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A generic widefield topographical and chemical photopatterning method for hydrogels.

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Physiologically relevant cell-based models require engineered microenvironments which recapitulate the topographical, biochemical and mechanical properties encountered *in vivo*. In this context hydrogels are the materials of choice. However, shaping hydrogels at the cellular scale and tuning their chemical properties requires deep investment in custom chemistry and devices while more accessible ones lack a simple structuration and functionalization mean. Here, we show how the most commonly used hydrogels (i.e. Matrigel, Agar, PEG, Polyacrylamide) can be finely structured and spatially functionalized by exploiting oxygen and radical photochemistry together with a widefield patterned UV light illumination. Our generic hydrogel microfabrication platform can be used to grow neurons and cell lines onto chemically and topographically complex PEG gels, inside engineered Matrigel structures or within microfluidic chambers. Our findings demonstrate that oxygen-controlled polymerization and photo-scission unlock the engineering of hydrogels that lack a dedicated chemistry.

Hydrogels are commonplace in 3D cell culture i.e. the art of organ-izing cells by cultivating them in configurations that more closely mimic the in-vivo environment[1]. Whether to fill scaffolds with cells[2], exploit cell self-organization[3,4], or both[5], their high hydric content, modulable mechanical properties and access to chemical functionalization[3,6–8] make them a staple for most applications. Once structured and/or decorated with biomolecules, these meshes could form the much sought after micro-niches necessary to create organoids in a reproducible manner[5,9]. Light is the best candidate to cure, cleave or decorate hydrogels while avoiding physical contact, and now tremendous solutions exist[10].

Most light-tunable hydrogels are the results of proprietary hardware and chemistry[11] while common gels (Matrigel, Agar, Polyethylene glycol (PEG)) can only be cleaved by using time consuming raster scanning laser setups[12]. Generic solutions allowing widefield additive or subtractive manufacturing and decoration are lacking. We argue that such a platform should be compatible with common materials and embrace the day to day flow of modern biology.

In this report we explore how a rather simple setup combining patterned light illumination with oxygen permeable (PDMS) reactors can achieve micron-scale additive and subtractive manufacturing for PEG, Agar, Matrigel or poly-acrylamide. We also confirm that photo-linkers are effective in this framework by decorating bio-inert PEG gels to culture cell lines and neurons in controlled configurations.

Our new platform combines a UV illumination module docked to a microscope that shines spatially modulated light through gas permeable micro-reactors made of glass and PDMS **(Fig.1A)** Light is patterned via a digital micro-mirror device (DMD) allowing arbitrary forms and gray levels (via pulse width modulation) to be projected. The microchambers are made of two PDMS stencils stacked onto a microscopic glass-slide, their design is modular and their assembly simple **(Fig.1A and Supp.1)**.

In this configuration, it is normally not possible to cure photopolymerizable hydrogels up to the ceiling as the perfusing oxygen forms an inhibition layer (deadzone) close to the PDMS[13,14]. This effect disappears by perfusing an inert gas on top of the device: the cured hydrogels will then reach the ceiling forming pillars or channels in the chamber **(Fig.1B)**. Following this principle, it is possible to create hydrogel microfluidic devices which are semi permeable and support the generation of gradients **(Supp.1) (Supp. video 4)**.

On the other hand, under normal atmosphere it is possible to take advantage of the deadzone properties as it was previously demonstrated in other works[13].

Here we exploit the dependence of the deadzone thickness towards the photon flux for the realization of multi-height structures **(Fig.1B)**. Indeed the dead zone grows and thus the gel height diminishes with a weaker photon flux[13,15]. This can be achieved either by decreasing laser power **(Fig.1E)** or by projecting darker gray levels **(Supp. 2)**. In turn topographically structured hydrogels can be generated in a single exposure by projecting UV gray scale patterns.

It is important to note that the thickness of the gel is only imposed by the photon flux and not by the UV dose. In turn the crosslinking rate of the gel can be tuned independently of the height, through the insolation time. On top of photo crosslinking inhibition, oxygen is also necessary for the radical

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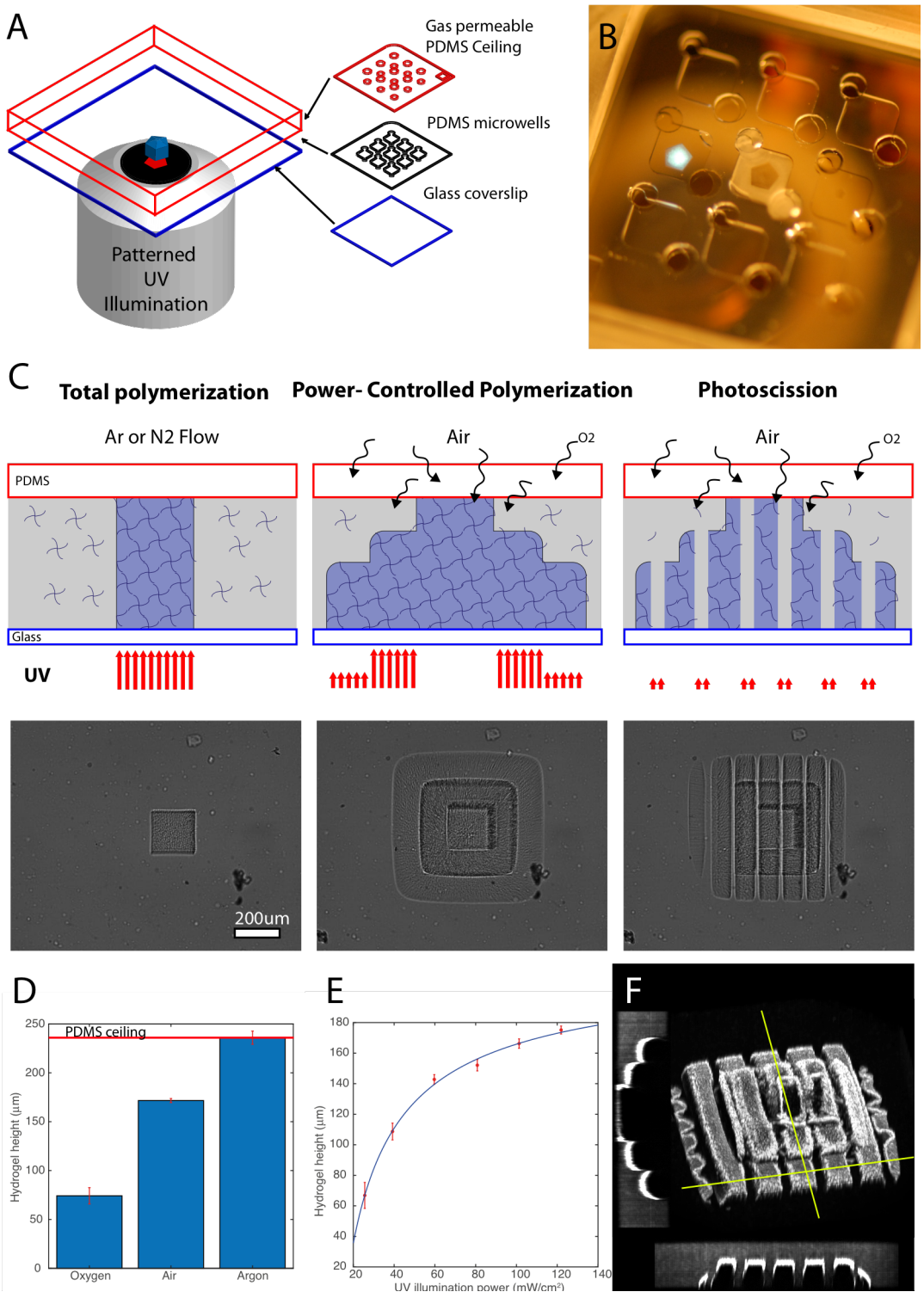

Figure 1 | Three structuration modes for a single setup. A, Schematics of the setup showing the patterned UV illumination coming through the microreactor composed of three layers: glass (blue), wells (black) and ceiling (red) made of stacked PDMS stencils. **B,** Close-up photography of a 9 wells microchamber substrate on top of the microscope with an ongoing insolation (left) and a transparent polymerized gel microstructure in an opaque solution (center). **C,** Fabrication of a sliced "Aztec-temple" obtained by three successive structuration modes. First a pillar was created under argon by total polymerization. Then, two stages were added by power-controlled polymerization. Finally, the gel was sliced by photo-scission. All steps were performed in the micro-reactor chamber made of glass (bright blue) and PDMS ceiling (red) with the gels represented in dark blue. Top: schematics of the 3 mechanisms in cross section view. Bottom: brightfield images of the hydrogel microstructure at each step of the process. **D,** Histogram showing the gel height as a function of the nature of gas perfusion and also displaying the position of the PDMS ceiling (red). Error bars represent the standard deviation of 32 experiments. **E,** Plot of the gel height as a function of photon flux in air atmosphere. Error bars represent the standard deviation of 32 experiments. **F,** 3D reconstruction of the final structure and orthogonal cross section views along the yellow lines.

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photo-scission of poly-ethylene chains[16,17]. We previously used this mechanism to degrade PEG-based brushes and generate proteins micropatterns in a dose dependent manner[17]. We now demonstrate that this same reaction can be harnessed to cleave hydrogels **(Fig.1B)**. In more detail, a water-soluble photo-initiator is first let to perfuse inside the cured gel. Then, by shining light with a very low power, and avoiding oxygen depletion within the insolated volume we enable photo-scission. This process leads to the liquefaction of the hydrogel in the entire insolated volume while leaving the unexposed area unaffected. We have successfully performed this experiment in most commonly used hydrogels, namely PEG, Matrigel, Agar and Poly-acrylamide. In the absence of oxygen depletion all the gels above were cleaved in times ranging from 30 minutes to 4 hours depending on their differences in chemistry hinting that this reaction might be generic **(Supp. video 1)**.

Based on our observations and the works previously mentioned[13–16], we propose that the competition between photo-crosslinking and photo-scission depends on the radical types that are generated in the insolation volume. Alkoxy (ROx) radicals induce photo-scission and are formed in the presence of oxygen while alkyl radicals, formed in depleted areas, are responsible for polymerization and more generally crosslinking. We switch between those two opposing reactions by tuning the oxygen profile and thus, the resulting radical composition in the insolation volume. These general principles allow for three distinct structuration means to be queued in a single platform **(Supp. video 2)**.

As a demonstration, we sequentially applied the three mechanisms described above on a soft (E<10 kPa[18]) photo-polymerizable 4-Arm-PEG-Acrylate gel. We cured a pillar (200 x 200 x 250 um) then added 2 additional stages (300 x 300 x 176 um and 950 x 950 x 100 um) and finally photo-scissioned bands of this 3D-structured hydrogel at specific locations **(Fig.1)**.

More complex hydrogels like Matrigel largely used in 3D cell culture can undergo the same queued structuration steps. In turn cells can be cultured in complex environments. As an example, we first prototyped PEG microfluidic channels under argon atmosphere into our devices allowing us to pour Matrigel in controlled areas **(Supp.1C)**. Thanks to the permeability of the gels, we secondly perfused a photo-initiator in order to cleave the Matrigel and PEG barriers altogether. Finally, we injected a cell-laden Matrigel solution that filled the void space resulting from the photo-scission. While it would seem appealing to photocleave cell-laden hydrogels we noticed toxic effects on the surrounding cells. This is probably due to the photo-scission byproducts diffusing throughout the long exposure. We expect that further understanding of the reaction will help circumventing this weakness which so far contrains us to the preparation of 3D micro-structured templates for further cell culture.

In our hands, it turns out to be a very powerful micro-structuration technique that works for any gel or combination of gels we tested so far.

Decoration after structuration

We documented the ability of our platform to structure hydrogels, we will now present our strategies to spatially functionalize these structures with biomolecules to promote cell adhesion.

Among hydrogels we can structure, 4-Arm-PEG-Acrylate is well suited for this task. It is easily shapeable by photopolymerization thanks to its acrylate moieties and in our hands, PEG turns out to be the quickest material to undergo photo-scission **(Supp. video 1)**. Furthermore, PEG is well known for having limited biological activity[3,10,19] making it the gel of choice for protein micropatterning and cell seeding.

We developed a protocol based on commercially available reagents Acryl-PEG-SVA linkers and Polylysin (PLL) **(Fig.2)**. Acryl-PEG-SVA is an hetero-bifunctional ligand with a radical-sensitive acryl moiety and an amino-reactive SVA moiety which has been already used for the micropatterning of hydrogels[20]. We attach the linker onto the hydrogel in a spatially controlled manner by the conjugated action of the acryl moiety and a photo-initiator. The photo-grafted SVA functions will subsequently react with the many amino-groups of the PLL. This locally reverses the PEG antifouling properties and permits protein adsorption and allows for cell adhesion [21].

The PLL incubation step might seem facultative as the SVA function would likely react with proteins directly. In our hands however it enhances cell adhesion. It also allows for long incubation steps, required for low concentration protein solution, and which exceed by far the half life of the SVA function in water. It is also worth noting that fluorescently tagged proteins have *de facto* fewer available amine groups.

Thanks to this digital maskless and contactless method, chemical patterns can be aligned onto soft prefabricated micro structured hydrogels with ease **(Fig.2B)**. The reaction is also dose-dependent allowing the generation of gradients of cell adhesion molecules **(Fig.2C)**. Seeded cells stay on the pattern for at least two weeks. Nonetheless, due to the use of a photo-initiator which can diffuse into the gel, the patterned gel will be crosslinked beneath the insolated area resulting in a change in opacity and likely rheology of the mesh. Various adhesion proteins can be adsorbed to the PLL using this technique. For example, we used fibronectin and laminin to hierarchically grow COS-7 cells and primary rat cortical neurons respectively onto 4 different micro-structures of 4-Arm-PEG-Acrylate hydrogels. COS-7 cells grew mainly according to the fibronectin micropatterns **(Fig.3A)**. Neurons on the other hand were influenced both by the 3D structure and the biomolecules. Indeed, while their cell bodies were mainly anchored to the laminin micropatterns growing neurites seemed to be influenced by the topography **(Fig.3B). (Supp. video 3)**.

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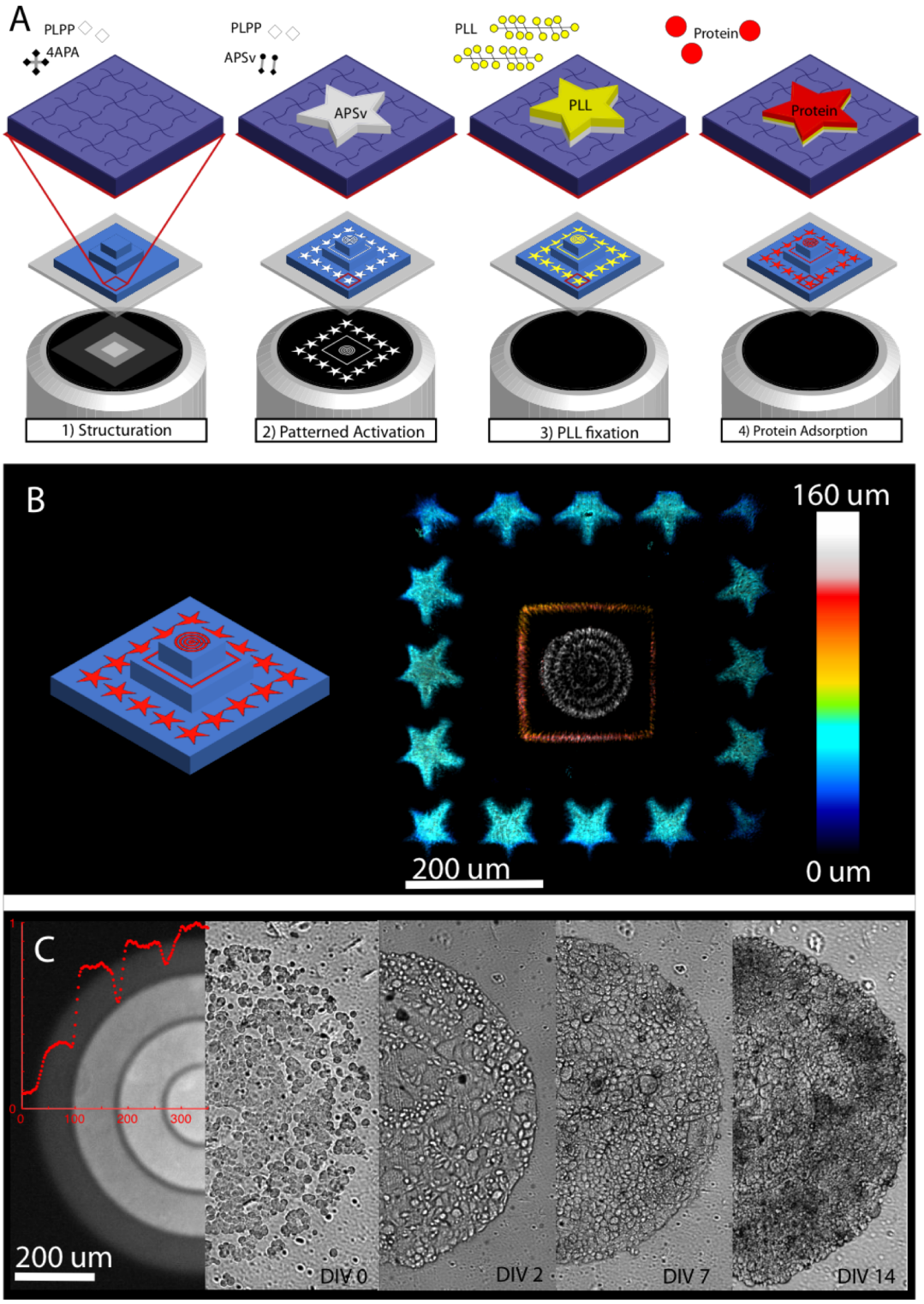

Figure 2 | Chemical photo-patterning, application to micro-structured gel decoration and grayscale patterning. A, schematics of the micro-structuration and decoration of an "Aztec temple" hydrogel. First, a gel is formed (dark blue) via height-controlled polymerization of 4-Arm-PEG-Acrylate (4APA) with PLPP as a photo-initiator. Then Acryl-PEG-SVA (APSv, white) is photo-grafted in the presence of PLPP according to the projected UV pattern. This allows for covalent ester-binding of polylysin (PLL, yellow) and the subsequent adsorption of biomolecules (red). **B,** Left panel: Schematic of the final topographically and chemically photopatterned "Aztec temple" hydrogel structure. Right panel: Z-color-coded confocal fluorescence microscopy image of the corresponding hydrogel structure. **c,** Left panel: epi-fluorescence microscopy image of the grayscale pattern of FITC-labelled PLL on a flat 4APA hydrogel. In overlay: radial fluorescence intensity profile (red) of the FITC signal. Right panels: bright field images of cell adhering on the corresponding pattern at day 0-2-7-14 respectively.

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This hints that competing effects of topographical and chemical cues governing 3D cell organization can be studied using this platform.

Outlook

By exploiting the unique chemistry of oxygen inside gas-permeable reactors combined with patterned UV illumination, we ended up with a very effective hydrogel design solution:

It generates topographically complex structures in one step. It gives new structuration means for common and staple hydrogels. It allows for decorating gels that are otherwise bio-inert. It is a time-efficient micro-fluidic system prototyping tool. It allows for cell culture in many different configurations due to fast trial and error procedures. This tool is fast, flexible and uses easily available materials.

This multi-purpose, multi-material solution allows to create complex and/or dynamic micro-environments for the cells to grow in. From the fabrication of fluidic devices, through the decoration of micro-structured PEG gels up to the photo-scission of Matrigel, this report shows how the control of oxygen's unique chemistry has yet to show the extent of its applications.

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Competing financial interests

A.P. and P-O.S. are employed by and V.S. is co-founder and shareholder of Alvéole (France).

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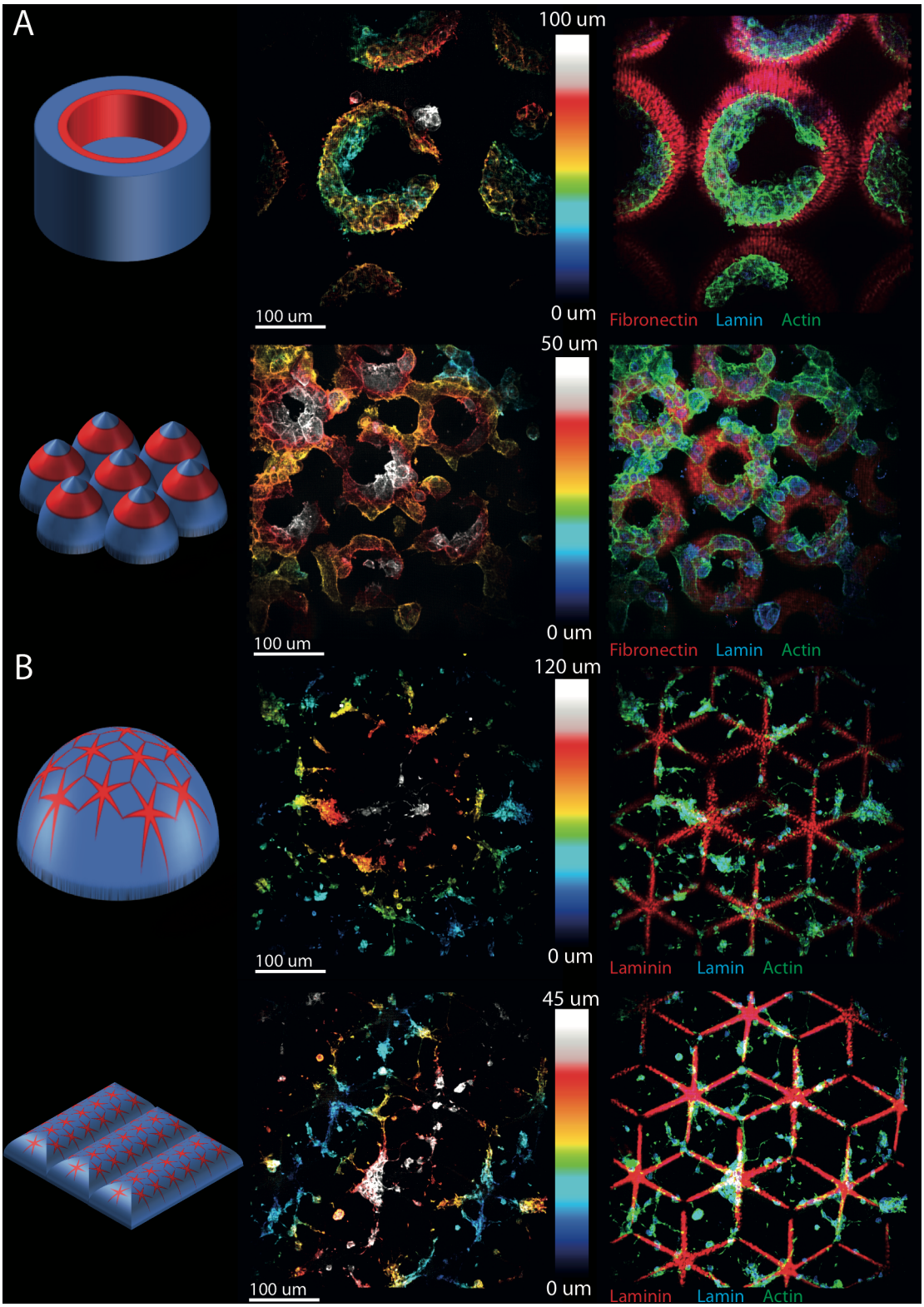

Figure 3: combining topography and decoration for cell culture. Left panel: schematic representation of topographically (blue) and chemically (red) photopatterned hydrogels. **Middle panel:** Z-color-coded confocal fluorescence microscopy images of COS-7 cells (A) and rat primary cortical neurons (B) seeded on the gels and stained for actin. **Right panel:** 3-color max-projection of confocal microscopy z-stack showing the patterned molecules (red), the actin cytoskeleton (green) and the nuclear envelope (blue).

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Material:

Experimental Setup: For all the experiments, we used a Nikon TI-E inverted microscope (Nikon Instruments) equipped with a DMD-based UV patterned illumination device (PRIMO, Alveole, France) together with its dedicated software (LEONARDO V3.3, Alveole). The wavelength of the illumination laser is 375 nm. 4X S-Fluor and 10X Plan Fluor (Nikon) were used to ensure efficient UV transmission. A homemade version of this illumination device was previously described in [18]. The patterns projected with PRIMO for all the experiments can be found in **Supp. 3**.

Modular Microreactors: We used custom PDMS chips made of two superposed PDMS thin films with holes fabricated by xurography (PDMS Stencils, Alveole, France, previously described in [18]). These stencils were stacked onto a 22x22 mm 170 um thick glass coverslip (Schott Nexterion, Schott Jena, Germany) forming PDMS microchambers of various geometries (**Fig 1 and Supp. 1**). Depending on the application, the upper PDMS film was removed after the building steps to ease the seeding of cells or beads. In gas controlled structuration experiments, the device is placed in a custom enclosure allowing argon or oxygen perfusion during the insolation steps.

Reagents were purchased as follow: 4-Arm-PEG-Acrylate MW 10K, Acryl-PEG-SVA MW 2K, 4-Arm-PEG-Amine MW 10K, 4-Arm-PEG-SAS MW 10K. Laysan Bio (Arab, USA). Matrigel Corning (USA). PLPP 1X (14.6 mg/mL) (Alvéole, France). Human Fibronectin (Roche). Laminin from mouse EHS, PolyLysine, PolyLysine FITC conjugate (P3543), NH4Cl, Triton X100, Paraformaldehyde, Bovine Serum Albumin (BSA), Phosphate Buffered Saline (PBS), N,N-DimethylFormamide (DMF), Dulbecco Modified Eagle Medium, NeuroBasal (Sigma Aldrich). Phalloidin-Alexa647 conjugate (Invitrogen), anti-Lamin (Abcam, ab16048) and Goat Anti-rabbit antibody Alexa 568 conjugate (Abcam, ab175471). 0.3 um diameter carboxy-fluorescent latex beads (Invitrogen).

Methods

Gas controlled/Total polymerization: to create the pillar shown in **Fig1** a solution consisting of 5% 4-Arm-PEG-Acrylate diluted in PLPP 1X was injected inside each well of a 9 wells microreactor. All the UV illumination steps were performed using a 4X objective. During argon perfusion, a square **(pattern 1 Supp. 3)** is insolated during 36 sec at max power: 128 mW/cm². To generate the histogram shown in **Fig.1D**, a solution consisting of 5% 4-Arm-PEG-Acrylate diluted in PLPP 1X was injected inside each well of a 9 wells microreactor. In each row of wells a pattern consisting of 32 wide squares **(pattern 2 Supp. 3)** was projected during argon perfusion, in air, and during oxygen perfusion at 128 mw/cm² for 20, 30 and 100 sec. respectively. The resulting gels were rinsed with PBS, incubated with 0.3 um carboxy-fluorescent beads and rinsed again with PBS. Their height was measured by focusing beads at the top and then at the bottom of the gels and deduced from the z stage displacement of the microscope.

Power controlled polymerization: to fabricate multistage structures as shown in **Fig.1**, a second stage **(pattern 3 Supp.3)** is insolated under air at 128 mw/cm² during 36 sec. around the pre-existing fully polymerized pillar described above. A third stage **(pattern 4 Supp.3)** is then photo-polymerized under air around the two first ones during 70 sec at 26,5 mw/cm². To generate the plot displayed in **Fig.1E**. a solution consisting of 5% 4-Arm-PEG-Acrylate diluted in PLPP 1X was injected inside each well of a 9 wells microreactor. In each well a pattern consisting of 32 wide squares **(pattern 2 Supp.3)** was projected under air at different powers and times: 128 mw/cm² 30 sec; 96 mw/cm² 36 sec; 78 mw/cm² 45 sec; 58 mw/cm² 60 sec; 38 mw/cm² 90 sec; 26.5 mw/cm² 135 sec. The resulting gels were rinsed with PBS, incubated with 0.3um carboxy-fluorescent beads and rinsed again with PBS. Their height was measured by focusing beads at the top and then at the bottom of the gels and deduced from the z stage displacement of the microscope.

Photo-scission and 3D imaging: to photocleave a hydrogel as shown in **Fig.1C** the gel is rinsed with PBS and incubated in PLPP1X. Photo-scission is achieved by insolating stripes **(pattern 5 Supp.3)** on the structure at 23 mw/cm² during 45 minutes. The resulting microstructured gel is rinsed with PBS, incubated with 0.3um carboxy-fluorescent beads and rinsed again with PBS. The 3D structure was imaged using a home made DMD based confocal microscope[22]

Chemical photopatterning: all structuration and decoration steps were performed with a 4X magnification objective.

Micro-structured gel decoration: to fabricate the decorated "Aztec temple" hydrogel shown in **Fig.2B**, a prepolymer solution of 5% 4-Arm-PEG-Acrylate diluted in PLPP 1X was polymerized and rinsed in PBS according to the first two steps of the previously mentioned protocol. PLPP 1X was then incubated inside the wells and a solution of 500 mg/mL Acryl-PEG-SVA MW 2K in DMF was added to reach a 10% vol/vol final concentration. Immediately after, a pattern consisting of stars, square and spirals **(pattern 6 Supp. 3)** was aligned and insolated onto the preexisting structure for 120 sec at 128 mw/cm² using the registration features of the Leonardo software. Immediately after, the gel was rinsed with PBS and a 1 mg/mL solution of PolyLysine FITC conjugate was incubated for 1 hour. The gels was rinsed with PBS and imaged using a DMD based confocal microscope[22]

Gray scale chemical patterning: a prepolymer solution of 5% 4-Arm-PEG-Acrylate diluted in PLPP 1X was injected inside one well of a 9 wells microreactors. A pattern consisting of a full white image **(pattern 7 Supp.3)** was insolated at 26,5 mw/cm² for 2 minutes. The gels were rinsed with PBS then incubated with PLPP 1X. A solution of 500 mg/mL Acryl-PEG-SVA MW 2K in DMF was added to reach a 10% vol/vol final concentration and a grayscale pattern **(pattern 8 Supp.3)** was insolated for 40 sec at 128 mw/cm². Immediately after the gel was rinsed with PBS and a 1 mg/mL solution of PolyLysine FITC conjugate was incubated for 1 hour. The gel was then rinsed with PBS and incubated with a 100 ug/mL solution of fibronectin for 10 minutes at 37C° prior cells seeding. The cell culture was then carried over for two weeks.

Topographically and chemically photopatterned hydrogels for cell culture:

All structuration and decoration steps were performed with a 4X magnification objective. The polymerization steps were done as follow for each specific structure:

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To generate the microstructures shown in **Fig.3**, a prepolymer solution of 5% 4-Arm-PEG-Acrylate diluted in PLPP 1X was injected inside one well of a 9 wells microreactors.

An all-white pattern **(pattern 7 Supp.3)** was insolated at 26.5 mw/cm² during 90 seconds. Then a second pattern consisting of wells **(pattern 9 Supp. 3)** was projected at 128 mW/cm² during 30 sec **(Fig. 3A top panel)**. Alternatively, a second grayscale pattern consisting of big domes **(pattern 10 Supp.3)** was projected at 128 mw/cm² for 3 minutes. **(Fig. 3B top panel).**

To generate Small domes **(Fig. 3A bottom panel)** and Waves **(Fig. 3B bottom panel)** grayscale patterns of domes wells **(pattern 11 Supp.3)** and waves wells **(pattern 12 Supp.3)** were insolated at 128 mW/cm² during 3 minutes. Afterwards a support pattern around the structure **(pattern 13 Supp.3)** was polymerized at 128 mW/cm² during 40 sec. in order to facilitate further neurons seeding. The gel was rinsed with PBS and incubated with PLPP 1X prior to decoration. A solution of 500 mg/mL Acryl-PEG-SVA MW 2K in DMF was added to reach a 10% vol/vol final concentration and a doughnut pattern for the wells **(pattern 14 Supp.3)** and small domes **(pattern 15 Supp.3),** an array of 6-pointed stars for the big domes and waves **(pattern 16 Supp.3)**, was insolated for 120 sec at 128 mW/cm².

Immediately after the gel was rinsed with PBS and a 1 mg/mL solution of PolyLysine FITC conjugate was incubated for 1 hour. The gel was then rinsed with PBS and incubated with a 100 ug/mL solution of fibronectin (Wells, small domes) or laminin (Big domes, waves) for 10 minutes at 37C° prior cells seeding. In wells and on small domes COS-7 cells were seeded in complete cell culture medium (DMEM, FBS). On big domes and waves E18 rat primary cortical neurons were seeded in complemented neurobasal. The cell culture was then carried for one day to allow cells to spread before fixing and imaging.

Cell staining and imaging : Cells were fixed with PFA 4% for 15 min and permeabilized by a 1% solution of Triton-X100 in PBS for 15 minutes before saturation with a 2% BSA solution for one hour. Finally, a solution of phalloidin-A647 (1/200), rabbit anti-lamin antibodies (1/500) in PBS 1% BSA was incubated for 2 hours. Finally, anti-rabbit-A568 secondary antibody was incubated (1/200) in PBS 1% BSA for 2 hours. The final structure and cellular organization was imaged in 3D with a home-made DMD based confocal microscope.[22]

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