Development of a single layer microfluidic device for dynamic stimulation, culture and imaging of mammalian cells

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Abstract

The possibility to tightly control the cellular microenvironment within microfluidic devices represents an important step forward precision analysis of cellular phenotypes *in vitro*. In particular, microfluidic platforms that allow both long-term mammalian cell culture and dynamic modulation of the culture environment can support quantitative studies of cells' responses to drugs and fate choices. Here, we report the design and testing of a novel microfluidic device of easy production (single Polydimethylsiloxane layer), which integrates a micromixer with vacuum assisted cell loading for long-term cell culture and dynamic mixing of culture media with 4 different inputs. Finite element modelling was used to predict the flow rates and device dimensions to achieve diffusion-based fluid mixing. The device showed efficient diffusion-based mixing and dynamic exchange of media in the cell trapping chambers. This work represents the first attempt to integrate single layer microfluidic mixing devices with vacuum assisted cell loading systems for long-term mammalian cell culture and dynamic stimulation.

Keywords: Microfluidics, micromixers, mammalian cells, single layer, PDMS.

Introduction

Culturing cells *in vitro* is of paramount importance in molecular biology to study cellular processes such as cell-cell interaction, responses to external stimuli (physical or chemical), cell-cell interaction, acquisition of genetic mutations over time and so on [1–3]. Traditional *in vitro* methods for cell culturing (i.e. in dishes) do not allow spatio-temporal control of the cellular environment and are therefore limited when it is important to study cellular response to dynamic perturbations in culturing conditions . Microfluidic devices find their advantage in using low sample volumes, while allowing precise control over cell culture environment and mimicking physiological conditions[4–7]. Recently, there has been an increased interest to employ microfluidic devices for a range of mammalian cells applications, such as high throughput single cell screening[8–10], parallelized drug screening[11], temporal modulations of inputs[12], easy-to-use perfusion for cell proliferation and differentiation[13,14] and automated feedback control of biological processes[15].

Dynamic drug stimulation, drug mixing and long-term continuous perfusion of cell culture can be of great importance for cell biology studies. This is often achieved by integrating in microfluidic devices active and passive micro mixers[16,17], and using multiple layers (which requires sophisticated mold preparation) and/or separate devices for multiplexer input control and cell growth, respectively, as in[11]. Trenzinger et al.[18] reported an automated microfluidic device with 48 independent chambers to culture both adherent and suspension cells in rapidly changing media conditions; in this case, mixing in implemented on chip, but the device is still complex to fabricate, as it includes actuated valves and multiple layers.

Kolnik et al[12] reported a multilayer microfluidic device with a built in function generator to create chaotic mixing between two different cell culture media. The device also used, for the first time, vacuum-assisted loading of cells in individual trapping chambers to protect them from the shear stress.

Single layer microfluidic platforms have been proposed to simplify fabrication and operation, and facilitate cell loading [19,20]. The single layer microfluidic devices serve the purpose for the long-term mammalian cell culture but lack the ability of dynamically mixing two or more cell culture media.

In this study we present a novel Polydimethylsiloxane (PDMS), single layer microfluidic device that can be employed to culture, for long term, mammalian cell lines and perfuse them with a combination of up to four cell culture media (possibly enriched with drugs). The device embeds a cell loading part, a diffusive mixing part and five cell culture chambers, adjoined to the perfusion channel (Figure 1). The cell loading design was inspired by the vacuum-assisted cell loading chip design by Kolnik et al[12]. The continuous perfusion of different cell culture media was achieved using four inlets, each of them regulated by software-controlled syringe pumps and connected to flow stabilizers and mixing serpentines structures. We show, both using finite element analysis and with experiments, that the device allows static mixing of different media, and fast media change in the culture chamber when varying the media provided to the inlets. Finally, the device was tested for cell viability by using two different mammalian cell lines. The device presented in this study has specific advantages such as single layer fabrication, diffusion-based mixing and easy cell loading. To our knowledge, this is the first study to use a single layer microfluidic device with an integrated vacuum assisted cell loading mechanism. It should be possible to easily modify the device to increase its drug screening capability by modifying the number of inlets/serpentines.

Materials and methods

Device design

The device consists of 5 consecutive cell culture chambers (footprint 360 µm × 360 µm, Figure 1a). The 'opening' that connects the chambers to the main channel is 200 µm × 200 µm. The height of the channel is 50 µm. Cells are loaded using a vacuum pump connected to the vacuum layer running parallel to the main channel, as shown in Figure 1b. Figures 1 c and d represent the flow stabilizers and serpentine structures for fluid stabilization and mixing, respectively. The distance between the vacuum layer and the trapping chambers is 80 µm (essentially a thin PDMS membrane).

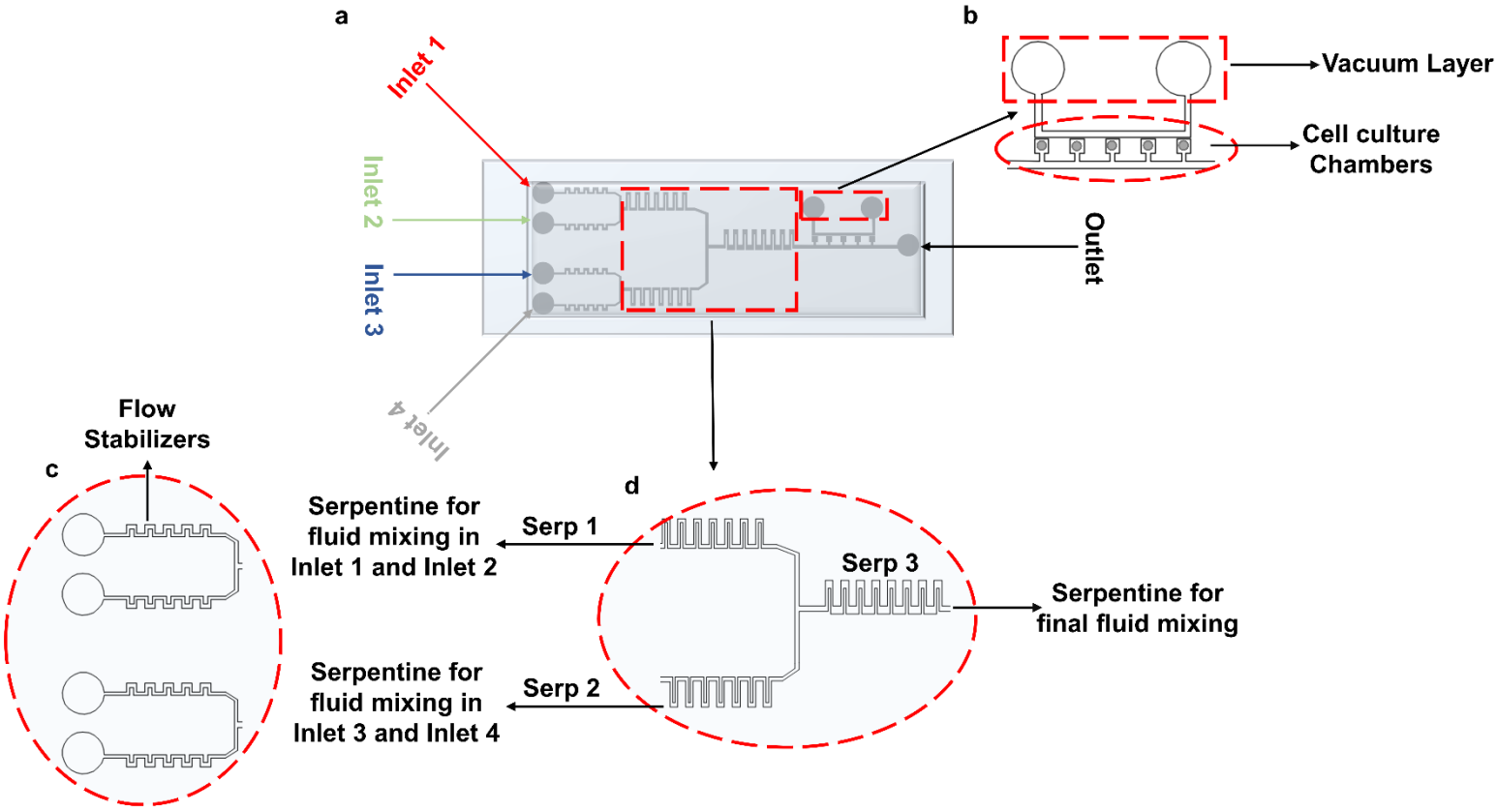

Figure 1. Microfluidic device design. (a) Graphical representation of the device bonded on glass slide. (b) Inlet part of the device with flow stabilizing serpentines. (c) Mixing serpentine serp 1 and serp 2 to facilitate diffusive mixing of cell media and drugs. (d) Serp 3 for enabling diffusive mixing of culture media coming from serp 1 and serp 2.

A combination of different cell culture media/drugs can be infused through the device using four inlets, as shown in Figure 1. Inlets 1 and 2 come together in the top serpentine (serp1) structure, and Inlets 3 and 4 come together in the bottom serpentine (serp2) for mixing purposes (Figure 1d). A larger serpentine structure (serp3) is connected at the junction of serp1 and serp2 for further mixing of the fluids coming from serp1 and serp2. The dimensions of the serpentine, the number of the serpentines required to achieve a complete mixing, fluid flow rates and the number of cell culture chambers were chosen given results from COMSOL simulations.

Device fabrication

The microfluidic device reported in this study was fabricated using mask less photolithography. Computer aided design software (CAD) AutoCAD was used to design

the input files; a negative photoresist SU-8 2050 (MicroChemicals GMBH, Stuttgart, Germany) was spin coated on a 4-inch silicon wafer (Figure 2a) at 3000 rpm for 30 seconds to achieve a height of 50 µm, as shown in Figure 2b. A pre-exposure bake was performed in two steps by placing the wafer directly on to the hot plate at 65 °C for 2 minutes and then at 95 °C for 9 minutes.

The baked silicon wafer was then exposed using a Heidelberg laser writer 'µPG101' (Heidelberg Instruments Mikrotechnik GmbH) using 30 mW of power at 10 % (Figure 2c). A post-exposure baking step was performed first at 65 °C for 1 minute and then at 95 °C for 7 minutes. The exposed wafer was than immersed in the SU-8 developer (MicroChemicals GMBH, Stuttgart, Germany) solution to remove the access photoresist using. The structure was obtained in the development step (Figure 2d) and we refer to it as the 'mother mold'. To further enhance the adhesion of structures to the wafer, a hard baking was done by placing the mother mold directly on to the hot plate at 200 °C for 10 minutes.

The final device was obtained by pouring PDMS on the mother mold with a base to curing agent ratio of 10:1 (Figure 2e). A degassing step was performed to remove the air bubbles by placing the mold with PDMS in a desiccator for 25 minutes. After degassing, the mother mold was placed in an oven at 70 °C for 120 minutes. The baked PDMS mold was autoclaved to achieve sterilization. The individual chips were realized by bonding the PDMS devices on a microscope glass slide (75 mm × 25 mm) using oxygen plasma at 100 % power as shown in Figure 2f.

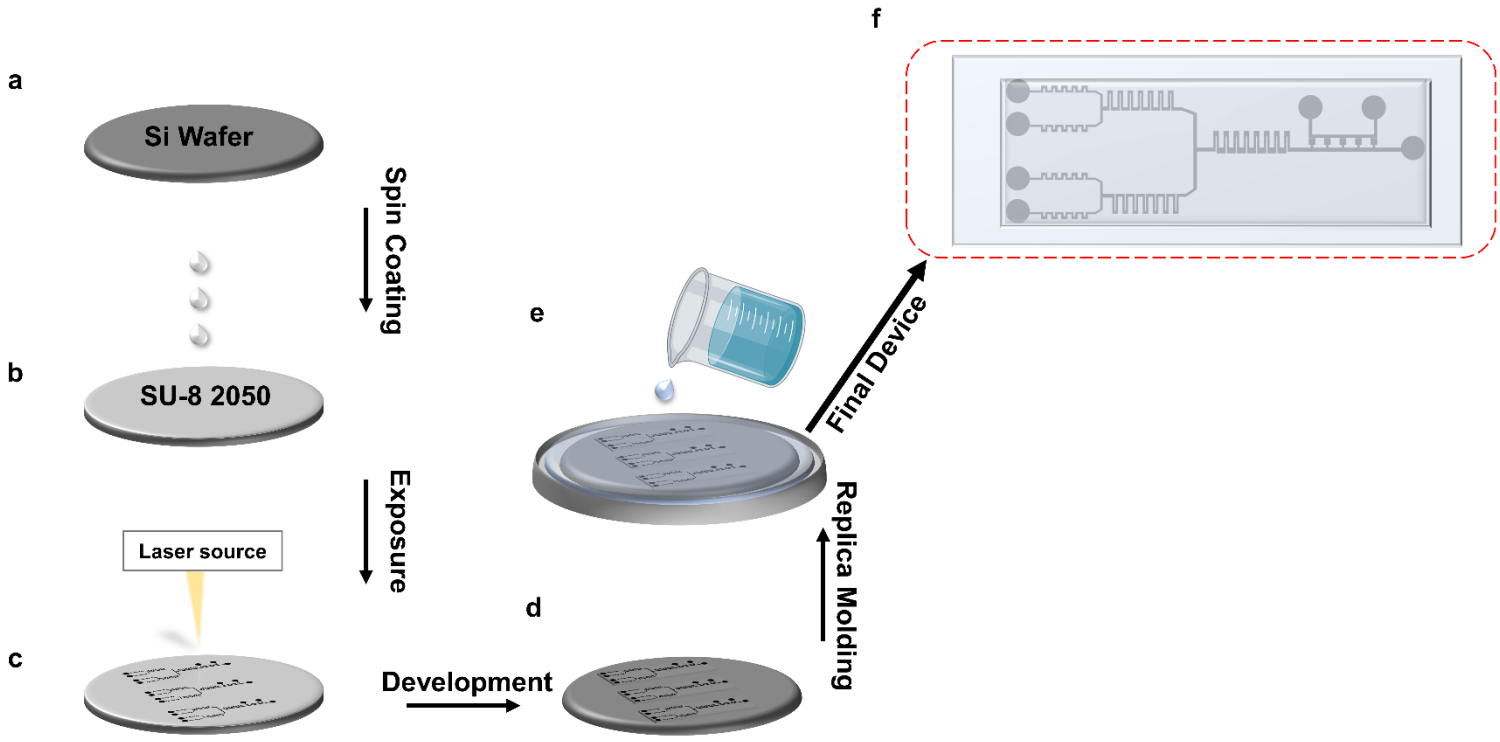

Figure 2. Device fabrication protocol. (a) 4-inch silicon wafer. (b) Spin coating Su-8 2050 negative photoresist on 4-inch silicon wafer. (c) Exposure of spin coated silicon wafer using laser source. (d) Removal of excess negative photoresist using Su-8 developer solution. (e) PDMS replica molding. (f) Final fabricated device bonded on glass slide.

Cell culture and device loading

Two mouse Embryonic Stem Cell (mESC) lines were used to test cells viability in the microfluidic device: REX-dGFP2 cell line[21] and MiR-290-mCherry/miR-302-eGFP cell line, called her for simplification DRC[22]. Before loading in the microfluidics device, mESCs were cultured on gelatinized tissue culture dishes at 37 °C in a 5% CO2 humidified incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (Sigma), non-essential amino acids, L-glutamine, sodium pyruvate, Penicillin-Streptomycin, 2-mercaptoethanol and 10ng/ml mLIF (Peprotech; 250-02). Once the mESCs in culture dishes reached confluency they were detached using 3 mL Gibco trypsin-EDTA (0.5%). 1 mL of detached cells suspended in trypsin were collected in a falcon and centrifuged at 120 RPM for 5 minutes. The supernatant was removed using a micropipette and the cells were then resuspended in 100 µL of the cell culture media. The cells were then loaded in to a 2.5 mL syringe avoiding any bubbles. The cell loading into

the microfluidic device was done in two steps. First, the chip was wetted by using the cell culture media. In the next step the syringe with cells was attached to the outlet and cells were infused into the chip. Once the channel was completely filled with the cells, a vacuum pump at a pressure of -80 kPa connected to vacuum layer was switched on to aspirate the cells into the chambers and to remove any air in the chambers. Once loaded, the device was infused with fresh media to clear the excess cells.

Mixing experiments and image capture

For experiments in Figures 4-7, and Figure S2, media was enriched with the fluorescence dyes Atto 488 (41051-1MG-F), Atto 647 (97875-1MG-F), Rhodamine B CAS-81-88-9 and Phosphate-buffer saline (RNBK3085), all obtained from Sigma Aldrich. The solutions for the media exchange and mixing experiments were prepared by pipetting dyes in the concentration of 1 $\mu l\ ml^{-1}$. The fluid samples used for the experiments were infused into the microfluidic device using syringe pumps (World Precision Instruments AL-1000) as shown in Figure 3. A customized Matlab code was used to run the pumps and change the flow rates during dynamic mixing and media exchange experiments.

The imaging experimental setup consisted of Leica LASX live cell imaging workstation on DMi8 inverted fluorescence microscope (Leica Microsystems Wetzlar, Germany). An Andor iXON 897 Ultra back integrated camera was used for imaging purposes. Images collected to test the efficacy of the device for the media exchange and mixing were recorded by using an objective lens of magnification 10x. The cell viability experiments' images were collected using a 20x magnification. The media exchange and mixing experiments in Figures 4-6 were 30 minutes long with images recorded at every 2 minutes, while that in Figure S2 were 145 minutes long with images recorded at every 5 minutes. For the cell viability experiments, microscopy images were collected every 24 hours. The experiments where the fluid concentration needs to be changed dynamically would require balancing of flow rates in the main junction i.e. the total flow rate at inlet 1 and 2 should always be equal to the total flow rate at inlets 3 and 4. The balancing of flow rate helps to avoid any back flow from inlets 3 and 4 towards the inlets 1 and 2 If the flow rate at inlet 1 and inlet 2 is 50 $\mu l\ hr^{-1}$ respectively (100 $\mu l\ hr^{-1}$ in total) then in order to have no back flow the total flow rate at inlets 3 and 4 should also be equal to 100 $\mu l\ hr^{-1}$. The experiments where flow rate at one of the inlets has to increased or reduced to increase or decrease the concentration of

the drug in the device the flow rate on the other inlet has to be adjusted to compensate for this change as shown in figure 7. This is where our chip design finds its advantage of not disturbing the over all flow in the device by controlling and mixing fluid flow rates in 2×2 configuration.

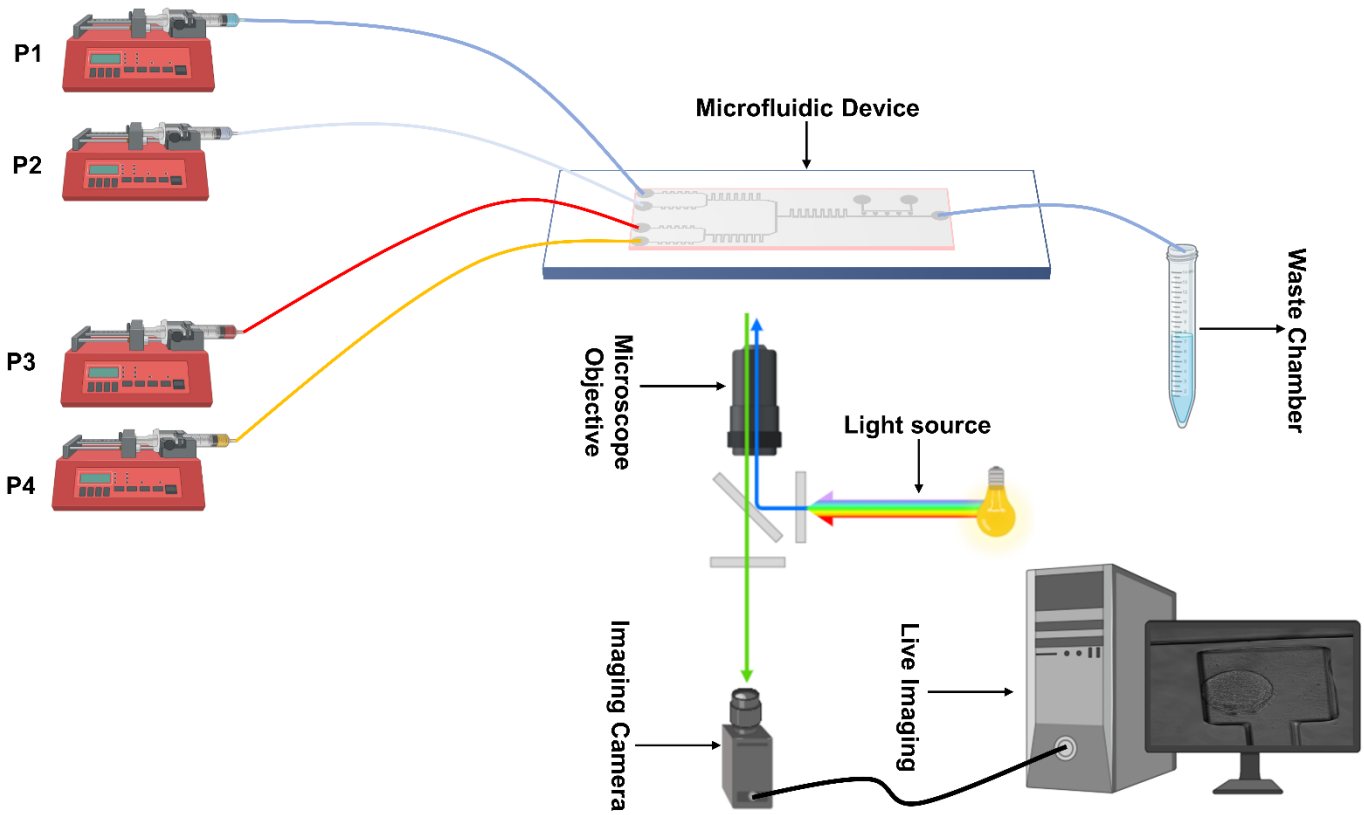

Figure 3. A schematic of the experimental setup for the multi input microfluidic device for in flow cell culture and drug delivery.

Diffusion and flow modeling

Fabrication of microfluidic devices requires resources and time not readily available. Hence, it is of utmost importance to that device design is optimized using all the available tools before the fabrication process is started. Computational fluid dynamics is one such tool that helps in the design optimization of the microfluidic devices. In order to verify the operating parameters of our device we used COMSOL 5.6 multiphysics software. The device design generated by AutoCAD as shown in Figure 4a was imported into COMSOL 5.6 for the simulation purpose. CFD model for our microfluidic device was based on solving

fluid flow by using laminar form of Navier-Stokes equation and modelling transport mechanism using mass transport equations. The fluid flow was modelled using 'laminar flow' built in Physics while transport mechanism was solved using 'transport of diluted species. . Initially, simulations using COMSOL multiphysics 5.6 were setup by using fully developed flow boundary condition at the inlets to accurately model the laminar flow regime. The mass transport in the serpentine and cell trapping chambers is dominated by diffusion and was modelled using transport of diluted species physics node. In order to validate the experiments the dyes were used at a concentration of 1 µl mL^{-1} in all simulations. The outlet boundary condition was set to be zero pressure. Diffusion coefficient values for Rhodamine B and Atto 488 were used to simulate diffusive mass transport in the device. The governing equations, fluid velocity magnitude and shear stress in serpentine structures, connecting channel and cell culture chambers are given in supporting information S1.

Results and Discussion

Diffusion Mixing Experiments

Firstly, we tested both via simulations and with experiments the device performance in mixing media from the different inlets (first just two, and then four). Finite element method was used to model fluid flow and mixing with in the microfluidic device. The simulation results revealed that the rectangular serpentine structures act as effective micromixers for two inputs, as shown in Figure 4b-e.

We then tested experimentally the mixing of two media, containing Atto 488 (green dye) or Rhodamine B (red dye), infused with flow rates between 50 – 100 µl hr^{-1} in inlets 1 and 4; inlets 2 and 3 were not used in these experiments and were closed (as represented by the cross-sign in Figure 4a). It was observed that, at 50 µl hr^{-1}, the two fluids flow side by side in the initial part of the serp 3 (simulation and experiment in Figure 3c and g, respectively) and mix completely as they reach the end of serp 3 and, ultimately, the cell culture chambers (simulation and experiment in Figure 4d/e, and h/i). Mixing at flow rates higher than 100 µl hr^{-1} might be possible by increasing the number of serpentine structures for high throughput applications.

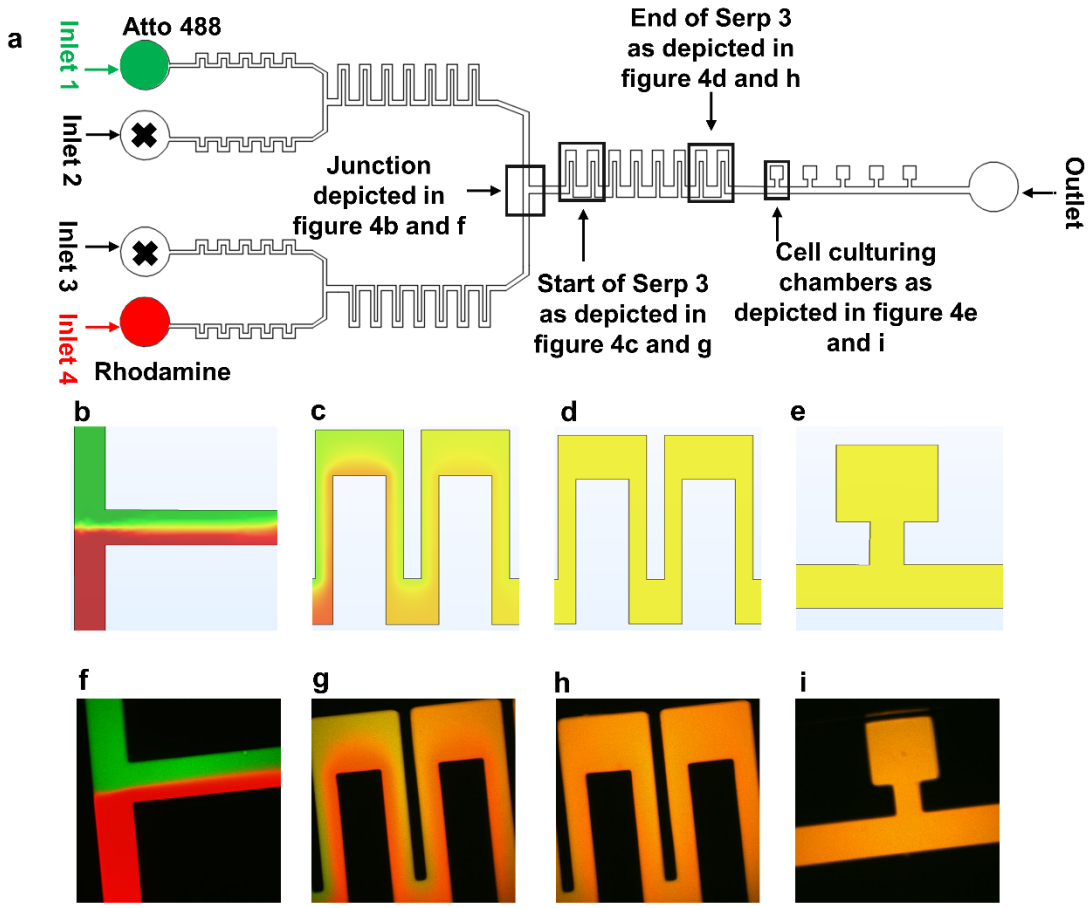

Figure 4. Mixing 2 different fluids using microfluidic mixer part of the device. The flow rate used for these experiments was 50 µl hr^{-1}. (a) Microfluidic device design with highlighted parts. (b-e) COMSOL simulation depicting diffusion-based mixing of two fluids using inlets 1 and 4. (f-i) Experimental results for static mixing using Atto 488 and Rhodamine B at 50 µl hr^{-1}

The device performance was further tested by mixing four different fluids; this was achieved by combining fluids in 2×2 configurations as shown in Figure 5a The experiments were setup by keeping the flow rate at 50 µl hr^{-1} at all the four inlets. The fluids coming from inlet 1 (Atto 647, blue dye) and inlet 2 (phosphate-buffered silane, PBS) were combined in the top junction as shown in Figures 5b (simulation) and h (experiment), and they were mixed via serp 1. Similarly, fluids coming from inlet 3 (Rhodamine B, red dye) and inlet 4 (Atto 488, green dye) came together at the bottom junction (Figures 5c and i, simulation and experiment respectively) and were mixed via serp 2. The final mixing step is performed by combining the mixed fluids at the junction of serp 1 and serp 2, and using serpentine 3 (serp 3).

As observed when mixing two fluids, there is a thorough mixing of the four fluids in serp 3, and in the cell culture chambers (Figures 5 g and m, simulation and experiment, respectively; differences in the experimental mixed media color are due to the fact that the colorless media used in experiments could not be represented in COMSOL, where a light blue color was instead used, and a light green color represents the Atto 488 dye).

These experiments revealed that mixing efficiency was highly dependent on the fluid flow velocity.

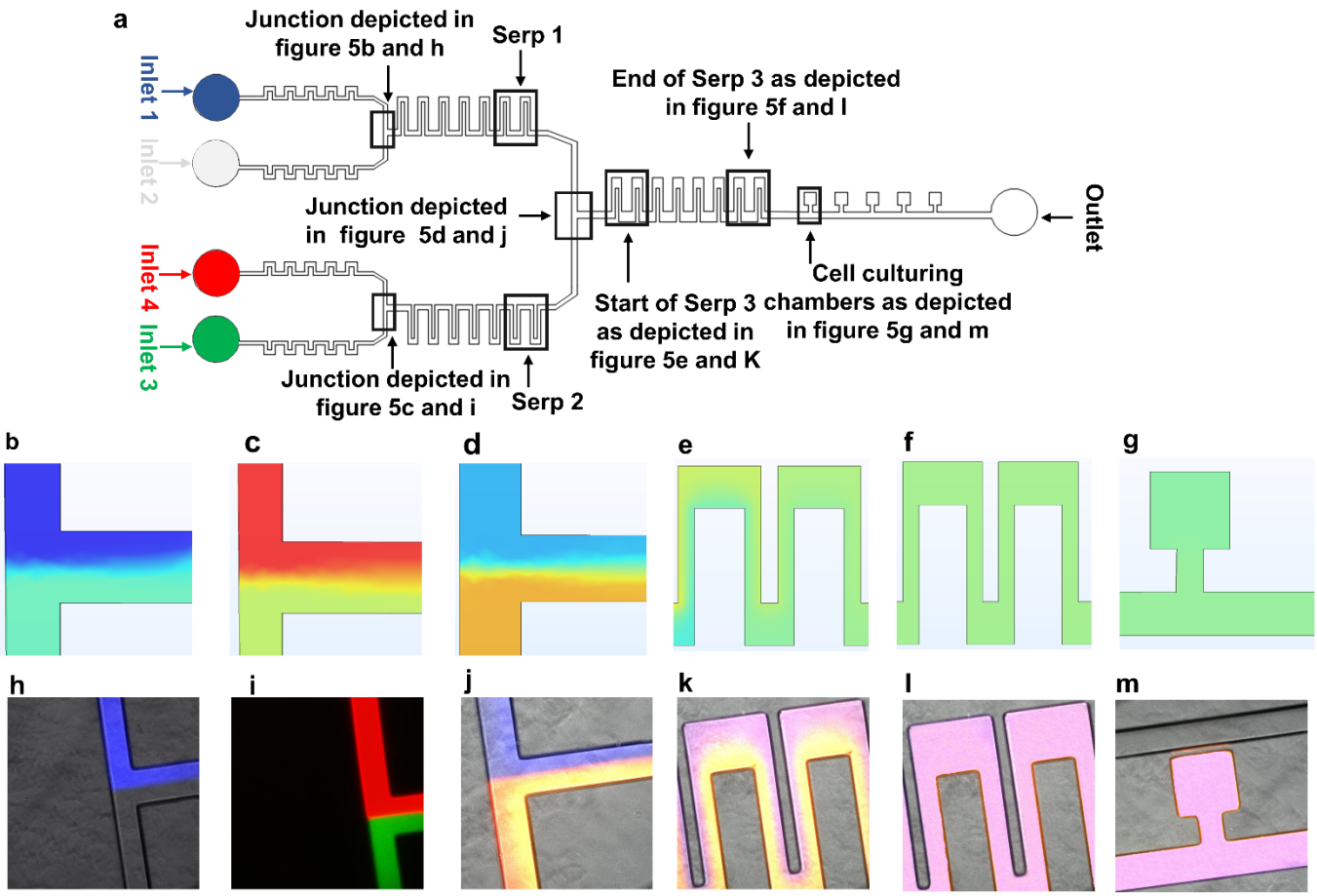

Figure 5. Mixing 4 different fluids using microfluidic mixer in 2×2 configuration. The experiments were designed to test the ability of the device to achieve mixing using serpentine structures. The flow rate at all the inlets was set at 50 µl hr^{-1}. (a) Microfluidic device design highlighting different parts of the device. (b-g) COMSOL simulation depicting diffusion-based mixing of four fluids using inlets 1-4. (h-m) Experimental results for static mixing using Atto 488, Rhodamine B, Atto 647 and PBS at 50 µl hr^{-1}.

We then tested our device for effective media exchange. We firstly set up simulations and experiments by using only two inlets, infused with two fluorescence dye solutions Rhodamine B (red) and Atto 488 (green) in PBS with a concentration of 1 µL ml^{-1} (Figure 6a). Initially, the pump controlling the syringe containing the green dye was set at 50 µl hr^{-1} and the pump controlling the red dye-containing syringe was switched off.

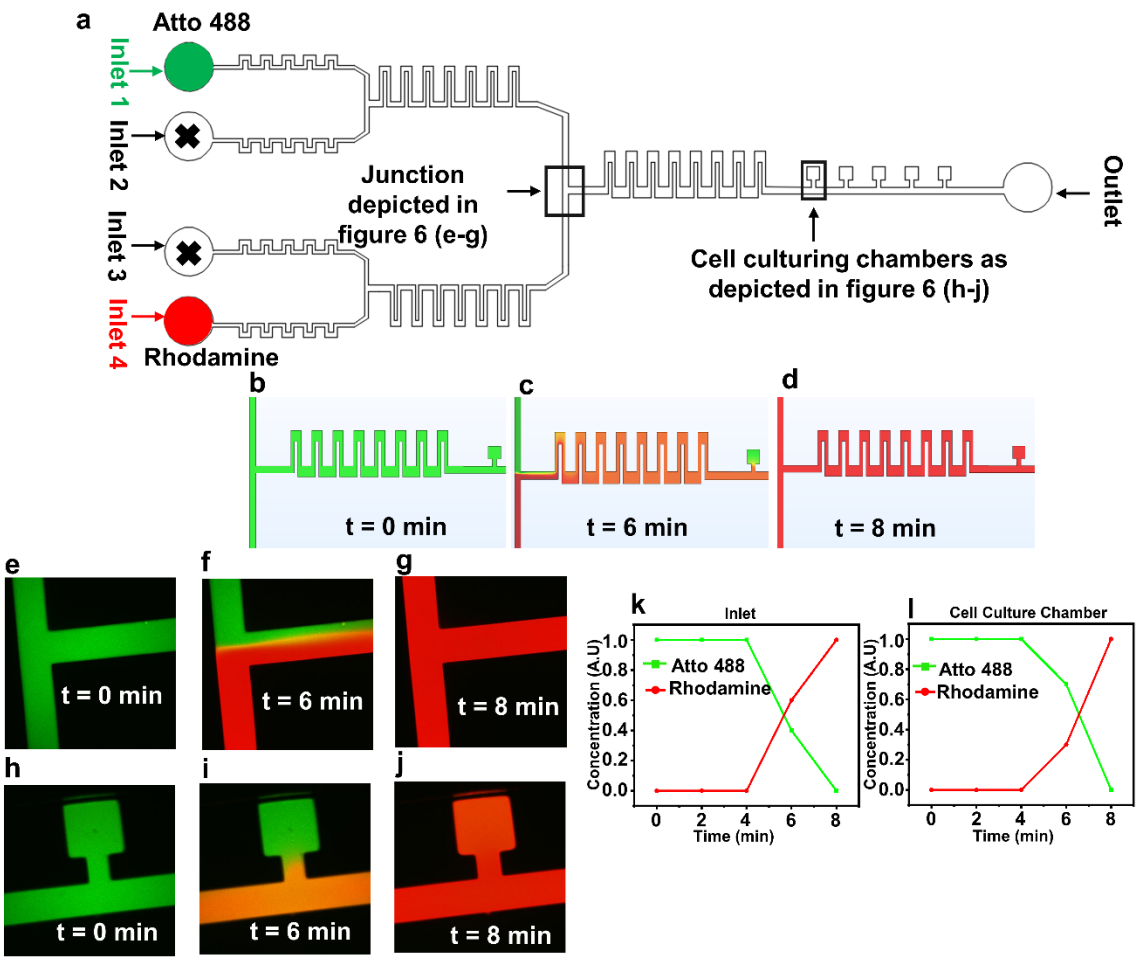

Figure 6. Media exchange experiments using Atto 488 and Rhodamine B. (a) Microfluidic device design with highlighted parts. The experiments were designed to test the dynamic media exchange ability of the device by using two inlets. (b-d) COMSOL simulation depicting diffusion-based media exchange between two fluids using inlets 1 and 4. (e-j) Experimental results depicting media exchange in time. (k-l). The concentration plots were generated by quantifying the dye concentration at the inlet junctions and within the cell culture chambers.

In this case, the entirety of the microfluidic device including cell culture chambers were completely filled with the green dye (t=0 minutes, Figures 6b -simulation- and e -

experiment-). We then switched off the pump infusing the green dye and switched on the pump with the red dye at 50 µl hr^{-1} at t=4 min. The red dye starts to take over the green dye and at t = 6 minutes in the junction (Figures 6 c, f), taking over the perfusion channel and the cell culture chambers at t = 8 minutes (Figures 6 g, j). Quantification of the fluorescence signals confirms successful media exchange, with a short time delay between switching of the pumps, and complete change of dye in the cell culture chambers (Figures 6 k and l). We also tested the media exchange, mixing and flow stability in longer experiments (5 hours vs 30 minutes). These experiments were designed keeping in mind the time lapse studies using different cell lines. These time lapse experiments can be 24-72 hours long and it is essential to have an understanding the dynamics of media exchange in cell culture chambers for these experiments as well. The experiments were setup at flow rate of 50 µl hr^{-1} and images were taken at every 5 minutes. These experiments proved the dynamic capability of the device to be used in long term experiments where the exchange between two different fluids happens after few hours compared to 30 minutes. The results of these experiments are shown in supplementary information S2.

The device performance was further assessed by setting up media exchange experiment utilizing all 4 inlets (Figure 7). Similar to two dye case, time dependent COMSOL simulations (Figures 7 b-d) were performed before setting up the experiments. Inlets 1-4 ware connected to the pumps infusing media containing Atto 647 (blue), PBS (colorless), Atto 488 (red) and Rhodamine B (green), respectively. Initially all the four solutions were infused at 50 µl hr^{-1} (time t = 0 min, Figures 7b and e). The pump infusing the blue dye (inlet 1) was then completely switched off and the flow rate at the pump infusing PBS was increased to 100 µl hr^{-1} to balance the flow rates at inlets 3 and 4.. At t = 4 min, PBS starts taking over the blue dye at the main junction (Figures 7 c and f), with the color of the mixed fluid altered at the end of serp 3 and in the cell culture chamber (Figures 7i and l). Complete media exchange is obtained at t = 8 min (Figure 7 d, g, j, m). Quantification of media fluorescence confirms successful dynamic exchange of mixed fluid, with a short delay between the time of pump switching, and the replacement of the media in the cell culture chamber.

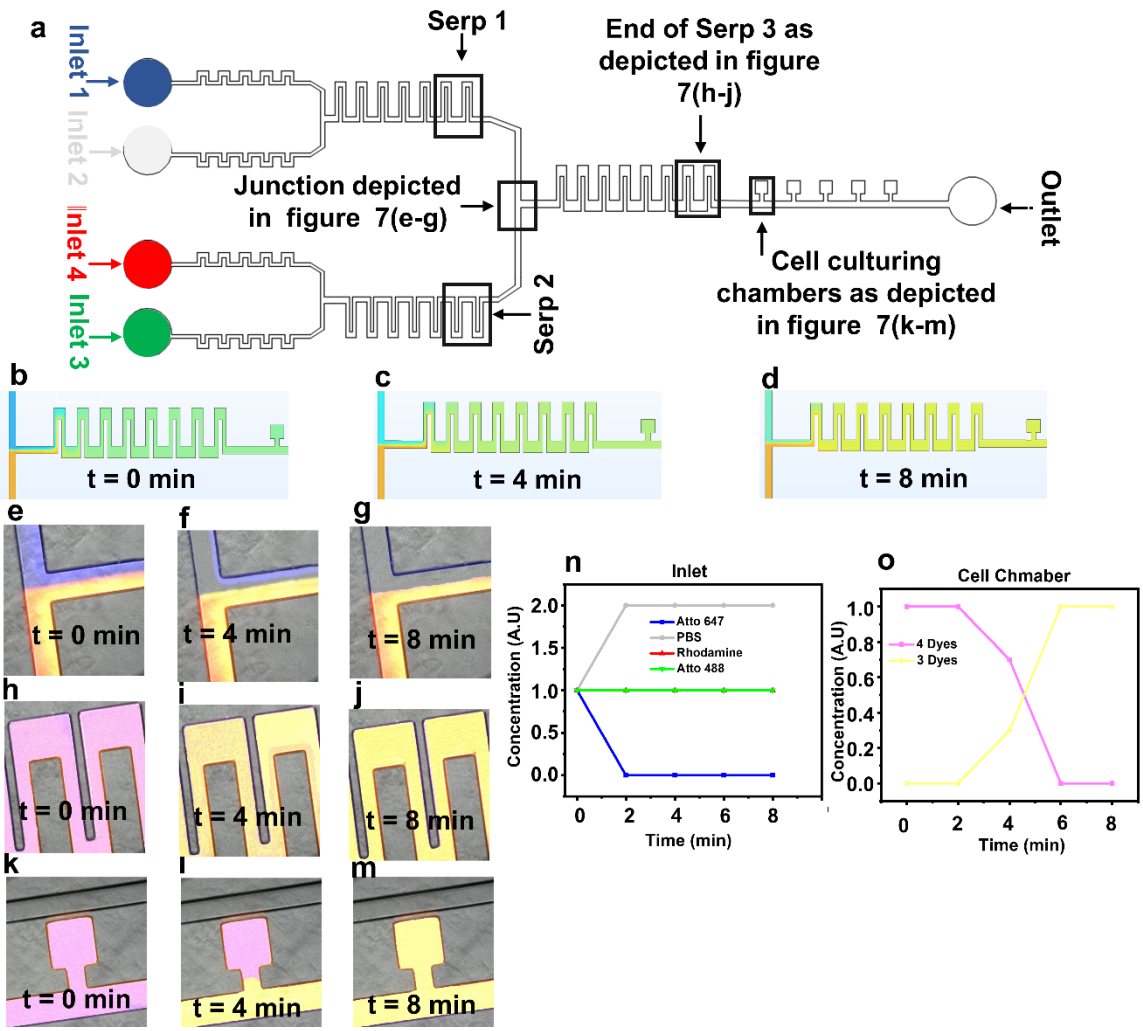

Figure 7. Media exchange experiments using Atto 647, PBS, Atto 448 and Rhodamine at 50 µl hr^{-1}. The experiments were designed to test the dynamic media exchange ability of the device. (a) Microfluidic device design with highlighted parts. (b-d) COMSOL simulation depicting diffusion-based media exchange between four fluids using inlets 1, 2, 3 and 4. (e-m) Experimental results depicting media exchange in time. The blue dye is switched off at t=0 min and the flow rate for PBS increased to 100 µl hr^{-1}. (n-o) Time vs concentration plots at the inlet junctions (n) and cell culture chambers (o). As the concentration of green and red dye in the channel is never changed in this experiment (flow rate fixed at 50 µl hr^{-1}) it is represented by the concentration value of 1 (n). The concentration of PBS in the channel becomes twice as the flow rate was increased to 100 µl hr^{-1} for flow balancing purposes; this is represented by the concentration value of 2 (n).

Cell Viability Experiments

We finally performed cell viability experiments to assess the suitability of our microfluidic device for long-term mammalian cell cultures. REX-dGFP2[21] and MiR-290- mCherry/miR-302-eGFP mouse embryonic stem cell lines were used for this purpose.[23,24] The microfluidic device with loaded cells was incubated at 37 °C for 24 hours with a continuous flow of media using a syringe pump at 50 µl hr^{-1}. The incubation allowed the cells to reach a certain confluency; images were then taken at 24 hours interval. The results in Figure 8 clearly show that both cell lines remain healthy and expand in our microfluidic device for at least 5 days.

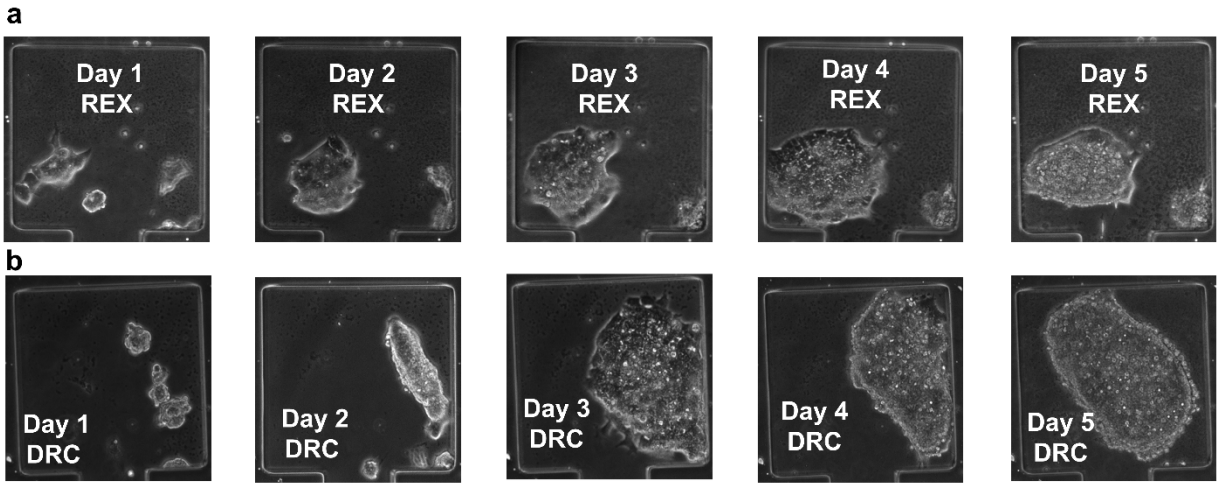

Figure 8. (a, b) Cell viability tests for REX-dGFP2 (REX, a) and MiR-290- mCherry/miR-302-eGFP (DRC, b) mouse embryonic stem cell lines.

Conclusions

Exposing cultured cells to a specific media in a stable and fixed amount of time is of utmost importance for many biomedical and biological studies.[25,26] The outcome of such studies can be adversely effected if the cells are not exposed to a certain type of media in the said amount of time or over exposed to a particular drug. It is therefore necessary to have a controlled and rapid mechanism of media exchange for microfluidic devices that are designed and fabricated for such experiments. In this study, we presented a novel single layer microfluidic device that combines a passive microfluidic micromixing mechanism with vacuum-assisted cell loading for long-term mammalian cell culture. Our device allows cell cultures with reduced shear stress and dynamical cell stimulation cells with up to 4 inputs, which can be automatically delivered to cells via computer-controlled pumps. The mixing of the fluids is achieved by

rectangular serpentine structures and at fluid flow velocities in the µl hr^{-1} range. FEM simulations performed using COMSOL and experiments showed successful fluid mixing and media exchange in cell trapping chambers at flow velocities between 50 -100 µl hr^{-1}; such flow rate is ideal for biological applications where it is important to avoid shear stressing of cells. Long term cell culture (5 days) of two different mammalian cell lines, in a fully controllable culture environment, was achieved.

As compared to previously reported devices with comparable specs, the one presented here is easier to fabricate and use, thanks to its single-layer design. The device design can be modified for different kind of cell lines by changing the device dimensions.

We foresee interest in using this and comparable devices in systems biology applications, where it is important to measure cell responses upon dynamic stimulations and derive/distinguish mathematical models of the systems and in synthetic biology, both to quantitatively characterize responses to inputs in synthetic gene networks and to implement external feedback control using microfluidics/microscopy platforms.

Author Contributions

AM: Experimental work, data analysis, and manuscript writing. EP: Experimental work and manuscript writing. ALR: Experimental work and manuscript writing AE: methodology. LM: methodology, supervision, manuscript review and writing, funding.

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Conflict of interest

The authors declare no conflict of interest.

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