# Evaluation of amyloid beta oligomers (AβO) effects on functional network integrity of rodent hippocampal neurons

## Benefits

1. Setting-up a relevant injury methodology with low non-cytotoxic concentrations of AβO
2. Recording of functional activity with AβO injury application
3. Evaluation of AβO effects using *in vitro* connectomic analysis (network analysis)
4. Long-term culture of neurons in a microfluidic chamber
5. Compatibility with High Throughput Assays

## Overview

Research to evaluate more effective treatments to Alzheimer disease (AD) requires relevant *in-vitro* models to understand pathophysiology of this disease. Microfluidic devices offer the possibility to co-culture several types of humans neurons (glutamatergic, gabaergic, astrocytes...), thus mimicking the physiological architecture of the brain. A large array of evidence indicates that amyloid beta oligomers (AβO) play a key-role in neuronal degeneration leading to neuronal cell death in AD. Neuroscience research requires new relevant *in vitro* model of AD to study and develop new therapeutics.
Thanks to NETRI's technologies, it has been possible to develop a brand-new neuronal culture system allowing long-term culture of neurons and perform functional activity. Using a deposition chamber enabling the seeding up to $10^5$ neurons (NF_1_CD_100) coupled with Microelectrode Array (MEA) system, NETRI could perform neuronal network analysis, in order to evaluate the status of the connectivity of the implied neurons.
Thanks to ETAP-Lab unique know-how in the production of soluble, stable and well-characterized human AβO, it has been possible to set-up a relevant injury methodology with low concentrations of toxins that are not cytotoxic.
This Application Note describes a peptide injury methodology to create a relevant *in vitro* AD model and to perform network analysis, post electrophysiology recording.

## Results

### Microfluidic device compatible with MEA system

#### Using a microfluidic device with MEA for neuronal activity recording

**In order to perform extracellular recording of the entire neuronal culture** in the chamber we have used the chip ref NF_1_CD_100 bonded on MEA substrate (Fig 1) seeded with rat hippocampal. Deposition chamber technology allows to precisely control the density and homogeneity of neurons seeded. Thanks to microfluidic architecture leading to avoid shear stress on cell culture and optimize media change, it has been possible to culture neurons on a long-term period, up to 24 days. Moreover, NETRI's devices are compatible with standard high throughput screening enabling use on ever cell culture automate. Neurons are in direct contact with electrodes on the MEA allowing to perform extracellular recording of the whole neuronal culture in the chamber. This microfluidic device architecture allows to control the homogeneous seeding of neurons with a targeted number of cells.

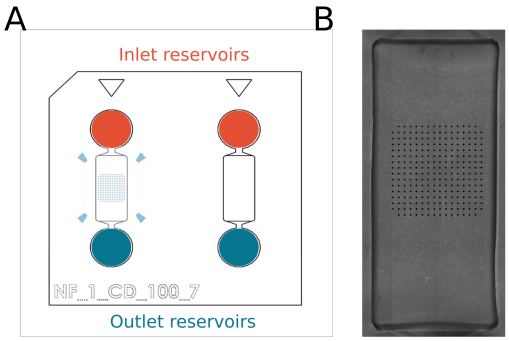


**Figure 1. Microfluidic device for electrophysiological recording. (A)** Schematic representation of a microfluidic chip (NF_1_CD_100) coupled with MEA for electrophysiology recording. **(B)** Picture in brightfield light of a device with MEA.

## Application of a peptide injury for modelling AD

### Relevant AβO synthesis

**ETAP-Lab's produced a new batch of AβO from human amyloid beta 1-42 monomers** and have been analyzed by Coomassie blue staining in SDS-PAGE, which demonstrates that oligomers contain a mixture of stable dimers, trimers and tetramers, as well as traces of high molecular weight oligomers (Fig 2-A).
Dot blotting analysis was performed to detect AβO using the anti-oligomer (A11) antibody that recognizes oligomers, but not fibrils. Only oligomer preparations were recognized by this antibody (Fig 2-B).

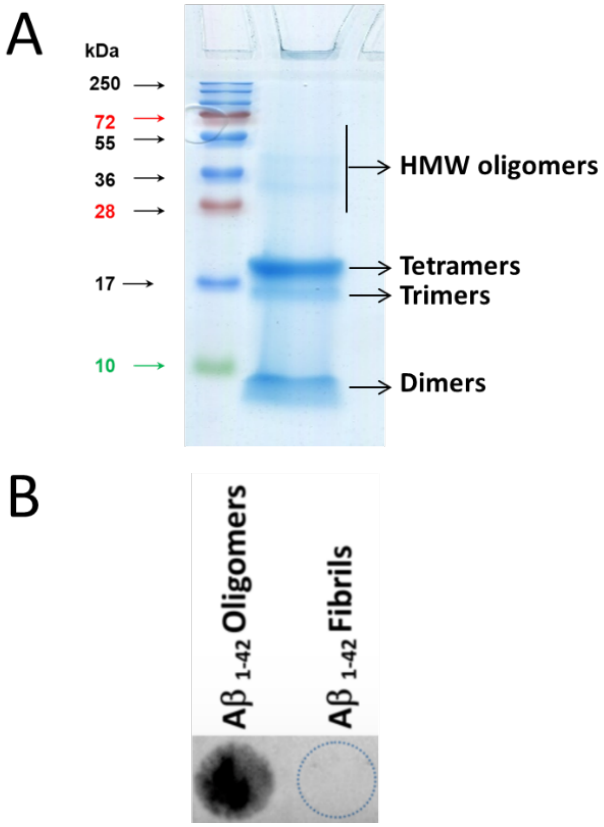


**Figure 2. Characterization of ETAP-Lab's AβO aggregates. (A)** Human AβO preparations were separated using 15% SDS-PAGE gel under non-reducing conditions. The gel profile revealed by Coomassie blue staining demonstrates that AβO aggregates are mainly composed of low-molecular-weight oligomers. **(B)** Dot blotting detection of Aβ oligomers and Aβ fibrils. AβO and Aβ fibrils were probed with the Rabbit Anti-oligomer (A11) antibody. Only oligomer preparations are recognized by this antibody.

### Dose-response of AβO injury effect on neuronal culture

**In order to validate AβO neurotoxicity,** ETAP-Lab tested the new batch of oligomer on primary cortical neurons on DIV5 (5 days *in vitro*) by Live/Dead cell viability assay. ETAP-Lab's AβO preparation induced a clear dose-dependent neurotoxicity while Aβ monomers (AβM) did not show any neurotoxic effect (Fig 3). However, AβO at 1µM did not induce a significant cytotoxic effect. Statistical analyses were performed using the StatView®5 statistical package (SAS Institute Inc., Cary, USA).

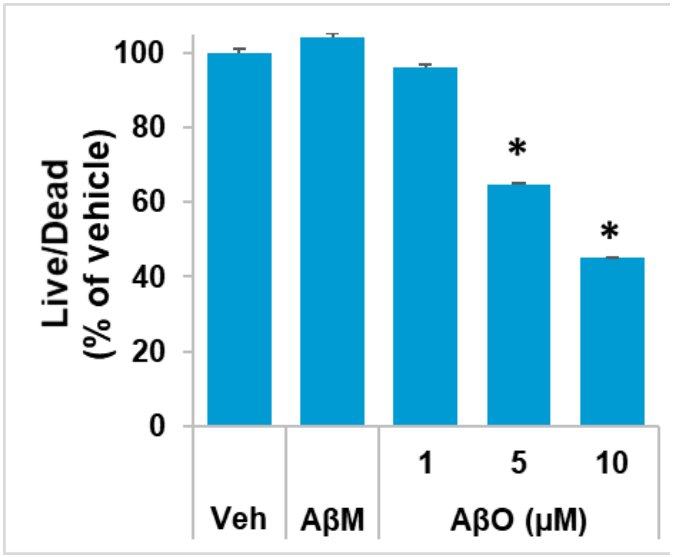


**Figure 3. Human AβO-induced dose-dependent neurodegeneration.** Data are expressed as percent of vehicle (set at 100%) and represent the mean ± SD (n=4/condition, N=3 independent experimentations) * P<0.0001 vs vehicle-treated cells (Scheffe's test)

## Evaluation of hippocampal neurons viability to AβO injury exposure

### Live-dead assay to quantify neuronal viability in microfluidic devices

Thanks to NETRI's microfluidic device, it has been possible to maintain neuronal culture during DIV21 to DIV24, with 3 media changes per week.

**To observe AβO effect on neurons,** we seeded rat hippocampal neurons from 18-day old embryos (OFA, Charles River Laboratories) following our Operating Protocol DR_3B_026.01. Before experiments, we have characterized neuronal culture by marking cells seeded on MEA with specific stain markers as MAP2 (1/50, in red) for neuronal maturation and GFAP (1/500, in green) for astrocytes (Fig 4-A). We also have maintained neuronal culture up to DIV24 and observed neuronal morphology with β-III-tubulin marker (1/200, in red) and astrocytes presence with GFAP marker (1/500, in green) (Fig4-B).

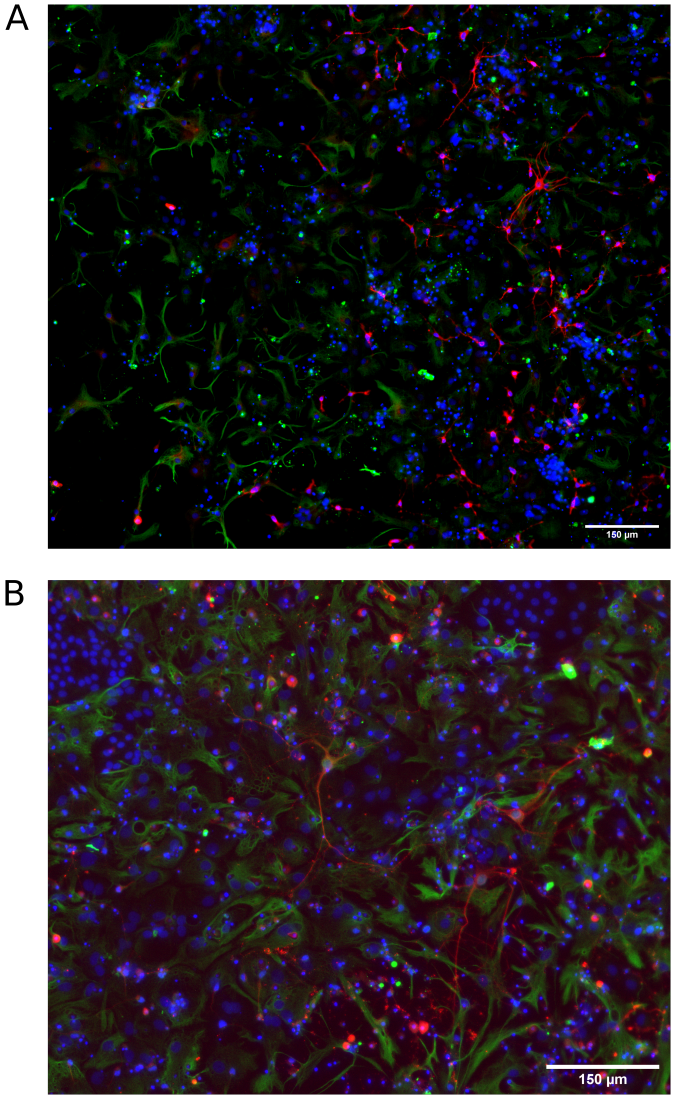


**Figure 4. Pictures of immunofluorescence characterization of hippocampal neurons in NF_1_CD_100.** **(A)** Hippocampal neurons seeded on MEA and marked at DIV21 with MAP2 (1/50, in red) for neuronal staining, GFAP (1/500, in green) for astrocytes and DAPI (in blue) for cell nuclei. **(B)** Hippocampal neurons marked at DIV24 with β-III-tubulin (1/200, in red) for neuronal staining, GFAP (1/500, in green) for astrocytes and DAPI (in blue) for cell nuclei.

**In order to quantify cell viability into the microfluidic device**, we have performed neuronal viability assay (LIVE/DEAD™ Viability/Cytotoxicity L3211, Thermo Fisher Scientific Inc,) with and without AβO application (Fig 5). With this assay, it is possible to discriminate live cells, stained with green and dead cells, stained with red. Images' analysis were performed with a proprietary software developed in collaboration with CIQLE, Lyon. The concentration of AβO at 1 µM was chosen because it does not induce cytotoxicity. In good accordance with ETAP-Lab data obtained on DIV5, AβO did not induce a significant cytotoxic effect on mature primary

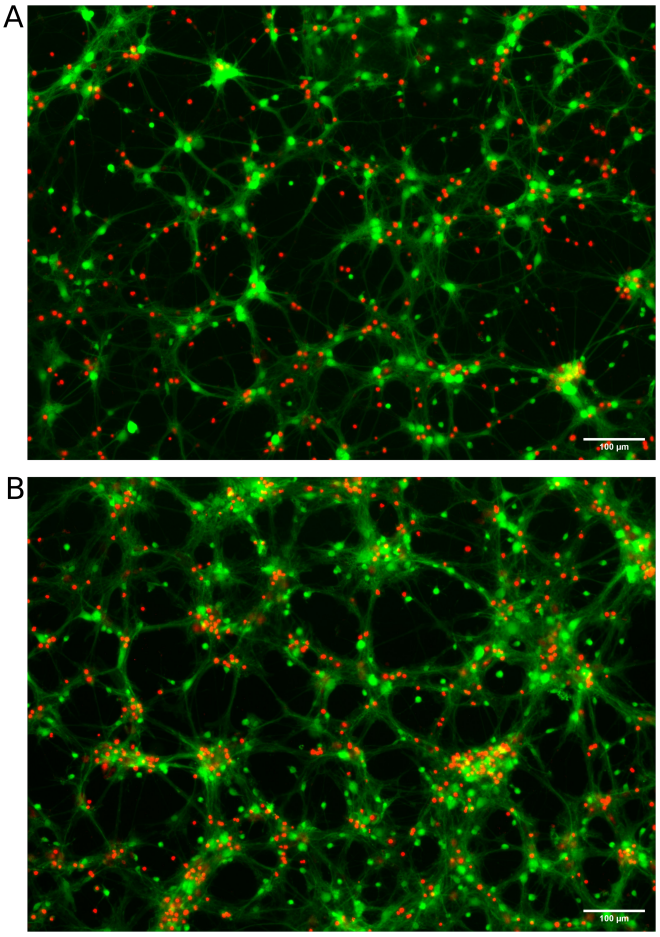


**Figure 5. Pictures[1] of Live/dead assay on neurons to observe cell viability.** **(A)** Neuronal viability assay (live cells in green and dead cells in red) performed on hippocampal neurons at DIV18 without peptide injury **(B)** and with 1µM of AβO injury application.

hippocampal neurons (DIV18) with a 92% Live/dead of vehicle. However, AβO clearly modified the visual aspect of cytoskeletal network with more neuronal clusters suggesting that neurons were suffering (Fig 5).

## Evaluation of functional activity after β-amyloid peptide injury exposure

### MEA system for neuronal activity recording

Cultured hippocampal neurons were recorded during 10 min with MEA2100-256-Systems from Multi Channel Systems (MCS).

All experiments were carried out with 256MEA100/30iR-ITO-w/o (MCS) consisting of 30-µm-diameter electrodes spaced by 100 µm.

Electrodes, head-stage (MEA2100-256-Systems) and software (Multi Channel Experimenter), used for recordings, are commercially available by MCS. Raw data were off-line filtered by a Bandpass Butterworth filter 2nd order (with cut-off frequencies of 100 Hz and 2500 Hz)

and plotted with an in-house customized algorithm software, (IVC) and an open access software (MEAnalyzer), both developed with Matlab (The Mathworks co.).
MEA technology allows to record extracellular functional and spontaneous activity of neurons as a marker of network connectivity[2].
For functional recording, two conditions were tested using two different concentrations of AβO: a control with a 0µM concentration and 1 µM concentration (Fig 6)[3].
During electrophysiological recording, neurons in culture showed functional activity represented with spikes. They are peaks detected by the algorithm. Every spike detected was marked with a red point (Fig 6-B-C-D).

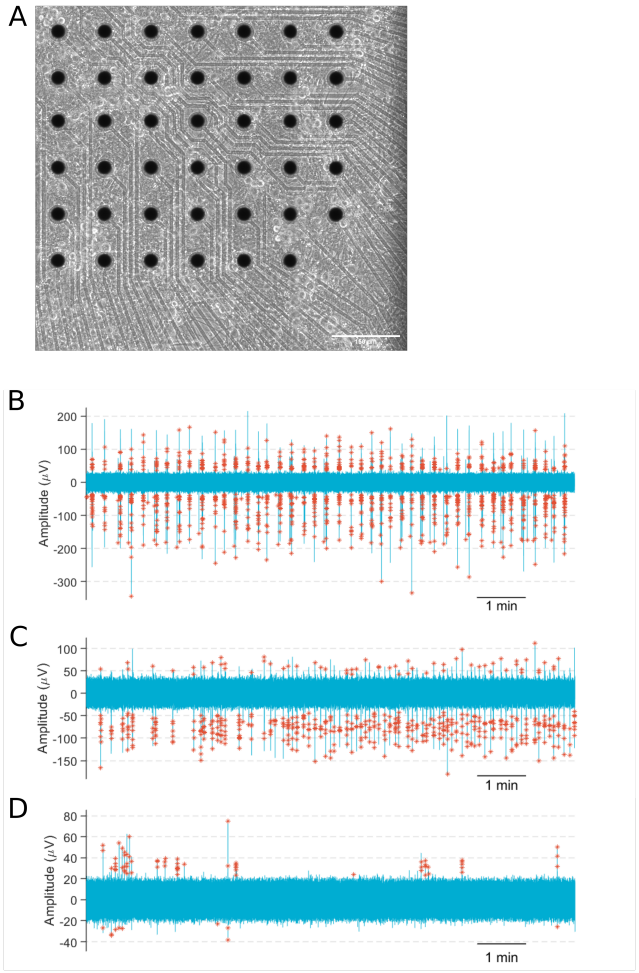


**Figure 6. Recordings with a MEA system in NF_1_CD_100 at DIV22. (A)** Picture in brightfield light of hippocampal neurons seeded in NF_1_CD_100 on MEA. Black points are electrodes. Recording plots of **(B)** condition 1 (control AβO=0µM), **(C-D)** condition 2 (control AβO=1µM). Red points represent every spike detected.

## *In vitro* connectomic algorithm for neuronal network analysis

Thanks to spikes data showed in Fig 6 B-C-D, it has been possible to create a matrix (raster plot) that shows the visualization of activity of each active electrode vs time (Fig 7 A). This graph allows to evaluate the synchronicity of a neuronal network, namely the capacity of neurons to have an activity at the same time. It clearly shows that the network of hippocampal neurons is synchronized without AβO (Fig 7 A-1) and desynchronized with 1µM AβO (Fig 7 A-2).

A neural network can be defined by nodes and interactions between them (Fig 7-B).
Cross Correlation is an algorithm representing the degree of neural network and allows to estimate the functional status of the network[4].
Network analysis show that the neural network has fewer connections with 1µM of AβO (Fig 7 B-2) than control condition (Fig 7 B-1). We conclude that AβO injury has a negative impact on neuronal network

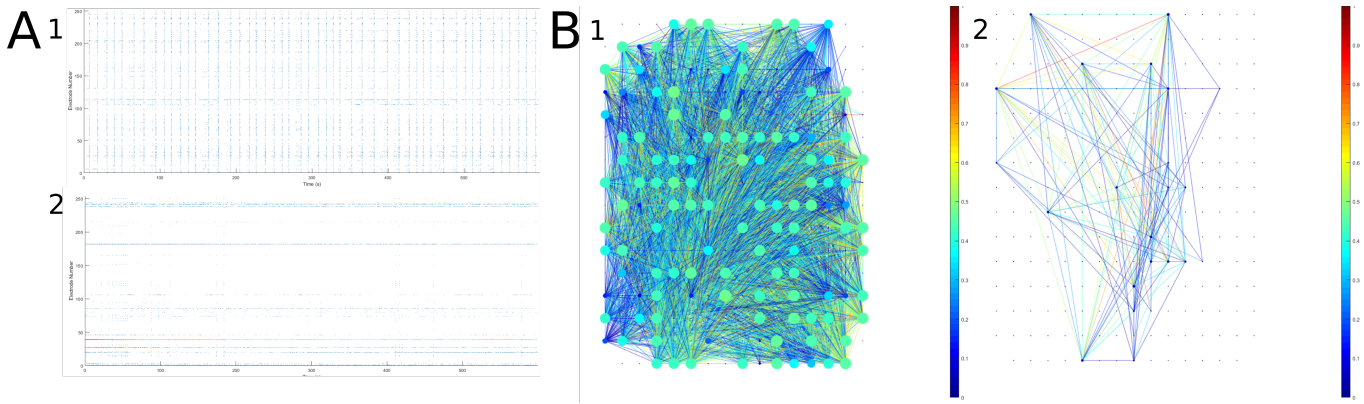


Figure 7. Network analyses of neuronal culture at DIV22 with an algorithm software. (A) Rasterplot with (A-1) a 0µM AβO concentration and (A-2) 1µM AβO concentration. (B) Cross-correlation graph with (B-1) a 0µM AβO concentration and (B-2) 1µM AβO concentration.

The optimal network structure is defined by quantitative parameters given by the **cross-correlation** algorithm and allows to estimate the quality of the network connectivity (Table 1). Results show that the network was poorly connected with the addition of 1µM AβO on neurons. Nodes were not connected enough to have a sufficient clustering coefficient (Table 1). There is a major difference in functional connectivity between condition with and without injury. Therefore, we can conclude that network connectivity is a relevant parameter showing information about neural network quality.

| | Control (0µM AβO) | 1µM AβO |
|---|---|---|
| Small-World Index | 3.4729 | NaN |
| Characteristic Path Length | 1.0327 | 1.4869 |
| Clustering Coefficient | 3.5864 | NaN |

Table 1. Quantitative data from cross-correlation analysis.

## Conclusions

This application note reports :

- Long-term culture is possible in Deposition Chamber technology developed by NETRI. Hippocampal neurons were mature and functional up to DIV24.
- Electrophysiology recording is compatible with microfluidic device and can be performed over time.
- Network analytics is a method allowing the discrimination of neural network structure and connectivity in a microfluidic device.

- Sub-cytotoxic concentrations of human AβO produced by ETAP-Lab disturb neuronal architecture and significantly impair network connectivity and functioning.
- This in vitro AD model could be a new relevant tool to evaluate the effect of a drug on neuronal functioning.

## Resources

*Available upon request*

- Technical Sheet NeurofluidicsTM-NB8L1NF_1_CD_100.
- Operating Protocol NeurofluidicsTM-NB8L1NF_1_CD_100.
- Human Amyloid-β oligomers: on demand.

## References

1. Schindelin, J. et al. Fiji: An open-source platform for biological-image analysis. *Nature Methods* **9**, 676–682 (2012).
2. Maccione, A. *et al.* A novel algorithm for precise identification of spikes in extracellularly recorded neuronal signals. *J. Neurosci. Methods* **177**, 241–249 (2009).
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4. Poli, D., Pastore, V. P. & Massobrio, P. Functional connectivity in in vitro neuronal assemblies. *Front. Neural Circuits* **9**, 1–14 (2015).