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Single Protoplasts Pickup System Combining Brightfield and Confocal Images

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***Abstract*—This paper presents a system that picks up protoplasts produced by removing the surrounding cell wall of root cells while preserving their positional information. The fundamental concept of this system involves scanning the root tip over time using a confocal microscopy to measure the positional information of each cell. Then, the protoplast pickup is conducted after switching to a brightfield microscopy to ensure the certainty of pickup. The system measures the position of single protoplasts, adjusts the position of the pipette using a 3-axis micromanipulator, and picks up the target protoplast using a microfluidic pump driven by a piezoelectric actuator. To automate this pickup process, we achieved calibration of the system. The fully automatic 3D calibration of the pipette tip was achieved, allowing 3D micromanipulation under the microscope with an accuracy of 3.1 μm in the XY-plane. Furthermore, by implementing multiple functions such as automatic detection of protoplasts, the process of protoplast pickup has been achieved.**

I. Introduction

One of the most talked-about global issues in recent years is the rapid growth of the world's population. According to the United Nations, the world population is expected to reach 9.3 to 10 billion by 2050 with a probability of 95% [1], which will increase the demand for food by 1.7 times compared to the 2010 level [2]. On the other hand, it is predicted that crop yields will decrease in many countries due to climate change such as global warming. The effects are substantial especially in the tropical regions where the risk of famine is high [3].

To solve this problem, we are focusing on regenerative ability of plants. Plant regeneration refers to the remarkable ability of plants to heal wounds that is caused by environmental stimuli such as herbivory, wind, and human-induced cuts [4]. By elucidating the mechanisms that improve plant regeneration, we hope to contribute to increasing the food supply by developing breeding methods for crops that can be harvested many times. Therefore, we decided to investigate plant regeneration at the single-cell level using *Arabidopsis thaliana*, which has been studied for several decades as a model plant for research on mechanisms of plant growth due to its simple root structure [5][6]. For single-cell level analysis, the single-cell transcriptome is commonly used as an analytical method [7]. However, the conventional method of cell collection has a major limitation, which is the loss of the original positional information of each cell [8]. The root tips are treated with cell wall lytic enzyme solution to produce protoplasts by removing the cell wall. Produced protoplasts are then collected through centrifugation, losing positional information. If the loss of the original positional information of each cell can be prevented, more detailed single-cell transcriptome analysis will be possible, providing important knowledge regarding the elucidation of mechanism of root growth. Moreover, it is expected to make a significant contribution to the field of plant regeneration and agriculture.

Based on the above, we developed an innovative system that allows for the isolation of protoplasts while preserving the original positional information within the root tip. To preserve the positional information of each cell, it is necessary to observe the process from the enzymatic treatment of the roots to the isolation of protoplasts over time. We measure the positional information by using a confocal microscopy to observe genetically engineered roots with fluorescence, and by performing three-dimensional scans at certain time intervals to track the position of each protoplast. While there are several conventional research on single cell pickers under a microscope [9][10], the focus is on isolating cells scattered in a 2D plane within a static environment. What makes 3D micromanipulation and automation even more challenging is that observation is limited to 2D planes under microscopy. Also, due to refraction of light under microscopy, the distance of the cells from the objective lens is difficult to measure. Moreover, there are no conventional research that effectively utilize confocal microscopy because the preservation of positional information of cells was not intended.

Therefore, our research introduces three significant innovations to pick up cells in 3D space within a dynamic environment. Firstly, we constructed a system integrating confocal microscopy and brightfield microscopy to simultaneously maintain positional information of cells and accurately pick up cells. Secondly, we achieved high-precision 3D micromanipulation through the development of fully automated pipette tip calibration and a high-resolution microfluidic pump. Thirdly, we automated the protoplast pickup by combining confocal and brightfield images using image processing. In detail, we report on a robotic system that can accurately pick up a single protoplast with a diameter of about 10 μm under an inverted microscope in a dynamic environment. We also propose an automated method to improve the accuracy and throughput of the pickup. We demonstrate proposed system on actual pickup experiments.

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II. System Overview

A. Single Protoplasts Pickup System

Fig.1(a) shows the configuration of the single protoplasts pickup system. This system consists of a 3-axis micromanipulator (Quick Pro, Micro Support Co., Ltd., Japan) with a piezo-driven microfluidic pump as an end-effector and a chip for root immobilization and observation. A camera for brightfield microscopy is attached to the eyepiece of an optical inverted microscope (ECLIPSE Ti-E, Nikon Co., Ltd., Japan), and another camera for confocal imaging is attached from another optical path. Taking advantage of the high-resolution in the Z-axis offered by confocal microscopy, we maintain the positional information of each cell through periodic three-dimensional scans. However, confocal microscopy only allows for fluorescence observation, making it impossible to simultaneously observe the pipette tip and the protoplasts for pickup reliably. To address this limitation, first the target protoplast is detected under confocal microscopy. Then the optical path is switched to brightfield microscopy, where cell is picked up based on the brightfield image. Furthermore, we achieved control of each device from a single program/UI, thus allowing for the acceleration of protoplast pickup experiments.

B. Piezo-driven Microfluidic Pump

A microfluidic pump was designed and fabricated by a 3D printer using a multilayer piezoelectric actuator (MPA-UA1, MESS-TEK Co., Ltd., Japan) that features high speed and high resolution. These features of the piezoelectric actuator are critical to controlling the pickup of very small moving protoplasts. The configuration of this microfluidic pump is as shown in Figure 1(b). Piezo actuator is connected to a 3D printed part, which is part of the fluidic channel. This part is connected to a deformable silicon tube. With this design, the displacement of the piezo actuator is converted into the displacement of the silicon tube. This results in a change in the volume within the fluidic channel, allowing for suction and ejection. Then, we evaluated the performance of this pump using a bead as shown in Fig.2. As a result, this piezo pump has a maximum suction volume of 12.5 nL for a maximum displacement of 20 μm, a theoretical resolution of 0.2 pL and a 90% response time of 50 ms. It provides sufficient performance to isolate protoplasts with a diameter of approximately 10 μm and a volume of 0.5 pL. In addition, a syringe pump is attached to the fluidic channel in this pump so that the fluidic channel can be filled with liquid.

C. Chip for Root Immobilization and Observation

We designed and fabricated a chip for root immobilization and observation with a 3D printed jig and a PDMS chip. The fabrication and assembling process of this chip is shown in Fig.3. The concept of this chip involves firstly placing the roots of *Arabidopsis thaliana* in the cavity of the PDMS chip. Secondly, this section is filled with a cell wall lytic enzyme solution, enabling the observation of produced protoplast under an inverted microscope. This process allows for the selective pickup of target protoplasts. Additionally, this chip achieves root immobilization by using agar, which is required for tracking of cell positions. Moreover, this chip has ejection wells, allowing the entire sequence of operations from picking up protoplasts from the root tip to ejecting them into the well to be conducted on the chip.

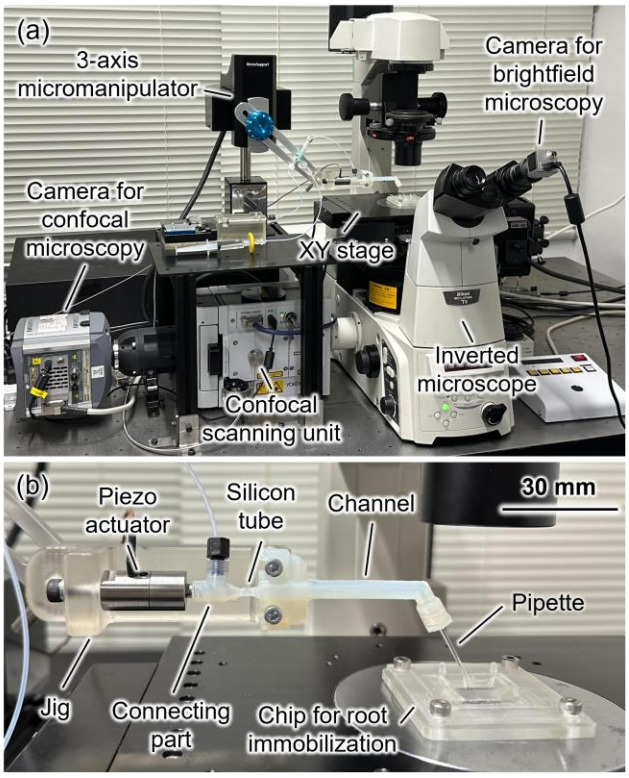

Figure 1. Photograph of single protoplasts pickup system. (a)The system overview. (b)Piezo pump and chip part.

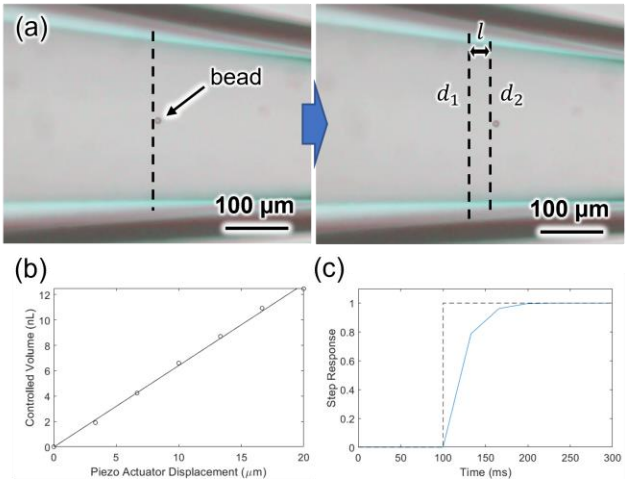

Figure 2. Evaluation of the volume, resolution, and response time of the piezo pump. (a)Experimental photograph visualizing the displacement of a bead. (b)Relationship between controlled volume and displacement of piezo actuator. (c)Response time with step input.

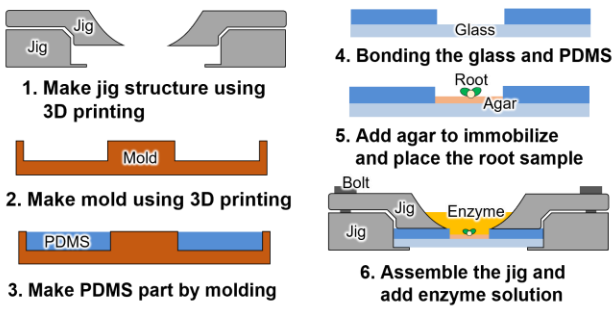

Figure 3. Fabrication and assembling process of the chip.

III. Calibration Method

A. Coordinate System

The coordinate system of the proposed system is set up as shown in Fig.4, and the system can be automated by determining the correspondence between each coordinate system through calibration. We propose two calibration methods for the microscope frame {M}, which is important for achieving the automation of this system. The first is the calibration to determine the correspondence between the microscope frame {M} and the pipette frame {P}. The second is between the microscope frame {M} and the confocal image frame {I}.

B. Fully Automatic 3D Calibration

We developed a method for fully automatic calibration to rapidly and accurately pick up protoplasts scattered in 3D space. Specifically, this method is based on the automatic determination of the homogeneous transformation matrix from the microscope frame {M} to the pipette frame {P} using image processing. To find the pipette tip position in microscope frame {M}, the main image processing methods used are shown in Fig.5(a). These include the use of YOLO [11], a deep learning object detection method, for pipette tip detection; template matching with multiple images, including those with focus deviations to detect pipette tip within microscope frame. Then center detection of the pipette tip is performed by detecting the contour and acquiring the minimum enclosing circle which is used for position of the pipette tip in XY-plane. Autofocusing performed using the sharpness measure of pipette tip. The best focus position of a pipette tip can be found, which is used as Z position of pipette tip. Using these algorithms pipette position in 3D space can be obtained, and the following calibration can be performed.

The actual calibration method involves first obtaining the best focus position of the pipette tip at multiple points in 3D space, as shown in Fig.5(b). In this process, the Z-axis of the microscope and the 3-axis micromanipulator are controlled, and the image processing as described above is utilized. The coordinates in the microscope frame {M} and the coordinates in the pipette frame {P} obtained during this process are stored in matrices $\boldsymbol{M}$ and $\boldsymbol{P}$, respectively. By using the pseudo-inverse matrix, the 4x4 three-dimensional homogeneous transformation matrix $\boldsymbol{T}$ can be calculated as

$$\boldsymbol{T = PM^T(MM^T)^{-1}}. \quad (1)$$

As a result, we were able to realize a function that enables movement of the pipette tip to a specific point on a focal plane with the best focus by using the coordinates of the microscope frame {M} and converting them to the coordinates of the pipette frame {P}. This enables the pipette tip to move to the target point at the shortest distance and at high speed and is expected to contribute greatly to speeding up the experiments. Furthermore, the accuracy of this calibration was evaluated by conducting three experiments in which taking the average of the deviations on the image from more than 20 target points. These experiments were performed under the same condition with an inverted microscope magnification of 100x. As shown in Fig.5(c), we achieved a high precision of 3.1 µm in the XY-plane, this calibration proves to be highly valuable for the experimental system. However, regarding the Z-axis direction, since the calibration is based on focusing, evaluating accuracy

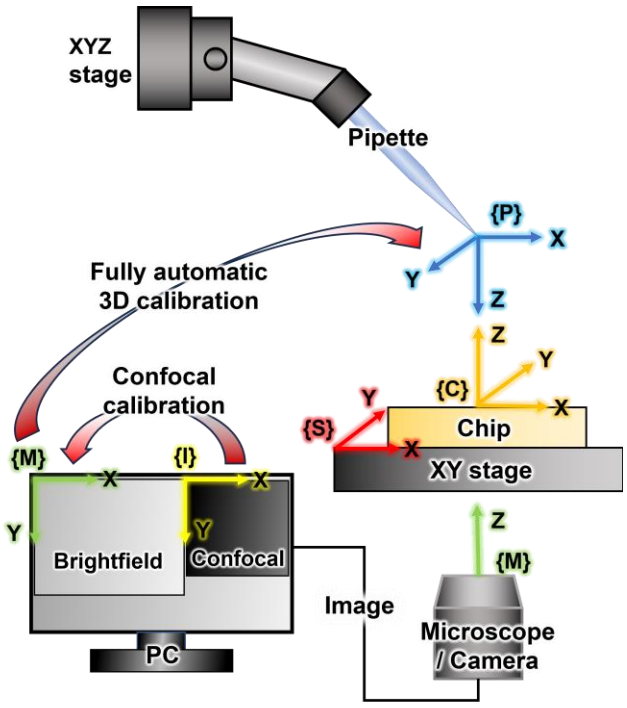

Figure 4. Illustration of the coordinate system and the coordinate transformation with fully automatic 3D calibration and calibration between brightfield and confocal images in single protoplasts pickup system.

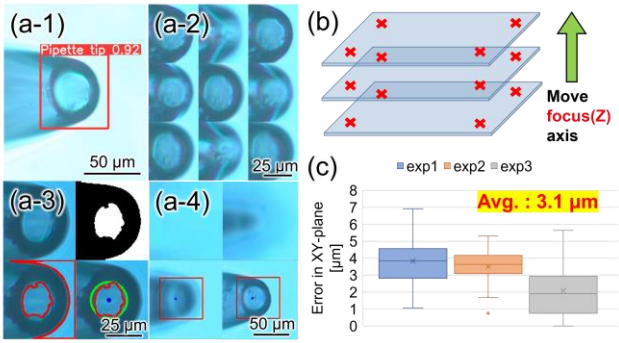

Figure 5. Fully automatic 3D calibration of the pipette tip; (a)Image processing for fully automatic 3D calibration. (a-1)Pipette tip detection using YOLOv5. (a-2)Template images with focus deviations for multiple template matching. (a-3)Center detection of the pipette tip using OpenCV. (a-4)Time series images of autofocus using the sharpness measure of the pipette tip. (b)Target planes and points to acquire the coordinates for fully automatic 3D calibration. (c)Accuracy of fully automatic 3D calibration in the XY-plane.

in absolute values is challenging and remains a future task. This calibration takes about 3 minutes for the whole process, but since it only needs to be done once before the experiment, the time constraint is not significant.

C. Confocal Calibration

We developed a method for calibration of two images from confocal microscopy and brightfield microscopy, which requires different image processing methods to detect the same object within the frame. The two cameras also have different number of pixels, scale, and angles. This calibration converts from the confocal image frame {I} to the microscope

frame {M} for positioning the pipette tip to a certain position after the protoplast is detected in the confocal image. The concept and process are as shown in Fig.6. Firstly, the centers of fluorescent beads scattered on a plane are recognized by contour detection after noise processing. Secondly, the correspondence is obtained by scaling, rotating, and translating the confocal image so that centers of all detected beads in the confocal image are matched to centers of some of detected beads in the brightfield image. Finally, using this scale, rotation and translation, positions in confocal image frame can be converted to positions in microscope frame.

IV. Functions for Automatic Pickup

A. Protoplast Detection in confocal image

We developed an automatic detection function for protoplast in confocal images as shown in Fig.7(a). This method consists of binarization of image, contour detection, and classification of roots and cells by the area. Additionally, protoplasts are circular in shape unlike cells. Therefore, only those with a circularity greater than a certain threshold are detected as protoplasts among the classified cells. The circularity is expressed as

$$\textbf{\textit{Circularity}} = 4\pi^{*}A/L. \quad (2)$$

Where the A and L are the area and length of detected contours, respectively.

Moreover, this method also includes the recognition of the protoplast which is the easiest to isolate among the protoplasts detected in this way by calculating the distance from other protoplasts. In this case, the protoplast that is the furthest away from other protoplasts is chosen to be isolated. In addition, when combined with scanning using confocal microscopy, the one with the highest central brightness in multiple confocal images is selected as the target plane for pickup. With this approach, it is possible to recognize the central plane of the target protoplast, contributing to improved stability in the Z-axis direction in the three-dimensional pickup plan.

B. Protoplast Detection in brightfield image

After detecting protoplasts in confocal images, the actual pickup is performed in brightfield microscopy, thus we have realized a detection of protoplasts in brightfield image. Specifically, protoplasts could be detected as shown in Fig.7(b) by using YOLOv5, which is strong in detecting small objects. The number in the image is the Confidence Score, and detected objects with a higher score are recognized as protoplasts. As a result, the protoplasts detected in the confocal image and the protoplasts detected in the brightfield image could be matched by the confocal calibration.

C. Detection of the Number of Protoplasts within Pipette

We have realized a function for detecting the number of protoplasts within the pipette during pickup, which is necessary for fully automated protoplast pickup. The basic algorithm of this sucking detection is shown in Fig.8(a), and the detailed algorithm and image processing method are shown below.

1. Acquire the original image before suction with the piezo pump.

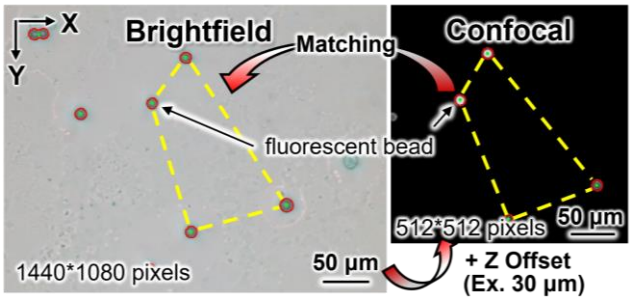

Figure 6. Concept of confocal calibration for matching two images.

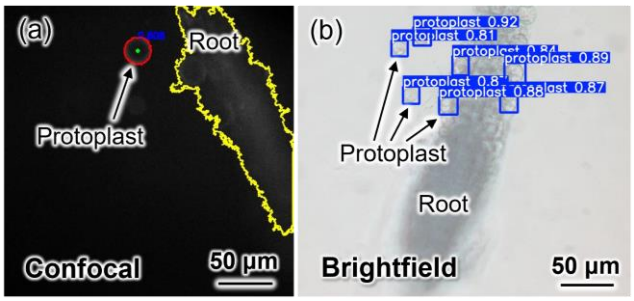

Figure 7. (a)Result of protoplast and root detection using contour detection with circularity in confocal image. (b)Result of protoplast detection using YOLOv5 with confidence score in brightfield image.

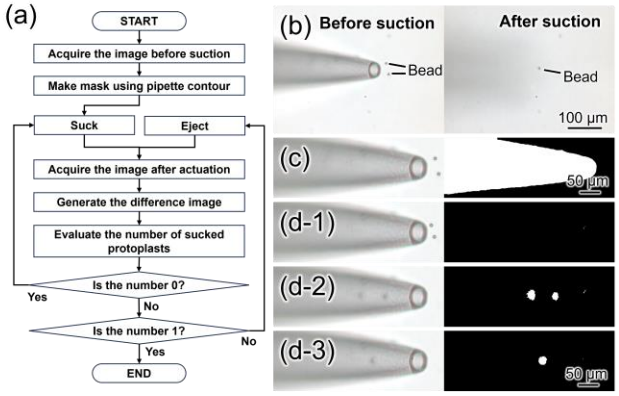

Figure 8. Detection of the number of protoplasts within the pipette using beads; (a)Basic algorithm. (b)Image with 2 beads before suction and image with one bead after suction. (c)Initial image and mask generated from it. (d) Time series images in brightfield and difference images from initial image. (d-1)After suction. (d-2)Suction of 2 beads. (d-3)Suction of 1 bead by ejection of 1 bead.

2. Detect the pipette tip in the original image using YOLOv5.
3. Estimate the region of image where pipette is from the detected position and size of the pipette tip and crop the image.
4. Binarize the cropped image using Otsu's method [12].
5. Detect the contour of the pipette after noise processing by morphological transformation in the binarized image.
6. Create a mask from the detected contour of the pipette.
7. Create the evaluation image (before) by overlaying the cropped original image and the mask.
8. Perform a suction with piezo pump and acquire a post image.

9. Crop the post image in the same way and overlay the mask on it to create the evaluation image (after).

10. Generate a difference image between the evaluation image (before) and the evaluation image (after).

11. Process the difference image with noise processing by morphological transformation and binarize it.

12. Detect contours in the binarized difference image and evaluate the number and position of sucked protoplasts.

13-1. If one protoplast is detected inside the pipette, the process is finished.

13-2. If multiple protoplasts are detected, perform ejection with piezo pump and repeat from 9.

13-3. If nothing is detected, repeat from 8.

Verification experiments for the sucking detection using the above algorithm were conducted using beads with diameters of 7 μm, as shown in Fig.8(b-d). As a result, the suction was detected when a single bead was picked up. Even when two beads in close proximity were sucked up together, one bead was ejected, and the other single bead was successfully isolated. This demonstrates that the feasibility of isolating a single protoplast has significantly improved, contributing to the automation of the pickup experiment.

V. Experiment and Result

We conducted an automatic pickup experiment of a single protoplast using the developed system. The algorithm of the overall experimental process is shown in Fig.9. The process begins with the preparation of the root and the setup of devices, followed by calibration to complete the system configuration. Subsequently, root tip is scanned at regular intervals using confocal microscopy. Then, this algorithm is employed to pick up the detected protoplasts as follows.

1. Scan the root tip with the confocal microscopy.

2. Detect the protoplasts in multiple confocal images.

3. Target the protoplast with the highest brightness at the center among those which are recognized as the easiest to separate in each confocal image.

4. Move the Z-axis of the microscope to the plane where the target protoplast is detected.

5. Switch the optical path to brightfield for pickup.

6. Detect the protoplasts in the brightfield image.

7. Match the target protoplast in the brightfield image.

8. Position the pipette tip to the detected position.

9. Pick up the target protoplast with the detection of the number of protoplasts within the pipette.

10. Retreat the pipette after the suction is detected.

As a result, the entire process from scanning using confocal microscopy to automatic pickup of detected protoplasts was successfully automated, as shown in Fig.10. Here, the ejection of the protoplast is performed through remote operation.

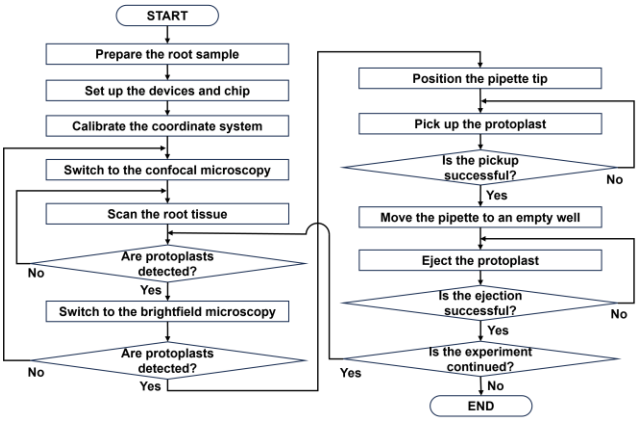

Figure 9. Algorithm of the overall experimental process.

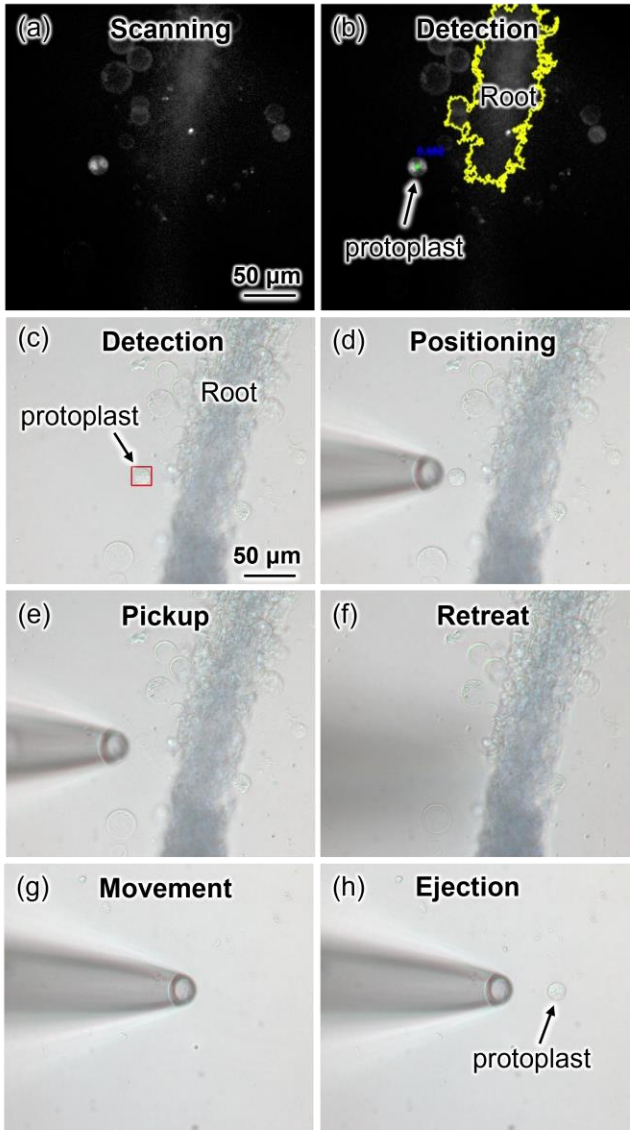

Figure 10. Result of automatic single protoplast pickup and ejection by remote operation; (a)Scanning the root with the confocal microscopy. (b)Protoplast detection in a confocal image. (c)Protoplast detection in a brightfield image. (d)Positioning the pipette tip. (e)Pickup of the target protoplast with piezo pump. (f)Retreat of the pipette after the pickup. (g)Movement of the pipette tip for ejection. (h)Ejection of the protoplast.

Furthermore, to understand how much the automation of the protoplast pickup contributes to improving throughput, we compared and evaluated the operation time using UI by remote operation with beads. In this case, we evaluated the time from detecting beads in a brightfield image to picking up a single bead because the time for scanning with a confocal microscopy and switching the optical path is fixed. The results are as shown in Fig.11, revealing that the pickup can be performed about 6~7 times faster than using UI by remote operation. Additionally, since the remote operation in this study was performed using the UI developed in this research, it is expected that the difference would be even greater in cases where each device is operated independently. The pickup time of about 30 second with remote operation would not be sufficient to pick up all protoplasts in time. Considering the possibility of multiple protoplasts being produced within one minute in this experiment, it is clear from this evaluation that automation of the protoplast pickup is essential. The automatic pickup succeeded 10 times out of 12 attempts, with a success rate of 83%. The two failed attempts involved a failed bead detection in the brightfield image and failed detection of the number of beads within the pipette.

VI. Conclusion and Future Work

In this study, a robotic system was developed to pick up produced protoplasts at the single-cell level while observing them under an inverted microscope. The system integrates a 3-axis micromanipulator, a microfluidic pump driven by a piezoelectric actuator, a chip for root immobilization and observation, an inverted microscope, and two cameras for brightfield microscopy and confocal microscopy. Additionally, we enabled the control of all these devices from a single program, contributing to the acceleration of experiments. We also proposed a method for fully automatic 3D calibration of a pipette tip using image processing. We also proposed a method for calibration between confocal and brightfield images. Moreover, we implemented protoplast detection and detection of the number of protoplasts within pipette during pickup. We confirmed that a single protoplast can be automatically picked up using this system. The system described above is the first of its kind, and we believe that the technology we developed for the three-dimensional pickup of single cells has broad applications. Since these calibration and automation methods are based on object detection by using YOLO and can be easily applied by using the respective learning models depending on target objects, there are various possibilities for development in 3D micromanipulation field.

Regarding the calibration, evaluation of accuracy in Z-axis direction is considered as a future task. Then, we plan to conduct further research on integrating AI and fluid simulations to design algorithms for improving the success rate as well as pickup speed of single protoplasts. Specifically, we are considering combining this method with protoplast tracking using MOT (Multiple Objects Tracking) methods such as DeepSORT [13] to recognize each protoplast by its ID. Moreover, this system would be able to pick up a single protoplast of 10 μm or less from a crowded environment by planning microscope magnification, pipette tip diameter, and pump flow rate considering the size of the target protoplast and its positional relationship to other objects.

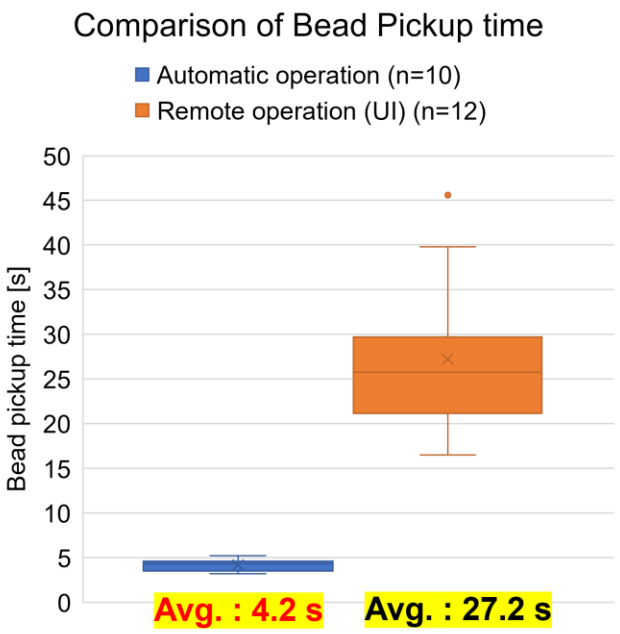

Figure 11. Comparison of bead pickup time from detecting beads in a brightfield image to picking up a single bead using UI by remote operation and automatic operation.

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