

Electronic Supplementary Information for

Multi-target Cell Therapy Using a Magnetolectric Microscale Biorobot for Targeted Delivery and Selective Differentiation of SH-SY5Y Cells via Magnetically Driven Cell Stamping

Hyunseok Song,^{a,b} Dong-in Kim,^{a,b} Sarmad Ahmad Abbasi,^{a,b} Nader Latifi Gharamaleki,^{a,b}

Eunhee Kim,^c Chaewon Jin,^d Samhwan Kim,^e Junsun Hwang,^{a,b} Jin-Young Kim,^{b,c} Xiang-Zhong

*Chen,^f Bradley J. Nelson,^f Salvador Pané, *^f and Hongsoo Choi*^{a,b,g}*

^a Department of Robotics and Mechatronics Engineering, Daegu Gyeong-buk Institute of Science and Technology (DGIST), Daegu, Republic of Korea

^b DGIST-ETH Microrobotics Research Center, DGIST, Daegu, Republic of Korea

^c IMsystem Co., Ltd., Daegu, Republic of Korea

^d Department of Division of Biotechnology, DGIST, Daegu, Republic of Korea

^e Brain Engineering Convergence Research Center, DGIST, Daegu, South Korea

^f Institute of Robotics and Intelligent Systems, ETH Zurich, Zurich, CH-8092 Switzerland

^g Robotics Research Center, DGIST, Daegu, Republic of Korea

Corresponding Author:

E-mail: mems@dgist.ac.kr (Hongsoo Choi), vidalp@ethz.ch (Salvador Pané)

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Figure S9. ME stimulation over 6 days; application of the AMF (30 mT, 30 Hz) for 30 min/ day with medium changes every 2 days.

Figure S10. The experimental set-up for ME stimulation (generated by the AMF). The system features an electromagnetic coil, a microcontroller, a coil driver, and a power supply.

Figure S11. (a) Distribution of MENPs and cells released from the SMMBs on ME stimulation, as revealed using an optical microscope (Leica, DMIL LED). The SMMBs are in contact with cells over the entire area wherein the cells are spread, including (b) inside the SMMB, (c) on cells near the SMMB, and (d) at the edge of the cell-spreading area. The SMMBs are marked with red arrows.

Supplementary Videos

Video S1. Manipulation of the stamping magnetoelectric microrobot (SMMB) under a rotating magnetic field (RMF).

Abbreviations

AMF: Alternating magnetic field

ATP: Adenosine triphosphate

BTO: Barium titanate (BaTiO₃)

CFO: Cobalt ferrite (CoFe₂O₄)

CNS: Central nervous system

EDX: Energy-dispersive X-ray

ME: Magnetolectric, Magnetolectricity

MENPs: Magnetolectric nanoparticles

NGF: Nerve growth factor

NSCs: Neuronal stem cells

PBS: Phosphate-buffered saline

PFM: Piezoresponse force microscopy

RMF: Rotating magnetic field

SEM: Scanning electron microscopy

SMMB: Stamping magnetolectric microscale biorobot

TEM: Transmission electron microscopy

VSM: Vibrating sample magnetometry

XRD: X-ray diffraction

METHODS/EXPERIMENTAL

Fabrication of core-shell CoFe₂O₄@BaTiO₃ MENPs. Core-shell CoFe₂O₄@BaTiO₃ MENPs were prepared as follows: Cobalt ferrite (CFO) nanoparticles (30 nm, catalog no. US3843; US Research Nanomaterials, USA) served as the magnetostrictive cores. A sol-gel technique was used to fabricate barium titanate (BTO) shells over CFO cores. The BTO precursor solution (with Ba and Ti ions) was prepared by mixing 30 mL of aqueous solution (DI) containing 0.029 g of barium carbonate (BaCO₃) and 0.1 g of citric acid with 30 mL of an ethanolic solution containing 1 g of citric acid and 0.048 mL of titanium isopropoxide (Ti(OCH(CH₃)₂)₄). The mixture was stirred at 90°C for 1 h to obtain complete sol. CFO nanoparticles (0.1 g) were dispersed in the sol (via sonication for 2 h) to form a gel, which was dried at 120°C overnight and then calcinated at 780°C for 5 h in a furnace to obtain CoFe₂O₄@BaTiO₃ nanoparticles.

Characterization of the core-shell CoFe₂O₄@BaTiO₃ MENPs. The surface morphology of the core-shell nanostructure was explored via field-emission transmission electron microscopy (FE-TEM HF-3300, Hitachi, Japan). The MENPs were mixed with ethanol and sonicated for 30 min to minimize aggregation. The suspension was drop-casted onto carbon-coated copper grids and dried. Elemental mapping were obtained by means of an energy-dispersive X-ray (EDX) analyzer coupled to the FE-TEM. The phase composition was confirmed by measuring the crystalline structure using an X-ray diffractometer (XRD Empyrean; Malvern Panalytical, USA) with a Cu radiation source ($\lambda = 1.542 \text{ \AA}$). The range of the diffraction scan (2θ) was varied between 20° and 70° at 2° min⁻¹ and a step size of 0.01°. Ferromagnetic hysteresis curves (M-H loops) for pure CFO nanoparticles and MENPs were obtained using a vibrating sample magnetometer (VSM 7407, Lake Shore Cryotronics, USA) as functions of the applied magnetic field up to 10 kOe. PFM measurements were performed using an atomic force microscope (AFM; NX10; Parksystem,

USA) equipped with a lock-in amplifier (SR-83; Zurich Instruments, Switzerland). AC and DC bias voltages were applied (using the contact mode) employing a Cr/Pt-coated Si probe (ElectriMulti75-G with a spring constant $k \sim 3 \text{ Nm}^{-1}$); this ensured careful control in the local contact force mode. The DC bias voltage wave (from -10 V to 10 V) was overlapped by an AC bias voltage of 0.5 V (delivered through the conductive probe contact tip) to measure the phase and amplitude response in the PFM mode. An external magnetic field of $1,000 \text{ Oe}$ (delivered by a permanent magnet under the sample holder) was applied to investigate ME coupling.

SH-SY5Y neuroblastoma cell culture. SH-SY5Y human neuroblastoma cells were grown as monolayers at 37°C in a humidified incubator under 5% (v/v) CO_2 . The growth medium was prepared by adding 10% (v/v) fetal bovine serum (FBS, Gibco Life Technologies, USA) and 1% (v/v) penicillin/streptomycin (P/S; Gibco Life Technologies, USA) to Dulbecco's minimum essential medium/nutrient mixture F-12 (DMEM/F-12; Gibco Life Technologies, USA). SH-SY5Y cells were seeded at $1.5 \times 10^4 \text{ cells cm}^{-1}$ and the medium changed every 2 days.

Fabrication of SMMBs. SH-SY5Y cells were dissociated by trypsinization (3 mL of 0.25% (v/v) trypsin-EDTA; Gibco Life Technologies) for 2 min . Trypsinization was stopped by the addition of 7 mL of culture medium. The cell suspension was centrifuged at 754 rpm for 5 min , and the supernatant was removed. The cell pellet was resuspended in 1 mL of medium, and a stock of $1 \times 10^6 \text{ cells mL}^{-1}$ was prepared after the cells were counted (Countes II automated cell counter; Invitrogen, USA). An MENP stock (1 mg mL^{-1} in culture medium) was also prepared. SH-SY5Y cell stock solution (1 mL amounts) was mixed with $0, 500, 1,000, 1,500, 2,000,$ and $2,500 \text{ ng}$ of MENPs to yield 10 mL stock solutions of SMMBs with different concentrations of MENPs. The suspensions ($100 \text{ }\mu\text{L}$ amounts) were seeded into (non-adhesive) U-bottomed 96-well plates

(~10,000 cells/spheroid) and incubated at 37°C under 5% (v/v) CO₂ for 4 days to generate SMMBs. Given the well U-profile, one SMMB formed in each well.

Cell viability (live/dead staining). The viability of the SH-SY5Y cells with MENPs in the SMMBs was evaluated using a Live/Dead Cell Imaging Kit (488/570) (Invitrogen, USA). The Live Green component was combined with the Dead Red component, and the solution was added to wells containing equal volumes of SMMB and medium followed by incubation for 15 min at room temperature. Live/Dead fluorescence images were obtained using a confocal microscope (LSM 780; Carl Zeiss, Germany)

Manipulation of SMMBs. Magnetic SMMB manipulation was performed using an electromagnetic steering system (OctoMag; Aeon Scientific, Switzerland). We verified targeted cell delivery and determined the maximum achievable velocities and step-out frequencies (Figure 3b and Figure S6). Eight electromagnetic coils in a hemispherical shape generated a three-dimensional magnetic field with five degrees of freedom (DOFs; three DOFs for positional control and two DOFs for orientation control).^{1,2} To visualize SMMB locomotion, each SMMB was placed in a conventional round Petri dish containing PBS. The SMMB was driven by an RMF of 15 mT at 5 Hz to verify targeted delivery, and at 5-20 mT and 1-39 Hz to determine the maximum velocities and step-out frequencies as the MENP concentrations varied. An optical microscope (acA1300-30uc; Basler, Germany) (top view) was integrated with the electromagnetic coils to visualize motion and determine SMMB velocity evaluated by analyzing time-lapse videos.

Multi-targeting therapy via stamping. SH-SY5Y cells (10,000) with 10 μM CellTracker Green CMFDA (Invitrogen) in 100 μL were seeded into U-bottomed wells (with and without MENPs) for fluorescence imaging of live cells after the multi-targeting test ('stamping'; retention of SMMBs on targets for extended times). The SMMBs were placed in hydrophobic,

biocompatible cell culture dishes (μ -Dish 35 mm, low; Ibidi, Germany) that were filled with medium and transferred to 1st target by an RMF of 35 mT at 1 Hz. The SMMB remained on 1st target for 12 h, then moved to 2nd target and (after another 12 h), to 3rd target, followed by 3 days of cell proliferation. All cells on targeted areas were observed via automated digital microscopy (Lionheart LX Automated Microscope, BioTek, USA) and the amounts were quantified using the ATP assay.

ME stimulation and quantitative evaluation. A single-coil electromagnetic system was used to generate the AMF for ME stimulation (Figure S10). The system featured a power supply, a microcontroller, a coil driver, an electromagnetic coil, and control switches. The sample was placed under the coil, and an AMF of 30 mT at 30 Hz was applied for 30 min/day for 6 days (Figure S9); the medium was changed every 2 days. The cells stained during SMMB fabrication were subjected to morphological analysis and observed under the Lionheart LX Automated Microscope (to compare the live-cell areas). The cell numbers after 7 days of ME stimulation were measured using the ATP assay; the groups were -AMF/-MENPs, -AMF/+MENPs, +AMF/-MENPs, and +AMF/+MENPs (Figure 3). The cells were covered with a 1:1 mixture of the ATP luminescence reagent (CellTiter-Glo 3D Cell Viability Assay; Promega, USA) and medium, followed by 5 min of mixing and 25 min of incubation. The luminescence of each sample was then recorded using a monochromator (Synergy HTX Multi-Mode Reader; BioTek, USA).

Immunofluorescence staining and confocal imaging. SH-SY5Y cells from the SMMBs were stained at 7 days after ME stimulation for β -III tubulin marker (TUBB3; Abcam, UK) and with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, USA) to visualize differentiated cells (green) and nuclei (blue), respectively; we sought to confirm differentiation of SH-SY5Y neuroblastoma cells after ME stimulation. SH-SY5Y cells were washed three times with PBS after

removing the medium and the cells fixed in paraformaldehyde (PFA, 4% (v/v) in PBS; Biosesang, Korea) for 30 min at room temperature. The cells were rewashed three times in PBS and permeabilized with Triton X-100 (0.5% (v/v) in PBS; Biosesang, Korea) for 1 h at room temperature followed by three washes in PBS and incubation in blocking buffer (normal goat serum 10% (v/v) in PBS; Vector Laboratories, USA) for 1 h at room temperature. A primary antibody against β -III tubulin was diluted 1:50 in blocking buffer, and cells were added, followed by overnight incubation at 4°C. The samples were washed with 0.2% (v/v) Triton X-100 twice and PBS once, and then stained (for 2 h at room temperature) with the secondary anti-rabbit antibody (tagged with Alexa Fluor 488; Thermo Fisher Scientific, USA) diluted 1:500 in blocking buffer, followed by three washes in PBS. DAPI solution (1:1,000) was added, followed by incubation for 10 min at room temperature (counterstaining). The cells were washed with PBS. All immunostaining procedures were performed in the dark to avoid photobleaching. Fluorescence images were obtained using a confocal microscope (LSM 780; Carl Zeiss, Germany) with multi-argon lasers operating in several fluorescence channels (405, 488, 555, and 639 nm). The fluorescence images were post-processed (via maximum intensity projection) using ZEN software (Carl Zeiss) to clearly reveal the neurite morphology. The images were acquired through two channels; Green (Alexa Fluor 488) for β -III tubulin and blue (Hoechst 33342) for DAPI.

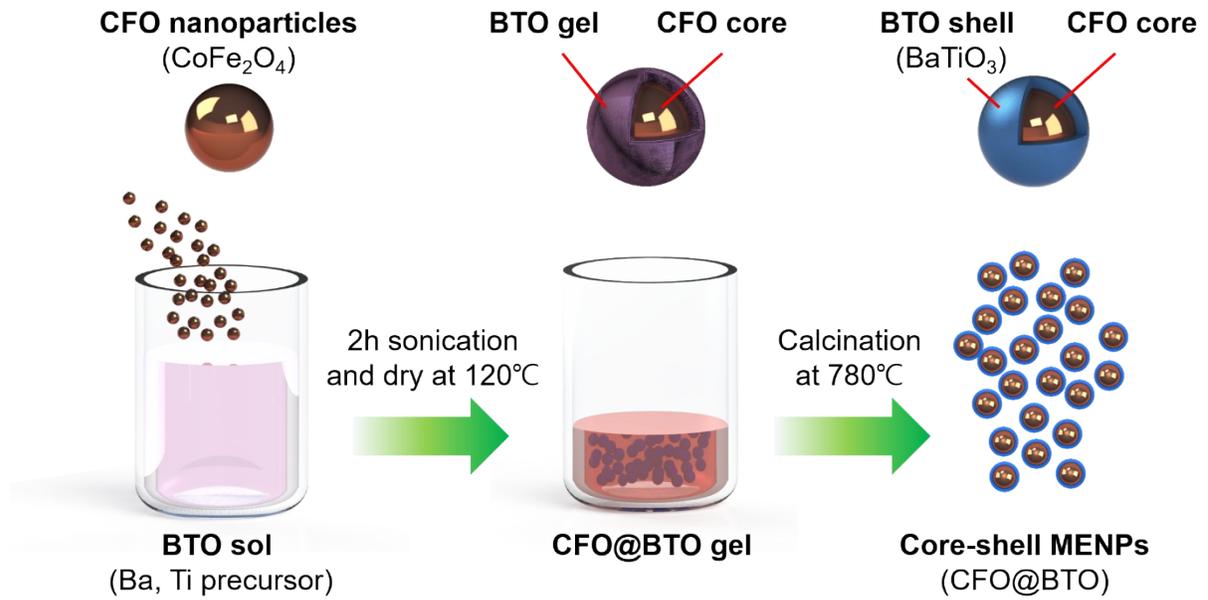


Figure S1. Schematic of fabrication of CFO@BTO core-shell magnetoelectric nanoparticles (MENPs) using a sol-gel method.

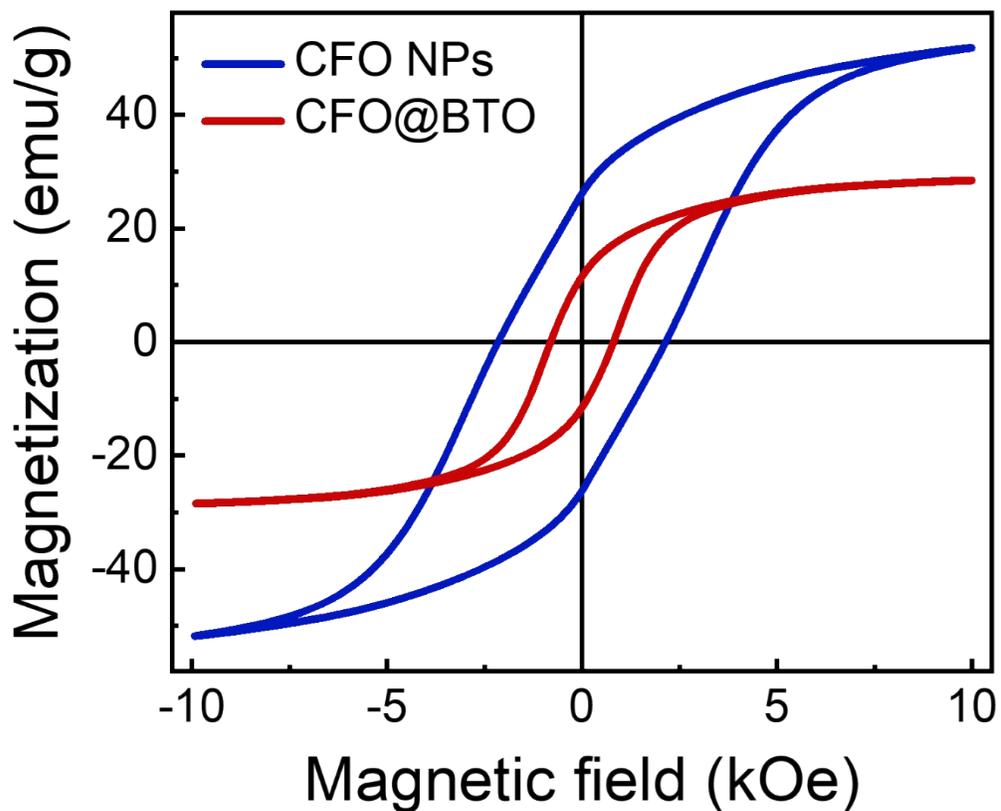


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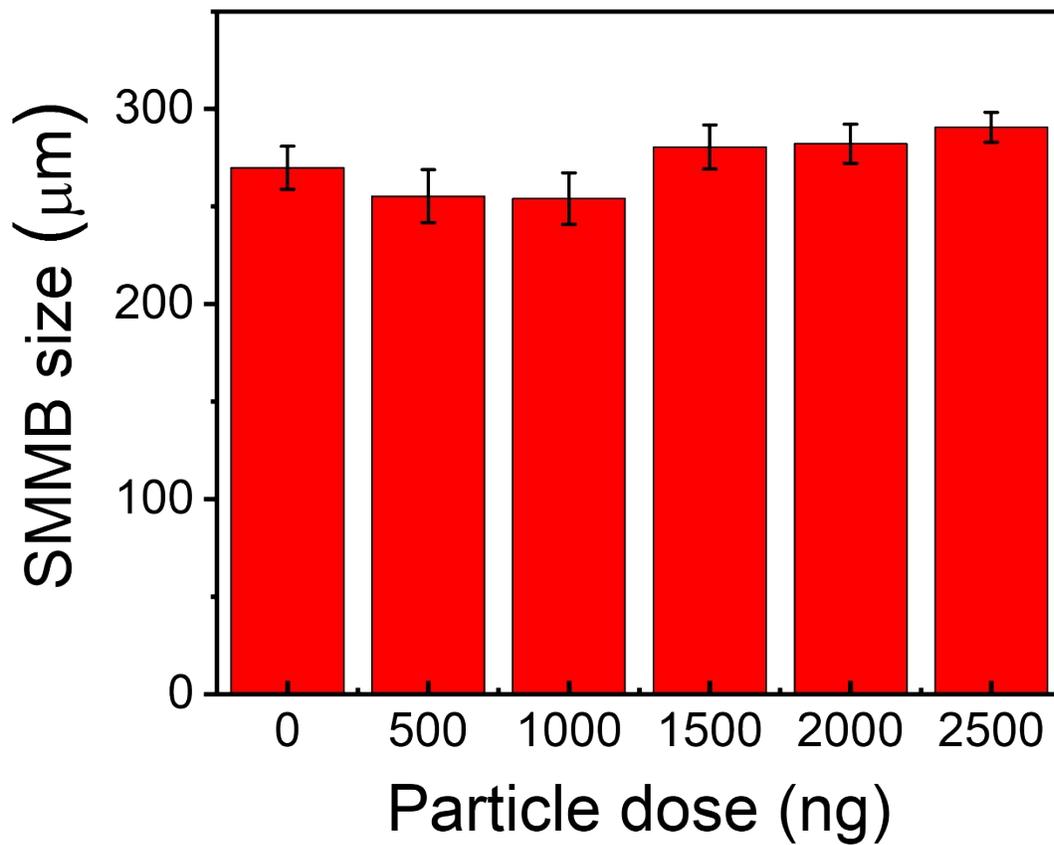


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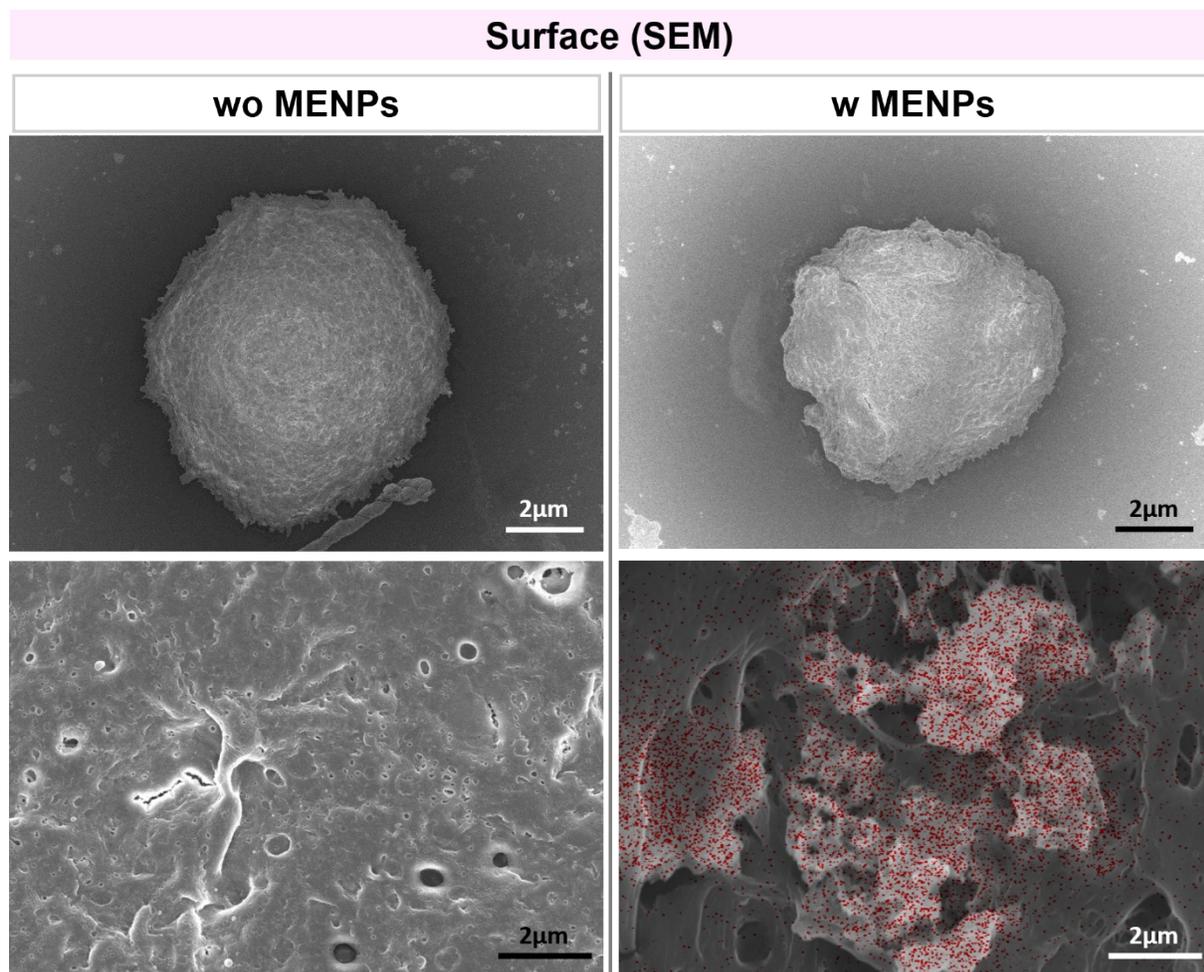


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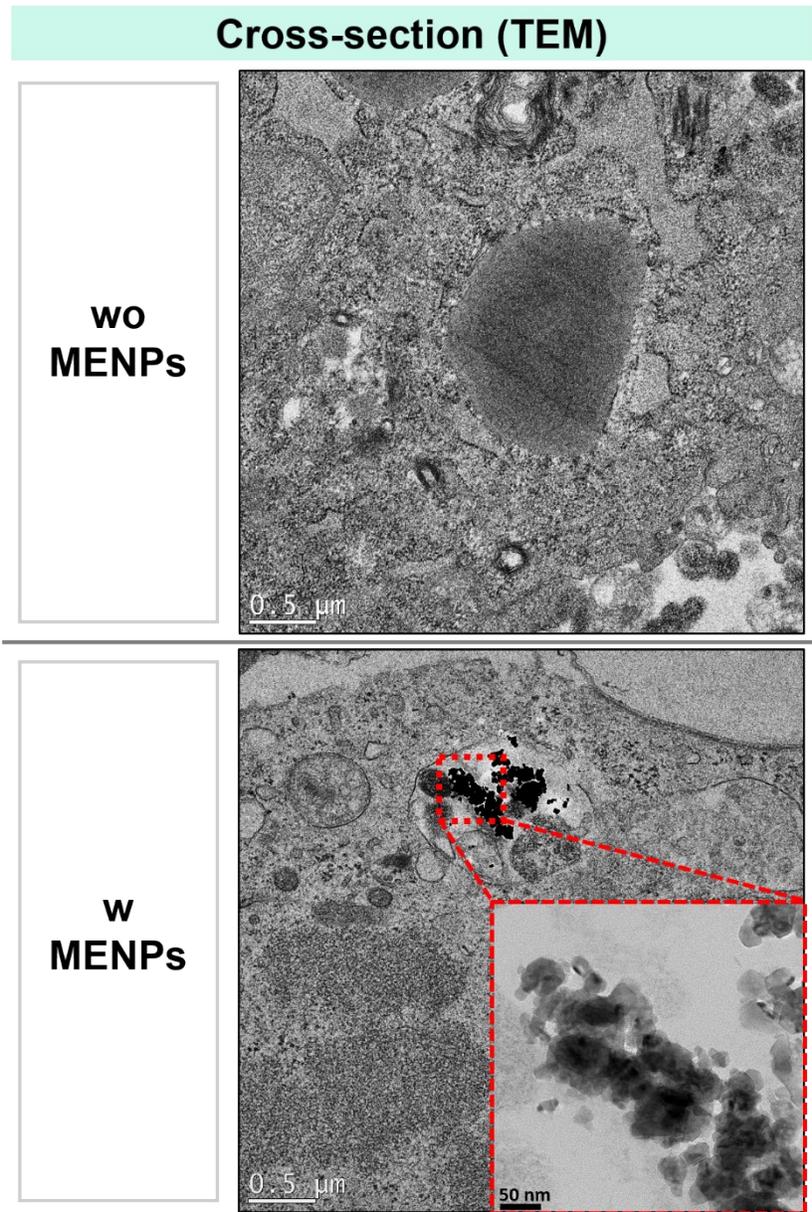


Figure S5. TEM images of SMMB cross-sections without and with 1,000 ng of MENPs.

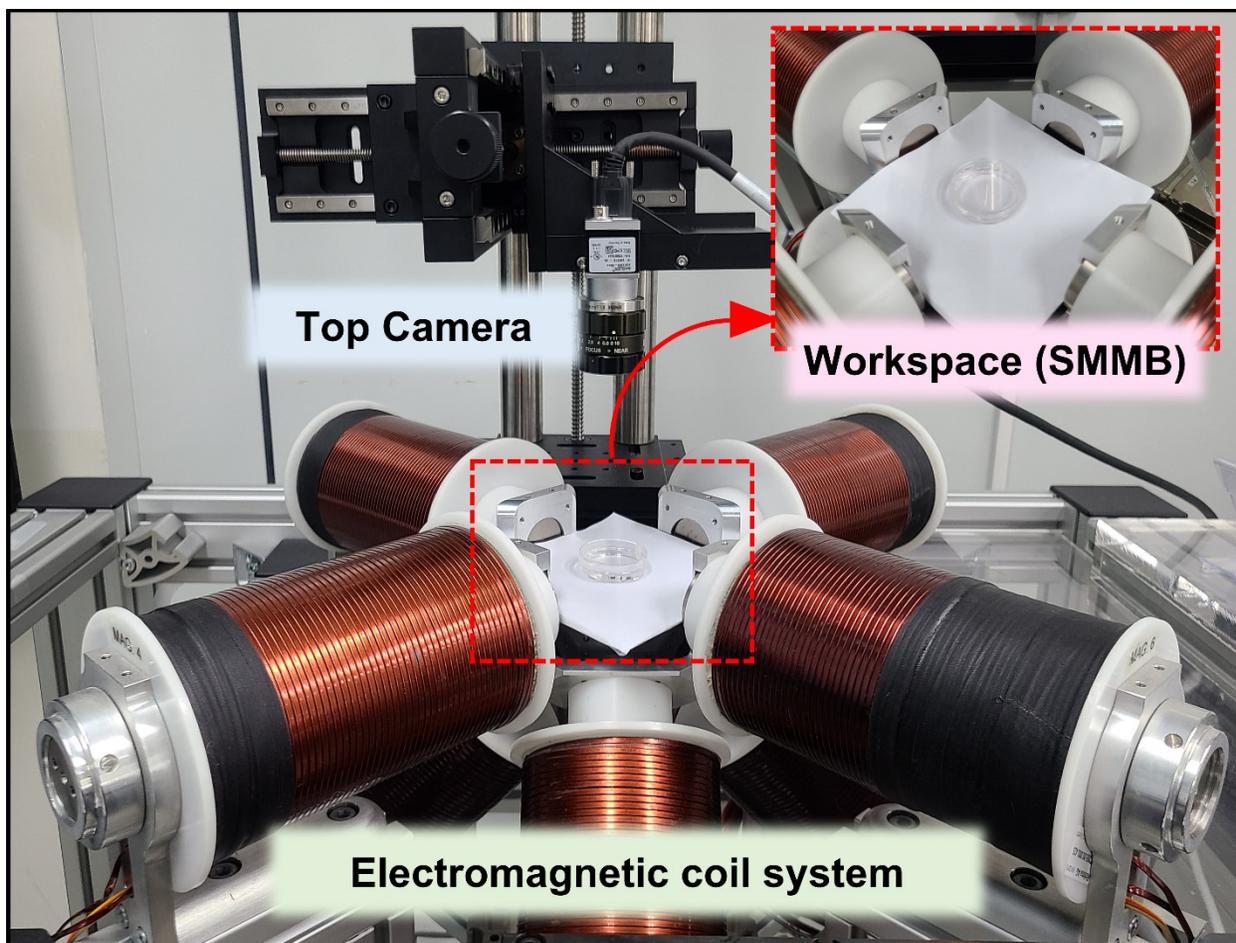


Figure S6. Experimental set-up for magnetic manipulation and SMMB velocity measurement using a rotating magnetic field (RMF) generated by the electromagnetic 8-coil system.

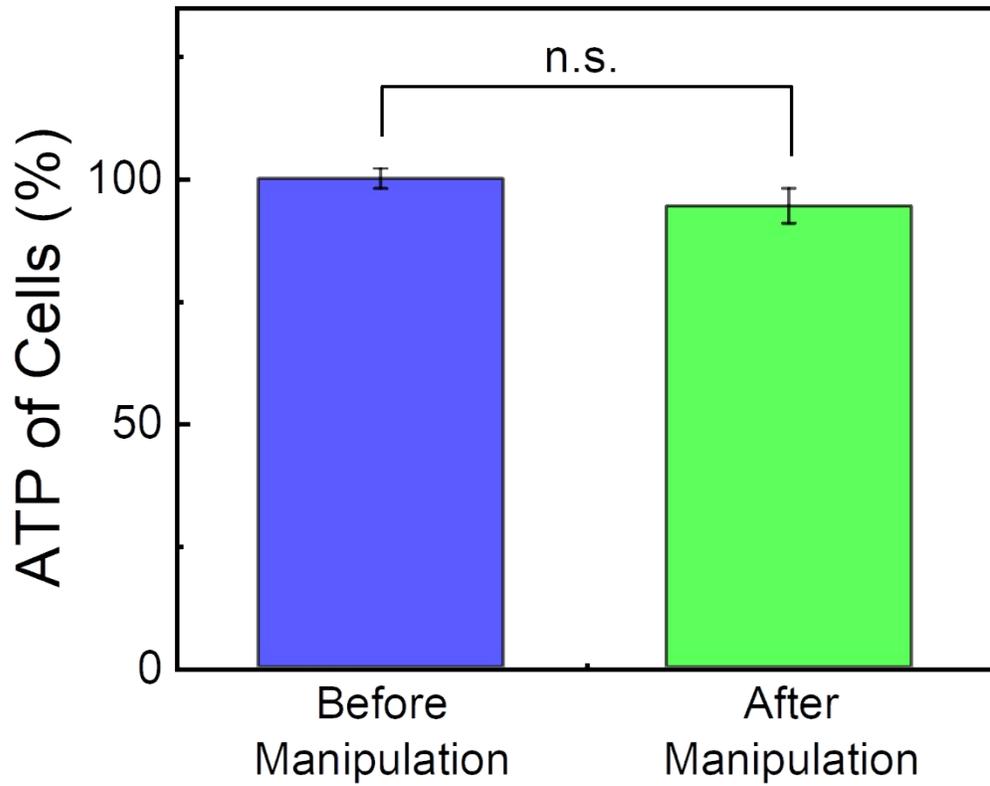


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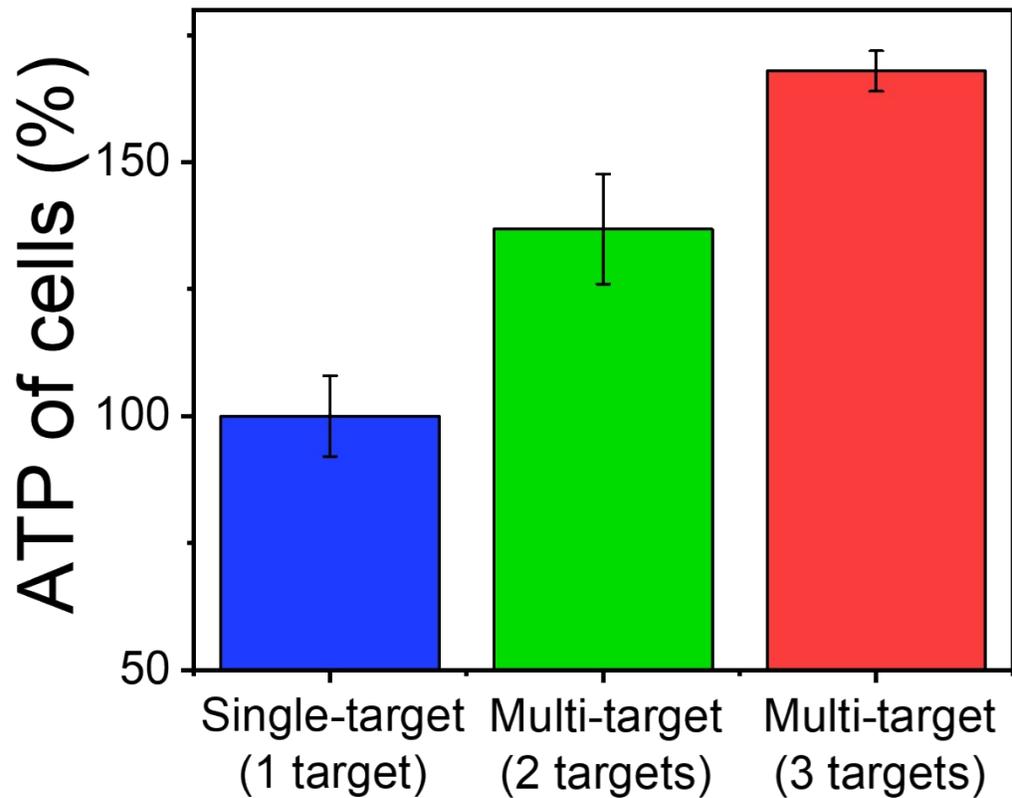


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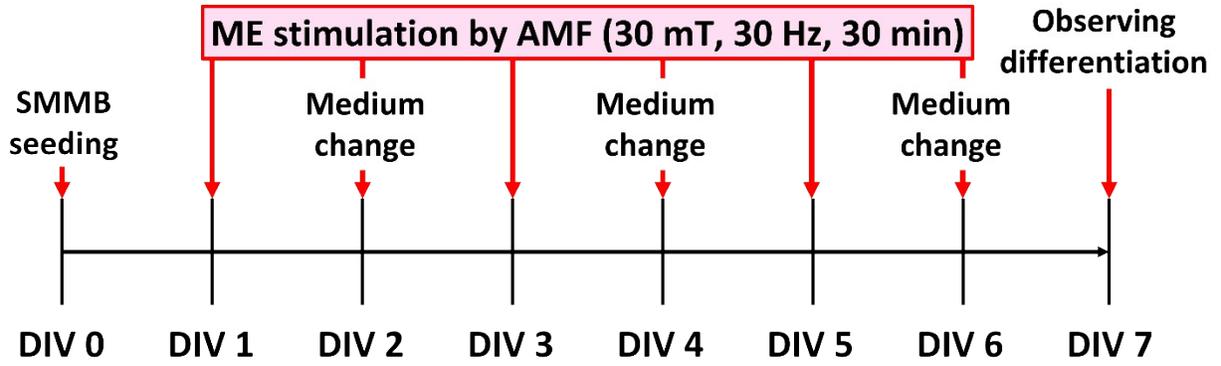


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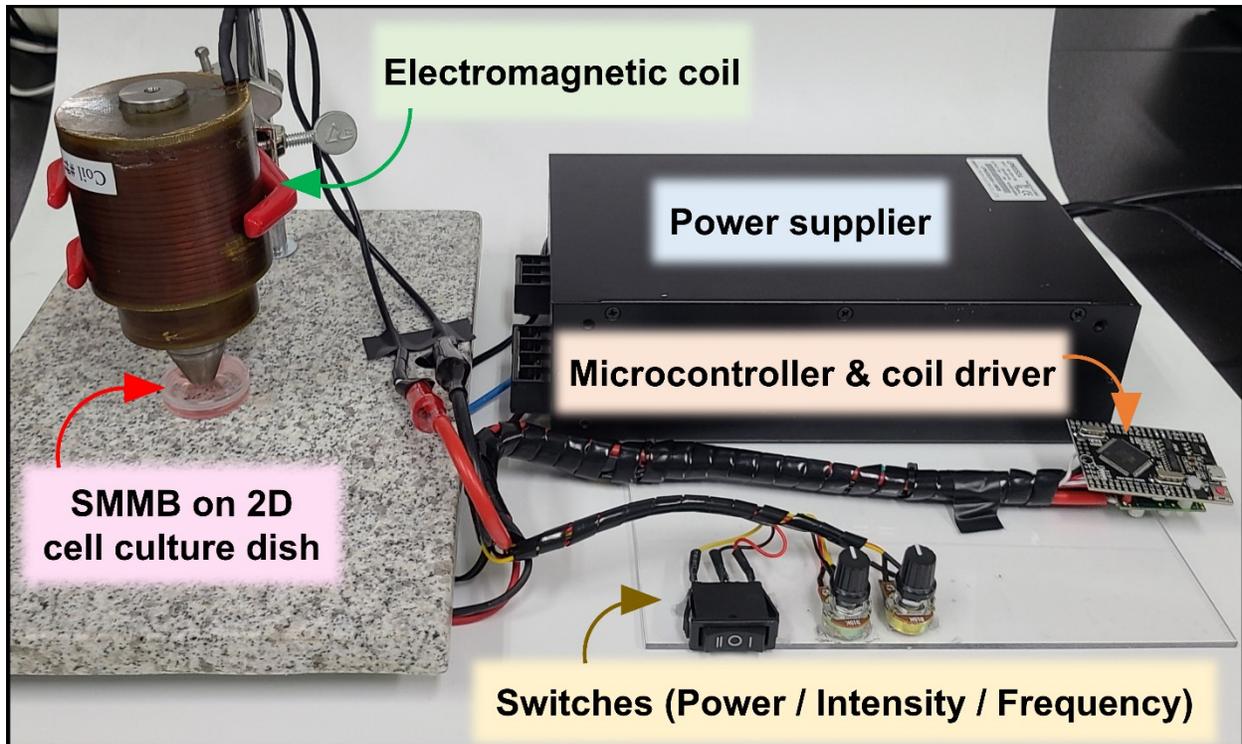


Figure S10. The experimental set-up for ME stimulation (generated by the AMF). The system features an electromagnetic coil, a microcontroller, a coil driver, and a power supply.

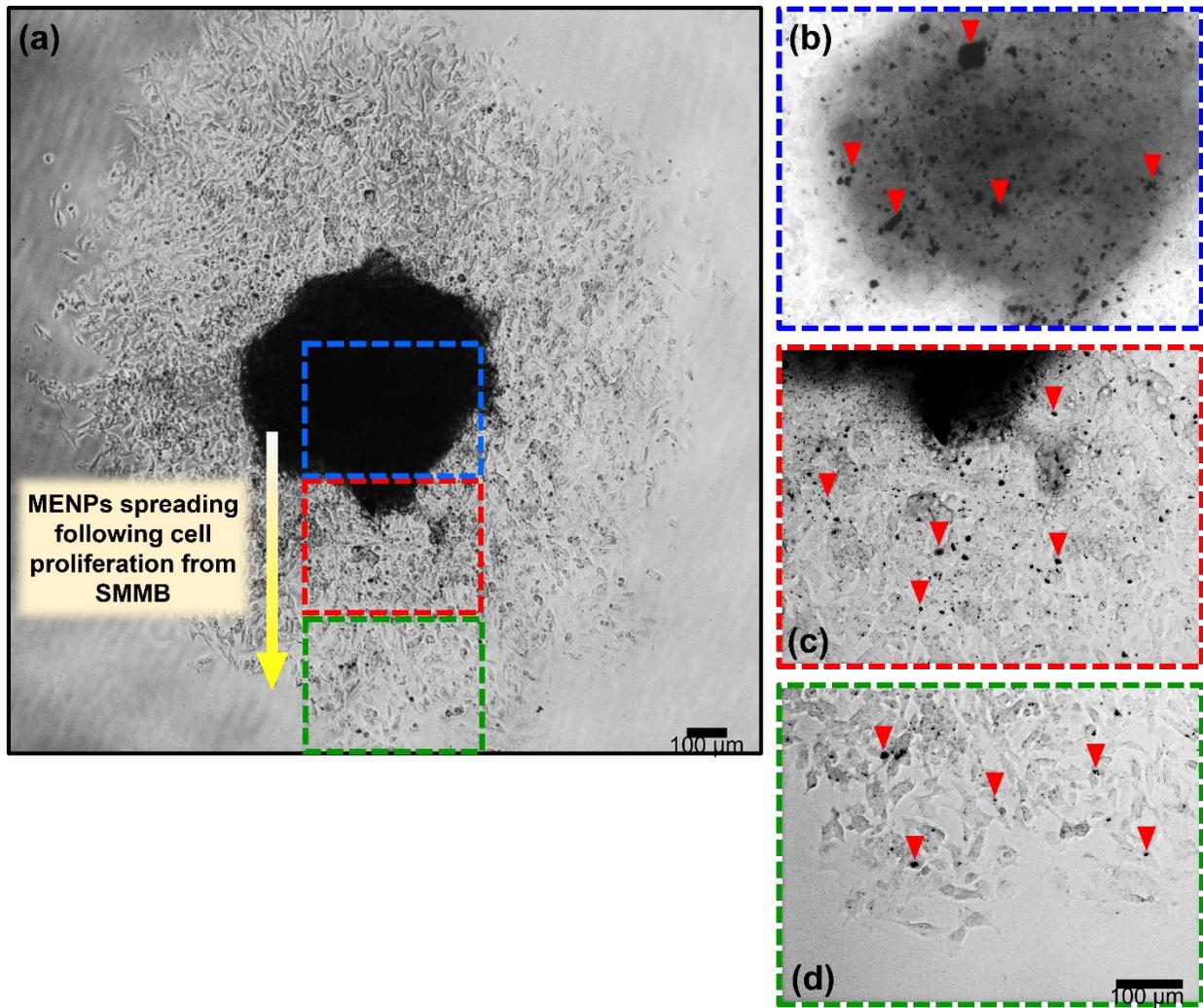


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