3D Synthetic Microstructures Fabricated by Two-Photon Polymerization Promote Homogeneous Expression of NANOG and ESRRB in Mouse Embryonic Stem Cells

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1. Introduction

In vivo, cells are arranged in a 3D multifunctional environment. The chemical and mechanical properties of this specific entourage influence their intracellular functioning. However, most of the currently employed approaches in cell- and tissue-based engineering studies still involve 2D surfaces, or monolayer cell cultures, that offer unnatural growth kinetics and cell attachments.[1,2] On 2D cultures, cells adhere to a plate, which restricts them to a flat shape. Moreover, these cultures lack a complex tissue-specific architecture leading to dramatic variation in the diffusion of chemical cues and cell-to-cell/cell-to-matrix interactions.[3] As a result, the molecular pathways that regulate cell behaviors are also altered, leading to distinct cellular phenotypes. Recent advances in cell biology, microfabrication techniques, and tissue engineering have enabled the development of a wide range of 3D cell cultures, including multicellular spheroids, organoids, scaffolds, hydrogels, and 3D bioprinting.[3] Nowadays, they have become a promising alternative for bridging the gap between in vitro cultures and living tissues as they exhibit protein expression patterns and intracellular junctions that are more similar to in vivo states.[4]

Currently, hydrogels are the most widely used system for 3D cell cultures to mimic extracellular matrix in vitro. For example, Matrigel, which is reconstituted from the mouse sarcoma and composed of laminin, entactin, collagens, and heparin sulfate proteoglycan plus an unknown mixture of growth factors and enzymes, is commonly used in biology.[5,6] It is important to understand that some properties of the hydrogels have major drawbacks, such as very low rigidity, which does not mimic the naturally stiff environment of some tissues.[7] To ensure optimal performance, swelling of these 3D biomaterials, their permeability to different molecules, interaction with media, and the immobilization of biomolecules have to be optimized.[8] Since culture composition is not clearly defined, these matrices lead to relatively poor reproducibility and lack of consistency between batches. Finally, the bigger or more complex the 3D volume is, the more difficult the extraction of cells for further experimentations.
becomes. All these limitations make hydrogels unsuitable for tissue modeling in vitro.[9] Consequently, chemically defined and reproducible culture systems are required to enable development of scalable and cost-effective 3D culture system.

3D synthetic microstructures, fabricated by two-photon polymerization (2-PP) photolithography, offer favorable cell responses due to tunable chemical, physical, and mechanical properties (reviewed by Hippler et al.).[8] 2-PP technology allows fabrication of volumetric structures of arbitrary shape by directly writing the intended geometry within a photosensitive material. Due to the unprecedented flexibility of 2-PP, matrix architecture and pore size can be controlled with a resolution down to 100 nanometers.[9] Indeed, structures with well-engineered nanotopographies have features on similar length scales to cellular components and have proven to be useful tools for recapitulating the cellular environment.[10] Additionally, artificial surface nanotopography helps maintain long-term human ESC pluripotency by inhibiting cell spreading, which makes the cells less flat, thus increasing the clone integrity.[11] 2-PP-fabricated structures by providing spatially defined adhesion sites allow communication between different cell types regarding proliferation and migration (e.g., by biological functionalization with several proteins).[12] Chemically defined media combined with the 3D architectures, which more accurately resemble the extracellular environment, offer a powerful tool to mimic specific tissues in vitro. The employment of necessary mechanical cues would have the important advantage of mimizing the use of biochemical molecules, which are otherwise necessary to regulate cellular phenotype.[13,14]

Mouse embryonic stem cells (mESCs) are a broadly used experimental model system to understand early mammalian development.[15,16] They are derived from the inner cell mass (ICM) of pre-implantation embryos and can be maintained in a pluripotent state in vitro using specific cues.[17] Two main pluripotent states have been described in vitro: the “naïve” state, which corresponds to blastocyst at days 3.5 (E3.5)–4.5 (E4.5) in vivo—and the “primed” state, which occur at a later developmental stage E5.5–6.5.[18,19] These two states are maintained in two different culture media in vitro: naïve in “2i” medium (for two small molecule inhibitors PD0325901 (PD) and Chir99021 (CH)) supplemented with leukemia inhibitory factor (LIF) and primed in serum supplemented with LIF (see details in the Experimental Section).[18] Although both naïve and primed mESCs have the potential to form all somatic cell lineages as well as germ cells, they are distinct in their morphological, epigenetic, and transcriptional characteristics. For example, naïve mESCs grown in 2i form compact dome-shaped colonies, whereas primed mESC colonies grown in serum + LIF are larger and flatter in shape.[20] The gene expression program of stem cells is maintained through the action of three key pluripotency transcription factors: octamer-binding transcription factor 4 (OCT4), SRY (sex determining region Y)-box 2 (SOX2), and homeobox protein NANOG.[19] They are required to establish the ICM identity. These key pluripotency regulators are fundamental for controlling the dual abilities of mESCs to self-renew and to differentiate in vivo and in vitro. In serum + LIF, mESCs express the NANOG protein in a heterogeneous manner, which is a manifestation of their dual potential for self-renewal and differentiation.[21,22] Its expression changes dynamically between two states: high NANOG, which possess high self-renewal efficiency, and low NANOG, which corresponds to increased differentiation capability. In 2i medium, mESCs display stronger self-renewal capabilities compared to mESCs cultured in serum + LIF. This is highlighted by NANOG homogenous expression, which resembles the epiblast of pre-implantation embryos.[23] Therefore, fractionating mESC populations cultured in two different conditions—serum + LIF and 2i—on the basis of heterogeneous NANOG expression has the potential to distinguish the naïve and primed states.

In recent years, a number of experimental strategies and computational models have been applied to reveal the molecular mechanisms and interactions that orchestrate pluripotency (reviewed by Martello et al. and Young et al.).[19,24] These strategies and models are based mainly on 2D cell cultures, in which cellular phenotypes are regulated by selective suppression and/or activation of key signaling pathways using growth factors and small molecules. Defining an optimal 3D model that best mimics the specificity of the in vivo microenvironment is a crucial step toward generating data that accurately reflects what occurs in embryos.[25] Several reports suggest that the critical signals for pluripotency maintenance likely depend more on spatial conformation changes rather than on extrinsic growth factors.[14,26] For example, Nava et al. used highly resolved “niche” in order to compare the expression of pluripotency and differentiation markers induced in mESCs, thereby showing the potential of 3D cultures for reducing the use of biochemical molecules.[21] However, none of the previous studies were able to distinguish between different mESC populations—naïve and primed—based on their pluripotent potential.

In this paper, we presented several designs of tailored 3D microstructure arrays using 2-PP photolithography and evaluated their impact on mESC pluripotency. We demonstrated that these microstructures maintained mESCs in a pluripotent state. In comparison to a 2D solid film, mESCs in 3D microstructure arrays exhibited a stronger signal intensity of three pluripotency markers—NANOG, OCT4, and estrogen-related receptor beta (ESRRB). Tracking the heterogeneity of the NANOG and ESRRB pluripotency factors by fluorescence microscopy allowed us to demonstrate that 3D culture reinforces naïve pluripotency of mESCs.

2. Results and Discussion

2.1. 3D Synthetic Microstructure Array Designs

The key aspect in the design of 3D microstructure arrays was to mimic the complex multifunctional environment required for promoting mESC adhesion and proliferation. Therefore, the design of the microstructures was motivated by the following reasons. It should: 1) be of highly precise micrometer shape (i.e., scale comparable to the cell size) with a roughness on the nanometer level (down to 100 nm) to allow for highly repetitive motifs for cells; 2) have a structural rigidity (as mechanical support); 3) be able to physically contain mESCs within their 3D microarchitecture, like individual niches (since mESCs have a round shape, microstructures with round/square form were prefered over rectangular/triangular microstructures); 4) have an open access geometry of 3D microstructures, which would allow the optical access; 5) allow for spatial cell distribution by gravity driven sedimentation into individual microstructures;
6) guarantee cell-to-matrix interaction in a 3D environment; 7) provide homogeneous dispersion of nutrients and chemical cues as well as gas exchange.

In order to evaluate the impact of microstructure architectures on mESC culture, we designed several concepts, including ring-like microwell (15 µm × 15 µm × 10 µm in terms of width × length × height), basket-like microwell (15 µm × 15 µm × 10 µm in terms of width × length × height), and micropillars (2 µm in diameter and 2 µm distance between pillars). Figure 1A schematically shows the three microstructure designs. Ring-like and basket-like microwell designs would allow for the allocation of each cell to an individual microwell. In micropillars, cells could hardly migrate due to a dimensional incompatibility enabling the planar cell distribution.

To recapitulate the cell-to-cell communication network, these structures were then assembled into tightly packed arrays. The number of microstructures in the array was motivated by a relatively large surface array (i.e., millimeter range) in order to perform cell population analysis; and should guarantee cell-to-cell interaction (i.e., cells in the neighboring structures). Therefore, individual structures have to be in direct contact. Besides the architectural aspects, the design of both the 3D microstructures and the array must also satisfy technological limitations of the 2-PP process. For example, high aspect ratio structures (1:10) should be avoided because microstructures might collapse after the development step. Large surface area elements (i.e., centimeter range) are also undesirable at the cost of a corresponding increase in overall fabrication time. The schematics and photographs of 3D microstructure arrays are depicted in Figure 1B,C, correspondingly.

2.2. Culture of mESCs in 3D Microstructure Arrays

To evaluate the biocompatibility of 3D microstructures (i.e., cell viability, adhesion to the 2PP-engineered microstructures and morphological compatibility), we first fabricated all three 3D microstructure arrays on a glass coverslip (see Experimental Section). After coating of 3D microstructure arrays with gelatin, mESCs were seeded and cultured under defined serum + LIF conditions for 5 days. The morphology of mESCs was evaluated daily by live microscopy. As an example, we show the mESCs grown in the ring-like microwell array in Figure 2. Rapidly growing colonies were observed from days 1 to 5 suggesting that the 3D microstructure arrays can maintain the growth of mESCs under serum + LIF condition.

We then assessed the stemness properties of the mESCs grown on the 3D microstructure arrays using alkaline phosphatase (AP) staining. AP is a unique and unambiguous pluripotent marker for all types of pluripotent stem cells, including ESCs, embryonic germ cells, and induced pluripotent stem cells (iPSCs), which allows for early identification of ESCs. Cell populations were maintained in all three 3D microstructure arrays (ring-like microwells, basket-like microwells, and micropillars) under serum + LIF conditions for 3 days. They formed distinct AP-positive colonies. The integrated...
intensities were similar to those colonies grown on 2D surface (i.e., glass coverslips) in serum + LIF. The quantification of integrated AP intensity at the single colony level are shown in Figure 3. To initiate differentiation on 2D surface (used as a control), mESCs were cultured on glass coverslips in LIF-free media (see Experimental Section). In this differentiation media, cells did not form colonies resembling ESCs and had less AP intensity (highlighted by light magenta color), indicating that these cells committed to differentiation. The quantification of single colonies revealed higher AP intensity for all three 3D microstructure arrays compared to mESCs on 2D surface. These initial experiments convincingly demonstrated that mESCs retained their self-renewal potential and remained undifferentiated in the 3D microstructure array under serum + LIF conditions.

2.3. 3D Synthetic Microstructures Promote NANOG Homogenous Expression

In order to assess the pluripotent state of the mESCs grown in 3D microstructures at the single cell level by high-resolution immunofluorescence imaging, we integrated 3D microstructures on a diagnostic microscopy slide (Figure 4A). Quantitative immunofluorescence analysis provides information, which includes location and distribution of protein levels in single cells across populations or relative amounts of two or more proteins within a single cell.\[^{22,28,29}\] The use of the slide significantly simplified the immunofluorescence imaging, since it was supplied with a thin pre-patterned hydrophobic epoxy resin mask incorporating ten individual wells. First, the mask enabled handling of several populations in parallel and subject each population to an experimental protocol in a systematic manner while decreasing reagent consumption (see Experimental Section). Second, it protected the microstructure arrays from damage during preparation of the slide for immunofluorescent imaging by eliminating the direct contact of the coverslip and microstructures. And finally, it enabled high-resolution imaging by providing the short working distance from the microscope objective to 3D microstructures.

To investigate the effects of 3D microstructures on the shape of stem cell colonies, mESC populations were maintained in all three 3D microstructure arrays (ring-like microwells, basket-like microwells, and micropillars) under serum + LIF conditions for 48 h. In the basket-like microwell array, cells dropped into individual microwells, settled down, adhered to the structure walls, and grew in height. In the ring-like microwell array, cells occupied not only the volume within the microstructures, but also the space in between (Figure S1A, Supporting Information 1). Cells adopted the form of the given internal volume of both the ring-like and basket-like microwell arrays and showed structural reorganization of subcellular microarchitecture. When cells were grown on the micropillar array and 2D layer (i.e., solid polymer film), they were flatter and did not reveal any defined axis formation or shape. Cells have also demonstrated a tendency to form diverse colonies within the 2PP-engineered microstructures. The Z-stack side-view projections of confocal images acquired on the microstructure arrays showed that cell colonies retained a spherical morphology typical for mESCs. A side-view projection of the ring-like microwell array is depicted in Figure S1B, Supporting Information 1 as an example. These results imply that the microstructures are able to guide the spatial organization of colony formation, thus limiting the need for selective cell seeding in stem cell culture systems.
We hypothesize that the ability of mESCs to grow inside the 2-PP-engineered microstructures better mimic the in vivo state, where cells are allowed to self-organize in a truly 3D multifunctional environment.

To probe the dynamics of the pluripotent states and examine whether the 3D architecture of microstructures rather than the material itself impacts expression levels of pluripotency factors, we compared mESC populations in all three microstructure arrays and on 2D solid polymer film (i.e., of the same material as the microstructures) under serum + LIF and 2i conditions. An optimized protocol for immunostaining is presented in Figure S2, Supporting Information. The expression of OCT4 and NANOG proteins was monitored at the single cell level. In agreement with previous studies, the distributions

Figure 3. A) Schematic view of the experimental design used for alkaline phosphatase (AP) staining. B) AP-stained mESC colonies cultured in three 3D microstructure arrays (ring-like microwells, basket-like microwells, and micropillars) and on the glass coverslips. Positive control: mESCs were maintained in serum + LIF medium. Negative control: mESCs were cultured in differentiation medium (see Experimental Section). Cell colonies were imaged 72 h after seeding. The scale bar is 50 µm. C) Quantification of AP intensity in mESCs at the single colony level. Quantification of AP-stained colonies from three independent experiments.
Figure 4. A) Schematic views of three 3D microstructure arrays (ring-like microwells, basket-like microwells, and micropillars), which were fabricated by two-photon polymerization (2-PP) photolithography in the wells of the diagnostic microscopy slide. The 2D solid polymer film was used as a control. B) Representative immunofluorescent images of NANOG (red) and OCT4 (magenta) expression in mESCs grown in 3D microstructure arrays and on 2D film in serum + LIF. As a control, cells were maintained on 2D film in 2i medium. DAPI was used as a nuclear counterstain. Scale bar is 10 µm. C) Quantification of immunofluorescent intensity for the NANOG (red) and OCT4 (magenta) at the single nuclear level in mESCs cultured in three 3D microstructure arrays (ring-like microwells, basket-like microwells, and micropillars) and on 2D solid polymeric film under serum + LIF and 2i conditions. Number of counted nuclei \( n = 100 \). Immunofluorescence staining was performed in triplicate. \( p < 0.0001 \) (Mann–Whitney test).
of expression values (black bars) of the two pluripotency markers—OCT4 and NANOG—on 2D solid film across the population were representative for each growth condition.\cite{18-22,36} Representative immunofluorescent images of individual cellular nuclei are shown in Figure 4B. In serum + LIF, NANOG protein had a high degree of heterogeneity, which is shown by a relatively broad distribution of expression values (black bars in Figure 4C, serum + LIF conditions). In contrast, OCT4 did not exhibit heterogeneity. As in pre-implantation embryos, OCT4 is present in all cells of the ICM until late blastocyst.\cite{31} NANOG heterogeneity among the cell populations was reduced on 3D microstructures, which is shown by a relatively narrow distribution of expression values (black bars in Figure 4C, serum + LIF conditions). Upon the transfer into 2i conditions, mESCs in 3D microstructure arrays and on the 2D solid film showed significantly higher mean expression levels of both pluripotency markers (Figure 4C). Moreover, the rather homogeneous expression levels of the NANOG transcription factor in 3D microstructures indicated that the cell populations possessed a stronger self-renewal ability (figure panel Figure 4B, 2i conditions). NANOG heterogeneity is an intrinsic state of cellular phenotypes, which shows a typical bimodal distribution both in vivo and in vitro. In vitro, \textasciitilde 80% cells show a relatively high level of NANOG, while the rest of 20% cells stay at low level of NANOG when cultured in serum + LIF conditions.\cite{32,33} Dual inhibition of the signaling glycosyn synthase kinase (GSK-3) and MEK signaling pathways in 2i medium converts mESCs into a state with more uniform and high NANOG expression.\cite{21,22} To normalize the intensity of immunofluorescence data, we used OCT4 marker. The quantified ratio between the integrated intensity of NANOG and OCT4 is presented in Figure S3, Supporting Information 3. As observed previously, mESCs in 3D microstructures have a more homogeneous ratio distribution, closer to distributions that were observed in 2i media. Interestingly, mESCs cultured in all 3D microstructure arrays in serum + LIF conditions expressed NANOG and OCT4 at levels comparable to those values in 2i medium (Figure S4, Supporting Information 4). These results indicated that mESCs show an enhanced responsiveness both to 3D microstructures and 2i medium conditions. It can be hypothesized that mESCs in 3D microstructure arrays might have a stronger self-renewal ability resembling more closely the ICM of pre-implantation embryos.\cite{23,34} Moreover, in 3D microstructure arrays there might exist a dynamic expression range, which is similar both for serum + LIF and 2i conditions. Therefore, the physical properties of 3D microstructures arise from their patterned structure, rather than an innate property of the material. Changing the constituent material or the surface chemistry may also impact the response (as is true for physical metamaterials), but the main effect is derived from the structure. Besides the 3D environment, the nanotopography of the 3D microstructure’s surface, as a result of the ellipsoidal two-photon absorption volume, may also amplify the expression levels of pluripotency markers.\cite{15,36} Our data demonstrated that in comparison to 2D surface, mESCs in 3D microstructure arrays exhibit a stronger immunostaining signal of two pluripotency markers—NANOG and OCT4—and show a more homogenous pluripotency state as highlighted by the expression of NANOG.

In order to reinforce our observations, we compared the expression of another key pluripotency factor—ESRRB—in two different culture media—serum + LIF and 2i—on 2D surface (i.e., solid film) and in 3D microstructures at the single cell level. ESRRB is involved in early development and reprogramming of mESCs from the primed to the naïve state. Interestingly, NANOG binds directly to ESRRB, enhancing binding of RNA polymerase II (a multiprotein complex that transcribes DNA into precursors of messenger RNA (mRNA)) and stimulating ESRRB transcription.\cite{37} Similar to NANOG, ESRRB is heterogeneously expressed in mESCs growing in serum + LIF conditions.\cite{18-40} Moreover, it co-fluctuates with NANOG protein levels: mESCs expressing high levels of NANOG also tend to show high levels of ESRRB and vice versa.\cite{40} Therefore, the heterogeneity of ESRRB in the stem cell population might be used to distinguish naïve and primed mESCs. Representative immunofluorescent images of individual cellular nuclei are shown in Figure 5A. Quantitative analysis confirmed that ESRRB is characterized by heterogeneous kinetics of gene expression (Figure 5B) similar to the levels of NANOG expression (Figure 4C). In comparison to the 2D solid film, mESCs in 3D microstructure arrays exhibit a stronger signal intensity of ESRRB and show a more homogenous expression of ESRRB (Figure 5A,B). This correlation between the cellular levels of ESRRB and NANOG suggests that high NANOG expression in mESCs may facilitate high ESRRB expression. However, it has a lower dynamic expression range when compared to NANOG. In this respect, heterogeneity in NANOG expression might be qualitatively distinct from heterogeneity in ESRRB. Similar correlations with embryonic expression programs have been reported in studies investigating the molecular changes accompanying heterogeneous expression of ESRRB and NANOG.\cite{37,38}

To conclude, all three designs of the microstructures, including ring-like microwells, basket-like microwells and micropillars, showed significantly higher mean expression levels of three pluripotency markers—NANOG, OCT4, and ESRRB—and more homogenous expression of two pluripotency markers—NANOG and ESRRB—than 2D solid film. However, mESCs in ring-like and basket-like microwells showed higher mean expression levels of three pluripotency markers, than micropillars. We assume that changes in morphology and consequential cytoskeletal rearrangements of mESCs, induced by the specific 3D geometry, might be responsible for promoting stemness maintenance.\cite{41,42} In micropillars, mESCs were not able to migrate to internal space of the pillars due to a dimensional incompatibility (pillars are 2 \( \mu \)m in diameter and the distance between pillars is 2 \( \mu \)m). This enabled the planar cell distribution similarly to 2D culture (Figure S1A, Supporting Information 1). Cells in 3D become less flat and developed matrix contacts in multiple planes inhibiting their spreading in 3D space.\cite{41} We also observed this tendency: mESCs adopted the form of the given internal volume (Figure S1A, Supporting Information 1). Thus, changes in cellular dimensionality and shape might be responsible for the enhanced expression of three pluripotency markers as components of the 3D microstructures interact with specific cell receptors, increase passive stretch of the cell membrane, and activate mechanosensitive pathways.\cite{42} Similar to our findings, other research groups
have found that 3D nichoid microstructures, fabricated by 2-PP photolithography, maintained the expression of the OCT4 pluripotency marker switched on in mESCs. In serum + LIF conditions, the OCT4 pluripotent marker was highly expressed in 3D-engineered nichoids and on 2D glass substrates throughout the 3 days culture period (78.80 ± 11.65% and 64.94 ± 22.24%, respectively). In the absence of LIF exogenous soluble factor, OCT4 expression was significantly greater in 3D nichoids compared to 2D glass substrate at day 7. The authors hypothesized that the pluripotency maintenance of cell populations may have arisen from the confinement effect due to the 3D architecture of the nichoid substrate, which induces the genetic reprogramming of cells by controlling their cytoskeletal tension.

We also think that nutrients and small molecular distribution within the microstructures, enabled by the 3D architecture, might enhance expression levels of NANOG, OCT4, and ESRRB while reducing NANOG and ESRRB heterogeneity among the cell populations. Round-shaped colonies are typical for mESCs. Cells located on the surface of a colony are highly exposed to the medium, whereas cells within the construct receive less oxygen and nutrients from the medium. Consequently, core and outer cells may experience different stages of differentiation, resulting in mixed populations. The 3D architecture of ring-like and basket-like microwells may serve for more thorough perfusion of the culture medium in the porous constructs and thus enhancing the delivery of the chemical inducers as well as oxygen transport. All these may lead to the favorable stemness maintenance in 3D microstructure arrays.

The exact molecular mechanism, which is responsible for stronger self-renewal ability of mESCs within 3D microstructures, is still unknown. Nowadays, several research groups fabricated stem cell niches (parallelepipeds with internal lattice-like structure) to show that mESCs, rat and human mesenchymal stromal cells maintained stemness potential more effectively than cells expanded on 2D glass substrates. Niche surfaces were also coated with hydrogels (hyaluronic acid–divinyl sulfone, glycidyl methacrylated–hyaluronic acid, and thiolated gelatin) to modify structure–mechanical properties: in all cases the 3D environment enhanced cell proliferation with respect to flat controls. In this paper, we demonstrated that ultraprecise 2-PP-engineered microstructures reinforce pluripotency maintenance of stem cells more closely to pre-implantation embryos. The physical containment due to the 3D architecture of the presented microstructures could explain this phenomenon, and thus could be a potent and sufficient cue for stemness maintenance of mESCs.

Additional studies are necessary to elucidate the mechanism(s) by which the physical constraints of 3D microstructures mediate the reprogramming effect, while potentially reducing the risks of exogenous conditioning for the industrial-scale expansion of stem cells.

3. Conclusions

We present a controllable engineered-niche system for studying the biophysical regulation of stem cell pluripotency. Revealing the molecular basis of mESC cellular heterogeneity in 3D culture conditions is not only important for understanding the flexible nature of the pluripotent state but might also serve as a model to understand heterogeneity in other systems (e.g., human ESCs and iPSCs). The design of tailored 3D microstructures and the array dimensions can be easily adapted to any cell type. Due to the unprecedented flexibility of the 3D patterning approach and superior advantages in terms of material characteristics, the entire manufacturing process can be performed in a time-efficient manner. Indeed, none of the currently available microfabrication approaches has been able to investigate cell
mechanical properties in 3D with the accuracy of 2-PP nanolithography. Thus, the technology is widely applicable to study other biological systems, for which 3D environment is of crucial importance for proper functioning.

4. Experimental Section

Design and Manufacturing of 3D Microstructure Arrays: A 3D computer-aided design program (Solidworks Corp., USA) was used for 3D model development of the microstructures. An original file (*.sldprt) of a 3D solid object was converted into surface tessellation language (STL) file for the “Photonic Professional GT” system (Nanoscribe GmbH, Germany). Two software packages, DeScribe 2.2.1 and NanoWrite 1.7.6, were used to control the system. To define the structural design, the input file (STL) was converted by DeScribe 2.2.1 to the Photonic Professional’s native data format (GWL), where the number of microstructures in the arrays as well the fabrication parameters, such as laser power, laser power scaling, line distance, and scanning speed of the laser focus, were configured. The generated GWL file with a code was then loaded for direct laser writing process by the control software NanoWrite for manufacturing. An example of a GWL code segment can be found in Supporting Information S5.

The manufacturing of 3D microstructure arrays was performed on a glass coverslip and diagnostic microscopy slide with a pre-patterned hydrophobic epoxy resin mask (the thickness may vary between 30 and 50 µm) with ten reaction wells (VWR International GmbH, Switzerland). Prior to patterning, the glass coverslip and slide were washed with acetone and isopropanol to clean the surface and increase the hydrophilicity of the surface before a drop of the photosensitive material (IPL-780 photoresist, Nanoscribe GmbH, Germany) was placed on the top. One droplet (a volume of a few microliters) was deposited manually on the glass coverslip or in the fluidic reservoirs of the diagnostic microscope slide using a pipette. Afterward, the coverslip/slide was fixed to the sample holder by a gluing tape and placed in a holder that fits into the piezoelectric stage. Microstructure arrays (one microstructure array per coverslip or well of the diagnostic slide) were written in a “bottom-up” sequence (i.e., the first layer was attached on the substrate surface). To minimize the optical aberrations related to the immersion-oil configuration and then get the best results in terms of resolution, the photosresist-immersion configuration (dip-in laser lithography, DILL) was used. The writing speed was 40 000 µm s⁻¹ for achieving completely crosslinked polymeric structures with well-defined 3D geometry and structural rigidity. To ensure that the polymerized material had a good connection to the substrate and to enhance the mechanical stability, the writing volume overlapped a few micrometers with the substrate. During the post-treatment, the uncured material was removed in a two-step development process: 1) 5 min in mr-DEV600 (Micro Resist Technology GmbH); 2) 15 min in 2-propanol. Finally, the substrate was dried with nitrogen gas. The 2D solid film (i.e., 170 µm thick macro-controlled pattern), which is used as a 2D control, was fabricated by placing a droplet (≈10 µL) of the same photosresist on a microscope slide between two quartz glass slides (170 µm thick). A quartz glass slides was placed on top to ensure a homogeneous photosresist distribution with no trapped air bubbles in the liquid photosresist. The microscope slide with liquid photosresist was then cured under UV light (365–405 nm) for 2 min (MA6, Karl Süss, Germany).

Cell Culture: The mESC line used in this study was E14Tg2A (CRL-1821, ATCC). In serum + LIF conditions, cells were cultured in DMEM (Invitrogen) supplemented with 15% fetal bovine serum (Gibco), 1 µL/mL LIF (EMD Millipore), 0.1 × 10⁻³ µM 2-β-mercaptoethanol (Thermo Fisher Scientific), 0.05 mg mL⁻¹ streptomycin and 50 µL mL⁻¹ penicillin (Sigma-Aldrich). In differentiation medium (i.e., LIF-free conditions), cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 0.1 × 10⁻³ µM 2-β-mercaptoethanol (Thermo Fisher Scientific), 0.05 mg mL⁻¹ streptomycin, and 50 µL mL⁻¹ penicillin (Sigma-Aldrich). In 2i conditions, cells were cultured in N2B27 buffer (Takara) complemented with 50 U mL⁻¹ of penicillin and 0.05 mg mL⁻¹ of streptomycin and the following inhibitors: PD0325901 (Millipore) at 1 × 10⁻⁶ M final concentration, CHIR99021 (StemCell Technologies) at 3 × 10⁻⁶ M final concentration, and 1000 U mL⁻¹ of LIF.

Biocompatibility Test of Microstructure’s Material: Before cell seeding, the glass coverslip (25 cm in diameter) with integrated 3D microstructure array was washed with 70% ethanol and sterilized by UV light (2.5 h). Afterward, it was coated by 0.2% gelatin (1 h) and placed in a Petri dish (4 cm in diameter). The cells were suspended in standard conditions (serum + LIF) and seeded at a density of 16 000 cells per cm². Media was changed after 24 h. All cells were grown at 37 °C in 5% CO₂ from day 1 to day 5 and analyzed continuously by bright field microscopy (Nikon Eclipse TS100).

Alkaline Phosphatase Staining: Glass coverslips (13 cm in diameter with or without integrated 3D microstructure arrays were washed with 70% ethanol, placed in the 6-well plate, sterilized by UV light (2.5 h) and then coated by 0.2% gelatin (1 h). The cells were suspended in standard conditions (serum + LIF medium) and seeded at a density of 5 500 cells per cm². Media was exchanged after 24 h. The alkaline phosphatase (AP) staining was performed 72 h after seeding using the Leukocyte Alkaline Phosphatase kit (Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions. AP staining images were captured by microscopy (Nikon Eclipse TS100). Image analysis was performed using Fiji software (https://imagej.net/Fiji). Integrated intensity measurements for the AP-stained mESC colonies were obtained after delineating the colony as the region of interest for segmentation. Only accurately segmented colonies were included in the analyses. 100 colonies from three independent experiments were analyzed for each sample. Scatterplots and statistical analysis of the data were generated using Prism 6 (https://www.graphpad.com/scientific-software/prism/). Mann–Whitney test was used to compute statistical significance. p-values of p < 0.05 were considered to be significant.

Immunostaining: Before cell seeding, the 10-well diagnostic microscopy slide with integrated 3D microstructure arrays were washed with 70% ethanol and sterilized by UV light (2.5 h). Afterward, it was placed in a standard grade plastic vessel with 3–4 mL of phosphate-buffered saline (1 × PBS) buffer to avoid medium evaporation. 3D microstructure arrays and 2D polymer film were then coated by 0.2% gelatin (24 h). Cell seeding was performed in two steps: 1) placing a 50 µL medium droplet per well; 2) injecting 1 µL with ≈100 cells above polymer foil or 3D microstructure array. Media was exchanged after 24 h. All cells were grown at 37 °C in 5% CO₂ from day 1 to day 2 and analyzed 48 h after seeding. Indirect immunofluorescence (IF) was performed 48 h post-seeding. Cells grown on microstructures were washed with 1 × PBS and fixed in 3.7% formaldehyde for 10 min. Cells were next washed in 1 × PBS three times and permeabilized with CSK buffer (100 × 10⁻³ M NaCl, 300 × 10⁻³ M sucrose, 3 × 10⁻³ M MgCl₂, 0.5% Triton X-100, 10 × 10⁻³ M PIPES, pH 6.8) for 5 min on ice. Cells were blocked in 2.5% BSA, 0.1% Tween 20 in 1 × PBS for 1 h at room temperature. The following antibodies were used: NANOg antibody (DA23, Cell Signaling, 1:500), OCT4 antibody (611202, BD Biosciences, 1:500), and ESRRB ((PP-H6705-00, R&D Systems, 1:500). Cells were washed three times in 1 × PBS with 0.1% Tween 20 prior to incubation with secondary antibodies, Donkey anti goat conjugated with Alexa Fluor 568 (Invitrogen, 1:400) for NANOg and Donkey anti mouse conjugated with Alexa Fluor 647 (Invitrogen, 1:4000) for OCT4 and ESRRB. Cells were washed three times in 1 × PBS with 0.1% Tween 20 and once in 1 × PBS, counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (100 µg mL⁻¹) for 10 min. Vectashield (antifade mounting medium) was used to reduce photobleaching.

Image and Image Analysis: Using sequential scanning mode images were acquired with Leica SP8-AOBS-CARS laser confocal microscope equipped with a 40 × 1.4 NA water HC PL APO CS2 objective. The same imaging parameters, such as laser intensity, gain, and pinhole, were set for all samples. Range indicator palette option was used to ensure that no oversaturated images were taken. Imaged colonies were randomly selected. Autofluorescence of the
material was evaluated with a multiphoton (MP) confocal microscope (Leica SP8 MP, Leica Microsystems, Germany). In particular, more autofluorescence of the photoreceptors in the blue and green regions of the emission spectrum and less autofluorescence in the far-red region were observed. To this end, excitation of the fluorophores conjugated to the secondary antibodies was implemented with wavelengths above 500 nm. Confocal images from an optical section located 1–2 μm from the top of the 3D microstructure arrays were saved independently in grayscale for each of the channels. Integrated intensity was measured from the nucleus sections, which were in focus, based on the delineated region of interest using DAPI staining free of confounding autofluorescence from the microstructures. Due to the autofluorescence of the 3D microstructure arrays, cells positioned at the beginning (i.e., at the bottom of 3D microstructures) and end of the Z-stack (i.e., top layer of cell colony) were excluded from the analysis. Image analysis was performed using Fiji software (https://imagej.net/Fiji). Briefly, integrated intensity measurements for the red (NANOG at 567 nm emission wavelength) and far red (OCT4 and ESRRB at 647 nm emission wavelength) channel were obtained after delineating the nucleus as the region of interest using DAPI staining for segmentation. Only accurately segmented nuclei were included in the analyses. 100 nuclei from each independent experiment were analyzed for each sample. Scatterplots and statistical analysis of the data were generated using Prism 6 (https://www.graphpad.com/scientific-software/prism/). Mann–Whitney test was used to compute statistical significance. p-values of p < 0.05 were considered to be significant. The quantitative immunofluorescence analysis was based on a number of assumptions: 1) the antibody is specific to the antigen, thus a blocking step was included in the protocol to avoid unspecific binding of the antibody to the antigens; 2) the antibody binds to all available specific antigens, consequently a permeabilization step was included in the protocol to allow the access of the antibody to the antigens through cell membrane and increase its chance of binding; 3) the intensity of the fluorescent signal is proportional to the concentration of the antigen; 4) there might be variability across samples, therefore to reduce this variability, when different cell lines were tested for their OCT4, NANOG, and ESRRB intensities, the whole experiment was performed in parallel including cell seeding, the complete protocol for fluorescent immunohistochemistry, and imaging steps.

Supporting Information

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

Acknowledgements

The authors thank the Ciaudo lab for the critical reading of the manuscript and for fruitful discussions. This work was supported by a core grant from ETH-Z (supported by Roche) and Olga Mayenfisch Stiftung. N.A.B. was supported by the European Union (EU) – Marie Skłodowska-Curie Individual Fellowship (project 792776, 8th Research Framework Programme HORIZON 2020). H.W. and R.A. were supported by the NCCR RNA and disease, and M.M. was supported by the Swiss National Science Foundation (grant 31003A_173120). The authors also express their gratitude to the Air Quality Control Laboratory, ETH Zurich (in particular, to Jean Schmitt), and the micro- and nanofabrication facilities at Birning and Rohrer Nanotechnology Center (BRNC), ETH Zurich for providing access to the equipment for 3D printing and assistance in 3D microstructure manufacturing. The authors are also thankful to the Scientific Centre for Optical and Electron Microscopy (ScopeM, ETH Zurich) for support provided in high-resolution intracellular imaging.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

N.A.B. and C.C. wrote the manuscript. N.A.B. designed and fabricated the 3D microstructure arrays. N.A.B. assembled all the figures. Biological experiments were supported by M.M., R.A., and H.W. All authors read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

3D microstructures, mouse embryonic stem cells, naïve and primed pluripotency, two-photon photopolymerization

Received: November 9, 2020
Revised: December 29, 2020
Published online: January 21, 2021

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