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Miniaturized genotoxicity evaluation system for fast biomaterial-related risk assessment

Varvara Gribova,^{*a,b} Jesus Manuel Antunez Dominguez,^c Alan Morin,^c Julia Sepulveda Diaz,^c Philippe Lavalle,^{a,b,d} Nihal Engin Vrana^{*d}

With the growing number of implanted medical devices in the world, their advanced risk assessment becomes of the outmost importance. Ideal implants should be biocompatible and do not promote any adverse effects such as cytotoxicity, uncontrolled inflammation, and foreign body response. In addition to being non-immunogenic and non-cytotoxic, the materials used for implantation should be non-genotoxic, i.e. do not promote mutations that can potentially lead to tumor formation. However, given the level of complexity of genotoxicity tests, they are not readily available to the biomaterials researchers, therefore this aspect is severely undertested in the literature. To solve this problem, we developed a simplified genotoxicity test that can be adapted by standard biomaterials laboratories. The test achieves a decrease by half of the testing time and requires significantly less material and space. Automatization option with a customized testing chamber architecture and a microfluidics-based control system is also designed and possible. This optimized microfluidic chip system can significantly improve the availability of genotoxicity tests for biomaterials developers, with the additional benefit of more in-depth observation and quantitative comparison due to the availability of processable image components.

Introduction

With the growing number of implanted medical devices in the world, their advanced risk assessment becomes of the outmost importance.¹ Ideal implants should be biocompatible and do not promote any adverse effects such as cytotoxicity, uncontrolled inflammation, and foreign body response.² In addition to being non-immunogenic and non-cytotoxic, the materials used for implantation should be non-genotoxic, i.e. do not promote point mutations that can potentially lead to tumor formation.³

In addition of being biocompatible, implantable materials are being modified to render them bioactive. For instance, researchers are working on new antibacterial and anti-inflammatory coatings, or are adding drug delivery functionalities.⁴⁻⁷ In this context, correct genotoxicity evaluation is crucial, as bioactive molecules can potentially interact with the genetic material.

However, there is a lack of technologies and standardization for evaluation of biomaterials used to produce medical devices.⁸ Although ISO norms, or OECD guidelines for genotoxicity, provide rules for preclinical characterization of materials, multiple adverse effects of implantable devices (infection, inflammation, rejection, tumor formation) show that this characterization is insufficient, and novel approaches are required.¹

Another problem, which precedes application of ISO / OECD norms, concerns preliminary steps of materials development by materials scientists.

For quite a long period of time during new material's development, the researchers do not characterize biological response to newly

developed materials, or perform simple tests such as cytotoxicity evaluation, followed by *in vivo* tests on rodent models. Genotoxicity evaluation is not usual in (bio)materials assessment by research laboratories, probably due to its relative complexity and the necessity of using pathogenic bacteria.

Ames test, or bacterial reverse mutation test, is a classic genotoxicity evaluation assay that was introduced by Maron and Ames in 1983.⁹ It consists in using amino acid-dependent strains of *Salmonella typhimurium* and *Escherichia coli*, which restore their capacity to synthesize essential amino acids when exposed to a mutagen. While the classic test is performed in Petri dishes, a variation of the test in multiwell plates, called Ames fluctuation test or Ames microplate assay, was also developed. These tests require 48 hours (or up to 5 days for Ames fluctuation test) of incubation and either a lot of Petri dishes or, in case of Ames microplate assay, a lot of pipetting work. As an alternative, chromosomal aberration assays with eukaryotic cells can be used, but such tests remain complex and unsuitable for materials scientists' needs.¹⁰

To address this lack and to overcome the difficulty of materials evaluation, some research projects targeted development of new systems allowing easier, faster, and cheaper materials assessment. Among them, PANBioRA project has been developing a modular system for preclinical material's evaluation using cutting edge technologies such as cytotoxicity monitoring using sensors,¹¹⁻¹² organ-on-a-chip models,¹³ and mathematical modelling.¹⁴⁻¹⁵

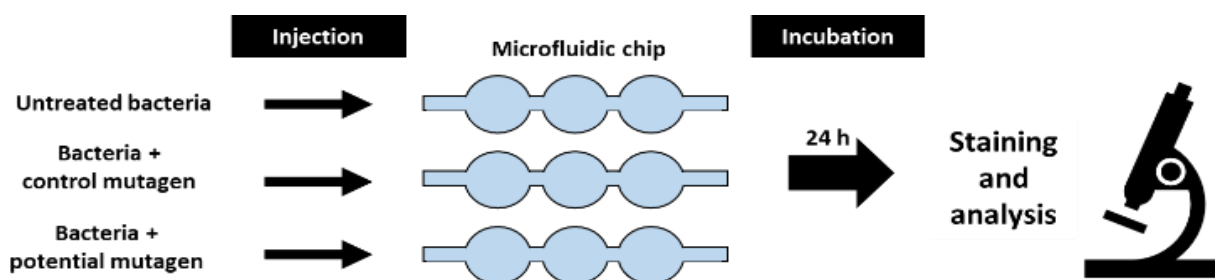


Figure 1. Schematic representation of the simplified, miniaturized Ames test. Untreated or pre-treated bacteria are injected into agar-coated microfluidic chip. After 24 hours of incubation, the cells are stained and observed using fluorescent microscope.

In the present work, we describe a new miniaturized and simplified genotoxicity testing system that saves place and can be observed using fluorescent microscopy after only 24 hours of incubation, reducing test time by two and allowing an easier readout which can be automatized by image analysis. In this test, *S. typhimurium* strain is pre-incubated with test chemicals and is deposited into agar-coated interconnected wells in a microfluidic chip, allowing to perform each test in triplicate using single injection (Figure 1). To our best knowledge, this is the first microfluidic-based genotoxicity test. While it can be performed manually by injecting the mixes into the device, at the end, we aim at generating a microfluidic module that will also minimize the contact of lab staff with genotoxic materials, chemicals and pathogenic bacteria. This will allow making the genotoxicity testing more accessible to material scientist and thus enable early detection of genotoxicity risks of promising biomaterials. Finally, the system can be applied to large populations of biomaterials in high throughput screening, taking genotoxicity testing to a completely new level.

Experimental

Materials

Oxoid #2 medium, minimal glucose agar plates, nutrient agar plates, Histidine/Biotine 0.5 mM solution, *S. typhimurium* TA100 strain, NaN₃ and mitomycin C, Moltox FT™ Mutagenicity Test Kit were purchased from Trinova Biochem (Germany). Agar was purchased from Sigma, PBS from Dutscher and SYTO™ 24 Green Fluorescent Nucleic Acid Stain from Invitrogen.

Elaboration of microfluidic chips

The chips were produced using stereolithography (SLA) 3D printing in BioMed Clear biocompatible resin (FormLabs™) with a printing resolution XYZ of 50 µm. Right after the chips are printed, resin is evacuated from inner channels by pushing air to prevent clogging. The chips are then submerged in isopropanol and stirred for 5 minutes. Finally, they are dried thoroughly and cured under UV light for 2 minutes. The chip design and features can be found in

Figure 2. After experiment preparation, the chip is sealed with transparent adhesive film (ARcare® 92524, Adhesives Research, Inc.) to close the open circuits while providing an observation window.

For automated microfluidic flow control, normally closed (NC) 2/2 valves (2/2-way Whisper Valve with media separation type 6712, Bürkert, Germany) are attached on top of the chip with double sided pressure tape (ARcare® 90106 NB, Adhesives Research, Inc.) with a EPDM rubber mat (EPDM rubber sheet SE95CC66, Solutions elastomères, France) for air-tight connection. A Mux Wire Valve controller (Elveflow, France) operated via the ESI software was used to control the 2/2 valves. Finally, a Cobalt autonomous pressure controller (Elveflow, France) was used for pressurizing the system. The pressure source is connected to the chip by using PMMA female luer lok connectors (Fluidic 302, Microfluidic ChipShop, Germany).

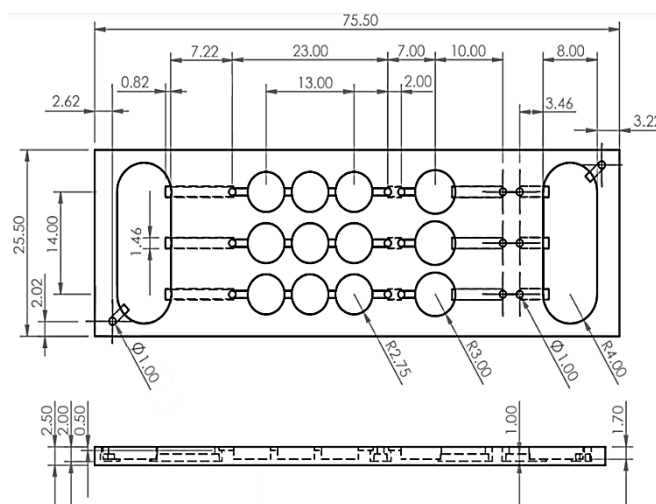


Figure 2. Detail of chip features of the microfluidic genotoxicity test. The test chip includes dedicated wells for agar, mixing chambers and elliptical reservoirs for reagents and waste, as well as one inlet and one outlet for injecting reagents, removing waste or to apply pressure. Chip dimensions are expressed in millimeters.

Table 1. Preparation of mixes for genotoxicity assay in Petri dish.

	Negative control	NaN ₃
PBS	600 µL	600 µL
H ₂ O	200 µL	-
NaN ₃ 15 µg/mL	-	200 µL
His/Biotin 0,5 mM	100 µL	100 µL
TA100	100 µL	100 µL

Table 2. Preparation of mixes for genotoxicity assay in 6-well plates

	Negative control	NaN ₃	Mitomycin C
PBS	5 µL	5 µL	5 µL
H ₂ O	30 µL	-	-
NaN ₃ 15 µg/mL Mitomycin 5 µg/mL	-	30 µL	30 µL
His/Biotin 0,5 mM	15 µL	15 µL	15 µL
TA100	100 µL	100 µL	100 µL

Bacterial assays

S. typhimurium TA100 strain bacteria were pre-cultured aerobically at 37°C in Oxoid medium. For this, one colony from previously prepared agar plate with streaked bacteria was transferred to 10 mL of medium and incubated at 37°C. Overnight culture was adjusted to OD₆₂₀ nm = 0.5 for the assays. For genotoxicity assay in Petri dish, the samples were prepared as indicated in Table 1, and 1 mL of the resulting mix was spread onto d = 10 cm Petri dish. The dish was dried and incubated at 37°C for 48h. The number of colonies per dish was quantified.

For genotoxicity assay in a 6-well plate, the volumes were adjusted for a smaller area (Table 2), the mixes were spread and incubated at 37°C for 24h, before being stained with 5 µM SYTO™ 24 dye for 10 minutes at 37°C and fixed with 4% PFA for another 10 minutes. After that, the samples were transferred upside down into a plate with glass bottom and observed using Zeiss LSM 710 confocal microscope. Fluorescence intensity was quantified from 3 images per condition using ImageJ software. The validation was performed using Moltox FT™ Mutagenicity Test Kit as a reference, according to manufacturer's instructions. Petri dish test and Moltox FT™ Mutagenicity Test were performed in parallel.

For the assays in a microfluidic chip, hot Minimal glucose agar (1.5 % agar, 2% glucose, 1x Vogel-Bonner medium E 9) was added to the wells and allowed to cool. Genotoxicity tests were performed in the wells filled with 27 µL of agar. Bacterial mix was prepared as indicated in Table 1, and the mix was injected into the chip until the wells were filled. After 24h of incubation at 37°C, the liquid in the wells was replaced by Syto 24 staining solution, then by PFA 4%, and the samples were observed using Zeiss LSM 710 confocal microscope. Fluorescence intensity was quantified from 6 or 9 images per condition using ImageJ software.

Results and discussion

Setup of the Ames test without top agar

The classic version of the Ames test includes mixing bacteria with melted top agar,⁹ although some protocols on the web omit this stage (Figure 3A). As an initial step, we established conditions for a successful no-top-agar Ames test (Table 1) that gave good difference between non-treated and NaN₃-treated samples (Figure 3B and C).

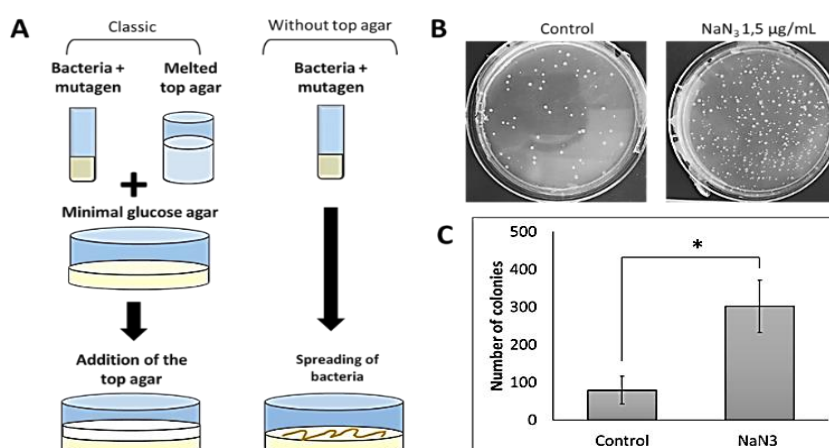


Figure 3. Setup of Ames test without top agar. (A) Comparison of the two protocols. (B) Images of bacterial colonies in 10-cm diameter Petri dishes after 48 h of incubation at 37°C. NaN₃ was used at 1.5 µg/mL. (C) Quantification of the number of bacterial colonies, averages from 3 independent experiments, * p<0.05.

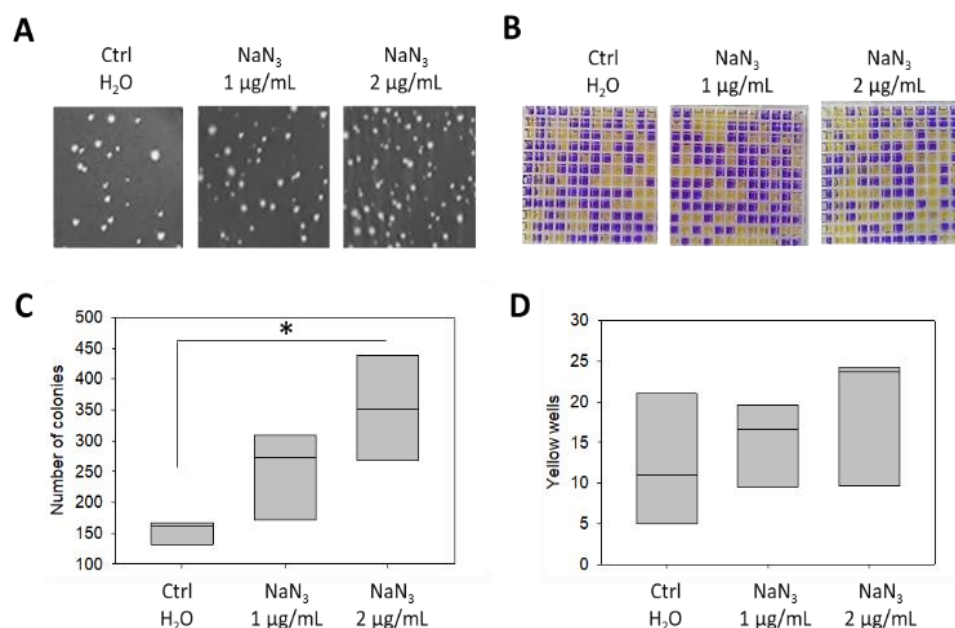


Figure 4. Comparison of Ames test without top agar with Ames fluctuation test. (A) and (C) Ames tests in Petri dishes. (B) and (D) Commercial Ames test using Moltox FT™ Mutagenicity Test Kit. Results of three independent experiments are shown (n = 3). *p < 0.05

Thus, 3 times more colonies were detected in NaN₃-treated condition, compared to untreated bacteria. The results were obtained after 48 hours of incubation. NaN₃, sodium azide, is a well-known powerful mutagen, so the appearance of mutant colonies in NaN₃-treated condition was expected.

The method was compared to a commercial Ames fluctuation test using Moltox FT™ Mutagenicity Test Kit (Figure 4). The results showed the presence of statistical difference between control and NaN₃ 2 µg/mL condition for Petri dish test (Figure 4A and C), as compared to Ames fluctuation test (Figure 4B and D). In addition, for Ames fluctuation test, there was an absence of statistical significance between control and NaN₃ condition, probably because of big variability of the control condition.

Mutagenicity detection by fluorescent microscopy

As a next step, we adapted the test to a 6-well plate format (Table 2), and detection was done after bacteria staining and observation by confocal microscopy. The results show a good contrast after only 24h, with 3-fold and 4-fold increase in fluorescence intensity for NaN₃ and mitomycin C-treated bacteria, respectively (Figure 5A and B). Thus, we demonstrate a totally new approach for genotoxicity testing, which takes only 24h and can be expressed as fluorescence intensity measured using ImageJ software. In addition, higher magnification images allow evaluation of bacterial morphology as a response to a mutagen. For instance, bacterial filamentation was observed in mutagen-treated conditions (Figure 5C).

Genotoxicity test miniaturization

To miniaturize the test, we used the microfluidic chip described in the Materials and Methods section (Figure 6A). Microfluidic chips were designed to comply with several technical needs. First, they had to include enough channels and wells to include the minimum number of conditions and replicates in a single experiment, thus improving experimentation time and yield of results compared to the standard Ames test, while being easily loaded with agar by hand pipetting. Second, their fabrication should be compatible with potential scale-up production methods, this parameter including as well the choice of material. Third, they had to be also compatible with microfluidic flow control equipment, in order to automate the whole process according to the user's lab equipment and needs.

Initially, the chips were developed through micro-milling using a polycarbonate substrate (Lexan™, Sabic Innovative Plastics). This method proved to be efficient in number, as the equivalent of 9 chips were produced in each batch, for a total time of 7 hours (approximately 45 min per chip). However, the procedure was then changed to 3D printing in BioMed Clear biocompatible resin (FormLabs™), and thus, the production time was reduced to 22 min per chip. This method decreased the surface roughness and implemented an updated design, including the reservoirs in the microfluidic chip. As opposed to micro-milling processing, 3D printing also allowed to have channels inside the volume of the chip, simplifying the preparation process to seal the bottom circuit needed in the micro-milled version.

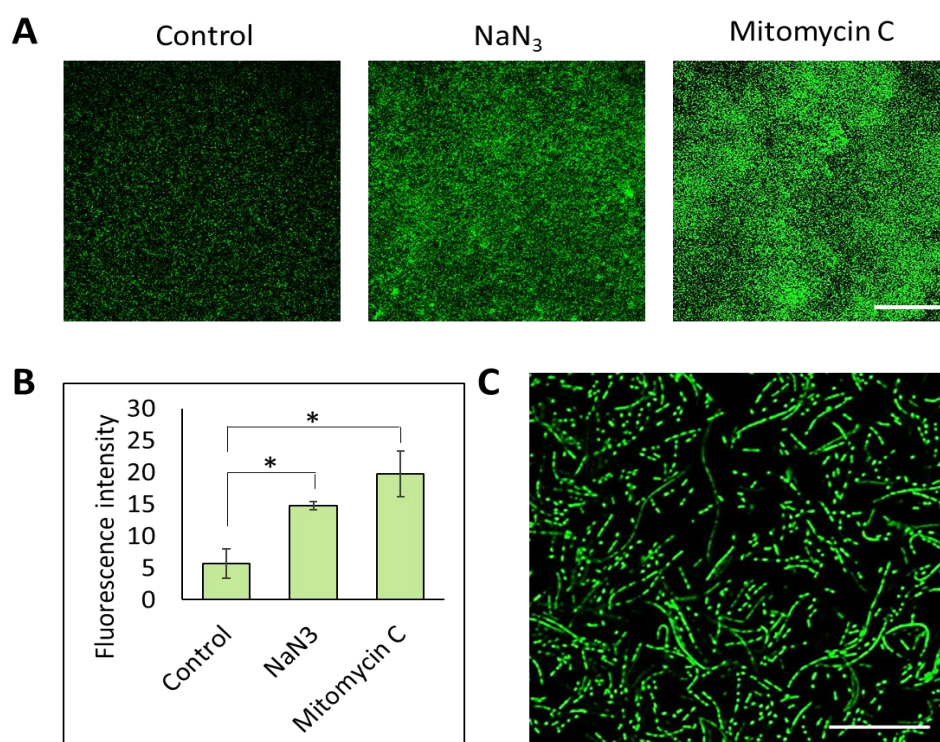


Figure 5. Mutagenicity detection by fluorescent microscopy. The test was performed in 6-well plate. After 24 hours of incubation at 37°C, bacteria were stained with Syto 24, fixed and observed using Zeiss LSM 710 confocal microscope. (A) Images taken with 10x magnification; scale bar corresponds to 400 μ m. (B) Quantification of fluorescence using ImageJ software, the averages from 3 independent experiments \pm SD, * p <0.05. (C) Images of NaN₃-treated sample taken with 40x magnification; scale bar corresponds to 50 μ m.

The resulting cartridges have an open circuit on the top side to fill the wells with agar and reagents (Figure 6A). The circuit consists of three parallel, independent channels connecting three consecutive circular well or culture chambers (to be filled with agar for bacterial culture). In each line, the set of three chambers is preceded by an additional well that act at the same time as a reservoir for test reagents and as a mixing chamber to homogenize the solutions perfused in the culture wells with such test reagents. The lines start and end in two common elliptical reservoirs, the one proximal to the mixing chambers act as a common reservoir for bacterial suspension, and the opposite one as a total waste container.

The bottom of the culture wells is filled with 27 μ L of minimal glucose agar. This volume was determined experimentally as being the optimal for both homogeneous well coating and for avoiding bubble formation during injection of the different liquid reagents. The agar coating was then left to cool down and solidify. Then, the cartridge was sealed with the transparent adhesive film to close the open circuits while providing an observation window. For experiments using automated microfluidic flow control, the valves were attached on top of the cartridge with double sided pressure tape. However,

this step is not necessary if the experiments are performed manually using a pipette.

For the genotoxicity test in the microfluidic chip (Figure 6A), bacterial suspensions prepared as indicated in Table 1 were injected. After 24 h, bacteria were labeled and observed using confocal microscope (Figure 6B), displaying significantly less fluorescence in control conditions compared to NaN₃-treated bacteria (Figure 6C). We thus demonstrate that fluorescent Ames test has the potential to be used for genotoxicity evaluation, making it faster and using less material and space. Moreover, the test allows observation of bacterial behavior/morphology (e.g., filamentation, Figure 5C), making it possible to have qualitative parameters in addition to quantitative measurements.

Microfluidic setup for automated flow assay

The microfluidic assay developed herein is designed to automate the Ames test. As mentioned above, this test is also designed to be compatible with pipette use in absence of flow controllers for a fully manual implementation.

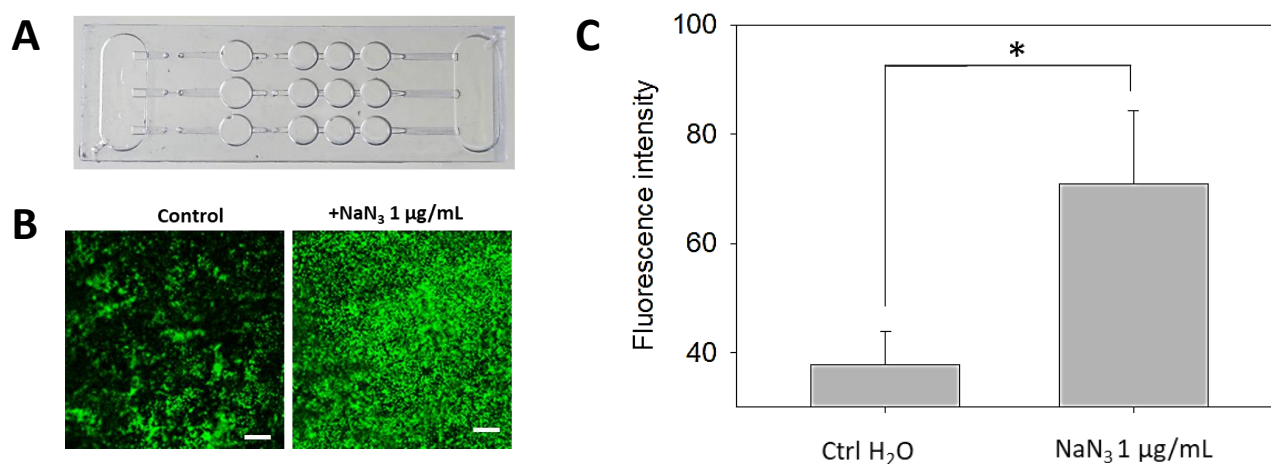


Figure 6. Genotoxicity test miniaturization using a microfluidic chip and fluorescence detection. (A). Image showing the final design of the microfluidic chip. (B) Images taken with 10x magnification; scale bar corresponds to 50 μm . (C) Quantification of fluorescence using ImageJ software, representative results from three independent experiments are shown, * $p < 0.05$.

The setup consists of an autonomous pressure source (Cobalt, Elveflow) to convey the liquids from the elliptical reservoirs into the mixing wells, then to the microfluidic culture chambers (Figure 7). The wells are filled with agar during the preparation phase, sealed with transparent film and loaded with the bacterial suspension and reagents. The valves are fixed on top of the chip with double sided tape, aligned with the orifices between the inlet reservoirs and mixing chambers. After connecting the pressure source to the inlet, the chip is ready to perform the automated experiments.

In order to minimize the size of the setup and its complexity, liquid control is only based on pressure in the final microfluidic system, which is described herein. The optimal pressure value was assessed through a series of experiments using a microfluidic flow sensor (MFS2, Elveflow) added to the setup for the optimization of the assay (Table 3). Bubble formation was evaluated in three channels of three consecutive wells for each of nine flow rate values in a range from 20 to 60 $\mu\text{L}/\text{min}$ in steps of 5 $\mu\text{L}/\text{min}$. The results showed that a flow rate of 25 $\mu\text{L}/\text{min}$ yields the least number of bubbles. This translates in a filling time of 108 s for one line comprising three wells using 45 μL of suspension.

Table 3. Assessment of the optimal filling time of the wells to avoid bubble generation in a flow-based microfluidic system. Results of each experiment (R1-R3) are indicated, the numbers correspond to the quantity of the bubbles. *Significantly different to 60 $\mu\text{L}/\text{min}$, #significantly different to 55 $\mu\text{L}/\text{min}$ ($p < 0.05$).

Flow rate ($\mu\text{L}/\text{min}$)	Pressure (mbar)	Filling time (s)	R1	R2	R3	Mean	SD
20	95	135	2	1	4	2.333	1.528
25 [#]	105	108	2	0	1	1.000	1.000
30 [*]	120	90	1	2	3	2.000	1.000
35	140	78	2	4	2	2.667	1.155
40	160	68	4	3	2	3.000	1.000
45	180	60	3	4	4	3.667	0.577
50	205	54	3	2	5	3.333	1.528
55	230	50	4	5	5	4.667	0.577
60	255	45	5	6	5	5.333	0.577

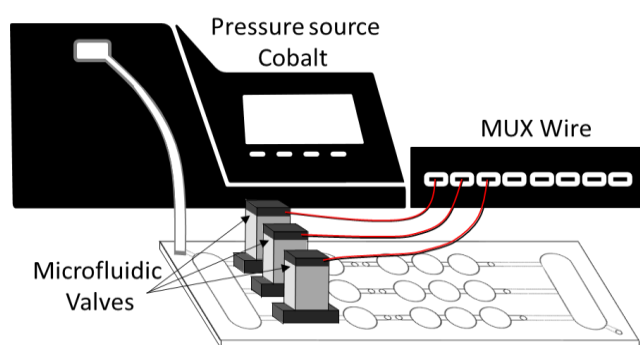


Figure 7. Microfluidic setup for automated delivery of bacterial suspensions treated or not with test reagents. The design integrates reservoirs in the microfluidic chip and the agar wells. The mounted Normally closed (NC) 2/2 valves open or close a specific circuit channel, alternating between positive control, negative control and test sample. Of note, the microfluidic chip and pressure source stand on the same surface to ensure the right pressure.

According to the previous results, pressure in the range of approximately 100 to 120 mbar can yield such filling time, if the chip and pressure source rest on top of the same surface, ensuring reproducible results for the same pressure values. The liquid filling sequence is activated through a constant pressure value and alternating liquid circulation by opening and closing the valves (Figure 8). For emptying the wells after inoculation, the pressure value is not critical, and higher pressures can be used to dry the agar faster. The agar is considered ready when no overflowing liquid can be observed in the agar wells, which might take different time according to environmental conditions.

The sequence can be repeated for a staining procedure after the agar is inoculated and incubated. In that case, the reservoir is filled with the corresponding dye and the process is repeated. Similarly, the washing process can be automatized with the same sequence.

Conclusions and Perspectives

Compared to the classic Ames test in Petri dishes, the system described in this work offers easier and faster genotoxicity evaluation, which also requires much less space. At the same time, it is also easier and faster than the Ames fluctuation test performed in 384-well plates. Moreover, the assay allows qualitative evaluation of bacterial morphology to assess bacterial reaction to treatment (e.g. filamentation).

Although in this work we used only one *S. typhimurium* strain, TA100, the test can be performed with other *S. typhimurium* strains containing different mutations. In addition, S8 enzyme which mimics metabolic activity of the liver can be used during pre-incubation step.

Two versions of the assay can be implemented: i) an automatable, flow-controlled version that allows for increased repeatability and reduced manipulation time from the user, and ii) a manual version that can be performed using a micropipette.

Our system will potentially allow biomaterials scientists to perform genotoxicity tests earlier during new materials development, and thus save time and money.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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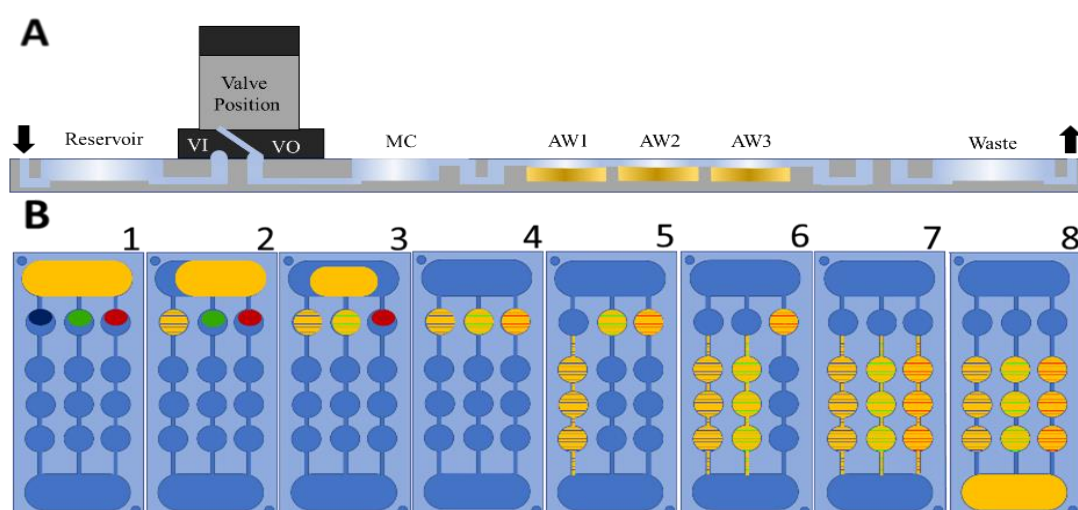


Figure 8. Microfluidic chip design. (A) Cross section of one of the circuit channels of the chip showing the channels and well connections. The inlet and outlet are represented by black arrows. The reservoir is shared by the three lines of the chip. Then the chip is connected to the outside through the valve inlet (VI) and outlet (VO), matching the valve position. In the three circuit lines, there is a mixing chamber (MC) to mix the sample, negative control and positive control with bacterial suspension. Three agar wells (AW) are the chambers where the bacterial culture will take place, connected to the waste reservoir that leads to the outlet of the chip. (B) Schematic of the liquid injection sequence. B1: Microfluidic chip ready to use after loading with the bacterial suspension in the reservoir (yellow), and the test reagents in the RCs (black, green, red). B2-B4: Pressure source is activated for the sequential injection of bacterial suspension into the three RCs containing the test reagents. B5-B7: sequential injection of the mixed reagents in their respective channels and AWs. The pressure source is stopped to allow sedimentation of the bacterial suspension. B8: AWs are seeded the suspension is then conveyed to the waste, pushing with air, to concomitantly dry the circuit lines in the same order they were filled.