple microparticle type distinction

5.2.1 Data analysis

The first experiment was conducted using a dataset of 120 seconds of back-scattered signal acquired in a previous work [38], considering 4 different conditions: "No particle trapped", "PMMA particle trapped", "Polystyrene particle trapped" and "living yeast cell trapped", in a dispersion of distilled water. All the particles present a similar size in order to exclude the hypothesis of particle differentiation due to the influence of the target size. After signal processing steps (section 4.2.2), the dataset was composed of back-scattered signal portions of 2 seconds. A more detailed description is provided on Table 5.1. From here, the unwrapped phase spectral representations were obtained for each class, presented in Figure 5.1. Finally, the set of phase-derived features

was extracted and each feature was individually evaluated in the statistical analysis stage, between the 4 classes and all possible pairwise combinations between these four classes (Figure 4.9).

Table 5.1: Dataset characterization after signal processing steps, for Experiment 1. Each sample corresponds to each 2 second portion of back-scattered signal. Sampling frequency = $5kHz$

Class	1	2	3	4
	No particle	PMMA particle	PS particle	Living yeast cell
Number of trapped Particles	16	16	18	16
Average number of signal portions per particle	59 ± 0	57 ± 2	54 ± 5	59 ± 2
Total number of 2-second signal portions	949	919	971	939

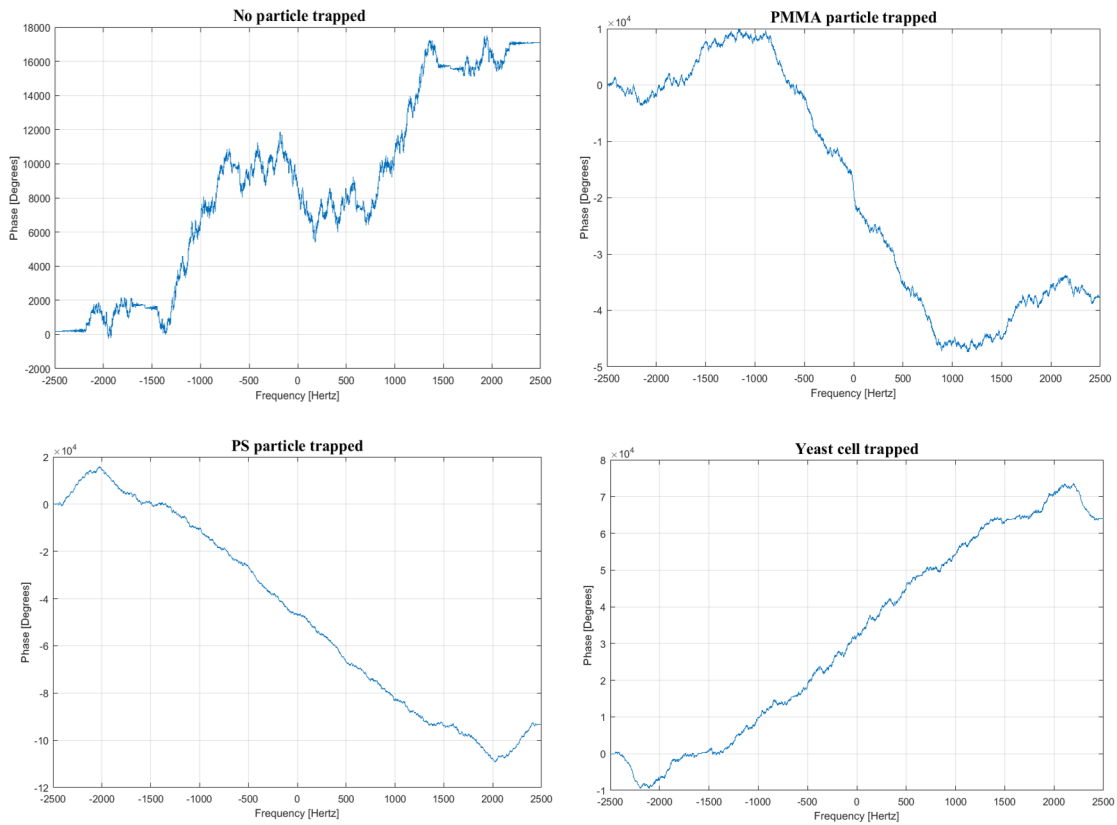


Figure 5.1: Examples of unwrapped phase spectrum obtained after signal processing and phase unwrapping procedures, for each condition evaluated - No particle trapped (Class 1), PMMA particle (Class 2), PS particle (Class 3) and Living yeast cell (Class 4).

5.2.2 Results and discussion

The p-value results obtained for each test applied (Kruskal-Wallis, Mann-Whitney) are presented on Table 5.2.

Table 5.2: Obtained p-value results of the statistical comparisons regarding 4 classes - No particle trapped (C1), PMMA particle trapped (C2), PS particle trapped (C3) and Yeast cell (C4) - using Kruskal-Wallis test and post-hoc pairwise comparisons (Mann-Whitney test) conducted on Experiment 1. A significance level of 0.05 was considered for all experiments. Results above the significance threshold (p-value > 0.05; two-tailed) are highlighted.

		Comparisons						
		4 class comparison	C1 vs C2	C1 vs C3	C1 vs C4	C2 vs C3	C2 vs C4	C3 vs C4
Phase-spectrum statistical features	STD	3,56E-07	0,011018	4,40E-05	2,94E-07	0,097085	6,99E-04	0,095683
	RMS	3,06E-06	0,004859	7,91E-09	6,28E-08	0,000433	5,33E-04	0,906811
	Skewness	0,994931	0,949289	0,809720	0,951387	0,863529	0,975206	0,818721
	Kurtosis	7,68E-08	2,49E-07	1,86E-08	4,28E-06	2,62E-06	0,023432	5,16E-08
	IQR	2,92E-05	0,514024	0,843034	2,23E-04	0,340428	2,77E-04	9,28E-06
	Entropy	3,56E-07	0,011018	4,39E-05	2,94E-07	0,097085	6,78E-04	0,095683
	Variance	0,022139	3,56E-06	1,25E-07	2,46E-08	0,001214	0,004327	0,617616
	Range	4,99E-07	0,019249	0,037887	0,066471	0,029264	0,069230	0,152176
FFT unwrapped phase coefficients	1st Coeff.	3,78E-05	2,27E-05	0,000662	6,75E-05	0,300538	0,215221	0,315900
	2nd Coeff.	4,88E-05	2,67E-05	0,000798	8,58E-05	0,297406	0,225228	0,314001
	3rd Coeff.	5,68E-05	3,07E-05	0,000882	9,42E-05	0,298746	0,226928	0,319003
	4th Coeff.	6,37E-05	3,41E-05	0,000928	0,000105	0,295516	0,230017	0,316912
	5th Coeff.	7,20E-05	3,87E-05	0,001018	0,000113	0,291044	0,233681	0,318884
	6th Coeff.	7,88E-05	4,17E-05	0,001132	0,000118	0,287435	0,236491	0,316734
	7th Coeff.	8,51E-05	4,61E-05	0,001181	0,000122	0,283343	0,239221	0,319812
	8th Coeff.	9,89E-05	5,29E-05	0,001295	0,000137	0,283396	0,239186	0,321885
	9th Coeff.	0,000108	5,69E-05	0,001377	0,000148	0,283208	0,242042	0,322487
	10th Coeff.	0,000119	6,31E-05	0,001490	0,000156	0,280896	0,251832	0,323876
	11th Coeff.	0,000136	6,81E-05	0,001606	0,000181	0,280146	0,252822	0,323997
	12th Coeff.	0,000149	7,28E-05	0,001716	0,000197	0,275409	0,253364	0,322126
	13th Coeff.	0,000158	7,69E-05	0,001767	0,000207	0,274142	0,256647	0,320923
	14th Coeff.	0,000179	8,59E-05	0,001896	0,000231	0,271147	0,257687	0,321223
	15th Coeff.	0,000195	9,01E-05	0,002058	0,000249	0,270937	0,259831	0,318704
	16th Coeff.	0,000213	9,31E-05	0,002221	0,000275	0,271749	0,260714	0,316079
	17th Coeff.	0,000228	9,86E-05	0,002360	0,000286	0,271697	0,265496	0,316675
	18th Coeff.	0,000229	9,94E-05	0,002408	0,000286	0,269189	0,271094	0,314119
	19th Coeff.	0,000243	0,000106	0,002549	0,000290	0,266257	0,276275	0,315484
	20th Coeff.	0,000251	0,000107	0,002641	0,000302	0,264658	0,282027	0,313764
	21th Coeff.	0,000259	0,000107	0,002641	0,000302	0,264658	0,287269	0,313764
	22th Coeff.	0,000279	0,000116	0,002858	0,000331	0,263527	0,287998	0,312877
	23th Coeff.	0,000301	0,000122	0,002973	0,000360	0,262809	0,287387	0,313823
	24th Coeff.	0,000333	0,000131	0,003219	0,000395	0,261377	0,291502	0,313409
	25th Coeff.	4,88E-05	0,000136	0,003497	0,000416	0,258784	0,297526	0,309696
	26th Coeff.	0,000380	0,000143	0,003638	0,000446	0,257013	0,299101	0,308992
	27th Coeff.	0,000398	0,000148	0,003870	0,000457	0,254625	0,305311	0,307003
	28th Coeff.	0,000428	0,000159	0,004167	0,000477	0,249969	0,304287	0,307120
	29th Coeff.	0,000463	0,000171	0,004491	0,000503	0,245690	0,307366	0,304674
	30th Coeff.	0,000498	0,000189	0,004726	0,000520	0,241801	0,308169	0,304326
	31th Coeff.	0,000519	0,000195	0,004952	0,000538	0,238290	0,309657	0,302357
	32th Coeff.	0,000548	0,000206	0,005196	0,000555	0,237669	0,310714	0,301664
	33th Coeff.	0,000571	0,000215	0,005321	0,000570	0,237382	0,311877	0,305197
	34th Coeff.	0,000618	0,000233	0,005627	0,000600	0,235431	0,317019	0,306595
	35th Coeff.	0,000656	0,000246	0,005858	0,000632	0,234342	0,316263	0,305430
	36th Coeff.	0,000708	0,000266	0,006137	0,000664	0,234626	0,318345	0,308435
	37th Coeff.	0,000744	0,000275	0,006431	0,000689	0,234106	0,305475	0,309051
	38th Coeff.	0,000793	0,000287	0,006769	0,000736	0,232033	0,338667	0,307617
	39th Coeff.	0,000867	0,000297	0,007188	0,000824	0,233256	0,340205	0,304152
	40th Coeff.	0,000881	0,000305	0,007471	0,000816	0,230488	0,342454	0,302126

The results obtained for both statistical tests are schematically presented in Figure 5.2. As observed in table 5.2, Skewness presented p-values ~ 1 for all comparisons evaluated. Since the obtained p-values are considerably different from the remaining features, this parameter was dis-

carded from the discussion and visual analysis. Excluding Skewness, all statistical features and FFT-phase coefficients were revealed to be significantly different when comparing the 4-classes of particles, which indicates the potential of unwrapped phase-derived features for trapped microparticles detection and discrimination techniques using OFT back-scattering signals. Besides, considering the binary combinations, the selected attributes were able to capture the presence of a trapped particle with great discriminative potential ($p\text{-value} < 0.001$). However, in discrimination between classes of trapped particles, the majority of statistical features failed to discriminate the PS particle from Yeast cell (Class 3 vs Class 4), while using the FFT-phase coefficients the comparisons between two synthetical particles (PMMA vs PS) and a synthetical versus biological particle (PMMA vs Yeast, PS vs Yeast) were not significantly different, which may be a consequence of the similar structural properties of the cells under analysis. Although the synthetic microspheres significantly differ from biological particles, yeast cells are one of the simplest eukaryotic organisms, only delimited structurally by a cell wall and constituted by a reduced number of organelles and structures. Since the synthetic particles analyzed did not undergo processes of surface functionalization, these are also characterized by a very simple structure. Considering that the scattering patterns and phase shifts present in the signal are expected to be related to the number of cell layers and structural properties that define different particle types, the poor separability of classes may be a consequence of the simplicity and high similarity of morphological and structural characteristics. To test this theory, analysis of back-scattered signals arising from more complex targets needs to be conducted. Nevertheless, the results obtained in the 4-class comparison reveal a considerable discriminative potential of the set of features, which is translated in very small $p\text{-value}$ results.

Since in the previous study conducted with the same dataset [38], equal statistical analysis methods were applied, it is possible to compare the results retrieved by the two different sets of parameters extracted from the back-scattered signal. This comparison is presented in Figure 5.3. In the previous approach, a total of 45 features was selected to characterize each class, based on time-domain (statistical and histogram-derived features) and frequency-domain (Discrete Cosine Transform (DCT)-derived features and Wavelet features) [38]. Comparing with the results obtained through the set of phase-based features, the statistical features calculated from the phase spectrum of the back-scattered signals presented better discrimination capability for the binary combinations, with one feature (Kurtosis) able to differentiate with statistical significance all binary classes. Regarding the FFT coefficients, a much better discrimination ability ($p\text{-values} < 10^{-06}$) for classes involving the “No particle” condition is presented in contrast with the results obtained for the DCT coefficients based on the magnitude of the signal, where such condition is associated with poor statistical significance (higher $p\text{-value}$). This is an important result since it reveals that the phase of FFT coefficients is able to retain important information of the particle and provide good discrimination performance in short duration signal portions (2 seconds), compared with the magnitude of the DCT coefficients, that is usually explored in detriment of phase. Besides, DCT is a technique well-known for its compression capabilities and ability to capture minimal periodicities of a signal [162, 38]. It is also worth noting that the most robust

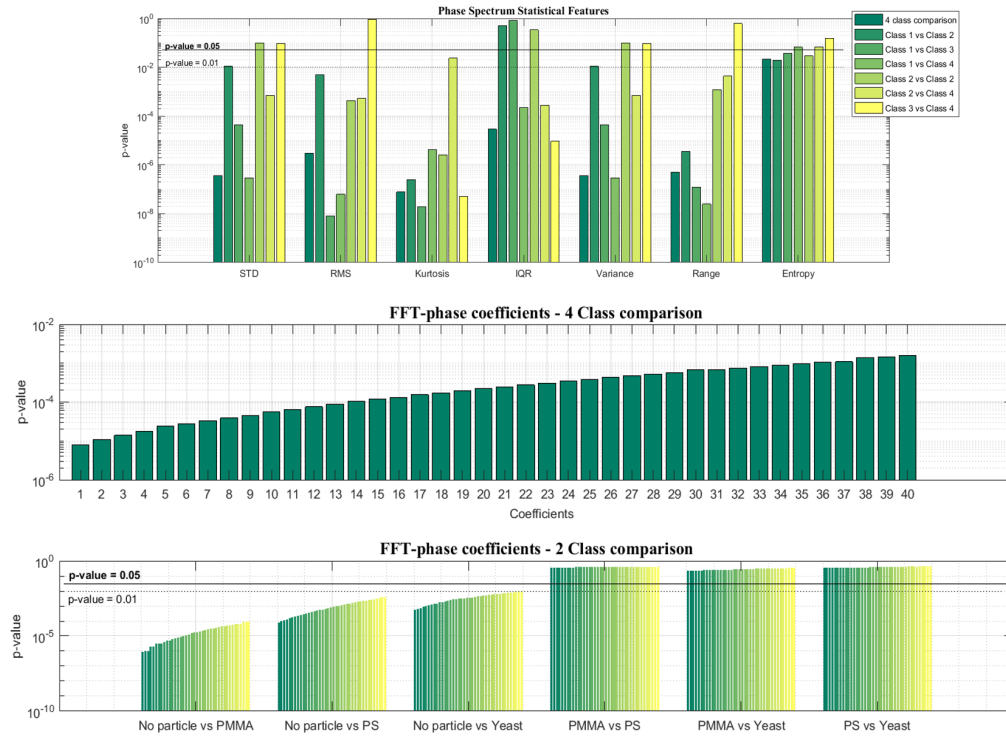


Figure 5.2: Graphical representation of the results obtained regarding the statistical comparisons involving 4 classes (Kruskal-Wallis test) and 2-classes (Mann-Whitney test), performed in experiment 1. (A) Results obtained for the first set of parameters: phase-spectrum derived statistical features. (B) Results obtained for the second set of parameters: Phase portion of the first 40 FFT coefficients. p-values obtained for each individual coefficient in 4-class comparison (top) and binary combinations (below).

feature of the previously extracted set of features (number of DCT coefficients that capture 98% of the total amount of energy of the original signal) failed to be statistically significant only for the comparisons between Class 3 (PS) and Class 4 (Yeast), the same combination where more of the present features failed to be statistically significant, which supports the theory that the signal reflected from such particles presents fewer differential patterns, not only in the phase spectral representation but also considering the time domain and magnitude spectral information.

5.3 Human cancer single-cell type identification

5.3.1 Data analysis

From experiment 1, a high degree of sensibility to detect the presence of trapped particles was demonstrated. However, the discrimination power was compromised possibly as a consequence of the simple and similar structural properties of the particles under analysis. Hereupon, it was expected that for more complex targets, such as mammalian cells, more evidence of phase shifts could be found. Since the heterogeneity degree is significantly higher, due to the wide variety of cellular structures (microtubules, microfilaments), organelles, and cellular layers, light-matter

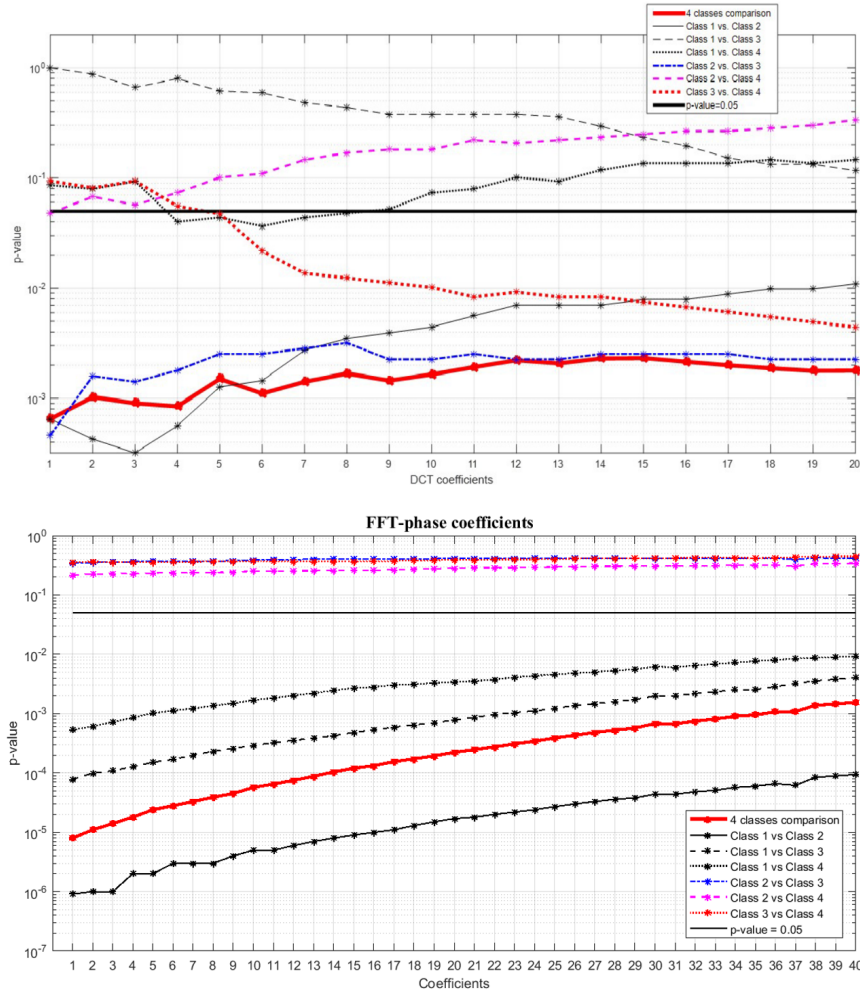


Figure 5.3: Graphical representation of p-values obtained from the statistical analysis to each coefficient extracted from the DCT transform applied to the original back-scattered signal, performed in the previous study with the same dataset (top) [38] and from the phase portion of FFT coefficient from the same signals, conducted in the present work.

interactions are theoretically enhanced. Therefore, to evaluate the micro-particle type discrimination performance of phase-based features in a scenario of increased complexity, the second experiment was conducted using back-scattered signals acquired from suspensions in PBS involving two types of mammalian cells derived from MKN45 gastric cell line - Mock and HST6 – with the same genetic background but displaying a different surface glycosylation profile, and synthetic PS particles. The dataset was composed of 80 seconds of the back-scattered signal acquired considering 4 distinct classes: No particle trapped (Class 1), Mock cell trapped (Class 2), HST6 cell trapped (Class 3), and PS cell trapped (Class 4). The complete dataset acquired in the previous work [19], after signal processing steps (section 4.2.2) is described on Table 5.3 and the phase spectral representations obtained for each class, after phase unwrapping, are presented in Figure 5.4. The set of phase-derived features was extracted and each feature was individually evaluated in the statistical analysis stage, between the 4 classes and the six possible pairwise combinations

between these four classes.

Table 5.3: Dataset characterization after signal processing steps, for Experiment 2. Each sample corresponds to each 2 second portion of back-scattered signal. Sampling frequency = $5kHz$. Obtained from [19]

Class	1 No particle	2 Mock tumor cell	3 HST6 tumor	4 PS particle
Number of trapped particles	13	15	15	10
Average number of signal portions per particle	34 ± 3	32 ± 5	31 ± 5	31 ± 6
Total number of 2-second signal portions	438	479	470	305

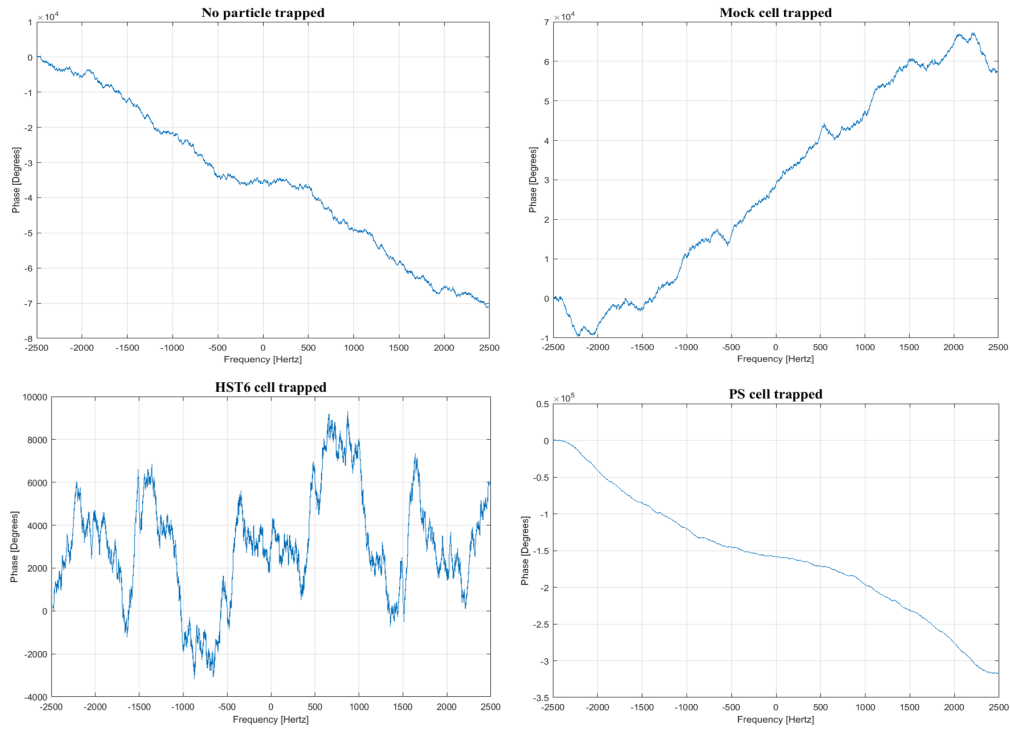


Figure 5.4: Unwrapped phase spectrum obtained for each condition evaluated - No particle trapped (Class 1), Mock cell (Class 2), HST6 cell (Class 3) and PS particle (Class 4).

5.3.2 Results and discussion

The p-value results obtained for each test applied (Kruskal-Wallis, Mann-Whitney) are presented on Table 5.4.

As observed in Figure 5.5, the most promising results are found for the second feature subset, where all FFT-phase coefficients present significant differences for the 4-class comparison and all the 6 binary combinations considered (p-value < 0.05). These showed to be suitable not only to

Table 5.4: Obtained p-value results of the statistical comparisons regarding 4 classes - No particle trapped (C1), Mock cell trapped (C2), HST6 cell trapped (C3) and PS particle trapped (C4) - using Kruskal-Wallis test and post-hoc pairwise comparisons (Mann-Whitney test) conducted on Experiment 2. A significance level of 0.05 was considered for all experiments. Results above the significance threshold (p-value > 0.05; two-tailed) are highlighted.

		Comparisons						
		4 class comparison	C1 vs C2	C1 vs C3	C1 vs C4	C2 vs C3	C2 vs C4	C3 vs C4
Phase-spectrum statistical features	STD	2,53E-08	4,83E-04	0,003956	0,040672	0,678747	0,134576	2,17E-05
	RMS	3,59E-05	1,81E-05	0,000569	0,002511	0,545831	0,566177	3,45E-07
	Skewness	0,837295	0,936374	0,089984	0,287451	0,875131	0,424718	0,440305
	Kurtosis	1,54E-07	4,06E-07	0,002721	0,467241	0,268305	0,171697	0,225892
	IQR	4,51E-07	0,008249	0,009006	0,040672	0,001263	0,231810	0,001463
	Entropy	2,53E-08	4,33E-04	0,003956	0,040672	0,678746	0,134576	2,17E-05
	Variance	1,93E-06	3,97E-05	2,83E-04	0,007076	0,102329	0,086131	8,23E-08
	Range	9,05E-04	0,002673	0,004395	0,002544	0,047535	0,063747	0,000897
FFT unwrapped phase coefficients	1st Coeff.	0.000498	0.006452	0.000452	0.014971	0.031498	0.028518	0.025754
	2nd Coeff.	0.000637	0.007031	0.000534	0.014644	0.032234	0.028294	0.025827
	3rd Coeff.	0.000721	0.007338	0.000604	0.014774	0.032607	0.028406	0.026338
	4th Coeff.	0.000761	0.007382	0.000634	0.015439	0.033442	0.028406	0.027194
	5th Coeff.	0.000829	0.007656	0.000695	0.015371	0.035087	0.028630	0.028065
	6th Coeff.	0.000878	0.008005	0.000737	0.015439	0.035247	0.028971	0.028327
	7th Coeff.	0.000933	0.008169	0.000799	0.015507	0.035087	0.028518	0.028387
	8th Coeff.	0.001056	0.008668	0.000876	0.015712	0.035569	0.028857	0.028854
	9th Coeff.	0.001146	0.008951	0.000929	0.015712	0.035976	0.028630	0.029014
	10th Coeff.	0.001220	0.009207	0.000985	0.015371	0.035894	0.028518	0.028741
	11th Coeff.	0.001367	0.009647	0.001053	0.015171	0.035650	0.029085	0.028933
	12th Coeff.	0.001473	0.010425	0.001069	0.015304	0.035327	0.029199	0.028963
	13th Coeff.	0.001539	0.010622	0.001113	0.015575	0.036303	0.030131	0.029818
	14th Coeff.	0.001710	0.011194	0.001202	0.015712	0.036883	0.030844	0.030654
	15th Coeff.	0.001834	0.012061	0.001231	0.015304	0.036883	0.030844	0.030266
	16th Coeff.	0.001969	0.012502	0.001268	0.015371	0.037176	0.030485	0.030099
	17th Coeff.	0.002075	0.013060	0.001313	0.015439	0.037302	0.032317	0.030991
	18th Coeff.	0.002074	0.013070	0.001316	0.015507	0.037896	0.031819	0.031119
	19th Coeff.	0.002164	0.013631	0.001393	0.015439	0.037811	0.031819	0.031104
	20th Coeff.	0.002164	0.013718	0.001373	0.015103	0.036967	0.032694	0.031067
	21th Coeff.	0.002228	0.013718	0.001373	0.015103	0.036967	0.032694	0.031073
	22th Coeff.	0.002358	0.014274	0.001461	0.015439	0.036386	0.032568	0.030751
	23th Coeff.	0.002484	0.014183	0.001515	0.015171	0.035650	0.032821	0.030373
	24th Coeff.	0.002678	0.014901	0.001603	0.015203	0.035327	0.032568	0.030146
	25th Coeff.	0.000637	0.015959	0.001654	0.014774	0.034531	0.032442	0.025827
	26th Coeff.	0.003019	0.016467	0.001732	0.014579	0.034452	0.032442	0.029452
	27th Coeff.	0.003114	0.016978	0.001764	0.014709	0.034413	0.034242	0.030081
	28th Coeff.	0.003296	0.017732	0.001864	0.015575	0.033983	0.034905	0.030507
	29th Coeff.	0.003475	0.018214	0.001955	0.015439	0.034061	0.035039	0.030539
	30th Coeff.	0.003677	0.018504	0.002095	0.015507	0.034061	0.035308	0.030791
	31th Coeff.	0.003809	0.019031	0.002159	0.015575	0.034178	0.035039	0.030974
	32th Coeff.	0.003912	0.019463	0.002199	0.015237	0.034610	0.035173	0.030910
	33th Coeff.	0.004009	0.019756	0.002278	0.015439	0.034611	0.034771	0.030889
	34th Coeff.	0.004336	0.021053	0.002500	0.015237	0.035007	0.035443	0.031342
	35th Coeff.	0.004587	0.022052	0.002605	0.015712	0.035650	0.034771	0.031825
	36th Coeff.	0.004863	0.022786	0.002788	0.015643	0.035731	0.034638	0.031597
	37th Coeff.	0.005067	0.023714	0.002863	0.015575	0.034927	0.034373	0.031196
	38th Coeff.	0.005324	0.024789	0.002942	0.015439	0.035488	0.034505	0.031551
	39th Coeff.	0.005609	0.025126	0.003014	0.015237	0.036303	0.034110	0.031596
	40th Coeff.	0.005668	0.025803	0.003089	0.015037	0.035813	0.034373	0.031485

identify the presence of a trapped particle but also to discriminate between biological (tumoral cells) vs synthetic (PS) particles and even between the two very similar biological cells (Mock and HST6). Comparing with the results of experiment 1, with simple microparticles, these results

show that the phase portion of FFT coefficients presents a higher discrimination power for complex biological particles. Since mammalian cells contain a wide variety of cellular structures (microtubules, microfilaments) and organelles with a complex, hydrodynamic outermost layer with phospholipids and proteins embedded, there is a significantly higher heterogeneity and complexity degree present, in comparison with simple synthetic structures. The differential surface glycosylation patterns on the cancer-derived cells probably originated different interactions of light with the glycans coat around each cell. Besides, the modified cell line can also present a different internal layer constitution, that can behave as resonant cavities when the light interacts with the cell. Thus, all of these factors seem to intensify the light-matter interactions during the light optical path, translated by distinct phase shifts in the back-scattered signal, that can be explored as biological signatures of the particles under analysis. Considering the results of the unwrapped-phase statistical features, the comparisons the comparison between the two tumoral cells trapped (Mock and HST6) was only statistically discriminated by IQR and Entropy. Although considerable differences are observed in the phase spectrum obtained, these were not significant considering the attributes evaluated. It was also observed that the binary comparison “Mock vs PS particle” was not discriminated by any of the statistical features extracted. Although there is a considerable difference between the two types of particles, since the tumor cell presents a higher heterogeneity degree and more complex structural properties, these were not captured by the statistical features used. It is worth noting that these results are similar to the previous experiment, where a biological (Yeast) particle was not discriminated from PS particles. In fact, in a general overview, the class involving PS particles is usually associated with poor discriminative results. Since this is a simple synthetic particle with a reduced heterogeneity degree, fewer interactions and reflective phenomena are expected and, consequently, less patterns are generated in the back-scattered. This is possible to visually observe not only in the sketches of the back-scattered signal acquired from each particle (Figure 4.3) but also in the unwrapped phase spectrum obtained 5.4. Consequently, the signal does not contain much discriminative information of the particle which turns the differentiation task more challenging.

Therefore, the FFT-phase coefficients showed to be more highly robust for biological particle detection and discrimination. This is an important outcome considering the current challenges of cancer and other disease detection methodologies based on single-cell fast screening. Since these are slight cellular changes, only affinity and biochemical assays, involving fluorescence, or highly sensitive spectral and imaging techniques are able to detect them. These are not ideal to be applied in portable and miniaturized bio-sensing devices, which prompts a crescent demand for simple and efficient techniques able to identify cancer cell alterations with high inter-cell similarity degrees. In this context, the sensitivity of these 40 individual features to cancer-associated glycoforms expressed at the surface presents a great potential for single-cell characterization methods such as cancer biomarker assays. Besides, considering that alterations in the glycosylation process are linked to tumor development, these can also be applied in personalized therapeutic approaches and early disease assessment methodologies.

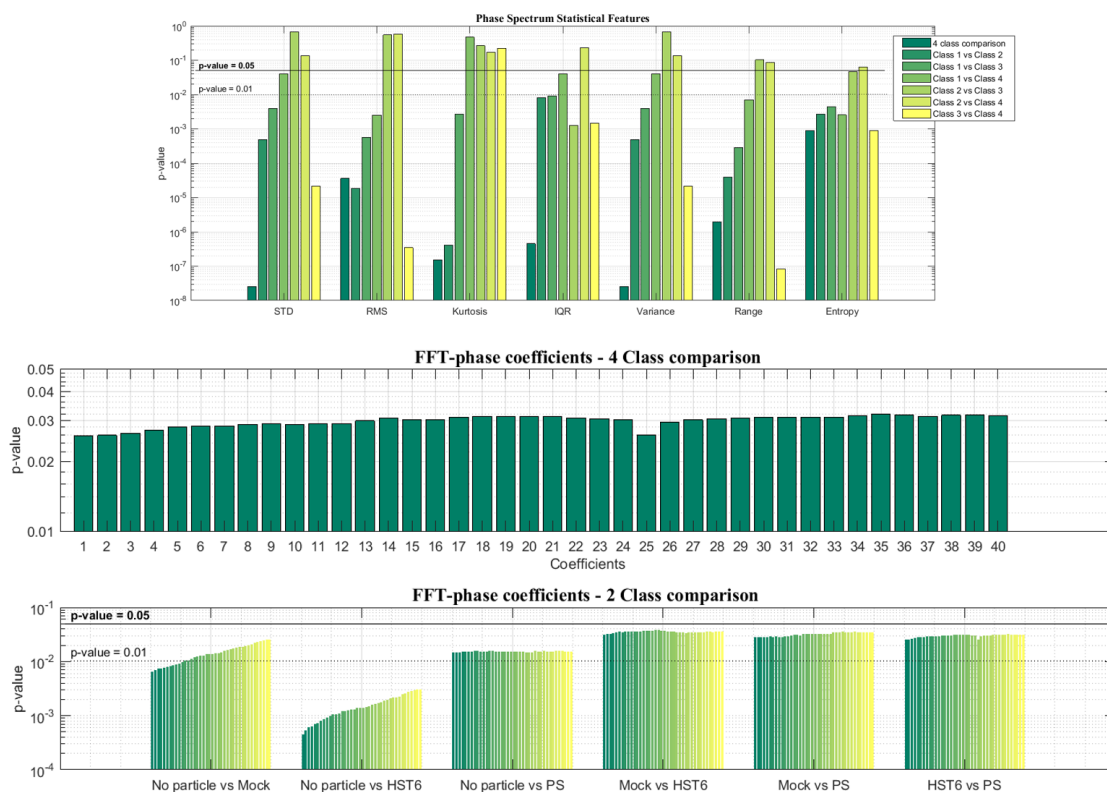


Figure 5.5: Graphical representation of the results obtained regarding the statistical comparisons involving 4 classes (Kruskal-Wallis test) and 2-classes (Mann-Whitney test), performed in experiment 2. (A) Results obtained for the first set of parameters: phase-spectrum derived statistical features. (B) Results obtained for the second set of parameters: Phase portion of the first 40 FFT coefficients. p-values obtained for each individual coefficient in 4-class comparison (top) and 6 different binary combinations (below).

5.4 Conclusions

This chapter presents the statistical analysis results to the set of phase-based features extracted from the two first experiments analyzed the present work, considering synthetic and simple biological microparticles and complex biological cells, whose reflected signal was acquired under optical trapping conditions. It was possible to conclude that the extracted set of features can provide a good discriminative power, for both 4-class and 2-class comparisons. The FFT-phase coefficients showed to be more sensitive and robust, in comparison with statistical features. Comparing the two experiments, better results were obtained for the most complex particles (Tumor cells) and biological media (PBS), where all 40 FFT-phase coefficients were capable to distinguish with statistical significance ($p\text{-value} < 0.05$) all the classes of particles considered, not only in 4-class comparison but also in the total pairwise combinations evaluated. Considering this, and the fact that one of the most simple particles tested, the PS microsphere, is associated with poor discriminative results of the features in both experiments, the proposed theory is reinforced - a higher heterogeneity degree, structural complexity and presence of surface components probably

intensifies the light-matter interactions during the light optical path, which is reflected in the form of phase-shifts in the back-scattered signal collected from the particles. This originates scattering patterns related with the intrinsic properties of each particles, thus representing a biological signature with great potential for single-cell characterization methods.

Chapter 6

Extracellular nano-vesicles detection and discrimination

After observing the robustness and discriminative power of unwrapped phase-based features considering the back-scattered signal of trapped microparticles (Experiments 1 and 2), the same approach was applied to evaluate the robustness of phase spectral features to detect and discriminate non-trapped extracellular nano-vesicles suspended in biological fluids, previously explored in iLoF technology development. Along this chapter, the results obtained are presented and the correlation of the conditions evaluated with real physiological scenarios is discussed.

6.1 Introduction

In the third experiment, the differentiation ability of the phase-based features was evaluated for back-scattered signals acquired by dipping the optical fiber tip in colloidal dispersions of highly complex biologic fluids, containing human-derived EVs obtained from the human-cancer cell line of experiment 2 (MKN45 gastric cell line) [129]. These present the same genetic background, but different surface glycosylation, mimicking cancer subtypes with different probabilities of metastasizing and associated with different clinical outcomes. Contrary to the previous experiments, a stable trapping of particles is not expected due to the small diameter of the particles (around 100 nm). Therefore, more challenging SNR conditions are introduced to test the robustness of the proposed set of features.

6.2 Data analysis

The discrimination power and sensibility of the extracted features were evaluated considering the presence of synthetic (PS) and biological (human cancer-derived EVs) nanoparticles in two types of biological-derived media: PBS (simple media) and cell culture medium supplemented with FBS (complex media). In previous work, 80 seconds of back-scattered signal derived from each dispersion were acquired [129]. These were processed according to the pipeline previously

described and the unwrapped phase representations were obtained for each class, presented in Figure 6.1.

6.3 Results and discussion

The p-value results obtained for each test applied are presented on Table 6.1. Due to the high p-value results obtained for Skewness in the experiments involving nanoparticles, this was discarded.

Table 6.1: Obtained p-value results of the statistical comparisons regarding 3 classes in PBS - PBS only (C1), Mock EVs (C2), HST6 EVs (C3) - and in FBS supplemented media- PBS only (C4), Mock EVs (C5), HST6 EVs (C6) - using Kruskal-Wallis test, and post-hoc pairwise comparisons (Mann-Whitney test) conducted on Experiment 3. A significance level of 0.05 was considered for all experiments. A significance level of 0.05 was considered for all experiments. Results above the significance threshold (p-value > 0.05; two-tailed) are highlighted.

		Comparisons							
		3 class comparison PBS	3 class comparison FBS	C1 vs C2 (PBS)	C1 vs C3 (PBS)	C2 vs C3 (PBS)	C4 vs C5 (FBS)	C4 vs C6 (FBS)	C5 vs C6 (FBS)
Phase-spectrum statistical features	STD	0.196533	1.04E-14	0.142622	0.069149	0.333971	1.53E-09	1.51E-14	0.000706
	RMS	0.152273	1.83E-14	0.102837	0.063217	0.201302	4.48E-10	2.15E-14	0.002661
	Kurtosis	0.112696	0.008092	0.161504	0.332694	0.036475	0.103185	0.447423	0.002587
	IQR	0.258567	1.78E-13	0.290506	0.096767	0.743240	7.45E-08	2.36E-13	0.000246
	Entropy	0.196533	1.04E-14	0.142622	0.069149	0.333971	1.53E-09	1.51E-14	0.000706
	Variance	0.915827	1.32E-05	0.121766	0.073056	0.256044	1.61E-10	4.57E-15	0.003030
	Range	0.185898	4.71E-15	0.076881	0.939732	0.785283	5.40E-05	3.50E-06	0.325862
FFT unwrapped phase coefficients	1st Coeff.	0.011928	0.000146	0.624942	0.009691	0.010246	0.003637	0.000131	0.020887
	2nd Coeff.	0.012728	0.000145	0.637763	0.011195	0.009845	0.003532	0.000133	0.021066
	3rd Coeff.	0.011966	0.000150	0.655694	0.010991	0.008941	0.003611	0.000133	0.021673
	4th Coeff.	0.013083	0.000163	0.645707	0.011404	0.010084	0.003773	0.000144	0.022045
	5th Coeff.	0.013976	0.000157	0.663728	0.012503	0.010205	0.00348	0.000143	0.022295
	6th Coeff.	0.013294	0.000160	0.651691	0.011780	0.010004	0.003611	0.000148	0.021735
	7th Coeff.	0.013206	0.000162	0.647699	0.011301	0.010327	0.003558	0.000147	0.022422
	8th Coeff.	0.013074	0.000161	0.642723	0.011273	0.010205	0.003584	0.000143	0.022422
	9th Coeff.	0.012761	0.000155	0.658702	0.010888	0.010044	0.003584	0.000136	0.022295
	10th Coeff.	0.012631	0.000158	0.656696	0.011247	0.009573	0.003480	0.000141	0.022868
	11th Coeff.	0.011947	0.000161	0.655694	0.010737	0.009123	0.003480	0.000139	0.023719
	12th Coeff.	0.011901	0.000157	0.656696	0.010838	0.008977	0.003454	0.000138	0.023062
	13th Coeff.	0.011988	0.000154	0.648696	0.010888	0.009086	0.003208	0.000136	0.023719
	14th Coeff.	0.011063	0.00016	0.649694	0.010248	0.008413	0.003305	0.000144	0.023520
	15th Coeff.	0.011137	0.000163	0.651691	0.010441	0.008345	0.003256	0.000147	0.023986
	16th Coeff.	0.010299	0.000160	0.648696	0.009373	0.008144	0.003137	0.000143	0.024460
	17th Coeff.	0.010359	0.000160	0.645707	0.009642	0.008012	0.003114	0.000146	0.024256
	18th Coeff.	0.010566	0.000161	0.647699	0.009964	0.008012	0.002978	0.000148	0.024803
	19th Coeff.	0.010783	0.000160	0.655694	0.010272	0.008012	0.003011	0.000147	0.024803
	20th Coeff.	0.010573	0.000169	0.655694	0.010224	0.007786	0.003068	0.000154	0.025361
	21th Coeff.	0.010827	0.000174	0.655694	0.010224	0.007786	0.003068	0.000154	0.025361
	22th Coeff.	0.010992	0.000174	0.658702	0.010688	0.007947	0.003208	0.000160	0.024942
	23th Coeff.	0.010661	0.000176	0.654692	0.010441	0.007738	0.003208	0.000160	0.025186
	24th Coeff.	0.010593	0.000175	0.650692	0.010200	0.007850	0.003161	0.000160	0.025361
	25th Coeff.	0.012728	0.000145	0.648696	0.010058	0.007979	0.003045	0.000163	0.025716
	26th Coeff.	0.010798	0.000177	0.656696	0.010441	0.007898	0.003001	0.000163	0.026147
	27th Coeff.	0.010969	0.000178	0.645209	0.010272	0.008295	0.002868	0.000163	0.027251
	28th Coeff.	0.010499	0.000181	0.642226	0.009987	0.007931	0.002826	0.000169	0.027251
	29th Coeff.	0.010634	0.000182	0.648696	0.010153	0.007947	0.002805	0.000169	0.027704
	30th Coeff.	0.009376	0.000195	0.643717	0.009417	0.006937	0.002934	0.000185	0.027552
	31th Coeff.	0.009205	0.000203	0.647699	0.009068	0.006966	0.003068	0.000192	0.027401
	32th Coeff.	0.008979	0.000197	0.649195	0.008961	0.006753	0.003068	0.000187	0.026878
	33th Coeff.	0.008979	0.000197	0.655694	0.009025	0.006683	0.003068	0.000187	0.026953
	34th Coeff.	0.007882	0.000196	0.665743	0.008165	0.005894	0.003068	0.000183	0.027027
	35th Coeff.	0.007718	0.000195	0.667760	0.007972	0.005820	0.003068	0.000181	0.027101
	36th Coeff.	0.008247	0.000197	0.667760	0.008126	0.006383	0.003091	0.000187	0.026657
	37th Coeff.	0.008784	0.000191	0.683982	0.008792	0.006518	0.003068	0.000183	0.026002
	38th Coeff.	0.008803	0.000182	0.676866	0.008855	0.006518	0.003045	0.000171	0.025859
	39th Coeff.	0.008758	0.000178	0.670790	0.008730	0.006572	0.003023	0.000163	0.026364
	40th Coeff.	0.009048	0.000175	0.664735	0.008688	0.006995	0.003000	0.000158	0.026364

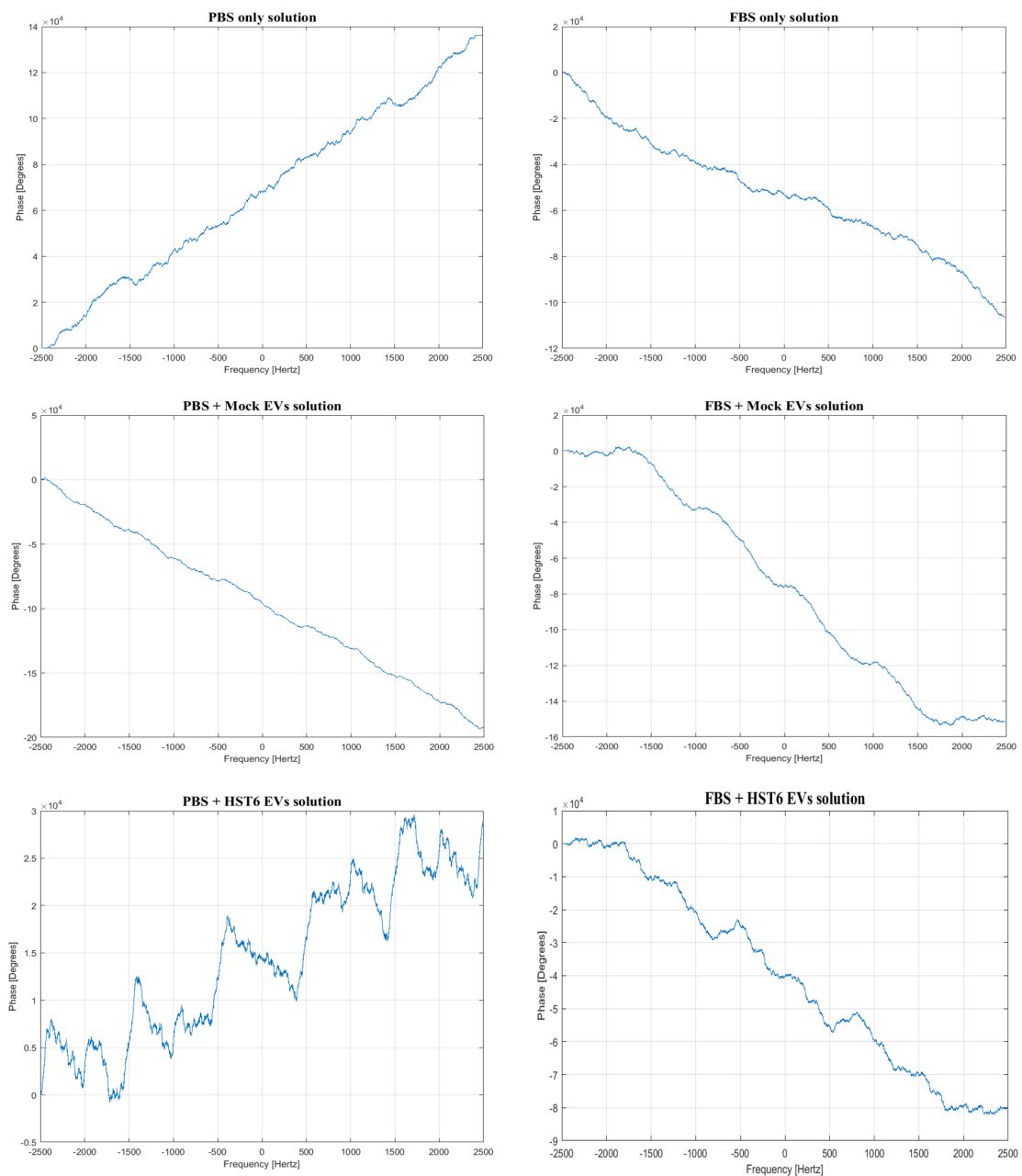


Figure 6.1: Unwrapped phase spectrum obtained for each condition evaluated - PBS only solution (Class 1), Mock EVs in PBS (Class 2), HST6 EVs in PBS (Class 3), FBS only solution (Class 4), Mock EVs in FBS (Class 5), HST6 EVs in FBS (Class 6).

The results are schematically presented in Figure 6.2.



Figure 6.2: Graphical representation of the results obtained regarding the statistical comparisons involving 3 classes (Kruskal-Wallis test) and 2-classes (Mann-Whitney test), performed in experiment 3, for the two biological media tested. (A) Results obtained for the first set of parameters: phase-spectrum derived statistical features. (B) Results obtained for the second set of parameters: Phase portion of the first 40 FFT coefficients. p-values obtained for each individual coefficient in 4-class comparison (top) and 6 different binary combinations (below).

Considering the 3-class comparison test, all the features extracted from back-scattered signals acquired from FBS supplemented media were revealed to be significantly different in statistical terms, between the conditions evaluated. In pairwise analysis, similar results were obtained. The majority of features extracted from such media present statistically significant results ($p\text{-value} < 0.05$) except for Kurtosis in comparisons involving Mock EVs presence (Class 4), Range in the comparison between HST6 EVs and PS particle (Class 5 vs Class 6), and combination 1 (No particle vs Mock) where none of the coefficients was statistically different. Therefore, besides detecting the presence of human dispersed EVs, the majority of phase-based features analyzed were also able to statistically differentiate the signals arising from the two types of EVs. Consid-

ering the challenging SNR conditions and high similarity between the two structures, these results reveal that phase features present high sensibility and robustness for nanoparticle detection and differentiation. Since the two different EVs are produced by cell models with the same genetic background, these present similar size and RI, differing only on the length of EVs surface glycans. This was confirmed in past studies [129], where the extracellular nano-vesicles were characterized through conventional methods considering morphological shape and aspect by Transmission Electron Microscopy (TEM) (Figure 6.3), size through Nanoparticle tracking analysis (NTA) technique, and macroscopic refractive index using a refractometer. It was concluded that despite HST6 EVs present a smaller diameter in relation to Mock EVs, on average, this difference was not significant in statistical terms so it is considered that the two types of EVs have a similar size, thus they cannot be effectively differentiated by this physical property. Besides, it was observed that independently of the type of EVs added to each solution, the differences in the final value of its average/macroscopic RI were not statistically significant, thus being approximately the same. Therefore, optical fiber sensors based on RI change measurement in the surrounding media to detect the presence of specific bio-species would not be enough to identify and discriminate the dispersed EVs and advances techniques such as NTA, based on mean size, without any previous information on sub-population origin, would also not be able to characterize the two different groups. In the present study, the only distinct characteristic – the surface glycosylation – showed to be enough to generate different phase patterns, successfully captured by the phase-based features analyzed.

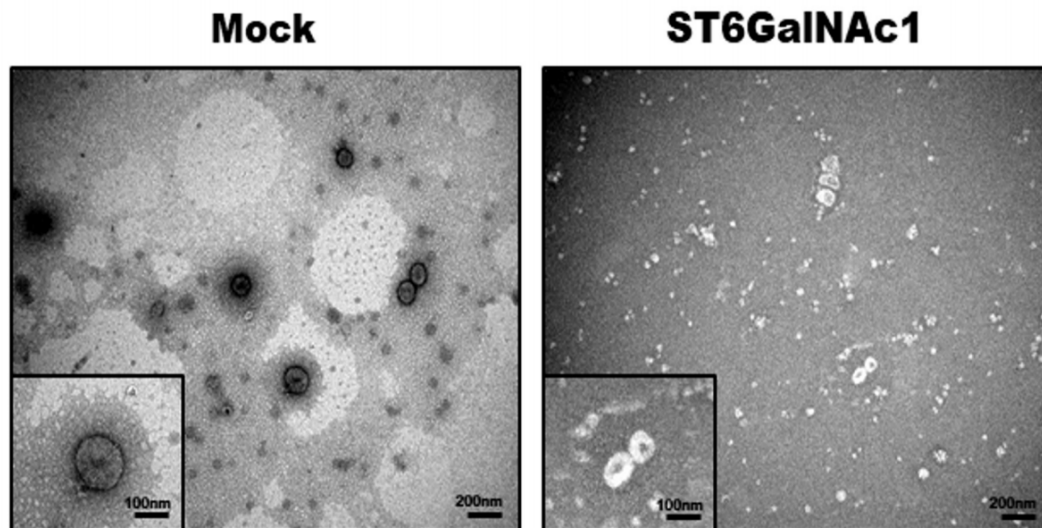


Figure 6.3: TEM images of the EVs isolated from MKN45 (Mock - empty vector) and MKN45 ST6GalNac-1 (ST6) transfected gastric cancer cell lines. Retrieved from [129]

Comparing the two culture media, the results are considerably different. While a high discrimination ability of the features was observed for signals acquired from FBS supplemented media, in PBS conditions, none of the analyzed statistical features was able to statistically discriminate the 3 classes considered. In binary comparisons, statistical significance was not found for combination 1 (No particle vs Mock), although combination 2 (No particle vs Mock) and combination 3

(Mock vs HST6) were successfully discriminated. It was expected that the discrimination ability decreased for the most complex media (cell culture medium supplemented with FBS) in comparison to PBS, since the amount of molecular components present increases, thus introducing more noise. However, the opposite was observed, which may be correlated with the ionic concentration and electrical conductivity of each media. According to the literature, PBS is defined by the electrical conductivity of, on average, 0.01 S/m while a standard cell culture medium has an electrical conductivity of 1.40 S/m and presents a higher electrical conductivity (values between $1.23 - 1.77\text{ S/m}$ for blood and plasma). A low conductivity media such as PBS induces an extended double layer of ions around each nanoparticle, which will generate a consequent reduction of EVs diffusion speed. This can be pointed to as a cause for the poor discrimination ability obtained since a reduced movement of particles leads to fewer intensity fluctuations and, consequently, less patterns contained in the back-scattered signals acquired. Besides, since one of the causes for phase-shifts is the heterogeneity degree of the particles and surrounding media, it is possible that the high heterogeneity and ionic concentration of FBS - containing several types of proteins (including hemoglobin), vitamins, growth factors, ions, and antibodies – enhanced the multiple scattering phenomena and phase-shifts occurrence, thus increasing the amount of information contained on the back-scattered signals. Finally, it is also important to analyze the concentration of nanoparticles used in this experiment. The dispersions evaluated were characterized by EVs concentrations of $3.45E + 07\text{ EVs/ml}$ and $4.38E + 07\text{ EVs/ml}$, for Mock and ST6 EVs respectively. Based on recent studies found in literature, there are several EVs related to important diseases (e.g. prostate cancer, Traumatic Brain Injury) that are present in blood or derivatives in similar concentrations. For instance, an average concentration of EVs associated with cancer spreading and progression between 10.00^{+07} particles/ml and 10.00^{+08} particles/ml was found in $100\text{ }\mu\text{l}$ serum samples belonging to patients with metastatic prostate cancer. Therefore, the features showed to be robust for the detection of nanoparticles at “naturally” occurring levels in the blood or derivatives (as serum or plasma). Considering the recent reports that point EVs present in such fluids as a suitable source of biomarkers, and the challenges in detecting particles with diameters in the region of $100\text{-}150\text{ nm}$, the proposed set of features present a great potential not only for early diagnosis of tumor subtypes and prediction of tumor metastasis, but also to be applied in biomarker-identification strategies.

6.4 Conclusion

This chapter presents the statistical analysis results of the experiment conducted to evaluate the robustness of the phase-derived set of features to discriminate highly similar human-derived EVs in colloidal dispersions of complex biologic fluids, whose signal was acquired in non-trapping conditions. Better discrimination ability obtained was obtained for back-scattered signals acquired in FBS supplemented media, probably due to the high heterogeneity and ionic concentration that enhanced the multiple scattering phenomena and phase-shifts occurrence. For this media, the majority of statistical features present a result below the significance threshold and the 40 FFT-phase

coefficients were able to statistically differentiate the three classes and all the binary classes analyzed. Therefore, the human dispersed EVs were statistically detected and differentiated through the set of features despite the challenging SNR conditions and high similarity between the two structures. The only distinct characteristic – the surface glycosylation – showed to be enough to generate different phase patterns in the collected back-scattered signal, which represents an important advance comparing with characterization techniques based on RI changes and morphological differences. Besides, the nanoparticles were detected in concentration values similar to the ones reported in cancer spreading and progression scenarios, which reveals a high sensibility and robustness of the phase-derived features for small particles detection in biological fluids, important to meet the requirements of selective and sensitive methods for biomarker-identification techniques.

Chapter 7

Conclusions and Future Work

In this chapter, the main conclusions of the present work are presented, the contributions provided are enumerated and future work is discussed.

7.1 Main conclusions

Along with the present study, we explored the potential of phase spectral information for the detection and discrimination of micro and nano (bio)particles present in different liquid suspensions used in biological assays. This was achieved through an exploratory statistical analysis to a set of phase-based features extracted from back-scattering signals collected through iLoF optical setup, considering three different experiments: (1) simple synthetic and biological microparticles in distilled water (PMMA, PS, Yeast), (2) highly similar complex human tumoral cells in PBS (Mock, HST6) and (3) cancer-derived extracellular nanovesicles (Mock EVs, HST6 EVs) in PBS and FBS biological liquid suspension media. The ability of each individual feature to discriminate between the different classes of liquid suspensions presented to the iLoF optoelectronic setup was evaluated in a series of 4-class and 2-class statistical comparisons for the micron-sized particle experiments (1 and 2) and 3-class and 2-class for the nano-sized experiment (3).

The results reveal that the phase-derived parameters are highly relevant and robust not only for micro and nanoparticle detection but also to distinguish different particles. It was observed that an increase in particle complexity and heterogeneity degree was associated with higher discrimination capability. This interesting observation is probably due to the increase in laser signal reflections over the different layers and sub-cellular organelles present in the more complex particles used, which have strengthening even more the amount of relevant information collected from the back-scattered signals. FFT-phase coefficients present a higher discriminative power than the statistical features of the signal analyzed for all experiments. In comparison with a set of time and magnitude-based features, previously used in iLoF studies and the majority of signal processing systems, better discrimination results were obtained regarding the statistical analysis conducted. This enhances the informative content of phase and potential to retain important biological patterns and supports the evidence of FFT-coefficients suitability to capture the predominant spectral

information contained in the original signal. This subset of features was capable not only to detect the presence of trapped particles and nanoparticles in complex solutions but also to successfully discriminate two highly similar human cancer cells and derived EVs. Considering that such particles present similar size, morphological and biochemical properties, and RI, only differing in the surface glycosylation patterns, this is an extraordinary outcome in view of the current challenges of cancer and other diseases detection methodologies based on single-cell fast screening and, specifically, using optical fiber for signal detection. The conventional procedures rely on the use of highly sensitive spectral and imaging techniques and complex functionalization essays that require transduction labeling elements in order to generate a physically readable signal. In the present method, the phase component of a signal arising from a trapped cell or from complex solutions containing nanoparticles was enough to allow cell and sub-cell vesicles detection and differentiation. The sensitivity of the analyzed features to cancer-associated glycoforms expressed at the surface presents a great potential for single-cell characterization methods such as cancer biomarker assays. Besides, considering that alterations in the glycosylation process are linked to tumor development, these can be promising for personalized therapeutic approaches and early disease assessment methodologies. Additionally, the features showed robustness for detection of biological nanoparticles at “naturally” occurring levels in the blood or derivatives, for complex media composed of different biological structures (vitamins, growth factors, ions, and antibodies), similar to physiological conditions. Considering the recent reports that point EVs present in blood or derivatives as a suitable source of biomarkers, and the challenges in detecting particles with diameters in the region of 100-150 *nm*, the differentiation ability of the presented set of features reveal a great potential to identify changes in composition and structural properties of molecules as a result of pathogenic processes or responses to therapeutic interventions, valuable information for disease diagnosis and prognosis. Although the importance of phase is recognized by several authors, the amount of research with respect to phase-based features is reduced. Considering the literature analyzed, this is the first time such information is explored for biological particle characterization through optical fiber-based tools. Considering the increasing demand for effective, discriminative features that provide a good performance of classification techniques, the results obtained demonstrate that the incorporation of phase-based features can extend the horizons for biophotonic signal processing systems, beyond the commonly applied temporal and magnitude spectral solutions. This is particularly important in the present area of device miniaturization and Lab-on-Fiber technologies, which require especially suited sensing technologies. Scattered signal processing methods are associated with a low computational cost and require less expensive and complex equipment. For instance, in this technique, the use of an optical fiber with a spherical polymeric lens on top and a photodetector was enough to capture relevant scattering data. The combination of advanced signal processing methods, able to capture and calculate several parameters from the output signal, with the inherent properties of optical fibers and, more specifically, OFTs can be a remarkable approach to discover biological signatures of main diseases and perform disease subtyping, as it has been demonstrated through the work developed by iLoF. Considering this, the present dissertation presents phase as a potential new contributor to obtain discriminative

light patterns and mathematical parameters strongly related to the structural properties of each cell and sub-cell vesicles, that can significantly enhance the automatic particle classification task to be deployed on the exciting vision of advanced, multifunctional and miniaturized “all-in-fiber” probes, for point-of-care diagnostic and *in-vivo* biosensing.

7.2 Contributions

The main contributions of this dissertation are:

- Pipeline for phase computation, processing and feature extraction.
- New insights on the information content of phase spectrum derived from back-scattering signals, and the relation with structural properties of biological particles.
- New evidences on the potential of phase information to derive features for classification problems, through a set of parameters statistically robust to identify and discriminate microscopic biological particles under optical trapping and extracellular nano-vesicles suspended in bio-fluids, at “naturally” occurring levels in the blood or derivatives (as serum or plasma).

The presented results are reported in a scientific article, entitled "Micron-Sized Bioparticles Detection through Phase Analysis of Back-scattering Signal from Optical Fiber Tweezers: An Exploratory Study", prepared to be submitted on the international peer-reviewed scientific journal *IEEE Transactions on Biomedical Engineering*.

7.3 Future Work

1. This work presents an exploratory approach to the use of features derived from phase-spectral information where the potential to detect and distinguish different particles in a variety of scenarios was evaluated through the application of statistical tests. Considering the results obtained, where a good discrimination power was demonstrated, the set of features is suitable to be used in more complex differentiation problems, for instance, to discriminate particles through AI-based algorithms, able to automatically identify the type of particle. Since such methods were applied in the iLoF technique, it would also be possible to compare the results obtained using phase information and the commonly derived time and magnitude spectral features. It involves following a pipeline of classifier optimization, testing, and training. Considering the procedures previously applied, the dataset to test the classifier was generated through a robust Leave-One-Out procedure where each particle was considered an object. Thus, a high number of evaluation runs was required. For instance, in the experiment using the two cancer cell models, 29500 evaluation runs were conducted [19]. Therefore, the application of AI algorithms could be time and computationally-consuming, so it was not possible to realize during this dissertation. However, it would be important

to continue evaluating the informative content of the phase for biological signals that were initiated in this work.

2. In order to study the phase information contained in the back-scattering signals, the FFT algorithm was selected, commonly applied for this purpose. However, different methods can also be used to explore phase. For instance, several reports use Hilbert transform to obtain the minimum-phase response from a spectral analysis [163]. Besides, Hartley transform and, more specifically, the Whitened Hartley spectrum, are also used for phase spectral processing in different applications such as audio and speech classification [164]. Furthermore, phase can also be analyzed indirectly by extracting information from the short-time Fourier phase, processing the group delay function, that constitutes the negative derivative of phase [165]. Therefore, it would be interesting to evaluate the discriminative potential of phase-based features extracted from such methods, in order to evaluate the most effective technique to apply on future feature extraction methodologies based on phase spectral information.
3. Considering the development of methods to be applied in biosensor devices, one important parameter is the Limit Of Detection (LOD), which corresponds to the smallest concentration of the analyte that the biosensor can detect. In this work, one single nanoparticle concentration was tested. In order to determine the LOD of the proposed method, decreasing analyte concentration values should be tested, to evaluate the discrimination power variation with reduced concentrations and compare such results with the LOD of conventional techniques. Since the features demonstrated to be robust for a concentration of nanoparticles similar to the plasmatic levels of cancer-related exosomes found in human plasma samples, it is expected that a satisfactory LOD for the detection of nanoparticles in complex solutions would be obtained.

Appendix A

Appendix

A.1 Code Architecture

The architecture of the code employed to perform each step of the signal processing and analysis pipeline (Section 4.2.2) is presented in Figure A.1

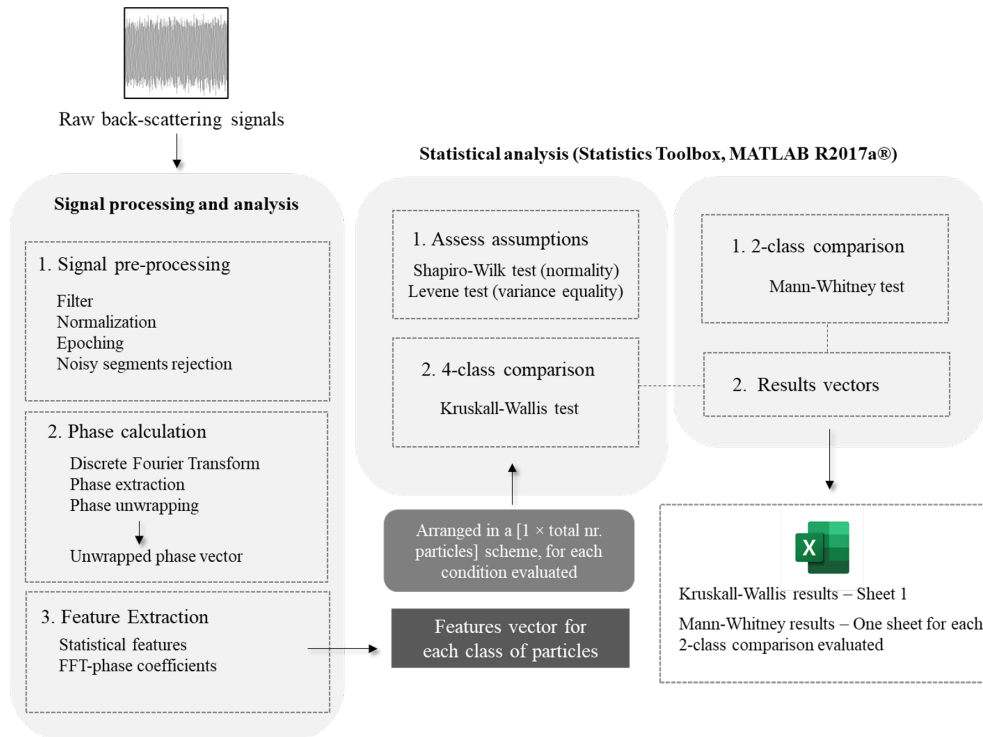


Figure A.1: Code architecture.

A.2 Unwrapping algorithm validation

The following code was created to conduct the unwrapping algorithm validation using synthetic signals.

```

%% Phase simulations
%GOAL: Apply phase calculation and extraction algorithm to a signal
%symmetrical around 0, shift the signal in time-domain and obtain:
% (1) Straight line phase spectrum
% (2) Changes in the slope

clear all ; close all ; clc

%% Create 3 signals from sinc function
fs = 100; % Sampling Frequency
t = -1:1/fs:1-1/fs; % Time vector
n=1:200 % Sample index

f=10 % Frequency
x1 = sinc(2*f*t)

td = 0.5 %Time delay
x2 = sinc(2*f*(t + td)) %Shift to the left
x3 = sinc(2*f*(t - td)) %Shift to the right

%Plot the original signal + delayed signals
plot(n,x1,n,x2,n,x3)
title('Sinc_signal_with_phase_delay_shifts')
xlabel('Sample_number')
ylabel('Amplitude')
legend('Original_signal','Signal_advanced','Signal_delayed');

%% Analyze signal in frequency domain
%SIGNAL 1
y1= fft(x1)
ly = length(y1);
f = (-ly/2:ly/2-1)/ly*fs;
tol = 1e-4;
y1(abs(y1) > tol) = 0;

phase = angle (y1) %Phase calculation
plot(f,phase)
xlabel('Frequency_(Hz)')

```

```

ylabel( 'Phase_(Radians) ' )
title( 'Wrapped_phase_spectrum ' )
unw_phase = unwrap(phase)      %Phase unwrapping
plot(f ,unw_phase)
xlabel( 'Frequency_(Hz) ' )
ylabel( 'Phase_(Radians) ' )
title( 'Unwrapped_phase_spectrum ' )

%SIGNAL 2
y2= fft(x2)
ly = length(y2);
f = (-ly/2:ly/2-1)/ly*fs;
tol = 1e-4;
y2(abs(y2) < tol) = 0;
phase2 = angle (y2)            %Phase calculation
plot(f ,phase2)
xlabel( 'Frequency_(Hz) ' )
ylabel( 'Phase_(Radians) ' )
title( 'Wrapped_phase_spectrum ' )

unw_phase2=_unwrap_( phase2)_____ %Phase unwrapping
plot(f ,unw_phase2)
xlabel( 'Frequency (Hz) ' )
ylabel( 'Phase_(Radians) ' )
title( 'Unwrapped_phase_spectrum ' )

%SIGNAL 3
y3= fft(x3)
ly = length(y3);
f = (-ly/2:ly/2-1)/ly*fs;
tol = 1e-4;
y3(abs(y3) < tol) = 0;
phase3 = angle (y3)            %Phase calculation
plot(f ,phase3)
xlabel( 'Frequency_(Hz) ' )
ylabel( 'Phase_(Radians) ' )
title( 'Wrapped_phase_spectrum ' )

unw_phase3 = unwrap ( phase3)    %Phase unwrapping

```

```
plot(f,unw_phase3)
title( 'Unwrapped_phase_spectrum' )
```

```
%% Observe the effect of noise
```

```
%Goal: Observe the effect of noise in phase unwrapping
```

```
clear all ; close all ; clc
```

```
%% Create a signal with added noise
```

```
fs = 100; % Sampling Frequency
```

```
t = -1:1/fs:1-1/fs; % Time vector
```

```
n=1:200 % Sample index
```

```
f=10 % Frequency
```

```
noise_variance = 0.7
```

```
td = 0.5 %Time delay
```

```
x2 = sinc(2*f*(t + td)) + noise_variance*randn(size(t));
```

```
x3 = sinc(2*f*(t - td)) + noise_variance*randn(size(t));
```

```
%Plot the signals
```

```
plot(n,x2,n,x3)
```

```
title( 'Sinc_signal+_noise_with_phase_delay_shifts' )
```

```
xlabel( 'Sample_number' )
```

```
ylabel( 'Amplitude' )
```

```
legend( 'Original_signal', 'Signal_advanced_0.5s', 'Signal_delayed_0.5s' );
```

```
%% Analyze signal in frequency domain
```

```
y2= fft(x2)
```

```
ly = length(y2);
```

```
f = (-ly/2:ly/2-1)/ly*fs;
```

```
tol = 1e-4;
```

```
y2(abs(y2) < tol) = 0;
```

```
phase2 = angle (y2) %Phase calculation
```

```
plot(f,phase2)
```

```
xlabel( 'Frequency_(Hz)' )
```

```
ylabel( 'Phase_(Radians)' )
```

```
title( 'Wrapped_phase_spectrum' )
```

```

unw_phase2 = unwrap (phase2)      %Phase unwrapping
plot(f,unw_phase2)
xlabel( 'Frequency_(Hz) ' )
ylabel( 'Phase_(Radians) ' )
title( 'Unwrapped_phase_spectrum ' )

y3=fft(x3)
ly=length(y3);
f=(-ly/2:ly/2-1)/ly*fs;
tol=1e-4;
y3(abs(y3)<tol)=0;

phase3=angle_(y3)_____ %Phase_calculation
plot(f,phase3)
xlabel( 'Frequency (Hz) ' )
ylabel( 'Phase_(Radians) ' )
title( 'Wrapped_phase_spectrum ' )
unw_phase3 = unwrap (phase3)      %Phase unwrapping
plot(f,unw_phase3)
title( 'Unwrapped_phase_spectrum ' )

%% Phase intelligibility ID
%Goal: Observe that phase contains information on the location
%of events in the time domain signal

clear all ; close all ; clc

%Signal 1 - Square wave pulse
t = linspace(0, 10E-4);           % Time Vector (0 - 10ms)
f = 4/max(t);                     % Frequency (Cycles/Timespan)
sqwave = sign(sin(2*pi*t*f));     % Signal
figure
plot(t, sqwave, 'k')
ylim(ylim*1.1)
xlabel( 'Time_(s) ' )
ylabel( 'Amplitude ' )

```

```

title ( 'Square_wave_pulse' )

A= fft (sqwave)
ly = length (A);
f = (-ly/2:ly/2-1)/ly*f;

tol = 1e-4;
A(abs(A) < tol) = 0;
ph1 = unwrap(angle(A))

random_vector = rand(1,100)

%random magnitude + unwrapped phase of the original signal
out2 = ifft (random_vector.*exp(j*ph1));

subplot (2,1,1)
plot (t, sqwave)
xlabel ( 'Time_(s)' )
ylabel ( 'Amplitude' )
title ( 'Square_wave_pulse' )

subplot (2,1,2)
plot (t, out2)
xlabel ( 'Time_(s)' )
ylabel ( 'Amplitude' )
title ( 'Reconstruction_from_true_phase_+_random_magnitude_vector' )

clear all ; close all ; clc

%SIGNAL 2 - asymmetric sawtooth waveform
fs = 1e3;
t = 0:1/1e3:1;
d = 0:1/6:1;
x = tripuls (t,0.2,-1);
y = pulstran (t,d,x,fs);
plot (t,y)
hold off
xlabel ( 'Time_(s)' )
ylabel ( 'Waveform' )

```

```

A= fft(y)
ly = length(A);
f = (-ly/2:ly/2-1)/ly*fs;

tol = 1e-4;
A(abs(A) < tol) = 0;
ph1 = unwrap(angle(A))
ph2 = angle(A)

random_vector = rand(1,1001)
%random magnitude + unwrapped phase of the original signal
out2 = ifft(random_vector.*exp(j*ph1));

subplot(2,1,1)
plot(t, y)
xlabel('Time_(s)')
ylabel('Amplitude')
title('Asymmetric_Sawtooth_Waveform')

subplot(2,1,2)
plot(t, out2)
xlabel('Time_(s)')
ylabel('Amplitude')
title('Reconstruction from true phase + random magnitude vector')

```


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