An automated microdrop delivery system for neuronal network patterning on microelectrode arrays

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Abstract

The aim of this work is to present a new technique for defining interconnected sub-populations of cultured neurons on microelectrode arrays (MEAs). An automated microdrop delivery technique allows to design and realize spatially distributed neuronal ensembles by depositing sub-nanoliter volumes of adhesion molecules in which neurons grow and develop. Electrophysiological tests demonstrate that functionally interconnected clusters are obtained and experimental results (both spontaneous and stimulus evoked activity recordings) attesting the feasibility of the proposed approach are presented. By means of the automated system, different and specific architectures can be easily designed and functionally studied. In the presented system the speed of drop deposition is about 30 drops/min; the mean diameter is 147 mm; typical cell survival time is 4–5 weeks. By changing drop size and spacing, investigations about how the network dynamics is related to the network structure can be systematically carried out.

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1. Introduction

The possibility of designing specific network architectures forcing neurons to follow a pre-defined structure has been a dream since the 1970 when the first attempts aimed at creating patterned networks were made (Letourneau, 1975). Cultured neurons on planar substrates that have “well defined” two-dimensional network architectures could give valuable insights into the cell-to-cell communication, network dynamics versus topology, and basic mechanism of synaptic plasticity and learning. Since the pioneering works of Letourneau many studies have been presented, using various methods, including surface modification by silane chemistry (Georger et al., 1992), photolithographic techniques (Corey et al., 1996; Ruardij et al., 2000; Torimitsu, 1990), deep-UV lithography (Dulcey et al., 1991), soft lithography (Branch et al., 1998). Soft lithographic techniques include microcontact printing and microfluidic channel flow patterning (Martinoia et al., 1999; Morin et al., 2006). Microcontact printing is the most popular soft lithographic technique. It is based on elastomeric stamps made from polydimethylsiloxane (PDMS) and it has been used to create patterns of a variety of organic molecules to promote or repulse cell adhesion on solid surfaces (Branch et al., 2001; Chang et al., 2003; Zhang et al., 1999). The key feature of this method is the capability to create patterns at the nanometer and micrometer scale (Kane et al., 1999), that is at a single cell or cell process scale (i.e. neurites guidance).

Another method working at the nanometer scale, namely dip-pen nanolithography, relies upon the use of AFM to create nanopatterns of proteins with a minimum feature dimension of 30 nm (Piner et al., 1999; Wilson et al., 2001). This method has been recently used to create high-resolution protein arrays to control cell attachment (Lee et al., 2002).

Although various nanomicro patterning techniques have been developed and utilized for patterning neuronal cell cultures (see for a review Li et al., 2003), it should be pointed out that, due to the inherent low density of such culture prepa-
rations, only few reports have demonstrated, for mammalian
neurons, the functional electrophysiological characterization of
these networks. Among them, some authors have used stan-
dard patch clamp techniques to probe synaptic connections and
assess cell-to-cell communication (Ma et al., 1998; Ravenscroft
et al., 1998). More recently, Vogt et al. (2005) studied synap-
tic plasticity in a network patterned with microcontact printing;
they observed short-term modulations (i.e. depression) similar
to those reported in brain slices.

Other authors proposed to couple micropatterning techniques
with extracellular recordings by means of microelectrode arrays
(MEAs). In this case micromachining or patterning is used to
define the network architecture by positioning cell bodies and
cell processes onto the microelectrodes. MEA devices consist of
60–100 electrodes (with diameter of 10–50 μm and interdistance
ranging from 50 to 500 μm) on a glass or silicon substrate. They
are widely used for electrophysiological recordings on “ran-
domly” grown large mammalian neuronal networks. Jimbo et
al. (1993) first monitored the electrical activity of patterned neu-
nal networks with MEAs. More recently, Suzuki et al. (2004,
2005) demonstrated the possibility of specifically designing a
neural architecture on MEA substrates; they were able to con-
trol the growth and connections of a hippocampal cell culture
by using a laser to dynamical ablate an agarose layer to create
channels and pits that entrap neurons and guide neuronal growth.
Other examples in the literature are related to the patterning or
confinement of neurons extracted from invertebrate (Fromherz,
2003; Claverol-Tinturé et al., 2005, in press). In this case, the
dimension of the neurons and the robustness of the experimental
preparation allow manipulation and electrophysiological moni-
toring.

In addition and in alternative to the previously introduced
patterning methods, low-cost and flexible procedures that apply
ink-jet printing deposition of proteins to modulate cell attach-
ment have been developed. For example, researchers at Clemson
University investigated the use of conventional ink-jet technol-
ogy to pattern surfaces with collagen in two dimensions to
target cell growth (Roth et al., 2004). They demonstrated, using
smooth muscle cells (SMCs), that ink-jet technology can be used
to create, reproducible, patterns of various geometries, with a
resolution of 350 μm, that support cell growth on agarose sub-
strates. Although the spatial resolution is not high, this technique
can be applied for neuronal patterning across the micro- to milli-
meter scale. Sanjana and Fuller (2004) recently reported about a
custom ink-jet printing method utilized for patterning dissociate
neurons. They realized microislands constituted by a mixture of
collagen and poly-t-lysine at micrometer scale (deposited dots
range from 100 to 500 μm) on glass or on substrates pre-treated
with poly(ethylene) glycol (PEG) acting as repulsive layer for
neurons. In this case too, electrophysiological recordings have
not been reported.

Following these works and the ink-jet printing principle,
we developed a new and simple microdeposition set-up based
on a piezo-dropper system and a motorized X–Y stage which
allows the deposition of tiny volumes (around 100 pl) of spe-
cific biomolecules such as proteins with a lateral resolution in
the micrometer range. In this work we used the microdeposition
set-up to pattern a MEA substrate with adhesion factors capable
of inducing functionally interconnected small sub-population of
neurons. The aim of the work was to constrain the connectivity
of the network at the level of a small population of neurons while
allowing the natural self-assembly of neuronal ensembles and
preserving the functionality (i.e. the spontaneous electrophysi-
ological activity) of the network. To demonstrate the feasibility
of such an approach, network activity was recorded at different
stages of development (i.e. days-in vitro, DIV): the first results
and analyses demonstrate that the microdrop delivery system
makes possible the study of the effects of network topology on
the electrical activity of neuronal networks coupled to MEA.

2. Materials and methods

2.1. Microdeposition set-up

The microdeposition set-up (Fig. 1a) consists of: (a) piezo-
electric droplet generator; (b) motorized stage; (c) optical video
system; (d) controlling software.

(a) The piezoelectric droplet generator (purchased from the
University of Bremen, Department of Chemical Engineer-
ing) consists of a borosilicate glass capillary with a nozzle
at one end, which is surrounded by a piezo-ceramic tube
and glued to it with a two component adhesive (Ulmke et
al., 2001a,b). The piezo-ceramic tube is connected to a high
voltage signal generator. By applying high voltage pulses,

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Fig. 1. (a) Microdeposition set-up and (b) drop deposition sequence. Scale bar
is 60 μm.
the tube, and consequently, the capillary are contracted, so that disturbances are generated in the liquid and droplets are ejected from the nozzle (Ulmeke et al., 2001a,b). The set-up has been equipped with two piezo droplet generators (nozzle diameter of 60 μm) with the corresponding driving electronics. This allows the deposition on the substrate of two different sample solutions in parallel. A light emitting diode is used to generate a stroboscopic light to visualize the droplets, thus allowing to adjust the driving pulse amplitude and length in order to generate spherical drops of known size (Fig. 1b). The driving electronics is connected to a computer by a data acquisition card (DAC NI-6711 by National Instrument, TX, USA) and controlled via software developed in LabVIEW (National Instruments). The glass capillaries are loaded from the upper side with small sample volumes (minimum volume of liquid needed for droplet generation is ~7 μl).

(b) The X–Y motorized stage is used to position and move the substrate below the droplet generator with micrometer accuracy. The micrometer screws can be driven either manually by a dial knob, or via software by a two-axes step motor controller card with USB interface (Eksma, Lithuania). The screw travel range is 25 mm and one step corresponds to 1.25 μm linear movement.

(c) The video system, consists of an optical zoom provided with a video camera with magnifying optics (Zoom6000, Navitar, USA). The total magnification ranges from 2.10 × 10 to 13.50 × with a 52 mm working distance. The video system is used to monitor the droplet generation and to facilitate the alignment of the droplet generator with the area of the substrate where the drops have to be deposited. A USB video acquisition card connected to the CCD camera is used for the acquisition of pictures and movies during the deposition.

(d) Software tools have been developed in LabVIEW (National Instruments) for the control of the two droplet generators and the motorized stage. The system is fully automated and specific patterns (i.e. drop position) can be generated by varying via software the amplitude and the duration of the voltage pulse, it is possible to generate droplets of different sizes, for example, by using a capillary with a nozzle diameter of 60 μm, droplets with a diameter varying in the range 40–80 μm are obtained.

2.2. Electrophysiology set-up

Primary cultures of cortical neurons were plated over two kinds of MEA (both from Multi Channel Systems (MCS); Reutlingen, Germany):

(i) 60 planar TiN/SiN microelectrodes, 30 μm diameter, 500 μm spaced;
(ii) 60 planar ITO (indium tin oxide) microelectrodes, 30 μm diameter, 200 μm spaced.

The experimental set-up is based on the MEA60 System (MCS, Reutlingen), and consists of: a microelectrode array, a mounting support with integrated 60 channels pre- and filter amplifier (gain 1200×); a stimulus generator (STG 1008), a general-purpose stimulator which generates current and voltage pulses to be applied to the electrodes; a control software for data acquisition and storage (MCRack), equipped with a data acquisition board for real time signal monitoring and recording. The set-up is mounted onto an anti-vibration table and hosted into a Faraday cage to minimize noise from the environment.

2.3. Experimental protocols

2.3.1. Microdrop deposition

The adhesion promoting factors used for the patterning were laminin (Sigma-L2020) and poly-l-lysine (either Fite labeled: Sigma-P3069, or not labeled). The adhesion factors were deposited both on glass coverslips to test the technique and on the two different types of MEAs.

Before the adhesion factor deposition, both the slides and the MEAs were extensively washed with MilliQ deionized water, dried at 60 °C and then sterilized at 121 °C for 2 h. The adhesion factor molecules were diluted in sterilized water to obtain the following final concentrations: laminin, 10 μg/ml; poly-l-lysine, 100 μg/ml. The solutions were usually stored at ~80 °C until their use.

Before use, the glass capillaries used for deposition were rinsed alternatively with ethanol and sterilized water and then filled with the solution containing the adhesion proteins. The generated droplets had a diameter size of about 60–80 μm corresponding to a volume in the range of 100–300 pl. It should be emphasized that the final bi-dimensional spot obtained on the substrate depends on the wetting properties of the surface (i.e. a more hydrophilic surface will favors smaller spots). Average deposition speed, that is limited by the dynamics of the XY translation stage, resulted in 30 drops/min.

In our case, two different types of adhesion layers were tested: the first one based on the deposition of a monolayer of poly-l-lysine; the second one based on the deposition of a double layer: laminin first and then poly-l-lysine. After protein patterning, the substrates were again sterilized with ethanol for 10–15 min and dried under laminar flow.

2.3.2. Cell culture

Primary cortical cultures were produced from brain tissue of Sprague–Dawley rats at embryonic day 18 (E18). The embryos were harvested by caesarian section from anesthetized pregnant dam, animal care followed standard procedures that are in accordance with institutional guidelines.

Cerebral cortices were isolated, chopped into small pieces and digested by 0.125% trypsin solution at 37 °C for 20 min. After blocking proteolitic enzyme with FBS 10% serum, the tissue was washed twice with serum-free medium and mechanically dissociated by gentle titration with fine-tipped Pasteur pipette. The resultant cell suspension, diluted at a final concentration of 1.5–2 × 10^6 cells/ml, was plated directly onto glass coverslips or MEAs from Multi Channel Systems.
Both substrates were pre-treated with patterned adhesion promoting molecules as described in the previous paragraph. To guide neurons attachment and neurite outgrowth it is possible to create various grids of differently spaced laminin and poly-l-lysine microdroplets, with different drop sizes and cellular densities. Microelectrode area and coverslip sub-area were covered with about 6–8 × 10^4 cells in a 40 μl volume.

Once the neurons were attached (2–3 h) the glass ring of MEA was refilled with 1 ml of culture medium while the coverslip was kept inside a Petri dish (Ø 100 mm) with 5 ml of culture medium. The cultures grew in neurobasal, serum-free medium, 2% B-27 supplement and 1% of Glutamax, in a humidified incubator with an atmosphere of 5% CO2 at 37°C. Every week one-half of the media was replaced with fresh one, no antimitotic drugs were used to eliminate glial cells from culture.

Spontaneous electrical activity recording was performed starting from the second week.

2.4. Data analysis

In order to process and analyze the data stream coming from the recording activity by the MEA-system, we used a custom developed software (Vato et al., 2004), implemented in Matlab© (The MathWorks, MA, USA).

Multi-site recorded data are converted into spike trains by means of spike detection techniques (Chapin et al., 1999; DeMarse et al., 2001; Marom and Shahaf, 2002; Wessberg et al., 2000). The used algorithm is based on a peak-to-peak threshold (Perkel et al., 1967) evaluated as a multiple of the standard deviation (S.D.) of the baseline noise (Jimbo et al., 1999). Data were analyzed in order to characterize the bursting and spiking behavior of the neuronal networks. In particular, we considered the mean firing rate (MFR), mean bursting rate (MBR), inter burst interval histogram (IBIH), and, in case of evoked response, the post stimulus time histogram (PSTH) (Rieke et al., 1997).

3. Results

We first verified the quality of the deposited drops in terms of spot dimension, spreading uniformity over the surface, and pitch distance between spots by using Fic labeled poly-l-lysine.

The neuronal network development was then monitored to validate the overlapping of cluster populations on the fluorescent spots. To this end, sequences of images (DIC and fluorescence microscopy) were captured by using a CCD camera mounted on an inverted microscope. The cultured neurons, which preferentially adhered to the circular area over the microelectrodes, showed a healthy morphology and, within 48 h after plating, started to generate processes that, in the following couple of days, established an interconnected grid of neuronal sub-populations. In these preliminary experiments a high density plating (i.e. 10^5 cells/mm^2) was preferred in order to favor the recording of electrophysiological spontaneous activity at early stages of the development. Moreover, in some cases, possible inaccuracy in the drop deposition (e.g. due to changes in the environmental conditions (humidity) or to the glass capillary response to the piezoelectric mechanical stimulus), caused adjacent clusters to merge inside the network.

3.1. Patterning on glass

The first tests were performed depositing drops of Fic-labeled poly-l-lysine on coverslips. Neurons were plated on the patterned substrates and incubated for several days to investigate the efficiency of the method. The initial cell adhesion distribution on the surfaces was uniform. After a couple of days a lot of neurons detached from the uncoated substrate and were eliminated with supernatant; others migrated towards the fluorescently labeled areas forming aggregated clouds of neurons. The stabilized neurons developed in correspondence of the deposited microislands of adhesion molecules, constituting neuronal aggregates of 60 cells per island, on average.

Quite interestingly, due to the relatively short distance among microislands, the neuronal ensembles tended to connect with each other. Results are shown in Fig. 2, where it can be speculated that for reciprocal distances of 100 μm or less (d2), they tend to collapse; on the contrary, for sufficient distance (d1) the clusters remain not-connected. Scale bar is 100 μm.

3.2. Device patterning

The deposition of only one adhesion factor (i.e. poly-l-lysine) on the MEAs resulted in insufficient cell adhesion, contrary to the results obtained on coverslips: a too low cell density was present on the electrode areas. This result was not unexpected since, at least in our experience, the general procedure for plat-
ing neurons on the MEA utilizes a double layer of laminin and poly-lysine (Chiappalone et al., in press; Jimbo et al., 1999). Therefore, following this approach, a double drop deposition was tested, that is, the two capillaries for deposition were used sequentially: the first one, filled with laminin solution, and the second one with labeled poly-lysine. The automated procedures and the possibility to visually align the MEA with the droplet generators, allows to precisely superimpose the two drops. The same patterning process was applied to the ITO and TiN microelectrode arrays (see Section 2). From the analysis of calibrated images, we derived a mean value of the spot diameter of 147.5 μm (standard deviation: 7.1 μm). Results are shown in Figs. 3 and 4.

The results reported in Figs. 3 and 4 show that cells are anchored on the substrate only in the areas where the adhesion proteins have been deposited. Among these areas the cells form interconnected sub-populations, which clearly appear during the later stages of the development. This process typically occurs by bridging gaps almost exclusively between nearest neighboring clusters. The interconnections between adjacent neuronal sub-populations is constituted by bundles of neurites (axons and dendrites). It should be pointed out that on the MEA surface the formation of neuronal clusters is less reliable. Unwanted peripheral clusters are obtained and less defined spot dimensions are formed (see Fig. 4). This is mainly due to the double layer deposition, which is necessary for appropriate adhesion onto and around the microelectrode, that still needs to be optimized.

3.3. Electrical activity characterization

In order to validate the capability of the new deposition technique to produce functionally interconnected neuronal sub-populations, we recorded the electrical activity from five preparations coupled to MEAs with different layouts.

We recorded spontaneous and evoked electrical activity at different DIVs (19, 25, 27 and 39) by using MEA 30/500 (i.e. 30 μm electrode diameter, 500 μm spaced) and MEA 30/200 (i.e. 30 μm electrode diameter, 200 μm spaced).

As an example, Fig. 5 shows the raster plots of the electrical activity corresponding to the cluster of Fig. 4 (15 recorded channels) during its development. In this case, the activity appears rather asynchronous till the late stage of development (39 DIV) where more bursting signals are activated.

A first evidence that electrophysiological activity is somehow correlated with the actual network architecture can be found by comparing Fig. 4 with Fig. 5. By observing the positions of the neuronal clusters in Fig. 4 and the electrophysiological recorded signals in Fig. 5, a qualitative correspondence between recorded signals and network morphology can be made: recorded signals (i.e. active electrodes) correspond to electrodes partially or totally covered by neuronal populations. As an example, if we consider electrode 58, a big cell assembly can be seen, the corresponding raster plot shows isolated spikes (19 and 25 DIVs) and bursting signals (27 and 39 DIVs). On the other hand, if the droplet does not cover the electrode (e.g. electrode 38), no electrical activity is recorded by the corresponding electrode. One should take into account that only well coupled neurons (i.e. with a high overlapping surface) can be recorded by this kind of microelectrodes (Martinoia et al., 2004).
Fig. 5. Raster plot of the correspondent electrodes of Fig. 4 at different ages: (a) DIV 19, (b) DIV 25, (c) DIV 27 and (d) DIV 39.

Fig. 6. Inter Burst Interval (IBI) distribution of the interconnected sub-population grown (left column; a, c, e and g) and of the randomly grown culture (right column; b, d, f and h) over a MEA 30/500 at different ages (DIV 19, 25, 27, 39).
Additionally, to quantify the spontaneous bursting activity, we evaluated the inter burst interval (IBI) distribution (Fig. 6) at different DIVs for recordings at different ages (same experiment for 5 min recording as partly shown in Fig. 5). This type of analysis could be utilized for comparing the results with a typical randomly grown culture at different stage of the in vitro development (Chiappalone et al., in press).

The proposed deposition technique gave interesting results (in terms of the obtained defined architecture) also when external stimulation was applied. Referring to the cluster of microelectrodes of Fig. 4, we applied voltage stimuli, i.e. a biphasic rectangular voltage pulse (width 250 μs; peak-to-peak amplitude 1.5 V; frequency 0.2 Hz) to electrode 67. The response of the network was analyzed by post stimulus time histograms (PSTHs) as shown in Fig. 7.

As it might be expected, electrodes 55 and 56, showed site specific response reflecting the functional topographical connectivity of the network: electrode 55, the one closer to the stimulating site, presents a fast response, whereas electrode 56, slightly far apart, presents a more delayed response. Moreover, it seems that the efficacy of the stimulation involves only the adjacent electrodes: electrodes 17, 28, 36, 37 present an attenuate response to the stimulus or, in some cases, no evoked activity (electrodes 38, 45, 47, 48).

These preliminary results open new possibilities in the study of network dynamics versus network architecture.

4. Discussion and conclusions

In this work we have presented a new, simple and flexible technique for defining functionally interconnected sub-populations of cultured neurons on MEAs. The automated microdrop delivery system which we used allows designing and realizing spatially distributed neuronal sub-populations by depositing sub-nanoliter volumes of adhesion molecules on which neurons grow and develop. The patterned neuronal ensembles spontaneously connect each others with no need for specific patterning for neurites outgrow and guidance. Our results demonstrate that, for distances between adjacent spots in the range of 50–150 μm, complex and electrophysiologically active neuronal networks can be created and maintained up to 40 DIV. The possibility to visually align the piezo-dropping system with a specific substrate (i.e. a MEA) allows the precise positioning of single drops on a given array, overlapping to the underlying electrodes. The resolution of the system is limited by the dimension of the nozzle and by the fluid-dynamic properties of the solution. It is known that with glass capillaries with a smaller aperture, drops as small as 10 μm can be generated; it is therefore possible to achieve a final spot diameter down to 30 μm, a size that is comparable with the electrode dimension. Moreover, the deposition set-up we developed allows fast, precise and reliable double drop deposition.

Interestingly enough, the recorded spontaneous and evoked electrophysiological activity on our neuronal cultures, demonstrates that it is possible to change the dynamics of the system by changing the topography of the network and that functional connectivity studies can be better performed on defined architectures than in random cultures.

By changing the architecture of the network, studies on the interplay between network morphology and electrophysiological activity (i.e. network dynamics) can be undertaken. By depositing different molecules, studies on the network development and network connectivity can be performed. Finally, specific hybrid system could be developed by patternning specific neuronal architectures (e.g. with a defined sensory input layer) coupled to microelectronic devices or MEAs for investigating the code of information in custom designed neuronal networks.

As a whole, we demonstrate that piezo-dropping systems provide a simple and efficient method for realizing defined neuronal architectures in conjunction with MEA techniques for extracellular recordings.
On the other hand our method shares the same advantages of other ink-jet based systems allowing a rapid prototyping, and the possibility to deposit drops on non-planar substrates. The simplicity of the system together with the possibility to automatically create a virtually unlimited number of patterns with mixtures of different biologically active molecules, makes it very attractive for neuroscientific investigation.

Additional and future works will be devoted to systematically study the interaction between neuronal ensemble during development at different spot dimensions and spot distances. Moreover, functional connectivity and interplay between morphology and network dynamics will be also investigated in more details.

References


