Block-Cell-Printing for live single-cell printing

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A unique live-cell printing technique, termed “Block-Cell-Printing” (BloC-Printing), allows for convenient, precise, multiplexed, and high-throughput printing of functional single-cell arrays. Adapted from woodblock printing techniques, the approach employs microfluidic arrays of hook-shaped traps to hold cells at designated positions and directly transfer the anchored cells onto various substrates. BloC-Printing has a minimum turnaround time of 0.5 h, a maximum resolution of 5 μm, close to 100% cell viability, the ability to handle multiple cell types, and efficiently construct protrusion-connected single-cell arrays. The approach enables the large-scale formation of heterotypic cell pairs with controlled morphology and allows for material transport through gap junction intercellular communication. When six types of breast cancer cells are allowed to extend membrane protrusions in the BloC-Printing device for 3 h, multiple biophysical characteristics of cells—including the protrusion percentage, extension rate, and cell length—are easily quantified and found to correlate well with their migration levels. In light of this discovery, BloC-Printing may serve as a rapid and high-throughput cell protrusion characterization tool to measure the invasion and migration capability of cancer cells. Furthermore, primary neurons are also compatible with BloC-Printing.

Current high-throughput screening of cell function and heterogeneity and in vitro cell-cell communication studies requires routine generation of large-scale single-cell arrays with high precision and efficiency, single-cell resolution, multiple cell types, and maintenance of cell viability and function (1, 2). Several approaches have been designed for this purpose, e.g., inkjet cell printing (3–6), surface engineering (7–15), and physical constraints (16–23). However, finding a method that completely satisfies the above requirements remains a challenge. Potentially useful and convenient tools may be available by adapting traditional printing tools to cell printing. In particular, woodblock printing is an efficient and convenient technology that revolutionized the printing world more than 1,800 y ago and was extended to microcontact molecular printing ~20 y ago (24). However, application of the block-printing concept to cells has never been achieved. The main challenges are (i) inking cells to their molds with precision and maintaining viability, (ii) evenly and gently applying and transferring the cells to a substrate and successfully lifting off the mold without detaching the cells, and (iii) maintaining cell functions after printing.

We report here the development and testing of a technology called “Block-Cell-Printing” (BloC-Printing), which involves directly inking cells to a predesigned mold and then transferring the cells to a substrate. We overcome the challenges described above by flow patterning, instead of pressing, the ink objects, as in woodblock or microcontact printing. By performing various validation experiments, we prove that BloC-Printing can achieve a maximum spatial resolution of 5 μm, has the ability to simultaneously handle multiple cell types, results in close to 100% cell viability, and requires a minimum turnaround time of ~0.5 h. The Block-Cell-Mold (BloC-Mold) is reusable for hundreds of printings. This approach does not require sophisticated equipment or large sample volumes. It is also straightforward and convenient, and it permits the subsequent culture of cells and image analysis under standard conditions.

Results and Discussion

Design and Operation of BloC-Printing. In a typical BloC-Printing process, the BloC-Mold, designed using AutoCAD (Autodesk) and fabricated by photolithography and polydimethylsiloxane (PDMS) molding techniques, was laid onto a Petri dish, glass slide, or other type of substrate, without thermal or oxygen plasma treatment. This formed an assembled BloC-Printing device with a network of microfluidic channels. A typical assembled BloC-Printing device is shown in Fig. 1A. After removal of air by vacuum pressure, cell culture medium is drawn into the channels by the application of negative pressure at the outlet (Fig. 1B, Lower). Then, the suspended cells are introduced into the BloC-Printing device from the inlet (Fig. 1B, Upper) and the flow of cells is driven by 1 psi vacuum pressure applied to the outlet. The flow force was carefully distributed to create a uniform flow of cells throughout the entire device. Single-cell traps were located along the sides of the flow channels with 3-μm gaps (Fig. 1C and SI Appendix, Fig. S1). The initial printings were performed with SKBR-3 breast cancer cells (American Type Culture Collection, ATCC).

There are two potential flow paths around a trap structure (Fig. 1D, Left). The wide side consists of a 22-μm gap and the narrow side consists of a 3-μm gap, and these are labeled as paths 1 and 2, respectively. The fluid resistance ratio of paths 1 to 2 is ~1:41, according to a theoretical calculation (SI Appendix, Fig. S24). Therefore, at low cell densities (<104 cells per mL), almost all cells will flow through the wide side of the trap area (Fig. 1D, Center Left), because of the Zweifach–Fung bifurcation law.

Significance

The ability of printing single-cell arrays with high precision and efficiency, single-cell resolution, multiple cell types, and maintenance of cell viability and function is essential for cell function and heterogeneity measurement. It is still hard for current methods to completely satisfy the above requirements. We report a unique live-cell printing technique, Block-Cell-Printing, that allows for convenient, precise, multiplexed, and high-throughput printing of functional single-cell arrays. Block-Cell-Printing has a minimum turnaround time of 0.5 h, a maximum resolution of 5 μm, and close to 100% cell viability. This method has been applied to study cell communications in heterotypic cell pairs with controlled morphology, characterize cell abilities to extend their membranes, and print primary neurons.

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The authors declare no conflict of interest.

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However, at high densities (>10⁶ cells per mL), the wide side may be temporarily blocked by a group of cells such that an individual cell may be forced into the narrow channel and trapped (Fig. 1D, Center Right and Movies S1–S4). Because of the flexibility of the cells, the flow force will immediately clear such edges, and (ii) the gas permeability of the PDMS material allowed the cells to “breathe” during the cell adhesion process. When the medium was refreshed via a specially designed gravity-induced flow, MDA-MB-231/green fluorescent protein (GFP) cells inside the BloC-Printing device were able to grow and migrate, demonstrating normal morphology, for more than 48 h (SI Appendix, Fig. S7). After removal of the BloC-Mold, the printed SK-BR-3 cells were also able to divide and propagate on the Petri dish for 5 d, indicating close to 100% cell viability (SI Appendix, Fig. S8).

**Various Single-Cell Arrays Generated by BloC-Printing.** Through precise placement of traps in the BloC-Mold, the spatial resolution of the printed cell array was approximately equivalent to the cell sizes, with an edge-to-edge distance of less than 30 μm vertically and 5 μm horizontally (Fig. 2 E and F). Fig. 2E and SI Appendix, Fig. S9 show the controlled edge-to-edge cell spaces of 30, 50, and 90 μm and printing efficiency of more than 96%. The corresponding precision of the cell positions was within ±2.5 μm both horizontally and vertically (SI Appendix, Fig. S10). Because of cell spreading and the cell membrane extension on the substrate, actual distances were slightly shorter in the finished arrays. Therefore, this method will be very useful to precisely generate density-controlled cell patterns (25).

Control of cell-pair distance is remarkably simple with BloC-Printing, providing a potential approach for studying cell–cell interaction (26, 27) and cell fusion (28). By designing trap pairs with edge-to-edge spacing from 5 to 20 μm (SI Appendix, Fig. S11A), corresponding cell pairs with more than 94% printing efficiency were obtained (Fig. 2F and SI Appendix, Fig. S11B). The fluctuation in cell spacing was within ±3 μm horizontally (SI Appendix, Fig. S12). Therefore, sophisticated and high-resolution single-cell arrays could also be made in various shapes, including a concentric square, a spiral square, shapes of the capital letters “THM,” an hourglass, a smiley face, and a ribbon (Fig. 2G and SI Appendix, Fig. S13). Moreover, the BloC-Printing approach also allows for flexibility in printing substrates. In addition to the Petri dish surface, direct printing on ultrathin glass (0.085–0.13 mm thickness) and elastic polyethylene naphthalate (PEN) membranes has also been achieved (SI Appendix, Fig. S14).

BloC-Printing has resolution limits of 5 μm horizontally and 30 μm vertically. In the cell pairing design, cell pairs are isolated (Fig. 2B, G, and SI Appendix, Figs. S11 and S13). The fluctuation in cell spacing was within ±3 μm horizontally (SI Appendix, Fig. S12). Therefore, sophisticated and high-resolution single-cell arrays could also be made in various shapes, including a concentric square, a spiral square, shapes of the capital letters “THM,” an hourglass, a smiley face, and a ribbon (Fig. 2G and SI Appendix, Fig. S13). Moreover, the BloC-Printing approach also allows for flexibility in printing substrates. In addition to the Petri dish surface, direct printing on ultrathin glass (0.085–0.13 mm thickness) and elastic polyethylene naphthalate (PEN) membranes has also been achieved (SI Appendix, Fig. S14).
Figure 2. Various cell arrays generated by BloC-Printing. (A) A bright-field image displays single-cell trapping efficiency in a 6 × 9 cell array. (B) Phase-contrast image of a printed 16 × 44 cell array. Bright field (C) and corresponding fluorescence image (D) of a 3 × 8 cell array. The cell microarray was stained with calcein AM (green) to show live cells and EthD-1 (red) to show dead cells (no dead cells appear in this array) immediately after BloC-Printing, to evaluate cell viability during the procedure. (E) Phase-contrast images of single-cell arrays with intercellular spacing of 30, 50, and 90 μm from left to right. (F) Phase-contrast images of cell pairs with intercellular spacing of 5, 10, and 20 μm from left to right, respectively. (G) Phase-contrast images of various single-cell arrays including a concentric square, a spiral square, capital letters “TMH” (abbreviation for “The Methodist Hospital”), an hourglass, a smiley face, and a ribbon (a bright-field image is also shown for the ribbon). SK-BR-3 cells were used in all images. (Scale bars: 50 μm.)

edge-to-edge distance according to standards in the literature (15, 20) rather than center-to-center, the cell spreading will decrease the final average spacing. Although we predesigned the cell array as 30, 50, and 90 μm edge-to-edge spacings (SI Appendix, Fig. S9) with an estimated cell size at 12 μm, the experimental results showed slightly larger cell spreading than the estimation and exhibited 27.1, 47.9, and 89.9 μm measured ones (SI Appendix, Fig. S10). Herein cells spread more for high-density arrays. Such a variation is most likely because high cell density promotes cell spreading, in light of the high growth factor concentration generated from neighbor cells.

Figure 3. Multiplexed cell arrays generated by BloC-Printing. (A) The BloC-Mold for patterning three types of cells. Red, green, and blue arrows represent the direction of flow of the three types of cells by dye color. (B) Patterning of a 3 × 2 single-cell microarray with red, green, and blue CellTracker-labeled SK-BR-3 cells. (C) Patterning of a 3 × 2 single-cell microarray with MDA-MB-231/GFP, MDA-MB-436/RFP, and MCF-7/GFP cells. Schematic (D) and corresponding micrographs (E) showing the whole process of patterning with two types of cells differentially labeled with green or red dyes. The numbers and arrows in D and E, respectively, represent time and direction of flow. Patterning of a ribbon (F) and cell pairing (G) with red and green CellTracker-labeled cells. The two right-hand panels in G are enlarged views of three cell pairs within the dotted box. (Scale bars: 50 μm.)
contacts) were created between DCs and RCs, using the multiplexed BloC-Printing approach. Dye transfer experiments showed that cells engaged in GJIC more readily through protrusion contact than through body contact (Fig. 4 C–E). Dye transfer was inhibited by the GJIC blocker carbenoxolone, indicating that transfer of dye was indeed occurring through connected protrusions (Fig. 4E and SI Appendix, Fig. S20). This discovery will be useful for future study of cell morphology- and protrusion-related GJIC (36).

Characterization of Cells’ Capability to Extend the Membrane. The ability of cells to generate membrane protrusions plays an important role in numerous biological activities, particularly in cell migration and invasion (37–40), which are mainly mediated by protrusions in the form of filopodia and lamellipodia (41). BloC-Printing provides a rapid and high-throughput method to characterize cell protrusions, including protrusion percentage and extension rate, and cell length, which are challenging to achieve using existing methods. A BloC-Mold containing a hook array with a longitudinal spacing of 200-μm was specifically designed to observe the extension of cell length (SI Appendix, Fig. S21A). Individual cells with long, thin protrusions were clearly visualized after on-chip culture (Fig. 5A and SI Appendix, Fig. S21B). Measurement after 3 h of culture was found to best represent the results as (i) extension of cell protrusions had almost stopped and (ii) cells began to move away from the trap with longer time in culture (SI Appendix, Fig. S21C). In such experiments, BloC-Molds could also be removed leaving the cell protrusions printed in the Petri dishes, if needed (Fig. 5B). We used this method to characterize protrusions of six types of breast cancer cells, including MDA-MB-231, MDA-MB-436, SUM 159, SUM 149, SK-BR-3, and MCF-7 (Fig. 5C).

For all six cell lines, the percentage of cells that elongated, the average cell length, and the average cell-extension rate were calculated and plotted in Fig. 5 D–F and SI Appendix, Fig. S22. It is not surprising that the percentages of cells that elongated (Fig. 5D) for the six cell lines correlate with their reported tumorigenicity (42), with invasiveness increasing from MCF-7 to SK-BR-3, SUM149, SUM159, MDA-MB-436, and MDA-MB-231. The same trend applied to the average cell length (Fig. 5E) and extension rate (Fig. 5F), when the averages were calculated for both elongated and nonelongated cells. In general, compared with luminal-like cancer cells, basal-like cancer cells, especially MDA-MB-231, MDA-MB-436, SUM149, SUM159, MDA-MB-436, and MDA-MB-231, the same trend applied to the average cell length (Fig. 5E) and extension rate (Fig. 5F), when the averages were calculated for both elongated and nonelongated cells. In general, compared with luminal-like cancer cells, basal-like cancer cells, especially MDA-MB-231, MDA-MB-436, and MDA-MB-436, had greater membrane elongation abilities, indicating their stronger migratory abilities (41). There was a slight change in the trend when the averages were calculated for only elongated cells, with MDA-MB-436 having the longest average length of protrusion (SI Appendix, Fig. S22); this seems reasonable, as cells are quite heterogeneous, and quantitation of cell invasiveness still remains a challenge given the complexity of the live-cell system.

BloC-Printing of Individual Primary Cortical Neurons. In addition to efficient printing of cancer and fibroblast cell lines, BloC-Printing can also be used for controllable printing of individual primary neurons. Positioning and addressing individual neurons are desirable for neuronal imaging and studies of signal transduction. Current methods are often limited by the difficulty of long term in vitro culture of individual neurons or the requirement of coculture with glial cells (43). Microfluidic devices have been described for culture of individual neurons for up to 11 d in vitro (DIV), without the use of any coculture or feeder layers (44). Such devices are still difficult to adapt to cell culture Petri dishes or substrates for measurement of neuronal activity because the neurons are retained in the PDMS device, and the PDMS material also requires complicated treatment. Herein, BloC-Printing was introduced to overcome such limitations. First, by heating the BloC-Mold at 110 °C for 60 min and then exposing it to UV light
The red dashed line indicates average cell length before extension of cell.

Fig. 5. Cells’ capability to extend membranes in the BloC-Printing device. (A) The morphology of MDA-MB-231/GFP cells after culture in the BloC device for 3 h. The hook, cell body, and cell membrane protrusions are indicated. (B) The morphology of the printed MDA-MB-436 cells on a Petri dish after removal of the BloC-Mold. (C) Representative images of six types of breast cancer cells after 3 h of culture in the BloC-Printing device. (D) Percentages of cells of the six selected cell lines that have extended their membranes. (E) The average extended cell length for the indicated cell lines. The red dashed line indicates average cell length before extension of cell protrusions. (F) The protrusion-generation rate calculated as the protrusion length divided by the cell extension time. More than 300 cells were counted following 3 h of BloC-Printing device culture. The averages in E and F were calculated for elongated and nonelongated cells combined. The error bars represent the SDs of three independent experiments. (Scale bars: 25 μm.)

for 12 h, one can sterilize and completely cross-link the PDMS. Such a step does not require days of solvent exchange treatment for PDMS, as with earlier studies (44). Second, stopped-flow incubation was adapted to the BloC-Printing of neurons to minimize outside interference and maintain localized concentration of secretions (43). As a result, individual primary rat cortical neurons were successfully cultured for up to 14 DIV in the BloC-Mold (Fig. 6A). The neurons showed normal morphology and clear neurite outgrowth. The confined cell-spraying channel also increased the possibility of autapse formation (6 and 11 DIV) (45). By controlling the number and spacing of hooks (SI Appendix, Fig. S23), single and paired neurons with highly branched dendrites could be obtained at 7 DIV (Fig. 6B). Because neurons adhere to the selected substrates, the fine axons and dendrites could be successfully printed to these substrates via BloC-Printing (Fig. 6C), facilitating future analyses, such as measuring electrical signals via patch-clamp technique.

Conclusions

In conclusion, a unique live single-cell printing method, BloC-Printing, has been introduced. The approach allows for convenient and highly efficient formation of multiplexed single-cell arrays with precise, adjustable cell spacing, sophisticated single-cell patterning, coculture of heterotypic cell pairs, and an elongated cell array. The BloC-Mold can be reused hundreds of times without loss of precision, and single cells can be directly printed on to commonly used materials, including PS and glass cell-culture dishes. This method has been applied to the study of GJIC in heterotypic cell pairs with controlled morphology, rapidly characterizing cells’ ability to extend membranes, and for controlled printing of individual primary neurons. In the future, BloC-Printing may be combined with well-established molecular printing technology (16, 22, 46) to obtain multiplexed single-cell arrays for high-throughput drug screening (47).

Materials and Methods

Design and Fabrication of the BloC-Mold. All designs were drawn with AutoCAD software and printed out as glass photomasks (Photo Sciences Inc.). PDMS BloC-Molds were fabricated by standard photolithography and elastomer molding. We used SPR 220–7 positive photoresist (MicroChem Corp.) to fabricate 12-μm thick channels and SU-8 3025 negative photoresist (MicroChem Corp.) to fabricate 17-μm thick channels. The SPR 220–7 photoresist was spin-coated onto a 4-inch silicon wafer (Silicon Quest International Inc.) at 1,500 rpm (Laurell Technologies Corp., Model: WS-400B-6NPP/LITEAS) for 40 s to form a layer ~12 μm thick. After baking at 75 °C for 3 min and then at 115 °C for 5 min, the wafer was cooled, exposed to UV light for 7 s, and developed. The SU-8 3025 photoresist was spin-coated onto a 4-inch silicon wafer at 4,000 rpm for 60 s to form a layer ~17 μm in thickness. After soft baking at 65 °C for 2 min and then at 95 °C for 10 min, the wafer was cooled and exposed to UV light for 6 s. It was then heated for postexposure baking at 65 °C for 1 min and at 95 °C for 3 min. After cooling down, the wafer was developed and heated for hard baking at 135 °C for 20 min. Finally PDMS (10A:1B; Dow Corning Corp.) was poured onto the photoresist mold and heated at 75 °C for 30 min. After curing, the PDMS was peeled off, cut to the appropriate size, and then punched to form the BloC-Printing device.

Cell Culture and Staining. The cell line SK-BR-3 (ATCC) was cultured in RPMI medium 1640 supplemented with 10% (vol/vol) FBS and 1% penicillin-streptomycin. The cell lines MDA-MB-231/GFP, MDA-MB-436/RFP, and MCF-7/GFP (Cell Bioblasts; SUM 159; Astarand); and HeLa cells and NIH 3T3 fibroblasts (ATCC) were cultured in Dulbecco’s modified Eagle medium supplemented with 10% (vol/vol) FBS and 1% penicillin-streptomycin. The cell line SUM 149 (Astarand) was grown in Ham’s F-12 medium (Life Technologies Corp.) supplemented with 5% (vol/vol) FBS, 1% penicillin-streptomycin, 5 μg/mL insulin, and 1 μg/mL hydrocortisone. All cells were grown in a humidified atmosphere of 5% (vol/vol) CO2 at 37 °C in the cell viability test (Fig. 2D), SK-BR-3 cells were stained with calcine AM and EthD-1 (Life Technologies Corp.), in accordance with the manufacturer’s instructions. In multiplexed BloC-Printing (Fig. 3), SK-BR-3 cells were stained with CellTracker Red CMTPX, CellTracker Green CMFDA, and

Fig. 6. BloC-Printing of individual primary cortical neurons. (A) Morphology of individual neurons from 1 to 14 DIV in the BloC-Printing device. Autapses are observed at 6 and 11 DIV. (B) Single and paired neurons in the BloC-Printing device at 7 DIV. (C) Individual neurons at 9 DIV on the BloC-Printing substrate after the removal of BloC-Mold. The width of channel is 42 μm. (Scale bars: 25 μm.)
CellTracker Blue CMAC (Life Technologies Corp.), following the manufacturer's instructions. In the dye transfer study (Fig. 4), fibroblasts were stained with 4 μM calcein AM for 15 min at 37 °C.

Preparation, Printing, and Culture of Primary Rat Cortical Neurons. Animal tissues were obtained following the protocol approved by Houston Method- odist Hospital Institutional Animal Care and Use Committee. Embryonic day 18 (E18) cortical rat neurons were prepared following established procedures (48). Briefly, cortical neurons were dissected from E18 Sprague–Dawley rats, and dissociated with trypsin to single cells. The dissociated cells were cul- tured within 4 h after dissection, and cell viability was determined to be greater than 95%. Cells were cultured in a medium consisting of neural basal media, B-27 supplement, and Glutamax (Life Technologies). To sterilize and reduce amounts of the un-cross-linked oligomer, which adversely affects neuron viability, before use, the BioC-Molds were heated at 110 °C for 60 min and then exposed to UV light (UV output: 13.9 W) overnight. The PS culture dish was first loaded with 100 μg/mL poly-lysine (Sigma) for 1 h. The poly-lysine solution was then aspirated, and the dish was rinsed once with double-distilled water. When the culture dish was dry, a degassed BioC-Mold was laid on the dish surface to form sealed channels. Cell-free medium was loaded into channels before cell loading. After individual neurons were trapped by the hooks, the medium was refreshed every 12 h.

BioC-Printing Cell Pairs for the Formation of Protrusions. Cell pairs with a protrusion-to-protrusion morphology were obtained using the strategy of sequential cell anchoring. Cells without fluorescent labels (RCs) were first loaded and anchored. After adhesion of the RCs to the substrate, cells labeled with calcein (DCs) were loaded and anchored. After culture, both classes of cells generated protrusions along the wall of the channel that contacted each other. Cell pairs showing body-to-body contact were obtained by simultane- ously loading RCs and DCs and anchoring them in trap pairs with 5 μm of spacing between traps. After cells had adhered for 2 h, they were polarized in the same direction. The BioC-Mold was then removed to allow anchored cells to spread and contact each other in a body-to-body arrangement.

Image Acquisition and Analysis. Bright-field, phase-contrast, and fluorescence images were obtained with an AMG EVOS II digital inverted fluorescence microscope, an Olympus IX81 inverted fluorescence microscope, and a Leica gated stimulated emission depletion super resolution microscope. Movies were filmed on the Olympus IX81 microscope.

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Supporting Information

Block-Cell-Printing for live single-cell printing

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Detailed BloC-Printing Procedures.

Before use, the BloC-Molds were cleaned with deionized water and then dried under pure N\textsubscript{2}. The devices were assembled by placing the BloC-Mold in contact with a polystyrene (PS) or glass dish, or soft polyethylene naphthalate (PEN) membrane (Life Technologies Corp.) and degassed in a vacuum container for 5 min. After degassing, culture medium was pumped into the channels with care to avoid bubble formation. For the cell test, adherent cells grown in culture dishes were detached with trypsin. After centrifugation at 1,000 rpm for 1 min, the cells were suspended in culture medium at a density of 10\textsuperscript{6}–10\textsuperscript{7} cells/ml. Suspended cells were placed in the inlet and pumped into the channels. Flow was maintained by connecting the outlets to a negative-pressure control system, and flow rates were kept to less than 100 μm/s to avoid damaging the cells. After cells were anchored by the traps, unanchored cells were washed away by replacing the cell suspension with culture medium. The negative pressure was turned off to allow in situ cell adhesion to the surface of the culture dish in a cell incubator with 5% CO\textsubscript{2}. After cell adhesion occurred (typically 30–60 min), the BloC-Mold was carefully detached from the cell culture dish to obtain the cell array. The BloC-Mold may be cleaned with isopropanol and water and reused hundreds of times without loss of function.

For sequential cell anchoring by long-tailed traps (Fig. 3F-G), the first cell type was loaded from top to bottom. After adhesion of the first cell type, the second cell type was loaded from the bottom to the top. Other steps were the same as described above.

Theoretical Calculation of Fluid Resistance.

The fluid resistance along Path 1 and Path 2 (Fig. S2A) was estimated using the previously described (1, 2) formula:

\[
\frac{R_2}{R_1} = \left(\frac{L_2}{L_1}\right) \cdot \left(\frac{a_2 + b_2}{a_1 + b_1}\right)^2 \cdot \left(\frac{a_1}{a_2}\right)^3 \cdot \left(\frac{b_1}{b_2}\right)^3 \cdot \left(1 + \frac{5a_1}{b_2} - \frac{5b_1}{6a_2}\right)
\]

Here, \(R_1\) and \(R_2\) are the fluid resistance of Path 1 and Path 2, respectively. Other variables comparing path 1 versus path 2, respectively, are path length \(L_1\) (18 μm) versus \(L_2\) (8 μm), path depth \(a_1\) (12 μm) versus \(a_2\) (12 μm), and path width \(b_1\) (22 μm) versus \(b_2\) (3 μm). Therefore, \(R_2\) is 41-fold greater than \(R_1\), and individual cells prefer to flow along path 1 following the Zweifach-Fung bifurcation law. When a concentrated group of cells is applied, temporary blockage of Path 1 forces cells to flow along Path 2, following the same law in a dynamic manner.

Fig. S1. Design and structure of a hook-shaped single-cell trap. (A) Detailed dimensions of the trap in a BloC-Mold. (B) Cross-sectional image showing the channel height of 12 μm.
**Fig. S2.** Flow mechanism in BloC-Printing. (A) Ratio of the fluid resistance around a single trap. The fluid resistance along Path 2 ($R_2$) is 41-fold greater than that along Path 1 ($R_1$), according to a theoretical calculation. (B) Due to the large gap in Path 1 (22 μm) and cell flexibility, even the largest cells (marked by asterisk, 26 μm in width) can smoothly flow through the channel without blocking it. (C) Trapping cells of four different sizes (indicated by numbers 1–4). (D) By slightly increasing the negative pressure (from −1 psi to −3 psi), trapped cells can be precisely positioned. SK-BR-3 cells are shown in all images, and scale bars are 20 μm.
**Fig. S3.** Large-scale image of single-cell trapping. The array is composed of $13 \times 18$ traps with SK-BR-3 cells. Scale bar is 40 $\mu$m.
**Fig. S4.** Adhesion of SUM 159 cells to substrates outside and inside the BloC-Printing device. Suspended cells are separately seeded onto polystyrene (PS) and glass substrates by randomly spreading with a pipette (A and C) or by precisely positioning with a BloC-Mold (B and D). Cell morphology is monitored for the first 2 h to assess cell adhesion.
Fig. S5. Adhesion of 3T3 fibroblasts to substrates outside and inside the BloC-Printing device. The same procedure was used as in Fig. S4.
Fig. S6. Analysis of the precision of BloC-Printing. (A) The 16 × 44 printed single-cell microarray, with yellow lines to indicate the fluorescence-intensity profiling. (B–C) A plot of cellular fluorescence intensity versus cell position. Due to cellular heterogeneity, variations in fluorescence were observed, but the positions of the cells are very reproducible, determined precisely by the designed trap microarray. SK-BR-3 cells were used, and the scale bar is 100 μm.
**Fig. S7.** Long-term cell viability and growth assay inside the BloC-Printing device with the integration of automatic flow of medium. (A) Scheme of the gravity-induced automatic flow of medium. Medium at the inlet is refreshed every 12 h. (B-D) MDA-MB-231/GFP cells continue to divide and propagate within the BloC-Mold for 48 h. (E) Striped cell pattern with 20-µm spacing is well retained after removal of the BloC-Mold at 48 h. (F) The spacing disappears due to cell migration 12 h after removal of BloC-Mold. (B–F) Left panels, bright-field image; right panels, fluorescence image.
Fig. S8. Long-term cell viability and growth assay outside BloC-Mold. Cells continue to divide and propagate on the polystyrene cell culture dish surface over 5 d of observation. SK-BR-3 cells were used, and scale bars are 50 μm.
**Fig. S9.** BloC-Printing spatial resolution. (A) A trap microarray containing three trap spacings of (from left to right) 30 µm, 50 µm, and 90 µm, respectively. (B) BloC-Printing efficiency of cells with varied trap spacings. The average printing efficiencies are 96% for both the 30-µm and 50-µm cell spacing, and 97% for the 90-µm cell spacing. SK-BR-3 cells were used in the experiments, and the error bars represent the standard deviations of three independent BloC-Printings.
Fig. S10. Precision analysis for three different cell spacings. (A) A schematic cell array with horizontal spacing (HS) and longitudinal spacing (LS) marked with solid and dashed lines, respectively. (B) Measured HS and LS of 50 cells in finished cell arrays. Because of cell spreading and cell membrane extension on the substrates, actual distances are shorter in the finished arrays. The average distances for the 90-μm array are 89.9 μm (HS, blue) and 88.1 μm (LS, dotted blue); the average distances for the 50-μm array are 47.9 μm (HS, green) and 48.5 μm (LS, dotted green); and the average distances for the 30-μm array are 27.1 μm (HS, red) and 27.1 μm (LS, dotted red). SK-BR-3 cells were used in the experiments.
Fig. S11. BloC-Printing of cell pairs. (A) Design of a BloC-Mold with trap pairs separated by 5 μm, 10 μm, and 20 μm (from left to right, respectively). (B) Efficiency of cell pairings in the three trap spacings. The average printing efficiency was 94% for the 5-μm trap spacing, 94% for 10-μm, and 95% for 20-μm. SK-BR-3 cells were used in the experiments, and error bars represent the standard deviations of three independent BloC-Printings.
Fig. S12. Precision analysis of cell pairs. (A) Schematic showing distance between paired cells. (B) Measured intercellular distances for 50 different cell pairs. Because of cell spreading and cell membrane extension on the substrate, actual distances are shorter in the finished arrays. The results show average distances of 2.1 μm (red), 7.2 μm (green), and 16.3 μm (blue) for 5-μm, 10-μm, and 20-μm trap spacings in the BloC-Molds, respectively. SK-BR-3 cells were used in the experiments.
Fig. S13. BloC-Printing generates sophisticated cell patterns. Polydimethylsiloxane (PDMS) BloC-Molds of designed patterns including a concentric square, spiral square, capital letters “TMH”, hourglass, face, and ribbon. Scale bars are 50 µm.
**Fig. S14.** BloC-Printing on selected substrates: a glass-bottom Petri dish from MatTek, versus a polyethylene napthalate (PEN) membrane surface. (A) The assembled BloC-Printing device consisting of BloC-Mold and glass-bottom dish. The channels are filled with red dye to aid visualization of microfluidic network. (B) MDA-MB-231 cells are patterned on the ultrathin glass surface of the glass-bottom dish after 40 min of trapping. (C) NIH 3T3 fibroblasts are patterned on the PEN surface, after 2 h of trapping. Scale bar is 40 μm.
Fig. S15. An enlarged cell trap for capturing relatively large cells.
Fig. S16. Long-tail trap. (A) Detailed dimensions of a single long-tail trap. BloC-Mold containing long-tail traps for patterning (B) a ribbon, and (C) a 5-μm cell pairing with cells of two different colors. Arrows (red and green) represent direction of flow red and green cells. Scale bars are 50 μm.
Fig. S17. Construction of protrusion-connected single-cell arrays. NIH 3T3 fibroblast cell protrusion connection arrays on (A) PS surface and (B) PEN membrane, cultured in the BloC-Printing device for 3 h, followed by removal of BloC-Mold. (C) HeLa cell protrusion connection arrays on PS surface, cultured in the BloC-Printing device for 24 h, followed by removal of BloC-Mold. Scale bars are 50 μm.
Fig. S18. Dual-direction flow allows patterning of multiple cell types. The microscopic images (left to right) show the BloC-Mold, the flow of the first cells arrayed, and the flow of the second cells arrayed. The design contains long-tailed traps to obtain protrusions of heterogenic fibroblast cell pairs. The arrows represent direction of flow, and the scale bar is 20 μm.
**Fig. S19.** Dye transfer via gap junction intercellular communications (GJIC). (A) The forward protrusion of a donor cell (DC) is in contact with the backward protrusion of a recipient cell (RC), and the RC fluorescent signal increases significantly after 10 min of calcein transfer. (B) Generation of a tunneling nanotube with minimal width of approximately 400 nm between a DC and a RC, after 180 min of dye transfer. (C) Calcein transfer through both forward and backward protrusions. The numbers represent time in minutes after protrusion contact. (A–C) Left panels, bright-field image; right panels, fluorescence image. Scale bars are 20 µm in (A and C) and 1 µm in (B).
Fig. S20. Dye transfer via GJIC-inhibited fibroblasts. Inhibition was carried out by treatment with 200 μM carbenoxolone (CBX). Representative bright-field and fluorescence images of GJIC-inhibited fibroblasts containing (A) body–to-body versus (B) protrusion-to-protrusion contacts. DCs, donor cell; RC, recipient cell. Scale bars are 20 μm.
Fig. S21. Extension of cell protrusions. (A) The BloC-Mold to measure membrane extension with a hook spacing of 200 μm. (B) MDA-MB-231/GFP cells showed elongated morphology 3 h after printing. (C) Some MDA-MB-231/GFP cells migrated from their original positions, indicated by the red dotted line, along channel walls 7 h after printing. Scale bars are 50 μm.
Fig. S22. Cell capability to extend membranes in the BloC-Printing device. (A) Average cell length of the elongated cells and (B) average protrusion extension rate of the elongated cells. More than 300 cells were counted after 3 h of BloC device culture. Error bars represent the standard deviations of three independent experiments.
**Fig. S23.** The BloC-Mold for neuron printing. Individual rat primary cortical neurons are trapped by hooks. Scale bar is 25 μm.
Supporting Information
Zhang et al. 10.1073/pnas.1313661111

Movie S1. Microscopic movie of cell loading and single-cell capture by a Block-Cell-Mold (BloC-Mold) with short-tailed hooks (10× objective lens).

Movie S2. Zoomed microscopic movie of cell loading and single-cell capture (20× objective lens).
Movie S3. Single-cell capture by a BloC-Mold with long-tailed hooks (20x objective lens).

Movie S4. Cell capture through reverse flow over hook pairs preloaded with cells on one side (20x objective lens).

Other Supporting Information Files

SI Appendix (PDF)