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RESEARCH ARTICLE

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High-throughput formation of miniaturized cocultures of 2D cell monolayers and 3D cell spheroids using droplet microarray

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Abstract

Most of the biological processes, including cell signaling, cancer invasion, embryogenesis, or neural development, are dependent on and guided by the complex architecture and composition of cellular microenvironments. Mimicking such microenvironments in cell coculture models is crucial for fundamental and applied biology investigations. The ability to combine different cell types grown as both two-dimensional (2D) monolayers and three-dimensional (3D) spheroids in specific defined location inside a microculture environments is a key towards in vitro tissue modeling and towards mimicking complex in vivo cellular processes. In this study, we introduce and investigate a method to create in vitro models of 2D cell monolayers cocultured with 3D spheroids in defined preorganization. We demonstrate the possibility of creating such complex cellular microenvironments in a high-throughput and automated manner by creating arrays of such droplets containing prearranged 2D and 3D cellular microcolonies. Furthermore, we demonstrate an application of this approach to study paracrine propagation of Wnt signaling between 2D and 3D cellular colonies. This method provides a general approach for the miniaturized, high-throughput, and automated formation of complex coculture cellular microarchitectures that will be useful for mimicking various in vivo complex cellular structures and for studying complex biological processes in vitro.

INTRODUCTION

In vivo, tissues are spatially organized across functionally and morphologically distinct but interacting compartments.[1,2] The hierarchical positioning or architecture of cells within the tissue determines cell-to-cell contacts and paracrine signaling gradients and ultimately defines the cellular phenotype and function of each tissue unit.[3–7] Therefore, to recreate in vitro multicellular interactive tissue-like models for fundamental research, regenerative medicine, and disease modeling, there exists a need for precise spatial positioning of cells.[8–10] Spatially separated coculture systems, where different cell types organized in different multicellular structures (such as two-dimensional [2D] or

three-dimensional [3D]) can grow and develop together and interact on cell–cell or paracrine levels, are important for various crucial biological applications. There are several platforms that have been developed for 2D/3D cell coculture systems. For example, coculture of 3D tumor spheroids and 2D stromal cells such as fibroblasts in hydrogels while being spatially separated showed better modeling of epithelial–mesenchymal transition and cancer invasion.[11–13] Inserts with permeable membranes (such as Transwell® plates) made for microtiter plates are often used for coculture of two and more types of cells for commonly used in vitro cell migration, adhesion, and invasion assays[14,15] or the creation of more complex cell models, such as the blood-brain-barrier (BBB).[16] However, most of these assays are performed on 2D monolayer cell culture models. Inspired by the Transwell® system, a membrane-bottomed microwell (MBM) for noncontact coculture of 2D–3D cells was developed. In this system, 2D cocultured feeder cells (STO, a mouse embryonic feeder cell line) were used to facilitate the successful formation and culturing of a spheroid culture of spermatogonial stem cells (SSCs) via paracrine signaling of STOs.[17] Although this system achieves spatial separation of the two cell types (2D cell sheets and 3D spheroids), this system requires a large number of cells and is not miniaturized. In another study by Järvinen et al., a microfluidic device was fabricated that allowed for concurrent coculture of human hepatocytes in the form of 2D monolayer and 3D spheroids. This format is required for assessing drug response concurrently in 2D and 3D cell culture environments. Cells behave and respond differently when they are cultured in 2D and 3D formats, with 3D cell culture models more closely representing in vivo situations.[18] However, microfluidic devices need to be highly customized and are complicated to prepare. Li and colleagues[19] created a concurrent 2D and 3D cell culture array using micropatterning in a culture dish, which allowed the formation of an array of islands containing cell monolayer on the bottom and spheroids on the top. In this method, 2D cell monolayers and 3D cell spheroids were cultured in the same solution environment, thus affecting the paracrine and signaling of cells of different morphologies. In another work, Stevens et al.[20] used a two-step 3D multicompartmental cellular patterning to concurrently encapsulate hepatic aggregates and endothelial cells in hydrogels in a controlled manner. Using this system, the authors demonstrated that cocultures of hepatocytes derived from primary or induced pluripotent stem cells with nonparenchymal cells can modulate hepatic functions. These studies show the importance of such in vitro 2D/3D cell coculture models for answering various biological questions. Despite the existence of platforms enabling concurrent coculturing of cells in 2D and 3D formats, no universal miniaturized and high throughput platform for precise localization of 3D spheroids and 2D cultured cells exists.

In this study, the droplet microarray (DMA) was used as the technology for growing cells in both 2D cell monolayers and 3D cell spheroids on the same microarray in nanoliter droplets in a high-throughput manner. The DMA is a miniaturized platform for cell culture and high-throughput screening developed by our group and applied in drug and transfection reagents screening,[21,22] cell culture and spheroid preparation,[23,24] as well as bacteria and antimicrobials screening.[25,26] The DMA has regular arrayed hydrophilic spots separated by superhydrophobic boarders. These spots are capable of confining droplets of nanoliter volumes. By using a commercial liquid dispenser to print cell suspension on the designated spots in the DMA, 2D or 3D cell cultivation in a low-volume and high-throughput manner can be achieved. Recently, we introduced a programmable Merging of Adjacent Droplets (proMAD) method for controllable merging of neighboring droplets on flat surfaces of DMA for the generation of multispheroid architectures.[27] Here, the proMAD method was used as a facile approach to spatially organize 2D cell monolayers and 3D spheroids in single droplets. Using this methodology, the potential for studying Wnt signaling was demonstrated. The proMAD technique for scaffolds-free high throughput combination of various tissue types can be applied for answering various biological questions.

RESULTS AND DISCUSSION

A method for creating an array of droplets containing coculture of 2D monolayer cells with 3D spheroids on demand was established in a high throughput manner. Parallel cultures of cells in 2D and 3D architectures via a two-step method were prepared. In this study, a DMA chip composed of an array of 14 × 14 hydrophilic square spots of 1 mm × 1 mm separated by 500 µm superhydrophobic borders was used. In the first step, 200 nl of medium containing 150 HepG2 cells was dispensed into every second column of DMA (Figure 1a and Supporting Information: Figure S1). Cells were cultured for 24 h to let them adhere to the surface. Afterward, 200 nl medium containing 200 HEK 293T was printed in the columns left empty during the seeding of HepG2 cells (Figure 1a). To let HEK 293 T cells form spheroids, the DMA slide was turned upside down to generate "hanging droplets" (Supporting Information: Scheme S1). Two days later HEK 293T cells formed spheroids, while HepG2 cells stayed attached to the surface. Therefore, an array containing both 2D monolayer cells and 3D cell spheroids was developed (Figure 1c). By addressing each droplet using an automated cell dispenser, it is easy to combine different cell types in 2D and 3D formats in separated droplets on one array (Figure 1b–e).

To combine cells grown in a monolayer with 3D spheroids, the previously developed droplet merging technique was used.[27] By dispensing 900 nl of cell culture medium into each of two neighboring droplets, the droplets can merge together over the hydrophobic border resulting in one droplet covering two hydrophilic spots (Figure 1a,g). Using this method, a coculture of cells in the form of 2D monolayer and spheroids in one droplet was developed (Figure 1f,g) in which cells from both culture types do not have physical contact but can have paracrine interactions (Figure 1h,i). Therefore, it was demonstrated that the droplet merging method can be used to combine 2D cell layers and 3D spheroids in a programable and high-throughput manner.

To study the merging process of two adjacent droplets, we explored the stability of droplets with different volumes using a contact angle meter and optical microscopy (Figure 2). In this work, the pattern of the DMA is composed of 14 × 14 hydrophilic square spots (1 mm × 1 mm each) that are separated by 500 µm

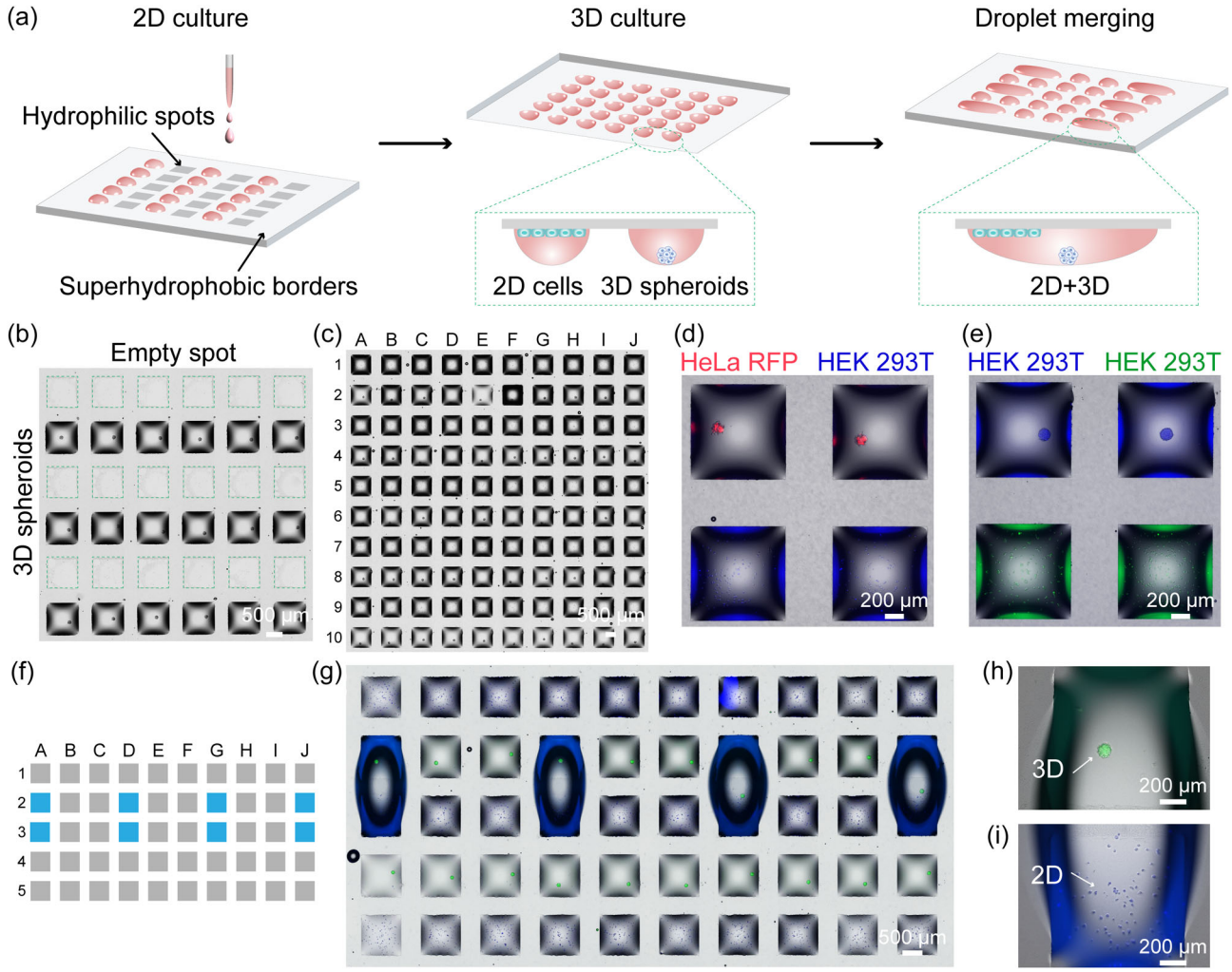

FIGURE 1 Engineering platform for parallel and combinatorial investigation of cells cultured in two- and three-dimensional (2D and 3D, respectively) cellular architectures. (a) Schematic representation of workflow for creating a 2D/3D cell array. First, cells were printed in designated rows and incubated overnight to allow the cells to form a 2D monolayer. Second, the remaining spots were filled with cell suspension and incubated in an inverted position ("hanging droplet" method) to obtain 3D cell spheroids. Finally, the adjacent droplets were merged to combine the 2D and 3D cell architectures. (b) Representative microscope image of a droplet microarray (DMA) setup containing HEK 293T spheroids on designated rows. (c) Representative microscope image of DMA containing both 2D and 3D cell culture in separated droplets. Rows 1, 3, 5, 7, 9 contain 2D cultured HepG2 cells, while rows 2, 4, 6, 8, 10 contain 3D HEK 293T spheroids. (d) Representative fluorescent image of adjacent droplets containing HEK 293T cells as 2D monolayer (blue) and HeLa spheroids (red). (e) Representative fluorescent image showing neighboring droplets containing HEK 293T cells as 2D monolayer (green) and HEK 293T spheroids (blue). (f) Schematic representation of DMA in which adjacent spots marked in blue were merged. (g) Microscope image of DMA with merged droplets containing both 2D HepG2 cells and 3D HEK 293T spheroids. Representative microscope image of 3D spheroids (h) and 2D cells (i) in one merged droplet.

superhydrophobic borders. By using a noncontact low-volume dispenser, it was possible to dispense volumes from 50 to 1100 nl in precise locations. The hydrophilic areas and superhydrophobic borders exhibit great differences in wettability whereby simple application of an aqueous solution results in immediate and spontaneous formation of multiple, separated droplets (Figure 2a). Combined with the noncontact liquid dispenser, solutions or media can be deposited individually and precisely in hydrophilic areas. Through additional dispensing of higher volumes, two separated droplets can be merged. As shown in Figure 2b,c, the 250 nl water droplets were well confined in the spots due to the limitation of superhydrophobic borders. These droplets are stable and separated by the hydrophobic regions. Upon increase of the volume of the droplet, the apparent water contact angle (θ_a) of the droplet begins to increase because of the nonwettable hydrophobic regions. Once the volume of each neighboring droplet reached 1100 nl, the two droplets fused into a new droplet (Figure 2d,e). The θ_a of the droplet ranged from 38° ± 4° to 127° ± 1° as the volume of the droplet increased, and the final merged droplet had an θ_a of 85° ± 1°. The possibility of merging different numbers of droplets, which demonstrates the flexibility and high combinatorial potential of the DMA platform, was shown (Supporting Information: Figure S2 and

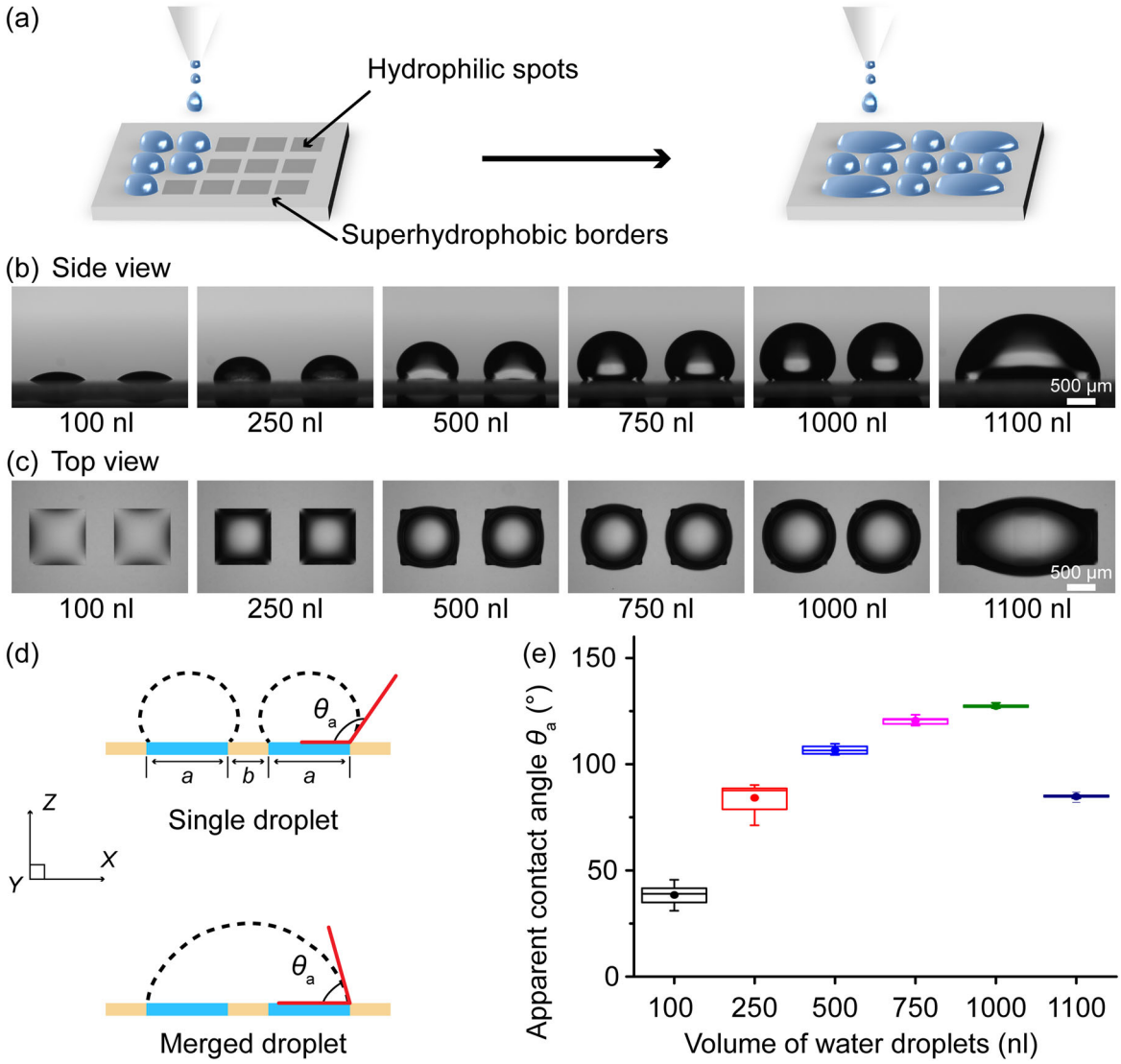

FIGURE 2 Controlled merging of two neighboring droplets. (a) Schematic representation of dispensing and merging of neighboring droplets using a noncontact dispenser. Side view (b) and top view (c) of water droplets of different volumes ranging from 100 to 1100 nl on two neighboring hydrophilic spots of DMA. The DMA contained square hydrophilic spots of 1 mm × 1 mm, and the superhydrophobic border between edges of two spots of 500 µm. (d) Schematic representation of single and merged water droplets confined on hydrophilic spots. (e) The graph showing apparent water contact angles (θ_a) of droplets with different volumes ranging from 100 to 1100 nl (n = 10). DMA, droplet microarray.

Supporting Information: Table S1). Taken together, these data demonstrate the feasibility and stability of merging adjacent droplets by controlling the droplet volume.

The viability of cells and intactness of 2D monolayer through the entire workflow of creating 2D/3D cell arrays were investigated (Figure 3). In this workflow, cells were incubated on the DMA for 24 h to allow them to adhere to the surface (Figure 3a). Therefore, whether these adherent cells would detach from the DMA surface after being cultured in an inverted position in "hanging drop" method was investigated (Figure 1a). As shown in Figure 3b, the cells stayed adhered to the surface after 3 days of being cultured in an inverted position. After 3 days of culture, on average of 1.5 cells detached from the DMA surface in which only 1% of the total number of cells in the droplet (~150 cells) were found as shown in Figure 3b,c. In addition, the process of droplet merging did not significantly affect cell adhesion and intactness of the cell monolayer (Figure 3d). The cell culture medium used during this process did not cause cell detachment from the surface of the DMA (Figure 3e).

As mentioned above, a controlled merging of individual neighboring droplets requires additional printing of considerable volumes, such as 900 nl (Figure 2). Thereby, these high volumes and the pressure used during printing can influence the viability of the cells that were precultured in the respective droplets (Figure 3f). To assess the influence of the printing procedure on cell viability while merging, HeLa cells were printed on individual spots in the DMA and cultured for 24 h in a monolayer culture. Cell viability was assessed

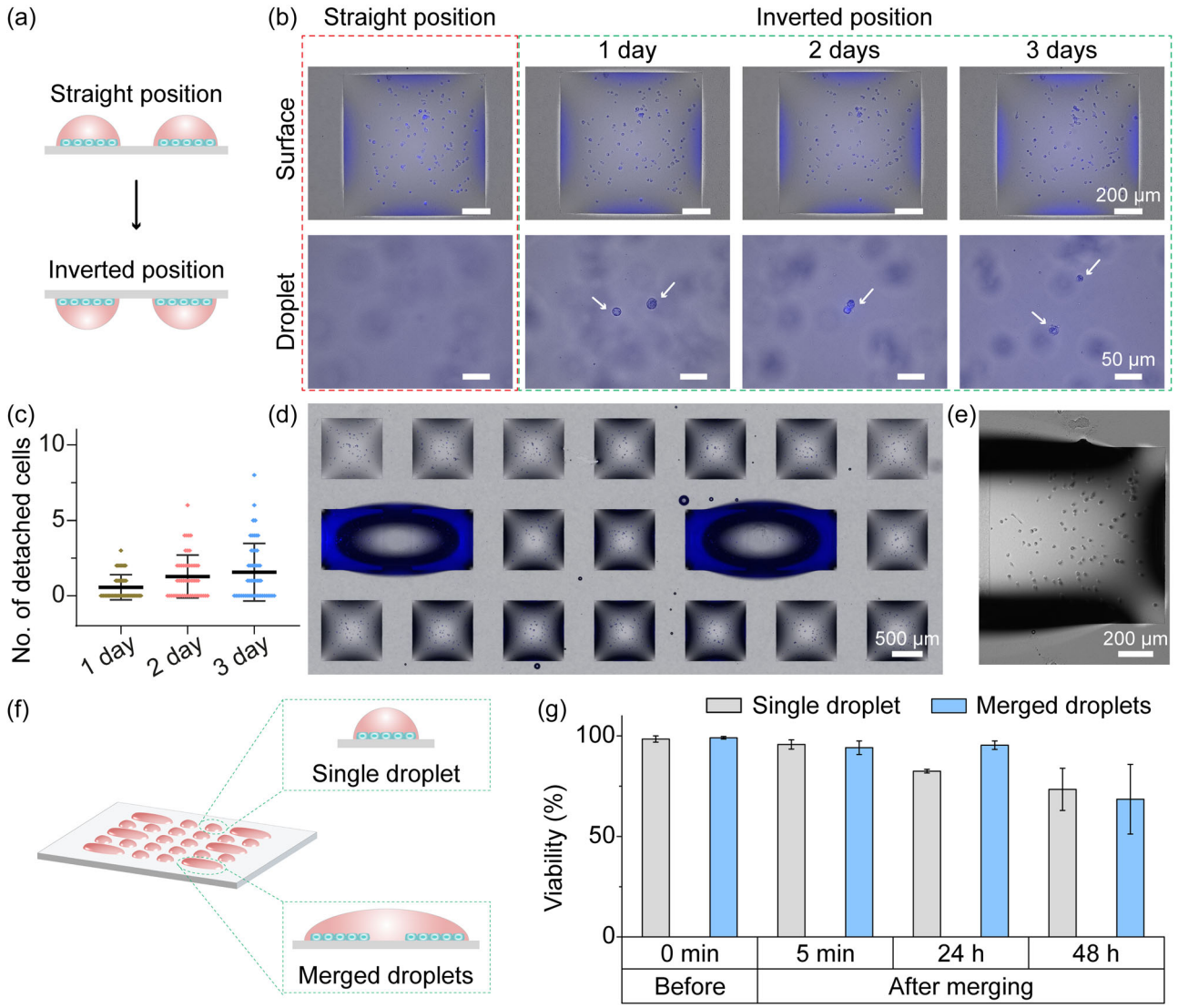

FIGURE 3 2D monolayer cell culture in merged droplets on the DMA. (a) Schematic representation of the workflow of culturing 2D monolayer cells in straight and inverted position. (b) Representative microscope images of HepG2 cells adhered on DMA (top panel) and detached cells in droplets after 1, 2, and 3 days of culture in inverted position (bottom panel). The white arrows show cells in the droplet, which detached from the surface. (c) The graph showing the number of cells detached from the surface 1–3 days after culturing cells on DMA surface in inverted position ($n = 50$). (d) Microscope image of DMA with single and merged droplets containing HepG2 cells cultured in monolayer. (e) Close up image of a single hydrophilic spot containing HepG2 cells in monolayer as a part of merged droplet. (f) Schematic representation of workflow of merging of two neighboring droplets containing 2D cultured cells. (g) Viability of HeLa cells on DMA before merging procedure and at 5 min and 24 and 48 h after the merging of two neighboring droplets. 2D, two-dimensional; 3D, three-dimensional; DMA, droplet microarray.

via Hoechst (total cell number) and propidium iodide (dead cells) staining before the merging procedure and at 5 min and 24 and 48 h after the merging of two neighboring droplets containing HeLa cells (Figure 3g). Viability was thereby estimated in single nonmerged (control) and merged droplets (sample). Cell viability before merging was about 98% in both control and sample droplets. Directly after the merging procedure, cell viability decreased slightly in control and sample droplets by 2% and 4%, respectively (Figure 3g). This decrease, even though very slight, might reflect the effect of evaporation or printing procedure on cells. No significant differences in viability of cells at 24 and 48 h after merging in control and sample droplets were detected (Figure 3f). This finding indicates that no long-term effects of the merging procedures on the viability of cells occurred. Taken together, our results demonstrate that the entire workflow of creating 2D/3D cell culture arrays (Figure 1a) does not produce any negative effect on cells.

As a next step, the possible effects of the culturing cells in straight (upright) and inverted positions on DMA in addition to the droplet merging process on the formation and integrity of cell spheroids were evaluated. The potential of the DMA to serve as a miniaturized microarray for the formation of 3D spheroids was recently demonstrated.[23] First, HEK 293T cells on the DMA in an

inverted position (Figure 4a) were cultured for 2 days. Without using any coating, the formation of several cell spheroids rather than a single spheroid per droplet and many cells attached to the surface of DMA was observed (Figure 4b). Afterward, hydrophilic spots of DMA were pre-coated with anticell adhesion coating to reduce the adhesion of cells on the surface (Figure 4b,c). Formation of a single spheroid per droplet after coating the spots with 25, 50, 75, and 100 nl of antiadhesive solution (details in Materials and Methods) after 2 days of culturing cells on the DMA in an inverted position ("hanging drop method") is shown in Figure 4c.

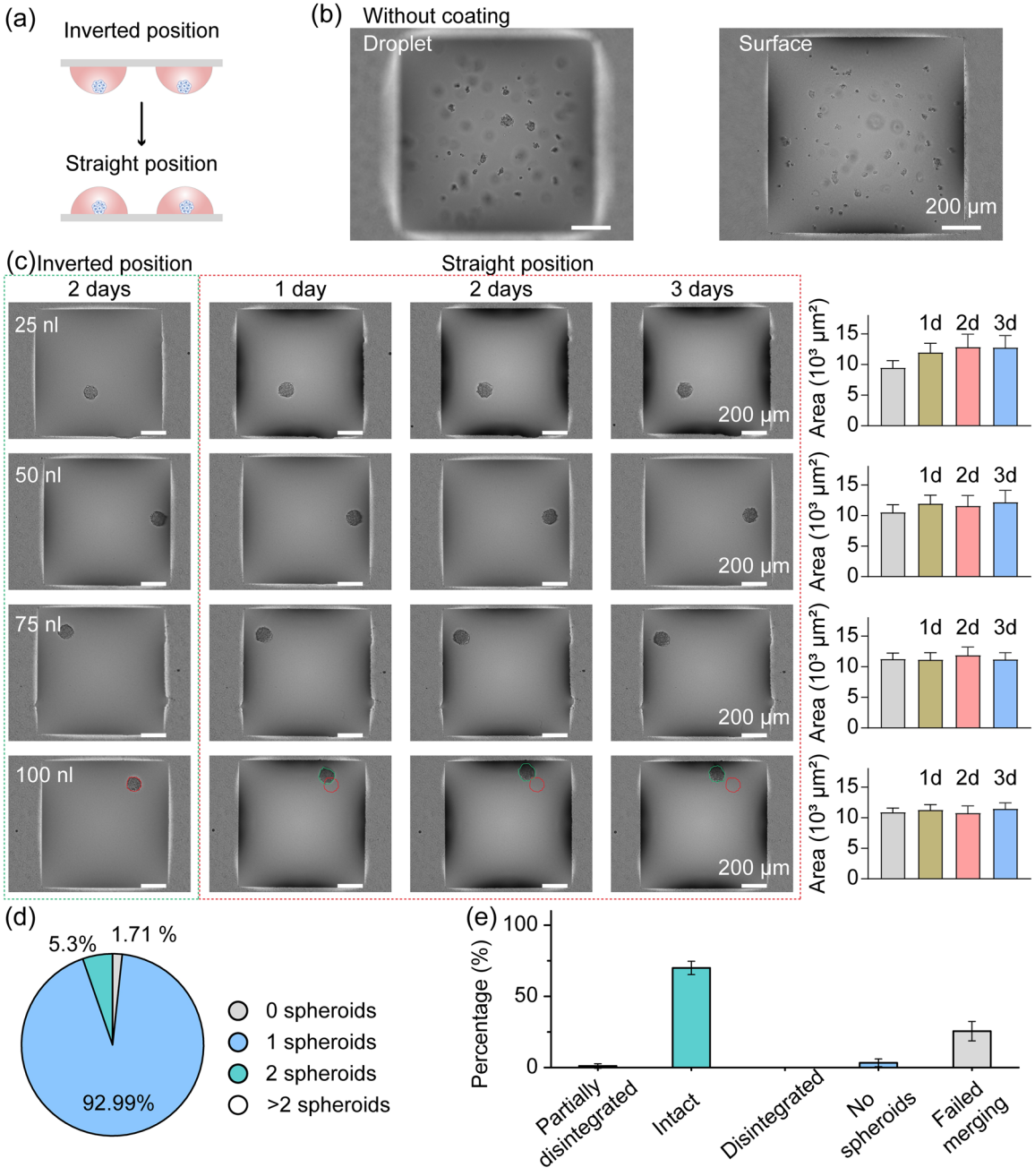

FIGURE 4 3D spheroid culture in merged droplets on the DMA. (a) Schematic representation of the workflow of culturing 3D spheroids in inverted and straight positions. (b) Cell clusters formed by HEK 293T cells in 200 nl droplet on the noncoated spots. (c) Representative microscope images of HEK 293T cell spheroids formed on spots coated with 25, 50, 75, and 100 nl of antiadhesive solution two days after cell seeding (left panel). Representative microscope images of spheroids cultured on DMA in a straight position ($n = 42$). The red and green circle sin the fourth row represent the original and current position of the spheroid, respectively, demonstrating that the spheroids do not adhere to the surface even after 3 days of culture in straight position. (d) Pie chart showing the percentage of droplets containing 0 (no spheroid formation), 1, 2, and more than 2 spheroids per droplet. (e) Graph showing the percentage of droplets containing intact, partially disintegrated, disintegrated, and no spheroids in successfully merged droplets in addition to the percentage of droplets that failed to merge. 2D, two-dimensional; 3D, three-dimensional; DMA, droplet microarray.

To investigate if formed spheroids can keep their integrity after culturing them in a straight position, spheroids in an inverted position (method of "hanging droplet") for 2 days were first formed and then cultured in a straight position for 3 more days (Figure 4a,c). It was observed that cell spheroids did not adhere or spread to the surface of DMA after 3 days of culture in a straight position. Moreover, these cell spheroids were not fixed on the DMA surface and freely moved within the droplet (Figure 4c). It was observed that the mean area of cell spheroids formed on 100 nl coating increased by 5.2% (from 10.8×10^3 to 11.4×10^3 μm^2) compared to 25.9% on 25 nl coatings. Thus, 100 nl of anticell adhesion coating was selected for all further experiments. Taking HeLa cells as a model, the array of single spheroids by dispensing 200 nl per hydrophilic spot was observed, and it was found that around 93% of spots contained a single spheroid (Figure 4d and Supporting Information: Figure S3). Next, we investigated the integrity of spheroids after merging of neighboring droplets containing spheroids (Supporting Information: Figure S3). After droplet merging, it was observed that no more than 1% of spheroids had disintegrated (Figure 4e), indicating that the droplet merging process has no effect on the integrity of 3D spheroids. Taken together, the formation of single-spheroid arrays can be demonstrated and culturing them in a straight position and merging two adjacent droplets does not compromise the integrity of spheroids.

To demonstrate the applicability of 2D/3D cell arrays on the DMA for the study of cellular signaling, we tested the propagation of Wnt signals between the cellular compartments. Wnt signaling plays an important role in multiple biological processes, including embryonic stem cell development, tissue regeneration, and cell differentiation.[28–31] Since the precise mechanism of how Wnt signals are propagated within tissues is still debated, it is important to have a platform that enables modeling the Wnt signaling in vitro by combining different types of cells on demand, for example, cells cultured in monolayer and spheroids. In our experiments, we used two different cell types in 2D/3D cell array: (1) a HEK 293T cell line expressing and secreting Wnt-3a protein and (2) a HEK 293T cell line harboring a TOP-GFP fluorescent reporter for monitoring activation of Wnt signaling in these cells.[32–35] First, a 2D/3D cell array containing HEK 293T spheroids producing Wnt-3a and a monolayer of Wnt reporter HEK 293T cells was generated (Figure 5a). For this, 200 HEK 293T-TOP-GFP cells in every second row of DMA were printed and allowed to attach for 24 h. Next, 200 Wnt-3a transfected HEK 293T cells were printed in the remaining rows in the DMA to generate Wnt-producing spheroids. Two days after spheroid formation, the droplets containing 2D Wnt reporter cells and Wnt-3a producing spheroids were merged pairwise in a high throughput manner. The activation of Wnt signaling was monitored 24, 48, and 72 h postfusion by measuring the fluorescence intensity of 2D cultured reporter cells (Figure 5b). The integrated density (the product of mean fluorescent intensity and area of cells) of reporter cells was 99.2 ± 38.0 and 297.1 ± 91.0 after 48 and 72 h droplet merging and coculture initiation, respectively (Figure 5b). After 72 h of coculture, the 2D reporter cells showed a clear increase in green fluorescent protein (GFP) fluorescence, indicating robust activation of Wnt signaling in 2D cultured cells (Figure 5c). To account for nonspecific background activation of Wnt signaling, fluorescence was also measured in HEK 293T-TOP-GFP cells that were combined with control HEK 293T cell spheroids not expressing Wnt-3a (Figure 5d). The control experiment showed only weak area of GFP intensity (Figure 5b,d) with the integrated densities of 8.1 ± 3.7, 18.5 ± 12.4, and 35.7 ± 14.5 at 24, 48, and 72 h postfusion, respectively.

We next tested the propagation of Wnt signaling from 2D-producing cells to 3D reporter spheroids (Figure 5e). Wnt signaling activity was monitored at 24, 48, and 72 h postfusion by measuring fluorescence intensities of 3D spheroids (Figure 5f). The integrated density of reporter spheroids was 407.3 ± 196.9 and 605.5 ± 214.8 after 48 and 72 h coculture, respectively (Figure 5f). After 72 h, the 3D cell spheroids showed robust GFP activation (Figure 5g). In the control experiment in absence of Wnt-3a ligand, the maximum integrated density was 68.2 ± 35.7 after 72 h of coculture (Figure 5h). In summary, it was demonstrated that a droplet merging method is a powerful tool for studying paracrine Wnt cell signaling between 2D and 3D cultured cells.

CONCLUSION

In this study, we demonstrated a method based on a droplet merging methodology for high-throughput generation of spatially organized 2D monolayer cells cocultured with 3D spheroids in individual submicroliter droplets on the DMA platform. An automated and programmable method for generating cell microenvironments by merging neighboring droplets on an array was developed. This method was used to create arrays of droplets containing both 2D cell layers and 3D cell spheroids. In this system, 2D and 3D cell colonies do not physically contact and interact through the paracrine signaling system. This 2D/3D cell array was used to demonstrate propagation of Wnt signaling from Wnt-3a ligand-producing spheroids to reporter cells grown in 2D monolayer and vice versa from ligand-producing cells grown in monolayer to 3D reporter spheroids. The described method can be used for mimicking complex cellular in vivo architectures in vitro through the spatial positioning of different cell types grown as both monolayers and 3D cell spheroids. Combined with the characteristics of microdroplets, we believe that our platform can be well combined with other biomaterials, such as hydrogels,[36–38] to model the complex interactions between cells and the extracellular matrix. The method is compatible with high throughput screenings and could be used for investigating various biological processes, including cancer invasion, cell signaling, and embryonic development that require complex coculture cell architectures and microenvironments.

METHODS

Cell dispensing and droplet merging: The pressure-based noncontact liquid dispenser I-DOT One (Dispendix GmbH; Stuttgart) was used to dispense medium or cell suspension with desired cell density in the

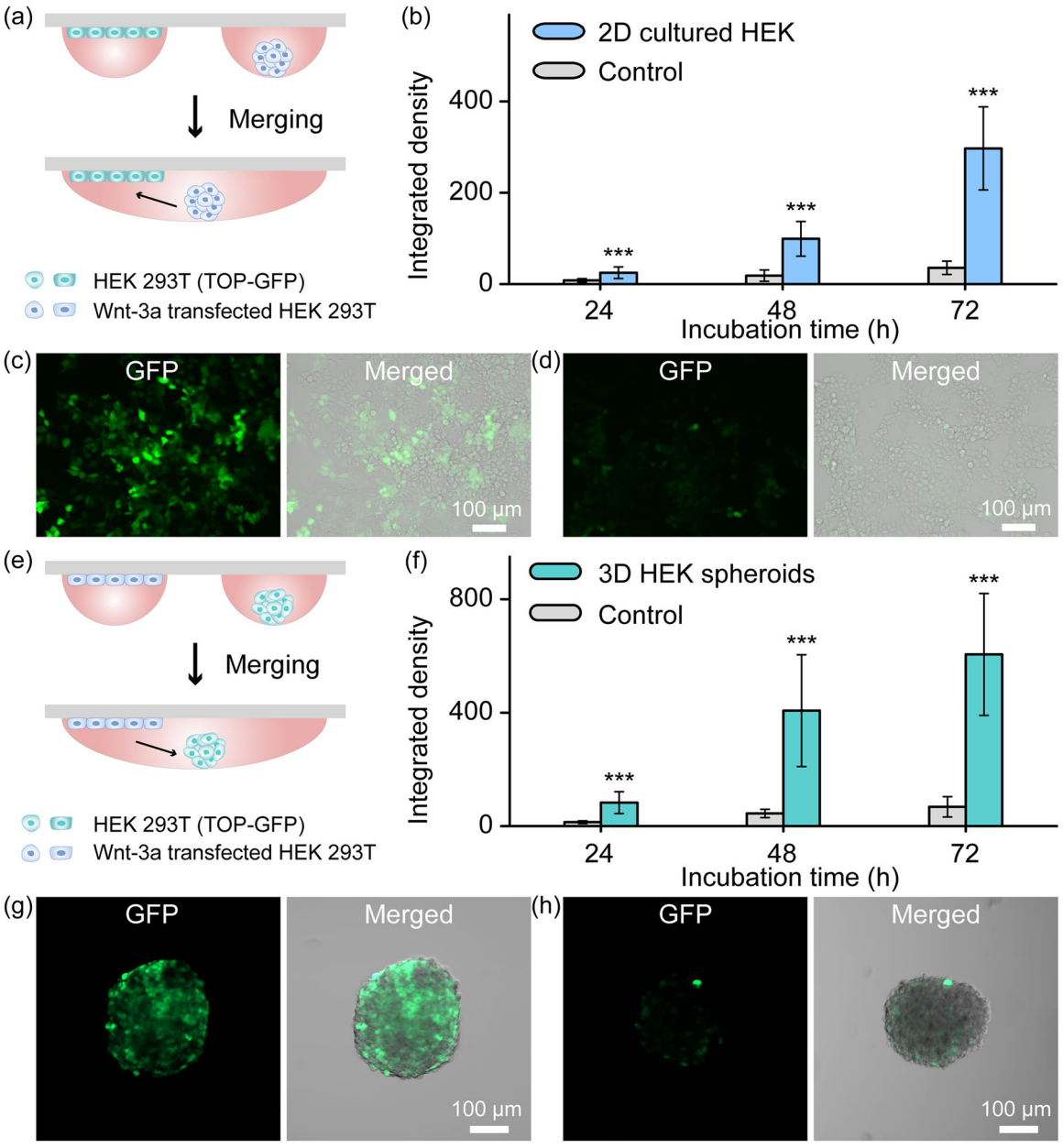

FIGURE 5 Paracrine propagation of Wnt-3a and activation of Wnt signaling in 2D/3D cell arrays. (a) Schematic representation of the workflow of coculture of the TOP-green fluorescent protein (GFP) fluorescent reporter cells cultured in 2D monolayer and Wnt-3a producing cell spheroids. (b) The graph showing the integrated density of 2D reporter cells after 24, 48, and 72 h of coculture. The integrated density was defined as the multiplication of the mean GFP fluorescence intensity and area of cells ($n = 30$, $^{***}p < 0.001$, one-way analysis of variance [ANOVA]). Typical fluorescence images of GFP activation (c) and the control assay (without Wnt-3a) (d). (e) Schematic representation of the workflow of coculture of Wnt-3a producing cells cultured in 2D monolayer and TOP-GFP reporter cells cultured as spheroids. (f) Graph showing the integrated density of 3D reporter spheroids after 24, 48, and 72 h of coculture. The integrated density was defined as the multiplication of the mean GFP fluorescence intensity and area of spheroids ($n = 10$, $^{***}p < 0.001$, one-way ANOVA). Typical fluorescence images of GFP activation (g) and the control assay (in absence of Wnt-3a) (h). 2D, two-dimensional; 3D, three-dimensional; DMA, droplet microarray.

volume of 200 nl per droplet on hydrophilic spots on the DMA. The DMA slides (Cat. No. G-np-301) were obtained from the Aquarray GmbH (Eggenstein-Leopoldshafen, Germany). The DMA slide used in this work had three arrays consisting of 196 hydrophilic spots (14 × 14). The hydrophilic spot is a square with a side length of 1000 µm, and the distance between the spot edges is 500 µm. To prevent the evaporation of the droplets during the printing procedure, a humidity level of 70% was maintained using a built-in humidifier. The cells were printed using the I-DOT pure plate with 90 µm orifice. Merging of individual droplets on the DMA was achieved by dispensing additional liquid (such as cell culture medium, staining solution, water, or phosphate-buffered saline [PBS]) in volumes ranging from 150 to 1000 nl into pre-existing 200 nl droplets. The entire cell printing process takes 2 min and the volume

of each droplet is 200 nl. And it takes 110 s to merge 20 groups of two adjacent droplets.

Cell culture: HeLa cells, HepG2, HEK 293T, and HEK 293T (TOP-GFP) were cultured in Dulbecco's modified Eagle medium (DMEM, 41966-029; Gibco™) supplemented with 10% fetal bovine serum (FBS, 10270-106; Gibco™) and 1% Penicillin/Streptomycin (15140-122; Gibco™). HeLa-RFP were cultured in DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 0.005% Puromycin Dihydrochloride (A1113803; Gibco™). The cells were split every 2–3 days. Cells were cultured on the DMA in DMEM cell culture medium containing 15% FBS. Cells were seeded as described in section "Cell dispensing and droplet merging." Cell culturing on DMA is described in sections "2D cell culture on DMA" and "3D cell culture on DMA."

2D cell culture on DMA: Before cell seeding in the DMA, slides were sterilized with ethanol for 10 min. For 2D cell culture HeLa, HepG2, or HEK 293T cells at a density of 7.5×10^5 cells/ml (~150 cells/droplet) were dispensed in 200 nl per spot. For the cell detachment experiment, 150 cells were seeded on the DMA and cultured for 1 day. After 1 day of 2D culture, the DMA slide was inverted (method of "hanging drop"). To form a 3D cell culture using the hanging drop method, the DMA slide was placed on a custom-made polytetrafluoroethylene (PTFE) table.[23] Humidity was set to 70% during the printing. The cells were cultured on the DMA at 37°C and 5% CO_2 inside 10 mm cell culture Petri dishes. To prevent evaporation during culturing, a wet humidifying pad was inserted in the lid of the Petri dish.

3D cell culture on DMA: To reduce cell adhesion on the DMA, each spot of the DMA slide was coated with 100 nl Anti-Adherence Rinsing Solution (Catalog #07010; STEMCELL Technologies Inc.). Before coating, DMA slides were soaked in absolute ethanol for 10 min to ensure that the slides were sterile. After the DMA slides were placed on a clean bench to dry sufficiently for 10 min, the spots of the slides were printed with 100 nl of anti-adherence rinsing solution by a noncontact liquid dispenser I-DOT One. The DMA slides were then placed on a clean bench to dry sufficiently for 10 min before they were used for 3D cell culture. For 3D cell culture, 200 nl droplets containing a suspension of HEK 293T cells in density of 1×10^6 cells/ml (~200 cells/droplet) were dispensed on DMA. Humidity during printing was set to 70%. To enable formation of 3D spheroids, the DMA slide was inverted immediately after printing and placed on an especially designed 3D printed table, allowing the culture of cells inside to form "hanging droplets."[23] The cells were culture for 48 h to form spheroids. To avoid evaporation during culturing, a wet humidifying pad was inserted in the lid of the Petri dish, and the Petri dish was filled with 10 ml of PBS. To investigate whether the formed spheroids could maintain their integrity after culturing in a straight position, 200 HEK 293T cells were seeded on coated or noncoated DMA wells. For the different groups, the volumes of Anti-Adherence Rinsing Solution used for coating were 0, 25, 50, 75, and 100 nl. Afterward, the DMA slide containing formed spheroids was placed in a straight position. The spheroids were monitored and imaged every day using microscopy (Keyence, BZ-X800).

Parallel and Combinatorial 2D/3D cell culture: For the parallel 2D/3D cell coculture, HepG2 cells (7.5×10^5 cells/ml) were first printed in 200 nl per droplet on the odd-numbered columns of the array and then cultured in a straight position for 24 h. HEK 293T cells (1×10^6 cells/ml) were then printed on the even-numbered columns and then cultured using the "hanging drop" method in an inverted position for 48 h (Supporting Information: Scheme S1). The even-numbered columns have been coated prior cell seeding with 100 nl of Anti-Adherence Rinsing Solution (Catalog #07010; STEMCELL Technologies Inc.). Afterward, the neighboring droplets containing a monolayer cell culture and spheroids were merged pairwise by dispensing 900 nl of cell culture medium into each droplet. To monitor and distinguish the cells from different spots, the cells were prestained with fluorescent dyes: CellTracker™ Blue CMAC (7-amino-4-chloromethylcoumarin; working concentration, 50 µM, C2110; Invitrogen™) and CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate; working concentration, 50 µM, C7025; Invitrogen™). Also, HeLa-RFP cells were used for the visualization. The resulting coculture with 2D/3D cell arrays were observed and imaged using Keyence (BZ-X800).

Viability staining: Cell viability was assessed by staining with Hoechst 33342 (H3570; Invitrogen™) and propidium iodide (P3566; Invitrogen™). Staining was performed by dispensing 50 nl of cell culture medium containing Hoechst 33342 (1:10,000; nucleus-total cell number) and propidium iodide (1:3000; dead cells). Cells were incubated with staining solution for 15 min in a cell culture incubator before imaging. ImageJ was used to assess number of dead cells and total cell number. Based on these results, the number of viable cells was calculated as the number of live cells (total number of cells minus number of dead cells) divided by the total number of cells.

Transfection of HEK 293T cells with Wnt-3a[27,34]: HEK 293T cells were transfected with 500 µg of *Mouse pCS2$^+$ Wnt3a* and 500 µg of *LacZ* plasmid in six-well plates using ScreenFect A according to the manufacturer's 1-step protocol (ScreenFect GmbH). As a control (without Wnt-3a), 1 µg of *LacZ* was transfected to HEK 293T cells. At 24 h posttransfection, cells were used as producers of Wnt-3a ligand.

Wnt signaling between 2D cell and 3D spheroids: HEK 293T Wnt-3a producing and stable transfected HEK 293T TOP-GFP reporter cell lines[34] were seeded according to the Supporting Information: Scheme S1. To achieve the fused droplets containing 2D Wnt reporter cell line and 3D Wnt-producing spheroids, the 200 nl of cell suspension containing HEK 293T TOP-GFP cells at a density of 1×10^6 cells/ml were printed on columns 1, 3, 5, 7, 9, 11, 13 for 2D culture and incubated for 24 h. Afterward, the Wnt-3a transfected HEK 293T cells (1×10^6 cells/ml) were printed on columns 2, 4, 6, 8, 10, 12, 14 for 3D culture as described in section "Parallel and Combinatorial 2D/3D cell culture" and incubated for 48 h. Next, the droplets from odd and even rows were merged pairwise to generate a 2D/3D cell array. To investigate the Wnt signaling, the 2D reporter cells were monitored every 24 h using fluorescence microscope (Keyence, BZ-X800) and the 3D spheroids were imaged using a confocal microscope (LSM 800 Confocal Laser Scanning Microscope; Zeiss Germany). The same method was used for the fused droplet with 3D Wnt reporter spheroids and 2D

Wnt-producing cells. The integrated density of the fluorescence image was measured by ImageJ (National Institutes of Health).[39] The microscope fluorescence images used for analysis of Wnt signaling activation had the same exposure time and threshold settings.

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CONFLICT OF INTEREST

The authors declare the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: in addition to being employed by the Karlsruhe Institute of Technology, A.A.P. and P.A.L. are (since March 2018) shareholders of Aquarray GmbH. H.C., T.T., X.W., J.W., and G.D. declare that there is no conflict of interest regarding the publication of this article. P.A.L. and G.D. are shareholders of ScreenFect GmbH.

ETHICS STATEMENT

The authors declare that this research has not been published elsewhere and that no data or images have been falsified. This research does not contain any experiments involving human subjects or animals. All authors have contributed to the submitted manuscript and are aware of the content of the manuscript. All authors were involved in critically revising the manuscript before submission.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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