Interaction and intelligence in living neuronal networks interfaced with moving robot.

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ABSTRACT

Neurons form complex networks and it seems that the living neuronal network can perform certain type of information processing. We are interested in intelligence autonomously formed in vitro. The most important features of the two-dimensional culture neural network are that it is a system in which the information processing is autonomously carries out. We reported previously that the functional connections were dynamically modified by synaptic potentiation and the process may be required for reorganization of the functional group of neurons. Such neuron assemblies are critical for information processing in brain. Certain types of feedback stimulation caused suppression of spontaneous network electrical activities and drastic re-organization of functional connections between neurons, when these activities are initially almost synchronized. The result suggests that neurons in dissociated culture autonomously re-organized their functional neuronal networks interacted with their environment. The spatio-temporal pattern of activity in the networks may be a reflection of their external environment. We also interfaced the cultured neuronal network with moving robot. The planar microelectrodes can be used for detecting neuronal electrical signals from the living neuronal network cultured on a 2-dimensional electrode array. The speed of actuators of moving robot was determined by these detected signals. Our goal is reconstruction of the neural network, which can process "thinking" in the dissociated culture system.

Keywords: Keywords: multi-electrode array, dissociated culture, synaptic potentiation, functional connection, cell assembly, scale-free network, miniature moving robot

1. INTRODUCTION

One of the features of the brain is a highly hierarchical system from the molecular level to the organ level. There have been many recent advances in methods for neuroscience research at the cellular level and the individual animal level, but there is still a gap between studies on higher brain functions and cellular function. Brain system is so complex. To bridge this gap, it will be important to simultaneously analyze molecular function and the dynamics of neuronal networks, attending to the relationship between each level. Another important features of the brain is that it is a nonlinear dynamical system. To elucidate the mechanism of information processing in brain system, we have to characterize network dynamics of living neuronal networks. A dissociated culture system developed on a multi-electrode array is particularly useful for these purpose. Dissociated neurons begin to elongate neurites on the multi-electrode array and construct a complicated living neuronal network.[1–8] Although their environmental is different from that in the brain, it seems that the dissociated neurons are equipped with the fundamental mechanisms necessary for the formation of functional networks. In addition, spontaneous ensemble electrical activities are observed in developed networks, which seem to be able to perform certain types of information processing. Functional assemblies of neurons in the brain have been reported to be critical for information processing,[9–11] and such functional assemblies of neurons can also exist in cultured living neuronal networks. The spatio-temporal patterns of activity in these networks might reflect functional neuron assemblies. We think these dynamic functional neuron assemblies correspond to some symbol of objects in outer world, and the modification of these assemblies corresponds to operation of symbols. We have hypotheses that cultured living neuronal networks possess fundamental system for intelligent behavior, and that a living

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neuronal network can generate intelligence if it interact to outer world. So, we are also developing the system in which living neuronal network interacts with outer world by the intermediary of moving robot. That idea was previously reported by Potter's group as Hybrot (Hybrid living+robotic).[12, 13] We also use similar configuration, but we provide a program which generates "premised control rules" for making a robot avoid obstacles, instead of making the living neuronal network generate such a rules autonomously. We are now trying to realize associative learning in vitro. We are interested in intelligence autonomously formed in vitro. Our ultimate goal is to make a living intelligent system in a culture dish.

2. METHODS

2.1. Primary culture of rat hippocampal neurons

The conduct of all experimental procedures was governed by the Guidelines for the Care and Use of Laboratory Animals of the AIST Kansai Center. The hippocampal neurons were prepared from Wistar rats on embryonic day 17 (E17) or E18 and cultured by a previously described method.[4, 14] Briefly, the hippocampuses of rat embryos were dissociated by treatment with 0.175% trypsin (Invitrogen-Gibco, U.S.A.) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) supplemented with 10 mM glucose at 37^oC for 10 min. They were then plated on a poly ethylene-imine- (or poly-D-lysine) -coated MED probe13 (Alpha MED Science, Japan), which has 64 planar microelectrodes on dish. Half of the culture medium was renewed every two days. The medium consisted of 45% Ham's F12, 45% Dulbecco's modified minimum essential medium (Invitrogen-Gibco, U.S.A.), 5% horse serum (Invitrogen-Gibco, U.S.A.), and 5% fetal calf serum (Invitrogen-Gibco, U.S.A.), supplemented with 100 U/ml penicillin, 100 ˚g/ml streptomycin (Invitrogen-Gibco, U.S.A.), and 5 ˚g/ml insulin (Sigma-Aldrich, U.S.A.). The dissociated neurons were cultured for 12-60 days at 37^oC in 5% CO2/95% air at saturating humidity.

2.2. Spike recording by the multi-electrode array

Spontaneous extracellular action potentials were recorded in the standard external bathing solution 10-60 days after the start of the culture. The standard external bathing solution contained (in mM) 120 NaCl, 3 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Na-Hepes (pH 7.3). The Mg^{2+}-free external bathing solution contained the same compounds except for the absence of $MgCl_2$. The osmolarity of each solution was adjusted to 300 mOsm with sucrose. The data were gathered with the MED64 system (Alpha MED Science, Japan) at a sampling rate of 10 or 20 kHz. All experiments were carried out at room temperature (20-25^oC). The recorded spikes were automatically analyzed using a program, MEDFAUST developed by us. Spontaneous events are detected when the amplitudes of spikes exceeded a pre-specified threshold. Detected spikes were classified by their amplitude -versus- decay-time distributions using modified k-means cluster analysis and classified to each neuron units. All these process performed automatically by MEDFAUST.

2.3. Connection map analysis

All possible combinations of pairs of spike trains recorded by the multi-electrode array were subjected to cross-correlation analysis. Then connectivity indices were calculated for each pair. The connectivity index is defined as follows:

$$Con.I. = A_{peak} \times \left(\frac{0.01 \times A_{peak}}{A_{total}} + \frac{1}{\triangle t + 1} + \frac{1}{Kurtosis} \right)$$

Where A_{peak} is an area within the 2 msec range around the peak of cross-correlofunction of the pair, A_{total} is the total area of cross-correlofunction, $\Delta - t$ is the distance of the peak of the cross-correlofunction from 0. The connectivity index indicates the degree of robustness of the relationship between the pair. The mean and standard error of all connectivity indices were calculated. When the value of the connectivity index exceeded a criterion, in this case, the mean plus standard error, the pair was assumed to be functionally connected. Each neuron is denoted as a small point in maps, and lines between the points represent the connectivity between the neurons indicated by these points. The color of the lines indicates the relative value of the connectivity index. Functional connections between all combinations of recorded spike trains are depicted simultaneously in

this 2-D map. In this study, we generated connection maps from data recorded for 10min, the bin width for calculation of the cross-correlation was 5 msec, and the range of the calculation of cross-correlofunction was 50 msec. Connection maps of serial experiments were expressed as follows. First, connection maps were generated for sequential recordings. Then each point indicating a neural unit in the second recording was shifted to the lower right. The parameters of classified APs, such as mean amplitudes, were compared with the parameters of the initial reference recording, and each point was identified as being the same as an individual unit seen in the initial recording. These units were then depicted at the same position as in connection map of the initial recording, and other units (including newly detected units) were left behind at the shifted positions. Units with more than 20 % fluctuation of the parameters in sequential records were identified as distinct neurons.

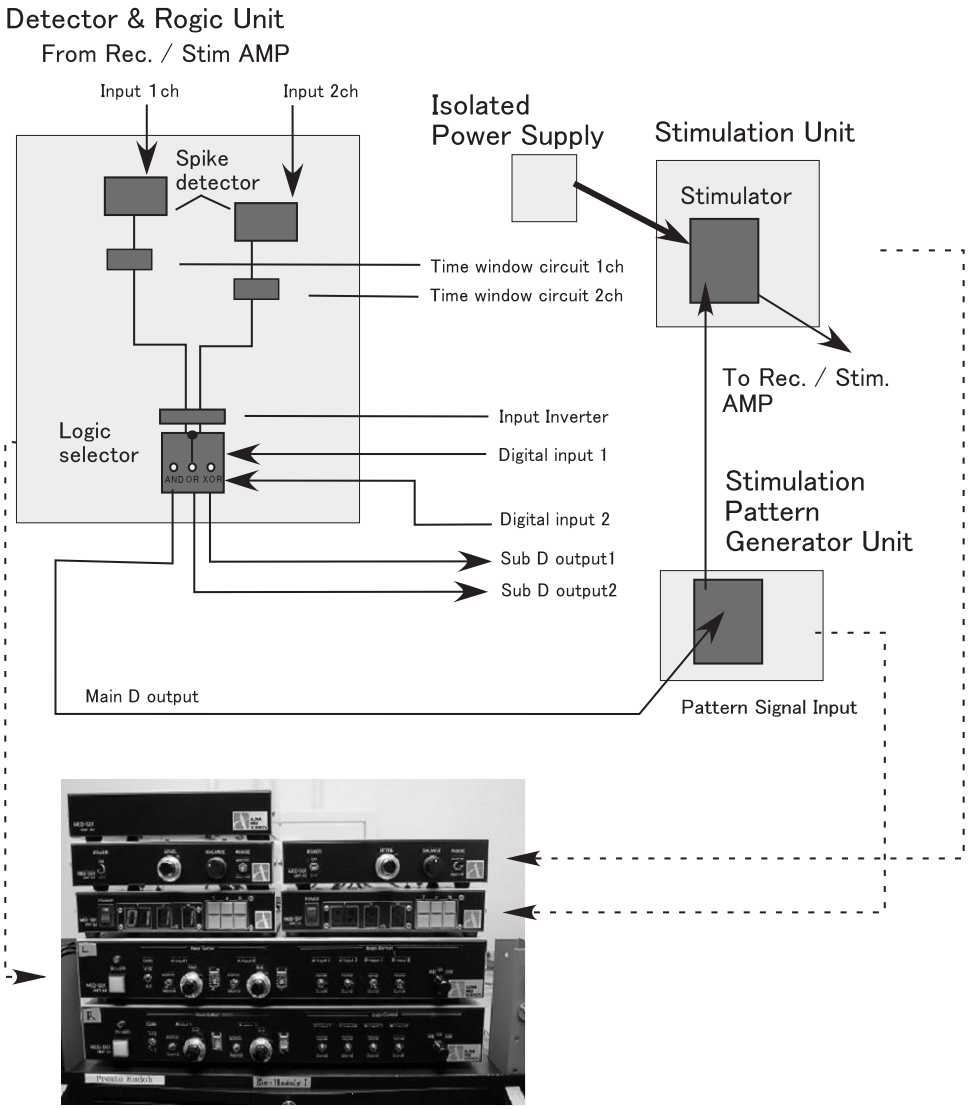

Figure 1. Real-time feedback stimulation system. This system composed by 3types of unit and the units can be combined one by one. Each "detector and logic unit" has 2 window discriminators and a logic calculator. The unit outputs trigger signal to "stimulation pattern generator unit". User can dtermin the frequency and the number of pulses using the patterngenerator unit.The stimulation unit is an isolator.

2.4. Real-time feedback stimulation

We designed real-time feedback stimulation system (fig. 1). The system was composed by 3types of unit and the units can be combined one by one. The detector and logic unit has two window discriminators and a logic calculator. User can select AND, OR and XOR, each combined with NOT as a logic calculator. For example, the unit output trigger signal only when both of two window discriminators output detection signal if AND is selected. The logic unit has two digital inputs and two digital output and it can output digital pulse determined by logical calculation to logic calculator in other units. Number and frequency of stimulation square pulses are determined by the stimulation pattern generator unit. The unit drive a stimulation unit, which has the function of isolator.

3. RESULTS AND DISCUSSIONS

3.1. Functional assembry of neurons by synaptic potentiation

The networks in rat hippocampus were once dissociated into each neuron and reconstructed their network on the dish with planer electrodes (fig.2). Neurons formed a network connecting to each other at synapses. The glutamatergic Spontaneous Post Synaptic Currents (SPSCs) were frequently observed in the rat hippocampal neurons, by whole-cell recording in a standard external solution.[4, 14, 15] These SPSCs elicit spontaneous action potentials. During development, the frequency and spatial distribution of the SAPs increased and gradually widened.

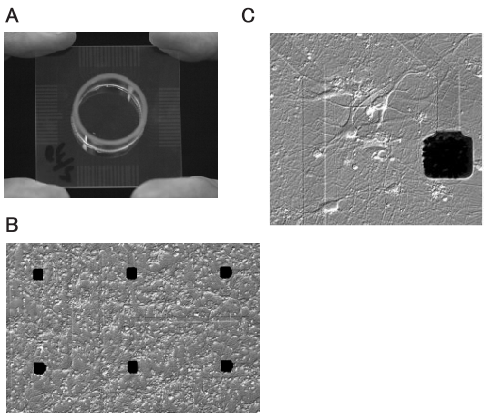

Figure 2. A. Culture dish with 64 planer electrodes (MED probe). **B.**Cultured neuronal network on multielectrode array. The black squares are electrodes. 200 μ m **C.**Expanded image of neurons. Black bar indicates 50 μ m. Neurons re-constructed complex networks *in vitro*.

From DIV15, the spatio-temporal pattern of the spontaneous action potentials (SAPs) segmented into several spatial clusters, and the SAPs clusters often overlapped each other, suggesting that distinct assemblies of neurons were organized into loosely defined subclasses of networks. The frequency of the SAPs saturated in the period of DIV20 to 30. To analyze functional connections between neurons, we developed a "connection map analysis". Fig.**??**)are examples of connectivity map of a living neuronal network, from rat hippocampus at embryonic day 17 and cultured for 26days in vitro. Each point indicates identified and distigushed neurons and lines represented for the connectivity between the neurons indicated by the points. Bold circles indicate neurons with several inputs and outputs, of which parameters concerned to waveform were stable. The right panel of figure **??** is a connection map calicurated from the data indicated in left panel. Sites with many spontaneous action potential also have a lot of links to other neurons. The connection map is calculated from 10-min recorded spontaneous activity, so this map corresponds to the overlapped images of all the transient activity pattern occurred during the recording period. The number of the incoming connections to each neuron varied even in a same cultured network in the range from only one to 102. The distribution of the number of incoming connections per neurons

approximated a power-law distribution rather than a Gaussian distribution (fig. 4). Though accurate fitting is difficult, we found that degree exponents $\gamma = 1.3$ in the case of the threshold (th) for the assumption of functional connections is 37 % of maximum value of the connectivity index (37 % corresponds to the mean plus the standard error in this data), $\gamma = 1.79$ for $th = 40$, $\gamma = 1.80$ for $th = 50$ %, respectively in the case of fig. 4. A power-law distribution is a typical feature of scale-free networks.[16,17] Hub-like neurons with many functional connections were also observed in the cultured networks. Because the hub-like neurons with many connections were observed in cultured network, the living neuronal network seems not to be a random network, but to be a scale-free network. In addition the connection map before and after the induction of the synaptic potentiation suggest that the functional connectivity between neurons changed drastically by the induction of synaptic potentiation in living neuronal networks cultured on a multielectrode array.

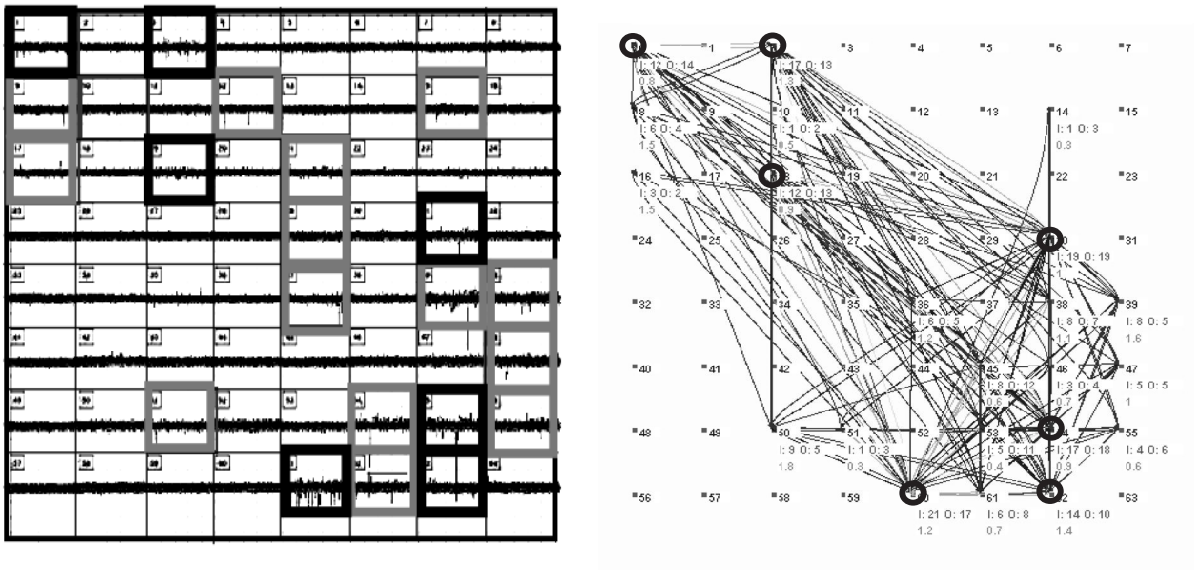

Figure 3. Functional connection between neurons. **Left.** Overlupped snapshot images of spontaneous action potentials (E18D26).**Right.** A connection map calicurated from the data indicated in left panel. Sites with many spontaneous action potential also have a lot of links to other neurons.

3.2. Drastic re-organization of functional assembry induced by feed-back stimulation

We set up the system in which the living neuronal network interacts to feedback stimulation system. In the experiment of Fig.6, only one stimulation pulse was applied to E20 in the cultured neuronal network when action potentials are detected from both of E48 and E11. We defined this experimental scheme as "2-detection and 1-stimulation scheme". This type of feedback stimulus was started on the 10th day in vitro (DIV10). In the case of the experiment, these spontaneous electrical activities synchronized highly and the stimulation frequently applied to the cultured network. The stimulation activated the network and next synchronized inputs at the two electrodes. The synchronised inputs triggered next stimulation. Thus, bursting-like stimulation pattern was generated by the interaction of network and the real-time feedback system. The important point is that the duration and frequency of this bursting stimulation are determined by the intrinsic state of living neuronal network. In other words, the living neuronal network can control these parameter of the stimulation (Fig.5). Consequently, even after the 24hr feedback stimulation, drastic change of activity pattern occurred. The example is shown in Fig.6. In fig.6 bold frames indicate the monitored electrodes and black square corresponds to a stimulation electrode, from which current stimulation applied to the network. Connection map analysis revealed that after "2-detection and 1-stimulation scheme" feedback stimulation caused suppression of spontaneous electrical activities in the network and induced drastic re-organization of functional connections between neurons, when

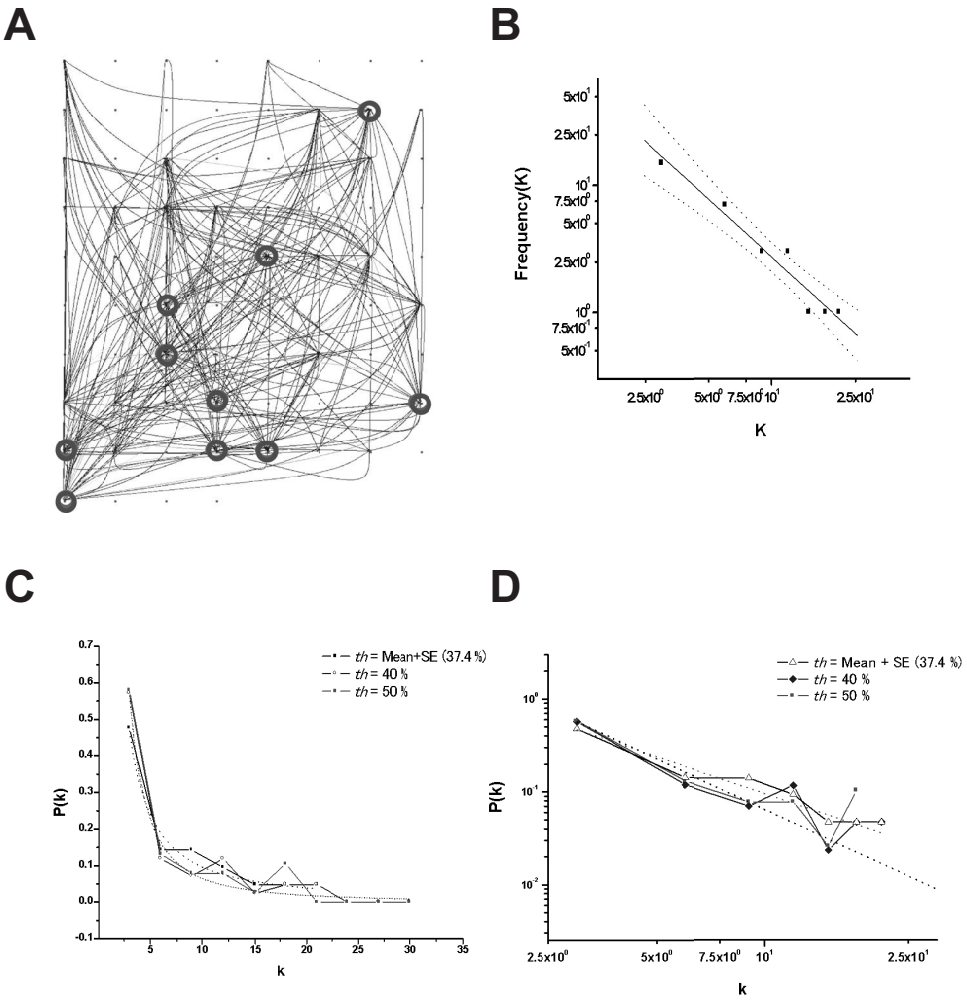

Figure 4. Another example of functional connections in a cultured neuronal network. **A.** Another example of a connection map. Each point in the map represents an identified neuron and the lines represent functional connections between these neurons. Bold circles indicate "hub"-like neurons, which neuron has more than ten inputs from other neurons. **B.** Scale-free feature of functional connections between neurons. Log-log plot of the distribution of the number of inputs. Fitted line and 95% confidence interval (dotted lines) are also indicated. In this example, the total number of input connections is 173 and the degree exponent is 1.52. **C.** The distributions of the number of the functional input connections in another neuronal network for three values of thresholds. P(k) gives the probability that a selected neuron has k functional input connections. Doted lines indicate the fitting curve. The total number of input connections is 264, in this example. **D.** Log-log plot of the distribution indicated in C. Doted lines indicate the fitting lines.

these activities are initially almost synchronized (Fig. 7 B). Interestingly, a particular neuron recorded from one of monitored electrode reduced its synchronous spontaneous activity , while another increased its activity. 24 hours' real-time feedback stimulation reduced synchronized activities in one of monitored electrodes, then we exchanged that monitored electrode to another electrode. neurons recorded from the electrode newly selected as monitor did not have many synchronized activities. Then after another 24 hours' feedback stimulation, the functional connectivities between neurons began to increase(fig.7 C).This result suggests that living neuronal network can re-organize their activity pattern depending on environmental I/O interaction. In other words, the

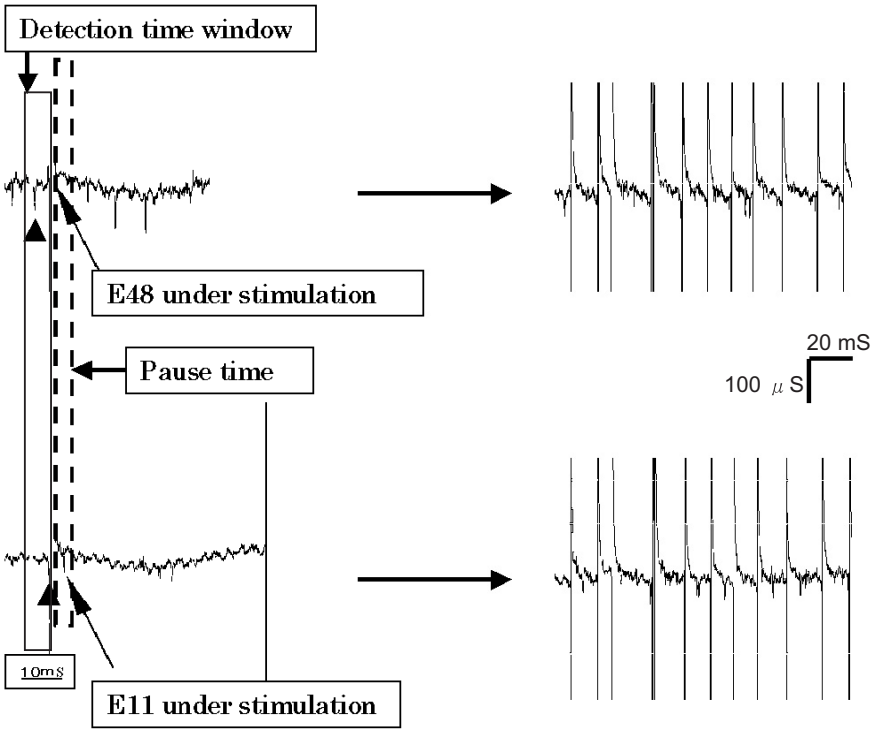

Figure 5. An example of stimulation pattern. Under "2-detection and 1-stimulation scheme", the real-time stimulation system moniters two electrodes simultaneously and began to apply stimulation to a selected electrodes when synchlonous inputs detected from these two selected electrodes.

cultured living neuronal network can embed "environmental pattern" into their functional activity pattern.

4. INTERACTION TO OUTER WORLD OF LIVING NEURONAL NETWORKS INTERFACED WITH MOVING ROBOT

In addition, developing the system in which living neuronal network interacts with outer world by the intermediary of moving robot. We use Khepera robot (K-Team), Lab View (National instruments) because Khepera can be programmed easily by LabView environment and MED64 system (Alpha MED Sciences) uses DAQ board of National instruments. The Hybrot (Hybrid living+robotic) idea was previously reported by Potter's group.[12, 13] We extended the idea and provided a program which generates "premised control rules" for making a robot avoid obstacles, instead of making the neuronal network generate such a rules autonomously. We think that it is impossible for living neuronal network to generate process for meaningful information processing autonomously. Creatures are also impossible to get such algorithm without emotional system, sense of pain, and so on. These basic systems have "meaning" from the point of survival. The value of the behavior is assessed on the basis of whether the behavior is suitable for survival or not. For example, there is one of premise rule that "avoiding a fire is good". This value is defined by evolutional selection. Creature with a behavior suitable to survive (avoiding fire in this case) is evolutionarily saved and creature with a behavior not suitable to survive (coming up to fire in this case) is culled out. As a result of that, the adequate judgement "avoiding a fire is good" is generated (fig.8). Thus, without premised rules which are generated by evolution, the base of intelligence cannot

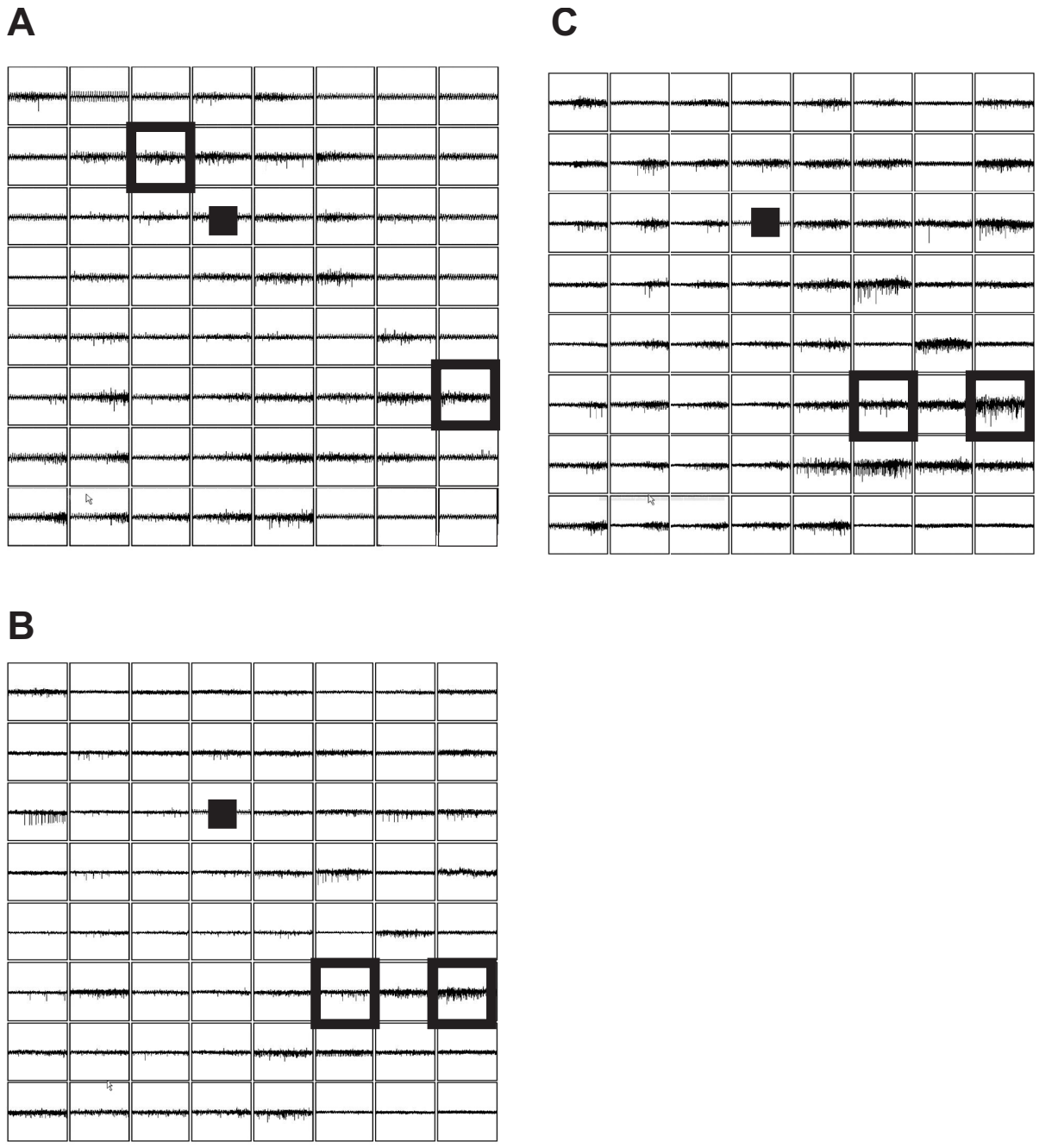

Figure 6. An example of drastic change in spatiotemporal pattern of spontaneous action potentials without stimulation . Each trace in squares indicates a signal from each electrode. **A.** Spontaneous action potentials before feed-back stimulation. **B.** Spontaneous action potentials after 24 hours feed-back stimulation. **C.** Spontaneous action potentials after another 24 hours feed-back stimulation with exchanging a detection electrode to another one.

emerge. From that view point, we are developing the living brain robot system with premised basic rules. The

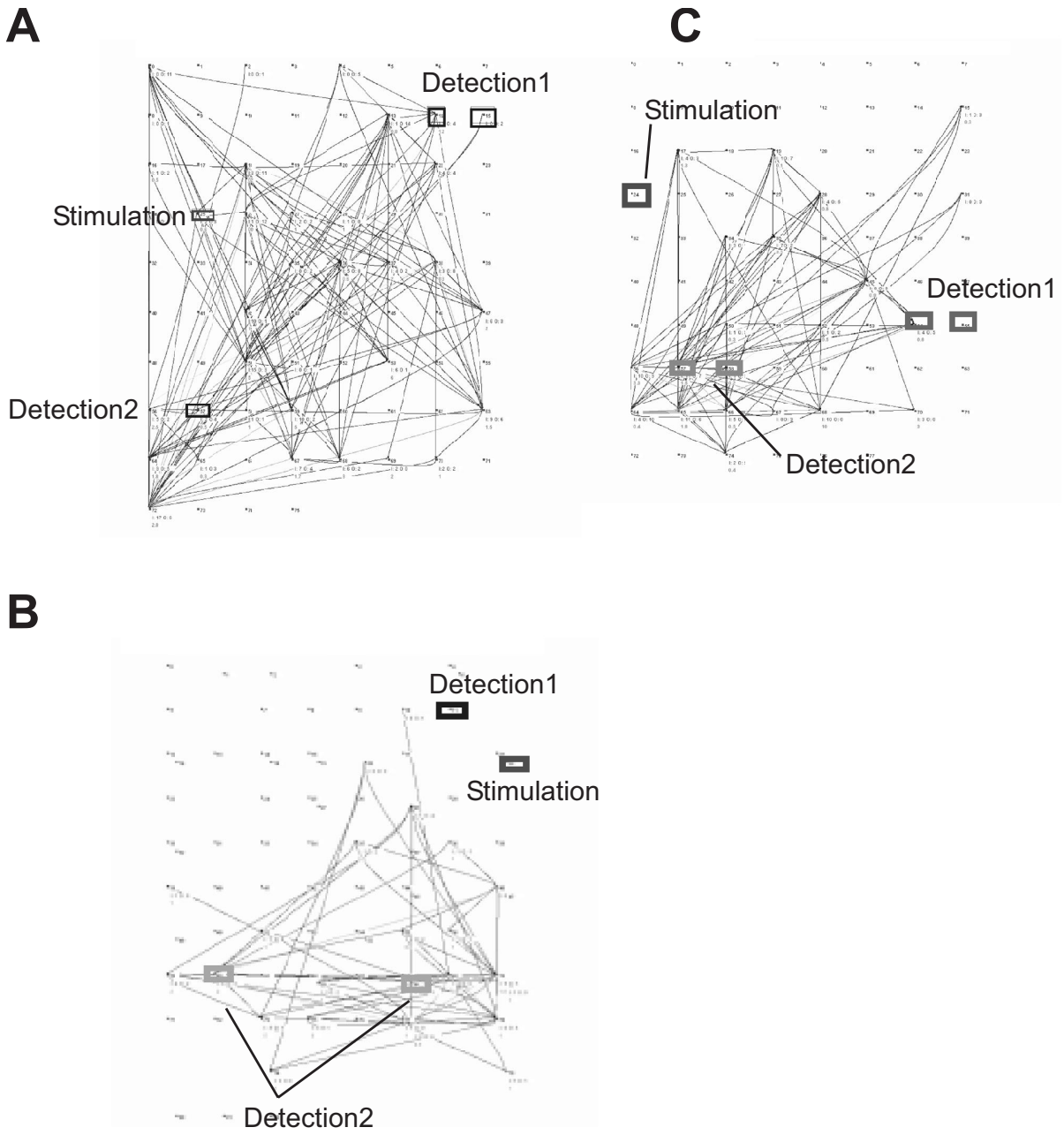

Figure 7. Connection maps calicurated from datumn indicated in fig.7. Bold squares indicated neurons near detection and stimulation electrodes.

premised rules are described by fuzzy logic and they play a role to generate instinctive behavior. If we observe adequate modification in the behavior of robot by interaction to outer world, can we define that phenomenon as the intelligence of living neuronal network? Our ultimate goal is to generate intelligence in culture dish and to observe it. For this purpose, we need a smart electrode array which allow us to perform stimulation restricted to the narrow range, a culture dish with a miniature maze in order to arrange neurons on purpose , and so on.

Microelectronics, MEMS, and nanotechnology are really critical to realize such items.

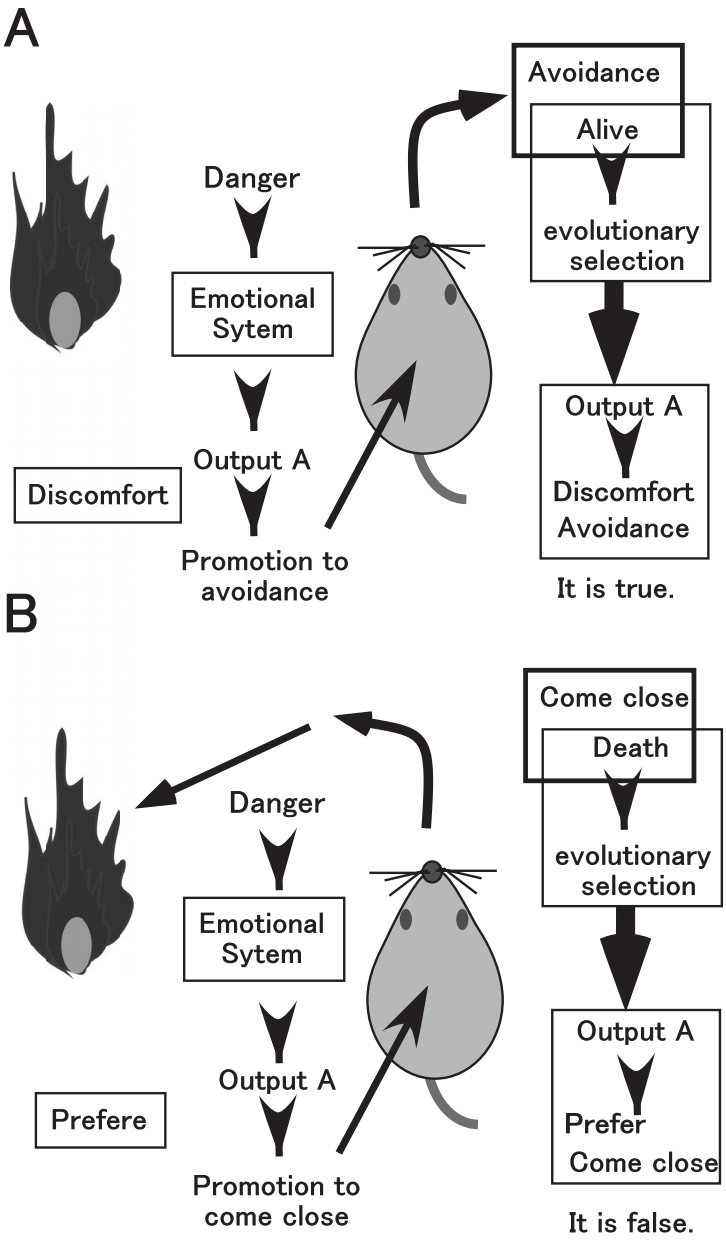

Figure 8. The value judgment on the basis of whether the behavior is suitable for survival is defined by evolutional selection. Creature with a behavior suitable to survive is evolutionary conserved (A). On the contrary, creature with a behavior not suitable to survive is culled out. As a result of that, the adequate judgement "avoiding a danger is good" is generated.

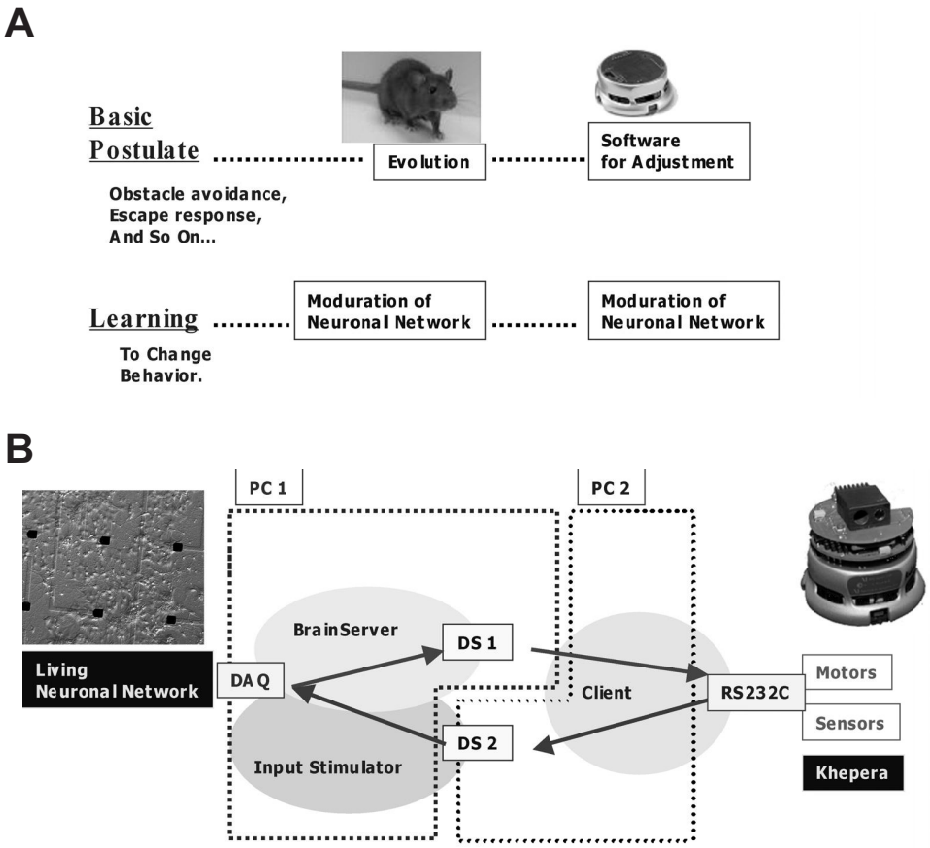

Figure 9. Living neuronal network with moving mini robot. **A.** Comparison between a creature and a robot with living neurons. **B.** Scheme of "Living Brain Robot System" .

REFERENCES

1. V. L. Dror Tal, Eyal Jacobson and S. Marom, "Frequency tuning of input-output relation in a rat cortical neuron in-vitro," *Neurosc. Lett.* **300**, pp. 21–24, 2001.
2. Y. Jimbo, H. Robinson, and A. Kawana, "Simultaneous measurement of intracellular calcium and electrical activity from patterned neural networks in culture," *IEEE Trans Biomed Eng* **40**, pp. 804–810, 1993.
3. H. Kamioka, E. Maeda, Y. Jimbo, H. Robinson, and A. Kawana, "Spontaneous periodic synchronized bursting during formation of mature patterns of connections in cortical cultures," *Neurosci Lett* **206**, pp. 109–112, 1996.
4. S. N. Kudoh and T. Taguchi, "Operation of spatiotemporal patterns stored in living neuronal networks cultured on a microelectrode array. 2003) 8(2):100-107," *Journal of Advanced Computational Intelligence and Intelligent Informatics* **8**, pp. 100–107, 2003.
5. J. Pine, "Recording action potentials from cultured neurons with extracellular microcircuit electrodes," *J Neurosci Methods* **2**, pp. 19–31, 1980.
6. S. M. Potter and T. B. DeMarse, "A new approach to neural cell culture for long-term studies," *J Neurosci Methods* **110**, pp. 17–24, 2001.
7. G. Shahaf and S. Marom, "Learning in networks of cortical neurons," *J. of Neuroscience* **21**, pp. 8782–8788, 2001.

8. D. A. Wagenaar, P. J, and S. M. Potter, "Effective parameters for stimulation of dissociated cultures using multi-electrode arrays," *J Neurosci Methods* **138**, pp. 27–37, 2004.
9. D. O. Hebb, *The organization of behavior*, (Wiley, New York, 1949):John Wiley, 1949.
10. Y. Sakurai, "Hippocampal and neocortical cell assemblies encode memory," *The Journal of Neuroscience* **16**, pp. 2809–2819, 1996.
11. E. Vaadia, I. Haalman, M. Abeles, H. Bergman, Y. Prut, H. Slovin, and A. Aertsen, "Dynamics of neuronal interactions in monkey cortex in relation to behavioural events," *Nature* **373**, pp. 515–518, 1995.
12. D.J. Bakkum, A.C. Shkolnik, G. Ben-Ary, P. Gamblen, T.B. DeMarse and S.M. Potter, *Removing some 'A' from AI: Embodied Cultured Networks. Embodied Artificial Intelligence.*, vol. 3139: 130-145., Springer, New York, 2004.
13. T. B. Demarse, D. A. Wagenaar, A. W. Blau, and S. M. Potter, "The neurally controlled animat: biological brains acting with simulated bodies," *Autonomous Robots* **11**, pp. 305–310, 2001.
14. K. SN and T. T., "A simple exploratory algorithm for the accurate and fast detection of spontaneous synaptic events.," *Biosens Bioelectron* **17**, pp. 773–782, 2002.
15. S. N. Kudoh, K. Kiyosue, and T.Taguchi, "A synaptic potentiation by a protein factor distinct from those induced by neurotrophins," *Int J Dev Neurosci* **20**, pp. 55–62, 2002.
16. A.-L. Baraba'si and R. Albert, "Emergence of scaling in random networks," *Science* **286**, pp. 509–512, 1999.
17. A. Re'ka and A.-L. Baraba'si, "Statistical mechanics of complex networks," *REVIEWS OF MODERN PHYSICS* **74**, pp. 47–97, 2002.