Engineered self-organization of neural networks using carbon nanotube clusters

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Abstract

A novel approach was developed to form engineered, electrically viable, neuronal networks, consisting of ganglion-like clusters of neurons. In the present method, the clusters are formed as the cells migrate on low affinity substrate towards high affinity, lithographically defined carbon nanotube templates on which they adhere and assemble. Subsequently, the gangliated neurons send neurites to form interconnected networks with pre-designed geometry and graph connectivity. This process is distinct from previously reported formation of clusterized neural networks in which a network of linked neurons collapses via neuronal migration along the inter-neuron links. The template preparation method is based on photo-lithography, micro-contact printing and carbon nanotube chemical vapor deposition techniques. The present work provides a new approach to form complex, engineered, interconnected neuronal network with pre-designed geometry via engineering the self-assembly process of neurons.
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Keywords: Neurons; Neural networks; Carbon nanotubes; Self-organization; Nano-topography; Cell patterning

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1. Introduction

In vitro cultured neuronal networks on multi electrode arrays (MEAs) are relatively simple, well-controlled systems suited for the investigation of neuronal network mechanisms [1]. Years of intensive research yielded a wealth of experimental evidence to support the notion that studies with such networks may provide valuable insight into brain processes [2–4]. Over the past decades intensive efforts were directed to the field of microelectrode engineering focusing on biocompatibility issues, improved electrode design, improved materials and cell positioning.

Cell patterning is particularly important for cultured neuronal network applications using MEA devices due to two main issues: First, to achieve good electrical recording capabilities it is essential to anchor the cells at the vicinity of the recording or stimulating electrodes. Otherwise, the effectiveness of the recording or stimulating electrodes is dramatically compromised due to background noise of nearby neurons and the conductance of the solution [5]. Second, cell patterning is important in order to study the relationship between network function and form [6] and to learn about networks repair and brain activity [7]. Cell patterning on micro-fabricated substrates is essential for a wide range of other applications such as cell based-sensors [8,9], and cell monitoring tools [10].

1.1. Self-organization of neural networks

A possible route to achieve cell organization is to harness natural neuronal self-assembly mechanism: at high cell densities neurons have a strong propensity to cluster [11,12]. This phenomenon is marked by an initial uniform network formation followed by a collapse into interconnected islands of very high cell density. The collapse occurs due to strong tension in the dendrite and axonal connections between the neurons in the system which overpowers cell adhesion to the substrate. Cell clusters achieved in this approach are useful for studying self-organization mechanisms in nervous systems but their positioning is accidental and poorly controlled (Fig. 1).
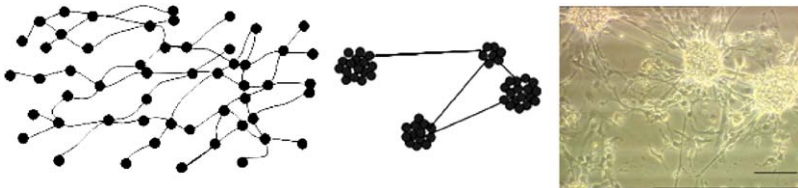

Fig. 1. Neural network collapse into isolated clusters due to tension. The first step of this process is based mainly on self-wiring of the neurons with only limited neuronal migration (left). In a later stage the network segregate into separate clusters (center). Neuronal clusters formed on a quartz substrate three days after incubation (right). Cell density was 1×10^3 cells/mm^2. Scale bar is 200 μm.

1.2. Cell positioning using micro-fabrication techniques

A more controlled approach for cell patterning consists of various micro-fabrication methods which are used to define regions for cell attachment. A straight forward approach to handle cell positioning consists of cell cages. Various such cages were devised consisting of micro-fabricated picket fences [13], or etched wells [14]. Placement of the cells in these wells is typically cumbersome and is limited to a small number of sites. Obvious alternative for the cell cages is to harness cells' natural affinity to specific surfaces as a self-assembly mechanism [15]. Such methods are primarily based on chemical and topographical surface modification which alter the material surface and influence the attachment and growth of cells.

In chemical modification the surface is patterned with heterogeneous regions with cell attracting and cell repelling chemistries. The chemistry may consist of a number of approaches such as hydrophobic and hydrophilic terminated self assembled monolayers (SAMs) [16], poly-L-lysine patterning (adhesion promoting protein) [6,17], and non-fouling gels [18]. "Programmable" attachment can also be realized by methods such as reduction of patterned SAMs [19] and thermo-responsive gels with micro-heaters [20].

More recently, nano-topography was demonstrated as an alternative effective method to control the attachment and growth of cells. Various studies have clearly demonstrated that surface texture at the scale of tens of nanometers to micrometers can influence the attachment of certain cells to surfaces and can be used as a mechanism to position cells at particular areas on a substrate [21]. It was shown that nano-scale textured silicon ("silicon grass") repelled transformed astrocytes cells, while attracting primary cortical astrocytes from neonatal rats. Both effects are attributed to the etched silicon surface [22]. The effectiveness of nano-surfaces for cell immobilization was attributed to topographical cues [9] such as directional physical signals transmitted from adhesion to actin filaments [23].

A common approach to realize such nano-topography is based on porous silicon (PS), chemically etched silicon, which offers significant advantages over bulk silicon surfaces for cell adherence and viability. No additional chemical modification, such as poly-D-lysine, is needed to support cell growth [24]. Alternative methods consist of etched oxidized silicon substrates [25] and patterned polystyrene substrates [23].

The demonstrated success of various rough substrates for cell patterning provides a clear motivation to explore other nano-topographical surfaces that may allow additional advantages over existing technologies such as substrate transparency, simplified fabrication processes, improved electro-chemical performances and improved biocompatibility. In particular, recent advances in nanotechnology offer opportunities to explore new technologies and expand existing tools beyond the conventional semi-conductor micro-fabrication technology. Here we describe a new method for neuronal cell patterning using nano topography realized by islands of high density fabrics made of carbon nanotubes (CNT). Carbon nanotube coated surfaces are biocompatible, provide an excellent surface for cell growth [26,27] and offer high specific capacitance crucial in electrochemical applications [28]. Indeed, carbon nanofibers coated surfaces were recently suggested as improved neural and

orthopedic implants [29]. In the presented method regular arrays of hydrophobic carbon nanotube islands (in the order of 100 μm) are grown on hydrophilic SiO_2 or quartz substrates to form preferred surfaces for neuronal adhesion. Neuronal clusters are formed as the cells migrate on the low affinity substrate (quartz) towards high affinity, lithographically defined carbon nanotube templates on which they adhere and assemble. Subsequently, the gangliated neurons send neurites to form interconnected networks with pre-designed geometry and graph connectivity. This process is distinct from previously reported formation of clusterized neural networks in which a network of linked neurons collapses via neuronal migration along the inter-neurons links. The template preparation method is based on photo-lithography, micro-contact printing and carbon nanotube chemical vapor deposition techniques. The present work provides a new approach to form complex, engineered, interconnected neuronal network with pre-designed geometry via engineering the self-assembly process of neurons.

2. Methods

2.1. Substrate fabrication

The realization of our new approach is based on a soft-lithography process to define regions with iron nano-particles acting as catalysts during the carbon nanotube growth (Fig. 2). The small dimension of the iron particles (several nm)
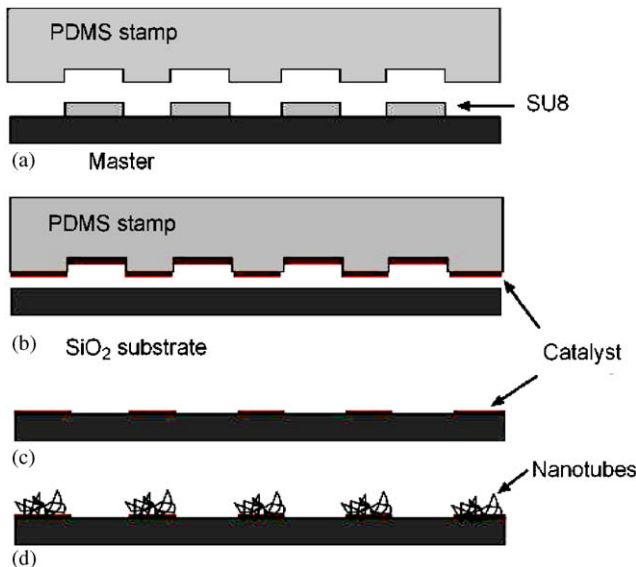

Fig. 2. Schematic drawing of major substrate preparation steps: (a) Molding a PDMS stamp from a rigid master. (b) and (c) Catalyst stamping. (d) CNT growth from catalyst particles.
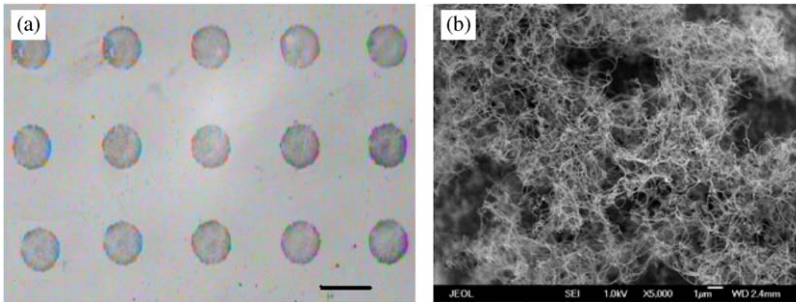
Fig. 3. (a) Optical image of islands of carbon nanotubes, synthesized by chemical vapor deposition from patterned iron catalyst (scale bar is 150 μm). The catalyst islands are achieved by a soft-lithography process using a molded PDMS stamp. (b) A high resolution scanning electron microscope (HRSEM) image of a carbon nanotube coated region.

determines the carbon nanotube diameters (several nm). The process consists of a stamping step, with a poly(dimethyl siloxane) (PDMS) stamp soaked with iso propyl alcohol (IPA) with iron nitrate catalysts.

A chemical vapor deposition (CVD) process is then used to synthesize carbon nanotubes by pyrolysis of carbon containing gases such as ethylene and methane at the catalyst sites on the substrate. During the hot growth process long CNT thermally fluctuate and eventually bind to each other to form a three-dimensional entangled matrix of CNTs (Fig. 3).

Preparation of the silicone stamp: a thick layer (14 μm) of SU8-2015 (Micro Chem) photoresist was patterned on a 4 inch polished n-type silicon substrate (Mitsubishi silicon America) using standard optical lithography. Feature height was verified by surface profiler. The stamp was prepared using a mixture of 10:1 silicone elestomer base, and curing agent (Sylgard 184, Dow Corning). 10 cm^3 of the mixture was poured into a rounded plastic mold which was tightly attached to a piece of the silicon master. The mold was then cured for 1 h at 100 °C and then extracted from the plastic mold. CNT were synthesized by a CVD process. A suspension of iron nitrate in IPA (0.1 g in 20 cm^3) was sonicated and centrifuged and was used as the catalyst for the CVD process. The catalyst was then put on the PDMS stamp and dried in air for 10 min. Cleaned quartz substrates (Electron Microscopy Sciences, cat 72250-03) were prepared by 1 min sonication in acetone followed by 1 min sonication in IPA and dried by air. The cleaned substrates were then attached to the PDMS stamp for few seconds. The substrates were put into a 1 inch quartz tube furnace in an atmosphere of hydrogen gas (1000 sccm for 10 min). The temperature was ramped up to 860 °C. Twenty sccm ethylene was used as the source gas, for 8 min. Finally the ethylene flow was terminated and the furnace turned off and cooled down to room temperature.

2.2. Cell cultures

Dissociated cortical cultures were prepared and maintained as follows: the entire cortices from one-day-old Charles River rats were finely chopped. The cortical tissue

was digested with 0.065% trypsin (Biological Industries, Beit Ha-Emek, catalog 03-046-1b) in phosphate buffered saline (Biological Industries, catalog No. 02-023-1a), for 15 min, followed by mechanical dissociation by trituration. Cells were resuspended in a modified essential medium with Eagle's salts (Biological Industries, catalog No. 01-025-1a) containing 5% horse serum (Biological Industries, catalog No. 04-001-1a), 1 mg/ml gentamycin, and 0.02 mM glucose. The cultures were maintained in growth conditions at 37 °C with 5% CO_2 and 95% humidity. Half of the growth medium was replaced twice a week. Pictures were taken by a camera (SC35, Olympus, Japan) mounted on an optical microscope (IX-70, Olympus), using the ×40 and ×100 magnifications.

3. Results

Neurons and glial cells were deposited on patterned quartz substrates and were incubated for several days to investigate the effectiveness of the method. After four days neurons have aggregated and accumulated on the CNT coated regions and the cell density on the CNT-free regions on the quartz was very low (Fig. 4). As the initial cell distribution on the quartz surface, before the incubation, was uniform it is clear that the cell's surface mobility and selective adhesion are the driving mechanism for the well organized placement of the cells at the CNT sites. As in previous reports the rough substrate serves to position cells at predefined locations.

Moreover, connections between the islands are clearly apparent and interconnected networks are formed following the exact pattern of the CNT templates. This process typically occurs by bridging gaps almost exclusively between nearest neighboring islands. The bridging consists either of an axon or bundles of axons and dendrites. In some cases the bridge is covered with clusters of cells. Interestingly,
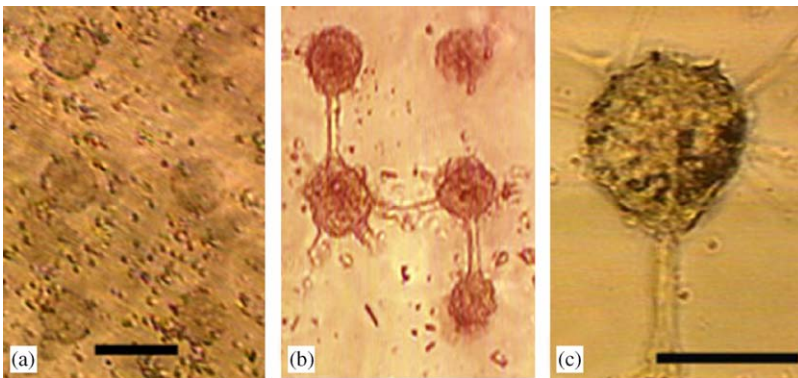

Fig. 4. Inverted microscope images of interconnected neuronal systems formed with CNT islands. (a) One hour after cell deposition cells are randomly distributed (scale bar is 150 μm). (b) After four days neurons form clusters at the CNT sites, which form connections between these islands. Some islands are not yet connected at this stage and several short and unconnected dendrites and axons are apparent. (c) Neuron cluster at a CNT island (scale bar is 100 μm). Cell density was 4.5×10^3 cells/mm^2.

these bridges form very efficiently over quartz surfaces which are apparently very poor surfaces for cell attachment. This observation strongly suggests that engineering the connectivity between neurons (using chemical template for example) is not necessary as these connections can be self-formed according to natural physical mechanisms.

3.1. Network evolution

Fig. 5 shows the evolution of a network over three days beginning with the fourth day after incubation. The data show that cells first aggregate at the CNT islands. As they complete this step axons and dendrites begin to form and to build connections
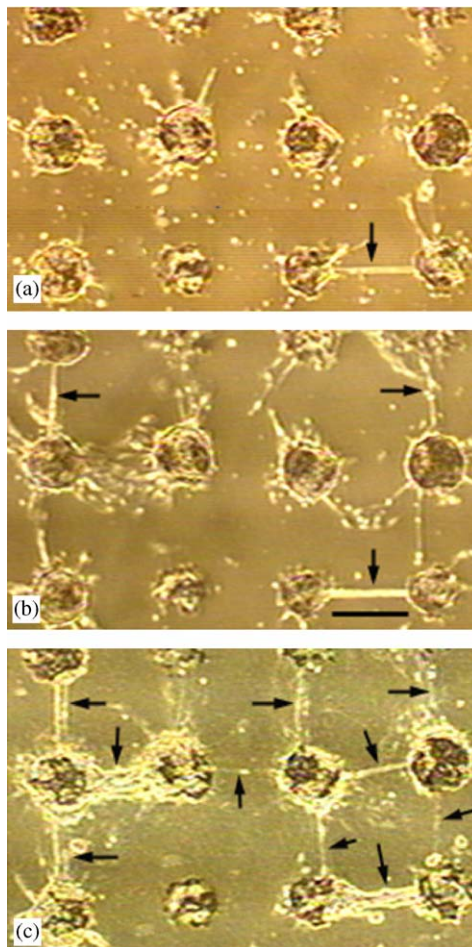

Fig. 5. Inverted microscope images of interconnected networks formed with CNT islands (top) 96 h, (center) 128 h and (bottom) 150 h after plating the cells on the substrate. Cell density was 1×10^3 cells/mm^2. Arrows indicate well defined connections between neuronal clusters. Scale bar is 150 μm.
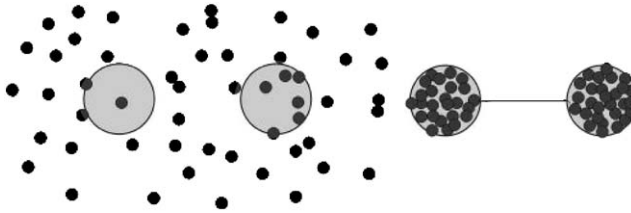
Fig. 6. Neural network formation from nucleation centers. In the first step neuronal migration on low affinity surface results in cell aggregation at high affinity regions (large, dark disks) (left). In a second step high density neuronal islands form well defined interconnection following the shortest link between islands (right). This process should be contrasted with the collapse process described in Fig. 1.

between neighboring islands. Eventually a single link is formed to connect nearest neighbors. The data in Fig. 5 also provide some insightful information about the development of these links. The links typically occur as two separate protrusions reach the same point. These two lines combine into a bundle and eventually stretch to form a tensed link between the two islands. Interestingly, the protrusions appear to be directed along the diagonal of the square island lattice. This issue is beyond the scope of this letter and will be discussed separately in a later publication.

The results presented above provide a first demonstration for engineered neuronal network formation from nucleation sites. This is a generic process and similar results may be obtained with other combinations of high and low affinity surfaces. Preliminary data show similar effects with poly-D-lysine islands on agarose surface. Unlike the clustering mechanism (discussed in Section 1) which begins with network connectivity and ends with a collapse (see Fig. 1), the mechanism described here is characterized by an initial cell organization followed by a network formation. The two mechanisms are distinct and the general principles are depicted in Figs. 6 and 1.

3.2. Cell density

Cell density and site density determine the number of cells per site. In Fig. 5 the plating density was 1×10^3 cells/mm^2 corresponding, in average; to 40 cells per island (distance between islands is $200 \, \mu$m and no cell death was apparent during the duration of the experiments). This value can be modified by changing island spacing or cell density. However, it is important to note that when trying to control this number one cannot ignore various processes that govern neural network formation such as cell's propensity to cluster. At high cell density ($\sim 1 \times 10^4$ cells/mm^2 [30]) neurons have a strong propensity to cluster [11,12] with typical cluster size of approximately $250 \, \mu$m which is comparable to the size of the CNT islands.

To study how this issue affects the performances of our substrates we have increased the cell density and repeated the experiments described above. Results with cell density of 4.5×10^3 cells/mm^2 are shown in Fig. 7. As in the low density case, over several days neurons form well structured systems following the template of the CNT islands. Over time (another three days of incubation) these well formed
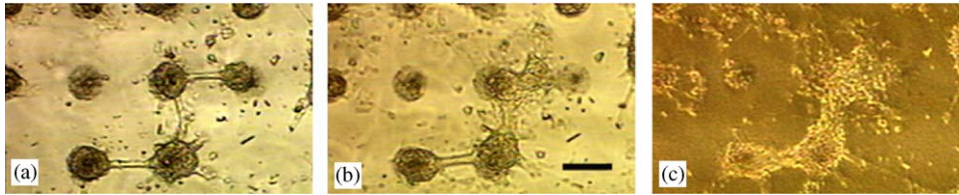
Fig. 7. Inverted microscope images of interconnected networks formed with CNT islands at a cell density of 4.5×10^3 cells/mm^2 at (a) 96 h, (b) 128 h and (c) 150 h after plating the cells on the substrate. At this high cell density mechanical tension overpowers cell adhesion to the CNT islands. Scale bar is 150 μm.

networks seem to "collapse" to big clusters (Fig. 7). At this high cell density mechanical tension overpowers cell-cluster adhesion to the CNT islands.

The results described above were obtained with surfaces with CNT mats without any additional chemical treatment prior to cell incubation. This fact is important if the CNT islands are to be used as electrodes as well as binding site. For recording electrodes the effect of additional coating of a thick protein layer (typically used to improve cell adhesion) on the capacitance is undesired. Patterned substrates with poly-D-lysine were tested and appear to have a slower time scale for engineered network formation: samples after six days of incubation show no apparent engineered network formation. It is conceivable that surfaces without poly-D-lysine allow much faster network formation compared to substrates with poly-D-lysine due to faster and more effective cell migration on the surface, better cell adhesion to uncoated three-dimension CNT matrix and ultimately better aggregation at the CNT sites. Further incubation of samples with poly-D-lysine allows network formation and overall behavior similar to the behavior of samples without poly-D-lysine. On both treated and untreated substrates neurons appear to survive over very prolonged periods of time (at least five weeks).

3.3. Electrical viability

To validate the electrical viability of the formed networks we have tested their electrical activity using the patch-clamp technique [31]. The cells show a normal generation of action potential with normal shape in response to electrical stimulations. These tests illustrate that the attachment to the CNT surface does not alter the normal functionality of the neurons. The nature of the electrical connectivity between islands and the effectiveness of the CNT islands as recording electrodes remains to be investigated.

4. Summary

To summarize, well defined engineered cultured neuronal systems were formed on high density carbon nanotube islands. The presented approach offers simplicity in fabrication and flexibility in choosing the substrate material. Self-assembly allows

the system to achieve optimal self-organization rather than forced unstable organization. We expect that the presented method will provide a powerful platform to study neuronal adhesion and neurite outgrowth. Due to the superior electrochemical properties of carbon nanotube electrodes, passivated electrical contacts to the CNT islands will also allow high quality neural recording.

Acknowledgements

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