

Supplementary Information

Arrays of Micro-cages by Two-photon Lithography for Spatially Organized Three-dimensional Neural Networks

2.2 3D microstructure

We used the direct laser writing (DLW) technology. This technology is based on a two-photon polymerization (2PP) reaction initiated in a photosensitive material in the focal spot of a femtosecond laser beam at a wavelength of 780 nm. Figure S1 illustrate the principle, as realized in the *Photonic Professional* system by *Nanoscribe GmbH*, Germany. The polymeric structure is directly written by moving the focal spot across the photoresist. An organic developer washes unexposed material away. Thereby 3D scaffolds can be fabricated on a glass substrate.

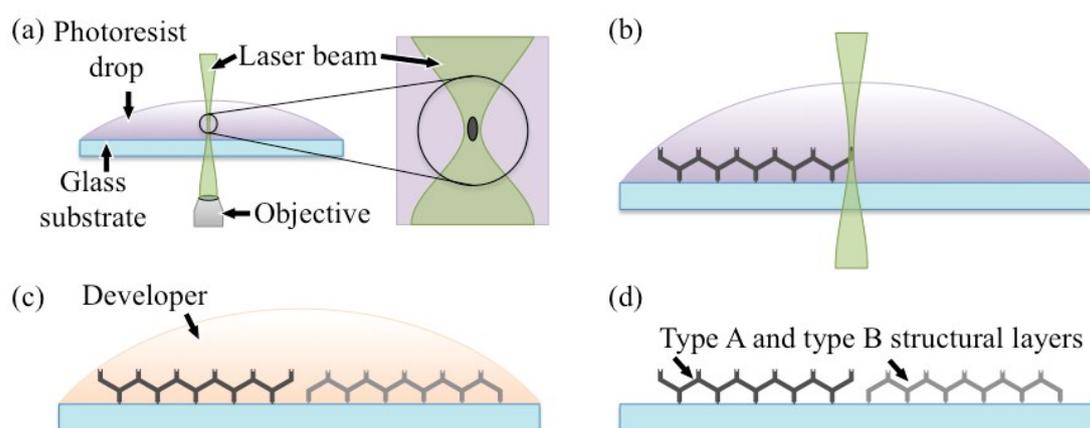


Figure S1: (a) Illustration of the two-photon polymerization concept with the laser-beam focus in a specific point (b) which is written in a drop of photoresist through a glass substrate. (c) Once the writing has been completed, unexposed photoresist is removed by a developer (d) in order to reveal the structures.

3.1. Structure optimization and stacking

Figure S2 illustrates the different generations of the scaffold structures. The first prototype (Figure S2a) was slightly too large and not robust enough. The second-generation prototype (Figure S2b) solved the stability problem by thicker edges. However, in this case, cells were able to pass through the relatively large hexagonal openings. To prevent this from happening, cell barriers were added to all hexagonal openings (Figure S2c). We also added alignment blocks in order to more comfortably manipulate the structure during stack assembly.

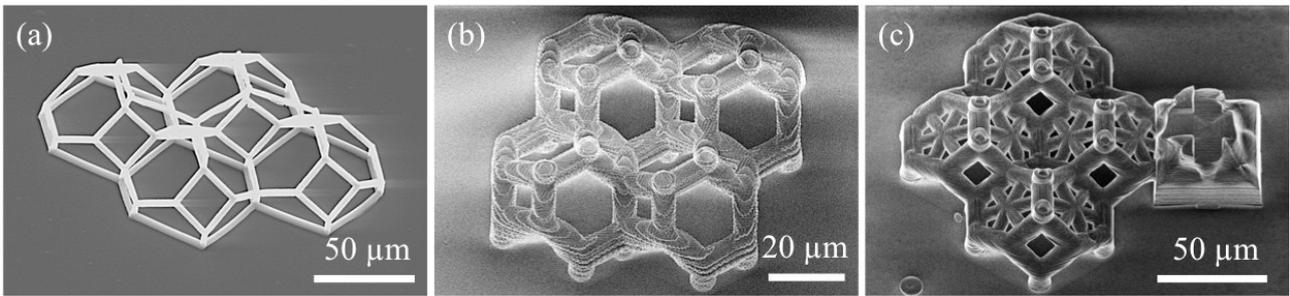


Fig. S2: Scanning electron micrographs of (a) the first structure realized for this study; (b) the second-generation structure with thicker edges; (c) third-generation structure with alignment block and cell barriers.

Structures made of 16 and 32 containers are realized from the basic unit structure with 4 containers, which is reproduced periodically in 2D with a writing overlap. As a consequence, some parts of the structures happened to be written two or three times. Misalignments during the rewriting process were determined to be small ($<1 \mu\text{m}$), as illustrated by **Figure S3** with connection holes, the most critical components of the structures.

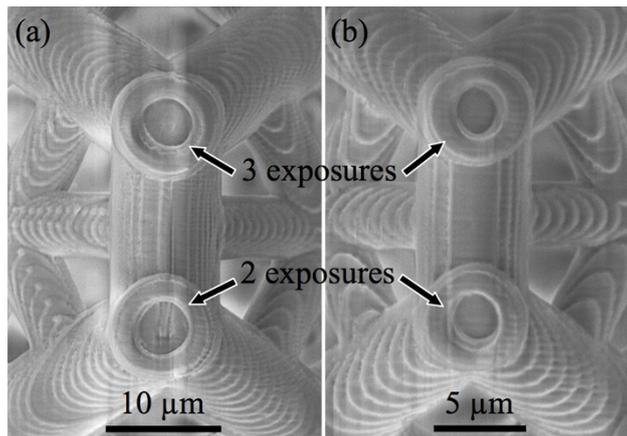


Figure S3. Scanning electron micrographs of connection holes of samples with (a) 50- μm -diameter and (b) 30- μm -diameter containers. Pictures show connection holes that were exposed twice and three times. The multiple exposures do not cause any obvious degradation in resolution.

The final 16-container structures are illustrated by **Figure S4** with (a,b) a Type B structure with its alignment blocks, connection holes and cell barriers; and (c,d) a flipped-over Type A structure showing its connection pins instead of holes.

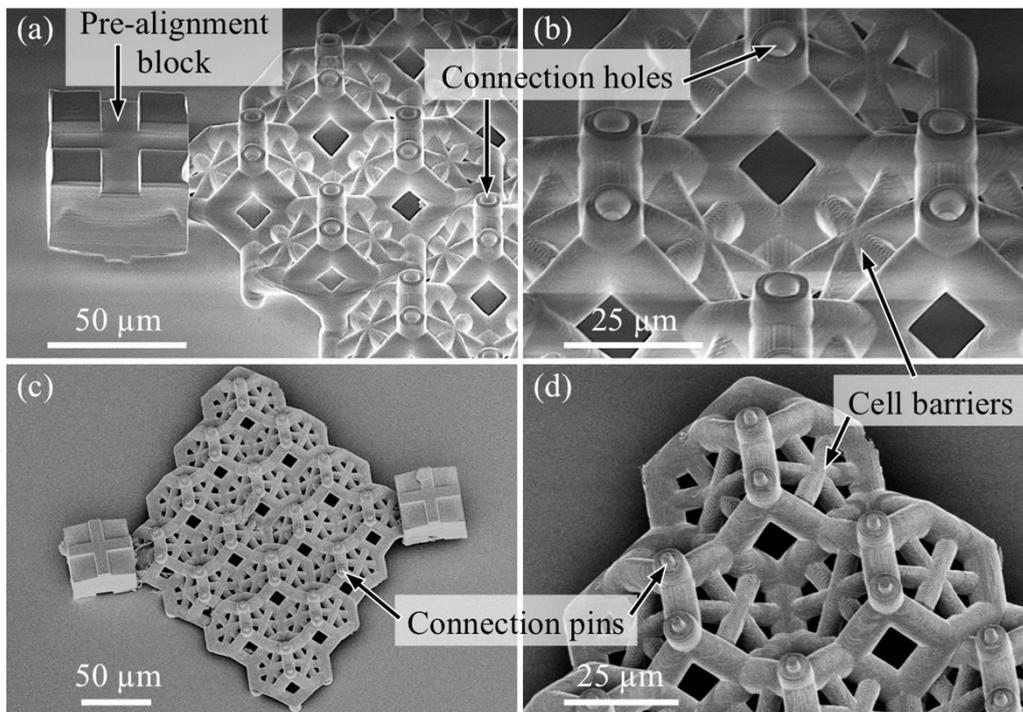


Figure S4. Scanning electron micrographs of (a) a close-up view of a Type B structure with 16 containers and alignment block and (b) cell barriers and connection holes. (c) Flipped-over Type A structure with 16 containers. (d) A close-up view of connection pins and cell barriers.

Figure S5 shows the storyboard for stacking two structures. In this illustration, a Type A structure is stacked with the micromanipulator onto a Type B structure.

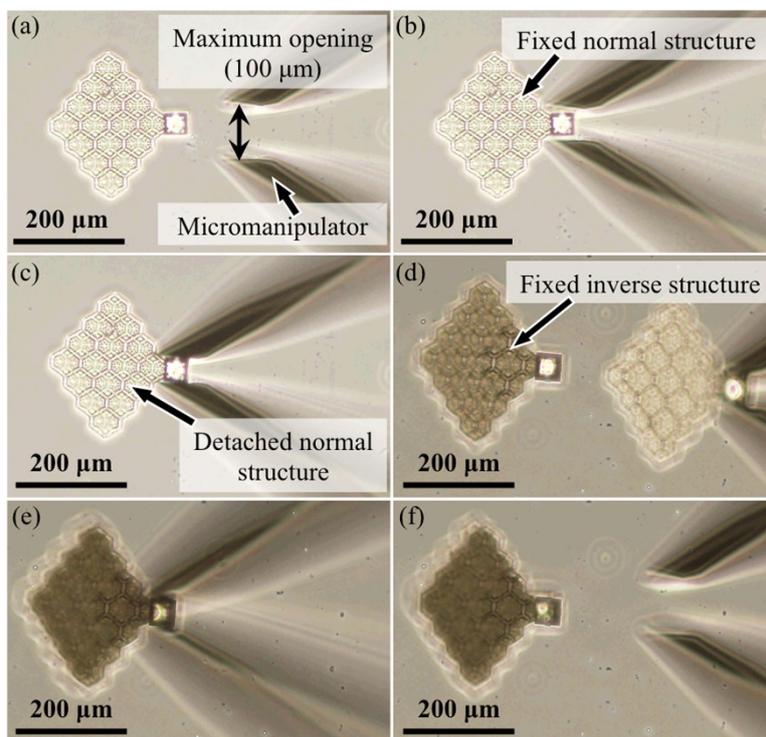


Figure S5. Storyboard of the structure stacking method. (a) First, the micromanipulator is moved close to the alignment block. The microtweezers are then opened to their maximum separation distance of $100\ \mu\text{m}$. (b) Next, they are moved forward to enclose the alignment block from two sides. (c) They are slowly closed until they grab the structure, detaching it from the glass substrate. (d) The grabbed structure is transferred and positioned over another structure. (e) It is then lowered until it is in contact with the structure below. (f) Finally, the micromanipulator is opened, releasing the structure, and is retracted.

3.2. Culture in 2.5D

To validate the concept of cell barriers, structures without (**Figure S6a**) and with (**Figure S6b**) cell barriers were realized. In the case of the structure without cell barriers, we observed that cells deposited from the suspension fall through the containers. Even if cells adhered to the scaffold structures and evolved within them, they tended to escape from them after stacking. In the case of structures with cell barriers, we observed that cells cannot escape from the container. They move over the cell barriers like over a substrate.

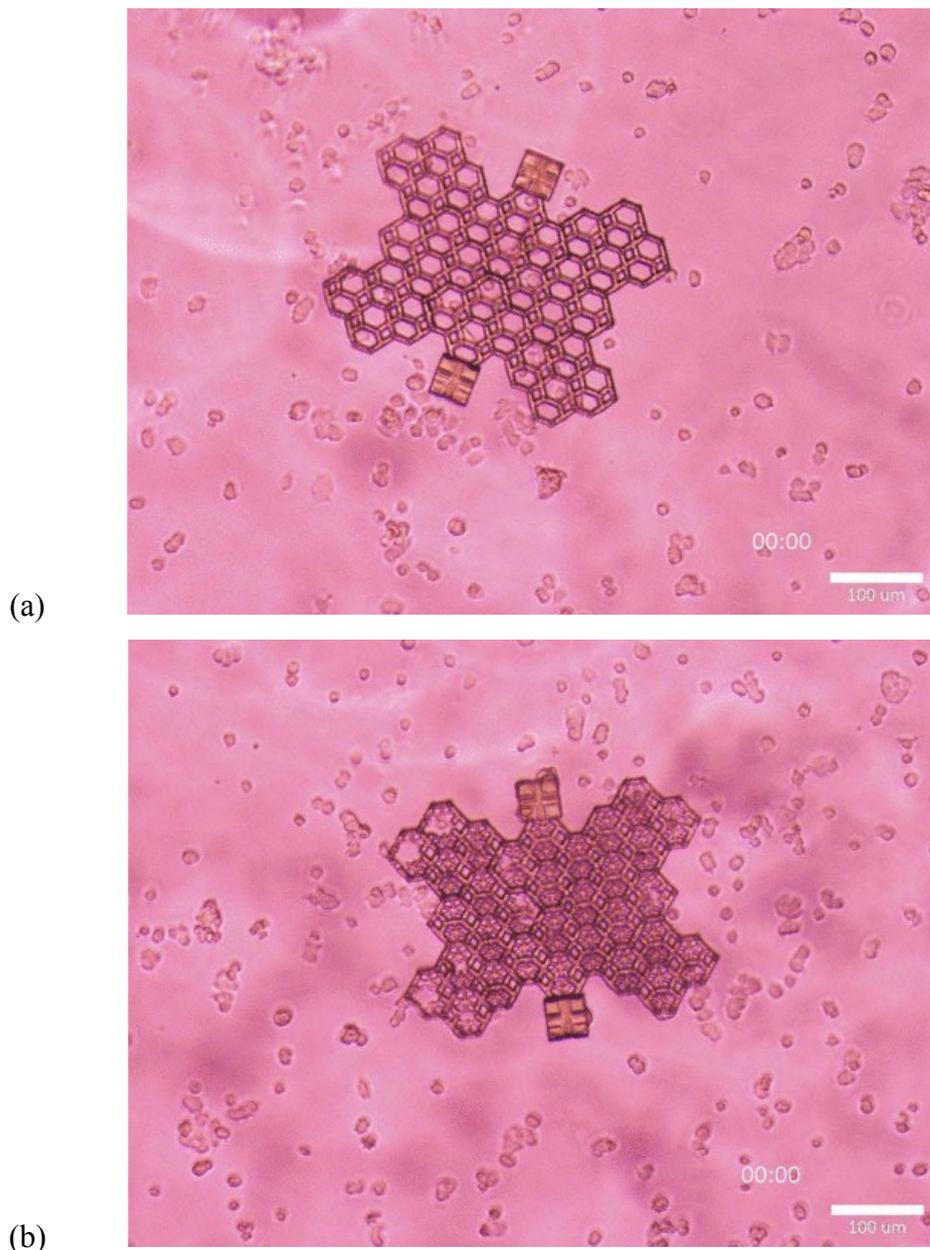


Figure S6. Time-lapse movies (double click on the gif files to watch the movie) of the cell development and migration on (a) scaffold structure without cell barrier and (b) structures with cell barriers during 3 hours.

3.3. Structure stacking for 3D culture

The experimental equipment used to stack structures with cells on top of each other is shown in **Figure S7**. It is composed of a microscope with motion control, controlled CO₂ concentration and thermal chamber, and a micromanipulator with its own motion control. The cell culture is performed directly on the glass substrate, with the silicone rubber frame defining the culture chamber walls.

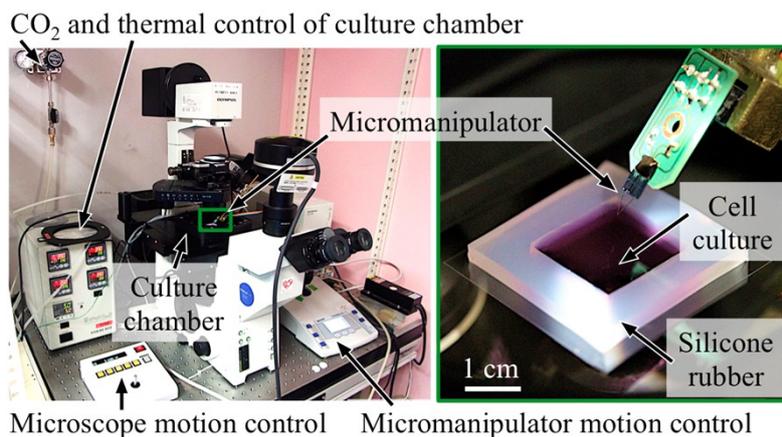


Figure S7. Photographs of the equipment used to stack structures with cells. (left) Culture chamber controlled with CO₂ and thermal control and a motion control for (right) the micromanipulator serving to stack structures.

Cell adhesion to microstructures is an important aspect of this study. It allows to keep structures stacked on each other. Proving the strong adhesion of cells, some structures were observed on the sidewall of the silicone rubber of the culture chamber. Due to insufficiently cautious medium addition, some structures were swept onto sidewalls of the silicone rubber frames, where they adhered. **Figure S8a** shows two such structures stuck on a silicone rubber wall. This adhesion is mediated by neurites and cell contacts, as illustrated by **Figure S8b**.

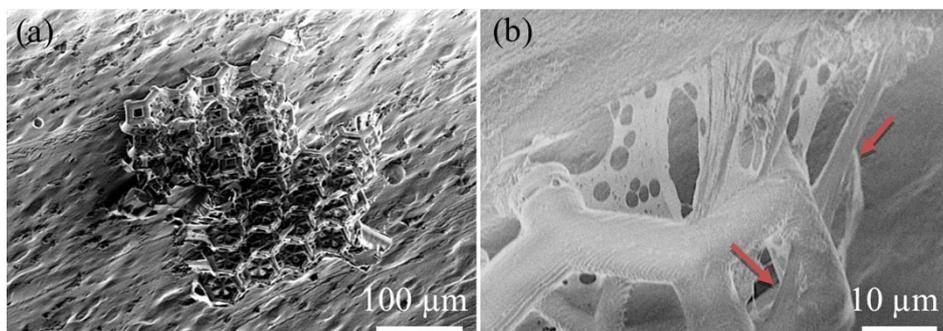


Figure S8. Scanning electron micrographs of (a) two structures adhering to the sidewall of the silicone rubber frame and (b) a close-up view of neurites (denoted by red arrows) mediating the adhesion between the structure and the silicone rubber surface.