ABSTRACT
We demonstrate the use of high-frequency electric fields to guide developing axons within a 3D matrix embedded in a microfluidic device. We have used compartmentalized microfluidic chips to seed neurons and let axons grow in collagen scaffolds placed in microfluidic channels with increasing heights and with patterned electrodes on the bottom. We show that AC electric fields are capable of guiding, enhancing, slowing and pushing up axons within the collagen matrix, which demonstrates the first contact-less guidance of axons in scaffolds.

KEYWORDS: Axon guidance, neural networks, dielectrophoresis, microfluidics

INTRODUCTION
The central nervous system (CNS) is a dense, layered, 3D interconnected network of neurons, and thus recapitulating that complexity for in vitro CNS disease models requires methods that can create defined neuronal networks in 3D. Typical systems for 3D control of axons use photopolymerization of adhesive cues within extracellular matrix (ECM) gels [1, 2], or, in some cases, 3D mechanical confinement [3] (Fig. 1). Unfortunately, these methods are typically constrained to use modified (rather than natural) ECM, and cannot control directionality nor speed of growth, which limits the value of those models and their network complexity to simple neuronal circuits. We previously introduced the use of AC electrokinetic forces to stop axonal growth on 2D surfaces, allowing creation of functional, configurable and directional neural networks [4]. Here we present, for the first time, a contactless method that provides the ability to guide axons within three-dimensional matrices.

EXPERIMENTAL
We have found that AC electrokinetic forces create axon-friendly repelling forces [4]. Because these forces extend in three dimensions, we hypothesized that we could control axons not just on surfaces [4], but also in 3D gels. We developed an electro-microfluidic device to compartmentalize axonal growth into 3D collagen gels (Fig. 2). We used this device to demonstrate four essential unit operations, in un-modified collagen, on axons that permit 3D control: axon blockage, speeding up axons, slowing down axons, and pushing axons up-and-over in 3D. We did this by coordinating control of electrode placement and steric channel constraints. Chips were functionalized with PDL and laminin to promote neuron adhesion in the microchannels. Scaffolds were selectively patterned in the grooves by capillary filling. Neurons were stained by Oregon green BAPTA 1 Calcium staining.
RESULTS AND DISCUSSION

First, when the channel height is <5 μm, activated electrodes apply electrokinetic repelling forces that, due to the channel’s steric constraints, the axons in gels cannot bypass. This thus provides a contactless, electrode-patterned and dynamic way to guide axons in a plane (Fig. 3). With deactivated electrodes (0 V), neurites grew parallel to the channel (median = 0°) and had only minor changes in growth direction that were equal on both sides (±10°). A similar deviation of growth direction could be found for an activated field with 1V<sub>p-p</sub>. However, the median angle is slightly shifted into the negative (~2.5°), meaning away from the electrodes. Electrokinetic forces at this voltage are minimal and the change is presumably not connected to electrokinetics. Application of 2V<sub>p-p</sub> led to turns of nearly ~70° in average. 3V<sub>p-p</sub> resulted in the largest turns of up to ~90° and parallel growth to the electrodes as it was observed in Fig. 3b. In the range from 2 – 3 V<sub>p-p</sub>, we modeled dielectrophoretic (DEP) forces, which is the electrokinetic force most likely responsible for the effect [4], and estimated a maximum DEP force of 66 pN acting on the growth cone [4]. This is in the same order of magnitude as traction forces that cause growth cone turning in the body [5]. Therefore, DEP is a likely able to cause the observed changes in growth direction. Finally, 4V<sub>p-p</sub> at the electrodes resulted in injury of the neurites and no angular deviation could be determined.

Second, by orienting the electrodes parallel to the axons (i.e., funnel), we find that the growth speed of the axons increases (Fig. 4) more than twice compared to pure collagen, which may be the result of spatial confinement of the cone probing area and, to our knowledge, is the first demonstration of a non-chemical and contactless approach to boost axonal growth rate [6]. We hypothesize that the growth cone needs less time for probing of the environment because AC electrokinetic forces limit the effective probing area to a 1-D line.

Third, by increasing the channel height to ~10 μm, we can significantly slow axonal growth as compared to growth on glass or in bare collagen (Fig. 5). Introduction of scaffolds in 10 μm-high microchannels resulted in a significant increase in extension length as shown in Fig. 5b (glass vs. 0 V). A potential explanation for growth promotion is the alignment of collagen fibers parallel to the growth direction as a result of the scaffold filling. Aligned collagen fibers provide a track-like mechanical guidance clue that promotes axon growth. At low voltages, the exerted force on the growth cone is easily surpassed by other guidance clues like substrate stiffness. This can explain why no influence on extension length can be observed at low voltages. However, at higher voltages, the growth cone is under a competition between physical microchannel confinement and electric field repelling, axons getting pushed up and away from the electrodes towards the channel top.
Finally, when the channel height is ~50 μm, we can push axons up (in z) and away from the electrodes (Fig. 6), creating a region where the axon is forbidden from entering and whose height increases with the electric field strength (Fig. 6c). The presence of deactivated electrodes or low field strengths did not influence the height of axons in the 50 μm-high channel, whereas applied voltages between 2 and 3 V_p-p led to a significant increase in height (Fig. 6c). By application of different voltages the z-deflection was tunable in a range h = 0 – 10 ± 2 μm. Similar to observations in our other experiments (Figs. 2–5), there appears to be a threshold minimum voltage where axon growth is influenced by the electrokinetic effects.

**CONCLUSION**

In conclusion, electric fields have the capacity to alter axonal growth in native 3D collagen scaffolds, and thus provide a toolbox for dynamic guidance, speeding up, slowing down and vertically repelling of axons. The combination of dynamic axon path finding and microfluidic compartmentalized chips now allow for the fabrication of neural networks that can mimic the realistic three-dimensional complexity found in vivo.

**REFERENCES**


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