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Intracellular Ca^{2+} Responses of 3D-Cultured Osteoblasts to Dynamic Loading*

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Abstract

In bone cells, intracellular Ca^{2+} (iCa^{2+}) functions as a second messenger in the mechanotransduction pathway. Its responses to mechanical stimulation can be observed microscopically on a rigid flat surface with a Ca^{2+} fluorescent indicator, generally, under fluid flow. However, bone cells are physiologically exposed to dynamic loading accompanied by bone matrix deformation. In this situation, microscopic methods of observing iCa^{2+} responses cannot be used because of the loss of focus or movement of cells out of the observation area. The purpose of this study was to develop a compact optical device for the observation of iCa^{2+} responses of osteoblasts to dynamic loadings accompanied by substrate deformation. This system comprised four light emitting diodes (LEDs) and a photodetector (PD) placed underneath a culture chamber, specifically designed for tissue-level iCa^{2+} observations. This device was used to study the frequency dependence of iCa^{2+} responses of osteoblasts to dynamic loading. MC3T3-E1 osteoblasts were cultured three-dimensionally in a collagen sponge scaffold with the fluorescent Ca^{2+} indicator Fluo-4 AM and mechanically stimulated by a 0.2% deformation of the sponge at 0.2, 2, or 20 Hz for 150 s. Our device succeeded in detecting temporal changes in the intensity of emitted fluorescent light, showing a frequency-dependent increase in fluorescence intensity. This device may contribute to further understanding of the mechanosensing and mechanotransduction mechanisms in bone under near-physiological conditions.

Key words: Intracellular Ca^{2+}, Optical Sensing, Dynamic Loading, 3D Culture, Osteoblasts

1. Introduction

Physical activities, such as walking, running, and jumping, may cause bone matrix deformation and fluid flow in the canaliculi and lacunae of the matrix [1] depending on the loading frequency [2, 3]. Consequently, bone cells in the matrix are mechanically stimulated by bone matrix deformation and load-induced fluid flow. Considering that bone cells are surrounded by this mechanical environment, observation of cellular responses to three-dimensional (3D) substrate deformation in the physiological range is essential to completely understand bone mechanobiology. In order to assess the mechano-responses of bone cells in a 3D environment, various bone-like constructs containing living bone cells have been experimentally developed *in vitro* [4-6]. These constructs could also be used as mechano-adaptable bone grafts, which are potentially useful in bone regenerative medicine.

Intracellular Ca^{2+} (iCa^{2+}) functions as a second messenger in the early stage of the mechanotransduction pathway in bone cells following mechanical stimulation [7-9]. Observing iCa^{2+} dynamics helps in understanding the mechanosensing and

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mechanotransduction mechanisms in bone. Fluorescence microscopes equipped with video analysis systems or laser confocal microscopes have generally been used to observe iCa^{2+} dynamics in bone cells in response to mechanical stimulation [10,11] in two-dimensional (2D) culture systems. However, these microscopic methods cannot be used for cells subjected to dynamic loading accompanied by substrate deformation because the cells become defocused or move out of the observation field due to 3D substrate deformation. This problem is related to the shallow depth and narrow range of microscopic fields. Therefore, the iCa^{2+} responses of bone cells to mechanical stimulation have been mainly observed by applying fluid flow to cells cultured on a rigid flat surface [12, 13]. Recently, Adachi et al. succeeded in microscopic observation of the iCa^{2+} response of an individual osteocyte in the bone matrix of embryonic chicken calvaria to a precisely defined static deformation of the matrix [14]. However, to date, no other study has reported the iCa^{2+} responses of bone cells to 3D substrate deformation caused by dynamic loading. Therefore, the objectives of this study were to develop a new compact optical device to observe the iCa^{2+} responses of osteoblasts to dynamic loading accompanied by 3D substrate deformation and to investigate the frequency dependence of iCa^{2+} responses to dynamic loading.

2. Materials & Methods

2.1 Cell culture

Approximately 16×10^6 MC3T3-E1 osteoblasts at a passage number of 8 were suspended in 0.6 ml of α-modified minimum essential medium (α-MEM) containing 10% fetal bovine serum and 1% antibiotic cocktail (100 U/ml penicillin and 100 μg/ml streptomycin). To create a bone-like construct, the cell suspension was gently and uniformly dropped onto the upper surface of a type I collagen sponge scaffold (L16 mm × W20 mm × t2 mm, pore size: 89 ± 28 μm; CollaCote, Zimmer Dental Inc., CA, USA), fixed in the culture chamber of a mechanical stimulator, and spontaneously sucked into the sponge, resulting in the distribution of the cells in the sponge as shown in Fig. 1. MC3T3-E1 cells were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The mechanical stimulator was sterilized using ethylene oxide gas prior to cell seeding. The cells were loaded with a fluorescent Ca^{2+} indicator, Fluo-4 AM (3 μM; Dojindo Laboratories, Kumamoto, Japan), in the medium 12 h after cell seeding and incubated for 1 h prior to dynamic loading. In this study, four experimental groups were prepared; one was used as a no-loading control and the other three were mechanically stimulated at different loading frequencies. Four samples were tested for each group.

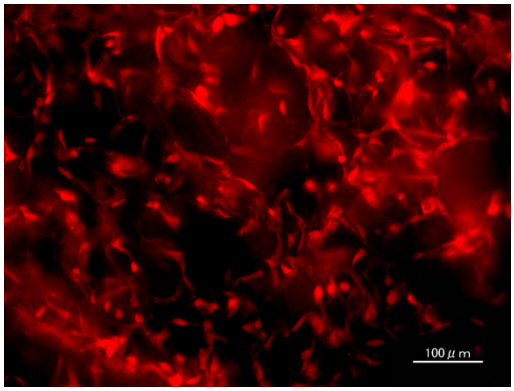

Figure 1 Microscopic picture of MC3T3-E1 cells seeded into a type I collagen sponge at a cell seeding density of 25000 cells/mm^3. The cells were stained with 1 μM Rhodamine 123 and observed using a fluorescence microscope (Keyence, BZ-9000). Observation depth: 180 μm (50 images taken at a depth interval of 3.6 μm are overlaid).

2.2 Dynamic loading

A dynamic load was applied to a bone-like construct by a piezo-type mechanical stimulator, as previously reported [15-17]. In brief, input voltage from a Windows-based computer is amplified by a high-speed amplifier and transferred to a bimorph-type piezoelectric actuator, resulting in the bending motion of the actuator. This motion of the actuator exerts mechanical compressive deformation on a construct in the culture chamber. The actuator is feedback-controlled using signals detected by an eddy current displacement sensor. In our study, a sinusoidal compressive deformation was applied to a construct for 150 s at 0.2, 2.0, or 20 Hz, with a peak deformation of 0.2% of the initial length of the scaffold in the loading direction. Osteoblasts in a construct were mechanically stimulated by substrate strain and fluid shear caused by flow of the culture medium under dynamic loading conditions [17], mimicking the mechanical environment in bone matrix *in vivo*. Therefore, the collagen sponge scaffold viscoelastically behaves in the medium and shows 0.75 kPa of apparent elastic modulus under the compressive loading condition with a peak deformation of 0.3% at 1 Hz [17]. The frequencies and magnitude of loading were selected with reference to the physiological and functional range of bone strain reported previously [18, 19].

2.3 iCa^{2+} monitoring

A compact optical device was developed to monitor the changes in iCa^{2+} concentrations in a construct under dynamic loading conditions. It was composed of four light-emitting diodes (LEDs; L490-03, Epitex, Kyoto, Japan), a silicon photodiode (PD; 57508, Edmund Optics, NJ, USA), a non-spherical condenser lens (focal distance = 13 mm), and two optical filters (1 and 2), all of which were packed into a cylindrical duralumin case of ϕ30 mm × H30 mm, placed underneath a construct in the culture chamber of the mechanical stimulator (Figs. 2 and 3). LEDs emitted the excitation light at 488 nm though the lens and filter 1 toward the bottom surface of the construct; the irradiated region had a diameter of approximately 5 mm. The excitation light passes through the construct with a thickness of 2 mm; therefore, the number of cells excited by the light is estimated to be approximately 1×10^6.

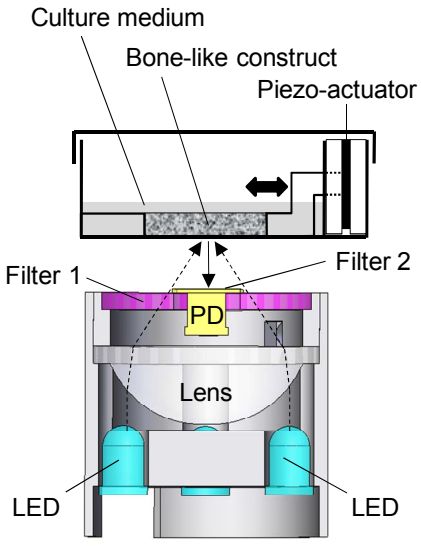

Figure 2 Configuration of the optical device for monitoring iCa^{2+} responses of osteoblasts under dynamic loading conditions accompanied by substrate deformation. Four LEDs excite the Ca^{2+} fluorescence indicator Fluo-4 in osteoblasts by irradiating a construct subjected to dynamic loading with a light of 488-nm wavelength. Fluorescent light of 518-nm wavelength emitted by Fluo-4 is detected by a PD positioned at the center of the device.

Figure 4 shows the transmittance properties of optical filters 1 and 2. The light emitted by the LEDs has a half-width of 30 nm around the peak at 490 nm. Only the LED light passes through filter 1 at wavelengths of approximately < 500 nm, which includes the excitation light at 488 nm. However, light at a wavelength of approximately > 500 nm passes through filter 2 on PD, which includes the fluorescent light, at a wavelength of 518 nm, emitted from the Ca^{2+} indicator in the irradiated region. Using these two filters, PD detects the fluorescence at an intensity equivalent to the iCa^{2+} concentration with a better signal to noise (S/N) ratio. To eliminate the effect of thermal drift from detected signals, the light of LEDs is pulsed with a duration of 225 ms, a duty ratio of 50%, and a maximum applied current of 24.5 mA. Voltage differences between the top and bottom of detected pulses are recorded and normalized by subtracting the average voltage difference during the initial non-loading period for 30 s, which is recognized as a background level. Finally, the normalized voltage differences are employed as fluorescence intensities.

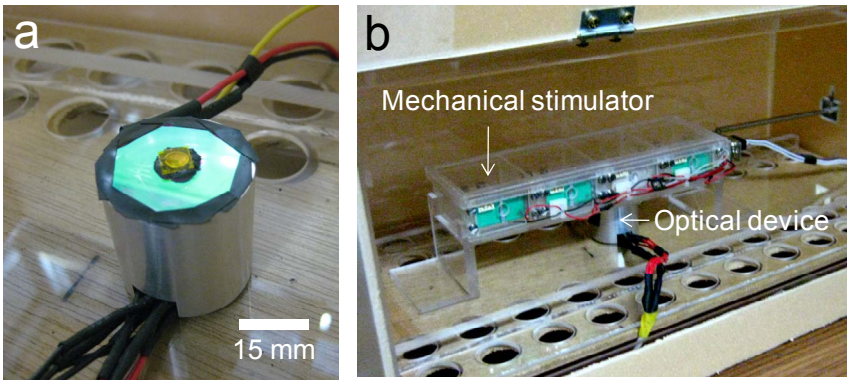

Figure 3 Photographs of the optical device. (a) Overall appearance of the device, (b) The device installed underneath the mechanical stimulator in a homeothermal container at 37°C.

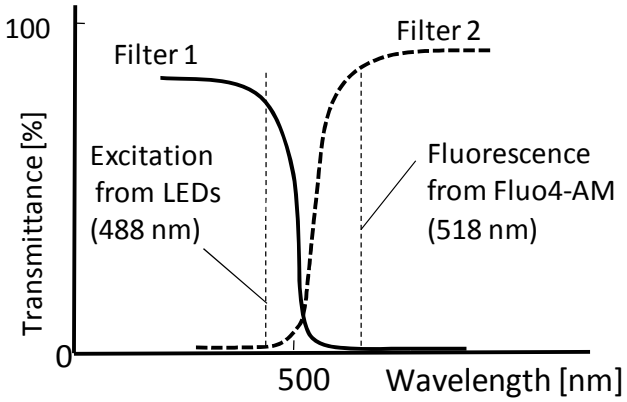

Figure 4 Transmittance properties of the optical filters 1 and 2 selected for Fluo-4. Light from LEDs (wavelength of approximately < 500 nm) passes through filter 1 while light from a construct (wavelength of approximately > 500 nm) passes through filter 2. The wavelength of the excitation light from the LEDs is 488 nm and that of the fluorescent light emitted by Fluo-4 is 518 nm.

Figure 5 shows the configuration of the experimental system. The emission of LEDs is controlled by a Windows-operated computer (PC) through an AD/DA interface board (16-bit resolution; DAQ Card-6036E, National Instruments, TX, USA). A signal detected by the PD is transferred to the PC through an amplifier (c9329, Hamamatsu Photonics, Shizuoka, Japan) and the interface board at a sampling rate of 667 Hz. In this study, the LEDs started to emit 30 s before applying the dynamic load and finished emitting 60 s after the end of the loading. All experiments were performed at a constant temperature of 37°C (Fig.3b). From the time-course data of fluorescence intensity, a total response time and accumulated fluorescence intensity during the response time were calculated and compared among the control and loaded samples. Response time was identified as the length of time for which the intensity exceeds the mean value plus one standard deviation of the no-loading control at each time point during the loading period. The accumulated fluorescence intensity is the sum of the intensities over the response time.

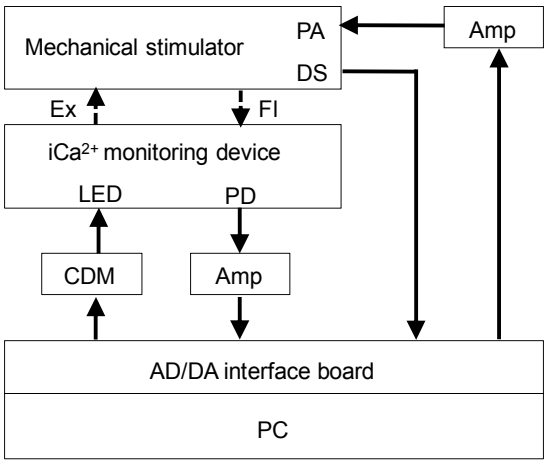

Figure 5 Configuration of the experimental system. PA: Piezoelectric actuator, DS: Displacement sensor, Ex: Excitation light, Fl: Fluorescent light, CDM: Current-drive module, Amp: Amplifier, PC: Personal computer.

2.4 Data analysis

In order to evaluate the statistical significance of any change in the fluorescence intensity parameters, analyses of variance (ANOVA) followed by Tukey HDS post-hoc test were conducted among the groups; the significance level was set at $p < 0.05$ using a statistics software package (KaleidaGraph, Synergy Software, PA, USA).

3. Results

Figure 6 shows the changes in fluorescence intensity at all the loading frequencies, representing the frequency dependence of the iCa^{2+} response of osteoblasts. The no-loading control cells did not show any obvious increase in fluorescence intensity over the observed time period. Larger increases of intensity were observed at higher frequency, particularly at 20 Hz, the intensity obviously increased just after the start of loading, and saturated at about 15 s after the start of loading, remaining at this level even after the loading ended.

The evaluation parameters obtained from the time course data also demonstrated the highest values in the 20-Hz loading group. The response times of the cells were 15.1%, 32.2%, and 71.2% of the total stimulation time in the 0.2, 2.0, and 20-Hz loading groups, respectively (Fig. 7a). The accumulated fluorescence intensities were 2.5, 7.1, and 20.0 mV in the 0.2, 2.0, and 20-Hz loading groups, respectively (Fig. 7b).

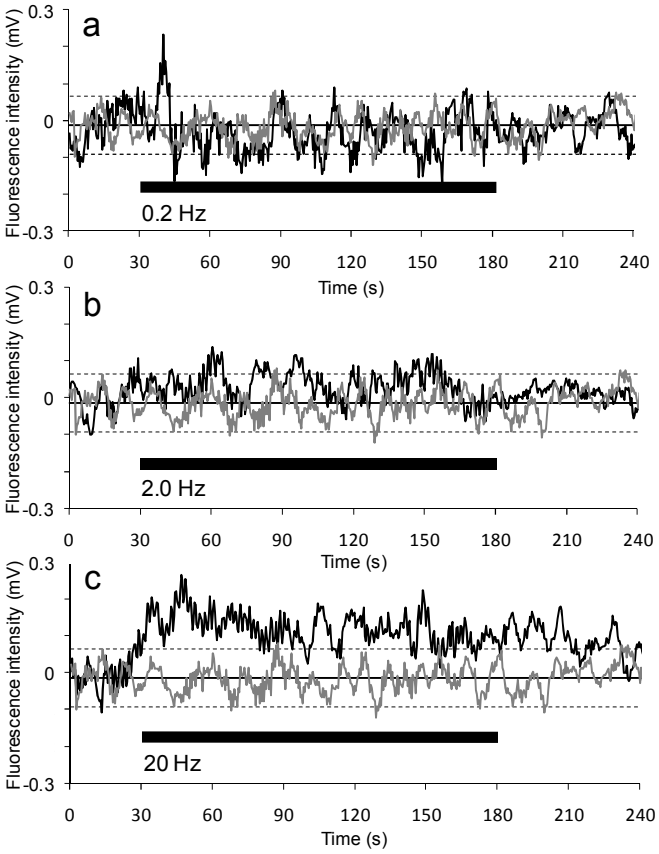

Figure 6 Changes in fluorescence intensity demonstrating intracellular Ca^{2+} dynamics in osteoblasts in response to (a) 0.2, (b) 2.0, and (c) 20-Hz dynamic loadings. Gray and black lines represent no-loading control and loaded sample, respectively. The lines are the averages of four samples. Horizontal black bars and dashed lines represent the periods of stimulation and one standard deviation of the no-loading control, respectively.

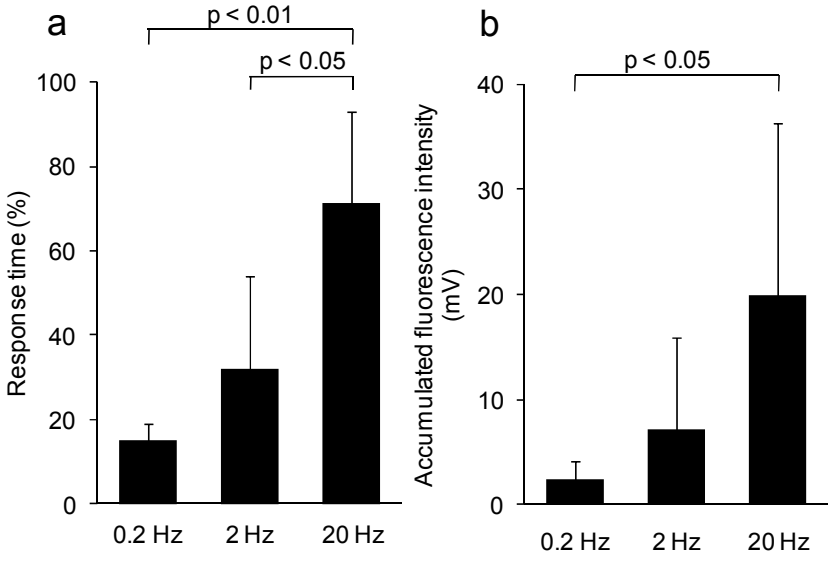

Figure 7 Comparison of intracellular Ca^{2+} responses among the 0.2-, 2.0-, and 20-Hz dynamic loadings. (a) Response time (percentage of the loading period), (b) Accumulated fluorescence intensity. The bars represent the average ± SD of four samples.

4. Discussion

Our results demonstrated the frequency-dependent iCa^{2+} response of osteoblasts to dynamic loading, suggesting that osteoblasts are more sensitive to deformation at high frequency than at low frequency. This finding supports findings of previous studies. Rubin et al. reported that dynamic loading elicits more bone ingrowth into a porous titanium implant *in vivo* at 20 Hz than at 1 Hz [20]. Walking-induced bone strain waveform involves not only high-magnitude, low frequency components derived from the walking cycle, but also low-magnitude, high frequency components over 30 Hz [18, 19]. Even with low magnitude, the higher frequency components involved in the walking-induced bone strain have strong stimulatory effects on the osteogenetic responses of osteoblasts [21]. Although the iCa^{2+} response is an early event of the mechanotransduction pathway in bone cells following mechanical stimulation [7-9], various simultaneous intracellular or downstream events acting through critical regulatory pathways, including the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway [22], the inositol trisphosphate (IP_3) pathway [12], and the nitric oxide (NO) pathway [23] occur together with iCa^{2+} responses toward adaptive osteogenesis. This suggests that each event could contribute to the frequency dependence of the osteogenic response. However, our results suggest at least a possibility that the iCa^{2+} response of osteoblasts is a major cause of the frequency dependence of the osteogenic response to dynamic loading. Nevertheless, it is necessary to investigate the frequency dependences of events other than the iCa^{2+} response to better understand the frequency-dependent response of osteogenic cells to dynamic loading.

Our result (Fig. 7b) suggests that the iCa^{2+} levels of osteoblasts respond non-proportionally to the frequency of loading. By applying mechanical stimulation to osteoblasts, stretch-activated Ca^{2+} ion channels on the cell membrane modulate the cell membrane voltage, consequently voltage-activated Ca^{2+} ion channels open and mobilize Ca^{2+} from extracellular fluid into the cell body [24]. It has been reported that the open–close behavior of voltage-activated ion channels shows a non-linear response to applied voltage, producing the stochastic resonance phenomenon [25]. This behavior may be explained using mathematical models of non-linear oscillation such as the FitzHugh–Nagumo model [26]. Although these models were originally proposed to theoretically explain the excitable systems of neural cells, they may also be applicable to the frequency-dependent iCa^{2+} response of bone cells to dynamic loading. Understanding the non-linearity of the frequency-dependent iCa^{2+} response helps us to determine the most effective frequency of loading to elicit the maximum osteogenesis in a limited period, providing useful information for bone regenerative medicine and bone tissue engineering.

In our bone-like constructs, a dynamic load generates oscillatory fluid flow synchronizing with loading waveform and substrate deformation [17]. Dynamic loading to bone induces fluid flow in the canaliculi and lacunae of the bone matrix [1], and fluid shear stress stimulates bone cells in the matrix, consequently eliciting their osteogenic responses. Fluid shear induces iCa^{2+} mobilization through stretch-activated Ca^{2+} channels on the cell membrane and from the iCa^{2+} stores [27], which drives several necessary intracellular signal-transduction pathways, resulting in adaptive bone formation in response to the mechanical environment [28]. The fluid shear stresses introduced in our constructs were estimated as 0.002 Pa at 0.2 Hz and 0.2 Pa at 20 Hz, by referring to our previous data obtained from the flow visualization patterns using microspheres [17]. Although these shear stresses are smaller than those of bone matrix, predicted as 0.8–3 Pa [2], their magnitude could be a dominant factor for the larger iCa^{2+} responses observed at higher loading frequencies [29]. However, even with the same magnitude of shear stress, iCa^{2+} response of osteoblasts still depends on the frequency of oscillating fluid flow [30, 31], suggesting the contribution of chemotransport [32]. Further studies including the precise determination of

local mechanical environments in a construct subject to dynamic loadings are needed to reveal dominant mechanical factors for the frequency-dependent mechanism of iCa^{2+} response of bone cells.

Our device succeeded in monitoring the iCa^{2+} responses to dynamic loading accompanied by 3D substrate deformation. Although the device cannot capture images of iCa^{2+} response in an individual cell, the average response of iCa^{2+} to dynamic loading can provide useful information leading to a full understanding of bone mechano-biology in a 3D environment. Fluo-4 AM was selected as the Ca^{2+} indicator in our device because of its high sensitivity to Ca^{2+}. However, this device can only measure the changes in iCa^{2+} concentrations relative to that in the control, whereas a two-photon excitation type Ca^{2+} indicator, such as Fura-2 AM, can measure the absolute values of iCa^{2+} concentration. It may be possible to modify our device so as to include this type of Ca^{2+} indicator by installing two types of UV-LEDs emitting light at different excitation wavelengths and optical filters for the selective passing of the two excitation lights.

In this study, a compact optical device using non-microscopic methods was developed to observe iCa^{2+} dynamics in osteoblasts under conditions of dynamic loading, accompanied by substrate deformation. Our device succeeded in detecting changes in iCa^{2+} concentrations in osteoblasts under conditions of 3D dynamic substrate deformation. To our knowledge, this is the first such instance. Furthermore, the device proved that 20-Hz loading is a stronger stimulant for the cells compared to 0.2 or 2.0-Hz loading. Our device may contribute to further understanding of the mechanosensing and mechanotransduction mechanisms in bone.

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