



An olfactory bulb slice-based biosensor for multi-site extracellular recording of neural networks

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ABSTRACT

Multi-site recording is the important component for studies of the neural networks. In order to investigate the electrophysiological properties of the olfactory bulb neural networks, we developed a novel slice-based biosensor for synchronous measurement with multi-sites. In the present study, the horizontal olfactory bulb slices with legible layered structures were prepared as the sensing element to construct a tissue-based biosensor with the microelectrode array. This olfactory bulb slice-based biosensor was used to simultaneously record the extracellular potentials from multi-positions. Spike detection and cross-correlation analysis were applied to evaluate the electrophysiological activities. The spontaneous potentials as well as the induced responses by glutamic acid took on different electrophysiological characteristics and firing patterns at the different sites of the olfactory bulb slice. This slice-based biosensor can realize multi-site synchronous monitoring and is advantageous for searching after the firing patterns and synaptic connections in the olfactory bulb neural networks. It is also helpful for further probing into olfactory information encoding of the olfactory neural networks.

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1. Introduction

The olfactory bulb (OB) is the initial brain site of the olfactory pathway and plays an important role in processing and relaying olfactory information from the sensory organ to several central targets (Sullivan and Dryer, 1996; Laurent et al., 2001; Lledo et al., 2005). Many studies have investigated odor-evoked responses of olfactory bulb neurons using extracellular unit recording or intracellular microelectrodes (Beuerman, 1975; Duchamp, 1982; Lam et al., 2000; Cang and Isaacson, 2003). Researchers have observed different oscillations in the main olfactory bulb during the experiments (Lam et al., 2000; Kashiwadani et al., 1999; Margrie and Schaefer, 2003; Yuan and Knöpfel, 2006; Beshel et al., 2007; Gire and Schoppa, 2008) or by computation simulation (Margrie and Schaefer, 2003; Bathellier et al., 2006), which contributes to the olfactory information processing and coding. Therefore, studies about the electrophysiological and synaptic properties of OB are increasing.

The former electrophysiology investigations about OB were usually on the responses of the *in vivo* animals to the odors (Beuerman, 1975; Duchamp, 1982; Lam et al., 2000; Cang and Isaacson, 2003),

whose results may be affected by anaesthesia, bleeding, respiration or movement of the animals. Now, the *in vitro* models, such as the isolated olfactory bulb tissues (Josephson et al., 2004) and slices (Yuan and Knöpfel, 2006; Gire and Schoppa, 2008; Keller et al., 1998; Scheidweiler et al., 2001; Heinbockel et al., 2007), have become the important objects for the physiological experiments.

According to the coupling principle of the cell and sensor, many types of tissue or slice-based biosensors have been developed by combining the tissues or slices with various sensors, such as light addressable potentiometric sensor (LAPS) (Liu et al., 2010a), field effect transistor (FET) (Hutzler and Fromherz, 2004) and microelectrode array (MEA) (Oka et al., 1999; Liu et al., 2010b). Thereinto, MEA has many advantages such as simplicity, convenience, low cost and multi-site measurement, so it is more widely used as a tissue-sensing system for extracellular recording, like hippocampus slice (Heuschkel et al., 2002), retina (Brivanlou et al., 1998), somatosensory cortex (Petersen and Diamond, 2000), visual cortex (Warren et al., 2001), cerebellum (Mapelli and D'Angelo, 2007) and olfactory mucosa (Liu et al., 2010a) based biosensor. Fig. 1 gives the basal system of extracellular measurement with tissue-based biosensor. When tissue or slice is placed on the surface of MEA, changes of the field potentials of the neurons will induce the MEA output to change, which is used to detect the extracellular potentials.

To our knowledge, no reports have been made to apply MEA into the OB slices. In the past researches, optical imaging (Senseman,

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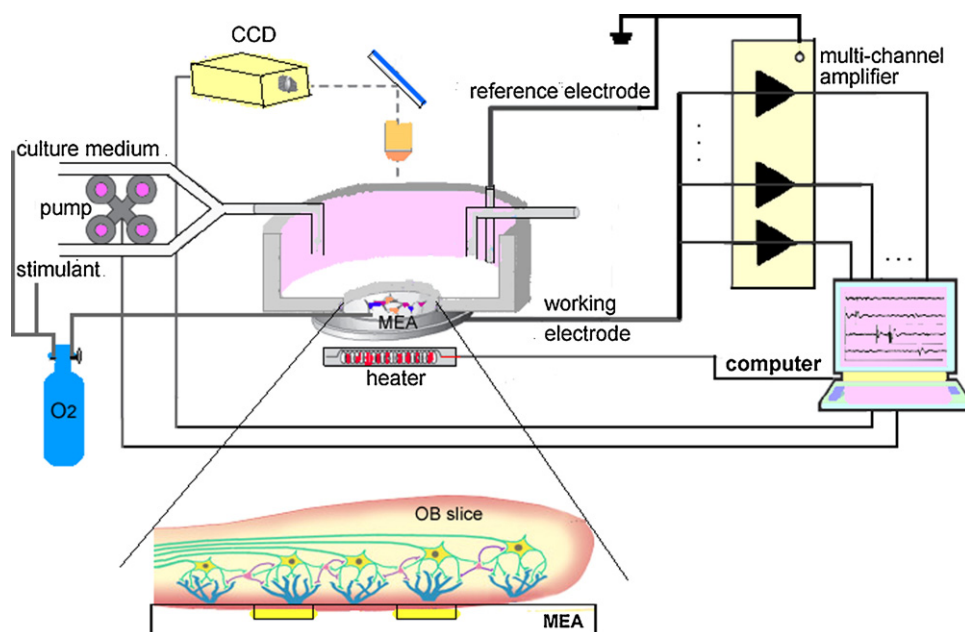


Fig. 1. Schematic drawing of testing system.

1996) and patch clamp recording (Karnup et al., 2006) were the main techniques for the electrophysiological monitoring on the olfactory bulb slices. In this paper, we take the acute OB slice as a novel sensing element of MEA chip for extracellular recording. This sensing element has the advantages that: (1) slice keeps the original neural networks and synaptic connectivity in the OB; (2) thickness of slice (300–400 μm) appears legible layered structure; (3) combination of slice and MEA adapts to multi-site extracellular measurement of neural networks. Placing the acute OB slice on MEA, we simultaneously obtained the multi-channel spontaneous signals and the responses induced by glutamic acid (Glu). This sensing technique can realize real-time monitoring with multi-sites, which is convenient for correlation analysis and is significant for further discussing the firing pattern of the OB.

2. Method and experiment

2.1. Device preparation

MEA fabrication was made according to the standard micro-electronic process flow, which has been detailedly narrated in some reports (Thiebaud et al., 1997; Oka et al., 1999). Here, the process was briefly described as follows. MEA fabrication was begun with a thick gold layer (2000–5000 Å) on the glass substrate (5 in. diameter, 500 μm thickness). Then evaporate a thin layer of chromium/titanium tungsten (100–500 Å) followed with sputtering photoresist on it. Next, use lift-off process to remove photoresist for getting the final electrode patterns (electrodes and traces). Finally, coat the surface with an insulating material such as polyimide or thick resist, and open the holes where electrodes are located. Fig. 2(a) is the micrograph of MEA.

After fabrication, carve the fabricated glass into unattached MEAs and connect MEA with printed circuit board (PCB) by spot welding. In the end, use epoxy resin to cover the welding strings and tailor-made conglutinate glass vessel. After epoxy resin solidifying, the device with a testing chamber can be used for measurement.

In the present study, the electrodes were electroplated with platinum black to decrease the noise and increase the ratio of signal to noise. The final electrodes with platinum black are presented in Fig. 2(a).

2.2. Slice preparation

Experiments were carried out in accordance with the guidelines of the local welfare committee. Rats (Wistar, about 3 weeks old, regardless of sex) were decapitated after anesthetized using 20% urethane (7 ml/kg), and an extensive craniotomy was made to remove the head carefully. Then the head was quickly placed in the oxygenated ice-cold (2–4 °C) artificial cerebrospinal fluid (ACSF) with the following composition (mmol/L): 124 NaCl, 26 NaHCO_3 , 3 KCl, 1.2 $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1.3 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 CaCl_2 , 10 glucose; the pH was usually adjusted to 7.4.

Next, the brain together with the olfactory bulbs and nerves, was separated into its two hemispheres. A transaction was made across the forebrain to isolate the olfactory bulbs. Then, every olfactory bulb was transferred to the flat roof of the vibratory microtome (Vibratome, 1000 Plus, Vibratome, USA) and cut into horizontal slices with the thickness of 300 μm . Slices with good configuration were stored in the ACSF continuously bubbled with a mixture of 95% O_2 and 5% CO_2 , and incubated at room temperature for at least 1 h for the further measurement.

To get the clear layers of OB slice and the orientation of electrodes on OB slice, Nissl staining was used to dye the slice after measurement.

2.3. Signal recording and processing

Measurements were carried out with the testing system in Fig. 1, which can perform 16-channel synchronous inspection in real-time. During experiments, we observed the extracellular electrical activities to evaluate the feasibility of MEA as the tissue slice-chip. In addition, we also investigated the effect of Glu on the signals and reviewed the detecting capability of this novel OB slice-based biosensor. Glu was dissolved in the ACSF for the final concentrations of 10 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$, 100 $\mu\text{mol/L}$, 200 $\mu\text{mol/L}$, 500 $\mu\text{mol/L}$, 1 mM and 5 mM.

In the present experiment, data were acquired simultaneously from 16 channels with a sampling frequency of 10 kHz. We recorded the raw data of slices in the normal ACSF as the control group, which was usually recorded for 1 h. The groups containing Glu were performed after recording for 20 min in the normal ACSF.

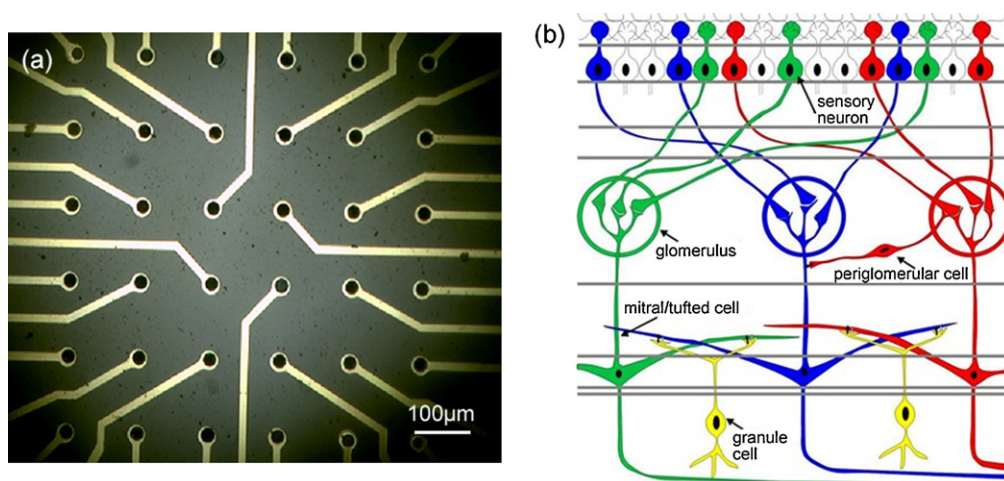


Fig. 2. (a) Photo of MEA after electroplating platinum black. (b) Schematic map of the neural network in the olfactory bulb.

Glu's action was controlled within 5 min. In the present study, curve fitting, raster plot and cross-correlation analysis were made with Matlab software.

3. Results and discussion

3.1. OB slice

In the mammalian OB, cell apoptosis and neural regeneration extensively exist for growth of OB. Studies have demonstrated that the apoptosis of the rat OB reached the most high at the postnatal day 5, then began to decrease (Fiske and Brunjes, 2001) going with neural growth. At the postnatal day 14–15, OB begins to grow when the developing nerves reach to the OB. At first, the big efferent nerves such as the mitral cells (MCs) come into being. Then the central cell cluster and external plexiform layer (EPL) gradually shape. In the end, the small juxtglomerular cells and granule cells (GCs) begin to form. Thus, OB of the rat basically contains several distinct and relatively simple layers (see Fig. 2(b)). In the present experiments, we can get the OB slice with distinct layers. Fig. 3 is the micrograph of OB slice with Nissl staining. According to the distribution of various neurons, OB slice mainly comprises olfactory nerve layer (ONL), glomerular layer (GL), EPL, mitral cell layer (MCL) and granule cell layer (GCL). In the picture, the blue dots are the orientation spots for identifying the layers of Fig. 4(a).

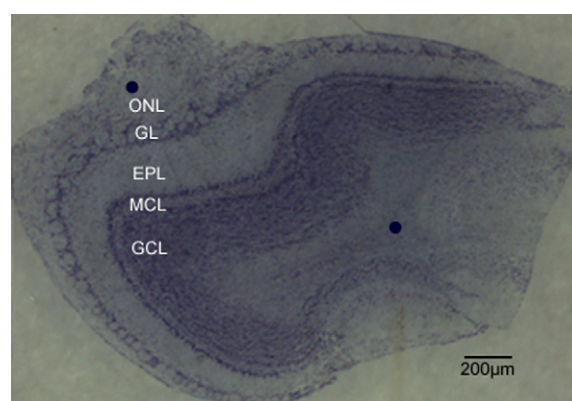


Fig. 3. Micrograph of OB slice with Nissl staining. ONL: olfactory nerve layer, GL: glomerular layer, EPL: external plexiform layer, MCL: mitral cell layer, GCL: granule cell layer. The blue dots are the orientation spots.

3.2. Synchronous recording with olfactory bulb slice-based biosensor

Combining the OB slice with MEA as shown in Fig. 4(a), multi-site signals can be simultaneously obtained through the multi-channel amplifier and acquisition system. After measurement, the OB slice was marked with micropipette at two positions

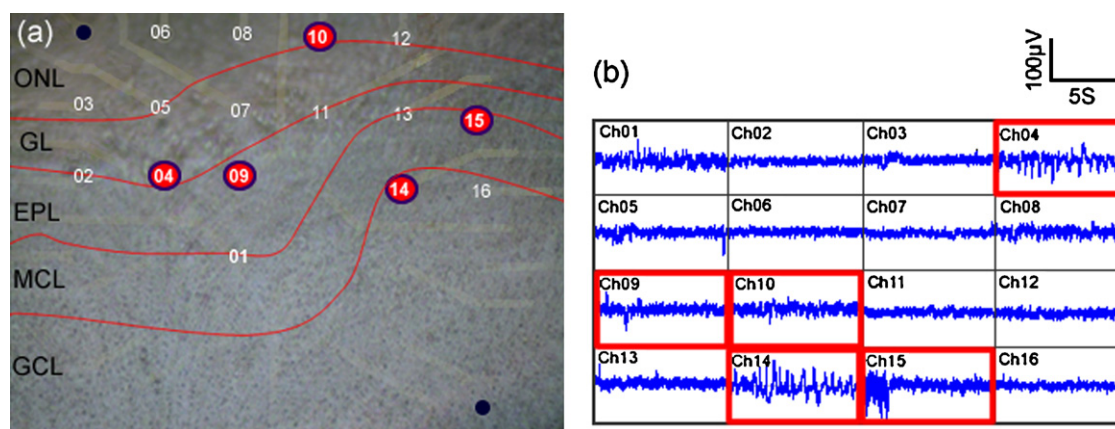


Fig. 4. (a) OB slice-based biosensor with MEA. Numbers are channel values corresponding to electrodes, and red curves are boundaries of different layers. (b) Spontaneous signals of OB slice from 16 channels. Signals in red frames are typical responses from different layers of OB slice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

shown as the blue dots in Fig. 4(a) and dyed with Nissl staining. According to the orientation spots of Figs. 3 and 4(a), we identified and presumably lined out the layers of OB slice on MEA (see Fig. 4(a)). Thereinto, the numbers are the channel values which electrodes correspond to, and red curves are the boundaries of different layers in OB slice. Thus we can obtain the signals of each electrode at different layer. Fig. 4(b) gives the 16-channel spontaneous signals by synchronous measurement. In the experiments, the maximum amplitude of the spontaneous signals of the OB slice can reach to 200 μ V, and the duration fluctuates from 15 ms to 300 ms. The firing rate of the different channels is dissimilar. Occasionally, burst will occur as shown in channel 15 of Fig. 4(b).

The signals in red frames of Fig. 4(b) are typical responses respectively from different layers (see the red discs in Fig. 4(a)). Linking the layers of Fig. 4(a) and signals of Fig. 4(b), it can be found that different spikes occurred in different layers. In the ONL and GL, negative spikes play the leading role. In the GL-EPL border and EPL, biphasic spikes and positive spikes begin to appear. Yet, in the MCL and GCL, positive spikes exhibit dominant. Usually, the negative charge is aroused by depolarization with ionic current influx, whereas the positive spikes are caused by the reverse phase. According to the results of (Aroniadou-Anderjaska et al., 1997), the negative charge may present in the GL and the positive charge may be recorded from the soma along the secondary or lateral dendrites in tufted cells or mitral cells (Karnup et al., 2006). In the present study, we observed similar phenomenon. In layered structures, both an active zone (sink) and a passive zone (source) are found according to the current source analysis (Kulics and Cauller, 1986; Taube and Schwartzkroin, 1988; Aizenman et al., 1996). Thereby, as a whole, we can take ONL and GL as the sink, and MCL and GCL as the source. Besides, we can also decompose them into two parts. At first, ONL, GL are taken as the first sink, and EPL as the first source. Then, EPL is taken as the second sink, and MCL, GCL as the second source. That is to say, EPL plays dual roles. This finding is very pivotal for the neural network modeling of the olfactory system in the future.

Compared with the former techniques for extracellular recording, the OB slice-based biosensor is more convenient to catch the various characteristics of potentials at the same time, which is important for neural network analysis of the OB slice.

3.3. Action of glutamic acid

Glutamic acid (Glu) is the primary excitatory neurotransmitter in the brain and spinal cord. Several studies demonstrated that Glu plays a crucial role in exciting mitral cells (Yuan and Knöpfel, 2006; De Saint Jan and Westbrook, 2005; Ennis et al., 2006) as well as granule cells (Heinbockel et al., 2007) in the main OB. In the present study, we observed the effects of Glu on the different layers of the OB slice.

Fig. 5(a) and (b) gives the statistic effect of different dosage Glu on the signal amplitude as well as frequency. The concentration of Glu includes 10 μ M, 50 μ M, 100 μ M, 200 μ M, 500 μ M, 1 mM and 5 mM. According to the scattering points, we made polynomial fitting with Matlab and got the fitting curves (red curves in Fig. 5(a) and (b)). Comparing Fig. 5(a) with (b), we find that Glu's effect on frequency is more obvious than on amplitude. But the action trends on amplitude and frequency are accordant. Under 100 μ M, Glu's action is subtle. Between 100 μ M and 1 mM, Glu exhibits distinctive effect and the concentration–effect relationship appears approximate linear. But over 1 mM, Glu's action becomes mild again. According to the effect of Fig. 5(a) and (b), we subsequently chose the moderate concentrations (100 μ M and 200 μ M) to present their action detailedly in Fig. 5.

Fig. 5(c), (d) and (e) gives the responses of 5 representative sites (see red discs in Fig. 4(a)), respectively under action of the normal

ACSF, 100 μ M and 200 μ M Glu, which shows that Glu has visible effect on all channels. It can be seen that Glu not only increases the amplitude of the signals (see Ch09 and Ch14) but also the firing rate (see Ch04, Ch09, Ch14, Ch15). When the firing rate becomes high enough, the spikes may break out in short time to form spike bursts (see Ch04, Ch09, Ch14, Ch15 in Fig. 5(e)). In addition, sometimes Glu can also excite some channels which do not bring spontaneous response in the normal ACSF (see Ch09 of Fig. 5). In the present experiment, Glu has weak effect on Ch10. It has reported that neuronal electrical activities include two main patterns: single spike firing and a series of spikes rhythmic firing quickly (Latham et al., 2000; Harris et al., 2001; Dhamala et al., 2004). That is to say, Glu can change the firing pattern of neurons at GL, EPL, MCL and GCL of OB.

In order to investigate the action of Glu on the firing rate of signals, we made the raster plot of Ch14 with Matlab software (see Fig. 5(f)). The results show that Glu makes the firing rate increase obviously, and also induces spikes to produce bursts. In the presence of 100 μ M Glu, bursts occasionally appear; but under action of 200 μ M Glu, bursts often take place.

Experiment results indicate that Glu has excitation action on the OB slice, which is accordant with the former conclusion (Yuan and Knöpfel, 2006; Heinbockel et al., 2007; De Saint Jan and Westbrook, 2005; Ennis et al., 2006). Nobbut, in the different layer, Glu's action behaves different degree. In the present experiment, Glu has notable effect on Ch04, Ch09, Ch14 and Ch15, which suggests that there be some synapses mediated by Glu in these positions. In the future, we also consider other drugs like GABA, 5-HT, DA, and the like to investigate other physiological mechanism.

3.4. Cross correlation analysis of multi-site signals

MEA has the virtue of simultaneous recording from multi sites, which is favorable to dig out the characteristics as well as the relationship among the multi-channel signals. Many researches based on MEA have investigated the synchronism and the parallel information between cells in the cultured neural networks (Segev et al., 2004; Eytan et al., 2004; Chiappalone et al., 2006) or *in vitro* tissues (Liu et al., 2010b). In the OB slice, the relationship between the distance and the spike occurrence in the glomeruli (Karnup et al., 2006), and the correlation between the synchrony and oscillations of the mitral cells (Galan et al., 2006) have been discussed. However, the recording sites by patch clamp are limited, and as a result the analyzed neurons are restricted. To investigate whether the spike firing from the different neurons of the OB slice has relationship, we surveyed the synchrony by computing the cross-correlation function of the signals recorded by MEA.

For continuous functions, $f(t)$ and $g(t)$, the cross-correlation is defined as

$$f(t) * g(t) = \int_{-\infty}^{\infty} f^*(\tau)g(t + \tau)d\tau$$

where $f^*(t)$ denotes the complex conjugate of $f(t)$. The formula essentially slides $g(t)$ along the t -axis, calculating the integral of the product of $f^*(t)$ and $g(t)$ at each position. Therefore, the cross-correlation is used to measure the similarity of two waveforms, and to find how much $g(t)$ must be shifted along t -axis to make it match to $f(t)$. That is to say, similarity as well as synchrony of two waves can be depicted by cross-correlation.

In the OB, synaptic contact may exist within glomeruli, between glomeruli and between mitral cells and granule cells. So we made cross-correlation analysis at 4 channels in Fig. 5 (Ch04, Ch09, Ch14 and Ch15, whose positions in OB slice are shown as the red dots in Fig. 4(a)). A segment of data with the length of 30 s was extracted. Fig. 6 is the results of cross-correlation analysis.

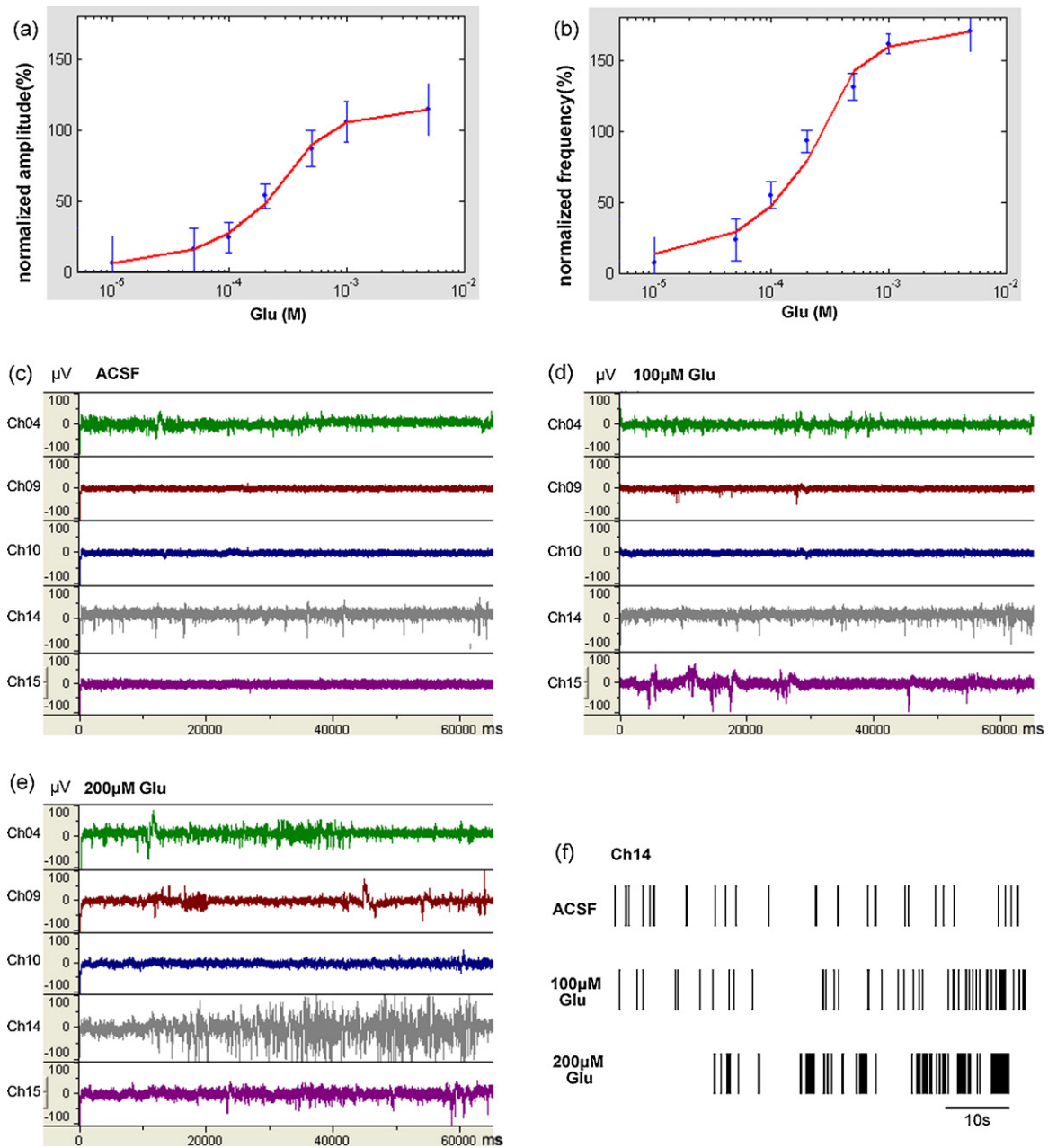


Fig. 5. (a) Dose–amplitude curve. (b) Dose–frequency curve. The amplitude and frequency are normalized based on that of spontaneous signals. Mean values were plotted ($n = 15$ per point) with error bars. The red smooth lines represent the curve fitting. (c–e) Signals from 5 channels respectively in the presence of (c) ACSF, (d) 100 μM and (e) 200 μM Glu. (f) Raster plot of Ch14 in the presence of ACSF, 100 μM and 200 μM Glu. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 6(a) is the cross-correlograms with lag time from -1 s to 1 s. For quantificational compare and synchrony analysis, we also give the histogram of electrode distribution and correlation coefficient when lag time is zero (see Fig. 6(b)). Before using Glu (see the purple curves in Fig. 6(a)), the signals between any two different channels have weak correlation. Well, the responses of Ch09, Ch14 and Ch15 exhibit good synchrony (see the first gram in Fig. 6(b)). These phenomena suggest that firing of Ch09, Ch14 and Ch15 is almost concurrent but the signal forms are not similar. Under the action of Glu, their cross-correlations appear more distinct and more extensive (see the red and blue curves in Fig. 6(a)). In the presence of 100 μM Glu, the peaks of the cross-correlation appear at a time-lag of zero, which indicates that firing in Ch09, Ch14 and Ch15 takes on favorable synchronous. Yet, to Ch09, Ch14 and Ch15, action of 200 μM Glu makes the peaks little shift and the curve smooth.

These results suggest that 200 μM Glu may enhance the connection between Channel 09, 14, 15. However, the cross-correlation of Channel 04 and the other channels is much small even in the presence of Glu. It can be speculated that Ch09, Ch14 and Ch15 may have some biological connection, but Ch04 has no connection with other channels in biology.

Past report (Karnup et al., 2006) depicted that the cross-correlation and the distance between two sites appeared some proportional, whose recording was limited within the glomeruli of the OB by patch clamp technique. Yet, in our experiment, data acquisition was made with MEA, which can cover more extensive area and layers of OB slice. The channels for cross-correlation analysis in the present study are positioned in the GL, EPL, MCL and GCL, which evaluated the relationship between different layers not the neurons within the glomeruli. According to the distribution of

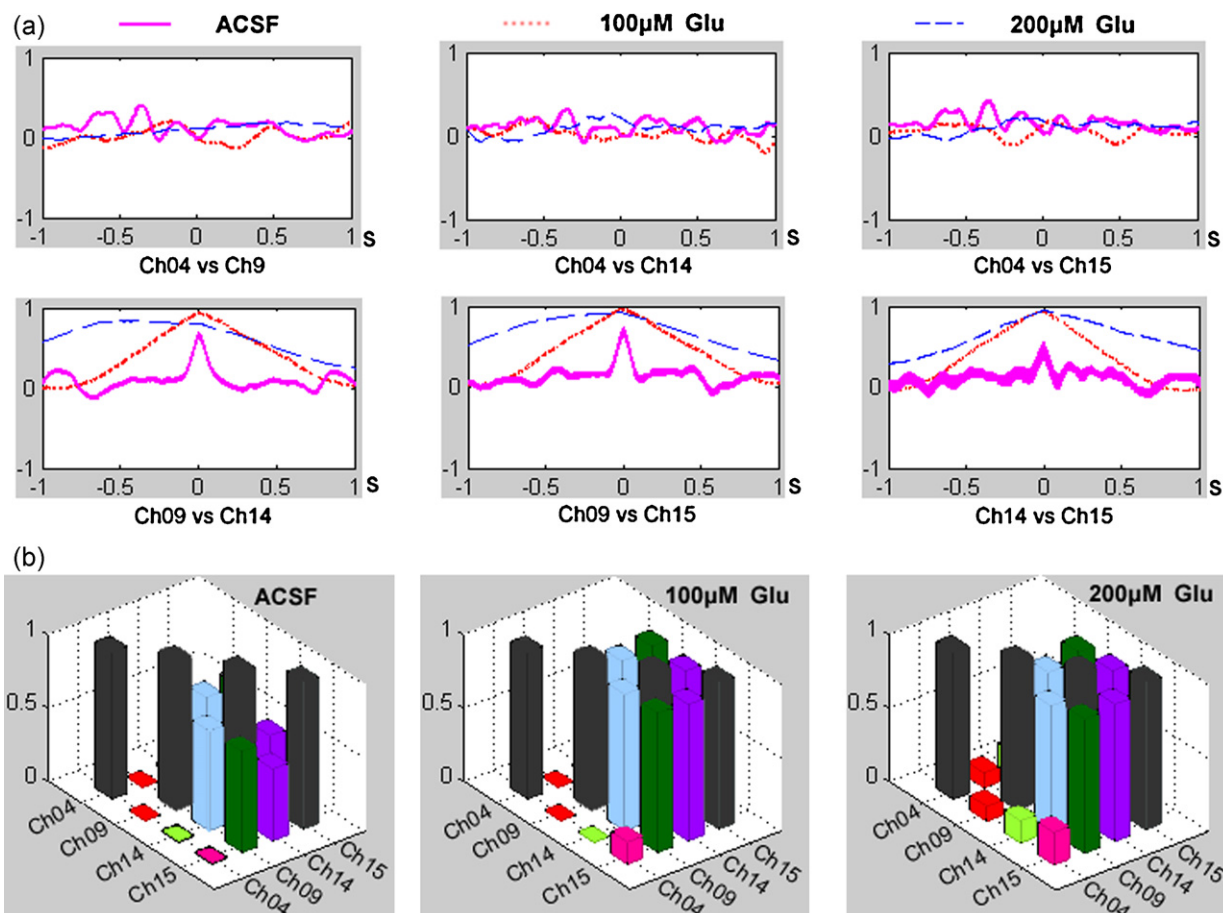


Fig. 6. (a) Histogram of electrode distribution and correlation coefficient (lag time is zero). The identical color represents compare of the same channels. (b) Cross-correlograms of signals. Purple, red and blue curves respectively represent the cross-correlations in the presence of ACSF, 100 μM and 200 μM Glu. Data for cross-correlation analysis come from 4 channels in Fig. 5 and are with a length of 30 s. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

electrodes in Fig. 4(a) and the cross-correlation result in Fig. 6, we find that the cross-correlation is not absolutely proportional to the distance between two positions. The possible cause is that two sites with biological connection may behave good synchrony or similar waveforms. For completely analyzing the correlation of different sites in the OB, we will redesign the pattern of MEA to acquire more information in the future.

Here, we can evaluate Glu' action on the different positions in the OB slice and the possible correlation of some sites. However, we can not conclude Glu excite one site to another by synaptic connection because Glu has excitation action on various neurons of the OB slice (Yuan and Knöpfel, 2006; Heinbockel et al., 2007; De Saint Jan and Westbrook, 2005; Ennis et al., 2006). The present results are elementary for the neural network study, and we need improve our device to realize the positioning irrigation to further investigate the action of Glu on the OB neural networks. Besides, under elicitation of *in vivo* successful experiments (Chaput and Chalansonnet, 1997; Laurent et al., 2001; Uva et al., 2006), we will construct another *in vitro* model including the olfactory mucosa and OB by coculture to study the response to odors. Moreover, we can also compare the data and methods of *in vitro* and *in vivo* model to review their different characteristics.

4. Conclusion

The novel OB slice-based biosensor for synchronous measurements can be used to detect the electrophysiological activities in the

neural networks and analyze the firing patterns of different neurons. Similarity as well as synchrony between two channel signals is the effective parameter for assessing the relationship among the different layers in the OB slice. Results of cross-correlation analysis about the simultaneous recording data show that most of the spontaneous potentials in the OB slice have weak correlation, and Glu can enhance the correlation of some channels but have no action on the correlation of other channels. This is favorable for digging out the synapses mediated by Glu. Therefore, combining multi-site recording by the slice-based biosensor with cross-correlation analysis is helpful for discussing the firing pattern of the spontaneous as well as induced potentials.

At present, we are designing 60-electrode microelectrode array and constructing 60-channel acquisition system, which is used to obtain more information in the future experiments. Furthermore, basing on our present work, we will develop a novel tissue-based biosensor with olfactory epithelium and OB tissue to investigate the information conduction and encoding in the presence of different odors.

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