Synaptrode: neural interface at the synapse level

Jasper Timmerman
NERF
Imec
Leuven, Belgium
jasper.timmerman@nerf.be

Joris De Wit
CBD
VIB
Leuven, Belgium
joris.dewit@kuleuven.be

Sebastian Haesler
NERF
Imec
Leuven, Belgium
sebastian.haesler@nerf.be

***Abstract*— Electrical recording and stimulation of neurons is used both by neuroscientists to gain insight into neural network dynamics and clinicians to diagnose or treat various neuropathologies. Current electrophysiology paradigms are limited in that subpopulations of neurons within the network cannot be selectively addressed without the use of difficult and low yield techniques such as optogenetically tagged recordings, not suitable for use in the clinic. With the synaptrode, we aim to address these limitations by adding versatility to the current electrophysiology hardware, without the need for auxiliary tools or modifications to the subject. This versatility consists of a manufacturing technology where electrodes make synapse-like connections with selected subpopulations of neurons enabled by the molecular code of cell adhesion molecules (CAMs). Using the molecular specificity of CAMs we can selectively induce presynaptic or postsynaptic connections to neurons, allowing separate monitoring and stimulation channels for efferent and afferent neuron-to-neural prostheses connections. The challenge we face is to create electrodes that are small enough to interface with single neurites and yet do not exhibit impedance values prohibitively large to reliably perform electrophysiology experiments. Combining the differential coding properties of CAMs immobilized at the electrodes with the ease and the speed of electrical readout of neuronal activity, we envision a technology that would enable accelerated research in closed loop electrophysiology paradigms for health, industry, and academic applications.**

I. Introduction

Gaining insight into information processing systems such as the brain requires analysis down to the physical level of implementation. For neural networks this corresponds to the structure-functional relationships of the constitutive neural subpopulations [1]. The structure-functional relationships of neurons are highly dynamic and regulated by a plethora of biological mechanisms that to date have not yet been exhaustively identified and/or elucidated [2]. However, the concerted action of cell surface proteins has been identified as an important determinant in cell type identity and connectivity [3]. Synaptogenic proteins are a diverse family of cell surface proteins that have been shown to induce synapse formation between specific neuronal subtypes. This specificity is codified in the homo-and hetero-dynamic interactions between the expressed synaptogenic proteins. For instance, the synaptogenic protein LRRTM2 induces excitatory synapse formation, but not inhibitory synapse formation [4]. In a seminal work in 2000 Peter Scheiffele showed that synaptogenic proteins retain their function when brought to expression in non-neuronal cells [5]. This phenomenon sparked great interest into synaptogenesis, inspiring research that showed synaptogenic proteins retain their function when only their extracellular part is immobilized on abiotic surfaces [6].

With the synaptrode project we aim to capitalize on these exceptional properties of synaptogenic proteins in the context of electrophysiology research. *In vitro* electrophysiology techniques using multi electrode arrays (MEAs) are ubiquitously used in clinical and research neuroscience, taking advantage of the phenomena associated with neuronal physiology to measure the activity of the neuronal cells [7]. Briefly, neuronal membranes depolarize via a flux of charged ions through ion channels in the membrane. These moving charges create a potential field that attenuates by distance and can be measured by nearby metal surfaces (electrodes) against a reference background [8]. This approach is used for both *in vivo* and *in vitro* paradigms with different geometrical constellations of the electrodes in the MEA. The higher the density of the electrodes on the surfaces, the higher the spatial resolution of the potential fields in the sample. However, downscaling of electrodes is limited by impedance increase, which makes electrodes less capable of distinguishing potentials from noise. Commercial passive MEA electrodes are usually not fabricated in sizes smaller than roughly 10 microns in diameter, because impedance becomes prohibitively high to perform reliable and repeatable experiments.

Although it has been shown that action potentials at the axon level can be measured by conventional electrodes, the large majority of electrophysiology experiments only measure cell body activity [9]. This is because the highest concentration of ion channels in the average neuron is localized to the axon hillock, at the transition between cell body and axon. At higher ion channel concentrations local charge flux during depolarization increases, resulting in a larger source potential. Because the potential attenuates with distance, the larger source potentials can be detected more easily by nearby electrodes. Synapses are submicron scaled biochemical signalling infrastructure. Ideally for single synapse resolution electrodes would be of similar size. Electrophysiological activity at the synapse level has, to our knowledge, never been reliably measured due to their submicron size, the lower local ion channel concentration, and the serendipitous nature of their physical location in the sample.

J. Timmerman is with Neuro-Electronics Research Flanders (NERF), 3001 Leuven, Belgium and KU Leuven, 3000 Leuven, Belgium (phone: +32491740792; e-mail: jasper.timmerman@nerf.be)

J. De Wit is with Vlaams Instituut Biotechnologie (VIB), 3000 Leuven, Belgium and KU Leuven, 3000 Leuven, Belgium

S. Haesler is with Neuro-Electronics Research Flanders (NERF), 3001 Leuven, Belgium, and with the Department of Neurosciences, KU Leuven, 3000 Leuven, Belgium (e-mail: sebastian.haesler@nerf.be)

To overcome the problems of higher impedance for small electrodes, lower source potential from the synaptic compartments, and their random localization we aim to create a physical connection between the synapse and the electrode. This physical connection needs to bring synapses in nanometer proximity to the electrodes, greatly limiting the attenuation by distance of the potential field generated by synapse activity, and specific, allowing only presynaptic synapses to form from a specific subpopulation of neurons. To achieve this, we will immobilize synaptogenic proteins to the electrode surface, the combinatorial effect of which will allow the electrophysiologist to selectively measure activity of neuronal subpopulations of interest at the synapse level.

II. Approach

As electrode material gold was selected, owing to its high conductivity and chemical properties. Gold is established as a biocompatible electrode material and at the same time allows chemical modification using established methods. Concerns over the impedance values of small gold electrodes we also considered another material. Vertically aligned carbon nanotubes (VACNTs) possess a 3D structure that reduces electrode impedance while at the same time acting as an anchor point for neural processes. These added benefits of the 3D structure makes up for the added difficulties in the fabrication process.

A. Gold electrodes

Gold MEA are fabricated via standard lithography processes. The circular MEA layout was codesigned with a polydimethylsiloxane (PDMS) microfluidic channel device (Fig 1) which divides the electrodes in an inner and outer compartment connected by microfluidic channels. Neurons are seeded inside the inner well and grow neurites towards the outer well. This isolates the cell bodies from the induces synaptic compartments, allowing reduced background noise levels and easier analysis of induced synaptic compartments.

Surface functionalization of the gold electrodes consists of immobilizing protein localized to the electrode surfaces. For the gold electrodes thiol chemistry is employed to create a biotin-tailed self-assembled monolayer (SAM) (Fig 2). The tetrameric biotin-binding neutravidin protein is used in a sandwich configuration to immobilize anti Fc antibody to the gold electrode surface. The anti-Fc antibody then captures the Fc fragment present on the n-terminus of the extracellular LRRTM2 constructs purchased from commercial suppliers or produced in-house.

B. VACNT electrodes

The VACNT electrodes are manufactured using standard silicon lithography processes, allowing precise 3D control over electrode topographical features. Briefly, after gold MEA fabrication, a catalyst layer is deposited on the electrodes and VACNT structures are grown via a thermal enhanced CVD process. Biotin linkers are grafted to the VACNT electrodes using EDC/NHS coupling chemistry. The tetrameric biotin-binding neutravidin protein is used in a sandwich configuration to immobilize anti Fc antibody to the VACNT backbone (Fig 3). The anti-Fc antibody then captures the Fc fragment that is present on the n-terminus of the extracellular LRRTM2 constructs that are procured from commercial suppliers or produced in-house. The combined biochemical and topological-mechanical cues of the protein-functionalized electrodes result in a close (<50 nm) physical connection between the electrode and the target neurons which will allow stable and specific membrane depolarization recording at the synapse level.

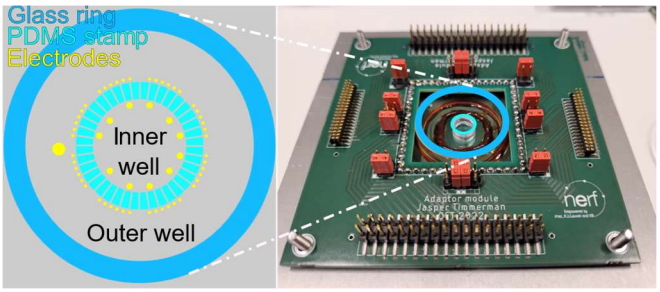

Figure 1. Left: top down schematic view of the circular design of the synaptrode MEA and PDMS microchannel device. Neurons are seeded in the inner well created by the PDMS stamp and grow to the outer well created between the PDMS stamp and the glass outer ring. Right: the electrodes of the synaptrode MEA can be addressed selectively and simultaneously using the adaptor module.

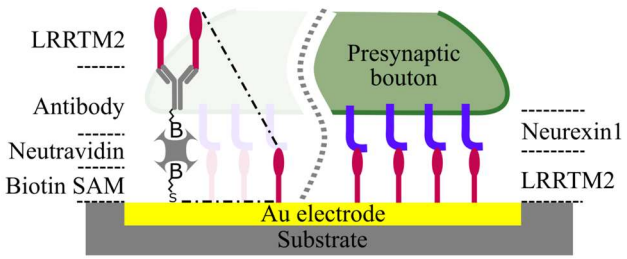

Figure 2. Schematic half-section of surface functionalized electrodes. Left: schematic of the chemical surface functionalization of gold electrodes (not to scale). The maximum height of the protein stack is ~30 nm. Right: schematic of an induced synapse like connection (not to scale). The synaptic cleft has a width of < 50 nm.

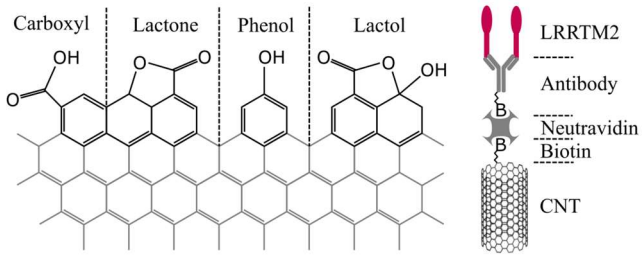

Figure 3. Left: Schematic of CNT backbone structure and impurities targeted for EDC/NHS coupling. Right: Schematic of the functionalized CNT backbone (not to scale).

III. RESULTS

An initial test was performed to confirm the synapse inducing capabilities of LRRTM2 for various samples. Primary mouse hippocampal neurons and human induced pluripotent stem cell-derived neurons (hIPSC neurons) were used in a co-culture experiment with LRRTM2 covered microbeads. The 5 micron diameter neutravidin-covered beads (sigma) were surface functionalized with a 10/1 ratio of LRRTM2/ fluorescent Fc tag protein. To analyze the results the cultures were fixed in PFA and stained for Tau (present in axons) and synapsin (present in synaptic compartments). The immunofluorescent images (Fig 4) were analyzed by creating a binary mask of both Tau and beads images. These masks were used to compare the fluorescence intensity of the synapsin stain at bead sites to non-bead sites. The analysis uses all the pixels that are left after applying the Tau mask and the beads mask and shows their intensity distribution on a log scale histogram. Then an equal number of pixels are randomly chosen from pixels that are left after applying the Tau mask and the inverse of the beads mask and displays the intensities on the same histogram. The result shows a bimodal distribution for the fluorescence intensity of the synapsin stain at bead sites compared to non-bead sites. For control beads surface functionalized with only fluorescent Fc fragment no increased synapsin staining fluorescent intensity is observed at bead sites. These results indicate that LRRTM2 is a good candidate for synapse induction on abiotic surfaces.

Next, we confirmed that the circular design of the chip and PDMS device enabled us to separate neurons from cell bodies and confirmed axons impinged on potential synapse inducing sites. Neurons were seeded in the central well of the PDMS stamp and allowed to grow without addition of any chemical cues to the culture. After one week in culture neurites were observed reaching the outer well of the sample. After two weeks the culture was fixed in PFA and stained for Tau. Immunofluorescent microscopy analysis revealed that sufficient neurites reach the outer well and cross paths with sites meant for synapse induction (Fig 5).

A. *Gold electrodes*

We analyzed how well protein could be immobilized on the gold surfaces. The analysis of the thiol SAM on gold proved challenging, as the bulk gold material quenches fluorescent signals, rendering all fluorescent markers unfit for analysis. Instead, we tried using chemiluminescent markers and DAB staining in combination with confocal microscopy and SEM imaging, which yielded inconclusive results. To confirm successful surface modification, secondary ion mass spectrometry (SIMS) was performed. This analysis revealed that protein immobilization was not successful and the protocol must be adjusted for gold functionalization.

B. *VACNT electrodes*

As mentioned, we are creating 3D VACNT electrodes in parallel to gold electrodes. The size of the structures can be brought down to about 10µm. We did some preliminary biocompatibility tests and confirmed that the VACNT structures are biocompatible and provide a topological cue to neurons as an anchoring point (Fig 6). To confirm our surface functionalization protocol for the 3D VACNT strucutures we used fluorescently tagged streptavidin to visualize immobilized protein on the VACNT surface. We found there was significantly higher fluorescent signal on chemically modified VACNT structures as compared to untreated structures (Fig 7), suggesting successful surface functionalization.

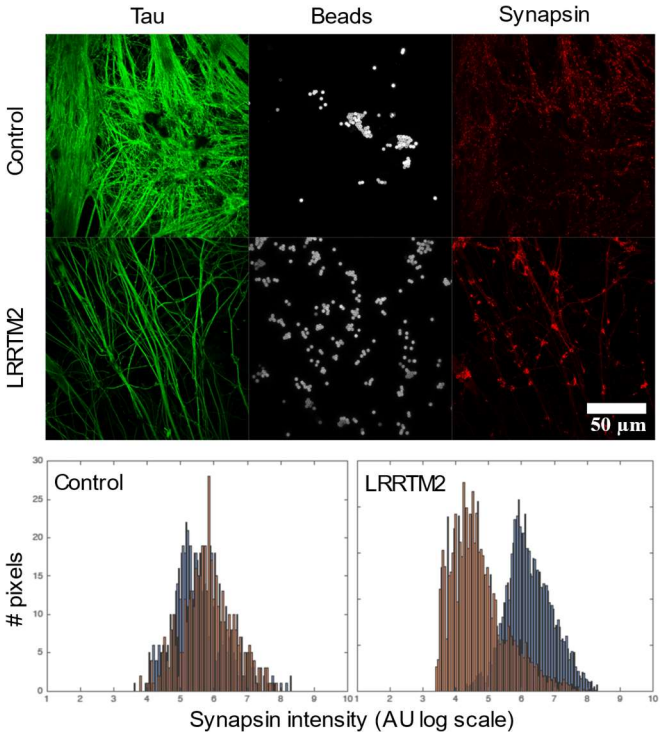

Figure 4. Coculture experiments with LRRTM2 covered beads. Histology shows Tau as a marker of the presence of axons, Beads shows the presence of beads in the sample, and Synapsin as a marker of synapse formation. The blue histograms show synapsin fluorescent intensity at locations where Tau and beads are both present, the red histogram shows synaspin fluorescent intensity where only Tau is present.

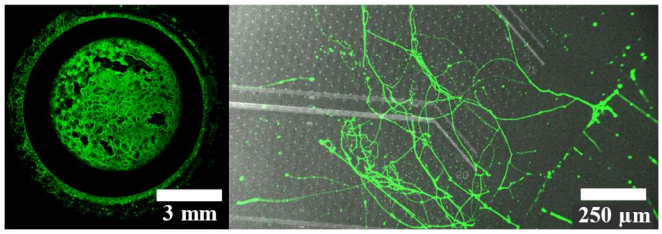

Figure 5. Immunohistochemistry images of Tau stained cultures after two weeks incubation in neurobasal medium. Virtually every microchannel shows signs of axons invading the outer well.

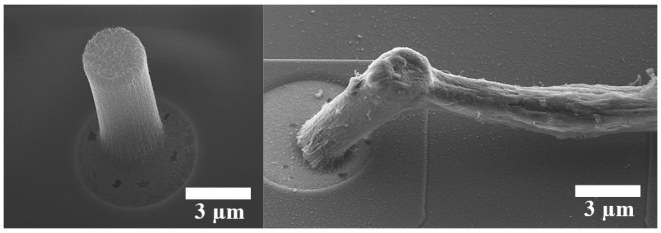

Figure 6. SEM images showing pristine VACNT electrodes on the left and a neurite anchoring to a VACNT structure on the right after two weeks incubation.

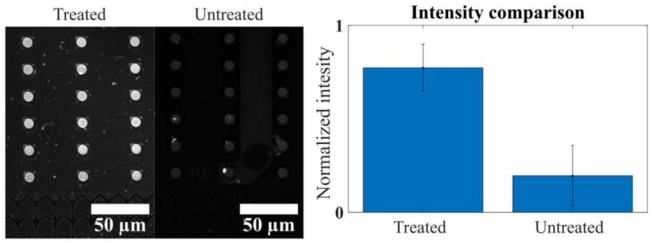

Figure 7. Validation of specific protein immobilization using fluorescently labeled avidin. CNT autofluorescence was verified in the control. VACNTs without a biotin linker yielded a low signal due to non-specific protein adsorption. Biotin-treated CNT structures, however, yielded a much stronger signal, indicating successful and specific immobilization of avidin.

IV. DISCUSSION

Synapse induction on microbeads was successful while induction on plain gold proved more difficult. SIMS analysis showed that our immobilization protocol was not successful and synaptogenic protein was not immobilized locally on the electrodes. Possible reasons for the failed functionalization could be the quality of the deposited gold or the quality of the chemicals used. In future experiments different depositing techniques will be used such as gold evaporation and electrodeposition combined with chemicals purchased from different suppliers. Alternatively, we want to continue pursuing the 3D VACNT electrode approach to create an optimal device to perform the synaptrode experiments.

Further into the future, the next steps for the synaptrode experiments are to perform electrophysiology experiments in combination with optogenetic tools and patch clamping to visualize the relationship between synapse activity and cell body activity. After these experiments are established, we want to combine different synaptogenic proteins at different concentrations to determine their efficacy and specificity profiles and create a library of optimal synaptrode configurations applicable for different research questions.

ACKNOWLEDGMENT

The authors would especially like to thank Dr. M. De Volder from the University of Cambridge (UK) for assisting with the growth of the VACNTs.

REFERENCES

[1] G. Fishell and N. Heintz, "The Neuron Identity Problem: Form Meets Function," *Neuron (Cambridge, Mass.),* vol. 80, no. 3, pp. 602-612, 2013, doi: 10.1016/j.neuron.2013.10.035.

[2] D. Schreiner, J. N. Savas, E. Herzog, N. Brose, and J. de Wit, "Synapse biology in the 'circuit-age'—paths toward molecular connectomics," *Current opinion in neurobiology,* vol. 42, pp. 102-110, 2016, doi: 10.1016/j.conb.2016.12.004.

[3] T. C. Sudhof, "Synaptic Neurexin Complexes: A Molecular Code for the Logic of Neural Circuits," *Cell,* vol. 171, no. 4, pp. 745-769, 2017, doi: 10.1016/j.cell.2017.10.024.

[4] J. de Wit *et al.*, "LRRTM2 Interacts with Neurexin1 and Regulates Excitatory Synapse Formation," *Neuron (Cambridge, Mass.),* vol. 64, no. 6, pp. 799-806, 2009, doi: 10.1016/j.neuron.2009.12.019.

[5] P. Scheiffele, J. Fan, J. Choih, R. Fetter, and T. Serafini, "Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons," *Cell,* vol. 101, no. 6, pp. 657-669, 2000, doi: 10.1016/S0092-8674(00)80877-6.

[6] E. J. Kim, C. S. Jeon, S. Y. Lee, I. Hwang, and T. D. Chung, "Robust Type-specific Hemisynapses Induced by Artificial Dendrites," *Scientific reports,* vol. 6, no. 1, pp. 24210-24210, 2016, doi: 10.1038/srep24210.

[7] M. E. Spira and A. Hai, "Multi-electrode array technologies for neuroscience and cardiology," *Nature nanotechnology,* vol. 8, no. 2, pp. 83-94, 2013, doi: 10.1038/nnano.2012.265.

[8] C. A. Thomas, P. A. Springer, G. E. Loeb, Y. Berwald-Netter, and L. M. Okun, "A miniature microelectrode array to monitor the bioelectric activity of cultured cells," *Experimental cell research,* vol. 74, no. 1, pp. 61-66, 1972, doi: 10.1016/0014-4827(72)90481-8.

[9] D. J. Bakkum *et al.*, "Tracking axonal action potential propagation on a high-density microelectrode array across hundreds of sites," *Nature communications,* vol. 4, no. 1, pp. 2181-2181, 2013, doi: 10.1038/ncomms3181.