

# Lab on a Chip

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## ARTICLE

## Testing A $\beta$ toxicity on primary CNS cultures using drug-screening microfluidic chips

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Open microscale cultures of primary central nervous system (CNS) cells have been implemented in microfluidic chips that can subject the cells to physiological fluidic shear stress conditions. Cells in the chips were exposed to differently aggregated forms of beta-amyloid (A $\beta$ ), i.e. conditions mimicking an Alzheimer's Disease environment, and treated with CNS drugs in order to assess the contribution of glial cells during pharmacological treatments. FTY-720, a drug approved for the treatment of Multiple Sclerosis, was found to play a marked neuroprotective role in neuronal cultures as well as in microglia-enriched neuronal cultures, preventing neurodegeneration after cell exposure to neurotoxic oligomers of A $\beta$ .

### Introduction.

Neurodegenerative diseases are characterized by dysfunction and death of specific neuronal populations and, most often, by the presence of intracellular and extracellular aggregates of otherwise physiological soluble proteins. The presence of such aggregates is a common mark observed in post-mortem tissues in several neurodegenerative disorders, such as Alzheimer's Disease (AD), Parkinson and Huntington's diseases and Amyotrophic Lateral Sclerosis [1]. Another common feature of neurodegenerative diseases relies on the key roles played by non-neuronal cells, i.e. glial cells such as astrocytes or microglia [2]. Indeed, in all three diseases, gene expression profiling has revealed deregulation of genes related to glial activation and inflammation [3].

Alzheimer's Disease is a devastating neurodegenerative disorder that in 2006 afflicted 26.6 million people with projections of a fourfold increase by 2050 [4]. It is characterized by a disruption of synaptic function leading to a progressive cognitive decline, memory deficits and behavioural changes. The main hallmarks of the pathology are extracellular accumulations of A $\beta$  in neuritic plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau protein, a microtubule-associated protein [5]. Activated glial cells are typically present in abundance around the amyloid plaques, and significant loss of neurons and synapses has been found in several regions of the diseased brain [6]. The crucial step in developing AD is related to the generation of toxic A $\beta$  from sequential proteolysis of the Amyloid Precursor Protein by  $\beta$ - and  $\gamma$ -secretases. The abnormal overproduction of A $\beta$  prompts its aggregation, at first in the form of small, soluble and potentially more neurotoxic oligomers [7], which eventually are recruited to form ordered, stable amyloid fibrils, which are deposited in neuritic plaques. The A $\beta$  cascade hypothesis considers the aggregation of A $\beta$  as the primary event that triggers a sequence of other neuropathological events leading to

neurodegeneration, such as synapse deterioration, oxidative stress, inflammation by the local activation of glial cells and formation of neurofibrillary tangles [5,8]. Neuroinflammatory processes, in which reactive glial cells release several pro-inflammatory mediators, are a common protective response to brain damage. However, these reactive inflammatory responses can turn into detrimental and contribute significantly to extend, rather than protect from, neuronal damage [9]. Notably, as a result of ageing, astrocytes abandon their housekeeping function and senesce, a process in which they stop dividing and start a path of destruction while secreting their inflammatory brew. Such senescent astrocytes peak up to high levels in the presence of A $\beta$  plaques [10]. On the other hand, microglia, which is considered the resident innate immune cells in the CNS, needed for phagocytosing apoptotic cells and limiting the damage to the adjacent tissue, may undergo prolonged activation and generate a chronic inflammation that contributes to the exacerbation of AD.

Extensive research has been conducted in the last decades to disclose the molecular mechanisms that lead to neuronal dysfunction and degeneration in AD, yielding significant advances in the understanding of the pathology [11-13]. However, the numerous and intricate signalling pathways that eventually cause neuronal loss are still not fully understood, and an effective cure is still unachieved [14]. Technology could provide breakthrough tools and methods to boost the research on neurodegeneration and to help deciphering the basis of the pathology. Through the use of microfluidics and soft lithography, a set of microfabrication techniques based on microstructuring elastomeric polymers that have enabled the development of devices such as micro cell culture analogues ( $\mu$ CCA) [15-17], the contribution of cell-cell interactions can be studied by allowing the communication of different brain cells through the diffusion of soluble factors [18], or through contact between specific cell parts (axon-glia [19], axon-neurons [20]). Furthermore, microsystems help presenting cells in situations

mimicking more closely their integration in the environment, including exposure to cues that vary in time and space as well as to gradients of molecules and secreted proteins from neighbouring cells and physiological shear stresses from fluid flow, in a controllable and reproducible fashion that cannot easily be achieved by standard tissue culture [16]. Finally, microsystems can also be used to link cell cultures with integrated analytical tools that can monitor the biochemical/biophysical processes that govern cell behaviour. Besides providing unique tools to investigate the molecular basis of the pathology, these techniques may help to accelerate drug development by providing alternative cell-based *in vitro* methods with enhanced predictability for initial screening, in order to prioritize treatments for further testing. Thus, biomimetic microsystems that reconstitute cell-to-cell communication are well suited for the development of *in vitro* models that mimic more closely the human tissue *in vivo*, with the additional advantage of using reduced amount of cells and reagents, therefore decreasing the impact on the use of animals or human tissue.

In this framework, the present work reports on a simple, yet efficient method to screen the effects of toxic agents and drugs on different populations of primary CNS cells. Microfluidic chips, operated by gravity-induced flow, have been designed to divide the flow of stimuli into 4 parallel streams that reach 4 cell chambers that can host different CNS cells populations. The stimuli (e.g. A $\beta$  species or drugs) are applied through a common inlet and distributed to the 4 chambers by perfusion achieved through a difference in volume level between the inlet and the outlets. The perfusion system is very easy to operate and the open culture chambers are extremely accessible for loading primary cells. This enables a straightforward scalability for high-throughput implementation and allows a reproducible evaluation of the influence of different cell types on neuronal viability. The use of these microfluidic chips in the context of Alzheimer's disease allowed to demonstrate a high toxicity of oligomeric A $\beta$  compared to the fibrillar form, and a protective effect of the anti-inflammatory drug FTY-720, when applied to neuronal cultures or microglia-enriched neuronal cultures.

## Experimental

### Fabrication of microfluidic chips.

Photolithography based on SU-8 photoresist was used to fabricate silicon moulds with depth features of 100  $\mu\text{m}$ , which will be the depth of the microfluidic channels. To fabricate the masters for replica molding, SU-8 photoresist was spin coated (500 rpm for 13 sec, and 2000 rpm for 30 sec) on silicon wafers and pre-baked at 65  $^{\circ}\text{C}$  for 5 min and at 95  $^{\circ}\text{C}$  for 30 min. The resist was exposed to 260  $\text{mJ}/\text{cm}^2$  UV light through ink-printed slides masks and post-baked at 65  $^{\circ}\text{C}$  for 5 min and at 95  $^{\circ}\text{C}$  for 12 min. After development, the wafers were washed thoroughly in isopropanol and dried with  $\text{N}_2$ . To facilitate peeling-off during the replication process, the silicon masters were coated with a Teflon-like layer,  $\text{CF}_x$ , deposited by plasma polymerization.

The patterns created in SU-8 were transferred to PDMS by replica molding. A mixture of polymer precursors was done by stirring vigorously 1:10 parts of base prepolymer:curing agent (Sylgard 184). The viscous mixture was poured on the masters, degassed under vacuum at room temperature for 1 h and cured at 65  $^{\circ}\text{C}$  for 4 h. Then, the PDMS wafer was cut to release the

individual microchips, which were punched to open up the cell chambers and the inlets-outlets for microfluidic flowing. Finally, the chips were covalently bonded to glass coverslips applying an oxygen plasma treatment (30 W, 30 sec, 5 sccm  $\text{O}_2$ ). Before cell seeding, the chips were sterilized by ethanol for 10 min, followed by UV treatment for 40 min.

### CNS cells isolation and culturing.

Primary cells were prepared from the hippocampi of 18 or 21-day-old fetal rats as described in [21]. For the neuronal cultures, E-18 dissociated cells were plated on the chambers of the chips coated with poly-L-lysine at 750 cells/chamber and maintained in Neurobasal medium supplemented with B-27, 0.5 mM glutamine and 12.5  $\mu\text{M}$  glutamate. Hippocampal mixed glia cultures from embryonic rat pups (E21) were grown in MEM supplemented with 20% FCS and 5.5 g/L glucose. Purified microglia were harvested by shaking 3-week-old cultures, seeded on the chips at 750 cells/chamber, and cultured in the same medium.

Primary neurons need to condition the medium with endogenous factors in order to differentiate. Flowing continuously fresh medium did not allow the cells to grow. Therefore, the chips were left in stationary mode during incubation and flow circulation was applied only to perform partial medium exchange after 3 days of culture and to flow the drugs and stimuli.

### Oligomeric and fibrillar A $\beta$ preparation.

To prepare oligomeric and fibrillar A $\beta$ , A $\beta_{1-42}$  (Anaspec) was first monomerized by dissolving it in pure hexafluoroisopropanol (Sigma) to obtain a 1 mM solution and then aliquoted in sterile microcentrifuge tubes. The hexafluoroisopropanol was removed under vacuum using a SpeedVac and the peptide film was stored desiccated at -80  $^{\circ}\text{C}$ . For each experiment, an aliquot of the dried HFIP peptide film was freshly resuspended in 100% DMSO to 5 mM, and further diluted to 100  $\mu\text{M}$  in F12 medium for preparing the oligomeric A $\beta$ , or in 10 mM HCl for the fibrillar form. Oligomers were incubated for 24h at 4  $^{\circ}\text{C}$ , and then centrifuged at 14000  $\times$  g for 10 min at 4  $^{\circ}\text{C}$ . The oligomers were collected from the supernatant after centrifugation [22]. Fibrils were vortexed for 15 sec immediately after dilution in HCl and incubated for 24 h at 37  $^{\circ}\text{C}$  [23].

### Calcium Imaging.

Cultures were loaded with 2  $\mu\text{M}$  Fura-2-AM in Krebs-Ringer solution for 40 min at 37  $^{\circ}\text{C}$ . The evaluation of the changes in intracellular calcium levels was done in an inverted microscope (Axiovert 100, Zeiss) equipped with Polychrome IV (TILL Photonics, Germany) light source. Fura-2 fluorescence images were collected with a PCO Super VGA SensiCam (Axon Instruments, CA, USA) and analyzed with TILL Vision Software (TILL Photonics, Germany). Single-cell 340/380 nm fluorescence ratios were acquired at a sampling frequency of 1 Hz.

### TEM and Thioflavin-T characterization.

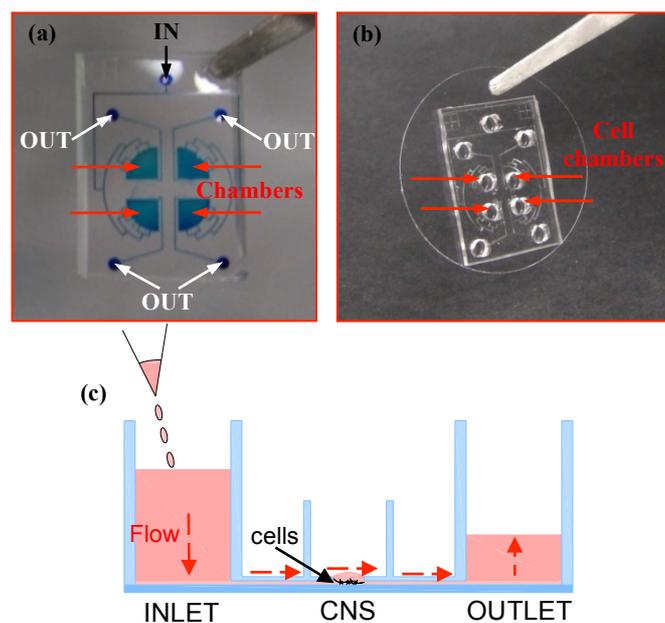
TEM images were acquired in a Philips CM10 TEM (FEI, Eindhoven, Germany) microscope. Fibrillar and oligomeric A $\beta$  preparations were diluted in Krebs-Ringer solution at 10  $\mu\text{M}$  concentration, deposited in a TEM grid for 10 min, and stained for 10 min using a saturated water solution of uranyl acetate.

For Thioflavin-T (ThT) assays, ThT (Fisher Scientific) 10  $\mu\text{M}$  was added to oligomeric or fibrillar A $\beta$  and monitored in a

(Perkin-Elmer LS50) spectrofluorometer. ThT fluorescence emission was measured with excitation at 450 nm and recording the spectrum between 465 and 565 nm with 5 nm slits.

### Toxicity tests.

Live cells were incubated for 20 min at 37 °C with a solution of propidium iodide, PI, (20 µg/ml) and Hoechst 33342 (8.1 nM), both from Invitrogen. Both dyes bind to DNA and are used to visualize cell nuclei. PI is an impermeable dye that enters the cell only upon membrane disruption, thus staining only the nuclei of dead cells. On the other hand, Hoechst 33342 permeates also in living cells and is therefore used to count the total cell number (live and dead cells) [24]. After PI and Hoechst 33342 incubation, the cells were washed in warm phosphate buffered solution and imaged under a Leica DMI 4000B fluorescence microscope. The neuronal loss was calculated by the ratio of PI positive cells to the total number of cells, given by Hoechst nuclei staining. For that purpose, images of the PI and Hoechst staining of the whole cell compartment were taken (see Supplementary Figure 1), and processed by ImageJ to perform an automatic counting of the number of nuclei stained with PI or Hoechst. The toxicity tests were performed in 8 chambers for each condition and the neuronal loss was given as the mean value and standard error of the 8 replicates, normalised to the cell death observed in non-treated neuronal cultures. Data groups were compared using one-way ANOVA with significance level of 0.05. Significant differences ( $P < 0.05$ ) are marked with an asterisk.

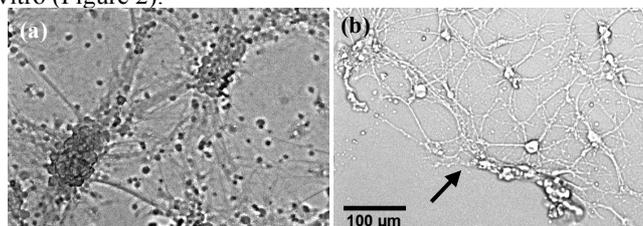


**Figure 1.** (a) PDMS chip before punching and mounting. The blue ink shows the path of the microfluidic channels from the inlet to the outlets; (b) final microfluidic chip mounted on a glass coverslip, and (c) illustration of the principle of operation via gravity-induced flow (CNS stands for Central Nervous System and indicates the chamber where primary cells are plated).

## Results and discussion

The microfluidic chips have been fabricated through replica moulding in PDMS of a master produced by SU8 lithography. Figure 1 shows the schematics of the chip configuration and the principle of operation. By applying a difference of liquid level between the inlet and the outlets, substances flow from the inlet, through the cell chamber and towards the outlets. The surface tension in the open cell chambers avoids the spread of liquid outside the confined area providing a microfluidic perfusion over the cell compartment when the difference in liquid height is applied. The chips consist of 4 chambers with an area of 4.6 mm<sup>2</sup>, placed in a circular disposition and connected by 50 µm wide, 100 µm depth microchannels. Different cell combinations can be plated in each cell chamber depending on the cells required for the experiments. To illustrate this fact, Supplementary Figure 2 shows bright field images of three whole chambers of one chip that contained a different cell combination. The PDMS chips are covalently bonded to standard glass coverslips (24 mm diameter), which allows plating the cells in direct contact with the glass, and ensures compatibility with high-resolution microscopy characterization. Stimuli, applied through one inlet, are divided in 4 parallel flows in 4 microchannels and driven into the cell culture chambers. Supplementary Video 1 shows the flux of blue ink through a chip with different cell numbers in each compartment (500, 1000, 1500 and 2000). It can be seen that the flow of blue ink reaches the cell chambers and starts displacing the medium remaining in the cell chambers at the same time. This guarantees that the same stimulus is given to each cell combination, allowing a reproducible and accurate control of the treatments applied. However, it is worthy to mention that the actual concentration of drugs and reagents might be slightly different than the nominally injected one, since the PDMS may capture some drugs and proteins; also, small hydrophobic molecules can diffuse into the material, thus possibly resulting in lower drug concentrations [25].

The open cell chambers improve the reproducibility of the cell density during plating, since the number of cells per chamber can be accurately controlled. The cell plating density has been adjusted to obtain homogeneous, differentiated cultures. Plating 750 neuronal cells isolated from the hippocampi of 18-day-old fetal rats on each chamber allowed the functional formation of a differentiated neuronal network, either for neuronal cultures or for co-cultures of neurons with microglia. Neuronal densities higher than 750 cells/chamber resulted in cell clustering after 2 weeks of neuronal differentiation. On the other hand, lower cell densities were not sufficient to achieve neuronal differentiation, probably because the cell density was too low for conditioning the medium with endogenous factors released from neurons during development, resulting in cell detachment after 7 days *in vitro* (Figure 2).

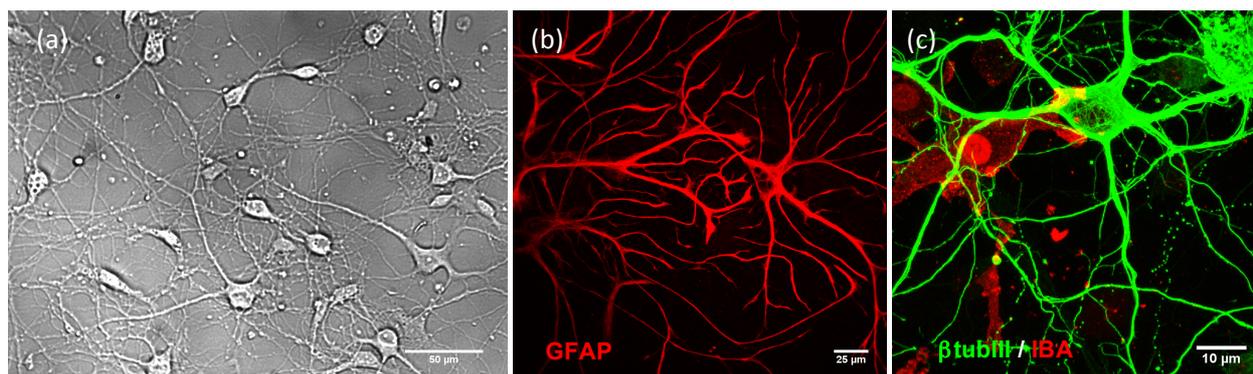


**Figure 2.** Cell clustering and damage on the microfluidic chips when plated at 1000 cells/chamber, DIV 17 (a), and cell detachment when plated at 500 cells/chamber, DIV 14, (b).

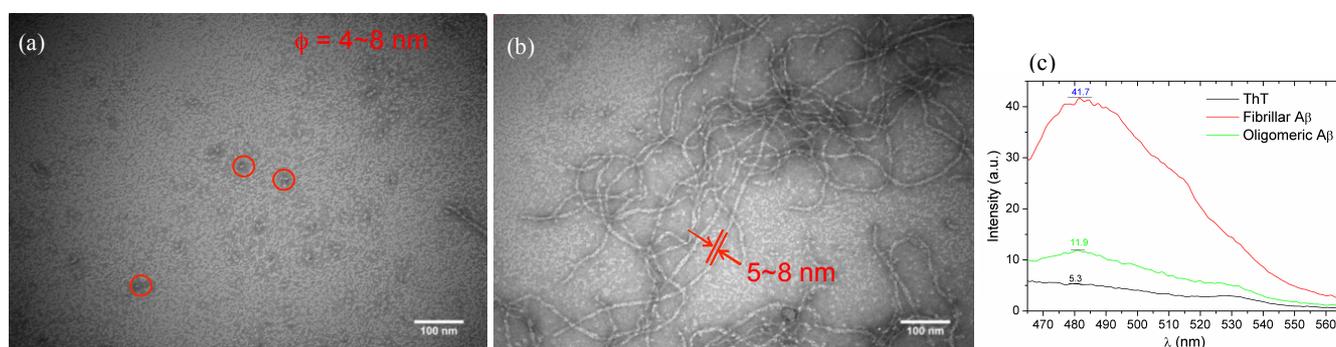
When 750 dissociated cells were plated in the chips and maintained for 20-21 days, the purity of the neuronal cultures was assessed at 70%, with about 30% of the plated cells being represented by astrocytes, as evaluated by counting the number of living cells after treating the cultures with 100  $\mu$ M glutamate for 24 h, which causes 100% of neuronal death. In the case of mixed neuron-microglia cultures, about 750 microglia cells were plated in each chamber on top of the neurons, and maintained for 24 h before the drug/toxicity tests. Figure 3 shows representative pictures of cultures plated in the chip and stained by immunocytochemistry with antibodies directed against GFAP for astrocytes, or with  $\beta$ -tubulin III and IBA1 for neurons and microglia, respectively. Neurons plated in the chip exhibited normal intracellular calcium transient response to 50 mM KCl depolarization (see supplementary Figure 3).

Figure 4 shows the characterization of the different species of A $\beta$  used for toxicity and drug testing. As observed by TEM micrographs, the fibrillar preparations consisted of 5-8 nm wide fibrils of several microns length, while the oligomeric preparations consisted of globular structures of diameter between 4 and 8 nm. The aggregation state of A $\beta$  was also assessed by Thioflavin-T (ThT) assay. ThT presents a fluorescence emission peak centered at 482 nm when it binds to fibrils. The peak was clearly observed in the fibrillar A $\beta$  and was absent for the oligomeric A $\beta$ , indicating that the oligomeric preparations contain only small globular aggregates of the protein.

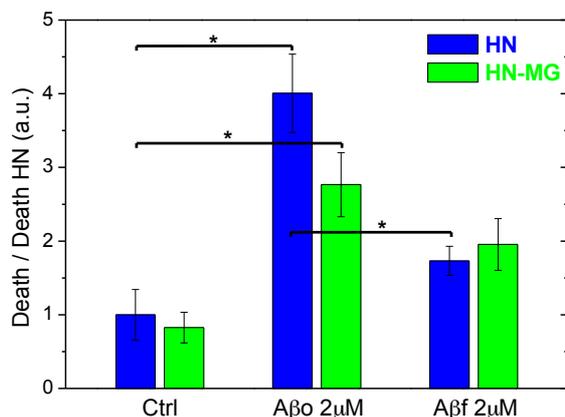
Oligomeric and fibrillar A $\beta$  preparations were diluted in conditioned neuronal medium at 2  $\mu$ M concentration, and loaded in the inlet port of chips hosting either neuronal cultures in all chambers, or microglia-enriched neuronal cultures in all chambers. To perform the toxicity assays, the same cell combination was plated in all chips to avoid possible effects of diffusion of endogenous factors that can be produced when the chips are kept in stationary mode during cell differentiation. The neuronal loss was evaluated 96 h after A $\beta$  exposure by calculating the ratio of PI positive cells to the total number of cells, given by Hoechst nuclei staining. As shown in Figure 5 (blue bars), in neuronal cultures, the neuronal loss caused by 96 h exposure to oligomeric A $\beta$  reached a 4-fold increase as compared to the control neuronal cultures, while the toxicity of the fibrillar form was much lower (below 2-fold increase). Significantly, the neurotoxicity induced by oligomeric A $\beta$  decreased when the neuronal cultures were enriched with microglia (green bars). This could be due to a major clearance activity of microglia triggered by small A $\beta$  aggregates. Indeed, we observed that when pure microglia cultures were exposed to oligomeric or fibrillar A $\beta$ , microglia presented more A $\beta$  attached to the membrane or internalized in the case of incubation with oligomers (Figure 6). Alternatively, the reduced number of damaged neurons in neuron-microglia co-cultures exposed to oligomeric A $\beta$  may be due to the well-known capability of reactive microglia to phagocytose dead or dying cells, thus resulting in progressive loss of damaged neurons. After exposure to the fibrillar form, however, the levels of toxicity were similar for either neuronal or neuron-microglia co-cultures.



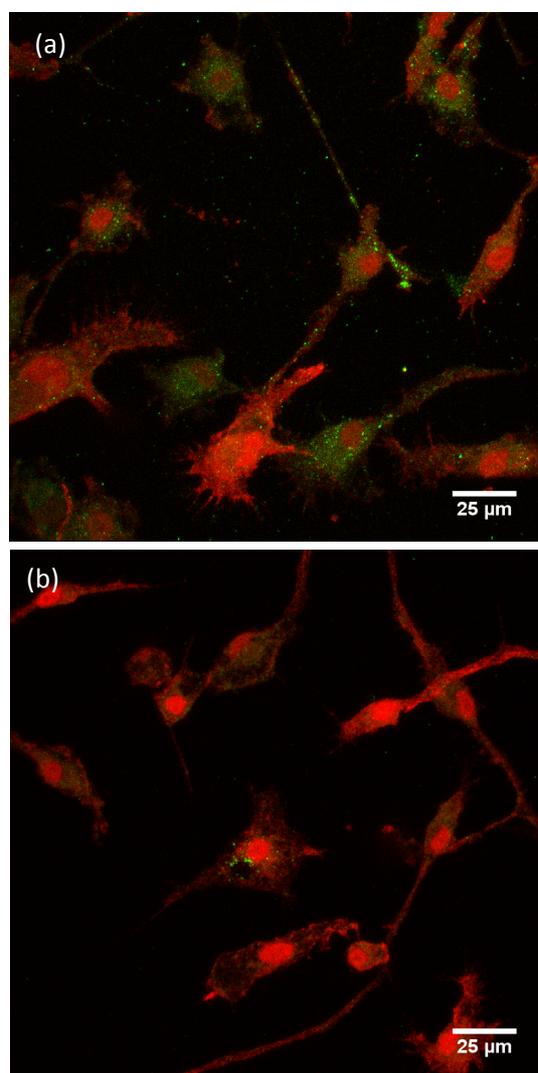
**Figure 3.** (a) Primary hippocampal neurons grown in the microfluidic chips after 7 days in vitro; (b) astrocytes present in the neuronal cultures at DIV 20, immunostained by GFAP; and (c) co-culture of hippocampal neurons (positive  $\beta$ -tubulin-III cells), DIV 17, and microglia (positive IBA1 cells), DIV 3.



**Figure 4.** TEM images of the oligomeric (a) and fibrillar (b) preparations of A $\beta_{1-42}$ . (c) Emission spectra of Thioflavin-T after binding to fibrillar and oligomeric A $\beta_{1-42}$ .



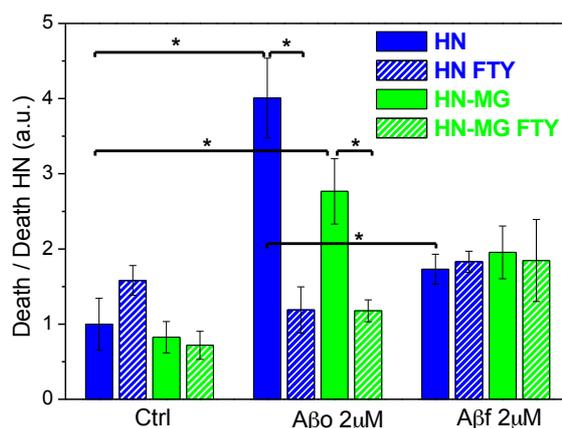
**Figure 5.** Neuronal death in 20-day-old neuronal cultures or in neuron-microglia co-cultures, 96 h after exposure to different Aβ<sub>1-42</sub> species. Neuronal loss is observed more prominently after exposure to Aβ<sub>1-42</sub> oligomers. (\* one-way ANOVA,  $p < 0.05$ ).



**Figure 6.** Representative images of pure microglia cultures incubated with oligomeric (a) or fibrillar (b) Aβ, fixed and immunostained with IBA1 (red) for microglia and 6E10 (green) for Aβ. Aβ oligomers more than fibrils attach to microglia.

We then assessed the possible neuroprotective activity of the anti-inflammatory drug used in Multiple Sclerosis (MS) FTY720. FTY720 is a S1P receptor agonist whose immunomodulating action has been proved effective for limiting the infiltration of autoreactive lymphocytes in the CNS, thus suppressing subsequent neuroinflammation. Recent works lighted hope on the therapeutic power of FTY720 for other CNS disorders [26-29]. The drug has been recently shown to prevent apoptosis after cerebral ischemia and proposed as a novel compound for the treatment of stroke [27]. Likewise, it has been demonstrated that FTY720 downregulates the production of several pro-inflammatory cytokines in microglia and proposed as a potent therapeutic agent in neurological diseases associated to microglial activation [28]. Moreover, FTY720 modulates the BDNF levels in brain cells, improving the symptoms of Rett syndrome [26], promoting the neuroprotective effects of microglia [28], and attenuating the neurotoxicity of oligomeric Aβ [29]. Remarkably, recent studies also indicate that FTY720 has a beneficial effect in restoring memory loss in AD rats [30, 31].

In view of these promising features, we were interested in evaluating whether the neuroprotection of FTY720 against oligomer-induced neurotoxicity could be further amplified by a synergistic action on microglia, and which was the effect on the fibrils-induced neurotoxicity. In order to assess the long-term neurotoxicity of Aβ in the different cell combinations the amyloid-induced neurotoxicity was also evaluated 96 h after exposure to 2 μM oligomeric or fibrillar Aβ in the presence of 200nM FTY720 (Figure 7). Interestingly, the toxicity of the oligomers was reverted to control levels upon FTY-720 treatment, in both neuronal and neuron-microglia cultures. Conversely, the drug did not significantly affect the toxicity of fibrils. These data indicate that FTY-720, a drug used for the treatment of MS targeted to glial cells, may play a neuroprotective role in AD context, acting primarily on neurons. These results are in favor of a direct interaction of the drug with neuronal cells or with Aβ, rather than an indirect action on glial cells by which it could prevent inflammation and/or promote the clearance of extracellular Aβ aggregates.



**Figure 7.** Long-term Aβ toxicity and rescue by FTY-720 in neuronal cultures or neuron-microglia co-cultures plated in the different chambers of microfluidic chips. (\* one-way ANOVA,  $p < 0.05$ ).

## Conclusions

Open microfluidic chips driven by gravity-induced flow are an easy-to-use alternative to standard cell cultures for increasing the throughput and reproducibility in the assessment of toxicity and drug efficacy in primary CNS cultures. They can also provide fluidic shear stress to the cells and, thus, a more physiological testing condition. Neuronal viability when cultures of primary CNS cells are challenged by different forms of beta-amyloid can be assessed using microchambers with a sufficient number of replicates for statistical analysis. As well, drug target cells can be identified by applying the same treatment to different combinations of cell co-cultures. Our demonstration of a predominant direct action on neurons, rather than a crucial contribution of microglia to the neuroprotective effect of FTY-720 in A $\beta$ -exposed neurons suggests FTY-720 as a promising candidate to directly prevent neuronal death in AD.

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## Notes and references

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