The design, construction and characterization of new nanovibrational bioreactors for osteogenesis

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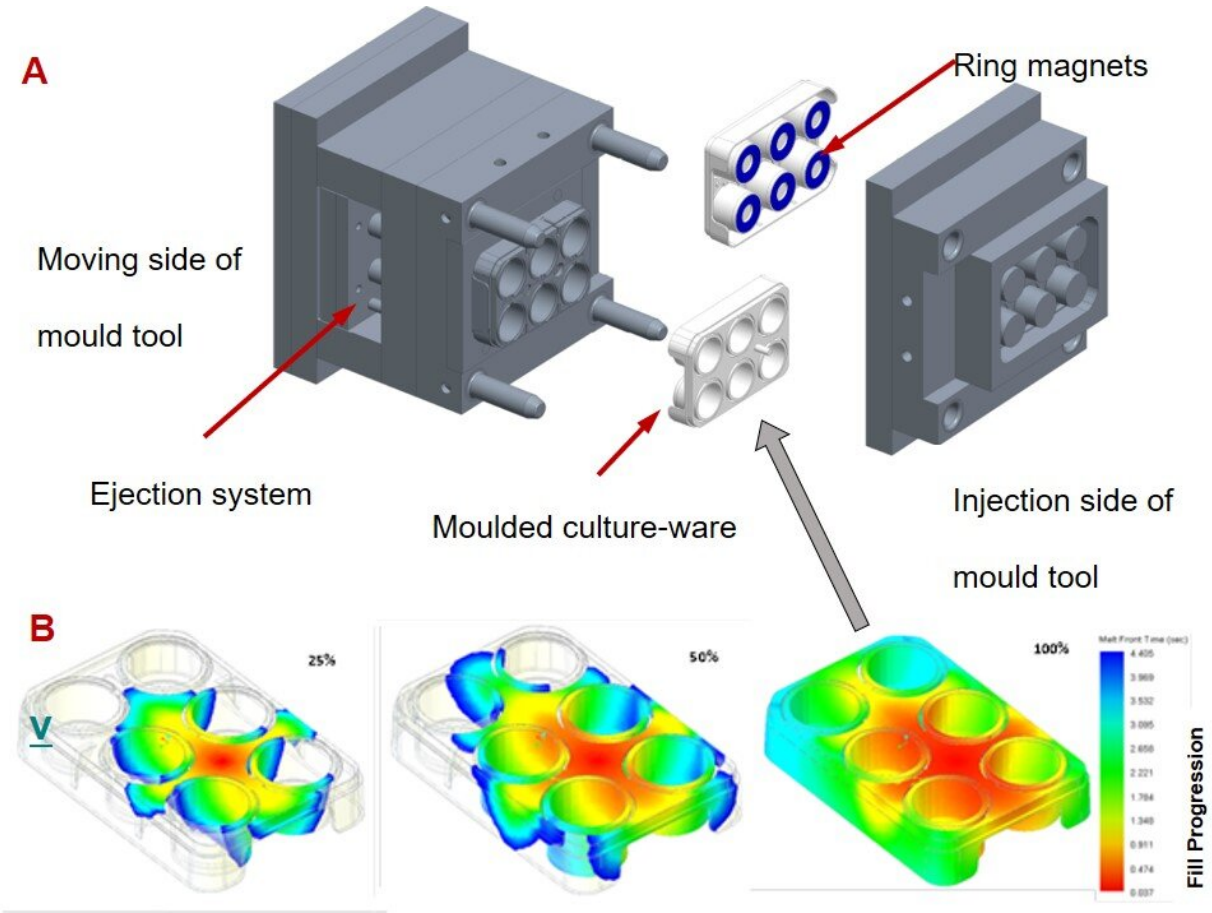


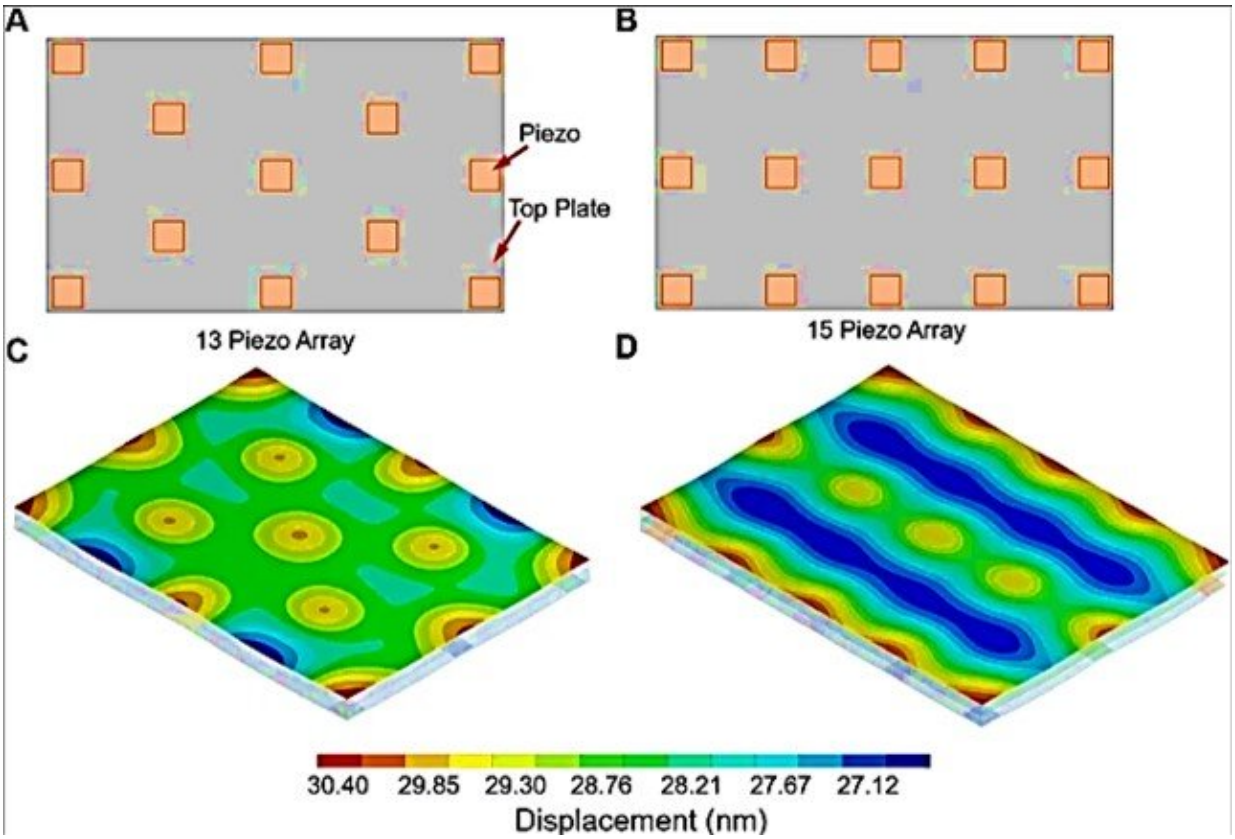
Injection mold tool design and mold fill analysis using simulations prior to manufacture (A) An exploded view of the mold tool with culture plate is shown to illustrate the major components of the mold interface and ejection system. (B) shows the mold fill analysis which estimates that the part cavity in the tool

should take 3.65 seconds to completely fill giving a defect free part. Credit: Scientific Reports, doi: 10.1038/s41598-019-49422-4

In regenerative medicine, scientists aim to significantly advance techniques that can control stem cell lineage commitment. For example, mechanical stimulation of mesenchymal stem cells (MSCs) at the nanoscale can activate mechanotransduction pathways to stimulate osteogenesis (bone development) in 2-D and 3-D culture. Such work can revolutionize bone graft procedures by creating graft material from autologous or allogenic sources of MSCs without chemically inducing the phenomenon. Due to increasing biomedical interest in such mechanical stimulation of cells for clinical use, both researchers and clinicians require a scalable bioreactor system to provide consistently reproducible results. In a new study now published on *Scientific Reports*, Paul Campsie and a team of multidisciplinary researchers at the departments of biomedical engineering, computing, physics, and molecular, cell and systems biology engineered a new bioreactor system to meet the existing requirements.

The new instrument contained a vibration plate for bioreactions, calibrated and optimized for nanometer vibrations at 1 kHz, a power supply unit to generate a 30 nm vibration amplitude and custom six-well cultureware for cell growth. The cultureware contained magnetic inserts to attach to the bioreactor's magnetic vibration plate. They assessed osteogenic protein expression to confirm the differentiation of MSCs after initial biological experiments within the system. Campsie et al. conducted atomic force microscopy (AFM) of the 3-D gel constructs to verify that strain hardening of the gel did not occur during vibrational stimulation. The results confirmed cell differentiation to be the result of nano-vibrational stimulations provided by the bioreactor alone.

The increasing incidence of skeletal injuries due to age-related conditions such as osteoporosis and osteoarthritis is a metric of the depleting quality of human life. The development of treatments for increased bone density or fracture healing are prime targets for the regenerative potential of mesenchymal stem cells (MSCs). Researchers have demonstrated controlled osteogenesis (development of bones) of MSCs via mechanical stimulation using several methods, including passive and active strategies. Passive methods typically alter the substrate topography to influence the cell adhesion profile, while active methods include exposure to varied forces from external sources.

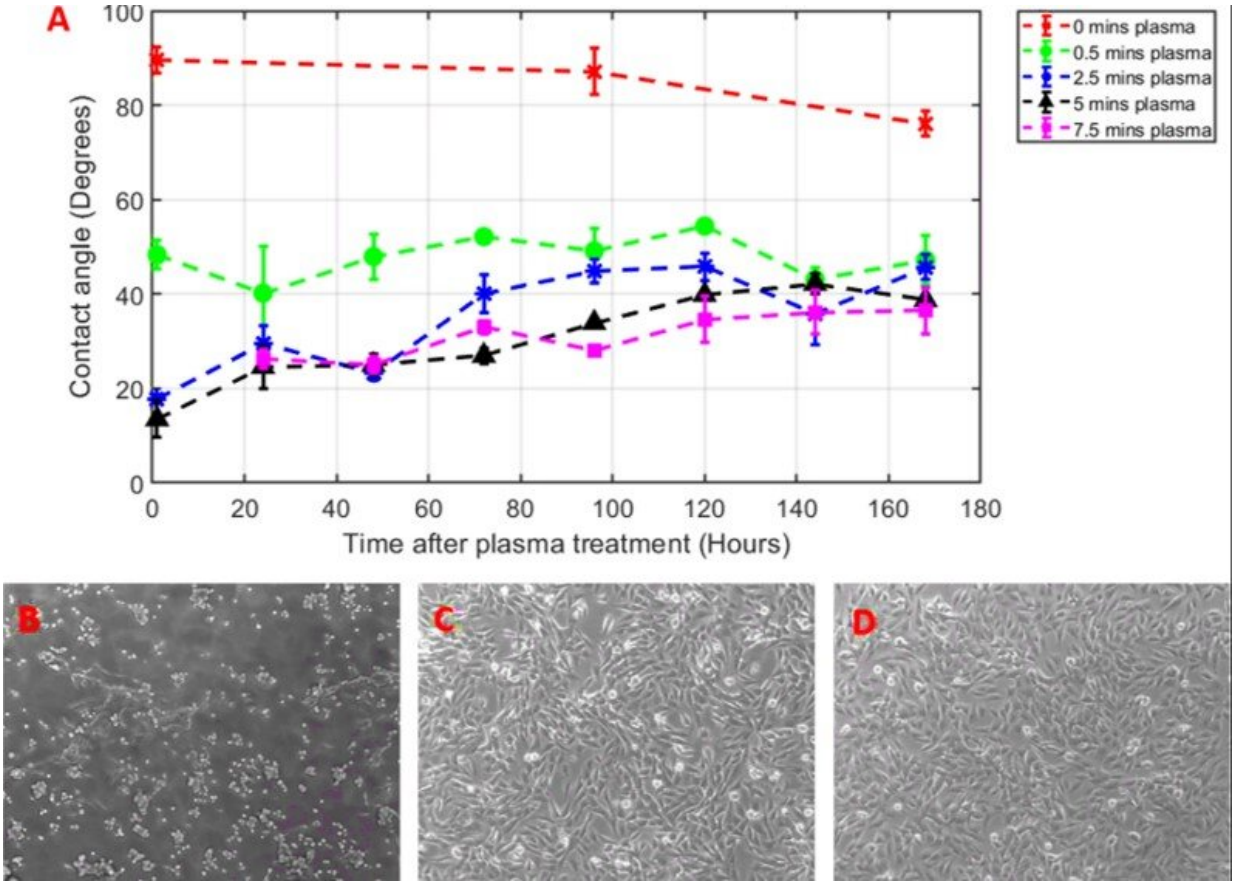


FEA analysis was performed in ANSYS workbench 17.1 to determine the harmonic response at 1 kHz on the thirteen and fifteen piezo array top plate arrangement. (A) Diagram of thirteen piezo array. (B) Diagram of fifteen piezo array. (C) Predicted nanoscale displacement of thirteen piezo array at 1 kHz. (D)

Predicted nanoscale displacement of fifteen piezo array at 1 kHz. Credit: Scientific Reports, doi: 10.1038/s41598-019-49422-4.

The present work by Campsie et al. intend to progress on pre-existing designs for the controlled osteogenesis of MSCs to construct a Good Manufacturing Practice (GMP) compatible system applicable for small scale clinical trials. Upon construction, the team used laser interferometry to accurately measure vibration displacement from the bioreactor's top plate and within the wells used for cultureware to validate the equipment they developed based on finite elemental analysis (FEA) models. The team used a direct digital synthesis waveform (DDS) generator and a reconstruction filter to remove high frequency components of the DDS output so as to generate a pure sine wave output of 1 kHZ for precise nanovibrations.

The research team validated the operation of the bioreactor system by performing biological experiments to quantify the osteogenic protein expression of MSCs exposed to nano-vibrational stimulation. They conducted AFM measurements on the collagen gel used in the experiments to determine that vibrations transmitted from the cultureware into the gel. Then they showed that the stiffness of the gel did not significantly increase in response to the nanovibrations that occurred.

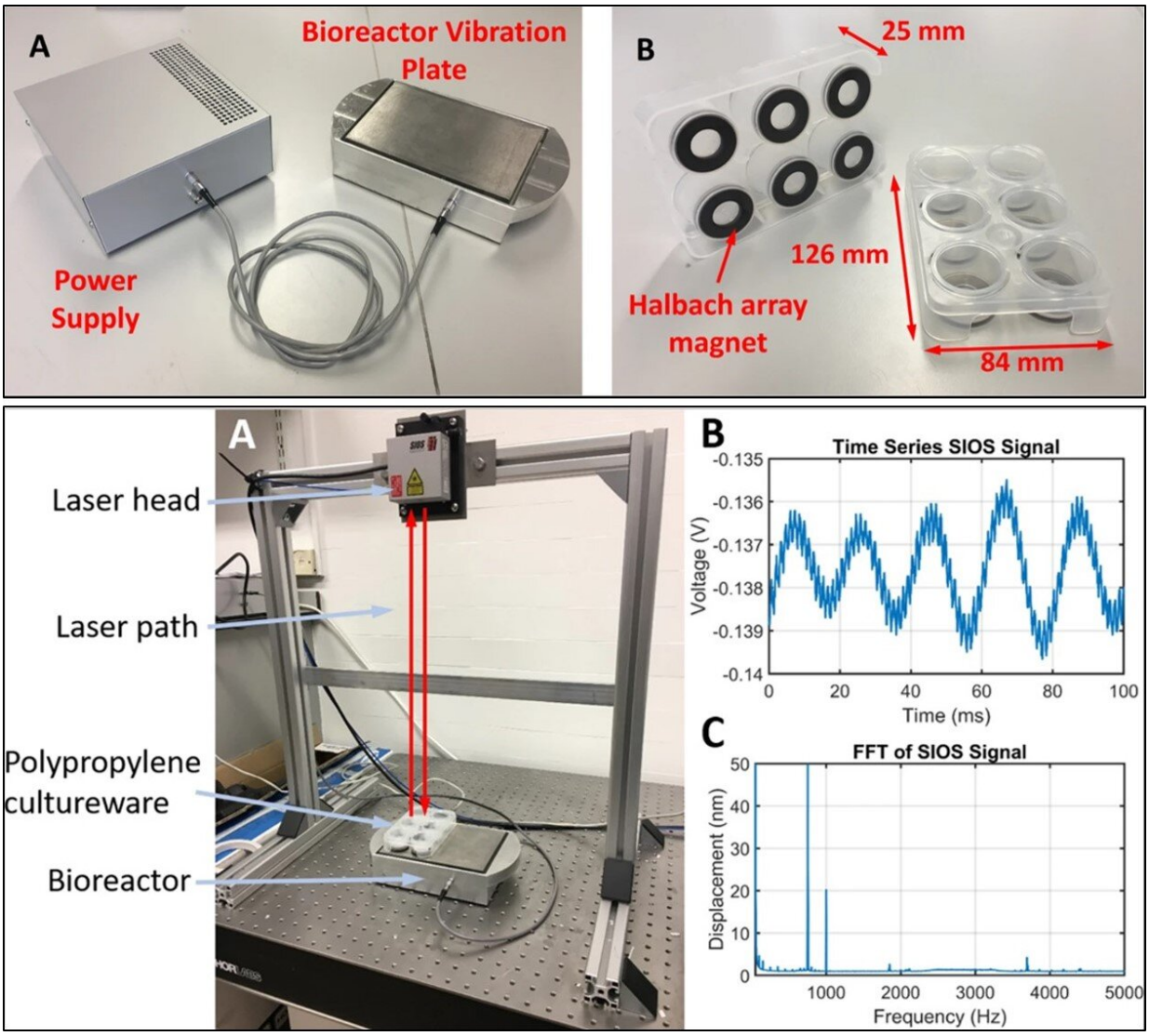


Water contact angle measurements of PP cultureware after different doses of plasma treatment and microscopy images of MG63 cells (osteogenic cells) on PP and polystyrene (PS) 6-well plates. A plot of WCA measurements post plasma treatment (A) shows that at least 30 seconds is required to significantly alter the WCA to a level that would allow cells to adhere and proliferate. Images of (B) non-adherence of MG63 cells on the PP 6-well plate prior to plasma treatment, (C) adhesion and proliferation of MG63 cells on plasma treated PP 6 well plate, and (D) MG63 cells cultured on a standard Corning PS 6-well plate. Credit: Scientific Reports, doi: 10.1038/s41598-019-49422-4.

Campsie et al. constructed the bioreactor with specific material choices and cultureware attachment to deliver optimal nanoscale vibrations between the frequencies of 1 Hz and 5 kHz. They ensured the resonant

frequency of the apparatus to be well above the frequency of operation to prevent resonant amplification or damping. To determine the appropriate dimensions of the device, the research team performed FEA using ANSYS Workbench software. The scientists created the bioreactors inexpensively by using 13 to 15 piezo arrays for its construction. The product design allowed distinct alternating bands of minimum and maximum displacement for cells to receive inconsistent levels of vibrations across the cultureware. The team estimated the intrinsic resonance frequency of the piezoactuators and other device components to understand their impact on the experimental setup.

The research team then modified the surface chemistry of the plastic cultureware to aid cell adhesion and proliferation using plasma surface activation to increase the surface energy of the polymer. After five minutes of air-based plasma treatment, they cultured human osteoblast-like cells to observe increased cell attachment to the cultureware. They measured the water contact angle of the polymer to determine the surface energy of the modification and surface wettability. The scientists demonstrated proof-of-principle on plasma activation of polymer cultureware and its impact on surface wettability for favorable cell attachment. They aimed to further develop cultureware surfaces similarly to ensure their stability and shelf life.



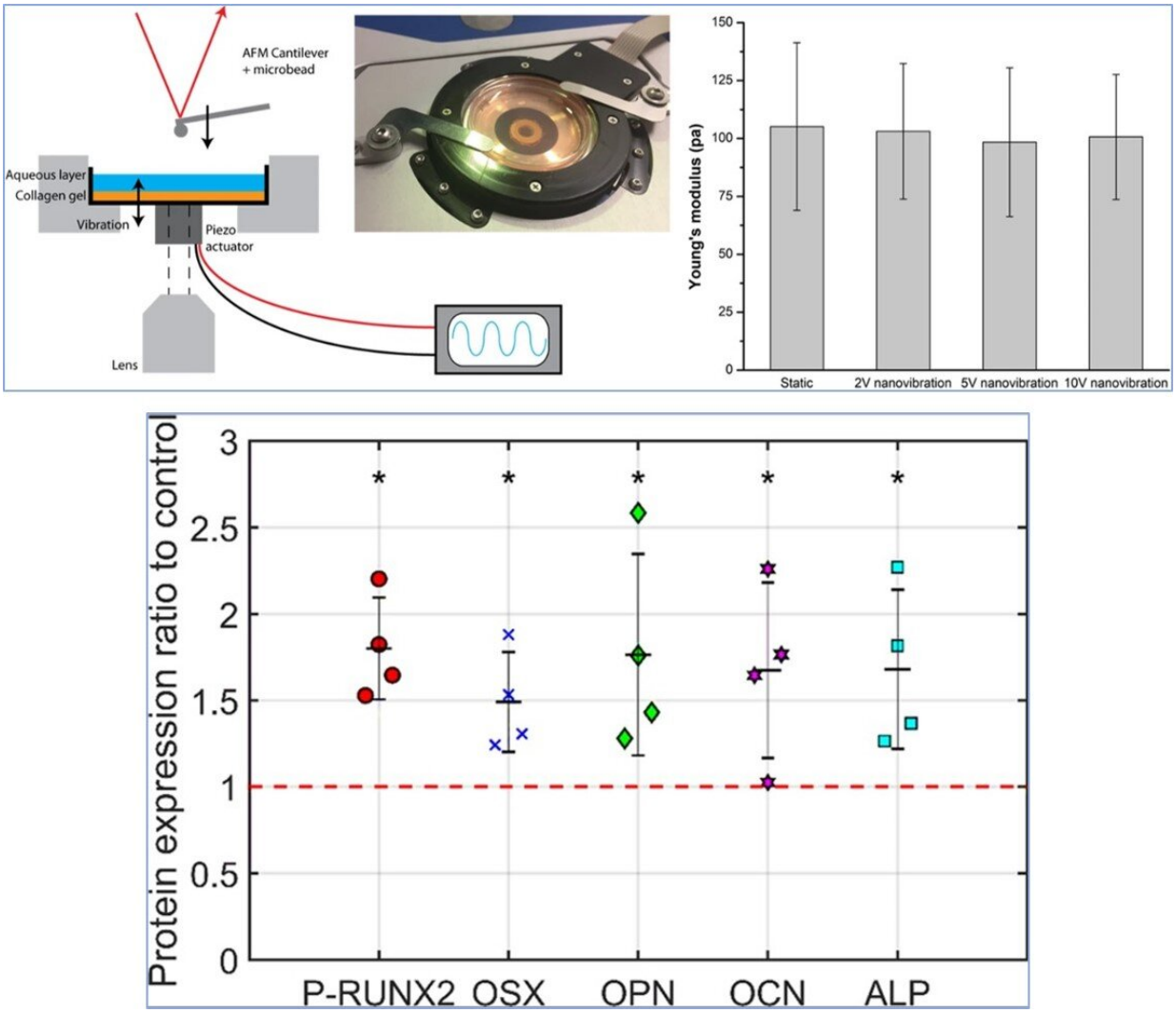
TOP: Bioreactor vibration plate with injection molded PP 6-well cultureware. (A) The improved version of the bioreactor has a lighter base, carrying handles and a recessed top plate, along with a power supply designed to output a sine wave of 1 kHz and 30 nm displacement amplitude. (B) Injection molded PP cultureware with incorporated halbach ferrite ring magnets in the base of each well. The thickness of the frame and walls of the wells is 1.5 mm. BOTTOM: Interferometer measurement setup and output signal. (A) To measure nanoscale displacements the interferometer emits a laser beam from the laser head which is reflected back to the photodetector (also within the laser head) off the object being measured. Analysis of the optical interference pattern produced allows the displacement to be obtained. (B) Example of the time series data measured by

the interferometer. (C) Example of an FFT analysis on the time series data. The 1 kHz peak of the bioreactor is clearly seen and there is also a large peak at 750 Hz, however, this signal is produced by the reference mirror of the interferometer which is constantly excited at a fixed frequency in order to obtain the control signals. Credit: Scientific Reports, doi: 10.1038/s41598-019-49422-4.

The research team significantly improved the design of the bioreactor in the present work to form a lighter base compared to the prototype they previously presented. They used an AD9833 power waveform generator for power supply with easy tuning and maintained appropriate filtering to derive a pure 1 kHz sine wave drive signal. The researchers obtained a power spectrum of the pre- and post-filtered signal to estimate the power spectral density of the generator. They verified the FEA modeling and calibration of the bioreactor using a laser interferometer to determine nanoscale changes in displacement. The scientists used prismatic reflective tape bonded to the bottom surface of each well to measure the cultureware well dimensions that were magnetically attached to the bioreactor.

This technology has huge scope to generate a 3-D mineralized matrix from MSCs seeded in a collagen gel to form bone scaffolds. For example, cultured cells received a periodic acceleration force during vibration, which acted on the cell membrane and cytoskeleton to induce osteogenesis. The effect could also be related to environmental stiffness within the cell culture media, impacting stem cell differentiation and inducing osteogenesis in MSCs instead. To differentiate the cause, Campsie et al. used AFM to detect any change in stiffness while they nanovibrated the collagen gel. They did not observe significant effects of strain hardening within the gel and the Young's modulus maintained values of soft collagen gels; thus attributing cell differentiation to

nanovibration alone.



TOP: AFM measurement of collagen gel during nanovibration. (A) A vibrating piezo actuator was attached to a Petri dish containing collagen gel with an AFM cantilever being used to measure any changes in gel stiffness during vibration. (B) Young's modulus was assessed via AFM for collagen samples which were nanovibrated at three different piezo amplitudes. Data are mean ± SD (n > 30). BOTTOM: Protein expression at three weeks of nanovibrational stimulation. Cells were stimulated for three weeks at a frequency of 1 kHz and 30 nm displacement alongside a non-stimulated control. Protein expression for RUNX2, OSX, OPN, OCN and ALP, was measured relative to static controls and

quantified using the LI-COR Odyssey system (LI-COR, Nebraska, USA). Statistically significantly higher expression was observed in the mechanically stimulated samples when compared to the non-stimulated control (indicated by red dashed line). Data are mean ± standard deviation, n = 4, stats Mann Whitney U-test *p