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Institute of Computational Biology (ICB)
Helmholtz Zentrum München
Master's Thesis in Bioinformatics

Single-cell analysis of cancer drug response using computer vision and learning algorithms on time-lapse micro-trench data

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**Wirkungsanalyse von Krebsmedikamenten in Einzeller
Auflösung durch die Anwendung von Computer-Vision-
und Machine-Learning-Algorithmen auf Microtrench-
Videoaufnahme**

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Selbständigkeitserklärung

Ich versichere, dass ich diese Master's Thesis selbständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

München, den 12. Februar 2018

Statement of authorship

I confirm that this master's thesis is my own work and I have documented all sources and material used.

Munich, February 11, 2018

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Abstract

Quantitative measurement of cancer drug response is essential to objectively gauge the efficacy of cancer drugs. So far, there has been no method to reliably track and quantitatively measure single-cell response of of cancer drug treatment over a long time (over 12 hours). A novel platform and image processing pipeline are presented in this thesis that enables the continuous observation of cell clones derived from individual non-adherent cells of the leukemia cell line AML-M5a MOLM-13. The platform employs arrays of micro-trenches optimized to observe cells for two consecutive generations. We demonstrate that automated image analysis is feasible and allows precise determination of the cell cycle distribution and sister cell correlations. A key feature of the platform is the direct and parallel observation of hundreds of cells in different cell cycle states. Here, we first studied the time distribution of a full cell cycle, i.e. the time between the first and second division. We then focused on the response dynamics of single cells to chemotherapeutic drugs and on the comparison of the response to these agents between a chemically synchronized and a non-synchronized population. We show that the time-to-death after induction of apoptosis using the anti-mitotic agent Vincristine and Daunorubicin exhibits dependence on cell cycle. The results were found consistent with experiments using cells that were synchronized using standard thymidine cell cycle arrest. Our approach also enabled a time correlation analysis, which showed that the time-to-death of sister cells is correlated. While the method enabled relatively easy and quasi-high-throughput analysis of cancer treatment *in vitro*, our pipeline could also be adapted in various contexts involving single-cell analysis in a micro-trench environment with only reasonable amount of modifications necessary.

Zusammenfassung

Quantitative Messung des Zell-Responses zu einem Krebsmedikament ist bedeutsam zur objektiven Messung von Wirksamkeit von dem Medikament. Bisher existiert keine Methode, die in der Lage ist, die Wirkung eines Krebsmedikaments in Einzeller Auflösung verlässlich in längerem Zeitraum (über 12 Stunden) zu tracken und messen. Wir stellen in dieser Arbeit neue Platform und Pipeline vor, die kontinuirliche Beobachtung von der bewegenden Zelllinie AML-M5a MOLM-13 in Einzeller Auflösung ermöglichen. Das Platform besteht aus den Microtrench-Arrays, die die Zellen optimal eingrenzen und daher zuverlässige Videoaufnahme und nachfolgende Auswertung ermöglichen. Dieses Platform nimmt die Videoaufnahme direkt und parallel auf, was zur hochdurchsatziger Eigenschaft des Platforms führt. Anhand von dieser Kombination von dem Platform und dem Bildverarbeitungspipeline führen wir folgende Analysen vor. Zuerst analysieren wir die Zellzyklusdauer aller gültigen Zellen. Wir analysieren danach die Zellaktion zu den ausgewählten Krebsmedikamenten Vincristine und Daunorubicin. Wir stellen fest, dass die Response-Dynamik zu dem Medikament von den Zellphasen abhängig ist. Diese und andere Resultate bezüglich Response-Dynamik sind in Übereinstimmung mit vorigen Ergebnissen, die die Validität unserer Platform und Bildverarbeitungspipeline bestätigen. Neben ihrer hochdurchsatziger Eigenschaft zeichnet sich unsere Methode aus durch ihre Anpassbarkeit. Durch das modulare Design von dem Pipeline lässt sich die Methode in verschiedenen einzellen Analysen in Microtrench-Arrays einfach anpassen.

Terminology and abbreviations

Following terms are used frequently in this thesis and therefore merit special explanation:

Name	Explanation
micro-trench	Single unit of micro-trench inside a well
wafer	The plate where the micro-trench is manufactured on
well	Macro-containment where wafer is located, usually part of larger wells installation (2 by 4 wells in our case).
sticky slide	The containment box where wells are located in
slice	An image
stack	An ordered sequence of images (usually coming from the same channel)
image position	Unique position which is captured by the camera.
	A stack represents sequence of images taken in the same image position
channel	A connection between data source (camera) and storage. Images coming from the same channel have same capture properties formatting.

Note that in this thesis, following things are used interchangeably:

- image and slice, while referring to an image particularly a capture image of the trenches and its processed versions.
- image and figure, while referring to an image shown in this paper in general.

Abbreviation	Full Form	Explanation
TTD	Time-to-death	See Subsection 3.4.4
DT	Death time	See Subsection 3.4.4
DivT	Division time	See Subsection 3.4.4
TIC	Time in cell cycle	See Subsection 3.4.4
CCD	Cell cycle duration	See Subsection 4.3
SDT	Sisters death time	See Section 4.4

Symbol	Explanation
$\mathbf{B}(x; a, b)$	Beta function
B_t	A 1-bit monochrome image taken at time t (t -th image)
$\rho_{X,Y}$	Pearson's correlation coefficient (PCC) of two random variables X and Y
$\text{cov}(X, Y)$	Covariance of two random variables X and Y
$d(v_i, v_j)$	L_2 distance between two objects
$d_G(G_i, G_j)$	Sum of intensity difference between two grayscale images
$\mathbb{G}_{x,y}$	Sobel gradient of pixel $M_{t,x,y}$
$\mathfrak{g}_{x,y}$	Quadratic Sobel gradient of pixel $M_{t,x,y}$
G_t	Grayscale image at time t
$G(x)$	One dimensional Gaussian function
$G(x, y)$	Two dimensional Gaussian function
$\mathbf{I}_x(a, b)$	Regularized Beta function
$I_t(x, y)$	Intensity function for pixel (x, y) of RGB image taken at time t M_t
$I_t^{cG}(x, y)$	Gaussian blurred intensity function for pixel (x, y) of M_t
$J_t(x, y)$	Brightness and contrast transforming function for pixel (x, y) of grayscale image taken at time t
M	A stack of images
M_t	An RGB image captured at time t
$M_{t,x,y}$	Pixel at (x, y) in M_t
$M_{t,x,y,1}$	First channel of pixel $M_{t,x,y}$
M_t^b	A b -bit encoded RGB image captured at time t
$M_{t,x,y,i}^m$	Pixel-wise mean-subtracted pixel $M_{t,x,y}$
$\langle M \rangle$	Pixel-wise mean image of stack M .
T_R	Gradient threshold value in a quadtree (used in RATS algorithm).
$\Phi_{RBF}(x_i, x_j)$	An RBF kernel function.

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Chapter 1

Introduction

Cancer is among the deadliest diseases ever known to human being. It is a leading cause of death in 2009, second only to cardiovascular diseases [1]. The numbers are disconcerting, especially in the developed world. In the United States alone, half of men and a third of women are expected to develop some kind of cancer. According to US government, in 2016 alone, an estimated 1,685,210 people will be diagnosed with cancer, while 595,690 more will be die from it [2]. Worldwide, the International Agency for Research on Cancer's GLOBOCAN series report that, in 2012, [3] there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis).

Among the most studied type of cancer is the Acute Myeloid Leukemia (AML), a cancer that affected about one million people globally and resulted in almost 150,000 deaths in 2016 [4]. A cancer of myeloid line of blood cells, it is mostly indicated by overabundance of myeloid cells in bone marrow. In the advanced stage of AML, the abnormal myeloid cells interfere with the production of normal myeloid cells [5] (Megakaryocyte, Erythrocyte, Basophil, Neutrophil, Eosinophil and Macrophage among others [6]) which, if left untreated, will be lethal to patient.

One of the special characteristics of the AML is its difference in curability in various stages of cancer. Early stage of AML is highly curable while advanced stage of AML is barely curable [7]. Deriving an early detection of the disease is thus of utmost importance to ensure patient's survival. Several methods are developed for early AML detection ranging from a screening-based [8] to a flow cytometry-based methods [9]. However, these methods either focus on population level thereby ignoring single-cell dynamics or are not high-throughput enough in analyzing cells in single-cell resolution.

1.1 Variability of cell-to-cell response towards cancer treatment

Cell-to-cell variability in response to external stimuli is a pervasive trait in cellular systems that prevails even in isogenic cell populations [10]. Here, heterogeneity might be caused by epigenetic modifications, differences in the cell cycle phase, or by intrinsic stochastic fluctuations in gene expression and biochemical regulation. The implications of heterogeneity for cancer progression and treatment are poorly understood. In some cases, heterogeneity is dominated by intrinsic fluctuations in the stochastic expression of key regulators

that randomly alter the sensitiveness of individual cancer cells [11]. Experiments and simulations show that variability in cell fate is sensitive to small stochastic increases in the levels of several regulator proteins and are transiently heritable to siblings [12, 13]. The profound consequences for cancer treatment have been subject to theoretical studies with several proposed models explaining the stochastic origins of cell-to-cell variability in cancer cell death decisions [14, 15].

Since cancer is an intrinsically highly diverse disease, tumors of any different histological type not only exhibit genetic diversity but also display their variation when exposed to all forms of chemotherapy [16]. Most state of the art chemotherapeutic drugs in clinical use, target rapidly dividing cells and trigger apoptosis. Vincristine (VCR), an antitumor vinca alkaloid, binds to tubulin and stops the dividing cell from separating its chromosomes during metaphase in M-phase. It is thus considered an M-phase dependent drug [17]. In contrast, Daunorubicin, an anthracycline aminoglycoside antineoplastic, intercalates on DNA and inhibits the function of the enzyme Topoisomerase II during transcription and replication. Daunorubicin is thus expected to act throughout the whole cell cycle and but especially strong during the DNA replication in the S-phase. Both drugs are used to fight hematopoietic disorders such as AML among other neoplasms (both drugs are further explained in Subsection 2.1.3). In the literature, a drug that interferes with the cell cycle is in general considered cell cycle dependent, but in practice it is not clear whether it only acts on a specific cell cycle phase, due to side effect toxicities. Based on this, it is hypothesized that both VCR and daunorubicin are cell cycle dependent.

1.2 Combination of the time lapse imaging and the microfluidics system for single-cell analysis of cancer

It is essential to understand sources of the heterogeneous response to cancer drug treatment in a therapy in order to design novel therapeutic strategies and potent agents that not only targets key signaling pathways with high specificity but also address the contextual role of cell cycle timing in the treatment. In this context, time-lapse imaging, which allows for the recording of accurate histories of individual cancer cell fates and cancer subpopulations, received increasing attention [18, 19, 20]. However, the study of the effect of particular cell cycle phases on cell response to cancer drug treatment requires that single cells are observed continuously throughout their lives including during cell division, drug addition, and death. For this, the single-cell level of detail has not been explored before.

The typical bottleneck for a high-throughput analysis for such is cell tracking. Different automatic tracking tools have been proposed [21, 22] and compared [23, 24]. But to achieve tracks for fast moving cells, high cell densities and long term identifications, manual tracking is often applied [25, 22] to generate tracks with maximal accuracy. For many approaches, the workload of manual tracking has to be compared to correcting erroneous track from tracking algorithms [23]. Among the techniques to capture non-adherent cells for long-term observation microfluidic devices [24] as well as micro-well platforms [25, 21, 22, 26, 27, 28] have been developed. Platforms that confine single starting cells and thus lead to spatially separated cell families (also called clones) are an especially useful tool to investigate cell cycle length, sister cell correlations, or the effect of cell cycle phases to cell-to-cell variability in a high-throughput manner.

1.3 Advances in microfluidics, image processing and machine learning

In the recent decades, as in many other fields, there are numerous groundbreaking advances in the fields of microfluidics, image processing and machine learning. The advances, coupled with general technological advances in computing power and energy efficiency, have made it possible for us to design almost completely automated analytical pipeline for single-cell microfluidic system. While not all methods and algorithms in this project are the most recent, many of them are state-of-the-art for the purpose and, more importantly, robust enough for the pipeline to work seamlessly. The explanation of every method used is available in Chapter 2.

1.3.1 Microfluidics

As the name suggests, 'microfluidics' concerns the manipulation of fluids in a small working dimension, typically starting from nanometers to lower millimeters [29]. In modern context, the entire fields tries to find application of micro-sized devices which hold and control the state of liquid [29] including cell culture medium. There are two categories of microfluidics: active and passive microfluidic devices. The separation is based on the device's ability to actively manipulate the flow and control of devices [30]. Active devices use micro-valves to perform sophisticated chemical processes [31]. This goes as far as reactions at individual cell level [32]. A passive device, which our micro-array system is, exploits on the other hand its physical property to provide rapid controlled environment for micro-sized experiments.

Active microfluidic methods for analysis and manipulation of biological cells have been done in various way and form. In 2003, Wheeler *et al.* developed a novel microfluidic device from poly-dimethylsiloxane using multilayer soft lithography technology for the analysis of single cells [33]. The microfluidic setup facilitates the passive and gentle separation of a single cell from the bulk cell suspension. This in turn enables the precise delivery of reagents as little as one nanoliter to the cell. In other use cases, the optical-based microfluidic methods have been used to sort cell with very high accuracy [34]. This family of method utilizes the fact that different dielectric particles respond differently to an applied light field [35]. Combined with the minuscule spatial setting, the methods are compatible for single-cell resolution analysis. For example, optical-based microfluidic methods have been used to sort cells with very high accuracy [34, 36, 37].

As a method, passive macrofluidic methods are mostly used to provide specialized environment in cell-size resoultion. For example, microfluidic settings have been used to keep spatio-temporal identity of single cell for the analysis of the underlying biological dynamics of the isolated cells [38, 39], which form the methods the design and synthesis of our micro-trench system are based on.

In the last decades, the recent advances in both passive and active microfluidics have created an entire field [29] with use cases ranging from *in vivo* imaging [40], single-cell analysis [33] to cellular biophysics [41]. This project leverages in these advances coupled with equally outstanding advances in image acquisition, image processing and machine learning methods which will be described in the subsequent subsections.

The use of microfluidics enables us firstly to study the distribution of division times among single cells and also to correlate the division times between sister cells, which are

genetically very similar. Secondly, the array of micro-trenches enabled the study of the response dynamics of single-cells to doxorubicin, a widely used chemotherapeutic drug, and the comparison of the response to this agent between a chemically synchronized and a non-synchronized population

1.3.2 Image Processing and Computer Vision

Before the runaway advance of deep learning methods in the last years, object and area detection methods generally rely on discriminating certain patterns and features in the picturek. In 1999, David Lowe proposed the method of image recognition using features similar to the ones observed in primate neuron [42]. Known as SIFT, these features are invariant to image scaling, translation, rotation and, partly, illumination changes and affine or 3D projection. In 2001, Paul Viola and Michael Jones proposed a method that was able to recognize face [43], and later, objects in almost real-time fasion [44]. Until the advent of deep neural network-based breakthrough in the early 2010s, these methods have been a go-to method for recognizing objects in an image.

1.3.3 Machine Learning

The breakthrough by Lowe increased the interest in the solution of vision pattern recognition based on human perception. This sparked interest in neural network-based model for image recognition. Due to advent in computing power in the early and later 2000s enabled the construction of larger and deeper neural network allowing for more general representation of features [45].

In 2012, the improvement of the best method over the last year's method in recognizing object in the annual ImageNet Large Scale Visual Recognition Challenge (ILSVRC), the world's most important general image recognition challenge at that time, has been dramatic. The improvement is marked by the utilization of deep neural network (AlexNet [46]). From that time on, many deep neural network architectures are born, including VGG Net (Simonyan and Zisserman, 2014 [47]), Google's Inception Net [48] and Microsoft's ResNet [49].

Since then, almost every new method, while basing itself on principles very similar to classical computer vision methods such as Viola-Jones and SIFT, is implemented using some variant deep neural networks.l

1.4 Related methods considered for the project

1.4.1 Cell death determination

Measuring cell death is a crucial part of the experiment, as the reliable determination of it is the basis of most analysis in this thesis. There are several other ways to measure cell deaths with varying complexity and accuracy. Each method contains certain assumptions of cell death.

For example, determining cell death by cell movement assumes death of a cell if no movement beyond random flux is observed in certain amount of time. This obviously has certain drawbacks, such as when the observation is done in non-static environment. Moreover, defining the limit of the random flux, above which a given cell is assumed to

actively move, is not a trivial task. Some kind of gold standard for a given cell line and environment has to be established manually, which is very time consuming. This fact is again made even more complicated by the fact that many cells show different movement pattern upon introduction of treatment. It is well known that some cells tend to move faster or slower under stress, the situation many cancerous cells in our experiment will experience upon addition of cancer drug treatment [50, 51, 52].

The second method is using cell size. During apoptosis, a cell blebs, shrinks and discharges pockets of containing organelles [53]. This is followed by nuclear fragmentation and chromatin condensation and finally the day of the entire nucleus. This has direct effect on cell size: as apoptosis proceeds, more parts of the cell are discharged from the main body thus reducing its size. Detecting cell death based on the size is however not verifiable as the process takes hours if not days and the definition of cell death is not generally agreed upon based on the size.

1.4.2 Other model cell lines

Some other cell lines were examined as potential probe cell line in this experiment. One of them is Jurkat Cell, a model cell commonly used to study T Cell Leukemia, T cell signalling mechanism and the expression of various HIV-related chemokines [54]. The cell line was considered since it is well-studied [55, 56]. This is especially true if we consider apoptotic mechanism of the cell line, a problem this project and other related projects by our and partner labs are trying to investigate. There are several seminal publications about the dynamics of apoptotic mechanism of Jurkat cells we could well compare our results to [57]. Samali *et al.* [58] even studies the dynamics of Caspase expression in Jurkat cells, a topic dealt a lot in this project as the chapters progress (see for example Subsection 2.1.3) while Kasai *et al.* considers the aspect of spindle checkpoint in the context of apoptotic cell death [59]. However, we figured out early on that the cell motility of the Jurkat cell line was increased dramatically (a phenomenon observed by others before us [60]) upon the introduction of chemotherapeutic treatment – the increase dramatic enough that the cells managed to escape the micro-trench it initially landed in.

1.5 Hypothesis

In this part, the hypothesis underlying the experiment is presented:

- **Cell-cycle dependency of cell response towards cancer drug treatments:** Since it is assumed that VCR acts only during M-phase in their cycling, we can expect that cells that are closer to M-phase will die earlier [18, 61]. We also expect that there is some degree of correlation of the response towards cancer drug treatments between sister cells.
- **Machine learning model of cancer cell deaths:** we can also expect that there are certain features taken from cell that correspond to some stage of programmed cell death. These features shall be partly learnable using some simple machine learning method.

1.6 The structure of the thesis

The thesis is presented as two closely-related things. Chapter 2 deals mostly with: (i) the experimental setting used in this project including the microfluidic system used, the cancer drug treatments used, the reporting signals used and the protocol of the experiment and (ii) the methods used to process and analyze the resulting images from the experiment. Chapter 3 describes the application of methods from Chapter 2 on the data coming from the experiments. In Chapter 4, selected findings from the analysis of the processed data are presented. Finally in Chapter 5, we discuss the results in relation to published findings that are related to ours. There we will also confirm the validity of our microfluidic method.

Chapter 2

Data and Methods

This project consists of three parts: (i) the problem statement regarding the dynamics of cancerous single-cells under pressure of treatment, (ii) the microfluidics which enables the single-cell protocol and (iii) the software implementation used to process and analyze the time-lapse data coming out of the experiment.

This chapter considers two aspects of the project: the experimental setting and the theoretical aspects behind the data analysis pipeline. In the first half of the chapter, we deal mostly with the experimental background and the underlying questions of single cell dynamics of cancer cell under stress with focus on chemotherapeutic pressure. In this part, the highlight of the experiment, the microfluidic system for cell containment is elucidated. In conjunction with the system, some biomedical and biochemical aspects of the experiment are also mentioned. This includes the drug, the auxiliary chemicals used in the experiment and the cell lines probed. The second part deals mostly with the quantitative methods and algorithms used to process data into meaningful observations. This part is opened with definitions used in the methods section.

2.1 Experimental setting and data

2.1.1 Cell culture

To enable cross reference and comparability of experiment results, a model cell line is used: the acute monocytic leukemia (AML) cell line **AML-M5a MOLM-13** [62]. The line used in our experiment was derived from the cell line initially described by Matsuo et al. in 1997 [62]. In the paper, the authors developed the line from the peripheral blood of a relapse patient with AML of subtype FAB M5a, which is characterized by predominantly monoblastic leukemia cells visible in pap smear [63]. Due to extensive research done on the cell line and the well-explained mechanism of the cell line dynamics and response towards cancer medications, the cell line is an ideal candidate for *in vitro* study of monocytic differentiation, leukemogenesis and treatment dynamics [62, 64, 65].

For our experiment, the AML-M5a MOLM-13 cell line was cultured in Gibco® RPMI 1640 GlutaMAX medium, produced by Life Technologies [66]. The medium is a popular choice in human cell biology for both experiments and biological syntheses using human cells and their derivatives [67, 68]. It is pre-supplemented with stable form of L-glutamine to prevent ammonia buildup, a common and serious problem in cell culture due to its cell toxicity[69]. The medium is further supplemented with Gibco® Fetal Bovine Serum

(FBS), also offered by Life Technologies [70], as supplement for the AML-M5a MOLM-13 cell culture.

2.1.2 Microfluidics

In our cases, the microfluidics is designed by the Soft Condensed Matter Group at the Faculty of Physics at the Ludwig-Maximilians-Universität München. There are several papers related to the system. For example, in 2013, Marel et al. proposed the method of creating micro-wells for single-cell containment based on three-dimensional poly(ethylene glycol)-dimethacrylate (PEG-DMA) microstructures [71]. Later on in 2015, Sekhavati et al. publishes the first design of micro-trench arrays that are used in our experiment [39, 30].

In order to track non-adherent cells in a label-free manner over several generations, a set of micro-trenches ($30 \times 120 \mu\text{m}$) out of PEG-DA (Polyethylene(glycol) Diacrylate), which can accommodate four to six cells are designed and fabricated. The platform facilitates cell tracking leading to the observation of hundreds of families of cells, derived from one single mother at each case.

First, the SU-8 (MicroChem Corp, USA) wafer was fabricated in a cleanroom facility using a ProtoLaser LDI system (LPKF Laser & Electronika, Naklo, Slovenia), with a 375 nm wavelength laser and 1 μm spot diameter. Polydimethylsiloxane (PDMS) prepolymer solution is mixed with the crosslinker in a 10:1 ratio (w/w) (Sylgard 184, Dow Corning, USA) and then degassed under vacuum. PDMS is then purred on the SU-8 wafer, degassed and cured in 50 °C. The resulting PDMS stamp is peeled off the wafer and cut into appropriate shapes. The PDMS pieces, with 25 1/4 pillars in height, are activated with argon plasma and then immediately placed upside down on a glass coverslip silanized with TMSPMA (3-(Trimethoxysilyl)propyl methacrylate, Sigma-Aldrich). A solution of PEG-DA (Mn=258) containing 2% v/v of the 2-hydroxy-2methylpropiophenone (both from Sigma-Aldrich, Germany) is freshly prepared and then a drop is deposited at the edge of the PDMS stamp. The PDMS stamp is filled by capillary force induced flow. PEG-DA is then polymerized in an UV-ozone cleaning system (UVOH 150 LAB, FHR, Ottendorf, Germany). Next, the PDMS stamps are peeled off and the resulting micro-trenches of cross-linked PEG-DA are dried in an oven (Binder GmbH, Tuttlingen, Germany) overnight at 50 °C. Finally, the slides are sonicated with 70% ethanol and distilled water before a sticky slide is attached on top (8-well sticky slide, ibidi GmbH, Munich, Germany).

The design of the micro-trench and the schematic representative of cell tracking are shown in Figure 2.2. After the manufacturing process the structure is divided into two main parts:

- The micro-trenches are the smallest structure of the setting, measuring about $120 \times 30 \mu\text{m}$. The base of the trench is made of Polyethylene (glycol) Diacrylate (PEGDA), an inert substance commonly found as construction material in microfluidic system [39]. Each well contains about 2400 micro-trenches contained in one containment box.
- The sticky slide contain 8 wells where the wafer containing micro-trenches is located. The sticky slide chosen for containing the wafers holding the micro-trenches is ibidi® sticky-Slide 8 Well (see Figure 2.1). In the project, each cell treatment is isolated

in one containment box. This ensures the separations of different chemicals used in each treatment.

Due to limitations in the image capturing coverage area, each well is further divided into eight image positions. Each image position represents an area the camera captures completely. In each well there are 7 to 8 image positions and experiment with eight wells, which results in 63 image positions in total. Tables 2.1 and 2.3 show the list of all image positions in the experiment and their drug concentrations.

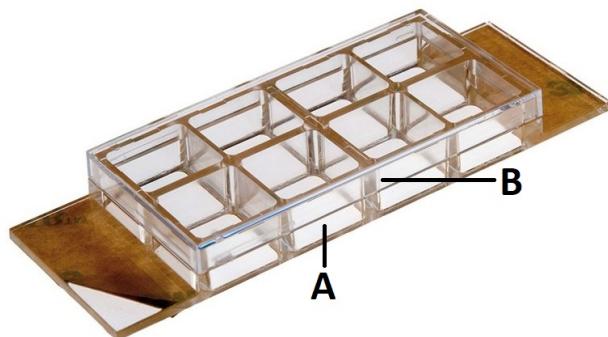


Figure 2.1: ibidi® sticky-Slide 8 Well. The base SU-8 wafer is located in each of the containment box (**A**): the SU-8 wafer (the part below the cap) is then fabricated in the surface of each containment box using nano photolithographic printing. The microfluidic system is then poured and stamped on top of the wafer. Note that each containment box is upside-open. (**B**): the cap is used to prevent the ingress of foreign materials into the medium. *Image taken and modified from ibidi GmbH's website.*

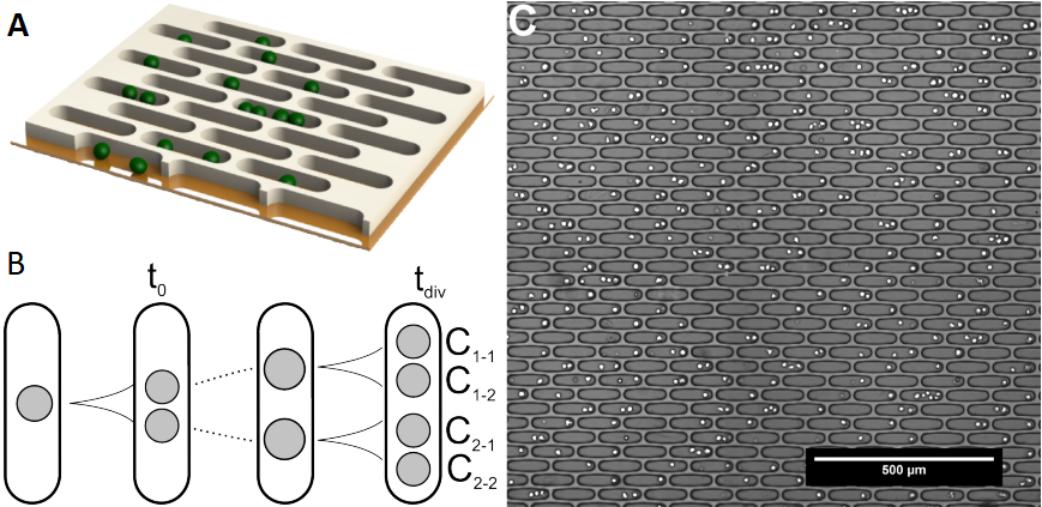


Figure 2.2: The structure of micro-trenches: (A) 3D model of a micro-trench surface with cells inside. (B) The schematic representation of a time-lapse in a trench. First, a singly-placed cell is tracked in a micro-trench. At time t_0 , the cell divides into two daughter cells. The two cells will be tracked until the time point each of the daughter cells divides at time t_{div} . Note the simplification of the sample. First, not every cell is singly-placed inside a trench. Indeed, not every trench is occupied by cells. Second, not every cell divides. Third, not every cell line observed has three generations in it. And finally, not all children divide synchronizedly. Indeed, this special case almost never happens in real life. (C) Sample view into the environment with cells occupying some micro-trenches. Here, the micro-trenches are $120 \mu\text{m}$ long and $30 \mu\text{m}$ wide. Note also the roundish characteristic of the cells taken in out-of-focus fashion. This improves the performance of the tracking algorithms. Figure taken from (Sekhavati, 2015) with our own text [30].

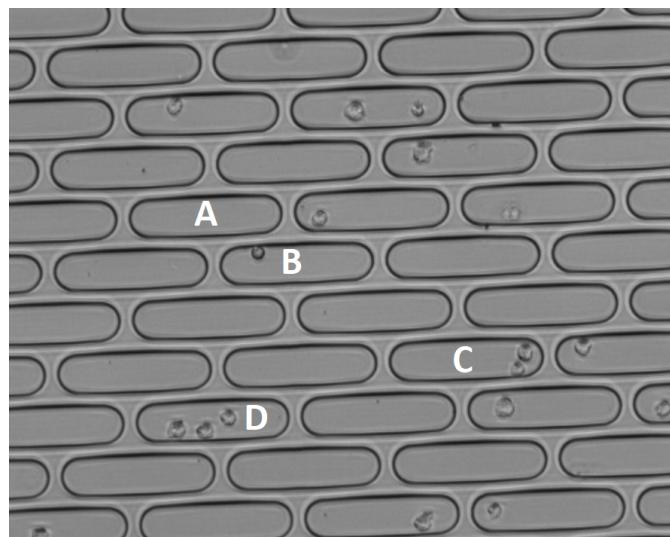


Figure 2.3: The typical view of a micro-trench setting. Some trenches contain no cell at all (A), several trenches contain exactly one cell (B) and a few more trenches contain two cells (C). In rare cases the trench may contain even more than two cells (D).

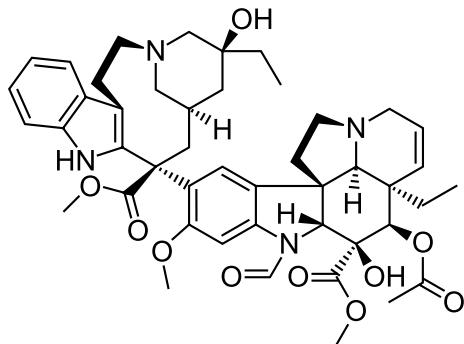
2.1.3 Cancer treatment regimes

As mentioned in previous chapters, the objective of the project pertains mostly the dynamics of single cancer cell under treatment. After mentioning the cell lines of interest (Chapter 2.1.1) and the microfluidic setup used to contain cancer cells in single-cell setting (Chapter 2.1.2), we arrived at the last aspect of the experimental setting: the chemical treatment used on the cells.

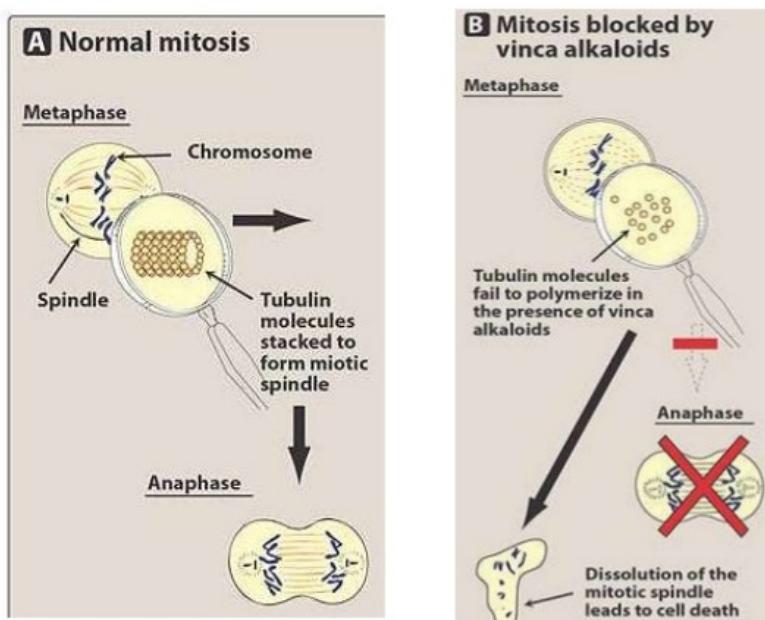
For cell cultures mentioned in the previous subsection, two drug treatment regimes are developed: Vincristine and Daunorubicin – both chemotherapeutic compounds well known for treating various kinds of cancer [72, 73, 74, 75].

Vincristine

Vincristine, a vinca alcaloid, is initially isolated from the Madagascar periwinkle *Catharanthus roseus* (basionym *Vinca roseus*, hence the name) [76]. It is mainly known as Tubulin polymerase inhibitor [77], a subclass of the mitotic inhibitor family of drugs [78]. Mechanistically, it prevents Tubulin polymerization in two ways. First, it binds elongating Tubulin polymer and reduces the affinity of the elongating polymer [79] towards Tubulin monomers therefore preventing the monomers to bind on the elongating polymer. Meanwhile, further elongation by the polymers is also prevented via allosteric inhibition. It has also been shown that Vincristine depolymerizes stable microtubuli in the axonal part of rats' neuronal cells [78]. Due to these mechanistic actions, the effect of Vincristine is most emphasized during the time of high Tubulin synthesis, e.g. during the separation of chromosomes in the Metaphase by means of tearing them with the simultaneous pulling and pushing mechanism of Tubulin poly- and depolymerization [80]. In the context of chemotherapy, Vincristine is often used as combination in CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) regime [81] against non-Hodgkin's lymphoma; MOPP [82], COPP [83] and BEACOPP [84] regimes against Hodgkin's lymphoma; and Stanford V regimes against acute lymphoblastic leukemia [85]. It is also used to certain degree as immunosupresant due to its mitotic inhibiting characteristics [86]. Figure 2.4 shows the structure of Vincristine and its mechanism of action.



(a)



(b)

Figure 2.4: (a) The molecular structure of Vincristine. (b) Visualization of Vincristine's mechanism of action. During normal metaphase, microtubuli elongate from centrosome towards cell equator while pulling apart fully replicated chromosomes. Vincristine and other vinca alkaloids prevent this from happening by competitive inhibition, allosteric inhibition and active depolymerization of extending microtubuli. Unsuccessful exit from the metaphase forces the cell to undergo programmed cell death.

Daunorubicin

Daunorubicin is initially isolated from bacterium *Streptomyces peucetius* [87, 88]. It is part of the anthracycline class of drug [75] extracted mainly from *Streptomyces* bacteria. Some well-known members of this class are Doxorubicin [89], Epirubicin [90] and Idarubicin [91]. Together, they are among the most effective cancer drugs ever deployed and are used towards more kinds of cancer than other group of chemotherapeutic agents [92, 93, 94]. Like many chemotherapeutic agents including Vincristine, anthracyclines attack cancerous tissues and cells by preventing their division [72]. Unlike vinca alkaloids

however, anthracyclines prevent the division by disrupting another mechanistic part of cell division: the DNA polymerization [75]. There are four ways anthracyclines disrupt DNA polymerization:

- Anthracyclines intercalate with base pairs involved in DNA polymerization thus preventing the extension of DNA strands [95].
- Anthracyclines covalently inhibit type II topoisomerase which is responsible for RNA and DNA supercoil relaxation [96]. Inhibition of type II topoisomerase causes supercoiled RNA and DNA to be inaccessible for initiation of duplication thus breaking the DNA [97].
- Anthracyclines induce oxidative stress on cancer cell organelles by generating free oxygen radicals. These radicals in turn damage DNA, proteins and cell membranes and initiate Caspase induced apoptosis [98, 99].
- Anthracyclines disrupt epigenetic, transcriptomic and DNA upstream regulations by removing histones from DNA strands [100]. This also exposes DNA strands to degradation factors such as DNA methylase [101] and oxidative damage [102]
- In the presence of formaldehyde, anthracyclines cross-link with the DNA covalently, creating cytotoxin that will disrupt DNA from functioning properly [103].

In normal chemotherapeutic regimes, both Vincristine and Daunorubicin are prescribed intravenously to patients [104]. Needless to say, both drugs will disrupt both cancerous and healthy cells. The effect is however mostly felt in actively dividing cells and organs such as blood cells and hair follicles due to chemotherapeutic agents' highly disruptive effect during cell division as mentioned above [104, 105]. Figure [106] shows the structure of Daunorubicin and its mechanism of action.

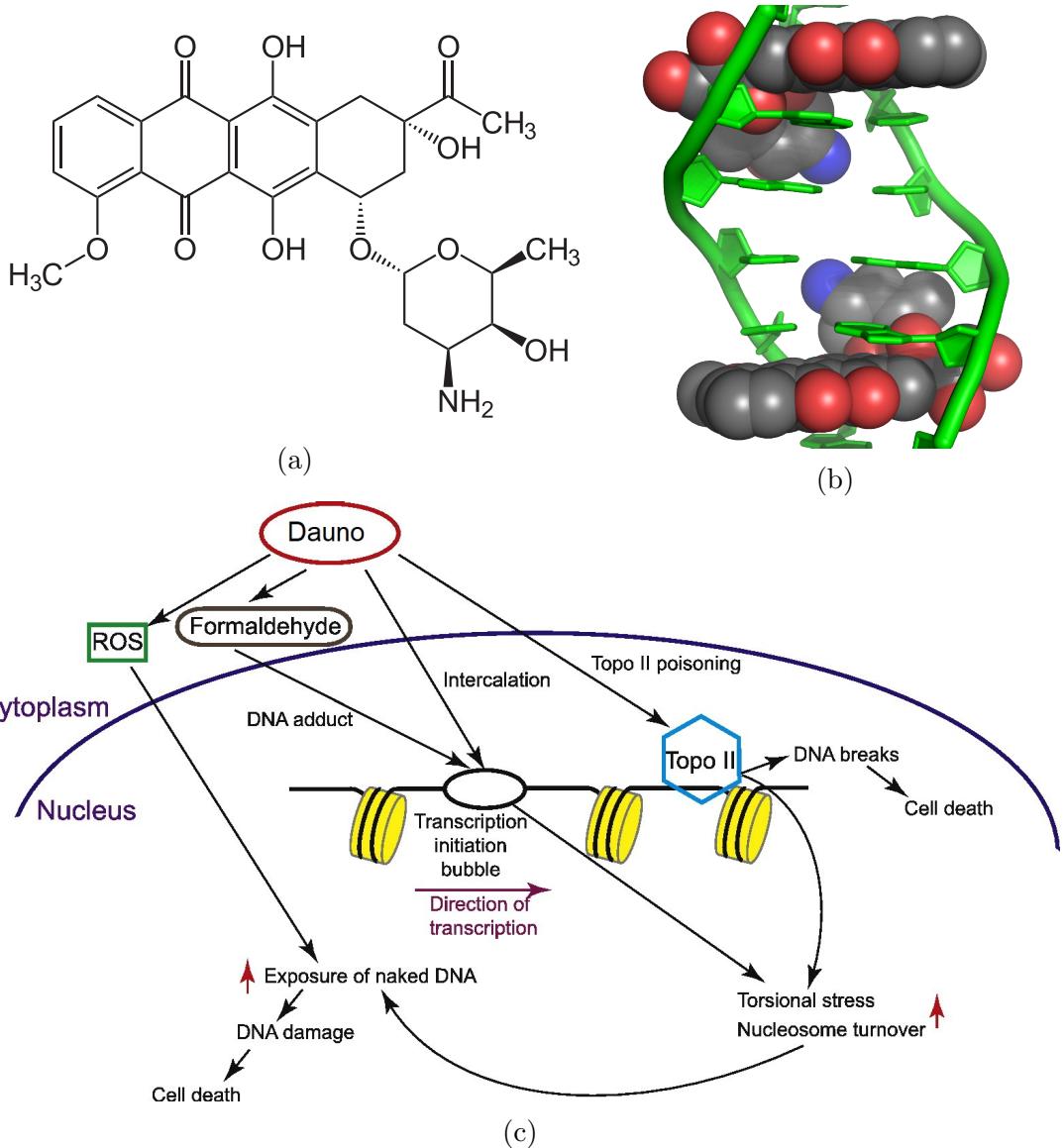


Figure 2.5: (a) The molecular structure of Daunorubicin. The planar structure of the drug fits into the small helix of the DNA strand, a phenomenon called "DNA intercalation" [107]. (b) Two anthracyclines intercalating with DNA double helix. (c) Schematic diagram of Daunorubicin's mechanism of action. From left to right: daunorubicin induces reactive oxygen species (ROS) which damage the DNA. Daunorubicin interferes with the DNA by (1) covalently crosslinking with the DNA mediated by formaldehyde and (2) intercalating DNA double helix. Daunorubicin also inhibits Topoisomerase II which prevents DNA supercoil's relaxation. Not shown in image: daunorubicin removes histone (shown in yellow in image) disrupting epigenetic regulations. Modified from (Yang et al., 2014 [106])

2.1.4 Cell death signal

As mentioned in Subsection 2.1.3, the introduction of cancer treatment disrupts mitotic cell activities which in turn activates cell death pathways. Two biochemical signals are selected to observe cell death caused by the disruption of mitotic process:

Caspase 3/7

Caspase 3 and 7 are both known as executioner Caspases in programmed cell death pathways [108]. For this project CellEvent Caspase3/7 vial from ThermoFisher Scientific is used [109]. Besides Caspase 3/7, the treatment consists also of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. This cell-permeable substrate is intrinsically non-fluorescent, because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of Caspase 3 or Caspase 7 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response. It has excitation/emission maxima of 502/530 nm (see Figure 2.6c).

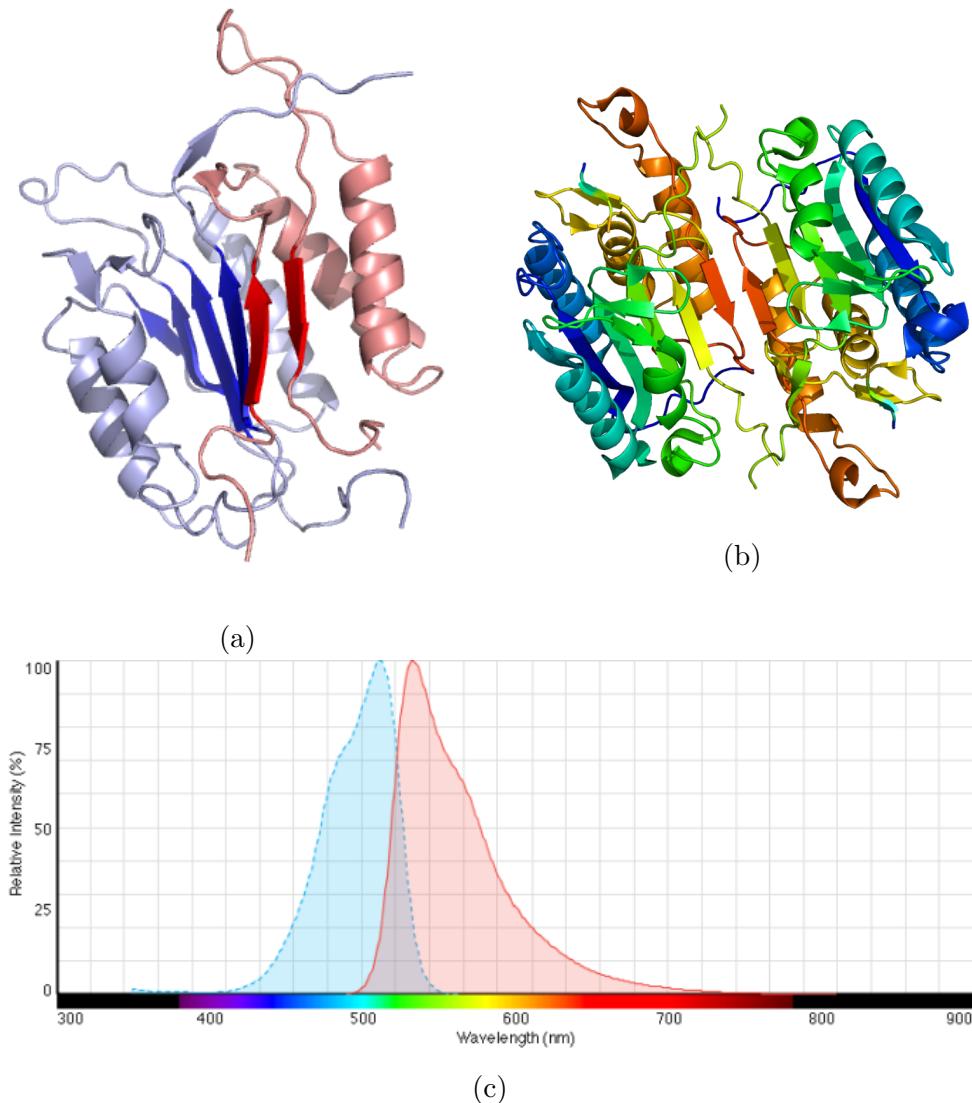


Figure 2.6: (a) The molecular structure of Caspase 3 heterodimer (b) The molecular structure of Caspase 7 homodimer. The p17 (light blue) and p12 (pink) subunits are shown. (c) The excitation (blue) and emission (red) spectra of Caspase3/7 vial used in the experiment. Notice the emission peaking around the 525-535 nm range in green channel. Image taken from ThermoFisher Scientific product specification page [109].

Propium Iodide (PI)

PI is a membrane impermeable marker and generally excluded from viable cells. Therefore it is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques. PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4-5 base pairs of DNA [110]. In aqueous solution, the dye has excitation/emission maxima of 493/636 nm (see Figure 2.7b). PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold. For this project PI vial from ThermoFisher Scientific is used [111].

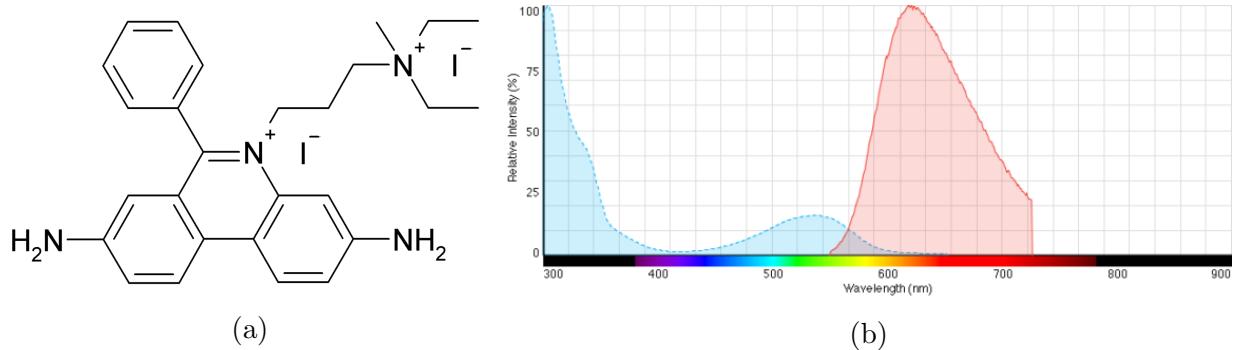


Figure 2.7: a) The molecular structure of Proprium Iodide (PI) (b) The excitation (blue) and emission (red) spectra of PI. Notice the peaking around the 630-650 nm range in red channel. Image taken from ThermoFisher Scientific product specification page [111].

2.1.5 Experiment setup and image capturing

In this project, two experiments are done:

Unsynchronized experiment.

In the unsynchronized experiment, all 8 wells of the ibidi® sticky-Slide 8 Well ('slide') are used with one micro-trench wafer in each well (see Subsection 2.1.2 for the description of the slide). Every well is poured with different concentrations of drugs. To cover a large concentration range, a logarithmic scale is used to select concentration starting with 1 nanomolar (nM) all the way to 1000 nM (see Table 2.1). The use of a logarithmic scale is also in line with the logarithmic nature of many enzymatic and molecular processes in cell biology [112, 113]. Following concentrations are used: 1 nM, 5 nM, 10 nM, 100 nM and 1000 nM of Vincristine; 10 nM and 100 nM of Daunorubicin; and no drug as control. The unsynchronized experiment concentration configuration can be seen in Table 2.1. The recording lasts for 45 hours. After 21 hours of recording, the drug treatment is introduced into each well. Table 2.2 shows the image capturing frequency for each image type.

Condition	Positions
A1: 1000 nM - VCR	1-8
A2: 100 nM - VCR	9-15
A3: 10 nM - VCR	16-23
A4: 1 nM - VCR	24-31
B4: 10 nM - Dauno	32-39
B3: 100 nM - Dauno	40-47
B2: 0 nM - VCR	48-55
B1: 5 nM - VCR	56-63

Table 2.1: Configuration table of treatments for the synchronized experiment and their positions in the well.

Image type	Purpose	Δ_t
In-focus brightfield (BF) image	Sanity check, micro-trench masking	30 minutes
Out-of-focus BF image	Image tracking, image analysis	15 minutes
Red fluorescence channel (PI)	Image tracking, image analysis	15 minutes
Green fluorescence channel (Csp3/7)	Image tracking, image analysis	15 minutes

Table 2.2: Image capture frequency of the experiment.

Synchronized experiment

The main difference between unsynchronized and synchronized experiments is the synchronization step. This process involves arresting cell cycle with the so-called "double thymidine block" protocol [114], which arrests cell development at G_1/S -phase by stopping DNA synthesis using double thymidine block, a well known DNA synthesis inhibitor [115]. Cells were released 3 h before the start of imaging and seeded in micro-trenches. The drug was added just before starting imaging

Like in the unsynchronized experiment, several concentration levels are tested in this experiment (see Table 2.3). The image capturing starts right after the cells are synchronized and lasts 24 hours. The image capturing frequency is exactly the same as in the unsynchronized experiment (see Table 2.2).

Condition	Positions
A1: 1000 nM - VCR	1-8
A2: 100 nM - VCR	9-15
A3: 10 nM - VCR	16-23
A4: 1 nM - VCR	24-31
B4: 10 nM - Dauno	32-39
B3: 5 nM - VCR	40-47
B2: 100 nM - Dauno	48-55
B1: 1000 nM - Dauno	56-63

Table 2.3: Configuration table of drug treatments for the synchronized experiment and their positions in the well.

Image capturing

Imaging was performed under an inverted Nikon Ti Eclipse microscope with a motorized stage (Tango XY Stage Controller, Märzhäuser Wetzlar GmbH & Co. KG, Germany), a CFI Plan Fluor DL 10X objective, a pco.edge 4.2 Camera (PCO AG, Kelheim, Germany) and a Lumencor Sprectra LED fluorescence lamp. For detection of the Caspase-3/7 and the PI marker, the following filters were used respectively: 474/27 nm and 554/23 nm excitation filters and 515/35 nm and 595/35 nm emission filters. Brightfield out of focus ($-20\ \mu\text{m}$) images were taken every 10 minutes and in-focus brightfield and fluorescence images every 30 minutes for 48 hours. Vincristine or Daunorubicin were added after 20 hours from the beginning of the imaging. During the recording samples were kept at a constant temperature of 37°C and CO₂ using an Okolab heating and CO₂ 2 box (OKOLAB S.R.L., NA, Italy). For the synchronized population, the double thymidine block protocol was followed. Briefly, MOLM-13s cells at the exponentially growing phase were incubated in blocking medium (culture medium supplemented with 2 mM Thymidine (CAS 50-89-5, Calbiochem®, Germany)) for 24 hours. Cells were then released and incubated in culture medium for 8 hours and finally incubated in blocking medium for 12 hours. After 2 hours, the synchronized population was seeded in the slide bearing the micro-trenches together with the markers and drugs at the same conditions as the unsynchronized population, and imaged for 24 hours.

2.2 Methods

This section contains formal definitions and methods used in this thesis.

2.2.1 Image channels and encoding

Several image standards and encodings are being processed in our pipeline. We start from the raw image captured by the camera going down to processed images (See Subsubsection 2.1.5 for detailed protocols on image acquisition). There are two brightfield channels (in-focus and out-of-focus channel) and two fluorescent channels (red and green) in this project. Out-of-focus brightfield images are used to track cells as their unsharpness can be exploited through image processing creating robust patch of cells, as shown by Buggenthin [116]. The red and green fluorescent channels capture fluorescence emission in 620-750 nm and 495-570 nm respectively (see Subsection 2.1.4).

- In-focus brightfield image, the focal distance of the camera d_f is exactly the same as the distance of the camera to the center of the cell d_m , i.e. $d_f = d_m$.
- In out-of-focus brightfield image, the difference of focal plane and center of the cells, $d_f - d_m$, influences the quality of the image in several ways (see Figure 2.8). It was determined that the ideal image for segmentation came from the setting with $d_f - d_m = -20\mu\text{m}$.
- The red fluorescent channel is used to capture emission from PI activation due to PI's DNA binding emission (see Subsection 2.1.5).
- The green fluorescent channel is used to capture emission from caspase3/7 activation due to DAVD peptide cleavage (see Subsection 2.1.5).

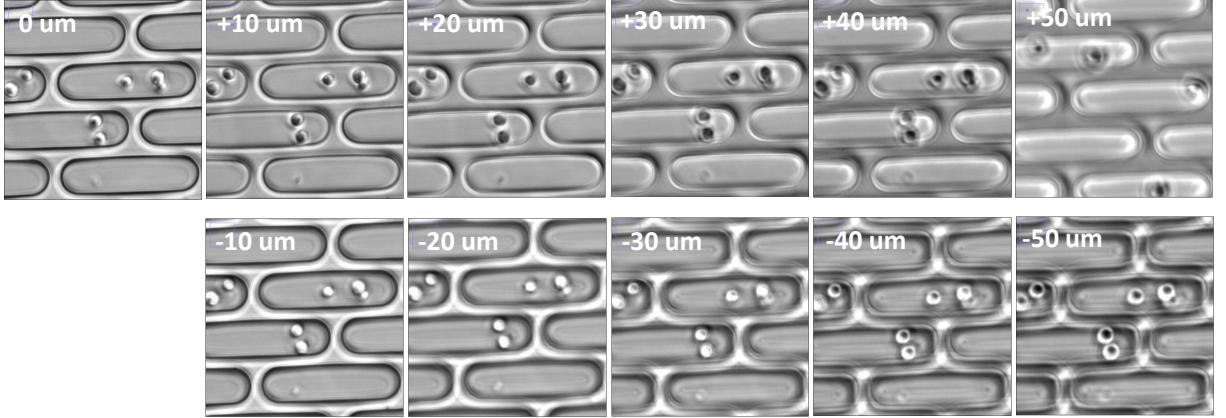


Figure 2.8: In-focus image and out-of-focus brightfield images taken at various focus levels. In the first row are images focused behind the microfluidics system while in the second row are images focused before the system.

The camera records TIFF images (see Appendix ?? for the detailed protocols) [117]. Each recorded stack consists of slice with one slice representing a single image capture at a time point. Each stack is encoded using RGB color model. This is also the case for the fluorescent image in which each slice shows the intensity in corresponding color channel (red, green or blue) as a monochromatic RGB image.

RGB color model represents the pixel as a combination of red, green and blue color. This encoding is able to represent any human visible color and is sufficient enough for most use cases [118, 119]. The most commonly used RGB encoding is the 8 bit encoding. Here, each pixel is represented as an RGB pixel having red, green and blue color values ranging from 0 to 255. Mathematically, this means that each pixel $M_{t,x,y}$ of a slice captured at time t can be represented as a triple, i.e.,

$$M_{t,x,y} := (M_{t,x,y,R}, M_{t,x,y,G}, M_{t,x,y,B}) \in [0 : 255]^3 \quad (2.1)$$

for 8-bit RGB encoding. Consequently, a slice M_t captured at time t of width w and height h is a 3-dimensional matrix of dimension $w \times h$, i.e.

$$M_i \in p_c^{w \times h \times 3}$$

with $p_c \in [0, 255]$. A stack (a sequence of slices) M of n slices is in turn a 4-dimensional matrix:

$$M \in p_c^{n \times w \times h \times 3}$$

To convert the RGB value of a pixel $M_{x,y}^a$ from one bit-encoding to another $M_{x,y}^b$, linear conversion is used:

$$M_{x,y}^b = (\lceil M_{x,y,1}^a \frac{2^b}{2^a} \rceil, \lceil M_{x,y,2}^a \frac{2^b}{2^a} \rceil, \lceil M_{x,y,3}^a \frac{2^b}{2^a} \rceil) \quad (2.2)$$

where a and b refer to the bit length of the source and target encoding respectively (commonly known as bid depth). Commonly used depths are 8, 16 (high color format), 24 (true color format) and 48 bits (deep color format) [120, 121, 122].

Sometimes, other type of encodings like grayscale and 1-bit monochrome are used. A grayscale image essentially shows the intensity of the pixels in an image. A grayscale

slice G can be represented as a matrix of integer, i.e. $G \in p_c^{w \times h}$. Like RGB image, linear scaling can be applied to transform grayscale images across bit depth:

$$G_{x,y}^b = \lceil G_{x,y}^a \frac{2^b}{2^a} \rceil \quad (2.3)$$

An RGB image can be transformed to a grayscale image by combining the intensity from every channel:

$$G_{x,y} = \frac{M_{x,y,1} + M_{x,y,2} + M_{x,y,3}}{3}$$

This however does not reflect human perception of light, as human eyes' spectral sensitivity is not uniform across the color spectrum [123]. Indeed, as Osorio and Vorobyev shows in 2005, each species has its own specific spectral sensitivity distribution [124], as can be seen in following figure:

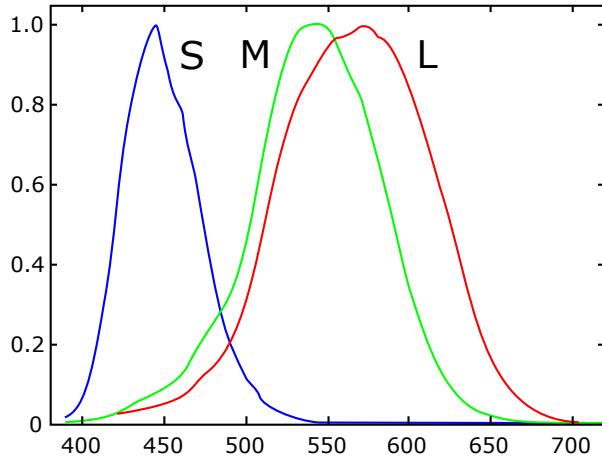


Figure 2.9: Spectral sensitivity of human. Note that the sensitivity distribution is maximum normalized. Each curve shows the relative sensitivity of each type of human cone cells (S, M and L types). The curve color represents the dominating absorbed color range of each cell type.

There are some conversions published on transforming RGB value to human intensity perception based on the measured spectral sensitivity [125, 126, 127]. Among the most commonly used is the *BT.601* standard from International Telecommunication Union (ITU). It recommends the following luminosity weight for RGB to grayscale conversion [126]:

$$G_{x,y} = \frac{0.299M_{x,y,R} + 0.587M_{x,y,G} + 0.114M_{x,y,B}}{3} \quad (2.4)$$

For following image:

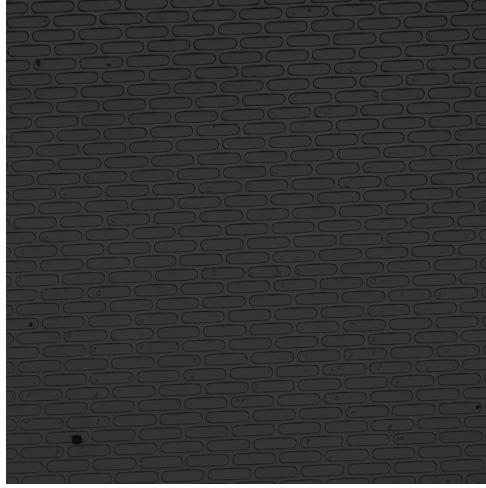


Figure 2.10: The in-focus image position 41 from unsynchronized experiment.

using Equation 2.4 to convert RGB to intensity , we can convert the image into grayscale image representing the intensity. The grayscale image has the intensity distribution as shown in following histogram:

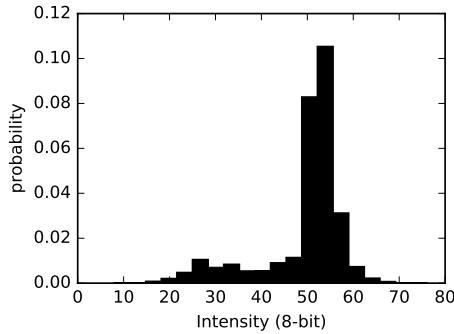


Figure 2.11: Normalized distribution of intensity of Figure 2.10.

Reverting back a grayscale image to an RGB image in turn only consists of applying grayscale value to each color component:

$$M_{x,y} := (G_{x,y}, G_{x,y}, G_{x,y})$$

A 1-bit monochrome image is encoded as a binary matrix:

$$B \in p_b^{w \times h} \wedge p_b \in \{0, 1\}$$

This encoding is useful when not using not the complete information of the image but rather separation of interesting parts in the image, since since it requires less memory (1 bit per pixel vs 24 bits per pixel of normal RGB image) and enables bitwise operation of the CPU which takes constant time [128]. Some use cases are for example region of interest (ROI) bounding and contour and boundary visualization [129].

The conversion from RGB to binary images can be done by defining a cutoff value c on one of the channels, e.g. for red channel, following conversion can be used:

$$B_{x,y} = \begin{cases} 1 & \text{if } M_{x,y,R} > c \\ 0 & \text{else} \end{cases}$$

Note that, a bit encoding of an image refers to unsigned encoding. Thus, an m -bit encoding allows value ranging from 0 to $2^m - 1$.

2.2.2 Intensity, brightness and contrast

Intensity, brightness and contrast can be modified to show and hide parts of an image. In this project, many image processing steps utilize the modification of them. Previously we defined intensity in image encoding. In this subsection we will define brightness and contrast and their adjustment.

Mathematically, we can represent brightness and contrast adjustment as a mapping between one domain to another. Let $l(M_{t,i,j})$ be a function that maps the RGB value of a pixel $M_{t,i,j}$ to its corresponding intensity I_t . Conveniently, we can use ITU's *BT.601* standard formula written in Subsection 2.2.1 to convert an RGB to grayscale image.

We then define the $I_t : [0 : w] \times [0 : h] \rightarrow \mathbb{N}_0$ function that maps the coordinate of a pixel to its corresponding intensity value i.e.

$$I_t(i, j) := l(M_{t,i,j})$$

Note that the codomain of the function I_t depends on the encoding used. For a 8-bit encoding this will be then $[0, 255]$.

An intensity transformation J_t is then defined as a linear transformation of I_t with **gain** and **bias** parameters α and β respectively:

$$J_t(i, j) := \alpha \cdot I_t(i, j) + \beta \tag{2.5}$$

with $\alpha > 0$ [130]. The gain and bias parameters are also known as **contrast** and **brightness** parameters. In this regard, increasing/decreasing brightness is equivalent to increasing/decreasing β . The same thing also applies for the contrast parameter, increasing α will increase the contrast of the image.

Predictably, doing transformation using transformation function J_t will inevitably cause the resulting intensity to be outside of the allowed value range $[0 : 2^m]$ for m -bit encoding. To understand this situation, we first have to consider the boundedness of eyes perception. The argumentation for boundedness can be shown by realizing that the excitation of a neuron follows a sigmoid function [131]. Thus given no impuls the neuron will stay in ground state, while very large impulse is bounded due to biochemical constraint of a neuron. Mapped in the context of a sigmoid function, a steady state corresponds to 0 while asymptotically unlimited excitation corresponds to 1. In our context, a non-excited state corresponds to 0 intensity while full-excitation corresponds to $2^m - 1$ intensity. Thus, the Equation 2.5 can be bounded by introducing upper and lower bounds of 0 and $2^m - 1$, i.e.

$$J_t(i, j) := \begin{cases} 2^m - 1 & \text{if } \alpha \cdot I_t(i, j) + \beta > 2^m - 1 \\ 0 & \text{if } \alpha \cdot I_t(i, j) + \beta < 0 \\ \alpha \cdot I_t(i, j) + \beta & \text{else} \end{cases}$$

We can for example, transform Figure 2.10 using $J_t(i, j)$ with $\alpha = 1$ and $\beta = 100$. The transformed image can be seen in Subfigure 2.12a with Subfigure 2.12c shows the intensity distribution of the transformed image. We can also try to modify α . Changing α to a value larger than 1 will scale the image's intensity and emphasize/de-emphasize image contrast. Subfigure 2.12c shows the transformed image for $\alpha = 3$ and $\beta = 0$. Subfigure 2.12d shows the intensity distribution of the transformed image.

See for example following image:

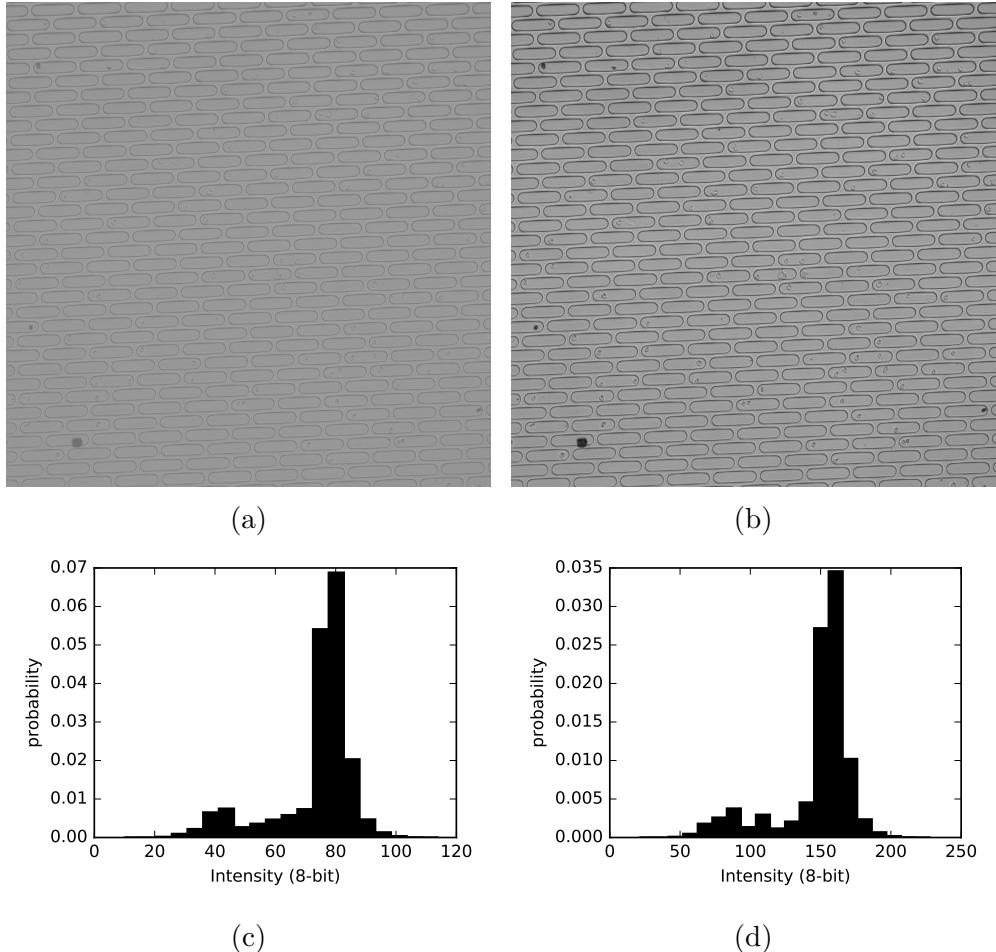


Figure 2.12: (a) The in-focus image position 41 from unsynchronized experiment transformed with $\alpha = 1$ and $\beta = 100$. (b) The in-focus image of position 41 from unsynchronized experiment transformed with $\alpha = 3$ and $\beta = 0$. Notice the contrast has increased significantly, especially around area with high intensity differential such us the margin of a micro-trench and the dark spot in the lower left part of the slice. (c) Normalized distribution of intensity of (a). (d) Normalized distribution of intensity of the (b). Note that the difference in height across bins in two histograms is mainly caused by the definition of bins in the histogram function.

Visually, brightness describes the level of visibility of a pixel. Adjusting β shifts the entire intensity distribution to the right by β ¹. On the other hand, contrast can be understood as the distance of closely resembling pixel [129]. Two pixels which are very similar will look very different given a high contrast.

¹Note the wording. For negative β , the shift is negative to the right, i.e. to the left

2.2.3 Robust automatic threshold selection (RATS)

Robust Automated Threshold Selection (RATS), based on description by Wilkinson and Schut [132], computes a threshold map for an image based on two criteria: pixel values, and pixel gradients [133].

The gradient is computed using the so-called Sobel kernel [134] commonly used in computer vision for edge detection as it particularly emphasizes edges in an image upon transformation. It is a discrete differential operator which computes an approximation of the gradient of the intensity function J .

Originally, the Sobel gradient $\mathbb{G}_{x,y}$ of coordinate (x, y) is defined as

$$\mathbb{G}_{x,y} := \sqrt{\Delta_x^2 + \Delta_y^2}$$

Whereas Δ_x and Δ_y are defined as

$$\Delta_x = \begin{bmatrix} +1 & 0 & -1 \\ +2 & 0 & -2 \\ +1 & 0 & -1 \end{bmatrix} * \mathbf{M}_t$$

and

$$\Delta_y = \begin{bmatrix} +1 & +2 & +1 \\ +0 & 0 & 0 \\ -1 & -2 & -1 \end{bmatrix} * \mathbf{M}_t$$

with \mathbf{M}_t denoting the image at time t and asterisk symbol $*$ denotes a two dimensional signal processing convolution [135]. For example, for a 3×3 matrix,

$$X_{3 \times 3} = \begin{bmatrix} 1 & 2 & 3 \\ 4 & 5 & 6 \\ 7 & 8 & 9 \end{bmatrix}$$

and Δ_x , the value of the middle pixel $(2, 2)$ after evaluating the convolution $\Delta_x * X_{3 \times 3}$ is,

$$\left(\begin{bmatrix} +1 & 0 & -1 \\ +2 & 0 & -2 \\ +1 & 0 & -1 \end{bmatrix} * \begin{bmatrix} a & b & c \\ d & e & f \\ g & h & i \end{bmatrix} \right) [2, 2] = i \cdot 1 + 0 \cdot h - 1 \cdot g + 2 \cdot f + 0 \cdot e - 2 \cdot d - 1 \cdot g + 0 \cdot h - 1 \cdot a$$

It has been shown, however, that only taking the quadratic gradient yields comparable result [132]. The Sobel operator is thus defined as quadratic gradient in Fiji RATS plugin, i.e.

$$\mathbb{G}_{x,y} = \mathbb{G}_{x,y}^2$$

Pixels having gradients smaller than threshold $\lambda\sigma$ are then removed, where σ is the noise (generally, standard deviation of the expected background is used, e.g. standard deviation of the whole image) and λ is the scaling factor (Wilkinson determined $\lambda = 3$ to be a good approximation [132]). Visually, this step can be understood as removal of background noise from the image.

To refine the filtering further, iterative correction is applied. First, a quadtree construction is applied in an image. A quadtree is a tree-like data structure in which each

node has exactly four children (Finkel and Bentley, 1974 [136]). In the context of an image, an image is recursively separated into smaller quadtree until the standard deviation of the intensity in every quadtree is lower than a pre-defined limit (usually the standard deviation of the intensity of the whole image σ). Figure 2.13 gives an example on how a quadtree is constructed from an image.



Figure 2.13: Example of quadtree. The left side is the quadtree representation of original image on the right. *Creative Commons courtesy of anonymous.*

In the second step, we attempt to remove the filter locally. To do that, we first define a local threshold T_R within the smallest square. For a quadrant, the threshold is computed in following way:

$$T_R = \frac{\sum(g_{x,y} \cdot M_{t,x,y})}{\sum g_{x,y}} \quad (2.6)$$

Where $\sum g_{x,y}$ the sum of all children quadrants' gradient. For the smallest quadrant, only $g_{x,y}$ is used instead, i.e.

$$T_R = \frac{g_{x,y} \cdot M_{t,x,y}}{g_{x,y}} \quad (2.7)$$

This threshold is used to filter pixels within a quadrant. Pixels with gradient value smaller than T_R of a quadrant are then filtered out. If T_R is very low that no point in a quadrant is filtered out, the T_R of the parent is taken instead. This step is iterated until a parent square with at least one filtered pixel is reached. Visually, the refining step is equal to filtering out the area of an image in which the pixels are mostly noise. In the initial global filtering process, many noisy pixels might not be filtered since they happen to have gradients larger than $\lambda\sigma$. Using this bottom-up approach these pixels will be eventually filtered out.

2.2.4 Contour holes filling

Filling micro-trench holes to create the contour is done in two steps:

1. Recognition of the outer part of micro-trench contours, followed by

2. Filling of such contour with a new neutral value (black).

Following image explains the definition of contours and their hierarchy:

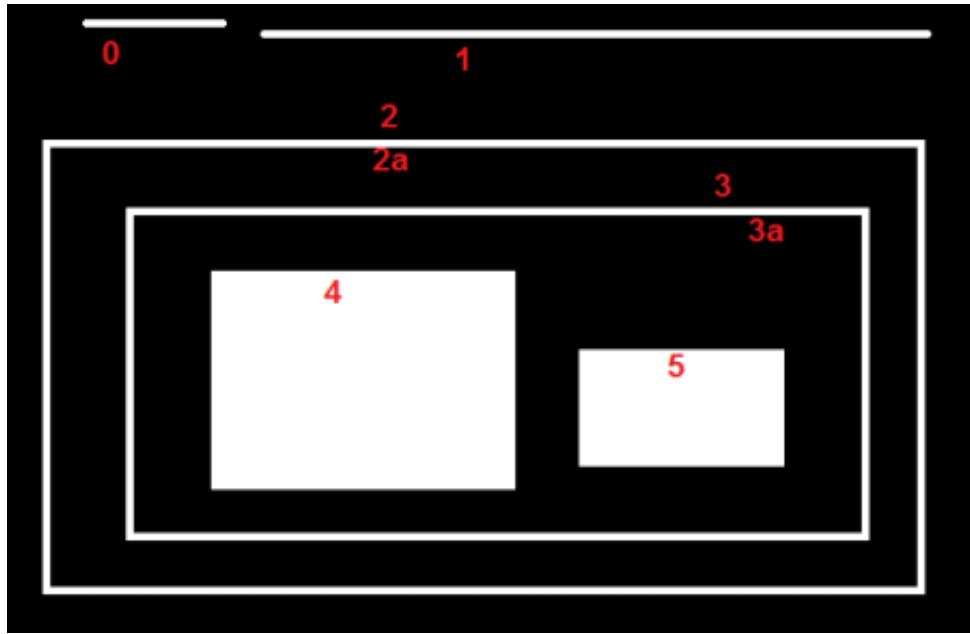


Figure 2.14: Example of contours and their hierarchy. **Contours 0, 1 and 2** are **outermost contours** and of **hierarchy-0**. **Contour 2a** is **child contour** and of **hierarchy-1**. **Contour 3** is child contour of **contour 2a** and of **hierarchy-2**. **Contour 3a** is child contour of **contour 3** and of **hierarchy-3**. Both **contours 4** and **5** are children of **contour 3a** and of **hierarchy-4**. All contours with same hierarchy are topologically equal. Contour Adopted from OpenCV tutorials by Bradski and Kaehler [137].

There are several well known algorithms for hierarchical contours recognition. In our case, the algorithm of Suzuki and Abe [138] is used. The algorithm works by walking along the border between two regions of different color. The border walking process is done hierarchically creating tree-like dependency structure of contours. We then take the outermost (hierarchically the highest) contours which are then filled. The filling in turn is simply done by assigning neutral value in every pixel located inside every outermost contour.

2.2.5 Gaussian blur

Gaussian blurring in the context of image processing is the process of applying Gaussian noise on an image. The Gaussian function $G : \mathbb{R} \rightarrow \mathbb{R}$ is a function defined as

$$G(x) = \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\frac{x^2}{2\sigma^2}}$$

In 2 dimensional setting, we extend the function to $G : \mathbb{R} \times \mathbb{R} \rightarrow \mathbb{R}$ defined as

$$G(x, y) = \frac{1}{2\pi\sigma^2} e^{-\frac{x^2+y^2}{2\sigma^2}} \quad (2.8)$$

Applying this function on a 2D image creates concentric circles centered around the center of the distribution with values around the center distributed normally. This means that, for an arbitrary σ and a uniform image with the intensity of n in every pixel, convolving the image with Equation 2.8 centered at point (i, j) yields a transformed image with the transformed intensity I^G of each point distributed normally around (i, j) with scaling of n and standard deviation σ :

$$I^G(x, y) = n \cdot \frac{1}{2\pi\sigma^2} e^{-\frac{(x-i)^2+(y-j)^2}{2\sigma^2}} \quad (2.9)$$

The idea of transforming an image with one selected center point is not very practical as the pixels far away from image will have intensity of almost zero. We can however, use the fact that the intensity of pixels around the center point fall exponentially the further the pixels are away to introduce a dependency of each pixel on its surrounding environment. This will introduce a blur due to pull-down effect of the neighboring pixels. Hence the name **Gaussian blurring**.

Mathematically, we can express the Gaussian blurring process as convolving the pixels around a pixel (x, y) and take the average of the convoluted image as the new intensity value in (x, y) . Using the continuous Gaussian intensity transformation defined in Equation 2.9, the Gaussian convolution function is defined as following:

$$I^{conv_G}(x, y) = \frac{\sum_{x-\lfloor d/2 \rfloor < i < x+\lfloor d/2 \rfloor} \sum_{x-\lfloor d/2 \rfloor < j < x+\lfloor d/2 \rfloor} I^G(i, j)}{(\lfloor d/2 \rfloor)^2}$$

with d denoting the L_1 distance of the blur. We can furthermore refine the convolution further by using L_2 distance (i.e. radius) instead:

$$I^{conv_G}(x, y) = \frac{\sum_{(i,j) \in C} I^G(i, j)}{\|C\|} \quad (2.10)$$

for $C := \{(i, j) \text{ where } d_{L_2}((x, y), (i, j)) \leq d\}$. It can be seen from the equation that increasing the radius d increases the pull-down effect on the pixel and hence increases the smoothness of in the image. We exploit this property to create regional gradient which will be used to normalize an image by its background gradient. By dividing the value of each pixel by its convoluted value we can correct the image by its global background noise. The Gaussian blur-corrected intensity I^{c_G} is thus defined as

$$I^{c_G}(x, y) = \frac{I(x, y)}{I^{conv_G}(x, y)} \quad (2.11)$$

Visually, the method can be understood as low pass filter. It removes the higher frequency 2D signals, i.e. the value with high local variance, resulting in an image with lower fidelity and local variance.

2.2.6 Contrast limited adaptive histogram equalization (CLAHE)

CLAHE, initially developed by Karel Zuiderveld in 1994 [139], is an instance of the class of algorithms called adaptive histogram equalization (AHE) commonly used to improve contrast in an image. An AHE algorithm generally works by transforming each pixel with a transformation function derived from the neighboring region instead of the whole image.

By doing this, an AHE algorithm can account for the variation of brightness and contrasts in areas of an image [140].

The distinction of CLAHE among other AHE algorithms is the contrast limiting property of the algorithm. Given a contrast distribution of an area around a given point, CLAHE conducts following steps:

1. First, the algorithm takes a predefined clipping value of the intensity histogram of the area around a center point.
2. The algorithm then calculates the intensity histogram of the area.
3. For some intensity values there will be more pixels than allowed with the intensity values. We call these intensity values the clipping intensity area.
4. Adapt the intensity histogram by reassigning the intensity of some pixels in the clipping intensity area uniformly across the range of intensity in the image (see upper part of Figure 2.15).
5. Repeat until for randomly selected areas in the image the clipping value constraint is satisfied, i.e. for every randomly chosen pixel, the intensity histogram contains no clipping intensity area.

Figure 2.15 visualizes the step 2, 3 and 4 of the algorithm description above. As can be seen in the area intensity CDF in the lower part of Figure 2.15, the algorithm results in a more equalized distribution of intensity. Visually, this increases the contrast and in turn sharpens the image. Figure 2.16 shows an area of the well before and after the application of CLAHE. Note the increased brightness and emphasized convolution effect around the micro-trench margin area.

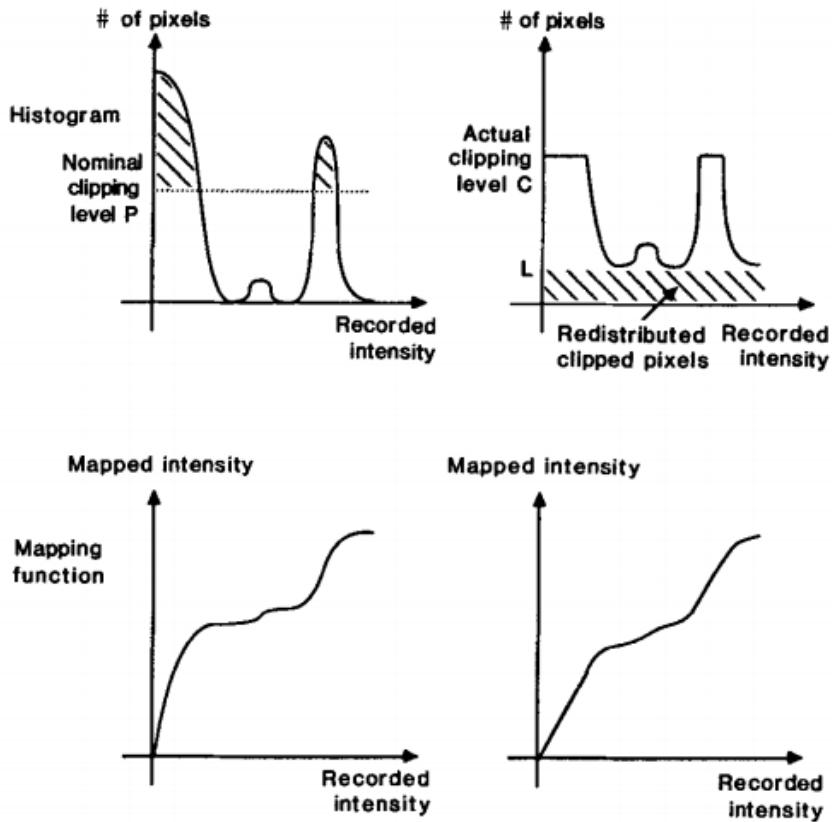


Figure 2.15: Visualization of the CLAHE algorithm taken from Pizer, Zuiderfeld et al., 1987 [140]. Note the smooth intensity CDF function (lower part of the figure) after reassigning some pixels with pixel values above the clipping value.

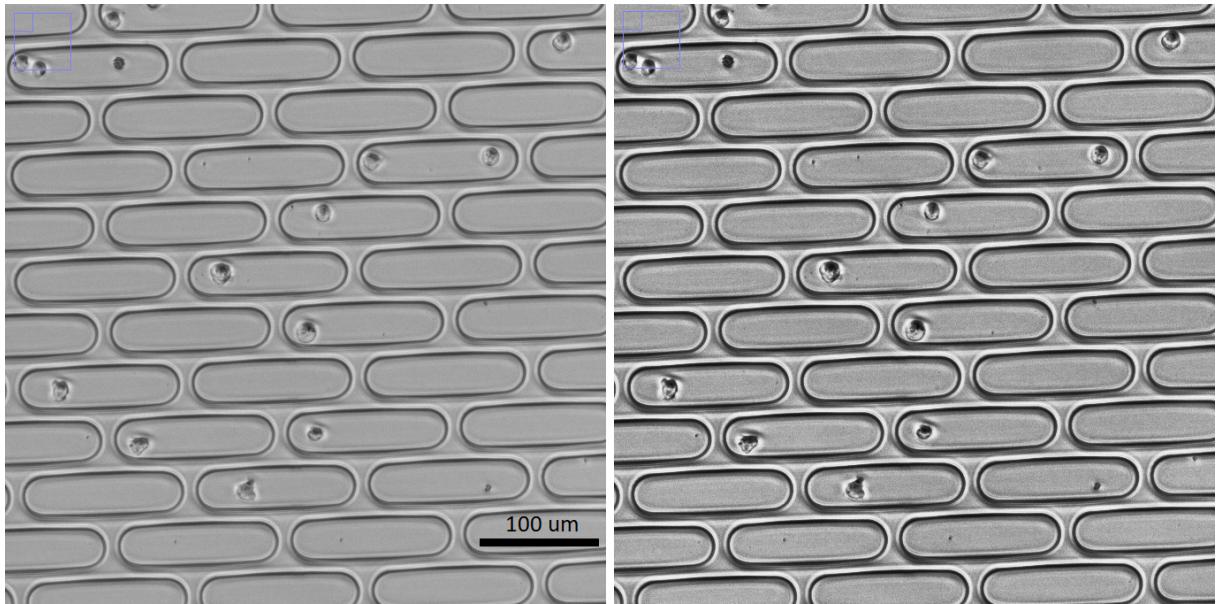


Figure 2.16: Example of the CLAHE algorithm applied on an image. The upper left corner of the in-focus brightfield channel of the image position 41 at time $t = 0$: before (left) and after (right) the application of CLAHE on the image. Scale bar on the left figure is $100\mu M$ long.

2.2.7 Subtraction by pixel-wise mean intensity

To subtract an RGB image at time M_t by its pixel-wise mean intensity $\langle M \rangle$, the average intensity for each pixel across all time points is calculated for a pixel. For a stack with n images, we can simply define the pixel-wise mean of the R channel for pixel (x, y) as following:

$$\langle M \rangle_{x,y,R} := \frac{\sum_{1 \leq t \leq n} M_{t,x,y,R}}{n} \quad (2.12)$$

The same applies to $\langle M \rangle_{x,y,G}$ and $\langle M \rangle_{x,y,B}$. For a grayscale stack we define analogously:

$$\langle G \rangle_{x,y} := \frac{\sum_{1 \leq t \leq n} G_{t,x,y}}{n} \quad (2.13)$$

The pixel-wise subtracted value of R channel is thus defined as point-wise operation for each pixel,

$$M_{t,x,y,R}^m := M_{t,x,y,R} - \langle M \rangle_{x,y,R} \quad (2.14)$$

The same applies to $\langle M \rangle_{x,y,G}$ and $\langle M \rangle_{x,y,B}$. The transformed RGB value is thus defined as

$$M_{t,x,y}^m := [M_{t,x,y,1}^m; M_{t,x,y,2}^m; M_{t,x,y,3}^m] \quad (2.15)$$

As for a grayscale image we define following:

$$G_{t,x,y}^m := G_{t,x,y} - \langle G \rangle_{x,y} \quad (2.16)$$

In the context of the single-cell enclosure on a wafer, this means that in the parts of images in which only dynamic movement of cells are observed, the pixel-wise mean intensity value of dynamic parts will be very low. Subtracting every pixel the area from every time point with the average will barely affect the original intensity value. On the other hand, applying the method on areas with static objects such as micro-trench wall and its surrounding reduces each pixel of the area with exactly the same intensity value as it barely changes during the experiment. This will nullify the static parts to large extent.

This method can be improved by iteratively repeating the calculation of $\langle M \rangle$. This will remove periodically static parts or static parts that abruptly moved (due to sudden shift in the well with regard to the camera for example). We call this algorithm **k-subtraction by pixel-mean intensity**. The lower the

```

Data:  $M_t$ 
Result:  $M_t^m$ 
Parameters:  $k$ 
for  $i = 0$  to  $k - 1$  do
    calculates  $\langle M \rangle$  from  $M_t$  ;
     $M_t^m = M_t - \langle M \rangle$  ;
     $M_t = M_t^m$  ;
end
```

Output: M_t

Algorithm 1: The k-subtraction by pixel-mean intensity algorithm

2.2.8 Cell recognition

Due to the cells' roundish shape, the **blob detection** family of algorithm is well-suited for recognizing cells. In our pipeline, we use the Laplacian of Gaussian (LoG) detector method.

Laplacian of Gaussian (LoG) detector

Laplacian of Gaussian (LoG), also known as Marr-Hildreth-Opreator [141], is among the first and still the most popular method for detecting blobs [118]. It is characterized by applying the second derivative of the σ -scaled Gaussian to detect a blob in an image.

To derive the method we first consider a 2-dimensional Gaussian kernel function:

$$G(x, y) = \frac{1}{2\pi\sigma^2} e^{-\frac{x^2+y^2}{2\sigma^2}} \quad (2.17)$$

As the name suggests, the representation of LoG operator is obtained by applying the Laplace-Operator Δ on the Gaussian,

$$L(x, y) = \Delta G(x, y)$$

This can be expanded into,

$$\begin{aligned} L(x, y) &= \frac{\partial^2 G(x, y)}{\partial x^2} + \frac{\partial^2 G(x, y)}{\partial y^2} \\ L(x, y) &= -\frac{1}{\pi\sigma^4} e^{-\frac{x^2+y^2}{2\sigma^2}} \left(1 - \frac{x^2+y^2}{2\sigma^2} \right) \end{aligned}$$

Figure 2.17 shows the representation of the LoG operator in 2D. Notice the inverse hat characteristic giving it the nickname **Mexican hat operator**.

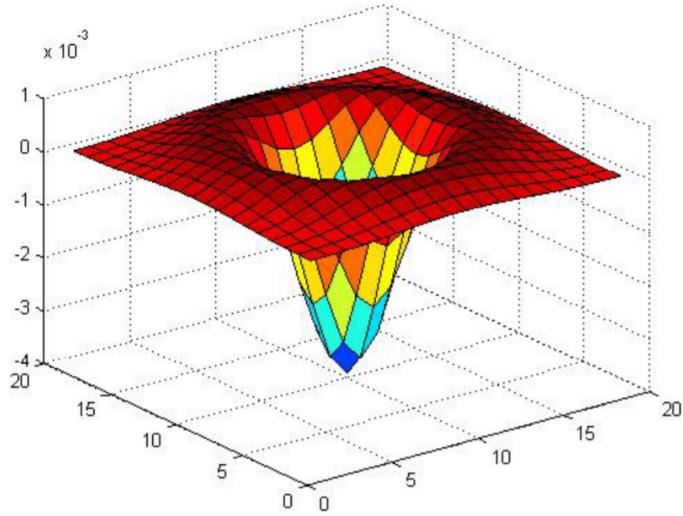


Figure 2.17: A LoG operator. Notice the lack of dimension in an operator as it transforms an image (in one space) into another image (still in the same space).

The operator is then applied on the two dimensional matrix of a grayscale image. To get maximum response from a blob, the zeros of the operator have to be aligned with

the circle to be detected [141]. Figure 2.18 shows an example of idealized circle with the diameter r and the corresponding response from the LoG operator on the surface of the circle. Applied to pre-processed out-of-focus image, LoG will return response with supremum in the center of cell sizes in similar fashion to the idealized circle.

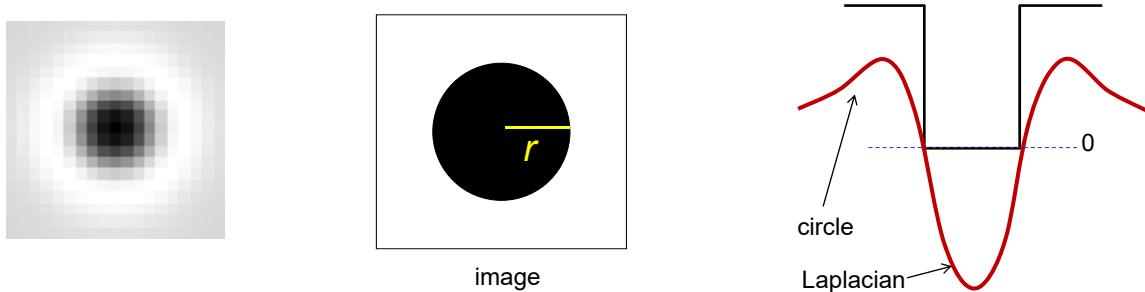


Figure 2.18: Example of applying the LoG operator on a 2D circle. The maxima will be achieved if the diameter of the LoG operator is roughly equal to the diameter of the cell. This however does not have to be the case. In case of non-perfect blob or blob with different diameter, local supremum can still be detected by the cell recognition algorithm [142].

2.2.9 Cell tracking

To track detected cells, the Linear Assignment Problem (LAP) tracker, by Jaqaman et al., 2018, is used. The method builds upon the cell recognition method explained in Subsection 2.2.8.

Linear Assignment Problem (LAP) framework of cell tracking

The algorithm follows closely the case of the linear assignment problem in bipartite graph. Given a bipartite graph $\mathcal{G}_b := \{\mathcal{G}_1, \mathcal{G}_2\}$ and the assignment cost function $c(v_i, v_j)$ for $v_i \in \mathcal{G}_1$ and $v_i \in \mathcal{G}_2$, find a bijection $f_{\mathcal{G}_b} : \mathcal{G}_1 \rightarrow \mathcal{G}_2$ so that the total cost function:

$$\sum_{v_i \in \mathcal{G}_1} c(v_i, f_{\mathcal{G}_b}(v_i)) \quad (2.18)$$

is minimized. As seen in Equation 2.18, the cost function and its corresponding constraint (bipartite assignment) is linear. Hence, the "linear" part of the assignment problem.

We can frame our cell tracking problem as a variant of the linear assignment problem. Recall that, for each slice, a set of blobs representing cells are detected. To track cells, each blob in a slice has to be connected with a blob in proceeding and succeeding slices (except in the case where a cell died).

Revisiting Subsection 2.2.8, it is obvious that upon cell detection the next step is to track single cell movements in a stack by assigning detected blobs \mathcal{G}_t at time t with the blobs \mathcal{G}_{t+1} detected at time $t+1$, for $t \in [1, n-1]$. To do that, first we define particle-to-particle cost function c . Jaqaman and his colleagues argued that, due to Brownian nature

of cell movement, the square distance of blobs or its derivation should be used as cost function [143]:

$$d(v_i, v_j) = \|v_i - v_j\|_2^2 \quad (2.19)$$

The algorithm is roughly divided into two main steps:

- Creation of track segments through frame-to-frame blobs linking.
- Gap closing and cell division inference to achieve the closing of the track segments.

Both steps are framed as linear assigned problems. In the first step, two consecutive slices M_t and M_{t+1} are optimized for the links. To do that, a $(m+n) \times (n+m)$ matrix \mathcal{C} is created, where m and n refer to the number of detected blobs in M_t and M_{t+1} respectively. The matrix contains four quadrants:

- The upper left quadrant ($m \times n$ elements) contains the costs of linking blobs in M_t to those in M_{t+1} , also known as the segment linking cost.
- The upper right quadrant ($m \times m$ elements) contains the costs of not linking blobs in M_t to any blobs in M_{t+1} , also known as the segment stop cost.
- The lower left quadrant ($n \times n$ elements) contains the costs of not linking blobs in M_{t+1} to any blobs in M_t , also known as the segment start cost.
- The lower right quadrant ($n \times m$ elements) is the auxiliary matrix used by the LAP framework as formalism for its algorithm. The matrix is created by transposing the upper right left quadrant and replacing all non-infinity cost with the minimal cost (by default 0).

The segment linking cost (upper left quadrant) is calculated as following:

$$c(v_k, v_l) = \begin{cases} (d(v_k, v_l) \cdot (1 + \sum_{\mathbb{f}} (3W_{\mathbb{f}} \frac{f_{\mathbb{f}1} - f_{\mathbb{f}2}}{f_{\mathbb{f}1} + f_{\mathbb{f}2}}))^2 & \text{if } d(v_k, v_l) \leq d_{max} \\ \infty & \text{else} \end{cases}$$

where \mathbb{f} refers to each feature penalty defined for the optimization and $W_{\mathbb{f}}$, $f_{\mathbb{f}1}$ and $f_{\mathbb{f}2}$ refer to the feature penalty factor, the value of feature \mathbb{f} of v_k and the value of feature \mathbb{f} of v_l respectively. Generally, only blob related features are used in the algorithm, e.g. total blob intensity, average blob intensity and minimum/maximum blob intensity. Note that:

- If the distance is larger than the pre-defined maximum distance d_{max} , the link is forbidden. A blocking cost (∞) is assigned between two blobs.
- If no penalty feature is introduced then the cost is simply the squared distance of two blobs.

The non-linking cost (the upper right and lower left quadrants) is calculated as follows:

$$c(v_k, v_l) = \begin{cases} 1.05 \cdot \mathbb{C} & \text{if } v_k = v_l \\ \infty & \text{else} \end{cases}$$

where C is the maximum value of the upper left quadrant of the matrix.

The optimization over the matrix is then solved with the Munkers & Kuhn algorithm [144], which solves the problem in cubic time $\mathcal{O}((m+n)^3)$. The algorithm returns assignment minimizing the sum of the assignment costs.

The interpretation of the cost functions is following: without any penalty, the optimization problem favors the solution which minimizes the sum of squared distance between two blobs. This is in line with previously mentioned assumption of the Brownian motion of cells. By adding feature penalties, we aim at favoring linking blobs that are more similar to each other. In brute single particle linking problems, spots are generally all the same, and they only differ by the coordinate. However, there is a variety of problems for which these feature penalties can add robustness to the tracking process. In our case for example, the cells might pose several features that change over time depending on the treatment and cell-dependent characteristics (the phenomenon we further investigate using machine learning methods, see Subsection 2.2.11 and Section 4.5).

Upon the linking of blobs into track segments, more refinement is then done to achieve globally optimal cell tracks configuration. To do this, three events are considered in our model:

- In case of *gap closing* events, the end of a track segment is linked to the start of another track segment.
- In case of *splitting* events, the start of one track segment is linked to non-terminal part of another track segment.

The matrix is created in similar fashion to the cost matrix in the first step, with the details best referenced directly to the article by Jaqaman et al. [143]. Also similar to the first part is the use of penalty features during segment creation. Unlike the first part however, the segment merging cost is blocking (∞) if two segments are separated by frames larger than pre-defined maximum number of frame gaps.

The optimal solution for the problem is again found using the Munkers & Kuhn algorithm [144].

2.2.10 Shift correction

Shift correction is done to correct the slice alignment within stacks. As mentioned in Subsection 2.1.5, the images were captured discretely at the frequency of $\frac{1}{10\text{min}}$ and $\frac{1}{30\text{min}}$ depending on the channel. During this time, the setup might move albeit very slightly causing very small shift. The bulk of the shift, however, happened during the introduction of the drug treatment. At this time, the drug is introduced to the medium using a pipette. The liquid released pushes the wafer somewhat causing noticeable shift in camera's field of view (see Table B.1 of Appendix B for the inferred shift in every image position).

Now, we consider the case in which the images are shifted in a time-lapsed movie during the introduction of drug treatment. No rotation of camera is assumed, hence there are only two degree of freedoms (vertical and horizontal). Thus, a shift can be defined as a vector movement \vec{v} of all points $x_{i,j} \in M_t$ in the time-lapse from time t to $t+1$. Given two degrees of freedom and discreteness of the problem due to pixel representation, the task is reduced to finding difference in x- and y-axis (δ_x and δ_y), so that the difference of transformed pixels at t and t_{i+1} are minimized, i.e.:

$$\arg \min_{\delta_x, \delta_y} \{d(M_t, M_{t+1}^{\delta_x, \delta_y})\}$$

Where $M_{t+1}^{\delta_x, \delta_y}$ are the entries of matrix M_{t+1} after applying the shift $\vec{v} := (\delta_x, \delta_y)^T$, i.e.

$$M_{t+1, x, y}^{\delta_x, \delta_y} = M_{t+1, x-\delta_x, y-\delta_y}$$

and the distance function d is defined as all-channel all-pixel sum of differences between two images:

$$d_{RGB}(M_i, M_j) = \sum_{c \in \{R, B, G\}} \sum_x \sum_y |M_{i,x,y,c} - M_{j,x,y,c}|$$

Since some pixels are lost from the field of view during a shift, only a subset of subsequent images is used to determine the shift, preferably those around the center point. This will allow the largest search space possible, since the distance to all four margins of the image is maximized at the center point. The search for the optimal (δ_x, δ_y) pair is implemented as a grid search along the x- and y-axis. An example of the search grid is shown in Figure 2.19. Algorithm 2 shows the pseudocode of the shift inference algorithm for RGB images.

Data: M_t, M_{t+1}

Result: M_t^m

Parameters: d, l

D distance matrix for various shifting configurations ;

$c := (c_x, c_y)$ coordinate of center pixel of M_t ;

$M'_t := M_t[c_x - l : c_x + l][c_y - l : c_y + l]$ sub-image of M_t centered around (c_x, c_y) ;

for $i = -d$ **to** d **do**

for $j = -d$ **to** d **do**

$c' := (c_y - i, c_y - j)$;

$M'_{t+1} := M_{t+1}[c'_x - l : c'_x + l][c'_y - l : c'_y + l]$;

$D[i, j] := d_{RGB}(M'_t, M'_{t+1})$

end

end

Output: $\arg \min_{i,j} \{D\}$

Algorithm 2: Shift inference algorithm for RGB images

Since the time-lapsed data consists mainly of grayscale image, the RGB encoding could be directly transformed to grayscale encoding (see Equation 2.4 of Subsection 2.2.1). Using the transformed method also speeds up the calculation process since the distance function only computes the difference of grayscale channel's values:

$$d_G(G_i, G_j) = \sum_x \sum_y |G_{i,x,y} - G_{j,x,y}|$$

For this case, the shift inference algorithm can simply be modified by replacing d_{RGB} with d_G .

Due to lost pixels around the margin of before and after images, only the overlapping part of both slides are included after the correction. Thus, for an inferred shift of (δ_x, δ_y) , the new dimension of the images is then $(m - \delta_x) \times (n - \delta_y)$. This change would then propagate to the other time-lapse images to maintain consistency of the images.

Ideally, the shift correction should be done for each time point to reduce the track dropout rate caused by image shifts. This is however computationally very expensive. Moreover, inferring the shift for every recording time is not really necessary since the biggest shift, as mentioned before, only happens right before and after the treatment. The difference between consecutive images of the image position 26 can be seen in 2.20. Here we can see that the major spike of difference is only observed upon the introduction of the drug treatment.

As described in Subsection 2.2.9, the tracking algorithm allows certain amount of tolerance represented as maximum distance d_{max} . In this regard, the frame shifts happening not during the drug treatment introduction are way within the tolerance of our tracking algorithm. As shown in Figure 2.21, the dropouts caused by frame shifts in the other time points are basically noisy dropout caused by random noises in the time-lapse movie being tracked as cells [143].

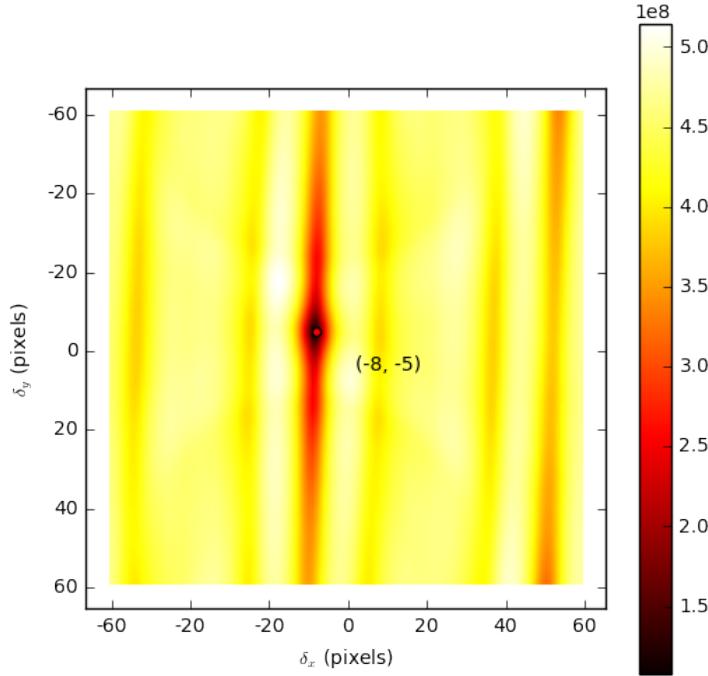


Figure 2.19: Search grid shift for the image position 26. The search was conducted for shift between the last time point before and the first time point after the drug treatment. The minimum is marked with thick black dot, which is returned after every grid-search call as inferred shift. In the position, the shift was inferred to be 8 pixels upwards and 5 pixels leftwards. Note the repeating pattern of relatively favorable configurations after approximately 50 horizontal and 100 vertical pixels caused by lattice nature of the micro-trenches.

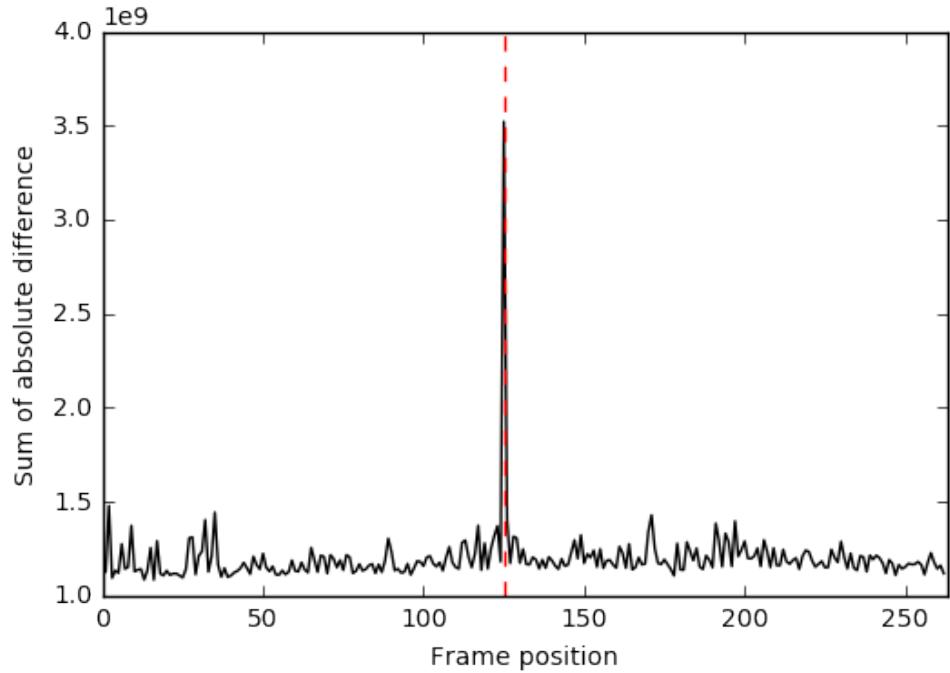


Figure 2.20: Pixel difference between consecutive frames in the image position 26. In most cases, the pixel difference between the frames is mainly caused by moving cells. The difference during the introduction of the drug treatment (red dashed line), on the other hand, is caused by physical shift of the frame. While moving cells mostly caused minimum noise-like pixel difference, the physical shift of field of view distorts the physical alignment and evokes immense pixel difference.

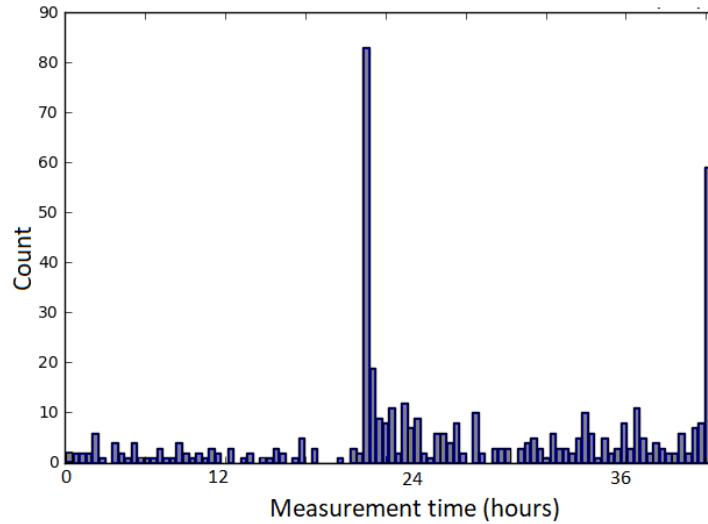


Figure 2.21: Distribution of tracking dropout rate in the image position 26. Note that the tracking dropout is defined as the last time point at which a cell is tracked. Thus the last time point of a valid cell's track also counts as a dropout. This is especially true after treatment where many cells died.

2.2.11 Support vector machine (SVM)

In machine learning, SVM is a construct which, given the training set $\mathbf{S} \subset \mathbf{D}$ with,

$$\mathbf{S} = \{\mathbf{x}_1, \dots, \mathbf{x}_{|\mathbf{S}|}\}$$

and corresponding target classes,

$$\mathbf{T} = \{y_1, \dots, y_{|\mathbf{S}|}\}$$

finds following things,

- A hyperplane that separates the input by its classes, so that every point belonging to one class is located on one side of the hyperplane. This hyperplane is, in turn, defined by,
- support vectors.

A hyperplane is defined as a set of points \mathbf{x} in \mathbf{S} satisfying following criteria,

$$\mathbf{w} \cdot \mathbf{x} - b = 0 \quad (2.20)$$

(See Figure 2.22) where \mathbf{w} and $\frac{b}{\|\mathbf{w}\|}$ denote the normal vector to the hyperplane and the distance of the hyperplane from the origin along the normal vector \mathbf{w} . For every class c_i , the set of data points satisfying criteria,

$$\mathbf{w} \cdot \mathbf{x} - b = c_i \quad (2.21)$$

are called support vectors. The distance from the hyperplane to support vectors is thus,

$$\frac{1}{\|\mathbf{w}\|}$$

For two-class classification, the classes are conventionally annotated as -1 and 1 . As the Equation 2.21 suggests, for every point beyond (seen from the perspective of hyperplane) the support vectors of the class $c = -1$, following inequality applies,

$$\mathbf{w} \cdot \mathbf{x} - b < -1 \quad (2.22)$$

The analogous applies to the class $c = 1$,

$$\mathbf{w} \cdot \mathbf{x} - b > 1 \quad (2.23)$$

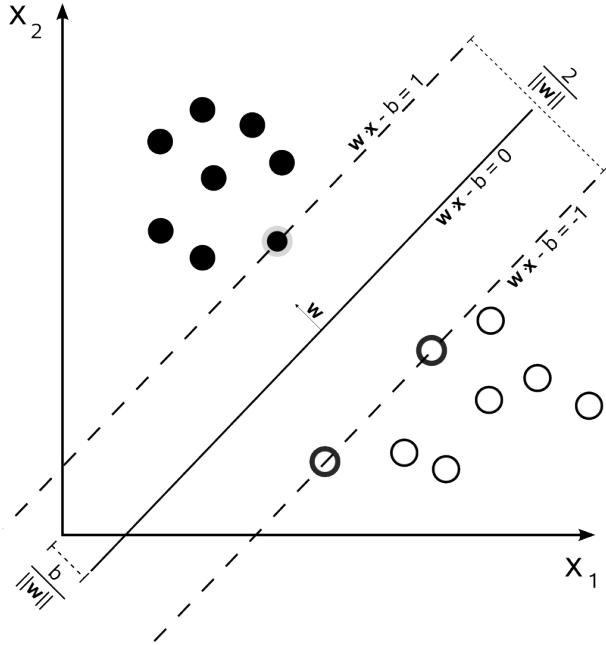


Figure 2.22: Illustration of a support vector machine in 2D. For the black class there is one hyperplane-defining point (marked with grey margin) while for white class there are two (marked with bold black margin). The support vector \mathbf{w} is maximized for each class.

Following large margin principle [145, 146], SVM tries to find support vectors that maximize $\|\mathbf{w}\|$. Given separability of the training data, the support vector \mathbf{w} can then be solved by solving following optimization problem,

$$\begin{aligned} & \underset{\mathbf{w}}{\text{minimize}} \quad \|\mathbf{w}\| \\ & \text{subject to } y_i(\mathbf{w} \cdot \mathbf{x}_i - b) \leq 1 \text{ for } i = 1, \dots, \|S\|. \end{aligned}$$

This hard-margin only converges only when the data are linearly separable in mapped space (also known as **feature space**). This is especially bad since many problems are not linearly separable in their original space. There are two fundamental ways of relaxing this problem to enable classification using SVM:

- Relaxation of the definition of the SVM by allowing data points to be misclassified.
- Blowing up the input space into sufficiently high dimensional features using the kernel trick.

Relaxation of SVM

A relaxation of above explained problem is known as soft margin SVM or ξ -SVM [147]. The problem allows misclassification of some training data. In SVM, misclassification occurs when a data point belonging to a certain class c_j is located **not** in the area defined by the margin $\mathbf{w}x_{c_j} = c_j$ for class c_j .

The problem is thus reduced to minimizing following term,

$$\left[\frac{1}{n} \sum_{i=1}^{|S|} \max(0, 1 - y_i(\mathbf{w} \cdot \mathbf{x}_i - b)) \right] + \lambda \|\mathbf{w}\|^2 \quad (2.24)$$

The term inside of summation is called **classification error**. For correctly classified class we have $\mathbf{w} \cdot \mathbf{x}_i - b \leq -1$ and $\mathbf{w} \cdot \mathbf{x}_i - b \geq 1$ for $c_i = -1$ and $c_i = 1$ respectively, i.e. the summation term is 0 for every correctly classified data point. The coefficient λ is regularization coefficient which penalizes the magnitude of normal vector \mathbf{w} . Note that the higher the dimension of \mathbf{w} the larger the penalty is. This is important for next part on **kernel methods**.

Minimizing Term 2.24 is equal to constraint optimization with differentiable objective function [148]. We can for example introduce a variable ζ_i defined as,

$$\zeta_i = \max(0, 1 - y_i(\mathbf{w} \cdot \mathbf{x}_i - b)) \quad (2.25)$$

This can be written as $y_i(\mathbf{w} \cdot \mathbf{x}_i - b) \geq 1 - \zeta_i$. Geometrically this is the maximum distance of a wrongly classified data point from the support vector. Using Definition 2.25 we can reduce optimization posed in Term 2.24 to,

$$\begin{aligned} & \underset{\mathbf{w}}{\text{minimize}} \quad \frac{1}{|S|} \sum_{i=1}^{|S|} \zeta_i + \lambda \|\mathbf{w}\|^2 \\ & \text{subject to} \quad y_i(\mathbf{w} \cdot \mathbf{x}_i - b) \leq 1 - \zeta_i \\ & \text{and} \quad \zeta_i \geq 0 \text{ for all } i. \end{aligned}$$

Kernel method

As mentioned before, the optimization problem for the standard SVM converges only in the case of linear separability of training data. While this mostly is not the case, Vapnik and Cortes [147] proposed the so-called **kernel trick**. It utilizes a kernel function Φ which maps the training set into high dimensional space representation. Essentially, a kernel function $\Phi : \mathbb{R} \times \mathbb{R} \rightarrow \mathbb{R}^+$ is a symmetric and non-negative function following the criteria by Mercer [149] defining it as, among others, general measure of similarity between two vectors.

In our case, we focus on two very well-known examples of kernel function, **the polynomial kernel function of order n**, defined as,

$$\Phi_{poly}(\mathbf{x}_i, \mathbf{x}_j) = (\mathbf{x}_i^T \mathbf{x}_j + r)^n \quad \text{with } r > 0 \quad (2.26)$$

and the **radial basis function (RBF)**, defined as,

$$\Phi_{RBF}(\mathbf{x}_i, \mathbf{x}_j) = \exp\left(\frac{\|\mathbf{x}_i - \mathbf{x}_j\|^2}{2\sigma^2}\right) \quad (2.27)$$

In both examples we can observe the assumed dimensionality of the target feature space. A polynomial kernel function of order n maps the training data to n -dimensional feature space. Thus, blowing up the training data into higher dimensions requires the explicit assignment of a very high n value. The radial basis function on the other hand does not assume any dimensionality (or rather, it assumes unbounded dimensionality) as

the Gaussian function used only assumes the input vectors \mathbf{x}_i and \mathbf{x}_j to be of the same dimensionality.

While assuming very high/unbounded dimensionality seems convenient at the start, this is not very straightforward, since:

- Given a non-powerful kernel function Φ that only maps the input into bounded number of dimension, the optimization problem may not converge if there is no representation that is mapable using the kernel function.
- Given a powerful kernel function Φ capable of blowing up the dimension to very high dimensions kernel might settle for an unnecessarily sparse dimensions reducing the generalizability of the problem.

Regularization

One solution is to introduce regularization. As already shown in Term 2.24, we can add a regularization term such as,

- L_1 -regularization: $\lambda|\mathbf{w}|$ [150], and
- L_2 -regularization (Tikhonov regularization): $\lambda\|\mathbf{w}\|^2$ [151].

Adding regularization will control against overfitted models by penalizing higher dimensional hyperplane \mathbf{w} as the error will get blown up with higher dimensions and the corresponding error minimizing hyperplane, are chosen.

Regressive SVM

SVM can be extended to enable regression. The method, initially proposed by Smola, Vapnik et al. [152] and commonly known as Support Vector Regression (SVR), modifies the optimization problem of standard SVM to:

$$\begin{aligned} & \underset{\mathbf{w}}{\text{minimize}} \quad \frac{1}{2}\|\mathbf{w}\| \\ & \text{subject to} \quad y_i - \langle \mathbf{w}, \mathbf{x}_i \rangle - \mathbf{b} \leq \epsilon \\ & \text{and} \quad \langle \mathbf{w}, \mathbf{x}_i \rangle + \mathbf{b} - y_i \leq \epsilon \end{aligned}$$

where \mathbf{b} and $\langle \bullet, \bullet \rangle$ refer to the intercept of a linear model and the inner product operator. Note the term

$$y = \langle \mathbf{w}, \mathbf{x}_i \rangle + \mathbf{b} + \sigma$$

being the term for standard linear model with intercept \mathbf{b} and error term σ .

2.2.12 Pearson's correlation coefficient (PCC)

In statistics, the PCC is a measure of the linear correlation between two random variables X and Y [153]. It has a value ranging from -1 to 1 . A correlation value of -1 denotes a perfectly inverse correlation, 0 denotes no linear correlation and 1 denotes a perfect positive correlation.

For two random variables X and Y , the PCC is defined as follows:

$$\rho_{X,Y} = \frac{\text{cov}(X, Y)}{\sigma_X \sigma_Y} \quad (2.28)$$

where $\text{cov}(X, Y)$ is the covariance of random variables X and Y , σ_X is the standard deviation of X and σ_Y is the standard deviation of Y .

The covariance of two random variables is in turn defined as

$$\text{cov}(X, Y) = E[(X - \mu_X)(Y - \mu_Y)] \quad (2.29)$$

with $E[X]$ denoting expected value of a random variable X . Combining Equations 2.28 and 2.29 and using definition of mean ($\mu_X = E[X]$) and standard deviation ($\sigma_X^2 = E[(X - E[X])^2]$) we can derive the formula of $\rho_{X,Y}$ to:

$$\rho_{X,Y} = \frac{E[XY] - E[X]E[Y]}{\sqrt{E[X^2] - [E[X]]^2}\sqrt{E[Y^2] - [E[Y]]^2}} \quad (2.30)$$

To calculate the p-value, we first calculate the t^* statistic, defined as

$$t^* = \frac{\rho_{X,Y}\sqrt{n-2}}{\sqrt{1-\rho_{X,Y}^2}} \quad (2.31)$$

where n denotes the number of observation. The p-value is then defined as probability of Student's t distribution T with $n - 2$ degrees of freedom having value x smaller than t , i.e.

$$p = P(Z \leq t^* \mid Z \sim T) \quad (2.32)$$

2.2.13 F-Test

In statistics, the F-Test is a statistical test in which the statistic $F_{X,Y}$ is assumed to be F-distributed under null Hypothesis, i.e. $F_{X,Y} \sim F(n_1, n_2) \mid H_0$ [153]. The distribution arise from the ratio of the χ -squared variance of two normally distributed random variables, i.e. for two random variables $U \sim \chi^2(n_1)$ and $V \sim \chi^2(n_2)$, the ratio

$$F = \frac{U/n_1}{V/n_2} \quad (2.33)$$

is then F-distributed. The symbols n_1 and n_2 denote the degree of freedom of U and V . Conducting an F-test is thus reduced to checking whether, given two distributions X and Y , the F statistic of two distributions is F distributed. To do that, we first compute the F statistic of both distributions,

$$F_{X,Y} = \frac{\text{var}(X)}{\text{var}(Y)} \quad (2.34)$$

with var denoting the variance of the distribution $\text{var}(X) = E[(X - \mu)^2]$ The degree of freedom of both distributions is defined as,

$$n_1 = |X| - 1 \quad (2.35)$$

$$n_2 = |Y| - 1 \quad (2.36)$$

The cumulative density function (cdf) F_{cdf} of the F-distribution is given by

$$F_{cdf}(x; n_1, n_2) = \mathbf{I}_{\frac{n_1 x}{n_1 x + n_2}} \left(\frac{n_1}{2}, \frac{n_2}{2} \right) \quad (2.37)$$

where \mathbf{I}_x is the regularized beta function

$$\mathbf{I}_x(a, b) = \frac{\mathbf{B}(x; a, b)}{\mathbf{B}(a, b)} \quad (2.38)$$

and \mathbf{B} is the beta function

$$\mathbf{B}(x; a, b) = \int_0^x t^{a-1} (1-t)^{b-1} dt. \quad (2.39)$$

Using the standard definition of one-sided right tail p-value we can derive for the p-value of the F statistic as following:

$$\begin{aligned} p_F &= Pr(Z \geq F_{X,Y} \mid H_0) \\ p_F &= 1 - F_{cdf}(F_{X,Y}; n_1, n_2) \end{aligned} \quad (2.40)$$

for $Z \sim F(n_1, n_2)$.

Chapter 3

Analytic Pipeline

In this chapter the image and data analytic pipeline is presented. Each pipeline of image processing analysis is elaborated with reference to publications and the methods from Chapter 2. Every method developed/used in the pipeline is brought forward and explained with references to scientific literature and the definitions brought forward in Chapter 2.

3.1 Image computing

To assess single cell characteristics (like its lifetime, time-to-death, division time, daughter cells and other information relating to its time- and generation-dependent cell cycle information), we track all cells in the brightfield channel from the start of the movie, assign cells to individual micro-trenches to e.g. filter out micro-trenches with multiple starting cells, and determine cell death via marker onset in the fluorescent channels.

Figure 3.5 shows how the image is sequentially processed from out-of-focus image to cell trees information.

3.2 Micro-trench masking

Various techniques could be applied to highlight certain areas in the image. In Subsection 1.3.2, several advances in computer vision methods are described. Furthermore, advances in the field of machine learning are chronicled in Subsection 1.3.3. While the collection of advanced methods for region and image detection abound [154, 155, 156], some simple interpretable methods could be used best to detect and mask the micro-trenches. In particular, we can see that the area around a micro-trench exposes strong intensity gradient (see Figure 3.1): the area around the margin of a micro-trench is much darker than the other parts of the well. This can be explained by the fact that the light is reflected less around the wall area and thus the intensity decreases. Additionally, the light beam coming out of the laser is not perfectly perpendicular to the well and thus the non-perpendicular reflection is not reflected back to the camera sensor.

Before us, there are several methods that exploit this kind of phenomenon. Cheng et al. [157] for example showed it is possible to recognize salient objects in images by using contrast and brightness adjustment. Our method on the other hand, goes further by improving robustness via a noise cancellation step (See 2.2.3).

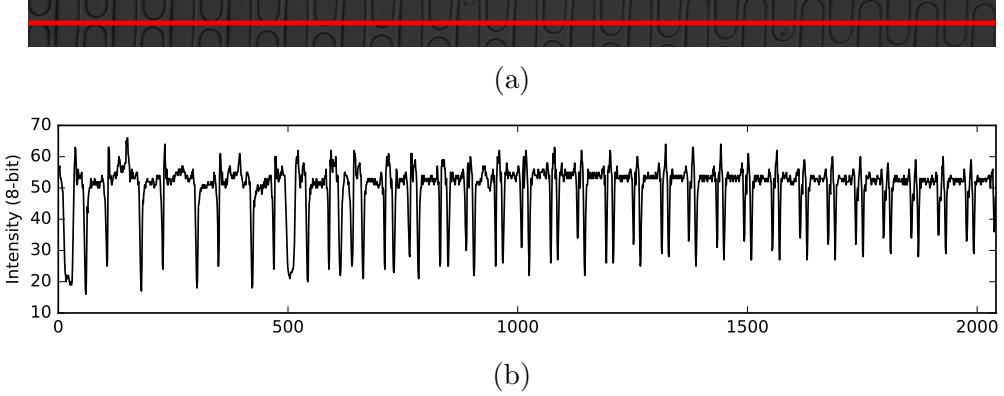


Figure 3.1: (a) The highlighted area around the 500-th column of the first slice of the image position 41. The range starts from the 450-th until the 550-th column. (b) The intensity of the red-marked area in (a), the region around micro-trench margin is indicated by the sudden drop of intensity. Note also the slow drift of the intensity as the pixel goes south (right side of the plot). This phenomenon is called *intensity gradient* (see Subsection 2.2.5 for more).

We could thus exploit this knowledge by designing a micro-trench masking algorithm as follows:

3.2.1 Brightness and contrast adjustment of in-focus image

First, we adjust the brightness and contrast so that the area far away from the margin is encoded as maximum intensity, while the area around the margin is encoded as minimum intensity. One simple and robust way to do this is to reduce the intensity of each position with the maximum intensity value of the region around the margin.

Mathematically, we can define two sets of points, \mathfrak{M} and \mathfrak{S} . \mathfrak{M} refers to the set of points around the trench margin while \mathfrak{S} denotes the set of points far away from it. This brings us to the following transformation,

$$J_i(i, j) := I_t(i, j) + \operatorname{argmax}_{(x,y) \in \mathfrak{M}} I_t(x, y)$$

creating transformed intensity I^t .

As can be seen in Figures 2.11 and 3.1, while sets \mathfrak{M} and \mathfrak{S} are locally separable, across the board this does not seem so clear. We can thus improve the transformation by also adjusting the contrast parameter α by increasing it so that $\alpha > 1$. This brings the pixels with similar intensity values around the decision boundary (somewhere between the two distributions in Figure 2.11) apart and thus ameliorates the determination of decision boundary by the user. Applying this, we have now the new transformation:

$$J_i(i, j) := \alpha I_t(i, j) + \operatorname{argmax}_{(x,y) \in \mathfrak{M}} I_t(x, y) \text{ with } \alpha > 1$$

creating transformed intensity I^t .

Note that this is still not a perfect transformation, as there are some pixels in \mathfrak{S} with intensity lower than the minimum intensity of pixels in \mathfrak{M} . Besides doing this manually, we also improve this by refining the transformed intensity I^t further with the next step: Robust Automatic Threshold Selection.

Subfigures 3.5i and 3.5j show images of micro-trenches before and after brightness and contrast adjustment in the image processing pipeline. While the example does not show clear difference on macro level, the algorithm removes local artifacts that may distort micro-trench masking process. The step is done manually for each image position using the **Bright/Contrast Adjustment** function in Fiji.

3.2.2 RATS denoising

After brightness and contrast adjustment, some parts inside the micro-trench still have pixels that were not transformed to complete white color (maximum intensity). This can be removed by the global and local noise correction through Robust Automatic Threshold Selection (RATS, see Subsection 2.2.3). The algorithm is applied on each adjusted brightfield image, which creates a binary image of the micro-trench margins.

Subfigures 3.5j and 3.5k show the input and output of RATS in the image processing pipeline. The noise pertaining in a micro-trench is removed upon application of gradient-based filtering. We can thus say that,

- the first part of RATS (g -filtering by $\lambda\sigma$) is designed to move global level noise which in this case means white noises coming from non-perfect reflection of light from the well.
- the second part of RATS (recursive g -filtering by T_r) is designed to remove local noise cause by local distortion due to location-specific artifacts (direction of incoming light not perfectly perpendicular with regard to micro-trench's base etc).

The step is done automatically using the **Robust Automatic Threshold Selection (RATS)** plugin in Fiji with following parameters:

Parameter	Value
noise_threshold	25
lambda_factor	3
min_leaf_size_pixels	408

3.2.3 Micro-trench mask filling

After applying brightness and contrast adjustment and RATS, the image now contains only the margin of micro-trenches. We can then fill these holes to create micro-trench masks. Subsection 2.2.4 describes the holes filling algorithm. The step is done automatically using the **Fill Holes** function in Fiji. Subfigures 3.5k and 3.5l show the input and output of the holes filling algorithm in the image processing pipeline. Upon holes filling, Each mask (appearing as a black rod-like form) is assigned a unique identity, which is then used as an identifier for tracks in both the brightfield and the fluorescent channel for filtering of cells and clones (see Sections 3.3 and 3.4).

3.3 Single cell tracking in the brightfield channel

To identify and track single cells from movie start, each out-of-focus brightfield image is processed in the following way (see Figure 3.5 for pipeline visualization):

3.3.1 Gaussian blurring

First, a Gaussian blur with a large radius of 50 pixel is applied to each brightfield image. The algorithm is explained in Subsection 2.2.5. Equation 2.10 of Subsection 2.2.5 is used to convolve every pixel in an image. The method results in the estimation of the image's background and global gradient. We then correct the input image against the background and gradient by dividing every pixel of the original image by the pixel in the same position in the convoluted image (see Equation 2.11 of Subsection 2.2.5).

This step frees the out-of-focus image from global background and gradient which may have been arisen during image acquisition process caused by non-homogeneous lightning and unsynchronized lightning for example. This step is done using **Gaussian blur** filter implementation in Fiji with $\sigma = 50$ as parameter.

Subfigures 3.5a, 3.5b and 3.5c depict the input of the algorithm, computed background and output of the algorithm in the image processing pipeline.

3.3.2 Local contrast normalization

After removing global background and gradient, we normalize the local contrast of each image(Zuiderveld, 1994 [139]). Subsection 2.2.6 explains the details of the algorithm. This separates foreground from background. Upon running the algorithms the cells will be visible as negative while other parts of the image (micro-trench, noises etc) disappear. Figures 3.5c and 3.5d depict the input and output of the algorithm in the image processing pipeline. This step is done using **Normalize Local Contrast** function in Fiji with following parameters:

Parameter	Value
block_radius_x	100
block_radius_y	100
standard_deviation	1

3.3.3 Mean intensity correction

To reduce noise from micro-trench margins in the foreground, we calculate the pixel-wise intensity average from all slices in one image position and subtract this from each image to generate a binary image with mainly cells in the foreground. The details of this step is explained in Subsection 2.2.7. This creates a mask of the cells. Equation 2.13 is used to calculate the mean while the correction is done using Equation 2.16.

Subfigures 3.5d, 3.5e and 3.5f depict the input of the algorithm, computed pixel-wise mean intensity and output of the algorithm in the image processing pipeline.

3.3.4 Cells recognition

After the image pre-processing, the image now shows only the negative of the cells (see Subfigure 3.5f). Subsection 2.2.8 explains the Laplacian of Gaussian (LoG) detector in depth. The cells are recognized using the **Downsampled LoG Detector** in the Fiji TrackMate plugin [142] with following parameters:

Parameter	Value
<code>pixel_width</code>	0.647
<code>pixel_height</code>	0.647
<code>voxel_depth_pixel</code>	1.000
<code>downsample_factor</code>	2
<code>blob_diameter_pixel</code>	15
<code>threshold</code>	0

Subfigure 3.5g shows the recognized cells from input image shown in Subfigure 3.5f.

3.3.5 Cells tracking

Upon detection, the cells are assigned to tracks and concatenated to cell trees. The details of the method used, the Linear Assignment Problem (LAP) Tracker [143], are elaborated in Subsection 2.2.9. The tracking is done using the **LAP Tracker** in the Fiji TrackMate plugin [142] with following parameters:

Parameter	Value
<code>pixel_width</code>	0.647
<code>pixel_height</code>	0.647
<code>voxel_depth_pixel</code>	1.000
<code>time_interval_second</code>	600
<code>frame_to_frame_linking_pixel</code>	25
<code>track_segment_gap_closing_pixel</code>	25
<code>track_segment_gap_closing_max_frame</code>	6
<code>track_segment_splitting</code>	True
<code>track_segment_splitting_pixel</code>	25

Figure 3.5h shows the tracked trajectory of the cells from Figure 3.5g.

3.4 Single cell tracking in the fluorescent channel and cell death signal determination

The main issue of fully tracking the cells using only brightfield images is the fact that the reliability of the tracking decreases as the cells are put under stress. Dying cells stop moving, become small and unstructured, and loose a distinctive bright signal in the brightfield channel, which makes them hard to track. The longer the experiment proceeds after treatment, the more likely that the cells will be lost from the tracking algorithm due to aforementioned factors.

We thus track dying cells in the fluorescent channels and concatenate the tracks with earlier tracks in the brightfield to determine time-to-death. Unlike in brightfield images, dying cells start emitting light under fluorescent light beam as they begin dying (see examples in Figure 3.2). This continues as the programmed cell death advances and the emitted light becomes even brighter (the progression of the emission follows roughly the step function, see Figure 3.4).

Knowing that at one point the disruption by the drug treatments induces cell death and thus emission of PI or Caspase fluorescent signals (see t_{PMP} in Figure 3.4), finding the cell death time is reduced to simply finding the time point at which the blob of the cell in fluorescent channels is detected. Take for example the Figure 3.2a. Here we see that on the upper right side of the image there are at least two blobs visible in the naked eye. We can thus do similar pre-processing step as that of brightfield images followed by image detection to detect at which time the onset happened (see Figure 3.5).

3.4.1 Brightness and contrast adjustment

This step is similar to the method used in micro-trench masking in Subsection 3.2.1. Brightness and contrast are adjusted to remove the artefacts on the wafer. Subfigures 3.5a and 3.5b show the input and output of the brightness and contrast adjustment step.

3.4.2 Mean intensity correction

Again, the method is the same as the method described in Subsection 3.3.3. The resulting image can be seen in Subfigure 3.5c.

3.4.3 Cells recognition and tracking

After the pre-processing steps, the images undergo the detection and tracking steps in a similar fashion as the brightfield images. Following parameters are used to detect the cells:

Parameter	Value
<code>pixel_width</code>	0.647
<code>pixel_height</code>	0.647
<code>voxel_depth_pixel</code>	1.000
<code>downsample_factor</code>	2
<code>blob_diameter_pixel</code>	30
<code>threshold</code>	0

The assumed diameter is larger since the emission spreads around the cell and thus makes the cell negative larger than the cell body itself. Following parameters are used to track the cells:

Parameter	Value
<code>pixel_width</code>	0.647
<code>pixel_height</code>	0.647
<code>voxel_depth_pixel</code>	1.000
<code>time_interval_second</code>	1800
<code>frame_to_frame_linking_pixel</code>	35
<code>track_segment_gap_closing_pixel</code>	35
<code>track_segment_gap_closing_max_frame</code>	2
<code>track_segment_splitting</code>	False
<code>track_segment_splitting_pixel</code>	25

Note that track splitting is disabled in the fluorescent tracking. The reason behind this is simply that the fluorescent signal is indicative of the cell death caused by the failure to undergo mitosis.

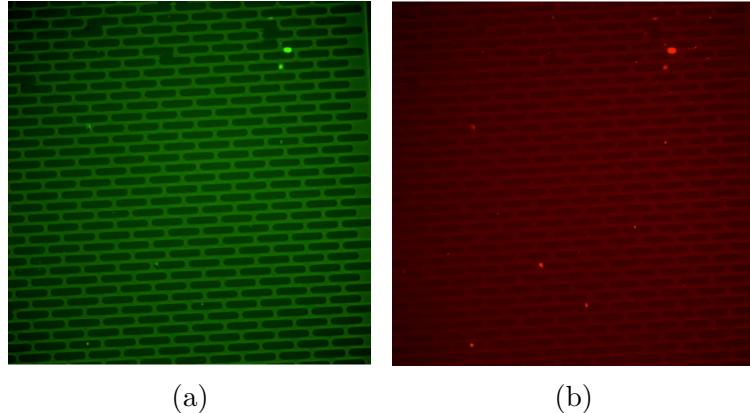


Figure 3.2: Example of images captured in (a) green and (b) red fluorescent channels. Upon programmed cell death, the bright emission is detected in corresponding channels coming from the activation of PI and Caspase 3/7 expression paths.

3.4.4 Cell death determination

After tracking the detected cells in brightfield and fluorescent images, the cell line track (in this project known as cell tree) is then assigned to the micro-trench it is located. This is done by looking at where the track is located in the mask image containing micro-trench masks. Now that we have mapping between the brightfield and fluorescent cell trees and the micro-tranch they are located in, we can combine the information from both tracks to create cell death signal-corrected tracking data (see Figure 3.9). Figure 3.3 shows how it is done. First, we have one bright field cell track associated with one micro-trench. The next step would be to find out whether there is one fluorescent cell track associated with the micro-trench. In case there is one, the death time of the cell is then updated to the start of fluorescent the cell track. However, since not every micro-trench is associated with one cell track (see Figure 2.3 for typical view of a well and Figure 4.2 for the occupancy distribution of the micro-trenches), several considerations have to be made. Following is the table of possible occupancy scenario and what to do with the data (BF: brigtfeld, FS: fluorescent) in the unsynchronized experiments:

Trees _{BF}	Children _{BF}	Trees _{FS}	Remark
0	0	NA	Do nothing, not relevant for analysis
1	1	NA	Do nothing, cell not dividing
1	2	0	Do nothing, cell not dying
1	2	1	Correct TTD; dead cell analyzed for TTD
1	2	2	Correct TTDs; dead cells analyzed for TTD and SC
1	3+	NA	Do nothing, some clones divided > 1 times
2+	NA	NA	Do nothing, too many cells

Table 3.1: Scenario for time-to-death (TTD) and sisters death time (SDT) correlation analysis of unsynchronized. Trees: number of cell tracks; Children: number of leaves. BF: brightfield; FS: fluorescent. Note that the cell tracks do not branch in FS.

The scenario for synchronized experiment is described in following table:

Trees _{BF}	Trees _{FS}	Remark
0	NA	Do nothing, not relevant for analysis
1	NA	Do nothing, cell not dividing
2	1	Correct TTD; dead cell analyzed for TTD
2	2	Correct TTDs; dead cells analyzed for TTD and SDT
3+	NA	Do nothing, too many cells

Table 3.2: Scenario for TTD and SC analysis of synchronized experiment. Note that in synchronized experiment, the number of cell track is equal to the number of leaves in BF.

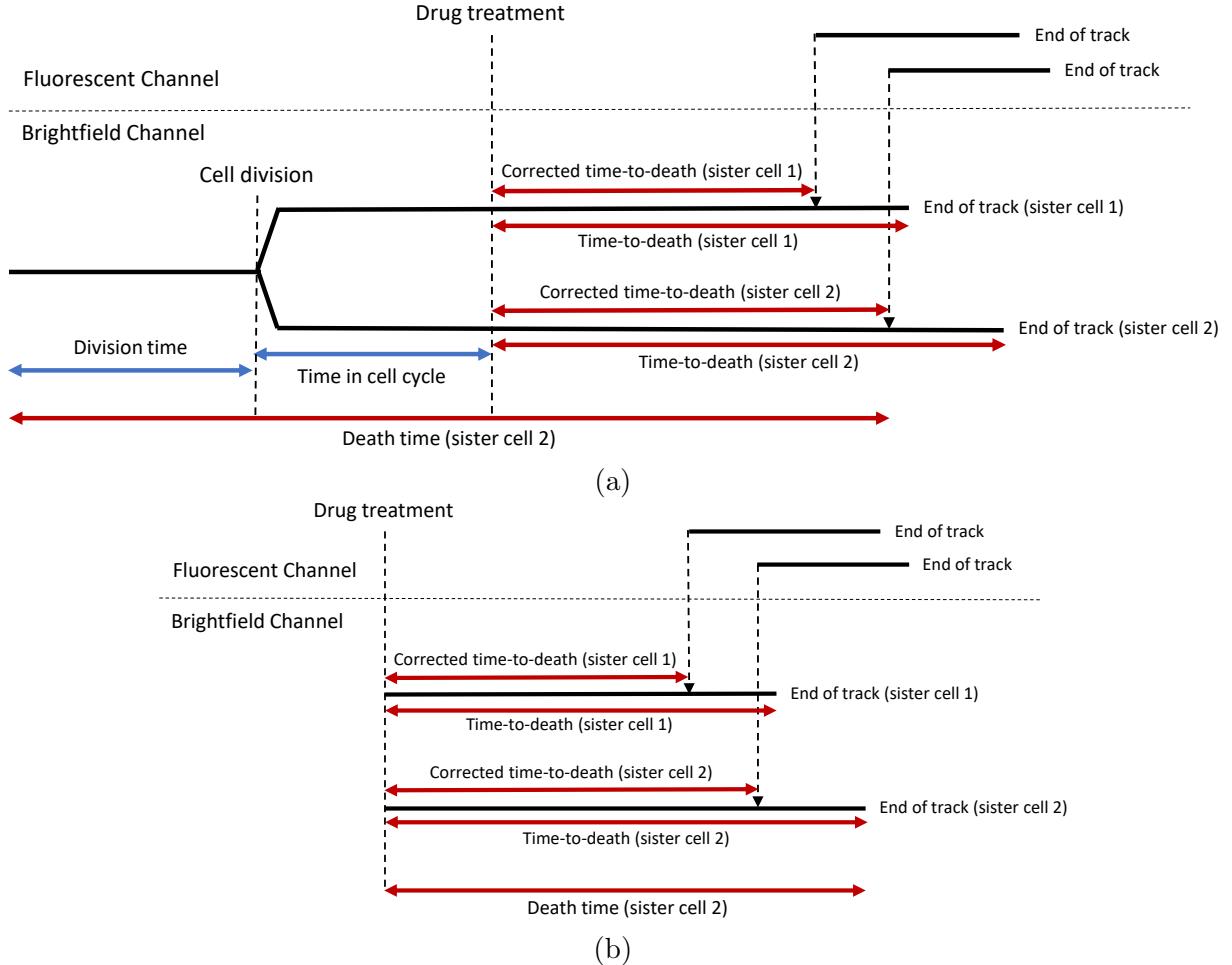


Figure 3.3: Schematic overview of cell tracking in unsynchronized and synchronized experiments. (a) Various definitions used in unsynchronized experiment. (b) Various definitions used in synchronized experiments. Note that in case micro-trench with two tracks in the fluorescent channel the assignment of the fluorescent to brightfield track does not affect time-to-death and sister-correlation analysis due to symmetric definition of time-to-death and time in cell cycle (i.e. the correlation of time-to-death between sister cells and time in cycle to time-to-death do not change no matter how the tracks assignment is done).

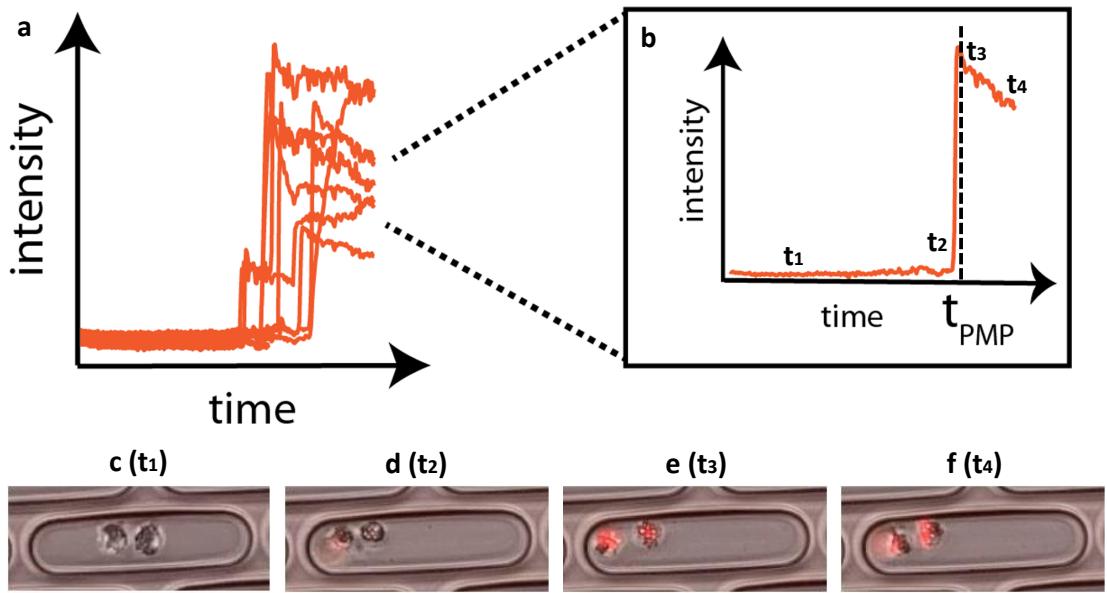


Figure 3.4: Illustration of the annotation of PI death signal emission as the cell death time (t_{PMP}). (a) Schematic plot of how the total emission intensity would look like if it's being tracked (image taken for one related experiment of Radler's lab). (b) The hypothetical progression of total intensity of *the cell on the left* upon treatment with some approximate time points representing various cell phases marked with t_1 , t_2 , t_3 and t_4 . Initially, the left cell behaves normally upon treatment (c/ t_1). Only upon reaching the mitotic phase, the addition of Vincristine/Daunorubicin disrupts the process. Failure of entering the next phase sets the cell onto programmed cell death (d/ t_2). As the programmed cell death process advances, the PI emission becomes stronger (e/ t_3). At this point, the cell recognition will identify the cell death signal as cell and thus represents cell death annotation. After a while, the emission will keep going on (f/ t_3). We are, however, only interested in the onset time (t_{PMP}) to determine the cell death time and correct the track data (see Figure 3.3). Note especially the difference of onset time between the left and right cells. At t_2 , the left cell has begun programmed cell death process while the right cell has not. This difference in time-to-death between sisters is one of the focuses of the project.

3.5 Pipeline visualization

The image processing pipelines for both brightfield and fluorescence channels can be seen in following figures:

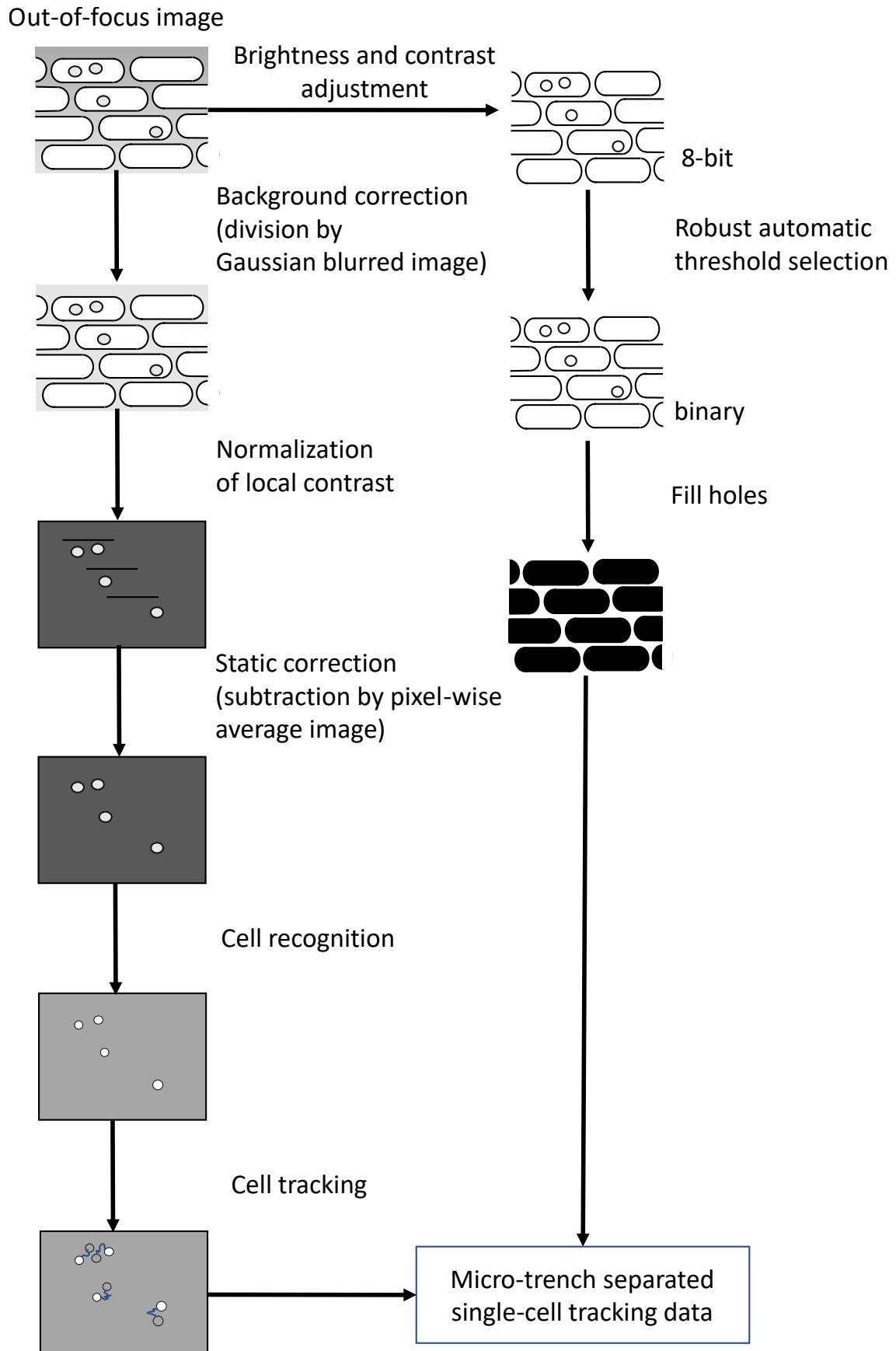
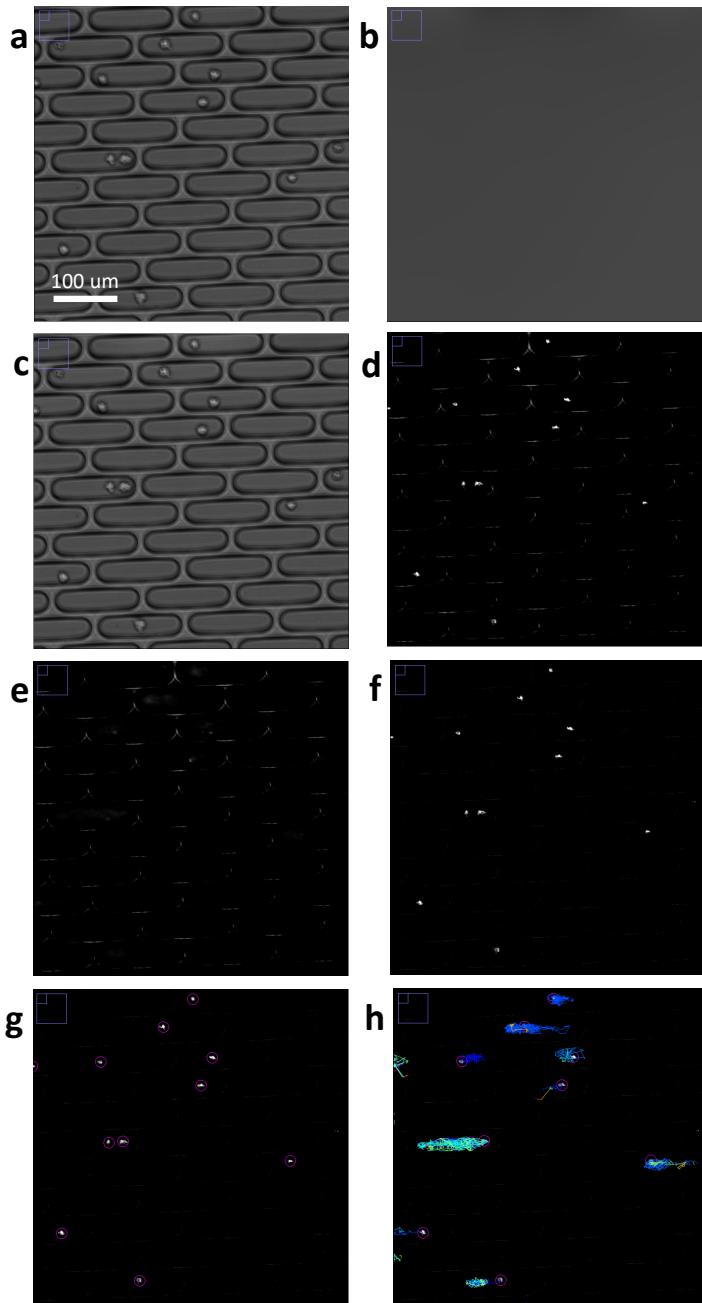


Figure 3.5: The image processing pipeline in the brightfield channel.

Image pre-processing and cell tracking:



Micro-trench masking:

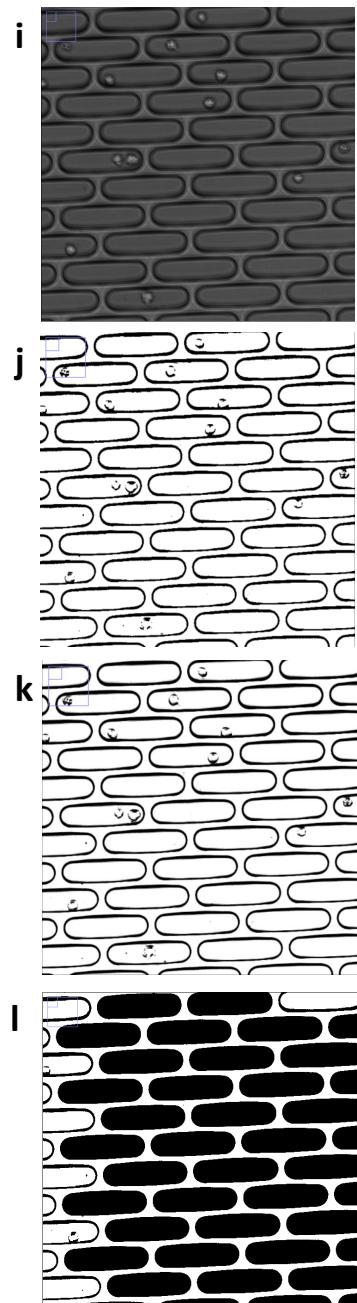


Figure 3.6: **Left:** an example of the image processing and the cell tracking in the bright-field channel. (a) The input image. (b) The result of Gaussian blur on input image. (c) The input image after correction with Gaussian blur. (d) The output of the local contrast normalization on (c). (e) The pixel-wise mean intensity image of (d). (f) The output of pixel-wise mean correction. (g) Detected cells in the the image (f). (h) The cell tracks superimposed on the image (f). **Right:** an example of the micro-trench masking pipeline in the brightfield channel: (a) The input image. (b) The input image after adjusting the brightness and contrast. (c) The output of RATS on image (b). (d) The output of the holes filling algorithm on (c).

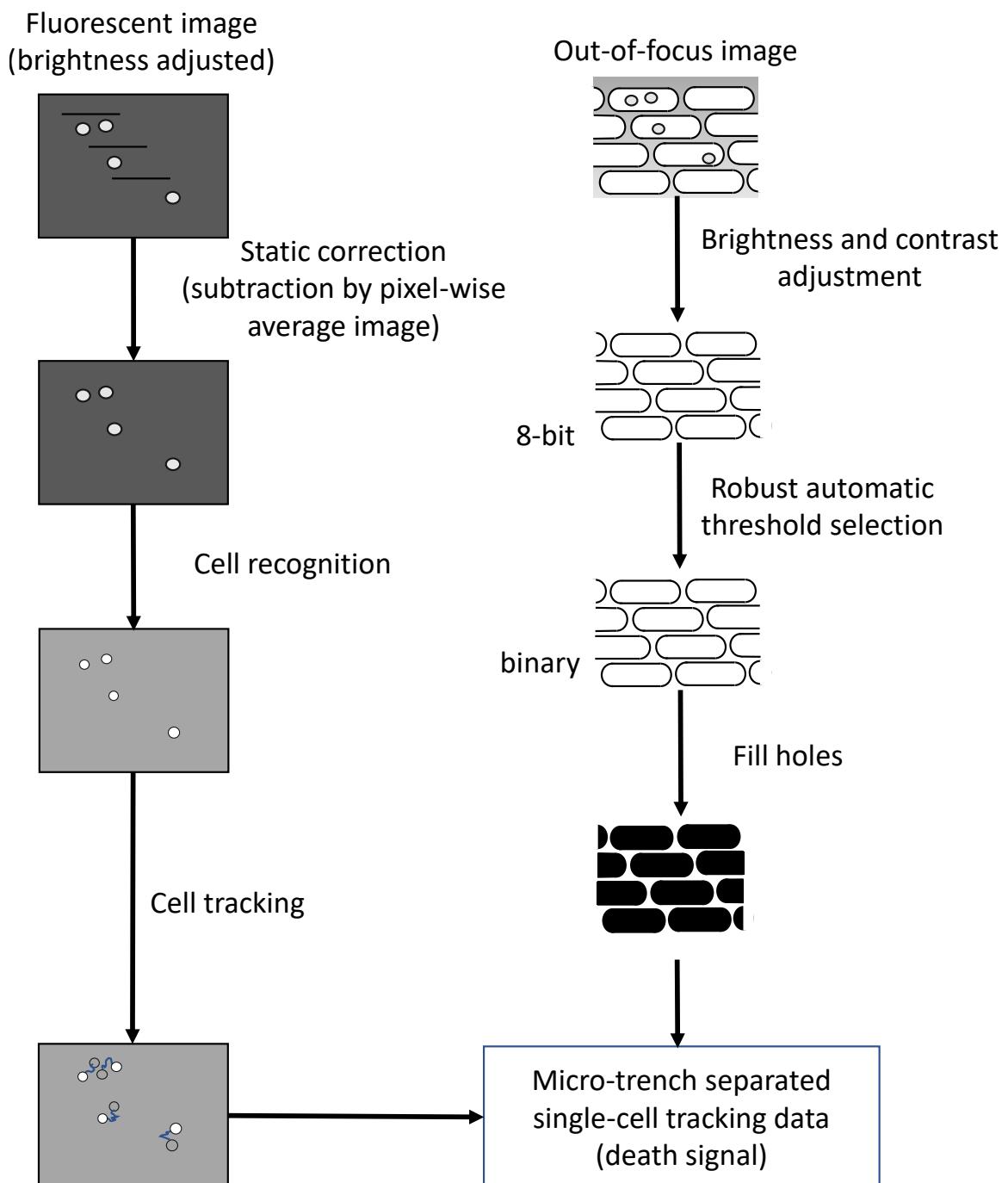


Figure 3.7: The image processing pipeline in the fluorescence channel.

Fluorescence image pre-processing:

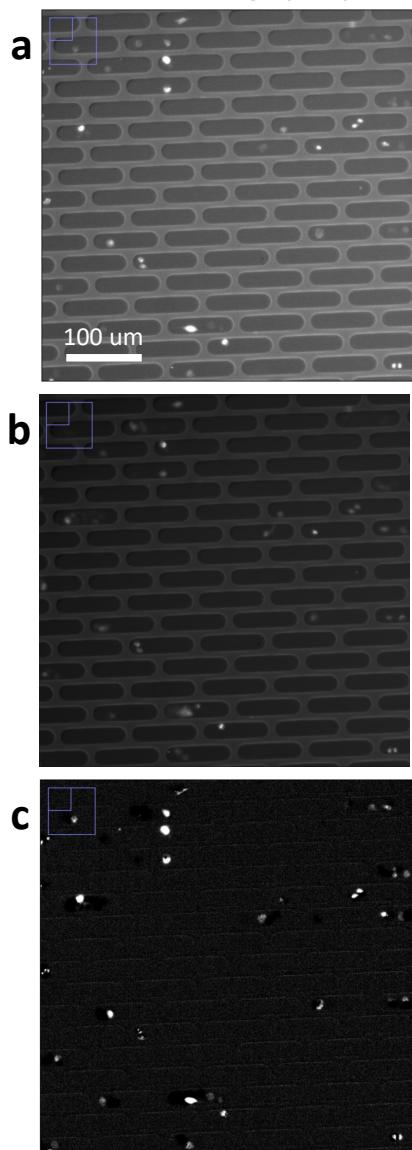


Figure 3.8: An example of the brightness and contrast adjustment in the fluorescence channel: (a) The input image (b) The input image after adjusting for the brightness and contrast. (c) The out of the pixel-wise mean correction on (b).

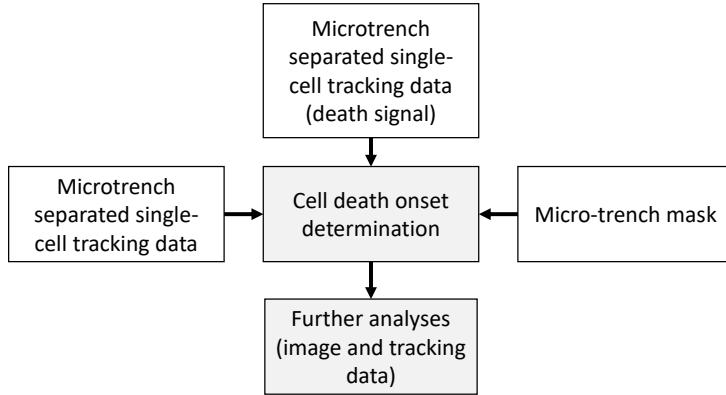


Figure 3.9: Combination of tracking data with cell death information creating cell death signal-corrected tracks data.

3.6 Implementation and availability

The image and tracking data is combined and post-processed using Python scripts utilizing OpenCV[137], NumPy [158], pandas [159], Matplotlib [160], Jython and the Trackmate [142, 161] plugin in Fiji [162]. The Python package scikit-learn is used for training and testing both support vector machine and random forest [163]. Our code is available at:

<https://github.com/raharjaliu/MA/tree/master/source>.

Chapter 4

Results

The pipeline described in Chapter 3 provides us with wealth of data to be analyzed. We present the characteristics of the analyzed cells in Section 4.1. The results of our analyses are presented in this chapter. Those are:

- The distribution of occupancy among micro-trenches using our single-cell tracking data (see Section 4.2).
- The variability of cell cycle times (see Section 4.3).
- The variability of time-to-death with time in cell cycle and between sisters. Here we look into the effect of the time in cell cycle, which roughly translates to the current cell cycle phase, on the response to the cancer drug treatment. We also analyze the variability of time-to-death between sister cells to get an understanding about heritable response towards the cancer drug treatment (see Section 4.4).
- Machine learning methods to investigate whether there are features that can predict the time-to-death at a given time point (see Section 4.5).

4.1 Characteristics of analyzed cells

Our experimental setup and analysis pipeline allows for a strict filtering of the data with respect to various requirements. Table 4.1 lists the requirements a cell tree has to fulfill for time-to-death (TTD) and sisters death time (SDT) correlation analyses in the unsynchronized experiment (see Section 4.4). The number of cells fulfilling requirements listed in the Table 4.1 can be seen in the Table ?? in Appendix B.3.

Category	Value	Explanation
Placement	Single	Cell is placed alone in a micro-trench
Division	1	Only one division in cell tree
DivT	$t_{div} < t_{treat}$	Mother cell divides before the treatment
Children's DT	$t_{death}^i > t_{treat}$	Both children die after the treatment

Table 4.1: Cell tree's requirements for TTD and SDT analysis in the unsynchronized experiment.

To investigate differential dynamics of cancer drug treatment between two sister cells, both cells have to be born before the treatment and die after the treatment. For the tracking data to be highly reliable, we focus on a singly-placed cell (see Table 4.2) with only one division, thus reducing mistracking error (see Subsection 4.2).

Table 4.2 lists down requirements for TTD analysis in synchronized experiment.

Category	Value	Explanation
Placement	Single	Cell is placed alone in a micro-trench
Branching	0	Cell does not divide
Death time	$t_{death} < 24$ hours	Cell dies eventually

Table 4.2: Cell tree's requirements for TTD analysis in the synchronized experiment.

For the synchronized experiment, only the TTD analysis is done, since imaging starts with drug addition and it can thus not be determined whether two cells placed in one micro-trench are sisters. We can however, exploit the fact that the doubly-placed cells in synchronized population are not sisters. By analyzing their death time (DT) correlation we can use it as control against the sister death time correlation acquired in unsynchronized experiment. Table 4.3 shows the protocol of non-sisters DT correlation analysis in synchronized experiment.

Category	Experiment	Remark
Placement	Double	Two non-sister cells are placed in a micro-trench
Branching	0	Cells do not divide
Death time	$t_{death} < 24$ hours	Cell dies eventually

Table 4.3: Setup for control non-sister death time (DT) correlation analysis in synchronized experiment.

For cell cycle duration (CCD) analysis, we require cell trees to span two full generations, i.e. the mother and at least one of the two daughter cells divides. Table 4.4 lists requirements a cell tree has to fulfill for the CCD analysis.

Category	Value	Explanation
Concentration	0 mM	Only cells in control treatment are used
Placement	Single	Cell is placed alone in a micro-trench
Division	2/3	At least one child divides
Generations	2	At least one child divides
DivT	$t_{div} < t_{treat}$	Mother cell divides before the treatment
Children's DT	$t_{death}^i > t_{treat}$	Children die after the treatment

Table 4.4: Cell tree's requirements for CCD analysis in the unsynchronized experiment.

4.2 Occupancy of micro-trench

The micro-trench setup is designed to maximize tracking accuracy and throughput: in principle every wafer should have as many micro-trenches as possible with as many singly-placed cells as possible. The reason for the single-placement preference comes from the

inherent uncertainty involved in tracking. Figure 4.1 explains the issue of ambiguity arising from multiple cell placement. There we see that the ambiguity occurs as soon as two cells are within possible moving range of each other. This ambiguity can be minimized by having one cell in a micro-trench.

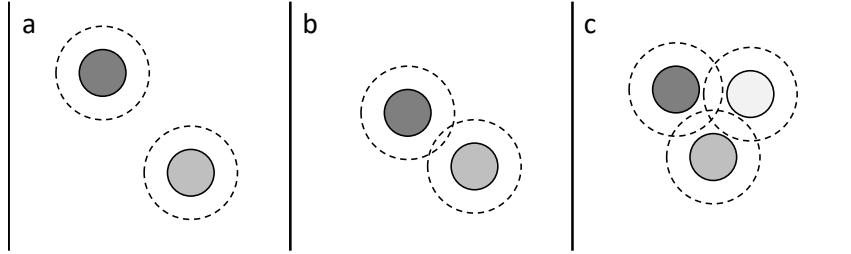


Figure 4.1: The ambiguity arising from multiple cell placements in a micro-trench. Each colored circle represents a cell and the dashed line enclosing the cell refers to assumed possible movement range. (a) Two cells which are far away from each other can be tracked uniquely in one recording interval. However, at one point, the cells may get close to each other. (b) Two cells that are within the movement range of the other cell might be tracked wrongly. This, however, is irrelevant for the time-to-death (TTD) analysis and the sister death time (SDT) correlation analysis. (c) In case of more than two cells the SDT and TTD analyses can be flawed by tracking errors.

Due to the independent nature of the cell placement in the micro-trench, the Poisson point process is suited to model the placement process [164] with the following assumptions:

- The cell placement rate is constant.
- The placement of one cell in a micro-trench does not affect the probability of another cell being placed in a micro-trench.
- The probability that a micro-trench receives a cell is proportional to the length of the observation.
- Two cells can't land in one micro-trench at exactly the same instant.

Since the placement process is Poisson, the probability of a micro-trench being placed with n cells is

$$P_{pois}(o(t) = n) = \frac{\lambda^n}{n!} e^{-\lambda} \quad (4.1)$$

We can compare this model with the experimental occupancy distribution. For the image position 41, each cell is tracked back to the initial time point. Figure 4.2 shows that the cell placement process is very well modeled using the Poisson process. Arguing in reverse, we can also say that the assumptions listed above which are necessary for the process to be Poisson seem to be met in our experiment.

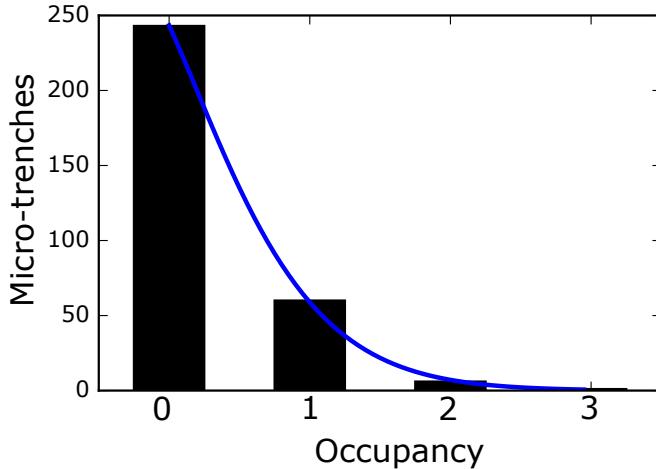


Figure 4.2: Occupancy distribution of micro-trenches in the image position 41. The blue line is a fitted Poisson distribution $P_{pois}(x)$ for x from 0 to 3. The fitting process yields fitted parameter $\lambda = 0.242$ which corresponds to the average placement of the micro-trench. This means that on average, less than one cell can be found in a micro-trench. Also, the fits with the observation confirming our assumption of the Poisson characteristics of the cell placement process.

On the down side, however, knowing the process is Poisson, increasing the number of singly-placed cells beyond this distribution is hard due to the nature of the process. Manually placing each cell takes a lot of time and may not work at all. Scaling the experiment thus, for now, translates directly to raise the number of micro-trench to increase the number of singly-placed cancer cells.

The complete occupancy statistics for every valid image positions in the unsynchronized and synchronized experiments can be seen in Table B.2 in Appendix B.

4.3 Variability of cell cycle duration (CCD) time

To conduct CCD analysis, cell trees fulfilling criteria in Table 4.4 are used. For each cell tree, we determine the first division time point t_0 , the second division of the first daughter cell at time t_1 , and the division of the second daughter cell at t_2 . Figure 4.3 shows an exemplary image series of one micro-trench filled with initially one cell which giving birth to four cells within the first 45 hours.

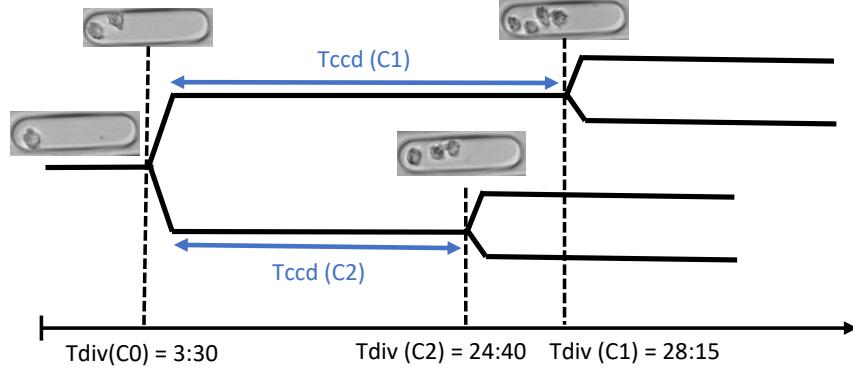


Figure 4.3: Schematic representation of each cell's division time T_{div} and cell cycle duration T_{ccd} of a cell tree. The accompanying insets show the recording of the cell tree in various time points (left to right): shortly after the start of the recording, after the first division, after the first division of the children and after the second division of the children. The timepoints are all in hours:minutes.

In unsynchronized and synchronized experiments, 58 and 13 sisters of singly-placed cells were observed for 45 and 24 hours to detect three cell divisions. Some distributional statistics are gathered for each experiment. The data were also fitted with gamma (dashed blue line, see Subfigures 4.4a and 4.4b) and lognormal (red line, see Subfigures 4.4a and 4.4b) distributions. Tables 4.5 and 4.6 list the findings for the unsynchronized and the synchronized experiments.

Category	Value
Size	116 cells (58 sisters pairs)
Mean T_{div}	20.9 hours
Median T_{div}	20.5 hours
Standard dev. of T_{div}	5.0 hours
Gamma – shape	5.7
Gamma – shift	8.5
Gamma – scale	2.2
Lognorm – shape	0.2
Lognorm – shift	-4.2
Lognorm – scale	24.6

Table 4.5: Results of CCD analysis in the unsynchronized experiment. The shape, shift and scale parameters refer to the parameters of gamma and lognorm distribution as defined in `scipy's gamma` and `lognorm` classes' `cfd` function.

Category	Value
Size	26 cells (13 sisters pairs)
Mean T_{div}	20.8 hours
Median T_{div}	21.1 hours
Standard dev. of T_{div}	1.3 hours
Gamma – shape	345.3
Gamma – shift	-4.3
Gamma – scale	0.1
Lognorm – shape	0.01
Lognorm – shift	-426.1
Lognorm – scale	446.9

Table 4.6: Results of CCD analysis in the synchronized experiment. The shape, shift and scale parameters refer to the parameters of gamma and lognorm distribution as defined in `scipy's gamma` and `lognorm` classes' `cfd` function.

The difference in size is attributable to the fact that unlike in unsynchronized experiment, the image capturing only last for 24 hours in synchronized experiment. Thus, assuming the cells are in various phase when the recording starts, only cells that have just divided at the start of the recording will ever divide twice (see the distribution of cell-cycle time). In a sense, the size difference confirms our finding regarding the duration of the cell cycle. The average cell cycle durations are also in line with previously done experiment by Sekhavati [30].

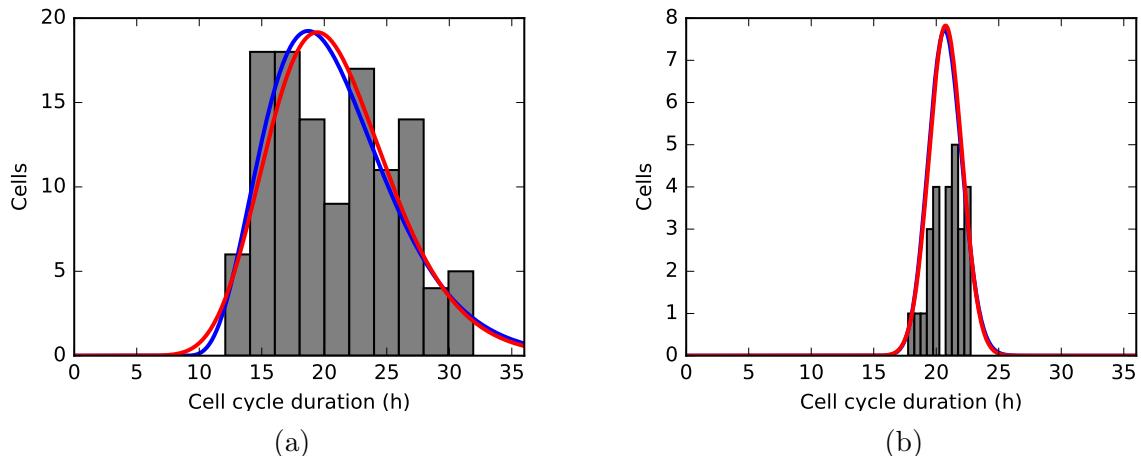


Figure 4.4: Measured distribution of cell cycle duration (CCD) T_{div} of (a) unsynchronized and (b) synchronized experiments from an ensemble of 116 and 26 cells showing a mean cell cycle time of (a) 20.90 hours with standard deviation of 4.98 hours and (b) 20.75 hours with standard deviation of 1.32 hours. The dotted red line corresponds to a lognormal fit and the dashed blue line to a gamma distribution fit. The difference in the distribution width from both experiments is caused by the fact that the synchronized experiment only lasts for 24 hours. The measurement is thus bounded at 24 hours.

We also analyzed the difference of CCD between two sisters. Subfigures 4.5c and 4.5a show the distribution of the difference in CCD between sister cells in unsynchronized and

synchronized experiments. Subfigures 4.5b and 4.5d show the correlation plots of T_{div} of sister cells (black circle) and randomly picked pairs (red triangle) from unsynchronized and synchronized experiments.

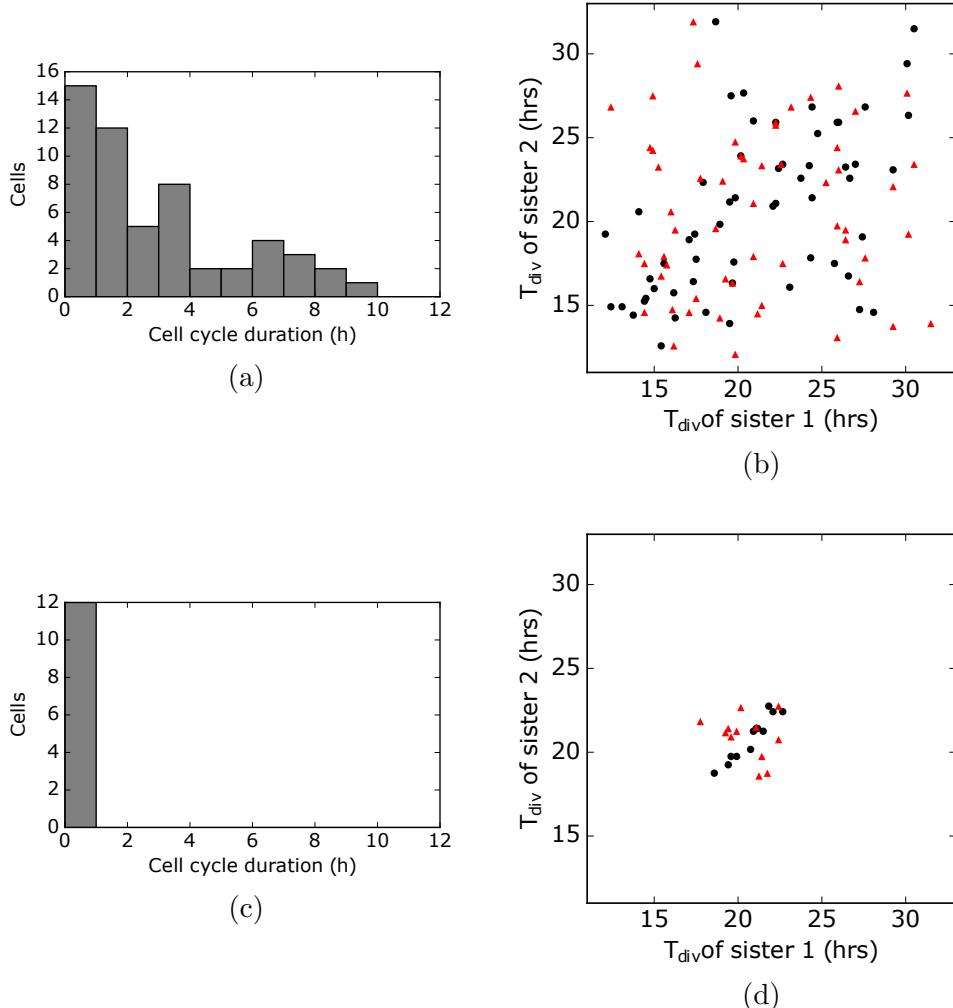


Figure 4.5: Difference of cell-cycle duration between sisters. Distribution of the difference of the cell cycle duration between sister cells in (a) unsynchronized and (c) synchronized experiment. Correlation plots of the cell cycle duration for sister cells (black circles) and randomly paired cells (red triangles) in (b) unsynchronized and (d) synchronized experiments. In unsynchronized experiment, the Pearson correlation coefficient for sister cells is $r = 0.525$ with $p\text{-value} = 2.66 \cdot 10^{-5}$, and for random-paired cells $r = 0.02$ with $p\text{-value} = 0.85$. In synchronized experiment, the Pearson correlation coefficient for sister cells is $r = 0.956$ with $p\text{-value} = 1.24 \cdot 10^{-6}$, and for random-paired cells $r = -0.48$ with $p\text{-value} = 0.109$.

4.4 Variability of time-to-death (TTD)

Generally, we are interested in the following aspects:

- Does the response of cancer cells depends on their cell cycle state?

- The heritability of cancer drugs treatments.
- Correlation between drug concentration and the response of AML-M5a MOLM-13 cell line.

To answer the questions we select cells fulfilling criteria in Tables 4.1 (unsynchronized experiment) and 4.1 (synchronized experiment).

4.4.1 Effect of the time in cell cycle on cancer drugs response

The measured time-to-death distribution is shown in Figure 4.6 for increasing Vincristine concentration. Figure 4.7 shows the measured time-to-death distribution for increasing Daunorubicin concentration. The blue curves represent the Kernel density estimation of the probability density function of each histogram.

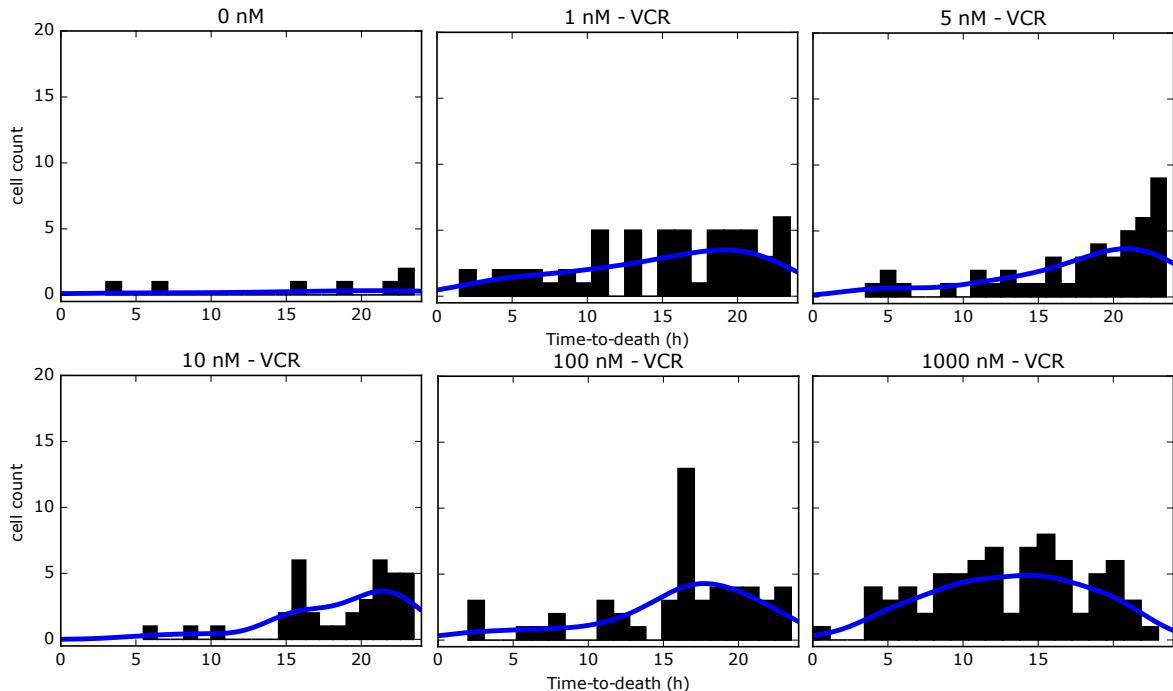


Figure 4.6: Time-to-death distribution of AML-M5a MOLM-13 cells for increasing concentration of Vincristine in the unsynchronized experiment. The upper left histogram is the distribution in the control.

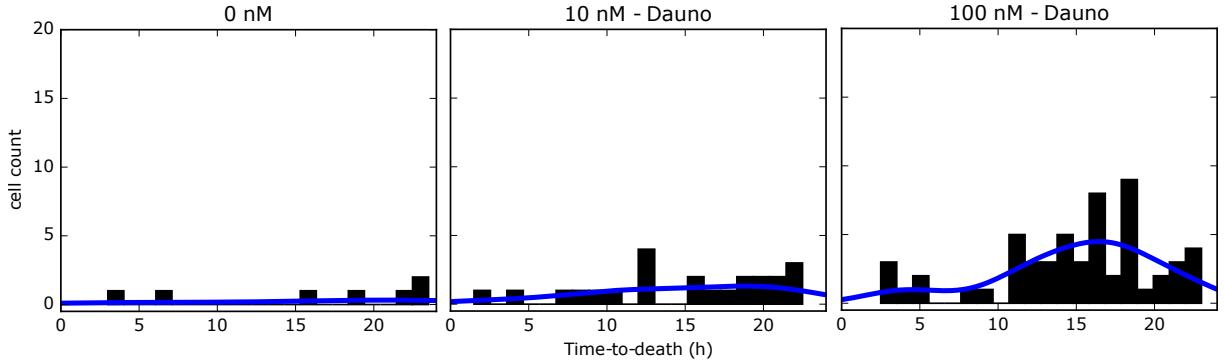


Figure 4.7: Time-to-death distribution of AML-M5a MOLM-13 cells with increasing concentration of Daunorubicin in the unsynchronized experiment. The leftmost histogram is the distribution in the control.

The plots in Figure 4.8 depict, for each Vincristine concentration, a scatter plot of the time passed in the cell cycle, i.e. the time passed from the division until the drug was added, with the time-to-death i.e. the time passed from the drug administration time point until the death of the cell. The scatter plots for every Daunorubicin concentration can be seen in Figure 4.9. The blue lines are best linear fits and show the dependence of the time-to-death on the time spent in the cell cycle. The colored areas indicate the cell cycle phase based on the division distribution in Subfigure 4.4a, the duration for each phase is calculated based on the phase durations proposed by T. S. Weber et al. [170]. We observed a negative correlation between the time in the cell cycle and the death time, which increased for increasing VCR concentrations (1-100 nM), but there was no correlation for the highest VCR concentration (1000 nM), indicating that in this high concentration side effect toxicities are prominent. The Pearson product-moment correlation test results are presented in Table 4.8. In the case of Daunorubicin (100 nM), we observed an even stronger negative correlation between the time spent in the cell cycle and the time-to-death in the case of Daunorubicin (100 nM). Especially the time-to-death of the cells that were in the S phase when the drug was added, the phase in which Daunorubicin should be more effective, had a larger deviation in comparison to the time-of-death of cells that were in the G1 or in the G2/M phase.

Treatment	r	p-value
Control (0 nM)	0.26	0.23
Vincristine 1 nM	-0.15	0.27
Vincristine 5 nM	-0.47	0.038
Vincristine 10 nM	-0.29	0.085
Vincristine 100 nM	-0.34	0.02
Vincristine 1000 nM	-0.00	0.97
Daunorubicin 10 nM	0.26	0.22
Daunorubicin 100 nM	-0.16	0.22

Table 4.7: The results of the Pearson product-moment correlation test (correlation coefficient r and p-value) for various concentrations in the unsynchronized experiment.

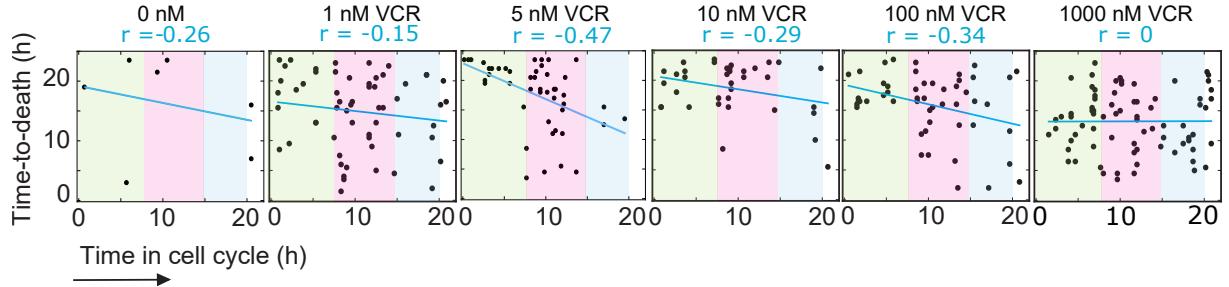


Figure 4.8: Correlation between the time in cell cycle and the time-to-death for every Vincristine concentration in the unsynchronized experiment. The concentration increases from left to right with the leftmost plot shows the correlation in the control.

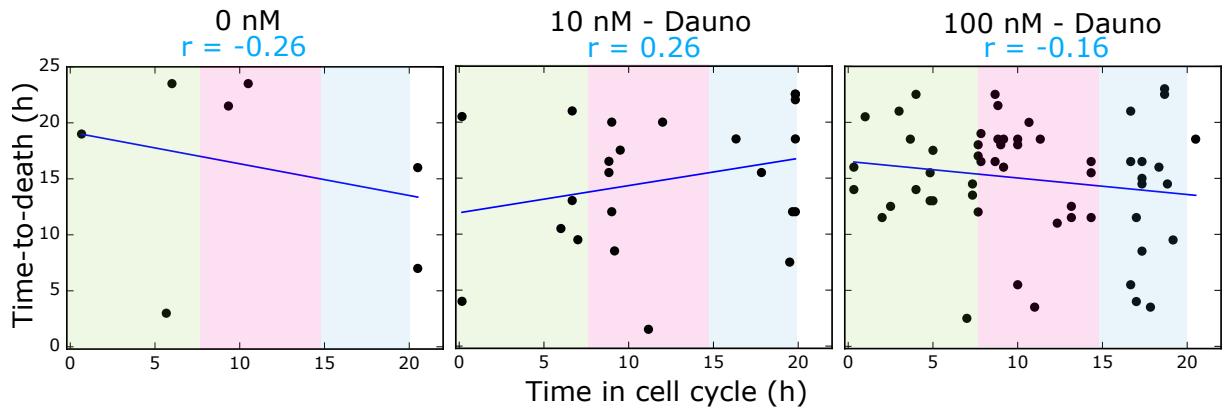


Figure 4.9: Correlation between the time in cell cycle and the time-to-death for every Daunorubicin concentration in the unsynchronized experiment. The concentration increases from left to right with the leftmost plot shows the correlation in the control.

We then compare the results to the synchronized experiment. In Figure 4.10, we plot the distributions of the time-to-death for every Vincristine concentration. Figure 4.11 shows the distribution of the time-to-death for every Daunorubicin concentration. The blue curves represent the Kernel density estimation of the probability density function of each histogram. For all drug concentrations the maximum of these distributions is near the 15th hour after starting imaging.

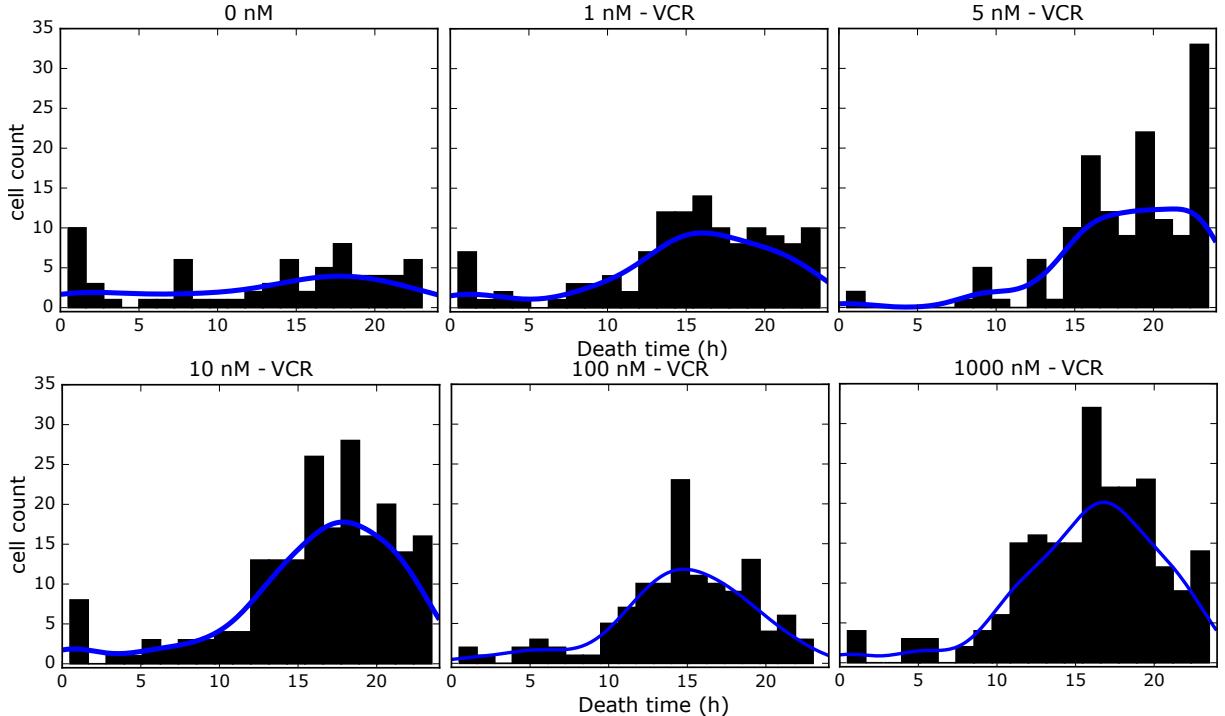


Figure 4.10: Time-to-death distribution of AML-M5a MOLM-13 cells for increasing concentration of Vincristine in the synchronized experiment. The upper left histogram is the distribution in the control.

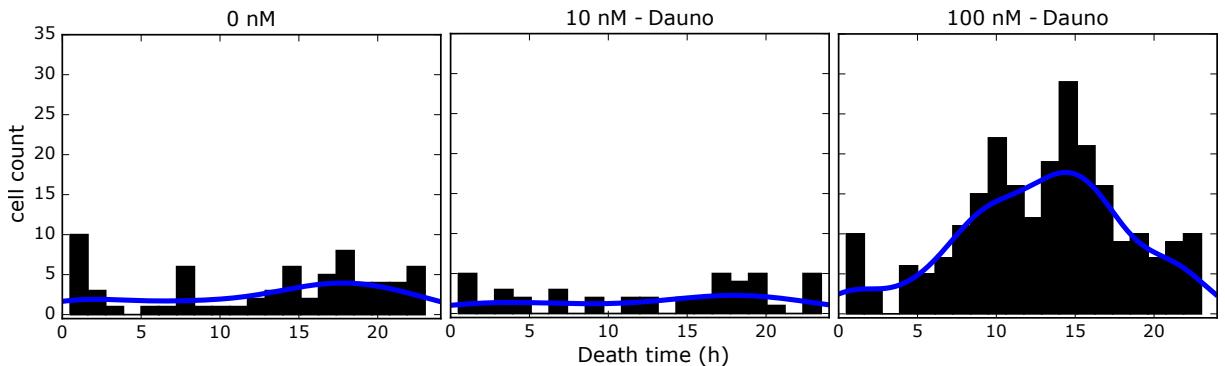


Figure 4.11: Time-to-death distribution of AML-M5a MOLM-13 cells for increasing concentration of Daunorubicin (left-right) in the synchronized experiment. The leftmost histogram is the distribution in the control

Figure 4.12, shows the death times between the synchronized (blue) and the unsynchronized (red) population. The distributions plotted are the normalized number of cells in all drug treatments, for all cells tracked. Apart from a peak at the beginning of the measurement in the synchronized population, the shape of the two distributions is equivalent. In the synchronized population less cells have a short time-to-death, especially evident in the VCR 1000 nM concentration (Figures 4.6 and 4.10), indicating that the synchronized population is more resistant to death.

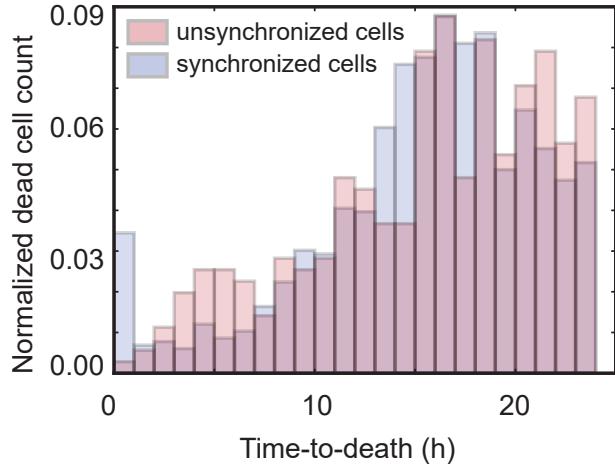


Figure 4.12: Normalized distribution of time-to-death of every analyzed AML-M5a MOLM-13 cells in the unsynchronized and synchronized experiments. The normalized distribution represents empirical probability density function for the survival rate of the cells in both experiments.

4.4.2 Heritability of cancer drugs response

To test heritability of time-to-death. The sisters death time(SDT)correlation and non-sister death time analyses are conducted. Only cell trees fulfilling the requirements listed in Tables 4.1 and 4.3 are analyzed. Figure 4.13 show scatter plots of the time-to-death between sister cells in an ensemble of all drug treatments. The Pearson correlation coefficient r is 0.52 (medium correlation) with p-value of $1.14 \cdot 10^{-6}$ for the sister cells of the unsynchronized population and 0.06 with p-value of 0.52 for the non-sister cells in synchronized experiment. The ellipses indicate the directionality of the correlation. Thus, the time-to-death was positively correlated between sister cells in unsynchronized experiment but not correlated between non-sister cells placed in the same micro-trench in the unsynchronized experiment.

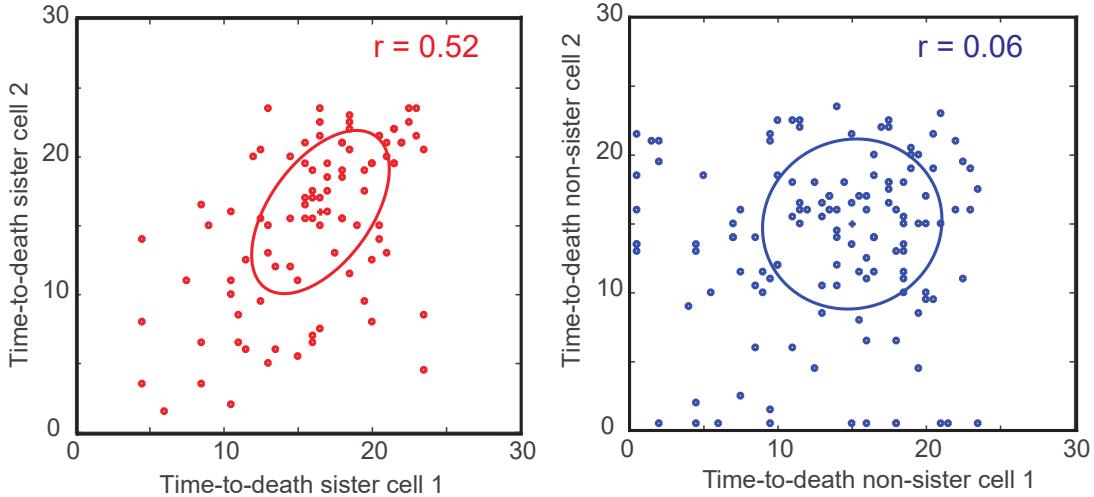


Figure 4.13: The correlation of measured time-to-deaths between two sisters in the unsynchronized experiment (left) and two unrelated cells in the synchronized experiment (right). The ellipses denote the directionality of the correlation and were calculated with the Principal Component Analysis (PCA).

4.4.3 Effects of cancer drugs concentration

To analyze the effect of cancer drug concentration on cell survival, we take the latest timepoint in both brightfield and fluorescent channels. For each image position i the number of cells with fluorescent signals n_e^i is calculated by using the LoG detector in Fiji. This number is then compared to the number of all cells in the image position i n^i . Let C be the set of image positions with cancer drugs concentration c . The percentage of death cells p_c for a given concentration c is thus defined as following:

$$p_c := \frac{\sum_{i \in C} n_e^i}{\sum_{i \in C} n^i} \quad (4.2)$$

For both the unsynchronized and the synchronized population, the percentage of dead cells as a function of drug concentration is shown in Figure 4.14. It shows the expected increase in death response with increasing dose of Vincristine. For the error bars, we hypothesize that the data follow the binomial distribution. For a confidence level of 95% the error is $1.96 \sqrt{\frac{p_c}{(1-p_c)n^i}}$.

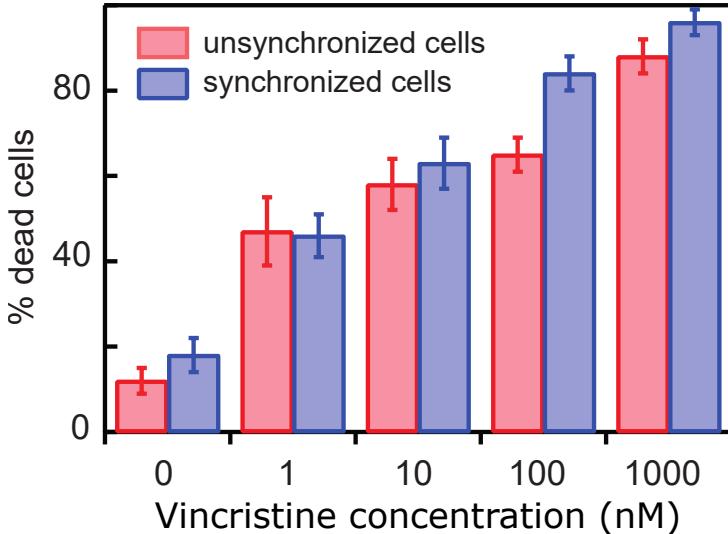


Figure 4.14: Percentage of dead cells in increasing Vincristine concentration in the unsynchronized and synchronized experiments. Note that even in the image positions without any cancer drug treatments some cell undergo programmed cell death and emit fluorescent signal.

4.5 Machine learning models of cell death

Is it possible to predict a given cell’s time-to-death using features coming out of our pipeline? To answer that, we use Support Vector Regression (SVR, see Subsection 2.2.11) to predict the time-to-death of a cell at a given time point. For the regression, we first take every valid time point for every valid cell tree. For every time point t , following features are used:

- The cell’s velocity at time t .
- Total brightfield intensity at time t .
- Total PI intensity at time t .
- Total Caspase intensity at time t .

The model is trained on 70% of data points from the unsynchronized tracking data and tested on the other 30%. Figure 4.15 shows the results of the analysis. We evaluate the performance of the regressor using the coefficient of the determination between measured and predicted time-to-deaths (TTD), the measured Pearson’s correlation between the real and predicted TTDs. Table 4.8 lists the evaluated measures for the model.

Measure	Value
R2 score	0.15
Pearson’s correlation coefficient (PCC)	0.33
p-value	$1.2 \cdot 10^{-257}$

Table 4.8: The results of the Pearson product-moment correlation test (correlation coefficient r and p-value) for various concentrations in the unsynchronized experiment.

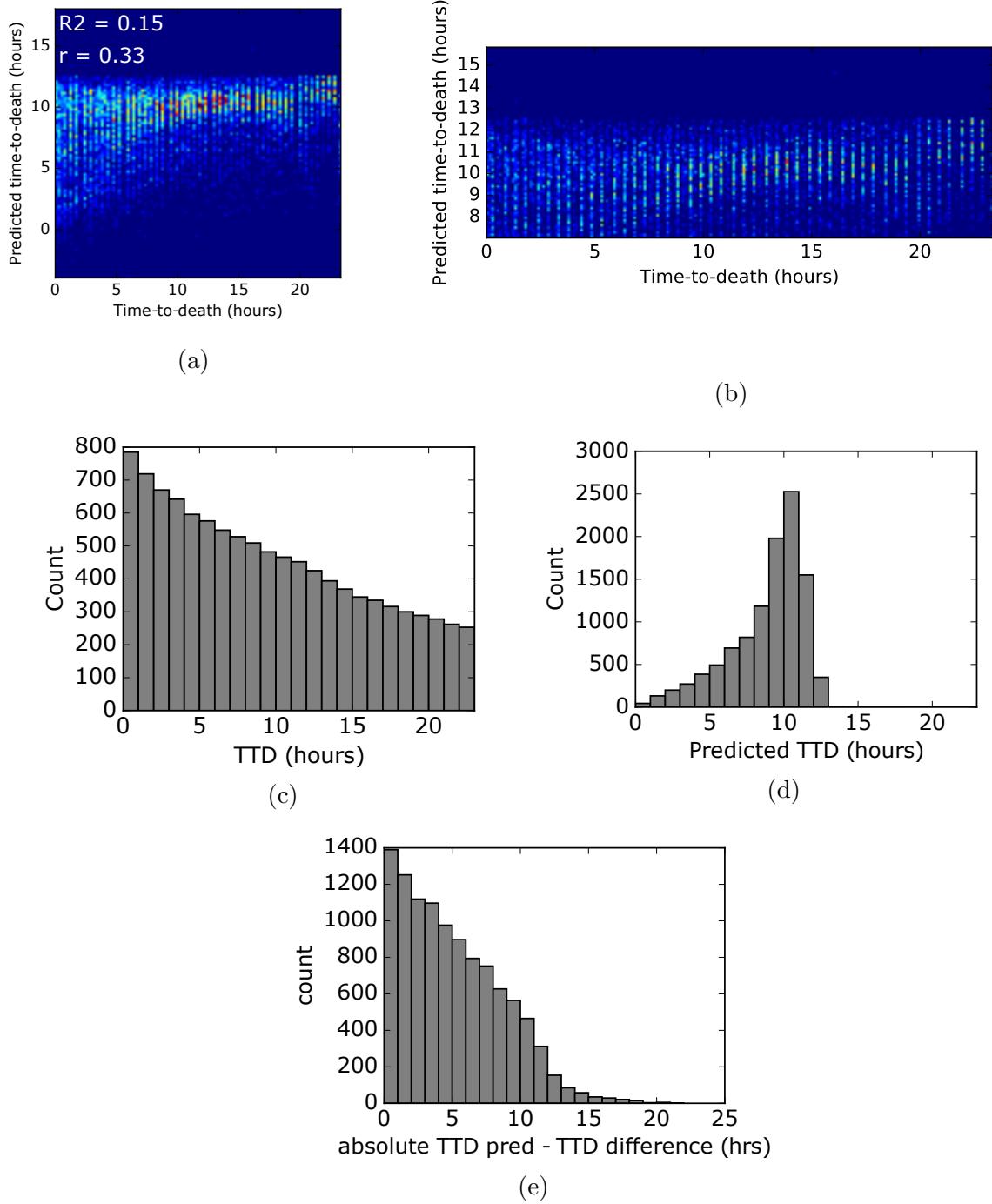


Figure 4.15: The results of TTD prediction using SVM: (a) The correlation plot of the TTD and predicted TTD in the unsynchronized experiment. The coefficient of determination of the predictor is 0.15 and the measured Pearson's correlation coefficient between the real and predicted TTD is 0.33 (medium correlation). (b) The correlation plot of the TTD and predicted TTD zoomed in the y-axis area between 0 and 16 hours. The predicted TTD values generally follow the real TTD in the later phase of the cell life (low TTD range). In the earlier phase of a cell's life however, the model could not predict the TTD at all as shown by the random prediction at 20+ hours aTTD range. (c) Distribution of TTD values in the test set. (d) The distribution of predicted TTD values in the test set. In line with the observation in the heatmap, the prediction levels off around 10 and there is barely any predicted TTD above 14 hours (e) The distribution of the absolute difference between the real and predicted TTD values.

Chapter 5

Conclusion and Outlook

We showed that arrays of micro-structured trenches provide a platform that enables a label-free method for tracking cells and for approximating the cell cycle phase without the use of molecular markers. Our approach allows to set individual clocks in single cells using the first division for each occupied trench as a starting point. This is highly accurate and overcomes typical drawbacks of low detection efficiency of fluorescence-based indicators such as the FUCCI marker, which usually have low transfection efficiency round 20-40%, and generally short duration of staining, namely 15 hours [172], which is no sufficient for our long-time measurements.

By compartmentalizing the cell population in small groups we enable an image-based cell tracking. Without the use of micro-trench, time-lapse observation of a cell population for very long hours in excess of 24 hours is impossible for both adherent and non-adherent cells since cells escape from the field of view very quickly, as fast as within a few hours. Even assuming that the cells move in random walk fashion, some cells are bound to move beyond the margin of an image position. Even if they came back to the field of view, this cell will not be recognized as a previously tracked cell. Over the long term, the number of cells that stay within an image position approaches zero.

In the context of the cell tracking, compartmentalization improves the tracking quality in two ways. First, it reduces the error of mixing identities of adjacent cells (Figure 4.1 visualizes why having two or more cells within moving distance from the other cells in one frame time may cause ambiguity during tracking). Second, it reduces computational time since the range of cell movement is limited by the micro-trench. For example, using micro-trenches we can use much lower maximum distance d_{max} in our cell tracking algorithm (see Subsection 2.2.9).

We also observe an interesting finding regarding the distribution of the cells inside the micro-trenches. The distribution follows closely the Poisson point process, which is widely used to model distribution of independently occurring events on a plane such as rainfall [173] and photon absorption on a surface [174]. Moreover, Othmer [175] shows that the Poisson point process explains well various biological dispersion phenomena, indicating that our finding aligns well with the previous findings in related processes.

The correlation between cancer drug concentration and cell survival is also shown to be inversely proportional in the logarithmic setting. This is for example in line with the Finding by Jackson and Bender [176] on the cytotoxicity of Vincristine. Similar finding was also reported by Kobayashi et al. [177].

In addition, we demonstrate the practicability of the micro-trench platform to deter-

mine the time-to-death after induction of cell death with Vincristine and Daunorubicin. At high concentration Vincristine stimulates microtubule depolymerization and mitotic spindle destruction. At lower clinically relevant concentrations, it blocks mitotic progression. As a result, we expected a negative correlation between the time spent in the cell cycle and the time-to-death in the case of VCR, since a cell that spent a long large amount of time in the cell cycle should be closer to the M-phase so it should have a shorter time-to-death i.e. it should die earlier. Indeed here we observed that the time-to-death negatively correlates with the time spent in the cell cycle and that this correlation becomes more prominent with increasing VCR concentration up to 100 nM, but there is no correlation at the highest concentration (1000 nM). The reason for no correlation in the 1000 nM might be side effect toxicities that happen through the whole cell cycle and as it has been shown before [18], after exposure to antimitotic drugs cells display complex fate profiles, such as unequal cell division producing aneuploid daughter cells, exiting the cell cycle without undergoing cell division (mitotic slippage), or exiting G1 and undergoing apoptosis or senescence. On the other hand, the correlation of the time-to-death with the time spent in the cell cycle is more prominent in the case of Daunorubicin (100 nM) and the deviation of the time-to-death for the cells in the S-phase during drug addition, is larger compared to the cells in G1 or G2/M phase (Figure 4.6).

The measured cell cycle durations, while sparse in the synchronized population, also follows previous findings regarding the cell cycle duration [30]. The underlying Gamma distribution, while not as obvious as previous finding by Sekhavati [30], still shows a semblance to a Gamma distributed variable. We also observe the significant relatedness of the measured cell cycle duration between sister cells vis-à-vis randomly selected pairs. This is shown by the significant difference in Pearson's correlation coefficient and its p-value between the two (see Subfigures 4.5b and 4.5d) showing the heritability of cell cycle duration. Axelrod [178] shows for example that cell cycle durations are passed down even multiple generations down the line [178].

The observed lognormal cell cycle duration distribution is well documented in literature, with both the size of mammalian cells as well as cell doubling times following a lognormal distribution [165, 166]. The underlying mechanism of cell division timing is an active field of research. It is however assumed that the so-called adder model accepted in bacteria [167] is also valid in somatic cells. In general size regulation can arise from various types of coupling between cell size, cell growth and cell cycle progression [168]. It has also been proposed, based on a coupled mathematical model of mammalian cell cycle and circadian clock, that the circadian clock triggers critical size control in the mammalian cell cycle and that it is more readily observed in cell trees that contain circadian rhythms [169]. In this context it is important that the adder mechanism also predicts exponential time correlation between sister cells as observed in our experiments [167].

We furthermore find that the sister cell correlation of the time-to-death differs from non-sister correlation of the time-to-death as shown by differing results in the unsynchronized experiment which are taken from sister cells and synchronized experiment which are taken from cells which happened to occupy the same micro-trench. This is not the case in the synchronized experiment. This, combined with related results regarding the correlation of the cell cycle duration between sister cells, confirm the hereditary factor playing a role in the cell response to cancer drug treatment.

At the same time the distributions of the time-to-death (average of all different drug concentrations) of the unsynchronized and the synchronized populations are similar, which

would suggest that synchronizing the cells with the double thymidine block did not affect their response to both tested drugs. Combined with the fact that thymidine block synchronizes the cell phase, this suggests that the double thymidine block procedure has an effect on the cell response heterogeneity to Vincristine and Daunorubicin. Several authors [14, 179] previously reported that sister cells undergo apoptosis synchronously. In another study [11], sister cell fate in response to TRAIL-induced apoptosis correlated, as did also the time-to-death between HeLa sister cells but this correlation decayed as a function of time since division, the time period tested was 8 hours. Based on this observation, the transient heritability in fate model was proposed, which states that protein synthesis promotes cell divergence so that sister cells soon become no more similar to each other than random paired cells.

5.1 Outlook

5.1.1 More advanced use cases for micro-trench experiments

In future work, the micro-trenches array can be used with more than one drug, namely for combination therapy, to determine the optimal administration timing of each drug. Microfluidic devices have facilitated single-cell studies and boosted the collection of quantitative experimental data, such as the measurement of single-cell mass with high accuracy [180]. Measuring the cell size or the volume added to the cell after its mother division are crucial quantitative data to elucidate the underlying mechanisms that drive cell division. The micro-trenches array coupled with time-lapse microscopy and automated image analysis is a step toward this direction.

5.1.2 Fully automated microfluidics pipeline

An interesting improvement possibility for the microfluidics would be designing cell placement process that is not bound by the Poisson distribution. Microrobotics has been an up and growing field in the recent years with new developments attempting to manipulate cells using micro-sized robots [181]. Some microrobots have found its use in non-invasive surgery for example [182]. Scaling up the process would mean that the bottleneck caused by the Poisson characteristics of cell placement may be resolved in the future.

5.1.3 Fully automated pipeline for single-cell analysis

One positive thing about the design of our data analysis pipeline is the modularity of its component. As mentioned multiple times in this thesis (in particular in Subsection 3.2, our method can well be improved by automatizing the micro-trench masking process. Although itself not a bottleneck of the process, as the entire dataset of 63 image positions could be processed in less than an hour, automating this process will save time and more importantly, clicking and dragging efforts, thus lets scientists do the things that matter more for them.

There are several methods that came to mind in improving the micro-trench masking, which generally can be separated in two groups: ML-based methods and non-ML-based methods. ML-based methods generally require either a manual annotation of correctly recognized micro-trench (a class of methods called supervised learning methods) or certain

prior knowledge regarding distinctive features of micro-trench that separate it from background image and/or the cells (a class of methods called unsupervised/semi-supervised learning methods).

In his master's thesis, Oliver Hilsenbeck introduced a masking tool that requires minimum amount of annotation data [183]. As one of recent series of groundbreaking papers, Jonathan Long proposes fully convolutional extension [155] of previously state of the art convolutional networks [47, 48] thereby improving the performance and speed of the process. Considering the relatively simple form of micro-trench, training a model for trench detection seems to be the next natural step to improve on the pipeline.

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Appendices

Appendix A

Source code and data

All our code are available in the github repository:

<https://github.com/raharjaliu/MA>.

Following scripts are particularly interesting:

- `fij.py` contains Jython implementation of the image pre-processing pipeline.
- `source/` contains various analysis scripts used in the post-tracking analysis.

The data used in this project are proprietary to the Soft Condensed Matter Group at the Faculty of Physics of the Ludwig-Maximilliäns-Universität München (the chair of Prof. Joachim O. Rädler). If any access to the data are required, please contact one of the following email addresses:

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Appendix B

Tables

B.1 Inferred shift in wells

Pos.	δ_x	δ_y	Pos.	δ_x	δ_y	Pos.	δ_x	δ_y
01	-2	-4	22	-7	-4	43	-7	-3
02	-3	-4	23	-7	-4	44	-7	-3
03	-3	-4	24	-9	-4	45	-7	-3
04	-3	-4	25	-9	-5	46	-7	-3
05	-3	-3	26	-8	-5	47	-7	-2
06	-3	-4	27	-8	-4	48	-4	-2
07	-3	-4	28	-8	-4	49	-4	-2
08	-4	-3	29	-8	-4	50	-4	-2
09	-4	-4	30	-8	-4	51	-4	-2
10	-4	-4	31	-8	-4	52	-4	-2
11	-4	-4	32	-8	-3	53	-5	-2
12	-4	-3	33	-9	-3	54	-5	-2
13	-4	-3	34	-8	-3	55	-4	-1
14	-5	-3	35	-8	-3	56	-2	-2
15	-5	-3	36	-9	-2	57	-3	-3
16	-6	-5	37	-9	-3	58	-3	-3
17	-6	-5	38	-9	-2	59	-3	-2
18	-6	-5	39	-8	-2	60	-3	-2
19	-6	-5	40	-7	-3	61	-3	-3
20	-6	-4	41	-7	-3	62	-3	-2
21	-7	-5	42	-7	-3	63	-4	-2

Table B.1: The inferred shift (in pixels) for each image position in unsynchronized experiment. The shifts are inferred using the shift correction algorithm explained in Subsection 2.2.10.

B.2 Micro-trench occupancy statistics

Pos.	Det.	Occ.	1	2	3	4+	Pos.	Det.	Occ.	1	2	3	4+
1	305	68	55	12	1	0	33	311	55	50	4	1	0
2	314	57	52	5	0	0	34	109	14	13	1	0	0
3	291	57	48	8	1	0	35	266	56	51	5	0	0
6	300	76	64	11	1	0	36	108	19	16	3	0	0
7	313	62	47	14	0	1	39	284	111	87	20	4	0
8	310	78	69	9	0	0	40	310	67	60	6	1	0
9	305	77	59	16	2	0	41	309	76	66	10	0	0
10	303	54	45	9	0	0	42	301	78	68	10	0	0
12	253	79	60	17	2	0	43	286	85	73	10	1	1
14	234	79	56	13	7	3	45	295	45	36	8	1	0
15	301	86	66	15	5	0	46	312	57	50	7	0	0
16	305	53	44	8	0	1	47	217	71	60	10	1	0
17	309	69	54	15	0	0	48	280	80	67	11	1	1
18	199	30	25	5	0	0	49	312	77	70	6	1	0
21	240	49	45	4	0	0	50	262	57	48	7	2	0
22	232	53	49	4	0	0	51	304	114	84	26	4	0
23	235	64	48	7	8	1	52	287	94	69	21	4	0
24	305	59	53	5	0	1	55	309	67	57	9	1	0
25	307	62	60	2	0	0	56	281	53	45	8	0	0
26	296	85	76	6	3	0	57	307	44	40	4	0	0
27	312	83	69	12	2	0	58	280	60	56	4	0	0
28	308	93	78	14	0	1	59	57	14	13	1	0	0
29	208	22	20	2	0	0	61	304	58	51	7	0	0
30	307	80	63	12	3	2	62	298	101	83	16	2	0
31	307	87	64	18	4	1	63	214	51	48	3	0	0
32	292	40	35	5	0	0							

Table B.2: The number of detected micro-trenches (Det.), occupied micro-trenches (Occ.), micro-trenches with one cell tree (1), micro-trenches with two cell trees (2), micro-trenches with three cells (3) and micro-trenches with more than 3 cells (4+) of the unsynchronized experiment.

Pos.	Det.	Occ.	1	2	3	4+	Pos.	Det.	Occ.	1	2	3	4+
1	294	40	38	2	0	0	35	314	72	58	14	0	0
2	305	31	30	1	0	0	36	310	73	59	14	0	0
3	310	66	51	14	1	0	39	312	106	98	7	1	0
6	311	26	24	2	0	0	40	310	72	60	10	2	0
7	313	16	16	0	0	0	41	297	68	57	9	2	0
8	280	70	63	7	0	0	42	309	55	49	4	2	0
9	299	31	29	2	0	0	43	307	40	37	2	1	0
11	290	50	46	3	1	0	45	300	52	44	6	2	0
13	310	76	63	12	1	0	46	298	59	53	5	1	0
15	263	32	27	4	1	0	48	308	63	57	5	0	1
16	305	50	45	5	0	0	49	314	72	63	7	2	0
17	307	71	66	4	1	0	51	314	51	42	9	0	0
18	314	79	65	11	3	0	52	313	40	37	2	1	0
21	314	71	62	9	0	0	54	319	52	46	6	0	0
22	309	70	65	5	0	0	55	304	55	51	4	0	0
23	306	53	47	5	1	0	56	311	101	89	10	2	0
24	302	69	61	8	0	0	57	310	63	51	11	1	0
27	294	98	75	18	5	0	58	309	32	28	4	0	0
28	311	71	58	11	1	1	61	309	51	48	2	1	0
30	303	59	51	8	0	0	62	304	43	38	5	0	0
31	305	56	50	6	0	0	63	294	8	8	0	0	0
32	306	65	57	7	1	0							
33	311	70	62	7	1	0							

Table B.3: The number of detected micro-trenches (Det.), occupied micro-trenches (Occ.), micro-trenches with one cell tree (1), micro-trenches with two cell trees (2), micro-trenches with three cells (3) and micro-trenches with more than 3 cells (4+) of the synchronized experiment.

B.3 Micro-trench yields statistics

Pos.	Cell trees	Singly-placed	One division	$T_{div} < T_{treat}$	$T_{eot} > T_{treat}$
1	82	55	9	8	0
2	62	52	11	7	3
3	67	48	10	10	0
6	89	64	19	15	8
7	80	47	8	8	1
8	87	69	21	21	8
9	97	59	7	6	2
10	63	45	12	9	2
12	100	60	14	12	6
14	116	56	14	14	7
15	111	66	11	9	7
16	64	44	11	9	3
17	84	54	7	5	3
18	35	25	3	2	2
21	53	45	14	12	8
22	57	49	12	9	4
23	90	48	6	4	1
24	67	53	10	7	4
25	64	60	16	14	7
26	97	76	16	10	3
27	99	69	16	11	8
28	111	78	15	13	8
29	24	20	0	0	0
30	105	63	19	16	8
31	116	64	15	12	6
32	45	35	2	1	0

Table B.4: The number of processed cells in the unsynchronized experiment for the time-to-death (TTD) analyses. From left to right the requirements become more stringent and only the cell trees fulfilling all criteria (rightmost column) are used for TTD analyses.

Pos.	Tree	Singly-placed	One division	$T_{div} < T_{treat}$	$T_{eot} > T_{treat}$
33	61	50	10	6	3
34	15	13	2	2	0
35	61	51	13	7	1
36	22	16	5	5	1
39	139	87	16	16	1
40	75	60	13	12	7
41	86	66	17	8	4
42	88	68	17	13	3
43	100	73	12	10	1
45	55	36	5	3	1
46	64	50	12	10	6
47	83	60	12	8	3
48	96	67	3	2	1
49	85	70	24	23	0
50	68	48	2	2	0
51	148	84	10	8	0
52	123	69	11	7	1
55	78	57	6	3	0
56	61	45	6	3	1
57	48	40	1	0	0
58	64	56	6	6	3
59	15	13	1	1	1
61	65	51	17	12	9
62	121	83	20	13	11
63	54	48	19	18	5

Table B.5: The number of processed cells in the unsynchronized experiment for the time-to-death (TTD) analyses. From left to right the requirements become more stringent and only the cell trees fulfilling all criteria (rightmost column) are used for TTD analyses.