CELL ORIENTATION CONTROL SYSTEM USING A ROTATING ELECTRIC FIELD

by

Chuan Jiang

A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Graduate Department of Mechanical & Industrial Engineering
University of Toronto

© Copyright 2014 by Chuan Jiang
Abstract

Cell Orientation Control System Using A Rotating Electric Field

Chuan Jiang
Master of Applied Science
Graduate Department of Mechanical & Industrial Engineering
University of Toronto
2014

The objective of this project is to design a cell orientation control system using a rotating electric field. In particular, the system utilizes two electrostatic phenomena known as electrophoresis and electro-rotation. The device used for creating the electric field was designed and fabricated using the MEMS fabrication technique. The cell orientation control system also includes a vision tracking system that senses the orientation of the cell and a PID controller. Overall, the system is able to control the orientation of the cell with zero steady state error. The objective of this project has been met.
Acknowledgements

First, I would like to express my sincere gratitude and deep appreciation to my advisor Professor J. K. Mills for his encouragement, guidance, and belief in my abilities through the course of this work. I would like to thank Prof. B. Benhabib and Prof. G. Nejat for devoting their time on reviewing my thesis and serve on my committee. I would like to extend my appreciation and sincere thanks to my colleagues in Nonlinear Systems Control Laboratory, Dr. I. Bahadur, Dr. H. Chu, Masih Mahmoodi, Stephen Kinio and Christopher Wong for devoting their time for providing their invaluable insights and comments.

I extend my deepest appreciation to my dear parents for their support and trust in me. I highly appreciate the financial support from the Department of Mechanical and Industrial Engineering at University of Toronto and Yong Jing Development Co. Ltd. which are much needed for completing this research.
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>Nomenclature</td>
<td>viii</td>
</tr>
<tr>
<td>1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Motivation</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Objective</td>
<td>1</td>
</tr>
<tr>
<td>1.3 Research Significance and Contribution</td>
<td>2</td>
</tr>
<tr>
<td>1.4 Outline of the Thesis</td>
<td>2</td>
</tr>
<tr>
<td>2 Literature Review</td>
<td>3</td>
</tr>
<tr>
<td>3 Methodology</td>
<td>13</td>
</tr>
<tr>
<td>3.1 DEP Theory</td>
<td>13</td>
</tr>
<tr>
<td>3.2 Overview of the System</td>
<td>18</td>
</tr>
<tr>
<td>3.3 Vision Tracking</td>
<td>21</td>
</tr>
<tr>
<td>3.4 MEMS Design and Fabrication</td>
<td>24</td>
</tr>
<tr>
<td>3.4.1 Overview</td>
<td>24</td>
</tr>
<tr>
<td>3.4.2 Design</td>
<td>24</td>
</tr>
<tr>
<td>3.4.3 Fabrication</td>
<td>27</td>
</tr>
<tr>
<td>4 Experiment Results and Discussion</td>
<td>29</td>
</tr>
<tr>
<td>5 Conclusion</td>
<td>37</td>
</tr>
<tr>
<td>5.1 Summary</td>
<td>37</td>
</tr>
<tr>
<td>5.2 Research Significance and Contribution</td>
<td>37</td>
</tr>
<tr>
<td>5.3 Future Improvement</td>
<td>38</td>
</tr>
<tr>
<td>Bibliography</td>
<td>39</td>
</tr>
</tbody>
</table>
## List of Figures

2.1 Outline of the Vision Tracking Algorithm from Sheng et al.[1] .................. 5  
2.2 Alignment of Three dimensional Electrodes by Tsukada et al.[2] ............... 6  
2.3 Frames of Video Showing the Function of Cell Processor by Park et al.[3] 7  
2.4 Microelectrodes created for manipulating cells[4] .............................. 8  
2.5 (A) Diagram of coplanar electrodes. (B) SEM image of a Titanium microsphere[5]. 9  
2.6 Cell recognition and tracking. Image sequence of cell recognition and tracking by Lu et al.[6] ................................................................. 10  
2.7 Image-processing sequence conducted for polar-body recognition and tracking by Leung et al.[7] ................................................................. 11  
3.1 Electro-rotation ................................................................. 14  
3.2 Angular Velocity of Cell vs. Electric Field Frequency ........................... 16  
3.3 Physical Hardware Layout of Cell Orientation Control System:(a) Function Generator. (b) Vision Camera. (c) MEMS Electrode (d) Inverted Microscope. ................................................................. 18  
3.4 System Block Diagram of Cell Orientation Control System .................... 19  
3.5 MEMS Electrodes ............................................................. 19  
3.6 Architecture of the Cell Orientation Control System ............................ 20  
3.7 Circular Hough Transform of Single Circle ....................................... 21  
3.8 Circular Hough Transform of Multiple Circles [8] .............................. 22  
3.9 Angle $\theta$ definition: $c_1$ and $c_2$ are the centers of the two blastomeres found by Circular Hough Transform ................................................................. 22  
3.10 Vision Tracking Testing with Cell Rotation Video [9] ........................... 23  
3.11 Overview of the MEMS Electrodes, [$\mu m$] ....................................... 24  
3.12 Geometry of the MEMS Electrodes .............................................. 25  
3.13 COMSOL Simulation Arrow Volume of Rotating Electric Field .............. 26  
3.14 COMSOL Simulation of Rotating Electric Field Strength at the Center of the Electrodes ................................................................. 26
3.15 Stages of MEMS Electrode Fabrication: (a) After Exposed to UV light (b) After Metal Deposition (c) After Lift-Off (d) After Dicing (e) After Wire-bonding (f) Final Device

4.1 Electro-Rotation of Yeast Cell

4.2 Theta and Applied Voltage Vs. Time in an Open Loop Test at Various Frequencies

4.3 Theta and Applied Voltage Vs. Time with A Step Input

4.4 Theta and Applied Voltage Vs. Time with a Ramp Input

4.5 Theta and Applied Voltage Vs. Time with A Sinusoidal Input

4.6 Theta and Applied Voltage Vs. Time with A Multi-Step Input

A.1 Top level Simulink Block Diagram of the Cell Orientation Control System

A.2 Simulink Block Diagram for the Vision Tracking Block

A.3 Simulink Block Diagram for the Controller Block
Nomenclature

$\epsilon_e$  Permittivity of the medium
$\epsilon_i$  Permittivity of the cell
$\epsilon_m$  Permittivity of the cell membrane
$\eta$  Viscosity of the medium
$\hat{j}$  The unit vector of the pitch axis
$\mu_C$  Induced dipole of the cell
$\Omega$  Angular velocity of the cell
$\omega$  Frequency of the electric field [rad]
$\sigma^*_C$  Effective permittivity of the cell
$\sigma_e$  Specific conductivity of the medium
$\sigma^*_e$  Effective permittivity of the medium
$\sigma_i$  Specific conductivity of the cell
$\sigma^*_i$  Complex equivalent conductivity of the cell
$\sigma_m$  Specific conductivity of the cell membrane
$\sigma^*_m$  Complex equivalent conductivity of cell membrane
$\tau_m$  Charging time of the membrane
$\theta_r$  Reference input for angular position of the cell
$\theta_s$  Sensed angular position of the cell
\[ \vec{\Gamma}_f \quad \text{Fluidic frictional torque} \]

\[ \vec{\Gamma} \quad \text{The torque exerted on the particle due to the rotating electric field} \]

\[ \vec{E} \quad \text{Applied Electric Field} \]

\[ a \quad \text{Radius of the Cell} \]

\[ d \quad \text{Thickness of the cell membrane} \]

\[ e \quad \text{Error between reference input and sensor value} \]

\[ E_{\text{rms}} \quad \text{The root-mean-square value of the electric field } \vec{E} \]

\[ f \quad \text{Frequency of the electric field [Hz]} \]

\[ G_m \quad \text{Area-specific conductance of the cell} \]

\[ K_d \quad \text{Derivative gain} \]

\[ K_i \quad \text{Integral gain} \]

\[ K_p \quad \text{Proportional gain} \]

\[ t \quad \text{Time} \]

\[ u(t) \quad \text{Controller’s output} \]

\[ U^* \quad \text{Clausius Mosotti function} \]

\[ V_g \quad \text{Induced membrane potential} \]
Chapter 1

Introduction

1.1 Motivation

Cellular surgeries demands a range of techniques for manipulating a single cell. In recent years, researchers have become particularly interested in the technique of cell rotation. Cell rotation has a range of applications. Amongst all, intracytoplasmic sperm injection (ICSI) and polar body biopsy are the two operations where cell rotation is found particularly useful. During the ICSI, polar body shall be oriented away from the injection needle. In polar body preimplantation genetic diagnosis (PGD), the cell shall be oriented in a way such that polar body can be extracted without any invasive damage to the cell components. However, such an operation has been conventionally practised manually by skilled operator. The operator uses a micropipette to repeatedly apply vacuum and release of the cell until the targeted cellular feature is visible and the cell is properly oriented. Such approach is time consuming and has a low success rate.

1.2 Objective

The objective of this project is to design a system that controls the orientation of the mouse embryo using electric forces. Particularly, positive dielectrophoresis attracts cell to the region of strongest electric field intensity, and electrorotation creates torque that causes the rotation of the cell. Furthermore, a polar body tracking algorithm is implemented to provide visual feedback of the angular position and velocity of the cell. Finally, a PD controller is designed to control the orientation of the cell by controlling the electric field intensity.
1.3 Research Significance and Contribution

This research significantly contributes towards providing a precision control in cell handling techniques. To our best knowledge and abilities, this is the first closed-loop cell orientation control system that uses an electric field as the means of actuation to date. Specifically, a cell orientation tracking algorithm was implemented and tested for sensing the angular position of the cell. A MEMS device was designed, fabricated and tested to create the rotating electric field needed for cell rotation. Finally, an integrated control system using PID controller was designed, implemented and tested to precisely control the orientation of the cell.

1.4 Outline of the Thesis

In Chapter 2, a literature survey is first presented to go over some previous attempts in the field of cell rotation. In Chapter 3, details of the system are presented including the related DEP theories, the system design and fabrication processes. In Chapter 4, the experiment results are presented and discussed. Finally in Chapter 5, concluding remarks are given highlighting the research significance and potential area for future improvements.
Chapter 2

Literature Review

The last several years has witnessed increasing interest in the development of methodologies for single cell surgery. Such techniques are required for various biological research techniques including: (i) removal of cell organelles [10], (ii) removal of cell components for the initiation of stem cell lines [11], (iii) transfer of RNA, DNA and proteins into the cell for the creation of transgenic organisms [12] (iv) delivery of non-biologics for intracellular sensing and probing [13]; and in medical processes: (i) removal of human embryonic cells formed during cell cleavage for Pre-implantation Genetic Diagnosis (PGD) and Pre-fertilization Genetic Diagnosis (PFD) [14]. Growing demand for such techniques, now carried out manually, mandates the development of automated processing systems to achieve high throughput and high success rates.

Controlling the cell orientation is an important technique in single cell surgery. For example, in polar body preimplantation genetic diagnosis (PGD), the cell shall be oriented in a way such that polar body can be extracted without any invasive damage to the cell components [14]. Vision based sensing will be utilized to determine the location of cell internal structures, and determine how to reorient the cell for perforation. It has been shown that cells may be reoriented through the application of rotating electric fields. As discussed by Huang et al.[4], the applied rotating electric field alters the charge distribution between the electrodes which subsequently induces a cell dipole. The dipole decays very rapidly once the field is removed. However, a moment can be imparted on the cell when the dipole is presented in an alternating electric field, leading to a small change in its orientation. With a pair of 90-degrees phase-shifted voltages applied on two perpendicular electrodes, one is able to orient the cell surface correctly for perforation in two dimensional plane.

Control of the orientation of trapped particles may be achieved through the use of polarized optical traps, i.e. Airas et al. [15]. Each of two or more optical traps is
generated with an independent linear polarization state with arbitrary orientation, which permits simultaneous independent polarization rotation control. The laser beam was modulated using a single reflective nematic liquid crystal with parallel alignment. With this approach, controlled displacement, orientation and rotation of birefringent particles is made possible. The orientation of objects within optical traps may be controlled through the use of the combination of point and elliptical tweezers, Mohantry et al.[16]. The relative intensity of these two optical traps may be changed by varying their focal spot size and the laser trap power. The orientation of the elliptical tweezers in the horizontal plane, and hence the orientation of the object in the plane, can be controlled. Hence, by adjustment of the relative power of the two tweezers, controlled three dimensional orientation of micro-scale objects can be achieved. In addition to the use of laser tweezers, a number of other approach have been proposed for cell orientation control. These approaches include flow control, Leung et al.[7], electromagnetic approaches Sebastian et al.[17]. Ouyang et al.[4] has employed a dielectrophoretic force field to rotate cells, but without active control of cell orientation.

**Electro-rotation and orientation tracking**

Sheng et al.[1] developed an automated electro-rotation measurement system of canola protoplasts in 1993. The rotating electric field is produced by a programmable four-phase sine wave generator. Video images of rotating cells are digitized and processed using a velocity measurement algorithm.

The electro-rotation subsystem consists of four gold coated stainless steel plate electrodes set at right angles to each other. The center of the cell loading chamber is loaded with a two-layer isotonic solution of equal volume (lower layer 0.7M sucrose, upper layer 8% sorbitol containing the cells mounted on a horizontal microscope stage.)

The cell rotational velocity measurement subsystem uses a centroid-boundary detection algorithm carried out in three steps: 1) image digitization, 2) edge detection and 3) cell rotational velocity measurement. Once the image is digitized, the “Region Growing by Pixel Aggregation” technique is applied to detect the edges of biological cells. This technique groups pixels or sub-regions into a larger region by appending neighbouring points to a set of seed points. The algorithm was shown to be able to determine two contours, the inner contour housing the chloroplasts body and the outer contour describing the outer cell membrane boundary as can be seen in figure 2.1. From the contour feature data, a vector \( \mathbf{r} \) between the center of gravity of the two contours are calculated. As the cell rotates, by calculating the change of direction of this vector, the rotational velocity
S in rad/s can be calculated as follows:

\[ S = \frac{2\pi (\alpha_m - \alpha_n)}{360 (m - n)T} \]  

(2.1)

where \( \alpha_m, \alpha_n \) are the angle displacement of direction vector for the \( m^{th} \) and \( n^{th} \) image frames and \( (m-n)T \) is the real time interval.

Three dimensional electro-rotation

Tsukada et al.\cite{2} reported an open-loop position control system of biological cell using electrostatic force in 2002. A rotating electric field was generated by applying 90 degrees phase-shifted voltage on four electrodes around the mouse embryo. Three dimensional rotation was generated by the alignment of eight electrodes with four on the top and two on the bottom as shown in figure 2.2. The rotation speed of embryo was measured by eye and stop-watch. The electrodes were fabricated by depositing chromium and aluminium on the crystal glass substrate. The thickness of the electrodes was about 1\( \mu m \). The gap bewteen opposite electrodes was 300\( \mu m \) or 150\( \mu m \). At a frequency of 500kHz, and the amplitude of 4\( V_{o-p} \) (the magnitude of \( V_{o-p} \) was not documented in the paper), the maximum rotational speed was recorded to be 100 deg/s at 4.5\( V_{o-p} \) and 80 deg/s at 4.5\( V_{o-p} \). When the amplitude exceeded 5\( V_{o-p} \), electrolysis occurred. In order to test the viability of the cell after electrorotation, in vitro fertilization and nuclear transplantation were performed on the cell. The result of IVF shows that electrorotation does not influence the success rate of mouse embryo fertilization. The result of nuclear
transplantation shows that the rotated embryo has a survival rate of 4.8% and 3.4% less than that of the control embryo at the Fused and Cleavage stage respectively. It has a survival rate of 8.7% less than that of the control embryos at the Mor/Bla stage. The sample size of the rotated embryos and control embryos is 88 and 82.

![Figure 2.2: Alignment of Three dimensional Electrodes by Tsukada et al.][2]

**Cell parameters measurement using electro-rotation**

Arnold et al.[18] had analysed and measured the properties of mouse embryo of zona-intact, zona-free cells and of the isolated zona pellucida in 1987. The zona-intact embryo is modelled as a zona-free embryo with additional surface conductances of 100, 250 and 400 nS. The zona-free embryo was modeled with a radius of 36µm; membrane capacity of 1.25µF/cm²; and resistivity of 400Ωcm². The internal conductivity was assumed to be 5mS/cm, and relative permittivity values of 70 were assumed for inside and outside the media. The experiment result shows that both zona-intact and zona-free embryos rotate in the opposite direction to the field over the 1-100kHz range of frequencies. It shows a good agreement with the theoretical prediction, except that the theoretical rotation rate is consistently higher by about 60%. It is suggested that this is due to the increase in hydrodynamic resistance above the theoretical value. The maximum rotating speed of a zona-intact embryo is observed to be 80deg/s at about 6.3kHz. The rotating field is produced by superposition of two linear, phase-quadrature, alternating fields at right angles with a spacing of 3.0 mm between electrodes.
Electro-rotation on an integrated cell processor

Park et al.[3] designed and fabricated an integrated cell processor for single embryo in 2005. The integrated cell processor can perform four functions which are cell transport, isolation, orientation and immobilization. Electro-rotation (ER) was applied as the mechanism for cell rotation. It was stated that high voltages may lead to joule heating of the electrode and irreversible membrane breakdown of the cell. The solution is assumed to have a conductivity of 0.9 S/m and a relative permittivity of 80. The calculated rotation speed at a range of 0.14-0.56 rad/s in the voltage of 1-2V at a frequency of 400kHz. The properties of the cells such as conductivity and permittivity are obtained from the work of Arnold and Zimmerman[18]. As can be seen in figure 2.3, it was observed that the cells rotated stably with a speed of $0.1 - 0.2 \text{rad/s}$ at a frequency of 400 kHz. The result shows good agreement with the experimental data. The viability of the cell was investigated after ER. In the case of cells in vivio, innumerable mitochondria inside the cells can be observed by staining with the fluorescent dye Rhodamin 123. If the cells are damaged by environmental changes, they enter into cell death processes, and the number of mitochondria will decrease markedly. In order to verify the degree of viability of the cells exposed to the electric field (2V, 400kHz for 60s), the cells are cultured in $M16^{24}$ solution for 15 and 300 minutes. It is observed that the number of mitochondria did not decrease. In addition, the cells treated by ER and cultured for 86 hours developed normally from the two-cell stage to the blastocyst, as did the control cells.

![Figure 2.3: Frames of Video Showing the Function of Cell Processor by Park et al.[3]](image-url)
Electro-rotation of pigmented cells

Ouyang et al.[4] had observed pigmented biological cells rotating in a rotating electric field. During the experiments, rotations were observed as stable and repeatable. It was also observed that the variation of frequency, amplitude and waveform of the applied voltage change the rotation speed accordingly. The micro-electrode geometry that was used is illustrated in figure 2.4. The two pair of electrodes are perpendicular to each other. Each semi circular electrodes has a diameter of 150µm; and the distance between the opposite electrodes is 200µm. The processes of fabricating the electrodes are as follow: firstly, gold was sputtered onto the glass wafer, then the patterns are created by exposing to UV light and by wet chemical etching process. A negative mold of a micro-channel was fabricated with photoresist on a PMMA substrate 2.4. Then, the channels was mounted on the mold and peeled off later after curing. Then, the inter-connectors were built in PDMS layer and served as inlet and outlet for the cell solution during manipulation process2.4.

![Figure 2.4: Microelectrodes created for manipulating cells][4]

Electro-rotation of titanium microspheres

In stead of using biological cells, Arcenegui et al.[5] conducted experiments of electro-rotation using titanium microspheres. Arcenegui et al. reported that the spheres rotate at the fastest rate at a frequency of the order of the reciprocal RC time constant for charging the particle double layer capacitance through the resistor of the electrolyte bulk [5]. A model for the electrical torque acting on a metallic sphere was presented. The electrode array as shown in figure 2.5 was made using standard lithography and consisted
of a 300nm thick gold layer on a glass substrate. Two different designs were used, with diagonal gap between opposite electrodes of 0.5mm or 1mm. Two pairs of phase shifted voltages of frequencies 10Hz to 100kHz were applied on the electrodes which generates a rotating electric field in the center of the electrodes. The rotational speed was calculated after the experiments by looking at the videos. The medium in which the particles was suspended during the experiment was KCl solution. Medium with different conductivity values were tested (0.9, 3.6 and 15.9mS/m).

Figure 2.5: (A) Diagram of coplanar electrodes. (B) SEM image of a Titanium microsphere[5].

Cell tracking algorithm

Lu et al.[6] developed a cell recognition and tracking technique in 2010. As illustrated in figure 2.6. This technique is semi-autonomous, in which the operator clicks on a cell to first create a region of interest (ROI). And then, the Hough gradient transform is applied to find the largest circle within the ROI [19]. After the center of the circle is located, by averaging the distances between each point to the circle center, the radius is calculated. The cell is then transported to the center of the image. A sum-of-squared-differences (ssd) tracking algorithm is used to track the cell during visual servoing [20].
Cell rotation with fluid flow and orientation tracking

Leung et al.[7] designed and implemented a three-dimensional rotation system of mouse embryo in 2012. The system uses fluidic flow produced by a standard holding pipette for cell rotation. A polar body recognition algorithm was implemented. If the polar body is not found in the image, out-of-plane rotation is performed until the polar body is clear in the image. Furthermore, a polar body tracking algorithm as seen in figure 2.7 was implemented to track the polar body while the cell rotates. If the polar body is found in the image plane, an in-plane rotation is performed such that the polar body reaches a desired orientation with respect to the cell.
Figure 2.7: Image-processing sequence conducted for polar-body recognition and tracking by Leung et al.[7]

The polar body tracking algorithm finds polar body when the control system begins. Binary image is obtained using Otsu adaptive thresholding [7]. Dilation and erosion is then performed to the binary image for noise removal. The cytoplasm contour and the cell origin O are then defined. Cytoplasm is tracked as a circle with radius R. To find the polar body, the rest of the binary image is surveyed to find multiple contours [7]. As the width of a polar body is always approximately less than 30 pixels, the polar body is found by searching for a feature less than 30 pixels.

To track the polar body as the cell rotates, a polar body tracking algorithm is applied. With the optical flow method, corner detection is performed on each image frame to compute feature points to track. The motion field vector \( f_j(i) \) of feature \( j \) at image frame \( i \) is formed by subtracting the embryo position \( O \) from each feature point position. Based on the change of vector \( f_j(i) \), the rotation angle \( \theta_1 \) can be found. The polar body position vector \( p(i) \) is initialized by subtracting the embryo position \( O \) by the polar body position \( P \). It is then updated by applying rotation matrix of angle \( \theta_1 \) to the polar body vector in the previous frame, \( p(i-1) \).

The fluidic flow is produced by a standard holding micropipette (Cook; tip diameter: \( 15\,\mu m \), tilting angle: 35 deg), connected to a 250\( \mu L \) glass syringe (Hamilton) [7]. By ejecting/intaking fluidic flow through micropipette near the embryo, a torque is exerted on the embryo causing its rotation motion. For out-of-plane rotation control, the speed of the linear stage which connects the piston of the syringe \( d \) has a piecewise controller
as follows [7]:

\[
d = \begin{cases} 
1 & \text{if } \alpha = 0 \\
kp_1(\alpha - \beta) & \text{if } 0 < \alpha < \beta \\
0 & \text{if } \alpha > \beta, \alpha(i - 1) < \alpha(i) \\
-1 & \text{if } \alpha > \beta, \alpha(i - 1) > \alpha(i) 
\end{cases}
\]  

(2.2)

where \( \alpha \) is the polar body length, \( \beta \) is a threshold of polar body length at which the polar body is determined to be in the image plane, \( kp_1 \) is the proportional gain. For an in-plane rotational control, a PD controller is applied with the form as follows [7]:

\[
d = kp_2(\theta_2 - 0) + kd_2(0 - \theta'_1)
\]  

(2.3)
Chapter 3

Methodology

The cell orientation control system is an integrated closed loop control system that controls the orientation of biological cell in real time. In this chapter, the theory of Electrorotation of particles will first be presented. And then an overview will be given to describe the hardware and software architecture of the system. Finally, the design and fabrication of the MEMS Electrodes will be presented.

3.1 DEP Theory

Dielectrophoresis (DEP) refers to the force exerted on the induced dipole moment of an uncharged dielectric and/or conductive particle by a non-uniform electric field [21]. The term “dielectrophoresis” was first introduced by H. A. Pohl in 1951. The phenomenological bases of his definition are catalogued below:

- Particles experience a DEP force only when the electric field is nonuniform [21].
- The DEP force does not depend on the polarity of the electric field and is observed with AC as well as DC excitation [21].
- Particles are attracted to regions of stronger electric field when their permittivity \( \varepsilon_2 \) exceeds that of the suspension medium \( \varepsilon_1 \), i.e., when \( \varepsilon_2 > \varepsilon_1 \) [21].
- Particles are repelled from regions of stronger electric field when \( \varepsilon_2 < \varepsilon_1 \) [21].
- DEP is most readily observed for particles with diameters ranging from approximately 1 to 1000\( \mu m \) [21].

As illustrated from figure 3.1, Electrorotation (ER) is the rotation of particle by the interaction between the surrounding rotating electric field and the polarization of the particle [21]. As early as 1892, Arno reported that small particles can be made to spin
when placed in a rotating electric field. This rotation is not synchronous. The angular velocity of the particle depends on the electric field magnitude square.

In the following, we summarize the theory of electro-rotation as described in [21]. The subject embryo can be modeled as a conductive sphere (cytoplasm) with radius $a$, specific conductivity $\sigma_i$, and permittivity $\epsilon_i$, surrounding by an insulating shell (cell membrane) of thickness $d$, specific conductivity $\sigma_m << \sigma_i$, permittivity $\epsilon_m$, area-specific conductance $G_m = \sigma_m / d$, and capacitance $C_m = \epsilon_m / d$. The cell is suspended in an aqueous medium with a permittivity $\epsilon_e$ and specific conductivity $\sigma_e$ and subject to a uniform alternating electric field $\vec{E}$. The induced dipole $\mu_C$ has the expression as below:[22]

$$\vec{\mu}_C = 4\pi a^3 \epsilon_e \vec{E} U^*$$  \hspace{1cm} (3.1)

where $U^*$ is the Clausius Mosotti function describing the polarizability of the complex cell:

$$U^* = \frac{\sigma_C^* - \sigma_e^*}{\sigma_C^* + 2\sigma_e^*}$$  \hspace{1cm} (3.2)

where $\sigma_C^*$ is the effective permittivity of the cell, which can be approximated by:

$$\sigma_C^* = \frac{aC_m^* \sigma_i^*}{aC_m^3 + \sigma_i^*}$$  \hspace{1cm} (3.3)
\(\sigma_i^*, \sigma_e^*\) and \(\sigma_m^*\) are the complex equivalent conductivities given by:

\[
\sigma_i^* = \sigma_i - j\sigma_i/\omega \tag{3.4}
\]
\[
\sigma_e^* = \sigma_e - j\sigma_e/\omega \tag{3.5}
\]
\[
\sigma_m^* = \sigma_m - j\sigma_m/\omega \tag{3.6}
\]

where \(j = \sqrt{-1}, \omega = 2\pi f\) and \(f\) is the frequency of the electric field.

Moreover, the torque exerted on the particle due to the rotating electric field \(\vec{\Gamma}\) has the expression as below:

\[
\vec{\Gamma} = 4\pi a^3 \epsilon_e \text{Im}(U^*) E_{rms,j}^2 \tag{3.7}
\]

where \(\hat{j}\) denotes the unit vector of the pitch axis, and \(\text{Im}(U^3)\) denotes the imaginary part of the Clausius Mossotti factor, \(E_{rms}\) is the root-mean-square value of the electric field \(E\).

Assumed under constant angular velocity \(\Omega\), the rotational torque is balanced by fluidic frictional torque \(\vec{\Gamma}_f\) given by:

\[
\vec{\Gamma}_f = 8\pi \eta \Omega^3 \hat{j} \tag{3.8}
\]

where \(\eta\) is the viscosity of the medium.

In equilibrium,

\[
\vec{\Gamma} \cdot \vec{\Gamma}_f = 0 \tag{3.9}
\]

Substituting equation 3.7 and 3.8 into equation 3.9 yields the rotational velocity \(\Omega\):

\[
\Omega = -\frac{\epsilon_m \text{Im}(U^*) E_{rms}^2}{2\eta} \tag{3.10}
\]

Moreover, the induced membrane potential \(V_g\) can be approximated by:

\[
V_g = \frac{1.5aE}{\sqrt{1 + (\omega \tau_m)^2}} \tag{3.11}
\]

where the charging time of the membrane \(\tau_m\) is given by:

\[
\tau_m = aC_m \left(\frac{1}{\sigma_i} + \frac{2}{\sigma_e}\right) \tag{3.12}
\]
At low frequencies approximately between 1 and 100 kHz, the cell undergoes an anti-field rotation [18]. The period of the field frequency that creates the fastest rotation is equal to the time-constant of the cell membrane [18]. At intermediate frequencies above 300kHz, the cell exhibits co-filed rotation and reaches to a local maximum of rotational speed [18]. At high frequencies above 100Mhz in a weekly conductive medium, the cell also experience co-field rotation because the electric conductivity of the cell membrane is three times higher than that of the medium [18]. Based on the cell parameters described in [18], and equations 3.1 to 3.12, the rotation rate $\Omega$ of a rotating mouse embryo is plotted against frequency $f$ for different values of medium conductivity $\epsilon_e$:

![Figure 3.2: Angular Velocity of Cell vs. Electric Field Frequency](image)

The steady-state rotation rate of a mouse embryo was thus simulated according to the theory presented above. The cell parameters used in the simulation was based on what is presented by Arnold et al. in [18]. It can be seen from figure 3.2 that two peaks are observed in the low frequency region and the megahertz region which represent the anti-field rotation and the co-field rotation. The maximum rotation rate in the low
frequency region is about $360\,\text{deg/s}$ at a frequency between 1 to 100kHz. Furthermore, low frequencies are preferred for cell rotation for its low demands on the experiment equipment. Therefore anti-field rotation at low frequencies is suitable for cell rotation. It is noted that the medium conductivity also makes an impact on the spectrum of the angular velocity. In practise, there exists a certain degree of uncertainty in median conductivity. But the cell orientation control system shall have a robust performance with the PID controller which will be presented in the next chapter of this document.
3.2 Overview of the System

The goal of the system is to control the orientation of the biological cell using a rotating electric field. The electric field is produced by four parallel electrodes aligned horizontally with 4mm spacing between the opposite ones. The physical hardware of the system are shown in figure 3.3. The vision tracking system which consists of an inverted microscope (Nikon Ti), a camera (PixeLink PL-A741) and a cell orientation tracking algorithm, which senses the angular position of the cell as feedback signal to the controller.

![Figure 3.3: Physical Hardware Layout of Cell Orientation Control System](image)

The overall control architecture is shown in figure 3.4. The PID controller takes the reference angular position and the current angular position of the cell as input, and generates a voltage signal to the function generator. The function generator (Agilent 33522A) then generates a pair of 90 degrees phase-shifted voltages on the MEMS electrode as seen in figure 3.5 to create a rotating electric field with the desired magnitude and direction. The cell orientation control system is synchronized in real-time at a rate of 10Hz. The rotating electric field causes the cell to rotate until it reaches the desired angular position.
Figure 3.4: System Block Diagram of Cell Orientation Control System

1. Microscope with camera
2. PC with Controller
3. Function Generator
4. MEMS Electrode
5. Cell rotation

a. Track cell position
b. Generate Voltage Input
c. Generate electric field
d. Rotate cells

Figure 3.5: MEMS Electrodes
The controller used in the cell orientation control system is proportional-integral-derivative (PID) controller. It is a generic feedback controller that calculates the error between the measured angular position of the cell and its desired angular position. The overall control block diagram is shown in figure 3.6.

The PID controller takes error $e$ as the controller’s input and create $u(t)$ as the controller’s output. The equation for a PID controller is given as follow:

$$u(t) = K_p e(t) + K_i \int_0^t e(\tau) d\tau + K_d \frac{d}{dt} e(t)$$  \hspace{1cm} (3.13)$$

where

$K_p$: Proportional gain, a tuning parameter

$K_i$: Integral gain, a tuning parameter

$K_d$: Derivative gain, a tuning parameter

$e$: Error $= \theta_r - \theta_s$

$\theta_r$: Reference input for angular position of the cell

$\theta_s$: Sensed angular position of the cell

$t$: Time

Figure 3.6: Architecture of the Cell Orientation Control System
3.3 Vision Tracking

In order to track the angular position of the cell, a vision tracking system was designed and implemented. A mouse embryo in 2-cell stage contains two spherical blastomeres inside the cell membrane. The goal of the vision tracking system is to track the two circles (the blastomeres) inside the cell. Once the two circles are tracked, by connecting the coordinates of the two blastomeres, the planer angular position of the cell is defined. Specifically, circular Hough transformation was used to define the centres and the radius of the circles [23].

The objective of circles tracking is to find the parameter \((a, b, R)\) for the circles [8].

\[
x = a + R\cos(\theta) \tag{3.14}
\]
\[
y = b + R\cos(\theta) \tag{3.15}
\]

If the radius of the circles are known, then the objective is to find the parameter \((a, b)\) which is the coordinates of the centers. As shown in figure 3.7. Parameter circles are generated with each point from the image as the center. With a Hough accumulation array, the center of the tracked circles can be found as the intersection of all parameter circles.

![Figure 3.7: Circular Hough Transform of Single Circle](image)

Multiple circles with the same radius can be found with the same technique as shown in figure 3.8. Overlap of circles can create extra circles which can be removed by matching to circles in the original image [8].
As shown in figure 3.9, the angle of the cell $\theta$ is defined as the angle of the vector connecting the centers of the two blastomeres $c_1$ and $c_2$. To avoid singularity, the value of $\theta$ is defined to be from 0 to 180 degrees. The vision tracking algorithm was tested using a cell rotation video obtained by Bahadur [9] previously using the technique of fluid flow as described by Leung et al.[7]. As can be seen in figure 3.10 the two blastomeres inside the mouse embryo were successfully tracked by the algorithm throughout the rotation. Then, a vector indicating the angular position of the embryo were defined by connecting the centres of the blastomeres.

Figure 3.9: Angle $\theta$ definition: $c_1$ and $c_2$ are the centers of the two blastomeres found by Circular Hough Transform
Figure 3.10: Vision Tracking Testing with Cell Rotation Video [9]
3.4 MEMS Design and Fabrication

3.4.1 Overview

The purpose of this device is to create a two dimensional rotating AC electric field at the center of the MEMS electrodes. The MEMS electrodes are fabricated consists of a Cr/Au (20nm/200nm) layer created by lift-off. As can be seen from figure 3.11, the MEMS electrodes are bonded to the center of the wiring circuit board, with the center cut out thoroughly such that the device is visible under an inverted microscope. The copper surface is created by standard PCB surface etching. Wires are soldered onto the copper surfaces which connect to a multi-channel function generator.

![Figure 3.11: Overview of the MEMS Electrodes, [µm]](image)

3.4.2 Design

In the center of the MEMS electrodes lies four circular pads which create a rotating electric field in the center vacancy of the device. The electrode pads are 300µm in radius and the distance between the opposite electrodes are 400µm as can be seen in figure 3.12. Four wires act as transmitting lines connecting the electrode pads with the wire bonding pads.

In order to determine the field strength that the electrodes create in a 3-D space, simulations were performed in COMSOL. As can be seen in figure 3.13, the same electrode geometries was created in COMSOL. A pair of 90 degrees phase-shifted voltages were applied onto the electrode with an amplitude of 1V at a frequency of 50kHz. As a
result, at the center of the electrodes, the field strength of the rotating electric field was simulated to be approximately $2495 \text{V/cm}$ with an applied voltage of $1 \text{V}$ between each pair of electrodes as shown in figure 3.14.
Figure 3.13: COMSOL Simulation Arrow Volume of Rotating Electric Field

Figure 3.14: COMSOL Simulation of Rotating Electric Field Strength at the Center of the Electrodes
3.4.3 Fabrication

The fabrication of the MEMS electrodes was performed in a similar fashion as mentioned in [5]. The overall process flow is shown in figure 3.15. Firstly, HMPS primer and S1818 photo resist was subsequently deposited onto a 4 inch, 800\(\mu\)m thick, circular glass wafer by a centrifugal spinner. The wafer along with the photo mask were then transferred into a mask aligner in which the wafer will be exposed to UV light and forming the patterns.

![Figure 3.15: Stages of MEMS Electrode Fabrication: (a) After Exposed to UV light (b) After Metal Deposition (c) After Lift-Off (d) After Dicing (e) After Wire-bonding (f) Final Device](image)

The Cr/Au (20/200nm) layer was created by thermal evaporation with a thickness monitor. After the thermal evaporation, lift-off was performed by soaking the wafer with acetone, forming only the desired pattern on the glass wafer. The glass wafer was then diced by a dicing saw, forming 40 individual chips as can be seen in figure 3.15.d. Each glass chip was taken out and glued onto a pcb board made using the standard PCB manufacturing technique. As each wafer is able to produce over 40 chips, over 40 devices can be produced with the same number of PCB boards. Finally, wire bonding
was performed to create electric connection between the chip and the pcb board to create the final device for testing.
Chapter 4

Experiment Results and Discussion

In this chapter, the experiment results of cell orientation control is presented. Step inputs, ramp inputs, sinusoidal inputs and multi-step inputs were tested with PID controller. The cell orientation control system was tested with yeast cell instead of the mouse embryo due to its many advantages. Yeast cells are cheap, easy to obtain, and can be easily grown in large quantities. In contrast, mouse embryos are not available in large quantities. Testing with mouse embryos had been attempted. However, due to the small number of embryos available for testing, it was difficult to target and transfer the embryos. The testing with yeast cells however was successful. The yeast cells (Baker’s yeast, obtained in house) were grown in liquid cultures at 27°C for 2 hours prior to experiment. Before experiments, test samples were created by adjusting the electrical conductivity of the yeast extract to 0.267 mS/cm with distilled water and Hank’s Balanced Salt Solution (HBSS).

As shown in the figure 4.1, cell rotations were observed during the experiments when a rotating electric field was applied. The applied voltage between opposite electrodes were saturated at 5V to avoid electrolysis and damage to the cells. The frequency was chosen to be 400kHz. Both open-loop and closed-loop tests were carried out. A closed-loop PID controller was implemented and tuned using the standard PID tuning technique.
Figure 4.1: Electro-Rotation of Yeast Cell
Open-Loop Response

An open-loop test was carried out with a voltage of 3V applied for a period of 10 seconds. It can be seen from figure 4.2 that the rotation speed reaches steady-state shortly after the voltage is applied. This agrees with the theory presented previously that the fluid friction will be in equilibrium with the torque exerted by the electric field. It is noted that the rotating speed varies depending on the frequency of applied voltage which again agrees with the theory discussed previously. The rotating speed of the yeast cell was finally calculated to be 59.74 deg/s at 400kHz, 28.61 deg/s at 150kHz and 19.47 deg/s at 100kHz.

Figure 4.2: Theta and Applied Voltage Vs. Time in an Open Loop Test at Various Frequencies
Step Input Response

The step input response was tested with a PID controller at a frequency of 400 kHz as shown in figure 4.3. As given previously by equation 3.2, the output of the controller is expressed as follow:

\[ u(t) = K_p e(t) + K_i \int_0^t e(\tau) d\tau + K_d \frac{d}{dt} e(t) \]

where

- \( K_p \): Proportional gain, a tuning parameter
- \( K_i \): Integral gain, a tuning parameter
- \( K_d \): Derivative gain, a tuning parameter
- \( e \): Error = \( \theta_r - \theta(s) \)
- \( \theta_r \): Reference input for angular position of the cell
- \( \theta_s \): Sensed angular position of the cell
- \( t \): Time

Therefore, the gain parameters \( K_p, K_i \) and \( K_d \) need to be tuned such that the angular position of the cell quickly converge to the desired value without having a big overshoot. Finally, gain values were found as \( K_p = 0.2, K_i = 0.02 \) and \( K_d = 0.008 \). A step input of 30 degrees was applied at \( t = 10s \) to bring the cell from an angle of 40 degrees to 70 degrees. With the PID controller, the percentage overshoot was calculated as 21.4%; the settling time was calculated as 1.3 seconds and the rise time was calculated as 0.2 seconds. The small spikes observed in the steady state may be due to the accuracy of the vision tracking algorithm and the disturbance of the cell from the test environment such as the vibrations from the test bench. Overall the controller was able to track the step input and drive the position error to zero.
Figure 4.3: Theta and Applied Voltage Vs. Time with A Step Input
Ramp Input Response

The ramp input response was tested with a PID controller at a frequency of 400 kHz with $K_p = 0.2$, $K_i = 0.05$ and $K_d = 0.008$ as shown in figure 4.4. A ramp input with a slope of 4 deg/s was applied at $t = 10s$ for 5 seconds and a ramp input with a slope of -4 deg/s was applied at $t = 20s$ for 5 seconds. Overall the PID controller was able to track the reference ramp input closely without saturating the voltage input.

![Figure 4.4: Theta and Applied Voltage Vs. Time with a Ramp Input](image-url)
Sinusoidal Input Response

The sinusoidal input response was tested with a PID controller at a frequency of 400 kHz with $K_p = 0.2$, $K_i = 0.05$ and $K_d = 0.008$ as shown in figure 4.5. A sinusoidal input was applied at $t = 10s$ to bring the cell from an angle of 20 degrees to 60 degrees and finally back to its original position. Overall the PID controller was able to track the sinusoid input closely without saturating the voltage input.

Figure 4.5: Theta and Applied Voltage Vs. Time with A Sinusoidal Input
Multi-Step Input Response

The multi-step input response was tested with a PID controller at a frequency of 400kHz with $K_p = 0.2$, $K_i = 0.05$ and $K_d = 0.008$ as shown in figure 4.6. Several step inputs were applied with a step size of 20 degrees from $t = 10s$ to $t = 25s$. Overall the PID controller was able to track the reference inputs closely without saturating the voltage input.

Figure 4.6: Theta and Applied Voltage Vs. Time with A Multi-Step Input
Chapter 5

Conclusion

5.1 Summary

The objective of this project is to design a cell orientation control system using a rotating electric field. In particular, the system utilizes two electrostatic phenomena known as electrophoresis and electro-rotation. The electrodes were fabricated using PCB and MEMS fabrication techniques. The rotating electric field was generated by applying two 90 degree phase-shifted waveforms on two pairs of perpendicular aligned electrodes. The angular position of the cell was sensed by a vision camera with a cell orientation tracking algorithm based on Circular Hough Transform. Finally, a PID controller was used in the closed-loop system. It has been shown that the system is able to rotate the cell to a desired angular position with zero steady state error. Overall, the objective of the system has been met.

5.2 Research Significance and Contribution

This research significantly contributes towards providing a precision control in cell handling techniques. Specifically, this is the first closed-loop cell orientation control system that uses an electric field as the means of actuation. Throughout this research process, the following key contributions have been accomplished:

- Design and fabrication of a MEMS device which can produce the rotating electric field for cell-rotation using a function generator.

- A vision tracking system utilizes a vision camera and an algorithm based on the Circular Hough Transform which tracks the angular position of cells under an inverted microscope.
• A closed-loop control system that can precisely control the orientation of biological cells in a two dimensional plane.

5.3 Future Improvement

Based on the research conducted and possible future directions presented in literatures, these are the potential areas to be explored in the future regarding the cell orientation control:

• Testing with mouse embryos with the current system. As mentioned previously, due to the availability of mouse embryos, yeast cells were tested with the current system. As presented in the literature, many open-loop tests of electro-rotation of mouse embryos have been done in the past. With a trained operator and an appropriate operating environment, mouse embryo can be tested in a closed-loop system.

• Design and fabrication of a MEMS device that can rotate cells in three dimensional space using an electric field. This task is particularly challenging as three dimensional devices are generally difficult to fabricate. Other approaches have been found in the literature such as using the fluid flow to control the orientation of cells in three dimensional space [7]. The design and fabrication of MEMS devices for electro-rotation in three dimensional space is still to be explored in the future.
Bibliography


Appendices
Appendix A

Simulink Block Diagram

Figure A.1: Top level Simulink Block Diagram of the Cell Orientation Control System
Figure A.2: Simulink Block Diagram for the Vision Tracking Block

Figure A.3: Simulink Block Diagram for the Controller Block
Appendix B

Embedded Matlab Source Code

B.1 Waveforms Generation

%% Embedded Matlab Function for Generating Waveforms

function y = fcn(u, enable, freq)
coder.extrinsic('num2str');
coder.extrinsic('visa');
y=0;
if enable == 0;
  vusb = visa('AGILENT', 'USB0::2391::8967::MY50005289::0::INSTR');
  fopen(vusb)
  fprintf(vusb, 'source1: apply: sin 10e3, 0.01, 0')
  fprintf(vusb, 'source2: apply: sin 10e3, 0.01, 0')
  fclose(vusb)
elseif enable && u>0 && u<=5,
  amp=num2str(u);
  f=num2str(freq);
  vusb = visa('AGILENT', 'USB0::2391::8967::MY50005289::0::INSTR');

  % sin wave, 10khz, amplitude of 1 Vpp, and a DC offset of 0 V
  % fprintf(vusb, 'source2: apply: sin 10e3,1,0')

  cmd1 = ['source1: apply: sin', ',', 'f', ',', 'amp', ',', '0']
  cmd2 = ['source2: apply: sin', ',', 'f', ',', 'amp', ',', '0']
  % open visa usb connection to 33522a function generator
fopen(vusb)

% generate two wave forms to function generator
fprintf(vusb,cmd1) % source1 = sin(wt)
fprintf(vusb,cmd2)
fprintf(vusb,'source2:phase:synchronize ')
fprintf(vusb,'source2:phase 90 deg') % source2 = cos(wt)

fclose(vusb)
elseif enable && u<0 && u=-5,
    amp=num2str(-u); % the positive amplitude
    f=num2str(freq);
    vusb = visa('AGILENT','USB0::2391::8967::MY50005289::0::INSTR');

    cmd1 = ['source1:apply:sin',' ',f,' ','amp',' ',0];
    cmd2 = ['source2:apply:sin',' ',f,' ','amp',' ',0];
% open visa usb connection to 33522a function generator
fopen(vusb)

% generate two wave forms to function generator
fprintf(vusb,cmd1) % source1 = sin(wt)
fprintf(vusb,cmd2)
fprintf(vusb,'source2:phase:synchronize ')
fprintf(vusb,'source2:phase -90 deg') % source2 = -cos(wt)

fclose(vusb)
;
else
    ;
end

B.2 Circles Tracking

% Embedded Matlab Function for Circles Tracking
function [circen, radius] = fcn(u,x,y)
rad_range = [10,40];
L=5; % number of points
num_pts = 0;

coder.extrinsic('imfindcircles');
circen = zeros(L,2);
radius = zeros(L,1);
n=zeros(1,2);

[c_track, r_track] = imfindcircles((u(y(1):y(2),x(1):x(2))),...
rad_range,'ObjectPolarity','dark','Sensitivity',0.7,'Method',...
'twostage','EdgeThreshold',0.25);

n=size(c_track);

num_pts = n(1);

if isempty(c_track),
;
else
    circen(1:num_pts,:) = c_track;
    radius(1:num_pts,:) = r_track;
end

function [centers, r_estimated, metric] = imfindcircles(varargin)
%IMFINDCIRCLES Find circles using Circular Hough Transform.
parsedInputs = parseInputs(varargin{:});

A = parsedInputs.Image;
radiusRange = parsedInputs.RadiusRange;
method = lower(parsedInputs.Method);
objPolarity = lower(parsedInputs.ObjectPolarity);
edgeThresh = parsedInputs.EdgeThreshold;
sensitivity = parsedInputs.Sensitivity;

centers = [];
r_estimated = [];
metric = [];
% Warn if the radius range is too large
if (numel(radiusRange) == 2)
    if ((radiusRange(2) > 3*radiusRange(1)) || (radiusRange(2) - radiusRange(1) > 100))
        warning(message('images:...imfindcircles:warnForLargeRadiusRange', upper(mfilename), ...
        'Rmax < 3*Rmin', '(Rmax - Rmin) < 100', '[20 100]', ...
        upper(mfilename), sprintf('%\t[CENTERS1, RADI1, METRIC1]...
        = IMFINDCIRCLES(A, [20 60]);\n\t[CENTERS2, RADI2,...
        METRIC2] = IMFINDCIRCLES(A, [61 100]);'))
    end
end

% Warn if the minimum radius is too small
if (radiusRange(1) <= 5)
    warning(message('images:...imfindcircles:warnForSmallRadius', upper(mfilename)))
end

% Compute the accumulator array
[accumMatrix, gradientImg] = chaccum(A, radiusRange, ...'
    'Method', method, 'ObjectPolarity', ...'
    objPolarity', 'EdgeThreshold', edgeThresh);

% Check if the accumulator array is all-zero
if (~any(accumMatrix(:)))
    return;
end

% Estimate the centers
accumThresh = 1 - sensitivity;
[centers, metric] = chcenters(accumMatrix, accumThresh);

if (isempty(centers))
    return;
end
%% Retain circles with metric value greater than threshold
idx2Keep = find(metric >= accumThresh);
centers = centers(idx2Keep,:);
metric = metric(idx2Keep,:);

if (isempty(centers)) % If no centers are retained
    centers = [];
    metric = [];
    return;
end

%% Estimate radii
if (nargout > 1)
    if (length(radiusRange) == 1)
        r_estimated = repmat(radiusRange, size(centers,1), 1);
    else
        switch (method)
            case 'phasedcode'
                r_estimated = chradiiphcode(centers, ...
                                          accumMatrix, radiusRange);
            case 'twostage'
                r_estimated = chradii(centers, ...
                                      gradientImg, radiusRange);
            otherwise
                iptassert(false, 'images: ...
                                imfindcircles:unrecognizedMethod ');
        end
    end
end

function parsedInputs = parseInputs(varargin)
narginchk(2,Inf);
persistent parser;

if (isempty(parser))
    parser = inputParser();

    parser.addRequired('Image', @checkImage);
    parser.addRequired('RadiusRange', @checkRadiusRange);
    parser.addParamValue('Method', 'phasecode', @checkMethod);
    parser.addParamValue('ObjectPolarity', 'bright');
    parser.addParamValue('EdgeThreshold', []);
    parser.addParamValue('Sensitivity', 0.85, @checkSensitivity);
end

% Parse input, replacing partial name
% matches with the canonical form.
if (nargin > 2) % If any name-value pairs are given
    varargin(3:end) = remapPartialParamNames...
    ({'Method', 'ObjectPolarity', ...
      'EdgeThreshold', 'Sensitivity'}, ...
    varargin{3:end});
end

parser.parse(varargin{:});
parsedInputs = parser.Results;

% If Rmin and Rmax are the same then set R = Rmin.
validateRadiusRange();

function tf = checkImage(A)
    allowedImageTypes = {'uint8', 'uint16',...
    'double', 'logical', 'single', 'int16'};
    validateattributes(A,allowedImageTypes,{'nonempty',...
    'nonsparse','real'},mfilename,'A',1);
    N = ndims(A);
    if (isvector(A) || N > 3)
Appendix B. Embedded Matlab Source Code

```matlab
error(message...
    ('images:imfindcircles:invalidInputImage')));
elseriF (N == 3)
    if (size(A,3) ~= 3)
        error(message...
            ('images:imfindcircles:invalidImageFormat')));
    end
end
tf = true;
end

function tf = checkRadiusRange(radiusRange)
    if (isscalar(radiusRange))
        validateattributes(radiusRange,'numeric',...
            {'nonnan', 'nonsparse', 'nonempty', 'positive',...
            'finite', 'vector'},mfilename,'RADIUS_RANGE',2);
    else
        validateattributes(radiusRange,'numeric',...
            {'integer', 'nonnan', 'nonsparse',...
            'nonempty', 'positive', 'finite',...
            'vector'},mfilename,'RADIUS_RANGE',2);
    end
    if (length(radiusRange) > 2)
        error(message...
            ('images:imfindcircles:unrecognizedRadiusRange')));
    elseif (length(radiusRange) == 2)
        if (radiusRange(1) > radiusRange(2))
            error(message...
                ('images:imfindcircles:invalidRadiusRange')));
        end
    end

tf = true;
end

function tf = checkMethod(method)
```

validatestring(lower(method), {'twostage', 'phasecode'}, ...
    mfilename, 'Method');

tf = true;
end

function tf = checkSensitivity(s)
    validateattributes(s, {'numeric'}, {'nonempty', 'nonnan',...
        'finite', 'scalar'}, mfilename, 'Sensitivity');
    if (s > 1 || s < 0)
        error(message ... ('images:imfindcircles:OutOfRangeSensitivity'));
    end
    tf = true;
end

function validateRadiusRange
    if (length(parsedInputs.RadiusRange) == 2)
        if (parsedInputs.RadiusRange(1)...
            == parsedInputs.RadiusRange(2))
            parsedInputs.RadiusRange...
                = parsedInputs.RadiusRange(1);
        end
    end
end