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Microfluidic-based platform for tumour spheroid culture, monitoring and drug screening

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Development of novel cellular models that can replace animals in preclinical trials of drug candidates is one of the major goals of cell engineering. Current *in vitro* screening methods hardly correspond with the *in vivo* situation, whereas there is lack of assays for more accurate cell culture models. Therefore, development of automated assays for 3D cell culture models is urgently required.

In this work, we present a SpheroChip system: microfluidic-based platform for long-term 3D cell culture and analysis. The system is compatible with commercially available microplate readers and provides continuous, *in situ* monitoring of tumour spheroids cultured on a chip. The microfluidic chip consists of cell culture microchambers and hemispherical microwells connected with a concentration gradient generator. HT-29 and Hep-G2 cells were successfully cultured as tumour spheroids in SpheroChip and metabolic activity of cells was monitored for up to two weeks by *in situ* fluorimetric measurements. Cellular response to anticancer drug was observed using SpheroChip. The experimental setup provided unique possibility of observation of dynamic changes of metabolic activity of one culture during sequencing days after drug dosage. According to this new approach, unknown phenomena of cellular response to anticancer drug were observed, such as increase of metabolic activity shortly after drug dosage. Moreover, influence of a second dose of a drug was evaluated. The SpheroChip system can be used by researchers working on drug screening, evaluation of anticancer procedures and chemiresistance phenomena.

Introduction

Nowadays, drug development sector faces a quandary between throughput and accuracy of accessible *in vitro* screening assays. A majority of the applied tests base on monolayer (2D) cell culture, which is an easy to handle model suitable for automation and is willingly used in High Throughput Screening (HTS) systems.[1] However, 2D culture lacks essential interactions present *in vivo*, which strongly limits a prediction of an effect of a drug on humans.[2] Therefore, increasing attention is paid on development of three-dimensional cellular models.[3] 3D cellular models mimic spatial cell-to-cell interactions present *in vivo*, which are proven to influence intracellular pathways.[4] Thus, application of 3D models for drug screening leads to results that can predict *in vivo* situation more accurately.[3]

Among 3D cellular models spheroids gain increasing interest. Tumour Spheroid is considered as the best cellular model for cancer research developed so far.[5] There are several factors which make morphology and physiology of tumour spheroid similar to a tumour *in vivo*, *i.e.* network of cell-cell interactions, three-dimensional structure, presence of natural extracellular matrix and nutrients, metabolites and oxygen gradients.[6] The tissue structure determines growth rate of a tumour as well as response to anticancer drugs.[7] Despite numerous and known for decades advantages of the spheroid model, its widespread use for drug screening is still limited. There are a number of methods of spheroid cultivation,[5,8] among which there is

increasing participation of microfluidic-based methods.[9-22] However, quantitative determination of cellular response in 3D arrangement is still problematic and not fully satisfying.[3, 23, 24] Microfluidic devices mostly have been used for spheroid formation[9-14, 16, 20, 21, 25, 26] and/or cultivation.[9-13, 20-22, 26] Few of them were used for spheroid analysis[18, 21, 22, 24] and none utilized instrumental method *in situ*. There is no microfluidic solution for spheroid formation, long-term culture and automated analysis on chip combined in one device. Moreover, there is only one spheroid-based solution compatible with microplate readers, utilizing a hanging drop cultivation method.[27]

In this work, we propose a microfluidic-based bioanalytical platform for long-term cultivation and observation of tumour spheroids. Our SpheroChip system is suitable for dual type of analysis: (1) microscopic observations of cellular morphology and (2) fluorescent staining as well as fluorimetric measurements *in situ* using commercially available microplate readers. We applied a simple spheroid formation and cultivation method, which was successfully used in our previous work.[28] We improved a cell culture zone by changing of a shape of microwells. In a SpheroChip, microwells for spheroid formation are hemispherical. This was possible due to application of a clean-room independent fabrication method, which was developed by us and can be easily adapted in small laboratories.[28, 29] Cell culture zones were grouped into microchambers. Dimensions and arrangement of microchambers were identical with wells of a standard 384-well plate. Therefore, SpheroChip can be applied in widely accessible instruments. Two human cell lines (HT-29 colon carcinoma and Hep-G2 liver carcinoma) were successfully cultured as spheroids using SpheroChip.

Design of the SpheroChip system provided effective medium exchange and precise control over spheroid environment. It is a significant advancement comparing with existing methods, *i.e.* hanging drop method, which principle disables total medium exchange. Moreover, the applied protocol made possible to monitor of metabolic activity of cultured cells continuously. This enabled novel approach to drug activity evaluation. One population of spheroids could have been monitored for several days after drug dosage. Profiles of time-dependent drug sensitivity were plotted for different concentrations of a model anticancer drug: 5-fluorouracil. This provided novel insights into cellular response to external factors. For example, we observed the effect of acquired resistance to higher drug concentration and the increase of metabolic activity shortly after drug dosage. The proposed technique can be helpful in revealing phenomenon of chemiresistance of colon cancer,[30] as dynamic changes of cellular activity can be detected. Additionally, the applied protocol enabled monitoring of drug activity within different dosing regimens. There was no method for quantitative analysis of response of one population of spheroids to sequencing doses of a drug, so far. Effects of dosing is subject, that is highly required, but still missing in drug development prior to clinical trials.[1] To sum up, in this paper we present a novel bioanalytical platform that can provide novel insights into tumour physiology and mechanisms of response to anticancer drugs.

Experimental

Fabrication

The microfluidic chip was fabricated in PDMS (Sylgard 184, Dow Corning) according to the previously described protocol.[28, 29] The first master was micromilled in a poly(methyl methacrylate) (PMMA) slab using CNC micromilling machine (Minitech Machinery Co.). The second master was obtained by replica moulding in PDMS. PDMS was prepared by mixing pre-polymer and the curing agent in the weight ratio of 9:1. The PDMS mixture was poured over the PMMA structure and cured for 3 h in 70°C. The PDMS replica was peeled off and placed in the oven set to 100°C for 48 h thermal aging, and thus the second master was obtained. The second master was used for replica moulding in 9:1 PDMS. Up to 20 sequencing moulding steps could have been performed using one thermally aged master. A replica of the second master was peeled off from the mould and cut to a proper shape. Thus, a bottom layer of the chip was obtained. A cover layer was made of a plane PDMS slab, which contained drilled ports for tubings. Both layers were connected by bonding using oxygen plasma treatment (Plasma Preen System Inc. II 973). The chip was cut to a bottom layer shape and tubings were connected. The resulted chip is presented in Figure 1.

The positioning plate was fabricated in a poly(ether ether ketone) slab using CNC micromilling machine (Minitech Machinery Co.). Cavities for chip positioning were blackened with pieces of black printed paper.

Chip operation

The microfluidic chips were operated using syringe pumps (NE 1000 New Era Pump Systems Inc.). During incubation or measurements tubings were sealed by PDMS-filled needles.

Prior to cell culture, the chips were sterilized by exposure to UV light (Black Ray) for 20 min, and then flushed with 70%$_{vol}$ ethyl alcohol (POCh, Poland).[28, 31, 32] To prevent cell attachment, 0.5%$_{weight}$ solution of poly(vinyl alcohol) (PVA, Sigma Aldrich) in DI water was sterilised using PTFE syringe filter (pores of 0.2 μm, Sigma-Aldrich) and introduced to the microchannels and incubated for 1 h. Then, the system was filled with cell culture medium and warmed-up by placing in CO_2 incubator (HERAcell 150, Thermo Scientific).

Cell culture and observation

Two cell lines were used for experiments: HT-29 human colon carcinoma cells and Hep-G2 human liver carcinoma cells (both lines purchased from American Type Culture Collection). HT-29 culture medium was based on RPMI Medium (Sigma-Aldrich) supplemented with 5%$_{vol}$ of Fetal Bovine Serum (Gibco), 1%$_{vol}$ of 25mM L-glutamine (Sigma-Aldrich) and 0.6%$_{vol}$ of 100mM penicillin and streptomycin (Sigma-

Aldrich). Hep-G2 culture medium was based on MEME Medium (Sigma-Aldrich) supplemented with 10%$_{vol}$ of Fetal Bovine Serum (Gibco), 1%$_{vol}$ of 25mM L-glutamine (Sigma-Aldrich) and 1%$_{vol}$ of 100mM penicillin and streptomycin (Sigma-Aldrich). Routine passages of both lines were performed at 80-95% confluence using TrypLE™ Express (Gibco) and Phosphate Buffered Saline (Sigma-Aldrich).

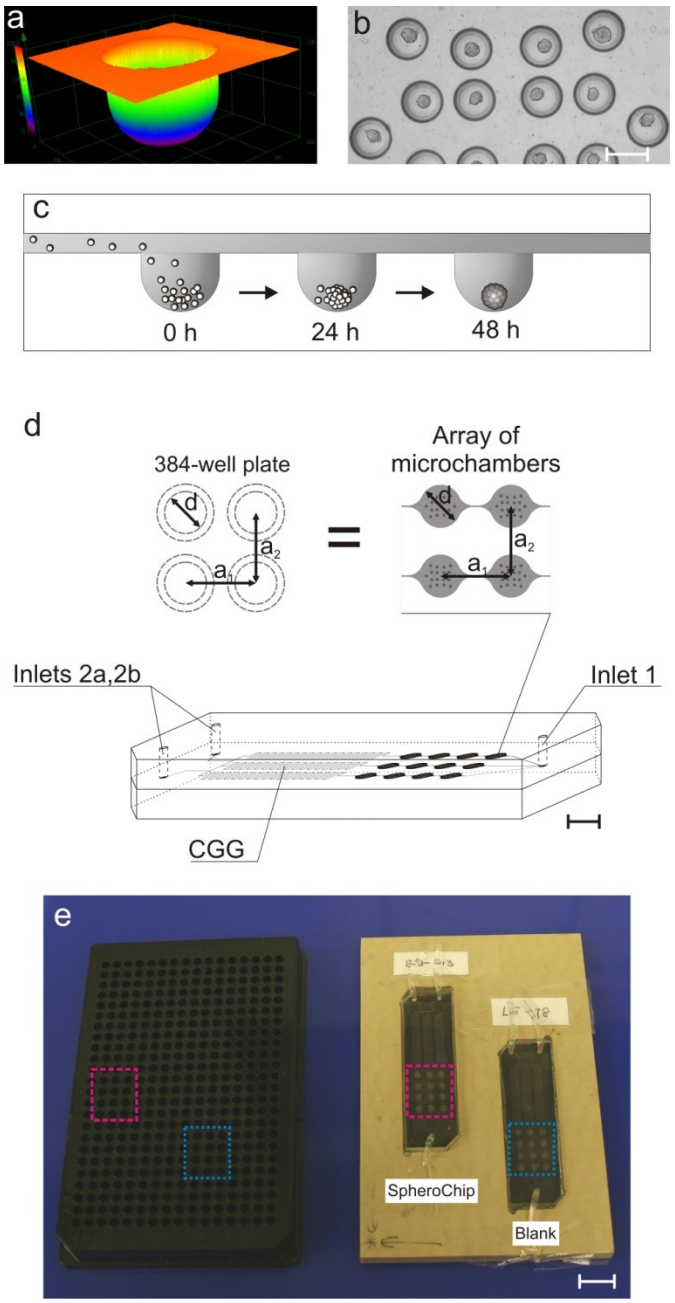

Figure 1. Construction of the SpheroChip system. (a) Confocal laser microscope profile of a hemispherical microwell for spheroid formation and culture. (b) Micrograph of uniformly distributed spheroids in centres of microwells; HT-29 cells, 2nd day of culture; scale bar corresponds to 200 µm. (c) Cross-section of SpheroChip and scheme of spheroid formation. (d) Design of SpheroChip; diameters (d) and offsets (a_1, a_2) between microchambers are the same as adequate dimensions of 384-well plate. (e) Chips placed in the positioning plate and comparison with 384-well plate.

Cell suspension was prepared according to the protocol previously described.[28, 32] Density of cell suspension was measured using Countess Cell Counter (Invitrogen). Cell suspension of $1 \cdot 10^6 - 5 \cdot 10^6$ cells/mL was used as an inoculum for on-chip experiments. Cells were introduced to the microsystem using syringe pumps. The system was sealed and placed for incubation in CO_2 incubator. Medium was exchanged daily with a constant flow rate of 4.5 µL/min for 15 minutes.

Observations of cell cultures were carried out by an inverted fluorescence microscope coupled with a CCD camera (Olympus). CellSens image analysis software (Olympus) was used for data acquisition and analysis. Micrographs of culture progress were taken each day of culture, and sizes of spheroids (surface areas) were measured. Assuming spherical shape of spheroids, their volumes were estimated and growth curves were plotted (see Figure 4).

Spectrofluorimetric measurements

All spectrofluorimetric measurements were carried out using Varian Cary Eclipse Fluorescence Spectrophotometer equipped with Microplate Reader (Agilent). Alamar Blue (AbD Serotec) was used as a fluorescent indicator of metabolic activity of cells.[33] 10%$_{vol}$ of Alamar Blue was added to cell culture medium and left for incubation with cells. Petri dish cell culture was incubated for 16 hours with Alamar Blue, while on-chip culture was incubated for 10 minutes (see *Metabolic activity monitoring* in *Results and discussion* section). Medium with 10%$_{vol}$ of Alamar Blue incubated for the same time without cells was used as a blank. Measurements were carried out using following parameters: excitation wavelength of 552 nm, and emission wavelength of 582 nm. After the measurement, medium was exchanged in a microfluidic chip.

Drug activity evaluation

5-fluorouracil (5-FU, Sigma-Aldrich) was used as a model anticancer drug.[28] Microfluidic chips with compact spheroids of uniform size distribution (48 hours after seeding) were chosen for drug screening experiments. Solution of 5-FU in HT-29 cell culture medium was introduced to the selected chip via 2a inlet (see Figure 1), while pure medium (for no-drug control) was introduced via 2b inlet. Each stream was introduced with a flow rate of 2 µL/min, which gives a total flow rate of 4 µL/min. Drug introduction lasted 15 minutes. After that, the chip was sealed and incubated for 24 hours. Next, medium was exchanged via 2a and 2b inlets, and measurements were performed every day. For each concentration of 5-FU, at least three experiments were performed.

At the end-point of each culture, a fluorescence-based live/dead cell viability assay using Calcein-AM and Propidium Iodide (Sigma-Aldrich) was performed.

Data analysis

Results of spectrofluorimetric measurements of on-chip cultures were processed according to a following scheme:
(1) $I_{relative} = I_{measured} - I_{blank}$
(2) $I_{av(control)} = (\sum I_{relative(control)})/4$,
where $I_{relative(control)}$ was relative intensity of microchambers without drug.
(3) $I_n = (I_{relative}/I_{av(control)})\cdot 100\%$
To sum up, I_n values were presented as percentage of a control. No-drug control was performed for each chip separately. Similarly, blank probe was measured each time.

Computational modelling

Fluid flow in the designed structure was simulated by computer modelling using MEMS Module of COMSOL Multiphysics software.

Results and discussion

Design

The design of the SpheroChip system (Figure 1) was meant to meet following requirements: (1) long-term culture of spheroids; (2) possibility of control over extracellular environment by total medium exchange; (3) controllable distribution of tested drugs; (4) microscopic observation of the culture and (5) compatibility with a microplate reader for assay automation. The design of a spheroid culture microchamber was based on our previous chip.[28] Each microchamber was 50 μm deep and contained 18 microwells of diameters of 200 μm and depths of 150 μm (Figure 1: a-c). This design provides isolation of a culture from shear stress caused by medium flow, while diffusion-based mass exchange is fast enough for effective nutrition and waste removal.[28] The improvement of a present design lays in application of hemispherical microwells (Figure 1: a). The microwells were obtained using a ball-end mill in a fabrication process. The hemispherical shape of microwells resulted in more effective aggregation of cells and centred position of resulted spheroids (Figure 1: b). Spheroid culture microchambers were placed in an array of 3 series, each containing 4 microchambers. Each series was connected with a separate outlet of a concentration gradient generator (CGG, Figure 1: d).[31] The design of the chip provided uniform cell distribution among series of microchambers: standard deviation of average spheroid dimensions in individual microchambers did not exceeded 30% (slightly misaligned chips) in the whole chip. Additionally, only those chips with standard deviations less than 20% were chosen for experiments.
Microchambers were also designed as measurement cells for spectrofluorimetric microplate readers. Diameters of microchambers (2.67 mm) were exactly the same as wells of a 384-well plate. Similarly, off-sets between the microchambers and wells were the same (4.5 mm, see Figure 1: d). An integral element of the system was a positioning plate fitting into a microplate reader. The positioning plate contained two hollows for SpheroChip placement. When chips were fitted to the positioning plate, arrays of microchambers were in positions corresponding with B10 to D13 and J5 to L8 wells of 384 well plate (Figure 1: e). Thus, each reading corresponded to 18 microwells, so a signal from one microchamber was resultant from 18 spheroids.
For proper positioning of SpheroChip in the reader, thicknesses of PDMS layers were considered. The bottom layer, containing microchannels and microchambers, was *c.a.* 3 mm thick. Thus, microchambers were on a proper height in the reader. The cover PDMS layer should have been thick enough for tight tubing placement and thin enough for proper culture oxygenation.[28, 32] During our preliminary measurements it was found, that thickness of the cover PDMS layer ranging from 2.7 to 8.0 mm, does not influence results of measurements. Therefore, thickness of the cover layer was set to *c.a.* 5 mm.

Experimental setup

To verify an experimental setup for long-term monitoring of spheroids on-chip, following issues were addressed: (1) usefulness of a measurement cell; (2) measurement parameters; (3) correctness of chip operation and (4) suitability of selected reagent for spheroid monitoring. First, two model solutions were prepared basing on Alamar Blue mixed with cell culture medium. The first probe was incubated overnight with HT-29 cells on a Petri dish (off-chip reduced reagent). The second probe was incubated for the same time without cells (native reagent).
Excitation and emission spectra of these probes were taken in a standard 1 cm^2 quartz cuvette as well as in a SpheroChip (Figure 2). Wavelengths for excitation and emission were set to 552 nm and 582 nm, respectively. It was confirmed, that the building material of a SpheroChip measurement cell (PDMS) does not affect spectra of used probes within selected parameters (see comparison between a cuvette and a chip in Figure 2).
The next experiment was designed to verify correctness of operation of a concentration gradient generator (CGG). Operation of a CGG structure designed for SpheroChip was initially modelled using COMSOL Multiphysics software (Figure 3: b). Total flow rates ranging from 2 to 20 μL/min were analyzed and total mixing was observed in the middle channel for each case. A maximal total flow rate for cell-containing SpheroChips was 5 μL/min, thus this value was selected for laboratory experiment of CGG performance. Probes described above (native reagent – C_0, and off-chip reduced reagent – C_1) were introduced into SpheroChip via CGG inlets with a flow rate of 2.5 μL/min each. As a result, three concentrations were expected in the series of microchambers: C_1, $C_{0.5}$ and C_0, (see scheme in Figure 3: a). The chip was sealed, placed in a positioning plated and introduced into a microplate reader. The result of the experiment is presented in Figure 3: c. Each point represents an average from 4 microchambers of one series. Error bars correspond to standard deviation. As it can be seen, measured

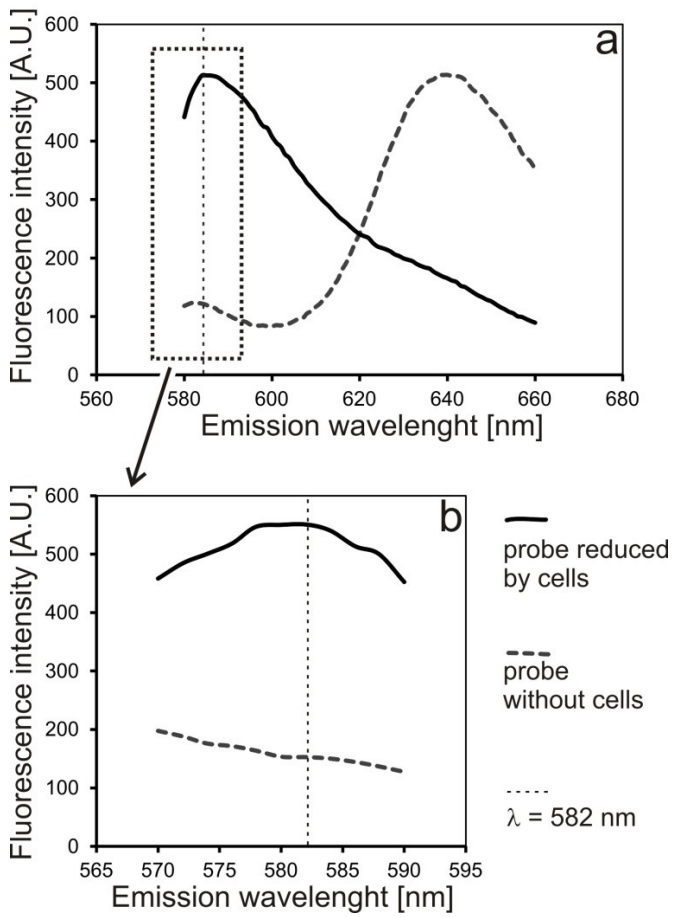

Figure 2. Comparison of emission spectra of Alamar Blue reduced by cells (off-chip) and native form of Alamar Blue in cell culture medium (without cells). Measurements performed: (a) in a standard 1 cm quartz cuvette and (b) on microfluidic chip placed in a microplate reader. Excitation wavelength = 552 nm.

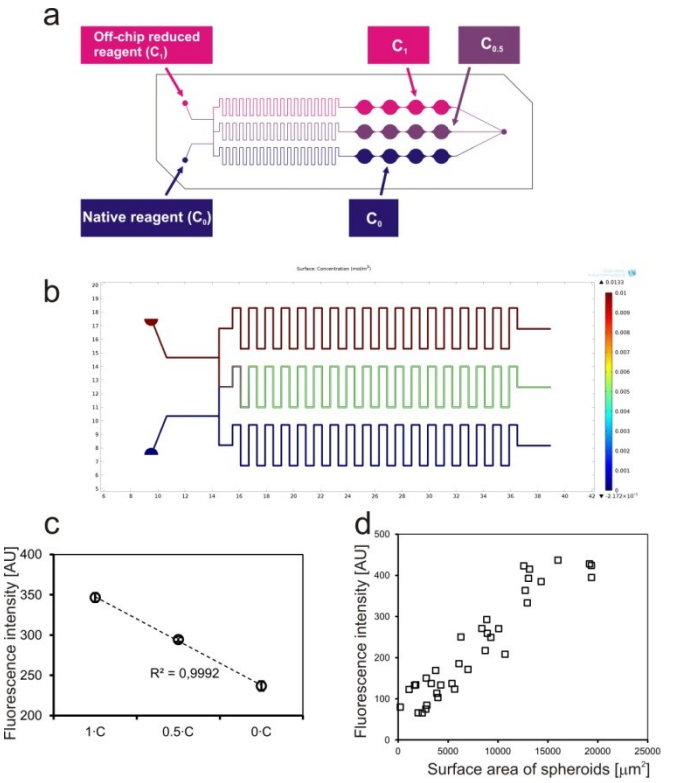

Figure 3. Proving of the principle of measurements: (a) scheme of an experiment; (b) results of computational modelling of a CGG structure; (c) results of spectrofluorimetric measurements of the chip containing solutions as shown on scheme "a". (d) Dependence of fluorescence signal of one microchamber on average size of spheroids in this chamber (on-chip culture).

values are arranged linearly. It is an evidence, that operation of the microfluidic chip and detection cells are consistent with expected ones.

The last experiment planned to test the experimental setup, was verification of dependence between the amount of cells and fluorescence intensity. Three SpheroChips containing HT-29 spheroids of different diameters were used in this experiment.

The measurement was performed according to the protocol given in Experimental section. Next, the areas of spheroids stained with Calcein-AM were measured. An average size of spheroids was calculated for each microchamber. The results were plotted on a graph presented in Figure 3: d. It was confirmed, that fluorescence intensity detected in the microchamber was directly proportional to a size of spheroids. Hence, basic principle of drug-screening experiments was verified and its correctness was confirmed.

Spheroid culture

The principle of spheroid formation and culture is presented in Figure 1: c. First, all microchannels of the system were filled with cell suspension, and afterwards, cells began to sediment into the microwells (0 h). During the first incubation period (24 h), cell aggregation was observed. At this point unaggregated cells were rinsed with fresh medium flow. During the next 24 hours spheroid compaction occurred (48 h) and it was considered as the start point of spheroid culture and spheroid-based experiments.

In this work, two cell lines were cultured as tumour spheroids inside SpheroChip: HT-29 human colon carcinoma cells and Hep-G2 human liver carcinoma cells (Figure 4). Both cell lines were successfully cultured for over one week and spheroid growth was observed. Growth rate of HT-29 cells was higher than Hep-G2 cells. Live/dead cell viability assay performed at the end-point of culture confirmed viability of cultured spheroids. Therefore, the designed microfluidic system proved to be suitable for long-term three dimensional culture of different cell lines.

Metabolic activity monitoring

Prior to drug screening experiments, regular cultures of HT-29 spheroids were monitored using the designed experimental setup. It was observed, that incubation time was crucial for results of measurement. Resazurin is a reagent of Alamar Blue and is reduced by metabolically active cells to fluorescent resorufin. Resorufin can be further reduced to nonfluorescent hydroresorufin.[33] The effect of over-reduction of Alamar Blue was not observed for Petri dish culture of HT-29 cells during applied time of incubation (up to 16 hours). On the other hand,

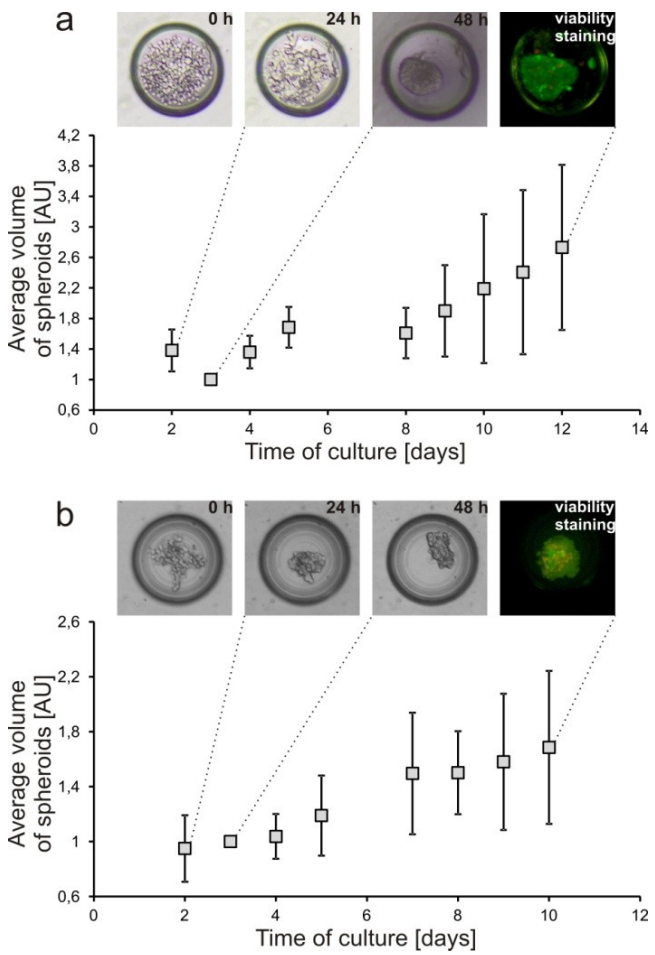

Figure 4. Growth of (a) HT-29 and (b) Hep-G2 spheroids in a microfluidic chip. Growth curves expressed in volumes of biomass normalized to 1 on the second day of culture (time of compact spheroid formation). Sequencing micrographs present: (0 h) cells seeded to a microwell, (24 h) cell aggregation, (48 h) compact spheroid formation, and (viability staining) live/dead cell viability assay at the end-point of culture.

the same time of incubation of cell-containing chip resulted in lower signal of fluorescence comparing to a control without cells. For confluent Petri dish culture, the ratio of surface with growing cells to volume of medium was 0.24 mm^{-1}, whereas for a microchamber of SpheroChip the ratio was over 10 times higher and came to 3.24 mm^{-1}. Therefore, local resazurin run-down effected in faster over-reduction in a microfluidic environment. It was confirmed by observation of fluorescence signal of cell-containing SpheroChip. Measurements were taken every 2 minutes for the first 40 minutes of incubation with Alamar Blue, and every 10 minutes for the next 2 hours. It was observed that, in most cases, signal of fluorescence decreased after 1 hour of incubation. However, for the most dense cultures, maximum signal was detected about 20 minutes from incubation start, and after 1 hour decreased to values beyond the reference. Therefore, the incubation time of 10 minutes was selected as optimal for on-chip measurements. Thus, on-chip measurements could be performed much faster than analogous Petri dish ones, where required incubation periods are counted in hours.

Regular on-chip HT-29 spheroid cultures were monitored for 12 days according to the protocol given in the Experimental section. It was observed that alterations between individual microchambers of cultures without drug did not exceed 20%.

Drug screening

Drug screening experiments were performed according to the following timeline: (day 1) cell seeding; (day 2) measurement, medium exchange; (day 3) measurement, drug dosage; (day 4 – day 11) measurement, medium exchange and (day 12) measurement, live/dead cell viability staining. Application of a CGG structure provided possibility of holding control (no drug) culture in each chip subjected to a drug. As all results were compared to a control, it was important to select only perfectly aligned chips with uniformly seeded cells. Very important feature of the SpheroChip system is possibility of total medium exchange from spheroids' environment. According to the results of COMSOL Multiphysics simulations, time needed for total equalization of glucose concentration by diffusion in the microwell is less than 20 seconds.[28] Therefore, environment of spheroids' surroundings can be precisely controlled during the experiment. Possibility of dynamic changes of extracellular environment, depending on the design of the experiment, is one of the unique and highly advantageous features of the presented system. This can be used for considerations of drug concentrations consistent with ADME profiles.[1]

In our study, 5-fluorouracil was dosed in a concentration range of 0.125 – 1 mM. Same concentrations of 5-FU were applied to Petri dish monolayer cultures (see Electronic Supplementary Information). Petri dish experiments revealed that 5-FU concentration of 0.25 mM or higher is lethal for monolayer cultured HT-29 cells after at least 72 hours from dosage. On the other hand, viability staining of spheroids (Figure 6) shown that spheroids are resistant to higher concentrations of 5-FU (up to 1 mM).

The biggest advantage of the presented system is possibility of continuous, long-term monitoring of viability of spheroids subjected to anticancer drug. To present unique possibilities of the presented system, we decided to apply 24 hour incubation of a drug, followed by no-drug period of time for recovery. This was to present, that applying certain dosing regimens one can mimic changes of the local drug concentration subjected to distribution and elimination processes *in vivo*.[1] This is an advancement over existing methods, which base on one dose of a drug, followed by a single analysis step, often connected with cell culture end. In a contrary, SpheroChip enables for continuous observation *in situ* of cellular response for a long period of time.

In Figure 5, the results of fluorescence-based monitoring using a microplate reader are presented. Relatively high error bars could be caused by population alterations combined together with inevitable differences in spheroid diameters (up to 20 %, see *Metabolic activity monitoring* section). It was observed, that spheroids subjected to 0.125 – 0.5 mM of 5-FU demonstrated increase of metabolic activity 24 hours after dosage. According

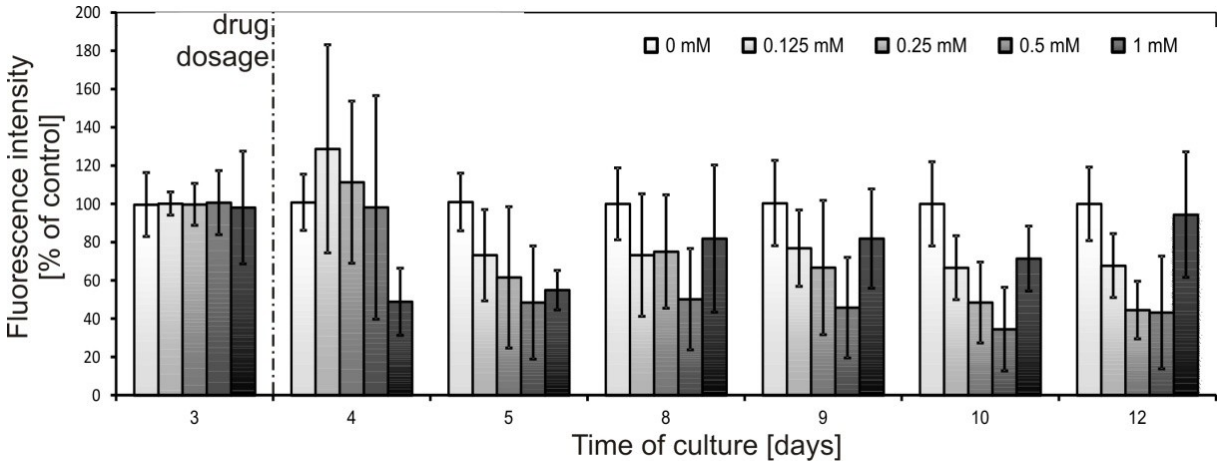

Figure 5. Results of spectrofluorimetric monitoring of HT-29 spheroids exposed to 5-fluorouracil (one dose). Probe "0 mM" was reference without drug, which was considered as 100% of a signal.

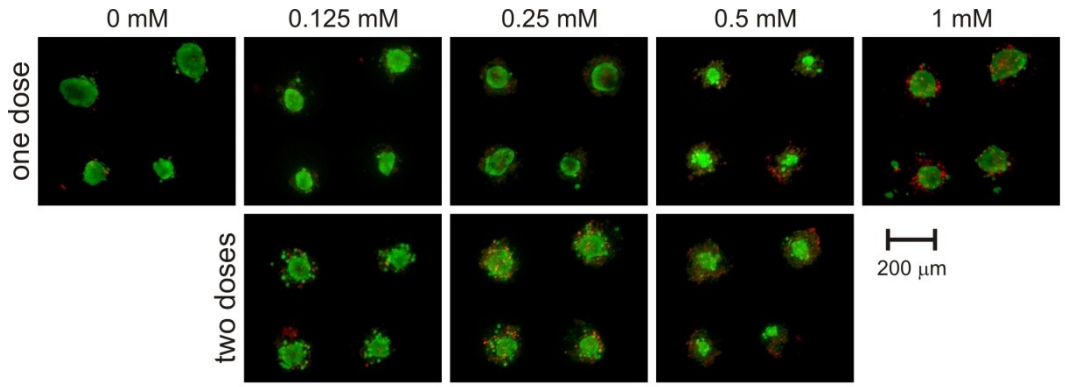

Figure 6. End-point microscopic analysis of viability of on-chip HT-29 spheroids subjected to 5-fluorouracil of different concentrations. Measurements performed 9 days from the first dose of a drug (12th day of culture).

to the experimental protocol (see Experimental section), whole amount of the drug was removed from a cell culture zone prior to introduction of Alamar Blue. Therefore, this sudden metabolic activity decrease could not have been affected by presence of the drug in cell culture medium during measurement procedure. Thus, there should be some inner effects causing growth of metabolic activity of a spheroid in a response to 5-fluorouracil. No literature reporting similar effect could have been found, mostly because of the assay procedures that apply only end-point type of analysis.[34]

After initial increase in 24 hours after drug dosage, metabolic activity was decreasing during following days. Signal decreased more rapidly for higher concentrations of the drug. 9 days after drug dosage (12th day of culture) metabolic activity reached 68 ± 16 % of a control for 0.125 mM, 45 ± 15 % of a control for 0.25 mM and 43 ± 29 % of a control for 0.5 mM. These results were in correlation with live/dead staining presented in Figure 6. Spheroids subjected to 0.125 mM 5-FU remained integral, but their growth was limited and thus, detected signal was lower than control. For concentrations of 0.25 and 0.5 mM spheroid disintegration was observed and was proportional to concentration applied.

Different response was observed for 1 mM 5-FU (Figure 5). 24 hours after 1 mM 5-FU dosage spheroids presented strong decrease of metabolic activity to a value of 49 ± 17 % of a control (no increase was observed). During next days the metabolic activity was growing, and 9 days after drug dosage reached 94 ± 30 % of a control.

Microscopic observations confirmed high viability and integrity of these spheroids (Figure 6). Presence of unaggregated, necrotic cells can lead to a conclusion, that outer layer of spheroid was affected by the drug, while inner cells remained unaffected. Intrinsic or acquired resistance of colon cancer cells to 5-fluorouracil is phenomenon widely reported in literature.[30, 35-39] Moreover, there are reports on drug resistance of HT-29 cells enhanced by higher drug concentrations.[36] However, none of these effects have been satisfyingly explained.[30, 38, 39] Our SpheroChip system provides novel procedure of drug activity monitoring, and thus, can be beneficial for researchers working on chemiresistance of cancer cells. Using the SpheroChip system, one can observe dynamics of cellular response rather

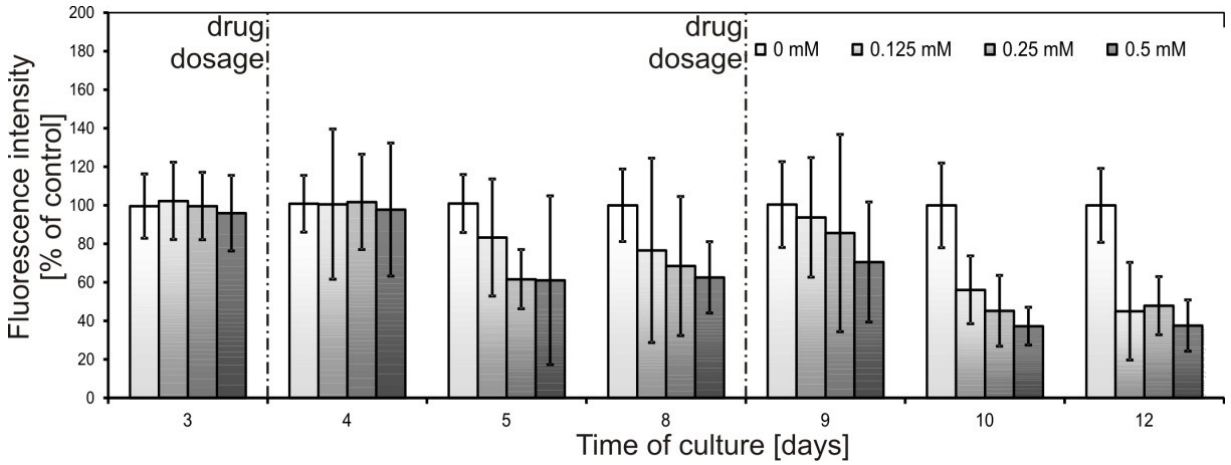

Figure 7. Results of spectrofluorimetric monitoring of HT-29 spheroids exposed to 5-fluorouracil (two sequencing doses). Probe "0 mM" was reference without drug, which was considered as 100% of signal.

than viability at the endpoint. This can be useful for studies on mechanisms of cytotoxicity and resistance.

Another advantage of the SpheroChip system is possibility of observation of spheroids' response to repeated dose of a drug. During anticancer treatment, environment around cancer tumour is dynamic, concentration of a drug varies according to the ADME profile and dose of a drug is usually repeated. Unfortunately, these features are mostly ignored in current *in vitro* tests.[1, 34] Our motivation was to develop a system, that can be used for mimicking *in vivo* dosing regimens. We verified the effect of dosing on cellular sensitivity to 5-FU. The step of adding of a second dose of a drug was added to the protocol described above on the 8th day. Concentrations of 5-FU, which were tested in this regime, ranged between 0.125 and 0.5 mM. The results of spectrofluorimetric monitoring are presented in Figure 7. Similarly to the first dose, the second dose of a drug resulted in increase of metabolic activity 24 h after dosage (9th day of culture, Figure 7). During following days, successive metabolic activity decrease was observed. For 5-FU concentration of 0.25 mM or 0.5 mM no significant differences have been observed between one- or two-doses treatment: metabolic activity at the endpoint of culture was 48 ± 15 % of a control for two doses of 0.25 mM (comparing to 45 ± 15 % of a control for one dose treatment), and 38 ± 13 % of a control for two doses of 0.5 mM (comparing to 43 ± 29 % of a control for one dose treatment). Slightly stronger effect of a doubly dosed drug was observed for 0.125 mM 5-FU. Metabolic activity for two doses at the endpoint of culture reached 45 ± 25 % of a control, comparing to 68 ± 14 % of a control for one dose treatment. These results are in a good correlation with microscopic observations of live/dead cell viability assay (Figure 6). Micrographs of one- or two-dose treated spheroids for 0.25 mM and 0.5 mM are similar. Strong difference can be seen between spheroids subjected to 0.125 mM 5-FU. One dose treatment resulted in small but integral spheroids. On the other hand, two doses of 0.125 mM caused noticeable spheroid disintegration and presence of apoptotic and necrotic unaggregated cells. Therefore, morphology of spheroids doubly treated with 0.125 mM 5-FU was similar to those treated once with higher doses of 5-FU. This experiment proved usefulness of the SpheroChip system for research on influence of dosing on anticancer therapy effectiveness.

Conclusions

In the presented research we developed and tested a microfluidic-based platform for long-term three-dimensional cell culture and monitoring. The SpheroChip system consists of disposable microfluidic chips for cell culture and a positioning plate, which enables analysis using a commercially available microplate reader. To our best knowledge, it is the first microfluidic-based microplate compatible system for *in situ* spheroid analysis.

We performed series of experiments proving usability of the system for spheroid culture, monitoring and analysis. Self-assembled spheroids were formed using simple principle of adherent cell culture on non-adhesive surfaces. Three-dimensional microfluidic structures were fabricated using simple, clean-room independent method. Resultant hemispherical microwells provided effective formation of spherical aggregates and thus led to high uniformity of spheroids. Two human cell lines: HT-29 colon cancer cells and Hep-G2 hepatocytes have been successfully cultured as spheroids using SpheroChip. Cellular metabolic activity was monitored continuously using a fluorimetric microplate reader. Application of a concentration generator structure provided no-drug control and two drug concentrations on each microfluidic chip. 5-fluorouracil was used as a model anticancer drug for HT-29 cells, and alterations in metabolic activity depending on drug concentrations could have been observed. Moreover, chemiresistance phenomenon was observed for high drug concentration. We proposed novel method of presenting results – we plotted time-dependent changes in metabolic activity rather than standard dose-response curves. Possibility of

monitoring of one culture during sequencing days gives novel insight into drug sensitivity/resistance phenomena. For example, growth of metabolic activity was observed in a response to 5-FU during 24 hours, followed by subsequent decrease – phenomenon impossible to observe by end-point type of assays. Thus, the proposed system can be particularly beneficial for research on mechanisms of cytotoxicity. Also repeated doses of a drug could have been investigated using SpheroChip system. It was observed, that low concentrations of 5-FU are more effective, when dose is repeated.

To sum up, the presented bioanalytical platform gives novel opportunities of research on three-dimensional cellular models. Compared to the conventional methods, the SpheroChip system enables for *in situ* and instrumental analysis of three-dimensional cellular model cultured in controllable conditions. Additionally, time-dependent data plot is possible for each culture, unlike end-point analysis used in conventional methods. Simplicity of fabrication and operation of the chip reduces costs of experiments which determines possibility of use in biological laboratories. Moreover, the time of an assay is reduced comparing to the macro-scale, therefore more experimental data can be obtained. The SpheroChip system provides on-line monitoring of three-dimensional cellular model, and therefore, can be used for novel experimental approaches.

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Notes and references

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