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SHORT COMMUNICATION

Microscreening toxicity system based on living magnetic yeast and gradient chips

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Abstract There is an increasing demand for easy and cost-effective methods to screen the toxicological impact of the growing number of chemical mixtures being generated by industry. Such a screening method has been developed using viable, genetically modified green fluorescent protein (GFP) reporter yeast that was magnetically functionalised and held within a microfluidic device. The GFP reporter yeast was used to detect genotoxicity by monitoring the exposure of the cells to a well-known genotoxic chemical (methyl methane sulfonate, MMS). The cells were magnetised using biocompatible positively charged PAH-stabilised magnetic nanoparticles with diameters around 15 nm. Gradient mixing was utilised to simultaneously expose yeast to a range of concentrations of toxins, and the effective fluorescence emitted from the produced GFP was measured. The magnetically enhanced retention of the yeast cells, with their facile subsequent removal and reloading, allowed for very convenient and rapid toxicity screening of a wide range of chemicals. This is the first report showing magnetic yeast within microfluidic devices in a simple bioassay, with potential applications to other types of fluorescent reporter yeast in toxicological and biomedical research. The microfluidic chip offers a simple and low-cost screening test that can be automated to allow multiple uses (adapted to different cell types) of the device on a wide range of chemicals and concentrations.

Keywords Toxicity screening · GFP reporter yeast · Magnetic retention · Microfluidic devices

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Introduction

There is an urgent need for simple toxicity screening systems for environmental and regulatory reasons. For instance, the introduction of the new European Union regulations for the Registration, Evaluation, Authorisation and Restriction of Chemicals which has placed a duty on manufacturers to assess the safety of the chemicals they manufacture, including formulations, for both humans and the environment [1]. There is a profound difference with respect to monitoring the safety of individual chemicals compared to mixtures, as synergistic effects from several compounds in a formulation or product can occur. Approved animal-based toxicity testing methods are expensive, and (Q)SAR methods are not designed to test

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synergistic effects in mixtures. This creates a need for simple screening methods to identify products that may be of concern. Although fully automated commercial screening systems based on 96-well plate systems have been developed for the pharmaceutical industry, these are not relevant for companies that just want to check different formulations of a relatively low number of chemicals.

Toxicity screening using yeast is widely employed for detecting oxidative stress factors [2], genotoxic chemicals [3] or endocrine-disrupting chemicals [4]. Recombinant budding yeasts *Saccharomyces cerevisiae* have been developed, containing fluorescent markers such as green or red fluorescent protein (GFP or RFP), and have been proven to be extremely useful for microscreening because they release the fluorescence without the necessity of adding any substrates. For example, GreenScreen™ yeast cells have been genetically modified to express the GFP whenever the cells carry out repair of DNA damage, and this has been used to detect genotoxicity and cytotoxicity simultaneously [3]. Under genotoxic conditions, the fluorescence emission increases. GreenScreen™ is a well-established yeast reporter, and it has been employed for a range of applications including screening of industrial products and environmental samples [5, 6].

The incorporation of such screening tools within a miniaturised system has many advantages including a small sample and reagent volumes as well as the biomimetic microenvironment within microfluidic systems, which is ideal for microorganism maintenance. The microfluidic environment with its inherent high surface area-to-volume ratio provides a tool that creates a more in vivo-like cellular microenvironment in vitro than current methodology offers. A number of elegant microfluidic cell-based handling applications have been described for drug development, tissue engineering, molecular diagnostics and biosensors [7–9]. Microfluidic systems have been used to analyse single cells, including bacterial, fungal, yeast and mammalian cells [10–14]. It has also been shown that the gradient mixing can be employed to facilitate the screening process by automatically providing the range of reagent concentrations [13, 15].

Yeast cells are small (<12 μm) and can thus be easily loaded into microfluidic devices. However, it can be challenging to retain the cells within the device and in a viable condition [14]. One method of overcoming this problem is to coat the yeast with magnetic nanoparticles [16] and then employ magnetic fields to retain them inside the microfluidic devices [17].

In this communication, we present a simple, low-cost method for "on-chip" testing of toxicity, incorporating both gradient mixing of toxins and magnetic GFP reporter yeast cells retained and released by the application of external magnetic fields.

Experimental procedures

Chemicals and materials

GreenScreen™ yeast strains, specialist resuscitation and assay media were supplied by Gentronix Ltd. (Manchester, UK). A DNA-repair-competent strain of the brewer's yeast *S. cerevisiae* was employed as the host strain for a reporter of DNA repair activity (the "test" strain). Viability test was carried out using fluorescein diacetate (F7378, Sigma-Aldrich). On exposure to a genotoxic agent, the cells become increasingly fluorescent as GFP accumulates. MMS (# M4016) was employed as the genotoxic standard and dimethyl sulfoxide (DMSO # D8418) as diluent (purchased from Sigma-Aldrich).

Microfluidic devices manufacture

"Glass-on-glass" and "polydimethylsiloxane (PDMS)-on-glass" microchips were designed with multi-gradient pattern (Fig. 1a). The design was drawn using auto-CAD software and transferred by a commercial process (J. D. PhotoTools, Oldham, UK) to a film photomask. Crown white glass (B270) plates coated with chrome and photoresist (Telic Co., CA, USA) were contacted with a photomask design and exposed to UV radiation. The plates were then treated with photoresist developer followed by chrome etch solution (Rohm-Haas Ltd. UK). The exposed glass channels were etched at a rate of 4 μm/min in a 1% hydrofluoric acid/5% ammonium fluoride solution at 65 °C. A pine tree pattern of channels were designed in order to get a gradient from two inlets (one containing culture medium and the other containing a high concentration of standard or test sample). Channels (40 μm wide and 80 μm deep) with three series of serpentines consisting of four completed loops and the yeast chambers (up to 400 μm wide and 40 μm deep) were fabricated by wet etching the glass [14]. After a cleaning process, the etched plates were thermally bonded (595 °C for 3 h) to top plates with drilled access holes.

The fabrication of the PDMS glass microchips used the same methodology as described above for the glass base plates. Briefly, these microchips were developed using the etched glass bonded with two consecutive PMDS layers. PDMS (Sylgard 184 kit, IPA, HMDS) were polymerase in two layers. The upper layers of PDMS (0.1 and 5 mm, respectively) were polymerised and hardened for 2 h at 90 °C and plasma-bonded via Oxigen to the glass (Fig. 1a).

TFZL tubing (i.d. 1/16 in, Upchurch Scientific) was used to interface the chip with syringes containing culture medium.

Microscreening bioassay

The magnetisation of the GFP report yeast with nanoparticles, and the posterior viability test of the cells are

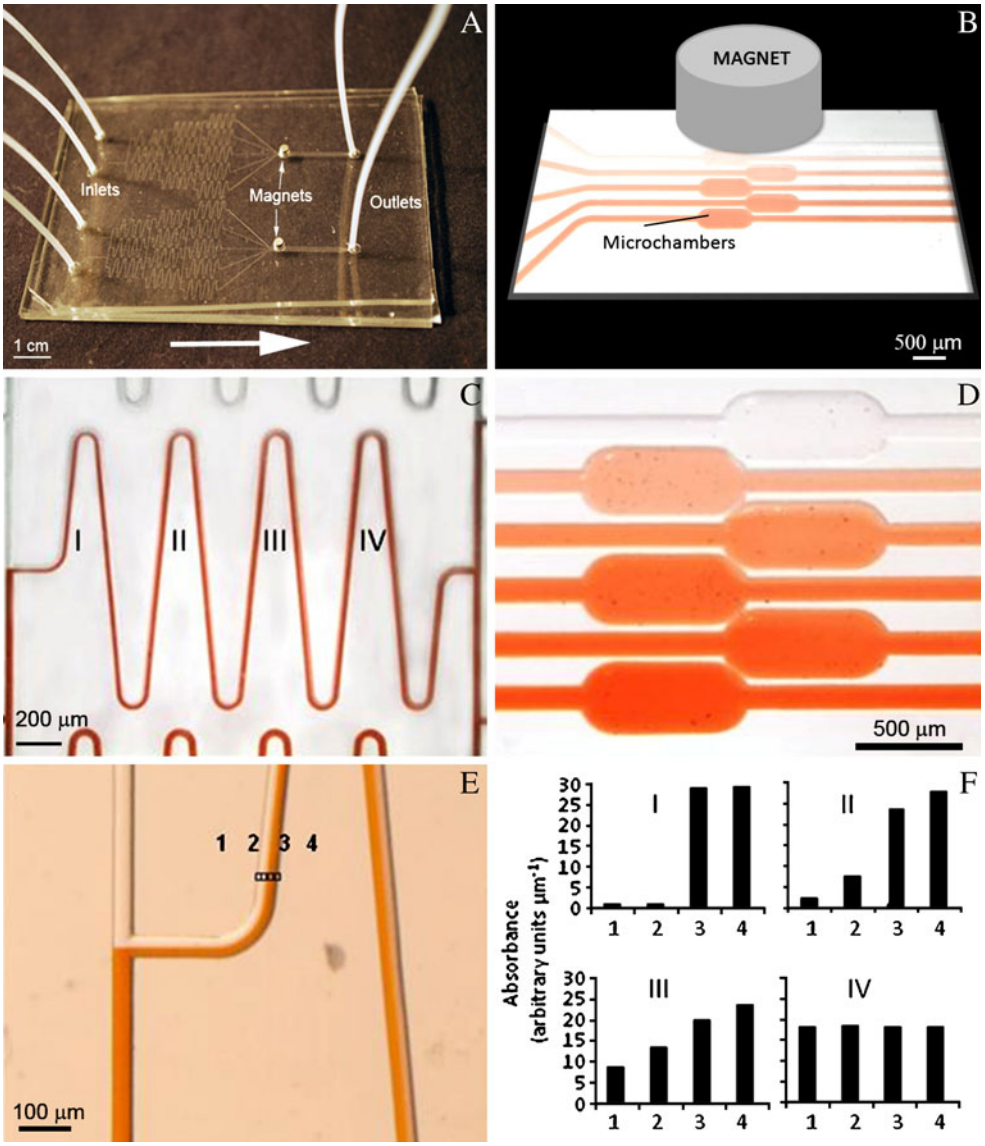

Fig. 1 The gradient microscreening system. **a** PDMS glass microscreening system. Two parallel systems are used, one to test an unknown sample and the second with a reference. The magnets were placed above the chambers to retain the yeast. *Arrow* shows the laminar flow direction. **b** A scheme showing the position of the magnet on the top of the chambers. **c** The details of the serpentine pattern visualised with safranin (dye) showing the four loops that contain each serpentine. **d** Generated concentration gradients in the microchambers visualised with red ink (safranin) at the working flow rate. **e** The first step in the pine-tree-shaped pattern, indicating the squares where the intensity (densitometry) was analysed to corroborate diffusion and complete homogenisation. **f** Densitometry of the squares at each loop indicating laminar flow and posterior diffusion at each serpentine

described in the Electronic Supplementary Material Fig. 1S. Based on the previous prototype, the devices allowed the performance of toxicity bioassays by placing the microchip under an inverted microscope, such that all the chambers could be observed at the same time at 40 times magnification. The specific assay culture medium was then pumped into the microfluidic device at a very low flow rate of 0.1 µL min^{-1} using two pumps (KDS-200CE, KD Scientific®). One inlet of the system was connected to a syringe containing culture medium + MMS at 0.005%, while the other inlet was connected to a syringe with culture medium and diluent (DMSO) (see Fig. 1SA).

Aliquots (50 µL) of magnetic yeast suspension were pumped into the microchip. The yeast were trapped in the chambers, and then the culture media (with or without the addition of MMS) was passed over the cells for 14–16 h at 25 °C. After exposure to MMS, yeast were excited at 485 nm, and the fluorescence emission detected at 520 nm under an inverted microscope (Olympus IX71) using a ×4 objective (UPLFLN). The induction of GFP fluorescence in exposed yeast was compared to the constitutive expression of GFP in the control cells, as they maintain their DNA integrity.

Fluorescence emission was quantified as intensity per area using Cell D™ software. Ten squares (5,000 μm^2) were analysed per chamber, obtaining mean intensity per treatment. Comparisons of the fluorescence intensity were performed by analyses of variance followed by Dunnett's T_3 post hoc test, using SPSS 16.0 software. Genotoxicity was corroborated when significant differences appeared ($p < 0.05$) between those cells exposed to MMS and those under the control condition (2% DMSO only). When the bioassay is finished, the yeast is easily discarded from the devices by removing the magnets on the top of the chambers and flowing culture medium through the channels and chambers. After that, the systems are ready to re-load yeast and start a bioassay again with very good reproducibility.

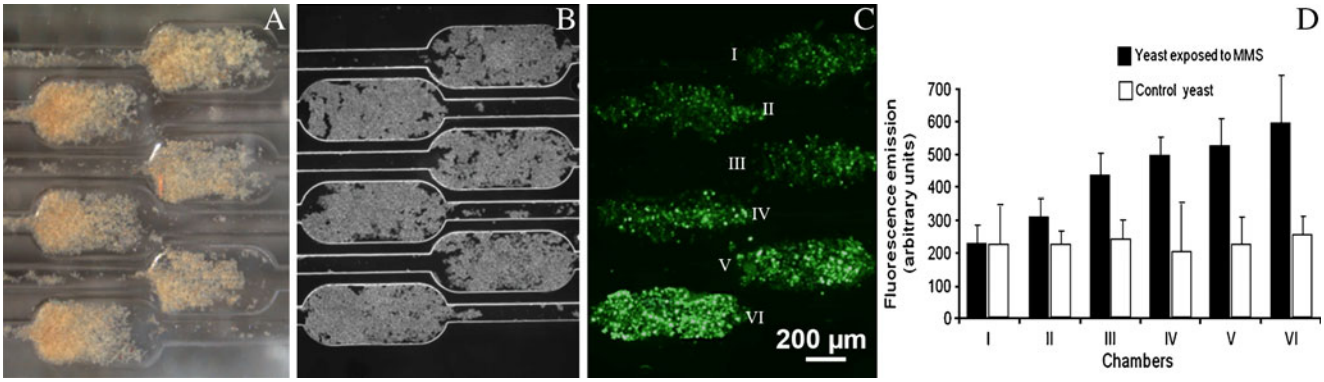

Fig. 2 A six-concentration microscreening system. **a** Yeast cells are loaded into the system and retained via the external magnetic field; **b** after the bioassay (14 h), yeast remained in the chamber (light microscopy image); **c** fluorescence emission from yeast, showing the GFP expression and intensity correlating with the amount of genotoxic compound. **d** Fluorescence quantification (intensity per square millilitre, mean ± SD). The concentration range of MMS were 0, 28, 56, 112, 225 and 450 µM (*black columns*). White columns correspond to a control parallel system (not shown); chamber was exposed only to the diluent (culture media containing 2% DMSO)

Results and discussion

The microfluidic device, designed to generate concentration gradients and based on magnetically functionalised GFP reporter yeast, was evaluated using both glass and PDMS glass microchips. The devices were found to maintain a concentration gradient (Fig. 1c–f) and to retain the cells within the microchambers (Fig. 2a). The glass microchips were found to be more robust for handling, although the multilayer PDMS glass devices offer great potential to develop 3D microscreening devices with low cost of production.

Visualisation of concentration gradient was performed in the system by using the dye safranin instead of the genotoxic agent (Fig. 1c, d). The serpentine pattern increases the length of the channels, allowing the lateral diffusion and complete homogenisation for each concentration before reaching the chambers containing the yeast (Fig. 1c). As can be seen from Fig. 1d, the concentration gradient was well established in chambers that housed the yeast cells. A direct magnetisation of yeast with magnetite nanoparticles were achieved previous to the bioassay (Fig. 1SB, C). The nanoparticles were located at the surface of the cell walls and did not internalise into the cells (Fig. 1SC).

In this work, the utilisation of magnetically retained living fluorescent yeast was found to overcome cell retention problems commonly reported in microfluidic devices [14]. Yeast was successfully retained by placing a small neodymium magnet on the top of the chambers (Figs. 1b and 2a). The removal of the magnet led to sweeping of the yeast cells out of the microchip. The fluorescence emitted by GFP reporter yeast used indicates that the gene expression and synthesis of GFP were not affected either by the coating or the devices.

A genotoxicity bioassay was developed in the microscreening device, with a proportional increase of fluorescence upon increasing the genotoxic compound and statistically significantly higher than the control system (Fig. 2c, d).

At the level of the microchambers where the yeast are maintained, the speed of the culture medium flow was at 21 $\mu m s^{-1}$, calculated as the flow rate/cross-sectional area. At this speed, the flow allows the magnetic fields to retain the yeast inside the chambers. As expected, an increase in fluorescence intensity was observed towards the chambers where the yeast was exposed to the higher concentration of genotoxic agent (MMS, Fig. 2d). Using selected areas for fluorescence intensity quantification, it was possible to clearly differentiate between the cells exposed to MMS and those in the control chamber (the yeast strain exposed to DMSO alone). Yeast exposed to the lowest concentrations of MMS showed the lowest values in arbitrary units of 75.57±15. Then, the increasing concentration generated by the gradient system proportionally increased the fluorescence until values of 455±55 were reached (Fig. 2).

The methodology developed was found to offer an effective way of locating magnetically functionalised GFP reporter yeast on the chip, including magnet-facilitated loading of the cells, their retention during the toxicity tests and their release upon removal of the magnetic field (Fig. 1SB). Further optimisation of flow designs was carried out in order to fine-tune the system and avoid any uneven distribution of cells within all chambers. Based on its dynamic, this microscreening system presents several advantages over the static plate-based bioassays. It allows simple testing of different formulations and concentrations, regulated fresh nutrient and analytes inputs, adaptability to diverse conditions, keeping diverse cells types in optimum conditions and potential portability.

This screening test can be easily automated to allow multiple uses in biomedical, industrial and environmental monitoring studies. Miniaturisation of toxicity screening methods is very promising for toxicity testing of new formulations and for environmental monitoring. Future work will involve the spatial reduction of the microfluidic pattern and increase the number of toxicities to be tested in parallel.

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