A novel hanging spherical drop system for the generation of cellular spheroids and high throughput combinatorial drug screening

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Experimental Section

Fabrication of patterned SH surfaces: SH polystyrene (PS) surfaces were produced using a one-step phase-separation methodology under ambient condition. A solution of 70 mg.mL^{-1} was prepared dissolving the PS granules (Styrolution PS 158K) in tetrahydrofuran (THF from Fluka, p.a. >99.5%) solvent for 2 hours to form a pre-solution at ambient temperature. Then, 1.3 mL of ethanol (from Panreac) was added into 2 mL of PS pre-solution prepared and the mixed solution was stirred. A necessary volume to cover the PS substrate with this mixture was dipped onto a cleaned PS film (from Goodfellow, UK, with 0.25 mm of thickness, ref. ST311190/1). The substrate was tilted in order to remove the solution and then was immersed in ethanol for 1 min. Afterwards the surface was dried at ambient temperature and porous PS surface was obtained.

Micro-indentations over the SH surfaces were produced using a microhardeness tester equipment (Leica VMHT 30) applying loads of 2942 mN with a dwell time of 10 s. A sharp rigid Vickers diamond pyramid indenter (included angle α=136º) penetrated into the surface with a constant load during a complete loading–unloading cycle producing individual indentations. To produce the arrays to sustain droplets of cell culture medium, the micro-indentations were placed in the SH surface at a distance of 5 mm from each other, with a square configuration, in the total of 16 spots for each condition.

Surface characterization: Water contact angle measurements were also performed at room temperature using an OCA 15 plus goniometer (DataPhysics). The values were obtained by the sessile drop method. The used liquid was ultrapure water and the drop volume was 3 μL. At least five measurements were carried out.

The surface morphology of the SH sample and the spheroid formation was observed using a Leica Cambridge S-360 scanning electron microscope (SEM). All samples were coated with a conductive layer of sputtered gold. The SEM micrographs were taken at an accelerating voltage of 15 kV and at different magnifications.

Cell Expansion and Cell Culture: The immortalized mouse lung fibroblast cell line L929 was purchased from the European Collection of Cell Cultures and was used as well established cell line for viability studies. Cells were expanded in low glucose Dulbecco's Modified Eagle's Medium (DMEM), from Sigma-Aldrich, supplemented with 3.7 $g \cdot L^{-1}$ sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and 1% antibiotic/antimycotic (final concentration of penicillin 100 $units.mL^{-1}$ and streptomycin 100 $mg.mL^{-1}$; Gibco) at pH 7.4. Cells were grown in 75 cm^2 tissue culture flasks and incubated at 37 °C in a humidified air atmosphere of 5% CO_2. Every 3−4 days, fresh medium was added. At 90% of confluence, cells grown in tissue culture flasks were buffered with phosphate buffer saline (PBS) and subsequently detached by a chemical procedure with 0.05% trypsin-EDTA solution for 5 min at 37 °C in a humidified air atmosphere of 5% CO_2. To inactivate the trypsin effect, cell culture medium was added. The cells were then centrifuged at 300 g and 25 °C for 5 min and the medium was decanted. Cell suspensions with distinct densities were prepared.

Monolayer Cell Culture: 1 mL of supplemented DMEM containing different cell suspensions was placed in a non-treated surface of 48-well cell plate (in triplicate). The plates were incubated at 37 ºC in a humidified air atmosphere of 5% CO_2 at different incubation days. The fluorescent images of live/dead assay, were obtained by removing the cell culture media, and wash the culture plates carefully with PBS. Then, 1 mL of PBS containing 2 µL of calcein-AM and 1 µL of propidium iodide dyes were added to each well to stain the live cells with green and dead with red. After 10 minutes at 37 ºC, samples were extensively washed with PBS solution and immediately visualized in the dark by transmitted light microscopy (Axio Imager Z1m, Zeiss). ImageJ software (NHI, USA) was used to quantify the cells adhered on the well plate.

Cytotoxicity of 2D cell culture: The 2D monolayers of cells were tested for cytotoxicity using alamarBlue colorimetric assay. Briefly, different cells density were placed in a treated surface 48-well cell culture plate (in triplicate) and incubated at 37 °C and 5% CO_2. After 48 hours of

culture, the assay was performed, protected from light. The culture medium was removed and 500 µL of supplemented DMEM containing 10 % v/v alamarBlue solution was added to each well (n=9). Samples were then incubated in the dark at 37 °C and 5% CO_2. After 4 h, 100 µL of each well (in triplicate) was transferred to a 96-well plate. The absorbance was monitored at 570 nm and 600 nm using a microplate reader (Synergy HT, Bio-TEK).

On-chip spheroids formation: A hanging drops approach used for the formation of spheroids was performed using SH surfaces patterned with adhesive micro-indentations. Prior to cell seeding, the SH surfaces were sterilized by UV radiation for 30 min. Suspensions with different fibroblasts-like cell densities were prepared. Droplets of 5 µL of cell suspensions (in triplicate) were placed over the micro-indentations of the platforms. The platform with different cell suspensions was inverted 180º and incubated for 48 hours at 37ºC in a humidified 5% CO_2 atmosphere. The platform containing droplets of cell suspensions was fixed into the lids of polystyrene petri dishes, and the bottom part was filled with cell culture media, in order to create a saturated environment and avoid the evaporation of the droplets. The representative images of the formed spheroid in the droplet with 40000 L929 cells after 48 hours of incubation (Figure 2) were taken using scanning electron microscope (JSM-6010LV, JEOL), transmitted light microscopy (Axio Imager Z1m, Zeiss) and inverted confocal microscopy (Leica, TCS SP8). For the SEM analysis, after the spheroid formation, 5 µL of 10% formalin was dispensed to the droplets. The spheroids were dehydrated with a series of increasing ethanol concentrations (50%, 70%, 80%, 95% and 100%). Live/dead assay was assessed. 2 µL of PBS containing calcein-AM and propidium iodide was added to the spheroids. DAPI and phalloidin assay was also performed. DAPI stains preferentially double-stranded DNA by delineating cells nuclei in blue and phalloidin stains with the red colour the actin filaments of the cells and is used to evaluate the function of cytoplasmic actin. Prior to staining, the culture medium was removed and 5 µL of 10% formalin was dispensed to the droplets. Then, 2 µL of PBS containing DAPI and phalloidin was added.

Drug screening tests: After 48 hours of cell culture for spheroids formation, 2 µL of doxorubicin (Doxorubicin hydrochloride, Sigma-Aldrich) with different concentrations (0.1 $mg.mL^{-1}$, 0.01 $mg.mL^{-1}$ and 0.001 $mg.mL^{-1}$) were dispensed in the different droplets. The chips were incubated for 24h at 37ºC in a humidified 5% CO_2 atmosphere. The liquid content of the droplets with cell

aggregates were carefully exchanged with PBS. Afterwards, 2 μL of PBS containing calcein-AM and propidium iodide was added to each condition to staining the spheroids. The same procedure was made to 2D cell culture.

3D spheroids analysis: Image analysis tool (WCIF ImageJ software, NHI) was used to evaluate the percentage of live/dead cells of each droplet condition. Each spheroid image obtained from confocal microscope was divided in green and red channels and then transformed in a gray scale (grey scale intensity 0-255, in which 0 correspond to black colour and 255 white colour). The percentage area of the cells in each channel was calculated and divided for the number of total analysed area. The same threshold criterion was used in all images.

Statistical analysis: The statistical analysis of both cell cultures data was performed using two-way analysis of variance (ANOVA) with Bonferroni post-test using GraphPad Prism 5.0 software. The adopted nomenclature was the following: statistical differences in grouped by time point analysis were marked with (**) and (***), which stand for p-value < 0.01 and $p < 0.001$, respectively. For evaluate the statistical differences relating to the time point before, the symbol "#" was used to represent 3D spheroid. Double symbols (##) represent $p < 0.01$ and triple symbols (###) represent $p < 0.001$. All results were presented as mean ± standard deviation.