Supplementary Information for

Long-term and label-free monitoring for osteogenic differentiation of mesenchymal stem cells using force sensor and impedance measurement

Zhizhong Zhang[†], Tianyang Zheng[†], Rong Zhu*

State Key Laboratory of Precision Measurement Technology and Instruments, Department of Precision Instrument, Tsinghua University, Beijing, China

** Corresponding author:*

Postal address: Department of Precision Instrument, Tsinghua University, Beijing, China, 100084

Tel: +86-010-62788935

E-mail: zr_gloria@mail.tsinghua.edu.cn

[†] *The authors contributed equally to this work.*

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Note 1. Growth of ZnO nanorods on microelectrodes

ZnO nanorods were grown using a wet chemical method with the assistance of an applied electric-field. The whole microchip was immerged in an equal molar aqueous solution (0.015 M) of $Zn(NO_3)_2 \cdot 6H_2O$ and HMTA ($C_6H_{12}N_4$) heating at 75 °C. An AC signal (0.5–1 MHz, 3.6–4.5 V_{pp}) generated by a Function Generator (Tektronix, AFG 3252) was applied on the anode, the cathode was connected to the ground. The gate electrode was connected to a DC voltage of 40 mV (generated by a DC power supply DH1715 A-3). After about 5 hours of the growth process, the chip was picked up from the solution, rinsed with deionized water using ultrasonic cleaning and dried in air.

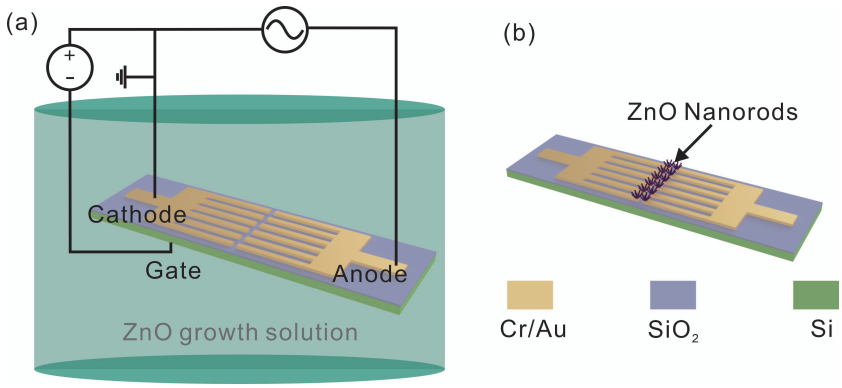

Figure S1. (a) Schematic view of the growth of ZnO nanorods. The two top electrodes are defined as the cathode and anode. The bottom doped silicon wafer with low resistivity (0.008–0.02 Ω cm) is defined as the gate. (b) Schematic view of ZnO nanorods growing on and between the cathode and anode.

Note 2. Long-term stability of the nano CTF sensor

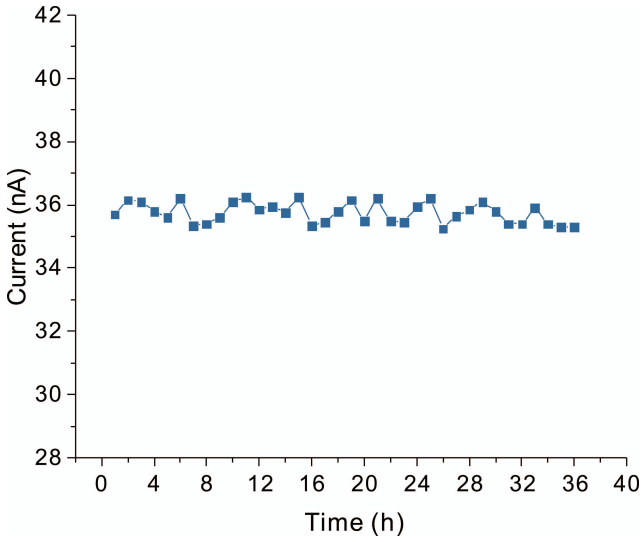

Figure S2. Long-term stability test of the nano CTF sensor for 36 h. The sensor was immersed in osteogenic medium and tested in the cell culture environment at 37°C. The standard deviation was less than 4.5%.

Note 3. The staining protocols

Cell viability assay

Cell viability test and the morphology observation were performed with Calcein-AM (Dojindo, Japan) dye (excitation=450-490 nm, emission maximum=515 nm). Green fluoresce could be emitted when the cell was viable. After washing the MSCs with phosphate buffered saline (PBS), 2 μmol/L Calcein-AM were added into the culture medium and the cells were incubated at 37 °C for 15 minutes. Fluorescence microscope (DM2500, Leica, Germany) was used for the observation.

Alkaline phosphatase (ALP) activity

The culture medium was removed from the chip surface, and then the MSCs were gently washed twice with PBS. MSCs were fixed with 4% paraformaldehyde (Beyotime, China) for 20 minutes. Then remove 4% paraformaldehyde and gently washed MSCs three times with PBS. The fixed MSCs were incubated in ALP assay solution (Beyotime, China) for 10 minutes at room temperature in the dark. Finally, the staining solution was removed from the chip surface and the MSCs were gently washed at least 4 times with DI water. The staining results were observed using a microscope and analyzed with an open source software ImageJ.

Alizarin red staining characterization

Absolute ethanol was employed to fix the MSCs at room temperature for 30 minutes after washing with PBS and then removed the ethanol from the chip surface. Afterwards, waiting for around 10 minutes until chip surface was completely evaporated, Alizarin Red S Working Solution (0.1% wt, Sigma-Aldrich, Germany) was applied to stain the fixed MSCs for 20 minutes. Finally, the MSCs were gently washed with DI water.

Immunocytochemistry assay

For immunostaining, paraformaldehyde (PFA, 4%, Beyotime, China) wa**s firstly used to fix** MSCs for 20 minutes. Then, the cells were permeabilized for 15 minutes

with 0.2% Triton X-100 (Sigma-Aldrich, Germany**) solvated** in PBS. In order to block non-specific staining of antibodies, cells were incubated for 2 hours at room temperature in blocking buffer (PBS+0.2% Triton X-100+3% goat serum (Gibco, USA)**)**. Afterwards, primary antibody anti-osteopontin (Abcam, USA) solved in blocking buffer (1:200) was applied for incubating cells at 4 °C overnight. Then an appropriate diluted (1:100) FITC-conjugated secondary antibody (Abcam, USA) was employed at room temperature for 2 hours in the dark to mark the cells with fluorescent dyeing.

Note 4. Impedance measurement on ECIS microelectrode array chip

As **Figure S4**(a) illustrated, the measuring system was connected to the electrochemical workstation (PARSTAT 4000), in which AC signals with a peak to peak voltage of 20 mV_{pp} and the frequencies from 1 kHz to 1 MHz were applied to the counter electrodes while the output signals were read out from the sensing electrodes.

After adding MSCs suspension into the sample pool, a 24-hour real-time measurement of the MSCs impedance spectrum was carried out at 37 °C under a 5% CO_2 atmosphere in the cell culture incubator. **Figure S4**(b) showed the measured impedance spectra. The impedance magnitude at 50 kHz was shown in **Figure S4**(c) and exhibited an increasing tendency over time. In the first 5 hours, the impedance increased rapidly correlating to the cell attaching onto the substrate. Afterwards, the impedance signal continued to increase with a lower speed due to the cell spreading and proliferation. The inset image indicated the coverage ratio of live MSCs at 8 hours was around 40%. At 24 hours, the impedance signal tended to be stable corresponding to full coverage of cells on the microelectrodes. The coverage ratio of MSCs was raised up to nearly 90% at 24 hours after seeding, as the inset fluorescent image of Figure 3 (c) shown. The impedance results were consistent with the results of the nano CTF sensor.

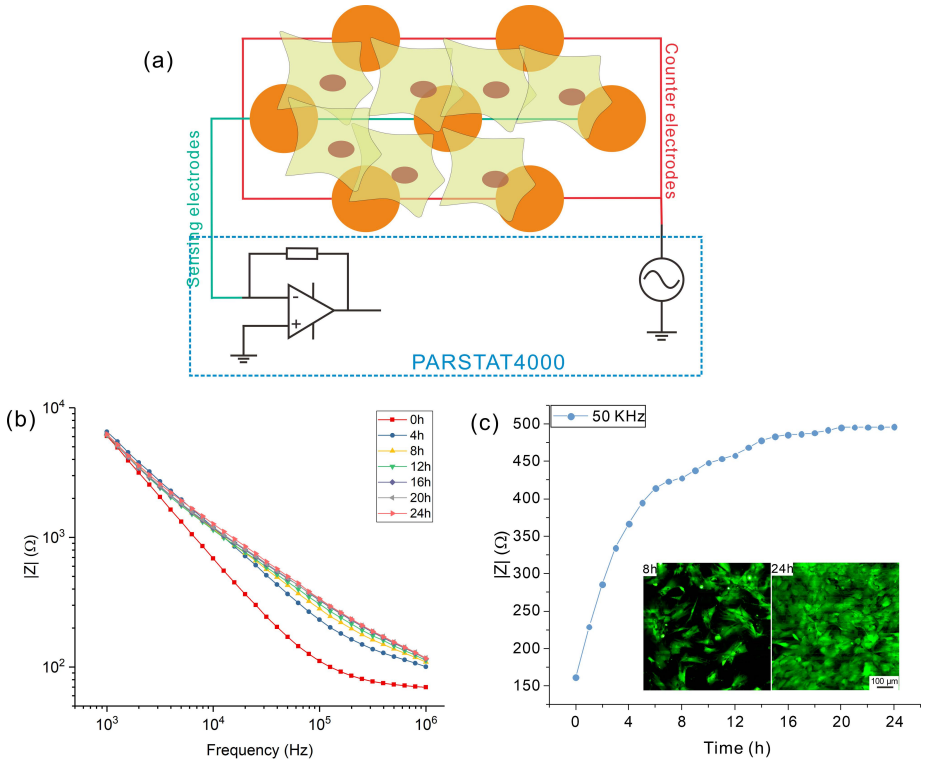

Figure S3. (a) Schematic of impedance measurement with ECIS microelectrode array chip. The counter and sensing electrodes were connected to AC signal and an amplifier respectively using PARSTAT4000 electrochemical work station. (b) Impedance measurement during the MSCs attachment and spreading. (c) Real-time impedance tracing for cellular behaviors of MSCs from initial seeding to cell adherence. The insets were the Calcein-AM fluorescent images taken at 8 hours and 24 hours respectively. The staining protocol was shown in **Supplementary Note 3**.

Note 5. Control measurements for non-induced MSCs in proliferation

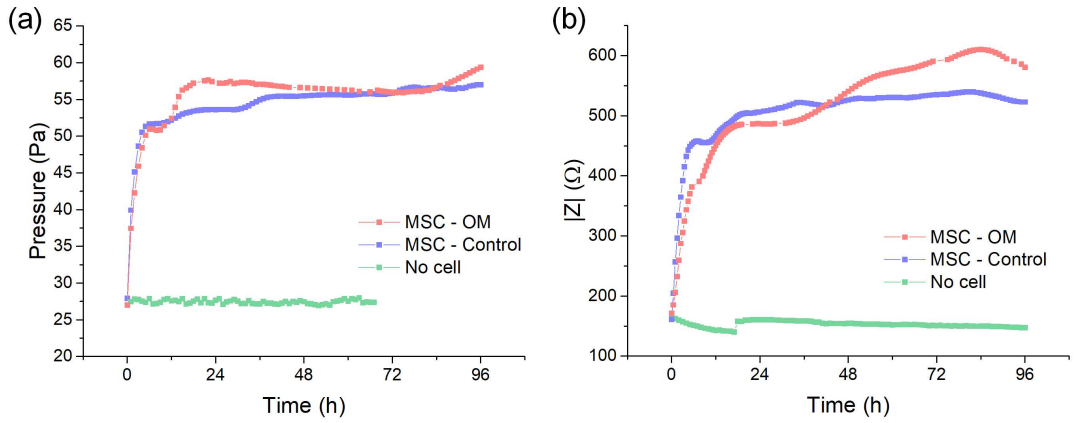

Figure S4. The (a) cell traction force and (b) ECIS monitoring results for the MSCs cultured with osteogenic medium (OM), normal medium (Control) and without cell (No cell).

Note 6. The processing method of phase contrast image

Using a phase contract microscope, the cell profiles and cell aggregations could be transferred to the different sensitivity of the light in the images. As a result, the cell coverage areas as well as aggregation areas could be extracted from these images by using image processing. The aggregation ratio was defined as the ratio between the aggregation areas and the coverage areas.

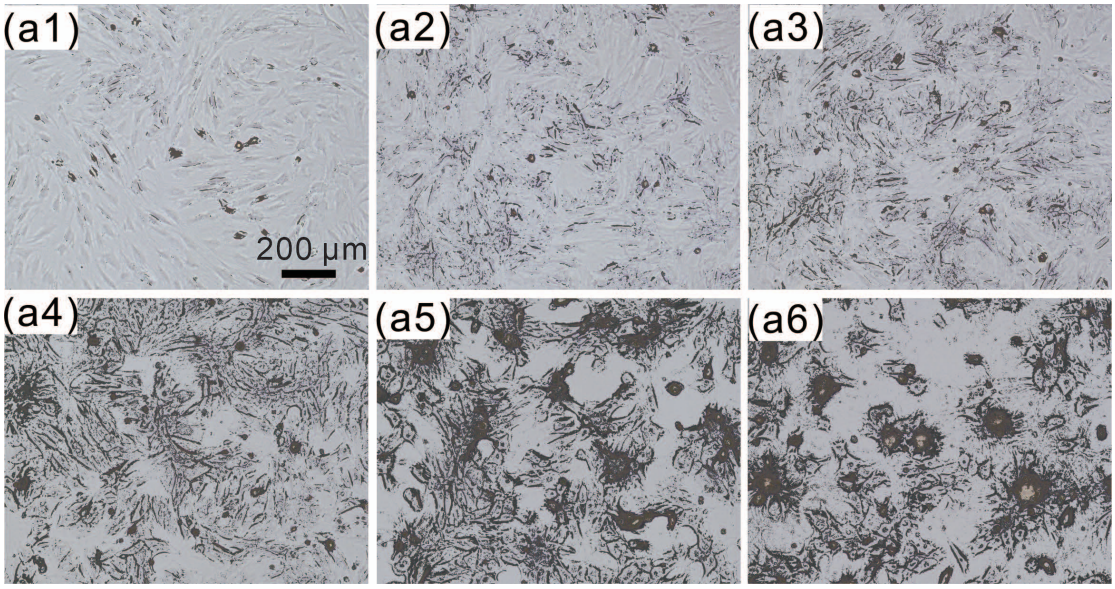

Figure S5. Image processing using an open source software ImageJ to extract the cell coverage areas and the aggregated cell areas shown in the phase contrat images of **Figure 3** (a1-a6). The areas were extracted via thresholding method to transfer the RGB images to grayscale maps. Set an appropriate threshold, the aggregated cell areas was transferred to black and leave out the background areas as white. With the same approach, the cell coverage areas in the phase contract images were also extracted.

Note 7. MSCs ALP staining

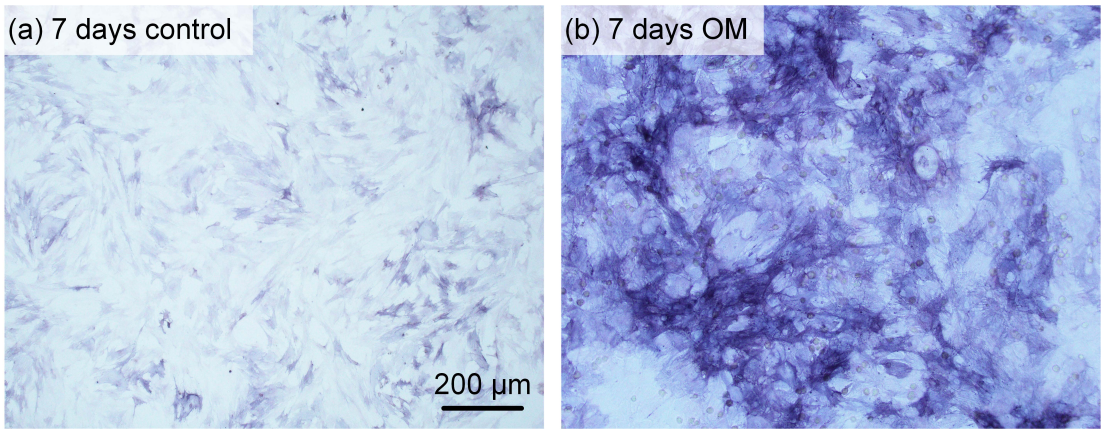

Figure S6. ALP staining results on day 7 in the cases of (a) control culture and (b) OM culture. The staining protocol was shown in **Supplementary Note 3**.

Note 8. Immunocytochemistry assay of osteopontin

The expression of osteopontin, an osteogenic marker was evaluated by immunocytochemistry at the end of day 14 of culture with different induced conditions (OM and control).

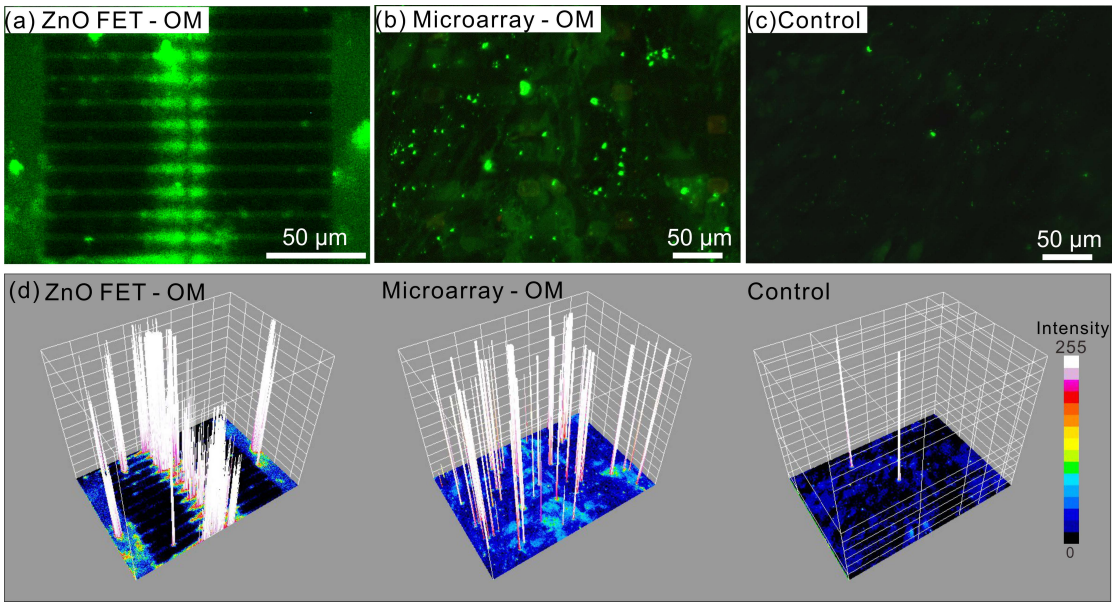

Figure S7. Immunofluorescence images of osteopontin for MSCs on the nano CTF sensor (a) and ECIS microelectrode array chip (b) at day 14. The immunopositive expressions of osteopontin were observed. By the contrast, few of cells was positively expressed for osteopontin in the control group (c). (d) Quantitative analyses to the intensities of green fluorescence shown in (a-c).

Note 9. MSCs alizarin red staining

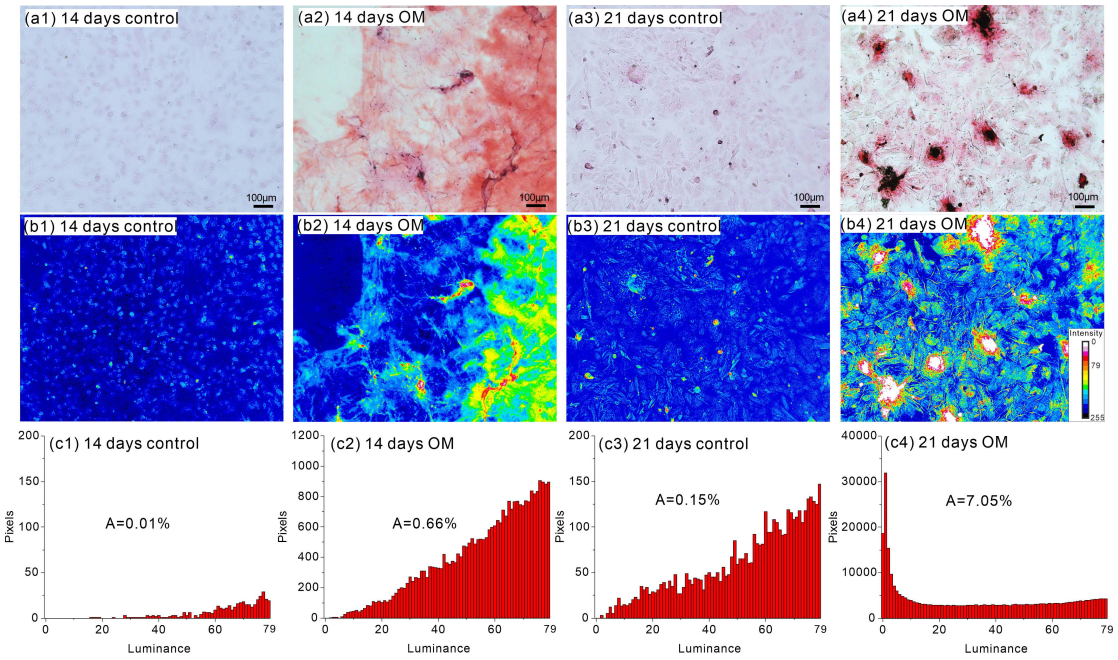

Figure S8. (a1-a4) Alizarin red staining results on Day 14 and Day 21 in the cases of control culture and OM culture. The staining protocol was shown in **Supplementary Note 3**. (b1-b4) Image processing for staining intensity analysis with thresholding method. The images in (a1-a4) were firstly transfered to greyscale images and normalized. Dark areas with low luminance represented the stained areas. In order to exhibit the staining intensity, luminance maps were transferred from these greyscale images and the results were shown in (b1-b4). The color gradient respresented the different luminaces with diverse staining intensity. The luminace lower than the threshold of 79 indicated stained nodual structures. The pixels with different luminace were transfered to histograms (c1-c4). The variable A represented the stained area proportion.

Note 10. Long-term stability evaluation for the nano CTF sensor

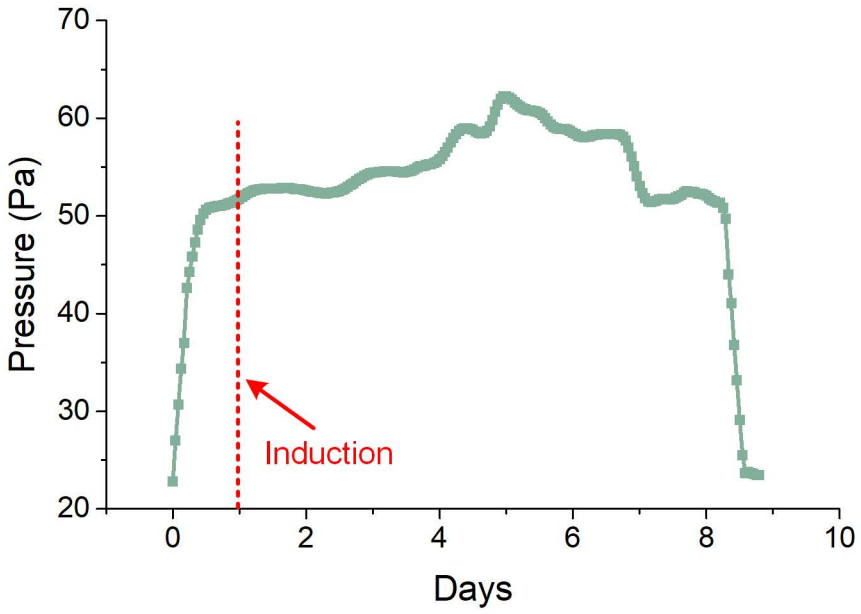

Figure S9. Long-term stability evaluation for the nano CTF sensor. At the beginning, MSCs were seeded on the CTF sensor. After 9 days, the MSCs migrated and deviated from the CTF sensor, and the force signal dropped down. The force signal detected by the nano CTF sensor at the beginning (day 0) and the end (day 9) were almost same, which indicated a good long-term stability of the CTF sensor.