2013 IEEE/RSJ International Conference on
Intelligent Robots and Systems (IROS)
November 3-7, 2013. Tokyo, Japan

Piezoelectric Inkjet-based One Cell per One Droplet Automatic Printing by Image Processing*

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***Abstract*—Piezoelectric inkjet printer technology recently has gained attention and was utilized eject particles and cells of several tens of μm scale, which is large compared to colloid used in printer ink. However, the inkjet head was originally design for color ink. Therefore, problems, such as, head clogging and trajectory error occurred. In this study, those problems were addressed, and optimal condition for stable cells ejection was verified. Furthermore, one cell per one droplet printing method was established by observing cells position on the inside of inkjet head. Finally, automatic one cell per one droplet printing system was successfully developed by processing image of the inkjet head to automatically detect cells position. The automatic one cell per one droplet printing system was successfully developed with 98% successful ratio.**

I. Introduction

Single cell analysis are key component in the understanding of biological process and basic biological concept. As research in the study of single cell become more complex, cell handling system role becomes more important. In a study requiring precise single cells placement, including research involving in vitro cardiomyocytes network [1], single cells positioning proved to be difficult. On the other hand, researches about cell-to-cell differences are becoming increasingly important. For example, in the analysis of antibiotic response, resistance could originate from a sub-population, which forms the basis of the subsequent main population [2]. In such case, precise single cell patterning device which capable of patterning large number of single cells on arbitrary position is needed.

On the other hand, inkjet printing technology had been adapted in the field of biotechnology to print protein and cells [3,4,5,6,7,8,9,10,11,12,13]. Inkjet printers are commonly used to print documents and photographs in home and offices from a few decades ago. This technology works by separating a tiny drop of liquid that are positioned onto desired position. Inkjet printer technology main advantages are its high speed printing ability, thus there had been effort to construct functional tissue three-dimensionally [14]. However, these studies utilized the modified commercial inkjet printer, which was originally designed to print colloid particles, such as color ink. Therefore, when particles considerably larger than colloid particles were used instead of color ink, critical problems arose. These problems include inkjet head clogging, trajectory errors, and unstable ejection of liquid suspensions.

In this study, the development of one cell per one droplet printing system is the target. In order to realize this target, necessary condition for cell printing by piezoelectric inkjet technology was investigated. Piezoelectric inkjet printer technology was utilized to eject particles and cells of several tens of μm scale, which is large compared to colloid in printer ink. Stability of the ejection and trajectory errors was evaluated, and necessary condition to prevent inkjet head clogging was also investigated using beads of 10 and 20 μm in diameter [15]. Afterwards, beads were replaced with cells whose mean diameter was 21 μm, and we verified that the experiment condition for beads also applied to the experiment for cells. Furthermore, one cell per one droplet printing system was realized by detecting cells position inside inkjet head [16]. Finally, automation of the one cell per one droplet ejection system was performed using the automatic cell detection system to automatically detect cells position inside inkjet head.

This method is very useful and promising not only for biofabrication, 3D tissue construction, cell printing, but also for a number of biomedical application, such as bioMEMS and lab on a chip research field.

II. Piezoelectric Inkjet Printer Head Structure and Piezoelectric Element Control Method

A. Inkjet Head Structure

Fig. 1 shows the piezoelectric inkjet head, which was used for ejecting droplets. The device was fabricated with the assistance of Microjet Corporation and consisted of a glass

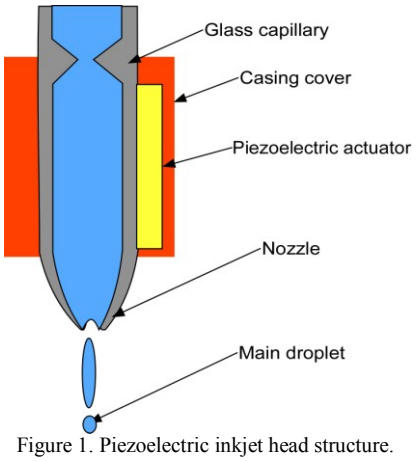

Figure 1. Piezoelectric inkjet head structure.

*Research supported by JSPS, MEXT KAKENHI Grant Number 20034017, 21676002, 21225007, 21111503, 23111705, and the Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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tube with a piezoelectric actuator attached to a fabricated flat surface. When voltage was applied to this piezoelectric actuator, the piezoelectric effect caused it to bent inward and generated a wave inside the pressure chamber that propagated to the inkjet head tip, causing a droplet to be ejected. Such droplets were initially cylindrical, but under the influence of surface tension and air resistance, they separated into a main droplet and several smaller satellite droplets. The main droplet usually remained the largest. Since it was also the fastest, it was used to identify droplet speed and the droplet flight angle as well as other droplet ejection characteristics. In this study, influence of the nozzle diameter was investigated using nozzle diameters of 40, 60, and 80 μm.

B. Piezoelectric Element Control Method

In this study, inwardly bent piezoelectric element refers to "push", while returning movement of piezoelectric element refers to "pull". According to the order of the "push" and "pull" movement, there are two methods to drive the piezoelectric element, the pull-push method and push-pull method.

Pull-push method is commonly used in commercial piezoelectric inkjet printer. The pull movement of the piezoelectric element produces negative pressure inside the inkjet head chamber, which in turn pull the meniscus layer at the tip. Without the push movement, the meniscus layer would oscillate back and forth. However, the push movement at the correct timing creates positive pressure which caused resonance vibration of meniscus layer. Meniscus layer was pushed even further, thus ejecting the column of liquid as droplet. Because of the resonance vibration movement of the meniscus, pull - push method resulted in the formation of a thin, high velocity fluid column. Fig. 2 (a), (c) shows the droplet formation process for the pull - push method.

On the other hand, push - pull method drives the piezoelectric by a push movement followed by a pull movement. The push movement of the piezoelectric element produces positive pressure inside the inkjet head chamber, which in turn push the meniscus layer. The pull movement at the correct timing creates negative pressure which in turn pulls the meniscus layer, thus breaking the meniscus layer and ejecting liquid droplet through the nozzle. Compared to pull - push, the push - pull method produces bigger and slower droplet. Fig. 2 (b), (d) shows the droplet formation for the push - pull method. For commercial piezoelectric inkjet printer, smaller droplet is more advantageous as that means higher printing resolution. Therefore, for commercial piezoelectric inkjet printer, pull - push method is used instead of push - pull method.

III. PIEZOELECTRIC INKJET-BASED STABLE BEADS EJECTION

For large particles ejection, stable ejection could not be obtained by only voltage amplitude and pulse width adjustment. To evaluate stability of ejection, variation in the droplet flight angle was used. Droplet flight angle θ is defined as the angle of the main droplet path with vertical line. Unstable ejection is shown by bigger variation of the droplet flight angle.

A. Factors Effecting Stable Beads Ejection

Droplet was formed from the pressure generated by piezoelectric effect, which forced the droplet out against the surface tension. Stable droplet ejection could be achieved if meniscus layer break evenly during droplet formation. Large particles suspension cause unstable ejection by breaking the balance of this meniscus layer during the droplet formation process. Therefore, stable ejection is considered to be connected to the ratio of the ejection meniscus layer area to particles area. Fig. 3 shows the illustration of the particles area and ejection meniscus layer area. In this study, each factor causing a change in this ratio was investigated, and necessary experiment conditions to produce stable ejection of large particles were also investigated. Factors effecting this ratio are as follows: (1) Inkjet head diameter and particles diameter, (2)

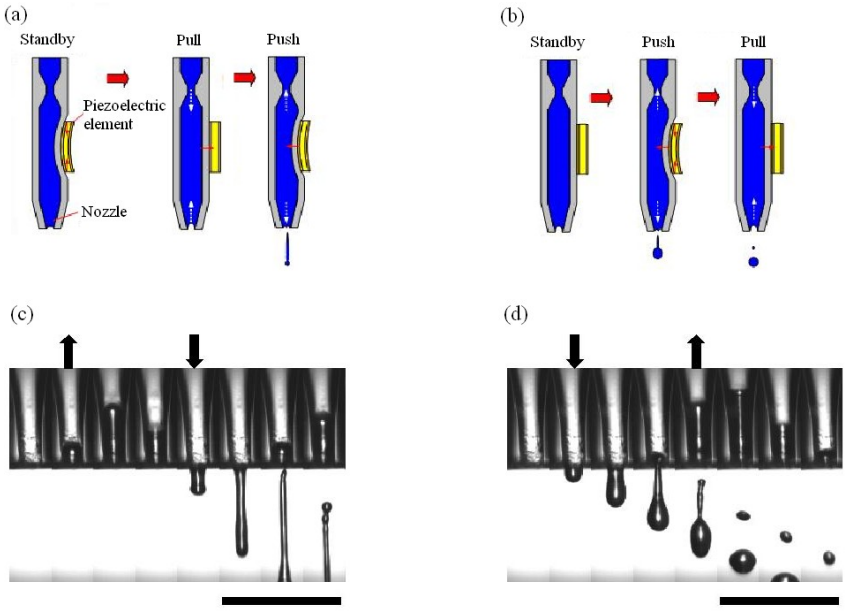

Figure 2. Piezoelectric element control method: (a) pull-push method and (b) push-pull method. (c) 50 μs/frame image of pull-push method ejection and (d) 50 μs/frame image of push-pull method ejection. Scale bar: 500μm.

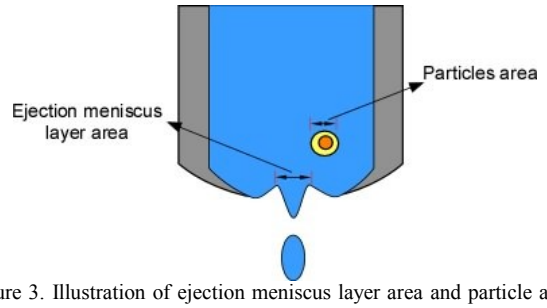

Figure 3. Illustration of ejection meniscus layer area and particle area. Bigger ratio results in more stable ejection.

piezoelectric element driving method, and (3) particles suspension concentration. Bigger inkjet head diameter and smaller particles diameter mean the ratio of ejection meniscus layer area to particles area is also bigger, which in turn means more stability for ejection of large particles. On the other hand, as can be observed from Fig. 2, pull - push method and push - pull method generates different size droplet column, which means the ejection meniscus layer area is also different. Furthermore, particles suspension concentration effects this ratio by aggregation of the particles near the tip, thus leads to more unstable ejection.

B. *Experimental Setup*

Investigation of stable ejection was conducted using polystyrene beads of diameters 10 μm and 20 μm. Beads particle precipitation speed in 1:1 mix of water and ethylene glycol was found to be 1.1 μm/s. The maximum permissible deviation of droplet placement in a printer is ±10 μm. For inkjet head-to-printing surface distance of 1.0 mm, the maximum permissible flight angle was calculated to be 0.57°. Based on this calculation, the maximum permissible flight angle in these experiments was set to 0.6°.

C. *Results and Discussion*

1) *Relation between inkjet head diameters and bead diameters*

For these experiments, three inkjet head of 40 μm, 60 μm, and 80 μm sizes were fabricated. With these heads, 20 μm polystyrene beads suspension of 0.1% concentration was loaded into each head, and the droplet flight angle was measured. Pull - push method was used as the driving force of the piezoelectric element. Fig. 4 shows the plotted graph for 40 μm and 60 μm inkjet head, where vertical axis represents the flight angle while the horizontal axis represents 200 droplets data values. The data from three experiments were plotted in each of the graphs.

These figure clearly showed that smaller inkjet head diameter resulted in greater fluctuation of the droplet flight angle θ. This fluctuation of droplet flight angle was evaluated by calculating the 3σ, where σ is the standard deviation of the droplet flight angle. Table 1 (a) shows the variation in droplet flight angle (3σ) for each of the three inkjet head and different concentration of beads suspension. Measurements were conducted for variation of the droplet flight angle between the value 0.3° and 1.0°. "NG" marked showed that the experiment could not be conducted because clogging occurred, thus preventing droplet ejection.

Next, to determine the influence of beads diameter changes, experiments were also conducted at the same

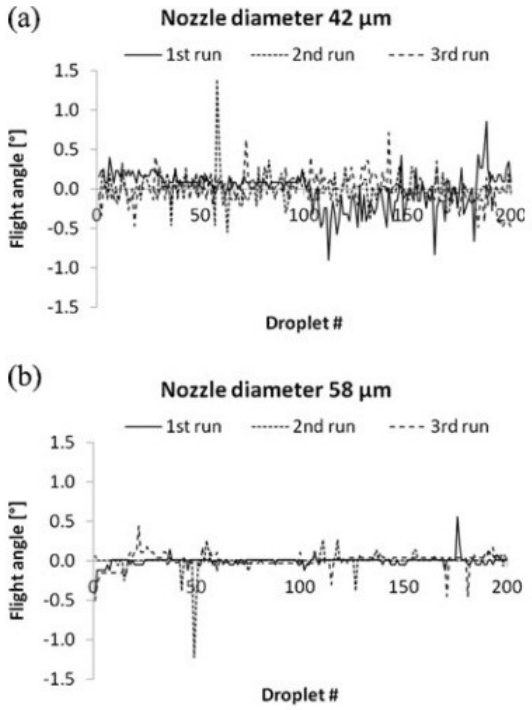

Figure 4. Results of droplet flight angle θ measurements.

condition for suspension of beads with diameter of 10 μm. Table 1 (b) shows the results for these experiments. There was no clogging for 10 μm beads, unlike the 20 μm beads. Additionally, compared to the same concentrations for 20 μm beads, variation of the droplet flight angle also decreased.

2) *Comparison of pull-push and push-pull method*

Same experiments were carried out using push-pull method as the driving pulse of the piezoelectric element. Fig. 5 shows the comparison of the plotted droplet flight angle for pull-push method and push-pull method. Table 2 shows the analysis results for the variation of the droplet flight angle for the push-pull method. The data clearly demonstrated that the push-pull method resulted in lower variation in droplet flight angle compared to the pull-push method. Push-pull method could even eject high concentration particles of up to ten times higher than the pull-push method. Therefore, the push-pull method was judged to be more suitable for stable droplet ejection with large particles suspension. Additionally, particles aggregation for the push-pull method was considerably fewer compared to the aggregation for the pull-push method.

IV. PIEZOELECTRIC INKJET-BASED CELL PRINTING

In previous chapter, optimal ejection condition for large particles was established for beads. Ejection stability only depends on the balance shape of the meniscus layer, thus stability only depends on the position and sizes of the particle, not on the shape and hardness. Therefore, ejecting condition established for beads in the previous chapter was considered applicable for cells ejection with piezoelectric inkjet head.

Experiments were carried out with cells suspension as the replacement of the beads suspension. Furthermore, the method to print one cell per one droplet was also clarified and one cell

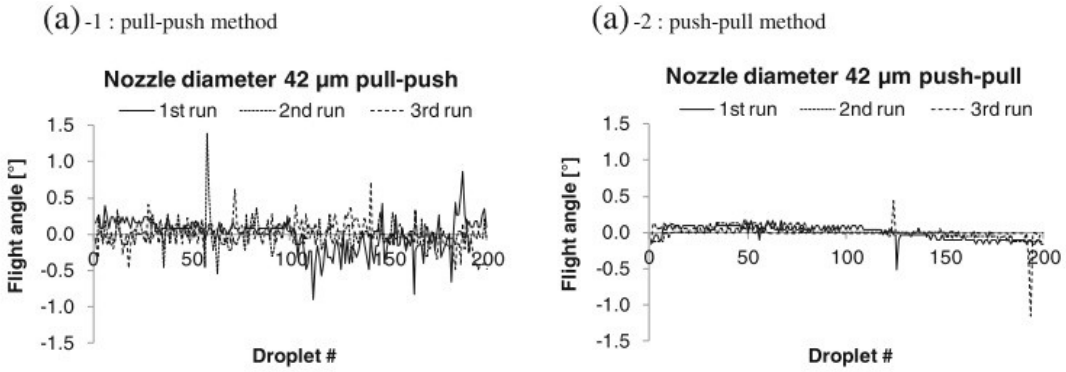

Figure 5. Comparison of results for droplet flight angle θ measurements between pull-push and push-pull method.

Table 1. Variation in droplet flight angle (3σ) measurement results for pull-push method.

(a) Beads diameter 20 μm

Inkjet head diameter	Beads concentration					
	no beads	0.1%	0.5%	1.0%	5.0%	10.0%
42 μm	0.14	0.59	NG	NG	NG	NG
58 μm	0.09	0.32	0.47	0.72	1.34	-
82 μm	0.10	-	0.15	0.31	0.47	1.14

(b) Beads diameter 10 μm

Inkjet head diameter	Beads concentration				
	0.1%	0.5%	1.0%	5.0%	10.0%
42 μm	0.52	1.00	-	-	-
58 μm	0.23	0.49	0.56	0.66	1.03
82 μm	-	-	0.29	0.54	0.78

Table 2. Variation in droplet flight angle (3σ) measurement results for push-pull method.

a) Beads diameter 20 μm

Inkjet head diameter	Beads concentration					
	no beads	0.1%	0.5%	1.0%	5.0%	10.0%
42 μm	0.16	0.27	NG	NG	NG	NG
58 μm	0.10	-	-	0.25	0.49	0.74
82 μm	0.10	-	-	-	0.29	0.33

b) Beads diameter 10 μm

Inkjet head diameter	Beads concentration				
	0.1%	0.5%	1.0%	5.0%	10.0%
42 μm	-	0.25	0.43	0.92	1.66
58 μm	-	-	-	0.30	0.53
82 μm	-	-	-	-	0.28

per one droplet printing was carried out while monitoring the image from the CCD camera.

A. Cell Ejection Stability Investigation

Instead of beads suspension, SF9 insect cells suspension was used in these experiments. SF9 insect cells suspended in the physiological saline at the 1 x 10^5 cells/mL concentration were used. This cell concentration correlates to around 0.05 cells per droplet based on volume calculation. Relatively low cells concentration was used in order to prevent cells aggregation, which can cause clogging at the tip of the inkjet head. Mean size of the SF9 cells was 21 μm, with size range from 12 μm to 35 μm.

B. One Cell per One Droplet Printing

1) One cell per one droplet ejection principle

When cells suspension was loaded onto the piezoelectric inkjet head, the formed droplet may contain no cell, one cell, two cells, and so on. In order to successfully print one cell per one droplet, the number of cells that a droplet contains must be detected beforehand. There is a certain area at the inkjet head tip where cells were ejected. This area will be called "ejection area" here on in this study. One cell per one droplet printing could be achieved by observing cells position inside the inkjet head. Number of cells in this area is the number of cells contained in the droplet. When only one cell was in this ejection area, which means the droplet will contain one cell, the inkjet head is positioned to the target printing location and the signal was sent to the piezoelectric element to eject the droplet. When more than one cell or no cell existed in ejection area, the inkjet head is positioned to the discard location and the droplet is discarded.

2) Inkjet head mapping method and results

Fig. 6 illustrates the method used for the ejection area mapping. First, each cells position near the tip of the inkjet head was recorded. Next, ejection signal was sent to the piezoelectric element to eject one droplet. After the ejection, cells, whose position was recorded before the ejection, was checked whether it had been ejected along with the droplet. In case it was ejected, a plus sign (+) was put onto the recorded position. In case it was not ejected, a cross sign (×) was put onto the recorded position. After 100 particles ejection, the area with only the plus sign (+) was marked as the Region A, the ejection area. While area with both the cross sign (×) and the plus sign (+) in it was marked as the Region B. In other words, Region A is the area where all of the particles will be ejected, while Region B is the area where some particles will be ejected and some particles will not be ejected. Because Region B is the area where cells ejection could not be determined, when one or more cells existed in the Region B, the number of cells was uncertain. Therefore, to successfully detect one cell per one droplet ejection, cells must not exist in the Region B and only one cell exists in ejection area, Region A. Thus, smaller Region B and bigger Region A condition is favorable for the one cell per one droplet printing experiment.

Inkjet head mapping was conducted with both methods of piezoelectric driving pulse, pull-push and push-pull method. Fig. 7 shows the mapping results for the (a) pull-push method and for the (b) push-pull method. The figure shows that for the conventional pull-push method, Region B exists within Region A, while for push-pull method, Region B does not exist or is negligible. From these experiments results, it can be concluded that push-pull method is more appropriate for one cell per one droplet printing.

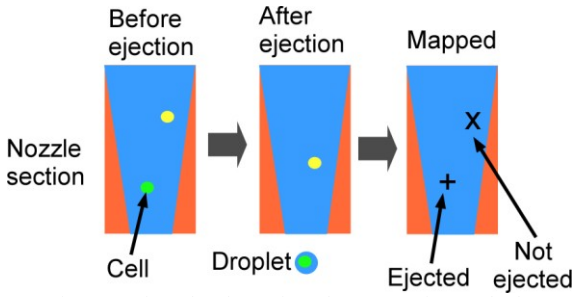

Figure 6. Ejected and not-ejected area mapping method.

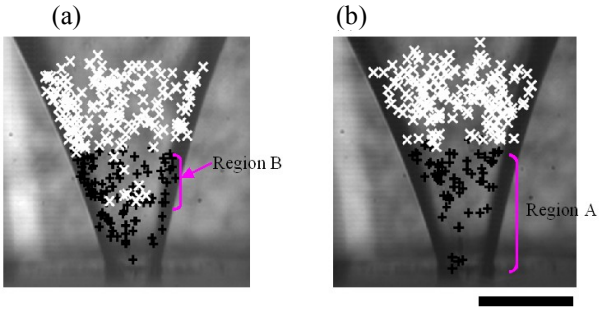

Figure 7. Mapping results of cell ejection with (a) pull-push method and (e) push-pull method. Scale bar: 50μm.

3) One cell per one droplet printing results and discussion

Fig. 8 shows the results of the one cell per one droplet printing, positioned on a 5 x 5 grid pattern with a spacing of 500 μm between each point. Experiments were conducted with the push-pull method, and a human operator constantly monitoring the image from camera to judge whether the one cell per one droplet ejection condition is fulfilled or not. When the one cell per one droplet condition was fulfilled, the human operator gives the signal to the computer to position the inkjet head on the target printing location and eject the droplet which contains one cell, thus successfully carried out one cell per one droplet printing.

Position error of the printed cells was found to be 75 μm. This resulted from the following 3 factors: droplet flight instability, mechanical device, and cell movement inside the liquid droplet. From previous section, droplet flight instability was measured to be around 5 μm, and error from the mechanical device was around10 μm. Therefore, error from the cell movement across the liquid droplet accounts to around 60 μm of positioning error.

V. Automation of One Cell per One Droplet Printing

Up to this point, one cell per one droplet printing was successfully done. However, the printing was carried out while a human operator constantly monitoring the condition inside the tip of inkjet head. The manually operated device has little value in productivity and efficiency. Therefore, the necessary equipment and knowledge for the automation of the one cell per one droplet printing was investigated and automatic cell detection system was constructed using open source image processing library, OpenCV [17].

A. Cell Detection by Image Processing

To detect cells position inside inkjet head, cells pixel must be extracted from the image sent from CCD camera.

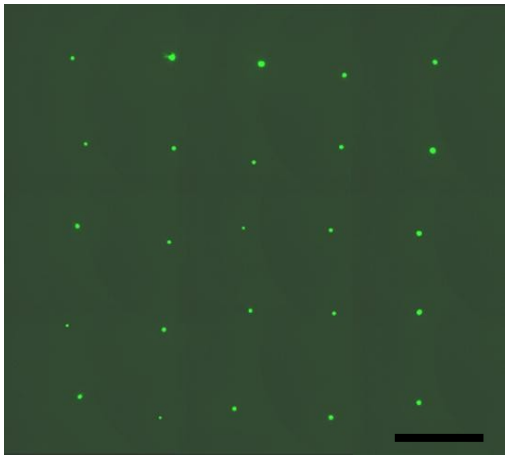
Figure 8. SYTO Green stained cells placed on a 5 × 5 grid. Scale bar: 500 μm.

Background subtraction method can be used for extraction of foreground objects pixel [18]. The background subtraction method extracts the pixel by beforehand determining a background model which contains no foreground object (only the medium without cells). After that, foreground objects pixel can be determined by comparison of background model and image from the CCD camera.

Foreground objects means suspended particle in the medium, which consist of single cell, cells, and debris. In order to successfully print one cell per one droplet, automatic cell detection system must successfully separate single cell from cells group and debris. In order to separate these particles, cells main visual characteristics, its size and shape was evaluated. The following conventional formula (1) was used to evaluate cells shape by its roundness.

$$R = 4\pi A/p^2 \times 100 \quad (1)$$

R is the roundness value of the object in [%], A is the area of the object in [m^2], and p is the perimeter of the object in [m].

Cells were observed under microscope and the image was sent to the computer for size and roundness evaluation. The following graph in Fig. 9 shows SF9 cell size and roundness distribution. Based on this graph, cells size and roundness threshold was decided. Relatively very high value of roundness threshold value was taken in order to achieve higher ratio of one cell per one droplet detection. SF9 size threshold was set to between 15 and 30 μm, and its roundness threshold was set to more than 80%. For SF9 cells whose roundness value is high, separating single cell from cells group and debris using this technique proved to be very effective.

B. Automatic One Cell per One Droplet Printing System

The inkjet head is first positioned on a "discard area". Discard area is an area where droplets are discarded because the one cell per one droplet printing condition doesn't match. When one cell per one droplet condition matched, inkjet head was positioned on the printing target position. With this flow of operation, automatic one cell per one droplet printing system was developed. Fig. 10 shows the automatic one cell per one droplet printing system.

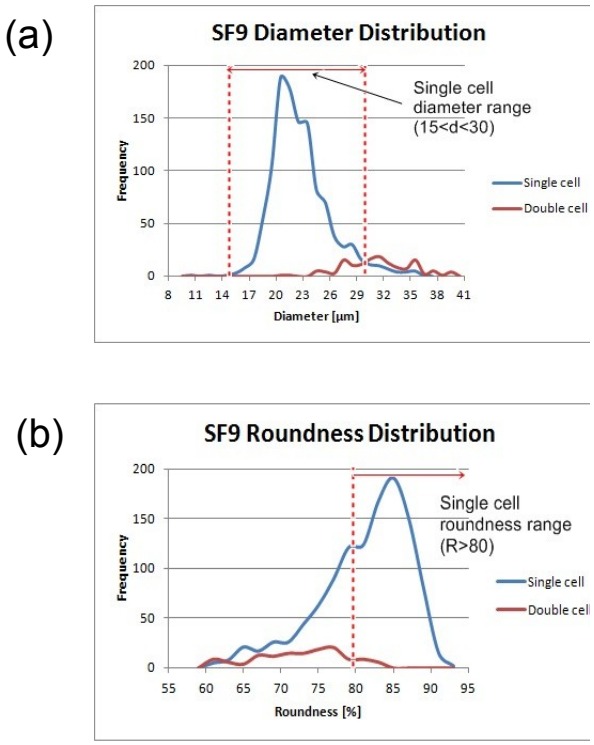

Figure 9. SF9 cell size and roundness distribution.

C. *Printing Results and Discussion*

Using the automatic one cell per one droplet printing system, each single cell was positioned on 5 x 5 grid pattern. By evaluating the correct number of single cell of each dot, 98% of successful printing rate was obtained. Significantly high one cell per one droplets ratio was obtained.

VI. Conclusion

In this study, piezoelectric inkjet printer was used to print large particles, beads and cells, and the condition for stable ejection was verified. Additionally, single cell printing method using this inkjet printer was investigated, and by combining this method and automatic cell recognition system, automatic single cell printing was achieved with 98% ratio of successful printing. Considering the potential of this technology regarding printing speed, this technology may play an important role for single cells study and tissue engineering in the future.

Acknowledgment

We are grateful to Lilian Kaede Komaba for her technical assistance in cell culturing and Funakoshi Kei for his advice.

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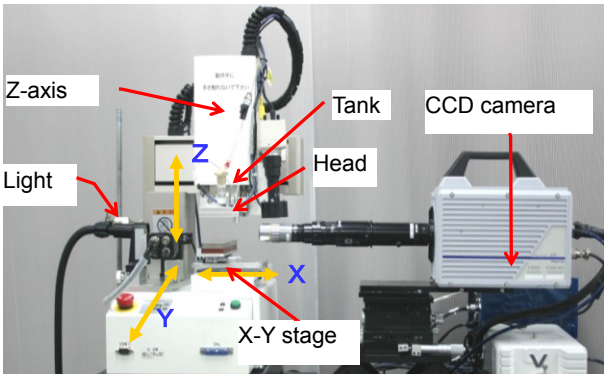

Figure 10. Automatic One Cell per One Droplet Patterning System. a) Discard area. b) Target area. c) Inkjet head. d) Lens for high speed camera.

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