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Use of a highly parallel microfluidic flow cell array to determine therapeutic drug dose response curves

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Abstract A high-throughput, microfluidic flow cell array (MFCA) system has been modified to enable drug screening against small-volume cell-, and tissue cultures. The MFCA is composed of a 3D channel network that simultaneously flows fluids through forty-eight 830 μm by 500 μm flow cells, which physically divide and fluidically seal an existing culture into multiple compartments when docked onto the surface of a cell or tissue culture dish. The modified system provides temperature (37 °C) and CO_2/pH level controls, while continuously flowing solutions (media or other liquid such as drug suspensions) over the cells/tissues. These assays were enhanced and validated using inverted microscopy and fluorescent staining techniques which also allow real time viability and toxicity assessments. This work presents the results of this new generation *in vitro* drug testing assay performed using this modified MFCA system. This setup allows the testing of 48 drug combinations on 48 different cell-, tissue specimen at once under flow conditions. All 48 flow cells were utilized to test 5 different concentrations of cisplatin (CDDP). CDDP solutions in various concentrations were continually flowed over cultured human ovarian cancer cells for 48 h. Viability assessments were performed using red-orange calcein and SYTOX ® Green nucleic acid stains. Cells were imaged at the beginning and end of the experiment (48 h). In order to compare and validate MFCAs suitability as drug screening assay, MTT assays were performed on cells. We found that both, MTT and MFCA assays generated dose-response curves with similar profiles. Innovative advantages of the MFCA system include the ability of handling smaller amounts of solutions compared to conventional and current state of the art drug screening and cell viability/toxicity methods. It also provides the ability to continually deliver fresh solution to the cell samples, while eliminating wastes that are produced. Based on our here reported findings MFCA may have a strong potential of providing a more physiological model than current state of the art static MTT assays.

Keywords Microfluidics · Personalized medicine

1 Introduction/background

The pharmaceutical industry can expect to spend approximately $5 billion and 10–12 years on the development of any novel single drug. This cost is attributable to the fact that 95% of drugs initially developed fail to advance through all stages of clinical testing to final market approval (Harper 2013; Yang et al. 2012). Even though most of these drug-screening procedures rely on animal models, as predictors for human drug target validation, they are generally expensive and often only produce poor and very slow results with weak predictability in regards to outcomes of subsequent clinical trials. If human cells-, and tissues could be analyzed in more *in vivo*-like conditions, developments in combinatorial chemistry and biology could be used to generate more rapid and accurate high-throughput screening (HTS) techniques (Yang et al. 2012). Cell-based assays in commercially available 384-well and higher density plates for parallel screening assays

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have some practical limitations such as the control of evaporation in open wells (i.e. MTT assays) with volumes that are continually getting smaller (Sundberg 2000; Wu et al. 2010) and the static conditions that deplete drugs and nutrients while allowing wastes to build up contaminating results and lacking resemblance to processes in a living organism. The static nature of current assays requires repeated manipulation such as media changes that translate into a higher risk of cell contamination (Wu et al. 2010) and shock to cells due micro-environmental variations often leading to experimental failure (Sittinger et al. 1997). These disruptive changes in the culture environment (e.g. pH, temperature, CO_2) have been demonstrated to considerably alter cell physiology influencing drug treatment outcomes (Wu et al. 2006). Flow-based assays could potentially overcome these limitations and support a more robust, realistic and predictive cell environment by providing: a more stable culture micro-environment utilizing an uninterrupted supply of necessary nutrients and waste removal (Wu et al. 2006), compensation for evaporation, and additional functionalities (e.g. gradients of drug concentrations) produced by the construction of a circulatory system (Yang et al. 2012). Cell based assays have several advantages such as providing direct information on compounds that involve cell permeability and also the potential to obtain additional information on acute cytotoxicity directly related to the compounds being screened (Florento et al. 2012). However, studies indicate that preserving the 3-dimensional cellular makeup and micro-environment will translate into biologically more pertinent tumor models (Astolfi et al. 2016). Thus, effective screening systems should have the capability of testing 2D and 3D samples. A variety of 2D microfluidic systems have been used to study the responses of cell populations to reagents while performing cell cytotoxicity analysis. The review by Rothbauer et al. has recently discussed the use of microfluidic cell arrays for the specific analysis of cancer cell line responses to chemotherapeutics (Rothbauer et al. 2015). As a specific example, Song, et al. have presented a microfluidic device that allows the seeding of MCF-7 (breast cancer cells) and determining the effects of two different chemotherapy drugs (mitomycin C and tamoxifen). The microfluidic results agreed with results obtained from similar experiments using conventional culture plates (Song et al. 2010). Similar works have created chamber arrays that create concentration gradients in order to provide a range of concentrations of chemotherapeutics. One example is Hung, et al., whose device has satisfactorily demonstrated its ability to generate a concentration gradient (Hung et al. 2005a) and seed and grow HeLa (Human Carcinoma) cells to confluency (Hung et al. 2005b). However, the effects of chemotherapeutics on these cells has not yet been reported. Another, Kim, et al. have also been able to create concentration gradients with the added ability to perform combinatorial drug treatment (Kim et al. 2012). They were able to expose PC3 prostate cancer cells to combinations of either doxorubicin or mitoxantrone and TRAIL (TNF-alpha Related Apoptosis Inducing Ligand) in sequential or simultaneous fashion. Even though all these results suggest that these devices can provide reliable drug screening assessment, the need for a cell seeding/loading procedure is still a major disadvantage. The seeding of the cells is done by flowing the cells into the microfluidic devices, which can result in a distribution in the number of cells (Rothbauer et al. 2015). Another major limitation that these microfluidic devices have is their inability to test 3 dimensional samples. Upadhyaya et al. has worked in a 3-layer device in which cells can be grown on a top layer while being treated through a nanoporous membrane directly below it. The nanoporous layer (also known as the middle layer) is controlled via external electric fields created in the bottom layer. This device has been able to precisely control amounts of reagents delivered to specific spots/groups of cells on the top layer; however, providing a continuous flow is not possible as it will eventually cross-contaminate neighboring spots. Then, a microfluidic device that will provide favorable conditions for cell growth, while allowing the continuous flow of reagents and having potential to be used with 3 dimensional samples is still needed. Cell seeding/loading is also a problematic procedure that should be avoided as part of the device design. Thus, there is still a need for a robust, simple microfluidic system that can use the advantages of microfluidics with the advantages of standard cell culture or tissue analysis.

To meet these needs for high-throughput screening (HTS) tools with more accurate predictability, a three-dimensional, highly parallel microfluidic delivery system has been developed for use with human tissue- or other cell cultures. This microfluidic flow cell array (MFCA) has been fabricated by embedding microfluidic channel networks in polydimethylsiloxane (PDMS). This MFCA consists currently of 48 flow-cells that can form individual chambers as they are docked over a cell culture using conventional Petri dishes (Fig. 1a–d). Each of these flow cells is linked to a unique set of inlet and outlet fluidic ports, so is thus capable of addressing individual spots (Fig. 1a). The flow cells formed on the Petri dish are 830 μm long, 500 μm wide, and 250 μm high, and have 300 μm square inlets and outlets. The 48 parallel flow-cells are spread over a total area of 35 mm^2 in a 6 × 8 grid (as shown in Fig. 1b). The MFCA is docked onto a Petri dish or sample surface (Fig. 1e) to form the flow-cells. Once the flow-cells are completely and independently sealed, fluids can be flowed through the MFCA into the 48 independent flow-cells, cross the sample surface and then exit the flow-cell (Fig. 1a, c). An inverted microscope and a fluorescent light source have been integrated with the system to allow permanent analysis of cell culture samples quantitatively and qualitatively (Fig. 1a, f). The MFCA further employs a parallel testing setup that permits the comparison of the chemical responses of multiple cell

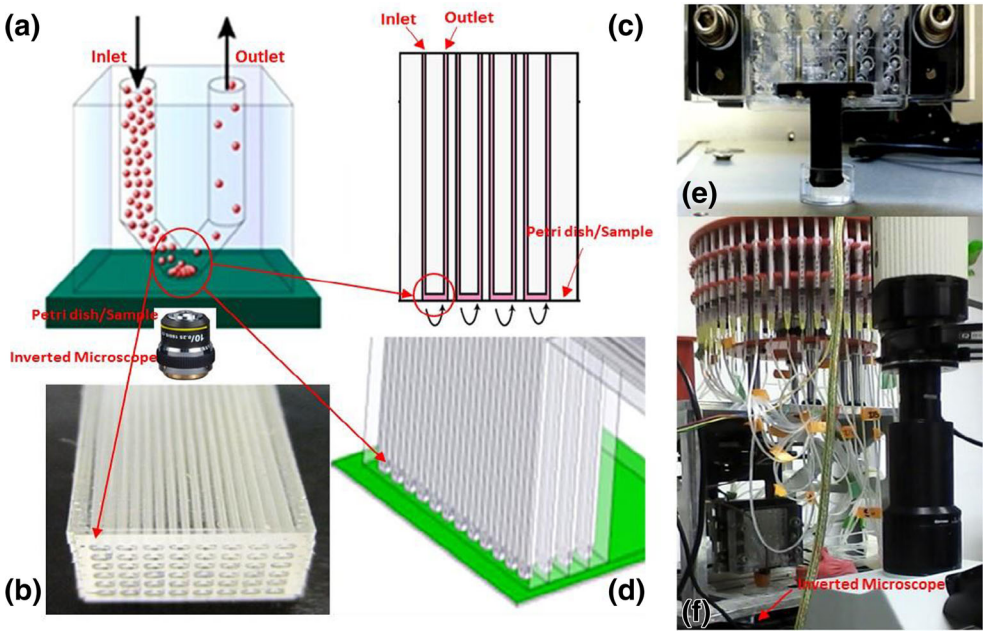

Fig. 1 The MFCA system emphasizing the mode of operation and modifications **a** Cartoon of a single flow cell showing operation of the MFCA and how spots/cells are isolated from one another after complete docking on a cell culture dish or other substrate. **b** Photograph of the tip of the MFCA that comes in contact with the assay surface and forms 48 independent flow-cells. **c** Cartoon of a 2D crossectional view of the MFCA docked over a sample surface showing the way fluids flow once the flow-cells are formed. **d** A 3D rendering of the 48-flow-cell array. **e** Close-up of the MFCA inserted into the instrument that docks the MFCA and generates flow. **f** Entire setup with thermocouple attached to the MFCA and the vertical syringe pump connected to the back of the MFCA (inlet side)

populations to multiple solutions within a closely controlled environment. This system has successfully demonstrated that it can be used to print (referred to a continuous flow microspotter or CFM) or deposit fluorophores, proteins, antibodies, and ligands, in addition to the patterning of cellular microarrays of multiple different cells lines into conventional tissue culture wells (Chang-Yen et al. 2006; Eddings et al. 2009; Natarajan et al. 2008a, b). The CFM/MFCA has also proven its usefulness for high-throughput multiplexed protein deposition by generating uniform spot morphology, spot-to-spot uniformity, and concentration control. This concentration control is possible due to careful flow control allowing ligands to reach optimal deposition conditions (Natarajan et al. 2008b).

Considering the different characteristics and features the CFM system displays, we hypothesize that it will provide a reliable, unique and predictive drug-screening platform for the identification of more effective and less toxic drugs and drug combinations. Using CFM-based treatment recommendations, novel therapies could be personally tailored to each patient and the type and stage of their disease. In light of clinical challenges, including limitations in obtaining vast amounts of specimens for additional testing other than for diagnostic purposes, this system recognizes clinical reality by only requiring small numbers of cells or minute amounts of tissues for testing. To validate our hypothesis, we are focusing on targeting epithelial ovarian cancer (EOC) cells. EOC affects over 21,000 women each year in the U.S. alone (Society 2015) and, based on the lack of early detection as well as the development of drug resistance during the course of treatment, there has not been an improvement in morbidity and mortality (Society 2015). Resistance after an initial treatment with platinum-based drug combinations such as Carboplatin/Paclitaxel is commmon (Sato and Itamochi 2014; Bookman and First-line 2012; Omura et al. 1986). Thus, EOC demands urgent improvements in drug screening tools to appropriately aid physicians in their therapeutic decision making process.

Here we report a modified CFM system, which has been converted into a MFCA and is used to screen for drug effects of various concentrations of CDDP on an ovarian cell culture. This study will explore the capabilities of this system by directly comparing it to traditional MTT assays. The expected biological insight from this work is that dynamic flow systems provide more "*in vivo* like" conditions and generate better or at least similar responses to static cell cultures (Kim et al. 2013), and can do so with much smaller biological samples in a multiplexed fashion. The effect of continuous flow on the transportation of cisplatin and its cellular uptake will be assessed in a multiplexed fashion (48 simultaneous wells). This dynamic capability is expected to render a more physiologically accurate assessment as it includes more variables such as drug delivery and waste removal than static assays (Kim et al. 2013).

The MFCA system will continually flow different concentrations of CDDP over 48 parallel microscopic samples of ovarian cancer cells while integrating it with a high resolution and real time fluorescent microscopy techniques.

2 Materials and methods

2.1 MFCA configuration

The MFCA was constructed by modifying a commercial CFM (Wasatch Microfluidics, Utah). Both systems are built around a microfluidic, polydimethylsiloxane (PDMS) MFCA that deposits 48 spots in a 7 mm by 5 mm area. The MFCA is placed in an instrument that connects two standard 96-well microtiter plates to a 48-syringe pump (Fig. 2) to the MFCA from which samples can automatically be pumped through the flow cells in the MFCA. A sample volume of 300 μL is required for each flow cell. An X-Y-Z automated sample handling and positioning system and an automated cleaning system allow for multiple flow cell or printing operations to be completed on 1″ by 3″ microscope slides or other substrates.

To adapt the CFM system to cell culture, additional features have been added and changes have been made in order to provide the suitable temperature, pH level, bubble-free environment, and flow rate. Changes to the cleaning protocols were also made as some reagents (NaOH, Sigma-Aldrich Missouri USA) proved to be highly basic and harder to rinse off the system.

2.1.1 Temperature

2.2 Temperature regulation of MFCA system

The CFM system was modified to include a temperature control system that keeps the temperature within the cell culture dish constant at 37 °C. This temperature control system is composed of a ring built out of Sylgard 184 PDMS (Shin-Etsu, Tokyo Japan), a flexible silicone rubber heater (SRFG)

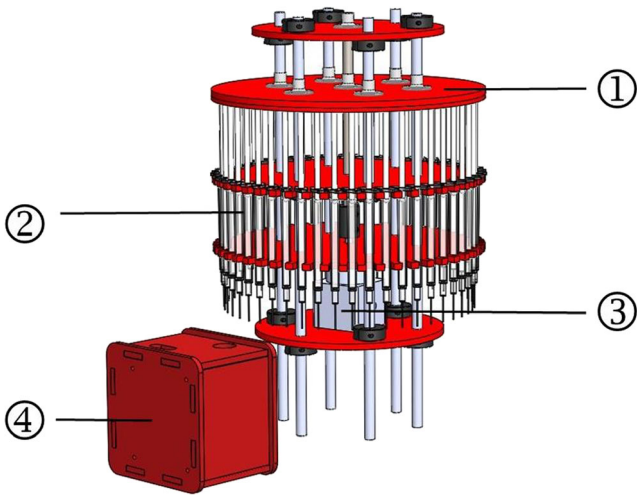

Fig. 2 Vertical syringe pump. Depiction of the main components of the vertical syringe pump and a color rendition of the positioning of the 1 CC syringes. 1. Linear motion bearing and force plate (red); 2. 1 cm^3 syringes; 3. Stepper motor; 4. Control box with Arduino Uno and motor driver

(Omega, Connecticut USA), a "K" thermocouple with an immersion probe (Item # 6JHW5, Grainger, USA), a CN 4000 digital PID temperature controller (Omega, Connecticut USA), and a PS 280 low DC voltage power supply (Tektronix, Oregon USA). PDMS was mixed at a base and cross-linker ratio of 10:1 and poured into a 50 mm tall and 12 mm diameter cylindrical mold. A flexible silicone rubber heater was immediately placed inside this cylindrical mold with the uncured PDMS mixture. The mold, PDMS, and heater were then placed inside an IC-62 oven (American Scientific Products, Ohio USA) at 40 °C and the PDMS was allowed to cure inside for an hour. The heater was serially connected to the power supply and the PID temperature controller, which was set to 37 °C. The thermocouple was taped to the bottom end of the MFCA in order to place it immediately adjacent to the patch of cells to be treated. The flexible silicone rubber heater was placed and taped around the inner circumference of the PDMS ring (Fig. 1f).

2.3 Temperature regulation of MTT assays

Plates were maintained in an incubator at 37 °C before and after treatment.

2.3.1 pH and CO_2 control

MFCA system The role of CO_2 is to support maintenance of the pH inside a conventional cell culture incubator. To enable such CO_2 control, both ends of a latex balloon were removed creating two openings (approximately 1 and 2 in.). The biggest opening was stretched around the PDMS ring heater and the narrow end enclosed the lower end of the MFCA. Vinyl electrical tape (3 M, 35-Orange-3/4) was utilized to tightly seal the balloon around the MFCA upon docking over the cell culture dishes. Thus, creating an airtight environment that would keep the CO_2, temperature, and humidity controllable around the MFCA. Once assays were completed, the pH of the solution was measured by raising the MFCA under continuous flow. At the end of the experiments, the MFCA was raised and a combined drop from all the flow cells was collected on pH test paper and an average measurement of the pH was made. Due to the small amounts (a few microliters) of the DMEM-CDDP solutions in each flow cell, measuring the pH in each flow cell was not practical.

Additional modifications to the system to provide pH and CO_2 control were also tested, but discarded. Flowing a 95% oxygen and 5% CO_2 gas mixture lowered the pH of the medium in the entire system to acidic levels (i.e. pH = 5), likely due to the imbalance in the relative liquid and gas volumes compared to the incubator setup for the MTT assays described below (i.e. conventional incubators have a large gas volume over the culture dish, the MFCA has none). Since the medium was kept enclosed in an airtight environment (syringes and

tubing in the MFCA system), the pH levels were expected to remain at 7.5 considering that media was constantly being refreshed and waste was being removed at the same rate. In addition, the chamber (heater ring plus latex balloon) surrounding the cell culture dish creates a barrier to gas transport and humidity loss and should keep the humidity, pH, and CO_2 levels in acceptable ranges for cell growth.

Oxygen was not added to the flow lines or controlled in any way. It should be noted that PDMS, the material from which the MFCA is made, is highly gas permeable ($D_{oxygen\ in\ PDMS} = 3 \times 10^{-5}$ cm^2/s) (Oppegard et al. 2009) and likely allows sufficient oxygen to reach the cells.

MTT assays Even though it has been reported that SKOV-3 cells poorly mimic the genetic composition of ovarian tumors, they belong to the group (SKOV-3 and A2780) of most used cell lines in ovarian cancer panels. SKOV-3 cells are human epithelial adenocarcinoma cells derived from ascites, a late-stage hallmark of this disease. These cells were chosen as representative for the clinical fact that most patients are diagnosed at later stages. For these experiments, 96-well plates containing human SKOV 3 cells were kept at pH 7.4 by maintaining CO_2 at 5% in the incubator (Thermo Scientific, Illinois). Identical pH, O_2 (95%) and CO_2 levels were maintained during all drug treatment experiments.

2.3.2 Flow control

MFCA system vertical syringe pump Microfluidic systems can be hampered by air bubbles present in the system, and hence bubble entrapment in the flow cells was also an early problem identified during our experimentation using MFCA. A peristaltic pump was initially used to create flow in the MFCA system. The negative pressures associated with cycling or pulling of the fluid led to the formation of bubbles in every flow cell, making this approach unsuitable for driving flow in the system. Using the peristaltic pump to only push flows through the MFCA still generated bubbles, but the needed low flow rates were not obtainable in a 48 channel pump, so we switched to syringe pumps. No commercially available syringe pump can actuate 48 syringes simultaneously. To reduce the occurrence of bubbles in the flow system and meet the needed low flow rates, a new vertical syringe pump was designed and built to simultaneously pump all 48 channels. The pump orients vertically forty-eight 1cm^3 syringes (Becton Dickinson, BD) equidistant from a central axis (Fig. 2). The vertical syringe orientation helps to prevent bubbles from entering and traveling through flow lines, provides equal force to each syringe, and reduces the syringe pump footprint by 80%. An Arduino Uno microcontroller running the pump (shown in Fig. 2) controls a stepper motor (Kysan,1,124,090) through a motor driver (SainSmart, TB6560), which drives the lead screw mechanism forcing a circular plate against the syringe plungers. Volumes, flow rates and experimental parameters are entered into an Arduino computer interface. The pump was calibrated and found to have performance similar to commercial pumps and better than 5% reproducibility.

Despite the use of a vertical syringe pump, bubbles were found to occasionally form and linger within the flow cells. To determine which flow cells were affected by the bubbles, the video (still images every hour) of each flow cell was analyzed and flow cells that exhibited any bubble activity were eliminated from later analysis.

MTT assays Even with the absence of continuous flow in these assays, bubble formation has occurred as drug is added into each of the wells. No additional measures were taken to evade this problem other than careful pipetting.

2.4 Cell culture

SKOV3 epithelial cells are derived from the ascites of a patient diagnosed with ovarian adenocarcinoma and were purchased from ATCC, Manassas VA. Cells were maintained in DMEM (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, MO) after seeding 4 × 10 (Sittinger et al. 1997) cells in 40 mm diameter tissue culture dishes (Techno Plastic Products -Switzerland) and incubated at 37 °C in a humidified atmosphere. For the MTT assays, 10,000 cells/well were seeded in the 60 internal wells (no wells on the edge of the plate were used) of 3 different 96-well plates. For the MFCA experiments, the MFCA was placed directly on the cell culture dish after priming.

2.4.1 Drug treatment

MFCA To perform a drug screening assay using the MFCA system, a logarithmic dose range of the drug was prepared and presented to cells using flow the 96-well plates (MTT) and the MFCA. The CDDP drug mixtures were prepared by diluting appropriate volumes of 1 mg/ml of CDDP in DMEM to prepare the following solution concentrations: 0.02 mg/ml, 0.005 mg/ml, 0.00125 mg/ml, 0.000313 mg/ml, and 7.813×10^{-5} mg/ml. These concentrations were selected after multiple previous MTT assay trials suggested that this range covered the relevant responses of the SKOV3 cells in the MFCA. The solutions were then each loaded into one of 48 1 mL syringes and placed in the pump.

Each of the 5 different concentrations of the CDDP-DMEM mixtures were assigned to 8 specific, spatially distributed flow cells of the MFCA (40 total) in order to reduce location effects on cell response. Eight flow cells were exposed to DMEM only and functioned as negative controls. Syringes each filled with the appropriate concentration of drug and DMEM were loaded onto the vertical 48-syringe pump.

The MFCA was primed by filling the tubing connecting the syringes to the MFCA with 0.3 ml of CDDP-DMEM solution. The MFCA was then docked to the center of the Petri dish containing cultured cells until the cells in each flow cell were isolated from each other, as shown in Fig. 3. This procedure was performed in small steps (a few microns) with a constant force of 8.15 N. The vertical 48 syringe pump was set to flow 25 μl at a rate of 300 μl/h of the CDDP-DMEM to fill up the flow cells with their corresponding concentration. Finally, the pump was set to flow at a rate of 0.01 μl/h (equivalent to 10% of the total volume of every flow cell each hour) for the remainder of each experiment (48 h).

MTT assay MTT assays were repeated 18 times at each concentration using human SKOV 3 ovarian carcinoma cell cultures. The MTT cell proliferation assay was performed following manufacturer's instructions. Human SKOV3 ovarian carcinoma cells were seeded in 96-well tissue culture plates (Olympus Plastics) at a density of 10,000 per well in 100 μl of DMEM. Cells were placed in each of the 144 wells to be used (10 wells in each of the 6 rows in 3 different 96-well plates) while columns and rows around the edges of the plates were left blank. The plates were allowed to incubate at 37 °C under a humidified atmosphere with 5% CO_2 for 24 h. Appropriate amounts of sterile 1 mg/ml CDDP solution, saline solution, and DMEM were utilized to prepare 500 μl of the following concentrations: 0.02 mg/ml, 0.01 mg/ml, 0.005 mg/ml, 0.0025 mg/ml, 0.00125 mg/ml, 0.000625 mg/ml, 0.000313 mg/ml, and 7.81×10^{-5} mg/ml. The edge wells (in each row) were not treated and were used as DMEM controls. The experimental wells were treated with 100 μl of solution. Each column received the same concentration of drug with the concentration decreasing from left to right. The plates were then incubated for 48 h after the drug was loaded.

2.5 Image acquisition automation and stain application in MFCA assays

After removing cell cultures from the incubator, but prior to docking them with the MFCA, 2 μl of calcein red-orange dye (ThermoFisher Scientific, C34851) was added to each cell culture dish and left to incubate for 10 min. Then, the media in each treated dish were removed and 2 ml of fresh DMEM at 37 °C were added. After initially docking the MFCA, pictures from all 48 flow cells were taken, and the system was set up to take pictures of each flow cell every hour using an inverted microscope with a magnification of 10X. To enable image capture of the flow cells with the MFCA docked, the dial-illuminator and the condenser parts of the microscope were removed and were replaced by a camera extension tube and a camera. The camera was directly connected to a computer running Metamorph software (Molecular Devices, Sunnyvale CA). The coordinates for each of the 48 flow cells were recorded in Metamorph and settings were registered to take individual pictures of all the flow cells every hour. ImageJ (National Insitutes of Health, Bethesda MD) was then utilized to create image sequences (videos). These videos were then further analyzed in order to identify the presence of any bubbles inside flow cells. Flow cells containing bubbles were then excluded from final cell viability calculations.

After 48 h, the MFCA was lifted from the cell culture surface and calcein red-orange dye was reapplied (2 μl). SYTOX ® Green dye (ThermoFisher Scientific, S7020) was also added (1 μl) and both dyes were allowed to incubate for 10 min. Then, the entire volume of media was replaced with 2 ml of fresh, sterile 37 °C DMEM and additional images of every flow cell were taken. First, fluorescence images were taken with an excitation wave length of 577 nm to observe the live cells marked with calcein red-orange, and immediately following, images at a wavelength of 504 nm were taken of the SYTOX ® Green to identify non-viable cells. Both pictures obtained from each flow cell were merged using ImageJ and the numbers of live and dead cells were counted in order to determine the drug response of the cells in each flow cell. A custom Matlab (Matworks, Natick MA) code (see supplemental material) was written to integrate pictures from all 48 different spots into the same image, showing the complete array (48) of flow cells (Fig. 3). These pictures were then converted into binary (black and white) pictures and cropped in order to analyze a central rectangular area (380 μm by 570 μm) of

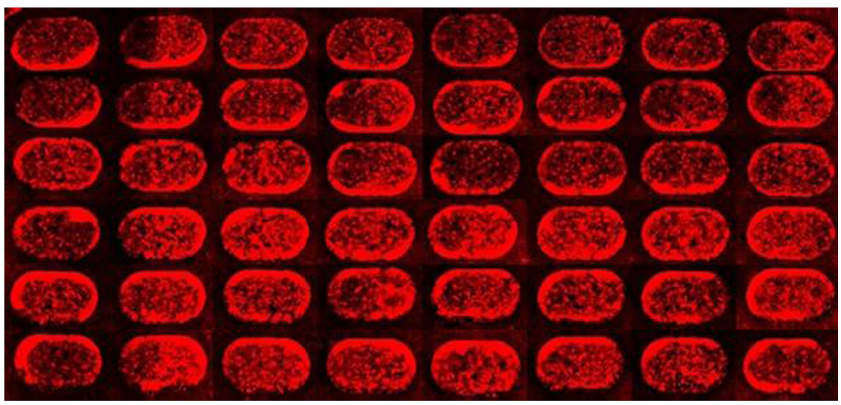

Fig. 3 Combined images of all 48 flow cells harboring human ovarian carcinoma (SKOV3) cells immediately after docking of the MFCA onto the culture dish. Red-orange Calcein dye marks viable cells, whereas live cells appear red. Pictures of every flow cell were taken individually and combined to generate this image

each well. White areas were considered representative of live cells whereas black areas depicted dead cells; these values were then used to calculate the viability percentage after 48 h. The percentage of viability for each flow-cell was calculated by using the number of cells found initially (time = 0 h) in each flow cell and then divided by the number of cells remaining after 48 h (Eq. 1). These individual values were then used to calculate average values for each drug concentration group. This procedure also yielded an average viability percentage for the control group, which was used to calculate final normalized viability percentages for the each of the non-control flow cells. The final normalized viability percentages for each flow-cell were calculated by dividing percentage viability from each flow cell (found using Eq. 1) by the average percentage viability of the control flow cells. A typical MTT assay, though, is based only on endpoint data. Thus, since MTT assay viability percentages are calculated only using the absorbance readings obtained at time = 48 h, MFCA viability percentages were also calculated in a similar fashion. Thus, a second calculation of viability percentages in the MFCA was performed using the live (white) area ratios for treatment concentration groups calculated using only pictures taken at time = 48 h. First, an average live (white) area measurement was calculated for the control (no drug) group. Then, viability percentage values were calculated for each flow-cell by finding the ratio of the average live (white) area values to the average of the control group. These individual percentage viability percentages were used to calculate the standard error. Finally, Welch's *t*-test was performed using these ratios and a significance level of $\alpha = 0.05$ was used. Percentage values were plotted using a log scale for the concentrations (*x*-axis), and a sigmoidal curve was fit through the plotted points using Matlab.

$$Viability\% = \frac{(Live\,white)\ area\ at\ t = 48\ hours)}{(Live\,white) area\ at\ t = 0\ hours)} \quad (1)$$

2.6 Colorimetric assay and plate reading

NAD(P)H-dependent cellular oxidoreductase enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-ditetrazolium bromide to its insoluble formazan, which has a purple color. Thus, a purple color in cells indicates that they are alive. The MTT dye was prepared by diluting 5 mg of MTT dye in 1 ml of PBS. Twenty μl of this dye solution was added to every well and allowed to incubate and crystallize for two hours. Media was removed and 100 μl of DMSO was added. Finally, the three 96 well-plates were placed in a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale CA, USA. Absorbance values were averaged for each concentration group, including the control (no drug) group. The viability percentage values were calculated as described for the secondary MFCA data processing method.

3 Results

The microfluidic flow cell array (MFCA) system was successfully modified and set up to test 48 drug samples on 48 independently isolated cell specimens. Five different concentrations of CDDP were continually flowed and tested over cultured human ovarian cancer cells for 48 h with multiple wells experiencing the same drug concentration, giving replicates in a single experiment. Differences in cell response were measurable.

Specifically, it was found that the MFCA provided favorable conditions to incubate SKOV-3 Human ovarian carcinoma cells. Figure 3 shows that each flow cell initially holds 300–500 cells depending on confluency and the uniformity of the cell seeding in the particular case of SKOV-3 cell cultures. Figure 4a shows a zoomed image of a typical control flow cell containing SKOV-3 cells dyed with red-orange calcein immediately after the MFCA was docked onto the cell culture dish (time = 0 h). Figure 4b shows the same flow cell

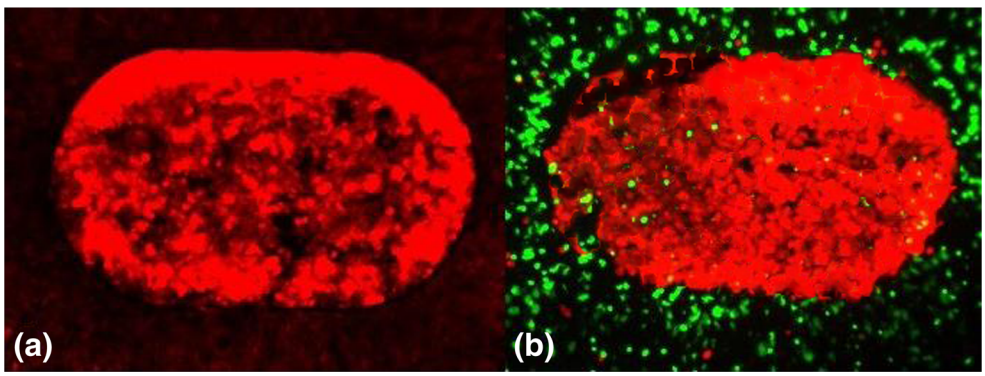

Fig. 4 Single flow cell: **a** viable human ovarian carcinoma (SKOV-3) cells dyed with red orange calcein immediately after initial docking ($t = 0$). **b** The same flow cell area now additionally dyed with SYTOX ® Green nucleic acid stain (green) after 48 h of DMEM flow. The flow cell has been removed allowing cells under the MFCA to also be stained and rinsed to remove any detached cells or debris

48 h later following continuous flow of DMEM at 0.01 μl/h. Live (red) and dead (green) cells are marked showing that general cell viability in the flow cell was maintained over the entire time period of the experiments (48 h). As an example of general cell culture success, row 5 in Fig. 5 shows the flow cells that were kept as controls (No drug treatment) in later drug exposure studies. The calculation of viability percentages indicated that these flow cells maintained an average of 90.4% of SKOV-3 cells alive after 48 h with a standard error of 4.95%. Visual observation of the different control pictures and percentages show that some cells died during the experiment, but others grew to keep the overall viable cell count fairly stable. Note that the MFCA was docked over the surface of a cell culture dish previously seeded with SKOV-3 cells. The solid parts of the MFCA docked over some areas covered with healthy cells. These cells were not only damaged by the pressure applied by the solid PDMS, but they were also excluded from the flow cell as shown in Fig. 4b. Cells outside the flow cell are all dead. Cells inside the flow cells survived normally with only a few dead cells present. Some cells were found to accumulate at the walls of the flow cells, which is caused by the sliding of the MFCA printhead on the surface of the culture dish during the docking procedure. Figure 5 shows a compilation of the entire 48 flow cells after 48 h and it can be observed that these accumulated cells survived and experienced growth.

3.1 Temperature and pH control in the MFCA system

The average temperature during the experiments was measured to be 36.5 °C (35 °C – 36.5 °C), assuring physiologically compatible temperatures for the cells. pH level testing was performed at the end of each experiment (time = 48 h) and ranged between 7 and 7.5. Indicating that the observed pH level in the cell culture medium within the flow cells was in the physiological range. Control flow cells in this particular experiment displayed an average viability of 90.4% after 48 h, suggesting that the temperature and pH ranges were appropriate.

3.2 Bubble-free environment in MFCA system

For the drug exposure experiments, a video of the entire 48-h experiment was captured and analyzed in order to exclude the flow cells that showed evidence of bubbles within the flow cell. Flow cells 1G, 3C, 3D, 4B, 5A, and 6C in Fig. 5 are representative examples of the effect of bubbles. The video revealed that cells trapped within the bubbles for extended periods were usually removed and not viable, but as depicted in the video and Fig. 5, some cells continued to grow in areas immediately adjacent to the bubbles (e.g. flow cell 3C). Cases in which bubbles were found to form and remain in the same position for over an hour resulted in cell death. In some cases, bubbles rapidly traveled (< an hour) through the flow cell and

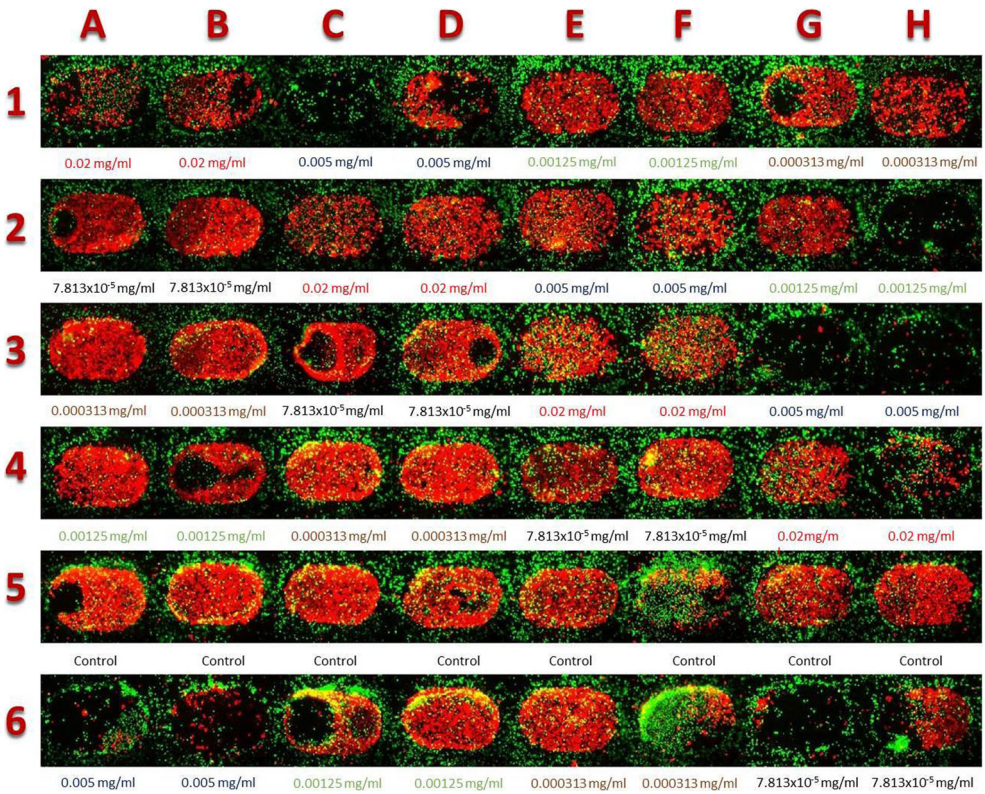

Fig. 5 Human ovarian carcinoma (SKOV-3) cells after 48 h of treatment in the MFCA with different concentrations of DMEM-CDDP solutions. Red-orange calcein and SYTOX® green nucleic acid stain were used to mark live and dead cells. The CDDP concentration of each flow cell is specified below every flow cell and each concentration has been assigned a particular color for easy identification. All concentration values are in units of mg/ml

caused no visible damage to the cells. In flow cells in which the entire cell population experienced cell death, (flow cells 1C, 2H, 3G, and 3H), bubbles were observed and lingered for over two hours within the flow cells. Therefore, cell death in the MFCA may potentially be caused by a combination of factors that include: the presence of bubbles within the flow system and their residence time within the flow system. The video shows that the number, locations, sizes and residence times of bubbles present within the flow cells varies greatly. All these variables have an impact on cell viability that also varies from minimal cell death (<10%) to complete depletion of cells (100%).

Based on these findings and the uncontrollable variability caused by bubbles, all flow cells presenting any bubbles were disqualified and eliminated from the calculation of viability percentage values for resulting dose-response curves. 5 out of 6 of the DMEM-CDDP treatment groups had at least 50% (4 out 8) of valid flow cells at time = 48 h. However, one treatment group only had 2 out of 8 valid cells because of bubbles. The elimination of bubble containing cells did not appear to affect the results, as will be discussed later.

3.3 Cell viability assessment in MFCA assays

Assembled images of the SKOV-3 cells exposed to a range of drug concentrations using the MFCA over 48 h are shown in Fig. 5. These images were converted into the cell viability measurements as described in the method. Figure 6 shows the dose response results for the MFCA assay (green and red) calculated in the two different ways. First, for the analysis in which only results at time = 48 h were considered, the green curve revealed that the average cell viability percentages were 63%, 61%, 103%, 104%, and 104% at 0.02 mg/ml, 0.005 mg/ml, 0.00125 mg/ml, 0.000313 mg/ml, and 7.81×10^{-5} mg/ml CDDP concentrations, respectively compared to control. Following the same order, the standard error values were 8.34%, 4.69%, 0.89%, 4.52% and 3.2%, while the *p*-values were 0.007, 0.007, 0.541, 0.565, 0.565, respectively. Then, the calculation of viability percentages considering cell numbers obtained at both time = 0 h and 48 h produced the following results: 59%, 56%, 95%, 94%, and 94%. The corresponding standard deviations were 7.87%, 4.58%, 0.71%, 4.31%, 2.64% and *p*-values were found to be 0.012, 0.010, 0.408, 0.598, and 0.543 for the 0.02 mg/ml, 0.005 mg/ml, 0.00125 mg/ml, 0.000313 mg/ml, and 7.81×10^{-5} mg/ml CDDP concentrations, respectively.

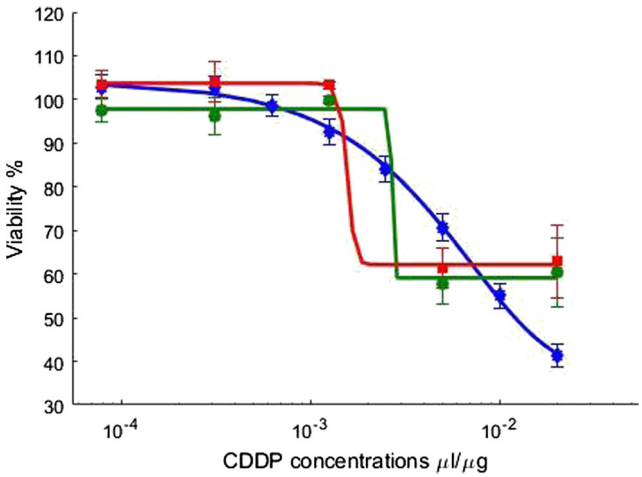

Fig. 6 Dose-Response curves for: MTT (blue) and MFCA assays (green and red). Both assays were performed using SKOV-3 cells and different concentrations of CDDP within the same domain. Both MFCA assay curves correspond to the same experiment, they just represent two different ways to calculate viability percentage values. Red curve represents the percentages calculated without the taking into account pictures taken at time = 0 h. while the green curve utilizes pictures obtained at time = 0 h

3.3.1 Cell viability assessment in MTT assays

A dose-response curve was plotted for the MTT assays performed as shown in Fig. 6 (blue). These assays included the 5 concentrations used in the MFCA assays as well as 3 additional concentrations chosen based on the highest and lowest concentration values used. The mean viability percentages were 41%, 55%, 71%, 84%, 93%, 99%, 103%, and 103% at 0.02 mg/ml, 0.01 mg/ml, 0.005 mg/ml, 0.0025 mg/ml, 0.00125 mg/ml, 0.000625 mg/ml, 0.000313 mg/ml, and 7.81×10^{-5} mg/ml CDD concentrations, respectively. Following the same order, the standard error values were calculated to be 2.6%, 2.9%, 3%, 3%, 3%, 2.5%, 2.6%, and 2.8%, while the *p*-values were found to be equal to $2.17\text{x}10^{-13}$, $1.55\text{x}10^{-10}$, $1.24\text{x}10^{-6}$, 0.002, 0.145, 0.766, 0.537, and 0.557, respectively when compared to control.

4 Discussion and conclusion

This study has determined that the MFCA can independently isolate 48 groups of approximately 400 SKOV-3 cells in microscopic flow cells. The present setup has the ability to provide incubator like conditions while allowing continuous flow of different reagents. Control (culture media only) flow cells have been able to culture cells for 48 h with an average viability percentage of 90.4%. Continuous flow treatment with CDDP have rendered similar dose-response curves to those obtained from MTT assays.

The overall goal of this project was the successful integration of the MFCA with a conventional inverted microscope to create 48 observable, individual flow cells capable of assessing cell responses to the provided environment. This would allow for multiplexed cell interrogation in a controlled flow environment. Conventional 2-D cell cultures could be rapidly converted into a high-throughput cell-based assay.

The MFCA was successfully mounted to a microscope with sufficient resolution and magnification (10X) to readily allow the automated observation of the growth and development of the cells being cultured and treated in each flow cell simultaneously. The ability to capture time lapse videos of each individual flow cell allows users to determine cell responses to modifications of their environment in real time. The MFCA setup has the potential to be mounted on a variety of inverted microscopes. This capability could improve the way drug screening is currently performed; cells will no longer have to be treated and incubated for set periods of time. Instead dose-response curves could be generated and observed in much shorter intervals of time (i.e. every hour) until a sufficient response is obtained. These modifications will save both time and money as drug screening assays can be performed for the minimum length of time. They will not have to be repeated as often, and reduced amounts of reagents will be consumed.

The MFCA and the associated environment were modified to allow for cell growth. Specifically, temperature control was added and shown to keep the area immediately around the cell culture dish at 36° +/− 0.5 °C, which is appropriate for maintaining mammalian cells and tissues. The temperature can be adjusted if a different temperature is desired. Flow and shear conditions across the different flow-cells were also shown to be adjustable over a wide range from no flow to flow sufficient to remove all cells from the surface (data not shown). For the SKOV-3 cells tested, conditions for normal growth were determined through multiple experiments (data not shown) and found to be optimal on the cell culture dishes used for a continuous slow flow rate of 0.01 μL/h. This flow rate generates a shear stress two orders of magnitude below the shear stress value that endothelial cells have been shown to resist (Papaioannou and Stefanadis 2005). The MFCA can also generate higher shear rates, though larger syringes (i.e. 3 cm^3 and 10 cm^3) may be required for longer-term studies (i.e. 72 h). Studies have demonstrated that mechanical forces associated with blood or other flow (i.e. wall shear stresses) affect different cellular processes in multiple types of cells (including endothelial cells), which lead to biophysical, biochemical, and gene regulatory responses. In the particular case of the ovarian surface, cells have to resist the pressures produced by the flow of peritoneal fluid and shear stress caused by the peritoneal surface motion(peristalsis) (Avraham-chakim et al. 2013), which has also been suggested to play an important role in the control of carcinomatosis development (Carmignani et al. 2003). Studies have also shown that the aspect ratios of epithelial cancer cells (EOC) increased while exposed to higher shear stress values (Avraham-chakim et al. 2013). The MFCA can potentially test these hypotheses.

Elimination and prevention of bubble formation was an important aspect related to creating appropriate cell growth conditions. Early experiments showed that bubbles could impact viability of cultured cells and could remove vast amounts of attached cells. The goal of preventing bubble formation while providing continuous flow was partially achieved by using a custom-built, vertical 48-syringe pump, but bubbles still occasionally affected some of the flow cells. Bubble detection could likely be automated in the future by integrating bubble traps (Johnson et al. 2009). Even after removing some flow cells from further data analysis, a robust number of flow cells for each concentration was found to be free of any environmental disturbances and useful data could be obtained.

Once development and basic validation were completed, the MFCA was used to construct drug dose response curves. The results shown in Fig. 6 indicate that the MTT and MFCA drug assays found similar dose-response curves, regardless of the way viability percentages were calculated, which suggests that both screening systems are comparable and can be used to generate actionable data. All three curves demonstrate a decline in viability percentage in a similar concentration interval suggesting that the MFCA system has the potential to produce similar dose-response curves. However, the MFCA assay shows less viability percentage decrease than in the case of the MTT assay. The MFCA-assay viability percentages for the 2 highest treatment concentrations are on average 20% higher than the viability percentages for similar concentrations in the case of the MTT assay. This suggests that the constant flow of fresh culture media (including drug) has a limiting effect on the cytotoxic capabilities of CDDP, validating our expectation of modified results under flow conditions. While the implication for this difference is not yet clear, it is clear that the results are at least as useful as the MTT assay. One limitation of this result for the MFCA is that in order to identify the median lethal dose (LD-50) using the MFCA system, the use of higher and wider chemotherapeutic concentration ranges than that used with MTT assays may be necessary, if this resistance to cell death appears in other assays as well. Future work will use MFCA printheads with larger arrays (i.e. 96 flow-cells) which will enable additional testing groups that will help answer this particular question.

Statistical analysis indicated that the two highest concentration groups displayed statistically valid p-values (<0.05) when compared to the control (and the first three groups), but the other three lowest concentration groups did not. The results clearly show that a significant difference in cellular response can be measured when using the MFCA, and the inflection points of the curves match well. The standard error values in the MFCA data are somewhat larger than for the MTT data, suggesting that better control still needs to be instituted for the MFCA. Possibly issues with bubbles and image collection and processing may have led to the larger standard error values, but it may just be that the much smaller number and variability of starting cells in the MFCA leads to more statistical variation. Higher starting cell culture

confluency may resolve this issue, but leads to more lethargic cells. Further work to reduce the number of flow cells compromised by bubbles and increasing the number of flow cells available within the MFCA array (new MFCA with a larger array) will likely help with these statistical questions as well.

In conclusion, results presented in this work demonstrate the basic capability of the MFCA as an innovative, new screening tool for use with cell culture or printed cells (Davidoff et al. 2014). The ultimate possibilities of this platform are significant with potentially larger numbers of flow cells allowing HTS of cell based assays, new applications being developed (such as drug and particle toxicity), the direct use of primary patient derived cells and tissues, and the subsequent personalized medical treatments. Compared to existing static systems, many of which are capable of multiple parallel assays, the MFCA has the potential to better mimic natural cell conditions due to its ability to continually replace nutrients and evacuate waste throughout the experiment and create a flowing, dynamic environment like in the body. The MFCA also avoids evaporation problems. The very small volumes in the MFCA should also enable the use of the system with precious samples consisting of only very small numbers of cells, a clinically relevant and frequent problem. When compared to commercially available microfluidic live cell analysis plates (i.e. CellASIC) the MFCA offers advantages that include: reusability, low cost, and higher-throughput. MFCA printheads are reusable and can withstand frequent use (one 48 h experiment every 3 days) for an average of 6 months, while the CellASIC plates are disposable. Overall, the results presented here suggest that there are opportunities for using the MFCA with cells for screening applications.

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