Computer vision guided embryo biopsy

Wang, Zenan

2015

Wang, Z. (2015). Computer vision guided embryo biopsy. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65779>

<https://doi.org/10.32657/10356/65779>

COMPUTER VISION GUIDED EMBRYO BIOPSY

WANG ZENAN

*Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy*

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING
NANYANG TECHNOLOGICAL UNIVERSITY

December 2015

Abstract

Embryo biopsy, which is a process to remove a part of materials from a cell, is a necessary step of preimplantation genetic diagnosis (PGD). The rapid advancement of the PGD technique has been calling for easy, fast and precise automated embryo biopsy techniques to improve the efficiency, the throughput, and the survival rate of the PGD. In this thesis, a computer vision guided embryo biopsy system is introduced. This research is devoted to demonstrate several techniques that contribute towards the fully automated embryo biopsy system, including the embryo autofocusing, the embryo image segmentation, the embryo orientation, and the zona pellucida (ZP) dissection.

A fast and robust autofocusing algorithm consisting of an automatic focus window selection method and an optimum searching algorithm based on the shuffled frog leaping (SFL) algorithm is proposed. The size-adjustable focus window is capable of improving the accuracy of the focus measurement function (FMF) graph of the target object by focusing only on the informative portion of the image, thus leading to an accurate embryo position estimation in Z axis. The proposed improved shuffled frog leaping (ISFL) searching algorithm is able to bypass local maxima and reach the global maximum in the non-unimodal FMF graph, which effectively identifies the most suitable focal plane for single blastomere.

A technique that is able to select a suitable biopsy position on the zona pellucida automatically is also developed. The proposed technique consists of a method of estimating the elliptical internal and external ZP boundary and two methods of suitable ZP dissection position selection. The validity of this technique is demonstrated by both quantitative and qualitative results. The

elliptical ZP boundary estimation can also be applied to measure zona pellucida thickness variance (ZPTV) and other ZP morphology measurements.

A 3-point contact automated three-dimensional cell rotational system for single cell manipulation is developed. A high resolution in-house rotational stage and cellular structure recognition algorithms are designed and developed to facilitate the visual servo control. The system is capable of orienting a zebrafish embryo to the ideal viewing cross-section that is aligned with the focal plane of the microscope.

Based on the investigations in the axial and lateral oscillations of the piezo-driven micropipette in cell membrane injection and dissection, a piezo-driven lateral-oscillation micro-cutter is proposed to perform both single-direction and three-dimensional cell dissection. The proposed technique is superior to the classic manual technique in many ways such as higher precision, lower skill requirement and shorter processing time.

佛陀彌阿無南

*We are what we think. All that we are
arises with our thoughts. With our thoughts
we make the world.*

—*Buddha*

*To my dear Mom and Dad,
and
my beloved wife and son.
May they have joy and happiness in abundance.*

Acknowledgments

I would like to express my great gratitude to my supervisor Dr. Ang Wei Tech. This thesis would not have been accomplished without his theoretical enlightenment and critical suggestions. Without his help and encouragement, I would never reach this far on the academic road.

I am deeply grateful to research fellow Dr. Zhao Su, research associate Mr. Shee Chen Yap and Yan Naing Aye for their great support throughout my years of candidature. Their constructive suggestions helped me solve lots of technical problems.

I am also thankful to the technicians at Robotics Research Center for all the tedious administrative work that they have done for me, particularly Lim Eng Cheng, Tan Siok Kuan, Agnes and You Kim San.

I extend my greatest gratitude to my dear parents for their countless and invaluable caring and providing. I would also thank my wife for her support, love and encouragement and my newborn son for the joy and motivation he brought to me.

I would also like to thank my colleagues, Carlo Tiseo, Er Jie Kai, Lim Zhen Yi for the enjoyable time at RRC.

Contents

Acknowledgment	ii
Table of Contents	iii
List of Figures	vii
List of Tables	x
List of Symbols and Abbreviations	xi
1 Introduction	1
1.1 Research Motivation	1
1.2 Research Objectives	3
1.3 Technical Challenges	5
1.4 Research Scope	5
1.5 Dissertation Outline	6
2 Literature Review	8
2.1 Embryo Autofocusing	8
2.2 Embryo Image Processing	11
2.3 Cell Orientation	13
2.4 Zona Pellucida Dissection Techniques	15

2.4.1	Chemical Zona Pellucida Breaching	15
2.4.2	Laser Zona Pellucida Drilling	18
2.4.3	Mechanical Zona Pellucida Dissection	19
3	Autofocusing Technique Under Microscope	24
3.1	Autofocusing Window Selection	25
3.1.1	Related Researches On Autofocusing Window Selection	25
3.1.2	Focus Window For The Entire Embryo	26
3.1.3	Focus Window For A Target Blastomere	28
3.2	Autofocusing Searching Algorithm	29
3.2.1	Optimum Search Methods For Autofocusing	30
3.2.2	Shuffled Frog Leaping Algorithm	32
3.2.3	Improved Shuffled Frog Leaping Algorithm	35
3.3	Experimental Evaluation	39
3.3.1	Autofocusing Window Selection	40
3.3.2	Autofocusing Search Algorithm	42
3.4	Summary	46
4	Embryo Image Processing	48
4.1	Methodology	49
4.1.1	Zona Pellucida Boundary Estimation	50
4.1.2	Position Selection With Partial Image Stretching . . .	57
4.1.3	Position Selection With Value Adjustment	62
4.2	Experiment Results	66
4.2.1	ZP Boundary Estimation	68
4.2.2	Suitable ZP Dissection Position Selection	69
4.3	Discussion	70
4.4	Summary	72

5	Three-dimensional cell rotation	74
5.1	System Design	75
5.1.1	System Setup	75
5.1.2	Rotational Stage Design	77
5.1.3	The 3-Point Contact Cell Rotation Model	79
5.1.4	Operation Sequence For The Cell Rotation Control	85
5.2	Cell Rotation Control	87
5.2.1	Cellular Structure Recognition	87
5.2.2	Cell Rotation About The X Axis	89
5.2.3	Cell Rotation About The Z Axis	93
5.3	Experimental Results And Discussion	95
5.3.1	Cellular Structure Recognition	96
5.3.2	Cell Rotation About the X Axis	97
5.3.3	Cell Rotation About The Z Axis	99
5.4	Summary	100
6	Piezo-driven Zona Pellucida Dissection	102
6.1	Zona Pellucida Cutting Methodologies	103
6.2	Experiment Setups	105
6.3	Beneficial Oscillation In Piezo-assisted Cell Injection	105
6.3.1	Experimental Methods	105
6.3.2	Experiment Result and Discussion	108
6.4	Piezo-driven Zona Pellucida Dissection	116
6.4.1	Thermal Effects On Cutting	116
6.4.2	Investigation of Cutting Effect	119
6.4.3	Result And Discussion	122
6.4.4	Application of Lateral Oscillated Cutter	128
6.5	Summary	131

7 Conclusion	133
7.1 Summary	133
7.2 Contributions	136
7.3 Future Work	137
A Embryo Image Processing Results	140
Bibliography	151

List of Figures

1.1	Embryo biopsy in PGD.	2
2.1	Embryo image segmentation methods.	12
2.2	Cell orientation techniques.	14
2.3	Methods of zona pellucida opening.	16
2.4	Chemical zona pellucida breaching	17
2.5	Laser zona pellucida dissection	19
2.6	Mechanical zona pellucida dissection	20
2.7	Active mechanical zona pellucida injection/dissection	21
2.8	Mechanical zona pellucida dissection techniques.	22
3.1	Comparison between two focus windows for the entire embryo.	27
3.2	Focus window for a target blastomere.	29
3.3	Frog leaping rule.	38
3.4	Autofocusing with the all-inclusive focus window.	41
3.5	Autofocusing with the proposed focus window.	42
3.6	Comparison of the Fibonacci and ISFL algorithms.	43
3.7	Schematic of fish embryo and the corresponding FMF graph.	45
3.8	The portions of cytoplasm in different focal planes.	46
3.9	Performance on the FMF graph.	46
4.1	Function flow chart of embryo image processing	51

4.2	Embryo center estimation	52
4.3	Embryo boundary estimation	54
4.4	Zona pellucida boundary estimation	56
4.5	Zona pellucida boundary estimation	58
4.6	Zona pellucida partial stretching	59
4.7	Unequal sampled and stretched image	60
4.8	Edge and intensity weighted image	61
4.9	Edge and intensity image of ZP	64
4.10	Preliminary edge and intensity boundary of ZP	65
4.11	Value Adjusted boundary of ZP	67
4.12	Indication of perivitelline space	68
4.13	Image processing algorithm testing	69
4.14	Suitable ZP dissection position	70
4.15	Proposed algorithm VS Karlsson's	71
4.16	Inaccurate boundary with correct indication	72
5.1	Schematic illustration of the 3-D cell rotation process.	76
5.2	Embryo manipulation system setup.	77
5.3	The three-dimensional model of the rotational stage.	78
5.4	The positions of three contact points.	80
5.5	The contact forces and torque acting on the embryo.	82
5.6	Operation sequence for the cell rotation.	86
5.7	System control architecture.	87
5.8	Image segmentation of the target internal cellular structure.	90
5.9	Diagram of cell rotation about the X and Z axis.	92
5.10	Plot of the A values with angular speed of the rotational stage.	93
5.11	Cell rotation about the Y axis.	94
5.12	Cell release and in-plane rotation	96

5.13	Predictability evaluation	98
6.1	Axial and Lateral Oscillations	103
6.2	Experiment setups	106
6.3	Piezo-injector for axial vibration	107
6.4	First and second natural frequency of micropipette	108
6.5	Displacement measurement by using interferometer	109
6.6	Deformation vs axial amplitude.	110
6.7	Amplitude of a lateral vibrating micropipette tip in air.	111
6.8	Amplitude of a lateral vibrating micropipette tip in water.	112
6.9	Deformation vs lateral amplitude.	113
6.10	Cell deformation during injection	114
6.11	Membrane cutting-up	115
6.12	Solid-like Voigt model	117
6.13	Loop of lost energy	118
6.14	Loop of lost energy comparison	119
6.15	Design of the lateral-driven micro-cutter	120
6.16	Vibration of a lateral vibrating microneedle immersed in water	121
6.17	Design of the axial-driven micro-cutter	121
6.18	Ratio of split expansion to microneedle displacement	123
6.19	Deformation under axial-driven direct cutting	125
6.20	Ratio c in several cell cutting conditions	126
6.21	Suction and destructive effect	128
6.22	Investigation of whirling effect using ink	129
6.23	Four types of cutts generated by lateral-oscillated micro-cutter.	130
7.1	Process flow of blastomere extrusion	139

List of Tables

3.1	Comparison between SFL and ISFL	40
3.2	Comparison between two focus windows on 10 embryo samples	42
3.3	Comparison between Fibonacci and ISFL on Ackley function .	44
4.1	Table of success rates	73

List of Symbols and Abbreviations

φ	Angular displacement of the rotational stage
(c_x, c_y)	Estimated center of an embryo
(x_a, y_a, z_a)	Real-world coordinates of point a
(x_b, y_b, z_b)	Real-world coordinates of point b
α	Angular acceleration
β	Angular change of a cell
η	Viscosity coefficient
γ	Strain
μ, μ_r	Friction coefficient
ω_r	Preset angular velocity fo the rotational stage

ω_{rp}	Real-time angular velocity fo the rotational stage
ω	Frequency
σ	Stress
τ	Torque
θ_e	Origin angle of an elliptical boundary
θ_{phase}	Phase delay
θ	Rotation angle
x_i	Position of i^{th} frog
a_e	Major radius of an elliptical boundary
a	Acceleration
A	Area
b_e	Minor radius of an elliptical boundary
c	Splitting effect ratio
d_n	The n^{th} sampling point along a line
D_d	Displacement of the micro cutter

D	Distance from the center of a sphere
d	Step size of a frog
E_y	Young's modulus
e	Deformation ratio
E	Elastic modulus
f_i	Correspondent fitness value
FMF	focus measure function
f	Force
g	Gravitational acceleration
$I_{cartesian}$	Image in Cartesian-coordinate format
$ICSI$	Intracytoplasmic sperm injection
I_{polar}	Image in polar-coordinate format
$ISFL$	Improved Shuffled Frog Leaping
I	Moment of inertia
k	Iteration number

L	Length of the diameter of the embryo
m_f	Numbers of memeplexes
m	Mass
n_f	Numbers of frogs in each memeplex
n	Numbers of samples
N	Normal force
O_c	Center of a sphere
P_i	Weight of a frog
PGD	Preimplantation genetic diagnosis
PSO	Particle Swarm Optimization
P	Pushing force
$Point\ a, b, c$	Three contact point on a fish embryo
q	Numbers of frogs in each submemeplex
Q	Predefined maximum number of evolutions
r_{c1}	Internal radius of the corona

r_{c2}	External radius of the corona
r_1, r_2, r_3	Random value between 0 and 1
R_p	Distance between the center of a cell and the center of the petri dish
R	Radius of a sphere
r	Radius of contact area
s_s	Scaling factor
SFL	Shuffled Frog Leaping
s	Width of the split
v	Velocity
x_{IP_1}	Intersection point 1
x_{IP_2}	Intersection point 2
x_{lb}	Minimum boundary of a frog's feasible position
x_{ub}	Maximum boundary of a frog's feasible position
x_B	Frog with local best fitness value
x_W	Frog with local worst fitness value

x_X	Frog with global best fitness value
x	Contact interference
y_{LB}	Lower boundary line of the embryo image
y_{UB}	Upper boundary line of the embryo image
$z_{best_sharpness}$	Position in z-axis corresponding to the position with best sharpness value
ZP	Zona pellucida

Chapter 1

Introduction

1.1 Research Motivation

Preimplantation genetic diagnosis (PGD) has been introduced for more than twenty years with its first publication in 1991 [1]. PGD is a prenatal diagnosis process that analyses embryos that grown in vitro for well-defined genetic defects. After the PGD, only those embryos without any defect are transferred back into the womb [2]. PGD mainly treats two groups of patients: the individuals with high chances of giving birth to a baby with genetic diseases and patients who are older than 37 and request embryo screening to make sure the chromosome aneuploidies to increase the chance of their continuing pregnancies [3].

Oocytes prepared for PGD are normally obtained through ovarian hyperstimulation, intracytoplasmic sperm injection (ICSI), and embryo culture. Cell biopsy is a process of removing a part of materials from a cell for diagnosis, as shown in Figure 1.1. There are three types of biopsies that can be used for PGD based on the development stages of an embryo. Polar-body biopsy is applied to extract the polar bodies from oocytes, which is

Figure 1.1: Embryo biopsy in PGD.

used for the diagnosis of genotype and single-gene disorders of an oocyte. The technique was first developed by Verlinsky et al. [4]. By applying this technique, Rechitsky [5] was able to detect genetic disorders in 157 out of 160 sample oocytes. Blastocyst biopsy is applied in the very late yet still safe stage to perform biopsy on an embryo. Although it has the advantage of obtaining more cells for analysis, clinics do not normally prefer this type of biopsy, because it leaves little time for diagnosis as the embryos have to be transferred back to the womb before day 6 of its development [6]. The most widely adopted biopsy for PGD is cleavage-stage biopsy. This type of biopsy is performed on a third-division (D3) embryo, normally on the third day after the insemination [7].

A D3 embryo has more than six blastomeres, out of which one or two blastomeres are extracted for diagnosis. Currently, embryo biopsy in PGD is completed by highly trained embryologists using manually controlled micro-manipulators under a microscope. Manual cell manipulations have stringent skill requirements and require long training time. They also suffer from low success rate (16%-47%)[8], low reproducibility and low precision. All these drawbacks of conventional embryo biopsy are calling for an automatic micro-

surgery system for embryo biopsy imminently.

1.2 Research Objectives

Besides the preparation and further development of an embryo, an embryo biopsy comprises five major steps that relate to cell manipulation. The first step is to adjust the microscope, so both micropipettes and the embryo are in the field of view and the focal plane of the microscope. The second step is to identify a suitable embryo biopsy position on the embryo, normally the perivitelline space, to minimize the undesired damage to the embryo. The third step is to orient and immobilize the embryo, so the target perivitelline space is aligned with the micro-cutter and the embryo is ready for dissection. The fourth step is to perform the zona pellucida dissection, where the zona pellucida is breached, burned or cut open depending on the chosen dissection approaches. The final step is to perform blastomere aspiration, where one or two blastomeres are removed from the embryo through the opening on the zona pellucida. In order to automate the process of embryo biopsy, a microsurgery system should be capable of performing all these tasks automatically. The aim of this research is to propose a microsurgery system that performs the first four aforementioned steps of the embryo biopsy process automatically. There are six research objectives listed as follows:

1. To improve the consistency and repeatability of the embryo biopsy procedure by employing an automatic microsurgery system. This effectively helps prevent errors caused by human fatigue, habits of different operators and other human involvement factors;
2. To improve the accuracy and precision of the focusing process of microscopy. The mbryo and micropipette focusing process is commonly

performed by an experienced operator by looking into the eyepiece of the microscope to search for the sharpest image of the target object. The low accuracy of the sharpest image identification and the low-precision micropipette position adjustment will be improved by employing computer vision and visually servoed micromanipulators;

3. To improve the controllability and reproducibility of embryo orientation. Comparing to a trial-and-error based approach for embryo orientation, a predicable embryo rotation approach can help achieve a more reproducible and more precise embryo orientation;
4. To reduce the total time required for the embryo biopsy procedure. Time is crucial to each embryo biopsy session, as long-time exposure to the environment may introduce bacteria or cause degradation to the embryos. Thus, reduction in process time efficiently lowers the probability of getting affected samples in the uncertain environment;
5. To implement a cost effective yet automatable approach to realize the zona pellucida dissection. Current zona pellucida dissection approaches, such as the mechanical zona dissection, chemical breaching and laser burning, are facing problems of either low controllability or expensiveness, or both. These problems can be solved by employing a piezo-driven zona pellucida dissection system;
6. To drastically reduce the training time required to perform embryo biopsy. The proposed system does not necessarily require an embryologist to operate as it is designed to be fully automatic, though an embryologist is preferred. A non-embryologist technician may be trained to perform the operation within a very short period of time.

1.3 Technical Challenges

To realize the automation of the embryo biopsy process, there are several main challenging technical issues:

1. High accuracy in all degrees of freedom is required: $0.2\ \mu\text{m}$ RMS error in translational motion and 0.02° RMS error in rotational motion. This ensures the precision of the embryo biopsy process.
2. The automatic/robotic cell micromanipulation must be performed in real time to ensure the precision. Therefore, fast and accurate auto-focusing and visual recognition system is required to allow real-time ($\geq 30\ \text{Hz}$) processing and control.
3. Vision recognition algorithm must be able to perform on low-contrast and non-uniform brightness (Hoffman Modulation Contrast) microscopic image.
4. The embryo is very fragile, thus zona pellucida dissection should cause minimum invasion to the embryo.

1.4 Research Scope

Automation of embryo biopsy is a very broad topic and can be realized through various approaches. In this dissertation, the author limits the scope as follows and validates all algorithms/mechanisms using Zebrafish embryo:

1. To propose a fast and robust autofocus algorithm, including window selection and maximum searching algorithm, for cell manipulation under microscope. The proposed algorithm is necessary to automate the first step of the embryo biopsy process.

2. To propose a D3 embryo image processing algorithm for selection of a zona pellucida dissection position on a cleavage-stage embryo. The proposed algorithm automates the second step of the embryo biopsy process.
3. To design and develop a rotational stage for three dimensional embryo orientation. This proposed method realizes the automation of the third step of the embryo biopsy process.
4. To investigate the beneficial oscillation of the injection micropipette of the piezo-driven micro-dissection system.
5. To design and implement a piezo-driven zona pellucida dissection system. The proposed system makes it easier to accomplish the automation of the fourth step of the embryo biopsy process.

1.5 Dissertation Outline

The remaining contents of this thesis is organized into the following chapters as follows:

Chapter 2 provides a literature review on existing embryo biopsy related methods proposed by researchers around the world. Four topics are mainly reviewed; they are autofocusing in microscopic image, embryo image processing, cell orientation, and zona pellucida dissection techniques.

Chapter 3 proposes an autofocusing algorithm that includes an intuitive window selection algorithm and a robust maximum searching algorithm based on improved shuffled frog leaping (ISFL).

Chapter 4 introduces the first automatic segmentation method for identifying both the internal and external boundaries of the zona pellucida using

edge detection and ellipse fitting techniques from a single focused embryo image. A suitable zona pellucida dissection position for embryo biopsy can be selected automatically by applying the proposed method.

Chapter 5 introduces a system with multiple techniques that can perform three-dimensional rotational control of single embryos. An in-house designed and developed rotational stage for a microscope is also introduced. A Zebrafish embryo image segmentation algorithm is also proposed to validate the cell orientation system.

Chapter 6 presents the result of the investigation of the beneficial oscillation of the micropipette in zona pellucida dissection. A piezo-driving zona pellucida dissection device is also proposed to enable effective and safe zona dissection for embryo biopsy.

Chapter 7 summarizes all the work done in this dissertation. Future works towards computer vision guided embryo biopsy is also suggested in this Chapter.

Chapter 2

Literature Review

2.1 Embryo Autofocusing

Automated cell manipulation has attracted lots of researchers' attentions during the past years due to its improvement in efficiency and accuracy. Recently, encouraging results have been demonstrated by several successful in vitro fertilization (IVF) related cell manipulations involving visual servoing [9, 10, 11]. In order to ensure the accurate visual servoing, autofocusing is one of the most critical steps to be performed in real-time to restrict the object in the depth of field (DoF).

There are two main categories of autofocusing methods: active autofocusing and passive autofocusing [12]. Active autofocusing is a reflection-based approach [13], where a sensor or measurement device, such as a Laser [14] or ultrasound sensor [15], is utilized to estimate the distance between targeting object and the lens. Although it can achieve high speed autofocusing by using an independent focusing feedback loop, due to the presence of multiple reflections, the true fiducial reflection may be confounded leading to partial focusing. Passive autofocusing, on the other hand, is an image based method

that depends only on the image information, or figure of merit (FOM), which can be obtained using image processing techniques, to determine the focal plane. The FOM includes image contrast, resolution, entropy or spatial frequency content [16, 17]. One parameter of the FOM, the sharpness of image, is measured using the focus measure function (FMF). The global best sharpness value is obtained by performing a search for the maximum value from a set of scanned images[18]. Passive autofocusing has a drawback of being time consuming, due to the time required for the acquisition and the processing of multiple images; however, its accuracy can be assured. The passive autofocusing system for a micro-object consists of three components: focusing window selection, image sharpness measurement and global peak search.

In real-time operation, a suitable and consistent focusing window must be selected, prior to image acquisition for the entire image set, to define the focusing region. An appropriate focusing window selection significantly reduces the number of pixels, which leads to less calculation and higher efficiency. An elaborate focusing window can also improve the reliability of the autofocusing system by separating the background from the target region, thus leading to an improvement in system accuracy. Central region and multi-region are the very commonly used window selecting approaches [19]. The multi-region approach is superior to the central-region approach in capturing of the main body. The drawback of the multi-region approach is that it compromises the accuracy of the autofocusing system as the image sharpness is calculated based on a weighted multi-region average and the image variation range is also reduced. There are also other approaches, such as face detection, skin detection, etc. However, their applications are strictly limited to portrait photos.

An image is analyzed to calculate the sharpness measurement using a

FMF. Different FMFs are applied according to the image acquisition method, i.e. different microscopic filters. Sun et al. [17, 20] and other researchers [18, 19] demonstrated that normalized variance (VAR) was superior to other FMFs on images acquired from phase contrast and differential interference contrast (DIC) microscopes. Both VAR and standard deviation (STD) are able to provide accurate and consistent sharpness measurement of bright field images [21]. Vollath's F4 [22] method, which is a correlation-based focus that measure F4, performed better than others in fluorescence in situ hybridization (FISH) image processing. In terms of speed, the Brenner method [23], which calculates summation of the square of the difference between two neighbor pixels, and VAR are the most straightforward methods that are suitable for conventional automated microscopes [16].

The global peak search algorithm is necessary to indicate the best in-focus lens position, which corresponds to the best sharpness measurement value. There are several global peak searching algorithms, such as the hill climbing method [24], which belongs to the local search family; the Fibonacci searching algorithm [25], which is a sorted array searching technique; and particle swarm optimization (PSO) [26], which is an evolutionary algorithm. Since the sharpness value obtained by the embryo autofocusing system is non-unimodal, the optimization algorithm must be capable of escaping from the local maximum to reach its ultimate target.

In summary, current autofocusing technique has two major drawbacks: (1) the inaccurate window selection, which causes the high computational load and the inaccurate estimation of the FMF value of the target object, (2) the ineffective global peak searching, which may fail in non-unimodal searching due to local maximum trapping. Aiming to improve the current autofocusing approach and make it more suitable for the particular application, the

autofocusing of an embryo and micropipettes, an intuitive window selection algorithm and a robust autofocusing searching algorithm based on improved shuffled frog leaping (ISFL) will be introduced in the next chapter.

2.2 Embryo Image Processing

Region-based and edge-based methods are the most commonly used image segmentation methods [27]. A major drawback of region-based segmentation is the difficulty in identifying accurate borders of objects when they are diffused. Classic region-based segmentation is not suitable for embryo images due to their complexities. Edge-based segmentation, on the other hand, has the challenges of thresholding and linking after high gradient value searching. In many cases, threshold values are set arbitrarily or derived experimentally.

Currently, there is only a limited number of segmentation algorithms that are able to perform zona pellucida recognition on a Day-3 embryo. The shape of the zona pellucida can be approximated by an ellipse [28]. Karlsson's group [29, 30] introduced zona pellucida detection using region-based segmentation methods with a probabilistic procedure, as shown in Figure 2.1(a). The background of the image had to be removed to facilitate external boundary detection. The internal boundary of the zona pellucida was identified by separating the zona pellucida from the embryo. In cases of the appearances of artifacts, a penalty function was manually applied to remove small artifacts. Large artifacts must be covered with masks manually to avoid false detections. The iterative energy minimization method, active contour [31], was applied to zona pellucida segmentation [32]. Surrounding artifacts, debris and spermatozoa in the zona pellucida area must be removed before the application of active contour; otherwise the algorithm will be con-

fused by local energy minima, which hinders the algorithm from rapid and robust convergence. Automatic and semi-automatic ellipse model-based approaches on Day-5 embryo (blastocyst) zona pellucida recognition were also introduced by Yee's and Filho's groups, respectively [33, 34]. However, the internal structure of a Day-5 embryo was very different from a Day-3 embryo; therefore, their techniques may not work well on Day-3 embryos.

Figure 2.1: Embryo image segmentation methods. (a) Region-based segmentation [29]. (b) Active contour based segmentation [32]. (c) Minimum cost searching based segmentation [35].

So far, no attempt at recognition of the location of the perivitelline space has been reported. Giustia's group proposed a segmentation and 3D reconstruction method by converting the segmentation problem into a minimum cost searching problem [35, 36] (shown in Figure 2.1(c)). The method was able to recognize the zygotes of oocyte or blastomeres of embryos, but not the zona pellucida or perivitelline space. 3D reconstruction of the embryo from a stack of embryo images with different focal planes is also relatively complex and computationally intensive. Although it has the potential to identify the perivitelline space by combining this method with other zona pellucida recognition algorithms, application of this technique is limited only to the segmentation of Hoffman Modulation Contrast (HMC) microscopy

images. Similarly, Feng et al. performed blastomere recognition by using an embryo video clip, which can also be considered to be a set of images [37]. The method also required a considerably long processing time. Tian et al. proposed another method that was able to count the number of blastomeres in an embryo using only a single image [38]. However, the exact position of the blastomeres cannot be identified. In summary, to the best of our knowledge, no one has proposed any method that can detect the perivitelline space position using only a single image.

2.3 Cell Orientation

In order to fully automate the cell manipulation process, both cell injection and intracellular structure biopsy require cells to be rotated in three dimensions for access to target cellular structures. Orientation control of biological cells plays a very important role in cell manipulation as it is necessary for much biomedical research, such as cell position control in Zebrafish embryo injection [39], polar body orientation in intracytoplasmic sperm injection (ICSI) [40, 41, 42, 43], and perivitelline space orientation in embryo biopsy for preimplantation genetic diagnosis (PGD) [44, 45, 46]. As increasingly more attention is paid to the applications of rotational cell manipulation, an automated three-dimensional cell rotation approach with high success rate, high precision and high reproducibility becomes highly desirable.

The most commonly used approach for cell rotation employs a glass micropipette [47], manipulated manually by a skilled operator for the repeated vacuum aspiration and release of the cell until the desired orientation is achieved. Manual cell manipulation requires extensive experience to conduct while suffering from low precision, low success rate and low controllability

due to its trial-and-error nature. Any improvement to the success rate and speed of operation largely depends on the skill of the operator.

Figure 2.2: Cell orientation (a) using optical tweezers [48] (b) using rotational stage [9]. (c) using rotational plate designed for fish embryo[39] (d) using fluidic flow [49].

Several cell rotation approaches have been introduced in the literature recently. Optical tweezers utilizing a focused laser beam to realize the rotation of microscopic objects [50] were used to orientate individual cells [51, 48, 52, 53, 54]. However, their applications were limited by low controllability about the cells' pitch axis and the size limitation of micro objects that can be manipulated [55]. A rotational stage and a through-hole embryo holder facilitated polar body orientation in ICSI [9, 11, 56] and also fish embryo injection [39], as shown in Figure 2.2 (b) and (c). However, this system and technique was limited to rotation around an axis perpendicular to the field of vision of the microscope. Strong static magnetic fields [57, 58] demonstrated the capability of rotating cells. However, the magnetic fields' alignment restricted the achievable cell orientations. Dielectrophoresis (DEP) was introduced to rotate cells using a non-uniform electric field [59, 60, 61]. However, the electric field required for cell rotation can damage cells significantly [62]. Fluidic flow control was introduced to perform three-dimensional mouse oocyte rotation for polar body orientation [49]. However, the actual forces applied on the object are difficult to predict, and the proposed method

was not a dependable approach to orienting a cell.

The aforementioned methods are incapable of producing predictable three-dimensional cell rotation essential for ICSI and PGD. In order to overcome the shortcomings of these aforementioned methods, such as low precision, low controllability, low reproducibility and low predictability, a system that allows three-dimensional rotational control of single embryos/oocytes is introduced in this thesis.

2.4 Zona Pellucida Dissection Techniques

PGD technique has been applied to diagnose and eliminate genetic-disorder transmission from parents to their off-spring for more than twenty years. The cleavage stage biopsy is a two-step procedure, including zona pellucida breaching and removal of one or two blastomeres from a day 3 embryo. Zona pellucida dissection process is most commonly implemented by utilizing acidified medium (e.g. Tyrode's solution) or laser technology in many research centers [63]. In some institutes, it is done by mechanical means [64], as illustrated in Figure 2.3.

2.4.1 Chemical Zona Pellucida Breaching

Chemical zona breaching, first described in 1986 [65], uses acidic Tyrode's solution with a pH value of 2.3 to breach a hole on the zona pellucida, shown in Figure 2.4. Acidified medium zona breaching, a very simple procedure in terms of its equipment setup [45, 66], is commonly done by using three pipettes with different inner diameter: a ϕ 20 μm pipette for embryo immobilization, a ϕ 5-7 μm pipette for acid releasing and drilling and a ϕ 40 μm pipette for aspiration or biopsy [7]. Though it is simple in setup, it requires

Figure 2.3: Methods of zona pellucida opening. (a) Chemical breaching. (b) Laser burning. (c) Mechanical cutting.

high technical skills to deal with embryos exposed to acidified solution. An appropriately sized zona opening needs to be made within a limiting duration. Since it is extremely difficult to control the amount of solution applied to the embryo, the size of the opening is almost uncontrollable. The average diameter of the hole drilled on the zona pellucida is $20\text{ }\mu\text{m}$ with a wide range from 10 to $36\text{ }\mu\text{m}$ [68]. As the human zona pellucida is quite resistant to acid [69] and the acid may be diluted by the surrounding medium, quite an amount of acidic Tyrode's solution is required to breach the zona pellucida. In addition, an excessive amount of acidic solution may result in decreas-

Figure 2.4: Chemical zona pellucida breaching[67].

ing the pH value of the surrounding culture medium and also increasing the chance of injecting acid into the perivitelline space [70]. Furthermore, acid exposure also leads to immediate cell lysis, as even subtle destruction to the cell can interfere with the development and implantation in the future. Thus, embryos are operated separately and immediate washing is required to remove residual acid [68]. Human zona has a stratified bilayered structure that consists of an outer layer that can be easily removed by acidified medium, and an inner layer that does not always make the zona breaching a easy task [65, 71]. The oocyte or the blastomeres are prone to lysis once the inner layer is drilled through. Besides the manipulations of a micropipette to control the amount of acids, choosing the target site of zona breaching also takes time. The drilling can only be applied in the area that is between blastomeres or in front of anucleate fragments, so to avoid excessive damage to the cell from the acidic medium. Research shows that the chemical drilling procedure takes a highly skilled embryologist about 30 s to 2 mins to complete [7].

2.4.2 Laser Zona Pellucida Drilling

Laser-assisted zona opening has been an alternative to chemical zona drilling for more than two decades[72][73]. Specifically configured laser beams, with various wavelengths, can be utilized to create minimal superficial damage to the zona pellucida, which helps in the polar body and embryo biopsy, as shown in Figure 2.5. The first reported laser used to drill the zona pellucida is a UV-laser with 193 nm wavelength. Researchers declared that this laser beam did not interfere with the embryo development in vitro or in vivo at all [72][74]. However, since the DNA absorbs the UV radiation, thus, to prevent genetic damage, the wavelength should be further separated from the maximum absorption wavelength of DNA, 260 nm. Two types of laser beams in the infrared region are studied for drilling purpose, a 2.9 μm erbium-YAG contact laser [75] and a 1.48 μm non-contact diode laser [76][77]. Although the erbium-YAG contact laser is claimed to be safe [78], the 1.48 μm non-contact diode laser is preferred to perform assisted hatching and fertilization, as it can complete every single task that erbium-YAG contact laser does, with the advantage of being a non-contact approach. In the laser-assisted zona opening procedure, the oocyte or embryo is positioned at the coordinate where the zona pellucida is under the target or laser beam by manipulating the X-Y stage of the microscope. By adjusting the irradiation time, the size of the opening can be controlled; the longer the irradiation time is, the bigger the size of the hole is. Normally, a 5-10 μm in diameter trench-like hole can be perforated in 10-15 ms[76].

Laser drilling has several drawbacks. Besides its high cost, it is considerably complicated in setup. The trench-like hole it creates exposes the internal cellular structures of the embryo to the environment, which increases the chance of cell lysis.

Figure 2.5: Laser zona pellucida dissection [79].

2.4.3 Mechanical Zona Pellucida Dissection

Mechanical partial zona pellucida dissection methods can be categorized into passive and active approach.

Passive Cutting

A micropipette is forced through the zona pellucida and rubbed against the holding pipette to perform the dissection manually in passive approaches, as shown in Figure 2.6. This approach is also applied in assisted hatching [80]. Conventional partial zona dissection (PZD) pierces the zona pellucida with a very thin microneedle; the needle penetrates the zona pellucida from the 5 o'clock position to the 7 o'clock position of the embryo. The site of piercing is normally the perivitelline space. Then the embryo is taken away by the micropipette, which later presses and rubs the zona pellucida of the embryo against the holding pipette till the zona pellucida sandwiched between micropipette and holding pipette is completely abraded to form a slit [66]. Unlike the laser technique, a slit is created by the passive mechanical cutting technique. Comparing to a trench-like hole, a slit can protect the internal cellular structure from exposure better. The setup for PZD is rela-

tively simple, but it also requires highly skilled operators. Theoretically, the length of the arc between two holes created by the microneedle on the zona pellucida decides the size of the slit, as the holes are the slit end. Malter [81] claimed that the slit never exceeded $20\text{ }\mu\text{m}$ in size and was hard to be found after culturing the cell in most cases. Another work by Payne's group [82] made larger openings up to $40\text{ }\mu\text{m}$ with $2\text{ }\mu\text{m}$ in width in their experiment.

There are several drawbacks which make this technique undesirable and difficult to be automated. This technique is highly skill dependent; and at the same time very time consuming and tedious. It is very hard to ensure the accuracy as the force applied to the zona pellucida cannot be measured precisely. In addition, lots of computing power is required to perform vision recognition and tracking for the rubbing action, which makes automation of this task very difficult.

Figure 2.6: Mechanical zona pellucida dissection [83].

Active Cutting

A vibrational cutter, such as a glass pipette, metal microneedle, or silicon blade, is utilized to dissect or penetrate the zona pellucida in active cutting approaches. Both penetration and dissection, shown in Figure 2.7(a) and (b), are successfully performed with the active mechanical approach using an axial vibrating micropipette. The piezo-driven micropipette on a microma-

Figure 2.7: (a) Active mechanical oocyte injection[42]. (b) Active mechanical zona pellucida dissection [84].

nipulator is advanced against the embryo to slit the zona pellucida. Because of the eccentricity and the elasticity of the micro-cutter, it is prone to cause embryo lysis due to the high-amplitude vibration of the cutting pipette tip. The amplitude of the vibration is also difficult to control precisely and can only be estimated roughly. To mitigate the large displacement at the tip, the tip is commonly partially filled with mercury [85][86]. However, as a toxic material, use of mercury is limited, though only $0.6 \mu\text{g/l}$ was detected in the ICSI injection drop, which is within the range of mercury concentration of the blood in a healthy human, 0.3 to $1.05 \mu\text{g/l}$ [87].

Active mechanical partial zona dissection (PZD) has been widely applied.

Figure 2.8: Mechanical zona pellucida dissection techniques: (a) PiezoDrill, a piezo-driven microinjector [88]. (b) Liquid jet [89] (c) DC motor-driven micropipette[90] . (d) Ultrasonic cell microinjector[88].

The approach generally requires a vibrating actuator that is made of multi-layer piezoelectric material. A micro-needle or pipette is attached to the free end of the piezo-actuator, which drives the needle in the axial direction. The other end of the piezo-actuator is attached to a micromanipulator or a casing that is attached to the micromanipulator, which is used to precisely control the cutting site on the zona pellucida, as shown in Figure 2.8(a). Although other techniques, such as the liquid jet [89] [shown in Figure 2.8(b)], the DC motor-driven micropipette [90] [shown in Figure 2.8(c)], and the ultra-slow cell biopsy [91] are also introduced, they are not widely applied in PZD due to large vibration amplitude, lack of accuracy, and unbearable time consumption respectively. It is proven that piezo-driven zona pellucida perforation has its significant benefit of reducing the linear-translation injection force by 52

% to 94.9 % as vibrational frequency increases from 3.1kHz to 14 kHz [92]. More recent researches have extended the cutting frequency to ultrasonic level and applied active cutting to micro-dissection. In ultrasonic vibration micro-dissection (UVM) on tissue, the acceleration of the cutter exceeds the elasticity threshold of the tissue and causes the fracture [93], and it is proven to be effective. Ultrasonic cell microinjection, based on UVM, applied on zebrafish oocyte was introduced [88][94] [shown in Figure 2.8(d)], and it was claimed that lateral oscillation was mitigated and ultrasonic cutting force played a very important role during the penetration.

Active mechanical PZD has its advantages of low cost, easy controllability and simple setups. Similar to laser zona opening, which is a heat absorption process, zona pellucida also absorbs heat caused by friction and expansion and contraction of the zona pellucida [95]. Thus, to minimize the possibility of excessively damaging the embryo, the heat intensity and dissection position must be taken into consideration. Compared to the axially vibrating pipette, the laterally vibrating pipette generates less heat. Although a number of researchers considered the lateral vibration as harmful, it is still debatable, as no one has separated the axial vibration from the lateral vibration to prove that yet. In this case, the author believes the lateral vibration actually plays a very important role in zona injection and dissection. In this thesis, an investigation in beneficial vibration is carried out and lateral vibration is utilized to perform the zona pellucida dissection.

Chapter 3

Autofocusing Technique Under Microscope

In cell manipulation under microscope, it is critical to bring the object into sharp focus and remain in the focal plane to enable accurate positioning and manipulation. In order to automate the manipulation process, autofocusing is necessary to maintain the object feature within the depth of field (DoF) for accurate visual servoing. Four micro objects in the embryo biopsy procedure require visual servoing: the holding micropipette, micro-injector, aspiration micropipette and the embryo. In manual focusing procedure, the objective lens of a microscope is tuned to search for the image of the target object with the sharpest outline edges. There are several parameters that significantly affect the autofocusing results: the window selection, the optimum searching for sharpness measurement, and the focus measure function (FMF). In this chapter, an intuitive window selection algorithm and a robust autofocusing searching algorithm based on improved shuffled frog leaping (ISFL) are introduced.

3.1 Autofocusing Window Selection

In this section, two autofocus windows are proposed to facilitate embryo autofocusing and blastomere autofocusing.

3.1.1 Related Researches On Autofocusing Window Selection

Selecting a suitable focus window for the micro-object autofocusing system does not only reduce the computational load, but also improves the sharpness of the object of interest. Various FMFs [96, 97, 98, 99], defocus analyses and correlation algorithms [100, 101, 102, 103] have been proposed and compared by researchers. Although certain FMFs, for instance, VAR and Tenengrad [104], are capable of handling most tasks of image focusing, they do not provide ideal result in some situations, such as low-light condition, noisy background, etc. Due to the limitation of the FMF, a focus window is utilized to improve the accuracy, consistency and speed of the autofocusing system. A common assumption made on the object of interest is that it is often in the center of the image. The central region is widely used as the focus window to evaluate the degree of focus of micro-objects [20]. An inverse ‘T’ shape focus window is applied to define the area for golden multi-region focusing method [105]. This method performs well in capturing the main body, but its way of calculating the sharpness by averaging the weighted multiple regions’ values deteriorates the system accuracy. Zhu et al. [106] proposed the focus window selection using a Gaussian non-uniform sampling algorithm [107]. The algorithm keeps the central area of the image unchanged with high resolution; meanwhile, the foreground and the background of the image are unequally sampled, thus all information of the image is taken into

account. However, the method still takes the image center as the main target and is too computationally intensive for a real-time autofocusing. Similarly, Wang et al. [108] proposed dynamic autofocusing region selection based on an improved particle swarm optimization, where the searching algorithm was improved to limit the focus window on the most information intensive area. If this method is applied to an embryo image, the focus window is most likely to be set in the center of the embryo instead of the whole embryo. Pattern recognition methods such as face detection and skin detection are helpful in window selection. However, their applications are very limited.

The aforementioned autofocusing window selection methods are only applicable to images with the object of interest located at an expected position. In practice, the embryo may not be always in a particular position. Moreover, the main purpose of autofocusing is to identify the Z axis position of the object of interest, thus a focus window that is focused on a more specific object is preferred. According to the application, e.g. the whole embryo autofocusing or the blastomere autofocusing, the autofocus window should be able to vary its position and size. Therefore, in this chapter, a novel autofocus window selection method for embryo and blastomere is presented.

3.1.2 Focus Window For The Entire Embryo

The autofocusing on the entire embryo is required to align the holding pipette and micro cutter with the embryo. In embryo autofocusing, the entire embryo is the object of interest. However, if a focus window encloses the entire embryo, the sharpness measurement will be affected by other undesired object, such as blastomeres and artifacts. This is because the FMF information of the internal cellular structure is more outstanding than that of the cell membrane. In order to obtain a smooth unimodal curve of sharpness mea-

surement values, ideally, a focus window should cover only the area of interest. The outer boundary of the embryo is the area that provides most of the sharpness measurement data that is important for the identification of the Z axis position, thus the focus window proposed for the embryo autofocusing is a corona-shape window covers only the outer boundary of zona pellucida.

Figure 3.1: Comparison between two focus windows for the entire embryo. Window 2 provides a sharper peak FMF value.

There are three major steps of defining the autofocus window for the entire embryo: coarse focal plane estimation, fine focal plane estimation, and sharpness measurement. The coarse focal plane estimation is achieved by using a focus window that encloses the entire embryo, for example, the ‘Window 1’ illustrated in Figure 3.1. In practice, if the resolution of the image is not too high to significantly slow down the computational speed, the entire image can be fed into the FMF instead of using a focus window. The approximating focal plane is at the position that has the highest sharpness value. The image corresponding to the approximating focal plane is applied to search for a corona-shaped focus window. The position of the corona shape is defined by an ellipse, which is used to estimate the external boundary of

the zona pellucida. The detailed procedure of the external zona pellucida boundary recognition will be discussed in Chapter 4. The elliptical edge of the zona pellucida boundary is dilated by an arbitrary number of pixels based on the image size to create the corona-shape focus window for embryo autofocusing. The sharpness measurement is calculated only on those pixels enclosed by the defined focus window. By searching the global optimum FMF value, the final focal plane can be identified. As shown in Figure 3.1, the global best sharpness value is shifted to the left of the curve when 'window 2' is implemented, which provides a much more accurate focal plane for Z axis positioning.

3.1.3 Focus Window For A Target Blastomere

A blastomere that is close to the zona pellucida dissection position is usually chosen as the target blastomere. As least one blastomere needs to be aspirated upon zona pellucida dissection, thus the ability of focusing on a particular blastomere is necessary. The ideal focus window for a blastomere should only include a single blastomere with minimum foreground and background information. However, due to various shapes of blastomeres and the possibility of having more than one blastomere in a field of view, the focus window can be selected to exclude background noises and uninteresting objects to a certain extent. Two important factors must be obtained to define the focus window: the internal boundary of the zona pellucida and the zona pellucida opening position. The internal boundary is used to exclude background noise created by the zona pellucida. The opening position is used to estimate the center of the focus window. The detail procedures of obtaining the internal zona pellucida boundary and suitable zona pellucida opening position will be discussed in Chapter 4. Since most blastomeres are round, a

round focus window can be utilized to cover the object of interest. The circle is defined by finding the circumscribed circle of three points: the origin point of the elliptical internal zona pellucida boundary, the suitable zona pellucida opening point and a point along the internal zona pellucida boundary that is 90 degrees counterclockwise away from the suitable zona pellucida opening point. As shown in Figure 3.2, although the selected focus window that

Figure 3.2: Focus window for a target blastomere.

covers parts of blastomeres that are considered as background noise, because the majority of the image is occupied by the target blastomere, the FMF value will not be significantly affected.

3.2 Autofocusing Searching Algorithm

In this section, an evolutionary algorithm, the shuffled frog leaping (SFL) method, is implemented to perform the optimization search. A novel improvement on SFL is introduced to facilitate autofocusing in global optimum searching. A comparison between performance of the improved SFL and the

traditional SFL has been performed using five test functions. The FMF values obtained from an embryo are also used to compare the improved SFL and the traditional Fibonacci algorithm. Autofocusing is achieved by adjusting the position of the motor-driven objective lens iteratively till it reaches the optimum focal plane position, which is identified by the optimum FMF values obtained from the proposed optimization searching algorithm.

3.2.1 Related Researches On Optimum Search Methods For Autofocusing

An ideal search algorithm should have a high convergence rate and be suitable to different FMF. Fibonacci search algorithm [109] was applied by Liu et al. [110] and Yeo et al. [104] to search for the best FMF value of the blood smear image and tissue image respectively to evaluate the best focal position. Mountain-climbing servo(MCS) [111] and hill-climbing search(HCS) [99] were also implemented for fast optimum searching. Both MCS and HCS consist of a large-step-size peak approaching and small-step-size best position searching. A second degree polynomial is obtained from more than two sharpness measurements to estimate the next lens position, thus to determine the best focus position [112, 113]. In this iterative search method, the first derivative is set to zero to solve for the next lens movement. However, the optimum position is not guaranteed, as when the set of acquired images is not within the DoF, the resulting polynomial may have a negative second derivative. Yazdanfar et al. [13] developed a similar method using only three images to indicate the focal plane. However, the Lorentzian function they employed to approximate the Brenner gradient limits the application of this method, as in real practice, not all types of FMF can be approximated with the Lorentzian function. Furthermore, Geusebroek et al. [114] utilized an

incremental scanning method, which moves the motor of the objective lens by a constant pre-defined interval till it reaches the target position with the best sharpness value. Since both the backlash and the step size of the motor can significantly influence the autofocusing result, the method consumes more time to compensate the backlash and performs more iterative scanning. So far, the above-mentioned search algorithms suit best or are only suitable for unimodal FMF, which is not the case in the embryo and blastomere autofocusing. For example, the phase transition effects shown in the literature [114] gave multiple local peaks in its FMF chart. Dust and other artifacts can also introduce local FMF peaks. Moreover, when performing autofocusing on blastomeres local peaks may also appear as blastomeres are in different focal planes. A filter is normally implemented to suppress the image noise prior to the image sharpness measurement. However, some local peaks remain, as they are not noise in a sense that the FMF does have an expected high value at that focal plane. For instance, a morphological pre-filter and a focus window were used to filter out the dust appearing in the image in the literature [115]; however, it does not guarantee a unimodal FMF outcome. Due to the existence of the local peaks, unimodal search methods do not guarantee to indicate the global optimum FMF measurement or the focal plane position. For example, in Fibonacci search method, when the step size of the focus motor is small, we normally have a large set of data, which is time-consuming for exhaustively searching. Fibonacci search was based on an assumption of a unimodal profile to narrow the data set down to the peak. Fibonacci search only evaluates at two points within a predefined interval, which must be large enough to include the expected maximum value. As the search goes on, the interval shortens. The search will not stop until length of the interval is less than a predefined threshold, however, search may be

terminated at a point in a misleading interval. In addition, the Fibonacci search does not perform the search in a completely random way, this is another reason it is often trapped in local maximum. To solve the problem of searching the global optimum FMF out of a multimodal sharpness measurement plot, improved shuffled frog leaping (SFL) algorithms for fast and robust optimum value searching are introduced in this section.

Evolutionary algorithms have been proven very effective to perform approximating solutions to all types of optimization problems. Such an algorithm is very robust because it does not make many assumptions about the fitness landscape prior to the estimation. Particle swarm optimization (PSO), a stochastic optimization technique inspired by social behaviors of bird flocking, has been utilized in many application areas [116, 117]. Wang et al. [108] implemented PSO on their window selection procedure and obtained encouraging results. Besides PSO, there are several types of evolutionary-based optimization algorithms: genetic algorithms, memetic algorithms, ant-colony optimization and shuffled frog leaping algorithms. Recently, SFL, an algorithm inspired by the memetic evolution of a group of frogs seeking for the position of maximum amount of food, has been applied to many applications of image processing, such as image enhancement [118], image segmentation [119], edge detection [120], image thresholding [121], etc. [122, 123]. Though the performances of both PSO and SFL are efficient to deal with all types of problems, SFL outperforms PSO in term of processing time [124], which is very important in real-time processing.

3.2.2 Shuffled Frog Leaping Algorithm

Shuffled Frog Leaping(SFL) is a comparably new meta-heuristic algorithm for optimum searching. It models and mimics the food searching behavior of

frogs. It has the advantages of the social behavior-based PSO algorithm and the genetic-based memetic algorithm (MA). SFL has been widely applied in many areas, such as project management, multi-user detection in communication systems, water resource distribution, etc. In the past five years, its application in image processing has been prevalent [122, 125].

The basic shuffled frog leaping algorithm [126] evolves periodically in a memetic evolutionary manner. A meme, which is similar to the gene in MA but evolves through communication and simulation, is the smallest unit of culture and communication information carried by a frog. The total frogs in the swamp are divided into several memeplexes, from which several frogs in a memeplex form a submemeplex that evolves independently. A frog leaps with a step, determined by the best position in submemeplexes or the swarm, to adjust its position. Upon several iterations of evolvments, the swarm is reshuffled for further evolvments.

The first step of SFL is the partition of memeplexes. Suppose there are F frogs in the swamp represented as $X = \{x_i, f_i, i = 1, 2, \dots, F\}$, where x_i is the position of the i^{th} frog, and f_i is the correspondent fitness value. The frog swarm is first sorted from the largest to the smallest according to individual fitness value. x_X defines the position of the frog with best performance. The whole frog population F is divided into m_f memeplexes: $Y_1, Y_2, \dots, Y_{m_f}$, with n_f number of frogs in each memeplex, such that

$$[x_i, f_i | x_j = x_{i+m_f(j-1)}, f_j = f_{i+m_f(j-1)}, j = 1, 2, \dots, n] \quad (3.1)$$

where $i=1, 2, \dots, m_f$.

Each memeplex has a submemeplex, which contains q frogs, where $q < n_f$. The member selection for a submemeplex is random in a way that a frog with better fitness value has a higher possibility of being picked up. A weight P calculated through a triangular probability distribution, as shown in (3.2), is

assigned to each frog.

$$P_i = \frac{2(n+1-i)}{n(n+1)}, i = 1, 2, \dots, n_f \quad (3.2)$$

Since frogs from a memplex have been sorted, the first frog should have the best weight, $P_i=2/(n_f+1)$ according to (3.2). The frogs with the best and worst fitness value in the submemplex are defined as x_B and x_W , thus $x_B = x_1$ and $x_W = x_q$. The frog with the worst fitness value will be updated by letting the frog leap by a step. The step is defined as follows:

$$d^{k+1} = \begin{cases} \text{Min}\{\text{Int}[r_1^k(x_B^k - x_W^k)], d_{max}\}, & \text{for positive step} \\ \text{Max}\{\text{Int}[r_1^k(x_B^k - x_W^k)], -d_{max}\}, & \text{for negative step} \end{cases} \quad (3.3)$$

where $\text{Int}(\cdot)$ is the round function, $\text{Min}(\cdot)$ and $\text{Max}(\cdot)$ are applied to find the minimum and maximum value. A maximum step d_{max} is defined to constrain the step; a random value, r_1 , set between 0 and 1, is applied for random selection, and k is the iteration number.

Once the step has been defined, the frog with the worst fitness value leaps one step, which is computed as

$$x_q^{(k+1)} = x_q^k + d^{(k+1)} \quad (3.4)$$

If $x_q^{(k+1)}$ is within the feasible space and it has a better fitness value, the new frogs in submemplexes return to their memplexes, which are sorted again in decreasing order. After a defined number of iterations of memetic evolution, the whole swarm is shuffled and new memplexes are generated. By checking the best frog position of the population, P_X , with the convergence criteria, the algorithm decides when the evolution stops. In case the P_X does not meet the convergence criteria, the process stops when it reaches the predefined number of function evaluations. In the situation that $x_q^{(k+1)}$ is not within the

feasible space or the fitness value is worse than before, the step is defined as follows:

$$d^{k+1} = \begin{cases} \text{Min}\{\text{Int}[r_2^k(x_X^k - x_W^k)], d_{max}\}, & \text{for positive step} \\ \text{Max}\{\text{Int}[r_2^k(x_X^k - x_W^k)], -d_{max}\}, & \text{for negative step} \end{cases} \quad (3.5)$$

If the newly defined $d^{(k+1)}$ still does not lead to a desirable $x_q^{(k+1)}$, the new position will be given to the worst frog by:

$$x_q^{(k+1)} = x_{lb} + \text{Int}[r_3^k(x_{ub} - x_{lb})] \quad (3.6)$$

where x_{ub} and x_{lb} are the maximum and minimum boundaries of a frog's feasible position.

3.2.3 Improved Shuffled Frog Leaping Algorithm

Since its first introduction by Eusuff and Lansey a decade ago, various improvements have been implemented on the algorithm. Zhen et al. [127] improved the SFL by balancing the memplexes and updating all frogs in each memplex. The population is divided into memplexes with frog members with balanced fitness values, i.e. if two memplexes are generated from four frogs, the first memplex has the best and worst frogs, and the second memplex has the other two frogs. The new division of the frog swarm benefits the internal learning process. Instead of updating frogs from the submemplex, all frogs from a memplex are updated by leaping a step, which is calculated as the summation of a portion of the step in the last iteration and a portion of the position difference between the target frog and a better randomly selected frog. The all-frog updating method can prevent searching from falling into local minima. However, the method increased the computational load. Jiang et al. [128, 129] proposed a folding dividing group method, which also

generated the submemplexes in a balanced way. The division of the whole population was also completed by distributing frogs uniformly. On top of that, the proposed method added in simplex method to calculate a second best solution, which positively influenced the step with iterations. However, employing simplex method in each iteration increased the processing time. Zhang et al. [125] improved the SFL with a cognitive behavior, where the step is calculated as the summation of local and global difference between the best and worst frog position. Fan et al. [130] further improved the SFL by setting the leaping step with a combination of the ethnic best, global best frog and a diversity factor. However, the method focused more on the local searching than the global searching, which increased the chance of local trapping. Luo et al. [131] modified the SFL with a similar approach but added in a factor that relates the frog's step to its previous position. The proposed method increased convergence rate but led to less local maxima searching.

In the original SFL algorithm, the initialization of the frog population is completed by random selection, which generates a non-uniform population. The population can be initialized by evenly dividing the space into several parts and performing random selections in each part[128]. This method is adapted to obtain a uniform population. On top of that, in this section, a more effective step calculation is introduced as a novel improvement on the SFL.

In order to avoid local optima trappings, the original SFL employs several techniques. For example, the submemplex is introduced to weaken the effect of a local optimum in the memplex by applying a probability weight using (3.2). The step is also calculated in a sequential order, which prioritizes the influences of local optima over global ones. Only when the local leaping towards the optimum cannot meet positive evolution or the frog leap out of

the feasible space, the global optimum is used to calculate the evolution step. The step is calculated by either best position in a memplex or be a global best. However, the frog's behavior should be affected by both of them. The effects of them are dynamic. The proposed evolution step can be calculated as follows

$$d^{k+1} = \begin{cases} \text{Min}\{\text{Int}[\frac{(Q-k)r_1^k}{Q}(x_B^k - x_W^k) + \frac{kr_2^k}{Q}(x_X^k - x_W^k)], & d_{max}\}, \\ & \text{for positive step} \\ \text{Max}\{\text{Int}[\frac{(Q-k)r_1^k}{Q}(x_B^k - x_W^k) + \frac{kr_2^k}{Q}(x_X^k - x_W^k)], & -d_{max}\}, \\ & \text{for negative step} \end{cases} \quad (3.7)$$

where Q is the predefined maximum number of evolutions and k stands for the k th evolution, and r_1 and r_2 are independent random values ranging between 0 and 1. Thus, formula (3.3) and (3.5) will be replaced by (3.7) in the ISFL. There are several advantages of the proposed step. The frogs perform both local and global searching, thus the chance of trapping into the local optimum is smaller. As the evolution goes on, the frog's behavior is increasingly affected by the global best till the last evolution, where the step is calculated without applying local optimum. As shown in Figure 3.3(a), the original frog leaping step can be obtained from either local evolution, d_1 , or global evolution, d_2 , depending on the FMF and space constraint. The proposed frog leaping step, d_3 , shown in Figure 3.3(b), has a higher chance to be within the green shaded area at first and gradually gains tendency to move into the blue shaded area. Since both the proposed method and the uniform population distribution are helpful in avoiding local optimum converging, the use of submemplexes is not necessary. By dropping the submemplexes from the algorithm, the computational load is reduced.

Figure 3.3: (a) Original Frog leaping rule. (b) Frog leaping rule with local and global learning.

To verify the performance of the ISFL, the ISFL is applied to five test functions for optimizations. The five test functions are as follows:

(A) Ackley function:

$$f(x_0 \cdots x_n) = -a \exp\left(-b \sqrt{\frac{1}{n} \sum_{i=1}^n x_i^2}\right) - \exp\left(\frac{1}{n} \sum_{i=1}^n \cos(cx_i)\right) + a + \exp(1) \quad (3.8)$$

minimum at $f(0, \cdots, 0) = 0$

where $x_i \in [-5, 5]$, $a=20$, $b=0.2$ and $c=2\pi$.

(B) Levy function:

$$f(x_1 \cdots x_n) = \sin^2(\pi\omega_1) + \sum_{i=1}^{n-1} (\omega_i - 1)^2 [1 + 10 \sin^2(\pi\omega_i + 1)] + (\omega_n - 1)^2 [1 + \sin^2(2\pi\omega_n)] \quad (3.9)$$

$$\omega_i = 1 + \frac{x_i - 1}{4}$$

minimum at $f(1, 1, \cdots, 1) = 0$

where $x_i \in [-10, 10]$.

(C) Rosenbrock Function:

$$f(x_1 \cdots x_n) = \sum_{i=1}^{n-1} (100(x_i^2 - x_{i+1})^2 + (1 - x_i)^2) \quad (3.10)$$

minimum at $f(1, 1, \dots, 1) = 0$

where $x_i \in [-2.048, 2.048]$.

(D) Rastrigin Function:

$$f(x_1 \cdots x_n) = 10n + \sum_{i=1}^n (x_i^2 - 10 \cos(2\pi x_i)) \quad (3.11)$$

minimum at $f(0, \dots, 0) = 0$

where $x_i \in [-5.12, 5.12]$.

(E) Griewank Function:

$$f(x_1 \cdots x_n) = 1 + \frac{1}{4000} \sum_{i=1}^n x_i^2 - \prod_{i=1}^n \cos\left(\frac{x_i}{\sqrt{i}}\right) \quad (3.12)$$

minimum at $f(0, \dots, 0) = 0$

where $x_i \in [-512, 512]$.

The population size is 100, with $m=10$ and $n=10$. After 10 memetic evolutionary steps, memeplexes are shuffled. The maximum total number of function evaluations is 50. Table 3.1 shows the experiment results of applying SFL and ISFL to the aforementioned test functions.

3.3 Experimental Evaluation

In this section, both the autofocusing window selection algorithm and the global searching algorithm for autofocusing have been evaluated.

Table 3.1: Comparison between SFL and ISFL

Function	Algorithm	Average optimal solution	Standard deviation	Optimal solution in theory	Processing time saving
Ackley function	SFL	8.107510^{-4}	0.0024	0	24%
	ISFL	1.839710^{-5}	7.115910^{-5}		
Levy function	SFL	2.575510^{-4}	0.0016	0	8%
	ISFL	1.233010^{-5}	5.901010^{-5}		
Rosenbrock Function	SFL	0.1850	0.4063	0	30%
	ISFL	0.0039	0.0162		
Rastrigin Function	SFL	0.0059	0.0087	0	21%
	ISFL	0.0048	0.0044		
Griewank Function	SFL	0.0091	0.0126	0	21%
	ISFL	0.0079	0.0059		

3.3.1 Autofocusing Window Selection

As mentioned in 3.1.2, the focus window can affect the accuracy of the estimation of the Z-axis the embryo position for immobilization and zona pellucida dissection. In this section, two focus windows, the all-inclusive focus window and the proposed size-adjustable focus window are compared with each other. Several sets of embryo images are taken for the comparison. Each frame of an image set is taken after the focal plane is moved down by $2\mu\text{m}$ from the focal plane of the previous image frame. Two focus windows are implemented on the same set of embryo images to generate two sets of FMF values. After searching for the global peak of each image set, the microscope objective lens is brought to the position that is corresponding to the peak

FMF value. The injection micropipette is then moved into the focal plane and pushed against the cell membrane. The accuracy of the focus window is examined by recognizing the dent at the contact point of the injection micropipette and the cell membrane. A symmetric dent demonstrates that both the embryo membrane and the injection micropipette are in the focal plane. On the other hand, if an asymmetric dent appears, it is safe to say that the embryo membrane is not in the image plane that contains the injection micropipette. Since it can be confirmed that the micropipette is in the focal plane, that means the cell membrane is out of the focal plane.

As shown in Figure 3.4, the all-inclusive focus window provided a set of FMF values that had a peak value at the 475th frame. An asymmetric dent is illustrated in top view image of the embryo. The front view illustration indicates the actual position of the contact point on the cell membrane.

Applying the proposed adjustable focus window provided a set of FMF values that had a peak value at the 448th frame of the image set, as shown in Figure 3.5. The top view image and front view illustration of the embryo demonstrate a symmetric dent on the cell membrane and the position of the contact point.

Figure 3.4: Autofocusing with the all-inclusive focus window, which gives a peak index value of 475. However, the peak index does not correspond with the focal plane of the embryo membrane.

Figure 3.5: Autofocusing with the proposed focus window.

Since the distance between two consecutive frames is $2\text{ }\mu\text{m}$, the estimation given by the all-inclusive focus window is off the focal plane by $54\text{ }\mu\text{m}$ (27 frame difference from 475^{th} to 448^{th} frame), which is about 5% of the diameter of an embryo. Ten embryos were used to perform the comparison between the two focus windows. The proposed window was able to identify the focal plane accurately, while the all-inclusive focus window had an average deviation of $52\text{ }\mu\text{m}$, as illustrated in Table 3.2. This deviation can significantly affect the controllability of the embryo biopsy process in the latter stage.

Table 3.2: Comparison between two focus windows on 10 embryo samples

Embryo sample No.	1	2	3	4	5	6	7	8	9	10
Deviation in frames(index)	27	26	26	26	26	25	26	25	27	26
Deviation in distance(μm)	54	52	52	52	52	50	52	50	54	52

3.3.2 Autofocusing Search Algorithm

Fibonacci searching is one of the most popular global maximum searching algorithms[109, 110]. In this experiment, the proposed ISFL algorithm is implemented on the Ackley function and the FMF graph, obtained from microscopic images of a fish embryo, to perform comparisons with the Fibonacci

Figure 3.6: Testing data generated by Ackley function and comparison of the Fibonacci and ISFL algorithms. The Fibonacci algorithm is trapped at the local peak.

searching algorithm.

Performance on the Ackley function

The occurrence of a multi-peak FMF graph is very common. That may be because of different positions of blastomeres in Z-axis and existence of dust, debris or other artifacts in the medium. The Ackley function was used to simulate the multi-peak curve and to compare the performances of the Fibonacci and the ISFL. The searching domain was between -15 and 30 units. The searching domain was equally sampled at 4500 positions, and the reciprocal values of the outputs from the function formed the testing data set.

As shown in Figure 3.6, the Fibonacci searching algorithm was finally

trapped in the local maximum at position 1404. However, the ISFL algorithm was also able to bypass the local maximums and reach the global peak at position 1501. The details of the comparison are listed in Table 3.3 below.

Table 3.3: Comparison between Fibonacci and ISFL on Ackley function

Function	Algorithm	Parameter settings	Optimum position	Processing time
Ackley function	Fibonacci	Length of uncertainty: 0.1 Distinguishability constant: 0.01	1404	0.013091s
	SFL	No. iteration before shuffling: 2 No. of evolution: 3	1501	0.031573s
	ISFL	No. iteration before shuffling: 2 No. of evolution: 3	1501	0.030937s

Performance on the FMF graph

A stack of microscopic images of partial-cleavage-stage zebrafish embryo is firstly obtained by moving the objective lens downwards with a $2\text{ }\mu\text{m}$ step size. The FMF graph was obtained by performing normalized variance in the focus window area of each image, as illustrated in Figure 3.7.

The focus window was set on top of the cytoplasm portion of the embryo image, as shown in Figure 3.8. As the objective lens was moving down, two different parts of the cytoplasm were brought into focal plane at different image frames. The top portion was in focus at the 637^{th} frame, and the

Figure 3.7: Schematic of fish embryo and the corresponding FMF graph.

lower portion was brought into focal plane at the 702nd frame, which makes them 130 μm apart.

Both the Fibonacci and the ISFL algorithm were applied on the FMF graph with the same settings used for the Ackley function. As illustrated in Figure 3.9(a), the FMF graph has two local peaks corresponding to the FMF value of frame 702 and the frame with artifacts respectively. The Fibonacci algorithm was trapped in local maximum, as shown in Figure 3.9(b), whereas the ISFL was able to find the global maximum.

Figure 3.8: The portions of cytoplasm in different focal planes.

Figure 3.9: Performance on the FMF graph: the Fibonacci method is trapped in the local peak, while the ISFL can reach the global maximum.

3.4 Summary

An intuitive focus window selection algorithm and a robust autofocusing searching algorithm based on an improved shuffled frog leaping algorithm have been introduced in this chapter. The size-adjustable focus window is

capable of improving the accuracy of the FMF graph of the target object by focusing only on the more informative portion of the image. This is necessary for estimation of the Z-axis position of the embryo's outer boundary, which is essential to perform embryo immobilization and zona pellucida dissection. An ideal FMF graph is a unimodal curve whose peak can be identified easily. However, that is not always the case, as the curve may have multiple peaks. The proposed ISFL searching algorithm is able to bypass local maxima and reach the global maximum in the non-unimodal FMF graph, which effectively identifies the most suitable focal plane for a single blastomere. In addition, the improved shuffled frog leaping algorithm has proven to be more robust, accurate and efficient than the original SFL.

Chapter 4

Embryo Image Processing

In vitro fertilization (IVF) is a widely applied clinical technique to help infertile couples conceive when normal medical treatment proves to be ineffective. A successful IVF cycle consists of successful oocyte fertilization and embryo implantation that leads to a healthy offspring. The success rate of IVF can be significantly improved by selecting high-quality fertilized embryos prior to implantation. Selection methods include both invasive and non-invasive procedures. An embryo can be examined non-invasively by evaluating and cataloguing its morphological features [32, 132]. The morphology of the zona pellucida (ZP) is a key indicator of the quality of an embryo as it will have direct contact with the uterus wall. For example, if the ZP is too thick or has hardened, assisted embryo hatching is required to help the blastocyst escape through the ZP before transferring to the uterus to increase its implantation capability. Recent studies have identified zona pellucida thickness variation (ZPTV) as a key morphological feature that distinguishes high-quality embryos [133, 134, 135]. Invasive examination, also known as pre-implantation genetic diagnosis (PGD), is performed when the embryo is suspected to be afflicted by genetic diseases[136]. Embryo biopsy is an essential step prior

to PGD, where a portion of the ZP is dissected and one or two blastomeres are extracted from the embryo. The success rate of embryo biopsy is highly dependent on the position where ZP dissection is carried out, as an unsuitable dissection point will lead to embryo lysis [137, 70, 66]. The perivitelline space is often identified as the ideal dissection point for three of the most common dissecting techniques: mechanical cutting, chemical breaching and laser.

Cell manipulations for IVF, especially intracytoplasmic sperm injection and PGD, are labor-intensive processes. Experienced and skilled embryologists are normally required to perform both the embryo ZP measurement and PGD. For both procedures, it is necessary for the embryologist to recognize the ZP from an embryo image that typically has poor contrast. In order to expedite the PGD process and automate ZP recognition, the first automatic segmentation method for identifying both the internal and external boundaries of the ZP using edge detection and ellipse fitting from a single focused embryo image is proposed. Furthermore, the technique can also estimate a suitable position for ZP dissection.

4.1 Methodology

The suitable ZP dissection position is commonly defined as the perivitelline space of an embryo. We have developed two segmentation methods to identify the suitable ZP dissection position. The perivitelline space, boundary of blastomeres and boundaries of the ZP will first have to be identified for the realization of position selection. Edge-detection and ellipse fitting techniques are applied for internal ZP boundary detection. Regional intensity recognition, classic edge detection and the value adjustment algorithm are applied to

estimate the location of the blastomere boundary and thus the perivitelline space position. The overview of the proposed method is illustrated in the function flow chart in Figure. 4.1.

4.1.1 Zona Pellucida Boundary Estimation

Recognition of the internal ZP boundary is necessary to identify the perivitelline space position. In this section, a method to perform elliptical estimation of both the internal and external ZP boundary based on edge detection with value adjustments is proposed.

Preliminary Embryo Center Estimation

The preliminary center of the embryo is defined as the mass center of the largest connected component of the binary image of the embryo. The original image is converted into a grayscale image, and then smoothed by an average (pillbox) filter with a radius of 5 pixels. The Sobel edge detector is used to obtain the edges of the smoothed image because it is less sensitive to noise [27]. A threshold value for Sobel edge detection is selected automatically by using a Bayes minimum-error decision rule with a cutoff value. The cutoff value is calculated as four times the signal-to-noise ratio. Since the intensity gradient changes of the internal structure of an embryo are more distinctive than that of the ZP region, more edge information can be obtained from the blastomere region. We can assume that most of the edges detected in this stage are the edges of blastomeres. Using image-closing (image dilation then erosion) and hole-filling techniques, all the edges together can be connected. Boundary clearing and image opening (image erosion then dilation) is then applied on the binary image to smooth out the boundary of the largest component. Simultaneously, unwanted smaller particles normally obtained

Figure 4.1: Function flow chart of embryo image processing.

from the detection of artifacts, debris and spermatozoa are also removed. The mass center of the largest connected component of the binary image,

(c_{x1}, c_{y1}) , is calculated and indicated in Figure 4.2(c).

Figure 4.2: (a) Grayscale image. (b) Sobel edge detection. (c) Application of image-closing and hole-filling on edge image to find mass center (c_{x1}, c_{y1}) . (d) Preliminary embryo center (c_{x1}, c_{y1}) .

Estimation Of The External ZP Boundary

It is challenging to automatically choose a suitable threshold for the diffused external boundary of ZP due to the unclear edge features. The threshold value is normally set through a trial-and-error approach. A biased threshold is likely to be obtained when the entire embryo image is taken into account

for threshold seeking. When adaptive edge detection is applied, the intensity gradient values of the embryo's internal structure dominate the gradient matrix. This domination makes the intensity gradients of the external ZP boundary negligible. Thus it is critical to select a suitable area, where the ZP and minimal internal embryo structure appear, for ZP segmentation. The area of internal structure of the embryo is estimated using a circular model. Since the setup for embryo image acquisition remains unchanged within a laboratory, i.e. the same camera, microscope, magnification lens and light source are used during the operation; and all embryo samples are in the cleavage stage, it is safe to assume that the sizes (in pixels) of these embryos all fall within a certain range. Hence, around the estimated center (c_{x1}, c_{y1}) , a corona to define an area that covers the ZP was drawn. The radius of the internal boundary of the corona, r_{c1} , has been set as the minor radius of the ellipse that can encircle the binary image shown in Figure 4.2(c). Through experiments, it is found that r_{c1} can be set to be between 68 and 73 pixels without affecting the result for all of our image samples. The external boundary of the corona has a radius r_{c2} , which equals to either twice of r_{c1} or r_{c1} added to the shortest distance from the inner circle to the boundary of the image, whichever is smaller, as illustrated by the red solid circle in Figure 4.3(b). The area that is enclosed by the corona is then mapped onto polar coordinates about the mass center using (4.1).

$$I_{polar}(\theta_e, r_{c2}) = I_{cartesian}[c_{x1} + r_{c2}\cos(\theta_e), c_{y1} + r_{c2}\sin(\theta_e)] \quad (4.1)$$

where $0 \leq \theta_e \leq 2\pi$.

Canny edge detection is applied to obtain the edge image of the image in polar coordinates, see Figure 4.3(d). Thresholds used for Canny edge detection are determined automatically. The higher threshold value is set as the seventieth percentile of the intensity gradient values in the edge image. The

Figure 4.3: (a) Estimation of embryo center and exclusion circle. (b) The corona that covers external boundary. (c) Polar-coordinate image of corona covered portion. (d) Canny edge detection. (e) Canny edge detection and edge indicator of external boundary. (f) Canny edge detection and edge indicator of external boundary with the value adjustment algorithm. (g) Cartesian-coordinate of external boundary indicator. (h) Elliptical fitting of external boundary and real embryo center (c_{x2}, c_{y2}) .

lower threshold is set as forty percent of the higher threshold. Both global and local edge detections have been performed on the image. In local edge detection, an averaging filter is used to blur the image. The smoothed image is equally divided into 8 sections and subjected to Canny edge detection individually. The preliminary estimation of the ZP external boundary is defined by the position of the last non-zero pixel in each column of the edge image. The average value (av_1) and the maximum difference value (mdf_1) between the position values are calculated. Position values that are not within the range $[av_1 - \frac{mdf_1}{3}, av_1 + \frac{mdf_1}{3}]$ will be replaced by av_1 , followed by the smoothing of the estimated ZP external boundary by a median filter. The resulting estimated boundary image is transformed back to Cartesian coordinates, and all the points of the estimated boundary are fed into an ellipse fitting model to generate an elliptical ZP external boundary. Figure 4.3(h) shows the final estimated elliptical ZP external boundary with center (c_{x2}, c_{y2}) .

Estimation Of The Internal ZP Boundary

The area encircled by the elliptical external boundary is first transformed into a polar coordinate image. As the external boundary has a major and a minor radius, the variable radius d instead of r_{c2} is used:

$$d(\theta_e) = \frac{a_e b_e}{\sqrt{(b_e \cos(\theta_e))^2 + (b_e \sin(\theta_e))^2}} \quad (4.2)$$

where a_e , b_e and θ_e are the major radius, minor radius and the origin angle of the external elliptical boundary respectively.

The variable d is then equally sampled n times, where n is the length of the major axis in pixels. Using equation (4.3), the polar coordinate image I_{polar} can be obtained. The transformation process is illustrated in Figure

Figure 4.4: Illustration of polar-coordinate transformation along external boundary using $d(\theta_e)$ and n sampling points.

4.4.

$$I_{polar}(\theta_e, d_n) = I_{cartesian}[c_{x2} + d_n(\theta_e)\cos(\theta_e), c_{y2} + d_n(\theta_e)\sin(\theta_e)] \quad (4.3)$$

where $0 \leq \theta_e \leq 2\pi$. To minimize the undesired edge information in the ZP, the image is cropped by 5 pixels from the bottom. Canny edge detection is applied to perform both global and local edge detection to obtain the edge image. The position of the last non-zero pixel in each column of the edge image is defined as the preliminary estimated internal boundary, as shown in Figure 4.5(d). The estimated internal boundary is subjected to the value adjustment algorithm. The average, av_2 , is calculated as the average value of top 50 percentile of all boundary values in each column as dented blastomere boundaries tend to be mistaken for the internal ZP boundary. The difference of maximum and minimum boundary value is assigned to mdf_2 . If a boundary value is closer to the maximum boundary value, which means that the value is most likely obtained from the edge of the internal boundary, the value adjustment exclusion range is defined as $[av_2 - \frac{mdf_2}{2}, av_1 + \frac{mdf_2}{3}]$; otherwise, the value is most likely obtained from the edge of blastomeres, and thus the range was defined as $[av_2 - \frac{mdf_2}{5}, av_1 + \frac{mdf_2}{2}]$ to mitigate the influence of dented blastomeres' edges. Once a boundary value is outside the defined range, it will be replaced by the average of the target boundary value and av_2 . The

value adjustment is completed by applying a medium filter to smoothen out the boundary indicators. The coordinates of the boundary indicator are then transformed back to Cartesian coordinates for ellipse fitting, where the final estimation of internal ZP boundary is derived. As shown in Figure 4.5(h), the center of the internal boundary, (c_{x3}, c_{y3}) is not always the same as the center of the external boundary.

4.1.2 ZP Dissection Position Selection With Partial Image Stretching

Cartesian-to-polar Coordinate Transformation

The first step for position selection is to transform the area encircled by the estimated internal boundary ellipse into the polar coordinate image using unequal sampling. The number of samples taken in each direction is determined by the number of pixels that are covered by the radius from the origin, (c_{x3}, c_{y3}) , in that direction. There are two main reasons why unequal sampling is critical. Firstly, equal sampling causes pixels close to the minor axis of the ellipse to be significantly stretched especially if the major axis is much longer than the minor axis. It is inevitable that the estimated internal elliptical boundary encircles a portion of ZP, which will be exaggerated if it is near the minor axis. For example, the internal ZP boundary is pulled up due to pixel stretching as shown in Figure 4.6(b) and (c). Since the intensity and contrast of ZP is very similar to that of the perivitelline space, to differentiate ZP and perivitelline space becomes very difficult. Secondly, applying an arbitrary sampling size may cause image shrinkage near the major axis, which results in loss of image information. The number of sampling points is defined as variable D , which can be calculated using equation (4.2), where

Figure 4.5: (a) Polar-coordinate image of internal structure. (b) Global Canny edge detection. (c) Global and local Canny edge detection. (d) Edge indicator of internal boundary. (e) Edge indicator of internal boundary with the value adjustment algorithm. (f) Cartesian-coordinate of internal boundary indicator. (g) Elliptical fitting of internal boundary. (h) Internal and external elliptical boundary.

a , b and θ_e are the major radius, minor radius and the origin angle of the internal boundary ellipse respectively. The maximum and minimum D values equal the major and minor radius respectively. Since the size of the resulting polar-coordinate is $720 \text{ pixel} \times a \text{ pixel}$, whenever D is smaller than the major radius a , only pixels at the bottom of the image are filled with sampled pixels from the Cartesian-coordinate images. Other pixels are filled with zeros or black pixels. In other words, the size ratio between the perivitelline space and the ZP is maintained after the coordinate transformation.

Figure 4.6: (a) Cartesian coordinate image with internal boundary ellipse. (b) Polar coordinate transformation by a (major radius) sampling points. (c) Polar coordinate transformation using unequal sampling.

Partial Polar-coordinate Image Transformation

The perivitelline space located at the bottom of the polar-coordinate image is the part of the image that is of interest. The perivitelline space can be differentiated from other structures by utilizing its properties of low contrast and small variation in intensity. In order to make the perivitelline space more distinctive, the polar-coordinate image is stretched unequally by taking more sampling points from the bottom of the image after replacing all black pixels with the average image intensity. As shown in Figure 4.7, the perivitelline space portion in the partial stretched image is significantly magnified.

Figure 4.7: (a) Unequal sampled image. (b) Unequal stretched image.

Edge And Intensity Detection

Once the area containing the perivitelline space is enhanced by partial image stretching, the next task is to recognize the features of suitable ZP dissection positions. There are two key features of the stretched image that are of interest: the edge and the pixel intensity. HMC microscopy provides images with 3D appearance by converting invisible phase gradients into variations in light intensity. Boundaries of blastomeres normally appear brighter or darker than other parts of the image. The distinctive brightness and dark-

ness are utilized to identify part of blastomeres' boundaries. Pixel intensities in each column are sorted first. Only the coordinates with intensity values that are within the top and bottom five percent brightness are recorded and used to form the intensity boundary image, as shown in Figure 4.8(a). We also applied Canny edge detection on the partially stretched image and the resulting edges were dilated. As the intensity does not provide more accurate boundary information than the edges, white pixels are assigned with different weights. A white pixel in the intensity boundary image and in the edge image has a weight of 1 and 3 respectively. Thus in the combination image, each white pixel has a maximum possible weight of 4 and minimum possible weight of 1.

Figure 4.8: (a)Intensity boundary image. (b) Edge detection of stretched image. (c) Combination of (a) and (b).

Template Image Correlation For Position Selection

The final suitable dissection position is achieved by correlating a template image with the intensity-edge image. The template window used for correlation is a trapezoidal shape with a height equal to one third of that of the intensity-edge image's and one of two arbitrary base widths 30 and 90 pixels in length. When correlating the template window with the bottom portion of the intensity-edge image, the position with the minimum correlation value is considered to be the most suitable position. If there is more than one position having the same minimum correlation value, the longest consecutive positions with minimum correlation values are identified. The midpoint of these positions is considered to be the final position. The coordinate of the position is then transformed back to Cartesian coordinate and a curve is drawn to indicate the suitable ZP dissection position, as shown in Figure 4.12.

4.1.3 ZP Dissection Position Selection With Value Adjustment

Blastomere boundary estimation by edge detection

After obtaining the polar-coordinate image using the aforementioned method in Section 4.1.2, the image is extended by 4 to 8 pixels at the bottom to compensate for the inaccuracy of the estimating ellipse. This image is then cropped by one-third from its top to eliminate both the zero padding and pixels with high intensity gradients. Two averaging (pillbox) filters with radii of 5 and 3 pixels are applied to the top half and the bottom sixth of the image respectively, resulting in a double-layer filtered image. The averaging filter with 5-pixel radius is applied to mitigate the threshold bias due to the

high intensity-gradient changes at the top of the image. All noise caused by small artifacts at the bottom of the image is diminished by applying the averaging filter with 3-pixel radius. The resulting image is then subjected to global and local Sobel edge detection. The global edge detection is applied to this 2-layer smoothed image. The local edge detection is applied to a 3-layer smoothed image, obtained by smoothing the unfiltered portion of the aforementioned 2-layer smoothed image with an averaging filter with 2-pixel radius. The results are shown in Figure 4.9.

Improvement On Blastomere Boundary Estimation By Intensity Recognition

The lighting feature of Hoffman modulation contrast (HMC) described in Section 4.1.2 is utilized to further improve the accuracy of the blastomere boundaries obtained by edge detection. The top half of the image is smoothened by an average filter with 5-pixel radius to eliminate high brightness variations. Intensity values of each column are sorted and indexed from low to high. Due to the position of the light source, the dominating intensity in each column is different, i.e. there is higher pixel intensity in the area facing the light source. The dominating intensity is determined by comparing the average intensity of the column with a threshold value of 0.5. If the average intensity is above or equal to 0.5, the dominating intensity is defined as bright, otherwise, it is considered to be dark. When dominating intensity is bright, the top 3 percent of bright pixels in a column will contribute to the intensity boundary image as shown in Figure 4.10. On the other hand, if the dominating intensity is dark, the top 3 percent of dark pixels is taken as the boundary pixels. The blastomeres' boundary is initially defined by the last non-zero pixel positions of the edge image. As shown in Figure 4.9(f), yellow

columns indicate columns without any non-zero pixel. Those columns are filled with the last white pixel of the corresponding column from the intensity boundary image, as shown in Figure 4.10(b). In the case that there are remaining columns without any white pixel, the boundary values for those columns are defined as the average of the neighboring non-zero values.

Figure 4.9: (a)Extended and cropped image. (b) Two-layer smoothed image. (c) Global edge detection. (d) Local edge detection. (e) Combination of (c) and (d). (f) Initial blastomeres boundary with empty column.

Boundary Value Adjustment And Template Image Correlation

In order to eliminate abrupt and unexpected variations and to compensate the inaccuracy of the boundary estimation, a boundary value adjustment method was developed. The resulting boundary image shown in Figure 4.10(c) is filled up with white pixels above the boundary line, giving Figure 4.11(a).

Figure 4.10: (a)Top-half smoothed image. (b) Intensity boundary image. (c) Blastomeres boundary before value adjustment.

The image is then extended by 10 black pixels to facilitate a 5×5 -image closing to eliminate those thin lines, as shown in Figure 4.11(b). A boundary value is defined as unexpected and subject to change when it meets following criteria:

1. The position of the boundary value is not within the range $[LM-s, LM+s]$, where LM is the local average value of a 90-pixel-wide image block where the considered pixel is located; and s is the global standard deviation.
2. The boundary value varies more than $s/2$ from its previous boundary

value.

Columns with boundary values satisfying both criteria are replaced by the boundary value of the previous column. The value adjustment algorithm is performed on the boundary image twice, once starting from the right side and the other starting from the left side to obtain two new boundary images, shown in Figure 4.11(d) and (e). Weight correction is then applied by first comparing the values in both boundary images, with the larger boundary value of the column of interest assigned with a weight of $2/3$ and the lower boundary value with a weight of $1/3$. The final boundary position is obtained by the summation of the two weight-corrected values. Pixels above the boundary lines are then filled up with white pixels for template correlation. The same template correlation method described in Section 4.1.2 is applied with a triangular template to the resulting boundary image. The final ZP dissection position in Cartesian-coordinates is indicated as shown in Figure 4.12.

4.2 Experiment Results

We have validated the proposed approach on 45 microscopy images. Each image contains a single embryo with an internal ZP boundary radius of 70 ± 5 pixels. Most of these images have one or more features that are undesirable for image segmentation. These undesirable features include low signal-to-noise ratio, low resolution, low contrast, large artifacts and small artifacts, etc.

Figure 4.11: (a) Filled-up image. (b) Image closing. (c) Boundary image. (d) Value adjustment from left to right. (e) Value adjustment from right to left. (f) Combination of (d) and (e). (g) Filled up image for template correlation.

Figure 4.12: (a) Position selected by implementing ZP dissection position selection with partial image stretching with correlating template in the lower left corner. The inset is the correlation template. (b) Position selected by ZP dissection position selection with value adjustment with correlating template in the lower left corner. The inset is the correlation template.

4.2.1 ZP Boundary Estimation

The internal and external ZP boundary have been manually indicated using ellipses placed by an expert. The estimated ZP boundary ellipses from the proposed method are compared with the manually recognized ZP boundary ellipses. A MatchScore, calculated using (4.4), is applied to define the success of the automatic segmentation.

$$MatchScore = \frac{n(MS \Psi AS)}{n(AS)} \times 100\% \quad (4.4)$$

where MS is the manually segmented boundary ellipse by the expert and AS is the automatically segmented boundary ellipse by our proposed method; $n(AS)$ is to calculate the number of pixels of AS and $n(MS \Psi AS)$ is to calculate the number of true results. $MS \Psi AS$ is defined as a true if in each particular angle, there is an intersection with MS within a 2-pixel vicinity of AS . As illustrated in Figure 4.13, the cyan squares indicate MS pixels, the

red squares indicate AS pixels, the beige squares indicate the neighboring pixels of the target AS pixel and the black arrow points to the origin of the ellipse. If the beige color area covers the target MS pixel, $MS\Psi AS$ is defined as true; otherwise, it is defined as false. The automatic segmentation is deemed to be successful when the MatchScore achieved larger than 85%. Out of 45 images, the ZP boundaries of 43 images were correctly segmented, giving a success rate of 96%. Failures of image segmentation are mainly due to the low signal-to-noise ratio which causes false edge detections along the internal ZP boundary (see Figure A.26 and Figure A.41 in Appendix A).

Figure 4.13: (a) Example of function $MS\Psi AS=TRUE$. (b) Number of successful segmented images *vs.* MatchScore.

4.2.2 Suitable ZP Dissection Position Selection

The suitable ZP dissection positions are manually selected by experts using arcs, as shown in Figure 4.14. Some embryos have one or more suitable ZP dissection positions, whereas, some have no obvious ZP dissection position. We deemed our testing result successful if the cutting position recommended by our system lies within the manually selected arc.

Figure 4.14: (a) Embryo with many suitable ZP dissection positions. (b) Embryo with one suitable ZP dissection position. (c) Embryo with no distinctly suitable ZP dissection position.

Partial Image Stretching

Our ZP dissection position selection approach with partial image stretching successfully indicated a suitable ZP dissection position in 42 of 45 embryo images, achieving a 94% success rate (failure image can be found in Figure A.17, Figure A.22 and Figure A.38 in Appendix A).

Value Adjustment

The ZP dissection position selection method with the value adjustment algorithm was able to achieve the same success rate by successfully selecting suitable positions in 43 of 45 embryo images (refer to Figure A.17 and Figure A.22 in Appendix A).

4.3 Discussion

The ZP boundary estimation method proposed is a fully automatic approach. When edge values that exceed a certain range are replaced by adjusted values,

the accuracy of the boundary estimation is not easily affected by artifacts appearing in the image. Compared to Karlsson's method [30], our approach is able to estimate the boundary without manual mask insertion, as shown in Figure 4.15. This is due to our proposed built-in value adjustment algorithm. As the proposed method is based on edge detection, it can also be applied to bright field embryo images.

Figure 4.15: (a)Image with artifacts. (b) Karlssons method. (c) Karlssons manual modification. (d) Our proposed method.

The testing of our ZP dissection position selection algorithms was dependent on the ZP boundary estimation algorithm. However, this does not

mean that a low *MatchScore* will lead to failure in our position selection algorithm. For example, though the manually-drawn internal boundary ellipse has a *MatchScore* less than 85%, as shown in Figure 4.16, the resulting position selected for ZP dissection is still acceptable. This is because the internal boundary ellipse encircled a portion of perivitelline space.

Although both approaches achieved similar success rates, the positions selected for suitable ZP dissection may be different, as shown in Figure 4.12. By comparing the accuracy of positions recommended by both methods, it has been discovered that the first method is superior to the second in low-contrast image segmentation, while the second method performs better on embryos with more complex internal structure. Therefore, users can select the more appropriate approach according to the situation encountered.

Figure 4.16: (a)Image with MatchScore less than 80%. (b) ZP dissection position selection.

4.4 Summary

In this section, a method to automatically select a suitable ZP dissection position from single embryo image has been presented . The proposed method

consists of elliptical internal and external ZP boundary estimation and two methods of suitable ZP dissection position selection. We have validated our techniques on 45 images and obtained the success rates shown in Table. 4.1:

Table 4.1: Table of success rates

Method	Success rate
ZP boundary estimation	96%
ZP dissection position selection with partial image stretching	94%
ZP dissection position selection with value adjustment	96%

Even though our initial primary task is to select the suitable position, the elliptical ZP boundary estimation can also be applied to ZPTV and other ZP morphology measurements. Furthermore, the computationally inexpensive nature of our methods allows for fast position selection prior to automatic PGD.

Chapter 5

Three-dimensional cell rotation

A predictable three-dimensional cell rotation is essential for ICSI and PGD. So far, existing researches have not demonstrated an effective way of performing the embryo orientation. In this chapter, a system with multiple techniques that can perform automated three-dimensional rotational control of a single zebrafish embryo is proposed. A 3-point contact method that allows the three-dimensional cell rotation is introduced. The system employs a custom designed rotational microscopy device and a microscope stage to enable two-dimensional cell rotations in the X-Y plane. Used in conjunction with a pair of capillary glass pipettes, the contact points between a cell and micropipettes and the contact point between the cell and the petri dish established a 3-point contact that allows the embryo to be rotated in the third dimension.

For an oocyte, its cellular structure can most easily be discerned with a particular cross-sectional view. The focal plane of the microscope must thus be aligned with the cutting plane of this cross-section. This can be done by rotating the cell about the axes lying within the focal plane. To automate this process, a method using visual recognition and tracking of target cellular structures to recognize when the cross-section and the focal plane have come

into alignment needs to be developed. A pattern recognition algorithm (dimension measurement) was developed and applied to test the performance of the system. The visual servo control was successful in orienting the fish embryo such that the yolk and the cytoplasm are viewed at the ideal cross-section, with the cytoplasm facing the cell's 3 o'clock direction from its top view. A schematic illustration of the process of rotating a cell in the three dimensions is given in Figure 5.1.

5.1 System Design

5.1.1 System Setup

Figure 5.2 shows the setup of the system, which consists of a standard upright microscope (Nikon, Eclipse FN1, Japan) equipped with a motorized X-Y stage (Narishige, ITS-FN1, Japan), a motorized Z axis (Märzhäuser Wetzlar, ECO-MOT, Germany), a custom-designed rotational stage, and a 659x490-pixel CCD camera (Basler, scA640-74gc, Germany) for visual servo control of embryo position and orientation. The XY stage has 30 mm travel range with 0.02 μm resolution and $\pm 1 \mu\text{m}$ repeatability, while the Z stage has 0.002 μm resolution. Images are taken through objective lens with magnification factor of 4 and a 0.1 numerical aperture (Nikon, Plan Fluor, Japan), and were processed by the computer in real time (30 Hz). A pair of micropipettes (Eppendorf; tip diameter: 10 μm , tilt angle: 35°) are mounted on a pair of 3-DOF motorized stages (National Aperture Inc., MM-4M-F, USA) for the establishment of the rotational axis of the embryo. The linear micromanipulator has 0.1 μm resolution (0.124 μm per count) and 50 mm travel range.

Figure 5.1: Schematic illustration of the 3-D cell rotation process.

Figure 5.2: Embryo manipulation system setup.

5.1.2 Rotational Stage Design

To realize the three dimensional embryo rotations with the proposed 3-point contact approach, a rotational stage is a necessity. In order to meet the requirements of the micro-manipulation system and suit the characteristics of the upright microscope, the rotational stage conforms to the following requirements: (1) no obstruction of the microscope's field of view; (2) low height to ensure that the sample is within the depth of field of the microscope and allow sufficient working space for operation; (3) a compact structure to fit into the existing translational stage; (4) fast and smooth rotational motion. As no existing rotational stage satisfies all of the requirements, it is necessary to custom-design to realize the cell rotation method described.

Figure 5.3: The three-dimensional model of the in-house made rotational stage.

A CAD image of the resulting rotational stage is shown in Figure 5.3. The rotational stage consists of a stepper motor with planetary gearheads with reduction ratio of 275:1, a pair of spur gears with gear ratio of 8.5:1 that transmits the rotational motions, and a commercially available Petri dish that loads the sample cells. The height of the rotational stage (excluding the Petri dish) is 2 mm, which is the height of the loading plate of the existing microscope platform. Thus, the introduction of the rotational stage does not compromise the depth of field of the microscope. Furthermore, since a commercially available Petri dish is installed on the stage, the image quality is ensured. Fast and smooth rotational motion in compact structure was achieved through the use of the spur gear pair and a 0.3 mm Teflon washer. The rotation stage is capable of a maximum rotational speed of $1440^\circ/\text{sec}$

with a high rotational positioning resolution of 0.02° .

5.1.3 The 3-Point Contact Cell Rotation Model

The three points of contact of the 3-point cell rotation model are illustrated in Figure 5.4(a). Points a and b serve to immobilize the embryo along the X-axis; and point c (surface contact) provides the torque to rotate the embryo. When an embryo is positioned along the X-axis of the Petri dish, the friction force exerted on point c of the embryo is perpendicular to the line connecting points a and b , and a torque to rotate the embryo about the X-axis is generated.

Establishment Of Three Contact Points

To determine the positions of points a and b , the uppermost (UB) and lowermost (LB) boundaries of the embryo are first recognized by computer vision, as shown in Figure 5.4(b). The coordinates of points a and b along the Y-axis are the average of that of the two boundary lines. A line parallel to the X-axis with the same Y-coordinate intersects with the cell boundary at two points. The X coordinates of points a and b are the coordinates of those intersection points (IP_1 and IP_2) $\pm l$, respectively, where l is less than $3\ \mu\text{m}$ in this case.

The Z-coordinates are found by applying an autofocusing algorithm to find the image plane with the best sharpness value. The accuracy of the selected Z-coordinates largely depends on the type of computer vision used, and is important as an additional push-down or lift-up force will be exerted on the cell during rotation if the contact points deviate too significantly from the center of the cell. The most common window selection for autofocusing is to draw a window that covers the entire target object. However, the sharpness

Figure 5.4: (a) Three contact points on the rotating cell and the angular displacement, β , about X -axis. (b) Determination of the coordinate of the axle points a and b in X - Y plane and the distance R_p between cell center O_c and Petri dish center O_p . (c) Determination of the coordinate of the axle point a and b in Z axis by applying window 2 (green).

information derived from the internal cellular structure of the embryo tends to dominate that from the embryo's membrane. As the sharpness of the cell membrane provides the most pertinent information about the appropriate Z-coordinate to use, the corona shape focusing window is selected as the area around the cell membrane. Figure 5.4(c) shows that the selection of focusing windows affects the shape of the distribution curves produced by the focusing measurement function. Window 2, a coronal window, was found to be much more accurate in focusing on the embryo's membrane. More detailed elaboration of the method can be found in Chapter 3.

The coordinates of point a ($[x_a, y_a, z_a]$) and b ($[x_b, y_b, z_b]$) can be describe as follows:

$$x_a = x_{IP_1} + l \quad (5.1)$$

$$x_b = x_{IP_2} - l \quad (5.2)$$

$$y_a = y_b = \frac{y_{UB} + y_{LB}}{2} \quad (5.3)$$

$$z_a = z_b = z_{best_sharpness} \quad (5.4)$$

Cell Rotation Model

Rolling friction is a key factor mediating the rolling motions of the embryo. Rolling friction on the round object can be modeled by applying the ideas presented in recent publications [138, 139]. Two factors are responsible for generating the frictional forces: the frictional torque about the axis opposing the cell's rotational motion, and the deformation of the cell. In the experiment, the cell is assumed to be an elastic sphere. Since the axle (i.e. micropipette) does not penetrate the cell, and it is significantly thinner than the diameter of the cell ($\approx 1:120$), the frictional forces exerted on the cell

due to contact with the micropipettes are very small. Thus, the torque τ at the axle can be neglected. Deformation is represented as the flattening of the cell. The relationship between rolling friction and angular acceleration is derived as follows. Figure 5.5 shows the normal contact force, N , and torque,

Figure 5.5: Enlarged view of the contact forces and torque acting on the embryo. O_c and R are the center and radius of the cell respectively. N , f , τ and α are the normal contract force, friction force, axle torque and angular acceleration respectively. C is the effective point of contact, which has a distance D from O_c . x and r are the contact interference and radius of contact area respectively

τ , acting on the cell. Letting m be the mass of the cell and considering the equilibrium condition in the vertical direction, the normal contact force generated at the effective point of contact between the cell and Petri dish C , can be evaluated to be

$$N = mg \quad (5.5)$$

The frictional force tangent to the Petri dish's surface is then

$$f = ma = \mu N = \mu mg \quad (5.6)$$

By Newton's second law, the relationship between angular acceleration and resulting torques is formulated and we get

$$ND + \tau - f(R - x) = I\alpha = \frac{2}{5}mR^2 \times \frac{\alpha}{R} = \frac{2}{5}mR \times \frac{f}{m} = \frac{2}{5}Rf \quad (5.7)$$

where D is the perpendicular distance between the vector N and the cell center, O_c ; D can be estimated to be half of the radius of the contact interference, r .

$$D \cong \frac{r}{2} = \frac{\sqrt{R^2 - (R - x)^2}}{2} \quad (5.8)$$

x is the contact interference; R represents the radius of the cell; I is the moment of inertia of the cell; and α is the angular acceleration of the cell. Substituting (5.5), (5.6) and (5.8) into (5.7) gives

$$\mu_r = \mu = \frac{\sqrt{R^2 - (R - x)^2}}{2 \times (\frac{7}{5}R - x)} \quad (5.9)$$

Note that μ_r is the coefficient of rolling friction, which can be found if x and R are known.

A zebrafish embryo on a Petri dish can be considered to be a spherical object that is pushed against a hard flat surface by the force of gravity. In this process, the sphere may transit among three different phases: the elastic compression phase, the elasto-plastic compression phase and the restitution phase. Since critical deformation of the embryo is not expected to occur, the deformation of the embryo can be considered to be only the result of elastic compression. When only elastic deformation occurs, the Hertz law can be applied to obtain pushing force, P :

$$P = k_1 x^{3/2} \quad (5.10)$$

where

$$k_1 = \frac{4}{3}E\sqrt{R} \quad (5.11)$$

and E is the equivalent elastic modulus [140] that can be calculated from

$$\frac{1}{E} = \frac{1 - v_1^2}{E_1} + \frac{1 - v_2^2}{E_2} \quad (5.12)$$

where E_1 , E_2 , v_1 and v_2 are the elastic moduli and the Poisson's ratios of the sphere and the flat surface, respectively. The equation of motion of the embryo can be given as

$$m\ddot{x} = mg - k_1x^{3/2} \quad (5.13)$$

When the acceleration of the embryo is zero, the equation can be written as

$$mg = k_1x^{3/2} \quad (5.14)$$

Thus by substituting (5.11) and (5.12) into (5.14) we obtain

$$x = \left[\frac{3mg(E_1 + E_2 - v_2^2E_1 - v_1^2E_2)}{4E_1E_2\sqrt{R}} \right]^{2/3} \quad (5.15)$$

The diameter of a zebrafish embryo, including the chorion, is approximately 1.2 mm; the density of a single cell is approximately 1.079 g/mL [141]. Using these figures, the approximate mass of the embryo can be calculated. The Poisson's ratio and Young's modulus of a zebrafish embryo are determined experimentally to be 0.48 and 1.51 MPa respectively [142, 143]. The equivalent material properties of plastic are 0.35 and 2.6GPa respectively. Taking 9.8 N/kg for g , the contact interference x is found to be 0.2816 μm . The value for μ_r can then be obtained, and substituting into (5.6) and (5.7), α is calculated to be 18.2 rad/s^2 . If the angular velocity of the cell is small, say 0.036 rad/s (the angular velocity setting of our system), the time required for acceleration is 0.003 s. Since the acceleration time period is so short, we can assume the cell rolls immediately when the rotational stage starts to rotate.

Therefore, we can estimate the relationship between angular displacement of cell, β , about the X axis and rotational stage angular displacement, φ [see Figure 5.4(b)], as:

$$\beta = \frac{\varphi R_p}{R} \quad (5.16)$$

where R_p is the distance between cell and Petri dish center.

5.1.4 Operation Sequence For The Cell Rotation Control

Our cell rotation control process is illustrated in Figure 5.6. The cell rotation process starts by aligning the rotational axis of the rotational stage with center of the microscope's field of view. Next, the X-axis of the stage is advanced in the positive direction. During batch cell manipulation, fluid dispensed from a micropipette can be used to draw a circular cell loading area around the center of the Petri dish, O_p [see Figure 5.4(b)], by rotating the stage by 360 degrees. The radius of the circle is approximately R_p . In our experiment, we load a cell at a position along the X-axis, with the center of the cell a distance R_p away from O_p . Determination of the positions of points a and b using the aforementioned technique (see Figure 5.4(b) and (c)) allows the two micropipettes to move towards the corresponding points by a distance of x_d in Figure 5.7 to immobilize the target embryo and establish the 3-point contact necessary for rotation. Measurement of the cellular structure is used to determine if the cell needs to be rotated about the X axis or can proceed to adjusting in-plane orientation immediately. If the measurement of the cellular structure does not meet the predefined requirement, the rotational stage is activated to enable cell rotation about the X axis by closing switch S_1 in Figure 5.7. Visual servoing is used to control the rotational stage's rolling

Figure 5.6: Operation sequence for the cell rotation.

of the cell until the measurement of the cellular structure meets the imposed requirement. Once cell rotation about the X axis is completed, the cell is released from the micropipettes. The cellular structure angle is measured and checked against the predefined requirement to determine if cell rotation about its Z axis should be performed. In-plane rotation control involves the use of the micromanipulator for cell release, the XY stage for translational motion and the rotational stage for rotational motion (the rotational stage is connected to switch S_2 in Figure 5.7). The cellular structure is monitored visually to track its angular position which will be used by the rotational stage for angle control and the XY stage for cell translational displacement tracking. Once the angle meets the user's requirement (e.g. aligning with X-axis or 3 o'clock position of the cell), the in-plane cell rotation process stops; and the cell is ready for further operation, such as injection and biopsy.

Figure 5.7: System control architecture. The X-Y-Z manipulator is for rotational supporting points positioning. The X-Y stage is for translational motion. The rotational stage is for cell rotation about the X axis (switch S_1) and in-plane cell orientation (switch S_2). x_d , O_d , A_d and θ_d are desired micropipette position, desired embryo position on the X-Y stage, the desired area measurement value and the desired angle for the cytoplasm., respectively.

5.2 Cell Rotation Control

5.2.1 Cellular Structure Recognition

Orienting the internal cellular structure to a desired orientation is the main purpose of three-dimensional cell rotation technique. The cellular structure can be the polar body of an oocyte [49], the perivitelline space of an embryo [144], etc. In our experiment, the target cellular structure is the yolk and the cytoplasm of a zebrafish embryo. Microinjection of zebrafish embryos typically targets its yolk or cytoplasm [145]. Our system serves to orient

the yolk and the cytoplasm to facilitate the injection. Although a precise orientation need not be achieved for microinjection of zebrafish embryos to be successful, it may be more critical in other micro-operations, hence the value of our 3-D rotation technique. In this chapter, work done on the zebrafish embryos serve to validate the performance of the three-dimensional rotation system. The cellular structure recognition algorithm identifies the current position of the embryo. If the embryo is properly oriented around the X axis with the ideal cross-section aligned with the focal plane, the system proceeds to perform cell rotation about Z-axis. Otherwise, the necessary rotation about the X axis is performed before proceeding with the rest of the procedure.

A binary image of the embryo is obtained by applying adaptive Otsu thresholding [146] on the original image. A series of morphology processing including image closing (dilation then erosion), boundary pixel elimination and image opening (erosion then dilation) are applied to the binary image to eliminate boundary pixels, noises and small particles. The binary image of the chorion (the outer membrane of the cell) is removed to form a binary image of the target internal cellular structure by searching for the largest connected binary component, as shown in Figure 5.8(c). After image closing and the filling of holes, the binary image of the yolk and the cytoplasm is obtained. The center of mass of the yolk is estimated to be the center of mass of the area of the hole in the binary image in Figure 5.8(c). The presence of the hole is due to strong light reflection off the yolk portion of the cell. The center of mass of the cytoplasm is taken to be the center of mass of the remaining portion of the binary image after dilating the hole in Figure 5.8(c).

The area of (number of pixels covered by) the binary image of the yolk

and the cytoplasm is considered as cellular structure measurement value, A . The value of A changes when the cell is rotated about the X axis and reaches a maximum magnitude when the ideal cross-section is aligned with the focal plane. Our algorithm is designed to search for the largest A value. The in-plane rotational angle, θ , is defined as the angle between the X axis and the line joining CM_1 and CM_2 . Note that θ is calculated after cell rotation about the X axis has been completed and the cell has been released by the micropipettes.

5.2.2 Cell Rotation About The X Axis

The cell needs to be rotated about the X-axis to find the orientation at which A is at its maximum. As the micropipettes are oriented with their lengths parallel to the X-axis, the cell is first loaded along the positive direction of the X-axis with a distance R_p away from the center of the Petri dish such that a tangential force along the Y-axis can be generated from the contact with the Petri dish to cause a torque that effects the desired rotation, as illustrated in Figure 5.9.

The micropipettes then move to points a and b , as illustrated in Figure 5.4, to establish the axis of rotation. Experimental results proved that rolling friction was sufficient to make the embryo roll on a dry Petri dish when no buoyant force is present. However, when water is introduced to the Petri dish, the embryo experienced a buoyant force that significantly reduced the normal force generated at the contact surface, leading to a significant reduction in rolling friction. In addition, the thin water layer between the embryo and the Petri dish provided lubrication causing the real friction coefficient to be smaller than μ_r . In order for sufficient deformation and normal force to be generated, the embryo needs to be pushed downwards against the Petri dish.

Figure 5.8: Image segmentation of the target internal cellular structure. (a) Original image. (b) Binary image. (c) Binary image after removal of chorion. (d) Morphological processing (image closing and hole filling) of (c) to obtain the area A . (e) Identification of CM_1 , the estimating center of the yolk (the area of the hole in (c)) and CM_2 the estimating center of the cytoplasm (the area obtained by image dilation on (c)). (f) Determination of direction of the cytoplasm, θ , by connecting CM_1 and CM_2

As the effect of this thin layer of water between the embryo and the Petri dish on the friction coefficient is difficult to measure, the embryo is pushed down by an x value, which is set experimentally to twice of the original x value, to compensate for the decrease in the friction coefficient.

For a desired angular velocity of the cell, ω , rolling about the X-axis of 0.036 rad/s, the corresponding angular velocity of the rotational stage, ω_p is 4.5 rad/s, obtained using (5.16), with R_p and R being 7 mm and 0.6 mm

respectively. The A value, which is used to visually servo the rotational stage, is the input to the rotation controller. A piecewise controller controls the real-time angular velocity of the rotational stage, ω_{rp} , by switching between a few states:

$$\omega_{rp}^{i+1} = \begin{cases} \omega_p & \text{if } A_{max} \neq A_{peak}, \\ k[A_i - 0.996 \times A_{max}]\omega_p & \text{if } A_i > 0.996 \times A_{max}, A_i \leq A_{i-1} \\ 0 & \text{if } A_i \leq 0.996 \times A_{max}, A_i \leq A_{i-1} \\ -k[A_{max} - A_{end}]\omega_p & \text{if } A_i \leq A_{max}, A_i > A_{i-1} \end{cases} \quad (5.17)$$

where A_{max} is the maximum value of A_i , A_{peak} is the value of A at the peak and A_{end} is the final A value when the rotational stage stops. A_{max} is set to be positive infinity till it receives the first measured A value. The system starts the rotation of the cell with an angular velocity of ω_p to search for the first peak of the plot of the A_i , as shown in Figure 5.10. Once the first peak value is found, the rotational motion slows down to an angular velocity of $k[A_i - 0.996 \times A_{max}]\omega_p$ with the k adjusted to a value of 0.0002. The rotational stage starts its motion in the counterclockwise direction [see Figure 5.11(a-c)]. As the cell rotates, the control system continually notes the value of A_i , and once its value drops below $0.996 \times A_{max}$, the rotation stops [see Figure 5.11(d)]. A rotation with an angular velocity of $-k[A_{max} - A_i]\omega_p$ in the clockwise direction is initiated to compensate for the overshoot.

In rare situations, the cell may not rotate with the rotational stage due to insufficient rotational friction. When the computer vision algorithm does not detect any changes in A_i and θ_i (do not exceed certain threshold) for more than 2 seconds (60 frames), both micropipettes are lowered along Z-

Figure 5.9: (a) Diagram of cell rotation about the X axis. The cell is R_p away from the Petri dish center O_p (Top view,). (b) Diagram of cell rotation about the Z axis (Top view). (c) Diagram of cell rotation about the X axis (Front view).

axis by $0.2 \mu\text{m}$ to provide the friction necessary to effect the rotation. The situation in which the cell is perfectly aligned along the X-axis such that no change in area nor angle can be expected to be observed is deemed extremely improbable and is not considered in the experiment.

Figure 5.10: Plot of the A values with angular speed of the rotational stage: ω_{rp} switches among four states (in four different colors) depending on the value of A . Phase 4 partially overlaps Phase 2 with opposite direction of ω_{rp}

5.2.3 Cell Rotation About The Z Axis

When attempting to release the cell at the completion of cell rotation about the X-axis, the cell may adhere to one of the micropipettes when they are retracted, causing in-plane rotation of the cell when the rotational stage is activated. To achieve successful cell release and a correct cell angular recognition, the micropipettes and the rotational stages are activated simultaneously, as shown in Figure 5.12(a-d). The successful release of the cell

Figure 5.11: Cell rotation about the X axis. (a) Initial position with $A_i = 3.27 \times 10^4 \text{pixel}^2$. (b-c) The cytoplasm is partially in the focal plane, thus the A_i is increasing from $3.48 \times 10^4 \text{pixel}^2$ to $3.70 \times 10^4 \text{pixel}^2$. (d) The cytoplasm is totally in the focal plane and A_i reaches it maxima, $3.81 \times 10^4 \text{pixel}^2$.

is verified using computer vision by determining the cellular structure angle. The cellular structure angle is determined through image segmentation as aforementioned [see Figure 5.8 (f)]. If the cell is in contact with one of the micropipettes, it will rotate about the contact point when the rotational stage is in motion. If the cellular structure angle does not change significantly as the rotational stage rotates, the cell is considered to have been completely released from the micropipettes. At this point, in-plane rotation of the cell

can be performed. The desired outcome is to orient the cytoplasm of the cell to point in its 3 o'clock direction (i.e. θ in Figure 5.8 equals to 0°), as shown in Figure 5.12(f-g). Prior to translational and rotational position control, the system conducts coordinate transformation calibration.

$$\begin{bmatrix} x \\ y \end{bmatrix} = s_s \begin{bmatrix} \cos\theta & -\sin\theta \\ \sin\theta & \cos\theta \end{bmatrix} \begin{bmatrix} x_c \\ y_c \end{bmatrix} + \begin{bmatrix} \Delta x \\ \Delta y \end{bmatrix} \quad (5.18)$$

where $(x, y)^T$ is the target cell coordinate in world frame, s is the scale factor, $(x_c, y_c)^T$ is the target cell coordinate in the rotational stage frame, and $(\Delta x, \Delta y)^T$ is the coordinate O_p in the world frame, as shown in Figure 5.9 (b). As the embryo is stationary relative to the Petri dish and the rotational stage is capable of moving with high precision, open-loop control was applied to move the X-Y stage and the rotational stage. Once the rotational stage stops, the X-Y stage is moved to the coordinate found from (5.18). The angular velocity of the rotational stage was set to be 4.17×10^{-2} rad/s (equivalent to 15.02° per second), which is also the angular velocity of the rotating fish embryo. Thus the system takes approximately 12 seconds to complete a 180-degree cell orientation with a resolution of 0.5° per frame.

5.3 Experimental Results And Discussion

All zebrafish embryos used this experiment were collected in accordance with standard embryo preparation procedures at the blastula stage (2 hours after placed in room temperature). The Petri dish was filled with water to a depth of about 5 mm. A total of 20 and 40 fish embryos were used to evaluate the cellular structure recognition algorithm and the three-dimensional cell rotation respectively.

Figure 5.12: (a-d) The process of cell release. Red arrows indicate motion direction of the micropipettes and rotational stage. (e) Initial cell position with cell angle indication line. (f-g) Cell positions with angles of 36.8° and 21.6° respectively. (h) Final cell position.

5.3.1 Cellular Structure Recognition

The cellular structure recognition algorithm was evaluated using 20 off-line captured videos of rotating fish embryos. Fish embryos were rotated in three dimensions with manual control of the angular velocity of the rotational stage. All operations, including cell rotation about the X-axis, cell releasing and cell rotation about the Z axis, were captured in a video at 30 fps. The algorithm is deemed to be successful if the algorithm is capable of the following: (a) Indicating the frame in which the area of the yolk and the cytoplasm from the binary image reaches its maximum value. (b) Monitoring the cellular structure angle changes during cell release while providing the desired in-plane rotation angle θ_d , and recognizing the failure of the cell to rotate with the rotation stage. The system was able to achieve a success rate of 100% from the tests. The lighting of the microscope has significant effects

on the image processing of the cellular structure. A low lighting decreases the light reflection area, and can cause inaccurate or failed angle detection. Therefore, the lighting of the microscope requires proper calibration prior to conducting the experiment.

5.3.2 Cell Rotation About the X Axis

For cell rotation about the X-axis, the success rate of rotating the yolk and the cytoplasm into the X-Y plane, the predictability of the procedure, and the amount of time required were evaluated. The cell was positioned in the loading area with the ideal cross-section misaligned with the focal plane of the microscope. The orientations used were random. Rotation about the X-axis was considered to be successful if the system properly oriented the cell based on the maximum- A -value criterion.

The sample size for the validation of cell rotation about the X-axis was 40 embryos. The system achieved a success rate of 92.5%. One of the failures occurred when the cytoplasm of the fish embryo was abnormally small, and the system was not able to identify a distinct maximum value of A . Instead of obtaining the maximum A value from the binary image of the profile of the cytoplasm and yolk as expected, the maximum A value was obtained from the profile of the yolk alone. The other two failures occurred when the embryo got detached from one or both micropipettes. The accidental release of the embryo may be due to improper alignment of the two micropipettes, or due to insufficient force applied on the cell for axial immobilization.

The predictability of the system is defined as the ability of the system to produce changes in the value of A in a deterministic manner. In this case, a plot of the values of A should form a sinusoidal graph. Figure 5.13 shows the plot of values of A over cell rotation of 360-degrees. The image frames

possessing peak values of A occur when the ideal cross-section and the focal plane of the microscope are aligned. Conversely, the minimum values of A were obtained when the yolk and cytoplasm overlapped each other in the line of sight of the microscope, i.e. the focal plane was orthogonal to the ideal viewing cross-section. 20 embryos were used to evaluate the system's predictability with the camera being set to a lower frame-capturing rate. Experimental results showed that all plots of 20 sets of A values are of a sinusoidal form; and all the peak A values corresponded to images where the internal cellular structures were in focus. To reduce the invasiveness of the system, the force exerted on the cell by the micropipettes was not strong. The loose contact points may cause the cell to slip off the axis of rotation, leading to out-of-plane rotation when the cell is rotated more than 360° . However, since the in-focus image can always be found within 180° of rotation, the slippery contact point problem does not affect the predictability of the system. The shortest and longest times required to properly orientate

Figure 5.13: Predictability evaluation: plot of A values with embryo images corresponding to the peak and valley values.

the cell were 7s and 53s. The average time required for a successful X-axis rotation is 21s. The process can theoretically take up to 88 s to complete, and this occurs when the ideal viewing cross-section happens to be oriented just shy of the initial orientation of the cell, and the rotation used to find it is initiated in the opposite direction. However, since we intentionally oriented the cell away from the ideal viewing cross-section, such a situation did not occur in the experiment.

5.3.3 Cell Rotation About The Z Axis

The success rate of the overall in-plane cell rotation system is defined as the ability of the system to release the cell completely upon completion of X-axis rotation and to orient the cytoplasm to the target position, defined as 3 o'clock position (i.e. θ equals to 0°). In addition to the 40 trials following the X-axis rotation, each of these 40 embryos was also used to perform an additional in-plane rotation test. Thus a total of 40 and 80 trials were performed to evaluate the cell release system and the in-plane rotational system, respectively. The rotational direction of the in-plane rotation stage is determined such that if the initial position of the cytoplasm of the cell is in the first or second quadrant, the cell is rotated clockwise; otherwise, it is rotated counterclockwise. Out of the 80 trials, the shortest and longest times required for successful cytoplasm orientation were 3s and 15s respectively. For the case when 15s was required, the cytoplasm's position was opposite of the desired position; the time the process took to complete was longer than what was predicted theoretically as the latter did not include the time for required acceleration and deceleration of the rotation stage. The average time required to rotate the cytoplasm to its 3 o'clock position from its initial position was 6s. The average angular error of the final positions in the 80 trials was 0.3° ,

with a maximum error of 0.5° . Therefore, the in-plane cell rotation function achieved a success rate of 100%. The system was able to completely release 39 out of 40 cells, achieving an overall in-plane cell rotation system success rate of 97.5%.

Based on the average time required for individual tasks, the total average time required for the three-dimensional system to achieve the cell orientation is 31 seconds, including an average 4-second period needed for cell releasing. The moment of inertia plays a very important role in rotation about both the X axis and Z axis. The rotational speed about the X-axis was set through trial and error to minimize the overshoot of the rolling motion due to the inertia of the cell. Since the medium, water, has low viscosity, proper tuning of the angular acceleration, speed and deceleration was also critical to avoid overshoot for the in-plane rotation due to the inertia of the water.

The safety of the system was evaluated through careful observation of the membranes of 40 embryos at a higher magnification factor. Although zebrafish embryos are relatively large, their membranes are fragile and can be damaged easily. Of the 40 embryos, none sustained observable damage to its membrane upon completing the 3-dimensional cell rotation process, confirming the non-invasiveness of the system.

5.4 Summary

In this chapter, a 3-point-contact automated three-dimensional cell rotational system for single cell manipulation was presented. A high resolution in-house rotational stage and cellular structure recognition algorithms were developed to facilitate the visual servo control. The system is capable of orienting a zebrafish embryo to the ideal viewing cross-section that is aligned with the

focal plane of the microscope. The cellular structure recognition algorithm achieved a 100% success rate with an image capturing rate of 30 fps. A 3-point-contact cell rotation model enabled X-axis rolling motion of the cell, achieving a success rate of 92.5%. The in-plane orientation of the cell had a success rate of 97.5% and an average accuracy of 0.3° . The average processing speed was 31 seconds/cell. The system allows highly precise, highly controllable, highly reproducible and predictable three-dimensional rotational control of single embryos/oocytes. Its application can be extended to micro manipulations of other types of biological cells, such as human or mouse embryos/oocytes. The system also provides a starting point for automating other cell-manipulation-related tasks, such as ICSI and embryo biopsy.

Chapter 6

Piezo-driven Zona Pellucida Dissection

To this day, all piezo-driven micropipettes used for zona piercing [147], zona drilling [148], partial zona dissection [85] and zona biopsy [149] are driven by a piezo actuator excited in the axial direction. As the micropipette has an extremely thin and long end section, it is possible that the success of piezo-assisted perforation is because of the lateral oscillations, which transferred to and amplified at the tip of the micropipette when the micropipette is excited in the axial direction.

This chapter is devoted to investigation of the application of lateral oscillation in cell membrane injection and dissection. The possibility of using only lateral oscillation for cell membrane dissection will be demonstrated through experiments. Zebrafish embryo membrane cutting with different frequencies and amplitudes has been performed with the proposed piezo-driven micro-cutter.

6.1 Zona Pellucida Cutting Methodologies

One of the major regulators of cell structure and function is force. Changes in force transmitted to cells may change their movement, enzyme secretion and their future development [150]. In order to protect the cell from degeneration and lysis, the force applied to the cell during cutting should be maintained as small as possible to prevent excessive mechanical stimulus on the cell membrane and interior. There are two vibrational cutting methodologies that can be used to cut the zona pellucida: The axial-vibrational and the lateral-vibrational cutting, shown in Figure 6.1.

Figure 6.1: (a) Axial Oscillation (b) Lateral Oscillation.

In order to design an effective zona pellucida cutter, the benefits of each method must be studied. Till now, there is no study of which oscillation contributes more to membrane penetration. Microdynamics of the piezo-driven pipette have been investigated by Ediz's group [151, 152], which claims that the natural frequency of the pipette can be reduced by filling mercury in the micropipette tip. Even the driving oscillation is in axial direction, lateral oscillation has much larger amplitude than axial oscillation does, and it plays a more important role in membrane piercing. In their experiment, axial

displacement has an amplitude of $\pm 80\text{nm}$, which is considered not enough to pierce through the $8\text{ }\mu\text{m}$ -thick zona. However, according to the theory of vibration-assisted machining (VAM), small amplitude and high frequency can reduce the piecing force and resistance [153], thus the contribution of axial oscillation cannot be denied. Also, the above-mentioned hypothesis is considered not comprehensive as analysis was only performed on the effect of liquids inside but not surrounding the pipette. The experiments on effect of surrounding liquids provided contradictory results [154]. Though the insertion of mercury does mitigate the resonance frequency of the pipette tip, the reduced frequency causes lower damping of the pipette vibration in surrounding medium. The combination of these two effects shows larger amplitude on pipette oscillation caused by piezo pulses. Mehdi et al. considered both internal and external fluidic effects on the micropipette and they concluded that it was the high shear force made the perforation happen [155]. So far, there is still no generally accepted conclusion on how the use of mercury improves the oscillation, so as to achieve better results. Gan et al. [156] have also modeled and simulated the mouse zona piercing process and held the same opinion as Ediz [152]; in this work, the lateral oscillation plays the most important role. In addition, ultrasonic piezo-driven microinjection shows that ultrasonic cutting force results in the piercing, according to ultrasonic vibration micro-dissection theory [157]. In Huang's experiment [157], the lateral oscillation is treated as a harmful factor that needs to be controlled to the minimum amplitude possible [94]. In order to confirm the effects of lateral oscillation and then to apply it further to facilitate embryo dissection, investigations in the lateral oscillation of the piezo-driven micropipette through experiments are reported in this chapter.

6.2 Experiment Setups

The experiment set-up is shown in Figure 6.2. On the top of the upright microscope (Nikon, Eclipse FN1, Japan), a 659x490-pixel CCD camera (Basler, scA640-74gc, Germany) is mounted for image capturing. Both the holding pipette and the micro cutting needle are fixed on a pair of 3-DOF micromanipulators (National Aperture Inc., MM-4M-F, USA). A microsyringe pump (Eppendorf, Celltram Vario 5176, Germany) is connected to the holding pipette to providing suction force for embryo immobilization. For lateral vibration driving, a piezoelectric shear actuator with free stroke of 10 μm in two axes (Physik Instrumente, CA-Shear P152.10, Germany) is used to provide a wide range of frequencies with high amplitudes. The shear actuator can produce high-frequency oscillations directly without preloading structures [158]. Therefore, the system can be made very compact. For axial vibration, a piezo actuator (Physik Instrumente, P885.51, Germany) with 15 μm free stroke and 70 kHz resonant frequency is installed. A PDX voltage amplifier (Newcastle Innovation Ltd, PDX150, Australia) is used to provide -30V to 150V voltage for the piezo actuators.

6.3 Beneficial Oscillation In Piezo-assisted Cell Injection

6.3.1 Experimental Methods

For the axial vibration experiment, a flat-tip microinjection pipette with inner diameter of 20 μm is inserted into the syringe needle with gauge size 17 and bonded using Loctite 431. The tip of the micropipette outside the needle tube is approximately 450 μm long. The modified syringe needle with

Figure 6.2: Experiment setups

micropipette inserted is then glued on the bottom of the piezo shear actuator, with the pipette axis aligned with the moving direction of the actuator (see Figure 6.3). The length-to-diameter ratio of the injection pipette tip is about 15:1 ($450\ \mu\text{m}$: $30\ \mu\text{m}$). Finite Element Analysis (FEA) has been conducted using ANSYS software package. The short tip of the micropipette has been modelled with one end fixed and the other end free. The modal analysis results have shown that the first two natural modes of the pipette have the first and second bending mode shape, with natural frequencies of 137.6 kHz and 839 kHz respectively (see Figure 6.4). The first longitudinal vibration mode is found at 1.08 MHz. Since the driving frequencies of the proposed experiments are below 40 kHz, which are far away from the first natural frequency, it is safe to assume that the short micropipette tip follows the

movement of the steel needle, which means there is no lateral vibration when the micropipette is driven along the axial direction. As the needle has been firmly attached to the actuator, we can assume that the short micropipette tip moved together with the actuator end surface as a rigid body during the experiment.

Figure 6.3: Piezo-injector for axial vibration.

For the lateral vibration investigation, the injection micropipette (about 63 mm long) was directly glued on the bottom of the piezo actuator, leaving a 51-mm-long tip as a free end. The driving direction of the actuator was perpendicular to the axis of the pipette, to excite transverse vibration modes of the pipette. A fiber-optic displacement sensor (Philtec, RC12-T, USA) was also set up to measure the displacement of the pipette, as shown in Figure 6.5. The interferometer with a resolution of $0.08 \mu\text{m}$ detected the displacement of the actuator and converted it to an analog voltage signal.

To investigate the impact of oscillation directions of the microinjection pipette in zona pellucida dissections, a series of experiments has been per-

Figure 6.4: First and second natural frequency of micropipette

formed using the aforementioned system. Zebrafish embryos have been selected as the injection targets. The diameter of a zebrafish embryo is about $1200\ \mu\text{m}$ with chorion and $600\ \mu\text{m}$ without chorion. The thickness of the zebrafish membrane is approximately $3\ \mu\text{m}$ [159]. The flat-tip injection pipette has an inner diameter of $20\ \mu\text{m}$, which is slightly larger than those used for human oocytes due to the similarly larger size of zebrafish embryo. Various frequencies, from 0 Hz up to 40,000 Hz, have been used to investigate the pipette vibration in the experiment.

6.3.2 Experiment Result and Discussion

Three batches of zebrafish embryos were injected for each type of vibrations during the experiment. Each batch was performed with different frequencies in a random sequence. Cell injections with an inactive piezo-actuator (0Hz) were performed frequently to ensure the embryo membranes maintain the same properties throughout each experiment.

Direct measurement of the force exerted at the tip on the cell membrane

Figure 6.5: Displacement measurement by using interferometer.

is extremely difficult,, especially in such a high-frequency vibration. Thus we perform an indirect measurement of the force to quantify the performance of the piezo-assisted injection, as the deformation ratio e . The deformation ratio e right before penetration is defined as ,

$$e = \Delta L / L \times 100\%, \quad (6.1)$$

where ΔL is change in diameter in the injection direction and L is the original diameter. The assumption is that all embryos are in perfect spherical shape.

Axial oscillation

The effect of axial vibration is investigated by oscillation frequency up to 40 kHz and amplitude up to 17.28 μm . Figure 6.6 shows the result of the experiment. Deformations caused by various frequencies and amplitudes are all within the range of 30% to 45%.

The membrane deformation ratios of embryos injected by active piezo actuator are compared to those injected by an idle piezo actuator (0 Hz; the

Figure 6.6: Deformation vs axial amplitude.

process could be considered a conventional embryo injection). There is no significant difference in cell deformation between high and low frequency or amplitude, as shown in Figure 6.6; values of deformation ratio e at different amplitudes and frequencies are all clustered around 0.35. It is generally known that axial vibration is often very small in amplitude due to the limitation of design of micro piezo-driving system, thus it is very difficult to measure directly on the pipette tip. Because of the special design of the experiment setup, the displacement can be measured indirectly on the actuator end by using laser displacement sensor. The maximum amplitude we obtained was $17.28 \mu\text{m}$ at a frequency of 4.15 kHz. We believed that such a high amplitude was caused by reaching the harmonic frequency of the whole injection structure of the system. However, even at this high frequency and amplitude, which is normally very difficult to generate by a small piezo actuator, there was no sign of early penetration of the embryo membrane. It is worth mentioning that the amplitude is much larger than $3 \mu\text{m}$, the thickness

of cell membrane. At a driving frequency of 40 kHz, the vibration amplitude reduced to 200 nm. The deformation ratio at penetration still showed no significant variation from the deformation ratio without vibration (33%). It can be concluded that with the provided amount of axial vibration, the influence on membrane penetration during injection is negligible. Although, axial with much larger amplitude and higher frequency might help the penetration, it becomes very difficult to realize experimentally.

Lateral Oscillation

Figure 6.7: Amplitude of a lateral vibrating micropipette tip in air.

When the pipette is driven in the lateral direction from the base, flexural vibration modes will be excited, resulting in much higher oscillation amplitudes at the tip (the free end) comparing to the input amplitude. When the driving frequency is close to one of the natural frequencies of the pipette, the resulting vibration will be further amplified. Due to the size and trans-

parency of the pipette tip, direct measurement of high-frequency displacement is challenging. In the experiment, the tip displacement was estimated by monitoring the envelope width of the moving tip under the microscope. Figure 6.7 shows the amplitudes of the pipette tip at a few typical frequencies with relatively high driving voltages. It can be seen that at 10 kHz the amplitude reached $212\mu\text{m}$, which was much greater than the largest axial value we obtained earlier. Note that the amplitudes shown in the figure includes the diameter of the pipette tip, which is about $30\mu\text{m}$. Hence, the actual oscillation amplitude should be $30\mu\text{m}$ less than what is shown. Considering the real situation during injection, part of the pipette tip is immersed in water, which introduces a damping effect and reduces the vibration amplitude. Therefore, the amplitudes of a lateral vibrating micropipette in water were also measured (see Figure 6.8). As expected, the amplitudes were reduced by about 20 percent. However, high amplitudes could still be obtained.

Figure 6.8: Amplitude of a lateral vibrating micropipette tip in water.

A series of experiments with different frequencies and amplitudes was performed. The deformation ratios are measured and plotted in Figure 6.9. It was found that in many cases the deformation ratios were reduced to be in the range of 2% to 8%. This proves that lateral vibrations are more effective than axial ones in the cell membrane piercing process. The amplitudes given in Figure 6.9 are the output vibration amplitudes of the piezo actuator, instead of the actual amplitude of the pipette tip, as it is difficult to measure using the aforementioned method when the amplitude is small. Thus we cannot conclude that with the same amplitude, higher frequency makes cell piercing easier. However, it is obvious from the 20 kHz fitting line in Figure 6.9 that with a certain frequency, higher amplitude makes less deformation on the cell wall.

Figure 6.9: Deformation vs lateral amplitude.

Figure 6.10 shows two penetration examples, with and without lateral ultrasonic vibrations. It is obvious (in Figure 6.10(b)) that the pipette with lateral vibrations penetrates the cell membrane much easier than the non-

Figure 6.10: Cell deformation during injection, (a) with inactive piezo-actuator (b) with 20 kHz and about 30 μm peak-to-peak amplitude lateral oscillation. Frame 2 and 3 of (a) and (b) show significant deformation differences.

vibrating or the axial vibrating micropipette does.

Comparing to axial vibration, cell deformation was reduced by applying the lateral vibration. As the lateral vibration contributes more to the piercing, we believe that it is the cutting action caused by this vibration that helps penetrate the embryo membrane.

Figure 6.11 illustrates an extreme case in which the micropipette cut the cell membrane open when the pipette tip vibrated at 10 kHz with a very large amplitude of about 400 μm . in Image A, at the moment the pipette was in contact with the cell membrane, there was no deformation on the cell. As the pipette being pushed forward, we can see crimples at point 1. The crimples became much clearer at point 2, which means cytoplasm inside the cell leaks out causing imbalanced pressure inside and outside the cell. Thus we can be sure that the cell has been damaged somewhere on the cell mem-

Figure 6.11: Membrane cutting-up by high-amplitude vibration at 10kHz, input voltage 118 V.

brane. Image D further confirms the damage as the micropipette touches the core of the cell at point 3, which also verifies the cutting position is at the pipette-membrane contacting point. One can learn from this phenomenon that excessive vibration amplitude is unwanted for embryo injection as it may cut a large opening on the cell membrane and damage the cell. However, the phenomenon shows the potential of applying the lateral vibrational micropipette on cell membrane dissection.

6.4 Piezo-driven Zona Pellucida Dissection

There are two major environmental changes that may significantly affect a cell's future development: the force and the heat. Both of the factors and the potential of applying the lateral-vibrational micropipette in zona pellucida dissection are analyzed in this section.

6.4.1 Thermal Effects On Axial And Lateral Vibrational Cutting

Environmental changes may significantly affect the future development of the cell, thus heat generated due to friction and expansion and contraction of the cellular structure must be considered before selecting a suitable cutting method. Viscoelasticity is considered a living cell property when modeling a sinusoidal force applied to a cell [95, 160, 161]. Since a cell exhibits a viscoelastic behavior, the membrane and the cytoskeleton can be represented by either a Maxwell model or a Voigt model. In a fluid-like viscoelastic Maxwell model, a spring and a dashpot in series are used to model the cell, while in a solid-like Voigt model, a spring and a dashpot in parallel are used [162]. The viscoelasticity is approximated using a solid-like Voigt model, as shown in Figure 6.12, in this case. The basic Voigt model equation is:

$$\sigma = E_y \gamma + \eta \frac{d\gamma}{dt} \quad (6.2)$$

where E_y , η , σ and γ are the Young's modulus, viscosity coefficient, stress and strain, respectively. As the force applied on the embryo is a harmonic oscillation, the stress can be expressed as follows:

$$\sigma = \sigma_0 \sin(\omega t) \quad (6.3)$$

Figure 6.12: Solid-like Voigt model.

By substituting (6.3) into (6.2), we have

$$\gamma = \frac{\sigma_0}{E_y \sqrt{1 + (\omega \frac{\eta}{E_y})^2}} \sin(\omega t - \theta_{phase}) \quad (6.4)$$

where $\theta_{phase} = \tan^{-1}(\omega \frac{\eta}{E_y}) = \tan^{-1}(\omega \tau)$.

By taking the time derivative of (6.4), we have

$$\frac{d\gamma}{dt} = \omega \gamma_0 \cos(\omega t - \theta_{phase}). \quad (6.5)$$

It can be seen that there is a phase delay θ_{phase} between the strain γ and stress σ . Therefore, the strain and stress actually have a hysteretic relationship as shown in Figure 6.13. The energy lost in the form of heat can be represented by the area bounded by the stress-strain loop. The area can be calculated as follows:

$$\begin{aligned} W_{heat} &= \oint \sigma d\gamma \\ &= \int_0^{\frac{2\pi}{\omega}} \omega \sigma_0 \gamma_0 \sin(\omega t) \cos(\omega t - \theta_{phase}) dt \\ &= \frac{1}{2} \omega \sigma_0 \gamma_0 \int_0^{\frac{2\pi}{\omega}} [\sin(2\omega t - \theta_{phase}) + \sin \theta_{phase}] dt \\ &= \pi \sigma_0 \gamma_0 \sin \theta_{phase} \end{aligned} \quad (6.6)$$

Figure 6.13: Loop of lost energy.

Equation (6.6) provides a quantitative result for heat generated during the vibrational cutting. Thus one can find out that the amount of heat generated highly depends on the vibration mode. In axial vibration, viscoelasticity is determined by Young's modulus E_y and the viscosity coefficient η of the embryo. In lateral vibration, the shear modulus G and the shear viscosity coefficient λ define the viscoelasticity. Young's modulus E_y is three times the shear modulus G ; and the same holds for viscosity coefficient ($\eta = 3\lambda$), as shown in Figure 6.14. Thus with same frequency and same strain applied, the axial cutting generates roughly three times more heat than the lateral cutting does. Therefore, in order to maintain minimum change in the surrounding environment in terms of force and temperature, the lateral vibration should be selected to perform the zona cutting. In order to prove the efficiency of the lateral cutting method, both axial and lateral cutting methods are tested in the experiment.

Figure 6.14: Loop of lost energy comparison.

6.4.2 Investigation of Cutting Effect

Experimental Methods

For the lateral vibration investigation, a 25.4 mm tungsten microneedle (TED Pella, 13570-10, USA) was fixed on an in-house-made holder, which is directly glued to the shear actuator, as shown in Figure 6.15. A portion of 3 mm of the microneedle is inserted into the holder, fastened by two set screws and reinforced by super glue. The driving direction of the actuator was perpendicular to the axis of the microneedle to excite lateral vibration modes of the microneedle. The tip displacement was estimated by recognizing the width of the envelope in microscopy images of the vibrating tip. The average intensity of ten images was used for the measurement in case of missing capture of a particular movement. The number of pixels between the lowermost and uppermost pixel provides the displacement of the tip, given that each pixel is

Figure 6.15: Design of the lateral-driven micro-cutter.

equivalent to $0.7035 \mu\text{m}$ in real-world dimension. The measurements of several typical frequencies with different amplitudes are illustrated Figure 6.16. As the tip of the microneedle is $0.6 \mu\text{m}$ thick, the amplitude of displacement should be the overall displacement value minus the thickness of the tip. Five frequencies, 5.3 kHz, 13.6 kHz, 22.6 kHz 25.4 kHz and 33.5 kHz, with various tip amplitudes have been tested in lateral-vibration-driven membrane dissection. These frequencies are chosen because of their capabilities of making the microneedle achieve considerably high tip displacement. Three cells are tested for each unique combination of frequency and amplitude.

For the axial vibration experiment, the tungsten needle is inserted into a modified 21-gauge syringe filled with superglue to mitigate the lateral vibration. The syringe is then fixed into the flexure, fastened by set screws and superglue, as shown in Figure 6.17.

The axial tip displacement was not measured due to technical difficulty,

Figure 6.16: Vibration of a lateral vibrating microneedle immersed in water.

Figure 6.17: Design of the axial-driven micro-cutter.

but the lateral tip displacement was calculated by using the aforementioned method. As the effect of axial oscillation was proven to be insignificant, thus only a few frequencies with relatively large lateral amplitudes have been tested to compare the dissection effect with the pure lateral oscillation.

Experiment Procedure

The procedure of the experiment is described as follows:

1. Align the microneedle tip with the vertical tangent point of cell membrane using focusing method.
2. The piezo-driven microneedle with a relatively high vibrational amplitude is pushed against the cell membrane at a speed of $6 \mu\text{m/s}$ till it penetrates the membrane. This is used to test the direct cutting effect of piezo-driven microneedle.
3. Move the vibrational microneedle or micropipette upward or downward at a speed of $6 \mu\text{m/s}$ to test the splitting effect after penetration.

6.4.3 Result And Discussion

Four batches of fertilized zebrafish embryos, more than 260 embryos in total, were used for the experiment. Only those providing clear penetration images and staying in focus throughout the membrane dissection process were considered as valid samples. The sample size for lateral-vibrational cutting used in this experiment is 192 embryos. Each unique combination of waveform, frequency and amplitude was performed on three samples. Combinations of frequency and amplitude were chosen randomly for each batch. To make sure the properties of the embryo membrane did not change significantly, cell membrane penetrations with non-vibrating microneedle were performed frequently, and all experiments were carried out within eight hours of the embryo harvest.

Equation (6.1) was used to quantify the deformation under direct lateral-vibration zona cutting. The ratio of split expansion to microneedle displacement was used to quantify the splitting effect of the piezo-cutter, and it is defined as follows:

$$c = \Delta s / D_d \times 100\%, \quad (6.7)$$

where $\Delta s (= s_2 - s_1)$ is the change of width of the split after lateral-directional movement of the microneedle and D_d is the lateral displacement of the piezo-driven microneedle, as illustrated in Figure 6.18. The ratio c is a value between 0 and 1, where value 1 indicates that the microneedle is able to split every part of the membrane along its moving path; in contrast, value 0 means the microneedle has no splitting capability at all.

Figure 6.18: Ratio of split expansion to microneedle displacement.

The Direct Cutting Effect of The Piezo-driven Microneedle

As mentioned in the previous section, it was the shear cutting force that helped the piezo-driven micropipette penetrate the cell membrane. In this experiment, to further investigate the effect of the cutting force, a microneedle with a $0.6\text{-}\mu\text{m}$ -thick tip was used as a cutter to perform cell membrane cutting. The effect of cutting or gashing motion in lateral direction was investigated by exciting the microneedle with frequencies up to 33.5 kHz, and amplitudes up to $40\text{ }\mu\text{m}$. Both sinusoidal and square waveforms were tested in this experiment. Most deformation ratios e obtained from various combinations of frequency and amplitude fell in the range of 0.43% to 0.48%. Since the deformation on the cell membrane was too small under 200-times magnification, tried our best to count the number of pixels covered by the

concavity in the axial direction, only 7 pixels to 10 pixels could be measured. We rounded up the number to avoid ambiguity. Since one pixel is merely $0.7035\ \mu\text{m}$ in the real world, the error of deformation ratio obtained can be considered to be less than 0.05%. In comparison, the average deformation ratio e of 12 non-vibrational injections was 3.8%, which is about 9 times bigger than the deformation ratio obtained from lateral vibration-assisted cutting. By visual inspection, the opening created by the vibrational cutter was very close to the amplitude of the vibration, which proved that the direct lateral vibrational cutting can achieve a desired zona opening. Although the square waveform should generate twice the energy that sinusoidal waveform could provide, the deformation ratio did not show any significant difference. We believe that it was because the deformation was already too small and the membrane of the fish embryo was too thin for us to identify the difference.

The cutting effect of the square-waveform driven axial-vibrational micro-cutter was also investigated. Since it had been concluded that axial vibration did not play the critical role in the membrane penetration, thus the effect of solely axial-vibrational cutting was not tested. However, the effect of combination of the lateral and axial vibration was tested (the lateral oscillation always comes with the axial oscillation due to the eccentricity of the needle). Since the harmonic frequencies had changed when driving the microcutter in the axial direction, it was very difficult to obtain the same lateral amplitude with same frequency as driven in lateral direction. Only in few frequency bands, amplitudes can reach the expected value. No significant differences between the effect of axially driven cutting was found in selected frequency bands from 5.3 kHz to 22.6 kHz, which could induce lateral amplitudes that were large enough. Figure 6.19 (a),(b) and (c) illustrate the membrane deformations when the microcutter was driven by square waveform in the axial

direction, sinusoidal waveform in the lateral direction and square waveform in the lateral direction respectively, with a frequency of 5.3kHz and a lateral tip amplitude of 10 μm . One can notice that there was no significant difference among the resulting deformations, but all of them were much smaller compared to the deformation caused by injection without vibration. This further confirms that in spite of the direction of the driving force, as long as the lateral vibration is introduced, the deformation caused by the membrane dissection is reduced significantly.

Figure 6.19: (a) Injection with axial square-waveform excitation. (b) Injection with lateral sinusoidal-waveform excitation. (c) Injection with lateral square-waveform excitation with a frequency of 5.3 kHz and a lateral-tip amplitude of 10 μm . (d) Injection without vibration.

The Splitting Effect of The Piezo-driven Microneedle

In the biopsy process of PGD, an opening up to $40\ \mu\text{m}$ long and $2\ \mu\text{m}$ wide is introduced to the zona pellucida [82]. Though a $40\ \mu\text{m}$ opening can be achieved precisely by using the aforementioned lateral cutting method, it is believed that with the same frequency, lower amplitude causes less heat energy, thus minimizing the risk of embryo defects and lysis. There are two methods of achieving a $40\ \mu\text{m}$ opening using low-amplitude vibration (less than $40\ \mu\text{m}$). One method is multiple attempts of the aforementioned cell perforation. However, the small opening is very difficult to be identified once the micro injector is pulled out from the embryo. In addition, to prevent the injector from slipping, the best position to perform cell perforation should be the center of the zona convex. Therefore this method makes it difficult to achieve precise cutting. The other method is to cut the zona open by dragging the injector up or down after insertion into the cell; thus it requires the vibrational microneedle to be able to split up the zona pellucida once it penetrates the zona. In this experiment, the splitting capability of the vibrational

Figure 6.20: Ratio c in several cell cutting conditions.

microneedle has been quantified using a split-expansion-to-tip-displacement ratio c . Since lateral vibration is proven to be the beneficial vibration for

cell membrane penetration, the experiments mainly focused on the splitting effect of lateral vibration. Figure 6.20 illustrates key variables ($s1$, $s2$, D) of ratio c in some typical situations. A square waveform was used to drive the microneedle. All values of ratio c obtained from 96 embryo samples were close to zero. The expansions of the opening during the splitting process were too small to be differentiated (one or two pixel differences with possible measurement error from the image). Therefore, one can conclude that the lateral vibration does not split the cell membrane along its movement once it has penetrated the cell. Several experiments with axial square-wave driven microneedle were also conducted to test the splitting effect. No significant difference in effectiveness was observed. It may be due to the small displacement amplitude in the axial direction that may not cause enough destructive effect to the structure of the cell membrane. However, big amplitude in axial direction is not recommended as it was found that when driven in axial direction, the whirling effect was much larger than when driven in lateral direction with the same frequency. For example, as shown in Figure 6.21, when the microneedle is dipped, driven by 33 kHz and 15 μm excitement in the axial direction, into the medium, the cell which was not attached to the holding pipette experienced suction force from the tip of the microneedle. Eventually the cell was penetrated by the cutter as it moved towards the needle at a high speed. The destructive effect was also observed once the needle broke into the embryo, where the yolk of the embryo was blended completely.

The whirling effect is also clearly illustrated in Figure 6.22. The microneedle was driven in the axial direction with 30 kHz frequency but with a much smaller amplitude. When the piezo-driven micro-needle was turned on, ink flowed towards the tip of the micro-needle and then dispersed along the axis of the needle. The ink can be previewed as the internal fluid of an

Figure 6.21: Suction and destructive effect: The cell is at rest at 0 s. When the actuator is activated, the cell starts to move towards the microinjector with an accelerating speed, due to dragging force caused by the whirling effect. The cell is in contact with the microinjector at 0.32 s, when the zona was pierced. From 0.46 s onwards, the destruction of the cell occurs.

embryo. At a high frequency, if the amplitude is big enough, the internal fluid will be drained off very quickly. Therefore, the axial vibration of the microcutter should be prevented to minimize the damage to the cell.

6.4.4 Application of Lateral Oscillated Cutter

Three-dimensional piezo-assisted zona dissection(PZD) was introduced as a new technique by creating a V shape opening or a cross shape slit on the zona pellucida for embryo biopsy [163] or assisted hatching [163, 164].

A three-dimensional PZD produces a comparably larger opening by creating a second slit perpendicular to the first slit. The variance of the cross opening is a V shape opening, which allows insertion of a 25 to 30 μm micropipette to perform blastomere extraction. The original three-dimensional PZD was performed using the mentioned passive mechanical method [(see Figure 2.3(c)]. The embryo is stabilized by the holding pipette. The first

Figure 6.22: Investigation of whirling effect using ink: The ink starts to move towards the tip of the injector right after the vibration starts. When the ink reaches the injector tip, it starts to disperse along the microinjector at 10 s.

guiding PZD was created by piercing the largest perivitelline space at 12 o'clock position. The embryo is then moved and rotated until one end of the guiding opening is in the focal plane of the microscope. Once the end of the opening is in focus and at 12 o'clock position, the second opening is made by introducing the fine pipette into the first slit and moving through the perivitelline space, then out from the 10 o'clock position. The V-shaped cut creates a triangular flap, which makes the insertion of suction pipette much easier. However, the process of the making a cross or a V-shaped cut is very complex, time consuming and has a stringent skill requirement.

The three-dimensional PZD can be achieved by applying the proposed lateral-vibrational cutter. The cutter is capable of performing the task as the maximum length of the slit required is about 25 to 30 μm , which is within the displacement range of the oscillating needle tip. The piezo-driven cutter is able to cut four types of zona opening as shown in Figure 6.23. The unidirectional cut, illustrated in Figure 6.23(a), is performed by directly

Figure 6.23: Four types of cuts generated by lateral-oscillated micro-cutter: (a) Unidirectional cut. (b) T-shaped cut. (c) X-shaped cut. (d) V-shaped cut.

pushing the vibrating micro-cutter against the cell membrane. The X-shaped opening, as shown in Figure 6.23(b), was made by performing another cut perpendicular to the first slit by exciting the second shear actuator on the piezo stack. The T-shaped cut is very similar to the X-shaped cut but was done by aligning the end of the second slit with the midpoint of the first slit. The V-shaped cut shown in Figure 6.23(d) was achieved by starting two slits at the same position. Since zebrafish embryos were used in the experiment, one may notice that the triangular flap was curled up instead of covering the zona opening. That is because the opening is much smaller compared to the size of the embryo and the cell membrane is too thin; the tension from the cell membrane pulls the slit open, and meanwhile the heat generated by the vibration may also affect the property of the flap.

Comparing with the manual zona dissection technique, the proposed technique has several distinctive advantages. It does not require rotation or positioning of the cell, as the dissection can be completed by only controlling the position of the micro-cutter. The proposed technique is much more pre-

cise and accurate, as the amplitude and the position of the cutter are closely monitored by computer vision. Unlike the manual dissection technique, the proposed one does not have stringent requirements on skill and experience, as it is more ready to be automated. In addition, the proposed technique can be completed within a much shorter period of time comparing to the manual zona dissection technique.

6.5 Summary

In this chapter, both axial and lateral oscillations of the piezo-driven micropipette in cell membrane injection and dissection have been investigated through experiments. Experimental results demonstrated that the lateral oscillation contributed more to the membrane perforation and dissection than the axial did. The lateral oscillation of the piezo-driven micropipette is considered beneficial as it generates less heat and allows direct and precise control. On the other hand, axial oscillation can cause harmful whirling effects to the cell internal fluid leading to cell lysis during the dissection process. Unlike the longitudinal-driven lateral oscillation, the transverse-driven, or direct-control, lateral vibration can reach a much higher lateral displacement with lower axial displacements and less collateral oscillations due to the eccentricity of the micropipette. In addition, the experimental results indicate that high amplitude with low frequency is preferable for both injection and dissection. Therefore, a novel piezo-driven lateral-oscillating micro cutter with a new dissection technique has been proposed to perform cell membrane dissection. The proposed micro-cutter is capable of performing both single-direction dissection and three-dimensional dissection. Since the new dissection technique does not require reorientation of the cell, the dissection

process is simplified in terms of both position control and image processing, which push the dissection process one step forward towards automation.

Chapter 7

Conclusion

7.1 Summary

This thesis presents a computer-vision-guided micromanipulation system for embryo biopsy in the preimplantation genetic diagnosis (PGD). The comprehensive literature review in this dissertation elaborated the drawbacks and shortcomings of current techniques that are used in embryo biopsy. This research mainly aims at four tasks in the automated embryo biopsy procedure, including: the microscopic autofocusing technique, the image segmentation for selection of the suitable position for embryo biopsy, the embryo orientation system and the active embryo dissection technique.

An autofocusing window selection algorithm and a robust autofocusing search algorithm have been introduced. The proposed autofocusing window, with its size-adjustable feature, is capable of selecting the more informative portion of the image for the three-dimensional position estimation of the micropipette and the embryo. The autofocusing window is able to provide more accurate focus measurement data by eliminating insignificant image information or noises. In addition, it can estimate the in-plane coordinate

of the object of interest. Together with the proposed autofocus search algorithm, the positions of the micropipette, embryo and blastomere in the Z axis can be obtained. The searching algorithm is based on an improved shuffled frog leaping algorithm (ISFL), which performs well in non-unimodal optimum value searching. Experiments using zebrafish embryo were carried out to validate the autofocus algorithm. Experimental results have shown exemplary performance in Z-axis position estimation and in multimodal focus measurement function graphs. Comparing with the existing central-region based focus window, which provided a focus window that was off the real focal plane by more than 5% of the diameter of the embryo, the proposed method improved the accuracy and precision by choosing a focus plane with a maximum error that was less than 1% of the embryo's diameter.

A novel image-segmentation method for recognition of the zona pellucida and the perivitelline space of a Day-3 embryo was introduced. The proposed method is capable of selecting a suitable position on the zona pellucida for embryo biopsy. The image segmentation can be achieved by two techniques: the perivitelline space recognition with partial image stretching and the perivitelline space recognition with value adjustment algorithm. The image-stretching technique has the advantage of dealing with low contrast image segmentation, while the value-adjustment technique outperforms the image-stretching technique in embryo images with more complex internal structure. The experimental results showed that the zona boundary estimation algorithm achieved a 96% success rate, and both the perivitelline techniques achieved a 94% success rate. Although the system's success rate has not achieved 100%, by applying this technique, the outcome of each embryo biopsy will no longer depend on the skill and experience of an individual embryologist. Therefore, the consistency and repeatability of the embryo

biopsy process have been improved. In addition to the application in embryo biopsy, the technique has potential to achieve automatic zona pellucida thickness variation measurement, which is a key morphological determinant of a high-quality embryo.

A three-dimensional cell orientation system for positioning and orienting single embryos was developed. The orientation system is capable of orienting the zebrafish embryo to a certain angle at which the target cellular structure is in the focal plane. In order to achieve rotation of an embryo, a high-resolution rotational stage designed and developed in house was employed. In addition, cellular structure recognition algorithms were also developed to facilitate the visual servoing of the embryo rotation system. Highly controllable, highly precise and highly reproducible and predictable embryo positioning and orienting can be achieved by applying the proposed system. Experimental results demonstrated that the system was able to achieve an average in-plane accuracy of 0.3° and an average processing speed of 31 s/cell, which is 22.5% faster than manual operation (average 40 s/cell). Both the rotational stage and the cellular structure recognition algorithm can be applied to manipulation of single oocytes and embryos to build a foundation for automated intracytoplasmic sperm injection (ICSI) and embryo biopsy in preimplantation genetic diagnosis (PGD).

A piezo-driven lateral-oscillation micro-cutter is proposed to perform both single-direction and three-dimensional cell dissection. The design was based on investigations in the axial and lateral oscillations of the piezo-driven micropipette in cell membrane injection and dissection. Having shown that the lateral oscillation is more beneficial and contributes more to the membrane perforation and dissection than the axial oscillation does through experiments, a two-axis shear-force piezo stack was employed for the transverse-

driven cutter. Applying the proposed micro-cutter achieves a more precise, less skill-intensive and time-efficient dissection process than applying the classic manual technique. In addition, adopting the proposed piezo-driven cutter can save more than 90% the cost in setup, comparing to the Laser drilling technique(costs more than 80k USD), yet can perform an accurate 3D dissection, which is a safer dissection technique that is difficult to be achieved by using Laser drilling technique.

7.2 Contributions

This research significantly contributes to the embryo biopsy system for PGD by proposing various essential techniques that are required for an automated embryo biopsy. Towards the ultimate objectives of improving the success rate of PGD by improving the consistency, accuracy, controllability and reproducibility of an embryo biopsy system and reducing the operation time, the key contributions accomplished in the thesis are as follows:

- Introduced a focus-window selection algorithm for autofocusing on an embryo and blastomeres under microscope.
- Proposed a maximum searching algorithm based on an improved shuffled frog leaping algorithm to achieve accurate global optimum searching in multimodal focus measurement function graphs.
- Developed an image segmentation algorithm for zona pellucida recognition.
- Proposed an image processing method for recognition of the perivitelline space of an embryo for biopsy position selection.

- Designed and developed a high-resolution (0.02°) and a high-speed ($1440^\circ/sec$) rotational stage for a microscope.
- Introduced a visual-servo three-dimensional embryo orientation system that can complete an embryo orientation in an average 31-second period.
- Investigated the beneficial vibration of a micropipette in the perforation and dissection of the zona pellucida and designed the lateral-vibrational piezo-driven zona pellucida dissection system.

7.3 Future Work

As mentioned in Section 1.2, this research only focuses on the four steps of the embryo biopsy. The automation of the last step, blastomere extraction, is yet to be explored. In addition, upon embryo biopsy, automation of blastomere sorting and collection and embryo culturing are also interesting research topics. Since many techniques used in automated embryo biopsy can be applied to other cell manipulation operations, such as intracytoplasmic sperm injection, the proposed techniques can be modified and improved in the future to serve other purposes. Several areas are of interest for further studies are lists as follows:

- Test the automated embryo biopsy system using mouse embryos.
- Blastomere extraction
 - Following the V shape cutting, blastomere removal using extrusion method can be performed. In manual manipulation, a blastomere can be seen protruding from the opening spontaneously [165, 166];

this is because the internal structures are bounded by the zona pellucida prior to zona cutting. After gentle pressure exerted on the zona pellucida, this protruding blastomere is the first one to be squeezed out through the opening. As more pressure is applied, one more cells can be removed from the embryo.

- An automated blastomere removal technique mimics the manual procedure and improves on its efficiency, precision and accuracy. The first step of automated extrusion blastomere removal is embryo orientation. After the opening is cut as described, the opening is rotated to 12 o'clock position. The second step is the exertion of pressure on the ZP with the blastomere being monitored. The pressure applied to the embryo is estimated by the cell deformation. The blastomere that is squeezed out from the embryo can be identified by recognizing the position of the blastomere with respect to the zona pellucida. The final step is pressure relief and release of the embryo from the holding pipette. The process of the blastomere removal is described briefly in Figure 7.1.

- Evaluation of the embryo biopsy system based on the embryo survival rate and birth rate. As the ultimate target of the system is to increase the birth rate after PGD, it is necessary to obtain the data of birth rate to make the study comprehensive.
- Design and develop a piezo-driven injection/dissection device that can be used for different applications. Besides the tungsten micro-needle, the piezo-driven device should also be able to drive a micropipette that is connected to the microinjector. Therefore, the device can also be applied to sperm immobilization and sperm injection in ICSI.

Figure 7.1: Process flow of blastomere extrusion: (a) ZP dissection and retraction of the holder and the cutter. (b) In-plane embryo orientation. (c) Embryo immobilization. (d) Exerting pressure on the embryo. (e) Extruded blastomere detection.

- A dynamic model of the lateral vibrational injector/cutter can be developed to minimize the vibration, thus to minimize the damage to the cell. Force and heat transferred to the embryo have always been the problems that affect dissection results and the cell's viability. Further studies on the dynamics of the piezo-driven cutter can help researchers understand the dissection process better, thus to achieve desired ZP dissection or injection results with minimum energy being transferred to the embryo.

Appendix A

Embryo Image Processing Results

All image are presented in following format:

External ZP Boundary	Internal ZP Boundary	Suggested Dissection Position by Partial Image Stretching	Suggested Dissection Position by Value Adjustment
-------------------------	-------------------------	---	---

Figure A.1

Figure A.2

Figure A.3

Figure A.4

Figure A.5

Figure A.6

Figure A.7

Figure A.8

Figure A.9

Figure A.10

Figure A.11

Figure A.12

Figure A.13

Figure A.14

Figure A.15

Figure A.16

Figure A.17

Figure A.18

Figure A.19

Figure A.20

Figure A.21

Figure A.22

Figure A.23

Figure A.24

Figure A.25

Figure A.26

Figure A.27

Figure A.28

Figure A.29

Figure A.30

Figure A.31

Figure A.32

Figure A.33

Figure A.34

Figure A.35

Figure A.36

Figure A.37

Figure A.38

Figure A.39

Figure A.40

Figure A.41

Figure A.42

Figure A.43

Figure A.44

Figure A.45

Bibliography

- [1] A. H. Handyside, E. H. Kontogianni, K. Hardy, and R. M. Winston, “Pregnancies from biopsied human preimplantation embryos sexed by y-specific DNA amplification,” *Nature*, vol. 344, pp. 768–770, 1990. 10.1038/344768a0.
- [2] K. Sermon, “Current concepts in preimplantation genetic diagnosis (pgd): a molecular biologist’s view,” *Hum. Reprod. Update*, vol. 8, pp. 11–20, 2002. 10.1093/humupd/8.1.11.
- [3] L. Wilton, R. Williamson, J. McBain, D. Edgar, and L. Voullaire, “Preimplantation of aneuploidy using comparative genomic hybridisation,” *Reprod. BioMed. Online*, vol. 4, p. 13, 2002.
- [4] Y. Verlinsky, S. Rechitsky, S. Evsikov, M. White, J. Cieslak, A. Lifchez, J. Valle, J. Moise, and C. Strom, “Preconception and preimplantation diagnosis for cystic fibrosis,” *Prenatal Diagnosis*, vol. 12(2), pp. 103–110., 1992.
- [5] S. Rechitsky, “Accuracy of preimplantation diagnosis of single-gene disorders by polar body analysis of oocytes,” *J. Assist. Reprod. Genet.*, vol. 16, pp. 192–198, 1999. 10.1023/A:1020312823155.

BIBLIOGRAPHY

- [6] A. Veiga, “Laser blastocyst biopsy for preimplantation diagnosis in the human,” *Zygote*, vol. 5, pp. 351–354, 1997.
- [7] A. De Vos and A. Van Steirteghem, “Aspects of biopsy procedures prior to preimplantation genetic diagnosis,” *Prenatal Diagnosis*, vol. 21, no. 9, pp. 767–780, 2001.
- [8] I. M. Bait Bahadur, *An Automated Microsurgery System for Embryo Biopsy*. PhD thesis, 2013.
- [9] L. Zhe, Z. Xuping, C. Leung, N. Esfandiari, R. F. Casper, and S. Yu, “Robotic icsi (intracytoplasmic sperm injection),” *Biomedical Engineering, IEEE Transactions on*, vol. 58, no. 7, pp. 2102–2108, 2011.
- [10] K. K. Zhang, Yi. Tan and S. Huang, “Vision-servo system for automated cell injection,” *Industrial Electronics, IEEE Transactions on*, vol. 56, no. 1, pp. 231–238, 2009.
- [11] L. Zhe, Z. Xuping, C. Leung, N. Esfandiari, R. F. Casper, and S. Yu, “Automated cell manipulation: Robotic icsi,” in *Robotics and Automation (ICRA), 2011 IEEE International Conference on*, pp. 2540–2545, 2011.
- [12] L. Shih, “Autofocus survey: a comparison of algorithms,” vol. 6502, pp. 65020B–65020B–11, 2007.
- [13] S. Yazdanfar, K. B. Kenny, K. Tasimi, A. D. Corwin, E. L. Dixon, and R. J. Filkins, “Simple and robust image-based autofocusing for digital microscopy,” *Optics Express*, vol. 16, no. 12, pp. 8670–8677, 2008.

BIBLIOGRAPHY

- [14] Y. Liron, Y. Paran, N. G. Zatorsky, B. Geiger, and Z. Kam, “Laser autofocus system for high-resolution cell biological imaging,” *Journal of Microscopy*, vol. 221, no. 2, pp. 145–151, 2006.
- [15] N. El-Zehiry, M. Yan, S. Good, T. Fang, S. Zhou, and L. Grady, “Learning the manifold of quality ultrasound acquisition,” *Med Image Comput Assist Interv.*, vol. 16, pp. 122–130, 2013.
- [16] L. Firestone, K. Cook, K. Culp, N. Talsania, and K. J. Preston, “Comparison of autofocus methods for automated microscopy,” *Cytometry*, vol. 12, no. 3, pp. 195–206, 1991.
- [17] Y. Sun, S. Duthaler, and B. J. Nelson, “Autofocusing in computer microscopy: Selecting the optimal focus algorithm,” *Microscopy Research and Technique*, vol. 65, no. 3, pp. 139–149, 2004.
- [18] V. Della Mea, F. Viel, and C. A. Beltrami, “A pixel-based autofocus technique for digital histologic and cytologic slides,” *Computerized Medical Imaging and Graphics*, vol. 29, no. 5, pp. 333–341, 2005.
- [19] Y. Peng and J. Wei, “Autofocusing region selection for computer vision,” in *Signal Processing, 2008. ICSP 2008. 9th International Conference on*, pp. 1364–1367, 2008.
- [20] Y. Sun, S. Duthaler, and B. J. Nelson, “Autofocusing algorithm selection in computer microscopy,” in *Intelligent Robots and Systems, 2005. (IROS 2005). 2005 IEEE/RSJ International Conference on*, pp. 70–76, 2005.
- [21] M. Yu, M. Han, Y. Shee, and W. Ang, “Autofocusing algorithm comparison in bright field microscopy for automatic vision aided cell

BIBLIOGRAPHY

- micromanipulation,” in *Nano/Molecular Medicine and Engineering (NANOMED), 2010 IEEE 4th International Conference on*, pp. 88–92, 2010.
- [22] A. Santos, O. De., C. Solorzano, J. J. Vaquero, N. Malpica, and F. Del Pozo, “Evaluation of autofocus functions in molecular cytogenetic analysis,” *Journal of Microscopy*, vol. 188, no. 3, pp. 264–272, 1997.
- [23] J. Brenner, B. Dew, J. Horton, T. King, P. Neurath, and W. Selles, “An automated microscope for cytologic research a preliminary evaluation,” *J Histochem Cytochem*, vol. 24, pp. 100–111, 1976.
- [24] I. Tsamardinos, L. Brown, and C. Aliferis, “The max-min hill-climbing bayesian network structure learning algorithm,” *Machine Learning*, vol. 65, no. 1, pp. 31–78, 2006.
- [25] J. Kiefer and J. Wolfowitz, “Stochastic estimation of the maximum of a regression function,” *Ann. Math. Statistics*, vol. 23, pp. 462–466, 1952.
- [26] J. Kennedy and R. Eberhart, “Particle swarm optimization,” in *Neural Networks, 1995. Proceedings., IEEE International Conference on*, vol. 4, pp. 1942–1948 vol.4, Nov 1995.
- [27] R. Gonzales, R. Woods, *Digital Image Processing. Second edn.* USA: Prentice Hall, 2004.
- [28] A. Beuchat, P. Thevenaz, M. Unser, T. Ebner, A. Senn, F. Urner, M. Germond, and C. Sorzano, “Quantitative morphometrical characterization of human pronuclear zygotes,” *Human Reproduction*, vol. 23, no. 9, pp. 1983–1992, 2008.

BIBLIOGRAPHY

- [29] A. Karlsson, N. Overgaard, and A. Heyden, “A two-step area based method for automatic tight segmentation of zona pellucida in HMC images of human embryos,” in *Scale Space and PDE Methods in Computer Vision* (R. Kimmel, N. Sochen, and J. Weickert, eds.), vol. 3459 of *Lecture Notes in Computer Science*, pp. 503–514, Springer Berlin Heidelberg, 2005.
- [30] A. Karlsson, N. Overgaard, and A. Heyden, “Automatic segmentation of zona pellucida in HMC images of human embryos,” in *Pattern Recognition, 2004. ICPR 2004. Proceedings of the 17th International Conference on*, vol. 3, pp. 518–521 Vol.3, Aug 2004.
- [31] C. Xu and J. Prince, “Snakes, shapes, and gradient vector flow,” *Image Processing, IEEE Transactions on*, vol. 7, pp. 359–369, Mar 1998.
- [32] E. Morales, D.A. Bengoetxea and P. Larranaga, “Automatic segmentation of zona pellucida in human embryo images applying an active contour model,” in *Medical Understanding and Analysis* (S. M. J. Hoey, ed.), (Dundee, Scotland), pp. 104–116, 2008.
- [33] E. S. Filho, J. Noble, M. Poli, T. Griffiths, G. Emerson, and D. Wells, “A method for semi-automatic grading of human blastocyst microscope images,” *Human Reproduction*, 2012.
- [34] P. Yee, D. Saeedi and J. Havelock, “An automatic model-based approach for measuring the zona pellucida thickness in day five human blastocysts,” in *International Conference on Image Processing, 2013. Computer Vision, and Pattern Recognition (IPCV)*, 2013.
- [35] A. Giustia, G. Corani, L. Gambardella, C. Magli, and L. Gianaroli, “Blastomere segmentation and 3D morphology measurements of early

BIBLIOGRAPHY

- embryos from hoffman modulation contrast image stacks,” in *Biomedical Imaging: From Nano to Macro, 2010 IEEE International Symposium on*, pp. 1261–1264, April 2010.
- [36] Giustia, C. Giorgio, M. Luca, C. Maria, and G. Luca, “Segmentation of human zygotes in Hoffman modulation contrast images,” in *Proc. of Medical Image Understanding and Analysis (MIUA)*, 2009.
- [37] F. Ning, D. Delhomme, Y. LeCun, F. Piano, L. Bottou, and P. Barbano, “Toward automatic phenotyping of developing embryos from videos,” *Image Processing, IEEE Transactions on*, vol. 14, pp. 1360–1371, Sept 2005.
- [38] Y. Tian, F. Yin, Y.B.and Duan, W. Wang, W. Wang, and M. Zhou, “Automatic blastomere recognition from a single embryo image,” *Computational and Mathematical Methods in Medicine*, vol. 2014, 2014.
- [39] H. Huang, D. Sun, J. Mills, and S. H. Cheng, “Robotic cell injection system with position and force control: Toward automatic batch biomanipulation,” *Robotics, IEEE Transactions on*, vol. 25, pp. 727–737, June 2009.
- [40] X. Liu, Z. Lu, and Y. Sun, “Orientation control of biological cells under inverted microscopy,” *Mechatronics, IEEE/ASME Transactions on*, vol. 16, pp. 918–924, Oct 2011.
- [41] P. Stein and R. M. Schultz, “Chapter 14 - ICSI in the mouse,” in *Guide to Techniques in Mouse Development, Part A: Mice, Embryos, and Cells, 2nd Edition* (P. M. Wassarman and P. M. Soriano, eds.), vol. 476 of *Methods in Enzymology*, pp. 251 – 262, Academic Press, 2010.

BIBLIOGRAPHY

- [42] N. Yoshida and A. C. F. Perry, “Piezo-actuated mouse intracytoplasmic sperm injection (ICSI),” *Nat. Protocols*, vol. 2, no. 2, pp. 296–304, 2007. 10.1038/nprot.2007.7.
- [43] L. Van der Westerlaken, F. Helmerhorst, J. Hermans, and N. Naaktgeboren, “Intracytoplasmic sperm injection: position of the polar body affects pregnancy rate,” *Human Reproduction*, vol. 14, no. 10, pp. 2565–2569, 1999.
- [44] I. M. Bahadur and J. K. Mills, “Overview of the automation of embryo biopsy for preimplantation genetic diagnosis,” *International Journal of Mechatronics and Automation*, vol. 1, no. 3, pp. 201–212, 2011.
- [45] L. Gianaroli, “Preimplantation genetic diagnosis: polar body and embryo biopsy,” *Human Reproduction*, vol. 15, no. suppl 4, pp. 69–75, 2000.
- [46] P. Braude, S. Pickering, F. Flinter, and C. M. Ogilvie, “Preimplantation genetic diagnosis,” *Nat Rev Genet*, vol. 3, no. 12, pp. 941–955, 2002. 10.1038/nrg953.
- [47] Y. Anis, M. Holl, and D. Meldrum, “Automated selection and placement of single cells using vision-based feedback control,” *Automation Science and Engineering, IEEE Transactions on*, vol. 7, July 2010.
- [48] Y. Liu and M. Yu, “Investigation of inclined dual-fiber optical tweezers for 3d manipulation and force sensing,” *Opt. Express*, vol. 17, pp. 13624–13638, Aug 2009.
- [49] C. Leung, Z. Lu, X. Zhang, and Y. Sun, “Three-dimensional rotation of mouse embryos,” *Biomedical Engineering, IEEE Transactions on*, vol. 59, pp. 1049–1056, April 2012.

BIBLIOGRAPHY

- [50] S. K. Mohanty and P. K. Gupta, “Laser-assisted three-dimensional rotation of microscopic objects,” *Review of Scientific Instruments*, vol. 75, pp. 2320–2322, Jul 2004.
- [51] S. Bayoudh, T. Nieminen, N. Heckenberg, and H. Rubinsztein-Dunlop, “Orientation of biological cells using plane-polarized Gaussian beam optical tweezers,” *Journal of Modern Optics*, vol. 50, pp. 1581–1590, Oct. 2003.
- [52] S. Sato, M. Ishigure, and H. Inaba, “Optical trapping and rotational manipulation of microscopic particles and biological cells using higher-order mode Nd:YAG laser beams,” *Electronics Letters*, vol. 27, pp. 1831–1832, Sept 1991.
- [53] Y. Liang, Y. Huang, Y. Lu, M. Hou, and J. A. Yeh, “Cell rotation using optoelectronic tweezers,” *Biomicrofluidics*, vol. 4, no. 4, 2010.
- [54] F. Sheu, T. Lan, Y. Lin, and C. Chen, S.and Ay, “Stable trapping and manually controlled rotation of an asymmetric or birefringent microparticle using dual-mode split-beam optical tweezers,” *Opt. Express*, vol. 18, pp. 14724–14729, Jul 2010.
- [55] M. Wu, “Optoelectronic tweezers,” *Nat Photon*, vol. 5, no. 6, pp. 322–324, 2011.
- [56] X. Liu and Y. Sun, “Visually servoed orientation control of biological cells in microrobotic cell manipulation,” in *Experimental Robotics* (O. Khatib, V. Kumar, and G. Pappas, eds.), vol. 54 of *Springer Tracts in Advanced Robotics*, pp. 179–187, Springer Berlin Heidelberg, 2009.

BIBLIOGRAPHY

- [57] Y. Eguchi, M. Ogiue-Ikeda, and S. Ueno, “Control of orientation of rat schwann cells using an 8-t static magnetic field,” *Neuroscience Letters*, vol. 351, no. 2, pp. 130 – 132, 2003.
- [58] M. Ogiue-Ikeda and S. Ueno, “Magnetic cell orientation depending on cell type and cell density,” *Magnetics, IEEE Transactions on*, vol. 40, pp. 3024–3026, July 2004.
- [59] J. L. Sebastian, S. Munoz, M. Sancho, G. Martinez, and G. Alvarez, “Electromechanical effects on multilayered cells in nonuniform rotating fields,” *Phys. Rev. E*, vol. 84, p. 011926, Jul 2011.
- [60] J. Park, S.-H. Jung, Y.-H. Kim, B. Kim, S.-K. Lee, B. Ju, and K.-I. Lee, “An integrated bio cell processor for single embryo cell manipulation,” in *Intelligent Robots and Systems, 2004. (IROS 2004). Proceedings. 2004 IEEE/RSJ International Conference on*, vol. 1, pp. 242–247 vol.1, Sept 2004.
- [61] M. Nishioka, S. Katsura, K. Hirano, and A. Mizuno, “Evaluation of cell characteristics by step-wise orientational rotation using optoelectrostatic micromanipulation,” *Industry Applications, IEEE Transactions on*, vol. 33, pp. 1381–1388, Sep 1997.
- [62] D. S. Gray, J. L. Tan, J. Voldman, and C. S. Chen, “Dielectrophoretic registration of living cells to a microelectrode array,” *Biosensors and Bioelectronics*, vol. 19, no. 12, pp. 1765 – 1774, 2004.
- [63] E. Iwarsson, H. Malmgren, and E. Blennow, “Preimplantation genetic diagnosis: twenty years of practice,” *Seminars in Fetal and Neonatal Medicine*, vol. 16, no. 2, pp. 74–80, 2011.

BIBLIOGRAPHY

- [64] K.-H. Cui, P. Verma, and C. Matthews, "Hatching rate: An optimal discriminator for the assessment of single-blastomere biopsy," *Journal of Assisted Reproduction and Genetics*, vol. 10, no. 2, pp. 157–162, 1993.
- [65] J. W. Gordon and B. E. Talansky, "Assisted fertilization by zona drilling: A mouse model for correction of oligospermia," *Journal of Experimental Zoology*, vol. 239, no. 3, pp. 347–354, 1986.
- [66] J. Selva, "Assisted hatching," *Human Reproduction*, vol. 15, no. 4, pp. 65–68, 2000.
- [67] J. Krussel, H. Huang, C. Simon, B. Behr, A. Pape, Y. Wen, P. Bielfeld, and M. Polan, "Single blastomeres within human preimplantation embryos express different amounts of messenger ribonucleic acid for beta actin and interleukin 1 receptor type i," *Journal of Clinical Endocrinology and Metabolism*, vol. 83, no. 3, pp. 953–959, 1998.
- [68] J. Cohen, M. Alikani, J. Trowbridge, and Z. Rosenwaks, "Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis," *Human Reproduction*, vol. 7, no. 5, pp. 685–691, 1992.
- [69] J. Gordon, L. Grunfeld, G. Garrisi, B. Talansky, S. Richard, and N. Laufer, "Fertilization of human oocytes by sperm from infertile males after zona pellucida drilling," *Fertility and sterility*, vol. 50, no. 1, pp. 68–73, 1988.
- [70] G. Garrisi, B. Talansky, L. Grunfeld, V. Sapira, D. Navot, and J. Gordon, "Clinical evaluation of three approaches to micromanipulation-

BIBLIOGRAPHY

- assisted fertilization,” *Fertility and Sterility*, vol. 54, no. 4, pp. 671–677, 1990.
- [71] M. Tucker, N. Luecke, S. Wiker, and G. Wright, “Chemical removal of the outside of the zona pellucida of day 3 human embryos has no impact on implantation rate,” *Journal of Assisted Reproduction and Genetics*, vol. 10, no. 3, pp. 187–191, 1993.
- [72] D. Palanker, S. Ohad, A. Lewis, A. Simon, J. Shenkar, S. Penchas, and N. Laufer, “Technique for cellular microsurgery using the 193-nm excimer laser,” *Lasers in Surgery and Medicine*, vol. 11(6), pp. 580–586, 1991.
- [73] Y. Tadir, J. Neev, P. Ho, and M. Berns, “Lasers for gamete micro-manipulation: Basic concepts,” *Journal of Assisted Reproduction and Genetics*, vol. 10, no. 2, pp. 121–125, 1993.
- [74] N. Laufer, D. Palanker, Y. Shufaro, A. Safran, A. Simon, and A. Lewis, “The efficacy and safety of zona pellucida drilling by a 193-nm excimer laser,” *Fertility and sterility*, vol. 59, pp. 889–895, 1993.
- [75] H. Strohmer and W. Feichtinger, “Successful clinical application of laser for micromanipulation in an in vitro fertilization program,” *Fertility and sterility*, vol. 58(1), pp. 212–214, 1992.
- [76] M. Germond, D. Nocera, A. Senn, K. Rink, G. Delacraz, T. Pedrazzini, and J. Hornung, “Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 m diode laser beam,” *Human Reproduction*, vol. 11, no. 5, pp. 1043–1048, 1996.

BIBLIOGRAPHY

- [77] T. Ebner, M. Moser, and G. Tews, “Possible applications of a non-contact 1.48 um wavelength diode laser in assisted reproduction technologies,” *Human reproduction update*, vol. 11, no. 4, pp. 425–435, 2005. Cited By (since 1996): 7 Export Date: 12 September 2011 Source: Scopus.
- [78] S. Antinori, C. Versaci, P. Fuhrberg, C. Panci, B. Caffa, and G. Gholami, “Seventeen live births after the use of an erbium-yttrium aluminum garnet laser in the treatment of male factor infertility,” *Human Reproduction*, vol. 9(10), pp. 1891–1896, 1994.
- [79] M. Montag, R. Klose, M. Koster, B. Rosing, K. Van der Ven, K. Rink, and H. Van der Ven, “Application of non-contact laser technology in assisted reproduction,” *Anwendung von Non-Kontakt-Lasertechnologien in der assistierten Reproduktion*, vol. 24, no. 1, pp. 57–64, 2009.
- [80] A. De Vos and A. Van Steirteghem, “Zona hardening, zona drilling and assisted hatching: New achievements in assisted reproduction,” *Cells Tissues Organs*, vol. 166, no. 2, pp. 220–227, 2000.
- [81] H. Malter and J. Cohen, “Partial zona dissection of the human oocyte: a nontraumatic method using micromanipulation to assist zona pellucida penetration,” *Fertility and sterility*, vol. 1, no. 1, pp. 139–148, 1989.
- [82] D. Payne, K. McLaughlin, H. Depyper, C. Kirby, G. Warnes, and C. Matthews, “Experience with zona drilling and zona cutting to improve fertilization rates of human oocytes in vitro,” *Human Reproduction*, vol. 6, no. 3, pp. 423–431, 1991.

BIBLIOGRAPHY

- [83] S. Sun, J. Choi, J. Son, J. Joo, G. Ko, and K. Lee, “The effect of long zona dissection using icsi pipettes for mechanical assisted hatching in vitrified-thawed blastocyst transfers,” *Journal of Assisted Reproduction and Genetics*, vol. 29, no. 12, pp. 1431–1434, 2012.
- [84] K. A. Kelley, *Chapter 13 - Enhancement of IVF in the Mouse by Zona-Drilling*, vol. Volume 476, pp. 229–250. Academic Press, 2010.
- [85] Y. Kawase, T. Iwata, O. Ueda, N. Kamada, Y. J. K. Tachibe, T. and Aoki, and H. Suzuki, “Effect of partial incision of the zona pellucida by piezo-micromanipulator for in vitro fertilization using frozen-thawed mouse spermatozoa on the developmental rate of embryos transferred at the 2-cell stage,” *Biology of Reproduction*, vol. 66, no. 2, pp. 381–385, 2002.
- [86] Y. Kimura and R. Yanagimachi, “Intracytoplasmic sperm injection in the mouse,” *Biology of Reproduction*, vol. 52, no. 4, pp. 709–720, 1995.
- [87] K. Yanagida, H. Katayose, H. Yazawa, Y. Kimura, K. Konnai, and A. Sato, “The usefulness of a piezo-micromanipulator in intracytoplasmic sperm injection in humans,” *Human Reproduction*, vol. 14, no. 2, pp. 448–453, 1999.
- [88] H. Huang, J. Mills, C. Lu, and D. Sun, “A universal piezo-driven ultrasonic cell microinjection system,” *Biomedical Microdevices*, vol. 13, no. 4, pp. 743–752, 2011.
- [89] J. Hirvonen, P. Ronkanen, T. Ylikomi, M. Blauer, R. Suuronen, H. Skottman, and P. Kallio, “Microcutting of living tissue slices and stem cell colonies by using mechanical tool and liquid jet,” in *Biomed-*

BIBLIOGRAPHY

- cal Robotics and Biomechatronics, 2008. BioRob 2008. 2nd IEEE RAS and EMBS International Conference on*, pp. 612–617.
- [90] A. F. Ergenc and N. Olgac, “New technology for cellular piercing: rotationally oscillating mu-injector, description and validation tests,” *Biomedical Microdevices*, vol. 9, no. 6, pp. 885–891, 2007.
- [91] T. I. Hagen and R. F. Gunter, “Biopsy on living cells by ultra slow instrument movement,” *Journal of Physics: Condensed Matter*, vol. 18, no. 18, p. S627, 2006.
- [92] X. Zhang, M. P. Scott, C. F. Quate, and O. Solgaard, “Microoptical characterization of piezoelectric vibratory microinjections in drosophila embryos for genome-wide rnai screen,” *Microelectromechanical Systems, Journal of*, vol. 15, no. 2, pp. 277–286, 2006.
- [93] L. Sun, H. Wang, L. Chen, and Y. Liu, “A novel ultrasonic micro-dissection technique for biomedicine,” *Ultrasonics*, vol. 44, Supplement, no. 0, pp. e255 – e260, 2006. Proceedings of Ultrasonics International (UI5) and World Congress on Ultrasonics (WCU).
- [94] H. Huang, J. K. Mills, and D. Sun, “A new piezo-driven ultrasonic cell microinjection system,” in *Information and Automation (ICIA), 2010 IEEE International Conference on*, pp. 18–23.
- [95] K. Ebina, H. Hasegawa, and H. Kanai, “Investigation of frequency characteristics in cutting of soft tissue using prototype ultrasonic knives,” *The Japan Society of Applied Physics*, vol. 46, no. 7b, 2007.
- [96] M. Subbarao, T. Choi, and A. Nikzad, “Focusing techniques,” *Optical Engineering*, vol. 32, no. 11, pp. 2824–2836, 1993. 10.1117/12.147706.

BIBLIOGRAPHY

- [97] S. Jutamulia, T. Asakura, R. Bahuguna, and P. C. De, G, “Autofocusing based on power-spectra analysis,” *Applied Optics*, vol. 33, no. 26, pp. 6210–6212, 1994.
- [98] M. Subbarao and J. K. Tyan, “Selecting the optimal focus measure for autofocusing and depth-from-focus,” *Pattern Analysis and Machine Intelligence, IEEE Transactions on*, vol. 20, no. 8, pp. 864–870, 1998.
- [99] K. Choi and S. Lee, J. and Ko, “New autofocusing technique using the frequency selective weighted median filter for video cameras,” *Consumer Electronics, IEEE Transactions on*, vol. 45, no. 3, pp. 820–827, 1999.
- [100] M. Subbarao and Y. Liu, “Accurate reconstruction of three-dimensional shape and focused image from a sequence of noisy defocused images,” vol. 2909, pp. 178–191, 1997.
- [101] S. K. Kim, S. R. Park, and J. K. Paik, “Simultaneous out-of-focus blur estimation and restoration for digital auto-focusing system,” *Consumer Electronics, IEEE Transactions on*, vol. 44, no. 3, pp. 1071–1075, 1998.
- [102] A. W. Lohmann, D. Mendlovic, and Z. Zalevsky, “Digital method for measuring the focus error,” *Applied Optics*, vol. 36, no. 28, pp. 7204–7209, 1997.
- [103] I. Raveh, D. Mendlovic, Z. Zalevsky, and A. W. Lohmann, “Digital method for defocus corrections: experimental results,” *Optical Engineering*, vol. 38, no. 10, pp. 1620–1626, 1999. 10.1117/1.602215.
- [104] T. Yeo, S. Ong, Jayasooriah, and R. Sinniah, “Autofocusing for tissue microscopy,” *Image and Vision Computing*, vol. 11, no. 10, pp. 629–639, 1993.

BIBLIOGRAPHY

- [105] Q. Li, Z. Xu, H. Feng, and S. Dai, "Autofocus area design of digital image system," *Acta Photonica Sinica*, vol. 12, no. 32, pp. 1499–1501, 2003.
- [106] K. Zhu, W. Jiang, Z. Gao, X. Zhou, and J. Zhang, "Focusing window choice and parameters determination in automatic focusing system," *Acta Optica Sinica*, vol. 6, no. 26, pp. 777–780, 2006.
- [107] Q. Wang, J. Liu, and Y. Lin, "Study on unequally spaced sampling parameters in rosette scanning imaging subsystem," *Infrared and Laser Engineering*, vol. 31, no. 4, pp. 322–325, 2002.
- [108] W. Wang, Yanfang. Jiang and Y. Zheng, "Dynamic autofocusing region selection based on improved PSO," in *Image and Signal Processing (CISP), 2010 3rd International Congress on*, vol. 6, pp. 2760–2764.
- [109] G. Beveridge and R. Schechter, *Optimization: Theory and practice*. New York: McGraw-Hill Inc, 1970.
- [110] X. Y. Liu, W. H. Wang, and Y. Sun, "Dynamic evaluation of autofocusing for automated microscopic analysis of blood smear and pap smear," *Journal of Microscopy*, vol. 227, no. 1, pp. 15–23, 2007.
- [111] K. Ooi, K. Izumi, M. Nozaki, and I. Takeda, "An advanced autofocus system for video camera using quasi condition reasoning," *Consumer Electronics, IEEE Transactions on*, vol. 36, no. 3, pp. 526–530, 1990.
- [112] F. Svahn, *Tools and methods to obtain a passive autofocus system*. Linkping University, 1996.

BIBLIOGRAPHY

- [113] O. A. Osibote, R. Dendere, S. Krishnan, and T. S. Douglas, “Automated focusing in bright-field microscopy for tuberculosis detection,” *Journal of Microscopy*, vol. 240, no. 2, pp. 155–163, 2010.
- [114] J.-M. Geusebroek, F. Cornelissen, A. W. M. Smeulders, and H. Geerts, “Robust autofocusing in microscopy,” *Cytometry*, vol. 39, no. 1, pp. 1–9, 2000.
- [115] V. Hilsenstein, “Robust autofocusing for automated microscopy imaging of fluorescently labelled bacteria,” in *Digital Image Computing: Techniques and Applications, 2005. DICTA '05. Proceedings 2005*, pp. 15–15.
- [116] S. Naka, T. Genji, T. Yura, and Y. Fukuyama, “A hybrid particle swarm optimization for distribution state estimation,” *Power Systems, IEEE Transactions on*, vol. 18, no. 1, pp. 60–68, 2003.
- [117] T. S. Lim, V. C. Koo, H. Ewe, and H. Chuah, “A sar autofocus algorithm based on particle swarm optimization,” *Progress In Electromagnetics Research B*, vol. 1, pp. 159–176, 2008.
- [118] Z. Zeng, L. Lu, and Y. Wang, “An enhancement of ladar image based on SFLA algorithm,” *Springer Berlin Heidelberg*, vol. 278, 2013.
- [119] A. Ladgham, F. Hamdaoui, A. Sakly, and A. Mtibaa, “Fast mr brain image segmentation based on modified shuffled frog leaping algorithm,” *Signal, Image and Video Processing*, pp. 1–8, 2013.
- [120] J.-s. Wang and Y. Zhang, *Research on Edge Detection Algorithm of Rotary Kiln Infrared Color Image*, vol. 7004 of *Lecture Notes in Computer Science*, ch. 45, pp. 370–377. Springer Berlin Heidelberg, 2011.

BIBLIOGRAPHY

- [121] M. H. Horng, “Multilevel image threshold selection based on the shuffled frog-leaping algorithm,” *Journal of Chemical and Pharmaceutical Research*, vol. 5, no. 9, pp. 599–605, 2013.
- [122] Z. Wang and X. Sun, “Image watermarking scheme based on shuffled frog leaping algorithm,” in *Knowledge Acquisition and Modeling Workshop, 2008. KAM Workshop 2008. IEEE International Symposium on*, pp. 239–242, Dec 2008.
- [123] X. Li, L. Liu, N. Wang, and J. Pan, “A new robust watermarking scheme based on shuffled frog leaping algorithm,” *Intelligent automation and soft computing*, vol. 17, no. 2, pp. 219–232, 2011.
- [124] E. Elbeltagi, T. Hegazy, and D. Grierson, “Comparison among five evolutionary-based optimization algorithms,” *Advanced Engineering Informatics*, vol. 19, no. 1, pp. 43–53, 2005.
- [125] X. Zhang, G. Hu, Xuemei. and Cui, Y. Wang, and Y. Niu, “An improved shuffled frog leaping algorithm with cognitive behavior,” in *Intelligent Control and Automation, 2008. WCICA 2008. 7th World Congress on*, pp. 6197–6202.
- [126] M. Eusuffa, L. Kevin., and F. Pashab, “Shuffled frog-leaping algorithm: a memetic meta-heuristic for discrete optimization,” *Engineering Optimization*, vol. 38, no. 2, pp. 129–154, 2006.
- [127] D. Zhen, Ziyang. Wang and Y. Liu, “Improved shuffled frog leaping algorithm for continuous optimization problem,” in *Evolutionary Computation, 2009. CEC '09. IEEE Congress on*, pp. 2992–2995.

BIBLIOGRAPHY

- [128] J. Jiang, P. Ma, X. Gao, J. Li, and F. Zhao, “An improved shuffled frog leaping algorithm,” *Journal of Information and Computational Science*, vol. 10, no. 6, pp. 1665–1673, 2013.
- [129] J. Jiang, Q. Su, M. Li, M. Liu, and L. Zhang, “An improved shuffled frog leaping algorithm,” *Journal of Information and Computational Science*, vol. 10, no. 14, pp. 4619–4626, 2013.
- [130] L. Fan, TangHuai. Lv and J. Zhao, “Improved shuffled frog leaping algorithm and its application in node localization of wireless sensor network,” *Intelligent Automation and Soft Computing*, vol. 18, no. 7, pp. 807–818, 2012.
- [131] P. Luo, Q. Lu, and C. Wu, “Modified shuffled frog leaping algorithm based on new searching strategy,” in *Natural Computation (ICNC), 2011 Seventh International Conference on*, vol. 3, pp. 1346–1350.
- [132] E. Van Royen, K. Mangelschots, D. De Neubourg, M. Valkenburg, M. Van de Meerssche, G. Ryckaert, W. Eestermans, and J. Gerris, “Characterization of a top quality embryo, a step towards single-embryo transfer,” *Human Reproduction*, vol. 14, no. 9, pp. 2345–2349, 1999.
- [133] A. Gabrielsen, P. Bhatnager, K. Petersen, and S. Lindenberg, “Influence of zona pellucida thickness of human embryos on clinical pregnancy outcome following in vitro fertilization treatment,” *Journal of Assisted Reproduction and Genetics*, vol. 17, no. 6, pp. 323–328, 2000.
- [134] A. Gabrielsen, S. Lindenberg, and K. Petersen, “The impact of the zona pellucida thickness variation of human embryos on pregnancy outcome in relation to suboptimal embryo development. a prospective

BIBLIOGRAPHY

- randomized controlled study,” *Human Reproduction*, vol. 16, no. 10, pp. 2166–2170, 2001.
- [135] M. Palmstierna, D. Murkes, G. Csemiczky, O. Andersson, and H. Wramsby, “Zona pellucida thickness variation and occurrence of visible mononucleated blastomeres in preembryos are associated with a high pregnancy rate in ivf treatment,” *Journal of Assisted Reproduction and Genetics*, vol. 15, no. 2, pp. 70–75, 1998.
- [136] K. Sermon, A. Van Steirteghem, and I. Liebaers, “Preimplantation genetic diagnosis,” *The Lancet*, vol. 363, no. 9421, pp. 1633–1641, 2004.
- [137] M. Boada, M. Carrera, C. De La Iglesia, M. Sandalinas, P. Barri, and A. Veiga, “Successful use of a laser for human embryo biopsy in preimplantation genetic diagnosis: Report of two cases,” *Journal of Assisted Reproduction and Genetics*, vol. 15, no. 5, pp. 302–307, 1998.
- [138] S. Krasner, “Why wheels work: A second version,” *The Physics Teacher*, vol. 30, no. 4, pp. 212–215, 1992.
- [139] A. Domenech, T. Domenech, and J. Cebrian, “Introduction to the study of rolling friction,” *American Journal of Physics*, vol. 55, no. 3, pp. 231–235, 1987.
- [140] W. Chang, I. Etsion, and D. Bogy, “An elastic-plastic model for the contact of rough surfaces,” *Journal of Tribology*, vol. 109, no. 2, pp. 257–263, 1987.
- [141] W. H. Grover, A. K. Bryan, M. Diez-Silva, S. Suresh, J. M. Higgins, and S. R. Manalis, “Measuring single-cell density,” *Proceedings of the National Academy of Sciences*, vol. 108, no. 27, pp. 10992–10996, 2011.

BIBLIOGRAPHY

- [142] L. Lin, Y. Yu, A. Liu, and P. . Yap, “Non-contact measurement of young’s modulus on single living cells using hydrostatic pressure in a microchamber,” in *the Eleventh International Conference on Miniaturized Systems for Chemistry and Life Sciences, Paris, France*, 2007.
- [143] D. Kim, Y. Sun, S. Yun, B. Kim, N. Chang, S. Lee, and B. Nelson, “Mechanical property characterization of the zebrafish embryo chorion,” in *Engineering in Medicine and Biology Society, 2004. IEMBS '04. 26th Annual International Conference of the IEEE*, vol. 2, pp. 5061–5064, Sept 2004.
- [144] J. Bahadu, Issam. M. Mills, “Overview of the automation of embryo biopsy for precimplantation genetic diagnosis,” *International Journal of Mechatronics and Automation*, vol. 1, pp. 201–212, 2011.
- [145] Q. Xu, “Microinjection into zebrafish embryos,” in *Molecular Methods in Developmental Biology* (M. Guille, ed.), vol. 127, pp. 125–132, Humana Press, 1999.
- [146] N. Otsu, “A threshold selection method from gray-level histograms,” *IEEE Trans. Systems, Man and Cybernetics*, vol. 9, no. 1, pp. 62–66, 1979.
- [147] D. Dozortsev, T. Wakaiama, A. Ermilov, and R. Yanagimachi, “Intracytoplasmic sperm injection in the rat,” *Image Processing, IEEE Transactions on*, vol. 6, pp. 143–147, May 1998.
- [148] L. Devito, C. Fernandes, I. Blanco, P. Tsuribe, and F. Landim-Alvarenga, “Use of a piezo drill for intracytoplasmic sperm injection into cattle oocytes activated with ionomycin associated with roscovi-

BIBLIOGRAPHY

- tine,” *Reproduction in Domestic Animals*, vol. 45, no. 4, pp. 654–658, 2010.
- [149] A. Norhazlin, J. and Nor and K. Ko, “Biopsy of blastomeres from cleavage-stage mouse embryos with eppendorf piezoxpert,” in *Eppendorf, E.A.P. Headquarters, Editor. 2013: Malaysia*).
- [150] P. A. Janmey and C. A. McCulloch, “Cell mechanics: Integrating cell responses to mechanical stimuli,” *Annual Review of Biomedical Engineering*, vol. 9, no. 1, pp. 1–34, 2007. PMID: 17461730.
- [151] K. Ediz and N. Olgac, “Microdynamics of the piezo-driven pipettes in icsi,” *Biomedical Engineering, IEEE Transactions on*, vol. 51, pp. 1262–1268, July 2004.
- [152] K. Ediz and N. Olgac, “Effect of mercury column on the microdynamics of the piezo-driven pipettes,” *Journal of Biomechanical Engineering*, vol. 127, no. 3, pp. 531–535, 2005.
- [153] D. Brehl and T. Dow, “Review of vibration-assisted machining,” *Precision Engineering*, vol. 32, no. 3, pp. 153 – 172, 2008.
- [154] M. Fan, Z. C. Feng, Y. Agca, and J. K. Critser, “Vibration study of the piezodriven pipettes immersed in viscous liquids,” *Journal of Applied Physics*, vol. 100, no. 7, pp. –, 2006.
- [155] M. Karzar-Jeddi, N. Olgac, and T. Fan, “Dynamic response of micropipettes during piezo-assisted intracytoplasmic sperm injection,” *Phys. Rev. E*, vol. 84, p. 041908, Oct 2011.

BIBLIOGRAPHY

- [156] Y. Gan and Z. Chen, “A study of the zona piercing process in piezo-driven intracytoplasmic sperm injection,” *Journal of Applied Physics*, vol. 104, no. 4, pp. –, 2008.
- [157] H. Huang, H. Su, H. Y. Chen, and J. K. Mills, “Piezoelectric driven non-toxic injector for automated cell manipulation,” in *MMVR’11*, pp. 231–235, 2011.
- [158] S. Zhao, Y. N. Aye, C. Y. Shee, I.-M. Chen, and W. T. Ang, “A compact 3-dof compliant serial mechanism for trajectory tracking with flexures made by rapid prototyping,” in *Robotics and Automation (ICRA), 2012 IEEE International Conference on*, pp. 4475–4480, May 2012.
- [159] Y. Tan, D. Sun, and W. Huang, “A mechanical model of biological cells in microinjection,” in *Robotics and Biomimetics, 2008. ROBIO 2008. IEEE International Conference on*, pp. 61–66, Feb 2009.
- [160] A. G. Beysens, D. Forgacs and J. Glazier, “Embryonic tissues are viscoelastic materials,” *Canadian Journal of Physics*, vol. 78, no. 3, pp. 243–251, 2000.
- [161] E. Baesu, R. Rudd, J. Belak, and M. McElfresh, “Continuum modeling of cell membranes,” *International Journal of Non-Linear Mechanics*, vol. 39, no. 3, pp. 369 – 377, 2004.
- [162] H. Karcher, H. Lammerding, Jan. and Huang, R. T. Lee, R. D. Kamm, and M. R. . Kaazempur-Mofrad, “A three-dimensional viscoelastic model for cell deformation with experimental verification,” *Biophysical journal*, vol. 85, no. 5, pp. 3336–3349, 2003.
- [163] J. Cieslak, V. Ivakhnenko, G. Wolf, S. Sheleg, and Y. Verlinsky, “Three-dimensional partial zona dissection for preimplantation genetic diagno-

BIBLIOGRAPHY

- sis and assisted hatching,” *Fertility and Sterility*, vol. 71, no. 2, pp. 308–313, 1999.
- [164] S. Kahraman, K. Yakin, E. Donmez, H. Samli, M. Bahce, G. Cengiz, S. Sertyel, M. Samli, and N. Imirzalioglu, “Relationship between granular cytoplasm of oocytes and pregnancy outcome following intracytoplasmic sperm injection,” *Human Reproduction*, vol. 15, no. 11, pp. 2390–2393, 2000.
- [165] G. Levinson, R. Fields, G. Harton, F. Palmer, A. Maddalena, E. Fugger, and J. Schulman, “Reliable gender screening for human preimplantation embryos, using multiple dna target-sequences,” *Human Reproduction*, vol. 7, no. 9, pp. 1304–1313, 1992.
- [166] J. W. Gordon and I. Gang, “Use of zona drilling for safe and effective biopsy of murine oocytes and embryos,” *Biology of Reproduction*, vol. 42, no. 5-6, pp. 869–876, 1990.