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Nanoliter Hydrogel Array for Cell Screening and Cell Spheroid Sorting

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The transition from two-dimensional (2D) to physiologically relevant three-dimensional (3D) cell models has revolutionized biomedical research. Hydrogels are frequently used to produce 3D models for tissue engineering, disease modeling, and high-throughput screenings (HTS). However, integrating 3D cultures into HTS workflows presents challenges, including automation compatibility and cost constraints. Addressing these challenges requires innovative approaches that enable miniaturization, automation, and cost reduction while maintaining experimental fidelity. The Droplet Microarray platform, based on hydrophilic-superhydrophobic surface patterning, facilitates the formation of nanoliter-hydrogel arrays containing cells or spheroids. This method allows dispensing of hundreds of nanoliter-hydrogel droplets with precise control over volume and cell density, reducing reagent consumption and offering high-throughput applications. Here, we demonstrate stable nanoliter-hydrogel arrays on a chip, enabling experimental procedures such as washing and medium immersion. Our approach demonstrates that spheroid-containing droplets can be gelled at any point of the experiment, allowing for the fixation of cell structures on the surface. The selective gelation of individual droplets enables spheroid sorting by stabilizing desired droplets while pooling the others. This method holds the potential for HTS and miniaturized workflows in 3D microenvironments, thereby advancing research in different fields such as cell, cell spheroid, or organoid screenings, drug screenings, and precision medicine.

1. Introduction

Cells in tissues and organs exist in a three-dimensional (3D) microenvironment with complex interactions between cells and extracellular matrix (ECM).[1] Therefore, the conventional two-dimensional (2D) monolayer culture methods do not capture characteristics of cells in their in vivo environment. In order to bridge the gap between in vitro and in vivo conditions, there has been a transition from the 2D cell culture models to 3D models to generate more physiologically relevant data.[2,3] 3D cell culture models are divided into two main categories: scaffold-based methods and scaffold-free approaches.[4] Scaffold-free systems rely on self-assembly of cells into 3D structures, achieved by culturing cells using the method of "hanging drop" or in ultra-low attachment plates that promote assembly of cells into cell spheroids.[5] Scaffold-based approaches involve utilization of 3D matrix, such as hydrogels, for incorporation of cells. Hydrogels are continuously evolving and have become the preferred choice for establishing novel 3D systems in the tissue engineering field, culture of organoids, bioprinting, etc. Furthermore, the challenges of incorporating 3D cultures into high-throughput screening workflows have been addressed through the utilization of hydrogels as supportive scaffolds for cells. In this study, we have combined hydrogels as scaffolds for culturing cells with nanoliter array format on the Droplet Microarray (DMA) platform.

Hydrogels are hydrophilic materials composed of 3D networks of crosslinked synthetic or natural polymers. They can absorb substantial volumes of water and undergo swelling while retaining structural integrity.[6] They are often used as analogs of ECM and sometimes are functionalized with ECM components.[7,8] The crosslinked structure of hydrogels resembles the fibrillar architecture found in native tissues, creating a supportive scaffold for cells that offers mechanical support similar to that of the ECM.[9] This support is important for cell adhesion, growth and the overall maintenance of cellular functions.[10] As a result, hydrogels closely replicate the functionality of the native ECM by surrounding and instructing cells. Hydrogels are suitable for applications in the fields of drug delivery,[11] wound healing,[12]

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tissue engineering,[13,14] disease modeling including cancer[15] and high-throughput drug screening.[16] Besides offering structural support to cells, 3D hydrogel matrices can be used to confine cells in compartments to study the interactions between cancer cells and other cell types, including immune cells[17] and stromal fibroblasts.[18] For example, Huang et al., integrated hydrogels with incapsulated cells into microfluidic device for precise control of cell culture microenvironment.[19] Ding et.al., added cells from primary tissues to Matrigel to generate high-throughput microfluidic-based droplet Micro-Organospheres with the goal of facilitating drug screening and precision oncology treatment.[20]

However, the incorporation of 3D cultures into high-throughput screening (HTS) workflows is challenging due to the required compatibility of hydrogels with automated liquid handling equipment,[16] particularly because of the higher viscosity of hydrogel-like matrices, such as collagen or Matrigel. In addition, polymerization of these gels is sensitive to temperature and other parameters, therefore, requiring precise environmental control during printing. Miniaturization of such HTS combined with hydrogel-like matrices is even more challenging due to the small volumes.[21]

The Droplet Microarray (DMA) platform offers a solution for conducting miniaturized and high-throughput cell-based assays. The DMA platform is a miniaturized chip in the size of a microscope glass slide with an array of hydrophilic spots separated by superhydrophobic borders. Hydrophilic-superhydrophobic pattern enables the formation of hundreds of nanoliter droplets per chip, which can be used for culturing cells. Due to nanoliter volumes of the formed droplets, the DMA chip allows for significant (100–1000 folds) reduction of cells and reagents required for the experiments. The DMA platform has been used for cultivation and high throughput screening of various cell types including bacteria,[22] stem cells,[23] standard cell lines, chronic lymphocytic leukemia (CLL) cells,[24] and also for generation and testing of 3D cell spheroids using the hanging drop method.[25,26]

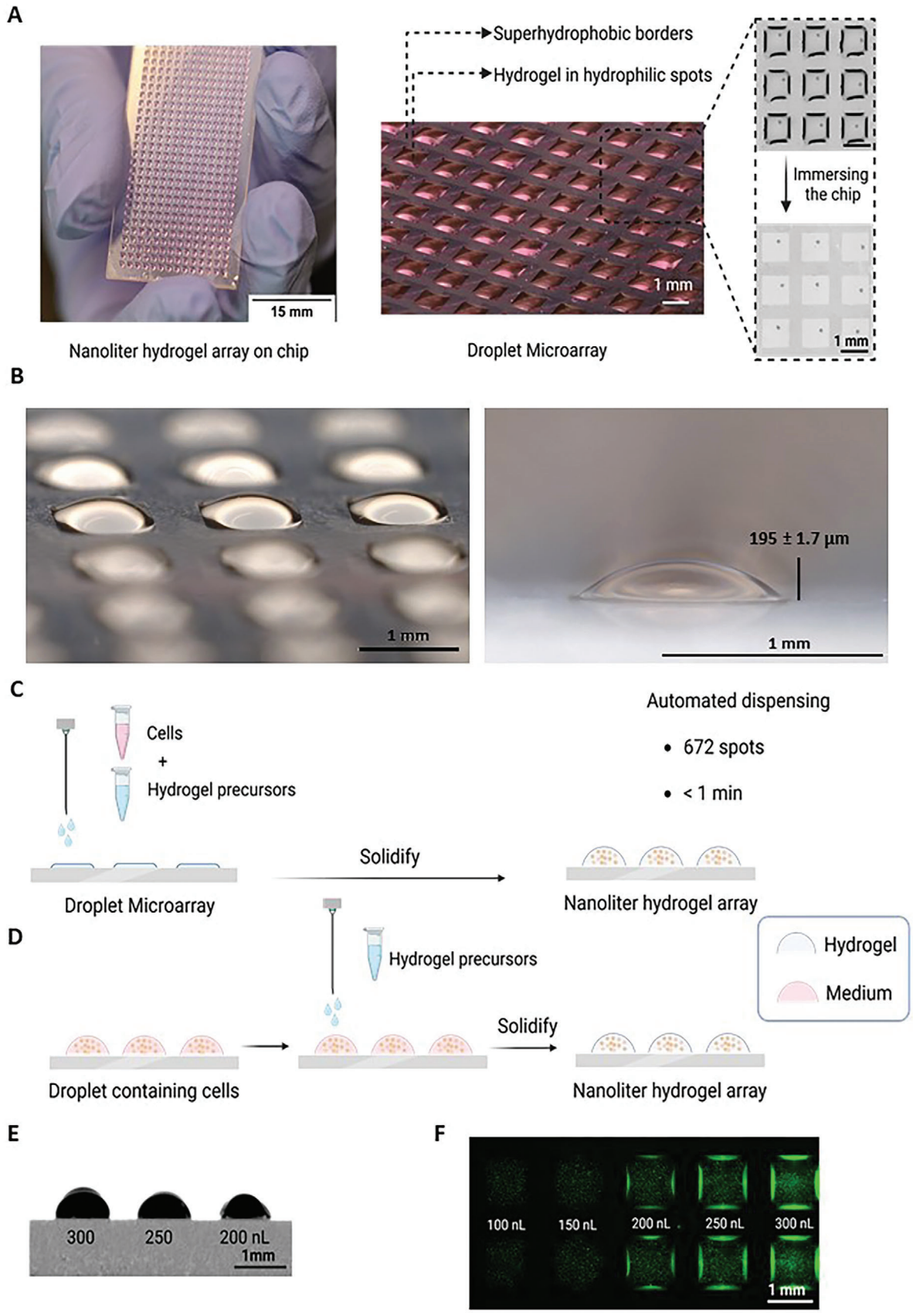
In this study, we have developed a nanoliter hydrogel array, a homogeneous and stable array of nanoliter-volume hydrogels incorporating cells or cell spheroids, introducing hydrogel-based 3D cell culture into the droplet microarray for HTS workflows for the first time. These arrays can be efficiently and precisely dispensed on a DMA chip in an automated and high-throughput manner. We have demonstrated the automated dispensing of 672 hydrogel pads, completed in less than 1 min. We have shown that this array is stable on the surface of the chip, and can be cultured in its own medium droplet for hydrogel, enabling compartmentalization for screening of multiple conditions. We have demonstrated the possibility to compartmentalize cell spheroids into hydrogel pads at any time point of the experiment, which opens possibilities for variety of protocols and procedures, including long-term culture, sorting of spheroids of interest and automated high throughput imaging of hydrogel arrays, without the risk of losing the cells or cell structures that are not adhered to the surface such as cell spheroids or organoids.

2. Results

In this study for the first time, we present a nanoliter hydrogel array for high throughout culture and screenings of cells in 3D hydrogel scaffolds in nanoliter volumes on the Droplet Microarray (DMA) platform (**Figure 1**). In this platform in opposition to liquid droplets, we form 3D hydrogels in hydrophilic spots of the DMA chip (Figure 1A,B). Hydrogel pads are separated by superhydrophobic borders and can be individually addressed for testing various conditions, for example drugs (Figure 1B). In addition, being stable on the surface of the DMA chip, the nanoliter hydrogel array is compatible with washing and submerging in media of the whole DMA chip keeping cells and 3D spheroids in intact positions (Figure 1A right panel).

In this study, we used a Droplet Microarray (DMA) chip containing 672 square hydrophilic spots in an array of 14 × 48 spots, each measuring 1 mm × 1 mm, and a Dextran-PEG hydrogel system. We selected this particular hydrogel for its slow-gelling properties, which enhance its printability and compatibility with automated liquid handling devices. We have explored two possibilities to incorporate cells into nanoliter hydrogel pads on DMA (Figure 1C,D). In the first method, the cells of the desired density were pre-mixed with hydrogel precursors, and 150 nL of the mixture was dispensed onto DMA spots using a non-contact liquid dispenser (Figure 1C). In the second approach, liquid media droplets containing cell suspension or spheroids are gelated at any desired time point by dispensing the hydrogel precursors onto the cell-containing droplet on the DMA (Figure 1D). Both approaches have been optimized in this study. We have achieved the printing of 672 homogeneous hydrogel pads of 150 nL volume in less than 1 min. The homogeneity of hydrogel volumes was validated by measuring the height of the hydrogel pads (Figure 1B; Figure S1, Supporting Information). Figure 1F demonstrates that we can control the printed volumes and number of cells incorporated into hydrogel pads. In addition to 150 nL hydrogel droplets accommodated by 672-spot DMA slides, larger volumes can be used on DMA platform containing larger hydrophilic spots. Alternative DMA slide configurations with 320 spots and a working volume of 1 µL and 80 spots with a working volume of 5 µL, can potentially accommodate more cells or larger 3D cellular structures. Representative images of hydrogel pads of 1 and 5 µL volumes are shown in Figure S4 (Supporting Information).

Having optimized the nanoliter hydrogel array dispensing process, we expanded on our previous work, where we exclusively cultured and screened cells in liquid media on the DMA platform, as detailed in earlier publications.[24,27] In this study, we further explored cell culture within the hydrogel, comparing it with conventional cell culture in liquid media on a DMA chip. Jurkat human T lymphocyte cells were pre-mixed with hydrogel precursors, Dextran, and PEG-link, and 150 nL of this mixture containing on average 300 cells was dispensed into every hydrophilic spot on a DMA slide. After 45 min of solidification, 150 nL of the culture medium was dispensed on top of each hydrogel pad (**Figure 2A**). After that, the viability of cells cultured in hydrogels for 4 days without medium exchange was assessed. Every 24 h cells were stained by dispensing of 50 nL of the staining solution, containing Hoechst 33342, Calcein-AM and Propidium Iodide (PI), to each hydrogel pad. Cell viability was calculated by dividing the number of live cells (Calcein positive) by total number of cells (Calcein positive + PI positive). The cells cultured in hydrogel pads showed the viability of 94.6, 93.5 and 91.3 in 24, 48 and 72 h of incubation respectively and the cells cultured in liquid medium showed the viability of 98, 90.7 and



88.8 in 24, 48 and 72 h of incubation, respectively. For both liquid medium and hydrogel-based droplets cells maintained high viability within the first 72 h of culture (Figure 2C). Following a 96 h culture period, we noted a decrease in cell viability in both liquid and hydrogel medium, which is expected due to the absence of medium exchange. Jurkat cells were cultured both in hydrogel pads and in liquid medium on the same chip, as previously described. After dispensing, the slide was placed in a humidity chamber and imaged using brightfield microscopy. Following imaging, the slide was incubated in a standard cell culture incubator. Cells were imaged every 24 h in both the hydrogel pads and liquid medium. As shown in Figure 2D, the cells proliferated the same in both environments. Our results indicate that nanoliter hydrogel array can be employed for cell culture on the DMA platform, similarly to previously demonstrated liquid droplet arrays.

To evaluate the stability of the hydrogels on the DMA slide, hydrogel precursors mixed with cells were dispensed on a DMA slide, following the previously described procedure (**Figure 3**A). Afterward cells were stained with Hoechst and Calcein-AM to enable their visualization. The nanoliter hydrogel array was then washed with PBS twice and intactness of hydrogels on DMA chip was evaluated by comparing positions and number of cells before and after the washing steps (Figure 3). Figure 3C,D show an array of hydrogels and close up of a single hydrogel before and after the immersion to PBS, respectively. As shown in Figure 3E, the number of cells in the same hydrogels did not change after the washing step. In addition, we demonstrated that the positions of the cells remained unchanged after washing (Figure 3F) by modeling the position of each cell before (green) and after (red) washing and overlaying the images using a representative hydrogel spot (Figure S2, Supporting Information). The yellow spots in the overlay indicate cells that remained in the same position, reflecting a complete overlap of their pre- and post-washing positions. Taken together, these results indicate that the number and position of cells did not change during washing procedure, indicating that hydrogels are stable on the hydrophilic spots of the DMA chip.

As a next step, we have demonstrated the possibility of gelation of the liquid droplets on the DMA chip at any time point of an experiment (Figure 1C). For this, we have chosen liquid media droplets containing 3D spheroids. The potential of the DMA platform to create a single 3D cell spheroid per droplet using the hanging drop method has been demonstrated in several of our studies,[25,26,28,29] however, all these studies have been conducted exclusively in a liquid medium and this is the first time we have used hydrogel on the DMA slide to encapsulate cell spheroids. In this procedure, an anti-adherence rinsing solution is applied to coat hydrophilic spots and minimize cell adhesion on DMA spots. First, the anti-adherence rinsing solution is dispensed onto the DMA spots and allowed to dry. Then, a cell suspension is dispensed onto the treated DMA spots, and the slide is immediately inverted after dispensing. The inverted slide is then placed on a "Spheroid table" to culture cells in the hanging drop method. Here we formed a single spheroid array using breast cancer cell line MCF-7 (**Figure 4**). Before demonstrating the possibility of gelation of droplets with spheroids, we first checked if the hydrogels were stable on the DMA surface even after the coating of hydrophilic spots with an anti-adherence solution used in the protocol for the formation of the single spheroid array (Figure S3A, Supporting Information). We successfully demonstrated the intactness of hydrogels on the coated hydrophilic spots after conducting washing steps by comparing the number and positions of cells before and after the washing (Figure S3B,C, Supporting Information). Next, to generate spheroids, MCF-7 cells were cultured on the DMA slide using a hanging drop method for 48 h allowing for the formation of a single 3D spheroid per liquid media droplet. The MCF-7 cells were dispensed onto spots coated with the anti-adherence solution, which prevented the cells from attaching to the surface. Instead, the cells began attaching to each other, forming cell aggregates, as shown in Figure 4B. Over time, the cell aggregates became more compact, resulting in the formation of roundish spheroids. After 48 h of incubation, hydrogel precursors were dispensed into each liquid droplet on the DMA chip keeping the ratio necessary for the gelation (Figure 4A). To demonstrate the gelation of the droplets containing spheroids and showcase their stability on the slide during washing procedures, the DMA slide containing spheroids in hydrogels was imaged before and after immersion in PBS (Figure 4D). We have shown that spheroids stayed at the same positions after the washing (Figure 4D), which was not the case for the spheroids in liquid droplets, that were washed away after the immersion in the liquid (Figure 4C). Therefore, we have demonstrated the gelation and stability of hydrogels formed by dispensing of hydrogel precursors in the droplets on DMA slide. In addition, the gelation of droplets and immersion of the slide in PBS improved imaging by eliminating the edge reflection observed in images taken before immersion in PBS (Figure 4D). This approach enables the fixation of spheroids immediately after their formation in liquid media, making it suitable for a variety of applications. These include fixation, staining, and washing procedures for immunofluorescence (IF), extended culturing by immersing the DMA slide in cell culture media, and the addition of other layers of media and/or hydrogels to facilitate co-culture, among many others. Furthermore, the DMA platform can accommodate larger spheroids and organoids by utilizing slides with larger hydrophilic spots, capable of holding greater volumes (Figure S4, Supporting Information). This versatility makes DMA a highly

Figure 1. Nanoliter hydrogel array on the DMA platform. A) Photos of nanoliter hydrogel array formed on DMA containing 672 spots (left and middle panel). The DMA platform contains hydrophilic square-shaped spots measuring 1 mm × 1 mm, with a superhydrophobic border separating adjacent spots. On the right panel the photos of 3 × 3 array of hydrogel pads containing MCF-7 cell spheroids before (right panel up) and after (right panel down) immersing into the medium demonstrate that the hydrogels remain stable on the spots. B) Microscope image of an array of hydrogel pads on a DMA slide, each with a volume of 150 nanoliters; scale bar: 1 mm (left panel). Photo of a single hydrogel pad with a vertical dimension of 195 µm (standard deviation of 1.77); scale bar: 1 mm (right panel). C) Hydrogel precursors can be mixed with a cell suspension and dispensed onto the 672 spots DMA slide using a liquid dispenser in less than 1 min. D) Hydrogel precursors can be dispensed onto cell-containing droplets on the DMA at any point during their culture, leading to the gelation of the droplets. E) and F) Side (E) and top view (F) of hydrogel droplets of different volumes ranging from 100 to 300 nL formed on the DMA chip. Microscope image of Jurkat human T lymphocyte cells stained with Calcein incorporated in hydrogel pads.

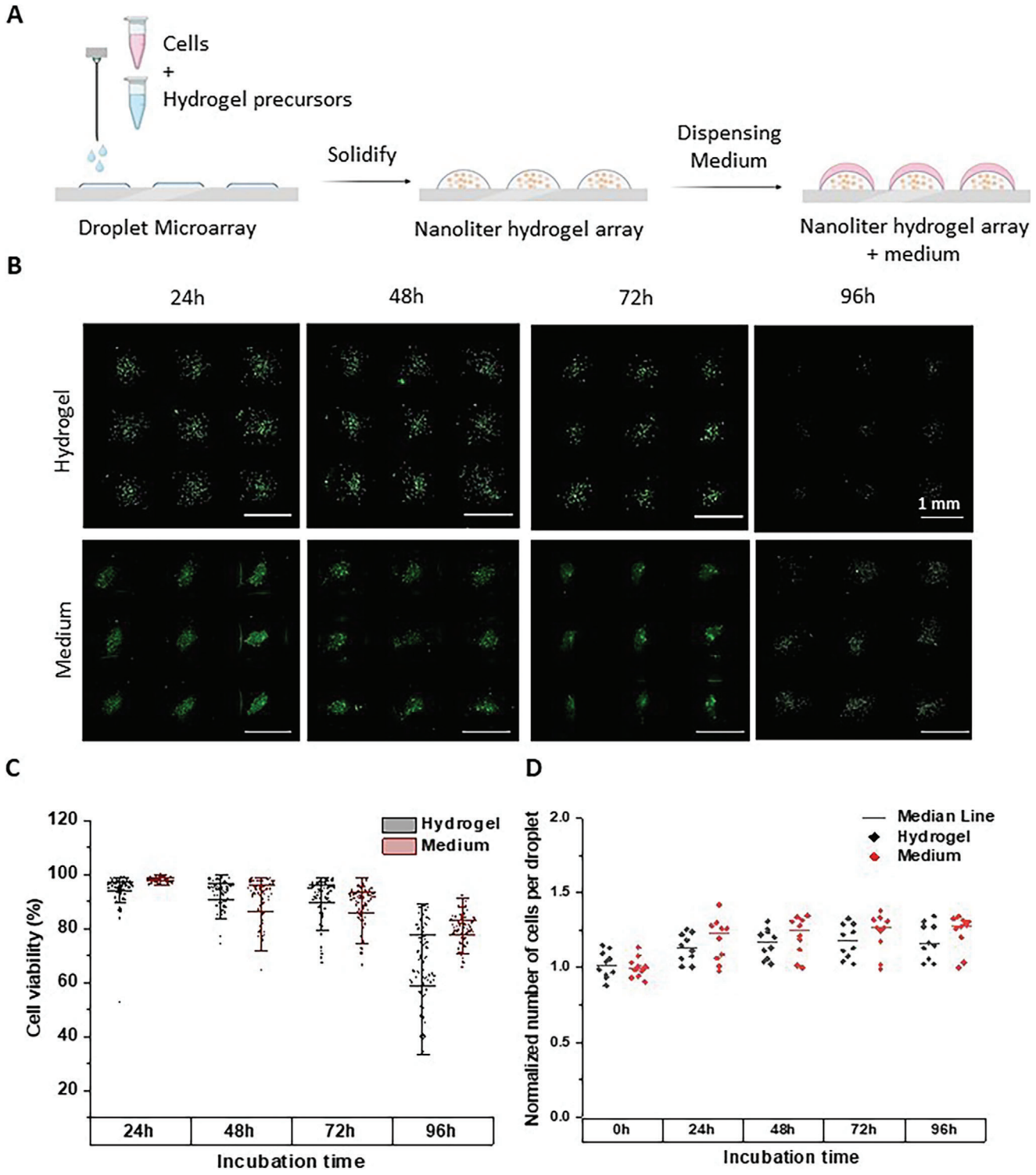
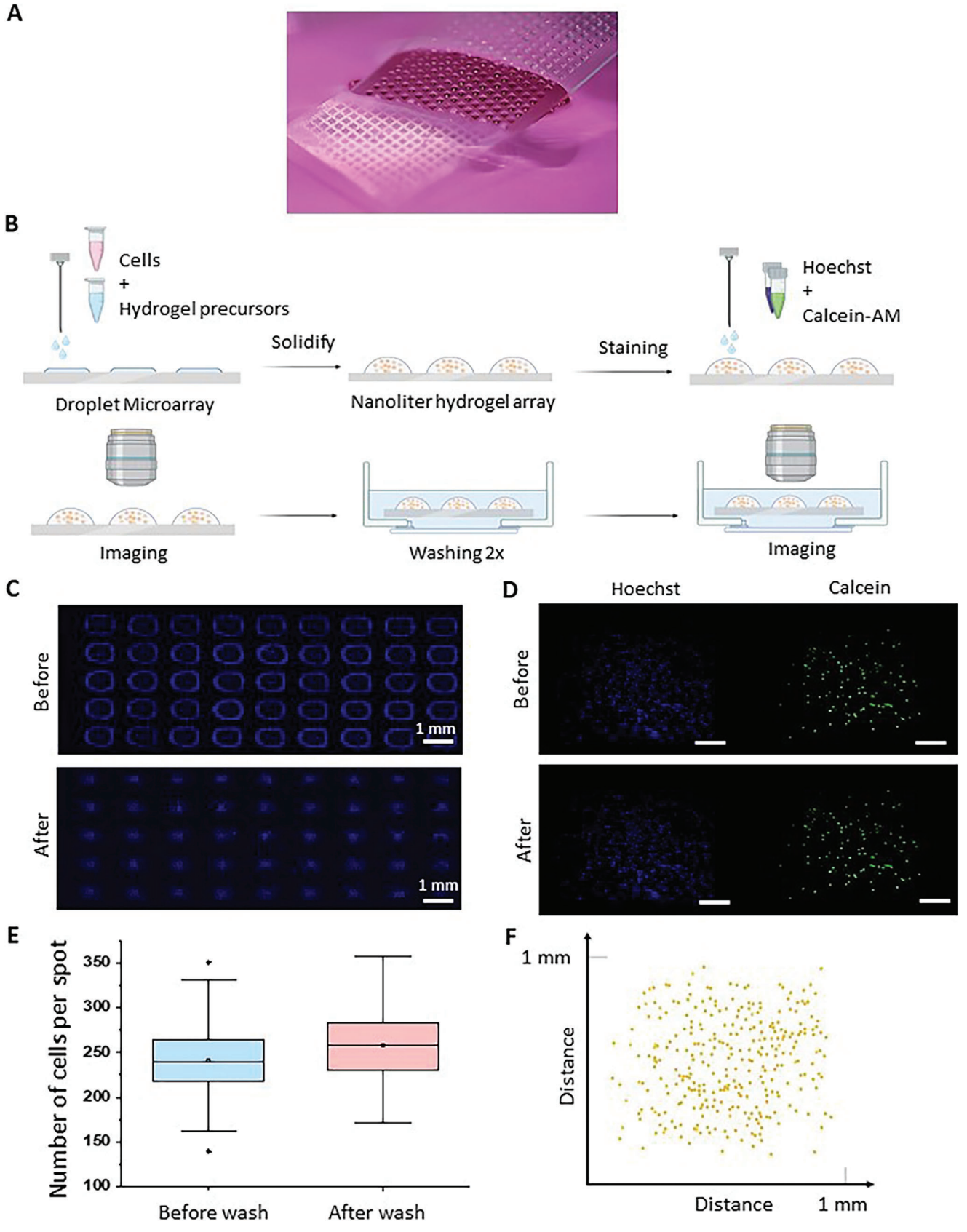


Figure 2. Comparison of cell culture in nanoliter hydrogel array and an array of liquid droplets. A) Schematic representation of the workflow for creating a nanoliter hydrogel array containing cells. A suspension of cells was pre-mixed with hydrogel precursors and the mixture was dispensed onto DMA spots in a volume of 150 nL. After the hydrogel solidified for 45 min in a cell culture incubator, 150 nL of culturing medium was dispensed on top of each hydrogel pad. B) Representative fluorescence images of a 3 × 3 of nanoliter hydrogel (top panel) and liquid droplet (bottom panel) containing Jurkat cells, stained with Calcein-AM after 24, 48, 72, and 96 h of culture. Scale bar: 1 mm C) Viability of cells cultured in hydrogels and liquid droplets for 24, 48, 72, and 96 h. D) Proliferation of cells cultured in hydrogel pads and liquid medium droplets over 96 h. Each condition was tested in 12 spots.



customizable and adaptable platform for diverse biological applications.

In our next step, we demonstrated the selective sorting of cell spheroids by gelation of the spots of choice followed by washing off the content of the rest of the spots on the DMA platform (**Figure** 5A). This approach allows for precise control over which spheroids are retained and which are removed, a feature that has significant implications for downstream applications. First, MCF-7 cells were used to generate an array of cell spheroids on the DMA platform using the hanging drop method. After 48 h of incubation, the cell spheroids were formed. To introduce selective differences, a staining solution containing Calcein-AM was dispensed into the droplets containing spheroids in a pattern spelled "KIT", representing the authors' affiliation (Figure 5B). Following a 30 min incubation period, hydrogel precursors were dispensed in the spots containing the Calcein positive spheroids. After 1 h of incubation, the DMA slide was submerged in one well of a 4-well plate containing PBS. Then, the slide was transferred to another well of the same plate filled with PBS and imaged (Figure 5C), revealing that the cell spheroids encapsulated in hydrogel remained attached to the DMA slide. The spots without Calcein-positive spheroids were left un-gelated, and the spheroids in these spots were collected in the first well of the 4-well plate through a simple washing procedure. Demonstrating the ability to selectively fix and sort spheroids based on their state and location on the DMA platform. This demonstrates the successful selective gelation of chosen spots and collection of spheroids of interest. The selective encapsulation of spheroids in hydrogel on the DMA platform can be particularly useful in applications such as drug screenings and investigation of signaling pathway. For instance, in the previous studies, DMA platform was used for high-throughput drug screening, demonstrating its capability to assess drug effects on spheroids.[29] Furthermore, propagation of Wnt signaling in spheroids using the platform was also studied, highlighting its utility in analyzing complex biological processes.[26] By leveraging the selective patterning approach described in this study, it becomes possible to isolate and analyze spheroids that respond to specific treatments. For example, spheroids treated with a particular drug could be selectively encapsulated, allowing for targeted studies of signaling pathways in these spheroids, enhancing the precision and efficiency of downstream analyses.

As a next step, we have demonstrated the possibility of using a gelation-degelation cycle for sorting the spheroids encapsulated in hydrogel pads (Figure 5D). MCF-7 cell spheroids were first generated on the full array of 672 spots on the DMA platform. Spheroids were stained with Calcein AM for visualization purposes (Figure 5E). Afterward, the hydrogel precursors were dispensed in a pattern of two arrays of 7 × 7 spots (Figure 5F). Following gelation, the slide was washed with PBS, resulting in washing off all the spheroids that were in liquid droplets, leaving the spheroids in hydrogel pads intact on the chip (Figure 5F). Next, the dextranase enzyme was dispensed on all hydrogel pads, followed by 45 min of incubation. The DMA was then washed with PBS removing all the spheroids from the surface, proving degelation of hydrogels containing spheroids (Figure 5G). The presented approach holds potential for selective and programmed fixation and sorting of cell spheroids on the DMA platform.

3. Conclusion

The demand for high-throughput culturing and screening of cells continues to grow across various fields, from drug discovery to monitoring of cell behavior and cancer research.[16,30–33] Culturing cells in 3D microenvironments, such as hydrogels, offers a more physiologically relevant setting compared to traditional 2D cultures and can improve high-throughput screening (HTS) outcomes.[34] However, incorporating 3D cell cultures into miniaturized and automated workflows remains a challenge.[35]

Our study addresses these challenges by using the DMA platform to establish a homogeneous nanoliter volume array of hydrogels containing cells or spheroids in a fast, automated, and high-throughput manner. We have successfully demonstrated automated dispensing of hydrogels creating 672 homogeneous hydrogels of 150 nL volume in less than a minute time. By demonstrating the formation of a hydrogel array with precise control over volume and cell density, we highlighted the platform's potential to advance high-throughput screening in 3D cell culture.

Miniaturizing the hydrogel array down to 150 nL per spot offers 2–3 orders of magnitude reduction in reagent consumption compared to traditional microtiter plates: from ≈150 µL in 96 well plates and 25 µL in 384 well plates. In addition to saving on reagents and compounds, the need for at least 10–100 times fewer cells per experimental point, provides a solution for screening rare and limited cells, such as patient-derived cells from solid tumor biopsies.

The stability of the nanoliter hydrogel array during washing steps opens the possibility for immobilizing cells or individual cell spheroids in precise locations on the DMA slide during washing and staining procedures, which is not possible with liquid media droplets containing suspension cells or non-attached spheroids. In addition, the possibility of gela-

Figure 3. Validation of the stability of hydrogel pads on the DMA slide during the washing procedure. A) A photograph of the nanoliter hydrogel array immersed in a culture medium. B) Schematics representation of the experimental workflow for validation of hydrogel intactness on the DMA slide. First, the hydrogel array was dispensed on the DMA chip, after the gelation the cells in hydrogels were stained and the hydrogel array was imaged, then the whole array was immersed in PBS and washed twice, finally, the array was imaged again to compare number and position of cells before and after the procedure C) Microscope images of nanoliter hydrogel array before (upper panel) and after (lower panel) the washing steps. D) Microscope image of a single hydrogel containing cells before (upper panel) and after (lower panel) the washing steps. The scale bar: 200 µm. E) Graph showing the number of cells cultured in 84 hydrogel pads on the DMA slide before and after immersion in PBS. F) Overlay of cell positions before and after washing steps, depicted schematically based on microscopy images of a representative hydrogel. Fully overlaid cells, indicated in yellow, demonstrate that cell positions remained unchanged post-washing. The corresponding schematic images before and after the washing step are presented in Figure S3 (Supporting Information).

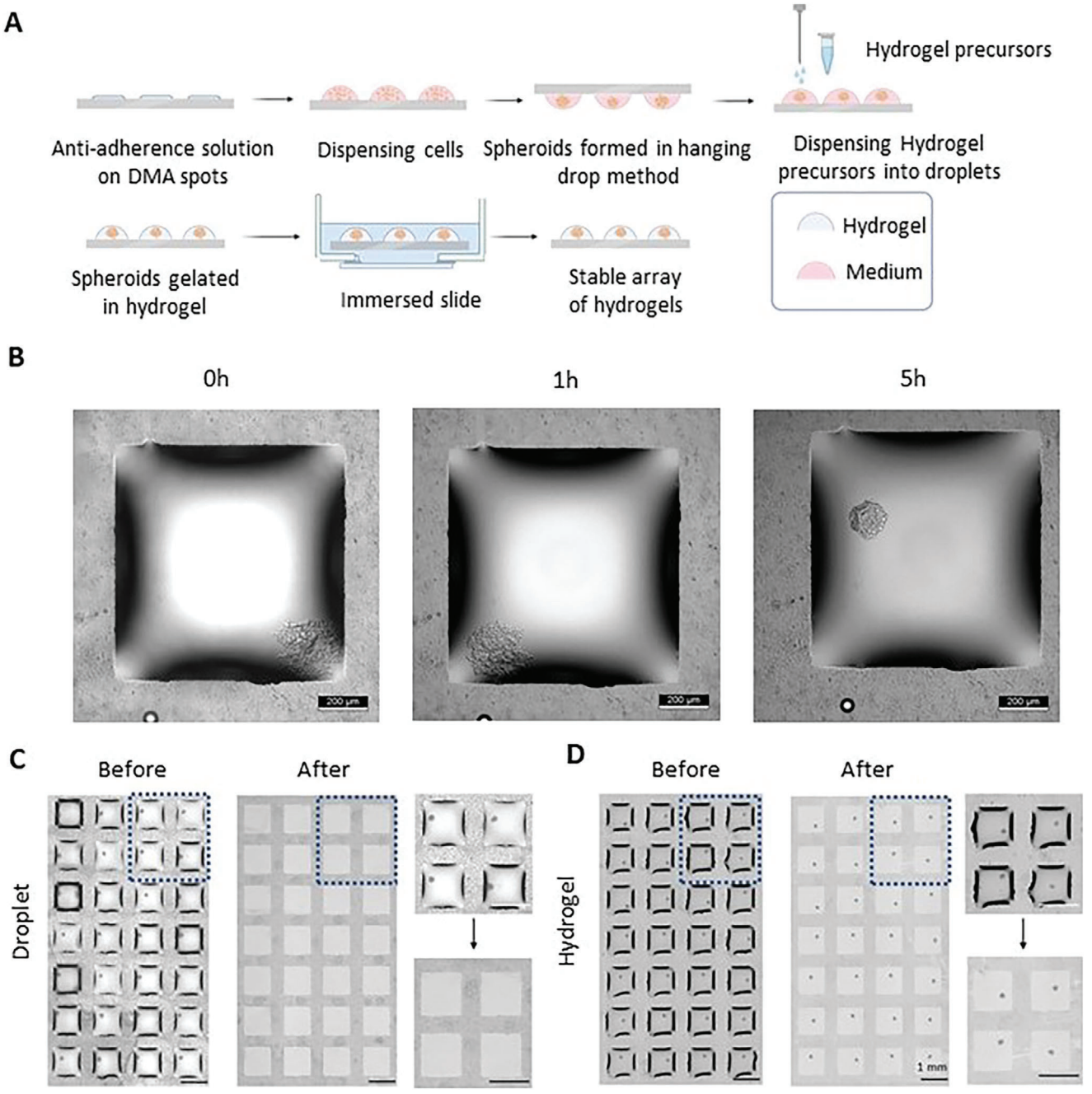


Figure 4. Gelation of the single spheroid array on the DMA slide. A) Schematic representation of the workflow for the generation and subsequent gelation of the single spheroid array. MCF-7 cells were dispensed into a hydrophilic spot coated with an anti-adherence solution and cultured for 48 h in an inverted position. Afterward, the hydrogel precursors were dispensed into each droplet. B) Formation of a single cell spheroid on a spot of the DMA slide. After dispensing, the cells begin attaching to each other (0 h), forming a cell aggregate after 1 h, which becomes more compact after 5 h, leading to the formation of a single roundish cell spheroid on the DMA slide. The gelation and immersion of the slide in PBS and comparison presence and positioning of single spheroids inside hydrogels before and after the washing. C,D) Microscope images of array of 4 × 7 liquid droplets (C) and hydrogels (D) containing single spheroids before and after immersion in PBS. Scale bar: 1 mm.

tion of liquid droplets at any time point of an experiment provides flexibility in experimental design. Furthermore, the demonstrated selective gelation of spheroids of interest inside nanoliter droplets enables on-chip spheroid sorting and spheroid patterning.

We believe that our approach can be adopted for miniaturized screening workflows in 3D microenvironments, for such assays as cell migration and invasion tests, spheroid sorting, patterning, or for drug sensitivity tests in the field of functional precision oncology.

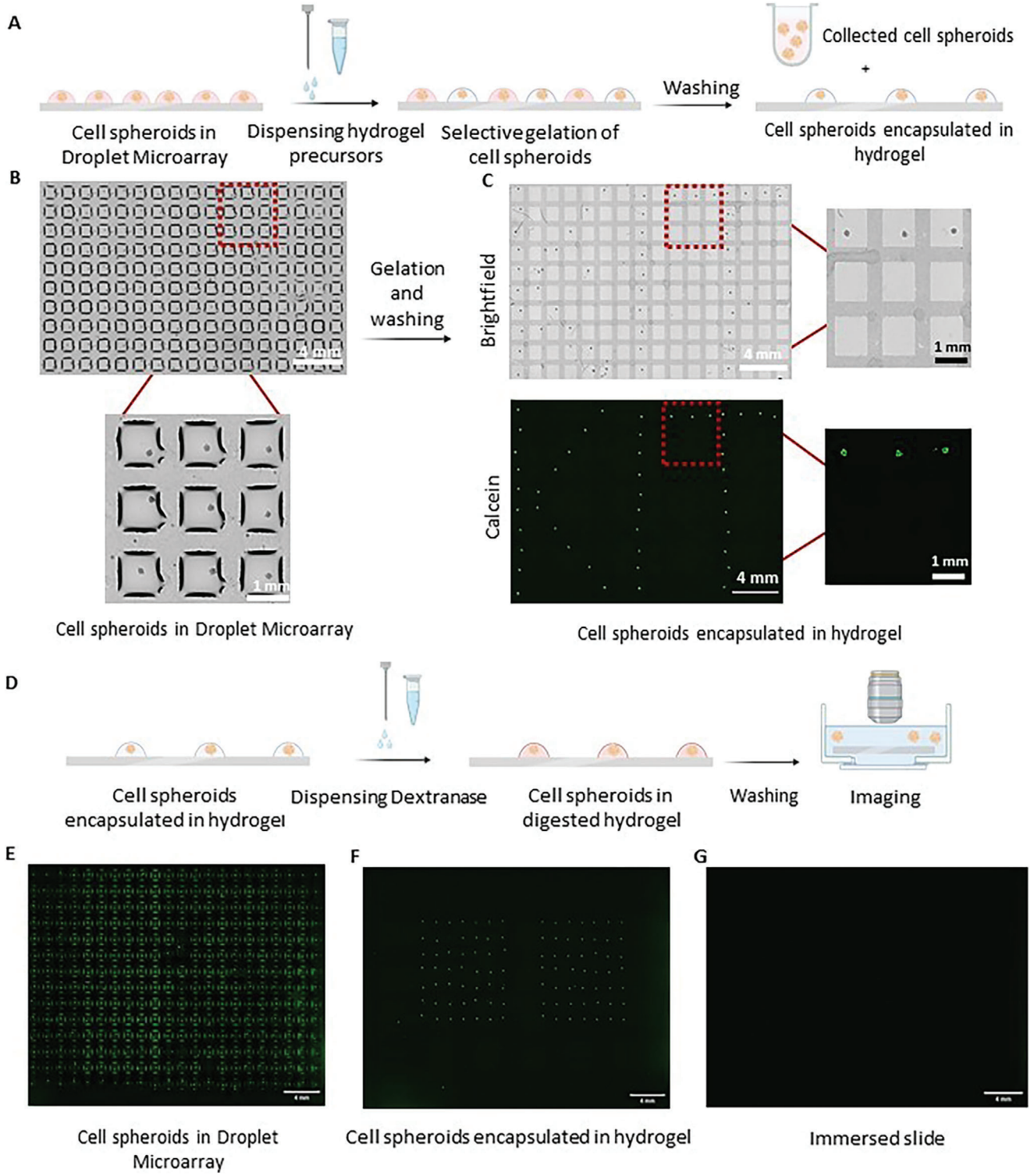


Figure 5. Sorting of cell spheroids. A) Schematics representation of the workflow for selective gelation of droplets containing spheroids on the DMA slide. B) Microscope image of the array of 10 × 17 droplets containing cell spheroids (upper panel), and close-up of an array of 3 × 3 spots before gelation (lower panel). C) Brightfield (upper panel) and fluorescent (lower panel) microscope image of the array of 10 × 17 spots on DMA showing cell spheroids encapsulated in hydrogel after the selective gelation and washing off spheroids from not gelated droplets. D) Schematics representation of the workflow for digestion of hydrogel and returning the hydrogel pads to the liquid state. E) Fluorescent microscope image of the array of 22 × 14 droplets containing cell spheroids, stained with calcein. F) Fluorescent microscope image of 22 × 14 spots of DMA slide with cell spheroids encapsulated in hydrogel after immersion of the slide in PBS. G) Fluorescent microscope image of DMA slide after digestion of the hydrogel-containing cell spheroids by Dextranase, immersed in PBS. Scale bar: 4 mm.

4. Experimental Section

Cell Culture: Jurkat human T lymphocyte cell line was cultured in RPMI-1640 medium (Gibco, Life Technologies, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Sigma–Aldrich, USA) and 1% of penicillin/streptomycin (Gibco, Life Technologies, USA). The cells were maintained in T25 flasks at 37 °C in an atmosphere of 5% CO_2 and split regularly every 2–3 days when the cells were reaching a cell density of 3×10^6 cells mL^{-1}.

MCF-7 human breast cancer cells (generously donated to our research laboratory by Dr. Oliver Kassel research group, Institute of Biological and Chemical Systems (IBCS), Karlsruhe Institute of Technology (KIT)) were cultivated in RPMI-1640 medium supplemented with 10% of heat-inactivated FBS and 1% of penicillin/streptomycin. The cells were split every 2–3 days once reaching 80% confluency and were incubated at 37 °C with 5% CO_2.

Preparing Hydrogel on DMA Platform: The DMA slides were purchased from Aquarray GmbH (Eggenstein-Leopoldshafen, Germany). The DMA slide (Catalogue number G-np-102) with 672 hydrophilic spots (1 mm × 1 mm) was used throughout the study. Initially, DMA slides were sterilized by immersion in 100% ethanol for 1 min, followed by drying under a sterile bench for 20 min before use, according to the manufacturer's instructions.

Hydrogels were prepared using the 3-D Life Dextran-PEG Hydrogel SG Kit (#G92-1, Cellendes GmbH, Germany) containing PEG-Link, 10× CB pH 7.5, water and SG Dextran. Gel formation was performed by mixing 35.5 µL of water, 8 µL of 10x CB, and 6.7 µL of SG-Polymer Dextran. Then, 40 µL of cell suspension (cell concentration: 2×10^6 cells mL^{-1}, corresponding to 300 cells per spot) was added, and thoroughly mixed, followed by the addition of 10 µL of crosslinker (PEG-link), resulting in a total volume of 100 µL. The mixture was immediately mixed by pipetting. Using an automated non-contact liquid dispenser, I-DOT One (Dispendix GmbH, Germany), the hydrogel was dispensed directly onto hydrophilic spots in a volume of 150 nL. Humidity was set to 70% during dispensing. The DMA slide was then incubated for 45 min in a cell culture incubator in humidity Petri dish, which is a 10 cm Petri dish filled with 2 mL of PBS and covered with a lid containing humidifying pad. After gel formation, 150 nL of complete RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin was dispensed on top of hydrogel using Certus FLEX liquid dispenser (Gyger Fluidics GmbH, Switzerland). Afterward, the DMA slide containing hydrogels covered with the medium was placed into a 10 cm humidified Petri dish. Alternatively, after gel formation, the slide was placed in a sterile 4-well plate and the well was filled with complete RPMI-1640 medium until the slide was fully immersed. Subsequently, the plate was placed in a cell culture incubator.

To digest the hydrogels, 19 nL of Dextranase enzyme (#D10-1, Cellendes GmbH, Germany) was dispensed on hydrogel pads followed by 45 min of incubation in a cell culture incubator.

Formation of Nanoliter Hydrogel Array Containing 3D Cell Spheroids: The DMA slides were immersed in 100% ethanol and dried on a clean bench for 20 min. Anti-adherence rinsing solution (STEMCELL Technologies Inc., Canada) was used to coat hydrophilic spots and to reduce cell adhesion on DMA spots. 50 nL of anti-adherence rinsing solution was dispensed on each spot using I-DOT One dispenser. The DMA slides were then dried under clean bench for 15 min before being used for cell culture. Then, 200 nL of MCF-7 cell suspension with cell density of 2.5×10^6 cells mL^{-1}, corresponding to 500 cells per spot, was dispensed by I-DOT One dispenser on each spot under 70% humidity condition. The slide was inverted immediately after dispensing and placed on a "Spheroid table" (Catalogue number: AQP-0006, Aquarray GmbH, Germany) designed to enable the cultivation of DMA slides upside-down, facilitating the formation of 3D cell spheroids using the hanging drop method. The "spheroid table" with a slide on it was placed in a 10 cm Petri dish filled with 10 mL of PBS and covered with lid containing humidifying pad. The cells were cultured for 48 h to allow the formation of spheroids. After the single spheroid array was formed, 21.24 µL of 10x CB (pH 7.2), 17.8 µL of SG Dextran and 26.56 µL of PEG-Link were mixed thoroughly and immediately dispensed into the droplets containing cell spheroids. Finally, the slide was incubated for 2 h in a cell culture incubator to ensure hydrogel formation.

Viability Staining and Microscopy: Staining was performed by dispensing 50 nL of PBS containing Hoechst 33342 (10 mg mL^{-1}, Invitrogen, USA), Calcein-AM (1mg mL^{-1}, Invitrogen, USA), and Propidium Iodide (1mg mL^{-1}, Invitrogen, USA). Cells were incubated with a staining solution for 30 min in a cell culture incubator.

To conduct microscopy, the slide was placed in a 4-well plate with humidity pads and sealed with parafilm to minimize evaporation during imaging. Cells were monitored and imaged using a Keyence BZ-X810 microscope (Keyence, Japan) and an automated screening microscope Leica 3D Thunder imager (Leica, Germany) for brightfield and fluorescent imaging. The data analysis was carried out using ImageJ/Fiji (National Institutes of Health Inc, USA).

The images of the DMA slide containing hydrogel pads, representing the vertical dimension, were captured using a Keyence VHX-7000 microscope (Keyence, Japan).

Data Analysis: The image analysis to estimate the number of cells per spot was conducted using ImageJ/Fiji software (National Institutes of Health Inc, USA). First, the image underwent an 8-bit conversion, followed by manual adjustment of the threshold. After that, the "Watershed" algorithm was applied to segregate cells. To quantify stained cells per spot, the "Analyze particle" function was used. The "Batch macro" function was used for automatic analysis of images.

The calculations and plots were generated using Origin 2020b (OriginLab Corporation, USA).

Creation of Figures: Illustrations were created and designed using Biorender.com.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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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. M.S. declares that there is no conflict of interest regarding the publication of this article.

Data Availability Statement

The data that support the findings of this study are available under DOI 10.35097/rysyac0xmreqfmv3.

Keywords

3D cell culture, automation, droplet microarray, high-throughput, hydrogel, miniaturization, spheroids

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