Miniaturized stimulator for imaging of live cell responses to high frequency mechanical vibration

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Abstract. Cellular mechanobiology is highly important for tissue development and disease formation. However, lack of proper tools limit investigation of the cellular responses to different mechanical cues. High frequency (HF) vibration has already been applied in different cellular applications, but the knowledge of the stimulation effect on cells is limited. To meet this challenge, we designed a HF vibration stimulator for combined mechanical manipulation of live cells and high-resolution light-microscopy. Our system utilizes a commercial miniatyrized speaker to vibrate a 3D printed sample vehicle horizontally. Technical tests demonstrated excellent performance at lower frequencies (30 Hz-60 Hz), enabling even high magnitude (HMHF, $\boldsymbol{G}_{peak} \geq 1\ \boldsymbol{G}_{peak}$) method. Real-time acceleration measurement and light-microscopy both revealed accurately and precisely produced low magnitude (LMHF, $\boldsymbol{G}_{peak} < 1\ \boldsymbol{G}_{peak}$) vibrations. With our system, we could observe cellular responses to the LMHF (0.2 $\boldsymbol{G}_{peak}$, 30 Hz) vibration. In this paper, we introduce an inexpensive stimulation platform for the mechanobiology research of different cell applications.

Keywords: high frequency vibration, live cell imaging, mechanotransduction.

1 Introduction

Tissues and cells are subjected to varying mechanical stimuli in our bodies and these physical cues alter the cellular and tissue physiology. However, mechanistic details of the cellular force sensing are poorly understood and novel techniques are needed to unravel the mechanotransduction machinery. A high frequency (HF, >30 Hz) mechanical vibration offers a way to study, how impact-like mechanical stimulation influences cells. The method is popular in bone tissue engineering [1-6] but has also been tested for other tissue engineering applications [1-2, 7-9].

A major advantage of the HF vibration is that it is easily applicable for different adherent cell types, but the optimization of the stimulation parameters (magnitude, frequency) present a significant challenge of the method. The stimulation has been applied mostly with the low magnitude (LMHF) method [1, 3-6, 8], although also the high magnitude (HMHF) method has been used more recently [2, 4, 8]. The most often the stimulation has been applied between 10 Hz-200 Hz frequencies [2-5, 7-9], yet even

higher frequencies ranging from 400Hz - 10 kHz have attracted interests [5-6]. For example, in bone tissue engineering the method has lead in highly varying proliferation responses, and benefits to the osteogenic differentiation have been reported [2-5].

The above summarized HF vibration studies have utilized varying linear actuators, such as vibration sensors [3], shakers [5], piezos [6] and speakers [1-2], for studying cellular responses. However, current methods often require weeks of cell culture before analysis, thus making the optimization of the stimulation parameters both inefficient and challenging. Observing the cellular responses immediately after the HF vibration with light microscopy could provide new information on both the mechanocoupling and the mechanical force transduction of cells. This is necessary for deepening our understanding on the effects of mechanical manipulation on cellular physiology. Devices has already been introduced for the purpose. These systems utilize commercial speaker and optical disk reader to produce even HMHF vibration in room temperature (RT) [10-11]. However, previous studies have demonstrated the need to conduct the experiments in controlled cell culture conditions [12-13].

In this paper, we introduce a 3D printed stimulator platform, which combines the HF vibration and high-resolution light microscopy. Our aim was to design a device, which has a wide stimulation performance range in + 37˚C. The accuracy and precision of the system were tested with acceleration measurement and real-time imaging. We tested the device and imaged living cells before and immediately after the HF vibration. Our stimulator is an additional tool for cellular mechanobiology research, with the aim to provide a significantly more comprehensive knowledge of the direct stimulation effects to the cells.

2 Material and Methods

2.1 Stimulator design and working principle

The stimulator was designed with SolidWorks (Dassault Systémes Corp., MA, USA) to fit on a commercial inverted microscope unit (ZEISS LSM 780 LSCM, Carl Zeiss AG) and to accept coverslip cell chambers (ø=3 cm, Aireka Cells, China) (Fig. 1A). We ordered 3D printing of the system (i.materialise, Netherlands). A sample vehicle accepts single sample holder (Aireka Cells, China) and is tightly attached against a commercial speaker (ø=5 cm, Partco Oy, Finland). Supportive plastic plates with miniaturized wheels (Roco, Germany) on their bottom provided stable horizontal movement (y-axis) of the vehicle on the stage adapter. The sample movement was measured with an accelerometer (3axis, ADXL325, Analog Devices). The stimulator was used with a user interface (LabVIEW, National Instruments) in temperature-controlled conditions provided with the microscope system (Incubator XL S1, ZEISS LSM 780 LSCM) and its software (Zen Black, Carl Zeiss AG) (Fig.1B).

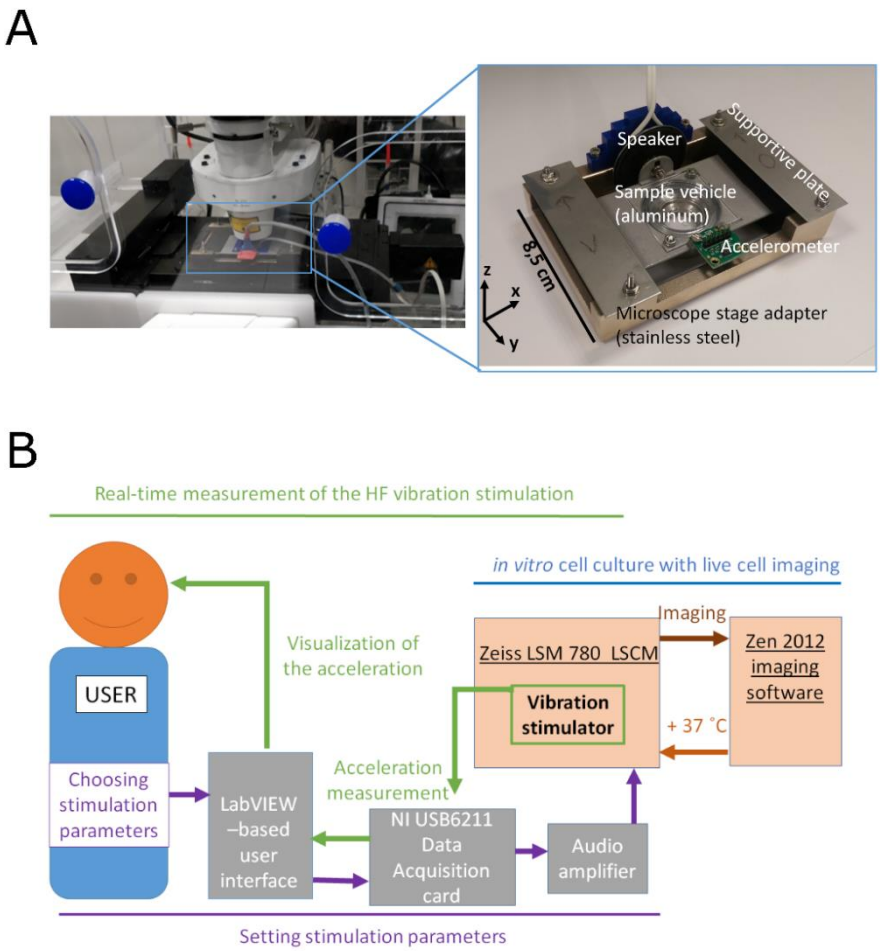


Fig. 1. A) Representative images of the stimulator and its placement on a commercial microscope unit. B) To produce the desired HF vibration, the AC-signal parameters (voltage, frequency, duration) were set with a user interface, after which it was amplified (Pro-Ject Box-Design Amp Box S2, Pro-Ject Audio Systems, Austria). The stimulation was monitored and recorded with the user interface.

2.2 Performance range

The performance range to produce sinusoidal HF vibration of the sample vehicle (m=104 g) at a frequency range of 30-200 Hz was evaluated by increasing the AC voltage input of the stimulator and evaluating the time domain representations and power spectral density (PSD) estimates of three-dimensional accelerations. They were determined from the recorded acceleration data with a matlab-script (MATLAB R2014a (32-bit), MathWorks, MA, USA). The vibration magnitude ($\boldsymbol{G}_{peak}$) maxima were considered

either if the main channel movement (Y Ch) stopped increasing or was deformed, or the movement to other directions (X Ch, Z Ch) either increased higher than that of the main channel or exceeded the 0.5 $\boldsymbol{G}_{\text{peak}}$. The stimulator performance was tested inside the large chamber incubator (Incubator XL S1, Carl Zeiss AG) of ZEISS LSM 780 LSCM microscope (Carl Zeiss AG) first in RT and in +37 ˚C after one hour of preheating.

2.3 Accuracy and precision of the LMHF vibration

Accuracy and precision of the sinusoidal HF vibration were tested in +37 ˚C with measuring acceleration and real-time imaging of the sample movement. For the acceleration measurement, the accuracies of the LMHF (0.5 $\boldsymbol{G}_{\text{peak}}$, 30 Hz, 60 Hz) vibrations were evaluated for the measurement channels (X Ch, Y Ch, Z Ch) from their maximum $\boldsymbol{G}_{\text{peak}}$ values and frequency contents. The precisions were tested with six stimulation repetitions, consisting of several stimulation bouts each (n=2-10). For the imaging, a line sample (Au, width=50 µm) was printed on the cover glass (Carl Zeiss AG) and assembled into the sample holder (Aireka Cells). With rotated image scanning area (90 ˚) in-plane differential interference contrast (DIC) image was taken (25x/0.8 Im Korr DIC UV M27, Carl Zeiss AG). Thereafter, time lapse with thin imaging area (2x512 px; frame rate: 265 FPS) was taken during two cycles of the LMHF (0.8 $\boldsymbol{G}_{\text{peak}}$; 30 Hz) vibration. The time lapse was converted to montage (Fiji), from which average gray scale intensities were measured before and after two stimulation bouts to determine the z-focus change. Misplacements (x-axis) from the initial position were measured as the distances of the sample line edge with respect to the image scanning area edge. Maximum sample displacement (µm) during the vibration were measured and compared with the theoretical values obtained from the measured acceleration data after acceleration to amplitude conversion.

We tested, if the system enables observing of fast cellular responses to the LMHF vibration (0.2 $\boldsymbol{G}_{\text{peak}}$, 30 Hz; 110 µm displacement). During six short stimulation repetitions, we imaged (C-Apochromat 63x/1.20 W Korr M27) a time lapse (113,98 s, frame rate 2.1 FPS) of living epithelial cells (MDCKII) expressing H2B-EGFP (nuclei) and E-cadherin-DsRed (cell-cell junctions) that were cultured on the cover glass (Carl Zeiss AG) and assembled into the sample holder (Aireka Cells). For the visualization, brightness and contrast were manually adjusted (Fiji) close to saturation limits.

3 Results

3.1 At low frequencies stimulator produces even HMHF vibration

The best performance range occurred at frequencies less than 70 Hz, after which the movement to other directions increased with respect to the main axis (Fig. 2.). The environmental temperature had no effects to the maximum $\boldsymbol{G}_{\text{peak}}$ values, with the exception at the 30 Hz and 80 Hz frequencies, but it affected the amount of movement to other directions (Fig. 2.).

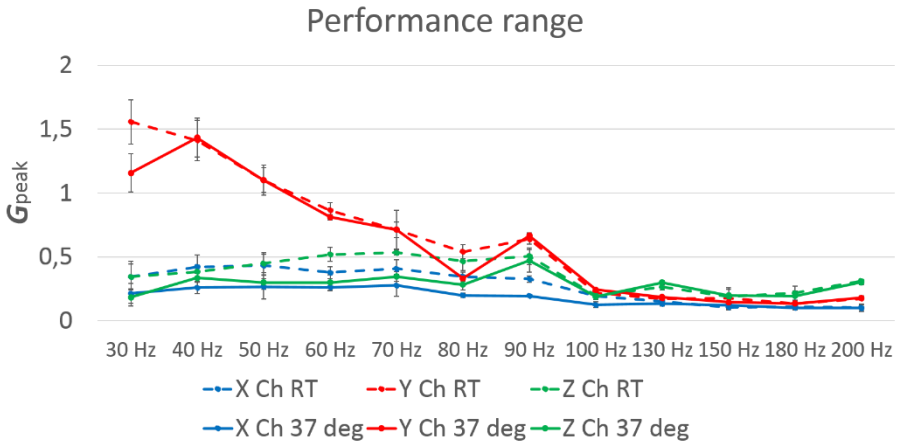
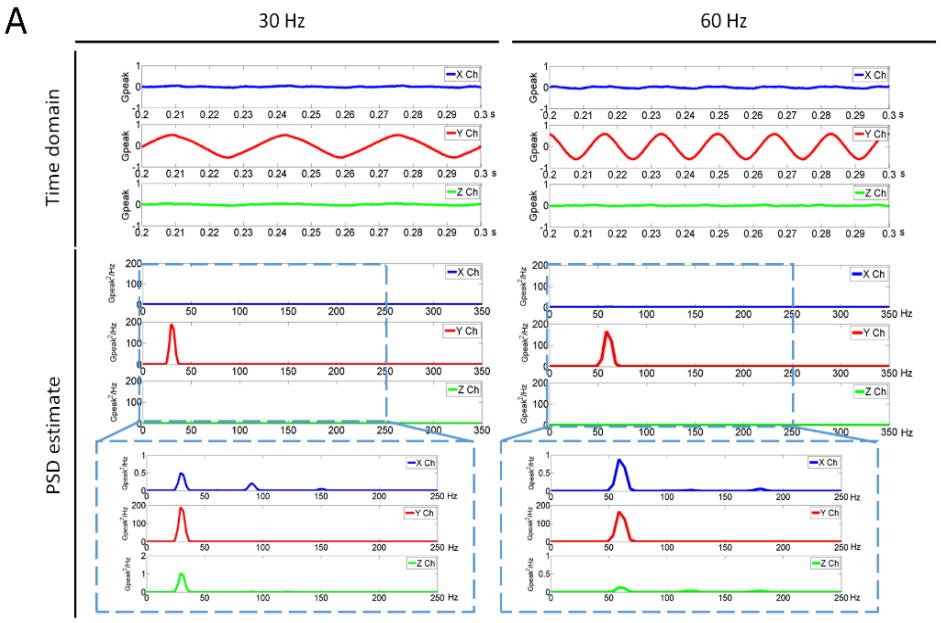


Fig. 2. On the usable performance range (30-70 Hz), the stimulator produced even HMHF vibration of the main axis (Y Ch), which magnitude increased at 30 Hz frequency even to 6-fold higher than those of other axes (X Ch, Z Ch). The maximum $\boldsymbol{G}_{peak}$ values decreased at +37 ˚C only at 30 Hz frequency, but the higher temperature decreased the movement of other axes with approximately 50 %.

3.2 Acceleration and imaging data both demonstrate accurately produced LMHF vibration

The recorded acceleration data demonstrated accurately produced LMHF vibration at both stimulation frequencies (Fig. 3A.). Representative DIC images illustrated adequately tracked real-time movement of the Au-printed sample during the sinusoidal LMHF vibration (Fig. 3.B). The image scanning area restricted observing of the peak-to-peak displacements with higher $\boldsymbol{G}_{peak}$ values.


A
30 Hz
60 Hz
Time domain
PSD estimate
X Ch
Y Ch
Z Ch
Gpeak
Gpeak²/Hz
0.2 0.21 0.22 0.23 0.24 0.25 0.26 0.27 0.28 0.29 0.3 s
0 50 100 150 200 250 300 350 Hz
0 50 100 150 200 250 Hz

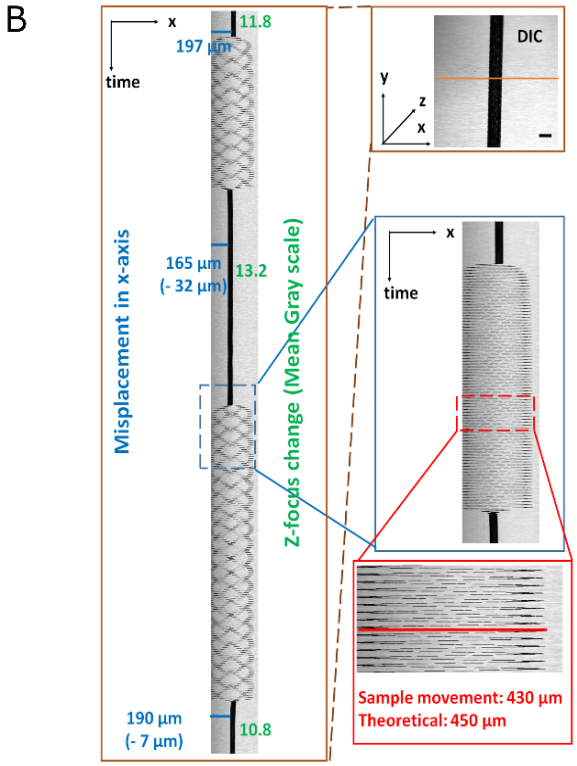
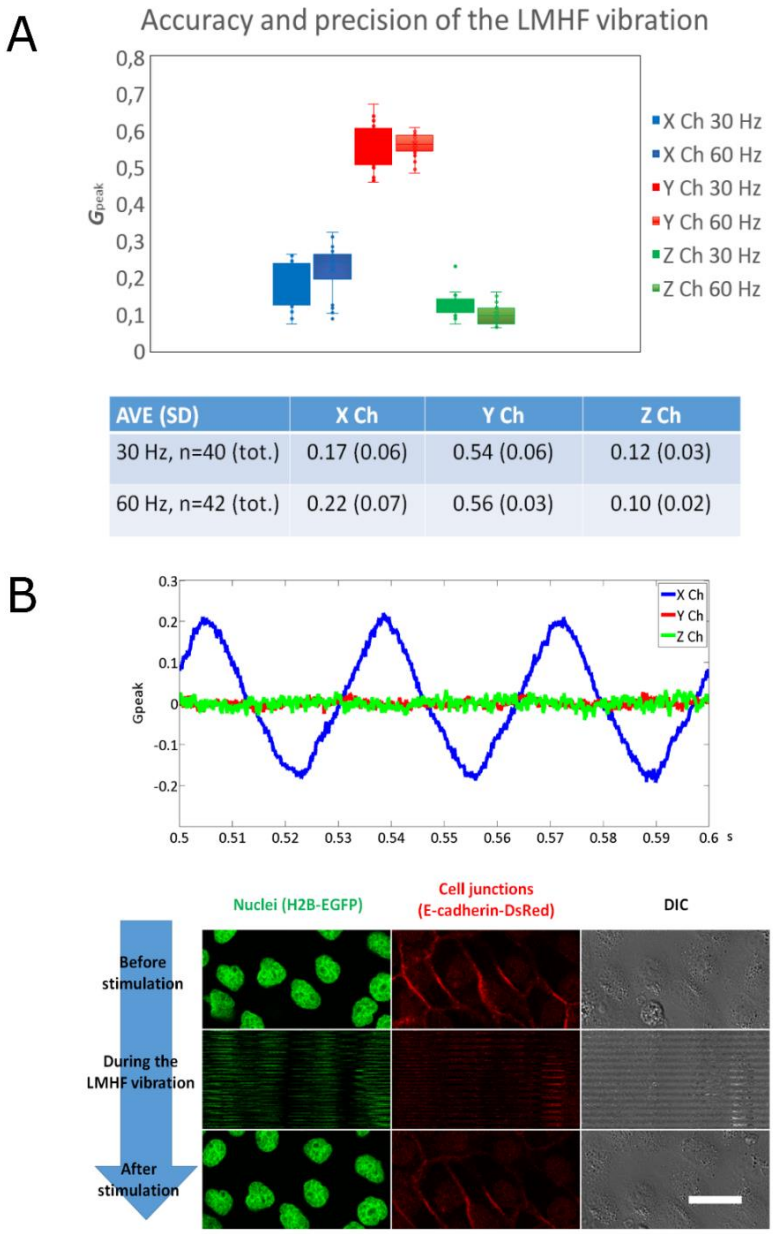

B
x
time
11.8
197 µm
165 µm
(- 32 µm)
13.2
190 µm
(- 7 µm)
10.8
Misplacement in x-axis
Z-focus change (Mean Gray scale)
DIC
y
z
x
Sample movement: 430 µm
Theoretical: 450 µm

Fig. 3. A) Time domain representations (0.5 $\boldsymbol{G}_{\text{peak}}$) and PSD estimates (200 $\boldsymbol{G}_{peak}^{2}/\text{Hz}$) of the acceleration data showed at 30 Hz 60 Hz frequencies sinusoidal LMHF vibrations of the main axis without movement to other directions. B) According to real-time imaging of the sample (orange line), two repetitions of the sinusoidal LMHF (0.82 $\boldsymbol{G}_{\text{peak}}$, 30 Hz) vibration caused only minor misplacement from initial location (blue) with invariant z-focus (green). Moreover, the sample movement (red) had 93 % fit to the theoretically calculated peak-to-peak displacements of the sinusoidal waveform. Scalebar 50 µm.

3.3 High precision enables observing of cellular responses to the LMHF vibration

Acceleration results combining data from multiple stimulation bouts demonstrated accurately and precisely produced LMHF vibrations at both frequencies (Fig. 4A.). The image field was precisely preserved after six LMHF vibration cycles, yet observing of the sample movement during the LMHF vibration was prevented by the limited imaging speed (Fig. 4B.).



AVE (SD)	X Ch	Y Ch	Z Ch
30 Hz, n=40 (tot.)	0.17 (0.06)	0.54 (0.06)	0.12 (0.03)
60 Hz, n=42 (tot.)	0.22 (0.07)	0.56 (0.03)	0.10 (0.02)

Fig. 4. A) The LMHF (0.5 $\boldsymbol{G}_{peak}$) vibration was accurately produced towards the main axis (Y Ch; 0.54-0.56 $\boldsymbol{G}_{peak}$, 108-112 %) with less than 22 % of sideways movement (X Ch) and 40 % of bouncing movement (Z Ch) with respect to the main axis. Thinner quartiles of the box plots of the 60 Hz frequency demonstrated more precisely produced LMHF vibration when compared to the 30 Hz frequency. B) Our system enabled observing of local biological fast cell responses to the LMHF (0.2 $\boldsymbol{G}_{peak}$, 30 Hz) vibration. Scale bar 30 µm.

4 Discussion

The HF vibration affects the cellular physiology, such as proliferation [3-5] and differentiation of stem cells [1-8, 14]. Actin cytoskeleton and nucleus have been found crucial for the mechanocoupling of the stimulus, together with their interaction through Linker- of Nucleoskeleton and Cytoskeleton (LINC) complex [4, 14]. However, a wider utilization of the method requires still a better understanding of the stimulation effects to the cellular mechanobiology.

Our stimulator produced horizontal HF vibration accurately and precisely, especially when applied at the 60 Hz frequency. On specific frequencies, high magnitude method (HMHF) were enabled reaching even higher magnitudes than previously reported with corresponding stimulators designed for live cell imaging [10-11]. Currently the performance sufficiently covers needs set for the stimulator, while low magnitude method (LMHF) has been reported in many cell studies [3-6]. However, increasing the magnitudes from the current would probably be needed to detect immediate cellular responses to the stimulus. With the current frequency range our stimulator covers the most common stimulation frequencies of bone tissue engineering only partially [2-5], failing to produce e.g. nanoscale mechanical vibrations [6]. The linear actuator sets the highest performance limitations, and thus it should be chosen accordingly to meet the cell application -specific stimulation needs.

Currently, our stimulator enabled tracking of the real-time movement of the sample during the HF vibration similarly to previously introduced devices [10-11]. However, imaging speed prevented us from observing movements of larger areas. This would be essential to observe real-time stimulation effects in the cell culture. To our knowledge, neither stimulation initiated cellular deformations nor fluid flow have been studied with high-resolution light-microscopy [10-11], but mathematical models have suggested their occurrence [3, 15-17]. Consequently, imaging of the live cells with higher imaging speeds could provide us with new knowledge on the effect of the different loading parameters in the stimulus response.

The higher environmental temperature had only minimal effects to our stimulator's performance. Environmental factors (e.g. temperature and gas concentrations) are crucial for the cellular functions even in static cell cultures [12-13]. Consequently, the miniaturized size of the device enables performing of cell stimulations in more favorable cell culture conditions than the previous devices [10-11]. However, to study the cellular responses even more reliably would require adding a light-weighted heating plate in the proximity of the sample and replacing the current sample holder with a miniaturized and portable cell incubation chamber [13] to provide more precisely controlled cell culture conditions.

In this study, we realized a portable and easy-to-use mechanical stimulator. The HF vibration is produced on the commercial microscope system both accurately and precisely, though the sample vehicle design and the linear actuator should be optimized to enhance the performance further. Our initial findings with live epithelial cells suggest that, after development of more adequate control of the cell culture conditions, our device could be beneficial for many areas of the mechanobiology research.

5 Conclusion

We have designed a tool for the mechanobiology research, which enables observing of fast biological cellular responses to the dynamic HF mechanical vibration with high-resolution light-microscopy. In future, our system will be a good platform for real-time observation of the cellular responses to mechanical stimulation.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

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