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UNIVERSITY OF CALIFORNIA,
IRVINE

Developing a Multimodal System to Study Cellular Injuries, with the Focus on Traumatic
Brain Injury

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Engineering

by

Pegah Pouladian

Dissertation Committee:
Professor Daryl Preece, Chair
Professor Michelle Digman
Professor Bernard Choi

2023

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DEDICATION

This thesis is dedicated to my family for their continuous support, motivation, and valuable advice. My academic achievements are undoubtedly influenced by them, and this dedication is a small token of appreciation.

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude to everyone who contributed to the realization of this dissertation.

Firstly, I extend my deepest appreciation to my co-advisor, Dr. Michael W. Berns, R.I.P.. His significant contributions to the field of biomedical engineering and his unwavering support have left an indelible mark on my academic journey. Dr. Berns' passing is a great loss to the scientific community, and he is dearly missed.

I am indebted to my supervisor, Dr. Daryl Preece, for his guidance, mentorship, and continuous support throughout my research endeavors. His expertise and encouragement have played a pivotal role in shaping the direction of my work.

Special thanks go to Veronica Gomez Godinez, Chungho Sun, and Nicole Wakida for their substantial contributions and support. Their expertise and willingness to share knowledge have been instrumental in overcoming various challenges.

I would like to acknowledge the invaluable assistance and collaboration of my lab mates, particularly Nic Perez and Janelle Ho. Their insights have enriched my research experience.

I extend my sincere appreciation to the Air Force Office of Scientific Research (AFSOR) for their generous funding, which played a pivotal role in supporting and advancing my research endeavors. The financial assistance provided by AFSOR has been instrumental in the successful execution of my projects, contributing to the broader scientific knowledge in the field.

acknowledge the publishers who have granted permission for the incorporation of my previous work into this dissertation. This research would not have been possible without their collaboration and support.

I would also like to express my heartfelt gratitude to my family — my dad, my mom, and my sister — and my dear friends for their unwavering support, understanding, and encouragement throughout my academic journey.

Finally, I thank the OPTICA publishing group, JPhys Photonics, Frontiers in Bioengineering and Biotechnology, and SPIE for permission to include content from my previously published work in [83, 84, 82, 81, 114, 112]

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PATENTS

- Berns, M. W., Wakida, N., Preece, D., Yamauchi, T., Pegah Pouladian (2022). U.S. Patent Application No. 17/468,223.

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ABSTRACT OF THE DISSERTATION

Developing a Multimodal System to Study Cellular Injuries, with the Focus on Traumatic Brain Injury

By

Pegah Pouladian

Doctor of Philosophy in Biomedical Engineering

University of California, Irvine, 2023

Professor Daryl Preece, Chair

Traumatic Brain Injury (TBI) is a significant public health concern. The Centers for Disease Control and Prevention reported a notable increase in TBI-related incidents from 2006 to 2014. TBI occurs when an external force disrupts the normal function of the brain, leading to a range of outcomes, from mild to severe, with consequences ranging from full neurological recovery to mortality. Despite advances, TBI remains a leading cause of physical impairment and death, particularly among young people.

This work focuses on developing a multimodal microscopy system that includes a custom-made, cost-effective Quantitative Phase Microscope (QPM) that is a pivotal tool in label-free imaging of transparent specimens, especially in cell biology. This microscopy system supports fluorescence imaging, enhancing flexibility for studying cellular structures and dynamics.

The microscopy system is a promising tool for simulating traumatic brain injury in vitro, as it is integrated with a Laser-Induced Shockwave (LIS) system. The LIS system generates controlled shockwaves for brief shear stress applications to replicate conditions similar to those experienced in TBI. Optical Trapping (OT), using continuous laser light manipulation, is also combined with the LIS system, creating a versatile platform to investigate the interplay between shockwaves and optical trapping effects on biological specimens, particularly for

single-cell manipulation. This integrated approach significantly improves the understanding of dynamic cellular responses, morphological changes, and intracellular dynamics under controlled conditions.

A section of the thesis is dedicated to quantitative phase image processing, providing methodologies to convert raw camera images into precise heightmaps, and addressing challenges in cell segmentation and feature extraction. The chapter aims to equip researchers with scientifically accurate tools for analyzing cellular structures.

In the final chapter, we investigate the response of astrocyte cells to laser-induced shockwaves, focusing on morphological characteristics such as surface area, volume, and circularity. We chose astrocytes as the focal point of this investigation because astrocytes undergo a defense mechanism called astrogliosis when triggered. The experimental setup involves control and shockwave-exposed groups in a custom-made heating chamber with tightly controlled environmental conditions. Statistical analysis reveals significant differences in cellular parameters, providing insights into the impact of shockwaves on astrocyte cell morphology. This chapter contributes to understanding cellular responses to mechanical stimuli and opens avenues for further investigations into underlying mechanisms. Overall, the thesis offers a scientific exploration of advanced microscopy techniques and their applications in studying cellular dynamics and responses.

Chapter 1

Quantitative Phase Microscopy (QPM)

1.1 Motivation

Quantitative Phase Microscopy (QPM) has become an essential tool in microscopy, providing label-free imaging of transparent specimens, especially in cell biology and related fields. The QPM enables the reveal of the intricate details of living cells and tissues without invasive techniques such as labeling or staining. Traditional optical microscopy often fails to deliver the necessary contrast and resolution for such investigations. QPM fills this gap by quantifying the phase changes that occur when light interacts with the specimens. It provides vital information about cellular structures, behaviors, and overall health.

This chapter discusses the principles of Quantitative Phase Microscopy (QPM) and its various applications. It presents a custom cost-effective QPM system that supports both quantitative phase imaging and fluorescence, offering enhanced flexibility for studying cellular structures and dynamics. The system is compatible with common glass-bottom cell culture

dishes, simplifying specimen preparation and making QPM more accessible in cell biology research.

1.2 Quantitative Phase Microscopy

Visualizing and imaging cellular material poses significant challenges due to the inherent lack of contrast in translucent specimens, particularly when working with live cells. While staining techniques can enhance contrast in fixed specimens, they are usually not feasible for live cells. To address this problem, various forms of phase microscopy have been developed to manipulate the optical path and improve contrast to visualize viable cellular samples [72, 10]. The use of quantitative phase imaging (QPI) enables nondestructive imaging of transparent structures in 2D, 3D, and 4D formats, which provides valuable quantitative insights based on intrinsic contrast. [80].

When it comes to visualizing cellular material, understanding light's interaction with specimens is crucial. Stained samples absorb some light waves, which can change the amplitude of emergent light waves. These variations in amplitude can create differences in brightness and color that are visible to the human eye. However, unstained samples do not absorb light at the visible wavelengths used in microscopy, which means that there is minimal change in amplitude. Consequently, unstained specimens appear translucent, which makes it difficult to discern their morphology [24].

Despite this, when light passes through a translucent sample, it experiences a phase shift known as "phase retardation." This phase shift is caused by the sample's impact on the propagation of light waves. Thicker sample regions induce greater phase retardation, and the relative phase shift, measured in radians, determines the extent of this phase shift. It is worth noting that unlike amplitude variations, differences in phase are imperceptible to the

naked eye or photographic film [88].

Optical phase microscopy methods were developed to overcome the challenge of visualizing phase properties in unstained cellular material. These methods work by converting phase properties into amplitude differences that can be visually detected [24].

1.2.1 Optical Phase Microscopy

Several forms of optical phase microscopy have been employed for examination of live cells. The major types of phase microscopy techniques are Zernike phase microscopy, differential interference contrast (DIC) microscopy, Hoffman modulation contrast (HMC), and quantitative phase microscopy (QPM) [126, 71, 40, 72, 10, 24].

Zernike Phase Microscopy

The Zernike phase microscope, introduced by Fritz Zernike, employs a phase plate to induce a specified wavelength fraction of the phase shift in the light passing directly through a sample. This results in destructive interference, creating intensity contrast that reveals details of otherwise transparent cellular specimens. This innovation revolutionized phase object imaging without the need for staining or tagging [127]. However, Zernike phase microscopy is associated with the appearance of light halos at the edges of the sample, which can hinder accurate boundary localization, particularly when quantifying cell size and structure [126, 24].

Differential Interference Contrast (DIC) Microscopy

DIC microscopy, developed by George Nomarski, utilizes a modified Wollaston prism to detect optical gradients in specimens and convert them into intensity differences. This technique provides 3D "shadow relief" images and superior resolution compared to Zernike phase contrast microscopy. It also allows for optical sectioning and minimizes halo effects at the edges of the specimen. However, DIC microscopy is costly to set up, requires high incident light levels, and may not be suitable for imaging with plastic culture dishes [71, 24].

Hoffman Modulation Contrast (HMC)

Hoffman modulation contrast microscopy, similar to DIC microscopy, produces 3D-like images with good resolution and visibility. It is suitable for imaging through plastic culture ware, and it is widely used in this context. However, the technique lacks the precise location of the image details, which can complicate spatial navigation through the specimens. Like DIC microscopy, HMC involves additional optical components and is relatively expensive to implement [40, 24].

Quantitative Phase Microscopy (QPM)

Quantitative phase microscopy (QPM) is an innovative approach that combines the qualitative attributes of previous phase imaging techniques with the capability to provide quantitative information about specimen phase characteristics. Unlike conventional optical phase microscopy, where the components of the amplitude and phase image are inseparable, QPM allows the separation of these components in the generated images [24]. QPM involves the calculation of a "phase map" from a set of images obtained under standard bright-field microscopy. A computational algorithm is applied to analyze these images, allowing quan-

tification of the phase change induced by the specimen. QPM offers a simple yet effective way to perform phase-based analysis of cell structure, morphology, and composition [72, 10].

Full-field quantitative phase imaging (QPI) methods can be categorized into two primary classes: those based on phase shifting and those rooted in off-axis holography. In contrast to conventional holography, which captures both phase and amplitude information, QPI specifically extracts the phase map of an object while decoupling it from intensity data [74].

In the first approach, QPI images are obtained directly in the focal plane using in-line geometries coupled with temporal phase shifting. Multiple intensity images are recorded as the time delay of the reference is incrementally adjusted. The irradiance at the detector can be expressed as:

$$I(x, y) = I_0 + I_1 + \sqrt{2I_0I_1(x, y)} \cos(\omega\tau + \phi(x, y)) \quad (1.1)$$

where I_0 and I_1 are the irradiances for the reference and object fields, ω is the angular frequency of the optical field, τ is the time delay between the two waves, and $\phi(x, y)$ represents the phase map [80].

The phase image ϕ can be computed from four intensity measurements corresponding to $\omega\tau = 0, \pi/2, \pi, 3\pi/2$ as:

$$\phi = -\arg(I_0 - I_\pi, I_{3\pi/2} - I_\pi) \quad (1.2)$$

In the spatial case of phase-shifting interferometry, the transport of intensity equation relates the axial gradient of intensity to the transverse Laplacian of the phase. Normally, two intensity images (in focus and slightly out of focus) suffice to obtain the Laplacian of the phase image. This approach is straightforward but struggles with low-frequency recovery

and is valid mainly for low-resolution imaging due to its small-angle approximation [80].

Off-axis interferometry exploits spatial modulation and provides a phase map from a single intensity recording. The irradiance of the interferogram is given by:

$$I(x, y) = I_0 + I_1 + \sqrt{2I_0I_1} \cos(\alpha x + \phi(x, y)) \quad (1.3)$$

where α is the spatial frequency introduced by the off-axis angle θ [80].

The key advantage of off-axis methods is their single-shot capability, which enables high-speed imaging. However, this comes at the cost of a limited space-bandwidth product. The choice between phase-shifting and off-axis methods depends on specific applications.

Furthermore, the use of neural networks for the phase retrieval of intensity signals is an intriguing area of exploration in QPI [92, 87].

1.3 Quantitative Phase Microscopy's Applications

One of the main applications of QPM is in the study of cell morphology and dynamics. By providing quantitative measurements of cellular structures, QPM offers insights into the structural changes that occur in cells under different conditions [24]. For example, QPM has been used to investigate the volume and morphology of erythrocyte cells in different osmotic environments [24]. This information can be valuable to understand the mechanisms behind diseases such as anemia and sickle cell disease.

QPM also has applications in the field of cancer research. It can be used to study changes in cell morphology and refractive index, which can provide insights into the early detection and monitoring of cancer [28]. For example, QPM has been combined with waveguide

trapping techniques to study changes in red blood cell morphology during planar trapping and transportation [1]. This research has the potential to contribute to the development of new diagnostic tools for cancer.

In addition to its applications in cell biology and cancer research, QPM has also been used in materials science and engineering. It can be used to study the dynamics of thin films and provide detailed information about the underlying physics of thin films [117]. QPM has also been combined with digital holography to create a quantitative phase contrast confocal microscope, which offers high contrast intensity images and optical sectioning capabilities [57]. Phase reconstruction methods in QPI can be broadly categorized into two groups: interferometric methods and propagation-based non-interferometric methods. These methods each have their trade-offs in terms of image acquisition speed, horizontal resolution, temporal sensitivity, and spatial sensitivity. Classical interferometric methods often require extensive and costly setups, but they produce results that are not influenced by the reconstruction algorithms associated with propagation-based methods. This makes them suitable for applications involving slowly-varying phase objects, such as densely populated cell cultures. Interferometric methods benefit from the use of spatially and/or temporally incoherent light sources, which enables QPI to achieve spatial resolution comparable to that of bright-field imaging while simultaneously eliminating speckle noise. This combination of features makes this custom QPM system both cost-effective and capable of providing high-quality quantitative phase measurements, making it a valuable tool for various applications [99].

1.4 Interferometry

Quantitative Phase Microscopy (QPM) uses interferometry as its fundamental technique to capture and analyze phase changes in light resulting from its interaction with a specimen. The Linnik interferometer [56], is the primary instrument employed in QPM due

to its effectiveness and widespread application in microscopy and metrology systems. The interferometer consists of several essential components, each serving a unique role:

- **Beam Splitter:** The key component of the Linnik interferometer is the beam splitter, a partially reflective optical element. It splits the incoming light into two beams, which are the sample beam and the reference beam. This splitting process is crucial to the measurement process that relies on interference.
- **Sample:** The term "sample" refers to the object or specimen that is being examined. It plays a pivotal role within the interferometer, as it introduces phase alterations into the sample beam by interacting with the light. Changes in the refractive index and thickness of the sample are responsible for these phase alterations.
- **Reference Beam:** The reference beam plays a vital role as a stable baseline for measuring the phase changes caused by the sample. It is important to note that this reference beam doesn't interact with the sample beam. As a result, any phase changes detected in the sample beam can be confidently attributed to the sample itself.
- **Reflecting Surfaces:** Reflecting surfaces play a vital role in accurately redirecting both sample and reference beams towards the imaging and detection apparatus. These surfaces are positioned strategically to ensure the correct alignment of the beams, which enables interference to occur.
- **Imaging/Detection System (Camera):** The imaging and detection system, typically consisting of a camera, is responsible for capturing the interference patterns that emerge when the sample and reference beams interact. These patterns contain valuable information about the optical properties of the sample.

The list of components mentioned covers the fundamental elements required. However, depending on the setup and application, additional components or accessories are incorporated.

Here are some commonly used additional components:

- **Illuminating Light Source (e.g., LED):** The illuminating light source, such as an LED (Light Emitting Diode), provides the light that is split into the sample and reference beams. The quality and stability of this light source are crucial to obtain accurate interferometric measurements. Therefore, it is important to ensure that the light source is of high quality and remains stable throughout the measurement process.
- **Polarization Control Elements:** In certain cases, researchers may manipulate the polarization state of illuminating light or reference beam using polarization control elements such as polarizers and waveplates. This is particularly important when studying anisotropic samples or when polarization information is required.
- **Environmental Control:** Environmental control chambers or systems can be integrated to maintain stable conditions during imaging experiments that require precise control over temperature, humidity, or gas composition.
- **Objective Lenses:** In microscopy, the quality of objective lenses plays a vital role in capturing high-resolution images. Objective lenses with specific numerical apertures and magnifications are selected on the basis of the imaging needs.
- **Autofocus Systems:** In setups for automated microscopy, autofocus systems can be incorporated to maintain optimal focus when imaging dynamic samples or performing time-lapse imaging.
- **Computer and Software:** A computer equipped with specialized software is typically used to control the microscope, capture images and perform data analysis. This software may comprise features for phase reconstruction and quantitative analysis.

1.5 Custom Quantitative Phase Microscope Setup

The quantitative phase microscope that was developed in the lab had initially a different configuration, compared to the current setup. In the following section, both configurations are explained.

1.5.1 Initial Setup

Quantitative Phase Microscopy Setup

To acquire interference images for quantitative phase microscopy (QPM), a red-light LED with a 633 nm wavelength is used as the light source. The beam splitter separates the LED light into two beams: the reference beam and the sample beam. Each beam is focused on the reference mirror and the sample through their respective objective lenses. For 20x magnification, two identical dry-immersion objectives (*PLN 20X, Olympus*) are used, and for 40x magnification, two identical water-immersion objective lenses (*LUMPLFL 40X, Olympus*) are used. The power of the red-light beam at the sample objective lens's back aperture is 0.32 mW. A 12-bit CCD camera (*acA1920-40um, Basler AG*) captures the interference images produced by the reflected beams from the sample and the reference mirror, with a maximum frame rate of 41 fps. The data is then transferred to a PC for further analysis.

The QPM setup also includes a feedback control system designed to precisely adjust the Optical Path Difference (OPD). The feedback system consists of a laser source with a 1.3 μm wavelength (*S1FC1310, Thorlabs*), an infrared Photo-Detector (PD) (*62-271, Edmund Optics*), a Piezoelectric Transducer (PZT) (*AE0505D08F, NEC TOKIN*), and a DSP-based signal demodulation circuit. The PZT in the reference arm can manipulate the OPD with a resolution of less than 1 nm and a maximum variation range of 440 nm at a frequency of approximately 500 Hz [121].

Fluorescence Microscopy Setup

For fluorescence microscopy, a blue-light LED with a 475 nm wavelength is used. The LED is positioned in front of a condensing lens and a mirror with 20% reflectance, which stimulates fluorescence in the sample. This light is directed through the same objective lens used for QPM. The fluorescence light intensity at the sample objective lens's back aperture can reach up to 4.4 mW. The system allows for the interchange of the LEDs according to the specific dye used. The emitted fluorescence light passes through the objective lens and mirrors before being captured by the camera.

The schematic of QPM and fluorescence imaging is shown in Figure 1.1.

Imaging Acquisition and Processing

The image acquisition and processing are done through the QPMAcquisition GUI, which has been developed by the Hamamatsu company. The software enables capturing the quantitative phase, phase contrast, differential interference contrast simultaneously, and fluorescence images semi-simultaneously.

1.5.2 Current Setup

Quantitative Phase Microscopy Setup

To ensure the effective operation of the initial setup, with the integration of a laser-induced shockwave and an optical trapping system from below, custom-made dielectric mirrors were employed (refer to Figure 1.2). These mirrors exhibit a 50% reflectance in the visible light spectrum and offer approximately 92% transmission at a wavelength of 1030 nm. As a result, the LED light from the Quantitative Phase Microscopy (QPM) was reflected by these mirrors

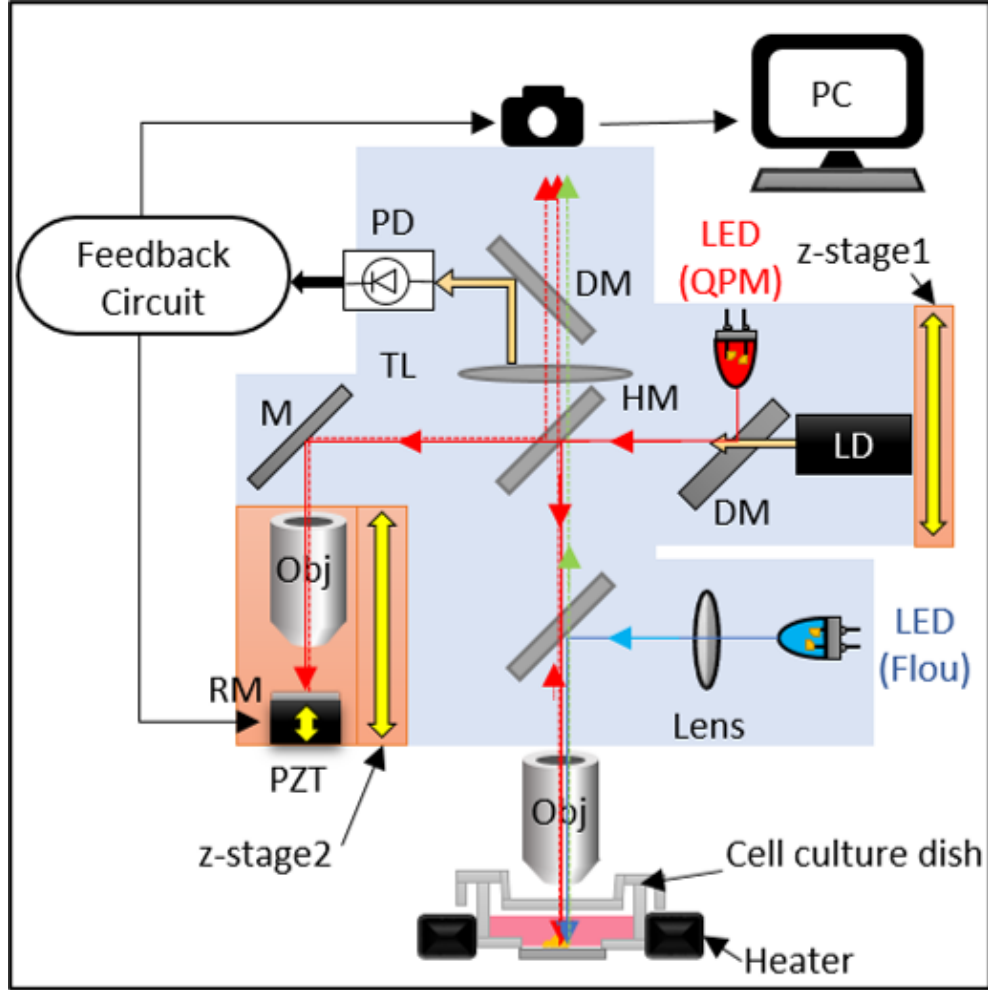


Figure 1.1: Quantitative phase and fluorescence microscope setup. The setup includes a photodiode (PD), light-emitting diode (LED), laser diode (LD), reference mirror (RM), half mirror (HM), dichroic mirror (DM), mirror (M), tube lens (TL), and objective lens (Obj) [123]. Reflected beams are colored with a stripe pattern to prevent confusion.

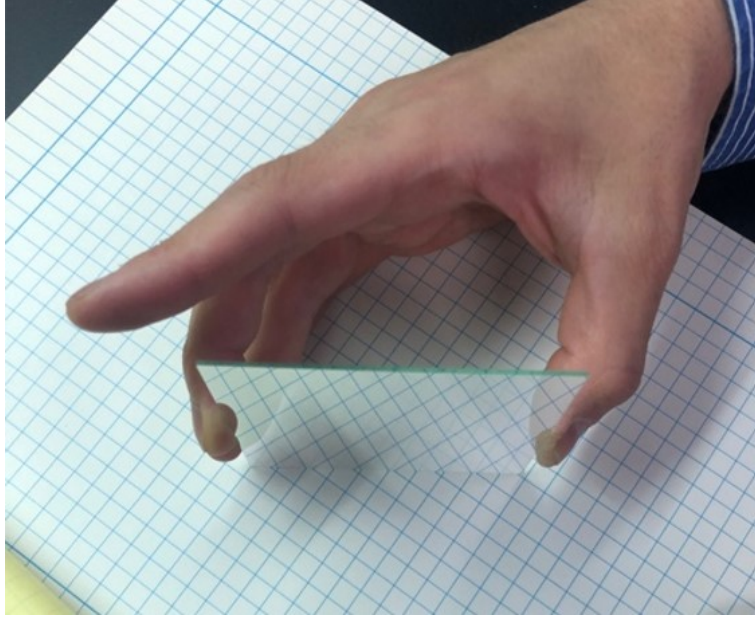


Figure 1.2: Custom-made dielectrics manufactured by Hamamatsu with a reflectance of 50% in visible light and approximately 92% transmission percentage at 1030 nm.



Figure 1.3: The mirrors are cut into 2×2 mm pieces and glued to bottomless cell culture dishes

and captured by the camera, while the laser light passed through the mirrors, enabling focus on the cell culture media. These dielectric mirrors were precision-cut into 2×2 mm squares and securely affixed to bottomless cell culture dishes (depicted in Figure 1.3).

The thickness of the mirrors led to a notable loss of laser power, as each laser pulse resulted

in the formation of cracks on the mirror's surface. This rendered the mirrors non-reusable and significantly impacted the efficiency of both time and cost, necessitating the creation of new dishes for each experimental run.

Moreover, the mirror's thickness imposed restrictions on the selection of objective lenses capable of effectively focusing the laser beam. Specifically, these objective lenses needed to maintain a working distance exceeding 1 mm, which, in turn, limited the range of objective lenses that could be employed. Furthermore, this higher working distance correlated with a lower numerical aperture (NA). While this attribute proved suitable for laser-induced shockwave experiments, it posed challenges for the integration of a robust optical trapping setup.

As a result of these limitations, the initial setup was modified to optimize the current configuration for quantitative phase imaging, even in the absence of highly reflective surfaces. Consequently, imaging can now be performed using conventional glass-bottom cell culture Petri dishes. The current setup, as illustrated in Figure 1.4, does not have a feedback mechanism. Instead, the PZT is linked to a function generator that generates a sine wave with a frequency of 5 Hz, an offset of 5 volts, and a peak-to-peak amplitude of 8 volts. In the current setup, both the reference and sample beams reflect from glass surfaces. The reference beam reflects from the coverslip attached to the PZT, while the other beam is reflected from the glass surface at the bottom of the dish.

Fluorescence Microscopy Setup

For fluorescence imaging, in contrast to the initial setup, the illumination and the imaging is done from the bottom of the dish. An LED illumination system (*X-Cite XYLIS, Excelitas*) is used to provide excitation light. A holder for the filter set cube is positioned beneath the cell culture dish. The excitation light is guided through the filter set cube and

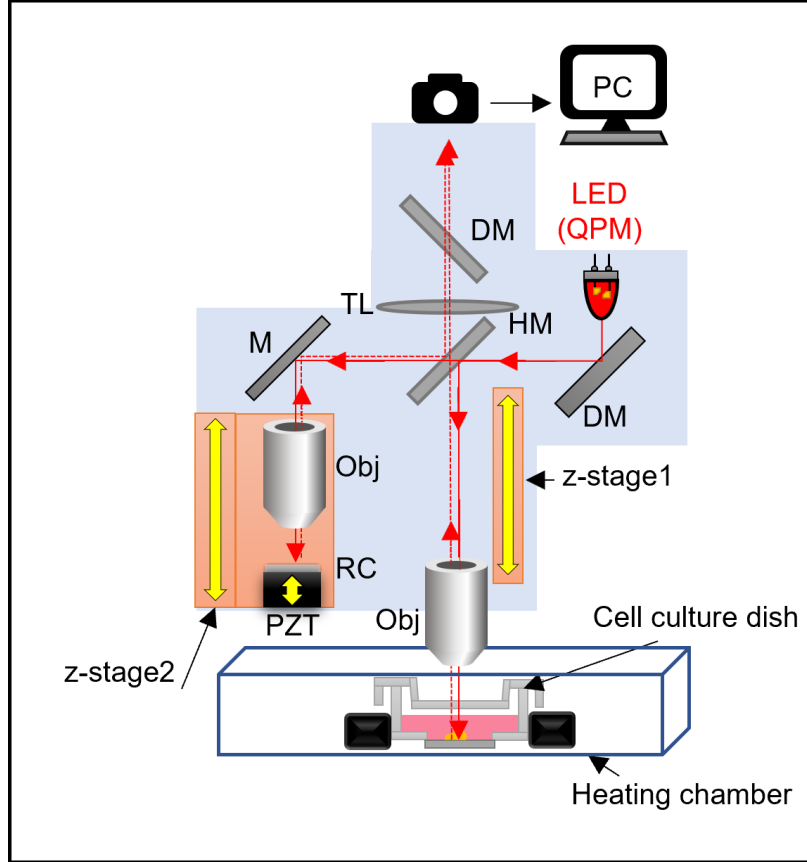


Figure 1.4: Current QPM setup. The setup includes a light-emitting diode (LED), reference mirror (RM), half mirror (HM), dichroic mirror (DM), mirror (M), tube lens (TL), and objective lens (Obj) [123]. Reflected beams are colored with a strip pattern to prevent confusion.

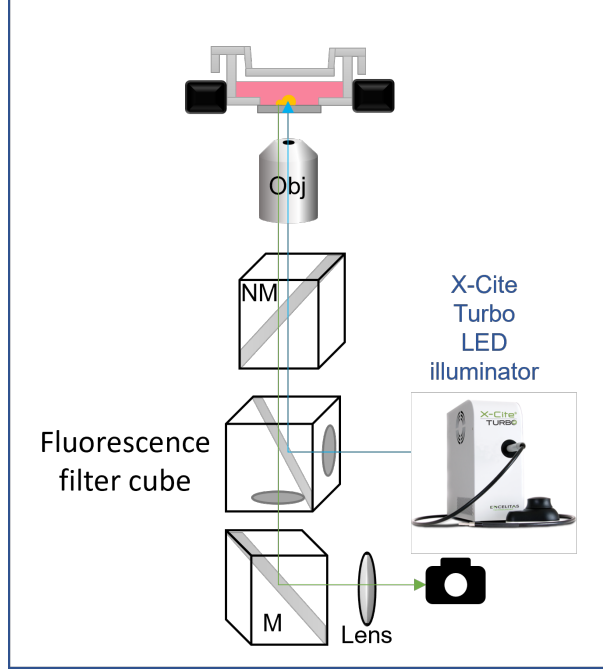


Figure 1.5: Current fluorescence microscopy setup. This setup includes a LED Illumination System, reference mirror (RM), fluorescence filter set cube, mirror (M), focusing lens, and objective lens

focused to illuminate the cells from the bottom with a 40x Objective lens (*Plan-Apochromat 40x/1.3 Oil, Carl Zeiss*). Following the interaction, the emitted light is captured by a 12-bit CMOS camera (*acA3088-57um - Basler ace*), positioned beneath the cell culture dish. The schematic of the current fluorescence imaging modality is illustrated in Figure 1.5.

1.6 Discussion

The development of the custom Quantitative Phase Microscope (QPM) outlined in this chapter marks a notable advancement in cell biology research. Its non-invasive imaging capabilities offer researchers unprecedented opportunities to explore transparent specimens, providing valuable insights into cellular structures and dynamics.

This custom quantitative phase microscopy (QPM) system offers a significant cost advantage

compared to other QPMs on the market. The primary reasons for its cost-effectiveness are two-fold. First, instead of utilizing expensive laser sources [60] or spatial light modulators (SLMs) [129], which are commonly employed in many QPM systems, this custom QPM relies on economical LED sources and a straightforward Linnik interferometer configuration.

Furthermore, the design of this system has a unique feature that sets it apart. Both the sample and reference arm beams pass through the same objective lens, which has the benefit of reducing aberrations in the obtained images. This is a critical advantage for achieving high-quality, distortion-free phase measurements.

In our initial setup, a critical issue that needed addressing was the thickness of the mirror beneath the sample dish. The mirror’s relatively high thickness not only resulted in the attenuation of laser power but also caused damage to the mirror itself, making precise control of laser energy at the focal point a challenging task. Moreover, the thicker mirror limited our choice of objective lenses for focusing the laser beam, necessitating a working distance of more than 1 mm. As a result, this constrained the optical resolution of the cells and their internal organelles. However, through a series of enhancements, optimizations, and the implementation of more effective algorithms, we successfully overcame these challenges. We now have a custom QPM system that is compatible with commonly used glass-bottom cell culture dishes. This significant advancement simplifies specimen preparation, eliminates the need for specialized dishes, and greatly expands the accessibility of QPM technology to a broader range of researchers, representing a substantial improvement in our experimental setup for this thesis.

The custom QPM system also needed to be cost-effective while maintaining flexibility. The integration of a fluorescence modality added versatility, enabling the study of dynamic cellular responses and specific fluorescent markers. The system uses broad-spectrum LED illumination, with the flexibility to adapt to different fluorescent dyes and experimental requirements.

While the custom QPM system has made substantial strides, there are areas that warrant attention for further improvement. A significant challenge in obtaining optimal interference images is the manual adjustment of 3 micrometers: the objective lens focus, and the z-stage 1 and z-stage 2 micrometers (as depicted in Figure 1.4). Precise alignment is crucial, as any deviation can lead to images being out of focus, low-quality interferences, and hinder the unwrapping of phase information. The time-consuming nature of this manual adjustment underscores the need for future automation to streamline the imaging process, reduce the potential for human error, and enhance overall efficiency.

In conclusion, the custom Quantitative Phase Microscope (QPM) proves to be a valuable tool for researchers in cell biology and related fields. Overcoming challenges such as mirror thickness, its adaptability, integration of a fluorescence modality, and compatibility with common glass-bottom dishes contribute to its significance in advancing quantitative phase imaging. Ongoing efforts to address challenges like noise reduction, image quality enhancement, and the automation of focusing ensure that the system remains a practical and reliable choice in label-free microscopy, providing a versatile platform for various scientific investigations.

Chapter 2

Laser-Induced Shockwave Dynamics in Cellular Research

2.1 Motivation

This chapter explores the Laser-Induced Shockwave (LIS) system's setup, operation, and integration with quantitative phase microscopy. The LIS system generates controlled shockwaves, exposing cells to brief, sudden shear stress applications.

In addition, our experimental setup includes optical trapping (OT) using continuous laser light. Optical trapping allows for the manipulation of microscopic particles.

The integration of optical trapping with the LIS system creates a versatile platform for studying the interplay between laser-induced shockwaves and optical trapping effects on biological specimens. This combined approach enhances our ability to investigate dynamic cellular responses, morphological changes, and intracellular dynamics in a controlled manner, providing valuable insights for biophotonics and neuroscience research.

2.2 Laser-Induced Shockwave

When laser light is pulsed and exceeds the optical breakdown threshold of a fluid, it sets off a series of events that involve nonlinear absorption and the creation of plasma through photo-ionization. This happens when the intense laser light interacts with the fluid and ionizes its atoms or molecules, leading to a highly energized plasma state. The unique pressure and temperature conditions inside the plasma, as opposed to the surrounding fluid, lead to the formation of a cavitation bubble within the fluid and plasma expansion [53, 84]. Researchers have made significant efforts to understand laser-induced shockwaves, which are a natural consequence of this complex interaction. They have studied various aspects of these shockwaves, including their pressure rise time and how they depend on factors such as laser pulse energy and duration [78].

The knowledge gained from studying laser-induced shockwaves has practical applications in diverse fields, including biology, materials science, physics, chemistry, and aerospace engineering. These shockwaves have been used in applications such as hardness measurement, nanoparticle separation and analysis, photomask cleaning, and the study of plasma expansion [25, 68, 47, 73]. A detailed understanding of laser-induced plasma and shockwaves is crucial not only for scientific progress but also for enabling various medical and industrial applications [37].

2.2.1 Laser-Induced Shockwaves and Traumatic Brain Injury

Laser-induced shockwaves (LIS) have recently gained significant attention due to their versatile applications in the realm of biological research, particularly within the intersection of biophotonics and neuroscience. These shockwaves are produced when a high-energy laser pulse interacts with a target, leading to the formation of microbubbles and the propagation

of shockwaves through the surrounding tissue, thereby inducing a range of biological effects. An intriguing consequence of LIS is their ability to precipitate rapid changes in intracellular calcium levels, a phenomenon thoroughly examined by Selfridge et al. within the context of a model for traumatic brain injury (TBI) [90].

This discovery holds particular importance since disturbances in intracellular calcium signaling have been directly linked to the pathophysiology of TBI, affecting neuronal functionality and viability [118].

Furthermore, astrocytes, the most numerous cells in the central nervous system (CNS), exhibit dynamic responsiveness to the mechanical forces, notably shear stress [114], that can be generated by LIS. Reactive astrogliosis, the process through which astrocytes react to neural tissue damage, has emerged as a topic of growing significance, offering potential strategies for mitigating the progression of brain injuries. The functional responses of astrocytes exhibit variance depending on the severity of CNS insults, involving distinct morphological changes and the uptake of extruded materials [94].

In conclusion, Laser Induced shockwave has been shown to be a promising technique to simulate traumatic brain injury for in vitro studies, leading to a better understanding of the underlying mechanisms and potential therapeutic interventions.

2.2.2 Laser-induced breakdown

In the context of laser-induced breakdown in transparent liquids, especially water, a complex interplay of mechanisms, such as photoionization, inverse Bremsstrahlung absorption, and cascade (avalanche) ionization, leads to the formation of a plasma [48]. The characteristics of water in this context are often modeled as an amorphous semiconductor, and the band gap energy Δ of water, which is the energy required for electrons to transition to an excitation

band, is 6.5 eV [89, 119, 36, 70].

Photoionization or tunneling generates quasi-free seed electrons in the conduction band, which are essential for initiating avalanche breakdown. Impurities in the liquid can facilitate the generation of seed electrons, reducing the breakdown threshold. Seed electrons, in turn, absorb laser photons via inverse Bremsstrahlung absorption, gaining kinetic energy. When the kinetic energy is beyond the effective ionization potential, impact ionization occurs, producing additional seed electrons and starting a cascade with an exponential rise in electron population. Loss mechanisms, including inelastic collisions and electron diffusion, contribute to a threshold irradiance for breakdown, leading to plasma formation [106]. This process heats the focal volume, causing rapid temperature and pressure increases [109].

Spectroscopic measurements have determined that the maximum temperature within the breakdown plasma typically falls within the range of 6000 K to about 15,000 K for picosecond and nanosecond laser pulses [109]. However, for sub-picosecond pulses that lead to breakdown without detectable light emission, temperature values remain undetermined.

In addition to temperature, pressure within the breakdown plasma is of interest, even though it cannot be directly measured. Pressure can be inferred from factors such as temperature rise or the initial velocity of the shock wave at the plasma's edge. The pressure within the breakdown volume rises rapidly, reaching its maximum at the end of the laser pulse. This pressure disturbance propagates into the surrounding liquid and can be measured using techniques such as fast hydrophones or optical methods, producing a pressure pulse or shock wave [107].

Shock Wave Propagation and Bubble Dynamics

When investigating laser-induced breakdown, it was soon noticed that shock waves were produced, visible in photographs or shadowgraphs of the breakdown area [11]. By the use of

photography and shadowgraphy, the propagation of the shock front and bubble wall motion have been determined by direct evaluation. The pulse duration of the shock wave or its precise form can only roughly be estimated from its width on photographic or streak images due to the complicated image-forming process involving the camera settings. However, this is possible with optical deflection measurements [108].

The dissipation of the shock wave can be quantified via the measurement of the shock front velocity when measured as a function of propagation distance. The energy loss is high immediately after detachment from the plasma and gets almost negligible as soon as the propagation velocity reaches the linear sound speed in the liquid [106].

Following the detachment of a shock wave from the plasma generated by a laser, there exists a residual volume characterized by high pressure and temperature, which is not in thermal equilibrium with its surroundings. This volume undergoes expansion against the ambient static pressure, leading to the conversion of potential energy into the creation of a spherical bubble, often containing water vapor [9, 12]. If the final volume, when the energy of the plasma is used up, is a spherical bubble of maximum radius R_{max} , as often seen in experiments, the energy stored in the bubble, E_B , is given by:

$$E_B = \frac{4\pi}{3}(p_{\text{stat}} - p_v)(R_{\text{max}})^3 \quad (2.1)$$

After the bubble has reached the maximum radius, the interior pressure is p_v (2.33 kPa in water at 20°C), much smaller than the outer pressure p_{stat} (normally about 1 bar = 100 kPa), and the bubble will be compressed back to a small volume, inverting the expansion. As the bubble attains its maximum radius, it undergoes a dramatic collapse, characterized by the emission of a luminous flash [17] and the release of a shock wave into the surrounding liquid [51]. This shock wave contributes significantly to the dissipation of energy, leading to the subsequent rebound of the bubble, which expands to a smaller maximum radius. It's

important to note that bubble collapse is inherently unstable, as it may be subject to the Strube instability [98].

The dynamics of these spherical bubbles are mathematically described by several equations, with one of the simplest being the Rayleigh model [85]. This model factors in variables like the liquid's density and the pressure differential between the inside and outside of the bubble, offering insights into the bubble's behavior and evolution over time [52].

Researchers have achieved high-speed photography, capturing bubble collapse at rates of up to 100 million frames per second (FPS) [55]. Notably, these bubbles tend to slightly elongate during the collapse, especially when generated by slightly elongated laser plasmas. This elongation adds to the complexity of understanding the dynamics of bubble collapse.

Scientists have found that shock wave emission during laser-induced bubble collapse in water is a complex and intricate phenomenon. By comparing experimental data with Gilmore's theoretical model [55], it was observed that the model effectively describes bubble collapse and shock wave emission. The use of a high-speed fiber optic hydrophone provided valuable insights into shock wave dynamics, with recorded rise times potentially underestimating their actual speed.

Furthermore, former studies have revealed that when a bubble collapses near a boundary, it may result in the emission of different shock waves with varying strengths and complex dynamics, adding an additional layer of intricacy to the study [15]. These observations suggest that shock wave dynamics during bubble collapse are highly dependent on the properties of nearby surfaces, such as solid boundaries or free surfaces, offering further avenues for exploration and enhancing our understanding of the underlying physics [15]. High-speed imaging techniques, along with the interplay between bubble size and shock wave parameters, contribute to a more detailed understanding of the processes in this field of study.

2.2.3 Laser-Induced Shockwave Specifications

In order to accurately calculate the specifications of the laser-induced shockwave, a thorough characterization of the laser's properties is essential. This process necessitates a comprehensive understanding of the laser beam's characteristics. To ascertain the shape of the beam, an image of the focus of the beam is captured by the quantitative phase microscope's camera. The intensity profile of the focal spot is analyzed by fitting a Gaussian function, which yields the FWHM of 3.1 microns. Figure 2.1(a) displays the image of the laser beam's focus, while Figure 2.1(b) displays the intensity profile and the fitted Gaussian.

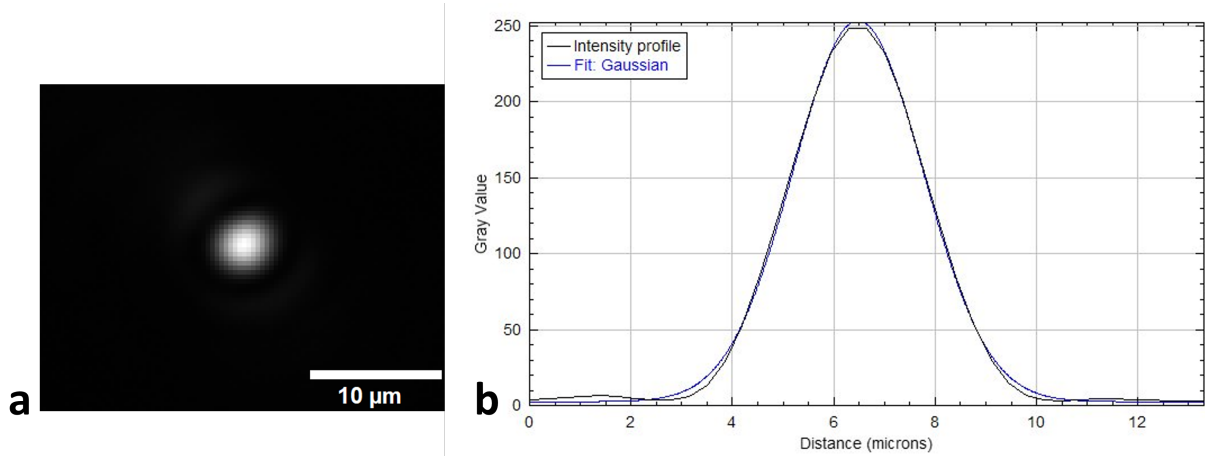


Figure 2.1: a) Image of the laser beam's focus b) Intensity profile of the beam's focus and a fitted Gaussian function

To accurately calculate the pulse power, the laser is set on the pulse mode and is triggered by the function generator at its nominal frequency (2000 Hz). To measure the average power, the thermal sensor is placed at the aperture of the objective lens. Figure 2.2 displays the measured average power vs different angles of half-wave plate. By the use of a half-wave plate, the laser power is set right after the threshold that leads to 100 percent LIS with each pulse, the measured power is 120 milliwatts.

In order to calculate the pulse power, the average power is divided by the frequency (2000)

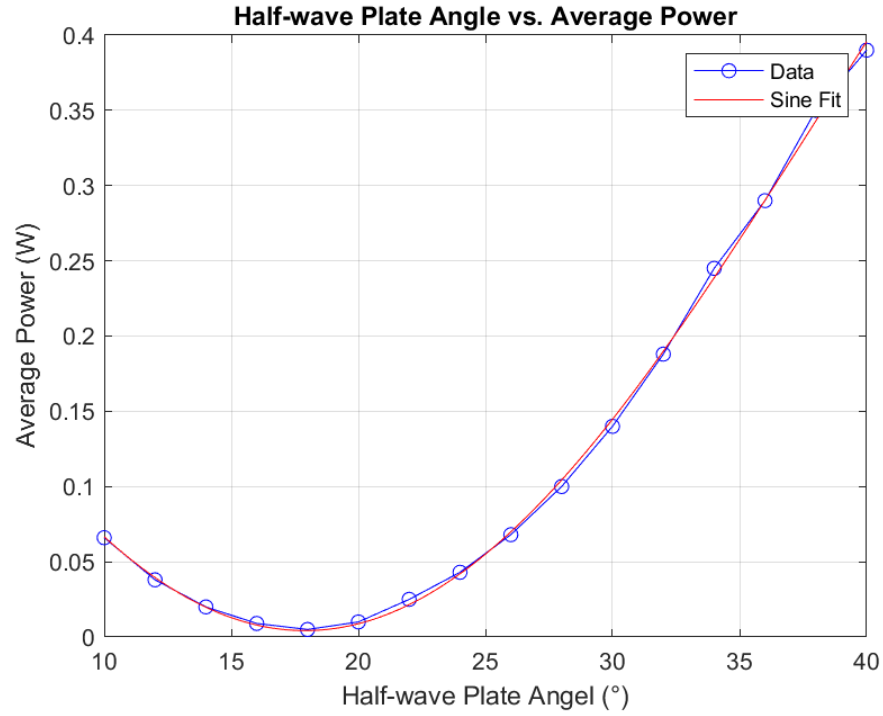


Figure 2.2: Time averaged power vs half-wave plate angle and sine fit

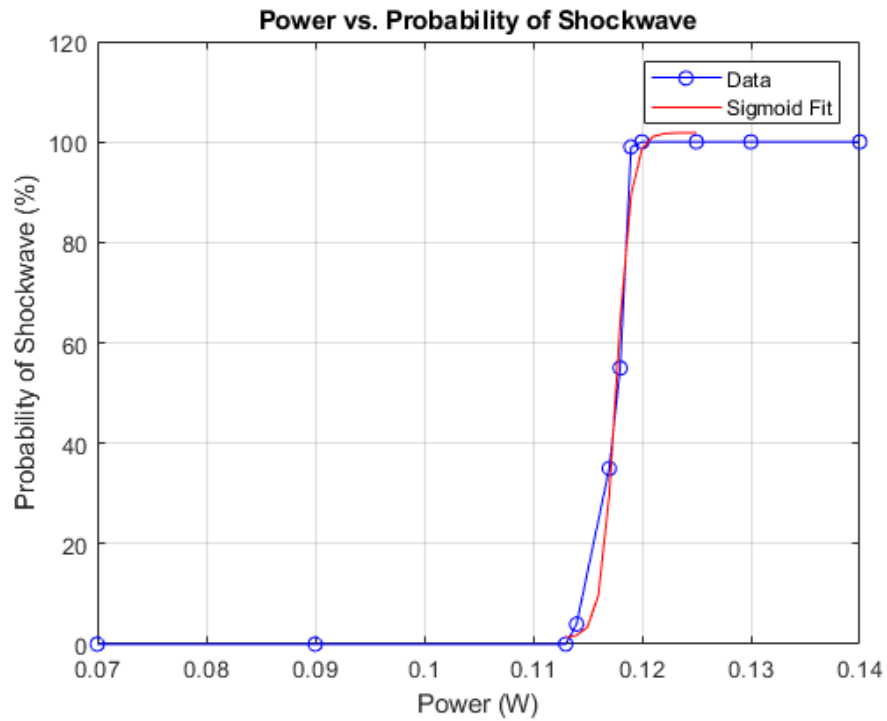


Figure 2.3: Probability of shockwave vs Time averaged power and sigmoid fit

and multiplied by the pulse duration (1.5 ns), which leads to :

$$E_{\text{pulse}} = \frac{\text{average power}}{\text{pulse freq}} = \frac{0.120W}{2000Hz} = 60\mu J$$

$$P_{\text{pulse}} = \frac{E_{\text{pulse}}}{\text{pulse length} \times \text{pulse freq}} = \frac{0.120W}{1.5ns \times 2000Hz} = 40kW$$

Considering the FWHM of the beam, the beam radius can be calculated as:

$$W = \frac{\text{FWHM}}{2\sqrt{2\ln(2)}}$$

$$W \approx 1.274 \times 10^{-6} \text{ m}$$

Finally, to calculate the peak irradiance: The peak irradiance (E_{peak}) at the focal spot represents the maximum intensity of the laser beam. It can be calculated using the formula:

$$I_{\text{peak}} = \frac{2P_{\text{pulse}} \times (1 - \text{loss})}{\pi W^2}$$

$$I_{\text{peak}} \approx 1.02 \times 10^{16} \text{ W/m}^2 = 1.02 \times 10^{11} \text{ W/cm}^2$$

This calculation takes into account the pulse power and the beam radius and also the loss of power through the objective lens, which for the case of the objective lens used in this study is 35%. The breakdown threshold exhibits considerable variation, with typical threshold values of approximately 10^{11} W/cm^2 for pulses lasting a few nanoseconds and 10^{13} W/cm^2 for 100-femtosecond pulses, as reported by Vogel (2005) [111]. These disparities stem from the statistical nature of the breakdown process and the differing experimental conditions employed in various studies. In the context of the laser specifications in this study, which feature a 1.5 ns pulse width, a threshold of $1.02 \times 10^{11} \text{ W/cm}^2$ aligns with findings in other research studies.

Determining the breakdown threshold can be quite challenging, but measuring the extent

of the plasma is comparatively straightforward. The length of the plasma along the laser beam's direction depends on both the laser pulse's energy and its duration. When comparing picosecond pulses to nanosecond pulses, picosecond pulses typically yield longer plasma lengths. However, when we normalize the data to the threshold energy, longer nanosecond pulses result in larger plasma lengths due to a higher volumetric energy density [111]. According to measurements from previous studies [110], we can expect the plasma length in our study's setup to fall in the range of 10 to 20 microns.

To be able to measure the energy of the cavitation bubble, we need to determine the maximum bubble radius. The relationship between the maximum bubble radius and the energy of laser pulses, whether they are in the nanosecond (ns) or picosecond (ps) range and are focused into the water, is examined by Vogel et al. [106]. It is evident that the maximum bubble size increases in proportion to the laser pulse energy, especially when the energy is well above the breakdown threshold. This proportional relationship is observable in the slopes of the curves, which are approximately one-third [106]. Therefore, for the pulse energy in our system, the maximum bubble radius would be around 21 microns.

To calculate the E_B , we will refer to Equation 2.1:

- Interior pressure, $p_v = 2.33 \text{ kPa}$ - Outer pressure, $p_{\text{stat}} = 100 \text{ kPa}$ - Maximum bubble radius,

$$R_{\text{max}} = 21 \mu\text{m}$$

$$E_B = \frac{4\pi}{3}(100 \text{ kPa} - 2.33 \text{ kPa})(21 \mu\text{m})^3$$

$$E_B = \frac{4\pi}{3}(97.67 \text{ kPa})(9261 \mu\text{m}^3)$$

$$E_B \approx 3.78 \text{ nJ}$$

So, the resulting energy of the bubble is approximately 3.78 nJ.

Temperature

The potential concern regarding cavitation bubble-induced mixing achieved by generating a laser-induced plasma arises from the possibility of direct harm caused by the plasma. Although the plasma can attain high temperatures [97], it is typically quite small, with a diameter of less than $20\text{ }\mu\text{m}$, a short lifespan of roughly 20 ns, and a rapid cooling process upon expansion. Nevertheless, it's probable that an amount of fluid equivalent to the plasma volume will be thermally affected. Experimental investigations indicate that, under the laser parameters employed in this study, the plasma volume can be described as an ellipsoid approximately $13\text{ }\mu\text{m}$ in length and $8\text{ }\mu\text{m}$ in diameter, resulting in a plasma volume of around $2000\text{ }\mu\text{m}^3$ [22, 104]. This implies that there is a ratio of over 1000:1 between the volume of mixed fluid (approximately 2 mL) and the volume of reactants destroyed during plasma formation. Additionally, even if the entire energy of the $60\text{ }\mu\text{J}$ laser pulse is concentrated within the mixed fluid volume of about 2 mL, the resulting temperature increase does not exceed 5 degrees Kelvin.

2.2.4 Laser-induced shockwave setup

As depicted in Figure 2.4, for Laser-Induced Shockwaves (LIS) setup, a Q-switched diode-pumped solid-state (DPSS) laser (*Flare NX, Coherent*). This laser emits pulses with a duration of 1.5 ± 0.2 nanoseconds at a wavelength of 1030 nanometers and can operate at frequencies up to 2000 Hz. To generate laser pulses, the laser is connected to a function generator GFG-8015G (*GW Instek*), producing a 5-volt square wave to trigger the laser pulse generation.

After passing through a 3x beam expander, the laser power is controlled by a half-wave plate polarizer and a polarized beam splitter. The remaining portion of the beam is reflected by a near-infrared (NIR) mirror and focused onto the cell culture medium from underneath using

an oil immersion objective lens (*Plan-Apochomat 40x/1.3, Zeiss*).

The laser power is measured at the back aperture of the objective lens with a thermal sensor S425C (*Thorlabs*), connected to the Optical Power and Energy Meter Console PM400 (*Thorlabs*).

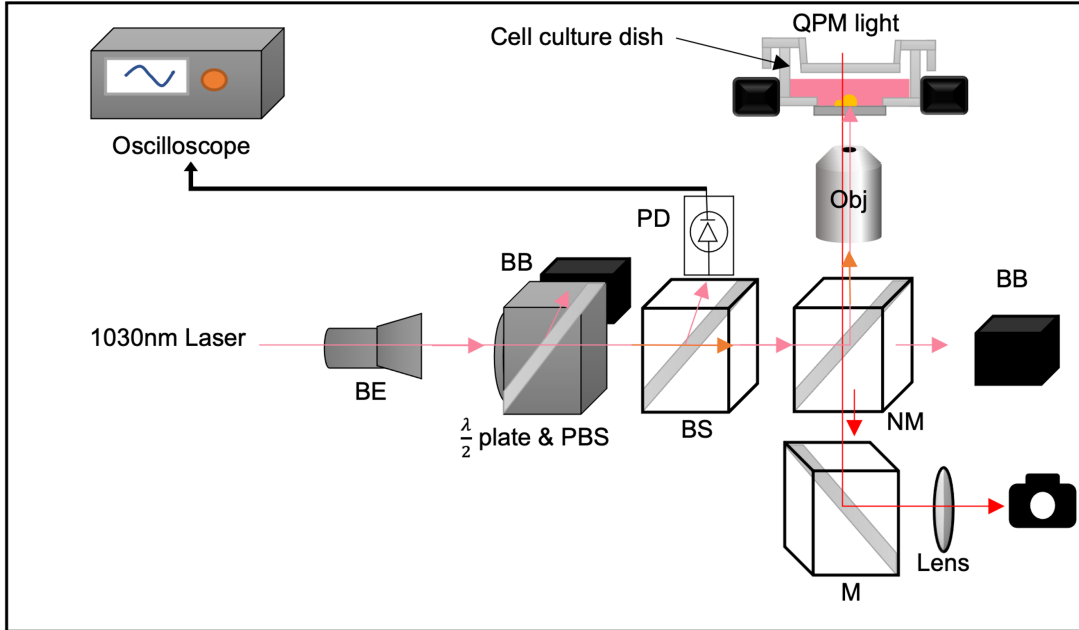


Figure 2.4: Laser-induced shockwave setup

2.3 Optical Trapping

The investigation into optical forces, stemming from the exchange of momentum between light and matter, has been a focal point of scientific exploration [46]. With the advent of intense and coherent light sources [91], Arthur Ashkin and his collaborators embarked on groundbreaking experiments that utilized lasers to adeptly trap and manipulate particles, atoms, and biological matter [4, 7, 5, 21, 20, 8, 6].

In its simplest form, an optical tweezer or an optical trap is fashioned using a single laser beam focused to its diffraction limit, creating the necessary light intensity gradients for

three-dimensional particle confinement [112]. Over time, optical tweezers have evolved into sophisticated instruments capable of diverse manipulations in various environments [77, 32].

The operational principle of optical traps relies on a delicate balance between two types of optical forces: scattering forces, propelling objects along the direction of light propagation, and gradient forces, pulling objects along the spatial gradient of light intensity [4, 7]. When gradient forces exceed scattering forces, an object is drawn to the point of highest intensity formed by focused light, achieving stable three-dimensional trapping [7]. In practical implementations, a laser beam is tightly focused by a high-numerical-aperture microscope objective to generate the requisite spatial gradient in light intensity for a stable trap [7].

In the proximity of this focus, the optical trap behaves akin to a linear "Hookean" spring, exerting forces on an object proportionate to its displacement from the trap's center [4].

While the optical forces generated are generally insufficient to stably trap biological macromolecules themselves, they prove more than adequate for manipulating microscopic dielectric objects, such as micron-sized polystyrene beads, biochemically linked to molecules of interest [4]. In single-molecule optical tweezer experiments, these beads function as handles for a biological system within a sample chamber, allowing calibrated forces to be exerted on the molecule through attachment to a surface, a second bead held at the end of a micropipette, or a second optical trap [4, 67]

2.3.1 Optical Trapping setup

Figure 2.5 illustrates the optical trapping (OT) setup, which involves a continuous laser (*Ytterbium Fiber Laser, IPG Photonics*), emitting continuous laser light at a wavelength of 1064 nanometers. The laser beam undergoes adjustments through a beam expander and a gimbal mirror to control focus. A 4f imaging system is employed to maintain the focus

position as it changes. This system consists of two lenses separated by their focal lengths, forming an intermediate image of the laser beam. The first lens, L1, creates a Fourier transform of the laser beam at its focal plane. The intermediate image is then magnified by a second lens, L2, to reproduce the original laser beam. This arrangement ensures that changes in the focus position are compensated, maintaining a stable trapping environment. The laser beam, directed by a NIR mirror, passes through a polarized beam splitter and focuses onto the cell culture medium from underneath using an oil immersion objective lens (*Plan-Apochromat 40x/1.3, Zeiss*).

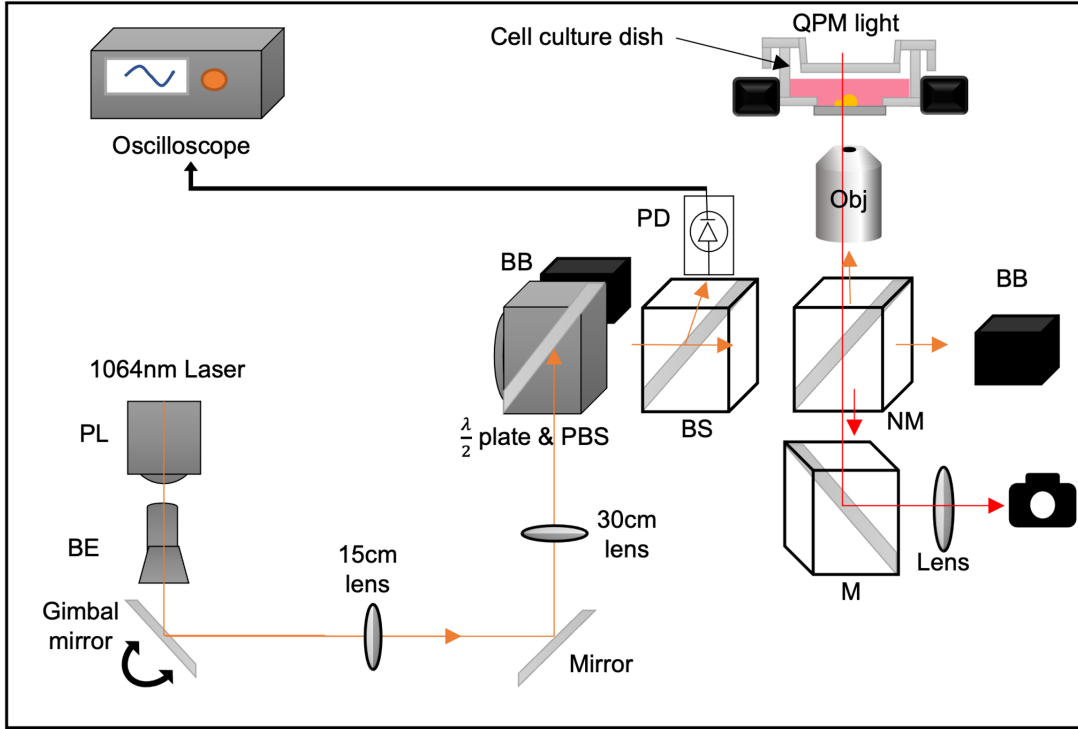


Figure 2.5: Schematic representation of the optical trapping (OT) setup. A continuous laser beam, undergoes adjustments for focus control. The 4f imaging system, comprising lenses L1 and L2, ensures stable focus position. The laser beam, directed by a NIR mirror, passes through a polarized beam splitter and focuses onto the cell culture medium from underneath using an oil immersion objective lens

2.4 Discussion

The Laser-Induced Shockwave (LIS) system, discussed in this chapter, generates controlled shockwaves with precise temporal and spatial characteristics. This capability replicates the mechanical forces associated with TBI, enabling a closer simulation of traumatic brain injuries. The integration of the LIS system with a quantitative phase microscope enhances the experimental setup, facilitating the quantification of morphological changes in cells subjected to shockwaves and improving the reliability of results.

Within the broader context of cell manipulation, the multimodal setup depicted in Figures 2.4 and 2.5 adds a unique dimension. The optical trapping (OT) modality, part of this setup, allows for the precise trapping of beads. These beads become versatile tools for applying minor forces to cells, enabling controlled manipulation to study cellular responses to subtle mechanical stimuli. Furthermore, the optical trapping system facilitates the ingestion of bacteria or other microorganisms into cells optically, providing a novel avenue for exploring cellular interactions.

The combination of the LIS system with optical trapping transforms the experimental platform into a multimodal tool for cell manipulation. The shockwave component applies controlled shear stress, while the optical trapping modality broadens the possibilities by introducing minor forces or microorganisms to cells optically. This multimodal capability enhances the versatility of the system, offering researchers a comprehensive toolkit for investigating various aspects of cellular behavior and response.

It is noteworthy that, due to the unavailability of fast cameras, fully characterizing the LIS system, including direct measurement of the maximum bubble radius, proved challenging. Nevertheless, our study utilized estimations with reliable accuracy, significantly contributing to the comprehensive evaluation of shockwave parameters and enhancing the interpretation of the LIS impact on biological systems. Additionally, the measured power threshold for

shockwave initiation aligns seamlessly with existing research, underscoring the scientific rigor and validity of the experimental framework.

In summary, the integration of the LIS system with optical trapping enhances the precision of TBI simulation while establishing a multimodal tool for comprehensive cell manipulation studies. This synergistic approach allows for a broader range of experimental paradigms, advancing research at the intersection of biophotonics and neuroscience.

Chapter 3

Advancing Cell Injury Research: A Multimodal Microscopy System Approach

3.1 Motivation

This chapter delves into the combined use of Quantitative Phase Microscopy (QPM), Laser-Induced Shockwave (LIS), Optical Trapping (OT), and Fluorescence Microscopy to explore cell injury dynamics. Single cell manipulation, propelled by tools such as laser tweezers, has become a pivotal topic in cell biology research. LIS, a promising technique for non-destructive cell detachment and membrane permeability alteration, presents a valuable avenue for further exploration.

The chapter aims to address the methodological gap in precisely measuring LIS-induced damage. By integrating QPM, the research provides a systematic approach to capture 3D images and quantifying changes in membrane and internal cell structure after LIS. The

study focuses on astrocyte and PTK2 cell responses, offering insights into morphological transformations and height changes.

This work contributes to the ongoing exploration of cell injury mechanisms using advanced technologies. The ability to observe and quantify cellular responses in real time positions this research as a valuable resource for understanding the nuanced dynamics of cell injury and repair. The findings lay the groundwork for further refinements and potential correlations between protein signaling changes and cellular responses to injury.

3.2 Integration of Quantitative Phase Microscopy and Laser-Induced Shockwave

3.2.1 Introduction

In the realm of single cell manipulation, where researchers analyze cell characteristics ranging from adhesion to intracellular movements, biophotonic tools such as laser tweezers have emerged as key players [34, 14, 44, 38]. These tools, utilizing focused lasers for single-cell manipulation, represent crucial developments for the future of cell biology [42, 41]. Within this field, the mechanical impact of Laser-Induced Shockwave (LIS) on cells has become a focal point of interest [96]. Hosokawa et al. demonstrated the potential of LIS by using a femtosecond laser to generate shockwaves, enabling non-destructive cell detachment [42]. Furthermore, Nd:YAG laser-driven flyer plate techniques have been used to induce LIS in endothelial cells, offering a reproducible method to study cell membrane injuries and detachment [95].

The impact of LIS extends beyond detachment, displaying increased permeability of the plasma membrane and stratum corneum, presenting a promising avenue for the noninvasive

introduction of various chemicals into cells [124, 29]. Moreover, LIS has proven effective in modeling traumatic brain injury (TBI) both in vivo and in vitro, demonstrating its versatility [69]. This technique, applied to the skin surface of the mouse brain, has provided insights into brain tissue responses, although the cellular changes during and immediately after injury remain challenging to study [102]. Furthermore, LIS has been instrumental in subjecting brain cells to conditions resembling blast-induced traumatic brain injury, allowing precise control over shear stress [35, 19]. Despite these advancements, a notable methodological deficiency lies in the lack of precise direct quantitative measurement of LIS-induced damage [90].

In this context, the study presented here bridges this gap by introducing a Quantitative Phase Microscopy (QPM) system. QPM, an emerging label-free method, employs interferometry to precisely quantify phase shifts caused by transparent cell samples [43, 74, 122]. This chapter details the application of QPM to enable quantitative phase imaging of cells before, during, and after LIS exposure. By capturing 3D images of LIS injury and quantitatively measuring membrane changes and internal cell structures, the study addresses the methodological deficiency in LIS damage quantification. The integration of QPM into the LIS study framework offers a comprehensive approach to understanding cellular responses, positioning this research at the forefront of unraveling the intricacies of cell injury and repair processes.

3.2.2 Method

Laser-induced shockwave was applied in the astrocyte medium, while Quantitative Phase Microscopy (QPM) captured both quantitative phase and phase-contrast (PhC) cell images at 40x magnification. The recording spanned 20 minutes before the laser-induced shockwave application to 80 minutes after. To evaluate the impact of the laser-induced shockwave on cell

injury, changes in height were assessed by calculating the difference between resultant height values before and immediately after the shockwave. The phase-contrast and differential interference contrast images were derived computationally by differentiating the phase map produced by QPM [66] and subtracting a wavefront shearing operation from the original wave [65], respectively.

3.2.3 Results

Figure 3.1(a) illustrates a phase-contrast view of astrocytes' field of view (FOV) 80 minutes post-laser-induced shockwave. The shockwave occurred on the right side of the FOV, and the red concentric circles indicate radii of $75\mu m$ and $150\mu m$ centered the laser focus. Figures 3.1(b-e) present the PhC image of the cell within the black rectangle at various time points relative to the shockwave; negative time denotes moments before the shockwave. Although the phase-contrast images prior to and immediately after the shockwave do not reveal substantial damage, relying solely on them is inadequate to confirm the cell's unaffected state, as they lack quantitative depiction of height changes. Therefore, quantitative phase images were used to calculate the cell height at different time intervals post-shockwave.

Figure 3.2 displays measurements of height changes for the cell, with purple and green lines signifying cell borders in earlier and later instances. Comparison of images from 20 minutes preceding the shockwave reveals no flattening or upward movement in the cell (Figure 3.2(a)). Differentiating the height of the cell just prior to and after the shockwave indicates that there are no notable changes resulting from shockwave-induced shear stress (Figure 3.2(b)). However, after 80 minutes, substantial morphological transformations were observed in the cell (Figure 3.2(c)). This figure shows a substantial change in cell morphology over an extended period following the shockwave. The cell seems to have morphologically flattened and migrated towards the adjacent deteriorating cell, hinting at potential phagocytic interactions.

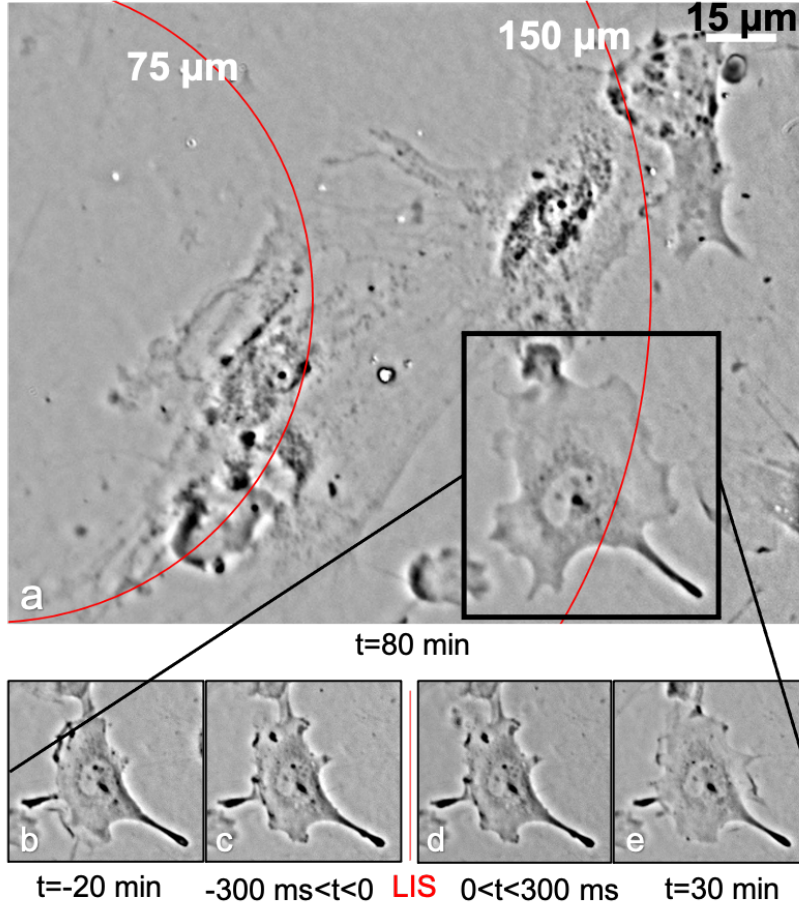


Figure 3.1: PhC image of astrocyte cells. (a) Whole FOV of cells 80 minutes after LIS. The red circles are concentric with LIS and have radii of 75 μm and 150 μm . (b-e) The PhC image of the cell in the black rectangle, at different time points with respect to LIS. The negative sign means the time before LIS.

Prior studies have also demonstrated astrocyte phagocytic responses to injured neighboring cells [115, 114].

A small FOV, encompassing the cell in Figure 3.1 and its neighboring dying cell, was chosen. QPM images of the cells from just prior to LIS to 80 minutes after, at different time points, were selected. Figure 3.3 depicts a montage of the cellular interactions with a real height colormap. The time indicated in each frame is the time with respect to LIS, and time zero is just prior to LIS. The mass being transferred between two cells have been pointed with white arrows and can be seen in different time frames. 24 minutes after LIS, there may

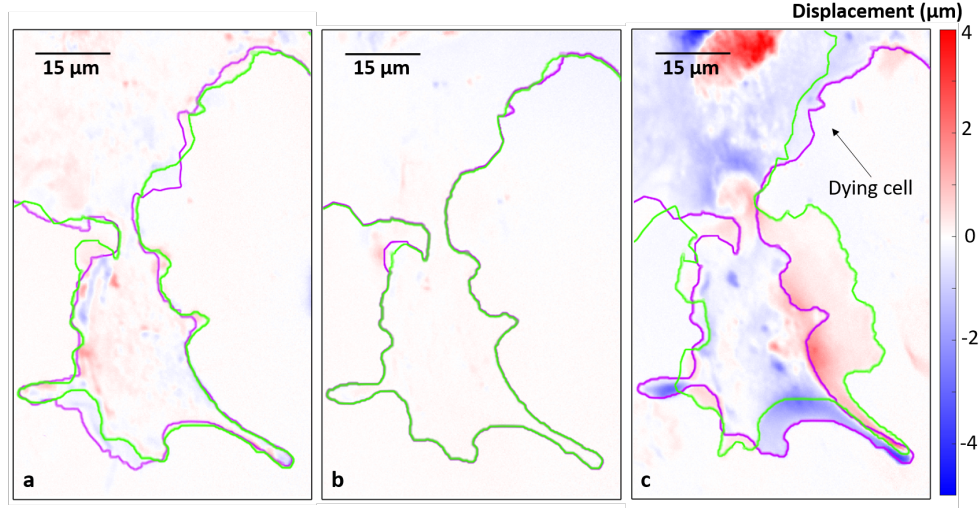


Figure 3.2: Height changes in the cell, indicated with a black rectangle in Figure 3.1, (a) from 20 minutes before to right before LIS, (b) from less than 300 ms before LIS to less than 300 ms after LIS, (c) from right before to 80 minutes after LIS. All of the figures are calculated by taking the differences of the cell's height at two indicated time points. The purple and green lines show the cell border in the former and later time points.

be a vacuole formation. The red arrow is pointing to the possibly formed vacuole. Figure 3.4 displays the phase contrast image of the same FOV, for the time of possible vacuole formation.

To showcase the system's capacity for producing repeated injuries, a sequence of two successive laser-induced shockwaves (LIS) was applied with a 1-second interval within the same region of the astrocytes' medium. Figure 3.5 displays a series of reconstructed 3D images spanning the pre- and post-LIS effects on cells in response to these laser-induced shockwaves (denoted as t_1 for the first LIS and t_2 for the second LIS).

These 3D images were generated employing quantitative phase images through the interactive 3D surface plot plugin of ImageJ. Within this sequence, minimal to no discernible LIS effects were observed following the initial shockwave. However, noteworthy effects became evident in the central cell (labeled as cell #1) after the second LIS, as amplified in Figure 3.5(a,d-f). Specifically, Figure 3.5(d) illustrates the deformation brought about by shear stress right after the second LIS. Furthermore, significant changes were observed in the nucleus and

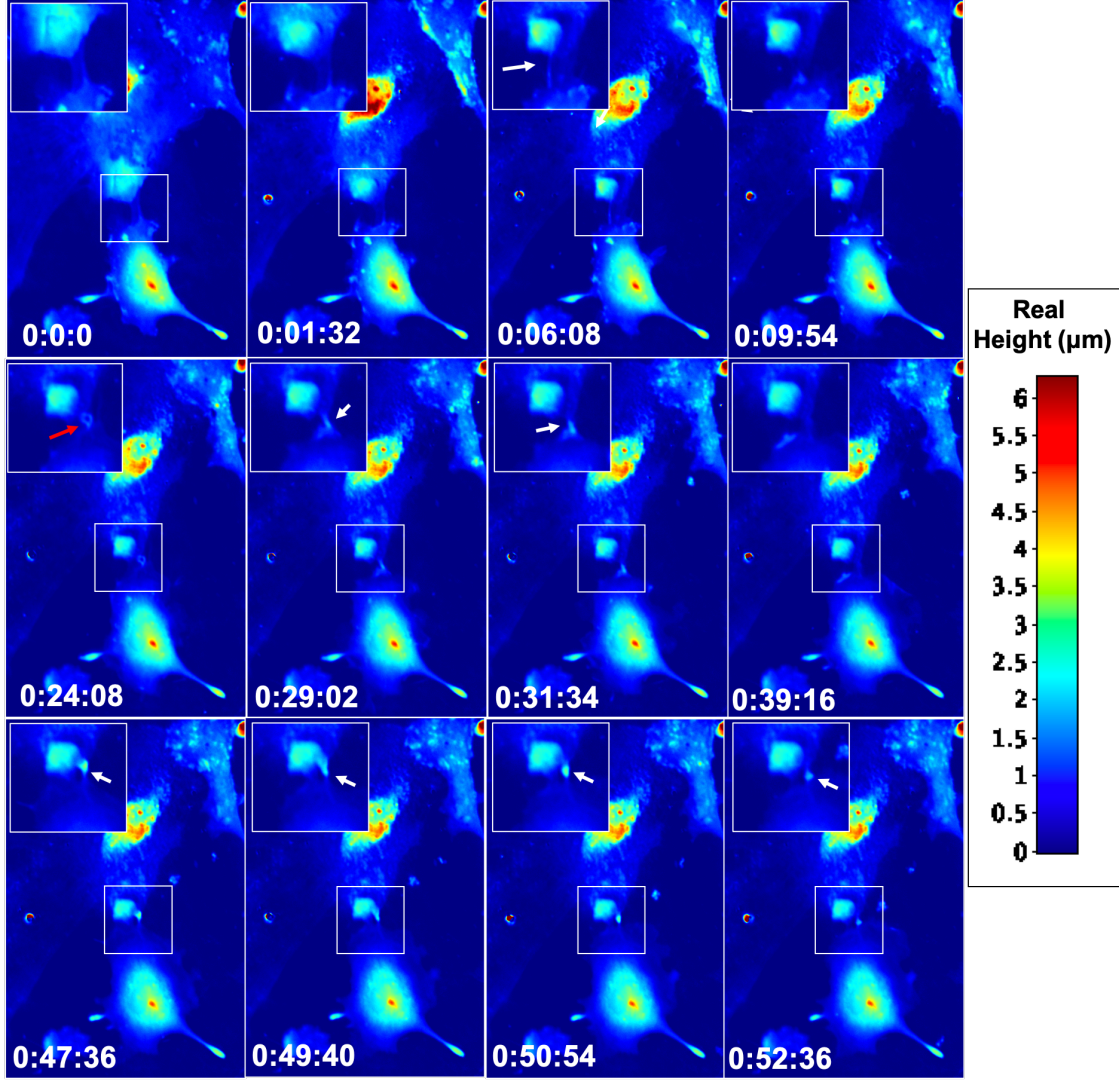


Figure 3.3: A montage of the cells' interaction with a real height colormap. The time indicated in each frame is the time with respect to LIS, and time zero is just prior to LIS.

cytoplasm of cell #2.

In this cell, distinct changes in the cytoplasm appeared as granulation or fragmentation of cellular components. Despite the absence of distinct internal cellular changes discernible in the QPM images following the first LIS, substantial changes emerged in the nucleus (central area) of the cell after the second LIS. Figure 3.6 demonstrates the z-displacement of the cells from 1 s to 41 s after the second LIS. PhC images of the FOV 1 s and 41 s after the 2nd LIS can be seen in 3.6(a) and 3.6(b) respectively. The displacement in the whole field of view is

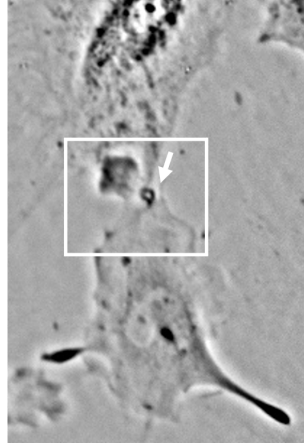


Figure 3.4: A PhC image of the same FOV in Figure 3.3 showing the possible vacuole formation.

shown in Figure 3.6(c).

Changes from $2.5\ \mu\text{m}$ decrease to $2\ \mu\text{m}$ increase in different parts of the cell #1 can be seen. Cell #2 seems to be forming vacuoles, as there is an approximate $0.5\ \mu\text{m}$ increase in spherical structures. These structures match those observed in the cytoplasm of the same cell in Figure 3.5 This response was not seen in the left neighboring cell, despite the fact that this cell was closer to the center of LIS compared to cell #2. This may be because some cells may be more prone to damage.

Similarly, LIS was applied to the culture medium containing PTK2 cells, as shown in Figure 3.7. Recordings commenced 15 minutes before LIS application and continued for 45 minutes thereafter. PhC, DIC, and QPM images were captured under 20x magnification during the LIS application. Figures 3.7(a-d) present PhC and DIC images before and after LIS, respectively. Figure 3.7(e) displays quantitative height changes caused by LIS in PTK2 cells. A gradual decline in height changes is noticeable as one moves away from the LIS center. Cells marked with a black rectangle appear to be in the telophase of the mitosis cycle. While PhC and DIC images suggest minimal change in these cells, the QPM image reveals significant height changes due to LIS. Figure 3.7(f) offers the height profile of a cell along a radial vector (represented by a black line in Figure 3.7(e)) from the LIS epicenter,

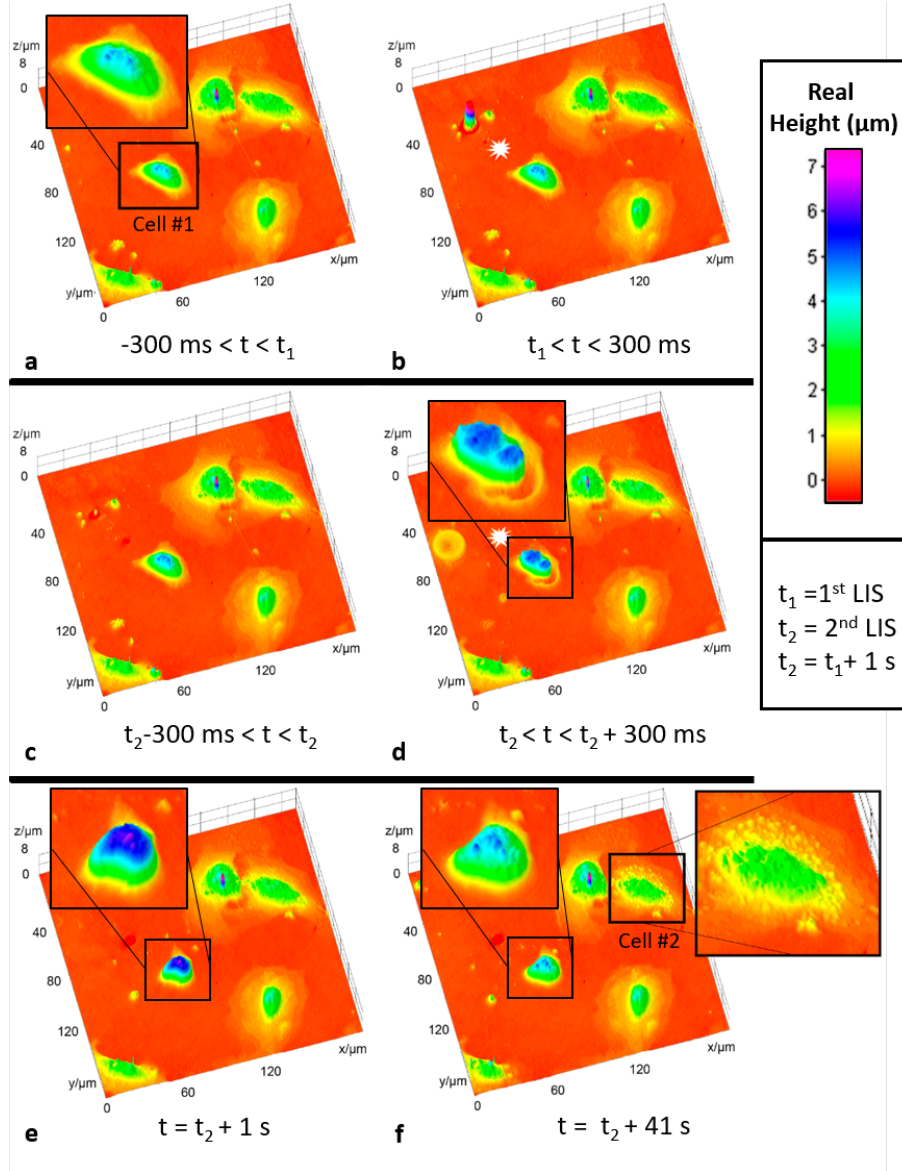


Figure 3.5: Reconstructed 3D image of the cells in the field of view. The center of the LIS has been shown with a white star. Cell #1 and cell #2 are magnified in some of the sub-figures. The t indicates the time that each figure has been recorded, that is (a) less than 300 ms before the first LIS, (b) less than 300 ms after the first LIS, (c) 1 s after the first LIS and less than 300ms before the second LIS, (d) less than 300 ms after the second LIS (This image shows how cell #1 has been pushed away from the center of the LIS instantaneously.), (e) 1 s after the second LIS, (e) 41 s after the second LIS. Vacuoles that have been formed in the cell #2 can be clearly seen in this image.

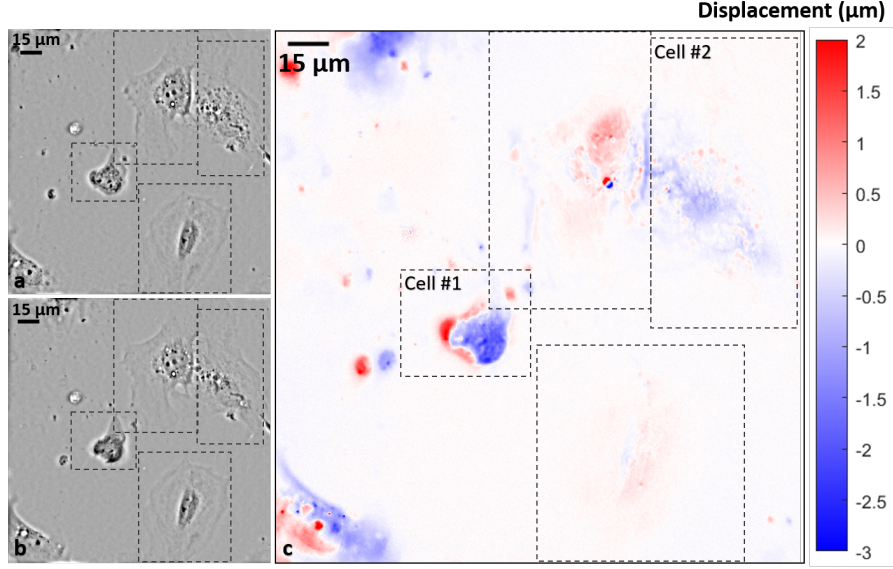


Figure 3.6: Cellular morphological changes from 1 s to 41 s after the second LIS. **(a)** PhC image of the FOV, 1 s after the second LIS. **(b)** PhC image of the FOV 1 s after the second LIS. **(c)** Z-displacement of cells from 1s to 41s after the second LIS. The center of LIS is shown with a white star. In different parts of cell #1 significant changes from 2.5 μm decrease to 2 μm increase is measured. Cell #2 seems to be forming vacuoles, as there is an approximate 0.5 μm increase in spherical structures. There are small height changes observed in other cells, on the order of magnitude of hundred nanometers.

both before and after the LIS. This profile enables precise measurement of cell height changes in the direction of the LIS.

3.3 Integration of Quantitative Phase Microscopy and Optical Trapping

3.3.1 Introduction

The success of optical trapping (OT) experiments is highly dependent on understanding the characteristics of the objects under investigation, including their shape and refractive index. Consequently, the integration of OT with technologies that provide access to this information

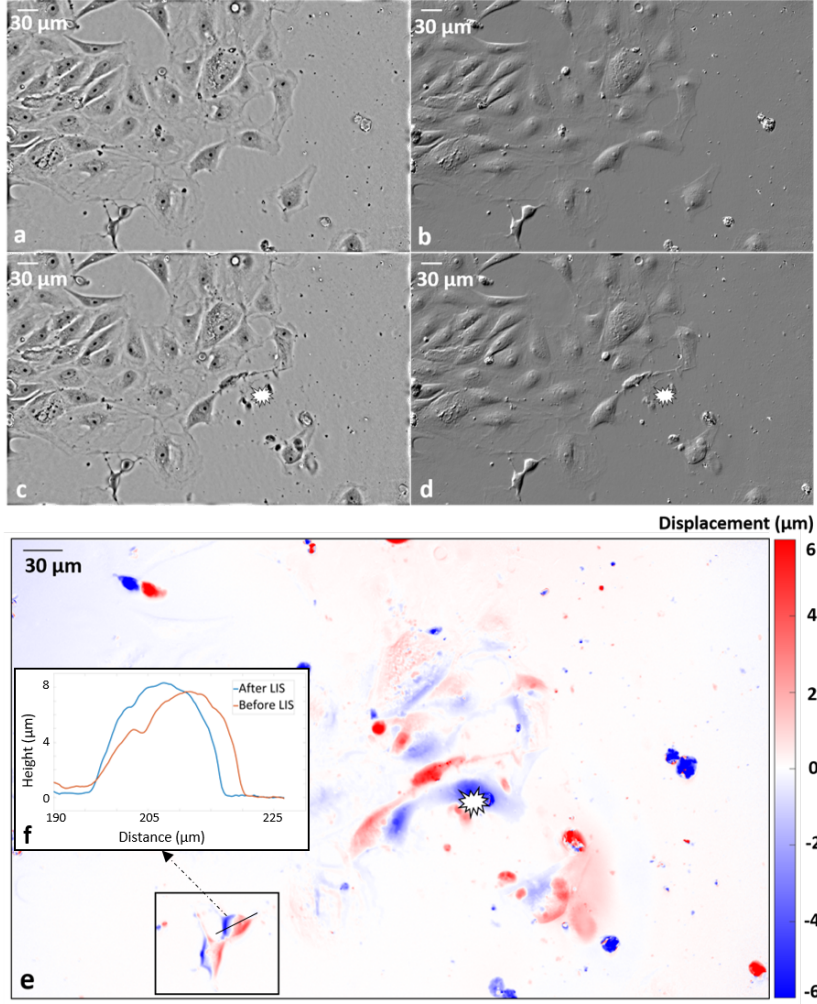


Figure 3.7: Images of PTK2 cells with 3 different modalities. The center of the LIS has been shown with a white star. **(a)** PhC image before LIS, **(b)** DIC image before LIS, **(c)** PhC image after LIS, **(d)** DIC image after LIS, **(e)** Quantitative height changes from before to after LIS. Significant height changes from LIS can be seen in the cells indicated with a black rectangle; these height changes cannot be detected in PhC and DIC images. **(f)** The height profile of the cell along a radial vector, shown with a black line in (e), from the LIS epicenter, before and after the LIS.

is a natural step. Various optical techniques have been employed to glean insights into phase changes induced by the passage of light through transparent microscopic objects. These techniques encompass digital holographic microscopy (DHM), quantitative phase microscopy (QPM), and quantitative phase imaging (QPI).

Typically, they employ interferometry-based imaging methods to extract multidimensional

information about samples, including their three-dimensional morphology, refractive index, and associated dynamics[101, 84, 27]. The integration of optical trapping and quantitative phase microscopy offers a powerful approach to understanding cellular properties and behavior. By simultaneously manipulating and interferometrically measuring cells, it provides valuable insights into refractive index and morphological changes, serving the fields of biophotonics and cellular studies. Despite current challenges, ongoing advancements in digital equipment, processing, and new technologies are likely to drive further integration and open new possibilities for cell probing, especially at the nanoscale [61].

3.3.2 Biological Applications

While many OT experiments employ dielectric microspheres, biological studies on the micrometer scale often involve objects with complex shapes and varying refractive indices over space and time. In this context, the combination of QPM and OT proves beneficial for discriminating "trappable" objects and understanding the effects of optical forces on the specimens. QPM also serves as a tool to assess the possible photodamage caused by the trapping beam, enabling adaptive trapping of biological samples and feedback on the consequences of optical trapping [79, 30].

Integrating OT with QPM facilitates quantitative investigations into cellular forces, morphological changes, and mechanical properties. This integration has been applied to study cellular deformation, elasticity, and axial changes in red blood cell (RBC) shape induced by OT. It allows simultaneous measurement of refractive index and elasticity, providing valuable insights into the biophysical changes of cells [18]. Furthermore, this integration can enhance QPM imaging systems by introducing mechanical actuation and rotation, enabling tomographic measurements for a comprehensive 3D view of refractive index and morphology [50].

3.3.3 Experimental Results

Optical tweezers serve as a versatile tool for investigating astrocyte responses to injuries through various methodologies. The detailed information of the developed optical trapping setup can be found in Section 2.3.1 of Chapter 2 of the thesis.

Our experimental approach involves the optical trapping of silica beads, which are subsequently pushed towards astrocyte cells to impart subtle forces, reaching as low as 0.1 femto-Newtons. Optical tweezers are employed to trap beads and apply subtle forces to cells. For instance, Figure 3.9 illustrates an application where a $10\mu m$ bead is trapped and gently pushed towards an astrocyte cell [81]. This capability allows for controlled manipulation and examination of cellular responses under precisely regulated forces. The combined utilization of optical trapping techniques and QPM provides a powerful platform for investigating astrocyte behavior in response to mechanical stimuli, offering valuable insights into their biomechanical characteristics and potential implications for neurobiological phenomena.

Another strategy entails trapping deceased neurons or astrocyte cells and maneuvering them in proximity to healthy astrocyte cells. The phagocytic response of astrocyte cells to injuries in the vicinity of neighboring cells has been previously elucidated in studies such as Wakida et al. (2018) [115]. Our experimental setup enables the assessment of this response at distinct distances from an intact astrocyte cell. Figure 3.8 presents reconstructed 3D images, captured with Quantitative Phase Microscopy (QPM), depicting an optically trapped deceased astrocyte positioned at varying distances relative to a healthy astrocyte cell.

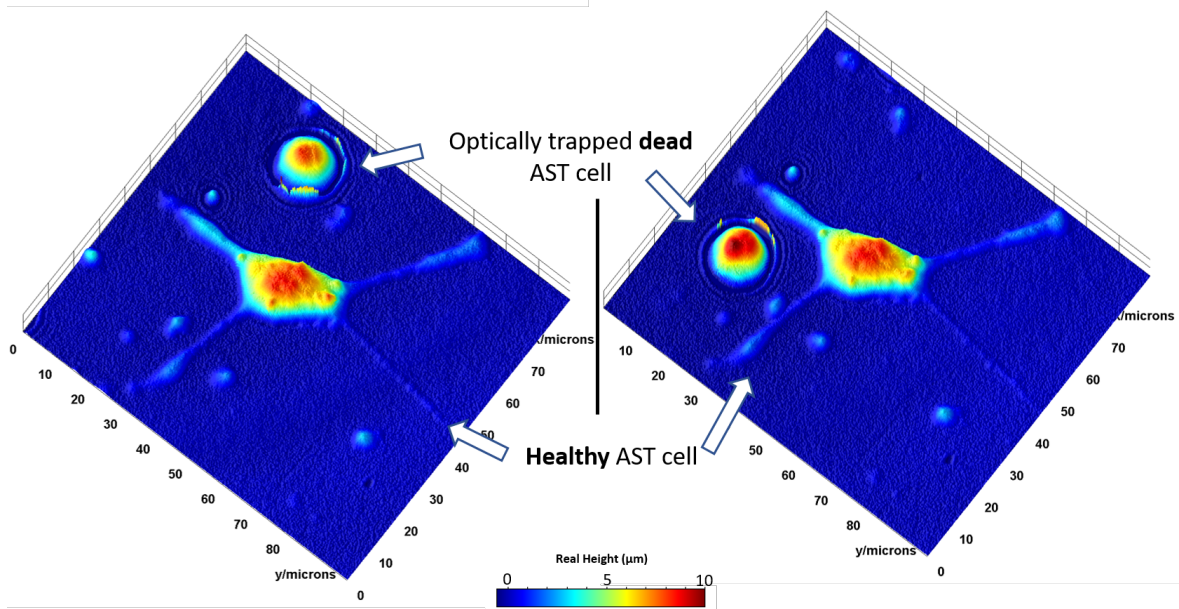


Figure 3.8: Reconstructed 3D images of astrocyte cells. A dead astrocyte cell was placed at different positions relative to an intact astrocyte cell using optical tweezers [82]

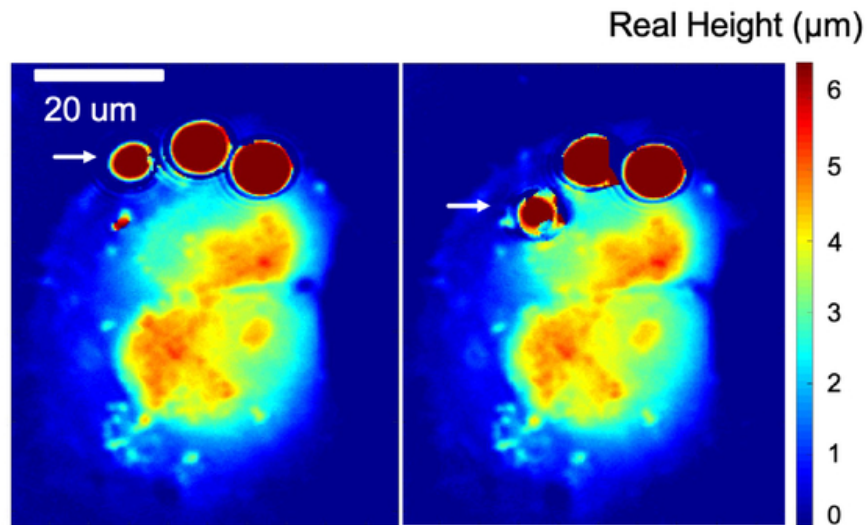


Figure 3.9: Quantitative phase images of trapping and pushing a 10μm bead towards an astrocyte cell [81].

3.4 Integration of Quantitative Phase Microscopy and Fluorescence Microscopy

The detailed configuration of the QPM/Fluorescence microscopy setup is provided in Section 1.5.2 of Chapter 1 of this thesis. Utilizing MDF-GFP Filter set (*Thorlabs*) (CWL = 469 nm, BW = 35 nm), both green fluorescent beads (G1000, Fluorescent Polymer Microspheres (Aqueous)) and Fluo 4am-tagged astrocyte cells were imaged during LIS experiments.

Green fluorescent beads served as the imaging target for both fluorescence and QPM under LIS. In Figures 3.10(a,b) and 3.10(c,d), fluorescence and quantitative phase images of the beads are presented before and after a laser-induced shockwave. The displacement measurements derived from these images are illustrated in Figure 3.10(e). Notably, minimal displacement is observed away from the center of LIS, contrasting with the pronounced bead movements in the proximity of the LIS center. Due to the rapid expansion and contraction of the bubble lasting only tens of microseconds, the camera's 3.3 fps rate captures the bead movements only after they have been pulled back due to bubble contraction.

To further validate our fluorescence microscopy setup and assess experimental outcomes, astrocyte cells were treated with Fluo-4 AM dye to measure intracellular calcium activity. Previous studies, including the work by Gomez et al. [35], reported a rapid increase in calcium activity in Schwinn cells and neurons following LIS. In our study, simultaneous QPM and fluorescence imaging of AST1 cells during shockwave application allowed us to observe and quantify intracellular calcium dynamics.

The quantitative analysis of mean intensity values within the Region of Interest (ROI), covering astrocyte cells as shown in Figure 3.11, affirmed a significant surge in calcium concentration coinciding with LIS application. This finding supports the immediate impact of shockwaves on intracellular calcium dynamics, consistent with the results reported by

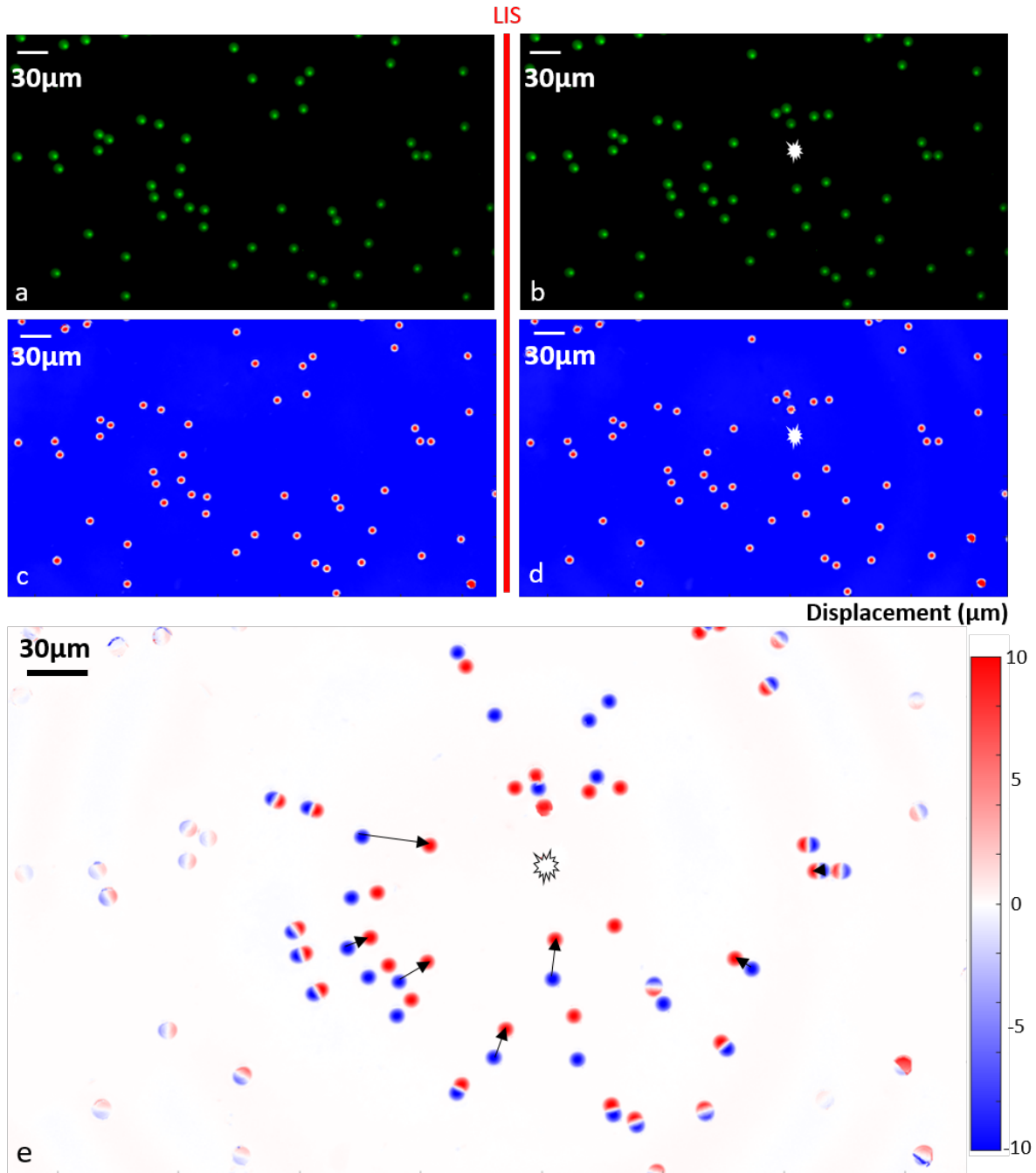


Figure 3.10: Experiments with fluorescent beads. The center of LIS is marked with a white star, and the scale bar is 30 μm . (a) Fluorescence image of beads before LIS. (b) Fluorescence image of beads after LIS. (c) QPM image of beads before LIS. (d) QPM image of beads after LIS. (e) Displacement measurements derived from Figure (c,d). Black arrows indicate the direction of bead movement caused by LIS.

Gomez et al. [35].

3.5 Discussion

In this chapter, a novel technique was introduced, which allows for the investigation of cellular injury caused by Laser-Induced Shockwave (LIS), using the combination of Quantitative Phase Microscopy (QPM) and LIS. The integration of these techniques fills critical methodological gaps, enabling accurate measurements of LIS-induced damage at both qualitative and quantitative levels.

The use of QPM enables high-resolution, three-dimensional imaging of cells, providing valuable insights into alterations in membrane behavior, nucleus and cytoplasm structure, as well as cell thickness. This label-free imaging modality offers a systematic and real-time approach to capture cellular responses during and after LIS application. The ability to observe progressive shifts in cell morphology over time, as demonstrated in Figure 3.1, highlights the nuanced and dynamic nature of cellular responses to injury, suggesting potential connections with neighboring cells.

Furthermore, the simultaneous imaging of cells during LIS application facilitates immediate capture of cellular changes, allowing for accurate quantification of damage. While traditional imaging modalities like Phase Contrast (PhC) and Differential Interference Contrast (DIC) images may indicate stability, QPM reveals significant thickness changes induced by LIS, as depicted in Figure 3.7. This underscores the importance of QPM in discerning subtle cellular alterations that might be overlooked by conventional imaging techniques, particularly in cells undergoing dynamic processes such as mitosis.

The integration of QPM into the LIS study framework provides a comprehensive and multidimensional approach to understanding cellular responses, positioning this research at the

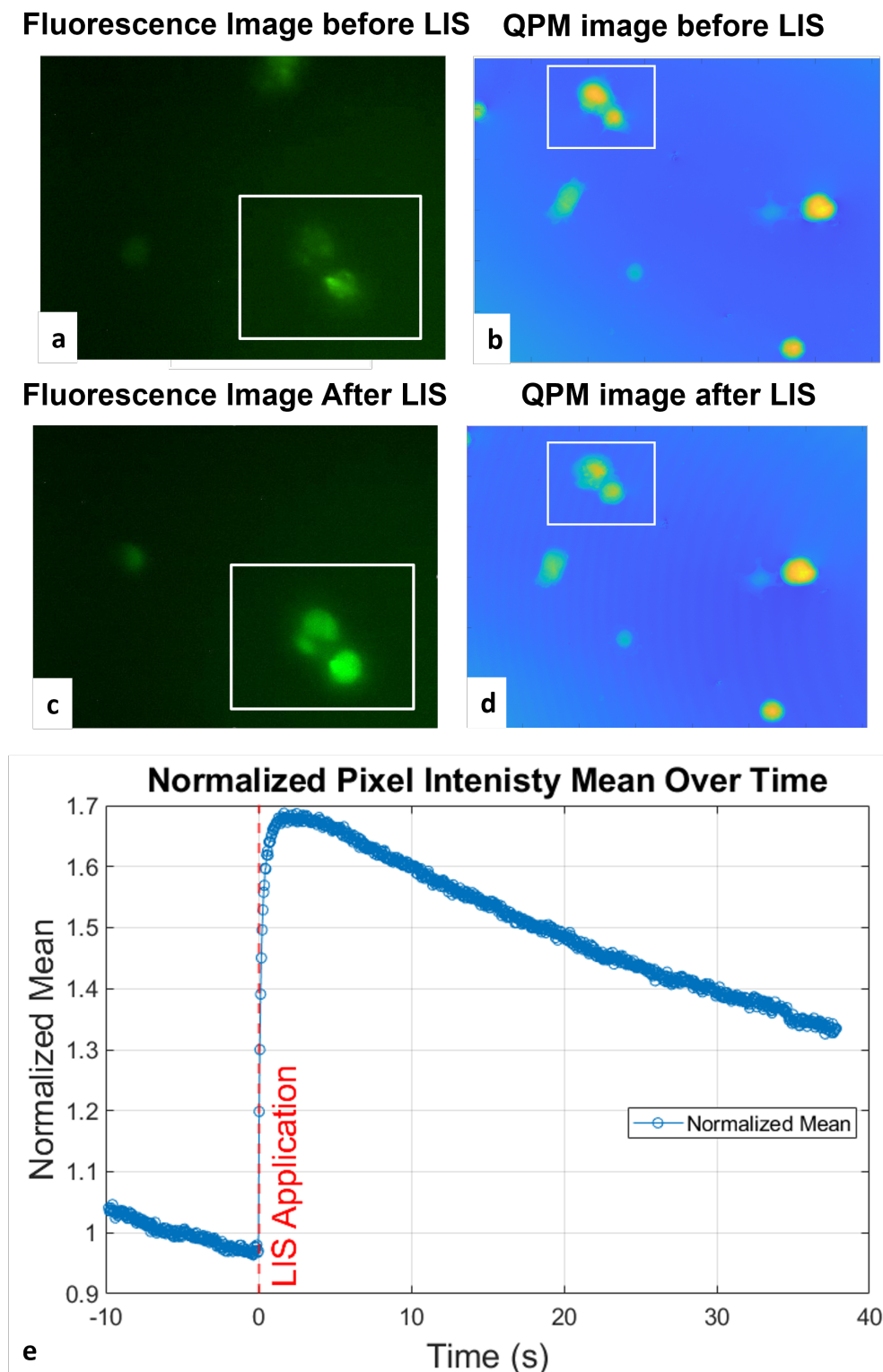


Figure 3.11: Fluorescence and QPM images of AST1 cells during Laser-Induced Shockwave (LIS). The Region of Interest (ROI) encompasses astrocyte cells.

forefront of unraveling the intricacies of cell injury and repair processes. Moreover, the system's proficiency in capturing quantitative phase images, phase-contrast images, and fluorescence images, provides an additional modality for comprehensive cellular study. This opens avenues for exploring correlations between protein signaling changes and the dynamics of membrane, cytoplasm, and nucleus in response to injury.

In addition to LIS and QPM, Optical Trapping (OT) augments the system's capabilities. OT facilitates controlled manipulation of objects, such as silica beads and deceased cells, allowing precise examination of cellular responses under regulated forces. Figure 3.9 exemplifies the application of OT, demonstrating the trapping and pushing of a bead towards an astrocyte cell. This technique broadens the scope of the system, offering insights into cellular behavior under precisely controlled conditions.

The multimodal nature of this system offers a holistic approach, capturing detailed 3D images and exploring dynamic interactions between cells and trapped objects. This versatility makes the system well-suited for assessing cellular repair over extended periods, providing valuable insights into astrocyte behavior and potential implications for neurobiological phenomena. Overall, the integration of LIS, QPM, OT, and fluorescence imaging enhances the comprehensiveness of the experimental setup, paving the way for nuanced investigations into cellular dynamics and responses.

Chapter 4

Quantitative Phase Image Processing

4.1 Motivation

This chapter delves into the detailed realm of quantitative phase image processing. The underlying motivation for this chapter is to provide an in-depth understanding of how to convert raw camera images into final heightmaps, and further explore the processes of cell segmentation and feature extraction from these heightmaps. Accurately identifying and outlining individual cells is extremely important for image-based research in biology and biomedicine. Here cell segmentation approaches come into play. To extract useful information from vast amounts of data generated by advanced microscopic imaging techniques, computerized image segmentation algorithms are necessary. Subsequently extracted features from these images hold the key to understanding the complex world of cellular structures. Features such as dry mass, surface area, and volume open doors to diverse analyses, ranging from cell tracking to investigating cell behavior. The main goal of this chapter is to provide practical insights and methodologies for researchers and practitioners to overcome the challenges associated with quantitative phase image processing. The chapter aims to explain the process of converting

interference images into precise heightmaps and provide detailed methods for cell segmentation and feature extraction. By doing so, it intends to contribute to the development of reliable tools for analyzing cellular structures in a scientifically accurate manner.

4.2 From Interference Images to Precision Heightmaps

Phase unwrapping is a fundamental process that finds significant relevance in the analysis of quantitative phase microscope images obtained using an interferometer. These interference images serve as a gateway to understanding the topography of various samples. To extract precise heightmaps from these images, a multi-step approach is necessary. First, the interference images are transformed into wrapped phase images. However, this wrapped representation introduces phase ambiguities due to the cyclic nature of phase values. Resolving these ambiguities through phase unwrapping is a crucial step, as it allows for the conversion of wrapped phase into meaningful heightmaps, facilitating the comprehensive study of samples in different scientific fields. Various methods aim to address the challenges associated with phase unwrapping and resolve phase ambiguities caused by the wrapping of phase values.

Goldstein’s branch cut method is a commonly used and versatile algorithm that has found applications in geophysics, optics, and computer science [125]. This method offers several advantages. For example, Goldstein’s method demonstrates versatility and broad utility, making it widely applicable in various fields. Furthermore, When applied under appropriate conditions, Goldstein’s method can provide highly accurate phase unwrapping results, making it suitable for precision applications.

In addition to Goldstein’s algorithm, various other phase unwrapping methods have been proposed to enhance performance and efficiency. These methods have their own set of

pros and cons. For example, the graph-cut phase unwrapping method, which incorporates horizon and unconformity constraints, has been effective in some applications [120]. Similar to Goldstein’s method, it may be sensitive to noise and require significant computational resources for large datasets. The deep GAN-based InSAR phase unwrapping is another algorithm that offers enhanced quality and reduced processing time by combining different methods [116]. However, the use of deep learning techniques may necessitate substantial computational power and extensive training data. Furthermore, this approach might be less interpretable compared to traditional algorithms. The network programming-based phase unwrapping method can be named as another method that employs network programming techniques to solve the phase unwrapping problem [23]. While effective, it may require users to possess a solid understanding of network programming concepts, making it less accessible to those without such expertise. Moreover, dual-frequency phase unwrapping is a widely used method with the capability to unwrap phases with significant discontinuities or separations [45]. However, its applicability may be constrained by the availability of dual-frequency data. Also, the phase-gradient-jump connections method has been proposed to efficiently reduce computational complexity compared to traditional phase unwrapping methods [128]. Nevertheless, it might still be sensitive to noise and exhibit limitations concerning dataset size. The fusion cut-line and quality-maps method combines various techniques to improve the accuracy of 2D phase unwrapping [39]. However, this method may necessitate the integration of multiple software tools and workflows, potentially increasing complexity.

While a variety of phase unwrapping methods offer valuable solutions, it’s crucial to consider their limitations alongside their advantages. These limitations include sensitivity to noise, computational complexity, specific expertise requirements, and challenges in interpretability.

In our experimental setup, we employ two identical optical paths—one reflecting from the sample and the other from a reference arm. The Golstein method for phase unwrapping was

chosen based on its ability to effectively handle complex fringe patterns, provide unbiased phase unwrapping, work well in noisy conditions, process data efficiently, and its broad applicability in scientific research.

4.2.1 Interference Images to Wrapped Phase Images

The system used in this study uses a phase-shifting algorithm to calculate the wrapped phase images. The optical path length is periodically shifted in interference images. The phase is calculated by the intensity of the 30 consecutive images. The wrapped phase images are constructed from the fringe images by the algorithm written in [103]. Figure 4.1 refers to an example of the result of this process. One of the interference images used to construct the phase image is shown in Figure 4.1(a). The resulting wrapped phase image is depicted in Figure 4.1(b).

4.2.2 Phase Unwrapping Process

Upon processing the interference images to generate a wrapped phase image, the subsequent steps involve unwrapping the phase using the Goldstein method in MATLAB, as detailed by Carey Smith in 2023 [93].

Goldstein phase unwrapping algorithm

The Goldstein method is a widely recognized technique for phase unwrapping in radar interferometry [125]. It is particularly useful for accurate and efficient two-dimensional phase unwrapping. This method has been instrumental in remote sensing and geophysical applications, allowing researchers to obtain precise elevation maps from interferometric radar data. The Goldstein method operates by considering the phase differences between neigh-

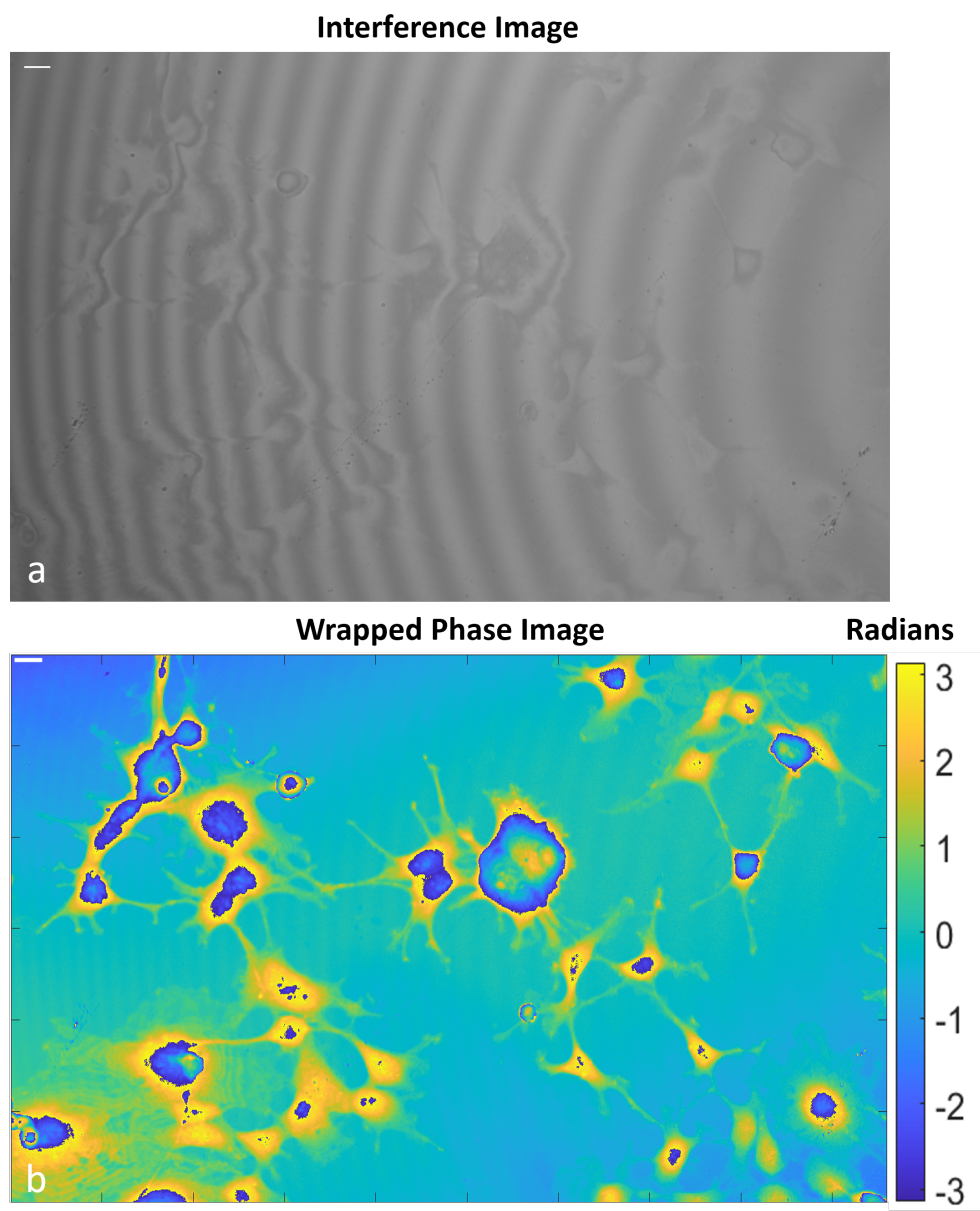


Figure 4.1: An example of the processing of interference images into wrapped phase image, a) one of the interference images, b) the resulting wrapped phase image. The scale bar is 20 microns.

boring pixels, intelligently identifying branch cuts, and iteratively unwrapping the phase to reconstruct the absolute phase distribution. This approach has proven effective in addressing phase ambiguities and generating high-quality unwrapped phase images. The Goldstein branch-cut phase unwrapping algorithm is an essential method for retrieving unwrapped phase information from two-dimensional phase data in various scientific and engineering contexts. The code provided by Carey Smith, implements this algorithm and includes a main code, called GoldsteinUnwrap2D [93], which leverages several auxiliary functions to perform the unwrapping process. The algorithm includes a series of key steps:

Phase Residue Calculation ('PhaseResidues' function): This function computes the phase differences between neighboring pixels in the wrapped phase image and marks the residues on the top-left corners of 2x2 regions. These residues are crucial for identifying branch cuts in the phase image.

Branch Cut Generation ('BranchCuts' function): After the phase residues are determined, this function generates branch cuts based on the residue information. These branch cuts separate regions with phase ambiguities. The algorithm ensures that branch cuts connect to an artificial border, that is defined by a binary mask to avoid isolated regions.

Flood Fill Unwrapping ('FloodFill' function): With the branch cuts in place, the 'Flood-Fill' function performs the phase unwrapping. It starts at a user-specified seed point and iteratively unwraps the phase while avoiding branch cuts. Using a flood-fill approach, the unwrapped phase values are gradually filled in. In this approach, adjoining pixels are unwrapped based on their phase values and magnitudes. The process continues until all unwrapped regions are connected.

Figure 4.2 shows an example of the initial phase and magnitude of the wrapped phase image, before further processing into an unwrapped phase image. Figure 4.3(a) shows the calculated phase residues and Figure 4.3(b) depicts the resulting branch cuts that are generated using

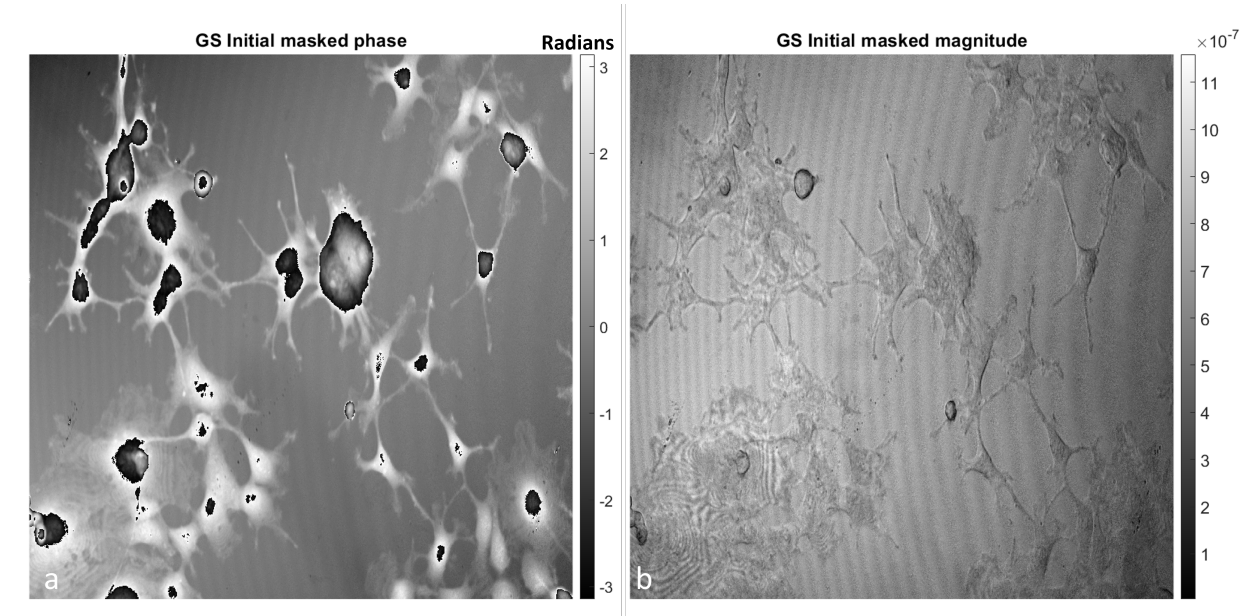


Figure 4.2: Initial a) wrapped phase b) magnitude

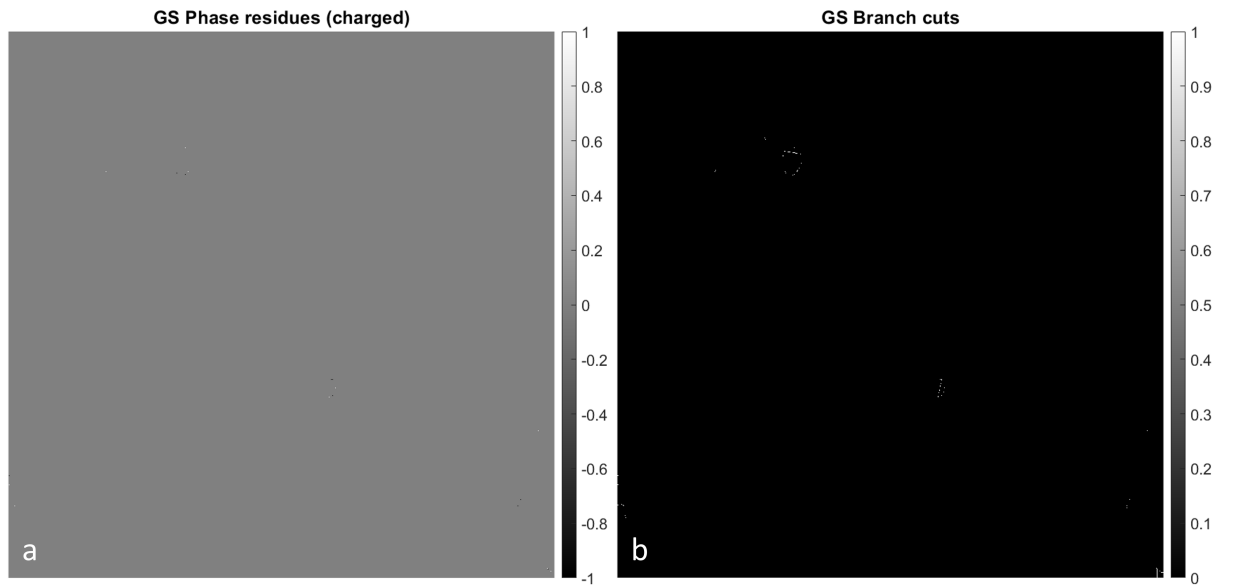


Figure 4.3: a) Phase residues B) Branch cuts

the phase residue information. After choosing a seed point for the algorithm manually, the program will calculate the final unwrapped phase image. Figure 4.4 displays the final image of the unwrapped phase for the image of the wrapped phase depicted in Figure 4.1(b).

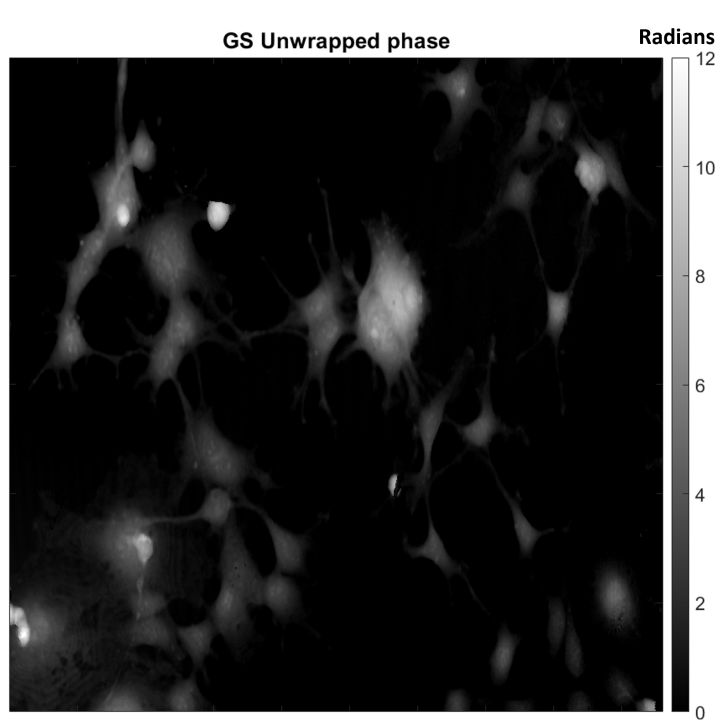


Figure 4.4: The resulting unwrapped phase image

4.2.3 Sample Thickness Estimation

Based on the resulting unwrapped phase, the optical height (OH) is measured by Eq. 4.1 [121]:

$$OH = \frac{\lambda_c}{2\pi} \times \frac{\theta}{2} \quad (4.1)$$

In this equation, θ represents the unwrapped phase, and λ_c is the center wavelength of the illuminating light used to capture the interference images, which in our case is the center wavelength of the illumination LED, and it is equal to 633 nm. To estimate the real height (RH) of the sample, we need to consider its optical height (OH) and the difference in the mean refractive index between the sample and its surrounding media. For example, if we take astrocyte cells, their mean refractive index can be estimated as $\bar{n}_{cell} \approx 1.37$ [121] while the medium has a refractive index of $n_{medium} \approx 1.337$ [54]. To calculate the real height of a

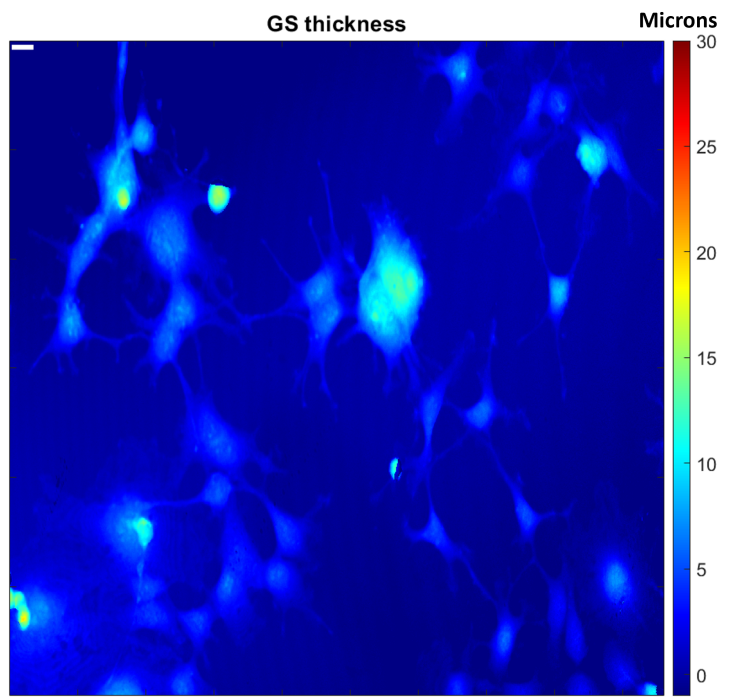


Figure 4.5: Real height of the astrocyte cells captured with QPM. The scale bar is 20 microns.

sample, we can use Eq.4.2 [121]:

$$RH = \frac{OH}{\bar{n}_{cell} - n_{medium}} \quad (4.2)$$

Based on the calculations above, the final height map of the example field of view is depicted in Figure 4.5.

4.3 From Heightmaps to Cell Segmentation and Feature Extraction

The field of cell biology has evolved significantly since the establishment of cell theory in the 19th century, which recognized the cell as the fundamental unit of life [2]. Light microscopy, in particular, has played a pivotal role in advancing cell biology [113], building upon the pio-

neering work of Antoni van Leeuwenhoek in the 1670s. Presently, cell biologists have access to a diverse array of advanced microscopic imaging techniques that allow for the visualization of cellular phenomena, surpassing the classical diffraction limit of light. These techniques generate extensive and complex datasets that have outgrown manual management, processing, and analysis. Consequently, the application of computational techniques for these tasks has become indispensable for the advancement of cell biology [76, 100, 86, 26, 63].

One of the primary challenges in cell studies is image segmentation, a critical step that involves the precise delineation of cell boundaries. Accurate cell delineation is pivotal for subsequent analyses, regardless of whether they focus on nanoscale details or encompass broader millimeter-scale perspectives [13].

Cell segmentation refers to the process of partitioning an image or series of images into distinct regions that correspond to individual cells. The main objective of cell segmentation is to detect and delineate each cell present in an image for further analysis. This step is crucial in image-based research in the field of biology and biomedicine. It is widely used in various biological and biomedical studies, including cell tracking, cell enumeration, investigation of cell behavior, and other forms of cellular analysis. To perform cell segmentation, it is essential to create an accurate mask that outlines the cell boundaries. Two critical factors are taken into account when developing a cell segmentation method: the ability to identify the borders of each cell correctly and the ability to differentiate between adjacent cells. Various methods have been tested and evaluated, and in the following sections, we will discuss some of these approaches in detail.

4.3.1 Approaches for Cell Segmentation Procedure

Various plugins, such as MorphoLibJ, Stardist, and local thresholding followed by Classic Watershed, were tested for different fields of view (FOV) containing astrocyte cells. The

study aimed to evaluate their capabilities in identifying astrocyte cells and accurately masking their borders. These image-processing techniques were combined with the 'regionprops' function in MATLAB to facilitate the isolation of individual cells for further analysis. Each tested approach was assessed based on its ability to differentiate neighboring astrocyte cells and its effectiveness in generating precise cell masks, particularly with respect to cellular processes.

Watershed

The watershed algorithm is a popular method for segmenting images in computer vision and image processing. This algorithm works particularly well when identifying objects or regions in an image that have clear boundaries. The algorithm mimics a flooding process, and it interprets pixel intensities as topographical elevations. This idea was first introduced by S. Beucher and C. Lantuéjoul [105]. The process starts by placing markers in the image at the highest and lowest intensity points, and these points guide the flooding process, assigning regions with common markers the same label. The output is a segmented image with clear boundaries shown by watershed lines. The watershed algorithm is used in many areas, including medical imaging for cell segmentation and tumor detection, as well as in geological and satellite image analysis.

Program Overview: Fiji/ImageJ has a watershed tool that can be accessed in the binary section. The tool works by treating the input image as a topographic surface with water sources placed at regional minima using the conventional watershed model. As the algorithm floods the relief, it creates dams where the water sources converge. However, this program has limited options for adjustments.

MorphoLibJ (Distance Transform Watershed 3D)

Program Overview: Distance Transform Watershed 3D is a segmentation tool that is available for free and open-source. It uses a combination of distance transform and watershed segmentation concepts to perform the segmentation. The distance transform feature computes the distance of each voxel to the nearest background voxel. The watershed algorithm utilizes these distance values as the water levels flood the image from local minima until the watershed lines are formed. Users have the option to customize the distance map and the watershed settings, including dynamic and connectivity settings.

Stardist

Program Overview: Stardist is a deep learning-based image segmentation tool that can be easily installed as a plugin for Fiji/Image J. It employs a fully convolutional neural network to detect and delineate objects using a unique "star-complex" shape model. This shape model uses a star-convex polygon concept which enhances segmentation accuracy, thanks to the presence of multiple "arms" in the segmentation shape. Additionally, the model utilizes a probability map to further refine the segmentation, estimating the likelihood of each pixel belonging to the object. Finally, a binary mask is generated that identifies the object pixels, and post-processing parameters such as non-maximum suppression (NMS) settings can be adjusted by users, including probability/score and overlap threshold.

Figure 4.6 presents a sample field of view (FOV) and the outcomes yielded by various methodologies. Despite meticulous parameter tuning to optimize accuracy, none of these techniques exhibited promising results. In Figure 4.6(c), the classic watershed plugin result is depicted, wherein the algorithm fails to effectively distinguish between neighboring astrocyte cells. Subsequently, Figure 4.6(d) showcases the outcome of employing the Stardist plugin. While this algorithm excels in segmenting circular cells, it proves inadequate when confronted with

more intricate cellular shapes, often failing to encompass the entire cell as a coherent entity. Finally, in Figure 4.6(e), an analogous behavior to the classic watershed is observed, with the method falling short in identifying cell boundaries, resulting in the inaccurate segmentation of neighboring cells.

4.3.2 Final cell segmentation procedure

After processing the interference cell images for each cell culture dish at various time points into final real height maps, they are loaded as image sequences in ImageJ. At each time point, rectangles are drawn manually around the cells to determine their bounding box. The 'measure' tool located in the 'analyze' section is used to measure the position, height, and width of each rectangle. The resulting data is saved as CSV files and loaded in MATLAB to crop the real height map images. The cropped images, each containing one cell at a certain time point are then batch-processed using macro in ImageJ. The code can be found in the Appendix section of the thesis. This code applies the "Auto Local Threshold" on images, using the "Median" method with a 55-pixel radius. The process is followed by one round of erosion to eliminate noise and small particles, and one round of dilation. When cells within the bounding box are closely packed, the process of isolating the desired cell may lead to generating more than one object in the mask. One solution is to import the masks into MATLAB for further image processing. The 'regionprops' function is used to identify the object with the highest surface area, which corresponds to the main cell in the image. The other objects are eliminated, resulting in a mask with only one object that corresponds to the desired cell. Figure 4.7 displays two cells and their respective masks. Each cell has three corresponding images: a grayscale image, a mask image created with ImageJ, and a final processed image generated with MATLAB.

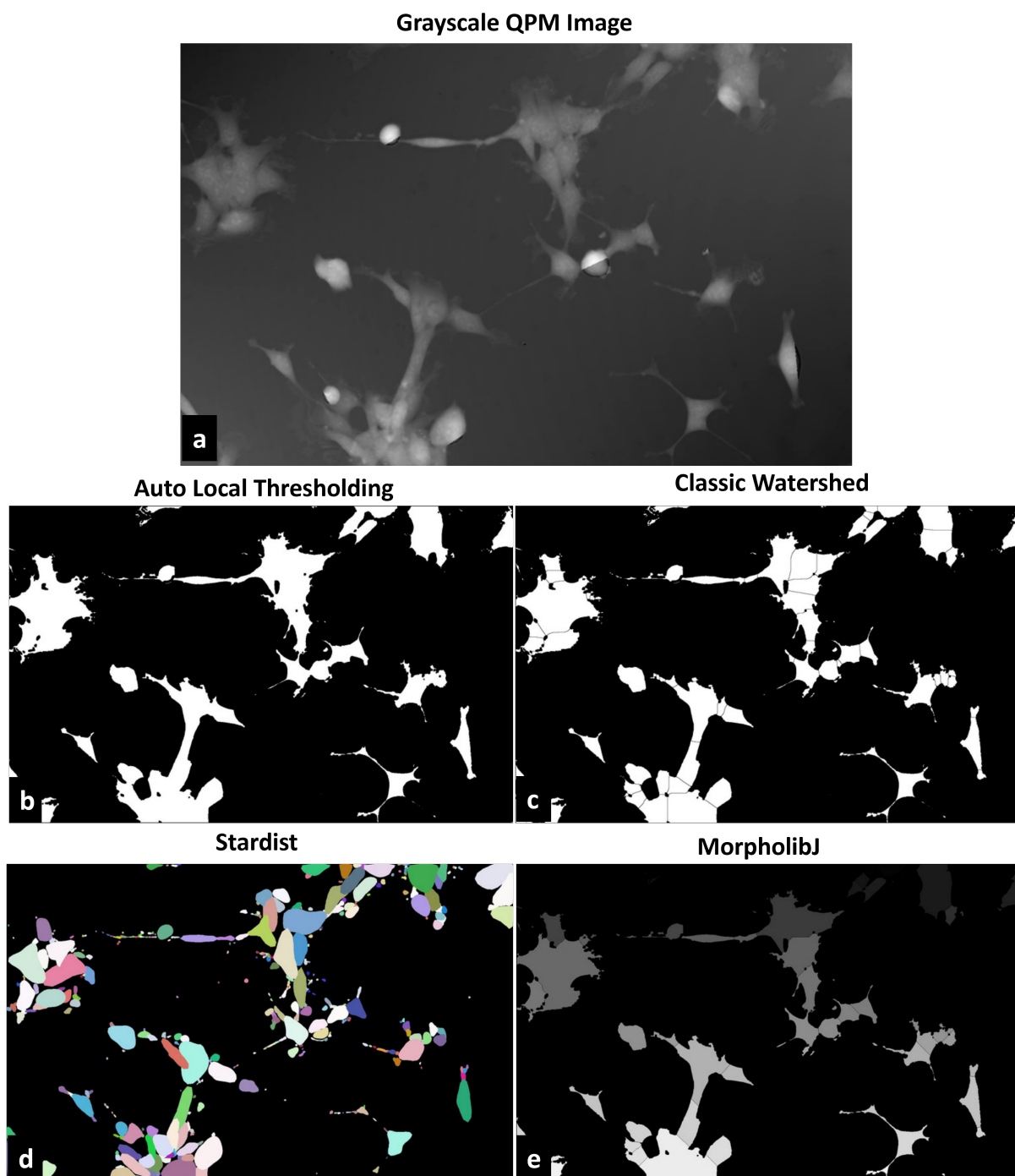


Figure 4.6: Comparison of various cell segmentation approaches on a representative FOV. a) The original grayscale FOV. b) Application of auto local thresholding and subsequent post-processing to eliminate small particles. c) Output of the classic watershed method. d) Segmentation achieved with the Stardist approach. e) Result obtained using the MorpholibJ algorithm.

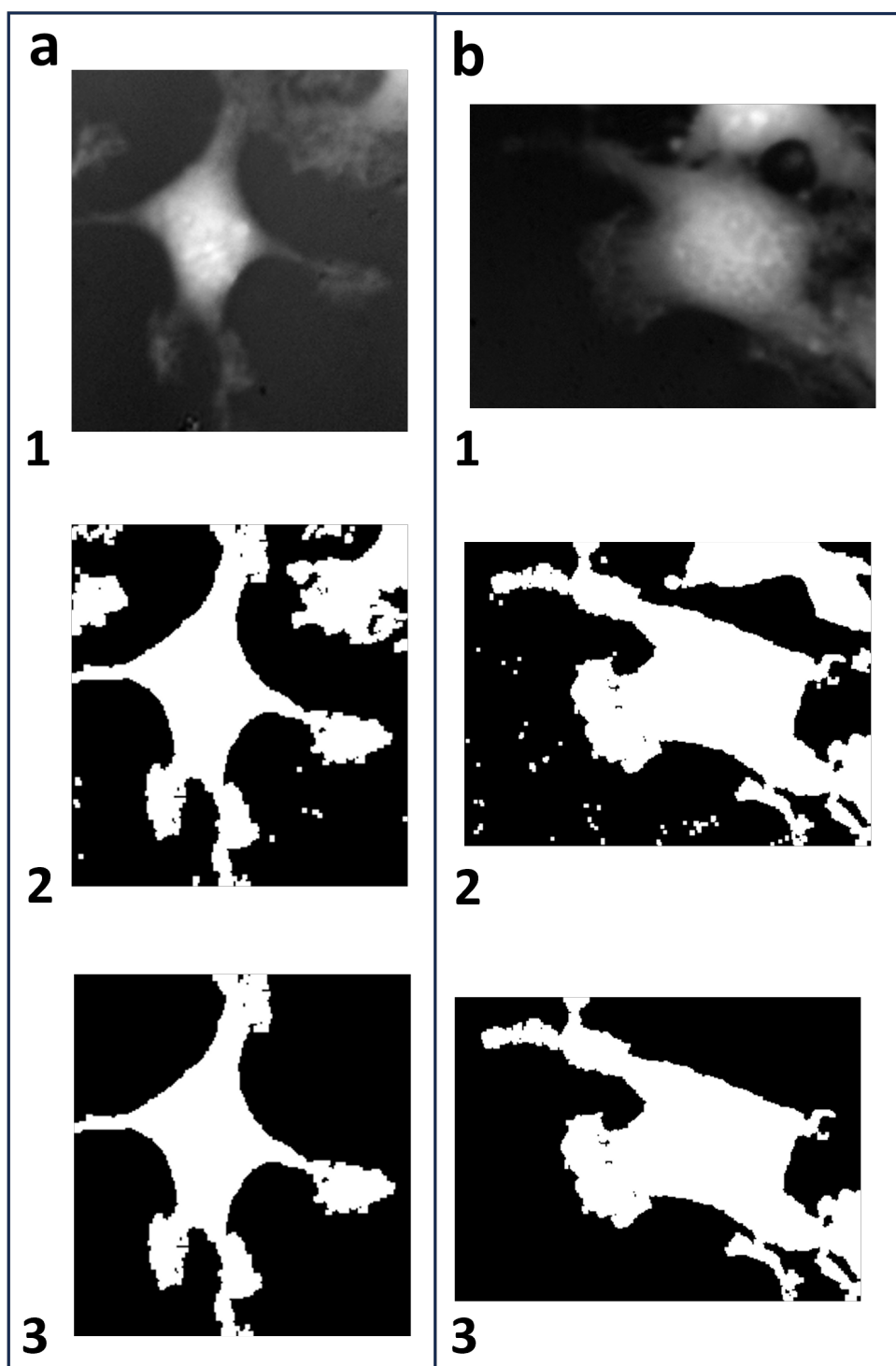


Figure 4.7: Two example cells (a,b) and their respective masks. Each cell has three corresponding images: (1) a grayscale image, (2) a mask image created with ImageJ, and (3) a final processed image generated with MATLAB.

4.3.3 Background Correction

To eliminate any background interference caused by uneven exposure, a mask generated through ImageJ processing is utilized as a guide for calculating the average background height. The mask includes all particles within the bounding box, and the remaining part of the image represents the background. The calculated average is then subtracted from the entire image, resulting in the background-corrected image shown in Figure 4.8. Figures 4.8(a,b) show the resulting masks obtained from ImageJ and processed using MATLAB, respectively. Figures 4.8(c,d) show the original cell image and the background-corrected image, respectively, along with the height value of a point on the background provided for reference in both images.

4.3.4 Feature Extraction

Several morphological features are measured from the phase and height map images, including dry mass (DM), surface area (SA), surface area to volume ratio (SAV), surface area to dry mass ratio (SDM), sphericity index, height variance (HV), height kurtosis (HK), height skewness (HS), eccentricity (Ecc), projected area (PA), volume divided by the projected area (VoldivArea), circularity (Cir), perimeter (perim) and perimeter divided by area (perimdivarea). All features, except volume divided by area, circularity, perimeter, and perimeter by area, are based on the parameters described in [33]. A detailed explanation of the features is described below:

- **Dry Mass:** The dry mass of the cell is proportional to its height profile. The relationship is determined by the refractive increment (α), which is typically constant for the type of cell under investigation. This measurement is crucial in biological research as it helps scientists understand the cellular composition and relative proportions of

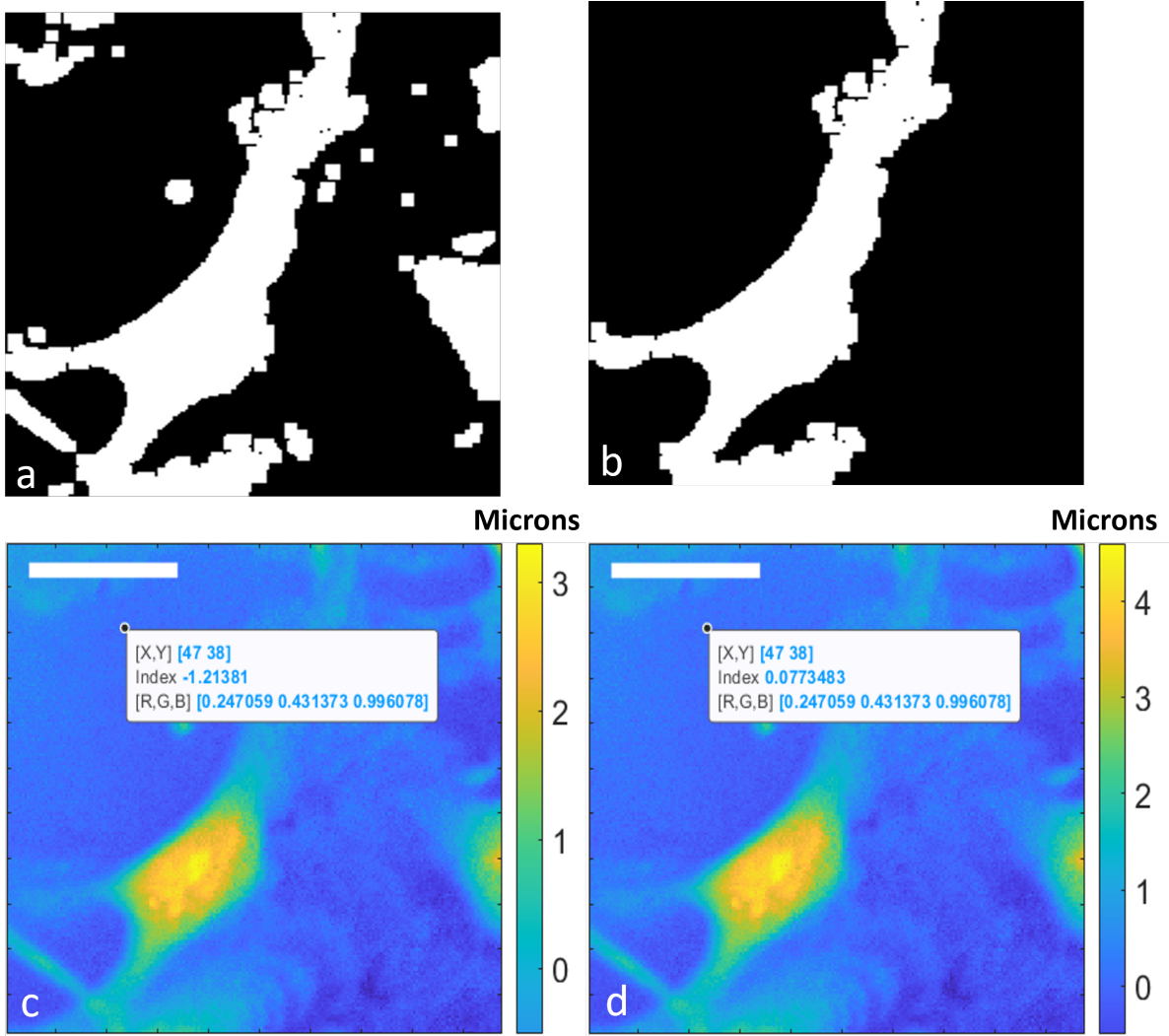


Figure 4.8: (a) The resulting masks obtained from ImageJ, (b) The mask processed using MATLAB, (c) the original cell image (d) the background-corrected image. The height value of a point on the background is provided for reference in both images. The scale bar is 20 microns.

different components. Dry mass is calculated using the formula:

$$\text{Dry mass} = \frac{1}{\alpha} \int (H) ds \quad (4.3)$$

where H is the height of the cell at each point. For astrocytes, α can be estimated as $1.8 \times 10^{-4} \text{ m}^3 \text{ kg}^{-1}$, and ds is the differential area element.

- **Volume:** The volume is calculated by integrating the height profile over the cell area:

$$\text{Volume} = \int (H ds) \quad (4.4)$$

where ds is the differential area element.

- **Surface Area from Height Profile (SA):** This is derived from the height profile and provides information about the variation in the cell's surface.
- **Projected Area (PA):** The cell's projected area is calculated by counting the number of pixels within the cell mask and multiplying it by the pixel size. This provides the two-dimensional surface area.
- **Surface Area to Volume Ratio (SAV):** This ratio is obtained by dividing the surface area by the volume calculated earlier.
- **Surface Area to Dry Mass Ratio (SDM):** Similar to the above, this ratio is found by dividing the surface area by the dry mass.
- **Sphericity Index:** The sphericity index measures how closely the cell approximates a perfect sphere. It is calculated using the formula:

$$\text{Sphericity} = \pi^{1/3} \frac{(6H)^{2/3}}{\text{Surface Area}} \quad (4.5)$$

A perfect sphere has a sphericity value of 1.

- **Height Variance (HV):** Height Variance (HV) is a parameter that measures the spread of height values within the cell. It provides insights into how varied the heights of different regions within the cell are. HV can be calculated as the variance of the height values over the cell area using the following formula:

$$HV = \frac{1}{n-1} \sum_{i=1}^n (H_i - \mu_H)^2 \quad (4.6)$$

Where: HV is the Height Variance, n is the number of data points (height values), H_i represents the individual height value, μ_H is the mean of the height values.

- **Height Kurtosis (HK):** Height kurtosis (HK) is a parameter that measures whether the height distribution within the cell (height distribution) is more peaked or flat compared to a normal distribution. It quantifies the shape of the height distribution. HK can be calculated using the formula for kurtosis:

$$HK = \sum_{i=1}^n \left(\frac{H_i - \mu_H}{\sigma_H} \right)^4 \quad (4.7)$$

Where: HK is the Height Kurtosis, n is the number of data points (height values), H_i represents the individual height value, μ_H is the mean of the height values, σ_H is the standard deviation of the height values.

- **Height Skewness (HS):** Height Skewness (HS) is a parameter that quantifies the asymmetry of the height distribution within the cell. It describes whether the distribution is skewed to the left or right. HS can be calculated using the formula for skewness:

$$HS = \sum_{i=1}^n \left(\frac{H_i - \mu_H}{\sigma_H} \right)^3 \quad (4.8)$$

Where: HK is the Height Kurtosis, n is the number of data points (height values), H_i represents the individual height value, μ_H is the mean of the height values, σ_H is the standard deviation of the height values.

- **Eccentricity:** Eccentricity quantifies how much the cell's shape deviates from a perfect circle. It is determined by finding the maximum and minimum radii ($r_{\max}$ and $r_{\min}$) of the cell's boundary and applying the formula:

$$\text{Eccentricity} = \frac{r_{\max} - r_{\min}}{r_{\max} + r_{\min}} \quad (4.9)$$

- **Projected Area to Volume Ratio (PAV):** This ratio quantifies cell flatness. It is calculated by dividing the projected area (2D area) by the volume of the cell (3D measurement).
- **Circularity:** Determined using the circularity feature in MATLAB's 'regionprops' function. It evaluates the similarity of the cell's shape to that of a perfect circle. This measure ranges from 0 to 1, with 1 representing a perfect circle.
- **Perimeter (Perim):** The perimeter represents the total length of the outer boundary of the cell. It is calculated as:

$$\text{Perimeter} = \int dl \quad (4.10)$$

where dl is the differential arc length along the boundary.

- **Perimeter-to-Area Ratio (PerimDivArea):** Represents the ratio of the perimeter to the cell's area. It is calculated as:

$$\text{Perimeter-to-Area Ratio} = \frac{\text{Perimeter}}{\text{Cell Area}} \quad (4.11)$$

- **Complexity Score:** The convex hull represents the smallest convex polygon that encloses the given cell, providing insight into the cell's overall spatial characteristics. This metric is derived using the convex hull algorithm, which calculates the difference between the projected area of a cell and its convex hull. The result is obtained by dividing this difference by the area of the convex hull shape:

$$\text{Complexity Score} = \frac{\text{Convex Hull Area} - \text{Cell Area}}{\text{Convex Hull Area}} \quad (4.12)$$

Table 4.1 shows the corresponding feature values for each image along with a grayscale image of a cell at two different time points and their corresponding final masks, represented in Figure 4.9.

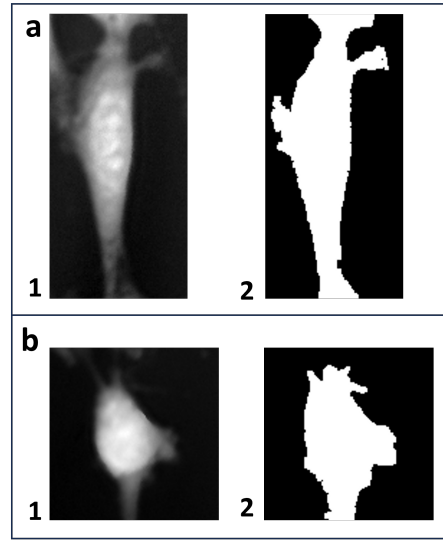


Figure 4.9: (a,b) Two grayscale images of a cell in two different time points: before LIS and 2 hours after LIS. (1,2) The corresponding final masks.

4.4 Discussion

In this chapter, we have explored methodologies for quantitative phase image processing that enable the extraction of valuable cellular features. These techniques provide a deeper

Parameter	2 hours after LIS	Before LIS
circ	0.44407	0.21979
volume	2061.7792 μm^3	1779.61 μm^3
PA	508.4975 μm^2	802.13 μm^2
drymass	11.4543 p gr	9.8867 p gr
SA	14729.0367 μm^2	19845.6655 μm^2
SAV	7.1438 $1/\mu\text{m}$	11.1517 $1/\mu\text{m}$
SDM	1285.8926 $\mu\text{m}^2 \text{ pgr}$	2007.3048 $\mu\text{m}^2 \text{ pgr}$
PAV	0.24663 $1/\mu\text{m}$	0.45073 $1/\mu\text{m}$
Sphericity	0.00083486 $\mu\text{m}^{1/3}$	0.00041451 $\mu\text{m}^{1/3}$
HV	9.8839 μm^2	1.7837 μm^2
KT	65.4439 $1/\mu\text{m}^4$	3895.9334 $1/\mu\text{m}^4$
SK	32.9157 $1/\mu\text{m}^3$	370.8449 $1/\mu\text{m}^3$
Ecc	0.26292	0.44856
perim	361.4214 μm	643.1615 μm
perimdivarea	0.71076 $1/\mu\text{m}$	0.80182 $1/\mu\text{m}$
complexityscore	0.19663	0.42817

Table 4.1: Comparison of Features for Cell Shown in Figure 4.9, Before and 2 Hours After LIS

understanding of the structure and behavior of cells, making significant contributions to cell biology research.

The ability to extract features such as cell area, dry mass, and volume from quantitative phase images is crucial in cell biology [62]. These features offer quantitative and reproducible metrics for understanding various cellular characteristics, opening doors to a plethora of applications. The analysis of these features can serve as the foundation for comprehending cellular injuries, cell behavior, cell cycle progression, and responses to external stimuli or drugs.

While the methods discussed in this chapter are highly effective for many aspects of cell biology research, there are challenges in the context of cell segmentation, particularly when dealing with astrocytes. Astrocytes play a vital role in the central nervous system and exhibit dynamic morphological changes in response to various stimuli [49]. Accurate quantification of astrocyte reactivity necessitates precise cell segmentation and the identification of features

distinguishing reactive from non-reactive cells. Astrocyte cell segmentation presents two significant challenges. The first is creating a mask that distinctly separates cell borders, and the second is defining the borders of cell clusters. To address these challenges, we adjusted parameters, including thresholding parameters in ImageJ. While results were generally acceptable for most cells, there is room for improvement. For instance, in Figure 4.7, the final mask for cell 'a' accurately delineates cell borders, but for cell 'b', the neighboring cell on the bottom right remains unmasked. Additionally, the developed algorithm necessitates manual outlining of cell bounding boxes, which enhances accuracy but is time-consuming. A combination of thresholding the entire image using ImageJ, followed by a watershed algorithm and particle detection with 'regionprops' in MATLAB, was employed to automate bounding box outlining. However, the watershed algorithm often led to poor border masking of astrocytes.

Regarding feature extraction, our results show promise in extracting various features from cells. For example, comparing the cell before a shockwave (Figure 4.9(a)) with the cell two hours after the shockwave (Figure 4.9(b)), as presented in Table 4.1, reveals changes in eccentricity and circularity, indicative of elongation. However, astrocyte reactivity is more complex, requiring the development of features capable of capturing intricate morphological changes.

Deep learning and machine learning techniques have shown promise in addressing cell detection and feature extraction challenges [3, 58]. These methods have the advantage of being able to adapt to data patterns, reducing the need for complex handcrafted algorithms [3]. However, there are several hurdles associated with these techniques.

One major challenge is the requirement for extensive and diverse training data. Deep learning models rely on large amounts of labeled data to learn and generalize effectively. Obtaining such datasets can be time-consuming and resource-intensive. Another challenge is the fine-tuning of model parameters and architecture. Finding the optimal configuration for a deep learning model can be a complex and iterative process. It often requires experimenta-

tion and computational resources to train and evaluate different models. Interpretability is also a concern when using deep learning techniques. These models often function as black boxes, making it difficult to understand the reasoning behind their decisions. This lack of interpretability can be problematic, especially in critical applications where explanations are necessary. Furthermore, deep learning methods are not one-size-fits-all and may require customization for specific tasks and datasets. Different cell detection and feature extraction tasks may have unique requirements and characteristics that need to be considered when designing and training deep learning models [3].

In conclusion, the methods discussed in this chapter empower researchers with powerful tools to extract valuable features from quantitative phase images, enhancing our understanding of cellular behavior. Moreover, for astrocyte cells and similar cases where reactivity plays a crucial role, exploring novel features can open new avenues for quantifying complex cellular responses.

Chapter 5

Astrocytes Morphology Analysis

5.1 Motivation

This chapter is motivated by a quantitative investigation into how astrocyte cells respond to laser-induced shockwaves, specifically focusing on measurable changes in their morphology. The analysis focuses on key morphological parameters, such as dry mass, volume, projected area, and circularity, both before and after exposure to shockwaves. The experimental design comprises two groups: a controlled group under condition similar to the incubator and a laser-induced shockwave group exposed to a shockwave under identical condition, with rigorous control of humidity and CO₂ levels for consistency. The imaging process, employing quantitative techniques like phase unwrapping and cell selection, allows for precise data processing. The subsequent statistical analysis quantifies significant differences in cellular parameters, providing concrete insights into the impact of shockwaves on astrocyte cell morphology. In summary, this chapter seeks to quantitatively explore the effects of laser-induced shockwaves on astrocyte cells, offering measurable insights into cellular responses to mechanical stimuli and laying the foundation for further quantitative investigations into the

underlying mechanisms.

5.2 Introduction

Traumatic Brain Injury (TBI) stands as a critical public health concern, with a notable surge in TBI-related incidents reported by the Centers for Disease Control and Prevention from 2006 to 2014 [31]. This injury occurs when an external force disrupts the normal function of the brain, leading to a spectrum of outcomes, from mild to severe, with consequences ranging from full neurological recovery to mortality. Despite advancements, TBI remains a leading cause of physical impairment and death, particularly among the youth.

Severe damage, the chief cause of death or physical impairment after TBI, happens after the primary injury. This damage is referred to as a secondary injury, which is characterized by an increase in intracranial pressure followed by a decrease in cerebral perfusion and ischemia [31]. Existing treatments have yet to conclusively prevent secondary injury, highlighting the necessity for a deeper understanding of the cellular mechanisms at play, particularly at the molecular and cellular levels [31].

Various models have been proposed to simulate TBI, with blast-induced TBI (bTBI) gaining prominence, particularly in the context of contemporary issues such as explosive devices directly impacting the brain [64]. Among the methods used to induce blast-like conditions, Laser-Induced Shockwaves (LIS) has proven to be an effective *in vivo* and *in vitro* model, allowing for the controlled application of shear stress to cells [64]. While this method has successfully simulated TBI at the cellular level, the study of cellular changes during and immediately after the injury remains a challenging endeavor.

Astrocytes, the most numerous cells in the central nervous system (CNS), play a pivotal role in maintaining CNS homeostasis and supporting neuronal functions [75, 94]. These

star-shaped cells regulate ions, water and blood flow, recycle neurotransmitters, and provide essential nutrition to cells. In response to CNS injuries, astrocytes engage in a process known as reactive astrogliosis. This dynamic process involves a spectrum of responses depending on the severity and proximity of the insult, leading to heterogeneous and variable cellular hypertrophy among reactive astrocytes [94]. The degree of morphological changes and interaction of astrocyte processes directly correlate with the extent of hypertrophy, making the morphological heterogeneity of astroglia a critical aspect of astrogliosis [94].

Studies have shown that astrocytes respond to mechanical forces, specifically shear stress, which can be generated by LIS [114, 59, 84]. Reactive astrogliosis has gained significance in the context of neural tissue damage, offering potential strategies for mitigating the progression of brain injuries. The degree of astrogliosis, marked by morphological changes and the uptake of extruded materials, varies with the severity of CNS insults [94]. Understanding how astrocytes dynamically respond to mechanical forces, becomes paramount in advancing our comprehension of brain injuries and potential avenues for therapeutic interventions.

The study presented herein introduces a comprehensive system that combines LIS with Quantitative Phase Microscopy (QPM) to enable quantitative phase imaging of astrocyte cells before, and after LIS exposure. By capturing 3D images of LIS-induced injury and quantitatively measuring changes in the cell membrane and internal cell structure, this system provides an unprecedented tool for studying astrocyte responses to shear stress caused by LIS at the cellular level. Leveraging the emerging label-free method of QPM, the integrated approach allows real-time monitoring of astrocyte membrane dynamics, cell shape alterations, and intracellular damage and recovery [114, 59].

The examination of how astrocytes react to laser-induced shockwaves (LIS) reveals immediate and sustained morphological transformations, providing insights into the dynamic adaptations of these cells. Following LIS, astrocytes swiftly shift to a more circular shape, accompanied by alterations in volume, surface characteristics, and height features. Subse-

quent analysis conducted 2 hours post-LIS indicates enduring changes, suggesting continuous cellular adjustments and regulatory processes. Statistical analysis underscores significant alterations in circularity, volume, surface area, and other features, highlighting the dynamic responses to mechanical disturbance. Mann-Kendall Tau-b test results affirm the robustness of the observed trends. The dynamics of surface area imply a cellular response geared towards enhancing chemical interactions, while an increased projected area and area-to-volume ratio indicate a flattening response to LIS.

The findings from this research illuminate significant morphological changes in astrocytes in response to LIS, contributing to the growing body of knowledge on how mechanical stimuli impact astrocyte morphology [114, 59]. These insights hold promise for further investigations into the underlying mechanisms of astrocyte responses to mechanical forces and the development of targeted therapeutic strategies in the context of traumatic brain injuries and related neurological disorders. The integrated LIS and QPM approach offers a powerful tool for 3D imaging and quantitative measurement of astrocyte morphological changes, providing deeper insights into cellular dynamics and potential therapeutic interventions [114, 59].

5.3 Average profile of Astrocytes

In order to get an average profile of astrocyte cells, a set of 16 astrocyte images was captured utilizing Quantitative Phase Microscope (QPM) equipped with a 40x objective lens. Utilizing the QPM, the thickness of each cell was computationally derived. The subsequent image analysis was conducted using Image J, and surface profiles were generated using the "Intensity Profile".

For each of the 16 astrocytes under investigation, the QPM was instrumental in constructing a height profile along both maximal and minimal distances from the center of mass to the

cell's edge. Distinct mean lengths were calculated for the long and short lines, amounting to approximately $56.55 \mu m$ and $32.25 \mu m$, respectively. Through individual scaling of each array to the computed mean size using interpolation in MATLAB, the mean thickness was determined for both short and long lines. The resulting average dimensions of the modeled cells are visually represented in 5.1 (A).

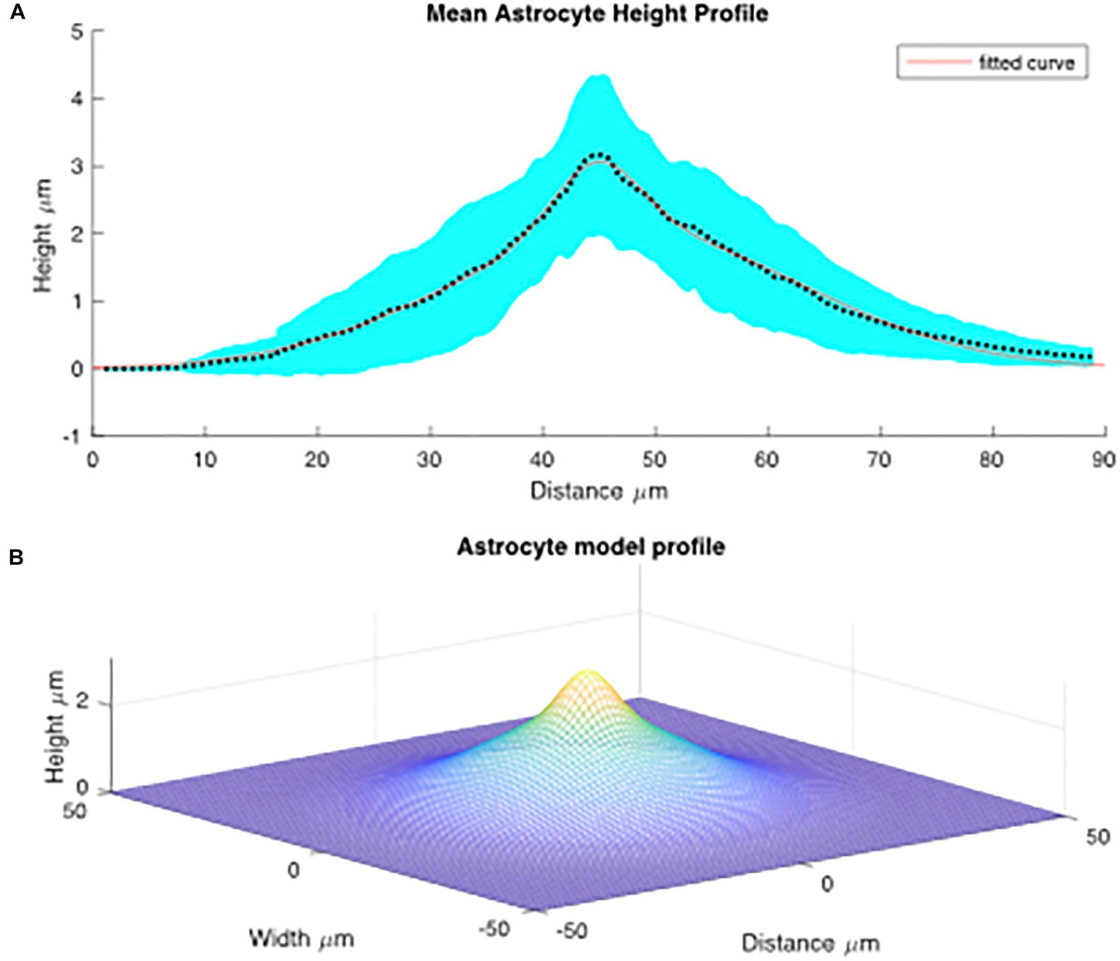


Figure 5.1: (A) Mean height profile of astrocytes derived from QPM data depicted by the dotted line. The red line denotes the fitted Gaussian function. The blue shaded area is the standard error derived from the QPM height profiles. (B) Model of the average astrocyte height profile used for modeling fluid flow over the cells[114]

This methodology not only facilitated the estimation of astrocyte height but also provided a comprehensive understanding of their morphological characteristics. The quantitative data

obtained through QPM, combined with advanced computational analyses, contributes valuable insights into the physical properties of astrocytes, thereby enhancing our understanding of the forces they experience in their microenvironment.

5.4 Comprehensive Morphological Analysis of Astrocyte Responses to LIS

5.4.1 Experiments protocol

AST Cell Preparation

An established astrocyte type-I (Ast-1) line (clone CRL-2541) was received directly from ATCC. Ast-1 cells were cultured in advanced DMEM media supplemented with 2% FBS, 1% glutamax, and 0.2% gentamicin/amphotericin B. Ast-1 cells were plated onto glass-bottom 35mm imaging dishes. Alternatively, imaging dishes were coated by incubating them for 1 hour in a 1:60 dilution of matrigel:DMEM to promote cell adhesion.

Control group

Astrocyte cells were maintained and observed within a temperature-controlled chamber designed specifically for the Quantitative Phase Microscope setup. The chamber's environmental conditions, including the composition of the air, were precisely controlled using an Ibidi gas mixer, which ensured a stable and controlled atmosphere. The key parameters that were regulated included the humidity level, which was maintained at a constant 80 percent, and the carbon dioxide (CO₂) concentration, which was set at 5%. These controlled conditions were essential to replicate the natural environment of these cells and minimize possible stress

or alterations in their behavior.

During the observation period, the astrocyte cells were subjected to a time-lapse imaging protocol. Every 20 minutes, the cells were imaged and this process continued for a total duration of two hours. We ensured that there was no interference or disruption to the cells' normal activity or behavior. This stringent approach allowed us to capture and analyze the astrocyte cells' activities and interactions in their near-native state, providing valuable insights into their functions and responses under controlled conditions. In total, 84 cells from 3 separate plates were analyzed and further processed for cell segmentation and feature extraction.

Laser-induced shockwave group

The astrocyte cells were kept under similar conditions as the control group, except for being exposed to a laser-induced shockwave. This experiment involved a continuous imaging process, which started 20 minutes before the shockwave and lasted for 2 hours afterwards. The resulting quantitative phase images were analyzed to detect and measure various aspects of cell morphology.

To evaluate the effect of the shockwave on the cells, we focused on their morphology before and 10 minutes, and 2 hours after the shockwave. By doing so, we could identify any rapid or gradual changes or responses induced by the shockwave. This approach allowed us to gain a deeper understanding of how cells react and adapt to sudden environmental changes under these unique experimental conditions. A total of 204 cells collected from four dishes were subjected to analysis.

5.4.2 Image processing

To acquire the wrapped phase images, the raw interference images were processed using MATLAB, following the algorithms described in chapter 4 of the Thesis. The cells within the field of view (FOV) were manually identified using ImageJ. Subsequently, binary masks for each individual cell were generated by applying a local thresholding method with the median filter in ImageJ. These binary masks were then imported into MATLAB for additional image processing tasks, which were executed using MATLAB.

5.4.3 Morphological Features

Several morphological features are measured from the height map images. These features provide valuable insights into various aspects of cellular biology.

- **Dry Mass:** The dry mass of a cell is proportional to its height profile and is determined by the refractive index of the cell. It plays a crucial role in understanding the composition of cells and the relative proportions of different cellular components.
- **Volume:** The volume of a cell provides insights into its overall size and shape. It is a fundamental parameter for understanding cell biology.
- **Surface Area from Height Profile (SA):** SA, derived from the height profile, describes the variations in a cell's surface and overall surface texture.
- **Projected Area (PA):** PA represents the two-dimensional surface area of the cell. It is determined by counting pixels within the cell mask. PA is used to assess the planar size of the cell.
- **Surface Area to Volume Ratio (SAV) and Surface Area to Dry Mass Ratio:** These ratios offer insight into the relationship between the surface area of a cell and its

internal content. A higher SAV may indicate a more extensive cell membrane relative to its volume, while the Surface Area to Dry Mass Ratio reflects the distribution of dry mass across the cell's surface area.

- **Sphericity Index:** The sphericity index measures how closely a cell approximates a perfect sphere. It is essential for understanding cell shape and deviations from a spherical structure.
- **Height Variance (HV):** HV quantifies the variability in height values across the cell. Higher HV values indicate greater height variation within the cell, potentially reflecting structural complexities or cellular processes.
- **Height Kurtosis (HK):** HK measures the shape of the height distribution within the cell. Deviations from a normal distribution can indicate irregularities in cell structure.
- **Height Skewness (HS):** HS quantifies the asymmetry of the height distribution. It helps identify whether height values are skewed to the left or right within the cell. Changes in HS may suggest structural alterations.
- **Cell Eccentricity (Ecc):** Cell eccentricity quantifies how much a cell's shape deviates from a perfect circle. Deviations from a circular shape can provide information about cellular processes or structural changes.
- **Projected Area to Volume Ratio (PAV)** PAV quantifies cell flatness, allowing researchers to assess whether cells are more planar or three-dimensional in shape.
- **Circularity (Circ):** Circularity measures the similarity of a cell's shape to that of a perfect circle. Higher circularity values indicate a closer resemblance to a circular shape. Deviations from circularity can suggest alterations in cell structure.
- **Perimeter (Perim):** Perimeter (perim) is a feature that represents the total length of the outer boundary of a cell. It provides information about the cell's extent and

boundary complexity. Changes in the perimeter may indicate variations in cell size or irregularities in cell shape.

- **Perimeter-to-Area Ratio (PerimDivArea):** PerimDivArea is a feature calculated by dividing the perimeter of a cell by its area. This ratio offers insight into the cell's boundary irregularity relative to its overall size. Higher values may suggest a more complex and convoluted cell boundary compared to its size.
- **Complexity Score:** Computed as the ratio of the difference between the area of the cell's convex hull and the cell area to the cell area itself. A lower score signifies a smoother and less intricate cell shape.

These features collectively contribute to our understanding of cell biology, enabling researchers to assess the effects of various conditions, treatments, or genetic manipulations on cellular structure and function.

5.5 Statistical Analysis

The statistical tests conducted to assess the changes in astrocyte cell parameters within the control and LIS group were performed through MATLAB, using the Wilcoxon signed-rank test, a non-parametric test suitable for paired data. Since the measurements were obtained from the same set of cells during the imaging period, the data are inherently dependent on each other. The Wilcoxon signed-rank test is well-suited for such paired comparisons, providing a robust analysis that does not assume a normal distribution of the data. This method accounts for the paired nature of the observations, making it appropriate for detecting subtle changes in the astrocyte cell parameters over time.

The Mann-Kendall Tau-b test [16] was also performed for data trend analysis. This is a non-parametric statistical method employed for the detection of monotonic trends in time

series data. It specifically assesses the presence of an upward or downward trend over time without assuming any underlying distribution of the data. The Taub statistic represents the magnitude of the trend, and the p-value indicates the statistical significance of that trend.

5.5.1 Control Group

In the control group, astrocyte cells were subjected to two hours of imaging, and the average value for each feature for the total of 125 cells, is summarized in Table 5.1. Various parameters were measured to assess any potential changes during this imaging period.

After two hours of imaging, a comparison of feature values was conducted, as presented in Table 5.2. The test statistics, p-values, and the determination of significant differences were reported for each feature. Despite this analysis, no statistically significant changes were observed in any of the features within the control group. The lack of statistical significance suggests that the astrocyte cells' morphology and characteristics remained stable during the experimental timeframe.

In conclusion, the data from the control group indicate that, no significant changes occurred in the astrocyte cells after two hours of imaging. This stability in cellular features provides a baseline for comparison with experimental groups, allowing for a more accurate assessment of any observed effects or changes due to LIS.

5.6 Laser-Induced Shockwaves (LIS) Group

The investigation of astrocyte responses to laser-induced shockwaves (LIS) has revealed profound morphological alterations, providing intricate insights into the dynamic cellular adaptations. Analyzing various morphological features immediately after LIS and 2 hours

Parameters	Average Value (n=84)
Circularity	0.24813
Volume	3054.7392 μm^3
Projected Area	1125.4119 μm^2
Dry Mass	16.9708 p gr
Surface Area	29107.4126 μm^2
Surface Area to Volume Ratio	9.6821 $1/\mu\text{m}$
Surface Area to Dry Mass Ratio	1742.7744 p gr/ μm^2
Projected Area to Volume Ratio	0.37841 $1/\mu\text{m}$
Sphericity	0.00024686 $\mu\text{m}^{1/3}$
Height Variance	4.4972 μm^2
Height Kurtosis	4375.1226 $1/\mu\text{m}^4$
Height Skewness	879.4492 $1/\mu\text{m}^3$
Eccentricity	0.28863
Perimeter	833.5168 μm
Perimeter to Projected Area Ratio	0.74369 $1/\mu\text{m}$
Complexity Score	0.38273

Table 5.1: AST1 Cells Various Features Average Values

post-LIS has elucidated the immediate responses and the persistence of changes, offering a comprehensive understanding of astrocytic behavior under mechanical stress.

The morphological changes observed in astrocytes following LIS reveal immediate adaptive responses and persistent alterations. The cells exhibit a rapid transition to a more circular shape, as evidenced by increased circularity. Simultaneously, an increase in volume and changes in surface characteristics, including surface area (SA) and surface area to volume ratio (SAV), signify dynamic adaptations aimed at withstanding the mechanical stress induced by LIS.

Height characteristics, such as height variance and non-uniformity, suggest rapid cytoskeletal rearrangements and structural adaptations. Furthermore, alterations in sphericity indicate a shift towards a more spherical cell shape, potentially enhancing cell stability.

A 2-hour post-LIS analysis reveals sustained changes in surface area related parameters, volume, and height characteristics, indicating ongoing cellular adjustments. The persistence

Parameters	P-Value	Sig.	Test Stat.	Mean Diff.
Circularity	0.71459	No	1703	0.0066564
Volume	0.87245	No	1749	-5.8727 μm^3
Projected Area	0.71959	No	1865.5	-1.9396 μm^2
Dry Mass	0.87245	No	1749	-0.032626 p gr
Surface Area	0.92538	No	1806	316.3684 μm^2
Surface Area to Volume Ratio	0.56207	No	1655	0.49498 $1/\mu\text{m}$
Surface Area to Dry Mass Ratio	0.56207	No	1655	89.0972 $\mu\text{m}^2/\text{p gr}$
Projected Area to Volume Ratio	0.78559	No	1846	0.017124 $1/\mu\text{m}$
Sphericity	0.96087	No	1796	3.8919e-07 $\mu\text{m}^{1/3}$
Height Variance	0.057451	No	1359	0.4453 μm^2
Height Kurtosis	0.43512	No	1960	572.6861 $1/\mu\text{m}^4$
Height Skewness	0.9183	No	1762	-164.8099 $1/\mu\text{m}^3$
Eccentricity	0.2167	No	2062	-0.015709
Perimeter	0.99644	No	1786	-10.0189 μm
Perimeter to Projected Area Ratio	0.23904	No	2049	-0.01669 $1/\mu\text{m}$
Complexity Score	0.10076	No	2153	-0.017269

Table 5.2: Significance Test Results for the Control Group in 2 Hours

of these alterations suggests a prolonged cellular response, possibly involving regulatory processes aimed at restoring homeostasis Table 5.4.

In Table 5.5, the statistical parameters provide a detailed insight into the morphological changes in astrocytes 1 minute before Laser-Induced Shockwave (LIS) compared to 2 hours after LIS. Notably, several features exhibit significant alterations during this time frame, shedding light on the dynamic responses of astrocytes to the mechanical perturbation induced by the shockwave.

Among the noteworthy changes, the circularity of astrocytes increases significantly (p -value = 0.0001094), indicating a shift toward a more rounded cell shape. Concurrently, there is a considerable decrease in both cell volume (p -value = 0.034178) and dry mass (p -value = 0.034178), suggesting potential cellular contraction or changes in density. The increase in sphericity (p -value = 0.024559) further supports a trend toward a more spherical cellular morphology, indicative of a response to mechanical stress or injury.

Examining the outer characteristics of the astrocytes, both perimeter (p -value = 0.001608) and perimeter-to-area ratio (p -value = 0.0019983) significantly decrease. This reduction implies a more compact cell structure or altered cell-cell interactions. Additionally, the increase in kurtosis (p -value = 0.011555) suggests changes in the height distribution within the cell, indicating potential alterations in cellular structure or response to the shockwave.

The observed morphological adaptations may be integral to the cellular response mechanism, influencing cellular mechanics, cell-cell interactions, and potentially contributing to the overall functionality of astrocytes. Understanding these changes is crucial for deciphering the nuanced ways in which astrocytes respond to mechanical stress, and it opens avenues for further investigations into the underlying cellular processes driving these morphological alterations. The Mann-Kendall Tau-b test results (Table 5.7) underscore the presence of significant trends in various morphological features, consolidating the robustness of the observed changes. Key variables, such as volume and surface area features, and height variance, manifest noteworthy trends, emphasizing the biological significance of these alterations. Figure 5.2 represents the average values of surface area, surface area to volume ratio, volume, and projected area to volume ratio at different time points, measured in minutes after LIS. The fitted line in the figure was obtained using the `fitlm` function in MATLAB to fit a linear regression model. Upon analyzing Figure 5.2 and Table 5.7, we can observe a significant increase in surface area and surface area divided by volume. This peculiar trend may indicate a cellular response aimed at enhancing chemical interactions with the surrounding environment. Additionally, it is noticeable that the projected area and projected area to volume ratio are also showing a significant increase over time, suggesting that the cells are getting flatter in response to LIS. These dynamics highlight the complexity of the cellular response to LIS, emphasizing the need for further investigation into the underlying biological mechanisms driving these morphological changes.

The observed morphological changes in astrocytes in response to LIS hold profound biological

significance. The shift towards a more circular cell shape, reduced volume, and alterations in surface characteristics may indicate an orchestrated effort to enhance cell stability and mitigate the impact of mechanical stress. The simultaneous changes in height characteristics, such as increased variance and non-uniformity, suggest dynamic cytoskeletal rearrangements and structural adaptations to the shockwave.

Furthermore, the persistence of these morphological adaptations over time underscores the resilience and regulatory capacity of astrocytes. The maintained alterations in sphericity, eccentricity, and boundary features may reflect the ongoing role of astrocytes in responding to and mitigating mechanical perturbations, emphasizing their dynamic nature and ability to adapt to external stimuli.

Table 5.6 provides insights into the significant changes in cellular morphological features at various time points following LIS, compared to the state 1 minute before LIS. The highlighted cells (in red) indicate significant alterations in the respective features. The consistent significance in circularity and sphericity throughout the observed time points suggests a sustained change in cell circularity, indicating potential alterations in cell shape stability and dynamics. Features associated with the ratio of area to volume of the cells like SAV and PAV, or height distributions, such as variance, kurtosis, and skewness, exhibit significant changes post-LIS. However, after the 100 to 120-minute mark, no further significant changes are observed when compared to the pre-LIS measurements. This observation underscores the plasticity of the cells; despite undergoing substantial morphological changes and stress, they demonstrate recovery. However, the sustained significance in certain features underscores their potential role in cellular adaptation and functional implications during and after LIS-induced stress.

In summary, the expanded analysis of astrocyte responses to LIS reveals a multifaceted and dynamic cellular adaptation to mechanical stress. The initial acute responses are followed by sustained alterations, suggesting a complex interplay of protective mechanisms and cellular

Parameters	P-Value	Sig.	Test Stat.	Mean Diff.
Circularity	0.00056679	Yes	7545	0.029897
Volume	1.51E-08	Yes	5676	-352.0049 μm^3
Projected Area	1.27E-10	Yes	15884	-167.5746 μm^2
Dry Mass	1.51E-08	Yes	5676	1.9556 p gr
Surface Area	2.62E-10	Yes	15790	-3708.6028 μm^2
Surface Area to Volume Ratio	9.60E-17	Yes	17470	-2.0859 $1/\mu\text{m}$
Surface Area to Dry Mass Ratio	9.60E-17	Yes	17470	-375.4581 $\mu\text{m}^2/\text{p gr}$
Projected Area to Volume Ratio	2.20E-29	Yes	19956	-0.088707 $1/\mu\text{m}$
Sphericity	4.83E-23	Yes	2110	0.00019486 $\mu\text{m}^{1/3}$
Height Variance	8.99E-26	Yes	1594	2.1892 μm^2
Height Kurtosis	3.47E-23	Yes	18828	-3588.9706 $1/\mu\text{m}^4$
Height Skewness	5.91E-07	Yes	14671	-580.7853 $1/\mu\text{m}^3$
Eccentricity	0.050085	No	8801	0.0094032
Perimeter	3.87E-07	Yes	14739.5	-128.5676 μm
Perimeter to Projected Area Ratio	0.23904	No	2049	-0.01669 $1/\mu\text{m}$
Complexity Score	0.10076	No	2153	-0.014833

Table 5.3: Statistical Parameters for the cells 1 Min Before LIS Vs. 10 min After LIS

remodeling. The interconnectedness of morphological features, temporal dynamics, and functional implications underscore the intricate nature of astrocyte responses and pave the way for further exploration into the molecular mechanisms governing these adaptations. This expanded interpretation enhances our understanding of astrocyte biology in the context of mechanical perturbations and lays the groundwork for future investigations into the broader implications for central nervous system dynamics.

5.7 Discussion

This study aims to explore the morphological changes occurring in astrocyte cells in response to laser-induced shockwaves, providing valuable insights into the dynamics of cellular responses to mechanical stimuli. The motivation behind this research lies in comprehending the microscopic consequences of traumatic events, particularly traumatic brain injury (TBI), at the cellular level. Focusing on astrocytes, the most abundant cells in the cen-

Parameters	P-Value	Sig.	Test Stat.	Mean Diff
Circularity	0.41917	No	9773	0.010624
Volume	5.80E-12	Yes	16267	-398.7118 μm^3
Projected Area	6.72E-05	Yes	7090	114.8401 μm^2
Dry Mass	5.80E-12	Yes	16267	-2.2151 p gr
Surface Area	6.97E-07	Yes	6266	2445.7871 μm^2
Surface Area to Volume Ratio	5.61E-21	Yes	2522	2.8767 $1/\mu\text{m}$
Surface Area to Dry Mass Ratio	5.61E-21	Yes	2522	517.8092 $\mu\text{m}^2/\text{p gr}$
Projected Area to Volume Ratio	1.52E-25	Yes	1636	0.092696 $1/\mu\text{m}$
Sphericity	2.35E-21	Yes	18465	-0.00016332 $\mu\text{m}^{1/3}$
Height Variance	2.18E-17	Yes	17617	-1.9532 μm^2
Height Kurtosis	8.76E-18	Yes	3204	7017.1477 $1/\mu\text{m}^4$
Height Skewness	0.001124	Yes	7705	538.0122 $1/\mu\text{m}^3$
Eccentricity	0.035607	Yes	12229	-0.015711
Perimeter	0.074821	No	8951	47.807 μm
Perimeter to Projected Area Ratio	5.31E-05	Yes	13867	-0.035515 $1/\mu\text{m}$
Complexity Score	0.0015005	Yes	13135	-0.032056

Table 5.4: Statistical parameters for the cells 10 min after LIS Vs. 2 Hr after LIS

tral nervous system, we simulate TBI conditions in vitro by subjecting cells to controlled shear stress through laser-induced shockwaves. For accurate measurement of morphological alterations, we utilize quantitative phase microscopy (QPM). Our specific interest in astrocyte cells stems from their propensity to undergo astrogliosis, a triggered response to injury, with previous studies establishing morphological changes associated with this process. This research framework provides a coherent exploration of how astrocyte cells adapt morphologically to mechanical stress, shedding light on potential implications for understanding cellular responses to traumatic brain injuries.

The experimental setup, involving a control group and a laser-induced shockwave group, ensures a systematic exploration of the effects of shockwaves on astrocyte morphology. For the control group, environmental conditions, such as humidity and CO2 levels, were controlled in order to minimize external factors that could influence cell behavior. Image processing techniques, including phase unwrapping and cell segmentation, and feature extraction contribute to the precision of data analysis. Utilizing the QPM images of astrocyte cells comprehensive

Parameters	P-Value	Sig.	Test Stat.	Mean Diff
Circularity	0.0001094	Yes	7189	0.040521
Volume	0.034178	Yes	12243	-46.707 μm^3
Projected Area	0.082363	No	11921.5	-52.7344 μm^2
Dry Mass	0.034178	Yes	12243	-0.25948 p gr
Surface Area	0.0026958	Yes	12988	-1262.8157 μm^2
Surface Area to Volume Ratio	0.21533	No	9409	0.79084 $1/\mu\text{m}$
Surface Area to Dry Mass Ratio	0.21533	No	9409	142.3511 $\mu\text{m}^2/\text{p gr}$
Projected Area to Volume Ratio	0.33316	No	9638	0.0039881 $1/\mu\text{m}$
Sphericity	0.024559	Yes	8557	3.1541e-05 $\mu\text{m}^{1/3}$
Height Variance	0.057276	No	12060	0.23605 μm^2
Height Kurtosis	0.011555	Yes	8323	3428.1771 $1/\mu\text{m}^4$
Height Skewness	0.85525	No	10609	-42.7731 $1/\mu\text{m}^3$
Eccentricity	0.59318	No	10906	-0.0063078
Perimeter	0.001608	Yes	13118	-80.7606 μm
Perimeter to Projected Area Ratio	0.0019983	Yes	13064	-0.028057 $1/\mu\text{m}$
Complexity Score	1.86E-06	Yes	14480	-0.046889

Table 5.5: Statistical Parameters for the cells 1 min before LIS Vs. 2 Hr after LIS

characteristics of astrocytes were measured, including an average height profile and average of parameters, such as dry mass, volume, and circularity, provide a comprehensive view of astrocyte morphology.

The experiments' protocol, including the control and laser-induced shockwave groups, ensures a systematic and controlled approach to studying astrocyte responses. The use of time-lapse imaging and careful monitoring of environmental conditions enhances the reliability of the data. The results from the control group indicate stability in astrocyte morphology over the two-hour imaging period. This provides a crucial baseline for comparing with the laser-induced shockwave group, where dynamic and sustained changes are observed.

The investigation into astrocyte responses to laser-induced shockwaves (LIS) uncovers immediate and sustained morphological changes, providing insights into dynamic cellular adaptations. Following LIS, astrocytes rapidly transition to a more circular shape, accompanied by changes in volume, surface characteristics, and height features. A 2-hour post-LIS anal-

Parameters/Time after LIS	1"	10"	20"	40"	60"	80"	100"	120"
Circularity	1	1	1	1	1	1	1	1
Volume	0	1	1	1	0	0	1	1
Projected Area	1	1	1	1	1	1	1	0
Dry Mass	0	1	1	1	0	0	1	1
Surface Area	1	1	1	1	1	1	1	1
Surface Area to Volume Ratio	1	1	1	1	1	1	0	0
Surface Area to Dry Mass Ratio	1	1	1	1	1	1	0	0
Projected Area to Volume Ratio	1	1	1	1	1	1	0	0
Sphericity	1	1	1	1	1	1	1	1
Height Variance	1	1	1	1	1	1	0	0
Height Kurtosis	1	1	1	1	1	0	0	1
Height Skewness	1	1	1	1	1	1	1	0
Eccentricity	0	0	0	0	0	0	0	0
Perimeter	1	1	1	1	1	1	1	1
Perimeter to Projected Area Ratio	0	0	0	0	0	1	1	1
Complexity Score	0	0	0	1	1	1	1	1

Table 5.6: Significance table of changes for each feature at various time points (in minutes) compared to 1 min before LIS

ysis reveals persistent alterations, suggesting ongoing cellular adjustments and regulatory processes. Statistical analysis highlights significant changes in circularity, volume, surface area, and other features, emphasizing dynamic responses to mechanical perturbation. Mann-Kendall Tau-b test results confirm the robustness of observed trends. Surface area dynamics suggest a cellular response to enhance chemical interactions, while increased projected area and area-to-volume ratio indicate flattening in response to LIS. Overall, these morphological changes signify astrocytes' multifaceted and adaptive nature, enhancing our understanding of their responses to mechanical stress and paving the way for further exploration into molecular mechanisms and broader implications for the central nervous system. Biologically, these findings indicate a remarkable plasticity in astrocytes, actively adapting to mechanical stressors. The sustained changes in various features suggest intricate molecular and cellular mechanisms at play. Further investigations into mechanotransduction pathways are crucial to unravel the underlying processes driving these observed adaptations.

Parameters	Tau-b	P-Value	Trend
Circularity	0.3333	0.4524	No significant trend detected
Volume	-1	0.0085	Significant trend detected
Projected Area	1	0.0085	Significant trend detected
Dry Mass	-1	0.0085	Significant trend detected
Surface Area	1	0.0085	Significant trend detected
Surface Area to Volume Ratio	1	0.0085	Significant trend detected
Surface Area to Dry Mass Ratio	1	0.0085	Significant trend detected
Projected Area to Volume Ratio	1	0.0085	Significant trend detected
Sphericity	-1	0.0085	Significant trend detected
Height Variance	-1	0.0085	Significant trend detected
Height Kurtosis	1	0.0085	Significant trend detected
Height Skewness	0.4667	0.2597	No significant trend detected
Eccentricity	-0.4667	0.2597	No significant trend detected
Perimeter	0.6	0.1329	No significant trend detected
Perimeter to Projected Area Ratio	-0.7333	0.0603	No significant trend detected
Complexity Score	-0.8667	0.0242	Significant trend detected

Table 5.7: Mann-Kendall Tau-b Test Results. This test shows whether there is a significant trend for a feature over the period of 20 minutes to 2 hours after LIS.

In conclusion, laser-induced shockwaves induce dynamic and lasting morphological changes in astrocytes, shedding light on the intricate cellular responses to mechanical stimuli.

Overall, the scientific rigor, systematic approach, and thorough analysis presented in this chapter contribute to the advancement of knowledge in the field of astrocyte biology and cellular responses to mechanical stimuli. The findings have implications for traumatic brain injuries and provide a foundation for future research in this critical area of neuroscience.

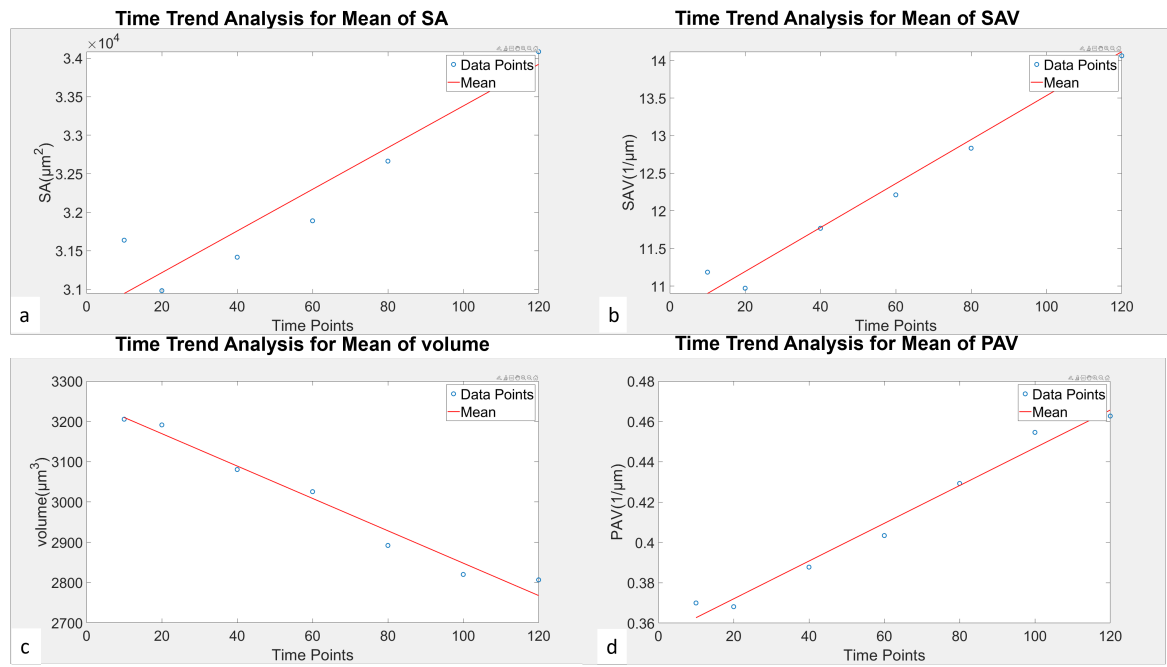


Figure 5.2: Time Trend Analysis for the mean value of various features. The time points show the time after LIS in minutes.

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Appendix A

Supplementemtry Codes

A.1 ImageJ Macro Code for Batch Processing

The cropped images, each containing one cell at a certain time point are then batch-processed using the following code in ImageJ, to create masks:

```
run("Auto_Local_Threshold", "method=Median_radius=55  
parameter_1=0_parameter_2=0_white");  
setOption("BlackBackground", true);  
run("Erode");  
run("Dilate");
```

A.2 Matlab Code for Converting Interference Images to Heightmaps

```
clear all  
close all
```

```

dish=17;
imagenum=10;
% step1 : phase shifting
for i=1:imagenum
    fileorig= strcat(num2str(i),'.tif');
    nframe = 30;
    numImg = 1;
    lambda = 0; % Define the threshold for SPUD
    paraboloid_coeff = -19; % Please edit here to get a flattened image;
    imtemp = imread(fileorig,1);
    [xSize ySize] = size(imtemp);
    inputImg = zeros(xSize,ySize,nframe);
    h = figure;
    for nn = 1:numImg
        for ii = 1:nframe
            imtemp = double(imread(fileorig,ii+(nn-1)*nframe));
            inputImg(:, :, ii) = imtemp;
        end
        comp = fringes2phase(inputImg);
        xMat = repmat((1:xSize)'-xSize/2,1,ySize);
        yMat = repmat((1:ySize)-ySize/2,xSize,1);
        comp_correct = comp .* exp(1i*paraboloid_coeff*...
            (xMat.*xMat+yMat.*yMat)/(1000*1000));
        % figure (2); imagesc(angle(comp_correct));
        comp_oversample = zeros(xSize,ySize);
        comp_small = comp_correct(1:4:end,1:4:end);
        comp_oversample(xSize*3/8:xSize*3/8+xSize/4-1,...
            ySize*3/8:ySize*3/8+ySize/4-1) = comp_small;
        fft3 = abs(fftshift(fft2(comp_oversample)));
    end

```

```

[xtmp ytmp] = find(fft3==max(fft3(:)));
xDiff = (xtmp - (xSize/2+1))/4;
yDiff = (ytmp - (ySize/2+1))/4;
comp_correct2 = comp_correct .*...
exp(-1i*2*pi*(xDiff*xMat/xSize+yDiff*yMat/ySize));
comp_correct2 = comp_correct2 ./ sum(comp_correct2(:));
phaseOutput(:, :, nn) = angle(comp_correct2);
psi = double(phaseOutput(:, :, nn));
psi = 2*pi./(max(psi(:)) - min(psi(:))) .*(psi - min(psi(:)) - pi);
imagesc(psi)
end

% Golstein phase unwrapping
IM= comp_correct2;
im_mag = abs(IM); %Magnitude image
im_phase = angle(IM); %Phase image

% Replace with your mask (if desired)
im_mask = ones(size(IM));
mag_max = max(im_mag(:));
%indx1 = find(im_mag < 0.1*mag_max);
%im_mask(indx1) = 0;
if(~exist('im_mask', 'var'))
    im_mask = ones(size(IM)); %Mask (if applicable)
end

% Compute the residues
residue_charge = PhaseResidues_r1(im_phase, im_mask);
%figure; imagesc(residue_charge), colormap(gray), axis square,...
axis off, title('GS_Phase_residues_(charged)'); colorbar;

```

```

% Compute the branch cuts
max_box_radius=floor(length(residue_charge)/2);
%max_box_radius=4 % Maximum search box radius (pixels)
if(~exist('max_box_radius','var'))
    max_box_radius=4; % Maximum search box radius (pixels)
end

branch_cuts = BranchCuts_r1(residue_charge, max_box_radius, im_mask);
%figure; imagesc(branch_cuts), colormap(gray), axis square,...
axis off, title('GS_Branch_cuts');
colorbar;

im_mask(branch_cuts) = 0;
im_mag1 = im_mag.*im_mask;

%Manually (default) or automatically identify starting seed point
if(1) % Chose starting point interactively
    im_phase_quality = im_mag1;
    minp = im_phase_quality(2:end-1, 2:end-1); minp = min(minp(:));
    maxp = im_phase_quality(2:end-1, 2:end-1); maxp = max(maxp(:));
    figure;
    imagesc(im_phase_quality,[minp maxp]), ...
    colormap(gray), colorbar, axis square, axis off;
    title('Phase_quality_map');

    [xpoint,ypoint] = ginput(1);
    colref = round(xpoint);
    rowref = round(ypoint);

    close; %Close the figure;
else % Chose starting point = max. intensity

```

```

[r_dim , c_dim]=size(im_phase);
im_mag1(1,:) = 0;
im_mag1(r_dim,:) = 0;
im_mag1(:,1) = 0;
im_mag1(:,c_dim) = 0;
[rowrefn,colrefn] = find(im_mag1 >= 0.99*mag_max);
rowref = rowrefn(1);
colref = colrefn(1);
end
% Unwrap
if(exist('rowref','var'))
    im_unwrapped(:,:,i) = FloodFill_r1(im_phase, im_mag, branch_cuts, ...
    im_mask, colref, rowref); % Flood fill phase unwrapping
else
    im_unwrapped(:,:,i) = FloodFill_r1(im_phase, im_mag, ...
    branch_cuts, im_mask); % Flood fill phase unwrapping
end
resultss= im_unwrapped(:,:,i);
nan_indices = find(isnan(resultss));

% Replace NaN elements with the mean of the four surrounding elements
for k = 1:length(nan_indices)
    [row, col] = ind2sub(size(resultss),...
    nan_indices(k));
    surrounding_elems =resultss(max(1, row-1):...
    min(size(resultss,1), row+1),...
    max(1, col-1):min(size(resultss,2), col+1));
    surrounding_mean = mean(surrounding_elems...
    (~isnan(surrounding_elems)));

```

```

    resultss(row, col) = surrounding_mean;
end

im_unwrapped(:,:,i)= resultss;
% Display results
%figure;

imagesc(im_unwrapped), colormap(gray), colorbar, axis square,...
axis off, title('GS_Unwrapped_phase');
results(:,:,i)= im_unwrapped(:,:,i).*633/(4*pi*0.033*1000);
OPD(:,:,i)= results(:,:,i)*0.033*10^(-6);
Phase(:,:,i)= im_unwrapped(:,:,i);
resultss=results(:,:,i);
figure('WindowState','maximized');
imagesc(resultss), colormap(jet), colorbar, axis square,...
caxis([-1 30]), title('GS_thickness');
saveas(gcf,'thickness_' + string(fileorig));
I2 = 255*(resultss - min(results(:)))...
./ (max(resultss(:)) - min(resultss(:)));
I2 = cast(I2,'uint8');
imwrite(I2,'myImage_' + string(fileorig));
close all
end

save(strcat('results', num2str(dish), '.mat'),'results','-mat')
save(strcat('phase', num2str(dish), '.mat'),'im_unwrapped','-mat')
save(strcat('OPD', num2str(dish), '.mat'),'OPD','-mat')

```

A.3 MATLAB Code for Cropping the cells and Making the Final Masks

```
%%Cellcrop:
clear all,
close all,
load results17.mat
dish=17;
LIScent=imread("LIScent.tiff");
save('LIScent.mat','LIScent','-mat')
pixelsize=0.35;
filename = 'Results17.csv';
% Read the CSV file
data = readtable(filename);
% Get unique slice numbers
sliceNumbers = unique(data.Slice);
% Initialize the matrix
matrixSize = size(data, 1);
numColumns = 4; % X, Y, XM, YM
Imcropmatrix = zeros(matrixSize/size(sliceNumbers,1),...
numColumns, numel(sliceNumbers));

% Loop through each slice number
for i = 1:numel(sliceNumbers)
    sliceData = data(data.Slice == sliceNumbers(i),...
    {'BX', 'BY', 'Width', 'Height'});
    Imcropmatrix(1:size(sliceData, 1), :, i)...
    = table2array(sliceData);
end
```



```

imshow(LIScent)

[xpoint,ypoint] = ginput(1);
for k=1: size(Imcropmatrix,1)
for im=1: size(sliceNumbers,1)
    celldist(k)= sqrt((Imcropmatrix(k,1,im)+ ...
    Imcropmatrix(k,3,im)/2- xpoint)^2+...
    (Imcropmatrix(k,2,im)+ Imcropmatrix(k,4,im)/2- ypoint)^2)*pixelsize;
    my_im = strcat('s',num2str(im),'_',num2str(k));
    D= results(:, :, im);
    orig3Dim.(my_im) = imcrop(D,Imcropmatrix(k, :, im));
    I = 255*( orig3Dim.(my_im) - min( orig3Dim.(my_im)(:))) ./ ...
    (max( orig3Dim.(my_im)(:)) - min( orig3Dim.(my_im)(:)));
    I = cast(I, 'uint8');
    imwrite(I, 'grayim'+ string(im)+'_' + string(k) +'.tif')
end
end

save(strcat('orig3Dim', num2str(dish), ...
'.mat'), 'orig3Dim', '-mat')
save(strcat('Imcropmatrix', num2str(dish), ...
'.mat'), 'Imcropmatrix', '-mat')
save(strcat('celldist', num2str(dish), ...
'.mat'), 'celldist', '-mat')
save(strcat('deadcropmatrix', num2str(dish), ...
'.mat'), 'deadcropmatrix', '-mat')

```

A.4 MATLAB Code for Feature Extraction

```
clear all
```

```

close all

pixel_size = 0.35 ;% Change this value if necessary
dx=pixel_size;
dy=pixel_size;
alpha= 0.18;

features = { 'circ', 'voldivarea', 'flatness',...
'roundness', 'volume', 'PA', 'drymass',...
'SA', 'SAV', 'SDM', 'PAV', 'Sphericity',...
'HV', 'KT', 'SK', 'Ecc', 'perim', 'perimdivarea' };

Units = { '1', [ '1/' char(181) 'm' ], '1', ...
[ '1/' char(181) 'm' ], [ char(181) 'm^3' ], ...
[ char(181) 'm^2' ], 'p-gr', [ char(181) 'm^2' ], ...
[ '1/' char(181) 'm' ], [ 'p-gr/' char(181) 'm^2' ] ...
..., [ '1/' char(181) 'm' ], [ char(181) 'm^(1/3)' ], ...
[ char(181) 'm^2' ], [ '1/' char(181) 'm^4' ], ...
[ '1/' char(181) 'm^3' ], '1', [ char(181) 'm' ], ...
[ '1/' char(181) 'm' ] };

datanum=222;

for i = 12:datanum

filename = strcat('Imcropmatrix', num2str(i), '.mat');

if isfile(filename)

load(filename);

filename = strcat('orig3Dim', num2str(i), '.mat');

load(filename);

filename = strcat('finalmask', num2str(i), '.mat');

load(filename);

filename = strcat('bgc3D', num2str(i), '.mat');

load(filename);

for k=1: size(Imcropmatrix,1)

```

```

for im=1: size(Imcropmatrix,3)
my_im = strcat('s',num2str(im),'_',num2str(k));
Z=zeros(size(bgc3D.(my_im)));
Z(find(finalmask.(my_im)==1))= bgc3D.(my_im)...
(find(finalmask.(my_im)==1));
nc= size(find(finalmask.(my_im)==1),1);
[m, n] = size(Z);
volume(k,im)= sum(sum(Z)).* pixel_size ^ 2;
PA(k,im)=sum(sum(finalmask.(my_im))).* pixel_size ^ 2;
voldivarea(k,im)= volume(k,im)./PA(k,im);
labeled_image1=finalmask.(my_im);
cell_props31= regionprops3(labeled_image1,Z, ...
{'Volume', 'SurfaceArea', 'Centroid', 'WeightedCentroid'});
cell_props1= regionprops...
(labeled_image1,Z, ...{'Area', 'Centroid', 'WeightedCentroid', ...
"MaxFeretProperties", "MinFeretProperties", 'Circularity'});
min_diameter = cell_props1.MinFeretDiameter;
max_diameter = cell_props1.MaxFeretDiameter;
flatness(k,im) = max_diameter / min_diameter;
circ(k,im)=cell_props1.Circularity;

drymass(k,im)= 1/alpha * volume(k,im)/1000;
[delta_hx, delta_hy] = gradient(double(Z));
integrand = sqrt(1 + (delta_hx .* pixel_size).^2 +...
(delta_hy .* pixel_size).^2);
SA (k,im)= sum(integrand(:))+ PA(k,im);
roundness(k,im) = (4 * pi * volume(k,I'm)) ...
/ SA(k,im)^2;
SAV(k,im)= SA (k,im)/volume(k,im);

```

```

SDM(k,im)=SA (k,im)/drymass(k,im);
PAV(k,im)=PA(k,im)/volume(k,im);
Hmean(k,im)= sum(sum(Z))/nc;
Sphericity(k,im)=pi^(1/3)*(6* Hmean(k,im))^(2/3)...
/SA(k,im);
HV(k,im)=1/(nc-1)* sum(sum((Z(find(finalmask.(my_im)...
==1))- Hmean(k,im)).^2));
KT(k,im)= sum(sum((Z(find(finalmask.(my_im)==1))...
- Hmean(k,im)).^4)/HV(k,im)^4);
SK(k,im)= sum(sum((Z(find(finalmask.(my_im)==1))...
- Hmean(k,im)).^3)/HV(k,im)^3);
Ecc(k,im)= (max_diameter-min_diameter)/...
(max_diameter+min_diameter);
boundaries = bwboundaries(finalmask.(my_im));
outerBoundary = cell(1, 1);

% Find the longest boundary (outer boundary)
maxPerimeter = 0;
for h = 1:length(boundaries)
    boundaryCoords = boundaries{h};
    perimeter = sum(sqrt(diff(boundaryCoords(:, 1)).^2...
+ diff(boundaryCoords(:, 2)).^2));

    if perimeter > maxPerimeter
        maxPerimeter = perimeter;
        outerBoundary{1} = boundaryCoords;
    end
end

```

```

% Calculate the total protrusion length based on the outer boundary
perim(k,im) = maxPerimeter;
perimdivarea(k,im)=maxPerimeter/PA(k,I'm);

end

end

    for f=1:numel(features)
variableName = features{f};

    % Save the variable
    save(strcat(variableName, num2str(i), '.mat'), variableName, '-mat');
    % Clear the variable
    eval(['clear_', variableName]);
    end

end

end

```