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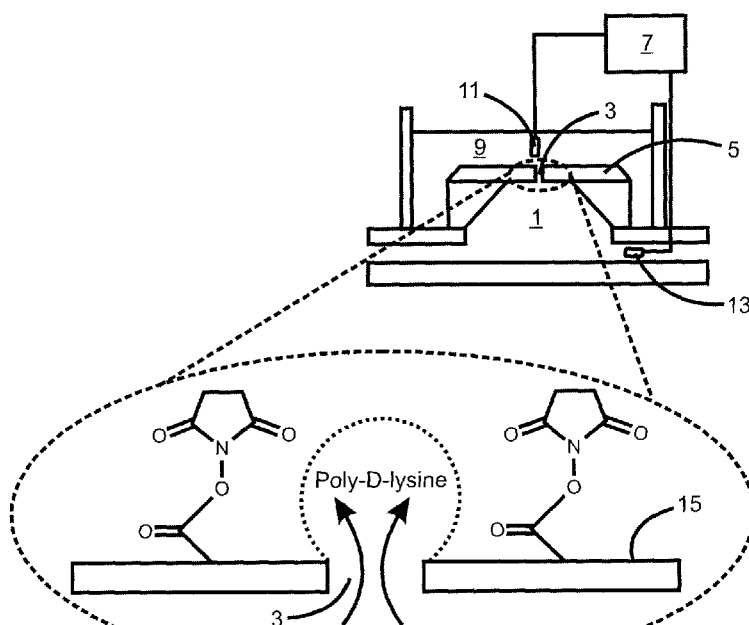


Fig. 1A

(57) Abstract: A method of producing a radial chemical gradient on a surface of a substrate involves controllably forcing a predetermined amount of a chemical compound through an orifice in the substrate. The orifice connects a surface on which the gradient is produced to another surface of the substrate and the chemical compound is forced through the orifice in a direction from the other surface to the surface on which the gradient is produced. The chemical compound is allowed to diffuse on the surface radially away from the orifice and the compound reacts with the surface to form the radial chemical gradient on the surface disposed around the orifice.



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SURFACE CHEMICAL GRADIENTS

Cross-reference to Related Applications

This application claims the benefit of United States Provisional Patent Application
USSN 61/684,178 filed August 17, 2012, the entire contents of which are herein
5 incorporated by reference.

Field of the Invention

The present invention relates to methods of modifying surfaces of substrates and
modified surfaces prepared by such methods. In particular, the method relates to
methods of producing chemical gradients on surfaces and to surfaces having chemical
10 gradients prepared thereon.

Background of the Invention

Chemical gradients are not only interesting systems for fundamental studies, but
also play a key role in defining and regulating biological phenomena. Surface-immobilized
chemical gradients are important systems employed in the study of molecular recognition
15 of the cellular microenvironment that triggers growth, differentiation, and communication.
Of particular interest is the study of cell patterning or guidance, which has applications
that span from fundamental studies to numerous biochip technologies.

Due to its simplicity, chemical gradients are generally prepared by diffusing the
desired compound in a highly porous matrix such as hydrogels. Although this method has
20 been employed in numerous studies in the literature, chemical gradients are not stable
over time due to the continued diffusion of the unbound molecules. In addition to the
obvious limitations of this method, the desired compound inside the matrix is not
immediately accessible at the matrix surface.

The preparation of covalently immobilized surface gradients has also been
25 reported for the simplest possible case. That is, surface concentrations that change only
in one dimension over large surface areas (mm or higher) can be obtained by immersing
the substrate at a given rate in a solution containing the desired molecule and allowing it
to react chemically with the surface. However, this method is not suitable for chemical
gradients at the sub-millimeter scale. Moreover, this approach cannot be applied to the
30 fabrication of multidimensional surface-chemical gradients which are highly desirable for
cell placement on biochip growth substrates such as those in planar patch-clamp chips.

Techniques for preparing radial surface gradients have also been developed. A syringe pump may be used to place a droplet of a chemical solution at a spot on a surface permitting the droplet to radially expand away from the spot by diffusion, further assisted by the pressure of adding more droplets to the spot (Spencer 2004; Spencer 5 2009). A chemical may be print stamped on to a surface as a round spot or ring away from which the chemical diffuses to create a radial gradient (Morgenthaler 2008). A fill system using a nozzle with multiple apertures may be used to spray a chemical solution on to a surface in a desired pattern to form a chemical gradient on the surface (Jenkins 2005). In general, a variety of strategies for forming chemical gradients on surfaces are 10 known (Ruardy 1997; Singh 2008). While effective for some applications, these techniques are generally unsuitable for producing radial chemical gradients centered on an orifice, particularly micron and sub-micron orifices, in a substrate. In particular, they present the major difficulty of accurately aligning the gradient around the orifice.

Patch clamp technologies are also known in the art (e.g. Huang 2005; Huang 15 2002; Kelly 2002; Vogel 2003). In one variation (Huang 2005) a cell is moved, positioned and sealed over a hole in a substrate by using mechanical, electroosmotic or electrophoretic techniques. A coating with a specific binding member (e.g. ligand, receptor, antibody, etc.) can be localized around the holes to induce adhesion and create a better seal between the cell membrane and substrate surface. However, such 20 technology does not use a gradient, which is desirable to enhance a self-guided placement/attachment over the hole.

There remains a need for a simple, effective method of preparing surface chemical gradients, particularly radially symmetric surface chemical gradients centered around an orifice in a substrate or radially asymmetric surface chemical gradients 25 emanating from an orifice in a substrate, particularly for use in patch clamp applications directed to studies of molecular recognition of biological systems.

Summary of the Invention

There is provided a method of producing a radial chemical gradient on a surface of a substrate, the method comprising: controllably forcing a predetermined amount of a 30 chemical compound through an orifice in the substrate, the orifice connecting a first surface on which the gradient is produced to a second surface of the substrate, the chemical compound being forced through the orifice in a direction from the second surface to the first surface; and, allowing the chemical compound to diffuse radially away from the orifice through which the compound is being forced, the compound reacting with

the first surface to form a radial chemical gradient on the first surface disposed around the orifice, the gradient having lower concentrations of the chemical compound at distances farther from the orifice.

There is further provided a substrate comprising a radial chemical gradient thereon produced by a method of the present invention.

Means for controllably forcing the chemical compound through the orifice include, for example, mechanical pumping, capillary action with or without the aid of electro-wetting, electrically driven methods or combinations thereof. Capillary action or electrically driven methods are preferred. Electrically driven methods including, for example, electrophoretic and electroosmotic techniques, require an ionically conductive medium on both sides of the surface. Electrically driven methods are highly desirable since these methods can not only drive precise quantities of the desired compound through the orifice but are also suitable for orifices with micron and sub-micron diameters where mechanical pumping would require prohibitively high pressures. Moreover, electrical pulses with sufficiently high potential differences have the added advantage of removing small air bubbles trapped at or within the orifice. Electroosmotic behavior is observed when the surface of the channel is charged either intrinsically or due to surface modification. Moreover, this behavior is predominantly obtained when the charged surface is free of adventitious organic compounds and when the diameter of the channel is sufficiently small. Electrophoretic behavior is observed when the surface of the channel is not charged (as it is the case in plastics) or not sufficiently charged (as may be the case for silicon-nitride). Electrically driven methods may use applied voltages in a range of about 10-200 V (e.g. about 100 V) at pulse rates of about 10-5000 msec (e.g. about 100-3000 msec) with polarity reversal to force the chemical compound back through the orifice after the chemical reaction at the surface. Other schemes may be advantageous depending on the configuration and end-goal.

Geometry and surface chemistry (e.g. hydrophilic and hydrophobic properties) within the orifice has a large effect on the flow of the chemical compound through the orifice when capillary action or electrically driven methods are used. The orifice preferably has a diameter in the micron or sub-micron range, for example, in a range of from about 1 nm to about 1000 μm , more preferably in a range of from about 10 nm to about 500 μm . For application to patch-clamp chips, the diameter is preferably between about 1 and 2 μm , though it may be smaller or larger depending on the type of cell interrogated.

The chemical compound to be imprinted as a chemical gradient may be in the form of a solution or neat liquid, aerosol or gas. Characteristics of the chemical gradient can be controlled by the viscosity of the solution, the amount of solution being passed through, pH and temperature of the solution. Solution viscosity controls the diffusion rate of the solution that has passed through the hole and this can be adjusted by choosing a solvent with the appropriate viscosity and/or dissolving an inert polymer (e.g. poly(ethylene glycol) (PEG)). The amount of solution that is forced through the orifice can be adjusted by varying the potential difference that is applied and by the length of the electrical pulse at a given ionic strength and viscosity. In instances where the reactive groups on the surface are susceptible to hydrolysis as it is the case for NHS activated carboxylic acid groups, pH and temperature have an influence on the chemical kinetics and this ultimately controls the geometry of the imprinted chemical gradient as the forced solution diffuses away from the orifice.

The chemical gradient may be radially symmetric or asymmetric disposed around the orifice. Higher concentrations of the compound in the gradient will be found closer to the orifice since the areas of the substrate surface in proximity to the orifice are in contact with the compound for a longer period of time.

Asymmetric gradients may be formed by controlling the direction of diffusion of the chemical compound on the first surface. For example, asymmetric gradients may be formed by inhibiting and/or encouraging diffusion of the chemical compound in certain directions away from the orifice. Inhibition of diffusion may be accomplished by any suitable means, for example by placing physical or chemical features in the diffusion path to guide diffusion in certain directions. In the case of electrically driven methods, placing an electrode laterally away from the orifice but close to the surface on which the gradient is being produced would encourage diffusion in the direction of the electrode. Thus, in electrically driven methods, biasing of the gradient in a particular direction may be performed under the influence of an electric field. Asymmetric gradients may be particularly desirable not only for cell placement but also axon guidance. In one embodiment, the chemical gradient is centered on the orifice.

Any suitable chemical compounds may be used to form gradients on the surface of the substrate. The type of compound is limited by its ability to diffuse along and react with the surface. Further, the compound used will depend on its ultimate application as the compound should possess reactive or bioactive moieties that can be used to immobilize objects of interest (e.g. cells via cell adhesion) on the substrate over the orifices. Suitable chemical compounds include, for example, cell adhesion promoters

such as poly-D-lysine, poly(ethyleneimine), collagen, fibronectin, laminin and of particular interest, peptides that contain the RGD motif. These peptides are able to bind to the integrin receptor on the surface of the cells thereby inducing cell adhesion via a molecular recognition event. Furthermore, the integrin receptors are in close proximity to ion channels and therefore provide the means to position the ion channels on or next to the orifice. This is extremely important for electrophysiology since ion channels govern the electrical activity of neurons and their abnormal behavior give rise to neurological diseases.

Preferably, the chemical compound is immobilized on the surface of the substrate. In some embodiments, the chemical compound may react directly with the substrate surface to be bound thereon. In other embodiments, the surface may be chemically or physically prepared in order to react with the chemical compound. An example of chemical preparation includes modifying the surface with functional groups, for example, N-hydroxysuccinimide (NHS) ester groups, maleimide groups, epoxy groups, azide groups (for "click chemistry"), etc.

Substrates may comprise any suitable material, for example, silicon, glass, quartz or plastic (e.g. polystyrene, polycarbonate, polyimides, etc.). The substrate may be in the form of cell culture plates, biochips (e.g. planar patch-clamp arrays, multi-electrode arrays), chemical sensors, etc.

The present invention is particularly suited to patch-clamp array technologies. The patch-clamp technique, employing a fine-aperture (usually 1-2 μm) glass micropipette filled with an electrochemically conductive physiological medium, is used to contact individual cells cultured in vitro and record the electrophysiological activity of either individual ion channel proteins, or the entire cell. Ion channels are specialized transmembrane proteins that selectively control the flow of specific ions through the membrane, thereby allowing cells to control their membrane potential. This process enables cells to be "excitable", to generate action potentials to process and transmit information in many biological systems including, but not limited to the nervous system. Neuropathologies are associated with altered electrophysiological activity such as synaptic dysfunction and altered ion channel activity; in vitro disease models are often used to assess therapeutic strategies by studying those particular ion channels. Because of its superior sensitivity and spatial resolution compared to other techniques, the patch-clamp technique is considered the gold standard of ion-channel electrophysiology and is essential to ion-channel pharmacology. It is however a slow and cumbersome technique that requires high technical skill. To increase throughput, planar patch-clamp chips have

been developed and integrated into Automated Patch-clamp Systems (APS) (Dunlop 2008). In those chips, the aperture at the apex of the pipette is replaced by an approximately micron size orifice in a membrane that separates the culture vial from a second bath containing the same electrochemically conductive physiological medium as found inside the pipette. APSs critically increase throughput for primary drug screening, but they can only interrogate isolated cells from suspensions. Moreover, these cells are from cell lines that have homogenous over-expressed ion channels. For this reason, and since every function in the brain and nervous system is effected by networks of cells, these are not particularly biologically relevant models of disease. This is particularly important for secondary drug screening, since therapeutic development requires that the mechanism of action of the drug candidate be understood. This is why patch-clamp array chip technology has been proposed where several orifices are integrated in the patch-clamp chip, each connected to a dedicated subterranean microfluidic channel to enable the simultaneous monitoring of several neurons cultured on-chip and engaged in network activity (Mealing 2005; Mealing 2011).

Additionally, the present method is well suited to provide chemical gradients on Multi-Electrode Arrays, a commercially available type of neurochip that allows the electrophysiological monitoring of large networks of electrogenic cells (Fejtl 2006), albeit not at the resolution of individual cells or ion channel proteins. Multi-electrode arrays could be equipped with perfusion holes for the creation of chemical gradients for positioning cells over or near the electrodes in a predefined pattern.

The present method and surfaces are particularly useful for producing surface-immobilized chemical gradients especially for the study of molecular recognition of biological events. Of particular interest is the study of cell patterning and/or axon guidance, which has a large number of applications that span from fundamental studies to biochip technologies.

Synthetic cellular networks can be used as *in vitro* brain models, to simulate neurological dysfunction, and to evaluate therapeutic strategies to restore those functions. These studies may help health researchers better understand the mechanisms of action of neurological diseases, and pharmacologists to discover new drugs. Brain models may also be subjected to various substances affecting their normal spontaneous activity, and the altered function of the model allows one to infer the properties of that substance. For example, sampling the bodily fluid of a patient (i.e. cerebrospinal fluid) on the model and observing an altered behavior of the network that is the signature of a disease may lead to diagnosis. Sampling a treatment on the model may also be an

effective means of assessing its efficacy for the patient. Also, sampling food or water extracts on the model may be an effective and sensitive means of detecting neurotoxins or assessing food safety.

5 Synthetic cellular networks can additionally be implanted *in vivo* as either grafts to replace damaged tissue, or as functional interfaces with prostheses. For example, it is well known that severed nerves often cannot reconnect without intervention, and grafts of tissue cultured *in vitro* and organized in a morphologically compatible manner may result in restored function. The present method could therefore be used to synthesize a cellular network *in vitro* resulting in a tissue that can be lifted from the substrate and implanted in
10 *vivo* for restorative purposes.

Neural implants are routinely implanted in certain patients to alleviate the symptoms of disease (i.e. epilepsy or depression) or restore lost function (i.e. bladder control), but the electrical-nerve interfaces are of poor quality and durability due to the accumulation of scar tissue around the implant. Again, synthetic tissue cultured *in vitro* on
15 the implant before implantation may lead to the development of a better interface.

Finally, cell positioning may be used for the purpose of cell sorting (Yamamura 2005). In the present method, the combination of cell positioning and the specific interaction with an antigen may result in the identification of specific cells within a larger population.

20 It is an important advantage that the present method is self-aligning, that is, the gradient may be centered on the orifice since it uses the orifice for diffusion of the chemical compound. By contrast, most printing methods may be able to achieve high resolution and similar gradient shapes, but they face the difficulty of alignment. The dispensing of fluids from any kind of printer is generally limited to a resolution of about 20
25 μm therefore printing cannot achieve the precision needed for aligning synthetic cellular networks on patch-clamp array chip orifices, given that the cells of interest can be roughly 10 μm and the orifice 1 μm in diameter. For those commercial printing products that can print with nm resolution, e.g. dip-pen nanolithography, although these devices have nanometer resolution, they cannot easily print large areas. For instance, it would be very
30 impractical, if not impossible, to print a 10 μm spot using commercial printing products that can print with nm resolution. Thus, it is an important advantage that the present invention can easily produce radial chemical gradients aligned on an orifice and having a resolution of gradient concentrations in a range of about 500 nm to 10 μm .

Further features of the invention will be described or will become apparent in the course of the following detailed description.

Brief Description of the Drawings

5 In order that the invention may be more clearly understood, embodiments thereof will now be described in detail by way of example, with reference to the accompanying drawings, in which:

Fig. 1A depicts a schematic diagram illustrating a method of the present invention for immobilizing a radial gradient of poly-D-lysine on a silicon chip;

10 Fig. 1B depicts a fluorescent image of the gradient of fluorescently labeled poly-D-lysine formed on the silicon chip as illustrated in Fig. 1A centered on an orifice; and,

Fig. 2 depicts images of primary rat cortical neurons cultured on orifices of a silicon chip on which a radial gradient of poly-D-lysine has been formed as depicted in Fig. 1A and Fig. 1B.

15 Description of Preferred Embodiments

Example 1: Formation of radial gradient of poly-D-lysine on a silicon chip

Referring to Fig. 1A, an electroosmotic device is depicted in which solution **1** of poly-D-lysine is provided below silicon chip **5** having micro-sized orifice **3**. The chip separates the poly-D-lysine solution **1** from poly-D-lysine-free solvent **9** above the chip in order to provide a concentration difference of poly-D-lysine across the orifice. Solution **1** and solvent **9** are kept separated to prevent mixing except through the orifice. Power supply **7** provides a potential difference across electrodes **11** and **13**. Molecules of poly-D-lysine are forced through the orifice driven to the positively charged electrode **11** above the chip provided the channel is negatively charged (see inset). Poly-D-lysine then
20 diffuses radially away from the orifice and reacts with upper surface **15** of chip **5**, which has been modified chemically with highly reactive N-hydroxysuccinimide (NHS) ester groups that have been immobilized on the chip's surface. These highly reactive NHS ester groups react with amino groups of the poly-D-lysine forming stable amide linkages, thereby immobilizing the poly-D-lysine on the chip.
25

It will be apparent to those skilled in the art that the position of electrode **11** has an influence on the way poly-D-lysine diffuses from the orifice. If electrode **11** is positioned directly above the orifice, the resulting gradient is expected to be substantially symmetrical. Should electrode **11** be positioned substantially on one side of the orifice, the gradient will be biased towards it. Additional electrodes, or electrodes of various shapes and sizes, may be advantageously positioned in the bath in order to produce chemical gradients of various shapes. Generally, it will also be apparent to those skilled in the art that other methods, such as aspiration, may influence the diffusion of chemical compounds away from the orifice, and therefore affect the shape of the chemical gradient being immobilized on the surface of the substrate.

More details are as follows.

Preparation of the chip surface:

Silicon chips were coated with a thin film of silicon dioxide using a Plasma Enhanced Vapor Chemical Deposition at 350°C and 40 W power with gas flow rates set at 500 sccm N₂O, 54 sccm SiH₄ and 1100 sccm He and a pressure of 1500 mTorr. The deposition rate was approximately 350 Å/min to yield 200 nm films. Subsequently, the chips were cleaned in a standard piranha solution at 120°C for at least 2 hours. The dry substrates placed inside a chamber containing two microcentrifuge tubes with 15 µL of 3-aminopropyltriethoxysilane (APTES) and allowed to react for 4 hours under vacuum. The resultant amino modified substrates were reacted with a freshly prepared solution of 10 mg/mL poly(methacrylic acid) (MW = ~100,000, Cat. # 00578, Polysciences Inc. USA) in 0.1M borate buffer pH = 8 in the presence of 9.6 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Aldrich, Oakville, ON, Canada) and 8.6 mM N-hydroxysuccinimide (NHS, Aldrich, Oakville, ON, Canada). The substrates were washed with a solution of 0.1% sodium dodecylsulfate (SDS) in PBS pH = 7.4 (Aldrich, Oakville, ON, Canada), rinsed in MilliQ™ water and dried under a stream of nitrogen after the 2 h reaction. The carboxylic acid terminated substrates were activated (formation of surface NHS esters) in a solution containing 9.6 mM EDC and 8.6 mM NHS in nitromethane for 2 hours. The freshly activated substrates were stored in a desiccator and employed immediately in the preparation of gradients.

Preparation of the gradient:

400 µL of PEG (M_n = 950-1050, 2.2 g/mL) in 0.2 M borate buffer pH 8 is placed on the top surface of chip **5**. 80 µL of a solution prepared by mixing 25 µL of poly-D-lysine

hydrobromide (20.1 mg/mL) in MilliQ™ water, 15.4 µL of poly-D-lysine labeled with Alexa Fluor™ 555 (2.65 mg/mL) in MilliQ™ water and 40.4 µL of PEG ($M_n = 950-1050$, 2.2 g/mL) in 0.2 M borate buffer pH 8 is placed on the bottom surface of the chip as depicted in Fig 1A. The top electrode is carefully positioned above the hole while ensuring it is immersed in the solution. The bottom platinum electrode **13** is in contact with the solution in the fluidic channel. Top platinum electrode **11** is positioned with the aid of a 3-axes micrometer driven translation stage. A potential difference of 100 V is applied for 100 msec and the plug of polylysine is allowed to diffuse and react with NHS-activated surface **15** for 1000 msec before applying a potential difference with reverse polarity for 3000 msec. The sample chip **5** is immediately rinsed and immersed sequentially in the following solutions: 1) 0.1%SDS in PBS pH = 7.4 for 5 min; 2) 0.1M sodium phosphate buffer pH = 5.76; and, 3) 1 M ethanolamine pH = 8.5 for 5min. In all cases, the sample is rinsed with MQ water after treatment with each of the above solutions. Finally the sample is dried with a stream of nitrogen. Alternatively, gradients can be prepared without mounting the silicon chip to substrate with fluidics. That is, the chip is held with an appropriate holder that allows the placement of small volumes (3 µL) of solutions (same as above) at the orifice of the chip. In this case, the volume of the solution is small enough that it stays in place. The holder can be designed in such a manner that the bottom platinum electrode is aligned and close enough to the orifice to allow contact with the 3 µL solution. The top platinum electrode can be position with a translation stage in the same manner as before ensuring that it is in contact with the 3 µL solution.

Fig. 1B depicts the radial chemical gradient of poly-D-lysine formed on the silicon chip centered on the orifice. It is evident from the intensity that there is greater concentration of poly-D-lysine near the center where the orifice is located, and that the concentration falls off gradually farther from the orifice. The diameter of the gradient is about 25 µm at half-height.

Example 2: Immobilization and culturing of cells on patch-clamp array chips prepared with a radial gradient of poly-D-lysine around an orifice

Primary rat cortical neurons were cultured on the orifices of silicon chips prepared with a radial gradient of poly-D-lysine as described in Example 1. The chips were stained with calcein (cell viability) and with RH-237 (a membrane stain) to detect where the cells were growing.

Thus, embryonic day E18 rat cortical neurons were harvested as a suspension with a stock density of 1.3×10^6 cells/ml in a growing medium that comprised EMEM supplemented with 21 mM glucose, 10% fetal bovine serum, and 10% horse serum.

5 Chips were sterilized for 30 minutes using 70% ethanol and subsequently transferred into a 24-well plate. Cells were plated by placing 500 μ l of the above stock cell suspension in each well. Cell cultivation was carried out in an incubator set at 37.3°C and 5.3% CO₂. Half of the culture media was replaced after one week of plating (7 DIV). After 14 days in culture, cells were stained as described below. Bath Medium (BM) was prepared as follows: 140 mM NaCl, 3.5 mM KCl, 0.4 mM KH₂PO₄, 20 mM HEPES, 5 mM
10 NaHCO₃, 1.2 mM MgSO₄, 1.3 mM CaCl₂, and 15 mM glucose. Calcein-AM (MolecularQ2 Probes, C-3100), was used as an indicator to access cell viability. A 5 mM stock solution was prepared in DMSO with vigorous vortexing. The stock solution was then diluted to a 40 μ M sub-stock in BM. Cells were subsequently stained using Calcein-AM at 5 μ M. A 10 mM stock solution of RH-237 was prepared and subsequently diluted to 50 μ M in BM for
15 cell staining. Each sample was placed in an individual well of a 24-well plate. 350 μ L of 5 mM calcein were placed in each well before incubation for 30 min at 37°C in the dark. Calcein was then replaced by the same volume of RH-237, followed by incubation for 10 min before rinsing to minimize nonspecific background fluorescence. Finally, 450 μ L BM was added to each well.

20 Fluorescence and reflection images of samples were obtained using an LSM-410 Zeiss confocal microscope equipped with a Krypton/Argon laser and a LSM objective inverter. For each dye, excitation wavelength and emission filter were appropriately selected and images of both dyes were collected sequentially. Calcein was excited with the 488 nm wavelength of the laser and an emission filter with a bandwidth of 515-540
25 nm was used. For the RH-237 conjugate, the excitation wavelength of 568 nm and a long-pass emission filter with a cut-off at 610 nm was used. Reflection images were collected using the 568 nm line of the laser with no filter in front of the photomultiplier tube (PMT). Reflection and fluorescence images of the same field were merged using Northern Eclipse software.

30 Fig. 2 depicts optical images A) of the cells on the chips, and fluorescent images of the stained cells where B) are calcein-stained cells and C) are RH-237-stained cells. The arrows indicate the location of orifices. It is evident from Fig. 2 that a cell is localized over the orifice, demonstrating the efficacy of the poly-D-lysine gradients prepared in the manner described in Example 1 for localizing cells cultured on the silicon chip. This
35 should permit monitoring the activity of individual cells engaged in network activity at the

high resolution when employing a multi-orifice patch-clamp chip. Chemical gradients of appropriate biomolecules can also be employed in axon guidance since they mimic molecular signaling necessary for forming neural networks. Asymmetric gradients are particularly useful in this regard.

5

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20 Other advantages that are inherent to the structure are obvious to one skilled in the art. The embodiments are described herein illustratively and are not meant to limit the scope of the invention as claimed. Variations of the foregoing embodiments within the scope of the disclosed invention will be evident to a person of ordinary skill and are intended by the inventor to be encompassed by the following claims.

Claims:

1. A method of producing a radial chemical gradient on a surface of a substrate, the method comprising: controllably forcing a predetermined amount of a chemical compound through an orifice in the substrate, the orifice connecting a first surface on which the
5 gradient is produced to a second surface of the substrate, the chemical compound being forced through the orifice in a direction from the second surface to the first surface; and, allowing the chemical compound to diffuse radially away from the orifice through which the compound is being forced, the compound reacting with the first surface to form a radial chemical gradient on the first surface disposed around the orifice, the gradient
10 having lower concentrations of the chemical compound at distances farther from the orifice.
2. The method according to claim 1, wherein the compound is controllably forced through the orifice by mechanical pumping or capillary action.
3. The method according to claim 1, wherein the compound is controllably forced
15 through the orifice by an electrophoretic or electroosmotic technique.
4. The method according to any one of claims 1 to 3, wherein the first surface is chemically modified with a functional group that reacts with the chemical compound.
5. The method according to claim 4, wherein the chemical compound comprises a cell adhesion promoter.
- 20 6. The method according to claim 4, wherein the chemical compound comprises poly-D-lysine, poly(ethyleneimine), collagen, fibronectin, laminin or a peptide containing a RGD motif.
7. The method according to claim 4, wherein the chemical compound comprises a peptide containing a RGD motif.
- 25 8. The method according to any one of claims 1 to 7, wherein the chemical gradient is shaped to form an asymmetric gradient by controlling direction of diffusion of the chemical compound on the first surface.
9. The method according to any one of claims 1 to 8, wherein the substrate is a silicon, glass or quartz chip.

10. The method according to any one of claims 1 to 9, wherein the substrate is a patch-clamp chip.
11. The method according to any one of claims 1 to 9, wherein the substrate is a patch-clamp array chip.
- 5 12. The method according to any one of claims 1 to 9, wherein the substrate is a multi-electrode array.
13. The method according to any one of claims 1 to 9, wherein the substrate is a neural implant.
14. The method according to any one of claims 1 to 13, further comprising
10 synthesizing a tissue on the substrate.
15. The method according to claim 14, further comprising implanting the tissue *in vivo*.
16. The method according to any one of claims 1 to 13, further comprising growing cells on the substrate.
17. The method according to claim 16, further comprising sorting the cells on the
15 substrate.
18. A substrate comprising a radial chemical gradient thereon produced by a method as defined in any one of claims 1 to 13.

1/3

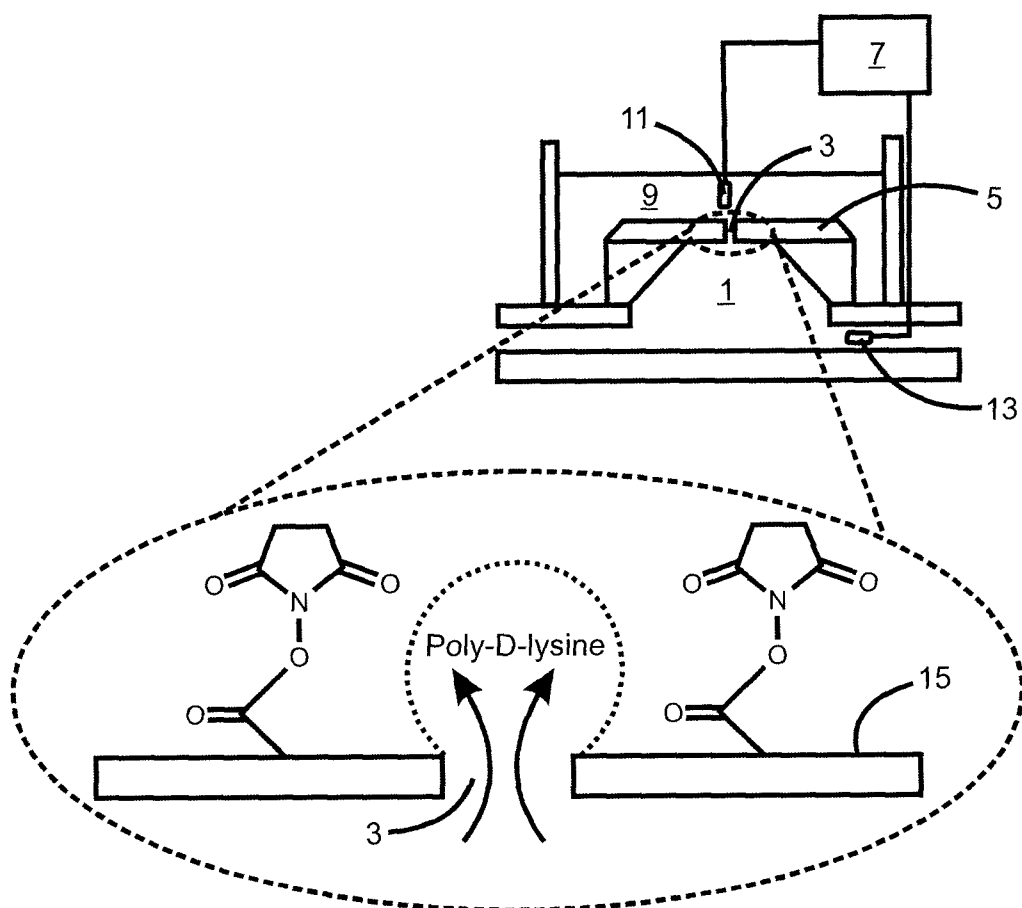


Fig. 1A

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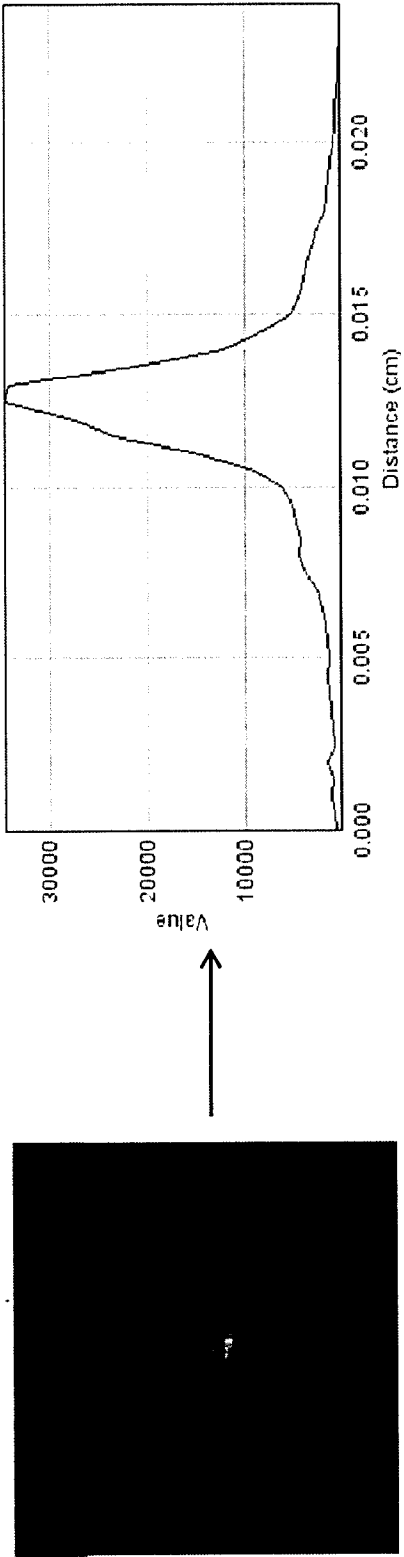


Fig. 1B

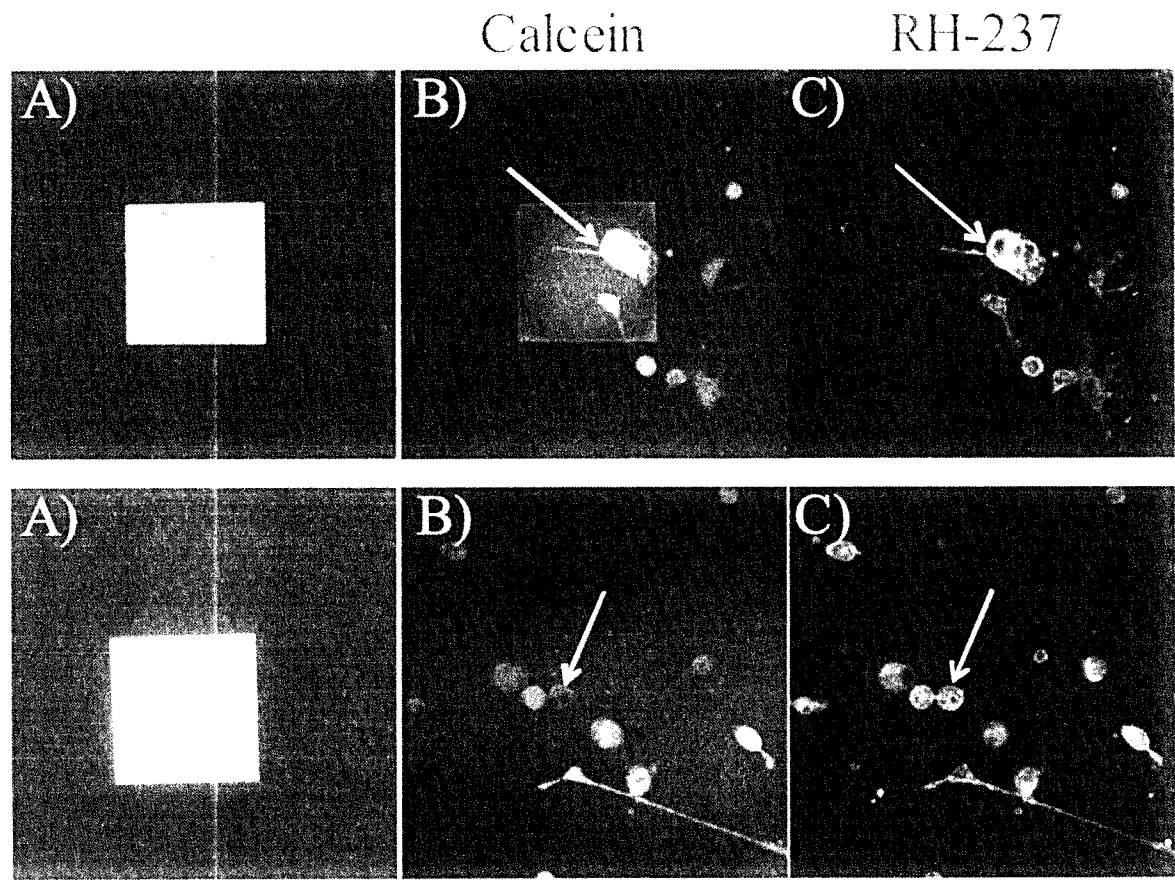


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2013/000716

A. CLASSIFICATION OF SUBJECT MATTER IPC: B01J 19/26 (2006.01) , A61L 27/38 (2006.01) , C07K 17/14 (2006.01) , C12N 5/0793 (2010.01) , C12N 11/00 (2006.01) According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) B01J 19/26 (2006.01), A61L 27/38 (2006.01), C07K 17/14 (2006.01), C12N 5/0793 (2010.01), C12N 11/00 (2006.01) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Canadian Patent Database, Total Patent, google (radial, gradient, orifice, patch clamp, surface, chemical)														
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>A</td> <td>US 2002/0164777 (Kelly et al.) 7 November 2002 (07-11-2002) * cited by applicant * * abstract; Figure 1; paragraphs [0002], [0004], [0011], [0013], [0017]; claims *</td> <td>1-18</td> </tr> <tr> <td>A</td> <td>US 2003/0015495 (Genzer et al.) 23 January 2003 (23-01-2003) * cited by applicant * * abstract; paragraphs [0002], [0006]-[0008], [0027], [0031]; claims *</td> <td>1-18</td> </tr> </tbody> </table>			Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	A	US 2002/0164777 (Kelly et al.) 7 November 2002 (07-11-2002) * cited by applicant * * abstract; Figure 1; paragraphs [0002], [0004], [0011], [0013], [0017]; claims *	1-18	A	US 2003/0015495 (Genzer et al.) 23 January 2003 (23-01-2003) * cited by applicant * * abstract; paragraphs [0002], [0006]-[0008], [0027], [0031]; claims *	1-18			
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A	US 2003/0015495 (Genzer et al.) 23 January 2003 (23-01-2003) * cited by applicant * * abstract; paragraphs [0002], [0006]-[0008], [0027], [0031]; claims *	1-18												
☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex. <table border="1"> <tbody> <tr> <td>* Special categories of cited documents :</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </tbody> </table>			* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means		"P" document published prior to the international filing date but later than the priority date claimed	
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Date of the actual completion of the international search 29 October 2013 (29-10-2013)		Date of mailing of the international search report 31 October 2013 (31-10-2013)												
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2013/000716

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