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

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

Keywords: Lab-on-Chip, Microfluidics, Clonogenic test, CFU-GM, CBMNC

1. Introduction

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

One of the fields which could mainly benefit from the paradigm shift towards *in vitro* experimentation is toxicology, i.e. the study of drug toxicity (Gad, 1990). In particular, clonogenic assays are thought to better bridge the gap between toxicity experienced *in vivo* and toxicity effects on cells cultivated in the laboratory (cytotoxicity). Clonogenic assays are cytotoxicity tests based on the rate of survival of colony forming units (CFUs) in the presence of several drug concentrations, thus differing from traditional proliferation assays which take into account the mere increase in cell number. Specific advantages of clonogenic assays are related to higher accuracy and predictivity with respect to traditional proliferation tests (Pessina et al., 2004).

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

In 2004 Pessina and co-workers optimized a new protocol for CFU-GM assay based on the use of 96-well plates in place of standard Petri dishes thus reducing complexity and costs of the original test.
However, the assay still remains time-consuming. Colonies are generally counted only upon 14 days of incubation. In addition, the assay implies the dispersion of cells within a semisolid culture medium, which inhibits a pre-determined localization of colonies (within dishes or wells), which all must be manually found by a trained operator (Pessina et al., 2001).
Our goal was to develop a microfluidic device able to miniaturize the current state-of-art paradigm of *in vitro* hematotoxicity of xenobiotics protocol. Indeed, a further miniaturization of the original assay, down to the micrometer scale, will provide at the same time advantages in terms of cost reduction (cells, drugs, cytokines, etc.) and decrease of testing time (colony forming process). To this aim we designed a novel Lab-on-Chip (LoC) platform enabling for trapping, feeding and growing of multipotent monoclonal stem cells.

2. Materials and Methods

2.1 Lab-on-Chip design and fabrication

A grid-like distribution of micro-chambers was envisaged within the LoC device to organize the spatial distribution of cells and, in turn, to obtain a univocal correspondence between colonies and culture chambers.
For this purpose we designed a microfluidic platform divided in two functional areas: a serial dilution generator (Jeon et al., 2002; Kim et al., 2008; Lee et al., 2009) and a culture region (Fig. 1). The first one consists of a collection of channels (70µm high and 70µm wide) arranged in a resistive flow-based scheme to generate six linear dilutions of drugs (concentration ranging from 1 to 0, step 0.2) from two input ports to the culture region. This area is useful to evaluate the maximum tolerated dose of drugs on bone marrow progenitor cells.
Downstream, the culture region consists of 180 cubic chambers (side 150µm) divided in 6 units, each composed by 3 lines of ten culture chambers.
The microfluidic device is made of polydimethylsiloxane (PDMS) by means of standard soft lithography techniques. Briefly, a 4" (~10cm) silicon wafer, microstructured with two photoresist types, served as master mold to cast a thin layer (about 1mm) of liquid PDMS, being curing agent and pre-polymer mixed in a ratio 1:10 (w/w). A 3 hours thermal treatment in an oven at 65°C was applied to achieve a complete crosslinking. Subsequently, the cured PDMS stamp was removed from the mold and bonded, upon an air plasma treatment, to a previously casted slab of PDMS (thickness 4 mm) to close the fluidic features. The assembly was trimmed and drilled in correspondence of 4 inputs and 6 outputs, by means of biopsy punchers having diameters of 0.5 and 3mm, respectively. Finally, the LoC device was bonded to a histology glass slide upon a further air plasma treatment, sterilized through autoclaving (121°C, 20 min, wet cycle) and subsequently dried overnight at 80°C to recover PDMS hydrophobicity after plasma treatment, thus minimizing cell adhesion.

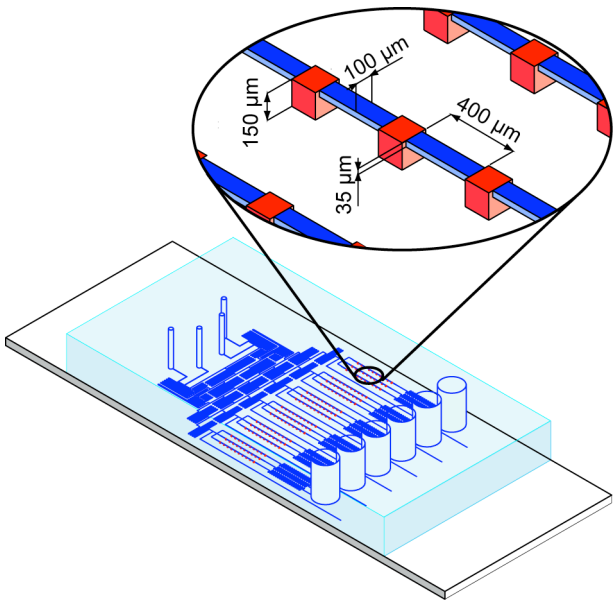

Fig. 1. The layout of the Lab-on-Chip device is shown together with a detail of a group of chambers within a culture unit. Two main input ports (together with two secondary ports) deliver dilutions of chemicals (100%, 80%, 60%, 40%, 20%, CTRL) to 6 culture units (30 chambers each).

2.2 Cell expansion and characterization

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

Most of the wash was removed by pipette leaving 1–2 ml behind so the cell pellet was not disturbed and was then resuspended in the remaining medium. Fresh IMDM with 1% l-glutamine and 10% FBS was added to the cell suspension and then centrifuged as above described. All the reagents were purchased from EuroClone (Italy). Cells were then resuspended in Complete Medium (CM), consisting of IMDM, containing 30% FBS, 1% L-Glutamine, 1% Penicillin/Streptomycin solution and 10 ng/ml recombinant human granulocyte/ macrophage colony-stimulating factor (r hu GM-CSF, RELIATech, Germany) to evaluate their number and viability.

Subsequently, CFU based tests were performed by using pooled donor cord blood-derived CD34+ cells, purchased frozen from Life Technologies, USA. The cells were stored and thawed according to the same protocol applied for CB-MNCs.

2.3 Cell seeding within the LoC platform

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

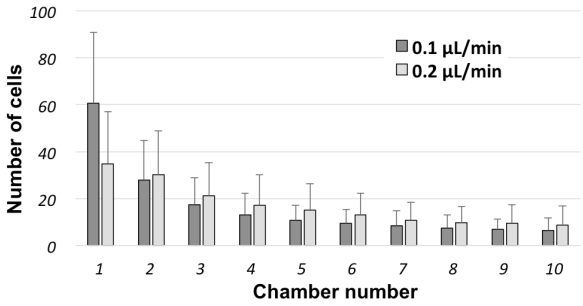

Fig. 2. Content in terms of cell number for each of the 10 chambers encountered by cells in their stream. Each device has 18 decades of chambers, and experiments were carried out for 9 and 8 replicates for 0.1 and 0.2 µl/min flow rates, respectively. Data is presented as mean ± standard deviation.

The cell distribution for both conditions tested is depicted in Fig. 2, which shows a decrement in population along chambers in the same fluidic path. In particular, the number of cells present in each chamber at the end of the seeding process resulted 14.2 ± 26.2 and 16.8 ± 18.7 for flow rates of 0.1 and 0.2µl/min, respectively. Although these values were similar, the higher flow rate provided a significantly lower number of empty chambers (24.4 ± 22.8 vs 11.4 ± 9.5), while reducing the seeding time (2.5 vs 5 hours). Thus 0.2 µl/min was chosen as seeding condition for the following experiments and corresponding data was used in the statistical model.

2.4 Statistical model of the clonogenic assay

In general, the toxicity effect of a drug molecule tested through a clonogenic assay shows up through a smaller number of CFUs with respect to a control condition wherein no drug is administered. For a suitable statistical analysis, we modeled this situation as if the drug was able to decrease the probability of any single cell to be clonogenic, while keeping the number of cells constant.

We denote with $\mathbf{p_0}$ the probability of any cell to form a colony in the control. According to

the results obtained with standard macroscale experiments, in our case study $\mathbf{p_0}$ was set equal to 1/146. We then set a hypothesis test to determine if there is statistical evidence to state whether the probability **p** of a cell to form a colony when drug is administered is smaller than the probability $\mathbf{p_0}$ in the control. Hence, in the null hypothesis we have $\mathbf{p}=\mathbf{p_0}$, while in the alternative hypothesis we have $\mathbf{p}=\mathbf{p_1}<\mathbf{p_0}$.

The seeding process suggested us to model the number of cells injected in each chamber as a random variable with a probability distribution depending on the position of the chamber in the corresponding line. Moreover, all the chambers in the chip were treated as independent variables, and all the lines and units were considered to have the same distribution of cells. From the data collected in the seeding characterization tests (Fig. 2, perfusion rate 0.2µl/min) we computed the empirical distribution of the number of cells for each position within the culture line (constituted by 10 chambers).

The probability to find a colony in each chamber was obtained by modelling the number of clonogenic cells per chamber with a binomial law, having the same number as the average number of cells in that chamber and probability equal to $\mathbf{p_0}$. Then, the distribution of positive chambers (*chambers+*, *i.e.*, chambers containing at least one colony) was computed for a chip with **n** chambers (or **n/10** culture lines). Simulations were run through the R environment (software version 2.13.1), and a set of 10000 repetitions was enough to obtain stable distributions.

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

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

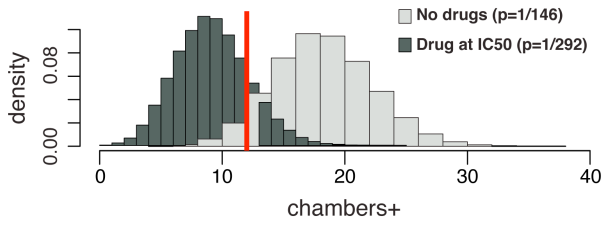

Fig. 3. Probability distributions of the number of chambers+ within the chip both when no drug (light grey; probability of proliferation $\mathbf{p_0}$=1/146) and a drug at a concentration corresponding to the IC50 (dark grey; $\mathbf{p_1}$=1/292) are administered. The vertical red line indicates the threshold **C**=12 used for the critical region of the test.

2.5 Implementation of the microscale clonogenic assay

CD34+ cells were seeded into the LoC device at a flow rate of 0.2 µl/min. Upon 2.5 hours, CM was prepared and perfused into the device at the same flow rate throughout the entire culture period (14 days). At specific time points (days 1, 5, 7 and 14) pictures from all culture chambers of the LoC device were taken to monitor possible variations in cell number. The results were compared to control experiments, consisting of devices perfused with culture medium free from cytokines.

CFU potential of CD34+ cells within the microfluidic device was also assessed in both conditions of absence and presence of a drug with a recognized hematotoxicity in clinical use. In particular, 1200 ng/ml (200X) of Paclitaxel (Toronto Research Chemicals, Canada) dissolved in dimethylsulfoxide (DMSO, Serva, Germany), were added to CM in order to have a final drug concentration of 6 ng/ml and a DMSO percentage of 0.5% (v/v). Paclitaxel was delivered to cells through the input ports through continuous perfusion. The culture was carried up to 7 days, and pictures of each chamber were taken at days 1, 5 and 7.

Pictures from the control device were taken at the same time-points and used as positive control.

3. Results and Discussion

The 2-weeks culture period within the microfluidic platform provided an increasing number of CD34+ cells in each chamber from day 1 to day 14 when perfused with CM, being the initial number of cells per chamber 13.1 ± 15.3 and the final 24.4 ± 21.0. As shown in Fig. 4 the number of cells did not change significantly along the culture in the control experiment.

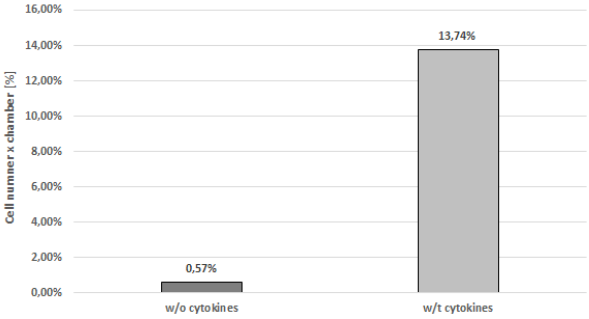

Fig. 4. Increase in cell number in a period of 14 days in culture in the LoC device. When cytokines are perfused together with the culture medium, the increase in cell number is significantly higher than in the case of perfusion with pure culture medium.

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

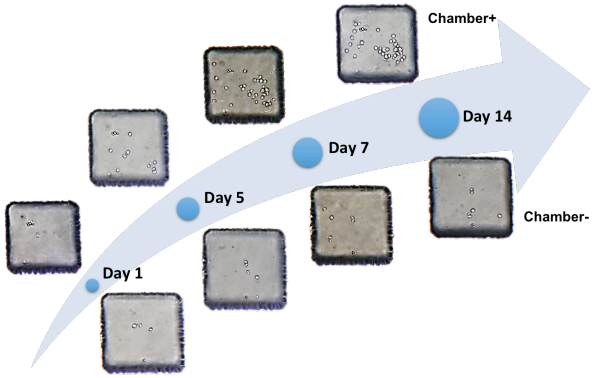

Fig. 5. Examples of a chamber containing at least one CFU (top) and no CFU (bottom).

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

i. relative cell number increase > 25%;
ii. absolute cell number increase > 4.

The number of resulting chamber+ was 15.33 ± 6.11 (n=3), in agreement with the statistical model.
Furthermore, as preliminary test, the number of chamber+ was also assessed in presence of Paclitaxel, a prototype toxicant molecule whose dosage was chosen, in agreement with our previous findings corresponding to half maximal inhibitory concentration (IC50). The number of chamber+ in absence of any toxicant substances was 14 (positive control), while chamber+ number was reduced to 6 when the drug was delivered. Fig. 6 shows a graphical representation of the distributions of chamber+ (green), chamber- (yellow) and empty chambers (red) for both cases.

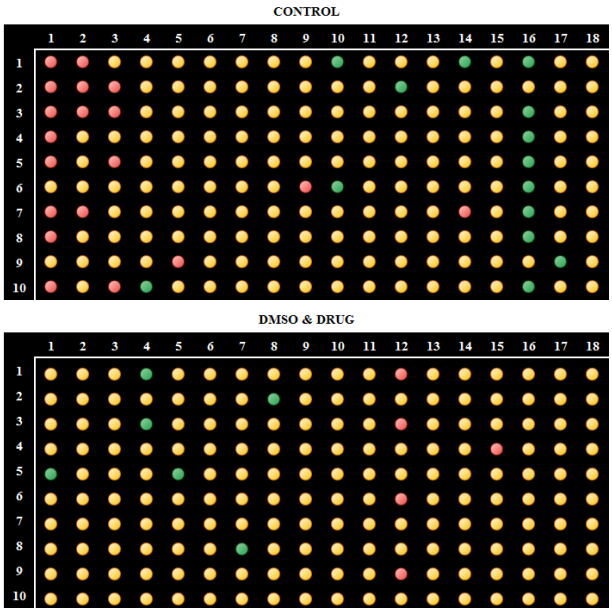

Fig. 6. Representation of positive (green), negative (yellow) and empty (red) chambers.

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

4. Conclusions

The proposed LoC device demonstrated its suitability as platform for CFU-based assays. The average number of colonies formed after five days of culture (15.33 ± 6.11) was adequate to detect the presence of a toxic dosage (IC50) of a drug. The assay time was dramatically reduced in two ways: i) the culture time decreased from 14 to 5 days, and ii) the colony identification was reduced to microscopy acquisitions on a well-defined plane (chamber/channel fluidic layer).
The device was provided with a serial dilution generator unit, able to precisely deliver to culture units predetermined concentration of soluble factors. However, the number of chambers implemented for these experiments (n=180) was only enough to catch the effect of a single test condition – namely the concentration of the administered molecule – and this unit was not used. Neverthless, by simply increasing the number of chambers contained in each culture unit (e.g. to 180 or more), the device could be exploited to fully implement a miniaturized clonogenic test directly on-chip in an automated manner.

Acknowledgements

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