Electronic Supplementary Information

Protein microswimmers capable of delivering cells for tissue engineering applications

*Takaaki Kurinomaru, Akiko Inagaki, Masamichi Hoshi, Chikashi Nakamura, and Hironori Yamazoe**

1. Materials and methods

1.1. Preparation of cross-linked albumin film containing magnetic nanoparticles

A cross-linked albumin solution was prepared as previously described.[1] Briefly, bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS, pH 7.4) to yield a 30 mg/ml solution, which was then reacted with 215 mM ethylene glycol diglycidyl ether (EGDE; Wako, Osaka, Japan) with vigorous stirring for 24 h at 25ºC. The reaction mixture was dialyzed for 3 days at room temperature against Milli-Q water using cellulose tubing (molecular weight cutoff = 12 kDa; Nihon Medical Science, Gunma, Japan) to remove unreacted EGDE. The reaction mixture was then adjusted to a final concentration of 20 mg/ml by adding Milli-Q water, sterilized by filtering through a 0.22 μm filter, and stored at 4ºC. The protein concentration was determined by measuring the absorbance at 280 nm using Nanodrop One (Thermo Fisher Scientific, Inc., MA, USA). The resultant cross-linked albumin solution and 5 mg/ml dextran-coated magnetic Fe_3O_4 nanoparticles (MNPs; nanomag®-D-spio, average size = 20 nm; Micromod Partikeltechnologie GmbH, Rostock, Germany) were mixed at a volume ratio of 9:1, into which glycerol was added at final concentration of 0.475%. Then, 2 ml of the mixture was poured into a silicon mold (2.1 × 3.1 cm^2; Matsuno Industry Co., Ltd., Osaka, Japan) and dried overnight in a thermo-hygrostat (SH-222; ESPEC Co., Ltd., Osaka, Japan) at 37ºC and 50% relative humidity (RH) to form the water-insoluble albumin films containing MNPs. Free-standing films were obtained by peeling the formed films from the molds. Then, the films were cut into specific shapes using a biopsy punch (Kai Industries Co., Gifu, Japan) or a CO_2 laser marker (LP-400; Panasonic, Osaka, Japan). The morphological details of the films were characterized using a confocal laser microscope (Optelics C130; Lasertec, Yokohama, Japan). The data of film thickness was averaged for the measurements of 30 samples. The magnetic properties of MNPs-containing albumin film were measured using a vibrating sample magnetometer (VSM-5; TOEI Industry Co., Ltd., Tokyo, Japan) at room temperature.

Tiny albumin films were prepared using the inkjet printing technique according to the previously reported method, since small-size films below 400 μm are difficult to prepare using the cutting free-standing films method mentioned above.[2] Briefly, a small drop of cross-linked albumin solution containing MNPs and glycerol was deposited onto the silicon sheet using piezoelectric printhead (IJHB-1000; Microjet Co., Ltd., Nagano, Japan). The driving of the printhead was controlled by drive electronics (IJHC-10; Microjet Co., Ltd). After depositing the protein drop, the substrates were placed into a thermo-hygrostat at 37ºC and 50%RH and dried overnight to form the tiny albumin films. The prepared films were detached from the substrate by immersing in ethanol.

1.2. Binding of cell membrane anchoring reagent to albumin-based films

The albumin films containing MNPs, suspended in ethanol, were placed into centrifuge tubes and gathered at one place using a neodymium magnet. After removing the ethanol, the films were immersed in 2% BSA in PBS. The films were blocked with BSA for 30 min to prevent the attachment of films to the wall surface of the tubes and were subsequently washed with PBS three times. Then, the films were immersed in 100 μM cell membrane anchoring reagent (CMAR; SUNBRIGHT OE-040CS; M_n = 4000, NOF Co., Tokyo, Japan) solution in PBS with 2% DMSO for 30 min at room temperature. The resultant CMAR-albumin film conjugates (i.e., protein swimmer) were washed with PBS three times and stored at 4ºC.

The binding of CMAR to the surface of the albumin films was measured by surface plasmon resonance (SPR) using a Biacore 2000 instrument (GE Healthcare Bio-Sciences Corp., Uppsala, Sweden). Cross-linked albumin solution was poured onto a bare gold substrate (SIA kit Au; GE healthcare Bio-Sciences Corp.) and incubated for 30 min at room temperature to allow for the adsorption of cross-linked albumin molecules on the gold surface. After washing with Milli-Q water three times, the substrates were placed into the thermo-hygrostat at 37ºC and 50%RH overnight to form the albumin film on gold substrates. The resultant substrate was docked in the instrument, and 10 μM CMAR in running buffer (PBS containing 2 % DMSO) was injected at 25ºC for 30 min at a flow rate of 5 μl/min. Then, the running buffer was added to the substrate for 3 min.

1.3. Cells and cell culture

Human THP-1 monocytes (RCB1189; Riken cell Bank, Tsukuba, Japan) were maintained in RPMI 1640 supplemented with 10% fatal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Mouse NIH3T3 fibroblasts (RCB1862; Riken Cell Bank) were grown on culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Mouse stromal PA6 cells (RCB1127; Riken Cell Bank) were grown on culture dishes in Alpha Minimal Essential Medium (α-MEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. All experiments were conducted with the corresponding medium, whereas the co-culture of NIH3T3 with PA6 was conducted with the medium used for NIH3T3. All of the cells were cultured in a humidified 5% CO_2 incubator at 37ºC. Observation of cells was performed using a phase contrast microscope (IX70; OLYMPUS, Tokyo, Japan).

1.4. Capturing of cells by the protein microswimmer

Cultivated non-adherent THP-1 cells were collected by centrifugation, washed with PBS, and resuspended in serum-free RPMI 1640. Adherent NIH3T3 and PA6 cells were collected by adding a 0.25% trypsin and 1 mM EDTA solution and resuspended in serum-free DMEM containing 10 mM EDTA. EDTA was added to prevent the cell-to-cell interactions via cadherins. For cell capture by protein swimmer, one protein swimmer was mixed with approximately $1 \times 10^5 - 1 \times 10^6$ cells in a small chamber, followed by incubation for 1 h, which included turning the chamber upside down every 10 min. After removing the supernatant containing the uncaptured cells, the cell-capturing protein swimmer was washed with PBS and suspended in culture medium. CMAR-devoid protein swimmers, prepared by omitting the binding of the CMAR to the albumin films, were used as controls in the cell-capturing assay.

1.5. Detachment behavior of non-adherent cells from the protein swimmer

The cells captured by the protein swimmer were detached from the swimmer spontaneously over time. For the quantitative analyses of detachment of non-adherent THP-1 cells from protein swimmer, the CMAR-carrying albumin films were prepared on the plates using the same procedure as that for the preparation of protein swimmer. The cross-linked albumin solution containing MNPs and glycerol was poured onto a multiwell plate (0.95

cm^2/well; Thermo Fisher Scientific Inc.) and dried overnight to form the albumin film on the plate, followed by the binding of CMAR to the film surfaces. Then, THP-1 cells suspended in serum-free RPMI 1640 were seeded into the plates at a density of 5×10^4 cells/cm^2 and incubated for 10 min at room temperature to immobilize the THP-1 cells on CMAR-carrying surfaces. After washing with PBS, the serum-free medium was removed and replaced with culture medium for THP-1 cells. At various predetermined times (2, 4, 6, 8, and 10 h), the supernatants containing the detached cells were removed from the plates and fresh medium was added. The numbers of immobilized cells were counted under a phase contrast microscope at each time point. The detachment ratio was estimated by dividing the number of immobilized cells after culturing for the predetermined time period by the number of cells post-immobilization. The viability of detached cells was evaluated by staining the cells with calcein-acetoxymethyl ester (calcein-AM; Dojindo Laboratories, Kumamoto, Japan) and propidium iodide (PI; Dojindo Laboratories) according to the manufacturer's instructions.

The reusability of the protein swimmer was also investigated by using THP-1 cells and CMAR-carrying albumin films-coated plates. THP-1 cells were immobilized on the CMAR-carrying plates and then cultured overnight to allow the detachment of all immobilized cells. The plates after cell detachment were washed with PBS, immersed in 70% ethanol for 1 h at room temperature, then washed again with PBS five times for regeneration. This procedure was repeated four times.

1.6. Transfer printing of adherent cells captured by the protein swimmer on the substrate

Culture dishes were immersed in the 100 μg/ml polyethylenimine (PEI; number-average molecular weight: 60 kDa; Sigma) solution in PBS for 30 min at room temperature. After washing with PBS three times, the PEI-coated dishes were immersed in the 10 μg/ml bovine plasma fibronectin (FN; Invitrogen, CH, USA) solution in PBS for overnight at 4ºC. The protein swimmers that had previously captured the adherent cells, including NIH3T3 and PA6 cells, were placed onto fibronectin-coated surfaces and their position was fixed using a neodymium magnet placed under the dishes to avoid any movement of the swimmer during the culture. Then, the protein swimmers were incubated overnight (12-18 h) in the culture medium to transfer the cells captured by the swimmer to the FN-coated dishes. After removing the protein swimmers without loading the cells from dishes using a pipette, the transferred cells were observed using a phase-contrast microscope. The viability of the transferred cells was evaluated by staining the cells with calcein-AM and PI.

1.7. Interaction force measurement using atomic force microscopy

The interaction force between the cells and CMAR in the protein swimmer was measured by mechanically detaching the cells using an atomic force microscope (AFM) in accordance with the previous reports.[3, 4] Arrowhead nanoneedles were fabricated by etching the pyramidal tips of standard AFM silicon cantilevers (ATEC-Cont; NanoWord, Neuchâtel, Switzerland) into an arrowhead-shaped end using a focused ion beam (SMI500; Hitachi High-Tech Science Corporation, Tokyo, Japan). The width and height of the arrowhead was 2.4 and 2 μm, respectively. The needle was approximately 10 μm in length and 400 nm in thickness. Spring constants, 1.22 N/m, were determined using the thermal fluctuation method prior to each experiment.[5] The prepared nanoneedles were cleaned by immersing them in a sulfuric peroxide mixture [4:1 (vol/vol) of sulfuric acid and 30% hydrogen peroxide solution] at 55ºC for 30 min and washing twice with Milli-Q water.

The CMAR-carrying albumin films were prepared on the culture dish (9 cm^2) using the procedure described in Section 1.5. Then, NIH3T3 cells suspended in serum-free DMEM were seeded onto the dishes at a density of 1×10^4 cells/cm^2, followed by a 10-min incubation for cell immobilization. After washing with PBS, the serum-free medium was removed and replaced with culture medium for NIH3T3 cells. The arrowhead nanoneedles were inserted into

the single immobilized cell at various incubation periods using the AFM system (Nanowizard II BioAFM; Bruker, Berlin, Germany) equipped with a CellHesion module, which enables the probe to move vertically up to 100 μm. Both the approach and retraction of the arrowhead nanoneedle were performed at a velocity of 5 μm/s. The force exerted on the cantilever during the insertion and retraction process was monitored using force spectroscopy mode. The set point in the approach process was set to a maximum force of 300 nN. All procedures were conducted in a temperature-controlled chamber at 37ºC.

1.8. Remote manipulation of cells using a magnetically propelled protein swimmer

The transportation of cells from cell reservoir (a departure point) to target site (an arrival point) was conducted using a protein swimmer. The experimental setting is schematically shown in Fig. S8 (ESI). Two silicon frames (diameter, 8.5 mm; height, 1.5 mm) were placed on the culture dish (9 cm^2); each frame was used as a departure and arrival point. The surface of the arrival point was coated with PEI and FN, as described in Section 1.6. After filling the dish with serum-free DMEM containing 10 mM EDTA and 0.005 wt% Tween-80, a concentrated cell suspension of NIH3T3 cells was added into the silicon frame of the departure point. Then, a 1 mm-diameter circular protein swimmer was added to the dishes, moved toward the departure point by applying a magnetic field, and placed on the cells for 10 min at room temperature to capture the NIH3T3 cells. The protein swimmer was placed into the dishes immediately after (within 10 min) the addition of NIH3T3 cells into the silicon frame, in order to carry out cell capturing before the adhesion of the cells to the dish. The cell-capturing swimmer was then pulled up from the frame of the departure point and moved inside the frame of the arrival point using the magnetic force. After replacing the serum-free medium with culture medium for NIH3T3 cells, the protein swimmer at the arrival point was incubated overnight for the NIH3T3 cells captured by the swimmer could be transferred to the FN-coated dishes, as described in Section 1.6. The external magnetic field was produced using a cylindrical neodymium magnet (diameter, 17.5 mm; height, 20 mm; magnetic induction, 540 mT; Niroku Seisakusho Co., Ltd., Kobe, Japan).

Cell manipulation inside the enclosed space was conducted using the protein swimmer. A cell culture flask (25 cm^2) was coated with PEI and FN. The resultant flask was maintained vertical and a neodymium magnet was placed on the side of the flask. After filling the flask with culture medium for NIH3T3 cells, a NIH3T3 cell-loaded circular protein swimmer (2 mm-diameter) was added to the flask. After attaching the protein swimmer to the side of flask using magnetic attraction, the cells captured by swimmer were transferred to the side wall of the flask.

Single-cell manipulation was conducted using a tiny protein swimmer prepared using the inkjet printing technique, as described in Section 1.1. The culture dish (21 cm^2) was coated with BSA by immersing the dish in the 2% BSA solution for 30 min. After filling the dish with serum-free RPMI 1640, a small number of THP-1 cells and a tiny protein swimmer were added to the dish. The protein swimmer was magnetically directed toward the target THP-1 cells, which were captured and transported via magnetic steering using a small neodymium magnet (diameter, 1 mm; height, 10 mm; magnetic induction, 210 mT; NeoMag Co., Ltd., Ichikawa, Japan). The translocation of single cells by the tiny protein swimmer was observed and recorded using a phase contrast microscope equipped with a color CCD video camera (VB-7010; Keyence, Osaka, Japan).

1.9. Fabrication of cellular constructs composed of multiple cell types

A two-dimensional patterned cell co-culture was created using a protein swimmer. A cell culture dish (9 cm^2) was coated with PEI and FN. After filling the dish with culture medium used for NIH3T3 cells, a NIH3T3 cell-loaded annular shaped protein swimmer (outer diameter 2 mm, inner diameter 3 mm) was added to the dish and placed on the FN-coated surface. Subsequently, a PA6 cell-loaded circular protein swimmer (1 mm-diameter) was added to the

dish and magnetically guided towards the inside of the annular ring. The resultant two swimmers were incubated overnight while maintaining their initial positions to ensure that the cells captured by the swimmers were transferred to the FN-coated dishes.

Multilayered cell assembly was also created using a protein swimmer. A larger NIH3T3 cell-loaded circular protein swimmer (3 mm-diameter) was added to the PEI/FN-coated dish containing the culture medium, placed on the FN-coated surface, and incubated overnight to ensure that the NIH3T3 cells captured by the swimmer were transferred onto the dishes. After removing the protein swimmer without loading the cells from dishes using a pipette, a smaller PA6 cell-loaded circular protein swimmer (1 mm-diameter) was added to the dish and magnetically guided towards the NIH3T3 cells-attached region. The protein swimmer placed on the NIH3T3 cells was incubated overnight to ensure that the PA6 cells captured by swimmer were transferred onto the NIH3T3 cells. To validate the patterns in the co-culture, NIH3T3 and PA6 cells were stained with red and green fluorescent dyes, respectively, using PKH26-red (Invitrogen), PKH67-green (Invitrogen), and CFSE-green (Invitrogen), prior to cell-capturing by the protein swimmer. The created cellular constructs were observed using an IX70 fluorescent microscope.

2. Supplementary figures

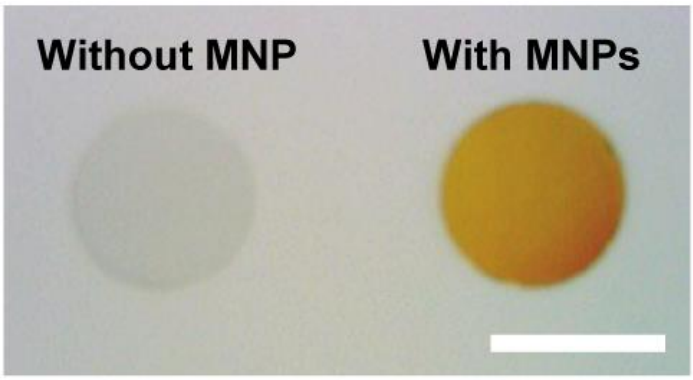

Figure S1. Image of free-standing albumin films with and without MNPs. Scale bar: 3 mm.

Magnetic properties of albumin film containing MNPs

Figure S2 shows the magnetic hysteresis curve of an albumin film containing superparamagnetic MNPs. The film containing MNPs showed superparamagnetic behavior, as evidenced by the negligible coercivity and remanence on the magnetization curve. The saturation magnetization value of the film was 0.67 emu/g, which was much lower than that of pure MNPs (69 emu/g), due to the nonmagnetic behavior of the albumin matrix that surrounded the MNPs.

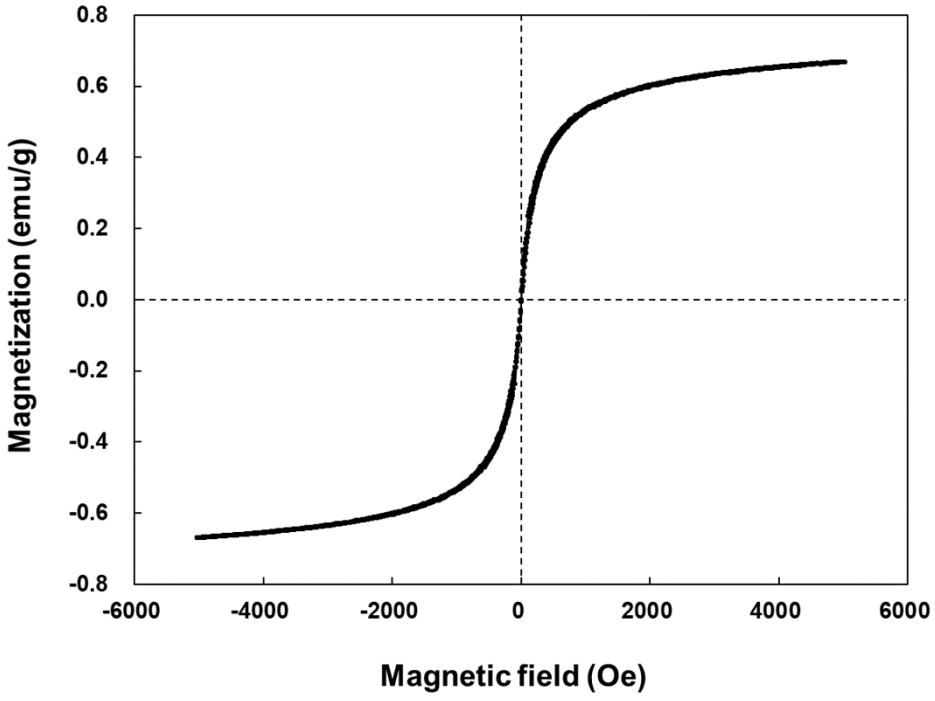

Figure S2. Magnetic hysteresis curve of albumin film containing MNPs.

Binding of CMAR to albumin-based films

The amine-reactive group in CMAR reacts with the ε-amines of the lysine side chains and the α-amines at the N-terminus of albumin. To examine the binding of CMAR to the surface of the albumin film, SPR was performed using a gold substrate coated with albumin film. As shown in Fig. S3, the SPR signal sharply increased upon the introduction of the CMAR solution. A net SPR signal increase by CMAR binding can be observed after replacement with running buffer.

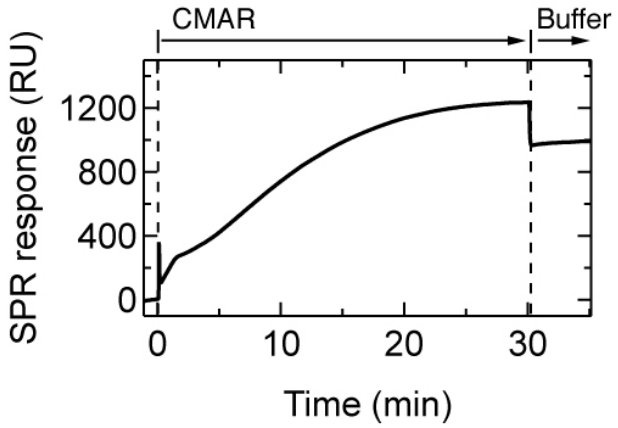

Figure S3. SPR sensorgram during exposure of CMAR to albumin film surface. Dashed lines indicate the starting points for the CMAR and buffer injection.

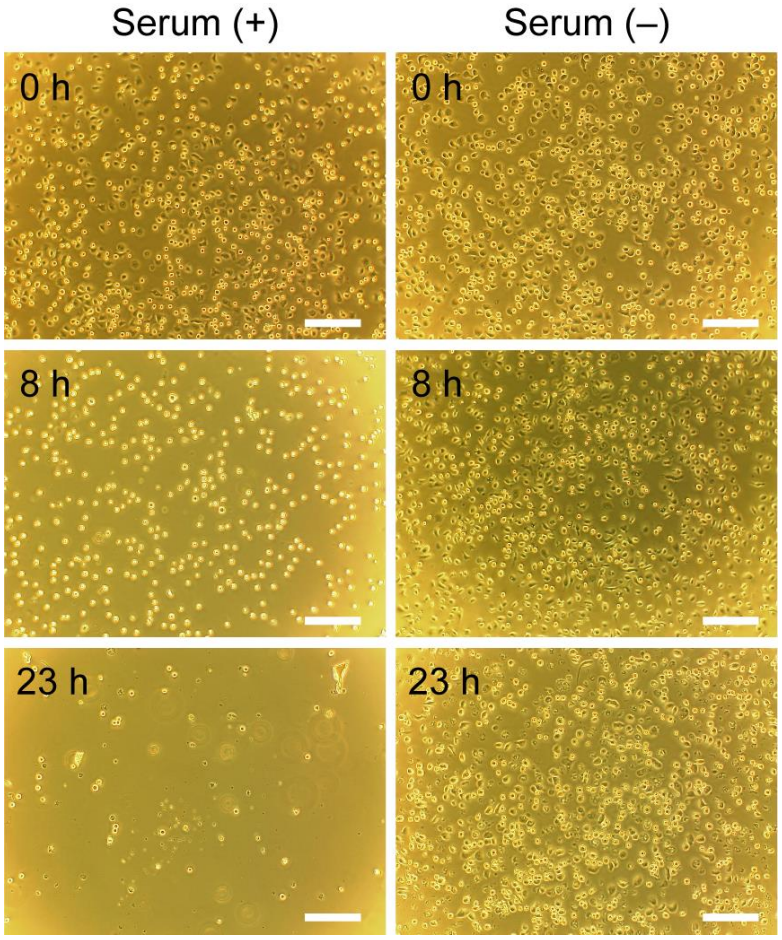

Figure S4. Phase-contrast micrographs of THP-1 cells captured by the CMAR-carrying surfaces before and after cultivation in medium with or without serum for 8 and 23 h. Time-dependent cell detachment was observed when cultured in the medium with serum, but not in the serum-free medium. Scale bar: 200 μm.

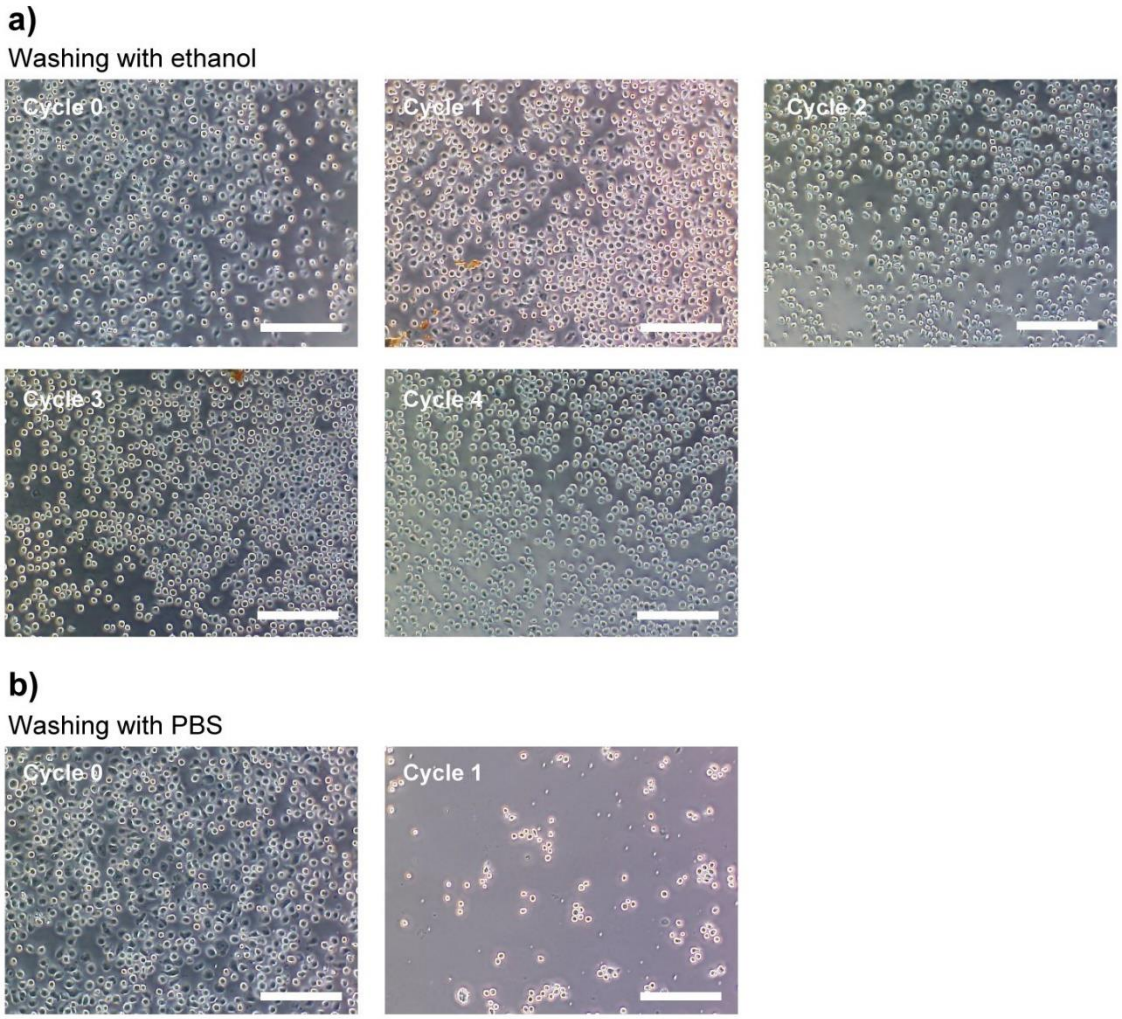

Figure S5. Phase-contrast micrographs of THP-1 cells captured by the CMAR-carrying surfaces before and after repeated cycles of cell capture-and-detachment. Regeneration of CMAR surface for cell-capturing can be achieved by washing with ethanol (a), but not with PBS (b). Scale bar: 200 μm.

Transfer printing of cells using protein swimmer with non-planar surface

When a small droplet of cross-linked albumin solution was deposited onto the substrate for the preparation of the small-size films, the formed films did not have flat surface, with a peripheral region that was thick, while the thickness in the central region decreased (Fig. S6a). This shape is commonly observed in droplets deposited on the substrate, which is known as the coffee stain phenomenon.[6] As shown in Fig. S6b, we failed to produce the precise cellular pattern when a protein swimmer with a non-planar surface was used. Therefore, in this study, we employed another strategy, i.e. the preparation of large free-standing films with a flat surface and subsequently cutting of the films, to obtain a protein swimmer with flat surface.

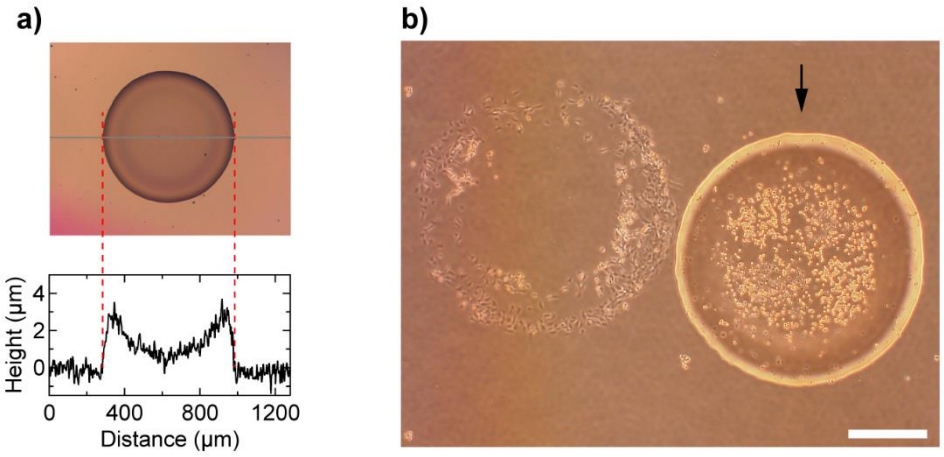

Figure S6. a) Microscopic image of the albumin film formed on the substrate showing the thickness profile. b) Phase-contrast micrograph of transferred NIH3T3 cells on the substrate using the protein swimmer with a non-planar surface. The arrow indicates the protein swimmer after cell transfer printing, in which residual cells were observed in the central region. Scale bar: 500 μm. Please note that protein swimmers with different sizes were used for the experiments in (a) and (b); they had similar morphological features.

Interaction force measurement using atomic force microscopy

Figure S7 shows an example of a force-distance curve during needle approach and retraction. The needle was moved toward the cells from 100 µm above and retracted to the original height after a dwell time of 1 min. The penetration of the needle into the cell was confirmed by the approach force curve.[7] During the retraction of the nanoneedle, peaks were observed when the cells were detached from the substrate. Detachment of the cells was confirmed by phase-contrast microscopy. The minimum of the retraction curve peak was termed as interaction force.

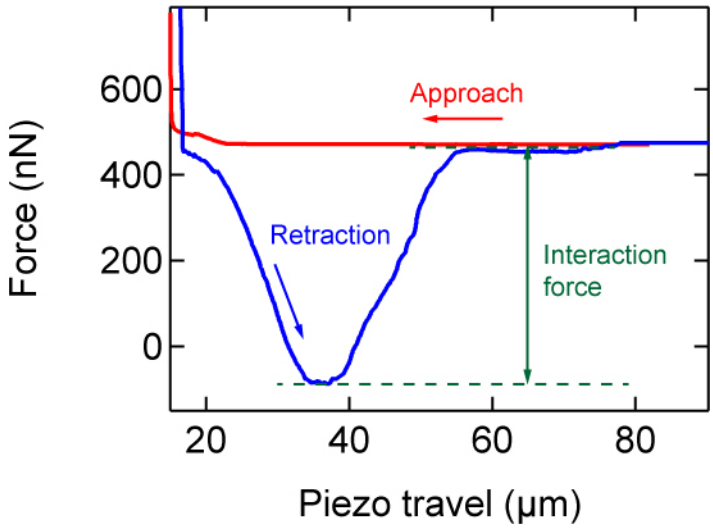

Figure S7. The representative force-distance curve during needle approach and retraction.

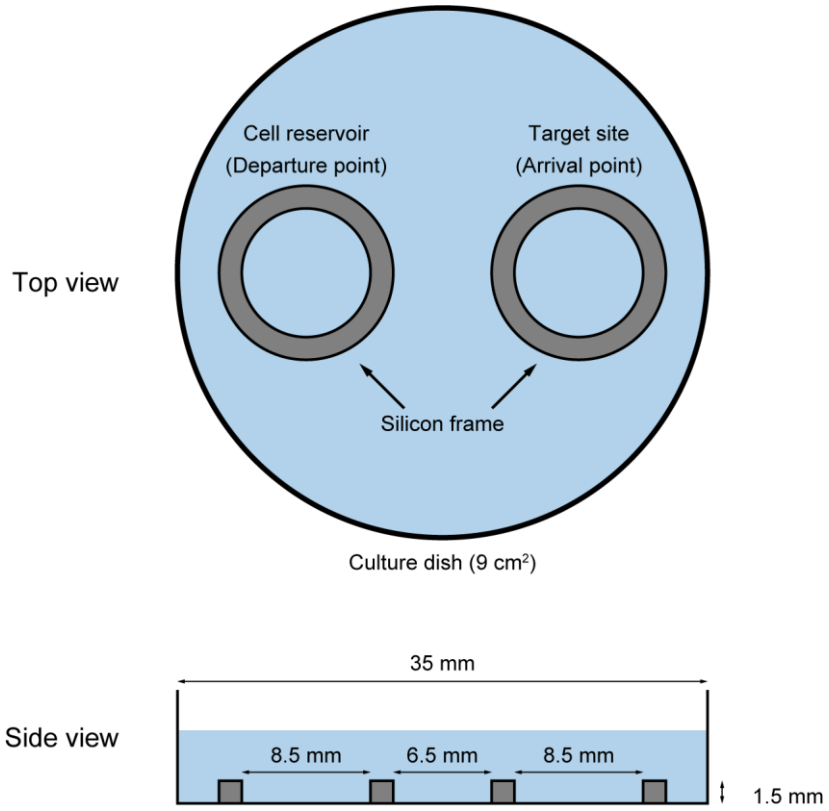

Figure S8. Schematic illustration of experimental design for the transportation of cells from the cell reservoir (a departure point) to the target site (an arrival point) using the protein swimmer.

Movie S1: Behavior of cells captured by protein swimmer during a 16 h culture. Images were obtained every 5 min using the CytoSMART^{TM}2 system (Lonza, Basel, Switzerland). Scale bar: 100 μm.

Movie S2: Magnetic guidance of the protein swimmer in the vertical and horizontal directions.

Movie S3: Magnetic guidance of the protein swimmer inside the flask. Transparent solution was used to allow for observation of the movement of the protein swimmer inside the flask in the movie; the actual experiment for cell transfer printing was carried out using the normal culture medium containing phenol red.

Movie S4: Capturing of a single cell by a tiny protein swimmer under magnetic control.

References

1 H. Yamazoe, T. Ichikawa, Y. Hagihara, Y. Iwasaki, *Acta Biomater.*, 2016, **31**, 231-240.
2 H. Yamazoe, *Biomaterials*, 2018, **155**, 1-12.
3 R. Kawamura, M. Mishima, S. Ryu, Y. Arai, M. Okose, Y. R. Silberberg, S. R. Rao, C.

Nakamura, *Langmuir*, 2013, **29**, 6429-6433.
4 S. Ryu, Y. Hashizume, M. Mishima, R. Kawamura, M. Tamura, H. Matsui, M. Matsusaki, M. Akashi, C. Nakamura, *Biochem. Biophys. Res. Commun.*, 2014, **451**, 107-111.
5 J. L. Hutter, J. Bechhoefer, *Rev. Sci. Instrum.*, 1993, **64**, 1868-1873.
6 R. D. Deegan, O. Bakajin, T. F. Dupont, G. Huber, S. R. Nagel, T. A. Witten, *Nature*, 1997, **389**, 827-829.
7 I. Obataya, C. Nakamura, S. Han, N. Nakamura, J. Miyake, *Nano Lett.*, 2005, **5**, 27-30.