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Three-dimensional neural cultures produce networks that mimic native brain activity

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ABSTRACT

Development of brain function is critically dependent on neuronal networks organised through three dimensions. Culture of central nervous system neurons has traditionally been limited to two dimensions, restricting growth patterns and network formation to a single plane. Here, with the use of multichannel extracellular microelectrode arrays, we demonstrate that neurons cultured in a true three-dimensional environment recapitulate native neuronal network formation and produce functional outcomes more akin to *in vivo* neuronal network activity.

MAIN TEXT

Functional electrophysiological recordings are used *in vivo* to interrogate neural networks and understand neural coding of information (Colom *et al*., 2006; Magill *et al*., 2000; Rajan *et al*., 2007). A fundamental constraint of *in vitro* neuroscience research is the limited networking capacity of neurons in 2D culture systems. Engineered 3D neural constructs *in vitro* mimic the natural cellular environment more accurately than traditional 2D substrates

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(Edelman & Keefer, 2005), with single cell function investigated in these substrates with patch clamp electrophysiology (Irons *et al*., 2008; Ma *et al*., 2004; Xu *et al*., 2009) and calcium fluorescence imaging (Bosi *et al*., 2015; Ma *et al*., 2004). Although network activity can be inferred from single cell response to addition of glutamate or GABA, or antagonists thereof (Bosi *et al*., 2015; Ma *et al*., 2004), the observation of overall network activity requires simultaneous electrophysiological recordings from multiple sites (Odawara *et al*., 2013; Odawara *et al*., 2016; Odawara *et al*., 2014).

Toward developing a 3D neural culture model, Pautot *et al*. (2008) constructed an ordered colloidal substrate within which neurons could be seeded. Placing these colloidal neural structures atop microelectrode arrays (MEA), Frega & Tedesco *et al.* developed an electrophysiological recording system from an ordered neuronal construct (Frega *et al*., 2014; Tedesco *et al*., 2015). However, neurites in such colloidal constructs can only extend along bead surfaces, so the bead-to-bead connections limit neurite interactions between the highly segregated neurons. Given the spatial arrangement of bead-to-bead connections between stacked layers, interconnectivity is inherently higher within a layer than between layers (Frega *et al*., 2014; Pautot *et al*., 2008). Yla-Outinen *et al*. completed neural recordings from contiguous protein based Puramatrix gels on an MEA, although, as stated by the authors, synchronous spiking indicative of neuronal network activity was not observed (Ylä-Outinen *et al*., 2014).

We have developed a method for recording multichannel electrophysiological data from a 3D culture environment composed of a collagen gel, which allows unrestricted cell positioning and neurite outgrowth, and more naturally recapitulates network formation of brain tissue. Using a protocol previously described (Bourke et al., 2014), embryonic day 18 rat hippocampal neurons were isolated and dissociated, with all experiments carried out in accordance with the *Australian code for the care and use of animals for scientific purposes* (National Health and Medical Research Council). Neurons were either seeded on MEA's

(60MEA200/30iR-ITO-gr, Multi Channel Systems) in a two-dimensional monolayer, or encapsulated within a three-dimensional gel composed of 400 μg/mL collagen (Corning, #354236) and 100 μg/mL extracellular matrix protein (Merck Millipore, 08-110) at 400,000 cells/mL. Arrays were coated with pol-L-ornithine (Sigma Aldrich, P4957, 0.01%) followed by laminin (Thermo Fisher, 23017-015, 20μg/mL), each for 4 hours at 37^{o}C prior to seeding cells in 2D at 30,000 cells/cm^{2}. Three-dimensional cultures were constructed in culture dishes and transferred to MEA's for electrophysiological recordings. Constructs were maintained in medium containing neurobasal medium, 1% N2 supplement, 2% B27 supplement, 100 U/mL penicillin, and 100 μg/mL streptomycin (media reagents from Thermo Fisher), with approximately 70% of medium replaced every 3 to 4 days throughout culture. At 35 days in culture, spontaneous electrophysiological activity of the hippocampal neurons was recorded from 2D (Fig 1A) and 3D (Fig 1B) cultures at 25 kHz with a 60 channel microelectrode array system (USB-MEA system with MEA1060 amplifier and MC Rack software, Multi Channel Systems). During recordings, cells were perfused with a solution of (in mM) 137 NaCl, 1.5 $CaCl_2$, 5.4 KCl, 0.44 KH_2PO_4, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 0.3 Na_2HPO_4, 4 $NaHCO_3$, 5.6 D-glucose, and 10 HEPES, at pH 7.4. In response to glutamate addition (200 μM L-glutamic acid, Sigma Aldrich), neurons in both 2D (Fig 1C) and 3D (Fig 1D) increased their firing rate and gradually decreased in spike amplitude, indicating the presence of glutamate sensitive neurons. In all cases, activity returned following washout with perfusion solution. Imaging of 2D and 3D networks was completed at 35 days in culture, with samples fixed in 10% formalin for 20 min (2D) or overnight (3D) and stained with neuron specific TuJ1 primary antibody (Covance MMS-435P, 1:1000) followed by Alexa Fluor 488 secondary antibody (Thermo Fisher, 1:500). Samples were counterstained with DAPI and imaged on a Zeiss confocal microscope (Fig 2).

Striking differences in timing and duration of neuronal bursts were observed between 2D and 3D cultures. Neuronal activation originated from spontaneously active pace-making regions within both 2D and 3D cultures. In 2D, activity flowed directly across the plane of the culture resulting in a short repetitive temporal bursting pattern (Fig 1E), with each

activation of the pace-making region resulting in one short burst at each electrode. In contrast, the 3D cultures exhibited bursts of extended duration separated by quiescent periods (Fig 1F). The extended bursting activity in three dimensions arises from oscillatory activation through multiple network pathways with different path lengths, and indicates extensive network formation throughout the collagen gel. Voltage trigger levels were set to detect neural spikes on multiple channels using in-house software (Matlab), with strong synchronicity in spike activity observed between channels in both 2D (Fig 1G) and 3D (Fig 1H) constructs, confirming functionally mature networks in both culture conditions.

Activation patterns were found to be consistent over extended recording periods in both 2D (Fig 3A) and 3D (Fig 3B) cultures, with synchronicity of spike times across multiple electrodes indicating fully connected neuronal networks. The extended bursting pattern observed within the non-restrictive 3D collagen gel contrasts with previously reported spiking patterns within colloidal 3D constructs. The colloidal constructs of Frega *et al.* (2014) exhibited periods of both short neuronal bursts and extended bursting patterns, as would be expected for a series of discrete stacked 2D layers with limited inter-layer functional connectivity. In contrast, the *in situ* neuronal networks of the hippocampus (Colom *et al*., 2006), and networks between the subthalamic nucleus and cortex (Fig 1 inset) (Magill *et al*., 2000), each exhibit extended neuronal bursting patterns that more closely match the extended bursting of networks formed in the 3D collagen environment (Fig 1F & 3B). The purely extended bursting pattern in the unrestricted 3D collagen environment suggests that the contiguous gel has a more fully interconnected neural network, and thereby produces network function more akin to neuronal networks within the brain.

With unrestricted cell positioning and neurite outgrowth, the 3D collagen culture model reported here transforms intercellular interactions from a planar 2D array to three dimensions. Interrogating intercellular communication within these neural constructs using microelectrode arrays reveals network formation and function that mechanistically models

archetypal network activity within the brain. This functional 3D neuronal networking model provides a readily adaptive system for investigating functional network formation and plasticity, and will provide insight into mechanisms behind networking disorders such as epilepsy and schizophrenia. In conjunction with patient-derived cells as a neuronal cell source, the 3D networking model presented here will provide a vehicle for personalised drug screening for individuals afflicted with neural networking disorders.

AUTHOR CONTRIBUTIONS

J.B., A.Q. and C.O. contributed to methods development. J.B. and A.Q. performed cultures and collected the electrophysiological data, J.B. completed the data analysis. SD performed the confocal imaging, and all authors contributed to project planning and manuscript preparation.

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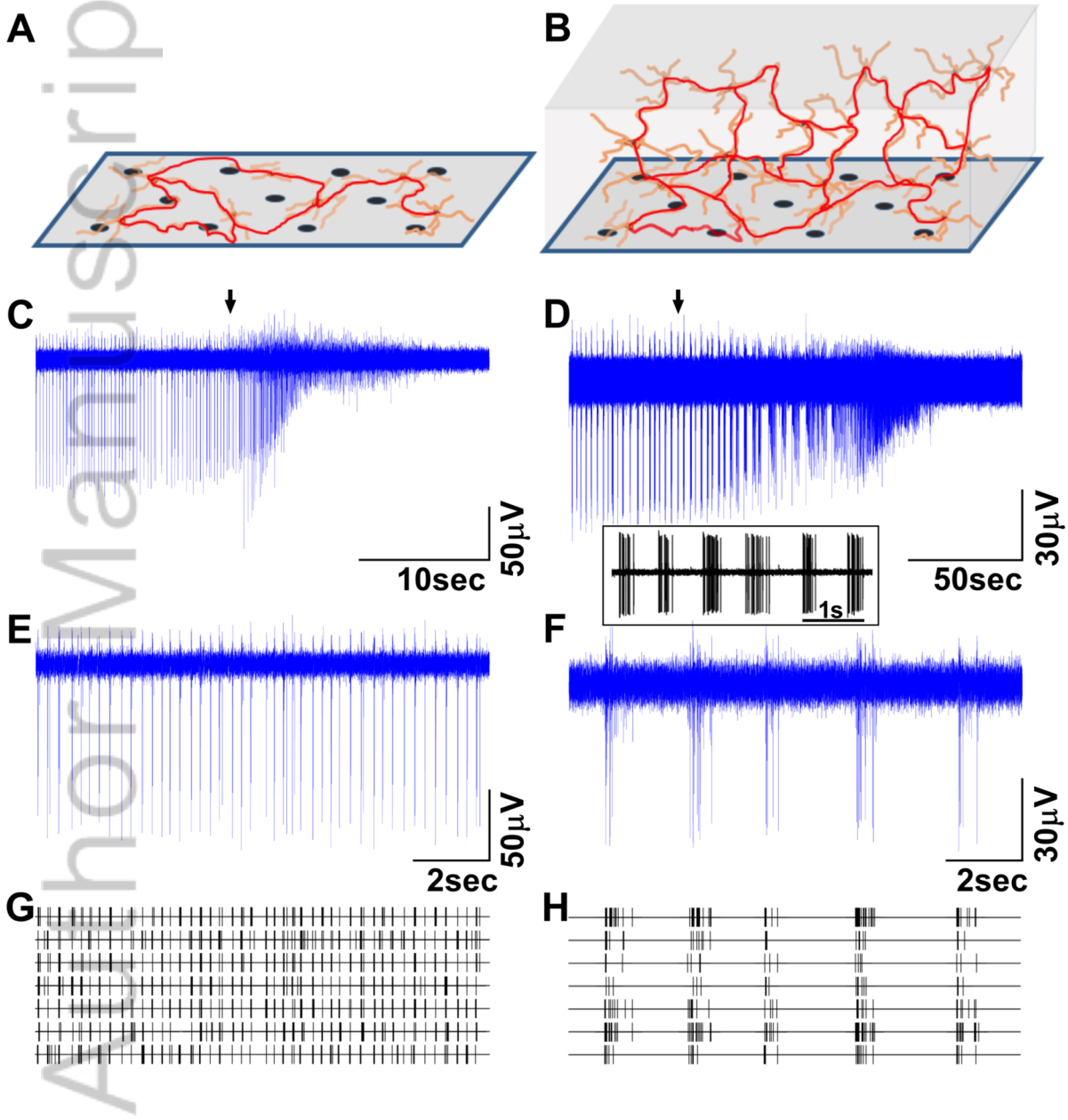
Figure 1: Network formation in (A) two and (B) three dimensions, the z-direction providing an added dimensionality of neural network connectivity (red indicates network activation). Typical MEA recordings from neuronal networks upon K-L-glutamic acid addition (200 μM) as indicated by arrows in (C) 2D and (D) 3D neuronal cultures, confirming presence of functional glutamatergic neurons, with the extended response time in 3D due to delayed diffusion of glutamate into the 3D gel. Typical single electrode recordings from (E) 2D and

(F) 3D cultures with comparable time-scales illustrate the short fast bursting of 2D (inter-burst interval 245 +/- 71 ms for this trace) and extended bursting pattern of 3D cultures (inter-burst interval 2395 +/- 388 ms and burst duration 408 +/- 116 ms for this trace). Inset is an *in vivo* recording from the rat subthalamic nucleus for comparison, adapted from Magill *et al.* (Magill et al., 2000), note the similarity between 3D collagen gel bursting patterns and *in vivo* network activity. (G,H) Synchronised spike timing of multiple channels matched to timing of traces in E and F respectively. Values presented as mean +/- standard deviation.

Figure 2: Neuronal networks imaged at 35 days in culture in (A) 2D and (B) 3D cultures, with TuJ1 (green) and DAPI (blue) (A & C-G). The depth color-coding of the 3D confocal images (B) illustrates neuron position throughout the gels, with slices at various depths also presented (C-G). Neurons in slices at different depths (C-G) are each indicated in the depth color coded image (B) as labelled (a'-j'). Scale bars 50μm.

Figure 3: Typical single electrode recordings in (Ai) 2D and (Bi) 3D cultures. Synchronicity within raster plots of spike times across multiple electrode in (Aii) 2D and (Bii) 3D cultures indicate fully connected neural networks in both culture conditions. Note the fast spiking patterns in 2D cultures and slower, highly consistent bursting patterns in 3D networks.



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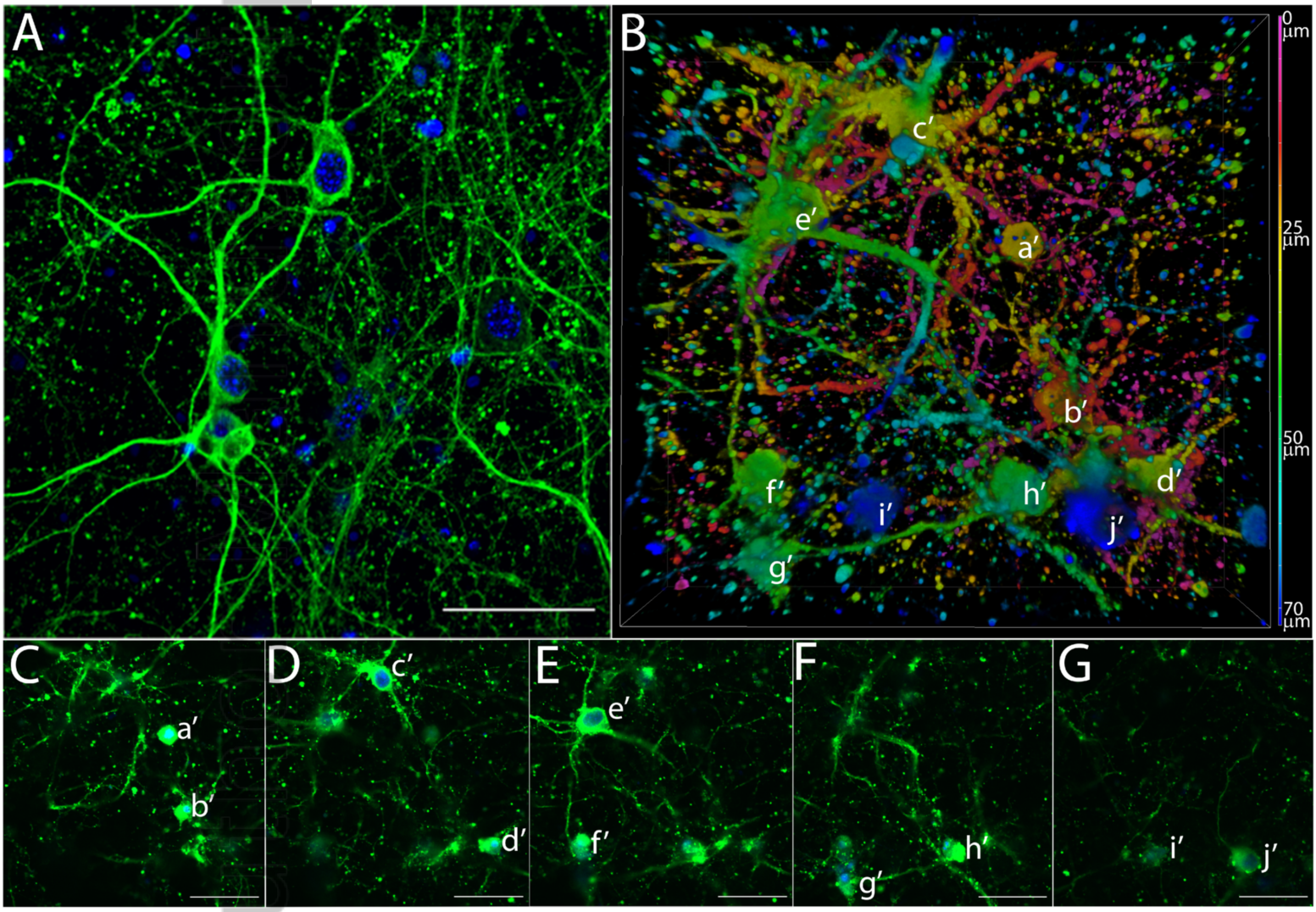

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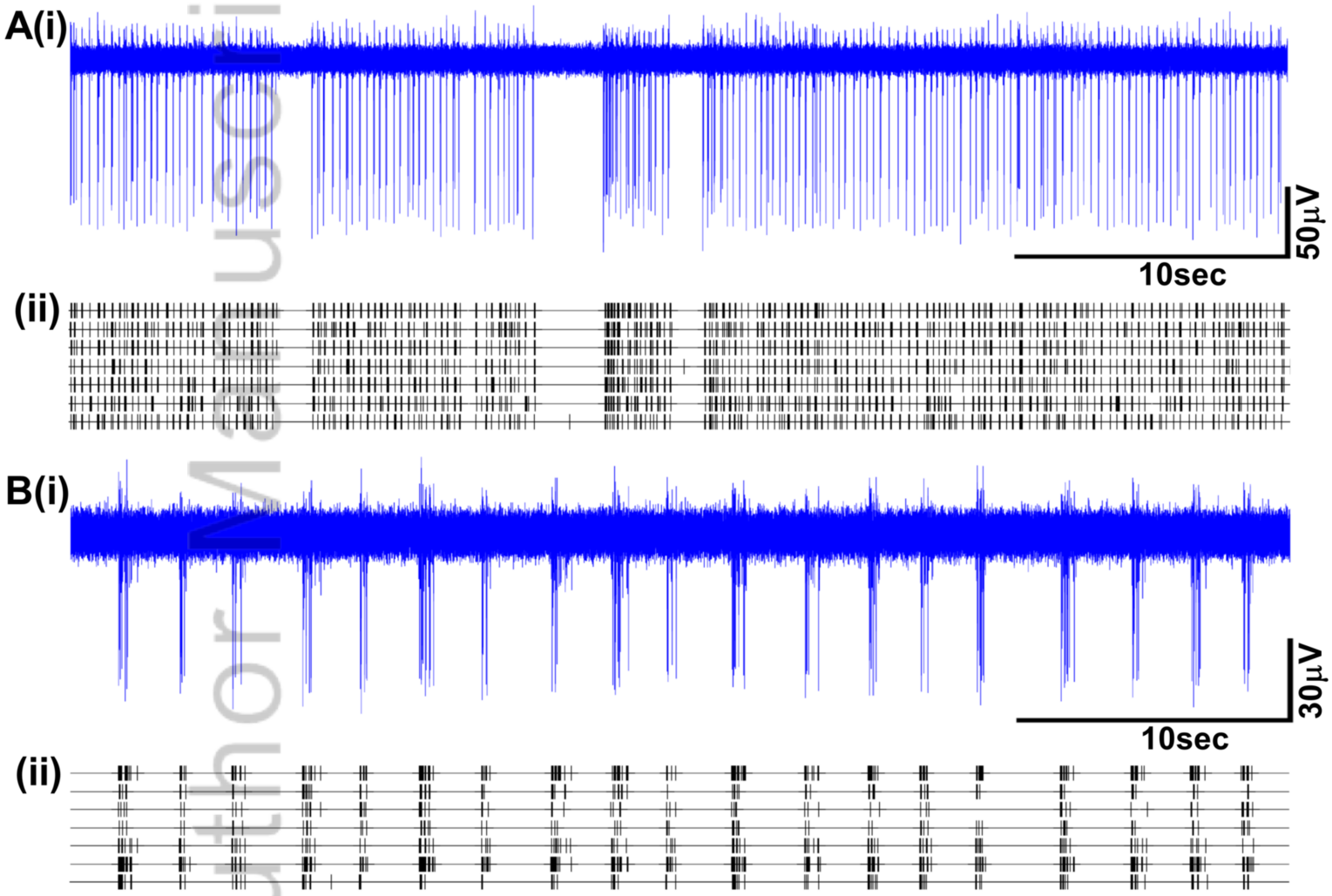

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