



ADDIS ABABA UNIVERSITY
ADDIS ABABA INSTITUTE OF TECHNOLOGY

MASTER THESIS

**Segmentation of Sonicated Blood Cells and Ultrasound
Contrast Agent Microbubbles from Time-Lapse
Microscopic Images**

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for the degree of Master of Science*

in

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(Imaging and Instrumentation Stream)
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Addis Ababa Institute of Technology
Addis Ababa University

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I, Mohammed Aliy, declare that this thesis titled, “Segmentation of Sonicated Blood Cells and Ultrasound Contrast Agent Microbubbles from Time-Lapse Microscopic Images” and the work presented in it are my own. I confirm that:

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Name (Advisor)	Signature	Date

Chief of department or graduate program coordinator

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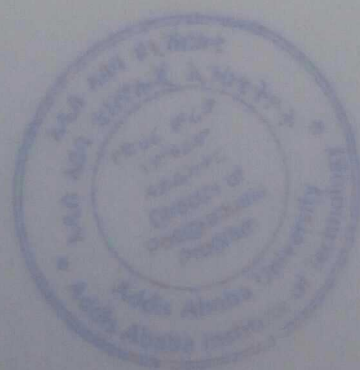
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“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein

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Addis Ababa Institute of Technology

Abstract

Center of Biomedical Engineering
Medical Imaging and Instrumentation Stream

Master of Science

Segmentation of Sonicated Blood Cells and Ultrasound Contrast Agent Microbubbles from Time-Lapse Microscopic Images

by Mohammed Aliy

Blood cells and ultrasound contrast agent microbubbles behave strangely when subjected to an ultrasound field. They migrate towards the nodes of the ultrasound wave and form circular patterns. However, differentiation between blood cells and ultrasound contrast agent microbubbles can be easily determined via time-lapse analysis of the pattern formation. Ultrasound contrast agent microbubbles migrate towards the wave nodes very quickly and form tightly packed clusters. In contrast, formation of tightly packed clusters in the case of blood cells is unlikely based on the sonophore model theory of cells. Moreover, the interaction of the microbubbles with the blood cells and the surrounding medium is not fully understood. To study the behavior and interaction of sonicated blood cells and microbubbles on the time-lapse microscopic images, there is a need to define a contour around the cells and the microbubbles. To do so, first, the microbubbles and the cells should be segmented from the rest of image content. In this regard, this thesis devised a scheme that combines features of the Laplacian of the Gaussian detector and a modified form of the Contrast Limited Adaptive Histogram Equalization (CLAHE) technique for effective analysis of time-lapse microscopic images. The scheme is tested on three datasets (one synthetic and two real) and its subjective and objective performance is found quite pleasing. Absence of ground truth for the real datasets makes the evaluation of the segmentation scheme merely subjective. Objective evaluation is only performed on the computer-simulated time-lapse images. The average segmentation sensitivity, specificity and accuracy of the proposed algorithm are valued around 0.96, 0.91 and 0.95 on the synthetic dataset out of a unit scale, respectively. The result generated could be a crucial input for effective particle tracking and sizing studies.

Keywords: Segmentation, Red Blood Cell, Ultrasound, Microbubbles, Image Processing, Contrast Agent, LoG, CLAHE

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List of Abbreviations

2D	Two Dimensional
3D	Three Dimensional
4D	Four Dimensional
AD	Analog to Digital
AHE	Adaptive Histogram Equalization
CCD	Charge Coupled Device
CEUS	Contrast Enhanced Ultrasound
CHT	Circular Hough Transform
CLAHE	Contrast Limited Adaptive Histogram Equalization
CMOS	Complementary Metal Oxide Semiconductor
CPU	Central Processing Unit
CRT	Cathode Ray Tube
CT	Computed Tomography
DoG	Difference of Gaussian
DoH	Difference of Hessian
DSI	Dice Similarity Index
FN	False Negative
FP	False Positive
GMM	Gaussian Mixture Model
GPU	Graphics Processing Unit
GT	Ground Truth
GUI	Graphical User Interface
HE	Histogram Equalization
HSL	Hue Saturation Luminance
IDE	Integrated Development Environment
IEEE	Institute of Electrical and Electronics Engineers
JI	Jaccard Index
LoG	Laplacian of Gaussian
LUT	Look-Up Table
MI	Mechanical Index
MRFM	Markov Random Field Models
MRI	Magnetic Resonance Imaging
PET	Positron Emission Tomography

PhD	Doctor of Philosophy
RBC	Red Blood Cells
RGB	Red Green Blue
ROI	Region of Interest
RSF	Region Scalable Fitting
SEM	Scanning Electron Microscope
SNR	Signal to Noise Ratio
SONAR	Sound Navigation and Ranging
SPECT	Single Photon Emission Computed Tomography
SPM	Scanning Probe Microscope
TEM	Transmission Electron Microscope
TN	True Negative
TNR	True Negative Rate
TP	True Positive
TPR	True Positive Rate
USCA	Ultrasound Contrast Agent
USCAM	Ultrasound Contrast Agent Microbubbles
WBC	White Blood Cells

1

Introduction

1.1 Background

Nearly three and half centuries have passed since Antony Van Leeuwenhoek first established a benchmark for biological imaging by discovering “little animals,” present-day microbes, using his microscopes. His breakthrough paved a road for scientists and biologists to study tiny structures of living things. At that time, Leeuwenhoek’s main curiosity was to magnify his “little animals,” protozoa and bacteria, and study their structure, and he was the first one to see and describe sperm and blood cells (Croft, 2006; Dobell and van Leeuwenhoek, 1960). Since then, scientists and technologists have devoted and carried out remarkable works to magnify and study micro (small) level events and macro (large) level objects and structures.

Micro level imaging technologies such as Scanning Probe Microscope (SPM), acoustic microscope, Transmission Electron Microscope (TEM) and Scanning Electron Microscope (SEM) help scientists and researchers to study immensely smaller resolution structures or objects (Croft, 2006). On the other hand, macro level imaging technologies such as X-ray, Computed Tomography (CT), Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), Ultrasound and Single Photon Emission Computed Tomography (SPECT) help the physicians and researchers to understand the anatomy and physiology of normal and abnormal internal hidden organs/tissues of human being (Dhawan, 2011). As well, macro level imaging technologies such as astrological telescopes help cosmologists to see distant cosmic objects (Russ, 2016).

After the World War II, a new imaging technique called ultrasound emerged as a treatment and diagnostic tool. The Currie brothers' discovery of piezoelectricity makes the imaging application of ultrasound more fascinating (Waingankar et al., 2015). Today, ultrasound is used to see the internal organs/tissues of a human being in real time. The basic working principle behind the ultrasound imaging device is as follows: a sound wave is sent to the target organ, the organ reflects or scatters back the part of the sound wave back to the source or detector. This reflected back ultrasound beam is used to construct the image of the targeted organ or tissue (Sehmbi and Perlas, 2015; Hendee et al., 2003).

To generate an image which has a good contrast, the organ or the tissue needs to be a good reflector or scatterer. Otherwise, it's necessary to administer contrast enhancing microbubble spheres prior to ultrasound imaging. Contrast enhancing microbubble spheres are good for reflecting or scattering ultrasound wave. Studies also show the promising therapeutic aspects of the ultrasound contrast agent microbubble spheres, and even today, it remains an active research area; their interaction with ultrasound wave and cells at a micro level needs a careful study to examine their pros and cons (Postema, 2017). Therefore, it's ideal to set up a microscopic laboratory to capture, store and study the activities of the spheres.

The revolution of digital and information technology after the second half of the 20th century dominates the traditional analog way of imaging and simplifies how images are acquired (both in time and process), processed, stored, displayed and transmitted from one place to the other (Trussell and Vrhel, 2008). For example, studying microscopic level cell activities would be impossible without the the presence of high frame rate cameras. Some cellular activities may begin and end within a fraction of microseconds and would be challenging to be captured by the human visual system. Or, some cellular activities may take days, weeks even months to happen; these are also difficult for a human to sit and watch all the activities for a longer time patiently. Or, if the images are not captured and stored, it will be necessary to set up a larger number of experiments to study a single cellular/ molecular activity. To alleviate the challenges, nowadays most molecular and cellular laboratory microscopes have been integrated with high frame rate cameras. These high speed cameras will help the researcher to capture the photographs of cellular or molecular micron-scale activities that occur within a fraction of second or a longer period of time and store them for off-line studies.

Not only these laboratory microscope mounted cameras but also other macro and micro scale imaging technologies/modalities produce immensely large quantities of digital images within a short period of time. All the acquired digital images may not contain useful information and worth to store, display or transmit from one place to the other. Thanks to the emergence of digital image processing, it has been a great relief for professionals who work in the field of computer vision system to use the acquired images effectively and resources efficiently (Dhawan, 2011; Marques, 2011).

Digital image processing is a manipulation of digital images using computers for the sake of improving the appearance of the image for human vision, making them suitable for storage

and transmission and shaping them for measurement and analysis (essential for digging the information they contain) (Marques, 2011; Russ, 2016). To achieve these tasks, there is no clearly stated process flow. The process flow varies from one application to another application. It mostly relies on the knowledge of the computer vision expert. For common understanding though, digital image processing is broadly classified into three categories; low-, mid- and high-level. Low-level digital image processing comprises all preprocessing operations such as contrast enhancement and noise reduction. Segmentation and extraction of attributes of the image such as creating contours or drawing edges around the region of interest on the image is categorized under mid-level digital image processing. The high-level is the most advanced level. It helps the computer vision experts to take measurements, analyze and interpret the contents of the image (Marques, 2011).

The main purpose of this thesis work is to find an efficient algorithm that segments/detects ultrasound contrast agent microbubble spheres and blood cells that are disturbed by ultrasound field from a series of photographs. The photographs are captured using a high speed digital camera, WAT 902HS Charge Coupled Device (CCD) camera (Watec Co., Ltd., Yamagata, Japan).

1.2 Statement of Problem

Experimental results from previous work by Mazzawi et al. (2015) showed that sonication of mixture of blood, polystyrene micro-spheres and ultrasound contrast agent forces particles and cells to migrate towards the nodes of the sound field. Particles and cells create a circular pattern as shown on the top row of Figure 1.1. When the sonication field is halted the particles/cells trapped at the nodes remain there and the rest wobble inside the ring. All blood cells, ultrasound contrast agent and polystyrene micro-spheres are responsible for the circular pattern formation.

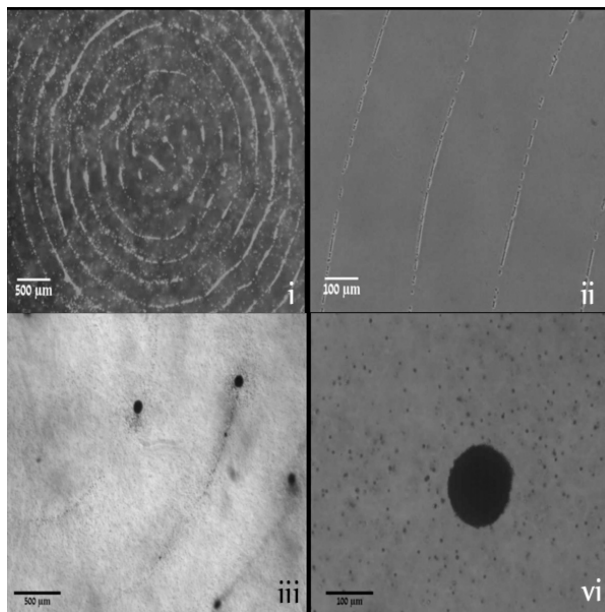


FIGURE 1.1:
Blood cells arranged in concentric circles inside the ring transducer after sonication, observed at $2\times$ (i) and $10\times$ (ii), and formation of tightly packed microbubble clusters at the nodes, when observed at $2\times$ (iii) and $10\times$ (iv) magnification (Mazzawi et al., 2015).

Looking separately, ultrasound contrast agent microbubbles behave differently. They accumulate in circles at the nodes faster than blood cells and polystyrene micro-spheres and create a strong clusters as illustrated on the bottom row of Figure 1.1 which is not possible in blood cells and polystyrene particles due to weak Bjerknes forces, i.e. sonophore model of cells does not allow strong cluster formation.

In addition to translation and cluster formation, the microbubbles show other non-linear interactions such as jetting, fragmentation, coalescence and cracking when subjected to ultrasound energy. Moreover, they pull, push or shear the nearby cell membrane to release their content when they act as drug or gene delivery agent (Postema, 2017; Postema et al., 2003).

The sequential photographs of such interactions can be captured using a high frame-rate camera mounted on a microscope for offline investigation. Having only the photographs is not enough to analyze the effect of ultrasound field on the contrast agent microbubble spheres, blood cells and cluster formation. The particles (the contrast agent microbubbles, the blood cells and the clusters) need to be separated from the rest of the photograph content using some kind of digital image processing algorithm.

1.3 Objectives

1.3.1 General Objective

The main objective of this thesis is to devise an efficient object identification and segmentation technique for time-lapse microscopic images of near-endothelial lining, highly dense blood cells and ultrasound contrast agent microbubbles that are disturbed by ultrasound field.

1.3.2 Specific Objectives

The specific objectives of the thesis are

1. To review high density cell or particle segmentation techniques that are used in cell or particle tracking projects.
2. To rank the best segmentation algorithm based on rigorous literature review.
3. To test the selected segmentation scheme on the simulated and real dataset used in the thesis work.
4. To propose a modified scheme addressing challenges with existing tracking algorithms.

1.4 Significance of the Study

The segmentation scheme proposed in this thesis work plays a crucial role for effective particle tracking and sizing. The microbubbles and the cells need to be linked from one frame to the other and expressed statistically. The backbone of any particle tracking algorithm in computer vision system is particle segmentation or detection. The thesis is one segment of the quest to study the interaction of sonicated ultrasound contrast agent microbubbles and blood cells with their surroundings.

1.5 Organization of the Thesis

This thesis is segregated into seven chapters, in sequential order, to help the reader to grasp and understand the work easily. From **Chapter 1**, the reader will get the overall background, the base problem, the purpose and the significance of the research work. On **Chapter 2**, ultrasound field concepts that are essential to the project (historical background, physics, interaction with biological medium and application in medicine) will be discussed. **Chapter 3** will give an explanation about blood cells, ultrasound contrast agents and effect of sound field on blood cells and ultrasound contrast agents. The next chapter, **Chapter 4**, is all about digital image processing and micron scale segmentation techniques. The research methodology, CLAHE-enhanced Laplacian of Gaussian, is elaborated in detail within **Chapter 5**. Inside **Chapter 6**, all the research achievements will be found with adequate explanation. The last chapter, **Chapter 7**, summarizes all the chapters prior to it, discusses the strong sides of the project, points out the limitations and lists future recommendations. To make the reader fully satisfied, some mathematical formulas, tables and codes are included in the form of Appendix at the end of the document.

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2

Ultrasound Field

The discovery of the ultrasound wave has set its footprints on the technological advancements of the era starting from the first quarter of the 19th century, specifically, in the areas of sound navigation and ranging (SONAR) and medicine. This chapter is included to give a comprehensive summary of ultrasound field and its application in medicine. Inside the chapter, historical background, physics fundamentals, artificial production, biological medium interaction and medical applications of ultrasound field are addressed.

2.1 History

The discovery of ultrasound dated back to 1794 when Italian biologist, Lazzaro Spallanzani, proved the ability of bats finding their flying path and prey through sending high frequency inaudible sound and hearing the reflected back echo. The process of finding the location of an object by sending a sound wave and hearing the reflected back echo is termed as echolocation (Dijkgraaf, 1960). After Lazzaro's demonstration of echolocation the Swiss physicist, Jean-Daniel Colladon, determined the speed of sound inside a water and measured the distance of an object using underwater sound wave. Indeed, he is the pioneer to flourish a bright future for sound physics and its application. Thereafter, scientists and researchers did a considerable work about sound vibration, transmission, propagation and reflection. Lord Rayleigh, researcher from England, is one of them who published a paper titled 'The Theory of Sound' in 1877. His paper gives a mathematical description about sound wave (Report of World Congress on Ultrasound in Obstetrics and Gynecology, 2013; Newman and Rozycki, 1998).

In 1876, the English scientist, Francis Galton, showed how to generate extremely high frequency inaudible sound waves via his invention, the Galton whistle (Baker, 2011). But the astonishing breakthrough happened in Paris, France in 1880 when the Curie brothers, Pierre Curie and Jacques Curie, noticed the piezoelectric effect of some crystals. The Curie brothers applied a mechanical pressure such as ultrasound wave on a quartz crystal and produced an electric potential. The process of applying mechanical pressure on a matter and producing electrical energy in response is termed as piezoelectricity (Waingankar et al., 2015; Hendee et al., 2003; Newman and Rozycki, 1998).



FIGURE 2.1:
Photograph of Curie brothers: Jacques Curie (left) and Pierre Curie (right)
(Madame S . Zak Lehrer, 1982).

A year later, in 1881, Gabriel Lippman showed mathematically the reverse of piezoelectric effect is also true, producing a mechanical stress on a crystal by applying electrical energy. Immediately, Curie brothers also confirmed his suggestion. The process of applying electrical potential on a matter and producing a mechanical stress/pressure in response is termed as converse piezoelectricity. Then after, tremendous amount of research has been done to exploit the application of piezoelectricity, converse piezoelectricity and ultrasound waves (Waingankar et al., 2015).

In the 1920s, Langevin found the devastation of schools of fishes in the sea when subjected to high intensity ultrasound field. He also felt the pain when he inserted his hand into an insonated water tank. This was, probably, the time when researchers understood the therapeutic medical application of ultrasound waves.

In the 1930s, researchers clearly understood the heating and disruptive effect of ultrasound energy and started to use it for therapeutic purpose to treat cancer and give physical therapy for different illnesses. In the 1940s, physicists and SONAR engineers advanced the application of ultrasound in medicine from therapeutic to diagnostic purposes. Physicist Dussik and his brother published an experimental paper in 1942, detailing an ultrasound transmission technique to locate a brain tumor and cerebral ventricles. Their result could be considered the first in the area of medical imaging using ultrasound energy (Newman and Rozycki, 1998).

Following Dussik's experimental work, ultrasound changed the imaging technology dramatically. In 1949, John Wild, utilized ultrasound to measure the thickness of the bowel to

decide if surgery is need or not. He was also the first to show the capability of the first generation of ultrasound, amplitude mode (A-mode) ultrasound, to tell whether a tissue is normal or cancerous. Later, in collaboration with engineer John Reid, he built a brightness mode (B-mode) medical echography device to display the location and reflectivity of an organ/tissue. In the 1950s, A-mode ultrasound became a tool in obstetrics and gynecology departments (Newman and Rozycki, 1998).

After the second half of the 19th century, different varieties of medical imaging ultrasounds have been designed. To name some of them; motion mode ultrasound (M-mode) and Doppler ultrasound are the common ones. The improvement of digital technology has played a great role on ultrasound imaging devices to render information in 3D and even in 4D form (Haidekker, 2013). Table 2.1 shows historical timeline of the ultrasound imaging device in parallel with advancement of the digital technology (Souquet and Bercoff, 2011).

Innovation	Period	Technology Enabler
Real time imaging	1970s	Microprocessor
Doppler imaging	1980s	Digital signal processing
Compound and harmonic imaging	1990s	Low cost AD converters
Portable devices	2000s	Miniaturization
Ultrafast imaging	2010s	GPU ^a and multi-core CPU ^b

TABLE 2.1: Technological advancement and ultrasound imaging technology over the timeline.

^aGPU - Graphics Processing Unit

^bCPU - Central Processing Unit

2.2 Wave Physics

Waves are classified into two broad categories depending on their transmission: mechanical waves and electromagnetic waves. The former needs a medium (gas, solid or fluid) to travel from one place to the other. The other, electromagnetic waves, can travel from one place to the other without any transmitting medium, i.e. they can travel through a vacuum. Sound is grouped under the erstwhile category, mechanical wave. It needs a transmitting medium to travel from one destination to the other.

Mechanical waves can be further classified as longitudinal and transversal waves depending on their propagation angle. If the propagation of the wave is parallel to the motions of the molecules of the medium, then the wave is termed as longitudinal wave, illustrated on Figure 2.2. This kind of mechanical wave is common in solids such as bone. The other mechanical wave, transverse wave, occurs when the motion of the molecules is perpendicular to the propagation of the wave and mostly take in gases, liquids and plasmas. Figure 2.3 depicts transversal wave.

Like any other wave, the ultrasound field exerts a force or pressure on matter. The pressure and force of sound wave are directly proportional to the square of its amplitude. Assume an ultrasound force is applied on a medium that is at equilibrium. Before the exertion of the force, the molecules of the medium are evenly distributed. When the sound energy is applied, the molecules of the medium start to concentrate near to the source of the energy and thereby creating high pressure near the area. This area is known as the zone of compression, see Figure 2.2. Immediately, the compressed and concentrated molecules start to migrate away from the source and create a low molecule density near to the energy source. The neighboring molecules move towards the less dense energy node to maintain the molecule distribution equilibrium by pushing the low density region away from the source. This moving low density molecules create an area which is known as the zone of rarefaction, illustrated on Figure 2.2. One cycle of an ultrasound wave constitutes one zone of compression and one zone of rarefaction.

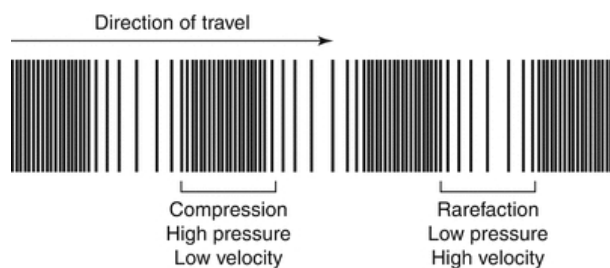


FIGURE 2.2:
Illustration of longitudinal wave and
ultrasound wave compression and
rarefaction (Sehmbi and Perlas, 2015).

Like other wave types, ultrasound has its own distinguishing characteristics: amplitude, velocity, wavelength and frequency. A distance covered by one ultrasound cycle is called the wavelength and represented by a symbol lambda, λ , illustrated on Figure 2.3. The second characteristic is frequency, ν . Frequency represents the number of cycles per unit time, usually inverse second (s). Multiplication of the frequency and wavelength will provide the velocity of the ultrasound wave, c . Sound waves move faster in dense materials (such as solids) compared to less dense materials (such as liquid, gas and plasma). It has a direct relationship with the square root of the compressibility of the material and indirect relationship with the square root of the density of the material. The velocities of the ultrasound waves in biological and non-biological materials is presented in Appendix A.1. The final ultrasound wave characteristic, amplitude, A , is the maximum distance traveled the wave from the center line of the wave - it gives the maximum height of the wave cycle in the ultrasound wave. It determines the energy, E , of the ultrasound wave ($E \propto A^2$).

A sound wave is segregated into three groups based on its frequency. The frequency range that is audible for the human ear is found between 20Hz and 20kHz. If the frequency is below 20Hz then the sound wave is called infrasonic and if the frequency is greater than 20kHz then the sound wave is called ultrasound. Ultrasound waves with frequencies between 1 and 30MHz are known as Diagnostic Medical Ultrasound.

Two or more ultrasound waves may interact with each other in two ways, constructively or destructively. If the interaction is constructive, the resultant wave amplitude is greater than the amplitudes of the interacting waves. On the other hand, the resultant amplitude is less

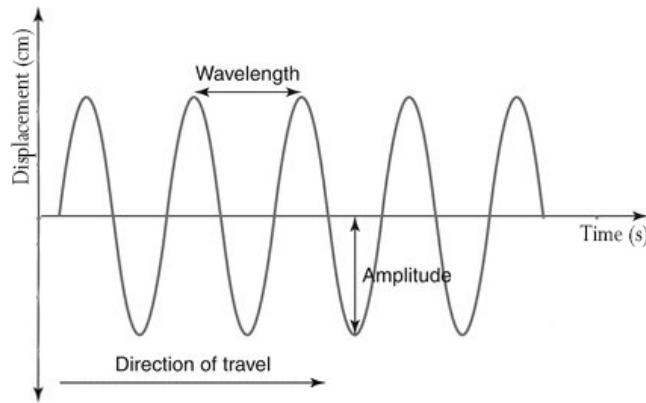


FIGURE 2.3:
Illustration of transversal wave
and some characteristics of
ultrasound wave (Sehmbi and
Perlas, 2015).

than that of the interacting waves amplitude if they interface destructively (Hendee et al., 2003).

The last but not the least essential concept related to sound characteristics is the Doppler effect. It is the foundation of Doppler ultrasound. Keeping the sound source stationary, the frequency of the reflected echo from the moving object is different from that of the incident frequency by a value commonly known as the Doppler shift frequency, v_D . The Doppler frequency can be positive or negative depending on the direction of movement of the moving object. If the object moves towards the sound source, the value of the Doppler shift frequency will be positive. Conversely, the value of the Doppler shift frequency becomes negative if the object moves away from the source (Haidekker, 2013).

An ultrasound wave propagates in beams. These beams carry energy when they pass through a medium. This energy is transferred to the medium in two forms: thermal (heating) or non-thermal (mechanical effect). As stated previously, the energy of the ultrasound field is directly proportional to the square of its amplitude. The rate of the energy that is carried away with the wave is called power (Hendee et al., 2003). The power of the acoustic beam is measured through the mechanical index (MI). It is a quite essential parameter when dealing with gas-filled biological organs and contrast agent microbubbles, since it is directly proportional to the negative maximum pressure and inversely proportional to the square root of the frequency of the ultrasound beam (Şen et al., 2015). Beams are concentrated in a small area and quantified using a term intensity. Intensity is a measure of power per unit area. Usually, intensity is described using some reference value. For example, in imaging ultrasound the reflected back sound intensity is used to construct the image of the targeted organ or tissue. This intensity is expressed numerically by comparing it with the sound intensity that is sent to the tissue or organ, using a decibel scale, dB. The ratio of the reference and the actual intensity varies with great orders of magnitude and it becomes logical to use a logarithmic expression to derive meaningful values (Hendee et al., 2003). Essential mathematical representations of an ultrasound wave is found on Appendix A.2.

2.3 Ultrasound Transducer

Naturally, animals such as bats are considered ultrasound transducers - they produce and sense ultrasound waves. It is also possible to generate ultrasound waves artificially using piezoelectric transducers. The term transducer represents any device that has the capacity to convert any energy from one form to the other. When piezoelectric crystals are under pressure/tension, they create a small electrical voltage difference. On the other hand, application of electrical energy on piezoelectric crystals creates mechanical deformation of the crystals, vibration, and resulting sound waves. The process of applying mechanical pressure on a crystal and producing a tiny electrical energy is termed as piezoelectricity. Conversely, applying an electrical energy and creating expanding and contracting mechanical pressure on a crystal is known as reverse/converse piezoelectricity. A simple visual representation of the two processes is shown on Figure 2.4. Common ultrasound transducers have both functionalities (electrical energy to sound wave and sound wave to electrical energy conversion). Ultrasound energy is generated, sent through a transporting medium, and the energy of the reflected mechanical wave is detected and changed into electrical energy.

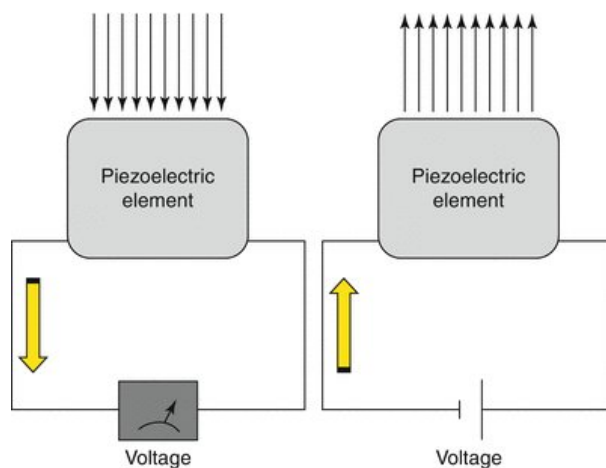


FIGURE 2.4:
Piezoelectricity and reverse
piezoelectricity of ultrasound
transducer (Sehmbi and Perlas, 2015).

The most important physical parameter of ultrasound transducers is their frequency. In diagnostic sonography, depending on their physical appearance and frequency range, transducer arrays are commonly classified into two types (Sehmbi and Perlas, 2015): linear array and curved array transducers, illustrated on Figure 2.5. In addition, application-specific transducers exist (Carovac et al., 2011). Linear array probes produce comparatively high frequencies between 8-12 MHz. They are used to produce a superficial, rectangular field of view and high resolution images of the organ/tissue. On the other hand, curved array probes produce comparatively low frequency intensity between 1 and 5 MHz and are used to penetrate the tissue deeper (imaging deeper organs/tissue). Usually the image is displayed as a sectoral field of view.



FIGURE 2.5:
Curved (i) and linear
(ii) ultrasound probes
(*Sehmbi and Perlas,*
2015).

2.4 Interaction of Ultrasound Wave with Biological Medium

When an ultrasound beam is allowed to pass through a biological medium, it will interact with the molecules of the tissues/organs in different ways. It may be absorbed, transmitted, scattered, reflected, diffracted or interfered (each other). The process of reduction of ultrasound energy on the way of propagation in a medium is called attenuation. The main contributors of the attenuation of the sound energy are absorption, scattering, refraction and interference (*Bryan, 2010*). The distance, the ultrasound beams travel inside a tissue, also has a pronounced factor for ultrasound attenuation. Based on Beer-Lambert's law, as the distance increases, the energy decreases exponentially. All of the energy may not be attenuated. In case of diagnostic ultrasound, the reflected back or the transmitted ultrasound intensity is required to provide information about the targeted tissue or organ.

The therapeutic effect of ultrasound waves mainly relies on its frequency and the absorption ability of the medium. When the energy is absorbed, it induces a heating effect on the molecules contained within the medium, causing the molecules to move randomly, i.e. the potential (compression) energy of the ultrasound wave will be changed to heat and kinetic energy. The kinetic motion of the molecules by itself induces a mechanical stress on the nearby molecules and facilitates the heating and destabilizing effect. Figure 2.6 shows the attenuation coefficient variation of different tissues and organs as a function of frequency.

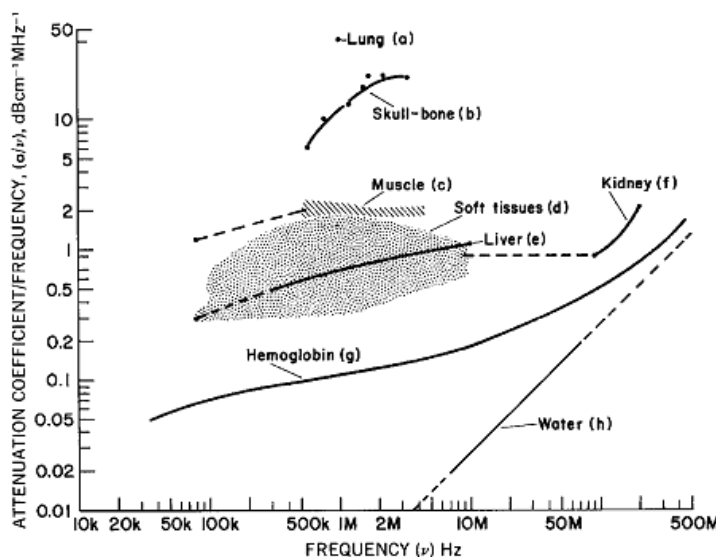


FIGURE 2.6:
Attenuation coefficient
variations of some
biological molecules,
tissues and organs
(*Hendee et al., 2003*).

The diagnostic application of ultrasound wave mainly depends on the reflected intensity of the sound wave from the interfaces. The incident beams of the ultrasound should be sent to the tissue with 90° angle with a tolerance of 3 degrees. Otherwise, the reflected beams may not provide the accurate information. When the incident ultrasound beams are sent at angle other than 90 degrees to the interface of a tissue, another phenomenon will be occurring – refraction. Referring Snell's law, when ultrasound beam travels from one medium to the other with an angle different from 90 degrees, the beam bends to the normal of the interfaces if the speed decrease as it passes the boundary. In contrast, the beam bends away from the normal if the speed of the ultrasound beam increases as it passes the boundary.

In case of 90° reflections, the reflected back intensity is affected by the acoustic impedance of the interfacing media. Acoustic impedance, z , is the product of the density, ρ , of the medium and the velocity, c , of the sound wave. When a large impedance mismatch occurs between interfacing media, a high intensity wave will be reflected back to the source/detector to provide information about the reflector, and the converse is also true. The remaining beam, the transmitted one, continues its journey until it encounters another reflector and sends back its information to the detector. The process continues until the image of the targeted organ/tissue is captured. The impedances of some selected materials is included in appendix [A.3](#).

One last phenomenon that biological obstacles impose on the ultrasound beam is called scattering. Scattering is the dispersing of the ultrasound beams in random directions. When the size of the obstacle is greater than that of the size of the wavelength and comparatively smooth, the reflected intensity holds its integrity. On the other hand, when the size of the wave length is small compared to the obstacle, the beam will be scattered in random directions.

2.5 Application of Ultrasound in Medicine

Ultrasound has been playing a great role in medicine since the earlier years of the second half of the 19th century. As it is noted on the historical background of this chapter, the application of ultrasound energy was started as a medical therapeutic tool to treat cancer and give physical therapy for different illnesses even though, later, its dominant application is diagnostic. Since then, many works have been done towards the imaging application, mainly. These days, its diagnostic application seems settled and researchers have focused their direction to exploit its therapeutic potential ([Haidekker, 2013](#)).

Medical imaging is the main application area of ultrasound. Starting from the simplest A-mode to the more sophisticated Doppler ultrasound, so far, different modes of ultrasound imaging devices have been designed. The earlier, A-mode (amplitude mode) ultrasound, was designed just to plot the characteristics of a single line through a tissue using only a single transducer. The concept of A-mode in combination with B-mode (brightness mode)

ultrasound forms the more advanced motion based ultrasounds (M-mode). B-mode ultrasound creates a multiple sequential 2D images of the tissue using multi-array transducers. Combining the sequential gray-scale images with continuous recording of the variation of the depth of the tissue using the concept of A-mode, physicians can study the motion and the physiological activities of organs, such as the opening and closing of the heart valves during blood pumping, in real-time.

The last mode, Doppler mode, uses Doppler effect to determine the speed of blood flow or other moving particles inside blood vessels. The important parameters, blood flow velocity and flow direction, can be calculated based on the value of Doppler shift frequency, v_D . This kind of ultrasonic echography is essential to deliver blood flow information in larger blood vessels. However, it fails when it comes to a capillary bed perfusion area. To alleviate this problem, microbubble contrast agents are administered to the blood stream to enhance the contrast of the real-time image of the blood flow near tissue perfusion sites (Carovac et al., 2011).

In addition to imaging, the microbubbles can be used as therapeutic tool by encapsulating a specific drug or gene and delivering it to a specific tissue. Upon arrival to the targeted tissue, the ultrasound field is applied to damage the microbubbles, allowing them to release the encapsulated drug or gene (Postema and Gilja, 2011).

The more recent and promising application area of ultrasound field is levitation. Levitation is the process of uplifting objects using some kind of energy. Researchers have proved the possibility of levitating smaller particles using acoustic energy. In the near future, blood samples and molecules might be levitated in the air using an acoustic field to study their structure and activities in a microgravity environment without the interference of microscopic slides or laboratory test tubes (Stephanie, 2017).

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3

Ultrasound Contrast Agents in Cardiovascular System

Comparing blood cells and ultrasound contrast agent microspheres, microspheres have the better reflecting and scattering property. To have a good contrast enhanced image near the tissue perfusion area, most of the ultrasound beams that are sent to the tissue must be reflected back to the transducer or detector. Due to the poor reflectivity of the blood cells, the contrast of the electrocardiography is also poor. That is the main reason why contrast agents are administered to the examinee before imaging for tissue perfusion and blood flow. In addition, there are ongoing research to use the microbubbles for therapeutic applications to deliver and release specific drugs to specific tissue upon sonication. In this chapter, fundamental concepts about the cardiovascular system (specifically blood vessels and cells), contrast enhanced ultrasound (CEUS) imaging, ultrasound contrast agent microbubbles (USCAM) and interaction of ultrasound field with USCAM and blood cells are presented.

3.1 The Cardiovascular System

Studying the anatomy and physiology of the human beings would be impossible without a systematic hierarchy of grouping – cells, tissues, organs, and systems. The basic hierarchy tier begins with cells. Cells become tissues when they are grouped together and specialized to perform some specific biological task. Groups of specialized tissues become organs

and organs become systems. The last tier, system, has 11 subdivisions: cardiovascular, digestive, respiratory, endocrine, integumentary, lymphatic, muscular, skeletal, nervous, renal and reproductive system (Enderle and Bronzino, 2012).

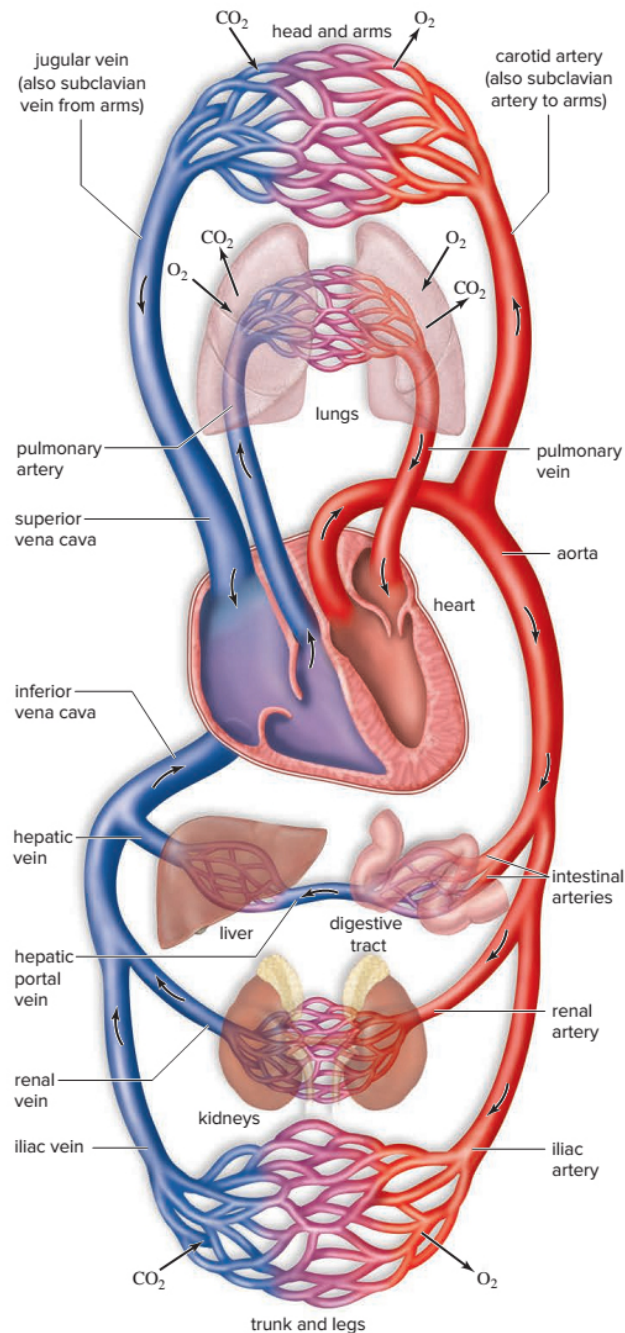


FIGURE 3.1:
Illustration of the cardiovascular system: the two main circulation routes (systemic and pulmonary), the heart, the lung, major route paths (arteries, veins and capillaries) that are extended to and away from the heart and perfusion and waste removal from major organs (Sylvia S. Mader and Windelspecht, 2017).

The cardiovascular system, also known as the circulatory system, is composed of the heart, the blood vessels, and the blood - illustrated on Figure 3.1. The closed network of endothelial tubes (blood vessels) provide a path to the blood to transport oxygen, nutrients and hormones from the heart to the rest of the body and returns back the waste products (e.g. carbon dioxide) from the whole body to the heart. This part of the circulatory system is known as systemic circulation. To take fresh oxygen from the ambient environment and

remove the waste gases from the body, there is also a special circulation which is known as pulmonary circulation. Pulmonary circulation takes place between the heart and the lung. Besides transportation, the cardiovascular system provides two essential functions: protection from pathogens and body regulation. The heart is the powerful organ in the circulatory system. The pressure of the blood that passes through the blood vessels is determined by the pumping effect of the heart (Kierszenbaum, 2013). The heart of normal 68.7kg adult man pumps, averagely, about 5 liters of blood in a minute (Bronzino and Peterson, 2015).

3.1.1 Blood Cells

When cells and surrounding substances form a group and specialized to do specific task or multiple tasks, they become a tissue. The human body is classified into four tissue types: connective tissue, muscular tissue, epithelial tissue and nervous tissue. Blood is categorized under the most abundant and widely distributed tissue type, connective tissue – it is a fluid type of connective tissue and accounts about $8\pm 1\%$ of the total body weight. Three type of blood cells (red blood cells (Erythrocytes), white blood cells (Leukocytes) and platelets (Thrombocytes)) are grouped together and specialized to multiple tasks to form this connective tissue. Bone marrow is the place where blood cells are produced. In addition to blood cells, blood also contains extracellular fibers (fibrinogen) and extracellular ground substances (fluids and proteins) (Enderle and Bronzino, 2012; Bronzino and Peterson, 2015; Johnson, 1991). Figure 3.2 summarizes the constituents of human blood.

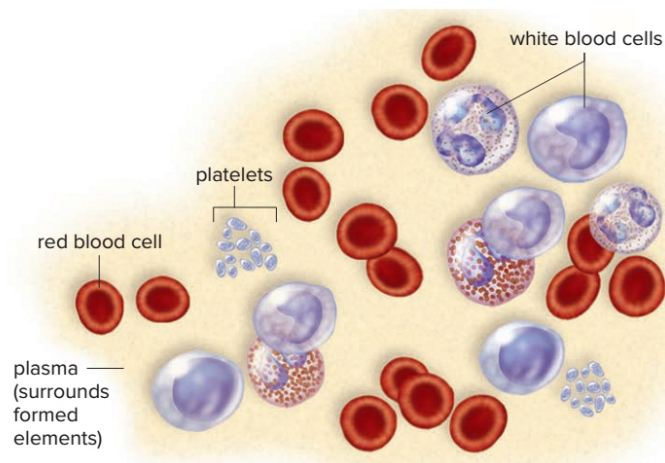


FIGURE 3.2:
Composition of blood: the formed elements (red blood cells, white blood cells and platelets) and plasma that surrounds the formed elements (Sylvia S. Mader and Windelspecht, 2017).

In adult body, approximately 5 liters of blood is found. When the blood sample is inserted into the centrifuge, the cells are allowed to sediment, and they will be segregated into different layers. Red blood cells (RBCs) are represented by the largest layer. They approximately cover 45% of the total sample volume. Studying RBCs using a scanning electron micrograph, illustrated on Figure 3.3, reveals structural and quantitative information; they have a biconcave disc shape, $7\text{ }\mu\text{m}$ - $8\text{ }\mu\text{m}$ diameter, $2\text{ }\mu\text{m}$ depth at the edges and immense amount of aggregate surface area ($200\times$ that of skin). Unlike other common cells, RBCs lack many of the organelles including the nucleus – only a plasma membrane is present. The cytoplasm of the RBCs contains a special protein which is known as hemoglobin (Johnson, 1991). The

red pigment that is present inside the hemoglobin protein is the main reason why RBCs and blood generally exhibits a red color (Sylvia S. Mader and Windelspecht, 2017).

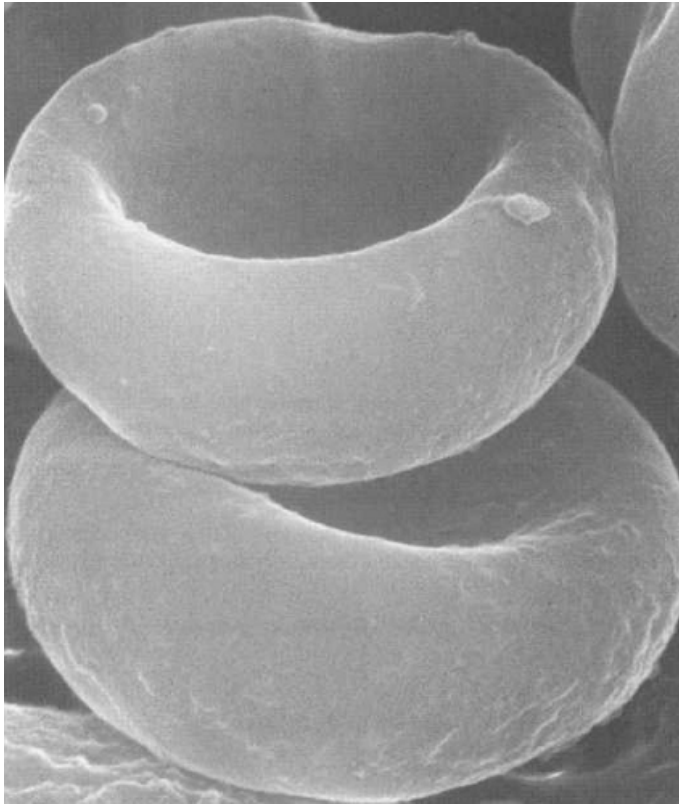


FIGURE 3.3:
Scanning electron micrograph
image of two normal and
overlapping red blood cells -
illustrating their biconcave disk
shape (Johnson, 1991).

Using the aid of hemoglobin, RBCs transport gases (mainly oxygen and carbon dioxide) throughout the cardiovascular system before the lifespan ends - approximate lifespan of RBCs is around 120 days. They pick up oxygen from the alveoli of the lung and transport them to the peripheral tissues, collect carbon dioxide from the peripheral tissues and return back to the alveoli of the lung through the two main circulation pathways (pulmonary and systematic circulation) (Johnson, 1991).

The second type of blood cells, white blood cells (WBCs), are the largest blood cells in size (Johnson, 1991). There are different varieties of WBCs: pathogen identifying (Agranulocytes) and disposing (Granulocytes) WBCs (Bronzino and Peterson, 2015). Among the disposing WBCs, neutrophils are the most abundant and have a diameter range of $12\text{ }\mu\text{m}$ - $15\text{ }\mu\text{m}$ (Johnson, 1991). Moreover, what makes them different from RBCs are their translucent appearance (difficult to distinguish them under microscope without staining) and the presence of a nucleus. Leaving aside their varieties, the ultimate function of all WBCs is protect the human body from external invaders (pathogens or infectious microorganisms). They are specialized to fight any kind of pathogen (e.g. bacteria or virus) or infected cell in general or specifically towards a particular type of pathogen or infected cell (Sylvia S. Mader and Windelspecht, 2017).

The last of the blood cell types, platelets, form from giant cell fragments and have a diameter of $2\text{ }\mu\text{m}$ - $4\text{ }\mu\text{m}$. Their main function is clotting blood. If one's blood vessel is cut, the

platelets will form a cluster mass in collaboration with fibrin, resulting in a clotting effect on damaged blood vessel. The clotting effect prevents the blood from continuous external flow and facilitates vascular tissue repair (Johnson, 1991).

Extracting blood cells from a blood sample leaves the aqueous and saline medium which is known as plasma. The most abundant constitute of the plasma is water – it covers around 91% of the total volume. The remaining volume is shared by proteins (albumin, globulins, and fibrinogen), minerals (inorganic ash), trace elements, electrolytes, fats, fat soluble vitamins, lipids, free fatty acids and extractives (glucose, other forms of carbohydrate, water soluble vitamins, enzymes, non-nitrogenous and nitrogenous waste products and biochemical constitutes). Plasma plays many essential functions in the cardiovascular system. It ensures the presence of normal mass exchange between the blood and interstitial fluid at the capillary bed, serves as a transporter of hormones and small or large biochemical substances (for instance, metal ions, fats and heavy metals), fights against external invaders in collaboration with WBCs, assists platelets to form a blood clot, keeps the fluid at a faintly alkaline pH (Bronzino and Peterson, 2015) and regulates body temperature (Sylvia S. Mader and Windelspecht, 2017).

3.1.2 Blood Vessels

There are three main piping pathways or vessels that allow the blood to flow through the two main cardiovascular circulation systems (pulmonary and systemic): arteries, veins and capillaries. As part of systemic circulation, a high pressure oxygenated blood is pumped from the heart, passes through artery and arterioles and reaches tissue capillaries. After nourishing the tissue at the capillary bed, the vein side capillaries transfer the low pressure deoxygenated blood to the venules, venules to the veins and the veins return the blood back to the heart and complete the cycle of the circulation. Regarding the other circulation type, pulmonary circulation, pulmonary arteries take the deoxygenated blood from the heart to the lung; pulmonary capillaries at the alveoli of the lung exchange the carbon dioxide gas with fresh oxygen; and lastly the oxygenated bloods flows towards the heart through pulmonary vein (Johnson, 1991; Enderle and Bronzino, 2012; Bronzino and Peterson, 2015; Sylvia S. Mader and Windelspecht, 2017).

Arterial vessel walls are designed to handle the pressurized blood that passes through them. The thick wall is made up of three layers. The inner-most portion of the vessel is covered by a thin layer of cells called the endothelium. Following the endothelium layer, at the middle, a thick smooth muscle and elastic tissue layer is found. A connective tissue that is wrapped around the middle layer forms the third layer. Similar to arteries, arterioles have a middle layer that is composed of smooth muscular and elastic tissues. Contraction and relaxation of the muscle fiber determines the constriction and dilation of the arterioles, respectively (Sylvia S. Mader and Windelspecht, 2017). The estimated internal diameter of the arteries and the arterioles of a normal adult person ranges between 10 μm - 3.1 cm (see Table 3.1).

Arterial Side		Venous Side	
Blood vessel type	Internal diameter	Blood vessel type	Internal diameter
Aorta	1 – 3 cm	Postcapillary venules	8 – 30 μm
Pulmonary artery	2.5 – 3.1 cm	Collecting venules	30 – 50 μm
Main branches	5 mm-2.25 cm	Muscular venules	50 – 100 μm
Large arteries	4 – 5 mm	Small collecting venules	100 – 200 μm
Medium arteries	2.5 – 4 mm	Terminal branches	200 – 600 μm
Small arteries	1 – 2.5 mm	Small veins	600 μm -1.1 mm
Tributaries	0.5 – 1 mm	Medium veins	1 – 5 mm
Small rami	250 – 500 μm	Large veins	5 – 9 mm
Terminal branches	100 – 250 μm	Main branches	9 mm-2 cm
Arterioles	25 – 100 μm	Vena cava	2.0 – 3.5 cm
Metarterioles	10 – 25 μm	Pulmonary veins	1.7 – 2.5 cm
Capillaries	3.5 – 10 μm		

TABLE 3.1: Approximate internal diameters of blood vessels of a healthy 68.7 kg person who has 5.2 L blood volume (Bronzino and Peterson, 2015).

Comparing the three blood vessels, capillaries are the smallest ones – their diameter is estimated between 3.5 and 10 μm (Bronzino and Peterson, 2015). The lining that covers the internal wall of the capillary vessels is a single layer of endothelium cells. This wall is designed in a way to facilitate easy exchange of nutrients, gases and waste between the blood and the surrounding interstitial fluid. A network of capillary vessels found inside tissues is called a capillary bed. Throughout the body there are a tremendous amount of capillary beds - these are the places where exchange of substances between the blood and the cells happens. The blood flow to these capillary beds is controlled by a special muscle called precapillary sphincters – when closed, blood flows towards the gas exchange.

The third type of blood vessels, veins, are the reservoir vessels – they hold about 70% of the total blood volume. As shown on Table 3.1, their internal diameter varies between 8 μm (Postcapillary venules) and 3.5 cm (vena cava). The deoxygenated blood that leaves the capillaries passes through different varieties of the vein before it reaches to the heart – starting from the venules (smallest vein) to the vena cava (the largest vein). The pressure inside veins is much smaller than that of the arteries and capillaries. Because of this and gravity effect, veins need to have a one-way valve in the inner most layer of their wall to prevent blood backflow. Similar to arteries, veins also have three layers: the inner most endothelium layer, the middle smooth muscle and the outer connective tissue. However, the middle and outer layers of the veins are not as thick as the arteries (Sylvia S. Mader and Windelspecht, 2017).

3.2 Contrast Enhanced Ultrasound (CEUS)

Some medical imaging modalities such as MRI, CT, and ultrasound need contrast agents to get the enhanced image of the targeted human organ or tissue (Wilson et al., 2009). Historically, researchers recognized the concept of contrast enhanced ultrasound (CEUS) in the late 1960s when agitated saline was injected into the aortic root and an unusual number of echoes were emerged from the undulating margins of the aortic root. Unknowingly, the gas bubbles inside the agitated saline were acting as a contrast agent (Faez et al., 2013). These days, conventional ultrasound imaging technologies are limited to static, dynamic and real time imaging of the internal organs and blood flow analysis in larger blood vessels but fail to give information regarding tissue perfusion. The remedy to this challenge is to administer microbubble contrast agents into the bloodstream (Wilson et al., 2009).

B-mode ultrasound shows the intensity of the reflected back ultrasound as a function of depth and displays the intensity value at the corresponding location, i.e. the imaging modality is highly dependent on the reflected beams. The intensity of the reflected ultrasound beam is mainly affected by the acoustic impedance of the tissues (Postema and Gilja, 2011). If the acoustic mismatch between interfaces of tissues is large, much of the incident ultrasound beam will be reflected back to the detector and the boundary between the interfaces will be clearly distinguished. For instance, the acoustic impedance mismatch between the air and the liver is big (approximately $1.6496 \times 10^{-4} \text{ kg/m}^2\text{s}$) since the impedance of air is immensely smaller than that of the liver. So, it becomes very easy to differentiate the boundary between the air and the tissue. On the other hand, if the acoustic impedance mismatch between the tissue interface is small, almost all of the beams will be transmitted and the transition boundary becomes difficult to delineate, e.g. the interface between blood and muscle (Hendee et al., 2003). Acoustic impedance mismatch calculation equations are stated on Appendix A.2. Values of acoustic impedances of some biological and non-biological materials are also stated on Appendix A.3

Ultrasound contrast agents were originally designed to enhance blood vasculatures, especially the arterioles with the estimated internal diameter of 40 μm . Currently, their application has been extended to image blood vessels and tissues at the capillary bed level. Capillary beds are the area where tissue perfusion occurs (Sylvia S. Mader and Windelspecht, 2017). The only ultrasound reflectors found in the capillary beds are the blood cells and the soft tissues of the targeted organs. Their acoustic impedance is approximately the same – in other words, their acoustic mismatch is very low. Therefore, differentiating the blood vessels and the soft tissues near this area becomes a challenging task. The remedy for this problem is administrating gas filled microbubble contrast agents into the blood stream intravenously. Figure 3.4 clearly shows the effect of contrast agent in ultrasound imaging - before and administration of the contrasting agent. The acoustic mismatch which is created between the microbubble and the blood or the soft tissues becomes huge and thereby increases the reflected ultrasound beams. As well, it provides a suitable situation to study tissue perfusion on B-mode ultrasounds (Hendee et al., 2003; Postema and Gilja, 2011; Bryan, 2010).

Ultrasound contrast agents are vascular contrast agents and cannot pass the endothelial wall of the blood vessel, i.e. they do not enhance interstitial fluid as MRI contrast agents do (Fanet, 2014; Bryan, 2010). After completing their function, except the core gas, all of the constituents of the contrast agent microbubbles will be removed from the blood stream by the hepatic and splenic reticuloendothelial system and takes approximately 30 min, though the imaging process is no longer than 4 min. And the lung removes the capsuled gas as part of its gas exchange with the ambient environment (Lee et al., 2016).



FIGURE 3.4: Clinical ultrasound images of Xenograft PC-3 tumor model before (A) and after (B) contrast agent enhancement. The arrow indicates the clear difference (Lee et al., 2016).

These days, contrast enhanced ultrasound is employed to image and study the heart, vessels, liver, pancreas, spleen, kidneys, gastrointestinal tract and brain. In addition, they are also helpful to measure tissue perfusion, detect abrasions in the liver, make angiogenesis assessment, characterize tumors, and detect sites of soreness (Postema, 2017)

In addition to imaging, ultrasound contrast agent microbubbles have been designed as a cavitation nucleus to transport drugs and genes to target normal or malignant tissue cells. These kind of contrast agent microbubbles are known as targeted microbubbles. Following their arrival, when they undergo ultrasonic field, they release their content (drugs and genes) to give drug or gene therapy for the targeted normal or cancerous tissue cells. The process of encapsulating drugs or genes within microbubbles, targeting a specific tissue cell, administering them into the bloodstream and giving a drug or gene therapy to the targeted tissue is called microbubble-mediated drug or gene delivery. Sonication of the microbubble, the process is called sonoporation, facilitates the microbubble-mediated drug/gene delivery by disturbing the microbubbles, maximizing the permeability of the blood vessels at the capillary bed (allow large molecules to stream towards the cells) and increasing the perforation of cell membranes (Lee et al., 2016). Targeted microbubbles are useful not only for drug/gene delivery but also for molecular imaging – modifying their encapsulating layer to target and enhance only specific tissue cells (Papadopoulou et al., 2014).

3.2.1 Ultrasound Contrast Agent Microbubbles (USCAM)

Commonly known ultrasound contrast agents are microbubbles. As the name indicates, their size is measured in micrometers – the estimated size is between 1 –10 μm . Recently,

contrast agent bubbles are being produced at the nanoscale (400 – 800 nm). Targeting a specific tumor, nanoscale bubbles are administrated into the bloodstream and accumulate themselves inside the tumor tissue – the process commonly known as passive targeting (Lee et al., 2016). Even though different varieties of microbubbles are found on market with varying applications, size and composition (Postema et al., 2003), their basic construction is the same. As illustrated in Figure 3.5, both targeted and non-targeted microbubbles have two main components: a core gas and an encapsulating shell. The core gas is the main determinant of the acoustic properties of the microbubble whereas the encapsulating shell controls the compressibility of the core gas, i.e. it determines the stiffness and viscoelasticity of the overall bubble (Paefgen et al., 2015). There is one additional feature, binding site, which is found on targeted microbubbles. Its functionality is to direct and attach the microbubbles to the targeted site within the body (Papadopoulou et al., 2014).

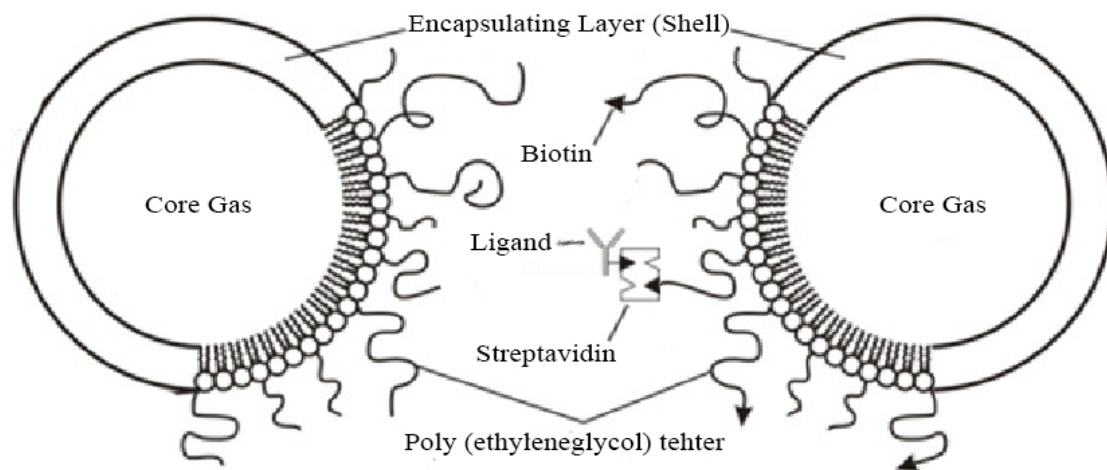


FIGURE 3.5: Illustration of a typical non-targeted (left) and targeted (right) ultrasound contrast agent microbubbles (Papadopoulou et al., 2014)

The gas of the microbubble need to be biodegradable (Postema and Gilja, 2011), biocompatible, less soluble in water/blood and biologically inert (highly stable). So far, ordinary air, heavy inert gases (such as perfluoropropane, perfluorobutane, perfluorohexane and sulfur hexafluoride) and nitrogen are being used to fill the core of the microbubbles. Heavy gases stay in the blood pool for a longer time and allow imaging for an extended time (Lee et al., 2016). In contrast, ordinary air is unstable and it quickly dissolves in the blood stream which shortens the lifetime of the microbubble (Paefgen et al., 2015).

The encapsulating shell is broadly categorized into two groups: hard shell and soft shell. The first category of encapsulating shells, hard shells, are constructed from low viscoelastic materials to decrease the compressibility (increase the stiffness) and increase the circulation time of microbubble. Materials such as polymers, denatured proteins and silica are used to construct such kind of shells. Microbubbles which have hard shell are used for high intensity applications. The second type of shells, soft encapsulating layers, are fabricated from thinner and more flexible materials such as surfactant molecules and phospholipids. The thinness and the flexibleness of the shell make the microbubble good oscillator for low

intensity ultrasound beam compared to that of hard shell microbubbles. Subjecting soft-shell microbubbles for high intensity ultrasound beam leads to disintegration of the bubble into smaller bubbles – not bursting as that of the hard-shell microbubbles (Paefgen et al., 2015).

Ligands (binding site) such as antibodies, proteins, peptides and natural or fabricated scaffolds are attached to the encapsulating layers of targeted microbubbles covalently and non-covalently depending on the function of the microbubble. Adding of the binding site on the microbubble can be achieved simultaneously with or after the production of the encapsulating layer. As illustrated on Figure 3.5, to form a connection between the ligand and the shell, there should be appropriate reactive moiety on the shell forming material. The reactive moiety can be biotin, a carboxylate group, thiol, or maleimide (Papadopoulou et al., 2014).

3.2.2 Interaction of Ultrasound field with USCAM and Blood Cells

Ultrasound contrast agent microbubbles have been playing a tremendous role in medical diagnosis (clinical or molecular imaging) and therapeutics (microbubble-mediated drug or gene delivery). Research towards USCAs is not settled yet - researchers, engineers and technologists have been stuck in their workshop and laboratory to exploit the future endeavors of it. In the near future, a single type USCA in collaboration with advanced instrumentation might play both the therapeutic and the diagnostic role as the same time to treat and image the most chronic illnesses such as cancer and cardiovascular diseases (Paefgen et al., 2015).

To design new USCAs and appropriate instrumentation systems, there is one big challenge - interaction of the ultrasound field with the contrast agent particles and tissue cells (especially that of blood cells) is not clearly or wholly understood yet. The occurrence of the interaction at a micro or nano level is the main reason for the study challenge. Thanks to the advancement of technology, optical imaging has made the study handy and straight forward. It enables the researcher to capture and store the images of the USCAs and blood cells, exposed to a particular ultrasound field, for offline study (Postema et al., 2003; Maz-zawi et al., 2015).

The behavior of cells, when exposed to an ultrasound field, is explained by the bilayer sonophore model – for this thesis, the cells are reduced to blood cells, specifically to red blood cells since RBCs constitute nearly half of the total blood volume. The model states that the ultrasound field creates a pulsating structure (from the surrounding dissolved gas) between the hydrophobic part (lipid monolayers) of the phospholipid bilayer plasma/cell membrane of the cell (see Figure 3.6). The pulsating structure is known as sonophore and has an estimated size of between 10-100nm. The sandwiched sonophore inflates and pushes

the phospholipid monolayer leaflets apart when the rarefaction ultrasound pressure is applied and deflates when the compressive ultrasound pressure is applied. The periodic inflation and deflation continues as long as the periodic ultrasound pressure is present. The pressurized sonophore enables the cell to act as mechanical oscillator, radiating the ultrasound pressure pulse to the surrounding medium and serving as the source of the intracellular cavitation activity.

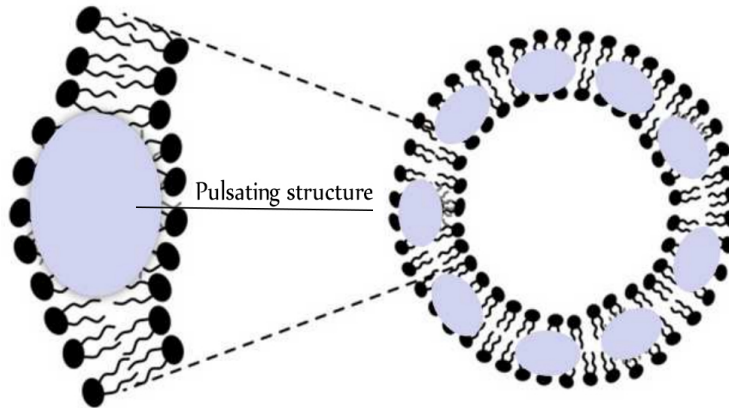


FIGURE 3.6:
Sonophore model -
pulsating behavior of
cells when they are
subjected to ultrasound
field (Mazzawi et al.,
2015)

From the sonophore model, two essential conclusions about cells and sonophores can be drawn. One, the secondary Bjerknes forces created by the sonophore is too weak since these forces are directly proportional to the fifth power of the radius of the gas bubbles. Second, cells will not create sonophores when aligned to the nodes of the ultrasound wave because the acoustic pressure at the nodes of the wave is zero (Mazzawi et al., 2015; Krasovitski et al., 2011).

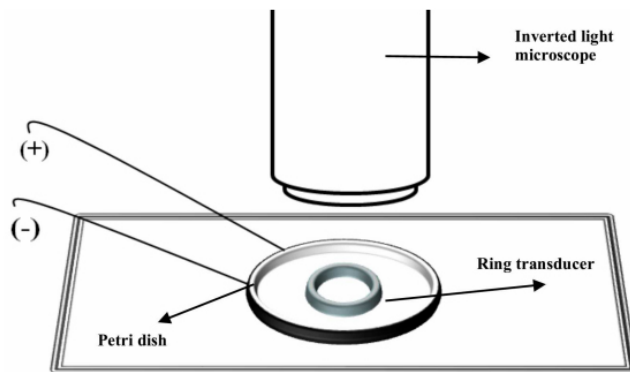


FIGURE 3.7:
Mazzawi et al. (2015)
experimental setup.

Mazzawi et al. (2015) made an experiment to investigate the response of living blood cells when they are subjected to ultrasound field using optical imaging technology. As shown on the simplified experimental setup illustration (Figure 3.7), a ring transducer (with the inner diameter of 28 mm, height of 10 mm and thickness of 2.1 mm) was placed at the center of the petri-dish (with diameter of 100 mm and height of 15 mm) and connected to power amplifier and arbitrary wave form generator which is tuned to 1 or 3 MHz frequency and 1 V or 10 V peak to peak amplitude. Focusing to the center, camera connected inverted light microscope (with a magnification of 2 $\times$, 10 $\times$ and 20 $\times$) was placed above the petri-dish to capture and store the events.

The mixture of three objects, microbubble ultrasound contrast agents, blood and polystyrene microspheres, were added to the petri dish and then the sonication process was started. The finding of the experiment is stated on the problem statement of this document (see section 1.2). This experiment might give a clear insight about the interaction of the intensity of the ultrasound beam with the blood cells and the microbubble microspheres.

The pressure of the ultrasound beam determines the oscillation behavior of USCAMs. When they are subjected to low acoustic pressure (mechanical index (MI)), they respond with linear oscillation. When the acoustic pressure (MI) becomes high, the oscillation of the microbubble becomes non-linear or asymmetric. Each microbubble has its own size-dependent resonant frequency. If a varying acoustic pressure is applied (starting from the lowest value to the highest) on smaller and larger microbubbles simultaneously, larger microbubbles respond with non-linear oscillations more quickly compared to smaller ones.

Following this asymmetric oscillation, the microbubble might go through one of the six non-linear phenomena. These phenomena are jetting, fragmentation, coalescence, cracking, translation and clustering. The ultrasound parameters (transmit frequency, acoustic impedance, pulse length, pulse repetition rate and transmit phase), the composition of the microbubble (composition of the shell, the bubble size, ratio between the gas and shell size and the gas) and properties of the medium (viscosity, surface tension and saturation) play an essential role for the occurrences of the listed phenomena.

Jetting, fragmentation and coalescence occur on released gases and thin shell (< 10 nm) microbubbles. When the microbubble collides with a boundary and collapse, it may be fragmented into smaller bubbles (the phenomenon is known as fragmentation) or the core gas may extrude from the microbubble as a funnel-shaped jet and escape through the nearby fluid toward the boundary (the phenomenon is called jetting). After fragmentation, two or more fragmented bubbles may come together to form a bigger bubble – the process is known as coalescence. Fragmentation and coalescence may occur at low ($MI < 0.3$), medium ($0.3 < MI < 0.7$) and higher ($MI > 0.7$) acoustic ranges whereas jetting occurs only on higher acoustic ranges.

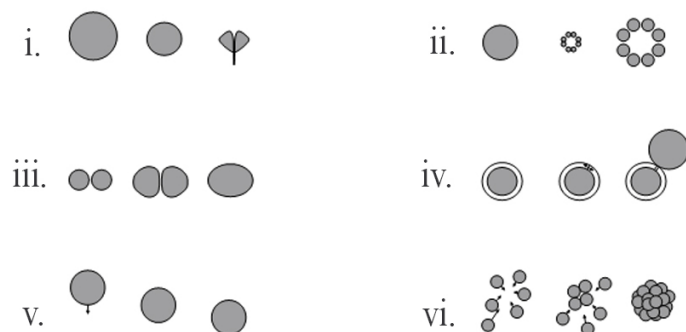


FIGURE 3.8:
Non-linear response of
ultrasound contrast agent
microbubbles when they are
subjected to ultrasound field.
Jetting (i), fragmentation (ii),
coalescence (iii), cracking (iv),
translation (v) and clustering
(vi) (Postema and Gilja, 2011).

When the microbubbles are exposed to a standing low, medium or high ultrasound wave ranges, they form a cluster on the quarters of the wavelength of the wave. The phenomenon happens on thin shell and thick shell (between 10 nm and 500 nm) microbubbles.

During acoustic wave expansion, sonic crackdown might occur on shells of the thin, thick and very thick (> 500 nm) microbubbles when a big pressure difference occurs between the inside and outside of the microbubble. The three acoustic ranges have the potency to create this phenomenon. If the applied acoustic pressure is beyond the critical deformation of the shell of the microbubble, a mechanical crack will form on the shell and the gas bubble will be extruded to the outside. Compared to the encapsulated gas, the released gas has high expansion frequency.

The last phenomenon, translation, takes place when the ultrasound beam forces the microbubble to move towards the blood vessel walls. The translation action is high during the contraction stage of the bubble. Regardless of the shell material and the acoustic pressure range, it happens all of the time (Postema and Gilja, 2011).

The process of facilitating cell membrane perforation, permeabilisation for a while and re-sealing the hole using acoustic energy is called sonoporation. Sonoporation is quite essential for targeted microbubble drug or gene delivery or therapy. Five mechanisms can explain sonoporation-induced drug or gene delivery: push, pull, jetting, shear and translation (see Figure 3.9).

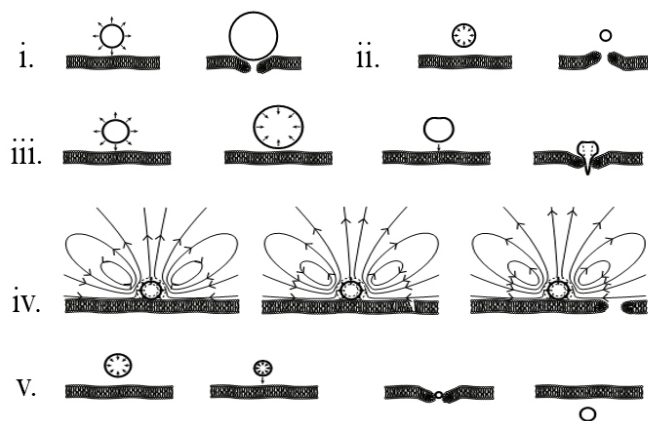


FIGURE 3.9:
Mechanisms of sonoporation
pushing (i), pulling (ii), jetting
(iii), shearing (iv) and
translation (v) (Postema,
2017).

If the microbubble is close enough to the cell membrane, it will push the cell membrane inwards during its expansion and pull outwards during its contraction. The pushing and pulling effect creates a hole through the cell membrane, but the discharging process requires another releasing mechanism. The third sonoporation mechanism, jetting, occurs at high acoustic MI range. It is good at creating a hole in the cell membrane and considered as the main sonoporation mechanism.

The moving fluid around the cell-fixed microbubble is responsible for the shear mechanism of the sonoporation. The fluid forces the oscillating microbubble to produce a shearing effect on the cell membrane and create a puncture. Similar to pulling and pushing sonoporation, another releasing mechanism is required to deliver the drug or gene.

Lipid encapsulated microbubbles might pass through the cell membrane (channels of the cell membrane) during the contraction stage of their oscillation. Since translation of the

microbubble requires low acoustic MI range, the damage that is caused by the internal cavitation of the bubble is rare (Postema, 2017).

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4

Detecting Micron-Scale Particles Using Digital Image Processing

It seems quite challenging to give a satisfactory definition for digital imaging using a single sentence. It covers vast areas (such as image acquisition, processing, storing, transmission and displaying) and touches several study disciplines (such as imaging physics, signal and image processing, imaging electronics, imaging instrumentation and computer vision). This chapter tries to cover, from a bird's eye view, the fundamental concepts of digital imaging and deeply reviews the state of the art micron-scale particle segmentation algorithms. Since the main significance of the thesis work is to enable researchers to track particles (sonicated blood cells and microbubbles) and study their behavior and interaction on time-lapse photographs of such events, the review mainly goes through detection/segmentation algorithms that are important for cell and particle tracking.

4.1 Digital Image

Image is the depiction, representation or reproduction of a real-world 3D scene or object (Bryan, 2010) onto a 2D, 3D or even 4D (time-varying 3D) ideal space using any kind of imaging device. The scene or the object could be hidden, visible or far from the imager or too small to be seen by the naked eye. An ordinary person might think cameras, video recorders, and scanners are the only devices capable of generating images. However, medical imaging

devices (such as X-ray, MRI, CT, ultrasound, SPECT and PET), optical devices (light microscopes and telescopes), special microscopes (electron microscope, scanning probe microscope, acoustic microscope and atomic force microscope) and aerial satellites can also produce images. Generally, the science of imaging is grouped into two broad categories: analog and digital imaging. Analog imaging is the traditional imaging technique and produces images that can be seen on paper, films, or CRTs. These days, analog images, specifically the ones based on film and CRT, appear to be out of the market and are totally overtaken by the digital ones (Dhawan, 2011; Russ, 2016).

The invention of computers and the ability to process analog images on computers are the main reasons why the concept of digital imaging came into existence. Roughly, digital imaging is defined as a field of study that deals with the acquisition, processing, displaying and storing of digital images (Dhawan, 2011).

A digital image is the representation of a real-world 3D scene or object on a discrete 2D or 3D space. It's also possible to produce artificial digital images using computers. Each discrete space is called pixel (picture element) when the representation is in two-dimensional space and voxel (volume element) when the representation occurs in 3D space. Assuming only 2D digital images, each pixel has its own discrete numerical value. This numerical value has a straightforward relationship with the intensity of the energy that is projected from the scene or the object. Equation 4.1 shows the mathematical (matrix) representation of a digital image (Dhawan, 2011; Marques, 2011).

$$I(x, y) = \begin{bmatrix} I(0, 0) & I(0, 1) & \cdot & \cdot & \cdot & I(0, N - 1) \\ I(1, 0) & I(1, 1) & \cdot & \cdot & \cdot & I(1, N - 1) \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot & \cdot \\ I(M - 1, 0) & I(M - 1, 1) & \cdot & \cdot & \cdot & I(M - 1, N - 1) \end{bmatrix} \quad (4.1)$$

where (x, y) stands for the coordinate of the pixel, $I(x, y)$ stands for pixel value (intensity) and $M \times N$ represents the total number of pixels that the digital image made up of.

4.1.1 Digital Image Formation

Formation of digital images requires three essential components: the object or scene, the energy source, and the imaging system that acquires, processes and displays the replica of the scene or the object (Marques, 2011). When it comes to medical image formation, the object or scene depicts internal organs of the body or microscopic images of the samples taken from the object; the energy sources can be electromagnetic or acoustic waves (acoustic waves in imaging explained in detail in Chapter 2); the imaging system includes the optical devices (such as mirrors and lenses), detectors and computers (for processing and displaying).

Electromagnetic waves are the main energy or radiation sources for many medical imaging modalities. Primarily, they interact with a biological medium in three ways. They may be reflected back (or scattered in random directions), transmitted through, or attenuated by the biological medium. The reflected or transmitted beam carries enough information about the targeted biological medium. For instance, magnetic resonance imaging uses radio waves to capture the anatomical and physiological features of a biological scene – it sends a radio wave to the patient, detects the reflected radio wave intensity, changes the intensity to electrical voltage and digitizes the electrical signal to form a digital image. On the other hand, X-ray and CT imaging devices use the transmitted x-ray intensity to record the information of the targeted biological organ or tissue.

In some medical imaging modalities, for example in nuclear imaging, the objects or the biological media act as the energy source. First, a radioactive element is administered to the patient targeting a specific organ or tissue. When the radioactive element reaches its destination, it emits a gamma ray that holds essential information about the targeted biological organ or tissue. The emitted radiation is then perceived by the external detector and converted into a digital image. PET and SPECT are categorized under this kind of medical imaging modality (Dhawan, 2011).

Optical devices such as lenses and mirrors are the main components of microscopes and cameras. Magnification of the biological scene, in addition to the redirection, diverging, and converging of the incident visible light beam towards the detector is achieved by the help of such optical devices. For example, ordinary light microscopes use lenses to magnify the specimen in question and mirrors direct the light beam to the desired direction (it could be the camera lens if the microscope is camera mounted) (Russ, 2016).

A detector is a device that changes the reflected, transmitted, or emitted electromagnetic radiation into electrical energy based on the intensity of the radiation to be perceived or interpreted as an image. The concept also works for ultrasound imaging – the reflected beam intensity is converted into electrical energy using piezoelectric detectors. In the imaging system, the position where the detector is placed varies from modality to modality. For example, the patient is placed between the source and the detector if the modality type is transmission-based; the source and the detector are placed at the same place if the imaging modality is reflection-based and the detector encircles the patient if the imaging modality type is emission-based (Dhawan, 2011).

The detector technology of imaging modalities has gone through some vivid technological advancements (from vacuum tubes to CCDs and complementary metal oxide semiconductors (CMOSs)). Understanding the working principle of charge coupled device (CCD) sensors might give a good insight towards present-day detector technologies. These detectors, CCDs, are filled with cells of light sensitive arrays called photosites. Each photosite will produce a voltage depending on the intensity of light that shines on it. The maximum intensity might be considered as bright or dark, and the minimum intensity will be the inverse. Four important parameters that describe the imaging detectors are resolution (the measure

of part of the scene that can be imaged on a single photosite), field of view (the size of the scene that can be recorded by the whole detector), sensitivity and signal to noise ratio.

Since the photosite-generated voltages are analog in nature, they need to be discretized and mapped onto a digital form – sampled in space and quantized in amplitude as illustrated on Figure 4.1. The sampling determines the total number of pixels that can be represented on the digital image and the quantization calculates the intensity or value of each pixel. The digitization process yields a rectangular matrix of pixels with corresponding pixel values. For example, the pixel values of 8-bit grayscale images are quantized between 0 and 255 (Marques, 2011).

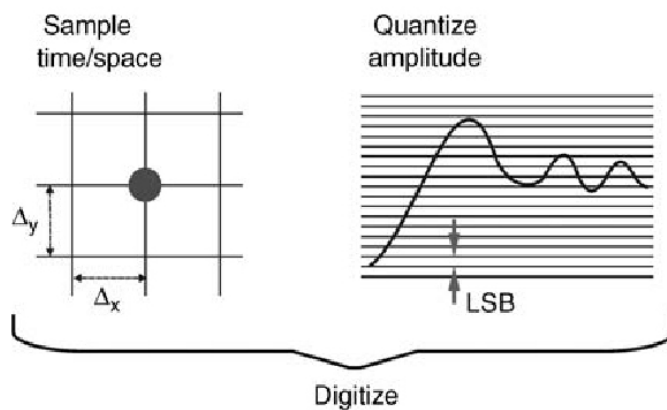


FIGURE 4.1:
Image digitization: spatial
sampling and quantization of
intensity (Marques, 2011).

4.1.2 Digital Image Types

The pixel values can be either a single element vector or a multi-element vector. Depending on the number of bits that represents the values of the elements of the vector, digital images are classified into three types: binary image, gray-level image and color image. The first two, binary and gray-level images, are represented using only a one-element vector whereas color images use either one-element vector and a look up table (LUT) or three-element vector. Figure 4.2 illustrates the stated digital image types.

Only one bit encodes the pixel values of a binary image, i.e. the data type of the image is logical (digital true or false). The brighter scene is represented by one, corresponding to a white color, while the darker scene is represented by zero, resulting in a black color. This kind of image representation is quite useful to set a clear delineation between the region of interest and the background, such as differentiating text from the background.

Most of the time, the pixel value of gray level images are represented by 8 bits, though there are also 16- and 32-bit representations. When the data type of the grayscale image is an unsigned integer represented by 8 bits, the minimum pixel value becomes zero (corresponding to a black color) and the maximum pixel value becomes 255 (corresponding to a white color). The rest, pixel values between 0 and 255, are filled with varying shades of gray.

RGB color images, sometimes also called true color images, require 24 bits per pixel to represent the three primary colors (red, green and blue), 8 bits for each color channel contained inside a single array. Each channel's pixel values range between 0 and 255, i.e. it allows the 2^{24} primary color combinations to form different varieties of true colors. Setting the pixel values of the red channel to 255 and the green and blue channels to zero produces a pure red color image – the concept works for all RGB color channels.

Another type of color image representation is indexing (pointing) the value of the pixel to the external color palette (color map) commonly known as the look-up table (LUT). This color palette is a $n \times 3$ matrix where the variable n is a pointer to the LUT and specifies the number of color variations. For instance, assume a pixel of an indexed color image has a value of 7 as shown on the bottom right side of Figure 4.2. The number 7 points to a specific color which is located at the 7th row of the LUT. The row contains three values whose values are ranged between 0 and 1 - 0.86 for red, 0.49 for green and 0.93 for blue (Marques, 2011).

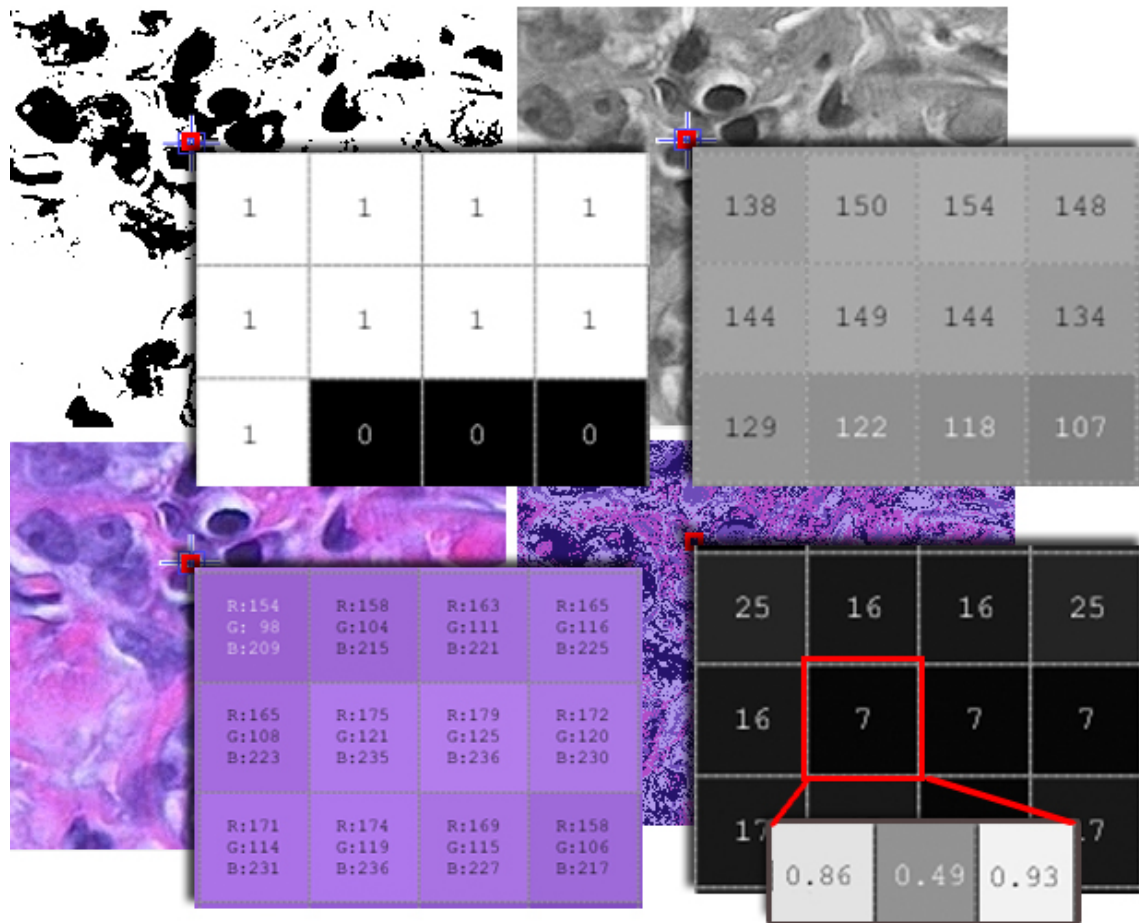


FIGURE 4.2: Types of digital images: binary (top-left), grayscale (top-right), RGB color (bottom-left) and indexed color (bottom-right). (Original image courtesy of Alan Partin, John Hopkins University)

4.1.3 Digital Image Processing

Different imaging technologies/modalities produce immensely large quantities of digital images within a short period of time. All the acquired digital images may not contain useful information, and worth to store, display or transmit from one place to the other. Thanks to the emergence of digital image processing, it helps professionals who work in the field of computer vision system to use the acquired images effectively and to use the computing resources efficiently.

Digital image processing is a manipulation of digital images using computers for the sake of improving the appearance of the image for human vision, making them suitable for storage and transmission and shaping them for measurement and analysis (essential for digging the information they contain) (Dhawan, 2011; Marques, 2011). To achieve these purposes, there is no clearly stated process flow. The process flow varies from one application to another application. It mostly relies on the knowledge of the computer vision expert. For common understanding though, digital image processing in computer vision system follows the process illustrated in Figure 4.3.

As a system, digital image processing requires an input image, and by using processing techniques that incorporate some kind of mapping function, a modified version of the input image is produced. Making a modification on the input image and producing a processed image is the responsibility of the mapping function. Depending on the type of mapping function used, digital image processing is classified into two types: global point operation and neighborhood-oriented operations. When the pixel values of the output image depend only on the corresponding pixel values of the input image, then the process is called global point operation. Operations such as global arithmetic, logical, histogram, geometric and gray-level transformation operations are categorized under this section. On the other hand, if the pixel value of the output image is depending on not only the corresponding pixel value of the input image but also the neighboring pixels, the process is called a neighborhood – oriented operation (or local operation). Special filters such as median and averaging filters are placed under this section.

The actual representation of digital images is in the spatial domain. However, sometimes it might be necessary to transform these spatial domain images into another domain such as frequency (e.g. cosine transform or wavelets) in order to make the processing easy and straightforward. Depending on the type of domain used, digital image processing is further classified into two forms: spatial domain and transform domain processing.

Digital image processing can also be classified as low-, middle-, and high-level processing depending on its complexity. Low-level digital image processing comprises all preprocessing operations such as contrast enhancement and noise reduction. Some images might be damaged by noise and their main content is corrupted, or some of their features might not be well emphasized or their contrast and illumination distribution are not suitable for human vision or even further machine processing. These challenges are the main reasons why

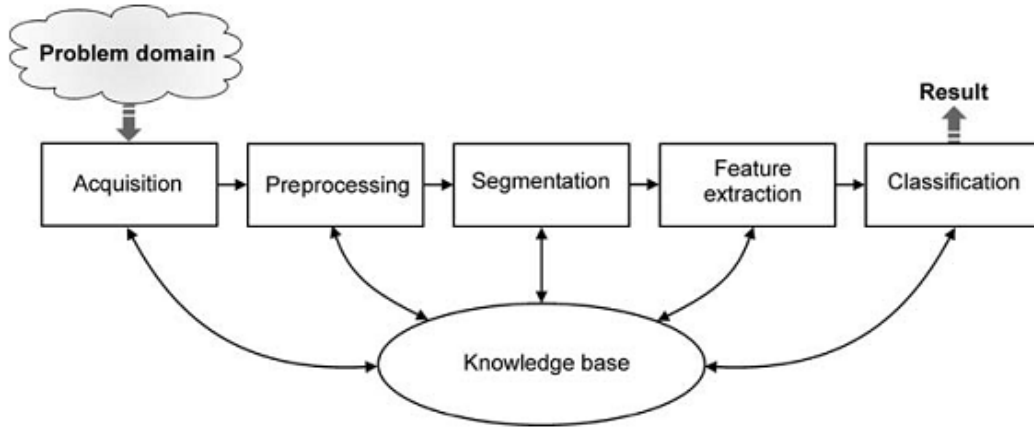


FIGURE 4.3: General process flow diagram of digital image processing in computer vision system (Marques, 2011).

preprocessing techniques are required for computer vision systems. Preprocessing operations transform images, making them useful for human eyes and suitable for the next part of image processing (mid-level digital image processing). Segmentation and extraction of attributes of the image such as creating contours or drawing edges around the region of interest on the image are categorized under mid - level digital image processing. The high level is the most advanced level. It helps the computer vision experts to take measurements, analyze and interpret the contents of the image (Marques, 2011).

Analyzing the available algorithms, the founding principle of color image and monochrome processing algorithms are almost the same. The majority of the color image processing algorithms are extensions of their gray-scale image counterparts. First, the channels of the color image are separated into individual monochrome images. Second, the monochrome algorithm is applied on each channel. And lastly, the processed monochrome images are recombined to form the processed color image. However, since all the channels do not contain the same information, applying the same monochrome algorithm might lead to information loss. To avoid such challenge, so far, two solutions have been proposed. The first solution is converting the RGB image into HSL (hue, saturation and luminance) to make it suitable for complex representation. However, the complex representation has its own drawback: it does not include luminance (intensity) component in its representation. The second potential solution is creating a representation of RGB images in the form of quaternions and trinions. This allows the color image to be processed holistically and avoids information loss by tackling the previous challenges. The respective Fourier transforms defined both in the trinion and quaternions domains allow holistic analysis of color images taking into account the intrinsic inter correlation information that is available within the different color channels. (Assefa and Krejcar, 2016; Assefa et al., 2011; Ell and Sangwine, 2007).

4.2 Micron-Scale Image Segmentation

Of the process flow diagram shown on Figure 4.3, segmentation is the most essential step in machine/computer vision system. It is a transition bridge between low level digital image processing and measurement, analysis or interpretation. Image segmentation is the chore of categorizing the most useful features of the image, region of interest (ROI), as foreground and the less useful features of the image as background. In other words, it is a process of partitioning the digital image into a set of objects whose union gives for the entire image (Dhawan, 2011; Marques, 2011).

Segmentation techniques are varied based on image type (such as binary, gray level or color), mathematical foundation (such as morphology, image statistics, graph theory), type of features (such as intensity, texture, pattern, color, motion) and approach (top down or bottom up or graph based). Yet, their final main goal is the same though - delineating a boundary between ROI and the background (Marques, 2011). So far, there is no clearly stated standard to categorize image segmentation techniques/algorithms.

Pham et al. (2000) tried to categorize digital image segmentation techniques into eight types: thresholding approaches, region growing approaches, Markov random field models (MRFM), classifiers, clustering approaches, artificial neural networks, deformable models and atlas guided models. The study presented a detailed explanation about the different approaches focusing on macro level image segmentation.

Another study by Meijering (2012) gave a summary of micro scale (cell) segmentation approaches that have been proposed since the 1960's. Based on the discussion presented in the paper, there are five such segmentation techniques: intensity thresholding, feature detection, morphological filtering, region accumulation, and deformable model fitting, and a comprehensive explanation of the methods was included.

A recent comprehensive manuscript by Russ (2016) has presented a detailed analysis on image segmentation techniques. The techniques discussed in this work were categorized into two broad classes: global and local. The former one assumes there is some kind of similarity between the features of interest when differentiating them from the background, i.e., it's quite easy to set a threshold between the foreground and the background. And the second one focuses on local relation of the pixels, i.e., the similarity and difference of the targeted pixel to the neighboring pixels.

Image segmentation algorithms also vary depending on the type of challenge need to be addressed. In general, determining a heterogeneous background, computing the signal to noise ratio, over-segmentation, under-segmentation, calculating the density of the object or ROI and properly updating the overall contrast of an image are considered the main challenges of image segmentation.

A simple search using key words "cell/particle" and "segmentation" or "particle/cell" on popular scientific work publishing websites reveals the availability of tremendous amount

of literature related to cell/particle segmentation and tracking. Since the main significance of segmenting time-lapse microscopic images is to link each and every cell and microbubble from frame to frame and study their motility, morphological change and interaction with each other or in the surrounding medium, only potential segmentation methods that are applicable for cell or particle (mainly for high density) tracking are briefly discussed in this thesis. Some cell or particle segmentation reviews are also presented.

Korzynska et al. (2009) made a comparison of three microscopic image segmentation techniques: reference method, morphological flattening and the watershed method. Of the three, the watershed method was found to be the best. The technique works as follows: first, a texture map is constructed from the image and secondly, a conventional watershed algorithm is applied. However, the watershed scheme requires a clear gradient difference between the objects and the background. When it comes to low magnification with highly dense particles, getting such a gradient difference is not guaranteed.

Meijering (2012) summarized the most common cell segmentation techniques of the past 50 years into five categories: intensity thresholding, feature detection, morphological filtering, region accumulation and deformable model fitting. The author discussed that when the magnification of the time-lapse images is relatively low, the particles or the cells cluster together and seem like one single object and makes the detection aspect challenging. For these type of hurdles, linear blob detectors such as the Laplacian of the Gaussian performs a relatively reliable job. But, they require post-processing procedures such as morphological closing, opening and/or filling.

For moderately high density of cells or particles Yang et al. (2016) proposed a segmentation technique called background reconstruction for a tracking project. Having about the same contrast was the assumption of the authors. They claimed that finding the difference of the two consecutive frames will remove the background and leave only the cells or the particles. Actually, the technique inclines towards background subtraction rather than construction and will not work when motility of the cells or the particles is relatively slow or no motion at all since the cells or the particles would be removed with the background. Likewise, Zhang et al. (2015) used somewhat similar approach but at this time instead of difference of frames, a previously stored background image was subtracted from the entire set of time-lapse images.

Wang et al. (2016) used level set active contour and a Circular Hough Transform (CHT) segmentation scheme to track highly dense colloids in time-lapse images with varying illumination. CHT is good enough to detect circle targets and less affected by noise. However, it misses main targets when the illumination is heterogeneous. Region scalable fitting (RSF) level set active contours are quite effective to handle such problems. The main drawback of the work was the computational time. It took about 28 minutes to segment and track a dataset (500 frames with a size of 256x336 pixels) using efficient processing machine (Intel Xeon 3.3 GHz CPU and 6 GB memory).

In addition, region scalable fitting level set active contours categorized under deformable model fitting can be used, but it requires prior knowledge about the object shapes to define an energy function. And, manual positioning of the initial contour is required (Brandes et al., 2015).

Brandes et al. (2015) presented the use of an unsupervised clustering algorithm, Gaussian mixture model (GMM) segmentation technique, to track non-rigid objects on time-lapse microscopic images. Obviously, the algorithm suffers the drawbacks of learned algorithms when the illumination is not consistent and the contrast is low. On their review, the authors emphasized this one important concept: that second derivative linear edge detection algorithms (Laplacian of Gaussian (LoG), Difference of Hessian (DoH), Difference of Gaussian (DoG)) are fast, easy to compute, and good enough to detect blobs.

To compare the state of the art tracking algorithms, an open particle tracking challenge was organized for the 2012 IEEE International Symposium on Biomedical Imaging (Chenouard et al., 2014). At the end of the challenge 14 teams submitted their tracking algorithm and results for comparison. For the detection part of the tracking algorithm different segmentation schemes were used to tackle different challenges (signal to noise ratio (SNR), dynamics and density). The segmentation schemes cover simple thresholding, local maxima finding, morphological processing, linear filtering (Gaussian, LoG and DoG), linear and nonlinear model fitting, centroid estimation and a combination of multiple schemes. Even though none of the tracking algorithms performed perfectly on all of the datasets, teams who used the LoG or Gaussian fit as their detection scheme took first place.

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5

Modified CLAHE and LoG Detector for Time-Lapse Microscopic Image Segmentation

Considering the literature review in Section 4.2 and intensive preliminary experiment on the available datasets using open source image analysis tool, **ImageJ**, and proprietary IDE, MATLAB, second derivative linear filters – especially Laplacian of Gaussian – is found to be quite effective towards the detection of time-lapse images of high density particles/cells and their clusters. Following the block diagram illustrated on Figure 5.1, this chapter explains the proposed procedures employed to segment time-lapse images of sonicated high density blood cells and ultrasound contrast agent microbubbles.

5.1 The Algorithm

The overall algorithm is designed to detect two objects (particles and clusters) separately and blend the detected objects together at the end of the process. To detect the smallest particles of the time-lapse images, only modified contrast limited adaptive histogram equalization (CLAHE) and array padding are applied. Conversely, detecting clusters requires a median filter before CLAHE and array padding.

The detector, LoG, is highly dependent on the size of the particles found on the time-lapse objects. Larger objects (clusters) need bigger sigma values while smaller objects (particles) need smaller sigma values. During the detection of the comparatively smaller clusters, some

of the smaller objects (particles) might be detected and act as a noise. To suppress them, there is a need to filter the time-lapse images using a 3×3 median filter.

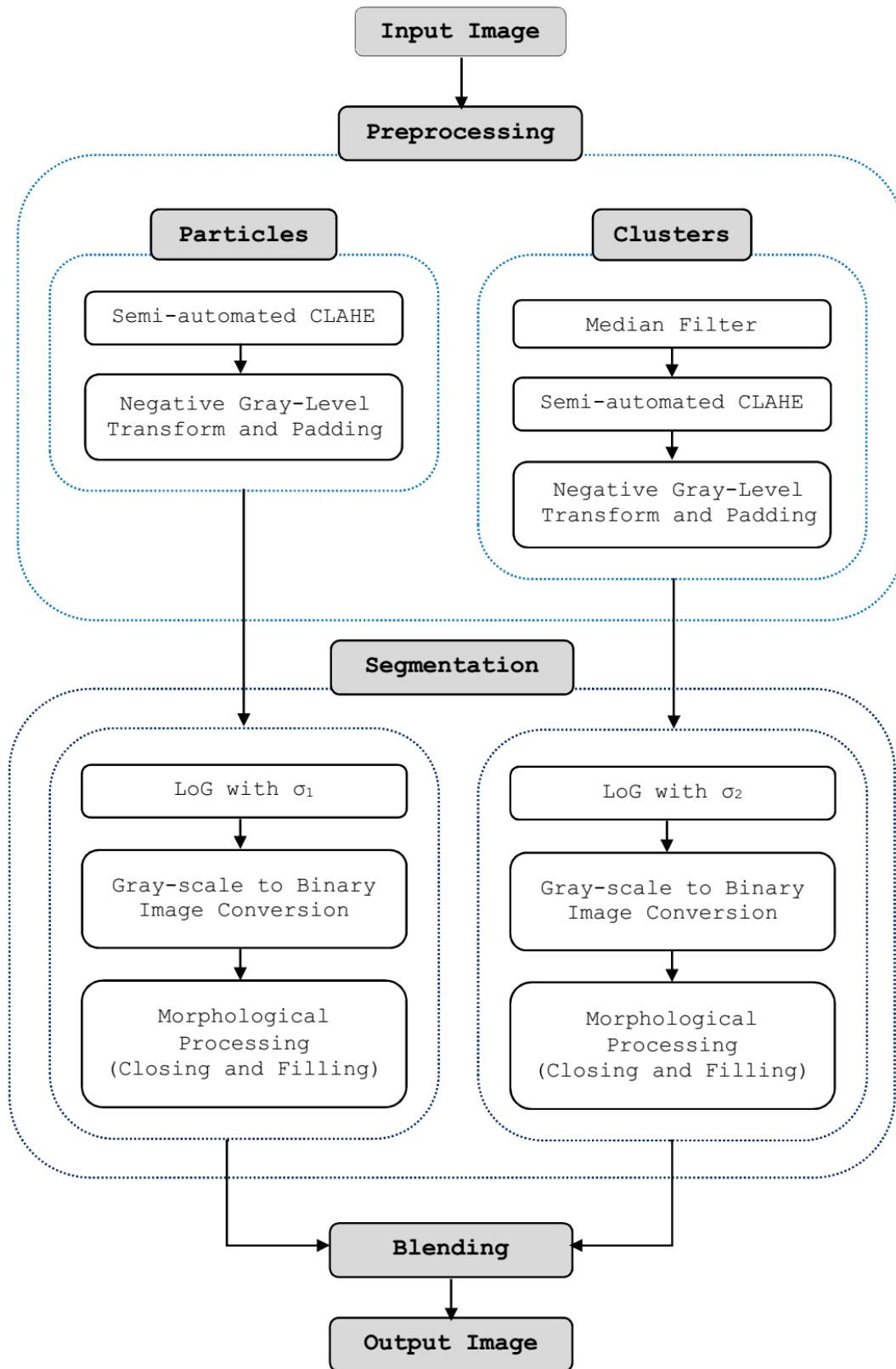


FIGURE 5.1: General block diagram of the proposed method.

The median filter replaces the center pixel with the median of the mask pixels as demonstrated on Figure 5.2.

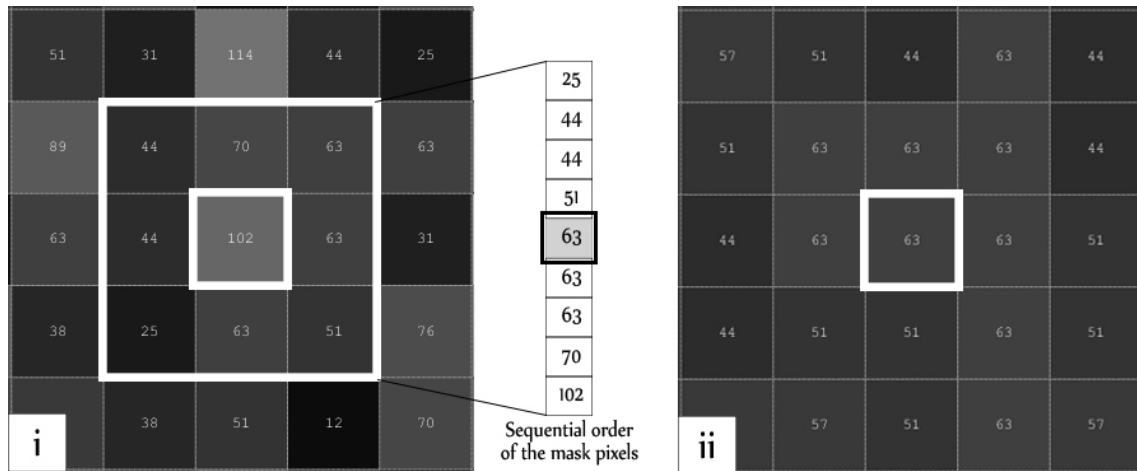


FIGURE 5.2: Replacing the gray value of the center pixel with the median of the mask. The larger white square represents the filter mask and the center white square represents the pixel that needs to be replaced (i). The output image after the center pixel value, 120, is replaced by the median of the mask, 63, (ii).

Due to the boundary effect of the LoG detector, particles or clusters at the edges of the image are not detected effectively. The simplest way of avoiding such a difficulty is padding all sides of the enhanced image with the median of the image, demonstrated on Figure 5.3. Padding each side of the time-lapse images with 50 pixels is sufficient for mitigating boundary effects.

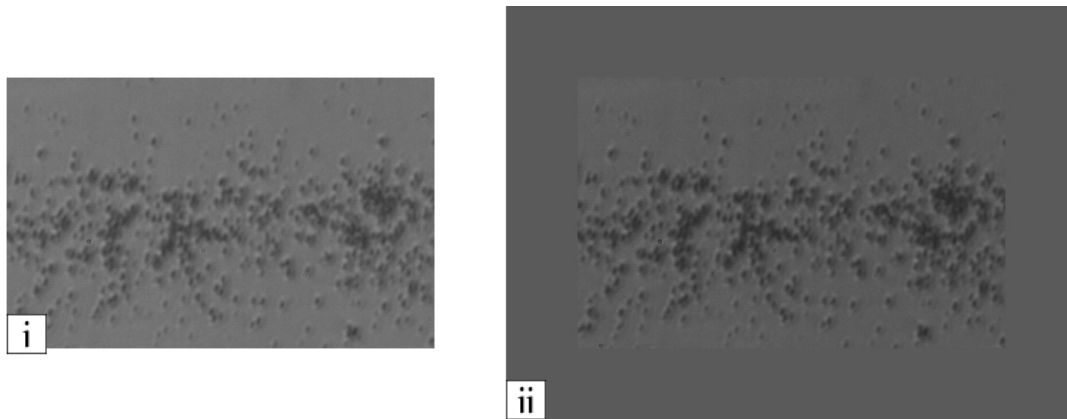


FIGURE 5.3: Padding the image to minimize the boundary effect of the LoG filter. Original image (i) and after padding with 50 pixels on each side (ii).

5.1.1 Contrast Limited Adaptive Histogram Equalization (CLAHE)

Adaptive histogram equalization (AHE) optimizes the contrast of an image locally using the concept of global histogram equalization (HE). First, the image needs to be partitioned into tiles (contextual regions) as illustrated on Figure 5.4. Then, the histogram equalization contrast enhancement technique is applied on every tile of the image. Finally, to avoid the

region boundaries between tiles, bilinear interpolation is applied on the boundary pixels (Pizer et al., 1987; Heckbert, 1994).

Both adaptive histogram equalization (AHE) and CLAHE techniques avoid the shortcomings of the global histogram equalization (HE) by optimizing the contrast of the image in question locally. These techniques follow three procedures, and the whole process, especially CLAHE, is depicted on Figure 5.4. First, the image need to be partitioned into tiles (rectangular contextual regions). Then, the histogram equalization enhancement technique is applied on each and every tile of the image. Lastly, to avoid the visibility of boundaries between tiles, bilinear interpolation is computed at the boundary pixels.

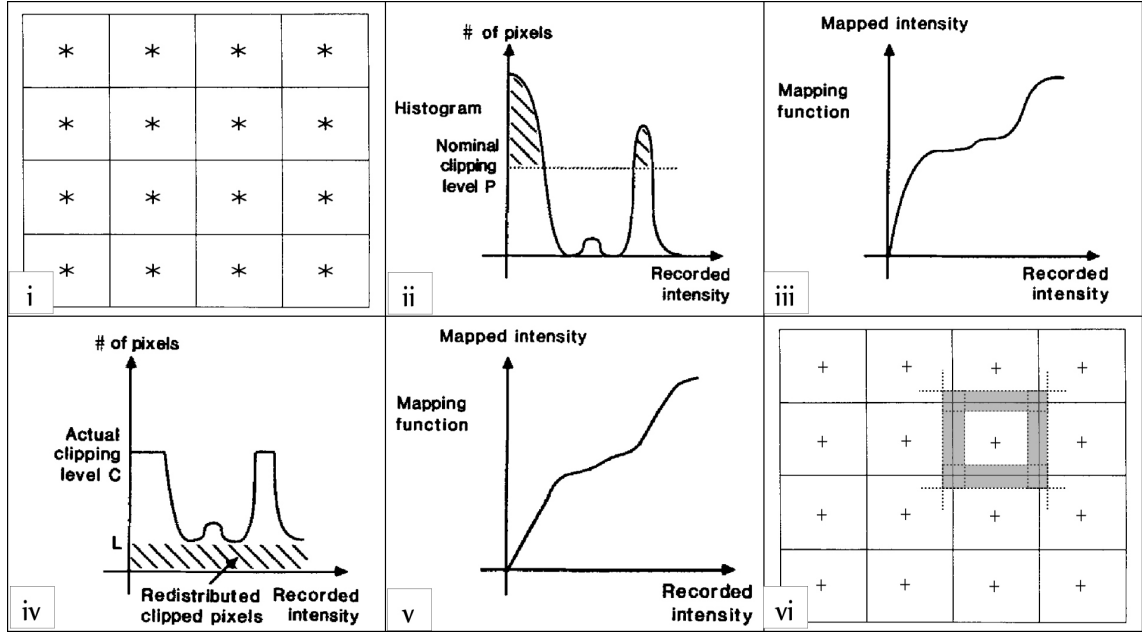


FIGURE 5.4: Contrast limited adaptive histogram equalization (CLAHE): contextual regions or tiles (i), histogram of the original image and its clip limit (ii), cumulative histogram before clipping (iii), clipped histogram (iv), cumulative histogram after clipping (v) and linear interpolation of tile border pixels (vi).

Histogram equalization is the process of redistributing the frequency of occurrence (histogram) of image gray-levels over the gray-level range in order to have a uniform or flat histogram distribution. As a system, any contrast enhancement algorithm needs an input image and a mapping function to produce the enhanced version of the input image. Here, the mapping function plays a pronounced role. The mapping function of histogram equalization uses the cumulative histogram of an input image as expressed mathematically in Equation 5.1 (Marques, 2011).

$$s_k = T(r_k) = \sum_{j=0}^k \frac{n_j}{N \times M} = \sum_{j=0}^k p(r_j), \quad 0 \leq k \leq L - 1 \quad (5.1)$$

where T stands for the mapping function, s_k stands for the transformed gray-level value of the original gray-level (r_k), n_j stands for the counts of j^{th} gray-level and N stands for the

total number of rows, M stands for the total number of columns and p stands for probability density function. The value of L represents the display range of the image (for example, its value becomes 256 for unsigned integer 8bit gray-scale images).

However, sometimes enhancing an image which has a highly uneven histogram distribution using global histogram equalization might lead to an unrealistic output image whose contrast is worse than the input image. In the input image, some gray-level values might have excessively high histogram peaks while some gray-level values might have small histogram peaks. Redistributing the highest peaks over the lowest histogram peaks using HE over-enhances the lowest peak gray-levels while under-enhancing the highest peak gray-levels, leading to a totally different output image. Figure 5.5-ii illustrates the scenario.

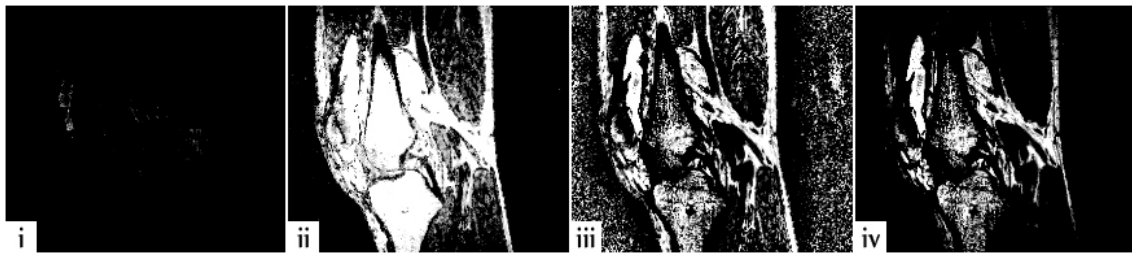


FIGURE 5.5: Effects of histogram equalization variations on an image of a human knee obtained using MRI: original image (i), contrast enhancement using HE (ii), AHE (iii) and CLAHE (iv) (Heckbert, 1994).

Adaptive histogram equalization rectifies the limitation of HE by reducing the problem domain locally, assuming the regions (tiles) have a somewhat ideal histogram distribution – at least, where the largest difference between the highest and the lowest histogram peak remains small. Even though AHE improves the contrast of the image under question much more effectively compared to the standard HE, still it has its own drawbacks. Most of the time, since the background pixels comprise the majority of the pixel count, they might over-enhance the background noise as demonstrated on Figure 5.5-iii. As a result, AHE images suffer from noise problems.

The remedy to the noise is the improved version AHE, contrast limited histogram equalization (CLAHE). The scenario is illustrated on Figure 5.5-iv. If a targeted contextual region (tile) is homogeneous, many of the pixels of the tile will be represented using only a small range of gray-levels rather the entire range. CLAHE controls the pixel value of the output image by clipping the highest histogram peaks and then equally redistribute the clipped pixels over the remaining gray-level ranges. In other words, clipping the histogram is achieved by limiting the slope of the mapping function (cumulative histogram). By definition, contrast enhancement is the slope of the mapping function that maps the input image to the output image, i.e. controlling the contrast of the image is the process of altering the slope of the mapping function. No enhancement occurs on the input image when the value of the slope is unity and greatly enhanced when the value of the slope is greater than one.

As stated in equation 5.2, the mapping function of the histogram equalization is a cumulative histogram. When it comes to AHE, the mapping function is modified slightly by adding two parameters: the size of the tile or region (RS) and the required gray-level display range (DR) of the output image. This change will modify Equation 5.1 as follows:

$$s_k = \frac{DR}{RS} T(r_k) = \frac{DR}{RS} \sum_{j=0}^k \frac{n_j}{NM} = \frac{DR}{RS} \sum_{j=0}^k p(r_j), \quad 0 \leq k \leq L-1 \quad (5.2)$$

Here, the ratio of the DR and RS becomes the slope of the mapping function (cumulative histogram) and differentiating this mapping function gives the histogram of the tile as shown in Equation 5.3.

$$\left(\frac{DR}{RS}\right) \frac{dT(r_k)}{dr_k} = \left(\frac{DR}{RS}\right) * histogram(r_k), \quad 0 \leq k \leq L-1 \quad (5.3)$$

That means, limiting the slope of the mapping function is another way of describing histogram clipping. Therefore, multiplying the slope with some constant number (x), as shown on Equation 5.4, provides a method to monitor the contrast of the image. The value of x is determined by the computer vision expert depending on the type of challenge that needs to be addressed.

$$(x) \left(\frac{DR}{RS}\right) \frac{dT(r_k)}{dr_k} = (x) \left(\frac{DR}{RS}\right) * histogram(r_k), \quad 0 \leq k \leq L-1 \quad (5.4)$$

When it comes to time-lapse images, assigning only one clipping factor for the whole image might not enhance the series of images as desired by the computer vision expert; moreover, finding individual clipping slopes for each and every image is quite tiresome.

To avoid this shortcoming, a new technique has been proposed in this thesis work - creating a relationship between the clipping factor of the CLAHE with the statistical properties of the image. After a series of trial and error experiments on Dataset I (see table 5.1), a working linear relationship between the clipping factors of the mapping function and the standard deviation of the image was found.

First, suitable clipping factors were experimented on the images that have the smallest and the largest standard deviation. Second, using the values of the two standard deviations (the smallest and the largest) and the corresponding mapping function clipping factors, a simple linear relationship was created. And lastly, the created linear relationship was applied on the entire time-lapse images to find a unique mapping clipping factor for every time-lapse image. To assist the experimenting process a simple graphical user interface (GUI) was created using ImageJ and MATLAB IDE. The code and the description of the GUI is given on Appendix B.2.

As illustrated on the Figure 5.6, images with a smaller standard deviations need higher mapping function clipping factors whereas images with higher standard deviations require smaller clipping factors.

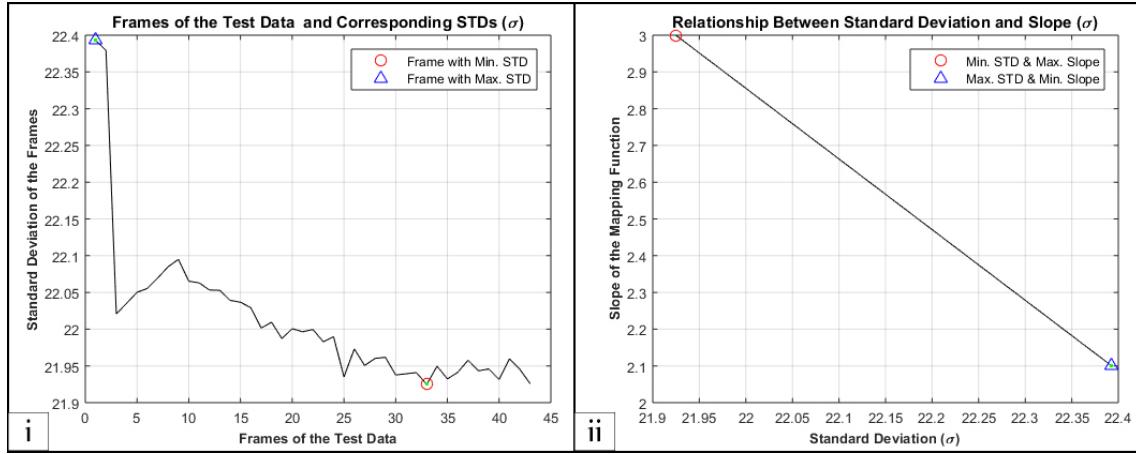


FIGURE 5.6: Frames of test dataset versus their standard deviations (STDs) (i) and relationship between the test time-lapse images standard deviation and corresponding mapping function slopes.

5.1.2 Laplacian of Gaussian (LoG) Edge Detector

As stated in Section 4.2, the intermediate section of the digital image processing comprises different categories of image segmentation algorithms. Computer vision experts consider the edge detection branch as the basic operation for detecting and outlining the most crucial boundaries of the image or the scene. The next image processing operations, advanced level processing, such as object counting, measuring, feature extraction and classification, are performed based on the outline created by the edge detector. In real-life scenes, images might exhibit heterogeneous illumination, various object sizes, noise, large contrast differences between the ROI and the background, and boundary effects which makes the detection operation quite challenging. As a remedy, preprocessing operations such as noise removal, illumination correction, contrast enhancement and padding are required.

The most commonly known edge detection algorithms utilize the first and the second derivatives of the intensity profile of the image. As demonstrated in Figure 5.7, the magnitude of first derivative edge detectors such as Roberts, Prewitt and Sobel are good at indicating of the presence of a boundary in the image. And, the sign of the second derivative edge detectors such as the Laplacian or the Laplacian of Gaussian indicates the state of the pixel (whether it is in the darker side or brighter side). In addition, the zero crossing between the positive and negative side can be considered as the center of the edge.

Among second derivative edge detectors, the Laplacian edge detector is isotropic (omni-directional), used to highlight rapid intensity changes and quite good at detecting blobs. However, it is highly sensitive to noise. Its variation, the Laplacian of Gaussian (LoG),

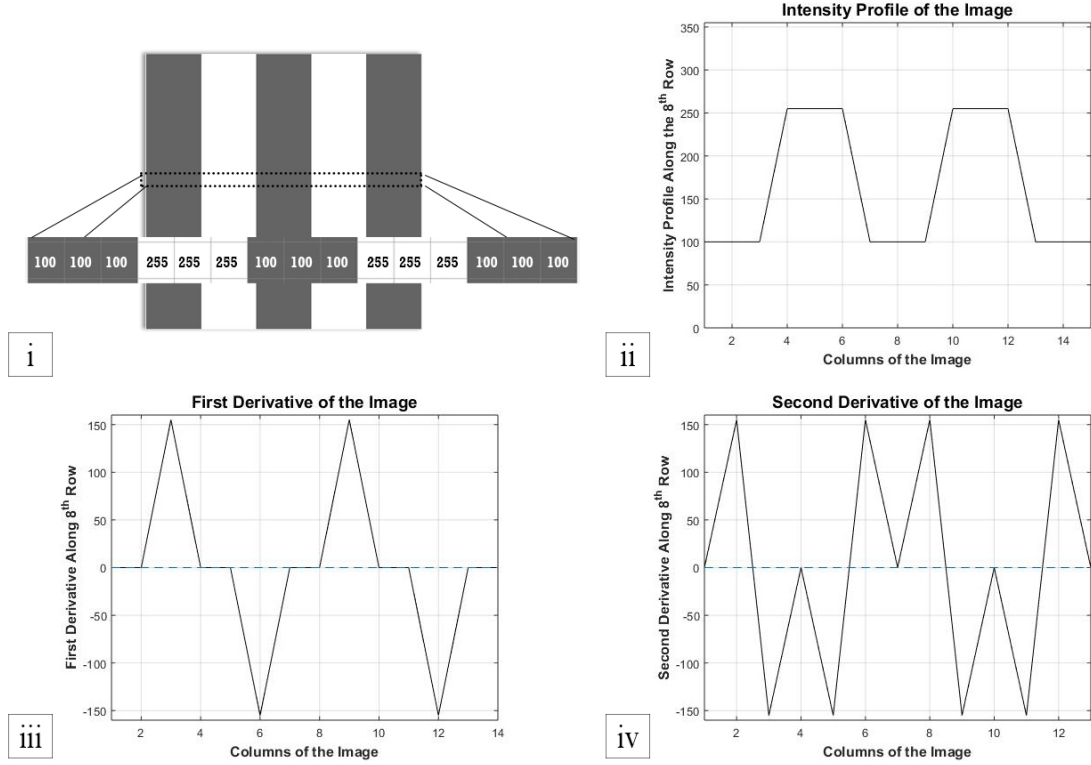


FIGURE 5.7: Derivative based edge detection: the intensity values of the 8th row of a simulated 15 × 15 grayscale image(i), actual(ii), first derivative (iii) and second derivative intensity profiles of the row.

suppresses the noise effect using a Gaussian low pass filter. The process of LoG edge detection can be achieved by either convolving the image with a Gaussian operator, $G(x, y : \sigma)$, (Equation 5.5) and applying the Laplacian operator (Equation 5.7), ∇^2 , on the result or directly convolving the image with the LoG operator (Equation 5.8), $\nabla^2 G(x, y : \sigma)$ (Kong et al., 2013).

$$L(x, y : \sigma) = I(x, y) * G(x, y : \sigma) \quad (5.5)$$

where $I(x, y)$ stands for the input image, $G(x, y : \sigma)$ stands for Gaussian operator (Equation 5.6), $L(x, y : \sigma)$ stands for the smoothened or filtered image and σ is the standard deviation of the the Gaussian operator.

$$G(x, y : \sigma) = \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\left(\frac{x^2+y^2}{2\sigma^2}\right)} \quad (5.6)$$

$$\nabla^2 = \frac{\partial^2 L}{\partial x^2} + \frac{\partial^2 L}{\partial y^2} \quad (5.7)$$

$$\nabla^2 G(x, y : \sigma) = \frac{x^2 + y^2 - 2\sigma^2}{\pi\sigma^4} e^{-\left(\frac{x^2+y^2}{2\sigma^2}\right)} \quad (5.8)$$

In addition to its smoothening effect, the value of the standard deviation of the Gaussian operator controls the size of the detected blobs. Equation 5.9 shows their estimated relationship. Moreover, the response of the Laplacian detector is strongly positive when the blob/object is dark and negative when the object/blob is bright.

$$\sigma = \frac{r}{\sqrt{2}} \quad (5.9)$$

where r is the value of the estimated blob radius.

5.2 Datasets

For this thesis work, three datasets have been used – one computer-simulated and two real datasets. The real datasets require some prior manual adjustments. First, only the part of the image that meets the objective of the thesis is cropped. Second, due to computational infrastructure limitation, randomly selected frames of the time-lapse images were tested, ensuring the inclusion of images with minimum and maximum standard deviations.

Dataset	No. of Frames	Extracted Frames	Magnification	fps ^a
Computer Simulated	20	20	-	-
Dataset I	43	43	-	-
Dataset II	6812	116	10×	2000

TABLE 5.1: Description of datasets used for the testing.

^afps - frame per second

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6

Results and Discussion

The proposed method has been tested on the three datasets considered in this thesis - one MATLAB simulated and two real time-lapse microscopic images. Toshiba Laptop (Intel Core i3 with a speed of 1.7GHz, 8GB RAM and Windows 8.1 Operating System), proprietary MATLAB IDE 2015a and open source ImageJ are the testing hardware and software platforms. The chapter presents the results of the algorithm, an objective evaluation on the simulated dataset, and a qualitative comparison of the proposed algorithm against other selected image segmentation algorithms previously proposed in the literature.

6.1 Test Results from the Datasets

The absence of ground truth (GT) for time-lapse microscopic images makes the evaluation of the proposed segmentation scheme merely subjective, i.e. evaluation of time-lapse microscopic images using objective metrics is quite difficult ([Chenouard et al., 2014](#)). As a result, it is required to generate synthetic time-lapse microscopy images using computers – this helps the computer vision expert not only to create the test images but also extract the ground truths of the time-lapse images at the same time.

For this thesis, 20 simulated time-lapse microscopy images were created using MATLAB (the code is found on Appendix [B.1](#)). The intensity of the background pixels was varied between 120 and 132. To simulate the illumination effect, all time-lapse images were partitioned horizontally into four sections and assigned a randomly distributed band of intensity values

for each partition (120-123 for the top, 120-126 for the second, 120-129 for the third and 120-132 for the last partition). Then, 600 particles with varying intensities (between 64 and 89) and radius (between 3 and 7 pixels) were dispersed randomly over the background. To put on the contrast effect, the final created time-lapse images were smoothed with a Gaussian filter (sigma value 3 pixels) to remove the sharp edges of the particles.

Generation of the ground truth follows the same procedure except the fixation of the background and the foreground intensity values to pure white (255) and pure black (0), respectively. Figure 6.1 demonstrates the procedure that is followed to create the synthetic time-lapse images and the ground truth (GT).

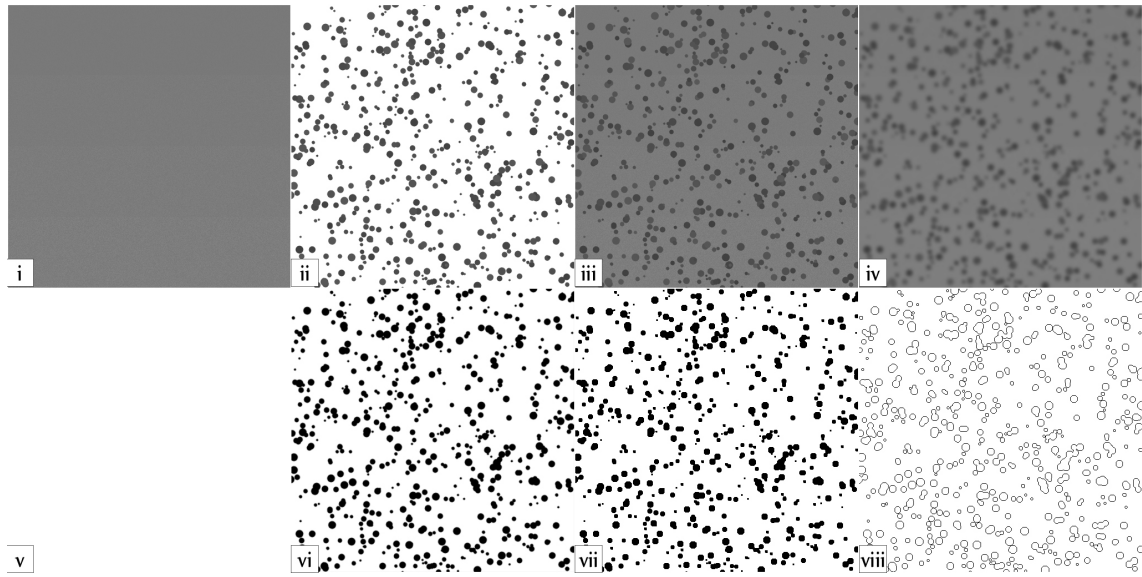


FIGURE 6.1: Synthetic dataset: background (i), particles on white background (ii), combined i and ii (iii) and smoothed with Gaussian blur filter (iv). Ground truth: gray-scale background (v), gray-scale particles over white background (vi), binary representation of ii (vii) and outline of vii (viii) .

Series of trial and error experimentation were performed on the images with minimum and maximum standard deviation to find the corresponding clipping factors of the CLAHE. Once the two clipping factors are found, the clipping factors for the rest of time-lapse images can be found by using a linear relationship (illustrated on Figure 6.2) created between the two pairs of standard deviations and clipping factors. The same procedure works for the other two datasets.

Using the assist of the GUI explained on Appendix B.2, the radius of the maximum blob-like structure of the synthetic dataset was found to be 9 pixels (since cluster formation is also possible in this synthetic dataset, it's ideal to get blob like structures that are larger than the individual particles) and its corresponding LoG sigma value was 6 pixels. As illustrated on the selected time-lapse images (Figure 6.3), the proposed scheme identified almost all the generated particles except some low contrast ones (particles which have an approximate intensity difference of 13-15 from the background).

The second dataset, Dataset I, includes images with minimum standard deviation(6.5031) at its 25th frame and its maximum (9.2469) at 2nd. The corresponding CLAHE clipping factors of these two images were 3.6 and 2.4, respectively. Similarly, using the GUI the actual radius of the blob-like structure was estimated at 17 pixels and its corresponding LoG sigma value was near 12 pixels.

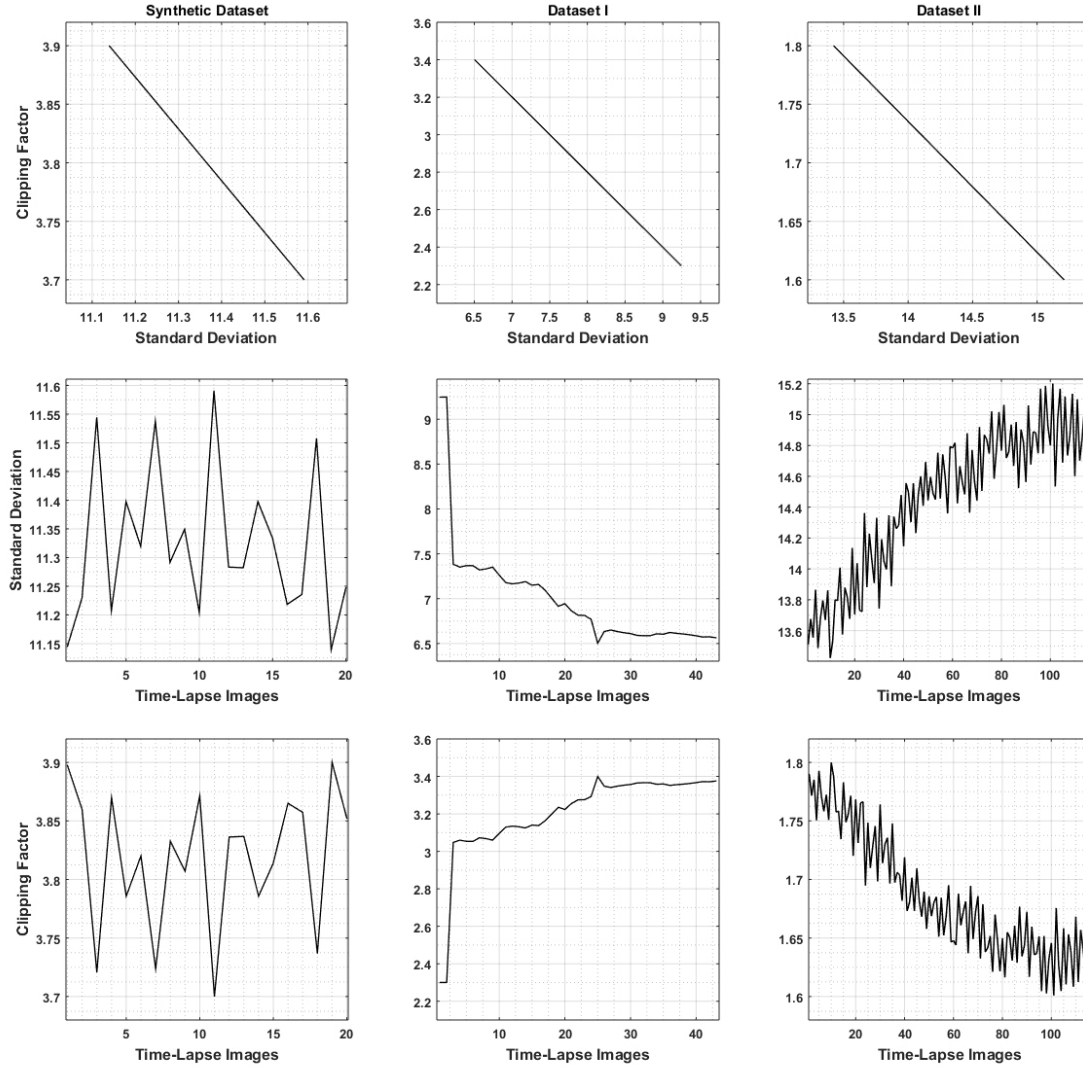


FIGURE 6.2: Relationship between standard deviation of the time-lapse images and clipping factor of CLAHE (top-row), time-lapse images and their corresponding standard deviations (middle-row) and clipping factor (bottom-row).

Analyzing the 43 time-lapse images of Dataset I subjectively, the proposed segmentation scheme correctly identified almost all the blob-like structures. Some blobs whose pixel values are nearly similar to the background failed to be detected but, in comparison with number of detected blobs, they are nearly null. Figure 6.4 illustrates the effect of the proposed segmentation scheme on the dataset.

The second real dataset, Dataset II, segmented effectively when the clipping factor of the

images with minimum and maximum standard deviations are fixed to 1.8 and 1.6, respectively and the maximum σ value for LoG detector to 8 pixels. The illustration on Figure 6.5 shows the performance of the proposed algorithm on the dataset.

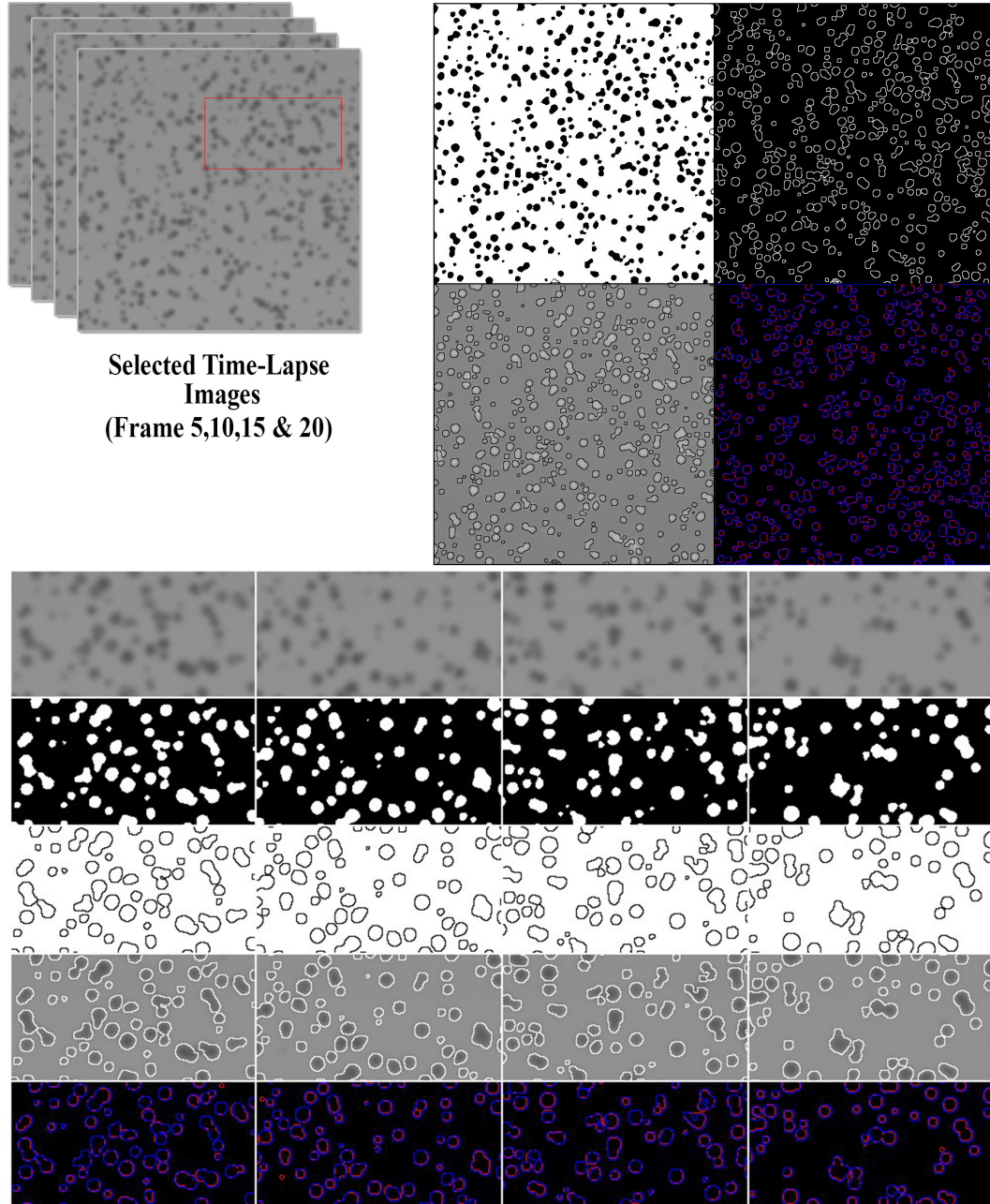


FIGURE 6.3: Result of the proposed segmentation scheme on the selected frames of the synthetic dataset. Most top-left: Stacks of selected frames, right to the stack: the proposed method on the 5th frame of the time-lapse images (black and white, outlined, overlaid and GT(red) over the method (blue)).

The bottom 5 rows - the zoomed version of the rectangle shown on the most top-left image stacks (red rectangle): input images (1st row), segmented black and white version (2nd row), segmented outline version (3rd row), segmented overlaid version (4th row) and the ground truth(red) and the method (blue) combined (last row) .

Fixing the clipping factors of CLAHE to a constant might give a clear insight why the linear relationship between the clipping factor and the standard deviation of the image is quite essential. Figure 6.6 illustrates the effect of having constant clipping factor for all time-lapse images. The use of large constant clipping factor might over-enhance the targeted time-lapse image and lead to noisy output. Low value constant clipping factors, on the other hand, under-enhance the image and the detector might fail to locate the required objects.

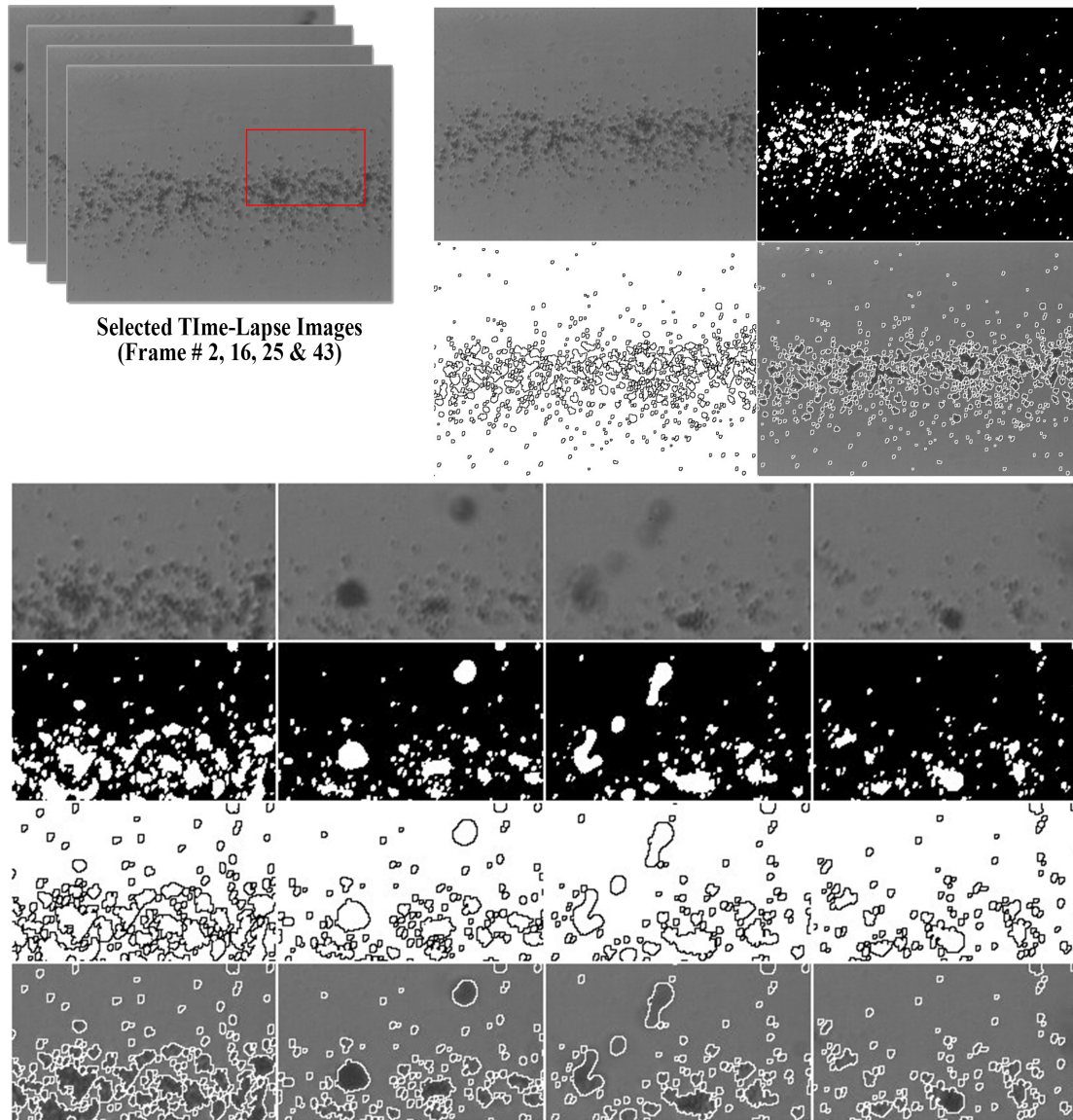


FIGURE 6.4: Result of the proposed segmentation scheme on the selected frames of the Dataset I. Most top-left: stacks of selected frames; right to the stack: the proposed method on the 2th frame of the time-lapse images (input, black and white, outlined and overlaid).

The bottom 4 rows - the zoomed version of the rectangle shown on the most top-left image stacks (red rectangle): input images (1st row), segmented black and white version (2nd row), segmented outline version (3rd row) and segmented overlaid version (4th row) .

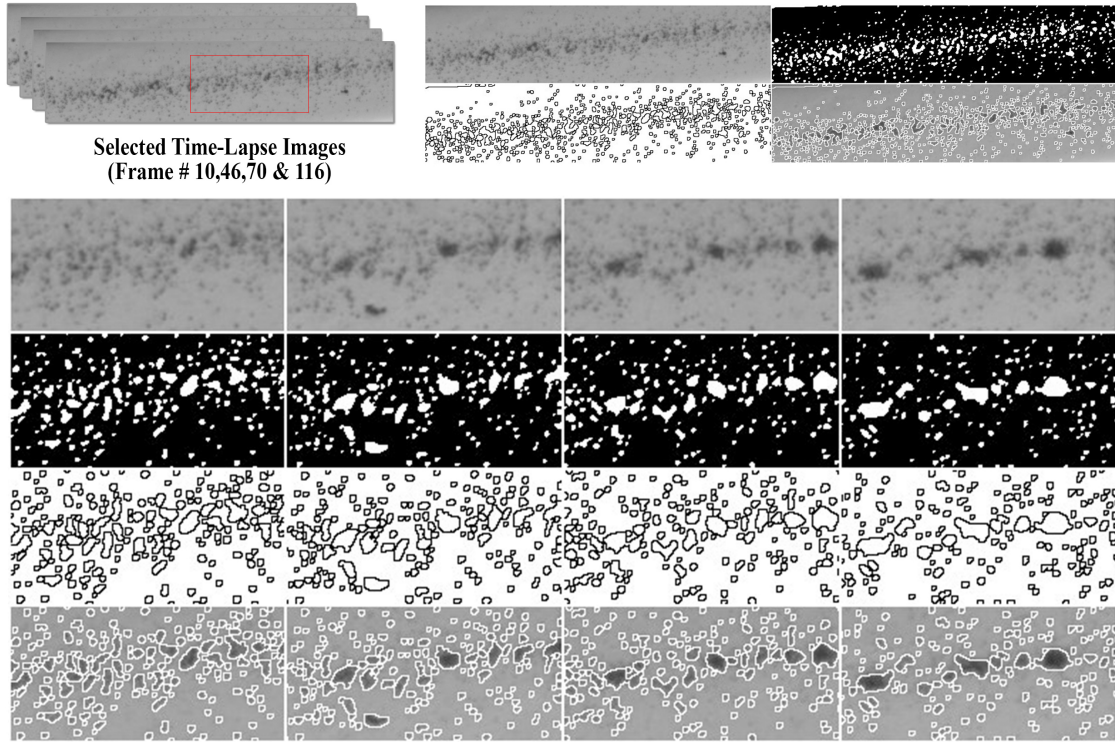


FIGURE 6.5: Result of the proposed segmentation scheme on the selected frames of the Dataset II. Most top-left: stacks of selected frames; right to the stack: the proposed method on the 10th frame of the time-lapse images (input, black and white, outlined and overlaid).

The bottom 4 rows - the zoomed version of the rectangle shown on the most top-left image stacks (red rectangle): input images (1st row), segmented black and white version (2nd row), segmented outline version (3rd row) and segmented overlaid version (4th row) .

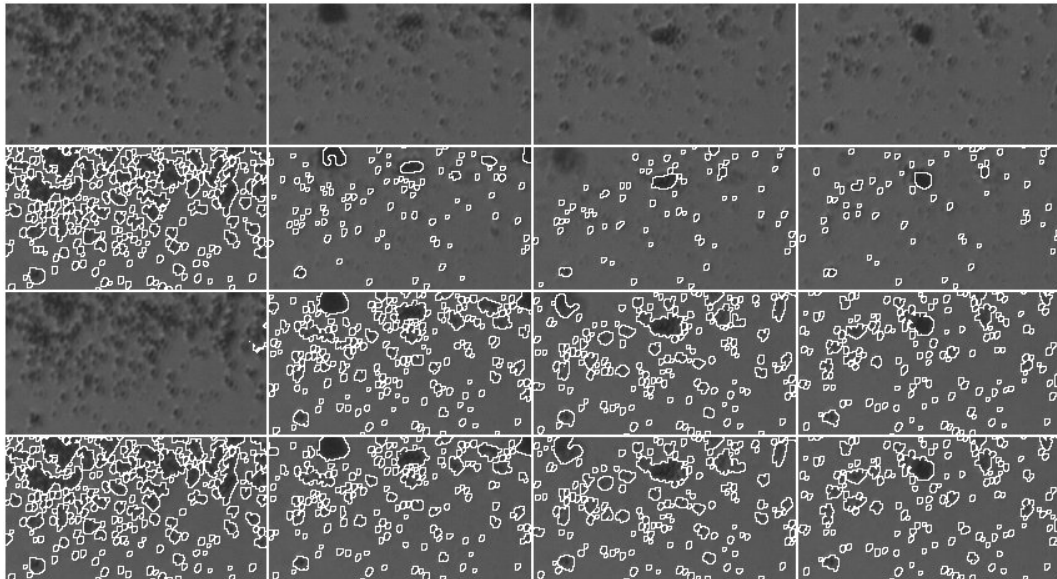


FIGURE 6.6: Effects of constant clipping factor: Input images - frame 2, 16, 25 & 43 (1st row), when the clipping factor of CLAHE is fixed to the smallest clipping factor of the method (2nd row), when the clipping factor of CLAHE is fixed to the maximum clipping factor of the method (3rd row) and the proposed method (4th row) .

6.2 Subjective Comparison

Figure 6.7 shows the subjective quality of the proposed segmentation scheme in comparison with potential ImageJ Otsu, Triangle, Yen and Gaussian Mixture Model (GMM). The pre-enhancement is kept the same for all segmentation algorithms.

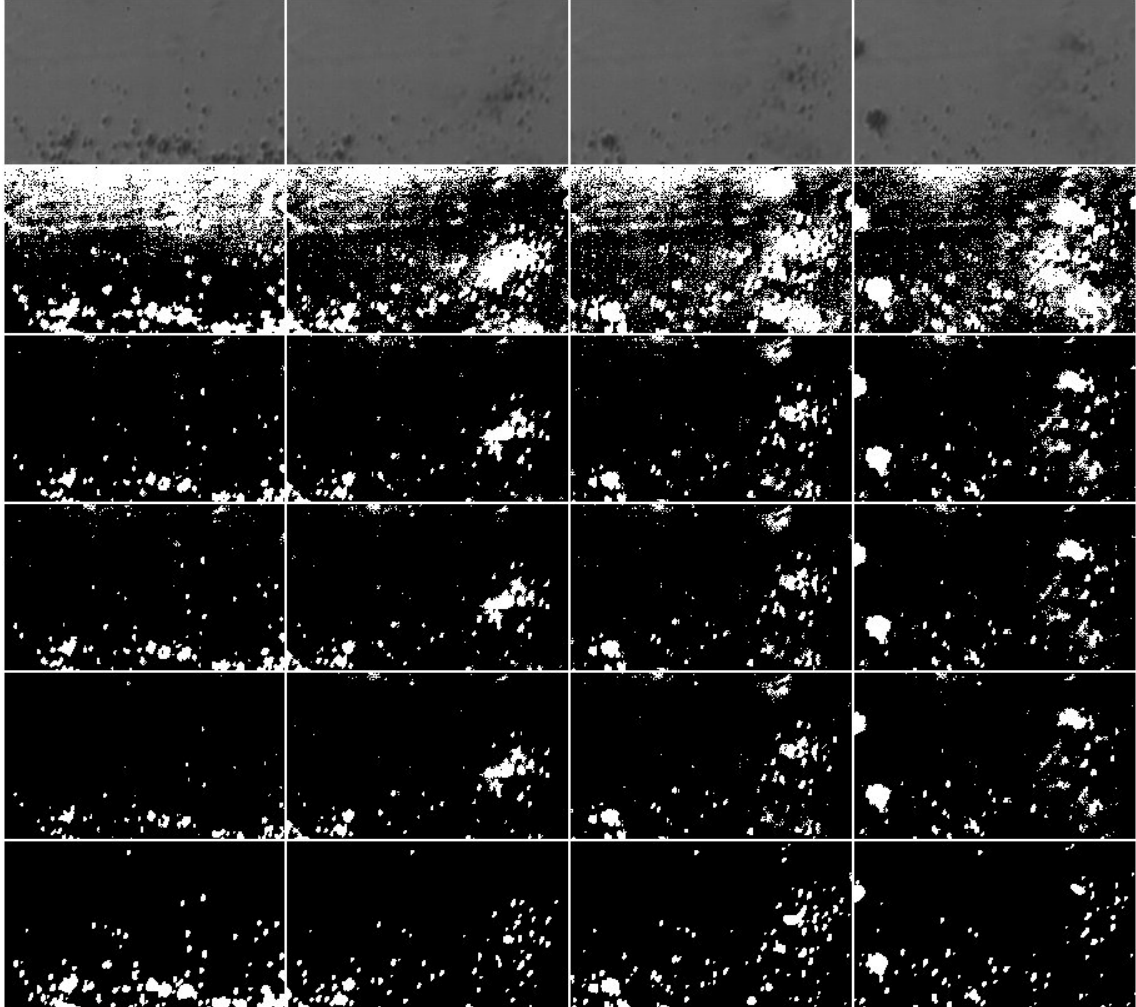


FIGURE 6.7: Different segmentation schemes: cropped input image (1st row), Otsu (2nd row), Triangle(3rd row), Yen (4th row), GMM (5th row) and the method (6th row) .

Visually, the last four rows seem quite similar. However, especially the third column reveals the drawbacks of the three segmentation schemes (Triangle, Yen and GMM). When the particles get more dense and the background intensity approaches the intensities of the particles, the algorithms failed to detect the particles individually and considered them as a cluster. Clearly, the Otsu algorithm could not segment such a complex dataset by itself. It is included in the comparison because it is simple, commonly known and considered as the first to be tested when new algorithms are designed.

6.3 Objective Evaluation

Objective evaluation of the efficiency of a segmentation algorithm without a ground truth is totally impossible. Here, only the computer simulated dataset is evaluated. For the evaluation four analysis metrics are used based on the assumption illustrated on Figure 6.8: Jaccard index (JI), Dice similarity index (DSI), true positive rate (TPR), true negative rate (TNR) and accuracy.

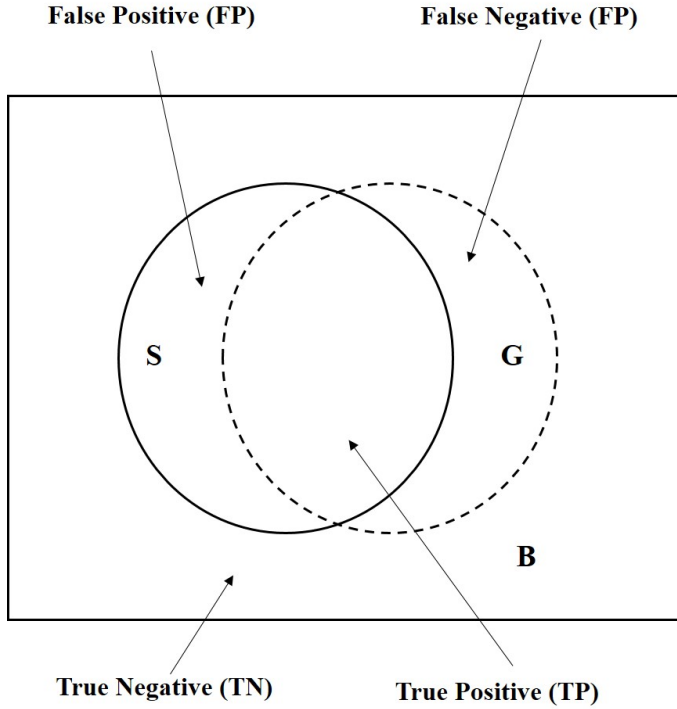


FIGURE 6.8:
Area enclosed by dashed lines represents the ground truth (G), area enclosed by the hard-line stands for the segmented result (S) and the remaining space represents the background (B).

The Jaccard index (*JI*) compares the segmented image pixels with the ground truth pixels to determine how many pixels are same and how many pixels are different. Its value lies between two values, 0 and 1. The value 1 shows the perfectness of segmentation algorithm and the vice versa. Mathematically,

$$JI = \frac{A(S \cap G)}{A(S \cup G)} \quad (6.1)$$

where $A(.)$ is an operation to count the pixels, S is the input (segmented) image and G stands for the reference image (ground truth) (Shi et al., 2014).

The second evaluation metrics, Dice similarity index (DSI), measures the spatial overlap between the segmented image and the ground truth. Similar to JI, DSI's value ranged between 0 and 1. When the number gets higher, the segmentation algorithm becomes highly efficient too. Mathematically (Zou et al., 2004),

$$DSI = \frac{2A(S \cap G)}{A(S) + A(G)} \quad (6.2)$$

The third, fourth and fifth evaluation metrics, true positive rate (TPR), true negative rate (TNR) and accuracy are employed to determine the sensitivity (Equation 6.3) and specificity (Equation 6.4) and accuracy (Equation 6.5) of the proposed algorithm, respectively. Sensitivity shows the ability of the algorithm to detect pixels which are part of the particles (foreground). Whereas, specificity describes the ability of the algorithm to identify non-particle (background) pixels as non-object (background) pixels (Wang and Zheng, 2013).

$$Sensitivity = \frac{TP}{TP + FN} \quad (6.3)$$

$$Specificity = \frac{TN}{TN + FP} \quad (6.4)$$

$$Accuracy = \frac{TP + TN}{TP + FP + FN + TN} \quad (6.5)$$

where TP , FN , TN and FP stand for true positive, false negative, true negative and false positive, respectively. The value of all the three metrics ranged between 0 and 1. As the value gets high, the performance of the algorithm gets high too.

Table 6.1 shows the objective evaluation result of the three ImageJ segmentation algorithms and the proposed method using the stated evaluation metrics. The proposed algorithm outperforms the three in JI, DSI, sensitivity and accuracy metrics. However, its specificity is the smallest one, though the average value (0.91) is respectable. The algorithm detection is not efficient enough to detect non-object pixels as non-object. But, the average accuracy (0.95) is quite promising.

Frame	Triangle					Yen					GMM					Method				
	JI ^a	DSI ^b	Se ^c	Sp ^d	Ac ^e	JI	DSI	Se	Sp	Ac	JI	DSI	Se	Sp	Ac	JI	DSI	Se	Sp	Ac
1	0.78	0.88	0.78	1.00	0.81	0.86	0.92	0.86	1.00	0.88	0.80	0.89	0.80	1.00	0.83	0.94	0.97	0.95	0.95	0.95
2	0.80	0.89	0.80	1.00	0.83	0.87	0.93	0.87	0.99	0.89	0.80	0.89	0.80	1.00	0.83	0.95	0.97	0.98	0.77	0.96
3	0.78	0.88	0.78	1.00	0.81	0.87	0.93	0.87	0.99	0.89	0.80	0.89	0.80	1.00	0.83	0.95	0.97	0.96	0.94	0.96
4	0.80	0.89	0.80	1.00	0.83	0.87	0.93	0.87	0.99	0.89	0.77	0.87	0.77	1.00	0.80	0.95	0.97	0.99	0.74	0.95
5	0.77	0.87	0.77	1.00	0.81	0.87	0.93	0.87	1.00	0.88	0.79	0.89	0.79	1.00	0.82	0.95	0.97	0.96	0.91	0.96
6	0.78	0.88	0.78	1.00	0.81	0.86	0.92	0.86	1.00	0.88	0.80	0.89	0.80	1.00	0.83	0.95	0.97	0.97	0.91	0.96
7	0.79	0.88	0.79	1.00	0.82	0.88	0.93	0.88	1.00	0.89	0.76	0.86	0.76	1.00	0.79	0.93	0.97	0.94	0.98	0.94
8	0.81	0.89	0.81	1.00	0.83	0.87	0.93	0.87	1.00	0.89	0.77	0.87	0.77	1.00	0.80	0.94	0.97	0.96	0.87	0.94
9	0.83	0.91	0.83	1.00	0.85	0.87	0.93	0.87	1.00	0.89	0.80	0.89	0.80	1.00	0.83	0.95	0.97	0.96	0.93	0.95
10	0.80	0.89	0.80	1.00	0.83	0.87	0.93	0.87	0.99	0.89	0.78	0.88	0.78	1.00	0.81	0.95	0.97	0.96	0.94	0.95
11	0.75	0.86	0.75	1.00	0.79	0.87	0.93	0.87	1.00	0.89	0.75	0.86	0.75	1.00	0.79	0.94	0.97	0.95	0.95	0.95
12	0.77	0.87	0.77	1.00	0.80	0.86	0.93	0.87	1.00	0.88	0.76	0.86	0.76	1.00	0.79	0.94	0.97	0.95	0.94	0.95
13	0.81	0.89	0.81	1.00	0.83	0.87	0.93	0.87	1.00	0.89	0.78	0.88	0.78	1.00	0.81	0.95	0.97	0.96	0.88	0.95
14	0.81	0.90	0.81	1.00	0.84	0.86	0.93	0.86	1.00	0.88	0.78	0.88	0.78	1.00	0.81	0.95	0.97	0.96	0.90	0.95
15	0.78	0.88	0.78	1.00	0.81	0.86	0.92	0.86	1.00	0.88	0.78	0.87	0.78	1.00	0.81	0.94	0.97	0.96	0.90	0.95
16	0.79	0.88	0.79	1.00	0.82	0.87	0.93	0.87	0.99	0.89	0.78	0.88	0.78	1.00	0.81	0.94	0.97	0.95	0.94	0.95
17	0.82	0.90	0.82	1.00	0.85	0.87	0.93	0.87	1.00	0.89	0.77	0.87	0.77	1.00	0.80	0.93	0.96	0.93	0.98	0.94
18	0.79	0.88	0.79	1.00	0.82	0.86	0.93	0.86	1.00	0.88	0.76	0.86	0.76	1.00	0.79	0.95	0.98	0.98	0.83	0.96
19	0.78	0.87	0.78	1.00	0.81	0.87	0.93	0.87	0.99	0.89	0.77	0.87	0.77	1.00	0.80	0.95	0.97	0.96	0.90	0.96
20	0.77	0.87	0.77	1.00	0.80	0.86	0.92	0.86	1.00	0.88	0.75	0.85	0.75	1.00	0.78	0.93	0.97	0.94	0.97	0.94
Average	0.79	0.88	0.79	1.00	0.82	0.87	0.93	0.87	1.00	0.89	0.78	0.87	0.78	1.00	0.81	0.94	0.97	0.96	0.91	0.95

TABLE 6.1: Objective evaluation of the algorithm on the synthetic dataset.

^aJaccard Index^bDice similarity index^cSensitivity^dSpecificity^eAccuracy

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7

Conclusion and Recommendation

This chapter sums up all the tasks accomplished in this thesis work, points out its limitations and mention gaps for future work.

7.1 Conclusion

The interaction of microbubbles with cells, each other, and the surrounding environment while subjected to ultrasound field is not fully understood yet. The occurrence of events at micro-level, the complex interaction, the speed at which the event occurs in conjunction with the imaging technology makes this study reasonably challenging. Optical imaging simplifies the challenge beyond one's imagination.

Designing microbubble-based ultrasound imaging contrast agents for the capillary bed, drug or gene capsules for drug or gene therapy and other unseen applications are the main reasons why studying the interaction of microbubbles with each other, with cells, and the surrounding medium is quite essential.

The microbubbles might burst into fragments, form clusters, translate towards the walls of the blood vessel or through cell membranes, form coalesce, or spit out their contents inside the cytoplasm or blood stream, or even push, pull or shear the cell membrane. Using optical imaging devices, a microscope and digital camera, large number of such activities can be captured and stored for offline analysis. The time-lapse images that are generated by the system are immensely large and it would be difficult to investigate each and every frame

manually. As a result, the need for automated or semi-automated computer software is mandatory.

This thesis focused on segmentation of sonicated blood cells and ultrasound contrast agent microbubbles from time-lapse microscopic images. To find suitable detection techniques, the state of art segmentation algorithms of low magnification and high density particles have been reviewed. Moreover, testing the dataset on the available ImageJ detection algorithms was also accomplished. After a series of trial and error experiments, the second derivative edge detector, LoG, performed comparatively well, visually.

The algorithm required preprocessing operations such as contrast enhancement and noise removal. The noise removal was achieved by a median filter and the contrast enhancement was done using a modified CLAHE method. Essentially, on this thesis the CLAHE was modified to be applicable for time-lapse microscopic images – a semi-automated linear relationship between the clipping factor of the CLAHE and the standard deviation of the time-lapse images was found.

Due to the unavailability of a ground truth for the real datasets that are employed for the thesis work, the evaluation of the performance of the algorithm was limited to subjective judgment – visual analysis. In reality, lacking a ground truth for real time-lapse images of high density and low magnification particles or cells is common.

In addition, to make the visual comparison ideal, other ImageJ segmentation algorithms (Otsu, Triangle, Yen and GMM) were also tested on the dataset. Indeed, the LoG detector is quite efficient, easy to compute and fast for high density and low magnification particle detection.

As for the objective evaluation, synthetic computer simulated time-lapse images were generated and evaluated using five metrics in comparison with three ImageJ segmentation algorithms. The proposed algorithm performed better than the rest with an overall accuracy of 0.95 from a unit scale.

7.2 Recommendation

Even though the segmentation scheme was designed to process large number of time-lapse microscopic images of sonicated blood cells and microbubbles, still, it remains semi - automated. The computer vision expert would first need to set the minimum and maximum clipping factors of the CLAHE and determine the upper sigma value of the LoG detector manually though the designed GUI simplifies the selection process. These two main challenges, automated identification of clipping factors and the upper sigma value, are open to be addressed in the future.

This thesis focused only on the detection part of the large project. Linking and taking measurements of the segmented objects are left for future work. Linking the segmented blood

cells, microbubbles and microbubble clusters from frame to frame is required to analyze and provide data such as their velocity, morphology change, and interaction with their surroundings.

These days, the advancement of optical technology allows the computer vision expert to capture images in color. Applying the proposed segmentation algorithm to color images requires a bit polishing. The color image should be separated into its channels, processed and recombined together. Or requires some kind of holistic color image processing algorithms. The recent potential holistic processing approaches, trinion and quaternion approaches, that are addressed by [Assefa and Krejcar \(2016\)](#); [Assefa et al. \(2011\)](#) might fill the gap.

References

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2011. The trinion Fourier transform of color images. *Signal Processing*, 91(8):1887–1900.



Further on Sound Wave

A.1 Ultrasound Speed in Selected Materials

Non-biological Material	Velocity (m/sec)	Biologic Material	Velocity (m/sec)
Acetone	1174	Fat	1475
Air	331	Brain	1560
Aluminum (rolled)	6420	Liver	1570
Brass	4700	Kidney	1560
Ethanol	1207	Spleen	1570
Glass (Pyrex)	5640	Blood	1570
Acrylic plastic	2680	Muscle	1580
Mercury	1450	Lens of eye	1620
Nylon (6-6)	2620	Skull bone	3360
Polyethylene	1950	Soft tissue (mean value)	1540
Water (distilled), 25°C	1498		
Water (distilled), 50°C	1540		

TABLE A.1: Approximate velocities of Ultrasound in selected biological and non-biological materials (Hendee et. al., 2003).

A.2 Some Fundamental Ultrasound Equations

1. Speed of ultrasound, c

$$c = v\lambda \quad (\text{A.1})$$

where, v is frequency and λ is wave length of the ultrasound wave.

2. Energy of ultrasound beam, E ,

3. Power of ultrasound beam, P

$$P = \frac{E}{t} \quad (\text{A.2})$$

where, E stands for energy and t stands for time.

4. Mechanical index, MI

$$MI = \frac{p_m}{\sqrt{v}} \quad (\text{A.3})$$

where, p_m stands for the maximum pressure in the medium and v stands for the frequency of the ultrasound beam.

5. Intensity of ultrasound beam, I ,

$$I = \frac{P}{A} = \frac{p_m^2}{2\rho c} \quad (\text{A.4})$$

where, P stands for power, A for area where the beam concentrates, p_m^2 for maximum pressure in the medium, c for speed of the beam and ρ for density of the medium. However, it's a common practice to express the intensity level of beams of ultrasound in decibels, dB . Decibels is just the ratio of the intensity of the incident beams to some reference intensity in logarithmic scale.

$$dB = 10 \log \frac{I}{I_o} = 10 \log \frac{E}{E_o} = 10 \log \frac{P}{P_o} = 20 \log \frac{p_m}{p_o} \quad (\text{A.5})$$

where, I , E , P and p_m are incident beams intensity, energy, power and maximum pressure respectively; and I_o , E_o , P_o and p_o stand for reference intensity, energy, power and reference pressure of the ultrasound respectively.

6. Acoustic impedance of materials, Z ,

$$Z = c\rho \quad (\text{A.6})$$

where, c is speed of of ultrasound beam and ρ is density of the transmitting medium. Acoustic impedance mismatch of the interfacing mediums determine the reflection

and transmission of the beam. Mathematically, reflection coefficient, α_R , and transmission coefficient, α_T , are expressed as follows

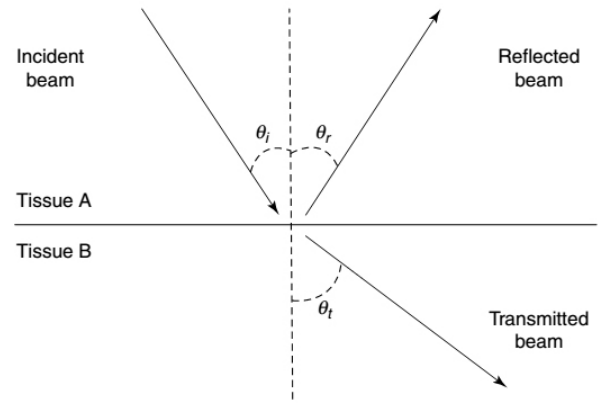
$$\alpha_R = \left(\frac{Z_2 - Z_1}{Z_2 + Z_1} \right)^2 \quad (\text{A.7})$$

$$\alpha_T = \frac{4Z_2Z_1}{(Z_2 + Z_1)^2} \quad (\text{A.8})$$

where, Z_1 and Z_2 are the impedances of the interfacing mediums. And, ideally, $\alpha_R + \alpha_T = 1$.

7. Beam reflection and refraction (Snell's law)

FIGURE A.1: Beam reflection and refraction (Snell's law) when ultrasound beam hits the interface of two tissues which have different acoustic impedances, i.e, speed of the incident beam, c_i , is different from the speed of transmitted beam, c_t .



$$\theta_i = \theta_r \quad (\text{A.9})$$

$$\frac{\sin \theta_i}{\sin \theta_t} = \frac{c_i}{c_t} \quad (\text{A.10})$$

where, θ_i , θ_r and θ_t are the angles of the incident, reflected and transmitted (refracted) beams measured from the normal of the tissues interface respectively.

8. Doppler shift frequency, v_D

$$v_D = 2v_o \left(\frac{s}{c} \right) \cos \theta \quad (\text{A.11})$$

where, v_o is frequency of the source ultrasound beam, s is speed of the moving object, c is the speed of ultrasound wave and θ is the angle between the moving object and the ultrasound transducer.

A.3 Acoustic Impedances of Materials

Material	Acoustic impedance ($\text{kg}/\text{m}^2\text{s} \times 10^{-4}$)	Material	Acoustic impedance ($\text{kg}/\text{m}^2\text{s} \times 10^{-4}$)
Air at standard temperature and pressure	0.0004	Water	1.50
Aluminum	18.0	Mercury	19.5
Brass	38.0	Fat	1.38
Aqueous and vitreous humor of eye	1.50	Brain	1.55
Blood	1.61	Kidney	1.62
Human soft tissue, mean value	1.63	Spleen	1.64
Liver	1.65	Muscle	1.70
Lens of eye	1.85	Skull bone	6.10

TABLE A.2: Acoustic impedances of some biological and non-biological materials.

B

Implementation Code

B.1 Code for the Synthetic Dataset and Its GT

```

1      % Download the export_fig function from MathWorks, extract it into a ...
      specified directory and add to the code as follows.
2      addpath(['Directory'])
3      % Clear the variables, close all the opened figures and clear the ...
      command window
4      clear
5      close all
6      clc
7      % rng helps to generate the same type of time-lapse images all the time.
8      rng(0,'twister');
9      % Generate the background of the time-lapse images
10     numOfFrames=20;
11     for i=1:numOfFrames
12         a = [120 120 120 120];
13         b = [123 126 129 132];
14         r1 = (b(1)-a(1)).*rand(128,512) + a(1);
15         r2 = (b(2)-a(2)).*rand(128,512) + a(2);
16         r3 = (b(3)-a(3)).*rand(128,512) + a(3);
17         r4 = (b(4)-a(4)).*rand(128,512) + a(4);
18         I(1:128,:,i)=uint8(r1);
19         I(129:256,:,i)=uint8(r2);
20         I(257:384,:,i)=uint8(r3);
21         I(385:512,:,i)=uint8(r4);
22         f=figure ('Visible','off','name','final');

```

```

23     imshow(I(:, :, i))
24
25     % Generate the particles that will be overlaid on background
26     nParticles = 600;
27     radii = zeros(nParticles, 1);
28     pos = zeros(nParticles, 2);
29     a = 0.25;
30     b = 0.35;
31     for iParticles=1:nParticles
32         allColours(iParticles,1) =(b-a).*rand(1, 1)+a;
33     end
34     allColours(:,2) = allColours(:,1);
35     allColours(:,3) = allColours(:,1);
36     for idx = 1:nParticles
37         pos(idx, :) = rand(1, 2)*2 - 1;
38         radii(idx) = rand * (1);
39     end
40     posmax = max(pos(:));
41     posmin = min(pos(:));
42     posnormalized = int16(512.0*double(...
43         (pos-posmin))/double((posmax-posmin)));
44     rmax=7;
45     rmin=2;
46     radiimax = max(radii(:));
47     radiimin = min(radii(:));
48     radiinormalized=int16((rmax-rmin)*(...
49         radii-radiimax)/(radiimax-radiimin)+rmax);
50
51     hold on
52     set(gca, 'visible', 'off')
53     % % Uncomment the following lines to generate the ground truth
54
55     % daspect([1, 1, 1])
56     % rectangle(...
57     %     'Position',[0 0 512 512],...
58     %     'Curvature', [0 0],...
59     %     'FaceColor', 'white',...
60     %     'EdgeColor', 'none');
61
62     for idx = 1:nParticles
63         rectangle(...
64             'Position',[posnormalized(idx, 1) - radiinormalized(idx), ...
65                 posnormalized(idx, 2) - radiinormalized(idx), ...
66                 2*radiinormalized(idx), 2*radiinormalized(idx)],...
67             'Curvature', [1 1],...
68             'EdgeColor','none',...
69             'FaceColor', allColours(idx,:)); % Change allColours(idx,:) ...
70             with 'black' to generate the background
71     end
72
73     % % Comment the following six lines to generate the ground truth
74     export_fig(['Frame' num2str(i)], '-tif');

```

```

72     h=imread(['Frame' num2str(i)], 'tif');
73     p=imgaussfilt(h,3);
74     hold off
75     imshow(p)
76     export_fig(['Frame',num2str(i)], '-tif')
77
78     % % Uncomment the following code to generate the ground truth
79
80     % export_fig(['GT' num2str(i)], '-tif');
81     % h=imread(['GT' num2str(i)], 'tif');
82     % p=im2bw(imgaussfilt(h,0.5));
83     % BWoutline = bwperim(p);
84     % hold off
85     % imshow(p)
86     % export_fig(['GTFilled',num2str(i)], '-tif')
87     % imshow(BWoutline)
88     % export_fig(['GTOutlined',num2str(i)], '-tif')
89     end

```

B.2 The GUI and its Code

Before using the GUI listed codes, it is required to download and appropriately setup MIJ java package (a package to integrate ImageJ with MATLAB) following the instruction stated on this [website:https://imagej.net/Miji](https://imagej.net/Miji). After the setup, initialize the package globally as follows. The directory need to be changed depending on the platform setting.

```

1  javaaddpath('C:/Program Files/MATLAB/MATLAB Production ...
    Server/R2015a/java/mij.jar')
2  addpath('C:/Users/Mamush-2016/OneDrive/Fiji/Fiji.app/scripts')
3  addpath('C:/Users/Mamush-2016/OneDrive/Dataset + Code/Code ...
    Folder/October/Final Project Design/Final/The Algorithm')
4  Miji(false);

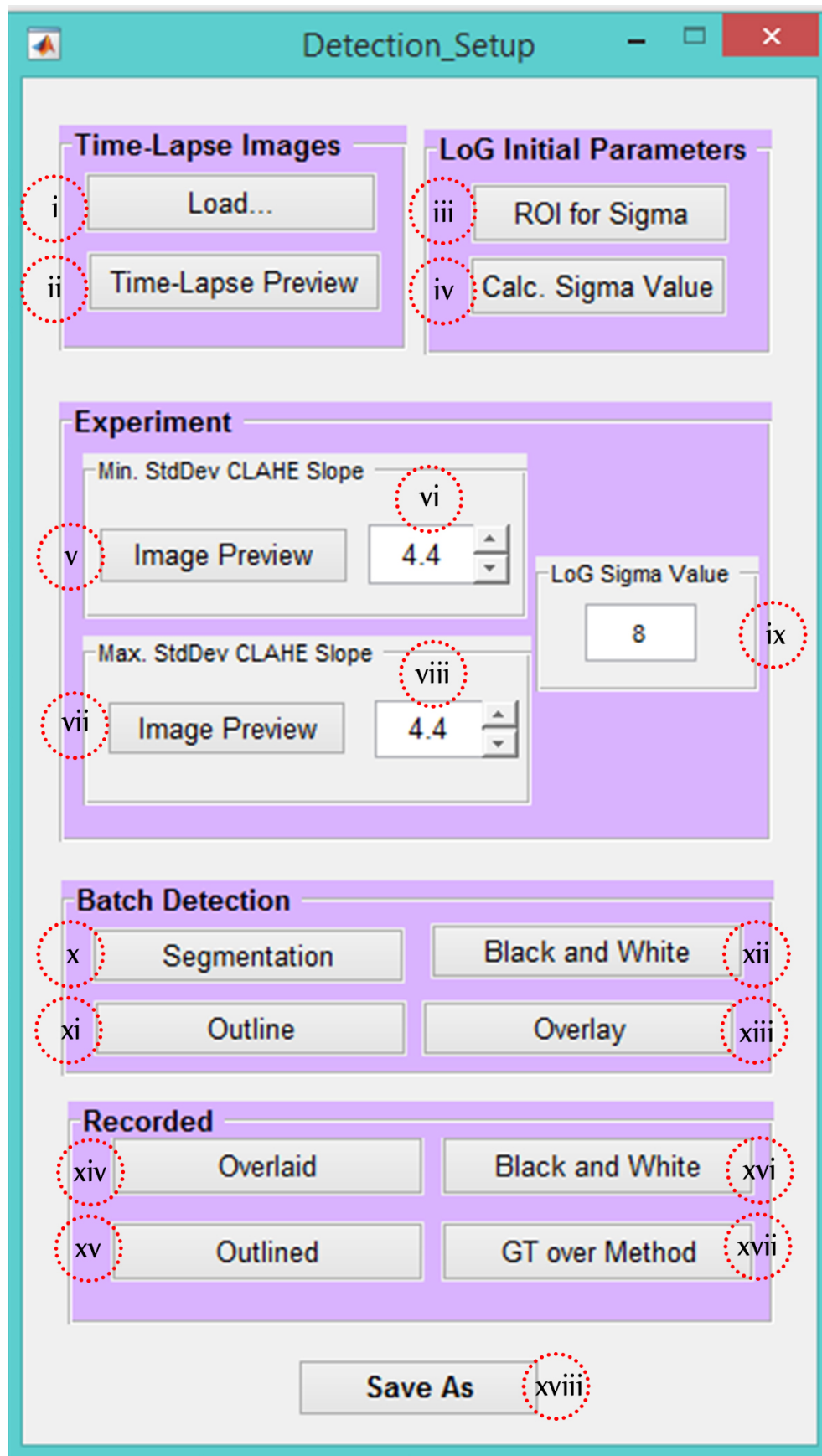
```

The following variables also need to be initialized globally inside the opening function of the GUI.

```

1  %Padding pixel Value
2  handles.n=50.5;
3  %Parameters for the CLAHE
4  handles.blocksize=127; handles.histogram_bins=256;handles.mask='*None*';
5  % Parameters of the LoG detetor
6  handles.sigmal=3;
7  handles.filterwidth=3;
8  handles.threshold=0;
9  handles.Δ=0;handles.mode=4;

```

FIGURE B.1: *The GUI.*

i. Loads time-lapse images

```

1 function loadImageSequenceButton_Callback(hObject, eventdata, handles)
2     MIJ.run('Image Sequence...')
3     I=uint8(MIJ.getCurrentImage);
4     handles.I=I;
5     MIJ.closeAllWindows
6     [handles.r handles.c handles.h]=size(I);
7     for i=1:handles.h
8         MIJ.createImage(uint8(I(:, :, i)));
9         MIJ.run('Measure');
10        Measurment=MIJ.getResultsTable;
11        StdDev(i)=Measurment(i,3);
12        MIJ.run('Close All');
13    end
14    [MinStdDev indmin]=min(StdDev);
15    handles.indmin=indmin;
16    handles.MinStdDev=MinStdDev;
17    handles.MinStdDev=MinStdDev;
18    [MaxStdDev indmax]=max(StdDev);
19    handles.indmax=indmax;
20    handles.MaxStdDev=MaxStdDev;
21    guidata(hObject, handles)

```

ii. Shows the preview of the loaded images

```

1 function PreviewButton_Callback(hObject, eventdata, handles)
2     if (isfield(handles, 'I'))
3         MIJ.createImage('Preview', uint8(handles.I), true);
4     else
5         errordlg('Load the time-lapse images first!', 'Error Msg. ');
6     end

```

iii. Helps to draw a re-sizable circular blob structure on the previewed time-lapse images in order to determine the upper sigma value.

```

1 function SelectROI_Callback(hObject, eventdata, handles)
2     [r c]=size(handles.I);
3     ROI=[0 r c 0; 0 0 r c];
4     MIJ.setRoi(ROI, 1);

```

iv. Calculate the upper sigma value from the the circular shape that is drawn on the previewed image and displays its value on xi

```

1 function SigmaValueButton_Callback(hObject, eventdata, handles)
2     handles.a=MIJ.getRoi(1);

```

```

3  val1=max(handles.a(1,:))-min(handles.a(1,:));
4  val2=max(handles.a(2,:))-min(handles.a(2,:));
5  val3=max([val1/2 val2/2])
6  sigmaVal=round(val3/sqrt(2));
7  set(handles.displaySigmaValue,'string',sigmaVal);
8  msgbox(['Real Radius: ',num2str(val3),' Calculated Sigma: ...
        ',num2str(sigmaVal) ],' Actual and Manipulated');

```

- v. Shows the preview of the time-lapse image with the smallest standard deviation.

```

1  function imageMinStdDev_Callback(hObject, eventdata, handles)
2      MIJ.run('Close All');
3      MIJ.createImage(handles.I(:,:,handles.indmin))

```

- vi. Helps to find the clipping factor of the image with the smallest standard deviation.

```

1  function sliderForMinStd_Callback(hObject, eventdata, handles)
2      MIJ.run('Close All');
3      minSlopeVal=hObject.Value;
4      set(handles.displayForMinStdDevSlope,'string',minSlopeVal);
5      sigma2=get(handles.displaySigmaValue,'String');
6      Imin=handles.I(:,:,handles.indmin);
7      MIJ.createImage(Imin)
8      parameterCLAHE = ['blocksize=',num2str(handles.blocksize),' ...
        histogram=',num2str(handles.histogram_bins),' maximum=', ...
        num2str(minSlopeVal), ' mask=' handles.mask];
9      MIJ.run('Enhance Local Contrast (CLAHE)',parameterCLAHE);
10     MIJ.run('Image Inverter');
11     J=uint8(MIJ.getCurrentImage);
12     Jpadded=padarray(J,[50 50],median(J(:)),'both');
13     MIJ.run('Close All');
14     MIJ.createImage(uint8(Jpadded));
15     parameterLoG1 = ['sigma=',num2str(handles.sigma1),' ...
        filterwidth=',num2str(handles.filterwidth),' threshold=', ...
        num2str(handles.threshold), ' Δ=',num2str(handles.Δ),' ...
        mode=',num2str(handles.mode)];
16     MIJ.run('Log Filter',parameterLoG1);
17     MIJ.run('Make Binary');
18     JLS=uint8(MIJ.getCurrentImage);
19     [JLSR,JLSC]=size(JLS);
20     JLScropped=imcrop(JLS, [handles.n, handles.n, JLSC - 2*handles.n, ...
        JLSR - 2 * handles.n]);
21     MIJ.run('Close All');
22     MIJ.createImage(uint8(Jpadded));
23     MIJ.run('Median...', 'radius=3');
24     parameterLoG2 = ['sigma=',num2str(sigma2),' ...
        filterwidth=',num2str(handles.filterwidth),' threshold=', ...
        num2str(handles.threshold), ' Δ=',num2str(handles.Δ),' ...
        mode=',num2str(handles.mode)];

```

```

25  MIJ.run('Log Filter',parameterLoG2);
26  MIJ.run('Make Binary');
27  JLL=uint8(MIJ.getCurrentImage);
28  [JLLR,JLLC]=size(JLL);
29  JLLcropped=imcrop(JLL, [handles.n, handles.n, JLLC - 2*handles.n, ...
    JLLR - 2 * handles.n]);
30  MIJ.run('Close All');
31  MIJ.createImage('Original',uint8(Imin),true);
32  MIJ.createImage('Small',uint8(JLLcropped),true);
33  MIJ.run('Binary Overlay', 'reference=[Original] binary=[Small] ...
    overlay=Red');
34  OV1=MIJ.getImage('Original-ovr');
35  MIJ.run('Close All');
36  MIJ.createColor('Overlay',uint8(OV1),true);
37  MIJ.createImage('Large',uint8(JLLcropped),true);
38  MIJ.run('Binary Overlay', 'reference=[Overlay] binary=[Large] ...
    overlay=Red');
39  OV2=uint8(MIJ.getCurrentImage);
40  MIJ.run('Close All');
41  MIJ.createColor('Final Overlay',OV2,true)
42  function displayForMinStdDevSlope_Callback(hObject, eventdata, handles)

```

vii. Shows the preview of the time-lapse image with the largest standard deviation.

```

1  function imageMaxStdDev_Callback(hObject, eventdata, handles)
2      MIJ.run('Close All');
3      MIJ.createImage(handles.I(:, :, handles.indmax))

```

viii. Helps to find the clipping factor of the image with the largest standard deviation

```

1  function sliderForMaxStd_Callback(hObject, eventdata, handles)
2      MIJ.run('Close All');
3      maxSlopeVal=hObject.Value;
4      set(handles.displayForMaxStdDevSlope,'string',maxSlopeVal);
5      sigma2=get(handles.displaySigmaValue,'String');
6      Imax=handles.I(:, :, handles.indmax);
7      MIJ.createImage(Imax)
8      parameterCLAHE = ['blocksize=',num2str(handles.blocksize),' ...
    histogram=',num2str(handles.histogram_bins),' ...
    maximum=',num2str(maxSlopeVal), ' mask=' handles.mask];
9      MIJ.run('Enhance Local Contrast (CLAHE)',parameterCLAHE);
10     MIJ.run('Image Inverter');
11     J=uint8(MIJ.getCurrentImage);
12     MIJ.run('Close All');
13     Jpadded=padarray(J,[50 50],median(J(:)), 'both');
14     MIJ.run('Close All');
15     MIJ.createImage(uint8(Jpadded));

```

```

16     parameterLoG1 = ['sigma=', num2str(handles.sigma1), ' ...
        filterwidth=', num2str(handles.filterwidth), ' threshold=', ...
        num2str(handles.threshold), ' Δ=', num2str(handles.Δ), ' ...
        mode=', num2str(handles.mode)];
17     MIJ.run('Log Filter', parameterLoG1);
18     MIJ.run('Make Binary');
19     JLS=uint8(MIJ.getCurrentImage);
20     [JLSR, JLSC]=size(JLS);
21     JLScropped=imcrop(JLS, [handles.n, handles.n, JLSC - 2*handles.n, ...
        JLSR - 2 * handles.n]);
22     MIJ.run('Close All');
23     MIJ.createImage(uint8(Jpadded));
24     MIJ.run('Median...', 'radius=3');
25     parameterLoG2 = ['sigma=', num2str(sigma2), ' ...
        filterwidth=', num2str(handles.filterwidth), ' threshold=', ...
        num2str(handles.threshold), ' Δ=', num2str(handles.Δ), ' ...
        mode=', num2str(handles.mode)];
26     MIJ.run('Log Filter', parameterLoG2);
27     MIJ.run('Make Binary');
28     JLL=uint8(MIJ.getCurrentImage);
29     [JLLR, JLLC]=size(JLL);
30     JLLcropped=imcrop(JLL, [handles.n, handles.n, JLLC - 2*handles.n, ...
        JLLR - 2 * handles.n]);
31     MIJ.run('Close All');
32     MIJ.createImage('Original', uint8(Imax), true);
33     MIJ.createImage('Small', uint8(JLScropped), true);
34     MIJ.run('Binary Overlay', 'reference=[Original] binary=[Small] ...
        overlay=Red');
35     OV1=MIJ.getImage('Original-ovr');
36     MIJ.run('Close All');
37     MIJ.createColor('Overlay', uint8(OV1), true);
38     MIJ.createImage('Large', uint8(JLLcropped), true);
39     MIJ.run('Binary Overlay', 'reference=[Overlay] binary=[Large] ...
        overlay=Red');
40     OV2=uint8(MIJ.getCurrentImage);
41     MIJ.run('Close All');
42     MIJ.createColor('Final Overlay', OV2, true)
43     function displayForMaxStdDevSlope_Callback(hObject, eventdata, handles)

```

ix. Display the maximum sigma value of the LoG detector

```

1 function displaySigmaValue_Callback(hObject, eventdata, handles)

```

x. Applies the proposed segmentation scheme on the loaded dataset

```

1 function Segmentation_Callback(hObject, eventdata, handles)
2     [r c h]=size(handles.I);
3     sigma2=get(handles.displaySigmaValue, 'String');
4     for i=1:h

```

```

5     MIJ.createImage(uint8(handles.I(:,:,i)));
6     MIJ.run('Measure');
7     stdDev=MIJ.getResultsTable;
8     minSlope=str2num(get(handles.displayForMinStdDevSlope,'string'));
9     maxSlope=str2num(get(handles.displayForMaxStdDevSlope,'string'));
10    slopeE=[minSlope maxSlope];
11    stdDevE=[handles.MinStdDev handles.MaxStdDev];
12    P=polyfit(stdDevE,slopeE,1);
13    slope=polyval(P,stdDev(i,3));
14    parameterCLAHE = ['blocksize=',num2str(handles.blocksize),' ...
        histogram=',num2str(handles.histogram_bins),' maximum=', ...
        num2str(slope), ' mask=' handles.mask];
15    MIJ.run('Enhance Local Contrast (CLAHE)',parameterCLAHE);
16    MIJ.run('Image Inverter');
17    J=uint8(MIJ.getCurrentImage);
18    Jpadded(:,:,i)=padarray(J,[50 50],median(J(:)),'both');
19    MIJ.run('Close All');
20    MIJ.createImage(uint8(Jpadded));
21    MIJ.run('Close All');
22    end
23    for i=1:h
24        MIJ.createImage(uint8(Jpadded(:,:,i)));
25        parameterLoG1 = ['sigma=',num2str(handles.sigmal),' ...
            filterwidth=',num2str(handles.filterwidth),' threshold=', ...
            num2str(handles.threshold), ' Δ=',num2str(handles.Δ),' ...
            mode=',num2str(handles.mode)];
26        MIJ.run('Log Filter',parameterLoG1);
27        MIJ.run('Make Binary');
28        JLS=uint8(MIJ.getCurrentImage);
29        [JLSR,JLSC]=size(JLS);
30        JLScropped(:,:,i)=imcrop(JLS, [handles.n, handles.n, JLSC - ...
            2*handles.n, JLSR - 2 * handles.n]);
31        MIJ.run('Close All');
32    end
33    handles.JLScropped=JLScropped;
34    for i=1:h
35        MIJ.createImage(uint8(Jpadded(:,:,i)));
36        MIJ.run('Median...', 'radius=3');
37        parameterLoG2 = ['sigma=',num2str(sigma2),' ...
            filterwidth=',num2str(handles.filterwidth),' threshold=', ...
            num2str(handles.threshold), ' Δ=',num2str(handles.Δ),' ...
            mode=',num2str(handles.mode)];
38        MIJ.run('Log Filter',parameterLoG2);
39        MIJ.run('Make Binary');
40        JLL=uint8(MIJ.getCurrentImage);
41        [JLLR,JLLC]=size(JLL);
42        JLLcropped(:,:,i)=imcrop(JLL, [handles.n, handles.n, JLLC - ...
            2*handles.n, JLLR - 2 * handles.n]);
43        MIJ.run('Close All');
44    end
45    handles.JLLcropped=JLLcropped;
46    guidata(hObject,handles)

```

xi. Generates the black and white version of the segmented dataset

```

1 function segmentationBlackAndWhite_Callback(hObject, eventdata, handles)
2     MIJ.createImage('Small',uint8(handles.JLScropped),true);
3     MIJ.run('Make Binary','method=Default background=Default calculate');
4     MIJ.run('Fill Holes','stack');
5     MIJ.createImage('Large',uint8(handles.JLLcropped),true);
6     MIJ.run('Make Binary','method=Default background=Default calculate');
7     MIJ.run('Fill Holes','stack');
8     MIJ.run('Image Calculator...')
9     BW=uint8(MIJ.getCurrentImage);
10    handles.BW=BW;
11    guidata(hObject,handles)

```

xii. Creates the outlines of the detected objects.

```

1 function Outline_Callback(hObject, eventdata, handles)
2     MIJ.run('Close All');
3     MIJ.createImage('Outlined',handles.BW,true)
4     MIJ.run('Outline');
5     handles.Outlined=MIJ.getCurrentImage;
6     guidata(hObject,handles)

```

xiii. Overlays the outline over the original (input) dataset.

```

1 function Overlaytwo_Callback(hObject, eventdata, handles)
2     MIJ.run('Close All');
3     MIJ.createImage('Input',handles.I,1);
4     MIJ.createImage('Outline',handles.Outlined,1);
5     MIJ.run('Make Binary','method=Default background=Default calculate');
6     MIJ.run('Binary Overlay','reference=[Input] binary=[Outline] ...
        overlay=White');
7     handles.Overlaid=MIJ.getCurrentImage;
8     guidata(hObject,handles)

```

xiv. Helps to preview the overlaid dataset.

```

1 function recordOverlay_Callback(hObject, eventdata, handles)
2     MIJ.createImage('Overlaid',handles.Overlaid,1);

```

xv. Helps to preview the black and white version of the segmented dataset.

```

1 function recordWhiteAndBlack_Callback(hObject, eventdata, handles)
2     MIJ.createImage('Outlined',handles.BW,true)

```

xvi. Helps to preview the outlined version the detected dataset.

```
1 function recordOutline_Callback(hObject, eventdata, handles)
2     MIJ.createImage('Outlined',handles.Outlined,true);
```

xvii. Prompts to load GT and overlays the segmented data over the ground truth (if available) in order to compare the accuracy of the proposed method, visually.

```
1 function GTandMethod_Callback(hObject, eventdata, handles)
2     MIJ.run('Close All');
3     MIJ.run('Image Sequence...')
4     handles.GT=uint8(MIJ.getCurrentImage);
5     MIJ.closeAllWindows
6     MIJ.createImage('Segmentation by the ...
7         Method',im2uint8(handles.BWoutline),true);
8     MIJ.createImage('Ground Truth',handles.GT,true);
9     MIJ.run('Binary Overlay', 'reference=[Ground Truth] ...
10         binary=[Segmentation by the Method] overlay=Red');
11     guidata(hObject,handles)
```

xviii. Prompts the user to saves the last selected image or stack of images

```
1 function SaveAs_Callback(hObject, eventdata, handles)
2     MIJ.run('Image Sequence... ', 'format=TIFF use save=[]');
```