Multiple Time Scales Observed in Spontaneously Evolved Neurons on High-density CMOS Electrode Array

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Abstract

Spontaneous evolution of neural cells was recorded around 4-34 days in vitro (DIV) with high-density CMOS micro-electrode array, which enables detailed study of the spatio-temporal activity of cultured neurons. We used the CMOS array to characterize 1) the evolution of activation patterns of each putative neuron, 2) the developmental change in cell-cell interactions, and finally, 3) emergence of multiple timescales for neurons to exchange information with each other. The results revealed not only the topology of the physical connectivity of the neurons but also the functional connectivity of the neurons within different time scales. We finally argued the relationship of the results with “functional networks”, which interact with each other to support multiple cognitive functions in the mature human brain.

Introduction

How can the gap between living and nonliving matter be bridged? Since 1987 when Artificial Life was launched by Christopher Langton, we have not answered this question. Rodney Brooks wrote in the paper (2001) that there are four possibilities why we still cannot make living machines: 1) An alife model is correct, but several parameters were set incorrectly; (2) an alife model needs more complexity; (3) we need more computational power; and (4) a new fundamental law in addition to the laws of physics is needed. In this paper, we search for the possibility of (2) yet unrevealed laws of neuro-dynamics, by studying cultivated neural cells on a glass plate.

Biological neurons are cultivated on a glass plate from neural “seeds”. The seeds develop into either neural or glia cells. Neurons have cell bodies, axons, and numerous dendrites, which the neurons use to connect with each other. A unique characteristic of the present study is that we record the action potential from neurons by using a CMOS array glass plate. As we will describe later, each CMOS is the same size order of the neurons. Therefore, by using the CMOS array, we can potentially accurately record the time series of each neural firing. A remarkable aspect of this biological neural network is the developmental process. The entire time course of the growing process can be recorded with the CMOS array. We analyze the time series data to characterize the developmental dynamics.

A disadvantage of this experiment is that we have no way of designating which neurons connect to which. We thus measure the information transfer from the time series to infer the neural connectivity. This method reveals not only the topology of the physical connectivity of the neurons but also the functional connectivity of the neurons within different timescales. A finding in this paper is that growing biological neurons use different time scales to exchange information with each other.

The paper is organized as follows. In section of Materials and Methods, the specifications of the CMOS array and the biological conditions for the neural cells are provided. The method for cultivating cells and associated techniques are also described. In section of Result and Discussion, the analyzed results are presented. The activation patterns of each cell are quantified with inter-spike intervals (ISIs). Cell-cell interaction is also inferred by transfer entropy (TE), which reveals that multiple functional networks emerge from the neural original network. Finally, in section of Conclusion, the paper is summarized, and future work is discussed.

Materials and Methods

To measure the electrical activity of cultured neurons, we used a high-density CMOS microelectrode array (Frey et al., 2010). The CMOS array is pictured in figure 1 (a). This array is an emerging instrument for investigating the spatio-temporal activity of cultured neurons in detail. The CMOS array has 11,011 recording sites with an inter-electrode distance of 18 µm, i.e., in the order of cell body size, and a sampling rate of 20 kHz. This high spatio-temporal resolution allows precise recording of action potentials from the identified cell bodies of neurons. Using this high spatial resolution, we localized neural somata and recorded their activity.

1 All procedures were approved by the institutional committee at the University of Tokyo, and were performed in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Japanese Physiological Society.
This CMOS array is superior to conventional microelectrode array (MEA) (Sun et al., 2010; Eytan and Marom, 2006; van Pelt et al., 2004) in respect of spatio-temporal resolution: The locations of recording sites in conventional MEAs are predetermined, with an inter-electrode distance of 200 µm, so that it is difficult to identify signals from an individual cell, and neurons far from these recording sites are not included. Alternatively, optical imaging can be used to study the Ca++, dynamics of any neuron of interest; however, the temporal resolution is not high enough to characterize the action potentials of each neuron.

Figure 1: High-density CMOS array (a) Appearance. (b) Interval of each electrodes is close to the cell body size.

**Dissociated neural culture** The neural cultures were prepared from the cerebral cortex of E18 (embryonic 18) Wistar rats. The cortex was triturated with trypsin and dissociated cells were plated and cultured on high-density CMOS microelectrode arrays coated with polyethyleneimine and laminin. For the first 24 h, the cells were cultured in neurobasal medium containing 10% horse serum, 0.5 mM GlutaMAX and 2% B-27 supplement. After the first 24 h, half of the medium was replaced with growth medium in the form of Dulbecco’s modified Eagle’s medium with 10% horse serum, 0.5 mM GlutaMAX, and 1 mM sodium pyruvate. During the cell culturing, half of the medium was replaced once a week with the growth medium. The cultures were placed in an incubator at 37 °C with an H2O-saturated atmosphere consisting of 95% air and 5% CO2. We prepared for two conditions with different densities of neurons. The denser chips had 35,000 cells plated, denoted as Chip#1D and Chip#2D, while the sparser ones had 14,000 cells, termed Chip#1S and Chip#2S.

**Recording of neural activity** Neural activity was recorded with high-density CMOS microelectrode arrays. Before the neural somata activity was recorded, almost all of the 11,011 electrodes were scanned to obtain an electrical activity map with which we estimated the locations of the somata. A scan session consisted of 95 recordings; each recording was conducted for 60 sec with about 110 electrodes that were selected randomly, while avoiding overlap with already selected electrodes. An electrical activity map was obtained from the scanned data by calculating the average height of the spikes for every electrode. We assumed that the neural somata were near the local peaks in the Gaussian-filtered electrical activity map. About 100 of the higher-level peaks were selected, and then the nearest electrodes were selected for recording. If the number of local peaks was fewer than 126, then all the peaks were selected. The electrical activity of the selected electrodes was recorded for 30 min. All recordings were done at a 20-kHz sampling rate using the LimAda spike detection algorithm (Wagenaar et al., 2005) with a threshold of 5. Unexpected double-detected spikes were removed from the data before the analysis was conducted.

**Results and Discussion**

**Simple observation of activity patterns**

First, we observed the activation patterns of the neurons by examining the time series of the neural spikes. Figure 2 shows examples of raster plots of Chip#1D and Chip#1S. Chip#2D and Chip#2S showed similar tendency to their respective counterparts (data not shown). The data plotted here are a compressed version of the raw data with a different bin-length, which is denoted by ∆t. Namely, the spikes within the same bin are regarded as one spike. If ∆t is smaller, the time series represents single spikes generated from each single neuron, while a larger ∆t represents the macroscopic behavior created by an ensemble of neurons. In the figure 2, the data are plotted in Δt = 0.6 ms and 100 ms. At the top of each raster plot, the activation ratio is displayed. We chose Δt = 0.6 ms and 100 ms as the examples by two reasons; a single spike of neurons lasts around 1 ms, while, observably from figure 2, synchronous activation of neurons is detected around Δt = 10–100 ms. Therefore, we took Δt = 0.6 ms to capture interaction between individual cells (microscopic), while Δt = 100 ms is to observe collective activities generated from an ensemble of neurons (macroscopic).

As shown in figure 2(a) right, the Chip#1D neurons at days in vitro 7 (DIV 7) are activated intermittently in synchronization. This activation pattern was burst synchronization, in which an ensemble of neurons has active and silent phases; in the active phase, synchronous activation of the neurons is intermittently observed. The burst synchronization is a typical activation pattern observed in cell cultures (Maeda et al., 1995). On the left of figure 2(a), the silent phase of the neural activation is magnified with Δt = 0.6 ms. Neural spikes are observed sparsely, which suggests that each neurons was activated independently from others.

On the other hand, at the later stage (DIV 14) of the Chip#1D, burst synchronization is not observed (figure 2(b) right). Instead, we observed each neurons shows different activation patterns; i.e., some neurons were activated...
almost all the time, some showed spikes less often, and others remained silent. These neurons seem to be activated at different frequencies. The left of figure 2(b) shows that, at $\Delta t = 0.6 \text{ ms}$, a single spike is followed by another, which suggests that one single spike can activate another. In contrast, Chip#1S showed the burst synchronization throughout the entire recording period (figure 2(c) and (d)).

To summarize the results thus far, burst synchronization was observed in two conditions; in the earlier developmental stage of the dense cell condition, or in the sparse cell condition. In those cases, any single spikes hardly activate another. In the later stage of the dense cell condition, the neurons spiked at various frequencies, where single spikes induce others. This tendency is explained by the maturity of synapses. At the earlier developmental stage, the synapses are not mature; therefore, a single spike cannot activate another. Still, if the neurons send spikes at the same time (synchronous bursts), the signals become enough strong to activate each other. In contrast, at the later stage of development, the synapses were enough strong to transmit a single spike from one neuron to another.

**Single cell activity evaluated with ISI**

In the next step of the analysis, we quantified the activation patterns of each neuron. Therefore, we analyzed the inter-spike intervals (ISI) distribution of the neural activity. Figure 3 (a) depicts examples of ISI distribution recorded from putative single neurons cultured in Chip#1D. In the earlier culture stage (DIV 7; figure 3 (a) left), exponential decay was observed, while the neurons tended to obey the power law at DIV 14 (right figure). To quantify this tendency, we plotted the ISI frequency on a logarithmic scale, and we fit the slope with a straight line by the least squared method. If the ISI distribution follows the power law, the distribution should be a straight line, so that the R-squared value is an index of fitting the power law.

Figure 3(b) shows the average of the R-squared values over each neuron; the values tended to increase in Chip#1D, Chip#1S and Chip#2S. Especially, Chip#1D shows a drastic increase at DIV 10, which suggests the neural activity became closer to the power law. Chip#2D also shows a higher R-squared value throughout the entire recording period. The recordings for Chip#2D started from DIV 17, which explains the result is similar to the later stage of Chip#1D.

The power law means that each neuron exhibited a wide range of ISIs. It may be related to the observation from the raster plots (figure 2), which showed that a wide range of frequencies between neurons were observed at the later development stage. To sum them up, a broader range of time scales likely emerges after synaptic maturation. However, from this ISI analysis, it is not possible to understand how those cultured neurons interact with each other to generate the activity patterns. In the next part, we then investigated neuron connectivity.

![Figure 3](image)

**Cell-cell interaction inferred with transfer entropy**

We used transfer entropy (TE) to estimate the effective connectivity for transferring information from one neuron to another. TE measures directed information transfer, which detects causal relationship between two time series (Schreiber, 2000; Lizier et al., 2011; Staniek and Lehnertz, 2008; Bertschinger et al., 2008). For instance, higher TE from one neuron to another indicates that the first neuron strongly affects the second. Therefore, TE enables us to find the functional synaptic connectivity. We defined the TE, and then applied it to artificial neural networks to ensure the validity of TE to estimate effective connectivity. Finally, we applied TE to the cultured neural cells to infer their topology.

**Definition of transfer entropy** Information is measured with Shannon entropy, which quantifies the amount of uncertainty associated with a system $X$. Specifically, Shannon entropy of a system $X$ is defined as:

$$H(X) = -\sum_{x \in X} p(x) \log p(x),$$

(1)
Figure 2: Examples of the raster plot of the cultured neurons. The X axis denotes time (s). The Y axis represents the indexes of the recording channel, where one channel can be considered as one neural cell. The subfigures at the top of the raster plots shows the spike rate. (a) - (d) Results for Chip#1D and Chip#1S with different DIVs. $\Delta t = 0.6$ ms (macroscopic) and 100 ms (macroscopic).

where $p(x)$ denotes the probability of $x$ ($x$ is an event of $X$). To evaluate the dependency between $X$ and $Y$, mutual information ($MI$) is defined as follows:

$$MI(X, Y) = H(X) - H(X|Y) = H(Y) - H(Y|X). \quad (2)$$

$H(X|Y)$ means conditional entropy, i.e., the uncertainty of $X$ when $Y$ is known. Therefore, $MI(X, Y)$ suggests a decrease in the uncertainty of $X$ when $Y$ is known. $MI(X, Y)$ measures the dependency between $X$ and $Y$; so that this variable cannot quantify a causal relationship between them.

TE measures the causal relationship between $X$ and $Y$ by calculating the past history. The TE from $X$ to $Y$ is denoted by $TE_{X \rightarrow Y}$, which is written as:

$$T_{X \rightarrow Y} = H(y_{n+1}|y^{(k)}_n) - H(y_{n+1}|y^{(k)}_n, x^{(l)}_n)$$

$$= \sum_{n=0}^{N-1} p(y_{n+1}, y^{(k)}_n, x^{(l)}_n) \log \frac{p(y_{n+1}|y^{(k)}_n)}{p(y_{n+1}|y^{(k)}_n, x^{(l)}_n)} \quad (3)$$

where $n$ is the current time step, and $y^{(k)}_n$ and $x^{(l)}_n$ are the past variables with length $k$ and $l$ respectively (i.e., $y^{(k)}_n = \{y_n, y_{n-1}, ..., y_{n-k+1}\}$ and $x^{(l)}_n = \{x_n, x_{n-1}, ..., x_{n-l+1}\}$). When the next step of $Y$ ($y_{n+1}$) is conditioned from the past history of $X$ ($x^{(l)}_n$), then $H(y_{n+1}|y^{(k)}_n, x^{(l)}_n)$ takes a smaller value than $H(y_{n+1}|y^{(k)}_n)$. If $y_{n+1}$ is independently determined from the past history of $X$, then the two components will have the same value. Therefore, $TE_{X \rightarrow Y}$ measures the causal-ity of $X$ to $Y$.

**Settings of artificial neural networks** TE analysis was first applied to a computational neural model. The model was built around Izhikevich neurons connected through artificial synapses (Izhikevich, 2003). The Izhikevich neurons form a simple model of cortical neurons that is implemented by a system of two differential equations modeling the membrane potential and the refractory period. When the membrane potential reaches a threshold value (for instance, 30 mV), a spike is emitted. This spike is transferred to post-synaptic neurons through some shared synapses. The voltage on arrival is the original spike strength, modulated by the efficacy of the synapses. For instance, an initial spike of 30 mV traveling on a synapse with an efficacy of 0.5 delivers a voltage of 15 mV to the post-synaptic neuron. Every synapse has a delay of 1 ms between the time of emission and the arrival of a spike.

The complete model is composed of seven neurons: four input neurons receiving randomly generated external stimulations, two internal neurons and one output neuron. The parameters for the Izhikevich neurons correspond to the regular spiking model ($\alpha = 0.02, b = 0.2, c = -65 mV$ and $d = 6$). Different types of connectivity patterns have been tested, ranging from fully inter-
connected to sparse (figure 4 (a)–(c)). The strength of the
connection is randomly assigned based on uniform dis-
tribution. Every update of the model represents a 0.1-ms
step in time, which ensures the model’s mathematical sta-
bility. The total duration of a test is 1000 s, which corre-
sponds to 10,000,000 updates of the model.

**Estimated connectivity of the artificial network** From
the time series of the artificial neural activity, we cal-
culated the TE from one neuron to another. Using the
TE, we estimated the network structure of the artificial
neurons. A synaptic connection from one to the other was
assumed when the TE between two neurons was higher
than the threshold. Then, we compared the topology
of the reconstructed network and the original network
shown in figure 4 (a)–(c). The number of false edges for
each topology is shown in figure 5. ∆t in this figure is
the same one as previously used, which represents
the bin-length of the compressed time series. This figure
shows that the optimal parameter set to reconstruct the
original topology depends on the dimension of the past
variables (k and l) and ∆t.

The result ensures that the effective connectivity to trans-
mit signals by TE is estimated. However, a good approx-
imation depends on the dimension and time scale ∆t. We
used various ∆t to get a good approximation of the effec-
tive connectivity in the cultured neurons.

**Estimated connectivity of cultured neurons** Figure 6
shows some examples of the estimated network structure
of neurons. This depicts the network of Chip #1D DIV 14
with different ∆t (t = 0.6 ms, 1 ms and 10 ms). An edge is
drawn if the TE from one neuron to another is higher than
the threshold, which was set to 0.00001. The threshold is
determined arbitrarily, but to display dynamical change
in connectivity patterns. The first observation about this
figure is that different topology is structured depending
on ∆t. As is shown with the artificial neural network,
each connection may not have the same optimal ∆t to
estimate connections. Based on (Oka and Ikegami, 2013),
we used the optimal ∆t to understand the information
flow in the network.

**Optimal time scale to convey information**
As observed, the cell-cell interaction estimated from the TE
analyses depends on the time scale ∆t, so that we evaluated
the optimal ∆t to estimate the network structure better. We
defined the optimal ∆t as ∆t* that maximizes TE. ∆t* was
calculated for each directed pair of neurons. Information
was considered to be transferred most effectively with the
∆t*. Therefore, the ∆t* exhibits effective synaptic connec-
tivity from each neuron to another.

Figure 7 (a) and (b) depict ∆ t* from one neuron to an-
other in Chip#1D and Chip#1S respectively. Edges are
drawn if TE at the $\Delta t^*$ is larger than the threshold (= 0.00001; same as the one used above). The color indicates the value of $\Delta t^*$, where red represents $\Delta t^*$ smaller than 10 ms and blue indicates larger. We defined $\Delta t^* = 10$ ms as a threshold to assign color, because burst activity was observably detected with $\Delta t$ larger than 10 ms (figure 2). Hereafter, we call $\Delta t > 10$ ms “the smaller $\Delta t^*$”, and $\Delta t \geq 10$ ms “the larger $\Delta t^*$”, where we divide the activity pattern into microscopic and macroscopic using this threshold. Additionally, figure 8 shows the $\Delta t^*$ distribution.

Figure 7 (a) and 8 (a) show that, at an earlier stage of the development of Chip #1D, a larger $\Delta t^*$ is mainly observed. The number of the smaller $\Delta t^*$ increases at DIV 12, which means a causal relationship between each neuron is observed within a shorter period. This is consistent with the observation in figure 2 (b) that a single spike activates another neuron within a short period. For Chip#2D, a smaller $\Delta t^*$ was observed throughout the entire recording period (figure 8(b)). Chip#2D was recorded beginning at DIV 17, which is relatively later compared with those of the other chips. That can explain why Chip#2D was similar to the later stage of development of Chip#1D.

Interestingly, the smaller $\Delta t^*$ observed in Chip#1D and Chip#2D coexisted with the larger $\Delta t^*$ (figure 8 (a) and (b)). This suggests that neural activity occurs at a wider range of time scales. Figure 7 (a) shows that the intervening neurons have red and blue edges, which implies microscopic and macroscopic activity to exchange information with each other. However, the frequency of the smaller $\Delta t^*$ in Chip#1S and Chip#2S was low throughout the entire recording period (figure 7(b), 8(c-d)). Still, similarly to the result for the dense cell condition, as DIV increased, the number of the smaller $\Delta t^*$ grew.

Conclusion

We characterized neural activity cultured on a high-density CMOS array. The neurons showed different activation patterns depending on the density and age (days in vitro; DIV). The neurons did not receive external inputs, but still spontaneously evolved. The neurons were cultured in two different conditions, i.e., dense or sparse.

When the cell density was high, the neurons showed burst activity first, and afterward, each neural cell activated at a different frequency (figure 2 (a), (b)). Some were activated all the time, some showed spikes intermittently, and others remained silent. The ISI distribution of each cell became closer to the power law as the DIV increased (figure 3), which suggests a single cell evolved to show a wider range of time scales. These results suggest that neural activity exhibits a wider range of time scales as the synapses mature. This tendency was shown clearly with TE (figure 7). At the earlier stage of development, TE was mainly optimized at a larger time scale, while, at the later stage, TE was also optimized at a smaller time scale (figure 8).

A mature human brain is a collection of functional networks, each of which corresponds to a different cognitive function (Fair et al., 2009). In this paper, we insist that even a neural network on a glass plate spontaneously develops “functional networks”, which can be distinguished in terms of the time scale determined by effective information transfer. Without relevant sensory input, we cannot say the networks are functional in the proper context of brain science; however, we speculate that the spontaneous development of “functional networks” is a candidate for the brain functional network. In future work, we will connect the neurons with a navigation robot to see how the functional networks actually “function” as cognitive modules.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research (Studies on Homeo-Dynamics with Cultivated Neural Circuits and Embodied Artificial Neural Networks; 24300080), Grant-in-Aid for Scientific Research (23680050), and Denso Corp. Eiko Matsuda thanks the Japan Society for the Promotion of Science for research funding.

References


Figure 7: Network structure obtained with $\Delta t^*$ from one neuron to another. The TE threshold for drawing edges is also equal to 0.00001. The color indicates the value of $\Delta t^*$, where red represents a $\Delta t^*$ smaller than 10 ms, and blue is for a larger $\Delta t^*$.


Figure 8: Distribution of $\Delta t^*$ with different DIVs. The X axis denotes $\Delta t^*$, while the Y axis shows the frequency of $\Delta t^*$ for each connection of neurons. (a) Chip#1D often shows smaller $\Delta t^*$ after DIV 12, while (b) Chip#2D displays smaller $\Delta t^*$ throughout the entire recoding periods. (c) (d) Smaller $\Delta t^*$ is observed less often. Still, $\Delta t^*$ decreases as DIV increases.