

1 **Lab-on-Chip for Testing Myelotoxic Effect of**
2 **Drugs and Chemicals**

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1 **Abstract**

2 In the last twenty years, one of the main goals in the drug discovery field has been the
3 development of reliable *in vitro* models. In particular, in 2006 the European Centre for the
4 Validation of Alternative Methods (ECVAM) has approved the Colony forming Unit-
5 Granulocytes-Macrophages (CFU-GM) test, which is the first and currently unique test applied to
6 evaluate the myelotoxicity of xenobiotics *in vitro*. The present work aimed at miniaturizing this *in*
7 *vitro* assay by developing and validating a Lab-on-Chip (LoC) platform consisting of a high
8 number of bioreactor chambers with screening capabilities in a high-throughput regime.

9

10 **Keywords**

11 *Lab-on-Chip*

12 *Microfluidics*

13 *Clonogenic test*

14 *CFU-GM*

15 *CBMNC*

16

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1 Introduction

2 In the last twenty years, one of the main goals in the drug discovery field has been
3 the development of reliable *in vitro* models. Being currently the *in vivo* testing
4 phase still necessary – to approve new drugs it is necessary to perform *in vivo*
5 studies including at least two animal species, and employing various dosing
6 schedules, depending on the molecule to be approved (Boorman et al. 1982) –
7 research has been focusing on the development of new platforms able to replace,
8 or reduce, the traditional *in vivo* trials at least in the early stages of the drug
9 discovery process. Advantages of the *in vitro* approach mainly rely on economical
10 and ethical standpoints. Indeed, *in vitro* testing presents numerous advantages,
11 namely the reduction of model-to-model variability, ease of data collection, less
12 requirements in terms of personnel training, reduction in the amounts of reagents,
13 miniaturization and automation (Hartung and Daston 2009).

14 One of the fields which could mainly benefit from the paradigm mutation towards
15 *in vitro* experimentation is toxicology, i.e. the study of drug toxicity (Gad 1990).

16 In particular, clonogenic assays are thought to better bridge the gap between
17 toxicity experienced *in vivo* and toxicity effects on cells cultivated in laboratory
18 (cytotoxicity). Clonogenic assays are cytotoxicity tests based on the rate of
19 survival of colony forming units (CFUs) in the presence of several drug
20 concentrations, thus differing from traditional proliferation assays which take into
21 account the mere increase in cell number. Specific advantages of clonogenic
22 assays are related to higher accuracy and predictivity with respect to traditional
23 proliferation tests (Pessina et al. 2004).

24 To date, the European Centre for Validation of Alternative Methods (ECVAM)
25 has approved only one clonogenic test (DB-ALM protocol 101, available on
26 <http://ecvam-dbalm.jrc.ec.europa.eu/>). This particular test aims at characterizing
27 the acute effect of toxicants on granulocytes and macrophages (GM) progenitor
28 cells derived both from bone marrow and umbilical cord blood, under maximally
29 stimulatory cytokine concentrations. The system is based on counting CFUs
30 formed by the surviving progenitors after xenobiotics exposure.

31 In 2004, Pessina and co-workers optimized a new protocol for CFU-GM assay
32 based on the use of 96-well plates in place of standard Petri dishes thus reducing
33 complexity and costs of the original test.

1 However, the assay still remains time-consuming. Colonies are generally counted
2 only upon 14 days of incubation. In addition, the assay implies the dispersion of
3 cells within a semisolid culture medium, which inhibits a pre-determined
4 localization of colonies (within dishes or wells), which all must be manually
5 found by a trained operator (Pessina et al. 2001).
6 Our goal was to develop a microfluidic device able to miniaturize the current
7 state-of-art paradigm of *in vitro* hematotoxicity of xenobiotics protocol. Indeed, a
8 further miniaturization of the original assay, down to the micrometer scale, will
9 provide at the same time advantages in terms of cost reduction (cells, drugs,
10 cytokines, etc.) and decrease of testing time (colony forming process). To this aim
11 we designed a novel Lab-on-Chip (LoC) platform enabling for trapping, feeding
12 and growing of multipotent monoclonal stem cells.

13 **2 Materials and Methods**

14 **2.1 Lab-on-Chip design and fabrication**

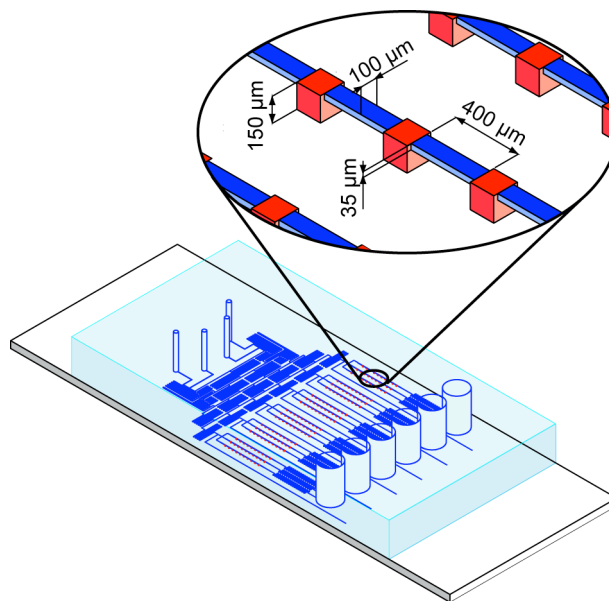
15 A grid-like distribution of micro-chambers was envisaged within the LoC device
16 to organize the spatial distribution of cells and, in turn, to obtain a univocal
17 correspondence between colonies and culture chambers.

18 For this purpose, we designed a microfluidic platform divided in two functional
19 areas: a serial dilution generator (Jeon et al. 2002; Kim et al. 2008; Lee et al.
20 2009; Occhetta et al. 2015a) and a culture region (Fig. 1). The first one consists of
21 a fluidic network of channels (70 μ m high and 70 μ m wide) arranged in a resistive
22 flow-based scheme to deliver six linear dilutions of drugs (concentration ranging
23 from 1 to 0, step 0.2) from two input ports to the culture region. This area was
24 integrated envisioning the possibility to test the cytotoxic effect of different dose
25 of drugs on bone marrow progenitor cells within a single device.

26 Downstream, the culture region consists of 180 cubic chambers (side 150 μ m)
27 divided in 6 units, each composed by 3 parallel lines of ten culture chambers.

28 The microfluidic device is made of polydimethylsiloxane (PDMS) and fabricated
29 by means of standard soft lithography techniques. Briefly, a 4" silicon wafer,
30 microstructured with two photoresist types, served as master mold to cast a thin
31 layer (about 1mm) of liquid PDMS, being curing agent and pre-polymer mixed in
32 a ratio 1:10 (w/w). A 3 hours thermal treatment at 65°C was applied to achieve a

1 complete crosslinking. Subsequently, the cured PDMS stamp was removed from
2 the mold and bonded, upon an air plasma treatment, to a previously casted slab of
3 PDMS (thickness 4 mm) to close the fluidic features. The assembly was trimmed
4 and drilled in correspondence of 4 inputs and 6 outputs, by means of biopsy
5 punchers having diameters of 0.5 and 3mm, respectively. Finally, the LoC device
6 was bonded to a histology glass slide upon a further air plasma treatment,
7 sterilized through autoclaving (121°C, 20 min, wet cycle) and subsequently dried
8 overnight at 80°C to recover PDMS hydrophobicity after plasma treatment, thus
9 minimizing cell adhesion.



10
11 **Fig. 1** The layout of the Lab-on-Chip device is shown together with a detail of a group of
12 chambers within a culture unit. Two main input ports (together with two secondary ports) deliver
13 dilutions of chemicals (100%, 80%, 60%, 40%, 20%, CTRL) to 6 culture units (30 chambers each)

14 **2.2 Cell expansion and characterization**

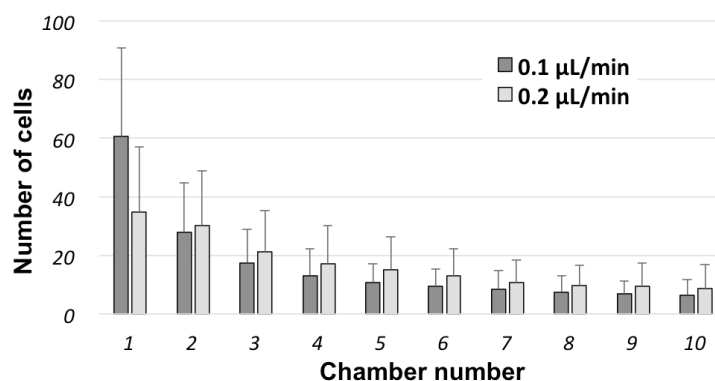
15 As a source of stem cells, the mononucleated cell fraction from human umbilical
16 cord blood (CB-MNCs) purchased frozen from Lonza (Swiss) was used. The
17 cells, stored in liquid nitrogen until use, were thawed according to a previously
18 described standard operating procedure (Pessina et al., 2004). Briefly, thawed
19 cells were transferred from the cryovial to a 15 ml conical tube and swirled while
20 warmed thawing medium (Iscove's Modified Dulbecco Medium (IMDM)
21 supplemented with 10% Fetal Bovine Serum (FBS) and 10 U/ml Dnase I) was
22 added drop by drop to the cells. The cell suspension was then centrifuged at 200g
23 for 15 min at room temperature.

1 Most of the wash was removed by pipette leaving 1–2 ml behind so the cell pellet
2 was not disturbed and was then resuspended in the remaining medium. Fresh
3 IMDM with 1% l-glutamine and 10% FBS was added to the cell suspension and
4 then centrifuged as above described. All the reagents were purchased from
5 EuroClone (Italy). Cells were then resuspended in Complete Medium (CM),
6 consisting of IMDM, containing 30% FBS, 1% L-Glutamine, 1%
7 Penicillin/Streptomycin solution and 10 ng/ml recombinant human granulocyte/
8 macrophage colony-stimulating factor (r hu GM-CSF, RELIA Tech, Germany) to
9 evaluate their number and viability.
10 Subsequently, CFU based tests were performed by using pooled donor cord
11 blood-derived CD34+ cells, purchased frozen from Life Technologies, USA. The
12 cells were stored and thawed according to the same protocol applied for CB-
13 MNCs.

14 2.3 Cell seeding within the LoC platform

15 Preliminary tests were carried out to establish a correlation between cell seeding
16 conditions and number of cells trapped in each chamber. For this purpose CB
17 MNCs were used. Aliquots of 30 μ l of cell suspension (concentration of 500,000
18 cells/ml) were simultaneously injected into both input ports through a dual
19 channel syringe pump at a flow rate of either 0.1 or 0.2 μ l/min. Upon complete
20 seeding, a picture of each chamber was taken through a phase contrast
21 microscope, and the number of cells contained in each chamber was directly
22 quantified. For these experiments, a number of 9 and 8 replicates (n=9 and n=8)
23 were used for 0.1 and 0.2 μ l/min, respectively.

24



25

26 **Fig. 2.** Content in terms of cell number for each of the 10 chambers encountered by cells in their
27 stream. Each device has 18 decades of chambers, and experiments were carried out for 9 and 8

1 replicates for 0.1 and 0.2 $\mu\text{l}/\text{min}$ flow rates, respectively. Data is presented as mean \pm standard
2 deviation

3

4 The cell distribution for both conditions tested is depicted in Fig. 2, which shows
5 a decrement in population along chambers in the same fluidic path. In particular,
6 the number of cells present in each chamber at the end of the seeding process
7 resulted 14.2 ± 26.2 and 16.8 ± 18.7 for flow rates of 0.1 and 0.2 $\mu\text{l}/\text{min}$,
8 respectively. Although these values were similar, the higher flow rate provided a
9 significantly lower number of empty chambers (24.4 ± 22.8 vs 11.4 ± 9.5), while
10 reducing the seeding time (2.5 vs 5 hours). Thus 0.2 $\mu\text{l}/\text{min}$ was chosen as seeding
11 condition for the following experiments and corresponding data were used in the
12 statistical model. Immediately after seeding, CB MNCs viability within the device
13 was estimated to be higher than 90% (see Supplementary Information).

14 **2.4 Statistical model of the clonogenic assay**

15 In general, the toxic effect of a drug molecule tested in a clonogenic assay results
16 in a smaller number of CFUs with respect to a control condition, wherein no drug
17 is administered. For a suitable statistical analysis, we modeled this situation as if
18 the drug was able to decrease the probability of any single cell to be clonogenic,
19 while keeping the number of cells constant.

20 We denote with \mathbf{p}_0 the probability of any cell to form a colony in the control. This
21 value does not depend on the chamber volume and it is also independent of time,
22 thus it is an intrinsic property of the cell donor. According to the results obtained
23 with standard macroscale experiments, in our case study \mathbf{p}_0 was set equal to
24 $1/146$. We then set a hypothesis test to determine if there is statistical evidence to
25 state whether the probability \mathbf{p} of a cell to form a colony when drug is
26 administered is smaller than the probability \mathbf{p}_0 in the control. Hence, in the null
27 hypothesis we have $\mathbf{p}=\mathbf{p}_0$, while in the alternative hypothesis we have $\mathbf{p}=\mathbf{p}_1<\mathbf{p}_0$.

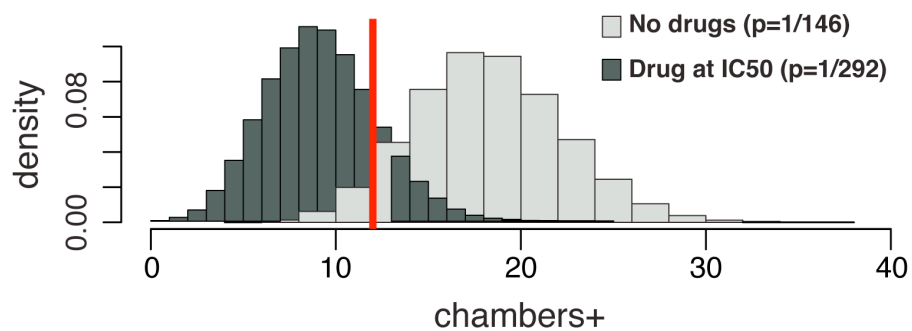
28 The seeding process suggested us to model the number of cells injected in each
29 chamber as a random variable with a probability distribution depending on the
30 position of the chamber in the corresponding line. Moreover, the numbers of cells
31 in the chambers were treated as independent variables, and all the lines and units
32 were considered to have the same distribution of cells. From the data collected in
33 the seeding characterization tests (Fig. 2, perfusion rate 0.2 $\mu\text{l}/\text{min}$) we computed

1 the empirical distribution of the number of cells for each position within the
 2 culture line (constituted by 10 chambers).
 3 The probability to find a colony in a chamber was obtained by modelling the
 4 number of clonogenic cells per chamber with a binomial law, having size equal to
 5 the average number of cell in that chamber and probability equal to p_0 . Hence, the
 6 number of positive chambers (*chambers+*, i.e. chambers containing at least one
 7 colony) in a chip composed by n chambers ($n/10$ culture lines) is defined by a
 8 sum of n independent and identical distribution binomial random variables whose
 9 parameters are those defined above. Since the probability distribution of this
 10 random variable does not belong to any family of distributions known in statistics,
 11 its density cannot be expressed analytically but it must be computed through
 12 simulations. These simulations were run through the R environment (software
 13 version 2.13.1), and a set of 10000 repetitions was enough to obtain stable
 14 distributions.

15 A critical region for the hypothesis test was built as it follows: the null hypothesis
 16 (i.e. a cell is clonogenic with a probability equal to p_0) has to be rejected
 17 whenever the number of *chambers+* in the chip is less than a threshold C . The
 18 threshold value depends upon the significance level α of the test and the number
 19 of chambers in the chip n . For instance, by setting $\alpha=0.05$, in our chip ($n=180$) we
 20 obtained $C=12$.

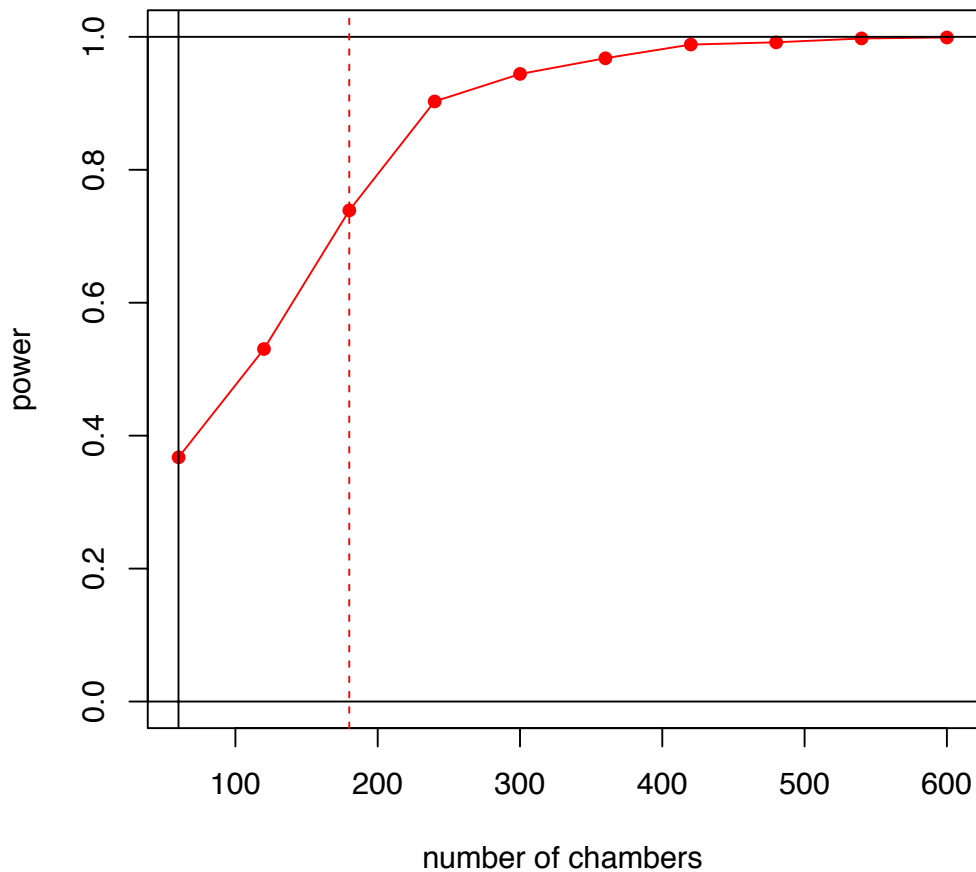
21 Furthermore, we computed the power of the test for different values of p_1 , that is
 22 the probability of having less than C *chambers+* due to the effect of the
 23 administered drug ($p_1 < p_0$). In Fig. 3, for instance, we show the probability
 24 distribution of the *chambers+* for a particular choice of $p_1 = 1/2 p_0 = 1/292$,
 25 corresponding to the ideal IC50 for a drug molecule. In this case, the power
 26 resulted equal to 73.8%.

27



28

1 **Fig. 3.** Probability distributions of the number of chambers+ within the chip both when no drug
 2 (light grey; probability of proliferation $p_0=1/146$) and a drug at a concentration corresponding to
 3 the IC50 (dark grey; $p_1=1/292$) are administered. The vertical red line indicates the threshold
 4 $C=12$ used for the critical region of the test
 5
 6 The power of the test computed above is only valid when the number of chambers
 7 in the chip is equal to 180. In Fig. 4 we show how the power of the test depends
 8 on n . The vertical red dotted line represents the current chip with $n=180$, whose
 9 power is equal to 0.738. The power rapidly approaches its maximum value when
 10 the number of chambers increases (it exceeds 0.9 already with $n=240$).



11
 12 **Fig. 4.** Power of the test as a function of the number of chambers in the chip (n). The vertical red
 13 dotted line represents the current chip with $n=180$, whose power is equal to 0.738.

14 **2.5 Implementation of the microscale clonogenic assay**

15 CD34+ cells were seeded into the LoC device at a flow rate of 0.2 $\mu\text{l}/\text{min}$. Upon
 16 2.5 hours, CM was prepared and perfused into the device at the same flow rate

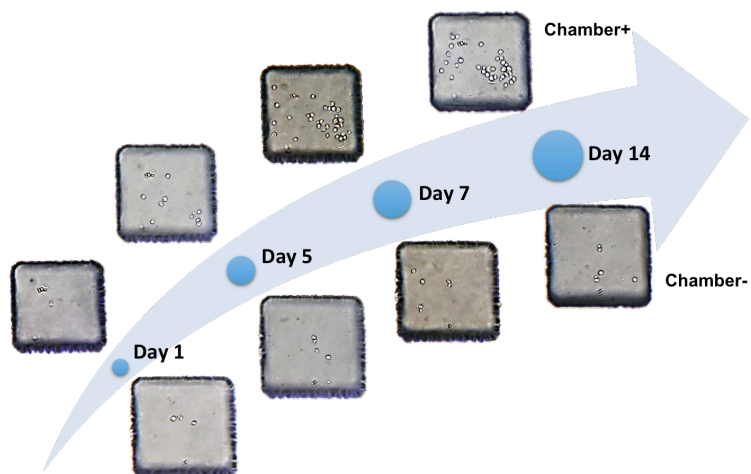
1 throughout the entire culture period (14 days), according to previous results in a
2 similar platform (Occhetta et al. 2015b). At specific time points (days 1, 5, 7 and
3 14) pictures from all culture chambers of the LoC device were taken to monitor
4 possible variations in cell number. The results were compared to control
5 experiments, consisting of devices perfused with culture medium free from
6 cytokines.
7 CFU potential of CD34+ cells within the microfluidic device was also assessed
8 both in the absence and presence of a drug with a recognized hematotoxicity in
9 clinical use. In particular, 1200 ng/ml (200X) of Paclitaxel (Toronto Research
10 Chemicals, Canada) dissolved in dimethylsulfoxide (DMSO, Serva, Germany),
11 were added to CM in order to have a final drug concentration of 6 ng/ml and a
12 DMSO percentage of 0.5% (v/v). Paclitaxel was delivered to cells through the
13 input ports through continuous perfusion. The culture was carried out up to 7
14 days, and pictures of each chamber were taken at days 1, 5 and 7. Pictures from
15 the control device were also taken at the same time-points and used as positive
16 control.

17 **3 Results and Discussion**

18 The 2-weeks culture period within the microfluidic platform provided an
19 increasing number of CD34+ cells in each chamber from day 1 to day 14 when
20 perfused with CM with cytokines, being the initial number of cells per chamber
21 13.1 ± 15.3 and the final 24.4 ± 21.0 . Conversely, the number of cells did not
22 change significantly along the culture period in the control experiment without
23 cytokines (with a percentage increase of only 0.57%).

24 In particular, we noticed an increased in the number of cells from day 1 to day 5
25 (21.0 ± 19.0), thus suggesting the possibility to reduce the assay time by
26 identifying the chamber containing colonies (positive chambers, chamber+)
27 within a shorter five days period of culture *in vitro*.

28



1
2 **Fig. 5.** Examples of a chamber containing at least one CFU (top) and no CFU (bottom)

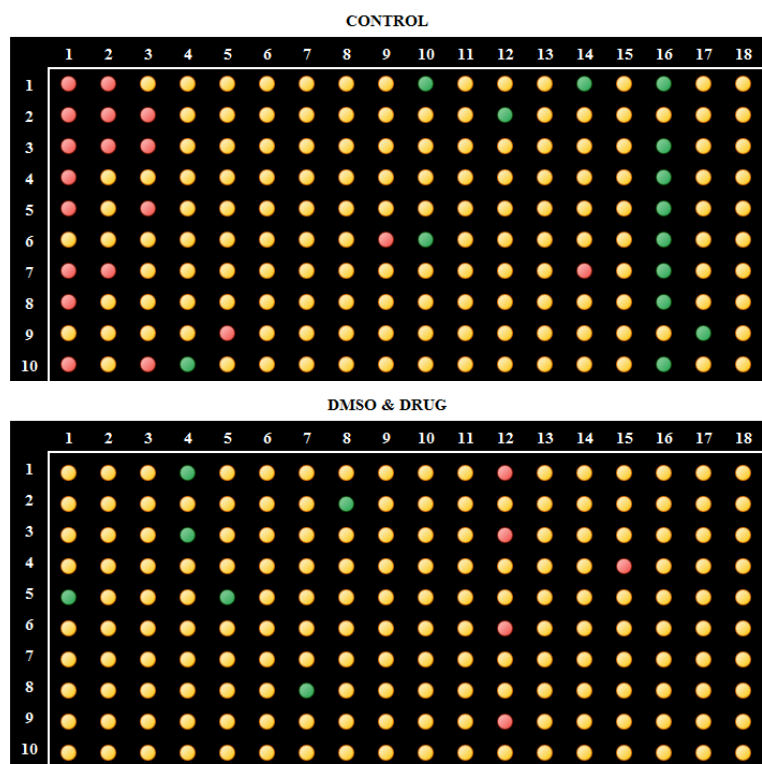
3
4 The number of chamber+ was inferred from pictures for both conditions. Fig. 5
5 shows the content of two exemplifying chambers. In particular, a chamber was set
6 positive in case the following conditions were simultaneously true:

- 7
8 *i. relative cell number increase > 25%;*
9 *ii. absolute cell number increase > 4.*

10
11 The number of resulting chamber+ was 15.33 ± 6.11 (n=3), in agreement with the
12 statistical model.

13 Furthermore, as preliminary test, the number of chamber+ was also assessed in
14 presence of Paclitaxel, a prototype toxicant molecule whose dosage was chosen,
15 in agreement with our previous findings corresponding to half maximal inhibitory
16 concentration (IC50). The number of chamber+ in absence of any toxicant
17 substances was 14 (positive control), while chamber+ number was reduced to 6
18 when the drug was delivered. Fig. 6 shows a graphical representation of the
19 distributions of chamber+ (green), chamber- (yellow) and empty chambers (red)
20 for both cases.

21



1
2 **Fig. 6.** Representation of positive (green), negative (yellow) and empty (red) chambers

3
4 This result suggests that the device, together with the implemented statistical
5 model, allows identifying the toxicity of a test molecule at a specific
6 concentration.

7 **4 Conclusions**

8 The proposed LoC device demonstrated its suitability as platform for CFU-based
9 assays. The average number of colonies formed after five days of culture ($15.33 \pm$
10 6.11) was adequate to detect the presence of a toxic dosage (IC50) of a drug. The
11 assay time was dramatically reduced in two ways: i) the culture time decreased
12 from 14 to 5 days, and ii) the colony identification was reduced to microscopy
13 acquisitions on a well-defined plane (chamber/channel fluidic layer).

14 The device was provided with a serial dilution generator unit, able to precisely
15 deliver to culture units predetermined concentration of soluble factors. However,
16 the number of chambers implemented for these experiments ($n=180$) was only
17 enough to catch the effect of a single test condition – namely the concentration of
18 the administered molecule – and this unit was not used. Nevertheless, by simply
19 increasing the number of chambers contained in each culture unit (e.g. to 180 or
20 more), the device could be exploited to fully implement a miniaturized clonogenic

1 test directly on-chip. As described, the increase of the number of culture chambers
2 is also expected to improve the power of the statistical model; indeed, finer cutoff
3 thresholds could be considered to exclude chambers initially containing a number
4 of cells statistically different from the expected one.

5 Remarkably, a straightforward integration of this miniaturized model with a real-
6 time acquisition system for the automatic detection and quantification of colonies,
7 easily feasible thanks to the pre-determined and ordered positions of CFUs within
8 the optically transparent device, would represent a huge step towards an automatic
9 and user-friendly implementation of the clonogenic test.

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