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**DESIGN AND CONTROL OF A PIEZO-DRIVEN FLEXURE
MICROMANIPULATOR FOR THE EMBRYO BIOPSY**

ZHANG ZE ZHONG
SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING
2019

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MICROMANIPULATOR FOR THE EMBRYO BIOPSY**

ZHANG ZE ZHONG

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Master of Engineering

2019

Statement of Originality

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Zhang Zezhong

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TABLE OF CONTENTS

ABSTRACT.....	i
LIST OF FIGURES	iii
LIST OF TABLES	vi
CHAPTER 1 Introduction.....	1
1.1 Background	1
1.2 Objectives.....	3
1.3 Scope	3
1.4 Organization	4
CHAPTER 2 Literature Review	6
2.1 Embryo Properties.....	7
2.1.1 Cleavage Divisions, Blastocyst Stage and Hatching	7
2.1.2 Embryo Mechanical Properties	9
2.2 Embryo Biopsy Approaches.....	11
2.3 Embryo Biopsy Techniques	16
2.3.1 Chemical Drilling	16
2.3.2 Laser-Assisted Biopsy	17
2.3.3 Mechanical Biopsy	19

2.4 Flexural Mechanism	24
2.4.1 Flexure Hinge	24
2.4.2 Flexure Mechanism Designs	26
2.5 Summary	28
CHAPTER 3 System Design	30
3.1 System Overview	30
3.2 Embryo Dissection Strategy	33
3.3 Piezo-Driven Flexure Micromanipulator	35
3.3.1 Micropipette Dynamics	35
3.3.2 Flexure Mechanism Design	38
3.3.3 Micropipette Connector	42
3.4 Summary	43
CHAPTER 4 Vibration Control of Micropipette Tip	45
4.1 Experimental Setup	45
4.2 Experimental Results.....	48
4.2.1 Lateral Vibration.....	48
4.2.2 Axial Vibration	53
4.3 Discussion	55
CHAPTER 5 Embryos Dissection	57
5.1 Experimental Setup	57
5.2 Experimental Results.....	59

5.3 Discussion	61
CHAPTER 6 Conclusion and Future Work.....	64
6.1 Conclusion.....	64
6.2 Future Work	65
REFERENCES	67

ABSTRACT

Trophectoderm (TE) biopsy is the most common method of embryo biopsy for Pre-implantation Genetic Testing (PGT). One of the critical problems in TE biopsy is how to dissect the zona pellucida (ZP) membrane of the embryo with minimal cell deformation, which requires high-precision micromanipulation. Instead of going with the more common approach of burning embryo by laser, we adopted a cheaper and more easily controlled piezo-driven mechanical approach by using the high-frequency axial and lateral vibration of the end-effector, the micropipette tip. It is challenging to control vibrations of the tip since the slender tip is easy to involve bending. This thesis focuses on the design and control of a piezo-driven flexure micromanipulator to control the vibration of micropipette tips and perform the embryo dissection.

The designed micromanipulator consists of a piezoelectric actuator, a flexure mechanism, a micropipette, and a connector. A two-stage embryo dissection strategy is proposed. First, we penetrate a hole on the surface of the embryo in the axial direction, and then enlarge the hole by lateral cutting. By utilizing the resonant vibration at corresponding driving frequencies in different axes, the flexure mechanism is designed to guide the micropipette tip to accomplish the desired vibrations described in the strategy.

A method is proposed to control the vibrations of the micropipette tip and to dissect the embryo based on the micromanipulator. By setting the parameters of driving frequency of 4, 400 Hz and the driving voltage of 50 V of the piezoelectric actuator, mouse embryos are dissected with little cell deformation (less than 5 μm) under the proposed procedure, in which the driving voltage is applied suddenly and a slight cell deformation is pre-pressed (3~5 μm).

In this work, a reliable piezo-driven flexure micromanipulator has been developed. It dissected the mouse embryo with an effective control method. It can be utilized to decrease the cost and to simplify the process for future embryo biopsy.

LIST OF FIGURES

Figure 2 - 1 The early human implantation development in vitro [6].	7
Figure 2 - 2 Blastocyst scheme [6].	8
Figure 2 - 3 Embryo hatching scheme [6].	9
Figure 2 - 4 The analysis for the cell elastic problem in Aspiration model [10].	10
Figure 2 - 5 Indentation of a cell membrane by a micropipette [11].	10
Figure 2 - 6 Procedures of laser-assisted polar body biopsy [12].	12
Figure 2 - 7 Cleavage-stage biopsy [7].	13
Figure 2 - 8 Schematic comparison between two TE biopsy ways, (a) day 3 hatching, day 5 biopsy; (b) simultaneous hatching and biopsy on day 5[3].	15
Figure 2 - 9 Schematic for chemical ZP drilling[35].	16
Figure 2 - 10 ZILOS-tk laser [37].	17
Figure 2 - 11 Isotherm Rings [38].	18
Figure 2 - 12 Schematic of laser-assisted biopsy.	18
Figure 2 - 13 Passive mechanical drilling [11].	20
Figure 2 - 14 (a) Active mechanical oocyte injection [4]; (b) Active mechanical ZP dissection [40].	21
Figure 2 - 15 Conventional piezo-drill system [47].	22
Figure 2 - 16 Ultrasonic cell injector [49].	23
Figure 2 - 17 Different types of flexures hinges [58].	25
Figure 2 - 18 Beam flexure design. [58].	26
Figure 2 - 19 Flexure linear spring stage [67].	27
Figure 2 - 20 Schematic of a preloading piezo-driven flexure mechanism [58].	28

Figure 3 - 1 (a) Experimental setup. (b) Piezo-assisted biopsy system. (c) Base holder 1. (d) Base holder 2.	30
Figure 3 - 2 Connected power devices for the piezoelectric actuator.....	32
Figure 3 - 3 Flow chart of hardware for cell dissection.	32
Figure 3 - 4 Schematic for dissection strategy.....	33
Figure 3 - 5 The desired three-dimensional peak-peak vibration amplitudes of the micropipette tip respects to the driving time.	34
Figure 3 - 6 (a) The indentation of the pressed ZP membrane; (b) A simplified dynamic model of the interaction between the micropipette and the ZP. [51].....	36
Figure 3 - 7 (a) Schematic of the front view of the flexure structure, with the parameters needed to be determined. (b) Side view. (c) The single unit of the flexure hinge. (d) The prototype.	38
Figure 3 - 8 Schematic of the relative position for the biopsy system and embryo.....	39
Figure 3 - 9 The modal analysis of the flexure structure.	40
Figure 3 - 10 Schematic of the micropipette connector.....	42
Figure 3 - 11 Schematic of the dynamics model for the flexure mechanism and the micropipette connector.....	42
Figure 4 - 1 Schematics of the flexure installation for monitoring different lateral vibration amplitudes of the micropipette tip. (a) The out-of-plane, y-axis direction; (b) The in-plane, x-axis direction.	45
Figure 4 - 2 Schematic for the submerged depth of the micropipette tip.	46
Figure 4 - 3 One blurred image of the lateral vibration in the band of 6,000 to 12, 500 Hz.	47
Figure 4 - 4 The x-axis LVAs cures with respect to the driving frequencies, under different driving voltages.....	48

Figure 4 - 5 The y-axis LVAs curves with respect to the driving frequencies, under different driving voltages.....	49
Figure 4 - 6 The comparing between the x -axis and y -axis LVAs at 50 V.....	50
Figure 4 - 7 The LVAs curves with respect to the driving voltage, in both x and y directions.	50
Figure 4 - 8 Lateral vibration images in different frequencies at 50 V.....	51
Figure 4 - 9 Peak-peak axial vibration amplitudes at different driving voltages.....	52
Figure 5 - 1 Experiment setup for embryo dissection.	55
Figure 5 - 2 The schematic of experimental procedures for the embryo dissection.....	56
Figure 5 - 3 Successful mouse embryo (day3.5) dissection with a pre-pressed deformation and suddenly applied driving voltage.	57
Figure 5 - 4 Failed embryo dissection case because of no pre-pressed deformation....	58
Figure 5 - 5 Failed embryo dissection case because of no suddenly applied driving voltage.	59
Figure 5 - 6 The inverted microscope.	61

LIST OF TABLES

Table 2 - 1 A comparison of different flexures geometries [56]	25
Table 3 - 1 Micropipette Resonant Frequency.....	38
Table 3 - 2 Dimensions of Biopsy System.....	39

CHAPTER 1 Introduction

1.1 Background

It is becoming popular to test genetic diseases for embryos created by In Vitro Fertilization (IVF) by utilizing Preimplantation Genetic Testing (PGT). The primary goal of PGT is to identify genetic defects in embryos before transferring them to the uterus, thus decreasing pregnancy terminations and births with genetic abnormalities [1]. It is mainly used for those women in their later 30's and 40's who have a significantly lower pregnancy rate than women who are below 35 years old, or for those couples whose children may be highly puzzled by severe Mendelian disorders, structural chromosome abnormalities or mitochondrial diseases [2].

Embryo biopsy, one of the critical steps of the PGT procedure, is to remove a part of living cell samples from an embryo for further diagnosis. It can be classified into three approaches according to the development stages: polar body (PB) biopsy, cleavage stage biopsy, and blastocyst biopsy. PB biopsy utilizes the by-products of the oocyte in the meiotic division [3]. It can provide only genetic information from the mother part. The cleavage stage and blastocyst biopsy approaches collect inner cells of the embryo in the cleavage stage and blastocyst stage, respectively, as shown in Figure 2 - 1. These two biopsy approaches can provide intact information from both parents. With research on biopsy impact on PGT, the blastocyst biopsy is viewed as the safest approach among various biopsy approaches [4].

The assisted hatching is one of the core steps of embryo biopsy, which is to dissect the zona pellucida (ZP) membrane of the embryo with proper trauma. ZP membrane is a protective shell of the embryo in the early stage, as shown in Figure 2 - 2. Naturally, the

Chapter 1 Introduction

inner cells of embryo break out the ZP membrane when the embryo develops to day 5 or day 6, as shown in Figure 2 - 3. The embryo dissection makes this procedure happen easily and increases the opportunity to be pregnant for the patient.

Currently, the embryo dissection is manipulated by experienced doctors using glass micropipettes. However, it is difficult to train a good operator. The manual manipulation usually costs much time and causes relatively significant deformation of the embryo in the dissection, which may generate a harmful effect on the subsequent development of embryos. Besides, the performance of human doctors varies from time to time, which may bring low reproducibility and low precision to biopsy.

The piezo-driven high-speed devices can be used to help doctors manipulate the embryo with less deformation of embryos than manually low-speed one [5]. However, it is a thorny problem to control the vibration of the micropipette tip in high frequency because of convoluted reasons, such as the slender shape of the micropipette tip and the resonance of assisted device. Therefore, the vibration control of the micropipette tip is critical if we want to utilize its high-speed effect.

Some researchers added the mercury drop at the micropipette tip to decrease the lateral vibration of micropipette tip [5] [6], by utilizing the damping effect. However, one apparent limitation for mercury to be used in cell dissection is its toxicity. It is easy to damage embryos if the mercury drop is not appropriately controlled.

Flexure mechanism is also applied to control the vibration of the micropipette tip [7]. With the characteristics of rigid architecture and no error accumulation, flexure mechanisms are widely employed in high-precision applications [8] [9]. It can be used to limit or amplify the vibration along different axes by adequate design.

Chapter 1 Introduction

Some clinics adopt laser-assisted techniques to dissect embryos. Though the laser-assisted biopsy is easy to use, the qualified laser-assisted devices are expensive. The high treatment fee prevents many families from taking a try.

Therefore, it is necessary to develop a reliable assisted micromanipulator to help doctors dissect the ZP membrane easily and make it available to more patients.

1.2 Objectives

This research aims to develop an assisted device that can contribute to the embryo biopsy. There are two primary objectives listed as follows:

1. To develop a piezo-driven flexure micromanipulator.
2. To develop a control method to perform embryo dissection.

1.3 Scope

Based on the work of other researchers in this project, such as the microscope monitoring system and cell holding system, the author focuses on the development of a piezo-driven flexure micromanipulator and the corresponding control method to dissect the embryo. The scope is as follows:

Piezo-Driven Flexure Micromanipulator

The core of the manipulator is the flexure mechanism. The vibration control of the micropipette tip is difficult because the slender tip is easy to involve bending. A flexure mechanism is designed and fabricated to guide the lateral and axial vibrations of the micropipette tip.

Control Method

Chapter 1 Introduction

A vibration control method for the micropipette tip is developed. The peak-peak vibration amplitudes in the axial and lateral directions are critical operational parameters for embryo dissection. Hence, the author developed a method to control the peak-peak vibration amplitudes accurately and smoothly.

Validation Experiments by Dissecting Mouse Embryos

Mouse embryos are used to validate the dissection performance. Mouse embryos are suitable to replace human embryos to validate the micromanipulator by considering its similarity to human embryos in size and elastic properties, and advantages of easy collecting. A dissection procedure is proposed, and relative operational parameters are determined based on the mouse embryos in this work.

1.4 Organization

The organization of this report is listed as follows:

Chapter 1 provides the background, objectives, scope, and challenges of the work. A brief outline of the organization of the report is stated.

Chapter 2 reviews embryo properties, embryo biopsy approaches, different biopsy techniques, and the principles and the design of flexure mechanisms. The current research gap and the proper technique for this work are analyzed.

Chapter 3 presents the overall system and propose a strategy for dissection. The micropipette dynamics is analyzed. The piezo-driven flexure micromanipulator is designed according to the desired vibrations, which consists of a piezoelectric actuator, a flexure mechanism, a biopsy micropipette, and a connector.

Chapter 1 Introduction

Chapter 4 accomplishes the vibration control for the micropipette tip. The experimental setup and results of the micropipette vibration tests in the liquid medium are presented. A control method for the micropipette tip is developed.

Chapter 5 validates the dissection performance based on mouse embryos. An effective dissection procedure is summarized. Two critical factors in dissection, the pre-pressed cell deformation and the suddenly applied driving voltage, and their contributions are discussed. Some possible improvements are listed for better performance.

Chapter 6 concludes results and suggests future work.

CHAPTER 2 Literature Review

In this chapter, a review of essential background and techniques is given to establish some basic understanding. These include a review of:

1. Embryo properties, including the development process and mechanical properties;
2. Embryo biopsy approaches classified by development stages;
3. Embryo biopsy techniques;
4. Flexure mechanism properties and designs.

An embryo is an early stage of development of a multicellular diploid eukaryotic organism [10]. In the natural development, the embryo goes through cleavage divisions to blastocyst, then to hatching. The embryo biopsy for PGT also happens during the stage before hatching. It is critical to know the development process and mechanical properties of the embryo to design the proper assisted biopsy device.

Also, Chapter 2 reviewed three biopsy approaches classified by embryo development stages and introduced three different biopsy techniques. After comparing the pros and cons, the piezo-assisted biopsy, an active mechanical method, is selected as the target approach. Besides, the core part of piezo-driven flexure micromanipulator is the flexure mechanism that guides the end-effector to achieve high-precision movements. Therefore, the flexure mechanism's properties and some designs that are related to this application scenario are reviewed at the end of Chapter 2.

2.1 Embryo Properties

2.1.1 Cleavage Divisions, Blastocyst Stage and Hatching

Figure 2 - 1 shows the early human implantation development. Image (a) shows that the first cleavage takes place, 16-20 hours post-fertilization, and the embryo divides at 20 hours intervals after that. Image (b), (c), and (d) show that individual blastomeres can be monitored clearly until 8 - cell stage, the 8 – cell stage is usually on the third day since fertilization.

Figure 2 - 1 The early human implantation development in vitro [11].

In the Image (e), tight junctions form around the 16 – 32-cell stage of development and the embryo becomes a tight ball of cells known as a ‘morula.’ During the stages shown in Image (f) – (g), the embryo continues to divide, now more quickly, and fluid begins to accumulate inside the embryo forming the blastocoelic cavity, which subsequently expands, giving rise to an expanded blastocyst. In Image (h), it comes to the sixth day, and the blastocyst hatches from the zona pellucida (ZP) to begin implantation [12].

When it comes to the 4th day after the fertilization, peripheral cells of the morula begin to compact each other. The two to four innermost cells of the previous morula develop to the inner cell mass (ICM) of the blastocyst. The actual embryo will develop solely from these cells, the embryoblast. At the same time, the outer cell mass is formed, consisting of many flat cells, which is called trophoblast. The infantile part of the placenta and the fetal membranes will arise from the trophoblast [11]. Figure 2 - 2 shows the distribution of the cells of the embryo at the blastocyst stage.

Figure 2 - 2 Blastocyst scheme [11].

Around the end of the fifth day, the embryo frees itself from the enveloping pellucid zone (same with zona pellucida, abbreviated as ZP). It is supported by the enzymes that dissolve the ZP membrane at the abembryonic pole. This “first birth” is called the hatching, which is shown in Figure 2 - 3.

Figure 2 - 3 Embryo hatching scheme [6].

2.1.2 Embryo Mechanical Properties

Three popular existing bio-capsule elastic models are proposed to analyze mechanical properties of the embryos, which are the mechanical contact model, micropipette aspiration model, and the bio-membrane point-load model. These models can help us obtain some mechanical properties of the embryos and are beneficial for us to analyze the piezo-driven piercing and cutting. Meanwhile, they have limitations and are not fully appropriate for our scenario.

The mechanical contact models, such as the Hertzian model [13] and the Sneddon model [14], view the deformed object as an elastic solid. However, the relatively large compression of the embryo in experiments does not obey the small deformation assumption of the mechanical contact models. Finally, the mechanical contact models assume there is a local geometry change, and the global geometry of the deformed object is unchanged, however, the deformation of the embryo is not limited to the contact area but will influence other volumes of the whole embryo. Thus, the mechanical contact models are not applicable to this scenario.

The micropipette aspiration model makes use of the deformation of the cells and sucking pressure to calculate the mechanical properties of the cells. Usually, the micropipette

sucks the testing cell in its axial direction, and the camera takes photos to record the deformation of the cell, as illustrated in Figure 2 - 4. This process is some different from our scenario, and we only aim to hold the embryo and try to keep the embryo's shape as unchanged as possible. Therefore, the micropipette aspiration model cannot be used directly to our application.

Figure 2 - 4 The analysis for the cell elastic problem in Aspiration model [15].

The bio-membrane point-load model is a modified model of the micropipette aspiration model presented in [16], as illustrated in Figure 2 - 5. It analyzes the penetration force and cell deformation during the low-speed piercing process. The F and Young's module of mouse oocytes and embryos are calculated within this model. According to their computing and experiment results, the ZP puncturing force is $13 \mu\text{N}$ for mouse embryo and $7.5 \mu\text{N}$ for oocytes. The calculated mouse embryo ZP elastic modulus is 42.2 kPa .

Figure 2 - 5 Indentation of a cell membrane by a micropipette [16].

2.2 Embryo Biopsy Approaches

Embryo biopsy is the indispensable part of for the PGT, which is to obtain a part of active living cell samples for further genetic diagnosis. According to the development stage, there are three different biopsy approaches Polar body biopsy, Cleavage stage biopsy, and Blastocyst (Trophectoderm) biopsy. This section introduces the pros and cons of these three biopsy approaches.

- **Polar Body Biopsy**

Polar bodies (PB) are by-products of the oocyte in the meiotic division [3]. PB biopsy works since the first polar body of the mature oocyte contains 23 bivalent maternal chromosomes. This unique structure is useless for the following embryo development. Thus, it can be removed and used for genetic testing or aneuploidy screening of the oocyte before fertilization [17] [18]. On fertilization, a second polar body, containing a complement of 23 maternal chromatids, is extruded from the oocyte and can be used for testing [12].

During the polar body biopsy, as illustrated in Figure 2 - 6, the first step is to find and rotate the oocyte cell to position two polar bodies aligned in one focal plane (Image A), then opening a hole in the Zona Pellucida using 2 or 3 laser shots (Image B), finally aspirating the polar body to the micropipette through the hole (Image C). Those aspirated polar bodies are displayed separately for further processing (Image D). The second step, to open a hole, is the critical step which has a significant influence in the biopsy, three standard technical methods will be discussed in the next section.

Figure 2 - 6 Procedures of laser-assisted polar body biopsy [3].

PB biopsy only removes the waste part, two polar bodies, from the oocyte. There is no ethics concerning due to its operation object is an oocyte instead of an embryo. Thus, PB biopsy is encouraged as a valuable alternative to cleavage biopsy because the human embryo operation is not allowed in some countries [19] [3]. However, the applicability of PB biopsy has always been under debate. It has an apparent weakness that PBs biopsy method cannot detect the mitotic and paternally derived aneuploidies because the detected information is only from the maternal part. PB biopsy has been used only 10~15% of all biopsy procedures performed in Europe during the last decade [4].

• **Cleavage-Stage Biopsy**

Compared to the PB biopsy, the cleavage-stage biopsy can offer intact information from both parents. In the cleavage stage, on the day 3 after oocyte fertilization, individual cells of the cleaving embryo can be distinguished easily, which is around 6~8 cells stage showed in the Image (d) of Figure 2 - 1. Individual cells in the embryo begin to compact each other after day 3, and the connection between two closed cells will form, after that, it will become challenging to pierce and to strip intact individual cell from cells group. Therefore, the cleavage-stage biopsy is chosen at the end of day 3 after fertilization. The localization and the zona pellucida opening ways of the cleavage-stage biopsy are similar to those of polar body biopsy, and the aspiration is illustrated in Figure 2 - 7. A sampling micropipette is stabbed into the embryo, and a single nucleated blastomere is removed by the suction force.

Figure 2 - 7 Cleavage-stage biopsy [12].

However, a theory for the embryo development is that embryo cells are relatively fragile at the preimplantation stage since cell differentiation processes and embryonic genome activation have not finished yet. Thus, it may cause irreparable hurt for the downstream development process if one inner cell is removed. Such influence is also reflected by the

relatively lower blastocyst rate of cells after cleavage stage biopsy with respect to those intact cells, as reported in several papers [20] [21] [22].

There are only 6~8 cells in the cleaving embryo. If one cell is stripped at this time, it causes a significant proportion mass loss for the embryo and may generate unpredictable potential harm for future development [23] [24]. Strong evidence was provided by Scott Jr. [25] in 2013 that cleavage-stage biopsy dramatically reduced embryonic reproductive potential.

- **Trophectoderm Biopsy**

Trophectoderm (TE) biopsy, also named as blastocyst biopsy, typically takes place on the day five or six after fertilization and the biopsied samples are some trophoctoderm cells, as illustrated in Figure 2 - 8. TE biopsy approach was a momentous breakthrough in modern IVF. It is gradually taking the place of the PB biopsy and Cleavage biopsy. The first live birth following the TE biopsy technology was reported in 2005 by Kokkali and colleagues [26] and by McArthur and colleagues [27]. However, one of the critical limiting factors for TE biopsy technology is the high standard for blastocyst culture and cryopreservation. Regardless, TE biopsy can provide a higher live birth rate per embryo transfer than cleavage biopsy as long as the required environment is prepared well enough [4]. Several papers in the literature reviewed no blastocyst degeneration after biopsy [27] [28] [29], and the survival rate after warming is always higher than 95% [27] [30] [31].

Two different methods have been published to perform trophoctoderm biopsy in Figure 2 - 8. The first one was put forward by McArthur and colleagues [27]. A hole is drilled in the zona pellucida on day 3, and the biopsy of hatching trophoctoderm cells is through that hole. Hatching can either occur far from the ICM or involving the ICM itself [4].

The second method was provided by Capalbo [32] and operated simultaneous ZP opening and TE cells biopsy. The embryo will not be biopsied until it is reaching full expansion on day 5 or day 6 after fertilization, as illustrated in Figure 2 - 8(b).

Figure 2 - 8 Schematic comparison between two different TE biopsy ways, (a) day 3 hatching, day 5 or day 6 biopsy; (b) simultaneous hatching and biopsy on day 5 or 6. [4]

Compared to the cleavage stage biopsy, the blastocyst stage biopsy keeps the embryo intact until day 5 or day 6, without disturbing the development of inner cells. Even the first operation approach in Figure 2 - 8 (a), it only operates at ZP area instead of touching inner cells. Besides, it is relatively easier to operate in the blastocyst stage than PB or cleavage-stage because the embryo becomes mature to implantation on day 5 or day 6, it tends flows out of ZP membrane. What is more, there are up to 300 trophoblast cells, and only a tiny fraction of whole cells is removed. The trophoblast cells will develop to placenta instead of the fetus after implantation. A real milestone is that Scott Jr. and colleagues verified blastocyst stage biopsy generate no effect on embryo viability and implantation potential [25].

2.3 Embryo Biopsy Techniques

For human embryo, its diameter is about $\varnothing 100\text{ }\mu\text{m}$ [33]. The outermost ZP membrane is a highly elastic layer composed of glycoproteins surrounding an embryo [34]. There are usually three ways to create an opening on the surface of the embryo cell: Tyrode's chemical drilling [35], laser-assisted method [36], and mechanical method [37].

2.3.1 Chemical Drilling

Since ZP membrane can be dissolved in a low-pH solution, the acid Tyrode (AT) solution has often been used to clean the ZP membrane of animal and assist fertilization [38] for decades. It is also used clinically to separate a human eight-cells to single blastomeres for preimplantation genetic testing in the 1990s [39] [40].

Figure 2 - 9 Schematic for chemical ZP drilling [40].

Traditionally, as shown in Figure 2 - 9, embryos are stabilized by one holding pipette at 9 o'clock orientation, and another $10\text{ }\mu\text{m}$ drilling micropipette containing AT solution gets close to 3 o'clock orientation, closing to an area of empty perivitelline space. A $20\sim 30\text{ }\mu\text{m}$ diameter hole is created by blowing the AT solution to the ZP area. Embryos should be washed several times before they are transferred to the cultivation system.

Chemical AT solution is a simple method used for decades; however, the main concern is that its potential influence of wrong operation for the development of the embryo. The extra volume of AT solution or improper washing both harm inner cells of the embryo.

Besides, research shows that it usually takes a highly skilled operator 30 s to 2 mins to complete the whole process [41]. Based on these drawbacks, it is not so widely used in embryo biopsy these years.

2.3.2 Laser-Assisted Biopsy

The laser-assisted biopsy is a non-contact technology. It utilizes the heat of a laser beam to burn the cell and then create a through-hole. The famous laser device is the product of Hamilton Thorne, ZILOS-tk laser. As Figure 2 - 10 shows, it can be easily installed

Figure 2 - 10 ZILOS-tk laser [42].

on most inverted microscopes with different objective lenses. The laser-assisted technology can be used in embryo hatching, trophectoderm biopsy, and blastomere biopsy. It does not need too much manual preparation; if the embryo cell is focused on the plane, the laser can fire and open required holes. Thus, the operating time is short, and the embryo is exposed to the non-incubator environment for a short time, which is beneficial to further embryo cultivation.

Figure 2 - 11 Isotherm Rings [43].

The potential harmful heat effect is the most concerned point for laser-assisted technology. As illustrated in Figure 2 - 11, the innermost ring is with the highest temperature, the orange ring is the “drilling ring,” which will open the hole. According to the study of Jerome Conia [43], the safe set up for the embryo is with laser pulse duration ≤ 5 ms and laser power ~ 100 mW. However, Taylor and his colleagues highlighted that when varying the laser pulse power from 20 mW to 400 mW, there is no significant adverse effect on outcomes after biopsy [44].

While the laser-assisted technique is developed fast these years since its convenience, high efficiency, and accuracy, it has several shortcomings. The laser beam runs through

Figure 2 - 12 Schematic of laser-assisted biopsy.

the hole and creates the trench-like holes, which increases the chance of cell lysis. As illustrated in Figure 2 - 12, the length of the burned run-through hole is about 75 μm . However, the required length of the embryo trauma is less than 30 μm , roughly estimated from the size of natural hatching, as shown in Figure 2 - 1 (h). Besides, lasers are expensive; for instance, the price of the standard product of Hamilton Thorne is 40,000 Singapore dollars, which is much higher than other biopsy techniques. The high cost of lasers prevents its promotion to more clinics and patients.

2.3.3 Mechanical Biopsy

The mechanical biopsy is a contact way to open the outmost ZP membrane and obtain cell samples. Its primary operations include penetration and dissection. According to the actuation ways, it can be divided into passive and active approaches.

Passive approach

A penetration micropipette pierces through the ZP and cell membrane to perform the dissection manually in passive cutting approach, and the cell is fixed by a holding pipette, as illustrated in Figure 2 - 13. In the experiments, mouse embryos are often used to test because of their similarity with human embryos. According to the study of Yu Sun and his colleagues [16], the penetration deformation of the oocyte is 42.2 μm , and the piercing force is 7.21 μN ; for the mouse embryo, the penetration deformation is 52.3 μm , and the piercing force is 12.7 μN . In Figure 2 - 13, Image (a) shows the static mouse oocyte, and Image (b) shows the deformation of oocyte increases with the piercing force of pipette increasing. The micropipette can penetrate the oocyte when the force increases upper to the critical threshold. Image (c) and (d) show the similar processes of the mouse embryo.

Figure 2 - 13 Passive mechanical drilling [16].

The device of passive mechanical approach is simple, yet it gradually discarded because of some problems. It requires highly skilled operators and consumes too much time. It is challenging to guarantee accuracy since the force applied to ZP is hard to control by operators. The deformation of the cell is also too large to be accepted, which may generate negative to subsequent embryos development. Therefore, alternative devices need to improve these drawbacks.

Active approach

The piezo-driven micropipette, a high-frequency vibration cutter, is utilized to penetrate and dissect the ZP membrane in the active approach. The deformation of oocyte/embryo decreases dramatically if the micropipette pierces into the cell with a high-speed axial vibration, as shown in Figure 2 - 14 (a) [5]. The piezo-driven micropipette can also be applied to remove the ZP material by rubbing the ZP area repeatedly, as shown in Figure 2 - 14 (b) [45].

Figure 2 - 14 (a) Active mechanical oocyte injection [5]; (b) Active mechanical ZP dissection [45].

The mechanics of piezo-assisted penetration is analyzed in the previous work [16] [46]. When the force applied to the local cell membrane is upper to the critical threshold of the cell membrane, it will be ruptured. The piezoelectric actuator helps the micropipette to pierce the cell with high speed. When the high-speed micropipette contacts with cell, the impact causes high acceleration to the local contacting membrane, which is beneficial to rupture the latter one with little cell deformation.

However, one common problem for piezo-driven micropipette is that high-frequency reciprocation along the axial direction is prone to excite uncontrollable lateral vibration since the micropipette tip is slender. It is difficult to control the precise axial and lateral vibration of the piezo-driven micropipette.

Besides, the effect on lateral vibration is strongly controversial among different researchers. Early researchers think the lateral vibration of the micropipette tip is

harmful to embryo penetration. They tried many methods to minimize lateral vibration. Some researchers add small amount mercury in the micropipette tip, as illustrated in Figure 2 - 15, to decrease the lateral vibration and increase the piercing success rate dramatically [47] [48] [49]. Ediz's group [50] developed a micro-dynamics model of the piezo-driven micropipette and claimed that mercury influenced nature frequency of the pipette because of the increasing mass. Gan's work [6] proved Ediz's point of view. However, due to the toxicity of mercury, this method was gradually abandoned by later researchers.

Figure 2 - 15 Conventional piezo-drill system. [51]

Haibo Huang's group insists the lateral vibration of the micropipette tip is detrimental [52]. Instead of using mercury, he designed a simple mechanism holder between the micropipette and piezoelectric actuator [53], as illustrated in Figure 2 - 16. This device is to centralize the piezoelectric oscillation power on the injection micropipette. It controls the lateral vibration amplitude of micropipette tip around $30\text{ }\mu\text{m}$ and the axial one less than $0.7\text{ }\mu\text{m}$ in the water medium. However, cell deformation is still apparent during penetration. Plus, the device fails to control the micropipette accurately. The lateral vibration is too large, and the axial one is small.

On the contrary, some researchers hold the view that lateral vibration is useful. Zenan Wang's study [54] claims that lateral vibration is more beneficial than axial vibration in piezo-assisted Intracytoplasmic Sperm Injection (ICSI). According to the latest research from Yu Sun's group, the huge lateral vibration ($> 10 \mu\text{m}$) is harmful, but little lateral vibration ($1\sim 2 \mu\text{m}$) is helpful for embryo penetration in ICSI. Their device limited lateral vibration within $2 \mu\text{m}$ and achieved a relatively large axial vibration, $1.2 \mu\text{m}$ at 18 kHz [7]. They penetrate the cell very well. However, the little lateral vibration limits the ability to cut the cell ZP membrane. Besides, it is not economical to prepare auxiliary devices for the operating system, such as the voltage amplifier which can supply a broad bandwidth (up to 18 kHz) is expensive.

Figure 2 - 16 Ultrasonic cell injector. [53]

Compared with the chemical and laser-assisted techniques, piezo-assisted active mechanical biopsy devices are easy to use and with low cost. Currently, there are no devices that can achieve penetration and dissection with little cell deformation, which require an accurate micropipette vibration control in both axial and lateral directions. In

the latest research work, the flexure mechanism is integrated into the biopsy system as the guide mechanism to increase the controllability of micropipette. The flexure mechanism will be reviewed in the next section.

2.4 Flexural Mechanism

The flexure mechanism cannot rotate or translate directly like rigid bodies since the lack of various joints, such as prismatic joints, revolute joints, universal joints, and spherical joints. Such movements are mostly achieved by the deformation and different combinations of flexure hinges.

2.4.1 Flexure Hinge

Different profiles of flexures, such as circular [55] [56] [57], corner-filletted [55] [58] [59], and conic [55], have been developed till now, as shown in Figure 2 - 17. Their sensitive axes are along the Z direction in the Image (a) of Figure 2 - 17, and the sensitive movements are bending about the sensitive axis. The sensitive axis is defined as the axis along or around which the stiffness is minimum [60]. The non-sensitive axis is defined as the axis along or around which the stiffness is relatively large. Stress is usually concentrated at the surface of the thinnest part, which limits the maximum bending. In the flexure mechanism design, the deformation of the sensitive axis should be utilized to achieve the desired movements [60]. The thickness t , length l , radius r , and width b are the most critical parameters that influence the properties of the flexure mechanism. It should achieve a balance between the stiffness in various directions, mechanism weight and volume, and the complexity of manufacturing in the flexure mechanism design.

The circular hinge provides the high out-of-plane stiffness and the small in-plane deformation. Circular flexures have been applied in many situations [9], and many theoretic equations have been provided through a series of papers [57].

Figure 2 - 17 Different types of flexures hinges. [9]

Corner-filleted flexures have relatively low bending stiffness, making them proper to the high displacement institution. For the same direction, the elliptical flexure has much lesser maximum stress in the corner and thus, has better flexibility with longer fatigue life. There are also some other types of flexures, such as parabolic and hyperbolic. Table 2 - 1 provides a brief comparison for their stiffness, motion accuracy, and maximum stress. The detailed information can be found in reference [61] and [62].

Table 2 - 1 A comparison of different flexure geometries [9]

Flexures	Stiffness		Motion accuracy	Max. stress
	In-plane	Out-of-plane		
Circular	High	High	High	High
Corner-filleted	Low	Low	Low	Low-med
Elliptical	Low-med	Low-med	Med-high	Low
Parabolic	Med-high	Med-high	Med-high	Med
Hyperbolic	High	High	High	Med-high

Figure 2 - 18 Beam flexure design: (a) trimetric view of constant cross section corner-fillet flexure beam, (b) trimetric view of corner-fillet serial-compliant flexure, (c) top view, (d) top view of applied load, (e) expanded the view of corner-fillet flexure hinge. [9]

Double-hinged flexures (see in Figure 2 - 18) with a rigid connection link in the center section have been used in designing Micro/Nano positioners [63] [64] [65]. The vertical stiffness, in the Z direction, of the beam flexure can be increased by thickening the center section to create a double-hinged flexure, as shown in Image (b). Increasing the flexure height h is helpful to increase the vertical stiffness, but with a cost of the additional weight of the flexure mechanism. Decreasing the flexure length L (as a result of the flexure thickness T) increases the vertical stiffness as well. However, a shorter thinner beam will have higher stress concentration than a longer thicker beam flexure of equal stiffness. A detailed comparison between single beam flexure and double-hinge flexure can be found in reference [66].

2.4.2 Flexure Mechanism Designs

Different flexure mechanism designs have been presented for many applications. In this report, 2-DOF flexure mechanisms that create the precise linear movements are mostly desired for the embryo biopsy. Most of them are planar monolithic structures that are

cut off from a plate by electro-discharge machine (EDM), laser or waterjet cutting machines.

In Figure 2 - 19 (a), a simple four-bar linkage using flexure hinges is used as a flexure prismatic joint in the micro-motion stage [8] [67]. A parasitic motion is perpendicular to the working direction of the linear spring stage. Then, a compound linear spring stage is provided to avoid the parasitic motion in Figure 2 - 19 (b) [8].

Figure 2 - 19 Flexure linear spring stage. [8]

By utilizing the basic unit of four-bar linkage shown in Figure 2 - 19 (a), a piezo-driven flexure-guided mechanism is provided, as shown in Figure 2 - 20 [9]. The deformation of the flexure is exaggerated in the diagram. A preload is added to the piezoelectric actuator to guarantee that the flexure rebounds immediately under high frequency. The required preload can be calculated from dynamic sample mass and its acceleration and can be achieved using the fasteners, metal shims, and torque wrench. As the cases in Figure 2 - 19 (b) and Figure 2 - 20 shown, the flexure mechanism can be applied to many application scenarios.

Figure 2 - 20 Schematic of a preloading piezo-driven flexure mechanism. [9]

2.5 Summary

In this chapter, the properties of the embryo, the embryo biopsy approaches and technologies, and the flexure mechanism are reviewed comprehensively. We choose the proper biopsy approach and the relative technique and state the current research gap.

Trophectoderm biopsy, as illustrated in Figure 2 - 8(a), is selected as the operation procedure because it is relatively safe and includes intact genetic information. For the other two approaches, PB biopsy is abandoned since it can only provide maternal genetic information; cleavage-stage biopsy causes a significant proportion of mass loss and reduces embryonic reproductive potential dramatically [25].

For three biopsy techniques: chemical drilling, laser-assisted biopsy, and mechanical biopsy, researchers conclude that they yield similar implantation and pregnancy rates if they are appropriately executed [68]. There is no universal selection for clinics as well, depending on different operating habits and financial ability. Chemical drilling is a traditional method which has been used for decades. The main limitations are that it requires highly skilled operators and costs much time (30 ~ 120 seconds). Laser-assisted device burns the embryo to achieve cell penetration and dissection. The operation for laser-assisted is easy and quick (less than 3 seconds). However, it is too expensive to be

afforded by all clinics. The mechanical biopsy includes the passive approach and the active approach. The passive approach requires the simplest equipment but usually generates significant deformation of the manipulated cell. The active approach, usually driven by piezoelectric actuator, can decrease the deformation dramatically. The operation is easy and fast (less than 3 seconds). Hence, we choose the piezo-driven active mechanical technique as the desired solution. The technical challenge for this solution is that it is difficult to control the vibration of the end-effector, micropipette tip, under the high-frequency axial actuation because the tip is slender and easy to involve bending. However, vibration control is vital for embryo dissection.

With the characteristics of rigid architecture, no backlash, no error accumulation, and compatibility, flexure mechanisms are widely employed in high-precision scenarios. It can be combined with the piezoelectric actuator to guide the end-effector, as examples shown in Figure 2 - 20. Its properties are mostly influenced by the shape and combination of the hinge. Hence, we choose the flexure mechanism to accomplish the precise vibration control of the micropipette tip.

To sum up, the current research gap is to achieve the dissection on the embryo ZP membrane with an economical technical solution. This work adopts a piezo-driven and flexure guidance method to control the vibration of the micropipette tip and to achieve the embryo dissection.

CHAPTER 3 System Design

3.1 System Overview

A system that consists of multiple components is needed to monitor, transfer, and manipulate the cell. The experimental setup is illustrated in Figure 3 - 1(a), which includes a microscope, a digital camera, a 3-DOF manipulator, a holding micropipette, a piezo-driven flexure micromanipulator, and two base holders for testing. The details of the micromanipulator and two installation ways are illustrated in Figure 3 - 1 (b) - (d). We define the x -axis and y -axis as in-plane and out-of-plane lateral directions, respectively. The z -axis is the axial direction, as shown in Figure 3 - 1 (b).

Figure 3 - 1 (a) Experimental setup. Through the digital camera and the microscope, a magnified view of the cell held by the holding micropipette and the micropipette tip used for dissection can be monitored. (b) Piezo-driven flexure micromanipulator, consisting of a piezoelectric actuator, a flexure mechanism, a micropipette connector, and an end-effector, the micropipette. (c) Base holder 1: for monitoring the out-of-plane (y -axis) lateral vibration of the micropipette tip. (d) Base holder 2: for monitoring the in-plane (x -axis) vibration of the micropipette tip.

In Figure 3 - 1 (a), the objective lens with a magnification of 10× is for monitoring cells. The digital camera records videos and images of the micropipette tip and cells with 97 frames per second (FPS). The 3-DOF manipulator controls the translation movements of the holding micropipette. The holding micropipette provides a negative pressure liquid to hold cells tightly.

Figure 3 - 1 (b) shows the piezo-driven flexure micromanipulator. The piezoelectric actuator is preloaded with the torque wrench. The peak-peak vibration amplitudes of the micropipette tip in three directions need to be tested, two lateral directions and one axial direction. Because the microscope can only monitor the vibration of the micropipette tip from top to down, there are two base holders for tests of two lateral directions, respectively, as shown in Figure 3 - 1 (c) and (d).

The Origio MAH-SM-0 assisted hatching micropipette is chosen as standard manipulation micropipette in this research. The inner and outer diameter of the micropipette tip is Ø 8 µm and Ø 10 µm, respectively. The length of the tip is 0.5 mm.

The Noliac NAC2002-H4 piezoelectric actuator is selected since its high blocking force F_b (387 N), the small volume (3 mm × 3 mm × 4 mm), and high response rate (up to 20,000 Hz). It can output a 1-DOF axial movement. The maximum peak-peak voltage $V_{\max}$ of the piezoelectric actuator is 60V in order to achieve the full stroke x_o , 2.9 µm. In practice, the stroke of the piezoelectric actuator x_p is usually lower than the full stroke and can be estimated as

$$x_p = \frac{V_{\text{dri}}}{V_{\max}} \times \frac{F_b - F_p}{F_b} \times x_o \quad (3 - 1)$$

where V_{dri} and F_p are the driving voltage and the preload of the piezoelectric actuator, respectively.

Figure 3 - 2 Connected power devices for the piezoelectric actuator.

The connected devices and wires connection are illustrated in Figure 3 - 2. The two-channel oscilloscope monitors the input and output voltage to guarantee the accurate driving source for the piezoelectric actuator. The function generator creates a sinewave voltage with a peak amplitude of 3 V and a driving frequency of 0 to 150 MHz. The voltage amplifier amplifies the signal with 20 times rates. Then the maximum driving voltage applied to the piezoelectric actuator can be up to 60 V. The flow chart of hardware connection for cell dissection is illustrated in Figure 3 - 3. The piezo-driven flexure micromanipulator transfers the 1-DOF output movement to the 2-DOF controllable vibration at the micropipette tip, which was used to dissect the cell. Cell dissection is monitored through the microscope.

Figure 3 - 3 Flow chart of hardware for cell dissection.

3.2 Embryo Dissection Strategy

According to the previous discussion, the trophectoderm (TE) biopsy approach is selected as the standard procedure. An opening channel on the surface of the ZP membrane should be dissected on day 3.5. A two-stage embryo dissection strategy is proposed, as shown in Figure 3 - 4. In stage 1, a hole is penetrated on the ZP membrane by utilizing the high-speed axial vibration of the micropipette tip, as shown in Figure 3 - 4 (a). In stage 2, the hole is enlarged to a long strip opening channel by the lateral cutting of the micropipette tip, utilizing the excited lateral resonant vibration of flexure mechanism. The flexure needs to be appropriately designed to guarantee that the resonance is only excited along with the y-axis. The coordinate defined in Figure 3 - 4 is constant with those in Figure 3 - 1.

The blue sphere, the transparent shell, and the blue tube represent the inner embryo cell, the zona pellucida, and the micropipette tip, respectively. The transparent tube in Figure 3 - 4 (b) represents the shadow of the micropipette tip during lateral vibration.

Figure 3 - 4 Schematic for the embryo dissection strategy. (a) Stage 1: Penetrate a hole on the surface of embryo ZP membrane, by the high-speed axial (z-axis) vibration; (b) Stage 2: Enlarge the opening channel by the y-axis lateral vibration of the micropipette tip.

The human embryo diameter is around $\varnothing 100 \mu\text{m}$ [69]. According to the human embryo hatching status shown in Figure 2 - 1 (h), the natural hatching opening is about one-third of embryo diameter, which is $30 \mu\text{m}$. Therefore, it is reasonable for the micropipette to create a $20 \sim 30 \mu\text{m}$ opening for assisted hatching.

Figure 3 - 5 shows a desired three-dimensional peak-peak vibration amplitude of the micropipette tip, which satisfies the desired vibrations of the dissection strategy. The figure is obtained based on the analysis of the mechanism proposed in Section 3.3, and it is validated by the experimental phenomenon in Section 4.2. In stage 1, the micropipette tip is pushed to the maximum position along the z -axis direction with high speed. No resonance has been excited in y -axis or x -axis directions during the first driving cycle. Its displacement can be estimated through Equation (3 – 1). Taking the preload $F_p = 120 \text{ N}$ (mentioned in Section 4.1) and the driving voltage $V_{\text{dri}} = 30 \text{ V}$ as an example, the transient displacement of the micropipette tip in the z -axis direction is around $1.2 \mu\text{m}$.

Figure 3 - 5 The desired three-dimensional peak-peak vibration amplitudes of the micropipette tip respects to the driving time, which satisfies the vibration requirements of the dissection strategy. In stage 1, the

high-speed axial (z -axis) vibration is responsible for penetrating a hole. In stage 2, the micropipette vibrates along with y -axis because of the excited resonance of the flexure mechanism, which is responsible for enlarging the hole.

In stage 2, after several driving cycles (less than 0.1 second), the axial vibration of micropipette tip gradually decreases to a stable state, since the stiffness of micropipette connector is much lower than the stiffness of the flexure mechanism in the axial direction (see the dynamics model illustrated in Figure 3 - 11). On the other hand, the y -axis resonance of the flexure mechanism can be excited when driving frequencies are close to its y -axis lateral resonant frequencies. Then we utilize y -axis lateral vibration to enlarge the hole. Considering only one lateral vibration is required according to the dissection strategy, the ideal design for the flexure mechanism is that the y -axis resonant frequency is far away from x -axis resonant frequency, to guarantee the resonance along two directions will not be excited at the same time.

3.3 Piezo-Driven Flexure Micromanipulator

The piezo-driven flexure micromanipulator consists of a piezoelectric actuator, a flexure mechanism, a micropipette connector, and a micropipette. In this section, the flexure mechanism is designed based on the dissection strategy and the micropipette dynamics. The micropipette connector and the simplified dynamics model of the system are also analyzed.

3.3.1 Micropipette Dynamics

It is easy to excite uncontrollable bending when the driving frequencies are close to resonant frequencies of the micropipette tip. The objective of this section is to build the dynamic model of the micropipette tip and to calculate its resonant frequencies; then we

can avoid those resonant frequencies as far as possible in the design of the flexure mechanism and the selection of the driving signal.

Figure 3 - 6 (a) The indentation of the pressed ZP membrane; (b) A simplified dynamic model of the interaction between the micropipette and the ZP. [7]

Figure 3 - 6 (a) is a schematic to describe the phenomenon when the micropipette tip presses the embryo. Figure 3 - 6 (b) is a simplified dynamic model relative to the interaction between the micropipette and the ZP established in Mehdi's work [46].

According to the Euler-Bernoulli beam theory [70], the movement equation of the micropipette tip can be written as

$$EI \frac{\partial^4 v}{\partial z^4} + F \frac{\partial^2 v}{\partial z^2} + \rho A \frac{\partial^2 v}{\partial t^2} + c \frac{\partial v}{\partial t} = 0 . \quad (3 - 2)$$

Boundary conditions are

$$v(0, t) = 0, \frac{\partial v(0, t)}{\partial z} = 0, \frac{\partial^2 v(L_{tip}, t)}{\partial z^2} = 0, \frac{\partial^3 v(L_{tip}, t)}{\partial z^3} = 0 \quad (3 - 3)$$

where z is the position along the axial direction of the micropipette tip before the shoulder; v is the lateral vibration displacement of the micropipette tip; E and I are the elastic modulus and the inertia area moment, respectively, of the micropipette; F is the axial force seen by the micropipette when in contact with the embryo; ρ and A are

density and crossing area of the micropipette, respectively; c is the viscous damping coefficient; and L_{tip} is the length of the micropipette tip.

According to the derivation work in Sun's group [7], assuming the micropipette tip is in free-vibrating status ($F = 0$, $c = 0$), then Equation (3 - 2) can be simplified as

$$EI \frac{\partial^4 v}{\partial z^4} - \rho A \omega^2 v = 0 \quad (3 - 4)$$

where ω is the vibration frequency of the micropipette tip. Rewriting Equation (3 - 4),

$$\frac{\partial^4 v}{\partial z^4} - \beta^4 v = 0 \quad (3 - 5)$$

Where $\beta^4 = \frac{\rho A \omega^2}{EI}$, from which resonant frequencies can be derived as

$$\omega_n = (\beta_n L_{\text{tip}})^2 \sqrt{\frac{EI}{\rho A L_{\text{tip}}^4}} = k_n^2 \sqrt{\frac{EI}{\rho A L_{\text{tip}}^4}} \quad (3 - 6)$$

where k_n is the wave number. The general solution of Equation (3 - 4) is

$$v = A \cosh \beta z + B \sinh \beta z + C \cos \beta z + D \sin \beta z \quad (3 - 7)$$

Substituting boundary conditions into Equation (3 - 7), the characteristic equation is obtained as

$$1 + \cos(\beta_n L_{\text{tip}}) \cosh(\beta_n L_{\text{tip}}) = 1 + \cos(k_n) \cosh(k_n) = 0 \quad (3 - 8)$$

where k_n is the wave number. We can find the resonant frequencies are sensitive to the length of the micropipette tip, L_{tip} , through Equation (3 - 6) [7]. A shorter L_{tip} can increase resonant frequencies, ω_n , which makes the tip more challenging to be excited. Thus, a shorter length of micropipette tip increases its stability in the vibration but also increases the fabricating difficulty.

The first three values of wave number k_n are 1.875, 4.694, 7.854 according to Equation (3 – 8) [7]. The material properties of micropipette is $\rho = 2,300 \text{ kg/m}^3$ and $E = 64 \text{ GPa}$ borosilicate glass. The geometric parameters include outer diameter $D_o = 10 \text{ }\mu\text{m}$, inner diameter $D_i = 8 \text{ }\mu\text{m}$, $A = 2.83 \times 10^{-11} \text{ m}^2$, $L_{\text{tip}} = 0.5 \text{ mm}$, and $I = \frac{\pi}{64} (D_o^4 - D_i^4) = 2.90 \times 10^{-22} \text{ m}^4$.

By substituting micropipette parameters and the first four wave numbers into Equation (3 – 6) [7], we analytically solved the first three resonant frequencies, as listed in Table 3 - 1. Therefore, the driving frequency and the lateral resonant frequencies of the flexure mechanism should be in the range of 0 ~ 237.5 kHz.

Table 3 - 1 Micropipette Resonant Frequency

Mode	1	2	3
Resonant Frequency (kHz)	237.5	1488.4	4167.1

3.3.2 Flexure Mechanism Design

The flexure mechanism design is critical to guide the micropipette to achieve the desired vibrations described in stage 2 of the dissection strategy. Based on previous work [7], [9], we designed a flexure structure to guide the injection micropipette, as illustrated in Figure 3 - 7.

The device is fabricated by wire electrical discharge machining from an integral piece of 304 stainless steel, one suitable material widely used for manufacture of medical devices. Its monolithic structure eliminates the nonlinearities caused by wear, backlash, and friction.

Figure 3 - 7 (a) Schematic of the front view of the flexure structure, with the parameters needed to be determined. (b) Side view. (c) The single unit of the flexure hinge. (d) The prototype.

Figure 3 - 8 illustrates the relative position for the biopsy system and the embryo cell. In this flexure structure, the out-of-plane stiffness in the y -direction is lower than the in-plane stiffness in the x -direction. Thus, it is easier to excite the resonant vibration in y -direction than in x -direction.

Figure 3 - 8 Schematic of the relative position for the biopsy system and the embryo.

Considering the strategy illustrated in Section 3.2, the design objective is to separate the y -axis resonant frequency and x -axis resonant frequency of the flexure structure as far

as possible. Meanwhile, they should be far away from the resonant frequencies of the micropipette. This objective is achieved by adjusting six primary parameters shown in Figure 3 - 7, including the corner radius r , corner filleting radius r_f ; the number n , length l , thickness t , and gap Δ of the hinge.

Table 3 - 2 Dimensions of Biopsy System

Corner radius r	1 mm
Corner filleting radius r_f	0.5 mm
Hinge number n	8
Hinge length l	6 mm
Hinge thickness t	1 mm
Hinge gap Δ	4 mm
Micropipette length L_{pip}	45 mm
Micropipette tip length L_{tip}	0.5 mm
Micropipette tip outer diameter Φ_{tip}	$\varnothing$ 10 μm

We do the modal analysis simulation for the structure iteratively to optimize its geometrical dimensions; the optimal results are shown in Figure 3 - 9. The six primary parameters are listed in Table 3 - 2. The overall size of the flexure structure is 35 mm $\times$ 5 mm $\times$ 39 mm, which are length L , width W , and height H , respectively, as shown in Figure 3 - 7.

Figure 3 - 9 The modal analysis of the flexure structure, showing the mode shapes, translational resonant frequencies, and rotational resonant frequencies.

The mode shapes of flexure structure at first five resonant frequencies are shown in Figure 3 - 9. When the driving frequency is around 3,100 Hz, only the vibration along the y-axis is excited, which is the desired vibration for lateral cutting. Meanwhile, the excitation of this y-axis resonant vibration does not excite the resonance of micropipette, since 3,100 Hz is much lower than the first resonant frequency of micropipette, 237.5 kHz mentioned in Table 3 - 1. What is more, a lower driving frequency solution requires a narrower bandwidth voltage amplifier, which can decrease the cost of the whole system compared to the higher driving frequency one.

Therefore, in subsequent experiments, although vibration tests are executed in the band of 0 to 20 kHz, fine adjustment of the lateral vibration of the micropipette tip is concentrated around the driving frequency of 3 kHz.

3.3.3 Micropipette Connector

The connecting structure combines the flexure mechanism and the micropipette, as shown in Figure 3 - 10, which consists of a nut, a hollow pin, and a rubber ring. The principle is that, when the nut is tightened, it presses the hollow pin downside as the two big red arrows shown in Figure 3 - 10 (b). Then the hollow pin presses the rubber ring and creates a slight deformation. The deformation of the rubber ring causes uniform pressure and friction to the exterior wall and the micropipette, as the small red arrows are shown. By utilizing the friction, the rubber ring connects the flexure mechanism and the micropipette.

Figure 3 - 10 Schematic of the micropipette connector. (a) Picture of a system prototype. (b) Cross-sectional view of connecting structure (without the nut). Two big red arrows represent the vertical pressure applied from hollow pins to rubber ring; small red arrows represent the uniform pressure (friction) that connects the micropipette and flexure structure.

This connecting structure is widely used in various situations that required connecting and sealing tasks. This standard design is chosen in this research since it ensures the repeatability of the experiments, and the simple structure is easy to replace.

Figure 3 - 11 Schematic of the dynamics model for the flexure mechanism and the micropipette connector. The flexure mechanism and the micropipette connector can be simplified as the dynamic model shown in Figure 3 - 11. As mentioned in the dissection strategy in Section 3.2, in the axial direction, the stiffness of the rubber ring K_R is much lower than the stiffness of the flexure structure K_F ; the damping of the rubber ring c_R is also much lower than the stiffness of the flexure structure c_F . Thus, the micropipette cannot move with the flexure mechanism synchronously under high-frequency reciprocation in the axial direction. The axial vibration of micropipette is gradually approaching a stable state, as shown in stage 2 in Figure 3 - 5. What we utilize for the penetration is the high-speed axial vibration of the micropipette tip in stage 1.

3.4 Summary

In this chapter, the setup of the whole system is introduced. We propose a two-stage embryo dissection strategy, in which one hole is penetrated by the axial vibration of the micropipette tip, and then, the hole is enlarged by lateral cutting.

The piezo-driven flexure micromanipulator is developed. We analyze the dynamic model of the micropipette tip and calculate its resonant frequencies. A flexure structure

is designed and fabricated based on the above analysis and the dissection strategy. The geometric parameters of the structure are optimized by iterative modal analysis simulation. In the end, the micropipette connector is introduced, and the simplified dynamic model of the connector and the flexure is analyzed.

To sum up, the theoretical analysis, design work, and fabrication for hardware part are accomplished in this chapter. It provides experimental setups and guidance for the subsequent experiments. The control method and validation experiments are executed in the following chapters.

CHAPTER 4 Vibration Control of Micropipette Tip

In this chapter, a set of experiments test the three-dimensional peak-peak vibration amplitudes of the micropipette tip in the liquid medium. With amounts of useful data, we found the driving frequency and the driving voltage are two critical variables that influence their vibration performance. The desired vibrations in the dissection strategy are achieved, and an effective control method is summarized.

4.1 Experimental Setup

As above mentioned, three-dimensional vibration amplitudes of the micropipette tip need to be tested. Since the microscope can only monitor the vibration of the micropipette tip in the horizontal direction, from top to downside, two experiment setups are prepared for the x -axis and y -axis lateral vibration amplitudes. The schematics for the relative position and the XYZ coordinates are shown in Figure 4 - 1 (a) and (b), respectively. The x -direction and y -direction are in-plane and out-of-plane lateral directions, respectively. The z -direction is the axial direction. The tilt angle θ is 14.4 degree which is designed to make the micropipette avoid the edge of the dish, and to leave the micropipette tip within the depth of view of the microscope, for easy monitoring.

The vibration amplitude is recorded by the digital camera with a low frame per second (97 FPS). Therefore, when the micropipette vibrates, its vibrational envelope is recorded as blurred edges, as shown in Figure 4 - 8. By measuring the distance between two most outside edge of the vibration-caused blurred micropipette, the peak-peak vibration amplitudes can be obtained. This method requires that the measured driving frequency should be much higher than and not the integer multiple of the sampling frequency of

the camera. Since the driving frequencies are integer multiples of 10 Hz, we choose 97 Hz, the largest prime number within the adjustable sampling frequencies of the camera, as the sampling frequency. In this way, the driving frequency can be avoided as much as possible, which is an integer multiple of the sampling frequency.

Figure 4 - 1 Schematics of the flexure installation for monitoring different lateral vibration amplitudes of the micropipette tip. (a) The out-of-plane, y-axis direction; (b) The in-plane, x-axis direction.

Based on the theoretical analysis in Chapter 3 and pre-experiment results, the driving frequency and the driving voltage of the piezoelectric actuator are found as two primary variables that influence the vibration performance. According to the proper installation of hardware as illustrated in Figure 3 - 7 and Figure 3 - 10, a series of tests are executed in the liquid medium, as shown in Figure 4 - 2. We adjust the driving frequency in the band of 10 Hz to 20, 000 Hz, with 10 Hz per step, under different driving voltage of piezoelectric actuator: 35 V, 40 V, 45 V, 50 V, and 55 V, in order to find the optimal combination of driving frequency and driving voltage to achieve the desired vibrations described in the dissection strategy.

Some critical experimental considerations in manipulation are listed below.

- (1) The piezoelectric actuator should be positioned accurately in the central axis of the flexure mechanism. The unaligned installation can generate a negative impact on the vibration performance.
- (2) The preload of the piezoelectric actuator should be one quarter to one half of its blocking force [71]. Enough preload is beneficial to guarantee the flexure structure move with the same frequency of the piezoelectric actuator; however, it decreases the stroke of the piezoelectric actuator proportionally. In this work, 120 N is preloaded to the piezoelectric actuator, about one-third of its blocking force, by the precise torque wrench. Correspondingly, its theoretical full stroke decreases to 2 μm from the original 2.9 μm , calculated by the Equation (3 – 1).

Figure 4 - 2 Schematic for the submerged depth of the micropipette tip.

- (3) The depth of the micropipette tip submerged in the liquid medium should be constant in all tests, as shown in Figure 4 - 2. The submerged depth influences the interaction mass and dynamic properties between the micropipette and saline water, furthermore, causes some slight influence on vibration results.
- (4) The saline water is selected as the medium since it has similar mechanical properties compared to the cultivating medium of embryos.

4.2 Experimental Results

4.2.1 Lateral Vibration

We test the peak-peak lateral vibration amplitudes (LVAs) in both x -axis and y -axis directions under different driving frequencies, in the band of 10 Hz to 20,000 Hz, and different driving voltages, from 35 V to 55 V. When the driving frequency is in the band of 3,600 Hz to 5,000 Hz, the LVAs of the micropipette tip in both x -axis and y -axis directions varies from 0 to 30 μm . It basically satisfied our requirements, and it is possible to select a proper driving frequency. The other driving frequencies are abandoned due to various reasons.

When the frequency is under 3,600 Hz, the maximum LVAs of micropipette tip is no more than 10 μm , mostly less than 5 μm , which is small to be ignored when compared to the desired LVAs, 20 ~ 30 μm .

When the driving frequency is in the band of 6,000 Hz to 12,500 Hz, the LVAs change abruptly and randomly, which is difficult to conclude a stable and repeatable vibration curve. Besides, many recorded images are not clear, shown in Figure 4 - 3; the blurred image means that the micropipette tip is not focused correctly. Considering the depth of view of the microscope is around 100 μm , it indicates that the LVAs in two directions are both significant, which is not the desired result.

Figure 4 - 3 One blurred image of the lateral vibration in the band of 6,000 to 12, 500 Hz.

What is more, the cavitation phenomenon is found when the driving frequency is around 7, 000 Hz, which introduces more uncontrollable factors to the interaction between the micropipette and the liquid medium. Meanwhile, the shrill noise caused by the piezoelectric actuators during this range is uncomfortable for human beings.

When the driving frequency increases up to 13, 000 Hz, though the noise gradually decreases; the LVAs are not stable, either. What is more, the higher driving frequency requires a broader bandwidth of the voltage amplifier, which increases the cost of the system significantly.

Based on the above reasons, the driving frequencies in the band of 3, 600 to 5, 000 Hz are chosen to do the subsequent fine-tune experiments. The vibration amplitudes have been tested three times under each combination of driving frequency and driving voltage. The average of the three measurements is used for the below figures. The x -axis LVAs curves rise and fall with respect to the driving frequency, under different driving voltages, as shown in Figure 4 - 4. The x -axis LVAs are almost not excited in the band of 4,400 to 4, 600 Hz, under whichever driving voltage.

Figure 4 - 4 The x -axis LVAs curves with respect to the driving frequencies, under different driving voltages.

The y -axis LVAs curves with respect to the driving frequency, under different driving voltages, are shown in Figure 4 - 5. The y -axis LVAs reach the peak at the frequency around 4,400 Hz.

Figure 4 - 5 The y -axis LVAs curves with respect to the driving frequencies, under different driving voltages.

Taking the curve at 50 V as an example, two curves along x -axis and y -axis directions are compared in Figure 4 - 6, some typical points are marked. The main lateral vibration direction changes with the change of the driving frequency. For example, at the driving frequency of 4,400 Hz, the y -axis LVA is 28 μm ; the x -axis one is 1 μm . However, at the driving frequency of 4,800 Hz, the y -axis LVA is 2 μm ; the x -axis one is 13 μm . This phenomenon matches the modal analysis simulation in Section 3.3.2, which indicates the lateral resonant vibration of the flexure mechanism along with different directions, can be excited at the proper driving frequencies.

Figure 4 - 6 The comparing between the x -axis and y -axis LVAs at 50 V.

What is more, LVAs are influenced by the driving voltage. Specifically, some typical frequencies are selected to show the relationship between LVAs and the driving voltage in both lateral directions. In Figure 4 - 7 (a), the LVAs in two lateral directions are both excited and increase monotonously with the increase of the driving voltage. However, in Figure 4 - 7 (b), the x -axis LVAs are close to zero at the driving frequency of 4,500 Hz since x -axis vibration is not excited, on the contrary, the y -axis LVAs are the main vibration at this frequency, and it increases monotonously.

Figure 4 - 7 The LVAs curves with respect to the driving voltage, in both x and y directions. (a) At the driving frequency of 4,700 Hz; (b) At the driving frequency of 4,500 Hz.

Two rules are concluded according to Figure 4 - 6 and Figure 4 - 7. First, the driving frequency decides whether the resonant vibration is excited or not. Second, the LVAs increase monotonously with the increase of the driving voltage, when the resonant vibration is excited.

The driving frequencies in the band of 4,400 to 4,500 Hz and the driving voltage of 50 V are selected as the driving parameters for subsequent embryo dissection experiments. With these driving parameters, the y -axis LVAs are much more significant than the x -axis LVAs. The former is suitable to create the 20 ~ 30 μm opening, and the latter is close to zero.

Figure 4 - 8 Lateral vibration images in different frequencies at 50 V.

Figure 4 - 8 shows some recorded images of LVAs in both x -axis and y -axis directions. The peak-peak vibration amplitudes are obtained by utilizing the marked values minus the diameter of the micropipette tip, $\varnothing 10 \mu\text{m}$. Different from the severely blurred image in Figure 4 - 3, edges of the micropipette tip in Figure 4 - 8 can be classified clearly.

4.2.2 Axial Vibration

The peak-peak axial vibration amplitude is not as apparent as the lateral one. Considering the preload, the maximum theoretical output stroke of the piezoelectric actuator is only $2 \mu\text{m}$ as above mentioned, under the situation without load and in low-frequency actuation. Thus, the axial vibration amplitude of the micropipette tip should be small since no axial resonant vibration of flexure mechanism or micropipette is excited when the driving frequency is in the band of 3, 600 Hz to 5, 000 Hz.

In experiments, the axial vibration is relatively apparent only at the initial moment of changing the driving voltage, as shown in Figure 4 - 9. After a very short time, the axial vibration seems like “disappearing,” because the resolution of our digital camera ($0.5\ \mu\text{m}/\text{pixel}$) cannot detect the small vibration amplitude. The experiment phenomenon supports the analysis of the axial vibration in Figure 3 - 5 and the explanation of the dynamic model in Figure 3 - 11.

Figure 4 - 9 Peak-peak axial vibration amplitudes at different driving voltages, at the driving frequency of 3, 800 Hz. (a) still; (b) 30 V; (c) 35 V; (d) 40 V; (e) 45 V.

At the moment of changing the driving voltage, it actually changes the peak-peak axial vibration amplitude of the micropipette tip, as described in stage 1 in Figure 3 - 5. Then the high-speed axial vibration is utilized to penetrate a hole.

Figure 4 - 9 shows a series of images for the same micropipette driven at 0 V, 30 V, 35 V, 40 V, 45 V, at the driving frequency 3, 800 Hz. The peak-peak axial vibration amplitudes are roughly measured since the resolution of the camera ($0.5\ \mu\text{m}/\text{pixel}$) is not high enough to test the accurate value, but the phenomenon indicates similar vibration rule with the lateral vibration. The peak-peak axial vibration amplitude increases monotonously with the rising of the driving voltage.

The 3,800 Hz is chosen since the axial vibration is a little more apparent at this frequency than at other driving frequencies. The vibration rule also adapts to other driving frequencies.

4.3 Discussion

In this series of experiments, the vibration control method for the micropipette tip is concluded as, for the axial direction, the peak-peak vibration amplitude can be adjusted by changing the driving voltage. For the two lateral directions, firstly, adjust the driving frequency to let the flexure mechanism vibrate in the desired lateral direction, and to make its peak-peak amplitude close to the desired value. Then fine-tune the driving voltage to achieve the accurate control of the lateral vibration of the micropipette tip. Figure 4 - 4 and Figure 4 - 5 can be referred to choose the proper values for driving frequency and driving voltage.

We also conclude some rules of thumb based on the vibration testing results. First, the driving frequency decides whether the lateral resonant vibration of the system is excited or not. The proper driving frequencies are found to excite the system to achieve desired vibrations. Usually, the change of driving frequency leads to a quick and relatively significant change of the peak-peak lateral vibration amplitudes.

We must mention that, for the driving frequency that can excite lateral resonant vibration, there is some difference between the simulation results (3,100 Hz) and the experimental data (4,400 ~ 4,600 Hz). The main reason for the difference is that the simulation model is only the flexure structure, without considering the piezoelectric actuator and the micropipette connector. The experimental data are adopted as the standard in the subsequent experiments.

Second, in both lateral and axial directions, the peak-peak vibration amplitude of the micropipette tip increases monotonously with the increase of the driving voltage. Meanwhile, we found the adjusting of driving voltage changes the peak-peak vibration amplitudes slightly and smoothly, without the abrupt jumping. Thus, the driving voltage can be used to fine-tune the peak-peak vibration amplitudes.

What is more, the submerging depth of the micropipette tip is a variable for the control of the vibration of the micropipette tip. It is recommended to maintain a constant depth during the dissection.

To sum up, the accurate vibration control of the micropipette tip along three directions based on this piezo-driven flexure micromanipulator can be achieved by following the above method. Furthermore, we will do embryo dissection experiments.

CHAPTER 5 Embryos Dissection

In this chapter, a set of experiments test the embryos dissection performance of the piezo-driven flexure micromanipulator based on the accurate vibration control of the micropipette tip. The dissection refers to the penetration and the lateral cutting of the embryo ZP membrane. It is successful if an opening is created with little cell deformation. An effective dissection procedure is summarized, and results show the embryo can be dissected successfully.

5.1 Experimental Setup

Based on the results of vibration experiment, the driving frequency at 4, 400 Hz, and the driving voltage at 50 V are selected to manipulate the cell. The experimental setup shown in Figure 5 - 1 is inherited from the vibration tests setup shown in Figure 4 - 1 (b), since the y-axis lateral vibration is the primary one utilized for embryo cutting. We are able to monitor cell trauma from this perspective. The manipulated cells are mouse morula-stage embryos developed to day 3.5 ~ day 4.

Figure 5 - 1 Experiment setup for embryo dissection.

The experimental procedures in Figure 5 - 2 are listed below.

- (1) One drop of medium concluding one cell is positioned at the center of the dish, shown in Figure 5 - 2 (a).
- (2) Move the holder to hold the cell.
- (3) Add saline water to the dish until the dish is full.
- (4) Move the holder up to the horizontal level of the micropipette
- (5) Move the micropipette close to the cell. The micropipette, the embryo cell, and the holder should be aligned on the same focus plane.
- (6) Power on the piezo-driven flexure micromanipulator and manipulate the cell.

Figure 5 - 2 The schematic of the experimental procedures for the embryo dissection.

Through various experiments, two key points are concluded for successful dissection.

- (1) Move the micropipette and press the cell, generating a slight pre-pressed cell deformation on the surface of the embryo ZP membrane. Usually, a proper deformation is 3~5 μm for mouse morula-stage embryos.
- (2) Apply the driving voltage suddenly. The procedure is that the micropipette contacts the cell first, after then, power on the piezoelectric actuator. The driving voltage increases from 0 V to 50 V within one driving cycle (1/4400 second). The key point is to utilize the high-speed initial driving pulse of the piezo-driven flexure micromanipulator. The opposite operation is to power on the piezoelectric actuator first, after then, move the micropipette to manipulate the cell.

5.2 Experimental Results

With the well-trained driving frequency of 4, 400 Hz and driving voltage of 50 V, the proper pre-pressed cell deformation and the suddenly applied driving voltage, the piezo-driven flexure micromanipulator dissects the embryo with a little deformation, no more than 5 μm , as shown in Figure 5 - 3.

Figure 5 - 3 Successful mouse embryo (day3.5) dissection with a pre-pressed deformation and suddenly applied driving voltage. (a) Image before applying sudden driving voltage, the micropipette presses the ZP membrane of mouse embryo with a proper pre-pressed deformation; (b) Image after applying sudden driving voltage, an opening is created with little deformation.

Compared to the passive penetration, which is shown in Figure 2 - 13 (d) with a $50\text{ }\mu\text{m}$ huge deformation, the piezo-driven flexure micromanipulator improves the dissection performance significantly.

In contrast, some cases failed either because a proper pre-pressed cell deformation is missed or because the driving voltage is not applied suddenly. For the case in Figure 5 - 4, the micropipette just contacts the embryo, without a proper pre-pressed cell deformation, then it fails to create an opening after applying the sudden driving voltage.

Figure 5 - 4 Failed dissection case because of no pre-pressed cell deformation. (a) Image before applying sudden driving voltage. The micropipette does not press properly on the surface of the embryo ZP membrane; (b) Image after applying sudden driving voltage. The micropipette fails to create an opening; the embryo remains intact.

Figure 5 - 5 shows another kind of failed example; the micropipette is driven to the high-frequency-vibrating state firstly instead of applying the driving voltage suddenly. It fails to create an opening even though it has pressed the embryo with an apparent deformation.

Figure 5 - 5 Failed dissection case because of no suddenly applied driving voltage. (a) Image before the micropipette manipulates the embryo. The piezoelectric actuator has been applied driving voltage, and the micropipette is in a vibrated state; (b) Image after the micropipette manipulates the embryo. The embryo remains intact even though an apparent deformation has been pressed.

In fact, it is easy to dissect the embryo ZP membrane with a little deformation if the piezo-driven flexure micromanipulator is utilized with a set of well-trained parameters (4,400 Hz and 50 V), a proper pre-pressed cell deformation (3~5 μm), and the suddenly applied driving voltage. In our experiments, the first four dissection cases of eight embryos failed due to bad-trained parameters or abovementioned reasons. The rest four embryos are all manipulated successfully with the correct parameters and procedure.

In addition, we must mention that the proper driving frequency, the driving voltage, and the pre-pressed cell deformation need to be retrained for embryos in different stages because their mechanical properties are different. The above parameters only adapt to mouse morula-stage embryos.

5.3 Discussion

In this series of experiments, the proposed dissection strategy in Section 3.2 is validated. The well-trained piezo-driven flexure micromanipulator dissects the morula-stage embryo successfully. A set of parameters, driving frequency of 4, 400 Hz and driving

voltage of 50 V, is trained to achieve the desired vibrations. A practical dissection procedure which stresses on the pre-pressed cell deformation (3~5 μm) and the suddenly applied driving voltage is provided.

The pre-pressed deformation performs two functions during the dissection. First, the initial high-speed peak-peak axial vibration amplitude is pretty small (around 2 μm), as shown in either stage 1 in Figure 3 - 5 or the measured data in Figure 4 - 9. Thus, a pre-pressed deformation can guarantee the micropipette tip contacts the cell ZP membrane closely. Second, small pre-pressed deformation of cell generates an elastic force which contributes to the penetration.

The suddenly applied driving voltage works because it provides a high-speed pulse to the micropipette tip. In fact, what matters is the changing rate of the driving voltage. In this work, since the function generator adjusts the driving voltage slowly by manually turning the knob, we can only utilize the voltage change at the moment of power-on. If one generator can change the voltage fast enough to provide high acceleration and high speed to the micropipette tip, it can also utilize the suddenly changed driving voltage to contribute to penetration. For example, if one generator can increase the driving voltage from 5 V to 55 V within a driving cycle, such as 1/4400 second, it should perform similarly to our loading way, which is suddenly applied driving voltage from 0 to 50 V.

Besides the successful experience, there are some limitations for the system, and relative solutions are discussed below.

- (1) The primary lateral vibration and its interaction with the cell in the y-axis direction cannot be recorded directly from the current view. Based on the phenomenon in Figure 5 - 3 (b), one opening does exist on the surface of the ZP membrane, but it is difficult to measure the size and shape of the opening.

One possible solution is to rotate the manipulated embryo, forcing the trauma to face to the monitoring direction of the microscope. This approach requires robust and accurate cell rotation techniques, in case the inner materials of opened embryos leak out.

- (2) The limited embryo manipulation space and the low magnification of the lens restrict the design of the peripheral system and the cell experiment procedure. The lenses with higher magnification can offer more explicit images. However, they usually have larger dimensional sizes and leave less space for micromanipulation than the lenses with lower magnification. In the upright microscope system, the selected objective lens with a magnification of 10× is a trade-off between the image quality and embryo manipulation space.

Compared to the upright microscope, a better choice for monitoring system is the inverted microscope, as shown in Figure 5 - 6. The noticeable feature of the inverted microscope is that lenses are located under the operation stage, in which the action of dissection and monitoring are divided into two separated space. Then, it is possible to choose a lens with a higher magnification without interfering dissection.

Figure 5 - 6 The inverted microscope.

CHAPTER 6 Conclusion and Future Work

6.1 Conclusion

This work aims to develop a reliable piezo-driven flexure micromanipulator, focusing on the vibration control of the micropipette tip and the dissection for the ZP membrane. The conclusions are summarized below.

A piezo-driven flexure micromanipulator is developed, which consists of a piezoelectric actuator, a flexure mechanism, a micropipette, and a connector. The flexure mechanism is the core part of the manipulator. Its key parameters are determined through iteratively model analysis simulation. It is fabricated by wire electrical discharge machining, from an integral piece of stainless steel.

A control method for vibration of the micropipette tip is proposed. By adjusting the driving frequency of the piezoelectric actuator, the lateral resonant vibration of the flexure mechanism can be excited, which is used to guide the lateral vibration of the micropipette. By adjusting the driving voltage, the peak-peak vibration amplitudes in axial and lateral directions can be fine-tuned. Those amplitudes increase monotonously with the rising of the driving voltage. The optimal parameters in this work are with the driving frequency of 4,400 Hz and with the driving voltage of 50V.

Mouse morula-stage embryos are dissected with little cell deformation. A dissection procedure based on this micromanipulator and the control method is provided. Two critical operational points are that the driving voltage should be applied suddenly, and the pre-pressed cell deformation is 3~5 μm for this work.

The optimal parameters for the dissection of other cells may vary due to different mechanical properties. However, the critical factors for cell dissection are the driving frequency, the peak-peak amplitude and the change rate of the driving voltage, and the proper pre-pressed cell deformation. This piezo-driven flexure micromanipulator can be extended to more cells if the parameters are appropriately retrained.

6.2 Future Work

Several areas are valuable for further studies are listed below.

1. To combine the vision-guided force detection work [72] with the current work to control the press force on the cell surface quantitatively. As mentioned in Section 5.3, a proper pre-pressed cell deformation is necessary for effective piercing and cutting. However, precise micro-force sensors are expensive and difficult to be applied in such situations. Han's work [72] in vision-guided force detection utilizes the deformation and the mechanical properties of the cell, to estimate the force pressed on the cell, which makes the force measurement easy and fast. It will be valuable to apply the vision-guided force detection to develop a more accurate and more robust piezo-driven flexure micromanipulator.
2. To develop a reliable cell rotation algorithm and procedure for cell micromanipulation. In this work, the accurate lateral vibration of the micropipette tip for dissection has been achieved, however, the size and shape of the opening are not analyzed since current experimental setup cannot record the trauma directly. If the manipulated embryo can be rotated to the proper orientation for monitoring, then clear feedback of dissection performance can be obtained, which is beneficial to optimize the piezo-driven flexure micromanipulator to create an opening with controllable size and shape.

3. To simulate the interaction between the micropipette tip and the cell based on the abovementioned dynamic model, not only the modal analysis of the flexure mechanism.

A systematic multi-physics simulation will be beneficial to improving the mechanical design and optimizing the design of the experiment in this work.

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