Plant Cell Injection Based on Autofocusing Algorithm

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***Abstract*—Plant cell injection is difficult due to the complicated structure of cells. Possesses great challenges to carry out the injection task automatically because of the large area it covers and the multiple layers of cells. In this paper, an automatic injection system based on the microscopic focus measurement is developed to automate injection process and also to enable the flexible selection of the target cells. A new strategy is presented to overcome the problem of that the micromanipulator and cells can not be in the same focal plane during the injection process. The depth information which is the key unknown variable needed to accomplish the injection process is estimated by the autofocusing algorithm. Complex biomanipulation task, like physical transfer of material into various locations within the plant cells, is possible and the successful rate is quantified.**

***Index Terms*—microinjection, autofocusing.**

I. Introduction

Microinjection is a technique to deliver small volume of genetic substance into living cells. It has been widely used in gene injection, transgenic organism production,in-vitro fertilization and drug development [1]. Studies of cells property also requires an high successful injection rate in biological research. While most cell injection operations are still performed manually, few automatic microinjection systems are developed. In the traditionally manual microinjection process, it takes months or even longer to train an professional operator. However, even for an experienced operator, the accuracy and successful rate can not be assured. This is due to the high requirement of accuracy for both position and force. It is hard for a human to reach the target position with the perfect force consistently. Automating the microinjection process is the desirable way to improve the success rate.

The automatic microinjection systems developed by several researchers are commonly composed of an injection unit, a holding unit and a vision unit. The information obtained by the vision unit is used to control the injection process ([2], [3]). The cell's mechanical property is characterized by combining the information from the vision unit and the injection unit ([4], [5], [6]). A force sensing component is incorporated in the injection unit. These works mainly deal with single cells or a batch of single cells. Both the target cell and the micromanipulator can be clearly seen under the microscope. However, the injection of plant cells is impossible by using these methods because of the structural complexity of the plant cells and a large layout of the cell images which cause the vision problem of the micromanipulator during the injection process [7]. The vision of the cell and the micromanipulator can not been seen at the same position of the microscope objective.

In this paper, an automatic injection system based on the microscopic focus measurement is developed to automate the cell injection and also to enable flexible selection of the target cells. The focus measurement provides the depth information which is one of the key variable needed to accomplish the injection process. The system is capable of performing automatic injection on plant cells by a visual servoing control scheme. Complex biomanipulation task, like physical transfer of material into various locations within the plant cells, can be automated and the successful rate is increased.

This paper is organized as follows. Section II indicates the specific problems during the microinjection for plant cells. Section III describes the setup of the automatic injection system and the strategy of accomplishing the automatic microinjection for plant cells. The measurement of focusing is presented to solve the main problem indicated in Section II. Section IV presents the experiment setup and results. Section V concludes the paper.

II. Microinjection for Plants

Biomanipulation task involves such operations as positioning, holding and injecting materials into various locations in cells. With respect to the objective lens of the microscope, the location of the micromanipulator and specimen plane are different, i.e. the micromanipulator and cells can not be in the same focal plane. The view of the micromanipulator is also blocked by the thickness and the large area of the plant cells, as shown in Fig. 1.

Comparing the injection for plant cells with the injection for a single cell shown in Fig. 2, the plant cells cover a larger area of the microscope slide than a single cell. Furthermore, plant cells are distributed in multiple layers and it is not realistic to separate them into individual layers or individual cells. Thus, the inverted microscope, which looks up at the specimen with its objective lens, is not suitable for the plant cell injection. Because only the first few layers at the bottom of the plant can be seen from the inverted microscope, while

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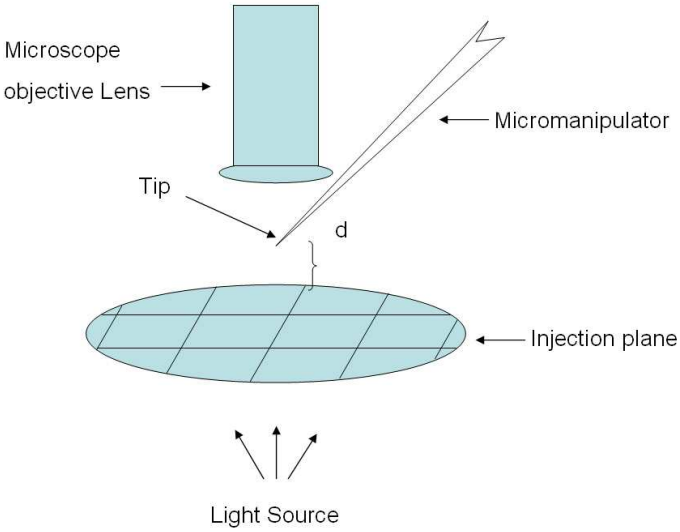

Fig. 1. Illustration of the injection for plant cells

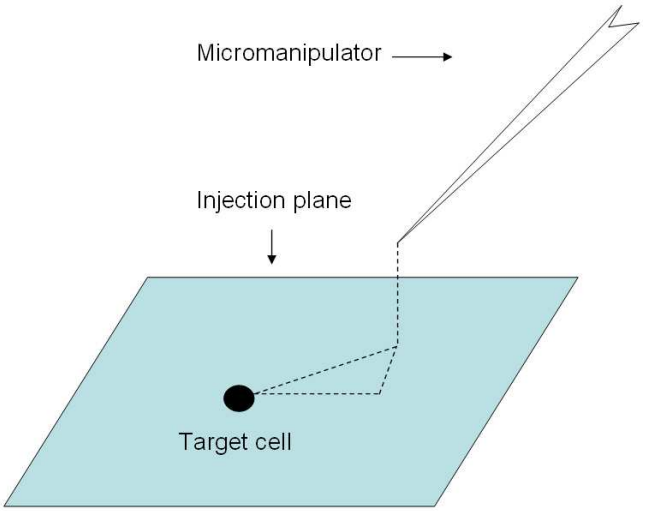

Fig. 2. Illustration of the injection for a single cell

the injection is actually carried out on the first few layers of the top. The plane can be seen and the plane can be injected are not the same. Because of this inconsistency, the target cell is never reached by the micromanipulator. Therefore, an upright microscope is chosen,i.e. the microscope looks down at the specimen. However, it results in another problem. The large area of the plant cells obstructs the light coming from the bottom of the microscope. There is not enough light for the tip of the micromanipulator to be seen from the microscope when the distance between the tip and the injection plane is too close. When this distance is hundreds of micrometers, i.e. d as shown in Fig. 1, the tip of the micromanipulator can be clearly seen by the microscope since more lights can be provided to the tip through the gap between the tip and the cells plane. However, at this time, the image of the plant cells are so blur that cells can not been distinguished individually while the image of the tip is in focus. So it is impossible to get an image which has both plant cells and tip included to accomplish the injection task.

In order to position the cells and the tip of the micromanipulator, the focused image of cells and the focused image of tip are needed. They are captured by the camera separately. The depth d, as shown in Fig. 1, is unknown. The estimation of the depth d becomes a main problem to fulfill the injection task for plant cells. While the difference between the target cell and the tip on the injection plane (2D plane) can be measured directly by image processing, the information of depth d is impossible to measure directly. Since the degree of focus of an image is related to the depth d, an automatic focusing algorithm is used to estimate the depth and a strategy to achieve injection task for plant cells is developed.

III. Design and Implementation

A. System setup

The automatic injection system is composed of an injection unit, a vision unit and an autofocusing unit. Fig. 3 shows the setup of the system.

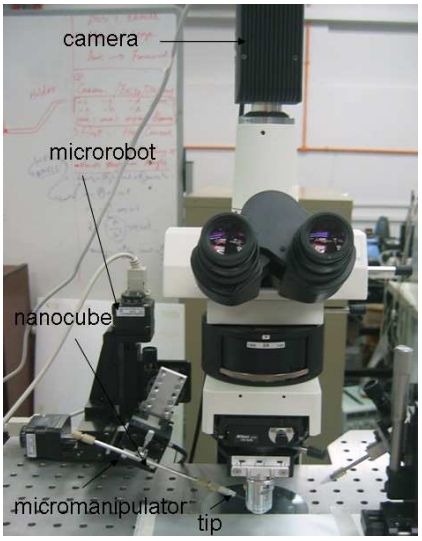

Fig. 3. Automatic injection system

The injection unit includes a sterile femtotip, a micromanipulator, a 3D piezoelectric actuator and a high precision 3D microrobot. The sterile femtotip is held by the micromanipulator. The micromanipulator is installed on the 3D piezoelectric nanopositioning cube which has a workspace of $100\times100\times$ μm, with resolution of 1 nm. The 3D piezoelectric nanocube is driven by the PZT-servo controller.The 3D microrobot is mounted by three motorized translation stages which have a travel range of $50\times50\times50$ mm, with a resolution of 1.25 μm. The two phase stepping motors mounted on the three translation stages have 200 steps per revolution. The piezoelectric nanocube and the microrobot are combined as shown in Fig. 3.

The vision unit consists of an upright microscope, a CMOS camera and a host computer. The upright microscope (Eclipse FN1 of Nikon) is used with a 20 times magnification objective

lens. The sample is stuck on the microscope slide by the gel. The Basler exA640-180m camera, mounted on the rear port of the microscope, has a maximum frame rate of 176 frames per second. Frames grasping, control of the micromanipulator are performed on the host computer.

The autofocusing unit is composed of a stepping motor installed on the fine focus knob of the microscope and a host computer. The stepping motor controls the movement of the objective lens with a resolution of 0.5 μm. The autofocusing algorithm which is used to find the best focused image in a set of images is performed on the host computer.

B. Automatic Focusing and Injection Strategy

As illustrated in Section II, the depth d is estimated using automatic focusing algorithm. The automatic focusing algorithm involves the region of the scene that is to be focused and a sharpness function which measures the degree of focus on an image quantitatively [8]. The Tenengrad method is one of the best sharpness function for obtaining the depth information [9]. In the Tenengrad method, the Sobel edge detection masks I_x, I_y are used as the operators to find the horizontal and vertical gradients. I_x and I_y are defined by

$$\mathbf{I_x} = \begin{pmatrix} -1 & 0 & 1 \\ -2 & 0 & 2 \\ -1 & 0 & 1 \end{pmatrix}, \mathbf{I_y} = \begin{pmatrix} 1 & 2 & 1 \\ 0 & 0 & 0 \\ -1 & -2 & -1 \end{pmatrix}$$

The sharpness for every image f_k is defined as

$$L_k = \sum\sum S_k(x,y), for S > T, \tag{1}$$

where

$$S_k(x,y) = \{\nabla {S_x}^2 + \nabla {S_y}^2\}_k, \tag{2}$$

T is the threshold value which is set to zero as suggested by Krotkov [10]. ∇S_x and ∇S_y are computed by

$$\begin{aligned}\nabla S_x = {} & f(x-1,y+1) + 2f(x,y+1) + f(x+1,y+1) - \\ & f(x-1,y-1) - 2f(x,y-1) - f(x+1,y-1)\end{aligned}$$

$$\begin{aligned}\nabla S_y = {} & f(x-1,y-1) + 2f(x-1,y) + f(x-1,y+1) - \\ & f(x+1,y-1) - 2f(x+1,y) - f(x+1,y+1)\end{aligned} \tag{3}$$

$f(x,y)$ are the gray value on the image pixel position (x,y). Then, the best focused image is f_k, when L_k is maximized.

A set of images are obtained by varying the position of the objective lens. The sharpness, i.e. degree of the focus of every image, changes accordingly. A figure of the magnitude of the image sharpness against the objective lens position can be obtained. As the objective lens travels down towards the specimen, a bell-like shape should be achieved for the figure. The global maximum of the sharpness function corresponds to the best focused image.

Based on the automatic focusing algorithm described, an injection strategy is developed in this paper to accomplish the microinjection task for plant cells.

In order to know the position (x,y) both for the target cell and the tip, the focused image of cells and the tip should be found before the injection started. The injection process can be performed in the following 7 steps: (1) Adjust the fine focus knob to get a clear focused image of the tip. At this time, the depth d could be thousands of micrometers. The plant cells can not be seen at all because of the large value of d. (2) Adjust the fine focus knob to move down the objective of the microscope while the tip is moved down accordingly to make sure a clear image of tip is maintained. The movements are stopped when a blur scene of cells come to be seen. The depth d which is as small as hundreds of micrometer at this time is what we are going to estimate in the next step. The blur scene of the cells helps to obtain a sharpness value which is considered as the starting point of the objective lens. (3) Moving down the objective lens through the stepping motor with known steps, a set of images with different degree of focus are saved on the hard disk of the host computer for processing. It usually takes the computer 9-10 seconds to process the images using Tenengrad method to find the best focused image. A graph of the magnitude of the image sharpness against the objective lens position is plotted. The image with the maximum sharpness magnitude have the best quality. The injection is inspected through this image. (4) With the known moving steps of the stepping motor in (3), the distance for the objective lens to travel in order to see the focused cells is known. Fig. 4 shows that this distance is just the depth d which is also the tip needs to travel in order to inject into cells.

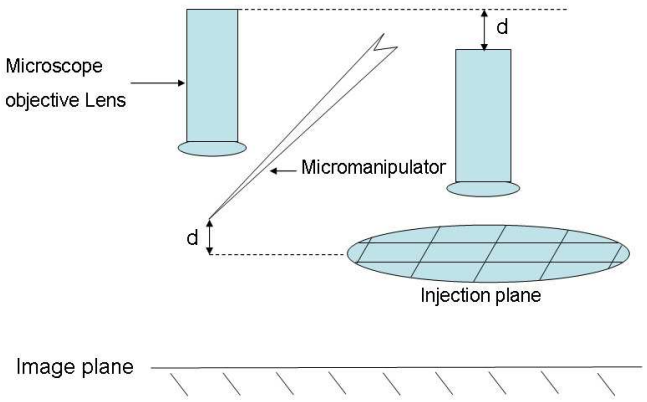

Fig. 4. The sketch of the injection strategy for plant cells

This reason is that no matter when it is tip in focus or cells in focus, the same image plane is shared. According to the basic image formation geometry, all the rays that radiate by the object and refracted by the lens converge on the image plane and forming a real image. If the CMOS sensor plane is not coincided with the image plane, the projection for a single point on the object becomes a small patch. The energy received from the object through the lens distributed over a circular patch on the sensor plane. It causes the whole image becoming blur and a defocused image is received by the sensor plane. Therefore, as long as the position of the camera is unchanged, the travel distance of the lens is the depth d between the tip and

the cells. (5) Travel back the objective lens to position the tip, (x_t, y_t) , in the focused image of tip and then travel down to the focused image of cells to find the target cell (x_c, y_c). The backlash is compensated to a resolution of 1 μm for the control of objective lens. (6) The tip of the manipulator is controlled by the microrobot to bring the tip from (x_t, y_t) to (x_c, y_c). Then, the tip is moved down $d - \Delta d$ by the microrobot, where Δd is to compensate the vertical distance in the injection process using piezoelectric nanocube. The pre-injection is finished. (7) The injection is executed by the piezoelectric nanocube by $2\Delta d$ along its x-axis with an angle of 30 degrees, as shown in the Fig. 5. In the experiment, an experience value of 20 μm is used for Δd.

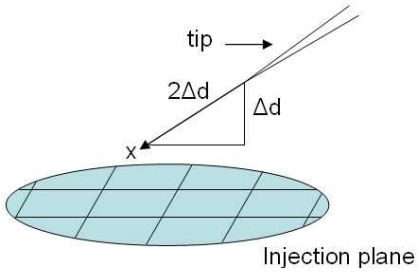

Fig. 5. The sketch of the injection by the tip of the manipulator

IV. Experimental Results

The experimental results demonstrate that the plant cell injection based on the autofocusing algorithm is successful. The Fig. 6, plotted during one of the experiments, shows that the sharpness value against the lens position of every image is distributed as we presented in the section III. The value of

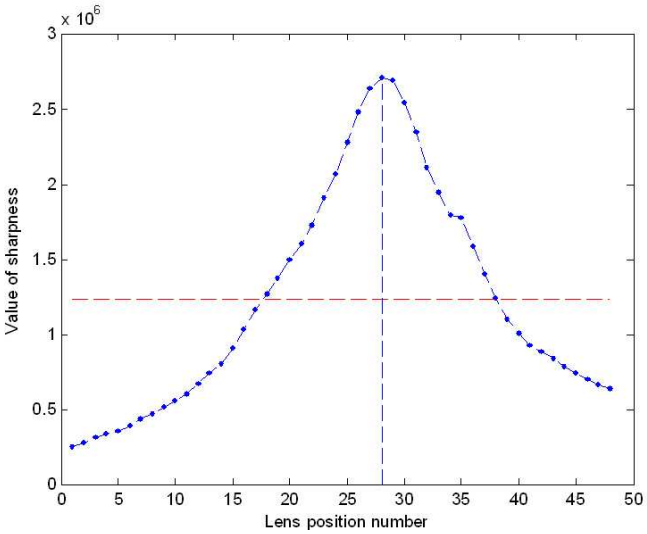

Fig. 6. The image sharpness vs the objective lens position

sharpness is computed in an interval of 12.5 μm of the lens position. The maximum value of the sharpness corresponds to the frame 28 which is proven to be just the best focused image on the host computer. Then, depth d is 350 μm. The tip travels d-Δd = 330 μm to get ready for the injection by the piezoelectric nanocube. From the experimental results, the range of d is 300 to 390 μm with an average of around 350 μm. The estimation resolution of d can be qualified to 25 μm which results from the mispositioning of the best focused image. The image at the starting point of the objective lens is shown in Fig. 7, the tip is clear but the cells are blur at this time.

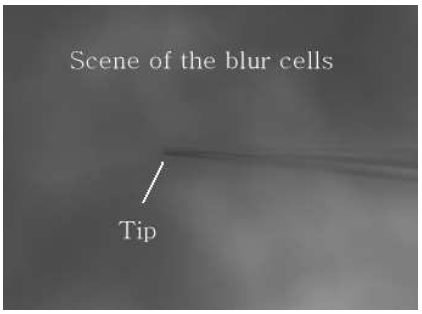

Fig. 7. The image shown at the starting point of the objective lens

After the depth d is estimated out, the injection is carried out. Fig. 8 shows the injection result. The tip of the manipulator penetrates into the target cell.

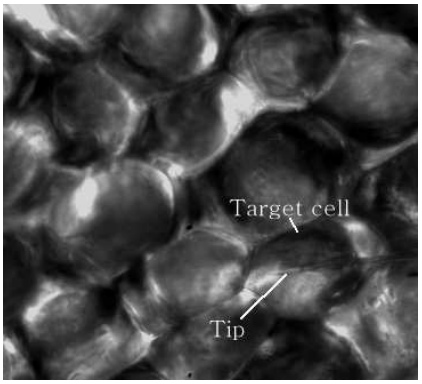

Fig. 8. Sketch of the injection result

V. Conclusions

An automatic injection system for plan cells was developed. An injection strategy for plan cells is proposed. The main problem during the injection is analyzed. An autofocusing method is used to find the depth between the tip of the micro-manipulator and the target cell which is the main specific issue for plant cell injection. Based on the good performance of the Tenengrad method in determing image sharpness, estimation of the depth is accurate enough for the microinject to proceed. Experimental results show that the automatic penetration into the plant cell is achieved by combining the injection strategy and the autofocusing method. Microrobot plays an important role in biomanipulation tasks.

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