Effect of Pulsatile Electric Field on Cultured Muscle Cells in Vitro

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ABSTRACT

An effect of an electric field on proliferation and on differentiation of cultured muscle cells has been studied *in vitro*. C2C12 (the mouse myoblast cell line originated with the cross-striated muscle of C3H mouse) was exposed to electric stimuli. In the first experiment, the adhered cells were exposed to the electric field between two electrodes made of platinum wire of 0.2 mm diameter dipped in the medium at 37 degrees Celsius for 72 hours. The electric pulses at a period of one second with a pulse width of 0.1 second were generated with a function generator. Variation was made on the pulse amplitude < 12 V. The number of adhered cells was counted after exposure to electric stimulation. In the second experiment, the cells were cultivated for 96 hours without electric stimulation in an incubator, after electric stimulation of 0.1 V for 72 hours. After incubation, the movement of myotubes was observed with electric stimulation at a period of one second with a pulse width of one millisecond of 30 V. The experimental results show that cells adhere and proliferate under electric pulses lower than 8 V, and that differentiation accelerates with the electric pulses of 0.1 V.

Keywords: Biomedical Engineering, Muscle Cells, Cell Culture, Electric Field, Differentiation and Proliferation

1. INTRODUCTION

The muscle tissue is exposed to electric pulses in the biological body. The movement is also controlled with the electric pulses. The biological systems have ability to optimize themselves to their environment. The optimum electric stimulation has a potential to control growth of the muscle tissue, which might contribute to regenerative medicine. In the present study, the effect of electric pulses on differentiation of muscle cells has been studied *in vitro*.

Behavior of biological cells depends on various environmental factors, such as electric [1-5], magnetic [6] and mechanical [7-9] fields. Electric pulses are generated with the ion movement through the membrane *in vivo*. The muscle tissue is controlled with electric stimulation under the nerve system *in vivo*. The electric stimulation has also been applied to a body in some medical treatments for rehabilitation.

Cell culture technique has been progressed and myoblasts have been clinically applied to ischaemic cardiomyopathy in the field of regenerative medicine. Acceleration technique for proliferation of cells has been studied to make the muscle tissue *in vivo* and *in vitro* [4]. Control methodology for proliferation and differentiation of cells would be applied to regenerative-tissue technology.

In the present study, the effect of a pulsatile electric field on proliferation and on differentiation of cultured muscle cells has been studied *in vitro*.

2. METHODS

Electric Field
The cells were cultured in a culture plate, which has six wells of a flat bottom without coating of collagen. The internal diameter of each well is 35 mm (Fig. 1a).

The electrodes are made of platinum wire of 0.2 mm diameter, and attached on a plate of polypropylene. When the plate covers the wells, a couple of electrodes in the rim position of each well generate an electric field in the medium (Fig. 1b). The wire has a square form in order to avoid concentration of the electric current, which might induce electrolysis. The cover plate has six square holes, through which the medium is observed during electric stimulation (Fi. 1a).

Electric pulses of a period of one second with a pulse width of 0.1 second were generated with a function generator (DF1906, NF Co. Ltd., Yokohama, Japan) (Fig. 2). Variation was made on the amplitude of the pulse between 0.1 V and 12 V.

Cell Culture
C2C12 (the mouse myoblast cell line originated with the cross-striated muscle of C3H mouse) was used in the experiments. The muscle cells were suspended in Dulbecco's Modified Eagle's Medium (D-MEM). The fetal bovine serum (FBS) was added to the medium with the volume rate in 10 percent of FBS and 90 percent of D-MEM. The suspension of four milliliter including eight hundred thousand of cells was poured into each well.

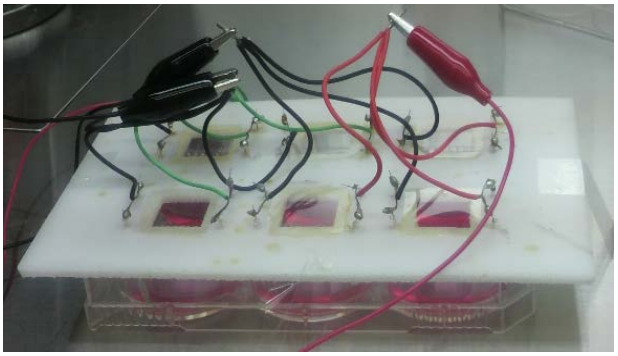

Fig. 1a: Six pairs of electrodes and wells for cell culture.

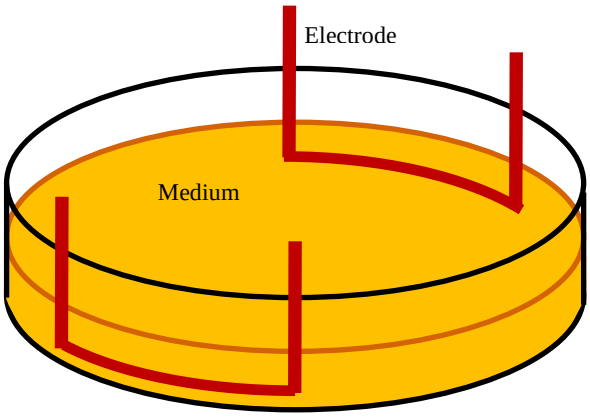

Fig. 1b: A couple of electrodes in the well.

To keep the temperature of 37 degrees Celsius and to keep the carbon dioxide content of five percent, an incubator was used for cultivation of cells. In the electric stimulation test, the electrodes were introduced into the incubator, and electric signals were introduced from the function generator outside.

Tolerance Test to Electric Pulses

After the cells were cultured in the incubator for 24 hours, electric pulses with the constant amplitude were applied continuously in the incubator for 72 hours (Fig. 2a). Variation was made in the amplitude of the electric pulse: 2, 4, 6, 7, 8, 9, 10 and 12 volts. In the control test, the cells were cultured without electric stimulation.

After electric stimulation, the medium was discarded and the number of cells adhered on the bottom of the dish was counted with the trypsin technique. The adhered cells were exfoliated with the medium including trypsin, and the suspension was introduced into a cytometry micro chamber to be counted under a microscope.

Myotubes after Electric Stimulation

After the cells were cultured in the incubator for 24 hours, the electric pulses with the constant amplitude of 0.1 V were applied to the cells in the incubator for 72 hours. In the control test, the cells were cultured without electric stimulation. After the continuous electric stimulation, the medium was changed to that with horse serum (HS) for differentiation (Fig. 2b). The volume ratio of HS in the medium is seven percent. The cells were cultured for successive 96 hours without electric

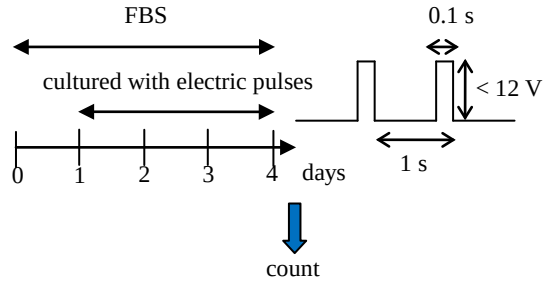

Fig. 2a: Protocol of tolerance test to electric pulses.

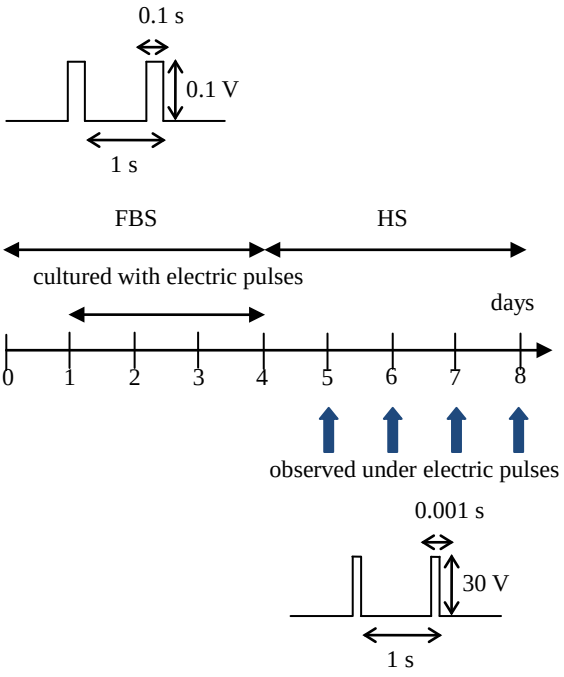

Fig. 2b: Protocol of the experiment of myotubes after electric stimulation.

stimulation, which means cells were cultured for eight days in total.

The movement of myotubes with the response to electric stimulation was observed under an inverted phase-contrast microscope once a day to examine their functional differentiation. The period, the width, and the amplitude of the electric pulse were one second, one millisecond, and 30 V, respectively. The electric stimuli were applied only for a short term for observation. The cells were not stimulated with the electric pulses in the rest of the cultivation term. Fig. 2b shows the protocol of the experiment.

3. RESULTS

Fig. 3 exemplifies cells in the control test without electric stimulation. The results show that cells proliferate to a confluent condition in four days.

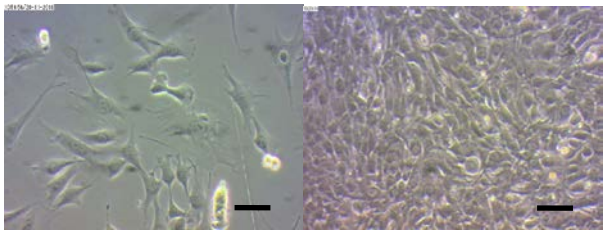

Fig. 3: Cells cultured without electric pulses. The bar indicates 0.1 mm. Left, 24 hours; Right, 96 hours.

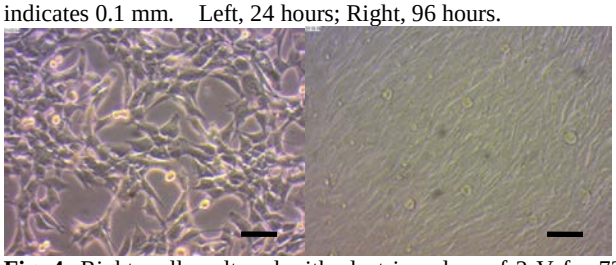

Fig. 4: Right, cells cultured with electric pulses of 2 V for 72 hours. Left, before electric stimulation. Bar indicates 0.1 mm.

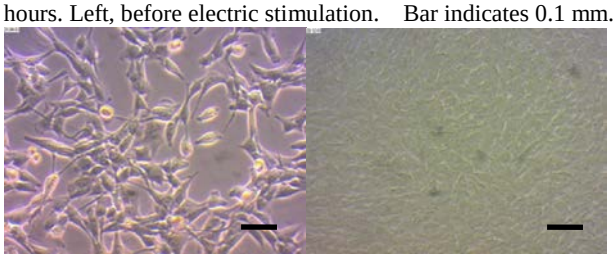

Fig. 5: Right, cells cultured with electric pulses of 4 V for 72 hours. Left, before electric stimulation. Bar indicates 0.1 mm.

Fig. 6: Right, cells cultured with electric pulses of 6 V for 72 hours. Left, before electric stimulation. Bar indicates 0.1 mm.

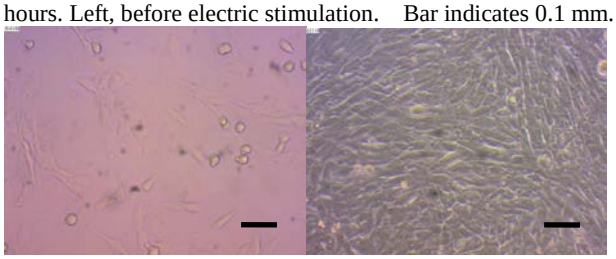

Fig. 7: Right, cells cultured with electric pulses of 8 V for 72 hours. Left, before electric stimulation. Bar indicates 0.1 mm.

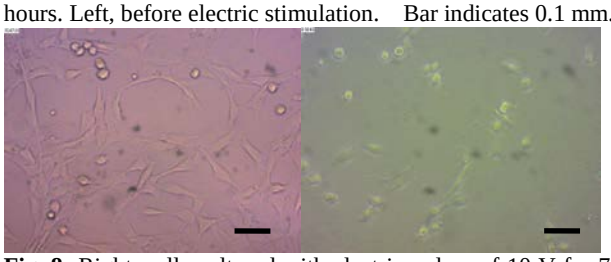

Fig. 8: Right, cells cultured with electric pulses of 10 V for 72 hours. Left, before electric stimulation. Bar indicates 0.1 mm.

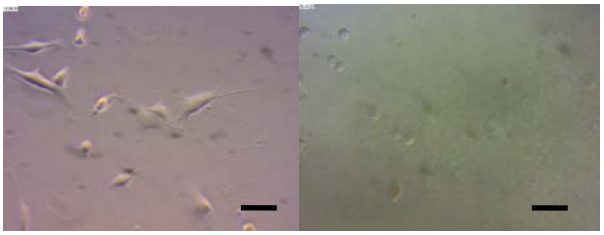

Fig. 9: Right, cells cultured with electric pulses of 12 V for 72 hours. Left, before electric stimulation. Bar indicates 0.1 mm.

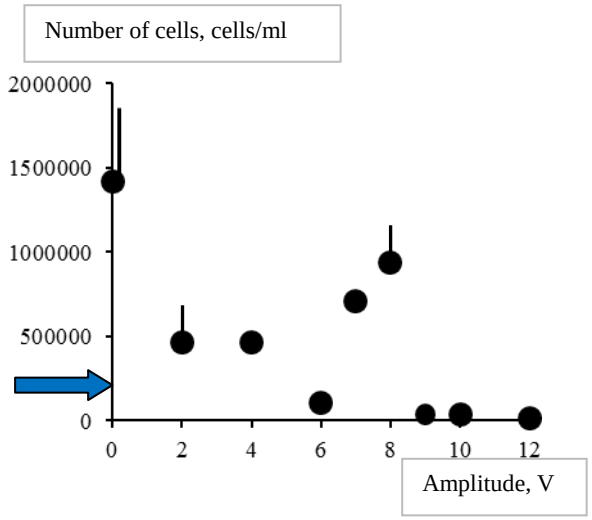

Fig. 10: Number of C2C12 after cultivation for 72 hours with electric stimulation. Arrow shows initial number.

Figs. 4-9 exemplify cells in the electric stimulation tests with the variation of the amplitude: 2 V, 4 V, 6 V, 8 V, 10 V and 12 V. The left and the right photos show cells before electric stimulation and those after electric stimulation, respectively. The figures show following results. Cells proliferate to confluent condition in four days, when the amplitude is smaller than 8 V. The number of cells does not increase in four days with the electric stimuli of the larger amplitude of 10 V and of 12 V, while several cells are adhering.

The numbers of cells after incubation for 72 hours with electric stimulation are collected in relation to the amplitude of the pulse in Fig. 10. The datum point shows the mean value of the three trials, and the top of the bar shows the maximum value in Fig. 10. The data scatter, but the number of cells is very small after the electric stimuli with pulses higher than 9 V.

Figs. 11-14 show myotubes formed after one, two, three and four days of incubation with horse serum, respectively. The myotubes in the control test and those in the electric stimulation test are displayed in A and in B of the figures, respectively. In every test, cells were morphologically differentiated to myotubes on the fifth day of culture. A lot of fused myotubes are shown in the stimulated cells (Fig. 14B).

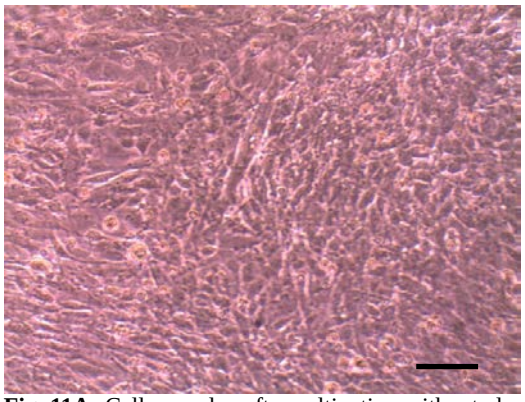

Fig. 11A: Cells one day after cultivation without electric pulses for 4 days. The bar indicates 0.1 mm.

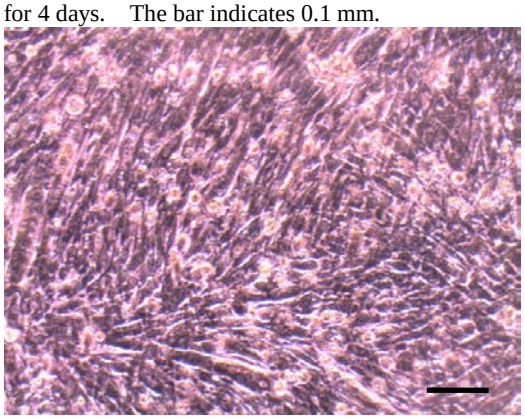

Fig. 11B: Cells one day after cultivation with electric pulses for 4 days. The bar indicates 0.1 mm.

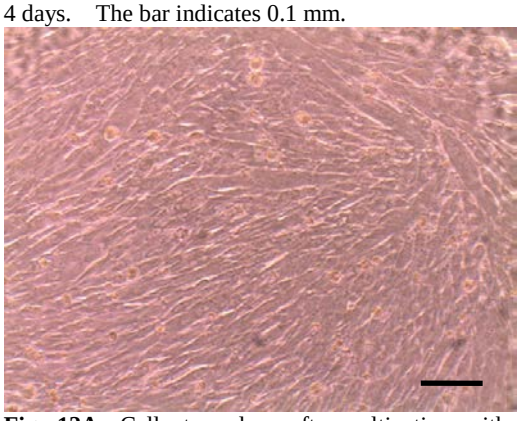

Fig. 12A: Cells two days after cultivation without electric pulses for 4 days. The bar indicates 0.1 mm.

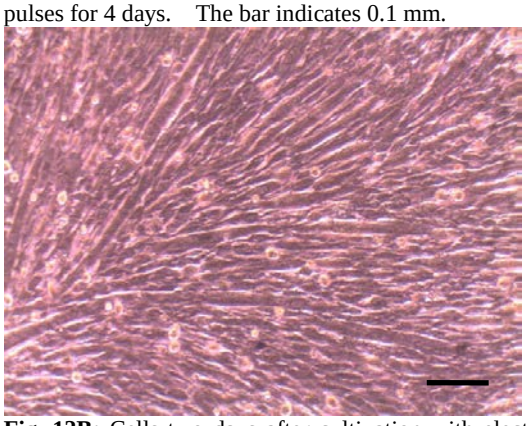

Fig. 12B: Cells two days after cultivation with electric pulses for 4 days. The bar indicates 0.1 mm.

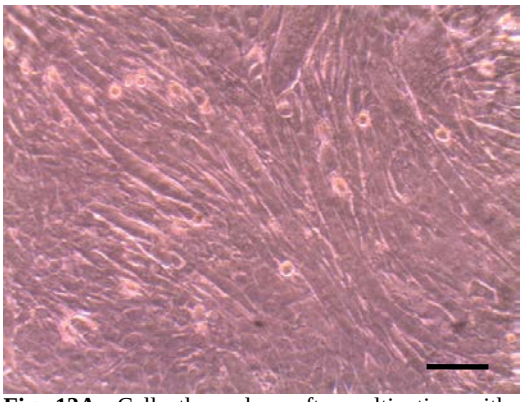

Fig. 13A: Cells three days after cultivation without electric pulses for 4 days. The bar indicates 0.1 mm.

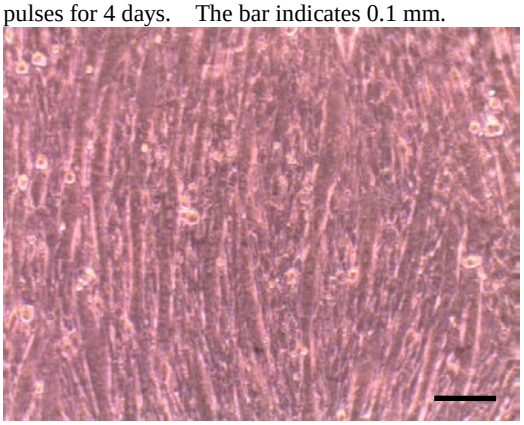

Fig. 13B: Cells three days after cultivation with electric pulses for 4 days. The bar indicates 0.1 mm.

Fig. 14A: Cells four days after cultivation without electric pulses for 4 days. The bar indicates 0.1 mm.

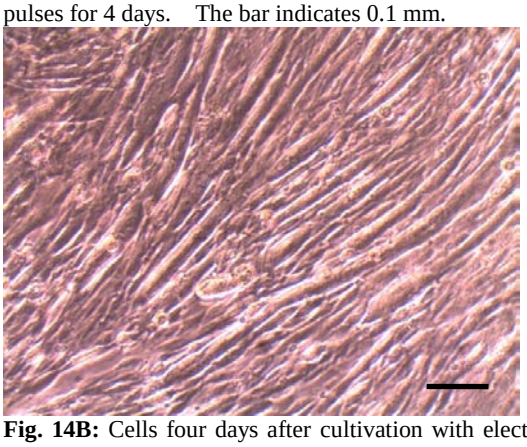

Fig. 14B: Cells four days after cultivation with electric pulses for 4 days. The bar indicates 0.1 mm.

The repetitive contraction was observed under the electric pulses on the eighth day of culture. The numbers of myotubes which shows repetitive contraction under the electric pulses in the stimulated cells and in the control cells are 106 and 20, respectively. The repetitive contraction of myotubes was more frequently observed in electrically stimulated cells than in control cells. The period of the repetitive contraction of myotubes was able to be controlled with the period of the electrical pulses applied.

4. DISCUSSION

The previous study shows that electric stimulation enhances differentiation of muscle cells [1]. Another previous experimental result shows that proliferation decelerates with the amplitude of electric pulses [5]. Another study shows that mechanical stimulation enhances differentiation of muscle cells [7] and improves the tissue-engineered human skeletal muscle [8].

Several factors might govern adhesion of biological cells. The previous study shows that electric stimulation can restrict adhesion of muscle cells [5]. Another study shows an electromagnetic field affects on the cell [6]. An alternating magnetic field might affect on adhesive molecules on the cell membrane. The mechanical stimulation also has potential to control adhesion and exfoliation of biological cells [7, 9].

The muscle tissue is daily exposed to the field of electric pulses, which are signals for the control of contraction. It serves a double purpose when the electric fields control regeneration of the tissue *in vivo*.

Several stresses might stimulate differentiation of cells. The electric stimulation might also affect myocytes to differentiate into myotubes. The change of environment stimulates the change of behavior of myocytes, so that myocytes differentiate into myotubes. The experimental results show that differentiation accelerates with the electric pulses.

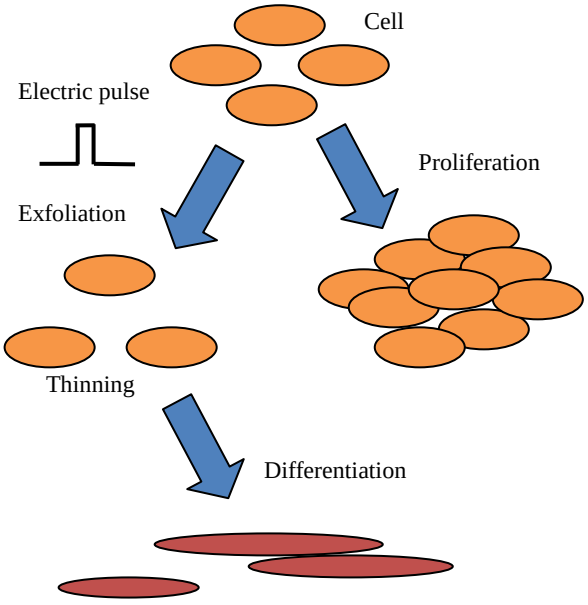

Fig. 15: Moderate electric stimulation might accelerate differentiation of myocytes.

An electrolytic process occurs around the electrode with the higher amplitude of pulses, which makes change of local pH in the medium. The environmental change restricts cell proliferation. The number of cells does not increase in four days with electric stimulation of the amplitude higher than 9 V in the present study. Too strong electric stimulation damages myocytes. Moderate electric stimulation, on the other hand, might accelerate differentiation of myocytes (Fig. 15). The electric stimuli decrease proliferation of cells. The electric stimuli also exfoliate several cells, which makes vacancy around the adhesive cell. The modification helps thickening of cells, and stimulates differentiation of cells. Differentiation might be optimization of cells to the changing environment.

The repetitive contraction proves functional differentiation of muscle cells. The present study shows the effect of electric stimulation on differentiation of muscle cells.

5. CONCLUSION

The effect of an electric field on differentiation of cultured myocytes has been studied *in vitro*. The experimental results show that cells adhere and proliferate under continuous electric pulses lower than 8 V, and that differentiation accelerates with the amplitude of electric pulses of 0.1 V.

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