Application of Lateral Oscillating Piezo-driven Micropipette in Embryo Biopsy for Pre-implantation Genetic Diagnosis

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Abstract—Biopsy of zona pellucida is a necessary step prior to pre-implantation genetic diagnosis (PGD). Besides traversing zona pellucida by applying laser or acidified medium (e.g. Tyrode’s solution), mechanical means is another safe approach. Traditional mechanical zona cutting requires highly skilled embryologist. It is very difficult to make the process automatic due to its complexity. The process can be enhanced by introducing piezo-driven cutter, which makes the cutting process more precise and introduces less cell deformation. In this paper, the application of lateral oscillation of the piezo-driven microneedle is introduced. We believe that further understanding and implementation of the lateral vibrational piezo-driven microucuter can be beneficial for more accurate and controllable mechanical cell biopsy.

Keywords—preimplantation genetic diagnosis, lateral piezo vibration, embryo biopsy, zona cutting, embryo biopsy, piezo-assisted biopsy

I. INTRODUCTION

Pre-implantation genetic diagnosis (PGD) has been introduced for more than twenty years with its first publication in 1991 [1]. PGD is a prenatal diagnosis process that analyses embryos grown in vitro for well-defined genetic defects. After PGD process, only those embryos without any defect are transferred back into the womb [2]. Embryo biopsy is a process to remove part of materials from a cell for diagnosis. There are three kinds of biopsies can be used for PGD based on the embryo development stage. The polar-body biopsy is [3] [4] applied in early development stage. The blastocyst biopsy[5] is applied in the latest development stage, which leaves little time for diagnosis. The most widely used biopsy for PGD is cleavage-stage biopsy. Cleavage-stage biopsy is performed on a third-division (D3) embryo obtained 3 days after the insemination [6]. A D3 embryo has more than six blastomeres, out of which one or two blastomeres are aspirated from the embryo. The cleavage stage biopsy is a two-step procedure, including zona pellucida breaching and removal of one or two blastomeres from a Day-3 embryo. Zona pellucida traversing process is most commonly implemented by utilizing acidified medium (e.g. Tyrode’s solution) or laser technology in many centers [7]. In some institutes, it is done by mechanical means [8].

Active zona cutting, using piezo-driven microneedle or micropipette, is an experimental proven[9] mechanical approach to complete embryo biopsy, as well as partial zona dissection for intracytoplasmic sperm injection. Kelley’s group[10] used a piezo-activate pipette to perform zona drilling on mouse embryos. A hole or split was introduced to the zona pellucida after performing three tangential passes with a piezo-active pipette on the zona pellucida. Because of the high elasticity property of the glass micropipette, large-amplitude lateral oscillations always come with the axial oscillation, which is in the same direction of the piezo-driving force. The lateral oscillation was once considered as harmful oscillation and cause of embryo lyses. Mercury can be filled into the micropipette to mitigate excessive vibrations, which has been proven by experiments [11]. However, the toxicity of the mercury is of big concern during the operation.

Two kinds of vibrations are introduced when the micropipette is driven by a piezo-driver: the axial and the lateral vibration. Till now, there is no unanimous conclusion on which vibration performs the cutting and penetration of the cell membrane. Ediz’s group [12] investigated the microdynamics of the piezo-driven pipette, they believed that the increasing mercury mass loading reduced the natural frequency of the micropipette. They claimed that lateral oscillation had much larger amplitude than the axial oscillation did in the experiment; implying lateral oscillation contributes more to the membrane piercing. The amplitude of axial displacement is merely ±80nm, which is considered not enough to pierce the 8 μm-thick [13] zona pellucida. Gan’s group[14] also holds the same opinion as Ediz’s after conducting the simulation on mouse zona piecing process, which shows that the lateral oscillation contributes to the destruction of the membrane more than axial vibration does. Although, ultrasonic piezo-driven microinjection proposed that the piecing of a cell membrane is a result of ultrasonic cutting force [15], and the lateral oscillation is treated as harmful factor and needed to be minimized [16], our investigation in the effects of lateral vibration [17] concluded that the cutting force of lateral vibration played a constructive role on the cell membrane piercing. It was the amplitude of the vibration contributed to the membrane destruction rather than the frequency. In addition, the heat generated by axial and lateral vibration for membrane cutting can also be qualitatively evaluated by using Voigt model [18], which indicates that the heat generated by axial vibration is three times more than that generated by lateral vibration. That adds points to our
assumption that the lateral oscillation is beneficial oscillation in the process of membrane perforation.

Till now, all piezo-driven micropipettes used for zona piercing [19], zona drilling [20], partial zona dissection [11] and zona biopsy[9] are driven by piezo actuator excited in axial direction. As the micropipette has an extremely thin and long end section, we believe that the successes of piezo-assisted injection are because of the lateral oscillations, which is excited and transferred to the tip of the micropipette when the micropipette is excited in axial direction.

This paper devotes to investigation in the application of lateral oscillation in cell membrane dissection. The possibility of using only lateral oscillation for cell membrane dissection has been demonstrated through experiments. Zebrafish embryos membrane cutting tests with different frequencies and amplitudes have been performed with the proposed piezo-driven micro-cutter.

II. METHOD

A. Experiment Setups

![Experiment Setups](image)

The set-up of this experiment is shown in Figure 1. On the top of the upright microscope (Nikon, Eclipse FN1, Japan), a 659x490-pixel CCD camera (Basler,scA640-74gc, Germany) is mounted for image capturing. Both holding pipette and the micro cutting needle are fixed on a pair of 3-DOF micromanipulators (National Aperture Inc., MM-4M-F, USA). Microsyringe pump (Eppendorf, Celltrum Vario 5176, Germany) is connected to the holding pipette to provide suction force for embryo immobilization. For lateral vibration driving, a piezoelectric shear actuator with free stroke of 10 μm in two axes is used (Physik Instumente, CA-Shear P152.10, Germany) to provide wide range of frequencies with considerable high amplitudes. For axial vibration, a piezo actuator (Physik Instumente, P885.51, Germany) with 15μm free stroke and 70 kHz resonant frequency is installed. A PDX voltage amplifier (Newcastle innovation Ltd, PDX150, Australia) is used to provide -30V to 150V voltage for the piezo actuators.

For the lateral vibration investigation, a 25.4 mm tungsten microneedle (TED Pella, 13570-10, USA) was fixed on the in-house-made holder which is directly glued to the shear actuator, as shown in Figure 2. A portion of 3 mm of the microneedle is inserted into the holder fasten by two set screws and reinforced by super glue. The driving direction of the actuator was perpendicular to the axis of the microneedle to excited lateral vibration modes on the microneedle.

![Lateral oscillating micro-cutter](image)

Figure 2. Lateral oscillating micro-cutter

The tip displacement was estimated by recognizing the width of the envelope in moving tip’s microscopy image. The average intensity of ten images was used for the measurement in case of missing capture of a particular movement. The number of pixels between the lowermost and uppermost pixel provides the displacement of the tip, given each pixel is equivalent to 0.7035 μm in real world dimension. The measurement of few typical frequencies with different amplitudes are illustrated Figure 3. As the tip of the microneedle is 0.6 μm thick, the amplitude of displacement

![Vibration of a lateral vibrating microneedle immersed in water](image)

Figure 3. Vibration of a lateral vibrating microneedle immersed in water
should be overall displacement value minus the thickness of the tip.

Five frequencies, 5.3 kHz, 13.6 kHz, 22.6 kHz 25.4 kHz and 33.5 kHz, with various tip amplitudes have been tested in lateral-vibration-driven membrane dissection. These frequencies are chosen because of their capabilities of making the microneedle achieve considerably high tip displacement. Three cells are tested for each frequency and amplitude combination.

For the axial vibration experiment, the tungsten needle is inserted into a modified gauge 21 syringe filled with superglue to mitigate the lateral vibration. The syringe is then fixed into the flexure fasten by set screws and superglue, as shown in Figure 4.

![Piezo Stack, Screw, Microneedle, Flexure](image)

Figure 4. Axial oscillating micro-cutter

The tip displacement in axial was not measured due to technical difficulty, but the lateral tip displacement was calculated by using the same aforementioned method. As the effect of axial oscillation was proven to be insignificant, thus only few frequencies with comparably larger lateral amplitudes have been tested to compare the dissection effect with the lateral-driven ones.

B. Experiment Procedure

The procedure of the experiment is described as follows:
1. Align the microneedle with the vertical tangent point of cell membrane,
2. The Piezo-driven microneedle is pushed against the cell membrane at a speed of 6 μm/s till it penetrates the membrane. This is used to test the penetration effect of piezo-driven microneedle before penetration.
3. Move the microneedle or micropipette upward or downward at a speed of 6 μm/s to test the cutting effect after penetration.

III. RESULT AND DISCUSSION

Four batches of fertilized zebrafish embryos, more than 260 embryos in total, were used for the entire experiment. Only those providing clear penetration images and staying in focus throughout the membrane dissection process were considered as valid samples. The sample size for lateral-vibrational cutting presented in this paper is 192 embryos, with three samples performed for each unique combination of waveform, frequency and amplitude. Combinations of frequency and amplitude were chosen randomly for each batch. To make sure the property of the embryo membrane did not change significantly, cell membrane penetration with non-vibrated microneedle were performed frequently, and all experiments were carried out within eight hours from the embryo harvest.

The ratio of embryo deformation right before penetration and ratio of split expansion to microneedle displacement were used to quantify the effect of the piezo-cutter, and they are defined as follows:

\[
a = \frac{\Delta L}{L} \times 100\% \tag{1}
\]

where \(\Delta L\) is depth of the concave right before penetration in axial direction and \(L\) is the original diameter of the cell, as illustrated in Figure 5.

![Deformation ratio a](image)

Figure 5. Deformation ratio a

\[
c = \frac{\Delta s}{D} \times 100\% \tag{2}
\]

where \(\Delta s(= s_2 - s_1)\) is the change of width of the split after lateral-directional movement of the microneedle and \(D\) is the displacement that the piezo-driven microneedle moved in lateral direction, as illustrated in Figure 6.

![Split expansion to tip displacement ratio c](image)

Figure 6. Split expansion to tip displacement ratio c

A. The direct cutting effect of the piezo-driven microneedle

In [17], we suspected that it was the shear cutting force that helped piezo-driven micropipette penetrate the cell membrane. In the experiment, to further investigate the effect of the cutting force, a microneedle with tip of 0.6 μm thick was used as a cutter. As the thickness of the tip is much thinner comparing to the cell diameter, we believe it should have more obvious cutting outcomes. The effect of cutting or gashing motion in lateral direction was investigated by exciting the microneedle with frequency up to 33.5 kHz, and amplitude up to 40 μm. Both sinusoidal and square waveforms were tested in this experiment. Most of deformation ratios \(a\) obtained from various combinations of frequency and amplitude fell in the
range of 0.43% to 0.48%. Since the deformation on the cell membrane was too small under 200 times magnification, given our best to count the number of pixels covered by the concave in axial direction, only 7 pixels to 10 pixels could be measured. We rounded up the number to avoid ambiguity. Since one pixel is merely 0.7035 μm in the real world dimension, the error of deformation ratio obtained can be considered as less than ± 0.05%. In comparison, the average deformation ratio σ of 12 non-vibrational injections was 3.8%, which is about 9 times bigger than the deformation ratio obtained from lateral vibration-assisted cutting. This result double confirms our finding that lateral oscillation plays an important role in cell penetration.

Though the square waveform should generate twice the energy that sinusoidal waveform can provide, the deformation ratio did not show any significant difference. We believe that it is because the deformation is already too small and the membrane of the fish embryo is too thin for us to identify the difference.

The cutting effect of the square-waveform driven axial-vibrational microcutter was also investigated. Since we had concluded that axial vibration did not play the critical role in the membrane penetration, thus the effect of solely axial-vibrational cutting was not tested; whereas the effect of combination of the lateral and axial vibration was tested (the lateral oscillation always comes with axial oscillation due to the eccentricity of the needle). Since the harmonic frequencies had change when driving the microcutter in axial direction, it was very hard to obtain the same lateral amplitude with same frequency as driven in lateral direction. Only in few frequency bands, we could get amplitudes we expected to achieve. No

![Figure 7](image)

Figure 7. (a) Injection with axial square-waveform excitation. (b) Injection with lateral sinusoidal-waveform excitation. (c) Injection with lateral square-waveform excitation with a frequency of 5.3 kHz and a lateral-tip amplitude of 10 μm. (d) Injection without vibration

significant differences between the effect of axial and lateral driven cutting was found in selected frequency bands from 5.3 kHz to 22.6 kHz, which could induce lateral amplitude that were large enough. Figure 7 (a),(b) and (c) illustrate the

![Figure 8](image)

Figure 8. Split expansion to tip displacement ratio c

B. The splitting effect of the piezo-driven microneedle

In the biopsy process of PGD, an opening up to 40 μm long and 2 μm wide is introduced to the zona pellucida[21]. Though a 40 μm opening can be achieved precisely by using the aforementioned lateral cutting method, it is believed that with the same frequency, lower amplitude causes less heat energy, thus minimizes the risk of causing embryo defects and
lysis. There are two methods of achieving a 40 μm opening using the low amplitude vibration (less than 40 μm). One method is multiple attempts of the aforementioned cell perforation. However, the small opening is very difficult to be identified once the micro injector is pulled out from the embryo. In addition, the cell perforation is best performed in the center of the zona convex to prevent injector from slipping. Therefore this method is difficult to achieve precise cutting. The other method is to cut the zona open by dragging the injector up or down after insertion into the cell, thus it requires that the vibrational microneedle must be able to split up the zona pellucida once it penetrates the zona.

In our experiments, we have quantified the splitting capability of the vibrational microneedle using a split-expansion-to-tip-displacement ratio $c$. The ratio $c$ is a value between 0 and 1, as shown in (2), where value 1 indicates that the microneedle is able to split every part of the membrane along its moving path; in contrast, value 0 means the microneedle has no splitting effect at all. Since the lateral vibration is believed to be the beneficial vibration for cell membrane penetration, our experiments mainly focused on the splitting effect of lateral vibration. Figure 8 illustrates key variables ($s_1$, $s_2$, $D$) of ratio $c$ in some typical situations. Square waveform was used to drive the microneedle. All values of ratio $c$ obtained from 96 embryo samples were very similar and there was no significant expansion of the opening during splitting process, which means that all values of ratio $c$ are close to zero. Therefore, we can conclude the lateral vibration does not split the cell membrane along its movement once it has penetrated the cell.

Several experiments with axial square-wave driven microneedle were also conducted to test the splitting effect. No significant difference in effectiveness was observed. It may be due to the small displacement amplitude in axial direction that may not cause enough destructive effect to the structure of the cell membrane. However, big amplitude in axial direction is not recommended as we found that when driven in axial direction, the whirling effect was much more phenomenal than driven in lateral direction with a same frequency. For example, as shown in Figure 9, when dip the microneedle, driven by 33 kHz and 15 μm excitation in axial direction, into the medium, the cell which was not attached to the holding pipette was experiencing suction from the tip of the microneedle. Eventually the cell was penetrated by the cutter as it moved towards the needle at a high speed. The destructive effect was also observed once the needle broke into the embryo. The yolk of the embryo was blended completely.

The whirling effect is also clearly illustrated in Figure 10. The micro-needle was driven in axial direction with 30 kHz frequency but with much smaller amplitude. When the piezo-driven micro-needle was turned on, ink flowed towards the tip of the micro-needle and then dispersed along the axis of the needle. The ink can be perceived as the internal fluid of an embryo. At a high frequency, if the amplitude is big enough, the internal fluid will be drained off very quickly.

![Figure 9. Suction and destructive effect](image)

![Figure 10. investigation of whirling effect using ink flow.](image)

I. Conclusion

In this paper, the shear cutting force of lateral oscillating piezo-driven micropipette is investigated. Based on our experimental results, we believe that lateral oscillation plays the most important role in cell membrane dissection. Although the lateral oscillation can be generated by exciting the piezo
actuator in axial direction, the axial oscillation can cause harmful whirling effect to the cell internal fluid leading cell lysis during the dissection process. In addition, the direction of lateral oscillation generated by axial excitement cannot be controlled nor the amplitude. In contrast, driving the cutter in lateral direction can achieve precise and desiring shape of the membrane opening as shown in Figure 11. The control of membrane dissection on Zebrafish embryo was challenging as the membrane is too thin, which make the pedal curl up upon the completion of membrane dissection. Piezo-assisted cutting makes mechanical cell membrane dissection a simple task to complete, which makes automatic cell membrane dissection possible. However, the lateral oscillating piezo-driven cutting requires more clinical data and further tests on zona pellucida of mouse embryo before claiming to be suitable for pre-implantation genetic diagnosis application.

Figure 11. (a) and (b) Single direction cutting. (c) T shape cutting. (d) Cross shape cutting.

REFERENCES


