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Paper

UV-SENSITIVE HYDROGEL BASED COMBINATORY DRUG DELIVERY CHIP (UVGEL-DRUGCHIP) FOR CANCER COCKTAIL DRUG SCREENING

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Ying-Ting Chen,[a] Venkanagouda S. Goudar,[a] Ren-Guei Wu,[a] Hsin-Yi Hsieh,[a] Chung-Shi Yang,[b] Hwan-You Chang,[c] Gwo-Bin Lee,[d] Chih-Ming Ho,[e] and Fan-Gang Tseng*[af]

Effective and efficient Treatment of diseases, such as HIV, cancers or hereditery diseases require accurate and precise control of combinatorial drug-dosage and their release. Here we introduce a simple photosensitive Poly (ethylene glycol) di acrylate (PEGDA) hydrogel based platform for high dynamic range testing of combinatorial cocktail drug screening using three chemical and two protein drug treatments for colon cancer. UV cross linked PEGDA hydrogel droplet arrays on a Teflon patterned glass substrate enabled a rapid yet accurate selection and dosage assignment of drugs. Precisely loaded cocktails of the anticancer drugs were simultaneously released in-parallel with the PEGDA hydrogel chips into 2D or 3D cultured HCT-8 colon cancer cells for combinatorial drug screening. We demonstrate the functionality of our UVgel-DrugChips 1000 fold range of concentrations for each of the five drugs in 30 seconds to find the optimized drug cocktail using a fractional factorial control system. Our device has low drug consumption, requiring only 12 nL per screening run per droplet. However, our UVgel-DrugChips were employed for finding the optimized drug cocktail by fractional search algorithm. Our cocktail drug response results for both 2D (cell viability is 7.3%) and 3D (cell viability is 10.8%) colon cancer cells were analogous to those found by conventional methods (6.8 and 9.3 respectively). In contrast to conventional method, our approach is faster, more effective, less time consuming and requires a lesser amount of drug volume.

Introduction

Conventional drug delivery techniques are time consuming, painful and they can cause side effects.[1] As such, there is a need for alternative, high throughput, cost effective, and user friendly laboratory drug screening methods for high efficient drug testing are in need.[2] Current trends in point-of-care, automatized and precise drug assay platforms are superior to the conventional techniques. In that they can achieve higher accuracy with lower drug dosage.[2] Microfluidic based platforms have been emerging as an efficient chip based assay for cell line tests. With their inbuilt advantages of low reagent consumption, precise control and high throughput scalability, and such device provide robust analytical tools for the investigating of complex biological processes at the cell level.[3-7] Target anticancer drugs have specific targeted sites against cancer types and locations. For example, *5-Fluorouracil* and *Capecitabine* are antimetabolites, Irinotecan is from plant alkaloid and acting as a topoisomerase inhibitor, *Folinic Acid* is a chemo protectant, which causes folic acid deficiency in cancer cells and leads to cell death, *Oxaliplatin* is alkylating agent for inhibiting cell cycles, *Bevacizumub* is a genetically modified drug for regulating angiogenesis by inhibiting vascular endothelial factor and *Cetuximub* is a protein based drug, which targets on endothelial growth factor receptor proteins.[58] These examples are some of the drugs approved by US drug agency for chemotherapy against cancer. However, none of the above drugs, on their own merits, can eliminate cancer thoroughly and efficiently.[57] Chemotherapies can reduce tumour burden within the sub clones by eliminating the highly proliferative cells by targeting on their RNA, DNA, enzymes, protein receptors and cell cycle, but when metastasis occurs, those cancer cells at the metastasis site do mutate. Hence, better and more effective drugs for treating metastatic cancer need to be developed. Sub-clonal diversity can be altered with chemotherapies, which allows for the selection of cells with additional genetic mutations to confer a survival advantage.[8]

Therefore, if one drug isn't enough to control these tumours, one may think of a combination of several drugs as a cocktail to potentially obtain better treatment result.[9] The Synergistic effect of multiple combination of drugs also allows lower dosage of drugs

[a] Engineering and System Science Dept., National Tsing Hua University, Hsinchu, Taiwan
[b] National Health Research Institutes, Zhunan, Miaoli, Taiwan
[c] Department of Medical Science, National Tsing Hua University, Hsinchu, Taiwan.
[d] Department of Power Mechanical engineering, National Tsing Hua University, Hsinchu, Taiwan.
[e] Mechanical Engineering Department, University of California, Los Angeles, CA, USA
[f] Applied Science Centre, Academia Sinica, Taipei, Taiwan.
† Electronic supplementary information (ESI) available. See DOI: 10.1039/

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but sufficient activity against many anticancer targets, which, in turn, also reduces the drug resistance effect on cancer cells.[10] Indeed, combinatory drugs have been reported to have higher efficacy and lower individual drug dosage needed in treating various diseases including cancers.[11] However, doses of drugs in the combination can also critically affect the efficacy or toxicity. Conventional drug cocktail optimization methods are not as cost effective due to their high drug cost owning to considerable volume ratio, time consuming routine process for large-scale assay and the limitation of dynamic ranges of drug concentrations.[12,13] In comparison, the feedback system control (FSC) technique is considerably less time consuming and cost effective as regard to labour cost.[14-17] This paper is the first time to demonstrate a lab-on-a-chip approach for efficient FSC based combinatorial drug screening.

To operate cocktail drugs in batch a sequential microfluidic processes were developed. For example, integrated micro valves were effectively employed to control and generate individual droplets of precise size and drug composition. The formed droplets can be selectively and sequentially used as drug cocktails.[18] The other process, which uses photo polymerization method in a microfluidic device to sequentially produce 5-fluorouracil loaded biocompatible poly (ethylene glycol) di acrylate (PEGDA) microspheres with mono disperse size distribution, has been reported to being used successfully for sustained drug releasing.[19] For instance, for protein drug delivery, microfluidic based polymeric capsules were prepared with high encapsulation efficiency.[20-28] for oral protein drug delivery, polymeric based drugs were produced.[30] For intracellular drug delivery, polymer nano capsules with different pH resistances were also prepared.[31] However, despite the success of the above methods, sequential processing for batch drug testing is having some unsolved challenges, which include variation on delivering time, drug combination complications and complex drug delivering networks/pathways to individual cell wells.[32]

On the other hand, a parallel process, bead-based assay platform was developed recently for cell drug testing.[33] However, the need for laser pulse actuation for breaking bead shells for releasing the drugs posed an additional issue. Therefore, heating and chemical reactions are bio-incompatible during laser activation. Furthermore, once the capsules are randomly assigned with drugs, it is not easy to select several different drugs in a combination of various concentrations on demand.

To alleviate these challenges, we propose a combinatory drug assay platform for cocktail drug testing by employing UV cross linked PEGDA hydrogel droplets to precisely release various cocktail ingredients from 5 different anticancer drugs for in-parallel drug testing against colon cancer cells. The platform incorporates the techniques of self-formation of drug/hydrogel micro-droplets and UV selective curing to desired drug dosage from obtained cocktails. Which is bio-compatible with high dynamic range (1000 folds) for cocktail drug assignation. These drug combinations can be simultaneously tested on both 2D and 3D cell cultures right after cocktails preparation without any time lag.

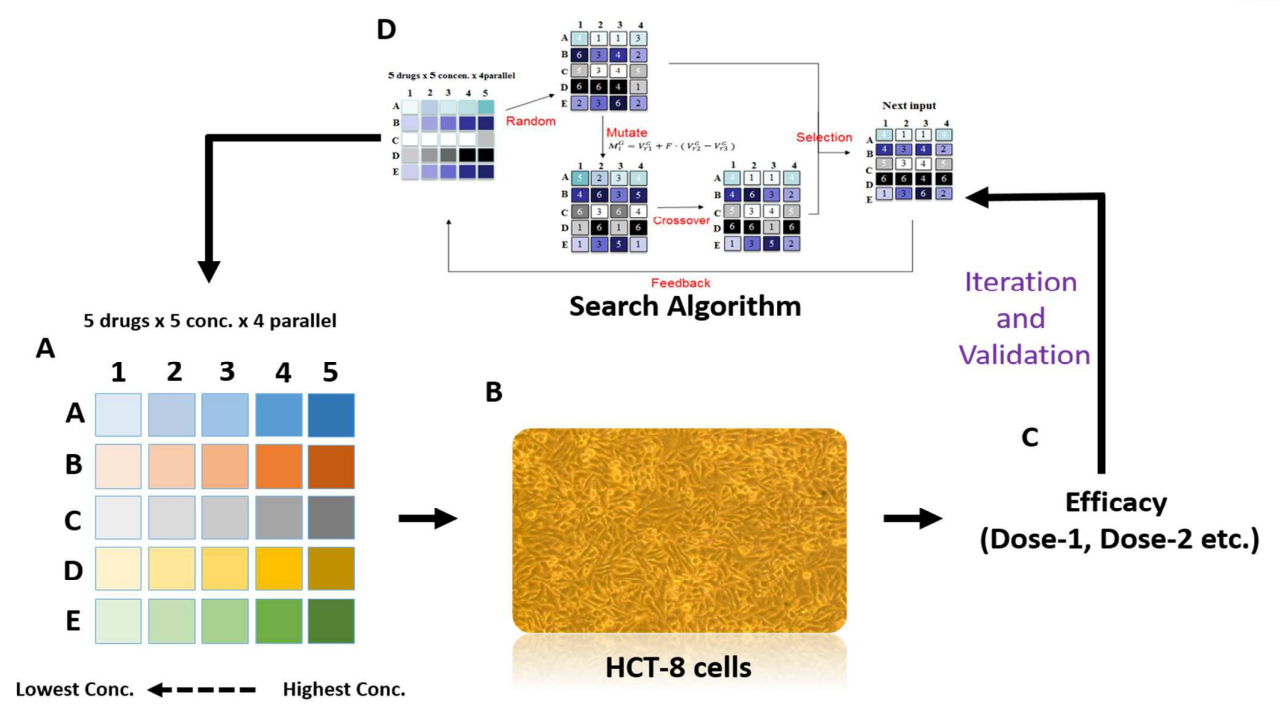

Fig. 1 Schematic representation of Feedback System Control (FSC).

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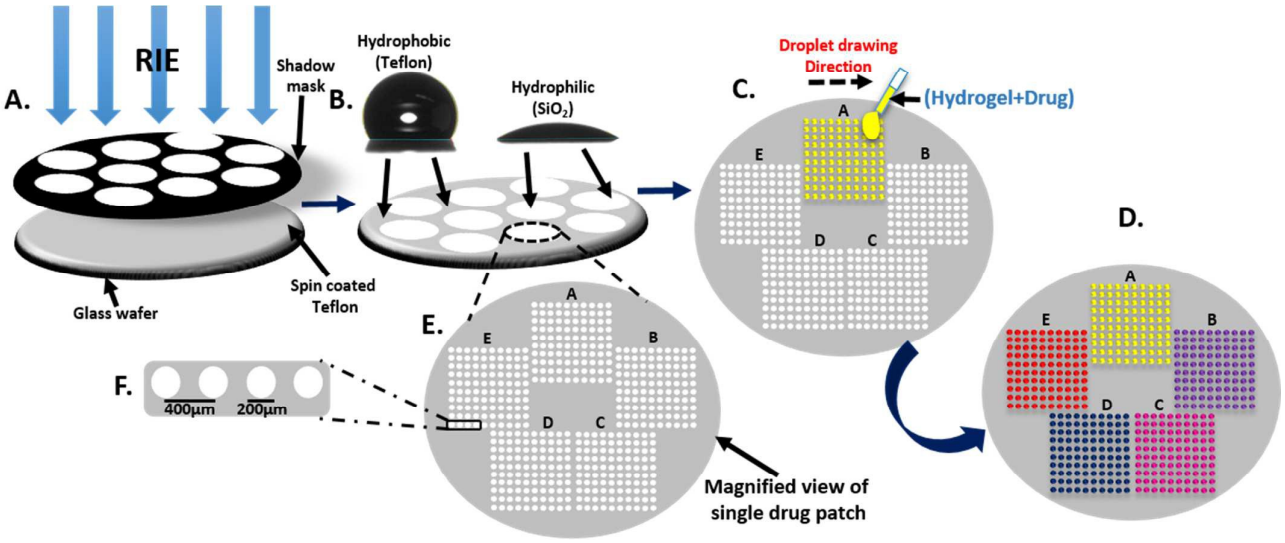

Fig. 2 Formation of a hydrogel array by rolling a droplet over a wettability contrast surface. Super hydrophilic array patterned on super hydrophobic Teflon coated substrate by RIE with the help of shadow mask (A and B). The water contact angle measurement represents the hydrophobic and hydrophilic regions (B). Single drug patch contains 5 different regions (i.e. A, B, C, D, E), each region contains 100 hydrophilic arrays around hydrophobic background (E). The magnified view represents, each array measures around 200µm and 400µm pitch to pitch distance (F). Then hydrogel+Drug solution were rolled carefully and manually along the surface (arrow shows direction of rolling) on respective area, the surface tension leads to the spontaneous array of completely separated micro droplets (C). In same way, 5 different hydrogel+Drug solutions were loaded on respective area (5 different colours represents the 5 different hydrogel+Drug solutions) (D).

The FSC Approach

Because of the efficacy and toxicity effects on the biological system, cell, organ and body, are not only functions of the drugs but also strongly affected by the doses. N^M combinations formed by M drug and N doses present a very large test parameter space. By integrating experiment and a search algorithm to form a feedback system control (FSC) loop, we have found that about 15 feedback loops can identify the optimal drug-dose cocktail out from 1,000,000 possible combinations.[14,17] Derived from the serial testing feedback loop concept, a much more efficient parallel search FSC technology has been demonstrated in cell line test and in preclinical *in vivo* experiment.[15,16] To make it more achievable for disease treatment purpose, differential evolutionary algorithms was proposed by Dr. F. Wei et. al. To find out the optimized combinatory drug system for best disease suppressing result in a very limited number of experiments.[29] Also, they reported a study using the FSC technique to investigate a biological system with Herpes simplex virus type 1 against five antiviral drugs. The finally searched combinations of drugs demonstrated a higher virus killing efficacy and lower individual drug dosage when compared to the effect of any individual drug with much higher dosage. While only fewer than 15 cycles of experiments were performed to obtain the final result. By considering the aforementioned study, we employed the similar search algorithm to our UVgel-DrugChip system for colon cancer cells drug screening. Initially, we set an IC_{50} value (half maximal inhibitory concentration) of each drug, as code 5, which is also the highest concentration of drug used in cocktail. Furthermore, to setup different and lower concentrations, one third concentration of code 5 is assigned to code 4 as a reduced dose. The dose will further be reduced to one third from 4, 3, 2 to 3, 2, 1 respectively, in a similar way, (Fig. 1) depicts the overall process for searching the optimized cocktail combinations. The system randomly picked up initial values for four parallel experiments, recorded as, V^G as shown in the first step. In the next step, the variation (also called mutation) is conducted according to the formula $M_i^G = V_{r1}^G + F \cdot (V_{r2}^G - V_{r3}^G)$, so that V^G can be converted to the M^G.

Then we crossover V^G and M^G, which means we randomly selected values from V^G and M^G for cross over and finally we can obtained a new combination values called T^G (after crossover). At last we compare the results of V^G and T^G groups, in respective parallel trial we chose the better readout as the next input V^{G+1}. We iterate the selection process for about five to eight times to obtain a convergence value of the testing result, which will finally lead to the final optimization value of the drug combination after several more feedback search cycles.

Experimental

Materials

Polyethylene glycol di acrylate (PEGDA) polymer (Fisher Scientific, Model B2200R-1, Pittsburgh, PA), 1X PBS buffer, pH 7.4 (gibco®, by life technologies), Irgacure 2959 (Sigma-Aldrich). 5-Fluorouracil (MW=130.1), Capecitabine (MW=359.4), Irinotecan (MW=558.64), Folnic Acid (MW=473.44), Oxaliplatin (MW=397.28) and Rhodamine 6G (MW=479) from Uni Ward Corp. Importantly, which all chemicals we used in UVGEL-DRUGCHIP preparation were dissolved and prepared in 1X PBS buffer solution.

Preparation of hydrogel

Now a days, hydrogels have been emerging among the best materials for controlled drug delivery and tissue engineering, because of their biocompatibility and biodegradability.[34-36] There

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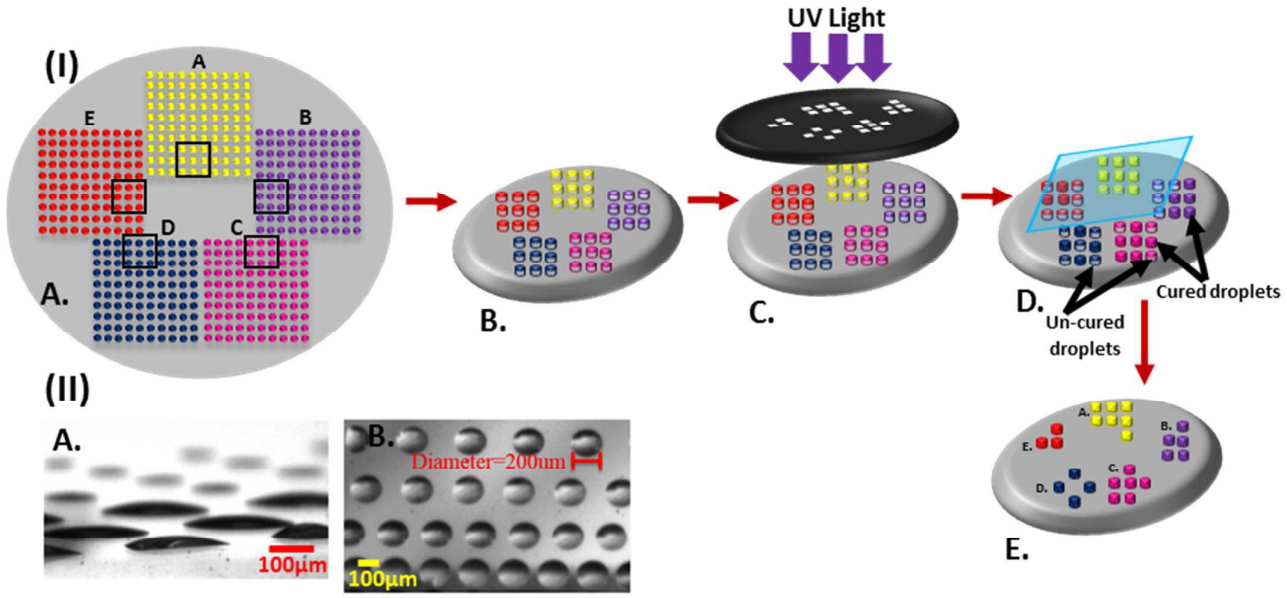

Fig. 3 Schematic representation of combinatorial cocktail hydrogel+Drug polymerization and selection of quantitatively varied drug carrying areas (I). The step (I, B) represents a selection area (black square box) from step (I, A). However, same procedure is fallowed for whole UVGel-DrugChip. Partially exposure to the UV light by using specially designed shadow mask (I, C). Then carefully wiped out the uncured hydrogel arrays by tissue paper without contaminating (I, D). Eventually, remaining UV cured arrays were quantitatively different drug carrying spots (I, E). The microscopic images of hydrogel array droplets (II). Higher magnification (II, A). Lower magnification (II, B).

are natural and synthetic hydrogels and polyethylene glycol (PEG) based gels are synthetic hydrogels. PEG gels are bio inert hydrogels and their chemical structures can be modified conveniently by adding copolymers and natural polymers to the side chains.[37-39] Polymeric hydrogels are also used in different stimuli responsive drug delivery systems, like, ATP,[40] temperature,[41] magnetic field,[42] mechanical signals,[43] redox[44] and ultrasound-triggered drug releasing systems.[45] Out of all PEG derivatives, PEGDA can be easily cross linked by UV irradiation,[46] and has been widely used in many biomedical applications due to its cytocompatibility, non-toxicity and ease of use.[47] In this study, we prepared 5 wt % of poly (ethylene glycol) di acrylate (PEGDA, Mw=700; Sigma-Aldrich) solution in 1X PBS buffer solution (total volume is 100ml) and agitated in an ultrasonic bath (Fisher Scientific, Model B2200R-1, and Pittsburgh, PA) for 15 minutes. Once the PEGDA polymer was completely dissolved, then mixed 0.25mg of photo initiator (Irgacure 2959, Sigma-Aldrich) in to the same solution. Further, solution were agitated again using the ultrasonic bath, and the pH was adjusted to 7.4. Importantly, Irgacure®2959 is more biocompatible against many type of cell lines than other UV photo initiators.[47-49]

Preparation of hydrogel+Drug complex

The above explained procedure was repeated to prepare 100ml of

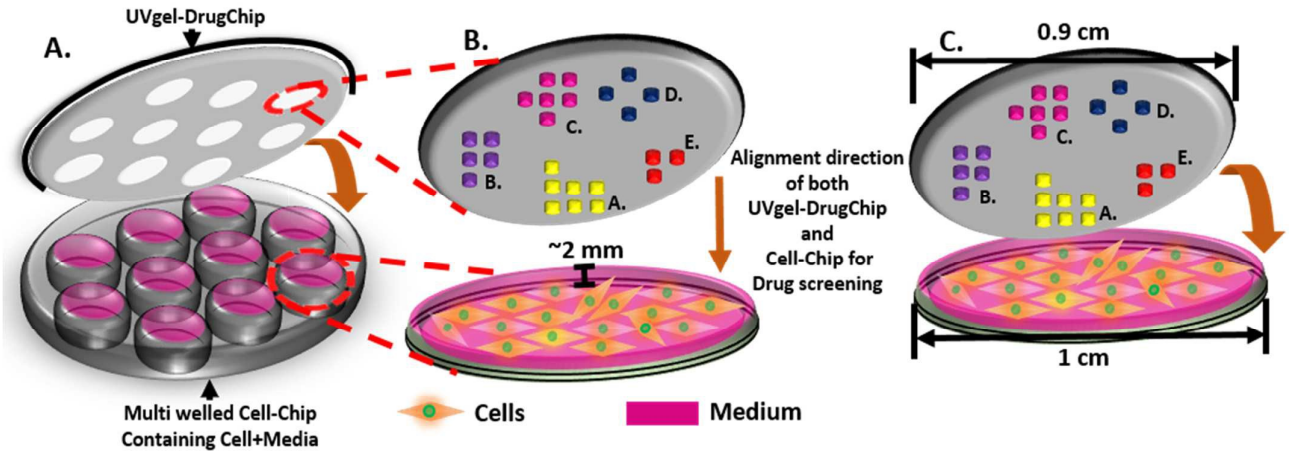

Fig. 4 The UVgel-DrugChip and the PDMS based multi welled Cell-Chip were aligned and combined face to face to release drugs by diffusion in to cell culture medium (A). The zoomed diagrams represents a single hydrogel drug patch and a single well containing media (pink colour) plus cells (yellow colour) (B). Arrow mark (coloured one) shows the alignment direction of both the UVgel-DrugChip and Cell-Chip for drug screening (B and C).

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hydrogel solution. Further, 100ml solution has been equally divided in to 5 parts. Additionally, according to the feedback system control (FSC) results, respective concentration of each drug [5-Fluorouracil, Capecitabine, Irinotecan, Folnic Acid, and Oxaliplatin] has been weighed separately and mixed in to the respective hydrogel solution. Further, individual hydrogel+Drug complex were used for drop casting.

Micro droplet Formation and UVgel-DrugChip preparation

We designed a wettability based contrast surface by arranging super hydrophilic defects on the super hydrophobic background which could cause spontaneous separation of liquids. firstly, piranha cleaned glass wafer were spin coated (60 Sec. at 3000rpm) by teflon (0.5 wt % 0f teflon were prepared in 1X PBS buffer solution). Further, teflon coated substrate were introduced to reactive ion etching [RIE] (Ar/O_2, 5sccm/10sccm, 80W) cabinet to form hydrophilic windows with the help of shadow mask (Fig. 2(A)). Further, pipette out the previously prepared hydrogel+Drug solution and rolling the solution across the super hydrophilic and super hydrophobic spots as showed in (Fig. 2(C)), hydrogel+Drug droplet arrays were rapidly formed with a volume variation less than 5%. However, each array is called as hydrogel+Drug patch (area is 0.9 cm^2) (Fig. 2(E)), which is consisting of 5 different regions (Fig. 2(E)). Each region has 100 droplets (each droplet size is 200 µm in diameter with 400 µm pitch to pitch distance) (Fig. 2(E and F)). Each region measured around 0.18 cm^2. Therefore these structures can be easily visible under naked eyes. Five different hydrogel+Drug complex solutions can be applied to the designated regions in a batch matter (Fig. 2(D)). The individual hydrogel+Drug complex where loaded in to the 5 separate regions respectively and manually without cross contamination (Fig. 2(D)).

Dosage selection by UV exposure

Further, once again, the hydrogels were selectively photo polymerized to assign corresponding dosages (Fig. 3 (I)(A-E)) by exposing to UV radiation at 15 mW/cm^2 intensity (by using, UV transilluminator, Daihan Scientific, Korea) with a 10 seconds curing time by using shadow mask (Fig. 3 (I)(C)). In next step, non-cured droplets (Fig. 3(I)(D)) were sucked out by the tissue paper through capillary effect while cured droplets remained on the chip (Fig. 3(I)(E)). The (Fig. 3 (I)(E) represents the withstand 5 different drug concentration regions in a single drug patch. Images from (Fig. 3(II)) shows the array of hydrogel droplets and each droplet has a diameter of 200 µm and a volume of 12 nL. The volume of the droplet were measured by taking droplet images (top and cross section views) under contact angle measurement instrument and further volume of droplet is calculated by using solid work software.

Cell-Chip preparation

However, same patterned design (as UVgel-DrugChip) were used to prepare multi welled Cell-Chip. Firstly, finest smooth surfaced metal mould were prepared. Then PDMS were prepared with standard procedure and poured on to the metal mould surface to get final

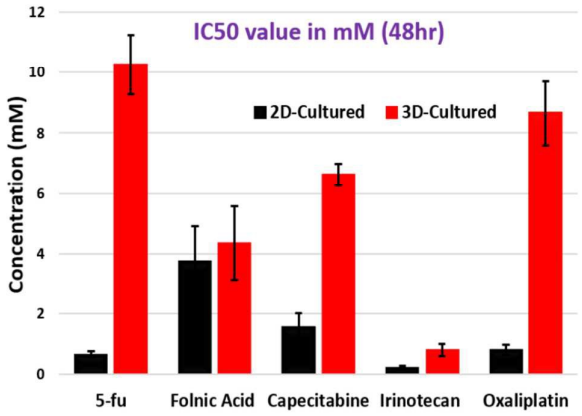

Fig. 5 The half maximal inhibitory concentration (IC50) values between 2D/3D cultures of individual drug.

multi welled Cell-Chip. Further, place it in oven at 80^0C for 3hrs. to cure. After curing, carefully PDMS were pealed out from the metal mould and bonded on glass wafer. Each well were measures (1cm diameter) little larger than that of each Hydrogel+Drug patch (0.9cm diameter) as showed in (Fig. 4(C)).

Cell Culture Condition

It is well known that many cancer cells lose some of their phenotypic properties when grown in vitro as 2D monolayers over a time. Also, 2D cultures lack the metabolic and proliferative gradients. Because of strong affinity of cells to artificial surface. Formation of tumour like 3D structures is highly inhibited in 2D monolayer cultures. In other words, 3D cultures closely mimic natural tissue and organs than cells grown in 2D. Also, there will be a natural cell to cell interaction in 3D cell cultures. In this study, HCT-8 cells were chosen to be the target cells and incubated for both 2D and 3D cultures for drug testing. For 2D culture, cells were initially cultured on 96 welled petri dish covered with 300 µL of culture medium. The culture medium was made from DMEM in the presence of 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Pen-Strep). 96 welled petri dish were incubated over night at 37^0C with 5% CO_2. In beginning, to do some basic and conventional experiments we used those 96 welled plates. But, mainly, we used PDMS based multi welled Cell-Chip for our most of the drug screening experiments, which is measure around 1cm diameter and holds approximately 155µl of medium.

For 3D culture, the hanging drop method is relatively simple and has been reported to have a reproducibility of almost 100% for producing one 3D spheroid per drop.[50] To produce 3D cultures, we used insphero gravity trap™ ULA and gravity plus™ plates with a cell stock containing 15,000 cells/ml by pipetting a 40 µl cell suspension in to the top side of the Hanging Drop 3D cell culture platforms. In order to prevent medium evaporation, 4 ml of distilled water was added into the peripheral water reservoir. The growth media was

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exchanged every other day by taking out 10 µl solution from a drop, and adding 20 µl fresh growth medium into the drop. After cells were incubated for 72 hours 200 µl cell medium were added to spheroid cells and then collected into gravity trap plate. Further, those cell spheroids were pipetted out and dropped in to multi welled Cell-Chip and also in to the 96 welled chip for further respective investigations.

Drug releasing process

Conventionally, to test all the combination of 5 drugs each with 5 different concentrations required at least 5^5 times operations. Through the search algorithm,[17,29] 5^2 times can reach the optimal value. The current platform not only can quickly ensemble all 5 drugs with desired concentration in each test but also can deliver in-parallel into each well for drug testing. In order to achieve high throughput process, as we mentioned earlier, patterned different areas on the glass substrate for cocktail drug delivery. Each area (0.18cm^2) contained hundreds of drug carrying hydrogel droplets with 5 different drug concentrations which is called a hydrogel patch (Fig. 4(A)).

As explained before, to define the dosage of each drug for a specific test, a shadow mask was employed to partially expose the selected area to cure drug carrying hydrogel droplets. Due to the discrete nature of hydrogel droplets, volume and ratio control can be very precise for the combination of 5 drugs. After hydrogel-drug droplet formation and cocktail dosage selection, UVgel-DrugChip were immersed in to the corresponded multi welled Cell-Chip for cocktail drug delivery at a controllable time pace, as shown in (Fig. 4(A and C)). As before mentioned, the well size (1 cm) of the cell chip is designed slightly larger than that (0.9 cm) of the drug chip. So, the area of one drug patch is large enough to be easily visible to necked eyes. Therefore, we aligned these two chips manually without using any alignment keys and any other specific instruments.

Measurement of Released Drug Concentration

We used five anticancer drugs and one fluorescent molecule to define the releasing rate: 5-Fluorouracil (MW=130.1), Capecitabine (MW=359.4), Irinotecan (MW=558.64), Folnic Acid (MW=473.44), Oxaliplatin (MW=397.28) and Rhodamine 6G (MW=479) (data is mentioned in supplementary information Fig.2). The released concentration of the drug was measured by a UV spectrophotometer. Initially, standard calibration curve were obtained by measuring absorbance from initial concentration of all 5 drugs. Furthermore, drug released from the PEGDA hydrogel into the cell medium was measured to determine the released drug concentration. The (Fig. 6(B)) shows the releasing rate of the different drugs from the hydrogel droplets. The releasing rate is defined as the percentage ratio of the released concentration to the loaded concentration, which is dependent on the molecular weight, molecular charge, diffusion coefficient and solubility of each drug.

In this work, we plotted graphs by using Origin.8 and Microsoft Excel software.

Results and Discussion

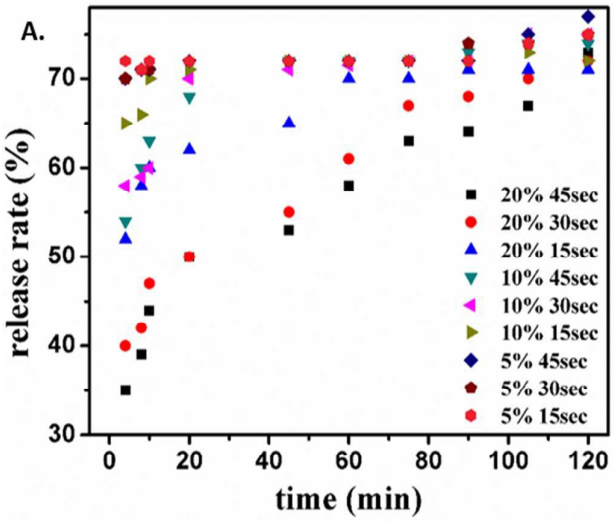
Cell Viability Test

Detailed process and results of cell viability test is mentioned in supplementary information (1).

Drug Sensitivity Test

In starting, cytotoxicity was tested individually for all five anticancer drugs (5-Fluorouracil, Capecitabine, Irinotecan, Folnic Acid and Oxaliplatin) in a conventional way (directly pipetting drugs in to 96 welled plates) against both 2D and 3D cultures of HCT-8 cell lines.

A.



B. Drug releasing test with 5% hydrogel and 15 seconds curing time

	5-fu	Capecitabine	Irinotecan	Folnic acid	Oxaliplatin
Molecular Weight of drug	130.77	359.35	558.64	473.44	397.28
IC_{50}Loading Conc. (mM)	0.67	3.75	1.56	0.267	0.97
Released Conc. (mM)	0.56	2.84	0.95	0.17	0.73
Amount of Drug released (µmole)	0.0868	0.4402	0.1472	0.0263	0.1131
Release rate (µmole/sec)	$2.8x10^{-3}$	$14.6x10^{-3}$	$4.9x10^{-3}$	$0.87x10^{-3}$	$3.77x10^{-3}$
% amount of drug released	83.00	75.80	61.20	63.40	76.10

Fig. 6 Drug releasing test. The graph represents drug releasing capacity of hydrogel with varying concentrations and with different curing time from 5% to 20% and 15 Sec. to 45 Sec. respectively, every data point is average of 3 repeated experiments (A). Table represents the individual releasing rate and amount of drug released (%) of 5 different drugs at 5% hydrogel and 15 sec. exposure time (B).

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Table 1. Cocktail drug screening result for 2D culture system. Top table (A) represents different concentrations (1-5) of different drugs (A-E). Below table (B) shows results of combinatorial (15 combinations) drug screening results of 2D cell culture against conventional culture method.

I. 2D Culture

(A)	Drugs	levels (mM)				
		1	2	3	4	5 (IC50)
A	5-fu	0.008	0.024	0.074	0.223	0.67
B	Folnic Acid	0.046	0.138	0.416	1.250	3.75
C	Capecitabine	0.019	0.058	0.174	0.522	1.568
D	Irinotecan	0.003	0.009	0.029	0.089	0.267
E	Oxaliplatin	0.012	0.036	0.108	0.324	0.973

(B)	Factor					readout (cell viability)	
run	A	B	C	D	E	cocktail chip	convent -ional
1	5	2	5	3	2	27.4	22.3
2	5	3	5	5	5	8.7	10.1
3	1	4	4	4	5	16.3	21.7
4	3	3	5	5	4	9.3	8.7
5	4	4	1	1	4	44.1	48
6	5	2	5	5	2	23.1	19.2
7	5	5	5	5	5	9	8.3
8	1	4	2	4	4	14.5	17.9
9	5	5	5	5	4	8.3	14.6
10	2	4	4	4	5	24.3	22.1
11	5	2	3	3	2	27.1	33.1
12	5	1	3	2	4	33.3	30
13	1	4	2	4	1	36.5	35.1
14	2	1	5	4	4	7.3	6.8
15	2	4	3	4	5	17.4	11.4

Table 2. Cocktail drug screening result for 3D culture system. Top table (A) represents different concentrations (1-5) of different drugs (A-E). Below table (B) shows results of combinatorial (15 combinations) drug screening results of 3D cell culture against conventional culture method.

II. 3D Culture

(A)	Drugs	levels (mM)				
		1	2	3	4	5 (IC50)
A	5-fu	0.127	0.380	1.140	3.420	10.260
B	Folnic Acid	0.054	0.161	0.484	1.451	4.353
C	Capecitabine	0.082	0.246	0.737	2.211	6.633
D	Irinotecan	0.010	0.030	0.090	0.269	0.807
E	Oxaliplatin	0.107	0.321	0.962	2.887	8.660

(B)	Factor					readout (cell viability)	
run	A	B	C	D	E	cocktail chip	Convent -ional
1	5	2	5	3	2	42.3	38.7
2	5	3	5	5	4	12.79	13.4
3	1	4	4	4	5	14.5	14.2
4	3	3	5	5	4	10.3	8.7
5	4	4	1	1	2	42.5	47.2
6	5	3	1	2	3	23.1	19.2
7	5	5	5	5	5	11.7	14.2
8	1	4	2	4	4	14.5	11.3
9	3	2	4	1	1	37.2	32.1
10	2	5	5	1	3	20.4	23.4
11	5	2	3	2	2	33.2	38.3
12	5	1	4	2	2	44.5	40.2
13	1	1	2	5	2	36.5	40.8
14	3	5	5	4	5	10.8	9.3
15	3	4	3	3	4	22.1	27.5

With this, we calculated and plotted half maximal inhibitory concentration (IC_{50}) as shown in (Fig. 5), in which the revealed IC_{50} values for anticancer drugs were significantly higher in 3D culture systems than 2D culture systems.

This result is mainly owning to the more drug resistance from the dense multicellular/multilayer structures in 3D cell spheroids. In this structure, naturally synthesized extracellular matrix promotes strong cell-to-cell interactions, migration, Ion transfer and cell-to-cell communication.[53] Which in turn may have caused significant retardation of drug penetration potential in to the core region of 3D cell spheroid. However, Folinic acid and 5-FU are the two protein based drugs we have used in our experiment. Actually, Folinic acid is marketed in the name of Leucovorine, which doesn't absorb UV light and so, it will not undergo further photolysis.[59] Also, experimental results (M. L. Pascu. *et.al.*) supports that, there will not be a structural change in 5-FU by irradiating with Hg lamp either N_2 laser beam in the UV spectral range. [60,61] Also, according to our experiment results IC50 values are similar (within 5% deviation) for all the 5 drugs by either the traditional pipetting way or the UV hydrogel method, which is suggesting that the similar effectiveness of both the methods in drug delivery. But, in our approach we used very less chemicals (drugs and medium) and rapid drug releasing is achieved by comparing to conventional way.

Released Drug Concentration Test

To understand the drug releasing rate from the UV-cured PEGDA hydrogels, we used 3 different concentrations (5, 10 and 20 w%) of PEGDA hydrogel with different UV curing timings (15, 30 and 45 Seconds). The Graphical representation in (Fig. 6(A)) shows the releasing rate of 3 different concentrations (5, 10 and 20 wt %) of PEGDA hydrogels with different UV curing times (15, 30 and 45 seconds). From the figure one can easily design the releasing rate and time period on demand by adjusting proper curing and concentration conditions of hydrogels.[54-56] Out of all the values, 5% hydrogel with curing time of 15, 30 and 45 Seconds were found to have greater drug releasing capacity i.e. nearly 80%. Therefore, we used 5% hydrogel and 15 Seconds curing time as a standard condition for test a releasing rate of 5 different drugs and the result is shown in (Fig. 6 (B)). The drug releasing rate is defined as the number of molecules released per second from the hydrogel in to the solution. The percentage ratio between the released concentrations to the loaded concentration gives the amount of drug released in to the medium. The releasing rate depends on the molecular weight of each drug. These values will be a calibration base for assigning drug releasing dose in the rest of the experiments in cocktail designs. Additionally, each drug has its own releasing rate (time constant) and effectiveness (IC50 value), and those basic properties have been carefully calibrated in Fig. 6 and Table 1 (A) and 2 (B) in this study, respectively. Therefore, the final cocktail releasing is normalized by these two factors to ensure the correct final real releasing dose in table 1 (B) and 2 (B). So both the

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releasing speed and drug effectiveness have been taken care at the same time in this study.

Cocktail Drug Testing

Once we have the IC_{50} value and releasing rate data of all 5 drugs, we can perform the cocktail fractional search algorithm to find out the optimized cocktail drug combination against colon cancer cells. Table I(A) shows the cocktail drug testing on 2D cell cultures. Each code (1, 2, 3, 4 and 5) on the top of the tables corresponds to the actual concentration of the 5 different drugs. Here, code 5 represents IC_{50} value of individual drugs and the concentration of the rest code will be one third of the previous one respectively. Drugs consist of various concentrations corresponding to the five different codes were used for further cocktail drug combination for drug screening. Table II(A) is also replication of the same process for 3D cell culture system. The remaining tables, Table. I(B) and II(B) are illustrate the 15 experimental runs with different permutation combinations of cocktail drug concentrations to test on both 2D and 3D cell cultures respectively. The values represents the cell viability for each cocktail testing result. Interestingly, run#7 (in red) indicated the applied dose of each drug combinations are at their highest value, but its efficiency in words of cell viability was worse than run#14 (green coloured) drug combinations with much lower total dosages. We can observe the same results in both 2D and 3D culture systems. In addition, cell viability and concentration of drugs is little higher in 3D cell culture than 2D cell culture system, as the drug penetration/inhibition issue mentioned earlier for 3D culture. We use the lowest survival rate (local minimum) of the drug testing result for defining the end point. To make sure this end point is correct, 5-6 more runs than the 14 runs was performed, which makes the end point clear at 14 runs. Therefore, we conclude that the run#14 (green coloured) is the best combination of drug cocktail to treat colon cancer in 2D or 3D culture system. At the same time, we also compared the cell viability obtained by a conventional technique and our technique. The readout (conventional) represents the viability of cells treated with drugs alone in conventional microtiter plates by direct drug pipetting process, serving as a reference data. Our Results are very much analogous to those by a conventional method. Importantly, 8-10 combinatorial assays can be performed by using our single UVgel-DrugChip. For more assays, several chips can be employed sequentially to accommodate the final desired numbers.

Conclusions

In summary, we have developed and optimized a simple method by employing UV selectable hydrogel droplets for cocktail drug screening (UVgel-DrugChip) against colon cancer. With the advantage of UV sensitive hydrogel, precise drug-loaded hydrogel droplet arrays were formed just by simply rolling a drop over wettability contrast surface. Those drug droplets can be further selected by UV-curing process of the hydrogel droplets for high dynamic range dosage assignation. In more words, the method is rapid and compatible with both adhesive (2D) and suspension (3D) cells system and does not require expensive or complicated fabrication and operations. We combined 5 drugs with 1000 folds dynamic range in 30 seconds with minimum drug consumption. We also examined and confirmed that the cell viability of 2D and 3D cultures searched by the employment of UVgel-DrugChip is quite close to the cell viability by the conventional drug screening method. The current platform provide a better operation of cocktail drug testing in three aspects over the traditional petri dishes ones: (1) Much less wastage of drugs during the drug testing (Now a days, in conventional way drug usage is 1-10 µl. But, our drug chip containing only 12 nL/drop. So, with this 10 fold of drug can be saved)., (2) More accurate drug releasing time/dosage control, (3) Reduce the labouring individual dosage preparation and drug-well registration issues from pipetting process (10-15 mins. Is needed for conventional 96 well experiment. In contrast, 1-2 mins. Is more than enough for our platform), and (4) Capable for programmed delay-time releasing process for each drug. Although this chip is still in the early stage of development, but with further advanced arrangements on different curing times or concentrations, the chip may have the potential for multiple drug releasing not only in precise dosage control, but also releasing time pace assignation, which will open up a new opportunity for advanced drug screening processes.

Acknowledgements

This work was financially supported by the Ministry of Science and Technology (MOST) of Taiwan under grant number MOST 104-2221-E-007-072-MY3, 104-2321-B-007-003, 103-2321-B-007-004 and Excellence University Centre Project for Biomedical Technology, NTHU, 2012-2015. Thank you very much to Dr. Helen Chang for grammatical correction.

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