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Droplet-Array (DA) Sandwich Chip: a Versatile Platform for High Throughput Cell Screenings Based on Superhydrophobic-Superhydrophilic Micropatterning

Anna A. Popova[§]*, Sebastian M. Schillo*[§]*, Konstantin Demir, Erica Ueda, A. Nesterov-Mueller and Pavel A. Levkin*[*]

[§]These authors contributed equally to this work

Dr. A. A. Popova, K. Demir, Dr. E. Ueda, Dr. P.A. Levkin

Karlsruhe Institute of Technology, Institute of Toxicology and Genetics, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

Dr. S. M. Schillo, PD Dr. A. Nesterov-Mueller

Karlsruhe Institute of Technology, Institute of Microstructure Technology, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

E-mail: levkin@kit.edu

Dr.P.A.Levkin

Karlsruhe Institute of Technology, Institute of Toxicology and Genetics, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

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Abstract

High throughput screening of live cells is a crucial technology that allows for the parallel functional evaluation of the influence of multiple factors on cell behavior and phenotype. In the last years due to the rapid expansion of bioinformatics and genomic tools, increasing throughput and decreasing screening costs became an essential milestone for research in this field. In current study we present a Droplet-Array (DA) Sandwich Technology – a miniaturized pipetting-free platform for cell-based high throughput screenings. DA is created by employing a superhydrophobic-superhydrophilic (SH-SL) surface patterning to create a surface that allows for the simultaneous formation of an array of thousands of separated microdroplets in precise locations and in single step without pipetting. Each of these droplets serves as a miniaturized reservoir for culturing cells. "Sandwiching" of a DA slide, where every droplet contains cell line, with a glass slide preprinted with a library of chemicals, Library-Microarray (LMA) slide, allows for the simultaneous addition of different substances into individual microdroplets on the array. The DA Sandwiching platform allows for (a) 1000 times less reagent consumption than a regular 96-well plate; (b) simultaneous initiation of a cell screening in thousands of droplets in precise locations on the array; (c) easy handling without multiple pipetting; (d) culturing cells in separated compartments; (e) compatibility with cells of adherent and non adherent nature; (f) spatial indexing of spots; (g) possibility to add reagents at any time point and retrieve the cells after culturing; (h) compatibility with standard screening microscopes. In the current study we demonstrate that DA Sandwich Chip can be applied for performing drug screens and gene overexpression experiments with

commonly used adherent cell lines and therefore can be adopted for various cell-based screening applications.

Introduction

In the post-genomic era with the rapid development of bioinformatics and molecular biology tools, cell-based screenings became an immensely important and widely used method. High throughput screenings (HTS) of live cells allow for the simultaneous evaluation of the effects of multiple factors like drugs, bioactive compounds, gene over expression and gene silencing on the phenotype of cells. HTS of immortalized cell lines,[4] primary cells,[7,8,9] human stem cells[3,10] and hiPSC[11] are widely used for fundamental and applied research in academia and industry in a variety of areas such as drug discovery.[5,6] In the past decade, the development of high content fluorescence imaging in parallel with sophisticated automatic microscopes and software made cell-based screenings an indispensable method in modern molecular biology.[1,2]

The majority of cell-based HTS are performed in 96- and 384-well microplates. However, HTS using microplates leads to high consumption of microplates, expensive reagents and valuable cells, as well as it requires multiple pipetting steps that often cannot be done without sophisticated and expensive robotics. These disadvantages resulted in the fact that HTS of live cells are currently available only to large pharmaceutical industry or a few HTS research centers worldwide.

In the past years, a lot of effort has been put towards the development of novel miniaturized platforms for cell-based assays.[12-35] For example, droplet microfluidic technology, which is based on encapsulating of cells in droplets formed in oil phase, allows for a dramatic decrease of reagent and cell consumption and is compatible with ultra high throughput.[12-14] SlipChip is a microfluidic platform that enables precise and parallel addition of nanoliter volumes of solutions into micro wells by sliding a top plate containing

corresponding wells preloaded with substances of interest.[15-19] Some groups have been working on the fabrication of miniaturized microwells on a standard glass slide.[20-24] In 2001, Sabatini and colleagues introduced cell microarray technology and a method of reverse transfection, where transfection mixtures were pre spotted onto a glass slide prior to seeding the monolayer of cells on it.[25] This innovative technique allowed spotting up to 10.000 transfection mixtures on a standard glass slide, increasing the throughput and decreasing the reagent consumption.[4,26-30] The group of Mano created an array of individual droplets containing cells by pipetting each individual droplet into hydrophilic spots on a superhydrophobic background. The method was applied for generating droplets containing spheroids for drug screening applications.[31,32]

The miniaturized platforms described above are promising alternatives to the microplate technology because they allow for a decrease of reagents and cell consumption. Nevertheless, risk of cross contamination and compatibility only with adherent cells (cell-based microarrays), requirement for multiple pipetting steps (DropArray[33] and miniaturized microplates[20-24]), using oil phase (droplet microfluidics,[12-14] SlipChip[15-19] and DropArray[33]) restrict the applicability of these platforms. Here we list a number of requirements a future screening platform should satisfy in order to become the next generation widely used technology for cell-based HT assays: (1) very small reagent and cell consumption; (2) compatibility with low and ultra high throughput; (3) culturing cells in separated compartments to avoid cross-contamination; (5) compatibility with cells of adherent and non-adherent nature; (5) parallel and simultaneous addition of chemical libraries as well as other reagents; (6) easy pipetting-free handling. Currently, no technology fulfills those requirements and there is a clear need for new developments to overcome the problems of the existing cell-based HTS platforms and make cell screenings affordable to every biological laboratory.

In this paper we describe a DA Sandwiching technology, a miniaturized platform for cell-based high throughput assays, which combines different advantages that can potentially solve the above mentioned problems of the existing HTS methods. We recently developed a novel method for creating precise superhydrophobic-superhydrophilic micropatterns on nanoporous polymer films.[36-38] Due to the extreme difference in wettability between superhydrophobic and superhydrophilic areas and the phenomenon of discontinuous dewetting,[37] aqueous solutions applied onto such surface spontaneously form an array of separated microdroplets (Figure 1). Each of these droplets can serve as a micro reservoir for culturing cells. In the current study we optimized the DA Sandwich method for cell-based screenings and demonstrated the possibility of (1) culturing adherent cell lines in individual droplet for at least 24 hours; (2) parallel addition of substances into individual droplets without cross-contamination; (4) performing drug treatments and gene overexpression in commonly used adherent cell lines. The DA cell screening platform enables HTS of cells in completely separated compartments and reduces reagent and cell consumption up to 1000 times (if compared to 96-well plate). Moreover, DA platform allows for parallel and simultaneous seeding of cells, addition of chemical libraries and initiation of HTS on the whole chip in thousands of microdroplets, which is not possible by most of the existing HTS technologies. Finally, the DA technology enables various read-outs including live/dead staining of cells in individual droplets and is compatible with standard screening microscopes.

Results and discussion

DA Sandwich platform

Schematic representation of the DA Sandwich platform and the corresponding HTS workflow are shown in Figure 1. The DA slide consists of an array of round superhydrophilic (SL) spots of 1 mm diameter separated by superhydrophobic (SH) borders (Figure 1a). The SH-SL patterned surface is prepared by applying a nanoporous 2-hydroxyethyl methacrylate

(HEMA) – ethylene dimethacrylate (EDMA) polymer layer onto a glass slide,[37,38] followed by modification of the polymer layer with alkyne groups. Photo-click thiol-yne reaction is used to create the SH-SL pattern of desired geometry (Figure 1a).[38] Due to the extreme difference in wettability of the SH and SL areas and the phenomenon of discontinuous dewetting,[37] aqueous solutions applied onto this pattern spontaneously form an array of separated transparent microdroplets (Figure 1b; Video S1, the images and video of droplets formation is made on black background). As a first step in DA Sandwich method workflow cells are seeded simultaneously on the whole DA slide using the effect of discontinuous dewetting by spreading cell suspension on patterned surface (Figure 1c, step 1). Library-Microarray (LMA) slide is prepared by printing drugs or transfection mixtures onto a simple glass slide using a non contact ultra-low volume dispenser in the geometry corresponding to the geometry of the DA slide (Figure 1c, step 1). As a next step simultaneous addition of substances into each individual droplet is performed by precise aligning and sandwiching of DA slide containing cells with LM slide containing compounds of interest (Figure 1c, step 2). Printed chemicals and transfection mixtures dissolve and diffuse into the individual droplets. After library transfer LMA slide is removed and DA slide containing cells is placed in a cell culture incubator (Figure 1c, step 3). As a read-out cells can be either directly subjected to live imaging (Figure 1c, step 4, upper panel) or after live staining by sandwiching DA slide containing cells with identical DA slide containing CalceinAM solution (Figure 1c, step 4, middle panel) or by immersing DA slide containing cells into CalceinAM solution (Figure 1c, step 4, lower panel).

Culturing adherent cell lines on Droplet-Array slide

First we evaluated viability and growth rate of cells cultured in individual droplets and compared to cells cultured in 24-well microplate. Human cervical carcinoma (HeLa) and human embryonic kidney (HEK293) cells were seeded on a DA slide by applying cell suspension onto the SH-SL patterned surface with SL circles of 1 mm diameter followed by

tilting the slide to allow the suspension to slip from the surface leading to spontaneous formation of an array of separated droplets containing cells (Figure 1c, step 1; Figure S2, Video S1). Volume of the droplets, calculated using droplet heights (Figure S1a,b) was 60 nl (Figure S1a). Cells were cultured in individual droplets for 24 hours. As shown on Figure 2a, both HeLa and HEK293 cells showed typical spread morphology after 24 hours post seeding (Figure 2a). To estimate growth rate and viability, cells were stained with Calcein using the sandwiching approach described below (Figure 1c, step 4, lower panel; Figure 2b). The doubling times of HeLa and HEK293 cells in individual droplets were 56 and 38 hours, respectively, while the viability of both cell types was 96 and 98%, respectively. Viability of cells cultured in a 24-well plate was comparable to that of cells cultured on a DA slide. However, cell proliferation in 24-well plate was about two times faster (Figure 2b), what might be explained by approximate 30 times lower medium volume per cell and 5000 times lower total medium volume per droplet compared to the 24-well plate.

Parallel addition of chemicals to individual droplets using the sandwiching method

As a next step we evaluated the process of parallel transfer of tiny amounts of chemical substances into individual droplets (Figure 2c). Usually, addition of minute amounts of reagents into the miniaturized cell culture systems is the most challenging step because of the difficulties associated with handling such small amounts. Parallel addition of reagents into thousands of miniaturized cell culture reservoirs is even more challenging but also immensely important in order to avoid time delays and discrepancies between different microwells. Library-Microarray (LMA) array was prepared by printing Rhodamine 6G in three different concentrations either onto a hydrophobic glass surface or into hydrophilic spots of SH-SL patterned surface. The DA and LMA slides were fixed in two parallel holders (Figure S2a,b) and aligned using a camera for controlling (Figure S2a,b). Followed the alignment the upper stage was brought down till the LMA slide came in contact with water droplets on the DA slide. The distance between two slides was carefully monitored using a side camera (Figure

S2a,b). The dye transfer rates from different surfaces were estimated by comparison of the fluorescence intensity of spots after transfer with fluorescence intensity of spots after direct printing of the dye (Figure S1c). Averaged transfer rates of different concentrations of Rhodamine 6G from hydrophobic glass and patterned surfaces were 71% and 56%, respectively. For all further experiments LMAs were prepared on hydrophobic glass slides. Figure 3b shows precise transfer of different dyes into individual droplets on the DA slide without cross-contamination. The droplet volume loss during the sandwiching process was found to be 26±16% (Figure S1c). This value depends on the surface properties of the LMA slide and probably can be reduced by increasing its hydrophobicity and water contact angle hysteresis.

As a following step, we employed the sandwiching approach for parallel addition of defined amounts of a drug into individual microdroplets containing live cells. First 25 ng of doxorubicin pro spot was printed onto a glass slide in a checkerboard pattern to form a LMA slide. Hela cells were seeded onto a DA slide to form an array of droplets. The DA and LMA slide were then sandwiched in order to deliver the preprinted doxorubicin into the individual microdroplets (Figure 2d). The sandwich was opened and cells were allowed to grow in an incubator in a humidified chamber for 18 hours followed by Calcein staining and a microscopic analysis. The results depicted in Figure 2d show that droplets treated with doxorubicin contained either no or only few live cells, while untreated droplets were fully populated with live cells based on Calcein staining (Figure 2d, Figure S3a). Comparison of cell viability in doxorubicin negative spots on DA slide from different locations (Cntr B and C) and on a control untreated DA slide (Cntr A) indicated that there was no cross-contamination between individual droplets during or after drug addition (Figure 3a,b).

As a next step we checked the possibility of addition of controlled amounts of drugs into individual droplets. Doxorubicin was printed onto a LMA slide in amounts ranging from 0,5 to 5 ng and transferred to droplets containing Hela cells by the sandwiching method. Viability

of cells was estimated 18 hours after drug addition by Calcein staining of the whole DA slide (Figure 1c, step 4, middle panel). As shown on Figure 3c and Figure S4, we observed concentration dependent effect of doxorubicin on cell viability (Figure 3c, Figure S4). In order to estimate concentration of doxorubicin in droplets we calculated transfer rate of doxorubicin by comparing the fluorescence intensity of spots after doxorubicin transfer to fluorescent intensity of spots after direct printing of the same amounts of the drug (Figure S1d). Our results indicated that the transfer of the drug was complete. Knowing the amount of doxorubicin and volume of the droplets (Figure S1a), final concentration of doxorubicin in individual droplets was estimated (Figure 3c). Our results showed that half maximal effective concentration (EC_{50}) of doxorubicin obtained using DA Sandwich Chip was 30 µM. EC_{50} of doxorubicin measured in 96-well plate with the same experimental set up (cell density per area, incubation time etc.) was 3 µM (Figure S3d), which is probably due to a higher volume to cell ratio in a well compared to a droplet, where the total amount of drug per cells is lower than in a well. The drug transfer experiments were also performed using doxorubicin in DMSO solution and showed similar results (Figure S3b).

In the next step, the sandwiching approach was evaluated for performing parallel gene over-expression in individual droplets. Although reverse cell transfection, introduced by Sabatini et al.,[25] is an excellent method for performing high throughput parallel transfection experiments on one glass slide, the absence of compartmentalization of individual spots leads to an inevitable problem of cross-contamination. The DA Sandwich method can potentially combine the advantages of reverse cell transfection and microfluidics where the experiments are highly compartmentalized. HEK293 cells were seeded on a DA slide and transfection was performed by sandwiching the DA slide containing cells with a LMA slide with pre-printed and dried transfection mixtures as described above. Transfection mixtures were composed of ScreenFectA transfection reagent, plasmid DNA and a dilution buffer containing sucrose. Transfection efficiency was estimated 24 hours after addition of transfection mixtures by live

imaging. As shown in Figure 3d, HEK293 cells were successfully transfected with H2B-YFP and H2B-RFP expressing plasmids with app. 20% transfection efficiency.

Conclusions

In the current study we show a novel miniaturized platform for high-throughput screenings of live cells. The DA Sandwich Chip is based on applying a sandwich approach for parallel and simultaneous addition of compound libraries printed on a glass slide to an array of individual separated cell containing microdroplets, spontaneously formed on a SH-SL microarray through the phenomenon of discontinues dewetting. We demonstrated the possibility of culturing commonly used cell lines, Hela and HEK293, in individual microdroplets. Cells proliferated and showed viability rates comparable with cells cultured by conventional methods. The process of transfer of printed chemical libraries from solid surface into individual droplets using sandwich approach was evaluated and applied for parallel drug treatment and transfection of cells in individual microdroplets. We also demonstrated the compatibility of the DA sandwiching method with live staining and standard screening microscopy. The DA sandwiching platform offers a number of advantages compared to existing screening platforms. For example it allows for about 1000 times less reagents consumption (compared to a standard 96-well plate); one step pipette-free seeding of cells on the whole chip; simultaneous initiation of screenings on the whole chip; culturing cells in completely separated miniaturized droplet microreservoirs; compatibility with cells of adherent and non adherent nature; spatial indexing of spots due to the array format; compatibility with standard screening microscopes and standard read-outs. We believe that the DA Sandwich platform can be adapted to a wide range of cell-based screening applications and can be used in routine screenings performed in industry and academia allowing for a significant decrease in reagent and cell consumption, and increase in

throughput. In addition, DA Sandwich platform enables screenings that are difficult or impossible to perform using conventional method, such as single cell analysis or HTS of rare and hard to expand cells, like primary or stem cells. Finally, DA Sandwich technology has a potential to be applied in clinical diagnostics and in personalized medicine where the pipetting-free sample multiplexing and the ability to use only minute sample quantities are crucial factors.

Supporting Information

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

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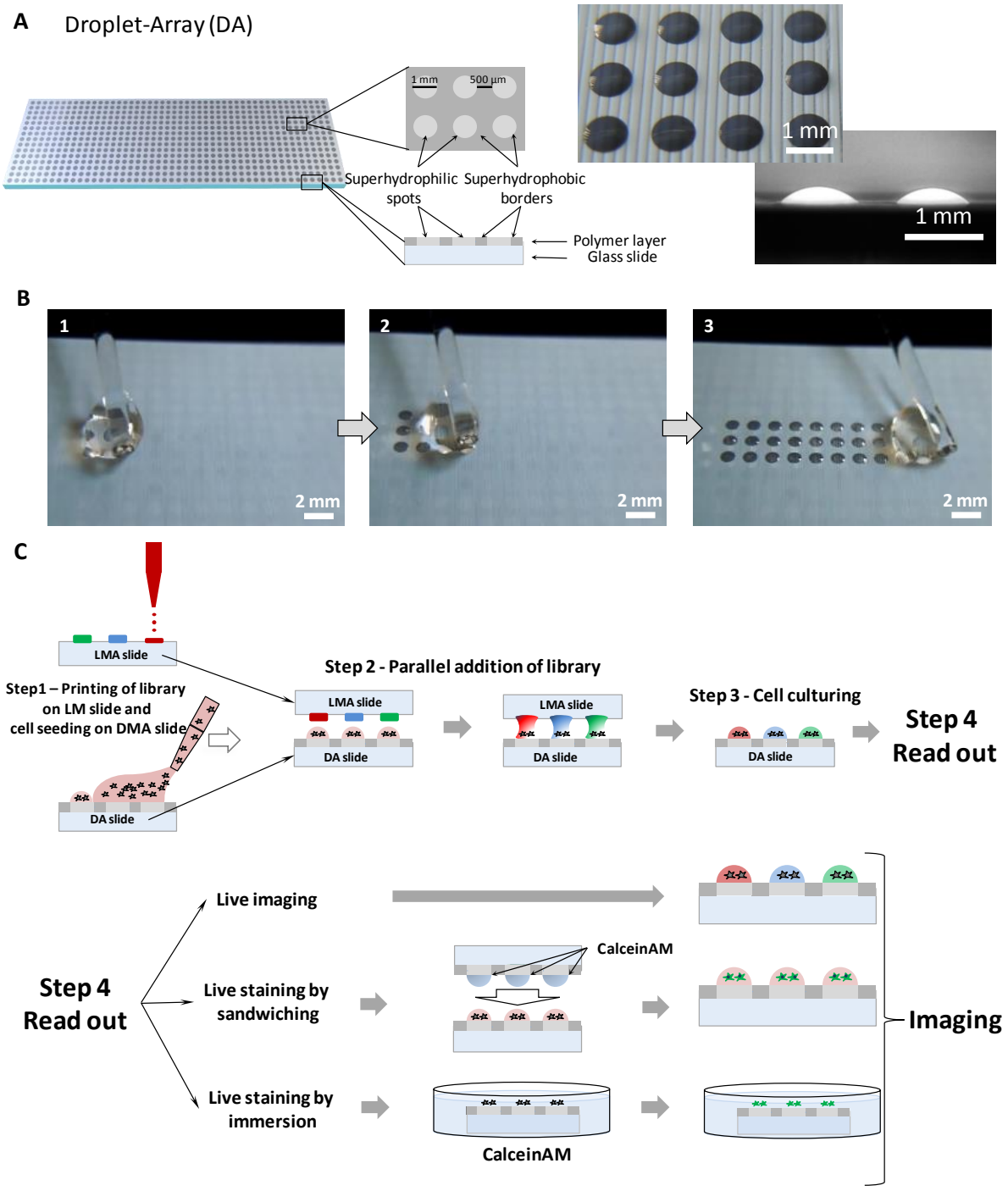

Figure 1. Droplet-Array (DA) Sandwich Platform. (a) Schematic representation of a DA slide (left) and images of droplets formed on a superhydrophobic-superhydrophilic pattern. (b) Snapshots of the process of discontinuous dewetting leading to the formation of an array of microdroplets (droplets are transparent; the image and video of droplets formation is made on black background for better contrast and representation). (c) Schematic of a workflow of cell-based screening using DA Sandwich Chip. LMA slide is prepared by printing of substances of

interest on a glass slide (Step 1); DA slide is prepared by seeding cells using the effect of discontinuous dewetting (Step 1). For parallel addition of library into individual dropelts LMA slide is aligned and sandwiched with DA slide containing cells (Step 2). After substances are transferred into droplets LMA slide is removed and DA slide is placed into cell culturing incubator (Step 3). As a read-out cells can be either directly subjected to live imaging (Figure 1c, step 4, upper panel) or after live staining by sandwiching DA slide containing cells with identical DA slide containing CalceinAM solution (Figure 1c, step 4, middle panel) or by immersing DA slide containing cells into CalceinAM solution (Figure 1c, step 4, lower panel).

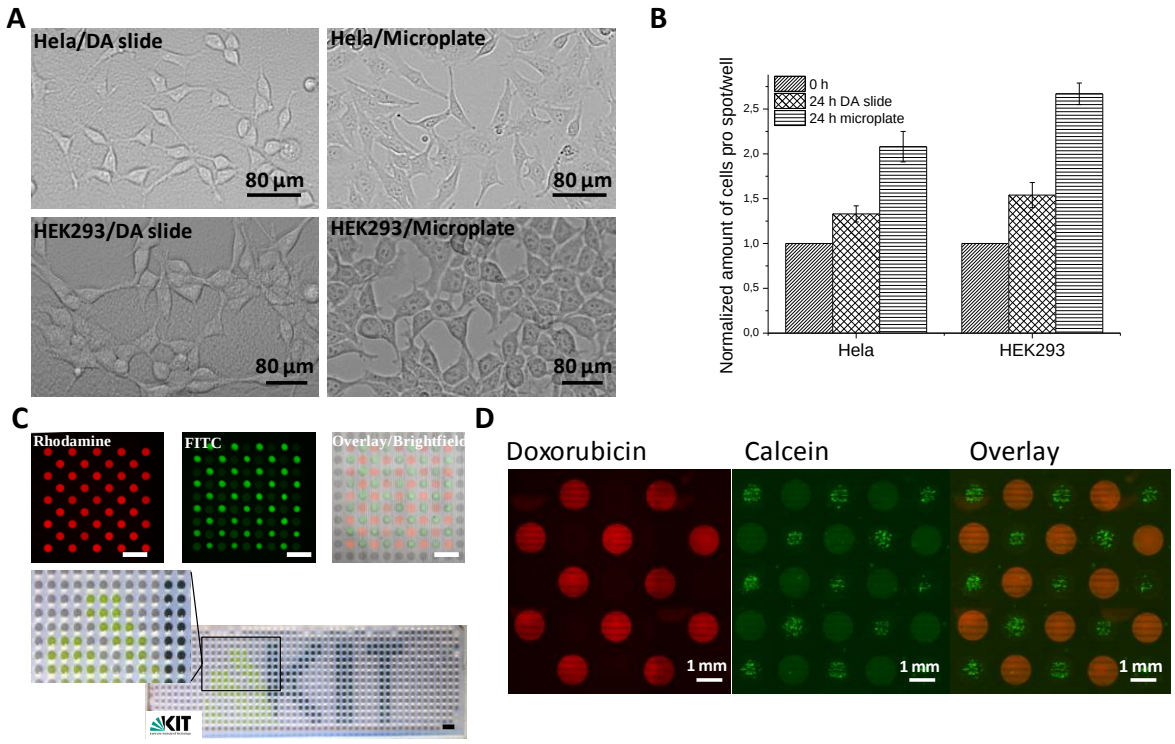

Figure 2. Culturing cells in individual droplets on DA slide and parallel addition of chemicals to individual droplets using the sandwiching method. (a) Microscope images of Hela and HEK293 cells cultured on a DA slide and microplate 24 hours after seeding. (b) Growth rates of Hela and HEK293 cells cultured on a DA slide and in a 24-well microplate. (c) Fluorescent microscope images of a DA after transferring an array of dyes into the individual microdroplets using the sandwiching method. Scale bars are 3 mm. (d) Fluorescence

microscope images of a DA slide containing Hela cells 18 hours after treatment with doxorubicin. Doxorubicin shows red fluorescence (left). Calcein stained Hela cells on the same DA slide (middle). Overlay of images of doxorubicin transfer and calcein staining (right).

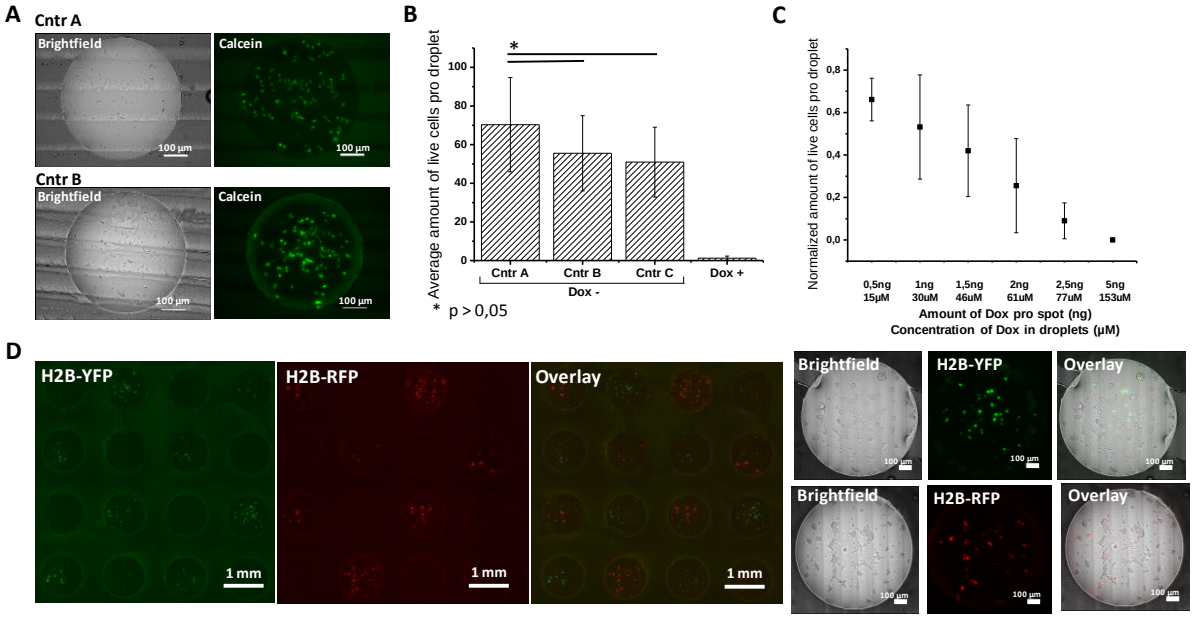

Figure 3. Parallel addition of drugs and transfection mixtures to individual droplets on DA slide using the sandwiching method. (a) Microscope images of individual doxorubicin-negative droplets. (b) Comparison of cell viability in doxorubicin negative droplets from different locations: Cntr A - droplets on a control untreated DA slide; Cntr B - doxorubicin-negative droplets located next to doxorubicin positive droplets; Cntr C - doxorubicin-negative droplets located at least two spots away from doxorubicin positive droplets. (c) Concentration dependent effect of doxorubicin on viability of HeLa cells 18 hours after treatment. Amount of live cells per droplet was normalized to amount of live cells in doxorubicin negative droplets. (d) Microscope image of a DA slide (left) and individual droplets (right) containing HEK293 cells transfected with pCS2-H2B-YFP (green) and pCS2-H2B-RFP (red) plasmid.

Droplet-Array Sandwich Chip is a miniaturized platform for cell-based high throughput screenings. It is based on sandwiching of glass slide with pre printed library and a superhydrophobic-superhydrophilic pattern, which consists of thousand simultaneously formed microdroplets containing cells. DA Sandwich Chip allows for one-step cell seeding, simultaneous initiation of screening and 1000 times less reagent consumption than a regular 96-well plate.

Droplet-Array Sandwich Chip

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