Robotic Manipulation and Characterization of Cells for Drug Screen and Clinical IVF Applications

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Abstract

Heterogeneity is the hallmark of cell biology. Robotic system and automated methods are required for characterization and manipulation of a large number of single cells for many important applications, such as characterizing cell-cell communication for screening drug effects, processing oocytes or embryos for cryopreservation, and analyzing sperm locomotion behavior for selection of a high-quality sperm for in vitro fertilization (IVF). However, current robotic characterization and manipulation systems have two major limitations in search-and-locating end-effector tips and determination of relative Z position between end-effector tips and single cells. This research has addressed these two limitations by developing an auto-locating end-effector tip method and two vision-based contact detection algorithms. By integrating the two new solutions, I have built two robotic system prototypes for microinjection of a large number of adherent cells and automatically processing oocytes or embryos for vitrification. The robotic microinjection system enabled automated gap junction measurement for repurposing antiarrhythmic drugs to identify optimal treatments for cardiac arrhythmias by assessing each drug’s efficacy on rescuing/enhancing gap junctions. Robotic vitrification relieves human operators from tedious and difficult manual steps, eliminating operator errors and inconsistencies. It also offers unparalleled timing control based on real-time embryo volume
monitoring. Automation enables the system to process multiple oocytes/embryos with high efficiency. In addition, automated methods were developed to quantitatively analyze sperm locomotion behaviors by tracking both sperm head and tail. Experimental results on automated sperm analysis revealed a significant correlation between sperm head velocity and tail beating amplitude. By combining automated sperm motility analysis with DNA assessment technique, this research offers a new solution for identifying single sperms of high quality for IVF applications. In the end, this research also reviewed future research directions in microsystems and nanoengineering for intracellular measurement and manipulations and discussed the promising research for the intracellular study.
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Contribution of Co-authors

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Professor Y. Sun was involved in project design and manuscript preparation. Discussions regarding research concepts, collection and interpretation of data were continuously conducted among all contributing authors.


Professor Y. Sun and Dr. R. Hamilton were involved in project design and manuscript preparation. V. Siragam was involved in cell preparation and data interpretation. Z. Gong, J. Chen, C. Leung, and Z. Lu helped in building the system hardware and programming software. Discussions regarding research concepts, collection and interpretation of data were continuously conducted among all contributing authors.


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To Qiaorong Fan, my dear and loving wife
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Chapter 1
Introduction

This chapter discusses the background knowledge for robotic single cell manipulations and characterization. In this work, adherent cells, oocytes/embryos and sperm cells are used as representative examples to demonstrate robotic and automated methods for characterizing cell-cell communication, picking-and-placing single cells, and analyzing sperm locomotion behaviors. A review of previous methods is also presented in this chapter.

1 Introduction

1.1 Single Cell Characterization and Manipulation

Population-based techniques, such as flow cytometry, in biological experimentation are unable to probe the rich information available from the study of single cells [1]. Heterogeneity is a hallmark of cell biology and is strongly evident in primary cell populations isolated from the same tissue [2] and well-established cell lines [3], [4]. Furthermore, supposedly identical clonal cell populations have been shown to deviate in their genetic expression [5] and response to environmental stimuli over generations of cell division [6]. This diversity has significant implications in coordinating multicellular behaviors and is of critical importance in developmental biology, pathobiology, and tissue engineering. For example, the heterogeneous responses to apoptosis-inducing drugs or ligands play a critical role in discovering new treatment for various diseases such as cancer, autoimmune disorders and neurodegenerative diseases [7]. Single-cell measurements are key to accurate characterization and modeling of heterogeneous responses. Therefore, single cell characterization and manipulations are necessary to understand the cellular basis for population behavior; and can also yield new methods for discovery of signaling pathway mechanisms and biochemical basis for cellular function [8].

Although single cell studies have attracted researchers’ extensive focus and efforts, sensing and quantifying single cell behaviors and properties from bulk analyses are not easy [9]. Their signals tend to be swamped by noise from the general population and the distinction between individual cells are blurred, making it difficult or impossible to understand which cell contributes to what effects in the entire cell populations or tissues [10] [11].
Recent advances in robotics and automation, especially at micro/nano scales, have demonstrated their feasibility and unique contributions in achieving high-throughput characterization and manipulations of single cells. Compared to manual operation, robotic and automated systems have the inherent advantages of high speed and high positioning accuracy. For example, microinjection is a widely used technique to deliver foreign materials (e.g., fluorescent molecules, DNA/RNA) into single cells. However, manual operation can only inject several to tens of cells [12], limiting its usefulness when a large number of single cells need to be tested. Furthermore, manual injection’s success rate and post-injection cell survival rate are largely dependent on the operator’s skills and can vary significantly from case to case.

In addition to the advantages in speed and accuracy, robotic and automated methods can provide objective results with improved reproducibility. For example, computer-aided sperm analysis can produce objective quantifying-matrices to assist doctors or embryologists to select high-quality sperm for in vitro fertilization (IVF). In contrast, manual selection of a single sperm is ad hoc, largely depending on the operator’s personal knowledge and expertise.

In general, biological cell lines in vitro can be classified into two groups: adherent cells and suspended cells. Adherent cells (e.g., cardiomyocytes, endothelial cells), as the major cell types derived mostly from vertebrates, are anchorage-dependent and need to be cultured on certain types of substrate that are specially treated to allow cell adhesion and spreading. In contrast, suspended cells (e.g., oocytes/embryos, blood cells, and sperm cells) are cultured in suspension mediums and do not require adhesion to the culture substrate. In the group of suspended cells, sperms are a special type of cells that can generate freely-swimming motion. Typical manipulations for adherent cells are microinjection and biopsy, whereas manipulations for suspended cells also include the pick-and-place or translocation of single cells. This research covers all typical cell manipulations including microinjection of adherent cells, pick-and-place of suspended cells manipulations, and analysis of sperm locomotion behaviors.

To assist single cell characterization and manipulation, this work focuses on the development of enabling micro-robotic systems and automated methods for characterizing cellular properties (e.g., cell-cell communication), analyzing cell behavior, and manipulating single cells. This research involves the development of the first-of-its-kind robotic adherent cell injection system for characterization of gap junctional intercellular communication, which has significant
implications for screening drug efficacy for cardiac diseases caused by the dysfunction of cell-cell communication. Additionally, this thesis also introduces a robotic system to pick-and-place mammalian oocytes or embryos for cryopreservation. Besides, this research also includes the development of computer vision algorithms for tracking both sperm head and tail to facilitate the analysis of sperm locomotion behaviors for IVF applications.
1.2 Characterization of Gap Junctional Intercellular Communication

Gap junctional intercellular communication (GJIC) exists in most mammalian tissues. Gap junction is a specialized intercellular channel formed by the juxtaposition of two half channels called connexons [13], [14]. Gap junctions are critical to several physiological roles, including impulse propagation in cardiac and neuronal tissue [15], regulation of embryonic development [16], homeostasis [17], and regulation of cellular proliferation [18]. Disorders of GJIC have been associated with many pathological conditions and vital diseases, such as cardiac arrhythmias [19], [20], which are a leading cause of cardiac morbidity and sudden death [21].

Gap junctions provide a direct pathway for electrical and metabolic signaling between connecting adjacent cells. Similar to ion channels, gap junctions are also ionic conduits [22]. However, unlike conventional ion channels, the selectivity of gap junctions for small ions (either positively or negatively charged) is minor compared to the selectivity of Na\(^+\) and K\(^+\) channels. The size of gap junction channels is much larger than that of conventional ion channels (100-150 Å vs. 3-5 Å) [23].

Gap junctions facilitate both diffusion of small molecules and conduction of ions between adjacent cells. Intercellular diffusion is attributed to essentially only through gap junctions, whereas sodium channels also contribute to conduction in the heart. In addition to ionic communication, gap junctions are molecular sieves that allow small hydrophilic chemical molecules, such as metabolites and second messengers (e.g., triphosphate inositol), to pass [24]. Gap junctions are regulated by gating that is referenced as open, semi-open, and closed configurations. When all the channels in a gap junction gate are closed, diffusion of permeants from cell to cell is halted. Experimentally, gating is commonly induced by imposing a voltage gradient across the junction. In addition to gating, the gap junction-mediated intercellular communication is also regulated by intracellular calcium, pH, and phosphorylation [25].

GJIC is regulated by the number of gap junction channels in the membrane, the functional state of gap junctions, and their permeability [26]. The measurement of GJIC has been studied for decades by using a number of methods, such as dye transfer through microinjection [27], the scrape/scratch method [28], electroporation [29], fluorescence redistribution after photobleaching [30], and conductance measurement by dual-cell patch clamp [31]. Among these techniques,
microinjection of membrane-impermeable, non-toxic tracers into single cells has been the most commonly used technique for identifying and mapping GJIC for a wide variety of cells [32]. The microinjection method is considered superior to other techniques because of the following reasons: 1) microinjection permits the correlation of morphological and functional data from individual cells; 2) the technique enables kinetic studies aimed at evaluating the transfer rate from one cell to another; and 3) in microinjection, the level of cell communication is expressed as number of dye-coupled cells, permitting the direct comparison of GJIC in different cell types [33].

However, microinjection has stringent skill requirements, low cell viability rate, and poor reproducibility [12]. Manual microinjection is typically used for injecting a few or tens of cells per experiment, limiting its usefulness when a large number of cells need to be tested for GJIC assessment. In this research, I have developed a robotic microinjection system and technique, for the first time, to enable the injection of hundreds of adherent cells per experiment rapidly and accurately. The automated system is operated via computer mouse clicking for indicating target cells for microinjection. Training a user who has no skills in microinjection takes ~15 min, and after a few hours of operation, the user can readily become proficient at operating the system to perform microinjection with high success rates.
1.3 Robotics and Automation for in vitro Fertilization

The World Health Organization reports that averagely 8~10% of couples worldwide experience infertility [34]. And data from the US National Centre for Disease Control shows that one out of eight North American couples seek medical treatment for infertility. Since the birth of Louise Brown, conceived using in vitro fertilization (IVF) pioneered by Noble Prize Laureate – Robert Edwards, the outcomes and impact of assisted reproductive theologies (ART) have been remarkable [35][36]. Despite the significant progress in ART, most operations in IVF clinics are still performed manually. Treatment outcomes largely rely on expert knowledge and experienced hands. To address the bottleneck problem in IVF, this research involves the development of robotic systems and automated methods aiming to standardize two IVF routine procedures: (1) vitrification of oocytes or embryos, and (2) analysis of sperm locomotive behaviors.

1.3.1 Robotic Vitrification of Mammalian Embryos

Cryopreservation of mammalian reproductive cells is an essential technique in IVF clinics [37][38]. Oocytes and embryos are routinely frozen and preserved for use in future. Nowadays, as more and more young ladies choose to focus on career development while they are young, cryopreservation is becoming a popular technology for preserving high-quality oocytes from their young age, allowing them to defer their family planning to the future [39]. Additionally, patients who undergo therapeutic procedures that can place their fertility at risk, such as chemotherapy, have the option of preserving their oocytes for future use through IVF cryopreservation. Furthermore, fertilized embryos are often more than needed for transferring back to mother uterus in one cycle of IVF treatment. The rest of the fertilized embryos are usually cryopreserved for future use. The length of time an embryo is frozen in liquid nitrogen has been shown not to have a significant impact on clinical pregnancy, miscarriage, implantation, or live birth [40].

Techniques of oocyte/embryo cryopreservation are classified into two categories, slow freezing and fast freezing (i.e., vitrification). Both techniques aim to minimize cell damage that is largely due to the formation of intracellular ice crystals at low temperature [41]. Slow freezing is a well-established technique developed during the early 1970s, which makes use of programmable sequences, or controlled cooling rates. During slow freezing, extracellular water freezes away from the embryo using a seeding technique, which creates an osmotic gradient that draws water
out of the cell until it finally freezes without the formation of intracellular ice crystals [42]. This procedure requires sophisticated equipment to control the freezing rate, which ranges between 0.3 and 1.0 µ°C/min, and produces a relatively poor survivability rate [43].

On the other hand, vitrification or fast freezing is a more effective cryopreservation method, first reported in 1985 [44]. Vitrification is considered superior to slow freezing because it vitrifies the oocyte/embryo with no crystal formation during freezing. The addition of cryoprotectants in vitrification increases the cytosol viscosity and makes the vitrified oocyte/embryo syrupy. When directly freezing oocytes/embryos in liquid nitrogen, the syrupy content inside the cell forms amorphous ice instead of ice crystals, which minimize the vital damage to the cell during freezing.

At present, oocyte/embryo vitrification is done manually in IVF clinics. An operator looks through the microscope eyepieces and manipulates oocytes/embryos using a Stipper® micropipette or mouth pipette. Manual operation for oocyte/embryo vitrification is a demanding and tedious task, for the following reasons:

(1) vitrification requires precise washing sequences and strict timings in each vitrification solution (VS) because the cryoprotectants (e.g., DMSO) are toxic and can significantly damage the cell viability if overexposed in vitrification solution;

(2) because of their small size (about 150 µm), oocytes/embryos can be difficult to observe and manipulate, especially when the oocytes/embryos locations are dynamically changing during micropipette aspiration and dispensing;

(3) washing oocytes/embryos with the highly viscous VS can cause osmotic shock to the cells, which can damage the cell survivability in vitrification;

(4) the manual process has stringent skill requirements, and success rate and cell survival rate vary across different operators. In IVF clinics, processing an embryo/oocyte in cryoprotectant medium typically takes a highly skilled embryologist 10 to 15 minutes.

To address the shortcomings of manual vitrification, this research focuses on the development of a robotic system for automatically pick-and-placing the oocytes/embryos through various vitrification solutions.
1.3.2 Quantitative Analysis of Sperm Locomotion Behavior

In natural fertilization, a healthy sperm overcomes the physiological and biological selection barriers, actively seeks out and fertilizes an egg. Sperm selection occurs naturally in this procedure. However, for couples having infertility issues, assisted reproduction technologies are required to address their reproductive needs. For example, in intracytoplasmic sperm injection (ICSI), an embryologist selects a single sperm cell and injects it directly into an oocyte (i.e., egg cell) to overcome issues such as male infertility [45]. These assisted reproduction technologies bypass the natural sperm selection barriers and demand expertise knowledge from the operator to select high-quality sperms. The criteria for sperm quality assessment provided by the World Health Organization are vitality, morphology, and motility [46]. A widely used method for sperm selection is motile sperm organelle morphology examination (MSOME) [47][48][49][50]. Sperm motility is also a commonly used criterion for sperm quality assessment. A motility grade is often used as a specified measure and classified into four grades:

- **Grade 1**: sperm with fast progressive movements;
- **Grade 2**: sperm with slow progressive movements;
- **Grade 3**: sperm with slow non-progressive movements (i.e., with curved motion);
- **Grade 4**: sperms are immobile and fail to move at all.

Besides sperm assessment based on morphology and motility, another method for selecting a healthy sperm is based on the analysis of sperm DNA integrity. Some of the DNA analysis methods assess sperm DNA quality directly, such as the TUNEL assay and the sperm chromatin structure assay. Some other methods indirectly measure sperm DNA quality. For instance, Huszar’s group recently proposed a hyaluronic acid (HA) assay [51]. Among a consecutive series of studies on HA-based sperm assay, researchers in Huszar’s group indicated that HA assay permits the selection of healthy sperm with no DNA damage [52][53]. In their research, sperms that bind to HA microdots are proven to have a higher level of DNA integrity compared to those unbound sperms. In clinical HA-based sperm selection, a number of healthy sperms’ heads bind to the HA microdot and lose their progressive movement despite vigorous tail beating. In this case, the sperm tail beating movement becomes the only indicator to differentiate the HA-bound sperms from each other.
The past few decades have witnessed the development of computer-assisted sperm analysis (CASA) methods for measuring both sperm morphology and motility [54]. CASA utilizes automated systems to digitize successive images of sperm, process and analyze the information, and provide the accurate and objective value for individual sperm cell. Since the 1970s, many algorithms have been developed to track sperm trajectories, measure sperm velocities, and analyze sperm morphology. Shi et al. reported a robust single-sperm tracking algorithm based on a four-class thresholding method to extract a single sperm in a region of interest [55]. The nearest neighbor method is complemented with a speed-check feature to aid tracking in the presence of additional sperm or other particles. In another study, Nafisi et al. demonstrated a template matching algorithm for sperm tracking. The algorithm is insensitive to image acquisition conditions [56]. Existing algorithms for sperm tracking are largely limited to sperm head tracking. The small size (≤ 1 μm in thickness) and low contrast of sperm tails under optical microscopy makes tracking sperm tails challenging.

This research also involves the development of a new automated analysis method by tracking both sperms’ heads and tails. With this automated method, users can obtain objective information about sperm locomotion behaviors. This research has significant applications to assist the selection of high-quality sperm by unifying the sperm motility analysis with DNA assessment techniques (e.g., HA assay) in IVF clinics.
1.4 Previous Methods for Robotic Cell Manipulation

The past decade has seen significant development of robotic cell manipulation systems and automated characterization techniques for patterning, grasping, measuring, and injecting individual cells [57][58][59][60]. In general, the strategies for single cell manipulations and characterization can be classified into five categories: mechanical, fluidic, electrical, optical and magnetic.

![Figure 1-1 - Schematic illustrations of typical cell manipulation strategies, and techniques for characterization and manipulation of single cells with mechanical strategy.](image)

The mechanical strategy, which directly acts on target cells with physical tools, is the oldest and still the most commonly-used method for single cell manipulation in biology labs [61]. In this category, micropipettes are often used to inject aqueous materials into single cells [62], pick-and-place single cells [63], and extract inner cell organelles or samples (e.g., polar body biopsy) [64]. In addition to conventional glass or plastic micropipettes, MEMS-based microgrippers have also been designed and fabricated for picking-and-placing single cells and characterizing their mechanical properties [65][66]. Mechanical strategy is the simplest approach and easy to use by directly touching the cells with physical force. However, direct interactions between single cells and micro tools can often introduce unexpected disturbances to cellular normal functions and
damage cell viabilities, especially when the experiments are conducted by users with little micromanipulation experience.

The second group of manipulation strategies use microfluidic devices to move single cells within constrained channels [67][68]. In microfluidic manipulations, researchers have applied flow-induced forces to characterize the cellular mechanical properties [69][70]. For automation in IVF, such as for vitrification of embryos and sperm quality assessment, microfluidic approaches have also become increasingly popular. The advantages of using microfluidic devices include the pre-patterned motion paths, minimal volume requirement, reduction of material costs, and decreased reaction time with high surface-to-volume ratio [71]. However, the difficulties in introduction and extraction of single cells on microfluidic devices greatly limit the practicality for targeting single cells of interest [72]. Moreover, the measured and processed single cells are difficult to be extracted from the large cell populations.

Different from mechanical and fluidic manipulations, which involve physical contact with single cells, electrical, optical and magnetic manipulations are capable of interacting with single cells in an untethered way. Electrical manipulations, such as dielectrophoresis (DEP) [73], generate a non-uniform electrical field to exert electrical forces on dielectric particles or cells. DEP manipulates cells without contacting them and is easy to control well [74]. However, the generated forces strongly depend on surrounding medium, cell electrical properties, cell size and shapes [75].

Optical manipulations, or optical tweezers, use a highly focused laser beam to produce an attractive or repulsive force depending on a refractive index mismatch to physically hold and move micro/nano-objects [76]. Although the optical tweezers can accurately position single cells with minimal invasiveness, this technique is limited in force generation (pN levels), and increasing laser power for larger force generation has the risk of laser-induced cell damage.

Magnetic nanoparticles, after introduced into cells, can be moved by magnetic forces generated by controlled magnetic fields. Because cells do not contain magnetic structures, the force is specifically applied to the internalized magnetic particles. Magnetic tweezers have been used for manipulating intracellular structures such as chromatin [77] and phagosomes [78]. Although most magnetic tweezers reported in literature apply forces in one direction only, there are a growing number of systems that allow 2D and 3D manipulation [79], [80]. One major limitation
of magnetic manipulation lies in the positioning accuracy and force resolutions. To solve this problem, pioneering researchers in Nelson’s group at ETH Zurich, Sitti’s group at Carnegie Mellon, and Kumar’s group at UPenn have worked to improve the control system for moving single [82] or multiple [83][84] untethered microrobots to assemble microobjects [85] or deliver drugs to single cells and tissues [86]. The magnetic microrobots had also been built with bioinspired flagellar shapes to increase their mobility in a liquid environment at micro/nano scale [87][88]. Japanese researchers at Nagoya University also combined the magnetic approach with microfluidic chips to build an on-chip microrobot system [89] for targeting single cell mechanical characterization [90].

Compared with manual operation and other cell manipulation strategies (e.g., microfluidics, magnetic tweezers, laser trapping and dielectrophoresis), robotic cell manipulation systems using mechanical strategies have been demonstrated to overcome their limitations (e.g., cell damage, cell loss, poor specificity) and be capable of conducting more complex tasks [57]. Direct manipulation using mechanical strategies, such as microassembly of microobjects, microinjection of cells, and pick-and-place of single cells, provides a more intuitive and straightforward solution.

Robotic micromanipulations using mechanical strategy can be further classified into three categories depending on their distinct feedback modes. The classifications of robotic manipulation using mechanical strategy are summarized in Table 1. Early studies in robotic micromanipulation used visual feedback for automation. Because all micromanipulation tasks are conducted under microscopes (either optical or electron microscope), visual feedback is the most useful approach for building closed-loop schemes because imaging feedback can provide plentiful information for tracking cell locations and positioning manipulation end-effectors. However, microscopy imaging often has a limited depth of field and a small field of view, posing critical challenges in searching and locating end-effectors both in the image plane and in depth. Therefore, early research with visual feedback are mainly focused on microassembly of relatively large objects or microinjection of large cells such as mouse oocytes and zebrafish embryos.
<table>
<thead>
<tr>
<th>Technical Classification</th>
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<tr>
<td>Force Feedback</td>
<td>MEMS-Based Devices</td>
<td>silicon-based MEMS devices measure forces at a high resolution of nano-Newton level; PDMS-based probes or micro-posts devices can also measure forces by visually tracking shape changes; fabrication processes are complex; devices are easy to damage.</td>
<td>microassembly; microinjection; cellular mechanical characterization</td>
<td>[91] [92] [93] [94] [95] [96] [97] [98] [99] [100]</td>
</tr>
<tr>
<td></td>
<td>Piezoelectric Materials (e.g., PVDF)</td>
<td>force sensing modules usually integrated on the micropipette holders or cell holding devices; relatively easier to fabricate; measurement resolution ranges from a few to several hundreds of micro-Newton.</td>
<td>microinjection; cellular mechanical characterization</td>
<td>[101] [102] [103] [104] [105] [106] [107]</td>
</tr>
<tr>
<td></td>
<td>AFM</td>
<td>highest force measurement resolution; limited applications; High equipment cost; AFM probes can be modified into various shapes; applications also performed under electron microscope</td>
<td>cellular or intracellular mechanical characterization; microinjection</td>
<td>[108] [109] [110] [111] [112] [113] [114] [115] [116] [117]</td>
</tr>
<tr>
<td>Electrical Feedback</td>
<td>Impedance Measurement</td>
<td>Electrode inserting into micropipette to measure the impedance change; Monitoring impedance change can detect cell-pipette contact, pipette tip breakage, and pipette clogging</td>
<td>microinjection; selectively isolate cells from cell cultures</td>
<td>[118] [119] [120] [121] [119]</td>
</tr>
<tr>
<td></td>
<td>I-V Curve Monitoring + Visual Feedback</td>
<td>electrode inserting into glass micropipette was used for monitoring cell-micropipette contact and realizing electroporation</td>
<td>microinjection of sea urchin eggs with fluorescent dye</td>
<td>[122]</td>
</tr>
<tr>
<td></td>
<td>Planar Patch-Clamp System + AFM</td>
<td>planar patch-clamp system can both record electrical signals and exchange intracellular solution; AFM generates force stimuli with controllable patterns and magnitudes; AFM scanning of the single cell provides detailed morphological information.</td>
<td>electrical recordings of whole-cell current of potassium ion channel</td>
<td>[123]</td>
</tr>
<tr>
<td>Visual Feedback</td>
<td>Computer Vision + Visual Servoing</td>
<td>No extra device/equipment cost; Optical microscopy has limited depth of field and small field of view; Z-position information can only be estimated; Applications limited to relative large objects or cells (e.g., embryos); Image-based visual feedback requires system calibration.</td>
<td>microassembly; microinjection; pick-and-place of single cells;</td>
<td>[124] [125] [126] [127] [128] [129] [130] [131] [132] [133] [134] [135]</td>
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As MEMS technology advanced during the first decade of the twenty-first century, force feedback was introduced into robotic micromanipulation by integrating MEMS-based force sensors into end effectors. The accurate and high-resolution force measurement provided an improved solution in the determination of relative Z positions between end-effectors and cells. Additionally, micromanipulation with MEMS-based force sensors also has the inherent advantages in characterizing the mechanical properties of single cells because the force-deformation curve can be easily achieved in experiments. Despite their significant advantages, MEMS-based force sensors did not reach the large-scale adoption in biological labs or clinics because of the customized force sensors’ time consuming and complex fabrication processes. As an alternative solution to MEMS-based force sensors, researchers also tried using piezoelectric materials (e.g., PVDF) to make cantilever structures on end-effector holders or thin films on cell culture devices for measuring forces. PVDF sensors are relatively easier to fabricate, but they have reduced force measurement resolution, often at several tens of micro-Newton. The third type of force feedback uses AFM (Atomic Force Microscopy) systems, which have very high force measurement resolutions. By modifying the AFM probes into special shapes (e.g., nanoneedle, nanofork, and nanoknife), Japanese researchers in Fukuda’s lab have conducted very interesting studies, such as the quantification of adhesion forces of single cells, single cell surgery with modified AFM nanofork or nanoknife. However, AFM is expensive and cannot achieve many other demanding manipulation tasks including microinjection and pick-and-place of cells, which limits its practicality in typical biological labs or regular clinics.

In addition to visual and force feedback, other researchers also used electrical feedback to assist in the detection of cell-micropipette contact. In this method, positive electrodes are often inserted into micropipettes and connected to electrical amplifying circuits and measurement equipment. The negative or ground electrodes are placed in the cell culture devices. The system monitors the changes in micropipette tip resistance/impedance to detect cell-micropipette contact, tip breakage or tip clogging. In addition to monitoring the changes of electrical signals, this method can also provide extra functions such as electroporation and patch clamping. However, robotic manipulations using electrical feedback usually require high-specification measurement equipment because the weak bioelectrical signals easily obscured by ambient electromagnetic noise. Moreover, the introduction of an electric field can also disturb normal cellular functions such as gap junctional intercellular communications.
Compared to force and electrical feedback, visual feedback works as the most basic approach to achieve robotic and automated cell manipulations. Robotic manipulation of cells with visual feedback does not require additional sensing or measurement equipment, making it the most suitable approach for large-scale adoption in biology labs and clinics from a cost standpoint.
1.5 Opportunities for Innovations in Robotic Cell Manipulation

Although many algorithms and techniques have been developed for robotic cell manipulations, several key issues still exist which limits the practical adoption of robotic cell characterization and manipulation technologies in biology labs and clinics. These technical issues are:

- In order to realize practical adoption in biology labs and clinics, complicated force sensors or electrical feedback should be avoided and research needs to be focused on visual feedback approaches.

- To achieve robotic manipulation with visual feedback, novel methods are required to search and detect low-contrast objects (e.g., end-effector tips with various shapes and fast-swimming motile sperms) in a small field of view under optical microscopy with a limited depth of field.

- To realize visual-servoing robotic manipulation, the relative vertical positions of end-effectors and cells (Z-position) must be accurately determined and the positioning errors in the X-Y plane must be well compensated.

- Novel robotic manipulation systems must have high throughput capability in order to achieve statistically significant results for investigating the heterogeneous cell behaviors.

- The cell’s response to external environment (e.g., embryo volume changes due to osmotic pressure) and behavior changes (e.g., sperm tail beating increases after binding to HA) must be closely monitored during cell manipulations in order to achieve the optimal performance for each target cells.

- The prerequisite step for all micromanipulations, which is how to locate end-effector tips, was performed manually. End-effectors, such as micropipettes and microgripppers, must be first searched and located under microscopy imaging before any micromanipulation task is performed. There is no robotic algorithm for automatically locating end-effector tips.
1.6 Research Objectives

In this research, I aimed to solve the aforementioned technical issues and use the new solutions to build robotic systems and automated methods for characterization and manipulation of single adherent and suspended cells. Specific objectives of this research include:

- To develop automated method for automatically locating end-effector tips.
- To develop computer vision based contact detection algorithms for determination of relative Z-positions of end-effector tips and single cells.
- To build an automated system for robotic injection of adherent cells to measure gap junctional intercellular communications.
- To achieve a high throughput in microinjection of adherent cells with a high success rate, a high reproducibility, and a high post-injection survival rate.
- To use the robotic microinjection system for screening the efficacy of selected drug molecules based on the measurement of gap junctional communications.
- To develop algorithms for controlled aspiration and positioning suspended cells inside a micropipette.
- To build an automated system for robotic processing of mouse embryos for vitrification.
- To develop 3D tracking algorithms for analyzing the morphological changes of oocytes or embryos during vitrification.
- To develop computer vision tracking algorithms for automated analysis of sperm head and tail locomotion behavior.
1.7 Dissertation Outline

An overview of the ensuing chapters is as follows:

Chapter 2 explains the new solution for automatically locating end-effector tips. Micropipettes and microgrippers were used as example end-effectors to evaluate the performance of proposed auto-locating method. Guidelines for implementation of this method are provided at the end of the chapter.

Chapter 3 presents a robotic injection system for characterization of GJIC. Two new vision-based contact detection algorithms are discussed in this chapter. The performance of the system is evaluated by injecting three distinct adherent cell lines. Preliminary testing results on screening the selected drugs are reported in this chapter.

Chapter 4 describes a robotic approach for automatically pick-and-place embryos for vitrification. The implementation of auto-locating end-effector tips method and vision based contact detection is discussed in this chapter. New methods for tracking embryos in vitrification solutions are explained. Test results on system operation speed, post-thawing survival rate, and development rate are also presented.

Chapter 5 reports automated analysis method for quantifying sperm locomotion behaviors. Experimental results on free-swimming sperm and HA-bond sperm are compared in this chapter, confirming the significant potentials to unify the sperm motility analysis with DNA integrity assessment.

Chapter 6 concludes the entire thesis work with a summary of contributions. Future research directions are also discussed in this chapter.
Chapter 2
Automatically Locating End-effector Tips in Micromanipulation

This chapter discusses an automated solution for automatically locating end-effector tips in micromanipulation. The performance of the automated function was tested by using various end-effectors under three major types of microscopy imaging modes. The newly developed auto-locating functions are proven to be effective for avoiding tip breakage, saving operation time, and eliminating human involvement. Special guidelines for implementation of this auto-locating function are provided at the end of this chapter.

The following section is based on the text from the following publication:

2 Automatically Locating End-Effector Tips in Micromanipulation

In robotic micromanipulation, end-effector tips must be first located under microscopy imaging before manipulation is performed. The tip of micromanipulation tools is typically a few micrometers in size and highly delicate. In all existing micromanipulation systems, the process of locating the end-effector tip is conducted by a skilled operator, and the automation of this task has not been attempted. This chapter presents a technique to automatically locate end-effector tips. The technique consists of programmed sweeping patterns, motion history image end-effector detection, active contour to estimate end-effector positions, autofocusing and quad-tree search to locate an end-effector tip, and, finally, visual servoing to position the tip to the center of the field of view. Two types of micromanipulation tools (micropipette that represents single-ended tools and microgripper that represents multi-ended tools) were used in experiments for testing. Quantitative results are reported in the speed and success rate of the auto-locating technique, based on over 500 trials. Furthermore, the effect of factors such as imaging mode and image processing parameter selections was also quantitatively discussed. Guidelines are provided for the implementation of the technique in order to achieve high efficiency and success rates.
2.1 Introduction

Micromanipulation tools such as micropipettes and microelectromechanical systems (MEMS) microgrippers are commonly used in the manipulation of micro-scaled objects. Locating the tip of these end-effectors under microscopy must be conducted before micromanipulation initiates. In previous manual and robotic micromanipulation, locating the tip of end-effectors (searching, positioning, and focusing) is a manual procedure performed by skilled operators. Because of the small size and fragility of micromanipulation end-effectors, manually locating end-effector tips has high skill requirements, is time-consuming, and can cause end-effector breakage. Despite the progress made in robotic micromanipulation [111][136][95][137][138], the automation of the procedure of locating end-effector tips has not been investigated. Most end-effectors used in micromanipulation have micrometer-sized tips (single or multi-ended). For instance, micropipette tips used in cell manipulation and microgrippers for assembly tasks are usually a few micrometers in diameter (see Fig. 2-1). The tiny tip of these end-effectors is difficult to locate, particularly under high magnifications in microscopy imaging. When the end-effector collides into other objects (e.g., wafer substrate, glass slide, petri dish, or other end-effectors) during the process of locating the end-effector and micromanipulation, the tip can be easily damaged and requires replacement. Hence, automation techniques to readily locate the end-effector tips are necessary to reduce human intervention and achieve autonomous robotic micromanipulation.

![Figure 2-1 - Example end-effectors used in micromanipulation. Their micrometer-sized tips must be located first before micromanipulation initiates. (a) Microgrippers for pick-and-place of small objects. The grasped particles are 10 µm in diameter. (b) Micropipettes for manipulating biomaterials.](image)
Automatically locating end-effectors requires visual detection and focus estimation to determine the end-effector’s position in three dimensions. Several techniques have been reported to visually detect and track micro objects. Ni et al. proposed an iterative closest point algorithm to track a microgripper’s position [139]. The algorithm requires the use of an additional dynamic vision sensor (silicon retina). This special hardware requirement makes the algorithm unsuitable for micromanipulation tasks that rely on standard vision systems. Microgrippers with complex features were also tracked using the template matching method for micro-assembly tasks [140][141]. Our experimental results indicate that the template matching is ineffective to track objects without distinct features (e.g., micropipettes). In another related work [142], a generalized Hough transform was applied to detect end-effector tips. However, the approach can only detect objects with regular shapes (i.e., lines or circular shapes). Algorithms based on one-class support vector machines [143], shearlet multiscale directional transform [144], and the Kalman filter [145] have also been used to detect or track objects under microscopy imaging. However, these algorithms are only suitable to process in-focus images. In the task of locating end-effector tips, the end-effector often is partially or entirely out of focus. In addition, the shapes and end-effectors’ direction of entering the field of view (FOV) also vary with different micromanipulation tasks. These unique requirements call for the development of techniques to automatically locate end-effectors under microscopy.

This chapter presents a technique that is capable of searching for out-of-FOV, out-of-focus, and low-contrast end-effectors. A detection algorithm based on a motion history image (MHI) and an active contour model is used to search for the end-effector. Through estimating the end-effector tip’s location and the use of an adaptive quadtree autofocus algorithm, the tip of the end-effector is detected and moved to the center of the FOV and brought in focus. This chapter describes in more detail algorithm comparisons and experimental results. Micropipettes and MEMS microgrippers are used as example end-effectors to evaluate the performance of the technique. Experimental results from the over 500 trials under three common imaging modes (bright field, differential interference contrast or DIC, and phase contrast) demonstrate that the technique is capable of automatically locating end-effectors under microscopy imaging with high efficiency and accuracy.
2.2 System Design

2.2.1 System Architecture

As shown in Fig. 2-2, the micromanipulation system setup consists of a standard inverted microscope (TE2000-S, Nikon) with motorized focus control and a CMOS camera connected...
Objectives of 4×, 10×, and 20× are used and have depths of field of 55.5, 13.5, 5.5 μm, respectively. An end-effector (i.e., a micropipette or a microgripper in this study) is mounted on a motorized 3-DOF micromanipulator (Sutter MP285) at a tilting angle of 30°. Movements performed in the automation procedure consist of X, Y, and Z translation motions of the end-effector as well as adjustment of the microscope’s focus.

### 2.2.2 Overall Sequence

The end-effector is initially at or above the focal plane. The search range is established within a 4-mm cubic workspace (−2 mm ≤ x ≤ +2 mm, −2 mm ≤ y ≤ +2 mm, 0 mm ≤ z ≤ +4 mm) [see Fig. 2-2(a)]. When setting up an end-effector in micromanipulation, it is feasible for the operator to readily position the end-effector tip to within this 4-mm cube workspace with unaided eyes and reasonable care. The automated procedure has two main steps: end-effector detection and autofocus adjustment. Fig. 2-3 summarizes the overall sequence.

(1) **End-Effector Detection**: In the detection step, the end-effector is swept in the X–Y plane. An algorithm based on an MHI is used to detect the presence of end-effector in the FOV. In some cases, the end-effector cannot be detected when it is far away from the focal plane. When this occurs, the focal plane is moved upwards, and horizontal X–Y sweep is repeated. The step size of focus adjustment depends on the depth of field of the microscope objectives. In our system, the step size for 4×, 10× and 20× objectives is set as 50, 10, and 5 μm, respectively. When a moving object is detected in multiple continuous frames of images during the sweeping of the end-effector, the end-effector is considered to be present in the FOV.
(2) **Autofocus Adjustment:** In the autofocus step, coarse and fine focus adjustments are conducted to focus on the end-effector tip. Coarse focus adjustment moves the focal plane in a large step size until the entire image produces a maximum focus measure value. In fine focus adjustment, a recursive quadtree autofocusing method is used to accurately focus on the end-effector tip. After the coarse and fine focus adjustments, the in-focus end-effector tip is moved to the center of FOV through closed-loop visual servo control. This centering step is designed to reduce search time when switching to a higher magnification objective.

2.3 **Key Methods for Auto-Locating End-effector Tips**

2.3.1 **End-Effector Sweep Pattern Design**

When an end-effector is initially mounted on the micromanipulator, the end-effector is often not within the FOV. However, the direction from which it will enter the FOV is usually known. Hence, our system moves the end-effector along this direction, and then, sweeps it perpendicularly [see Fig. 2-4(a)]. If the body of the end-effector instead of the tip enters the FOV, the system retracts the end-effector until the tip is found with the algorithm discussed in Section III-C. If the direction of entering the FOV is not known, the system moves the end-effector to the bottom left of the plane and sweeps it in a zigzag pattern [see Fig. 2-4(b)].

![Figure 2-4 - Sweep pattern in X–Y plane (top-down view).](image)

(a) When the direction of entering the FOV is known, end-effector is moved along the direction from which it enters the FOV, followed by sweeping perpendicularly. (b) When the direction of entering the FOV is not known, end-effector is swept in a zigzag pattern.
2.3.2 Computer Vision Based End-Effector Detection

When the end-effector is swept in the X–Y plane, the system uses a method based on an MHI to detect whether the end-effector has entered the FOV. The MHI-based detection is a view-based temporal method which is robust in representing movements and is employed in a variety of motion detection applications [146]. When the end-effector is out of focus, it appears blurry and the motion is not obvious to detect. Since the MHI-based method is able to enhance the motion representation by accumulating the end-effector’s movement for a period of time, it is suitable to use to detect the subtle motion of the end-effector. The original image is first denoised by using a Gaussian filter [147].

When the end-effector moves in the FOV, a silhouette image is obtained by subtracting two consecutive frames. The subtraction of two frames effectively suppresses the static noises from the background. The subtracted silhouette image is then binarized by applying a threshold to suppress background noise. When the end-effector moves, new silhouettes are captured and overlaid to the old silhouette that fades over time. The sequentially fading silhouettes record the motion history of the end-effector. Using this method, the MHI of the end-effector in the FOV is obtained [see Fig. 2-5(b) and (e)]. The sum of the pixel value in the MHI is used as a measure to determine the presence of the end-effector. If the sum of the pixel value is above a threshold (δ), the end-effector is considered to be present in the FOV. The threshold, δ, is calculated dynamically by analyzing 30 frames of the MHIs in which no end-effector is present.

\[ \delta = \mu + a \cdot \sigma \]  

where \( \mu \) is the average of pixel value sum of the MHIs in which no end-effector is present, \( \sigma \) is the standard deviation, and \( a \) is a preset parameter which determines the threshold value. By adding several times of standards deviations (i.e., \( a \cdot \sigma \)) to the mean value, the threshold is able to reject most of the MHIs that do not have the presence of the end-effector. Accordingly, false positive detection caused by random noise and the shadow of end-effectors is reduced.

The movement direction of the end-effector, if not known, can be derived by computing the gradients of the MHI image. The gradients are obtained by applying a 3×3 Sobel gradient filter to the MHI image. If the detected overall motion gradient correlates with the sweep pattern, the end-effector is confirmed to be present in the FOV. The system then proceeds to the next step.
(contour detection). Otherwise, motion detection is regarded as a false positive case, and the system returns to the sweep step until the end-effector is correctly detected.

### 2.3.3 Detection of End-Effector Contours

Once the end-effector is detected to be present in the FOV, the end-effector’s root and tip locations are estimated [see Fig. 2-5(c) & (f)]. In order to obtain a high estimation accuracy, the background is first removed by subtracting a background image from the current frame. The background image is recorded at the time when no end-effector is present in the FOV. The end-effector’s contour is then detected by using the active contour method [148].

An active contour is a continuous spline defined by \( v(s) = (x(s), y(s)) \), where \( x \) and \( y \) are image coordinates and \( s \in [0, 1] \). The active contour deforms in the spatial domain of an image to minimize

\[
E_{\text{snake}} = \int_0^1 E_{\text{int}}(v(s)) + E_{\text{ext}}(v(s)) \, ds
\]  

(2)

where \( E_{\text{int}} \) is the internal energy composed of the first and second derivatives of \( v(s) \), and \( E_{\text{ext}} \) is the external energy

\[
E_{\text{ext}}(v(s)) = -|\nabla(G_\sigma(x, y) * I(x, y))|^2
\]  

(3)

where \( \nabla \) and \(*\) are gradient and convolution operators, respectively, \( G_\sigma(x, y) \) is a Gaussian filter with standard deviation \( \sigma \), and \( I(x, y) \) is the image data. The active contour model is initialized by detecting a rough contour of the last binarized silhouette image overlaid on the MHI. Then, the initial contour was deformed in the background-removed image under the influence of the internal and external forces.

With the detected contour of the end-effector [see Fig. 2-5(c) & (f)], the average position of the contour points on image boundaries is taken as the position of the root of the end-effector. The point with the largest Euclidean distance to the root location is considered the end-effector’s tip position. For end-effectors with multi-ended tips (e.g., microgrippers), tip locations are detected separately.
Figure 2-5 - Detection of end-effector. Arrows in (b) and (e) show the motion gradient of the moving end-effectors. (a) Original image of a micropipette. (b) Corresponding MHI of the moving micropipette. (c) Tip detection result based on active contour is shown in a background-removed image. (d) Original image of microgripper. (e) Corresponding MHI of the moving microgripper. (f) Tip detection result based on active contour is shown in a background-removed image.

2.3.4 Autofocusing on End-Effector Tips

In this step, the focal plane is adjusted to focus on the end-effector tip. The normalized variance method [149] is used to calculate the focus measure. It compensates for the differences in average image intensity ($\mu$) among different images by normalizing the final output with the mean intensity [150]. The focus measure, $F$ changes as the system adjusts the focal plane. When $F$ reaches the global maximum, the end-effector is considered in focus.
\[ F = \frac{1}{W * H * \mu} \sum_{w} \sum_{h} (I(x, y) - \mu)^2 \]  

(4)

where \( W \) and \( H \) are the image width and height, respectively, \( I(x, y) \) is the pixel intensity at the point \((x, y)\), and \( \mu \) is the average image intensity.

With the completion of the aforementioned coarse focusing step, fine focus adjustment updates the most in-focus region, \( R_f \) until the region contains the end-effector tip. \( R_f \) is a region with a predefined size \( m \times n \) satisfying that the focus measure \( F \) of the region \( R_f \) is larger than any other region of the same size in the image.

The detection of \( R_f \) is performed by using a quad-tree recursive algorithm. The method recursively divides a region into four subregions with equal area. Out of the four subregions, the one with the highest focus measure \( F \) is further partitioned into four subregions. This procedure repeats recursively until the area of the subregion is equal or less than the area \( m \times n \) of \( R_f \). If the most in-focus region \( R_f \) is found to contain the end-effector body instead of the tip of the end-effector [see Fig. 2-6(a)], the focal plane is moved downwards since the end-effectors are always mounted at a tilting angle with tip side down in micromanipulation. The recursive detection of \( R_f \) and focal plane adjustment are then repeated until the end-effector tip is brought in focus [see Fig. 2-6(b)].

Figure 2-6 - Most in-focus region of the end-effector is detected by using a quad-tree recursive searching method. (a) The most in-focus region is detected to be on the end-effector body (not on the tip). (b) The tip is brought to focus (i.e., the most in-focus region is now on the tip).
2.3.5 Centering End-Effector Tips via Visual Servo Control

After the end-effector tip is brought in focus under a low-magnification objective, centering the tips is performed before the microscope switches to a higher magnification. By centering the tips under a low magnification, the end-effector is more likely to be present in the FOV when switching to a higher magnification. Experimentally, this step can significantly reduce the X–Y sweeping time required to locate the end-effector under higher magnifications. In the system, the in-focus end-effector tip is visually tracked and centered via closed-loop visual servo control.

Visual tracking algorithms can be classified into point tracking, kernel tracking, and silhouette tracking [151]. Representative algorithms were implemented in this study, and their performance was compared. In point tracking, the center point of the most in-focus region is detected and taken as the initial end-effector tip position. Since the end-effector was moved at a constant speed, the motion of end-effector tips was modeled as a constant velocity system. A Kalman filter is then applied to predict and optimize the end-effector tip position. In the kernel tracking category, template matching was tested to track end-effector tips. A template is manually captured, and the normalized cross-correlation [152] of the template with real-time images is calculated as a measure to detect the end-effector’s tip. Silhouette tracking is typically based on contour evolution and shape recognition. The active contour tracking approach was chosen and tested to detect the end-effector tip within a region of interest. For an end-effector with multi-ended tips, an average position of all tips was used as the overall tip position. Using the visually tracked tip position as feedback, an image-based PID visual servo controller was used to position the end-effector tip to the center of the FOV.
2.4 Experimental Results and Discussion

In the experiments, a micropipette (1 µm in tip diameter) and a microgripper were used as example end-effectors. The end-effectors were mounted along the X-axis with a tilting angle of 30° to the left side of the FOV. Before the automated procedure started, the end-effector tip was readily placed within the 4-mm cube workspace [see Fig. 2-2(a)] with unaided eyes, above the focal plane.

2.4.1 Overall Performance

![Graph showing time of locating end-effector tip]

**Figure 2-7** - Time of manually locating end-effector tip; auto-locating end-effector tip with unknown and known entering direction. Within each group (distance to initial focus plane), n = 20 (ten trials to locate micropipette tip and ten trials to locate microgripper tips).

The goal is to bring the end-effector tip to the center of the FOV under 4× magnification first, then under 10× magnification, and, finally, under 20× magnification. The experiments were categorized into five groups according to the end-effector’s initial distance (D) to the bottom of the workspace [see Fig. 2-2(a)].

The experiments were first conducted using bright field imaging under two conditions: with known and unknown direction of the end-effector to enter the FOV. Fig. 2-7 summarizes the
overall time spent on locating the end-effector and bringing its tip to the center of the FOV under 20× magnification. The average time of all five groups with unknown entering direction was 44.7 s, while the average time with known entering direction was only 31.2 s. These results demonstrate quantitatively that the overall locating time is significantly shorter (p< 0.001) by using the information of end-effector’s entering direction.

The task of locating end-effector tips in the five groups was also conducted manually by three skilled micromanipulation operators. The results shown in Fig. 2-7 demonstrate that the overall time to auto-locate end-effector tip, with or without known entering direction, is significantly shorter (p< 0.001) than in manual operation (averagely 64.8 s). In particular, in many micromanipulation tasks, end-effector’s entering direction is known. With known entering direction, the time required to auto-locate the tip is shorter by over 50% compared with manual operation, throughout the five groups. In all groups of experiments, the deviations of time were caused by D value differences and the differences in initial lateral distance of the tip to the FOV.

We also experimented using only 4× and 20× magnifications in the locating experiments. After locating the end-effector tips under 4× magnification, the system directly switched to 20× (instead of 10×). The average time of 100 trials (under bright field imaging) using only 4× and 20× magnifications was 44.8 s (with known entering direction), which is much longer than the average time (31.2 s) of auto-locating the tip by using 4×, 10×, and 20× magnifications sequentially. This is because the end-effector tip was sometimes out of the FOV when directly switching from 4× to 20×. When the tip was out of the FOV, the locating time was significantly lengthened because additional lateral sweeping and focus adjustments were needed under 20×. Experimental results also showed that the locating time under higher magnifications (i.e., 10×, 20×) is short (<5 s). This is because, after locating under the magnification of 4×, the end-effector tips are very close to the center of FOV. Accordingly, the searching time for end-effector tips is greatly reduced under higher magnifications.

2.4.2 Performance Under Different Microscopy Imaging Modes

The end-effector auto-locating technique was then tested under three different imaging modes (100 trials for each imaging condition): bright field, DIC, and phase contrast. The experimental results are summarized in Table 2.
Overall success rate refers to the rate of achieving the overall objective (locating and bringing the tip to the FOV of 20× magnification). The overall success rate under bright field imaging is higher than under DIC and phase contrast (97% versus 92% and 87%). This is because the depth of field of DIC imaging (2.6 µm for 20×) is smaller compared with bright field imaging (5.5µm for 20×), and a halo is present around the end-effector tip under phase contrast [see Fig. 2-8(c)]. The smaller depth of field in DIC caused the end-effector not always to be detected between two focus levels. The halo in phase contrast caused the system to sometimes fail to correctly focus on the end-effector tip. Thus, the success rate in the autofocus step under phase contrast was significantly lower than the other two imaging modes (89% versus 98%).

The tracking algorithms based on the Kalman filter, template matching, and active contour were evaluated under the three imaging modes by calculating the average tip tracking error across 200 frames during tip centering. The tip tracking error summarized in Table 2 is the Euclidean distance between the detected tip location and the actual tip position [see Fig. 2-8(c)]. The average tip tracking errors for Kalman filter tracking and active contour tracking under phase contrast are the largest because of the halo around the tip which significantly influenced the focus assessment and reduced the contour detection accuracy. In contrast, there is no significant performance difference among the three imaging modes for the template matching algorithm.
Figure 2-8 - Active contour detection under three different microscopy imaging modes. (a) bright field imaging, (b) DIC, and (c) phase contrast.

The experimental results also show that the active contour method outperforms Kalman filter tracking and template matching in terms of average tracking error for brightfield and DIC imaging. Optimal results for template matching are only possible when there are no significant changes in brightness and shadowing. Changes in scaling and rotation also can lead to false detection and loss of the tip position. Additionally, users may change end-effectors during a micromanipulation task. The slightly different shapes/dimensions across end-effectors of the same type can render template matching ineffective. Regarding Kalman filter tracking, it heavily relies on the result from the focus assessment step. However, the center point of the most in-focus region is not always the same as the end-effector tip location. In summary, the results demonstrate that active contour tracking under the bright field imaging mode was the most effective to locate end-effectors under microscopy.
2.4.3 Discussion

Table 3 - Effect of Different Threshold Values on Detection Time and Overall Success Rate
(Each Data Point in the Table Was from 100 Trials, All Under Bright Field Imaging)

<table>
<thead>
<tr>
<th>Threshold value (δ)</th>
<th>μ + 1σ</th>
<th>μ + 3σ</th>
<th>μ + 6σ</th>
<th>μ + 9σ</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHI detection time (sec)</td>
<td>17.4</td>
<td>23.2</td>
<td>30.6</td>
<td>42.7</td>
</tr>
<tr>
<td>Overall success rate</td>
<td>87%</td>
<td>95%</td>
<td>98%</td>
<td>99%</td>
</tr>
</tbody>
</table>

In the experiments for locating micropipettes under bright field imaging, 3 out of 100 trials failed. The three failure cases were caused by improper selections of the threshold value [δ in Eq. (1)] in MHI detection. To investigate the effect of threshold value selection on MHI detection, four groups of experiments were conducted with different threshold values. Table 3 summarizes the results from using different δ values.

When δ is set too low, the shadow of the micropipette body is detected by the MHI algorithm much earlier than the body itself. When this occurs, the active contour algorithm forms a contour around the shadow, and then, the recursive autofocusing algorithm locates a most-in-focus location on the shadow after the coarse focus adjustment. When the system lowers the focal plane in order to focus on the micropipette tip, the most-in-focus location on the shadow stays unchanged (because the shadow and the micropipette body are too far apart). In some cases, with too low a δ value set, the system was not able to locate the micropipette tip even after reaching the motion limit of microscope focus.

On the other hand, when the threshold value, δ is set too high, it takes much longer for MHI detection to complete although the overall success rate in locating the end-effector tip is higher [see Table 3]. A balance of MHI detection time and the overall success rate must be considered in practice.

In the microgripper experiments under bright field imaging, 5 out of 100 trials failed. Two failure cases were also caused by improper selection of the threshold for MHI detection. Other failure cases occurred in the group of experiments for locating microgripper tips assuming unknown entering direction, where other structures (rather than the gripping tips) appeared in the
FOV first, as shown in Fig. 2-9. Hence, at the completion of the auto-locating process, the first present structure was considered the microgripper tip and brought to the center of the FOV. This problem can be avoided in practice since the microgripper’s entering direction is typically known in a given micromanipulation system configuration.

![false tip detection](image)

**Figure 2-9 - Auto-locating microgripper tips failed due to other structure appeared in the FOV first.**

Based on the experimental results and observations, we attempt to establish a set of guidelines to implement the technique to auto-locate an end-effector in micromanipulation. First, the threshold value in MHI detection must be properly selected, with a balance of failure rates and time taken. Second, the threshold value selection is also sensitive to lighting. Histogram analysis in our experiments confirmed that an average value of pixel intensity, between 80 and 150, in the initial back-ground image should be used to mitigate the effect of lighting variations on MHI detection. This can be readily implemented in a control program to properly set lighting intensity in a micromanipulation system. Third, the task of auto-locating end-effector tips should be conducted under bright field imaging (versus DIC or phase contrast). Fourth, the auto-locating sequence should be performed under gradually increasing magnifications (e.g., 4×, 10×, and 20×) rather than under abrupt magnification changes (e.g., 4×, and then, immediately 20×).
2.5 Conclusion and Guidelines for Implementation

This chapter presented a technique to automatically locate an end-effector under microscopy imaging. Locating the end-effector tip is the very first step in either manual or automated micromanipulation. The technique described in this chapter is capable of searching for out-of-FOV, out-of-focus, and low contrast end-effectors. Experimental results demonstrate that the search time can be reduced by over 50% with the automated technique. The overall success rate of the locating system is 97% under the bright field imaging mode. Based on the experimental results and observations, the following guidelines are established to implement the technique of auto-locating end-effector tips in order to achieve a high efficiency and success rate.

1. The threshold value in MHI detection must be properly set ($[\mu + 3\sigma, \mu + 6\sigma]$).

2. Lighting intensity must be properly set, for instance, with the average value of pixel intensity in the initial background image set between 80 and 150.

3. Bright field imaging should be chosen (versus DIC and phase contrast) in the auto-locating process.

4. The active contour algorithm is most effective under bright field imaging to detect and track end-effector tips.

5. Abrupt magnification change should be avoided when switching from a low to a high magnification.

6. The prior knowledge of end-effector entering direction improves both completion time and the overall success rate.
Chapter 3
Robotic Microinjection of Adherent Cells

Compared to robotic injection of suspended cells (e.g., embryos and oocytes), fewer attempts were made to automate the injection of adherent cells (e.g., cancer cells and cardiomyocytes) due to their smaller size, highly irregular morphology, small thickness (a few micrometers thick), and large variations in thickness across cells. This chapter presents a robotic system for automated microinjection of adherent cells. The system is embedded with several new capabilities: automatically locating micropipette tips; robustly detecting the contact of micropipette tip with cell culturing surface and directly with cell membrane; and precisely compensating for accumulative positioning errors. These new capabilities make it practical to perform adherent cell microinjection truly via computer mouse clicking in front of a computer monitor, on hundreds and thousands of cells per experiment (vs. a few to tens of cells as state-of-the-art). System operation speed, success rate, and cell viability rate were quantitatively evaluated based on robotic microinjection of over 4,000 cells. This chapter also reports the use of the new robotic system to perform cell-cell communication studies using large sample sizes. The gap junction function in a cardiac muscle cell line (HL-1 cells), for the first time, was quantified with the system.

The following section is based on the text from the following publications:


3 Robotic Microinjection of Adherent Cells

3.1 Introduction

Intercellular communication is a critical part of cellular activities and coordinates cell functions [153]. Disorders of intercellular communication are responsible for diseases such as cancer [154], autoimmunity [155], and diabetes [156]. A standard technique for measuring intercellular communication is based on monitoring transfer of fluorescent molecules from an individual cell to adjacent cells through functional gap junctions (see Fig. 3-1). The quantitative measurement of dye transfer requires injection of fluorescent molecules into single cells. In present gap junction testing experiments, only a few or tens of cells can be injected due to the limitations of manual operation, posing a practical hurdle in attaining statistically significant data, for instance, for testing drug molecules on the alteration of gap junction function.

Figure 3-1 - Adherent cells (HL-1) under 20x magnification objective. The inset shows the schematic of a molecule passing through a gap junction from one cell to its adjacent cell.

Manually manipulating single cells is tedious and time-consuming and has high skill requirements. In manual operation, a skilled operator looks through the eyepieces of a
microscope while dexterously controlling multiple devices (e.g., micromanipulators, pump, microscope stage etc.). Robotic cell manipulation technologies progressed significantly over the past decade. The vast majority of demonstrated robotic systems focused on the manipulation of suspended cells (i.e., oocytes and embryos) [157]–[162]. Fewer attempts were made to automate the injection of adherent cells.

Most mammalian cells (e.g., cancer cells and cardiomyocytes) adhere to a culturing surface. Different from large suspend cells (e.g., mouse oocytes ~100 µm; zebrafish embryos ~1 mm), adherent cells are smaller in size and highly irregular in morphology (vs. spherical for oocytes/embryos), making robust pattern recognition difficult and automation challenging [163]. Additionally, adherent cells are only a few micrometers thick and vary significantly in thickness, posing more stringent requirements in robotic positioning.

A few joystick-based systems were demonstrated to assist operators for adherent cell microinjection [164], [120]. Long training, low success rates, and poor reproducibility make these systems incapable of injecting more than tens of cells per experiment. For example, the most popular commercial system for adherent cell injection is Eppendorf’s InjectMan system that requires an operator to look into the eyepieces of a microscope and perform microinjection by controlling joysticks. The injection success rate and cell survival rate of the system are 49.2% and 49.72%, respectively [164]. The only other joystick-based adherent cell injection system was demonstrated for the injection of fluorescent molecules into adherent cell lines with a 49% injection success rate [120]. Due to the difficulty of operating joysticks for fine positioning control, this system does not permit the injection of a high number of cells (only 82 cells injected). An automated system integrating a cell detection algorithm reported in [127] produced a success rate of 67% at a speed of 7-8 cells/minute.

In our previous work in the robotic injection of adherent cells [165], we attempted to transform joystick-based operation to computer mouse clicking. However, several practical limitations in the system required the operator to manually perform a few key steps using joysticks and looking into the eyepieces of the microscope. This hybrid way of system operation (mouse clicking in front of a computer monitor, and operating joysticks under a microscope) precluded the system’s potential for routine use in biology labs. Firstly, the system lacked the critical capability for automatically locating a micropipette tip. In adherent cell injection, the size of micropipette tip
must be kept within a few hundreds of nanometers to ensure a high cell viability. Locating the tip under optical microscopy requires high skills and extreme care since tip breakage can easily occur. Additionally, the small tip often gets clogged by large molecules or cell debris, necessitating regular replacement of micropipette tips (e.g., every 100 cells), demanding the system to possess the capability for locating micropipette tip automatically.

The second limitation in our previous system was the limited contact detection capability. In experiments, adherent cells are often cultured on gels or protein-coated (e.g., collagen) surfaces. Our previously reported contact detection algorithm works well on a bare culturing surface but has a low success rate on these coated surfaces. Furthermore, the previous contact detection algorithm is not applicable when cell confluency (i.e., density) is high (>80%). Thirdly, the previous system was not able to compensate for accumulated positioning errors, which made the system unable to inject more than 100 cells.

In this work, I present a new robotic system capable of injecting thousands of adherent cells. The system successfully addresses the limitations of our previous system prototype [15] and has the following new capabilities: automatically locating micropipette tips; robustly detecting the contact of micropipette tip with cell culturing surface and directly with cell membrane; and precisely compensating for accumulative positioning errors. These new capabilities, for the first time, make it practical to perform adherent cell microinjection truly via computer mouse clicking in front of a computer monitor, making the robotic system suitable for routine use in biology labs. I also demonstrate the use of the new robotic system to perform cell-cell communication studies with large sample sizes (over 1,000 cells vs. a few to tens of cells as state-of-the-art). Finally, the gap junction function in a cardiac muscle cell line (HL-1 cells), for the first time, was quantified with the new robotic system.
3.2 System Setup

![System setup diagram](image)

Figure 3-2 - System setup. (a) Robotic adherent cell injection (RACI) system. (b) A schematic showing of RACI system. (c) System control architecture.
This system uses well-calibrated air pressure to deliver aqueous solution into either the cytoplasm or nucleus of a cell. As shown in Fig. 3-2 (a) and (b), the system consists of a standard inverted microscope (Nikon TE2000-S, Nikon Microscopes) and a motorized X-Y translational stage (ProScan, Prior Scientific, Inc.). The X-Y stage has a travel range of 75 mm along both axes with a resolution of 0.01 μm, a maximum speed of 5 mm/s, and a repeatability of ±1 μm. A CMOS camera (acA1300-32gm, Basler) is connect to the microscope for visual feedback. A glass micropipette, laser pulled to have an outer diameter of 500 nm and an inner diameter of 300 nm, is mounted to a 4 degrees-of-freedom (DOF) DC-driven micromanipulator (MX7600, Siskiyou, Inc.) that has a travel range of 20 mm and a 0.1 μm positioning resolution along each axis. A host computer executing computer vision microscopy and motion control algorithms controls all hardware.

The robotic micromanipulator and X-Y stage are cooperatively controlled for positioning the micropipette along the XYZ axes and positioning cells in the XY plane, respectively. The overall control architecture of the system is summarized in Fig. 3-2 (c). Microscopy visual feedback is used to control the positioning of the micromanipulator and X-Y stage, forming an image-based visual servo control system.

When a micropipette is mounted on the system, the system detects the micropipette tip’s position and automatically moves it to the center of the field of view. The system is capable of detecting the contact in two modes, either on cell culturing surface or directly on the cell membrane, to determine the relative vertical distance between the micropipette tip and the cells in the Z direction. After the injection location (inside cytoplasm or nucleus) on each cell is selected via computer mouse clicking, the system controls the micropipette to deliver a precise volume into each cell and completes the injection of all selected cells following the shortest path. The system then controls the X-Y translational stage to bring cells in the next field of view under microscope imaging for injection.
3.3 Vision Based Contact Detection

3.3.1 Contact Detection on Cell Culture Surface

Figure 3-3 - Contact detection on cell culture surface. (a) Micropipette tip is lowered towards the cell culture surface while the X-Y stage moves along the X-axis from left to right. (b) Micropipette tip slides horizontally when it contacts the culture surface. (c) Schematic showing micropipette is lowered towards cell culture surface. (d) Schematic showing micropipette tip is sliding along the X-axis.

In order to inject materials into a cell, the relative vertical distance between the micropipette tip and the cell along the Z-direction must be accurately detected. Without the inclusion of an extra sensor (e.g., tactile or force sensors), which increases hardware complexity, two modes of computer vision based contact detection algorithms are developed to accurately determine the relative heights of the micropipette tip and adherent cells. This section describes the first mode, in which the system detects the initial contact of micropipette tip on the cell culturing surface that is protein coated (vs. bare surface).

Empty Region Detection: When cell confluency is lower than 80%, empty areas on the culturing surface can be detected and used for contact detection. The system divides the field of
view into sub-regions, the size of which is 100 pixels×100 pixels. The normalized variance of the pixel value, \( F \), is calculated for each region to represent its smoothness, according to

\[
F = \frac{1}{W \times H \times \mu} \sum_{w} \sum_{h} (I(x,y) - \mu)^2
\]  

(5)

where \( W \) and \( H \) are the region width and height, \( I(x,y) \) is the pixel intensity at the point \((x,y)\), and \( \mu \) is the average region intensity. This algorithm effectively compensates for differences in average intensity among different regions. The region with the lowest variance value is considered empty and used for performing contact detection.

**Contact Detection via Three-Dimensional Motion:** The system moves the detected empty region in the X-Y plane until it is directly under the micropipette tip [Fig. 3-3(a)&(c)]. The micropipette tip is then lowered by the micromanipulator along the Z direction. When the micropipette tip contacts the bare surface of a Petri dish, further vertical movement of the micropipette tip induces horizontal motion [166]. However, in cell culturing, the Petri dish surface is usually coated with gel or protein (e.g., collagen). The coating makes it difficult for the micropipette tip to ‘slide’ horizontally (it ‘stabs’ directly into the coated layer). Therefore, in this system, the X-Y stage is also servoed simultaneously to move along the direction in which micropipette tip enters the field of view, while the micropipette tip is being lowered by the micromanipulator. The X-Y stage’s motion enables the reliable generation of the pipette tip’s horizontal ‘sliding’ motion [Fig. 3-3(b)&(d)], resulting in highly reliable contact detection on protein-coated cell culture surfaces.

**3.3.2 Contact Detection on Cell Membrane**

When cell confluency is high, empty regions on the culture surface becomes unavailable; hence, the system performs contact detection by detecting direct contact of the micropipette tip on the cell membrane [see Fig. 3-4(a)]. After the operator selects a cell via computer mouse clicking, the system moves the cell to the micropipette tip position in the X-Y plane and then moves the micropipette tip downwards along the Z axis. When the micropipette tip contacts the cell membrane, the cell is deformed and a subtle motion appears around the contact point. To detect this subtle motion, we developed an algorithm based on motion history images (MHI). The MHI-
based method enhances motion representation by accumulating cell deformation over a period of time.

Figure 3-4 - Detection of contact on the cell surface (a) At high cell confluency, the system detects direct contact of the pipette tip on the cell surface. (b) ROI image shows cell being deformed: white dot indicates tip position. (c) Modified ROI removing the motion of micropipette tip. (d) Motion history image showing the deformation of the cell: red arrows indicate motion gradients.

A region of interest (ROI) is first obtained around the micropipette tip [Fig. 3-4(b)]. Within the ROI, the image is

\[ l(x,y,t) = b_t(x,y) + m_t(x,y) + n_t(x,y) \] (6)
where $b_t(x,y)$ is the static background; $m_t(x,y)$ is the moving object; $n_t(x,y)$ is the background noise; and $t$ is time. The moving object is extracted via taking the difference between consecutive frames.

$$D(x, y, t) = I(x, y, t + 1) - I(x, y, t) = M(x, y, t) + N(x, y, t)$$ \hspace{1cm} (7)

where $M(x, y, t)$ is the motion region; $N(x, y, t)$ is noise; and $D(x, y, t)$ is the difference image containing the moving object and background aberration due to motion and noise. Background aberration can lead to incorrect detection (e.g., motion ambiguity and distortion) and make detection fail when object motion speed is low.

In the system, the motion region $M(x, y, t)$ contains motion from both the lowering micropipette and cell deformation due to micropipette-and-cell contact. Hence,

$$M(x, y, t) = M_p(x, y, t) + M_c(x, y, t)$$ \hspace{1cm} (8)

where $M_p(x, y, t)$ is the micropipette lowering motion, and $M_c(x, y, t)$ is the motion from cell deformation. When the micropipette is moved along Z axis during contact detection, the tip’s location in the X-Y plane, $(x_p, y_p)$, is constant, and the lowering motion results from the defocusing phenomenon. Since a defocusing blurred image is equivalent to an exponential decay function [167], the micropipette lowering motion can be described as $M_p(x, y, t) = M(x_p, y_p, t_0) * e^{-t/\tau}$, where $M(x_p, y_p, t_0)$ is the initial micropipette’s motion before cell contact occurs. Thus, motion from cell deformation is

$$M_c(x, y, t) = M(x, y, t) - M(x_p, y_p, t_0) * e^{-t/\tau}$$ \hspace{1cm} (9)

After removing micropipette tip’s motion, the noise term $N(x, y, t)$ in the difference image can be removed by a Gaussian low-pass filter and image binarization. The binary image representing cell deformation-caused motion is

$$\Psi(x, y, t) = \begin{cases} 1, & \text{if } M_c(x, y, t) > \xi \\ 0, & \text{otherwise} \end{cases}$$ \hspace{1cm} (10)
The motion history image is

\[
H_\tau(x, y, t) = \begin{cases} 
\tau, & \text{if } \Psi(x, y, t) = 1 \\
\max(0, H_\tau(x, y, t - 1) - \gamma), & \text{otherwise}
\end{cases}
\] (11)

where the duration \( \tau \) determines the temporal extent of the movement, and \( \gamma \) is the decay parameter. The subtle cell deforming motion is greatly enhanced by adding the motion history information [see Fig. 3-4(d)].

The sum of pixel values in MHI, \( s(H) \), is calculated as a measure to determine the contact of cell membrane. If \( s(H) \) is above a threshold value \( \delta \), the micropipette tip is considered as contacting cell top membrane. The threshold \( \delta \) is calculated dynamically by analyzing MHIs in which the micropipette tip has not contacted cell surface.

\[
\delta = \bar{s} + a \cdot \sigma(s)
\] (12)

where \( \bar{s} \) is the average of MHIs’ sum of pixel values before cell contact occurs; \( \sigma(s) \) is the standard deviation; and \( a \) is a preset parameter which is experimentally determined. By adding \( a \cdot \sigma(s) \) to the mean value, the threshold \( \delta \) effectively rejects those MHIs that do not contain cell deformation. In addition, the motion gradient of the MHI is also used to avoid false positive detection. The motion gradient is computed by convolution with Sobel filters to yield spatial derivatives, \( F_x(x, y) \) and \( F_y(x, y) \). The orientation of the motion gradient at each pixel is

\[
\Phi(x, y) = \arctan\frac{F_y(x, y)}{F_x(x, y)}
\] (13)

As the micropipette tip touches the cell membrane, deformation occurs around the contact point. Accordingly, the motion gradients in MHI converge to the contact point.
3.4 Robotic Microinjection

Figure 3-5 - Robotic injection of adherent cells. (a)(b) When the micropipette is inserted into the cell, material deposition triggered by pressure pulses results in a “shock wave” motion inside the cell. (c) Fluorescence is present only in the cytoplasm. (d) Fluorescence is present only in the cell nucleus.

In this new robotic system, an operator performs every step throughout the microinjection process via computer mouse clicking. The operator readily selects injection locations (i.e., cell nucleus or cytoplasm) since the cell nucleus and cytoplasm are distinct on a computer monitor. The operator can select a single cell or multiple cells to inject within the field of view. During computer mouse clicking, cell templates centered at the mouse clicking positions are extracted. The templates are used for template matching to provide position feedback. After performing contact detection, the system moves the micropipette tip close to the first target cell and inserts the micropipette into the target cell along the tilting axis at the maximal speed. The pressure source is then triggered to apply a positive pressure to deposit the preloaded material into the cell. Material deposition results in a “shock wave” motion inside the cell, around the injection location [see Fig. 3-5(b)]. The volume of injected materials is precisely controlled by the
injection pressure magnitude and width. Although target cells are selected in a random order by
the operator, the system injects all cells along the shortest path. The system then moves to the
next field of view to continue the injection of more cells. The locations of injected cells are all
recorded by the system, permitting time-lapse imaging of every injected cell.

**Injection volume control.** Injection volume is determined by the injection pressure and pressure
“on” time (i.e., pulse width). Injection volume was experimentally measured. The micropipette
preloaded with distilled water was immersed into mineral oil. After 50 times of injection with a
pressure “on” time of 0.4 s, a water bubble was formed at the tip of the micropipette. The
injection volume was then calculated based on the size of the water bubble. Figure 3-6 shows the
calibrated relationship between injection pressure and injection volume.

To keep a high post-injection survival rate, the volume of injected foreign materials must not
exceed 5% of the cell’s cytoplasmic volume [165], and therefore not exceed 30 femtoliters (fL)
for most adherent cells. Accordingly, the injection pressure was set lower than 3,000 hPa in our
system. In addition to injection pressure, the pump also provides a constant positive pressure (50
hPa) throughout the entire microinjection process. The application of this positive pressure
ensures that there is a constant, gentle flow of solution from the micropipette, preventing
undesired dilution of the solution in the micropipette by the medium inside the Petri dish.
Moreover, the positive pressure also reduces the possibility of micropipette tip clogging by
foreign objects in the surrounding medium.

![Injection volume calibration](image)

Figure 3-6 Injection volume calibration results indicate that the injection pressure should
be set lower than 3000 hPa in order to achieve a high cell survival rate.
3.5 Experimental Results and Discussion

3.5.1 Performance of Locating Micropipette Tip

To evaluate the system’s performance of automatically locating micropipette, micropipette tip’s initial distance to the culture surface was divided into five groups. The task of locating micropipette tips in the five groups was also conducted manually by three skilled micromanipulation operators. Experimental results demonstrate that the overall time for auto-locating micropipette tip (30.2s in average) is significantly shorter ($p$-value < 0.001) than in manual operation (averagely 64.8s). Auto-locating micropipette tip was also tested under three imaging modes: bright field, phase contrast, and differential interference contrast (DIC). Although the phase contrast or DIC imaging modes are desired for producing a pseudo-3D view of cells, it was found in experiments that bright field imaging produced a high success rate because the micropipette tip appears more uniform and “halo” free under brightfield imaging.

3.5.2 Injection Speed

| Table 4 - Robotic Microinjection Speed of Three Different Cell Lines |
|-------------------------|----------------|---------------|-------------|----------|
| cell line              | HeLa | HEK293   | HL-1 | overall |
| injection number       | 2281 | 1068     | 1116 | 4465     |
| injection time (min)   | 97   | 48       | 52   | 197      |
| speed (cells/min)      | 23.5 | 22.3     | 21.5 | 22.7     |

Injection speed of the system was evaluated by injecting three different adherent cell lines: HeLa cells, HEK293 cells, and HL-1 cells. The number of injected cells and time consumed are summarized in Table 4. The injection time in Table 4 is the total experimental time for all steps including locating micropipette tip, contact detection, cell selection and injection. The results from the injection of over 4,000 cells show that the injection speed of the robotic system is consistent across different adherent cell lines. The average injection speed is 22.7 cells/min, which enables users to inject over a thousand of adherent cells within one hour.
### 3.5.3 Injection Success Rate and Cell Survival Rate

#### Table 5 - Injection Success Rate and Cell Survival Rate After Injection

<table>
<thead>
<tr>
<th>injection location</th>
<th>injection number</th>
<th>success rate</th>
<th>survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>into cytoplasm</td>
<td>1245</td>
<td>95.2%</td>
<td>97.2%</td>
</tr>
<tr>
<td>into nucleus</td>
<td>1036</td>
<td>97.5%</td>
<td>96.5%</td>
</tr>
</tbody>
</table>

In order to evaluate the injection success rate and cell survival rate, a Dextran-Rhodamine fluorescent dye was injected into the cytoplasm or nucleus of HeLa cells. The Dextran-Rhodamine dye was chosen because it is highly water-soluble and membrane-impermeable. Therefore, only the cells that are successfully injected with Dextran-Rhodamine are able to reveal red fluorescent color. The system permits material deposition into the cytoplasm [Fig. 3-5(c)] or into the cell nucleus [Fig. 3-5(d)]. When the deposition target (i.e., either cytoplasm or nucleus but not both) reveals strong red fluorescent signals, the injection was considered successful. Experimental results summarized in Table 5 show that the success rates for cytoplasmic (n=1,245) and nuclear (n=1,036) injection were comparable (95.2% and 97.5%). The success rate of nucleus injection is slightly higher than the cytoplasm injection. That is because the cytoplasm of the adherent cells appears flatter, while the nucleus is more like a bump shape. The micropipette tends to penetrate into the nucleus rather than the cytoplasm.

Cell post-injection viability was evaluated by using a cell viability assay kit (Viability/Cytotoxicity Kit, Life Technologies). The measured cell survival rate after cytoplasmic injection was 97.2% and after nuclear injection was 96.5%. The survival rate for nucleus injection is relatively lower because the volume of the nucleus is much smaller than the cytoplasm volume. Same amount of injected medium will impose greater disruptive effects on nucleus than on cytoplasm.
3.5.4 Characterization of Cell-Cell Communication

We used the robotic system to perform dye transfer experiments for measuring gap junctional intercellular communication (GJIC). In the dye transfer experiments, a membrane-impermeable HPTS (8-Hydroxypyrene-1,3,6-Trisulfonic Acid) fluorescent dye mixing with Dextran-Rhodamine dye was injected into a cell. HPTS fluorescent dye was chosen because of its small molecule size and membrane-impermeable property. The small HPTS molecule (MW=524.37 Da) can be transferred from the injected cell to adjacent cells through only gap junctions. In contrast, the Dextran-Rhodamine molecule, because of its large molecule size (MW=10,000 Da), cannot pass through gap junctions. Therefore, only the injected cell reveals a red fluorescent signal, while the injected cell, as well as a number of adjacent cells, reveal green fluorescent signal (see Fig. 3-7). The exact number of neighboring cells that can reveal green fluorescent signal is determined by the capability of the cells’ gap junctions. Thus, gap junctional intercellular communication was quantitatively investigated by measuring the dye transferred cell number (i.e., the number of cells uptaking fluorescent molecules from the injected cell through

Figure 3-7 - Only one cell is injected in an area, and fluorescence dye is transferred from the injected cell to adjacent cells through gap junctions.
gap junctions) and dye transferred distance (i.e., the furthest distance that the fluorescent dye can be transferred).

Gap junctional intercellular communication was investigated on three cell lines (i.e., Hela cells, HEK293 cells, and HL-1 cells). These cell lines are known to have an absent, moderate, and high expression of gap junctions, respectively. We also used 18-α glycyrrhetinic acid (18-αGA) to treat HL-1 cells as a demonstration of quantifying drug effect on regulating gap junction function. It is known that 18-αGA inhibits the role of GJIC in fibroblast growth [168], myoblast fusion [169], and trophoblast proliferation [170]. In our experiments, the robotic system was used to assess GJIC of three experiment groups with different doses (25, 50, or 100 µM) of 18-αGA treatment. Two control groups were also included. Control group 1 (0 µM in Fig. 3-7(b)) had no 18-αGA. Since the 18-αGA stocking solution was dissolved in DMSO, a second control group (DMSO in Fig. 3-8(b)) with only DMSO treatment was also examined.

The dye transfer distance was measured to quantitatively characterize the GJIC for the three cell lines. The histogram of dye transfer distance for the three cell lines shown in Fig. 3-8(a) reveals that HL-1 cells have higher GJIC than the other two cell lines. HL-1 is a cardiac muscle cell line. The strong GJIC of HL-1 cells is physiologically necessary for impulse propagation in cardiac tissue [171]. The impulse signal is passed efficiently through gap junctions, allowing the cardiac tissue to contract at the same tandem. In contrast, Hela cells, a cancer cell line derived from cervical cancer cells, express little GJIC, confirming they do not pass signals via gap junctions for cell coordination. Limited GJIC results in poor inhibition of cancer cell mitosis, causing tumor formation [172].

The experimental data in Fig. 3-8(b) shows that the number of HL-1 cells that uptook fluorescent dye from the injected cells decreased significantly with a higher dose of GJIC inhibitor. The results show that 100 µM of 18-αGA effectively blocked almost all the gap junctions. There is no significant difference between the two control groups of HL-1 cells (i.e., the first two bars in Fig. 3-7(b)).
Figure 3-8 - Results of dye transfer experiments. (a) Histogram of dye transfer distance for HeLa cells (n = 200), HEK293 cells (n = 200), and HL-1 cells (n = 400). (b) The number of HL-1 cells uptaking fluorescence dye from the injected cells. Over 200 cells were injected for each group.
3.5.5 Discussion

Microinjection of adherent cells permits the direct insertion of foreign materials (e.g., DNA/RNA, fluorescent dyes, quantum dots etc.) into single cells. It is a decades-old technology that is still widely used in cell biology for testing cell-cell communication, studying intracellular behavior, and gene transfection [173].

Manual microinjection of adherent cells and existing robotic system prototypes are limited to the injection of a few to tens of cells per experiment at best. This new robotic system described in this chapter is the first system capable of performing microinjection on hundreds and thousands of cells per experiment. The system is embedded with strong automation capabilities in every step of operation, enabling an operator to perform the entire microinjection process via mouse clicking in front of a computer monitor. Training of a user with no skills in microinjection takes 10-15 minutes, and after a few hours’ operation, the user becomes highly proficient at operating the system to perform adherent cell microinjection with high success rates.

3.6 Conclusion

This chapter described a robotic adherent cell injection system equipped with several key technologies that were recently developed. These new techniques include automatically locating micropipette tips, robustly detecting the contact of micropipette tip with cell culture surface and directly with the cell membrane, and precisely compensating for accumulative positioning errors. The high degree of automation of the system enables users with no microinjection training to perform large-scale cell injection with high success rates. System operation speed, success rate, and cell viability rate were quantitatively evaluated. Dye transfer experiments were conducted on different cell lines to establish a powerful assay for characterizing cell gap junction functions.
Chapter 4
Robotic Vitrification of Mammalian Embryos

This chapter reports on the first robotic system for vitrification of mammalian embryos. Vitrification is a technique used for preserving oocytes and embryos in clinical in vitro fertilization (IVF). The procedure involves multiple steps of stringently timed pick-and-place operation for processing an oocyte/embryo in vitrification media. In IVF clinics, the vitrification is conducted manually by highly skilled embryologists. Processing one oocyte/embryo takes the embryologist 15–20 min, depending on the protocols chosen to implement. Due to poor reproducibility and inconsistency across operators, the success and survival rates also vary significantly. Through collaboration with IVF clinics, we are in the process of realizing robotic vitrification (RoboVitri) and ultimately aim to standardize clinical vitrification from manual operation to fully automated robotic operation. This robotic system is embedded with two contact detection methods to determine the relative z positions of the vitrification micropipette, embryo, and vitrification straw. A three-dimensional (3-D) tracking algorithm is developed for visually served embryo transfer and real-time monitoring of embryo volume changes during vitrification. The excess medium is automatically removed from around the vitrified embryo on the vitrification straw to achieve a high cooling rate. Tests on mouse embryos demonstrate that the system is capable of performing vitrification with a throughput at least three times that of manual operation and a high survival (88.9%) and development rate (93.8%).

The following section is based on the text from the following publication:

4 Robotic Vitrification of Mammalian Embryos

4.1 Cryopreservation of Oocytes and Embryos

Cryopreservation of mammalian reproductive cells is an essential technique in IVF clinics [174]. Oocytes and embryos are routinely frozen and cryopreserved. Patients who undergo therapeutic procedures (e.g., chemotherapy) that can place their fertility at risk have the option of preserving their oocytes for use at a later time through IVF techniques. Moreover, fertilized embryos are often needed for more than one cycle of IVF treatment. The rest of the fertilized embryos are cryopreserved for future use [175].

The techniques of oocyte/embryo cryopreservation are classified into two categories: 1) slow freezing and 2) fast freezing (i.e., vitrification) [176]. Vitrification, or fast freezing, is proven to be the most effective method and was first reported in [177]. Vitrification is superior to slow freezing [44], because it vitrifies the oocyte/embryo with no ice crystal formation during freezing, resulting in higher cell survival rates. The addition of cryoprotectants in vitrification increases embryo viscosity and makes the vitrified embryos syrupy. When the vitrified oocytes/embryos are placed in liquid nitrogen, the syrupy content inside the cell forms amorphous ice instead of ice crystals, which minimizes the vital damage to the cell during freezing [178].

![Figure 4-1 - The schematic showing manual and RoboVitri approaches. Vitrification involves multiple steps of cell pick-and-place before freezing in liquid nitrogen.](image)


At present, oocyte/embryo vitrification is done manually in IVF clinics globally. An operator looks through the microscope eyepieces and manipulates oocytes/embryos using a micropipette. An oocyte/embryo is first picked up and removed from the culture dish and washed with the equilibrium solution (ES) and a series of vitrification solutions (VSs), as shown in Fig. 4-1. Within each step, timing control has been proven critical. After the many pick-and-place steps, the processed oocyte/embryo is placed onto a device called a vitrification straw. The volume of solution remaining around the oocyte/embryo on the straw must be minimal to ensure a high cooling rate [179]. The vitrification straw is then plunged into liquid nitrogen for freezing and long-term cryopreservation. Several commercial the VSs and protocols exist; however, their core steps are largely the same. All the protocols involve multiple washing steps with the ES and the VS, placing the vitrified oocytes/embryos on vitrification straws, and freezing the vitrification straws in liquid nitrogen. Manual oocyte/embryo vitrification is a laborious and demanding task due to the following reasons.

- There is a long learning curve, and intense focus is required for embryologists performing the manual method.

- The anti-freezing solutes [e.g., dimethyl sulfoxide (DMSO)] are toxic to oocytes/embryos. Therefore, the washing time in the VS is critical but can be difficult to strictly control by an operator.

- Because of their small size (~100 µm), oocytes/embryos can be difficult to detect and manipulate, especially when the medium surrounding the cells is dynamically changing (e.g., in viscosity) during micropipette aspiration and dispensing.

- The manual process has stringent skill requirements, and the success rate and cell survival rate can vary significantly across operators.

Over the past few decades, the robotics community has made significant progress in assisting/standardizing clinical procedures, from the transformative da Vinci surgical system to robotic systems under intensive development for manipulating single cells [180][181]. To realize RoboVitri for clinical use, a number of tasks must be tackled with clinically acceptable reliability, for instance, robust embryo tracking in three dimensions in media of different viscosities and autonomous transfer of processed embryos from liquid environments to the solid
vitrification straw tip. Furthermore, timing control in vitrification is much more stringent and critical than other cell manipulation tasks (e.g., intracytoplasmic sperm injection [157]) to achieve high cell development rates after embryo thawing.

Although automated vitrification was attempted using microfluidic approaches [72], [182], [183], embryo loss is a concern because of difficulties in loading and retrieving embryos onto and from the microfluidic devices. No attempt has been made to automate vitrification using a robotic approach.

In this chapter, I present a robotic system prototype for automated vitrification and thawing of embryos. The system has successfully addressed the major challenges in manual operation with the following technologies:

(1) automated locating of micropipette and vitrification straws;

(2) contact detection methods to determine the relative z-position of the micropipette tip;

(3) autonomous detection and tracking of embryos in three dimensions;

(4) robotically placing vitrified embryos onto vitrification straws and removing excess medium.

These core techniques make it practical to perform automated RoboVitri of embryos. The experimental results also demonstrate that the embryo survival and development rates after vitrification and thawing achieved by the robotic system were higher than those of the manual group. Automated vitrification would free up embryologists to focus on other tasks in IVF clinics and provide consistently high success and survival rates. With a higher throughput, automated vitrification also has the potential to become a standard tool for assessing existing vitrification protocols and developing new protocols.
4.2 System Overview

Figure 4-2 - (a) The RoboVitri system prototype. The inset is the custom-designed carrier plate. (b) The system control architecture.

As shown in Fig. 4-2 (a), the RoboVitri system is built around a standard upright microscope (Olympus SZX16, Olympus Canada, Inc.) that is equipped with motorized magnification control and motorized focusing. Mounted on the microscope is an XY-motorized stage (ProScan, Prior Scientific, Inc.), which has a travel range of 75 mm and a resolution of 0.01 nm along both axes. A custom-designed carrier plate [Fig. 4-2(a) inset] is placed on the XY stage to hold an embryo culture dish, a multi-well plate, and multiple vitrification straws. A three-degree-of-freedom robot (MP285, Sutter, Inc.) carrying a vitrification micropipette (tip diameter: 150 nm) is used to
manipulate embryos. A 25-µL glass syringe (Hamilton Company) is mounted on a linear stage (eTrack, Newmark System, Inc.) for controlled aspiration and dispensing of embryos into or out of the vitrification micropipette. A camera (scA1300-32gm, Basler, Inc.) is connected to the microscope to provide visual feedback. A host computer controls all hardware via our custom-developed control software.

The robotic micromanipulator and X-Y stage are cooperatively controlled for positioning the vitrification micropipette along the XYZ axes and positioning embryos in the x-y plane, respectively. The overall control architecture of the automated system is summarized in Fig. 4-2(b). The techniques for micropipette tip detection and embryo tracking are described in the “Contact Detection” and “3-D Embryo Tracking” sections, which provide position feedback to position the micromanipulator and the XY stage, forming an image-based visual servo control system.

In robotic vitrification, the system first performs an auto-locating of the end effector, a technique previously reported in Chapter 2, for the robotic system to automatically detect the micropipette tip. Two contact detection methods are used by the system to determine the relative z-position of the micropipette tip to the multi-well plate bottoms and the vitrification straw tip. During the washing steps, a 3-D tracking algorithm integrated with a Kalman filter is used to track the embryos. After washing in the ES and the VS, the robotic system automatically transfers the vitrified embryo out of the liquid environment onto the vitrification straw tip and removes the excess medium to realize the minimum volume requirement in vitrification. The vitrification straw carrying a vitrified embryo is placed in liquid nitrogen. Similar to the vitrification process, the frozen embryos are thawed and the system performance is evaluated by quantifying the post-thaw cell survival and development rate.
4.3 Key Methods for Robotic Vitrification

4.3.1 Contact Detection

Optical microscopy has a limited depth of field, making position detection along the z-axis difficult. The relative z-distance between the micropipette tip and the embryos must be accurately determined before cell manipulation can start. Since the embryos are always placed on the bottom of a multi-well plate or on the surface of the vitrification straw tip, the system determines the relative z-position of the micropipette tip by detecting its contact with the multi-well plate substrate and the straw surface.

![Diagram of contact detection](image)

Figure 4-3 - The detection of contact between the micropipette tip and the multi-well plate substrate and contact between micropipette tip and vitrification straw surface. (a) and (b) The schematic of contact detection on the plate substrate and vitrification straw, respectively. (c) and (d) The system moves the micropipette downward. (e) A further downward movement after contact induces the micropipette tip’s sliding motion on the plate substrate surface. (f) A further downward movement after contact deflects the vitrification straw tip, causing it to become out of focus.
During contact detection on the multi-well plate substrate, the system moves the micropipette tip downward to approach the plate substrate [Fig. 4-3(a) and (c)]. When the micropipette tip contacts the plate bottom [Fig. 4-3(a) and (e)], further downward movement induces the tip’s horizontal sliding motion on the plate substrate, which changes the tip’s position in the x-y plane and the imaging plane. The system detects the initial x-y position change and, thus, determines the initial contact of the vitrification micropipette tip with the plate substrate.

The detection of the micropipette tip contact on the vitrification straw surface is different from contact detection with the multi-well plate bottom. Vitrification straw tips are cantilevers in structure and have low stiffness compared with plate substrates. Therefore, instead of sliding on the soft vitrification straw surface, the micropipette tip’s further downward motion after initial contact deflects the soft straw tip [Fig. 4-3(b)]. When the straw is deflected by the micropipette tip, it becomes out of focus in imaging [Fig. 4-3(f)]. Based on the computed focus measure, the robotic system detects the contact between the micropipette tip and the straw tip surface. This contact detection step is critical before the robotic system can place the vitrified embryo onto the straw tip, which will be discussed in the “Placing Embryo on Vitrification Straw” section.

4.3.2 Embryo Tracking in Three-Dimensional Space

The system, after detecting the micropipette tip positions along the XYZ axes relative to the embryos, performs embryo pick and place to transfer the embryo from one type of solution to another (e.g., from the ES to the VS) via controlled micropipette aspiration. Due to the changes of fluid density and osmolarity, the embryo volume changes in the VS, resulting in variations of buoyancy. In addition, the fluidic flow from the micropipette dispensing also influences the embryo’s positions. Thus, the embryos move dynamically in 3-D space when transferred into a different VS. To avoid losing the embryo and to enable efficient pick and place for ensuring stringent time control in each type of cryoprotectants, the robotic system must be able to robustly detect and track embryos three-dimensionally.
Figure 4-4 - Embryo tracking in the VS. Left column: the original images with embryos at different heights. Middle column: the ROIs containing the tracked embryo target. Right column: the tracked embryo locations indicated by a red dot in the binarized image. (a) An embryo is dispensed out of micropipette into the VS solution. (b) An embryo is floating upward, due to buoyancy, whereas the system performs autofocus to control focus positions.

When an embryo is dispensed out of the micropipette, a region of interest (ROI) is extracted at the micropipette tip [Fig. 4-4(a)]. The ROI is denoised through the Gaussian smoothing method and binarized by applying Otsu’s adaptive thresholding. A morphological close operation is then performed to remove noise and small particles that may be present in the ROI. In the binarized image, the contour of the detected foreground object is computed. The embryo’s position in the image plane is detected by calculating the moment of the contour (see Fig. 4-4, right column). The ROI is then updated to be centered at the embryo’s centroid. The area of the embryo contour is also measured to reflect the embryo’s volume change. For subsequent frames of images, the system repeats the execution of the process to track the embryo’s position in the image plane (i.e., the x-y plane).

To track the embryo’s floating motion along the Z-axis, the system performs autofocus. The normalized variance method is used to calculate the focus measure. This method is chosen because it can effectively compensate for differences in the average image intensity ($\mu$) by normalizing the final output with the mean intensity. The focus measure $F$ changes as the embryo
moves in the z direction inside the VS solution. As the focal plane moves close to the embryo, the contents in the image increase, causing the focus measure to increase. The system adjusts the microscope’s focal plane according to maximizing the focus measure, and the position recorded by the encoder of the focusing motor on the microscope is taken by the system to be the detected z positions

$$ F = \frac{1}{W \times H \times \mu} \sum_{w} \sum_{h} (I(x, y) - \mu)^2 $$

(14)

where $W$ and $H$ are the ROI image width and height, respectively, $(I(x, y))$ is the pixel intensity at point $(x, y)$, and $\mu$ is the average pixel intensity in the ROI.

The tracked embryo positions from autofocusing can be inaccurate due to the delay of changing the focal plane. Therefore, a Kalman filter is applied to correct the detected embryo positions. The embryo movement along the z direction is mainly caused by the dynamic change in buoyancy. The dynamics of the embryo’s floating motion in the VS is

$$ \rho g V(t) - mg - F_d = m\ddot{x} + c\dot{x} $$

(15)

where $\rho$ is the liquid density, $g$ is the gravitational acceleration, $m$ is the mass of the embryo, $x$ is the embryo’s position along the z axis, and $c$ is the damping ratio of the liquid. Due to osmotic stress in the VSs, the embryo volume $V(t)$ changes over time [16]. The cell volume change in the VSs can be modeled as

$$ \frac{dV(t)}{dt} = -L_p \cdot A \cdot R \cdot T \cdot (M_s + M_n - \frac{n_s}{V_w} - \frac{n_n}{V_w}) $$

(16)

$$ \frac{dn_s}{dt} = P_d \cdot A \cdot (M_s - \frac{n_s}{V(t)}) $$

(17)

In this model, Eq. (16) describes the change of cell volume $V(t)$ over time as a function of the hydraulic conductivity $L_p$, surface area $A$, gas constant $R$, temperature $T$, intracellular permeating $M_s$, and non-permeating $M_n$ solute concentration in the osmoles, and the
extracellular permeating \( n_x \) and non-permeating \( n_n \) solute concentration. Equation (17) describes the change in the intracellular moles of permeating solute ns over time as a function of the DMSO permeability \( P_D \). When substituting \( V(t) \) into Eq. (17), the system analyzes the dynamics of the embryo’s 3-D motion and uses the Kalman filter to optimize the tracked results.

With the system dynamics modeled, the changes of embryo state in the VSs can be described by choosing the XYZ positions and velocities as the state variables

\[
X_k = A \cdot X_{k-1} + w_k \tag{18}
\]

where \( A \) is the state transition matrix, \( w_k \) represents the noises affecting the actual state of the embryo caused by the heterogeneous response of the individual embryos, and \( w_k \) is assumed to have a Gaussian distribution \( N(0,Q_k) \).

The embryo’s position is calculated according to

\[
Z_k = H \cdot X_k + v_k \tag{19}
\]

where \( H \) is the output matrix and \( v_k \) is the measurement noise, which is also assumed to have a Gaussian distribution \( N(0,R_k) \). \( R_k \) is chosen based on the estimate of how accurately the embryo’s 3-D positions are detected by image processing.

Based on the dynamic model, a priori estimate of the state is computed \( X_{k|k-1} = AX_{k-1|k-1} + w_k \). The error covariance is denoted as \( P_{k|k-1} \). The priori estimate for this covariance at time \( k \) is then determined by

\[
P_{k|k-1} = A \cdot P_{k-1|k-1} \cdot A^T + Q_k \tag{20}
\]

With the a priori estimate of the state \( X_{k|k-1} \) and the measurement \( Z_k \) (i.e., the detected embryo position from image processing), the real state of the embryo in the VS is optimized by

\[
X_{k|k} = X_{k-1|k-1} + K \cdot (Z_k - HX_{k|k-1}) \tag{21}
\]

where \( K \) is the Kalman gain and is given by
With the optimized embryo 3-D position $X_{k|k}$, the robotic system controls the micropipette tip to retrieve the embryo out of the solutions via controlled aspiration [184]. A biological advantage of the embryo detection algorithm is that it enables the system to determine the optimized processing time based on measuring the volume change of an embryo (i.e., individualized timing, which is not possible to achieve in manual operation). In the VS, the embryos shrink in the beginning due to osmotic pressure. Then they re-expand to equilibrate with the VS. Since the cryoprotectant in the VS is toxic, the equilibration with the VS should be avoided [185]. Therefore, the robotic system retrieves and transfers the embryo out of the VS once its minimum volume is reached, according to the 3-D tracking results.

### 4.3.3 Placing Embryo on Vitrification Straw

After washing the embryos in the ES and the VS under controlled timing, the vitrified embryos need to be placed on a vitrification straw tip. In this step, the excess medium must be removed from the vitrified embryo to ensure a high cooling rate in the liquid nitrogen. After detecting contact of the micropipette tip and the straw surface, the system dispenses the embryo with a relatively large volume of the VS solution onto the straw. The system then moves the micropipette tip on the straw surface away from the initial dispensing location to form a thin VS film [Fig. 4-5(a)]. The robotic system controls the motorized syringe to aspirate the VS until the volume of the embryo droplet stops changing [Fig. 4-5(b)]. When the medium is aspirated into the micropipette, a friction force acts on the embryo to keep it in the original place. To achieve this, the fluid speed from micropipette aspiration must be well controlled.

As shown in Figure 4-5(c), the embryo is also acted on by the drag force $F_d$ generated by micropipette aspiration flow

$$F_d = \frac{1}{2} \rho v^2 C_d A$$

where $t$ is the fluid density, $v$ is the fluid velocity controlled by the motorized syringe, $A$ is the cross-sectional area, and $C_d$ is the drag coefficient. If the drag force is too large, the embryo can
be undesirably moved together with the fluid into the micropipette. To keep the embryo in place on the straw, the drag force must be smaller than the friction force (i.e., $F_d < f = \mu mg$). Therefore, the aspiration flow rate should be below a threshold value. It was determined experimentally that the critical aspiration flow rate $v$ must be controlled to be lower than 240 $\mu$m/s. Below this threshold value, the minimum volume can be achieved reliably. After removing the excess medium from around the embryo, the straw is plunged into liquid nitrogen for freezing. The vitrification straw is then sealed with a plastic cap and put in a liquid nitrogen tank for preservation.

Figure 4-5 - A processed embryo placed on the vitrification straw tip. (a) An embryo is deposited onto the straw by dispensing the VS medium out of the micropipette. (b) The excess medium is removed by micropipette aspiration under a threshold flow rate to keep the embryo in place. (c) The schematic showing the embryo dynamics during micropipette aspiration.
4.4 Results and Discussion

In the experiments, mouse embryos were gathered from the Canadian Mouse Mutant Repository in the Toronto Centre for Phenogenomics (Toronto, Ontario). Embryos were produced by super-ovulating a female and collected ~1.5–2.5 days after conception, which corresponds to the embryos being in the with KSOM medium (EMD Millipore, Billerica, United States) in a 35-mm Petri dish and covered with mineral oil to prevent evaporation.

The VS typically contains anti-freezing agents or cryoprotectants such as DMSO, small molecular-sized glycols (e.g., ethylene glycol), or sucrose. In our experiments, the VS was made by diluting DMSO in KSOM medium at 20% concentration. The ES was at half the concentration of the VS (i.e., 10% DMSO). A multi-well plate (Repro Plate, Kitazato Corporation) was loaded with the ES and the VS for embryo washing. A standard vitrification straw (Cryotop, Kitazato Corporation) was used as the physical carrier to freeze embryos in liquid nitrogen. All vitrification experiments followed the Kitazato protocol by washing embryos in the ES and the VS for 12 min and 90 s, respectively. The robotic system can be readily reprogrammed to implement other vitrification protocols.

4.4.1 System Performance

The system throughput was evaluated by processing the mouse embryos at the two-, four-, and eight-cell stages. The capability of automated pick and place of single embryos enabled the robotic system to perform vitrification of multiple embryos in an optimally scheduled sequence. Since embryo equilibration in the ES costs minutes, after the first embryo was dispensed into the ES for equilibration, the robotic system moved back to the culture dish to pick up the next embryo and place it into the ES of another bath. Repeating this step to process six embryos in the ES, the system then retrieved the equilibrated embryos from the ES and washed them in the VS one by one, again following a prescheduled sequence. As a result, the system was able to process six embryos within 24 min. In comparison, in the manual implementation of the same vitrification protocol, it was only possible to process two embryos in the same time period, and the operator was fully occupied in the process.
### Table 6 - Embryo Vitrification Experimental Results

<table>
<thead>
<tr>
<th>Method</th>
<th>Success Rate</th>
<th>Survival Rate</th>
<th>Development Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group</td>
<td>N/A</td>
<td>100% (15/15)</td>
<td>93.3% (14/15)</td>
</tr>
<tr>
<td>Manual Group</td>
<td>83.3% (15/18)</td>
<td>73.3% (11/15)</td>
<td>90.9% (10/11)</td>
</tr>
<tr>
<td>Robotic Group</td>
<td>90% (18/20)</td>
<td>88.9% (16/18)</td>
<td>93.8% (15/16)</td>
</tr>
</tbody>
</table>

The success rate was also quantitatively evaluated. An experiment was defined to be successful when the system successfully processed the embryo within the given period of time for each step of a vitrification protocol. Manual vitrification experiments were also performed by three operators. The experimental results showed that the robotically vitrified group had a significantly higher success rate than the manual group (90% versus 83.3%, shown in Table 6). In manual vitrification, embryos could easily escape from operators’ monitoring when they floated upward in the VS, which was the major cause of failure. Embryo loss was effectively avoided by the system’s capability of 3-D embryo tracking in RoboVitri. However, failure in RoboVitri arose when an embryo floated in the VS solution and happened to drift into blind regions of the multi-well plate. The Repro Plate used in the experiments has inclined sidewalls that produce a dark blind region. Multi-well plates with vertical sidewalls can help reduce blind regions and, hence, system failure.

#### 4.4.2 Embryo Volume Measurement

In RoboVitri, the system is able to track embryos in 3-D space and monitor their volume change in real time for analyzing each individual embryo’s response to the VS. Fig. 4-6 shows the tracked embryo position and the volume change of three different embryos in the VS, measured by the robotic system. When washed in the VS, the embryos first experience a dehydration stage in which water molecules are drawn out of the cell, causing the embryo to shrink. When the embryo reaches its minimum volume, the toxic cryoprotectant solutes (e.g., DMSO) start to penetrate the cell membrane. The embryo ideally should be transferred out of the VS at its minimum volume.
Figure 4-6 - The 3-D-tracking results: (a) the z position change of a tracked embryo and (b) the volume change of three embryos in the same VS measured by the robotic system.
The experimental results summarized in Fig. 4-6(b) demonstrate that the embryos reach their minimum volume in the VS at different time points. This suggests heterogeneity in embryo dehydration timing even in the same VS. All existing vitrification protocols stipulate a fixed washing time for all embryos because measuring the individual embryo’s volume change is not feasible to achieve by human operators. In contrast, the robotic system is capable of measuring an embryo’s volume throughout the vitrification process and is able to retrieve each embryo from the VS at its minimum volume point. Therefore, the toxic effects from the cryoprotectant solute (i.e., DMSO) can be minimized as the embryo is taken out of the VS when embryo dehydration has just ended.

### 4.4.3 Post-Freezing Survival and Development Rates

The survival and development rates were defined to quantify the robotic system’s performance. Survivability was measured by examining the morphology of the embryo before and after freezing, as commonly performed in the literature (see [19]). Embryos were considered unhealthy/dead if they had an abnormal shape, membrane damage, leakage of cellular content, or degeneration of their cytoplasm.

![Example embryo images before and after vitrification.](image)

*Figure 4-7 - Example embryo images before and after vitrification.*

The development rate was determined by culturing the surviving embryos for an additional 24 h after thawing (Figure 4-7). If the cell number within the embryo increased, or if the embryo developed to the blastocyst stage, it was counted as developed. Control samples of non-vitrified embryos were also cultured to identify the base development rate of the mouse embryo.
population. Only embryos that had a healthy morphology after freezing were cultured following similar procedures to other vitrification studies [20].

The experimental results are summarized in Table 6. The robotically vitrified group had higher survival (88.9% versus 73.3%) and development rates (93.8% versus 90.9%) than the manually vitrified group. The higher survival and development rates produced by RoboVitri can be attributed to the optimized processing time achieved by the system through monitoring embryo volume changes in the VS. The ability of the system to effectively remove excess medium from around vitrified embryos on the straw (i.e., minimum volume vitrification for a higher cooling rate when freezing in liquid nitrogen) could also have enabled the robotic system to achieve higher embryo survival and development rates.

4.4.4 Discussion

Vitrification is an essential technique in IVF for preserving oocytes and embryos. A number of different the VSs and protocols (e.g., Kitazato, Origio, and Irvine) have been developed and are commercially available. All of these protocols require multiple steps of embryo washing in different types of the VS. With the automated capability, our robotic system can be programmed to test, optimize, and compare different vitrification protocols.

Besides embryo vitrification, oocyte vitrification is also important in IVF practice for preserving female fertility for future use. Oocyte vitrification requires more steps than embryo vitrification. For example, in the Kitazato protocol, oocyte vitrification involves three ES steps and two VS steps, whereas embryo vitrification only has one ES and VS step. The RoboVitri system can also be programmed to complete oocyte vitrification by simply repeating more washing steps because of its capability of automated pick and place and stringent time control.

Robotic cell manipulation relieves the human operator from tedious vitrification steps and eliminates manual operation-caused errors and inconsistencies. It also offers unparalleled timing control and the ability to leave a minimal volume of the solution on vitrification straws. Automation also enables the system to process multiple oocytes/embryos with high efficiency. Efforts will continue to develop the system into an ideal tool for standardizing oocyte/embryo vitrification in IVF clinics and to achieve improved cryopreservation outcomes.
4.5 Conclusion

This Chapter presented an automated RoboVitri system capable of processing embryos with different the VS. The system is integrated with two contact detection methods to determine the relative Z-position of the micropipette tip relative to embryos and vitrification straw tips. A 3-D embryo-tracking technique was developed to prevent embryo loss during multiple washing steps and achieve vision-guided embryo transfer. The 3-D embryo tracking technique also enables the system to monitor real-time embryo volume changes in the VS, permitting individualized time control for each embryo. In the step of placing an embryo onto the vitrification straw tip, the excess medium was automatically aspirated away from the vitrified embryo to obtain a high cooling rate. With these technical capabilities, the robotic system successfully achieved a high throughput and improved post-freezing survival and development rates compared to manual vitrification.
Chapter 5
Automated Analysis of Sperm Locomotion Behavior

Sperm selection plays a significant role in in vitro fertilization (IVF). Approaches for assessing sperm quality include noninvasive techniques based on sperm morphology and motility as well as invasive techniques for checking DNA integrity. In 2006, a new device using hyaluronic acid (HA)-coated dish for sperm selection was cleared by the Food and Drug Administration (FDA) and entered IVF clinics. In this technique, only sperms with DNA integrity bind to the HA droplet, after which these bound sperms stop revealing head motion and their tail movement becomes more vigorous. However, selecting a single sperm cell from among HA-bound sperms is ad hoc in IVF clinics. Different from existing sperm tracking algorithms that are largely limited to tracking sperm head only and are only able to track one sperm at a time, this chapter presents a multi-sperm tracking algorithm that tracks both sperm heads and low-contrast sperm tails. The tracking results confirm a significant correlation between sperm head velocity and tail beating amplitude, demonstrate that sperms bound to HA generally have a higher velocity (before binding) than those sperms that are not able to bind to HA microdots, and quantitatively reveal that HA-bound sperms’ tail beating amplitudes are different among HA-bound sperms.

The following section is based on the text from the following publication:

5 Automated Analysis of Sperm Locomotion Behavior

5.1 Introduction

The mechanisms of natural sperm selection are not well understood. It is accepted, however, that the sperm selection mechanisms play a significant role in the inheritance of superior health traits such as disease resistance, offspring survival, and fecundity [186][187]. In natural human conception, sperm selection occurs as a healthy sperm actively seeks out and fertilizes an egg. However, for couples having infertility issues, assisted reproduction technologies are required to address their reproductive needs. For instance, in intracytoplasmic sperm injection (ICSI), an embryologist selects a single sperm and injects it into an oocyte (i.e., egg cell) to overcome issues such as male infertility [35]. This IVF procedure bypasses the physiologic and biologic barriers for sperm selection and demands the operator to select high-quality sperms.

The criteria for sperm assessment provided by the World Health Organization are vitality, morphology, and motility [46]. In IVF, sperm selection is commonly based on sperms’ motility and morphology attributes. A widely used method for sperm selection is motile sperm organelle morphology examination [188][189]. Sperm motility is also a widely accepted criterion for sperm quality assessment. The past few decades have witnessed the development of computer-assisted sperm analysis (CASA) methods for measuring both sperm morphology and motility [190].

Recently, an emerging methodology was introduced for selecting viable sperms with a high level of DNA integrity. In order to noninvasively select a healthy sperm, Huszar’s group proposed the use of a hyaluronic acid (HA) assay [51]. HA is a linear polysaccharide in the extracellular matrix of cumulus oophorous around the oocyte and plays an important role in natural human fertilization [191]. A series of studies on HA-based sperm selection confirmed that the HA assay is able to select healthy sperms with no DNA damage [52][53], and has received FDA approval. In the HA assay, sperms that bind to HA microdots are proven to have a higher level of DNA integrity compared to those unbound sperms. When a sperm binds its head to an HA microdot, the sperm loses its progressive movement and the tail beating motion becomes more vigorous. The sperm tail beating amplitude becomes the only parameter to differentiate the HA-bound sperms from each other, calling for techniques to quantify HA-bound sperms’ tail beating motion.
Several algorithms have been reported for tracking multiple moving objects. Model-based tracking algorithms incorporate priori information about the objects to develop representations such as skin complexion [192], shape [193], kinematic skeleton [194], silhouettes [195], or layer information [196]. Appearance-based approaches apply recognition algorithms to track objects in eigenspace [197] derived from observations or in kernel space [198]. The correspondence of multi-object tracking becomes complex with the presence of occlusions, misdetections, entries, and exits of objects. Algorithms for solving the problems of nearby confuser and objects occlusion/overlap include deterministic methods [199], single object state estimation (e.g., Kalman filter [200], particle filter [201]), and multiple object data association and state estimation (e.g., global nearest neighbor method [202] and joint probability data association filter [203]). Due to color uniformity and shape similarity of sperms, multi-sperm tracking has not been well studied.

Several algorithms have been developed to track sperm trajectories, measure sperm velocities, and evaluate sperm energetics [54], [204], [205]. Shi et al. reported a single-sperm tracking algorithm based on a four-class thresholding method to extract a single sperm in a small region of interest [55]. The method is limited to tracking a single sperm and is incapable of multi-sperm tracking. Nafisi et al. demonstrated a template matching algorithm for sperm tracking. The algorithm is insensitive to image acquisition conditions [56]. However, this algorithm relies on user input to obtain the sperm’s initial position and cannot track multiple sperms. Existing algorithms for sperm tracking are largely limited to sperm head tracking. The small size (≤1 μm in thickness) and low contrast of sperm tails under optical microscopy make sperm tail tracking challenging. In our previous study [206], a maximum intensity region algorithm was developed for sperm tail tracking. The tracking algorithm, without proper filtering, can be susceptible to disturbances, such as overlapping of the target sperm with other sperms or debris and changes in lighting conditions.

In this chapter, I report an approach for tracking both sperm head and tail. This approach uses a motion template method to detect and track multiple moving sperms, and integrate a Kalman filter to the maximum intensity region algorithm to locate the sperm tail’s position. With the positions of the sperm head and tail detected, the sperm’s velocity and tail beating amplitude were measured. Experimental results demonstrate that there is a significant correlation between the sperm velocity and its tail beating amplitude. I also analyzed sperm motility and the tail
beating movement on HA coated dishes. I found that sperms with a higher level of motility are more likely to bind to the HA microdots, and the sperm tail beating amplitude significantly increases after a sperm binds to the HA microdots. Quantitative analysis of sperm tail’s beating amplitude can provide useful information for sperm selection.
5.2 System Setup and Experimental Design

Figure 5-1 - Experiment design. (a) and (b) Experiment 1: a sperm changed its position between two frames. (c) and (d) Experiment 2: a sperm was moving toward an HA microdot and then bound to the HA microdot.

Human sperm samples were tested under a standard inverted microscope (bright field imaging, Nikon TE2000-S). A 20× objective with a numerical aperture of 0.45 was used (CFI Plan Fluor ELWD, Nikon). A CMOS camera (601f, Basler; resolution: 640×480) was connected to the microscope to capture images at a frame rate of 30 frames/second.

We first investigated the correlation between sperm velocity and sperm tail beating amplitude. In this experiment, human sperms were placed in a Petri dish containing a standard medium (SpermCatch, NidaCon International). Mineral oil was used to cover the medium to prevent evaporation. Fig. 5-1(a) and (b) shows a sperm changed its position between the two image frames. Sperms that had linearly progressive movement for at least 2 s were taken into
consideration. To evaluate the linearity of the sperm motion, sperm’s average VCL and straight line velocity were calculated by measuring the sperm’s head position in each frame. Additionally, the sperm tail beating amplitude was measured using the sperm tail tracking algorithm.

The second experiment was designed to investigate the difference of sperm velocity and tail beating amplitude between HA bound sperms and those unbound ones. Human sperms were analyzed in a PICS1 dish (MidAtlantic diagnostic, Mount Laurel, NJ) with HA microdots coated at the dish bottom. The microdots were first hydrated by placing 10 μL droplets of human tubal fluid and were added with 10 μL SpermCatch. Human sperm was then placed on the HA microdots, which were covered with mineral oil to prevent the culture medium from evaporation. The experiment was conducted at room temperature. Some sperms were observed to bind to the microdots after 5 min. Fig. 5-1(c) shows that a sperm was moving toward an HA microdot, and Fig. 5-1(d) shows the same sperm bound to the HA microdot. When a sperm bound to the HA microdot, its head motion stopped and its tail beat vigorously. In contrast, sperms that might have DNA defects swam freely on top of the HA microdots without binding. In this experiment, the head velocity of HA bound sperms before binding and the head velocity of unbound sperms were measured and compared. The increase of sperm tail beating amplitude after a sperm binds to an HA microdot was also measured.

5.3 Computer Vision Methods for Sperm Tracking

5.3.1 Overview

In both experiments described in the previous section, the sperm velocity and tail beating amplitude are calculated in every frame of the image. The algorithm consists of three steps. The first step tracks the sperm head and uses its position to calculate the sperm curvilinear velocity (VCL), straight line velocity (VSL), and linearity of the sperm’s moving path. In the second step, the sperm tail region of interest (STROI) is extracted. STROI extraction is an extrapolation process that calculates the region in which the sperm tail is located by using information from the first step. The STROI is used to capture the tail tip region of the sperm. Once the STROI is found, the maximum intensity region (MIR) algorithm is used to locate a point on the sperm tail within the STROI. Finally, a Kalman filter is used to improve the accuracy of the located point on the sperm tail.
5.3.2 Sperm Head Tracking

![Sperm Head Tracking](image)

**Figure 5-2 - Tracking multiple sperm heads.** (a) Red dots are tracked sperm heads. (b) Corresponding motion history image.

There are typically multiple sperms moving randomly within a field of view. In order to detect the sperm heads simultaneously, a multi-target tracking algorithm was developed. For a specific single sperm, a silhouette image of this sperm is obtained by subtracting two consecutive frames. The silhouette image is then binarized by applying a threshold (i.e., 60) to suppress the background noise. When this sperm moves, new silhouettes are captured and overlaid to the old silhouette that fades over time. The time duration of every silhouette is set to be 0.5 s. The sequentially fading silhouettes record the motion history of this sperm. Using this method, a motion history image (MHI) [146] of all the moving objects in the same field of view is obtained, as shown in Fig. 5-2(b). The position of each moving object is calculated from its central moment in the MHI. Among these moving objects, there are some objects with very little motion caused by those sperms with extremely low motility or by Brownian motions of debris. These objects are excluded by applying morphological transformations (i.e., erosion and dilation) to the MHI. Figure 5-2(a) shows the detected moving sperms.

To track multiple sperms, the position history of each sperm is recorded and managed in the multiple sperm tracking algorithm, as summarized in Table 7. When $J$ sperms are detected at frame $i$, $P_{ij}$ represents the position of the $j$-th sperm at frame $i$. When a sperm moves close to the edge of the image and its moving direction is toward the boundary, it is considered swimming out of the field of view. The algorithm then terminates the corresponding data column for this
sperm. As shown in Table 7, the second column disappears after frame $i + 1$, which means that the second sperm has moved out of the field of view. On the other hand, when a sperm newly moves into the field of view, it is added into the data column. For instance, a new sperm moves into the field of view at frame $i + 3$. Correspondingly, a new column is added (see the last column in Table 7).

Table 7 - Dynamic Representation of Sperm Positions in Multi-Sperm Tracking Algorithm

<table>
<thead>
<tr>
<th>Frame No.</th>
<th>Sperm Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$i$</td>
<td>$P_{i1}$</td>
</tr>
<tr>
<td>$i+1$</td>
<td>$P_{i+1,1}$</td>
</tr>
<tr>
<td>$i+2$</td>
<td>$P_{i+2,1}$</td>
</tr>
<tr>
<td>$i+3$</td>
<td>$P_{i+3,1}$</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

To track an individual sperm, the direction vector of the sperm is used as a unique identifier to distinguish it from other sperms with similar color and shape. By denoting the position of the $j$-th sperm at frame $i + 1$ as $P_{i+1,j}$, the sperm’s direction vector is $D_{ij} = P_{i+1,j} - P_{i,o}$, where $o \in [1, J]$. We found experimentally that the maximum moving distance of fast sperms between two consecutive frames is approximate 3.86 μm (i.e., 8 pixels). Therefore, if the distance between the $j$-th sperm’s position at frame $i + 1$ and the $o$-th sperm’s position at frame $i$ is less than 8 pixels (i.e., $\|D_{ij}\| < 8$), these two sperms are recognized as the same sperm (i.e., $o = j$). There may be instances in which two or more sperms at frame $i + 1$ have a distance less than 8 pixels to the $j$-th sperm in frame $i$. The average movement direction in previous 30 frames, $\bar{D}_j$, is then used as a unique identifier to determine which sperm is the correct $j$-th sperm.
\[ \bar{D}_j = \frac{1}{30} \sum_{k=1}^{30} D_{l-k,j} \]  

(24)

The candidate sperm \( s \) that produces the minimum Euclidean distance value is considered the same \( j \)-th sperm in the previous frame \( i \)

\[
s = \min_{m \in [1,M]} \|D_m - \bar{D}_j\| 
\]

(25)

where \( M \) is in frame \( i + 1 \) the total number of sperms close to the \( j \)-th sperm (i.e., \( \|D_{ij}\| < 8 \)) and \( D_m \) is the distance vector between the candidate sperm and the \( j \)-th sperm.

Assume that the \( j \)-th sperm enters the field of view at frame \( i \) and swims out of the field of view at frame \( i + N \). With the sperm position detected in each frame, the travel distance of the \( j \)-th sperm between two consecutive frames can be determined from its direction vector \( D_{ij} \). The VCL, which is the average velocity of the sperm head along its actual curvilinear path, is

\[
VCL_j = \frac{1}{N} \sum_{k=0}^{N-1} D_{l+k,j} 
\]

(26)

The VSL, which is the average velocity of the sperm head along the straight line between its first and last detected position, is

\[
VSL_j = \frac{P_{l+N,j} - P_{lj}}{N} 
\]

(27)

The linearity (LIN) of the sperm’s curvilinear path is

\[
LIN_j = \frac{VSL_j}{VCL_j} 
\]

(28)

where LIN is the linearity measure (0 \( \leq \) LIN \( \leq \) 1). A higher LIN value means that the sperm’s moving path is more linear. Healthy energetic sperms with progressive/linear movement are desired (versus those traveling in circles for instance) in sperm selection. In our experiments, only those sperms having a LIN value greater than 0.9 were considered for further analysis.
5.3.3 Sperm Tail Tracking

After the sperm head position is detected, the sperm tail tracking algorithm extracts an STROI. As shown in Fig. 5-3(b), the STROI is determined using the sperm head position and the average direction vector of its movement. The average direction vector $D_j$ is used instead of the direction vector $D_{ij}$ because the sperm may exhibit abrupt changes in movement direction between two consecutive frames. By averaging the direction vectors of the sperm across a number of frames...
(e.g., 30frames), the effect of abrupt changes in the sperm moving direction between frames is mitigated and the extraction of STROI becomes more robust.

The STROI’s center position in the $i$ frame $T_{ij}$ is determined by subtracting a scaled value of the direction vector from the sperm head’s centroid

$$T_{ij} = P_{ij} - a \cdot \frac{\vec{D}_j}{\|\vec{D}_j\|}$$

where $a$ is a scalar value determined by the human sperm length. Under the 20× magnification, the average length of human sperms is approximately 90 pixels (i.e., $a = 90$). After the center position is found, a 25×25 region of interest is taken as the STROI. The size of 25×25 provides a sufficient tail search area that takes into consideration a range of sperm tail length variations and sperm tail beating amplitudes.

After finding the STROI, the algorithm verifies that a tail is present in the STROI. The fundamental feature of flicker is extracted by taking the absolute difference between several consecutive inverted grayscale image frames. A higher number of frames to form the flickering image results in more enhancement of the image contrast. On the other hand, too many consecutive frames would add too much sperm tail’s history information that can influence the sperm tail tracking accuracy. Experimentally, we determined that six consecutive frames were appropriate to use for forming a flickering image

$$f(i) = \sum_{k=0}^{5} |I(i - k) - I(i - k - 1)|$$

where $f(i)$ is the flickering image extracted at frame $i$, and $I$ represents the grayscale images containing the sperm of interest in frame $i$ to frame $i - 5$. Each pixel in the flickering image is squared to enhance the pixel values of areas in which the tail is present. The sum of the pixel value in the STROI of the $f(i)$ image is used as a measure to determine the presence of a sperm tail. If the pixel sum is above a specified threshold value, a tail is considered present. The threshold value was found experimentally by comparing the pixel-sum values of STROI images in which a tail exists against cases where no tail exists. An example flicker image is shown in Fig. 5-3(c). If the pixel sum is below a threshold value, no tail is found inside the STROI. This
situation can occur when the sperm of interest moves out of focus, resulting in the disappearance of the sperm tail.

Once the sperm tail is determined to exist within the STROI, the MIR algorithm uses the flickering image to locate a point on the sperm tail. By extracting the flicker feature of the sperm tail, as shown in Fig. 5-3(c), the position of the sperm tail can be detected. This approach overcomes the challenges that arise from the low contrast image of the sperm tail in a single frame. The algorithm first finds the location of maximum intensity within the 25×25 STROI of the flickering image. This is accomplished by evaluating the sum of the intensity values inside a 5×5 window at a spatial sampling interval of 5 pixels in both the x and y coordinates of the STROI flicker image. The center position of the 5×5 window with the highest intensity is considered the tail location (i.e., a point on the sperm tail).

The located point on the sperm tail is often inaccurate because the flickering image contains noises caused by some dark debris or by other sperms entering the STROI. Therefore, a Kalman filter is applied to correct the measured point on the sperm tail. In order to model the sperm tail motion, the sperm tail’s location and velocity in the image coordinate are chosen as state variables (i.e., $X = [x \ y \ \Delta x \ \Delta y]^T$). After the optimized sperm tail’s position is found, the sperm tail beating amplitude inside the STROI is computed. The relative position inside the STROI in frame $i$ is denoted by $PT_i$. The sperm tail beating amplitude $A$ is

$$A = \frac{1}{N} \sum_{i=1}^{N} \|PT_i - \bar{PT}\|^{31}$$

where $PT$ is the sperm tail’s average position inside the STROI and $N$ is the number of frames until when the sperm tail is successfully detected.
5.4 Experimental Results and Discussion

Figure 5-4 - Failure cases in sperm head tracking. (a) False positive detection of sperm head in shape-based tracking is caused by the stationary object/particle with a shape similar to sperm head. (b) False negative case: shape-based detection algorithm fails to detect the sperm head that has an abnormal head shape. (c) False positive detection of sperm head in MHI-based detection is caused by moving debris. (d) False negative case: MHI-based detection algorithm fails to detect stationary/slow-moving sperms.

The multi-sperm head tracking algorithm based on MHI was evaluated and compared with the multiple-object tracking algorithm which uses sperm head shape and the global nearest neighbor (GNN) data association method. Both algorithms were applied to the same video clips (476 s in duration) in which more than 200 sperms were present. The evaluation criteria are average tracking errors (ATE), false positive (FP) rate, false negative (FN) rate, false identified trackers (FIT), and false identified objects (FIO), as shown in Fig. 5-4 defined in [207].

Table 8 - Performance Comparison Between MHI-Based Multi-Sperm Tracking and GNN-Based Multi-Sperm Tracking

<table>
<thead>
<tr>
<th>Algorithms</th>
<th>ATE (µm)</th>
<th>FP</th>
<th>FN</th>
<th>FIT</th>
<th>FIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHI</td>
<td>0.85</td>
<td>3</td>
<td>15</td>
<td>33</td>
<td>28</td>
</tr>
<tr>
<td>Shape &amp; GNN</td>
<td>1.23</td>
<td>7</td>
<td>21</td>
<td>54</td>
<td>48</td>
</tr>
</tbody>
</table>
As summarized in Table 8 & 9, the average tracking error of MHI-based algorithm (0.85 pixel) is less than that of shape-based detection algorithm (1.23 pixel). Shape-based multi-sperm tracking algorithm also has a higher FP rate than the MHI-based tracking algorithm. The FP cases in shape-based tracking were mainly caused by stationary foreign objects (contaminant particles) with a similar shape as the sperm head [see Fig. 5-4(a)]. Since these objects were largely stationary, they had little effect on MHI-based tracking. The FP cases in MHI-based tracking were caused by cell debris moving with the fluidic flow when sperms were swimming nearby [see Fig. 5-4(c)]. The experimental results demonstrate that the shape-based tracking algorithm is more susceptible to background noise and is not always effective in filtering debris/particles that have similar shapes as the sperm head. On the other hand, the MHI-based multi-sperm tracking approach is more robust to background noise and disturbances from foreign stationary particles. The MHI-based tracking algorithm also outperforms the algorithm using GNN data association in terms of FIT and FIO. The GNN data association method only considers sperm head’s position in the last frame. Thus, it is not effective in dealing with abrupt movement changes between two consecutive frames. In the MHI-based algorithm, the effect of abrupt changes in sperm movement between frames is mitigated by averaging sperm’s movement direction vector across a number of frames.

It needs to be noted, however, that the MHI-based sperm head tracking algorithm as a higher value of FN than the shape-based multi-sperm tracking algorithm. This is because the MHI-based algorithm cannot detect stationary sperms. In IVF, only motile sperms are of interest for analysis. Therefore, this drawback of MHI-based sperm tracking algorithm does not constrain its applicability in sperm selection. The FN cases in shaped-based and MHI-based algorithms are shown in Fig. 5-4(b) and (d), respectively.

<table>
<thead>
<tr>
<th>Video</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>V4</th>
<th>V5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (sec)</td>
<td>3.83</td>
<td>6.67</td>
<td>3.83</td>
<td>4.60</td>
<td>3.87</td>
<td>4.56</td>
</tr>
<tr>
<td>MIR (pixels)</td>
<td>1.16</td>
<td>2.73</td>
<td>1.83</td>
<td>2.02</td>
<td>1.98</td>
<td>1.95</td>
</tr>
<tr>
<td>With Kalman (pixels)</td>
<td>0.97</td>
<td>2.53</td>
<td>1.05</td>
<td>1.39</td>
<td>1.25</td>
<td>1.43</td>
</tr>
</tbody>
</table>
The sperm tail tracking algorithm was evaluated by measuring the Euclidean distance error between the detected tail point and the actual sperm tail position. The actual position was carefully identified/input by a user for each frame via computer mouse clicking. Table 9 summarizes the average Euclidean distance error for five additional videos. The overall average Euclidean distance error for the MIR algorithm integrated with Kalman filtering is 1.43 pixels (0.69 μm), while the error for the MIR algorithm without Kalman filtering is 1.95 pixels (0.95 μm). This result indicates that Kalman filtering is effective in improving the sperm tail tracking accuracies of the MIR algorithm. When the MIR algorithm failed to track the sperm tail due to the overlap with other sperms or occlusion by debris, Kalman filtering was able to estimate the sperm tail’s position based on the motion model.

![Figure 5-5 - Correlation between sperm velocity and tail beating amplitude.](image)

**Figure 5-5 - Correlation between sperm velocity and tail beating amplitude.** Pearson’s correlation coefficient $r = 0.7225$.

To investigate the correlation between the sperm head velocity and the tail beating amplitude, we chose 30 sperms showing good progressive movements (LIN > 0.9). The tracking algorithms described in Section 5.3 were used to measure their head velocity and the tail beating amplitude. We processed the measured data using a linear regression model. As shown in Fig. 5-5, there is a statistically significant correlation between the head velocity and sperm tail beating amplitude. Bivariate association between sperm velocity and sperm tail beating amplitude was evaluated by
Pearson’s correlation coefficient. Tracking data captured on the 30 sperms show that Pearson’s correlation coefficient was 0.7225 and the \( p \)-value was lower than 0.0001. This result quantitatively demonstrates that the sperm’s head velocity is proportional to its tail beating amplitude.

![Figure 5-6 - Sperms on a PICSI dish with HA microdots. (a) Sperms binding after 5 min. (b) Sperms binding after 30 min.](image)

In the HA binding experiment, the heads of the HA-bound sperms became stationary, and their tail movements became more vigorous. The process of sperm binding to the HA microdots was recorded at 30 frames/s. Fig. 5-6 (a) and (b) show sperms binding to the HA microdot at 5 and 30 min, respectively. If a moving sperm was detected to stop in the region of HA microdots, this sperm was considered successfully binding to HA. In contrast, if a moving sperm passed the HA microdot and disappeared out of the image boundary, it was considered an HA-unbound sperm. During the process of sperm binding to the HA microdots, the head velocity of the HA-bound sperms before binding was measured. The HA-bound sperms were compared to those unbound ones in terms of their head speed. As in the first experiment, only those sperms that exhibited linearly progressive movement were analyzed. The result as shown in Fig. 5-7 demonstrates that the HA-bound sperms tended to have a higher head velocity than those unbound sperms. The HA-bound sperms had an average head velocity of 76.28 \( \mu \text{m/s} \) with a standard deviation of 21.25 \( \mu \text{m/s} \), while the average head velocity of those unbound sperms was 50.45 \( \mu \text{m/s} \) with a standard deviation of 15.52 \( \mu \text{m/s} \).
Figure 5-7 - Velocity comparison between the HA-bound sperms and unbound sperms.

We also observed in the HA binding experiment that after the sperms bound to the HA microdots, their tails beat more vigorously. The tail beating amplitude was measured on the same sperms before and after they bound to the HA microdots. Fig. 5-8 shows sperm tail beating amplitude before and after binding to the HA microdots. The average amplitude produced by these 30 HA-bound sperms was 5.31 μm (before binding) and 6.93 μm (after binding). These results, for the first time, quantitatively reveal an increase in sperm tail beating amplitude before and after a sperm binds to an HA microdot. The results also quantify differences in sperm tail beating amplitude across HA-bound sperms. The measurement of sperm tail beating amplitude can possibly be used as an additional criterion for sperm selection among HA-bound sperms.
5.5 Conclusion

This chapter presented visual tracking algorithms for tracking both the head and tail of motile human sperms. The sperm head tracking algorithm is capable of tracking multiple moving sperms with a high success rate. Based on the sperm head’s position and its motion direction vector, the STROI is located. In this region of interest, the MIR algorithm together with Kalman filtering determines the sperm tail position. The sperm head and tail tracking algorithms enabled a number of new findings. A significant correlation between sperm head velocity and tail beating amplitude was found, suggesting that stronger tail propelling produces a higher velocity. The results also reveal that sperms bound to HA generally have a higher velocity (before binding) than those sperms that are not able to bind to HA microdots. This discovery “unifies” the conventional sperm assessment criterion based on sperm velocity/motility and the most recent HA assay technique. Among the sperms bound to HA microdots, their tails produce different beating amplitudes. Measuring such amplitude differences quantitatively can possibly be used as a new, useful sperm selection criterion among HA bound sperms.
Chapter 6
Conclusions and Future Research

6 Conclusions and Future Research

This chapter summarizes the major contributions of this research and future research directions.

6.1 Contributions

In summary, this research focused on the development of new robotic systems and automated methods for characterization and manipulation of single cells (e.g., cardiomyocyte, embryo and sperm). This research has addressed two practical problems for robotic micromanipulations. With the new technical solutions, two robotic prototype systems have been designed and built for high throughput measurement of GJIC and automatically processing embryos for vitrification. Additionally, quantitative analysis of sperm locomotion behaviors revealed interesting correlations between sperm head movement speed and the tail beating motions. The combination of sperm locomotion analysis with HA assay provides new solutions to assist doctors or embryologists to select high-quality sperms for IVF applications.

The major contributions of this research are summarized as follows:

1. Developed the first automated method for locating end-effector tips. This method solved the long-lasting practical problem of how to search for and locate end-effector tips for all micromanipulation tasks. A summary of guidelines was provided for implementation of this automated method.

2. Developed two modes of computer vision based contact detection algorithms for determination of relative Z-positions of end-effector tips and single cells. Without the use of extra sensors (e.g., force sensors or electrodes), these vision based methods were able to determine the relative Z positions between end-effector tips and cells. The newly developed methods were integrated into the first-of-its-kind robotic adherent cell injection system and the robotic vitrification system.

3. Developed an automated system for robotic microinjection of adherent cells to measure gap junctional intercellular communications. The powerful adherent cell injection system
was made possible by integrating several new techniques including automatically locating micropipette tips, robustly detecting the contact of micropipette tip with the cell membrane, and precisely compensating for accumulative positioning errors. The system has been used to quantitatively measure GJIC for screening efficacy of selected drug molecules.

4. Developed an automated system for robotic processing of mouse embryos for vitrification. Robotic vitrification relieves the human operator from tedious vitrification steps and eliminates manual operation-caused errors and inconsistencies. It also offers unparalleled timing control based on monitoring embryo volume changes in real time.

5. Developed computer vision tracking algorithms for automated analysis of sperm head and tail locomotion behavior. The analysis of both sperm head and tail locomotion behaviors revealed a significant correlation between sperm head velocity and tail beating amplitude, suggesting that stronger tail propelling produces a higher velocity. The results also showed that sperms bound to HA generally have a higher velocity (before binding) than those sperms that are not able to bind to HA microdots. These results provided new information to assist the selection of high-quality of human sperm in IVF clinics.

6.2 Future Research

6.2.1 Improvement of the Present Research

Despite the significant progress of the present research, there are still many opportunities remaining for further improvement of the robotic systems and automated techniques for characterization and manipulation of single cells. Several exemplary future research topics are:

- Fully automated robotic injection system is required to screen a high number of FDA-approved drug molecules. In order to achieve a fully automated system, a robust single cell detection method needs to be developed to recognize the optimal injection locations on individual cells for injection.

- Gap junctions facilitate both diffusion of small molecules and conduction of ions between adjacent cells. Gap junction conductance measurement can be performed by using a double whole-cell patch clamp system or optical mapping technique. Future research
should involve the development of robotic automation approaches to achieve high throughput measurement of electrical conduction.

- To further increase the throughput of the robotic vitrification system, new functions such as controlled aspiration of multiple embryos at one time are needed. Along this direction, future research should focus on microfluidic modeling and controller design to accurately position multiple embryos with equal distance inside a micropipette.

- In the present robotic vitrification system, the last step still needs a technician to manually pick up and plunge vitrification straws with vitrified embryos into liquid nitrogen for cryopreservation. To fully eliminate human involvement in the vitrification procedure, future research can focus on the development of a robotic pick-and-place manipulator (e.g., a 3-DOF robotic arm with a gripper) to automatically freeze vitrification straws in liquid nitrogen.

- At present, the newly-developed robotic vitrification system was tested by using mouse embryos. Future work should include more testing on mouse oocytes for oocyte vitrification.

- The current robotic vitrification system is designed to retrieve the embryos out of VS at their minimum volume to reduce the toxic effects of DMSO. Future testing should include the performance comparison between the individualized time control in the robotic system and a fixed VS washing protocol to experimentally validate the theory of minimizing toxic effects by real-time monitoring oocytes/embryo volume change.

- The automated sperm analysis method in this research is able to extract the sperm tail beating amplitude and use it to investigate the sperm locomotion behaviors. However, the sperm forward movement also depends on the sperm beating frequency. Further investigation should be conducted to measure the sperm tail-beating frequency by developing new computer vision algorithms with high-speed imaging techniques (e.g., over 100 fps).
6.2.2 Research Outlook on Intracellular Measurement and Manipulation

Beyond single cell characterization and manipulation, there are an increasing number of researchers starting to focus on the measurement of intracellular properties and manipulation of sub-cellular structures or organelles. Inside a living cell, numerous biological processes and biochemical reactions occur in the subcellular organelles, which are often compartmentalized and dynamically change intracellular physical and chemical properties, for instance, temperature [208], [209], pressure [210], mechanical [211], [212] and electrical characteristics [213], pH [214], and concentrations of ions and other molecules [215], [216]. These processes, such as the production of ATP by mitochondria or protein synthesis by ribosomes, require intracellular homeostasis to maintain normal cellular functions. Therefore, it is not surprising that each property is strictly regulated and varies among different intracellular structures and organelles. Tracking the regulation of these quantities could reveal largely underexplored subcellular functions and mechanisms. Moreover, an increasing body of evidence has indicated close correlations between intracellular disorders and diseases. Thus, monitoring intracellular environments and quantitatively measuring intracellular properties would enable us to better understand subcellular activities and disease mechanisms and potentially develop new therapies via rescuing/altering subcellular functions.

Directly measuring the properties of organelles and intracellular structures is difficult. Early researchers attempted to use glass micropipettes and microelectrodes to measure cytoplasmic pH [217], pressure [218] and electrical properties [219]. However, these direct measurements were mostly made on an entire cell due to spatial limitations of these technologies. Compared to the measurement of whole cells (typically tens of micrometers in size), the characterization of organelles and intracellular structures requires finer spatial positioning accuracy and much more miniaturized sensing tips. In addition, signals measured from intracellular structures are often weak and differ minutely inside a cell. For instance, subcellular temperature variations in different locations in a cell are only within a tenth of one degree [220]. To measure intracellular properties, microsystems and nano-engineered tools developed for this purpose must have high measurement sensitivities and resolutions.

The past few years have witnessed exploratory efforts in the development of new tools and techniques for direct intracellular measurement and manipulation. For example, because of the
small sizes and unique electrical properties, nanowires [221] and nanotubes [222][223] have been used to build tethered probes for intracellular electrical measurement. AFM tips, modified via FIB or via direct assembly/growth of nanowires or nanotubes, have been used to quantify intracellular mechanical characteristics [224] [225]. Untethered nanoparticles and MEMS sensors, after being introduced into cells using manipulation techniques, have been used to measure other physical properties (e.g., temperature, pressure) and chemical properties (e.g., pH, Ca2+ concentration) inside a cell. By combining untethered sensors with tethered probes (e.g., SiO2 nanowires), intracellular activities have been transformed into photonic signals and measured via optical single cell endoscopes [226].

Existing intracellular research started with manipulating and measuring large organelles, such as cell nuclei, and then moved onto targeting smaller organelles, such as mitochondria. Many intracellular properties (e.g., pH and temperature) in existing studies have been measured in the cytoplasm. Future micro- and nanoengineered tools will become even finer in size and more powerful in function to monitor real-time changes of suborganelle signals, such as pH and temperature changes during ATP synthesis in mitochondria and calcium storage variations in the nuclear membrane, reticulum, and Golgi apparatus [227], [228].

New materials, such as graphene, may possibly help in the development of more accurate and sensitive measurement tools [229]. Graphene field effect transistors have been developed to monitor action potentials of cardiomyocytes extracellularly [230], [231]. Graphene-based sensors might be developed and delivered into single cells for intracellular electrical measurements. In addition to new materials, emerging imaging techniques might also significantly accelerate the advancement of intracellular measurement and manipulation capabilities. Studies using near-field imaging enabled by optical nanowires have demonstrated the ability to accurately detect fluorescent signals with higher resolutions [226]. These new imaging capabilities might enhance the observation and measurement of subcellular and suborganelle signal changes.

Presently, intracellular measurement and manipulation are manually conducted. Automation technologies can be developed to help minimize human errors and skill inconsistency [232]. Automation would allow researchers to more easily position tethered devices or more accurately move untethered sensors inside a cell. In manual microinjection, for example, the number of injected cells is limited to several or tens of cells[233]. To increase throughput, robotic
microinjection systems demonstrated in this research have shown the injection of over 1,000 cells within one hour [234]. The significantly higher throughput enabled quantitative characterization of gap junction function on a large cell population. To improve the performance of magnetic or optical tweezers, automated functions are under development to increase the spatial resolution and accuracy for the manipulation of single cells [235], [236]. These technologies have direct relevance and might be expanded to enhance intracellular manipulation and measurement.

Compared to measurements in single cells, the direct measurement and manipulation of subcellular structures and organelles remain largely underexplored. In the pursuit of better understanding of intracellular properties, the development of new microsystems and nanoengineered techniques would transform cell biology by enabling intracellular measurement and manipulation. These new tools would enable researchers to directly interrogate intracellular structures, explore the environment inside a cell, and observe and measure intracellular processes and activities with high spatial and temporal resolutions. The exciting era of intracellular measurement and manipulation has just begun.
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