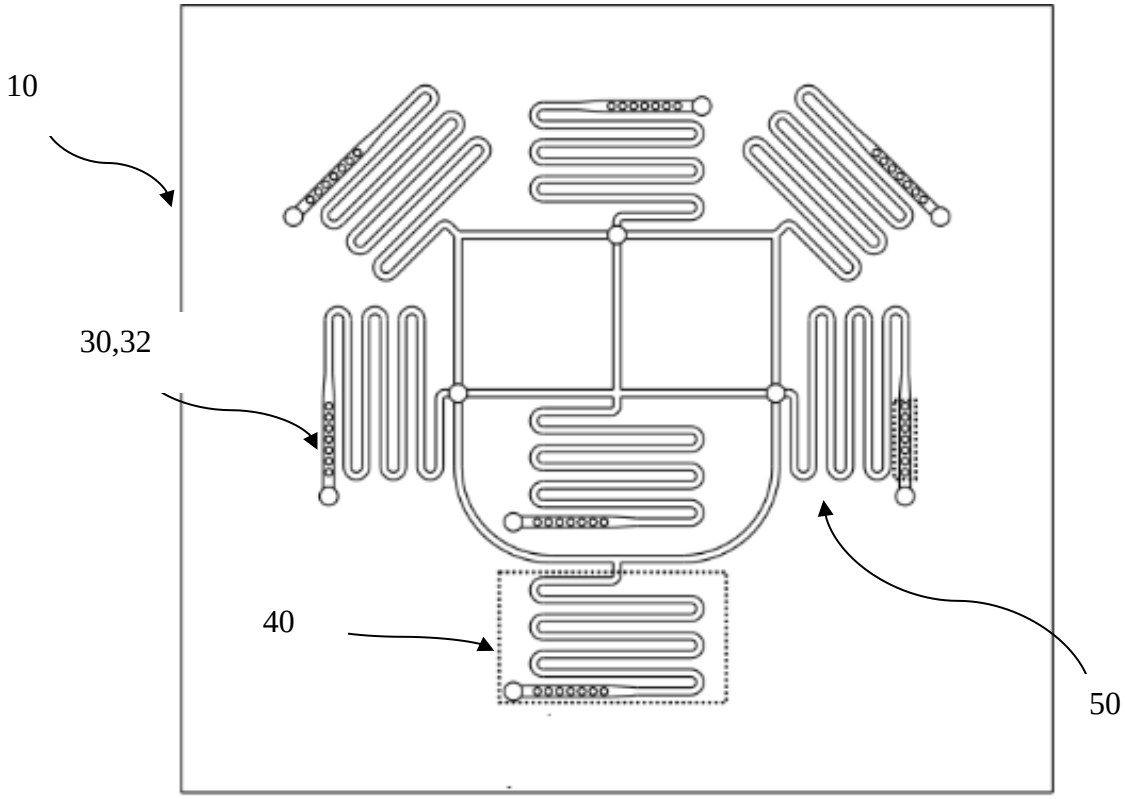


Figure 1a

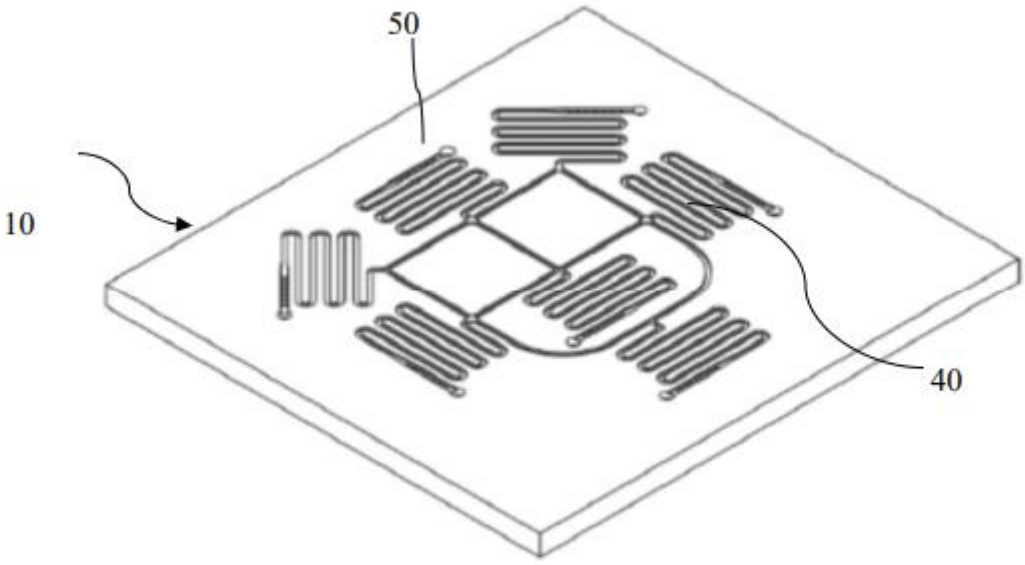


Figure 1b

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

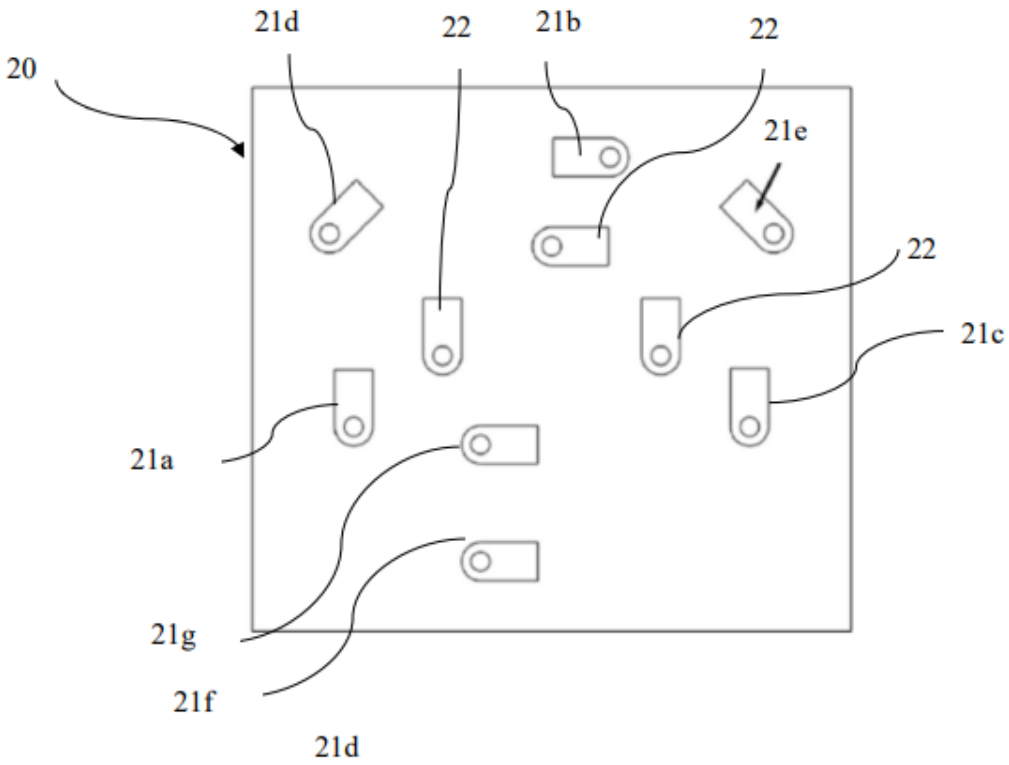


Figure 1c

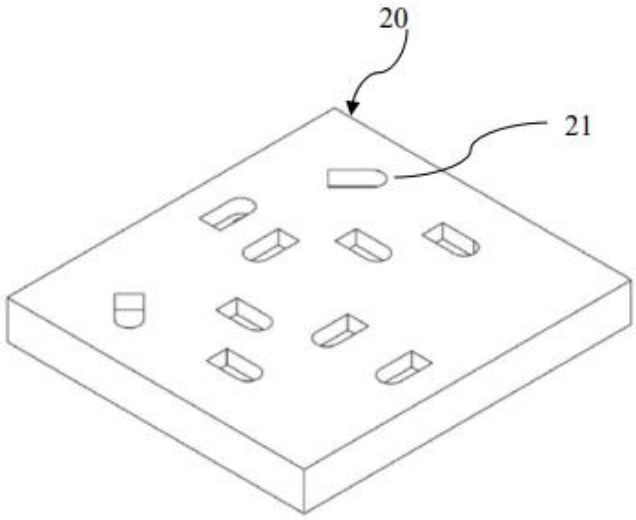


Figure 1d

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

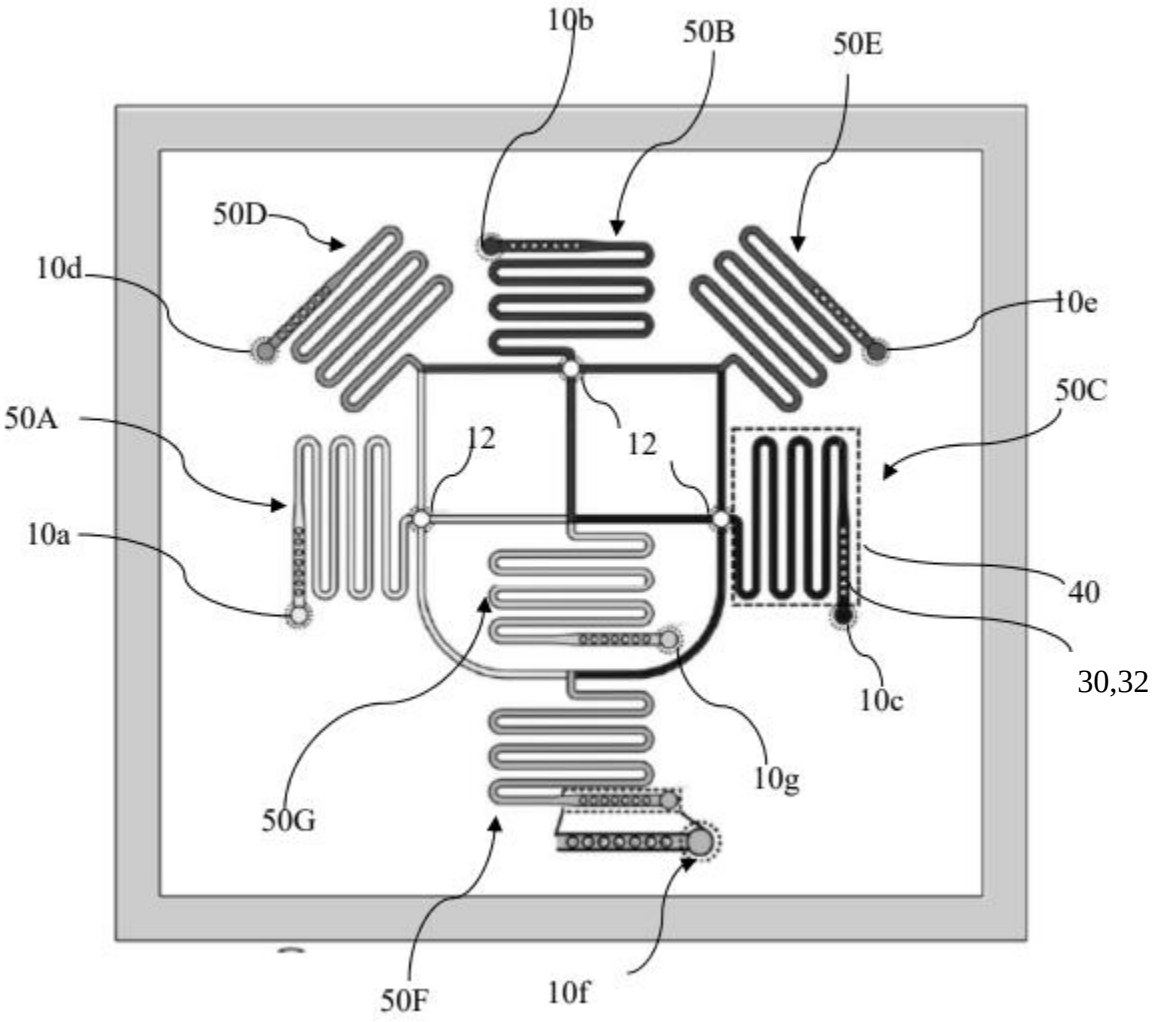
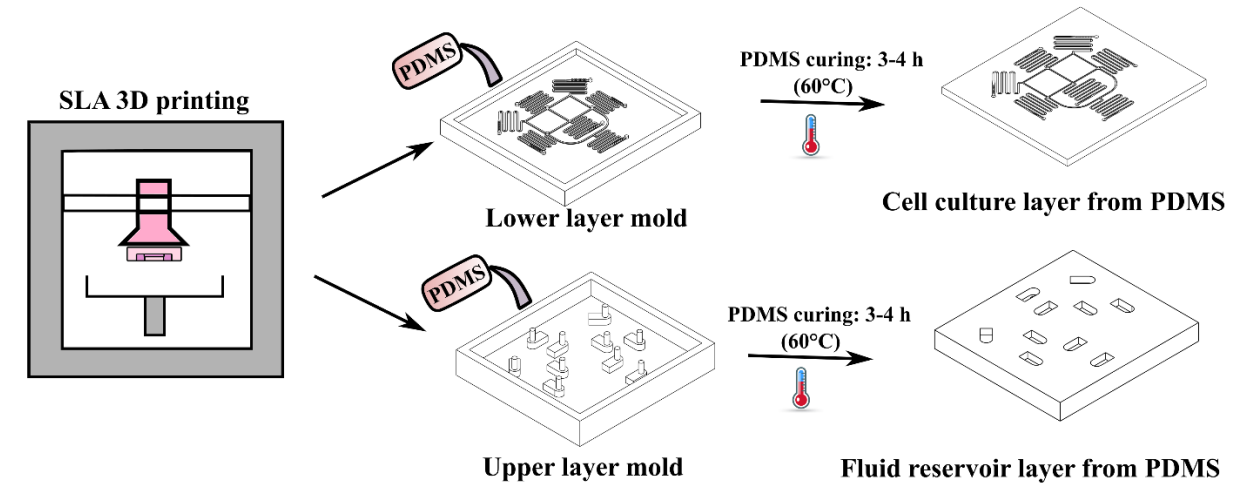
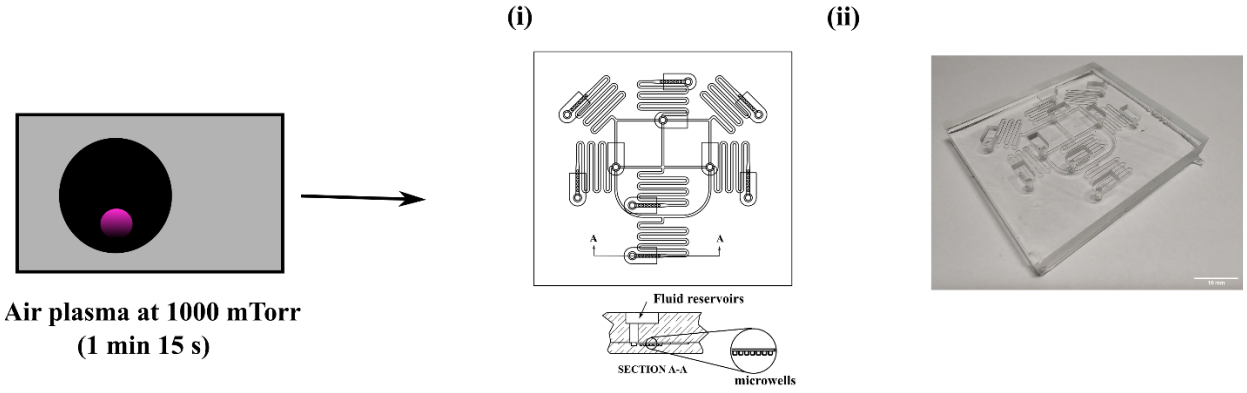
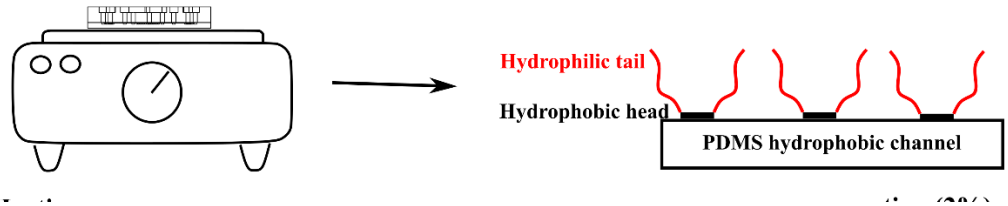


Figure 2

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606







M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

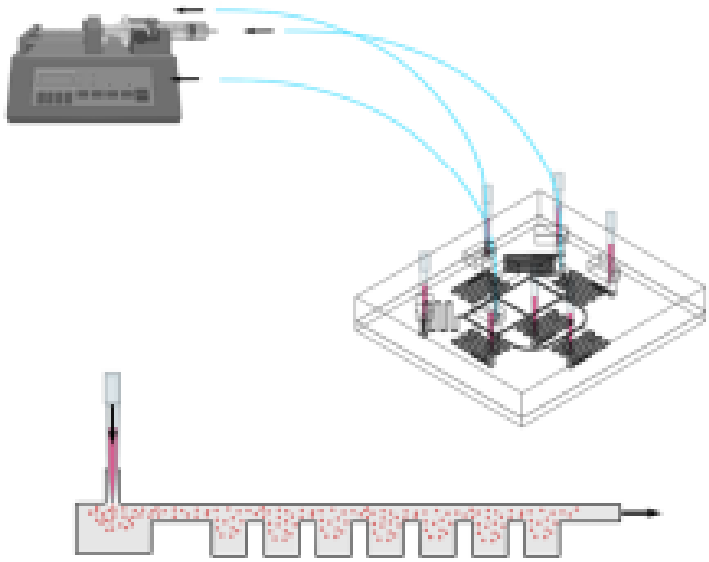


Figure 4 (a)

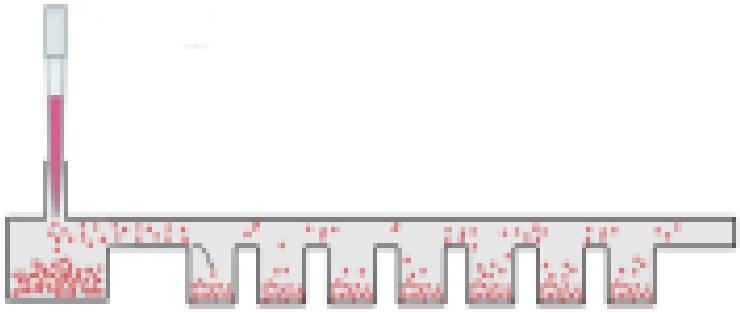


Figure 4 (b)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

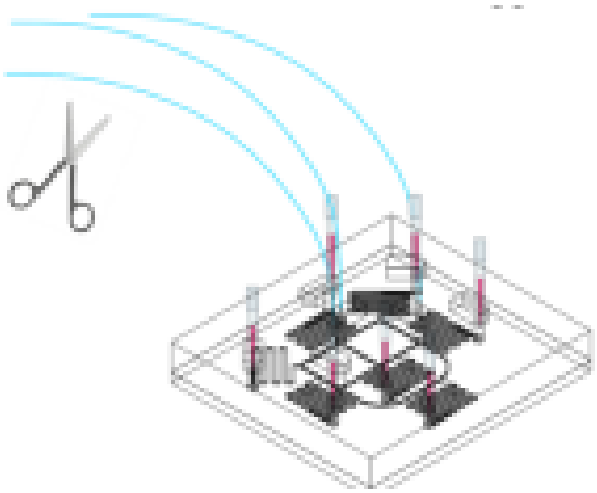


Figure 4 (c)

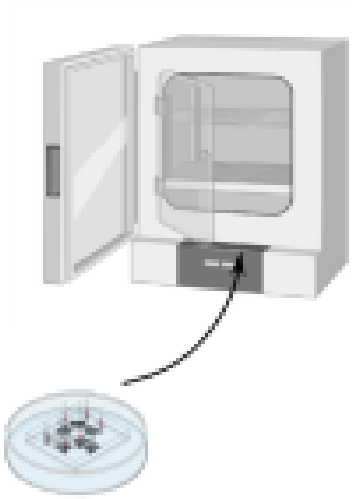


Figure 4 (d)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

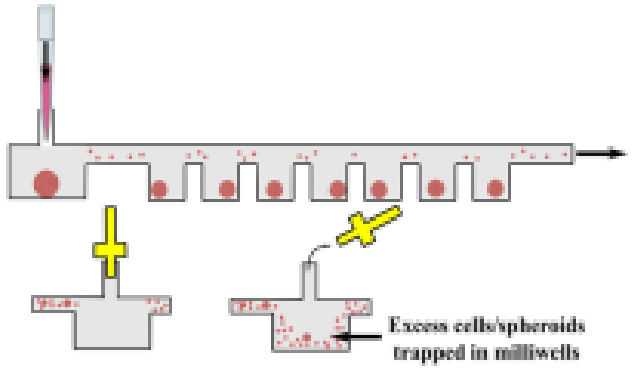


Figure 4 (e)

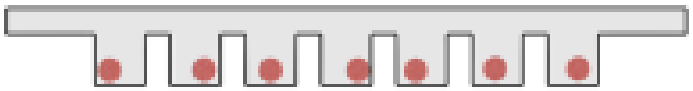


Figure 4 (f)

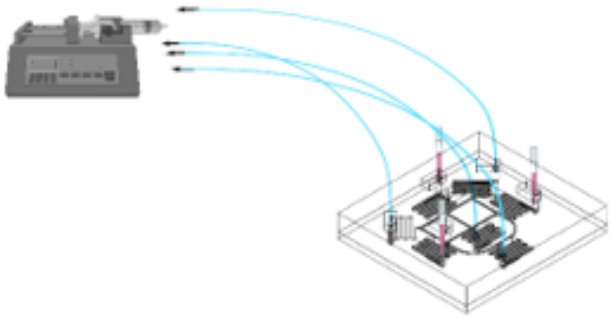


Figure 4(g)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

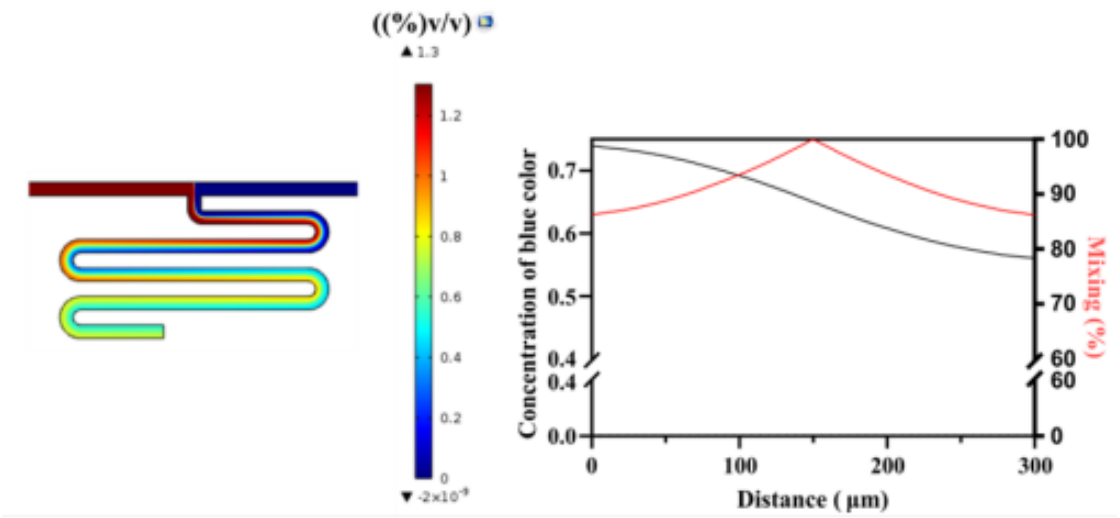


Figure 5 (a)

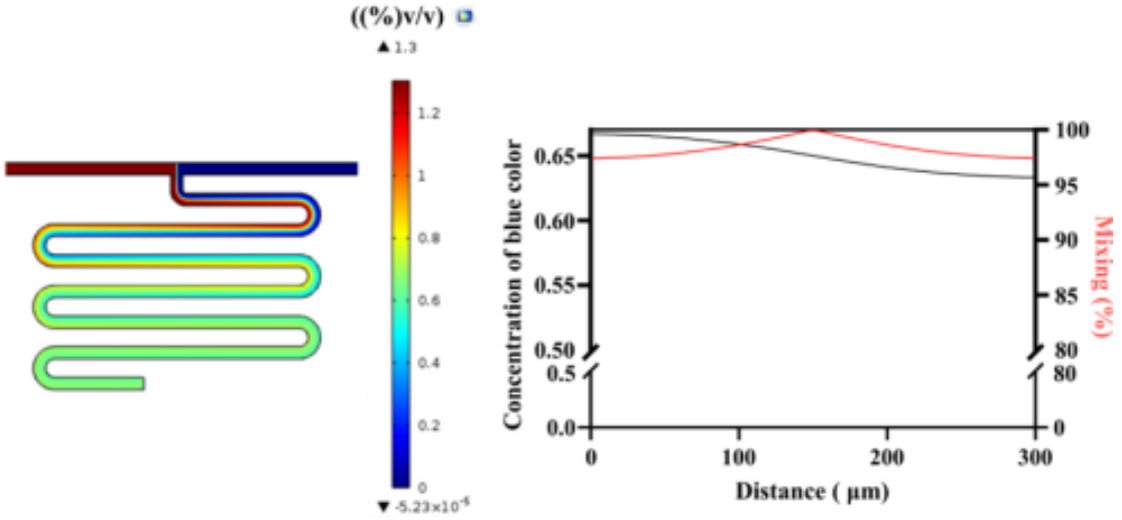
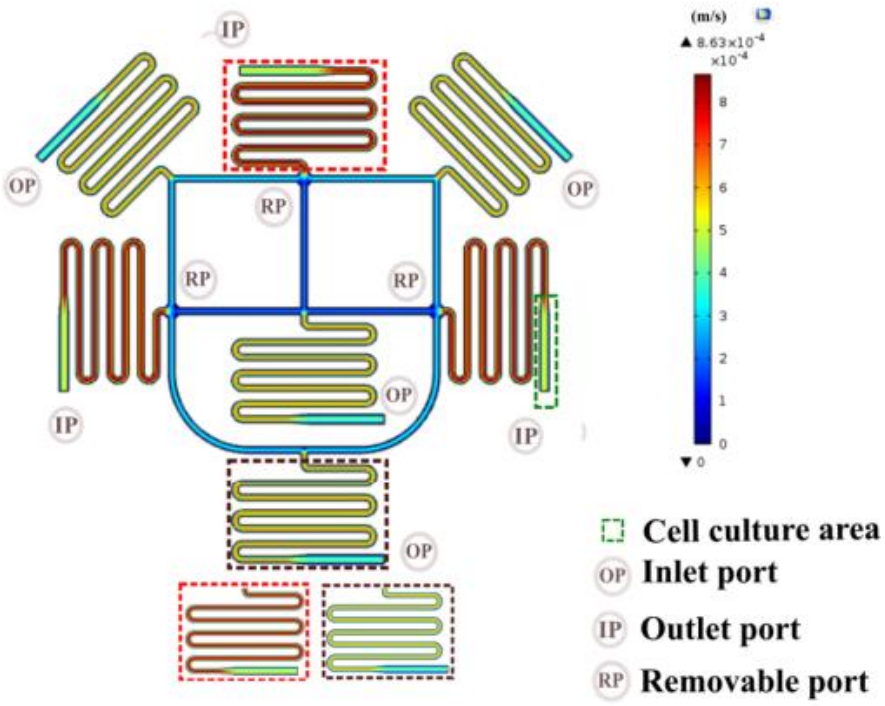


Figure 5 (b)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606



Parameter	Value (mm/s)
V_{max} in outlet cell culture area	0.4231
V_{max} in inlet cell culture area	0.561

Figure 5 (c)

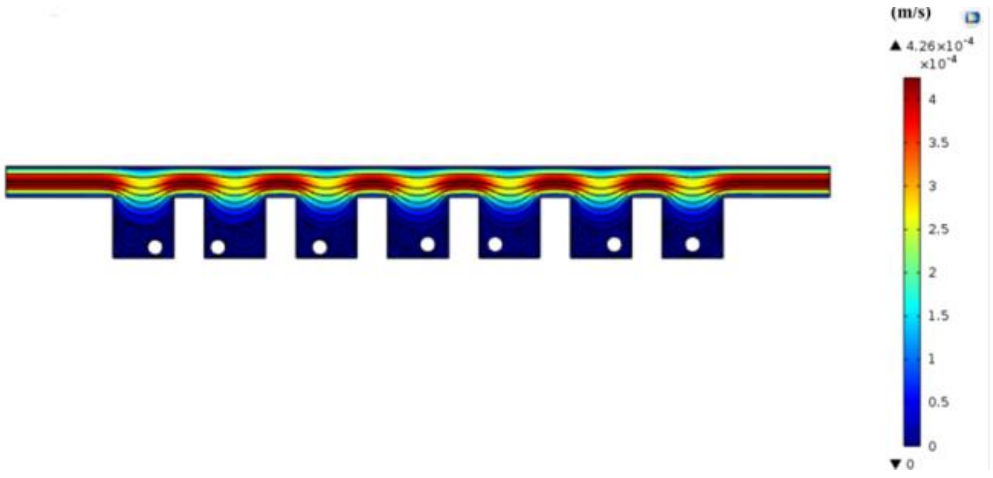


Figure 5 (d)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

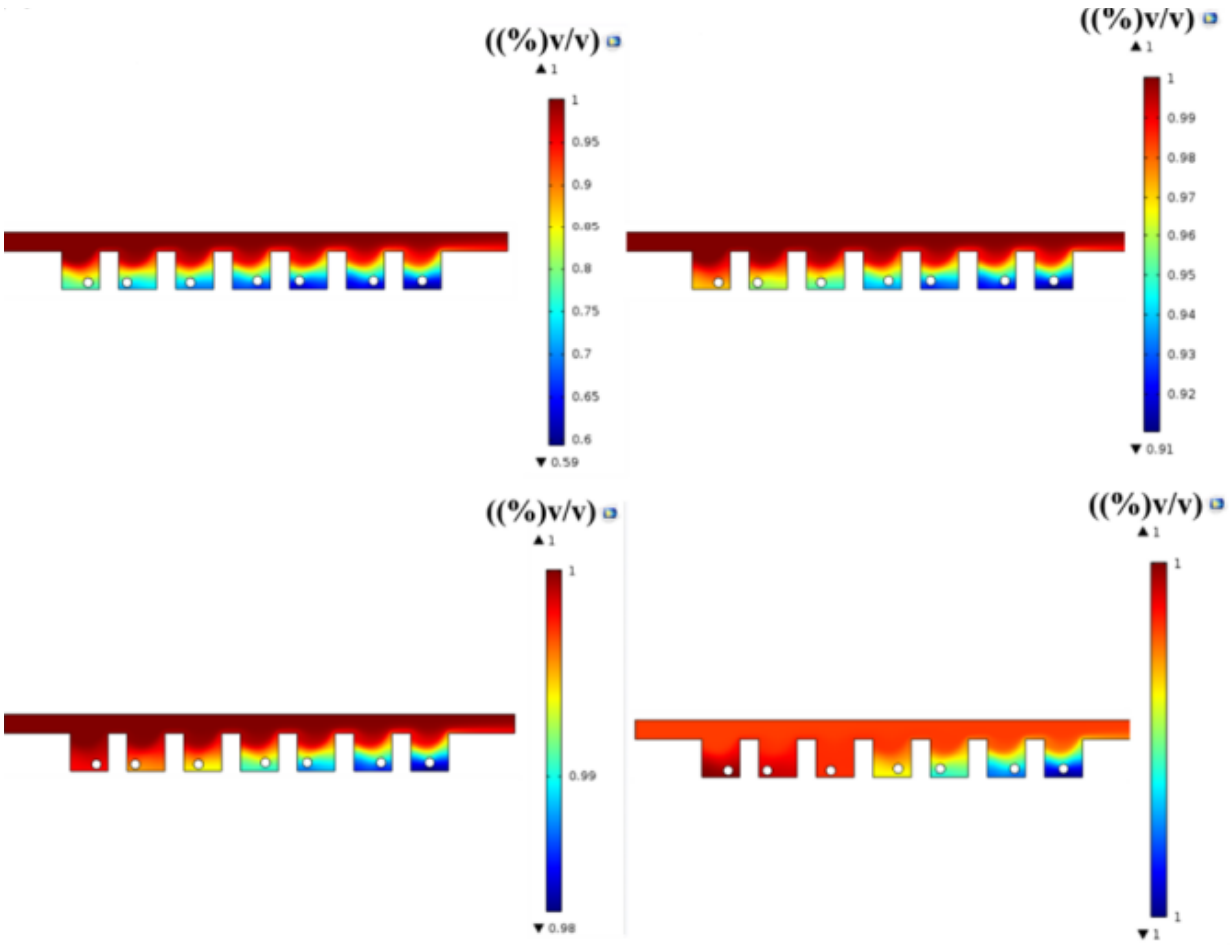


Figure 5 (e)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

100

Figure 6 (a)

100

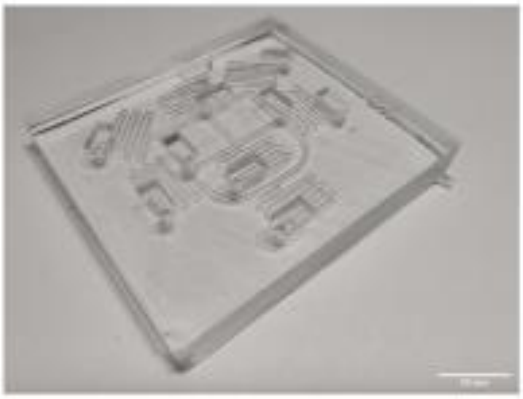


Figure 6 (b)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

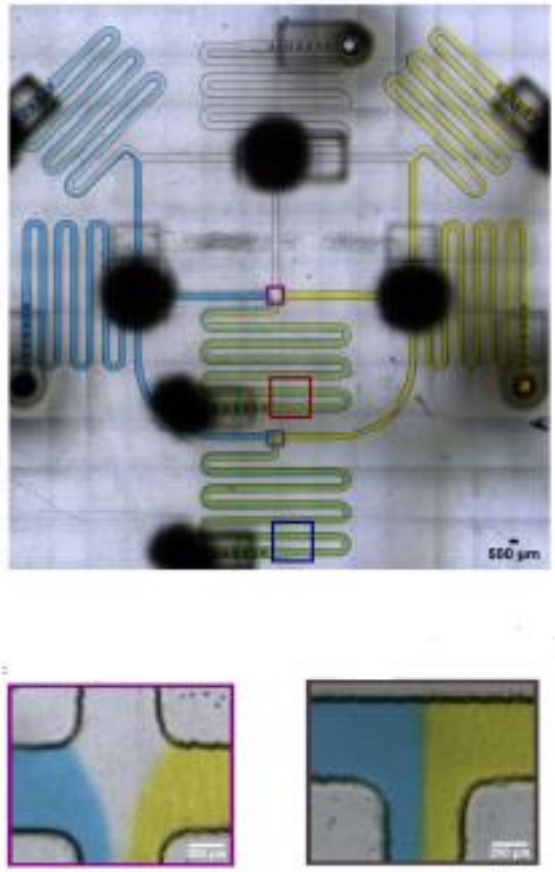


Figure 7 (a)

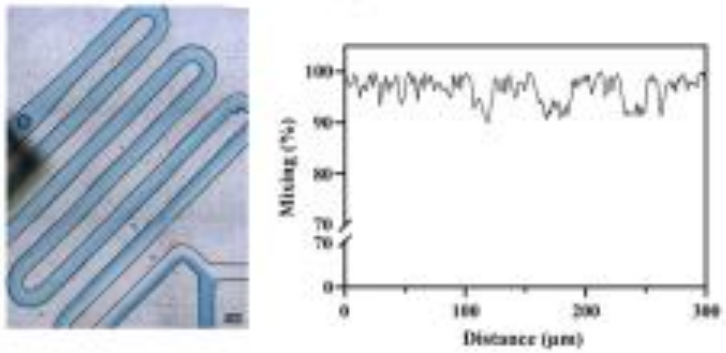


Figure 7 (b)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

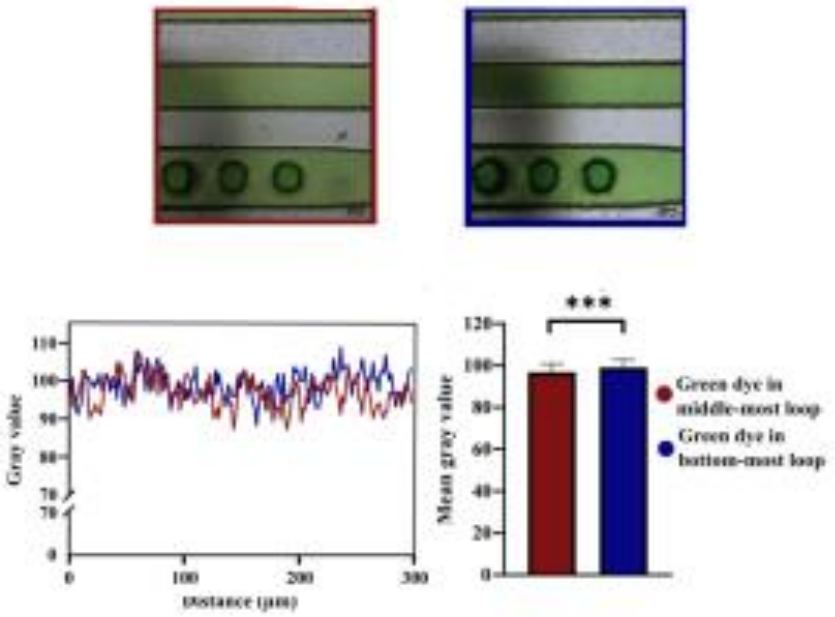


Figure 7 (c)

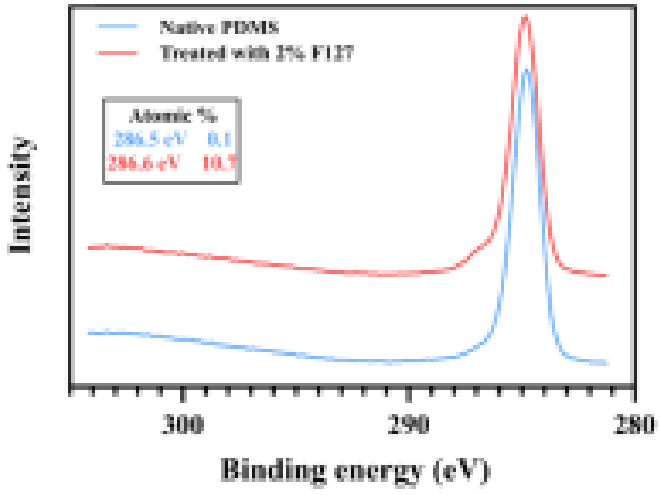


Figure 7 (d)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

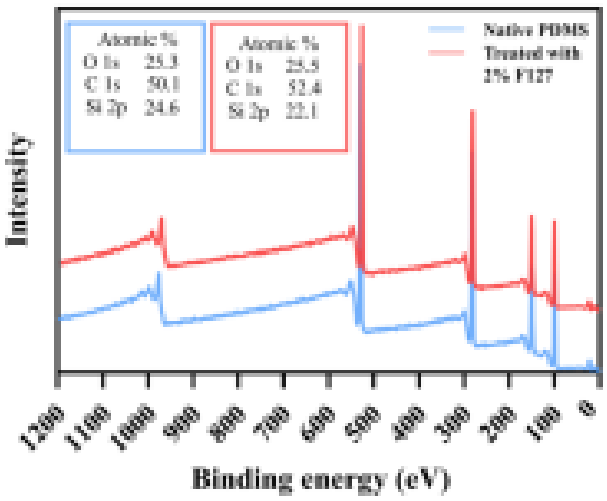


Figure 7 (e)

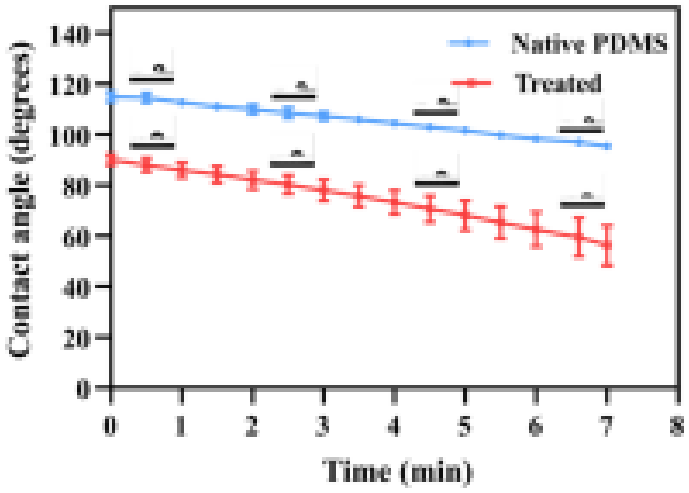


Figure 7 (f)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

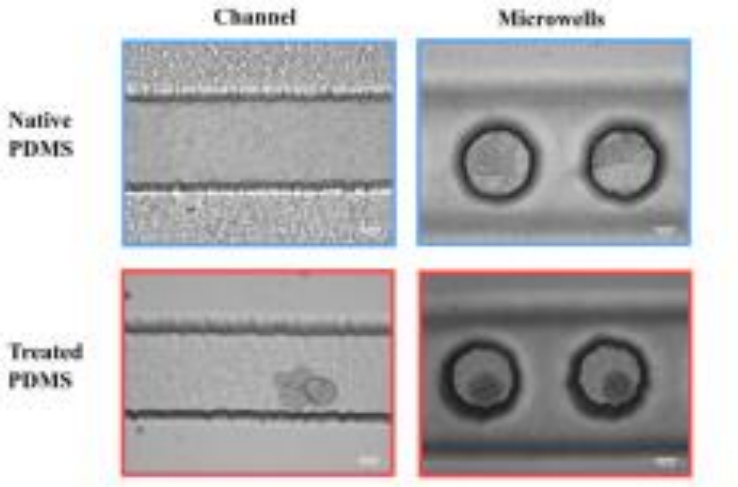


Figure 7 (g)

Figure 8 (a)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

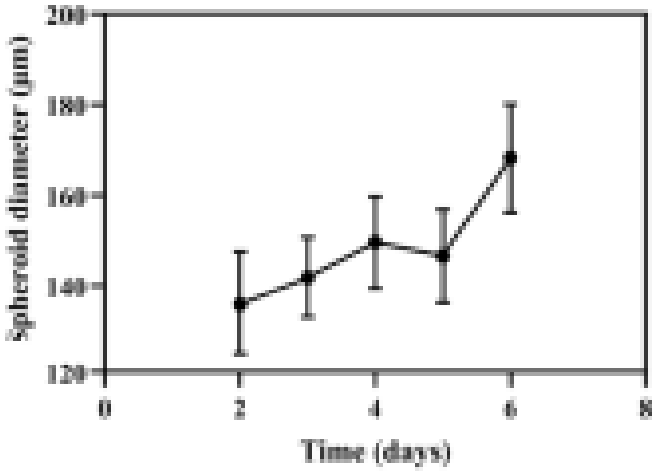


Figure 8 (b)

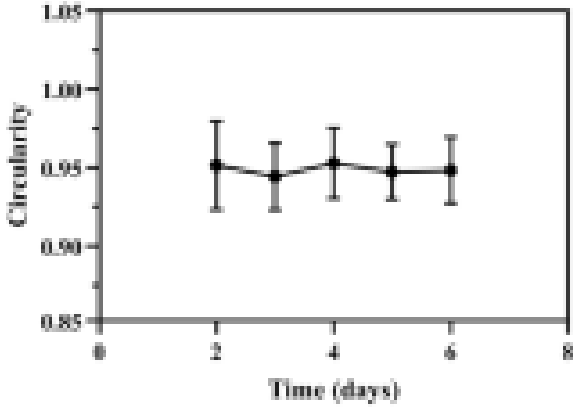


Figure 8 (c)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

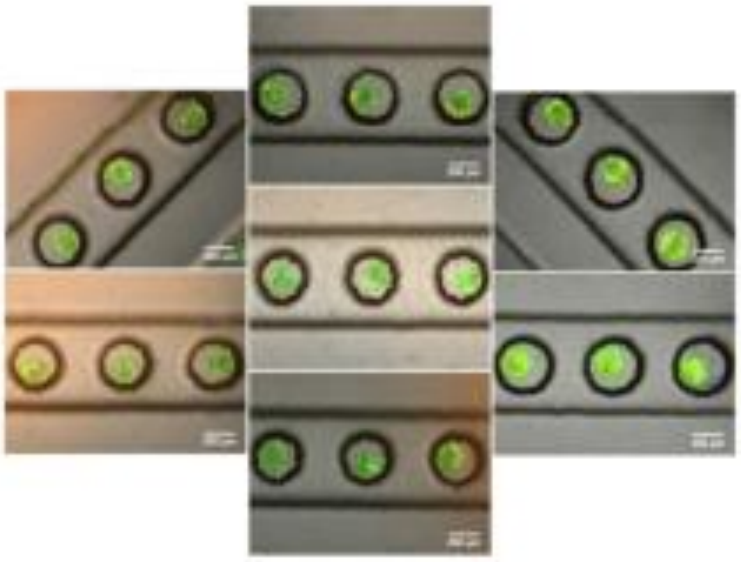


Figure 8 (d)

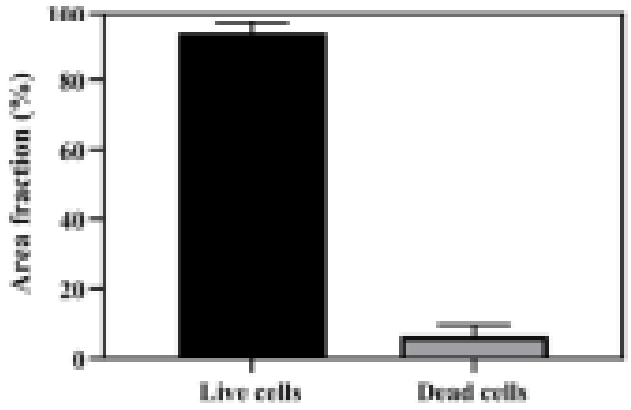


Figure 8 (e)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

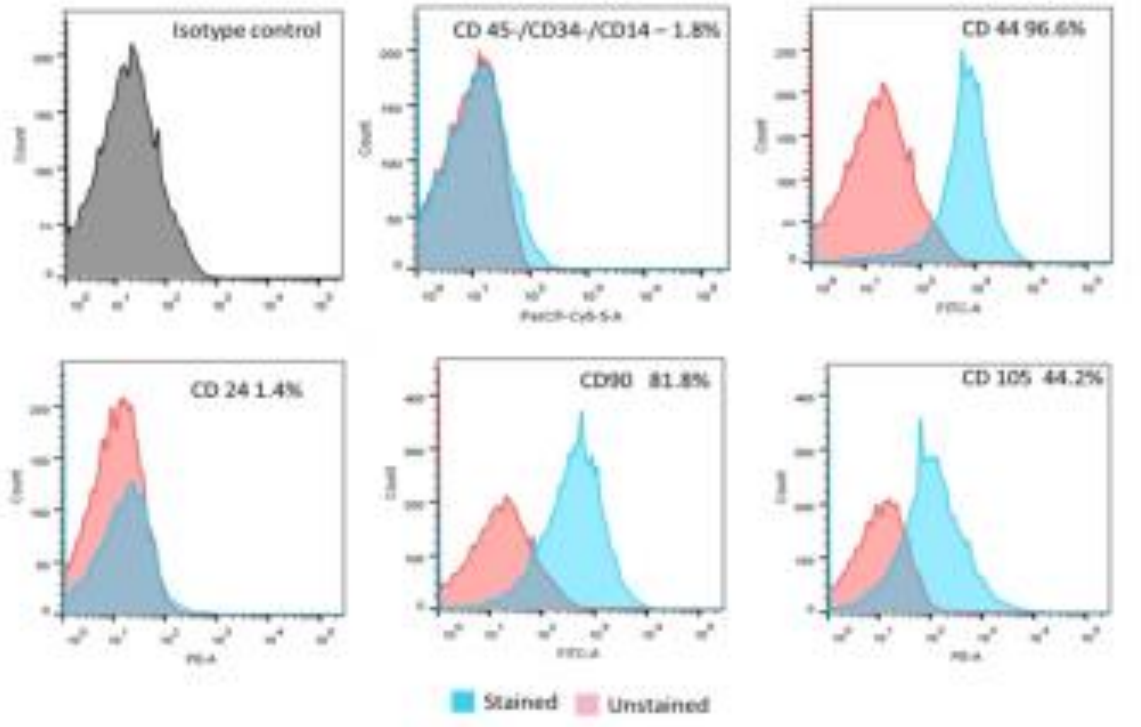


Figure 9

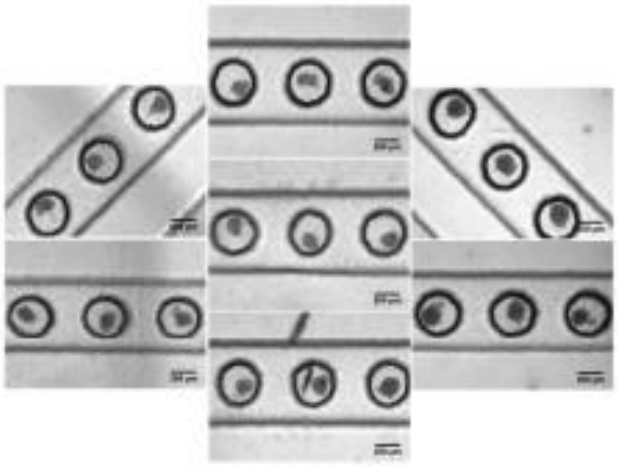


Figure 10 (a)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

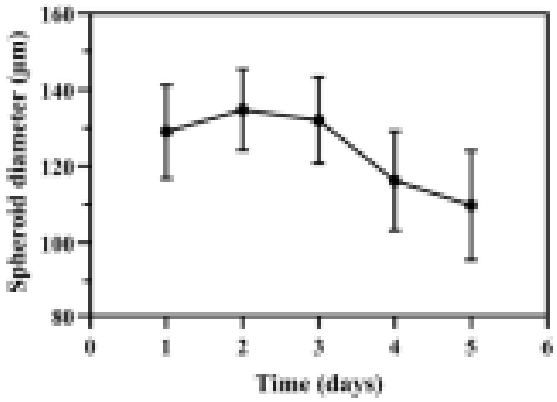


Figure 10 (b)

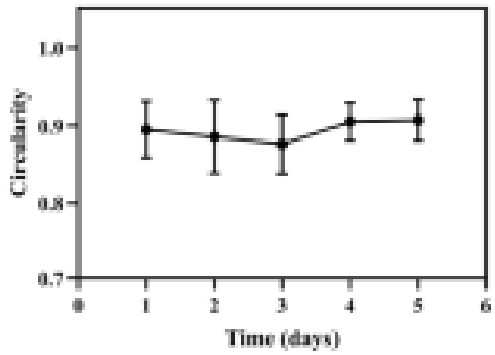


Figure 10 (c)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

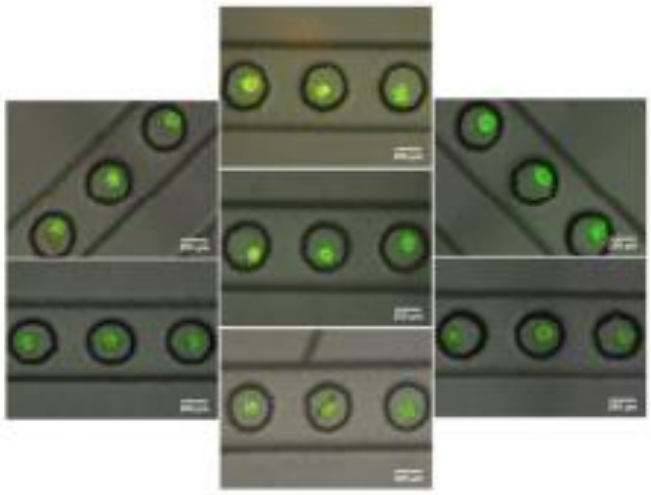


Figure 10 (d)

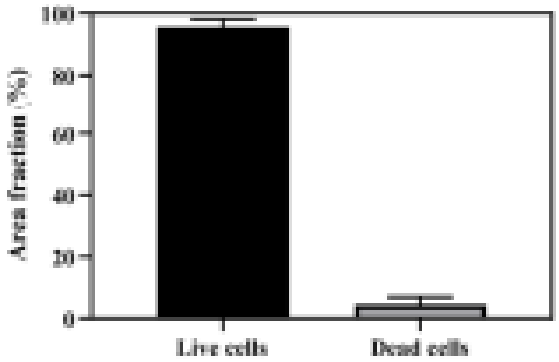


Figure 10 (e)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

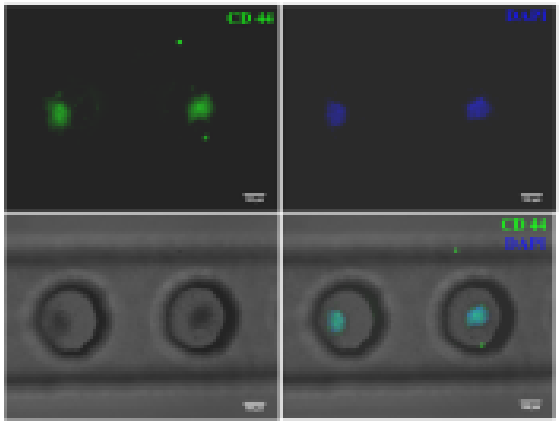


Figure 10 (f)

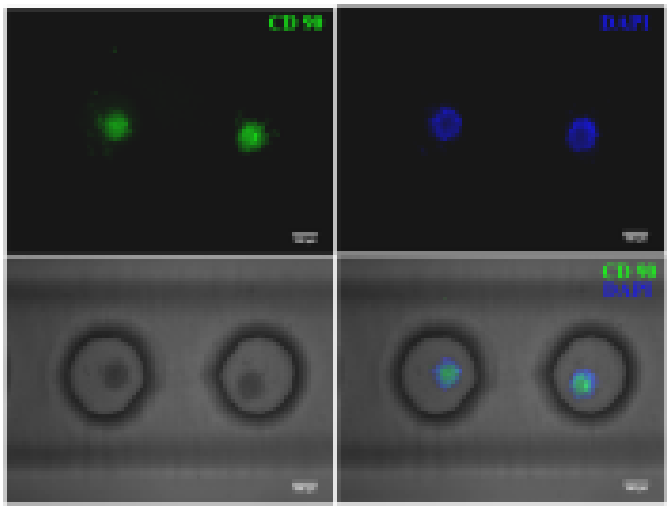


Figure 10 (g)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

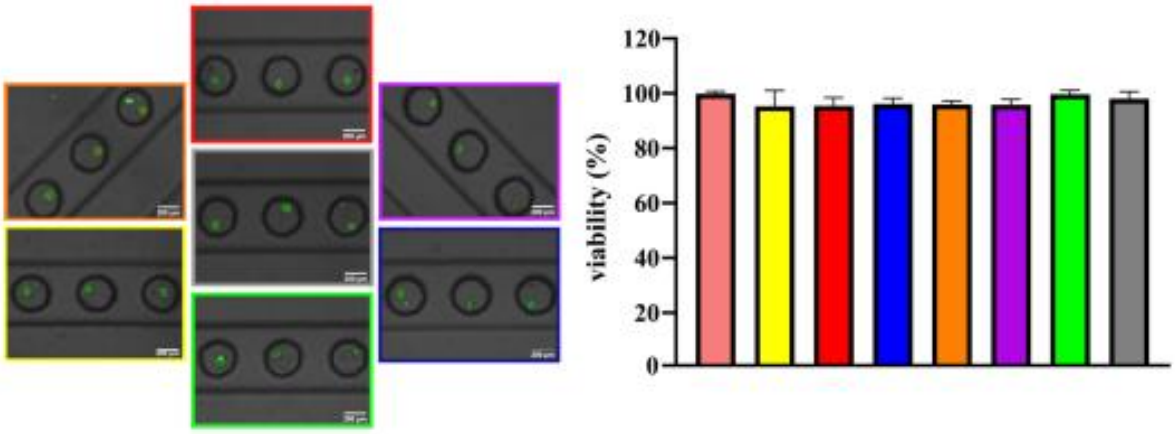


Figure 11 (a)

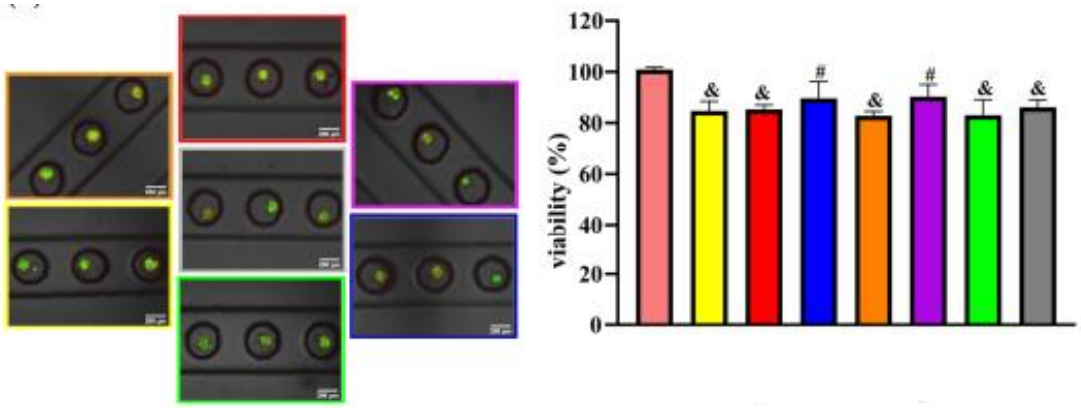


Figure 11 (b)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

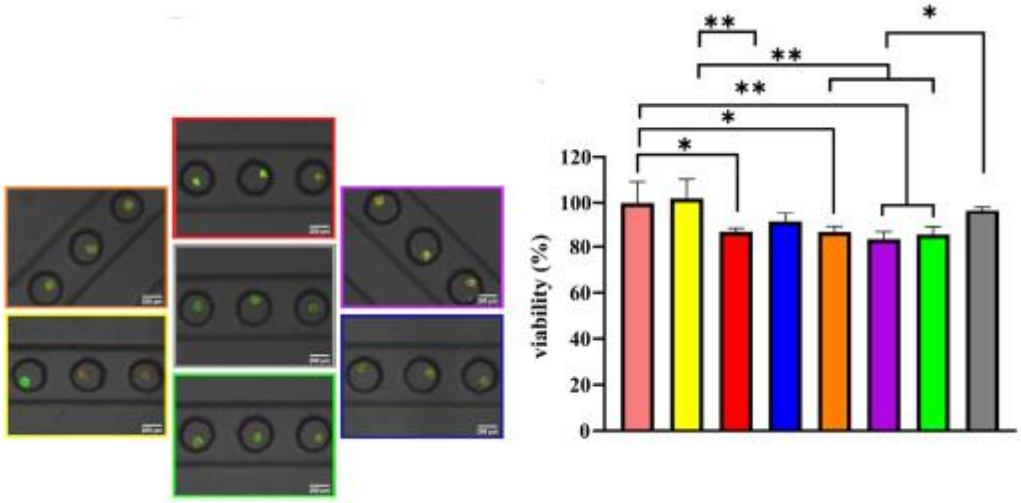


Figure 11 (c)

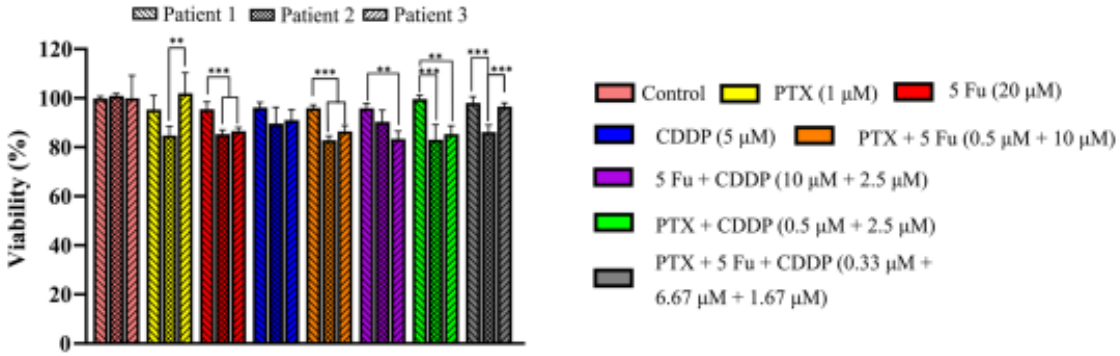


Figure 11 (d)

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

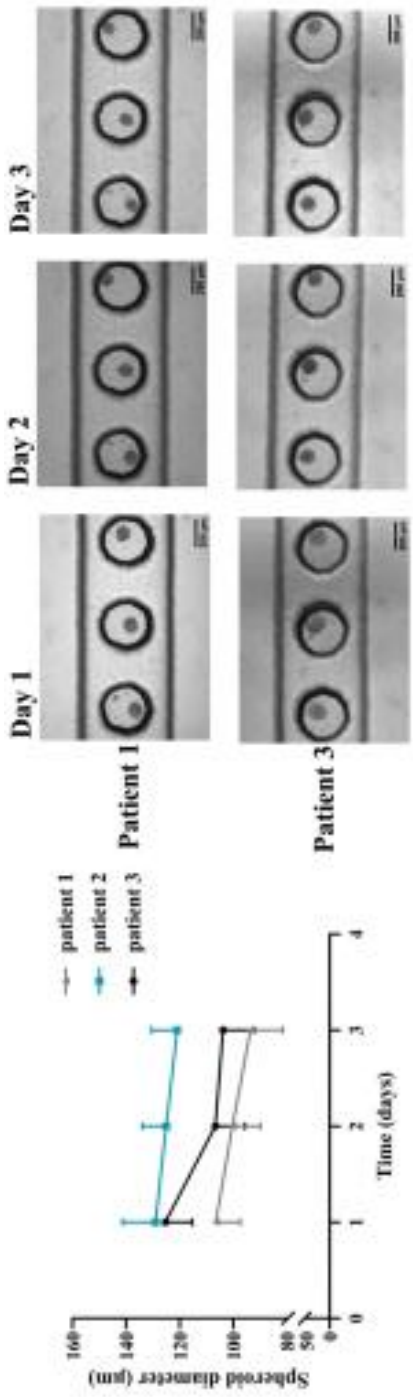


Figure 12

M. S. DEVI
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-1606

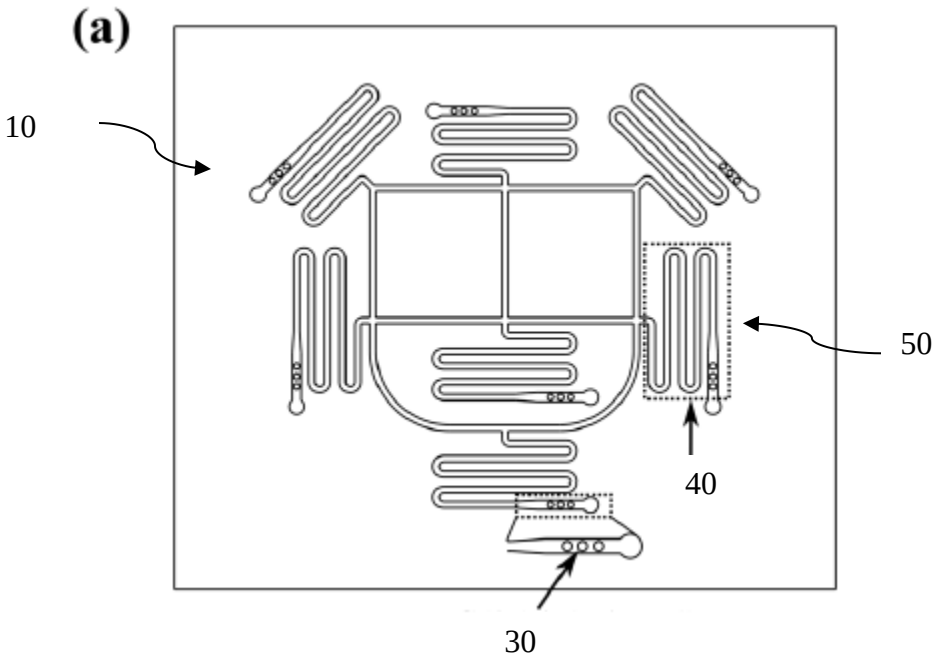


Figure 1a

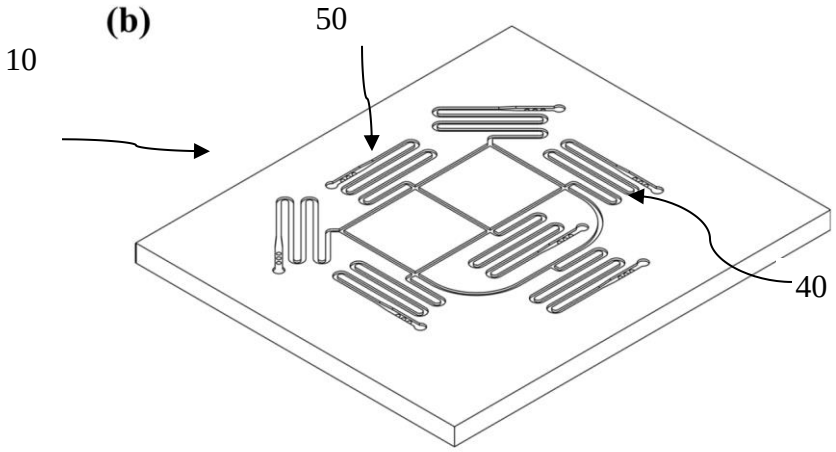


Figure 1b

Samrat Ganguly

SAMRAT GANGULY
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-2733

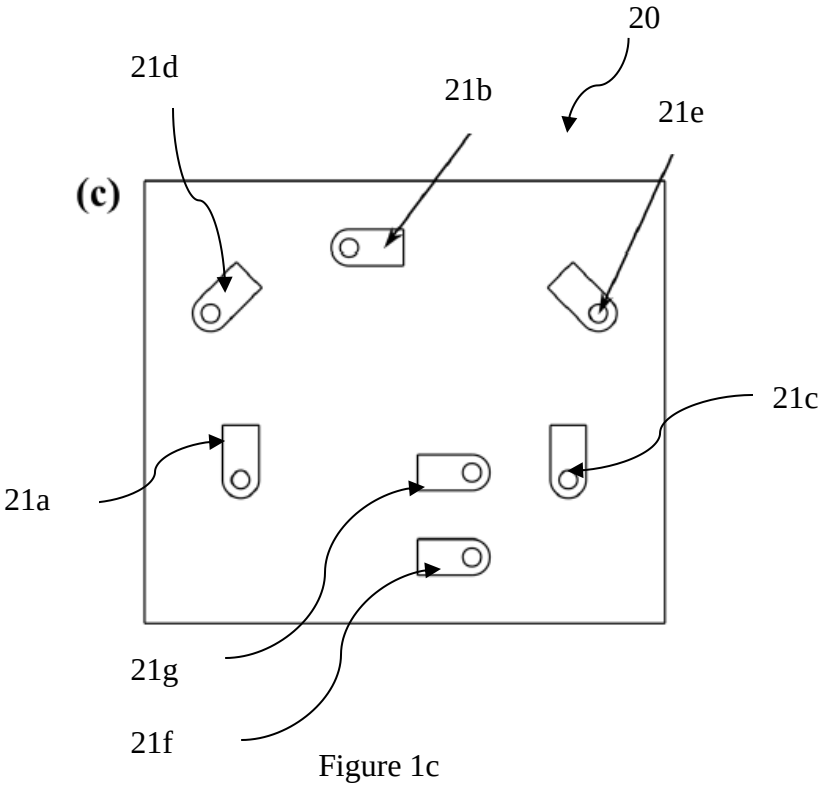


Figure 1c

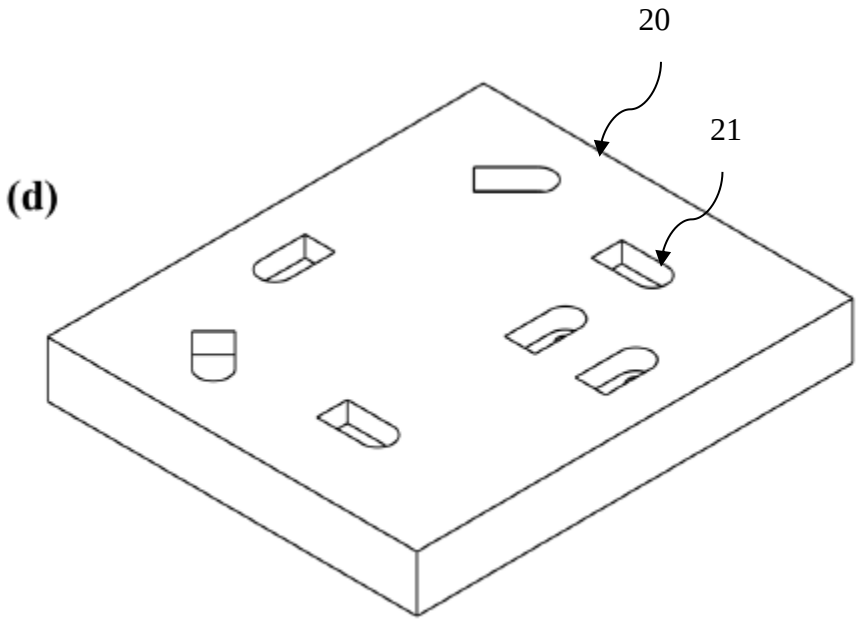


Figure 1d

Samrat Ganguly

SAMRAT GANGULY
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-2733

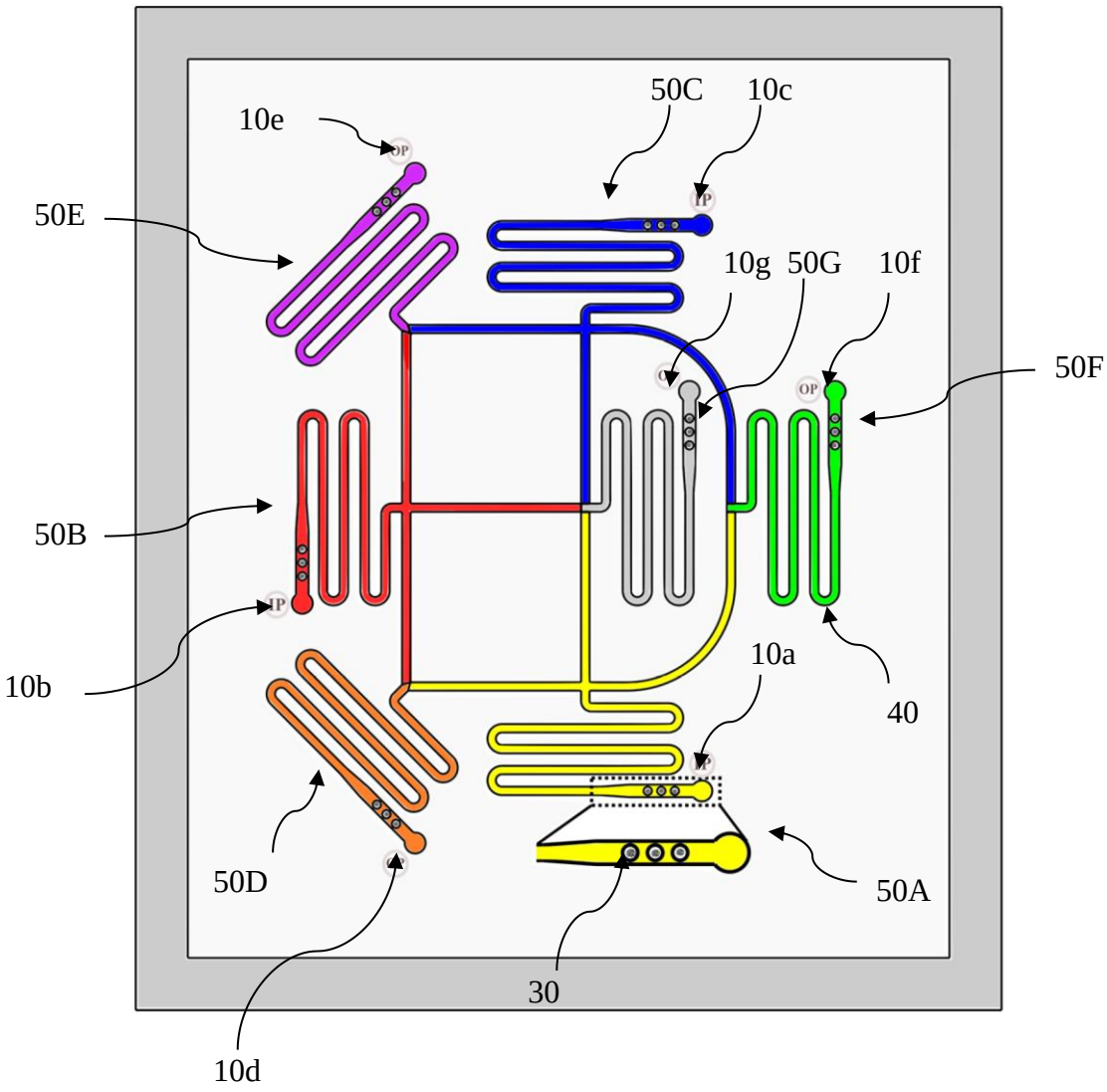


Figure 2

SAMRAT GANGULY
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-2733

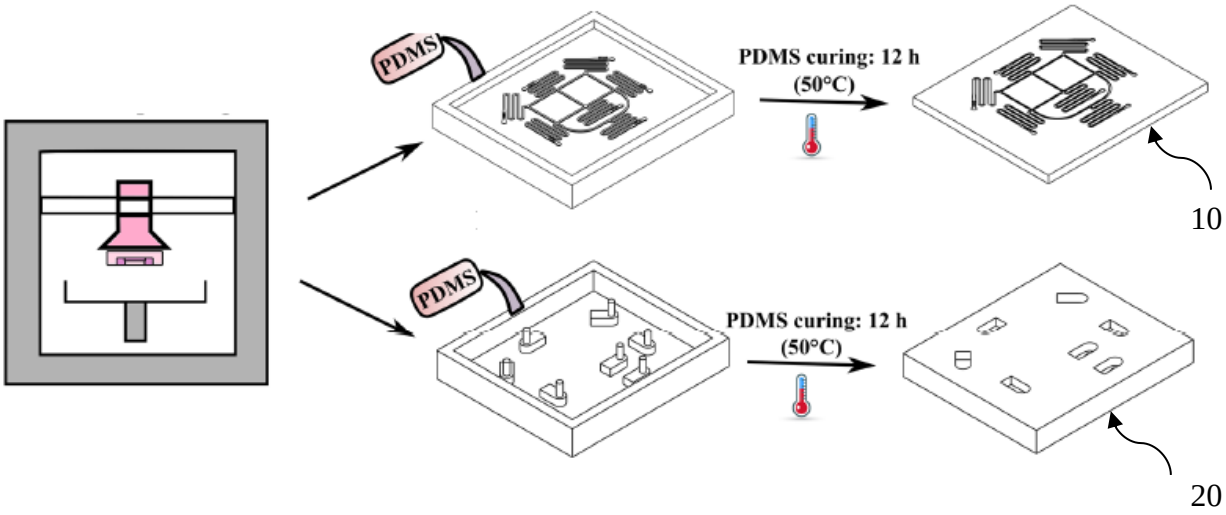


Figure 3a

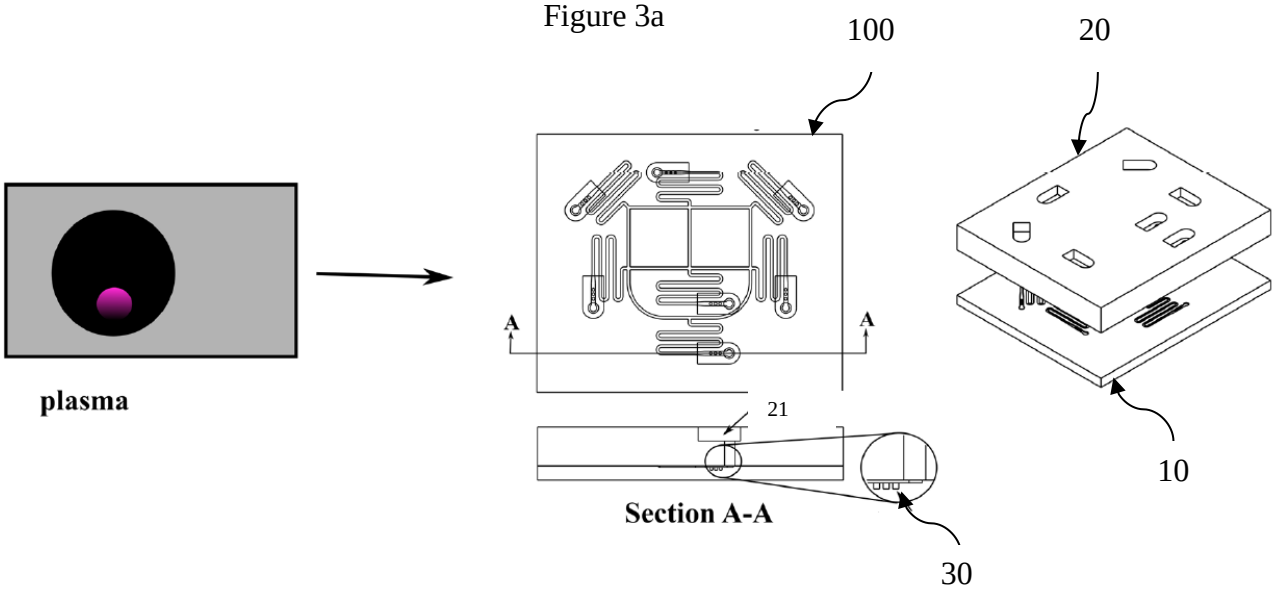


Figure 3b (i) Figure 3b (ii)

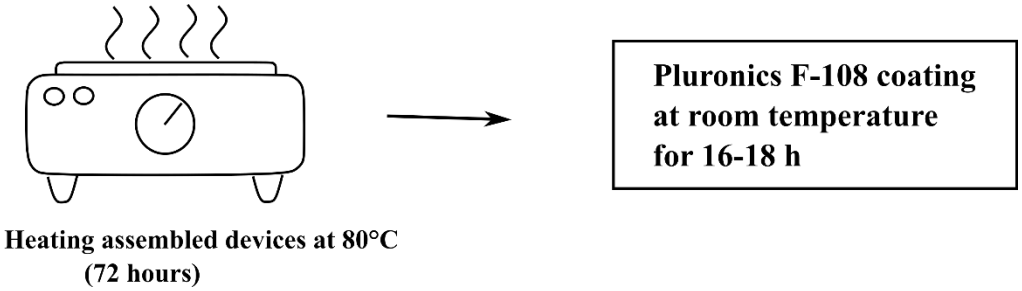


Figure 3c

Samrat Ganguly

SAMRAT GANGULY
OF K & S PARTNERS
AGENT FOR THE APPLICANT
IN/PA-2733

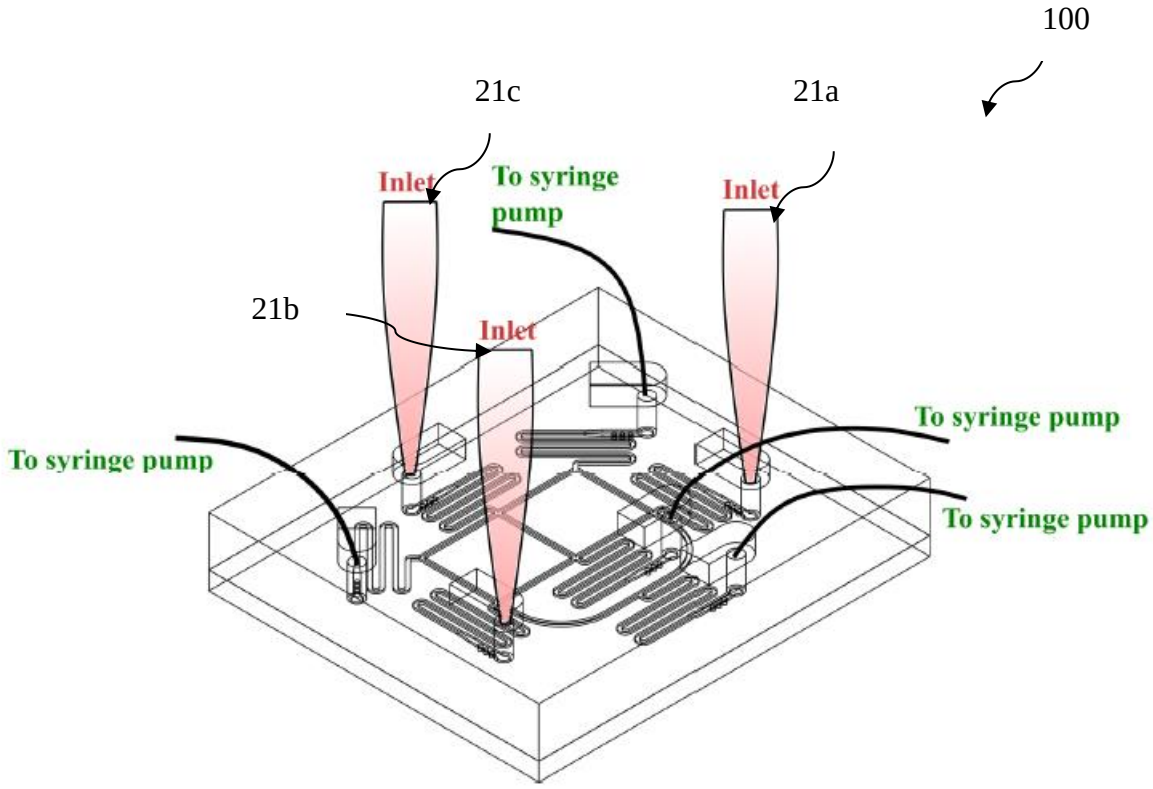


Figure 4

Samrat Ganguly

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FORM 2

THE PATENTS ACT 1970

[39 OF 1970]

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THE PATENTS RULES, 2003

COMPLETE SPECIFICATION

[See section 10 and Rule 13]

A MICROFLUIDIC DEVICE, METHOD OF FABRICATING AND ITS APPLICATION THEREOF

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The following specification particularly describes the invention and the manner in which it is to be performed.

FIELD OF INVENTION

The present invention pertains to the field of microfluidic platforms. More particularly, the invention relates to a microfluidic device and method of three-dimensional sample cell culturing and multi-drug testing using the device thereof.

BACKGROUND OF THE INVENTION

Cancer is a devastating disease that takes the lives of millions of people every year. Due to the heterogeneity in the underlying causes of cancer, chemotherapy with standardized drugs produces variable treatment outcomes and often results in treatment failure. This inherent variability of underlying causes of cancer has given rise to the development of personalized and precision medicine approach in which the diagnostic and therapeutic modalities being adopted are tailored for a specific patient. A popular approach in personalized and precision medicine for cancer treatment utilizes the identification of specific genetic mutations by DNA sequencing. Identification of specific genetic mutations can aid in selecting an initial treatment strategy, but it cannot predict the outcome of the treatment. Therefore, functional drug testing on patient-derived samples is necessary. Personalized functional drug testing is the assessment of various drug responses on the patient-derived samples. Besides guiding the initial therapeutic decisions, functional drug testing can also potentially predict the outcome of therapy.

One of the approaches for functional drug testing is *in vivo* based patient-derived xenograft models, which preserve the dynamic tumor microenvironment. However, they are expensive and take several months to develop. Thus, they cannot be used for guiding the initial treatment strategy. Further, two-dimensional well-plate based *in vitro* platforms have been used widely for testing drugs on patient-derived samples. However, such monolayer cell culture platforms cannot recapitulate important aspects of the native tumor microenvironment such as gradients of nutrients, pH, and oxygen. Further, the two-dimensional culture often results in underestimation of required drug concentration due to lack of cell-cell and cell-ECM (extracellular matrix) interaction. Additionally, 3D *in vitro* tumor culture based microfluidic platforms have emerged as an alternative to the aforementioned models. 3D culture-based microfluidic models generally include spheroid/organoid and hydrogel-based tumor culture. 3D cell culture-based microfluidic models

are more relevant for personalized functional drug testing as they retain some of the important aspects of tumor microenvironment such as (1) cancer stem cell population (2) drug resistance (3) cell-cell and cell-ECM interactions (4) gradients of nutrients, pH, and oxygen (5) tumor stroma by co-culture (6) necrotic cell population. However, such microfluidic devices are mostly static, limited by their single drug testing capability and their fabrication technique is expensive, time consuming, low-throughput, and incompatible with mass production. Due to the above disadvantages in the prior art, there is a need for the development of improved methods and fabrication technique for microfluidic based functional drug testing. Further, the conventional development of *in vitro* dynamic microfluidic platforms for personalized multi-drug combinatory drug testing, fails to overcome the aforementioned problems. Moreover, conventional platforms are not economical to manufacture.

Hence, the present disclosure is directed to solve one or more limitations stated above or any other limitations associated with the prior arts. Consequently, those skilled in the art will appreciate the present disclosure that provides many advantages and overcomes all the above and other limitations.

The information disclosed in this background of the disclosure section is only for enhancement of understanding of the general background of the invention and should not be taken as an acknowledgement or any form of suggestion that this information forms the prior art already known to a person skilled in the art.

SUMMARY

The one or more shortcomings of the prior art are overcome by a microfluidic device and a method of fabricating thereof as claimed and additional advantages are provided through the provisions as claimed in the present disclosure. Other embodiments and aspects of the disclosure are described in detail herein. The present disclosure provides a microfluidic device, a method of fabricating and its application thereof. A microfluidic device comprises a bottom layer defined with a network of microfluidic channels. The network of channels includes a plurality of primary channels, each defining a flow path for at least one fluid. Each of the plurality of primary channels includes an inlet end to receive the at least one fluid. Further, a plurality of secondary channels are provided each configured to receive a combination of fluids from at least two of the plurality of primary

channels. Each of the plurality of secondary channels is disposed at an intersection of at least two primary channels. Each of the plurality of secondary channels includes an outlet end to dispense the combination of fluids. The device further includes a top layer secured to the bottom layer covering the network of microfluidic channels. The top layer is defined with a plurality of inlet ports and outlet ports each in fluid communication with a corresponding inlet ends and outlet ends.

The present disclosure discloses a method of screening a drug or drugs of interest on a biological sample using the microfluidic device.

In an embodiment, the present invention also relates to use of microfluidic device for testing of various combinations of drugs on a biological sample.

In an embodiment, the network of microfluidic channels is defined with a segment of mixing path for premixing the at least one fluid.

In an embodiment, the mixing paths is a non-linear path comprising at least one of a curved, serpentine, and zig-zag path.

In an embodiment, each of the mixing path is provided with an array of microwells and atleast one milliwell.

In an embodiment, the microwells and milliwells have a bottom surface which aids in single spheroid formation.

In an embodiment, the top layer is provided with a plurality of auxiliary ports, each in fluid communication with each of the primary channels to remove air bubbles.

In an embodiment, each of the plurality of inlet ports, outlet ports, and the auxiliary ports are provided with a reservoir into which at least one fluid is introduced and collected.

In an embodiment, the device includes a plurality of syringe pumps, each in fluid communication with each of the reservoir for withdrawing and introducing at least one fluid.

In an embodiment, the bottom layer and the top layer are made from Polydimethylsiloxane (PDMS) elastomer.

The present disclosure also discloses a method of fabricating a microfluidic device. The method comprises initially mixing a Polydimethylsiloxane (PDMS) elastomer with a curing agent at a predefined mixing ratio to obtain a PDMS solution. Later, the PDMS solution is casted over moulds, and the PDMS solution forms the bottom layer and the top layer. Further, the PDMS solution in the moulds is cured at a first predetermined temperature for a first predefined duration. The bottom layer and the top layer are removed from the moulds. The removed bottom and top layers are treated with air plasma. Later, the top layer is secured on the bottom layer to form the microfluidic device. The secured and bonded device is coated with anti-fouling agent.

In an embodiment, the moulds are fabricated using additive manufacturing.

In an embodiment, the moulds are manufactured by a soft stereolithography 3D printing technique using a resin.

In an embodiment, the fabricated moulds are post-processed by initially washing the moulds in isopropyl alcohol (IPA) and later curing the washed moulds at a second predetermined temperature for a second predefined duration.

In an embodiment, the second predetermined temperature is 40°C-80°C, and the second predefined duration ranging about 10-60 minutes.

In another embodiment, the predefined mixing ratio ranges between 5:1 to 15:1(w/w).

In an embodiment, the moulds are manufactured by a resin which is methacrylate based transparent photosensitive resin.

In an embodiment, the first predetermined temperature is 50°C-80°C, and the first predefined duration ranges about 3 to 24 hours.

In an embodiment, the prepared bottom and top layers are treated with air plasma at pressure ranging between 600-1000 mTorr, power 10-200 W for 5-120 sec.

In an embodiment, the top layer is secured to the bottom layer by bonding and heating at 50-150°C for 1-72 hours.

In an embodiment, the method comprises coating the bonded bottom layer with the anti-fouling agent Pluronic block copolymer 0.1 – 10%(w/v) agent for 1-48 hours.

Additional features and advantages are realized through the techniques of the present disclosure. Other embodiments and aspects of the disclosure are described in detail herein and are considered a part of the claimed disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features and characteristics of the disclosure are explained herein. The embodiments of the disclosure itself, however, as well as a preferred mode of use, further objectives and advantages thereof, will best be understood by reference to the following detailed description of an illustrative embodiment when read in conjunction with the accompanying drawings. These figures have been provided by way of illustration and not by way of limitation. One or more embodiments are now described, by way of example only, with reference to the accompanying drawing in which:

Figure 1(a) illustrates a top view of a bottom cell culture layer of a microfluidic device of the present disclosure;

Figure 1(b) illustrates a perspective view of the bottom cell culture layer of the microfluidic device of Figure 1(a);

Figure 1(c) illustrates a top view of a top fluid reservoir layer of a microfluidic device of the present disclosure;

Figure 1(d) illustrates a perspective view of the top fluid reservoir layer of the microfluidic device of Figure 1(c);

Figure 2 illustrates the bottom cell culture layer comprising an array of microfluidic channels for multi-drug testing with inlet, outlet, and auxiliary ports of Figure 1(a);

Figure 3 illustrates a schematic view of method of fabrication of the microfluidic device of the present disclosure;

Figure 4 illustrates method of use: 4(a) cell seeding with a syringe pump 4(b) static phase for cells to settle down in microwells 4(c) silicon tube removal from chip 4(d) static incubation for 24 h 4(e) disconnection of tube luers from chip to capture excess cells and spheroid 4(f) Microwells without multiple spheroids in flat microwells 4(g) Drug perfusion scheme.

Figure 5 illustrates design validation of the device through computational simulations: 5**(a)-** Mixing simulation in short mixing paths. **5(b)-** mixing simulation in mixing paths of 57 mm. **5(c)-** velocity profile in channel network. **5(d)-** velocity profile and streamlines in microwells. **5(e)-** transport of drugs into microwells with time.

Figure 6(a) illustrates bottom layer mold fabricated by 3D printing soft-stereolithography;

Figure 6(b) illustrates assembled device used for multi-drug testing;

Figure 7 illustrates device characterization: **7(a)** brightfield image of chip in dynamic phase at 2 µl/min.

Figure 7(b) illustrates characterization of mixing (%): (i) brightfield image of blue dye (1.3% v/v) in dynamic phase (ii) variation in mixing (%) along a line at the end of mixing path

Figure 7(c) illustrates dissimilar green dye solutions: (i) brightfield image of green dye in middle-most mixing path (ii) brightfield image of green dye in bottom-most mixing path (iii) gray value variation along a line at end of mixing path (iv) mean gray value comparison of plotted values along a line.

Figure 7(d) illustrates X-ray photoelectron spectroscopy (XPS) C1s spectra of native and coated PDMS devices.

Figure 7(e) illustrates survey scan of native and coated PDMS

Figure 7(f) dynamic contact angle analysis of native and coated PDMS samples.

Figure 7(g) illustrates MCF-7 culture on coated and native PDMS devices.

Figure 8 illustrates biocompatibility evaluation of device using breast cancer cell line: **8(a)-** Brightfield image of spheroids on day 3; **8(b)-** spheroid size variation with time; **8(c)-** spheroid

circularity with time; **8(d)-** live/dead staining on day 6; **8(e)-** quantification of live/dead staining on day 6. Spheroid size, circularity, and viability have been reported as Mean ± SD. Only uniform sized tumor spheroids have been considered for analysis.

Figure 9 illustrates flow cytometry analysis of isolated population;

Figure 10 illustrates 3D primary oral tumor spheroid culture (a) brightfield image of spheroidspheroids in chip on day 3 **Figure 10(b)** illustrates spheroid size variation with time; **Figure 10(c)** illustrates spheroid circularity with time; **Figure 9(d)** illustrates live/dead staining on day 5; **Figure 10(e)** illustrates quantification of live/dead staining on day 5;

Figure 10(f) illustrates Immunofluorescence with CD 44;

Figure 10(g) illustrates Immunofluorescence with CD 90. This representative data is from patient 2 derived tumoroids;

Figure 11 illustrates Live/dead staining after drug testing: **11(a)** patient 1 spheroids; **11(b)** patient 2 spheroids; **11(c)** patient 3 spheroid; 11**(d)-** inter-patient comparison. (data normalized to control). ('&' shows $p<0.001$ with control, '#' shows $p<0.05$ with control); Graphpad Prism software was used to perform a one-way analysis of variance (ANOVA) followed by Tukey's post hoc to check the significance level. Significance was determined by $p<0.05$ (*), $p<0.01$(**), $p<0.001$ (***) Spheroid size, circularity, and viability have been reported as Mean ± SD. Only uniform sized tumor spheroids have been considered for analysis.

Figure 12 patient-specific spheroid size variation with time.

The figures depict embodiments of the disclosure for purposes of illustration only. One skilled in the art will readily recognize from the following description that alternative embodiments of the structures and methods illustrated herein may be employed without departing from the principles of the disclosure described herein.

DETAILED DESCRIPTION

At the very outset, it may be understood that the ensuing description only illustrates a particular form of this invention. However, such a particular form is only an exemplary embodiment, without intending to imply any limitation on the scope of this invention.

In the present document, the word "exemplary" is used herein to mean "serving as an example, instance, or illustration." Any embodiment or implementation of the present subject matter described herein as "exemplary" is not necessarily to be construed as preferred or advantageous over other embodiments.

While the disclosure is susceptible to various modifications and alternative forms, specific embodiment thereof has been shown by way of example in the drawings and will be described in detail below. It should be understood, however that it is not intended to limit the disclosure to the forms disclosed, but on the contrary, the disclosure is to cover all modifications, equivalents, and alternative falling within the spirit and the scope of the disclosure.

The terms “comprises”, “comprising”, or any other variations thereof, are intended to cover a non-exclusive inclusion, such that a setup, device or process that comprises a list of components or steps does not include only those components or steps but may include other components or steps not expressly listed or inherent to such setup or device or process. In other words, one or more elements in a system or apparatus proceeded by “comprises… a” does not, without more constraints, preclude the existence of other elements or additional elements in the system or apparatus.

Embodiments of the present disclosure relates to a microfluidic device. The microfluidic device of the present disclosure facilitates 3D cell culture of a sample efficiently and test combination of a plurality of drugs to determine the most effective drug combination and dosages for providing precision medical care. Generally, conventional microfluidic devices are mostly static, limited by their single drug testing capability and their fabrication technique is expensive. Moreover, the conventional microfluidic devices for *in vitro* personalized multi-drug combinatory drug testing, are expensive to manufacture.

Accordingly, the present disclosure discloses a microfluidic device 100 [alternatively referred as device 100]. The device 100 comprises a bottom layer 10 and a top layer 20. The bottom layer 10

is defined with network of microfluidic channels 50 comprising a plurality of primary channels 50A, 50B, 50C to receive at least one fluid and a plurality of secondary channels 50D, 50E, 50F, 50G disposed at an intersection of the at least two primary channels 50A, 50B, 50C configured to receive a combination of fluids from at least two of the plurality of primary channels 50A, 50B, 50C. The top layer 20 is secured to the bottom layer 10 covering the network of microfluidic channels 50. The device 100 allows three-dimensional cell culturing of the sample and functional precision drug testing.

Referring to Figure 1a to 2, the device 100 includes a bottom layer 10 defined with a network of microfluidic channels 50. In an embodiment, the bottom layer 10 acts as a platform of the device 100. The network of microfluidic channels 50 includes a plurality of primary channels 50A, 50B, 50C. Further, each of the plurality of primary channels 50A, 50B, 50C is defined with a flow path for at least one fluid. The plurality of primary channels 50A, 50B, 50C includes an inlet end 10a,10b,10c to receive the at least one fluid at one end. Further, the plurality of primary channels 50A, 50B, 50C includes auxiliary ends 12 at another end which serve as outlets during sample cell seeding and can be sealed during drug perfusion experiments. The auxiliary ends 12 also aid in removing air bubbles from the plurality of primary channels 50A, 50B, 50C during the experiments. In an embodiment, each of the plurality of primary channels 50A, 50B, 50C is disposed in spaced apart configuration with each other as shown in Figure 2.

The network of microfluidic channels 50 also comprises a plurality of secondary channels 50D, 50E, 50F, 50G. Each of the plurality of secondary channels 50D, 50E, 50F, 50G is configured to receive a combination of fluids from at least two of the plurality of primary channels 50A, 50B, 50C. Each of the plurality of secondary channels 50D, 50E, 50F, 50G is disposed at an intersection of at least two primary channels 50A, 50B, 50C and configured to receive the combination of fluids from the at least two primary channels 50A, 50B, 50C. Each of the plurality of secondary channels 50D, 50E, 50F, 50G includes an outlet end 10d, 10e, 10f, 10g at one end to dispense the combination of fluids received from the plurality of primary channels 50A, 50B, 50C. In an embodiment, at least one secondary channel 50D, 50E, 50F, 50G is disposed between intersection of at least two primary channels 50A, 50B, 50C so that the combination of at least two drugs can be tested in at least one secondary channel 50D, 50E, 50F, 50G. In yet another embodiment, the secondary channel 50D, 50E, 50F, 50G is disposed between intersection of the at least three

primary channels 50A, 50B, 50C so that the combination of at least three drugs is tested as seen in Figure 2. In an embodiment, the fluid is at least one of cell sample, cell culture medium, and drug or any other fluid relevant to present innovation.

In an embodiment, the network of microfluidic channels 50 is defined with a segment of mixing path 40 for premixing at least one fluid. The fluid mixing is facilitated by passive or active mixing elements. The mixing path 40 is a non-linear path comprising at least one of a curved, serpentine, and zig-zag path or any suitable mixing path 40. In an embodiment, each of the mixing path 40 is provided with an array of microwells 30 and atleast one milliwell 32. The array of microwells 30 and milliwells 32 have a flat bottom surface which aids in single spheroid formation when the sample is introduced. In an embodiment, the array of microwells 30 and milliwells 32 are formed within the at least one inlet end of the primary channels 50A, 50B, 50C and at least one outlet end 10d,10e,10f, 10g of the secondary channels 50D, 50E, 50F, 50G and below auxiliary ends 12. The microwells 30 are configured to trap the sample cells for spheroid formation and the milliwells 32 traps excess sample cells that are not required for drug testing.

In an embodiment, the microwells 30 and milliwells 32 are defined with a cross section. In an embodiment, the microwells 30 and milliwells 32 have cross section of at least one of circular, square, hexagonal, elliptical, rectangle or any suitable shape. The bottom surface of microwells and milliwells is flat, concave, U-shaped, V-shaped surface or any suitable surface. In an embodiment, a rectangular cross-section of the channelsis 400 × 200 μm. In an embodiment, the cylindrical microwells are 400 μm in diameter with 400 μm in depth. The milliwells are formed at bottom of the inlet end 10a,10b,10c and outlet end 10d,10e,10f, 10g of the primary channel 50A, 50B, 50C and secondary channels 50D, 50E, 50F, 50G, respectively. In another embodiment, the dimension ranges of the mixing paths 50 is indicated in the table below.

Channels width	10 um - 1 mm
Channel height	10 um - 1 mm
Microwell diameter	10 um - 700 um
Microwell height	10 um - 700 um
Milliwell diameter	1mm – 5mm
Milliwells height	10 um - 700 um

In an embodiment, three milliwells are formed at other end of primary channels 50A, 50B, 50C below auxiliary ends 12. In an embodiment, at least ten milliwells are formed having a diameter of 1 mm and a depth of 600 μm. These milliwells 32 are configured to capture loose cells of the sample and spheroids from the primary 50A, 50B, 50C and secondary channels 50D, 50E, 50F, 50G and overall channel network 50 after sample cell seeding, thus preventing multiple spheroids formation in microwells. In an embodiment, the mixing path 40 of the network of microfluidic channels 50 is defined with mixing paths allowing premixing the of drugs before they reach to microwells 30 and milliwells 32 containing cell spheroids. Moreover, mixing paths also increase hydraulic resistance of a fluidic circuit preventing sudden fluid flow due to variable fluid level in a plurality of reservoirs 21 provided on a top layer 20.

The device 100 further includes a top layer 20 secured to the bottom layer 10 covering the network of microfluidic channels 50. The top layer 20 is removably secured to the bottom layer 10. The top layer 20 defined with a plurality of inlet ports 21a, 21b, 21c and outlet ports 21d, 21e, 21f, 21g each in fluid communication with a corresponding inlet ends 10a, 10b, 10c and outlet ends 10d, 10e, 10f, 10g of the plurality of primary 50A, 50B, 50C and secondary channels 50D, 50E, 50F, 50G. In an embodiment, the top layer 20 is provided with a plurality of auxiliary ports (22), each in fluid communication with each of the auxiliary ends (12) of the primary channels 50A, 50B, 50C, to remove air bubbles. Each of the plurality of inlet ports 21a,21b,21c, outlet ports 21d,21e,21f, 21g, and the auxiliary ports 22 are provided with a reservoir 21 into which at least one fluid is introduced and collected. Each fluid reservoir 21 is configured to store and collect 25-50 μl of fluid volume. In one embodiment, the fluid reservoir 21 can hold 35 μl of fluid volume. The fluid reservoirs 21 serve dual purposes. The first is to prevent sample cell culture media from drying in overall of network of microfluidic channels 50 in bottom layer 20. The second purpose is to prevent air bubbles from going in the network of microfluidic channels 50 while exchanging fluids. In an embodiment, a plurality of syringe pumps may be provided such that each of the plurality of syringe pumps is in fluid communication with each of the reservoir 21 for withdrawing and introducing the at least one fluid in the plurality of primary 50A, 50B, 50C and secondary channels 50D, 50E, 50F, 50G. In another embodiment, the fluid can be withdrawn or introduced either manually or automated means.

In an example as shown in Figure 2, the microfluidic device 100 consists of three inlet ends of the plurality of primary channels 50A, 50B, 50C for receiving three different drugs perfusion. Accordingly, a drug combination exits from the device 100 through four outlet ends of plurality of the secondary channel 50D, 50E, 50F, 50G. In an embodiment, drug mixing ratio is 1:1. Each drug combination can be tested on a set of seven spheroids. Hence, at least forty-nine microwells have been made for seven drug combinations. Further, the sample cells of predetermined quantity are introduced through from each end of the mixing paths 40 of the network of fluidic channels 50 at a required flow rate using a syringe pump. The syringe pump is configured to withdraw fluid from auxiliary ends (12) of the plurality of primary channels 50A, 50B, 50C for a period. Subsequently, the syringe pump is stopped, and the device is kept idle for a period for sample cells to settle down at the bottom surface of each array of microwell and milliwells. Later, once spheroid forms, the drugs are introduced by the syringe pump to allow multiple drug testing on the sample cells. In an embodiment, the bottom layer 10 and the top layer 20 is made from Polydimethylsiloxane (PDMS) elastomer using casting process over moulds. Other suitable thermally curable materials, photocurable materials, hydrogels with physical or chemical cross-linking mechanism can also be used for casting.

The present disclosure further discloses a method of fabricating a microfluidic device 100. The method comprises initially mixing a Polydimethylsiloxane (PDMS) elastomer with a curing agent at a predefined mixing ratio to obtain a PDMS solution. In another embodiment, the predefined mixing ratio ranging between 5:1 to 15:1 (w/w). The PDMS solution is degassed before it is casted over moulds to form a bottom layer 10 and a top layer 20 of the microfluidic device 100. In an embodiment, the moulds are fabricated using additive manufacturing. In an embodiment, the moulds are manufactured by a soft stereolithography 3D printing technique using a resin in parallel orientation to build surface for superior surface finish of casting surface Other suitable 3D printing techniques include material extrusion, material jetting, and selective laser sintering. Other known microfluidic fabrication techniques such as photolithography, hot embossing, micro-milling may also be used for fabricating moulds for comparison with 3D printed moulds. In an embodiment, the moulds are manufactured by a resin which is methacrylate based transparent photosensitive resin, Any print material not inconsistent with the objectives of the present invention may be used. In some embodiments, the print material comprises an organic composition such as photocurable

organic polymeric composition or thermoplastic filaments, and polymeric powder materials. For example, in some embodiments, the print material can be made of acrylonitrile butadiene styrene (ABS) polymer, polylactic acid (PLA), polyethylene terephthalate glycol (PETg), Polyethylene terephthalate (PET), Poly(methyl methacrylate) (PMMA), polyether ether ketone (PEEK), polyaryletherketone (PAEK), polytetrafluoroethylene (PTFE), polyurethane (PU), Nylon, polycarbonate (PC), polypropylene (PP) and other suitable materials. Other suitable polymers may also be used as a print material. 3D printable materials such as metallic materials, composites, or hydrogels can also be used.

The present disclosure discloses a method for preparing the PDMS elastomer based multi-drug testing microfluidic device using 3D printed moulds. The 3D printed moulds are post-processed by initially washing the moulds in isopropyl alcohol (IPA) and later curing the washed moulds at a second predetermined temperature for a second predefined duration. The second predetermined temperature is 40°C-80°C, and the second predefined duration ranging about 10-60 minutes. The PDMS is casted over moulds at a first predetermined temperature for a first predefined duration. In an embodiment, the first predetermined temperature is 50°C-80°C, and the first predefined duration ranges about 3 to 24 hours.

Further, the bottom layer and the top layer are removed from the moulds. The removed bottom and top layers are treated with air plasma at pressure ranging between 600-1000 mTorr, power 10-200 W for 5-120 sec. Later, the top layer 10 is secured on the bottom layer 20 to form the microfluidic device 100. The top layer 10 is secured to the bottom layer 20 by bonding and heating at 50-150°C for 1-72 hours. The secured and bonded device 100 is coated with anti-fouling agent pluronic block copolymer with concentration ranging from 0.1 -10%(w/w) for time duration of 1 -48 hours.

In an embodiment, the size and dimensions of the device 100 and its components can be varied as per application requirements. In an example an overall size of the bottom layer is 48.2 × 43.8 × 1.4 mm and the top layer is 47.2 × 42.8 × 5.5 mm.

The present disclosure also discloses a method of screening a drug or drugs of interest on a biological sample using the microfluidic device (100). The said method comprises sterilization of

the microfluidic device 100, introducing a cell sample from each end of the mixing paths 40 of the network of fluidic channels 50 and withdrawing from auxiliary ports 22 and allowing the cells to settle at a bottom of the array of microwells 30 and milliwells 32. Further, culturing the cells in the said device 100 at standard incubation conditions to allow spheroid formation. Then, inducing microspheres and untrapped cells to flow towards plurality of auxiliary ports 32 due to gravity-based flow to make sure only single spheroid remains in each microwell 30 after atleast 12 h of the introduction of cell sample. Furthermore, adding the drug or drugs of interest into the said device (100 through inlet ports (21a, 21b, 21c) at least 24 h after introduction of the cell sample and allowing it to dynamically perfuse followed by incubation. Lastly, determining the most effective drug combination based on quantitative and qualitative biological assays.

In an embodiment, the biological sample may comprise of tumor cell sample. The said tumor cell sample (6000/µl) is introduced through 1 ml tips from each end of mixing paths 40 at 12 µl/min using a syringe pump (Fig. 4(a)). Further, the syringe pump withdraws fluid from three auxiliary ports for 2 min. Subsequently, the syringe pump is stopped, and the device is kept for 5 min for cells to settle down at bottom of each microwell array and milliwells (Fig. 4(b)). Next, the silicon tubes are cut keeping tube luers in removable port (Fig. 4(c)). Afterwards the device is kept in static incubation for 24 h for spheroid formation (Fig. 4 (d)). The next day, silicon tube luers are removed from holes which induces fluid flow capturing loose cells and s spheroids from channel network into three milliwells located below each silicon tube luers or auxiliary ports 22 (Fig. 4(e)). As excess cells are washed out, a single spheroid can be formed and maintained in each flat microwell throughout the culture duration (Fig. 4(f)). Further, Fig. 4 (g) shows drug perfusion scheme.

In an embodiment of the present invention relates to a method of screening drug or drugs of interest, the method comprising:

a. Sterilizing the microfluidic device 100;
b. Introducing a cell sample from each end of the mixing paths 40 of the network of fluidic channels 50 and withdrawing from auxiliary ports (22) and allowing the cells to settle at a bottom of the array of microwells (30) and milliwells (32);

c. Culturing the cells as defined in step b, in the said device (100) at standard incubation conditions to allow spheroid formation;
d. Inducing microspheres and untrapped cells to flow towards plurality of auxiliary ports (32) due to gravity-based flow to make sure only single spheroid remains in each microwell (30) after atleast 12 h of introduction of cell sample;
e. Adding the drug or drugs of interest into the said device (100) through inlet ports (21a, 21b, 21c) at least 24 h after introduction of the cell sample and allowing it to dynamically perfuse followed by incubation; and
f. Determining the most effective drug combination based on quantitative and qualitative biological assays.

In another embodiment, the method of preparation of the primary cell sample comprises:

a. Treating the patient-derived tumor sample with hydrogen peroxide solution for sterilization.
b. Mincing the said tumor cell mass mechanically into small pieces;
c. Digesting the minced cell mass as defined in step b, enzymatically along with frequent vortexing to obtain a cell suspension;
d. Filtering the cell suspension obtained in step c, through mesh with a pore size ranging from 40 – 100 µm;
e. Removing the RBCs from the filtered cells obtained in step d with an erythrocyte lysing buffer and obtaining a cell pellet;
f. Suspending the cell pellet obtained in step e, in a growth medium and propagating the cells at standard incubation conditions; and
g. Trypsinizing the cells obtained in step f and introducing them in the fabricated microfluidic device (100).

In another embodiment, the sterilization of microfluidic device (100) is done using UV radiation, chemicals, and steam.

In yet another embodiment, 10 -100 µl cell suspension containing 1000 – 50,000/µl of sample cells are introduced at the flow rate of 1 - 1000 µl/min.

In another embodiment, the standard incubation conditions include 37°C, 5 % CO_2, 95% humid environment.

In another embodiment, the spheroids formed have high circularity with mean circularity index in the range of 0.6 – 0.99.

In another embodiment, the circular spheroid formed are viable for at least 7 days.

Another embodiment, the drug or drugs of interest is perfused at the flow rate of 0.1 – 100 µl/min for 1 – 120 h or according to the clinical guidelines.

In an embodiment, the cells can be any cancer cells, or non-cancerous cells.

In another embodiment, the dosage of at least one or more drug of interest or their combination is determined/tested simultaneously in addition to the most effective drug combination. The dosage of drugs perfused may be determined by clinical pharmacokinetics or in vitro IC_{50} values or any other dosages relevant to present invention.

EXAMPLES

The following examples particularly describe the manner in which the invention is to be performed. But the embodiments disclosed herein do not limit the scope of the invention in any manner.

Example 1: Designing of mould of microfluidic device, its fabrication and characterization

1.1 Validation of design with computational simulations

Velocity simulations were carried out using commercial computational simulation software fluid mechanics module. Density and dynamic viscosity of fluid were 993 kg/m^3 and 8.9 × 10^{-4} Pas respectively. Boundary conditions were: inlet gauge pressure = 0 Pa, laminar outflow rate = 2 µl/min, wall = no slip. Isotropic diffusion coefficient of chemical species was considered 3 × 10^{-10} m^2/s. Concentration of three chemical species at three inlet ports (representing three drugs) was considered 1.3% (v/v). Steady state simulations were carried out for velocity and mixing, and transient simulations were carried out for 800 s for chemical species transport in microwells with step size of 10 s. Spheroids of 100 µm size were considered in microwells.

1.2 Fabrication procedure

Soft-lithography moulds for each layer of microfluidic device 100 was modelled with digital CAD. The modeled files were exported in stereolithography (.stl) file format... The moulds for top and bottom layer were printed using SLA apparatus in 100 and 25 µm layer height respectively. The moulds were printed parallel to build plate for better surface finish and accuracy. After printing, resin moulds were washed with isopropyl alcohol (IPA) for 20 min and cured at 60°C for 30 min. Next, PDMS base and curing agent were mixed at a ratio of 10:1 (w/w), degassed, casted over mould surface, and heated at 60°C for 3-4 h as shown in Figure 3a. Cured PDMS devices were treated with air plasma at 1000 mTorr and 30 W for 1 min 15 sec as shown in Figure 3b. Treated devices were bonded and heated at 100°C for 42 h for hydrophobicity restoration. Subsequently they were coated with 2% (w/v) pluronic F127 solution for 24 hr as shown in Figure 3c).

1.3 Mixing Characterization

Microfluidic device 100 was fabricated as described in section 1.2. The inventors of the present invention used food color dyes representing similar molecular weight to drugs used in present study. Blue food color dye (1.3 % v/v) containing Brilliant blue (molecular weight 792.9 g/mol) and yellow dye (2.7 % v/v) containing Tartrazine (molecular weight 534.36 g/mol) were prepared in deionized water. Blue (representing drug A), deionized water (representing drug B), and yellow color (representing drug C) were perfused from three inlets at 2 µl/min. Brightfield images of all mixing paths were captured. Quantification of blue dye and water mixing in one of the mixing paths was performed in ImageJ to verify the mixing percentage. Firstly, images were coverted to HSB (Hue, saturation, brightness) stack and saturation channel was selected. Next, gray values were plotted along a line at the end of mixing paths before reaching cell culture area. Mean gray value of the externally mixed blue color solution was measured in the channel. Mixing (%) was calculated according to equation as mentioned below.

$$mixing\ (\%) = \frac{I}{I_{ext}} \times 100$$

Where, I = Gray value of a pixel in a line at end of mixing path, I_{ext} = Mean gray value of externally mixed dye in channel

1.4 Surface Characterization

PDMS piece (0.5 × 1 cm) was cut from casted sample over 3D printed device. X-ray Photoelectron Spectroscopy analysis (XPS) was carried out on physically coated sample (2% (w/v) F127, 24 h) and non-coated PDMS sample with Al Kα X-ray source. X ray power was 225 W. C1s spectra was corrected by adjusting peak (C-C) to 284.8 eV (+4.2 eV). Survey scan was obtained from 0 – 1200 eV with 1 eV step size. Peaks were fitted using Guass-Lorentz algorithm.

PDMS piece (0.8 × 1 cm) was cut from casted sample over standard clear resin surface. Contact angle goniometer (Rame-Hart Instrument Company, Succasunna, NJ, Model 290-F4) was used to measure contact angle of 3 µl deionized water droplet on physically coated and native PDMS sample at 30 s interval for 7 min.

MCF-7 (NCCS, Pune, India) cells were cultured in T75 flask in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at standard incubation conditions. The cells were trypsinized (0.25% Trypsin–EDTA, Sigma-Aldrich, India) at 70-80% confluency and seeded in microfluidic device.

1.5 Results

1.5.1 Validation of device design through computational simulations

Design of the device was validated with computational simulations Firstly, mixing path length was decided based on mixing (%). The inventors of the present invention found mixing was incomplete in the serpentine length of 38 mm (Fig. 5(a)). However, it reached to ~ 97% in loop length of 57 mm (Fig. 5(b)). Hence mixing path length of 57 mm was decided. After finalizing the mixing path length, velocity simulations in the designed mixing path network were caried out. Velocity distribution in outlet cell culture and inlet cell culture areas was slightly different with the maximum velocity (V_{max}) in the outlet cell culture area = 0.4231 mm/s and inlet cell culture = 0.561 mm/s. (Fig. 5(c)). Moreover, deeper areas of microwells remain devoid of fluid flow protecting spheroids from shear stress due to dynamic perfusion (Fig. 5(d)). However, drugs penetrate thoroughly to 400 µm deep microwells within 800s with convection and diffusion

transport mechanisms (Fig. 5(e)). This also accumulates drugs surrounding spheroids recapitulating *in vivo* drug treatment.

1.5.2 . Device characterization

Fig. 7(a) shows the device in dynamic phase with color dyes and color dye and water interface in bottom-most and middle mixing paths respectively. Mixing characterization in blue dye mixing path indicated average mixing of 96.54 % closely matching with computational simulation results (Fig. 7(b)). Moreover, gray values at end of middle-most and bottom-most mixing paths were plotted along a line (Fig. 7(c)). Further, mean gray value for both of them were found significantly different validating dissimilar green dye solutions.

C1s XPS analysis of native PDMS surface demonstrated only one major peak at 284.8 eV which is due to $(CH_3)_2SiO$ groups present in PDMS. However, coated PDMS shows minor peak at 286.6 eV suggesting presence of CH_2CH_2O group present in pluronic F127 (Fig. 7(d)). In line with C1s spectra, survey scan showed increase in amount of atomic carbon and oxygen content due to presence of pluronic groups (Fig. 7(e)). Moreover, presence of pluronic groups rendered PDMS surface hydrophilic unlike native PDMS (Fig. 7(f)). Finally, we introduced MCF-7 cells in native and coated PDMS devices. We found cells attached on native PDMS channels and microwells. In contrast cells aggregated on pluronic coated devices (Fig. 7(g)). Hence, it was decided to coat PDMS devices with 2% (w/v) pluronic F127 for 24 h before introducing cells.

Example 3: Isolation of primary oral tumor cells, 3D cell culture in microfluidic device and its characterization

3.1 Isolation protocol of primary oral tumor cells from patient-derived biopsy samples

The current study involving human samples was approved by institute ethical committee of IIT Hyderabad (IITH/IEC/2022/04/03). Oral tumor samples not treated with any chemotherapeutic drugs from three patients (**Table 1**) undergoing surgery were transported in RPMI medium with 5% antibiotic–antimycotic solution (penicillin–streptomycin (P/S), Invitrogen, Thermo Fisher Scientific, India) after obtaining patient consent. Next, sample is mechanically minced in individual pieces of 3-4 mm before enzymatic digestion using collagenase-hyaluronidase solution

at 37°C for 2 h with frequent vortexing. Next, cell suspension was filtered through 100 and 40 µm nylon meshes. Subsequently, treatment with Ammonium-Chloride-Potassium (ACK) lysing buffer (5 min) at room temperature was done for removing RBCs from cell mixture. Finally, cell pellet is suspended in oral tumor growth medium. Cells were propagated at standard incubation conditions (37 °C, 5% CO2, and 95% humidity).

Table 1. Oral cancer patient details

Patient age (years)	**Sex**	**Tumor staging**	**Diagnosis**
Patient 1 (63)	Female	T3N1M0	Well differentiated squamous cell carcinoma
Patient 2 (46)	Male	T4N1M0	Well differentiated squamous cell carcinoma
Patient 3 (60)	Female	T3N1M0	Moderately differentiated squamous cell carcinoma

3.1 3D Cell culture in device

Fabricated device was coated with 2% (w/v) pluronic F127 at room temperature for 24 h. Next day, the device was sterilized first with UV and 70% ethanol solution for 10 min. Ethanol solution was replaced with cell culture medium before introducing 6000 cells/µl (20 µl) from each inlet at flow rate of 12 µl/min from 1 ml tips. Cells were allowed to settle at bottom of wells for 5 min before cutting silicon tubes from three outlets. Next, microfluidic device was cultured at standard incubation conditions undisturbed for 24 h for spheroid formation. Cell culture medium (DMEM + 20% FBS) was exchanged twice in a day. At the end of 5 days cell viability assay was performed.

3.2 Performing the live/dead assay

Firstly, device was washed with 1x PBS from all seven inlets. Next, solution of 1 µg/ml Calcein/AM (Invitrogen, India) and 4 µg/ml PI (propidium iodide) was prepared in PBS. Solution was introduced from all seven inlets before incubating device at 37°C for 30 min. Next, device was washed with 1x PBS and images were captured in EVOS M7000 fluorescent microscope. Quantification of images was done according to previous protocol based on area fraction. Viability (%) was calculated according to following equation in ImageJ. Individual spheroid images were cropped with 300 × 300 µm window size and area fraction of green and red channels were measured individually.

$$Viability\ (\%) = \frac{Area\ f\ raction_{green}}{Area\ f\ raction_{green} + Area\ f\ raction_{red}}$$

3.3 Results

3.3.1 Biocompatibility evaluation of device

Breast cancer cell line (MCF-7) was cultured in the developed device for biocompatibility evaluation. As shown in **Fig. 8 (a)** uniform sized spheroids could be formed in all wells with developed cell seeding protocol. We found the spheroid size remained under 200 µm during six days of culture (**Fig. 8(b)).** Moreover, mean spheroid size was found increasing over 6 days of culture. Mean circularity index remained 0.95 showing capability of flat bottom 3D printed microwells to form circular single spheroids (**Fig. 8(c))**. Moreover, MCF-7 spheroids were found maintaining high viability of more than 90% (**Fig. 8(d-e))**. Only uniform sized tumor spheroids have been considered for analysis.

3.3.2 Primary oral tumor cell culture in device

Next, primary oral tumor cells were cultured in the developed microfluidic device. Fig. 10(a) shows brightfield image of spheroids in chip on day 3. Mean diameter of primary cells was found

decreasing after day 2 (Fig. 10(b)). Moreover, mean diameter remained below 150 µmover 5 days of culture. Primary spheroids maintained mean circularity index close to 0.9 during culture (Fig. 10(c)). In addition, they maintained excellent viability (>90%) at the end of day 5 (Fig. 10(d-e)).

Example 4: Combinatory drug screening on patient derived oral tumor cells

Well-known oral cancer regimen including paclitaxel (1 µM), 5 fluorouracil (20 µM), and cisplatin (5 µM) was dynamically perfused at flow rate of 2 µl/min on day 3. Firstly, silicon tubes were attached at four outlet ports 21d, 21e, 21f, 21g and 1 ml tips were attached at inlet ports 21a, 21b, 21c for introducing three drugs. Next, syringe pump assembly with device was kept in incubator for 6 h. Syringe pump applies negative pressure to withdraw the drugs from three inlets towards four outlets. Next, drugs were washed with cell culture medium and device was kept in static incubation for 2 h before performing the cell viability assay.

Maximum plasma concentrations of each drug (at which the drugs are most effective on tumor) associated with clinically recommended dosages from Phase I/II clinical trials were used in present study.

4.1 Results

4.1.1 Drug testing results of three oral tumor patient spheroids

Oral tumor cells from three patient samples were isolated and cultured in the developed tumor-on-a-chip for drug testing. The inventors selected well-known oral cancer regimen including paclitaxel, 5 Fu, and cisplatin. The mixing paths premix the drugs for all three pair-wise combinations and a combination with all the three drugs. Pair-wise combinations in all three mixing paths result in the concentration of each individual drug reduced close to half. Similarly, in the central loop where all three drugs merge, the concentration of each drug reduces close to one-third.

Patient 1: Mean diameter of patient 1 tumoroids was reduced by 12% on day 3 (Figure 12). From day 1 onwards patient-1 derived spheroids maintained tight s junctions Besides, they formed the smallest diameter on day 1 among all three patient-specific spheroids. Despite their smallest size, they were highly resistant to all the drug combinations in the device (Figure 11(a)) (The inventors

found no significant difference between drug groups and control. However, inventors found several spheroids responded to paclitaxel (1 µM) monotherapy.

Patient 2: The inventors observed that the patient 2 spheroids showed reduction in mean diameter by 6% on day 3 (Figure 12) All drug groups produced significantly higher cell death compared to the control (Figure 11(b)). Moreover, there was no difference among individual drug groups. More interestingly, cisplatin resistance was observed in patient 2 spheroids. This resistance could not be overcome in pair-wise combination with either paclitaxel or 5 Fu. Therefore, pair-wise combination of all 3 drugs or pair-wise combination of paclitaxel + 5 Fu or paclitaxel and 5 Fu mono-drug therapies may be beneficial for patient 2.

Patient 3: The inventors found the highest reduction in spheroid size by 17% in patient 3 derived spheroids on day 3 (Figure 12) This correlated with brightfield images of spheroids on day 3 loosening up from the boundary (Figure 26) Patient 3 spheroids didn't respond to paclitaxel and cisplatin mono drug treatments. Moreover, treatment with all three drugs was also ineffective (Figure 11(c)). However, pair-wise combination of paclitaxel with either cisplatin or 5 Fu produced higher cytotoxicity than mono-drug therapy of paclitaxel. Besides, tumor response towards 5 Fu mono-drug therapy was better than paclitaxel. Hence, 5 Fu mono drug therapy or pair-wise combinations may be effective for patient 3.

Overall, the responses of all drug combinations were highly dissimilar among all three sets of patient samples suggesting inter-patient heterogeneity with clinically-relevant dosages (Figure 11(d) . More importantly, patients 1 and 2 having the same tumor differentiation status produced dissimilar drug responses on our platform.

ADVANTAGES

In an embodiment, the method of fabricating the microfluidic device is economical and satisfies the customizable need of the application.

The 3D printing-based fabrication method enhances the feasibility of mass production and commercialization of technology over other conventionally manufactured drug testing devices known in prior art.

The design of device allowed the testing of various combinations of drugs on / 3D spheroids. Thus, aid in discovering potential drug combinations with lower dosages and mitigating side effects.

The novel design of the device having mixing paths aids in efficient premixing of the drugs before they reach spheroids and also increases hydraulic resistance (than straight fluid circuit) preventing sudden movement of fluid due to mismatch of fluid levels in upper reservoirs.

The method of 3D cell culture and drug testing with presence of milliwell traps and auxiliary ports also aid in forming single spheroids by capturing loose spheroids from the channels after cell seeding.

Therefore, those skilled in the art will appreciate the present disclosure that provides many advantages and overcomes various limitations.

In the detailed description of the embodiments of the disclosure, reference is made to the accompanying drawings that form a part hereof, and in which are shown by way of illustration specific embodiments in which the disclosure may be practiced. These embodiments are described in sufficient detail to enable those skilled in the art to practice the disclosure, and it is to be understood that other embodiments may be utilized and that changes may be made without departing from the scope of the present disclosure. The following description is, therefore, not to be taken in a limiting sense.

Equivalents:

With respect to the use of substantially any plural and/or singular terms herein, those having skill in the art can translate from the plural to the singular and/or from the singular to the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for sake of clarity.

It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims (e.g., bodies of the appended claims) are generally intended as “open” terms (e.g., the term “including” should be interpreted as “including but not limited to,” the term “having” should be interpreted as “having at least,” the term “includes” should be interpreted as “includes but is not limited to,” etc.). It will be further understood by those within the art that if a

specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims may contain usage of the introductory phrases "at least one" and "one or more" to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles "a" or "an" limits any particular claim containing such introduced claim recitation to inventions containing only one such recitation, even when the same claim includes the introductory phrases "one or more" or "at least one" and indefinite articles such as "a" or "an" (e.g., "a" and/or "an" should typically be interpreted to mean "at least one" or "one or more"); the same holds true for the use of definite articles used to introduce claim recitations.

In addition, even if a specific number of an introduced claim recitation is explicitly recited, those skilled in the art will recognize that such recitation should typically be interpreted to mean at least the recited number (e.g., the bare recitation of "two recitations," without other modifiers, typically means at least two recitations, or two or more recitations). Furthermore, in those instances where a convention analogous to "at least one of A, B, and C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., "a system having at least one of A, B, and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). In those instances where a convention analogous to "at least one of A, B, or C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (e.g., "a system having at least one of A, B, or C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or "B" or "A and B."

While various aspects and embodiments have been disclosed herein, other aspects and embodiments will be apparent to those skilled in the art. The various aspects and embodiments

disclosed herein are for purposes of illustration and are not intended to be limiting, with the true scope and spirit being indicated by the following claims.

List of referral numerals:

Description	Reference Numeral
Microfluidic device	100
Bottom layer	10
Inlet of Primary fluidic channels	10a,10b,10c
Top layer	20
Plurality of reservoirs	21
Inlet port of the top layer	21a,21b,21c
Outlet port of the top layer	21d,21e,21f, 21g
Microwells	30
Milliwells	32
Auxiliary ports	22
Auxiliary ends in bottom layer	12
Mixing paths	40
Microfluidic channels	50
Primary channels	50A, 50B, 50C
Secondary channels	50D, 50E, 50F, 50G

Outlet of Secondary channels	10d,10e,10f,10g

We Claim:

1. A microfluidic device (100), comprising:

a bottom layer (10) defined with a network of microfluidic channels (50), comprising:

a plurality of primary channels (50A, 50B, 50C), each defining a flow path (40) for at least one fluid, wherein each of the plurality of primary channels (50A, 50B, 50C) includes an inlet end (10a,10b,10c) to receive the at least one fluid; and

a plurality of secondary channels (50D, 50E, 50F, 50G), each configured to receive a combination of fluids from at least two of the plurality of primary channels (50A, 50B, 50C), wherein each of the plurality of secondary channels (50D, 50E, 50F, 50G) is disposed at an intersection of at least two primary channels (50A, 50B, 50C), and each of the plurality of secondary channels (50D, 50E, 50F, 50G) includes an outlet end (10d,10e,10f,10g) to dispense the combination of fluids; and

a top layer (20) secured to the bottom layer (10) covering the network of microfluidic channels (50), the top layer (20) is defined with a plurality of inlet ports (21a,21b,21c) and outlet ports (21d,21e,21f, 21g), each in fluid communication with a corresponding inlet ends (10a,10b,10c) and outlet ends (10d,10e,10f,10g).

2. The microfluidic device (100) as claimed in claim 1, wherein the network of microfluidic channels (50) is defined with a segment of mixing path (40) for premixing the at least one fluid.
3. The microfluidic device (100) as claimed in claim 2, wherein the mixing path (40) is a non-linear path comprises at least one of a curved, serpentine, and zig-zag path.
4. The microfluidic device (100) as claimed in claim 2, wherein each of the mixing path (40) is provided with an array of microwells (30) and atleast one milliwell (32).
5. The microfluidic device (100) as claimed in claim 1, wherein the microwells (30) and milliwells (32) have a bottom surface which aids in single spheroid formation.

6. The microfluidic device (100) as claimed in claim 5, wherein the bottom surface of microwells (30) and milliwells (32) is flat, concave, U-shaped surface or any suitable surface for spheroid formation.
7. The microfluidic device (100) as claimed in claim 5, wherein the cross-sectional shape of the of microwells (30) and milliwells (32) is circular, squared, rectangular, hexagonal, elliptical and other polygonal shapes.
8. The microfluidic device (100) as claimed in claim 1, wherein the top layer (20) is provided with a plurality of auxiliary ports (22), each in fluid communication with each of the primary channels (50A, 50B, 50C) and auxiliary ends (12), to remove air bubbles.
9. The microfluidic device (100) as claimed in claim 5, wherein each of the plurality of inlet ports (21a,21b,21c), outlet ports (21d,21e,21f, 21g), and the auxiliary ports (22) are provided with a reservoir (21) into which at least one fluid is introduced and collected.
10. The microfluidic device (100) as claimed in claim 6, comprises a plurality of syringe pumps, each in fluid communication with each of the reservoir (21) for withdrawing and introducing the at least one fluid.
11. The microfluidic device (100) as claimed in claim 1, wherein the bottom layer (10) and the top layer (20) is made from Polydimethylsiloxane (PDMS) elastomer.
12. The microfluidic device (100) as claimed in claim 1, wherein at least one fluid is selected from cell sample, drug, cell culture medium, buffers, sterilizing agent, anti-fouling agents, and other fluid compositions for biological characterization.
13. A method of fabricating a microfluidic device (100), the method comprising:

mixing a Polydimethylsiloxane (PDMS) elastomer with a curing agent at a predefined mixing ratio to obtain a PDMS solution;

casting the PDMS solution over moulds, wherein the PDMS solution forms the bottom layer (10) and the top layer (20);

curing PDMS solution in the moulds at a first predetermined temperature for a first predefined duration;

removing the bottom layer (10) and the top layer (20) from the moulds;

treating the removed bottom layer (10) and the top layer (20) from the moulds with air plasma;

securing the top layer (20) on the bottom layer (10) to form the microfluidic device (100); and

coating the secured microfluidic device (100) with anti-fouling agent.

14. The method as claimed in claim 13, wherein the moulds are fabricated using additive manufacturing.
15. The method as claimed in claim 14, wherein the moulds are manufactured by a soft stereolithography 3D printing technique using a resin with orientation parallel to build surface.
16. The method as claimed in claim 14, wherein the fabricated moulds are post-processed by:

    washing the moulds in isopropyl alcohol (IPA); and

    curing the washed moulds at a second predetermined temperature for a second predefined duration.
17. The method as claimed in claim 16, wherein the second predetermined temperature is 40°C-80°C, and the second predefined duration ranging about 10-60 min.
18. The method as claimed in claim 13, wherein the predefined mixing ratio ranging between 5:1 to 15:1 (w/w).
19. The method as claimed in claim 13 wherein the moulds are manufactured by a resin which is methacrylate based transparent photosensitive resin,
20. The method as claimed in claim 13, wherein the first predetermined temperature is 50-80°C, and the first predefined duration ranges from 3 to 24 hours.
21. The method as claimed in claim 13, wherein the fabricated bottom (10) and top layer (20) are treated with air plasma at pressure ranging between 600-1000 mTorr, power 10-200 W for 5-120 sec.
22. The method as claimed in claim 13, wherein the top layer (20) is secured to the bottom layer (10) by bonding and heating at 50-150°C for 1-72 hours.
23. The method as claimed in claim 13, comprises coating the bonded bottom layer (20) with said anti-fouling agent Pluronic block co polymer 0.1 – 10% (w/v) agent for 1-48 hours.
24. A method of screening drug or drugs of interest, the method comprising:
    a. Sterilizing a microfluidic device (100) as claimed in claim 1;

b. Introducing a cell sample from cell seeding ports (21a, 21b, 21c, 21d, 21e, 21f, 21g) and withdrawing from auxiliary ports (22) and allowing the cells to settle at a bottom of the array of microwells (30) and milliwells (32);

c. Culturing the cells as defined in step b, in the said device (100) at standard incubation conditions to allow spheroid formation;

d. Inducing microspheres and untrapped cells to flow towards milliwells located below plurality of auxiliary ports (22) due to gravity-based flow to make sure only single spheroid remains in each microwell (30) after atleast 12 h of introduction of cell sample;

e. Adding the drug or drugs of interest into the said device (100) through inlet ports (21a, 21b, 21c) at least 24 h after introduction of the cell sample and allowing it to dynamically perfuse followed by incubation; and

f. Determining the most effective drug combination based on quantitative and qualitative biological assays.

25. The method as claimed in claim 24, wherein the preparation of the cell sample comprises:

a. treating the tumor mass with hydrogen peroxide solution for sterilization.

b. Mincing the said tumor cell mass mechanically into small pieces;

c. Digesting the minced cell mass as defined in step b, enzymatically along with frequent vortexing to obtain a cell suspension;

d. Filtering the cell suspension obtained in step c, through mesh with a pore size of 40 – 100 µm;

e. Removing the RBCs from the filtered cells obtained in step d with an erythrocyte lysing buffer and obtaining a cell pellet;

f. Suspending the cell pellet obtained in step e, in a growth medium and propagating the cells at standard incubation conditions; and

g. Trypsinizing the cells obtained in step f and introducing them in the fabricated microfluidic device (100).

26. The method as claimed in claim 24, wherein the sterilization of microfluidic device (100) is done using UV radiation, chemicals, and steam.

27. The method as claimed in claim 24, wherein 10 -100 µl cell suspension containing 1000 – 50,000/µl of sample cells are introduced at the flow rate of 1 - 1000 µl/min.

28. The method as claimed in claim 24, wherein cells can be any cancer cells, or non-cancerous cells.
29. The method as claimed in claim 24, wherein standard incubation conditions include 37°C, 5 % CO_2, 95% humid environment.
30. The method as claimed in claim 24, wherein the spheroids formed have high circularity with mean circularity index in the range of 0.6 – 0.99.
31. The circular spheroid formed as claimed in claim 24, are viable for at least 7 days.
32. The method as claimed in claim 24, the drug or drugs of interest is perfused at the flow rate of 0.1 – 100 µl/min
33. The method as claimed in claim 24, wherein dosage of at least one or more drug of interest or their combination is determined/tested simultaneously in addition to the most effective drug combination.

Dated 25th day of August 2022

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ABSTRACT

A MICROFLUIDIC DEVICE, METHOD OF FABRICATING AND ITS APPLICATION THEREOF

The present invention pertains to the field of microfluidic platforms. More particularly, the invention relates to a microfluidic device and method of three-dimensional sample cell culturing and multi-drug testing using the device thereof.

Figure 1.