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# Ultrasonic observation of intracellular differentiation of HDACi-treated C2C12 cultured cells and evaluation of artificial organs

HDACi 処理した C2C12 培養細胞の細胞内分化の超音波観測と人工器官の評価

Kazuki Ota[1†], Washiya Mamoru[1], Kyoichi Takanashi[1], Kazuto Kobayashi[2], Naohiro Hozumi[1], Sachiko Yoshida[1] ([1]Toyohashi Univ. of Technology; [2]Honda electronics Co., Ltd. )

太田　一城[1†]　鷲谷　衛[1]　高梨　恭一[1]　小林　和人[2]　穂積　直裕[1]　吉田　祥子[1] ([1]豊橋技術科学大学　[2]本多電子株式会社)

## 1. Introduction

Ultrasonic microscopy enables noninvasive measurement of living tissue and cells. Previous studies have shown that changes in intracellular structure, especially the cytoskeleton, during C2C12 myoblast differentiation can be observed[1]. C2C12 myoblasts have the characteristic of differentiating into muscle tissue and muscle cells having contraction ability depending on the differentiation condition. Globular actin present in the cell polymerizes with differentiation and becomes actin filament (F-actin) which plays a role of the cytoskeleton. Since the ultrasonic microscope can visualize F-actin, it is suitable for observation of cell differentiation[2].

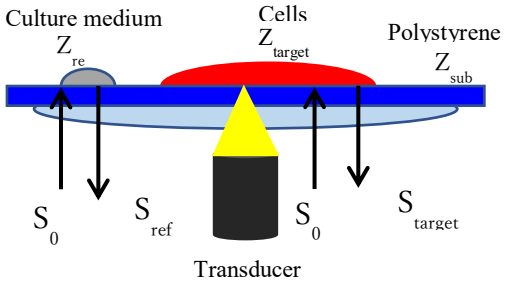
Recently it has been reported that the differentiation of C2C12 myoblasts into myotubes is promoted by HDAC inhibitors[3]. In this study, differentiation under the condition of C2C12 myoblasts with HDAC inhibitor treatment was observed using an ultrasonic microscope, and it was compared with the one with ordinary differentiation induction.

## 2. Methods

### 2.1 Acoustic living cell observation

To observe intracellular structure, the pulsed focus ultrasound (central frequency: 320 MHz) was transmitted and the reflection from the interface between cell and polystyrene film was received and interpreted into characteristic acoustic impedance (Fig.1). The 2D profile of acoustic impedance was acquired by mechanically scanning the transducer.

a.




b.

$$Z_{tgt} = \frac{1+\frac{S_{tgt}}{S_0}}{1-\frac{S_{tgt}}{S_0}} Z_{sub} = \frac{1-\frac{S_{tgt}}{S_{ref}}\frac{Z_{sub}-Z_{ref}}{Z_{sub}+Z_{ref}}}{1+\frac{S_{tgt}}{S_{ref}}\frac{Z_{sub}-Z_{ref}}{Z_{sub}+Z_{ref}}} Z_{sub}$$

Fig. 1 Diagram of breast cancer cells observation using acoustic impedance microscope (a). Waveform parameter extraction formula (b).

### 2.2 C2C12 cell culture and biological observation

C2C12 myoblasts (DS Pharma Biomedical Co.Ltd, Japan) were proliferated in Minimum Essential Medium Eagle with Hanks' salt (HMEM) supplemented with L-Glutamine, 10 % fetal bovine serum (FBS), some vitamins, amino acids, and 10 ml/L Penicillin-Streptomycin. To induce differentiation, the culture medium was replaced with serum-free medium. Cells were cultured and observed on a 50 μm polystyrene film. These cells were exposed to valproic acid (VPA) (0.1, 1 or 10 mM) for 24h, 4 days after the induction of differentiation. To observe specific protein expression by HDAC inhibitor, some cells were stained with myosin protein (MY-32) and actin-binding protein (α-actinin) and observed with a fluorescence microscope.

## 3. Results and Discussion

As a result of observation with a fluorescence microscope, a significant increase in the expression level of myosin protein (MY-32) and actin-binding protein (α-actinin), which are differentiation indicators, was observed immediately after treatment with HDAC inhibitor (Fig.2). This indicates that the HDAC inhibitor promoted differentiation, thereby enhancing cell fusion and contractility. Furthermore, in order to investigate the effect of HDAC inhibitor, it was observed with an ultrasonic microscope (Fig. 3). As a result, a large increase in the acoustic impedance value was observed as compared with untreated cells. This result indicates that not only cell fusion and contraction ability were acquired but also a cytoskeleton was formed in the cell. From this fact, it was thought that F-actin was formed in the cell and became a cytoskeleton, and the cell became fibrous myotubes.

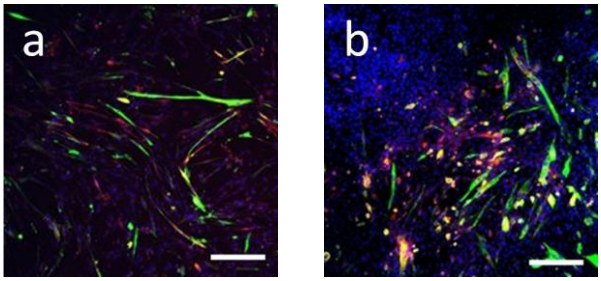
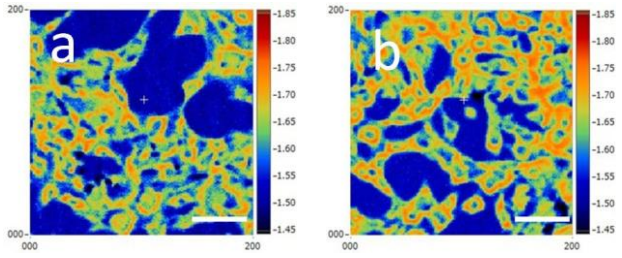



Fig.2 Investigation of the effect of HDAC inhibitor by fluorescence microscopic image. C2C12 myoblasts were stained after 5 days of differentiation induction. They were stained with α-actinin (red), MY-32 (green), nucleus (blue). (a)control. (b)VPA 0.1 mM treated cells. Scale bars, 200 μm.



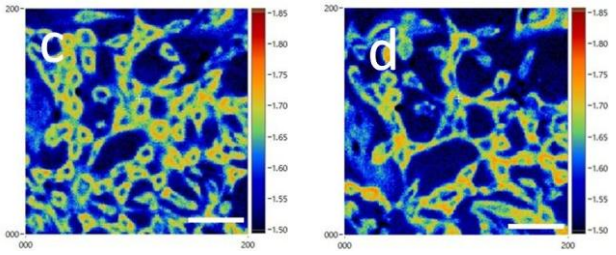




Fig. 3 Acoustic impedance image of C2C12 cells before and after HDAC inhibitor treatment. A, B: Images of 4 days after induction of differentiation before VPA treatment (A) and 5 days after induction of differentiation after VPA treatment (B) are shown. C, D: As a control, images of 4 days (C) and 5 days (D) after differentiation induction without VPA treatment are shown. Scale bars, 100 μm.

## 4. Conclusion

In observation of C2C12 cells treated with HDAC inhibitor by fluorescence microscopy, an increase in protein expression as an indicator of differentiation was observed, and an effect of myotubes differentiation by HDAC inhibitor could be observed. However, in this method, since it is necessary to fix and stain the cells, it was impossible to observe the same cell change. On the other hand, with the ultrasonic microscope, we could continuously observe non-invasively. As a result, it was possible to observe not only the change of the same cell but also the formation of the cytoskeleton which was not understood by the fluorescence microscope.
Presently used cell observation means are optical microscopes and fluorescence microscopes, but in this study, it was suggested that ultrasound microscopy is very useful for observing cell dynamics.

## References

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3. Ikeda, Kazushi, et al. "In vitro drug testing based on contractile activity of C2C12 cells in an epigenetic drug model." *Scientific reports* 7 (2017): 44570.