

Facile one step formation and screening of tumor spheroids in hanging droplets on the miniaturized Droplet-Microarray platform

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Abstract

Tumor spheroids or microtumors are important 3D *in vitro* tumor models, possessing *in vivo*-like tumor microenvironment. Microtumors are widely applied in the fields of fundamental cancer research, drug discovery and precision medicine. In precision medicine tumor spheroids derived from patient tumor cells represent a promising system for drug sensitivity and resistance testing. Current methods for 3D spheroids fabrication and screening require large number of cells and compounds and not always result in formation of a single spheroid per well. In this study we demonstrate an application of the Droplet Microarray platform, based on hydrophilic-superhydrophobic patterning, in combination with the method of hanging droplet for the formation of highly miniaturized single-spheroid-microarrays. We demonstrate formation of spheroids from several commonly used cancer cell lines in 100 nL droplets starting with as few as 150 cells per spheroid within 24 - 48 hours. We establish a highly miniaturized and fast assay for screening of anti-cancer compounds on tumor spheroids, which carries a potential to be adopted for routine workflows of high-throughput compound screening in 3D cell cultures.

Introduction

Tumor spheroids or microtumors consist of cancer cells that self-organize into three-dimensional (3D) tumor-like spherical structures.[1] They find wide application as 3D *in vitro* tumor models in the fields of fundamental cancer research, drug discovery and precision medicine.[2-4] Compared to 2D monolayer cultures, tumor spheroids closer represent *in vivo* like tumor microenvironment. Extracellular matrix (ECM) proteins produced by cells in three-dimensional structure, as well as cell-cell contact provide mechanical properties that are essential for morphology, expression profile, proliferation and drug response of cells residing in microtumor.[5][6-9] In addition, microtumor environment differs biochemically from 2D monolayer, and characterized by low oxygen, low pH and low glucose content.[10, 11] This different biochemical characteristics influence cell biology and have been shown to increase invasiveness, induce proliferation and drug resistance of cancer cells.[12-14] Limitation of oxygen and nutrients diffusion within a tumor spheroid results in developing of a hypoxic region and a necrotic core, similar to that in *in vivo* tumors.[4, 7-9, 15-17] Therefore, cells grown in spheroid models demonstrate properties that resemble those of cancer cells found in

naturally occurring tumors. This close resemblance of the *in vivo* tumor microenvironment makes tumor spheroids an important physiologically relevant *in vitro* model, which has been widely applied for testing of anti-cancer compounds, investigating the mechanism of drug resistance and studying growth kinetics and metabolism of tumors to mention a few examples.[18]

Drug sensitivity and resistance testing using tumor spheroids formed *in vitro* has shown drug responses similar to those found *in vivo*. [4, 8, 9] Previous studies showed reduced sensitivity of cancer cells towards anti-cancer therapy when cultured in 3D spheroids compared to 2D cell culture. Detailed investigation of mechanism of drug sensitivity difference between 3D and 2D environments revealed that cells cultured in 3D cell models possessed enhanced gene expression profile, which correlated with increased mutation rates and the ability of tumor cells for invasion and formation of metastasis. [3, 15, 19-21] Routine use of 3D tumor spheroids model for drug testing can result in reduction of false positive and negative results, therefore decreasing usually high failure rates and costs of drug development, and ultimately leading to reduction of unnecessary animal experiments. [3, 4, 21, 22] In order to employ spheroid models for routine screening applications, it is necessary to have reliable and affordable high-throughput platforms enabling formation, culturing and screening of hundreds of uniform spheroids in one-spheroid-per-well format.

Current standard methods for 3D spheroids fabrication include spinner flasks, bacterial grade dishes, ultra-low attachment (ULA) plates and method of hanging drop. [8, 17, 21-23] Using spinner flasks, bacterial grade dishes, and ultra-low attachment (ULA) plates result in formation of multiple spheroids per well/plate that show high variability in size. In addition, spheroids obtained from spinner flasks and bacterial grade dishes have to be manually plated into microtiter plates for screening applications, limiting their applicability for high-throughput screening applications. [17, 20, 22] Method of hanging droplet allows for the generation of homogeneous spheroids in one-spheroid-per-drop format and compatible with standard liquid handling robotics. [27] Usually it is performed using standard micro titer plate formats of 96- and 384-well plates and requires from 30 to 100 μL of culturing volume resulting in high consumption of compounds, reagents and valuable cells. In addition, it is necessary to transfer spheroids to a multiwall plate for imaging analysis.

Reduction of the screening costs by reducing consumption of compounds, reagents and cells is of ultimate importance for both industrial and academic environments. In addition, reducing cell numbers is especially critical in case of screening of spheroids derived from rare cells, such as primary patient tumor cells obtained via biopsy for example. Drug sensitivity and resistance test, where tumor spheroids derived from patient tumor cells are tested against a panel of anti-cancer drugs with a goal to identify suitable therapy for each patient, is a promising test in precision cancer medicine. [28] Therefore, a high-throughput platforms that allow for facile formation and screening of uniform spheroids in one-spheroid-per-well format in highly miniaturized format is critical for implementation of tumor spheroids as a model for routine screening applications.

A lot of progress has been made in developing novel miniaturized methods for the formation and screening of spheroids. Such miniaturized systems include microfluidic devices, scaffold-based microarrays and micro-engineered hanging drop arrays. [8] (21, 23, 25, 26) Microfluidic-based systems enable facile formation and long-term culturing of uniform spheroids. (16, 24) The main advantage of these systems is a possibility to create a complex perfusion organ-on-a-chip system. However, the drawback of microfluidic platforms is limited compatibility with screening of large compound libraries since drugs have to be introduced to the spheroids using valves. [16, 24] Scaffold-based microarrays and micro-engineered hanging drop arrays enable formation of homogeneous spheroids in volumes down to 1 μL using low cell numbers and demonstrate high compatibility with standard liquid handling robotics and

automated workflow.[21, 23, 25, 26] Some of these platforms, however, still have limitations. For example, it is challenging to retrieve spheroids from scaffolds for further analysis (22, 27) and some of the materials used in those platforms such as e.g. PDMS, showed non-specific absorption of compounds, especially in case of hydrophobic substances, resulting in decreasing of drug efficacy during the screening. (23, 27, Small molecule absorption by PDMS in the context of drug response bioassays.)

Recently, we developed the Droplet Microarray (DMA), a miniaturized platform for cell-based high-throughput screenings.[29-34] The Droplet Microarray consists of an array of hydrophilic spots on superhydrophobic background and exhibits extreme contrast in wettability between both of these areas. Due to this extreme contrast a high-density array of homogeneous, separated and stable nano-droplets can be formed on the surface of the Droplet Microarray. Each of these droplets can trap cells and serve as a reservoir for performing individual biological experiments. In the current study, we utilized the DMA platform to establish fast and highly miniaturized system for screening of single microtumors. We demonstrate formation of single-spheroid arrays from several commonly used cancer cell lines in 100 nL droplets within 24 - 48 hours starting only with 150 cells per spheroid. We perform drug treatment on microtumors formed from HeLa cells, and compare the response of HeLa cells cultured in 3D spheroids with cells cultured in 2D monolayer.

Results

Formation of spheroids on the DMA platform

DMA slide consists of a glass slide patterned with an array of hydrophilic spots (water contact angle (WCA) $\leq 10^\circ$)[30] separated by superhydrophobic (advancing and receding WCAs $\geq 150^\circ$ and sliding WCA $\leq 5^\circ$)[30] borders (Fig. 2a). In this work we used DMA slides with dimension of 25 x 75 mm containing 588 square hydrophilic spots with side length of 1 mm (Fig. 2a,b). Due to the extreme difference in water affinity of hydrophilic and superhydrophobic parts of the surface an array of separated and homogeneous droplets is formed on DMA surface spontaneously by applying aqueous solutions onto the surface (Fig. 2a)[30-32]. This effect is known as discontinuous dewetting[35]. Previously we demonstrated formation of arrays of homogeneous separated droplets containing cells using the effect of discontinuous dewetting[31]. Here, we utilized both discontinuous dewetting (Fig. 1a, upper panel) and a non-contact liter dispenser to print cells directly into each hydrophilic spot (Fig. 1a, bottom panel). In case of manual seeding, cells were seeded in approximately 80 nL droplets[31], in case of using dispenser, cells were seeded in 100 nL droplets. After cells were seeded, the DMA slides were turned upside down and placed on a slide holder to create an array of hanging droplets (Fig. 1a, 2b). The slide holder with the DMA slide was placed into a standard 10 cm Petri dish containing buffer and closed with a lid containing a humidified pad preventing evaporation of the droplets during culturing (see experimental section). Cells were incubated in a standard cell culture incubator. Using this simple and miniaturized system we were able to create a single-spheroid-array in nanoliter droplets within 24-48 hours (Fig. 2 c, d). Such single-cell spheroid array can be used for compound treatment followed by microscopy analysis directly on the DMA slide, where each spheroid can be addressed individually (Fig. 1b). Alternatively, spheroids can be easily collected from the wall-less surface by washing them down in Petri dish or other container for further experiments or analysis (Fig. 1c). Thus, DMA slides can be not only used as a platform for screening of spheroids, but also as a platform for fabrication of hundreds of homogeneous single spheroids that can be further used for various applications.

We successfully formed spheroids from several cell types including MCF-7, HEK293 and HeLa (Fig. 2c, 3). Size distribution of formed spheroids across an array has corresponded

to Poisson distribution with about 14% deviation of diameter and was comparable between both manual and automated seeding methods (Fig. 2d). Size of spheroids was dependent on initial cell number in the droplets and could be controlled by changing initial cell seeding concentration (Fig. 3c).

We monitored the growth and viability of spheroids over several days of culturing without medium exchange (Fig. 3 a, b, e, f). From 24 hours to 96 hours of culture the diameter of HeLa spheroids increased from $70\pm 10\ \mu\text{m}$ to $100\pm 15\ \mu\text{m}$, while that of HEK 293 decreased from 170 ± 10 to $140\pm 10\ \mu\text{m}$ and of MCF-7 decreased from $140\pm 20\ \mu\text{m}$ to $110\pm 20\ \mu\text{m}$ (Fig. 3 a, b). We observed that viability of spheroids formed from all three cell types was above 90% up to 7 days of culturing without medium exchange (Fig. 3 e, f). By addition of fresh medium using non-contact dispenser it might be possible to extend culturing time of spheroids beyond 7 days.

Drug treatment of spheroids on the DMA platform

As a next step we performed treatment of spheroids formed from HeLa cells with three different anti-cancer compounds (doxorubicin, oxaliplatin and 5-fluoracil) and compared dose-response of HeLa spheroids and HeLa cells grown in 2D monolayer (Fig. 4). HeLa cells were seeded using the liquid dispenser in amount of 150 cells per 100 nL droplets and incubated in hanging droplets for 48 hours to allow spheroid formation. Afterwards, spheroids were treated with drugs by dispensing 50 nL of compounds diluted in culture medium till desired concentrations (see experimental section) and incubating arrays for 72 hours. For 2D cell culture HeLa cells were dispensed with a liquid dispenser onto a DMA slide and incubated in the upright position for 5 hours to allow cells to attach to the surface, followed by treatment with the same concentrations of compounds as in the case of spheroids before culturing for 72 hours. Then cells were stained with Calcein and propidium iodide (PI) for visualization of live and dead cells, respectively. The dose-response curves are shown in Figure 4 and demonstrate a clear dose-dependent effect of all three compounds on viability of HeLa cells grown both in 3D and 2D formats (Fig. 4). HeLa cells grown in spheroids were more resistant to drug treatment compared to 2D monolayer culture (Fig. 4): for doxorubicin IC₅₀ were $\sim 6\ \mu\text{M}$ and $< 0.05\ \mu\text{M}$; for oxaliplatin $1.5\ \mu\text{M}$ and $\sim 0.05\ \mu\text{M}$; for 5-fluoruracil $1\ \mu\text{M}$ and between 0.1 and $0.5\ \mu\text{M}$ for 3D and 2D cell culture, respectively. Increased resistance of tumor spheroids to anti-cancer compounds compared to monolayer culture of the same type of cells was reported multiple times previously (Ma 2012, Multicellular tumor spheroids as an in vivo-like tumor model for three-dimensional imaging of chemotherapeutic and nano material cellular penetration; Mohapatra 2016 Spectral mapping of 3D multi-cellular tumor spheroids: time-resolved confocal microscopy; Shi 2018 Facile Tumor Spheroids Formation in Large Quantity with Controllable Size and High Uniformity).

Discussion and conclusion

In the current study a facile methodology for fast and miniaturized drug screenings using arrays of single cancer spheroids formed in nanoliter droplets on the Droplet-Microarray platform was demonstrated. Formation of spheroids from several commonly used cancer cell lines in 100 nL droplets within 24 - 48 hours starting only with 150 cells per spheroid was shown. To evaluate the possibility of miniaturized drug screenings on cancer cell spheroids, the drug treatment of HeLa cells-based spheroids was performed, and clear dose-responses to three different anti-cancer compounds were observed. The spheroids could be cultured for up to 7 days with over 90% viability without exchange of the medium. The size of spheroids could be controlled by varying the initial number of cells per a single nanoliter droplets. Established methodology offers a number of advantages compared to existing platforms. For example, **miniaturization** down to 100 nL per droplet can result in up to 99% savings of compounds,

reagents, cells compared to microtiter plates. The possibility to **use only 150 cells** per spheroid can be crucial when working with rare and hard to expand cells, such as primary patient derived tumor cells. This makes this system attractive for drug sensitivity and resistance tests performed in 3D on patient-derived tumor cells, which in turn an important milestone for personalized medicine applications. The DMA platform is uniquely suited for the formation of **single-spheroid microarrays** with **uniform spheroid** size as opposed to many other methodologies including ULA plates, where number and size of formed spheroids per well cannot be defined. Another advantage is the possibility to perform microscopy of spheroids directly on the DMA slides without the need to transfer them into microtiter plates. Due to the small droplet size the positioning of spheroids is fixed, which also simplifies both the microscopy and image processing. Last but not least, formed homogeneous cell spheroids can be conveniently collected into a flask for further experiments, which is important for various applications ranging from *in vitro* tests to regenerative medicine. Thus, we believe that the established single-spheroid microarrays can be adopted for screening workflows in fundamental research, in drug discovery and in the field of precision medicine.

Figures

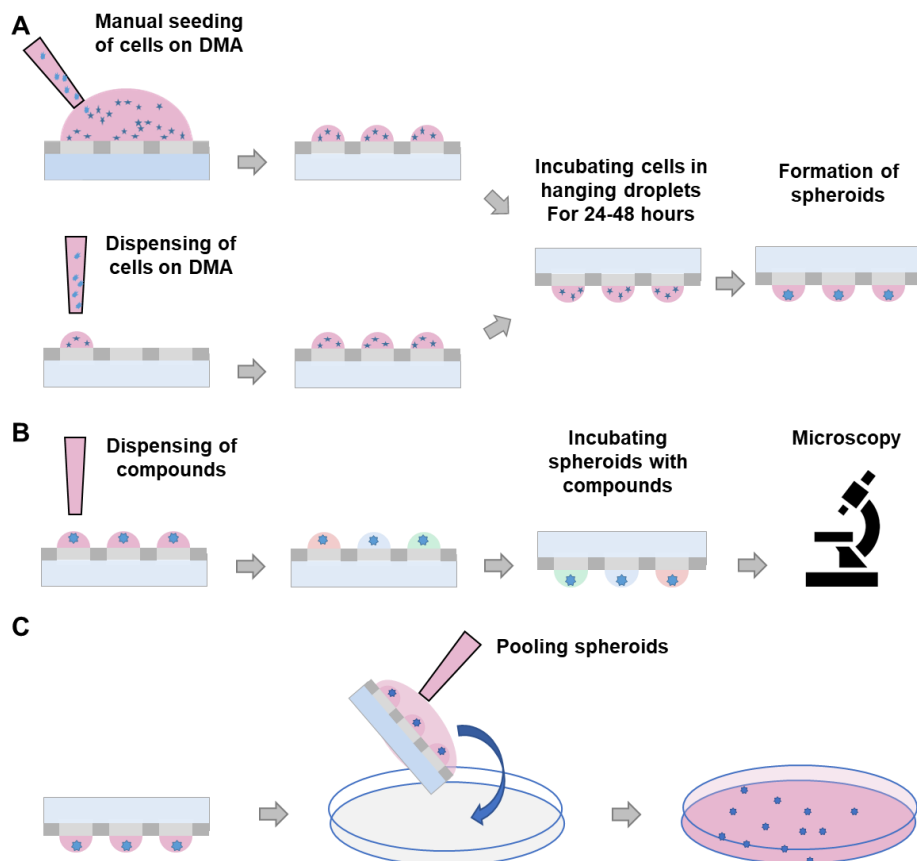


Figure 1. Schematics demonstrating the fabrication and screening of spheroids on the Droplet-Microarray (DMA) platform. (a) Manual cell seeding and seeding by dispensing followed by growing spheroids in hanging droplets. (b) Compound screening using single-spheroid-microarrays. (c) Pooling spheroids formed on the DMA platform.

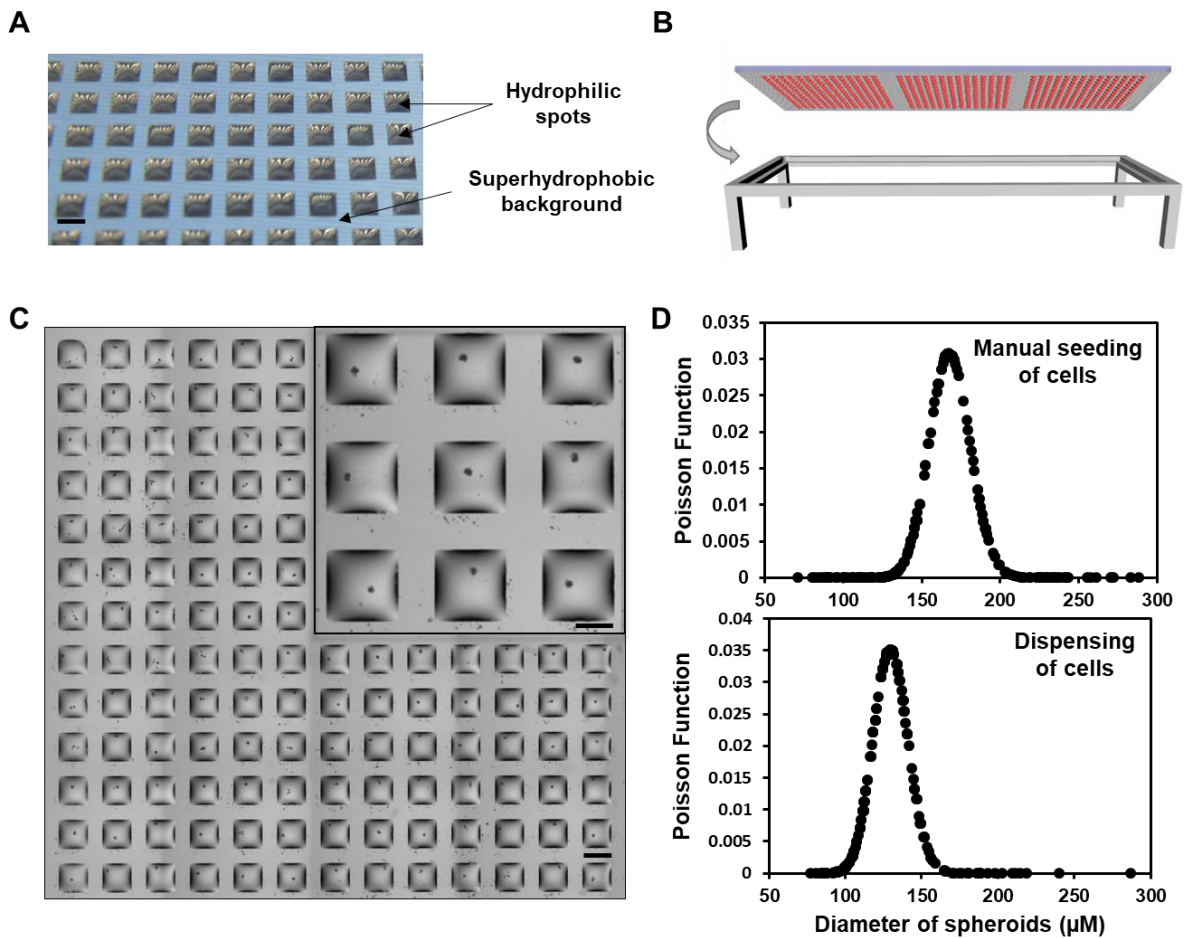


Figure 2. (a) Photograph of an array of water droplets formed on a DMA slide. Scale bar: 1 mm. (b) DMA slide in the hanging droplet format. (c) Microscope image of an array of 13 x 13 droplets containing single spheroids. Scale bar: 1 mm. Insert: 0.5 mm. (d) Graphs showing distribution of spheroids' diameters (196 spheroids formed on a single DMA slide). Spheroids were obtained by using manual seeding (top graph) and by cell dispensing (bottom graph).

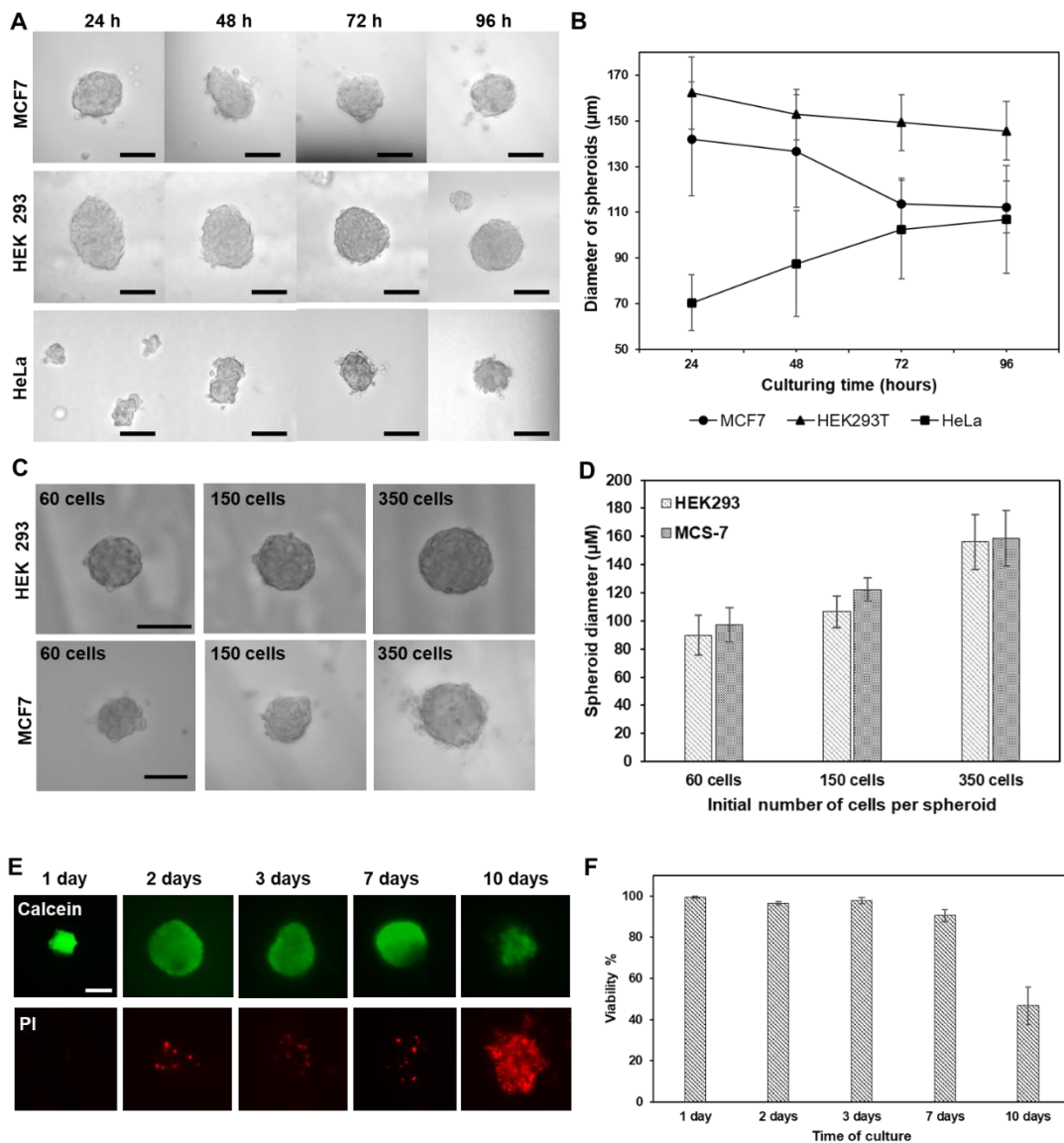


Figure 3. Fabrication and culturing of spheroids on the Droplet-Microarray (DMA) platform. (a) Brightfield microscope images of spheroid formed from MCF-7, HEK 293 and HeLa cells 24, 48, 72 and 96 hours after cell seeding. Scale bar: 100 μm . (b) Diameter of spheroids obtained from MCF-7, HEK 293 and HeLa cell lines cultured for 96 hours in 80 nL droplets. The initial cell number was on average 150 cells per spheroid. (c) Brightfield microscope images of spheroids formed from HEK 293 and MCF-7 cell lines starting from about 60, 150 and 350 cells per spheroid. Scale bar: 100 μm . (d) Diameter of spheroids obtained from HEK 293 and MCF-7 cell lines starting from different initial cell numbers. (e) Microscope images of HEK 293 spheroids cultured on the DMA for 1, 2, 3, 7 and 10 days without medium exchange. Spheroids were stained with Calcein AM and propidium iodide (PI). Scale bar: 100 μm . (f) Viability of spheroids formed from HEK 293 cells cultured on the DMA without medium exchange for 10 days.

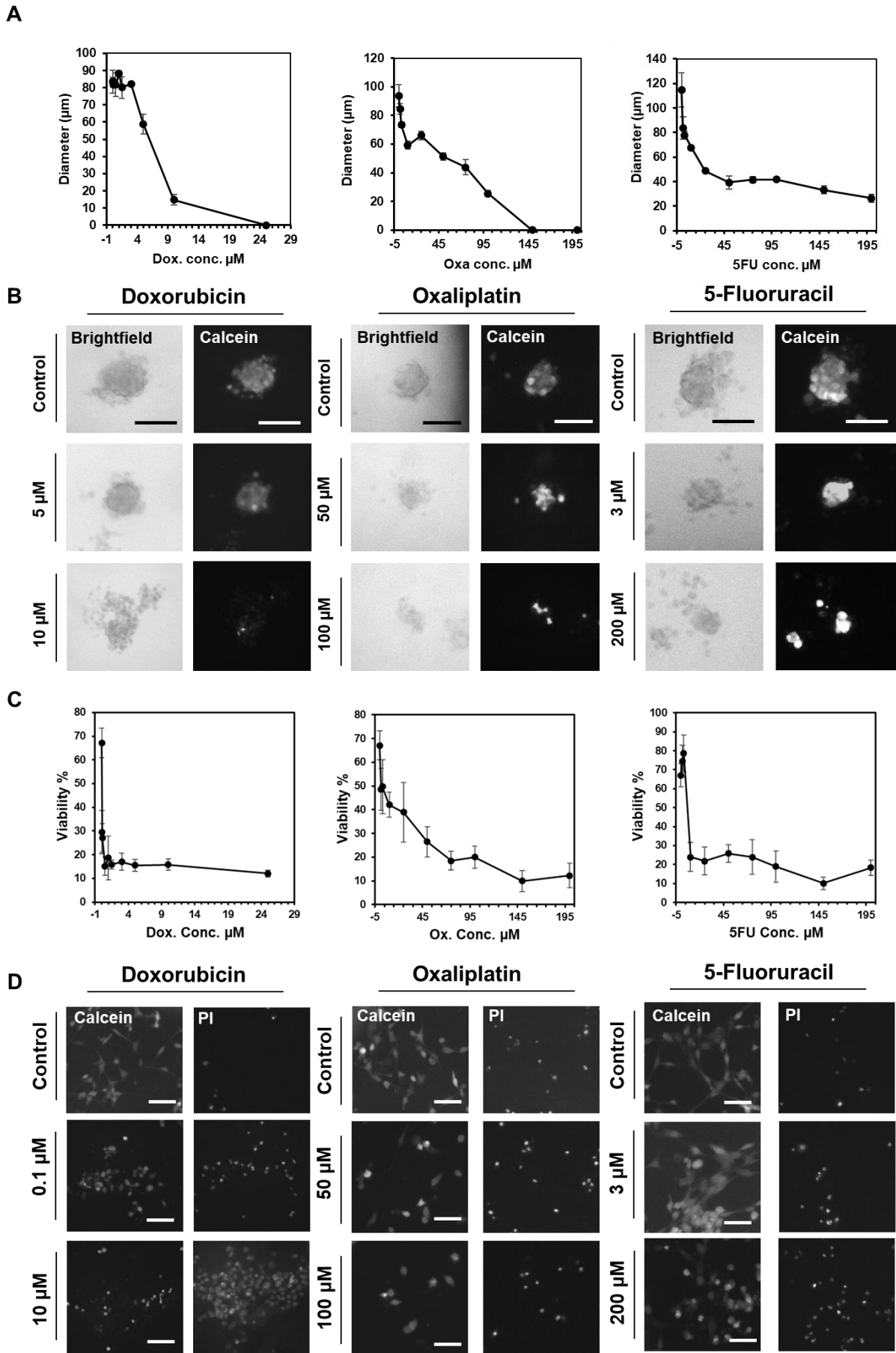


Figure 4. Treatment of spheroids on the Droplet-Microarray (DMA) platform with anti-cancer drugs. (a) Dose-dependent effect of doxorubicin, oxaliplatin and 5-fluorouracil on HeLa spheroids. Initial cell number: about 150 cells per spheroid. Spheroids were formed for 72 hours, followed by addition of drugs using non-contact liquid dispenser and incubating of spheroids for 48 h before staining and microscopy analysis. (b) Representative microscope images of HeLa spheroids treated with different concentrations of drugs. Scale bar: 100 μm . (c)

Dose-dependent effect of doxorubicin, oxaliplatin and 5-fluorouracil on HeLa cells cultured in 2D monolayer. Initial cell number: about 150 cells per spot. Cells were cultured on the DMA slide for 24 hours followed by addition of drugs using non-contact liquid dispenser and incubating of cells for 48 h before staining and microscopy analysis. (d) Representative microscope images of HeLa cells treated with different concentrations of drugs. Scale bar: 100 μm .

Experimental section

Cell culture

Hela, MCF7 and HEK293 T cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were cultured in 10 cm cell culture dishes and were split every 2-3 days.

Seeding cells and formation of spheroids on Droplet-Microarrays

DMA slides were purchased from Aquarray GmbH (Eggenstein-Leopoldshafen, Germany). Before cell culture DMA slides were sterilized with 70% ethanol and dried under the clean bench. For seeding on DMA slides cells were trypsinized and diluted with DMEM containing 15% FCS and 1% penicillin/streptomycin till concentration of $2,5 \times 10^5$ cells/mL and $1,5 \times 10^6$ cells/mL for manual and automated seeding, respectively. For manual seeding DMA slide was placed into Petri dish and 1,7 mL of cell suspension was applied onto each field containing 196 spots and incubated for 30 seconds. Afterwards Petri dish was slightly tilted letting big droplet of cell suspension roll off the slide, resulting in spontaneous droplet formation containing cells. For culturing cells in 2D Petri dish was closed with humidifying lid containing humidifying pad wetted with PBS to avoid evaporation and placed to cell culture incubator immediately. For culturing cells in 3D DMA slide containing cells was placed on specially designed table in upside down position. Table was placed into Petri dish containing 10 mL of PBS and closed with humidified lid. Petri dish was placed in cell culture incubator immediately after seeding. For automated cell seeding cells were dispensed in amount of 150 cells per 100 nL droplets with I-DOT dispenser (Dispendix GmbH). After printing DMA slide was placed in Petri dish either in upright or on the special table in upside down position for 2D and 3D cell culture, respectively.

Imaging of spheroids

For imaging DMA slides containing spheroids were placed either into Petri dish or a four-well dish (Thermo Scientific Nunc) in upright position. Imaging was performed with Keyence BZ-9000 (KEYENCE, Osaka, Japan) and with Olympus IX81 microscope (Olympus, Tokyo, Japan). The images were taken at $6,4 \times$ magnification, in three different channels: brightfield, mCherry for PI staining, and green fluorescent protein GFP, for CalceinAM staining.

Live/dead staining of spheroids

For live/dead staining of spheroids 50 nL of staining solution containing 1 $\mu\text{g/mL}$ propidium iodide (PI) (Invitrogene, Merelbeke, Belgium) and 1,5 $\mu\text{g/mL}$ CalceinAM (Thermo Scientific) in PBS was dispensed into each droplet using I-DOT dispenser (Dispendix GmbH). DMA slide was incubated in cell culture incubator for 15 minutes and imaging was performed as described in section 'Imaging of spheroids'.

Drug treatment of HeLa cells in 3D spheroids and in 2D monolayer culture

For drug treatment HeLa cells were seeded onto DMA slides by dispensing cells in amount of 150 cells per 100 nL droplets and incubated either in upright or upside-down position

for 2D and 3D cells culture, respectively. Spheroids from HeLa cells were grown for 48 hours before the drug treatment. For 2D cell culture experiments HeLa cells were incubated for 24 hours before drug treatment. Afterwards, different concentration of three compounds, doxorubicin (European Pharmacopolia Reference Standards, European Directorate for the Quality of Medicines & Healthcare, Strasbourg, France), oxaliplatin (XX) and 5-Fluoruracil (XX) were dispensed with I-DOT dispenser (Dispendix GmbH) into droplets containing HeLa cells either in spheroids or in 2D monolayer. Compounds were diluted in DMEM medium containing 15% FCS and 1% penicillin/streptomycin till concentrations 0.15, 0.3, 1.5, 3, 4.5, 9, 30 and 75 μM and dispensed in amount of 50 nL into droplets of 100 nL resulting in final concentration in droplets 0.05, 0.1, 0.5, 1, 1.5, 3, 5, 10 and 25 μM , respectively. After drug addition cells in both 2D and 3D cell culture were incubated for 48 hours. Afterwards, cells were stained with Calcein and PI and imaged as it is described in sections ‘Live/dead staining of spheroids’ and ‘Imaging of spheroids’, respectively.

Image analysis

For monitoring spheroid growth over a period of 5 days (Fig. 3b) and estimation of dependence of spheroid size on initial cell number per spheroid (Fig. 3d), diameter of spheroids was measured manually in ImageJ software in brightfield images (Fig. 2d). For estimation of viability of spheroids cultured over a period of 10 days, ratio of Calcein positive area to combined Calcein and PI positive area was calculated (Fig. 3f). The Calcein and PI positive areas for live and dead part of a spheroid, respectively, were estimated using ImageJ software. To estimate the effect of the drugs, diameter of spheroids was measured in fluorescent images acquired in GFP channel showing Calcein (live) staining (Fig. 4). For estimation of viability of HeLa cells cultured in 2D monolayer, number of live and dead cells was counted in images acquired in GFP and mCherry channels, respectively, using ImageJ software (Fig. 4).

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