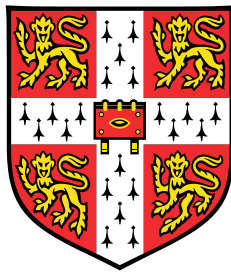


Using 3D image data to improve 2D Brightfield image segmentation

A method of 3D projection



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This dissertation is submitted for the degree of
Master of Philosophy

I would like to dedicate this thesis to my long-suffering supervisor, Y. Y. "Shery" Huang, for her help and advice. Without her guidance, this would not have been possible.

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements. This dissertation contains fewer than 15,000 words including appendices, bibliography, footnotes, tables and equations and has fewer than 150 figures.

Nicholas Piano
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And I would like to acknowledge the support and patience of my parents, my colleague Cristina Bertulli, and Xiaohao Cai.

Abstract

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Chapter 1

Introduction

1.1 Cell segmentation in microbiology

An important part of live cell microbiology is the accurate measurement and tracking of cell morphology during an experiment. Using a microscope, there are many different ways of observing the cells ranging from brightfield microscopy to 3D fluorescence reconstructions. Both 2D and 3D shape data from the cells along with their speed and directionality can provide information on the effectiveness of drugs or other agents in the experiment. The processing of cell data often relies on the quality of Cell Segmentation, or the automatic or manual differentiation of Objects of Interest, such as cells, from the background. Many algorithms and software packages, such as Cellprofiler and ImageJ, are used to segment cells automatically, yielding variable quality.

A key limitation in widely used software that this paper seeks to address is the inability to account for consistent features that cannot be easily located in 3D data. 3D image data, such as from a confocal microscope, contains information about an environment on many focal planes [ref]. Objects can appear blurred or in focus depending the current focal plane. Consequently, features that are useful for segmentation; dark edges, uniform bright interiors, and other features such as fluorescent markers placed within cells are subject to any fluctuations in focus or the movement of objects vertically in the environment. This prevents consistent segmentation of the cell.

The environment used in this study is a microfluidics chip built to simulate a human blood vessel. This type of chip, a microchannel framework printed on a PDMS substrate, is widely used in the medical industry to mimic body tissue [ref]. The 3D nature of this setup requires the use of confocal microscopy or similar methods to observe objects in all parts of the environment. In this case, a confocal microscope was used to record data in both a brightfield channel and a fluorescent GFP channel. A number of limitations on the

image quality, discussed in Chapter 2, prevent more powerful direct 3D methods to be used for segmentation. This necessitates a deeper investigation into how the currently available brightfield and GFP information can be used to segment the cells consistently.

1.2 Current work in context

The problem of consistently recognizing objects in a 3D environment using the brightfield is partially addressed in the 2009 study by Selinummi et al. [ref] They attempted to remove the problem of finding objects in 3D by studying the variance of vertical brightness profiles in the brightfield. This had the effect of simplifying 3D data into a 2D plane containing relevant object shapes, from which segmentation could be done more easily. Although this was effective in their case, it has several disadvantages that the current work aims to solve. Notably, it performs poorly in a multicellular environment, necessary for this and many other studies. This is discussed in more detail in the method in Chapter [ref, method].

The current work builds on the idea of studying vertical intensity profiles by applying this concept instead to the GFP. In the current data, the low quality GFP does not show the outlines of cells accurately. This is due to the internal cellular distribution of the GFP, described in Chapter 5. The central regions of the cell (not including the nucleus) are highlighted, so it can be used to locate the general bulk of the cell. The brightfield, on the other hand, shows the edges of the cell more clearly, but only if the cell is in focus (at the correct level). Edges of the cell start to fade along protrusions. In this study, any useful segmentation done must include accurate outlines of long cell protrusions to determine cell motility and behaviour.

The method described here uses the bulk of the cell visible in the GFP to locate the level in the data needed to ensure consistently clear dark edges in the brightfield, yielding accurate cell shape. The GFP and brightfield data represent the same physical space. This correspondence is exploited to allow information about the GFP channel to aid searching for features in the brightfield. The correct edges are located by building a vertical GFP intensity distribution for each pixel and selecting pixels from the brightfield data that match the level of the distribution peak. Several other properties of this GFP “profile” can be used to find different information about the cells. This is described in more detail in Chapter 6. This new method is one of image pre-processing; to prepare images for segmentation by software, in this case, Cellprofiler. It is not a method of segmentation, although as outlined in Chapter 7, it has the potential to become one.

1.3 Thesis outline

Firstly, a brief description of the microfluidics environment, its purpose, and its limitations will be given in Chapter 2. The system used and its properties are the original reason this study into image processing was undertaken. The 3D nature of the system prevents conventional 2D microscopy, and the live cell environment meant that more detailed cell fixing techniques could not be used. The lack of image quality and coarseness of the data led to the need to investigate if any useful information could be extracted reliably from it.

The current method depends heavily on the distribution of GFP within a cell. This, along with an understanding of cell shape and its optical properties and responses to the light used in the microscope is important for determining how useful this method can be in changing conditions. If the type of microscope or environment is changed, this method should be able to operate under a new set of parameters. A description of the places GFP can be found within a cell can inform adaptations of this method and help find conditions where the method could fail or have reduced effectiveness. An example of this is cytosolic versus cytoskeletal GFP, found in the central cytosol and the external cytoskeleton respectively. These two different distributions of GFP can effect this method which relies on peak positions of the profiles. Peaks in different distributions can give misleading answers regarding the positions of parts of the cell. A description of the brightfield reveals similar situations. A key example is the superposition of two objects in the environment. The brightfield light is mixed in the final data, so data representing superimposed objects cannot be divided into specific shapes of both objects. This limitation cannot be resolved with the GFP data. This and other problems will be described in Chapter 3.

To explain the context of this type image pre-processing, a background in image processing and some techniques used by Cellprofiler will be given in Chapter 4. Basic image features can be combined into larger features that can delimit objects and parts of objects. Software like Cellprofiler can use this information to measure shapes and other properties of cells in an image. It can also be used to track cells from one frame to another by looking for similar cell shapes and proximity. The method described here depends on the way a recognizer like Cellprofiler works, although with further work, the information used for pre-processing in this method could allow a more powerful segmentation algorithm to be created. This will be investigated finally in Chapter 7.

Chapter 6

Finally, testing will be used to compare this method to previous experimental work and commonly used methods of image pre-processing. Although this is not yet a fully capable segmentation method, the pre-processing can be compared by segmentation. Data from several experiments was collected and cancer cells within them segmented. Several properties

of the cancer cell shapes, such as the projected 2D area, can be used to compare images on their ability to represent the cells.

Chapter 2

The microfluidics environment

2.1 Purpose and the need for segmentation

The reason this environment was developed is to determine how cancer cells cross the endothelium (blood vessel wall cell barrier) during extravasation, or the stage of metastasis where cancerous cells that have already entered the blood stream from some initial location, make their way around the body, break through the blood vessel wall, and embed themselves in another type of tissue. Extravasation is a poorly understood process in cancer biology [ref], and this type of system could help to understand it by providing accurate data on the morphodynamics of the cells as they move in the simulation of a blood vessel.

To provide useful data, information about a great many cells must be gathered. This requires visually identifying cells and parts of cells and assessing their changing shapes, sizes, and speeds over time and in distinct regions of the environment. This can be done manually by keeping track of each cell and recording its progress over the course of the experiment. Unfortunately, this is impractical for the amount of data needed and would be too time consuming for a human to do. As such, it must be done automatically. This type of recognition done by a computer is known as segmentation, where cell shapes are separated and differentiated from the background. Many algorithms, techniques, and pieces of software exist to solve this with varying levels of success. They require a mathematical description of a set of images, and a way to consistently find features of the objects of interest that can be matched and measured.

Certain aspects of the environment used in the experiment and the methods by which it was imaged pose problems for accurate cell segmentation by disrupting the consistency that cell segmentation algorithms depend on. Traditional cell microscopy using glass slides and chemically fixed cells allows for high resolution images of cells that are consistently in focus. Factors affecting the quality in this case are the 3D structure of the channel, the

live cell environment where cells are free to move and interact, and the movement of the microscope hardware and cancer cells vertically (relative to the microscope objective) that causes object to fluctuate in and out of focus in the final data.

2.2 Description of the environment

The microfluidics environment used for this study consisted of a microchannel framework printed using soft lithography on a PDMS chip. PDMS, or Polydimethylsiloxane, is a silicone gel that can be molded and used to create microscopic structures, such as microscopic channels to use as environments for cells [ref]. This microchannel technique is widely used in the biomedical industry to mimic body tissue for complex *in vitro* studies. In this case, the channel was set up to model a human blood vessel. The diagram in Figure [ref] below shows part of the channel framework with two PDMS pillars on either side of a gap. On one side of the gap, a liquid medium meant to simulate blood plasma is pumped in where it can remain static, or be used to simulate the flow inside a blood vessel. On the other side of the gap, collagen gel, used to simulate the extra-cellular matrix surrounding a blood vessel, provides an anchor point for the endothelial cells, or cells found in the blood vessel wall, to attach to. These endothelial cells are added to the environment prior to the experiment. The experiment is monitored when cancer cells that are marked with a fluorescent GFP, or Green Fluorescent Protein, are added to the environment in the medium channel, from which they can cross the endothelial cell barrier into the collagen gel, as they might *in vivo* during the extravasation stage of metastasis [ref].

The entire channel is approximately 100 microns thick [ref]. this is roughly an order of magnitude greater than the width of a typical cancer cell used in the experiments [ref]. During the experiment the cells may be attached (flattened) and thus much thinner vertically [ref]. The width of the gap horizontally containing the endothelial cell barrier is between 100-200 microns [ref]. A typical blood vessel would be tubular, but this setup, while possible [ref], would be difficult to study and to image with a microscope. Opting instead for the simplified setup, the vertical wall of cells can be observed much more easily and still be used to provide information on how the cancer cells cross the barrier. More advanced setups are under development at the time of writing.

2.3 Confocal microscope and 3D structure

Image data were gathered using a confocal microscope. This allows a 3D space to be imaged by scanning over a volume and recording data at a number of discrete levels. Data can

also be recorded in a number of different channels. The data for these experiments used brightfield, or transmission, images and GFP fluorescence images. The light in brightfield images from different levels is subject to mixing due to the optics of the microscope objective, resulting in blurred representations of objects unless their level is in focus. In contrast, light from GFP inside cells can be isolated to a particular level using the pinhole mechanism of the microscope. The size of the pinhole determines the maximum resolution of the image. This isolation of the GFP channel levels can be used to build up a 3D model of the objects marked with GFP, which in this experiment is only the cancer cells. Cancer cells can then be distinguished from other types of objects in the experiment.

The 3D information from the GFP can be used to locate cancer cells within the environment, but the limits on the resolution prevent accurate outlines of the cells from being found. This is also dependent on the cells' internal distribution of GFP. The cancer cells in the current experiments had GFP embedded within the cytosol, or the fluid interior of the cell. From this, the general bulk of the cell and to a certain extent its general shape could be made out, but finer edges and protrusions could not be determined since the GFP did not penetrate the cytoskeleton or the outer wall of the cell. The method used to find cells and prepare the images for segmentation is directly dependent on this arrangement of the GFP. modifications can be made to account for other sources of fluorescence inside the cell. This is discussed more in Chapter [ref].

2.4 Live cell imaging limitations

Imaging live cells imposes limits on the techniques available since the cells must remain alive and be able to behave naturally for the entire duration of the experiment. This means they cannot be subjected to unnatural amounts of illumination from the laser used to stimulate fluorescence lest they absorb too much energy and overheat, causing erratic movements and finally death, making any data on their movements unusable. This limit on the amount of light that one is able to put into the cells to stimulate the GFP limits the amount of light that can be gathered from the cells. This lowers the brightness and contrast of the final data, making the location of finer details in a cell less certain.

The cells are also free to move about the environment. Tracking a single cell means it must remain in the field of view. The time period necessary to allow the cells to cross the endothelial barrier is between 10-15 hours, and the distance a cell can move in this time sets a lower limit on the size of the viewport, or the view of the microscope that is finally recorded as an image. A large viewport means a lower resolution, since the objective must move away from the sample. This sacrifices image quality per cell for information about

many cells. For this trade-off to be worthwhile, useful data must be extracted from the lower quality cell images. This is difficult for simple cell segmentation algorithms.

The movement of the cells is not restricted to the two dimensions of the viewport. The cells can move up and down in the environment (towards and away from the objective). This will move them between different focal planes. While all this data is recorded, cells cannot be guaranteed to stay on one level for the whole experiment. As a cell moves vertically, its representation in the image will change and properties of its edges will change their colour and texture. This breaks the feature consistency that segmentation relies on.

2.5 Autofocus and focal fluctuations

Apart from the behaviour of the cells, the microscope hardware must also operate reliably for the 10-15 hour time period. This is not always possible, and small changes in the alignment of different components will cause systematic errors to build up and compound over time. The result of the microscope imaging in one alignment while the instruments report another causes errors in the consistency of the image sequence. The focus will fluctuate visibly while an index representing the focal plane remains the same. This means such an index cannot be relied on to accurately report the location in the environment.

Software that is meant to solve this problem is known as Autofocus [ref]. Most commercial microscope setups come with this software preinstalled. It attempts to analyse the images gathered at regular intervals, for example, every frame, and determine the current focal plane in the environment from simple numerical properties. Such a property is the minimum entropy of the image [ref]. By keeping the minimum entropy constant, the image plane with the most similar entropy in the next frame can be found. This is set as the corresponding level so that representations of the objects remain constant. Other methods such as measuring the reflection of light from the cover slip of the sample are also used [ref]. Highly variable environments such as live cells can cause misleading calculations of a constant entropy, hence the unreliability of the level index. This makes segmentation more complicated.

Chapter 3

Background

3.1 Introduction

A comprehensive background on the key concepts that the method presented in this thesis is based on is essential for understanding its context and significance. Any physical or mathematical principles that might affect the performance of the method should also be understood. The previous chapter outlined some of the problems faced when attempting to segment cancer cells given a 3D live cell environment. These included autofocus fluctuations of the microscope and limits on light absorption by cells. This chapter gives some detail on topics including the GFP distribution within the cells, which plays a key role in the pre-processing method, basic image processing and segmentation and how these techniques are used in Cellprofiler and other segmentation software packages, and finally an introduction to the brightfield method on which the current method is based.

3.2 Cell microscopy, optical structure, and GFP distributions

Under a microscope, the visible parts of a cell, the optical properties of edges, and colours observed depend on the materials that it contains. A typical human cell is colourless and transparent [ref], but when light passes tangentially through the cell wall, it can be blocked and give the appearance of a dark edge. This leads to a projected (2D) shape under the microscope. This shape is a closed curve and thus has an interior (the material within the cell) and an exterior (the background). This is an assumption made throughout the segmentation process, since it is not possible for the cell to be an open shape. This is useful

for segmentation, because there is a natural boundary that allows the recognition to be restricted to closed curves made up of dark edges.

Cells can move in response to chemical stimuli [ref]. In order to move in their environment, a cell can change tension in its outer wall and form protrusions that stretch in the direction of a stimulus [ref]. This can allow the cell to propel itself and follow chemical gradients. These protrusions, if they can be measured, provide useful information about the behaviour of the cell in response to any agents that have been added to the system as part of the experiment. The microfluidics environment was designed with the addition of chemical agents in mind. Even without added stimuli, the purpose of the experiment is to be able to observe the cells moving and allow for their shapes to be measured. Accurate recognition of cell protrusions is a key aim of this study. Without it, cell behaviour cannot be judged in changing conditions.

Cells can be marked by injecting a variety of fluorescent proteins into different parts of their internal structure such as the internal cytosol, the cell membrane, and the nucleus. A common protein used is known as GFP, or Green Fluorescent Protein. It is usually expressed in the cytosol, a clear fluid surrounding the nucleus, occupying the bulk of the cell, but not the membrane or the nucleus [ref]. When a 3D reconstruction is created using a confocal microscope, this distribution of GFP will appear as a single bulk shape with a gap at the location of the nucleus. It notably does not contain information about the extremities of the cell such as protrusions. It can also be expressed in the cell membrane, leading to a hollow 3D shape that shows clearly the extremities of the cell.

3.3 Image processing and segmentation

An edge is defined as an intensity discontinuity in one dimension. Crossing the edge, the intensity can be plotted and represented as a mathematical relationship between the intensity and the path taken. Movement across a thin dark edge will show a decrease in intensity followed by a subsequent increase once the edge is passed. This mathematical model allows edges in a set of images to be found quickly and reliably if it is general enough. The ability to rapidly find edges in an image is a key tool in a segmentation program, since this is the most certain way of locating objects. An example of a widely used edge detection method is called the Canny filter. Proposed by John Canny in his 1986 paper, *A Computational Approach To Edge Detection* [italic]. It works by first smoothing the image to reduce noise, finding the intensity gradients of the image (edges will show a high intensity gradient), suppressing points with less than the maximum gradient, along with applying an absolute intensity threshold, and finally joining potential edge segments by removing those not connected to

strong edge segments. Other features such as corners can be modelled as superpositions of the simple edges in different orientations.

Another tool for recognition is the blob detector. "blob" is a technical term meaning an contiguous (interconnected) region of uniform (similar) colour. This could indicate a solid object or a region of background. Depending on the type of imaging, blobs that represent objects can either be light or dark, but both can be found by modelling the region as a 2D intensity discontinuity. This can be represented mathematically as a 2D Gaussian of arbitrary, but fixed, radius. Such a modelling function can then be convolved with the image in a similar manner to simple edges. Parts of the image that contain regions of colour of size proportional to the radius of the Gaussian will give a response to the convolution. The areas with the highest response are the most likely candidates for objects of the specified size.

Once blobs and edges have been found, it is possible that in a given cellular environment, cells will be found packed closely in dense clusters. In order to be differentiated from each other, boundaries between the cells must be drawn based on the image properties surrounding them. Often, cells are separated by sharp discontinuities such as dark edges, but they may be so close that optically, their intensity curves appear to transition smoothly from one cell to another. In this case, a variant of the watershed method is useful for separating them [ref]. like many image processing techniques, the watershed method models the intensity map of the image as a terrain, where the contour height at a point is proportional to its intensity. If one imagines the terrain as being slowly filled with a certain amount of water, highly divergent regions such as cell representations will stand out like hills and the point where each "hill" meets the water is the boundary of that cell. If the water is raised high enough, even smoothly transitioning boundaries between cells will appear to have a dividing line between them. This can be used to segment closely packed objects using their intensity peaks alone.

3.4 Cellprofiler and segmentation software

The last section details some of the methods used by Cellprofiler and other software packages, such as several ImageJ plugins, to segment cells. When Cellprofiler is provided with an image, it will first look for blobs of colour in the image. Dependent on the background, it will look for bright or dark blobs as potential candidates for objects. The edges close to these blobs are kept as potential object edges. The blobs of colour are then separated using a watershed method. Their contours are matched with the edge image and the space is filled in to form an object "mask". The mask is a binary representation the object of interest. Every point in the mask is considered part of the object, and all points outside are considered part

of the background. The watershed method allows adjacent masks to be separated. Every task that Cellprofiler performs on an image such as edge detection or blob detection is typically contained within a module that stores information about operations done to the image. These modules can be chained to compound effects and allow very specific workflows to suit all types of images. It can also combine images from several channels using basic mathematical operations.

Cellprofiler has a particularly useful module called "Secondary Objects". Rather than searching the image for objects from scratch, it can use information about previously found objects to search in their vicinity. If object centres are represented as points, Secondary Objects can search around the points and use information about the vicinity of the point to match similar patches of colour or edge profiles. This operation is very adaptable, and does not have a size preference, filling whatever space is available. This is highly valued since one of the driving philosophies of this work is that recognition should not be biased towards a particular size or shape. A disadvantage of this approach is a recognition that expands without limit. If there is no discernible boundary, there is no reason for the recognition to stop expanding into the background if it is of similar intensity. To clarify, if the background of the image and the interior of the cell have similar colours, and the edges of the cell are incomplete or have an inconsistent profile, the recognition could add parts of the background to the final mask of the cell. This can affect the method used, but eventually adding 3D information to this 2D method can solve the problem.

Another tool for segmentation is the Blow/Lasso tool in ImageJ. This treats the image as a "terrain", where the "height" of a pixel is proportional to its intensity. If an algorithm follows a path through the image, moving from one pixel to another can be assigned a cost based on the relationship between the intensities. An algorithm could be set up such that moving into dark edges could be very cheap, but moving out of them could be expensive, causing the path to follow a chain of edges closely. In a similar fashion, two points can be specified and the cost between them calculated [ref]. If one point is specified as the centre from which to search, the locus of points that share the same cost from the centre can yield a closed shape with dark edges such as a cell. While not reliable without tuning, the cost function can be made more specific to suit the application. Such a function can also be made to take account of multiple channels. Depending on how it is implemented, considering a central point can bias the shape towards a circular locus.

A notable disadvantage of Cellprofiler is the lack of comprehensive support for 3D environments and sets of images with 3D relationships. Although it can be made to behave in this way, images must be processed on an individual basis or in basic groupings, limiting the complexity of 3D operations. This makes true 3D segmentation difficult or impossible.

Nevertheless, Cellprofiler has a wide array of very powerful 2D operations. Thus an ideal solution is a method that is able to cast a 3D dataset in a 2D context in order to take advantage of 2D methods while preserving 3D data.

The final data output from Cellprofiler is a list of object masks along with some analysis of their shape such as their eccentricity or orientation (from a bounding ellipse). Cellprofiler does allow the segmentation of connected objects, such as protrusions, and can provide their measurements, but this can be unreliable since the properties of protrusions are hard to specify. A larger problem with Cellprofiler is the inability to adapt to inconsistencies. For example, the edges of a cell protrusion can have a very different appearance to the main body of the cell as the cell walls grow thinner. While the edge of a protrusion maybe remain visible towards its apex, the difference in edge properties might cause Cellprofiler to conclude that they are unrelated edges, and lead to a protrusion being rejected and not represented in the final data. This unreliability with regards to “tertiary object” like protrusions is insufficient for the accuracy required by this project. The method in Chapter [ref] will show how 3D information can enhance the recognition of tertiary objects.

3.5 Cell tracking

Cell tracking is any method of associating recognised objects in different images gathered at different times and assigning them same identity. This can allow properties of the cells such as area and position to be plotted over time and for trends to be observed. In a low density packing of cells, this can be as simple as finding the distance between objects in different frames and assigning the next iteration to be the object with the smallest distance from the starting point. In this study, connected iterations of a single physical object are referred to as “instances” as in “cell instance”. This is an important distinction, since a cell objects cannot be said to have an area or velocity since these properties change with time. Assigning an area to a particular cell at a point in time is equivalent to assigning the area to the cell instance. Thus a single cell can have many cell instances.

A popular tracking method is the LAP, or “Linear Assignment Problem”, framework tracking algorithm. Once two lists of objects and their positions in subsequent images are found, they can be connected and ranked by a likelihood of correspondence. This is done by first constructing and solving a $N \times N$ [maths] matrix of correspondence parameters where N is the number of objects considered. A similar matrix is constructed to represent the probabilities of cells merging or splitting. The final step reconciles the two images into a list of corresponding cell instances. This process can then be repeated for subsequent frames. This algorithm is employed by Cellprofiler as a module, so it can be applied to objects

found through recognition. Other properties of the cells such as changing shape and size can contribute to the likelihood of correspondence.

The method yields four important numbers, namely “LostObjectCount”, “NewObjectCount”, “SplitObjectCount”, and “MergedObjectCount”. A measure of the reliability of the tracking is given by the consistency of these numbers. For example, if NewObjectCount continues to increase throughout the time series, it is likely that objects that should be connected are being rejected and treated as new objects. This can happen if objects move a long distance, such as many multiples of their length, between frames. If the ratio of distance moved to time between frames is too high, the tracking algorithm may be too unreliable for use. In this case, manual tracking may be necessary to provide accurate information about cells.

LAP also helps to associate cells that disappear and reappear.

3.6 The Selinummi brightfield profile method

In their 2009 paper, *Bright Field Microscopy as an Alternative to Whole Cell Fluorescence in Automated Analysis of Macrophage Images* [italic], Selinummi et al. describe a method of using 3D brightfield information to aid segmentation instead of relying on GFP imaging of cells. The images for their study were gathered in a similar way to this study. A confocal microscope was used to scan a 3D environment gathering images in brightfield and GFP channels. Gathering data with a confocal microscope is slow and expensive, so if adequate cell data can be produced using a single channel, this can save time. They proposed using the amplitude of the variation of the brightfield intensity in the Z dimension as an indicator of the presence of an object at a particular XY location. This exploits the behaviour of the brightfield representations of objects as they move in and out of focus. As outlined in Chapter [ref], the brightfield does not store 3D information, but changes with the focal plane of the environment. An area of background will not vary across focal planes, but a location or single pixel containing an object will vary with focal plane.

The basis of the method is an inspiration for the current project. The vertical distribution of the brightfield intensities at a particular XY location, known as a “profile” in this study, shows how the features vary in intensity across Z.

Their results were promising for the data they gathered, but suffered when applied to the data from the current experiment. To test the method, they compared cell segmentation of the modified brightfield images to the GFP images of the same cells. They used the GFP segmentation as the “ground truth” of their testing. Ground truth is assumed to be the best representation of the object. This is a failing of the study since there are many details omitted

by the GFP, especially considering the variety of configurations cellular GFP can take, as discussed in Section [ref], earlier in this chapter. Segmentation was compared pixel by pixel using the “FScore”, or a compound ratio of the true and false positive scores.

There are several disadvantages of the Selinummi method, especially when applied to the current data. Firstly, the method was originally applied to a single cell environment with no visible materials other than cells. In contrast, the current environment is primarily composed of PDMS plastic. It is also a multicellular environment. The images thus contain many other objects in the brightfield that are not desired as part of the dataset, but they still have similar edges and strong variations in their intensity profiles. Regions of the image where any kind of object is found will appear bright. This can hinder accurate segmentation since unwanted objects are highlighted. Secondly, due to the brightfield variation in the images, strongly varying pixels can occur outside the true edge of the cell, causing these regions to be highlighted, and giving the cell a bright halo. This halo is picked up in the recognition and contributes to the measured area of the cell. This can cause an over-estimation of the cell area and a false representation of the cell’s shape. These problems must be addressed for a variant of this method to be useful.

Chapter 4

Method

4.1 Introduction to the method

This method is a successor to the Selinummi brightfield method described in the previous chapter. An aim in developing it was to improve on two key problems. The first was the unwanted highlighting caused by objects other than those stained with GFP, preventing accurate segmentation of a multicellular environment. The second was a halo effect on the cells as the variance of the brightfield extended beyond the true edge of the cell due to optical mixing of the light in the brightfield. The Selinummi method was originally intended to replace GFP segmentation in 3D environments [ref]. This was previously done by projecting the GFP in the Z dimension, creating a new image where the value of each pixel corresponded to the sum or mean value of the pixels in that single XY profile distribution.

The concept of this method is, instead of disposing of the GFP, to apply the variance method previously applied to the brightfield to the GFP itself. This yields a much more informative estimate of the position and shape of the cell. Due to the low quality of the GFP, the precise shape of the cell cannot be found, but the 3D information can then be used to search the brightfield data and construct an image such that every object of interest (marked with GFP) appears to be in focus. This is in effect a method of pre-processing, since the segmentation can then be done on the product by Cellprofiler or by other means in the manner of any other 2D image. In other words, the method proposed in this study casts 3D data in a 2D format that can be easily processed by well tested 2D algorithms. The method depends on three main parameters that can be varied to suit the application: R , ΔZ , and Σ . The next few sections will describe these parameters and their functions. They affect the linear smoothing of the original data, the focal plane of the outcome, and the final inter-level smoothing respectively.

While this projection of 3D data into a 2D context is the main method proposed, a further method of optimising the segmentation of the product using additional 3D data is included as it is important to the testing of the method. This optimisation uses the creation of artificial edges drawn on an image delimiting the boundary for cell segmentation of single cell or group of cells to prevent the segmentation of areas of the background with similar intensity profiles but low GFP intensity. This is an improved alternative to simply highlighting areas of the image with the GFP intensity [ref].

4.2 The GFP profile

The key component of this method is the GFP vertical intensity distribution or “GFP profile”. For a single pixel in XY, this appears as a single series of intensities spanning Z. These values can be smoothed both in XY and Z. Smoothing in Z is meaningful because of the spatial relationship between planes of GFP in the environment. Planes in the brightfield are not related spatially, as thus cannot be meaningfully smoothed. Any noise present between frames in the GFP can be minimised and properties such as the maximum value can be determined to sub-pixel accuracy via interpolation. A profile can also be found by considering a square or mask of pixels in XY combined through some operation such as a mean value for each Z level. The size of this mask can be set arbitrarily to the size of a part of a cell, but analysis in Section [ref] will show that such a method of generating the profile amounts to a type of linear image smoothing and yields a profile with similar properties to a profile generated using only the centre point of the mask. A size of between 3 and 5 pixels was chosen for the mask to allow smooth transitions in the final image. This parameter is important to the outcome of the method and is given the symbol R .

Similarly to the brightfield profile considered by Selinummi et al., measureable properties such as the variance, the location of the maximum value, and the mean value can be found for the GFP profile. The variance was found using the normalised profile, that is, the maximum value in each individual distribution is set to 1. Comparing profiles in an image can then be done using their mean value and variance. These are linearly related as shown in Figure [ref]. A background pixel will have a flat profile, giving a low variance and a high mean, since the majority of the distribution stays close to the maximum value. As peaks appear in the profile due to the presence of objects, the mean will decrease, but the variance will increase proportionally. In this way, pixels with varying profile strengths can show very clearly whether they contain an object. Figure [ref] also shows a collection of blue points indicating points manually chosen to be inside cells. These are clustered in the high variance - low

mean portion of the plot. The large cluster of points at low variance represent background pixels.

The most important part of the profile for the main method is the Z position of the maximum value. This indicates the peak intensity in the profile, and points to the most likely Z position of the part of the cell that contains the pixel in XY. This value can be used to search in the brightfield for an accurate cell representation by looking at the same level for the object in focus. Since the level indices are integers, the Z image was chosen by rounding the estimate of the Z position from the profile to the nearest integer. The more precise information is lost in the final image, but it can be stored elsewhere for further use.

4.3 Optimum features for cell recognition

By searching the brightfield with the GFP profile, the goal is to find the optimum features for segmentation. These include bright, smooth, uniformly coloured interiors surrounded by consistent, dark edges. Given a series of focal planes showing the object, it is likely that there is a single plane that contains the closest possible features to the ideal features required. This is assumed to be where the maximum GFP occurs. Unfortunately, the location of the maximum GFP for a cell shows an image that is ironically “too focussed”. When in the sharpest possible focus, a cell’s edges become very thin and bright. To provide a better image for segmentation, a constant value is added to the level determined by the GFP profile. This was determined empirically and set to be between 4-8 levels added to the profile estimate. This is the second parameter for the method and is given the symbol ΔZ .

For manual tracking, the accurate shape of the cell does not need to be known; it is better to see the interior of the cell clearly in order to make a good estimate of the centre to place a marker. The focal plane best for this type of observation also does not lie on plane with the maximum GFP. This shift is also empirically determined and is between 10-12 levels above the maximum GFP. This parameter is not crucial to the outcome of the method and is set by personal preference of the user tasked with finding the cells.

4.4 zMod and zBF

For each frame in time, the Z position of the maximum of the GFP profile is calculated for each pixel in XY. A new image is created, called “zMod”, where the value of each pixel proportionally represents a Z position in the environment. This is analogous to a terrain. The range of values lies between 0 and 1, but the intermediate values are discretised to multiples of $\frac{1}{\text{numberOfZPlanes}}$ in order to represent the full Z range of the experiment. Floating point

representation is easier to keep track of than integer representation in an image since the float value also works as a percentage height in the environment. This map of Z positions can be smoothed to make transitions between levels more gradual. The smoothing applied used a Gaussian blur with a radius of 3 pixels. This is the third important parameter and is given the symbol Σ .

The zMod image for each frame can be mapped to the entire set of brightfield data for the same frame by selecting a pixel value from the brightfield stack focal plane that corresponds to the Z index indicated by the zMod image. This produces the most important result of this study, the “zBF” image. It is a single 2D image for each frame containing representations of all objects in focus with clear edges and interiors. This works by only selecting pixel values from the levels where the objects are in focus. This does not correct the focus of objects not marked by the GFP. This image is used to segment all cells in the environment simultaneously independently of their level in the experiment using 2D segmentation techniques.

4.5 Artificial edges for segmentation: zMean and zEdge

A further improvement can be made to the segmentation. Part of the 3D data has already been used to correct the focus of objects marked with GFP. The part of the 3D data not used for this method is the mean (or equivalently the variance due to the linear relationship). This can indicate presence of an object more reliably than the absolute value of the GFP. Pixels with very different values in the GFP can have similar values in the mean image since the profiles are normalised such that only the shape of the distribution matters. If the mean of the normalised and inverted (since low mean/high variance indicates an object) GFP profile for each pixel in a frame is found, a new image, “zMean” can be made. Depending on the linear smoothing of the original data (the parameter R), the boundary of objects in the mean image can extend beyond the edges of objects in the brightfield.

The extra extent of the zMean image is exploited to provide a maximum boundary for segmentation, this is combined with zMod to produce outlines around cells. This does not require the edges to be followed correctly, but only needs to enclose the general shape of the cell. These shapes are then segmented to separate objects or even rough groups of objects. The outlines of the segmentation are used to generate artificial edges using a DoF (Difference of Gaussians) edge model. These edges cut through background values that are similar in intensity to the interiors of cells. If there is a gap in the cell edge with an intensity close to the background, the segmentation will expand into the background and cause a major overestimation of the area of the cell. This image is called “zEdge” and is the image for each frame used to provide the final segmentation data for each experiment.

4.6 Protrusion measurement

One of the main aims of this study is to accurately measure the lengths and orientations of the cell protrusions to gather useful data on cell behaviour. The protrusions were measured by plotting the outline of the cell on a polar graph and measuring the divergence from a smooth circular shape as measured from the calculated cell centre. The protrusions show as peaks in this plot and their length is measured from the tip of the peak to the mean radius, not the centre of the cell.

[PLOT SHOWING RADIAL DISTRIBUTION OF CELL BORDER WITH PROTRUSION PEAKS MARKED]

4.7 Summary of the method

To summarise the steps taken to pre-process 3D image data for segmentation:

1. Smooth the data to reduce noise.
2. For each pixel in XY for the 3D data, find the vertical intensity distribution in Z, or "profile", for each pixel and evaluate its properties.
3. Using the profile for each pixel, create an image where the value of a pixel is proportional to the Z level of the maximum intensity of GFP at that XY location. This is zMod.
4. For zMod, there are three parameters that must be chosen: R , ΔZ , and Σ . These affect the smoothing of the final image.
5. Map the Z values in zMod to the stack of image data for the brightfield. Select pixel values from the brightfield whose Z level corresponds to that indicated by zMod and place them into a new image, called zBF.
6. Although zBF now contains in-focus representations of each object marked with GFP, a further improvement can be made using the outlines of the GFP mean image. The mean image, zMean, takes the mean value of the normalised GFP profile as the value of each pixel. This gives a maximum extent of the cell and limits segmentation to a boundary to prevent it from extending into the background and incorrectly judging the area of a cell.
7. Finally, once segmentation is complete, cell properties such as project area can be obtained along with protrusion lengths and orientations. Protrusions are found by

radially plotting the distance of the edge from the centre point and designating outliers as protrusions.

Chapter 5

Results and discussion

5.1 Introduction

To say that this method is an improvement on previously available methods is an understatement. This must be shown using comparable data. The best way to provide this data is to attempt to segment images from the current experiment. The methods can be compared easily under similar conditions using the same dataset. A readily available property of the cell instances found is the projected cell area. Other properties are the number and orientation of the cell protrusions. These will also be compared to judge the difference between the methods.

[SIDE BY SIDE COMPARISON OF ZBF AND ORIGINAL BRIGHTFIELD]

Despite the improvements made, the method developed is not without error. There are parameters that have optimal ranges, but outside those ranges, the method can fail. Some physical properties of the environment can limit the effectiveness of the method, for example, if the level of the GFP is too low to generate an accurate estimate of the Z level, the focus of GFP marked objects will not be corrected accurately, and the results will not be useful. Additionally, some configurations of cells in close proximity can cause problems for this method. A clear example is when two cells lie vertically one on top of the other, causing two visible peaks in the GFP. This, and other examples are discussed in the last section [ref].

An important point when comparing different methods is the fact that there is no “ground truth”, or definitely correct answer to the question of the projected area of a cell, and despite the attempts of Selinummi et al. to define theirs as the segmentation of the projected GFP image [ref], all images are subject to error as are the results of their segmentation, and the results are judged subjectively. The only viable automatic means of judging different methods is to compare their consistency and repeatability. They could, however, be consistently and repeatably wrong. For this reason, the ground truth chosen for the comparison of the methods

shown here is the manual segmentation, or outlines of the cells drawn by eye on the zBF image. Only of sample of the frames are considered since manual segmentation is very time consuming.

Yet another problem, is that not even the manual segmentation can be considered the ground truth. The human eye cannot be trusted to determine the shape of an object which it is totally unfit to identify in an environment that does not lend itself to casual observation. Despite this obstructing piece of philosophy, the final judgement must lie with something, and the best thing available is human vision, which is not saying much in the microscopic world. We are left with no choice but to accept a system of pattern recognition whose highest recommendation is that it insists on discovering human faces wherever it looks [ref].

5.2 zBF parameters: R , Delta Z , Sigma

The three parameters that contribute to the generation of zBF must be carefully chosen, since a low quality result will be useless for segmentation. An analysis of the sensitivity of the result to changes in the parameters can be used to determine the optimum values. Below, some examples of parameters chosen outside the optimal ranges are shown and some consequences of their segmentation is investigated.

5.2.1 The radius of GFP linear smoothing: R

The parameter R is the radius of the linear smoothing filter applied to the GFP in order to generate a profile for a point in XY. The filter mixes values in XY, but not Z. It has the effect of reducing noise and making transitions between levels of neighbouring pixels more fluid. This helps to reduce the problem of cross-level artifacts, discussed in Section [ref]. In the case where neighbouring objects in XY has very different levels in Z, the level transition would be smoothed such that the point half way between them would be assigned to a level half way in Z. While this case has not been observed, it could lead to strange results, such as half of each cell being in focus, while the halves closest to the other cell are out of focus. This would depend on the radius of the linear filter, but at a certain point, the information is too smoothed and ceases to be useful. Below [ref] is an example of the linear filter set too high. The logical limit is a smoothing filter the size of the image itself in XY. This would simply yield the sum or mean of the GFP for each focal plane, which would not provide any more information than where most of the cells were found in the environment.

[EXAMPLE OF R TOO HIGH]

On the other hand, if the size of the linear filter is too low, such as a single pixel, the noise in the GFP would dominate the Z estimate, leading to highly fluctuating Z values across even a single cell. The result in the zBF image is an extremely noisy cell interior and edge, surrounded by an equally noisy background. This does not lend itself to accurate segmentation. Below [ref] is an example of the linear filter using a small R .

[EXAMPLE OF R TOO LOW]

[EXAMPLE OF VARYING THE PARAMETER R]

5.2.2 The brightfield level correction: ΔZ

The weakest part of the method is the spatial relationship between the brightfield and the GFP and the decision about the value of $[\Delta Z]$. The brightfield is subject to fluctuations from the autofocus and has no intrinsic spatial information. The most important piece of information relating the GFP and the brightfield is for a hypothetical fixed object in focus in the brightfield, the GFP representation will always correspond to that level of focus. This is because the position of the microscope hardware is constant for a single frame and is set by the autofocus estimate from the brightfield. Assuming the same physical space is represented in both, the GFP can be used to account for the autofocus fluctuations, but the relationship between the GFP representation and the appearance of an object in the brightfield is arbitrary. A cell may be “in focus” as far as possible, but still not be clearly visible by a human. This hinders manual tracking. optimum features for observation may not correspond to optimum features for segmentation. For tracking, it most effective to observe an object in a solid colour with or without clear edges since the precise shape is not necessary to indicate object centres. For segmentation, a clear object boundary with dark edges and a smooth, uniformly coloured interior yields the most accurate shape. This discrepancy is represented by the empirically determined value, delta-Z. Through further study, a value could be found automatically, but the focus level for optimum human observation is highly subjective.

[EXAMPLE OF VARYING THE PARAMETER DELTA Z]

5.2.3 The radius of level gaussian blur: Σ

[EXAMPLE OF VARYING THE PARAMETER SIGMA]

5.2.4 Final choice of parameters

The three variables used to produce zBF were fixed to the following values:

[TABLE OF VALUES WITH REASONS FOR THOSE CHOICES]

5.2.5 The subject of the background

It should be noted that background pixels in XY that contain very little GFP at any Z, and thus have very flat profiles, are still assigned a value for the Z position. This may appear misleading, as it can be highly random as the “maximum” of a flat distribution is given by the noise alone. This can be seen clearly in the PDMS pillars in Figure [ref], indicated by an arrow. While this might be thought to produce errors, other means of filtering background can be used that do not rely on the Z position, such as the mean image. It is more reliable to be unbiased when generating the zMod image since there is no certain way of determining whether a particular Z value should be assigned. If filtering must be done eventually, it can be done using other 3D data.

5.3 zMean and zEdge

The zEdge image is intended to improve the segmentation by bounding it.

Artificial edges can cut off parts of the cell that do not contain enough GFP.

[EXAMPLE OF IMAGE WITH A DROP IN GFP]

[EXAMPLE OF REDUCED EFFECTIVENESS FROM ZEDGE]

5.4 Comparison with common methods

Previous methods cannot account for the inconsistencies in the focus fluctuations and so are not really comparable in their quality. Below, some of the results from segmentation of images generated using several

[CONSTANT LEVEL TIME SERIES] [SELINUMMI METHOD TIME SERIES] [MAX GFP TIME SERIES] [ZBF TIME SERIES] [ZEDGE TIME SERIES]

[6 CELLS SAMPLED AT TIME INTERVALS SHOWING AREA FROM EACH METHOD] [RADIAL PLOTS OF 6 CELL INSTANCES SHOWING PROTRUSION ANGLES AND LENGTHS] [6 CELLS -> 12 GRAPHS OF CELL PROTRUSION LENGTH AND ANGLE TIME SERIES]

5.5 Errors and limitations

Several possible errors that would affect segmentation results must be mentioned for completeness. Some of these error have not been observed, but are possible given certain conditions. Errors can be caused by faults in the microscope hardware, irregularities in the

illumination of the environment for any reason, poor choices in imaging setup, or particular configurations of cells that cause the method to fail.

A controversial example of a possible error is a situation where two cells are superimposed in Z. In this case, a GFP profile for a single XY location could have two very prominent peaks. Currently, the method does not attempt to resolve this situation since it has not been observed in the current data, but trivially, it could be resolved by only taking account of the peak with the highest Z level, since the brightfield information is not 3D, and there is no way to recover any edge information about the lower cell if it is obscured. In this case, such a conflict could be noted and the GFP edge, which is not obscured, could act as a fallback for the segmentation. This information would allow the GFP edges, while they are suboptimal, to be incorporated into the outline of the rest of the cell if it is visible elsewhere in the brightfield. To reiterate, this situation has not been observed, but it is theoretically possible in such a 3D environment.

[HYPOTHETICAL DIAGRAM OF TWO SUPERIMPOSED CELLS]

In some cases, through a hardware fault or a temporary shift in the illumination of the environment, the level of the GFP intensity can drop in the whole experiment. This happened in one experiment done and caused the method to fail in a single frame, and subsequently rendered the area estimates in that frame for all objects unreliable.

[IMAGE SHOWING RESULT GIVEN LOW GFP -> 2 FRAMES]

If the illumination of the environment is not uniform, and regions at the top are significantly brighter than the bottom for example, when brightfield pixel values from different Z levels are brought together to generate zBF, it is possible that the intensities are so different as to be discontinuous, leading to the appearance of a false edge. This type of effect is shown in Figure [ref]. This can be alleviated partially by the choice of R or Σ , since both control to a certain extent the smoothing between levels. If transitions between levels are smoother, artefacts such as these are less likely to form.

[IMAGE SHOWING CROSS-LEVEL ARTEFACTS]

Finally, if the configuration of the microscope is chosen before imaging to yield generally low contrast brightfield images, edges may not be visible in any focal plane. In this case, the method of finding the in-focus representations of cells may not be very useful as no more information is revealed. In this case, an alternative might be use the segmentation of the GFP primarily and gather what results are available from the brightfield. As with hypothetical superimposed cells, edges from GFP and brightfield channels could be combined, but here it might be more effective to weight the edges found in GFP to be more reliable than those in the brightfield. This is subject for future work.

[IMAGE SHOWING LACK OF BRIGHTFIELD CONTRAST]

Chapter 6

Conclusion

6.1 Summary

Given the limitations on cell segmentation in 3D image data, the method described in this study performs well in overcoming problems and improves on the previously proposed method by Selinummi et al. by a large margin. It also compares favourably with previous attempts at segmentation using conventional means, such as Z projection of the GFP and segmentation at a constant Z level. It can also be used as a method to correct the autofocus fluctuations in brightfield data, which while not originally intended, is a natural consequence of the 3D data used.

6.2 Observations

6.3 Further work

Could be used to help locate seeds in images to simplify microscope imaging. Currently the method only does pre-processing. Can be used instead to do segmentation directly using more information than a current segmentation algorithm is able to use.

References

