MICROROBOTIC MANIPULATION AND CHARACTERIZATION OF BIOLOGICAL CELLS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy Graduate Department of Mechanical and Industrial Engineering University of Toronto

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Abstract

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Mechanical manipulation and characterization of biological cells have wide applications in genetics, reproductive biology, and cell mechanics. This research focuses on (1) the development of enabling microrobotic systems and techniques for automated cell microinjection and in situ mechanical characterization; and (2) the demonstration of molecule efficacy testing and cell quality assessment with the new technologies.

Targeting high-speed cell injection for molecule screening, a first-of-its-kind automated microrobotic cell injection system is developed for injecting foreign materials (e.g., DNA, morpholinos, and proteins) into zebrafish embryos (∼1.2 mm) and mouse oocytes/embryos (∼100 µm), which overcomes the problems inherent in manual operation, such as long learning curves, human fatigue, and large variations in success rates due to poor reproducibility.

Novel cell holding devices are developed for immobilizing a large number of embryos into a regular pattern, greatly facilitating sample preparation and increasing the sample preparation speed. Leveraging motion control and computer vision techniques, the microrobotic system is capable of performing robust cell injection at a high speed with high survival, success, and phenotypic rates. The mouse embryo injection system is applied to molecule testing of recombinant mitochondrial proteins. The efficacy of an anti-apoptotic Bcl-xL (ΔTM) protein is, for the first time, quantitatively evaluated for enhancing the development competence of mouse embryos.
For cell quality assessment, this research develops a vision-based technique for real-time cellular force measurement and \textit{in situ} mechanical characterization of individual cells during microinjection. A microfabricated elastic device and a sub-pixel computer vision tracking algorithm together resolve cellular forces at the nanonewton level. Experimental results on young and old mouse oocytes demonstrate that the \textit{in situ} obtained force-deformation data can be used for mechanically distinguishing healthy mouse oocytes from those with cellular dysfunctions. This work represents the first study that quantified the mechanical difference between young and old mouse oocytes, promising a practical way for oocyte quality assessment during microinjection.
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Chapter 1

Introduction

1.1 Background and Motivations

Recent advances in cell biology, such as molecule screening and assisted reproduction technologies (ART), demonstrate that increasingly complex micromanipulation strategies are required for manipulating individual biological cells. For example, cell microarray analysis [1,2], allowing for development studies and genome-wide analysis of transcriptional genotypes, requires patterning and immobilization of individual cells. In order to create transgenic organisms for genetic research such as gene identification and human disease studies, transgenic materials need to be introduced into single cells [3, 4]. In ART, a single sperm needs to be injected into an oocyte through intracytoplasmic sperm injection (ICSI) [5, 6]; and molecular targets that regulate embryo development need to be microinjected for screening purposes.

The development of cell manipulation systems capable of effectively patterning, grasping, characterizing, and injecting individual cells has evolved during the past decade [7–12]. The progress has been greatly facilitated by microrobotics and MEMS (microelectromechanical systems) technologies. As an advance on manual operation and other cell manipulation methods (e.g., laser trapping [13] and dielectrophoresis [14]), microrobotic cell manipulation has been demonstrated to overcome their limitations and be capable of conducting more complex
cell manipulation tasks [7]. MEMS-based cell manipulation tools are particularly powerful when integrated into microrobotic systems to directly interact with cells [8, 15, 16]. To further facilitate intelligent microrobotic cell manipulation, MEMS sensors providing microrobotic systems with cellular feedback, such as manipulation forces and cellular displacements are integrated into these systems [17–19].

Cell injection is a cell manipulation task widely used in genetics and ART. The injection targets are often embryos/oocytes of model organisms. The zebrafish and mouse are two widely used models for studying vertebrate development, genetics, and reproduction [20, 21]. The injection of a large number of zebrafish and mouse embryos within a short time window is required on a daily basis in many biological laboratories and biotechnology companies. Conventionally, cell injection is conducted manually by well trained technicians [22]. However, the laborious injection task easily causes fatigue in technicians and hinders performance consistency and success rates. Long learning curves, low throughput, and low success rates in manual operations call for the elimination of direct human involvement.

Many research efforts have been focused on developing microrobot-assisted (i.e., teleoperated) or semi-automated cell injection systems. Microrobots/micromanipulators were controlled by a human operator to provide “steady hand” and conduct human-in-loop mouse embryo injection [23–27]. An autonomous microrobotic cell injection system was developed using holding pipette for immobilizing a mouse embryo, and a visually servoed microrobot for automated cell injection [7]. However, switching from one embryo to another was conducted manually, and thus, injection was time consuming. A semi-automated MEMS-based high-throughput drosophila embryo injection system was reported recently [28], where a MEMS microneedle was used as an injector. A 3-DOF scanning stage was used for locating randomly dispersed embryos that were ‘glued’ on a glass slide, and another 3-DOF motion stage with the injector mounted was employed for injection. One drawback of this system is that manual alignment of the two stages was required before injection. The large alignment error would greatly influence the injection performance. More importantly, the low stiffness of the MEMS
injector requires that the hard embryo chorion be removed in order to facilitate the injection, which may affect subsequent embryonic development, making the system unsuitable for zebrafish or mouse embryo injection. In summary, existing systems cannot satisfy the demand for automated, high-throughput cell injection in the field of zebrafish and mouse studies. A fully automated, high-throughput cell injection system is highly desired.

Besides microrobotic cell injection, this research also developed techniques to enable mechanical characterization of individual oocytes/embryos during microinjection without requiring a separate process or system setup. In situ quantification of cellular mechanical properties during the injection process may prove subtle mechanical differences to be useful for embryo selection and health monitoring. In addition, quantification of cellular forces is also important in that cellular force feedback would enable force-controlled microrobotic cell manipulation and minimize injection-induced cell damage.

Existing techniques such as atomic force microscopy (AFM) [29, 30], laser trapping [31, 32], micropipette aspiration [33, 34], magnetic bead measurement [35, 36], and MEMS transducer based force measurement [8, 37] require a stand-alone setup, do not provide flexibility for being integrated into a cell injection system, and is not capable of in situ mechanical characterization of biological cells. As an integrative component of this research, the development of low-cost polymeric MEMS devices and experimental techniques was pursued to enable real-time cellular force measurements and in situ mechanical characterization.

### 1.2 Research Objectives

The objectives of this research include:

- To develop a microrobotic zebrafish embryo injection system including fast sample immobilization; and to demonstrate fully automated, robust injection at 15 zebrafish embryos per minute (compares favorably to the speed of manual operation) with high survival, success, and phenotypic rates.
• To extend the control architecture of the zebrafish embryo injection system to develop a microrobotic system for high-throughput mouse embryo injection at a speed of 12 embryos/minute (six times the speed of manual operation); and to apply the system to mitochondrial protein testing.

• To develop a MEMS-vision-based cellular force measurement technique to enable in situ measurements of cellular forces and mechanical properties of individual embryos; and to apply the devices and technique to characterize subtle mechanical differences between healthy mouse oocytes and those with cellular dysfunctions.

1.3 Dissertation Outline

An overview of the ensuing chapters is as follows: Chapter 2 describes the fully-automated microrobotic zebrafish embryo injection system and experimental results of morpholino injection for suppressing specific genes. Chapter 3 presents a vision-based contact detection technique for determining relative vertical positions of the end-effector and the micro object to be manipulated in microrobotic manipulation. Chapter 4 reports the development of an automated microrobotic mouse embryo injection system and experimental results of system performance evaluation. Chapter 5 presents application of the automated mouse embryo injection system to molecule testing of mitochondrial proteins. Chapter 6 presents a MEMS-vision-based cellular force measurement technique for resolving cellular forces at nanonewton level during microinjection and its application to in-situ differentiation of healthy mouse oocytes from those with cellular defects. The thesis is concluded in Chapter 7, with a summary, contributions of this research, and possible future research directions.
Chapter 2

Automated Microrobotic Zebrafish Embryo Injection

2.1 Introduction

Molecule screening at the single cell level, which is critical in molecular biology and drug discovery, requires that target molecules be introduced into single cells to permit cellular-function-targeted molecules to directly regulate cell development and their functions to be quantified. Several technologies exist for introducing foreign materials into a cell, such as electroporation [38, 39], viral vectors [40, 41], gene gun [42, 43], ultrasonics [44, 45], and MEMS-based injection [46, 47]. Compared to these techniques, microinjection with a single glass micropipette remains the most effective in terms of cell damage, cell viability, cell waste, effectiveness of delivering macromolecules, specificity, and freedom from concerns about phenotype alteration [48]. However, in order to enable fast, precise, and robust screening for molecular targets, the state-of-the-art manual injection must be replaced with fully automated operation.

For testing cellular responses to molecular targets and to obtain statistically significant data, the injection of thousands of cells needs to be conducted within a short time window
Figure 2.1: The structure of a zebrafish embryo.

(e.g., within 1.5 hr after fertilization, before the 16-cell stage for zebrafish embryo injection). Manual injection is not only slow; the laborious task of manual injection easily causes fatigue in injection technicians and hinders performance consistency and success rates. Efforts in automating cell injection have been continuous, resulting in a visually servoed system [7], a semi-automated system [28], and many tele-operated systems [23–27], to name just a few. These systems are limited in throughput and reproducibility as operator input (e.g., locating features and destinations) or operator involvement (e.g., switching from one cell to another or injector alignment) is still required.

Among many biological models, the zebrafish has emerged as an important model organism for developmental genetic studies as well as for drug discovery [49, 50]. Zebrafish embryonic development is remarkably similar to that of humans; however, zebrafish embryos are laid and fertilized externally, they develop rapidly, and the embryos are transparent (Figure 2.1), making it convenient to observe the movement and fate of individual cells during embryonic development [51, 52]. Molecular and genetic analyses of zebrafish embryogenesis depend on the injection of foreign materials into early zebrafish embryos [53, 54]. DNA injection is used to generate transgenic zebrafish lines, mRNA injection is used to overexpress gene-products in zebrafish embryos, and reverse genetic or loss-of-gene-function studies require the injection of antisense morpholino-modified oligonucleotides (morpholinos or MOs) to specifically inhibit
RNA splicing and/or translation in vivo [55].

Despite their relatively large size (~1.2 mm including chorion), zebrafish embryos have a delicate structure and can be easily damaged, making automated, high-throughput injection difficult. Specific challenges include: (i) to quickly (i.e., in seconds) immobilize a large number of zebrafish embryos; (ii) to automatically, robustly identify cell structures for vision-based position control and account for size differences across embryos; and (iii) to coordinately control two microrobots to maximize operation speed. Addressing these challenges, the objective of this research was thus to develop an effective massive sample preparation method and create a system that is capable of injecting a large number of embryos in the short time window. In this chapter, a microrobotic system for zebrafish embryo injection is presented, featuring full automation, high-speed sample immobilization, and high survival, success, and phenotypic rates.

2.2 System Design

2.2.1 System Architecture

The zebrafish embryo injection system, shown in Figure 2.2, employs two three-degrees-of-freedom microrobots (MP-285, Sutter; positioning resolution: 40 nm). Two motion control cards (PCI-6259 and NI PCI-6289, National Instruments) are mounted on a host computer (3.0GHz CPU and 1GB memory) where control algorithms and image processing algorithms operate. Visual feedback is provided through a CMOS camera (A601f, Basler) mounted on an optical microscope (SZX12, Olympus). An embryo holding device is attached to microrobot-1. A glass micropipette (TW120F-4, WPI), heated and pulled using a laser micropipette puller (Model 2000, Sutter), is connected to microrobot-2 via a micropipette holder (MPH412, WPI). A Venturi vacuum pump (UN816, KNF) provides negative pressure to immobilize embryos into regular patterns. A computer-controlled pico-injector (PLI-100, Harvard Apparatus) with a volume control resolution down to sub-picoliter provides positive pressure for material de-
position. To minimize vibration, all units except the host computer and pressure units are mounted on a vibration isolation table.

Figure 2.3 shows a screen capture of the control program interface. For fully automated injection, the system-level command buttons permit the user to start, pause/resume, terminate, and reset the system. The live image display area and the system status information window allow for visually monitoring the operation status. The two control panels provide the user with the option for tele-operated injection (i.e., using mouse clicks), alternative to fully automated operation. Users can also readily specify parameters through the control program interface, such as the number of embryos within a batch and camera control parameters.
Figure 2.3: Control program interface with an array of embryos immobilized on the embryo holding device. The embryo image was taken under 0.7×. For fully automated injection, the system-level command buttons enable the user to start, pause/resume, terminate, and reset the system.

2.2.2 Vacuum-Based Embryo Holding Device

Placing individual zebrafish embryos into a regular pattern significantly simplifies the embryo searching/positioning task, and therefore, increases injection speed. Vacuum-based immobilization was chosen since it could provide large immobilization forces to hold embryos in place during micropipette penetration. Low vacuum levels prove effective and do not produce undesired biological complications for further cellular development.

Figure 2.4(a) shows the vacuum-based embryo holding device which is constructed by conventional machining using phylcarbonate. Evenly spaced through-holes (diameter ~400µm)
Figure 2.4: Vacuum-based embryo holding device. Embryos are immobilized on individual through holes via a negative pressure. Extra embryos are flushed off the device. (a) Picture of a device (5×5 holes). (b) An array of immobilized embryos with continuous injection path labeled.

are connected to a vacuum source via a backside channel. Upon dispersing many embryos onto the device, a sucking pressure enables each through-hole to trap a single embryo. The extra non-trapped embryos are flushed away from the device. Figure 2.4(b) shows an array of immobilized zebrafish embryos. In practice, a negative pressure of 2-7 InHg proved effective in immobilizing zebrafish embryos without damaging the embryos. Upon cell immobilization, the system conducts injection continuously along the arrow labeled path.
2.2.3 Volume Control

Volume calibration is important for precisely depositing a specified amount of materials into individual cells such that dose effect can be investigated. Deionized (DI) water is used as an example in this section to describe the calibration of the relationship between injection volume, applied pressure, and pressure ‘on’ time. A drop of DI water pushed out of the injection micropipette forms a sphere at the micropipette tip. Injection volume is then calculated by detecting the diameter of the sphere via a Hough transform.

For a micropipette tip with an inner diameter of 10 µm, Figure 2.5 shows the relationship of injected volume versus pressure ‘on’ time corresponding to two different injection pressure levels. By controlling the pressure ‘on’ time, 3 nl materials were deposited into each zebrafish embryo in the experiments.

2.3 Control Flow of Automated Embryo Injection

2.3.1 Overall Sequence

A batch of zebrafish embryos, immobilized into a regular pattern on the embryo holding device, are placed on the microrobot-1 under the microscope. Automated injection starts with
Chapter 2. Automated Microrobotic Zebrafish Embryo Injection

Figure 2.6: Automated injection flow. Except for the task of bringing next embryo into the field of view (from (e) to (f)), control of both embryo positioning stage and microrobot is based on ‘looking-then-moving’. Top row: 3-D view. Bottom row: microscopic (image) 2-D view.

(a) The vertical height of the micropipette tip is determined with a computer vision approach. This step is required only once at the beginning of one batch. (b) Micropipette at the home position. The white curve outlines the recognized cytoplasm contour. The white dot represents the cytoplasm center. (c) Embryo is brought to the center of the field of view. Micropipette is positioned at the switching point. (d) Micropipette tip penetrates the embryo and deposits material at a pre-set destination in a specified volume. (e) Micropipette retracts out of the embryo. (f) Micropipette returns to the home position, and the next embryo is brought into the field of view. From (b) to (c), and from (e) to (f), the embryo positioning stage and the microrobot move in parallel to increase injection throughput.
vision-based contact detection, described in Chapter 3 to determine the vertical positions of
the micropipette tip and the surface of the embryo holding device (Figure 2.6(a)) with an ac-
ccuracy down to 0.2 µm. An embryo is recognized and brought to the center of the field of
view; simultaneously, the micropipette tip is moved by the microrobot-2 to a switching point,
$S$ that serves as an indicator of the boundary between inside and outside of an embryo and is
determined through the recognition of embryo structures. The micropipette tip penetrates the
chorion and deposits materials at the desired location within the embryo. In the experiments
presented in this chapter, the deposition destination was chosen to be the cytoplasm center,
where cytoplasm is defined as the combination of the yolk and the cell portion of a zebrafish
embryo (Figure 2.1). Upon retreating out of the embryo, the micropipette tip is moved to a
home position that is 1.4 mm above contact point, to prevent it from crashing into the next
embryo. In the meanwhile, the next embryo is brought into the field of view, the structures are
recognized, and the injection process is repeated until all embryos in the batch are injected.

Throughout the process, the microrobot-1 does not produce vertical motion while the micro-
robot-2 is servoed along three axes, as shown in Figure 2.6. For positioning each embryo
and controlling the motion of the injection micropipette, PID (proportional-integral-derivative)
controller is employed for servoing both microrobots that are operated in parallel whenever
possible (i.e., in Figure 2.6, from (b) to (c), and from (e) to (f)). Parallel operation of the
embryo positioning stage and the microrobot is maximized to increase injection throughput.

### 2.3.2 Image Processing: Recognizing Cell Structures

The purpose of recognizing detailed embryo structures is for determining deposition destina-
tions to guarantee a high reproducibility. In this study, the cytoplasm center ($O$ in Figure 2.7(b))
was chosen as the deposition destination. However, the recognition algorithm allows for choos-
ing a different destination, for example, closer to the yolk/cell interface to facilitate the diffusion
of injected molecules into the cell portion. The recognition of detailed embryo structures takes
45 ms on the host computer.
Pre-processing is conducted to obtain de-noised binary images. An image is first convolved with a low-pass Gaussian filter for noise suppression. The gray-level image is then binarized to a binary image using an adaptive thresholding method [56], in which a local threshold for each pixel is set to be the mean value of its local neighbors. The binary image is eroded to remove small areas that represent spurious features and then, dilated to connect broken segments that originally belong to one object. An example after pre-processing is shown in Figure 2.7(a).

Recognition of Chorion, Cell, and Yolk of the connected objects in the binary image, the one with the maximum area is recognized as the chorion. In Figure 2.7(b), the chorion is enclosed by its minimum enclosing circle. The second largest object in the image is the cytoplasm, the boundary of which is represented by a chain code contour. The boundary of the cytoplasm is often not fully connected (Figure 2.7(a)); however, a fully closed contour is important for the recognition of detailed cytoplasm structures including the yolk, the cell portion, and the yolk-cell interface. Thus, a convex hull [57] of the contour is constructed and used as initial positions for subsequent snake tracking [58]. Snakes, or active contours, are often used to locate object boundaries and track deformable objects. They are energy minimizing splines influenced by external constraint forces and image forces that guide snake points towards features such as lines and edges. The closed cytoplasm contour resulting from snake tracking is shown in Figure 2.7(b). The centroid of the contour, $O$ is recognized as the cytoplasm cent-
ter. The switching point, $S$ is then determined as the intersect point of the minimum enclosing circle and the horizontal line passing through the cytoplasm center. In order to distinguish the yolk from the cell portion to provide the flexibility for choosing a desired destination, the cytoplasm contour after snake tracking is fitted into an ellipse using a least squares method, and intercepted into two parts by the minor axis of the fitted ellipse. Based on the fact that the cell portion always has greater convex deficiency [57], the cell and yolk portions are distinguished. The recognized yolk/cell interface is shown in Figure 2.7(b).

### 2.4 Experimental Results and Discussion

#### 2.4.1 Materials

The zebrafish embryos used in the injection experiments were collected in the Hospital for Sick Children (Toronto, Canada) with standard embryo preparation procedures [59]. Animal protocols were approved by the Hospital for Sick Children’s Lab Animal Services’ Animal Care Committee. The outbred zebrafish embryos, which were not de-chorionized, were cultured in embryo media that contained 10 L reverse osmosis water, 3 g instant ocean salt mix, and 10 ml methylene blue solution.

For the ease of visually inspecting the injection effectiveness, fluorescent dyes (Rhodamine B, 100 µM) were injected into 350 embryos. To quantify the efficacy of the system for recapitulating mutant embryonic phenotypes, fluorescein-tagged morpholinos that target the gene no tail (ntl-MO, 5'-GACTTGAGGCAGCCATATTTCCGAT-3', 300 nM, Gene Tools) were injected into additional 210 embryos. The no tail gene product is required for tail formation in zebrafish [60]. Successful injection of ntl-MO should inhibit translation of the ntl gene product, resulting in the tail-less phenotype.
2.4.2 Results and Discussion

The collected embryos were spread on the surface of the embryo holding device together with embryo media. Applied negative pressure promptly immobilized individual embryos on top of each through-hole. The extra embryos were flushed off the device. The process of embryo immobilization was manually conducted, taking approximately 6-12 s.

The automated system continuously injected a total of 350 zebrafish embryos with fluorescent dyes and 210 embryos with ntl-MO, demonstrating an operation speed of 15 embryos/minute. The injection experiments were arranged in different mornings in a half-a-year period. Each morning, one or two batches of zebrafish embryos were injected. Normally, there were 25 embryos for each batch. The only exception was the first batch for ntl-MO injection with 10 embryos injected for trial.

The injected embryos were cultured at 32 °C. To determine survival rate and success rate (defined later), embryos injected with fluorescent dyes were inspected under a fluorescence microscope (IX81, Olympus). The embryos were excited by 540 nm laser light and observed through a tetramethylrhodamine Isothiocyanate (TRITC) filter set. Visual inspection was conducted right after injection, 24 hr after injection, and 48 hr after injection. To quantitate phenotypic rate (defined later), the embryos injected with ntl-MO were inspected under a bright-field microscope (SZX12, Olympus) 24 hr after injection and 48 hr after injection. Figure 2.8 shows the injected embryos and their subsequent development. The deposited fluorescent dyes (high-brightness) can be clearly observed in the area of the cytoplasm center, as shown in Figures 2.8(a) and 2.8(b). Diffused fluorescent dyes are observable 48hr after injection, as shown in Figure 2.8(c). Bright-field images of four no-tail fishes (24 hr after injection) are shown in Figure 2.8(d). Figure 2.8(e) shows a comparison of a no-tail fish 48hr after injection and control (wild-type).

To quantitatively evaluate the performance of the microrobotic injection system, three measures were defined. (1) Survival rate: This measure is defined as the ratio between the number of injected embryos that are capable of developing into larva and the total number of embryos
Figure 2.8: Development of zebrafish embryos injected with fluorescent dyes and ntl-MO. (a)-(c) show embryos injected with fluorescent dyes, (d)-(e) show embryos injected with ntl-MO. Dye injected embryos are shown immediately following injection (a), 24hr after injection (b), and 48hr after injection (c). (d) Ntl-MO injected embryos 24hr following injection. (e) Comparison of ntl-MO injected embryo (left) with uninjected control embryo (right) 48hr following injection.
injected, essentially representing the severity and frequency of cell damage from injection. With less cell damage caused in injection, the embryo is more likely to survive and develop normally to a fish. Based on the 350 injected zebrafish embryos, the microrobotic injection system produced an overall survival rate of 98±2%. (2) Success rate: This measure is defined as the ratio between the number of embryos with materials successfully deposited in the cytoplasm center and the total number of injected embryos. Essentially, this measure represents the reliability and the reproducibility of the system. It differs from survival rate in that it evaluates the correctness of locating the desired deposition destination. Visual inspection demonstrated that the overall success rate of the 350 injected embryos was 99±1%. (3) Phenotypic rate: This measure is defined as the ratio between the number of 48 hour-old embryos demonstrating a no-tail phenotype and the number of embryos with fluorescein-tagged ntl-MO deposited in the cytoplasm center. Essentially, this measure represents the readiness of the system for genetic studies. Based on the 210 ntl-MO injected embryos, the overall phenotypic rate was 98.5±1%. The detailed statistics of the three measures are given in Tables 2.1 and 2.2, demonstrating a high degree of reproducibility.

<table>
<thead>
<tr>
<th>Table 2.1: Statistics of fluorescent dye injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
</tr>
<tr>
<td>Number of injected embryos</td>
</tr>
<tr>
<td>Number of survived embryos</td>
</tr>
<tr>
<td>Number of successful injection</td>
</tr>
<tr>
<td>Survival rate (%)</td>
</tr>
<tr>
<td>Success rate (%)</td>
</tr>
</tbody>
</table>
Table 2.2: Statistics of ntl-MO injection

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of injected embryos</td>
<td>10</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>210</td>
</tr>
<tr>
<td>Number of no-tail fishes</td>
<td>10</td>
<td>49</td>
<td>49</td>
<td>49</td>
<td>49</td>
<td>206</td>
</tr>
<tr>
<td>Phenotypic rate (%)</td>
<td>100</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98</td>
<td>98.5±1.0</td>
</tr>
</tbody>
</table>

2.5 Conclusion

Leveraging computer vision and microrobotic control, the high-throughput automated cell injection system experimentally demonstrated the capability of injecting 15 zebrafish embryos per minute with a 98% survival rate, a 99% success rate, and a 98.5% phenotypic rate. The vacuum-based embryo holding device is capable of immobilizing a large number of embryos into regular patterns within seconds, dramatically shortening the sample preparation process. The recognition of embryo structures and precise motion control enable the automated system to precisely deposit a pre-specified amount of materials at a desired destination within the embryo. The application of the microrobotic zebrafish embryo injection system, which is autonomous in operation, fast in speed, free from fatigue, and provides unparalleled reproducibility, to biological and pharmaceutical research for timely injecting materials into a larger number of zebrafish embryos will facilitate large-scale screening of biomolecules or drug compounds.
Chapter 3

Contact Detection in Microrobotic Manipulation

3.1 Introduction

In microrobotic manipulation, an end-effector, such as a glass micropipette [7], a micro probe [61–63], or a microelectromechanical systems (MEMS) based microgripper [64] controlled by a microrobot is used to interact with micro objects under an optical microscope. An important operation in micromanipulation is the precise determination of the relative vertical positions of the end-effector and the micro object to be manipulated.

The schematic in Figure 3.1 shows the side view of a biological cell held by a vacuum-based cell holding device for microrobotic cell injection, in which the micropipette is required to penetrate the cell along the diagonal direction. To align the micropipette to the cell center (Figure 3.1), the relative vertical positions of the micropipette tip and the device surface must be precisely determined. Without loss of generality, detection of the contact between a micropipette tip and a substrate surface is taken as an example in this chapter to illustrate the presented technique.

Existing methods employ proximity sensors [65, 66], piezoresistive sensors [67, 68], or
piezoelectric touch sensors [69–71] to determine the relative vertical coordinates between the end-effector and the target surface. The integration of sensors with end-effectors is often difficult (e.g., using epoxy) and complicates system setup. As contact-type sensors at the micronewton levels are fragile and prone to damage, extra care must be taken in sensor overloading protection. Furthermore, reported contact detection resolutions using additional sensors are often limited to several micrometers, calling for methods capable of providing a better detection resolution without using additional sensors.

As microrobotic cell manipulation is universally conducted under an optical microscope that provides high-resolution, low depth-of-field visual feedback, it is highly desirable to utilize microscopy visual feedback for contact detection. Assuming that the micropipette tip and the target (e.g., glass slide surface) share the same focal plane, autofocusing algorithms [72, 73] can be used to independently servo the micropipette tip and the glass slide surface to bring them to a co-plane.

However, autofocusing algorithms are sensitive to feature selection variations and illumination conditions (angle of incidence and intensity) for calculating focus measures, making autofocusing-based contact detection unreliable. More importantly, the depth of field of mi-
croscope objectives is on the order of a few micrometers to tens of micrometers, which makes images of two objects with distinctly different world coordinates reveal sharpness over a distance of micrometers. Thus, autofocusing-based methods are not capable of precisely bringing the micropipette tip and the glass slide surface to an exact co-plane.

This chapter reports on a computer vision-based method that addresses the detection of contact between an end-effector and a target surface. The fundamental rationale is based on the experimental observation that when contact is established, further vertical motion of the end-effector (flexible or stiff) produces horizontal motion in the image plane. As shown in Figure 3.2, upon contact, further motion of the end-effector along the vertical direction (Z) is translated to horizontal motion along the X direction and reflects itself in the image plane. This general observation is not limited to contact between a micropipette tip and a glass slide surface although the contact between a micropipette tip and a glass slide is used as an example to illustrate the detection method, as long as the target surface roughness is not sufficiently high to significantly alter the horizontal motion of the end-effector or obscure the identification and tracking of the end-effector.

### 3.2 Contact Detection Analysis

Figure 3.3 shows a schematic consisting of an image plane, microscope objective, the end-effector (e.g., micropipette tip), and the target surface. Throughout the chapter, the world frame is denoted by X-Y-Z, and image plane by x-y. When the tip is controlled by a microrobot
Figure 3.3: Contact detection analysis. (a) Before contact. (b) After contact. (c) Horizontal sliding after contact.

to move downwards at a constant speed, the initial tip position, position before contact, exact contact position, and position after contact are denoted as Position 1, 2, 3, and 4 in the world frame and 1', 2', 3', and 4' in the image plane. ‘0’ is taken as the origin of the image plane, and ‘O’ the origin of the world frame.

Denote the distance between the tip at initial Position 1 and the target surface by $h$, the distance between the tip at Position 1 and the objective by $u$, and the distance between the image plane and the objective by $v$. The horizontal distance between the tip and the optical axis is denoted by $X_0$. In practice, the target surface is first brought into focus before contact detection is conducted; however, the initial position of the end-effector does not need to lie within the depth of focus.

Before contact is established, similarity of triangles gives

$$\frac{x}{X_0} = \frac{v}{u + Z} \quad (3.1)$$

Differentiating both sides yields

$$dx = -\frac{v \cdot X_0}{u^2} dZ \quad (3.2)$$

which implies that prior to contact, the $x$ coordinate values of the tip decrease in proportion to downward displacements along the $Z$ direction in the world frame.
Similarly, after contact is established,

$$dx = \frac{v \cdot \tan(\theta/2)}{u} \, dZ$$

(3.3)

implying that after contact, the $x$ coordinate values of the tip increase in proportion to downward displacements along the $Z$ direction in the world frame.

In summary, the brief analysis demonstrates that the $x$-coordinate values of the micropipette tip in the image plane decrease before contact and then increase after contact. When the micropipette tip reaches the minimal $x$-coordinate value in the image plane, initial contact occurs between the micropipette tip and the target surface. Thus, the relative vertical position between the end-effector and target surface is determined by monitoring the pattern change in the $x$-coordinate values of the end-effector, differing this contact detection method from autofocusing-based techniques.

### 3.3 Tip Area Identification

In order to identify the region of interest (ROI) surrounding the micropipette tip for subsequent contact detection that will be described in Section 3.4, an identification algorithm is developed. The algorithm distinguishes a moving object by subtracting the unchanged background from each frame of image. The micropipette, not required to be in focus, is moved by a microrobot.
Table 3.1: Preprocessing steps for tip area identification

<table>
<thead>
<tr>
<th>Step number</th>
<th>Processing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Contrast stretching, mapping the gray levels from the original range to a full range of [0, 255]</td>
</tr>
<tr>
<td>2</td>
<td>Adaptive thresholding</td>
</tr>
<tr>
<td>3</td>
<td>Morphological operations: erosion and dilation to remove small areas regarded as noises that produce many separate artificial objects, and to connect segments that originally are of one object.</td>
</tr>
</tbody>
</table>

horizontally (Y) at a constant speed without Z motion, producing motion along the y direction in the image plane (Figure 3.4). In practice, the Z-axis of the microrobot is aligned parallel to the optical axis by adjusting the microrobot base (X-Y); and the image plane x-y is aligned parallel to the X-Y plane by rotating the camera adaptor. Full-frame images (640×480) are processed in real time (30 Hz) for locating the ROI that contains the micropipette tip.

Denote image frames by \( I(x, y, t) \), where \( t=0, 1, 2, \ldots \). Each image frame is first convolved with a low-pass Gaussian filter for noise suppression. The resulting image is denoted by \( F(x, y, t) \). For each frame in an image sequence, its gray-level difference with respect to the very first frame \( (t=0) \) is

\[
D(x, y, t) = F(x, y, t) - F(x, y, 0)
\]  

(3.4)

Table 3.1 summarizes preprocessing steps that are applied to image \( D(x, y, t) \). Resulting images are shown in Figure 3.5.

As the micropipette continues to move along the Y direction, the number of connected entities represented by boundary chain codes [74] in images decreases dramatically. When the number of entities decreases by 90% in two successive frames, the entity with the maximum area is recognized as the micropipette. The tip is located on the rightmost end of the
Figure 3.5: Sequence for identifying the tip (i.e., determination of ROI). (a) Frame 0. (b) Frame 4. (c) Differentiation image between (b) and (a). (d) Image after contrast stretching of (c). (e) Thresholded image of (d). (f) Erosion image of (e). (g) Dilation image of (f). (h) ROI found at Frame 4.

micropipette in this example. A ROI (e.g., 100×80) shown in Figure 3.5(h) around the tip is then chosen for subsequent contact detection. Typically, the ROI is found at Frame 4 (t=4).

After the determination of ROI, the micropipette stops moving along the Y direction. It is then controlled to move along the downward direction (Z) at a constant speed to establish contact with the surface. In the subsequent contact detection process described in Section 3.4, image processing is only conducted inside the ROI to alleviate computation complexity and allow real-time performance (30 Hz).

### 3.4 Contact Detection

As shown in Figure 3.2, upon contact, the tip is located at a. After the establishment of contact, the tip slides horizontally from location a to b on the surface. Contact detection leverages such changes in the x coordinate in the image plane. As the brief analysis in Section 2 shows, physical contact occurs when the tip reaches its minimal x-coordinate value. After the identification of ROI, the micropipette moves downwards at a constant speed until its x-coordinate surpasses the minimal value by a few pixels (e.g., 6 pixels). Surpassing more pixels represents
larger micropipette tip deformations that can lead to micropipette breakage, but constitutes less of a concern for stiff end-effectors such as micro probes for MEMS and integrated circuit (IC) testing [63, 75]. During this process, the precise Z positions of the microrobot corresponding to each frame of image are recorded. Thus, the microrobot can precisely bring the micropipette tip back to the exact contact position after the completion of contact detection.

The processing sequence for contact detection is described in Table 2. Note that ROIs of images $I(x, y, t)$ rather than the differentiation images $D(x, y, t)$ are processed for all the five processing steps, including the first three steps of preprocessing. In Step 4, the micropipette tip is identified by searching for the object with the maximum area in the ROI. Inside the ROI (step 5), tasks include: 1) to determine the tip’s $x$-coordinate value using either a pixel-accuracy method or a sub-pixel-accuracy method that will be discussed in Section 3.4.1 and Section 3.4.2; 2) to compare the current $x$-coordinate value with the current minimal value and update the minimal value, if needed.

<table>
<thead>
<tr>
<th>Step number</th>
<th>Processing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gaussian low-pass filtering</td>
</tr>
<tr>
<td>2</td>
<td>Adaptive thresholding with the Otsu method</td>
</tr>
<tr>
<td>3</td>
<td>Morphological operation (erosion and dilation)</td>
</tr>
<tr>
<td>4</td>
<td>Tip’s $x$ coordinate in image update</td>
</tr>
<tr>
<td>5</td>
<td>If current $x$ coordinate exceeds the minimal value by 6 pixels, moving the micropipette back to the Z position of the contact point corresponding to the minimal value. Otherwise, update the minimal $x$ value and go back to Step 1</td>
</tr>
</tbody>
</table>
3.4.1 Tracking Tip with Pixel Accuracy

A representative experimental curve of micropipette tip’s $x$ coordinate changes vs. frame indices is shown in Figure 3.6. Point 1 corresponds to the first frame in the valley. Point 2 is one pixel above the valley. Similarly, Point 6 is five pixels above the valley. The valley lasts a number of frames between Point 1 and Point 2. Within this valley band lies the exact contact point that can be determined by interpreting (3).

(3) reveals that after contact, each increment of one pixel in the $x$ coordinate corresponds to an equal displacement along the $Z$ direction in the world frame. The frame index for the contact point (Point $P$ in Figure 3.6), $f_P$, can be obtained as $f_2-N$, where $f_2$ is the frame index for Point 2, and $N$ is the number of frames between Point 2 and Point 3. However, as the number of frames between Point 2 and Point 3 and the one between Point 3 and Point 4 are not strictly equal, numbers of frames per pixel step are averaged to reduce the error of locating $f_P$.

$$f_P = f_2 - (f_6 - f_2)/4 \quad (3.5)$$

where $f_6$ is the frame index for Point 6. Based on the determined $f_P$, the microrobot brings the micropipette tip back to the initial contact position according to the recorded positions that correspond to each frame of image.
Figure 3.7: Edge detection with moment invariance. Each square represents a pixel with different gray levels.

3.4.2 Tracking Tip with Sub-Pixel Accuracy

The accuracy using (5) to determine the contact point is limited due to the fact that numbers of frames between Point 2 and 3 ... and between Point 5 and 6 are slightly different. In order to further improve the detection accuracy, an edge detection algorithm based on moment invariance [76] is employed to track the micropipette tip with a sub-pixel accuracy.

A step edge in the absence of noise is characterized by a set of pixels having gray levels $L_i$ ($i=0, 1, 2, \ldots, n-1$) that are either monotonically non-decreasing or non-increasing. As shown in Figure 3.7, an ideal edge is a sequence of pixels with one gray level $h_1$, followed by a sequence of pixels with another gray level $h_2$, where $k$ denotes the edge location to be determined.

The first three moments of the input data are

$$m_j = \frac{1}{n} \sum_{i=0}^{n-1} L_i^j \quad j = 1, 2, 3 \quad (3.6)$$

The solutions of the edge are

$$h_1 = \bar{m}_1 - d \sqrt{p_2/p_1} \quad (3.7)$$

$$h_2 = \bar{m}_1 + d \sqrt{p_1/p_2} \quad (3.8)$$
\[ p_1 = \left[ 1 + s \sqrt{1/(4 + s^2)} \right] / 2 \] (3.9)

where

\[ s = \left( \overline{m}_3 + 2\overline{m}_1^3 - 3\overline{m}_1 \cdot \overline{m}_2 \right) / d^3 \]

\[ d = \sqrt{\overline{m}_2 - \overline{m}_1^2} \]

\[ p_1 + p_2 = 1 \]

Thus, the edge location is determined as

\[ k = p_1 \cdot n \] (3.10)

To obtain the \( x \) coordinate of the tip using the sub-pixel-accuracy method, a certain number (e.g., \( n=11 \)) of pixels along the \( x \)-axis are selected around the tip located with the pixel-accuracy method. Let the \( x \) coordinate of the tip located with the pixel-accuracy method be \( k_0 \). Pixels with \( x \) coordinates \( k_0-5, k_0-4, k_0-3, \ldots, k_0+3, k_0+4, \) and \( k_0+5 \) are used to calculate the \( x \) coordinate of the tip with sub-pixel accuracy according to (6)-(10). In Figure 3.6, the dashed curve shows the \( x \) coordinate values of the micropipette tip obtained via the sub-pixel-accuracy method.

The curve, however, is not smooth due to image noise. Around the valley point of this V-shaped, dashed curve also exist false spikes that affect the accuracy of contact point determination. Thus, to reduce the error, the original curve is divided into two parts from the valley point that has the minimal \( x \) coordinate of the tip, each side fitted into a straight line using the Huber method [77], which is a weighted linear least squares method. Let \( r_i \) represent the distance between the \( i \)th data point and the fitted line. \( \rho(r_i) \) is a distance function

\[ \rho(r_i) = \begin{cases} \frac{r_i^2}{2} & \text{if } r_i < c \\ c \cdot (r_i - c/2) & \text{else} \end{cases} \] (3.11)

The line is obtained by minimizing

\[ \varepsilon = \sum_i \rho(r_i) \] (3.12)
The constant $c$ limits the influence of outliers and was chosen to be 0.05 under the experimental conditions. The intersection point of the two fitted lines is taken as the contact point (Point $S$ in Figure 3.6).

### 3.5 Experimental Results

#### 3.5.1 Experimental Setup

The system, shown in Figure 3.8, consists of a stage holding a glass slide, an optical microscope (SZX12, Olympus) with a CMOS digital camera (A601f, Basler), and a three-degrees-of-freedom microrobot with a travel of 25 mm and a 0.04 $\mu$m positioning resolution along each axis (MP-285, Sutter). The microrobot is controlled via a motion control board (PCI-6259, National Instruments), carrying a glass micropipette (TW120F-4, World Precision Instruments) with a 5 $\mu$m tip and tilting angle from the target surface of the glass slide $\theta=20^\circ$. The system setup is mounted on a vibration isolation table.

In the beginning of experiments, the glass slide serving as the target surface was brought in focus and kept unchanged thereafter. In terms of microrobot motion, the micropipette was first moved laterally for ROI determination and then, moved vertically to establish contact. In order to evaluate the performance of the computer vision-based contact detection method, effects
Table 3.3: Effect of illumination intensity (speed: 58 µm/s, mag.: 9×)

<table>
<thead>
<tr>
<th>Intensity</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>pixel</td>
<td>sub-pixel</td>
<td>pixel</td>
</tr>
<tr>
<td>Mean (µm)</td>
<td>7410.1</td>
<td>7410.8</td>
<td>7410.8</td>
</tr>
<tr>
<td>S.d. (µm)</td>
<td>1.6</td>
<td>1.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 3.4: Effect of microrobot speed (mag.: 9×)

<table>
<thead>
<tr>
<th>Speed</th>
<th>14 (µm/s)</th>
<th>58 (µm/s)</th>
<th>380 (µm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>pixel</td>
<td>sub-pixel</td>
<td>pixel</td>
</tr>
<tr>
<td>Mean (µm)</td>
<td>7410.2</td>
<td>7410.7</td>
<td>7410.8</td>
</tr>
<tr>
<td>S.d. (µm)</td>
<td>1.4</td>
<td>1.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

of variations in illumination intensity, microrobot motion speed in lowering the micropipette, and magnifications of the microscope on detection accuracy were experimentally investigated. Experiments were repeated 50 times for studying each effect, amounting to a total of 1000 experimental trials. Tables 3.3, 3.4, and 3.5 summarize contact detection results in terms of the mean and one standard deviation (s.d.).

Firstly, the experimental results demonstrate that the sub-pixel accuracy method provides more accurate detection results than the pixel-accuracy method. This is attributed to the fact that the sub-pixel-accuracy method uses more data points for line fitting and thus, locates the contact point more accurately. Secondly, both algorithms are not significantly affected by illumination intensity variations (Table 3.3). Thirdly, a very high motion speed produces a high standard deviation (Table 3.4). Finally, under a higher magnification, both the pixel-accuracy
Table 3.5: Effect of magnifications (speed: 58 µm/s)

<table>
<thead>
<tr>
<th>Mag.</th>
<th>1.25×</th>
<th>2.5×</th>
<th>5×</th>
<th>9×</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pixel</td>
<td>sub-pixel</td>
<td>pixel</td>
<td>sub-pixel</td>
</tr>
<tr>
<td>Mean (µm)</td>
<td>7398.4</td>
<td>7409.2</td>
<td>7402.8</td>
<td>7410.8</td>
</tr>
<tr>
<td>S.d. (µm)</td>
<td>3.6</td>
<td>2.7</td>
<td>2.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

and sub-pixel-accuracy methods provide higher detection repeatability (Table 3.5).

The presented contact detection method was proven robust. In all the 1000 experimental trials, contact detection was achieved without the occurrence of micropipette tip breakage using both the pixel-accuracy method and sub-pixel-accuracy method. For the majority of the trials, the standard deviation of contact detection is smaller than 2 µm. When the motion speed was high (380 µm/s), the largest standard deviation occurred (7.9 µm for the pixel-accuracy method; 6.4 µm for the sub-pixel-accuracy method). This high motion speed produced a large vertical displacement (12.7 µm) along the Z direction between two successive frames of images, causing a large contact detection deviation. Additionally, an extremely high motion speed could cause damage to the delicate end-effector and/or target surface resulting from excessive impact.

### 3.5.2 Validation

#### Finite Element Simulation

A 3-D finite element model was constructed according to the geometries of the micropipette used in the experiments. Structural simulation was conducted using ANSYS, in which the material parameters of glass (Young’s modulus of 6.5 GPa, Poisson ratio of 0.2) for the micropipette were used. The relationship between contact forces and micropipette tip deformations...
Figure 3.9: Finite element structural simulation (tip deflection vs. applied force) of micropipette contact with a solid substrate. Unit is $\mu$m for deformations. Deflection in the figure is exaggerated for visualization purposes.

The simulation results are summarized in Table 3.6. A larger contact force results in a larger structural deformation (Figure 3.9). Corresponding to a contact force of 0.1 $\mu$N, the micropipette tip deforms by 0.24 $\mu$m.

<table>
<thead>
<tr>
<th>Force ($\mu$N)</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max. tip displacement ($\mu$m)</td>
<td>0.12</td>
<td>0.24</td>
<td>0.48</td>
<td>1.19</td>
<td>2.38</td>
</tr>
</tbody>
</table>
Table 3.7: Z Readings for contact points (µm)

<table>
<thead>
<tr>
<th>Trial group #</th>
<th>Balance-measured position</th>
<th>Pixel-accuracy method</th>
<th>Sub-pixel accuracy method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2961.0</td>
<td>2958.2</td>
<td>2961.4</td>
</tr>
<tr>
<td>2</td>
<td>3031.0</td>
<td>3029.4</td>
<td>3031.2</td>
</tr>
</tbody>
</table>

Calibration Experiments

In order to verify the accuracies of the contact points determined by the computer vision-based detection method, the glass slide was placed on an analytical balance for detecting contact forces (XP205, Mettler Toledo; resolution: 0.1 µN). In the validation experiments, the micropipette was controlled by the microrobot to move downwards at a speed of 14 µm/s. A magnification of 3.2× was used. Using the computer vision-based contact detection method, 100 experimental trials were conducted (50 using the pixel-accuracy methods; 50 using the sub-pixel-accuracy method) in each trial group (two trial groups amounting to 200 trials in total). The mean values of the located contact points are summarized in Table 3.7, where the ‘real’ contact positions were determined by reading a contact force change of the balance from zero to 0.01 mg (0.1 µN). Comparing the balance measured results with the results from the computer vision-based contact detection method, an accuracy down to 0.2 µm was achieved (i.e., difference between ‘real’ contact position and computer vision detected position).

3.6 Conclusion

This chapter presented a computer-vision based method for visually detecting the contact between an end-effector and a target surface under an optical microscope without using additional proximity or force/touch sensors. The rationale behind the algorithm is based on the fact that
after the establishment of a contact, further vertical motion in the world frame induces horizontal motion in the image plane. Without requiring the end-effector being in focus, detection starts with the determination of a region of interest, and then further detects the contact point using either a pixel-accuracy or a sub-pixel-accuracy method. Experiments demonstrated that the computer vision-based method is capable of achieving contact detection between a micropipette tip and a glass slide surface with an accuracy of 0.2 µm. The robustness of the contact detection method was experimentally demonstrated through varying several factors including illumination intensity, magnification, and microrobot motion speed. The presented contact detection method can be applicable to many microrobotic and nanorobotic manipulation scenarios, in which flexible or stiff end-effectors are operated under an optical or electron microscope.
Chapter 4

Automated Microrobotic Mouse Embryo Injection

4.1 Introduction

The mouse is a popular mammalian model organism which has been the primary animal for genetic studies and reproductive research. In reproduction studies, mitochondrial-associated recombinant proteins, as an example of many molecular targets, can be injected into mouse embryos to rescue oocyte fragmentation and avoid embryo demise, which is capable of enhancing embryo development in assisted reproduction. Microinjection of mouse embryos is also important for screening other molecular targets linked to study of basic biology of embryo development, such as neutralizing antibody, morpholinos, and expression vectors for small interfering ribonucleic acid (siRNA).

From a technical perspective, the mouse embryo (~100 µm in diameter) is much smaller than the zebrafish embryo, making the cell manipulation task more challenging. Although the overall configuration of the microrobotic zebrafish embryo injection system is applicable to mouse oocyte/zygote injection, there exist important differences that must be carefully considered: (1) the much smaller size of mouse embryo (~100 µm vs. ~1.2 mm zebrafish
embryos) demands the cell holding device to be miniaturized, which requires the development of a new microfabrication process for device construction; (2) mouse embryos are much more deformable and prone to damage (i.e., lysis), requiring intensive efforts to optimize system operation parameters to achieve a high survival rate; (3) mouse embryo injection requires the polar body (Figure 4.1) be positioned away from penetration site to avoid injection-induced cell damage. Thus, an automated cell orientation technique must be developed, which did not exist in our previous zebrafish system.

An automated mouse embryo injection system has been developed by extending the control architecture of the zebrafish embryo injection system. A novel glass cell holding device was microfabricated to immobilize many mouse embryos into a regular pattern. A visually servoed cell orientation technique as well as an in-house developed motorized rotational stage was integrated into the microrobotic system for fast embryo orientation control. Experimental results demonstrate that the system is capable of conducting high-speed, reliable mouse embryo injection with high survival and success rates.

4.2 Mouse Embryo Preparation

The mouse embryos used in this research were collected according to standard protocols approved by the Mount Sinai Hospital Animal Care Committee in Toronto. Young (8-12 weeks
old) imprinting-control-region (ICR) female mice (Harlan) were superovulated with 5IU of pregnant mare’s serum gonadotropin (PMSG) (Sigma) and 48hr later with 5IU of human chorionic gonadotropin (hCG) (Sigma), by intraperitoneal injection. The mice were subsequently mated with ICR males of proven fertility, and plugs were verified the next morning. *In vivo* fertilized embryos were collected from the mated female mice at day 0.5 post-coitum (p.c.) and cultured in potassium simplex optimization medium (KSOM) medium (Specialty Media). The average diameter of the mouse embryos was 98 μm.

### 4.3 System Architecture and Coordinate Frames

The microrobotic system (Figure 4.2) consists of a regular inverted microscope (TE-2000S, Nikon) with differential interference contrast (DIC) microscopy, a CMOS camera (A601f, Basler), a microfabricated glass embryo holding device, an in-house developed motorized rotational stage placed on a motorized X-Y translational stage (ProScan II, Prior) for embryo positioning and orientation control, an injection micropipette connected to a computer-controlled pressure unit (XenoWorks, Sutter) for material deposition, a 3-DOF microrobot (MP-285, Sutter) for controlling the injection pipette (45° tilting angle) to diagonally penetrate cells, an environmental chamber (Solent Scientific) to maintain cells at 37°, and a host computer (3.2 GHz CPU, 1 GB RAM) with a motion control board (PCIe-6259, National Instruments).

Figure 4.3(a) summarizes the coordinate frames of the system, including (1) $X_e$-$Y_e$-$Z_e$: coordinate frame of the end-effector (injection micropipette) attached to the 3-DOF microrobot; (2) $X_t$-$O_t$-$Y_t$: coordinate frame of the X-Y translational stage; (3) $X_r$-$O_r$-$Y_r$: coordinate frame of the rotational stage where $O_r$ is coincident with the rotational axis of the stage; (4) $X_c$-$O_c$-$Y_c$: coordinate frame of the camera; and (5) $x_i$-$o_i$-$y_i$: image plane.

The coordinates of a point $P$ in frame $c$ are denoted by $^cP = (x_c, y_c)$ (Figure 4.3(b)), and the coordinates of the corresponding mapped point $p$ in image frame $i$ are denoted by $^ip = (u, v)$. 
Figure 4.2: Microrobotic Mouse Embryo Injection System.

Figure 4.3: (a) Coordinate frames of the microrobotic system. (b) Image projection model between the camera coordinate frame and the image plane.
Scaled orthographic projection gives

\[
\begin{bmatrix}
  s_x & 0 \\
  0 & s_y
\end{bmatrix}
\begin{bmatrix}
  u \\
  v
\end{bmatrix}
=
\begin{bmatrix}
  x_c \\
  y_c
\end{bmatrix}
\]

where \(s_x\) and \(s_y\) are horizontal and vertical pixel sizes, which were calibrated off line.

### 4.4 Motorized Rotational Stage

In order to realize automated cell orientation, a compact motorized rotational microscopy stage, which is commercially unavailable was developed. Considering inverted microscopy characteristics and microrobotic system integration requirements, the cell orientation stage must: (1) be able to produce smooth and fast rotational motion; (2) not block the light path; (3) possess a sample clamping mechanism to make the cell sample close enough to the microscope objectives and therefore, within the working distance; (4) have a compact structure to permit the rotational stage to be readily mounted onto a commonly used XY microscopy stage. As no commercially available rotational stages meet these requirements, a rotational stage was developed to enable automatic cell orientation control.

Figure 4.4 shows a 3D model of the rotational stage that includes a stepper motor, a pair of spur gears (gear ratio: 2.78:1) for transmitting rotational motions, and a rotating sample holder with a sliding clamp that is capable of holding glass slides of different sizes. The stage does not introduce any obstruction into the optical path for cell observation. The choice of the gear pair provides more precise motion transmissions, smoother rotational motions, and more compact structures over other motion transmission mechanisms (e.g., belt-pulley). The sliding clamp is located on the very bottom of the rotating sample holder to fix a sample within the working distances of the microscope objectives. The rotational stage is capable of orienting samples with a positioning resolution of 0.08° and a maximum rotational speed of 1800 °/sec.
4.5 Microfabricated Glass Embryo Holding Device

As demonstrated in Chapter 2, the vacuum-based embryo immobilization employs an array of through-holes connected to a vacuum chamber for immobilizing individual embryos. The DIC microscopy used in the embryo imaging system requires glass to be chosen as the material for the construction of cell immobilization devices [78]. However, the construction of through-holes (e.g., 2-50 µm) on the glass material is challenging in the microfabrication process. Although laser micromachining can be used to drill high-aspect-ratio through-holes on glass substrates [79], laser micromachined through-holes have rough surfaces along vertical walls, and minute amount of debris can cause shadows around through-holes in imaging.

In this research, a novel glass embryo holding device is constructed by standard microfabrication. Micrometer-sized through-holes are formed on a standard cover slip (≤180 µm thick) using hydrofluoric acid (HF) wet etching. As illustrated in Figure 4.5, the device consists of a top glass layer with an array of through-holes, a bottom glass layer, and a PDMS spacer for
forming a vacuum chamber. Considering the size of mouse embryos (∼100 μm), the size of the through-holes is designed to be 35 μm-40 μm. The use of glass in the microscopy light path meets the requirement of DIC imaging. Standard cover slips (size: 22 mm×60 mm, thickness: ∼180 μm, Fisher Scientific) are used as the top glass layer. Microscope slides (size: 76 mm×26 mm, thickness: 1 mm, Fisher Scientific) are used as the bottom glass layer.

The fabrication process is summarized in Figure 4.6. In order to fabricate 35 μm-40 μm through-holes on a cover slip, a ∼155 μm deep cell holding cavity is first etched into the one side of the cover slip using HF wet etching, leaving a ∼25 μm thin layer of glass (Figure 4.6(a)). Evaporated metal layers of Cr/Au (30 nm/800 nm) plus hard-baked positive photoresist (S1818, Shipley) are used as etch masks [80]. The S1818 layers are capable of preventing penetration of HF solution through the Cr/Au layers.

Etching rates of cover slips as a function of HF concentrations were experimentally determined, as shown in Figure 4.7. No agitation was used during glass wet etching. High concentrations of HF cause rough bottom surfaces (inlet pictures in Figure 4.7) of the cell holding cavities. Resulting poor optical clarity is also not acceptable for cell imaging. It was found
that concentrations below 15% produced smooth surfaces under a 400× microscope objective (surface roughness $R_a \leq 100$ nm). A concentration of 14.3% was used in the final device fabrication, and the corresponding etching rate is 0.9 $\mu$m/min (Figure 4.7). Bottom flatness of the cell holding cavity was measured to be $\leq 0.8 \mu$m using a Wyko optical profilometer (Veeco Instruments).

After the formation of the cell holding cavity, S1818 on both sides is removed with acetone. The Cr/Au layers on the bottom side of the cover slip are then patterned using photolithography. A second time wet etching from both the top and bottom sides is conducted to form through-holes (Figure 4.6(b)). In the meanwhile, a 1.7 mm through hole is also formed on the cover slip to construct a connect port (Figure 4.8). Diameters of the etched micrometer-sized through-holes on each device are highly uniform with a standard deviation less than 0.5 $\mu$m. The thickness of the thin portion of the cover slip after through-hole formation is 10 $\mu$m-12 $\mu$m. Finally, S1818 and Cr/Au layers are removed, and the cover slip is cleaned in piranha solution.

The last step of fabrication is PDMS-glass bonding to form a vacuum chamber. PDMS
Figure 4.7: Cover slip etching rates as a function of HF concentrations.

Figure 4.8: A completed device. Inlet picture shows zoomed-in through holes.
prepolymer (mixing weight ratio of 10:1, Dow Corning) is spin-coated on a 1 mm thick glass slide at 500 rpm, and is then completely cured to form a spacer layer of $\sim 100 \, \mu m$ in thickness. The PDMS spacer layer is carefully cut with a scalpel, oxygen plasma treated, and bonded with the pattered cover slip. Figure 4.8 shows a ready-to-use cell holding device with the inlet showing the zoomed-in view of through-holes.

### 4.6 Deposition Volume Control

In order to guarantee viability of injected embryos and investigate dose effect of the injected materials, deposition volume should be accurately controlled, which is achieved by repeatably fabricating the injection micropipette and precisely regulating the pressure unit output (i.e., pressure level and deposition time).

The injection micropipette is pulled using a programmable micropipette puller (P-97, Sutter), and the tip of pulled micropipette is then abraded using a micropipette beveler (BV-10,
Sutter) to form a sharp tip and a 1.2 µm opening (inlet picture in Figure 4.9). This micropipette fabrication process is highly repeatable, providing precisely controlled shape and size of the micropipette tip.

For a micropipette tip with a 1.2 µm opening, the deposition volume as a function of the pressure level and deposition time was accurately calibrated by measuring the size of spherical droplet blown out of the micropipette tip using a Hough transform algorithm. Figure 4.9 shows a calibration data example of the deposition volume using deionized water. The resolution of material deposition volume was experimentally determined to be 1fL.

4.7 High-Speed Mouse Embryo Injection

4.7.1 Overall Control Sequence

A batch of mouse embryos are transferred and immobilized on the embryo holding device placed on the motorized rotational stage. Control flow of the injection process, shown in Figure 4.10, starts with vision-based contact detection [81] to vertically align the injection micropipette tip and the bottom surface of the cell holding cavity (Figure 4.10(a)). The injection pipette tip is then raised to a home position, \( H \) (Figure 4.10(b)). The determination of the home position will be described in Section 4.7.3.

Simultaneously, the first embryo is brought into the field of view. The cytoplasm center and the polar body center are identified visually by a human operator and input to the system through computer mouse clicking. The embryo is then brought to the center of the field of view by the X-Y translational stage. If the polar body faces the penetration site (e.g., Figure 4.10(b)), it is rotated away via visually servoed embryo orientation control, a topic to be described in detail in Section 4.7.5.

Once the embryo is oriented, if needed, the microrobot controls the micropipette to a switch point, \( S \). The \( X_e-Z_e \) coordinates of the switch point are determined by the embryo average radius \( r \) ((Figure 4.10(c)), and the \( Y_e \) coordinate is equal to the \( Y_e \) coordinate of injection
Figure 4.10: Overall control flow of microrobotic mouse embryo injection. (a) Contact between micropipette tip and cell holding cavity is detected using a vision-based algorithm. (b) The micropipette tip is elevated to a home position $H$, and the first embryo is brought into the field of view, recognized and centered. If the polar body faces the penetration site, the embryo is properly rotated through orientation control. (c) Micropipette is moved to a switch point, $S$. (d) The micropipette penetrates the embryo and deposits materials to the target destination. (e) The micropipette is retracted out of the embryo. (f) Micropipette is moved to the home position. Simultaneously, the next embryo is brought into the field of view.
destination. After the switch point, the micropipette diagonally penetrates the embryo and deposits materials at the injection destination (Figure 4.10(d)).

In the experiments presented in this chapter, the target destination for material deposition was selected as the cytoplasm center. Upon retracting out of the embryo (Figure 4.10(e)), the micropipette is moved back to the *home* position, $H$. The next embryo is then brought into the field of view (Figure 4.10(f)). This injection process is repeated until all the embryos in the batch are injected.

### 4.7.2 Control Architecture and Coordinate Transformations

There are three motion control devices in the system including the microrobot, X-Y translational stage, and rotational stage that are cooperatively controlled. Each motion device is regulated via proportional-integral-derivative (PID) close-loop position control. Visual feedback is used for identifying cellular structures (e.g., cytoplasm and polar body) and for guiding the operation of the microrobot, X-Y translational stage, and rotational stage, forming a vision-guided “look-and-move” system.

The control architecture is described in Figure 4.11. An image space task planner receives
visual feedback (image coordinates of cytoplasm center $i^{c} p_{c}$ and polar body center $i^{p} p_{p}$) and makes decisions to generate reference signals $(p_{1}, p_{2}, \theta)$ for the three closed-loop PID position controllers. A virtual switch, $K$ controls the transition from separate control of the X-Y stage and the rotational stage (state 1) to simultaneous, cooperative control of the X-Y and rotational stages (state 2). Simultaneous control of the two stages occurs during automatic embryo orientation.

The system autonomously conducts transformations among multiple coordinate frames (#1, #2, and #3 in Figure 4.11). Transformation #1 is between frames $i$ and $e$, transformation #2 is between frames $i$ and $t$, and transformation #3 is between frames $r$ and $t$.

Denote by $^e R_{i} \in \mathbb{R}^{2 \times 2}$ the rotation matrix of frame $i$ with respect to (w.r.t.) frame $e$. Denote by $^e t_{i} \in \mathbb{R}^{2}$ the origin location of the frame $i$ w.r.t. frame $e$. Transformations #1 and #2 are

$$
^e P = ^e R_{c} i^{c} P + ^e t_{c} \quad (4.2)
$$

$$
^i P = ^i R_{c} i^{c} P + ^i t_{c} \quad (4.3)
$$

Rewriting (4.1) as

$$
S^{i^{c}} P = ^c P \quad (4.3)
$$

where

$$
S = \begin{bmatrix}
s_{x} & 0 \\
0 & s_{y}
\end{bmatrix}
$$

and substituting (4.3) into (4.2) yields

$$
^e P = ^e R_{c} S^{i^{c}} P + ^e t_{c} \quad (4.4)
$$

$$
^i P = ^i R_{c} S^{i^{c}} P + ^i t_{c} \quad (4.5)
$$

According to the coordinate frames defined in Figure 4.3(a),

$$
^e R_{c} = ^{r} R_{c} = \begin{bmatrix}
0 & 1 \\
1 & 0
\end{bmatrix} \quad (4.6)
$$

For transformation #3,

$$
^i P = ^i R_{r} i^{r} P + ^i t_{r} \quad (4.7)
$$
where

\[
^t R_r = \begin{bmatrix}
\cos \theta & -\sin \theta \\
\sin \theta & \cos \theta
\end{bmatrix}
\]

and \( \theta \) is the orientation angle of frame \( r \) w.r.t. frame \( t \).

Since \( S \) is known from off-line pixel calibration, the transformation between the image frame and the microrobot frame is uniquely established upon the determination of \( ^e t_c \) and \( ^t t_c \). When the micropipette tip is identified (Section 4.7.3), \( ^e t_c \) is solved from a determined pair of \( ^e P \) and \( ^t P \). When the cytoplasm center of an embryo in the field of view is identified (Section 4.7.4), \( ^t t_c \) is solved from a determined pair of \( ^t P \) and \( ^t p \). Transformation #3 is calibrated through on-line visually servoed embryo orientation control (Section 4.7.5).

### 4.7.3 Contact Detection and Home Position Determination

After the initial contact is detected, the system automatically determines the \( X_e-Y_e-Z_e \) coordinates of the home position, \( H \) ((Figure 4.10(b)). The \( X_e-Y_e \) coordinates of the contact point are taken as the \( X_e-Y_e \) coordinates of \( H \), and the \( Z_e \) coordinate of \( H \) is set at 110 \( \mu \)m, which is slightly greater than embryo diameters, above the contact point. This height prevents the micropipette from crashing into the embryos during embryo orientation and switching.

### 4.7.4 Embryo Structure Identification

The determination of the injection destination (cytoplasm center) and the polar body center for orientation control is conducted by a human operator and input to the microrobotic system via computer mouse clicking in the graphical user interface of the control software.

When the polar body of an embryo is within the depth of field, the human operator directly selects image coordinates of the cytoplasm and polar body centers. When the polar body is not visible (out of focus), the embryo is focally scanned by the \( z \)-motor on the microscope. During this scanning process, the human operator identifies and selects the polar body center.
Figure 4.12: Mouse embryo orientation. (a) Side view and (b) top view of the embryo and injection micropipette before orientation. (c) Top view of the embryo after orientation. Polar body is now at 12 o’clock position.

The control software accepts the image coordinates from user input and the vertical $Z_e$ coordinate of the polar body center from the encoder feedback of the $z$-motor on the microscope. The image coordinates of the cytoplasm and polar body centers are converted into Cartesian frame coordinates via coordinate transformations #1 and #2 (Figure 4.11). The system sets $Z_e$ coordinate of the injection destination (i.e., cytoplasm center) as the average embryo radius $r$ above the bottom surface of the cell holding cavity (Figure 4.10(d)).

4.7.5 Visually Servoed Embryo Orientation Control

Before the injection micropipette penetrates an embryo, the embryo must be properly oriented. When the polar body appears in the space of quadrant II in Figure 4.12(a), there are risks of either direct polar body penetration or large stress induced polar body damage. The desired target orientation is either 12 o’clock or 6 o’clock (Figure 4.12(c)).

Since an embryo within a batch is rarely coincident along the rotational axis of the rotational stage (Figure 4.13), coupled translational motions during rotation cause the embryo to move out of the field of view. Thus, the system conducts 3-DOF cooperative control of the X-Y translational stage and the rotational stage, shown in Figure 4.11, to bring the embryo back into the field of view after orientation. This control method permits high-speed orientation of
Figure 4.13: Image-based visual servoing keeps the embryo inside the field of view during orientation for calibrating coordinate transformation #3.

cells [82].

In Figure 4.11, coordinate transformation #3 between the frames of the rotational stage and X-Y translational stage is calibrated by image-based visual servoing of the X-Y stage for always keeping the embryo inside the field of view during cell orientation (Section 4.7.5). Although the slow dynamic response of visual servoing due to low vision sampling frequencies (30 Hz) result in a low speed for cell orientation, this calibration procedure is only required/ conducted on the first embryo that requires re-orientation for an entire batch of immobilized embryos, since pitches (300 μm) between adjacent embryos are accurately known.

**Feature Tracking**

The system selects a portion of the polar body including abundant edge information as a tracking target to provide the visual servo controller with position feedback in the image coordinate frame. A sum-of-squared differences (SSD) algorithm with a translation-rotation-scaling (TRS) motion model [83] is employed for tracking the target image patch. The SSD objective
Figure 4.14: Image-based visual servoing of the X-Y translational stage.

The function is

\[ SSD(u) = \sum_{x \in T} \left[ I(f(x, u), t_n) - I(x, t_0) \right]^2 \]  

(4.8)

where \( I(x, t_0) \) is the intensity of point \( x \) in the template image \( T \), \( I(f(x, u), t_n) \) is the intensity of point \( x \) in the rectified image with motion parameters \( u \) at time \( t_n \), and \( f(x, u) \) is the TRS motion model. By minimizing (6.1), \( u \) can be incrementally calculated. The increment \( \Delta u \) between two time instants is

\[ \Delta u = -\Sigma^T (M_0^T M_0)^{-1} M_0^T [I(f(x, u), t_n) - I(x, t_0)] \]  

(4.9)

where \( M_0 \) is an off-line computed constant matrix depending on the template image gradient and the TRS model, and \( \Sigma \) is a matrix determined by the TRS model. With a template of 31x31 pixels used in experiments, real-time visual tracking is achieved (calculation of \( \Delta u \) takes 20.5 ms).

**Calibration of Coordinate Transformation #3 and 3-DOF Position Control**

For rotating the first embryo that requires re-orientation, an image-based visual servo controller (Figure 4.14) is initiated to control the motorized X-Y stage for keeping the visually tracked image patch inside the field of view and ultimately, bringing the cytoplasm center to the image center. The system records the two angular positions \( \theta_1 \) and \( \theta_2 \) and the corresponding coordinates \( ^{'} t_{r1} \) and \( ^{'} t_{r2} \) of the X-Y stage before and after orientation and substitutes them into (4.7)
to determine \( P \), which completes the calibration of coordinate transformation #3. The calibrated model of coordinate transformation #3 is used for mapping an orientation angle into translational distances to compensate for rotation-induced translational motions.

Since this calibration process only requires to be conducted once for the entire batch of embryos, the control system is then switched back to separate control of the X-Y stage and the rotational stage (i.e., \( K \) switches to and stays at state 1 in Figure 4.11). Other embryos within the same batch that require re-orientation are oriented via closed-loop position control at a high speed.

### 4.8 Experimental Results and Discussion

Micropipettes with an inner diameter of 1.2 \( \mu m \) and a sharp angle of 10° were used for injection. All the embryos were injected in the time window of 1–3 hr post-collection. PBS buffer was injected for system performance evaluation, and the deposition volume was regulated by the computer controlled pressure unit at a resolution of 1 fL. 20× DIC objective (numerical aperture: 0.45, depth of field: 3.7 \( \mu m \)) was used for embryo imaging. The vertical and horizontal pixel sizes were calibrated to be \( s_x = s_y = 0.5 \mu m \).

#### 4.8.1 Embryo Immobilization

Devices with arrays of 3×3 and 5×5 through-holes were used for immobilizing mouse embryos (98±2 \( \mu m \)). The average diameter of the through holes is 37±0.5 \( \mu m \). Low pressures of 1.6 kPa-2.2 kPa were experimentally determined to be effective for holding the cells in place with sufficient forces during micropipette penetration.

For devices with 5×5 through-holes, a batch of mouse embryos more than the number of through-holes (e.g., 30-35) are transferred to the cell holding cavity (Figure 4.15(a)). With the application of a negative pressure, each through-hole traps a single cell. The immobilization process costs approximately 10 s. Extra untrapped cells are removed using a transfer pipette
Figure 4.15: Immobilization on a 5×5 array of mouse embryos. (a) 30 mouse embryos are transferred to the cell holding cavity. (b) With the application of a low sucking pressure (1.8 kPa), through-holes trap individual cells. 19 cells are immobilized within 5 s. (c) 25 cells are immobilized within 10 s. A transfer pipette is used to remove extra untrapped embryos. (d) The immobilized 5×5 array of mouse embryos. The complete process including removal of extra untrapped cells takes 31 s.

(Figure 4.15(c)). The complete process including the removal of extra cells typically takes 31 s for devices with an array of 5×5 through-holes.

When a small number of embryos need to be injected, devices with a lower number of through-holes (e.g., 3×3) are used. In this case, exactly the same number of cells are transferred to the cell holding cavity. In Figure 4.16(a), only 4 cells of the 9 delivered cells are within the field of view. Due to the small number of through-holes and the smaller cell holding cavity, the 9 through-holes are capable of rapidly immobilizing 9 cells without requiring the delivery of extra cells. Therefore, the step of removing extra untrapped embryos is not needed. The
Figure 4.16: Immobilization on a 3×3 array of mouse embryos. (a) 9 embryos are delivered into the cell holding cavity. 5 of them are out of field-of-view. (b) A 2 kPa sucking pressure initiates cell immobilization. (c) Last untrapped embryo is moving toward the final open through-hole. (d) The immobilized 3×3 array of mouse embryos. The complete process takes 12 s.

complete process for immobilizing 9 cells into a 3×3 array takes approximately 12 s.

4.8.2 System Performance Evaluation

During system development, 306 mouse embryos were injected. Figure 4.17(a) shows a penetrated embryo with the micropipette tip at cytoplasm center. Through these trials, an injection speed of 200 µm/s and a retraction speed of 500 µm/s were found to be optimal in terms of minimizing injection-induced embryo lysis.

To quantify system performance, the microrobotic system injected additionally 240 embryos with PBS buffer, demonstrating an average injection speed of 12 embryos/min vs. 2 embryos/min in typical manual operation by a proficient technician. The injected embryos were cultured inside a 37 °C incubator with 5% CO₂ for 4 days to allow the embryos to de-
Figure 4.17: Experimental results. (a) Micropipette tip at cytoplasm center before material deposition. (b) Microrobotically injected embryos developing into the blastocyst stage.

Furthermore, two measures were defined. **Success (non-lysis) rate**: this measure is defined as the ratio of the number of injected embryos without lysis to the total number of injected embryos, essentially representing the severity and frequency of the injection-induced embryo lysis. Based on visual inspection of the 240 embryos right after injection, the microrobotic injection system produced a success rate of $98.9 \pm 1.5\%$, a higher rate than the best success rate (90%) achieved by proficient injection technicians with over 12 years’ experience.

**Survival (blastocyst formation) rate**: this measure is defined as the ratio of the number of injected embryos developing into the blastocyst stage to the total number of injected embryos, quantitating the negative impact of microrobotic injection on embryonic development. For the determination of the survival rate, control groups of non-injected embryos were cultured at the same condition (KSOM, 37°C, 5% CO$_2$) to eliminate the embryo quality difference across embryo batches. Based on the 240 injected embryos, the system produced a survival rate of $89\pm3.5\%$, comparable with the best survival rate ($\sim80\%$) achieved by proficient injection technicians. Table 4.1 summarizes the statistics of success and survival rates of mouse embryos with PBS injection.
Table 4.1: Statistics of success and survival rates of mouse embryos with PBS injection

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of injected embryos</td>
<td>18</td>
<td>18</td>
<td>27</td>
<td>27</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>240</td>
</tr>
<tr>
<td>Number of non-lysis embryos</td>
<td>18</td>
<td>18</td>
<td>26</td>
<td>27</td>
<td>50</td>
<td>49</td>
<td>49</td>
<td>237</td>
</tr>
<tr>
<td>Number of blastocysts</td>
<td>16</td>
<td>15</td>
<td>24</td>
<td>23</td>
<td>46</td>
<td>45</td>
<td>44</td>
<td>213</td>
</tr>
<tr>
<td>Success rate (%)</td>
<td>100</td>
<td>100</td>
<td>96.3</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>98</td>
<td>98.9±1.5</td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>88.9</td>
<td>83.3</td>
<td>92.3</td>
<td>85.2</td>
<td>92</td>
<td>91.8</td>
<td>89.8</td>
<td>89±3.5</td>
</tr>
</tbody>
</table>

Of the injected 546 embryos, it was found that the polar body of 157 embryos (28.8%) lied out of the initial focal plane and therefore, required focal scanning for locating the polar body. Of the 546 embryos, the polar body of 45 embryos (8.2%) appeared in the space of quadrant II (Figure 4.12(a)), which required automated re-orientation.

Experiments demonstrate that the visual servo controller for calibrating coordinate transformation #3 is capable of keeping the target image patch inside the field of view at an orientation speed of 15 °/s. Within the same batch of embryos, for the rest of the embryos that require re-orientation, the cooperative controller of the X-Y stage and rotational stage oriented the cells at a speed of 720 °/s.

4.8.3 Discussion

Enabled by the microfabricated embryo holding devices and vision-position based control of multiple motion control devices, the microrobotic mouse embryo injection system is capable of fast immobilization, switching, orientation, and injection of mouse embryos. Requiring minimal human involvement (a maximum of three times computer mouse clicking per embryo), the automated system is independent of operator skills for injection and immune from human fatigue.
The high injection speed (12 embryos/min), success rate (98.9%), and survival rate (89%) demonstrate that the system is moving towards practical use to change how mouse embryos are injected. Except the micro devices for immobilizing embryos and the motorized rotational stage for orienting cells, the system contains no other custom developed components and presents literally no difference in hardware compared to a conventional injection system, which is an advantage promising its use in biology laboratories and mouse core facilities.

4.9 Conclusion

This chapter presented an automated mouse embryo injection system with a high degree of automation through vision-position based control of multiple motion control devices (a 3-DOF microrobot, a motorized X-Y translational stage, and a motorized rotational stage). A new system architecture was developed to enable fast embryo immobilization and automatic embryo orientation control. A microfabricated glass embryo holding device was used to immobilize a large number of embryos into a regular pattern within seconds. An in-house developed motorized rotational stage was employed for automatic embryo orientation via 3-DOF cooperative control. The experimental results demonstrate that the automated system is capable of conducting high-speed, reliable mouse embryo injection at a speed of 12 embryos/min with a high success rate (98.9%) and survival rate (89%).
Chapter 5

Molecule Testing with Microrobotic Mouse Embryo Injection

5.1 Introduction

Mammalian preimplantation embryo development is prone to high rates of early embryo arrest, particularly under current in vitro culture conditions. There are several possible underlying causes for embryo demise, for example, DNA damage, altered embryo metabolism, and the effect of suboptimal culture media, all of which contribute to an imbalance in gene expression and the failed execution of basic embryonic decisions [84]. An increasing body of evidence indicates that cell fate is determined by the outcome of specific intracellular interactions between pro- and anti-apoptotic proteins, many of which are expressed during oocyte and pre-implantation embryo development [85, 86]. It has been demonstrated that some human and murine oocytes display an imbalance in the endowment of maternally stored products and organelles. Specifically, mitochondrial abnormalities often exist in humans and strains of mice whose oocytes undergo excessive apoptotic fragmentation [87, 88].

Mitochondria, in addition to their critical role in the production of cellular energy, are also the source of key molecules involved in the regulation of programmed cell death (i.e.,
apoptosis) [89]. Proteins that propagate mitochondrial activities are candidates to affect oocyte health and subsequent embryonic development. Injection of healthy mitochondria isolated from murine embryonic stem or granulosa cells was demonstrated effective to rescue oocyte fragmentation in mice and facilitate the progression of zygotes throughout early cleavage stages [90,91]. However, mitochondria injection can alter the embryonic genome and have deleterious effect on physiological functions [92–94].

The Bcl-2 family of cytoplasmic proteins plays a central role in regulating apoptosis [95–98]. Many Bcl-2 family members function at the level of the mitochondria by regulating the permeability of the mitochondrial membrane by either forming or blocking pores on the mitochondrial membrane [99,100]. Opening these pores results in transitional changes in mitochondrial membrane potential accompanied by the release of several death-inducing factors from the mitochondrion into the cytoplasm. Several anti-apoptotic Bcl-2 family members can block the induced decrease in mitochondrial membrane potential [101] and inhibit the activation of apoptotic caspases by maintaining death inducing proteins within the mitochondrion [102].

Bcl-x is one of the anti-apoptotic Bcl-2 proteins that are most abundantly expressed in the oocyte [103–108]. Previous studies have showed that Bcl-x splicing is altered in the subset of fragmenting embryos, as both murine and human fragmented embryos up-regulate the pro-apoptotic form of Bcl-xS [85, 86]. Moreover, interfering with Bcl-x splicing by antisense oligonucleotides, resulting in depletion of Bcl-xL, is associated with increased cell death and decreased total number of cells in murine blastocysts [109]. These results suggest that the Bcl-x protein might be important for embryo survival. In contrast to mitochondria, the recombinant format of the Bcl-x protein has a terminal half-life resulting in its clearance prior to implantation; thus, its addition is aimed at providing transient support during the time most susceptible to embryo demise and will not result in any genetic modification of offspring. Screening these molecular targets by microinjection promises improved embryo development and clinical pregnancy outcomes in assisted reproduction.

High speed, high reproducibility, and high survival rate upon injection are critically im-
important for molecule testing. However, due to the inherent difficulty of manipulating small-sized (80-100 μm), delicate mammalian embryos, current manual injection suffers from a long learning curve, low success rates, and low reproducibility arising from human errors and inconsistency, which is a serious hurdle of determining the efficacy of the mitochondrial proteins for embryo rescue. This chapter presents the application of the automated micorobotic mouse embryo injection system to injecting the recombinant Bcl-xL protein for enhancing the preimplantation embryo development competence.

5.2 Hypothesis

The primary aim of this study was to assess the feasibility of enhancing early embryo development competence using automated injection of anti-apoptotic proteins. It is widely recognized that the balance of pro- and anti-apoptotic proteins contributes to oocyte quality and subsequently embryo development. Previous research has demonstrated that murine embryos of imprinting-control-region (ICR) genetic background have a tendency to arrest prior to the blastocyst stage when cultured in human tubal fluid (HTF) medium, known as a suboptimal culture condition. The arrest could result in less than 40% of the cultured embryos developing to the blastocyst stage. In contrast, potassium simplex optimization medium (KSOM) better supports the development of the ICR embryos and provides a blastocyst formation rate of ≥80%. Furthermore, 24-hr culture of ICR embryos in HTF medium causes downregulation of the Bcl-x protein at the 2-cell stage [110].

The hypothesis was that the suboptimal culture conditions alter the balance of pro- and anti-apoptotic proteins, which ultimately results in embryo demise; however, microinjection of the anti-apoptotic recombinant Bcl-x protein into preimplantation embryos is capable of rescuing the embryonic arrest, leading to improved blastocyst formation rates.
5.3 Materials and Methods

5.3.1 Mouse Husbandry and Embryo Preparation

All animal protocols were approved by the Mount Sinai Hospital Animal Care Committee and met standards for ethical animal treatment. All mice were housed in the mouse facility of Toronto Center for Phenogenomics with free access to food and water, and kept on a 12 hr-12 hr light-dark cycle. ICR mice were used in the experiments. The mouse embryos are prepared following the procedure described in Chapter 4 (Section 4.2).

5.3.2 Injection Materials

A recombinant Bcl-x protein, Bcl-xL (ΔTM), truncated at the carboxyterminal transmembrane domain, was injected into mouse embryos at the 1-cell stage. The Bcl-xL (ΔTM) protein was obtained from Dr. Newmeyer’s group [111, 112] with an original concentration of 16 µg/µl, and then diluted with injection buffer (Specialty Media) to 1.6 µg/µl. 3 µl of the diluted protein solution was injected into individual embryos. Injection buffer (Specialty Media) with 1.6 µg/µl BSA was also injected into ICR embryos as control groups.

5.3.3 Culture Conditions

The ICR mice exhibit a high rate of embryo arrest and increased cell death when cultured in the HTF medium, which is commonly used in in-vitro fertilization (IVF) clinics. In this study, the HTF medium was used as a mild adverse culture model for creating embryo defects and to determine the efficacy of Bcl-xL (ΔTM) on supporting preimplantation development under this mildly adverse culture condition. KSOM medium, which better supports the embryonic development, was also used to culture uninjected embryos as control groups.

Before experiments, 20 µl droplets of HTF (Irvine Scientific) or KSOM (Specialty Media) medium were set up in 35 mm petri dishes (Sarstedt), covered with mineral oil (Sigma), and
pre-equilibrated overnight in a humidified 37 °C incubator with 5% CO₂. All the collected embryos were cultured in KSOM droplets until injection. After injection with either Bcl-xL (ΔTM) protein or buffer with BSA, embryos were cultured in HTF medium with 10-14 embryos per group. In addition, uninjected embryos were also cultured in HTF and KSOM droplets as control groups.

5.3.4 Automated Microrobotic Mouse Embryo Injection

Micropipettes with an inner diameter (I.D.) of 3 µm and a sharp angle of 20° were used for microinjection. The micropipette fabrication method was described in Chapter 4 (Section 4.6). Cell holding devices with 5×5 through-holes were used for immobilizing embryos with sucking pressures of 1.6 kPa-2.2 kPa. The optimized injection speed of 200 µm/s and retraction speed of 500 µm/s were used for minimizing injection-induced embryo lysis. 400 embryos were injected with Bcl-xL(ΔTM) protein, and another 400 embryos were injected with injection buffer solution. All the embryos were injected within the time window of 1~3 hr post-collection.

5.3.5 Embryo Progression Analysis and Quality Evaluation

*In vitro* cultured embryos were assessed daily from the 1-cell stage to the blastocyst stage. Survival (blastocyst formation) rates were calculated as the ratio of embryos developing into the blastocyst stage at day 4.5 post-coitum (p.c.) to the total number of embryos in the culture group. The final values were mean values of the blastocyst formation rates of different culture groups.

Assessment of embryo quality at the blastocyst stage is commonly performed by quantitating the total cell number (TCN), cell death index (CDI), and mitotic index (MI) of individual blastocysts. The CDI is defined as the ratio of the number of apoptotic cells to the total cell number of the blastocysts. The MI is defined as the ratio of the number of mitotic cells to the total cell number of the individual blastocyst. At day 4.5 p.c., cultured embryos were fixed in
formalin for 15 minutes, and then counterstained with 4′-6-Diamidino-2-phenylindole (DAPI) for 10 minutes. The stained embryos were finally mounted with a 1:1 glycerol:PBS solution onto microscope slides. The slides were stored at 4 °C until analysis. Embryos were analyzed under a fluorescent microscope (Axioplan, Zeiss). Each embryo was assessed for TCN and numbers of apoptotic and mitotic cells.

5.3.6 Reactive Oxygen Species (ROS) Content Measurement

Early mammalian embryos are susceptible to damage caused by excessive reactive oxygen species (ROS) [113, 114]. Suboptimal culture conditions may result in alteration of embryo metabolism and elevate intracellular production of ROS which will in turn negatively affect the embryonic development. It has been demonstrated that in vitro culture in HTF medium yields greater superoxide anion production at the time of genomic activation (i.e., 2-cell stage) which may contribute to lowered developmental competence of embryos [115].

In the experiments, ROS content of the injected and uninjected embryos was measured at the 2-cell stage to determine if the injection of Bcl-xL (ΔTM) is capable of alleviating the oxidative stress resulted from suboptimal HTF culture. The level of ROS content was quantified using the dichlorodihydrofluorescein diacetate (DCHFDA) method [116, 117]. A 1 M stock solution of H2DCFDA (Molecular Probes) was prepared in dimethylsulfoxide (DMSO, Sigma) and diluted to 0.01 M before the start of each staining. The dye was then added to a double-well culture dish containing HTF or KSOM with 0.1% BSA with a final concentration of $1 \times 10^{-5}$ M. The samples were stained for 15 minutes in a humidified incubator at 37°C, 5% CO2, washed through several drops of HTF or KSOM, and placed in a 20 µl drop of HTF or KSOM on a 0.2 mm Hiraki depression slide. Live imaging was conducted on a deconvolution microscope (IX70, Olympus) with a fluorescein isothiocyanate (FITC) filter. Ten 1 µm optical sections for each sample were obtained. Average fluorescent intensity of the ten optical sections was determined using an image analysis program (SoftwoRx, Applied Precision Inc.), which was regarded as the ROS content for individual embryos.
Figure 5.1: Success rate comparison of automated mouse embryo injection and manual injection. The data of manual protein injection was provided by Dr. Marina Gertsenstein at the Toronto Center for Phenogenomics. Dr. Gertsenstein has over 12 years’ experience in microinjection.

5.3.7 Statistical Analysis

Experimental data were analyzed using either student’s t-test or one-way ANOVA (SigmaStat 3.5, Systat Software Inc.), as appropriate. In one-way ANOVA, post-hoc pairwise comparison was conducted using either Holm-Sidak or Dunn’s method. Experimental results were plotted as means ± one standard deviation.

5.4 Experimental Results

5.4.1 Success (Non-Lysis) Rates

The microrobotic injection system generated high success (non-lysis) rates for both protein and buffer injection, owing to its high reproducibility and optimized operation parameters (i.e., sucking pressures, injection and retraction speeds). Figure 5.1 illustrates the success rates
5.4.2 Survival (Blastocyst Formation) Rates and Embryo Quality Parameters

After injection, protein-injected and buffer-injected embryos were cultured at 10-14 embryos per group to reach the blastocyst stage. Figure 5.2 shows the experimental results of the survival (blastocyst formation) rates and their statistical analysis output. 74±5.6% of the embryos
Figure 5.3: Nuclear staining pictures of the blastocysts from (a) KSOM control groups, (b) HTF control groups, (c) HTF + buffer groups, and (d) HTF + protein groups. The dead and miotic cells are respectively labeled by solid and hollow arrows in (b).

(n=302), injected with Bcl-xL (ΔTM) protein and cultured in HTF (‘HTF + protein’ in Figure 5.2), formed blastocysts in contrast to 49.1±8.7% of the embryos (n=307) injected with buffer solution and cultured in HTF (‘HTF + buffer’ in Figure 5.2). These results suggest that injection of the Bcl-xL (ΔTM) protein is capable of significantly improving blastocyst formation of the ICR embryos in HTF.

In addition, KSOM medium better supported the development of uninjected ICR embryos with a survival rate of 85.4±7.4% (n=273), while HTF culture condition only allowed 51.2±9.1% of the uninjected embryos (n=273) to develop into the blastocyst stage, proving that HTF is a suboptimal culture medium. Although the embryos injected with buffer solution expressed slightly lower developmental competence than the uninjected HTF control groups (‘HTF control’ vs. ‘HTF+buffer’: 51.2±9.1% vs. 49.1±8.7%), there is no significant difference (p=0.344) between the two data sets, demonstrating that the microrobotic injection system...
does not impose significant negative effect on embryo development.

Quality of the formed blastocysts was assessed by quantitating the total cell number (TCN), cell death index (CDI), and miotic index (MI). All embryos reaching the blastocyst stage by day 4.5 p.c. were analyzed. Figure 5.3 illustrates the nucleus-stained blastocysts from the injected and control groups. Data of the cell quality parameters are illustrated in Figure 5.4.
and summarized in Table 5.1. The mean TCN of embryos from ‘HTF + protein’ groups (63.2±6.1%, n=44) are significantly higher (p<0.001) than those of embryos from ‘HTF + buffer’ (49.7±7.7%, n=32) and ‘HTF control’ (53.1±4.5%, n=35) groups, suggesting that injection of the Bcl-xL (ΔTM) protein greatly enhances the embryo quality, further confirming the ability of Bcl-xL (ΔTM) protein to protect the embryos from damage induced by HTF culture condition.

The mean CDI and MI of embryos from the ‘HTF + protein’ group are significant lower (p<0.001) than those of embryos from ‘HTF + buffer’ and ‘HTF control’ groups, indicating higher quality of the protein-injected embryos than the buffer-injected and uninjected ones. The data of cell quality parameters also demonstrate that embryos cultured in KSOM commonly have better quality than the ones cultured in HTF (Figure 5.4 and Table 5.1). The cell quality parameters of the embryos from ‘HTF + buffer’ groups are comparable to the embryos from HTF control groups, verifying that microrobotic injection does not significantly impair embryo quality.

### 5.4.3 Reactive Oxygen Species (ROS) Content

Another important measure of embryo health is ROS content. The ROS levels in 2-cell embryos from injected and control groups were examined using the DCHFDA method. Figure 5.5 shows the DCHFDA staining pictures of 2-cell embryos from the injected and control groups. Quantitative results of the measured ROS content are shown in Figure 5.6. Embryos from the ‘KSOM control’ group had a significantly lower ROS content (2.6×10⁶±4.7×10⁵, n=15) than the ones from the ‘HTF control’ group (4.1×10⁶±5.4×10⁵, n=15), showing the same trend as previous studies [115]. The embryos injected with Bcl-xL (ΔTM) protein had a significantly lower ROS level (2.9×10⁶±3.6×10⁵, n=15) than the ones from the ‘HTF + buffer’ (4.2×10⁶±5.8×10⁵, n=15) and ‘HTF control’ (4.1×10⁶±5.4×10⁵, n=15) groups. These results demonstrated that the Bcl-xL (ΔTM) protein is capable of effectively alleviating the oxidative stress imposed on the embryos by the suboptimal HTF culture environment.
Figure 5.5: DCHFDA staining pictures of 2-cell embryos from (a) ‘KSOM control’ groups, (b) ‘HTF control’ group, (c) ‘HTF + buffer’ group, and (d) ‘HTF + protein’ group. Fluorescence intensities were measured to quantify ROS content. Higher fluorescent intensity represents higher ROS content.

Figure 5.6: ROS content levels quantitated by fluorescent intensity measurement (* p< 0.001 compared to the ‘HTF control’ and ‘HTF + buffer’ values).
5.5 Discussion and Conclusion

Anti-apoptotic proteins are promising candidates for enhancing the developmental competence of *in vitro* cultured embryos. However, the testing of these molecular targets has been severely hampered by the lack of techniques for high-throughput delivery of molecules into embryos. Compared to other molecule delivery techniques such as electroporation, gene gun, viral vectors, mechanical microinjection is the most effective means to break the barrier of thick zona pellucida and introduce molecules into mammalian embryos. However, the state-of-the-art manual injection does not meet the requirements of molecule testing tasks, in terms of operator skill dependence, throughput, success and survival rates, and reproducibility. To address these limitations, an automated microrobotic mouse embryo injection system was developed and applied to mitochondrial protein testing.

The microrobotic injection system performed injection of Bcl-xL (ΔTM) protein and buffer solution at a speed of 12 embryos/min (vs. 2 embryos/min in manual injection). High accuracy and consistency of the microrobotic system produced higher success rates (protein injection: 79.8±6.2%, buffer injection: 82.8±5.6%) than manual injection (~50%), greatly improving the efficiency of protein testing. It was found that the injection of protein/buffer produced higher lysis rates (protein injection: 20.2%, buffer injection: 17.2%) than the injection of pure PBS (1.1%). This is mainly due to the use of larger injection needles (3 µm I.D.) in protein/buffer injection than in PBS injection (1.2 µm I.D.). Additionally, higher viscosity of the protein/buffer solution than PBS required higher injection pressures for material deposition, which also possibly induced higher lysis rates.

The experimental results of protein testing indicate that the injection of Bcl-xL (ΔTM) protein can improve the development competence of mouse embryos. Further screening of Bcl-2 family proteins (e.g., Bag-1, Bcl-xES, and Aven) using the automated microrobotic injection system would lead to the selection of most efficacious protein on improving embryo survival and promise clinical applications in assisted reproduction.
Chapter 6

In Situ Mechanical Characterization of Mouse Oocytes

6.1 Introduction

One important procedure in assisted reproduction technologies (ART) is assessing the reproductive quality of oocytes for in vitro fertilization (IVF). The state-of-the-art morphology analysis method [118, 119] fails to provide definitive prediction for the oocyte quality, causing low pregnancy rates and therefore, imposing extra difficulties on the follow-up reproductive studies.

Emerging techniques for oocyte quality assessment include preimplantation genetic screening [120, 121], near-infrared spectroscopy based metabolomic profiling [122–124], and polscope-based spindle imaging [125, 126]. Despite tremendous advances made by these techniques, they are limited by: (1) the invasive deoxyribonucleic acid (DNA) sampling procedure that may impair the oocytes and result in lower development competence [127]; or (2) the requirement of specific analysis equipment [122–126] and complex spectral data analysis [122–124]. ART demands efficient and low-cost methods for oocyte quality investigation.

It is well known that a variety of cellular functions, such as cell division, gene expression, signal transduction, and apoptosis, greatly depend on mediation and regulation of mechanical
signals (i.e., forces and stresses), and mechanical properties of cell membrane and intercellular proteins and fluid [128–130]. Similarly, a wide range of human diseases are also closely correlated with variations of the mechanical properties of cells [129, 131, 132], suggesting mechanical characterization of biological cells a possible candidate for disease state detection. In this context, the mechanical property (e.g., force-deformation data) of oocytes may provide additional cues for oocyte defects, such as mitochondrial leakage and DNA damage. Hence, it was motivated to develop suitable mechanical characterization techniques for exploring the feasibility of using mechanical cues for the purpose of oocyte quality evaluation.

For mechanical characterization of a living cell, the cell must be deformed in some way and the applied forces/stresses and cell deformations accurately measured. Experimental techniques for cellular force measurement include micropipette aspiration [34], optical tweezers [31], atomic force microscopy (AFM) [133], magnetic bead measurement [35], and microelectromechanical systems (MEMS) transducer based measurement [8, 18], among which MEMS force transducers outperform other tools due to their cost-effectiveness and flexibility for system integration. However, the construction of the MEMS force sensors are typically based on silicon micromachining [8] that requires sophisticated equipment and much precessing effort. Furthermore, issues such as biocompatibility and operating in an aqueous environment for biological cells to survive often pose stringent challenges and intricacies in MEMS design, material selection, and microfabrication.

Instead of using silicon-based MEMS transducers, polymeric materials such as polydimethylsiloxane (PDMS) and polyacrylamide (PAM) have been widely employed as passive deformable force sensors for cellular force measurements, due to their high transparency, low stiffness, and biocompatibility. Although PDMS or PAM flexible substrates are used for characterizing cellular traction forces by visually tracking local deformations of the substrate [134, 135], the continuous deformation model of the substrate requires heavily complex computation to interpolate measured local discrete deformations into global continuous deformations.

Based on the same concept, researchers conceived innovative PDMS micro-post structures
Figure 6.1: Cellular force measurement using low-stiffness elastic posts during microinjection.

as force transducers, enabling measurement of local traction forces generated by adherent cells [9, 136–138]. The devices can be easily constructed using the soft-lithography technique [139]. Image processing techniques were used for measuring the PDMS post deflection, and only a simple cantilever mechanical model is needed for mapping post deflections into cellular forces.

Most recently, such micro-post devices were modified by integrating magnetic nanowires into individual micro-posts so that external magnetic field induced forces can be applied to the cells [140, 141]. The cellular retraction force response to the applied forces was examined, providing a new way to investigate cellular locomotion behavior under mechanical stimuli. Although the polymeric micro-post devices can both apply mechanical stimuli to adherent cells and simultaneously measure their traction forces, they do no permit characterizing mechanical properties of suspended cells such as oocytes/embryos.

Considering in vitro oocyte fertilization is often achieved via intracytoplasmic sperm injection (ICSI), it would be convenient and efficient to mechanically characterize oocytes during the ICSI process. This chapter presents a vision-based cellular force measurement technique and its application to in situ mechanical characterization of mouse oocytes during microinjection for assessing mouse oocyte quality. A PDMS cell holding device (Figure 6.1) and a sub-pixel visual tracking algorithm are used together to visually resolve applied forces to a sin-
gle oocyte with nanonewton force resolutions. Experimental results demonstrate that the in situ obtained force-deformation data could be useful for distinguishing healthy oocytes from those with compromised cellular functions during microinjection, without requiring a separate mechanical characterization process. Follow-up structural analyses of the zona pellucida (ZP) and fluorescence analysis of filamentous actin (F-actin) content verify that structural differences of the ZP and cytoskeleton exist between healthy and defective oocytes, which is speculated to result from oocyte defects.

6.2 Hypothesis

Defective oocytes often contain compromised mitochondria, insufficient maternal endowment of proteins, and/or transcripts leading to chromosomal aneuploidy, particularly evident with aging [142, 143]. Thus, it is anticipated that these molecular events may have impact on the cytoskeleton. For instance, compromised mitochondrial function alters oocytes’ metabolic activities, such as cytoskeletal motor protein behavior and intracellular transport.

The hypothesis to test is that cellular force-deformation measurements can provide additional information for detecting oocyte dysfunctions and therefore, helping better select healthy oocytes for ICSI and subsequent implantation.

6.3 Materials and Methods

6.3.1 Mouse Oocyte Preparation

In this research, oocytes from young (8-12 weeks old) and old (40-45 weeks old) imprinting-control-region (ICR) female mice (referred as young and old oocytes in the rest of this chapter) were used as a comparison model to investigate the feasibility of using mechanical cues to distinguish healthy oocytes from defective ones. 40-45 weeks old ICR female mice are near the end of their reproductive lifespan. Their oocytes and corresponding embryos reveal com-
promised developmental competence due to multiple cellular defects, such as meiotic irregularities and mitochondrial dysfunction [142]. The old mouse model has been widely used in reproductive biology as an analogue to human female infertility due to a high maternal age (≥35 years) [88]. The oocytes were prepared following the same superovulation and cell collection procedures described in Chapter 4 (Section 4.2). No male mating was conducted. The average diameter of the mouse oocytes was 96 µm.

6.3.2 Working Principle of Vision-Based Cellular Force Measurement

Vision-based force measurement techniques are capable of retrieving both vision and force information from a single vision sensor (CCD/CMOS camera) under microscopic environments [144–146]. For cellular force measurement during cell manipulation, this concept is realized by visually tracking structural deformations of an elastic cell holding device, and subsequently, transforming material deformations into forces.

The cell holding device, as shown in Figure 6.2, integrates an array of cavities (180 µm in diameter) for accommodating individual cells. Inside each cavity, low-stiffness posts (45 µm high and 12 µm in diameter) are arranged in a circular pattern to support the oocyte during microinjection. Figure 6.1 schematically illustrates the working principle of the cell holding device for vision-based cellular force measurement during oocyte injection. While the micropipette injects individual oocytes inside these cavities, applied forces are transmitted to the low-stiffness, supporting posts. In real time, a sub-pixel visual tracking algorithm measures post deflections that are fitted into an analytical mechanics model to calculate the force exerted on the oocyte. The current post arrangement employs the minimal number of supporting posts (e.g., three) for securely immobilizing the oocyte during microinjection (Figure 6.1), maximizing the post deflections. Thus, a maximized force measurement sensitivity, defined as the ratio of post deflection to corresponding indentation force, is achieved.
6.3.3 Experimental Setup

The mouse embryo injection system (Figure 6.3), described in Chapter 4, is employed for in situ measuring the cellular forces during microinjection. It consists of the PDMS cell holding device, an inverted microscope with a CMOS digital camera, a 3-DOF micromanipulator for controlling the micropipette motion, a motorized X-Y stage for positioning cell samples, and a temperature-controlled chamber to maintain cells at 37 °C.

6.3.4 Device Fabrication and Characterization

The cell holding device (Figure 6.2) was constructed with PDMS via soft lithography [139]. Figure 6.4 illustrates the device fabrication process. Briefly, PDMS prepolymer prepared by mixing Sylgard 184 (Dow Corning) and its curing agent with a weight ratio of 15:1, was poured over a SU-8 mold (SU-8 50, MicroChem) made on a silicon wafer using standard photolithography. After curing at 80 °C for 8 hr, the PDMS devices were peeled off the SU-8 mold. The depth of the cavity and protruding posts is 45 µm, and the diameter of the posts is 12 µm (Figure 6.2). In order to make the PDMS surface hydrophilic, the devices were oxygen...
Figure 6.3: Experimental setup for cellular force measurement during microinjection.

Figure 6.4: Cross-section view of the device fabrication process.
plasma treated for 10 s before use.

Nanoindentation was used to determine the Young’s modulus of the cell holding device. Five cell holding devices were calibrated using a nanoindentation instrument (TI-750 Ubi nanomechanical test instrument, Hysitron). Figure 6.5 shows a calibration curve of applied forces vs. displacements. The determined Young’s modulus value is 524.7 kPa ± 22.1 kPa (n=5).

6.3.5 Force Analysis

Figure 6.6 shows a snapshot captured in the cell injection process. The microrobot controls an injection micropipette to exert an indentation force to a mouse oocyte, deflecting the three supporting posts on the opposite side. Post deflections, measured by a visual tracking algorithm that will be discussed in Section 6.3.6, are fitted to an analytical mechanics model to obtain contact forces between the oocyte and posts. Based on the contact forces, the indentation force applied by the micropipette on the oocyte is determined through the following force analysis.

The oocyte is treated as elastic due to the fact that quick indentation by the micropipette does not leave sufficient time for cellular creep or relaxation to occur. Consequently, the in-
Figure 6.6: Indentation forces deform the mouse oocyte and deflect three supporting posts.

Figure 6.7: Injection force analysis. (a) Force balance on the cell under indentation. (b) Post deflection model.

Injection force, $F$ is balanced by the horizontal components, $f_{hi}$ of contact forces between the oocyte and supporting posts (Figure 6.7(a)),

$$F = \sum_{i=1}^{3} f_{hi}, \quad i = 1, 2, 3$$  \hspace{1cm} (6.1)

In the device configuration, the radius of the oocyte (~48 µm) is larger than the depth of the cavity and posts (45 µm), resulting in an initial point contact between the oocyte and supporting posts before post deflections occur. However, the high deformability of mouse oocytes makes cell membrane conform to the posts when an injection force is applied to the oocyte. It is assumed that the contact forces are evenly distributed over the contact areas. Thus, the horizontal components, $f_{hi}$ are expressed by a constant force intensity, $p_{hi}$ and a contact
length, $a_i$ (Figure 6.7(b))

$$f_{hi} = p_{hi}a_i, \quad i = 1, 2, 3$$

(6.2)

Note that drag forces applied to the supporting posts by the fluidic environment were safely ignored, which were determined to be at a force level of $10^{-16}$ N using the fluidic drag model [147].

Slope $\theta$ of the posts’ free ends shown in Figure 6.7(b) was measured to verify the validity of linear elasticity that requires small structural deflections. The maximum slope was determined to be $11.1^\circ$, which satisfies $\sin \theta \approx \theta$; thus, the small deflection assumption of linear elasticity holds [148]. Therefore, the relationship of the horizontal force intensity, $p_{hi}$ and post deflections can be established [148].

$$p_{hi} = \frac{\delta_i}{40a_i(1+\gamma)(2H-a_i)} + \frac{8(a_i^3+8H^2a_i-6H^2a_i^2)}{3\pi ED^4}$$

(6.3)

where $i = 1, 2, 3$; $\delta_i$ is the horizontal deflection; $H$ and $D$ are post height and diameter; $E$ and $\gamma$ are Young’s modulus and Poisson’s ratio ($\gamma = 0.5$ for PDMS [149]).

Combining (6.1)-(6.3) yields the injection force applied by the micropipette to the oocyte.

$$F = \sum_{i=1}^{3} \frac{\delta_i a_i}{40a_i(1+\gamma)(2H-a_i)} + \frac{8(a_i^3+8H^2a_i-6H^2a_i^2)}{3\pi ED^4}$$

(6.4)

In (6.4), the unknown parameters are post horizontal deflections, $\delta_i$ and the contact length, $a_i$. Experimentally, imaging with a side-view microscope confirmed that the contact length, $a_i$ increases at a constant speed, $v_i$ for a given indentation speed. Hence, $a_i = v_it$, where $t$ denotes time.

Note that for a constant indentation speed of the micropipette, the variation speed of contact length $a$, $v_i$ varies for different oocytes. At 60 $\mu$m/sec used throughout the experiments, $v_i$ of the tested mouse oocytes were measured to be 0.8 $\mu$m/s-1.2 $\mu$m/s. Interestingly, the sensitivity of the mechanics model (6.4) to variations in $v_i$ is low. The injection force varies only by 1% when $v_i$ changes from 0.8 $\mu$m/s to 1.2 $\mu$m/s. Thus, the average value of the measured speeds, 1 $\mu$m/s was used to calculate injection forces for all the oocytes.
6.3.6 Real-Time Sub-Pixel Visual Tracking of Post Deflections

In order to accurately track post deflections, a sub-pixel visual tracking algorithm is developed. The task is two fold, to track image patches of the top surfaces of supporting posts as well as to accurately detect the circular center positions. Gradient-based tracking algorithms such as optical flow [150] are not capable of providing robust tracking performance. This is because gradient-based algorithms assume pixel intensities translated from one frame to the next remain constant (i.e., the brightness constancy assumption). However, shadows resulting from post deflections render this assumption invalid.

In this study, a template matching algorithm with template update is used to track the motion of the supporting posts, providing processing areas for subsequent least squares circle detection (LSCD) to determine posts’ center positions. Template matching with constant template update permits small changes in image patterns between successive frames of images; therefore, is capable of robustly tracking the top surfaces of the three supporting posts. Accumulative errors caused by updating templates are eliminated in the subsequent detection of circular centers using the LSCD algorithm.

Pixel intensity in an image can be represented by \( I_n(x) \), where \( x = (x, y)^T \) is the pixel position in the image coordinate frame and \( n=0, 1, 2, ... \) is the image frame number. Initially, a square patch (i.e., the initial template) of 80×80 pixels, containing the post top surface is acquired for each of the three supporting posts. The initial template is denoted by \( T_0(x_T) \), where \( x_T = (x_T, y_T)^T \) is the pixel position in the template coordinate frame. Then, the template used in the \( n \)th frame is \( T_n(x_T) \). Within each frame of image, the sum-of-squared-differences (SSD) correlation measure is calculated to locate the best match within a search window of 150×150 pixels. The displacement of the tracking target in the \( n \)th frame of image, \( \Delta x_n = (\Delta x, \Delta y)^T \), can be obtained by computing

\[
\Delta x_n = \arg \min_{\Delta x} \sum_{x_T \in T_n} [I_n(x_n + x_T + \Delta x) - T_n(x_T)]^2
\]  

(6.5)

where \( x_n \) is the template position in the coordinate frame of the \( n \)th image. The template is
Figure 6.8: Circular center detection of a post top surface. (a) Original image patch. (b) Resulting image patch after Canny edge detection. (c) Rightmost portion of the circle used for circle fitting. (d) Final fitted circle.

updated after each template matching operation by selecting the current best match

\[ T_{n+1}(x_T) = I_n(x_n + x_T + \Delta x_n) \]  

The time complexity of the algorithm depends on sizes of the template and search window. In this study, the template size is determined by the area of post top surfaces. A large enough search window was chosen to handle larger displacements, provided that images can be processed in real time (30 Hz) including image patch tracking and circular center detection.

After tracking the image patches that contain post top surfaces, the circular center positions are detected by the LSCD algorithm. Canny edge detection of a tracked image patch (Figure 6.8(a)) results in an edge image (Figure 6.8(b)). The curve edge of post top surface is then extracted for circle fitting. During curve edge extraction, only the rightmost portion of the circle (Figure 6.8(c)) was selected for circle fitting. The selection criterion is to minimize the error in the circle detection process. The left half (edge 2 in Figure 6.8(b)) was discarded as it is distorted and blurred due to the fact that the view was blocked by a portion of the cell membrane (edge 1 in Figure 6.8(b)), which can cause significant errors in circle fitting. Edge
3 is the shadow contour that was also discarded.

The extracted curve edge points were then fitted to a circle using a least squares fitting algorithm. Denote the coordinates of edge points as \((x_i, y_i)\), \(i = 0, 1, 2, \ldots, n\). The circle fitting algorithm minimizes the sum of squares of algebraic distance

\[
O(a, b, R) = \sum_{i=1}^{n} \left[ (x_i - a)^2 + (y_i - b)^2 - R^2 \right]^2
\]  

(6.7)

where \(a\) and \(b\) are coordinates of the circle center, and \(R\) is the circle radius. Rewriting (6.7) yields an objective function

\[
O(A, B, C) = \sum_{i=1}^{n} (z_i - Ax_i + By_i + C)^2
\]  

(6.8)

where \(z_i = x_i^2 + y_i^2\), \(A = -2a\), \(B = -2b\) and \(C = a^2 + b^2 - R^2\). Differentiating (6.8) with respect to \(B\), \(C\), and \(D\) produces a linear equation set, from which the center coordinates and radius of the circle are obtained. Figure 6.9 shows the tracked image patches and LSCD detected post top circles.

### 6.3.7 Zona Pellucida (ZP) Electron Microscopy

ZP is a unique extracellular membrane (8-10 µm thick in mouse oocytes/embryos) surrounding the oocyte/embryo, which is composed of several types of glycoproteins arranged in a delicate filamentous matrix [151]. The ZP structure significantly contributes to the mechanical stiffness of the oocyte [8,152]. To further understand the cause of possible subtle mechanical differences of healthy and defective oocytes, ZP surface morphology and glycoprotein structures were imaged using electron microscopy.

ZP surface morphologies of young and old oocytes were analyzed by scanning electron microscopy (SEM) imaging. Oocytes at 2 hrs post-collection were mounted on a Thermanox plastic coverslip (Fisher Scientific), fixed for 1 hr 2% glutaraldehyde in 1% sodium cacodylate buffer, postfixed for 1 hr with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, and dehydrated in an acetone series of increasing concentration, according to the protocol reported...
in [153]. After dehydration, the oocytes were CO₂ critical point dried in a polythene chamber, mounted on the specimen holder, coated with gold, and observed in an environmental SEM (XL-30, Philips).

Structural analysis of ZP glycoproteins was conducted via transmission electron microscopy (TEM) imaging of ZP cross-sections, following the method reported in [154]. Young and old oocytes at 2 hrs post-collection were fixed for 1 hr 2% glutaraldehyde in 1% sodium cacodylate buffer, postfixed for 1 hr with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, embedded in agarose, cut into 100 nm thick sections, and observed in a TEM (CM-100, Philips).

### 6.3.8 F-Actin Staining

Besides ZP, oocyte mechanical properties are also regulated by the cytoskeleton. In this study, F-actin content of the young and old oocytes was quantified by fluorescence microscopy. F-actin of the oocytes was stained by fluorescein isothiocyanate (FITC) conjugated phalloidin. Oocytes at 2 hrs post-collection were washed with phosphate buffered saline (PBS), fixed for 10 minutes with 4% formaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and stained with a 5 µg/ml phalloidin-FITC solution in PBS for 60 minutes at room temperature. The concentration of 5 µg/ml and the staining time of 60 minutes were experimentally determined to guarantee the saturation of all binding sites for phalloidin-FITC [155]. Between consecutive steps, PBS washing was conducted three times. Nuclei of the oocytes were also stained with 4’-6-Diamidino-2-phenylindole (DAPI) for control purpose.

Finally, the stained oocytes were finally mounted with a 1:1 glycerol:PBS solution onto microscope slides, and analyzed on a deconvolution microscope (Olympus IX70, Applied Precision Inc.) with a FITC filter. Ten 1 µm optical sections for each sample were obtained. Average fluorescent intensity of the ten optical sections was taken as the F-actin content.
6.3.9 Statistical Analysis

Experimental data were analyzed using student’s t-test (SigmaStat 3.5, Systat Software Inc.). Plots with error bars represent means ± one standard deviation (s.d.).

6.4 Experimental Results

All the experiments were conducted at 37 °C inside a temperature-controlled chamber. With a 40× objective (NA 0.55), the pixel size of the imaging system was calibrated to be 0.24 µm×0.24 µm. Micropipette tips used for indenting mouse oocytes were 5.3 µm in diameter.

The template matching algorithm and the LSCD algorithm together cost 22.3 ms for processing each frame of image, proving the real-time capability of the vision-based force measurement system. Figure 6.9 shows tracking of deflected posts. The tracking resolution was determined to be 0.5 pixel (e.g., 0.12 µm).

6.4.1 Force-Deformation Data

Twenty young oocytes and twenty old oocytes were delivered onto the cell holding device using a transfer pipette and then indented. The micropipette was controlled to indent each oocyte by
Figure 6.10: Force-deformation curves of young (blue) and old (red) oocytes.

Figure 6.11: Means ± one standard deviations of force-deformation curve slopes from young and old oocytes (* p<0.001).
Figure 6.12: Distribution histogram of the slopes of the force-deformation curves from (a) young oocytes and (b) old oocytes. There is a small overlap (4.4 nN/µm-4.8 nN/µm) of slopes between young and old oocytes.
25 µm at 60 µm/s. During the indentation process, force data were collected in real time (30 data points per second). Figure 6.10 shows force-deformation curves of both young and old oocytes. The horizontal axis represents cell deformation, \( d = d_1 + d_2 \), where \( d_1 \) and \( d_2 \) were defined in Figure 6.7. The vertical axis shows vision-based cellular force data. With the current cell holding devices and imaging system, the force measurement resolution was determined to be 2 nN.

One can note that most of the force-deformation curves of young and old oocytes separate themselves into two distinct areas with a slight overlap of a few curves. It was also observed that during microinjection only ZP is deformed when the cell deformation is less than 11.7 µm± 1.4 µm (n=40), corresponding to region I in Figure 6.10 where ZP stiffness is dominant. After the cell deformation is beyond 11.7 µm± 1.4 µm (region II in Figure 6.10), both ZP and cytoplasm are deformed. Thus, the force-deformation data in region II reflects the overall stiffness of the ZP and cytoplasm.

Slopes of the force-deformation curves were calculated using linear regression, which is considered as oocytes’ overall stiffness by assuming linear elasticity of the oocytes. Figure 6.11 shows the means ± one standard deviations (s.d.) of the slopes of young and old oocytes. It was found that old oocytes have significantly lower stiffness (p<0.001) than young oocytes (young oocytes: 6.4±1.3 nN/µm, old oocytes: 3.3±0.9 nN/µm). Figure 6.12 illustrates the distribution histogram of the slopes of the force-deformation curves. There is a small overlap (4.4 nN/µm-4.8 nN/µm) of the slopes or stiffness values. 10% (n=20) of the young oocytes and 15% (n=20) of the old oocytes falls into this overlapping.

6.4.2 ZP Structure Analysis

In order to probe the cause of the detected mechanical changes in old oocytes, ZP thickness, surface morphology, and cross-sectional glycoprotein structures of young and old oocytes were analyzed by optical microscopy, SEM, and TEM imaging. ZP thickness of 16 young oocytes and 10 old oocytes was measured under an optical microscope (Nikon TE-2000S) with 400×
In Situ Mechanical Characterization of Mouse Oocytes

Figure 6.13: Representative SEM images of ZP surfaces of (a) young oocytes and (b) old oocytes.

SEM imaging of ZP surfaces (Figure 6.13) demonstrate that young and old oocytes reveal different surface morphologies. All observed young oocytes (n=8) have a ‘spongy’ surface comprising of multiple layers of networked glycoproteins with numerous pores (Figure 6.13(a)), while only 20% of the old oocytes have similar surfaces. The other 80% of the old oocytes show a compact and rough surface without pores (Figure 6.13(b)). The different ZP surface morphologies indicate different structures of ZP glycoproteins in young and old oocytes, which were quantitatively analyzed via TEM imaging.

Figure 6.14 shows TEM cross-sectional views of the ZP glycoprotein structures from young and old oocytes. The density of ZP glycoproteins was quantitated using image processing techniques. An adaptive thresholding algorithm [56] was used to recognize the glycoprotein structure areas (black areas in Figure 6.14(d)). The area ratio of glycoprotein structures to the total image is defined as the relative density of glycoproteins. The final relative density value of each oocyte was obtained from five different ZP regions by averaging. As shown in
Figure 6.14: Representative TEM images of cross sections of ZP glycoprotein structures (C: cytoplasm). (a) Young oocytes. (b) Old oocytes. (c) A sub-region from (a) for image processing. (d) Binary image after adaptive thresholding of (c).

Figure 6.15: Relative density of ZP glycoprotein structures of young and old oocytes (* p< 0.001).
Figure 6.16: F-actin staining images of (a) young and (b) old oocytes. Green: F-actin. Blue: nucleus.

Figure 6.17: F-actin content of young and old oocytes (* p< 0.001).

Figure 6.15, the glycoproteins in old oocyte ZP are significantly sparser (p<0.001) than those in young oocyte ZP. It is believed that the sparser ZP glycoproteins in old oocytes result in lower ZP stiffness than young oocytes, which mechanically differentiates young and old oocytes in region I of the force-deformation data (Figure 6.10).

6.4.3 F-Actin Content

Figure 6.16 shows the F-actin staining picture of young and old oocytes, where the green and blue channels respectively represent F-actin and nucleus. Higher fluorescent intensity of the
green channel indicates higher F-actin content. The fluorescence analysis results (Figure 6.17) indicate that old oocytes contain significantly less (p<0.001) F-actin than young oocytes, which can be possibly responsible for the stiffness difference in Region II of the fore-deformation data (Figure 6.10).

6.5 Discussion

Mechanical properties of individual biological cells plays a key role in understanding cellular structures and functions, predicting their response to mechanical stimuli, and correlating mechanical properties to disease states [131, 156]. Characterizing mammalian oocytes during ICSI without a separate characterization process promises a useful and low-cost approach to detect potential oocyte defects and select high-quality oocytes for subsequent IVF and implantation. Targeting in situ distinguishing healthy oocytes from those with compromised cellular functions, a vision-based cellular force measurement technique was developed to resolve nanonewton-level cellular forces and characterize the stiffness of oocytes.

Using young and old mouse oocytes as a comparison model, the cellular force measurement technique was proved to be effective for resolving subtle mechanical changes of old oocytes, due to structural changes of the ZP and cytoskeleton. These cellular structure changes are speculated to result from the aging-induced defects of old oocytes. Previous studies have demonstrated that all ZP glycoprotein genes (ZP1, ZP2, and ZP3) downregulate in oocytes of old C57BL/6 mice [142], which may explain the lower glycoprotein density of old oocytes observed in the experiments. In addition, cytoskeleton-related genes, such as Krt8 and Myo10 also have a lower expression in the old C57BL/6 mouse oocytes [142]. Krt8 is a member of the type II keratin gene family, and its protein product forms intermediate filaments of the cytoskeleton. Myo10 is a gene for encoding Myosin-X, which is an actin-based motor protein involved in cell motility. Researchers in reproductive biology are still trying to uncover downregulated genes in old oocytes responsible for F-actin expression, which would interpret the
low F-actin content in old oocytes. Further studies are required to more clearly decipher the regulation pathways of these downregulated genes in mouse oocytes in order to better understand the connection of oocyte defects and ZP/cytoskeleton structure changes.

The vision-based cellular force measurement platform is not scale dependent although it was applied to characterizing mouse oocytes. Different from mouse oocytes, the majority of suspended cell lines have a much smaller size (e.g., fibroblasts are 15 µm in diameter). The presented PDMS cell holding device that was constructed using soft lithography, can be scaled down to accommodate cells of smaller sizes. Soft lithography permits the construction of PDMS structures with an aspect ratio up to 10:1 (post height vs. post diameter) via process optimization. For example, a cell holding device with supporting posts of 10 µm in height and 2 µm in diameter (aspect ratio: 5:1; mechanical stiffness of each post: 1.2 nN/µm), based on a 0.5 pixel visual tracking resolution obtained in this study, has the capability of visually resolving forces down to 145 pN with a 40× objective. Thus, the device design and real-time visual tracking algorithm provide a highly cost-effective, yet powerful experimental platform for single cell studies with a sub-nanoNewton force measurement resolution.

Possible insignificant error sources of the cellular force measurement, which were not considered in this study, include: (1) the assumption that the contact forces between the cell and supporting posts are even distributed over the contact areas; (2) the use of average value of $v_i$; (3) the Young’s modulus calibration uncertainty (4.2%) of the PDMS cell holding device; and (4) the visual tracking error for post deflection measurements ($\leq 0.5$ pixel).

### 6.6 Conclusion

This chapter presented a vision-based cellular force measurement technique to measure cellular forces applied to single biological cells through the use of a microfabricated PDMS device and a sub-pixel computer vision tracking algorithm with a resolution of 0.5 pixel. Based on visually tracked deflections of elastic, low-stiffness supporting posts, quantitative force mea-
measurement results on mouse oocytes were obtained through an analytical mechanics model. Experimental results demonstrate that the system is capable of performing robust cellular force measurements at a full 30 Hz with a 2 nN resolution.

The technique was applied to *in situ* characterization of mouse oocytes and proved useful to distinguish healthy oocytes from those with aging-induced cellular defects. The force-deformation data revealed that the old oocytes have lower relative stiffness than healthy young oocytes. Structural analysis of ZP and F-actin showed the mechanical difference between young and old oocytes can be due to the cellular structural changes, which could be a result from cellular defects (e.g., downregulation of certain genes) in old oocytes. This research represents the first study that quantified mechanical difference between young and old mouse oocytes, promising a practical way for oocyte quality assessment during microinjection.
Chapter 7

Conclusions

In this research, microrobotic systems for zebrafish embryo injection and mouse embryo injection were developed. Leveraging automatic control and computer vision techniques, the automated systems enable robust cell injection at a high speed. Importantly, the systems feature unparalleled reproducibility and success/survival rates resulting from the optimization of operation parameters. The microrobotic systems are amendable to use for operators without long training of microinjection and can be directly applicable to genetics, transgenics, and reproduction studies.

Application of the microrobotic mouse embryo injection system to molecule testing of anti-apoptotic mitochondrial proteins demonstrated that the Bcl-2 protein family member, Bcl-xL (ΔTM) is capable of rescuing embryo arrest at the preimplantation stage and significantly enhancing the blastocyst formation rates of mouse embryos cultured in suboptimal culture conditions. Further screening of the Bcl-2 family proteins using the automated microrobotic injection system would lead to the selection of most efficacious protein on improving embryo survival and promise clinical applications in assisted reproduction.

The vision-based cellular force measurement technique is capable of resolving cellular forces during microinjection with a nanonewton force resolution. In situ mechanical characterization of mouse oocytes using this technique revealed mechanical difference between
young and old mouse oocytes, which was due to structural changes of the zona pellucida (ZP) and filamentous actin in old mouse oocytes. The mechanical characterization technique is a promising approach for bolstering oocyte quality assessment during microinjection without requiring a separate characterization process or dedicated, expensive equipment.

7.1 Contributions

1. Development of the first microrobotic zebrafish embryo injection system including fast sample preparation; and demonstration of fully automated, robust injection at 15 zebrafish embryos per minute with survival, success, and phenotypic rates all close to 100%.

2. Development of a vision-based contact detection technique for microrobotic manipulation to precisely determine the relative vertical positions of the end-effector and the micro object to be manipulated.

3. Development of a microrobotic mouse embryo injection system featuring new types of microfabricated glass cell holding devices, visually servoed automatic cell orientation, high injection speed, and high success/survival rates.

4. Design and microfabrication of glass micro devices for rapid single cell immobilization in mouse embryo microinjection.

5. Development of a motorized rotational stage for inverted optical microscopy and a visual servoing control strategy for automatic cell orientation during microrobotic cell manipulation.

6. First quantification of the efficacy of anti-apoptotic protein, Bcl-xL (ΔTM) on rescuing mouse embryo arrest and enhancing embryo development competence, with the automated microrobotic mouse embryo injection system.
7. Development of a vision-based cellular force measurement technique for mechanical characterization of biological cells during microinjection.

8. First quantitative study of the mechanical difference of young and old mouse embryos using the vision-based cellular measurement technique for mouse oocyte quality assessment.

### 7.2 Future Directions

Many possibilities exist for further extending this research. Examples are:

1. To fully automate the microrobotic mouse embryo injection system to further improve the injection speed. This objective can be achieved by developing robust pattern recognition algorithms to identify cellular structures of mouse embryos and automatically determine the intracellular location for material deposition. A practically achievable injection speed can be 30 mouse embryos per minute (15 times manual operation speed).

2. To increase microinjection throughput by increasing the array size to 10 by 10 on the cell holding devices. This will permit the injection of 100 cells per batch.

3. To screen other anti-apoptotic mitochondrial proteins (e.g., Bag-1, Bcl-xES, and Aven) from the Bcl-2 family for selecting the most efficacious protein on improving embryo development, which will have both research value in the mouse model and clinical applications.

4. To develop cell mechanics models for interpreting force-deformation data collected from vision-based force measurements to extract Young’s modulus values of cells. Possibly, two separate models are required to quantify the Young’s modulus of the zona pellucida (ZP) and of the cytoskeleton.
5. To extend the automated microinjection technologies to other related cell manipulation tasks, such as automated enucleation, polar body extraction, blastocyst injection, and intracytoplasmic sperm injection (ICSI).
Bibliography


