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Research article

Classification of Spike Wave Propagations in a Cultured Neuronal Network: Investigating a Brain Communication Mechanism

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Abstract: In brain information science, it is still unclear how multiple data can be stored and transmitted in ambiguously behaving neuronal networks. In the present study, we analyze the spatiotemporal propagation of spike trains in neuronal networks. Recently, spike propagation was observed functioning as a cluster of excitation waves (spike wave propagation) in cultured neuronal networks. We now assume that spike wave propagations are just events of communications in the brain. However, in reality, various spike wave propagations are generated in neuronal networks. Thus, there should be some mechanism to classify these spike wave propagations so that multiple communications in brain can be distinguished. To prove this assumption, we attempt to classify various spike wave propagations generated from different stimulated neurons using our original spatiotemporal pattern matching method for spike temporal patterns at each neuron in spike wave propagation in the cultured neuronal network. Based on the experimental results, it became clear that spike wave propagations have various temporal patterns from stimulated neurons. Therefore these stimulated neurons could be classified at several neurons away from the stimulated neurons. These

are the *classifiable neurons*. Moreover, distribution of *classifiable neurons* in a network is also different when stimulated neurons generating spike wave propagations are different. These results suggest that distinct communications occur via multiple communication links and that *classifiable neurons* serve this function.

Keywords: cultured neuronal network; spike wave propagation; spatiotemporal form; classifying; multiple communications

1. Introduction

The brain is an intellectual information processing system [1–5]. How a neuronal network of ambiguously behaving neurons establishes a highly reliable information processing system, distinct communication, and organized communication links is an unanswered question. Despite many researchers attempting to solve this question, it remains a mystery.

In previous studies, factors such as spatiotemporal coding, the Synfire chain, and the spatiotemporal form of spike activity were considered the fundamental generators of natural intelligence in the brain [6–11]. However, basic communication functions between neurons have not been elucidated in these studies. Therefore, the abovementioned question still remains unsolved.

Recently, we focused on distinct and different communication to investigate the previously mentioned question [12–15]. In previous work [16], spike propagation as a cluster of excitation waves, termed as spike wave propagation, was observed in cultured neuronal networks. However, in those experiments, it was only observed that various spike wave propagations were generated in neuronal networks. The details of these mechanisms were still unclear.

To investigate these mechanisms, we simulated a 9 × 9 2D mesh neural network consisting of an integrate-and-fire model without leak. Resulting from this method, multiplex communication is possible at a success rate of 99% [17]. This result suggested that distinction of the spike wave propagation spatiotemporal form was the clue to classifying multiple communications in the brain. Here, we assume spike wave propagations are just communication events in the brain and attempt to prove this assumption. However, physiological experiments, analysis, and discussions about these events have yet to be reported [17].

In this study, we attempt to classify various spike wave propagation from different stimulated neurons in cultured neuronal networks, as well as discuss the implications of these classifying results in a view of brain communication. The authors' research group is presently studying the functions of neuronal networks by combining experiments with cultured neuronal networks with artificial neural network simulations. This paper corresponds to previous work on the ability of remote receiving neurons to identify two transmitting neuron groups stimulated in a neuronal network, i.e., 2 to 1 communication [17]. These mechanisms may be the basis of higher cortical functions.

The aim of this study is to investigate the most essential question in our study: to identify what the spatiotemporal form of spike wave propagation suggests in view of communication in brain physiologically.

2. Methods

2.1. Cell cultures

Cell cultures of hippocampal neurons were dissected from Wistar rats on embryonic day 18. The procedure conformed to the protocols approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology. Hippocampi were dissociated with 0.1% trypsin (Invitrogen; Tokyo, Japan) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline at 37°C for 15 min. The dissociated neurons were planted at a density of 3.3 × 105 cells/mm^2 in polyethylentimine-coated microelectrode array (MEA) dishes (MED-P515A, Alpha MED Scientific; Kadoma, Japan) with 8 × 8 planar microelectrodes. The size and spacing of the electrodes were 50 × 50 μm^2 and 150 or 450 μm, respectively. To position the neuronal networks in the central area of each MEA dish, a cloning ring with an inner diameter of 7 mm was used. The ring was removed the following day. Neurons adhered to the substrate of the MEAs, covering all electrodes.

Neurons were maintained at 37°C in a humidified atmosphere of 5% CO_2 and cultured for 21–40 days in Dulbecco's modified Eagle's medium (Invitrogen), which contained 5% horse serum and 5% fetal calf serum with supplements of 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml insulin. Half of the culture medium was renewed twice per week. In this study, four cultured cell samples at 22–50 days in vitro were prepared and are referred to as Cultures 1, 2, 3, and 4. Figure 1 shows a micrograph of the cultured neurons in an MEA.

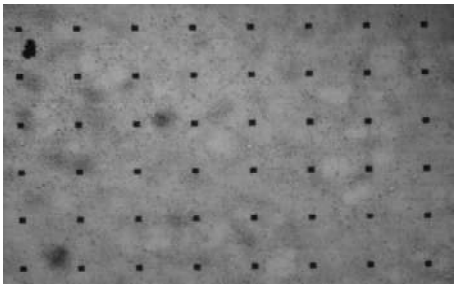

Figure 1. Micrograph of cultured neurons in an MEA (×20).

2.2. Stimulated spike recording

Stimulated spikes were recorded using MED64 (Alpha MED Scientific; Osaka Japan), an extracellular recording system with 64 electrodes (channels). The size of each electrode is approximately the size of a neuron. The recording was performed for 3 s at a sampling rate of 20 kHz.

A selected channel was stimulated at 5 ms after the start of the recording. The stimulation signal was a current-controlled bipolar pulse (positive, then negative) with a strength of 10 uA and a duration of 100 us.

Two to three channels in each culture were selected as the stimulation channels, and they were subjected to 10–15 recordings. In this study, the stimulated channels are referred to as StimA, StimB, and StimC. Incidentally, this study investigates whether the original stimulated channels (StimA, B, or C) can be identified from spike train at each channel (including multi-neurons), rather than by single neurons. Therefore, spike sorting was not performed.

2.3. Coding spike trains

The recorded spike trains were coded as follows: first, raster plots were generated by detecting peaks above a pre-specified threshold on each channel in the recorded spike responses [18]. Then, spike interval trains were calculated from the raster plot data.

2.4. Classifying procedure

Previously [16], effort was made to analyze the differences in the spike spatiotemporal pattern corresponding to the stimulated neuron using the dynamic time warping (DTW) method. This method uses a dynamic programming technique to find the minimum distance by stretching or shrinking the linearly or non-linearly warped time series and is thus useful for finding the optimal alignment between two non-uniform time series [19]. However, the DTW method does not offer an adequate resolution [20]. Therefore, the qualities of the analysis results were not enough to clarify whether multiple spike waves are classifiable.

The brain must have some physiological learning mechanism for classifying spike wave propagations with various temporal patterns. Considering previous experimental results, we used an analytical method with a learning algorithm instead of DTW. In the field of machine learning, back propagation, deep learning, etc. are well known. Though these methods, which imitate the behavior of physiological neuronal networks, are very effective for classifying various and complex data, the learning algorithm seems to be better suited for arranging physiological behavior to fit machine learning. Therefore, in this study, we use a simpler learning algorithm based on the arithmetical average method, which seems to have more compatibility with natural recognition (See Supplementary S-1).

The outline of classifying procedure is as follows.

Repeat for each 64 channel on MED64

(1) Spike train is learned by 5–10 spike temporal patterns with the same stimulated neuron (called neuron *A* temporarily). This spike train form is termed *Learning pattern A*.

(2) *Learning pattern B* (stimulated neuron is neuron *B*) are created by the same method as *Learning pattern A*.

(3) To find *classifiable neurons*, the resemblance of spike train (before learning) on trial (named *Trial Data*) and *learning pattern A or B* was estimated by the procedure described in Supplementary S-2.

3. Results

*****To explain the detection method of classifiable neurons, the results of Cultures 1 and 2 are in described in detail*.*

3.1. Culture 1

In Culture 1, 15 spike responses were recorded when channel 4 was stimulated. Five spike responses from the 15 were used for *Trial Data* named *Tr401, Tr402, . . . Tr405*, while the other 10 spike responses were used for *Learning Pattern 4*. Next, five *Trial Data* named *Tr2801, Tr2802, . . . Tr2805*, and *Learning Pattern 28* (channel 28 is stimulated) were created by the same procedure as *Tr401-Tr405* and *Learning Pattern 4*.

Figure 2 shows the result of the resemblance test for *Tr2801*. In Fig. 2b, which focused on channel 16, the mean value of *SpsetTrial* was significantly greater than that of *SpsetLocal* (see Supplementary S-2), when the stimulated neuron of the trial was different than that in the learning pattern. No significant difference was observed when the stimulated neuron of *Trial Data* was the same as in learning pattern (Figure 2a). This result suggested that the stimulated neuron of these *Trial Data* was not neuron 4. In other words, these *Trial Data* can be extracted from *Leaning Pattern 4* and the stimulated neuron 28 can be classified successfully as a neuron on channel 16. Therefore, this neuron was a *classifiable neuron*. In this trial, there were 14 *classifiable neurons*. Table 1a shows the number of *classifiable neuron* in each trial in Culture 1.

3.2. Culture 2

In Culture 2, *Tr1301*, *Tr1302*, . . . *Tr1305*, *Learning Pattern 13*, *Tr3001*, *Tr3002*, . . . *Tr3005, Learning Pattern 30*, *Tr5401*, *Tr5402*, . . . *Tr5405*, and *Learning Pattern 54* (the stimulated neurons were channels 13, 30, and 54, respectively) were prepared for experiments and learning patterns were created by 5-spike responses. Figure 3 shows the estimation result of the comparison for *Tr1304*. Sixteen *classifiable neurons* were observed through comparison with *Learning Pattern 54*

and 10 through comparison with *Learning Pattern 30*. Table 1b shows the number of *classifiable neurons* for each trial.

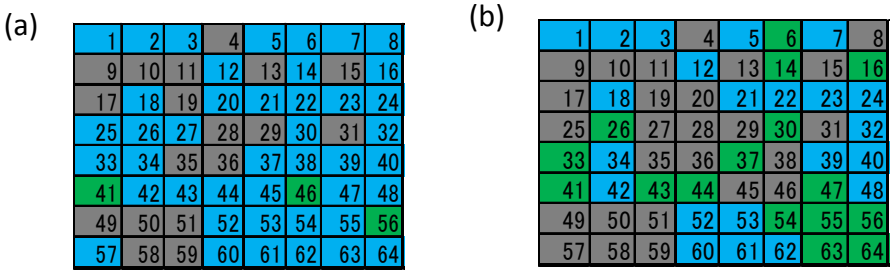

Figure 2. Estimation results of the comparisons for Tr2801. (a) Comparison with *Learning pattern 28* (b) Comparison with *Learning pattern 4*. Green cells indicate that the mean value of *SpsetTrial* was significantly greater than that of *SpsetLocal* (see Supplementary S-2). Blue cells indicate that *SpsetTrial* was not significantly greater than *SpsetLocal*. Gray cells indicate no spikes or that the number of spike was less than eight in the recording.

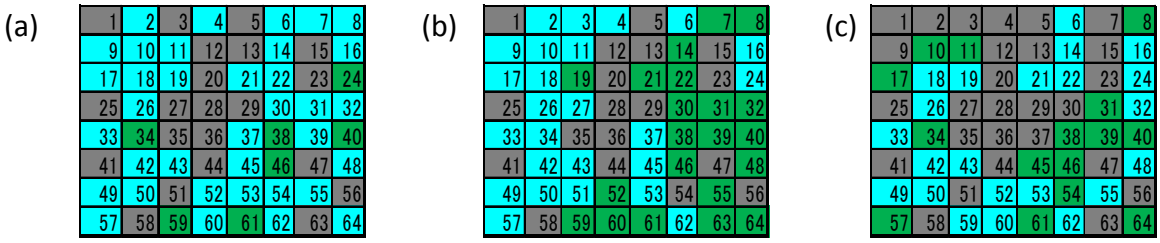

Figure 3. Estimation results of the comparisons for Tr1304. (a) Comparison with *Learning Pattern 13* (b) Comparison with *Learning Pattern 54* (c) Comparison with *Learning Pattern 30*. Green cells indicate the mean value of *SpsetTrial* was significantly greater than that of *SpsetLocal* (see Supplementary S-2). Blue cells indicate that *SpsetTrial* was not significantly greater than *SpsetLocal*. Gray cells indicate no spikes or that the number of spikes was less than eight in this recording.

3.3. Cultures 3 and 4

For Culture 3, channels 4 and 38 were stimulated. For Culture 4, channels 8, 10, and 57 were stimulated. The detection method of *classifiable neurons* in these cultures was similar to Culture 1 and 2. Therefore, in Culture 3 and 4, only the number of *classifiable neurons* for each *Trial Data* in Table 1c and 1d is shown.

3.4. Comparing with Spike Interval Shuffling data

As shown in Figure 2, Figure 3, and Table 1, *classifiable neurons* were observed in particular areas of neuronal networks. However, there was indication that these *classifiable neurons* were detected accidentally and purpose of the number of experiments performed was not to dispel this doubt. Therefore, we attempted to detect *classifiable neurons* from shuffled spike-interval sequence, called *Interval Shuffle* (*Int. Shuf*) [21], in parts of the trial data in Cultures 2 and 3.

The numbers of *classifiable neurons* from *Interval Shuffle* data were less than from original (non-*Interval Shuffle*) spike-interval data. In Culture 2, the difference between the two was significant ($p < 0.05$, as result of t-test). These results show that the detected *classifiable neurons* from the original spike data were not accidental.

Table 1. The number of *classifiable neurons* for each *Trial Data.*

a. Culture 1

Trial	Classification	
	vs ch 4 stim	vs ch 28 stim
Tr401	–	10
Tr402	–	9
Tr403	–	10
Tr404	–	17
Tr405	–	13
Tr2801	14	–
Tr2802	12	–
Tr2803	13	–
Tr2804	14	–
Tr2805	14	–

b. Culture 2

Trial	Classification		
	vs ch 13 stim	vs ch 54 stim	vs ch 30 stim
Tr1301	–	10	10
Tr1302	–	17	19
Tr1303	–	16	15
Tr1304	–	16	10
Tr1305	–	11	6
Tr5401	21	–	3
Tr5402	18	–	7
Tr5403	16	–	2
Tr5404	19	–	5
Tr5405	19	–	6
Tr3001	16	9	–
Tr3002	21	6	–
Tr3003	8	7	–
Tr3004	15	3	–
Tr3005	10	10	–

c. Culture 3

Trial	Classification	
	vs ch4 stim	vs ch 38 stim
Tr401	–	19
Tr402	–	25
Tr403	–	21
Tr404	–	24
Tr405	–	10
Tr3801	18	–
Tr3802	0	–
Tr3803	19	–
Tr3804	20	–
Tr3805	17	–

d. Culture 4

Trial	Classification		
	vs ch57 stim	vs ch08 stim	vs ch 10 stim
Tr5701	–	9	0
Tr5702	–	2	0
Tr5703	–	8	2
Tr0801	0	–	0
Tr0802	0	–	0
Tr0803	0	–	0
Tr1001	10	7	–

e. Culture 2 (*Int. Shuf*)

Trial	Classification		
	vs ch13 stim	vs ch54 stim	vs ch 30 stim
Tr1301	–	0	0
Tr1302	–	5	1
Tr1303	–	0	0
Tr1304	–	0	2
Tr1305	–	0	3
Tr5401	6	–	0
Tr5402	0	–	0
Tr5403	2	–	0
Tr5404	6	–	3
Tr5405	0	–	0

f. Culture 3(*Int. Shuf*)

Trial	Classification	
	vs ch4 stim	vs ch 38 stim
Tr401	–	3
Tr3805	0	–

4. Discussion

4.1. Discussion on the analysis results

Based on the experimental results, several *classifiable neurons* were observed in particular areas of neuronal networks. In detail, multiplexed spike wave propagation share several neurons and some may be used to classify different spike wave propagations. Accordingly, questions arose considering the distribution of *classifiable neurons*: do both *classifiable* and *non-classifiable neurons* exist in the same neuronal network?

The distribution of *classifiable neurons* is influenced by the distribution of synaptic weights in the neuronal network. It is well known that each neuron has an individually specific (intrinsic) synaptic weight and each neuron is considered *classifiable neuron* or not depending on conditions such as synaptic weights. In the physiological experiments, unlike the simulation experiments [17], it is difficult to determine weight distributions intentionally and only a limited number of realized weight distributions were observed. Therefore, distributions of *classifiable neurons* varied between different cultures.

In attempt to understand why *non-classifiable neurons* are intermingled with *classifiable neurons* are intermingled in the same neuronal network, three conditions of spike wave propagation scheme were presumed, as shown in Figure 4. For simplicity, it was assumed that all neurons were connected to neighboring neurons and spike waves spread radially from stimulated neurons. Due to the influence of the synaptic weight distribution in neuronal networks, each spike wave propagates with its own individual spatiotemporal pattern. Therefore, neurons sharing multiple spike wave propagations could be used to classify different spike wave propagations if a spike wave does not spread to neurons stimulated another spike wave each other (Figure 4 a1-2). However, if one spike wave spreads to neurons stimulated by another spike wave, as shown in Figure 4b, some neurons fire the same temporal patterns, even when a different neuron is stimulated. Results shown in Figure 2, Figure 3, and Table 1 suggest that this condition was realized in neuronal network used in these experiments.

Moreover, it was difficult to classify the stimulations of channel 54 and channel 30 in Culture 2, as fewer *classifiable neurons* were observed. The reason for this result was that spike waves spread to neurons that were stimulated by other spike waves, as shown in Figure 4c. Under this condition, some neurons fire the same temporal patterns, even when a different neuron is stimulated. Additionally, although we assume in this discussion that the spike waves spread in a simple radial direction, neurons are connected randomly in reality. Therefore, both *classifiable neurons* and *non-classifiable neurons* observed (Figures 2 and 3).

From Figures 3b and 3c, the distribution of *classifiable neurons* in *Learning Pattern 54* (stimulated neuron was ch54) was different from the distribution of *classifiable neurons* based on *Learning Pattern 30*. This phenomenon provides explanation for how spikes wave spread, as shown

in Figure 4. If a pair of naturally stimulated neurons generate two different spike waves, the distribution of these spike waves and the overlap area are different, thus reflecting the distribution of *classifiable neurons*. Consequently, the spatial distribution of *classifiable neuron*s in the network varies when there are multiple targets for spike waves.

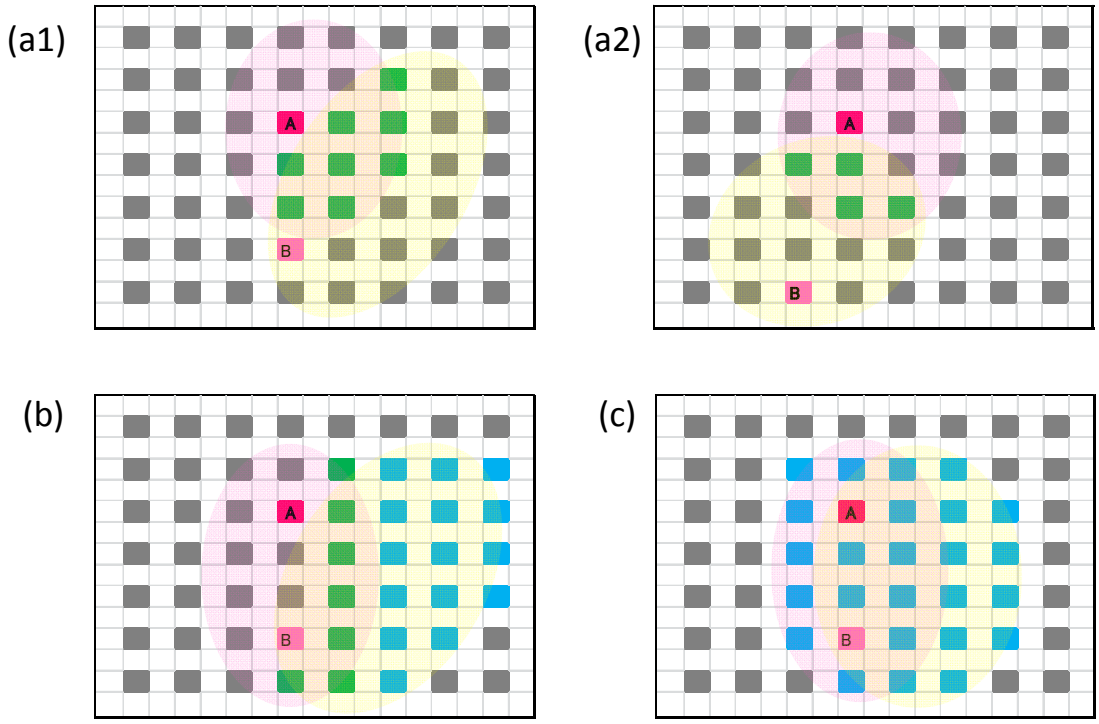

Figure 4 Condition of spike wave propagation scheme. (a1-a2) The spike wave generated from neuron A did not cover neuron B and spike wave generated from neuron B did not cover neuron A. In this condition, each spike wave was generated independently when neuron A or B was stimulated. Neurons overlapping both spike waves (green) generate different temporal patterns when the stimulated neuron was different Therefore, two stimulated neurons were classifiable in this area. If a pair of stimulated neurons generated two different spike waves, the distribution of these spike waves and the overlap area were different, thus reflecting the distribution of *classifiable neurons*. **(a2)** If the location of neuron B was different from **a1**, the spread and distribution of "green neurons," corresponding to the different overlapping areas. **(b)** Spike waves generated from neuron A covered neuron B; neuron B fired and spike wave were generated from neuron B. Under this condition, neurons indicated in blue fired in the same temporal pattern both when neuron A was stimulated and when neuron B was stimulated. Therefore, no difference was observed in the temporal pattern in this area. However, two stimulated neurons were classifiable (green). **(c)** Spike waves generated from neuron A covered neuron B and spike wave generated from neuron B covered neuron A. Both spike wave were generated from either stimulated neuron A or B. Hence, the temporal pattern was observed.

Furthermore, we investigated how multiplexed communication affects the processing of intellectual information in the brain. A simple multiplexed communication in the brain was modeled, as shown in Figure 5. The establishment of a virtual communication link from stimulated neurons to a particular area in the neuronal network was observed. Consequently, specific information was received in a particular area (Figure 5). We consider these processes as the fundamental mechanisms of intelligence in the brain. In fact, we hypothesize that the present model is valid not only for simple situations, but also for more complex similar situations.

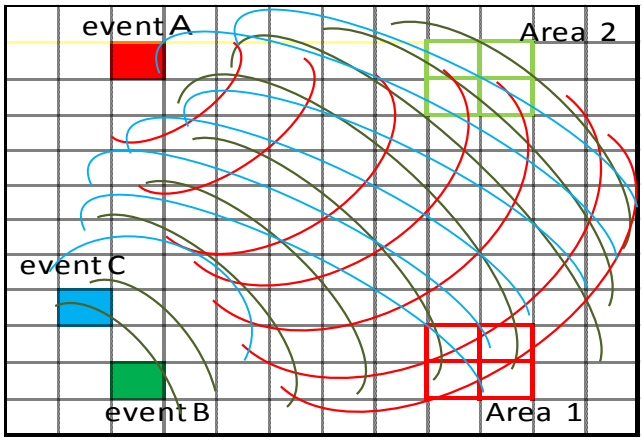

Figure 5. A sample of the multiplexed communication field in the brain. The figure shows events corresponding to stimulated neurons and spike wave propagations. In Area 1, event A was distinguishable from event C and in Area 2, event B was distinguishable from event C because *classifiable neurons* were concentrated in these areas. From a broad perspective, information for event A was receivable in Area 1 and information for event B was receivable in Area 2. Thus, two communication links from event A to Area 1 and from event B to Area 2 were extracted. In this case, event C was the comparison criterion of the spike spatiotemporal pattern of events A and B (if another event, such as event A or B, was the comparison criterion, the communication link for event C could also be extracted).

In contrast, for a few neurons, the mean value of *SpsetTrial* was greater than *SpsetLocal*. The mean value was significantly greater when both the trial pattern and the learning pattern were generated from the same stimulated neurons (Figures 2a and 3a). The results of these experiments suggest the possibility of the incorrect classification of some spike wave propagations. However, such neurons are fewer in number than *classifiable neurons* (when the stimulated neurons are different between the trial and the learning pattern). Therefore, the activities of such neurons may be masked by *classifiable neurons*. In brief, the trials successfully classified the entire neuronal network in a broad way and the experimental results reflect the distribution of synaptic weight in neuronal networks.

4.2. Function of classifiable neurons in the brain

The function of *classifiable neurons* was investigated in the brain. It was considered that *classifiable neurons* may participate in distinguishing different communications in the brain and that multiplexed spike wave propagations correspond to multiplexed communications in the brain. Some communications use the same neurons, as shown in Figure 4. In this case, the function of *classifiable neurons* was to classify multiple communications and recognize individual information. This function is similar to the multiplexed communication mechanism in artificial communication systems, such as mobile phones.

5. Conclusion

In this study, we classified various spike wave propagations individually generated from different stimulated neurons using an original spatiotemporal pattern matching the method of spikes in a cultured neuronal network. Based on the experimental results, *classifiable neurons* were observed in the neuronal network. We also confirmed that the spatial pattern of *classifiable neurons* within the neuronal network depended on stimulated neurons generating different spike wave propagations. These results suggest that distinct communications occur via multiple communication links in the brain and *classifiable neurons* play a significant role in this process.

Moreover, multiplexed communication scheme in the neuronal network were modeled in order to discuss the meaning of the multiplexed communication mechanism with regard to the management of intellectual information in the brain. The results of this study suggest that communication in the neuronal network is the basis of brain activity**.** This research provides a significant clue to solving one of the deepest mysteries of neuronal networks, namely, how seemingly ambiguous behavior among neurons leads to a reliable information processing system.

In this study, multiplexed communication is only modeled for one simple situation in a neuronal network. Because the comparable spatiotemporal patterns in the present analytical program are limited to two (events A vs B, A vs C, or B vs C), the resulting multiple analyzed spike spatiotemporal pattern includes only a pair of events (events A vs B, A vs C, or B vs C). Thus, the present multiplexed communication scheme is incomplete and further research is required to investigate situations with more than three events. Although the present scheme may be adequate for more complex situations as well, it is necessary to clarify these situations of multiple communications in the brain in future studies.

Lastly, the features of this paper are summarized as follows:

(1) To our current knowledge this study is the first attempt to investigate multiplex communication in a cultured neuronal network.

(2) Experiments and analysis correspond to a simulation experiment in 9 × 9 2D mesh neural network and sought to identify two transmitting neuron groups stimulated in a simulated

neuronal network, i.e., 2:1 communication [17].

(3) The results of this study show a signal transmission principle in neuronal networks which provides a possible solution to the mystery of the manner of reliable neuronal communication, which is thought to be the basis of brain activity.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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