[Ca$^{2+}$]$_i$ Profiles Extraction from Endothelial Cells Using Fluorescence Digital Images

by

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Abstract

The understanding about cells behavior and its interaction with the medium has become fundamental in order to design therapies and drugs to maintain health of living.

Endothelial cells cover the inner surface of blood vessels and cardiac cavities and they achieve a wide variety of functions as the vascular tone regulation, inflammatory reactions, fibrinolysis and coagulation. Proliferation and migration of endothelial cells are strongly regulated by its intracellular calcium ([Ca]$^{2+}$) concentration.

Those essential functions are altered when the endothelium is damaged by some diseases as diabetes mellitus which is a public health problem that is increasing at alarming rate not only in Mexico but also worldwide. Hence the importance of modeling the endothelial cells behavior under different stimuli.

In this thesis a proposal to automate the intracellular calcium profiles extraction from fluorescence image sequences of endothelial tissue is presented. Digital image processing techniques, as adaptive binarization, were joined with a multi-target tracking scheme supported by Kalman state estimation. Each component of the proposed system is explained below.

The system was tested with image sequences from two different stimuli. Results showed consistency in the extracted data and validated the need of the tracking scheme. Two important contributions of this work are the automatic relocation of the regions of interest assigned to the cells and the possibility of a massive data extraction.
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Chapter 1

Introduction

The knowledge about cells behavior and its interaction with the medium has become fundamental in order to design therapies and drugs which help to maintain health of human beings. Ca$^{2+}$ ion is the second transmitter most ubiquitous in human body, its role is very important in many functions as vital as the contraction of the cardiac muscle; regulation of body size, the conception process, the angiogenesis, inter alia.

Endothelial cells cover the inner surface of blood vessels and cardiac cavities and they achieve a wide variety of functions as the vascular tone regulation, inflammatory reactions, fibrinolysis and coagulation. Proliferation and migration of this cells are strongly regulated by its $[\text{Ca}]_{i}^{2+}$ concentration.

Over time several schemes to investigate the cell activity have been proposed. In the specific case of $[\text{Ca}]_{i}^{2+}$, initial schemes used to include optical and chemical methods. As science and technology advanced, electronic and computer science were gradually incorporated in tasks of digital images acquisition and processing. Nowadays the multiparametric analysis of the interaction between cells and Ca$^{2+}$ is a multidisciplinary work.
1.1 THE IMPORTANCE OF CALCIUM ION

1.1 The Importance of Calcium Ion

Most of us see the word calcium daily, whether it is read on the label of a multivitamin or seen on a carton of milk, but do not realize the complexity and vast array of functions that this simple divalent cation plays on a molecular level. The majority of people know Ca\(^{2+}\) is involved in bone development and in the prevention of diseases such as osteoporosis; however, Ca\(^{2+}\) is one of the most abundant signaling molecules found in the human body.

Over one hundred years ago, at the end of the 1800’s, Ringer et al demonstrated that calcium is necessary for a normal frog heart contraction. In 1940, it was found that a Ca\(^{2+}\) injection induced a contraction in a muscular fiber, but K\(^+\), Na\(^+\) and Mg\(^{2+}\) did not. Heilbrum emphasized the potential roles of calcium in several cellular functions; nevertheless, it took a long time for his ideas to be accepted. In 1960 Ebashi and Lipmann discovered a Ca\(^{2+}\) storage site in muscular fibers, the sarcoplasmic reticulum. They explained the exact mechanism of muscular contraction and the union of calcium with troponine protein role in the striated muscle contraction. After that, it was demonstrated that there are proteins able to join to Ca\(^{2+}\) in a variety of cells, this encouraged the analysis of calcium potential functions [1].

Nowadays it is known that Ca\(^{2+}\) ion has a preponderant role as second messenger in several signal transduction mechanisms. The signalization via Ca\(^{2+}\) is present in the control of different cellular processes [2]. From the beginning of a new organism at the time of fertilization; along the nerve processes that accompany it; with every beat of its heart and in every step that its muscles conduct, the calcium ion is involved in cellular homeostasis, translating external signals to the internal language of the cell and in this way the organism is able to respond, adapt and continue the phenomenon of life.

This ion, not only participates in the process of neurotransmitter release at chemical synapses and excitation–contraction coupling in different types of muscle; calcium is also essential for the activation of oocytes, restart the cycle of meiosis at
CHAPTER 1. INTRODUCTION

fertilization, embryonic development, control of vascular tone, proliferation and cell migration, angiogenesis (formation of new blood vessels), activation the phenomenon of synaptic plasticity, until the control of cell death or apoptosis [3].

Disruptions in Ca\textsuperscript{2+} signaling has been linked to the pathogenesis of numerous diseases such as, but not limited to Huntington's disease, Alzheimer's disease, cancer, congenital heart failure, and diabetes.

1.2 Intracellular Calcium Measurements $[\text{Ca}^{2+}]_i$

Due to relevancy of Ca\textsuperscript{2+} in biology, several techniques and methods have been developed and established to analyze cellular and intracellular activity mechanisms of calcium.

Since 1920 different approaches to measure $[\text{Ca}^{2+}]_i$, were proposed but just a few were successful. First reliable $[\text{Ca}^{2+}]_i$ measurement was done by Ridway and Ashley, they injected aequorin photoprotein in a muscular fiber [4]. After, on 80’s, Tsien et al produced a variety of fluorescent chemical dyes [5] [6]. These indicators have high affinity and one-to-one stoichiometry for Ca\textsuperscript{2+}. They display low affinity for Mg\textsuperscript{2+} and H\textsuperscript{+} and when Ca\textsuperscript{2+} binds to them, there are large absorbance and fluorescence changes. The indicator molecule consists of two parts: the Ca\textsuperscript{2+} -binding part that changes its shape when Ca\textsuperscript{2+} binds to it and which in turn alters the conformation of the fluorescent part of the molecule.

Since these indicators development, the study of cellular phenomena related with calcium has increased dizzily.

Furthermore, indicators using bioluminescent photoproteins were developed [7] [8] [9], these indicators have a low fluorescence dynamic range as disadvantage.
1.2. INTRACELLULAR CALCIUM MEASUREMENTS $[\text{Ca}^{2+}]_I$

1.2.1 Chemical Fluorescent Indicators

Fluorescent chemical indicators are the most used because its output signal (light) for a certain change of $[\text{Ca}^{2+}]$ is bigger than others indicators output, i.e., they are more sensitive. The election of suitable indicator depends on experiment conditions and objectives.

Indicators can be classified according to several characteristics, for example, they can be single or double excitation, ratiometric or non-ratiometric. Using their excitation/emission spectrum, indicators are classified as ultra violet wavelength and as visible wavelength indicators. Then, a brief description of indicators is given.

- **Ultra Violet (UV) Excitation Wavelength Indicators**
  They were the first and most popular chemical indicators. Although visible excitation wavelength indicators were produced, UV is still being used as quantitative and ratiometric indicators of Ca$^{2+}$. Their main disadvantage is the UV irradiation cytotoxicity.

  Quin 2, Indo 1, Fura 2, Indo 1FF, Mag-indo 1, Mag-fura 2, Mag-fura 5, Fura PE3, Bis-fura 2, C$_{18}$-fura 2, FFP18, FIP18, BTC are examples of this indicators class [1].

- **Visible Excitation Wavelength Indicators**
  These indicators have some advantages over UV excitation indicators due to its emissions are located in electromagnetic spectrum regions where cellular auto-fluorescence and background scattering are less severe, in addition, cytotoxicity of visible light is minor than UV ligth.

  Some visible excitation wavelength are fluo 3, Calcium green, Oregon green BAPTA, Calcium Orange, Calcium crimson, Fura red, Rhod 2, Dextran conjugates, Calcium green C$_{18}$, Fura-indoline-C$_{18}$.

For more information about indicators please go to Appendix A.
CHAPTER 1. INTRODUCTION

There are inconveniences and limits using fluorescent indicators, some of them are the intracellular buffering, cytotoxicity, damage and photobleaching, accumulation and non-homogeneous distribution indicator, adhesion with other ions and proteins and dye leakage \[1\].

1.2.2 Ratiometric Measurements

Ratiometric measurements are those where the ratio between emission signals is used for monitoring \([\text{Ca}^{2+}]\) with dual indicators. This kind of measurements has the following properties:

- Increases \([\text{Ca}^{2+}]\) sensitivity.
- Smooth photobleaching effects.
- Ratio is independent of:
  - Dye concentration.
  - Illumination intensity.
  - Length optic path.

Indicators used in ratiometric measurements have an isosbestic point as advantage \[10\]. Isosbestic point represents the wavelength, or frequency, in which a sample absorbance does not change during a chemical reaction or a physical change on it \[11\].

1.2.3 Fura 2

Fura 2 is the archetype of dual excitation indicators and allows ratiometric measurements. Maximum absorption peaks are located at 355 and 363 nm with saturated and free \(\text{Ca}^{2+}\), respectively. For ratiometric measurements excitation at 340 and 380 nm is preferred due to its absorption peak in free \(\text{Ca}^{2+}\) state is very close to its isosbestic point that is in 260nm. Emission is monitored in 510 nm \[12\].
1.2. **INTRACELLULAR CALCIUM MEASUREMENTS** \([Ca^{2+}]_i\)

![Graph](image1.png)

Figure 1-1: (A) Fura 2 excitation spectra. Green line shows calcium free state, blue line shows calcium saturated state and the isosbestic point is in red. (B) Typical signals obtained from a cell loaded with Fura 2 when is excited at 340nm and 380nm alternately; Action of agonist, ionophore and chelator is shown too [10].

### 1.2.4 Load Procedures and Measurement Techniques

There are several load procedures of dye in the study object. Some of them use chemical agents to make permeable the cell membrane. When is needed to load dye in large photoproteins, invasive techniques like microinjection with micropipettes have to be used. Other techniques requires more processes like washing, incubation and centrifugation, all with controlled temperature and pH for each indicator, but mostly, particularizing all for the different cells or tissues of interest [1] [10].

As for measurement, on one hand, there are no optical techniques based on electrophysiology to estimate Ca\(^{2+}\) from electrical currents generated by calcium dependent channels on cell membrane, there are also ion selective electrodes with a greater dynamic range than fluorescent indicators, nevertheless, its response is very slow (from 0.5 to 1 s for the electrodes while ms for fluorescent indicators); on the other hand, optical techniques includes confocal laser scanner microscopy, two photon confocal laser scanner microscopy, pulsed laser images to detect fast changes of calcium and flow cytometry. It is noteworthy that in order to explain the mechanisms of intracellular phenomena, a multiparametric analysis is needed [1].
1.3 Endothelium

Endothelium was considered as an homogeneous population of endothelial cells that conform an inert barrier between interstitial and vascular space, however, located in the interface blood-vessels, endothelium forms a multifunctional signal transducing surface that regulates cardiovascular homeostasis. Accordingly, a healthy endothelium serves as an interface regulating blood pressure, coagulation and fibrinolysis, vascular inflammatory reactions, permeability of the vessel wall, and angiogenesis. In addition, cardiac microvascular endothelial cells modulate myocardiac contractile behavior by releasing a number of paracrine mediators, such as nitric oxide (NO), endothelin-1 and prostanoids \[13\] \[14\]. Mean human endothelium covers an 350m$^2$ and its mass is around 110g with an approximate thickness of 0.3µm \[15\].

Endothelial phenotype differs between species, organs and vascular consecutive sections \[?\]. It has long been known that an increase in intracellular Ca$^{2+}$ concentration plays a key role in the intricate network of signal transduction pathways exploited by endothelial cells to maintain cardiovascular homeostasis. Due to its strategic location at the interface between the vascular wall and bloodstream, the endothelium is exposed to a myriad of transmitters (released by autonomic and sensory nerves or platelets), circulating hormones, autacoids, cytokines, growth factors, and drugs, as well as to mechanical stimuli, such as pulsatile stretch, shear stress, and changes in the local osmotic pressure. Moreover, vascular endothelial cells might serve as thermosensors and modulate peripheral vasoconstriction or vasodilation depending on the environmental temperature.

The majority of endotelial cells functions in cardiovascular system are modulated by the concentration of intracellular free calcium or the concentration of the principal intracellular calcium reservoir, the endoplasmic reticulum. The difference on its calcium concentration produces a gradient toward the cell's cytoplasmic interior \[16\], \[17\].

Calcium signaling starts when the vascular endothelium is activated, whether a
1.3. ENDOTHELIUM

chemical stimulus, when extracellular agonist interacts with its specific membrane receivers, coupled with G proteins or with tyrosine–kinase activity; or physical stimulus, when the mechano-receivers are activated as a consequence of the mechanical stress which generates the bloodstream flow over the arterial walls. Both stimuli promotes an intracellular calcium increase (from 100nM to 0.1mM approximately) [17], [18], [19]. Considering the asymmetric distribution of \([\text{Ca}^{2+}]_i\), a release of calcium from reticulum endoplasmic or the entry of calcium from extracellular medium though ion channels membrane are needed to produce an increase of cytosolic Calcium in endothelium.

The endothelial Ca\(^{2+}\) response may comprise an initial Ca\(^{2+}\) spike, shaped by Ca\(^{2+}\) mobilization from the intracellular Ca\(^{2+}\) reservoir, followed by an intermediate plateau level due to Ca\(^{2+}\) entry across the plasma membrane. However, at low–dose agonist stimulation, the Ca\(^{2+}\) signal may adopt an oscillatory pattern driven by the interplay between intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) influx from the extracellular space. Accordingly, in most microvascular endothelial cells, chemical stimulation does not elicit a remarkable Ca\(^{2+}\) inflow and results in either a transient Ca\(^{2+}\) spike or localized, high frequency Ca\(^{2+}\) oscillations, which are attributable to Ca\(^{2+}\) mobilization from the intracellular store. Conversely, when the Ca\(^{2+}\) signal is uniquely modeled by Ca\(^{2+}\) entry, a sustained and monotonic increase in \([\text{Ca}^{2+}]_i\) occurs. Mechanical stimulation may also affect vascular endothelium by causing an elevation in \([\text{Ca}^{2+}]_i\).

Diabetes mellitus type 2 is an important risk factor for the development of cardiovascular system pathologies due to structural and functional abnormalities in the heart and the micro and macro vasculature which increases the prognosis for the development of diseases such as hypertension, atherosclerosis, coronary artery disease or events such as heart attacks, sudden death and stroke [20]. Recent studies have shown that in diabetic patients, elevated blood glucose levels (hyperglycemia) chronic and/or acute generates a dysfunction of endothelial cells [21].

The endothelial dysfunction is characterized by abnormal vascular reactivity, decreased nitric oxide bioavailability, increased production of reactive oxygen species
CHAPTER 1. INTRODUCTION

and a change in vascular permeability, endangering the transport of nutrients, oxygen, hormones; and the removal of metabolic products. In this way the vascular endothelium is exposed to endogenous stimuli, cytokines, chemokines, among other hypoxic conditions [22].

It has also been found that alteration of the endothelium, produced by diabetes mellitus type 2, helps to the increase of procoagulant substances, pro-thrombotic, pro-inflammatory mediators and to the constriction of arterial smooth muscle [20] [23]. Most studies have focused on the development and progression of the damage caused by oxidative stress; however, other mechanisms that favors endothelial dysfunction have been reported [23].

Nowadays, the exact mechanism used by diabetes mellitus to cause dysfunctions of vascular endothelium still unknown. It is therefore necessary to continue research on the molecular mechanisms involved in this disease.

1.4 Problem Description

Significant endothelial cell (EC) damage is considered as the first event that leads in severe vascular disorders, such as ischemia, atherosclerosis and aneurysms [24]. Furthermore, it is well known that endothelial proliferation and migration are regulated by an increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) [14].

Figure 1-2: (A) Ratio images construction. (B) ROI selection in ratio images.
1.4. PROBLEM DESCRIPTION

Diabetes is a worldwide problem. In Mexico, incidence, prevalence and mortality are increasing at accelerated rate [25]. Cardiovascular Physiology Laboratory (CVPL-FMBUAP), of Medicine Faculty at Benemérita Universidad Autónoma de Puebla, has an special interest over studying and modeling endothelium behavior under different stimuli [14] [24] [26] [27] with the aim of explaining its regeneration mechanism. This would allow the design of drugs and therapies that could help in endothelium restoration which presents severe dysfunctions associated to diabetes [28] [29].

Methodology used in the laboratory generates and analyses experimental data in a reliable way, nevertheless, available analysis tools in CVPL-FMBUAP demand a big amount of time and human resources.

A description of the image sequence analysis process is given below.

1. As shown in Fig. 1-3, ratio image is obtained through a pixel to pixel ratio of images obtained with 340nm excitation over images obtained with 380nm. Then, if there are \( n \) images for excitation wavelength, there will be \( n \) ratio images.

2. Once ratio images has been made, some cells are selected and bounded in regions of interest (ROI’s), see Fig. 1-2.

3. Intensity mean from each selected ROI is obtained and registered.

4. Step 3 is repeated every frame of the ratio set before passing to step 5.

![Figure 1-3: Intracellular calcium profile example.](image-url)
5. One plot per ROI is done. Number of frames or time are located in abscissas axis, while ordinates shows the ratio mean intensity. That data representation for each cell is called $[\text{Ca}^{2+}]_i$ profile.

The above procedure is done in CVPL-FMBUAP using the RatioPlus plugin of ImageJ an open source digital image processing software [30].

1.4.1 Problem

Generally, sequences of more than five hundred images per excitation wavelength are obtained and around one hundred $[\text{Ca}^{2+}]_i$ profiles are extracted from a single experiment, i.e., typically, one hundred of ROIs are selected.

When endothelium is stimulated, fluorescence of its cells begin to oscillate, some of them start to move and they get out from its assigned ROI. As a consequence, fluorescence mean measurements are altered, and the physiologist is forced to verify and manually move the ROIs in each frame as needed, therefore, the data extraction process becomes tedious, impractical, and time consuming of computational and human resources.
1.5 Objectives

1.5.1 General

Automate the methodology of analysis for extraction of $[\text{Ca}^{2+}]_i$ profiles from fluorescence image sequences of endothelial cells using digital image processing, and tracking techniques.

1.5.2 Particular

- Implement and evaluate several binarization techniques to select one that has a good performance for the fluorescence images.

- Suggest and implement a scheme of multi target tracking scheme for endothelial cells.

- Join the image processing and the tracking stages to extract data from the image sequences.

- Design a graphical user interface to facilitate the intracellular calcium profiles extraction.

- Test and validate the proposed solution scheme.

1.6 Related Works and State of Art

As seen, almost in all eukaryote type cells, $[\text{Ca}^{2+}]_i$ signal regulates a wide range of essential processes. With the aim of studying and modeling calcium dynamics in a variety of cells some working groups has fused chemistry, optics, electronics and computer science giving as results several proposed systems and techniques. Techniques can be classified in many ways, herein are separated in optical and non optical. In the second category we can find mainly ion selective micro electrodes \cite{31,32,33}. 
CHAPTER 1. INTRODUCTION

This work belongs to optical techniques, where images analysis has played a crucial role, so we will discuss a brief about it in this section.

In the eighties, images with a one second temporal resolution were obtained, nevertheless, a lot of ionic changes are faster than this. In [34] a microscope system was designed and built to obtain multi-spectral images at a thirty frames per second. Two stages were included to this imaging fluorescent microscope, acquisition and processing. In the process of acquiring fast multi-spectral data, there was a loss in image quality. That was due to the decrease in image signal to noise ratio as the time exposure of each frame was reduced, as a consequence, resulting images were corrupted by several noise contributions and blurred temporally. The processing proposed for images consisted of four steps: (i) lag correction, used a linear predictor to find the contributions of the previous images in the present one and correct for it, (ii) normalization of data by multiplying all pixels of a given image by a equalizing factor, (iii) noise smoothing, using Poisson noise model and a local linear minimum mean square filter, and finally, (iv) a concentration gradient calculation via formula that included the ratio pixel by pixel of the preprocessed data and other calibration parameters taken at the moment of the experiments.

Since equipment for image processing used to be at the same place where experimental instrumentation and that limited the researchers access to the system, in [35] a similar system to [34] was proposed but they changed the pdp computer for a IBM compatible personal computer. A free acquisition software written in C was published, software had the possibility of implement segmentation and morphological analysis. Later, with the aid of photomultiplier tube and more optical instrumentation, the first intracellular calcium transients in rabbit heart were mapped and reported in [36].

In 2004, Sebille et al presented in [37] a computer program called HARVest of Elementary Events (HARVELE). The program achieved analysis of events on series of confocal images and followed sparks morphology from one site during several seconds. These events have been termed calcium sparks because of the spontaneous localized and transient elevations of fluorescence exhibited with the calcium increase.
The program was coded in the image processing language IDL 5.3 and it could be applied on a series of $n$ images of $512 \times 512$ pixels obtained from the same scanning line. The program was conformed by four modules. (i) The automatic sparks detection module whose goals were to calculate the fluorescence background (without events) and detect all the events exceeding a threshold value. The module carried out conventional filtering to increase signal to noise ratio and normalization based on image sequence standard deviation. (ii) The user intervention module provided possibilities for the user to directly interact with the signals selection, selecting or deleting events manually. (iii) Measurement and analysis module was used to extract position and morphological parameters on each selected area using binary masks. (iv) Storage of results module had the aim of store parameters an classify them according to a distribution patterns.

Continuing with the development of calcium sparks detection tools, in [38] a fully automatic approach for calcium spark events detection in confocal image system is proposed. The proposed system was composed of two units. (i) Index image extraction unit, this unit included the following submodules: denoising, averaging, thresholding, pixel statistics, potential sparks detection and index image generation. For denoising, each image was passed through a 3 by 3 median filter and a 3 by 3 smoothing spatial filter, and Otsu’s thresholding method was used. (ii) Calcium sparks detection unit had the following submodules: region of interest extraction, two layer thresholding, morphology and delay.

The optical instrumentation improvement allows that other techniques can be used. A multifocal multiphoton microscopy setup connected to an inverted microscope is used in [39] with an excitation laser of 1.7W that provided femtosecond pulses at a wavelength preset to 380 nm, a beam splitter, a XY scanning mirror system and a 12-bit CCD camera coupled that allowed rates up to 60 Hz. As for image processing, five stages can be identified. (i) Background normalization in wich the average background signal is estimated from the background information of the entire time series. (ii) Wavelet denoising, the 2D undecimated discrete wavelet transform is computed via
the *trous* implementation, the filter is applied using 2D convolution. (iii) Event Detection is performed on the DWT of the denoised images, a detection parameter $\tau$ to define the detection threshold is introduced. (iv) Event morphometry, a fitting procedure relied on a nonlinear least squares fit using a gradient expansion algorithm that is based on the Marquardt algorithm. (v) Error Estimation is performed using Monte Carlo simulation.

Vallmitjana et al present in [40] an automatic image processing method to analyze confocal calcium images of isolated cardiac myocytes. A feature extraction determines the temporal properties of the oscillations in the average fluorescence and its correspondence to the stimulation times and principal component analysis (PCA) was used to identify basic spatial modes in order to determine if the images present out-of-phase activity.

An automated pipeline for analyzing and identifying neuronal activity from calcium imaging data to investigate neuronal activity is presented in [41]. Wavelet transform and watershed are used in segmentation process, a 2D-Gaussian is fitted for each region of candidate cells. The transient analysis infers the transient events using sparse coding. The extraction of cellular signals is formulated as a sparse dictionary learning. Dictionary learning should allow to model fluorescence calcium images as a combination of few dictionary elements.
## 1.6. RELATED WORKS AND STATE OF ART

Table 1.1: Most important references summary in computer vision related to calcium at cellular level.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Samples Type</th>
<th>Objective</th>
<th>Image Processing Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>[34] 1989</td>
<td>-</td>
<td>Acquire fast multi-spectral data</td>
<td>- Lag correction</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Normalization</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Minimum mean square filter</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Gradient calculation</td>
</tr>
<tr>
<td>[35] 1991</td>
<td>-</td>
<td>Provide analysis system portability</td>
<td>- Segmentation</td>
</tr>
<tr>
<td>[37] 2004</td>
<td>Muscle cells (Skeletal, cardiac and smooth)</td>
<td>Ca$^{2+}$ Sparks analysis</td>
<td>- Morphological analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Normalization</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Automatic and user defined thresholding</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Morphological analysis</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Median filter</td>
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<td></td>
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<td></td>
<td>- Smoothing spatial filter</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Otsu’s thresholding</td>
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<tr>
<td></td>
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<td></td>
<td>- Two layer thresholding</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Morphological operations</td>
</tr>
<tr>
<td>[38] 2006</td>
<td>Skeletal muscle fibers</td>
<td>Ca$^{2+}$ Sparks analysis</td>
<td>- Background normalization</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Wavelet denoising</td>
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<td></td>
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<td></td>
<td>- Fitting</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Monte Carlo simulation for error estimation</td>
</tr>
<tr>
<td>[39] 2007</td>
<td>Muscle fibers</td>
<td>Cytosolic Ca$^{2+}$ release analysis</td>
<td>- Grayscale conversion</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Thresholding</td>
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<tr>
<td></td>
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<td>- Principal Component Analysis</td>
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<td></td>
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<td>- Wavelet transform</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Watershed segmentation</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- 2D Gaussian fitting</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Sparse dictionary</td>
</tr>
<tr>
<td>[40] 2010</td>
<td>Cardiac myocytes</td>
<td>Event detection</td>
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<td></td>
</tr>
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<td>[41] 2013</td>
<td>Hippocampal slice cultures</td>
<td>Neuronal activity identification and analysis</td>
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</tbody>
</table>
1.7 Thesis Organization

In the next chapter the reader will find a brief background which contains the basic concepts about digital image processing, later, the mathematical tools that were used to implement and test several adaptive thresholding techniques are also described. Subsequently morphological operations and the Kalman filter derivation are presented.

The third chapter is the core of this thesis work, the proposal to achieve the objectives is explained. It was divided in tasks. The first builds the ratio sequence, the second performs the binarization process, later, a multi-target tracking scheme aided with Kalman estimation is presented and a structure array for extracted data storage is explained.

The graphical user interface where all the blocks of the proposal were joined is presented in the fourth chapter. The functionality of the software application is described. Furthermore the validation results of the proposal obtained through two case-of-study are discussed.

Finally the conclusions of this work and the perspectives are mentioned in the fifth chapter.
In this chapter, a brief look over theoretical background is done in order to present the concepts and tools that were used in this thesis work. Nevertheless, in the references cited the reader can consult in more detail every topic that is mentioned in pages below.

As this work is based on image processing, the first section gives a basic description of digital images and describes the different kind of images that were used. Some statistical definitions were taken up to introduce the adaptive thresholding techniques based on local variance that were implemented and evaluated for fluorescence images. The next section is about morphology operators and finally a short review of Kalman filter is presented.
2.1 Digital Images: A brief description

The word pixel is an abbreviation of ‘picture element’. Indexed as an \((x, y)\) location from the origin of the image, it represents the smallest constituent element in a digital image, and contains a numerical value which is the basic unit of information within the image at a given spatial resolution and quantization level. Since digital images are stored as binary data, the digital image never fades or degrades over time. Commonly, pixels contain the color or intensity response of the image as a small point sample of colored light from the scene. However, not all images necessarily contain strictly visual information. An image is simply a 2-D signal digitalized as a grid of pixels, the values of which may relate to other properties than color or light intensity. The information content of pixels can vary considerably depending on the type of image to be processed [45]. In this thesis, we have used mainly the image types shown in Figure 2-1 and described below.

<table>
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<th>123</th>
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<th>17</th>
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<tr>
<td>80</td>
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<td>6</td>
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<td>20</td>
<td>190</td>
<td>115</td>
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(a) Intensity

<table>
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<tr>
<th>1</th>
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<th>1</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
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</table>

(b) Binary

<table>
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<tr>
<th>0.12</th>
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<th>0.74</th>
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<tr>
<td>0.64</td>
<td>1.27</td>
<td>1.61</td>
</tr>
<tr>
<td>2.03</td>
<td>0.02</td>
<td>1.99</td>
</tr>
</tbody>
</table>

(c) Special

Figure 2-1: Three different image types.

2.1.1 Intensity Images

The image data in an intensity (or grayscale) image consists of a single channel that represents the intensity, brightness, or density of the image. In most cases, only positive values make sense, as the numbers represent the intensity of light energy or density of film and this cannot be negative, so typically whole integers in the range of \([0...2^{k-1}]\) are used [43].
2.1.2 Binary Images

Binary images are a special type of intensity image where pixels can only take one of two values, black or white. These values are typically encoded using a single bit (0/1) per pixel. Binary images are often used for representing line graphics, archiving documents, encoding fax transmissions, and of course in electronic printing [43].

2.1.3 Special Images

Special images are required if none of the standard formats is sufficient for representing the images values. Two common examples of special images are those with negative values and those with floating-point values. Images with negative values arise during image-processing steps, such as filtering for edge detection, and images with floating-point values are often found in medical, biological or astronomical applications, where extended numerical range and precision are required. These special formats are mostly application-specific and thus may be difficult to use with standard image-processing tools [43].

2.1.4 Image Coordinate System

In order to know which position on the image corresponds to which image element, a coordinate system is used. Contrary to normal mathematical conventions, in image processing the coordinate system is usually flipped in the vertical direction; that is, the y-coordinate runs from top to bottom and the origin lies in the upper left corner. While this system has no practical or theoretical advantage, and in fact may be a bit confusing in the context of geometrical transformations, it is used almost without exception in imaging software systems [43].
2.2 Digital Image Processing

Digital image processing is the study of techniques for transforming a digital image into another, improved, digital image or for analyzing a digital image to obtain specific information about the image.

Computer vision, or machine vision, is another increasingly important area closely related to image processing, where an input image is analyzed in order to determine its content. The primary goal of computer vision systems is to analyze a digital image and infer meaningful information about scene depicted by the image.

A complete digital image processing system is able to service every aspect of digital image handling. The five typical stages in an image processing pipeline: image acquisition, image processing, image archival, image transmission, and image display. Image acquisition is the process by which digital images are obtained or generated. Image processing is the stage where a digital images is enhanced or analyzed. Image archival is concerned with how digital images are represented in memory. Image transmission is likewise concerned with data representation but places added emphasis on the robust reconstruction of potentially corrupted data due to transmission noise. Image display deals with the visual display of digital image data [42].

Digital images are used across an exceptionally wide spectrum of modern life. Ranging from digital cameras and cell phones to medical scans and web technology, digital image processing plays a central role in modern culture [42]. Digital image processing is cross disciplinary in nature. It uses ideas and techniques from optics, solid-state physics, electronics, computer architecture, software design, algebra, statistics, graph theory and more, and applies them to images from every field of the natural sciences and the technical disciplines. Knowledge of the application area, not only knowledge of image processing techniques, is required to obtain the best solution to a particular problem [44]. Some application examples are listed in Table 2.1.
### CHAPTER 2. BACKGROUND

<table>
<thead>
<tr>
<th>Field</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical diagnostic imaging</td>
<td>Computer tomography</td>
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<td>Digital subtraction angiography</td>
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<tr>
<td></td>
<td>Mammography</td>
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<td></td>
<td>Planar scintigraphy</td>
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<td></td>
<td>Emission computed tomography</td>
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<td></td>
<td>Ultrasound tomography</td>
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<tr>
<td></td>
<td>Magnetic resonance imaging</td>
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<td>Biological imaging</td>
<td>Analysis, classification and matching of 3-D genome topology</td>
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<tr>
<td></td>
<td>Automatic counting and classification of cell types and morphology</td>
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<td>Grow rate measurements</td>
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<td>Motility assay for motion analysis</td>
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<td>Human-machine interface</td>
<td>Gesture and sign language recognition</td>
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<td>Forensic medicine and Law</td>
<td>Fingerprint analysis</td>
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<tr>
<td>enforcement</td>
<td>Face recognition</td>
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<td>Signature verification</td>
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<td></td>
<td>DNA matching</td>
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<td></td>
<td>Automated reading of license plates</td>
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<tr>
<td>Automation and robotics</td>
<td>Automatic part recognition</td>
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<td></td>
<td>Quality inspection</td>
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<td></td>
<td>Process monitoring</td>
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<td></td>
<td>Virtual and augmented reality</td>
</tr>
<tr>
<td>Document processing</td>
<td>Optical character recognition</td>
</tr>
<tr>
<td>Material research</td>
<td>Texture analysis</td>
</tr>
<tr>
<td>Photography/cinematography</td>
<td>Image enhancement</td>
</tr>
<tr>
<td></td>
<td>Compositing and special effects</td>
</tr>
</tbody>
</table>

Table continues in following page.
2.3. **TIFF**

<table>
<thead>
<tr>
<th>Field</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publishing</td>
<td>Fabrication of synthetic scenes</td>
</tr>
<tr>
<td></td>
<td>Video archiving and transmission</td>
</tr>
<tr>
<td></td>
<td>Layout efficiency</td>
</tr>
<tr>
<td></td>
<td>Improved color separation</td>
</tr>
<tr>
<td></td>
<td>Printing</td>
</tr>
<tr>
<td>Remote sensing</td>
<td>Land cover analysis</td>
</tr>
<tr>
<td></td>
<td>Assess environmental damage</td>
</tr>
<tr>
<td></td>
<td>Weather observation and prediction</td>
</tr>
<tr>
<td>Communications</td>
<td>File compression</td>
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<td>Teleconferencing</td>
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<tr>
<td>Space exploration</td>
<td>Terrain rendering</td>
</tr>
<tr>
<td>Astronomy</td>
<td>Image enhancement and restoration</td>
</tr>
<tr>
<td></td>
<td>Automatic detection of cosmic phenomena</td>
</tr>
</tbody>
</table>

Table 2.1: Examples of image processing applications within various fields [44].

**2.3 TIFF**

In practice, image data must first be loaded into memory from a file. Files provide the essential mechanism for storing, archiving, and exchanging image data, and the choice of the correct file format is an important decision. Today there exist a wide range of standardized file format for almost every new developed application. For many projects the selection of the right file format is not always simple, and compromises must be made. The following are a few typical criteria that need to be considered when selecting an appropriate file format:

- Type of image
- Storage size and compression
CHAPTER 2. BACKGROUND

Figure 2-2: Structure of a typical TIFF file. A TIFF file consists of a header and a linked list of image objects, three in this example. Each image object consists of a list of “tags” with their corresponding entries followed by a pointer to the actual image data [43].

- Compatibility

- Application domain

TIFF format is a widely used and flexible file format designed to meet the professional needs of diverse fields. It was originally developed by Aldus and later extended by Microsoft and currently Adobe. The format supports a range of grayscale, indexed, and true color images, but also special image types with large-depth integer and floating-point elements. A TIFF file can contain a number of images with different properties. The TIFF specification provides a range of different sizes and representations together in a single TIFF file. The flexibility of TIFF has made it an almost universal exchange format that is widely used in archiving documents, scientific applications, digital photography, and digital video production.

The strength of this image format lies within its architecture, which enables new image types and information blocks to be created by defining new ”tags”. In this flexibility also lies the weakness of the format, namely that proprietary tags are not always supported and so the ”unsupported tag” error is sometimes still encountered when loading TIFF files [43].
2.4 Adaptive Thresholding

One of the first tasks to be undertaken in vision applications and image processing is to segment objects from their background. When objects are large and do not possess very much surface detail, segmentation is often imagined as splitting the image into a number of regions, each having a high level of uniformity in some parameter such as brightness, color, texture, or even motion [47]. Thresholding is one of the most important approaches to image segmentation in both static-scene and dynamic-scene analysis. The classic and the most straightforward approach to thresholding is based on comparing the grey level of each image pixel with the global threshold [46].

The single threshold technique is the simplest one; for a given source sample $S_{\text{input}}$ and a threshold value $T$ the output sample $S_{\text{output}}$ is either black or white, depending upon whether or not the input sample is below or above the threshold, see Equation 2.1.

$$S_{\text{output}} = \begin{cases} 
\text{black} & S_{\text{input}} < T \\
\text{white} & S_{\text{input}} \geq T
\end{cases} \quad (2.1)$$

Single threshold is solely dependent on proper selection of the threshold value, and does not produce good results in many cases, for example, when illumination is not sufficiently uniform.

![Figure 2-3: Single threshold result.](image)

Adaptive thresholding, also known as dynamic thresholding, is used to determine an appropriate threshold for a particular image. Adaptive thresholding is typically
based on statistical analysis of an image’s histogram, and seeks to determine an optimal split between clusters of samples in the data distribution [42].

The problem that arises when illumination is not sufficiently uniform may be tackled by permitting the threshold to vary adaptively (or “dynamically”) over the whole image. In principle, there are several ways of achieving this. One involves modeling the background within the image. Another is to work out a local threshold value for each pixel by examining the range of intensities in its neighborhood. A third approach is to split the image into subimages and deal with them independently [47]. Some techniques are discussed after mathematical definitions.

### 2.4.1 Definitions

For a given input image $I$, its *sum integral image* can be obtained setting to each new pixel the value of the sum of the upper and left pixels intensities from the original image. Mathematical description is shown in Equation 2.2

$$g(x, y) = \sum_{i=1}^{x} \sum_{j=1}^{y} I(i, j)$$

(2.2)

Equation 2.3 shows the local sum $s(x, y)$ definition as the sum of all the elements of the local window of $w \times w$ size, whose center on $(x, y)$ and with $c = \frac{w-1}{2}$.

$$s(x, y) = \sum_{i=x-c}^{x+c} \sum_{j=y-c}^{y+c} I(i, j)$$

(2.3)

Local arithmetic mean $m(x, y)$ is the pixel intensity average from $I$, within a $w \times w$ size window [49]. Equation 2.4

$$m(x, y) = \frac{s(x, y)}{w^2}$$

(2.4)

Local standard deviation $\sigma$ can be found from local variance [53], as in Equation 2.5

---

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2.4. ADAPTIVE THRESHOLDING

\[
\sigma^2(x, y) = \frac{1}{w^2} \sum_{i=x-c}^{x+c} \sum_{j=y-c}^{y+c} I^2(i, j) - m^2(x, y) \tag{2.5}
\]

Finally, local mean deviation is defined as follows \[49\].

\[
\partial(x, y) = I(x, y) - m(x, y); \tag{2.6}
\]

2.4.2 Niblack Technique

This technique determines the threshold \( T(x, y) \) using a \( w \times w \) size window as in Equation 2.7.

\[
T(x, y) = m(x, y) + k\sigma(x, y) \tag{2.7}
\]

Arithmetic mean and standard deviation are used to generate the threshold value according to the contrast of pixel neighborhood, \( k \) gain controls the adaptation control; \( w = 15 \) and \( k = -0.2 \) are preferred values \[49\] \[50\].

2.4.3 Sauvola Technique

In this technique, the threshold is computed using the same statistical measurements of Niblack, see Equation 2.8 for a \( w \times w \) size window.
CHAPTER 2. BACKGROUND

![Figure 2-5: Sauvola thresholding result.](image)

Figure 2-5: Sauvola thresholding result.

\[
T(x, y) = m(x, y) \left[ 1 + k \left( \frac{\sigma(x, y)}{R} - 1 \right) \right]
\]  

(2.8)

Where \( R \) represents the maximum value possible of standard deviation, i.e., for 8 bit images \( R = 128 \). \( k \) has an allowed range from 0.2 to 0.5 but \( k = 0.34 \) was determined experimentally to give the best results. In high contrast zones, this method behaves on a similar way to Niblack technique, but in low contrast zones the threshold goes below \( m(x, y) \) which helps to split on a suitable manner the dark objects from the background.

2.4.4 Romen Technique

![Figure 2-6: Romen thresholding result.](image)

Figure 2-6: Romen thresholding result.

This technique is supposed to be more efficient than the two above due to standard deviation is not used. Standard deviation is a costly computation. Local threshold is determined as in Equation 2.9 where \( k = [0,1] \).
2.4. ADAPTIVE THRESHOLDING

\[
T(x, y) = m(x, y) \left[ 1 + k \left( \frac{\partial(x, y)}{1 - \partial(x, y)} - 1 \right) \right]
\]

(2.9)

2.4.5 Jyh Technique

![Input and Output Images](image)

Figure 2-7: Jyh technique result.

Techniques above mentioned uses a moving square window of fixed size to get statistical information in order to adapt the threshold. This technique, conversely, is based on a fixed threshold proportional to the standard deviation of the whole image and adapts the radius size of a circle window, see Equation 2.10, giving a normalized image as result which is contrast enhanced. The final step is applying a single threshold in order to separate objects from the background.

\[
r(x, y) = \min_r \{ r > 0 \mid StD[R_r(x, y)] \geq T_{STD} \}
\]

(2.10)

Where

- \( r(x, y) \): radius of the circle window with center in \((x, y)\)
- \( StD \): local standard deviation
- \( R_r(x, y) \): circle region of radius \( r \) and centered in \((x, y)\)
- \( T_{STD} = k \times StD(I) \): single threshold, \( k = [0.2, 0.8] \)


### 2.4.6 Bradley Technique

![Input and Output Images](image)

Figure 2-8: Bradley thresholding result.

In this technique, the average of an $w \times w$ window of pixels centered around each pixel is computed which considers neighboring pixels on all sides. If the value of the current pixel is $t$ percent less than this average then it is set to black, otherwise it is set to white \[51\], see Equation 2.11.

$$T(x, y) = m(x, y) \left( \frac{100 - t}{100} \right)$$ (2.11)

### 2.5 Morphology

In the context of image processing, mathematical morphology treats image components as geometrical shapes. Mathematical morphology is concerned with how to identify, represent and process the shapes of objects within a scene. Morphology relies on several branches of mathematics such as discrete geometry, topology, and differential equations. It has produced sophisticated and efficient algorithms for such tasks as handwriting analysis, biomedical imaging, and others \[42\].

Morphological image processing is a type of processing in which the spatial form or structure of objects within an image are modified. Dilation, erosion, and skeletonization are three fundamental morphological operations. With dilation, an object grows uniformly in spatial extent, whereas with erosion an object shrinks uniformly. Skeletonization results in a stick figure representation of an object. The fundamental goals
of morphological processing are those of preprocessing, segmenting, and classifying images and image components.

The basic concepts of morphological image trace back to the research on spatial set algebra by Minkowski and the studies of Matheron on topology. Serra developed much of the early foundation of the subject. Steinberg was a pioneer in applying morphological methods to medical and industrial vision applications. This research work led to the development of the cytocomputer for high-speed morphological image processing [54].

### 2.5.1 Components

A component is a set of pixels where membership in the set is determined by both the color and the location of pixel. A binary image contains only two colors, which are classified as the foreground and the background. Any pixel that is a foreground pixel is a member of some component while no background pixel is part of a component. Foreground pixels that are spatially connected belong to the same component while foreground pixels that are not spatially connected are not part of the same component. This spatial connectedness is known as *connectivity*.

![Figure 2-9: Neighbors](image)

Consider two adjacent pixels within a binary image. A pixel $p(x, y)$ has neighboring pixels to the north, south, east and west that are given by locations $(x, y - 1)$, $(x, y + 1)$, $(x + 1, y)$, and $(x - 1, y)$, respectively. These neighbors form a set of four pixels known as the *4-neighbors of $p$*, that are denoted as $N_4(p)$. A pixel $p$ also
CHAPTER 2. BACKGROUND

has four neighbors that adjoin to the northwest, northeast, southwest and southeast. These neighbors constitute a set of four pixels which are denoted as \( N_d(p) \) where the subscript \( d \) stands for the diagonal neighbors of \( p \). The set of all eight is denoted as \( N_8(p) \) and is referred to as the 8-neighborhood of \( p \). The 8-neighbor set is the union of the 4-neighbor and diagonal neighbor sets: \( N_8(p) = N_4(p) \cup N_d(p) \). Figure 2-9 illustrates each of these sets.

![Diagram of neighbors and connected components](image)

(a) Binary source.  (b) 4-connected  (c) 8-connected

Figure 2-10: Connected components

A pixel \( p \) is said to be 4-adjacent to another pixel \( q \) if \( q \) is one of the 4-neighbors of \( p \). Is similar for 8-adjacent pixels. Pixels \( p \) and \( q \) are also said to be connected if there is a path of connected pixels extending form \( p \) to \( q \). A path is a sequence of pixels \( p_1, p_2, ..., p_{n-1}, p_n \) such that each pixel in the sequence is connected to the preceding and succeeding pixel in the sequence. Then it is defined a 4-connected component containing pixel \( p \) as the set of all pixels that are 4-connected to \( p \). Figure 2-10 illustrates how connectedness is used to identify components within a binary image.

### 2.5.2 Erosion and Dilation

The properties of a morphological filter are specified by elements in a matrix called a “structuring element”. In binary morphology, the structuring element contains only the values 0 and 1, i.e., \( H(x,y) \in \{0,1\} \), and the hot spot marks the origin of the coordinate system of \( H \). Notice that the hot spot is not necessarily located at the center of the structuring element, nor must its value be 1. Dilation and erosion are low level operations upon which many more sophisticated operations rely.
2.5. MORPHOLOGY

▷ Dilation

Dilation is the morphological operation that corresponds to our intuitive concept of “growing”. As a set operation it is defined as

\[ I \oplus H \equiv \{(p + q) \mid \text{for every } p \in I, q \in H\} \quad (2.12) \]

Thus the point set produced by a dilation is the sum of all possible pairs of coordinate points from the original sets \( I \) and \( H \).

Alternatively, one could view the dilation as the structuring element \( H \) being \textit{replicated} at each foreground pixel of the image \( I \) or, conversely, the image \( I \) being replicated at each foreground element of \( H \). Equation 2.13 shows this expressed in set notation.

\[ I \oplus H \equiv \bigcup_{p \in I} H_p = \bigcup_{q \in H} I_q \tag{2.13} \]

with \( H_p, I_p \) denoting the sets \( H, I \) shifted by \( p \) and \( q \), respectively.

▷ Erosion

Is the quasi-inverse operation of dilation, again defined in set notation as

\[ I \ominus H \equiv \{p \in \mathbb{Z}^2 \mid (p + q) \in I, \text{ for every } q \in H\} \quad (2.14) \]

This definition may appear quite cryptic but is simply explained as follows. A position \( p \) is contained in the result \( I \ominus H \) if, and only if, the structuring element \( H \)- when placed at this point \( p \)- is \textit{fully contained} in the foreground pixels of the original image; i.e., if \( H_p \) is a subset of \( I \). Equivalent to Equation 2.14, erosion can be defined as

\[ I \ominus H \equiv \{p \in \mathbb{Z}^2 \mid H_p \subseteq I\} \tag{2.15} \]
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2.5.3 Opening and Closing

Opening and closing are morphological processes based on dilation and erosion. Opening is used to separate a single component within a source image at narrow pathways between portions of the component. Closing has the opposite effect of joining two separate shapes that are in sufficiently close proximity. Opening and closing tend to otherwise leave the thickness of components unchanged, which makes these operations significantly different than either dilation or erosion [42].

Closing operation consists of dilation followed by erosion, it is expressed symbolically as

\[ G(x, y) = I(x, y) \bullet H(x, y) = [I(x, y) \oplus H(x, y)] \ominus \tilde{H}(x, y) \]

where \( H(x, y) \) is a \( L \times L \) structuring element, note that erosion is performed with the reflection of the structuring element. Closing of an image with a compact structuring element without holes, such as a square or circle, smooths contour of objects, eliminates small holes in objects, and fuses short gaps between objects.

An opening operation, expressed symbolically as

\[ G(x, y) = I(x, y) \circ H(x, y) = [I(x, y) \ominus \tilde{H}(x, y)] \oplus H(x, y) \]

where again, the erosion is with the reflection of the structuring element. Opening of an image smooths contours of objects, eliminates small objects, and breaks narrow strokes. The closing operation tends to increase the spatial extent of an object, while the opening operation decreases its spatial extent [54].

2.6 Kalman Filter

The Kalman filter is a state estimator which produces an optimal estimate in the sense that the mean value of the sum of the estimation errors gets a minimal value.
2.6. KALMAN FILTER

In other words, the Kalman filter gives the sum of squared errors, Equation 2.18, a minimal value.

\[
E[e^T_x(k)e^T_x(k)] = E[e^2_{x1}(k) + ... + e^2_{xn}(k)]
\]

(2.18)

where

\[e_x(k) = x_{est}(k) - x(k)\]

Kalman filter applies for tracking in any number of dimensions for the measurement and state space and for general dynamics models. The proper specification of the state vector, observation matrix, transition matrix, dynamics model, and measurement covariance matrix is needed.

2.6.1 Derivation of r-dimensional Kalman filter

Employing a weighted least-squares error estimate approach a physical sense can be given to Kalman filter. If \(Y_n\) represents the measurements, \(X^*_n,n-1\) represents the predicted state vector and \(X^*_n,n\) represents the desired estimate. There are two errors, one is the distance of \(X^*_n,n\) form \(Y_n\) and the other is its distance of \(X^*_n,n\) from \(X^2_{n,n-1}\). For the minimum least-squares estimate we should minimize the sum of the squares of the distances (errors) between the measurements and the best-fitting line (trajectory) to the measurements. One of the estimates, either \(Y_n\) or \(X^*_n,n-1\), will typically be more accurate than the other. For convenience let us say \(X^*_n,n-1\) is more accurate than \(Y_n\). In this case it is more important that \((X^2_{n,n-1} - X^*_n,n)^2\) be small, specifically smaller than \((Y_n - MX^*_n,n)^2\), where \(M\) is called the observation matrix and it fits the coordinate systems of measurements and estimate which not necessarily should be equal. This would be achieved if in finding the least sum of the squares of each of the two errors we weighted the former error by a larger constant than the later error. We are thus obtaining a minimization of an appropriately weighted sum of the two errors wherein the former receives a larger weighting. A logical weighting is to weight each
CHAPTER 2. BACKGROUND

term by 1 over the accuracy of their respective estimates as the Equation 2.19 does.

\[
E = \frac{(Y_n - MX_n^*)^2}{\text{VAR}(Y_n)} + \frac{(X_{n,n-1}^* - X_{n,n}^*)^2}{\text{VAR}(X_{n,n-1}^*)}
\]  

(2.19)

Thus, \(\text{VAR}(X_{n,n-1}^*) \ll \text{VAR}(Y_n)\) forces the error \((X_{n,n-1}^* - X_{n,n}^*)^2\) to be much smaller than the error \((Y_n - MX_{n,n}^*)^2\) when minimizing the weighted sum of errors \(E\) of Equation 2.19. The more accurate \(X_{n,n-1}^*\) the closer is \(X_{n,n}^*\) to \(X_{n,n-1}^*\), and the more important an error is, the smaller it will be made. In Equation 2.19 the two errors are automatically weighted according to their importance, the errors being divided by their respective variances.

The concepts introduced by equation above, are mathematically formalized using matrix notation as shown below.

\[
J = (Y_n - MX_{n,n}^*)^T R_n^{-1} (Y_n - MX_{n,n}^*) + (X_{n,n-1}^* - X_{n,n}^*)^T S_{n,n-1}^{-1} (X_{n,n-1}^* - X_{n,n}^*)
\]  

(2.20)

where \(R_n = \text{COV}(Y_n)\) and \(S_{n,n-1}^{-1} = \text{COV}(X_{n,n-1}^*)\). Now we are in a position to solve for \(X_{n,n}^*\), differentiating respect to \(X_{n,n}^*\), setting the resulting equation equal to zero in solving for \(X_{n,n}^*\).

\[
\frac{\partial J}{\partial X} = 2(Y_n - MX_{n,n}^*)^T R_n^{-1} (-M) + 2(X_{n,n}^* - X_{n,n-1}^*)^T S_{n,n-1}^{-1} = 0
\]  

(2.21)

Rewritting 2.21

\[
(S_{n,n-1}^{-1} + M^T R_n^{-1} M) X_{n,n}^* = S_{n,n-1}^{-1} X_{n,n-1}^* - M^T R_n^{-1} Y_n
\]

Then

\[
X_{n,n}^* = (S_{n,n-1}^{-1} + M^T R_n^{-1} M)^{-1} (S_{n,n-1}^{-1} X_{n,n-1}^* + M^T R_n^{-1} Y_n)
\]  

(2.22)
2.6. Kalman Filter

Using the matrix inversion lemma we have

\[
(S_{n,n-1}^* + M^T R^{-1} M)^{-1} = S_{n,n-1}^* - S_{n,n-1}^* M^T (R_n + M S_{n,n-1}^* M^T)^{-1} M S_{n,n-1}^*
\]

\[
= S_{n,n-1}^* - H_n M S_{n,n-1}^* \tag{2.23}
\]

Such that

\[
H_n = S_{n,n-1}^* M^T (R_n + M S_{n,n-1}^* M^T)^{-1} \tag{2.25}
\]

\[
S_{n,n-1}^* M^T = H_n (R_n + M S_{n,n-1}^* M^T) \tag{2.22}
\]

\[
H_n R_n = S_{n,n-1}^* M^T - H_n M S_{n,n-1}^* M^T \tag{2.26}
\]

Substituting Equation 2.23 in 2.22

\[
X_{n,n}^* = (S_{n,n-1}^* - H_n M S_{n,n-1}^*) (S_{n,n-1}^* X_{n,n-1}^* + M^T R_n^{-1} Y_n)
\]

\[
= X_{n,n-1}^* - H_n M X_{n,n-1}^* + (S_{n,n-1}^* - H_n M S_{n,n-1}^*) M^T R_n^{-1} Y_n
\]

using Equation 2.26

\[
= X_{n,n-1}^* + H_n Y_n - H_n M X_{n,n-1}^*
\]

\[
X_{n,n}^* = X_{n,n-1}^* + H_n (Y_n - M X_{n,n-1}^*) \tag{2.27}
\]

Equation 2.27 is the Kalman filter equation. Table 2.2 shows an equation summary for Kalman filtering 55.
Table 2.2: Kalman equation summary.

Predictor equation:
\[ X_{n+1,n}^* = \Phi X_{n,n}^* \]

Filtering equation:
\[ X_{n,n}^* = X_{n,n-1}^* + H_n (Y_n - M X_{n,n-1}^*) \]

Weight equation:
\[ H_n = S_{n,n-1}^* M^T [R_n + M S_{n,n-1}^* M^T]^{-1} \]

Predictor covariance matrix equation:
\[ S_{n,n-1}^* = \text{COV}(X_{n,n-1}^*) \]
\[ S_{n,n-1} = \Phi S_{n-1,n-1}^* \Phi^T + Q_n \]

Covariance of random system dynamics model noise vector \( U_n \):
\[ Q_n = \text{COV}(U_n) = E[U_n U_n^T] \]

Covariance of measurement vector \( Y_n = X_n + N_n^* \):
\[ R_n = \text{COV}(Y_n) = \text{COV}(N_n) = E[N_n N_n^T] \]

Corrector equation (covariance of smoothed estimate):
\[ S_{n-1,n-1} = \text{COV}(X_{n-1,n-1}^*) = (I - H_{n-1,M}) S_{n-1,n-2}^* \]

1 If \( E[U] = E[N_n] = 0 \).
Chapter 3

Proposed Approach Description

This chapter presents the proposed solution to achieve the general and particular objectives previously presented. The whole problem was divided in smaller parts represented in a block diagram that contains the essential components of the proposal, here the reader can find an explanation of each component.

Basically, the diagram consists of four stages. In the first stage the fluorescence image sequences are loaded and with those the ratio sequence is made, a comparison with Image J results can be seen. In the second stage an adaptive binarization process is performed to one of the initial image sequences, the implementation of several techniques was done and the comparison of the results is presented to explain why one of them was used and no the others. In the third block a tracking scheme based on logical intersections was developed, the scheme is enhanced with state estimation using Kalman filter. Finally, in the last one the feature extraction of the cell and the register of the important data are done, a description of the array of structures used by this stage is also given.
### 3.1 Diagram of Solution

As seen in section 1.5, the overall objective of this thesis work is to automate the methodology of analysis for extracting $[\text{Ca}^{2+}]_i$ profiles. The following block diagram represents the proposed solution.

![Block diagram of the proposed solution.](image)

**Figure 3-1:** Block diagram of the proposed solution.

Given two fluorescence image sequences as input (340 nm and 380 nm excitation wavelength image sequences), the ratio sequence is made in initial block. The 380 nm image sequence is adaptively binarized to segment the cell nuclei, then, the user selected cells can be tracked using and scheme based on logical intersections, a Kalman estimator protects the operation of this block when some cells disappear during some frames. In the last block a feature extraction is performed, the new position is sent as feedback to the tracking block and features are also stored since they are useful for cell behavior modeling, between these features there are the intracellular calcium measurements.

All the blocks were developed using MATLAB® R2013b and all were joined together in a graphical user interface (GUI) using GUIDE. Every block is explained with more detail in the sections below.
CHAPTER 3. PROPOSED APPROACH DESCRIPTION

3.2 Ratio Sequences Block

Two fluorescence image sequences that correspond to the $\lambda_{Ex} = 340$ nm and to the $\lambda_{Ex} = 380$ nm excitation wavelengths are used as inputs. Images, in TIFF format, are loaded to MATLAB® as two arrays of $m \times n \times k$ size of uint8 variable type. Where $m$ and $n$ are the image height and width correspondingly and $k$ represents the number of frames of the sequence. Once the sequences are loaded, a pixel by pixel ratio is done to produce a third image sequence called $Ratio$ sequence. Output sequence has the same size of the input ones, but its type of data is changed to double because of its dynamic range. See Figure 3-3a.

![Figure 3-2: Ratio sequence block.](image)

![Figure 3-3: (a) Ratio image sequence construction with two excitation wavelength sequences $\lambda_{Ex} = 340$ nm and $\lambda_{Ex} = 380$ nm. (b) Randomly selected ROIs.](image)

Ratio sequence is very important due to $[Ca^{2+}]_i$ profiles are going to be extracted from this images as intensity mean measurements. To validate the proposed ratio procedure, two data sets were compared. To create data sets, ten rectangular ROI’s were chosen, see Figure 3-3b, each one surrounding one different cell nucleus and their $[Ca^{2+}]_i$ profiles ($CP_{RP}$) were obtained using the $RatioPlus$ plugin from ImageJ.
3.2. RATIO SEQUENCES BLOCK

Bounding box sizes and positions were saved then loaded in MATLAB® and the same 10 calcium profiles (CP\textsubscript{M}) were obtained. Mean square error (MSE) was obtained later as following.

\[
MSE_i = \frac{1}{N}(CP_{RP} - CP_{M})(CP_{RP} - CP_{M})^T
\]  \hspace{1cm} (3.1)

Then the total mean square error is defined as follow

\[
TMSE = \frac{1}{N} \sum_{i=1}^{N} MSE_i
\]  \hspace{1cm} (3.2)

Where \( N = 10 \) in Equation 3.2. The selected ROIs, its position, size and mean square error are shown in Table 3.1. This error is attributable to the type data changes that take place, and its magnitude is not significant for the final measurements. In this way, the proposed Ratio Sequences Block has a good enough performance.

Table 3.1: Randomly selected ROIs and mean square error.

<table>
<thead>
<tr>
<th>ROI</th>
<th>Position ([x, y])</th>
<th>Size ([w, h])</th>
<th>MSE(_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>280, 219</td>
<td>39, 26</td>
<td>6.0599 \times 10^{-5}</td>
</tr>
<tr>
<td>2</td>
<td>277, 491</td>
<td>32, 25</td>
<td>9.5643 \times 10^{-8}</td>
</tr>
<tr>
<td>3</td>
<td>443, 145</td>
<td>36, 32</td>
<td>6.7804 \times 10^{-8}</td>
</tr>
<tr>
<td>4</td>
<td>352, 398</td>
<td>38, 35</td>
<td>7.4837 \times 10^{-8}</td>
</tr>
<tr>
<td>5</td>
<td>467, 21</td>
<td>39, 27</td>
<td>8.5509 \times 10^{-8}</td>
</tr>
<tr>
<td>6</td>
<td>297, 445</td>
<td>38, 23</td>
<td>8.5691 \times 10^{-8}</td>
</tr>
<tr>
<td>7</td>
<td>416, 305</td>
<td>34, 26</td>
<td>8.7202 \times 10^{-8}</td>
</tr>
<tr>
<td>8</td>
<td>206, 229</td>
<td>34, 32</td>
<td>8.7577 \times 10^{-8}</td>
</tr>
<tr>
<td>9</td>
<td>180, 138</td>
<td>38, 27</td>
<td>6.9980 \times 10^{-8}</td>
</tr>
<tr>
<td>10</td>
<td>49, 260</td>
<td>38, 34</td>
<td>9.2960 \times 10^{-8}</td>
</tr>
</tbody>
</table>

\[
TMSE = 6.1346 \times 10^{-6}
\]
3.3 Adaptive Binarization Block

Threshold process is carried out in order to separate, in an image, pixels which represent an object from those that represent the background. Generally two values are assigned to pixels, 0 for background and 1 for objects, or viceversa, giving as result binary images or two level images, this images are also called binarized.

In fluorescent images from endothelial tissue, the nuclei are the objects and the remaining pixels conform the background. The correct nuclei segmentation is important because it is used for next stages. Now, there are three image sequences available, the ones corresponding to $\lambda_{Ex} = 340\text{nm}$ and $\lambda_{Ex} = 380\text{nm}$ and the ratio sequence; all of them have an non-uniform illumination and also have low contrast between nuclei and background so it is needed to adapt the threshold of each pixel according its own and its neighbors intensity, so that adaptive binarization was chosen.

All the techniques cited in Section 2.4 were programmed. For performance evaluation images binarized automatically were compared with images binarized manually by experts in health science from the CVPL–FMBUAP, these last are also known as Ground Truth Segmentation. A graphical user interface was developed for manual binarization, Figure 3-5a, in this GUI it is possible to select with the mouse pointer all that could represent an “object” or “region of interest”. The binarized images, Figure 3-5b, were saved as *.mat files and these can be loaded to MATLAB® as a binary matrix.
3.3. **ADAPTIVE BINARIZATION BLOCK**

![Figure 3-5](image)

Figure 3-5: (a) GUI developed for ground truth segmentation. (b) Ground truth segmentation example.

Evaluation method was partially taken from [52]. To be able to compare the performance of each algorithm, the following aspects were quantified.

1. Successfully binarized objects percent \( (P_{sbo}) \): is the portion of objects in the manual binarization that are correctly binarized by the algorithm. According to this, the performance was classified as follow.

   - \([100 - 95]\)% Outstanding to excellent.
   - \([95 - 90]\)% Good to outstanding.
   - \([90 - 80]\)% Tolerable to good.
   - \([80 - 70]\)% Deficient to tolerable.
   - \([70 - 0]\)% Deficient

2. Deletion error \( (e_d) \): it occurs when the algorithm can not detect an object that is in the manual binarization (mb), i.e., a false negative.

3. Insertion error \( (e_i) \): it occurs when the algorithm detects an object that is not in the mb, i.e., a false positive.

4. Melting error \( (e_m) \): it occurs when the algorithm melts two or more objects from the mb into only one.
5. Splitting error ($e_s$): it occurs when the algorithm splits in two or more parts a single object from the mb.

6. Total of errors ($E_T$): it is the sum of deletion, insertion, melting and splitting errors.

7. Missclassified pixels percent ($P_{mcp}$): it is the portion, from all the pixels, which are misclassified by the algorithm. This pixels belong to the well binarized nuclei according to the mb. The closer to zero is $P_{mcp}$ the better performance the algorithm has.

Images given by CVPL-FMBUAP where used, its dimensions are $576 \times 768$ pixels and they have an 8-bit grayscale resolution. For $\lambda_{Ex} = 340$ nm and $\lambda_{Ex} = 380$ nm sample images, we chose those with low contrast and undesirable illumination effects, i.e., the worst cases. Ratio images were also selected, see Table 3.3. The evaluation had two goals, the first is to find the algorithm with the best performance and the second is to know which of the three sequences is the most convenient to work with.

In order to remove noise from the sample images and preserve its sharp high-frequency detail, a median filter with a window $7 \times 7$ size was applied to all the images before they went through the binarization process, see Figure 3-4. The gains of each thresholding technique were experimentally set for the fluorescence images case and once binarized, the images were conditioned using a series of morphological operations, finally nuclei were thresholded by area and eccentricity. The image conditioning was different for each binarization technique because its results was different too, some of them may include another median filter stage. Results are reported in following subsections.
### 3.3. Adaptive Binarization Block

<table>
<thead>
<tr>
<th>Original Image</th>
<th>Manual Binarization</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1 (img0-340_068)</td>
<td><img src="image1.png" alt="Image" /> <img src="image2.png" alt="BM Image" /> B1.a (bm_m_340_068)* <img src="image3.png" alt="BM Image" /> B1.b (Nucleos340nm_068)</td>
</tr>
<tr>
<td>I2 (img0-380_068)</td>
<td><img src="image4.png" alt="Image" /> <img src="image5.png" alt="Image" /> B2 (Nucleos380nm_068)</td>
</tr>
<tr>
<td>I3 (ratio 50)</td>
<td><img src="image6.png" alt="Image" /> <img src="image7.png" alt="Image" /> B3 (Nucleos50)</td>
</tr>
<tr>
<td>I4 (ratio 70)</td>
<td><img src="image8.png" alt="Image" /> <img src="image9.png" alt="Image" /> B4 (Nucleos70)</td>
</tr>
<tr>
<td>I3 (ratio 80)</td>
<td><img src="image10.png" alt="Image" /> <img src="image11.png" alt="Image" /> B5 (Nucleos80)</td>
</tr>
</tbody>
</table>

*Except this binary image, all of the remaining were manually binarized by the CVPL-FMBUAP.

Table 3.3: Original and manual binarized images used for the evaluation of the algorithms.
CHAPTER 3. PROPOSED APPROACH DESCRIPTION

3.3.1 Niblack Technique

For this technique it was used a \( w = 11 \) window size and \( k = -0.2 \). Window size was approximated based on the nuclei mean area from one manual binarized image and taking the supposition that they are in a square region, then \( w = \sqrt{A} \). For image B1.b, \( w = 13.8037 \), however, \( w = 11 \) gave better results and it is very close to 13.8037. The conditioning stage of this technique is shown in the Table 3.4.

\[
\begin{align*}
1. & \quad I_1 = I \ominus s_1 \\
2. & \quad I_2 = \text{median filter, } w = 7 \times 7 \leftarrow I_1 \\
3. & \quad I_3 = (((I_2 \oplus s_2) \bullet s_2) \oplus s_2) \bullet s_2 \\
4. & \quad \text{Threshold } (A > 30) \land (E < 0.999)
\end{align*}
\]

Table 3.4: Binary image conditioning scheme for Niblack technique.

Where \( s_1 \) and \( s_2 \) are structuring elements, they were built using the function \( \text{strel('disk',4,6)} \) and \( \text{strel('disk',1,6)} \) respectively. \( A \) represents the area and \( E \) the eccentricity of the segmented cells nuclei, and \( I_i \) is the processed image.

<table>
<thead>
<tr>
<th>Manual Binarization</th>
<th>( P_{sbo} (%) )</th>
<th>( e_d )</th>
<th>( e_i )</th>
<th>( e_m )</th>
<th>( e_s )</th>
<th>( E_T )</th>
<th>( P_{mcp} (%) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>81.2766</td>
<td>44</td>
<td>50</td>
<td>4</td>
<td>5</td>
<td>103</td>
<td>8.7741</td>
</tr>
<tr>
<td>B2</td>
<td>86.1272</td>
<td>24</td>
<td>95</td>
<td>1</td>
<td>2</td>
<td>122</td>
<td>7.2259</td>
</tr>
<tr>
<td>B3</td>
<td>84.2857</td>
<td>22</td>
<td>81</td>
<td>0</td>
<td>2</td>
<td>105</td>
<td>5.4502</td>
</tr>
<tr>
<td>B4</td>
<td>56.2500</td>
<td>7</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>34</td>
<td>0.9205</td>
</tr>
<tr>
<td>B5</td>
<td>80.7229</td>
<td>16</td>
<td>40</td>
<td>0</td>
<td>2</td>
<td>58</td>
<td>3.1835</td>
</tr>
<tr>
<td>B6</td>
<td>80.3571</td>
<td>11</td>
<td>55</td>
<td>1</td>
<td>1</td>
<td>68</td>
<td>2.5443</td>
</tr>
</tbody>
</table>

Table 3.5: Niblack technique performance.

Based on the \( P_{sbo} \) results, it can be seen that the performance of this technique is from tolerable to good, but is sightly better for \( \lambda_{Ex} \) than from ratio images, see Table 3.5. It can be seen that there is an important number of insertion errors, however, it is convenient to see images from Table 3.3 to realize that those insertion errors seems to belong to a nucleus although it was not included in the manual binarization. Concerning about the \( P_{mcp} \), the performance is better for ratio than for \( \lambda_{Ex} \) images.
3.3. **ADAPTIVE BINARIZATION BLOCK**

3.3.2 **Sauvola Technique**

A $w = 21$ size window and $k = 0.2$ were used, both quantities were obtained experimentally, meanwhile $R = 128$ because of 8-bit resolution images.

According with $P_{sbo}$, the technique showed a deficient performance for all the images, and there are a lot of errors, Table 3.6. $P_{mcp}$ percentage is acceptable, but in this case it does not make sense because the automatic segmented nuclei do not match with the ground truth segmentation ones. The conditioning stage is the same reported in Table 3.4.

<table>
<thead>
<tr>
<th>Manual Binarization</th>
<th>$P_{sbo}$ (%)</th>
<th>$e_d$</th>
<th>$e_i$</th>
<th>$e_m$</th>
<th>$e_s$</th>
<th>$E_T$</th>
<th>$P_{mcp}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.8511</td>
<td>233</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>236</td>
<td>11.5734</td>
</tr>
<tr>
<td>B2</td>
<td>0.5780</td>
<td>172</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>176</td>
<td>7.5100</td>
</tr>
<tr>
<td>B3</td>
<td>0</td>
<td>140</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>142</td>
<td>5.3607</td>
</tr>
<tr>
<td>B4</td>
<td>0</td>
<td>16</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>0.7078</td>
</tr>
<tr>
<td>B5</td>
<td>22.8916</td>
<td>64</td>
<td>82</td>
<td>3</td>
<td>3</td>
<td>152</td>
<td>6.7078</td>
</tr>
<tr>
<td>B6</td>
<td>23.2143</td>
<td>43</td>
<td>54</td>
<td>4</td>
<td>4</td>
<td>105</td>
<td>4.9084</td>
</tr>
</tbody>
</table>

Table 3.6: Sauvola technique performance.
3.3.3 Romen Technique

This technique showed its best performance using a $w = 25$ and $k = 0.0001$. The threshold is very proximate to the neighborhood intensity mean with that gain.

The image conditioning stage was different, the final threshold was only based in the area of the segmented nuclei. Assuming a normal distribution for the nuclei area, we changed the final threshold criterion, Table 3.7.

| 1.- | $I_1 = I \ominus s_1$ |
| 2.- | $I_2 = \text{median filter, } w = 7 \times 7 \leftarrow I_1$ |
| 3.- | $I_3 = (((I_2 \oplus s_2) \bullet s_2) \oplus s_2) \bullet s_2$ |
| 4.- | Threshold $\mu_A - 0.8\sigma_A \leq A \leq \mu_A + 0.8\sigma_A$ |

Table 3.7: Binary image conditioning scheme for Romen technique.

Where $s_1$ and $s_2$ are structuring elements, they were built using the function \texttt{strel('disk',4,6)} and \texttt{strel('disk',1,6)} respectively. $A$ represents the area of the segmented cells nuclei, $\mu_A$ and $\sigma_A$ are the mean and standard deviation of area,
3.3. ADAPTIVE BINARIZATION BLOCK

respectively.

<table>
<thead>
<tr>
<th>Manual Binarization</th>
<th>$P_{sbo}$ (%)</th>
<th>$e_d$</th>
<th>$e_i$</th>
<th>$e_m$</th>
<th>$e_s$</th>
<th>$E_T$</th>
<th>$P_{mcp}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>70.6383</td>
<td>69</td>
<td>65</td>
<td>3</td>
<td>0</td>
<td>137</td>
<td>12.7346</td>
</tr>
<tr>
<td>B2</td>
<td>79.7688</td>
<td>35</td>
<td>92</td>
<td>1</td>
<td>1</td>
<td>129</td>
<td>11.6304</td>
</tr>
<tr>
<td>B3</td>
<td>73.5714</td>
<td>37</td>
<td>134</td>
<td>0</td>
<td>0</td>
<td>173</td>
<td>12.3323</td>
</tr>
<tr>
<td>B4</td>
<td>43.7500</td>
<td>9</td>
<td>335</td>
<td>0</td>
<td>0</td>
<td>344</td>
<td>12.5970</td>
</tr>
<tr>
<td>B5</td>
<td>60.2410</td>
<td>33</td>
<td>329</td>
<td>0</td>
<td>1</td>
<td>363</td>
<td>13.9395</td>
</tr>
<tr>
<td>B6</td>
<td>42.8571</td>
<td>32</td>
<td>281</td>
<td>1</td>
<td>0</td>
<td>313</td>
<td>14.6096</td>
</tr>
</tbody>
</table>

Table 3.8: Romen technique performance.

Based on $P_{sbo}$, it can be seen that Romen technique has a deficient to tolerable performance for $\lambda_{Ex}$ images and a deficient performance for ratio images. Some of the $e_i$ correspond to apparent nuclei that were not considered in ground truth segmentation. See Table 3.8.

Figure 3-8: Automatic binarization frame (blue), overlapped with ground truth segmentation frame (red). Intersections between both frames can be seen in violet color.
3.3.4 Jyh Technique

As recommended in [52], a $T_{STD} = 0.3$ standard deviation threshold was chosen. A maximum window size $w_{max} = 25$ was selected, and for the single thresholding, we found the best results with $T_{gb} = 0.5$ experimentally. That threshold gives another threshold $T$ which works on the normalized image $I_N$ given by this algorithm.

$$\begin{align*}
T &= \min\{I_N\} + T_{gb}(\max\{I_N\} - \min\{I_N\})
\end{align*}$$

The conditioning image scheme for this technique is shown in Table 3.9. The structuring element $s_1$ was built using the function \texttt{strel('disk',1,6)}. $A$ represents the area of the segmented cells nuclei and $E$ its eccentricity.

1.- $I_1 = (((I \ominus s_1) \circ s_1) \ominus s_1) \circ s_1$

2.- Threshold $(A > 30) \& (E < 0.999)$

Table 3.9: Binary image conditioning scheme for Jyh technique.

In Table 3.10 it can be seen that, based on $P_{sbo}$, the performance for $\lambda_{Ex}$ images is better than for ratio images, although for both the $P_{mcp}$ is acceptable. It is important to say that this technique is computationally costly because it needs to get statistical information several times for each pixel.

<table>
<thead>
<tr>
<th>Manual Binarization</th>
<th>$P_{sbo}$ (%)</th>
<th>$e_d$</th>
<th>$e_i$</th>
<th>$e_m$</th>
<th>$e_s$</th>
<th>$E_T$</th>
<th>$P_{mcp}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>80.9</td>
<td>45</td>
<td>43</td>
<td>10</td>
<td>5</td>
<td>103</td>
<td>10.1</td>
</tr>
<tr>
<td>B2</td>
<td>79.2</td>
<td>36</td>
<td>98</td>
<td>1</td>
<td>3</td>
<td>138</td>
<td>9.6</td>
</tr>
<tr>
<td>B3</td>
<td>97.1</td>
<td>4</td>
<td>107</td>
<td>16</td>
<td>0</td>
<td>127</td>
<td>27.9</td>
</tr>
<tr>
<td>B4</td>
<td>81.3</td>
<td>3</td>
<td>460</td>
<td>1</td>
<td>1</td>
<td>465</td>
<td>21.1</td>
</tr>
<tr>
<td>B5</td>
<td>6.0</td>
<td>78</td>
<td>216</td>
<td>0</td>
<td>0</td>
<td>294</td>
<td>7.7</td>
</tr>
<tr>
<td>B6</td>
<td>91.1</td>
<td>5</td>
<td>370</td>
<td>1</td>
<td>5</td>
<td>381</td>
<td>20.1</td>
</tr>
</tbody>
</table>

Table 3.10: Jyh technique performance.
3.3. ADAPTIVE BINARIZATION BLOCK

Figure 3-9: Automatic binarization frame (blue), overlapped with ground truth segmentation frame (red). Intersections between both frames can be seen in violet color.

3.3.5 Bradley Technique

Window size for this technique was \( w = 17 \) and \( t = 10 \). Based on \( P_{sbo} \) we can say that its performance is from outstanding to excellent with both kind of images, but the quantity of \( e_i \) is greater for ratio images than for \( \lambda_{Ex} \) images, Table 3.11.

<table>
<thead>
<tr>
<th>Manual Binarization</th>
<th>( P_{sbo} (%) )</th>
<th>( e_d )</th>
<th>( e_l )</th>
<th>( e_m )</th>
<th>( e_s )</th>
<th>( E_T )</th>
<th>( P_{mcp} (%) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>98.7234</td>
<td>3</td>
<td>33</td>
<td>4</td>
<td>5</td>
<td>45</td>
<td>7.1452</td>
</tr>
<tr>
<td>B2</td>
<td>98.2659</td>
<td>3</td>
<td>97</td>
<td>2</td>
<td>3</td>
<td>105</td>
<td>6.7227</td>
</tr>
<tr>
<td>B3</td>
<td>97.8571</td>
<td>2</td>
<td>57</td>
<td>1</td>
<td>2</td>
<td>63</td>
<td>4.3755</td>
</tr>
<tr>
<td>B4</td>
<td>100.0000</td>
<td>0</td>
<td>282</td>
<td>1</td>
<td>1</td>
<td>284</td>
<td>6.5891</td>
</tr>
<tr>
<td>B5</td>
<td>98.7952</td>
<td>1</td>
<td>220</td>
<td>4</td>
<td>2</td>
<td>227</td>
<td>7.5580</td>
</tr>
<tr>
<td>B6</td>
<td>96.4286</td>
<td>2</td>
<td>245</td>
<td>3</td>
<td>0</td>
<td>250</td>
<td>8.3166</td>
</tr>
</tbody>
</table>

Table 3.11: Bradley technique performance.

The conditioning image scheme for this technique is the same used for Jyh technique, see Table 3.9.
**CHAPTER 3. PROPOSED APPROACH DESCRIPTION**

Figure 3-10: Automatic binarization frame (blue), overlapped with ground truth segmentation frame (red). Intersections between both frames can be seen in violet color.

### 3.3.6 Conclusions

Based on the reported above, it can be seen that Bradley technique showed the best performance for the endothelial fluorescence images binarization. The quantification of the performance allow us to say that $\lambda_{Ex}$ sequences are better than ratio sequence, in particular $\lambda_{Ex} = 340$ nm was binarized and used in the next stage, the multi-target tracking block.

For efficiency issues, a C executable file was programmed and was called from MATLAB\textsuperscript{®} using a MEX-file, code in Appendix B. This reduced the binarization and image conditioning processing mean time from 2.3 to 1.2 seconds.
3.4 Tracking Block

The purpose of this block is to give the updated tracking label of a given cell. Taking advantage of the slow cell movement a tracking based on intersections was implemented which proved to be a simple and fast solution. Frequently after the tissue is stimulated, many cells disappear only during some frames. When that happens, the first part of the block would detect no intersection, to solve this drawback, a Kalman filter was implemented. The position, velocity and acceleration of the cells are estimated in every iteration to be able to predict the position of the current cell in the case it has disappeared. A counter called reliability counter is used to control the quantity of times that is allowed a cell to disappear before it is considered as a unreliable measurement then labeled as OFFLINE, although that counter is updated in the Feature Extraction block, it is noteworthy that the counter enables the tracking block. From this section, when position of an object is mentioned, the centroid of the object is being referenced.

3.4.1 Intersection

As tracking block is based on logical intersections, it works iteratively with two frames of binarized and labeled images, $f_n$ and $f_{n-1}$. The main goal is to identify the selected cells from $f_n$ to $f_{n-1}$ through the correspondence of the labels called track_label.
A kernel is obtained for each selected cell with the intersection of the current cell with the $f_n$. When intersection detects only one object, the centroid of that object is obtained in order to get the tracking label in $f_n$. When more than one or no object is detected, the prediction equations of Kalman filter are used to estimate the position and to find the new label for the current cell in $f_n$.

Figure 3-12: Intersection illustration.

Once the new tracking label is found, a block called store data is responsible of store the position, and intensity measurement for each cell and sets a 0 in the flag\_lost vector. If no label is detected with intersections neither with Kalman estimation, it is assumed that the cell disappeared in that frame and the reliability\_counter is increased by one, when this counter is equal to the user defined reliability\_limit, the tracking is considered as unreliable and status is changed to OFFLINE and its new tracking label is left to search. The block is initialized with $f_n = 2$ and $f_{n-1} = 1$ and finishes with $f_n = n$ and $f_{n-1} = n - 1$. A pseudocode and an illustration of this sub-block is shown in Algorithm 1 and Figure 3-12, respectively.

**Algorithm 1** Intersection block pseudocode

**Inputs**

- bin\_340nm: Binarized image sequence
- nroi: number of ROIs or selected cells
- CELLS: Array of selected cell structures
- reliability\_limit: Reliability limit
3.4. TRACKING BLOCK

Output

n_track_label: Updated tracking label

cell: Updated cell structure

Process

\( f_n \leftarrow \text{Label(bin}_{340\text{nm}(m,n,k))} \)

\( f_{n-1} \leftarrow \text{Label(bin}_{340\text{nm}(m,n,k-1))} \)

for \((i=1,i++,\text{nroi})\) do

if \(\text{reliability\_limit}==\text{Cell}(i).\text{reliability\_counter}\) then

\(\text{Cell}(i).\text{status}\leftarrow\text{OFFLINE}\)

else

\(\text{kernel} \leftarrow \text{and}(f_{n-1}==\text{track\_label}, f_n)\)

\(\text{n\_o} \leftarrow \text{NumberOfObjects(kernel)}\)

if \(\text{n\_o}==1\) then

\(\text{cent} \leftarrow \text{Centroid(kernel)}\)

\(\text{n\_track\_label} \leftarrow f_n(\text{cent}(2),\text{cent}(1))\)

else

\(\text{x\_aux} \leftarrow \text{KalmanPredictor}\)

\(\text{n\_track\_label} \leftarrow f_n(x\_aux(2),x\_aux(1))\)

end if

\(\text{cell} \leftarrow \text{StoreData}(f_n==\text{n\_track\_label})\)

end if

end for
3.4.2 Kalman Estimator

Although the cell motion is relatively slow, its randomness makes that the tracking based on intersection could get in the kernel more than one or none object due to the uncertainty in the target trajectory or acceleration at any given time.

Model

Since it is extremely complex to find an exact mathematical model to describe the motion of cells, for tracking purposes, it was assumed that cells have a constant acceleration motion. The deterministic description in one dimension is shown in Equation 3.3

\[
\begin{align*}
    x_{n+1} &= x_n + T\dot{x}_n + \frac{T^2}{2}\ddot{x}_n + \frac{T^3}{3}J_n \\
    \dot{x}_{n+1} &= \dot{x}_n + \ddot{x}_n T + \frac{T^2}{2}J_n \\
    \ddot{x}_{n+1} &= \ddot{x}_n + TJ_n
\end{align*}
\]  

(3.3)

Where \( J_n \) is a random change in acceleration (jerk) occurring between time \( n \) and \( n + 1 \). The random jerk \( J_n \) has the auto correlation function given by Equation 3.4.

\[
J_nJ_m = \begin{cases} 
\sigma_J^2 & \text{for } n = m \\
0 & \text{for } n \neq m
\end{cases}
\]  

(3.4)

Hence \( J_n \) is characterized as white noise. Due to the tracking must be done for two dimensions, the state space model is
3.4. TRACKING BLOCK

\[
\begin{align*}
\begin{bmatrix}
    x \\
    \dot{x} \\
    \ddot{x} \\
    y \\
    \dot{y} \\
    \ddot{y}
\end{bmatrix}_{n+1} &= 
\begin{bmatrix}
    1 & T & \frac{T^2}{2} & 0 & 0 & 0 \\
    0 & 1 & T & 0 & 0 & 0 \\
    0 & 0 & 1 & 0 & 0 & 0 \\
    0 & 0 & 0 & 1 & T & \frac{T^2}{2} \\
    0 & 0 & 0 & 0 & 1 & T \\
    0 & 0 & 0 & 0 & 0 & 1
\end{bmatrix}
\begin{bmatrix}
    x \\
    \dot{x} \\
    \ddot{x} \\
    y \\
    \dot{y} \\
    \ddot{y}
\end{bmatrix}_n \\
&+ 
\begin{bmatrix}
    \frac{T^3 J_x}{3} \\
    \frac{T^2 J_y}{2} \\
    T J_x \\
    \frac{T^3 J_y}{3} \\
    \frac{T^2 J_y}{2}
\end{bmatrix}
\end{align*}
\]

\[
\begin{align*}
\begin{bmatrix}
    x \\
    \dot{x} \\
    \ddot{x} \\
    y \\
    \dot{y} \\
    \ddot{y}
\end{bmatrix}_n &= 
\begin{bmatrix}
    1 & 0 & 0 & 0 & 0 \\
    0 & 0 & 0 & 1 & 0 \\
    0 & 0 & 0 & 0 & 1
\end{bmatrix}
\begin{bmatrix}
    x \\
    \dot{x} \\
    \ddot{x} \\
    y \\
    \dot{y} \\
    \ddot{y}
\end{bmatrix}_n
\end{align*}
\]

(3.5)

With the sampling period \( T = 3 \), the observability matrix has full rank, i.e. \( \text{rank} = 6 \), therefore the proposed system model is observable and the Kalman filter can work correctly. Assuming statistical independence between the coordinates \( x \) and \( y \), the system covariance matrix is shown in Equation 3.6. The observation error covariance matrix is shown in Equation 3.7.

\[
Q = 
\begin{bmatrix}
    \frac{T^6 \sigma_{\dot{x}}^2}{9} & \frac{T^5 \sigma_{\dot{x}}^2}{6} & \frac{T^4 \sigma_{\dot{x}}^2}{3} & \frac{T^6 \sigma_{\dot{x} \dot{y}}}{9} & \frac{T^5 \sigma_{\dot{x} \dot{y}}}{6} & \frac{T^4 \sigma_{\dot{x} \dot{y}}}{3} \\
    \frac{T^6 \sigma_{\dot{x}}^2}{6} & \frac{T^5 \sigma_{\dot{x}}^2}{4} & \frac{T^4 \sigma_{\dot{x}}^2}{2} & \frac{T^6 \sigma_{\dot{x} \dot{y}}}{9} & \frac{T^5 \sigma_{\dot{x} \dot{y}}}{6} & \frac{T^4 \sigma_{\dot{x} \dot{y}}}{3} \\
    \frac{T^6 \sigma_{\dot{x}}^2}{3} & \frac{T^5 \sigma_{\dot{x}}^2}{2} & \frac{T^4 \sigma_{\dot{x}}^2}{1} & \frac{T^6 \sigma_{\dot{x} \dot{y}}}{9} & \frac{T^5 \sigma_{\dot{x} \dot{y}}}{6} & \frac{T^4 \sigma_{\dot{x} \dot{y}}}{3} \\
    \frac{T^6 \sigma_{\dot{x}}^2}{9} & \frac{T^5 \sigma_{\dot{x}}^2}{6} & \frac{T^4 \sigma_{\dot{x}}^2}{3} & \frac{T^6 \sigma_{\dot{x} \dot{y}}}{9} & \frac{T^5 \sigma_{\dot{x} \dot{y}}}{6} & \frac{T^4 \sigma_{\dot{x} \dot{y}}}{3} \\
    \frac{T^6 \sigma_{\dot{x}}^2}{6} & \frac{T^5 \sigma_{\dot{x}}^2}{4} & \frac{T^4 \sigma_{\dot{x}}^2}{2} & \frac{T^6 \sigma_{\dot{x} \dot{y}}}{9} & \frac{T^5 \sigma_{\dot{x} \dot{y}}}{6} & \frac{T^4 \sigma_{\dot{x} \dot{y}}}{3} \\
    \frac{T^6 \sigma_{\dot{x}}^2}{3} & \frac{T^5 \sigma_{\dot{x}}^2}{2} & \frac{T^4 \sigma_{\dot{x}}^2}{1} & \frac{T^6 \sigma_{\dot{x} \dot{y}}}{9} & \frac{T^5 \sigma_{\dot{x} \dot{y}}}{6} & \frac{T^4 \sigma_{\dot{x} \dot{y}}}{3}
\end{bmatrix}
\]

(3.6)
\[ R = \begin{bmatrix} \sigma_x^2 & 0 \\ 0 & \sigma_y^2 \end{bmatrix} \]  

(3.7)

**Statistical Parameters**

The parameters \( \sigma_{Jx} \) and \( \sigma_{Jy} \) belong to the randomness nature of the system, for this reason, they were extracted from a sequence of 100 images. Fifteen cells were randomly selected, and were manually tracked frame to frame to register its vectors position, \( x \) and \( y \). Three numerical derivatives were performed in order to get the jerk vector for each dimension, then the standard deviation of each jerk vector was obtained, finally \( \sigma_{Jx} \) is the average of those \( \sigma_{Jx_i} \), see Equations 3.8 and 3.9. The same process was done for \( \sigma_{Jy} \).

\[
\sigma_{Jx_i}^2 = \frac{1}{99} \sum_{i=1}^{100} (Jx_i - \bar{J}_x)^2
\]

(3.8)

\[
\sigma_{Jx} = \frac{1}{15} \sum_{n=1}^{15} \sigma_{Jx_n}
\]

(3.9)

The state estimation with prediction and correction is performed in every iteration once the current cell has been found with the aim of update the second and third order derivatives because a single prediction estimation is used only when intersection scheme fails to find the selected cell. For the observation error covariance matrix the standard deviations were set in such a way that the estimates were very close to measurements and that the influence of \( Q \) were incorporated to avoid singularities in the calculus. The parameters are shown in Table 3.12.
3.4. TRACKING BLOCK

Figure 3-13: Kalman filter simulation.

Figure 3-13 shows the simulation of Kalman estimator performance for $x$ and $y$ with a sine trajectory, awgn of $\mu = 0$, $\sigma = 0.5$ and $T_s = 0.001$. The Kalman filter was set with the parameters of Table 3.12 and $T = T_s$. It can be seen the natural lag of the estimate. A pseudocode of Kalman estimator implementation is shown in Algorithm 2 and the Matlab® code can be consulted in Appendix B. An enable parameter ($en$) was added to switch between prediction-correction and only prediction.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\sigma_{fx}$</th>
<th>$\sigma_{fy}$</th>
<th>$\sigma_x$</th>
<th>$\sigma_y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>0.0269</td>
<td>0.0993</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 3.12: Statistical parameters for Kalman filter.

**Algorithm 2** Kalman estimator pseudocode

**Inputs**

- A,H: Transition and observation matrices (model)
- Q,R: System and observation covariance matrices
- z: Measurement
- x: Past state estimate
- P: Error covariance matrix
- en: Enable corrector flag
CHAPTER 3. PROPOSED APPROACH DESCRIPTION

**Outputs**
- $x_{k.1}$: Updated state estimation
- $P_k$: Updated error covariance matrix

**Process**
- $x_k \leftarrow A^* x$
- $P_k \leftarrow A^* P^* A^T + Q$
- if $en == 1$ then
  - $K_k \leftarrow P_k^* H^T \text{inv}(H^* P_k^* H^T + R)$
  - n_track_label $\leftarrow x_k + K_k^* (z - H^* x_k)$
  - $P_k \leftarrow (I - K_k^* H) P_k$
- end if

A set of artificial images was made in order to test the Kalman filter estimation. The image sequence was designed to simulate the trajectory of a cell, the coordinates of the cell are determined mathematically in Equation \[3.10\]

\[
\begin{align*}
x &= c \\
y &= A \cos(2\pi ft)
\end{align*}
\]

(3.10)

Where $c$ is a constant or a bias, $f = 4$, $A$ was adjusted to produce a 36 pixels peak to peak amplitude and $t$ is a vector with the same number of elements than $x$. The area of the object in the images is the average cells area, see Figure \[3-14\]

Figure 3-14: Cell trajectory simulation.
3.4. TRACKING BLOCK

Figure 3-15: Trajectory estimation, (a) using prediction-correction in all frames and (b) only prediction in several frames.

The estimation was obtained with Kalman filter configured with the statistical parameters described above. In Figure 3-15a it can be seen the trajectory estimation using the whole scheme predictor–corrector, while in Figure 3-15b several missed points were used to test the only prediction estimation. The mean square error between trajectories in both cases is reported in Table 3.13. By using the centroid as the position of the cell, the reported error is tolerable since it is possible to find again the selected cell, thus the Kalman filter has a good performance.

<table>
<thead>
<tr>
<th></th>
<th>MSE (_x)</th>
<th>MSE (_y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prediction–Correction</td>
<td>9.6537</td>
<td>0.8958</td>
</tr>
<tr>
<td>Only Prediction</td>
<td>10.5347</td>
<td>0.8929</td>
</tr>
</tbody>
</table>

Table 3.13: Mean square error between trajectories.
CHAPTER 3. PROPOSED APPROACH DESCRIPTION

Tracking results are shown in Figure 3-16. It can be seen that the Reliability Counter is equal to three as the cell “A003” is not tracked after frame 4. In the image, cell nuclei are in black and the background in white.

Figure 3-16: Tracking results through six consecutive frames.
3.5  Store Data Block

This is the last block of the proposed solution. The block receives as one input the new tracking label, with that label the current cell is identified and some parameters as the position and area are extracted from the binarized image. Using current ratio frame, the intensity measurement is also done in this stage, pseudocode is available in Algorithm 3.

Algorithm 3 Store data block pseudocode

1: **Inputs**

2: cell: Current cell structure

3: n_track_label: Updated tracking label

4: fc: Frame counter

5: LM_fc1: Binarized and labeled image, frame fc

6: A,H,Q,R: Kalman estimator matrices

7: Ratio_frame: Ratio image, frame fc

8: **Outputs**

9: cell: Updated cell structure

10: **Process**
CHAPTER 3. PROPOSED APPROACH DESCRIPTION

11: if n_track_label == 0 then
12:   cell.track_label ←− 0
13:   cell.trajectory(fc,:)[state_estimate(4),state_estimate(1)]
14:   crop_ratio ←− CropRatio(bounding_box,cell.state_estimate)
15:   cell.Ca_profile ←− mean(crop_ratio)
16:   [cell.state_estimate,cell.covariance_matrix] ←− Kalman_Predictor
17:   cell.reliability_counter ←− cell.reliability_counter+1
18: else
19:   bin_object ←− LM_fc1 == n_track_label
20:   [centroid,area,bounding_box] ←− FeatureExtraction(bin_object)
21:   cell.track_label ←− n_track_label
22:   cell.measurement ←− f_n.centroid
23:   crop_ratio ←− CropRatio(bounding_box,cell.state_estimate)
24:   cell.Ca_profile ←− mean(crop_ratio)
25:   [cell.state_estimate,cell.covariance_matrix] ←− Kalman_Predictor_Corrector
26:   cell.reliability_counter ←− 0
27: end if

For ease and clarity in the data management, an array of structures was selected to store the useful information, the members of the structure are shown in Figure 3-17b.

More precisely, the members of the cell structure are

1. Tracking label: It is the tracking label of the cell in the current frame.
2. User label: It is a user defined label for the selected cell.
3. Bounding box: The position, width and height of the rectangular region of interest that surrounds the selected cell.
3.5. **STORE DATA BLOCK**

5. State estimate: The estimated position of the current cell.


7. Area: Area of the object that correspond to the current cell in the binary image.

8. Calcium profile: Intensity average from ratio image that belongs to the current cell, this is stored in a vector that builds the calcium profile.

9. Trajectory: The trajectory that is followed by the current cell, i.e., the position of the cell in each frame of the whole image sequence.

10. Reliability counter: If this counter is equal to the limit determined by the user the measurement is considered as unreliable and is suspended.

11. Status: The tracking status, ONLINE or OFFLINE. If some cell status is OFFLINE then the tracking routine is suspended for that cell.
Chapter 4

Results

The blocks previously presented were put all together and a software application was developed to facilitate the extraction of intracellular calcium profiles.

This chapter presents the application software and its functions are described. The application was tested with several image sequences from two experiment type and the results proved the consistency of the data obtained with the application. Two main advantages of the proposed scheme are also mentioned.
4.1 Software Application

The blocks that were presented in the past chapter were joined to conform a functional, easy and user friendly software application. It is important to say that the proposed blocks are the fundamental components to automate the methodology to extract $[\text{Ca}^{2+}]$ profiles, nevertheless, there are some secondary routines that must be programmed to achieve the functionality of the application. Those secondary routines can be built in different ways and it would be impractical get into detail, so they only will be mentioned. The application was built using the Matlab® GUIDE. The main screen is shown in Figure 4-1.

![Software application](image)

**Figure 4-1: Software application.**

With the button *Load Sequences* the two $\lambda_{Ex}$ image sequences can be loaded, then the binarized sequence is generated using the Bradley technique reported above.
CHAPTER 4. RESULTS

With the *New Roi* button the user can select a cell in the image axis, if *Selection Mode* ratio button is set as *Auto* a rectangular bounding box with the minimum area is defined, with *Manual* the user can define the ROI with the desired size. Only one cell per ROI is allowed, and a custom or default label can be assigned. With the *Select All* button all the detected cells are selected and its labels are assigned automatically. *Auxiliar Edges* plots in the image the edges of the cell nuclei in order to help the user to identify where the detectable cells are. Once the user has selected the desired cells, the *Start Analysis* must be clicked. An emerging window will ask to the user the *Reliability Limit* and the analysis starts. During the analysis, the $[Ca^{2+}]_i$ profiles are plotted and the tracking *Status* of the cells is shown. A pop up message is displayed when the analysis is complete. With *Save Data* button the data stored in the structure array can be saved as a *.xls file.

4.2 Testing

The intracellular calcium profiles are extracted with the aim of modeling the endothelium behavior under different stimuli. With that purpose the application was tested with several image sequences sources from two principal type of experiments: Endothelium under ATP stimulus and Endothelium under mechanical stimulus. The images were provided by the LCVF–BUAP.

4.2.1 ATP Stimulus

The methods of this experiment can be consulted in detail by the reader in [14]. The dimensions of the images are $640 \times 480$ pixels and they have *.TIFF format. During this experiment the cells have a little displacement and an intensity transient. Fifteen cells were selected randomly, Figure 4-2 and their intracellular calcium profile were extracted with the system proposed and also with ImageJ.
4.2. TESTING

Figure 4-2: Cells selected randomly on ratio image.

The mean square difference between both obtained data sets is shown in Table 4.1. Although small movements were registered in the cells trajectory, the calcium profiles are very similar and the mean square difference is small, as was expected. This demonstrate that this approach delivers consistent results. Figure 4-3 shows an example of the profiles comparison.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Mean Square Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2606e–05</td>
</tr>
<tr>
<td>2</td>
<td>5.1455e–06</td>
</tr>
<tr>
<td>3</td>
<td>1.1145e–04</td>
</tr>
<tr>
<td>4</td>
<td>1.2029e–05</td>
</tr>
<tr>
<td>5</td>
<td>3.4416e–06</td>
</tr>
<tr>
<td>6</td>
<td>5.6798e–06</td>
</tr>
<tr>
<td>7</td>
<td>1.1774e–05</td>
</tr>
<tr>
<td>8</td>
<td>2.2187e–05</td>
</tr>
<tr>
<td>9</td>
<td>6.0434e–06</td>
</tr>
<tr>
<td>10</td>
<td>3.3664e–06</td>
</tr>
<tr>
<td>11</td>
<td>1.8023e–05</td>
</tr>
<tr>
<td>12</td>
<td>4.0428e–06</td>
</tr>
<tr>
<td>13</td>
<td>3.3812e–06</td>
</tr>
<tr>
<td>14</td>
<td>2.1519e–06</td>
</tr>
<tr>
<td>15</td>
<td>6.0133e–06</td>
</tr>
</tbody>
</table>

Table 4.1: Mean square difference between calcium profile extracted with ImageJ and with the present approach. ATP stimulus experiment.
CHAPTER 4. RESULTS

To quantify the performance of the system with all the blocks working together, two figures of merit were proposed. The first was the percentage $S$ of tracked cells of the proposed approach that is defined in Equation 4.1, and the second was the density of tracked cells $\rho$ per frame, defined in Equation 4.2.

$S = 100 \times \frac{\text{Number of ONLINE cells at the end of the analysis}}{\text{Total number of selected cells}} \quad (4.1)$

$\rho = 100 \times \frac{\text{Number of ONLINE cells}}{\text{Number of binary objects}} \quad (4.2)$

Image sequences of two different ATP experiments with 200 images per excitation wavelength where analyzed. All the detected cells were selected to test the performance of this approach with different reliability limits (RL). The average results are shown in Table 4.2 and the average number of detected and selected cells was 190.
4.2. TESTING

<table>
<thead>
<tr>
<th>RL</th>
<th>$S$ (%)</th>
<th>$\rho_{\text{max}}$ (%)</th>
<th>$\rho_{\text{min}}$ (%)</th>
<th>$\bar{\rho}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>58.95</td>
<td>104.3956</td>
<td>67.1378</td>
<td>84.8415</td>
</tr>
<tr>
<td>5</td>
<td>63.68</td>
<td>104.3956</td>
<td>76.4045</td>
<td>89.4309</td>
</tr>
<tr>
<td>7</td>
<td>65.79</td>
<td>104.3956</td>
<td>79.7753</td>
<td>92.3110</td>
</tr>
</tbody>
</table>

Table 4.2: Average performance results with different reliability limits.

It can be seen that $\rho_{\text{max}}$ exceeds the 100%, as said before, this is due to some cells disappear in some frames causing a bigger number of ROIs than cells; for the same reason it is important to mention that a low $S$ is not necessarily related with a poor performance. In the other hand, the big amount of noise in some frames produces more binarized cells than ROIs.

4.2.2 Mechanical Stimulus

![Figure 4-4: Suggested zones.](image)

In this experiment a region of the tissue is damaged mechanically with a micro–pipette. Detailed experimental procedure is described in reference [26]. The image sequences has *.TIFF format and its dimension is $768 \times 576$ pixels.

Three zones were considered in the image as shown in the Figure 4-4, and three cells were selected per zone. The fluorescence and position of cells in zones 1 and 3 have a stable behavior while the cells in zone 2 have more movement and their
CHAPTER 4. RESULTS

fluorescence change more often. Some cells of the inner zone die and then disappear, this is reflected as an OFFLINE status.

Due to the cells moves slightly in the outer zones, it can be seen that the intracellular calcium profiles obtained with ImageJ and with the proposed approach are
very similar, Figure 4-5. The zone 2 results show a more notable difference in the structure of the \([Ca^{2+}]_i\) profiles and in the trajectory of the cells, Figure 4-6.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Mean Square Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5306e–05</td>
</tr>
<tr>
<td>Zone 1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>8.8160e–06</td>
</tr>
<tr>
<td>4</td>
<td>2.1e–03</td>
</tr>
<tr>
<td>Zone 2</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>2.3e–03</td>
</tr>
<tr>
<td>7</td>
<td>2.3425e–05</td>
</tr>
<tr>
<td>Zone 3</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>1.0412e–05</td>
</tr>
</tbody>
</table>

Table 4.3: Mean square difference between calcium profile extracted with ImageJ and with the present approach. Mechanical stimulus experiment.

Based on the mean squared difference of the results obtained from zones 1 and 3, Table 4.3, the proposed scheme delivers consistent results as showed in the previous subsection. While the results of the zone 2 allows to see the importance of tracking block since the structure in the profiles of calcium changes dramatically, it is noteworthy that there are patterns of intracellular calcium profiles associated with different physiological functions, so, a better profile extraction may help a better understanding of cellular behavior [14], [26].

<table>
<thead>
<tr>
<th>RL</th>
<th>(S(%))</th>
<th>(\rho_{max}(%))</th>
<th>(\rho_{min}(%))</th>
<th>(\bar{\rho}(%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>58.66</td>
<td>100.7117</td>
<td>58.0756</td>
<td>69.9652</td>
</tr>
<tr>
<td>5</td>
<td>60.42</td>
<td>101.0714</td>
<td>59.7938</td>
<td>73.1079</td>
</tr>
<tr>
<td>7</td>
<td>61.84</td>
<td>101.0714</td>
<td>61.1684</td>
<td>75.1659</td>
</tr>
</tbody>
</table>

Table 4.4: Average performance results with different reliability limits.

As for ATP experiments, two mechanical stimulus experiments with 200 frames per wavelength were used to test the approach. The average results are in Table 4.4.
where the average number of selected cells was 283. As expected, compared with the ATP experiments results, a lower performance can be seen and it is caused by the more random and more noticeable movement of the cells.

![Figure 4-7: Stressed system.](image)

Finally, the software application was stressed with complete image sequences, up to 750 frames, with all the detectable cells selected, up to 278, and a Reliability Limit of 3, see Figure 4-7. The intensive memory usage can be noted, however, the analysis finished successfully. This proves that the application can be used to extract calcium profiles massively which in turn would allow spatially model the behavior of endothelial cells.

### 4.3 Summary

A summary of the contributions of this work can be read below.

- Results obtained with the proposed solution are consistent.

- Adaptive binarization gives a segmentation based on local statistics to aid the tracking scheme.

- The automatic relocation of ROIs gives a better measurement of the relative intracellular calcium concentration.
4.3. SUMMARY

- The proposed scheme gives to the user the ability to perform the calcium profiles extraction not only from certain cells, but massively, this allows spatial modeling of cellular phenomena.

- The trajectory followed by cells can be stored to its posterior study.

- Less time consumption for calcium profiles extraction.
Chapter 5

Conclusions

It was proposed and validated a scheme to automate the methodology of intracellular calcium profiles extraction from fluorescence images of endothelial cells, fulfilling the overall objective of this thesis.

Adaptive binarization based on variance showed a good performance for fluorescence images and together with another digital image processing as nonlinear spatial filters and morphological operators it was possible to obtain a set of images that was useful as a basis to the multi–target tracking scheme.

Taking advantage of the slow movement of the cells, a multi-target tracking scheme was designed and implemented. The proposed scheme was based on logical intersections which is a very simple solution and it is supported by a Kalman estimator and a constant acceleration with random jerk model. That tracking scheme and the Kalman estimator were selected because the simplicity and efficiency would allow a real–time application.

A graphical user interface was developed and it was tested using several image sequences from two different types of experiments, one of them showed the extracted data consistency and the other validated the tracking need. The results allow us to say that the automatic relocation of the regions of interest and the possibility of massive data extraction are two important contributions of this work.
5.1  Future Work

A broad range of techniques are continually created in digital image processing with the aim of meeting needs in different areas of science. Fortunately, this work can be improved and the following is proposed as future work

- Develop a method to adapt the binarization automatically to different cell sizes.
- Add a watershed stage to enhance the segmentation process.
- Improve the memory management.
- Migrate the algorithms to Java programming language to add it as a plug of Image J. In this way the tool would be available as free software and could be enriched by a larger community.
Appendix A

Dual Excitation Ratiometric Indicators

Figure A-1: Dual excitation ratiometric indicators [10].
Appendix B

C and Matlab® Source Code

B.1 MEX function for Bradley Thresholding Technique

```c
#include <mex.h>
#include "matrix.h"
#include <stdio.h>
#include <math.h>
#include <conio.h>

void mexFunction(int nlhs, mxArray *plhs[], int nrhs, mxArray *prhs[])
{
    #define I_in(i,j) I_in[(i) + (j)*mrow] //Macros to define input Image
        with Matlab indexing format
    #define I_out(i,j) I_out[(i) + (j)*mrow]

    double *I_in,*I_out; // Pointers
    double t,w;       // parameters for binarization process
    double l,count,sum_e;//x1,x2,y1,y2;
    int mrow,ncol,i,j,iaux,jaux,x1,x2,y1,y2;

    if(nrhs>3||nrhs<3)
```
B.1. MEX FUNCTION FOR BRADLEY THRESHOLDING TECHNIQUE

{  mexErrMsgTxt("Invalid input.");
   return;
}
else if(nlhs>1)
{
    mexErrMsgTxt("Excesive outputs for this function.");
    return;
}
I_in=mxGetPr(prhs[0]); // Assign pointer data to I_in
mrow=mxGetM(prhs[0]); // Get I_in dimensions: number of rows
ncol=mxGetN(prhs[0]); // number of columns

    t=mxGetScalar(prhs[1]); // Assign pointer data to parameter t: binarization
    w=mxGetScalar(prhs[2]); // Assign pointer data to parameter w: window size
    l=floor(w/2);
    count=4*pow(l,2);

plhs[0]=mxCreateDoubleMatrix(mrow,ncol,mxREAL); // Declaration of output matrix
I_out=mxGetPr(plhs[0]); // Assign I_out data to output pointer

// Bradley Algorithm
    for (i=l;i<=mrow-l-1;i++){
        x1=i-(int)l;
        x2=i+(int)l;
        for (j=l;j<=ncol-l-1;j++){
            y1=j-(int)l;
            y2=j+(int)l;
            sum_e=0;
            for(iaux=x1;iaux<=x2;iaux++){
                for(jaux=y1;jaux<=y2;jaux++){
                    sum_e=sum_e+I_in(iaux,jaux);
                }
            }
        }
    }
B.2 Kalman Matrices Evaluation

%% Thesis
% Function that evaluates the system (A), observation matrix (H),
% system randomness (Q), and observation error covariance (R) matrix.
% where:
% 1. T : sampling period
% 2. sx : x position standard deviation
% 3. sy : y position standard deviation
% 4. ra : x direction random jerk
% 5. rb : y direction random jerk
% function [A,H,Q,R]=k_eval(T,sx,sy,ra,rb)

A=[1 T T^2/2 0 0 0 ;... % system
    0 1 T 0 0 0 ;...
    0 0 1 0 0 0 ;...
    0 0 0 1 T T^2/2 ;...
    0 0 0 0 1 T ;...
    0 0 0 0 0 1 ];

H=[1 0 0 0 0 0;... % observation matrix
B.3. **KALMAN ESTIMATION: PREDICTION–CORRECTION**

\[
0 0 0 1 0 0
\]

\[
w = [ra*T^3/3 \ ra*T^2/2 \ ra*T \ rb*T^3/3 \ rb*T^2/2 \ rb*T]
\]

\[
Q = w' * w;
\]

\[
R = [sx^2 0 ; 0 sy^2 ]; \text{ % assuming independence}
\]

## B.3 Kalman Estimation: Prediction–Correction

```matlab
%% Thesis
% Kalman estimator function Predictor/Corrector
%%
function [xk, Pk] = kalman_v1(A, H, Q, R, zk, x, P, flag)

[m, n] = size(Q);
I = eye(m, n);

% Only Prediction (when no cell is found)
    xk = A * x;
Pk = A * P * A' + Q;
if 1 == flag
% Correction
    Kk = Pk * H' / (H * Pk * H' + R);
xk = xk + Kk * (zk - H * xk);
Pk = (I - Kk * H) * Pk;
end
```

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