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Lab-made 3D printed stoppers as high-throughput cell migration screening tool

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ABSTRACT

Cell migration is a process that underlies the development and maintenance of multicellular organisms, with profound implications in various pathologies. The study of cell migration is fundamental in various fields of basic biology and pharmaceutical development. Wound healing assay is an indirect way to assess cell migration. Conventional methods, such as the scratch test, are inexpensive and easy to execute but have the disadvantages of being poorly reproducible and difficult to perform on a high-throughput scale. Meanwhile, commercial strategies are expensive. In the present work, we developed a lab-made wound healing assay device that is inexpensive, easy to handle, and reproducible. We designed 3D-printed stoppers compatible with cell culture in 96-well plates. These stoppers did not affect HaCaT cells viability. The stopper-produced initial wound size was reproducible on a high-throughput scale. Also, stoppers demonstrated their effectiveness to evaluate cell migration and allowed differentiating treatments with and without fetal bovine serum. Finally, proliferation assay was determined in this wound healing model. In conclusion, our lab-made 3D-printed stopper-based assay is a more economical alternative to currently available strategies for developing reproducible, high-throughput assays to assess cell migration and proliferation.

Introduction

Cell migration plays a key role in both physiological and pathological conditions. Wound healing assays are an indirect way of cell migration measurement, in which a cell-free region is created in a cell monolayer [1]. Cells migrate to cover the wound, and time-lapse images are recorded using a microscope. Based on these images, wound-healing rates can be calculated [2].

The scratch assay is the conventional and more used wound healing assay [3]. It consists of scratching, with a pipette tip, a monolayer of cells, once they have reached confluence [4,5]. Although its execution is very simple, the method has the disadvantages of low reproducibility and the difficulty of implementation in a high-throughput [1,6,7], microwell scale.

Many strategies have been proposed to solve this problem. For example, an 8-channel mechanical "wounder" device has been developed [8] to make reproducible scratches in 96-well plates. Other strategies commercially available include circular gel layers [9,10], silicon stoppers [6,11], and high-throughput scratching devices [12]. These strategies have the disadvantages of being expensive and may require sophisticated laboratory equipment for their implementation.

HaCaT cells are spontaneously immortalized human keratinocytes obtained from adult human skin [13]. This cell line presents mutations in p53 [14] and enhanced telomerase activity, both associated with its immortalization [15]. Also, HaCaT cells remain no-tumorigenic and their growth and potential differentiation are similar to normal human keratinocytes [13,16,17], so this cell line is a good model to study epithelial processes [18–20]. In the present work, we developed a

Abbreviations: BSA, bovine serum albumin; CAD, computer-aided design; DAPI, 4′,6-diamidine-2′-dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; EdU, 5-ethynyl-2´-deoxyuridine; FBS, fetal bovine serum; RZ, resazurin; SLA, stereolithography.

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reproducible and inexpensive lab-made device for wound healing assays. We designed 3D-printed stoppers, compatible with HaCaT cell culture and 96-well plates, demonstrating its effectiveness and reproducibility for wound healing assay, and its applicability for proliferation assay in HaCaT cells.

Materials and methods

Materials

3D-printing photopolymer resin (FLGPWH04, Standard White) was from Formlabs (Somerville, Massachusetts, USA). Cell culture supplies were from Gibco (Gaithersburg, Maryland, USA), Capricorn Scientific (Ebsdorfergrund, Germany), Sigma-Aldrich (St. Louis, Missouri, USA) and Applichem Laboratories (Darmstadt, Germany). RZ was from Sigma-Aldrich. Click-iT EdU Imaging Kit and DAPI were from Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Immortalized human keratinocytes, HaCaT cells [13], were kindly provided by Dr. Paola Hernandez [21], and cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin in a humidified incubator at 37°C and 5% CO_2.

3D-printed stoppers

Stoppers were produced using 3D printing SLA technology. The process of elaboration consisted of successive additions of layers of photopolymer resin, which were subject of photopolymerization point by point with a laser beam (gallium nitride solid-state laser; 405 nm wavelength; 96 mW power at print plane; 140 µm spot size) [22,23]. The software used for CAD modeling was Autodesk Inventor (AutoDesk, San Rafael, California, USA). The design consisted of a simple geometry-based in the dimensions of a single well from a 96-well plate (polystyrene 127.8 mm × 85.5 mm × 20.0 mm plate, with clear, circular, and flat-bottomed wells, 6.7 mm diameter and 11 mm height). We designed the stoppers (Supplemental Material, Stoppers3Dfile) to fit perfectly inside the well of a 96-well plate, like one described above allowing a correct alignment in the cavity with adequate space to fill the well with the cell-containing culture media. The stoppers (Fig. 1) consist of two sectors: the upper one, from the top to the middle, and the lower one, from the middle to the end. The upper sector consists of a thick cylinder (6.5 mm diameter, 7.9 mm height) with two opposite side gaps (3.3 mm diameter). The lower sector is a thinner cylinder (1.6 mm diameter, 3.1 mm height) that rests on the well's bottom surface. The printer model used was ~~A~~ Form 2 (Formlabs, Somerville, Massachusetts, USA) with a definition of 0.1 mm per layer. When the printing process finished, we cleaned the pieces discarding the resin excess by washing them with isopropyl alcohol. Before each experiment, we sterilized the stoppers by immersion in 70% ethanol for 10 minutes. Stoppers can be dried from 10 minutes to overnight, in a Petri dish under HEPA-filtered air.

Wound healing assay

In a 96-well plate with sterile stoppers, we seeded 4.5×10^4 cells per well and allowed them to attach for 24 h in a humidified 5% CO_2 atmosphere at 37°C. Then, we removed the stoppers and rinsed the cells with PBS. We acquired the "time 0" images using an Olympus IX-81 microscope. Immediately, cells were treated with culture media with or without 10% FBS for 24 h in a humidified 5% CO_2 atmosphere at 37°C. Afterward, we removed culture media and added 100 µL of PBS to capture the "time 24" images. We measured the wound areas tracing the cell-free area in captured images using FIJI ImageJ software [24], and calculated the percentage of wound closure as:

$$\%WC = \left[\left(A_{0h} - -A_{24h} \right) / A_{0h} \right] x100$$

being A_{0h} the area of wound measured immediately and A_{24h} the area of the wound measured 24 h after [2].

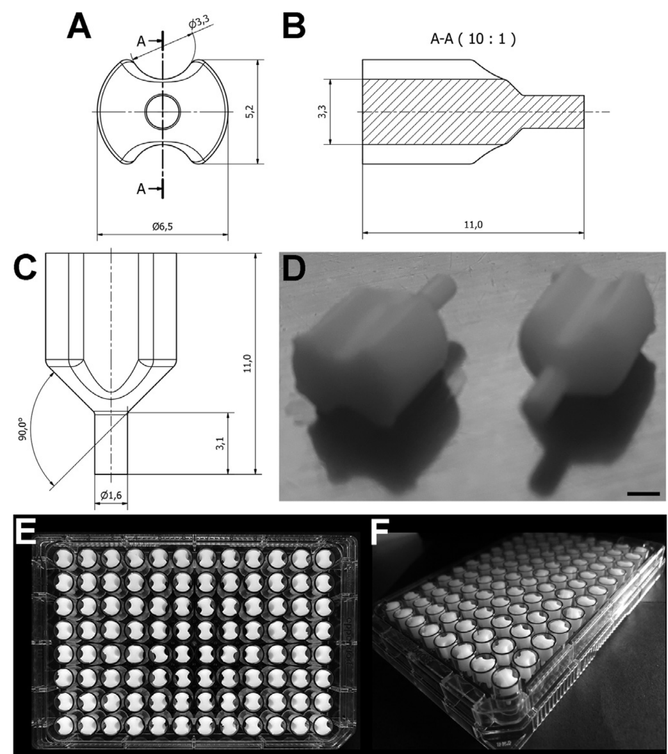

Fig. 1. Stopper design. Stopper vectorial design showing upper **(A)** and lateral **(B, C)** views. Dimensions are indicated in millimeters. **(D)** Representative picture of 3D-printed stoppers. Bar: 2 mm. **(E, F)** Upper and side views of a 96-well plate with the stoppers inside.

Viability test

Cells were seeded at 5×10^3 per well in a 96-well plate with or without sterile stoppers and allowed to attach for 24 h in a humidified 5% CO_2 atmosphere at 37°C. Afterwards, we removed stoppers and culture media. To determine cell viability by RZ, we added 100 μL of 0.025 mg mL^{-1} RZ (in sterile PBS) to each well, for further incubation at 37°C for 4 h. A microplate spectrophotometer (Varioskan Flash Microplate spectrophotometer; Thermo Fisher, Vantaa, Finland) measured the fluorescence at 530 nm of excitation wavelength and 590 nm emission wavelength.

Cell proliferation assay

We seeded cells at the confluence in a 96-well plate with or without sterile stoppers. Alternatively, we also seeded isolated cells without stoppers and allowed them to attach in a humidified 5% CO_2 atmosphere at 37°C. After 48 h, we removed stoppers and treated the cells with culture media with or without 10% FBS for 24 h in a humidified 5% CO_2 atmosphere at 37°C. Then, we rinsed the cells with PBS and added 10 µM EdU, and incubated them for 2 h. The modified thymidine analogue EdU is incorporated into newly synthesized DNA. DNA-incorporated EdU is subsequently labeled with a fluorescent dye by the "click" chemistry, allowing the detection of DNA synthesizing cells in a population [25].

According to Click-iT EdU Imaging Kits protocol [25], we performed the cell fixation, permeabilization, and EdU detection. Briefly, we fixed the cells in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, Missouri, USA), 15 min at RT at gentle stirring, and then washed two times with 3% BSA in PBS. We incubated the cells with 0.5% Triton X-100 in PBS for 20 min at RT, and gentle stirring. Then, we washed the cells two times with 3% BSA (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS. Afterwards, we added 25 µL of Click-iT reaction cocktail (containing Alexa Fluor 488-azide) to each well and incubated for 30 min at RT

protected from light. We washed the cells for 5 min with PBS. For DNA staining we added 0.1 μg mL^{-1} DAPI, incubated for 30 min at RT, and then washed with PBS two times, 5 min each. We acquired epifluorescence images for both fluorophores (Alexa Fluor 488 and DAPI) with an LSM 800 ZEISS microscope (Oberkochen, Germany).

Statistical analysis

We did all experiments with three biological replicas at least. For wound area reproducibility experiments, we performed 96 technical replicas, meanwhile for wound healing assay, we did 48 biological technical replicates and for cell proliferation assay we carried out 5 technical replicates. To analyze statistically our results, we used D'Agostino & Pearson and Shapiro-Wilk test for data normality behavior assessment, and Student's t-test for viability and wound healing experiments analysis. Also, we used one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests to examine differences between treatments ($p<0.05$) in the proliferation assay.

Results

Stoppers design

To perform reproducible and inexpensive wound healing assays in a high-throughput manner, we designed (Figure 1A-C) and 3D-SLA printed (Figure 1D) photopolymer resin-based devices, called stoppers, with the dimensions described in the Materials and methods section (Figure 1 A-C) to fit in each well of a flat-bottomed 96-well plate as described above. We design the stoppers fitting just right into each well and avoiding its movement laterally. The weight of the stopper makes that it remains in contact with the bottom despite the liquid inside the well. The two lateral gaps facilitate the exit of bubbles, enable to place and remove each stopper with a #5 tweezer.

Wound healing assay

The stoppers designed left cell-free areas (Figure 2A, left) with a mean of 0.79 ± 0.05 mm^2 (Figure 2A, right), and a normal data behavior ($p=0.5118$, D'Agostino & Pearson test; $p=0.4264$, Shapiro-Wilk test). The lab-made stoppers did not affect cell viability as the RZ method indicated (Figure 2B). Cells cultured without stoppers showed viability of 100.7 ± 7.3% whereas cells cultured with stoppers showed viability of 102.3 ±7.3%, with a Student's t-test $p=0.1993$ between them. To find out if stoppers are useful tools for wound healing assays, we compared the wound closure area after 24 h of stoppers removal in two culture conditions, one with 10% FBS and the other one in its absence (Figure 2C). Culturing cells 24 h after stoppers removal without FBS produced a percentage of wound closure area of 1.9 ± 0.6%, meanwhile, cells cultured with FBS showed a percentage of 39.0 ± 1.2% (Figure 2C, right). The difference between both groups was statistically significant with a Student's t-test $p<0.0001$.

Proliferation assay

To assess cell proliferation in our model, we carried out the proliferation assay based on EdU incorporation. We plated cells with stoppers and allowed them to remain confluent for 48 h. Afterwards, we removed the stoppers and let the cells migrate for 24 h. We analyzed EdU incorporation in the last 2 h with or without 10% FSB (Figure 3). We included two controls, one of them was a 48 h confluent cells group as a proliferation baseline control, and the second group was control of maximal proliferation capacity with isolated cells. The culture condition affected the proliferation of HaCaT cells at the $p<0.05$ level confirmed by one-way ANOVA test [$F(3, 16)=138.7$, $p<0.0001$]. Basal proliferation percentage of HaCaT cells cultured at confluence for 48 h determined was

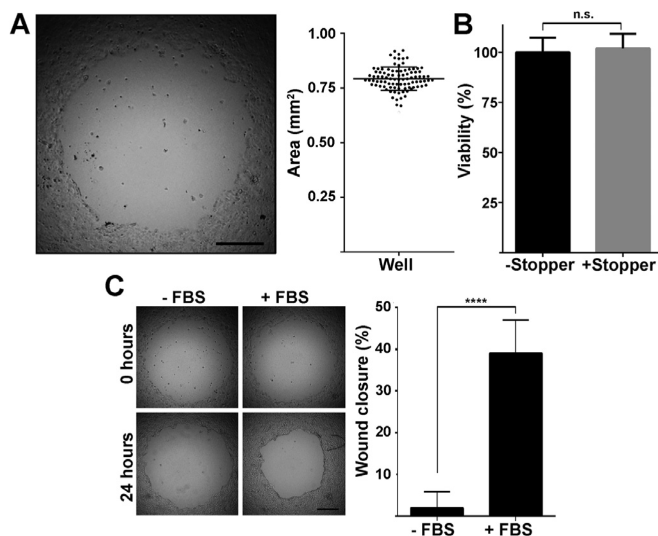

Fig. 2. Wound healing assay. A) A representative image of the cell-free area produced by the lab-made stoppers is shown (left). Bee swarm plot showing the distribution of values of the initial area (cell-free area left by the stoppers) in each well for a 96-well plate is shown at the right. **B)** Graphical view of cell viability (%) for cells cultured in the absence (-Stopper) or presence (+Stopper) of the lab-made stoppers. Values are mean ± SD. n.s.: not significant. **C)** Analysis of wound closure area (%) after 24 h of culture in the absence (-FBS) or presence (+FBS) of 10% fetal bovine serum. Representative images of each condition at times 0 and 24 h are shown (left). Mean ± SD of wound closure area (%) of each condition are presented. **** *p<0.0001*. Bar: 250 μm

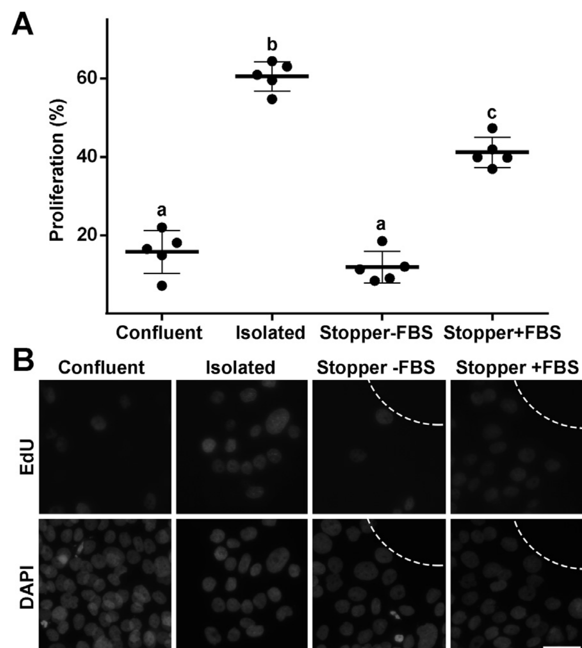

Fig. 3. Proliferation assay. The proliferative population of HaCaT cells cultured at a confluence, isolated or with stoppers in the absence (-FBS) or presence (+FBS) of 10% fetal bovine serum. **A)** Graphical view of proliferative population percentage (EdU/DAPI positive nuclei) of each condition. Values are mean ± SD. Lower case letters (a, b and c) in the graph indicate significant differences among groups (*p<0.0001*). **B)** Representative images of EdU and DAPI labeling of each condition are shown. The stopper border is depicted as a dotted line. Bar: 100 μm

15.7 ± 5.5%, statistically indistinguishable (Tukey's multiple comparisons test) from the proliferation percentage of those cultured without serum 24 h after stopper removal (11.8 ± 4.0%, Figure 3A). The isolated cell culture condition showed the maximal proliferation, with an EdU incorporation percentage of 60.5 ± 3.7% (Figure 3A), meanwhile, the proliferation percentage of cells cultured with FBS 24 h after stopper removal was 41.2 ± 3.9%.

Discussion

In this work, we designed and 3D-printed stoppers that are compatible with HaCaT cells culture in 96-well plates. The printed stoppers are inexpensive devices, reusable, and in our experience, an individual stopper can be reused at least 20 times without affecting its performance. In addition, our stoppers are produced using a 3D printer, a technology available in most universities and biological research institutes. Furthermore, these stoppers are easily sterilizable by simply soaking in 70% ethanol for 10 minutes and let them dry under HEPA-filtered air for 10 minutes.

The produced stoppers are easy to handle and lead to high reproducibility of the cell-free areas left in the 96-well plate, which makes them an excellent tool for high-throughput assays, a feature hardly reachable with scratching techniques [1]. The reproducibility of initial wound size is of great importance, traditional scratches with pipette tips can be highly variable in size (initial area mean ± SD: 2.15 ± 0.83 mm^2, Supplemental material Figure 1). As we show in the supporting information Figure 1, the percentage of wound closure for HaCaT cells in a scratch assay is dependent on the initial area of the wound. Conversely, with our stoppers, the variability in the initial size of the wound is lower (initial area mean ± SD: 0.79 ± 0.05 mm^2), which leads to independence of the percentage of wound closure on the initial size of the wound. This is important in experiments where it is desirable that the only dependent variable is the percentage of wound closure and the only independent variable is the culture condition. In addition, our stoppers produced a cell-free area with a reproducibility error lower than other available devices [26,27].

Wound healing assays are used to study the effects of stimulants or inhibitors of migration rate. Here we assessed two basic culture conditions, with and without FBS. 3D-printed stoppers were very useful to differentiate the percentage of wound closure area between these two conditions. Culturing HaCaT cells in absence of FBS produced almost zero wound closure after 24 h using our stoppers, making this condition the optimal baseline for assays with metabolites that are promising to increase cell migration. On the other hand, 24 h after stoppers removal and culture with 10% FBS, the resulting percentage of wound closure is useful for migration rate inhibitors studies. Comparing these results with those obtained with the classic scratch assay (Supplemental material Figure 2), the advantage of the use of stoppers become more evident. Although with the classical scratch test the wound closure percentage between the conditions with and without serum presented a statistically significant difference, the range of data for each condition was wide. This can be a problem when comparing experimental conditions where the challenge is less drastic than culture cells in the presence and absence of serum.

Wound healing involves two main cellular events, migration and proliferation [2,28]. The capability of performing proliferation assay in addition to wound healing assay is an additional advantage of the stopper-based assay. First, we were able to reproduce the proliferation behavior of a HaCaT monolayer. In isolated conditions near 50% of HaCaT cells incorporated EdU [29]. Also, proliferation contact inhibition is reached after 48 h of confluent culture for HaCaT cells [29]. After stopper removal and 24 h of additional culture with 10% FBS showed, for the first time to our knowledge, the proliferative population of the wound healing boundary for this cell line. This condition is useful for testing antiproliferative compounds. Meanwhile, 24 h of additional culture without FBS after stopper removal resembles the confluent monolayer proliferation capacity, making this culture condition a good choice for tests of compounds with a possible pro-proliferative activity.

In conclusion, the 3D-printed stoppers-based assay that we describe in this study is a more reproducible alternative for HaCaT cells to conventional scratching and a more economical alternative to commercially available devices for wound-healing assay.

Availability of data and material

All relevant data are within the manuscript and its Supporting Information files.

Authors' contributions

All authors contributed to the study conception and design. Silvina Acosta, Lucía Canclini, and Diego Alem performed material preparation, data collection and analysis. Silvina Acosta, Lucía Canclini, and Diego Alem wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no commercial or financial conflict of interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.slast.2021.10.003.

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