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Short communication

The fabrication of an olfactory receptor neuron chip based on planar multi-electrode array and its odor-response analysis

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ABSTRACT

A novel olfactory neurochip based on olfactory receptor neurons (ORNs) cultivated on the surface of the 60-channel planar multi-electrode array (MEA) devices was developed in this study. In order to investigate the odor-response characteristics of ORNs, two types of odorants at different concentrations were quantitatively pumped into the neurochip by a customized gas intake system, and the extracellular electrical activities of multiple ORNs were simultaneously recorded *in vitro*. Accordingly, the odor-response features of ORNs such as firing amplitude, firing threshold, firing rate as well as firing channels were analyzed qualitatively and quantitatively in terms of ORN spike trains. Especially, after introducing the classification algorithm based on the spike threshold, the odor-response maps from the multiple sites could be used to identify DL-limonene (LIM) and isoamyle acetate (ISO) odorants. These preliminary studies indicate that the ORN-based biosensor developed here has a potential capability of distinguishing different odorants as a true bionic electronic nose.

1. Introduction

The olfactory system is one of the chemosensory senses of very high sensitivity and specificity due to olfactory receptors (ORs) recognizing thousands of odor molecules by ORNs (Buck and Axel, 1991). ORNs used for sensing odors were reported and the odor-response of individual ORN was recorded by microelectrodes (Huotari, 2000; Rospars et al., 2000; Trotier and MacLeod, 1983). Furthermore, a biosensor combining insect ORNs with a field effect transistor (FET) was fabricated as an odor sensor (Schöning et al., 1998). Although their work has greatly contributed to the studies of olfactory mechanism and ORN biosensors, the *in vivo* techniques to distinguish different odors are merely elementary at present. Comparing with ORN biosensors *in vivo*, biosensors made of ORNs or cells transfected with ORs, or intact olfactory epithelium *in vitro* have several advantages of simplicity, controllability, noninvasive detection and dispensing with sophisticate surgical operation. Cells transfected with ORs used as sensitive elements *in vitro* were reported by quartz crystal microbalance (QCM) (Ko and Park, 2005; Sung et al., 2006) and surface plasmon resonance (SPR) (Lee et al., 2006). However, QCM and SPR cannot get transient neural electrical activity signals but some statistical average value. ORNs cultured *in vitro* on light-addressable potentiometric sensor (LAPS) devices was reported (Liu et al., 2006), but they just referred to a single cell other than ORN networks. In order to sensitively detect different odors by olfactory biosensors, a sensor array or a sensor with a high scanning speed which can acquire signals from numbers of cells is indispensable. When considering the simplicity of the detecting system, real time etc., currently, electrophysiological techniques are more feasible than other methods mentioned above (Ottoson, 1956; Scott and Scott-Johnson, 2002; Chiu et al., 1997; Delgado and Bacigalupo, 2004). Intact olfactory epithelium based on MEA was reported as an olfactory sensor element in our previous study and the recent study (Chen, 2007; Liu et al., 2010). Nevertheless, they were at the tissue level and devoid of effective analysis of how to use these signals. At the cellular level, a similar work was about cell-based olfactory biosensor on the planar millimeter-electrode device (Lee et al., 2009), where an olfactory receptor protein was expressed in kidney (HEK)-293 cells and then a micro-fabricated planar electrode was used as an electrophysiological recording tool for the cells. This method might have the difficulty of how to gain a great deal of functional olfactory receptors. Up to now, to the best of our knowledge, all these experiments related to the odor-response of ORNs *in vitro* have been very scarce, and even more preliminary than that *in vivo*.

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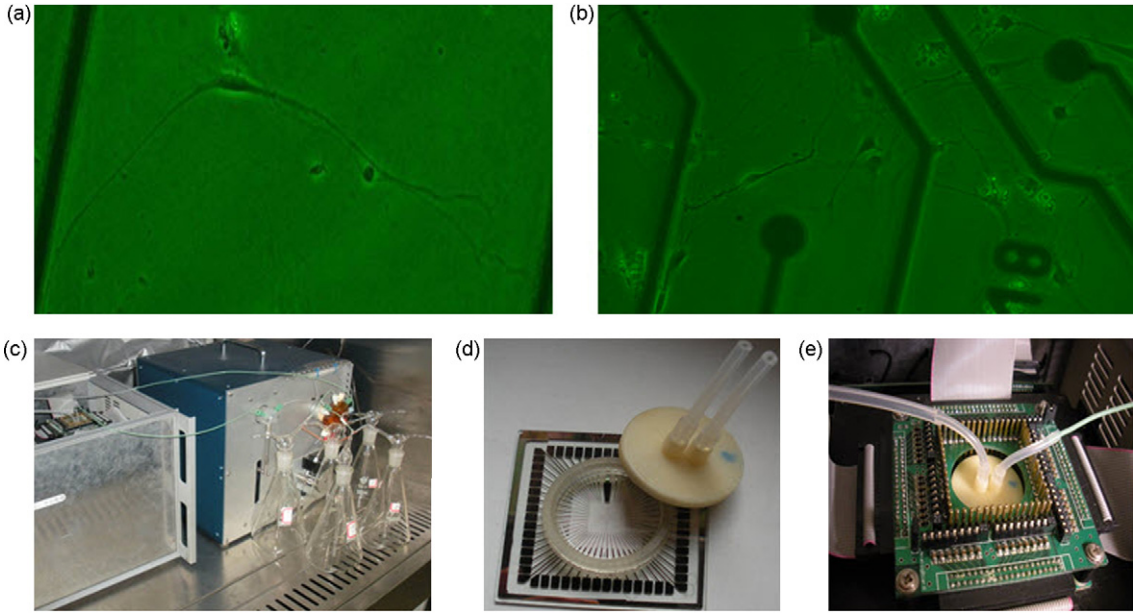

Fig. 1. (a) An individual bipolar ORN with motile cilia as long as 250 μm on the surface of MEA. (b) The neural network formed by several ORNs on the surface of MEA. (c) Gas intake system and MEA recording system. (d) Culture cells and the customized cover. (e) Interface circuits between olfactory neurochip and MEA recording system.

The purpose of this study is to probe a new way to fabricate biosensors based on cultivated ORNs and to develop a general neurochip platform along with some fundamental analysis methods for the research of the olfactory transduction mechanism based on its neural network. This would be a simple and convenient way to quantitatively measure the response of ORNs to various odors and assess the olfactory mechanism effectively.

2. Materials and methods

2.1. Cell culture

ORNs were obtained from 5 P0 Sprague–Dawley rats (provided by the Experimental Animal Center, Zhejiang Academy of Medical Science, Hangzhou, China). Approval for animal protocols was obtained from the Zhejiang University Animal Experimentation Committee. Firstly, rats were decapitated and pinned head dorsal up in a sylgard-coated petri dish filled with L-15 (Sigma). Then we dissected turbinates and septa away from surrounding tissues and place in eppendorf tube of neurobasal on ice and collected septa and turbinates placed in 4 °C. Thirdly, after rinsing twice and cutting to small pieces, put them into 0.125% trypsin (Gibco) to digeste at 37 °C for 25 min, then sucked out the trypsin, added DMEM (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco) to terminate digestion, agitated gently so as to loose the tissue and the cells. After 1000 r/min centrifugating for 10 min at 4 °C, removed supernatant, the cell pellet wasresuspend in 1 ml prewarmed ORN culture medium (neurobasal (Gibco) containing 100 u/ml penicillin (Invitmgen), 100 mg/l streptomycin (Invitmgen), 200 mg/l L-glutamine (Invitmgen), 25 ng/ml NGF (Sigma), 20 ml/l B27 (Gibco)) and plated on uncoated 35 cm petri dish for 8 h at 37 °C in 5% CO_2 incubator. The non-adhesive cell suspension was collected and then inoculated in MEA (MCS, 200/30iR-Ti-pr) and placed in the 5% CO_2 incubator (37 °C). The cultures were fed with OE medium every 3 days. In addition, to improve the biocompatibility of MEA, the surface of MEA was coated by the poly-D-lysine to facilitate cell attachment before cell culture (Ismail et al., 2003). Cultured ORNs on the surface of MEA are shown in Fig. 1a and b. The number of olfactory neurons in the MEA was evaluated to be about 50,000 after 3 days.

2.2. Gas intake system

To mimic a real circumstance, we exploited an automatic gas intake system consisting of microfluidic manipulation device (Fialab Inc., Fialab-3200), gas transport pathways and customized seal cover fit for the culture cell on MEA (Fig. 1c). The microfluidic manipulation device is a syringe pump with multiple units selecting different samples. The volume of gas injection can be precisely controlled by a high-resolution stepper motor. The seal cover customized by 3D printer (Stratasys Inc., Dimension Elite) was used to construct a gas chamber together with the MEA culture cell (Fig. 1d). For the one passway of the chamber, the odorants (or cleaning air) were imbibed from sample bottles, which filled with two kinds of odorants of different concentrations, and then went through the multiple units, finally reached the gas chamber via glue pipes. The other was an outlet passway connected to the open air outside the room (Fig. 1e). Pumping and recording were simultaneously controlled by a set of PC programs.

2.3. MEA recording system

We utilized a standard MEA (MCS Inc., 200/30iR-Ti-pr) as the recording unit and developed a customized recording system. To verify the availability of the MEA recording system, we performed a simulation test as well as a real physiological experiment. First, a signal of local field potential (LFP) combined with action potentials (APs) provided by a neural signal simulator (Cyberkinetics Inc., Cerebus 128-Channel Data Acquisition System) was used to test the performance of our MEA recording system. When the weak signals of APs mixed with LTP were input into the system, they were amplified and acquired by particular processing circuits (Fig. 4a). Furthermore, hippocampus neuron cells of rats were cultivated in the chamber of the MEA. After cells were cultivated for two weeks, spikes of hippocampus were recorded (Fig. 4b), and they could be sorted by the method which had been used in our previous studies (Ye et al., 2008). This system was also successfully used to record the spontaneous spikes of rat olfactory blub neurons cultured on MEA (Liu et al., 2009).

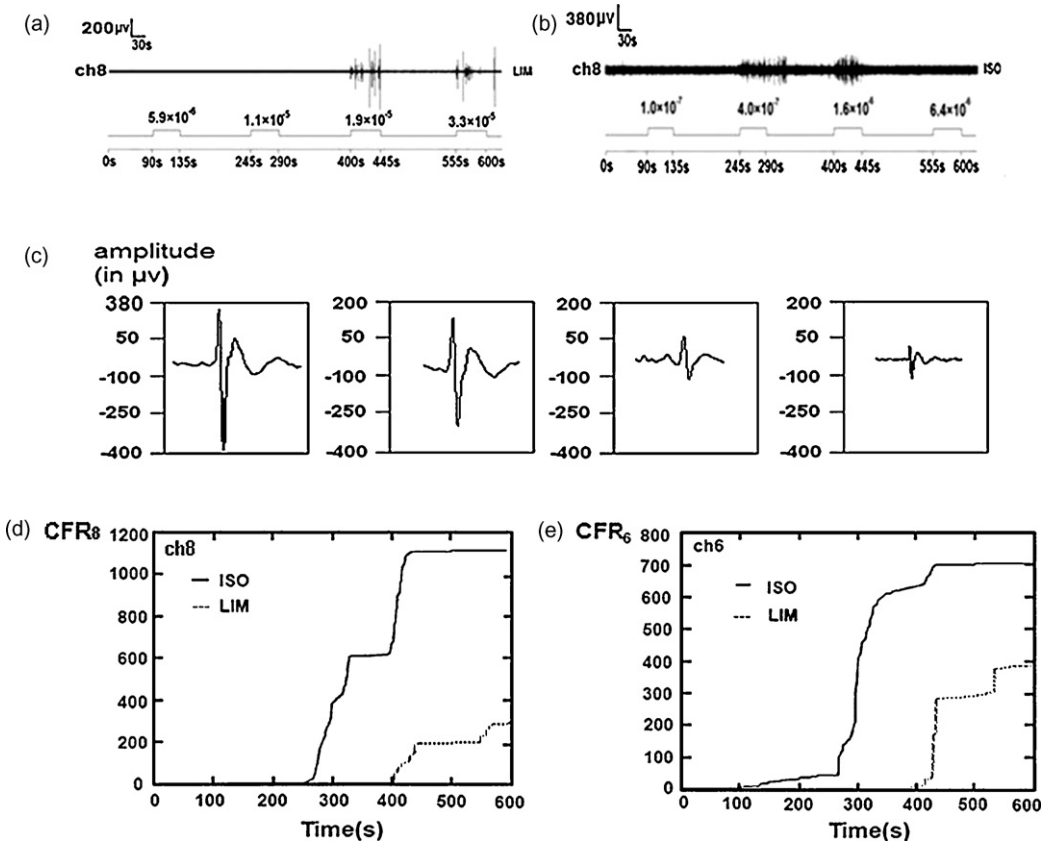

Fig. 2. Extracellular spike recording from MEA and $CFR_c(t)$ curves from two different batches of neurochips. (a) ORN responses of channel 8 to LIM. (b) ORN responses of channel 8 to ISO. (c) Four typical amplitudes of ORN spikes. (d) $CFR_8(t)$ curves of odor-response of (a) and (b). (e) $CFR_6(t)$ curves based on the data acquired from another chip.

2.4. Odor stimuli

As two pure odor compounds, LIM and ISO were selected from those previous tests in the rat according to their effectiveness and molecular structure (Duchamp-Viret et al., 1999). Considering that there were some critical uncertain factors such as the survivor time of ORNs *in vitro*, and even what effects on their activities when stimulating these cells for a long time, we tentatively selected a set of sensitive concentrations to test according to the previous studies (Duchamp-Viret et al., 1999). Stimuli (Table 1) were delivered about 45 s at 200 ml/min by the gas intake system to form odor pulses. In order to eliminate the interaction between the different inputs one after the other, the filtered air was injected into culture chamber alternately and the cleaning period was about 110 s. In the experiments, the test sequence was from low concentration to the high, and LIM was the first odorant under test and then ISO. All the tests were implemented by the same neurochip. The injection sequence was continuously carried out by the PC control program according to the test processes, which is consistent with a set of presetting time.

3. Results and discussion

In this section, firstly, several basic electrophysiological characteristics of the odor-response of ORNs are analyzed on the basis of the recording data from the one channel of MEA. After that, it presents an odor-response map of multi-channel data and their preliminary analysis results. Before the investigation of the characteristics of ORNs, the response time related with our current experimental system or method was discussed first. The time between the starting of imbibing odorants and neural spike occurring consisted of several processes such as gas dynamic exchanging in pipes and MEA culture cell chamber, odorant diffusion in liquid culture medium, as well as ORN cell intrinsic response time, which was estimated to be about 85 s and had been compensated in Fig. 2a and b.

3.1. Amplitude analysis of ORN spikes on MEA

The extracellular potentials were recorded by the planar MEA system. Fig. 2a and b shows the data from channel 8. The recording of the original 90 s could be viewed as the baseline of the recording system, in which some low frequency artifacts were filtered by a bandpass filter. Typical extracellular spike intensity is measured around dozens of microvolts by MEA. It is known that the data from the same recording site represent the electrical activities of the local group of neurons near the site, and the acquired potential amplitude on the electrode is inversely related to the distance between neurons and recording sites, so the amplitude of firing spikes is different. For our system, it showed that the amplitudes of spikes were about from 20 μV to 380 μV (Fig. 2c). This result indicates that the dynamical scope of the spikes recorded in these experiments is reasonable.

3.2. Threshold analysis of ORNs to the odors

For the LIM odorant, it was found that the firing spikes occurred when the odor concentration was gradually increased to 1.9×10^{-5} mol/l (400–455 s) (Fig. 2a). After the time 455 s, the firing disappeared gradually as the fresh air was being injected. Similarly, spike response recurred at 3.3×10^{-5} mol/l (555–600 s). For the other ISO odorant, typical firing states also occurred at two concentrations: firing began at 4×10^{-7} mol/l (245–290 s), and sustained at 1.6×10^{-6} mol/l (400–445 s) (Fig. 2b). However,

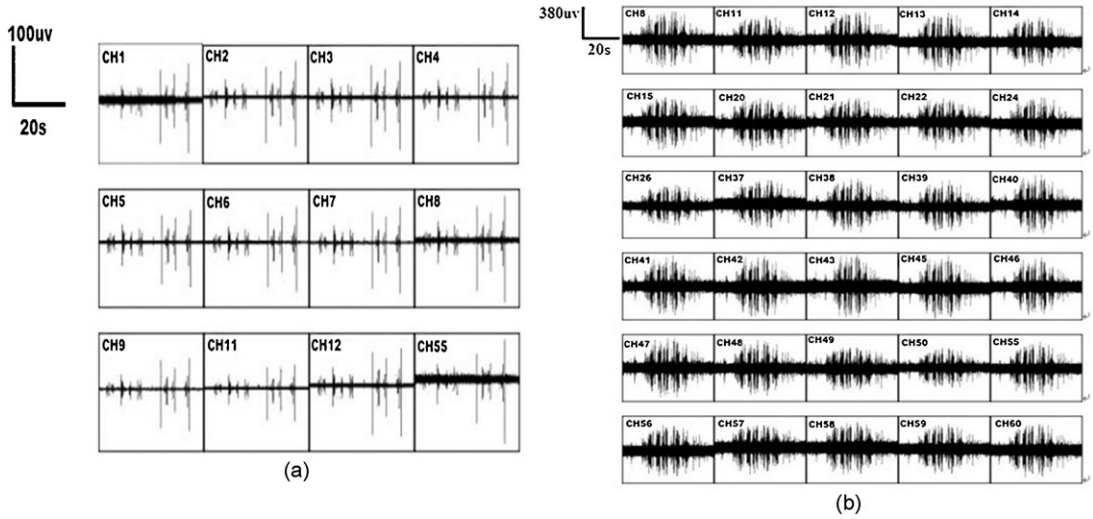

Fig. 3. Firing spikes map from multiple recording sites of planar MEA. (a) Responses of ORNs on the channels to LIM in 400–445 s. (b) Responses of ORNs on the channels to ISO in 400–445 s.

firing responses disappeared at 6.4×10^{-6} mol/l (555–600 s). The response threshold for LIM was about between 1.1×10^{-5} mol/l and 1.9×10^{-5} mol/l, which was higher than that of ISO about between 1.0×10^{-7} mol/l and 4.0×10^{-7} mol/l. Particularly, the ORN firing induced by ISO was inhibited when the concentration of the odor was higher than 1.6×10^{-6} mol/l. Different conditions might have effect on the odor-response thresholds of ORNs, and there were some differences in the distribution of thresholds due to the ORNs being cultured *in vitro* on the surface of MEA. The results suggest that the response of ORNs to different odors may have different thresholds and even the response to a certain odor may have a narrow range of concentration, and this experimental phenomenon was also reported in the former research (Rospars et al., 2000), which may be caused by the saturating effects of biomediators with respect to the chemical receptors of ORNs. According to the conversion, the current thresholds or detection limits for ISO and LIM in our study are about 50 mg/m^3 and 2.6 g/m^3 respectively. The ISO threshold is acceptable and promising as compared with 525 mg/m^3 stated by the report of the United State Dept. of Labor, but for LIM, it is far from 480 μg/m^3 required in the subreport on Limonene of the National Swedish Screening Programme 2004.

In our work, spontaneous spikes were sparse and even invisible (Fig. 2a and b). One reason is low survivors of isolated ORNs of rats *in vitro*, which make the density of ORNs on MEA is sparse, and their response need depend on the choice of the odor (Oka et al., 2004; Reisert et al., 2007). The other possible reason is that isolated ORNs *in vitro* are of low activities. These factors also made ORN firing rate lower than that *in vivo* (see the analysis in Section 3.3).

3.3. Statistic analysis of spike trains to the odors

There are several problems should be taken into account before this work. Firstly, according to our experimental procedure, the actual odor arriving the chamber was not a strict square pulses (Getchell and Shepherd, 1978). Secondly, the actual transient odor-response process of ORNs is a dynamic process which has some special physiological phenomenon such as firing latency (Duchamp-Viret et al., 1999). Thirdly, the samples under test were of limited concentrations, the reason of which was mentioned before in this paper. So we need a method from which not only the firing rate of the odor-response within interested intervals could be calculated, but also some dynamic information could be obtained, which maybe useful for the understanding of ORN encoding. Here, the method of cumulative firing rate (CFR) was introduced to characterize the dynamic process of the odor-response of ORNs, which is expressed as follow:

$$CFR_c(t) = \sum_{i=1}^{n} \delta(t - t_i),$$

where $CFR_c(t)$ is the cumulative firing rate of neuron populations, c represents the MEA channel number, t represents the time during the experiments, when $t = t_i$, $\delta(t - t_i) = 1$, which represents there is a spike at the time of t_i, otherwise, $\delta(t - t_i) = 0$ (Dayan and Abbott, 2001; Blejec, 2005). The $CFR_c(t)$ curves from two neurochips made in different time are shown in Fig. 2d and e, ORNs on the two neurochips were from two rats. The results of two experiments showed that the main electrophysiological characteristics were basically similar. In order to get the intervals when the odor-response reached steady-state, the segments of the curves with a steep slew rate could be selected to get the average firing rate (AFR). For LIM, AFRs were about 4.4 spikes/s in the first segment and 4.2 spikes/s in the second. While for ISO, AFRs were about 10.1 and 14.3 spikes/s respectively. The results showed that ORNs had different sensitivities to these odorants and for the different concentrations, at least for LIM, there was no monotonic relationship between AFR and concentration, this result was similar for the other channels in our experiments currently.

The dynamic characteristics of fitting curves of gas response are widely used as an effective method of feature-extracting for gas sensors (Nucci et al., 2003). Around the turning points of the curves where the odor concentrations were changing, more detailed information could be acquired and analyzed. Therefore, a set of curves will be found to fit these segments to improve the limited test point as well as odorants with subdividing concentrations, and even the mixtures of them or competing substrates need to be tested in our further study.

3.4. Odor-response map

Considering that odor quality is coded by the whole neuronal populations (Sandström et al., 2009), it is reasonable to analyze the odor-response of ORNs by means of counting all firing spike trains from every channel respectively, and then to integrate all these information together. According to classification algorithm based on the spike threshold (Ye et al., 2008), we selected all sites having response signals from 60 recording channels during the pulse period of 45 s from 400 s to 445 s (Fig. 3). We found that not all of the

same site could be excited by the two different odors. There were 12 channels with firing spikes for LIM and 30 channels for ISO, from which 4 channels of channel 8, 11, 12 and 55 were overlapped for both two odorants. These results basically tally with the conclusion of the ORN biochemistry studies (Malnic et al., 1999). It has been reported that there is an olfactory map existing in the olfactory neural system (Mori et al., 1999), and we found that the sites except for overlapping and no response exhibited good specificity towards these two odorants and this new ORN map from the channels of MEA could identify them in our experiments of different batches of neuralchips. Spatial resolution is one of the key advantages of MEA, so some spiking analysis methods can be used to extract and sort the different spikes from multiple neuron recording. By reorganizing these maps using multi-dimensions classification, different odor stimuli even their concentrations can be identified hopefully.

4. Conclusion

This work is a new attempt to develop a novel neurochip based on the combination of ORNs and MEA. The odor-response of ORNs *in vitro* was successfully recorded in certain concentration ranges, and the properties such as amplitude, threshold and average firing rate were also preliminarily analyzed. It showed that the main electrophysiological features of ORNs did not change a lot even they were cultured *in vitro* and the new ORN map from MEA channels could be used to identify two different odorants in our studies.

As a biosensor, due to being closer to the real human natural response, the MEA system should has many potential advantages of more sensitive and reliable performance, faster reaction, etc. over the existing e-nose based on the chemical gas sensors. The improvement of ORN biosensor sensitivity and detection threshold may be considered in the following several aspects such as increasing the degree of dissolution of these two organic compounds in the cell culture fluid, elimination of the dissolution procedure as well as changing the density of ORNs on MEA. With respect to the comprehensive platform for the ORN study and olfaction medical/biological research, under current experiment conditions, the cultured ORNs could survival for about 30 min. The further improvement of cell culture apparatus to keep the ORN-biomediators alive for a long time can made our current MEA system more stable and reliable to obtain large quantity of encoding information of ORNs (Josephson et al., 2004; Pinato et al., 2008), which is very meaningful in the research of olfactory neuroscience. Beside this, our system can also be used as neural recording and stimulation devices for general multi-channel neural electrophysiological research, and is low-cost compared with lots of current equipment such as patch clamp. Whether from the view of ORN biosensor or olfactory research platform, these two aspects are bound tightly, and we think that only through a controllable method to get a simple and observable olfactory network and obtain its corresponding electrophysiological signals can we get to know what happen among these cells, which is essential to form a controllable and perceptible ORN biosensor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.08.071.

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