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# Nanofiber Membranes with Controllable Microwells and Structural Cues and Their Use in Forming Cell Microarrays and Neuronal Networks

**Jingwei Xie**,
Department of Biomedical Engineering, Washington University, St. Louis, MO 63130 (USA)

**Wenying Liu**,
Department of Energy, Environmental and Chemical Engineering, Washington University, St. Louis, MO 63130 (USA)

**Matthew R. MacEwan**,
Department of Biomedical Engineering, Washington University, St. Louis, MO 63130 (USA)

**Yi-Chun Yeh**,
Department of Biomedical Engineering Washington University, St. Louis, MO 63130 (USA)

**Stavros Thomopoulos**, and
Department of Orthopaedic Surgery, Washington University, St. Louis, MO 63110 (USA)

**Younan Xia**
Department of Biomedical Engineering, Washington University, St. Louis, MO 63130 (USA)

Younan Xia: xia@biomed.wustl.edu

There is a strong demand for cell culture substrates with arrayed microwells and controllable structural cues in many biological applications.[1,2] For example, such substrates are needed for fabricating cell microarrays to be used in high-throughput screening of a large number of test samples, and this approach is beneficial to drug screening, toxicology, stem cell research, and cell therapy.[3] Similarly, formation of neuronal networks could be studied *in vitro* through the use of arrayed microwells connected by structural cues, and this approach can be employed to examine synapse formation as well as the development and function of neuronal assemblies.[4,5] Most studies involving the formation of cell microarrays and neural networks have concentrated on the patterning of cell attractive or repulsive materials at pre-defined locations of a flat or contoured surface.[6] In general, the procedures are typically complex, costly, time-consuming, and require sophisticated techniques such as photolithography, e-beam writing, and focused ion beam (FIB) machining in order to generate the nanoscale structural cues.[7–10]

In contrast to the aforementioned techniques, electrospinning is a simpler and more versatile nanofabrication technique that can be used to process many types of materials into long fibers with diameters ranging from tens of nanometers to several micrometers.[11] Owing to the high ratio of surface area to volume for a nanofiber and the one-dimensional morphology, electrospun nanofibers can mimic the architecture of the extracellular matrix (ECM), making them attractive for a broad range of biomedical applications.[10] Electrospun fibers are typically deposited on a conductive collector as a random, nonwoven mat. Several techniques have been developed to control the alignment and organization of the nanofibers. For example, electrospun fibers can be aligned into uniaxial arrays by

Correspondence to: Younan Xia, xia@biomed.wustl.edu.

manipulating the electric field or by applying a mechanical force.[13,14] However, little effort has been devoted to the fabrication of membranes with complex surface structures via electrospinning. One recent report described the use of a wire mesh and an array of square-shaped protrusions made of iron as collectors for the fabrication of nanofiber membranes with patterned and woven structures.[15] Another study demonstrated the use of two-dimensional arrays of pins as collectors to generate mesh-like nanofiber membranes over large areas.[16] The nanofiber membranes described in these reports, however, were essentially flat films with no microwells on the surface (limiting their potential as cell microarray substrates) and no biological applications was demonstrated.

Here we describe a new approach based on electrospinning for generating arrayed microwells on a nanofiber membrane whose surface also contains structural cues in the form of uniaxially aligned nanoscale features. Compared to the conventional microwells for cell culture, which are commonly used as individual, isolated containers, the arrayed microwells presented here could be used both individually and collectively. Specifically, with an array of stainless steel beads as the collectors for electrospinning, we demonstrated the fabrication of poly( caprolactone) (PCL) nanofiber membranes with arrayed microwells and controllable structural cues on the surface. We further demonstrated two applications for this novel class of nanofiber scaffolds: *i*) formation of cell microarrays; and *ii*) formation of neuronal networks.

The experimental setup is similar to what we used in previous studies with the exception of a new design for the collector (Figure 1a),[17–19] which can be easily assembled from stainless steel beads and reconfigured into different patterns. Figure 1b shows the distribution of electric field between the needle tip and the arrayed metal beads, obtained using the software COMSOL 3.3 (COMSOL Inc, Burlington, MA). Note that the electric field vectors above each bead point directly towards the surface of the bead, similar to a conventional collector. However, the electric field vectors above the gap region between two adjacent beads are split into two main streams, pointing towards each bead. This pattern suggests that the nanofibers deposited directly onto the beads will be randomly oriented while those deposited across the gap between adjacent beads will be uniaxially aligned. Figure 2a shows a photograph of a nanofiber membrane collected with a close packed array of stainless steel beads 2 mm in diameter. Figure 2b shows a typical scanning electron microscopy (SEM) image of the same membrane, illustrating a complex architecture composed of a hexagonal array of microwells interconnected through a network of uniaxially aligned nanofibers. The depth of the wells was 430 ± 8 μm, as measured by analyzing cross-sectional images of the membrane. When the collectors were constructed from stainless steel beads of 1 mm in diameter, the depth was reduced to 219 ± 6 n. Figure 2, c–f, shows SEM images of the regions indicated in Figure 2b at a higher magnification. It is clear that the nanofibers deposited on the surface of stainless steel beads were randomly oriented whereas those deposited across the gap between two adjacent beads were uniaxially aligned. There was also a short transition zone from random to uniaxial alignment. Figure 2f shows that the density of fibers was much lower across the void among three neighboring beads than other regions of the membrane and the fibers deposited in the void region were randomly distributed.

The first application we examined for this new class of nanofiber-based scaffolds was their potential as substrates in generating cell microarrays. Microwells are essential for the formation of cell microarrays as they can physically confine cells, inhibit cell migration, and, if necessary, prevent them from communicating with cells in adjacent wells. In order to use the microwells, the membrane needed to be flipped over after being de-attached from the collector. Here we demonstrated the formation of cell microarrays with MG63 cells as a model system.[20] As shown in Figure 3a, the cells could be directly loaded into each well

as a droplet of a cell-containing medium. The cell-laden droplet could maintain its spherical shape due to the geometric confinement of the well and the surface hydrophobicity of the nanofibers. After 2 h, the loosely attached cells were washed away using phosphate buffered saline (PBS) buffer. The cell-seeded membrane was immersed in the culture medium and incubated for another 24 h before the cells were stained in green with fluorescein diacetate (FDA). Figure 3b shows a fluorescence micrograph of a typical cell microarray fabricated using this new substrate. Each well contained ~45 cells and all of the cells were located inside the wells. The cell number per well could easily be varied by changing the density of cells in the seeding medium. Figure 3c shows a fluorescence micrograph of a different cell microarray, in which the number of cells per well was increased to ~150. The cells were still confined within the wells in this case. Figure 3d shows a fluorescence micrograph of a third sample, where the initial density of cells was similar to that in Figure 3c, but the incubation time was increased from one to three days. The cells in this sample were still physically confined within the wells and the cell microarray was well maintained, although some of the cells likely underwent proliferation during the culture. When cultured for a longer time, the cells would start to migrate from the wells to the regions between the wells. If needed, this problem can be solved by forming larger and/or deeper wells.

The second application we examined for the membranes is related to their potential use in generating neural networks. In this case, dorsal root ganglia (DRG) were seeded into the wells on a nanofiber membrane (pre-coated with polylysine and then laminin to improve neuron adhesion and neurite outgrowth) and incubated for 6 days.[21] The neurite field was stained with anti-neurofilament 200 for visualization. Figure 4a shows an overlay of an optical micrograph with a fluorescence microscopy image, illustrating that neurites emanated from the DRG main body in the microwell and formed a neuronal network after 6 days of culture. Neurites grew along the uniaxially aligned nanofibers between adjacent wells, replicating the alignment of the nanofibers underneath. Neurites extended from all wells, following the arrays of aligned fiber connecting adjacent wells and eventually generating a neuronal network (Fig. 4b).

To further explore the use of these membranes for generating neural networks, embryonic stem (ES) cells were cultured to aggregate into embryoid bodies (EBs) using the 4–/4+ protocol to initiate differentiation.[18] The EBs were then seeded into the wells on a nanofiber membrane and incubated in a neural basal medium with a supplement of B27 to assist the differentiation of ES cells into neural lineages. We then performed immunostaining with Tuj1, a neuron marker, after incubation for 14 days. Figure 4, c and d, shows fluorescence micrographs of the neuronal network fabricated using this approach. In one demonstration, the EB was confined to one of the wells and the extended neurites followed the fiber orientation into adjacent wells (Figure 4c). The neurites were uniaxially aligned in the region where the fibers were aligned. When the neurites reached the vicinity of an adjacent well, they displayed a haphazard orientation, presumably because the underlying fibers were randomly oriented. In another demonstration, the EB was seeded into a region where the fibers had been aligned into a uniaxial array (Figure 4d). The neurites from this EB initially extended along the direction of fiber alignment. When moving closer to the neighboring well, the neurites took a disordered arrangement. As the neurites left the neighboring well and entered another region of aligned fibers, they became aligned again to follow the underlying fibers. Based on these proof-of-concept experiments, it appears that culturing neurons or ES cells on the nanofiber membranes having arrayed and interconnected microwells is a feasible way to develop complex neuronal networks.

In addition, it is worth noting that both the distance between the neighboring wells and the diameter of the wells can be separately adjusted by changing the bead assembly arrangements and the bead diameters. Figure 5, a and b, shows SEM images of nanofiber

membranes that were fabricated using hexagonal arrays of stainless steel beads as collectors with different distances between neighboring beads. We have also demonstrated the fabrication of other types of arrays besides the hexagonal pattern. Figure 5c shows a nanofiber membrane which was obtained using a square array of stainless steel beads as the collectors. Figure 5d demonstrates the fabrication of a nanofiber membrane with a square array of stainless steel beads as the collectors in which there was a gradual increase in distance between the beads along one direction. The distance between the stainless steel beads for practical use should be kept below 4 mm and 8 mm for beads with diameters of 1 mm and 2 mm, respectively.

Two previous studies reported the fabrication of nanofiber membranes with ordered architectures.[15, 16] These studies, however, described membranes with flat surfaces lacking “dimple” structures and did not show any applications of the membranes. In the current study, we fabricated nanofibrous membranes with multiple wells that could be used to physically confine cells. To our knowledge, this is the first report on the use of electrospun nanofibers for the generation of cell microarrays and complex neuronal networks. Compared to other approaches, the nanofibrous membranes described here may be more relevant to the *in vivo* environment, as electrospun nanofibers can mimic the architecture of the native ECM. Based on previous studies, neurite growth follows the direction of the underlying fibers and laminin coating could enhance neurite outgrowth and guidance.[17–19] The present work is consistent with previous studies.[22, 23] We also showed that the neurites extended from one well to another along the aligned fibers in between, eventually forming complex neuronal networks. These networks could be useful for high-throughput applications in neurotoxicology and neurodevelopmental biology. In addition, the nanofiber membrane with wells connected to each other through the aligned fibers can be used to study cell-cell communication between the wells.

In summary, we have demonstrated a simple and versatile method for fabricating electrospun nanofiber membranes with arrayed microwells and controllable structural cues on the surface by using arrays of stainless steel beads as collectors for electrospinning. We have also shown that cell microarrays could be formed by culturing cells in the microwells. Furthermore, we have demonstrated the formation of complex neuronal networks on such membranes and that neurite outgrowth could recapitulate the structural cues of the underlying nanofibers. This novel class of electrospun nanofiber membranes may also find unique applications in a range of biological applications. For example, it is widely known that the standard two-dimensional monolayer culture condition is not a good mimic of the cellular environment *in vivo*. Since the microwells in our system is constructed from nanofibers that can more closely mimic the microenvironment in which the cells live *in vivo*, this new class of substrates may enable three-dimensional organotypic cell cultures and have the potential to provide biological insight not achievable before with conventional culture plates.

## Experimental Section

### Fabrication of the nanofiber membranes

The electrospinning setup is similar to what we used in our previous studies with the exception of the collector.[17–19] The polymer solution used for electrospinning contained 20 w/v% PCL (Sigma-Aldrich, St. Louis, MO) in a solvent mixture of dichloromethane (DCM) and dimethylformamide (DMF) (Fisher Chemical, Waltham, MA) at a volume ratio of 80:20. The collector was constructed from stainless steel beads with a diameter of either 1 mm or 2 mm. The fiber membranes were removed and transferred to culture plates and then fixed by Silastic Type A Medical Adhesive (Dow Corning Co, Midland, MI). The PCL

nanofibers were sputter-coated with gold prior to imaging by scanning electron microscope (200 NanoLab, FEI, Oregon) at an accelerating voltage of 15 kV.

### Formation of cell microarrays

The MG63 cell line obtained from American Type Culture Collection (Manassas, VA) was used to demonstrate the formation of cell microarrays. The cells were cultured in an alpha minimum essential medium ( -MEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% antibiotics (containing penicillin and streptomycin, Invitrogen). The medium was changed every other day and the cultures were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. A small droplet containing ~50 or ~150 cells was placed into each well on the membrane. After incubation for 2 h, the membrane was washed with PBS buffer to remove the loosely attached cells. Subsequently, the cell-laden membrane was immersed in the medium and placed into the incubator. After incubation for 24 h or 72 h, the living cells were stained with FDA (Sigma-Aldrich) and imaged with a fluorescence microscope.

### Dorsal root ganglia (DRG) culture and immunostaining

Embryonic day 8 chicks (E8, stage HH35-36) were removed from eggs and decapitated. DRG were dissected from the thoracic region and collected in Hank's buffered salt solution (HBSS) prior to plating. DRG were seeded onto the fiber membrane and incubated for 6 days in a modified neurobasal (NB) medium containing 1% ABAM, 1% N-2 supplement (Invitrogen, Carlsbad, CA), and 30 ng/mL Beta nerve growth factors ( -NGF) (R&D Systems, Minneapolis, MN). After incubation for 6 days, the DRG were immunostained with the marker anti-neurofilament 200 (NF 200) (Sigma-Aldrich). Briefly, the DRG were fixed in 3.7% formaldehyde (Fisher Chemical) for 45 min and permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 30 min. The samples were blocked with PBS containing 2.5% bovine serum albumin (BSA) (Sigma-Aldrich) for 1 h. Anti-NF 200 diluted with PBS containing 1.5% BSA was applied to the cells overnight at 4 oC. A secondary antibody, AlexaFluor 488 goat anti-mouse IgG (1:200, Invitrogen), was then applied for 1 h at room temperature. After staining, fluorescent images were taken using a QICAM Fast Cooled Mono 12-bit camera (Q Imaging, Burnaby, BC, Canada) attached to an Olympus microscope with OCapture 2.90.1 (Olympus, Tokyo, Japan).

### Embryoid body formation and immunostaining

The protocol for embryonic stem (ES) cell culture and embryoid bodies (EBs) formation was the same as previously described.[18] EBs were seeded onto a fiber membrane (either in the well or on the region of aligned fibers) and incubated with a neurobasal medium containing 2% of B27 supplement. After 14 days, immunohistochemistry was performed to visualize the spatial distribution of neurites using tubulin III (Tuj1) (1:1000; Covance, Berkeley, CA), a neuronal marker.[18]

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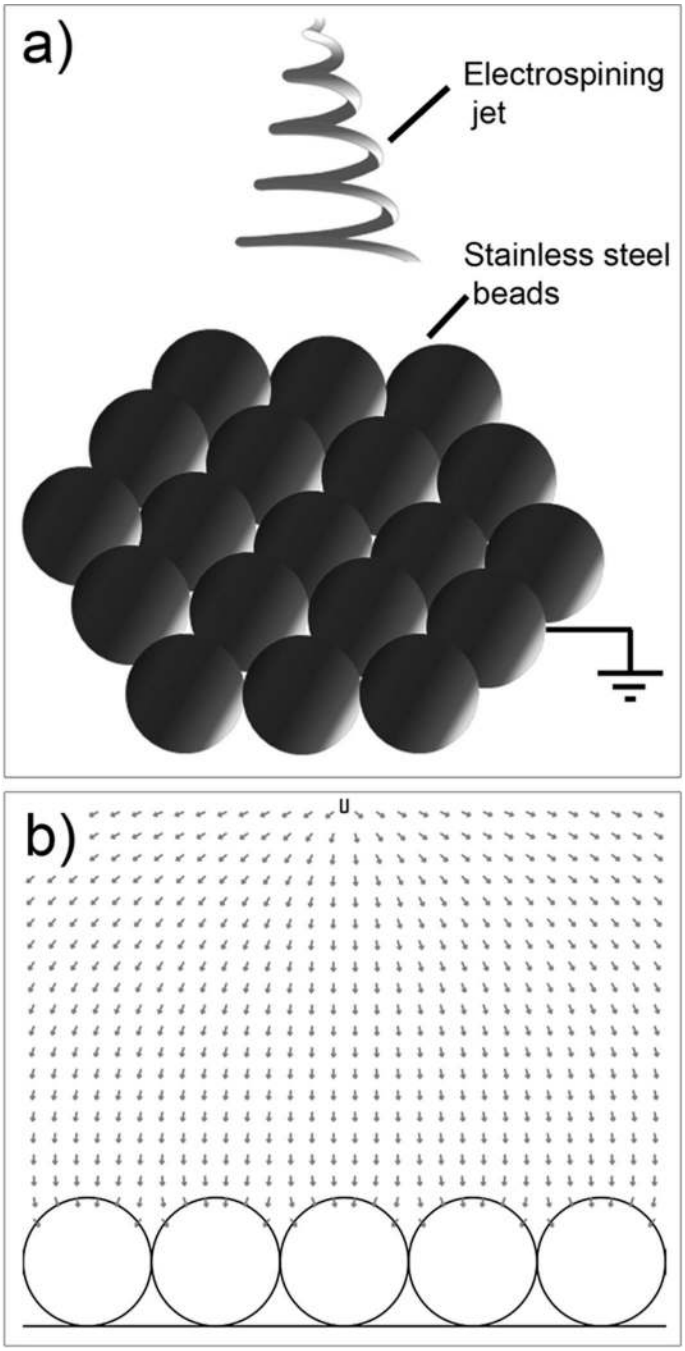


**Figure 1.**
(a) A schematic illustrating the electrospinning setup used for fabricating a nanofiber membrane with arrayed microwells and aligned structural cues on the surface, and (b) a simulated distribution of electric field vectors in the region between the needle and the collector.

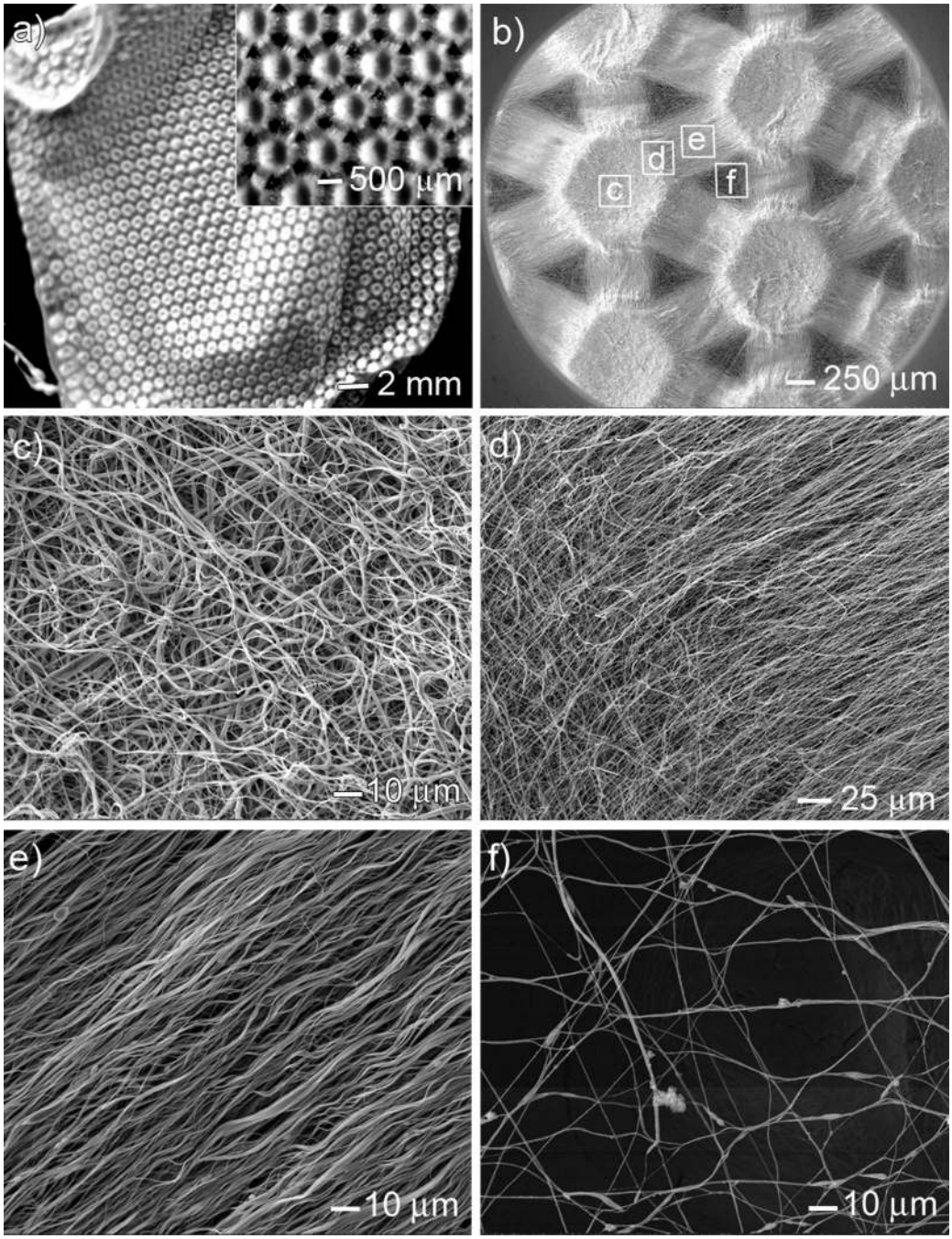


**Figure 2.**

(a) Optical micrographs and (b–f) SEM images of a membrane composed of PCL electrospun nanofibers. The inset in (a) shows an evidence for the formation of microwells on the membrane, as indicated by the shadows. This micrograph was taken by exposing the sample to a light source from the right-hand side. (c–f) SEM images taken from different regions as indicated in (b) and at higher magnifications. The background on the circumference of (b) is due to the presence of aperture.

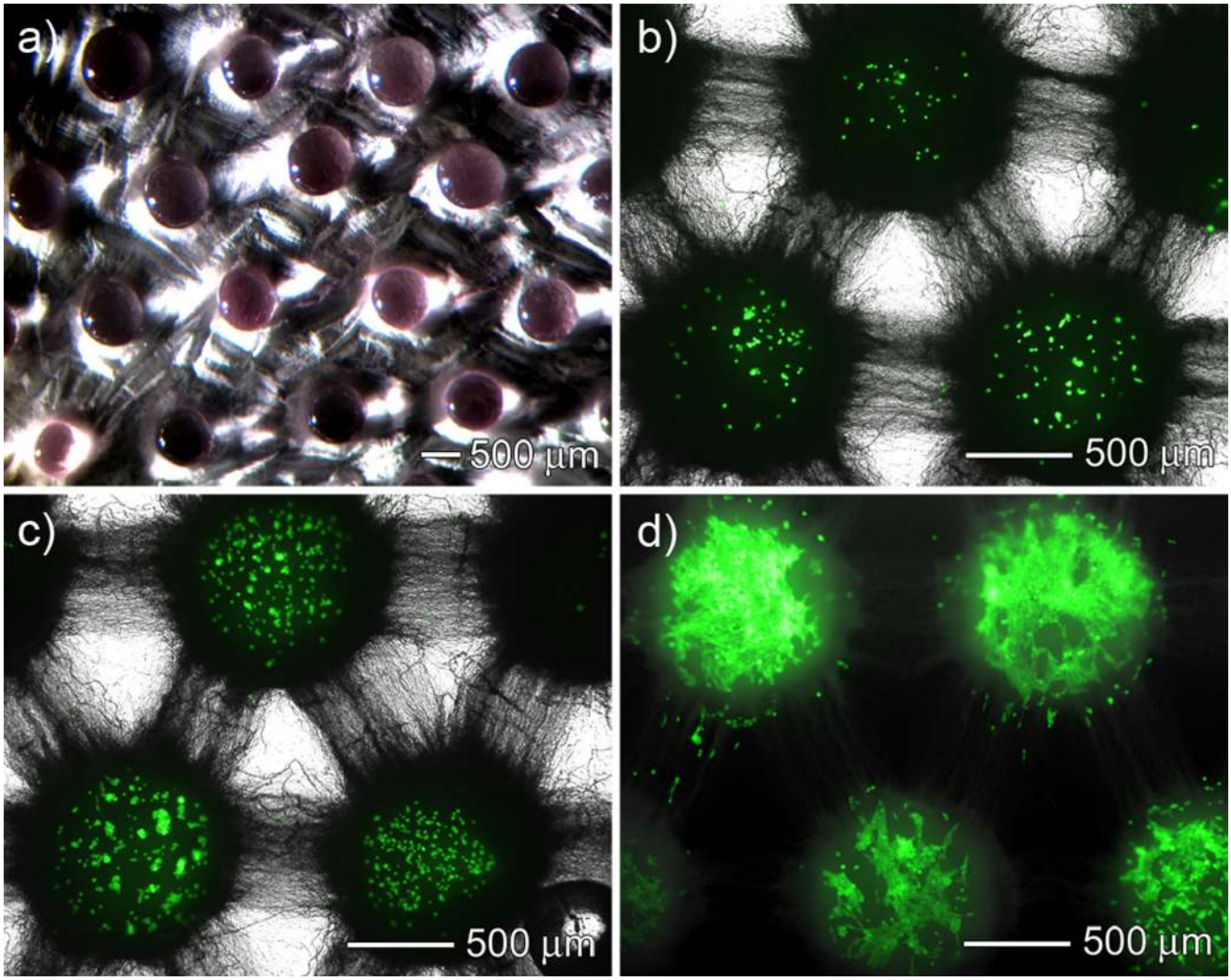


**Figure 3.**
(a) Optical micrograph illustrating the seeding of droplets (in purple color) containing MG63 cells into the microwells on a nanofiber membrane. (b) Fluorescence microscopy images showing MG63 cell microarrays after incubation for 1 day. (c) The same as in (b) except that the density of seeded MG63 cells was increased. (d) The same as in (c) except that the MG63 cells were incubated for 3 days. Live MG63 cells were stained in green with fluorescein diacetate.

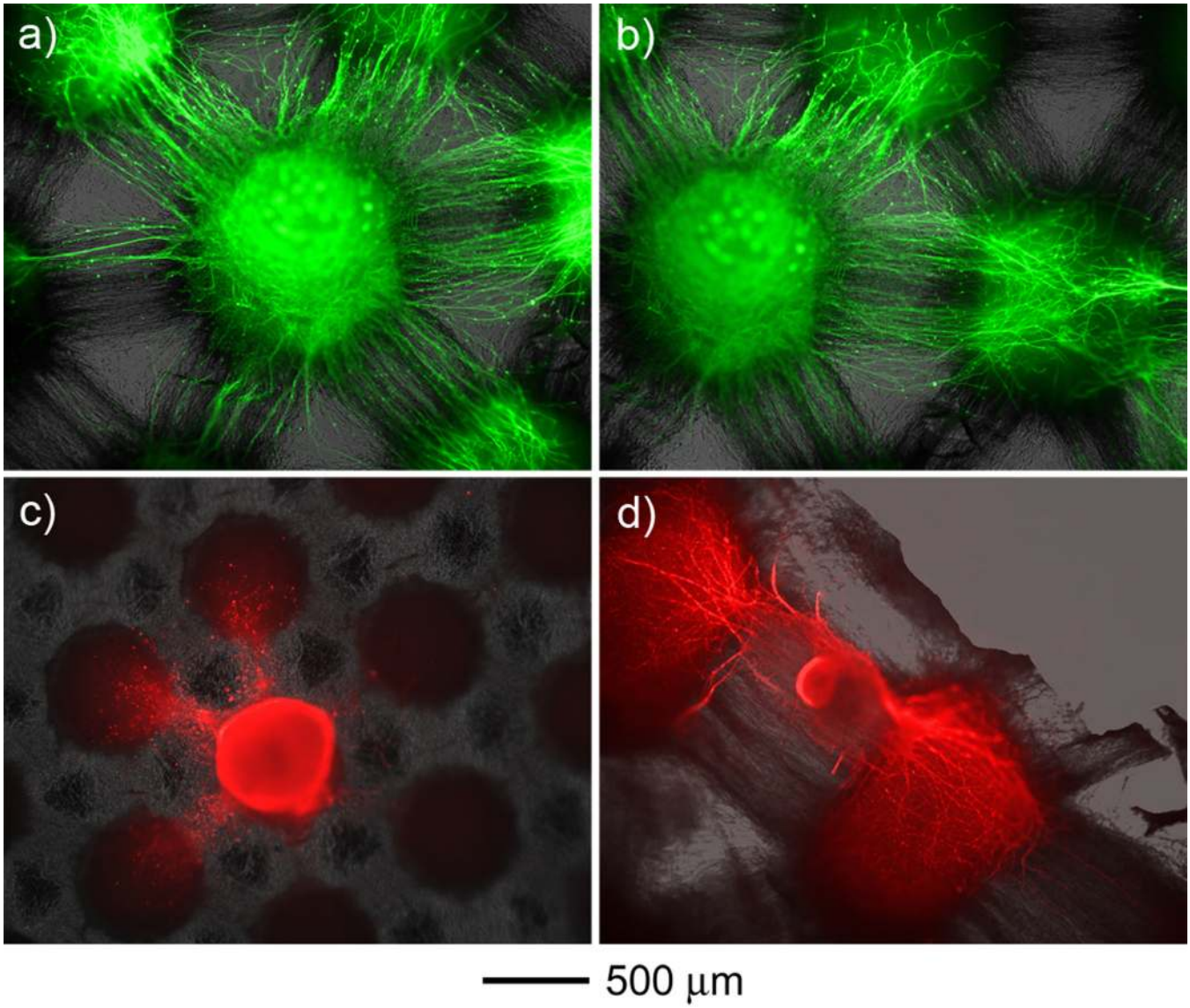


**Figure 4.**

(a) Optical and fluorescence microscopy images superimposed to show the DRG main body seeded in one well and the neurites extending from the main body to adjacent wells after culture for 6 days. The neurites grew along the uniaxially aligned nanofibers, replicating the structural cues of the underlying surface. (b) Images taken from a region next to that shown in (a), indicating that the neurites continued to grow along the uniaxially aligned nanofibers after passing the adjacent well and further extended towards a third well. (c) Optical and fluorescence microscopy images of an EB seeded in a well on a nanofiber membrane, superimposed to show the formation of a neuronal network as dictated by the underlying nanofibers. (d) The same as in (c) except the following differences: the diameter of the wells was larger, the distance between adjacent wells was larger, and the EB was seeded on the region of aligned nanofibers connecting two adjacent wells.

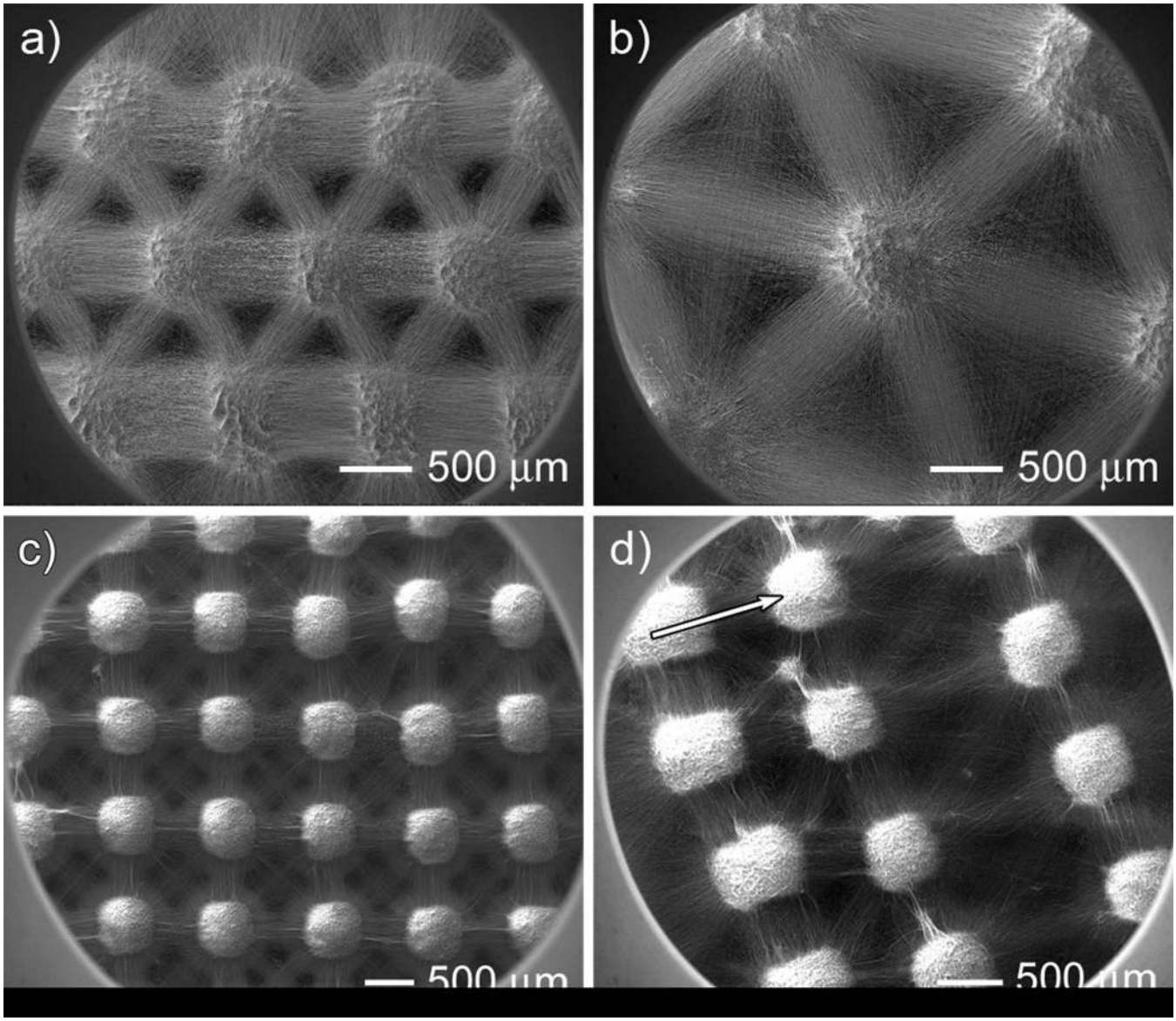


**Figure 5.**
SEM images showing the convex side of nanofiber membranes fabricated using: (a, b) hexagonal arrays of stainless steel beads as collectors with different distances between the neighboring beads, (c) a square array of stainless steel beads as the collector, and (d) a square array of stainless steel beads with a gradual increase in distances between adjacent beads along the direction indicated by the arrow. The background on the circumference of images is due to the presence of aperture.