Paper:

Biomodeling System – Interaction Between Living Neuronal Networks and the Outer World

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Rat hippocampal neurons reorganized into complex networks in a culture dish with 64 planar microelectrodes and the electrical activity of neurons were recorded from individual sites. Multi-site recording system for extracellular action potentials was used for recording the activity of living neuronal networks and for applying input from the outer world to the network. The living neuronal network was able to distinguish among patterns of evoked action potentials based on different input, suggesting that the living neuronal network can express several pattern independently, meaning that it has fundamental mechanisms for intelligent information processing. We are developing a "biomodeling system," in which a living neuronal network is connected to a moving robot with premised control rules corresponding to a genetically provided interface of neuronal networks to peripheral systems. Premised rules are described in fuzzy logic and the robot can generate instinctive behavior, avoiding collision. Sensor input from the robot body was sent to a neuronal network, and the robot moved based on commands from the living neuronal network. This is a good modeling system to analyze interaction between biological information processing and electrical devices.

Keywords: neuron, dissociated culture system, multi-electrode array (MED), fuzzy logic, moving robot

1. Introduction

Dissociated neurons in a culture dish elongate neurites and form mutual synaptic connections. Frequent spontaneous electrical activity is observed throughout culture period, even though their environmental is rather artificial. Complex networks of living neurons thus appear to conduct certain information processing. Especially, living neuronal networks may have a feature that they build and adjust themselves to their environment [1]. Dissociated neurons in a multi-electrode array are fully useful for clarifying the interaction between network dynamics and input from outer world.

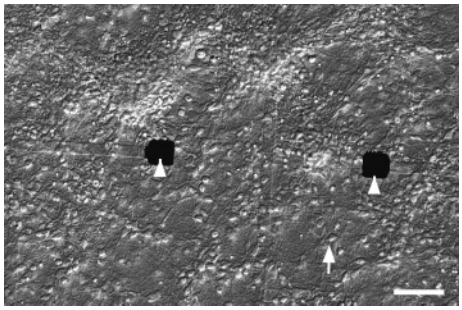

Fig. 1. Example of cultured living neuronal network (E17D10). The white bar indicates 50 μm. The MED probe has 64 planar microelectrodes (white arrowheads) arranged in an 8 $\times$ 8 grid on the culture dish. Neurons were cultured at high density and built a complex network on the culture dish. Example of neuron is indicated (white arrow).

Neurons reconstructed of complex networks (**Fig. 1**) and spontaneous spikes were frequently observed from 14 to 20 days in vitro (DIV) [2]. These spikes were action potentials spontaneously evoked by presynaptic neurons without external input from the outer world (**Fig. 2**). We have already shown in previous papers that long-lasting synaptic potentiation of spontaneous synaptic currents (SSCs) was induced by a Mg^{2+}-free condition in dissociated neuronal networks [3, 4]. Our results suggest a hypothesis that synaptic plasticity [5] modifies internal states organized by dynamic cell assemblies [6] in living neuronal networks. Indeed, such stable states were reported in rat hippocampal CA3 slices, and network activity wandered about several distinct states [7]. The result suggests that such small-scale networks have fundamental function for brain information processing.

It is likely that these dynamics are also modified by electrical input [8], and bidirectional (closed-loop) interaction of the neuronal network, and the outer world may generate new states containing information about the structures of outer world. The idea of developing a system for living neuronal networks to interact with the outer environment via small moving robot was first reported for

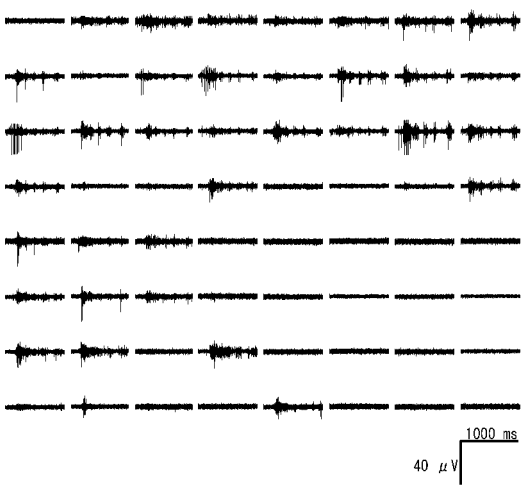

Fig. 2. Example of spontaneous spikes of action potentials recorded from the living neuronal network on the MED probe (E18D37). Traces correspond to the arrangement of corresponding electrodes. Action potentials were spontaneously evoked by presynaptic neurons without external input.

Hybrot (Hybrid living + robotic) by Potter's group [9, 10]. We think that it is impossible for living neuronal networks to generate meaningful rules autonomously without an assessor. Fundamental systems for evaluating behavior make "meaning" in response to outer world versus animal behavior. Instead of such evaluation systems, we provide a set of premised control rules, extending the Hybrot concept and providing a program that generates premised control rules for a robot to avoid collision. Premised rules are described by fuzzy rules and generate fixed behavior. We call a system with such premised control rules a "biomodeling system".

2. Methods

2.1. Primary Culture of Rat Hippocampal Neurons

The hippocampal region was dissected from Wistar rats on embryonic day 17 (E17) or E18 and neurons were dissociated. Procedure of primary culture of rat hippocampal neurons was conventional one and was previously described [11]. Briefly, rat hippocampal neurons were treated with 0.175% trypsin (Invitrogen-Gibco, U.S.A.) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS-minus, Nissui) supplemented with 10 mM glucose at 37°C for 10 min. Then cells were dissociated by gentle pipetting. Dissociated neurons were plated on a MED probe (Alpha MED Science, Japan), having 64 planar microelectrodes in the center of the culture dish. The MED probe was precoated overnight with 0.02% poly ethylene-imine. Using cloning ring, neurons were seeded in the central circular area of the MED probe at a density of 3200 cells/mm^2. The culture medium consisted of 45% Ham's F12, 45% Dulbecco's modified minimum essential medium (Invitrogen-Gibco, US), 5% horse serum (Invitrogen-Gibco, US), and 5% fetal calf serum (Invitrogen-Gibco, US), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen-Gibco, US), and 5 μg/ml insulin (Sigma-Aldrich, US). Half of the culture medium was changed to fresh every two days. Neurons were cultured for 60 days at 37°C in 5% CO_2/95% air at saturating humidity. All experimental procedures were governed by Guidelines for the Care and Use of Laboratory Animals of the AIST.

2.2. Measurement of Multiple-Site Extracellular Potentials via Electrode Array Dish

Spontaneous extracellular action potentials [12] were recorded in normal culture medium at 20-60 days in vitro. Extracellular potentials were collected through 64 electrodes simultaneously with the integrated MED64 system (Alpha MED Science, Japan) at a sampling rate of 10 kHz [12]. Experiments were conducted at room temperature (20-25°C). Control, such as stimulation sequences and sampling rates are done by MED64 conductor software (Alpha MED Science, Japan). Spontaneous spikes of action potentials were detected automatically by MEDFAUST, an analysis program developed at our lab. The amplitude threshold-based algorithm for detection was published elsewhere [13]. The threshold was determined to be 3 times of baseline noise during the preceding 100 ms time window. Detected spikes were classified into each neuron units by amplitude versus decay time distributions using k-means algorithms. Extra large spikes of stimulation artifacts were omitted.

3. Electrical Stimulation of Living Neuronal Network

3.1. Programmable Multisite Stimulation Head Amplifier for MED64 System

We designed the programmable multisite stimulation head amplifier for MED64 system (MED-H08). The amplifier included 1 channel stimulator for constant current stimulation and a circuit to switch electrodes for stimulation by extra eight-digit command input. MED-H08 enables us to record evoked action potentials through 64 individual electrodes and apply stimulus input to the living neuronal network through a selected electrode. It also records signals through the same electrode selected for stimulation, because the head amplifier switches the connection of the MED probe to the recording circuit from stimulation circuit immediately (**Fig. 3**). We developed "MultiStimulator" software for switching stimulating electrodes, using LabVIEW (National Instrument, US) and a multifunction data acquisition PC card (DAQ CARD 6020, National Instrument, US) to generate control signals for switching electrodes.

Experimental sessions were authorized by the MED64 conductor and triggered by TTL signals generated by the MultiStimulator.

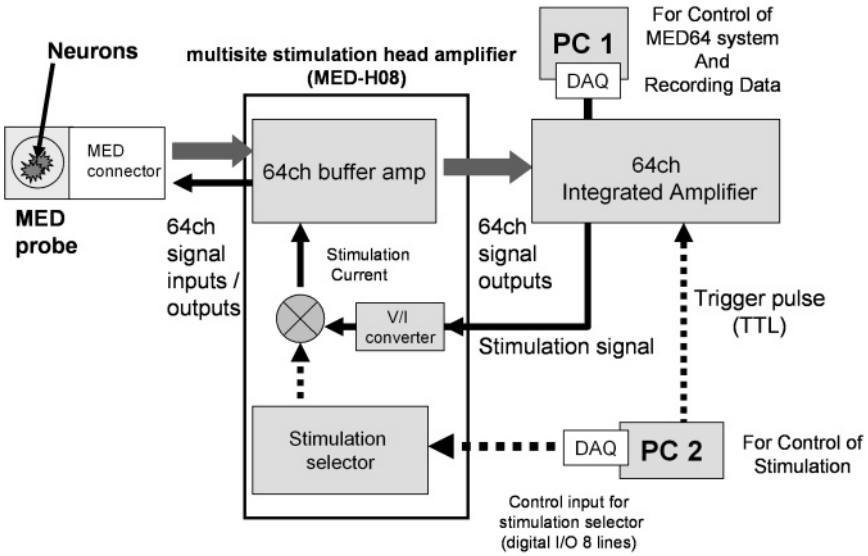

Fig. 3. System over view of multiple-site recording system for extracellular potentials. An experimental session was authorized by the MED64 conductor on PC1 and the session was triggered by a TTL signal generated by the MultiStimulator on PC2. Stimulation signals were generated by a built-in stimulation isolator consisting of a 64ch integrated Amplifier.

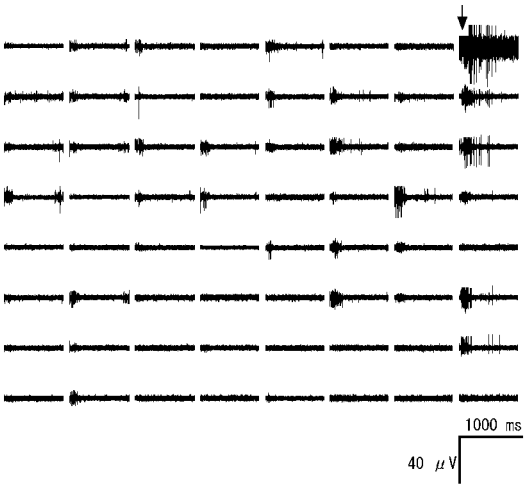

Fig. 4. Example of evoked action potentials recorded from a living neuronal network on a MED probe (E17D19). Traces correspond to corresponding electrodes. The arrow indicates electrical stimulation.

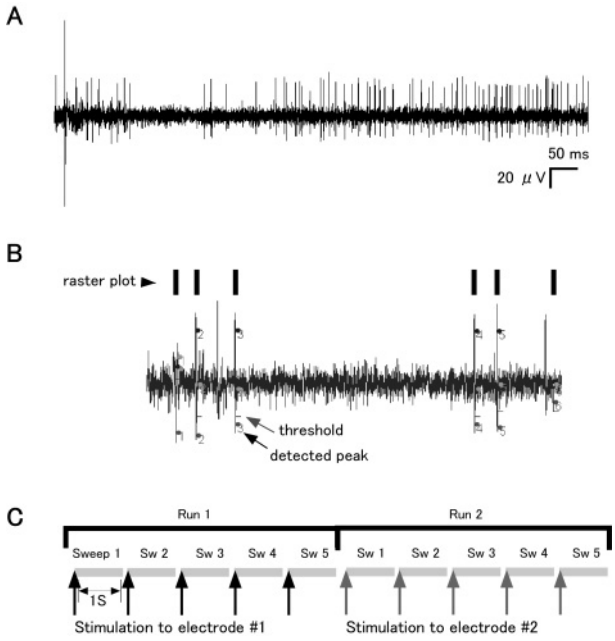

Fig. 5. A. Expanded trace recorded from the same cultured network as in **Fig. 4**. Evoked action potentials observed immediately after stimulation and after several hundred milliseconds. B. Action potentials automatically detected by MEDFAUST. Amplitude thresholds of detection were calculated automatically. Time stamps of action potentials are indicated as spike raster plots. C. Experimental scheme of current stimulation sequences for each 64 electrodes. During each run, the stimulation electrode was set to the electrode having the same number as the run. Sweep duration was 1 s, the interval between sweeps was 0 s, and the interval between runs was 0 S. All spikes were recorded from the same neurons in a culture dish (E17D19).

3.2. Distinct Spatiotemporal Patterns Evoked by External Input to Cultured Neuronal Network

After network reconstruction, spatiotemporal patterns of evoked action potentials were observed (**Fig. 4**). Current injection to an electrode simultaneously evoked numbers of action potentials at many recording sites, because neurons on a stimulation electrode triggered action potentials transmitted to other neurons via synaptic transmission. The stimulation current was injected into an electrode indicated by an arrow and a particular spatiotemporal pattern of action potentials was observed. Evoked action potentials were generally observed immediately after stimulation (early phase), and after several hundred milliseconds (late phase, **Fig. 5A**). Action potentials in the late phase were evoked by polysynaptic transmission and indicated the internal state of the neuronal network trig-

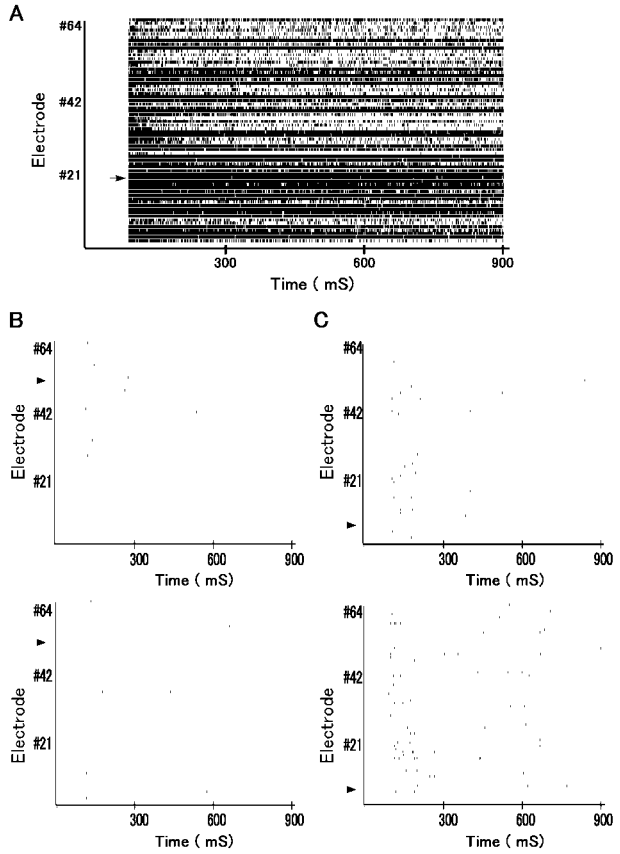

Fig. 6. A. Time stamps of evoked action potentials in each sweep of each run. Arrows indicate the electrodes in which notable spontaneous action potentials are observed. B. Action potentials in sweeps 1 (upper) and 3 (lower) evoked by current stimulation of electrode #51. C. Action potentials in sweeps 2 (upper) and 3 (lower) evoked by current stimulation of electrode #8. Stimulation electrodes are indicated by arrowheads. All spikes were recorded from the same neurons in a culture dish (E17D19).

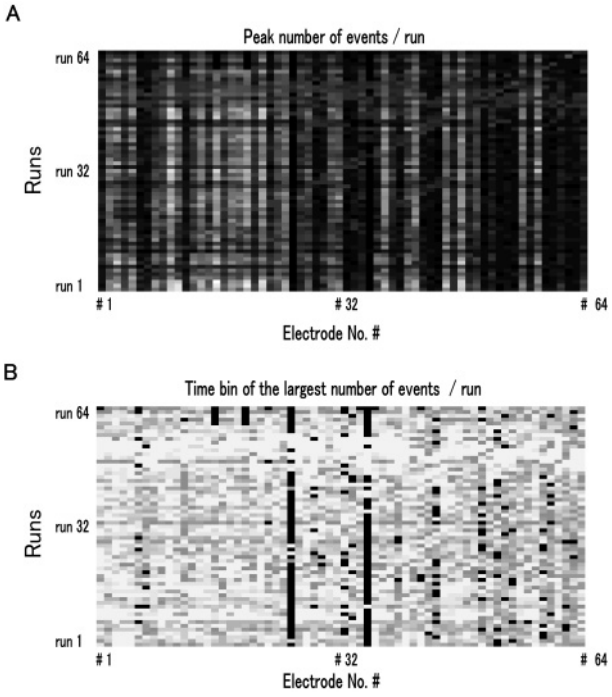

Fig. 7. A. Maximum number of action potentials of all time windows in all sweeps in each run. All spikes were recorded from the same neurons in a culture dish (E17D19). We generated a rate histogram of the number of action potentials in all sweeps per each run with 100 ms time windows. Boxes indicated the maximum number of events in a time window per run. White indicates the maximum number of events in all time windows in the dataset and black the minimum number of events. The maximum number of events was 31 and the minimum 3. During each run, the stimulation electrode was set to the electrode having the same ID number as the run number, (i.e., the stimulation electrode was #1 during run1). B. Latency of evoked action potentials. Boxes indicate data at the same position in panel A. Colors indicate the time window start time including the maximum number of events. White indicates a 0–100 mS time window and black 1900–2000 mS.

gered by stimulation.

Recorded action potentials were automatically detected by MEDFAUST and spike raster plots of a whole electrode array were generated by time stamps of action potentials (**Fig. 5B**). To elucidate whether a living neuronal network could distinguish among several patterns retrieved by input through individual electrodes, we applied current stimulation sequentially to each 64 electrodes. Using a programmable multisite stimulator, we recorded 5 sweeps of electrical activity evoked by current injection into a selected electrode. After 5 sweeps of recording, the stimulator changes from one stimulation electrode to another one and conducts the next 5 sweeps recordings (next "run"). During each run, the stimulation electrode was set to the electrode having the same ID number as the run number. Sweep duration was 1S, the interval between sweeps was 0 s, and the interval between runs was 0 s (**Fig. 5C**). **Fig. 6A** shows time stamps of evoked action potentials for each sweep of each run. In some electrodes, notable spontaneous action potentials were observed (e.g. the arrow in **Fig. 6A**). Evoked action potentials were masked by this spontaneous activity, elicited over the entire recording time. **Fig. 6B** and **C** show action potentials evoked by current stimulation of different electrodes. Spatio-temporal patterns of evoked action potentials were not same in detail in stimulation of the same electrodes due to spontaneous activity or different internal states of neurons, but the global trend in action potential patterns evoked by stimulation of the same electrode, while the patterns evoked by different stimulations were differed greatly from each other. **Fig. 7A** shows the sum of the number of action potentials in peak time windows of overall recording time in all sweeps in each run. It was not that a few sites were linked to a particular stimulation site, but a single site responded to multiple stimulations to different electrodes, suggesting that many sites were required for discriminations of several patterns. The variation of latency in action potentials evoked by stimulation was relatively small (**Fig. 7B**).

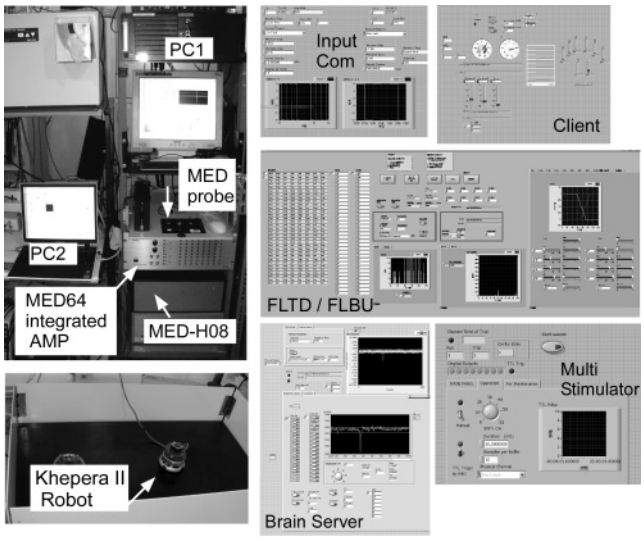

Fig. 8. Snap shots of biomodeling system and component soft wares.

The spatiotemporal pattern of evoked activity was formed relatively early, suggesting that it is necessary to analyze action potentials with short time window, in order to recognize the difference in the pattern of action potentials depending on synaptic delays or the number of intervenient synaptic connections. These results indicate that the living neuronal network can independently express several distinct spatiotemporal patterns evoked by stimulation of different electrodes.

4. Interaction with Outer World by Living Neuronal Networks Interfaced by Moving Robot

4.1. System Integration of Biomodeling System

We are developing a living brain robot system with premised basic rules described by fuzzy logic [14, 15]. We use a Khepera II robot (K-Team) for interfacing with a living neuronal network and the outer world and LabVIEW (National instruments) for the programming language. Khepera II is controlled via serial communication by virtual instruments (VI) of LabVIEW and MED64 system uses two DAQ boards of National instruments, also controlled easily by LabVIEW. The system consists of 5 independent programs and recording system for multiple-site extracellular potentials, and two computers (**Fig. 8**).

The "Brain Server" program records electrical potentials and detects action potentials. "FLTD / FLBU" programs decode signals. The "Client" program controls the robot. The "Input Com" program stimulates the neuronal network. For the first step, we used a stimulator with two fixed channels toggled for stimulation rather than the multisite stimulation head amplifier to simplify the system. Premised rules generate fixed behavior such as collision avoidance. We call such a system with living neuronal network, robot body and premised rules a "Biomodeling System" with a "top-down bio-processing" and a "bottom-up robot-processing". The robot has two Fuzzy controllers, – one for controlling the robot's actuators, fuzzy logic in a top-down process (FLTD) in top-down bio-processing, and another one for controlling the electrical stimulation to the neuronal network, fuzzy logic in a bottom-up Process (FLBU), in bottom-up robot processing (**Fig. 9A**). Programs exchange processing data information mediated by a datasocket transfer protocol (DSTP, National Instruments) [16]. The system uses 4 datasocket servers without buffering data, meaning there is a probability of lost data. It is the second-best policy for avoiding increased time delay between the living neuronal network and robot.

Brain Server, the processor for neuronal signals, receives eight inputs from the living neuronal network. Each input is the number of action potentials detected by eight adjacent electrodes within 50 ms time windows. Input Com, the stimulation controller, receives processed output of eight IR sensors from Khepera II. The fuzzy controller consists of 256 fuzzy rules with eight inputs. Each input has two types of fuzzy labels– high-frequency and low-frequency. The 256 fuzzy rules describe all classified patterns of action potentials in eight inputs. The membership function of fuzzy rules is setup as follows: The maximum frequency of the action potential in all electrodes was made the maximum of the horizontal axis of a membership function. The maximum membership function assigned to the high-frequency label was at three fourths of the points of maximum frequency, and the maximum of the membership function assigned to a low-frequency label was at one fourth of the points of maximum frequency. Fuzzy rules were set up as follows: Differences in the fuzzy number (μ) of each rule assigned to bringing an object close to the left and to the right side of the robot were calculated. We focused on rules with large differences. Rules with large fuzzy numbers for objects at the left correspond to particular patterns evoked by input related to left IR sensors. In contrast, rules with large fuzzy numbers for objects at the right side correspond to particular patterns related to right IR sensors. Cnsequents of rules for the left IR sensor, for example, were configured so that left actuator speed was high (**Fig. 10**).

4.2. Collision Avoidance via Living Neuronal Network

Neurons were dissociated and cultured on a dish, abolishing any inborn, physiological connections among them. Although self-organized structures of neuronal connections in culture are likely to be observed in an intact brain, adequate links between neuronal circuits and the external environment were corrupted. We think that it is impossible for a living neuronal network to autonomously generate processes for meaningful information processing without clues of some sort. Sentient beings also cannot obtain algorithms without inborn basics

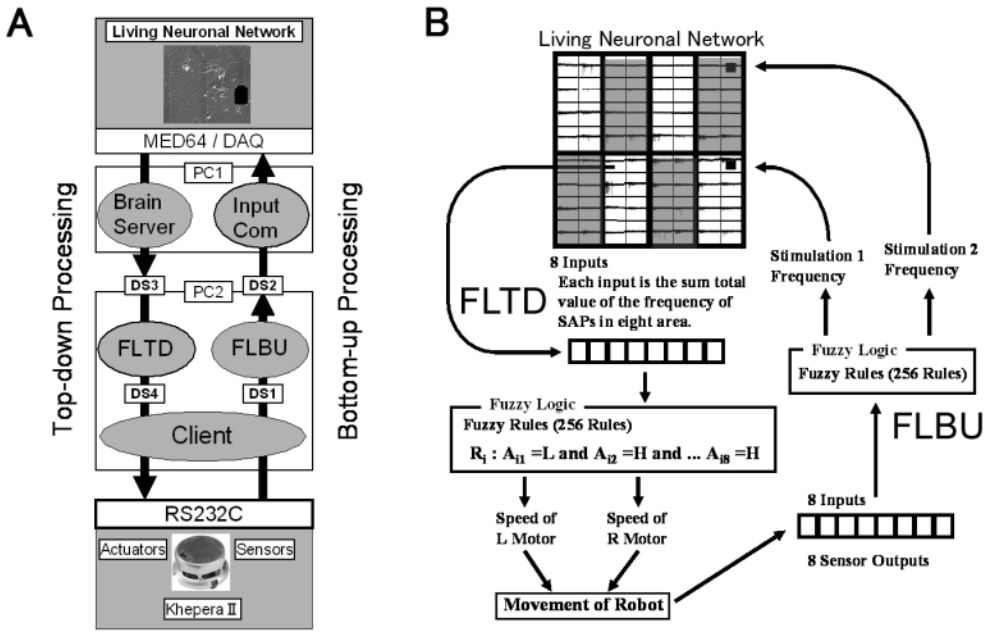

Fig. 9. A. Biomodeling system consistis of 5 independent programs distributed on two personal computers, recording system for multiple-site extracellular potentials. Program units exchange processing data information mediated by 4 datasocket servers, DS1 to DS4. B. Fuzzy control of biomodeling system. The processor for neuronal signals and the stimulation controller each receives eight inputs. The fuzzy controller consists of 256 fuzzy rules with eight inputs. Each input has two types of fuzzy labels high-frequency and low-frequency.

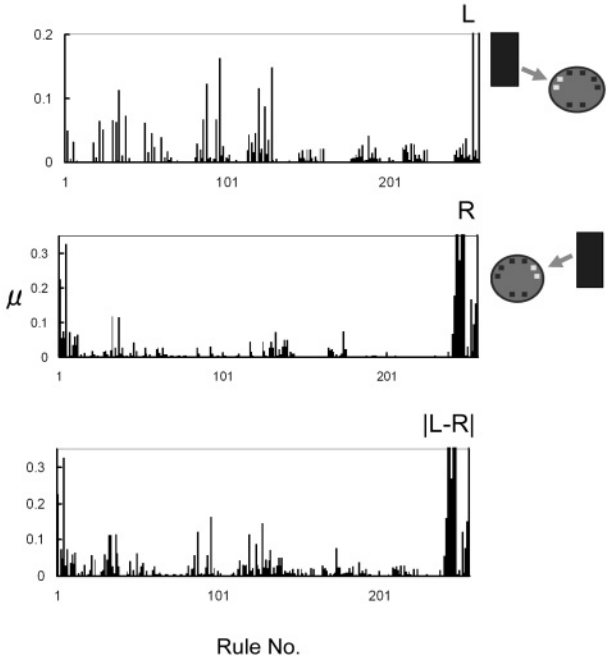

Fig. 10. Fuzzy rule setup. (L) Fuzzy number of each rule assigned to bringing an object close to the left. (R) Fuzzy number of each rule assigned to bringing an object close to the right. (|L-R|) Absolute differences in fuzzy numbers in different situations.

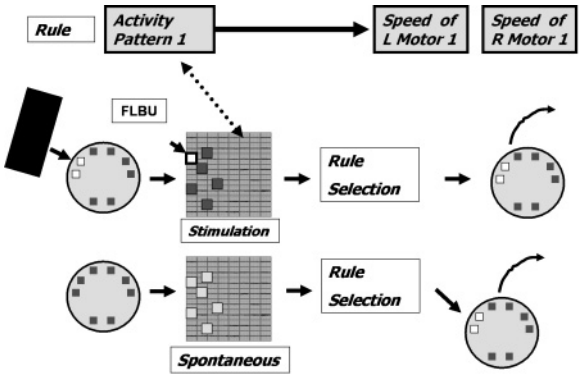

Fig. 11. Collision avoidance via a living neuronal network. When FLTD detects a particular pattern of network activity (Activity pattern 1) evoked by electrical stimulation of a particular electrode linked to high value of sensors on left, the actuator speed on the left is speeded up. In the case absence of sensors input, the robot is controlled by meaningless spontaneous electrical activity and moves spontaneously.

such as emotional system, a sense of pain, etc. These basic systems offer "meaning" from the viewpoint of survival. In information processing among sentient creatures, the value of behavior is assessed based on whether it is suitable for survival. We propose here a system in which a living neuronal network is connected to robot mediated by premised control rules. Such rules were described in two fuzzy processors.

When fuzzy processor, "FLTD", detects a particular pattern of network activity evoked by electrical stimulation of a particular electrode linked to the high value of sensors on either side, the actuator speed on that side is speeded up, i.e., input from the sensor from the robot is processed and transmitted to the living neuronal network by electrical stimulation, evoking a particular activity pattern of action potentials corresponding to the sen-

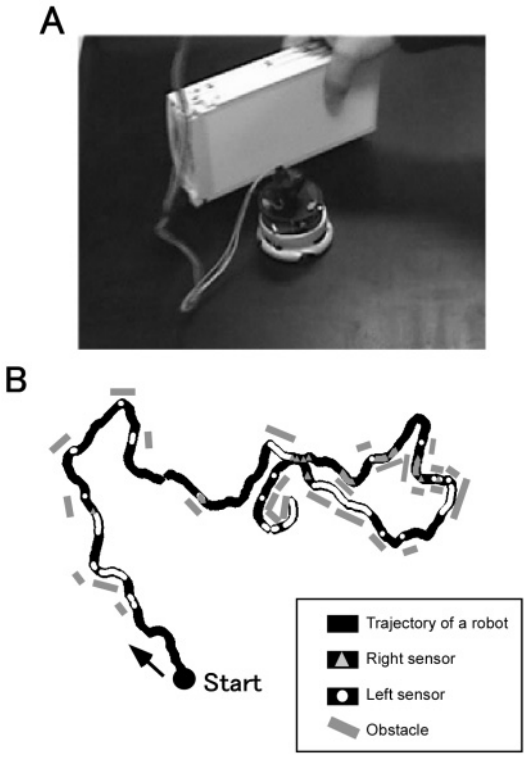

Fig. 12. A) A miniature moving robot Khepera II connected to a living neuronal network. B) Example of Khepera II robot trajectory with the living neuronal network (E18D96). The robot sometimes ignores sensor input, but it almost avoids collision. In this experiment, we placed and moved obstacles by hand to avoid neuronal death. Otherwise, when ignoring sensor input the robot collided with the obstacle, creating excessive stimulation harmful to neurons.

sor value. The system then recognizes such electrical activity patterns by fuzzy rules and determines actuator speed. If obstacles exist near the left, then the unit applies electrical stimulation to electrode 1, and so on. When the robot controller detects a particular pattern evoked by electrical stimulation of electrode 1, the left actuator is speeded up so the robot avoids a collision (**Fig. 11**). Where sensor input is absent, spontaneous electrical activity is generated by the internal state of the living neuronal network (**Fig. 2**). Spontaneous activity is classified into particular patterns, and the robot moves spontaneously. The response of the neuronal network to external input is determined by a combination of both spontaneous internal states of the network and a compulsory activated state. This is why the pattern evoked by electrical stimulation of a particular electrode is not constant (**Fig. 6**). In other words, the pattern is varied because it is the result of certain internal information processing of the living neuronal network, making robot behavior rather uneven, i.e., sometimes the robot did not change its direction despite the presence of an obstacle, just as an animal would (**Fig. 12B**). Sometimes the robot ignores the input of sensors, but it almost avoids collision. In our previous reports, the pattern of evoked action potentials is modified by a particular electrical pattern stimulation [17, 18]. We are interested in whether we can observe adequate modification in the behavior of this robot by its interaction with the outer world.

5. Conclusions

Dissociated culture system with a multi-electrode array is useful for elucidation of network dynamics of neurons. In this paper, we integrated a living neuronal network and a Khepera II robot using two types of fuzzy logic. Into what we call a "biomodeling system" in which "top-down bio-processing" send actuator signals to the robot from the living neuronal network and "bottom-up robot-processing" for electrical stimulation from robot to the living neuronal network. The system is a good modeling platform system for clarifying interaction between living neurons and the outer world and assessing the effects of the outer environment on the activity of the living neuronal system.

Acknowledgements

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1993-2004 Professor, Hannan University
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Main Works:
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• Japanese Neural Network Society (JNNS)
• The Japanese Psychonomic Society (JPS)
• The Vision Society of Japan (VSJ)
• The Institute of Systems, Control and Information Engineers (ISCIE)
• IEEE Systems, Man, and Cybernetics Society