Modelling Lymphocyte Proliferation on the Single Cell Level Based on Data From Time-lapse Fluorescence Microscopy

Liyan Liu

Submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

Department of Computing and Information Systems
THE UNIVERSITY OF MELBOURNE

April 2018
Copyright © 2018 Liyan Liu

All rights reserved. No part of the publication may be reproduced in any form by print, photoprint, microfilm or any other means without written permission from the author.
Abstract

Time-lapse fluorescence microscopy coupled with single-cell tracking and an appropriate data analysis method has become an increasingly important tool in cell biology. One important application of such single cell analysis is to develop models for immune lymphocyte responses. Lymphocyte proliferation is a fundamental characteristic of response to antigenic stimulation, and is an essential process to protect the host organism. Hence, it is important to understand the dynamics of this process. In this thesis, we study B cell responses to CpG stimulation using FUCCI transgenic mice, and mathematically model the single-cell fluorescence time courses, which are measured from the corrected fluorescence images with good quality.

However, time-lapse fluorescence imaging has an inherent problem in that the process of image acquisition distorts the original images (e.g., by introducing uneven illumination) and leads to inaccurate measurements of single-cell fluorescence levels. In order to obtain a correct interpretation of the true fluorescence time courses, we have proposed novel approaches to eliminate major acquisition effects. Specifically, we have first developed an uneven illumination correction algorithm and determined a quantitative measure from all the popular measures for performance evaluation on real fluorescence images, where the ground truth is not available. Then we have proposed a novel method to remove the acquisition effect due to overlapping emission spectra, and also developed a correlation-based approach for evaluating the correction results when reference objects are absent. Based on the corrected individual cell time courses, we have built a two-term sigmoid function and a piecewise exponential function for describing the cell behaviour through the cell cycle in FUCCI red channel and FUCCI green channel, respectively. Using these models, we can extract cell features such as cell cycle phases automatically, which form the basis for pattern recognition across a population of responding cells.

Our results show that the proposed correction methods exhibit superior performance to well-established methods and efficiently remove the bulk of acquisition effects. Using single-
cell time course models allows us to automatically identify transitions in cell cycle stages, and ultimately quantify relations between cell cycle progression in sibling cells. Moreover, we find that a global model can be shared by siblings, implying that the cell cycle phases of mother cells can be passed on to offspring and shared by sibling cells.
Declaration

This is to certify that

1. the thesis comprises only my original work towards the PhD degree,

2. due acknowledgement has been made in the text to all other material used,

3. the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

______________________________
Liyan Liu, April 2018
Acknowledgements

My past few years in the University of Melbourne has been a happy and fruitful period of my life. I would like to acknowledge people who supported and inspired me in the past. First of all, I would like to thank my parents, brothers and sisters for believing in me and taking care of me all the time. My boyfriend Yanchen is the main source of inspiration and motivation that helps me overcome difficulties in my study and life. He has always encouraged me whenever I feel exhausted and frustrated.

Next I would like to express my deepest gratitude to my primary supervisor, Prof. Christopher Leckie, for his patience and constant support during my study. Chris has provided me with the key insights to help establish the main direction of my research. I am deeply indebted to and owe my heartfelt gratitude to my secondary supervisor, Dr. Andrey Kan, for his excellent mentorship and constructive suggestions for this research. Andrey has always been there with good advice, inspired me with confidence and helped me greatly in reviewing my written reports and articles.

Furthermore, I would like to thank the staff of the collaborating Hodgkin Lab at Walter and Eliza Hall Institute of Medical Research (WEHI). As experienced researchers in the field of cell biology, Prof. Philip D. Hodgkin, John F. Markham and Jie H.S. Zhou at WEHI shared their cell videos and helped broaden my knowledge in cell biology. I am also grateful to Adrian Pearce, the chair of my Advisory Committee.

Finally, I would like to thank China Scholarship Council(CSC) for funding my postgraduate study at the University of Melbourne. Other people who contributed in one way or another include my friends from all over the world. Thank you Neelofar, Sam, Ping, Lunde, Jiazhen, Yanhui, Lakhami and Irum for always being supportive and spending great time together.
Preface

This thesis has been written at the Department of Computing and Information Systems, The University of Melbourne. Each chapter is based on manuscripts published or under review for publication. I declare that I am the primary author and have contributed to more than 50% of each of these papers.

Chapter 3 is based on the paper:


Chapter 4 is based on the paper:


Chapter 5 is based on the paper:

Contents

1 Introduction 1
  1.1 Research Scope .................................................. 2
  1.2 Motivation ...................................................... 4
  1.3 Contributions .................................................. 7

2 Background 9
  2.1 Methods of Single-cell Analysis ................................. 9
    2.1.1 Flow cytometry ............................................ 9
    2.1.2 Time-lapse fluorescence microscopy .......................... 11
  2.2 Digital Image Processing ...................................... 14
    2.2.1 Image Acquisition Process .................................. 15
    2.2.2 Alleviating Photobleaching ................................ 16
    2.2.3 Compensation for Shading Artefacts ......................... 17
    2.2.4 Removing Cross-talk Effects ................................ 24
  2.3 Cell Segmentation and Tracking ............................... 27
  2.4 Study of Lymphocyte Proliferation ............................ 28

3 Removal of Shading Effects 31
  3.1 Consequences of Shading Effects .............................. 31
  3.2 CJV-based Shading Correction Method .......................... 34
  3.3 Experimental Results and Discussion .......................... 35
    3.3.1 Tested Datasets ........................................... 35
    3.3.2 Implementation Details .................................... 38
    3.3.3 Performance Measure Decision ............................. 42
    3.3.4 Performance Evaluation Results ........................... 44
    3.3.5 Discussion ................................................ 51
  3.4 Conclusion ..................................................... 51

4 Removal of Cross-talk Effects 53
  4.1 Consequences of Cross-talk Effects ........................... 53
  4.2 Spectral Unmixing Model and Evaluation Methodology ........... 54
    4.2.1 Model of Spectral Unmixing ................................ 54
    4.2.2 Correlation-based Evaluation Methodology .................. 58
    4.2.3 Training Data Selection .................................... 59
  4.3 Evaluation Results and Discussion ............................ 62
    4.3.1 Image Preprocessing ....................................... 62
    4.3.2 Reference Intervals Identification ........................ 62
    4.3.3 Spectral Unmixing Scheme .................................. 64
    4.3.4 Experimental Results ...................................... 67
5 Modelling Single-cell Fluorescence Time Courses

5.1 Studies of Cell Kinetics

5.2 Methods

5.2.1 Image processing methods

5.2.2 Modelling single-cell fluorescence time courses

5.2.3 Statistical similarity test for progenitor cells and blasting cells

5.3 Results and Discussion

5.3.1 Fluorescence time courses acquisition

5.3.2 Patterns in dividing lymphocytes over generations

5.3.3 Progenitor cell follows regular division mechanism after G0/G1 phases

5.3.4 Maximum green fluorescence is correlated with the time Tm

5.3.5 Cell cycle phases exhibit strong correlation in sibling cells

5.3.6 Discussion

5.4 Conclusion

6 Conclusion and Future Research

6.1 Contributions

6.2 Future Work
## List of Figures

1.1 An example of a dividing B lymphocyte ................................. 2
1.2 A time-lapse fluorescence imaging system .............................. 3
1.3 One acquired time-lapse fluorescence image .............................. 5

2.1 Workflow of flow cytometry .............................................. 10
2.2 Schematic representation of fluorescence microscopy .......... 11
2.3 Acquired fluorescence images from three channels ................. 12
2.4 Two-colour cell cycle mapping using the FUCCI system .......... 13
2.5 Processes of mining single-cell fluorescence levels ................. 14
2.6 Fluorescence image acquisition process ............................. 15
2.7 Consequences of some major image acquisition effects ......... 15
2.8 Mono-exponential decay model of photobleaching kinetics ...... 18
2.9 Schematic diagram of some shading correction methods ......... 21
2.10 Effects of cross-talk on red fluorescence and green fluorescence .................................................. 25
2.11 A bright field cell image with segmentation and tracking .... 27
2.12 A cell lineage tree ...................................................... 28
2.13 Automated quantification of individual cell cycle phases .... 30

3.1 Shading effects on pixel intensity ....................................... 32
3.2 Shading effects on cell average intensity ............................ 33
3.3 Acquired images from videos where cells are placed in different well shapes ........................................ 36
3.4 Images representative of four real datasets .......................... 37
3.5 Empirical cumulative probability distribution of real and artificial datasets ............................................. 39
3.6 An image representative of four real datasets ....................... 41
3.7 Changes of CJV for real fluorescence images ....................... 50

4.1 Consequences of cross-talk effects .................................... 54
4.2 Diagram of the image acquisition process in time-lapse fluorescence microscopy ........................................... 55
4.3 Part of the captured fluorescence image ............................. 60
4.4 Schematic of identified reference intervals in mixed images ... 63
4.5 Defined reference intervals .............................................. 66

5.1 Modelling single-cell fluorescence time courses..................... 75
5.2 Statistical test for similarity between progenitor cells and blasting cells ............................................... 76
5.3 FUCCI reporter fluorescence time courses in dividing B cells ... 78
5.4 Reconstructed cell lineage tree for a tracked movie ............... 79
5.5 Photobleaching correction results .................................... 80
5.6 Shading correction results for one clone ............................ 81
5.7 Single-cell fluorescence time courses before and after image correction ............................................. 82
5.8 Single-cell fluorescence time courses from three types of cells ......................................................... 83
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.9</td>
<td>Dimensionality reduction using PCA for five extracted parameters</td>
<td>85</td>
</tr>
<tr>
<td>5.10</td>
<td>Distribution of activation time for progenitor cells</td>
<td>86</td>
</tr>
<tr>
<td>5.11</td>
<td>Histogram of proportions of individual cell cycle phases</td>
<td>86</td>
</tr>
<tr>
<td>5.12</td>
<td>Relationships between the activation time and cell lifetime</td>
<td>87</td>
</tr>
<tr>
<td>5.13</td>
<td>Relationship between cell cycle phases</td>
<td>90</td>
</tr>
<tr>
<td>5.14</td>
<td>Relations between the duration of S/G2/M in progenitor and blasting cells</td>
<td>91</td>
</tr>
<tr>
<td>5.15</td>
<td>Relationship between maximum green fluorescence $I_{\text{max}}$ and $T_m$ for all cells</td>
<td>92</td>
</tr>
<tr>
<td>5.16</td>
<td>Relations between cell cycle phases</td>
<td>93</td>
</tr>
<tr>
<td>5.17</td>
<td>Three fitting methods for two siblings</td>
<td>94</td>
</tr>
<tr>
<td>5.18</td>
<td>Relative change of AIC using global fitting and independent fitting</td>
<td>95</td>
</tr>
</tbody>
</table>
List of Tables

2.1 Summary of different retrospective shading correction methods .......................... 20
2.2 Summary of quantitative evaluation measures ..................................................... 22

3.1 Implementation details of shading correction methods ........................................... 40
3.2 Parameter values used in each shading correction method .................................... 43
3.3 Shading correction results for artificial dataset 1 ................................................. 45
3.4 Kendall’s tau coefficient between $L_2 - \text{norm}$ and other measures ..................... 46
3.5 Spearman’s correlation between $L_2 - \text{norm}$ and other measures ......................... 47

4.1 Requirements for training data selection ............................................................. 61
4.2 Identified reference intervals for each clone ......................................................... 64
4.3 Spectral unmixing results using brute force method ............................................. 65
4.4 The number of correlated reference intervals by selecting pixel intensity as the training data ................................................................. 67
4.5 The number of correlated reference intervals by selecting mean intensity as the training data ................................................................. 68

5.1 Correlation between cell cycle phases and total division time in all cells ................ 88
5.2 Correlation between maximum green fluorescence $I_{\text{max}}$ and $T_m$ in dividing cells. 91
5.3 Correlation between cell cycle phases and total division time in all sibling cells. .... 93
Chapter 1
Introduction

Biological cells are commonly referred to as the building blocks of life [44]. All living organisms including humans are made of cells, which are responsible for a wide range of functions including metabolism and immunity. The study of the functions and behaviour of cells plays a vital role in biological research, since it helps us understand how different cells behave in healthy and diseased states, and hence how we can protect living organisms when they are infected. In particular, the study of T or B lymphocytes has attracted considerable interest because these lymphocytes are major cellular components of the immune system response in mammals [4].

In order to model the behaviour of lymphocytes, it is first necessary to accurately measure key properties of individual cells over the course of their lifetime. An increasingly common approach for monitoring cells to make such measurements is the use of time-lapse fluorescence microscopy, as seen in Figure 1.1. From such videos, measurements such as cell size and cell intensity at regular time intervals can be obtained. However, the accuracy of these measurements can be limited by noise and other imaging artefacts that can affect image quality. Because of poor image quality, these measurements are usually skewed. The objective of this thesis is to study cell kinetics (e.g., the growth and division of cells) over time based on the estimated true cell intensities, after the image quality has been improved using automated image processing methods. We begin with an overview of this imaging technique and the motivation for our work in the following sections.
1.1 Research Scope

Time-lapse fluorescence microscopy can produce video of cells and monitor cell behaviour during their lifetimes for a prolonged period of time (e.g., several days). This long-term live cell imaging allows for the collection of different types of cell statistics such as cell positions, sizes, intensities, division times and trajectories. Time-lapse fluorescence imaging has proven to be an increasingly important tool in cell biology and has been used for various biological studies, including lymphocyte immune responses \[15\] in diseases such as malaria and diabetes \[25, 75\].

One of the important applications of time-lapse fluorescence microscopy is the study of lymphocyte proliferation. In response to pathogenic stimuli, lymphocytes undergo several rounds of divisions, and clear the pathogen. These complex dynamic processes are called lymphocyte immune responses \[8, 4\]. Lymphocyte proliferation is a fundamental characteristic of this response and an essential process to protect host organisms in mammals. By adding specific chemical compounds known as fluorescent markers or reporters to cells cultured in different wells, one can observe cell behaviour over time.

The main output of time-lapse fluorescence imaging is a sequence of raw images of cells. To measure single-cell features (e.g., the average intensity and total intensity of the fluorescent markers in cells) from the acquired images, the cells in each image need to be first segmented from the background and each other, and their positions tracked over time. These measurements form the basis for subsequent quantitative analysis of lymphocyte kinetics over each cell’s lifetime. However, the observed images are usually degraded due to the inherent problems of time-lapse fluorescence imaging in the process of image acquisition. This can result in inaccurate estimates of the measured cell features. Therefore, the observed images need to be corrected using appropriate image processing methods. The typical workflow of a time-lapse
fluorescence imaging and data analysis system is shown in Figure 1.2.

Figure 1.2: A time-lapse fluorescence imaging system. Cells tagged with chemical compounds are first placed in wells and then imaged for a long time to obtain multiple fluorescence images in different channels. Quantitative analysis of the measured cell features requires an appropriate combination of image processing method and semi-automated cell tracking system. Images courtesy of Hodgkin Lab, WEHI.

In this workflow, cells are first labelled with fluorescent chemical compounds and then randomly placed in physically separated compartments called wells at low density. The live cells are continuously imaged for several days using time-lapse fluorescence microscopy. Because of the multi-channel imaging mechanism, multiple time-series images in different channels (i.e., different wavelengths of light) are recorded. However, the process of image acquisition distorts the original images, so the obtained images need to be initially corrected by an image processing system. This system takes an image sequence as input and outputs a corrected image sequence with good quality. Next the corrected images are processed by a cell tracking system, which produces segmented cell outlines and links the positions of each cell over time to create the trajectories for each cell. As a result, single-cell intensity values and kinetics can be measured over time, which lays the foundations for a higher level analysis involving the
mathematical modelling of lymphocyte proliferation.

In this thesis, one of the key components in time-lapse imaging experiments is image processing. The role of the image processing system is to eliminate the major acquisition effects that arise from the time-lapse fluorescence imaging experiments. Then mathematical modelling can be applied to the estimated single-cell intensities in order to help understand the kinetics of the underlying cells.

1.2 Motivation

Live cell imaging, in particular observing fluorescent cells over prolonged periods of time, has revolutionized biological research [17, 18, 11]. This progress has been sustained by the ongoing introduction of new fluorescence reporters, as well as improvements in live-cell imaging hardware [64, 13, 49]. By attaching fluorescent reporters to cells, it is possible to monitor the expression levels of specific proteins in the cells using time-lapse fluorescence imaging. However, even with a well-established imaging platform and controlled experimental conditions, there are many multidisciplinary challenges in bridging the processes of image acquisition and biological knowledge discovery [32]. For example, recordings of lymphocyte clonal expansion in response to mitogenic stimuli potentially offers an unprecedented amount of information in terms of the level of the detail (the behaviour of individual cells over time), the complexity of data (the patterns across lineage relations), and the number of events (imaging large numbers of cells). Therefore, techniques for single cell tracking, accurate measurements of fluorescence levels, as well as appropriate data analysis methods are required in order to successfully extract biological insights from this large volume of raw data.

Early approaches to the problem of modelling cell kinetics and related biological processes from live cell microscopy images were heavily reliant on manual inspection and analysis of the imaging data. This severely restricted the level of detail that can be extracted, as well as the number experiments that can be analysed, which in turn limited the statistical significance of the biological models that could be acquired. To help overcome these restrictions, automated cell tracking has been a focus of research for decades [50], and has resulted in a selection of software packages for performing this task [76, 26, 82]. Once cell trajectories are reconstructed, it is then possible to measure fluorescence time courses for individual cells.

While automated cell tracking makes it possible to measure the proliferation and lifetimes of cells, it does not directly provide data about the timing and level of expression of key cell cy-
cle markers. Such data is essential for developing quantitative models of the processes driving cell kinetics. In order to take the next step in modelling cell kinetics, it is necessary to use the reconstructed cell trajectories as the basis for then measuring the time course of fluorescent cell cycle markers for individual cells. However, the accurate interpretation of these time courses is not straightforward, because of a variety of physical effects during image acquisition, such as uneven illumination, photobleaching or cross-talk [41, 43, 41, 6], which leads to a corruption of the fluorescence signal that originated from the cells. It appears that considerably less research has been applied to the problem of the systematic correction of such acquisition effects.

For example, uneven illumination is a major factor that distorts recorded images, as shown in Figure 1.3. It is evident that the intensity is much stronger in the image center than at the edges due to uneven illumination. Until recently, there has been no review of illumination correction methods in the context of time-lapse imaging. Only recently, there has been a publication comparing a limited number of methods [71], but still not assessing other methods that have been reported. This previous review is now complemented by our work (Chapter 3). In the literature, most proposed methods for addressing illumination correction [1, 77, 9] are designed for a particular type of imaging modality (e.g., magnetic resonance imaging) other than time-lapse fluorescence microscopy. Moreover, to the best of our knowledge, there has been no systematic assessment of the most appropriate measure for comparing the effectiveness of different illumination correction methods. Such an assessment is another contribution of our work to the problem of illumination correction (Chapter 3).

Figure 1.3: One acquired time-lapse fluorescence image of poor quality due to image acquisition effects. Image courtesy of Hodgkin Lab, WEHI.

In addition to uneven illumination, there are other image acquisition effects that adversely affect the subsequent measurements of cell features. For example, the fluorescent properties of
the attached fluorescent reporters enable cells to be viewed and distinguished from the background. However, these chemical compounds break down over time, resulting in a fading of the time-lapse fluorescence images. Another challenge is that the signals from different channels are mixed in each acquisition channel because of the overlapping emission spectra. Moreover, the evaluation of this correction result relies heavily on the availability of a ground truth or reference images, which is not feasible in many practical scenarios. This limitation motivates us to develop a novel quality evaluation approach that can be applied to real fluorescence images (Chapter 4). For all the above-mentioned acquisition effects, existing literature tends to focus on addressing individual effects, rather than treating all effects within a single theoretical framework [66, 6]. This has been another gap that we address in this thesis (Chapter 2).

Finally, Chapter 5 of this thesis is dedicated to the automated analysis of individual fluorescence time courses, which is another piece of the puzzle required for extracting knowledge from raw images. Analysis of single cell kinetic profiles has been somewhat overshadowed by the main challenge of tracking cells in the first place. With the increasing number of studies that result in fully tracked movies, there is now a growing effort being invested into the analysis of such data [67, 57, 34]. This kind of time-series analysis is particularly important because many previously recorded movies have only been lightly tracked manually, whereby certain cell events, such as division and death, were recorded, but time-courses of cell size or reporter expressions were not extracted [15, 24].

Moreover, most previous studies [70, 23, 24, 87] analyse cell data at the population level so that the properties of the individual cell behaviour over time are not able to be obtained. A direct observation [15] of cell behaviour is usually used for the estimates of cell features, which form the basis of further quantitative data analysis. This can require an impractical amount of manual work when processing thousands of fluorescence images. Thus, a method for automated cell feature extraction is urgently needed. The advent of cell tracking packages enables tracking and re-visiting of these movies, which in turn, requires adequate methods for time-series analysis of cell reporter expression over families of cells. This thesis provides a systematic framework for extracting such biological insights from live cell fluorescence imaging in the context of lymphocyte proliferation experiments (Chapter 5).

In this thesis, we first address the problems of how to compensate for the combination of major image acquisition effects and how to perform systematic performance evaluation on real data where a ground truth is absent. Then we perform automatic feature extraction from corrected images. Finally,
we conduct detailed quantitative modelling of the time-course behaviour of cell reporter expressions based on the corrected images. To the best of our knowledge, this is the first such attempt at time-course modelling for B lymphocyte proliferation studies.

1.3 Contributions

To address the challenges presented above, we develop a number of approaches specific to different stages of the image acquisition and analysis pipeline. Correcting the observed images of poor quality is a key step in the following quantitative analysis. Consequently, we propose novel correction methods and evaluation strategies for removing the effects of shading and cross-talk (see details in Sections 2.2.3 and 2.2.4). A reference-free evaluation approach is specifically designed for real fluorescence images after image correction. As an example application of biological studies, we study lymphocyte kinetics over time by mathematically modelling the estimated true single-cell fluorescence levels. Our results show that our models can be used as an automated method for cell feature extraction from tracked data.

In summary, our contributions and publications are as follows.

1. **Algorithm for automated shading correction:** We present a novel method for shading correction that does not require any parameter tuning except for a rough cell segmentation result. Furthermore, to the best of our knowledge, this is the only study in the literature to determine a unified reference-free based measure, which can be widely used for performance evaluation on time-lapse fluorescence images. The details of this algorithm are described in Chapter 3. The publication arising from this work is:


2. **A correlation-based method for evaluating spectral unmixing quality in the absence of reference objects:** A novel spectral unmixing method has been proposed to solve the problem of overlapping spectra, which can be used for quality evaluation on real data, where reference objects are not available for calibration. This method is presented in Chapter 4. The publication arising from this work is:

3. **Modelling lymphocyte proliferation at the single-cell level**: Based on the corrected images, we propose two simple nonlinear models to describe cell behaviour over time in the corresponding acquisition channels. These developed models enable automatic extraction of cell features from tracked data instead of using exhaustive manual annotation. This method is presented in Chapter 5. The publication arising from this work is:

*Liu, L., Zhou, J. H., Leckie, C., Hodgkin, P. D. and Kan, A., Modelling lymphocyte proliferation on the single level based on data from time-lapse fluorescence microscopy (In progress).*

Furthermore, this thesis provides a comprehensive overview of popular existing methods for eliminating prominent image acquisition effects (Section 2.2). By reviewing the strengths and weaknesses of these proposed methods, we identify the research challenges that have motivated us to develop novel image correction methods for use in time-lapse fluorescence imaging experiments. These newly developed methods are presented in the technical chapters (Chapter 3 and Chapter 4). Based on the data collected from corrected images, a novel quantitative analysis of cell kinetics is described in Chapter 5. Each of these technical chapters includes a brief introduction, motivation, methodology and discussion.

The background chapter (Chapter 2) is beneficial to the understanding of the subsequent sections, especially the technical chapters. As a result, we strongly advise readers who are not familiar with time-lapse imaging to grasp the basic knowledge in Sections 2.1 and 2.3. Readers new to image processing are also encouraged to read Section 2.2. Finally, we conclude by summarizing our contributions and possible future research directions in Chapter 6.
Chapter 2
Background

In this chapter, we present a survey on image processing and modelling techniques for lymphocyte proliferation studies. We start with a review of single-cell analysis methods, which combine imaging technology for recording the spatio-temporal dynamics during cell lifetimes with an appropriate data analysis approach (Section 2.1). Then we focus on a survey and in-depth analysis of existing image processing methods that are used to alleviate the consequences of image acquisition effects (Section 2.2). To further measure single-cell features, the methods of cell segmentation and tracking are introduced (Section 2.3). Finally, we overview the previous studies on modelling lymphocyte proliferation (Section 2.4). In particular, we highlight and motivate the open research problems that are addressed in this thesis.

2.1 Methods of Single-cell Analysis

In order to understand the process of lymphocyte proliferation, the technique of single-cell analysis has been employed to visualize cell behaviour over time and further measure relevant cell features (e.g., average intensity and cell size) during their lifetimes. Existing popular single-cell analysis methods include flow cytometry and time-lapse fluorescence microscopy [68], which are discussed at length as follows.

2.1.1 Flow cytometry

Flow cytometry is an automated instrument that allows simultaneous measurement of multiple single-cell characteristics as cells in suspension move one by one through an electronic detection apparatus, as shown in Figure 2.1. By taking advantage of fluorescent markers that bind to specific cellular molecules, flow cytometry can quantify structures and functions of individual cells since the markers may modify cell properties according to certain biochemical
reactions. In this case, cell populations and separate single cells can be characterized based on a wide range of biological properties. Hence, flow cytometry is widely employed in cell counting, cell sorting and biomarker detection.

A typical flow cytometry system consists of five main components: a fluids system, illumination system, optics system, electronics system and computer-based data analysis system. As shown in Figure 2.1, the sheath fluid surrounds the stained cells and carries them at a constant rate to pass single file through the illuminating beam. A laser light source is utilized to excite a fluorescent tag (e.g., a fluorescent antibody) bound to the cells in the specimen. Then the scattered light and emitted fluorescence are detected by the optics system. These detected analog signals are thereby converted into digital signals through the electronics system. By connecting flow cytometry to a computer, multiple biological parameters can be measured directly. More specifically, the scattered light is used to measure the intrinsic size and granularity of the cell, while fluorescence enables the measurement of the expression of specific proteins.

In summary, flow cytometry can process thousands of cells per second, but it is not suited to
following individual cells over time. Therefore, this technology is not adopted for quantitative analysis of lymphocyte proliferation at a single cell level. Instead, the other method of single-cell data analysis described in Section 2.1.2 has been used in our thesis.

2.1.2 Time-lapse fluorescence microscopy

Time-lapse fluorescence microscopy enables sustained visualization of small groups of single cells and the study of interactions between neighbour cells. It is based on the phenomenon of fluorescence, a process of light re-emission by the specimen in response to light absorption of a shorter wavelength. The molecules that exhibit fluorescence are termed fluorophores. Basically, the cells themselves do not contain a sufficient quantity of fluorophores so they cannot be differentiated from the background. Hence, certain fluorophores, acting as a marker, are attached to the target cells in advance. As seen in Figure 2.2, a bright lamp is used to illuminate the labelled specimen. With the light matching the excitation spectrum of the fluorophore, the emitted light from the specimen is collected by the objective and then passes through the dichroic mirror and the emission filter, thus reaching a detector (normally a digital camera) and being viewed. In this way, multiple time-series fluorescence images are acquired directly.

![Schematic representation of a fluorescence microscopy](image)

Figure 2.2: Schematic representation of a fluorescence microscopy. The specimen labelled by fluorescent markers is illuminated by a bright lamp. Light from the source passes a filter system through absorption and emission and is finally visualized by the detector. Schematics courtesy of Fluorescence microscope, Wikipedia [Creative Commons Licence].

The multi-channel fluorescence imaging mechanism enables us to acquire image sequences
in the bright field, fluorescence red and fluorescence green channels, as presented in Figure 2.3. Live B lymphocytes are placed in physically separated compartments called wells at low density. The medium around the cells may contain fluorophores that emit light at a weak level, known as background fluorescence. Because the fluorescence of the labelled cells is much stronger than the background fluorescence, it allows for the separation of objects of interest from the background. Generally, the cells are prepared by means of either staining or genetic modification. With the staining method, cells are placed in a solution containing a fluorescent dye (e.g., Hoechst 33342 or DAPI). In this way, the fluorophores from the dye are bound to certain cellular structures for viewing. In contrast, the genetic modification method makes use of fluorescent proteins such as red fluorescence protein (RFP) and green fluorescence protein (GFP), which are attached to other specific cellular proteins to form a fusion protein. This protein serves as a fluorescent probe in fluorescence microscopy. The target cells tagged with this probe will light up against a black background.

![Figure 2.3: One acquired image at one fixed time frame from three channels: (a) bright field, (b) fluorescence red and (c) fluorescence green. B lymphocytes are cultured in a rectangular well (red box) and pointed to by arrows. Original images courtesy of Hodgkin Lab, WEHI.](image)

Fluorescent probes as reporter molecules are powerful tools to detect the expression of genes and proteins in cells. There are a variety of fluorescent reporter proteins such as mCFP, GFP, RFP and dsRed. The fluorescent probe can be used to monitor the cell cycle phases, which are a series of events occurring in cells that lead to cell division and DNA duplication. Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) is a set of fluorescent probes that enables the monitoring of the cell cycle progression in living cells [64]. The FUCCI system employs both RFP and GFP fused to different regulators of the cell cycle: licensing factor Cdt1 and its inhibitor Geminin [86]. Moreover, Cdt1 and Geminin have opposing effects on DNA replication. In Figure 2.4, we can see the cell cycle mapping using the FUCCI system. Cdt1 peaks in the $G_1$ phase and declines after the initiation of the $S$ phase. As a result, only Cdt1 tagged...
with RFP is present, leading to red fluorescence within the nuclei. In the combined \( S/G_2/M \) phases, Geminin levels are high and only Geminin tagged with GFP remains, resulting in cells with green fluorescent nuclei.

Figure 2.4: Two-colour cell cycle mapping using the FUCCI system cited from [64]. The red fluorescence is indicative of the \( G_1 \) phase in which cell size increases, whereas the green fluorescence indicates the combined phases \( S/G_2/M \) in which DNA synthesis and cell division occur.

Time-lapse microscopy in conjunction with FUCCI reporter is not able to extract single-cell fluorescence levels over time. Thus, a semi-automated cell tracking method is introduced to monitor cell behaviour and further measure single-cell fluorescence time courses (Section 2.3). Based on the acquired fluorescence levels, the kinetics of B lymphocyte response progression in vitro can be studied. The study of lymphocyte responses is only an example application of our results. In this thesis, we focus on image processing for generic live cell imaging experiments, since the process of image acquisition corrupts the original image and thereby causes inaccurate measurement of single-cell fluorescence levels. The methods for eliminating these acquisition effects are elaborated in Section 2.2. In brief, the process of mining single-cell fluorescence time courses is shown in Figure 2.5. B lymphocytes are obtained from mice, labelled by FUCCI reporter and finally cultured in a plate with several wells. After image preprocessing, and segmentation and tracking, the single-cell fluorescence temporal profiles in both FUCCI red and FUCCI green channels are extracted, which facilitates quantitative analysis of the dynamics of individual cells through the cell cycle (Section 2.4).
2.2 Digital Image Processing

Fluorescence microscopy coupled with single-cell tracking and an appropriate data analysis method has proven to be an increasingly important tool in cell biology [15]. In particular, cell behaviour over the cell cycle can be analysed using the resulting fluorescence levels, since they are indicative of the internal cell state (i.e., the amount of the corresponding protein at a given time). However, the estimates of single-cell features such as average intensity and total intensity are adversely affected by the inherent problem of time-lapse imaging. Thus, it is essential to correct the observed image by means of an appropriate image processing method.

Let $U$ and $O$ be the true image and the observed image both of size $W \times H$. In one pixel location $(x, y)$ ($x = 0, ..., W - 1$, $y = 0, ..., H - 1$), their relation is $O(x, y) = F(U(x, y))$ where the function $F$ indicates the consequences of image acquisition effects. Given the observed image, the idea of image processing is to estimate the true image by inferring $F$. 

Figure 2.5: Mining single-cell fluorescence levels by time-lapse imaging experiment, image processing, segmentation and tracking, and cell cycle modelling, which are described in the corresponding sections of this chapter (Sections 2.1.2, 2.2, 2.3 and 2.4).
2.2.1 Image Acquisition Process

When time-lapse fluorescence microscopy is used in conjunction with FUCCI reporter, the observed images are corrupted by a number of artefacts in the process of image acquisition, as shown in Figure 2.6. In general, there are four major problems associated with time-lapse fluorescence imaging, as summarized below. Some major acquisition effects on the fluorescence image are shown in Figure 2.7.

1. **Photobleaching** [43] is the chemical destruction of fluorophores stimulated by excitation light. Because of this effect, the average fluorescence level of an image decays exponentially over time, and eventually reaches the level of background fluorescence, as seen in Figure 2.7(b).
2. **Shading** \[41\] refers to intensity inhomogeneity across the observed image. As a result, cell intensity varies within a non-uniform image, and from image to image. Figure 2.7(b) shows that it is much brighter in the center than the edges of the image.

3. **Cross-talk** \[6\] is caused by overlapping emission spectra, leading to more than one emitted fluorescence signal being collected in each acquisition channel. As is presented in Figure 2.7(c), when the cell sample is marked by FUCCI reporter, the signals recorded in FUCCI red channel originate from red fluorescence and green fluorescence, rather than red fluorescence alone.

4. **Autofluorescence** \[52\] is an intrinsic fluorescence in some proteins or medium other than reporter proteins in the cell. In most cases, it is assumed to be constant and removed in conjunction with cross-talk.

Another minor problem is induced by acquisition electronics (e.g., the digital camera) and comprises three effects: camera noise, offset and gain. Both the camera offset and camera gain are constant over time. In most cases, they are provided in the time-lapse imaging experiments. In terms of camera noise, Gaussian kernels are often used to reduce the noise. In the context of our thesis, this minor problem is not emphasized in the image processing method. Furthermore, photobleaching is time-dependent only, while shading relies on both time and position. In contrast, the effects of cross-talk and autofluorescence are assumed to be scalars and the same from image to image. These properties of image acquisition effects are beneficial to our newly proposed image processing method (Chapter 4). Meanwhile, relevant approaches for eliminating these image acquisition effects are described in Sections \[2.2.2\] to \[2.2.4\].

### 2.2.2 Alleviating Photobleaching

Photobleaching is an unwanted effect because it diminishes the level of available fluorophores and further leads to fluctuation in cell fluorescence levels. Thus, it is essential to eliminate bleaching effects beforehand. There are several techniques to slow down the rate of photobleaching in time-lapse imaging experiments. The presence of oxygen in the sample is one of the most important factors that influence photobleaching. In this case, argon \[72\] or nitrogen \[46\] flushing to deoxygenate stained samples may be used. An alternative way is to add anti-fading solutions (e.g., non-commercial substances containing N-propyl-gallate \[22\]).
Nevertheless, this method is not compatible with live cell imaging, as the added chemicals will either kill cells or influence cell behaviour.

One feasible approach to obtain a non-bleached image is to fit a mathematical decay model of bleaching kinetics. Generally, the photobleaching curve presents a negative exponential behaviour that could be considered as a generalization of a mono-exponential or multi-exponential function [43, 84]. Specifically, a mono-exponential decay model is commonly used due to its simplicity. It only depends on time and can be expressed by

$$ I(t) = I_0 \cdot e^{-a \cdot t} + b $$

where $I(t)$ refers to the average fluorescence level of objects of interest at time $t$. $I_0$ is the initial average intensity and the constant $a$ characterizes the decay rate of the fluorophore in the structure. Here $b$ represents the background fluorescence level.

If samples are stained, then photobleaching is present in both cells and background. In contrast, when the FUCCI reporter is used, it is the background in an image that gets bleached. Thus, the objects of interest in bleaching correction are the background regions. In each frame, the average fluorescence intensity in the background regions is calculated. The temporal evolution of the average intensity is shown in Figure 2.8. This curve can be fitted using non-linear least-squares regression with a mono-exponential model in Equation 2.1. It is commonly assumed that the decay rate is the same for all pixels in an image at time $t$. Thus, the fluorescence level for each pixel in the bleaching-corrected image $I_c$ at time $t$ can be formulated as

$$ I_c(x, y, t) = \frac{I(0)}{I(t)} \times I(x, y, t) $$

where $I(0)$ refers to the average fluorescence level for the initial image, and $I(x, y, t)$ represents the observed pixel value at time $t$. Besides, the correction results are evaluated by visually inspecting the bleaching rate. In practice, a bleaching correction plugin “Bleach Correction” built into image processing platform Fiji [65] has been adopted to solve this problem present in time-lapse fluorescence images [51], as addressed in Chapter 5.

2.2.3 Compensation for Shading Artefacts

In general, shading can be either object-independent or object-dependent. Object independent shading is caused by imperfect image acquisition, whereas object-dependent shading arises
from the preparation of objects by, for example, staining inhomogeneity. A prospective correction method can be applied to address object-independent shading by using a calibration slide and an acquisition protocol, but these are not always available. Hence, a number of retrospective methods have been developed to correct both object-dependent and object-independent shading when the only available data are the image itself.

Retrospective shading correction methods make few assumptions about the acquisition process and mainly rely on the information from the acquired images. The relationship between the acquired nonuniform image and the shading-free image can be formulated as

$$ N = S_M \odot U + S_A \tag{2.3} $$

where $N$ represents the nonuniform image, $U$ indicates the real uniform image, while $S_M$ and $S_A$ denote the multiplicative shading component and additive shading component, respectively. The notation $\odot$ refers to point-wise multiplication.

The goal of retrospective shading correction is to estimate both shading components from the acquired nonuniform image. In reality, this problem can be further simplified by assuming that only one shading component degrades the uniform image. Thus, the relationship between the nonuniform image and the uniform image could be expressed by a multiplicative or an additive model

$$ N = S_M \odot U \tag{2.4} $$

$$ N = U + S_A \tag{2.5} $$

Figure 2.8: Mono-exponential model for average fluorescence levels in FUCCI green channel.
where $U$ can be reformulated by the sum of a biologically relevant foreground fluorescence from cells $F$ and a background fluorescence $B$

$$U = F + B$$  \hspace{1cm} (2.6)

In summary, the image formation model can be classified into three categories, namely, multiplicative and additive, multiplicative only and additive only. For simplicity, most proposed shading correction methods are based on either multiplicative or additive models. Existing popular retrospective approaches include surface fitting (SF) [63], rolling ball algorithm (RB) [73], signal envelope estimation (SE) [62], homomorphic filtering (HF) [20], gradient distribution (GD) [85], entropy minimization (EM) [41], information minimization (IM) [42], nonparametric nonuniform intensity normalization (N3) [69] and an improved N3 correction method called N4ITK [78]. As summarized in Table 2.1, those methods adopt different image formation models, rely on different assumptions, include different numbers of tuning parameters and have different advantages and disadvantages.

The SF algorithm applies least-squares fitting of a polynomial or spline to estimate the shading component by selecting a number of points on the background manually or automatically. Manual selection is subjective and time-consuming, whereas automatic selection assumes good global support of the background, which is not always true. The idea of RB is to use morphological opening of a grey-scale image with a sphere (also called a top hat filter) to obtain an estimation of the background. Here, the assumption is that the size of the objects of interest is smaller than the background variation. SE removes the shading component by estimating the envelope of the image through iterative stretching. An implicit assumption involved here is that the objects are either all darker or all brighter than the background. These three methods can be seen in Figure 2.9.

The fourth correction method HF assumes that the shading is the low-frequency content that can be separated from the high-frequency spectrum of the true image. In some cases, due to the nature of the image, the spectra of shading and shading-free data cannot be separated, which makes it difficult to model the shading effect using low frequencies only. Thus, the accuracy of HF not only depends on the selection of an appropriate high-pass filter but also the nature of the image. A drawback of the HF method is that it can introduce halo artefacts on the boundaries of the image if optimal parameters are not obtained. GD eliminates the shading component by assuming that the image gradient probability distribution is sparse, which is not
<table>
<thead>
<tr>
<th>Methods</th>
<th>Model</th>
<th>Assumptions</th>
<th>Number of tuning parameters</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface fitting (SF)</td>
<td>Multiplicative or additive</td>
<td>The selected points can represent the global background</td>
<td>2</td>
<td>It performs well for images containing small-sized and medium-sized objects</td>
<td>The performance relies heavily on the selected points</td>
</tr>
<tr>
<td>Rolling ball (RB)</td>
<td>Multiplicative or additive</td>
<td>The size of objects of interest is smaller than the background variation</td>
<td>1</td>
<td>It is simple to implement and efficient</td>
<td>It fails to correct biomedical images with large objects</td>
</tr>
<tr>
<td>Signal envelope (SE)</td>
<td>Additive</td>
<td>The shading component is slowly-varying and can be estimated from the signal envelope</td>
<td>2</td>
<td>It does not require the objects of interest to be small in size and is simple to implement and efficient</td>
<td>The shading component is additive and additive</td>
</tr>
<tr>
<td>Homomorphic filtering (HF)</td>
<td>Multiplicative</td>
<td>The shading is low-frequency content which can be separated from the high-frequency spectrum of the true image</td>
<td>4</td>
<td>The shading field is not modelled by a parametric surface but estimated in the Fourier domain.</td>
<td>It produces halo artefacts on the boundaries when the parameters of the filter are not tuned appropriately</td>
</tr>
<tr>
<td>Gradient distribution (GD)</td>
<td>Multiplicative or additive</td>
<td>The image gradient probability distribution is sparse</td>
<td>5</td>
<td>Easy to implement and efficient</td>
<td>It is computationally expensive and heavily dependent on the parameters of the weighting function</td>
</tr>
<tr>
<td>Entropy minimization (EM)</td>
<td>Multiplicative and additive</td>
<td>Entropy increases after shading</td>
<td>0</td>
<td>No tuning parameters, and performs well for images with either large or small objects</td>
<td>High-order polynomial is required for accurate estimation of the shading field with small local variation</td>
</tr>
<tr>
<td>Information minimization (IM)</td>
<td>Multiplicative and additive</td>
<td>Entropy increases after shading</td>
<td>0</td>
<td>No tuning parameters and solely exploits the information naturally presented in the image.</td>
<td>It is optimization based and thus cannot guarantee a global minimum.</td>
</tr>
<tr>
<td>N3 ITK</td>
<td>Multiplicative</td>
<td>The true image intensities are independent identically distributed random variables</td>
<td>6</td>
<td>Improved convergence performance of the smooth shading field estimation compared to N3 and also widely applied to medical imaging</td>
<td>No prior knowledge on the intensity probability distribution of the image structures and widely applied to almost any MR images</td>
</tr>
</tbody>
</table>

*Table 2.1: Summary of different retrospective shading correction methods*
Figure 2.9: Schematic of (a) background point selection, (b) polynomial surface fitting and (c) top hat filter using a rolling ball.

always the case. This method is computationally expensive and relies on the parameters of the weighting function.

EM estimates the shading-free image by minimizing its entropy because it is assumed that shading leads to higher entropy. However, it suffers from a fundamental limitation as it encourages local optima in the optimization. In comparison with EM, IM also minimizes the entropy but estimates the inverse of the shading components using a combination of basis functions while the global intensity statistic is preserved. N3 was originally proposed to eradicate the multiplicative shading component in magnetic resonance (MR) images by maximizing the frequency content of the intensity distribution. The corresponding smooth shading field is supposed to be multiplicative and estimated by B-spline approximation. Compared to N3, N4ITK couples a robust B-spline approximation algorithm with an advantageous optimization strategy for shading field estimation. Both methods assume that the true image intensities are independent identically distributed random variables, whereas this is not the case for fluorescence images of cells as the intensity can vary considerably within cells. Moreover, the N3 algorithm may converge to local minima in the objective function and the N4ITK method is extremely computationally expensive. In particular, they require substantial manual work for
Table 2.2: Summary of quantitative evaluation measures

<table>
<thead>
<tr>
<th>Quantitative evaluations</th>
<th>Measure</th>
<th>Segmentation needed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference based evaluation</td>
<td>( L_2 )-norm</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Root mean squared error (RMSE)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Spearman’s correlation coefficient (( r ))</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Peak signal-to-noise ratio (PSNR)</td>
<td>No</td>
</tr>
<tr>
<td>Reference-free based evaluation</td>
<td>Standard deviation (( \sigma ))</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Coefficient of variation (CV)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Coefficient of joint variation (CJV)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Signal-to-noise ratio (SNR)</td>
<td>No</td>
</tr>
</tbody>
</table>

In order to show whether one correction method is feasible for fluorescence images and superior to other methods, numerous quantitative measures have been proposed. The evaluation methods can be categorized into qualitative and quantitative ones. The former is a subjective analysis, which compares the histogram or the intensity profiles before and after shading correction, while the latter adopts some numerical measure as an objective comparison. Hence, the quantitative evaluation is emphasized, which could be further divided into two major categories, namely, reference based and reference-free based evaluation. Here the reference means the true shading-free image or true shading component. In the following analysis, some popular quantitative performance measures for evaluation are listed and compared, as summarized in Table 2.2.

1. **Reference based evaluation** approach is employed when a reference is available. It includes four commonly used quantitative measures: normalized \( L_2 \)-norm [9], correlation coefficient, root mean squared error (RMSE) and peak signal-to-noise ratio (PSNR) [2]. The normalized \( L_2 \)-norm calculates the difference between the true shading-free image/shading field and the estimated one as

\[
L_2 = \min_w \sqrt{\frac{\sum_{i=1}^{M} \sum_{j=1}^{N} (w I_{est}(i,j) - I_{true}(i,j))^2}{\sum_{i=1}^{M} \sum_{j=1}^{N} (I_{true}(i,j))^2}}
\]  

(2.7)

where \( w \) is the normalization coefficient, since shading correction can lead to an arbi-
trary scaling of the reference image. While the optimization for the coefficient $w$ can be considered as a part of the correction algorithm rather than a performance measure, we define these performance measures exactly as they were defined in [2, 9]. $I_{est}$ and $I_{true}$ with size $M \times N$ represent the estimated shading-free image/shading field and the true shading-free image/shading field, respectively.

RMSE is used to measure the difference between the true and the estimated shading-free image/shading field defined as

$$
RMSE = \sqrt{\frac{\sum_{i=1}^{M} \sum_{j=1}^{N} (I_{est}(i,j) - I_{true}(i,j))^2}{M \times N}}
$$

Since we are interested in either a linear or nonlinear relationship, Spearman’s correlation rank coefficient ($r$) rather than Pearson’s correlation coefficient is adopted to show the similarity between the estimated image and the true one. PSNR is the ratio of the maximum possible pixel value $Max_{est}$ in an image to the root mean squared error $RMSE$

$$
PSNR = 20 \log \left( \frac{Max_{est}}{RMSE} \right)
$$

2. **Reference-free based evaluation** is applied in the absence of reference images. The shading correction results can be quantitatively assessed by estimating the intensity variation before and after correction. More specifically, most commonly used quantitative measures include standard deviation ($\sigma$) for the whole image, coefficient of variation (CV) [80], coefficient of joint variation (CJV) [80] and signal-to-noise ratio (SNR). CV is defined as a ratio of standard deviation $\sigma$ to mean value $\mu$ of one tissue class (C), namely foreground or background in an image

$$
CV = \frac{\sigma(C)}{\mu(C)}
$$

One drawback of CV is that the mean value of the class is changed but not the variance when the uniform additive intensity transformation occurs. Another drawback is that it is calculated for a single class, which makes it difficult to assess the overall correction quality for an image composed of many classes, particularly when CV improves for one class but not for others. In order to avoid these drawbacks, for an image composed of two classes ($C_1$ and $C_2$, e.g., cell and background), CJV has been proposed by estimating
the overlap between them

\[ CJV = \frac{\sigma(C_1) + \sigma(C_2)}{|\mu(C_1) - \mu(C_2)|} \]  

(2.11)

CJV is invariant to uniform additive and multiplicative transformations, but the disadvantage is that segmentation is required to obtain different classes in an image. Furthermore, it has been shown that the evaluation results depend on the accuracy of the segmentation [9]. Because true shading-free images are unlikely to be available for real data, CV and CJV cannot provide information on the amount of shading remaining after correction. In reality, a sound segmentation result is necessary. The disadvantage of reference based evaluation is that the true shading-free images or shading components must be known, which is not feasible for real data. Reference-free methods, in particular CV and CJV, require segmentation as a preprocessing step before evaluation.

Even though reference-free based measures could be applied to time-lapse fluorescence images, there is no consensus regarding which performance measure should be adopted. Apart from that, most shading correction approaches [5, 27, 80, 3] and evaluation strategies [1, 77, 9] are specially developed for MR images, which may be not suitable for adaptation to time-lapse fluorescence images. Moreover, some correction methods have many parameters to tune, increasing the difficulty of parameter setting. To solve these problems, we have developed a novel shading correction method that can be applied in time-lapse fluorescence images, and determined a quantitative measure for performance evaluation on real data (Chapter 3).

2.2.4 Removing Cross-talk Effects

FUCCI reporter is composed of two fluorescent proteins: green mAG1 and orange mKO2, which are suggestive of the \(G_1\) phase and \(S/G_2/M\) phases, respectively. Due to the overlapping emission spectra of these two proteins, the emitted light from both fluorophores are recorded in each acquisition channel when the marked cells are excited. In the context of single cell tracking and subsequent data analysis, cross-talk may cause an incorrect interpretation of the true signals. More specifically, Figure 2.10(a) shows that FUCCI red levels are high when FUCCI green levels peak. This deviates from the ground truth that one peaks while the other remains low, as seen in Figure 2.10(b). To address this problem, the method for cross-talk correction is called spectral unmixing.
Figure 2.10: Relationship between cell average red fluorescence and green fluorescence in (a) acquired mixed images due to cross-talk, and (b) spectral unmixed images after correction.

Based on a linear mixing model [88], the recorded fluorescence emissions can be modelled by a linear combination of the reference spectra of all involved fluorochromes as follows

\[ I_{\text{mix}} = A I_{\text{real}} + I_{af} \]  

(2.12)

Here \( I_{\text{mix}} \) and \( I_{\text{real}} \) represent real \( n \times m \) matrices whose entries are pixel intensities in the mixed and real images, respectively, \( n \) is the number of fluorophores present in the samples, and \( I_{af} \) denotes a real \( n \times m \) matrix whose entries are pixel intensities due to autofluorescence at each corresponding pixel location. Furthermore, \( A \) is a non-negative mixing matrix with \( n \times n \) mixing coefficients. When using FUCCI reporter, the fluorescence levels from two channels \( c_1 \) and \( c_2 \) can be reformulated as

\[
\begin{bmatrix}
I_{c_1}^{\text{mix}} \\
I_{c_2}^{\text{mix}}
\end{bmatrix}
= \begin{bmatrix}
A_{11} & A_{12} \\
A_{21} & A_{22}
\end{bmatrix}
\times
\begin{bmatrix}
I_{c_1}^{\text{real}} \\
I_{c_2}^{\text{real}}
\end{bmatrix}
+ \begin{bmatrix}
I_{c_1}^{af} \\
I_{c_2}^{af}
\end{bmatrix}
\]  

(2.13)

It is worth mentioning that autofluorescence is usually assumed to be constant for each image and thus neglected in the analysis. Given that both \( A \) and \( I_{\text{real}} \) are unknown, the idea of spectral unmixing is to recover each fluorophore from the mixed signals by minimizing some form of energy function or cost function based on the assumptions made. The simplest spectral unmixing method is automatic unmixing (AU) [45], which adopts a simple subtraction of the intensities of longer wavelength channels from those of shorter ones. In other words, the number of mixing coefficients is reduced. The assumption is that cross-talk is unidirectional,
which means only the shorter wavelength fluorophore is recorded in the longer wavelength channel. Other methods that have been used for unmixing are principal component analysis (PCA) \cite{74}, independent component analysis (ICA) \cite{30}, singular value decomposition (SVD) \cite{81} and non-negative matrix factorization (NMF) \cite{39}.

ICA requires sources to be statistically independent, while SVD takes orthogonal sources into consideration. In general, the true signals are not independent, and the use of PCA or ICA is not always appropriate. NMF is the most promising method since it requires few assumptions about the spectra and concentrations of fluorochromes. However, this method requires a specification of the initialization and convergence criteria. In order to overcome the problem of the sensitivity to initialization and slow convergence, several NMF algorithms \cite{60}, \cite{28}, \cite{10}, \cite{55} have been proposed based on diverse optimisation criteria by extending the work of Lee and Seung \cite{39}.

Each method takes a set of measurements as input and produces an estimated mixing matrix \cite{10} as output. The input measurements are called training data. Surprisingly, there has been no systematic description of how to generate the training data from images. Thus, it is unclear which strategy of training data selection could lead to higher performance of spectral unmixing. Furthermore, the quality evaluation of spectral unmixing is usually based on one or more reference objects: a part of the image is known to be fluorescent in one channel but non-fluorescent in another channel \cite{54}.

In some cases, these reference objects are not readily available. For example, in an experiment using a multi-colour fluorescence reporter such as FUCCI \cite{64}, it is not easy to include reference objects. If a FUCCI red/green mouse is used, the reference objects would require sacrificing two additional mice: FUCCI red and FUCCI green. In addition to ethical considerations, handling additional mice involves considerable additional work. Moreover, it was found that the mixing matrix estimated for one experiment is not necessarily optimal for another experiment even when the same reporters are used, presumably due to biological variability between mice, and the difficulty in preserving optical settings exactly between the experiments. Hence, we aim to address the challenge of spectral unmixing without reference objects. To this end, we develop a novel spectral unmixing method and perform quality evaluation in the absence of reference objects, as presented in Chapter \cite{4}.
2.3 Cell Segmentation and Tracking

Time-lapse fluorescence microscopy can produce cell fluorescence images and monitor the behaviour of a population of cells through the cell cycle. In order to obtain the degree of fluorescence within a single cell over time, cells need to be segmented from the background and each other, and then tracked. A cell segmentation method takes an image as input and produces a mask image by obtaining cell outlines. In this way, the background and the objects of interest can be distinguished based on the mask image. Then the cell features such as cell position, cell size and intensity values can be measured directly. Cell segmentation serves as a preliminary processing step in cell tracking systems. One major detection error in time-lapse imaging experiments is under-segmentation due to overlapping cells, as shown in Figure 2.11. Other problems include spurious detections and missed detections, which need manual correction. In terms of performance evaluation, the estimated boundaries are compared with the human plotted ones that are used as a reference. Ideally, the ground truth is used to evaluate the performance of segmentation algorithms.

Figure 2.11: A bright field cell image with segmentation and tracking. Cell outlines are marked in pink and the red line refers to the trajectory of cells between frames. Image courtesy of Hodgkin Lab, WEHI.

A cell tracking algorithm is used to monitor cell motions during their lifetime. Based on the acquired cell features during the segmentation step, the algorithm aims to identify the position of the target cell in subsequent frames repeatedly and link these cell positions to form a trajectory. A sound tracking method should be capable of locating cells that exhibit high levels of movement between frames. The estimated trajectory represents a cell’s motion over time. A number of cell tracking methods have been proposed and have become available within several software platforms. In particular, some popular packages are CellProfiler [7], LineageTracker [16] and TrackMate [76].
LineageTracker takes advantage of correlation scores to identify two-way matched cells in subsequent frames. This has been implemented in Fiji and used for cell tracking and data analysis in Chapter 4. Another open source Fiji plugin called TrackMate can track multi-channel time-series images, and provide visualization and analysis tools to analyse the measured cell features (e.g., cell average intensity and cell total intensity). One limitation is that the radius of all cells is assumed to be the same, which may lead to poor performance in the presence of large variations in cell morphology. Nevertheless, it works well for live B cell imaging due to small variations in cell radius. Furthermore, less manual correction work after automated tracking is required for TrackMate compared to LineageTracker. As a result, TrackMate has been employed to track B lymphocytes and study the resulting fluorescence levels in Chapter 3 and Chapter 5. Given the measured cell features in TrackMate, a cell lineage tree can then be constructed (see Figure 5.4 for an example lineage tree).

![Cell Lineage Tree](image)

Figure 2.12: A cell lineage tree consisting of mother cells (cell 0 and cell 2), dying cells (cell 1 and cell 4), and a lost cell (cell 3) that has not died at the end of the video.

### 2.4 Study of Lymphocyte Proliferation

Time-lapse fluorescence imaging has been applied to various biological areas, including stem cell biology [19], medical research [67], cell fate decisions [57, 11] and lymphocyte immune responses [58]. In this thesis, we emphasize one important application lymphocyte proliferation, since it is an essential process in lymphocyte immune responses.

In response to pathogenic stimuli, lymphocytes undergo several rounds of proliferation, cessation of division, and eventually death of the majority of newly generated cells. These processes are tightly regulated to clear pathogens while avoiding overspending the resources of the host organism. However, when dysregulated immune response occurs, the activated cells will not stop dividing, leading to a disease such as cancer in the host organism. Hence,
it is essential to understand the dynamics of proliferating lymphocytes. A number of studies have proposed various cell cycle models for monitoring cell dynamics over time, as a way of establishing the empirical distribution of the time spent in the individual cell cycle phases (e.g., G\textsubscript{1} and S/G\textsubscript{2}/M) \cite{70, 23, 24, 87, 15}.

Based on the cell cycle progression models, major analysis associated with immune cell dynamics includes stochastic variation in cell division time, kinetic relationship between cell cycle phases, and correlations between sibling cells. More specifically, Smith and Martin demonstrated that the time spent in the G\textsubscript{1} phase is highly variable, whereas the duration of the S/G\textsubscript{2}/M phases is approximately fixed \cite{70}. In contrast, a stretched model combined with FUCCI reporter, developed by Hodgkin and coworkers \cite{15}, concluded that the time spent in both G\textsubscript{1} and S/G\textsubscript{2}/M phases are highly variable, which contradicts the results in the Smith-Martin model. Besides, the stretched model showed that the different phases of the cell cycle are strongly correlated in either a cell population or sibling cells.

One major disadvantage of the proposed models is that most studies analyse cell data at the population level, while another disadvantage is that manual annotation is indispensable for the estimates of time spent in different cycle phases. Specifically, this manual annotation makes use of either the property of fluorescent reporter (e.g., FUCCI) itself or direct observation of cell dynamics in time-lapse imaging experiments. In either case, it brings exhaustive manual work when measuring time spent in different division phases at a single cell level. Meanwhile, despite the above-mentioned analysis, the mechanism that can explain the longer time spent before entering the S phase in the progenitor cell remains largely unknown. As is shown in Figure 2.13, the first division takes much longer than subsequent division rounds before moving into the combined S/G\textsubscript{2}/M phases. The question then arises as to whether progenitor cells and dividing cells in other generations follow the same division mechanism.

All of these limitations motivate us to develop a method for automatic feature extraction from the tracked single-cell fluorescence time courses using FUCCI reporter and semi-automated cell tracking. This method allows for automatic quantification of time spent in individual cell cycle phases. Due to the image acquisition effects present in fluorescence images, in this thesis we propose the methods for correcting prominent effects (Chapter 3 and Chapter 4). Based on the corrected fluorescence profiles, we have developed a mathematical model for describing the kinetics of B cell response in vitro (Chapter 5), as seen in Figure 2.13. Finally we perform analysis associated with FUCCI kinetics for entire lineage trees (Chapter 5). In particular, whether the progenitor cells follow the regular division mechanism after the
Figure 2.13: Automated quantification of individual cell cycle phases by modelling fluorescence time courses of (a) progenitor cell and (b) other dividing cell in FUCCI red channel and FUCCI green channel. Here $G_0$ indicates the quiescent stage in the progenitor cell.

$G_0/G_1$ phases is of great interest. Because of inconsistent results in previous proposed models, it is still worth validating the variation in cell cycle time and the relations between cell cycle phases. In summary, the study of lymphocyte proliferation can be approached using the steps as follows.

1. Measuring the single-cell fluorescence profiles using FUCCI reporter and cell tracking.
2. Estimating the true fluorescence levels by removing major image acquisition effects.
3. Developing a mathematical model for B cell dynamics through the cell cycle.
4. Analysing FUCCI kinetics for cells in entire lineage trees.
Chapter 3

Removal of Shading Effects

In this chapter, we present our first contribution: a novel shading correction method (Section 3.2) for eliminating the shading artefacts prominent in time-lapse fluorescence images, and a comprehensive quality evaluation on real data without ground truth (Section 3.3). An important contribution is that we have identified a quantitative measure (Section 3.3.3) that can be used for evaluation on real fluorescence images, which has not been discussed before. Furthermore, our proposed shading correction method does not require parameters to be tuned, which helps improve ease of use when processing large numbers of fluorescence images. Finally, we compare and evaluate the existing popular shading correction methods by conducting experiments on synthetic and real datasets (Section 3.3.4).

3.1 Consequences of Shading Effects

Compared to confocal microscopy, time-lapse microscopy is better suited for long-term imaging because it exposes the sample to less light. However, it suffers from the inherent problem of intensity inhomogeneity, also known as shading [41], or uneven illumination. Generally, there are four types of effects that cause the difference of cell intensities: natural variation between cells, position-dependent acquisition effects (i.e., shading), time-dependent acquisition effects [79] (i.e., photobleaching) and independent acquisition effects [6] (i.e., cross-talk). In order to analyse cell behaviour, we need to measure changes in natural cell fluorescence levels over time. Therefore, these acquisition effects need to be eliminated. In this thesis, we correct the observed images by removing photobleaching first (Section 2.2.2), then shading, and finally cross-talk (Section 4.2). Here we focus on shading removal.

Because of these shading artefacts, pixel intensities of the same tissue vary with its location, which can be seen in Figure 3.1. This will lead to inaccurate measurements of single-cell features such as the average intensity and total intensity of cells. Meanwhile, it has been the-
Figure 3.1: Shading effects on pixel intensity: (a) one fluorescence image captured from time-lapse fluorescence microscopy; (b) intensity profile showing the intensity variation along the red straight line in (a). The data points with relatively larger pixel intensities (spikes) represent the signal from cells, otherwise the intensities come from the background. This profile indicates that the cell pixel values near the center are elevated more than values near the corners due to uneven illumination. This is not expected in reality as cells are seeded at random. Image courtesy of Hodgkin Lab, WEHI.
oretically proven that shading has an effect on these single-cell features [12]. As is shown in Figure 3.2, there is a strong correlation (-0.33) between cell average intensity and cell location for one shaded image. Due to random cell placement, there should be no correlation between cell intensity and cell location. Thus, the value of -0.33 is not negligible since it may cause substantial variation in the average intensities. Moreover, many image processing applications such as segmentation, tracking or quantitative analysis are highly sensitive to shading artefacts. Hence, it can be important to eliminate the shading effects beforehand.

![Figure 3.2: Shading effects on cell average intensity of one shaded fluorescence image. Cells were automatically segmented using the thresholding method (Section 3.3.3), and visually inspected for segmentation quality. Cell location refers to the distance from the cell centroid to the image center. The red regression line shows the cell average intensity dependence on the cell location in the image.](image)

In this study, our aim is to choose an appropriate correction method from a number of proposed shading correction approaches for real data, where the ground truth is absent. Even though a great number of quantitative measures have been developed, there is no consensus regarding which one should be applied for evaluation on real data. Furthermore, most shading correction approaches [5, 27, 80, 78, 3] and evaluation strategies [11, 77, 9] are specially developed for MR images, which may be not suitable for adaptation to time lapse fluorescence microscopy images. Moreover, some correction methods have many parameters that need to be tuned, resulting in the difficulty of parameter setting. Consequently, it is not easy to choose
an optimal approach to deal with shading in time lapse fluorescence microscopy. Therefore, our purpose is to determine a quantitative measure for performance evaluation on real data.

### 3.2 CJV-based Shading Correction Method

In order to remove shading effects, existing popular approaches (Section 2.2.3) take advantage of different optimization criteria, e.g., minimizing the entropy or gradient of the corrected image. While there are many performance measures that focus on variation in pixel intensity, there are no methods that minimize the variance explicitly. In Section 2.2.3, we have shown that the CJV-based measure can be widely used for performance evaluation on real data. Therefore, we develop a novel shading correction method that aims to minimize the CJV value of the estimated uniform image. Recall that the relationship between the observed nonuniform image $N$ and the uniform image $U$ can be formulated as

$$N = S_M \odot U + S_A \quad \text{(3.1)}$$

In order to maintain the global intensity characteristics of the images, we model the smoothly varying shading fields using the same expressions as in

$$S_M(x, y) = 1 + m_1 x + m_2 y + m_3 xy + m_4(x^2 - W^2/12) + m_5(y^2 - H^2/12) \quad \text{(3.2)}$$

$$S_A(x, y) = a_1 x + a_2 y + a_3 xy + a_4(x^2 - W^2/12) + a_5(y^2 - H^2/12) \quad \text{(3.3)}$$

where $x$ and $y$ represent the pixel position in an image, and $W$ and $H$ refer to the image width and height, respectively. Considering both multiplicative $S_M$ and additive $S_A$ shading components, the estimated uniform image can be expressed by

$$\hat{U}(x, y) = \frac{N(x, y) - S_A(x, y)}{S_M(x, y)} \quad \text{(3.4)}$$

The idea of our novel shading correction method is to minimize the CJV value of the estimated uniform image by searching for the optimal additive parameters $a$ and multiplicative parameters $m$

$$CJV_{\min} = \min_{\{a, m\}} CJV \left( \hat{U}(x, y) \right) \quad \text{(3.5)}$$

This novel correction method is called CJVMA when both shading components are consid-
ered, otherwise it is called CJVM or CJVA when only multiplicative or additive parameters are
considered, respectively. We must perform segmentation for the acquired images beforehand.
For fluorescence images that are seriously corrupted, it is difficult and time-consuming to ob-
tain a good segmentation result. Thus, we aim to conduct rough segmentation and achieve
relatively good quality. The advantage of the CJVMA method is that no parameters need to
be tuned. It is a built-in plugin called “CJV Minimization” in Fiji. The source code and the
tested datasets mentioned above are publicly available at the website [1]. Specifically, these op-
timal parameters $a$ and $m$ are found by Powell’s multidimensional directional set algorithm
and Brent’s one-dimensional optimization method [61]. In order to achieve an iterative solu-
tion, three parameters are initialized. The shading coefficients $p$ which consist of $a_1 - a_5$ and
$m_1 - m_5$ are initialized to be zeros. The parameter $xi$ with size $10 \times 10$, an initial guess at the lo-
cation of the minimum value, is set to be ones. The tolerance $ftol$ for the optimization criterion
is $10^{-6}$.

3.3 Experimental Results and Discussion

In the absence of ground truth for real datasets, our quality evaluation strategy is to determine
one reasonable quantitative measure based on artificial datasets. In this case, we first gener-
ate artificial datasets that are representative of real datasets used in the experiments. Then
we determine a reference-free performance measure by comparing the consistency between
one reference-based baseline measure and all the others. In other words, the artificial datasets
are used to establish the most appropriate qualitative reference-free measure from all popu-
lar measures. Subsequently, we explicitly describe the implementation details of the existing
popular shading correction methods. In particular, our proposed algorithm CJVMA requires
prior segmentation of the cells from the background. By comparing and evaluating all the
above-mentioned shading correction methods, we determine an optimal approach to remove
shading for real fluorescence images according to the determined measure.

3.3.1 Tested Datasets

In this study, the proposed shading correction strategies are tested on both artificial datasets
and true fluorescence images. The real datasets were sampled from previously acquired multi-
channel videos [33]. In brief, Cpg-stimulated B lymphocytes were placed in wells at low den-

[1] https://github.com/LilyCrystal/CJVMinimization
sity and continuously imaged for several days. The role of the well is to prevent the cells from migrating outside the field of view. Ubiquitin-GFP mice were used in some videos, providing the fluorescence green signal. In this thesis, fluorescence ubiquitination cell cycle indicator (FUCCI) mice were used, in which case red and green fluorescence signals were recorded. In all cases, an image sequence in each channel was captured. It is convenient to refer to image sequences using well shapes (i.e., rectangular, hexagonal and square in Figure 3.3), which, however, do not have any biological significance in these experiments.

![Image](image-url)

Figure 3.3: Acquired images from videos where the cells are allocated in: (a) a rectangular well, (b) a hexagonal well and (c) a square well. The first two images are extracted from the transmission out of focus channel, whereas the last one is from the Ubiquitin-GFP channel. Several examples of cells are pointed to by arrows. Images courtesy of Hodgkin Lab, WEHI.

We have formed four real datasets reflecting the variability in experimental conditions. For each dataset, we randomly selected ten fluorescent images from one of the video sequences, as presented in Figure 3.4. Specifically, dataset 1 and dataset 2 contain images sampled from the video with rectangular wells in the FUCCI red channel and FUCCI green channel, respectively.
However, dataset 3 and dataset 4 consist of images sampled from the video with hexagonal wells and square wells in the Ubiquitin-GFP channel, respectively. Due to the low contrast between the background and cells (white dots) in Figure 3.4, the wells are barely visible for images in the fluorescent channels compared to images in the transmission channels. The difficulty of quality evaluation on real datasets lies in the lack of ground truth: shading-free images. An indirect way is to determine a reasonable quantitative measure from artificial datasets, which is then applied in real datasets.

![Figure 3.4: Images representative of real datasets 1 to 4 captured from: (a) rectangular well in FUCCI red channel, (b) rectangular well in FUCCI green channel, (c) hexagonal well in Ubiquitin-GFP channel and (d) square well in Ubiquitin-GFP channel. Images courtesy of Hodgkin Lab, WEHI.](image)

The synthetic images are generated using a previously published simulation algorithm with some modifications [40]. This image simulation algorithm is also used in other work [59]. Gaussian noise $\delta$ is added into the simulated images consisting of two classes: background and cells. In practice, we make the background follow a uniform nonnegative distribution.
The intensities in the background and cells vary. The illumination field \( S \) is simulated by a second-order polynomial with fixed parameters, and then applied to corrupt the simulated shading-free image by a multiplicative model rather than an additive one. The relationship between the nonuniform image \( N \) and the shading-free image \( U \) is given by

\[
N = S \odot U + \delta
\]  

(3.6)

We generated ten synthetic shading-free images, ten illumination images and ten resulting nonuniform images (1040×1388 pixels, non-normalized intensity between 0 and 65535) simultaneously. In this case, we can adopt reference-based quantitative measures to perform the evaluation for the synthetic datasets. Two artificial datasets are created by setting different parameters of the above-mentioned simulation algorithm. In order to ensure these datasets are representative of real data, we first aim to obtain appropriate parameters in this simulation algorithm by making the normalized empirical cumulative probability distribution of the artificial datasets follow that of the real datasets, as is presented in Figure 3.5. Then we exploit those parameters to produce the artificial datasets. In Figure 3.6, we show the shading-free image, corresponding shading field and resulting shaded image from artificial dataset 1 and artificial dataset 2. Next we introduce the implementation details of all the above-mentioned shading correction methods used in the experiment.

### 3.3.2 Implementation Details

For the shading correction methods that require parameter tuning, it is essential to find the appropriate parameter values of this method in order to obtain accurate correction results. As described in Table 3.1, the implementation details of the above-mentioned ten shading correction methods (Section 2.2.3) are summarized and compared regarding the tuning parameters, the application tools and the adopted image formation model. All of those algorithms are open source in Fiji [65], Matlab or MIPAV [48].

In the SF method, the shading field is obtained by fitting a parametric polynomial. Some methods such as “shading correction”, “polynomial_fit”, “fit_polynomial” and “nonuniform background removal” embedded in Fiji are widely used [37]. Here we choose the most accurate and least time-consuming algorithm “polynomial_fit” [3] to fit an Nth order Legendre

Figure 3.5: Empirical cumulative probability distribution of the normalized intensity from real datasets and artificial datasets: (a) artificial dataset 1 (red) and (b) artificial dataset 2 (green), indicating that they follow similar distributions.

The idea of the SE algorithm is to obtain the shading component through iterative stretching of a flexible surface until it satisfies the optimization criterion. This algorithm is implemented in Matlab, which requires the number of iterations $M$ and critical value $\delta$ for the op-
Table 3.1: Implementation details of shading correction methods

<table>
<thead>
<tr>
<th>Shading correction methods</th>
<th>Existing Parameters</th>
<th>Application tools</th>
<th>Alternative image model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface fitting (SFA)</td>
<td>( x\text{Order}, y\text{Order} )</td>
<td>“polynomial_fit” in Fiji</td>
<td>Additive</td>
</tr>
<tr>
<td>Rolling Ball (RB)</td>
<td>( R )</td>
<td>“Subtract Background” in Fiji</td>
<td>Additive</td>
</tr>
<tr>
<td>Signal Envelope (SE)</td>
<td>( M, \delta )</td>
<td>Matlab</td>
<td>Additive</td>
</tr>
<tr>
<td>Homomorphic filtering (HF)</td>
<td>( \gamma_H, \gamma_L, D_0, c )</td>
<td>“Homomorphic Filter” in Fiji</td>
<td>Multiplicative</td>
</tr>
<tr>
<td>Homomorphic filtering (GD)</td>
<td>( s, \alpha, D, M, c )</td>
<td>Matlab</td>
<td>Multiplicative</td>
</tr>
<tr>
<td>Entropy minimization (EM)</td>
<td>None</td>
<td>“Entropy Minimization” in Fiji</td>
<td>Multiplicative and Additive</td>
</tr>
<tr>
<td>Information minimization (IEM)</td>
<td>None</td>
<td>“Entropy Minimization” in MIPAV</td>
<td>Multiplicative or Multiplicative and Additive</td>
</tr>
<tr>
<td>Non-parametric non-uniform intensity normalization (N3)</td>
<td>( \theta, M, F, s, \beta, \phi, n )</td>
<td>“Inhomogeneity N3 Correction” in MIPAV</td>
<td>Multiplicative</td>
</tr>
<tr>
<td>N4ITK</td>
<td>( M, \beta, \phi, n, r, N )</td>
<td>“Inhomogeneity N4 Correction” in MIPAV</td>
<td>Multiplicative</td>
</tr>
<tr>
<td>CJV-based</td>
<td>( a_1...a_5, m_1...m_5 )</td>
<td>“CJV Minimization” in Fiji</td>
<td>Multiplicative or Additive or Multiplicative and Additive</td>
</tr>
</tbody>
</table>
Figure 3.6: One shading-free image (left), corresponding shading field (middle) and resulting shaded image (right) from (a) artificial dataset 1 and (b) artificial dataset 2, respectively. Here, the shaded image is approximately equal to the shading-free image multiplied by the shading field.

...
8-bit and 16-bit grayscale images. In the EM method, the correction is performed by minimizing the entropy after initializing five parameters $a_1 - a_5$ in the additive shading field and five parameters $m_1 - m_5$ in the multiplicative shading field. The former algorithm is implemented in Matlab and the latter in Fiji. The algorithm “Entropy Minimization” in the MIPAV software is a combination of IM and EM, which is renamed IEM here. No parameters need to be tuned for this algorithm except the selection of image models: multiplicative or additive and multiplicative.

Another algorithm in MIPAV is inhomogeneity N3 correction, an iterative approach that estimates the multiplicative shading field by setting seven parameters: 1) threshold $\theta$ used to differentiate background and foreground; 2) maximum number of iterations $M$; 3) convergence threshold $\beta$ between two iterations; 4) field distance $F$ over which the shading field varies; 5) subsampling factor $s$; 6) full width at half maximum of deconvolution kernel $\varphi$; 7) Wiener filter noise $n$. Next, N4ITK is publicly available through the Insight Toolkit of the National Institutes of Health and also built in MIPAV. Six parameters are required to be tuned: 1) maximum number of iterations $M$; 2) convergence threshold $\beta$ between two iterations; 3) full width at half maximum of deconvolution kernel $\varphi$; 4) Wiener filter noise $n$; 5) B-spline mesh resolution level $r$; 6) number of control points $N$ in B-spline.

In order to implement all the methods optimally for each dataset, we define the parameters by conducting extensive parameter tuning using grid search before evaluation. In Table 3.2, we determine the values of the tuning parameters for two artificial datasets and four real datasets. For the N3 method, the values for $\theta$ and $F$ are set automatically: minimum value $\text{Min}$ of current shaded image plus one and default. Both multiplicative and additive shading fields are considered for IEM algorithms, so we rename it IEMMA. In Table 3.2 symbol $-$ means that the parameters are fixed in the algorithms and do not need to be adjusted.

### 3.3.3 Performance Measure Decision

Due to the lack of ground truth for real datasets, we aim to determine the most appropriate reference-free based measure from artificial datasets and then apply this to the real datasets. Because of the segmentation-based performance measures, we need to conduct segmentation for each image from the real datasets, the results of which are further utilized for the segmentation-based shading correction method. Not every segmentation algorithm requires prior shading correction. Since we are interested in monitoring cell behaviour through the cell

Table 3.2: Summary of correction methods implemented and parameter values used for artificial and real datasets

<table>
<thead>
<tr>
<th>Shading correction methods</th>
<th>Tuning Parameters</th>
<th>Four real datasets</th>
<th>Two artificial datasets</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>xOrder, yOrder</td>
<td>2, 2</td>
<td>2, 2</td>
</tr>
<tr>
<td>RB</td>
<td>R</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>SE</td>
<td>R</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>HF</td>
<td>T, D, M</td>
<td>0.0001, 0.0001</td>
<td>0.0001, 0.0001</td>
</tr>
<tr>
<td>EM</td>
<td>None</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IEMMA</td>
<td>None</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>N3</td>
<td>M, F, E</td>
<td>θ</td>
<td>θ</td>
</tr>
<tr>
<td>N4</td>
<td>M, F, E</td>
<td>θ</td>
<td>θ</td>
</tr>
<tr>
<td>GYMA</td>
<td>None</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>EMMA</td>
<td>None</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Tuning Parameters: M, F, E, T, D, M, ϵ

Four real datasets: 20.0001, 0.15, 0.01, 3.4

Two artificial datasets: Min+1, 100.0001, Default, 2.5, 0.01
cycle in real datasets 1 and 2 only, we applied a single-particle tracking plugin called “TrackMate” (Section 2.3) in Fiji for these two datasets. TrackMate first detects cells, then segments cells and finally tracks cells. Generally, it works well for spot-like objects such as B lymphocytes. In practice, the Laplacian of Gaussian (LoG) detector [31] in this plugin is used for cell detection and segmentation, and thus we can achieve the segmentation results directly. The tuning parameters in the LoG detector are mainly the estimated cell diameter and the threshold for cell segmentation. In contrast, we applied a global histogram-derived thresholding method called "Auto Threshold" in Fiji for datasets 3 and 4 to segment cells of interest. In all cases, we manually validated that the segmentation results were of appropriate quality. For the artificial datasets, we could achieve the mask images directly during simulation since the ground truth is available. Our main aim is shading correction for the purposes of accurate measurement of cell fluorescence signals over time. Next, we conduct shading correction on two synthetic datasets including ten images each, and evaluate the quality using most proposed measures.

For each method, we calculate the mean value of each measure and then rank all the methods from the best to the worst. In Table 3.3, we show the rank of all the shading correction methods based on the values of the corresponding measures for artificial dataset 1 only. Rank “1” indicates the best quality among all, while “9” indicates the worst. This table shows that it is difficult to determine an optimal approach because the rank varies with performance measures. Here \( c \) and \( b \) refer to the measure from cell and background, respectively. Furthermore, \( \overline{CV}(\%) \) and \( \overline{\sigma}(\%) \) indicate the average of the relative change of coefficient variation (CV) and standard deviation (\( \sigma \)) from those two classes, respectively. The rank varies with measures, resulting in no consensus on which one should be used. Compared to reference-free measures such as RMSE and PSNR, the normalized \( L_2 \)-norm is resistant to scaling. Thus, we compare the consistency between other measures and \( L_2 \)-norm on artificial datasets using Kendall’s tau coefficient [35] and Spearman’s correlation coefficient, both of which reach relatively higher values in Table 3.4 and Table 3.5 for the two artificial datasets on average (0.639,0.808) when the CJV measure is used. This implies that the CJV based measure is strongly consistent with \( L_2 \)-norm.

### 3.3.4 Performance Evaluation Results

Quantitative evaluation is essential for the objective comparison of the results obtained by different shading correction methods. We conducted experiments on four real datasets by applying the above ten shading correction methods and perform evaluation based on the CJV
<table>
<thead>
<tr>
<th>Shading correction methods</th>
<th>( L_2 ) - norm</th>
<th>RMSE</th>
<th>PSNR</th>
<th>SNR</th>
<th>CV ( \tau )</th>
<th>CV ( \rho )</th>
<th>CV ( \phi )</th>
<th>CV ( \rho )</th>
<th>CV ( \phi )</th>
<th>CV ( \tau )</th>
<th>Number of wins</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RB</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>IEMMA</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>EM</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>CD</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>HF</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>SE</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>RB</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>SFA</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Number of wins</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
<td>( % )</td>
</tr>
</tbody>
</table>

Table 3.3: Shading correction results for artificial dataset 1.
<table>
<thead>
<tr>
<th></th>
<th>L2−norm</th>
<th>τ</th>
<th>τ</th>
<th>CV</th>
<th>CV</th>
<th>SNR</th>
<th>PSNR</th>
<th>RMSE</th>
<th>L2−norm</th>
<th>L2−norm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dataset 1</td>
<td>0.056</td>
<td>0.056</td>
<td>0.167</td>
<td>0.556</td>
<td>0.056</td>
<td>0.556</td>
<td>0.722</td>
<td>0.111</td>
<td>0.444</td>
<td>0.667</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>0.111</td>
<td>0.111</td>
<td>0.500</td>
<td>0.333</td>
<td>0.444</td>
<td>0.389</td>
<td>0.556</td>
<td>0.444</td>
<td>0.667</td>
<td>0.611</td>
</tr>
<tr>
<td>Mean values</td>
<td>0.084</td>
<td>0.084</td>
<td>0.334</td>
<td>0.445</td>
<td>0.250</td>
<td>0.473</td>
<td>0.639</td>
<td>0.278</td>
<td>0.556</td>
<td>0.639</td>
</tr>
</tbody>
</table>

Table 3.4: Kendall's tau coefficient between L2−norm and other measures for artificial datasets.
<table>
<thead>
<tr>
<th>Dataset</th>
<th>L2 norm</th>
<th>RMSE</th>
<th>PSNR</th>
<th>SNR</th>
<th>CV</th>
<th>σ</th>
<th>CV</th>
<th>σ</th>
<th>CIV</th>
<th>CIV (%)</th>
<th>CIV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.044</td>
<td>0.044</td>
<td>-0.400</td>
<td>0.467</td>
<td>0.317</td>
<td>-0.517</td>
<td>0.783</td>
<td>0.417</td>
<td>-0.767</td>
<td>0.808</td>
<td>0.808</td>
</tr>
<tr>
<td>2</td>
<td>0.083</td>
<td>0.083</td>
<td>-0.583</td>
<td>0.417</td>
<td>0.533</td>
<td>-0.517</td>
<td>0.683</td>
<td>0.583</td>
<td>-0.833</td>
<td>0.833</td>
<td>0.833</td>
</tr>
</tbody>
</table>

Table 3.5: Spearman's correlation between L2-norm and other measures for artificial datasets
value. We implement the CJVMA algorithm directly without manual tuning of parameters. Image noise is removed by “Gaussian Blur” in Fiji beforehand. In order to show whether the shading is efficiently removed, we apply the relative change of CJV to the comparative performance evaluation, which is defined as

$$CJV(\%) = \frac{CJV_o - CJV_c}{CJV_o} \times 100\%$$  \hspace{1cm} (3.7)

Here $CJV_o$ and $CJV_c$ refer to the CJV value of the original image and the corrected image, respectively. If one shading correction method succeeds in reducing shading, the relative change $CJV(\%)$ becomes positive. Hence, larger positive values imply better performance. The relative changes of CJV for the above-mentioned methods are given in the form of box-whiskers diagrams, illustrated in Figure 3.7. Each diagram shows the distribution of relative changes for 10 randomly selected real images. Since the CJVMA method aims to minimize the value of CJV directly, the relative change of CJV is the largest for these four real datasets. However, RB fails to correct real dataset 1 because the size of the object of interest is bigger than the background variation. EM and IEMMA do not work well for real dataset 1 in that a higher-order polynomial is required to estimate the shading field with a small local variation. Overall, we have the following conclusions:

1. The CJV measure can be generally applied in the performance evaluation on real data, where the ground truth is absent.

2. CJVMA performs best among all the existing shading correction methods for those four real datasets.

3. In general, CJVMA has a comparative advantage over all the abovementioned shading correction methods. The superiority lies in direct correction without parameter tuning, which saves time and manual work. However, the drawback is that segmentation is required beforehand.
Relative change of CJV for real dataset 1

(a)

Relative change of CJV for real dataset 2

(b)
Figure 3.7: Distribution of changes of CJV for real fluorescence images corrected by ten shading correction methods. Each boxplot shows the relative change of CJV for 10 real images. A larger value indicates higher performance of the method.
3.3.5 Discussion

In order to provide accurate measurements of the intensities of FUCCI reporters over the lifetime of each cell, we need to be able to compensate for errors in intensity measurements due to uneven illumination over the field of view. This is particularly important for time course measurements of individual cells, since each lymphocyte cell can typically move around within the field of view. Such movement can thus cause erroneous changes in the intensity measurements of the cell over time, and corrupt our ability to model the dynamics of the cell’s lifetime.

In the experiments in this chapter, we have quantitatively proven that the CJV measure can be generally used for quality evaluation of shading correction results on real images. To the best of our knowledge, we are the first example to determine this measure from all existing measures. Moreover, we have been able to demonstrate the advantages of the CJVMA correction method in terms of both accuracy, as well as the complexity of using this method in terms of the number of parameters that need to be adjusted. While CJVMA does not require any parameters to be tuned, we note that it does require a prior segmentation of the cells from the background and each other. A natural question to consider is whether this requirement for segmentation is reasonable in practice or not.

In the context of our overall goal of time-course modelling of lymphocytes, it is not unreasonable to make use of cell segmentation information, as this can be an important step that is needed for other relevant tasks, such as subsequent cell tracking for lineage tree construction. Consequently, the overhead of segmentation is not a major issue within this application context, but it needs to adapt to different shading levels across and within different videos.

An open question for future research is to consider how widely-applicable the results of this chapter might be to other types of live cell imaging domains. In particular, while our focus is on modelling B lymphocytes, the problem of measuring the intensity of FUCCI reporters also arises in the context of other cell types, such as cancer or embryonic stem cells. While such domains are outside the scope of this thesis, an interesting question for the future is to investigate whether or not the CJVMA approach will be similarly effective on other types of cells with different morphologies.

3.4 Conclusion

One of our aims in this thesis is to analyse cell behaviour over the cell cycle by extracting fluorescence time courses from time lapse microscopy. However, the imaging results can be
influenced by the inherent problem of acquisition effects such as cross-talk, autofluorescence, photobleaching and shading (Section 2.2). In order to achieve a correct interpretation of observed fluorescence levels, it is essential to address these effects before data analysis. We address the problem of cross-talk and autofluorescence in Chapter 4. In this chapter, our aim is to remove shading effects to avoid inaccurate intensity measurements for the subsequent quantitative analysis. Moreover, we deal with the problem of evaluation on real data without ground truth. In the aspect of performance evaluation, there is no consensus on which quantitative measure could be generally utilized in time lapse fluorescence images. In particular, some existing shading correction methods and performance evaluation strategies have been proposed for the MR imaging community, where the image formation model is different from microscopic images. One of our contributions is that we have identified that CJV is more reliable for lymphocyte microscopy images compared to the other measures. Hence, we have developed a novel shading correction method called CJVMA, which shows superior performance compared to the well-established methods for most datasets. Even though a prior segmentation is required, there is no need to adjust the parameters in this algorithm. Another contribution is that we have compared and evaluated state-of-the-art correction approaches on real datasets, and identified the most appropriate method for shading correction.
Chapter 4

Removal of Cross-talk Effects

In this chapter, our focus is to eliminate cross-talk effects in live cell fluorescence imaging and perform quality evaluation on real data in the absence of ground truth. In summary, our contributions are as follows: (1) We develop a spectral unmixing model based at the cell level by taking the effects of cross-talk and autofluorescence into consideration (Section 4.2.1). In order to obtain an accurate estimation of the mixing matrix in this model, we present four different strategies of training data selection (Section 4.2.3). (2) We propose a novel correlation-based evaluation approach by identifying an indirect ground truth dataset using what we call reference intervals, aiming to evaluate the quality of unmixing results in the absence of reference objects (Section 4.2.2). This evaluation method requires single cell segmentation and tracking, since the reference intervals are identified based on the mean intensity relationship of the cells. (3) We compare the unmixing results obtained by different spectral unmixing methods under different training datasets (Section 4.3).

4.1 Consequences of Cross-talk Effects

In the previous chapter, we considered the problem of illumination correction to address the effects of shading artefacts on the accuracy of time course fluorescence measurements. The second major image acquisition artefact that we consider is the effect of cross-talk. This artefact arises when multiple fluorescent channels are used (corresponding to multiple FUCCI reporters), and the emission spectra of the reporters overlap. In the context of single cell tracking and subsequent data analysis based on measurements of cell time-course intensities, cross-talk may cause an incorrect interpretation of the true signals. In Figure 4.1, we show the time course fluorescence intensity of the true signals S1 and S2. Since fluorescence time courses are indicative of cell events, in this example, we consider the problem of estimating the time of the onset of S1, which would be wrongly estimated to be $T_1'$ due to cross-talk. The data in this figure...
Figure 4.1: Motivating example - Time course fluorescence intensity of true signals S1 and S2, and estimated signal S1’. Times T1 and T1’ are the true onset of signal S1, and the estimated onset of signal S1, respectively.

is artificial but based on the real setup in practice. Hence, in order to construct meaningful models of cell cycle dynamics based on single-cell time-course intensity measurements, it is essential to first eliminate cross-talk effects by using the approach of spectral unmixing [36].

4.2 Spectral Unmixing Model and Evaluation Methodology

In this section, we first present a model of image acquisition in order to highlight the effects that need to be considered in the following analysis. We use this acquisition model to establish the spectral unmixing model based on the cell level. Next we introduce the correlation-based evaluation methodology for spectral unmixing. Finally, we show four approaches to selecting training data.

4.2.1 Model of Spectral Unmixing

Time-lapse fluorescence microscopy adopts a multi-channel imaging mechanism to record spatiotemporal molecular dynamics within cells. Due to uneven illumination, the luminance distribution becomes non-uniform within an image, and from image to image. Autofluorescence (AF), camera noise and constant offsets can further affect the acquisition of observed images. Hence, the process of image acquisition can be illustrated in Figure 4.2. The acquisition effects refer to AF, cross-talk, uneven illumination, camera noise and constant offsets. Furthermore, we adopt a common assumption that the effect of photobleaching is negligible compared to real fluorescence levels [52], [43]. Note that we consider fluorescence that has originated from
Figure 4.2: Diagram of the image acquisition process in time-lapse fluorescence microscopy. The red dotted line area indicates the relationship between mixed images and real images when only AF and cross-talk effects occur. $I_{af}$ refers to the intensity due to AF, $A$ indicates the mixing matrix, $L$ represents the shading effects, $\delta_n$ is the camera noise coefficient and $\delta_o$ refers to the constant offsets of the camera.

reporters, thus the real signal is not affected by photobleaching. Rather than recording two fluorophores simultaneously [6], the samples are excited with time-separated single wavelengths. Thus, we can acquire several images, each of which corresponds to a separate channel. In reality, each image is taken at a slightly different time, but we consider them to be of the same frame.

We focus on images from only two channels in the following analysis but our model can readily be generalized to large numbers of channels. Fluorescence multi-channel imaging enables us to acquire $T$ images with size $W \times H$ in each channel separately. Considering an image at frame $t$ in two channels, we can establish the relationship between real images and observed images by

$$I_{obs} = (AI_{real} + I_{af}) \odot L + \delta_n + \delta_o \quad (4.1)$$

where $I_{obs}$, $I_{real}$, $I_{af}$, $L$, $\delta_n$ and $\delta_o$ are real matrices with size $2 \times m$, and here $m \leq W \times H$. The notation $\odot$ refers to the point-wise multiplication. The rows of $I_{obs}$, $I_{real}$, and $I_{af}$ represent the pixel intensities for different channels. A 2D image is flattened into a row here. Furthermore, $A$ is a non-negative mixing matrix with size $2 \times 2$. The entries of $\delta_n$ indicate the camera noise at each pixel position. The matrix $\delta_o$ is composed of constant offsets. Here, we adopt a common assumption that uneven illumination and camera effects are addressed [60], thus $L = 1$, $\delta_n = 0$ and $\delta_o = 0$. So the above equation can be reformulated as

$$I_{mix} = AI_{real} + I_{af} \quad (4.2)$$

Here $I_{mix}$ and $I_{real}$ represent a real $2 \times m$ matrix whose entries are pixel intensities in mixed
and real images, respectively. $I_{af}$ denotes a real $2 \times m$ matrix whose entries are pixel intensities due to AF at each corresponding pixel location.

In the above model, the input is the pixel intensity. However, we aim to analyse the variation of the mean intensities for cells during their lifetimes, which implies that a *cell-level* spectral unmixing analysis can be applied. In particular, we want to obtain the spectral unmixing model when some *reference objects* can be estimated from the temporal fluorescence profiles of the cells. We now describe our approach to two subsequent types of analysis.

(1) **Cell-level Analysis**: We employ single cell segmentation and tracking for data analysis, thus generating the mean intensity of each cell. Therefore, we proceed with a cell-level model and relate it to the above pixel-level equations.

For one cell $i$ at frame $t$ in both the red and green channels, the mean intensity is defined by

$$I_{\text{real}}(i,t) = \frac{1}{M(i,t)} \sum_{(x,y) \in i} I_{\text{real}}(i,t)$$  \hspace{1cm} (4.3)

The entries of the $2 \times n$ matrix $I_{\text{real}}(i,t)$ are pixel intensities in the cell $i$ at frame $t$, where $n$ refers to the number of pixels. $I_{\text{real}}(i,t)$ is a $2 \times 1$ matrix, each element of which is the mean intensity for the cell in the corresponding channel. $M(i,t)$ represents the cell area, which is a constant for the cell $i$ but varies from cell to cell. By combining Equations 4.2 and 4.3, the mean intensity for cell $i$ in a real image can be given by

$$I_{\text{real}}(i,t) = \frac{1}{M(i,t)} \sum_{(x,y) \in i} A^{-1}(I_{\text{mix}}(i,t) - I_{af}(i,t))$$  \hspace{1cm} (4.4)

We assume that the mixing matrix is the same for all pixels in a cell at frame $t$, thus obtaining the expression

$$I_{\text{real}}(i,t) = A^{-1}(I_{\text{mix}}(i,t) - I_{af}(i,t))$$  \hspace{1cm} (4.5)

It is assumed that the mixing matrix $A$ is the same for cells from a single family (Section 4.3) at different frames and for a given cell over time, so we drop the indices

$$I_{\text{real}} = A^{-1}(I_{\text{mix}} - I_{af})$$  \hspace{1cm} (4.6)

Here $I_{\text{real}}, I_{\text{mix}}$ and $I_{af}$ are $2 \times p$ matrices, where $p$ is the number of measurements of mean intensities from different cells over their lifetimes. Hence, we can attain the same formula as
Equation 4.2 but on the level of mean intensities. More specifically, the mean intensities in channel $c_1$ and channel $c_2$ can be expressed by

$$
\begin{bmatrix}
I_{c_1}^{\text{mix}} \\
I_{c_2}^{\text{mix}}
\end{bmatrix} =
\begin{bmatrix}
a_{11} & a_{12} \\
a_{21} & a_{22}
\end{bmatrix} \times
\begin{bmatrix}
I_{c_1}^{\text{real}} \\
I_{c_2}^{\text{real}}
\end{bmatrix} +
\begin{bmatrix}
I_{c_1}^{\text{af}} \\
I_{c_2}^{\text{af}}
\end{bmatrix} \tag{4.7}
$$

Here $I_{c_1}^{\text{mix}}$, $I_{c_2}^{\text{mix}}$, $I_{c_1}^{\text{real}}$ and $I_{c_2}^{\text{real}}$ all refer to a real $1 \times p$ row vector whose entries are mean intensities in channel $c_1$ or channel $c_2$. The scalars $a_{11}$, $a_{12}$, $a_{21}$ and $a_{22}$ are called cross-talk coefficients specifying how the images $I_{c_1}^{\text{real}}$ and $I_{c_2}^{\text{real}}$ are combined to form the mixed images, each of which may contain some AF and some fluorescence of interest.

One of the previous works adopts the constraint that the columns of the mixing matrix add up to one \[60\]. In this case, those four coefficients can be reduced to two parameters. The authors of that work did not provide a justification for such a constraint, but we provide a justification as follows. Because the mean intensity is measured in arbitrary units, we can also convert the four coefficients to two by scaling the mean real intensities and the mean AF, thus obtaining the following expression

$$
\begin{bmatrix}
I_{c_1}^{\text{mix}} \\
I_{c_2}^{\text{mix}}
\end{bmatrix} =
\begin{bmatrix}
\alpha & 1 - \beta \\
1 - \alpha & \beta
\end{bmatrix} \times
\begin{bmatrix}
I_{c_1}^{\text{real},s} \\
I_{c_2}^{\text{real},s}
\end{bmatrix} +
\begin{bmatrix}
I_{c_1}^{\text{af},s} \\
I_{c_2}^{\text{af},s}
\end{bmatrix} \tag{4.8}
$$

where

$$
I_{c_1}^{\text{real},s} = (a_{11} + a_{21}) \times I_{c_1}^{\text{real}} \tag{4.9}
$$

$$
I_{c_2}^{\text{real},s} = (a_{12} + a_{22}) \times I_{c_2}^{\text{real}} \tag{4.10}
$$

We define $I_{c_1}^{\text{real},s}$, $I_{c_2}^{\text{real},s}$, $I_{c_1}^{\text{af},s}$, and $I_{c_2}^{\text{af},s}$ to be a real $1 \times p$ row vector, each entry of which is the scaled mean intensity from real images or AF images. The variables $\alpha$ and $\beta$ denote the nonnegative coefficients $\in [0, 1]$, expressed by $\alpha = \frac{a_{11}}{a_{11} + a_{21}}$ and $\beta = \frac{a_{22}}{a_{12} + a_{22}}$, respectively.

(2) Reference Objects Analysis: We define a cell as a reference object when its intensity in one channel equals zero but not in another channel. The reference object is characterized by $I_{c_1}^{\text{real}} = 0$ or $I_{c_2}^{\text{real}} = 0$. In this case, the scaled mean intensity in either channel equals zero.

When $I_{c_1}^{\text{real},s} = 0$, Equation 4.8 can be rewritten by

$$
I_{c_2}^{\text{mix}} = \frac{1 - \alpha}{\alpha} I_{c_1}^{\text{mix}} - \frac{1 - \alpha}{\alpha} I_{c_1}^{\text{af},s} + I_{c_2}^{\text{af},s} \tag{4.11}
$$

When $I_{c_2}^{\text{real},s} = 0$, we can reformulate Equation 4.8 as
\[ T_{mix1}^{1} = \frac{1 - \beta}{\beta} T_{mix2}^{2} - \frac{1 - \beta}{\beta} T_{af}^{2} + T_{af1}^{1} \]  

(4.12)

Both equations above show a linear relationship between \( T_{mix1}^{1} \) and \( T_{mix2}^{2} \). Thus, \( \alpha \) and \( \beta \) can be replaced by \( s_{1} \) and \( s_{2} \), where \( | s_{1} | = \frac{1 - \alpha}{\alpha} \) and \( | s_{2} | = \frac{1 - \beta}{\beta} \). The variables \( s_{1} \) and \( s_{2} \) represent the slope of the mean intensity relationship in the corresponding reference object. Therefore, the spectral unmixing model becomes

\[
\begin{bmatrix}
T_{mix1}^{1} \\
T_{mix2}^{2}
\end{bmatrix} =
\begin{bmatrix}
\frac{1}{1+|s_{1}|} & |s_{2}| \\
|s_{1}| & \frac{1}{1+|s_{2}|}
\end{bmatrix}
\times
\begin{bmatrix}
T_{real1}^{1} \\
T_{real2}^{2}
\end{bmatrix}
+ \begin{bmatrix}
I_{af1}^{1} \\
I_{af2}^{2}
\end{bmatrix}
\]  

(4.13)

Without knowing the mean AF, if the reference objects are available, then we can obtain the mixing matrix by estimating the slopes from two straight fitted lines. We define this approach of spectral unmixing as a *direct method*, which can be applied in the case of reference objects.

### 4.2.2 Correlation-based Evaluation Methodology

When no prior knowledge about the real images is known, there is no method available to evaluate the spectral unmixing results. Moreover, the scaled real images cannot be recovered based only on the known mixed images and the mixing matrix, as seen in Equations 4.8 and 4.13. However, if we know that the mean intensities in one channel are uncorrelated with those in the other (from the biology of the reporters), it is possible to estimate the quality of spectral unmixing. For example, when the estimated mixing matrix is \( B \), the estimated mean real images \( T_{real}^{'} \) can be expressed by \( T_{real}^{'} = B^{-1}T_{mix}^{'} + B^{-1}T_{af}^{'} \). It is noted that \( I_{af} \) is a random quantity, but we assume that \( I_{af} \) is constant. Thus, the estimated mean AF \( T_{af}^{'} \) is approximately constant. Then we can use what we call an *intermediate image* expressed by \( B^{-1}T_{mix}^{'} \) to represent \( T_{real}^{'} \), since the correlation will not be affected by \( T_{af}^{'} \). Therefore, we can obtain the uncorrelated components from the intermediate image.

As we discuss above, it is important to evaluate the spectral unmixing quality in the absence of reference objects. Thus, we identify a series of reference intervals for cells in the two channels based on the biological property of the FUCCI reporters [15], [64]. Provided that the spectral unmixing is performed effectively, the reference intervals have two key features: (1) there is little variation of the mean intensity in one of the channels; and (2) the mean intensity in one channel is not correlated with the corresponding mean intensity in the other channel.
The main procedure of quality evaluation is summarised as follows.

1) **Estimation of Mixing Matrix**: Before we perform spectral unmixing for the cells, it is necessary to select training data such as pixel-based intensity or cell-based intensity as input to estimate the mixing matrix. The more accurate the estimation is, the better the performance of cell-level spectral unmixing we can obtain. Hence, the selection of the training data becomes crucial.

2) **Cell-level Spectral Unmixing**: After the estimated mixing matrix is acquired, we apply it to cell-level spectral unmixing. Even though the scaled real images cannot be recovered, we can still obtain the intermediate images and thus estimate the quality of spectral unmixing.

3) **Correlation Test for Reference Intervals**: Once the mean intensities for cells in the intermediate images have been determined, we first calculate the Pearson’s correlation coefficient between two channels in each reference interval. Next we compute the P-value based on hypothesis testing on the correlation coefficient. If the P-value is less than 0.05, we decide that the mean intensity in one channel is correlated with that in the other channel. Using this correlation test on reference intervals, we can then evaluate each spectral unmixing method in terms of whether it minimises the number of correlated reference intervals.

### 4.2.3 Training Data Selection

Each spectral unmixing algorithm requires training data as input. One obvious strategy is to use all pixels from the video. However, we have acquired 1860 images with size 1388×1040 in each channel, which makes it time-consuming to select all pixels from all images as the training data. Therefore, we need to choose an appropriate strategy of training data selection.

In Figure 4.3, we show an example of a few clones: a set of cells that are descendants of the same starting cell. Prior to imaging, cells are randomly allocated to physically separated compartments called wells [13] at low density, resulting in many wells starting with a single cell. Therefore, we can use wells to visually identify clones. From this figure, we consider two sources of training data: pixel-based intensity and cell-based intensity, which can be selected either across the image or inside the segmented cells. Moreover, it is worth considering whether we could apply the training data to all the clones, which implies the mixing matrix for each clone is identical.

In the presence of the same mixing matrix for all clones then spectral unmixing can be performed for all the clones. This is called *all-clones unmixing*. If the mixing matrix is differ-
ent among the clones, we need to do spectral unmixing for each separate clone by selecting training data from that well of the clone. Thus, we define this situation as separate-clone unmixing. By considering pixel-based and cell-based intensity as the training data, four scenarios of training data selection are considered as listed below.

1) **Random Pixels**: In this case, pixel intensities are the source of the training data. For all-clones unmixing, we select pixels randomly within an image from random frames. However, for separate-clone unmixing, pixels are selected in the well belonging to that clone from random frames. This method is the simplest one, which requires the least effort.

2) **Segmented Cells**: Pixel intensities and mean intensities are both considered as the training data. In the case of all-clones unmixing, pixel intensities or mean intensities are randomly chosen from all the segmented cells from random frames. In contrast, pixel intensities or mean intensities are selected from the segmented cells belonging to that clone when using separate-clone unmixing. However, this method requires automatic cell segmentation in advance.

3) **Estimated Reference Objects**: We assume that it is possible to identify reference objects from cell morphology and general behaviours [15]. In this case, we can select pixel intensities or mean intensities as the training data. Considering all-clones unmixing, we estimate the reference objects by inspecting the video. After several repeated searches, the pixel intensities inside the reference objects or their mean intensities are selected as the training data. The difference for separate-clone unmixing is that we only estimate the reference objects in each separate clone. Overall, this approach does not require automatic cell segmentation but
requires manual effort.

4) Reference Intervals: We aim to identify the reference intervals where the mean intensities in different channels are expected to be uncorrelated. Here “expected” means that “expected based on our knowledge of the biology of this experimental system”. In practice, the mean intensities in this interval are likely to be correlated. We aim to make them uncorrelated after spectral unmixing. In other words, a reference interval can be either a correlated or an uncorrelated interval. The training data can be either the pixel intensities inside the cells or the mean intensities of the cells in the identified reference intervals. Using all-clones unmixing, the training data is randomly selected from all the clones. For separate-clone unmixing, only the cells from that well are considered. This method involves not only automatic cell segmentation but also cell tracking. Furthermore, some manual correction for segmentation and tracking are needed.

The requirements for those methods and the source of training data are summarised in Table 4.1. Since we have performed segmentation and tracking for the cells in advance, we adopt different spectral unmixing methods to compare the unmixing results. Our aim is to determine whether there is any difference in the unmixing results when different training datasets are selected. In particular, the direct method uses the estimated reference objects only to select pixel intensity or mean intensity for spectral unmixing.

Table 4.1: Requirements for different methods and the source of training data

<table>
<thead>
<tr>
<th>Methods of training data selection</th>
<th>Requirements</th>
<th>Source of training data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random pixels</td>
<td>No effort</td>
<td>Pixel intensity</td>
</tr>
<tr>
<td>Segmented cells</td>
<td>Automatic cell segmentation</td>
<td>Pixel intensity or mean intensity</td>
</tr>
<tr>
<td>Estimated reference objects</td>
<td>Manual effort to identify reference objects</td>
<td>Pixel intensity or mean intensity</td>
</tr>
<tr>
<td>Reference intervals</td>
<td>Automatic cell segmentation and tracking plus manual correction</td>
<td>Pixel intensity or mean intensity</td>
</tr>
</tbody>
</table>
4.3 Evaluation Results and Discussion

In this section, we first introduce how we perform image acquisition and preprocessing. We then describe how to identify the reference intervals for each separate clone. Next we describe the choice of spectral unmixing method and the source selection of the training data. Finally, the unmixing results under different training datasets and unmixing methods are compared using the correlation-based evaluation approach.

4.3.1 Image Preprocessing

In this study, we use a Fluorescence Carl Zeiss Microscope to image B lymphocytes stimulated with CpG [15]. The images were taken at 3 minute intervals. Each time point is also called a frame. We have obtained 1860 1388×1040 images in each channel, where 1860 refers to the total number of frames. Four images are acquired in four different channels: FUCCI red, FUCCI green, transmission in focus, and transmission out of focus. In the following spectral unmixing analysis, we focus on the images from the FUCCI red and FUCCI green channels.

All images are first corrected for field of view jittering using rigid image registration in the Fiji software package [65]. Next we do segmentation and tracking for cells after the images are smoothed with a rolling ball algorithm and Gaussian filter using the LineageTracker plugin [16]. Hence, we can obtain the outlines of the segmented cells. However, some manual correction is required when some cell outlines are wrongly detected. After correction, we should add the corrected cell to the link and perform automatic tracking again. In this case, we can obtain the accurate mean intensities for all the segmented cells.

To the best of our knowledge, there is no consensus regarding which method to use for illumination correction. Rolling ball filtering is one such method [2], and we adopt it here because it is readily implemented and requires only one parameter. Note that it is a heuristic method, and is not expected to perfectly correct uneven illumination effects. We have manually inspected selected images after filtering, and found the correction satisfactory.

4.3.2 Reference Intervals Identification

In order to evaluate the quality of spectral unmixing, we first identify a series of reference intervals based on the biology of the FUCCI reporters [15], [64]. As is shown in Figure 4.4, the Pearson’s correlation coefficients in (a) the red reference interval and (b) the green reference
Figure 4.4: Schematic of identified reference intervals for two different B cells in mixed images: (a) red reference interval; (b) green reference interval. A frame refers to the sequence number of the current image in a video. During those intervals, the mean real red intensities are expected to be uncorrelated with the mean real green intensities. The mean intensities are measured from the segmented cells.
interval are 0.1917 and 0.6208 respectively, which implies that the mean mixed intensities in the red and green channels are correlated. However, for real images, the mean intensities in those two channels are expected to be uncorrelated. Moreover, in the red reference interval, the mean real intensity is almost invariant in the red channel but varying in the green channel. Similarly, in the green reference interval, the mean real intensity varies little in the green channel but a lot in the red channel.

After inspecting the mean intensity relationship for the cells, we identify reference intervals for each clone. A correlated reference interval is an interval where the correlation between the mean intensities is statistically significant (P-value < 0.05), while the correlation coefficient is expected to be zero for an uncorrelated interval. In total, there are 26 identified reference intervals, half of which are red reference intervals and the other half are green reference intervals. In Table 4.2, we can see that the number of reference intervals varies between clones. Our theory and approach is general for multi-colour reporters, but the evaluation is performed using a FUCCI experiment as an example. An optimal spectral unmixing algorithm aims to minimize the number of reference intervals with statistically significant correlation coefficients.

Table 4.2: Identified reference intervals for each clone

<table>
<thead>
<tr>
<th>Clone</th>
<th>Red Channel</th>
<th>Green Channel</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>A5</td>
<td>6</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>A6</td>
<td>5</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>A3, A5 and A6</td>
<td>13</td>
<td>13</td>
<td>26</td>
</tr>
</tbody>
</table>

### 4.3.3 Spectral Unmixing Scheme

From the various spectral unmixing algorithms, we choose the multiplicative NMF algorithm with regularization and sparsity constraints (NMF_RS) [28] and automatic unmixing (AU, see Section 2.2.4) to do spectral unmixing, since the former is a popular method and the latter is the simplest technique. PCA and SVD have poor performance of spectral unmixing in our application and their results are not presented here. Note that we use the linear mixing model of AU to solve the equations rather than performing a simple subtraction [45]. Since the mean
intensities for the cells are obtained, we can enumerate all possible combinations of $\alpha$ and $\beta$ to find the best values of those two coefficients that minimize the number of reference intervals with non-zero correlation after spectral unmixing. This method is called *brute force*, which can obtain the globally optimal solution. Additionally, we adopt a direct method (in Reference Objects Analysis of Section 4.2.1) to perform spectral unmixing when the training data is selected from the estimated reference objects. In conclusion, the spectral unmixing results for the direct method, NMF$_{RS}$, AU and brute force method are compared based on different training data selection strategies.

Before spectral unmixing, we need to see whether the mixing matrix is the same for all clones. In order to perform this check, we obtain the relationship between the mean red intensity and the mean green intensity in the identified reference intervals. Three cases of the mean intensity relation are considered, as seen in (a), (b) and (c) of Figure 4.5. It is clearly shown that in all cases the strength of the linear association for Clones A3, A5 and A6 is different, which suggests that we should not use a single mixing matrix to do spectral unmixing.

In order to further demonstrate that spectral unmixing should be performed for each separate clone, we use the brute force method to obtain the minimum number of correlated reference intervals, as seen in Table 4.3. There are five correlated reference intervals after spectral unmixing based on the same mixing matrix for all clones, while in total there is only one correlated interval when we adopt a separate mixing matrix for Clones A3, A5 and A6. Since the minimum number of reference intervals with non-zero correlation is much higher when a single mixing matrix is used for all clones, we conclude that we should use a separate mixing matrix for each clone, showing that separate-clone unmixing is required.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Correlated red reference intervals</th>
<th>Correlated green reference intervals</th>
<th>Total correlated reference intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>A5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>All clones</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 4.3: Minimum number of correlated reference intervals by applying brute force method to separate clones and all clones
Figure 4.5: Relationship between the mean red intensity and the mean green intensity in red and green reference intervals for Clones A3, A5 and A6 before spectral unmixing. From top to bottom: (a) mean intensity from red reference intervals only; (b) mean intensity from green reference intervals only; (c) mean intensity from both red and green reference intervals.
4.3.4 Experimental Results

In the following analysis, we perform spectral unmixing based on each separate clone. We then sum up the number of reference intervals with non-zero correlation from each clone to obtain the total number of correlated reference intervals as the basis for the unmixing quality comparison. In addition, we compare all the unmixing results with the baseline, which refers to the number of correlated reference intervals for all the clones before spectral unmixing. In order to know whether we should select pixel intensity or mean intensity as the training data, we carry out the experiments for both cases. Since the training data is selected randomly, we report the number of correlated reference intervals using 95% confidence intervals after performing 20 experiments, as seen in Table 4.4 and Table 4.5. In addition, we also tried thresholded selection where a threshold is applied in all strategies to select the brightest pixels, but the results were not good and not shown here.

In Table 4.4 and Table 4.5, an entry marked by a − shows that this strategy for training data selection is not applicable. The direct method can only be applied on the estimated reference objects. Note that the brute force method is performed on the mean intensity but is shown in both tables as a basis for comparison. After comparing the spectral unmixing results, we can make the following conclusions:

Table 4.4: The number of correlated reference intervals (P-value <0.05) obtained by selecting pixel intensity as the training data under different spectral unmixing methods

<table>
<thead>
<tr>
<th>Spectral unmixing methods</th>
<th>Random pixels</th>
<th>Segmented cells</th>
<th>Estimated reference objects</th>
<th>Reference intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base line</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Direct method</td>
<td>−</td>
<td>−</td>
<td>7.2±0.4</td>
<td>−</td>
</tr>
<tr>
<td>NMF_RS</td>
<td>13±0.3</td>
<td>19±0.0</td>
<td>18±0.0</td>
<td>18.8±0.2</td>
</tr>
<tr>
<td>AU</td>
<td>11.2±0.2</td>
<td>11.9±0.4</td>
<td>10.5±0.3</td>
<td>10.2±0.3</td>
</tr>
<tr>
<td>Brute force</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>1</td>
</tr>
</tbody>
</table>

1. The number of reference intervals with non-zero correlation can be reduced from the
Table 4.5: The number of correlated reference intervals (P-value <0.05) obtained by selecting mean intensity as the training data under different spectral unmixing methods

<table>
<thead>
<tr>
<th>Spectral unmixing methods</th>
<th>Mean intensity selection strategies</th>
<th>Reference intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Random pixels</td>
<td>Segmented cells</td>
</tr>
<tr>
<td>Base line</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Direct method</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NMF_RS</td>
<td>–</td>
<td>20±0.0</td>
</tr>
<tr>
<td>AU</td>
<td>–</td>
<td>6.0±0.6</td>
</tr>
<tr>
<td>Brute force</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

1. base line 10 to 1 by using the brute force method.

2. We should not choose random pixels as the training data, since the number of correlated reference intervals are all above the baseline for NMF_RS and AU, showing the poor performance of spectral unmixing based on random pixel intensities.

3. In the case of pixel intensities and mean intensities, NMF_RS performs poorly for all the strategies of training data selection because the number of correlated reference intervals are all greater than 10.

4. Among all the strategies of training data selection, AU performs better in the case of mean intensities than pixel intensities, since the number of correlated reference intervals in the former are around 6 but 11 for the latter.

5. When the pixel intensities or the mean intensities are chosen from estimated reference objects, the number of correlated reference intervals obtained by the direct method are both around 7, showing a slight decrease from the base line but not much difference in the case of pixel intensities and mean intensities.
4.3.5 Discussion

While the problem of cross-talk between different channels in fluorescence microscopy is well-established, the problem of finding accurate and scalable methods for spectral unmixing in time-lapse fluorescence microscopy has not been widely studied. A major reason for this is that the interest in accurate modelling of the dynamics of fluorescent markers over the cell cycle for large numbers of cells is relatively new, due to the availability of video microscopy platforms that are capable of supporting large scale experiments. In the context of modelling the dynamics of fluorescent markers in individual cells, small errors in intensity arising due to cross-talk can have a significant impact on the accuracy of the fitted model parameters, and the consequent accuracy of the estimates of the duration of the difficult problems of the cell cycle.

In the experiments, we have made comparisons of the unmixing results obtained by the direct method, NMF_RS, AU and brute force. From our evaluation results we can conclude that: (1) We should use separate mixing matrices for separate clones to perform spectral unmixing. At present, it is not clear whether this is a result of a biological variation or spatial localization within the frame. Regardless of the reason, we find it beneficial to use a separate mixing matrix for each clone. (2) Our results suggest that utilizing reference intervals leads to a significantly improved unmixing quality. However, it requires automatic segmentation and tracking for cells, which are not always required for single cell data analysis, since manual tracking is sometimes sufficient [15].

4.4 Conclusion

In this chapter, we have presented a novel a spectral unmixing model that takes AF and cross-talk into consideration in time-lapse fluorescence microscopy. By building this model, we utilize training data to estimate the mixing matrix, which is then applied to the cell level. In addition, four different strategies of training data selection are explicitly illustrated. One novelty of our study is that we have proposed a correlation-based approach to evaluate the spectral unmixing results when reference objects are not available. The other novelty is that we adopt a linear mixing model to perform spectral unmixing on the level of cells rather than pixels. In particular, we have proposed that by using spectral mixing metrics for each separate clone we can achieve the best spectral unmixing results. Moreover, we have shown that by introducing the concept of reference intervals (i.e., the intervals in the cell cycle when the cell
average intensities in the red and green channels are expected to be uncorrelated), we can achieve highly accurate spectral unmixing results when no reference objects are available in the videos.
Chapter 5
Modelling Single-cell Fluorescence Time Courses

In this chapter, our aim is to quantitatively analyse cell behaviour over the cell cycle by modelling the single-cell fluorescence levels measured from the corrected image, in which all major image acquisition effects have been removed. In order to achieve our goal, we have addressed two major challenges in analysing such data: (1) we have developed a computational pipeline for correcting image acquisition effects (Chapter 3, Chapter 4, and Section 5.2.1), and (2) we propose a method for automatic feature extraction from tracked fluorescence profiles derived from a piecewise exponential model that characterises the single-cell fluorescence levels. Based on data that we can derive from multiple time-lapse videos, we demonstrate that our analysis pipeline is able to provide the basis for a statistical analysis to address several key questions about the biological processes associated with the lymphocyte cell cycle.

5.1 Studies of Cell Kinetics

Understanding mechanisms of lymphocyte response regulation is a major challenge in immunology, as elucidating this fundamental biological phenomenon will facilitate progress in developing strategies against auto-immune diseases and immunodeficiency conditions, as well as developing cancer immunotherapies. It has previously been demonstrated that dissecting the rules of response regulation in T and B cells in a controlled in vitro environment can lead to the development of mathematical models capable of accurate predictions in vivo \cite{70, 23, 24, 87, 15}. Long term live cell imaging coupled with single cell tracking enables a rich quantification of the response progression, especially when used in conjunction with fluorescence reporters. Hence, cell cycle kinetics can be visualized using FUCCI reporters.

Live cells are fluorescent in the red channel mostly during the $G_0/G_1$ phases and fluo-
cent in the green channel from the beginning of the S-phase. Here, we study the kinetics of B cell response in vitro using live cell imaging of FUCCI cells, and semi-automated cell tracking. The main question that we ask is how much more underlying complexity will be revealed by introducing more detailed measurements. In our case, the measurements comprise reconstructed lineage trees together with fluorescence levels for individual cells. We have collected data with FUCCI kinetics for entire lineage trees, as opposed to cycling cells as done previously [23, 24, 15]. In particular, we are able to dissect the kinetics of the progenitor cells early after activation. Moreover, we are able to include single cell fluorescence kinetics as measurable variables.

Generally, it takes a longer time for the progenitor cells to divide than the other dividing cells in subsequent division rounds. In this chapter, we address the hypothesis that extended division time is largely due to the activation stage leading to the first division, rather than the extended cell cycle. In order to test this hypothesis, we have analysed data from a standard lymphocyte proliferation assay complemented with cell cycle reporters. Moreover, we use computational acquisition correction methods and mathematical models contributed by this thesis. In the next section, we present details of our methodology. This is followed by results and discussion in Section 5.3.

5.2 Methods

5.2.1 Image processing methods

To measure the single-cell fluorescence levels, we first perform segmentation of the images in the transmission out of focus channel. Given the resulting cell boundaries, we track all image sequences in FUCCI red, FUCCI green and transmission channels simultaneously, by means of multi-channel TrackMate in Fiji. However, the acquisition effects such as photobleaching, shading and cross-talk lead to inaccurate measurement of cell fluorescence levels. In an image sequence, we assume that photobleaching is time-dependent only, shading relies on both time and position, and cross-talk is independent. Hence, we take advantage of a well-established frame-wise bleaching correction plugin called “Bleach Correction” in Fiji to eliminate the photobleaching effect first. As is discussed in Section 2.2.2, our goal is to fit an exponential model based on the time-course average fluorescence levels and then recover the bleaching-
corrected values. The simplex algorithm [56] is used for curve fitting and the relevant parameters are initial average intensities $I_0$, decay rate $a$ and background fluorescence level $b$, which are initialized to be $I_{last} - I_0, -10^{-4}$ and $I_{last}$, respectively. Here $I_0$ or $I_{last}$ represents the average fluorescence level of the initial image or the last image.

In terms of processing speed and efficiency, we adopt a signal envelope (SE) algorithm to remove the shading effect [62]. The parameters to be optimized in this algorithm have been explicitly described in Section 3.3.2. Based on the corrected fluorescence time courses of one clone, we define some stable reference intervals where the red fluorescence and green fluorescence are supposed to be uncorrelated, as explicitly described in Chapter 4. To solve the problem of cross-talk we use our proposed spectral unmixing method, which aims to minimize the number of uncorrelated intervals in the final fluorescence temporal profiles with all effects removed.

5.2.2 Modelling single-cell fluorescence time courses

Features such as the duration of cell cycle phases and maximum fluorescence levels have been commonly used to study the kinetic variation in cell cycle time. Nonetheless, the duration of these phases was obtained by direct observation in the previous studies [15, 23], leading to cumbersome manual work and inaccurate measurement. Moreover, it is improbable to measure the maximum fluorescence values by manual tracking. Therefore, we attempt to model the single-cell temporal profiles and thereby extract these features automatically in both FUCCI red and FUCCI green channels. As is described below, we adopt a two-term sigmoid function and a four piecewise exponential function to express red and green fluorescence time courses, respectively.

We have assessed several variations of phenomenological models capable of fitting to our data, and for the analysis presented here, we used the model that resulted in the smallest Akaike Information Criterion (AIC) [29]. Considering the bell-shaped relationships for dividing lymphocytes in FUCCI red channel, we employ a two-term sigmoid function, a widely used model for complex dose-response curves [53], to simulate cell behaviour through the cell cycle as

$$y(t) = \text{top} + \frac{\text{bottom}1 - \text{top}}{1 + \left(\frac{x(t)}{\text{midPoint}1}\right)^\text{slope1}} + \frac{\text{bottom}2 - \text{top}}{1 + \left(\frac{x(t)}{\text{midPoint}2}\right)^\text{slope2}}$$

(5.1)
In the case of progenitor cells, this model can be simplified as a four-parameter equation

$$y(t) = \text{top} + \frac{\text{bottom2} - \text{top}}{1 + (\frac{\text{midPnt}}{\text{slope2}})^2}$$  \hspace{1cm} (5.2)$$

where \text{top} refers to the maximum cell intensity, while \text{bottom1/2}, \text{midPnt1/2} and \text{slope1/2} indicate the minimum cell intensity, the time point corresponding to half of maximum and minimum values, and the slope for curve on the left/right, respectively. In contrast, we employ a four piecewise exponential function to model single-cell fluorescence levels in FUCCI green channel. To maintain continuity between adjacent curves, this model can be expressed as

\[
y(t) = \begin{cases} 
  a_0 & t_0 \leq t < t_1 \\
  a_0 \cdot e^{a_1 \cdot (t-t_1)} & t_1 \leq t < t_2 \\
  a_0 \cdot e^{a_1 \cdot (t-t_1) + a_2 \cdot (t-t_2)} & t_2 \leq t < t_3 \\
  a_0 \cdot e^{a_1 \cdot (t-t_1) + a_2 \cdot (t-t_2) + a_3 \cdot (t-t_3)} & t_3 \leq t \leq t_{\text{max}} 
\end{cases}
\]  \hspace{1cm} (5.3)$$

where \(t_0\) and \(t_{\text{max}}\) refer to the start time and end time of one dividing cell, respectively. Here \(t_1 - t_3\) indicate the turning points of these four piecewise curves. This model can be used to automatically quantify the transitions of the cell cycle phases. Most of our conclusions are based on the model in FUCCI green channel.

To facilitate curve fitting in the FUCCI green channel, we first convert the piecewise exponential functions in Equation 5.3 into linear ones using log transformations. Then we fit the piecewise linear models to the log transformed data. In contrast, we fit the observed data in the FUCCI red channel directly. In both cases, the objective functions can be formulated as

\[
f_{\text{red}} = \sum_{t=t_0}^{t_{\text{max}}} [I(t) - y(t)]^2 \hspace{1cm} \hspace{1cm} (5.4)\]

\[
f_{\text{grn}} = \sum_{t=t_0}^{t_{\text{max}}} [\log(I(t)) - \log(y(t))]^2 \hspace{1cm} \hspace{1cm} (5.5)\]

where \(I(t)\) refers to the observed data, and \(f_{\text{red}}\) or \(f_{\text{grn}}\) represents the sum of squared errors in the FUCCI red or green channel. We aim to search for the optimal fitting parameters by minimizing \(f_{\text{red}}\) or \(f_{\text{grn}}\). A nonlinear least squares algorithm called Levenberg-Marquardt [47] is used for curve fitting in the FUCCI red channel, while the linear least squares method [38] is adopted for linear model fitting in the FUCCI green channel. Thus, we can obtain the fitted curves for progenitor cells and blasting cells in Figure 5.1.
Figure 5.1: Modelling fluorescence time courses of progenitor cells and blasting cells in: (a)-(b) FUCCI red channel. (c)-(d) FUCCI green channel. The blue solid line represents the fitted data, whereas the dashed line refers to 95% confidence intervals.

The individual phases of single-cell division cycle could be automatically quantified based on FUCCI green fluorescence, which becomes more reliable than red fluorescence with noisier measurements [15]. The time between the beginning and turning point, where green fluorescence starts to increase, is indicative of the time in $G_0/G_1$ for progenitor cells but $G_1$ for blasting cells, while the remainder of the cell cycle time represents $S/G_2/M$. Here the maximum fluorescence level in the fitted model is denoted by $I_{max}$, and the time before reaching this maximum value is defined as $T_m$. The relationship between these features is further investigated in the following analysis.
5.2.3 Statistical similarity test for progenitor cells and blasting cells

In general, the progenitor cell takes longer time to enter $S/G_2/M$ phases than the blasting cell, which refers to the dividing cell in subsequent division rounds rather than the first round. One assumption is that the progenitor cell will not follow regular division cycle until activated in the $G_0/G_1$ phases. The key biological question that we aim to investigate based on our single-cell time course model is the whether the progenitor cells behave similarly to the other dividing cells in subsequent division rounds through the cell cycle. In this case, we take advantage of the fitted parameters from the green fluorescence model to compare the similarity between two cell groups: progenitor cells and selected blasting cells. The process of our data analysis is presented in Figure 5.2. Considering data from the $S$-phase onwards only, we first extract five relevant parameters: $a_1, a_2, a_3, t_2 - t_1$ and $t_3 - t_1$, and then accumulate them to form a $N \times 5$ array, which is finally reduced to two-dimensional data using PCA. Given the number of progenitor cells tracked (e.g., 15), we randomly select 15 blasting cells across all possible generations. Each of the blasting cells came from one of the corresponding sibling cells. Last we perform bivariate Kolmogorov-Smirnov statistical test for two-dimensional data from two cell types. A $P$-Value larger than 0.05 implies that the behaviour of progenitor cells and blasting cells are likely to be similar through the cell cycle. In the following section, we present the results of this statistical analysis of the feature data acquired from a large set of time-lapse microscopy videos, using the image processing and time course modelling pipeline we have described.

![Diagram](image)

Figure 5.2: Statistical test for similarity between progenitor cells and blasting cells.
5.3 Results and Discussion

In this section, we show how our mathematical models for corrected individual cell fluorescence profiles have been applied to a large set of multi-channel time-lapse fluorescence microscopy videos, and how the data collected can be used to answer a range of statistical questions concerning the cell cycle and proliferation of B lymphocytes.

We begin by describing our methodology for fluorescence time course acquisition for a population of responding cells (Section 5.3.1). Then we present the results of fitting single-cell fluorescence time course models to progenitor cells and blasting cells, in order to identify the main features that quantify the timing of the $G_0/G_1$ phases and the $S/G_2/M$ phases of each cell (Section 5.3.3). Moreover, we investigate the presence of each type of cell (i.e., progenitor, blasting and dying cells) in our data extracted from the fluorescence time courses (Section 5.3.2).

Based on the data extracted in Section 5.3.3, we then proceed to answer the following statistical questions concerning the behaviour of the cell cycle across various cell types and generations. These questions are motivated by the findings of earlier studies of lymphocyte kinetics, such as [15, 23]. Specifically, we address the following questions:

1. Do progenitor cells follow the same division mechanism as blasting cells, regardless of a longer time spent before entering the $S$ phase? (Section 5.3.3)

2. Does activation time affect cell lifespan in a cell lineage tree? (Section 5.3.3)

3. Is there a relationship between each of the cell cycle phases and the total division time? (Section 5.3.3)

4. Is there a relationship between the durations of the $S/G_2/M$ phases in the progenitor cells and their dividing daughter cells (also called blasting cells) in the first generation? (Section 5.3.3)

5. Is there a correlation between the maximum green fluorescence level and the time taken to reach this maximum level in dividing cells? (Section 5.3.4)

6. Are cell cycle phases correlated between sibling cells? (Section 5.3.5)
5.3.1 Fluorescence time courses acquisition

To understand proliferating lymphocyte dynamics we directly observe B cells isolated from FUCCI reporter mice over time. CpG-stimulated B lymphocytes were placed in physically separated compartments called wells at low density and continuously imaged for several days [14]. This type of stimulation enables us to track the major features of programmed immune response [15]. Because of the multi-channel imaging mechanism in time-lapse microscopy, we acquire image sequences in bright field, FUCCI red and FUCCI green channels, as seen in Figure 5.3(a).

![Figure 5.3: FUCCI reporter fluorescence time courses in proliferating lymphocytes and schematic of quantitative modelling and analysis in cell cycle time. (a) Time-lapse imaging of CpG-stimulated lymphocytes dividing twice. (b) Image processing pipeline of the acquired images (see the details in Results). (c) Detected fluorescence time courses in FUCCI red and green channels after image correction, and cell lineage relations for one clone, represented as a set of linked nodes. (d) Modelling single-cell fluorescence red/green profiles of progenitor cell (Cell 0) and blasts in generation 1 (Cell 2). The duration of both $G_1$ and $S/G_2/M$ phases can be automatically measured using the model in FUCCI green channel. Images courtesy of Hodgkin Lab, WEHI.](image)

In an attempt to record spatio-temporal molecular dynamics within cells, we use TrackMate in Fiji to conduct segmentation and tracking on these image sequences. Thus, we can obtain
the fluorescence time courses of all cells from each recorded video directly, as seen in Figure 5.3(c). At the same time, the cell lineage tree can be reconstructed, as shown in Figure 5.4.

Figure 5.4: Reconstructed cell lineage tree for a tracked movie. We mark all the dividing cells in orange, the dying cells in red and the cells with lost tracks in blue. This lineage tree shows that there are two progenitor cells at the start of time-lapse imaging, which are allocated in two different wells called A3 and B3.

However, due to the effects of photobleaching, shading and cross-talk in the process of image acquisition, the measurement of single-cell features such as average intensity and total intensity could be skewed \[^{79,6}\]. This will adversely affect the accuracy of single-cell temporal profile modelling. Consequently, we remove these effects beforehand using the proposed image processing pipeline, as described in Figure 5.3(b). More specifically, we employ a built-in plugin called “Bleach Correction” in Fiji \[^{65}\] to correct the effect of photobleaching.
In Figure 5.5, we show the bleaching correction results in FUCCI red and FUCCI green channels. By comparison, we conclude that the fluorescence images in the FUCCI green channel are more severely bleached than the fluorescence images in the red channel, and thus more closely tend to follow an exponential function. After bleaching correction, the fluorescence levels do not decay over time any more, implying a sound correction result.

![Bleaching Correction Diagrams](image)

Figure 5.5: Comparison of bleached and unbleached fluorescence levels in (a-b) FUCCI red channel and (c-d) FUCCI green channel.

Meanwhile, we use the proposed approaches in the previous Chapters 3 and 4 to remove the other two effects, i.e., shading and cross-talk. The shading correction results are shown in
Figure 5.6: We found that the corrected images are uniformly illuminated and the profiles of the corrected image do not vary with pixel location. This indicates that the observed image is approximately corrected. The spectral unmixing results can be seen in Section 2.2.4.

![Original Image](image1.png) ![Original Profile](image2.png)

![Corrected Image](image3.png) ![Corrected Profile](image4.png)

Figure 5.6: Comparison of (a) original and (c) shading-corrected images. The corresponding image profiles are shown in (b) and (d).

After removing all such image acquisition results, we can obtain the corrected single-cell fluorescence, as shown in Figure 5.7. By modelling the corrected single-cell fluorescence time courses as shown in Figure 5.3(d), we are able to quantify the transitions of cell cycle phases and further study the kinetics of B lymphocytes through the cell cycle. Overall we tracked 11 videos (around 20,460 frames) with 66 dividing cells and 63 dying cells from 15 lineage trees, corresponding to 15 progenitor cells.
5.3.2 Patterns in dividing lymphocytes over generations

In general, we define the cells into four categories: progenitor cell, blasting cell, dying cell and lost cell. Dying cell refers to the progeny dying before the end of the experiment, while lost cell refers to a cell whose track is lost. In a cell lineage tree shown in Figure 5.3(c), the root (Cell 0) represents the progenitor cell, the leaf could be either a lost cell (Cell 3) or dying cell (Cell 1 and Cell 4), whereas the parent after the first division round refers to the blasting cell (Cell 2). In this thesis, lost cells are not considered because we fail to completely track its behaviour in the recorded experiment time. In conformity with the previous findings, cells from different categories have distinct fluorescence temporal profiles. In particular, we search for patterns prominent across a population of responding cells.
As presented in Figure 5.8, we concluded that there exist three clusters in those three cell types on a population level. Especially, the cell intensity in FUCCI green channel increases after a duration of approximately constant fluorescence levels for both progenitor cells and blasting cells. By contrast, the red fluorescence does not vary at the beginning for progenitor cells but go up and down for blasting cells. The dying cell is characteristic of increased red fluorescence and roughly invariant green fluorescence.

Figure 5.8: Single-cell fluorescence time courses from three categories: (a) progenitor cell, (b) dividing cell, (c) dying cell.
To automatically classify the cell type we propose to obtain a classifier with high accuracy based on red fluorescence and green fluorescence. In principle, a cell could die while having high green fluorescence, but we did not observe this in the tracked videos and thus it is rare. In this case, the classification results in our data will not be affected. Here we randomly select 33 dying fluorescence data points to serve as training dataset, and the remaining 30 as the test dataset. We train our classifier model based on the selected mean intensities in FUCCI red and green channels using the “Classifier Learner” module in Matlab. The value of area under the receiver-operating-characteristic curve (AUC) of 0.966 indicates that the training data can be well classified by a decision tree classifier with 92.9% accuracy. This validation is merely to confirm those cell types in our data.

5.3.3 Progenitor cell follows regular division mechanism after $G_0/G_1$ phases

Normally the first division takes much longer than subsequent division rounds before entering the $S/G_2/M$ phases. It is assumed that some activation time is spent in the $G_0/G_1$ phases. Our aim is to explore whether the progenitor cells follow the same division mechanism as the blasting cells do regardless of time spent before the $S$ phase. In brief, the total division time in progenitor cells is assumed to be composed of $G_0/G_1$ and regular cell division phases ($S/G_2/M$). Hence, for each dividing cell, we extract five fitted parameters from the model of green fluorescence time courses in Equation 5.3.

This multi-dimensional dataset can be reduced into two-dimensional one using Principal Component Analysis (PCA) since the eigenvalues of the first two principal components are larger than the others, presented in Figure 5.9. Given 15 progenitor cells, we randomly select 15 out of the 66 data points in the blasting cells and thus conduct bivariate Kolmogorov-Smirnov statistical test, resulting in the hypothesis test $h = 0$ and $P$-Value $= 0.1788$. This implies that the progenitor cell and the blasting cell are most likely to comply with the same division rules through the cell cycle.

In progenitor cells, the duration of $G_0/G_1$ is denoted by the activation time, which equals the difference between total division time and the duration of $S/G_2/M$. This activation time appears to be a random variable as it is distinct for different progenitor cells based on its dis-
Figure 5.9: Dimensionality reduction using PCA for extracted five extracted parameters in dividing cells: (a) Eigenvalues corresponding to five parameters. (b) Two principal components.

Since there is no consensus regarding which cell cycle phase accounts for the majority variation in total division time, we also analyse the distributions of cell cycle phase proportions in progenitor cells and blasting cells. As revealed in Figure 5.11 rather than $G_1$ being responsible for the bulk of variation in cell cycle time, we found that $S/G_2/M$ is highly variable in itself and occupies a large proportion of total division time −78% for progenitor cells and −85% for blasting cells. Meanwhile, both activation time and $G_1$ exhibit stochasticity in the cell cycle time and hold a small proportion of the total division time. Our conclusions coincide with the results in the stretched model reported in [15].
Figure 5.10: Distribution of activation time for progenitor cells.

Figure 5.11: Histogram of proportions of individual cell cycle phases for (a) progenitor cells and (b) blasting cells.
Longer time spent in progenitor cell division motivated us to study whether the activation time affects cell life span in a cell lineage tree. This life span could be represented by average generations/time, defined as the normalized average depth/life time in a tree. We first analyse the relations between the activation time and average generations/life time of all cell lineage trees, shown in the top row of Figure 5.12. The results indicate that they are unlikely to be correlated. Then we intend to inspect if the duration of $S/G_2/M$ is influenced by the activation time in progenitor cells and blasting cells in generation 1. As seen in the bottom row of Figure 5.12, we demonstrate that the activation time has no impact on the duration of $S/G_2/M$ for these blasting cells, while there is a strong correlation (0.563) between them in progenitor cells.

Figure 5.12: Relationships between the activation time and (a) normalized average generations, (b) normalized average time (upper right), (c) $S/G_2/M$ in progenitor cells, and (d) $S/G_2/M$ in dividing cells at generation 1.
Finally we study the relationships between cell cycle phases and total division time on a population level. Scatter plots of times spent in all parts of the cell cycle are presented in Figure 5.13. The black straight line with a non-zero intercept was chosen as the simplest fitting model. It is clearly seen that the duration of both G1 (G0/G1) and S/G2/M are strongly correlated to the total division time in either blasting cells or progenitor cells. Furthermore, there is a weak correlation (0.440) between time spent in G1 and time spent in S/G2/M for blasting cells, shown in Table 5.1.

By contrast, the duration of the G0/G1 and S/G2/M phases are highly correlated for progenitor cells. We are also interested in the relationship between the duration of the S/G2/M phases in the progenitor cell and that in its dividing daughter cell at generation 1. Here only the progenitor cells that produce blasting cells are considered as our test data, seen in Figure 5.14(a). In summary, Figure 5.14(b) shows that they are not likely to be correlated because of a high P-Value (0.4028) in Table 5.1. Based on a Wilcoxon signed rank test, we can achieve a large P-Value (0.8135).

Table 5.1: Correlation between cell cycle phases and total division time in all dividing cells.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Cell cycle phases</th>
<th>Correlation</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progenitor cell</td>
<td>&lt; G0/G1, Total time &gt;</td>
<td>0.774</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>&lt; S/G2/M, Total time &gt;</td>
<td>0.886</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>&lt; G0/G1, S/G2/M &gt;</td>
<td>0.563</td>
<td>0.0289</td>
</tr>
<tr>
<td>Blasting cell</td>
<td>&lt; G1, Total time &gt;</td>
<td>0.617</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>&lt; S/G2/M, Total time &gt;</td>
<td>0.962</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>&lt; G1, S/G2/M &gt;</td>
<td>0.440</td>
<td>0.0013</td>
</tr>
<tr>
<td>Progenitor cell VS Blasting cell</td>
<td>&lt; S/G2/M, S/G2/M &gt;</td>
<td>0.270</td>
<td>0.4028</td>
</tr>
</tbody>
</table>
Figure 5.13: Relationship between cell cycle phases for progenitor cells (a-c) and blasting cells (d-f).
Figure 5.14: Relations between the duration of $S/G_2/M$ in progenitor cells and the corresponding blasting cells (b) by selecting $S/G_2/M$ values from progenitor cells with dividing daughter cells (a).

### 5.3.4 Maximum green fluorescence is correlated with the time $T_m$

For a population of dividing cells, we aim to explore the relations between maximum green fluorescence $I_{max}$ and the time spent before reaching the maximum, defined as $T_m$. In Figure 5.15 we show the scatter plots for all types of dividing cells. In Table 5.2 we conclude that $T_m$ and maximum fluorescence values are strongly correlated for progenitor cells and blasting cells. In other words, the longer $T_m$ lasts the larger the maximum value becomes. When we consider a population of proliferating cells, this correlation becomes weaker due to the longer activation time spent in progenitor cells.

Table 5.2: Correlation between maximum green fluorescence $I_{max}$ and $T_m$ in dividing cells.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Correlation</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All dividing cells</td>
<td>0.421</td>
<td>$0.001 &lt; P &lt; 0.01$</td>
</tr>
<tr>
<td>Progenitor cell</td>
<td>0.568</td>
<td>$0.01 &lt; P &lt; 0.05$</td>
</tr>
<tr>
<td>Blasting cell</td>
<td>0.566</td>
<td>$P &lt; 0.001$</td>
</tr>
</tbody>
</table>
5.3.5 Cell cycle phases exhibit strong correlation in sibling cells

In order to find out whether cell division phases are shared within siblings, we study sibling correlations in progression through the cell cycle. Each data point in Figure 5.16 represents the duration of one cell cycle phase in two siblings from one of 15 lineage trees. The correlations between division phases are present in these scatter plots and further quantified in Table 5.3. We conclude that the time spent in both $S/G_2/M$ phases and total division cycle for a population of siblings are highly correlated, as expected from previous studies [24][15]. Conversely, there is a lower degree of sibling correlation in the $G_1$ phase. Overall, these conclusions imply that the processes governing the variation in both division time and individual cell cycle phases of mother cells can be passed on to offspring and shared by sibling cells.

Figure 5.15: Relationship between maximum green fluorescence $I_{max}$ and $T_m$ for (a) progenitor cells, (b) blasting cells and (c) all dividing cells.
Table 5.3: Correlation between cell cycle phases and total division time in all sibling cells.

<table>
<thead>
<tr>
<th>Cell cycle phases</th>
<th>Correlation</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$&lt; G_1, G_1 &gt;$</td>
<td>0.652</td>
<td>0.0014</td>
</tr>
<tr>
<td>$&lt; S/G_2/M, S/G_2/M &gt;$</td>
<td>0.849</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>$&lt; \text{Total time, Total time} &gt;$</td>
<td>0.853</td>
<td>$P &lt; 0.001$</td>
</tr>
</tbody>
</table>

Figure 5.16: Relations between cell cycle phases: (a) $G_1$, (b) $S/G_2/M$ and (c) total division time for sibling cells.

Considering the similarity between siblings, we employ global fitting to model the green fluorescence temporal profiles by sharing either three parameters $a_0$, $t_1$ and $t_3$ or all parameters.
in Equation 5.3. One of the curve fitting results is presented in Figure 5.17. To compare those two global fitting methods with the independent fitting model, we evaluate the performance based on the relative change of AIC defined as

$$AIC(\%) = \left( \frac{AIC_i - AIC_g}{AIC_i} \right) \times 100\% \quad (5.6)$$

where $AIC_i$ and $AIC_g$ refer to AIC value of an independent fitting model and a global fitting model, respectively. A global fit is regarded as a good model when the value of $AIC(\%)$ becomes negative. The greater the negative value $AIC(\%)$ is, the higher performance the global model has than the independent fitting, otherwise lower performance. In Figure 5.18, we show the performance of two global fitting approaches in 20 siblings. Overall 3(0) out of 20 fitted curves using 3(all) shared parameters perform better than the independent fitting. Since the median value (1.80) is much closer to zero, we conclude that the model with three shared parameters, other than all shared parameters, can be applied in fitting the profiles of two siblings. As a consequence, we can replace the independent fitting method by the global model with 3 parameters shared, which makes curve fitting less time-consuming and more efficient.

![Figure 5.17: Three fitting methods for two siblings: independent (left), global with 3 parameters shared (middle) and global with all parameters shared (right).](image)

94
5.3.6 Discussion

In this chapter, we have made use of advances across technological domains, and supplement these tools with our methodology for automatic feature extraction from fluorescent time courses. Reconstructing lineages for responding B cells along with monitoring FUCCI fluorescence levels for individual cells have enabled us to quantify stochastic lymphocyte responses at a considerable level of detail.

It has been known for a long time [70] that upon activation, the time it takes newly activated cells to undergo the first division is considerably longer than subsequent division rounds. One possibility, especially in light of recently proposed stretched cell cycle model [15], could be that all parts of the cell cycle are elongated in this first division. An alternative possibility is that the difference between the first and subsequent divisions could be largely attributed to an "activation" stage followed by a "regular" division process.

Our data strongly supports the latter scenario, as $S/G_2/M$ times from the first and subsequent generations were statistically indistinguishable. This macroscopic observation has consequences for molecular studies of lymphocyte activation, as it suggests that the majority of activation-related transcriptional changes must be completed before proceeding into the first cell cycle. As a proxy, we termed the time before entering the first S-phase as "activation time". This activation time can serve as a measurable and biologically relevant random variable in mathematical models.

We have also provided evidence that despite cell to cell variability, the rules underlying the
autonomous part of lymphocyte response can be relatively simple in the sense that there are only a handful of underlying variables. For example, we found that peak intensity is related to timing of cell cycle phases, and sibling cells correlate in fluorescence kinetics. Our findings are in line with the theory behind several previous studies [15, 23], and provide an important validation for this research direction.

5.4 Conclusion

While we have explored a wide range of biological questions about specific aspects of B lymphocyte kinetics, these questions and the insights that we have extracted are primarily a starting point for future studies. Given the progress we have made in this chapter, a key challenge for future research is to work with the biologists who have helped guide our study, in order to identify new questions about B lymphocyte kinetics that can be analysed using the theoretical framework we have provided in this thesis. In addition, based on the empirical studies we have made of the straightforward weaknesses of the image correction and feature extraction methods we have proposed, an important direction for future research is to consider the interactions between different approaches to acquiring time-lapse fluorescence microscopy videos, and the difficult techniques for image correction and feature extraction.
Chapter 6
Conclusion and Future Research

Time-lapse fluorescence imaging combined with single cell tracking has been widely used in a number of biological studies such as cancer treatment [67], automated drug screening [21] and stem cell biology [19]. Apart from that, another important application is lymphocyte immune responses [8]. Understanding the mechanisms of lymphocyte response regulation has gained increasing importance due to its role in developing strategies against auto-immune diseases and cancer immunotherapies. Lymphocyte proliferation is a fundamental characteristic of immune responses and an essential process to protect the host in mammals. In order to understand this process, we monitor cell behaviour through the cell cycle using long-term live cell imaging in conjunction with semi-automated cell tracking. In general, this thesis focuses on the quantitative analysis of time-lapse fluorescence images with an application to lymphocytes. Due to the inherent problem of time-lapse fluorescence imaging, the major bottleneck in subsequent single-cell data analysis is the image processing methods for a correct measurement of cell fluorescence levels.

More specifically, one major challenge is that the observed images are corrupted by a number of image acquisition artefacts such as photobleaching, shading, cross-talk and autofluorescence. To alleviate the consequences of these effects, numerous image correction methods have been proposed. However, most previous studies focus on some specific effects rather than addressing the combination of all these effects. In particular, most shading correction algorithms are specially developed for MR images, which may not fit time-lapse fluorescence microscopy images. Furthermore, some shading correction approaches have many parameters that need to be tuned and the accuracy of these methods relies heavily on the parameter settings. When it comes to the performance evaluation on real cell imaging data, there is no clear consensus about which quantitative measure should be applied.

A second major challenge is the effect of cross-talk in image acquisition. Various spectral unmixing methods have been developed to solve the problem of cross-talk. Recall that the
idea of spectral unmixing is to recover the unmixed images by estimating the cross-talk coefficients of the mixing matrix based on a set of input measurements (Chapter 4). However, there has been little discussion into how to select the appropriate training data from images. As a result, it is unclear as to which data selection strategy leads to higher performance of spectral unmixing. Moreover, the quality evaluation of spectral unmixing depends on having one or more reference objects, which are not always readily available, particularly in experiments that involve multi-colour fluorescence reporters (e.g., FUCCI). Considering that the photobleaching effect is well studied and autofluorescence can be incorporated into the spectral unmixing model, our aim has been to solve the problems of shading artefacts and cross-talk effects in this thesis.

After the observed images have been corrected, we can then study the kinetics of lymphocyte proliferation through the cell cycle. Most proposed cell cycle progression models analyse data at a population level and require manual annotation to estimate the time spent in different cell cycle phases, which is time consuming and can lead to inaccurate measurements. Moreover, the conclusions obtained from previous models are inconsistent, and there has been little discussion about whether progenitor cells and other dividing cells follow the same regular division mechanism. This has become a focus of considerable interest because generally the progenitor cell takes a longer time to enter the S phase while the variation in its fluorescence time courses appears to be similar to cells in subsequent division rounds. Overall, our aim has been to develop a mathematical model for automatic feature extraction from the resulting single-cell fluorescence time courses.

6.1 Contributions

To achieve our goals, we first proposed a novel shading correction algorithm that is suitable for adaptation to time-lapse fluorescence images and comprehensive quality evaluation on real data, in which the ground truth is not available (Chapter 3). To the best of our knowledge, we are the first to determine a quantitative measure, called CJV, from almost all existing measures for performance evaluation on time-lapse fluorescence images. This is beneficial for an objective comparison between existing popular methods. Based on this measure, we have proposed a CJV-based algorithm that does not require any tuning of parameters. Extensive validation shows that our method performs better than other major well-established approaches on a range of real datasets tested.
Next we developed a spectral unmixing model by considering cross-talk and autofluorescence. By identifying an indirect ground truth dataset using what we call reference intervals, a new novel correlation-based evaluation approach has been proposed to evaluate the spectral unmixing results when reference objects are absent (Chapter 4). This spectral unmixing method is based on analysis at the level of cells rather than pixels, which becomes simpler and more computationally efficient. In order to obtain an accurate estimation of the cross-talk coefficients in the linear mixing model, we explicitly illustrated four different strategies of training data selection. Finally, we compared and evaluated spectral unmixing results obtained using different training strategies.

After the observed images are corrected, we can estimate the true single-cell fluorescence time courses together with reconstructed lineage trees. Based on the corrected individual cell fluorescence temporal profiles, we have developed a general mathematical model for describing the kinetics of B cell response in FUCCI red and FUCCI green channels, by means of different nonlinear functions (Chapter 5). Most importantly, we have demonstrated that the progenitor cells follow the same regular division mechanism as other dividing cells despite the longer time spent before entering the S phase. This kinetic analysis of progenitor cells is, to the best of our knowledge, the first example to prove the difference between the first and subsequent divisions. This difference can be largely attributed to an "activation" stage followed by a "regular" division process. We have also provided evidence that the rules underlying the autonomous part of the lymphocyte life cycle can be relatively simple. For example, we concluded that peak intensity is related to the timing of the cell cycle phases, and a global fitting model can be shared in sibling cells, implying the inheritance from mother cells and similarity between siblings.

6.2 Future Work

In this section, we highlight possible research directions for our future work as follows.

1. Even though there is no need to adjust the parameters in our CJV-based shading correction algorithm, a prior segmentation is required. However, for fluorescence images that are seriously corrupted, it is difficult and time-consuming to obtain a good segmentation result. Thus, a direction of future research is to achieve segmentations that are of sufficient quality for the purpose of CJV-based shading correction. Since the proposed method is computationally expensive, we can either improve the speed of optimization
in this algorithm or design a more efficient correction approach in our future work.

2. In principle, our novel spectral unmixing method has the potential to be more widely applicable to spectral unmixing for time-lapse fluorescence microscopy of other types of cells for which FUCCI reporters are available. A key challenge in this context will be to determine whether suitable uncorrected reference intervals can be identified for each fluorescent marker for the corresponding type of cell. In addition, one potential disadvantage of our novel spectral unmixing method is that automatic segmentation and tracking for each cell are required, which is not always required for single cell analysis since manual tracking can sometimes be sufficient. A direction for future work is to design a more efficient and theoretically sound algorithm that can work on the training data from random pixels or segmented cells, thus saving time and effort.

3. In the aspect of the kinetic study of B cell response in vitro, the mechanism that controls the division behaviour of progenitor cells over the cell cycle is worth further investigation. In particular, the difference between proliferating lymphocytes across generations is of great interest. We can also focus on the characteristics of the population of dying cells in order to understand the molecular regulatory mechanisms that control cell death.
Bibliography


[26] Oliver Hilsenbeck, Michael Schwarzfischer, Stavroula Skylaki, Bernhard Schaubberger, Philipp S Hoppe, Dirk Loeffler, Konstantinos D Kokkaliaris, Simon Hastreiter, Eleni Sky-


