

Interfacing insect neuronal networks with microelectronic devices

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List of abbreviations

2-D	two dimensional
AFM	atomic force microscopy
α - HRP	Anti horseradish antibody
AGL	<i>Aplysia</i> gonad lectin
AP	action potential
APTES	Aminopropyltriethoxysilane
C	Celsius
CaCl ₂	calcium chloride
Calcein AM	calcein acetoxymethylester
CC	current clamp
CNS	central nervous system
conA	concanavalin A
DIC	Differential interference contrast
DNT	<i>Drosophila</i> neurotrophin family
ECM	extracellular matrix
EGTA	ethylene glycol tetraacetic acid
FET	field effect transistor
FITC	fluorescein isothiocyanate
Fuca1-3GlcNAc	fucosyl 1-3 N-acetylglucosamine
Fra	Frazzled
g	gram
GI	giant interneuron
G Ω	Giga Ohm
HCl	hydrogen chloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hz	Hertz
HRP	horseradish peroxidase
KAsp	potassium aspartate
kDa	kilodalton
kg	kilogram

KCl	potassium chloride
LI	local interneuron
LNI	local non-spiking interneuron
LPCVD	low-pressure chemical vapor deposition
LSI	local spiking interneuron
MEA	multi electrode array
MetOH	methanol
μ CP	microcontact printing
μ L	microliter
μ m	micrometer
MIMIC	micromolding in capillaries
mbar	millibar
MgCl ₂	magnesium chloride
min	minute(s)
mL	milliliter
mm	millimeter
mM	millimolar
mOsm	milliosmol
ms	millisecond
mV	millivolt
MW	molecular weight
N	Newton
nA	nanoampere
Na ₂ -ATP	sodium adenosintriphosphate
NaCl	sodium chloride
NaOH	sodium hydroxide
Nd	Neodymium
Net	Netrin
nm	nanometer
NSS	normal salt solution
ONO	oxide-nitride-oxide (SiO ₂ -Si ₃ N ₄ -SiO ₂)
p.A.	pro analysi
PD	polydispersity

PDMS	Polydimethylsiloxane
PEG	poly(ethylene glycol)
PEO	poly(ethylene oxide)
pH	pondus Hydrogenii
PI	projecting interneuron
PlexiA	Plexin A
PNA	peanut agglutinin
PO	polyoxide
PSP	postsynaptical potential
RIE	reactive ion etching
rpm	rounds per minute
SAI	small ascending interneuron
SDM	standard deviation of the mean
SEM	scanning electron microscopy
Sema1a	Semaphorin-1a
Si	silicon
SiO ₂	silicon dioxide
star PEG	star shaped poly(ethylene glycol)
TAG	terminal abdominal ganglion
UV	ultra violet
VV	voltage clamp
W	Watt

1 Motivation

Neuronal networks form the basis of perception, task performance and behavior in animal systems. Many research hours are spent exploring both simple and complex nervous systems due to their high efficiency compared to current computer systems. Coordinated, parallel processing of electrical activity combined with an enormous amount of specific intra-network connections makes the brain remarkably efficient and thus interesting not only for neuroscientists but also for engineers. Neuronal circuits are functional entities within the brain, consisting of afferent and efferent neurons as well as interneurons and all their synaptic connections. Studies of neuronal networks focus on elucidating single neuron function as well as mechanisms of network formation, function and maintenance in order to understand data processing and transfer.

Cell culture methods are a powerful tool to study neuronal circuits. Beyond all whole unit experimental approaches (like computer tomography, electric encephalograms and positron emission tomography) and *in vitro* methods using tissue slices, primary cell culture allows the study of isolated functional units without confounding side input and offers the advantage of a manageable amount of neurons. Experiments on small, defined networks are highly relevant in neuroscience since they provide a direct and sensitive gateway to individual cells within a functional network thereby facilitating insights on task significance of single neurons within a network. As network architecture and neuronal connectivity are crucial for formation and shaping of network activity (Muller *et al.*, 1997, Manor *et al.*, 1999) neuronal patterning is a worthwhile tool to reduce network complexity and extract basic mechanisms. To date, vertebrate neuronal networks of defined architecture with good pattern compliance allow the investigation of synaptic interconnection for weeks and even months (Potter and DeMarse, 2001). Predefining neuronal networks by controlling neuronal adherence of individual cells and outgrowth of neurites is fundamental in studies of neuron-neuron and neuron-surface interaction as well as network formation. The precise control of cell position and connectivity provides a means to mimic *in vitro* a neuronal circuit as part of the natural tissue in order to

study the basic information in- and output as well as the processing steps. Due to their reduced complexity, isolated functional units like engineered neuronal networks facilitate the extraction of learning algorithms that natural systems are based on. The aim of such studies is to understand signal processing of neurons within a network by inducing adherence of single neurons to the substrate, guiding neurite outgrowth and thereby controlling cell-cell interaction by cellular patterning. The gained knowledge is valuable for biosensor applications as well as nerve prostheses or neurally-inspired computer algorithms (Wheeler, 2002).

Because of the high level of complexity in the nervous system, it is reasonable to study simpler model systems when investigating neuronal mechanisms. In invertebrates such as insects, the neuronal networks are extraordinarily efficient while being relatively simple. Insect models have been proven useful in a number of fields, e.g. adult neurogenesis (Cayre *et al.*, 2007) and genetics (Rubin *et al.*, 2000). Furthermore, because of the large neuron size (up to 100 μm) and the small amount of contributing neurons, these neurons can be isolated easily and offer the possibility to extract a functional network and rebuilt it *in vitro* as has been done for the mollusc *Helisoma trivolvis* (Syed *et al.*, 1993). Within the animal kingdom, insects have evolved particularly well-adapted sensory systems based on straight-forward neuronal circuits consisting of only a few specialized neuron types. Consequently, mimicking these circuitries could yield new information processing technologies and attempts to recreate these networks could provide fundamental information about network dynamics and developmental events along the way.

One example for a very effective natural mechanosensor is the cricket cercal somatosensory system. A specialized circuit of neurons in the terminal abdominal ganglion (TAG) of the cricket controls its highly sensitive, hair-based air flow-evoked escape reaction. The stimuli perceived by filiform hairs on the cerci are processed by interneurons in the TAG (Gras and Kohstall, 1998). Neurons from the TAG are large in diameter, easily accessible and highly specialized. The system has an easily traceable read-out system (one sensory neuron innervates one cercal hair [Paydar, 1999]) and neurons within a network are clearly arranged and individually retrievable in different animals. Each wind-sensitive giant interneuron displays an individual response type, threshold velocity and directional specificity (Bodnar *et al.*, 1991, Matsuura and Kanou, 2003, Kanou *et al.*, 2006, Jacobs *et al.*, 2008). To ensure an adequate behavioral reaction, the parameter of air currents such as direction and dynamics are encoded as firing rates of specialized interneurons such that within the TAG the sensory information is represented in functional maps as specific patterns of activity. Because of this extremely efficient information processing within this circuit, the cricket is able to detect air movements as small as thermal noise (Shimozawa *et al.*, 2003) and thus reliably distinguish between stimuli actuated by a

predator or wind. Investigating details of the information processing of this network is part of the EU project CILIA (Customized Intelligent Life-inspired Arrays) that deals with naturally inspired hair-based sensory systems. The cricket cercal system was chosen as the subject of the present work because of its high sensitivity and efficiency. The information processes of the circuits within the TAG ensure a precise and unambiguous stimulus coding and present thus an ideal model system for information processing as for instance solving details of signal processing of an engineered biosensor.

The requirements to implement such a system are tools to create defined functional networks and a read out system that allows for non-invasive long term recordings of neuronal networks. The field of neuroengineering using extracellular recording devices represents a powerful enhancement to classical cell culture experiments and offers the ability to gain important insights into network formation and dynamics. Extracellular recording techniques with field effect transistor arrays (FET) or multi electrode arrays (MEA) provide both an important tool to profit from the isolated and thus easily accessible individual neurons obtained by dissociating tissue and the possibility to study a neuronal population simultaneously over a very large time scale. Built by photolithographical means, considering electrical properties in the first range, microelectronic devices do not provide surface requirements for neuronal growth. Depending on the cell type, the requirements are specific biomolecules or protein patterns in order to gain control over network architecture. Because of the heterogeneous material components on the surface, one of the core challenges in the design of biosensors and biohybrid devices is the development of universal strategies for covalently attaching biomolecules to solid-state components (Medintz, 2006). Cell patterning, as introduced in this study, provides a means for a one-to-one neuron-electrode interfacing.

The present study describes a reliable method for the control and guidance of insect neuronal adherence and outgrowth in order to form simple model networks and selectively extract read-out information. For this purpose, a special surface coating of adherent binding sites in a spatially controlled, non-fouling star PEG background on glass surfaces has been developed. Maintaining the original physiological role of a single neuron within its functional unit is crucial when studying and reconstructing neuronal networks *in vitro*. Therefore, morphological and electrophysiological properties of pre-patterned neurons were compared to neurons grown on uniformly coated surfaces as part of this study. Detailed experiments of pre-patterned networks of cricket neurons from the TAG showed a strong morphological dependency on the patterned cues resulting in a shift to a more physiologically relevant morphological phenotype. This important finding facilitates the recreation of morphologically appropriate *in vivo* networks retaining at the same time basic electrophysiological properties. Non invasive investigation

methods as recording and stimulation with microelectronic devices are an important upgrade in neuronal network studies but still lack of reliable techniques to be interfaced with insect neuronal networks. For that reason, the present study includes a modification of the established star PEG coating method (see chapter 3 materials&methods) that was introduced to provide a technique for predefining neuronal networks on microelectronic devices.

Biomimetic systems such as bio-inspired sensors can profit from a thorough understanding of natural sensory systems like the cricket cercal system. Investigating sensory circuits not only leads to comprehension of their functional mode but more importantly provides a fundamental understanding of the extraordinarily efficient mechanism of information processing within the respective neuronal systems. The gained knowledge can be applied to increase signal-to-noise ratio in sensory prosthetic devices such as cochlea implants and help in the design of new neuron-based bio-arrays for pharmacological tools such as biochips for diagnostics and metabolism signaling.

2 Introduction

The mammalian brain is a unique structure that still leaves us with a lot of open questions after over a century of research. As basic mechanisms of neuronal activity and network dynamics are understood, they can yield new insights to the entire system. Although various basic principles have been decrypted, as new insights are discovered even more questions are added. In order to understand how the brain implements specific behavioral patterns and to identify the respective microcircuit and the individual responsible components, neuroscientists are trying to understand and integrate studies at all the relevant experimental levels including cellular, molecular studies and whole brain techniques. Different approaches exist to investigate brain function and neuronal network dynamics of both vertebrates and invertebrates. As for vertebrates, brain function and dysfunction mostly has exploited non-invasive use of functional imaging methods like positron emission tomography, functional magnetic resonance imaging, computer tomography, and electro-encephalography (Tracey *et al.*, 2007, Logothetis *et al.*, 2008, Horwitz and Smith *et al.*, 2008, Maddock *et al.*, 1999, Fletcher and Henson, 2001) or anatomical scanning electron microscopy studies like the study on the neural network on the basal ganglia synaptic connections (Smith and Bolam, 1990). However, detailed analysis revealing how neurons accomplish behavioral tasks of neuronal network intersignaling at the level of ionic currents, transmitters, and synapses is complicated or impossible in *in vivo* or *in situ* approaches.

Important basic principles governing biological behavior discovered in invertebrates were often found to be conserved and thus also found in vertebrates. For instance, the central pattern generator, first discovered in the crayfish (Hughes and Wiersma, 1960) and later on in the locust (Wilson, 1961), was also found in vertebrates (Grillner *et al.*, 2005). Electrical synapses between neurons that were first studied in crustacea (Zucker, 1972), are now known to exist in the mammalian cortex (Bennett, 1997). Studies of the vertebrate neuronal microcircuits are

technically challenging because of its high complexity and small neuron size, hence to date very few vertebrate microcircuits are understood.

2.1.1 Invertebrate neuronal networks

Invertebrate neuronal circuits, in contrast, are much simpler in various aspects making interpretation of their behavior more direct. These circuits are clearly arranged, possess specified cell types that can be uniquely identified from animal to animal and the total amount of neurons is smaller than in the vertebrate nervous system. Consequently, studies on invertebrate nervous systems offer a shortcut in fundamental studies of neuroscience and the gained knowledge can amend and ease studies on vertebrate systems. Reconstructing an isolated circuit *in vitro* allows addressing basic neurophysiological questions. Entire neuronal circuits and their individual components that control specific behavior patterns can be investigated by means of electrophysiology and pharmacology down to the single channel level. Several of such studies have already been performed on molluscs. By partially reconstructing the gill withdrawal reflex neuronal circuit of *Aplysia californica* *in vitro*, precise studies of habituation and sensitization were performed showing short-term homosynaptic depression and heterosynaptic facilitation in cell culture (Rayport and Schacher, 1986). Syed *et al.* (1993) identified a central neuronal network in *Helisoma trivolvis* consisting of aminergic and peptidergic interneurons and their follower cells *in vitro*. Because of their proved efficiency from the computational point of view those mechanisms have been conserved during evolution. Often, basic mechanisms of invertebrates and vertebrates are based on similar principles. Short-term homosynaptic depression was also found in neonatal rat spinal cord (Levtov and Pinco, 1992). Various insect behavioral networks are known to consist of a similarly manageable amount of neurons. The neuronal motor control of the locust jump was well identified by (Heitler and Burrows, 1977) and the network causing oviduct contraction of *Drosophila* has been refined by Rodriguez-Valentin *et al.* (2006). Such networks can be reconstructed and further investigated *in vitro*.

Primary cell cultures on the insect nervous system are well established. The insect central nervous system consists of ganglia with neuronal somata in the cortices that can be easily identified, removed and dissociated. The inner neuropil contains dendritic connections; paired connectives guide axon bundles between adjacent ganglia and nerves with efferents to the periphery and afferents to the CNS (Burrows, 1996). Five classes of neurons are existent within the ganglion: motor neurons, sensory neurons, local interneurons, intersegmental (projecting) interneurons, neurosecretory neurons and several glia cell types. Some of these cells can be easily identified due to their large size and precise position within the respective tissue.

2.1.2 Cricket cercal sensory system

Crickets have evolved a very complex sensory system that allows the detection of air movements in the direct surroundings. This sensory system consists of about 2000 densely packed mechano-sensory filiform hairs arranged on two cone-shaped abdominal cerci. In order to react to the respective stimuli the cercal hairs pivot in viscoelastic sockets. By force magnitude and direction applied by the air movement, the hairs are deflected with air velocities as little as 0.03 mm/s (Shimozawa *et al.*, 2003). Each hair projects its information via a sensory afferent neuron at the base of a hair cell (Gnatzy and Schmidt, 1971) to neurons of the terminal abdominal ganglion (Heusslein and Gnatzy, 1987). Numerous hair receptors innervate pairs of interneurons that are ascending to the thoracic ganglia and the brain. The afferents are monosynaptically connected to these interneurons using the neurotransmitter Acetylcholine (Ogawa, 2006). Within the TAG, the perceived air stimuli information is pre-processed in simple circuitries before it is forwarded to higher centers of the central nervous system as the thoracic and cephalic ganglia where the behavior of the cricket is controlled. Neurons of those ganglia are responsible for the coordination of avoidance and escape movements (Gras and Kohstall, 1998). The processed information results in escape motion to the opposite direction of the wind stimuli. Liebenthal *et al.* (1994) and Levi and Camhi (1995) propose that the resulting turning direction is determined by a comparison of total spike activity in specific neurons of the left side versus the activity in those neurons of the right side of the nerve cord. The ascending interneurons in the mesothoracic ganglion have a feedback function from the different motor centers as well as the notification of extero and entero receptor information of the body to the CNS. The respective circuitries within the TAG consist of local (LI) and projecting (PI) interneurons out of which the largest called giant interneurons (GI) are well studied (Edwards and Palka, 1974, Mendenhall and Murphey, 1974, Kanou *et al.*, 2004). The large diameter of their axons ensures a fast information transfer to higher centers of the CNS. Many GIs project intersegmentally and represent the key elements of the communication and processing of neuronal information between associative centers of the brain and the sensory and motor centers of the thoracic ganglia (Schoch *et al.*, 2005).

The cricket TAG consists of about 2000 neurons out of which 170-230 pairs of wind sensitive LIs (Baba *et al.*, 1995) and about seven pairs of wind sensitive GIs (Shen, 1983). Each wind-sensitive giant interneuron displays an individual response type, threshold velocity and directional specificity studied in detail in *Gryllus bimaculatus*. The nomenclature of the respective GI refers to its segmental origin (Jacobs and Murphey, 1987) as the TAG results from a fusion of five abdominal ganglia. GI 9-1 belongs to the phasic response type, firing 1 to 3 action potentials, GIs 8-1, 9-2, 9-3 are phasic-tonic, responding phasically to a weak stimulus but firing bursts to a

more intense stimulus. Finally, GIs 10-2 and 10-3 are called tonic type, as the response is always a burst independent on the stimulus strength (Kanou and Shimozawa, 1984, Kämper and Vedenina, 1998).

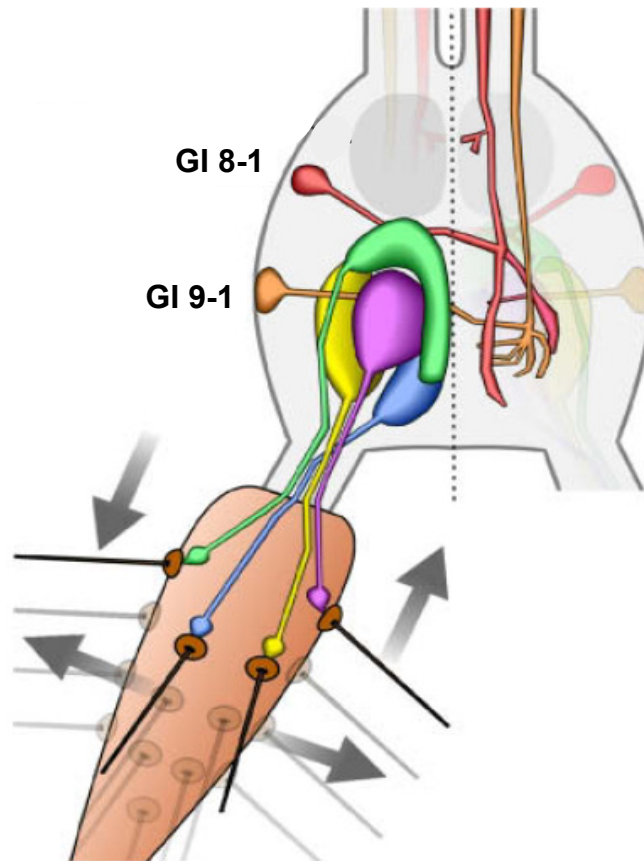


Fig.2.1. Schematic drawing of cricket cercal system illustrating the basis of neuronal selectivity (from Blagburn *et al.*, 2004). Four afferent neurons (blue, green, yellow, purple) shown exemplary with four specific wind directions and their respective projection fields within the neuropil of the terminal abdominal ganglion. Giant interneurons (GI 8-1 and GI 9-1) project contralaterally into a specific neuropil region.

Different LI types (at least 27) have been identified receiving cercal input, directly or indirectly, that transmit excitatory output to giant interneurons (GIs) and small ascending interneurons (SAIs) (Bodnar *et al.*, 1991, Bodnar, 1993, Baba *et al.*, 1995, Gras and Kohstall, 1998). Some of these neurons are non-spiking (LNI) displaying only graduated potentials without forming action potentials, even upon experimental stimulation, whereas some are spiking (LSI), both spontaneously and during wind stimulation. LNIs supposedly transfer information divergently to many GIs resulting in the influence of one LNI on several GIs depending on its synaptic strength simultaneously (Gras and Kohstall, 1998) (see Fig.2.2). LNIs possess larger soma

diameter and higher membrane potential (-45 ± 5 mV for LSI and -40 ± 5 mV for LSI). Most of the LSIs fire spikes spontaneously with amplitudes of 10-15 mV and frequencies of 9-30 Hz. LNIs and LSIs are probably wired in inhibitory pathways (Baba *et al.*, 1995) to sharpen the direction specificity of the downstream GI. Wind-sensitive interneurons were also found in the thoracic ganglion but these cells seem to play a minor role in motoric escape control (Hörner *et al.*, 1989).

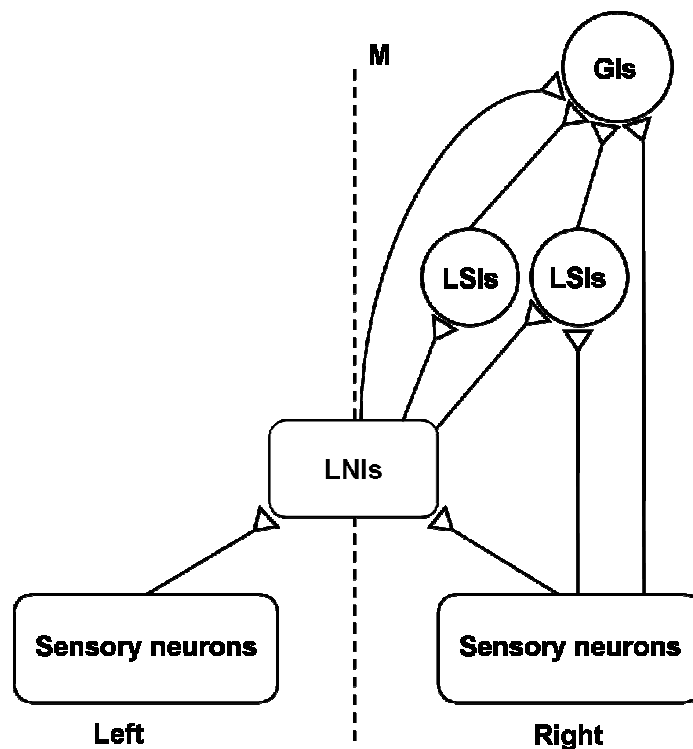


Fig.2.2. Model of the cercal sensory neuronal system. Sensory neurons project to LSIs (local spiking interneurons) and LNIs (local non spiking interneurons) that in turn project divergently to several LSIs. GIs (giant interneurons) integrate information from many LSIs. M : midline (Baba *et al.*, 1995).

Yono *et al.* found evidence that synchronous firing of specific pairs of giant interneurons that encode the wind direction ensure a more precise response due to their excitatory and inhibitory input from the same cercal sensory neurons. Thus, a specialized neuron functions potentially as a coincidence detector to encode the direction of the wind stimulus (Yono and Shimozawa, 2008).

The results of the neuronal circuit of the crickets escape reaction described above were gained from studies on the whole ganglion. From these studies important insights were won on the escape behavior of the cricket and on the different neuron types involved and their stimuli specificity as well as the receptive fields within the neuropils. To date, only one study on neurons that take part in the escape behavior of the cricket in cell culture has been published.

Kloppenborg and Hörner (1998) performed electrophysiological studies of identified neurons at the single cell level, however, without studying the interconnectivity of isolated functional networks. Hence, studies covering the whole system function yet need to be completed with data about neuronal networks on cellular level. Therefore, precise knowledge about neuronal adhesion, neurotransmitter specificity, ion channel distribution, specific excitatory and inhibitory connections and synaptic strength are missing. Network development, architecture and dynamics, in particular, remain to be elucidated.

2.1.3 Morphology of invertebrate neurons *in vivo* and *in vitro*

The majority of insect neurons *in vivo* are unipolar (Strausfeld, 1976, Laurent, 1999). However, this status is prevalently changed under cell culture conditions. Nevertheless, especially brain neurons seem to follow a strong intrinsic program and retain their *in vivo*-morphology, as for instance mushroom body neurons from *Acheta domesticus* (Cayre *et al.*, 1999) while other types are more influenceable by extracellular guiding cues, as neurons from terminal abdominal ganglion of *Gryllus bimaculatus* (see chapter 5). Uniformly coated cell culture surfaces, as classically used, are lacking specific adhesion cues and gradients establishing a guided pathway for growth cones as *in vivo*.

During natural development of a neuron a common branch sprouts from the neuronal cell body and splits subsequently into axon and dendrite. This process is similar to vertebrate neurons, where the axon also builds first, but both compartments arise independently from the cell body. Axon and dendrites in insects seem to share a gross of guiding molecules and the specific pathfinding is caused rather by temporal differences guidance molecule expression or variable trafficking of guidance molecules to the relevant compartments (Grueber and Jan, 2004). The growth cones possess sensory and motoric capabilities in order to trace the target tissue. The parameters influencing growth cone development are specific membrane cell adhesion proteins and receptors, as well as neurotransmitters that interact with special receptors and target cell activity. Action potentials, for instance were found to inhibit neurite outgrowth (Cohan and Kater, 1986). The entire growth process is regulated by calcium. It has been shown, that the Ca^{2+} -concentration in growing neurites is more than two times higher than in stable-state growth cones (Kater *et al.*, 1988).

Enzyme and mechanical treatment during cell isolation as well as the tissue type and age influence the neuritogenesis of dissociated neuronal cell cultures. Interaction with glial cells and the degree of axotomy also shape neurite formation. A remained axonal stump typically leads to reduced neurite growth in leech neurons (Schacher and Proshansky, 1983). For Retzius neurons in a leech preparation the critical factor for neurite shaping *in vitro* is the protein coated on the

cell culture substrate (Masudanakagawa and Wiedemann, 1992). Membrane contacts between neighboring cells also proved to enhance neurite outgrowth (Kirchhof and Bicker, 1992). Also, the admixture of specific additives has an impact on some neurons. Ecdysone, a steroidal molting hormone increases branching in cultured embryonic neurons of *Manduca sexta* (Oland and Hayashi, 1993). In a similar manner, the addition of 100 nM *Aplysia* gonad lectin (AGL) to *Aplysia* neurons in cell culture increases the number of neurites growing from the cell soma (Wilson *et al.*, 1992).

2.1.4 Primary cell culture

Cell culture approaches to investigate single cell signaling are valuable information sources to complete gaps in neurophysiological studies in reducing network complexity. Back in the 19th century the first cell culture was performed by Wilhelm Roux who extracted a portion of the medullary plate of an embryonic chicken (Alberts, 2008). Harrison established the first tissue culture in 1910 (Lees *et al.*, 1985). Since then, cell culture techniques have been refined and modern patch-clamp approaches combined with biochemical and imaging techniques helped to advance e.g. in drug screening, cancer research and genetic disorders.

Eckmann (2007) claims that the status of *in vitro* neuronal networks being decidedly different from *in situ*, enhances the interest of physicist because simplistic structure-function relations can be modeled. Isolated neuronal networks have the advantage of a controllable surrounding, where the environment can be kept constant or changed according to experimental requirements, e.g. addition of neuroactive substances, channel blockers, modified surfaces, and the organization of neuronal connections.

Distinguishing and dissociating the respective neurons are prerequisites for *in vitro* network formation. Likewise, the environment and physiological conditions need to be considered. A clear understanding of function of surface and soluble factors contributing to cell adhesion and neuritogenesis is central to the study of network formation and designing experiments of reconstructed networks.

2.1.5 Neurogenesis: adhesion molecules and neurotrophic factors

Adhesion of the cellular somata is the first step towards network formation as neurons are not vital without a permissive matrix. Likewise, within the vertebrate nervous system, chemoattractive and chemorepellent molecules serve insect neurons as guidance cues for axons and dendrites steering them toward their synaptic targets during developmental stages and ensure maintenance during adulthood. The growth cone guiding cues are highly conserved in

such diverse organisms as insects and vertebrates. The target recognition during axon and dendrite pathfinding is relaying on specific protein-receptor recognition. As all neural cell adhesion proteins are glycosylated (Matani *et al.*, 2007), glycan expression of a neuron functions as cell-type specific identity marker. Sugar recognition proteins are called lectins; each lectin binds specifically to oligosaccharide chains. Peanut agglutinin (PNA) for instance, binds to β -D-galactose, which is apparently lacking in glia cells, because PNA does not bind to glia. The highest concentration was found in neurite-neurite junctions. In *Drosophila*, PNA binds also to laminin (Montell and Goodman, 1988). In leech studies, ECM proteins that bind to PNA promotes bipolar outgrowth by inhibiting the sprouting of further neurites from the soma (De-Miguel *et al.*, 2002).

ECM molecules like laminin, netrin and semaphorin induce specific contact formation and spreading of neuronal processes in the invertebrate CNS. Other molecules like tenascin (Faissner *et al.*, 1990) are known to repel adhesion and/or neurite formation. Often ECM molecules can function as both, mediators and inhibitors in different tissues or in different developmental stages. A lot of knowledge about insect ECM and adhesion signaling was first discovered with genetic studies on *Drosophila melanogaster*. As most molecules involved in cell guidance are evolutionarily highly conserved (Dickson, 2002) and often were found in mammals after discovery in *Drosophila*, scientists assume their existence in other insect species. However, not for every protein it has been shown yet, whether it is existent in all insects. Laminin critically contributes to cell adhesion, cell morphology and cell-cell interaction in both, vertebrates and invertebrates. As a substrate adhesion molecule it promotes neurite outgrowth by targeting laminin molecules on adjacent cells and integrin receptors.

Semaphorin mediates and inhibits axon guidance. It occurs in secreted and membrane-bound form, and is known to cause collapsing of growth cones (Chilton, 2006). The transmembrane-protein Semaphorin-1a (Sema1a) has been shown to be involved in defasciculation of motor axon bundles in *Drosophila*. Sema1a binds to the receptor Plexin A (PlexA). Sema1a is also involved in synaptic formation (Cafferty *et al.*, 2006). A prominent example for an axon attraction mediating complex in *Drosophila* is the Netrin (Net)-Frazzled (Fra) signaling system. But netrins can act even so as repellent molecules e.g. for trochlear motor neurons in *Drosophila* (Winberg *et al.*, 1998). Slit has been identified as a repellent protein in the midline of *Drosophila* interacting with the repulsive guidance receptor Roundabout (Robo) which is also existent in mammals (Kim and Chiba, 2004). Robo is an important guidance receptor for both, dendrites and axons. Fasciclin I and II are expressed on specific subsets of axon pathways in grasshopper embryos (Bastiani *et al.*, 1987) during development. They might play an important role in selective fasciculation. It has been shown that Fasciclin II influences the pattern of synapse

formation in stabilizing growth cone contacts Winberg *et al.* (1998). They are good candidates for specific adhesion and/ or recognition molecules.

As recognition sequence vertebrates possess a specific carbohydrate moiety (L2/HNK-1) that is present on numerous neuronal surface glycoproteins. Similarly, one group of surface glycoproteins is sharing a neural-specific carbohydrate epitope in *Drosophila* and the grasshopper *Schistocerca nitens*. This epitope is fucosyl 1-3 N-acetylglucosamine (Fuca1-3GlcNAc) that is also present in plant glycoproteins as horseradish peroxidase (HRP). Anti-HRP antibodies (α -HRP) show that Fuca1-3GlcNAc is mainly found in neuronal tissue in a wide range of arthropods (Altmann *et al.*, 1999). On the neuronal membrane of grasshopper and *Drosophila* at least 17 different glycoproteins on the membrane are bound by α -HRP antibody (Snow *et al.*, 1987). Interestingly, most of the proteins bound by α -HRP are also bound by concanavalin A (conA), a plant lectin that is commonly used for substrate coatings of arthropod cell cultures as also in the present study; the epitope is probably the same carbohydrate. As it binds to such a variety of glycoproteins, using conA as coating protein it is assured, that no specific cell type is excluded from adhesion (by not offering the appropriate substrate). Furthermore, Aoki *et al.* (2007) demonstrated fucosylated, sialylated, hybrid, biantennary complex, and triantennary complex glycans on *Drosophila* embryonic neuronal membrane. This finding implies that arthropod and mammal glycobiology have more in common than expected (Varki *et al.*, 2008).

Neurotrophins are the main vertebrate secreted signaling molecules. They act during development, maintenance, and function of vertebrate nervous systems. They regulate axon growth, dendrite pruning, innervation patterning and protein expression, e.g. neurotransmitters and ion channels, but also cell apoptosis. In the mature nervous system, they control synaptic function and synaptic plasticity (for review see Huang and Reichardt, 2001). Neurotrophic interactions have been known to occur in *Drosophila*, but only recently a *Drosophila* neurotrophin family (DNT) has been identified (Zhu *et al.*, 2008). They are structurally related to all known neurotrophins and are highly conserved in insects. Zhu *et al.* presume that neurotrophins have been present in the common ancestor of all bilateral organisms.

2.1.6 Network development

For neuronal network studies and designing neuronal development, controlling synaptogenesis is crucial. Despite of being a field of high interest for neurobiologists, it has just begun to be elucidated. However, first insights are attained. In invertebrates electrical synapses are more

common than chemical. It has been shown with *in vitro* approaches that chemical synapses form preferentially, when neurites extend asynchronously and is only possible when neurites overlap while extending and both partners grow actively (Hadley *et al.*, 1983). In addition to these requirements also specific interactions on the molecular level are crucial for neuron-neuron connectivity. The interplay between adhesion molecules during network formation is very complex. Winberg *et al.* (1998) describe that the relative balance of Semaphorin II and Fasciclin II controls synaptogenesis and the relative balance of Semaphorin II and Netrins is crucial for target specificity. Finally, the specificity with which synaptic connections are formed between two neurons is dependent on their electrical activity and the synaptic receptors presented on the membrane.

Furthermore, dendritic morphology dramatically affects single cell electrophysiological response (Ascoli, 1999) and thus the network dynamics. Various publications have addressed the question why the neuronal dendritic structure is so highly branched. The complicated tree-like dendritic structure could be acting as a delay line consequently serving as a coincidence detector and for precise time computations (Ascoli, 1999).

Cuntz *et al.* also state that a single neurons specific dendritic shape strongly influences the networks neuronal computation. From their anatomical analysis of identified neurons of the fly visual system they draw the conclusion that all dendrites share the same growth program but the specific identity of a neuron is defined by the dendrite spanning field (Cuntz *et al.*, 2008). Spiking behavior of a neuron seems to depend of the ratio of somatic surface to dendritic surface (Ascoli, 1999, Larkman and Mason, 1990).

Extracellular recordings with multi electrode (MEA) and field effect transistor (FET) arrays proved to be important supplemental tools for *in vitro* studies of electrogenic cells. Non-invasive recordings with microelectronic devices add a multitude of new applications and experiment designs. The advantages are parallel recording on multi network or multi cell sites with a high spatial resolution, precise experimental control of the cellular microenvironment and lower reagent volumes compared to classical cell culture. Many interesting studies on network dynamics and synchronous firing have been published e.g. by Wheeler and coworkers (Chang 2001) and other groups (Li *et al.*, 2005, Chen *et al.*, 2006, Chiappalone *et al.*, 2007, Stegenga *et al.*, 2008, Berdondini *et al.*, 2009). However, the application of microelectronic devices also adds requirements, as the neurons must adhere close to the relevant electrical read-out site.

2.1.7 Cellular patterning

Studies on randomly formed neuronal networks from dissociated cells *in vitro* have helped to understand neuronal population dynamics such as for instance the maturation of synchronous firing behavior. In this case, input from outside the network of interest has to be silenced by drugs, all of which have various side effects (Varela *et al.*, 1999). It was only in 1975 that Letourneau developed a technique to pattern cells (Letourneau, 1975).

As the natural cytoarchitecture is lost during the dissociation of neurons, reconstruction of basic connection pattern is needed (Folch and Toner, 2000). The precise control of the architecture of multiple cells in culture and *in vivo* via precise engineering of the material surface properties is described as cell patterning (Jung *et al.*, 2001). The phenomenon of cells to orient in response to the underlying topography is commonly known as contact guidance (Singhvi *et al.*, 1994b), here it will be referred to as cellular patterning. Defined cell architecture can be achieved directly by positioning single cells and control their outgrowth by means of physical confinement to microconstructed wells and channels (Yap and Zhang, 2007) or “neurocages” (Tooker *et al.*, 2005). Cell position can also be manipulated by optical force as laser tweezers (Ashkin *et al.*, 1987) or by their adjustment to electrical field strength in nonuniform electric fields in dielectrophoretic approaches (Fiedler *et al.*, 1998).

However, classically cell patterning techniques have in common seeking to form a more specific interaction between surface molecules and cell membrane receptors to control cell position and shape, and eventually network formation for long-term studies. Chemical methods and clean room technology are used to create defined surface topology and/or chemistry. Surface structures can be formed using photolithographical (Scotchford *et al.*, 2003), softlithographical methods (Kane *et al.*, 1999, Whitesides *et al.*, 2001), and click-chemistry (Crescenzi *et al.*, 2007) that refers to simple reactions to generate surface coatings quickly from single units. The common principle of most techniques is creating patterns of cytophilic versus cytophobic surface. The application of the respective cytophilic or cytophobic substrate can predicate on specific membrane receptor / ECM molecule binding, electrostatic forces, or hydrophilic interaction.

Starting with a plain surface as glass or silicon and building an appropriate environment is considered as bottom up approach, whereas also top-down approaches have been introduced e.g. patterning using stencils (i.e. thin piece of material containing hollows) that were first used for selective metal vapor deposition. It can be applied to either add or remove material to/from the unprotected surface. Supplementary, it was also used for selective cell seeding (Jimbo *et al.*, 1993).

Photolithography was originally developed for semiconductor fabrication. A photoresist is applied onto the surface via spin-coating and locally exposed to UV light using a SiO₂/chromium mask. In a positive approach the photoresist regions become soluble upon light exposure and can be removed in the next step. In the negative approach the photoresist hardens and results in an image negative to the mask. This approach has been used for cell patterning for instance with two metaloxides (Scotchford *et al.*, 2003) as well as with hydrophilic aminosilane permissive for cell adhesion versus hydrophobic alkylsilane, repellent to cell adhesion (Healy *et al.*, 1996). This method is simple and the mask can be reused frequently. The limits of this technique are the high feature resolution (1-2 µm) and the usage of non organic surfaces. But reaching the aim of a specific cell-surface interaction by combining this method with protein coating would require an exposure of the protein to an organic solvent that is used to remove the unhardened photoresist. Nevertheless, photolithography is crucial for most approaches in cell patterning as it is forming the prerequisite for softlithography being used for creating topographical masters for molding.

The most prevalent softlithographical technique is microcontact printing, where bio-ink (mostly proteins) is stamped to the surface. The stamp is molded from a patterned Si-wafer typically with a thermocurable polymer like for example polydimethylsiloxane (PDMS). The polymer needs to be hardened by thermo- or photoreaction and can be peeled off. After incubation of the stamp with bio-ink, it is pressed for a defined time and with a defined pressure to the substrate, resulting in adsorption of the ink to the surface. Another softlithographical approach is called micromolding in capillaries (MIMIC) where elastomeric stamp with relief features (channels) is positioned on a plain or pre-treated surface. The channels allow for solution flow between the respective structures by capillary action. The material (e.g. protein) in the solution cross-links, crystallizes, cures, adheres, or deposits onto the surface of the substrate during a defined incubation time and thereafter the elastomeric stamp can be removed (Kim *et al.*, 1996). MIMIC can be used to form protein gradients on the surface, as for instance laminin for neuron growth (Dertinger *et al.*, 2002) and similarly for direct cell patterning. Populations of cells are flown into culture chamber in parallel streams and deposit selectively along streamlines which are controlled by channel geometry and flow rate.

To create a more stable pattern for long-term studies, proteins can be bound to solid surfaces covalently in contrast to non-covalent adsorption (van der Waals interactions, hydrogen bonding or electrostatic forces) (Yap and Zhang, 2007). Covalent linking is typically performed with bifunctional crosslinkers as silanes or silica-based linkers with a variable functional group at the opposed end. Kleinfeld *et al.* (1988) and Britland *et al.* (1992) used photochemical resist materials and lithographic masking techniques compatible with the silane chemistry and attached proteins pattern-wise.

A chemical surface approach which prevents unspecific cell and protein surface interaction is used typically with either poly(ethylene glycol) (Revzin *et al.*, 2003) or e.g. alkenethiols (Chen *et al.*, 1998) as cell aversive substrate. Also poly(methyl methacrylate) (PMMA) has been shown to resist protein adhesion (Woerz *et al.*, 2007). Several other molecules as carbohydrates (e.g. albumin, agarose, mannitol) (Falconnet *et al.*, 2006), acetat, paraffin, sulfonate-terminated alkylsilane self-assembled monolayers, poly(vinyl alcohol) and fluocarbon polymers (Folch and Toner, 2000) have been proposed to prevent protein adsorption and thus cell adhesion. There is a huge amount of variations to apply those molecules. Using gold-thiol binding, self-assembly of PEG-terminated alkanethiols was realized on gold combined with microcontact printing by Xia and Whitesides (1998) and Singhvi *et al.*, (1994a). Subsequently, applied self assembled monolayers can be patterned by photolithography, laser ablation, UV irradiation, electron beams, ion beams and scanning probe microscopes (Folch and Toner, 2000). Like cells, proteins and other molecules can be positioned single-wise onto surfaces. Dip pen nanolithography is an approach, where an atomic force microscope tip is used to transfer molecules to a surface via a solvent meniscus with a high feature resolution (Lee *et al.*, 2002). Commercial ink-jet printers were custom-changed and used to deposit alkanethiols onto gold substrates and different proteins onto silica supports (Pardo *et al.*, 2003). Ink-jet printing was even used to print bacterial colony arrays (Roth *et al.*, 2004). The obvious disadvantage is the feature size being larger than μ CP approaches ($\sim 100\ \mu\text{m}$). Turcu *et al.* (2003) and Sanjana and Fuller (2004) both used ink jet printing of proteins for the purpose of confining neuronal cells.

Each one of the presented approaches has advantages and disadvantages. When choosing the respective method, the scientist has to consider several prerequisites; starting with the appropriate protein, feature size and desired stability, different methods can lead to best results. It is crucial to choose the appropriate surface and bio-ink (e.g. respective ECM protein) for every cell type. Also, the degree of hydrophilicity of the surface (before and after the coating/patterning procedures) can have a strong impact on the outcome of the experiment.

In this study star-shaped PEG (star PEG) molecules were chosen as a non-fouling background because non-fouling or non-permissive materials like linear NH_2 PEG, tenascin, floursilane, polysiloxane, and SiO_2 known to be repellent to other cell types were not suitable for cricket neurons from the TAG that were used in this study as they are strongly adhesive and grew well on these materials. The star PEG molecules have six functionalized arms that bind to each other and form a dense layer on the surface. Having the advantage of change in functionality after the cross-linking reaction, the reactive arm can bind proteins applied a short time after coating of the star PEG (for detailed description see chapter 3 materials & methods).

As mentioned earlier, the cellular shape and the extent of neurite formation influences the network properties and its computational capability. Cell culture approaches combined with patterning techniques are very suitable for network formation and synaptogenesis studies as they offer a tool to direct growth and present controlled geometries of adhesive or permissive coatings. Predetermined environment of single cells or even cell compartments ensure control of each level relevant in synapse formation. Predetermination of adhesion and growth paths in form of cellular patterning results in single cell morphology changes and restriction of network connectivity pattern mimicking *in vivo* pathways. Insights are gained on how strong the pattern influences the neuronal outgrowth and the resulting morphology type.

2.1.8 Outline

In the present work, an approach to control insect neuronal adhesion and outgrowth (neuron architecture) on glass and silicon substrates was accomplished by means of μ CP of conA onto star PEG. The methods used are described in chapter 3 Materials & Methods. The patterning procedure as well as the resulting neuronal networks are described in chapter 4 “Ultrathin coatings with reactivity change by time enable functional *in vitro* networks of insect neurons” (Reska *et al.*, 2008). Additionally, a proof for functional chemical synapses formed within pre-patterned networks is presented.

Morphological and electrophysiological studies of pre-patterned cricket neuronal networks revealed basic electrophysiological characteristics and dependency on extrinsic cues as the presented patterns of single neurons. The apparent morphological shift and basic parameters of pre-patterned neurons in comparison to neurons cultured on uniformly conA-coated surfaces are part of chapter 5 “Surface growth restriction in insect neuronal cell culture promotes *in vivo*-like phenotypes” (Reska *et al.*, in preparation).

The continuative experiments in chapter 6 “Inverse patterning of cell-aversive star PEG for building neuronal networks on microelectronic devices” offer a possibility to move on to extracellular devices. This represents an upgrade for long-term studies where whole populations of neurons can be recorded simultaneously.

3 Materials and Methods

In order to offer a complete overview of the experiments in this study this section contains a detailed description of all procedures used. Reagents were purchased from Sigma unless otherwise noted.

3.1 Primary neuronal cell culture of cricket neurons

3.1.1 Animals

The cercal system offers a very fast and effective mechanosensory transduction triggering the escape reaction. For the cricket *Gryllus bimaculatus* (Fig.2.1) this neuronal system is well characterized. Additionally, this system consists of large and easily accessible neurons. For neuronal preparations male and female crickets of the species *Gryllus bimaculatus* were bred in a light:dark cycle 12:12 hours, fed with fresh apples, dog and fish food. Adult crickets were separated weekly from larvae in order to control the age of the animals. Animals were used for cell culture experiments within one week after adult molt.



Fig.3.1. Female cricket *Gryllus bimaculatus*.

3.1.2 Procedure of cell culture

As the first information processing of the stimuli that triggers the crickets escape reaction occurs in the neuronal networks of the terminal abdominal ganglion (TAG), neurons from this tissue were cultured and investigated. Neurons were dissociated and cultured according to methods described by Hayashi and Hildebrand (1990) and Kloppenburg and Hörner (1998) with slight modifications. The animals were cool-anesthetized at 4°C for about 60 min and decapitated prior to tissue preparation. All legs (and the ovipositor of female animals) were removed and the cricket was fixated dorsal side up to a preparation dish filled with normal salt solution (NSS) [NaCl 150 mM, KCl 5 mM, CaCl₂ 2 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM]. One horizontal cut and two cuts parallel to the sagittal axis (see Fig.3.2 a) enable the access to the abdominal part of the nervous system. The ventral part of the abdomen was opened, fixated to the preparation dish, and washed with NSS.

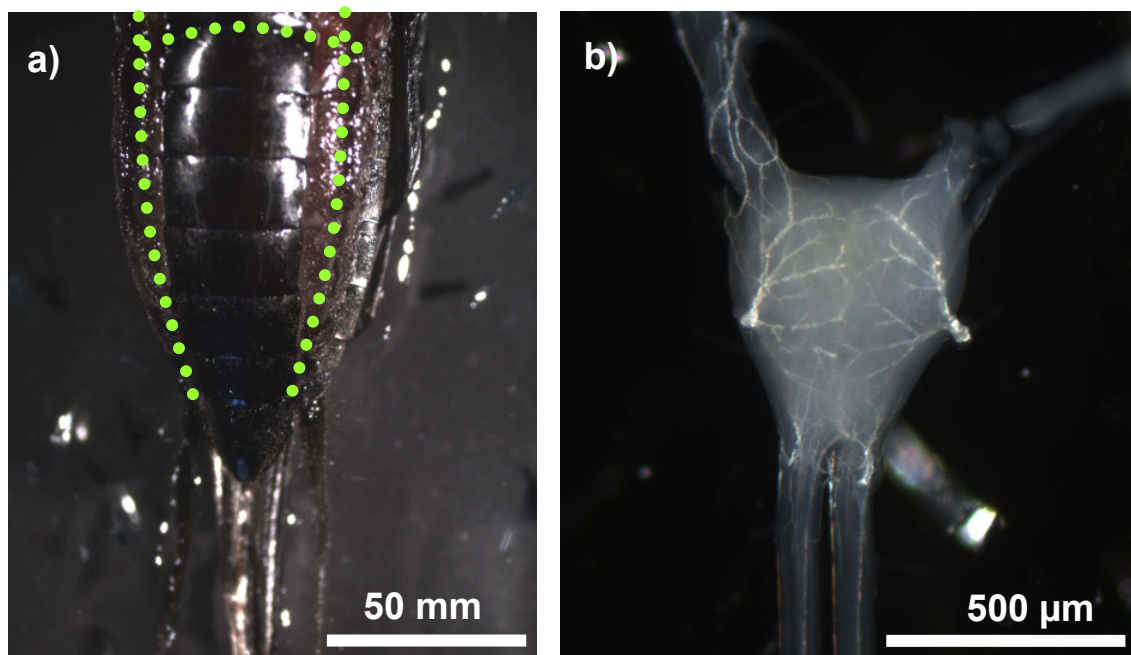


Fig.3.2. a) Ventral view on abdomen of *Gryllus bimaculatus*, dotted line indicates the cut lines. b) Isolated terminal abdominal ganglion (before desheathing), the two ceratal nerves furcate to upper left and upper right. The connectives lead to the subsequent abdominal ganglion.

The TAG and least one abdominal ganglion was excised by dividing both ceratal nerves and connectives and washed in sterile modified Leibovitz medium [(L-15, glucose (2 g/L), fructose (0.8 g/mL), proline (0.35 g/mL), imidazole (0.06 g/mL), glutamine (0.3 g/mL), adjusted to pH 7.0 with HEPES (360 mOsm/kg)]. Subsequent steps took place under semi-

sterile conditions working under a half-opened sterile hood in order to enable microscopic control during TAG preparation.

The TAG with one abdominal ganglion was fixated by the connectives and cercal nerves (see Fig.3.2 b) with steel pins to a preparation dish with PDMS bottom and filled with modified Leibovitz medium. Next, the TAG sheath was opened with two fine tweezers and peeled off. The neurons were dissociated enzymatically by 3 min incubation at 37°C in an enzyme solution (dispase 2 mg/mL; collagenase 0.5 mg/mL) followed by mechanical trituration with a silanized 200 µL tip. The enzyme reaction was stopped by transferring the TAG to pre-cooled modified Leibovitz medium, supplied with yeastolate (0.28 g/mL) and lactalbuminhydrolysate (1.4 g/mL) to prevent adhesion to the tubes, followed by a centrifugation (5 min, 8°C, 2800 rpm) collecting the cells at the bottom of the tube. To reduce cell suspension volume the supernatant was sucked off and withdrawn. Next, about 80 µL modified Leibovitz medium was added per substrate and neurons were plated on surfaces coated with conA or respective star PEG / conA pattern (see chapter 3.2.2). The neurons were left unmoved for 1.5 to 2 hours to adhere before the 35 mm Nunc Petri dishes containing the respective surface were filled with modified Leibovitz medium up to a volume of 3 ml and cultured in 95 % relative humidity at 29°C. As glass surfaces coated with star PEG and conA were more likely for dewetting or swim up, the medium had to be pipetted carefully holding the glass substrate down with the pipette tip. For morphological experiments neurons were cultured 5 days *in vitro*. Electrophysiological experiments were performed at different time stages. In order to keep a constant cell quality the culture medium was partially exchanged (1.5 of 3 mL) every 3-4 days. Usually neurons from one ganglion were plated on 3 surfaces. For the morphological experiments, during each independent preparation, 3 TAGs were pooled and seeded on 9 surfaces. Two of each conA grid pattern type (see section 3.6), two positive controls (coverslips uniformly coated with conA) and one uniformly star PEG coated surface as negative control.

3.2 Surface coatings for primary cell culture

3.2.1 Uniformly conA-coated surfaces

Glass coverslips (Ø15 mm, from Roth, Karlsruhe, Germany) were activated by oxygen plasma treatment in a plasma oven (Pico, Diener Electronic, Nagold, Germany) at 40 W in a vacuum of 0.8 mbar for 2 min. Next, 100 µL of a conA solution (0.2 mg/mL) in deionized water were

incubated for 60 min, subsequently washed three times with sterile deionized water and used within one week for cell culture experiments.

3.2.2 Surface coatings with star PEG / conA grid pattern

In order to study network architecture and role of network morphology, a functional cell culture, which provides long-term neuronal survival and control of growth and interconnection with adjacent neurons as well as electrophysiological activity and functional synaptic connectivity within the networks, is necessary. The star PEG / conA coating provides a means for creating geometrically-confined cellular networks on artificial surfaces. A six-armed star-shaped poly(ethylene glycol)-based polymer layer was used as an aversive background combined with the cell adhesive lectin conA used as standard protein coating in many insect neuronal cell cultures. The biological inertness of PEG is attributed to its hydrophilicity and charge neutrality (Revzin *et al.*, 2003).

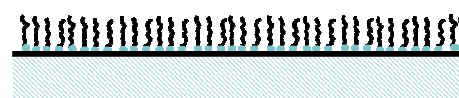
The single steps of the coating procedure are depicted schematically in Fig.3.3. Glass and silicon surfaces were cleaned by ultrasonification in isopropyl alcohol for 5 min and dried in a stream of nitrogen, then washed with deionized water and activated by a MetOH / HCl (1:1) treatment (30 min), washed and dried again. Directly after activation, surfaces were aminosilanized by vapor deposition in a glovebox (MB Braun 100) in an argon gas atmosphere prior to spin-coating. The substrates were placed in a desiccator adding 0.2 mL N-[3-(trimethoxysilyl) propyl]ethylenediamine evacuated with a pump until a vacuum of 4.5 mbar was reached and incubated for 1 h. For preparation of the star PEG film, isocyanate-terminated prepolymer (100 mg; MW 12 kDa, polydispersity (PD) 1.2) was dissolved in dry tetrahydrofuran (1 mL). After addition of deionized water (1:10), the solution was incubated for 5 min for pre-crosslinking, and subsequently applied to the surface. Spin-coating was performed at 2500 rpm for 40 s (Laurell, WS-400E-&NPP-Lite). The rotational speed of the surface during spin-coating and the properties of the star PEG solution (concentration, solvent used) define the thickness of the layer and ensure an even distribution of star PEG over the surface. The parameters used here resulted in star PEG coatings with a thickness of 30 ± 5 nm. The freshly prepared films were stored in ambient conditions for 2 h prior to microcontact printing (μ CP) of conA. After μ CP, the substrates were left for further cross-linking (see Fig.3.4) over night before they were used for cell culture experiments.

Surface activation

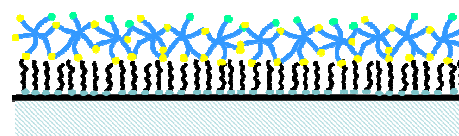


Glass / Silicon surface

Aminosilanization



Spin coating



Star PEG

Fig.3.3. Procedure of star PEG layer formation. First, the glass / Si surface is cleaned and activated with MetOH/HCl, next, aminosilane is vapor deposited. Finally star PEG is applied via spin-coating.

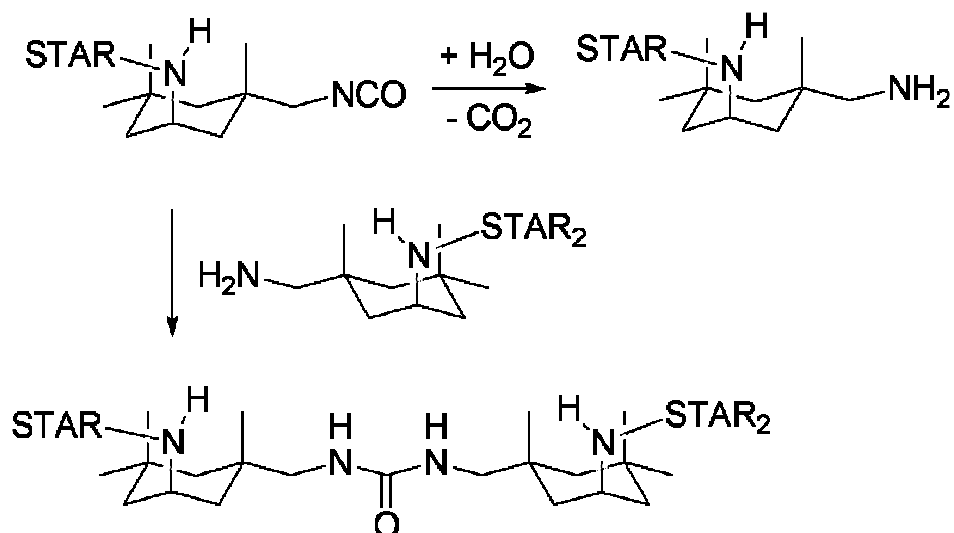


Fig.3.4. Cross-linking reaction of star PEG: Isocyanate reacts with water to form carbaminic acid which decarboxylates to an amino group. Other isocyanate groups react with these amines forming biocompatible urea groups (Groll, 2005).

3.3 Microcontact Printing

Diverse grid patterns were transposed to a chromium coated silicon wafer by electron beam writing. This wafer was used as a mask to fabricate the stamp master by UV photolithography on a Si [100] wafer coated with positive resist, and pattern transfer by deep Reactive Ion Etching (RIE) (2.5 μm deep) (Fig.3.5).

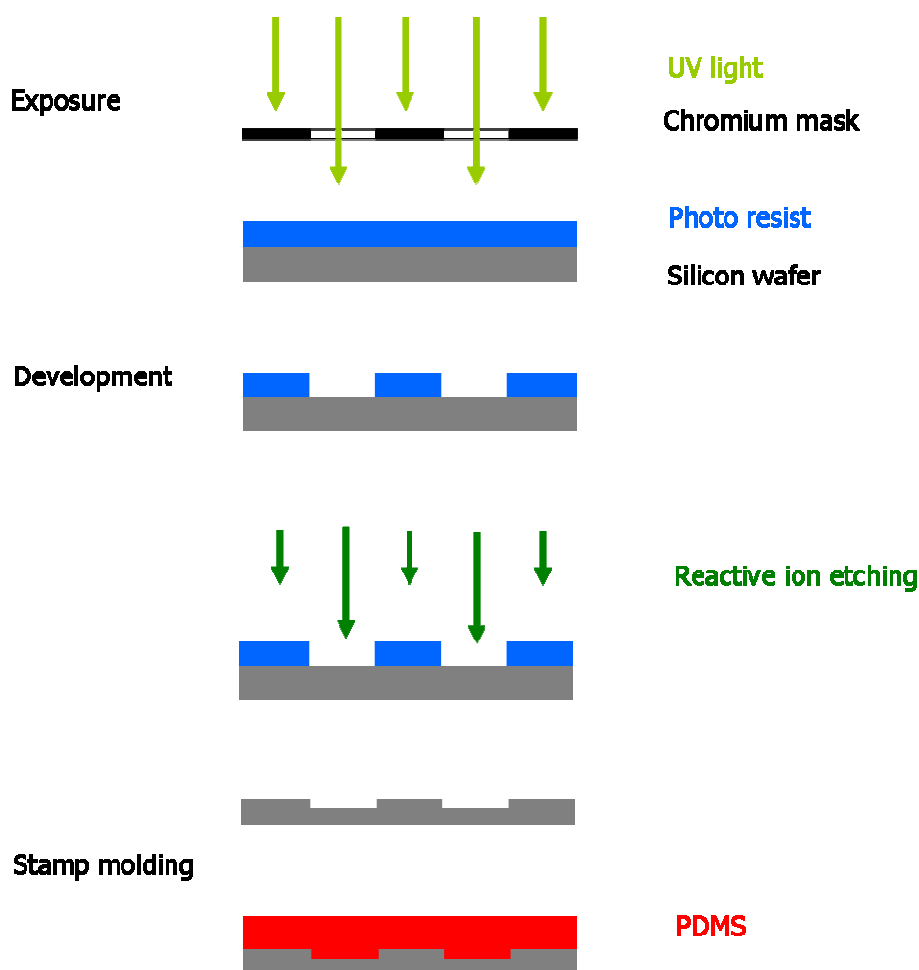
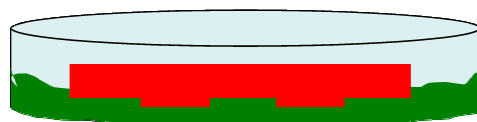


Fig.3.5. Creation of a master for molding PDMS microstamps. A photoresist coated Si wafer is developed through a patterned chromium mask. Next, the Si surface is patterned by RIE resulting in 2.5 μm ridges for PDMS molding.

Patterns of conA were generated by μCP (Xia & Whitesides, 1998) using microstamps polymerized on lithography masters. The Si stamp master was coated with fluorsilane [(tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane, from ABCR, Karlsruhe, Germany] as lubricant and could be reused more than 50 times. Polydimethylsiloxane (PDMS) stamps were fabricated by pouring Sylgard 184 (Dow Corning, Midland, Michigan, USA) with a 1:10 curing ratio onto these masters followed by a 12 h curing step at 80°C. Final curing, after

master stamp release, was performed for 1 h at 110°C. The stamps were inked by immersion in a conA solution (0.5 mg/mL deionized water) for 10 min, dried in a stream of nitrogen, and brought into conformal contact with the star PEG layer for 10 min.

Incubation in ink

PDMS microstamp

Ink solution (e.g. protein)

Dryment**Stamping**

Glass / chip surface

Removal of the stamp

Fig.3.6. Principle of microcontact printing (μ CP). The fabricated microstamp is incubated in an ink solution for 10 min. After drying in nitrogen flow it is brought into conformal contact with the surface for another 10 min resulting in a pattern-wise transfer of the ink onto the surface.

3.4 Parylene /star PEG patterning on microelectronic devices for extracellular recordings

Paired patch-clamp experiments represent a straightforward method to study the interconnectivity between two adjacent neurons. However, experimental success depends to a large degree on the grade of attachment of the neuron to the substrate, fitness of the neuron, and personal skills. Working with microelectronic devices, chemical modification of the chip surface turned out to be necessary not only for neuronal guidance in order to gain a control over the interconnection but also to increase the total number of neurons attached to the relevant recording sites on the electronic device by presetting the attachment areas. Extracellular measurements with microelectronic device arrays are not invasive and thus the failure rate due to manual handling is minimized. Due to these facts, the present study aimed to enable patterning on microelectronic devices with a one-to-one neuron-electrode connection and to create a close neuron electrode contact.

3.4.1 Microelectronic devices

Field effect transistor arrays are built up with clean room techniques. The chemically relevant chip surface consists of SiO₂. A layer stack of wet silicon oxide (250 nm) was used, low-pressure chemical vapor deposited (LPCVD) silicon nitride (130 nm) and LPCVD silicon oxide (100 nm) (ONO-stack). After deposition of this ONO-stack, the gate area was opened by a combination of dry etching and wet etching. A thin SiO₂ layer (10 nm) formed in a dry oxidation process at 820°C was used as gate dielectric. Transistor gates were arranged in a 4×4 matrix with a pitch of 200 µm (or 10×10 matrix with a pitch of 100 µm) in the center of a 5 mm×5 mm silicon chip (Offenhäusser *et al.*, 1997, Schäfer *et al.*, 2009).

MEAs were created by sputtering thin gold electrodes and feed lines (around 300 nm) on SiO₂ wafers. The electrode has typically a diameter between 10 and 30 µm. The distances between the electrodes are 100 µm or 200 µm. The insulation layer consists of an ONO-stack and protects the array from electrolyte solution. Only the respective recording site is opened by means of RIE (Krause, 2000, Ecken *et al.* 2003).

Both chip arrays were encapsulated with PDMS and glass rings to isolate the electric wires and form a cell culture chamber (Fig.3.7).

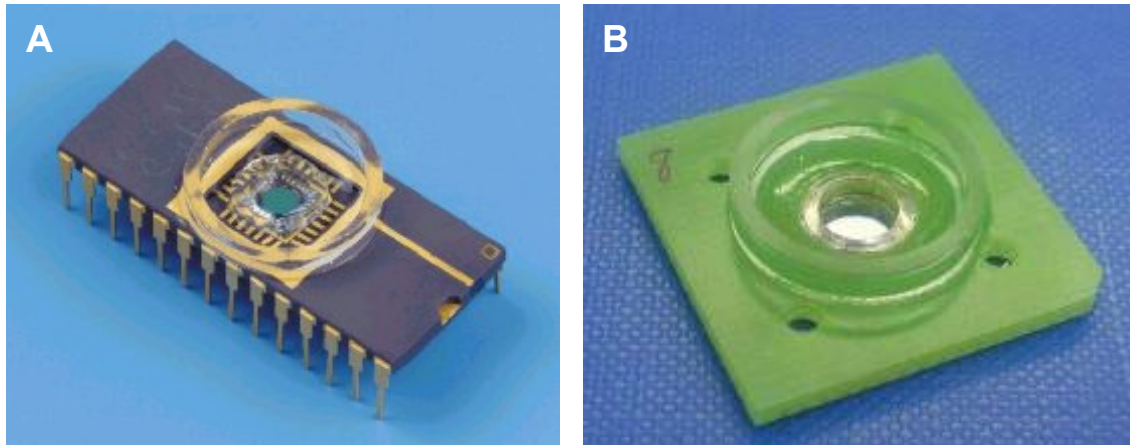


Fig.3.7. Two types of microelectronic devices. Both chips are encapsulated with glass rings that protect the electronic wiring and form a cell culture cavity. a) Field effect transistor array chip, b) Multielectrode array chip.

3.4.2 Parylene stencil production

Si wafers were fluorsilanized [(tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane, from ABCR, Karlsruhe, Germany] in an evacuated desiccator (45 mbar) in a glove box under argon gas atmosphere prior to parylene deposition. Parylene C [poly(monochloro-p-xylylene)] was chemically vapor deposited (PDS 2010 Labcoter, Speciality Coating Systems, Indianapolis, USA) onto fluorsilanized Si wafers in the clean room facilities of the IWE at RWTH Aachen University. The resulting thickness was around 4 μm . Subsequently, the wafer was coated with a photoresist (AZ5214) and patterned by RIE. Next, the photoresist was removed with acetone.

3.4.3 Plasma assisted removal of star PEG with parylene stencils

The activated glass or chip surface was aminosilanized and coated with star PEG as previously described. The parylene stencils were carefully detached from the wafer with tweezers and positioned under microscopically control onto the respective device. It was clearly visible on the surface whether it sealed properly to the surface.

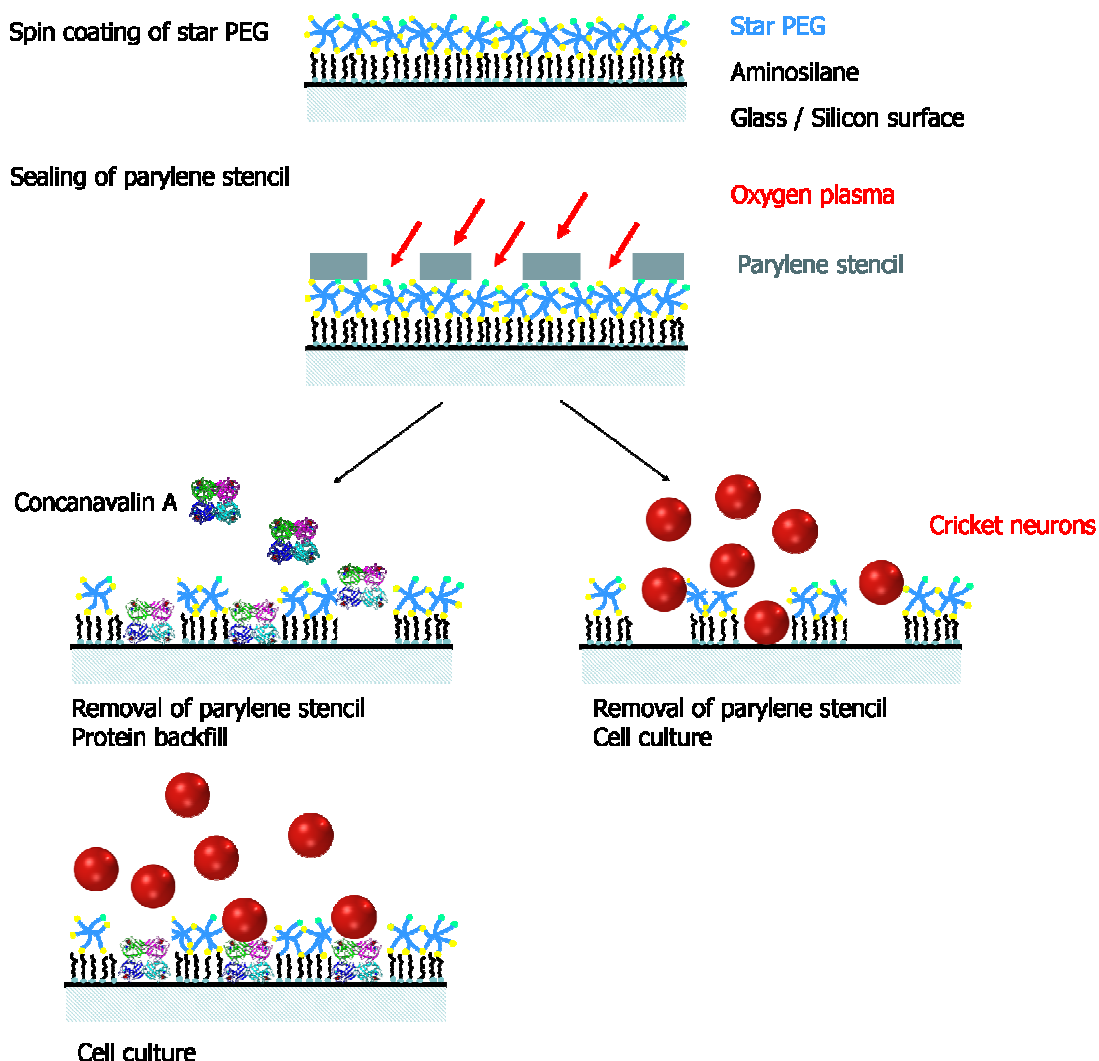


Fig.3.8. Schematic of plasma assisted removal of star PEG with parylene stencils. Star PEG layer applied by spin-coating is covered with a parylene stencil and removed pattern-wise by oxygen plasma. The subducted surface area is not cell repellent and can be used for cell culture or protein backfill prior to cell culture.

Subsequently, the substrates with positioned stencils were treated with oxygen plasma in a plasma oven (Pico, Diener Electronic, Nagold, Germany) at 40 W in a vacuum of 0.8 mbar. Optionally the surfaces were subsequently incubated with conA solution (200 $\mu\text{g}/\text{mL}$) for 60 min (see Fig.3.8).

3.5 Surface characterization

3.5.1 Ellipsometry

Ellipsometry is an optical technique that allows for the analysis of very thin films using polarized light to probe the dielectric properties of a sample. It is called “ellipsometry” because the state of polarized light is mostly elliptic. A linearly polarised light in form of a laser beam is directed to the surface and the state of polarisation of the light reflected from the surface yields information about the layers (Fig.3.8). This method is applicable for layers that are thinner than the wavelength of the light itself. Ellipsometry is used to determine properties of layer stacks of thin films and the interfaces between multiple layers. Depending on what information is available for the surface, a range of properties including the layer thickness, morphology, or chemical composition can be measured. The measured values for the polarisation state of the light are expressed in two variables Ψ and Δ . Because the analysis relies on the ratio of these two values, it can reach accuracy down to a single atomic layer or less and is very reproducible.

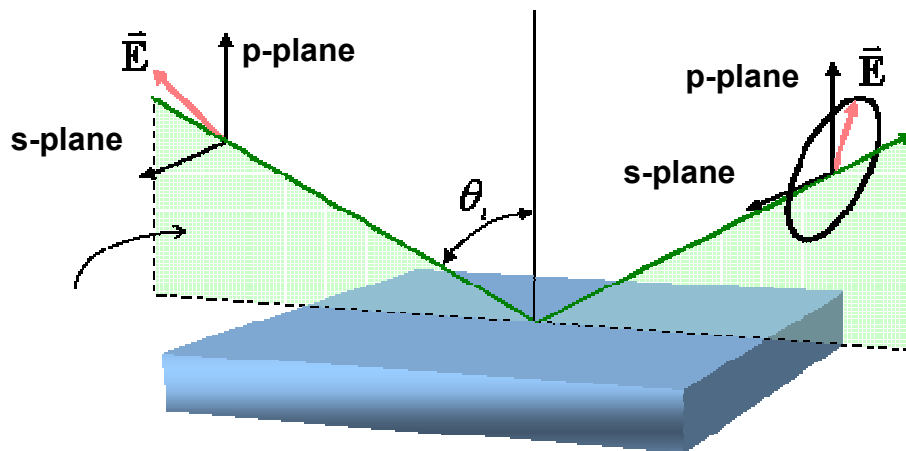


Fig. 3.9. Laser beam polarization during ellipsometry measurement. Polarized laser beam coming from left is changing its polarisation state from linear to elliptical due to surface properties. For any angle of incidence between 0° and 90° , p -polarized light and s -polarized light will be reflected differently. (source: University of Texas at Arlington, <http://www.uta.edu/optics/research/ellipsometry/ellipsometry.htm>)

Layer thicknesses were determined using a Nanofilm EP3 ellipsometer with a Nd:yttrium aluminum garnet (Nd:YAG) laser (532 nm) and EP3 View 2.05 software. The angle of

incidence was 70°C. As the refractive index of star PEG is in the proximity of SiO₂ (ca. 1.46), the measurement was performed assuming one continuous layer on Si.

3.5.2 Atomic force microscopy

Atomic force microscopy is used for investigation of surface topologies in the nanometer-range. As the measurement is performed purely by mechanical sweeping, this method is applicable for conductive and non conductive surface materials. A fine tip mounted on a flexible cantilever is scanning the surface line-wise with a scanning velocity of about 1 Hz resulting in an accuracy of up to 0.1 nm. As a consequence of the surface topology the cantilever is deflected and a laser beam that is pointed at the cantilever is reflected. The deflection of the laser beam is captured by photodetector and is transduced to a surface image. The exact movement control of the tip over the surface (or vice versa) is controlled by three dimensional piezoelectronic elements (Fig.3.9). The deflection of the cantilever is measured by detecting the reflected laser beam by a photodetector (Fig.3.10). Thus, the deflection of the cantilever gives rise to the surface properties. The small surface forces (ca. 10⁻⁸– 10⁻⁹ N) allow for a nondestructive investigation of relative soft materials. The cantilever experiences the resonance frequency depending on the force gradient, which is a function of the distance between tip and surface. Therefore, features about surface topography can be deduced from changes in the resonance frequency.

The laser beam that hits the tip is deflected stronger than the tip itself when the cantilever moves vertically, resulting in a high sensitivity perpendicular to the surface. Two photodiodes are arranged vertically and the potential difference between the two diodes correlates with a specific deflection in the z axis. The radius of curvature of the tip is typically smaller than 10 nm. Depending on the surface roughness this results in a lateral resolution of 0.1 – 10 nm.

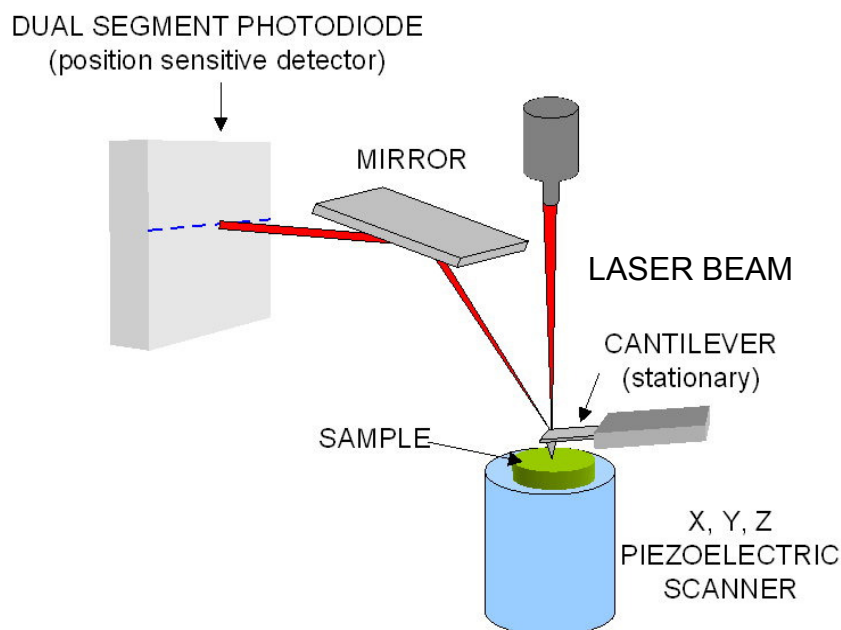


Fig.3.10. Schematic drawing of atomic microscope set-up. A piezoelectronic scanner is steering the surface for scanning it line-wise. Surface topology is measured indirectly by a photodetector measuring the deflection of a laser beam from the cantilever surface. (Image: Michigan Technological University, USA, <http://nano.mtu.edu/afm.htm/>).

A controller steers the movement of the scanner and of the tip and analyzes the signals. The resulting image is also influenced by other physical forces between the tip and the surface such as van der Waals forces (positive interaction) and e.g. Coulomb force (negative interaction). As the tip is in constant mechanical contact with the surface during measurements in the contact mode it can damage soft structures as protein layers. The dynamical tapping mode offers an important advantage compared to the contact mode because in this mode the cantilever is just tapping brought to vibration near its resonance frequency by means of an additional piezo crystal. The vibration amplitude serves as control variable. In contrast to SEM images, the AFM provides real three dimensional (3-D) surface profiles (Binning, 1985, Kollensperger *et al.*, 1999).

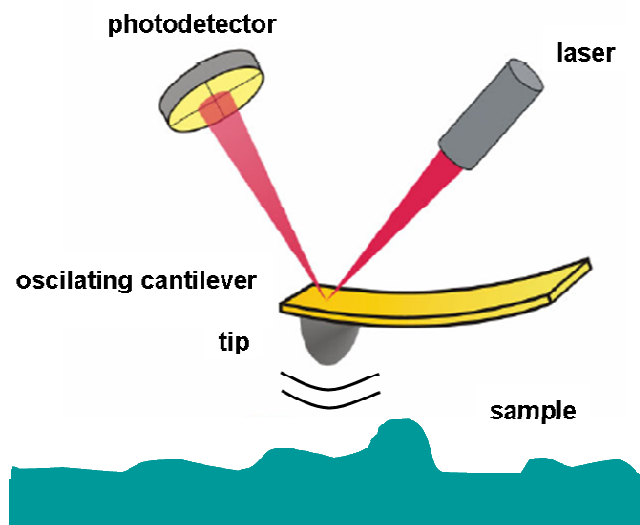


Fig.3.11. AFM tip scanning a surface in tapping mode. The AFM tip is mounted onto an oscillating cantilever. The deflection of the cantilever due to the surface topology changes the angle of the laser beam which is measured by a photodetector. (Schematic drawing modified from Roos *et al.*, 2008).

The atomic force measurements were performed with a Nanoscope IV atomic force microscope (Veeco, Mannheim, Germany) operating with the Nanoscope 5.30 software. The tips used in the AFM measurements were RTESPW from Veeco (Material: 1-10 Ω cm Phosphorus (n) doped Silicon) on a Micro Cantilever from Olympus (OMCL-AC160TS-W2). The resonance frequency was 300 kHz, spring constant 42 N/m.

SiO₂ substrates with respective star PEG pattern were glued with a foil to a holder that in turn was positioned under microscopical control onto a piezo scanner such that the relevant part of the pattern was underneath the AFM tip mounted to the cantilver. The piezo scanner including the substrate was placed in a faraday cage with a passive vibration-damping device.

The surface topography was analyzed with Nanoscope software. 3-D images as well as surface profiles were generated in order to investigate the protein transfer and thickness, star PEG layer thickness and homogeneity and to compare the different patterning methods.

3.5.3 Scanning electron microscope (SEM) measurements

SEM measurements

Scanning electron microscopy (SEM) is a method to investigate a sample surface yielding information about the surface topography, composition and other properties raster scan pattern. An electron gun produces a focused electron beam (primary electron beam) that passes through electromagnetic lenses and is directed rapidly over the surface. Entering a surface the electron beam generates many low energy secondary electrons. The intensity of these secondary electrons is governed by the surface topography of the sample.

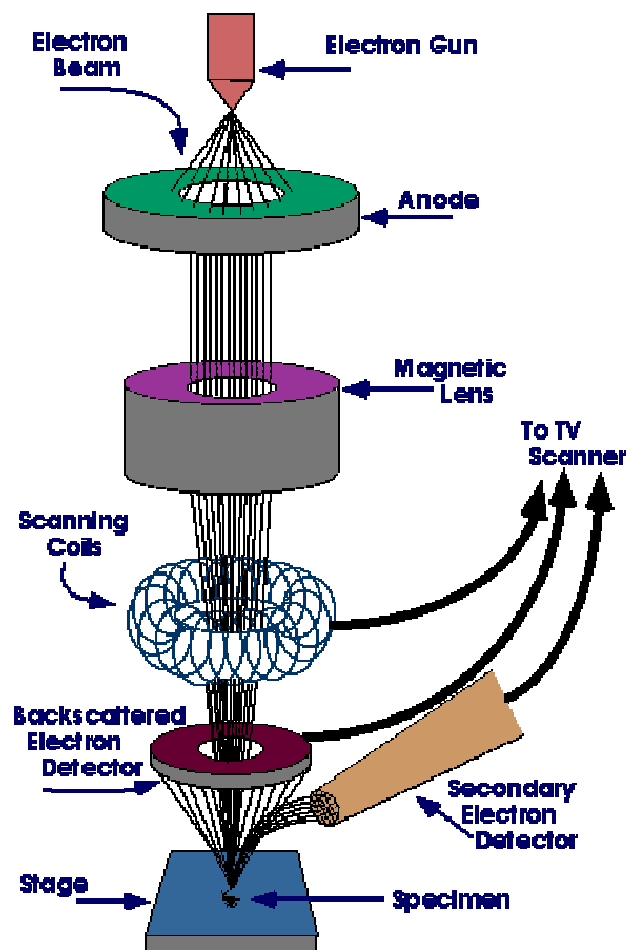


Fig.3.12. Schematic of a scanning electron microscope. Primary electrons are entering the sample surface ejecting secondary electrons from the surface that are detected and translated into an image. (Image: Purdue University, Indiana, USA, www.purdue.edu/REM/rs/graphics/sem2.gif).

An image of the sample surface is therefore constructed by measuring secondary electron intensity as a function of the position of the scanning primary electron beam. Secondary electrons are transmitted to an electron collector and amplified and produce finally an image

on the computer that appears to be 3 dimensional (see Fig.3.13). In order to prevent unspecific interactions the measurements are performed in high vacuum. Therefore the biological samples, as neurons have to be fixated and dehydrated before SEM measurements (Smith und Oatley, 2004). A Gemini 1550 scanning electron microscope (Zeiss, Oberkochen, Germany) was used for the present study, operating at 10 kV with In-Lense detector.

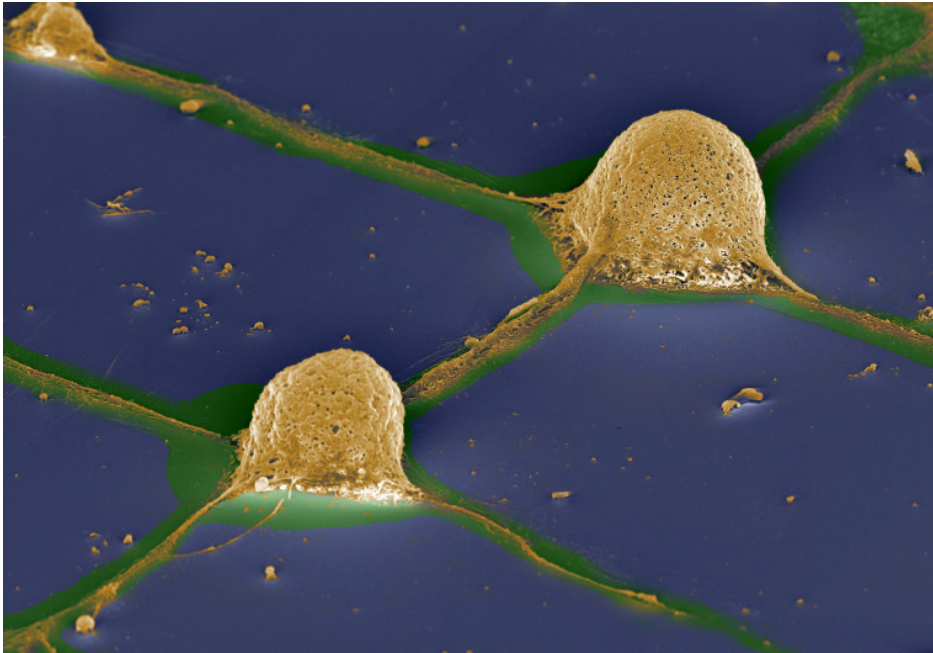


Fig.3.13. Colourized SEM micrograph of two cricket neurons grown on star PEG/ conA patterned surface (DIV 6). Green colour indicates conA on star PEG, blue colour indicates star PEG. SEM micrographs result in a 3-D like image.

Cell Fixation for SEM

For fixation, the cell-culture medium volume was substituted gradually with glutaraldehyde (3.5% in 20 mM HEPES, adjusted to pH 7.0 with NaOH) and incubated over night at 4°C. To dehydrate the cell material, ethanol (p.A. grade) was added and the concentration was gradually raised up to approximately 100%. After critical-point drying in ethanol, the substrates were sputtered with gold for 30 s at 50 mA under vacuum.

Critical point drying

Fixated substrates were dehydrated and critical-point dried prior to scanning electron microscopy that is performed in a vacuum chamber. The principle of critical point drying is based on the fact that during the critical point transgression of a medium no phase boundary between liquid and gas phase is existent (Dykstra, 1992). The liquid evaporates completely from the cell material during this procedure. The procedure used for critical point drying is described by Hoeller (2005). The probe chamber of the critical point dryer (BALTEC CPD030, from BALTEC, Liechtenstein) was filled with ethanol (p.A. grade), the substrates were placed in a coverslip holder and transferred into the chamber. Next, the chamber was cooled down to 10 °C and the ethanol was exchanged stepwise with liquid CO₂. Subsequently the chamber was heat up until the critical point was reached. At a temperature of 31°C and a pressure of 73.8 bar CO₂ reverted from liquid to gas phase resulting in a complete dehydration of the cellular material. The gas was carefully discharged and the dry substrates could be further prepared for SEM measurements. The substrates were sputtered with a Sputter Coater (BALTEC SCD004, from BALTEC, Liechtenstein) for 30 s at 50 mA with gold to increase the conductivity of the surface.

3.6 Data evaluation of morphological type distribution and statistical analysis

The single cell morphology of neurons with guided growth was compared to neurons grown on homogenously protein coated surfaces. After 5 days *in vitro* the respective cell culture preparations were evaluated using a Zeiss Axioscope equipped with an Axiocam digital camera. Images were taken using Zeiss Axiovision 4.5 software. Per preparation, one sample of each surface type was analyzed. I.e. one culture dish with two, four and eight growth paths (see Fig.3.14) and one uniformly conA and star PEG coated substrate, respectively, was counted. After 2-3 days of *in vitro* culture the neurons reached the final number of neurites that did not change while the neurites were elongating.

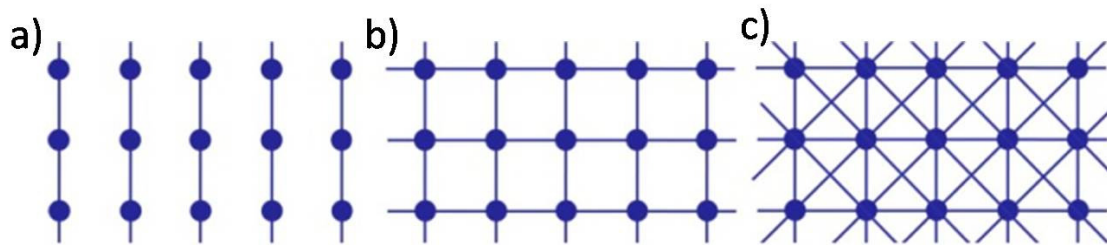


Fig.3.14. Three different pattern types for quantification of cell morphology development upon adhesion surface restriction. Nodes for neuronal somata with up to two (a), four (b) and 8 (c) possible growth paths.

The number of adherent neuronal cell bodies and the number of neurites originating from the cell body were counted. The percentages of outgrown neurites were analyzed with respect to the number of possibilities offered to the neuron. On the pre-patterned surfaces, only neurons adhering to nodes were taken into account. The major branches sprouting from the cell body were considered. A neuron that developed one or two main branches and multiple fine branches was considered unipolar or bipolar, respectively. All quantitative data are given as mean $\pm$ standard deviation of the mean (SDM). Error bars represent SDM. A two tailed t-test was used for statistical evaluation of measurements between two experimental groups.

3.7 Electrophysiology

Basic electrophysiological parameters from neurons on uniformly coated conA surfaces and conA / star PEG pre-patterned surfaces were collected.

For electrophysiological experiments the neurons were cultured for a time span between 1 and 15 days. Whole cell patch clamp recordings from the cell bodies were performed as described in Hamill *et al.* (1981) at room temperature in cell culture medium under optical control on the stage of an upright microscope (Zeiss) equipped with phase contrasts. The set-up was installed on a vibration-damped table (Newport, Darmstadt, Germany) and shielded by a custom-built Faraday cage. Micropipettes were pulled from borosilicate glass filaments (o.d. 1.5 mm, i.d. 0.86 mm; Sutter Instrument Co., Novato, CA, USA) using a horizontal Flaming/Brown micropipette puller (P-97, Sutter Instruments Co.) resulting in a pipette electrode resistance of 4–11 M Ω . The measuring constellation is presented in Fig.3.15.

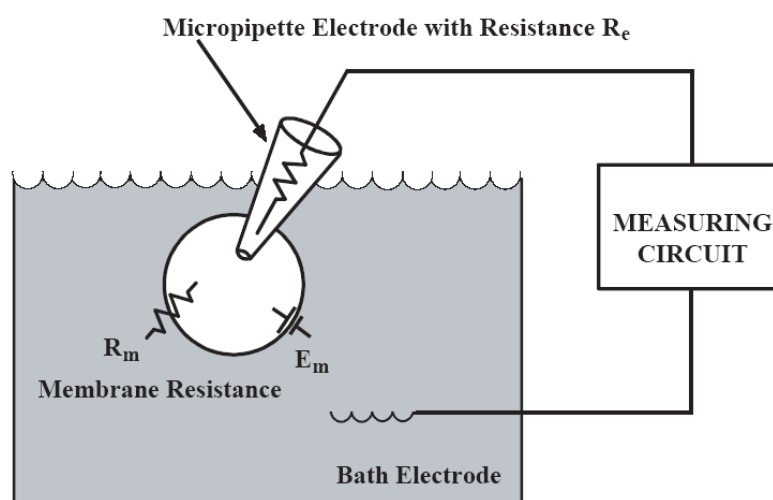


Fig.3.15. Measuring circuit of patch clamp experiment. A chlorinated silver wire is inserted into a patch pipette filled with intracellular patch solution. The electrode is mounted onto an amplifier head. (Image: the Axon guide, MDS Analytical Technologies).

The micropipettes were filled with intracellular-like solution (8 mM NaCl, 180 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 2 mM Na₂-ATP, 1 mM EGTA and 10 mM HEPES; around 390 mOsm/kg adjusted by equal shares glucose/fructose and pH 7.0 by NaOH). Currents and voltage changes were recorded in cell-attached patch configuration, through a EPC9/3 patch clamp amplifier controlled by TIDA 5 software (both HEKA Elektronik, Lambrecht, Germany). The micropipette which was filled with the intracellular-like solution was mounted in a pipette holder of the amplifier. It was connected to the amplifier via chlorinated silver wire within the pipette holder. A chlorinated silver wire in the cell culture dish filled with medium served as reference electrode. A slight pressure was applied to the

micropipette to avoid clogging due to debris. After immersing the micropipette into the bath solution, the offset potential was compensated and the resistance was observed as an indicator of the proximity of the electrode to the neuronal membrane. The micropipette was brought into conformal contact with the neuron under visual control with a 3 axis micromanipulator (Luigs&Neumann, Ratingen, Germany) while a constant pressure was applied to the electrode. Releasing the pressure usually facilitated the sealing to the membrane. Sometimes a gentle pressure had to be applied in order to gain a tight seal between the membrane and the micropipette. Once the micropipette had attached to the membrane and formed a Gigaseal (indicating seal resistance in the range of 1 G Ω) the fast capacitive currents (C_{Fast}) caused by charging of the pipette, as well as other parts of the setup and the connected membrane patch (see equivalent circuit Fig.3.16) were compensated.

Next, the neuronal membrane was opened with a gentle suction through the connected silicon tubing. Subsequently, the capacitive currents (C_{slow}) and the series resistance of the cell membrane (R_{ser}) were corrected with the series resistance compensation circuit of the amplifier. Also, the leak currents resulting from leakage through the patch boundaries and ionic currents were compensated. As the cricket neurons are a huge in diameter and thus have a large membrane surface, the leak was relative high, mostly around 5-10 nS. All these parameters were noted and considered as quality measure of the neuronal fitness and the performed measurement.

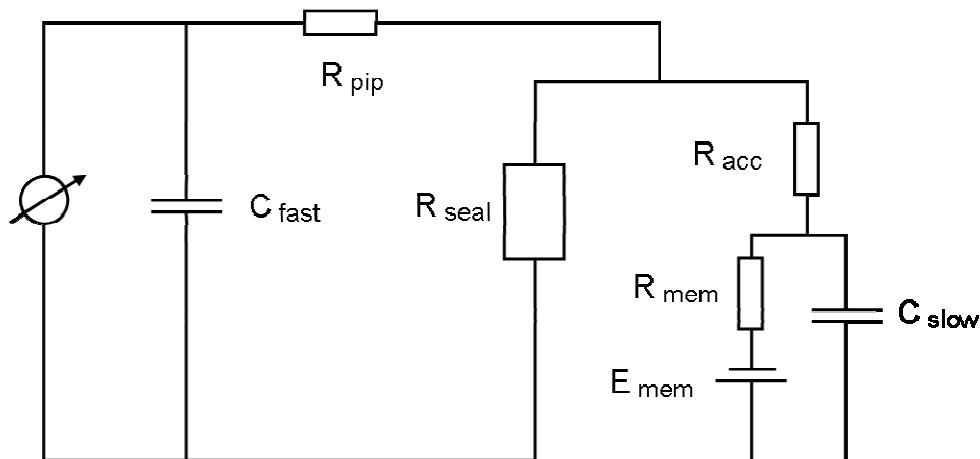


Fig.3.16 Equivalent circuit of patch clamp experiment. The pipette parameters consist of the access resistance R_{acc} , pipette capacitance C_{fast} and the resistance of the pipette (R_{pip}). The leak current is kept low when the seal resistance R_{seal} is high. As a thin insulating layer the membrane acts as a capacitor (C_{slow}) and is parallel in the circuit to the membrane resistance R_{mem} (Image: Vogt, 2006).

After achieving whole-cell configuration, first, the membrane resting potential was measured by a short measurement in current clamp mode without current injection. This parameter is an indicator for the neuronal fitness. For cricket TAG neurons it lies typically between -30 and -60 mV.

3.7.1 Voltage clamp experiments

After switching to voltage clamp (VC) mode resulting currents flowing through the neuronal membrane were recorded for each neuron. In the VC mode the neurons were clamped to a holding potential of -70 mV by injection of a constant hyperpolarising current. When the voltage is clamped, capacitive currents are eliminated, except for brief artefacts prior to new voltage step. The currents that flow are proportional only to the membrane conductance (except for the brief charging time during voltage switch) (see Fig.3.17). If channel gating is determined by the transmembrane voltage alone, voltage clamp offers control over the key variable that determines the opening and closing of ion channels (the Axon guide, MDS Analytical Technologies). The set of potential steps began with hyperpolarising potentials (-30 mV) and was repeated with an increment of 10 mV until reaching a potential of +100 mV lasting 100 ms each. The resulting membrane currents were recorded. The detected currents represent the ionflux through all activated ionic channels.

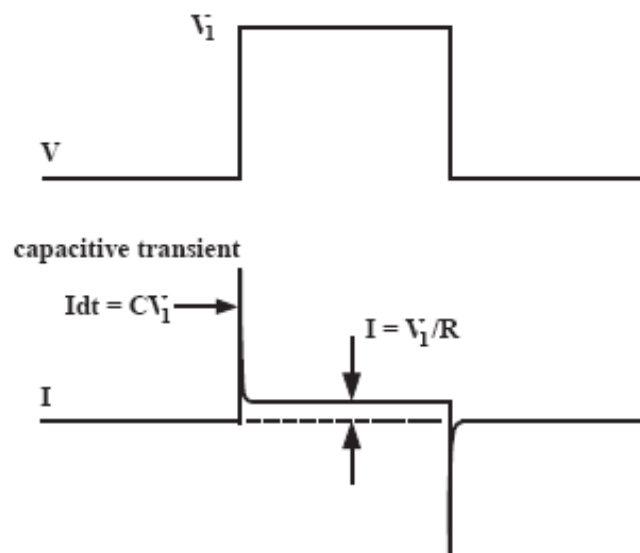


Fig.3.17 Characteristic current trend during a voltage clamp experiment. When the voltage is clamped capacitive currents are eliminated, except for brief artefacts prior to the new voltage step ($V \rightarrow V_1$, $V_1 \rightarrow V$) (the Axon guide, MDS Analytical Technologies).

Figure 3.18 presents a typical voltage clamp recording of a multi spiking cricket neuron. Repetitive inward currents that are represented as negative peaks are caused by the opening of voltage gated Na^+ channels stimulated above threshold.

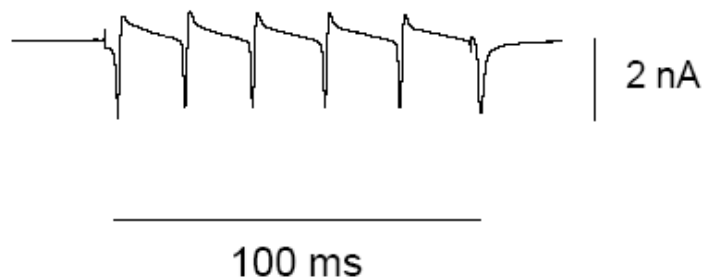


Fig.3.18 Typical voltage clamp recording of cricket neuron. Upon depolarization to -10 mV the neuronal membrane is discharged and voltage gated Na^+ channels lead to inward currents represented as downward peaks in the range of 2 nA.

3.7.2 Current clamp experiments

As next, current clamp (CC) recordings were performed. The current-clamp mode enables to detect changes of membrane potential after injection of rectangular current pulses and detect the ability of firing action potentials. Current was injected until a membrane potential of -70 mV was achieved. A pre-set stimulation protocol with increasing amplitudes of depolarizing and hyperpolarizing currents in 100 pA steps currents from -0.2 nA to 1 nA was applied and the potential changes of the membrane were measured. The stimuli were 100 ms long. A typical recording in current clamp mode is shown in Fig.3.19.

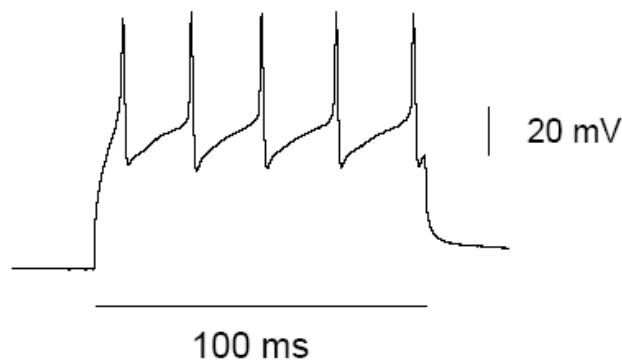


Fig.3.19 Typical current clamp recording of a multi spiking cricket neuron. A current stimulus of 700 pA elicits a row of action potentials with an amplitude of about 60 mV.

The data were sampled at 25 kHz and filtered at 10 kHz with a Bessel filter. The liquid-junction potential was calculated using JPCalcW 1.0 software (P.H. Barry, University of New South Wales, Sydney, Australia and Axon Instruments Inc., CA, USA) to be at most 3.7 mV and, hence, was not considered in our experiments.

Digitized signals were further analyzed by personal computer using TIDA software. Number and maximal amplitude of action potentials were read out.

3.7.3 Paired patch-clamp recordings

Paired patch-clamp recordings were performed in order to identify and characterize synaptical connections between adjacent neurons. Recordings were performed as described above. Apparently anatomically connected neuron pairs observed during light microscopy were chosen. First, single neurons were approached as described above with two independent micropipettes, each controlled with 3 axis micromanipulators (Luigs&Neumann, Ratingen, Germany) from opposed sides (Fig.3.20). Each neuron was checked for resting membrane potential and VC and CC protocols were performed. A CC protocol recording both neurons responses simultaneously while one of the neurons was stimulated by a current pulse of 0.3-0.5 nA. When one of the neurons was active the recording of the other neurons was checked for a shifted response in order to prove a functional connection. A characteristic recording of a pair of synaptically connected neurons is depicted in Fig.3.21. As a result to the stimulation pulse of 100 pA the presynaptical neuron (blue trace) fires two action potentials. The postsynaptical neuron was not firing action potentials when stimulated but showed clearly two excitatory postsynaptical potentials (EPSP) (red trace) as a response to stimulation of the presynaptical neuron.

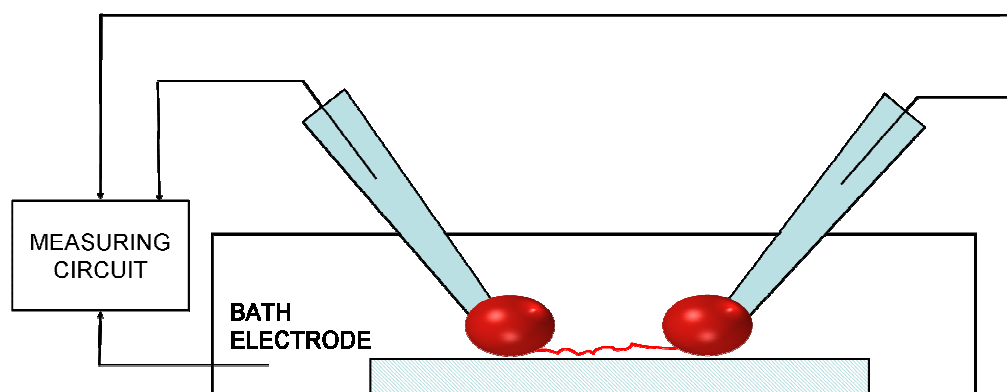


Fig.3.20 Schematic drawing of paired patch-clamp experiment. Two neurons that are anatomically connected are approached by patch-clamp electrodes. Both neurons are recorded simultaneously in whole cell modus while one neuron is stimulated.

Due to the extraordinary feature of insect cells to be able to attach to surfaces only via a minimal fraction of its membrane, performance of patch-clamp experiments proved to be very difficult. The cell-surface contact was studied in additional experiments by Total Internal Reflection microscopy (data not shown), which affirmed this observation.

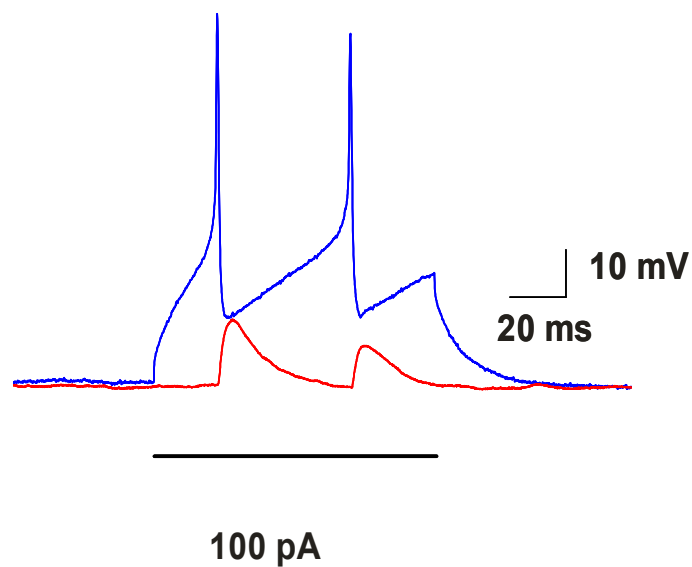


Fig.3.21 Example of a recording of two synaptically connected cricket neurons in current clamp mode. The presynaptic neuron (blue trace) fires two action potentials as a response to a 100 pA current stimulus, that in turn elicits two EPSPs of around 10 mV amplitude in the postsynaptic neuron (red trace)

4 Ultrathin coatings with reactivity change by time enable functional *in vitro* networks of insect neurons

Based on:

Ultrathin coatings with reactivity change in time enable functional *in vitro* networks of neurons

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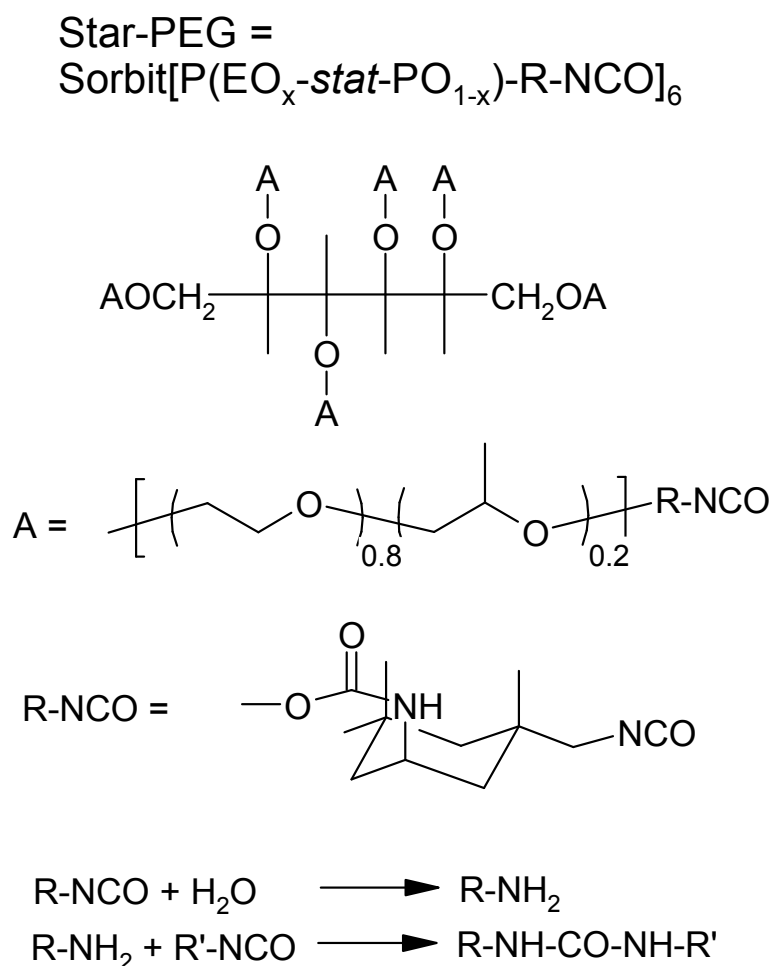
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Cellular processes such as sensing mechanisms and signal transduction have evolved to be highly specific and complex. In order to take advantage of these processes artificial environments must be designed to allow for precise control over cell behaviour and exploit cell function. For example, technological applications such as implantable devices and biosensors can be engineered using biomimetic approaches (García, 2006, Lim *et al.*, 2007, Shin *et al.*, 2003, Lutlof and Hubbell, 2005).

Combining a suited microfabrication technology with surface modification techniques provides adherent growing cells binding sites in spatially controlled regions with a non-fouling background (Falconnet *et al.*, 2007). Such biofunctional patterns on a protein-repellent background yield geometrically-confined cellular networks on artificial surfaces. This *in vitro* technique provides a means of systematic manipulation of the extracellular environment for investigating cell-surface and cell-cell interactions. Covalent immobilization of extracellular factors also provides pattern stability for long term cell-based experiments Nam *et al.*, 2006).

Most attempts to covalently link patterned cell adhesive molecules to substrates with a non-adhesive background rely on multi-step procedures (Falconnet *et al.*, 2007, Burnham *et al.*, 2006, Rozkiewicz *et al.*, 2006). Microfabrication techniques, such as photolithography, ink-jet technology, microfluidics and microcontact printing (μ CP), have been used for generating patterns of cells on artificial substrates (Park and Shuler, 2003, Yap and Zhang, 2007). μ CP is an especially valuable technique for surface pattern generation (Mrksich *et al.*, 1997, Ruiz and Chen, 2007) due to freedom in geometric pattern design, down to nanometer scale, and diversity in solutions that can be used as “ink”. Beyond spatial control, successful cellular patterning on artificial substrates also requires the precise control over surface chemistry. One of the core challenges in the design of biosensors and biohybrid devices is developing universal strategies for covalently attaching biomolecules to solid-state components (Mednitz, 2006).

Here, we present a coating system that combines non-fouling properties (Groll *et al.*, 2004) with a single-step covalent coupling scheme for attachment of biomolecules without further use of chemical crosslinkers. Ultra-thin hydrogel layers of isocyanate-functional star-shaped pre-polymers, based on poly(ethylene glycol), change chemical reactivity from amino-reactive, during layer preparation, to cell repellent, after hydrolysis of the isocyanate groups. We demonstrate the applicability of this system to biosensor technologies and biohybrid devices by geometrically controlling specific adhesion factors and growth of functional insect neuronal networks. Neurons are used for biosensing devices because they use electrical signals for task related information processing. When coupled to electronic devices, this signaling can be used for sensing. Here we used neurons from the terminal ganglion of crickets, *Gryllus bimaculatus*. These neurons control the highly sensitive, wind-evoked escape reaction of crickets and represent simple, functional networks



pre-polymer arms. This process is accompanied by a change in functionality from isocyanate towards urea bridges between the star molecules and remaining free amino groups over time. To ensure long-term stability of thin star PEG films on substrates such as glass or silicon, the polymer can be tightly linked to the support by aminosilanization of the substrate prior to coating. Here, we used silicon wafers and glass slides treated with N-[3-(trimethoxysilyl)propyl] ethylenediamine. Non-functionalized star PEG layers prevent unspecific protein adsorption and the adhesion of several human cell types under standard cell culture conditions over weeks of exposure (Groll *et al.*, 2005a).

Since not all isocyanate terminal groups are hydrolysed instantly upon contact with water, a portion of them will remain unreacted and available for further functionalization in freshly prepared films (Groll *et al.*, 2005b). This can be used to present specific cell adhesion molecules to control cell attachment and growth. Remaining isocyanate groups can be used to bind amino- or hydroxyl-functional compounds such as low molecular weight binding ligands or complete proteins through binding of peripheral lysines. Due to the higher nucleophilicity of amines, the aminolysis of isocyanates is generally some orders of magnitudes faster than the hydrolysis or even alcoholysis (Caraculacu and Coseri, 2001). Thus, hydrolysis of the isocyanate groups to amino functional groups is the rate limiting step of the crosslinking reaction within the star PEG film. This limiting step provides a time window of several hours in which the films bear isocyanate groups available for covalent binding of amino-functional compounds. We used this time window for covalently patterning of the lectin concanavalin A (conA) on freshly prepared films. This protein supports the adhesion of insect neurons (Hayashi and Hildebrand, 1990).

Patterning of conA was achieved by means of μ CP (Xia and Whitesides, 1998, Phillipsborn *et al.*, 2006). A PDMS stamp provided a rectangular grid pattern of 6 μ m lines with a side length of 50 and 100 μ m. Each intersection had a node of 20 μ m in diameter designed to capture cell bodies of cultured cricket neurons. At ambient conditions, the star PEG needed a minimum of 1h crosslinking time after coating to reach enough mechanical stability such that the printing procedure did not result in mechanical deformation and topological structuring of the films. However, due to the crosslinking within the film, the concentration of isocyanate groups decayed with time. Therefore, μ CP was performed at various time intervals after coating. Protein immobilization and pattern formation without mechanical deformation was best when the stamping was performed 2h after coating (determined by scanning force microscopy, SFM, and scanning electron microscopy, SEM; supporting information Fig.S1) and was used as the standard procedure for all further experiments.

The stability of the printed micropattern was tested by washing experiments. When conA was stamped onto fully crosslinked star PEG films, the weakly physisorbed proteins could be washed off,

whereas the conA that was brought in contact with the star PEG as described above could not be washed, indicating covalent binding of the protein to the layer (supporting information Fig.S2). The SEM measurement showed a smooth star PEG surface on which a conA grid pattern was deposited by μ CP (supporting information Fig.S1B). This substrate had been used in cell culture for 2 days before imaging by SEM, further demonstrating the stability of our surface coating system and the micropattern under cell culture conditions.

Both the SEM and the SFM images also reveal that more protein is deposited at the edge of the structure compared to the middle of the nodes and lines. Since the protein is transferred from aqueous solution to the hydrophobic PDMS stamp by incubation, partial dewetting may occur during drying of the stamp resulting in accumulation of proteins at the edges of the stamp structure. Hence, the rim of the structure transfers more protein.

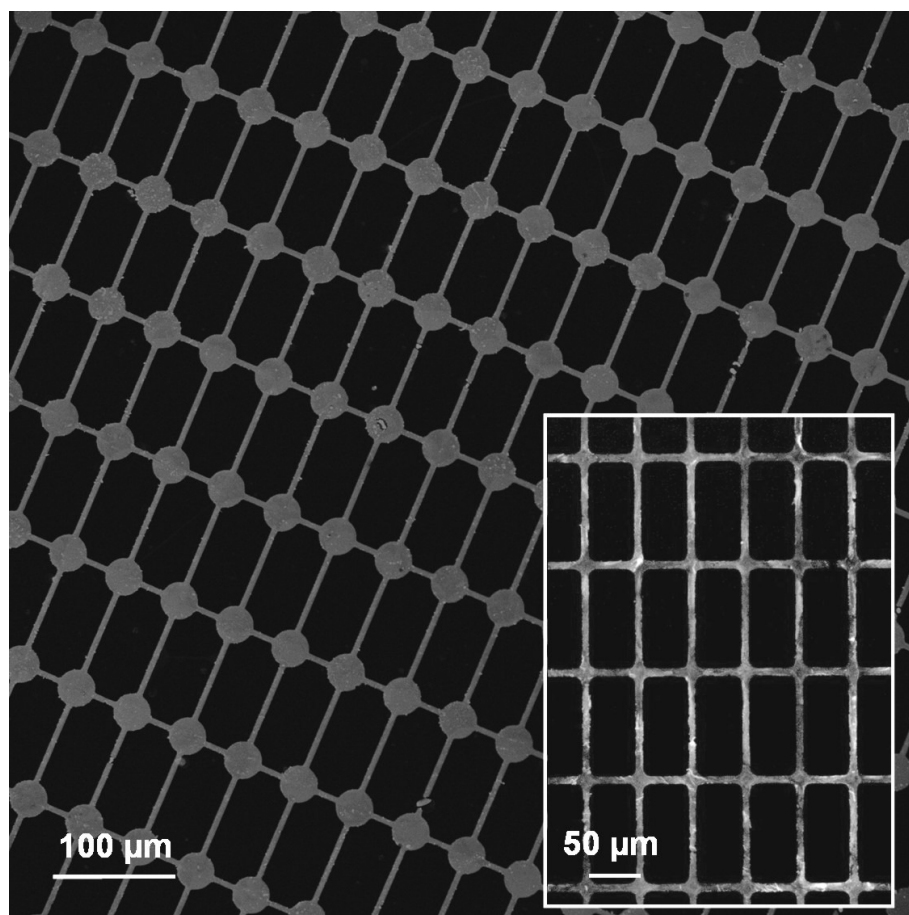


Fig.4.2. Confocal laser scan image of rhodamine red labelled conA printed on a star PEG surface (line width 5 μ m, nodes width 20 μ m; grid pattern 50 x 100 μ m). The inset shows printed FITC labelled conA (line width: 6 μ m; nodes width: 14 μ m; grid pattern 50 x 100 μ m).

The conA transfer to the star PEG surface was continuous over large sample areas as determined by stamping rhodamine red and FITC-labelled conA and imaging with confocal laser scanning microscopy (Fig.4.2). Although there were variations of protein density within the pattern, the pattern was transferred continuously over large surface areas. Corresponding results have been obtained by ellipsometry mapping of conA-structured surfaces (supporting information S3).

Studies on vertebrate neurons have shown geometrically controlled adhesion and mimicking of neuronal networks on artificial surfaces (Chang *et al.*, 2001, Vogt *et al.*, 2003). In contrast, only few studies (Krull *et al.*, 1994) have been published that deal with insect neurons although these cells are highly promising candidates for the formation of task related functional and biomimetic neuronal networks on artificial substrates. Sensing mechanisms in insects are controlled by small, but highly specialized nervous systems. The neuronal tissue consists of a low number of cells. Hence, the number of neurons involved in the circuitry for signal processing is small. For example, during wind evoked escape reaction of crickets the signals are propagated by only 20 interneurons to other centers of the brain (Ogawa *et al.*, 2006). In cell culture, these neurons are easy to handle and stable against environmental changes. They form simple but well working *in vitro* neuronal networks. Thus, the approach to grow a small number of cricket neurons in geometrically controlled patterns is a highly attractive alternative to generate biomimetic neuronal networks on artificial surfaces. Patterned adhesion and growth of insect neurons enables the study of how cellular circuits of defined cell populations transfer and process information. Moreover, analytical devices that employ insect neurons combine sensitivity with robustness. Insect cells are also highly adhesive to various surfaces. In order to take advantage of the cellular network, cell adhesion and neurite outgrowth has to be precisely controlled and directed by an appropriate surface modification. So far, this prerequisite has been the predominant reason why geometrically controlled network formation of insect neurons on artificial surfaces could not be achieved.

Neurons of crickets terminal ganglia were seeded onto prepatterned surfaces after full crosslinking of the star PEG films according to methods described previously (Hayashi and Hildebrand, 1990, Kloppenburg and Hörner, 1998). The cells did not adhere on the unmodified star PEG layers, but adhered preferentially to the conA grid pattern and showed first neurites within 12h (data not shown). SEM and phase contrast micrographs of cricket neuronal cells revealed precise geometrical restriction of the neurite growth to the conA-modified surface (Fig.4.3). Neurites often followed the structure at the edge of the patterned lines (see nodes in Fig.4.3A) in accordance with the higher amount of deposited protein at the edges. The neurite length extended over several hundred micrometers resulting in tremendous neurite trees (Fig.4.3B). The neurons conserved this distinct morphology for up to 3 weeks in cell culture indicating an outstanding stability and biocompatibility of the surface modification. The presented surface modification operated effectively for very large

surface areas as documented by phase contrast microscopy over square millimeter sized surface areas (supporting information Fig.S4). Slight variations of protein density in the overall homogeneous protein pattern documented by fluorescence microscopy did not significantly affect the adhesion of the cricket neuronal cells.

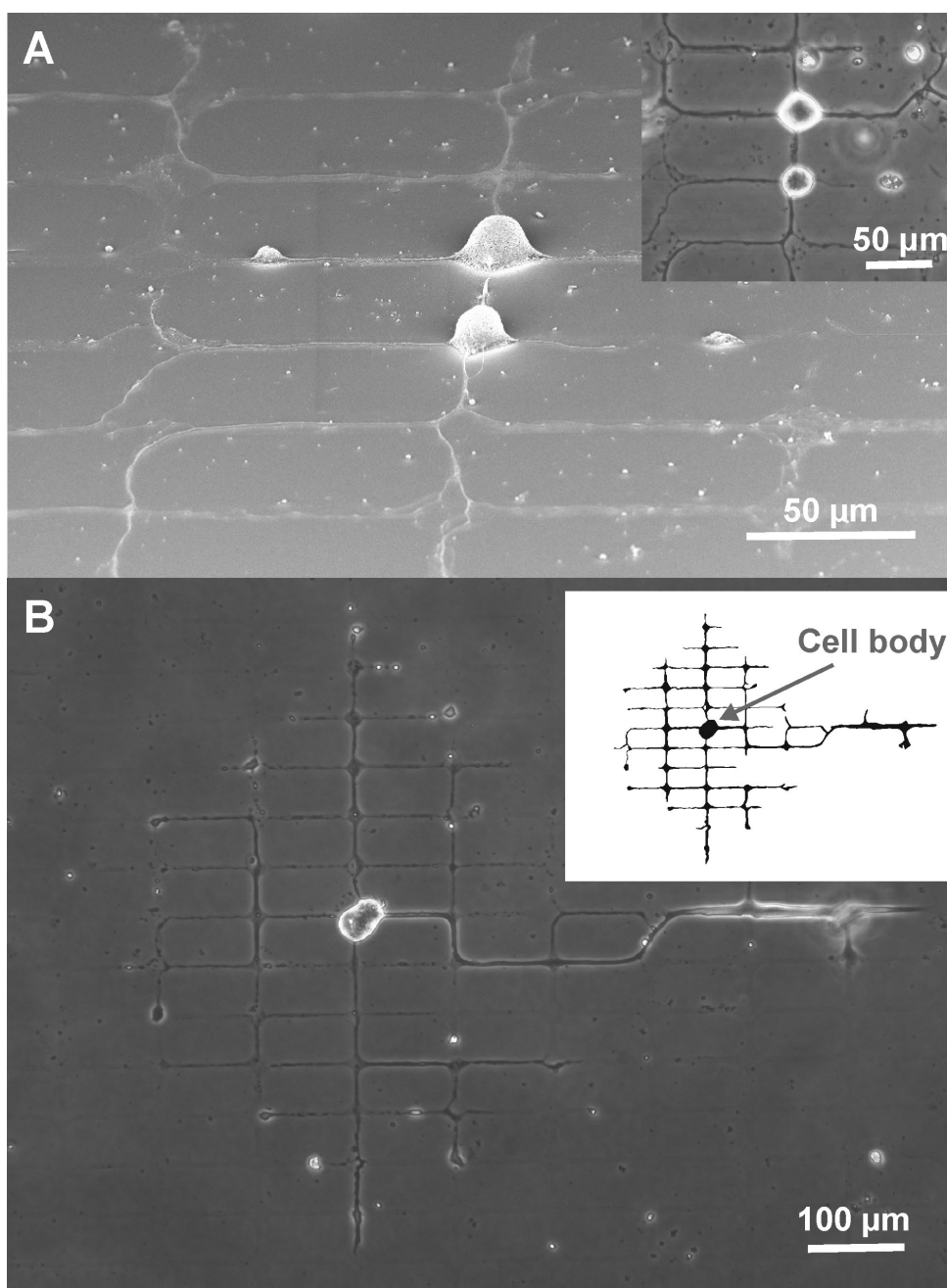


Fig.4.3. Pattern fidelity of cricket neurons grown on a conA / star PEG surface after 8 (A) and 10 (B) days in culture visualized by SEM (A) and phase contrast light microscopy (B). The inlet in Figure 4A shows the same 2 cells before sample preparation for SEM in cell culture by phase contrast light microscopy. Line width: 6µm, node width: 25µm, node distance: 50µm and 100µm.

Besides the generation of a defined morphology the cricket neurons also retained function, as evidenced by electrical activity. Whole cell patch-clamp recordings showed that membrane potential, amplitude and frequency of action potentials (APs) for patterned neurons were in good correspondence to APs for both *in vivo* and unpatterned *in vitro* cricket neurons (data not shown). Double patch clamp recordings revealed cell-cell communication between pairs of morphologically connected neurons via electrical signals (Fig.4.4). Chemical synapses mediate unidirectional signal propagation between two adjacent neurons via an asymmetric interface. The membranes of the pre- and postsynaptic neurons at this interface contain different molecular components and complexes. Triggered by a change in membrane voltage, e.g. APs, the presynaptic neuron secretes signaling molecules (neurotransmitter) into the interface, which bind to receptors of the postsynaptic neuron. The open probability of nearby ion channels in the post-synaptic cell membrane is altered and results in a voltage change (postsynaptic potential, PSP) (Kandel and Siegelbaum, 2000). These signals were recorded by measuring the membrane potential of two connected cells within the network simultaneously, while one of the neurons was stimulated by defined current pulses generated by an operational amplifier. Stimulation of the upper neuron failed to elicit a response in the adjacent neuron (data not shown). However, stimulation in opposite direction resulted in presynaptic APs (black line) of the lower neuron triggering graduated postsynaptic potentials (PSPs, dashed line) in the upper neuron. The first two APs of the lower neurons produced PSPs with amplitudes up to 10mV in the upper neuron, while the response from the following APs was possibly masked by postsynaptic repolarization. Hyperpolarizing presynaptic stimuli did not affect the postsynaptic neuron (data not shown). The synaptic delay between the peak of the presynaptic AP and begin of PSP was approximately 1 ms, which is in the typical range for chemical synapses. The double patch clamp recordings demonstrate the existence of functional synapses in the patterned cricket neuronal networks. These results also emphasize the suitability of the surface modification to control the growth of neuronal networks and excellent biocompatibility of the star PEG system.

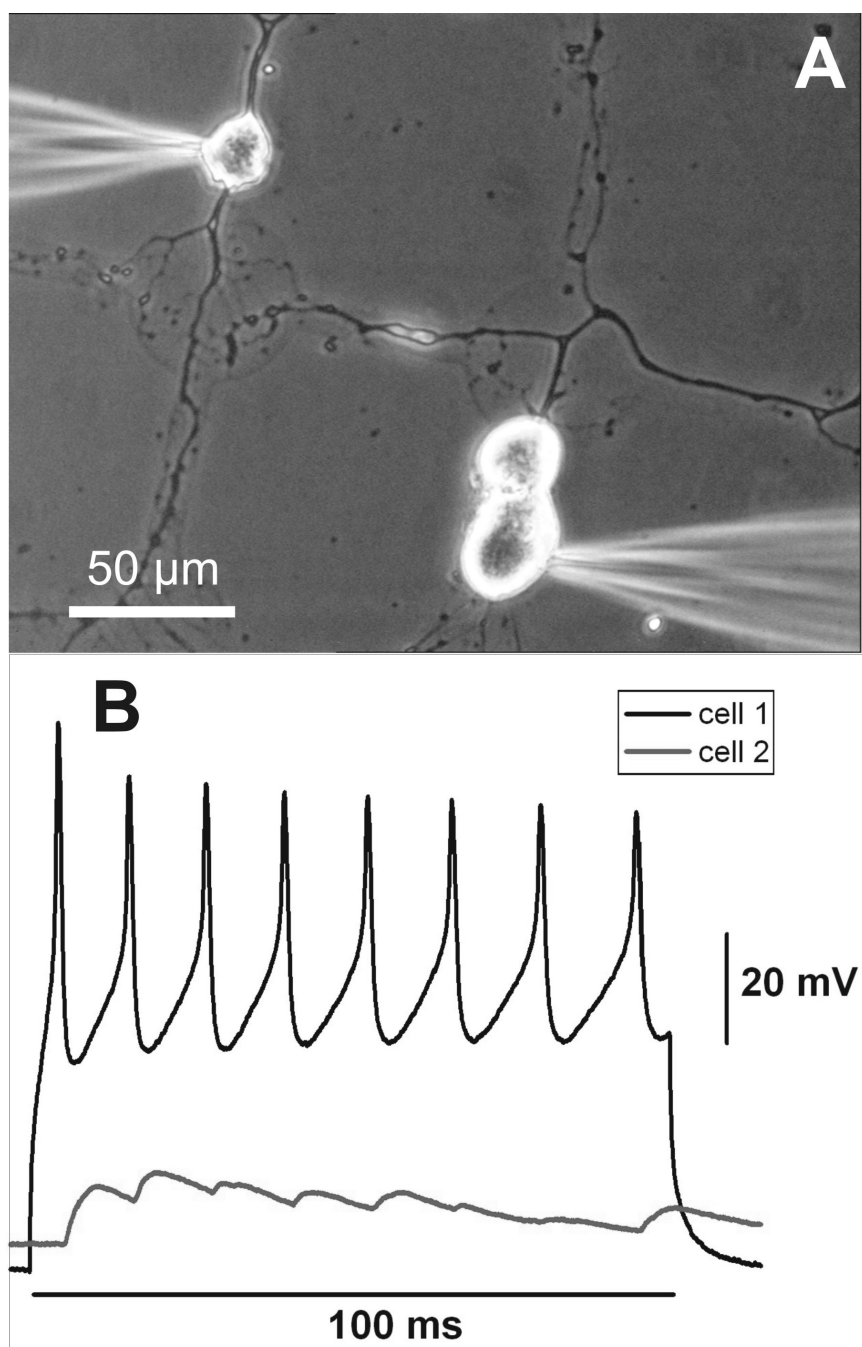


Fig. 4.4. A: Phase contrast light microscopy of double patch clamp recording obtained from a morphological connected pair of 2 cricket neurons after 5 days in culture on a conA / star PEG surface. The image shows the exact position of the patch clamp pipettes during the recording (upper left and lower right). B: Stimulation of the presynaptic cell led to action potentials in this cell (black line) followed by correlated and summated postsynaptic potentials in the postsynaptic cell (grey line), although the neuronal cell bodies have a distance of more than $100\mu\text{m}$, indicating that the synaptic contact is localized on the neurites. The synaptic latency was approximately 1ms, which is a typical value for a chemical synapse.

In conclusion, this study presents a powerful and universal surface modification for the generation of specifically interacting cell culture substrates and biosensors. Biofunctional molecules can be directly introduced without the need for further process steps or chemical crosslinkers due to a unique intrinsic property of this material, in which reactivity changes from highly reactive isocyanate groups into non reactive and cell repulsive. This feature enables binding of both amino and alcohol groups facilitating coupling of various molecules to our material. The applicability of this system to bio-systems is demonstrated by the development of a reliable method for the geometric growth control of cricket neuronal cells through simple microcontact printing and covalent binding of conA onto star PEG films. In cell culture, the insect neurons regenerated tremendous neurite trees exclusively along the protein micropattern. Patch clamp recordings evidenced that the surface modification did not affect the electrophysiological parameters of the neurons. Moreover, we demonstrate the first direct correlation between presynaptic stimulation and postsynaptic events in a patterned insect neuronal cell culture. These results are essential for the construction of defined insect neuronal networks *in vitro*. The broad applicability of the reactivity change from reactive isocyanate groups to an amino group containing cell repellent coating makes star PEG a highly effective and versatile coating system for applications that require the interfacing of biological molecules with artificial surfaces such as biosensors, cell based assays and implantable devices.

MATERIALS AND METHODS

Substrate preparation and star PEG film formation

Samples have been prepared as previously reported (Groll *et al.*, 2004, Groll *et al.*, 2005b). Briefly, glass and silicon substrates were cleaned by ultrasonication (acetone, water, and isopropanol; 1min each), dried in a stream of nitrogen, and activated by a UV/ozone treatment (12 min). Directly after the activation, substrates were incubated in a solution of N-[3-(trimethoxysilyl)propyl] ethylenediamine (0.2 ml) in dry toluene (50 ml) for 2h in a inert gas atmosphere. Then, the samples were washed several times with dry toluene and dried in a stream of nitrogen prior to spin-coating. For preparation of the star PEG film, isocyanate terminated pre-polymer (100 mg; MW = 12 kDa, PD = 1.2) was dissolved in dry tetrahydrofuran (1 ml). After addition of demineralized water (9 ml), this solution was gently stirred for 5 min, completely wetting the substrate. Spin-coating was performed at 2500 rpm for 40 s (R60, Convac). These freshly prepared films were stored at ambient conditions for 2 h prior to μ CP. The parameters chosen for the casting process in this study resulted in star PEG coatings with a thickness of 30 nm. Layer thicknesses were determined using a Nanofilm EP3 ellipsometer with NdYAG Laser (532 nm) and EP3 View 2.05 software. The angle of incidence was 70°.

Microcontact printing (μ CP)

Polydimethylsiloxane (PDMS) stamps with grid patterns (line width: 6 μ m, node width: 20 μ m, node distance: 50 μ m and 100 μ m) were fabricated by pouring Sylgard 184 (Dow Corning, Midland, Michigan, USA) onto silicon masters (fabricated by standard lithographic procedures as described elsewhere [Xia and Whitesides, 1998]) followed by a 12 h curing step at 80°C. Final curing, after master stamp release, was performed for 1 h at 110°C. The stamps were inked by immersion in a conA solution (0.5 mg in 1 ml purified water) for 10min, dried in a stream of nitrogen and placed for 10 min onto the star PEG coating.

Patch clamp recordings

Whole-cell patch-clamp experiments were performed as described previously (Vogt *et al.*, 2003). The culture medium was replaced by a normal salt solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl₂ and 10 mM HEPES; ~390 mOsm/kg adjusted by glucose and pH 7.0 by NaOH). Pipettes were pulled from borosilicate glass filaments (o.d. 1.5mm, i.d. 0.86mm; Sutter Instrument Co., Novato, CA, USA) using a Flaming/Brown micropipette puller (P-97; Sutter Instruments Co., Novato, CA, USA) resulting in a pipette electrode resistance of 4–11 M Ω . Glass pipettes were filled with internal solution (8 mM NaCl, 180 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 2mM Na₂-ATP, 1 mM EGTA and

10 mM HEPES; ~390 mOsm/kg adjusted by equal shares glucose/fructose and pH 7.0 by NaOH). Whole-cell patch-clamp recordings were made using an EPC9/3 amplifier controlled by TIDA 5.x software (both HEKA Elektronik, Germany). The data were sampled with 25 kHz and filtered at 10 kHz with a Bessel filter. The liquid junction potential was calculated using JPCalcW 1.0 software (P.H. Barry, University of New South Wales, Sydney, Australia and Axon Instruments Inc. California, USA) subtracted from the measured membrane voltages. Electrophysiological experiments were performed under optical control (Olympus IX 50 inverted microscope equipped with phase-contrast). All experiments were performed at room temperature.

Cell fixation for SEM

For fixation, the cell culture medium volume was substituted gradually with glutaraldehyde (3.5% in 20 mM HEPES, adjusted to pH 7.0 with NaOH) and incubated over night at 4°C. To dehydrate the cell material, ethanol p.A. was added and the concentration was gradually raised up to approximately 100%. After critical-point-drying in ethanol, the substrates were sputtered with gold for 30s at 50mA under vacuum.

SUPPORTING INFORMATION

The transfer, film thickness, and morphology of the protein were investigated. The SFM image (Fig.S1A) shows the border between unmodified star PEG (top right) and the area where conA has been stamped onto the coating (bottom left). As depicted in the height profile, the average thickness of the deposited protein films was between 2 and 3 nm, resembling a monomolecular layer of proteins. The SEM measurement (Fig.S1B) showed a smooth star- PEG surface (bottom left) on which a conA grid pattern was deposited by μ CP (right). This substrate had been used in cell culture for 2 days before imaging by SEM, further demonstrating the stability of our surface coating system and the micropattern under cell culture conditions. The prior use of the substrate in cell culture and fixation steps for imaging also explains the cell debris (middle part of the picture) and dirt particles on the star PEG film.

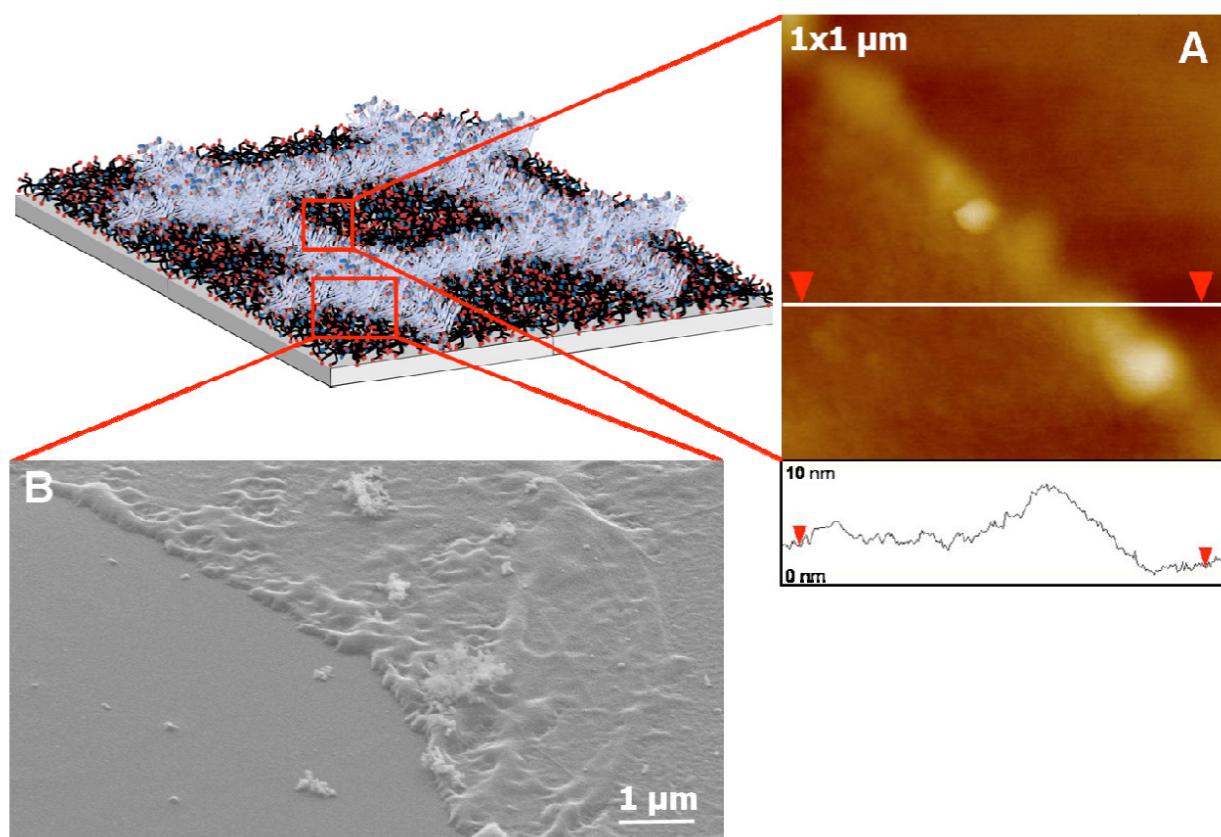


Figure S1. Idealized scheme of the conA grid pattern on the star PEG coating. The two insets show a SFM (A) and a SEM (B) image of boundary between star PEG and conA. The SEM picture was taken from a substrate that has been used for cell culture experiments before demonstrating the long term pattern stability. Z-limit of the SFM picture is 40 nm, the height of the profile 10nm. SEM measurements were performed using a Zeiss Gemini LEO 1550 operating at 10 kV. SFM investigations were obtained with a nanoscope IIIa (Digital Instruments) operating in Tapping ModeTM. The oscillation frequency for Tapping ModeTM was set in the range of 320 – 360 kHz depending on the Si cantilever ($k \sim 50$ N/m, Nanosensors).

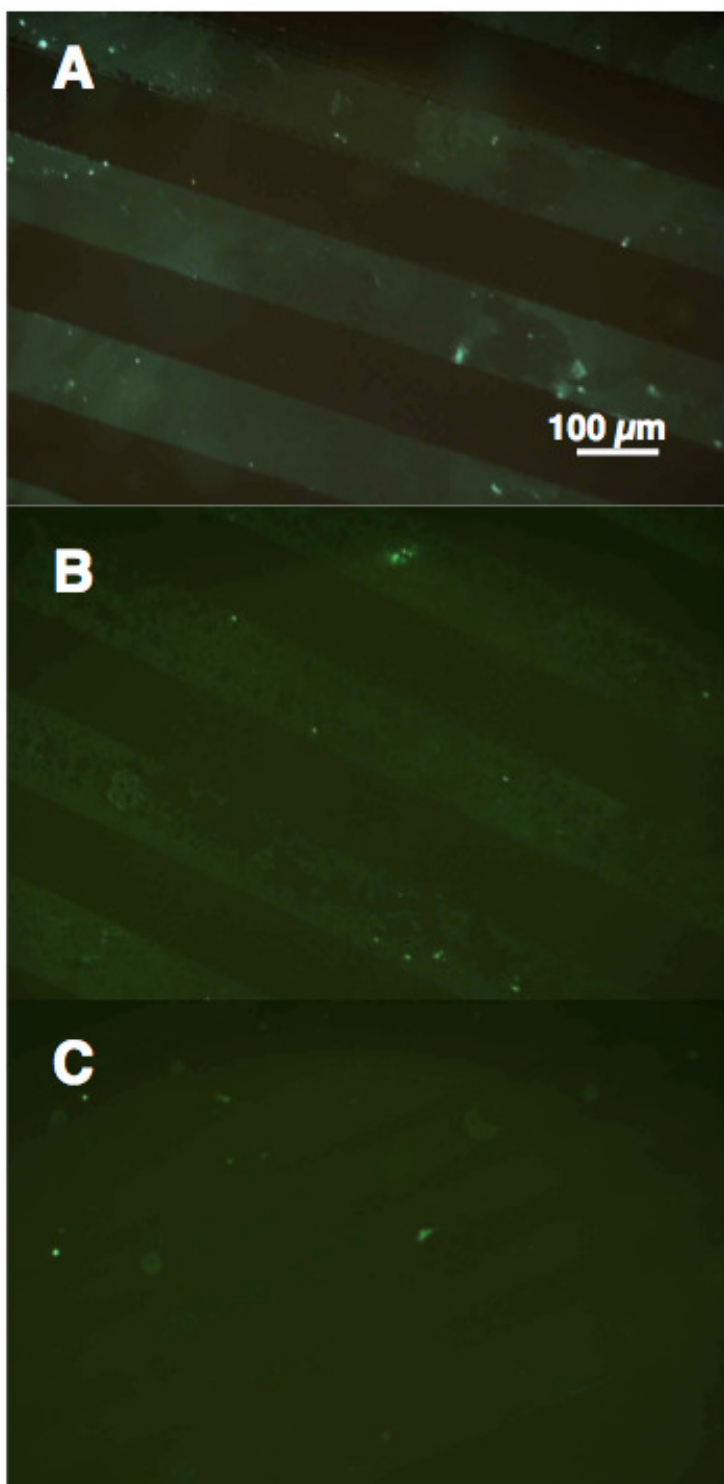


Figure S2. These fluorescence microscopy images show examples of the washing experiments mentioned in the manuscript. Panel (A) shows conA stamped onto a star PEG film 2 h after coating without washing, whereas panel (B) shows the same substrate after washing with PBS buffer 16h after coating. This step washes off loosely bound protein and only covalently bound protein remains on the substrate. Panel (C) shows conA stamped onto a star PEG film 16 h after coating. The structure is hardly visible in the image (lines from bottom left to top right). The transferred proteins were apparently loosely bound onto the surface and could be washed off. All pictures were taken with the same integration time.

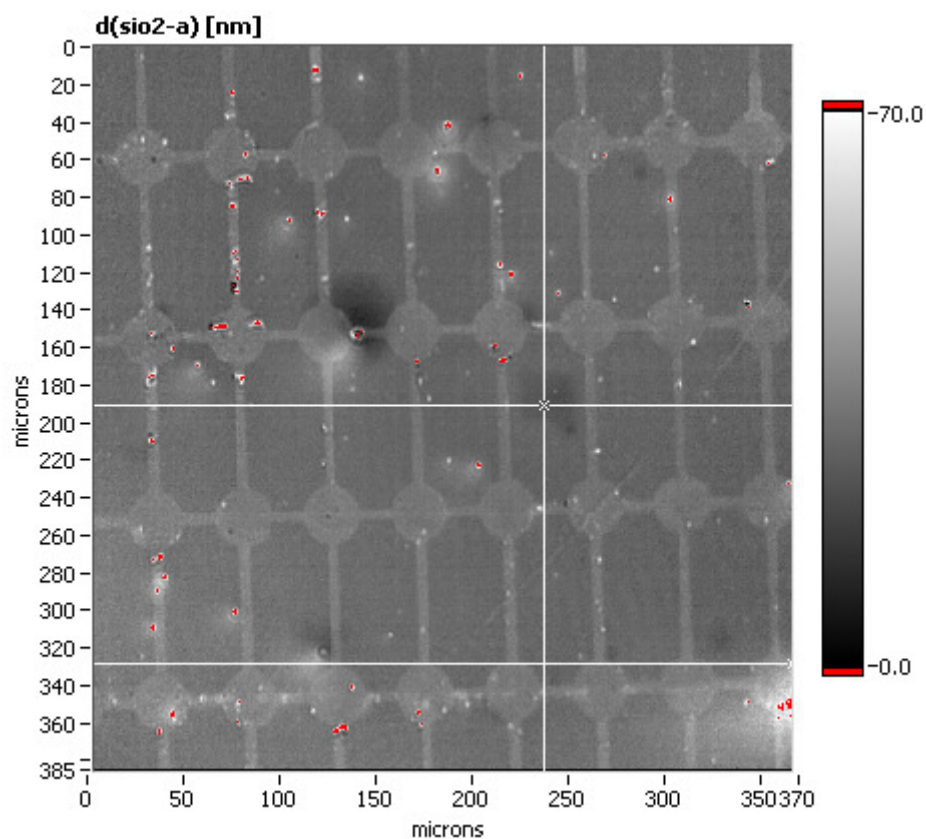


Figure S3. Ellipsometry mapping of a conA patterned star PEG surface (grid pattern; line width: 6 μm ; nodes width: 20 μm ; grid pattern 50 x 100 μm). The pattern transfer was detected in accordance to the fluorescence microscopy images. The red dots origin from dust contaminations. The image has been taken using a monochromatic ellipsometer operating at 532 nm wavelength with a lateral resolution of 1 μm .

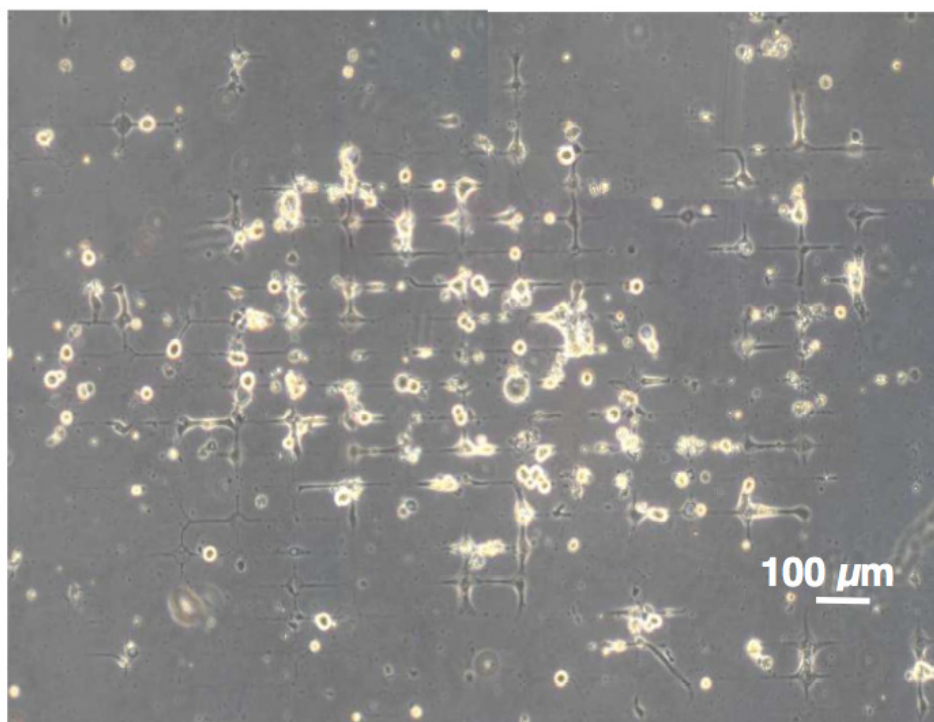


Figure S4. Composition of several light microscopy images to give an overview about the neuronal pattern formation on the substrates. The picture was taken after 3 days of cell culture.

5 Surface growth restriction in insect neuronal cell culture promotes *in vivo*-like phenotypes

Based on:

Surface growth restriction in insect neuronal cell culture promotes *in vivo*-like phenotypes

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In preparation

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ABSTRACT

In vitro neuronal cultures are often used to simplify neuronal networks in order to better understand complex biological information processing systems. Insects represent a unique model system of neuronal processing since their central nervous system is easily accessible, contains moderately simple networks, and single neurons can be identified and isolated for studies of controlled environmental input. However, *in vitro* culture of insect neurons often results in cell morphologies uncharacteristic of those found *in vivo*. In this study, we analyzed primary neurons of the cricket *Gryllus bimaculatus* terminal abdominal ganglion on surfaces of various 2-D geometries with the intention of reprogramming the outgrowth of dissociated neurons using simple *in vitro* guiding cues. Presenting cell adhesion-promoting lectin patterns on an aversive background, we investigated the surface pattern influence on single neuron morphology and electrophysiological characteristics. By restricting the neurite outgrowth to defined line and grid structures, the cell morphology was shifted from the *in vitro*-like multipolar type towards the physiologically relevant uni- or bipolar outgrowth. The amount of neurons sprouting multipolar processes changed from $89\pm4\%$ on uniformly coated surfaces to $40\pm19\%$ on pre-patterned surfaces whereas unipolar growth rose from $5\pm4\%$ to $28\pm9\%$ on the defined patterns. Furthermore, we found that the number of possible pathways available for neurite growth within the pattern had no significant effect on this morphological shift. Basic electrophysiological parameters, such as resting membrane potential and response type distribution, were found to be the same for guided and unguided neuron populations. Our results suggest that simple guiding cues presented on the culture surface influence the neurite regeneration to a more *in vivo*-like morphology compared to unguided growth morphology on uniformly coated surfaces. However, this morphological shift did not affect the basic electrophysiological properties in cell culture, suggesting that genetic pre-programming has a stronger influence on electrophysiology than morphology.

INTRODUCTION

Primary neuronal cell cultures from invertebrates have been used as model systems enhancing our understanding of basic neurobiological mechanisms for almost two decades due to the simplicity and availability of their nervous systems. Notably, Rayport and Schacher were able to reconstruct the simple withdraw circuit of *Aplysia* in the mid-eighties (Rayport and Schacher, 1986). Furthermore, comparisons of vertebrate and invertebrate nervous systems have shown many common genetic and functional characteristics (Laurent, 1999, Selverston, 1999, Sanchez-Soriano *et al.*, 2005, Cayre *et al.*, 2002). These studies suggest that we can use *in vitro* invertebrate networks to model neuronal network function and formation in higher ordered animals. *In vitro* neuronal insect culture systems also present an excellent model system for neuronal cell-based sensors or coincidence detectors for applications in comparative and biomedical research. Understanding single steps in the complex process of network development also increases our understanding of *in vivo* neuronal regeneration, which could be used to develop strategies for nerve regeneration and tissue engineering.

When studying *in vitro* neuronal networks, maintaining *in vivo* conditions regarding the morphology of single neurons, their interconnections, and their physiology improves the functional relevance of the study. Both the synaptic junctions between adjacent neurons within the network and the branching pattern of a single neuron play critical roles in the electrical properties of a neuron as well as in its ability to compute and store information within a functional network (Hausser *et al.*, 2000, Koch and Segev, 2000). In order to fully comprehend network activity, it is crucial to gain control over connectivity patterns. Establishing simple one-to-one connection patterns will help us understand network dynamics. Guiding neurite outgrowth is the first step towards building a controlled and functional network of primary neurons *in vitro*.

Although the insect nervous system consists of a relatively small number of neurons within one functional unit, these systems are highly efficient in processing information. The house fly *Musca domestica* controls the direction of flight with only few neurons (Ruffier *et al.* 2004), and locusts precisely trigger their escape jump with a network on the order of tens of neurons (Heitler and Burrows, 1977). The cercal system, which crickets use to detect changes in environmental air flow, consists of fine mechanosensory hairs at the cerci (Gnatzy and Schmidt, 1971) which stimulate sensory afferents projecting into the terminal abdominal ganglion (TAG). The perceived stimuli are assumed to be pre-processed by interneurons in the TAG (Gras and Kohstall, 1998) to provide fast information processing leading to an appropriate escape reaction in case of predator approach. The wind sensitive giant interneurons (GI) within the TAG code for specific wind directions and velocities ensuring a precise escape motion (Bodnar *et al.*, 1991, Matsuura and Kanou, 2003, Kanou *et al.*, 2006).

The neurons in the TAG of crickets are anatomically and physiologically well-characterized *in situ* and therefore provide an excellent base for analysing the influence of various cell culture parameters on vitality and functionality of this system. The typical unipolar invertebrate neuron morphology is characterized by a single process arising from the cell body which then branches to generate axons and dendrites from a common main branch. However, some sensory neurons are bipolar. *In vivo* multipolar neurons in the central nervous system have not been described to date (Grueber *et al.*, 2005) although this morphology is frequently observed *in vitro* (Sanchez-Soriano *et al.*, 2005, Howes *et al.*, 1991). Insect neurons exhibit a unique morphology combined with highly specific physiological properties (Burrows, 1996); thus, neuronal morphology should be considered when investigating information processing of neuronal networks *in vitro*.

We were interested in guiding neurons and controlling their basic outgrowth to offer a means for the generation of simple networks based on neurons of TAG of crickets. While using a chemical surface patterning approach including star-shaped poly(ethyleneglycol) (star PEG) and concanavalin A (conA) described previously (Reska *et al.*, 2008), we observed a striking reduction in the number of neurites when comparing the patterned to the unpatterned neurons in culture. Therefore, we examine in the present study the influence of 2-D geometrical structures on the cell morphology and electrophysiological characteristics to determine if changes in morphological type are accompanied by changes in functional characteristics.

MATERIALS AND METHODS

Male and female crickets, *Gryllus bimaculatus* were bred with light:dark cycle 12:12 hours. Cells were isolated from adult animals within one week after adult molt. Reagents were purchased from Sigma unless otherwise noted.

Cell culture of cricket neurons

Neurons of crickets terminal ganglia were cultured according to methods described previously by Hayashi and Hildebrand (1990), Kloppenburg and Hörner (1998) with slight modifications. The terminal ganglion of *Gryllus bimaculatus* was excised, washed, and desheathed. The neurons were dissociated by enzyme treatment (dispase 2 mg/mL; collagenase 0.5 mg/mL, 3 min at 37°C) followed by trituration with a silanized 200 μ L tip. The enzyme reaction was stopped by addition of pre-cooled medium supplied with yeastolate (0.28 g/mL) and lactalbuminhydrolysate (1.4 g/mL) followed by a centrifugation (5 min, 8°C, 2800 rpm). The neurons were plated on surfaces coated with conA or respective star PEG / conA patterns and cultured in modified Leibovitz medium [(L-15, glucose (2 g/L), fructose (0.8 g/mL), proline (0.35 g/mL), imidazole (0.06 g/mL), glutamine (0.3 g/mL), adjusted to pH 7.0 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Roth) (360 milliosm/kg)] in Nunc 35 mm Petri dishes containing a volume of 3 mL in 95 % relative humidity at 29°C.

For the experiments, 17 independent preparations have been carried out, counted and statistically evaluated. For each preparation, three animals were seeded on nine surfaces; two of each conA grid pattern type (Fig.5.1), two positive controls (coverslips coated uniformly with conA) and one negative control (uniformly star PEG coated surface).

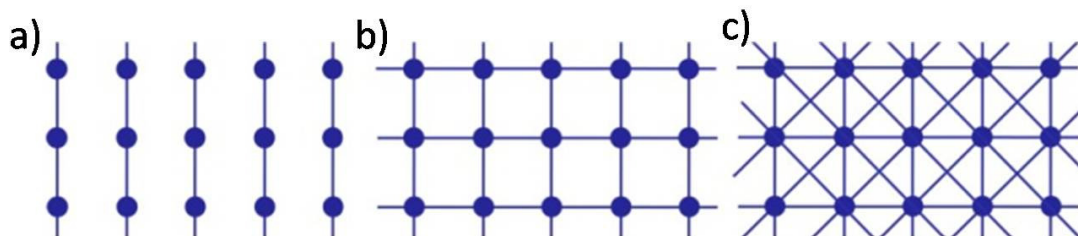


Fig.5.1. Three different node and line patterns provided for neuronal growth. 30 μ m nodes offer two, four or eight possible growth directions in form of 4 μ m wide lines.

Uniformly conA-coated surfaces

Glass coverslips were activated by oxygen plasma treatment. 100 μ L of a conA solution (0.2 mg/mL) in deionized water were incubated for 60 min and subsequently washed three times with sterile deionized water.

Surface coatings with star PEG / conA grid pattern

Samples have been prepared as previously reported (Reska *et al.* 2008). Briefly, glass and silicon surfaces were cleaned by ultrasonification in isopropyl alcohol for 5 min and dried in a stream of nitrogen, then washed with deionized water and activated by a MetOH / HCl (1:1) treatment (30 min), washed and dried again. Directly after activation, surfaces were aminosilanized in a glovebox (MB Braun 100) with N-[3-(trimethoxysilyl) propyl] ethylenediamine (0.2 mL) for 1 h in an argon gas atmosphere prior to spin-coating. For preparation of the star PEG film, isocyanate-terminated prepolymer (100 mg; MW 12 kDa, polydispersity (PD) 1.2) was dissolved in dry tetrahydrofuran (1 mL). After addition of deionized water (1:10), the solution was incubated for 5 min, and applied to the surface. Spin-coating was performed at 2500 rpm for 40 s (Laurell, WS-400E-&NPP-Lite). These freshly prepared films were stored in ambient conditions for 2 h prior to microcontact printing (μ CP) of conA. After μ CP, the surfaces were left for further cross-linking over night before they were used for cell culture experiments.

Microcontact Printing

Patterns of conA were generated by μ CP (Xia & Whitesides, 1998) using microstamps polymerized on lithography masters. Diverse grid patterns were transposed to a chromium coated quartz wafer by electron beam writing. This wafer was used as a mask to fabricate the stamp master by UV photolithography on a Si [100] wafer coated with positive resist, and pattern transfer by deep Reactive Ion Etching (2.5 μ m deep). After coating with fluorsilane [(tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane, from ABCR], this Si master could be reused more than 50 times. Polydimethylsiloxane (PDMS) stamps were then fabricated by pouring Sylgard 184 (Dow Corning, Michigan, USA) with a 1:10 curing ratio onto these masters followed by a 12 h curing step at 80°C. Final curing, after master stamp release, was performed for 1 h at 110°C. The stamps were inked by immersion in a conA solution (0.5 mg/mL deionized water) for 10 min, dried in a stream of nitrogen, and brought into conformal contact with the star PEG layer for 10 min. Three different conA grid patterns were

used for morphology type classification: 4 μm wide lines with 30 μm nodes providing two growth directions (Fig.5.1 a), a grid of 4 μm lines and 30 μm nodes (four possible growth directions) (Fig.5.1 b) and a grid with crossing lines (Fig.5.1c), having at most eight possibilities for neurite outgrowth.

Calcein staining

Live cell staining was performed by adding calcein-AM solution to 1.5 mL cell culture medium resulting in a working concentration of 2 μM .

Microscopy and Statistical Analysis

Cell culture preparations were evaluated using a Zeiss Axioscope equipped with an Axiocam digital camera. Images were taken using Zeiss Axiovision 4.5 software. Per preparation, one sample of each surface type was analyzed. Neurons were counted after 5 days of *in vitro* culture as the neurons reached their final shape at this time point but were not contacting each other. It was observed that neurons did not change their polarity after that time in culture. We counted the number of adherent neuronal cell bodies and the number neurites originating from the cell body. The percentages of outgrown neurites were analyzed with respect to the number of possibilities offered to the neuron. On the pre-patterned surfaces, only neurons adhering to nodes were taken into account. The major branches sprouting from the cell body were considered. A neuron that developed one or two main branches and multiple fine branches was considered unipolar or bipolar, respectively. All quantitative data are given as mean $\pm$ standard deviation of the mean (SDM). Error bars represent SDM. A two tailed t-test was used for statistical evaluation of measurements between two experimental groups.

Electrophysiology

Whole cell patch clamp recordings from the cell bodies were performed at room temperature in cell culture medium on the stage of an upright Zeiss microscope equipped with phase contrast. Patch pipettes were pulled from borosilicate glass capillaries using a horizontal Flaming/Brown micropipette puller (P 97, Sutter Instruments, USA). The resistance of the pulled electrodes ranged between 8 and 15 Ω . An intracellular solution containing 150 mM KAsp, 6 mM MgCl_2 , 1 mM EGTA, 1 mM CaCl_2 , 1 mM Na_2ATP and 10 mM HEPES, adjusted to pH 7.0 (360 mOsm) was used. The signals were sampled at 25 kHz and amplified with an

EPC9/3 amplifier (HEKA Electronic, Lambrecht, Germany). A 10 kHz Besselfilter was used. The liquid junction potential was calculated using JPCalcW 1.0 software (P.H. Barry, University of New South Wales, Sydney, Australia and Axon Instruments Inc. California, USA) to be at most 3.7 mV and, hence, was not considered in our experiments.

RESULTS

Morphological characteristics

Using surface patterns of different geometrical structures, we examined whether and to what extent the regrowth of axotomized neuronal insect cells *in vitro* is influenced by geometrical guiding cues. For this purpose, we used four different surfaces. The first surface was uniformly coated. The other three surfaces presented a pattern of concanavalin A on a protein repellent star PEG background with up to two, four or eight potential pathways for neurite outgrowth (see materials & methods section). Neuronal morphology was classified as multipolar, bipolar or unipolar according to the number of primary neurites as seen in Fig.5.2. Counting the cell bodies and respective primary neurite number on each pattern type, the percentage of unipolar, bipolar, multipolar or neurons without neurites was determined and related to the total cell number. Neurons with at least three primary neurites were considered multipolar. Using this straight-forward approach, we show that such unspecific external cues are sufficient to shift the basic cell morphology in *in vitro* culture towards a typical physiological shape.

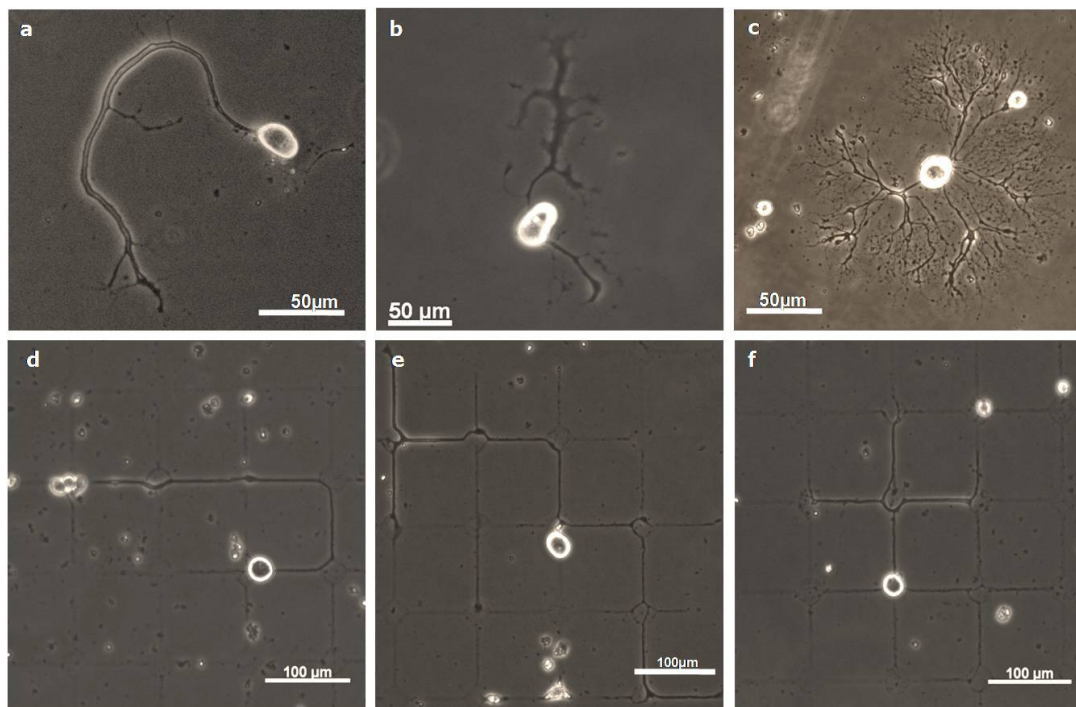


Fig.5.2. Representative images of the three different cell morphologies of cricket neurons grown on planar conA-coated glass surfaces: (a) unipolar with one neurite spreading from the soma, (b) bipolar with two main branches (c) and multipolar. Similarly on star PEG /conA pre-patterned surfaces with four possible paths: (d) unipolar, (e) bipolar, and (f) multipolar.

On uniformly conA-coated surfaces, neurons generally developed multipolar neurite extension in accordance with previous findings for locust neurons (Kirchhof and Bicker, 1992) and cockroach neurons (Smith and Howes, 1996). Neurons from this class exhibited numerous fine neurites that formed a meshwork around the soma (Fig.5.2 c). Unipolar neurons had a single long primary neurite with branchings at the distal end (Fig.5.2 a). The average number of adherent neurons on uniformly coated glass slides was 103 ± 36 while only 45 ± 13 adhered to star PEG / conA pre-patterned surfaces in spite of plating the same total number of neurons during the preparation. A possible explanation for this disagreement might be a difference in total adhesive surface available for cell attachment (100% attachment area was available for uniformly conA covered surfaces versus 8%, 11% or 16% attachment area available for each pattern type, respectively). Neurons that were observed over several days after initial adhesion on nodes of pre-patterned surfaces did not appear to migrate. In some cases, the cell soma shape became more rounded over time, but the soma diameter usually remained constant (data not shown). The dissociation method during cell isolation from the tissue was intended to axotomize the neurons such that all of the neurons initially adhered without an axon. However, in the rare case of a neuron with a remaining axonal stump, the axon regrew from this point. As this effect was observed on both patterned and unpatterned surfaces to the same extent and primary neurites deriving from such stumps could not be distinguished from rebuilt primary neurites, this aspect was disregarded. Normally, neurons started to form neurites after several hours *in vitro*. While some neurons grew neurites after 1-2 hours, others only grew neurites after up to 24 hours. If neurite growth did not appear by 24 hours, these neurons did not grow processes at all. Larval neurons showed less outgrowth than neurons from adult animals and thus young adult animals (within one week after their final molt) were used for these experiments.

The typical multipolar outgrowth displayed by neurons plated on unpatterned uniformly conA-coated surfaces is shown in Figure 5.2 c. A neuron was considered multipolar having at least three neurites. Figure 5.2 a shows the unipolar type with one primary neurite and Figure 5.2 b shows a representative bipolar neurite with two main branches. Only few neurons regrew those morphologies on uniformly coated conA surfaces, in spite of their predominance *in vivo*. On the pre-patterned surfaces, the same three outgrowth morphologies as on uniformly coated surfaces were observed. Figure 5.2 d-f presents examples of neurite outgrowth on grid structures with four possible outgrowth paths. The unipolar neuron in Figure 5.2 d grew one main branch to the right side and two fine neurites to the left and bottom line. Two main branches grew and spread over more than 3 quadrants from the cell soma of the bipolar neuron in Figure 5.2 e. Figure 5.2 f shows a multipolar

neuron with four equally thick neurites from the cell soma in each direction forming also secondary neurites.

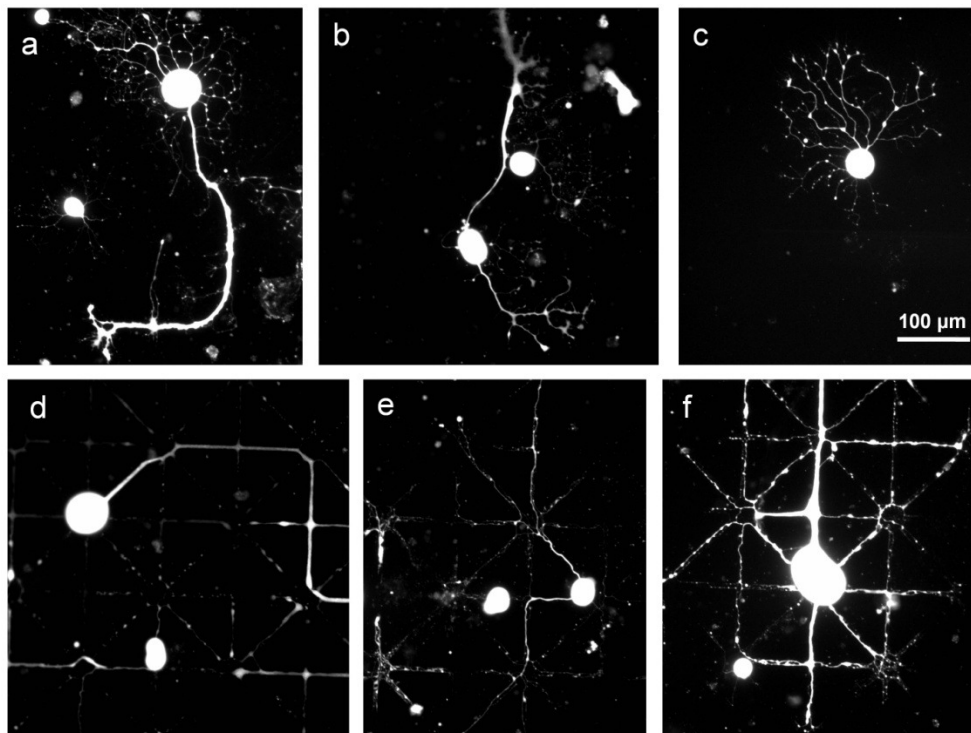


Fig.5.3. Calcein stained cricket neurons on conA-coated glass slides (a-c) and star PEG / conA patterns (d-f). Neurites are visualized down to filopodial level on uniformly coated surfaces and on patterns restricted to pre-defined structures. (a) and (d) unipolar neurons, (b) and (e) bipolar neurons (c) and (f) multipolar neurons.

To ensure fine neurites sprouting from the cell soma were not missed when classifying the neuronal morphology type, neurons were stained intracellularly using calcein-AM. The staining revealed fine filopodial structures sprouting from the main neurite that could not be resolved by light microscopy (see Fig.5.3 a) confirming the existence of main thick branches as well as fine dendritic-like meshes (Fig.5.3 c) and the confinement of all neuronal processes to the conA patterns on star PEG background (Fig.5.3 e,f). On uniformly conA-coated surfaces, the typical neurite meshwork displayed fine neurites as well as filopodial structures (Fig.5.3 a,b,c). On pre-patterned surfaces, the neurite outgrowth was strictly limited to line structures whereas thickened varicosities and small mesh-like structures also were formed on node structures (Fig.5.3 g).

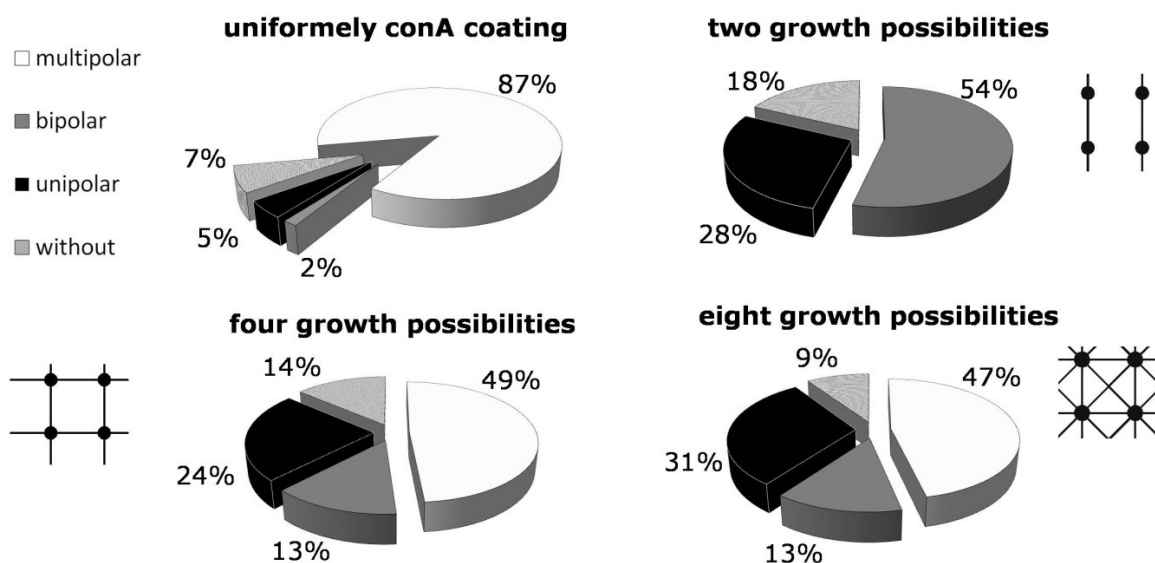


Fig.5.4. Comparison of percentages of each morphological type on different surface pattern. The amount of uni- (black) and bipolar (grey) growth types increased significantly on patterned surfaces while the multipolar type (white) decreased. This effect is independent on the number of potential paths for neurite extension.

Figure 5.4 displays the respective percentage of each neuron morphology type on the surfaces with variable potential outgrowth paths. The multipolar morphology significantly dominated the morphology of neurons grown *in vitro* on uniformly coated surfaces. $87 \pm 5\%$ of all neurons on uniformly conA-coated surfaces were multipolar. Only $5 \pm 4\%$ developed a unipolar morphology although this morphology is predominant *in vivo* and only $2 \pm 1\%$ exhibited a bipolar morphology. 7% of the neurons showed no neurite extensions. As presented in Fig 5.4 b-c, the percentage of unipolar and bipolar neurons increased on star PEG / conA patterned surfaces compared to uniformly conA-coated surfaces. When presented with a pattern of two growth paths from each node, $28 \pm 9\%$ of the neurons grew unipolar, $54 \pm 12\%$ grew bipolar processes and $18 \pm 10\%$ had no neurites (Fig. 5.4 b). Since the line pattern only presented two possible growth paths, the multipolar class was not present. On the grid pattern with two lines crossing each node (four possibilities), $49 \pm 10\%$ of the neurons grew in a multipolar manner, $24 \pm 9\%$ were unipolar, $13 \pm 6\%$ neurons displayed bipolar morphology and $14 \pm 7\%$ had no processes (Fig.5.4 c). On pre-patterned surfaces with eight potential paths for neurite extension, similar results were obtained: $47 \pm 12\%$

multipolar, $31 \pm 10\%$ unipolar and $13 \pm 6\%$ bipolar neurons were observed while $9 \pm 9\%$ did not develop any processes (Fig.5.4 d).

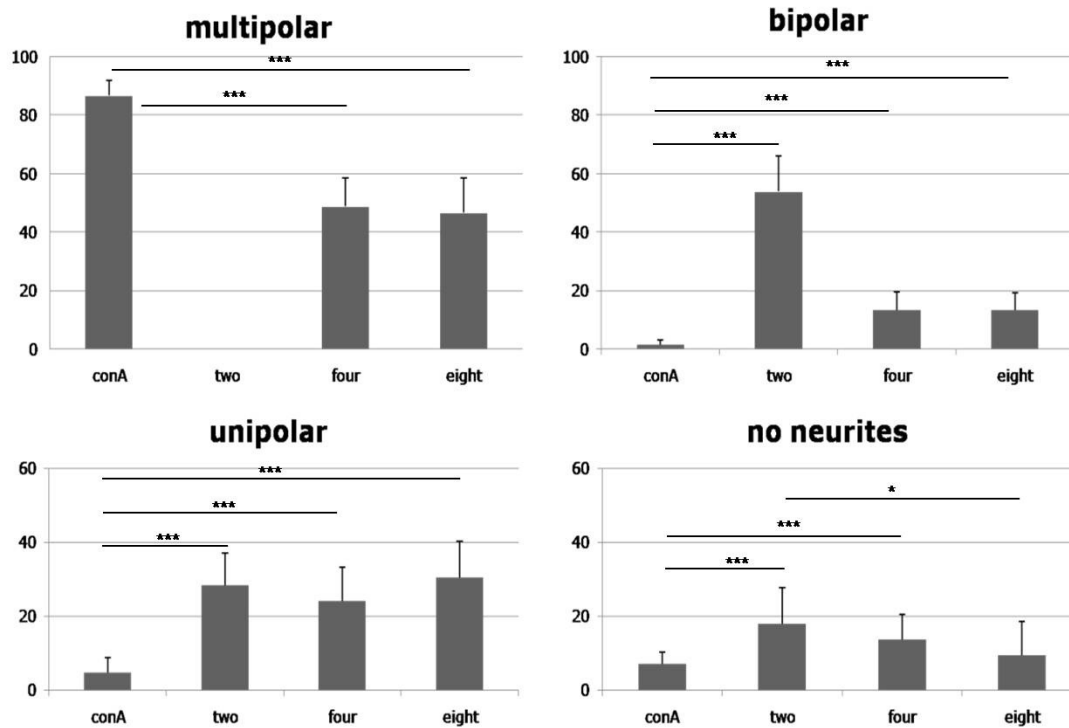


Fig.5.5. Comparison of cell morphology types depending on surface pattern. The amount of all morphological outgrowth types differs significantly between patterned and un-patterned surfaces but is not dependent on the amount of outgrowth possibilities presented. The bipolar type is outstanding high on the two possibility-pattern as it was the maximal neurite number possible in this case.

When comparing the amount of a particular morphological type on varying surfaces (as shown in Fig.5.5), the direct influence of the amount of paths offered for neurite outgrowth can be exposed. The fraction of multipolar neurons is significantly lower on any of the patterns independent of the amount of outgrowth paths presented ($p < 0.005$) compared to conA. Unipolar neurons show the opposite effect in that their number increases significantly on patterned surfaces ($p < 0.005$). A similar situation was observed for bipolar neurons where their number increased greatly on surfaces with two growth possibilities. This increase in bipolar neurons is likely due to inclusion of the multipolar growth type due to a lack of available adhesive area. However, the bipolar morphology still remained significantly higher on surfaces with four or eight possible growth paths compared to unrestricted growth on uniformly conA-coated surfaces ($p < 0.005$). The number of neurons without neurites was significantly higher on pre-patterned surfaces offering two and four possible paths ($p < 0.01$).

The pre-patterned surfaces significantly promoted the growth of unipolar and bipolar cell morphologies. In all, these results indicate that the use of patterned surfaces induces a significant shift from the non-physiological multipolar morphology to a more *in vivo*-like unipolar or bipolar morphology.

Electrophysiological characteristics

Whole cell patch-clamp experiments of cricket neurons displaying various numbers of potential paths and uniformly conA-coated surfaces were performed to investigate the general fitness of the neurons and compare basic electrophysiological parameters as well as the distribution of different response types. Neurons grown in culture were electrically active during a range of two days up to three weeks after plating. Four different response types were identified (see Fig.5.6). Spontaneously active neurons that were silent after clamping their membrane potential to -70 mV and responded with a row of action potentials to current stimulation (200 pA) were termed multi spiker (Fig.5.6 a). Single spikers fired one single action potential under the same experimental conditions (Fig.5.6 d). Also higher stimulation currents (up to 1 nA) did not increase the number of action potentials. Non spiking neurons showed an outward current but were not excitable at all upon stimulation (Fig.5.6 c). A recording of a periodic signal with decreasing amplitude (like a damped signal) was assigned the damper response type (Fig.5.6 b).

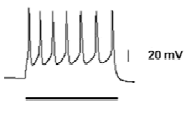
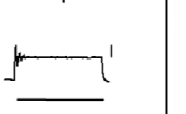
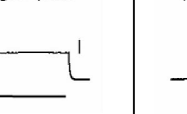
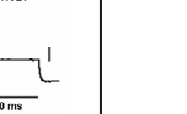
		multi spiker a) 	damper b) 	single spiker c) 	non spiker d) 
percentage	conA	43%	13%	21%	23%
	pattern	32%	12%	26%	30%
M _{pot}	conA	-48±7 mV	-39±6 mV	-40±7 mV	-39±9 mV
	pattern	-46±9 mV	-45±5 mV	-39±8 mV	-42±10 mV
AP amplitude	conA	68±20 mV	45±16 mV	32±7 mV	
	pattern	59±16 mV	32±6 mV	33±13 mV	

Fig.5.6. Electrophysiological response types on uniformly conA-coated (white background) and pre-patterned (grey background) surfaces. The distribution of response types and the mean resting potential as well as the action potential amplitude did not vary between the neurons grown on uniformly conA-coated surfaces compared to neurons on pre-patterned surfaces.

The largest fraction of neurons on both surface types were multi spiking (43% on conA and 32% pre-patterned) whereas the damper signal was the smallest fraction for both surface types (13% conA and 12% pre-patterned). Single spikers and non-spikers were in the mid range with 21% conA and 26% pre-patterned and 23% conA and 30% pre-patterned, respectively. These results reveal that the distribution of the response type does not differ in the population of neurons grown on uniformly coated compared to neurons grown on pre-patterned surfaces.

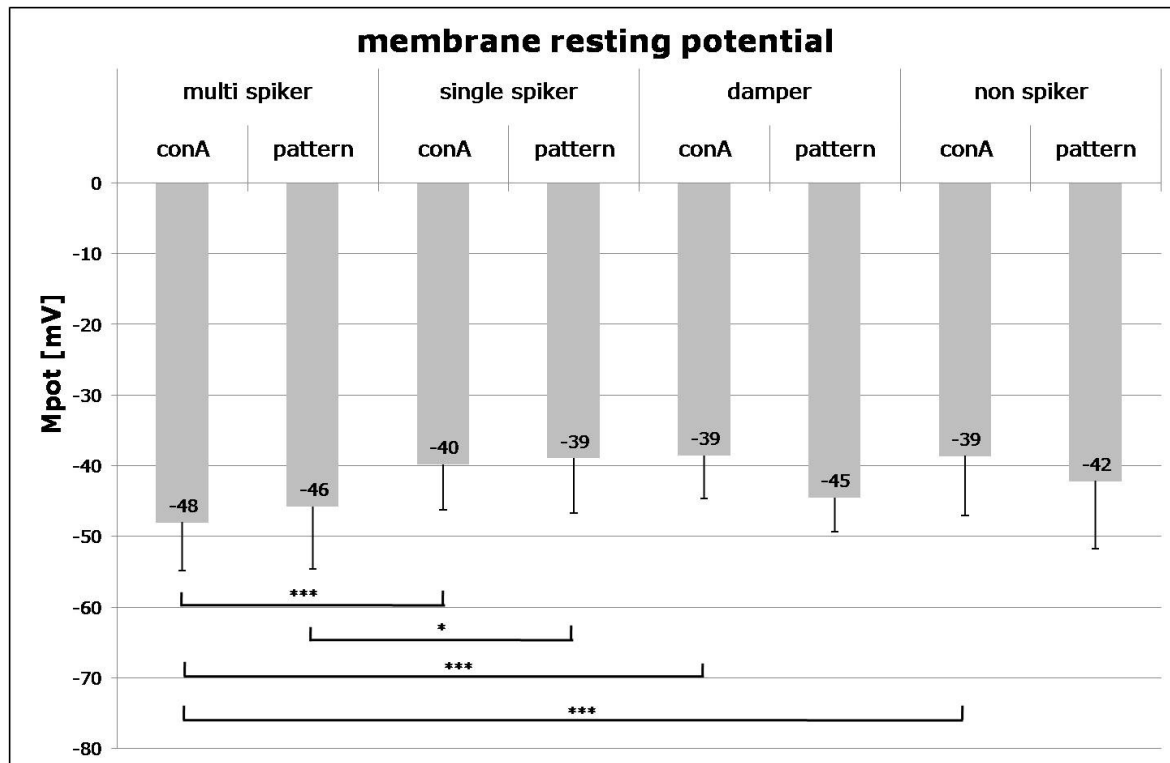


Fig.5.7. Resting membrane potentials of the four electrophysiological response types. The resting potential of the multi spiker differs significantly from the single spiker on uniformly conA-coated and on star PEG / conA pre-patterned surfaces.

The mean resting potential of multi spikers on uniformly coated conA surfaces was -48 ± 7 mV while it was -45 ± 9 mV on pre-patterned surfaces (Fig.5.7). Compared to the single spiker, this value was significantly lower for both neuron populations (single spiker on conA -40 ± 7 mV, on pattern -39 ± 8 mV). For multi spikers on conA, the difference was also significant when compared to damper and non spiker cells. On pre-patterned surfaces, a difference in resting potential comparing -46 ± 9 mV with -45 ± 5 mV and -42 ± 10 mV was also found, but it was not significant. The damper had a mean resting potential of -39 ± 6 mV on conA and -45 ± 5 mV on the patterns, whereas the non spiker had -39 ± 9 mV on conA and -42 ± 10 mV on the patterns (see Fig.5.7).

The mean action potential amplitude of a multi spiker was 68 ± 19 mV compared to 33 ± 7 mV of the single spiker and a maximum 45 ± 16 mV of the damper with a stimulation of 1 nA on uniformly coated surfaces. Comparable parameters were found on the pre-patterned surfaces such that the mean amplitude of a multi spiker was 59 ± 16 mV compared to 33 ± 13 mV of the single spiker and a maximum 32 ± 6 mV of the damper (Fig.5.8). The multi spiker amplitude differed in both cases significantly from the single spiker amplitude (Fig.5.8).

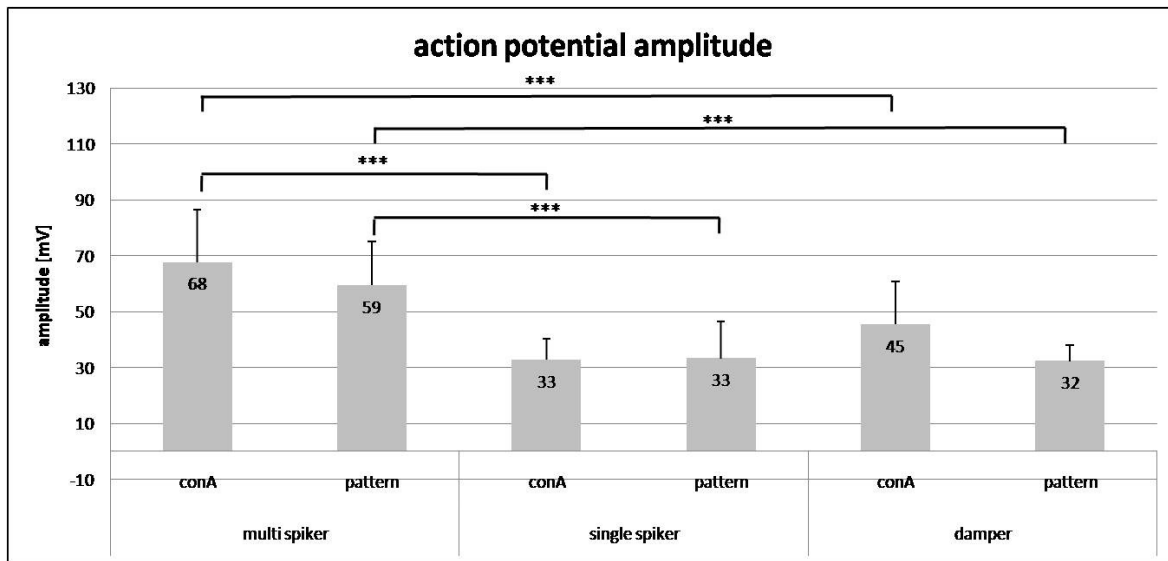


Fig.5.8 Maximal action potential amplitude upon 1 nA current stimulation of electrophysiological response types with evoked action potentials on conA and patterned surfaces.

The width of action potentials at half maximal amplitude was comparable for all spiking response types and varied between 1.4 and 2 ms. The damper had the lowest value (1.4 ± 0.2 ms on conA-coated surfaces and 1.4 ± 0.4 ms on pre-patterned surfaces).

The spike frequency of dampers (195 ± 36 Hz for uniformly conA-coated surfaces and 205 ± 40 Hz on pre-patterned surfaces) was much higher than the spike frequency of multi spikers (66 ± 32 Hz for uniformly coated surfaces and 64 ± 42 Hz on pre-patterned surfaces). Again, the values for neurons on uniformly conA-coated compared to pre-patterned surfaces did not show a significant difference. The width of action potential at half maximal amplitude upon 1 nA current stimulation also did not differ between the respective response types on conA compared to pre-patterned surfaces.

In all, the electrophysiology data suggest that the type of surface used (pre-patterned or uniformly conA-coated) has no significant effect on the electrophysiological behavior of these cells.

DISCUSSION

In the present study, we address the question of whether unspecific 2D-geometrical guiding cues in cell culture can support the restoration of a functional *in vivo*-like morphology pattern and whether this restoration is accompanied by a shift in electrophysiological behavior. To investigate neuronal processing *in vitro*, it is preferable to achieve a simplified yet close to *in vivo*-situation. Our results show that introducing a pattern to a dissociated culture of cricket neurons leads to a distribution of morphological cell types that is closer to the original *in vivo* situation, still keeping the basic electrophysiological properties found in the unpatterned population of cultured neurons. These findings are an important step in the challenge of controlling neuronal adhesion and network formation in order to investigate neuronal information processing *in vitro* and its potential use in bio-engineering and prosthetic research.

Morphology

In nature, insect neurons wire their information through unipolar primary connections with wide-spread dendritic endings. The targeting is triggered by specific adhesive and repellent molecules like laminin and tenascin, respectively. Among insects this guiding signaling has been studied in detail for *Drosophila melanogaster* (Van Vactor and Lorenz, 1999, Dickson, 2002), but it has also been studied in other species (Krull *et al.*, 1994). It is generally accepted that neuronal targeting is similar among insects. As observed by many investigators, including the present study, the morphology of neurons in culture does not necessarily conform to *in vivo* morphology. This discrepancy can partly be explained with the fact that uniformly coated surfaces with adhesion permissive molecules (e.g. laminin, poly-lysine, and conA which are typically used for insect cell culture) do not provide either specific receptor interactions or gradients, which determine neurite sprouting or growth direction *in vivo*. This lack of extrinsic guiding cues and spatial restrictions as well as neighboring neurons leads in most cases to unspecific multipolar outgrowth. In our guiding approach within the cell culture system, we included the extrinsic cues by adding conA tracks on a repellent star PEG background for neuronal outgrowth. ConA is a lectin typically used in insect cell culture, which binds glucose and mannose residues found uniformly distributed on the membrane of neurons. In introducing this simple geometric restriction, we are able to encourage a more *in vivo*-like neuronal growth.

Generally, axotomized insect neurons in cell cultures sprout numerous neurites from the cell body. In our culture system, the amount of multipolar neurons was as high as $87 \pm 5\%$ on

conA-coated surfaces but only $5\pm 4\%$ uni- and $2\pm 1\%$ bipolar neurons was found. Previous studies on interneurons dissociated from the terminal abdominal ganglion from crickets, also report rarely identifying uni- or bipolar cells (Cepok, 1999). Many other studies with invertebrate cell cultures have lead to similar results (Kirchhof and Bicker, 1992, Kim and Wu, 1991, Pfahlert and LakesHarlan, 2008). In contrast to cell culture, multipolar neurons have never been described in the intact ganglia. As Howes *et al.* point out, their appearance in culture may reflect random growth in the absence of guidance cues or target tissues (Howes *et al.*, 1991). Nevertheless, insect cell culture systems with opposite results were also presented (Cayre *et al.*, 1999, Angevin *et al.*, 2000). Thus, *in vitro* conditions apparently do not influence the neuronal properties equally pivotal to all cell types. Several investigators speculate that if the same surface and medium for all cell types are used, intrinsic cues must be responsible for such morphological cell type stability (Beadle, 2006). Indeed, some neurons seem to have a predetermined, intrinsic program specifying their outgrowth pattern without external guiding cues. For instance, mitotic neuroblasts already determine neuritic patterning of progeny for some cell types from rat superior cervical ganglia (Wolf *et al.*, 1996). In leeches, some neuron types maintained their typical outgrowth pattern while other neuron types grew a pattern distinctly different from the pattern within the ganglion during normal development (Acklin and Nicholls, 1990). In our study of neurons from the terminal abdominal ganglion of crickets, no such predetermination could be observed. The basic morphological properties on the contrary seemed to be highly influenced by the surface they were seeded after dissociation.

Nevertheless, the eventual morphology of dissociated neurons also depends on the preparation technique, e.g. enzyme and mechanical treatment, age of the animal, and type of neuronal tissue. Surface changes such as receptor density or remaining glial cell fragments after different dissociation treatments could be responsible for the phenotypic plasticity (Smith and Howes, 1996). According to Kirchhof and Bicker, there are two main factors that influence cellular morphology of *Schistocerca gregaria in vitro*: the presence of primary neurite stump and membrane contacts between neighboring cells. In agreement with that statement, Ayali *et al.* report that frontal ganglion neurons of locusts showed mostly multipolar growth after a full loss of their original neurites (Ayali *et al.*, 2002). In our preparation method, most neurons were fully axotomized and a regrowth from a stump was rarely apparent. Yet, this condition was found to the same degree on uniformly coated and pre-patterned surface, so this special case of neurite growth could be neglected in our statistical evaluation. As we were interested mainly in the influence of the surface contrast and pattern, we seeded the neurons in a low density to exclude the input of neighbouring neurons.

Also medium composition and additives have an impact on neuronal outgrowth. For example, the addition of embryonic nervous tissue or hemolymph (Kirchhof and Bicker, 1992) to culture medium reduces the branching pattern of the cultured neurons. These findings indicate that a mixture of natural extrinsic factors, applied in a rather unspecific way, can lead towards a change in favour of the *in vivo* characteristics. Even *in vivo*, extrinsic factors might influence the neuron extension during development stronger than previously thought. For instance, glial cells seem to play an important role in axon growth and guidance of *Drosophila* and *Manduca sexta* neurons (Smith *et al.*, 1991, Booth *et al.*, 2000, Shepherd, 2000, Oland and Tolbert, 2003). In vertebrate neuronal cell cultures, glial cells support neuronal growth and signaling but also influence and restrict growth and connectivity patterns. In the presented culture system, the amount of non-neuronal cells was small on both the uniformly coated and pre-patterned surfaces in the beginning of culturing, mostly appearing in clusters, and their number seemed to decrease in time. The variability in extrinsic factors was kept constant on all surfaces so that the morphological shift could be attributed to geometrical restrictions.

For invertebrates, the complex signal processing that is responsible for the neurite sprouting, steering and internalizing is not yet completely understood. Many authors address the question to what extent the morphology is preset by intrinsic factors. Acklin and Nicholls found out that the disruption of microtubular structures did not affect the later outgrowth of specific patterns *in vitro* (Acklin and Nicholls, 1990). Glycosylation of proteins also plays an important role in surface recognition. There are at least 17 different glycoproteins on the neuronal glycocalyx of *drosophila* and locust neurons (Snow *et al.*, 1987). Jacobs and Lakes-Harlan described the distribution of lectin binding sites within the metathoracic ganglion of the locust *Schistocerca gregaria* using lectin staining (Jacobs and Lakes-Harlan, 1997) showing a heterogenic distribution of glycoproteins, indicating that glycosylation in insects is tissue specific and also varies throughout the cellular compartments. Although these studies give some insights into the outgrowth process, it is still to be answered whether *in vivo* the growth pattern reflects pre-determined factors in the absence of neuronal stimuli, why it differs from cell type to cell type, what intrinsic factors lead to morphological distinction, and how the intrinsic signaling is controlled. For our study, we simplified the neuron-surface interaction such that geometry alone changed the morphology of the neurons.

Advances in technology have provided a means to study the response of cells to geometrical constraints. A lot of attempts to control the neuronal outgrowth of primary vertebrate neurons have been published over the last years (Kleinfeld *et al.*, 1988, Wheeler *et al.*, 1999, Vogt *et al.*, 2003, Sanjana and Fuller, 2004). However, only two of these studies describe morphological characteristics of guided neurons (Clark *et al.*, 1993, Lochter *et al.*, 1995).

Their results correspond with our finding that neurons shape neurite elongation upon restriction tracks and thus form a different morphology type and suggest that neuritogenesis *in vitro* may be controlled by adhesive surface pattern similar to those *in vivo*. In addition to the previous studies, we could show that not only the obvious geometrical restriction in form of offered paths influences the outgrowth, but the presentation of alternating unspecific permissive and non-permissive cues changes the apparent ambition of neurons to spread neurites in all possible direction paths. Instead, in spite of up to eight possible growth paths presented, the more physiologically relevant unipolar or bipolar morphology was displayed.

Electrophysiology

While changing the distribution of morphological types within the neuronal population in favor of the more natural situation, the neuronal guiding in the present study does not affect the basic electrophysiological parameters. The same four response types that were identified on uniformly conA-coated surfaces were found on star PEG / conA patterned surfaces in a similar distribution (Fig 5.6). The resting potential of each response type was comparable on conA and its guided counterpart. The mean maximal action potential amplitude was slightly smaller on pre-patterned surfaces but did not differ significantly. The morphological diversity of neurons in culture does not necessarily have an effect on their physiology. These results imply that the change in morphology to a more *in vivo*-like shape does not affect the electrophysiological properties of cultured neurons. Extrinsic cues strongly influence the morphology, but the receptor and channel specificity seems to be rather intrinsically controlled. However, the transformation of non-spiking to spiking soma is typical for axotomized neurons in culture (Roederer and Cohen, 1983, Gundel *et al.*, 1989). While most neurons in the intact ganglia have non-spiking cell somata, a high percentage of the investigated cells in the present study were electrically active (77% on uniformly coated and 70% on star PEG / conA patterned surfaces). For isolated neurons from *Schistocerca gregaria* and *Periplaneta americana*, previous studies also demonstrated an increased excitability such that the normally non-spiking insect soma generates action potentials (Gundel *et al.*, 1989; Lees *et al.*, 1985; Bodnar *et al.*, 1991; Bodnar, 1993). Evidence of formation of new ionic channels in the membrane of insect neurons in cell culture (Tribut *et al.*, 1991, Angevin *et al.*, 2000) could possibly explain the shift from non-spiking somata to spiking somata.

The average resting potential of all firing neurons in our cell culture system was -44 ± 8 mV, which is comparable to *in vivo* values of -45 ± 5 mV for wind-sensitive spiking interneurons within this ganglion. Non-spiking interneurons display *in vivo* a membrane potential of

-40±5 mV (Baba *et al.*, 1995), comparable to the value of -41±9 mV in our study. The most frequent response type, the multi spiker, had a significantly lower resting membrane potential compared to the single spiker on both culture surface treatments ($p < 0.005$ on uniformly coated conA surfaces and $p < 0.05$ on pre-patterned surfaces). As firing behavior is determined by the ion channel equipment of a cell, this fact could explain the difference in resting membrane potential. A low expression of Na⁺, or an overexpression in K⁺-channels could potentially be the reason for a small, subthreshold potential displayed by the non-spiking response type.

Our results show that influencing outgrowth patterns with simple 2-D guiding cues and taking advantage of the phenotypic plasticity can lead to a shift towards a more natural connection pattern without influencing the electrophysiological properties. Thus, this cell culture system including a cellular patterning approach makes *in vitro* work applicable for approaches where simple constructed networks with identifiable connections could serve for information processing studies.

6 Inverse patterning of cell-aversive Star PEG for building neuronal networks on microelectronic devices

Based on:

Inverse patterning of cell-aversive Star PEG for building neuronal networks on microelectronic devices

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ABSTRACT

Engineering neuronal networks on microelectronic devices with the goal of investigating biological information processing has risen to become an important field in current research. Cellular lithography is the critical step in directing neuronal growth on a chip in order to achieve a geometrically defined neuronal network that can be studied non-invasively. Some cell types are adherent to typical insulation materials used for microelectronic devices and thus complicate one-to-one neuron-sensor interfacing. Amongst those cellular systems are primary insect neurons, which represent a model system closing the gap between highly complicated operational mode of higher vertebrates and simplified cell line systems. The presented method offers a means to interface such strongly adherent cell types with electronic devices within pre-defining cytophobic structures providing the possibility for direct contact between the cell and sensor or the ability to adsorb proteins patternwise on the sensor surface. Our approach combines a cell and protein aversive coating based on functional star-shaped poly(ethyleneglycol) precursors (star PEG) with a parylene C stencil and plasma-assisted star PEG removal. Defined geometrical constraints of star PEG combined with an adhesive protein (concanavalin A) adsorbed on the surface lead to network formation and long-term cell compliance in our primary insect neuronal cell culture system.

INTRODUCTION

Extracellular recording using microelectronic devices offers a powerful tool to investigate neuronal network development, network activity, and spatiotemporal dynamics. Multi electrode arrays (MEA) and field effect transistor (FET) arrays present two methods to measure network behavior superior to imaging methods and invasive techniques like patch clamping in that they are non invasive, do not interfere chemically (e.g. voltage dyes), and offering synchronous multi-site recording. MEA based studies on network activity have emerged in the last decade providing new insights in information encoding (DeMarse *et al.*, 2001), neural plasticity (Arnold *et al.*, 2005), learning processes (Potter *et al.*, 2003, Stegenga *et al.*, 2008) and bursting synchronization (Esposti and Signorini, 2008). However, the specificity in network connections, branching processes, and the significance of such mechanisms for network activity remain to be clarified in the field of neuronal information processing. A one-to-one neuron sensor connection would allow addressing individual neurons within a functional circuit while the entire network behavior is monitored.

Patterning methods can provide control over cytoarchitecture in order to create exact geometrical surface constraints and thus predict cell body position on the respective sensor area and outgrowth patterns. Although many attempts and successful studies of patterned neuronal networks have been published to date, each cell system displays individual requirements. Published patterning methods on electronic devices cover chemical modification and physical confinement approaches. All approaches seek long-term compliance of the network to the pattern and a tight neuron to surface contact. Therefore, physical confinements with topographical constraints such as polyimide fences (Zeck and Fromherz, 2001) or parylene cages (Tooker *et al.*, 2005) were developed. However, the production is complicated and positioning of cells requires in some cases manual manipulation. Micropatterning by means of soft lithography is a well-established method for cellular patterning of cell lines and primary neurons (Kleinfeld *et al.*, 1988, Kane *et al.*, 1999, Falconnet *et al.*, 2006). Sophisticated procedures have been designed for specific cell-cell and cell-material interaction studies. Some lithographical techniques were implemented for engineering neural networks on microelectronic devices. Aligned printing combined with suitable surface chemistry for covalent binding offers a promising approach to selectively control neuronal cell bodies to attach to the electrode sites. James and colleagues used aligned printed poly-L-lysine structures for controlled growth of cultured neurons on planar MEAs (James *et al.*, 2000). Other approaches include photoresist processes (Chang *et al.*, 2001) and surface chemistry using epoxysilane (Nam *et al.*, 2006).

However, depending on the cell culture system, the introduced patterning techniques can suffer from shortcomings, e.g. denaturation of applied proteins due to solvents used for photoresist removal in lithographical approaches. Also, the protein transfer during μ CP can denature the adhesive protein and result in inhomogeneous layers. Furthermore, a large neuron-sensor distance due to the applied protein is disadvantageous for the signal-to-noise ratio while proteins, as well as any other molecule, bound to electrode surface can increase the electrode impedance. For some cell types such as insect neurons, typical microelectronic device materials are adhesion permissive and thus do not provide pattern confinement in spite of the application of cell adhesive molecules. When the array background does not sufficiently prevent adhesion, the neurons might migrate and lose contact to the relevant sensor area.

Recently, we published a patterning method using six-arm isocyanate functional poly(ethyleneglycol) prepolymers (star PEG) (Reska *et al.*, 2008). These coatings reliably prevent cell adhesion and protein adsorption (Groll *et al.*, 2005a, Groll *et al.*, 2005b) while at the same time offer the possibility of binding proteins covalently due to their reactivity change with time. Here, we describe a method that combines the cell-repulsiveness of the star PEG system with a plasma-patterning technique that allows for physisorption of adhesion mediating proteins directly onto the electrode without an intermediate polymer film to ensure a tight neuron-sensor contact when applying to microelectronic devices. Parylene stencils were used to remove star PEG pattern-wise from the surface using oxygen plasma. We removed the parylene stencil and coated the surface with a concanavalin A protein solution. The surface area that was exposed to oxygen plasma was subtracted from star PEG and thus presented for protein adsorption. In contrast to standard microcontact printing techniques, this method provides the advantage of keeping the protein under typical physiological conditions in all steps of protein patterning. Insect neurons from the terminal abdominal ganglion (TAG) of the cricket *Gryllus bimaculatus* were then seeded on the patterned devices showing guided growth and pattern compliance. This cell culture approach is a promising model system since the insect central nervous system possesses a relatively small amount of neurons within a functional unit compared to mammalian system while still performing high-level information processing. In fact, the cercal system controlled by neurons from the TAG, which evokes the escape behavior of crickets, is one of the most sensitive sensory systems known. Only the star PEG coating system has so far been able to prevent unspecific adhesion and growth of these cells *in vitro*. Overall, this procedure provides a means for neuron-neuron and neuron-surface interaction studies that are crucial for neuron-based biosensors or biomedical devices.

MATERIALS AND METHODS

Multi electrode array

The MEAs were manufactured using standard silicon technology. The gold interconnecting lanes, microelectrodes (ME) and bondpads were fabricated on glass wafers using a 5 nm Titanium layer as adhesion promoter. The chips consisted of 64 MEs arranged in an 8×8 array (100 or 200 μm pitch). The surfaces were passivated by an ONO layer (500 nm SiO_2 / 500 nm Si_3N_4 / 100 nm SiO_2). For details of the fabrication and the encapsulation process see (Krause *et al.*, 2000, Ecken *et al.*, 2003). The final PECVD oxide layer (300 nm) was deposited to provide a glass-like surface for cell culturing.

Planar FET

The p-channel FET devices were fabricated as described before (Offenhäusser *et al.*, 1997). Here we used an improved version: a second implantation step for drain-source doping reduced the series resistance of the contact lanes and, thereby, decreased the noise level of the p-channel transistor. After deposition of the ONO-stack [(silicon oxide (250 nm), low-pressure chemical vapor deposited (LPCVD) silicon nitride (130 nm), and LPCVD silicon oxide (100 nm)] the gate area was opened by a combination of dry etching and wet etching. A thin silicon dioxide layer (10 nm) formed in a dry oxidation process at 820°C was used as gate dielectric. Transistor gates were arranged in a 4×4 matrix with a pitch of 200 μm (or 10×10 matrix with a pitch of 100 μm) in the center of a 5 mm×5 mm silicon chip.

Star PEG film formation

Samples have been prepared as previously reported (Groll *et al.*, 2005c, Reska *et al.*, 2008) (see Fig.6.1). Briefly, glass and chip surfaces were cleaned by ultrasonification in isopropyl alcohol for 5 min and dried in a stream of nitrogen, then washed with deionized water and activated by a MetOH / HCl (1:1) treatment (30 min), washed and dried again. Directly after activation, substrates were aminosilanized in a glovebox (MB 100, MBraun, Garching, Germany) with APTES (N-[3-(trimethoxysilyl) propyl] ethylenediamine) (0.2 mL) for 1 h in an argon gas atmosphere prior to spin-coating. For preparation of the star PEG film, isocyanate-terminated prepolymer (100 mg; MW 12 kDa, polydispersity (PD) 1.2) was dissolved in dry tetrahydrofuran (1 mL). After addition of deionized water (1:10), the solution was incubated for 5 min, and applied to the substrate. Spin-coating was performed at

2500 rpm for 40 s (WS-400E-&NPP-Lite, Laurell, North Wales, USA). These freshly prepared films were stored in ambient conditions for at least 12 h prior to usage. The parameters chosen for the casting process in this study resulted in star PEG coatings with a thickness of 30 ± 5 nm.

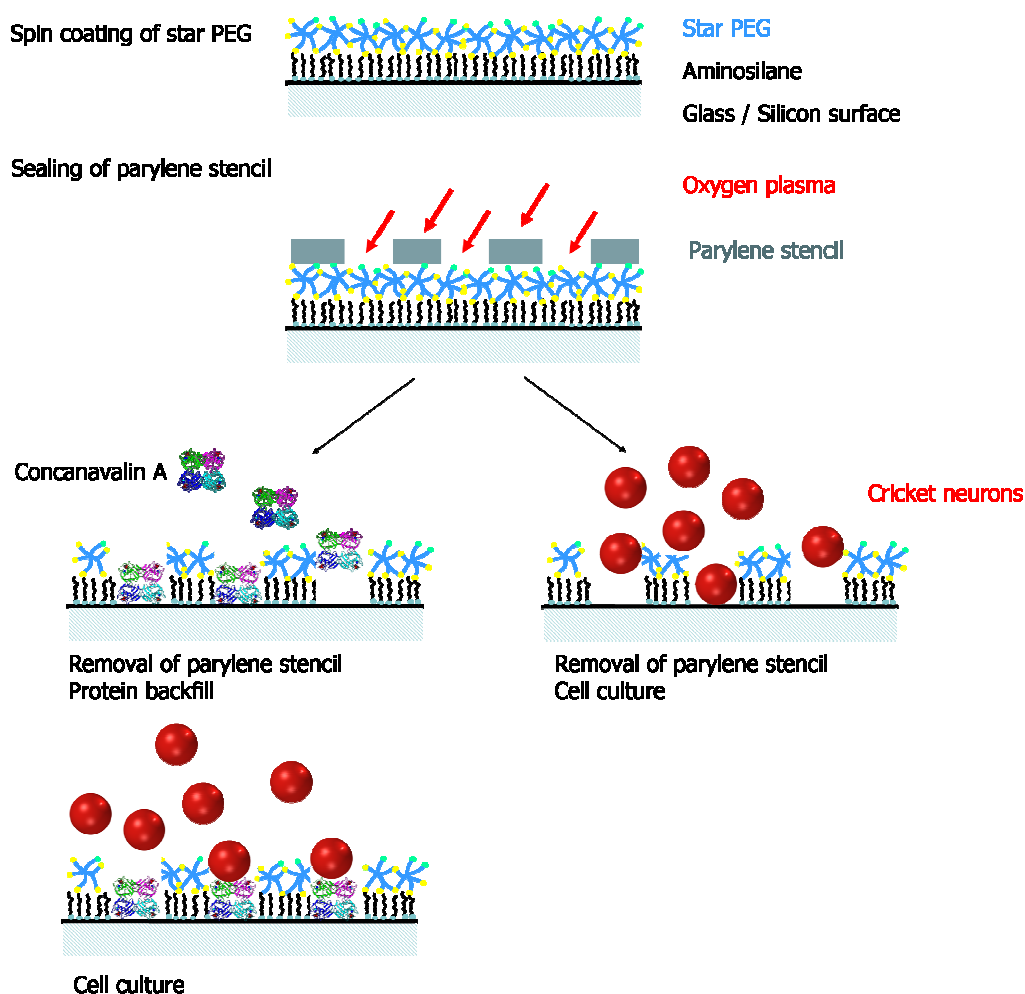


Fig. 6.1. Procedure of pattern formation on silicon surface and microelectronic devices. Prior to star PEG coating, the surface was aminosilanized. Star PEG was applied via spin-coating. After respective cross-linking time, a parylene stencil was aligned to the surface and star PEG was removed pattern-wise by oxygen plasma treatment. After stencil removal, the surface area was optionally backfilled with concanavalin A solution for 60 min and exposed to neuron cell culture.

Parylene stencil production

Parylene C [poly(monochloro-p-xylylene)] was deposited by chemical vapor deposition (PDS 2010 Labcoter Specialty Coating Systems, Indianapolis, USA) in the clean room facilities of the IWE, RWTH Aachen without an adhesion promoter directly on a 2" silicon wafer resulting in a thickness of about 4 μm . It was coated with a positive photoresist (AZ5214) and patterned by reactive ion etching. The photoresist was removed with acetone.

Alignment of stencils and oxygen plasma treatment

The parylene stencils were carefully detached from the wafer with tweezers and positioned under microscopically control onto the respective device. It was clearly visible on the surface whether it sealed properly to the surface. Subsequently, the substrates with positioned stencils were treated with oxygen plasma (Pico, Diener Electronic, Nagold, Germany) at 40 W in a vacuum of 0.8 mbar. Optionally, the surfaces were incubated with concanavalin A solution (200 $\mu\text{g/mL}$) for 60 min.

Cell culture

Female and male crickets *Gryllus bimaculatus* (within one weeks after their adult molt) were used for the cell culture experiments. Neurons were dissociated as described below and kept in culture for up to two weeks at 29°C and a relative humidity of 95%. The animal was cool-anesthetized at 4°C for about 30 min. After decapitation and removal of all legs, it was fixated ventral side up inside and part of the rope ladder-like central nervous system was removed including the terminal abdominal ganglion with three abdominal ganglia. It was transferred under a sterile hood and after several washing steps the terminal abdominal ganglion was desheathed. Then, the ganglion was incubated in 500 μL enzyme solution (collagenase 0.5 mg/mL, dispase 2.0 mg/mL) in silanized 1.5 ml Eppendorf-tube for 3 min at 37°C. After mechanical dissociation by 10 fold trituration with a silanized 200 μL tip it was added to 10 mL cooled modified Leibovitz medium in order to stop enzyme reaction. Next, it was centrifuged 10 min at 2500 rpm (8°C), the supernatant was withdrawn and 70 μL of modified Leibovitz medium [(L-15, glucose (2 g/L), fructose (0.8 g/mL), proline (0.35 g/mL), imidazole (0.06 g/mL), glutamine (0.3 g/mL), adjusted to pH 7.0 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, from Roth) (340 mOsm/kg)] per substrate was added trituated once again ten times with a silanized 200 μL tip and the cell suspension was plated

on the respective surface; usually one ganglion was plated on three substrates. For adhesion the surfaces were left unmoved for 90 min and filled up with modified Leibovitz medium after the adhesion time.

Ellipsometry

Layer thicknesses were determined using a Nanofilm EP3 ellipsometer with a Nd:yttrium aluminum garnet (Nd:YAG) laser (532 nm) and EP3 View 2.05 software. The angle of incidence was 70°C.

AFM

AFM measurements were performed with a Nanoscope IV (Veeco, Mannheim, Germany) with the software Nanoscope 5.30 in tapping mode (RTESPW tips, Veeco) with a scanning rate of 0.33 Hz.

Fluorescence images

Flourescence micrograph of conA Rhodamine backfill was imaged with a Zeiss microscope and Axiocam and Axiovision software with an excitation wavelength of 540 nm.

RESULTS AND DISCUSSION

With the presented approach, we were able to create star PEG patterns on microelectronic devices (see Fig.6.2) and achieve morphologically-controlled neuronal culture with reliable pattern compliance (Fig.6.6, Fig.6.7). This technique offers an important supplement in the field of patterning proteins and cells by means of soft- and photolithography because it enables geometrically controlled protein adsorption without rough pre- or post-treatment of the surface ensuring physiological conditions.

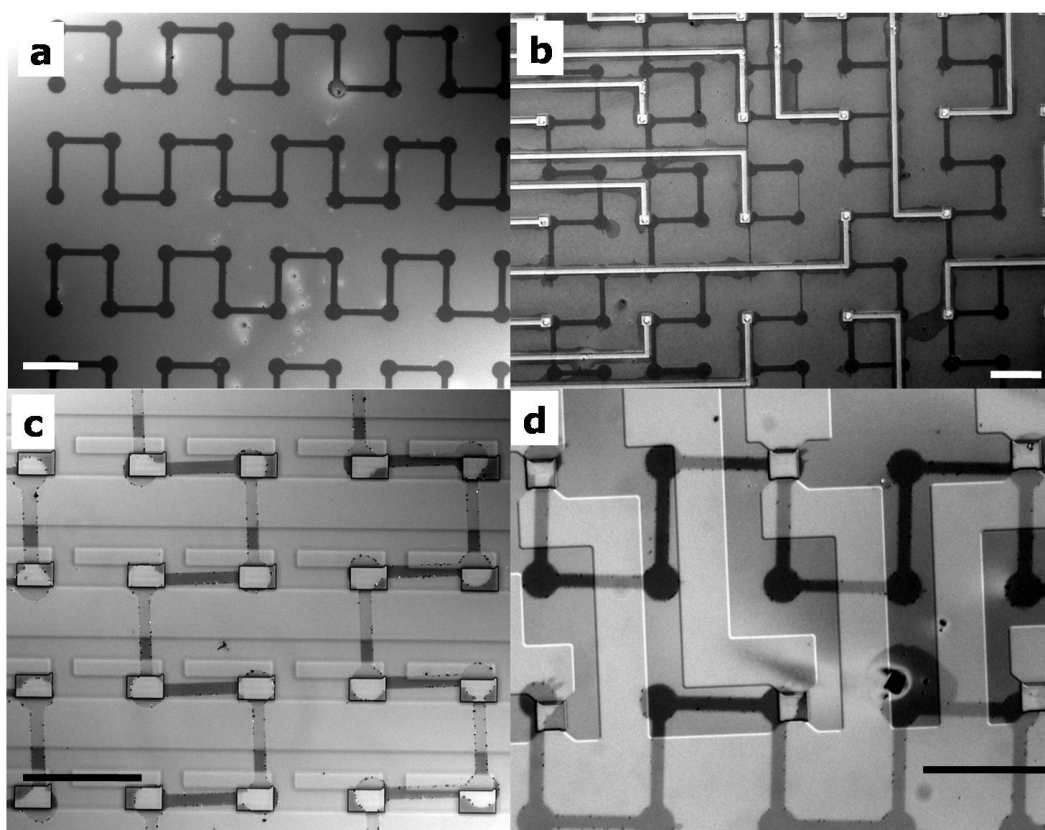


Fig.6.2. Differential interference contrast micrographs of star PEG coated surfaces treated with oxygen plasma through parylene stencil. Scale bars indicate 100 μm .

- (a) Plain silicon oxide surface, dark area is star PEG-free.
- (b) Multielectrode array with concanavalin A backfill. Star PEG pattern is aligned to the gold electrodes.
- (c) Planar FET array with 10x10 matrix. Pattern is aligned to the gold electrodes. Dark area is star PEG-free. The gate area within this star PEG free area interferes differently with light because of material differences.
- (d) Planar FET array with 4x4 matrix. Pattern is aligned to the gold electrodes. Dark area is star PEG-free. The gate area interferes differently with light because of material differences.

The results were obtained by using parylene stencils on star PEG background. Star PEG is a protein and cell repellent material with long-term stability and biocompatibility protein and cell repellent material (Groll *et al.*, 2005a, Reska *et al.*, 2008) and is thus suitable for various biological approaches on glass, silicon, and microelectronic devices. The parylene stencil was formed by means of photolithography using a positive photoresist and reactive ion etching. When sealing the parylene stencil to the respective surface, the uncovered area was subtracted from star PEG during oxygen plasma treatment while the parylene covered area was kept protected. The pattern transfer was geometrically well defined to the stencil structure as parylene seals tightly to the surface being mechanically very flexible (Wright *et al.*, 2008). Ellipsometry measurements of the patterned structure achieved on SiO₂, clearly displayed the height differences between the protected and unprotected surface area after oxygen plasma treatment (Fig.6.3). As the refractive index of star PEG is in the proximity of SiO₂, the measurement was performed assuming one continuous layer on Si. While the deepened structures were in the range of 10 nm according to the oxide thickness, the background was in the range of 45 nm indicating a total star PEG thickness of approximately 35 nm.

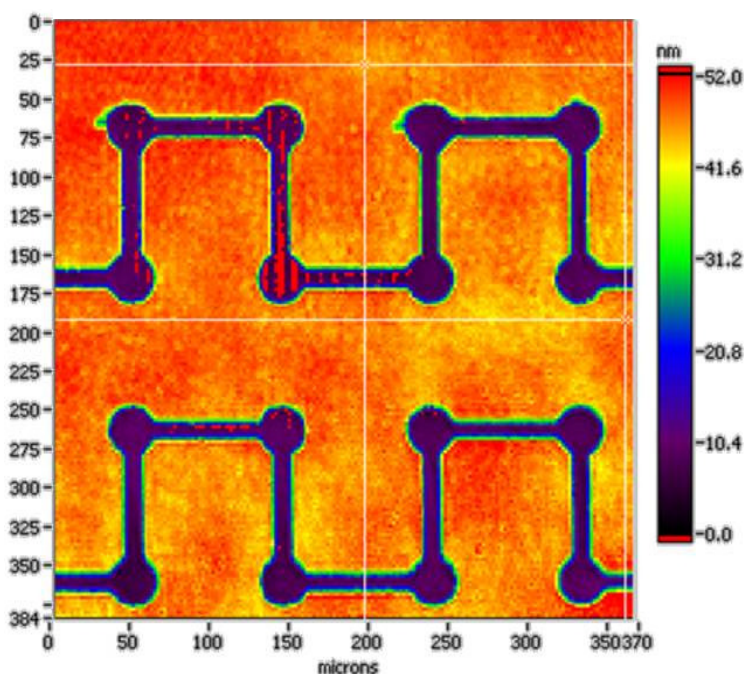


Fig.6.3. Ellipsometry measurement of star PEG parylene pattern without protein backfill. The surface thickness was measured assuming only one layer with the refractive index of 1.46. The height profile reveals the complete removal of star PEG from the unprotected area. Background height of ~ 45 nm is an assembly of 10 nm oxide and 30 ± 5 nm star PEG layer. The height of the area exposed to oxygen plasma is in the range of 10 nm.

The AFM measurement confirmed the surface topology and displays a smooth edge (Fig.6.4). Due to the protein aversive property of star PEG, the protein adsorption was only facilitated on the oxygen treated, unprotected area resulting in a pattern negative to the parylene stencil geometry. Protein backfilling of rhodamine labeled conA confirmed the aversity of the star PEG background and confinement to star PEG-free regions (see Fig.6.5).

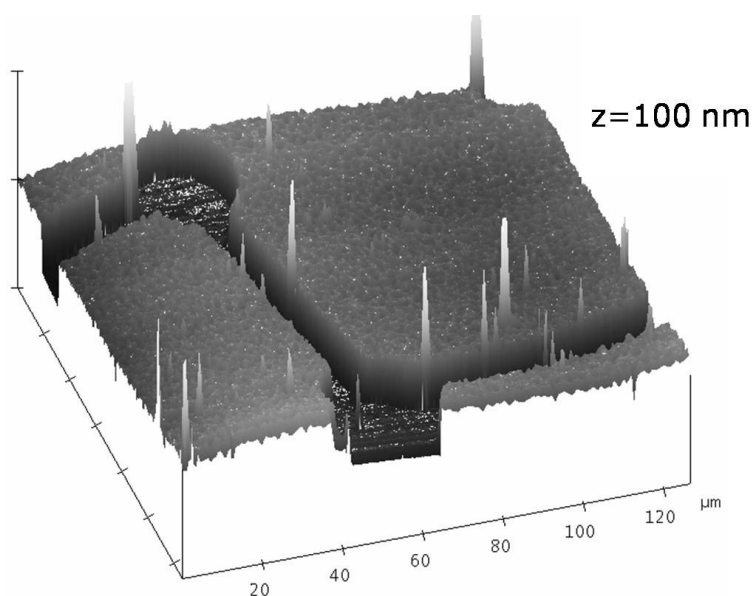


Fig.6.4. Atomic force microscopy of star PEG, parylene pattern on SiO₂ substrate. The AFM measurement confirmed a homogeneous layer thickness of 35 nm.

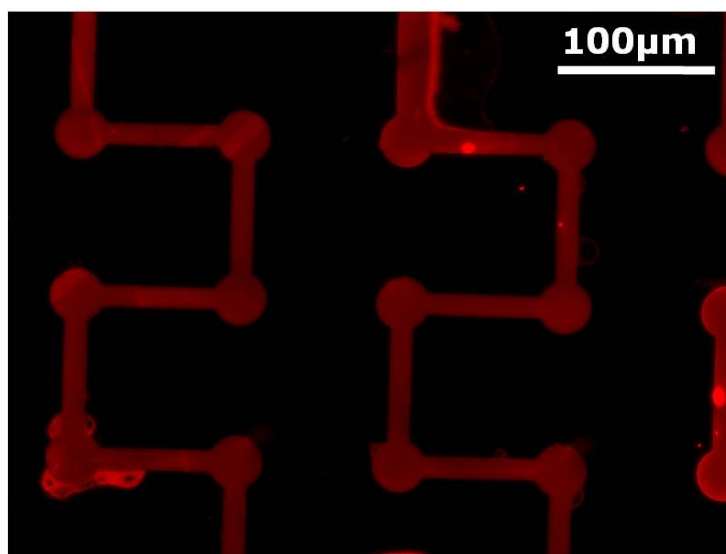


Fig.6.5. Fluorescence micrograph of star PEG layer on silicon oxide, patterned via parylene stencil and exposed to oxygen plasma. Backfilled with Rhodamin labeled concanavalin A. Area covered by parylene resisted protein adsorption.

Patterning on microelectronic devices can require enhanced sophistication as the surface of such devices is not always planar and various materials are combined in the fabrication process. Generally, our previous approach using μ CP of conA on an applied star PEG layer is suitable for cell patterning on microelectronic devices (Fig.6.8). However, it does not provide a well-defined surface topology and chemistry as in the currently presented method. Very adhesive cell types, like insect neurons require an aversive background as they potentially adhere to the entire surface area. However since they grow in a rather small density, the probability of neuron-sensor area contact is low and thus the adhesive area needs to be defined. Furthermore, with the presented method these neurons can be plated directly on the star PEG pattern without protein backfilling such that the neuron-sensor area distance can be minimized. As insect neurons have a rounded shape and do not seal strongly to the surface this distance reduction can be highly valuable in recording and stimulation experiments. A tight membrane-electrode contact is an important parameter for signal-to-noise ratio in extracellular measurements (Sorribas *et al.*, 2001). Previous studies using dorsal root ganglion cells show that this distance can be minimized to 37 nm where further reduction is probably hindered by the glycocalyx. Sorribas and colleagues demonstrated that the neuron-surface distance is about 40 nm when coating with (N-[3-(trimethoxysilyl) propyl] ethylenediamine) (APTES).

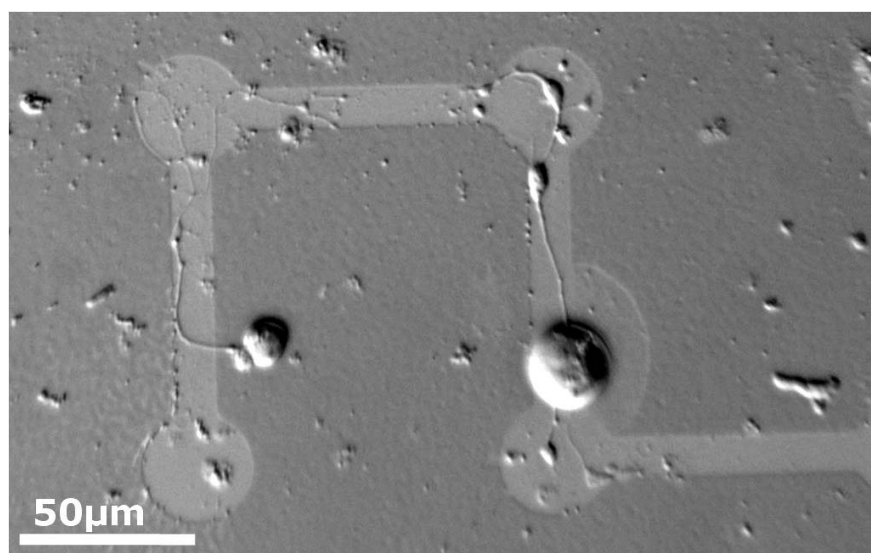


Fig.6.6. Differential interference contrast micrograph of cricket neurons grown on a star PEG / parylene pattern with a concanavalin A backfill (6 days *in vitro*). Neurons adhered to concanavalin A coated structures and grew neurites along the respective paths. Neuronal cell body on the left hand side is not adhered but was observed to float anchored only by the neurite (typical for cricket neurons, data not shown).

Figure 6.6 shows two cricket neurons following the applied star PEG pattern with conA backfill. The larger cell soma on the right side adhered to the patterned line and re-grew neurites towards the adjacent node structure. The neuron on the left side did not adhere (the cell soma was observed to be floating when taking this image). Cricket neurons tend to anchor the cell soma via their neurites probably detaching while neurites grow out. Similarly, several neurons presented in figure 6.7 adhered nearby to an electrode of the MEA; one neuron grew visible neurites along the presented pattern lines.

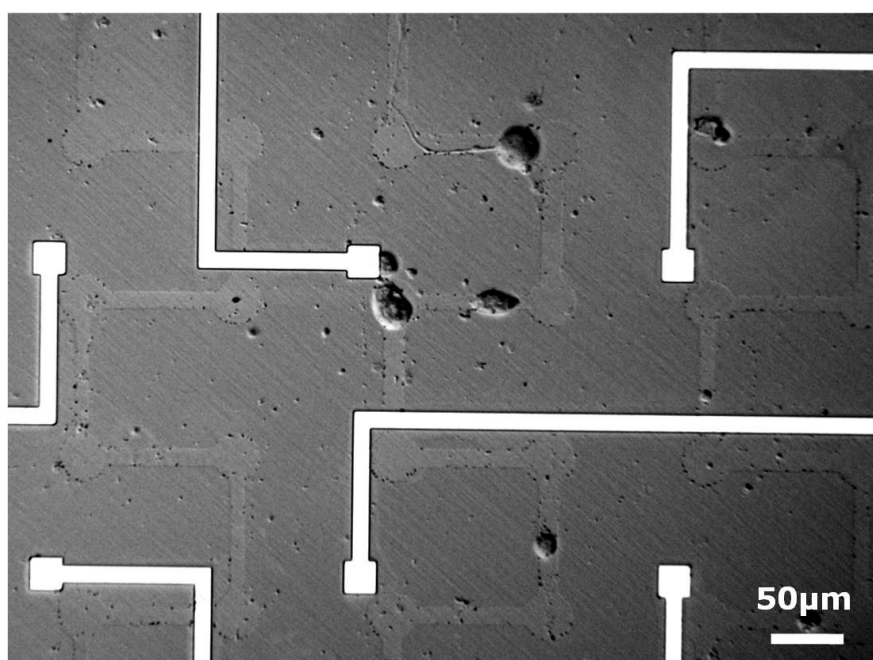


Fig.6.7. DIC micrograph of cricket neurons grown on star PEG with aligned parylene patterning with a concanavalin A backfill on multi electrode array (4 days *in vitro*). Neurons adhered to concanavalin A coated structures and grew neurites along the respective paths avoiding the star PEG covered areas.

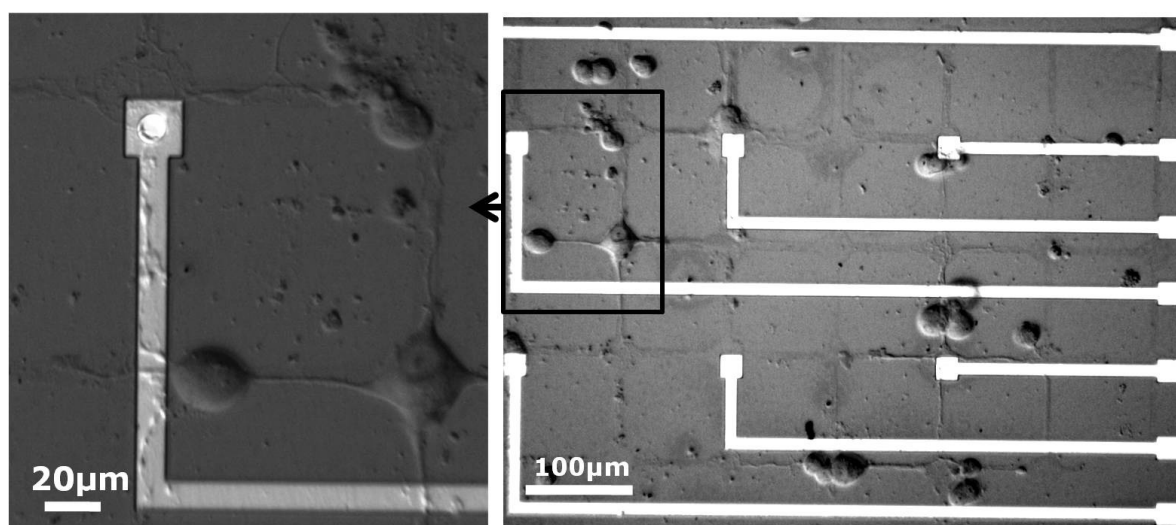


Fig.6.8. DIC micrograph of cricket neurons grown on star PEG with aligned microcontact printing of concanavalin A on multi electrode array (5 days *in vitro*). Neurons adhered to concanavalin A coated structures and grew neurites along the respective paths avoiding the star PEG covered areas. Several neuron cell bodies are found in proximity to electrodes. Inset: Enlarged section of MEA: neurites overgrowing electrode area are visible.

In neuronal network studies performed with microelectronic devices, the geometrical control of cell adhesion sites as well as growth paths should be developed. Clearly defined network geometry provides a basis for the formation of neuronal model systems with reduced complexity and one-to-one connections. A one-to-one neuron-sensor contact provided by patterning techniques on microelectronic devices as presented in this study is important when investigating dynamics or development of neuronal networks, such that synchronously multi-site recordings of individual neurons and neuronal compartments can be addressed. Using an appropriate surface coating with defined geometry, reliable control over cell adhesion and network formation can be reached.

7 Discussion

The present thesis deals with the challenge of patterning insect neuronal networks on standard cell culture surfaces and microelectronic devices in order to enable the recreation of selected neuronal circuitries for information processing studies. The approaches accompanying this study aimed to advance the investigation of neuronal networks that perform excellent information processing *in vivo* and are thus worthwhile to be mimicked for engineered systems. The requirements for such an attempt are the reliable control of neuroarchitecture to build defined neuronal networks and a high sensitive multi-site recording system. Additionally, the constructed neuronal networks need to be coupled to the respective read out system. The established methods available for cellular patterning still suffer from insufficiencies concerning the universal applicability for various cell systems and surface materials. Particularly for insect neurons, a need for a reliable patterning method currently exists. Information processing studies of networks *in vitro* are meaningful because *in vitro* systems represent a simplified copy of parts within a system. Complex processing steps are classically measured *in situ*, but within the entire system, the information input is overwhelming and too complex to draw straight-forward conclusions. Cell culture approaches enable studies in a defined environment and controlled parameter manipulation. Many studies were accomplished with insect neuronal preparations, but to date, no studies about the influence of geometrical constraints on neuritogenesis and targeting of synaptic partners have been accomplished.

Insect neuronal networks are convenient in handling but also provide highly efficient information processing. As many basic neurophysiological mechanisms are conserved, insights obtained in invertebrate studies are similar between higher-ordered and simple organisms. Insects and vertebrates share many common principles in neurogenesis and physiology. However, insect neurons are highly adhesive to various substrates unlike embryonic vertebrate neurons, which are classically for cell culture approaches. Therefore, insect neuronal patterning requires more differentiated coating materials and thus more steps than the methods developed for vertebrate neuronal patterning in that the background must be made non-adhesive. For this

purpose, a new approach with covalent binding of conA as standard protein supporting adhesion of insect neuronal cultures to an aversive background has been established here. Although a large amount of potential repellent substrates have been developed (see chapter 2 introduction), none of these substrates are proven suitable for repelling insect neurons. Poly(ethyleneglycol) (PEG) has been known since the nineties to prevent protein adsorption and therefore preventing cell adhesion due to protein binding (Holmberg, 1992). PEG prevents protein adsorption due to interactions with aqueous solutions which likely provide a thermodynamically-favorable reversal of protein adsorption from the PEG molecules. Linear PEG molecules, however, seem to have a steric arrangement, probably due to laterally bending, that is not sufficiently aversive for insect neurons, allowing some protein attachment to the surface. It is unclear why insect neurons are strongly adherent to various substrates, such as glass, polystyrene and silicon oxide, compared to vertebrate neurons. But, many vertebral cells isolated from other tissues, such as cardiovascular or orthopedic tissues, also adhere to these materials. The reason for this difference could be due to the secretion of a particularly large amount of extracellular matrix proteins by insect neurons. During the dissociation process of cell isolation, the neuronal glycocalyx does not seem to be digested through enzyme treatment as the adhesion of insect neurons *in vitro* takes place within one hour after plating. This time would not be sufficient for expression of new glycosylated proteins. Another possible explanation is that vertebrate neurons may have developed a specialized process for neuronal adhesion that could be less reliant on proteins (glycoproteins) and more reliant on highly glycosylated proteins (proteoglycans) which might be repelled by negatively charged substrates such as glass and silicon oxide. In spite of the strong adhesion of these neurons to numerous substrates, the star shaped functionalized PEG molecule does prevent insect neuronal adhesion reliably. The potential to combine this surface coating with protein patterns regardless of the strongly anti-adhesive properties due to a change in reactivity over time makes this molecule particularly interesting for many applications. Patterns of conA supporting cell culture have been realized using such substrates. This patterning technique is universal as the coupling of conA is based on the reaction of isocyanate with an amino group and thus can be used with any other protein.

As the physiology of single neurons determines the overall neuronal network behavior and potentially influences firing properties, basic morphology and electrophysiological characteristics of insect neurons on pre-patterned surfaces were investigated. The *in vitro* situation for other insect culture systems is also known to not encourage the growth of uni- or bipolar neurons, which are the predominant morphology type *in vivo*. Trying to gain insights into the information processes of the cercal system networks, it is important to respect physiological connectivity patterns. Geometric and molecular cues *in vivo* guide pathfinding of neurites and thus network formation. The additionally observed intrinsic control over the

morphological development of individual neurons is still unclear. However, creation of simple, pre-defined growth paths for insect neurons *in vitro* mimics *in vivo*-pathfinding and facilitates precise neuritogenesis and neuronal connections. When the growth of cricket neurons from the terminal abdominal ganglion was geometrically controlled, a dramatic reduction in branching patterns of neurons was observed. Quantification of the total neurite number of individual neurons on various 2-D geometries revealed an apparent shift in the distribution of morphological types towards more physiologically-relevant neuron types compared to classic culture conditions. ConA presents an ideal candidate to favor neuron adhesion rather than adhesion of glia since the potential recognition sequence (fucosyl 1-3 N-acetylglucosamine) is more frequently found on the neuronal membrane. In addition, lectin-binding studies on locusts have shown that conA binding is not neuron-type specific and thus does not introduce a pre-selection of neuron adhesion. Therefore, conA is suitable for investigations of all neurons in the terminal abdominal ganglion. Axotomy during tissue preparation seems to lead to a shift in ion channel distribution and thus an increase in excitability of the cell soma. This fact should be kept in mind when investigating electrophysiological network characteristics as activity influences the network connectivity patterns as well as synaptic strength. However, investigations into basic electrophysiological parameters of patterned neurons revealed that electrophysiological behavior of patterned neurons did not differ significantly from their counterparts grown on uniformly conA-coated surfaces. In fact, a general population of cultured neurons was retained in patterned cultures.

Creating *in vitro* network models, the functionality of anatomical connections between cultured neurons should be ensured. For molluscs, reconstructed, functional copies of identified networks *in vitro* have been created, but so far reconstructed networks have not been achieved for insects. Electrical and chemical synapses have been shown to redevelop *in vitro* (Ganforina, 1999). Functional pairs of neurons prove the feasibility of *in vitro* neuronal network recreation. Furthermore, exploring the influence of geometrical constraints on targeting of synaptic partners helps to elucidate the extent to which synaptic partners are pre-determined. Therefore, whole cell double patch-clamp experiments were performed in the present study to investigate the connectivity of adjacent cell pairs. Out of tens of experiments, only few pairs of functionally coupled neurons were found. A large failure rate can be assigned to using paired patch clamp being a very sophisticated method in combination with the fact that insect neuronal cell bodies typically adhere only slightly to the respective substrate. These neurons are rather anchored via the neurites. Future experiments should investigate the extent to which the specificity of neuron-neuron contacts was responsible for anatomically but not physiologically connected neuron pairs. Nevertheless, these experiments prove that insect neurons can form functional connections *in vitro*. Non-invasive methods such as calcium imaging and extracellular recordings

using microelectronic devices could potentially increase the successful recordings of functional connections.

Recordings with microelectronic devices have become very popular over the last years as chip array techniques enrich the scientific field with a powerful means to investigate biological systems without strong chemical and mechanical interference. Neuronal networks require especially sophisticated methodologies as their formation (including adherence of individual neurons, outgrowth and specific interconnections), network electrophysiological behavior and migration should be investigated at different developmental levels to monitor single events during network formation and thus do not tolerate invasive techniques such as patch clamp recordings. Gaining geometric control over the network architecture optimizes the amount of sensor sites contacting neurons thus increases the spatial resolution and improves the signal-to-noise ratio because of the proximity to the relevant sensor site. Additionally, an advanced method to interface the neuronal networks to the microelectronic devices is needed to ensure a low neuron to sensor distance for potential recording and stimulation experiments. With a negative lithography approach starting with the established star PEG approach, we took advantage of the known properties of star PEG while making use of the extraordinary adhesion properties of the insect neurons to grow directly on the chip array surface. This method provides a promising means to design specific surface geometries with defined chemical properties for various protein and cell interaction studies. By this means, the coupling of other cell culture systems to microelectronic devices can be achieved in a controlled manner.

In conclusion, geometric control of neuronal cultures facilitates studies of network characteristics *in vitro*. For the first time, a reliable patterning method for standard cultures as well as cultures on microelectronic devices for insect neurons is presented. Extensive experiments on morphology and electrophysiology of cricket neurons from the TAG prove the functionality and physiological relevance of such constructed networks.

8 Summary

Information processing systems in animals have evolved to be extraordinarily efficient. The present work deals with the challenge of designing neuronal networks *in vitro* in order to mimic these sensory systems. The cercal sensory system of crickets is one of the most sensitive sensory systems known. Neurons from the terminal abdominal ganglion (TAG) are responsible for the first information processing of the cercal sensory system that yields the escape reaction. Using *in vitro* systems, information processing steps within the neuronal networks can be studied without disturbing side input, and network formation can be monitored over developmental stages. Knowledge gained from such studies offers a thorough understanding of biological processes during network formation and function, and this knowledge can be implemented in engineering of prostheses and biosensors.

Construction of functional *in vitro* neuronal networks requires a reliable and universal cell patterning method in order to enable the reconstruction of an *in vivo*-like network with appropriate connection patterns as well as interfacing with a sensitive recording system. Microelectronic devices provide a non-destructive way of studying networks of neurons over the long-term with the additional advantage of simultaneously recording several single neurons within the network. However, interfacing neurons with microelectronic devices is challenging, as the device must provide a growth-permissive surface and the possibility to guide neuronal growth. This work presents an approach to control insect neuroarchitecture *in vitro* on both glass surfaces as well as silicon devices. Using isocyanate functionalized star-shaped PEG which changes reactivity over time, a patterning method can be developed such that covalently bound cell adhesive proteins are surrounded by cell and protein repulsive background. A geometrical patterning protocol using microcontact printing was initially used to control network formation of TAG neurons. The initial patterning method was modified further to offer the possibility to control neuronal growth on to extracellular recording devices. In the further developed method, star PEG is applied uniformly on the device surface and is subsequently removed by means of oxygen plasma, which can be combined with backfill of an adhesive protein to the inverse

pattern. The second approach is especially interesting due to the reduction in neuron-electrode distance by removing the PEG layer.

Detailed investigations of the pre-patterned neurons proved that patterning supports a more *in vivo*-like morphology in the cell culture system and the physiology is maintained. The investigation of basic electrophysiological characteristics of single neurons showed that neurons from the TAG are more excitable than *in vivo* neurons, which is typical for insect neurons after axotomy. However, compared to neurons grown on un-patterned, uniformly coated substrates with the same adhesive protein, the patterned neurons show the same distribution of electrophysiological response types and basic electrophysiological characteristics. Additionally, paired patch clamp experiments providing information about synaptic connectivity showed that functionality is restored within reconstructed, patterned networks.

In all, this work takes a step forward in the development of cell-based microsystems and biosensors, which seek to mimic natural information processing systems. The results presented here indicate that geometric patterning of insect neurons can simplify identifying network connectivity and modulate morphological characteristics of neurons to a more *in vivo*-like condition without modifying the electrophysiological characteristics of the neurons.

9 Zusammenfassung

Im Laufe der Evolution entwickelten natürliche sensorische Systeme der Tiere eine besonders effiziente Informationsverarbeitung. Ziel der hier vorgestellten Arbeit war es, neuronale Netzwerke *in vitro* zu konstruieren, um diese sensorischen Systeme nachzuahmen. Das Cerkalsystem der Grille ist eines der empfindlichsten sensorischen Systeme überhaupt. Neurone des Terminalganglions (TAG) stellen die erste Stufe der Informationsprozessierung des Cerkalsystems dar, welche zum Fluchtverhalten führt. *In vitro* können wichtige Schritte der Informationsverarbeitung innerhalb der neuronalen Netzwerke ohne störende Einflüsse untersucht und die Netzwerkentstehung über einzelne Entwicklungsstadien beobachtet werden. Ergebnisse solcher Untersuchungen liefern ein grundlegendes Verständnis von biologischen Prozessen der Netzbildung und Netzwerkfunktion, die in die Entwicklung von Prothesen und Biosensoren implementiert werden können.

Die Konstruktion von funktionellen neuronalen Netzwerken *in vitro* erfordert eine zuverlässige und universell einsetzbare Zellmusterungsmethode, um die Rekonstruktion von *in vivo* ähnlichen Netzwerken mit vergleichbaren Verknüpfungsmustern zu ermöglichen. Zusätzlich ist es notwendig, diese an ein empfindliches Ableitsystem zu koppeln. Mikroelektronische Elemente bieten eine zerstörungsfreie Untersuchungsmethode von neuronalen Netzwerken über einen längeren Zeitraum. Gleichzeitig liefern sie die Möglichkeit viele Neurone innerhalb eines Netzwerkes simultan abzuleiten. Die Kopplung von Neuronen an mikroelektronische Elemente ist jedoch sehr anspruchsvoll, da die Oberfläche dieser Elemente die Möglichkeit zur Zelladhäsion und Kontrolle des Neuritenwachstums liefern muss. Hierzu wurde in dieser Arbeit eine Methode zur Kontrolle der Neuroarchitektur von Insektenneuronen *in vitro* auf Glas und Siliziumoberflächen etabliert. Dabei wurde ein sternförmiges Polyethylen Molekül (star PEG) verwendet, welches mit Isocyanat funktionalisiert ist. Die Reaktivität des Moleküls ändert sich im Laufe der Zeit, so dass ein Oberflächenmuster aus kovalent gebundenem, zelladhäsivem Protein auf zellabweisendem Untergrund entsteht. Eine geometrische Musterung mit dieser Methode wurde benutzt, um das Wachstum von Neuronen des TAG zu steuern. Die etablierte

Musterungsmethode wurde anschließend weiterentwickelt, um das Neuronenwachstum und die Netzbildung auf extrazellulären Ableitelektroden zu ermöglichen. In dieser Methode wurde das star PEG gleichmäßig auf die Elektrodenoberfläche aufgetragen und im Anschluss mittels Sauerstoffplasmabehandlung gemustert entfernt. Das Vorgehen kann mit einer Proteinbeschichtung des invertierten Musters kombiniert werden. Diese Methodenweiterentwicklung wird durch die Reduzierung des Abstandes zwischen Neuronen und Elektroden für vielerlei Anwendungen besonders attraktiv.

Detaillierte Untersuchungen von gemusterten Neuronen haben gezeigt, dass die Musterung eine *in vivo*-ähnliche Morphologie im Zellkultursystem unterstützt und die Physiologie dabei erhalten bleibt. Zusätzlich hat die Bestimmung von grundlegenden elektrophysiologischen Eigenschaften von Einzelneuronen gezeigt, dass Neuronen des TAG stärker erregbar sind als Neurone *in vivo*, was charakteristisch für axotomierte Insektenneurone ist. Im Vergleich mit Neuronen, die auf ungemusterten Substraten mit gleichmäßiger Proteinbeschichtung gewachsen sind, zeigen die gemusterten Neurone die gleichen grundlegenden elektrophysiologischen Eigenschaften und Verteilung der elektrophysiologischen Antworttypen. Doppel Patch-clamp Experimente zeigen zudem, dass die Funktionalität von rekonstruierten, gemusterten *in vitro* Netzwerken wiederhergestellt werden kann.

Zusammenfassend schafft diese Arbeit eine wichtige Grundlage für die Entwicklung zell-basierter Mikrosysteme und Biosensoren, welche die natürliche Informationsverarbeitung imitieren. Die hier vorgestellten Ergebnisse legen nahe, dass die geometrische Musterung von Insektenneuronen *in vitro* dazu verhelfen kann, Netzwerkverbindungen zu identifizieren und die Morphologie der Neurone ihrem physiologischen Zustand näher zu bringen, ohne die elektrophysiologischen Eigenschaften zu verändern.

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