Surface-Functionalized Self-Standing Microdevices Exhibit Predictive Localization and Seamless Integration in 3D Neural Spheroids

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Brain organoids is an exciting technology proposed to advance studies on human brain development, diseases, and possible therapies. Establishing and applying such models, however, is hindered by the lack of technologies to chronically monitor neural activity. A promising new approach comprising self-standing biosensing microdevices capable of achieving seamless tissue integration during cell aggregation and culture. To date, there is little information on how to control the aggregation of such bioartificial 3D neural assemblies. Here, the growth of hybrid neurospheroids obtained by the aggregation of silicon sham microchips (100 × 100 × 50 μm³) with primary cortical cells is investigated. Results obtained via protein-binding microchips with different molecules reveal that surface functionalization can tune the integration and final 3D location of self-standing microdevices into neurospheroids. Morphological and functional characterization suggests that the presence of an integrated microdevice does not alter spheroid growth, cellular composition, nor functional development. Ultimately, cells and microdevices constituting such hybrid neurospheroids can be disaggregated for further single-cell analysis, and quantifications confirm an unaltered ratio of neurons and glia. These results uncover the potential of surface-engineered self-standing microdevices to grow untethered 3D brain tissue models with inbuilt bioelectronic sensors at predefined sites.

1. Introduction

3D neural cell assemblies are rapidly emerging as more comprehensive in vitro model systems to understand neurological diseases and develop potential cures.[3] Developments across the fields of biotechnology, tissue engineering, biomaterials, and microtechnology, have led to in vitro models ranging from multilayer 3D cell cultures[2] to small self-standing cell aggregates called spheroids,[3] up to complex brain organoids derived from human pluripotent stem (hPSC) cells.[4] Albeit grown in an artificial in vitro environment, the shift from conventional 2D neural cultures to 3D models was shown to better mimic the complexity of intertwined 3D networks found in the brain.[5] hPSC-derived brain organoids can indeed recapitulate several aspects specific to human brain development at the level of gene expression,[6] cell-type differentiation and network formation,[7] and can express phenotypes of human brain diseases when generated from patient-derived hPSC-cells.[4a,8] Following these results, 3D neural cell assemblies have raised a large interest for the study of human brain diseases and therapies. Furthermore, these models can overcome certain limitations of currently used animal models, such as low experimental accessibility for functional studies,[9] low sample size, low reproducibility and, above all, poor translational relevance of screening results to humans.[5]

The routine experimental use of 3D brain tissue models, however, remains largely unpractical for applications in drug discovery. On one side, intermodel variability and unmonitored cellular viability can affect the reliable generation of complex 3D brain tissue model systems. For instance, as 3D models become critically sized, the low diffusion of nutrients and oxygen tends to induce the formation of a necrotic core,[10a,5] with consequent losses in cellular viability. On the other side, available biosensing technologies are not yet adapted for the routine monitoring of biosignals such as neural activity inside individual 3D models. This hinders studies aiming toward a better understanding of the emergence of spontaneous neural activity in these models as well as their optimization to reliably generate electrically active brain tissue models for functional assays.

Over the last few years, researchers have been working on protocols for cultivating organoids with minimal variability, mainly focusing on homogenizing morphologies.[4b] As far as monitoring brain organoids, current major biosensing
approaches include the use of implantable bioelectronic probes, optical confocal microscopy applied on pregrown 3D models, and organ-on-a-chip engineered systems obtained by growing cells on microfabricated devices.\[10\] Similarly as 2D neural cultures, 3D cultures can grow on top of biosensing devices, such as multielectrode arrays for multisite extracellular electrophysiology.\[15\] However, this approach requires that each 3D cell culture model grows on a dedicated biosensing device, thus challenging the development of low-cost consumable biosensing chips and constraining sample number. Alternatively, implantable silicon (Si) probes widely used for animal experiments were used to record neural activity in 3D models.\[12\]

The invasiveness of these implantable devices and consequent tissue-damage, however, leads to low yield electrical read-outs and restricts measures to acute (nonchronic) experimental conditions. Finally, light scattering in thick tissue hampers resolution and sensitivity of optical technologies, such as Ca^{2+} confocal microscopy, nanoparticles with fluorescent reporters or biosensing methods based on surface-enhanced Raman scattering.\[10\] Besides, without slicing the 3D model, optic-based technologies are limited to measures on the superficial, outer layer of the sample, and are subject to alignment issues which makes them impractical to study large number of samples.

A promising alternative approach to overcome these issues consists in the development of bioartificial 3D brain model systems with seamless tissue-integrated biosensing artificial microdevices, without perturbing 3D model in terms of development, morphology, composition and functionality. Optically interfaced self-standing microscopic silicon particle devices internalized in cells\[14\] or even in embryos\[15\] were proposed. These micrometric intracellular devices were demonstrated for tasks such as cell tracking using a barcode system,\[16\] intracellular pressure sensing\[17\] or to implement multitasking delivery systems.\[18\] Because of alignment and light scattering issues, the functional performances of these very small area optically interfaced devices were demonstrated so far only on isolated cells or 2D cell cultures. Another study used commercial radio frequency identification (RFID) chips of 460 × 480 μm2 in size integrated into reaggregated iPSC-derived endoderm spheroids to demonstrate phenotypic screenings of a pool of RFID-modified cells.\[19\] Moreover, these cells on microdevices were demonstrated to promote adhesion and long-term viability of neuronal and glial cells in cell cultures.\[20\] In order to handle microchips for the successive steps, devices were finally released in deionized water and sterilized under hood UV for 1 h.

To investigate whether surface functionalization of the device can promote aggregation of cells around the microchips, we tested three different adhesion-promoting molecules typically used in neural cultures and that have shown no interference with recording capabilities.\[25\] namely, Matrigel (MG), poly-d-lysine (PDL), and poly-dl-ornithine (PDLO). MG is a solubilized basement membrane matrix, mainly containing extracellular matrix components such as laminin, collagen I, and entactin.\[26\] and widely used as a biologically active embedding scaffold suitable for 3D neural cell culture.\[2c,4a,27\] PDL and PDLO are also routinely used to promote adhesion and differentiation of primary neurons on glass or Si substrates through an increase of surface charge density.\[28\] These three molecules can bind onto the APTES layer, as evidenced by the contact angle measured on clean SiO2 surfaces (Figure 1B). The APTES coating on SiO2 surfaces increases the contact angle from 78° to 47°, a value in line with APTES deposition from aqueous solutions.\[29\] Matrigel binding increases slightly the surface hydrophobicity to 60.2°, which is to be expected for a gel-like structure, while PDL and PDLO significantly decrease the contact angle to 11.2° and 13.6°, respectively. As evidenced by the contact angle measured at different times over 28 days (Figure S1, Supporting Information), the presence of amine-terminated silanes after APTES deposition allows for proteins to covalently bind on the surface, which in turn provides a stable interface over time.\[30\]

As illustrated in Figure 1C, primary cortical neurons prepared from rats at embryonic day 18 (E18) were seeded in ultralow attachment 96-well plates and added with single Si microchips in each well. Despite involving different coatings on the devices and steps of manual manipulation of cells and with cells on cell viability, 3D morphology and network functionality are still unexplored. To address these questions, here we investigate the 3D assembling of neurons with microfabricated Si sham devices, and the growth of bioartificial hybrid spheroids from rat primary cortical neurons.

### 2. Results and Discussion

#### 2.1. Spontaneous Aggregation of Neurons and Si Microchips Forms Hybrid Neurospheroids

We first fabricated silicon dummy microchips (or µchips) to study their aggregation with primary neuronal cells and the growth of bioartificial hybrid neurospheroids. The fabrication process of these generic Si microchips is depicted in Figure 1A and detailed in the Experimental Section. This rather simple process includes a single photolithography and a step of deep reactive ion etching of the 50 μm thick Si substrate, and yields to ≈2000 microchips per cm2. Once structured, devices were top-side coated with a layer of silicon dioxide (SiO2) and of (3-aminopropyl)triethoxysilane (APTES) through atomic layer deposition (ALD) processes. The APTES was previously shown to stabilize protein immobilization on different substrates, including silicon, silicon dioxide, and polydimethylsiloxane, which in turn supports adhesion and long-term viability of neuronal and glial cells in cell cultures.\[24\]

**Figure 1.** A promising alternative approach to overcome these issues consists in the development of bioartificial 3D brain model systems with seamless tissue-integrated biosensing artificial micro devices, without perturbing 3D model in terms of development, morphology, composition and functionality. (A) A circuit architecture for large-scale radiofrequency (RF) based, low-power active complementary metal–oxide–semiconductor (CMOS) microdevices (100 × 100 × 50 μm3) providing integrated circuits for extracellular sensing of neural activity in organoids. These dimensions were chosen so that microdevices would be smaller than a typical rat cortical spheroid (typically 200–300 μm in diameter at 21 days in vitro\[15\]), yet large enough to integrate all the required circuits for wireless biosensing.\[21–23\] While bioelectronic and wireless technologies to realize such microdevices are under study, to date there is very little information on how to integrate extracellular Si microchips into neuronal 3D cell aggregates. Further, the potential effects of aggregating artificial Si micro devices...
microchips, this protocol reliably yielded bioartificial 3D neurospheroids (between 78% and 87% for \( n = 4 \) trials and over 96 plated wells per trial). As a step forward, production could be automated and scaled up by using pick-and-place and microfluidic techniques.

2.2. Morphology Evolution of Developing Hybrid Neurospheroids

The morphology of developing neurospheroids aggregated with differently coated microchips was characterized by optical microscopy imaging at multiple time-points and over 21 days of in vitro (DIV) culture. These results provide an overall initial evaluation of whether the presence of the Si microchip might affect spheroid growth. As shown in Figure 2, the quantification of the circularity and the mean radius does not reveal major differences among developing spheroids with and without microchips (see Figure 2C for an example), neither among spheroids integrating microchips with different coatings. Spheroids form and maintain a circular shape (circularity ratio \( >0.84 \) after 3 DIVs) and grow progressively over the course of 3 weeks, reaching a plateau in their mean radius at around 14 DIVs, corresponding to \( \approx 500 \, \mu \text{m} \) in diameter. The dimensional increase observed between 3 and 14 DIVs (about \( 200 \, \mu \text{m} \) in diameter) is most likely due to an increase of the intercellular spacing which mainly results from network formation and astrocyte proliferation. Slight intertrial differences (Figure S2, Supporting Information) are most likely due to variations in the ratio of astrocytes and neurons among primary cell culture preparations.

Overall, these results indicate that neither the presence of the microchip nor its functionalization affects the spheroid growth and its roundness, suggesting that the device does not disrupt the general morphology of self-aggregating spheroids.

2.3. Surface Functionalization of Si Microchip Determines Its Location inside Neurospheroids

To investigate the extent of the Si microchip integration in the forming neurospheroid, optical microscopy imaging of the wells was used over 3 weeks. To quantify this integration, we defined a parameter, the internalization degree (ID), corresponding to the ratio between the distance from the spheroid center to the microchip center \( D_{\text{ph} - \text{to-microchip}} \) and the radius of the spheroid \( r_{\text{sph}} \) (Figure S3, Supporting Information)

\[
\text{ID (\%)} = \frac{D_{\text{ph} - \text{to-microchip}}}{r_{\text{sph}}} \times 100
\]  

An ID between 0% and 75% corresponds to the case where the microchip is located in the center of the spheroid; an ID between 75% and 125% to the case where the microchip is located in the periphery of the spheroid; while an ID above 125% indicates that the microchip is not integrated in the spheroid. In Figure 3 we report the internalization degree quantified for each condition (No coat., MG, PDL, and PDLO) in the case of one representative experimental trial (refer to Figure S4, Supporting Information, for all other trials). Results show a marked difference in the microchip integration depending on the functionalization used. Microchips without any coating (red) tend to remain on the surface of the spheroid and in some cases (between 12% and 33%, all trials and all time points included) they are not even in contact with the spheroid. This suggests the presence of a weak adhesion of the microchip that might have detached upon handling of the multiwell plate. In the case of Matrigel coating (blue), the majority of microchips, particularly from DIV14 to DIV21, is integrated in the inner part of the periphery of the spheroids, suggesting a much stronger interaction between cells and microchips. Finally, in the case of PDL
and PDLO (green and yellow, respectively), the vast majority (between 65% and 100%, for all trials and all time points) of the microchips is instead internalized inside the spheroid. These results reveal the important role of the surface chemical properties of the microchips for the 3D assembling and growth of bioartificial hybrid neural constructs. Since these results were obtained by quantifying parameters computed from the planar projection of the 3D spheroid in the well, we qualitatively verified them by scanning electron microscopy (SEM), which allows direct 3D visualization of the spheroids with microchips.

Figure 4 displays representative examples of spheroids at different developmental time points for different functionalization conditions. These 3D images confirm optical imaging results on the different levels of microchip integration depending on the surface functionalization. In particular, uncoated Si microchips remain on the external surface of the spheroid, with a few neurites holding the device in place. This leads to a weak integration of the microchip into the spheroids and to a high number of devices detaching from the spheroid. Matrigel-coated microchips remain on the periphery of the spheroid, but exhibit more neurites that keep the device in place, which in turn supports a stronger interaction. Finally, for PDL and PDLO conditions, SEM images display much more neurite growth on the microchip at early time points and a higher level of microchip integration: at 21 DIV it is barely possible to distinguish the presence of the device in the neurospheroid.

These results indicate that surface functionalization allows to control the assembly of 3D bioartificial neuronal constructs with different levels of in-tissue integrated self-standing microdevices. Controlling the microdevice position in the 3D neural aggregate is particularly relevant for brain organoids. For instance, a microdevice with pH sensing capabilities internalized in the center of brain organoid would allow routinely monitoring the diffusion of nutrients and oxygen to detect the formation of a necrotic core, which in turn would provide crucial information to optimize organoid cultures efficiency.[1b] Besides, being able to control the placement of microdevices at different locations inside organoids would provide access to different cellular layers to routinely monitor neural activity or the chemical microenvironment, thus overcoming limitations of current imaging techniques for high-throughput read-outs.

2.4. Neuronal and Astrocytic Cell Composition of Hybrid Neurospheroids

Using immunofluorescence analysis of neuronal and glial markers, we further investigated whether the presence of Si microchips might affect spheroids cellular composition. Neurospheroids at DIV5 and DIV21 from each condition were fixed, stained, and analyzed through confocal microscopy (Figure 5). For the sake of simplicity, only control wells and wells containing a microchip functionalized with PDL are reported in the figure, while all images acquired from neurospheroids grown with microdevices with the other coatings are available in Figure S5 of the Supporting Information. At DIV5, spheroids exhibit globular patterns of β-III-tubulin neuronal staining (red), and fewer glial fibrillary acidic protein (GFAP)-positive astrocytes (green), while cells are highly packed in the structure, as evidenced by Hoechst staining (blue). At DIV21, both neurons and astrocytes express more elongated neurites, bundles, and form a complex network, while the distance between nuclei increases under the effect of astrocyte proliferation and neurite extension complexity. No evident difference is observed in the composition and maturation of the network with respect to the presence of the microchip, independently of the coating used (see also Figure S5, Supporting Information). These qualitative imaging data suggest that the Si microchip does not alter astrocyte proliferation, neurite branching nor the overall development of 3D neural constructs.
2.5. Expression of Spontaneous Neural Activity in Hybrid Neurospheroids

The integration of Si microchips might affect the expression of spontaneous neural activity. To verify this, we used calcium dye confocal microscopy imaging and compared the developmental activity in control spheroids without microchips with bioartificial hybrid spheroids. Given the little influence of coatings observed on neurospheroid cellular composition (Figure 5), only PDL-coated devices were used. After one week in culture and throughout three weeks, Ca$^{2+}$ oscillations represented as fluorescent variations are detected (Figure 6A–C). The pharmacologically induced KCl depolarization confirms that this calcium activity is associated with neuronal activity. At DIV7, neurospheroids both with and without microchips exhibit signs of neuronal activity on a few regions of interest (ROI). At this stage of development, the activity is sparse, both spatially and temporally, and no synchronous activity is detected (Figure 6A). Upon network development, at DIV14, all spheroids exhibit more active ROIs, with in some cases the appearance of synchronous activity among distant ROIs (Figure 6B). After three weeks, neurospheroids tend to express a sustained spontaneous activity both in terms of numbers of active soma as well as in the frequency of spikes (Figure 6C). Figure 6D shows that the number of active ROIs for each tested spheroid increases over developmental time, and very similar activity levels are observed between control spheroids and spheroids containing a microdevice. This suggests that the presence of the Si microchips does not perturb the spontaneous functional development of neurospheroids over the observed experimental time window of three weeks.

2.6. Disaggregating Bioartificial Neurospheroids for Single-Cell Analysis

Finally, we explored whether cells and devices of bioartificial hybrid neurospheroids can be separated for further single-cell analysis. By using a papain-based tissue dissociation, we found that it is possible to disaggregate formed spheroids to collect living single cells separated from microchips (Figure 7A). To demonstrate whether the single cell suspension obtained from the disaggregation can be used for further single-cell analysis, we quantified by fluorescence-activated cell sorting (FACS) the ratio of neurons and glial cells in neurospheroids at DIV28 with and without microchips. As shown in Figure 7B, no differences between the two conditions were found. On average, spheroids without microchips are composed of 53.1% neurons (identified as NeuN$^+$ cells) and 35.4% astrocytes (identified as GFAP$^+$ cells), while spheroids with microchips are composed of 51.5% neurons and 39.6% astrocytes (see details in Table S1, Supporting Information). Thus, besides confirming that the presence of microchips does not alter the spheroid cellular...
composition as previously observed by immunofluorescence analysis (Figure 5), this quantification demonstrates the feasibility of single-cell analysis on bioartificial hybrid spheroids.

3. Conclusion

The present study focuses on the integration of Si microchips into neuronal 3D cell aggregates through the assembling of primary cortical cells and microchips and the growth of hybrid neurospheroids over 21 DIVs. The presence of Si microchips of $100 \times 100 \times 50 \, \mu m^3$ does not affect the developing 3D morphology, cellular composition and the development of spontaneous neural activity. By immobilizing various adhesion-promoting proteins on Si microdevices, our results reveal the role of the surface-chemical properties of these microchips in driving their assembling with cortical cells and their incorporation inside spheroids. In particular, uncoated microchips have poor interaction with cells. Matrigel-coated microchips remain on the periphery of the spheroid, while PDL- and PDLO-coated microchips are integrated inside the spheroid.

Together, our results support the feasibility of a new generation of 3D brain tissue models with tissue-integrated biosensing microscale devices. This could provide built-in functionalities for routinely monitoring neural activity at sub-millisecond resolution and from deep inside organoids for behind the performances of currently available technologies, or for multiparametric monitoring of other biosignals (e.g., pH, temperature) during 3D model culture or assays. Here we focused on microscale Si devices having a square area of $100 \times 100 \, \mu m^2$ in size and $50 \, \mu m$ in thickness that were previously determined from the physical constraints of an RF biosensing circuit that we proposed for this application.\cite{21} However, shape and dimensions of microscale devices could provide additional degrees of freedom to tune the device integration inside 3D models and need to be evaluated in parallel with CMOS circuit design. Additionally, future studies need to focus on the assembling of bioartificial 3D models with human-derived neurons, extend the experimental time-window up to over several months and evaluate the microdevice performances to establish an efficient built-in-tissue seamless bioelectronic interface with normal neural tissue without glial scarring characteristics.

4. Experimental Section

**Silicon Microchip Fabrication**: The fabrication of Si microchips requires standard microelectronic processes (Figure 1A). First, squares of $100 \times 100 \mu m^2$ of 100 nm sputtered aluminum were patterned via lift-off (Microchem S1813 photoresist) onto an N-type 50 $\mu m$ silicon wafer (Si-Mat) and acted as a mask for the silicon etching step (Figure 1A-1). The thin silicon wafer was then transferred onto a thicker substrate by means of a dissolvable glue (Xtal bond SPI 555, Figure 1A-2). A standard Bosch process at 5 $^\circ$C allowed to etch through the 50 $\mu m$ thick wafer (ICP-RIE Si 500, SE Tech instruments) (Figure 1A-3).

**Surface Modifications—APTES**: Following this step, both SiO$_2$ (10 nm) and (3-aminopropyl)triethoxysilane (APTES, 40 cycles, dose 30") were deposited through atomic layer deposition (FlexAL, Oxford Instruments)
onto the substrate (Figure 1A-4). Finally, the microchips were released in deionized water in a glass vial. To avoid having molten glue residues in the vial, the deionized water was cold (4 °C) and a gentle ultrasound was applied on the vial for a few seconds to completely release the small silicon microchips into the vial (Figure 1A-5). The microchips were then rinsed twice in deionized water before being sterilized under hood UV for 1 h (Figure 1A-6).

**Protein Coatings:** For each trial, the microchips were separated in different sterile vials. For protein immobilization, water was replaced with either 800 μL of poly-lysine (0.1 mg mL⁻¹, Sigma P0671), poly-d-lysine (0.1 mg mL⁻¹, Sigma P6407) or Matrigel (0.5%, Corning 354230), and incubated overnight at 37 °C and 5% CO₂. The next day, the vials were rinsed three times with sterile Milli-Q water. In the end, one vial per condition was obtained: microchips without coating (No coat.), microchips with MG coating, microchips with PDL coating, and microchips with PDLO coating.

**Characterization of Surface Properties:** To characterize surface wettability and their stability over time, water droplet contact angle measurements were performed onto macro pieces of silicon using Dataphysics OCAH200 contact angle instrument equipped with a 2/3″ measure. Measurements were performed on macro pieces of silicon using Dataphysics OCAH200 contact angle instrument equipped with a 2/3″ measure. Five contact angle measurements were made using 5 μL droplets of water. The contact angle was measured from the surface of the droplet to the substrate. A minimum of 24 wells was plated. After dissection, cortices were placed in the digestion solution and incubated in water bath at 37 °C for 30 min. Few milliliters of complete NB + FBS (10%) were added to the cell solution, centrifuged at 1200 rpm for 5 min, and the supernatant was removed. The cell pellet was resuspended in fresh complete NB + FBS (10%) and gently pipetted for no more than ten times with P1000 pipette. The solution was filtered with a cell strainer (Biologix 15-1040, 40 μm pore size), centrifuged at 700 rpm for 7 min, and the supernatant was removed. The cell pellet was resuspended in complete NB. Cell viability at the time of isolation was determined by a Trypan Blue Exclusion Assay (Sigma T8154). Cortical cells were then seeded at a density of 6500 cells in 35 μL medium in ultralow attachment plates (GravityTRAP ULA plate 96-wells). In order to avoid bubbles in the wells, first 25 μL of warm NB was plated in the wells, then the plate was centrifuged at 250 g for 2 min, before adding 6500 cells in 50 μL in each well. Following cell plating, silicon microchips were seeded by carefully pipetting 1 μL of water in each glass vial, visually inspecting that it contained only one device, before plating it one by one in the wells. For each condition (control, microchip with and without protein), a minimum of 24 wells were plated. Device-less wells were replated after inspection. After a few hours, the

**Spheroids Formation:** All animal procedures carried out in this work were approved by the institutional IIT Ethics Committee and by the Italian Ministry of Health and Animal Care (Authorization No. 110/2014-PR of the 19th of December 2014).

Primary neuronal spheroids were established from cerebral cortices of Embryonic day 18 (E18) Sprague–Dawley rats and maintained at 37 °C in a humidified atmosphere of 5% CO₂. The following solutions and media were used: Hanks Balanced Salt Solution (HBSS) (Sigma H8648), digestion solution – Trypsin (0.125%, Thermo Fisher Scientific 25050014) in HBSS + DNase (0.25 mg mL⁻¹, Sigma D5025) in HBSS 5 × 10⁻³ M CaCl₂; complete Neurobasal medium (NB, Thermo Fisher Scientific 21103049) supplemented with B27 (2%, Thermo Fisher Scientific 17504044), Glutamax (1%, Thermo Fisher Scientific 35050038), and penicillin/streptomycin (1%, Sigma P4333); FBS (Sigma F7524). Briefly, embryos were removed and decapitated, brains were extracted from the skulls and placed in cold HBSS. After dissection, cortices were placed in the digestion solution and incubated in water bath at 37 °C for 30 min. Few milliliters of complete NB + FBS (10%) were added to the cell solution, centrifuged at 1200 rpm for 5 min, and the supernatant was removed. The cell pellet was resuspended in fresh complete NB + FBS (10%) and gently pipetted for no more than ten times with P1000 pipette. The solution was filtered with a cell strainer (Biologix 15-1040, 40 μm pore size), centrifuged at 700 rpm for 7 min, and the supernatant was removed. The cell pellet was resuspended in complete NB. Cell viability at the time of isolation was determined by a Trypan Blue Exclusion Assay (Sigma T8154). Cortical cells were then seeded at a density of 6500 cells in 75 μL medium in ultralow attachment plates (GravityTRAP ULA plate 96-wells). In order to avoid bubbles in the wells, first 25 μL of warm NB was plated in the wells, then the plate was centrifuged at 250 g for 2 min, before adding 6500 cells in 50 μL in each well. Following cell plating, silicon microchips were seeded by carefully pipetting 1 μL of water in each glass vial, visually inspecting that it contained only one device, before plating it one by one in the wells. For each condition (control, microchip with and without protein), a minimum of 24 wells were plated. Device-less wells were replated after inspection. After a few hours, the

**Figure 5.** Fluorescence imaging of cortical spheroids with and without microchip. Confocal projections at DIV5 and DIV21 of spheroids reveal the presence of central nervous system (CNS) cell types, including neurons (β-III-tubulin, red) and astrocytes (GFAP, glial fibrillary acidic protein, green). Nuclei are labeled with Hoechst (blue). Images are merged with and without bright field (BF). Data show a proliferation of neuronal connection between DIV5 and DIV21, and a great increase in GFAP-expressing astrocytes in the network in a similar fashion between spheroids with and without microchips functionalized with PDL. Images acquired for all conditions (Ctrl, No coating, MG, PDL, and PDLO) are available in Figure S5 of the Supporting Information. Scale bar: 50 μm.
plates were centrifuged at 250 g for 2 min before being kept in incubator for 21 days. At DIV 5, 10, 14, and 19, the medium was partially replaced with 30 µL of fresh complete NB.

**Morphology Assessment—Optical Microscopy:** At DIV 5, 10, 14, 17, and 21, plates were retrieved from the incubator and images were taken from all wells using a Leica DMI 6000 B inverted microscope with a ×10 objective. At each time point, the circularity of the spheroids, its area therefore its mean radius and the distance between the center of the device compared to the centroid of the spheroid were measured using ImageJ software (Figure S3, Supporting Information). Contaminated wells, dirty wells (residues), and wells containing more than one device, represented ≈8–15% of all wells. Besides, at early time points, wells containing more than one single spheroid were also excluded. Overall, between 13 and 23 wells were considered at each time point for each condition and each trial (three distinct trials, at different time during the year and with distinct animal dissection).

**SEM:** At DIV 5, 10, 14, 17, and 21, six spheroids per condition (Ctrl, No Coat., MC, PDL, PDL0) were fixed in paraformaldehyde (PFA, 2% v/v, Santa Cruz Biotechnology 30525-89-4) and glutaraldehyde (GA, 2% v/v, Sigma G5882) in phosphate-buffered saline (PBS) (Thermo Fisher Scientific 10010056) for 2 h, followed by three washes in PBS. The samples were postfixed in osmium tetroxide (1%) in Milli-Q water for 2 h and washed with Milli-Q water. The spheroids were subsequently dehydrated with a series of 10 min incubations in rising concentrations of ethanol in water solutions (from 30% to 100%), 1:1 ethanol:hexamethyldisilazane (HMDS, Sigma-Aldrich), and HMDS (100%) and dried overnight in air. Finally, the samples were sputtered with Au (10 nm) and analyzed by SEM (FEI NanoLab 600 dual beam system).

At DIV 5 and DIV 21, a few spheroids from each condition were fixed in PFA (2%) and GA (2%) (see previous section). All the following steps were performed on a shaker at 4 °C. The following antibodies were used: mouse antitubulin β3 (BioLegend 801213, 1:50), rabbit anti-GFAP (DAKO Z0334, 1:250), Alexa488 goat antirabbit (Invitrogen A11034, 1:200), and Alexa647 goat antimouse (Invitrogen A21236, 1:200). Spheroids were permeabilized and blocked with Triton X-100 (TX, 1%, Sigma T9284), normal goat serum (10%, Sigma C9023), and bovine serum albumin (BSA, 4%, Sigma A9647) in PBS (B-PBT) for 2 h, and subsequently incubated in primary antibodies diluted in B-PBT overnight. Spheroids underwent two 2 h washes with TX (0.2%) in PBS (PBT), followed by one 2 h PBT wash. Spheroids were incubated with secondary antibodies in B-PBT overnight. Spheroids underwent two 2 h PBT washes and
were incubated with Hoechst (BD Biosciences 561908, 1:300) in PBT for 1 h and returned to PBS. Spheroids were kept in PBS and transferred to glass-bottomed confocal dishes for imaging. All images were acquired with 40x objective lenses using a Leica SP5 inverted confocal microscope (Leica Microsystems). For each condition, the maximum intensity projection of a Z-stack from approximately half spheroid (from the bottom to the maximum diameter) is displayed.

**Activity Assessment:** Calcium dye Fluo-4 AM (ThermoFisher 14201) was reconstituted in dimethyl sulfoxide according to manufacturer’s instructions. Spheroids were incubated with Fluo-4 AM at the final concentration of 2.5 µg mL⁻¹ in extracellular saline solution (HEPES 10 × 10⁻³ M, D-glucose 5.5 × 10⁻³ M, NaCl 145 × 10⁻³ M, KCl 5 × 10⁻³ M, CaCl₂ 2 × 10⁻³ M, MgSO₄ 1 × 10⁻³ M, pH 7.3–7.4) and incubated at 37 °C in the dark for 15 min. Spheroids were consequently washed twice in extracellular saline solution and transferred in 300 µL of the latter in a chamber with glass bottom for live imaging. Live imaging was performed with inverted confocal microscope Nikon A1 coupled with a Nikon Objective 20x Plan Apo A.N.0.75 (Nikon Instruments S.p.A.), using a 488 nm laser for the green fluorescent dye. The focus plane was made on top of the spheroid, and the pinhole adjusted so that each manually selected ROI (5 µm in diameter) could be reasonably be associated as the contribution of a single soma in the spheroid. Each recording session lasted 10 min and consisted of 8 minutes of unperturbed activity, followed by injection of KCl (2 × 10⁻³ M) to activate voltage-gated calcium channels and depolarize the cells. Fluorescence intensity from various ROIs was then exported from Nikon imaging software NIS-2 and analyzed with OriginLab. For each recorded spheroid, the average number of active ROI on the focus plane considered was counted. Spheroids with less than three active ROI or for which the KCl control did not have a depolarization effect, were not considered.

**Single-Cell Analysis by FACS:** Spheroids at DIV28 were desegregated with the Papain Dissociation System (Worthington Biochemical, LK003150) according to the manufacturer’s instructions. Briefly, 156 spheroids per condition were pooled in a tube (one tube per condition), washed with PBS and placed in the papain solution (20 units mL⁻¹ papain, 0.005% DNase). The tube was incubated at 37 °C with constant agitation (550 rpm) for 1 h. Every 10 min, spheroids were triturated with P200 pipette and the solution re-equilibrated with 95% O₂:5% CO₂. After 1 h, while the freed devices remained at the bottom of the tube, the single cell suspension was transferred to a new tube and centrifuged at 300 g for 5 min at room temperature. The pelleted cells were resuspended in albumin-inhibitor solution with DNase (according to the manufacturer’s instructions) and centrifuged at 1200 rpm for 5 min. For FACS analysis, the pelleted cells were fixed in PFA (4%) for 20 min at room temperature, washed and centrifuged at 1200 rpm for 5 min. The pelleted cells were permeabilized with Triton X-100 (0.05%) in PBS for 20 min at room temperature, washed with cold FACS buffer (0.05% Triton X-100 and 0.5% BSA in PBS) and centrifuged at 1200 rpm for 5 min. The pelleted cells were stained with mouse anti-Neuronal Nuclei (NeuN, Alexa Fluor 488 conjugated, Millipore MAB377X, 1:1000) and mouse anti-GFAP (Alexa Fluor 647 conjugated, Millipore MAB377X, 1:1000) and mouse anti-GFAP (Alexa Fluor 647 conjugated, BD Pharmigen 561470, 1:250) in FACS buffer for 30 min on ice in the dark. Cells were washed, centrifuged at 1200 rpm for 5 min and resuspended in 200 µL of FACS buffer. FACS analysis was performed with BD FACSAria III cytometer (BD Biosciences), at least 10 000 events for each dot plot were acquired and data were analyzed with CellQuest software.

**Statistical Analysis:**

1. Preprocessing of data (e.g., transformation, normalization, evaluation of outliers): For cell culture analysis, wells excluded from the study were: dirty wells (residues, contamination), wells containing more than one device, wells containing more than one single spheroid 24 h after seeding.
2. Data presentation (e.g., mean ± SD): Droplet contact angle: mean ± SD (Figure 1B; Figure S1, Supporting Information).

3. Circularity and spheroid radius: mean ± SEM (standard error of the mean) (Figure 2; Figure S2, Supporting Information). Internalization degree: mean ± SD (Figure 3; Figure S4, Supporting Information). For FACS analysis, percentage of gated events compared to total events is represented (Figure 7; Table S1, Supporting Information).

4. Sample size for each statistical analysis, a) droplet contact angle: \( n = 5 \) per surface treatment and per time point (Figure 1B; Figure S1, Supporting Information). b) Circularity, mean spheroid radius (Figure 2; Figure S2, Supporting Information), and internalization degree (Figure 3; Figure S4, Supporting Information): three trials were performed (1 trial = set of 21 DIV-long cultures from 1 sacrificed animal, at different times during the year), with for each trial 24 wells dedicated per surface treatment. Without excluded wells (contaminated, etc. see previously), one obtains \( n = [13, 23] \) for each data point represented. c) FACS (Figure 7; Table S1, Supporting Information): 1 trial with 156 spheroids per condition and at least 10 000 events acquired for each dot plot.

5. Statistical methods used to assess significant differences with sufficient details (e.g., name of the statistical test including one- or two-sided testing, testing level (i.e., alpha value, \( \alpha \)-value), if applicable post hoc test or any alpha adjustment, validity of any assumptions made for the chosen test): not applicable.

6. Software used for statistical analysis: Matlab, OriginLab, CellQuest (FACS).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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