Automation of Single Cell Manipulation for Embryo Biopsy

by

Christopher Yee Wong

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

© Copyright 2017 by Christopher Yee Wong
Assisted reproductive technologies (ART) are a host of technologies and procedures related to fertility and reproduction. Preimplantation genetic diagnosis (PGD), a procedure within ART, is a form of embryo biopsy in which a single cell, called a blastomere, is non-destructively extracted from the embryo for genetic analysis. PGD procedures are currently performed manually with low success rates, potentially caused by operator performance variability and contamination through handling, and are financially, physically, and psychologically taxing for the patient. The automation of PGD can increase the procedure repeatability and hence improve overall success rates. This thesis presents novel methods for the automation of certain PGD embryo biopsy procedures using robotic micromanipulators, computer vision and automation algorithms. This dissertation is separated into three main chapters, each pertaining to the automation of the three main phases of PGD biopsy:

The first step in PGD biopsy automation is automated embryo position and orientation control using independently controlled parallel plates. Computer vision algorithms are used for tracking the embryo position and orientation. A novel 3D mapping technique for use with Hoffman modulation contrast microscopy is described and used for ablation zone optimization. Open and closed loop position and orientation control is demonstrated using the parallel plates.

The second step of PGD biopsy automation performs a novel two-stage optimization for laser zona drilling to reduce embryo heating. The first stage uses computer vision algorithms to identify embryonic structures and determines the optimal ablation zone
farthest away from critical structures such as blastomeres. The second stage combines a genetic optimization algorithm with a thermal model of LZD to optimize the combination of laser pulse locations and pulse durations.

The third step of PGD biopsy automation involves blastomere extraction using the displacement method. A process flow for blastomere extraction automation is developed, and implemented using algorithms for non-vision-based feedback micropipette position control, and computer vision algorithms for blastomere extraction event detection and blastomere tracking during retrieval.

For each PGD biopsy step, automation experiments were performed on mouse embryos. Successful demonstration of the above methods proves the feasibility of biopsy automation, and presents a step towards fully automated ART.
This thesis is dedicated to the order of graduate students against the abolition of free food.

Acknowledgements

First, I would like to express my sincerest gratitude to my advisor Professor James K. Mills for his guidance, insight, direction and encouragement throughout my doctoral studies. If it were not for him, my journey would have been much harder and misguided. Furthermore, I would like to thank Professor Pierre Sullivan and Professor Goldie Nejat for devoting their time to reviewing my research and providing insightful comments and criticism. This thesis would not be possible without the support of my labmates Steven Kinio, Ihab Abu Ajamieh, Gilbert Jiang, Adam Le, and Masih Mahmoodi. A special mention goes to Steven for letting me bounce ideas off of him, getting on my nerves, going for all those Tim Hortons runs, and letting me drag you around in the quest for free food. Furthermore, thanks to Emily Monroy from the University Health Network Animal Research Centre, and Jodi Garner and Dr. Bin Gu from the Rossant Laboratory for providing me with much needed help on the biology side this thesis, and for providing me with embryos for my experiments. I must thank my parents for enduring my absence from home for so many years but still giving me warm welcomes whenever I go back, and my brother for being someone I look up to who inspired me to do a PhD and whom I turn to for advice. And finally, I must thank my friends for being supportive and giving me encouragement throughout this endeavor; especially my teammates on New Dragons Racing Club for becoming my family away from home; Dr. Paraish Misra for the long talks about life, academia, and the wonders of science; my good friend Caffeine for helping me stay awake both when I needed to and when I tried to sleep; Karen Lai for all those weekends spent keeping me company and accountable while I wrote; and, last but not least, Wendi Zhou for making sure I stayed on track even when things got tough and that I was well fed whenever I worked extra long hours. This thesis was funded in part by the Natural Sciences and Engineering Research Council of Canada, and Fonds de recherche du Québec - Nature et technologies.


Table of Contents

Abstract ii
Acknowledgements iv
Table of Contents v
List of Tables viii
List of Figures ix
Nomenclature xiv

1 Introduction 1
  1.1 Research Objectives and Methodology 3
  1.2 Overview of Biopsy Techniques 4
    1.2.1 Cell Localization, Immobilization and Patterning 4
    1.2.2 Embryo Orientation Control 8
    1.2.3 Zona Breaching 10
    1.2.4 Blastomere Extraction 14
    1.2.5 Computer Vision in Cell Micromanipulation 16
  1.3 Research Significance and Contributions 20
  1.4 Outline of Dissertation 22

2 Embryo Position and Rotation Control 24
  2.1 Rotation Method 25
    2.1.1 Fluid Rotation 26
    2.1.2 Parallel Plate 27
  2.2 Experimental Setup 30
  2.3 Rotation Tracking 31
  2.4 3D Mapping and Zona Breaching Optimization 35
2.4.1 3D Mapping ............................................. 35
2.4.2 Ablation Zone Optimization ................................. 39
2.4.3 Orientation Control Strategy ................................. 40
2.5 Parallel Plate Manipulation Experiments ....................... 42
   2.5.1 Open Loop Control ..................................... 42
   2.5.2 Closed Loop Control .................................... 43
2.6 Chapter Conclusion ........................................... 46

3 Optimization and Automation of Laser Zona Drilling .......... 48
   3.1 Thermal Modeling ........................................... 50
      3.1.1 Laser Ablation Simulation ............................. 50
      3.1.2 Temperature Measurement Experiments .................. 54
      3.1.3 Effect of LZD parameters ............................... 56
   3.2 Selection of Optimal Ablation Zone ......................... 57
      3.2.1 Blastomere Segmentation ............................... 58
      3.2.2 Zona Pellucida Segmentation ........................... 59
      3.2.3 Ablation Zone Optimization ............................. 61
   3.3 Coverage Optimization ...................................... 62
   3.4 Automated Zona Ablation Experiments ....................... 65
   3.5 Chapter Conclusion .......................................... 68

4 Automated Blastomere Extraction ............................... 70
   4.1 Experimental Setup ......................................... 71
   4.2 Extraction Procedure Overview .............................. 72
   4.3 Displacement Pressure Modeling ............................ 76
   4.4 Computer Vision Algorithms ................................. 78
      4.4.1 Micropipette Calibration and Control .................. 79
      4.4.2 Blastomere Extraction Event Detection ................. 84
      4.4.3 Blastomere Retrieval .................................. 85
   4.5 Automated Extraction Experiments ......................... 87
   4.6 Chapter Conclusion .......................................... 89

5 Concluding Remarks ........................................... 91
   5.1 Summary and Contributions ................................ 91
   5.2 Future Research Directions ................................ 95
      5.2.1 Expansion of Orientation Tracking and 3D Reconstruction ... 95
      5.2.2 Embryo Survival Rate Validation ....................... 95
5.2.3 Error Diagnostics and Correction .......................... 96
5.2.4 Expansion to Turnkey System ............................... 96

Bibliography ............................... 98
List of Tables

3.1 Maximum sensor point temperature in °C ................................................. 57
3.2 Genetic optimization parameters .......................................................... 66

4.1 Automated extraction experiment results ................................................. 88
4.2 Post-tuning subset of automated extraction experiment results .............. 88
List of Figures

1.1 Embryo biopsy: (a) mouse embryo early developmental stages [1], (b) 4-cell cleavage stage embryo, (c) PGD biopsy overview 2
1.2 Embryo immobilization techniques using light vacuum: (a) traditional holding pipette, and (b) patterning device using through holes and light vacuum [2] 5
1.3 Magnetically-driven microtools (MMT) developed by Hagiwara et al. [3]: (a) MMT design, (b) Enucleation process using MMTs 6
1.4 Working principle of: (a) optical tweezers [4], (b) optoelectronic tweezers (OETs) [5], (c) standing surface acoustic waves for object manipulation [6] 7
1.5 Orientation control of embryos using (a) fluid flow from offset micropipette to generate a shear force [7], (b) custom built rotating stage [2], (c) rolling embryo inside a microfluidic device [8], (d) 3D DEP using carbon-embedded conductive C-PDMS [9] 9
1.6 Zona breaching using (a) mechanical microneedle [10], (b) laser zona drilling viewed under scanning electron microscopy [11] 11
1.7 Comparisons of diameters of oocytes in (A) absence of sucrose, (B) presence of sucrose at 0.5 M and (C) 0.25 M. Scaling bar = 20 µm. Arrows indicate drilled holes. Concentric circles indicate the heat map of the focused laser beam [12] 13
1.8 Aspiration-based methods for blastomere extraction 14
1.9 Other extraction methods: (a) blastomere extraction using displacement method [13] and (b) polar body extraction using optical tweezers [14] 15
1.10 Computer vision algorithms for: (a) micropipette tracking using template matching [15], (b) autofocusing [16], (c) detection of blastomere extraction using the aspiration method [17] 17
1.11 Use of computer vision for embryo segmentation: (a) 3D segmentation of a 4-cell embryo [18], (b) selection of zona position for zona breaching [19] 20
2.1 Schematics for (a) fluid rotation [7] and (b) mechanical traps [20] . . . . 26
2.2 Trap designs: (a) cylindrical posts, (b) rectangular vanes, (c) large wells.
   Posts and vanes are pressed down to show 3D aspect of traps. . . . . . . 26
2.3 Embryo rotation using parallel plates: (a) setup schematic, (b) position
   coordinate system, (c) orientation coordinate system . . . . . . . . . . . 28
2.4 Deformation analysis: (a) deformation model for embryos, (b) effect of
deformation on embryo circumference for initial embryo height $h_0 = 100\mu m$ 29
2.5 Experimental setup: (a) schematic of setup and (b) fabrication of the
   upper and lower plates . . . . . . . . . . . . . . . . . . . . . . . . . . . . 30
2.6 Time lapse of a rotating 2-cell embryo. Segmented embryo and blastomeres
   are outlined in white. Embryo center is shown with a white triangle,
   whereas the blastomere center is marked with a yellow circle. . . . . . . 32
2.7 Geometrical modeling of 2-cell embryo using a capsule model; (a) definition
   of the coordinate system and parameters for rotation tracking, (b) overlay
   of the capsule model projection on a microscope image . . . . . . . . . . 33
2.8 Validation of geometric model approach using a CAD model of a 2-cell
   embryo. Angles listed are set values vs geometric model-derived values.
   Embryo center is shown with a white triangle, whereas the blastomere
   center is marked with a yellow circle. . . . . . . . . . . . . . . . . . . . . 33
2.9 Plot of embryo orientation during tracking experiment. Letters mark the
   subfigures in Figure 2.6. . . . . . . . . . . . . . . . . . . . . . . . . . . . . 34
2.10 Superimposed time-lapse images of embryo rotating while rolling . . . . 36
2.11 Overview of the subtractive method for 3D mapping . . . . . . . . . . . 37
2.12 (a,b) Computer generated 3D map of a 4-cell embryo in (a) SolidWorks
   and (b) reconstructed blastomeres using 3D mapping algorithm; (c,d) Re-
   constructed 3D map of blastomeres from Figure 2.10 with (c) blastomeres
   only, (d) zona partially removed to reveal blastomeres for demonstration
   purposes . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 38
2.13 Optimal ablation zone for (a) 4-cell embryo CAD model, and (b) 2-cell
   embryo from Figure 2.10. Blue indicates the zona, red indicates the blas-
   tomeres, and the green indicates the optimal ablation zone. . . . . . . . . 40
2.14 Blastomere extraction step with extraction micropipette on the right side 41
2.15 Overview of the orientation control strategy . . . . . . . . . . . . . . . 41
2.16 Open loop control using (a) translation only and (b) rotation only motion 43
2.17 Block diagram of control for closed loop control . . . . . . . . . . . . . 44
2.18 Time lapse of a closed loop embryo position control experiment. “o” indicates start point, “x” indicates end point.

2.19 Closed loop embryo rotation control experiment: (a) Time-lapse of one experiment, green line depicts eccentricity using an arbitrary scale, red cross indicates $u^{upper}$; (b) Blastomere eccentricity over the course of the experiment.

3.1 (a) Zona breaching terminology definition, (b) embryo with opening after undergoing LZD.

3.2 Simulation setup in COMSOL Multiphysics. Block represents water, cylinder represents beam waist, points represent sensor points, beam convergence/divergence shown using discretized disks: (a) single pulse modeling, (b) multi-pulse modeling (beam convergence/divergence not shown), (c) close up of multi-pulse laser ablation pattern and sensor points.

3.3 Temperature response at the beam center from a 600 $\mu$s laser pulse.

3.4 Plot depicting the maximum temperature increase above ambient given the distance from laser center for different laser pulse durations simulated in COMSOL Multiphysics (shown up to distance of 25 $\mu$m).

3.5 Experimental setup for (a) laser zona drilling experiments with embryos, (b) custom resistive heater with fixed channel height for RhB calibration.

3.6 a) A time lapse series of 10 images taken at 2015 fps of a 600 $\mu$s laser pulse, b) Comparison of experimental and height-integrated simulation temperature response at beam center, c) Vertical temperature distribution.

3.7 Temperature profile of the zona ablation zone using uniformly spaced LPL grid with fixed pulse durations. Asterisk marks denote temperature sensor points.

3.8 Blastomere segmentation: (a) original image, (b) standard deviation transformation map with values capped at threshold for demonstration purposes, (c) thresholded mask containing only largest 8-connected object, (d) segmented blastomeres, with estimated embryo center calculated using the centroid of the blastomere mask.
3.9 Zona segmentation: (a) annulus indicating predicted zona location, (b) segmented zona and blastomeres, (c) annulus section converted to polar coordinates, (d) outer zona segmentation: energy map in polar coordinates with dilated blastomere mask and lowest cost path, (e) inner zona segmentation: Canny edge map in polar coordinates with blastomere and outer zona removed .................................................. 60

3.10 Final OAZ after optimization and the corresponding blastomere outline. Location of OAZ may change depending on OAZ width ............... 62

3.11 Effect of different laser pulse durations on laser pulse location layout when calculating the constraints on the minimum and maximum number of laser pulses during optimization ............................................. 64

3.12 Automated optimized LZD experiment: (a) 4-cell embryo, red outline represents magnified area in subsequent subfigures, (b) evolution of the population fitness value during coverage optimization, (c) estimated coverage pattern after optimization, (d) optimized laser pulse locations and corresponding boundaries for inner zona and blastomeres, (e) opening created by automated LZD, (f) comparison of predicted and actual zona ablation 67

4.1 Embryo biopsy extraction techniques: (a) 4-cell cleavage-stage embryo with an opening in the zona created by zona breaching, (b) aspiration method, (c) displacement method, (d) optical tweezer method for polar body biopsy .......................................................... 71

4.2 Automated blastomere extraction experimental setup overview ........ 72

4.3 Blastomere extraction procedure overview ............................... 73

4.4 Detailed flowchart of blastomere extraction automation with associated computer vision algorithms shown in pink rectangles .................. 74

4.5 Schematic of extraction system for pressure modeling. Fluid in blue, air in red. ................................................................. 77

4.6 (a) Calculated static system pressure during a 30 s displacement phase and infusion rate, (b) Simulated system response time to changes in pressure and relief valve ......................................................... 79

4.7 Coordinate transformation for camera frame to micromanipulator frame . 79
4.8 Micropipette segmentation: (a) original image of holding micropipette, (b) Canny edge detection, (c) closing image $I_{BL}$ using pseudo edges, (d) outline of the micropipette with side walls split into $s_L$, $s_R$ and tip, (e) holding micropipette with orientation and tip location, (f) micropipette moved to $p_2$ during the calibration, (g,h) segmentation of (g) zona drilling and (h) blastomere biopsy micropipettes.

4.9 Micropipette calibration demonstration mimicking the micropipette motion for automated blastomere extraction. White scale bar represents 100 $\mu$m.

4.10 Blastomere extraction event detection: (a) location of ROI, (b) time lapse of ROI for event detection, (c) plot of mean intensities in ROI during extraction process.

4.11 Blastomere tracking during retrieval phase: (a) pre-extraction image without micropipette in view with retrieval ROI outlined in red, (b) post-extraction example with embryo and micropipette mask outlines in white and ROI outline in red, (c) ROI post-subtraction, (d) blastomere segmentation, with $(x_b, y_b)$ marked by a red cross, after intensity threshold and removing micropipette and embryo.

4.12 Time lapse of automated blastomere extraction experiment: (a-b) Phase I Preparation, including motion planning and insertion of micropipette, (c-e) Phase II Blastomere Displacement, where the blastomere is extracted via displacement, (f-i) Phase III Blastomere Retrieval, where the blastomere is tracked and successfully retrieved by the micropipette.

4.13 Example of retrieval failure through micropipette blockage.
# Nomenclature

## Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART</td>
<td>Assisted reproductive technologies</td>
</tr>
<tr>
<td>AT</td>
<td>Acid Tyrode’s</td>
</tr>
<tr>
<td>AZ</td>
<td>Ablation zone</td>
</tr>
<tr>
<td>DEP</td>
<td>Dielectrophoresis</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DOF</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>ESHRE</td>
<td>European Society of Human Reproduction</td>
</tr>
<tr>
<td>FGD</td>
<td>Prefertilization genetic diagnosis</td>
</tr>
<tr>
<td>fps</td>
<td>Frames per second</td>
</tr>
<tr>
<td>HMC</td>
<td>Hoffman modulation contrast</td>
</tr>
<tr>
<td>HOT</td>
<td>Holographic optical tweezer</td>
</tr>
<tr>
<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilization</td>
</tr>
<tr>
<td>LPL</td>
<td>Laser pulse location</td>
</tr>
<tr>
<td>LZD</td>
<td>Laser zona drilling</td>
</tr>
<tr>
<td>MEMS</td>
<td>Microelectromechanical system</td>
</tr>
</tbody>
</table>
MMT Magnetically-driven microtool
OAZ Optimal ablation zone
OET Optoelectronic tweezers
OT Optical tweezer
PDMS Polydimethylsiloxane
PGD Preimplantation genetic diagnosis
PVS Perivitelline space
RhB Rhodamine B
SAW Standing acoustic waves
ZB Zona breaching
ZP Zona pellucida

**Position and Orientation Control**

$\alpha$\_\text{cur}$ \quad $Current blastomere shape eccentricity
$\alpha$\_\text{des}$ \quad $Desired blastomere shape eccentricity
$\lambda$\_\alpha$ \quad $Embryo rotational rolling slippage coefficient

$\alpha$\_\text{E}$ \quad $Blastomere eccentricity major axis angle
$\alpha$\_\text{E}$ \quad $Embryo orientation
$\Delta t$ \quad $Time step
$\dot{\alpha}$ \quad $Embryo rotational velocity
$\dot{p}$ \quad $Embryo Cartesian velocity
$\dot{u}_\alpha$ \quad $Parallel plate resultant rotational velocity input to embryo
$\dot{u}_p$ \quad $Parallel plate resultant translational velocity input to embryo
$\hat{i}$ \quad $Unit vector for x-axis
$\hat{j}$ \quad $Unit vector for y-axis

$x_v$
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_p$</td>
<td>Embryo Cartesian rolling slippage coefficient</td>
</tr>
<tr>
<td>$\phi$</td>
<td>Embryo orientation (latitude)</td>
</tr>
<tr>
<td>$\phi_0$</td>
<td>Initial embryo orientation (latitude)</td>
</tr>
<tr>
<td>$\phi_1$</td>
<td>Intermediate embryo latitude during reorientation</td>
</tr>
<tr>
<td>$\psi$</td>
<td>Embryo orientation (tilt)</td>
</tr>
<tr>
<td>$p$</td>
<td>Embryo Cartesian position</td>
</tr>
<tr>
<td>$p_{1\text{rev}}$</td>
<td>Translational displacement of embryo for a single revolution</td>
</tr>
<tr>
<td>$P_{MB}$</td>
<td>Position of motion boundary</td>
</tr>
<tr>
<td>$p_{\text{cur}}$</td>
<td>Current position</td>
</tr>
<tr>
<td>$p_{des}$</td>
<td>Desired position</td>
</tr>
<tr>
<td>$q$</td>
<td>Embryo pose</td>
</tr>
<tr>
<td>$u, u^\text{lower}, u^\text{upper}$</td>
<td>Parallel plate position, split into lower and upper plates</td>
</tr>
<tr>
<td>$u_\alpha$</td>
<td>Parallel plate resultant rotation input to embryo</td>
</tr>
<tr>
<td>$u_p$</td>
<td>Parallel plate resultant Cartesian translation input to embryo</td>
</tr>
<tr>
<td>$\theta$</td>
<td>Embryo orientation (longitude)</td>
</tr>
<tr>
<td>$\theta_0$</td>
<td>Initial embryo orientation (longitude)</td>
</tr>
<tr>
<td>$A_0$</td>
<td>Cross-sectional area of embryo</td>
</tr>
<tr>
<td>$AZ_c$</td>
<td>Set of points representing the ablation zone candidate</td>
</tr>
<tr>
<td>$B$</td>
<td>Set of blastomere points</td>
</tr>
<tr>
<td>$C$</td>
<td>Embryo circumference</td>
</tr>
<tr>
<td>$d$</td>
<td>Distance between ablation zone candidate $AZ_c$ and blastomeres $B$</td>
</tr>
<tr>
<td>$d_{MB}$</td>
<td>Distance between embryo and motion boundary</td>
</tr>
<tr>
<td>$e_e$</td>
<td>Blastomere eccentricity error</td>
</tr>
<tr>
<td>$e_i$</td>
<td>Integral position error</td>
</tr>
</tbody>
</table>
\( e_p \) \hspace{1cm} \text{Proportional position error}

\( e_{p,\text{mean}} \) \hspace{1cm} \text{Mean proportional position error}

\( h \) \hspace{1cm} \text{Compressed height of embryo}

\( h_0 \) \hspace{1cm} \text{Initial height of embryo}

\( K_{\epsilon} \) \hspace{1cm} \text{Blastomere eccentricity error constant}

\( K_i \) \hspace{1cm} \text{Integral error constant}

\( K_p \) \hspace{1cm} \text{Proportional error constant}

\( L \) \hspace{1cm} \text{Blastomere capsule model major axis length}

\( L_C \) \hspace{1cm} \text{Embryo middle section length during compression modeling}

\( L_{xy} \) \hspace{1cm} \text{2D Projection of blastomere capsule model major axis length}

\( m \) \hspace{1cm} \text{Blastomere capsule model minor axis length}

\( m_{xy} \) \hspace{1cm} \text{2D Projection of blastomere capsule model minor axis length}

\( P_{\{\theta,\phi}\} \) \hspace{1cm} \text{2D projection of embryo image taken at angles \( \theta \) and \( \phi \)}

\( r \) \hspace{1cm} \text{Distance between embryo centroid and blastomere centroid}

\( r_{xy} \) \hspace{1cm} \text{Projection of distance between embryo centroid and blastomere centroid}

\( u_{x, y}^{\text{lower}} \) \hspace{1cm} \text{Lower parallel plate Cartesian coordinates}

\( u_{x, y}^{\text{upper}} \) \hspace{1cm} \text{Upper parallel plate Cartesian coordinates}

\( V \) \hspace{1cm} \text{Volume used for 3D mapping of embryo}

\( V_{\{\theta,\phi\}} \) \hspace{1cm} \text{Rotated and extruded projection} \ P_{\{\theta,\phi\}}

\( x, y \) \hspace{1cm} \text{Embryo Cartesian coordinates}

\( ZP_{\text{inner}} \) \hspace{1cm} \text{Set of points for zona pellucida inner boundary}

\( ZP_{\text{outer}} \) \hspace{1cm} \text{Set of points for zona pellucida outer boundary}

\( \text{mod}() \) \hspace{1cm} \text{Modulus function}
Zona Breaching

\( \alpha \)  Laser beam absorptance

\( \delta_i \)  Time delay between \( i \)th laser pulse

\( \gamma \)  Apparent lighting in Hoffman modulation contrast images

\( \overline{g}(T) \)  Normalized \( g(T) \) by channel height

\( \sigma \)  Scaling parameter in calculation of image energy map \( E \)

\( \vec{q} \)  Heat flux

\( a, b \)  Weighting functions for laser zona drilling coverage optimization

\( B_0 \)  Subset of blastomere points \( B \) directly facing ablation zone candidate \( AZ_c \)

\( d_{emb} \)  Embryo diameter

\( dur_i \)  Pulse duration of \( i \)th laser pulse

\( E \)  Energy map of image

\( G(I) \)  Calibration of Rhodamine B fluorescence intensity to temperature

\( g(T) \)  Inverse function of \( G(I) \)

\( G_r \)  Gradient operator in radial direction

\( h \)  Laser beam waist cylinder height

\( I \)  Fluorescence intensity value

\( I_{polar} \)  Image after polar coordinate transformation

\( I_{sim,int} \)  Fluorescence intensity value from simulation, integrated over height

\( I_{std} \)  Image of standard deviation values

\( J \)  Objective function for coverage optimization

\( k \)  Coefficient of thermal conductivity

\( k_{att} \)  Laser beam attenuation factor
\( loc_i \)  
Location of \( i \)th laser pulse

\( n \)  
Number of laser pulses

\( P \)  
Laser beam power

\( p \)  
Laser beam pulse duration

\( P_{\text{edge}} \)  
Percentage of ablation zone edge ablated

\( P_{\text{thresh}} \)  
Threshold of minimum \( P_{ZP} \) value

\( P_{ZP} \)  
Percentage of ablation zone ablated

\( r_{\text{beam}} \)  
Laser beam waist cylinder radius

\( r_{\text{inner}} \)  
Radius of inner zona boundary annulus

\( r_{\text{mutation}} \)  
Mutation rate for genetic algorithm

\( r_{\text{outer}} \)  
Radius of outer zona boundary annulus

\( S_0 \)  
Laser beam waist heat source

\( S_{\pm} \)  
Laser beam converging/diverging heat source

\( T \)  
Temperature

\( T_0 \)  
Initial temperature

\( T_{\text{boundary}} \)  
Boundary temperature

\( T_{\text{int}} \)  
Temperature value, integrated over height

\( T_{\text{max}} \)  
Maximum temperature increase of blastomeres

\( T_{p,i} \)  
Temperature response of \( i \)th laser pulse with \( p \) pulse duration

\( T_{\text{sim}} \)  
Simulated temperature response

\( T_{\text{tot}} \)  
Total temperature response

\( w \)  
Width of ablation zone

\( z_{\text{beam}} \)  
Laser beam \( z \) position

\( n \)  
Index of refraction
Blastomere Extraction

$\alpha, \alpha_i$  
Micropipette orientation (global and local)

$\alpha_{init}$  
Initial guess of micropipette orientation

$\alpha_{threshold}$  
Threshold for micropipette orientation

$\beta_i$  
Angle between micropipette minor axis and point along micropipette side $s_{ordered}(i)$

$\delta_e$  
Number of points used in estimation of micropipette orientation $\alpha_{init}$

$\delta_s$  
Number of points used in calculation of local micropipette orientation $\alpha_i$

$\hat{J}_m$  
Micropipette $y$-axis unit vector

$\vec{r}_i$  
Vector between micropipette centroid $C$ and point along $s_{ordered}(i)$

$C$  
Centroid of micropipette

$c_L, c_R$  
Centroid of micropipette left and right sidewalls

$d_1, d_2$  
Distance parameters for micropipette insertion

$d_x, d_y$  
Distance thresholds used during blastomere retrieval

$I_{UL}, I_{UR}, I_{BL}, I_{BR}$  
Images with respective edges filled in

$n$  
Number of moles

$P$  
System pressure

$p$  
Micropipette tip location

$p^c, p^m$  
Micropipette tip location in camera or micromanipulator coordinate frame

$R$  
Ideal gas constant

$R^m_c$  
Rotation matrix from camera coordinate frame to micromanipulator coordinate frame
$s_{cm}^n$ Scaling factor for camera coordinate frame to micromanipulator coordinate frame

$s_{L}, s_{R}$ Ordered set of points for left and right sidewalls of micropipette

$s_{ordered}$ Ordered set of points for micropipette sidewall

$T$ Coordinate transformation between from micromanipulator frame to camera frame

$t$ Elapsed time

$V$ System volume

$V_{holder}$ Volume of micropipette holder in extraction system

$V_{micropipette}$ Volume of micropipette without liquid in extraction system

$V_{syringe}$ Volume of syringe in extraction system

$V_{tubing}$ Volume of tubing in extraction system

$x_b, y_b$ $xy$-coordinates of extracted blastomere

$x_p, y_p$ $xy$-coordinates of micropipette tip
Chapter 1

Introduction

The miniaturization of technology has enabled countless advances in science, especially in the field of biology. Specifically, manipulation at the microscale introduced the ability to interact with individual biological cells and even intracellular components. From a technological perspective, a subset of the field of micromanipulation deals specifically with the manipulation of single cells, which has seen many advancements in recent years, both in the development of novel methods and the automation of single cell manipulation [21–23]. One such field that has benefited from these advances is the field of assisted reproductive technologies (ART). ART is a host of technologies and procedures related to fertility and reproduction [23–25]. A certain class of ART procedures focuses on extrauterine manipulation of an embryo at a very early development stage, commonly known as \textit{in vitro} fertilization (IVF). Certain IVF procedures include intracytoplasmic sperm injection (ICSI) [26], assisted hatching [27,28], and preimplantation genetic diagnosis (PGD) [29–31].

In \textit{in vitro} fertilization, an oocyte, the term for an unfertilized egg, is inseminated using ICSI, where a sperm is injected directly into the oocyte using a small sharp needle. Once fertilized, embryo development starts at the single cell stage called a zygote, and is kept in an incubator that mimics physiological conditions for continued development outside the human body. The early developmental cycles of a mouse embryo are shown in Figure 1.1(a). The embryo enters the cleavage stage once it starts to divide into the 2-, 4- and 8-cells stages. The structure of a cleavage-stage embryo can be seen in Figure 1.1(b). Once the embryo reaches the 8-cell stage, the blastomeres compact and the embryo develops into a morula, and then into a blastocyst, at which point the embryo is transferred back into the uterus. The developmental cycles shown in Figure 1.1(a) occur in a timely manner, where each stage corresponds to approximately 1 day.

Preimplantation genetic diagnosis, an additional procedure in \textit{in vitro} fertilization,
extracts 1-2 blastomeres from a cleavage-stage embryo to permit chromosomal and genetic analysis. This chromosomal and genetic analysis can be used for multitude of purposes [30, 32], ranging from social sexing, to the detection of genetic diseases, e.g.: retinoblastoma, cystic fibrosis and sickle-cell disease. A compiled list of disorders can be found in a 10 year study of recorded PGD operations by the European Society of Human Reproduction and Embryology (ESHRE) [32]. Thus, only embryos that are free of genetic disorders, or are of the right sex, are implanted into the uterus. The use of PGD has been shown to improve implantation rates compared to standard IVF [29], improving clinical pregnancy rates and decreasing odds of miscarriage [33]. The caveat is the need to perform the biopsy non-destructively such that the embryo remains viable, as an embryo would be used for implantation only if deemed healthy. The embryo biopsy process can also be used for other purposes. For example, extracted blastomeres can also be used to generate human embryonic stem cells [34].

PGD is performed *in vitro*, where the embryo is located outside the human body in a petri dish. There are two elements to PGD: embryo biopsy and genetic analysis. Embryo biopsy involves physically creating an entry point into the embryo and extracting a blastomere, whereas genetic analysis, as the name implies, involves using techniques such as fluorescence *in situ* hybridization or polymerase chain reaction to analyze the extracted blastomere’s genome or chromosomes. In further detail, there are three main
Chapter 1. Introduction

steps to the PGD biopsy procedure [35], outlined in Figure 1.1(c): 1) Embryo position and orientation control, where the embryo is manipulated and rotated until a blastomere has been chosen for extraction and positioned correctly for subsequent steps; 2) Zona breaching, where a section of the zona pellucida (ZP), the outer shell of the embryo, is perforated in such a way that creates an opening for tools to enter the embryo and/or blastomeres to exit the embryo, and does not cause irreversible damage to the embryo; 3) Blastomere extraction, where 1 or 2 blastomeres are extracted from the embryo for genetic analysis.

A review of 10 years of PGD data in 2011 revealed that the clinical pregnancy rate after PGD was only approximately 27% [32]. Currently, embryo biopsy is performed manually by highly trained embryologists. While there are a multitude of reasons for low pregnancy rate beyond the biopsy procedure itself, automation of the embryo biopsy procedures could increase the clinical pregnancy rate. Automation would eliminate the subjective bias and error of human operators, the potential for chemical or biological contamination, and even the need for highly trained embryologists, which are all possible failure modes for an ART procedure. The introduction of automation would allow for higher throughput, decreasing the cost of the procedures and increase accessibility to these technologies for society. Automation of the biopsy task, by removing the human operator and instead allowing computers to control the micromanipulators with the aid of sensory feedback such as computer vision, would allow for more efficient, effective and improved embryo biopsy.

1.1 Research Objectives and Methodology

The overarching research objective of this thesis is the automation of certain embryo biopsy procedures through demonstration that these procedures are feasible without human intervention. These biopsy procedures include adapting established methods used in micromanipulation for automation, or by developing novel techniques. Specifically, these are the embryo biopsy procedures used in, but not limited to, preimplantation genetic diagnosis, outlined in Figure 1.1(c). Thus, the research presented in this thesis aims to:

1. Automate embryo position and orientation control

2. Automate zona breaching

3. Automate blastomere extraction
The objective is to allow complete computer control when performing each procedure. Additionally, the introduction of automation to these procedures allows for optimization of the procedure parameters with the goal of making the procedure more efficient, effective and safe. Automation also allows novel methods and procedures, which would otherwise not be possible to perform manually, to be developed. This research aims to further the nascent field of PGD automation by developing novel computer vision algorithms for task planning and cell tracking, reorientation of the embryo for use with optimal control strategies, and automating the biopsy task itself. Each biopsy procedure is broken down and analyzed for potential avenues of automation.

Experiments are performed in vitro with an inverted microscope using robotic micromanipulators, which allow position control of tools in a similar fashion to human operators, microscope objective-embedded laser for zona breaching, and other specialized tools that will be detailed in each chapter. Feedback is obtained through images from a CMOS camera attached to the microscope, or through encoders on the micromanipulators. Computer vision is also used to analyze the embryo structures and the performance of the procedure itself. All actuation is computer controlled with automated decision making and feedback.

1.2 Overview of Biopsy Techniques

“If I have seen further, it is by standing on the shoulders of giants.”

— Sir Isaac Newton

In the last few years, automation in the field of embryo manipulation has progressed immensely [7, 8, 36–40], creating automated injection systems [41–43] and algorithms for automated embryo image analysis [18, 19, 22, 44]. This section details important characteristics and recent advances in micromanipulation and ART techniques, specifically those relevant to PGD biopsy. The purpose is to review the state of the art, and examine current issues and missing knowledge within the state of the art.

1.2.1 Cell Localization, Immobilization and Patterning

The ability to manipulate objects at the microscale with high levels of precision is crucial to the success of any task related to individual cells. Micromanipulation includes relocation, immobilization and reorientation of cells. Advancement in different fields
have led to new and innovative ways of micromanipulation, including the introduction of microfluidic channels, optical tweezers, and microelectromechanical systems (MEMS).

First and foremost, in order to perform any operations on a biological cell, the first task is to position the cell in a desired location, typically in another tool’s workspace. In clinics performing ICSI and PGD, an embryo is typically held in place using a holding micropipette attached to a micromanipulator [Figure 1.2(a)]. Light negative pressure is applied to keep the embryo from moving while other tools interact with the embryo. This rudimentary technique requires manually locating both the pipette tip and embryo, and subsequently moving both objects together. Some devices have been designed to automatically locate, pattern and immobilize embryos for manipulation, removing the need for the operator to manually perform these actions.

Large scale patterning can be achieved using through-holes in a microfluidic device connected to a vacuum source [2, 41, 43, 45]. Embryos slated for microsurgery are pulled towards the through-holes at the bottom of the substrate as liquid is aspirated [Figure 1.2(b)]. Low suction is desired to prevent pulling the embryo into the through-holes, but at low vacuum pressure and flow rates, the adhesion force between the embryo and the substrate may prevent the embryo from patterning properly. This problem was alleviated by controlled vibration of the microfluidic device using a piezoelectric actuator [45]. Devices such as these are useful for high throughput processing of single cell manipulation while reducing the need to change tool tips between every procedure in order to avoid cross contamination. One drawback is the inability to control the exact orientation of each individual patterned cell, which is paramount for certain single cell surgery applications.

MEMS devices have been created for contact-based manipulation. Hagiwara et al.
created two three degree of freedom (XY-translation and Z-rotation) magnetically-driven microtool (MMT) for enucleation of oocytes (Figure 1.3) [3, 46]. To reduce MMT-substrate adhesion, Hagiwara vibrates the substrate at high frequency, similar to [45], to achieve extremely precise positioning accuracy of the MMT. Successful oocyte enucleation was demonstrated using this system. Similarly, Sun et al. used a MEMS probe to measure the mechanical properties of an embryo [47], which could be used to provide force feedback during manipulation. While novel and versatile, these systems are complicated and require very specialized equipment, which reduce their usefulness. Furthermore, to achieve wide-spread use, the equipment must be low-cost and easily disinfected to reduce cross contamination between samples.

Optical tweezers (OT), first demonstrated in 1970 by Ashkin [48], uses the refraction of light through an object to manipulate microsized particles [Figure 1.4(a)]. \( F_{\text{grad}} \) is the gradient component of radiation pressure perpendicular to the light source, whereas \( F_{\text{scat}} \) is the scattering component of light parallel to the direction of light. \( F_a \) and \( F_b \) are the resultant force vectors from two refracted rays of light from the same source through the same particle, and tends to pull the particle towards the center of the beam. Given the need for light to refract through the microparticle, the particle must allow light to pass, though modulation of the light path have allowed metallic particles to be manipulated as well. Furthermore, the larger difference in indexes of refraction of the particle and the surrounding medium, the greater the effect of the optical tweezers. Given the potential of optical tweezers, its use expanded dramatically to span multiple domains of science, especially biology [4].

For the first time, optical tweezers provided the capability to manipulate biological material in a controlled and precise fashion [49–51]. Wu et al. investigated the dynamics
Chapter 1. Introduction

Figure 1.4: Working principle of: (a) optical tweezers [4], (b) optoelectronic tweezers (OETs) [5], (c) standing surface acoustic waves for object manipulation [6]

of optical tweezers for automated cell transport using vision feedback and path planning [52]. Particles inside cells can be manipulated as well. Il’ina et al. used optical tweezers to extract polar bodies from embryos during PGD [14]. Single-beam optical tweezers can only grasp one object a time, thus limiting its uses. The introduction of holographic optical tweezers (HOTs), using computerized spatial light modulators, allows one light beam to manipulate multiple objects simultaneously. Furthermore, the use of optical tweezers in biology has been criticized for potential photochemical damage to cells. Koss et al. demonstrated the use of HOTs to manipulate beads in a different patterns to indirectly grip biological cells, lowering the possibility of photochemical damage [53]. The beads are arranged in different configurations, the most robust being two 3D triangular patterns to restrict the 3D motion of the trapped cell. While optical tweezer systems are costly, they possess the advantage of non-contact based manipulation, which minimizes the risk of cross contamination.

Similar to optical tweezers, optoelectronic tweezers (OETs) use projected optical images on a photosensitive substrate to locally create ”virtual” electrodes, as shown in Figure 1.4(b) [5]. The electrodes then use the well known particle manipulation method called dielectrophoresis (DEP) to attract or repel dielectric particles, which include biological cells. By controlling the projected optical images, the electrodes change shape correspondingly and effectively shifting particles trapped in the localized electric field. This indirect manipulation method once again lowers the possibility of photochemically damaging cells, but presents possible damage via electric fields.

The Pennsylvania State University BioNEMS Group developed an acoustic tweezer system that allows manipulation of microsized objects using surface standing acoustic
waves (SAW) generated by interdigital transducers deposited on a piezoelectric substrate [Figure 1.4(c)] [6, 54]. SAWs were used for 3D manipulation of cells for patterning. Objects manipulated ranged from a single bovine red blood cell (diameter of 6\(\mu m\)) to a single Caenorhabditis elegans organism (length of 1 mm). Even after exposing the organisms to 10 minute of SAW, no significant physiological damage was found, and the temperature increase in the medium stabilized at 31°C. Non-contact methods of manipulation have a significant advantage over contact-based methods, as non-contact methods eliminate the risk of chemical or biological contamination or cross-contamination.

1.2.2 Embryo Orientation Control

While positioning and immobilization of the embryo is important to single cell surgery, the orientation of the embryo during surgery is of utmost importance as well to maximize procedure efficacy and safety [55, 56]. Embryo rotation has been achieved using various different methods. Traditionally, there are two methods utilized by most fertility clinics to rotate the embryo until an adequate orientation has been achieved. The first is repeatedly alternating between capture and release of the embryo using a holding micropipette, which haphazardly rotates the embryo in a random fashion. The second involves using fluid flow from a micropipette near but slightly offset from the center of the embryo [Figure 1.5(a)]. The flow causes shear forces on the embryo, inducing a rotating motion. While less haphazard than the first method, there is no orientation strategy other than waiting until the embryo happens to be orientated adequately.

Leung et al. combined the second conventional rotation method of using offset fluid jets with computer vision to automatically control the orientation of mouse oocytes. This was achieved through controlling the relative location of the pipette to the embryo using computer vision and adjusting the flow rate through the micropipette. Leung et al. was able to demonstrate automated rotation of an oocyte to a predetermined orientation based on the location of the polar body with respect to the camera [7]. The presented control algorithm is broken down into two stages: out-of-plane rotation and in-plane rotation. This condition refers to whether or not the polar body is initially in view. This work is a prime example of applying automation to a conventional method.

Using a custom-built microscope stage [Figure 1.5(b)], Liu et al. was able to quickly reorient oocytes patterned using a grid of vacuum holes to a desired orientation by rotating the entire stage mount [2]. Although this system presents a very simple solution to reorientation of embryos in a high-throughput setting, several problems arise. Firstly, any wires or tubing connected to the rotating stage may become entangled, limiting range
Figure 1.5: Orientation control of embryos using (a) fluid flow from offset micropipette to generate a shear force [7], (b) custom built rotating stage [2], (c) rolling embryo inside a microfluidic device [8], (d) 3D DEP using carbon-embedded conductive C-PDMS [9] of motion of the stage. Secondly, the stage is restricted to rotation along one axis, thus full orientation control of the embryo is not possible.

Shin et al. devised an new strategy for controlling the orientation of embryos by analyzing the problem as a classic rolling ball problem with a fixed end point [Figure 1.5(c)], using microfluidic channels as the input force and assuming that the embryo rolls along the bottom of the channel with slippage [8]. The planning algorithm analyzes the embryo as it travels through the microfluidic channel, so when the embryo has reached the working area, the location of the polar body is already known and the algorithm can then decide the control inputs to reorient the embryo to the desired position. Other microfluidic devices have been designed for the rotation of cells as well [57–60]. Rolling of embryos can also be achieved using physical means. Wang et al. used micropipettes to drag a zebrafish embryo along the bottom of a petri dish [39, 61], whereas a pair of magnetically-driven microtools (MMTs) described in Section 1.2.1 was used to pinch a mouse embryo in between two MMTs to rotate the embryo [37, 46].

Dielectrophoresis, the generation of a force on a dielectric particle in the presence of non-uniform electric fields, has been shown to affect biological cells and is often used
in cell trapping or separation. By combining specific electrode patterns with control of the electric field frequency, Jiang showed that it is possible to control the orientation of cells [40, 62]. Four electrodes arranged in a planar pattern unfortunately only results in planar rotation. Huang et al. extended this concept to three dimensional electrodes using carbon-embedded conductive polydimethylsiloxane (PDMS) posts, and was able to achieve full three dimensional rotation, as shown in Figure 1.5(d) [9].

As mentioned previously, optical tweezers can be used to manipulate and pattern cells fairly easily. Optical tweezers controlling the movement of external beads can easily rotate the object they are grasping, but rotating objects directly with optical tweezers is not as straightforward. Paterson et al. successfully rotated different objects by interfering an annular shaped light path with a reference beam, creating a spiral of traps [63]. Furthermore, Dasgupta et al. demonstrates the capability of optical tweezers for rotating red blood cells in a controlled manner [64]. Xie et al. used two offset OT beams to rotate cells both in-plane and out of plane [51].

1.2.3 Zona Breaching

Once the embryo has been located and manipulated to be placed in an optimal position, the next step in embryo biopsy is to create an entry point to access the intracellular material. This process, called zona breaching or drilling, involves full thickness removal or perforating of a small section of the zona pellucida. The ultimate measure of a biopsy technique is the embryo viability post-manipulation. A breaching technique that grants the operator access to the intracellular material but destroys the embryo as a whole in the process is undesirable in PGD. Thus, the goal of research in zona breaching techniques aims to find the least damaging method to the embryo and the biopsied material. Currently, zona breaching typically falls under 3 different categories [35]: chemical, mechanical and thermal.

Chemical breaching uses the chemical acid Tyrodes (AT) or digestive enzymes such as pronase. Chemical zona drilling is performed by blowing the chemical over the breaching location using a drilling pipette (inner diameter of 5-6 µm) and waiting for the ZP to erode. Immediately after the ZP has been breached, the chemical is sucked back into the pipette and the embryo is thoroughly washed to remove any residue. The use of AT has been criticized for the acidity of the chemical used (pH = 2.4) and its potential danger to the embryo. Embryo development retardation has been associated with AT when compared to laser drilling [65,66], and lower success rates compared to mechanical zona drilling [67,68].
Mechanical drilling includes the use of a sharp needle to create tears in the ZP [Figure 1.6(a)] and can be further divided into passive or active mechanical drilling. In passive drilling, the needle is stationary and is simply pressed into the ZP until puncture occurs. In active drilling, the needle vibrates at high frequency using piezoelectric actuators whether in the longitudinal or transverse directions. The mechanical stress and the heat generated imposed on the embryo from this method is large, and is detrimental to the survival of the embryo. Bahadur demonstrated the construction of a piezo-driven micro-cutter using a lateral cutting motion to mechanically perforate the ZP [10].

Finally, thermal breaching includes the use of both contact and non-contact methods to focus laser pulses to locally ablate the ZP, shown in Figure 1.6(b) [14, 38, 66, 69]. According to ESHRE, the use of this procedure, known as laser zona drilling (LZD), has been increasing in recent years, accounting for 48% of all recorded PGDs in 2000 to 72% in 2007 [32]. LZD is achieved by focusing a beam of laser light on the ZP. The absorbed energy heats up the focal point extremely quickly to very high temperatures, leading to immediate ablation of the local area. Adjusting the laser beam parameters such as wavelength, output power and pulse duration all contribute to the diameter of the resultant hole, and the energy absorbed by the surrounding areas [70]. Compared with chemical and mechanical zona breaching methods, LZD is much simpler and yields results faster, while achieving higher rates of success [66, 69].

The introduction of non-contact lasers in micromanipulation has allowed more precise operations at the microscale, particularly in the ablation of cellular material. In ART, the partial or complete removal of the cellular wall sections is a pertinent step in assisted hatching or embryo biopsy procedures respectively. Some studies have compared the safety and efficacy of LZD to other zona removal methods [66, 69]. By choosing a wavelength with high absorptance in water and focusing the laser in a specific location on the
zona pellucida (ZP), the temperature will rise locally. Beyond a certain species-dependent temperature threshold [71], the zona will be ablated, creating an opening. Multiple ablative pulses can be used to create larger openings to allow access to intracellular material or to thin the zona.

Laser ablation has been criticized since it may harm embryos and their constituents, causing laser-induced cytotoxic thermal damage or damage to DNA. Initially ultraviolet lasers were used, but susceptibility of DNA to damage caused by light at ultraviolet wavelengths caused concern. Modern IVF lasers now use wavelengths in the near infrared (1460-1480 nm), given the strong absorption of this wavelength in water molecules. Whereas most lasers now used in LZD have been shown to be free of DNA mutagenic effects [70,72,73], one of the main concerns is possible hyperthermia of the critical components inside the embryo [74]. Douglas-Hamilton and Conia analyzed the heat generated in zona laser ablation, modeling the temperature gradient with variable output power and pulse duration [11]. This model allows the prediction of the effect of laser on the surrounding areas and is useful for determining safe breaching locations on the ZP. Certain commercial laser ablation systems provide a series of concentric circles, where each circle corresponds to a precomputed prediction in temperature increase, superimposed on the image of the cell during ablation to assist embryologists gauge the proper position for laser ablation (Figure 1.7).

Several studies investigated the effects of different LZD parameters on LZD performance. Research Instruments Limited, the manufacturer of the Saturn 5 family of laser systems for zona breaching, conducted a study to determine the total energy required to ablate similar sized holes in the ZP at different laser powers (175 mW, 300 mW and 400 mW) [75]. Their findings indicate that higher power outputs, applied over a shorter time duration, requires less energy to create ablations of similar size, thus generating less heat and lowering the possibility of any negative thermal effects. Taylor et al. investigated the effects of laser pulse duration, and concluded that within the scope of their pulse durations (0.604 ms, 0.708 ms and 1.010 ms), there is no significant difference on the development of the embryo to the blastocyst stage [76]. Il’ina et al. analyzed the effect of energy on embryo hatch rate for a femtosecond laser system, and saw no adverse effects [70].

Given the concern due to heat-related damage, Hartshorn, Anshelevich and Wangh used heat shock protein hsp70i to gauge whether or not 8-cell embryos undergo heat shock during LZD using three 1 ms pulses from a non-contact 1480 nm laser beam [74]. Levels of hsp70i detected in blastomeres from embryos that underwent LZD (24 ± 16 copies) were similar to those in the control group (15 copies). The blastomeres directly
adjacent to the breach zone had similar levels of \textit{hsp70i} to the other blastomeres in the same embryo. Conversely, embryos that were heat shocked by incubating at 43°C for 30min had significantly higher levels of \textit{hsp70i} (1331 ± 238 copies). Furthermore, rate of development was unchanged in the group that underwent LZD, whereas cell cycle arrest is a known effect of hyperthermia. The study concludes that LZD with a 1480 nm laser does not induce a heat shock response in the embryo under these conditions. While the study has been criticized for using multiple orders of magnitude difference in time exposure of the heat shocked case versus the time scale of LZD, the study still demonstrates that levels of \textit{hsp70i} are similar in both the LZD and control cases.

Although the focus of this section aims to examine different breaching techniques and their performance, there exists other methods nonspecific to breaching techniques that lower the possibility of collateral damage to the embryo and biopsied material. For example, Li \textit{et al.} report that the addition of sucrose to the culture medium induces oocyte shrinkage in volume relative to the perivitelline space (PVS), possibly via hyperosmotic effects [12]. This effect is shown in Figure 1.7. The increased perivitelline space allows a larger distance between the oocyte and the breaching site, decreasing the chance of collateral damage such as laser-induced cytotoxic thermal damage from laser ablation techniques. The use of sucrose was not shown to have harmful effects, and in fact increased the success rate of embryos compared to standard LZD IVF.

Despite these attempts to increase laser safety, current LZD techniques still require a human to perform LZD by gauging the distances visually and use subjective decision making based on experience. Many manuscripts cite minimization of embryo temperature increases as an important factor in LZD [11, 66, 71], often by increasing the distance
between the laser and the critical components. Local embryologists claim that maximum peak temperatures close to 50°C are acceptable, but one should aim to minimize any temperature increase. General guidelines exist for performing embryo biopsies [35], but the exact procedure is highly dependent on the operator, leading to inconsistent results between patients.

Despite advances in zona breaching, the introduction of automation to zona breaching has not yet been demonstrated or reported in literature. This presents a major gap in the automation of embryo biopsy, and a critical step that must be achieved.

1.2.4 Blastomere Extraction

Following zona breaching, the next step in embryo biopsy is the extraction of intracellular material. In PGD, the extracted material is typically one to two blastomeres to be used for genetic analysis in later steps. Three main techniques are used in embryo biopsy that have been reported in literature and used in clinics: aspiration, displacement and optical tweezers.

The most rudimentary and widely-used technique recommended by ESHRE guidelines [35], known as the aspiration technique, simply involves aspirating the polar body or blastomere using a micropipette inserted through the ZP into the PVS [Figure 1.8(a)]. When a smaller perforation is desired, the extrusion-aspiration method is used where a blastomere can be partially extracted by using a pipette to physically squeeze the embryo, compressing the PVS, and pushing a blastomere out [Figure 1.8(b)]. Both methods exert large amounts of mechanical stress to both the embryo and the blastomere, which may cause cell rupture or invoke an undesirable mechanobiological response resulting in reduced embryo viability.
To reduce the amount of mechanical stimuli required to extract a blastomere, a technique was developed called the displacement method [77]. By injecting a sterile medium into the embryo, the injected fluid causes a blastomere to be displaced and forced out through the opening in the ZP [Figure 1.9(a)]. Wang et al. reports that the displacement technique is easier and faster for embryologists, and increases embryo viability [13, 77]. Criticisms of this technique include the lack of control and possible contamination of embryo via tainted injection medium or the pipette tip, but the aspiration technique is equally likely to suffer from these side effects.

The third common extraction technique discussed in the literature is the use of optical tweezers. The advantage of optical tweezers have been outlined previously, and are suitable for performing PGD and prefertilization genetic diagnosis (FGD), which is equivalent to PGD for unfertilized oocytes. Il’ina et al. successfully performed an entirely non-contact embryo biopsy to extract polar bodies and trophectoderm cells using laser ablation and optical tweezers [Figure 1.9(b)] [14, 38]. Unfortunately, optical tweezer use for blastomere biopsy has not been reported, and may be a result of the low force output of optical tweezers unable to overcome the adhesion force between blastomeres.

Another method used rapid repeated aspiration and expulsion of the embryo using a Stripper Micropipetter (Origio) to extract blastomeres from 8-cell cleavage stage embryos. The repeated aspiration and expulsion motion would cause spontaneous release of a blastomere after a few cycles [78]. According to the Cedillo et al., this method was able to shorten biopsy time 5-fold compared to conventional aspiration methods (179.2 ± 17.5 s vs. 897.7 ± 34.0 s), while requiring a smaller opening in the zona. While this method has many advantages, notably the smaller zona opening and technique simplicity, it requires very rapid and precise control of fluid in and out of the micropipette, and is
somewhat unstable in the sense that the embryo may be pushed out of view.

Other non-conventional methods for extracting cell material have been developed by other researchers. One particular tool for cell enucleation, the removal of the nucleus from a cell such as an oocyte, has been performed using untethered magnetically driven microtools [3], which was first described in Figure 1.3 in Section 1.2.1. Two independently controlled MMT devices were moved in concert to pinch and shear the nucleus from oocytes.

While the method plays a large role in the successful extraction of material, other conditions may aid their removal. For example, the use of Ca\(^{2+}\)/Mg\(^{2+}\)-free medium reduces the membrane adhesion between blastomeres, allowing for significantly easier and faster removal of blastomeres which lowers the probability of cell rupture [79]. Embryos exposed to this chemical did not display any abnormalities and developed normally to the blastocyst stage. Use of Ca\(^{2+}\)/Mg\(^{2+}\)-free medium is considered a standard procedure in FGD/PGD [35].

Although some papers describe techniques that could be used in the automation of blastomere extraction, such as certain vision algorithms outlined in Section 1.2.5, no literature currently exists that demonstrate the automation of blastomere extraction procedure. Thus, a key step towards the full automation of embryo biopsy is still missing.

### 1.2.5 Computer Vision in Cell Micromanipulation

Stepping towards fully automated biological micromanipulation requires incorporating the use of sensory feedback in the controller design and decision-making process. Cameras have long since been used for visual feedback in mobile and stationary robotic systems for navigation, servoing and decision making [80, 81]. Thus, the integration of computer vision algorithms is an logical step towards automation of cellular-level micromanipulation.

#### Object and Micropipette Tracking

Typically, computer vision algorithms can be used as visual feedback for control or tracking of cells or manipulators in the current field of view [82–84], or can also be used for autofocusing of objects [16, 85]. Zhang et al. automatically tracked sperm cells using computer vision, and used visual feedback to control their position inside a micropipette after aspiration. Otsu adaptive thresholding is used to create a binary image which separates the foreground from the background of the original image, and contour tracking is then used to determine the location of the sperm. Additional steps were included to ren-
Figure 1.10: Computer vision algorithms for: (a) micropipette tracking using template matching [15], (b) autofocusing [16], (c) detection of blastomere extraction using the aspiration method [17]

Under the tracking algorithm robust to system vibration and visual contaminants. Rather than using contour tracking, Wang et al. used texture to differentiate somatic cells from background, reporting better results than when using traditional Otsu method [86].

Template matching, a method that attempts to match a reference image to the current field of view for segmentation, is sometimes used for tracking micropipettes as shown in Figure 1.10(a) [15, 83, 84, 86]. This method allows vision-based feedback for real-time closed loop position control of micropipettes. While vision feedback is often used for control of micropipettes and micromanipulators, these methods may fail when the object to be tracked is blocked or obscured [84].

Locating objects in the focal plane is important, and sometimes simply involves moving the stage to search in the XY plane [83]. Sometimes objects may be out of focus, or located at different focal planes [Figure 1.10(b)]. Many computer vision algorithms rely on using sharp images of objects in focus to function properly, thus it is paramount for objects to be consistently in focus despite dynamic systems where objects move around while being manipulated. For fully autonomous systems, this requires the use of autofocus algorithms, which automatically adjust the focal plane of the microscope until the objects are in focus based on the edge sharpness value [85]. Bahadur and Mills developed a robust autofocus algorithm using particle swarm optimization to automatically determine the focal depth with the maximum sharpness value [16].

During the blastomere extraction step, it is useful to determine when a blastomere has been successfully extracted from inside the embryo. For the aspiration extraction method, Paranawithana et al. reported using the Kanade-Lucas-Tomasi Feature Tracker computer vision algorithm on previously recorded video to detect when a blastomere has been extracted from the embryo and aspirated into the micropipette, as shown in Figure 1.10(c) [17]. Dong et al. used electrodes located both in a micropipette and on a petri
dish, and measured changes in voltage to detect when a cell has been aspirated [87]. As the cell is aspirated and forms a seal on the micropipette, the circuit resistance increases as current is forced to pass through the cell rather than through the comparatively less resistive culture medium. No vision algorithms have been reported that could be used for the other extraction methods mentioned in Section 1.2.4, which is required for the automation of blastomere extraction using those methods.

**Embryo Segmentation and 3D Reconstruction**

Not only can computer vision be used to detect the embryo itself, it can also be used to differentiate between the different parts within the embryo, a process known as segmentation. For example, the zona pellucida can be segmented separately from the blastomeres [19]; or each individual blastomere can be detected [88]. Most segmentation methods use edge-based techniques [18, 89], such as Canny edge detection [19], and sometimes complemented with Hough transform [40, 41]. Image texture, a measure of image properties such as the local standard deviation of pixel intensities, can also be used in segmentation [86]. Once the different parts of the embryo are segmented, there are many different applications possible, such as embryo grading, determining the mechanical properties of cells, or observing the relative spatial positioning of the different embryonic components for optimizing embryo biopsy.

Santos et al. published a review on the power of computer vision in IVF to automatically grade the quality of a potential embryo in hopes of lowering chances of failed implantation or multiple pregnancies [22]. Automatic embryo grading eliminates the subjective bias of embryologists in the evaluation process by using repeatable measurement methods and objective grading criteria which can be quantified numerically. The grading criteria involves measuring embryo parameters such as zona pellucida thickness, cell number and symmetry, and fragmentation amount [90–92], or using time-lapse analysis of embryos [93–95]. In the specific case of embryo biopsy, embryo grading allows choosing a suitable blastomere for extraction, rather than arbitrarily picking one that may not be healthy.

Cell membrane dynamics can be modeled by analyzing the deformation of the cell membrane under an applied force [84]. Membrane dynamics can be used to identify the age of the mouse [20]. Ammi et al. analyzed the cell membrane using the *Snakes* active contour technique during perforation to not only determine the applied force, but to also detect zona pellicuda penetration by using the relative velocity of the membrane and the injection pipette [96]. In a similar method, Shojaei-Baghini et al. applied a known vacuum force to an embryo and measured the depth of embryo from aspiration to
determine the mechanical properties of the embryo [15]. Using a more indirect approach, Liu et al. developed a computer vision algorithm to simultaneously measure the applied mechanical force and the resulting deformation using the deflection of PDMS posts [20, 97].

Although 2D imaging has defined general microscopy, there are vast amounts of information that can be gained from 3D imaging of cells. Pseudo-3D models of cells can be generated by assuming spherical symmetry, as was done in [96] for haptic applications, but certain applications require high-fidelity 3D models of cells and their components. 3D reconstruction of cell bodies involves the acquisition of many image slices at different focal planes (also known as focus stacks or Z-stacks) and reconstructing a model of the cell by combining these slices. There exist many imaging methods to generate the 3D structure of objects, such as confocal or fluorescence microscopy, light sheet microscopy, scanning electron microscopy. These methods typically require expensive equipment. This section will focus on non-destructive optical methods. In regular bright field microscopy, observation of near-transparent samples such as cells are difficult without staining. The use of contrast illumination such as differential interference contrast (DIC) or Hoffman modulation contrast (HMC) allows easier analysis of near-transparent objects without staining.

For example, cell morphology and motility cannot be truly understood without a 3D analysis. By combining time-lapse information with a full 3D model of the cell generated from DIC microscopy, Soll et al. was able to analyze the motility of organelles and map their movements over a period of time [98]. In polar body biopsy, the location of the polar body may be hidden from view. Leung et al. devised a control strategy to locate the polar body in an oocyte by rotating the embryo until the polar body appears [7], but a 3D mapping of the embryo would be useful in determining the polar body’s location prior to manipulation.

Giusti et al. was able to generate Z-stacks of embryos at the 4-cell stage with 71% accuracy by using a graph-based method for segmenting blastomeres, then generating a surface to recreate the 3D model, as shown in Figure 1.11(a) [18, 89]. Furthermore, using Local Binary Patterns for texture detection, Giusti et al. was able to reliably detect the location of pronuclei in 3D by combining information from multiple focal planes [99]. When analyzing the information gains of different features during classification, it was noted that certain features from adjacent focal planes provided high levels of information. Similarly, Pederson et al. was able to generate 3D reconstructions of 4-cell embryos using variational level sets by modeling the ZP and the blastomeres as elastic objects with surface tension [100].
Other optical methods are also used to generate 3D models of embryos. A full-field optical coherence tomography method was used to quickly obtain 3D models of the embryo [101]. The advantage of contrast imaging methods lies in the ability to quickly produce images. Segmentation using confocal microscopy may take long periods of time as each segment is generated using raster scanning [102]. Furthermore, some specimens may not be able to exhibit fluorescence signals.

Segmentation of embryo components allows spatial analysis of the different components. Certain operations in ART require specific orientations of embryo components to minimize damage or maximize effectiveness. For example, the largest gap between the cytoplasm and zona pellucida in an oocyte can be easily determined by the location of the polar body orientation during perforation [7, 55, 56]. Analysis of the relative positioning of the blastomeres and the zona pellucida can allow the gap between the two to be maximized to minimize the danger of damaging the blastomeres during zona breaching, as seen in Figure 1.11(b) [19, 44]. Once the optimal orientation has been determined, the embryo can be manipulated using methods described in Sections 1.2.1 and 1.2.2 such that the ART tools are aligned with the optimal location for interacting with the embryo.

1.3 Research Significance and Contributions

The introduction of PGD was able to improve the success rates of IVF [29]. Unfortunately, despite the first PGD procedure being performed in 1988 [103], PGD is still performed manually. Success of the procedures are highly dependent on the skill of
the embryologist. One could hypothesize that the automation of PGD could improve the success rate of PGD and IVF procedures even more. Despite advances in ART techniques and the development of some automated systems for embryo micromanipulation [42,43,104], many techniques outlined in Section 1.2 are still performed manually, use technology that are prohibitively expensive or specialized, or only present small advances to a larger problem.

The overarching goal of this thesis aims to address gaps in the state of the art of ART by developing methods for the automation of embryo biopsy techniques for use in preimplantation genetic diagnosis using conventional or low-cost technologies. Specifically, technologies related to each embryo biopsy procedure outlined in Figure 1.1(c): embryo position and orientation control, zona breaching and blastomere extraction. This thesis provides first-of-its-kind demonstration for the automation of laser zona drilling and for blastomere extraction. The outcome of this research will advance the knowledge in underrepresented areas of automated single cell surgery, as most research dedicates itself to automation of cell positioning and reorientation. Although this work is tied to specific applications, the methods can be expanded and adapted to other areas of single cell surgery and micromanipulation. The main contributions of this thesis also include:

- Novel low-cost method for the manipulation of human-sized embryos using individually-actuated parallel plates
- Development of rotation tracking algorithm for 2-cell embryos using a geometric model
- Creation of a 3D mapping method of embryos using Hoffman modulation contrast microscopy
- Texture-based segmentation of blastomeres using computer vision algorithms
- Optimization of laser zona drilling based on embryo morphology
- Formulation of a process flow for blastomere extraction automation using feedback control
- Development of computer vision algorithms for blastomere extraction event detection and blastomere retrieval
- Demonstration of zona breaching automation and blastomere extraction automation feasibility using mouse embryos
1.4 Outline of Dissertation

This thesis contains three major chapters, examining automation of each of the three embryo biopsy procedures. Chapter 2 focuses on novel methods related to embryo position and orientation control, including imaging, analysis, and manipulation. The goal of position and orientation control is to maneuver the embryo in such a way that achieves two objectives: 1) to determine which blastomere to extract and the location along the zona to perform zona breaching, and 2) to align the tools for zona breaching and blastomere extraction with the chosen blastomere and zona breaching location. Achieving these two objectives minimizes the damage potentially caused by subsequent biopsy procedures through proper positioning of the embryo relative to the biopsy tools. A pair of independently controlled parallel plates is used to generate rolling motion of the embryo in an effort to control the position and orientation of the embryo. The performance of the parallel plate system is characterized and closed loop embryo position and orientation control is demonstrated. Computer vision algorithms are used to track the position and orientation of the embryo, and to generate a 3D map of the embryo. This 3D map allows spatial analysis of the different embryo components and selection of optimal positioning and orientation for subsequent biopsy procedures.

Chapter 3 describes the automation and optimization of zona breaching using laser zona drilling. The goal is to minimize the temperature increase of the blastomeres while creating an entry point into the embryo during zona breaching. This entry point permits the next biopsy procedure, blastomere extraction, to occur. Temperature increase is minimized through the use of a two-stage optimization. The first stage uses computer vision algorithms to identify embryonic structures and determines the optimal ablation zone farthest away from critical structures such as blastomeres. The second stage combines a genetic algorithm with a thermal model of LZD to optimize the combination of laser pulse locations and pulse durations.

Finally, Chapter 4 automates blastomere extraction using the displacement method. As the name implies, the blastomere extraction procedure involves removing a blastomere from the inside of the embryo, ultimately to be used for genetic analysis step of PGD. A process flow for blastomere extraction automation is developed with the use of feedback control. Automation is implemented using algorithms for non-vision-based feedback micropipette position control, a simplified gas model for fluid pressure control, and computer vision algorithms for blastomere extraction and retrieval.

For each of the three biopsy procedures, validation experiments are performed on mouse embryos to examine the feasibility of biopsy automation. Successful experiments
demonstrate that the above methods are indeed feasible, and present steps toward realizing fully automated ART.
Chapter 2

Embryo Position and Rotation Control

Single cell manipulation, the process of physically moving and interacting with a single biological cell, is a very active field [21,23]. Many researchers are developing novel tools using new technologies for both manual and automated manipulation [43,46]. Assisted reproductive technology (ART) procedures, which sometimes involve *in vitro* manipulation of embryos to aid fertility and reproduction, are inherently dangerous for embryos, which are delicate and fragile. Any mishandling can lead to the death of an embryo. Given that ART procedures are taxing for the patient physically, psychologically and financially, it is in everyone’s best interest to make these procedures as safe and effective as possible. One of the easiest ways to improve ART procedures is to position and orient the embryo correctly prior to any proceeding with any operation [35,56].

In preimplantation genetic diagnosis (PGD), a cleavage stage embryo undergoes non-destructive biopsy to have 1-2 blastomeres removed for genetic analysis. This genetic analysis allows the presence of genetic or chromosomal diseases in the embryo to be detected, such that only healthy embryos are implanted in the uterus. There are three main steps in PGD biopsy: embryo position and orientation control, zona breaching, and blastomere extraction. In the first step of PGD biopsy, embryo position and orientation control, the embryo is manipulated and physically moved to achieve two objectives: 1) to determine which blastomere to extract and the location along the zona to perform zona breaching, and 2) to align the tools for zona breaching and blastomere extraction with the chosen blastomere and zona breaching location. Traditional manual embryo rotation is a trial and error process, as outlined in Section 1.2.2, typically involving haphazard maneuvers in the hopes of manipulating the embryo to a more favorable position, which can often take a long time. The introduction of automation allows these
manipulations to be directed, precise, and efficient, ensuring that the embryo is properly oriented, and not kept at unfavorable conditions outside of the incubator for longer than needed. Automation can be applied to conventional manipulation methods, as has been demonstrated by Leung et al. [7], but often times new technologies and methods are developed to demonstrate different possibilities.

In this chapter, novel methods for in vitro manipulation of human-sized embryos for the purposes of repositioning and reorientation are described. The goal of the work presented in this chapter is to fulfill the two objectives of embryo position and orientation control outlined above in an automated fashion using computer vision and computer-controlled actuation. In Section 2.1, a short investigation into fluid-based rotation is described and drawbacks are mentioned. Then the main novel system of using two parallel plates for embryo manipulation is described, and the governing kinematic equations and characteristics are derived. In Section 2.2, the experimental setup, along with the parallel plate fabrication, is outlined. A method for the rotation tracking of 2-cell embryos using a geometric model is derived in Section 2.3. In Section 2.4, a non-destructive method for generating a 3D map of an embryo using only Hoffman modulation contrast microscopy is described. A zona breaching optimization algorithm uses the generated 3D model to maximize the space between the blastomeres and the zona, achieving the first objective of embryo position and orientation control. A reorientation control strategy is described to complete the second objective of embryo position and orientation control. Finally, both open loop and closed loop parallel plate characterization experiments using mouse embryos are reported in Section 2.5. Successful closed loop position and rotation control experiments demonstrate the feasibility of the parallel plates method for position and orientation control of embryos. Work presented in this chapter has been presented at a conference [105] and will be submitted for publication in a peer-reviewed journal.

2.1 Rotation Method

In this section, two methods for the repositioning and reorientation of embryos are investigated. The first method involves reproducing the results of Leung et al. [7], which uses offset fluid jets to generate a shear force and rotate the embryo, with the addition of mechanical traps to immobilize the embryo. The second method, a novel method discussed in the rest of the chapter, places the embryo in between two independently actuated parallel plates to generate rolling motion of the embryo. The method is described and the governing kinematics equations are derived.
Chapter 2. Embryo Position and Rotation Control

2.1.1 Fluid Rotation

Embryo rotation control is first attempted using fluid drag to impart a rotational moment on the embryo, such as a fluid expelled from a micropipette offset from the embryo, as demonstrated in Figure 2.1(a) by [7]. This method also pushes the embryo away from the field of view, sometimes unpredictably, requiring compensatory motion from the microscope stage. To prevent embryo translational motion, the use of mechanical traps are investigated. Mechanical traps, different from suction traps [2,45], would ideally only prevent translational motion but still allow rotational motion. Similar posts to the ones described by Liu et al., who used polymer polydimethylsiloxane (PDMS) posts to verify the elasticity of embryo membranes as shown in Figure 2.1(b) [20], would be used.

Several designs, shown in Figure 2.2, are tested. Posts were approximately 80-100 µm in height and 30 µm thick. The large wells were 80-100 µm deep, and 120-200 µm in diameter. For the cylindrical posts and rectangular vanes, an embryo would be placed in the center of the traps, and the gap in between the traps would allow fluid flow to go past and impart rotational motion on the embryo. For the large wells, the embryo would sit inside them, and fluid flow would travel over the top of the well to rotate the embryo directly, or indirectly by generating vortices similar to the methods described by [57,106].

Mechanical traps were originally created using PDMS via soft lithography techniques.

Figure 2.1: Schematics for (a) fluid rotation [7] and (b) mechanical traps [20]

Figure 2.2: Trap designs: (a) cylindrical posts, (b) rectangular vanes, (c) large wells. Posts and vanes are pressed down to show 3D aspect of traps.
The low working focal length of the microscope required the use of a thin PDMS base for the posts, which resulted in a fragile platform that was difficult to handle and often tore during setup. To remedy this problem, hot embossing was used to imprint the posts into a standard petri dish made of stiff polystyrene. Both PDMS and hot embossed polystyrene posts and wells did not perform as expected. Preliminary tests are performed using glass beads, and while the traps prevented translation, rotational motion was also impeded and the beads did not reliably rotate smoothly. Given the poor rotational performance and fabrication difficulty, this research path was abandoned.

2.1.2 Parallel Plate

An alternative method for rotation control in this thesis places the embryo between two independently-actuated parallel plates and uses the relative translation of the two plates to generate rolling motion of the embryo [Figure 2.3(a)]. This method is similar to the well-studied ball-plate problem using parallel plates [107,108], and can be applied to the manipulation of embryos. Rotating embryos by rolling has been reported using microfluidic channels [8], specialized microgrippers [37,46] and contact rolling using a micropipette on significantly larger (1 order of magnitude) zebrafish embryos [39, 61]. In this thesis, we aim to apply the well-studied ball-plate problem using parallel plates for automated position and rotation control of mouse embryos, fabricated using inexpensive and readily available components. The goal is to achieve automated embryo rotation control for use with human-sized embryos without introducing overly complex and specialized devices.

In the parallel plate system described here, the upper plate has three translational degrees of freedom ($X Y Z$ axes), while the bottom plate only has two ($X Y$ axes). For the purpose of kinematic analysis, only the $X Y$ axes of each parallel plate are considered, and the embryo is assumed to be in simultaneous contact with both plates. The kinematic equations (2.1)-(2.5) govern the embryo motion under parallel plate manipulation, using the coordinate system shown in Figure 2.3. The embryo is modeled as a driftless nonholonomic system with the following state variables:

\[
q = \begin{bmatrix} p \\ \alpha \end{bmatrix}, \quad p = \begin{bmatrix} x \\ y \end{bmatrix}, \quad \alpha = \begin{bmatrix} \theta \\ \phi \end{bmatrix}
\]

(2.1)

where $q$ is the pose of the embryo, defined by the position $p$ split into $x$ and $y$ coordinates, and orientation $\alpha$ split into longitude $\theta$ and latitude $\phi$ [Figure 2.3(c)]. $u^{upper}$ and $u^{lower}$ represents the position of the upper and lower plates respectively in Cartesian coordinates, and are considered the control inputs:
Chapter 2. Embryo Position and Rotation Control

Figure 2.3: Embryo rotation using parallel plates: (a) setup schematic, (b) position coordinate system, (c) orientation coordinate system

\[
\mathbf{u}^{\text{upper}} = \begin{bmatrix} u_x^{\text{upper}} \\ u_y^{\text{upper}} \end{bmatrix}, \quad \mathbf{u}^{\text{lower}} = \begin{bmatrix} u_x^{\text{lower}} \\ u_y^{\text{lower}} \end{bmatrix} \tag{2.2}
\]

Resultant control inputs of the embryo are proportional to the relative motion of the two plates, and can be split into translation \( \mathbf{u}_p \) and angular \( \mathbf{u}_\alpha \) components.

\[
\mathbf{u}_p = \frac{1}{2}(\mathbf{u}^{\text{upper}} + \mathbf{u}^{\text{lower}}), \quad \mathbf{u}_\alpha = \frac{1}{2}(\mathbf{u}^{\text{upper}} - \mathbf{u}^{\text{lower}}) \tag{2.3}
\]

It is important to note that this separation of translation and rotation components allows independent control of the two. As can be seen in (2.3), \( \mathbf{u}^{\text{upper}} \) and \( \mathbf{u}^{\text{lower}} \) plate motions that are equal in magnitude but opposite in direction would render \( \mathbf{u}_p = 0 \) and only rotate the embryo, whereas plate motion equal in both magnitude and direction would render \( \mathbf{u}_\alpha = 0 \) and only impart translational motion of the embryo. Any deviation from these two cases would result in simultaneous translation and rotation of the embryo, defined by:

\[
\dot{\mathbf{p}} = \lambda_p \mathbf{u}_p \tag{2.4}
\]

\[
\dot{\mathbf{\alpha}} = 2\pi\lambda_\alpha \begin{bmatrix} -\sin \theta/\cos \phi & \cos \theta/\sin \phi \\ -\cos \theta/\cos \phi & -\sin \theta/\sin \phi \end{bmatrix} \frac{\mathbf{u}_\alpha}{C} \tag{2.5}
\]

where \( \lambda_p \) and \( \lambda_\alpha \) models the reduction in transferred translational or rotational rolling motion from slippage respectively, and \( C \) is the embryo circumference perpendicular to the axis of rotation. The slippage coefficients \( \lambda_p \) and \( \lambda_\alpha \) may be different, as a result of the embryo being carried by the bottom plate as it translates, or the embryo is pushed as a result of Couette flow between the parallel plates. Experimental measurements observed \( \lambda_p, \lambda_\alpha \in [0.3, 1] \) and changes from experiment to experiment. Shin et al. observed \( \lambda = 0.6 \) for embryo rolling along the bottom of a microfluidic channel [8].
As defined in (2.5), embryo rotation, given a certain plate input $\dot{u}_a$, is affected by embryo circumference $\dot{\alpha} \propto \frac{1}{h} \dot{u}_a$. To achieve contact-based rolling, the parallel plates may deform the embryo [Figure 2.4(a)], leading to changes in $C$ and affecting the relationship between plate motion and embryo rotation. To determine changes in $C$ from compression, the embryo is modeled as a sphere filled with an incompressible fluid and an elastic outer membrane, similar to a water-filled balloon [47, 109, 110]. Thus, volume is constant whereas surface area is variable depending on the embryo shape due to deformation. Using planar analysis, this embryo model would have constant area $A_0$ and variable circumference $C$ based on the embryo height $h$. In a simplified planar analysis, we assume that the deformed embryo shape is similar to that of a capsule, notably a rectangle with semicircles at the ends with a deformed embryo of height $h$, middle section length $L_C$, and semi-circle radius $h/2$ [Figure 2.4(a)]. Assuming constant area $A_0$, the relationship between $h$ and $L_C$ can be found using (2.6) by solving for $L_C$. Circumference $C$ is defined generally by (2.7). By combining (2.6) and (2.7), the resulting $C$ can be calculated using (2.8).

\[
A_0 = \text{constant} = \frac{\pi}{4} h_0^2 = \frac{\pi}{4} h^2 + L_C h \tag{2.6}
\]

\[
C = 2L_C + \pi h \tag{2.7}
\]

\[
C = \frac{\pi h_0^2 - h^2}{2} + \pi h \tag{2.8}
\]

where $h_0$ is the initial undeformed embryo height. For $h_0 = 100 \ µm$, the relationship between percentage change in circumference versus embryo height $h$ is plotted in Figure 2.4(b). For a 10 $µm$ deformation where $h_0 = 100 \ µm$ and thus $h = 90 \ µm$, there is a 0.54 $\%$ increase in $C$, which would lead to an angular error of $\epsilon_{\alpha} = 1.8^\circ/\text{rev}$. Thus for small deformations, there is a negligible effect on rotation kinematics from changes in embryo circumference.
Figure 2.5: Experimental setup: (a) schematic of setup and (b) fabrication of the upper and lower plates

2.2 Experimental Setup

A schematic of the experimental setup is shown in Figure 2.5(a). Experiments are performed on a standard inverted microscope with Hoffman modulation contrast capability (Nikon Ti-U) with a robotic micromanipulator (Scientifica Patchstar Micromanipulator) to control the motion of the custom fabricated upper plate [Figure 2.5(b)]. A two degree of freedom (DOF) motorized stage (Prior Proscan III) on the microscope actuates the bottom plate. The micromanipulator has three Cartesian (XYZ-axes) translational DOF, and the XY-axes are aligned with those of the camera frame and the motorized stage. Thus, both upper and lower plates combine for a 5-DOF system, but only XY-axes of micromanipulator are used during embryo manipulation. Images are captured at 1920 px x 1080 px using a standard camera (QImaging optiMOS) with a 10x magnification microscope objective, leading to a pixel density of 1.539 px/µm. Equipment control and automation algorithms are implemented in commercial software Mathworks MATLAB. Misalignments between the upper plate XY-axes, the bottom plate XY-plane and the camera can be corrected by manually measuring the angular offset and adjusting the inputs $u^{upper}$ and $u^{lower}$ accordingly.

The upper plate system, as shown in Figure 2.5(b), is fabricated using an aluminum rod, a ball-joint linkage, and a 15 mm x 25 mm x 1 mm glass slide as the upper plate. The upper plate is affixed to the ball-joint linkage using epoxy. The bottom plate is a 75 mm x 50 mm x 1 mm glass slide with PDMS walls to create a well and retain liquid. It should be noted that the upper plate should be submerged in liquid, else there may be random hydrodynamic effects during manipulation. The bottom plate was taped onto the stage to prevent slippage. Prior to embryo manipulation, upper plate alignment is achieved manually by making light contact between the two plates, allowing the ball-joint linkage to automatically correct any misalignment of the two plates. While
still in light contact, the ball-joint linkage is then fixed in place by applying hot melt adhesive on the ball joint to prevent rotation. This turns the system into a high stiffness joint, which is required to eliminate joint play and backlash during upper plate control. Correct spacing is achieved by manually measuring focal plane distance between the two planes using marks made on the corresponding surfaces, and upper plate contact is further determined through observation of embryo motion. Embryo motion response to plate motion differs when the plate is near the embryo compared to when the plate is in contact with the embryo. While a certain amount of embryo deformation is unavoidable to achieve contact-based rolling, excessive deformation could plastically deform or rupture the embryo, and should be avoided. It should be noted that the spacing in between the parallel plates (approximately 100 µm) is large enough such that micropipettes used for blastomere extraction (14 - 45 µm in diameter) are able to fit in between the plates and perform blastomere extraction as needed during the subsequent biopsy steps.

2.3 Rotation Tracking

To achieve robust rotation control required for automation, closed loop feedback control must be used. This requires the development of a method to derive and track the orientation of an embryo. While most manuscripts report rotation control of oocytes with polar bodies [2,7,8,39,61], PGD is performed on cleavage stage embryos, and thus the methods developed in this chapter aim to apply rotation control to cleavage-stage embryos. Unlike oocytes with distinctive polar bodies, cleavage-stage embryos lack a reliable marker for embryo orientation, rendering rotation tracking very difficult. In this section, a method for tracking the orientation of 2-cell cleavage stage embryos using a geometric model is described.

The first step to tracking embryo rotation was to develop a method for segmenting the different components of the embryo. Automatic embryo analysis and different methods for segmenting embryo components have been reported by others [18,19,22,44,100,111]. The strength of these methods lies in the segmentation of each individual blastomere. While useful, segmenting each individual blastomere is not necessary for the proposed rotation tracking method. Instead, blastomeres are segmented as a whole, using a texture based method described in detail in Section 3.2.1, simplifying the computation. Conversely, the zona pellucida is segmented using Canny edge detection applied with the MATLAB edge() function and closed using morphological operations. The embryo center was estimated as the centroid of the segmented embryo. Figure 2.6 shows an example of cleavage-stage embryo rotation.
Figure 2.6: Time lapse of a rotating 2-cell embryo. Segmented embryo and blastomeres are outlined in white. Embryo center is shown with a white triangle, whereas the blastomere center is marked with a yellow circle.

Geometrical Modeling

The orientation of a 2-cell embryo can be derived with the aid of a geometric model. In the case of 2-cell embryos, the two blastomeres together are modeled as a capsule (similar to a cylinder with spherical ends) with major axis $L$ and minor axis $m$, as shown in Figure 2.7(a). The embryo is considered as a spherical rigid body. The geometric model and segmentation of the different embryonic components allow two factors to be analyzed: 1) the relative motion between the centroid of the embryo and centroid of the blastomeres, and 2) the change in the observed projection of the blastomeres. Two angles of rotation $\theta$ and $\phi$ define the longitude and latitude of the blastomere respectively, with a third angle $\psi$ defined as the angle between the $xy$-plane and $L$, as shown in Figure 2.7(a). Angle $\psi$ represents orientation of the blastomeres within the embryo. $r$ is the vector between the center of the embryo to the centroid of the blastomere. Images taken during microscopy capture the projection of the embryo in the $xy$-plane as seen in Figure 2.7(b), thus these images depict $L_{xy}$, $m_{xy}$, and $r_{xy}$, the projections of $L$, $m$ and $r$, respectively, in the $xy$-plane.

The three angles $\theta$, $\phi$ and $\psi$ can be solved for by comparing the projected lengths $L_{xy}$, $m_{xy}$, and $r_{xy}$ to their respective full lengths $L$, $m$ and $r$. And thus, the orientations of the embryo and blastomere can be identified and tracked. Angle $\theta$ is solved by determining the angle of vector $\vec{r}_{xy}$ to the $x$-axis, as given by (2.9). Angle $\phi$ is determined by comparing the projection $r_{xy}$ to $r$, using (2.10). Angle $\psi$ is calculated by observing the relationship between $L$ and $L_{xy}$. Since the blastomeres are modeled as a 3D capsule, when $\psi \neq 0$, a portion of the minor axis $m$ is shown in the projection $L_{xy}$, as described
Figure 2.7: Geometrical modeling of 2-cell embryo using a capsule model; (a) definition of the coordinate system and parameters for rotation tracking, (b) overlay of the capsule model projection on a microscope image.

Figure 2.8: Validation of geometric model approach using a CAD model of a 2-cell embryo. Angles listed are set values vs geometric model-derived values. Embryo center is shown with a white triangle, whereas the blastomere center is marked with a yellow circle.

by (2.11). Angle $\psi$ can thus be solved using (2.12).

\[
\theta = \tan^{-1} \frac{\vec{r}_{xy} \cdot \hat{j}}{\vec{r}_{xy} \cdot \hat{i}} \tag{2.9}
\]

\[
\phi = \cos^{-1} \frac{|r_{xy}|}{|r|} \tag{2.10}
\]

\[
L_{xy} = (L - m) \cos \psi + m \tag{2.11}
\]

\[
\psi = \cos^{-1} \frac{L_{xy} - m}{L - m} \tag{2.12}
\]

where $\hat{i}$ and $\hat{j}$ are the unit vectors for the $x$- and $y$-axes respectively. To validate the geometric model, a 3D CAD model of a 2-cell embryo is constructed and rotated to different known positions. The geometric model approach is applied to these different
positions and the angles are calculated (Figure 2.8). The model-derived angles match closely with the set angles, verifying that the proposed approach is valid. Discrepancies in angle values are attributed to information lost during digitization of the images.

**Rotation Tracking Experiments**

Rotation tracking using the geometric model is applied to multiple rotating embryos at different angular velocities. A set of embryo images is shown from a video in Figure 2.6 and the corresponding tracked angular positions are shown in Figure 2.9. To determine \( L, m \) and \( r \) used in the calculations for angles \( \phi \) and \( \psi \), a calibration step must first be performed by fully rotating the embryo in all axes to be able to image and determine the maximum \( L_{xy}, m_{xy}, \) and \( r_{xy} \), and assume that these values are in fact the true \( L, m \) and \( r \). A thorough calibration must be performed, as inaccuracies in the values obtained for \( L, m \) and \( r \) may cause errors in rotation tracking.

A major drawback to using projections is the inability to determine the sign for angles \( \phi \) and \( \psi \). Thus, angles \( \phi \) and \( \psi \) are restricted to \([0°, 90°]\). In the particular case of angle \( \theta \), since the blastomere and cell centroids are very close to each other, small spatial variations cause large changes in \( \theta \) angle values, as seen between frames 11 and 13 in Figure 2.9. Assuming constant inputs and accounting for minor disturbances in the system, one would expect the embryo to continue rotating with generally the same angular pattern. This phenomenon is observed in the cyclical nature of the angle values in Figure 2.9, further validating the proposed rotation tracking method. To circumvent the apparent angle discontinuity and improve range of motion tracking, it may be possible to use information regarding the system input and use state estimators such as Kalman filtering. These experiments demonstrate that the proposed geometric model is capable of providing orientation feedback for 2-cell embryos. Unfortunately, this method cannot...
be applied to 4- or 8-cell embryos, as the geometries of 4- and 8-cell embryos are not unique.

## 2.4 3D Mapping and Zona Breaching Optimization

As mentioned at the beginning of this chapter, there are two objectives of embryo position and orientation control: 1) to determine which blastomere to extract and the location along the zona to perform zona breaching, and 2) to align the tools for zona breaching and blastomere extraction with the chosen blastomere and zona breaching location. This section delves deeper into how these objectives are achieved. The first objective requires complete knowledge of the layout of the embryo in order to maximize the distance between the blastomeres and the zona. This can be achieved by first generating a 3D map of the embryo [Section 2.4.1], and then using the 3D map for zona breaching location optimization [Section 2.4.2]. While certain groups have reported the zona breaching location optimization by only analyzing the current field of view [19], this optimization results in only a local optimum, as a better optimum may be out of view. A full 3D map of the embryo would allow a global optimum to be found. The second objective uses the result of the optimization to reposition and reorient the embryo according to a reorientation control strategy [Section 2.4.3].

As a note, the chosen method of zona breaching in this thesis is laser zona drilling. Thus, the terms zona breaching location and ablation zone are used interchangeably. The optimal zona breaching location will also be referred to as the optimal ablation zone (OAZ) in the following sections.

### 2.4.1 3D Mapping

Compared to microscopy methods that can achieve high resolution images in 3D (e.g.: confocal microscopy [102], holographic microscopy [112] and sheet light microscopy [113]), conventional microscopy is at a major disadvantage as captured images are only a two-dimensional (2D) projection of 3D objects. Much useful information is lost during conventional microscopy imaging. Despite these disadvantages, it is possible to generate 3D reconstruction of embryos using conventional microscopy. This section describes a method to reconstruct a 3D map of the embryo structures for analysis via a series of 2D images taken using Hoffman modulation contrast microscopy at regular angular intervals. Any rotation method may be used. In this thesis, parallel plate motion is used to roll the embryo to obtain images at different angular positions, similar to the timelapse shown
Figure 2.10: Superimposed time-lapse images of embryo rotating while rolling in Figure 2.10. The advantage of this method is that no specialized imaging equipment is needed.

The proposed 3D mapping method uses images taken at specific known angular intervals using Hoffman modulation contrast microscopy. In ideal non-slip rolling conditions, change in embryo orientation $\Delta \alpha$ can easily be tracked by the fact that $\Delta \alpha = 2\pi \lambda \Delta p / C$, where $C$ is embryo circumference defined in (2.7), and slippage $\lambda = 1$. Unfortunately, experiments reveal that $\lambda \neq 1$, and variable from trial to trial. For the examples shown below, one full embryo revolution is segmented manually, as shown in Figure 2.10. The measured distance for one revolution $\Delta p_{1\text{rev}}$ can then be used to calculate slippage $\lambda$. Alternatively, within a single revolution, the specific angular interval between frames $\Delta \alpha$ is calculated by interpolation using the linear displacement of the embryo centroid $\Delta p$, according to $\Delta \alpha = 2\pi \Delta p / \Delta p_{1\text{rev}}$.

Computer vision processing algorithms are used to segment the different structures of the embryo, notably the blastomeres, inner zona boundary and outer zona boundary. Segmentation algorithms are described in detail in Section 3.2. In summary, the blastomeres are segmented in bulk using image texture, as blastomeres have high local intensity variance comparatively to all other components. Conversely, both the inner and outer zona boundaries were segmented using edge-based methods in combination with morphological operations. Binary masks of the different embryo structures are then generated using these segmentations. Using these masks, a subtractive method is used to generate the 3D model of the embryo. Briefly, the subtractive method starts with a solid initial volume $V$, and progressively removes parts of $V$ based on the projections of the different segmented structures at different angles. The subtractive method will now be described in detail and is illustrated in Figure 2.11:

1. A volume $V$, initially a solid volume, has parts progressively removed to form the 3D generated map

2. Each image in the series of images taken over one revolution represents a 2D pro-
Figure 2.11: Overview of the subtractive method for 3D mapping

1. Projection of the embryo $P_{\{\theta, \phi\}}$ at a specific orientation, where the subscripts $\{\theta, \phi\}$ denote the angles at which the projection is taken as defined in Section 2.1.2.

2. Projection $P_{\{\theta, \phi\}}$ is extruded perpendicularly to fill a similar sized volume as $V$.

3. The extruded projection is then rotated according to angles $\{\theta, \phi\}$ to match the orientation of the projection relative to the embryo for the rotated extruded projection $V_{\{\theta, \phi\}}$. Single pixel artifacts appearing from the rotation step are removed using image erosion and then dilation.

4. $V_{\{\theta, \phi\}}$ is inverted to obtain $V'_{\{\theta, \phi\}}$

5. $V'_{\{\theta, \phi\}}$ is subtracted from the total volume $V$

6. Steps 1-6 are then repeated for all available projections.

It is important to note that a rotated extended projection (Step 4 of Figure 2.11) will have edges that either extend outside of $V$ or do not reach the edges of $V$. Areas that extend outside of $V$ are ignored. Given the nature of the subtractive method, if the working volume $V$ is sufficiently large, the missing overlaps of $V_{\{\theta, \phi\}}$ and $V$ are not crucial for the reconstruction to be successful. It is possible to account for reconstruction inaccuracies by using non-binary subtraction during reconstruction, and choosing a threshold where enough projections display the same shape (e.g.: 80% of projections display the same shape).

Reconstruction fidelity is verified using a 4-cell embryo model generated in SolidWorks. Images are taken at different angles and then reconstructed using the above
Figure 2.12: (a,b) Computer generated 3D map of a 4-cell embryo in (a) SolidWorks and (b) reconstructed blastomeres using 3D mapping algorithm; (c,d) Reconstructed 3D map of blastomeres from Figure 2.10 with (c) blastomeres only, (d) zona partially removed to reveal blastomeres for demonstration purposes.

Figure 2.12 shows the comparison between the SolidWorks model [Figure 2.12(a)] and the corresponding reconstructed 3D map of the blastomeres [Figure 2.12(b)]. Surface reconstruction of the 3D model is performed in ImageJ software using the Volume Viewer plugin. In this example, 24 images are taken at 15° intervals to create a 141 x 141 x 141 voxel (volume pixel) mapping of the embryo. Although it may seem like a low amount of voxels, the resolution would lead to 0.7 µm x 0.7 µm x 0.7 µm per voxel, assuming it fully encompasses a 100 µm diameter embryo. These set of images requires 34 s to process in MATLAB using multicore processing on a desktop PC with an Intel Core i7-4770 CPU at 3.40 GHz and 24 GB of RAM. Needless to say, reconstruction fidelity and computation time are directly proportional to the number of pictures available for reconstruction and the volume resolution.

Given that the 3D reconstruction relies on projections, certain features, specifically valleys, are not be reconstructed properly. These enclosed valleys, which are indents between multiple objects such as 3 touching blastomeres as seen in the bottom left section of Figure 2.12(b), appear flat after a certain depth. The lack of these valleys would change the apparent distance between the blastomeres and the zona, but would only affect ablation zone optimization, described in Section 2.4.2, in specific cases.

Next, the 3D reconstruction algorithm is applied to a 2-cell mouse embryo, as shown in Figure 2.10. The generated 3D map, which uses 74 projections and a V with 201 x 201 x 201 voxels, can be seen in Figure 2.12(c). Figure 2.12(d) shows the entire reconstruction with the zona partially removed to reveal the blastomeres for demonstration purposes. Similar valleys are seen between the two blastomeres and the polar body. There also exists striations in the reconstruction as a result of the discretization of V. As mentioned previously, increasing the number of voxels would improve the smoothness of the recon-
struction. Thus, using this mapping method, embryos can undergo 3D reconstruction, which can be used for analysis as shown in the next section.

2.4.2 Ablation Zone Optimization

The first objective of embryo position and orientation control is to determine which blastomere to extract and the location along the zona to perform zona breaching. As mentioned previously, the chosen method for zona breaching is laser zona drilling (LZD), which uses a laser to ablate the zona and discussed further in Chapter 3. With the knowledge of the embryo layout from the 3D map generated in Section 2.4.1, the zona breaching location can now be optimized to maximize the distance between the blastomeres and the zona, which leads to minimizing the temperature increase of the blastomeres during laser zona drilling. The location along the inner boundary of the zona $ZP_{inner}$ that maximizes the distance between the blastomeres $B$ and the zona is called the optimal ablation zona (OAZ). By generating a 3D map, ablation zone optimization can now be performed in 3D to find a global optimum, which would complete the first objective of embryo position and orientation control.

In LZD, a specific sized section of zona is ablated to create an entry point into the embryo, known as the ablation zone. Thus, ablation zone optimization is performed not by maximizing the distance of a single point, but maximizing the minimum distance from the blastomeres to the entire ablation zone. During optimization, a candidate ablation zone $AZ_c$ is defined as a subset of adjacent points within a radius $r_{AZ}$ of the $AZ_c$ center, located along the inner zona boundary $ZP_{inner}$. The optimal ablation zone (OAZ) is the candidate ablation zone that maximizes the minimum Euclidean distance $d$ to the blastomeres $B$. A brute force search steps through all possible $AZ_c$ along $ZP_{inner}$ to determine the OAZ:

$$\text{maximize } d \min d(AZ_c, B)$$
subject to $AZ_c \in ZP_{inner}$

(2.13)

To speed up the brute force search, a look up table for $d$ is populated during the optimization so that repeated calculations are not necessary. Figure 2.13 shows the OAZ locations for the 4-cell CAD model shown in Figure 2.12(a), and the 2-cell mouse embryo shown in Figures 2.10 and 2.12(d). Since the OAZ is defined symmetrically about a center, the OAZ can be referenced using only a single point, namely the OAZ center. Although three angles are technically required to fully define the orientation of the embryo, given that only a point is used to define the OAZ, a simpler two-angle model,
Chapter 2. Embryo Position and Rotation Control

40

Figure 2.13: Optimal ablation zone for (a) 4-cell embryo CAD model, and (b) 2-cell embryo from Figure 2.10. Blue indicates the zona, red indicates the blastomeres, and the green indicates the optimal ablation zone.

namely longitude $\theta$ and latitude $\phi$ as defined by $\alpha$ in (2.1), is sufficient. This simplification is important as it is one less variable required for trajectory planning during reorientation. This concludes the first objective of embryo position and orientation control.

2.4.3 Orientation Control Strategy

With the location of the OAZ known, the second objective of embryo position and orientation control is to align the tools for zona breaching and blastomere extraction with the OAZ. Thus, the next step is to reorient the embryo such that the OAZ is in position for subsequent PGD biopsy steps, namely LZD and blastomere extraction. In our experimental setup, we assume the micropipette for blastomere extraction is located on the right side of the embryo and aligned horizontally (Figure 2.14), thus the embryo should roll to the right side edge of the upper plate with OAZ orientation $\theta = 0^\circ$ and $\phi = 0^\circ$ to allow the micropipette to easily access the embryo. This also requires the embryo be close to the edge of the upper plate to allow the micropipette to reach the embryo. Orientation control strategies for ball and plate problems have been studied extensively [8,107,108]. Here, we introduce a simple orientation strategy that brings the embryo to a final position that satisfies the following criteria:

- Reorient embryo such that OAZ is at desired $\theta = 0^\circ$ and $\phi = 0^\circ$
- Position embryo near edge of upper plate

The proposed orientation strategy contains the following steps when starting from an initial position $\theta = \theta_0$, $\phi = \phi_0$, and is illustrated in Figure 2.15:
Chapter 2. Embryo Position and Rotation Control

Figure 2.14: Blastomere extraction step with extraction micropipette on the right side

Figure 2.15: Overview of the orientation control strategy

1. Roll embryo left \( u_{x,1} \) such that \( \theta \rightarrow 90^\circ \), where \( \phi \rightarrow \phi_1 \)
2. Roll embryo up/down \( u_{y,2} \) such that \( \phi \rightarrow 0^\circ \) (\( \theta = 90^\circ \))
3. Roll embryo right \( u_{x,3} \) until distance \( d_{MB} = \frac{\pi}{2} r \) from motion boundary (\( \theta = 90^\circ \), \( \phi = 0^\circ \))
4. Roll embryo up/down \( u_{y,4} \) such that \( \phi \rightarrow 90^\circ \) (\( \theta = \) undefined)
5. Roll embryo right \( u_{x,5} \) such that \( \theta \rightarrow 0^\circ \), \( \phi \rightarrow 0^\circ \)

All required motions are precomputed given initial angles \( \theta_0 \) and \( \phi_0 \). \( \phi_1 \) is an arbitrary intermediate stage as a result of Step 2 where \( \theta \rightarrow 90^\circ \). All plate motions required for each step are determined using (2.14). Directionality of steps depends on where the embryo is located in the camera field of view, such that the maneuver minimizes the chance of the embryo exiting the field of view.

\[
u_{x,1} = -\text{mod} \left( \frac{\pi}{2} - \theta_0, 2\pi \right) \ast r \]
\[
u_{y,2} = \pm \phi_1 r, \text{ where } \phi_1 \text{ is determined using (2.15)}
\]
\[
u_{x,3} = p_{MB,x} - d_{MB} - p_{x,3}
\]
\[
u_{y,4} = \pm \frac{\pi}{2} r
\]
\[
u_{x,5} = \frac{\pi}{2} r
\]
\[ y_1 = r \sin \theta_0 \cos \phi_0 \]
\[ z_1 = \sqrt{r^2 - y_1^2} \]
\[ \phi_1 = \text{atan2}(z_1, y_1) \]

where \( r \) is the radius of the embryo, \( \text{mod()} \) is the modulus function, \( p_{MB,x} \) is the \( x \) location of the motion boundary, \( p_{x,3} \) is the \( x \) coordinate of the embryo during Step 3. This orientation strategy provides a framework to reorient the embryo in an optimal fashion for the purposes of PGD biopsy, and can be performed in an open loop or closed loop fashion.

### 2.5 Parallel Plate Manipulation Experiments

In this section, the parallel plate system is characterized experimentally using a series of maneuvers with mouse embryos. Both open loop and closed loop control maneuvers are performed to demonstrate capability of the manipulation system for embryo position and orientation control. The application of these experiments is to ultimately apply the orientation control strategy defined in Section 2.4.3 using parallel plate manipulation to reorient the embryo to achieve the second objective of embryo position and orientation control.

#### 2.5.1 Open Loop Control

Multiple maneuvers are performed to evaluate the performance of the parallel plate system in an open loop fashion. Two special maneuvers, translation-only and rotation-only maneuvers, are possible given the independent control of the upper and bottom plates, as described by equation (2.3). Translation-only experiments occur when both plates move simultaneously in the same direction to negate any rotational motion. Figure 2.16(a) plots the motion of the embryo during a 520 μm x 260 μm rectangular maneuver. Only the corner points of the test maneuver are tracked. Translation-only motion is very repeatable, with a mean error \( e_p = 0.96 \) μm. This error is a result of slight embryo drift. As seen in the figure, motion was slightly angled as a result of misalignment of the two plates. No embryo rotation was observed, demonstrating that translation-only motion is feasible using this actuation method. Translation-only maneuvers are useful for LZD, where the embryo needs to be repositioned for different laser pulses without inducing any changes in orientation.
Figure 2.16: Open loop control using (a) translation only and (b) rotation only motion

A series of rotation-only experiments were performed, where the parallel plates moved at the same speed but in opposite directions. As the name of the maneuver implies, the embryo would ideally rotate without any translation relative to the camera frame. Rotation-only motion is useful to keep the embryo in view while rotating. Plates alternated between moving left/right and up/down directions for 520 $\mu$m in the $x$- and $y$-directions respectively. Figure 2.16(b) shows position tracking of one such experiment, where each data point represents one motion. A mean embryo motion $\Delta p = 7.5\%$ of the input plate motion was observed (average of 20 $\mu$m of embryo translation over 260 $\mu$m of theoretical embryo motion, if the parallel plates were not canceling out embryo motion).

Other open loop maneuvers are performed, including upper plate only and lower plate only motion. Similar to the results of the rotation-only experiments, embryo motion encountered drift, irregular slippage and motion bias. Given the erratic and unpredictable motion of the embryo during non-translation-only experiments, open loop control is deemed unfeasible. This points to the need for closed loop control.

### 2.5.2 Closed Loop Control

Closed loop control is often used to counteract disturbances in systems, model inaccuracies and generally unpredictable dynamics. The end result is usually a more accurate system response than open loop control and a higher chance to achieve the desired results. Given the inadequacy of open loop control for embryo manipulation using parallel plates, closed loop control is implemented and demonstrated using position and rotation control experiments. In the parallel plate system setup for embryo manipulation, closed loop feedback is achieved using computer vision. Embryo position and orientation are extracted from images taken from a camera, and compared to the desired position and orientation values. A controller attempts to correct position and orientation errors by actuating the parallel plates. A block diagram of the closed loop control is shown in
Position Control

Closed loop embryo position control experiments are performed. Only the upper plate is used in these experiments for the control of embryo motion. Embryo position feedback is obtained by segmenting the embryo as a whole and using the centroid as the embryo center. A simple proportional-integral (PI) control is implemented for manipulator velocity control, defined by (2.16):

\[
\begin{align*}
e_p[t] &= p_{des}[t] - p_{cur}[t] \\
e_i[t] &= e_p[t] \Delta t + e_i[t - 1] \\
\dot{u}_{\text{upper}}[t] &= K_p e_p[t] + K_i e_i[t]
\end{align*}
\]

where $K_p$, $e_p$, $p_{des}$ and $p_{cur}$ are the proportional error constant, position error, desired position and current position, respectively; $K_i$, $e_i$, and $\Delta t$ are the integral error constant, integral error and time step size, respectively. Figure 2.18 shows an example of a position control experiment where the embryo follows the path of a circle with a 200 $\mu$m diameter over 25 s. A mean error $e_{p,\text{mean}} = 12.0$ $\mu$m is observed throughout the maneuver. It is important to note that the controller uses non-optimal parameters, as the purpose was to simply demonstrate closed loop control feasibility. Properly tuned $K_p$ and $K_i$ constants could improve controller performance. Multiple trials are performed, and results are very repeatable. These experiments demonstrate that closed loop position control is indeed feasible, and provides significant improvements over open loop control.

Rotation Control

In order to demonstrate the capacity of parallel plates for closed loop orientation control, embryo rotation control experiments are performed. For demonstration purposes, a simplified but representative maneuver using a simplified orientation feedback is used.
Figure 2.18: Time lapse of a closed loop embryo position control experiment. “o” indicates start point, “x” indicates end point

Similar to the geometric model outlined in Section 2.3, the blastomeres of a 2-cell embryo are segmented as a whole, and the eccentricity of their shape is tracked as the orientation state variable. Eccentricity, in simplified terms, is a measure of roundness. Using the shape of the blastomeres as a guide, the goal of the embryo rotation control experiments is to minimize the eccentricity of the blastomeres by stacking the blastomeres on top of each other along the z-axis. An example of one such maneuver is shown in Figure 2.19.

The blastomere eccentricity is calculated by first segmenting the blastomeres using a texture segmentation algorithm described in Section 3.2.1, and then blastomere eccentricity $\epsilon$ and major axis angle $\alpha$, are calculated using the MATLAB function `regionprops()`. In Figure 2.19(a), eccentricity value $\epsilon$ is graphically represented by the green line using an arbitrary length scale, whereas the major axis angle $\alpha_\epsilon$ is the angle of the green line with respect to the x-axis. A simple proportional control is used to drive the upper plate along $\alpha_\epsilon$ based on the difference between current eccentricity $\epsilon_{\text{cur}}$ and desired eccentricity $\epsilon_{\text{des}}$, as defined by (2.17) and is represented in each frame by a red cross:

\[
\epsilon_\epsilon[t] = \epsilon_{\text{cur}}[t] - \epsilon_{\text{des}}
\]

\[
u^{\text{upper}}[t] = K_\epsilon \epsilon_\epsilon[t] \begin{bmatrix} \cos \alpha_\epsilon \\ \sin \alpha_\epsilon \end{bmatrix}
\]

(2.17)

where $K_\epsilon$ is the proportional error constant, and $\epsilon_\epsilon$ is the eccentricity error. The maneuver
Chapter 2. Embryo Position and Rotation Control

Figure 2.19: Closed loop embryo rotation control experiment: (a) Time-lapse of one experiment, green line depicts eccentricity using an arbitrary scale, red cross indicates \( u_{upper} \); (b) Blastomere eccentricity over the course of the experiment

is considered completed when the blastomere eccentricity \( \epsilon_{cur} < \epsilon_{des} \), where \( \epsilon_{des} = 0.5 \). Blastomere eccentricity values over the course of the maneuver shown in Figure 2.19(a) are given in Figure 2.19(b). Repeated tests show these results to be very reproducible, and demonstrate that the parallel plate system can be used for closed loop orientation control of embryos.

2.6 Chapter Conclusion

In summary, a novel parallel plate system was introduced for the position and orientation control of human-sized embryos, the first step of PGD biopsy, and demonstrated using
mouse embryos. In the parallel plate setup, the embryo is placed in between the parallel plates and modeled as a driftless non-holonomic system. Kinematic equations for parallel plate manipulation were derived. A geometric model was developed for rotation tracking of a 2-cell cleavage stage embryo. A non-destructive 3D mapping algorithm of the embryo using Hoffman modulation contrast microscopy was developed by compiling images taken at specific known angle intervals and using a subtractive method. This mapping was then used for optimization of the zona breaching location by determining the location on the zona that maximizes the distance between the blastomeres and the zona. Parallel plate characterization experiments were also performed on mouse embryos. Open loop control tests produced inconsistent results, which indicated that closed loop control is required. Closed loop position and rotation control using computer vision feedback was demonstrated, and determined to be feasible and repeatable. Thus, by combining the algorithms developed in this chapter with closed loop control, position and orientation control of the embryo can be achieved.
Chapter 3

Optimization and Automation of Laser Zona Drilling

Single cell manipulation often requires accessing the inside of a cell, particularly in embryo biopsy, where a cell is removed from inside the embryo. Preimplantation genetic diagnosis (PGD), a form of embryo biopsy that removes a blastomere from a cleavage-stage embryo for genetic profiling, is a delicate procedure with potentially severe consequences. In order to gain access to the embryo internals, a pathway must be made through the outer shell of the embryo, also known as the zona pellucida (ZP) as shown in Figure 3.1(a). This process of generating the pathway is called zona breaching (ZB), and involves removing parts or tearing of the zona to create an opening. Zona breaching is the second step in PGD biopsy, where the first step involves repositioning and reorienting the embryo, and the third step extracts a blastomere from the embryo. Given that these operations are currently performed manually with low success rates, the overall thesis objective is to demonstrate the feasibility of automating different portions of the embryo biopsy procedure. Specifically, this chapter outlines methods for the automation of zona breaching.

As reviewed in the biopsy techniques overview in Section 1.2.3, different methods exist to perform zona breaching [35], such as using acid Tyrode [69], a sharp micropipette [10] or a non-contact laser [11, 71], each with their own drawbacks. Traditionally, these operations are typically performed using acid Tyrode or a sharp micropipette, but recent years have seen a major switch to the use of non-contact lasers, a procedure called laser zona drilling (LZD) [32]. The mechanics behind LZD involve using a laser with high absorptance in water to quickly increase the temperature in a localized area [Figure 3.1(b)]. Beyond a certain threshold temperature, the zona will become ablated, and a gap will be created in the zona. By using multiple laser pulses in a close proximity to each other,
Figure 3.1: (a) Zona breaching terminology definition, (b) embryo with opening after undergoing LZD

an opening can be created in the zona to allow access to the blastomeres [Figure 3.1(c)]. Using LZD to remove enough zona to gain access to the embryo interior is also known as full thickness ablation. The biggest drawback of LZD is the possibility of thermal damage to the embryo given that LZD will locally raise temperatures in excess of 200°C. While tools have been created to allow embryologists to attempt to minimize this heating, manually performing LZD still requires the use of subjective decision making based on experience. The application of automation to LZD would lead to objectively-determined LZD parameters to increase embryo safety through minimizing unwanted temperature increase and procedure repeatability. The two methods for minimizing temperature increases are to either position the laser pulses further away from the blastomeres, or use a shorter pulse duration.

In this chapter, methods for the optimization of multi-pulse LZD are described and demonstrated for automated zona breaching. The goal of LZD optimization is to minimize the temperature increase of the blastomeres while creating an adequately sized hole for blastomere extraction, the third step of PGD biopsy. LZD parameters include the number of laser pulses, the location of each pulse and the pulse duration of each pulse. First, the thermal effects of LZD are modeled and analyzed both in simulation and experimentally, and are described in Section 3.1. Second, ablation zone optimization is performed in Section 3.2 by analyzing the relative spatial positioning of different embryonic structures, where the point along the zona farthest away from the blastomeres is chosen as the optimal ablation zone. Third, coverage optimization, described in Section 3.3, applies the thermal model to determine the optimal set of LZD parameters using genetic optimization to minimize heating at the blastomeres. Finally, successful automated zona ablation experiments are presented in Section 3.4, demonstrating that automating laser zona drilling is feasible. Work presented in this chapter has been presented at a conference [114] and published in a peer-reviewed journal [115].
3.1 Thermal Modeling

In order to optimize LZD and minimize the heating of the blastomeres, the heating effect from a laser pulse must first be understood. This heating is described using a thermal model. The purpose of a thermal model is to be able to theoretically predict the heating effect of LZD accurately without the need for sensors. As mentioned previously, zona ablation occurs once the local temperature of the zona surpasses a specific temperature threshold [71]. Therefore, a thermal model of LZD is useful to predict how much of the zona will be ablated, specifically how far away from the beam center, and the temperature increases in the surrounding area. This prediction allows LZD to be optimized based on calculation of the shape of the ablation zone and the increase in temperature experienced by the embryo for a set of laser pulses. The temperature responses of a single pulse case and multi-pulse case are modeled in this section.

3.1.1 Laser Ablation Simulation

Simulation Setup

A laser analysis approach similar to that performed by Tadir and Douglas-Hamilton on a 1480 nm laser [71] is used and implemented in the simulation package COMSOL Multiphysics (Stockholm, Sweden), as shown in Figure 3.2. The environment is modeled using a 500 µm x 500 µm x 285 µm cube of water with an initial temperature \( T_0 = 37^\circ C \), and constant boundary temperatures of \( T_{\text{boundary}} = 37^\circ C \). In vitro fertilization (IVF) and PGD procedures are typically performed in petri dishes in small droplets of biopsy medium, which have very similar physical properties to water. Thus, to maintain similar laser performance between procedures, the embryo should be placed at the bottom of the petri dish to minimize extraneous laser beam absorption by the surrounding medium. Thus, given the diameter of a mouse embryo \( d_{\text{emb}} \), the center plane of the embryo and the laser beam focal point would be located at \( z_{\text{beam}} = \frac{1}{2} d_{\text{emb}} \) from the bottom of both the petri dish and the simulation volume. One study states that the average diameter of a mouse embryo is \( d_{\text{emb}} \approx 70 \mu m \) [116], and thus \( z_{\text{beam}} = 35 \mu m \) is used for these simulations. The only mechanism of heat transfer modeled within the water is conduction [11], as defined by (3.1):

\[
\vec{q} = -k \vec{\nabla} T
\]  

(3.1)

where \( \vec{q} \) is the heat flux, \( k \) is the thermal conductivity of the medium, and \( \vec{\nabla} T \) is the temperature gradient. Convective heat transfer is not considered, as the time frame in
Figure 3.2: Simulation setup in COMSOL Multiphysics. Block represents water, cylinder represents beam waist, points represent sensor points, beam convergence/divergence shown using discretized disks: (a) single pulse modeling, (b) multi-pulse modeling (beam convergence/divergence not shown), (c) close up of multi-pulse laser ablation pattern and sensor points

which laser ablation occurs is very small. Similarly, radiative heat transfer is assumed negligible and ignored. The embryo is assumed to have similar thermal properties as water [11], with variable thermal conductivity $k_{\text{water}}$ according to the default definition in COMSOL, outlined in (3.2):

$$k_{\text{water}} = -0.869 + 8.95 \times 10^{-3}T - 1.58 \times 10^{-5}T^2 + 7.98 \times 10^{-9}T^3$$  \hspace{1cm} (3.2)

The profile of a laser beam is similar to that of an hourglass, where the light rays converge conically towards a focal point, after which the light rays will diverge conically. At the focal point, rather than an infinitesimally small convergence point, the laser beam waist is modeled as a cylinder with radius of $r_{\text{beam}} = 2 \, \mu\text{m}$ and height of $h = 12 \, \mu\text{m}$ [71]. In simulation, these cylinders are modeled as a heat source. These cylinders can be placed at different locations to model a single laser pulse or an array of laser pulses, each with individually configured timing and pulse duration [Figure 3.2(c)], depending on the case analyzed. Absorptance $\alpha$ at 1480 nm is assumed to remain constant at $\alpha = 27 \, \text{cm}^{-1}$, which results in the upper boundary of the temperature response. The laser beam waist heat source $S_0$, derived in [71], is expressed as a Gaussian beam and governed by (3.3):

$$S_0 = \frac{\alpha k_{\text{att}} P}{\pi r_{\text{beam}}^2} e^{-2(r/r_{\text{beam}})^2}$$  \hspace{1cm} (3.3)

where $P$ is the beam power, $k_{\text{att}}$ is the beam attenuation factor, $r_{\text{beam}}$ is the beam radius, and $r$ is the radial distance from the beam center. Beam attenuation factor through the column of water is assumed to be $k_{\text{att}} = 0.82$ [71]. For simplicity, the laser beam waist was modeled as a uniform beam, rather than as a Gaussian one. This simplification results in generating the upper bound of the temperature response. Furthermore, the
converging and diverging of the laser beam will also contribute to the thermal response, and is modeled using (3.4):

\[ S_\pm = \frac{\alpha P}{2\pi(1 - \cos\theta)} \frac{1}{r^2 + z^2} e^{-2\left(\frac{\arctan(r/z)}{\theta}\right)^2} e^{\pm \alpha \sqrt{r^2 + z^2}} \]  

(3.4)

Where the beam half angle \( \theta \) is calculated from \( \text{NA} = n \sin \theta \), where \( \text{NA} = 0.6 \) for the modeled laser described in Section 3.1.2 and \( n \) is the index of refraction of water \( n_{\text{water}} = 1.33 \). The converging and diverging heat sources are discretized using a series of thin cylinders with appropriate heat source coefficients, and shown in Figure 3.2(a). Rather than using Gaussian functions, the converging/diverging heat sources were each modeled uniformly by integrating the source function over the radius of the cylinder. The resulting temperature response is measured at multiple points, labeled as “sensor points”, which are typically located at the closest edge of the blastomeres to the ablation zone. The exact distribution of laser pulses and sensor points will be outlined in Section 3.1.3.

**Superposition approximation method of thermal response**

In order to reduce the computational load when performing repeated calculations of multi-pulse laser zona drilling during optimization, a linear superposition approximation of the thermal response is derived. For a series of \( n \) laser pulses delivered sequentially, the temperature response of a single laser pulse is denoted by \( T_{p,i}(t, x) \), where \( p \) is the pulse duration, \( i \in 1...n \) is the \( i \)th laser pulse, \( t \) is the time after the start of the \( i \)th laser pulse and \( x \) is the distance from the \( i \)th laser pulse beam center. \( T_{p,i}(t, x) \) is zero for negative values of \( t \). Assuming a linear response, the total temperature response \( T_{tot} \) from multiple laser pulses can then be calculated by superposition of each individual laser pulse using (3.5):

\[ T_{tot}(t, x) = \sum_i T_{p,i}(t - \delta_i, x) \]  

(3.5)

where \( \delta_i \) is the time delay until the start of the \( i \)th laser pulse. The temperature response of a single sensor point to eight 100 \( \mu \)s laser pulses using the superposition approximation is compared to a full COMSOL simulation. The time delay \( \delta_i \) between laser pulses is 5 ms. The mean percentage error between the full simulation and approximation method was \(<2\%\), demonstrating that the superposition approximation method is a valid approach.

Furthermore, the temperature response at the beam center from a single 600 \( \mu \)s laser pulse, as shown in Figure 3.3, returns to within 0.25°C of ambient temperature in 15
ms, thus we assume that given sufficient time between laser pulses $\delta_i$, successive pulses will not have any significant cumulative effect on each other while remaining within acceptable biopsy times. Thus a temporal analysis is not necessary, only the maximum temperature experienced is required. A look up table can then be generated beforehand and used during optimization to quickly determine the effect of a laser pulse given the distance from the beam center. The range of allowable laser pulse durations used in the subsequent LZD optimization is 100 $\mu$s to 500 $\mu$s in 50 $\mu$s increments, and then 500 $\mu$s to 800 $\mu$s in 100 $\mu$s increments. This range encompasses typical laser pulse durations used in clinical work [71,76,117]. The temperature response of each laser pulse duration in the allowable range is simulated, and the maximum temperature is recorded at each distance and shown in Figure 3.4. These assumptions allow an accurate method to gauge the relative improvement between different LZD schemes without relying on computationally intensive simulations.
3.1.2 Temperature Measurement Experiments

To validate the simulation model of a single laser pulse, a method to measure the transient temperature response of a laser pulse using a high speed camera is devised. The temperature response of the 1480 nm 300 mW laser system XYClone from Hamilton Thorne Biosciences, Inc. (Beverly, USA) is measured using the thermosensitive fluorescent dye Rhodamine B (RhB) from Sigma Aldrich (St-Louis, USA). Fluorescence images are captured using a QImaging optiMOS high speed camera (Surrey, Canada) mounted on a Nikon Ti-U inverted microscope (Tokyo, Japan). The experimental setup for LZD experiments is shown in Figure 3.5(a). A 500 mM RhB solution is calibrated using similar methods to [118] to a range of 30-90°C using a type K thermocouple. A custom-built resistive heater is placed over a 170 µm channel of RhB [Figure 3.5(b)], which replaces the petri dish and embryo shown in Figure 3.5(a), and a temperature calibration is achieved by heating the RhB solution to a certain temperature and then measuring the fluorescent signal. According to simulation results and explained by [71], water near the focal point becomes superheated but does not boil, thus the RhB calibration is extrapolated beyond 90°C using an exponential function.

Figure 3.5: Experimental setup for (a) laser zona drilling experiments with embryos, (b) custom resistive heater with fixed channel height for RhB calibration
Figure 3.6: a) A time lapse series of 10 images taken at 2015 fps of a 600 µs laser pulse, b) Comparison of experimental and height-integrated simulation temperature response at beam center, c) Vertical temperature distribution

Figure 3.6(a) shows a time lapse of a 600 µs laser pulse captured at 2015 frames per second (fps). There is a sudden decrease in RhB signal intensity as the laser pulse locally increases the water temperature, and then dissipates to the surrounding area. Using the RhB calibration, the fluorescent signal to a single laser pulse is converted to the temperature response, shown in Figure 3.6(b). The peak temperature measured (73°C) is significantly lower than that observed in simulation (370°C). This is result of the fluorescence signal captured by the camera being an integration of the signal over the entire channel height. Thus, any high local temperature changes will be masked by surrounding ambient signal, rendering depth analysis difficult, as depicted by Figure 3.6(c).

In order to correlate experimental and simulation results, the simulated response $T_{\text{sim}}(x, y, z)$ must be flattened in the z-axis using (3.6), which represents the image a camera would capture. Note that the simulation setup was modified to match the reduced experimental channel height of 170 µm. First, the standard RhB calibration $G(I)$ as presented previously is inverted to give the fluorescence intensity as a function of temperature $g(T)$, and then normalized by the channel height to achieve the fluorescence intensity per micrometer $\bar{g}(T)$. The normalized RhB calibration $\bar{g}(T(x, y, z))$ is then used to convert the temperature at a specific point $T(x, y, z)$ to a simulated fluorescent intensity value for that point, and then each point is integrated over the channel height to achieve the flattened simulated fluorescent intensity value $I_{\text{sim,int}}(x, y)$. Finally, the simulated intensity is converted back into a temperature value using the standard RhB calibration $G(I)$. 
\[ I_{\text{sim,int}}(x, y) = \int_z \bar{g}(T_{\text{sim}}(x, y, z)) \, dz \]

\[ T_{\text{int}}(x, y) = G(I_{\text{sim,int}}(x, y)) \quad (3.6) \]

Figure 3.6(b) compares the experimental temperature response at the center of the pulse to the height-integrated simulation results. Given that the experimental and integrated-simulation results are quite close, it may be possible to infer that the experimental temperature response may be similar to that seen in simulation. One caveat is that inference of localized temperature response from integration is not unique, as slightly different temperature distributions may yield the same integrated result.

It is interesting to note that although the pulse duration is 600 \( \mu s \), the integrated temperature peaks are only observed at approximately 1-1.2 ms. This delay is an artifact of ambient temperature in the channel height masking small local changes. Reducing the channel height would minimize this effect. The difference in response can be further explained by the fact that the current method in integrating simulation results assumes that the RhB fluorescent intensity can be linearly modeled based on the contribution of each vertical element \( dz \), which may not be entirely accurate. Furthermore, the difference in time constant of the temperature responses may also be indicative of deficiencies in the simulation model, such as the assumption that convective heat transfer is negligible. We can conclude from the similar responses exhibited by the experimental and integrated simulation results that the two models support each other.

### 3.1.3 Effect of LZD parameters

In the simulation model, the ablation zone is modeled as an arc of which the width is determined by the diameter of a biopsy micropipette (40 \( \mu m \)) and the depth by the average mouse zona thickness (7 \( \mu m \)) \[116\]. Sensor points span the width of the ablation zone, spaced 5 \( \mu m \) apart and follow the contour of the blastomere edge closest to the ablation zone in a 2-cell embryo, similar to what is shown in Figure 3.1(a).

A previous study observed that zona ablation occurs at specific temperatures for different embryos \[71\]. Thus, the hole diameter created by laser ablation can be predetermined for each pulse duration. Based on a fixed pulse duration, uniformly-spaced laser pulse locations (LPLs) were placed to cover \( \geq 95\% \) of the entire ablation zone. Two case studies using LPL layouts generated are analyzed using pulse durations of 100 \( \mu s \) [Figure 3.7(a)] and 600 \( \mu s \) [Figure 3.7(b)]. The goal is to determine the difference in thermal
Chapter 3. Optimization and Automation of Laser Zona Drilling

(a) Pulse duration of 100 $\mu$s  
(b) Pulse duration of 600 $\mu$s

Figure 3.7: Temperature profile of the zona ablation zone using uniformly spaced LPL grid with fixed pulse durations. Asterisk marks denote temperature sensor points.

<table>
<thead>
<tr>
<th>Location $(\mu m, \mu m)$</th>
<th>100 $\mu$s</th>
<th>600 $\mu$s</th>
<th>Location $(\mu m, \mu m)$</th>
<th>100 $\mu$s</th>
<th>600 $\mu$s</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1, -2)</td>
<td>50.99</td>
<td>62.54</td>
<td>(25, -6)</td>
<td>41.85</td>
<td>47.46</td>
</tr>
<tr>
<td>(5, -3)</td>
<td>48.75</td>
<td>60.53</td>
<td>(30, -4)</td>
<td>43.23</td>
<td>54.34</td>
</tr>
<tr>
<td>(10, -4)</td>
<td>43.23</td>
<td>54.34</td>
<td>(35, -3)</td>
<td>48.75</td>
<td>60.53</td>
</tr>
<tr>
<td>(15, -6)</td>
<td>41.85</td>
<td>47.46</td>
<td>(40, -2)</td>
<td>50.99</td>
<td>62.54</td>
</tr>
<tr>
<td>(20, -9)</td>
<td>40.88</td>
<td>42.28</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: Maximum sensor point temperature in $^\circ$C

The maximum temperature experienced by each sensor point was calculated using the look up table generated in Figure 3.4, and tabulated in Table 3.1. As depicted in Figures 3.7(a) and 3.7(b), the bottom left corner of the ablation zone begins at coordinate (1, 1). Even though both zona ablation patterns achieve the goal of $\geq 95\%$ coverage, we can see a difference in the maximum temperature at the sensor points. Thus, the next step is to optimize the zona ablation pattern without any restrictions on the number of ablation pulses, the pulse locations or the pulse durations.

3.2 Selection of Optimal Ablation Zone

An ablation zone (AZ) is defined as the area of the zona that will be ablated to create the opening into the embryo for biopsy during zona breaching, as shown in Figure 3.1(a). The first step to minimizing heating of the blastomeres during LZD is to select an AZ such that it maximizes the distance from the blastomeres. The AZ that maximizes this
distance would be designated as the optimal ablation zone (OAZ). The proposed approach for the selection of the OAZ is detailed in this section.

The OAZ is determined by first segmenting the zona and blastomeres using computer vision algorithms, and then using the segmentation to search for an optimal location on the zona that maximizes the distance between the ablation zone and the blastomeres. Thus, the thermal effects of laser zona drilling on internal cell structures will be minimized. The size of the ablation zone is determined by the goal of LZD. Here, the goal is to create a full thickness ablation of the zona for embryo biopsy, where the width of the ablation zone is determined by the given operation, such as accommodating the width of a micropipette or a blastomere. All algorithms are implemented in MathWorks MATLAB.

3.2.1 Blastomere Segmentation

Many researchers have reported the segmentation of blastomeres using edge-based techniques to determine the location and shape of each individual blastomere [18,44]. Edge-based techniques suffer from poor segmentation in sub-optimal conditions. For the purpose of LZD optimization, the location of each individual blastomere is not important, but rather the collective outline of all blastomeres and their relative shape to the zona. It is noted that blastomere segmentation is improved using texture-based segmentation, as blastomeres have significantly different image texture compared to the zona, perivitelline space and the image background (Figures 3.1(a) and 3.8). A simple method for the analysis of texture descriptors in image analysis consists of using a local standard deviation of intensity values is described.

Figure 3.8(a) shows the initial image of a 2-cell cleavage stage embryo. The first step in texture-based blastomere segmentation is to generate a new image, denoted $I_{std}$, using a moving-window standard deviation of the image. This is accomplished by using the stdfilt() function from the MATLAB Image Processing Toolbox with a window size of 5x5 pixels on the original image [Figure 3.8(a)] to generate $I_{std}$ [Figure 3.8(b)]. Binary thresholding and image closing is then applied to $I_{std}$. The threshold value is chosen empirically as the 98th percentile of standard deviations values, such that the blastomeres are segmented without requiring image dilation to join fragmented segmentations caused by higher thresholds. Note that the threshold value may change depending on the system setup. Next, only the largest 8-connected object is kept and all holes are filled. The image is then eroded to disconnect any thin extremities and once again filtered for the largest 8-connected object. The final binary thresholding is shown in Figure 3.8(c) and the
Figure 3.8: Blastomere segmentation: (a) original image, (b) standard deviation transformation map with values capped at threshold for demonstration purposes, (c) thresholded mask containing only largest 8-connected object, (d) segmented blastomeres, with estimated embryo center calculated using the centroid of the blastomere mask

resulting blastomere segmentation is shown in Figure 3.8(d).

Texture-based thresholding as a segmentation method has the advantage of fast processing time, compared to other graph search edge-based methods. A mask is generated using the perimeter of the segmented blastomeres, which will be used in subsequent steps. Furthermore, the estimated embryo center is defined as the centroid of the blastomere mask as shown in Figure 3.8(d), and is calculated by averaging the position of all points in the mask.

### 3.2.2 Zona Pellucida Segmentation

Unlike the blastomeres, there is insufficient textural difference between the zona pellucida and image background to accurately use texture-based segmentation. Thus, an edge-based approach must be used. Given that the diameter of the embryo is fairly consistent, an annulus of fixed radii is used to predict and create a region of interest around both the inner and outer boundaries of the zona $ZP_{\text{inner}}$ and $ZP_{\text{outer}}$ respectively [Figure 3.9(a)]. The center of the annulus is the estimated embryo center, and the inner radius $r_{\text{inner}}$ is 30 µm whereas the outer radius $r_{\text{outer}}$ is 60 µm. The area enclosed by the annulus is transformed into polar coordinates $\theta \in [0; 2\pi]$ and radius $r \in [r_{\text{inner}}; r_{\text{outer}}]$. To reduce computation time, $\theta$ is sampled using 185 discrete points, and $r$ using the number of pixels between $r_{\text{inner}}$ and $r_{\text{outer}}$.

**Outer Zona $ZP_{\text{outer}}$ Segmentation**

The original image [Figure 3.8(a)] is first preprocessed using background subtraction by subtracting the mean intensity of the image to provide greater edge contrast, before applying polar coordinate transformation [Figure 3.9(c)]. Outer ZP segmentation is per-
Figure 3.9: Zona segmentation: (a) annulus indicating predicted zona location, (b) segmented zona and blastomeres, (c) annulus section converted to polar coordinates, (d) outer zona segmentation: energy map in polar coordinates with dilated blastomere mask and lowest cost path, (e) inner zona segmentation: Canny edge map in polar coordinates with blastomere and outer zona removed.

Formed using a very similar method to what is proposed by Guisti et al. [89], which uses an energy transformation based on the gradient of the image and the apparent lighting caused by Hoffman Modulation Contrast (HMC) imaging technique [89]:

\[
E(\theta, r) = \left(1 + e^{\frac{1}{2} \left( \cos(\theta-\gamma) \cdot G_r(I_{\text{polar}}) + \sin^2(\theta-\gamma) \cdot |G_r(I_{\text{polar}})| \right)} \right)^{-1} \tag{3.7}
\]

where \(E\) is the energy map in polar coordinates scaled to the interval \([0, 1]\) using a decreasing sigmoid function, \(I_{\text{polar}}\) is the polar coordinate representation of the image as seen in Figure 3.9(c), \(G_r\) is the gradient operator in the radial direction, \(\gamma\) is the angle of apparent lighting in HMC images, and \(\sigma\) is a scaling parameter typically set to \(1/5\) of the image’s dynamic range [89].

A directed graph search algorithm, such as MATLAB function \texttt{graphshortestpath()}, is used to find the path with the lowest energy using only adjacent pixels. To prevent possible confusion between the edges of the blastomeres, and inner and outer zona boundaries, the blastomere mask from Section 3.2.1 is dilated to partially mask the inner zona and then given very high energy values. Thus, the graph search ignores these areas during the graph search and follows the outer zona boundary. The resulting energy map and shortest path are shown in Figure 3.9(d).
Inner Zona $ZP_{inner}$ Segmentation

Similar to segmentation of the outer zona, the inner boundary of the zona is segmented using a graph search method. Conversely, background subtraction is not performed prior to polar coordinate transformation for inner zona segmentation. Once polar coordinate transformation is applied to the annulus region [Figure 3.9(c)], rather than converting the image using an energy-based metric, MATLAB’s Canny edge detection function `edge()` is used directly on the image. Edges inside the blastomere are removed by using the blastomere mask generated in Section 3.2.1, and edges in a close neighbourhood of the outer zona (10 pixels is used) and beyond are removed. Finally, using the same graph search function, the lowest-cost path is found and is designated as the inner boundary of the zona [Figure 3.9(e)]. Figure 3.9(b) shows the resulting segmentation of both the inner and outer zona boundaries, and the blastomeres.

3.2.3 Ablation Zone Optimization

Three sets of points are generated from the each of the previous segmentation processes [Figure 3.9(b)]: an outline of the blastomeres $B$, the inner boundary of the zona pellucida $ZP_{inner}$, and the outer boundary of the zona pellucida $ZP_{outer}$. By using these three sets of points and analyzing their relative structure and positioning, the optimal ablation zone can be determined. Prior to the optimization steps, the width $w$ of the ablation zone is defined, as determined by the desired operation such as inserting a micropipette of a fixed width.

A candidate ablation zone $AZ_c$ is defined as a subset of adjacent points of the required width $w$ along the inner zona boundary $ZP_{inner}$. A set of points $B_0$ is a corresponding subset of $B$ of width $w$ and directly opposite of $AZ_c$. The optimal ablation zone $OAZ$ is the candidate ablation zone $AZ_c$ that maximizes the minimum Euclidean distance $d$ to $B_0$. Variable definitions are shown in Figure 3.10. A brute force search steps through all possible $AZ_c$ along $ZP_{inner}$ to determine the OAZ:

$$\text{maximize } \min_d d(AZ_c, B_0)$$

subject to $AZ_c \in ZP_{inner}$

$$B_0 \in B \quad (3.8)$$

Once the OAZ is selected, the ablation zone profile is expanded to include the full thickness of the zona, as shown in Figure 3.1(a). This method is superior to point-based OAZ selection as the width of an ablation zone is an important factor, as edge points
contribute to potential overheating of the blastomeres. A point-based optimum may not be suitable once the full width of the ablation zone is taken into account. This situation is highlighted in Figure 3.10, where the location of the OAZ is different for two different OAZ widths of $15 \mu m$ and $40 \mu m$, as the location of the $15 \mu m$ OAZ would be less suitable to create a $40 \mu m$ opening. Another method to approach selection of the OAZ could be to maximize the mean distance (or the mean distance of the lower percentile) between the ablation zone and the blastomeres, instead of the absolute minimum distance between $B_0$ and $AZ_c$.

3.3 Coverage Optimization

With the OAZ selected, the second phase for minimizing blastomere heating is to optimize how LZD is performed on the OAZ. The optimization variables, also known as the LZD parameters, include the number of laser pulses $n$, and the corresponding pulse locations $loc_i$ and pulse durations $dur_i$ for each laser pulse where $i \in 1...n$. This phase is called coverage optimization, as it is similar to treating the OAZ as a map, and ensuring that the entire map is covered by areas that will be ablated according to the temperature threshold for ablation outlined in Section 3.1. The purpose of coverage optimization in multi-pulse laser ablation is to determine the optimal LZD parameters to maximize the zona removed within the ablation zone, while minimizing both ablation outside the ablation zone, and the temperature increase experienced by critical components inside the embryo. The optimization can be expressed as (3.9), where the objective function $J$ is defined by (3.10):

$$\arg \min_{n, \ loc,\ dur} J(n, \ loc, \ dur)$$  \hspace{1cm} (3.9)
where $a$ and $b$ are weighting functions, $P_{ZP}$ is the percentage of the ablation zone that will be ablated, $P_{edge}$ is the percentage of the surrounding ZP outside of the AZ that will be ablated, $P_{thresh}$ is the minimum $P_{ZP}$ to not incur a penalty, and $T_{max}$ is the maximum temperature at the blastomere points $B_0$, which is calculated based on the temperature increase $T(loc_i, dur_i, B_0)$ of every point in $B_0$ to all $n$ laser pulses using the temperature-distance plot shown in Figure 3.4.

The ablation zone is subdivided into a grid consisting of 1 $\mu$m x 1 $\mu$m elements, where each element is a possible laser pulse location (LPL). The minimum and maximum number of pulses $n$ are constrained during optimization, and are determined by calculating the ablated hole diameter created by the largest and smallest allowable pulse durations respectively, and determining how many pulses are required to achieve 100% coverage. As mentioned in Section 3.1.1, the range of allowable laser pulse durations is 100 $\mu$s to 800 $\mu$s, and the relationship between distance, pulse duration and maximum temperature is shown in Figure 3.4. A previous study analyzing the effect of LZD determined that dissolution of the mouse ZP occurs at approximately 130$^\circ$C for the range of pulse durations used here [71]. Thus, to determine the percentage of ZP ablation, any area with a maximum temperature over 130$^\circ$C would be considered ablated. Figure 3.11 illustrates this process for an arbitrarily-sized ablation zone and pulse duration. The ablated areas are points where the maximum temperature is beyond the ablation threshold of 130$^\circ$C. Furthermore, since the transient temperature effects are being ignored, laser ablation occurs at the laser pulse locations in no particular order.

Optimization is performed using the MATLAB genetic algorithm function `ga()`. Genetic algorithms are a stochastic optimization method that follow a distinct structure, namely the evolution of a population of individual candidate solutions that transform through random mutation and exchanging genetic material with other candidates. The benefits of genetic algorithms are the fact that the cost function derivatives need not be evaluated, which may be time consuming if evaluated numerically. Furthermore, the use of search heuristics and stochastically generating individual solutions provides an intuitive approach to coverage optimization. Further details on genetic algorithms may be
Figure 3.11: Effect of different laser pulse durations on laser pulse location layout when calculating the constraints on the minimum and maximum number of laser pulses during optimization.

Examined in [119]. In the optimization algorithm proposed here, the genetic material for each individual consists of a discretized ablation zone grid, defined by zeros for neutral elements, and non-zero pulse durations for each LPL. The following defines the operators used in the genetic optimization:

1. Population creation: for each individual, randomize $n$, $loc_i$ and $dur_i$.
2. Mutation function: each grid element in each individual has probability $r_{mutation}$ of mutating. If mutation occurs, that element has a 50% chance of having no laser pulse, or 50% chance of becoming an LPL with a random non-zero pulse duration.
3. Crossover function: a new individual (child) is generated with two random individuals (parents), where each grid element/gene has an equal probability of inheriting either parent’s value.
4. Elite individuals: The top 5% of the population with the best fitness score are considered as elite individuals, and automatically survive to the next generation without undergoing mutation or crossover, though they may act as parents for new children.
For example, a $5 \mu m \times 3 \mu m$ candidate ablation zone is represented using a 5x3 matrix. Each element of the matrix is a gene, totaling 15 genes for this candidate. The following is a gene representation of the candidate ablation zone:

<table>
<thead>
<tr>
<th>$i$</th>
<th>$loc_i$</th>
<th>$dur_i$</th>
<th>$\begin{bmatrix} 0 &amp; 0 &amp; 0 &amp; 500 &amp; 0 \ 0 &amp; 300 &amp; 0 &amp; 0 &amp; 0 \ 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 \end{bmatrix}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(2,2)</td>
<td>300 $\mu$s</td>
<td>0 0 0 500 0</td>
</tr>
<tr>
<td>2</td>
<td>(4,1)</td>
<td>500 $\mu$s</td>
<td>0 300 0 0 0</td>
</tr>
</tbody>
</table>

Whereas most genes are neutral elements, the two non-zero elements represent laser pulse locations. Every individual in a population is scored using the fitness function $J$ defined by (3.10). The fitness of a population is determined by the individual that minimizes the fitness function. The genetic algorithm terminates when the population fitness remains unchanged after a fixed number of generations. Optimizations are performed on a desktop PC with an Intel Core i7-4770 CPU @ 3.40 GHz and 24 GB of RAM. One full optimization typically requires 240 seconds to reach a local minimum with the parameters listed in Table 3.2, up to a maximum of 280 seconds if the optimization reaches the maximum number of generations. Better solutions may be obtained with larger population of individuals or increasing the maximum number of generations, but there exists a tradeoff between minimal increases in performance versus an increase in computation time before converging.

While an average of 240 seconds may be somewhat longer compared to the time required for a human to perform the same operation, the purpose of optimization is to perform the operation in a better and safer manner. Although the aim is to always minimize embryo exposure outside of the incubator, buffered biopsy media are able to easily maintain the required pH levels for the duration of automated and optimized LZD. Furthermore, the execution of the optimization algorithm is performed using MATLAB scripts, i.e.: uncompiled code, thus optimization of the code itself or compiling of the scripts could possibly decrease the run time significantly. Moreover, considering the bulk of the computation is used for calculating the fitness values of each individual within a population, the use of parallel computing could significantly reduce the computation time, essentially dividing the total run time by the number of cores used.

### 3.4 Automated Zona Ablation Experiments

Initial experiments using mouse embryos are performed as a proof of concept to verify the functionality and feasibility of the LZD optimization and automation algorithms described in Sections 3.2 and 3.3. The experimental setup is similar as the one outlined in Section 3.1.2. Eight experiments are conducted, all with similar results. The blas-
<table>
<thead>
<tr>
<th>Parameter Name</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population Size</td>
<td>600</td>
</tr>
<tr>
<td>Maximum Number of Generations</td>
<td>20</td>
</tr>
<tr>
<td>Typical Ablation Zone Grid Size</td>
<td>20 µm x 7 µm</td>
</tr>
<tr>
<td>Mutation Rate $r_{mutation}$</td>
<td>15%</td>
</tr>
<tr>
<td>Minimum required coverage percent $P_{thresh}$</td>
<td>95%</td>
</tr>
<tr>
<td>Percentage of population considered elite</td>
<td>5%</td>
</tr>
</tbody>
</table>

Table 3.2: Genetic optimization parameters

tomere and ZP segmentation algorithms are tested on many other previously-acquired images. While blastomere segmentation using the proposed method is very effective and repeatable, accurate ZP segmentation remains more difficult and is highly affected by object focus, sometimes requiring refocusing of the embryo and restarting the segmentation process. The use of autofocusing algorithms for cleavage-stage embryos has been demonstrated [16] and could be used solve this issue, but is not implemented given the scope of this thesis. In these proof of concept experiments, success in automation of LZD is measured in the complete ablation of the OAZ and access to the blastomeres without inducing a significant increase in temperature at the blastomeres.

Before beginning the optimization procedures, the ablation laser center is first determined using a built-in alignment laser. By turning off all background illumination so that only the alignment laser is detected by the camera, the 50th percentile of the image intensity is used to segment the laser and the center of mass is deemed as the center of laser. Once an embryo is moved into the camera field of view, LZD optimization begins and searches for the optimal ablation zone and optimal laser pattern for ablation within the OAZ.

Figure 3.12 demonstrates one example of a 4-cell mouse embryo undergoing the optimized and automated LZD procedure. The evolution of the population fitness value during coverage optimization is shown in Figure 3.12(b). For this particular case, the genetic optimization reaches a local minimum after 16 generations due to insignificant change in the fitness value. The optimized laser pattern uses seven laser pulses ranging from 100 to 350 µs. The pulse locations, along with the coverage pattern, are shown in Figure 3.12(c). Given that the minimum coverage percentage is 95%, a number of points in the AZ fall below the temperature threshold for ablation and thus are predicted to be unablated during LZD. These points are marked with black squares in Figure 3.12(c). The highest peak temperature experienced by the blastomeres is estimated to be 54.7°C, which is close to the allowable temperature range in LZD according to local embryologists. This peak temperature corresponds to the variable $T_{max}$ according to the coverage...
Figure 3.12: Automated optimized LZD experiment: (a) 4-cell embryo, red outline represents magnified area in subsequent subfigures, (b) evolution of the population fitness value during coverage optimization, (c) estimated coverage pattern after optimization, (d) optimized laser pulse locations and corresponding boundaries for inner zona and blastomeres, (e) opening created by automated LZD, (f) comparison of predicted and actual zona ablation optimization method and COMSOL simulation model described in Section 3.3. Mimicking the standard manual LZD procedures outlined in [71], and using the same COMSOL model in Section 3.1 and embryo layout in Figure 3.12, LZD using 800 µs pulse duration without optimization is estimated to cause the blastomeres to experience a maximum temperature of 66°C. Furthermore, an embryologist may ablate closer to the blastomeres to ensure adequate zona removal during manual operation, which would in turn increase the maximum temperature further.

For the experiment setup used, the LZD laser is fixed in the camera frame. Hence, the embryo itself must be translated such that the laser pulse locations can reach the laser beam center. This is achieved through the use of a computer controlled motorized microscope stage. Desired stage movement is calculated using the difference in laser beam center and laser pulse locations. Stage acceleration is lowered to minimize shifts in embryo position during LZD, as the embryos are free floating in buffered culture media. Despite lowering stage acceleration, minor drift compensation is necessary as embryos may drift while LZD optimization occurs due to random motion. No major embryo drift or shift in blastomere locations is observed during LZD experiments. To overcome minor drift, another image is taken just prior to ablation and the embryo center is recalculated. Laser pulse locations are updated to reflect the embryo drift. Future embryo biopsy platforms
can employ methods to immobilize the embryo such as base-mounted vacuum ports [45]. Care must be taken to not interfere with the segmentation algorithms. Automated LZD is then performed using the compensated stage movements with a small pause at the end of each movement to allow settling of any motion. As mentioned previously, the laser pulse locations are ablated in no particular order. Beyond the time taken for the optimization to complete, the laser center calibration, drift compensation and actual laser ablation procedure occur in a matter of seconds.

As shown in Figure 3.12(e), openings in the zona using automated LZD are created, albeit somewhat different from the predicted opening. A comparison is noted in Figure 3.12(f) by overlaying the generated ablation zone grid shown in Figure 3.12(c) over the embryo image Figure 3.12(e). This overlay allows visual identification in Figure 3.12(f) to determine whether a point was predicted to undergo ablation that did or did not, or was not predicted to undergo ablation at all. It is interesting to note that the unablated areas in the OAZ are concentrated along the inner zona. This phenomenon arises since the optimization algorithm aims to minimize the temperature increase of the blastomeres. Thus, LPLs will tend to be placed farther away from the inner zona boundary to increase the distance between LPLs and the blastomeres, resulting in the OAZ points closest to the blastomere to be just above the temperature threshold for ablation. This in turn increases the possibility that ablation does not occur during experiments if either the calculated temperature or threshold estimation is not entirely accurate.

Discrepancies between the predicted and actual zona opening may be attributed to inaccuracies in the thermal model described above, and the assumed threshold for zona ablation. The simulated coverage percentage predicted a 96% opening of the zona (relative to the OAZ), but post-experiment image analysis indicates that only 86% of the OAZ was ablated. If the temperature ablation threshold is set to the upper bound of the reported values in [11], then the predicted coverage falls to 91%, somewhat closer to the experimental results. Despite the lower coverage percentage, access to the blastomeres is still possible given that a portion of the unablated area pertains to the width, whereas, for the most part, full-thickness zona removal is achieved. Thus, this automated and optimized LZD experiment is still considered a success.

### 3.5 Chapter Conclusion

In summary, a thermal model for laser zona drilling was generated in simulation and verified experimentally using a novel analysis method involving thermosensitive fluorescent dye and a high speed camera. This model allowed the building of a characterization
look up table correlating the maximum temperature increase, distance from beam center and laser pulse duration. Computer vision algorithms were developed for the segmentation of the blastomeres and zona pellucida for use in automated analysis of the relative spatial structure between the two to determine the optimal ablation zone. Next, given the optimal ablation zone and the laser pulse characterization, a method for coverage optimization using genetic algorithms was described to determine the optimal configuration of laser pulses to minimize temperature increase at the blastomeres. Finally, laser zona drilling automation and feasibility of these methods were successfully verified with mouse embryo experiments, where adequately-sized holes were created using the outlined automated procedures without human intervention. Although the proposed optimization algorithm applies to full thickness ablation of cleavage-stage embryos in PGD, these methods can be applied to a wide range of procedures that requires zona ablation, whether for different embryo developmental stages, embryo species, laser ablation systems, or ablation procedures such as assisted hatching.
Chapter 4

Automated Blastomere Extraction

While many micromanipulation procedures involve injecting cells with foreign materials [42,43,56], extraction of material is also important for embryo biopsy. In preimplantation genetic diagnosis (PGD), a form of embryo biopsy, blastomeres are extracted from the embryo. DNA analysis of the extracted blastomeres allows genetic profiling of the embryo to detect the presence of any genetic diseases. The PGD biopsy procedure has three main steps, embryo reposition and reorientation, zona breaching and blastomere extraction. These procedures are currently performed manually with low success rates [32]. Following the overall thesis objective of automating PGD biopsy procedures, this chapter describes methods for the automation of the third and final step of PGD biopsy: blastomere extraction.

Blastomere extraction, as the name implies, involves non-destructive removal of a single blastomere from the embryo without damaging the blastomere or the embryo itself. The extracted blastomere would then be used later for DNA analysis for genetic profiling of the embryo. A multitude of methods have been developed for the extraction of cells during embryo biopsy, where a selection is outlined in Figure 4.1. Traditionally, a blastomere micropipette is used to apply very low suction to a single blastomere to pull it out of the embryo [35]. This is known as the aspiration method and is shown in Figure 4.1(b). The micropipette may also be used to compress the embryo from the outside to partially squeeze the blastomere out of the opening of the embryo for easier aspiration. Although this method has been used extensively, the procedure imparts large mechanical stress on both the embryo and the blastomere, potentially causing irreversible damage. Another method, known as the displacement method, extracts a blastomere by inserting a smaller micropipette into the embryo and injecting a fluid to displace the blastomere and push it out of the embryo [77], and is depicted in Figure 4.1(c). This method is potentially simpler and involves less mechanical stress on the embryo and the blastomeres. Finally,
optical tweezers, which use the refractive properties of light to optically trap and move particles [4], has been used to extract polar bodies and trophectoderm cells during other stages of embryo biopsy [14, 38], as shown in Figure 4.1(d). Given the simplicity and robustness of the displacement method, the displacement method is used as the method of choice for automation.

Figure 4.1: Embryo biopsy extraction techniques: (a) 4-cell cleavage-stage embryo with an opening in the zona created by zona breaching, (b) aspiration method, (c) displacement method, (d) optical tweezer method for polar body biopsy

In this chapter, the automation of blastomere extraction using the displacement method is described and demonstrated using proof of concept experiments. The goal of blastomere extraction automation is to successfully extract 1-2 blastomeres without human intervention. Section 4.1 describes the experimental setup. Section 4.2 outlines the extraction procedure and the process flow. Section 4.3 derives a gas model used for system pressure calculation. Section 4.4 details various computer vision algorithms used in the automation process, namely micropipette calibration for position control, blastomere extraction event detection, and blastomere tracking during retrieval. Finally, Section 4.5 reports successful results from proof of concept experiments for automated blastomere extraction using mouse embryos, demonstrating that blastomere extraction automation is indeed feasible. Work presented in this chapter has been presented at a conference [105] and will be submitted for publication in a peer-reviewed journal.

4.1 Experimental Setup

The experimental setup is shown in Figure 4.2. Experiments are performed on a standard inverted microscope (Nikon Ti-U) using two robotic micromanipulators (Scientifica Patchstar) with three degrees of freedom (DOF) each. Micromanipulator position control is achieved using a built-in controller, such that no extra action is required other than inputting the desired position to the micromanipulator. Embryos are manipulated using standard IVF holding micropipettes and zona drilling micropipettes (Sunlight Medical Inc.) and mounted on micropipette holders (Sutter Instruments Micropipette Holder).
Holding micropipettes are driven pneumatically using a manually adjusted digital pressure controller (Sutter Instruments XenoWorks Digital Microinjector). Zona drilling pipettes (outer diameter $d = 8 - 10 \mu m$) are used for blastomere displacement and retrieval. Pneumatic pressure is used to drive the zona drilling pipette using a 10 mL syringe (Air-Tite Norm-Ject) mounted on a computer-controlled syringe pump (New Era NE-500). A solenoid valve (McMaster-Carr 5760T123) is used as a relief valve, and is controlled using a microcontroller (Arduino Uno). The zona drilling pipette for extraction, solenoid valve, and syringe are connected using a series of tubing and tubing adapters. Zona breaching is performed using a 20x magnification objective with a built-in laser (Hamilton-Thorne XYClone). This procedure, also referred to as laser zona drilling (LZD), uses short pulses of laser to locally ablate the zona [115]. Images are taken using a camera (QImaging optiMOS) with a resolution of 1920 px x 1080 px and a resulting pixel density of 3.25 px/$\mu m$. All algorithms are implemented in Mathworks MATLAB.

Mouse embryos at the 4- and 8-cell stages are used in feasibility experiments as they are appropriate analogs to human embryos.

### 4.2 Extraction Procedure Overview

This section provides an overview of the blastomere extraction automation procedure and process flow. The extraction procedure is split into a manual phase (preparation) and three automated phases (preparation, blastomere displacement and blastomere retrieval). An overview of the entire extraction process is summarized in Figure 4.3. In the procedures reported here, the embryo is held on the left side, and that the extraction pipette is located on the right side of the embryo, as seen in Figure 4.3. A detailed flowchart
with process timing and success/failure conditions is shown in Figure 4.4. Briefly, each phase has specific tasks:

0. Manual Phase - Preparation:
   - Bring embryo into view
   - Perform zona breaching

1. Automation Phase I - Preparation:
   - Load micropipette with fluid
   - Move micropipette into embryo through opening

2. Automation Phase II - Blastomere Displacement:
   - Inject fluid into embryo
   - Detect blastomere extraction event

3. Automation Phase III - Blastomere Retrieval:
   - Track blastomere position
   - Retrieve blastomere

**Manual Preparation**

The manual preparation phase is used for setting up the experiment. The micropipettes are loaded onto the micromanipulators with the holding micropipette on the left, and the extraction micropipette on the right. The micropipettes and the embryo are manually brought into view and into the same focal plane. The embryo is held in place using the holding pipette at low vacuum (approximately -8 hPa). An opening in the zona for micropipette insertion and blastomere extraction is created using manual laser zona drilling. In these experiments, the coordinates of the zona opening are determined manually by the user. Once fully autonomous embryo biopsy systems are implemented in the future, the coordinates of the opening would be determined automatically during the LZD step,
Figure 4.4: Detailed flowchart of blastomere extraction automation with associated computer vision algorithms shown in pink rectangles

as demonstrated in [115], and thus would be used for automated extraction. This marks the end of manual procedures. All following procedures are performed automatically without human intervention.

Automation Phase I - Preparation

At the start of the automated preparation phase, a calibration process, detailed in Section 4.4.1, is performed to generate a coordinate transformation between the micromanipulator encoders and the micropipette tip position. This calibration procedure allows accurate encoder-based position control of the micropipette tip by specifying the desired tip position in the camera frame. After this calibration process, an image of the camera field of view without the micropipette in view is taken for use with the blastomere tracking algorithm used during Phase III - Blastomere Retrieval procedure. With the
micropipette still outside of the embryo, the micropipette is preloaded with biopsy media by withdrawing syringe pump at a rate of 2 mL/min for 45 s.

With the micropipette loaded with media, the micropipette insertion path is then automatically generated using the current position of the micropipette and the coordinate of the embryo zona opening, as shown by points A → D in Automation Phase I portion of Figure 4.3 where $d_1 = 60 \, \mu m$ and $d_2 = 30 \, \mu m$. Micropipette motion from point C to point D occurs at a reduced speed to minimize damaging the blastomeres. Since the embryo and micropipettes are located in the same focal plane, motion planning is restricted to the XY-plane only. Using the coordinate transformation, the micromanipulators move the micropipette tip along the generated path to insert the micropipette into the embryo.

**Automation Phase II - Blastomere Displacement**

Once the micropipette is inserted into the embryo, the blastomere displacement phase begins. The syringe pump begins to infuse and increases the system air pressure to inject fluid into the blastomere. The 10 mL syringe infuses at a rate of 1.8 mL/min. Blastomere extraction event detection, a custom computer vision algorithm described in further detail in Section 4.4.2, is used to determine if a blastomere has been displaced from the embryo. If no blastomere extraction event is detected using the extraction event detection algorithm, the infusion rate increases by 0.3 mL/min every 5 seconds. After 30 seconds, if no extraction event is detected, infusion stops and the relief valve is opened, and the displacement process is deemed to have failed. If an extraction event is detected, infusion stops, the micropipette is immediately withdrawn from the embryo, and the relief valve cycles to reset the system to atmospheric pressure. The blastomere displacement process is then considered successful and the extraction process will move onto Phase III - Blastomere Retrieval.

**Automation Phase III - Blastomere Retrieval**

Once a blastomere is displaced successfully, as detected by the extraction event detection algorithm, the blastomere retrieval process begins. The same micropipette used for displacement is also used to capture the extracted blastomere using aspiration. Phase III begins with achieving aspiration by withdrawing the syringe pump at a rate of 2.5 mL/min.

A blastomere tracking computer vision algorithm, described in Section 4.4.3, tracks the desired contact point of the extracted blastomere $(x_b, y_b)$, defined by the $x$-coordinate of the right-most edge and the $y$-coordinate of the centroid of the blastomere. Coordinates
are shown in the Automation Phase III portion of Figure 4.3. The retrieval algorithm generates a path for the micropipette tip \((x_p, y_p)\) based on the relative distance with respect to the blastomere contact point \((x_b, y_b)\) according to the conditional statements defined by (4.1):

\[
\begin{align*}
    x_p &= \begin{cases}
        x_p, & \text{if } (x_p - x_b) \leq d_x \\
        x_b + d_x, & \text{otherwise}
    \end{cases} \\
    y_p &= \begin{cases}
        y_p, & \text{if } |y_p - y_b| < d_y \\
        y_b, & \text{if } |y_p - y_b| \geq d_y
    \end{cases}
\end{align*}
\]

where \(d_x = d_y = 50\) px are distance thresholds in \(x\)- and \(y\)-axes respectively. If the distances between \(x_p\) and \(x_b\), and \(y_p\) and \(y_b\) are within these thresholds, as outlined by the green square in Figure 4.3, then no micropipette motion will occur. This is to prevent excessive repositioning of the micropipette as the blastomere is pulled towards the micropipette. If micropipette repositioning is required, then the micromanipulator will actuate according to the coordinate transformation. The blastomere retrieval phase loops between tracking the blastomere contact point using computer vision and repositioning the micropipette tip at approximately 4 Hz until either retrieval process is deemed successful, or 30 s has elapsed at which point the retrieval will be deemed failed. The retrieval process is deemed successful and the blastomere is retrieved if \(x_b, y_b, x_p,\) and \(y_p\) are within the thresholds \(d_x\) and \(d_y\), and there is no relative motion between the two.

### 4.3 Displacement Pressure Modeling

The core mechanism used in the blastomere extraction procedure is the control of fluid in and out of the extraction micropipette. This is used for withdrawal/aspiration of fluid during Phase I Preparation and Phase III Blastomere Retrieval, and infusion/expulsion of the fluid during Phase II Blastomere Displacement. While biopsy media is loaded into the micropipette and is used to interact with the embryo, air is the transmission medium between the syringe pump and biopsy media (Figure 4.5). Fluid movement in and out of the extraction micropipette is achieved through a pressure differential between the extraction system and the atmosphere created by changes in syringe volume using a syringe pump, and controlled in an open loop fashion.

Without proper pressure control, the micropipette may expel or withdraw fluid uncontrollably, potentially causing the embryo to rupture or be aspirated into the micropipette. Since the air is a compressible gas, the extraction system can be modeled using the ideal
Figure 4.5: Schematic of extraction system for pressure modeling. Fluid in blue, air in red.

gas law given by (4.2):

\[
P V = n R T \quad (4.2)
\]

where \( P \) is gas pressure, \( V \) is system volume, \( n \) is the number of moles of air present in the system, \( R \) is the ideal gas constant, and \( T \) is the gas temperature. Assuming that the system is closed, such that the number of moles \( n \) and temperature \( T \) do not change, (4.2) can be rewritten as (4.3) to compare the relationship between the pressure and volume at different states. This allows calculation of the required syringe pump motion to achieve the desired pressure:

\[
P_1 V_1 = P_2 V_2
\]

\[
V_i = V_{\text{syringe},i} + V_{\text{tubing}} + V_{\text{holder}} + V_{\text{micropipette}}, \quad i = 1, 2 \quad (4.3)
\]

where system volume \( V_i \) includes the syringe volume \( V_{\text{syringe},i} \), tubing \( V_{\text{tubing}} \), micropipette holder \( V_{\text{holder}} \), and the micropipette itself \( V_{\text{micropipette}} \). Volumes are defined in Figure 4.5. Only the syringe volume \( V_{\text{syringe},i} \) is considered variable, as all other volumes are assumed fixed. Typically, \( P_1 = 1 \) atm as the system will be starting from rest. Operating pressures reach a maximum of 49 kPa according to calculations for the change in system volume for our experimental setup over a 30 s displacement phase [Figure 4.6(a)]. Note that this value represents system pressure at the end of the 30 s extraction process, whereas extraction typically occurs well before the time limit of 30 s. Certain assumptions were also made: fluid level in the micropipette does not change, and that the tubing is infinitely stiff. These assumptions imply that \( V_{\text{tubing}} \) and \( V_{\text{micropipette}} \) remain constant, and that there were no reactionary volume changes to system pressure, which would result in system pressures lower in magnitude than the calculated pressure values. The benefits of this pressure model is twofold: first, system pressure can be calculated and controlled in an open loop manner; second, it is simple to achieve similar pressure profiles across
Moreover, given that air is compressible, fluid motion may experience significant lag without proper system pressure control. In this thesis, we define lag as the time delay between changes in the syringe pump, i.e.: direction or flow rate, and corresponding changes in direction or flow rate of fluid going in or out of the micropipette. For example, a system with significant lag would experience a large temporal delay between starting syringe pump infusion, and the point in time at which fluid starts to expel from the micropipette tip; or conversely, the micropipette continues to expel fluid long after stopping the syringe pump. These issues are related to the fact that air is compressible. To alleviate problems associated with pneumatic lag, a computer controlled solenoid relief valve is added to the system (Figure 4.2) to quickly return system pressure to atmospheric and reliably stop any injection or aspiration process using the micropipette. A reliable pressure reset allows achieving repeatable desired system pressures.

To investigate the responsiveness of air as a transmission medium at such a small scale, especially when using a relief valve to quickly equalize system pressure to atmospheric, simulations are performed using COMSOL Multiphysics. Figure 4.6(b) shows a simulated system pressure response at the micropipette tip to demonstrate the speed at which pressure changes from either syringe infusion or opening of the relief valve. System inlet pressure $P$ is ramped up from 0 to 40 kPa from $t = 0.02$ s to $t = 0.08$ s, and then a step function drops the system pressure to 0 kPa at $t = 0.1$ s. According to simulations, the system will still respond to changes in pressure within tens of milliseconds, which is acceptable for the tasks performed in this thesis.

## 4.4 Computer Vision Algorithms

In this section, key computer vision algorithms used in the automation of blastomere extraction are described. Section 4.4.1 outlines a method for micropipette position control in the camera frame. Section 4.4.2 describes the blastomere extraction event detection algorithm, which is used in Automation Phase II Blastomere Displacement. This detection algorithm determines when a blastomere has been successfully displaced from the embryo, so that the next extraction phase may begin. Section 4.4.3 describes a method for tracking the position of a free-floating blastomere once it has been displaced, used in Automation Phase III Blastomere Retrieval. The position of the displaced free-floating blastomere must be known so that the extraction micropipette may be repositioned in close proximity to the blastomere for retrieval.
Chapter 4. Automated Blastomere Extraction

Figure 4.6: (a) Calculated static system pressure during a 30 s displacement phase and infusion rate, (b) Simulated system response time to changes in pressure and relief valve

Figure 4.7: Coordinate transformation for camera frame to micromanipulator frame

4.4.1 Micropipette Calibration and Control

One of the key aspects of automation and control is feedback. Without feedback, a system does not know how it is performing and cannot adjust otherwise. Since micropipettes are the main tools used in the proposed automation procedures, determining their location relative to the embryo and controlling them in the camera frame is paramount. Real-time image recognition algorithms exist [15,83], typically using pattern/template matching to detect the tip of the micropipette. These methods may encounter problems if debris adheres to the micropipette tip and thus changing the tip shape, or if objects block the micropipette tip from view [84], such as during the blastomere extraction in PGD.

In this thesis, a pre-procedure calibration is used to provide a coordinate transformation from the micromanipulator encoders to the micropipette tip location in the camera...
frame. This method allows the micropipette to be controlled in the camera frame without
the use of vision feedback during the extraction procedure, but still requires computer vi-
sion algorithm to perform the pre-procedure calibration. The computer vision algorithms
are used to segment and determine the position and orientation $\alpha$ of the micropipette tip,
and correlate its position in the camera frame to the micromanipulator encoder position
to obtain the coordinate transformation $T$ (Figure 4.7). The following sections detail the
micropipette segmentation algorithm and the calibration procedure.

**Micropipette Segmentation**

Micropipette segmentation is a method using computer vision to detect the micropipette
in the camera frame, and is performed in 3 steps. Briefly, starting with the micropipette
shown in Figure 4.8(a), the first step is to use MATLAB’s edge detection function `edge()`
to produce the binary image of edges, as shown in Figure 4.8(b). Second, the micropipette
is segmented through the use of pseudo edges, morphological operations and blob detec-
tion [Figure 4.8(c)]. Finally, the outline of the segmented micropipette is used to calculate
the micropipette tip location and orientation [Figure 4.8(d)].

To close the micropipette outline, as seen in Figure 4.8(c), the image borders are
artificially considered as edges. Since the direction of the micropipette is not known a
priori, all possibilities must be considered. Four images are generated $I_{UL}$, $I_{UR}$, $I_{BL}$, and
$I_{BR}$, where the subscripts denote which image borders are considered as pseudo edges
(e.g.: $I_{UL}$ designates that the upper and left image borders as pseudo edges, whereas
$I_{BR}$ designates the bottom and right image borders). Image closing is then performed all
four images $I_{UL}$, $I_{UR}$, $I_{BL}$, and $I_{BR}$ by first dilating the edge features to join nearby edge
segments, applying the pseudo edges on the designated image borders, and finally filling
the closed contours using the MATLAB function `imfill()` [Figure 4.8(c)]. It is important
to note that the image borders are considered in pairs, as a micropipette emerging from
the corner of the image may come in contact with two borders, but considering all four
borders as pseudo edges at once would fill the entire image. Once filled, all four images
are combined and the largest 8-connected object is singled out as the micropipette. The
image is eroded to counteract the previous dilation, and an outline of the micropipette
is generated using the MATLAB function `bwperim()`. Pseudo edge border pixels were
removed so that the micropipette outline does not close.

Micropipettes used in embryo biopsy are typically axisymmetric, with relatively par-
allel and straight side walls. These side walls are used to determine the orientation $\alpha$ of
the micropipette. While it is possible to estimate the orientation of the micropipette by
using the angle of the major axis of the segmented micropipette, this method is found
Figure 4.8: Micropipette segmentation: (a) original image of holding micropipette, (b) Canny edge detection, (c) closing image $I_{BL}$ using pseudo edges, (d) outline of the micropipette with side walls split into $s_L$, $s_R$ and tip, (e) holding micropipette with orientation and tip location, (f) micropipette moved to $p_2$ during the calibration, (g,h) segmentation of (g) zona drilling and (h) blastomere biopsy micropipettes to be inaccurate for situations where the micropipette is not perpendicular to the image boundaries, as one side would be overrepresented. The perimeter of the segmented micropipette is ordered starting from one end to the other, and labeled as the set of points $s_{ordered}(i), \ i \in 1...n$. A rough estimate of the orientation $\alpha_{\text{init}}$ is first calculated by averaging the slopes between points $s_{ordered}(1)$ and $s_{ordered}(1+\delta_e)$, and $s_{ordered}(n-\delta_e)$ and $s_{ordered}(n)$, where $\delta_e = 400$ is the number of points used for orientation estimation. A more accurate estimation of $\alpha$ is achieved by locally calculating $\alpha_i$ using a subset of $s_{ordered}$ and a moving window, and then averaging all $\alpha_i$ values, according to (4.4):

$$
\alpha_i = \tan^{-1}\left(\frac{s_{ordered,y}(i+\delta_s) - s_{ordered,y}(i-\delta_s)}{s_{ordered,x}(i+\delta_s) - s_{ordered,x}(i-\delta_s)}\right)
$$

(4.4)

where $\delta_s$ is the sampling length. A value of $\delta_s = 61$ points is found to adequately smooth out small disturbances but able to discern larger changes in angle. All points in $s_{ordered}$
Chapter 4. Automated Blastomere Extraction

that did not fulfill the criteria $\alpha_{\text{init}} - \alpha_{\text{thresh}} < \alpha_i < \alpha_{\text{init}} + \alpha_{\text{thresh}}$ are removed when calculating the orientation of the micropipette, where $\alpha_{\text{thresh}} = 15^\circ$. This effectively removes the front tip of the micropipette or ignores debris attached to the micropipette to create a set of points containing only the side walls of the micropipette, which is subsequently split into left $s_L$ and right $s_R$ sides for calculating $\alpha$, as shown in Figure 4.8(d).

To distinguish between left $s_L$ and right $s_R$ sides of the micropipette, a vector $\vec{r}$ is created between the centroid $C$ and every point in $s_{\text{sides}}$. For each $\vec{r}$, the angle $\beta$ between $\vec{r}$ and the micropipette $y$-axis unit vector $\hat{\vec{j}}_m$ in Figure 4.8(d), which is offset $90^\circ$ from $\alpha$, is calculated and used to classify that point as belonging to $s_L$ or $s_R$ depending on the value of the $\beta$, according to (4.5):

$$ \beta_i = \cos^{-1} \left( \frac{\vec{r}_i \cdot \hat{\vec{j}}_m}{|\vec{r}_i| \cdot |\hat{\vec{j}}_m|} \right), \quad r_i \in s_{\text{sides}} $$

if $\beta \geq 90^\circ$, $s_{\text{sides}}(i) \in s_L$

if $\beta < 90^\circ$, $s_{\text{sides}}(i) \in s_R$

(4.5)

To determine the location of the micropipette tip, the centroids $c_L$ and $c_R$ of $s_L$ and $s_R$, respectively, are calculated. From the midpoint of vector $\vec{c_Lc_R}$, a line is drawn with orientation $\alpha$. The intersection of this line with $s_{\text{ordered}}$ is considered the tip of the micropipette $p$, shown in Figure 4.8(e). This algorithm is successfully applied to two other micropipette types without modification, as shown in Figures 4.8(g) and 4.8(h).

Calibration Procedure

Using the segmentation to determine the location and orientation of the micropipette in the camera frame, the coordinate transformation can now be built using the calibration procedure. The calibration procedure is used to generate the coordinate transformation between the micromanipulator frame of reference to the camera frame (or vice versa) by using two points known to both reference frames [Figures 4.8(e) and 4.8(f)]. This is useful as automated manipulation is performed in the camera frame, but the micromanipulator may not be aligned with the camera workspace, or that the spatial relationship may not be known. The calibration procedure is as follows:

1. Remove all objects from view
2. Move micropipette to an arbitrary but known position deep in the camera image [Figure 4.8(e)]
3. Apply segmentation algorithm described above and obtain micropipette tip location
Chapter 4. Automated Blastomere Extraction

4. Move micropipette a known distance in camera image [Figure 4.8(f)]
5. Apply recognition algorithm and obtain \( p_2 \) only in both frames
6. Calculate the transformation \( T(p^c) \) using (4.6)

\[
\zeta^f = \tan^{-1}(p^f_{2y} - p^f_{1y}, p^f_{2x} - p^f_{1x})
\]

\[
R^m_c = \begin{bmatrix}
\cos(\zeta^m - \zeta^c) & -\sin(\zeta^m - \zeta^c) \\
\sin(\zeta^m - \zeta^c) & \cos(\zeta^m - \zeta^c)
\end{bmatrix}
\]

\[
p^m_{\text{offset}} = p^m_1 - s^m_c R^m_c p^c_1
\]

\[
p^m = T(p^c) = s^m_c R^m_c p^c + p^m_{\text{offset}}
\]

where superscripts \( f \), \( c \), or \( m \) denotes the frame of reference for a generalized frame, the camera frame, or the micromanipulator frame, respectively; \( p \) is the micropipette tip location; \( \zeta \) is the angle between the two calibration points; \( \tan^{-1} \) is the two-argument arctangent function to obtain angles \(-180^\circ < \zeta^f \leq 180^\circ\), as opposed to the standard one-argument arctangent function \( \tan^{-1} \) to obtain angles \(-90^\circ < \zeta^f < 90^\circ\); \( R^j_i \) is the rotation matrix from frame \( i \) to frame \( j \); \( p^m_{\text{offset}} \) is the offset of the micromanipulator frame origin to camera frame origin; and \( s^j_i \) is the scaling factor from frame \( i \) to frame \( j \). The scaling factor used is \( s^m_c = \frac{1\, \mu m}{3.248 \, \text{px}} \), given the camera resolution and microscope objective magnification used.

Micropipette Positioning Control Experiments

The performance of the micropipette position calibration is verified using positioning experiments, demonstrating that real time vision feedback is not required for the level of precision required for embryo biopsy. The positioning control experiment mimics micropipette path for the extraction step in PGD, which involves insertion of a micropipette into the embryo through the opening in the zona. Figure 4.9 demonstrates a semi-automated blastomere extraction experiment using a mouse embryo with an opening created by laser zona drilling. After performing the micropipette calibration described above, the micropipette follows an automatically generated path in the camera frame based on the relative location of the micropipette and the embryo opening, and then is slowly inserted into the embryo through the opening. Position error between the coordinate transformation and manual determination of the pipette tip location in the camera frame was no more than 3 \( \mu m \). Although real-time vision feedback control could correct these errors, the accuracy of the proposed offline calibration is acceptable for the preci-
Chapter 4. Automated Blastomere Extraction

4.4.2 Blastomere Extraction Event Detection

During Phase II Blastomere Displacement, it is important to be able to determine when a blastomere has been extracted from the embryo. Without a method to determine whether displacement was successful or not, too much fluid could be injected into the embryo, causing it to rupture or displacing more blastomeres than intended. Both these cases would irreversibly damage the embryo. As outlined in Section 1.2.5, certain extraction feedback algorithms exist for aspiration of cells, but they are not suitable for displacement extraction. A custom image-based analysis method for blastomere extraction event detection for use with the displacement method is described below.

First, a region of interest (ROI) is placed at the opening of the embryo with only a small portion of the blastomeres in view [Figure 4.10(a)]. During extraction, the blastomere is slowly pushed towards the zona opening as fluid is injected into the embryo, and enters the ROI over the course of a few seconds. Eventually, the blastomere will reach a critical point and rapidly exit from the zona opening and disappear from the ROI within a time interval as short as 0.3 s. This sequence can be seen in Figure 4.10(b). It is apparent that blastomeres are darker than the rest of the embryo and the background. Thus, detection of the blastomere extraction event is performed by observing the ROI mean intensity (ROIMI) and detecting any rapid large changes in intensity [Figure 4.10(c)]. As the blastomere enters the ROI, the ROIMI decreases. Conversely, when the blastomere is extracted and exits the ROI, the ROIMI increases. The extraction event detection algorithm calculates the percentage difference between the ROIMI of the current frame to the lowest ROIMI of the last $n$ frames ($n = 35$ frames in the example shown in Figure 4.10). A percentage difference in mean intensity beyond a threshold (0.025 in the example shown) would be considered a successful extraction event.

The lowest ROIMI of the last $n$ frames is used rather than averaging the ROIMI as it
4.4.3 Blastomere Retrieval

During Phase III Blastomere Retrieval, the blastomere has been displaced and is now free-floating in the surrounding media. Given that the goal of PGD is to perform genetic analysis of the extracted blastomere, the blastomere must be retrieved. During this phase, a custom computer vision algorithm, based on image subtraction and blastomere segmentation, is used to track the blastomere position for automatic retrieval. Most objects in the camera field of view are stationary during the retrieval phase, as the only objects that change positions are the blastomeres inside the embryo, the displaced...
Figure 4.11: Blastomere tracking during retrieval phase: (a) pre-extraction image without micropipette in view with retrieval ROI outlined in red, (b) post-extraction example with embryo and micropipette mask outlines in white and ROI outline in red, (c) ROI post-subtraction, (d) blastomere segmentation, with \((x_b, y_b)\) marked by a red cross, after intensity threshold and removing micropipette and embryo

blastomere and the micropipette. Thus, image subtraction of the current frame from an image taken prior to extraction is used to determine object motion [120]. This pre-extraction image is taken without the micropipette in view [Figure 4.11(a)]. Briefly, additional steps are taken to determine the blastomere location, including image intensity difference thresholding, object masking, and finally blob detection.

Image subtraction combined with intensity difference thresholding is used to detect changes in the camera field of view, which indicate moving objects. Unfortunately, these two steps alone would also detect changes in the micropipette position and within the embryo itself, such as the missing blastomeres. Masks are generated to filter out the embryo and micropipette images and hence ignore them during image comparison [Figure 4.11(b)]. The embryo mask is generated using a 100 µm x 100 µm (325 px x 325 px) square centered over the embryo. The micropipette mask used is a fixed-width rectangle of 18.5 µm (60 px) that extends to the edge of the screen and is positioned using the micropipette tip location. To reduce computational load, pixels left of the embryo opening are cropped out to create an ROI, as the blastomere do not float in that direction after being displaced. Once the two images are subtracted [Figure 4.11(c)], thresholded, and the embryo and the micropipette masked, the blastomere is located by determining the
Chapter 4. Automated Blastomere Extraction

Figure 4.12: Time lapse of automated blastomere extraction experiment: (a-b) Phase I Preparation, including motion planning and insertion of micropipette, (c-e) Phase II Blastomere Displacement, where the blastomere is extracted via displacement, (f-i) Phase III Blastomere Retrieval, where the blastomere is tracked and successfully retrieved by the micropipette.

largest 8-connected blob using the MATLAB function `regionprops()` [Figure 4.11(d)]. Blastomere right edge $x$-coordinate $x_b$ and centroid $y$-coordinate $y_b$, marked by a red cross, are determined using blastomere centroid and shape, and used in (4.1) in Section 4.2 to calculate the distance threshold during blastomere retrieval.

4.5 Automated Extraction Experiments

To validate the proposed automation procedures and algorithms, automated blastomere extraction is performed on 4- and 8-cell mouse embryos as feasibility and proof of concept experiments. A total of 34 trials are performed. The initial 18 trials are used to tune extraction parameters, e.g.: adjusting syringe pump parameters and distance threshold values, and the latter 16 trials are used to test the performance of automated extraction after tuning. Figure 4.12 shows a time-lapse of one such experiment, successfully demonstrating the full extraction process of micropipette insertion, displacement extraction and finally blastomere retrieval. The full breakdown of the automated extraction
experiments is reported in Table 4.1, with the subset of post-tuning results in Table 4.2. The tables report the distribution of trials according to the number of blastomeres extracted per trial, the corresponding number of successful detections of blastomere displacement using the blastomere extraction event detection algorithm reported in Section 4.4.2, and the corresponding number of successful blastomere retrievals. The success rate of blastomere displacement is 72%, which only includes trials that successfully displaced 1-2 blastomeres. The success rates of blastomere extraction event detection and retrieval are 88% and 73% respectively, but include both cases where 1-2 and 3+ blastomeres are displaced as they are valid attempts for demonstrating these phases. Given the sequential nature of these phases, success rates are reported as a percentage of number of attempts for a particular phase, and not of the total number of trials performed. While automated blastomere extraction is performed successfully and deemed feasible, many observations were made.

Figure 4.13 shows one trial that fails to retrieve the blastomere, as a small cell fragment enters the micropipette and creates a blockage before the micropipette is able to retrieve the displaced blastomere. Although this is the only occurrence of this phenomenon in the trials performed, issues such as this prevent the procedure from being
successful. Furthermore, as seen in Tables 4.1 and 4.2, many trials resulted in the extraction of 3 or more blastomeres. This issue stems from strong inter-blastomere adhesion, which could be alleviated with the use of Ca\(^{2+}\)/Mg\(^{2+}\)-free medium [79]. Some issues may arise during retrieval if a group of adherent blastomeres is extracted rather than a single blastomere, which may be the case during two blastomere biopsy. While there are no issues segmenting and tracking the group of blastomeres, the desired contact point may be different from the actual contact point during retrieval. If there is a sufficiently large difference between the coordinates of the desired and actual contact points, it would lead to erroneous micropipette motion after capture, as the algorithm attempts to correct a constant error. Although observed in some experiments, this is solved by expanding the \(d_x\) and \(d_y\) distance thresholds for motion.

Once a blastomere has been extracted and is free floating, the blastomere may travel in the \(z\)-direction and move away from the focal plane. In most trials, \(z\) displacement of the blastomere was not an issue, as the retrieval process captured the blastomeres before any significant \(z\) displacement, or the micropipette aspiration kept the blastomere in the focal plane. In one trial in which the blastomere displaced significantly in the \(z\)-axis, the micropipette simply waited in position close to the shadow of the blastomere until the blastomere was pulled back into the focal plane and was eventually retrieved successfully.

### 4.6 Chapter Conclusion

In summary, methods for the automation of blastomere extraction, the third and final step of PGD biopsy, were developed and validated using feasibility experiments with mouse embryos. The displacement method, as described by [77], was chosen as the extraction method of choice. Fluid is injected into the embryo, which pushes a blastomere out of the embryo. A multi-phase process flow for the automation of blastomere extraction was described, with conditions for successful progression. A simplified pressure model using the ideal gas law was derived to calculate system pressure, and simulations demonstrate
the responsiveness of using air as a transmission medium. Computer vision algorithms were used for the pre-procedure calibration to achieve automated micropipette control, for the detection of the blastomere extraction event, and finally tracking the blastomere during post-extraction retrieval. Proof of concept automated blastomere extraction experiments were performed on 4- and 8-cell cleavage stage mouse embryos to demonstrate the feasibility of this method. Successful experiments were observed with success rates ranging from 72% to 88% depending on the extraction phase, and thus proving that automation is feasible for extraction and retrieval of cells during biopsy, which has not been demonstrated previously.
Chapter 5

Concluding Remarks

5.1 Summary and Contributions

Assisted reproductive technologies (ART) are a host of technologies and procedures related to fertility and reproduction. The field of single cell manipulation deals with the micromanipulation of individual biological cells that range from 1 µm to 1 mm in diameter. Embryo biopsy, a class of micromanipulation involving removal of material from inside an embryo in a non-destructive manner, is procedure within in vitro fertilization (IVF), a series of benchtop micromanipulation procedures for embryos related to fertility and reproduction. This dissertation presents novel methods for the automation of single cell manipulation methods for embryo biopsy. Specifically, embryo biopsy for preimplantation genetic diagnosis (PGD), a procedure that permits genetic analysis of an embryo to detect the presence of specific genetic disorders. There are three main steps in PGD biopsy: embryo position and rotation control, zona breaching, and blastomere extraction. The goal of automation is to improve the currently low success rates of IVF and PGD by replacing human operators. Thus, eliminating the possibility of failure related to human error such as mistakes, or chemical or biological contamination, which would lead to increasing procedure safety and throughput. The overarching goal of this research is to demonstrate the feasibility of PGD embryo biopsy automation. While the automation methods developed in this thesis are derived for the embryo biopsy portion of PGD, they are not restricted to this application and can be applied in other fields.

A comprehensive literature review on the state of the art embryo biopsy techniques was first presented, focusing on single cell manipulation for position and orientation control, zona breaching, blastomere extraction, and finally computer vision algorithms. Deficiencies in methods or gaps in knowledge were highlighted. While ART is an active field, there is a significant lack of research in the automation of embryo biopsy procedures.
This research develops novel methods directed at pushing the knowledge for automation of the three PGD biopsy tasks outlined, whether it is novel methods or algorithms for automation, or adapting conventional techniques for automation. The dissertation was separated into three chapters, each dealing with a step in PGD biopsy.

**Embryo Position and Orientation Control**

First, an analysis into the automation of embryo position and rotation control was performed. The main goal of embryo position and rotation control is to optimize the position of the embryo such that subsequent biopsy procedures are safer and more efficient. Mouse embryos were used. While embryo *in vitro* manipulation itself is not novel, a novel method to achieve embryo position and orientation control using parallel plate system was developed. Parallel plate manipulation induces controlled rolling of the embryo in a manner such that embryo position and orientation can be controlled independently. A detailed analysis for manipulating cleavage-stage embryos using parallel plates was derived. Furthermore, a method for 2-cell embryo orientation tracking was developed using a geometrical model. The two blastomeres were modeled as a capsule, and the capsule projections were used to derive the embryo orientation. The geometrical model was validated against a CAD model with set orientations, and applied to experiments of rotating 2-cell embryos.

Given the main goal of embryo position and rotation control for optimizing the position of the embryo for subsequent biopsy procedures, this includes maximizing the distance to the blastomeres when performing zona breaching. To perform this optimization, a knowledge of the entire embryo layout must be known. A 3D mapping algorithm using a series of Hoffman modulation contrast images obtained at known angular intervals capable of 3D reconstruction of the embryo was developed. This 3D mapping reconstructed the blastomeres and zona separately, allowing a global optimization to determine the optimal ablation zone, the location along the zona that maximizes the distance to the blastomeres. An orientation control strategy to reorient the embryo was derived, which prescribes a series of motions that repositions the optimal ablation zone to a prescribed position, where the micropipette is located for subsequent biopsy steps.

Embryo manipulation using parallel plates was characterized using open loop experiments. Certain experiments showed excessive drift, irregular slippage and motion bias, leading to erratic and unpredictable manipulation of the embryo. To counteract these issues, closed loop position and rotation control experiments were performed using vision feedback to track the position and orientation of the embryo. Closed loop control experiments were successful and repeatable, demonstrating the feasibility of parallel plates for
embryo manipulation.

**Zona Breaching**

Second, optimization and automation of zona breaching was performed. The objective of zona breaching is to create a perforation in the zona in a safe manner to allow access to the blastomeres. The method used for zona breaching is laser zona drilling (LZD), which uses a series of deliberately positioned short high-powered laser beam to locally increase the temperature and subsequently ablate the zona. While mostly considered safe, LZD risks damaging the embryo and the blastomeres by raising temperatures excessively. The goal of LZD optimization is to perform zona breaching while minimizing temperature increase of the blastomeres. To perform LZD optimization, a thermal model of LZD was developed in COMSOL Multiphysics. The effect of single and multiple pulses was analyzed. Given the extremely transient nature of a laser pulse, it was determined that only the peak temperature was required for analysis, and that multiple laser pulses did not induce compounding effects given realistic working timescales. A lookup table for determining the peak temperature depending on beam distance and pulse duration was created using the simulation model. A series of temperature measuring experiments were performed using Rhodamine B, a thermosensitive fluorescent dye. By capturing the fluorescence intensity response of Rhodamine B to a single laser pulse using a high speed camera, the temperature response could be derived by comparing to a temperature calibration. Temperature response measurements were similar to results derived from the temperature simulation model.

Using the thermal model, the first step of LZD optimization is to determine the location along the zona that maximizes the distance between the zona and the blastomeres, called the optimal ablation zone (OAZ). Rather than optimize for a single point along the zona, small sections of zona equal to a specified width were compared. A non-point-based comparison is superior as ablation zone width is a possible factor for the selection of the OAZ. The blastomeres, inner zona boundary and outer zona boundary were segmented separately using computer vision algorithms. A brute force search then compared the distances between each candidate ablation zone along the zona to all the blastomere points to determine the OAZ. With the selection of the OAZ, coverage optimization was performed to optimize the laser parameters to fully ablate the OAZ while minimizing temperature increase of the blastomeres. Laser parameters include the number of laser pulses, and the location and pulse duration of each individual laser pulse. A genetic algorithm was used to optimize the laser parameters, and was shown to increase blastomere temperatures less than a standard evenly-spaced grid approach to LZD. Al-
gorithms were written to automatically reposition the embryo between each laser pulse for multipulse LZD. A series of LZD experiments with mouse embryos using the proposed optimization and automation algorithms were performed successfully. Adequately sized holes were made that allowed the insertion of a micropipette for subsequent biopsy steps, with calculated temperature increases within allowable ranges, demonstrating the feasibility of LZD automation.

**Blastomere Extraction**

Finally, the automation of blastomere extraction step was demonstrated using the displacement method, where a micropipette was inserted into the embryo through a perforation in the zona and fluid was injected into the embryo to push a blastomere out. Given that blastomere extraction is a multi-phase process, a process flow to integrate automation into this procedure was developed. Blastomere extraction automation was separated into three phases: preparation, blastomere displacement, and blastomere retrieval. The preparation phase involves a calibration step to achieve encoder-based position control of the micropipette, and insertion of the micropipette into the embryo; blastomere displacement phase involves fluid injection to displacement the blastomere; and blastomere retrieval phase captures the free floating blastomere using the micropipette through aspiration.

Fluid injection and aspiration control through the micropipette was achieved using a syringe pump to control system pressure. A simplified pressure model was derived for calculating the system pressure given changes in syringe pump volume for open loop control. A series of computer vision algorithms were developed to aid in blastomere extraction automation. Micropipette position control was achieved using a pre-procedure calibration method for real time encoder-based feedback rather than vision-based feedback. Computer vision algorithms were used to segment the micropipette and determine the tip location and orientation. Micropipette position control was used to automatically insert the micropipette into the embryo for blastomere displacement, and to retrieve the embryo during blastomere retrieval. A blastomere extraction event detection algorithm was used to detect when a blastomere was displaced from the embryo such that the next phase of blastomere extraction could begin. Detection was performed by observing changes in mean pixel intensity of a region of interest located at the embryo opening. During blastomere retrieval, when the blastomere has been displaced and is now free floating, computer vision was used to track the location of the blastomere through image subtraction so that the micropipette could be repositioned in a close vicinity to capture the blastomere. Automated blastomere extraction experiments were performed success-
fully on 4-cell mouse embryos, a process that has not yet been reported in the literature by others, demonstrating that automation is indeed feasible.

5.2 Future Research Directions

As is with all research, this thesis simply represents a small step towards achieving fully automated embryo biopsy procedures, where automated biopsy itself is a small step toward other grander goals. There are many improvements, unanswered questions and gaps in knowledge that remain to be filled, a few of which are outlined here.

5.2.1 Expansion of Orientation Tracking and 3D Reconstruction

Devising a strategy for the orientation tracking of 4- and 8-cell blastomeres is paramount to advance blastomere biopsy, especially in orientation control and 3D reconstruction discussed in Chapter 2. The capability to identify individual blastomeres and track their movement would be a useful tool to improve orientation tracking and 3D reconstruction. Individual blastomere motion tracking could be performed with the use of state estimators such as a Kalman filter. Then, by analyzing the motion of the blastomeres relative to each other and their location inside the embryo, it is possible to deduce changes in embryo orientation.

Once tracking the motion of individual blastomeres is implemented, a more accurate 3D reconstruction of the embryo can be achieved. The current method for generating a 3D map of the embryo, as mentioned in Section 2.4.1, contains artifacts, notably the lack definition in the valleys in between the blastomeres. Individually segmenting and tracking blastomeres would allow reconstruction of each blastomere. Then, by combining each individual reconstruction, a full 3D map of the embryo can be generated. This method of generating the 3D mapping of the embryo would eliminate certain reconstruction artifacts, such as the lack of valleys, and provide more accurate representation of the embryo for further processing.

5.2.2 Embryo Survival Rate Validation

Many of methods developed in this thesis were demonstrated for feasibility only. Once these methods have been refined further, it is important to verify whether or not automation of embryo biopsy techniques is safe and in fact do improve embryo biopsy outcomes,
whether in safety or efficacy. At the very minimum, experiments should be performed to verify embryo survival and developmental rates until the blastocyst stage. A large sample size of embryos should be used to generate statistically relevant data. While the first step may be to demonstrate that the automation procedures themselves have high success rates, the ultimate goal of embryo biopsy and assisted reproductive technologies is to improve the clinical pregnancy and birth rates.

5.2.3 Error Diagnostics and Correction

No system is perfectly reliable. At one point or another, any system will encounter problems and fail. One hallmark of a more sophisticated and robust system is the ability to detect and adapt when certain parameters do not coincide with regular operating conditions, which may occur from a process failure, or simply a new case that was never accounted for. This is similar to a higher level closed loop system with feedback.

Automated systems can have three levels of error diagnostics. At the most basic level, a system should be able to recognize when a failure has occurred, stop itself and then alert a human operator. A smarter system would attempt to recover itself from the failure so that the process can resume. Finally, the highest level of error diagnostics would allow the system to learn from failures and recovery attempts to self-teach what appropriate methods of recovery to use in the future.

In the case of automated embryo biopsy, any of the mentioned procedures in this thesis could encounter countless number of possible avenues for failure. One prime example given in Figure 4.13 of Section 4.5 for automated blastomere retrieval experiments points to a failure where the improper cell or debris was aspirated by the micropipette, preventing the desired blastomere to be retrieved. A system with integrated error diagnostics would first detect that the improper object is retrieved, stop the process, expel the debris and then continue with the retrieval. Similar failure recovery modes could be implemented in an automated embryo biopsy system, which is paramount for turnkey systems. Similarly, during laser zona drilling, if an LZD operation does not create an adequate opening in the zona, the system could respond by using more laser pulses to ablate the rest of the zona.

5.2.4 Expansion to Turnkey System

On the technological side of embryo biopsy automation, there are two main problems that truly inhibit the automation of embryo biopsy into a turnkey system: cross contamination and throughput. Both these problems must be tackled from two sides: the
biopsy procedures themselves, and process flow before and after the biopsy itself.

When performing embryo biopsies themselves, there is always the possibility of cross contamination if a contact-based method is used and the tool is not changed in between biopsy operations. A prime example is if the extraction pipette is used to extract blastomeres from multiple embryos without being changed. A process that requires changing of tool ends, or waiting for decontamination would reduce capability for increased throughput. A non-contact based method such as using LZD and optical tweezers would be ideal as there would no longer be tool ends that require changing or decontamination before moving onto the next embryo for biopsy. Conversely, a potential non-contact manipulation method could involve both optical tweezers and dielectrophoresis to allow reorientation of the embryo.

As for increasing throughput, embryos can be loaded and patterned using vacuum based ports such as those shown by Bahadur et al. [45] and Liu et al. [121], but into a series of individual chambers. The use of light-based non-contact tools means that the embryos can be enclosed in individual chambers to eliminate cross-contamination. After extraction, microfluidics could be used to separate the blastomere or trophectoderm cells from the embryo into a separate isolated chamber that could integrate the genetic analysis procedures on-chip, or at least simplify retrieval. This would create a streamlined process that would be more practical for commercialization.
Bibliography


drilling by non-contact infrared laser or acid tyrode’s on the development of human biopsied embryos as revealed by blastomere viability, cytoskeletal analysis and molecular cytogenetics,” *Reproductive biomedicine online*, vol. 11, no. 6, pp. 697–710, 2005.


[75] A comparison of different power levels used by laser systems in the IVF laboratory, Research Instruments Limited, June 2012.


