

Olfactory epithelium biosensor: odor discrimination of receptor neurons from a bio-hybrid sensing system

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Abstract Bio-hybrid systems provide an opportunity for integrating a living bio-active unit and a proper biosensing system, to employ the unique properties of the bio-active unit. The biological olfactory system can sense and identify thousands of trace odors. The purpose of this study is to combine olfactory epithelium with microelectrode array (MEA) to establish an olfactory epithelium-MEA hybrid system to record the odor-induced electrophysiological activities of the tissue. In our experiments, extracellular potential of olfactory receptor neurons in intact epithelium were measured in the presence of ethyl ether, acetic acid, butanedione, and acetone, respectively. After the odor-induced response signals were analyzed in the time and frequency domain, the temporal characteristics of response signals were extracted. We found that olfactory epithelium-MEA hybrid system can reflect the *in vitro* odor information of different signal characteristics and firing modes *in vitro*. The bio-hybrid sensing system can represent a useful instrument to sense and detect the odorant molecules with well recognizing patterns. With the development of sensor technology, bio-hybrid systems will represent emerging and

promising platforms for wide applications, ranging from health care to environmental monitoring.

Keywords Olfactory epithelium · Microelectrode array (MEA) · Bio-hybrid sensor · Cell-based biosensor · Receptor neuron

1 Introduction

In most vertebrates, the mechanism of signal detection and transduction in olfaction is an electrophysiological process. The olfactory initial sensing occurs in the receptor neurons of olfactory epithelium, where odorants interact with the olfactory receptors specifically in the ciliary membrane of the receptor neurons, initiating the olfactory signal transduction cascade and contributing to depolarization (Firestein 2001; Buck 2004). Action potentials evoked by odor-induced depolarization propagate through the axons of receptor cells to the olfactory bulb, where odorant information is further processed and transmitted to the brain, and the smell information forms in the brain ultimately.

Odorants commonly contain a series of functional chemical groups and some of them serve as ligands to combine with the specific receptors. The interactions between the odorant molecules and olfactory receptors exert combinatorial effects which are specific in the epithelium (Nef et al. 1992; Lledo et al. 2005). Therefore, biological olfactory system has high sensitivity and specificity to perceive and discriminate a large number of odors in the environment. Envisioning high-potential applications in a wide range of fields, ranging from environmental monitoring to medical diagnosis, researchers recently investigated and designed different electronic noses, depending on absorbability or catalysis properties of sensitive materials for specific odors (Gopel et al. 1998; Rock et al. 2008; Glatz and Bailey-Hill 2011). However, these odor-sensitive materials are far from

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matching the performances of natural olfaction system, which owns olfactory receptor neurons with intact structure and function.

Recent progress in cell culture and micro-fabrication technologies has contributed to the development of the bio-hybrid systems for the functional characterization and detection of drugs, pathogens, toxicants, and odorants (Rudolph and Reasor 2001; Wang and Liu 2009). Living cells have the potential to serve as sensors, naturally integrating the response to stimuli to generate predictions about cell fate (Park and Shuler 2003; Albrecht et al. 2005; Ho et al. 2006). Compared to sensitive materials of traditional electronic nose, bio-active units extracted from primary sources and cultured *in vitro*, have higher sensitivity, better selectivity, and faster response (Marrakchi et al. 2007; Ko and Park 2005; Lee et al. 2009). Furthermore, the extracellular activities related to cellular functions can be directly detected by microelectronic sensor chips. In our previous study (Liu et al. 2006; Liu et al. 2010), we have explored the olfactory cells as sensing elements for bioelectronic nose, and managed to establish the bioelectronic nose by combining the microelectrode array (MEA) with intact olfactory epithelium, which was extracted from a primary olfactory system, keeping its structural and functional integrity. In this study, olfactory epithelium and MEA hybrid system are used to investigate the possibility of detecting and discriminating real-time extracellular signals in the presence of odor stimulations, taking advantage of high sensitivity and selectivity of olfactory epithelium.

The olfactory epithelium is a specialized epithelial tissue that is directly involved in smell. It includes three main cell types: olfactory receptor neurons, supporting cells and basal cells (Fig. 1). The olfactory receptor neurons are the main sensing cells whose axons penetrate into the central nervous system. They contain bio-active units which determine

olfactory sensory transduction. All of the transduction molecules are located in the thin cilia of the olfactory neurons (Buck 2004; Bear et al. 2007). In our study, we used MEA to record odor response signals of the olfactory epithelium. The electrophysiological activities of olfactory receptor neurons can be recorded in the form of transmembrane potential with MEA. Thus, the specific response of olfactory epithelium can be detected and analyzed when the membrane depolarization is triggered due to the interaction between odorants and cilia.

The present study aims at evaluating the performance of this bio-hybrid system concerning odor discrimination. After odor-induced stimulation, MEA was used to record the electrophysiological activity of the olfactory receptor neurons in the epithelium and the extracellular potentials were consequently analyzed. We also extracted and analyzed the characteristic response signals, thus achieving an effective odor discrimination.

2 Materials and methods

2.1 Fabrication of the microelectrode array

MEA is a utility multichannel sensing device that can simultaneously record a multisite tissue electrical activity, so it is widely applied to detect tissue electrophysiological signals and to study their firing mechanisms (Hierlemann et al. 2011; Frega et al. 2011; Daus et al. 2012). The fabrication and preparation procedures for MEA were similar to those described in our previous works (Liu et al. 2010).

Briefly, a layer of Ti (30 nm) was deposited onto the glass substrate for 30 min to enhance adhesion of the Au layer (300 nm). Then, the photoresist was spin-coated onto the metallic layer and exposed to ultraviolet light under the mask with defined electrode layout. As a result, the metal without protection was removed, while the electrodes and interconnections were left. Subsequently, the Si_3N_4 layer (500 nm thick) for electric isolation of interconnections was deposited onto the chip by plasma enhanced chemical vapor deposition (PECVD) process. Finally, the electrode pattern and external contact pads were formed by wet etching in 3–5 % HF solution. A micrograph of MEA with a 6×6 array pattern is shown in Fig. 2a. The electrode diameter is 30 μm with a 200 μm center-to-center spacing, which permits to avoid electric interferences between neighboring electrodes.

In addition, the microelectrode was electrodeposited by platinum black to improve the signal-noise-ratio of the chip. It was electrodeposited at a constant voltage of 0.1 V for 25 s (EG&G Princeton Applied Research, M273A) with electrodes immersed in the petri dish solution medium

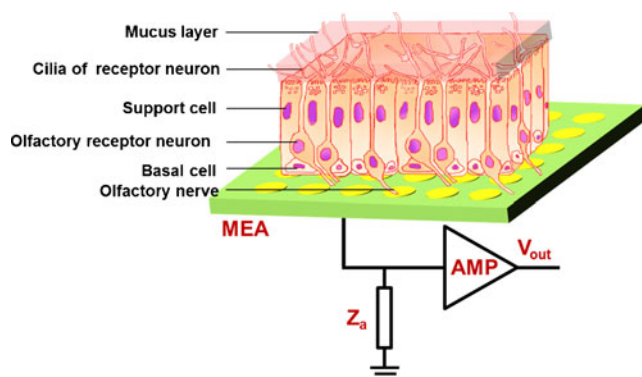


Fig. 1 The olfactory epithelium-MEA hybrid system. Odor-induced extracellular potentials of olfactory receptor neurons in intact epithelium can be recorded by the hybrid system. Z_a and AMP stand for resistance and amplifier which are components of the amplifying circuit in the USB-ME16-FAI system. V_{out} represents the amplified signal transmitted to computer

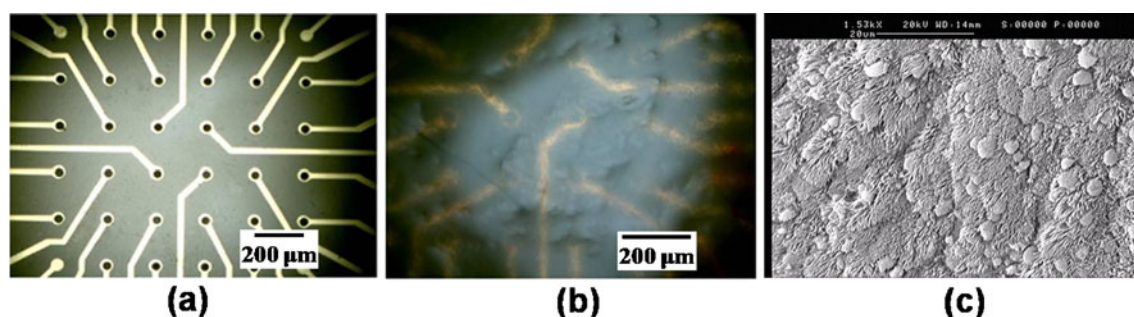


Fig. 2 Olfactory epithelium on MEA. **(a)** MEA pattern. **(b)** Microelectrodes distribution under olfactory epithelium. **(c)** Expanded cilia of the olfactory epithelium under SEM

(1 % chloroplatinic and 0.01 % acetate). Before tissue experiments, MEA was rinsed by deionized water and sterilized with ethanol.

2.2 Isolation and fixation of olfactory epithelium

Sprague Dawley rats, with weight about 250 g, were euthanized with intraperitoneal injections of Equithesin (a mixture of chloral hydrate, magnesium sulfate and pentobarbital sodium, 10 ml/kg). The head was hemisected in a midsagittal plane with the blade passing between the septum and the lateral wall. The olfactory epithelium covering on the septum was removed from underlying cartilage and bone carefully.

The isolated epithelium (about 5 mm×5 mm) was rinsed with Ringer's solution and placed with cilia receptors side up on the sensor surface (Fig. 2b). By means of scanning electron microscope (SEM, SU-70, Hitachi) observation, we found that olfactory cilia formed a dense meshwork expanding naturally on the olfactory epithelium (Fig. 2c). After rinsing, the solution was removed from the MEA petri dish and the tissue was fixed by a plastic ring-shaped frame covered with a tightly stretched piece of mesh. The olfactory epithelium was kept in standard perfusate containing (in mmol/L): 100 NaCl, 5 KCl, 3 MgSO₄, 1.8 CaCl₂, 25 NaHCO₃, 25 Glucose, and bubbled with 5 % CO₂ with a pH of 7.3±0.1. The olfactory epithelium was then allowed to sit for 5–8 min when it tightly coupled with the sensor before undergoing odor stimulations.

2.3 Odor stimulation

Odorants of ethyl ether, acetone, acetic acid, and butanedione (Sigma-Aldrich, America), were diluted to 10 μM by standard perfusate as stimulus, respectively. In our previous studies, the odorants of acetic acid and butanedione have already been used as odor stimuli to the olfactory epithelium and recorded the odor response signals (Liu et al. 2006; Liu et al. 2010). Acetic acid is an organic acid with a distinctive pungent odor, which is the main component of vinegar of

daily condiment. Butanedione is a natural byproduct of fermentation, occurring naturally in alcoholic beverages and is added to some food to impart a buttery flavor. In this study, ethyl ether and acetone were added as new stimuli, in order to study the discrimination of odors by olfactory epithelium. Ethyl ether is a highly volatile, flammable liquid with a characteristic odor, which has certain narcotic effect on mammals. Acetone is produced and disposed of in the human body through normal metabolic processes, which has potential to cause reproductive problems.

The experiments were based on the following steps. First, electrophysiological activity of olfactory epithelium was recorded for 5 min in absence of odors. Then one odorant perfusate was injected into the Petri dish by a peristaltic pump and a selection valve, recording the signal for 5 min. After achieving the odorant response, it was washed out by standard perfusate for three times. Subsequently, another odorant was injected. The experimental environment and odorants perfusate were controlled by a environmental control system and a auto-injection system. The whole recording system was placed in a shielding box to avoid external electromagnetic interference.

2.4 Signals acquisition and analysis

The USB-ME16-FAI (MCS, Reutlingen, Germany) system was used to record signals in real time. The odor response signals of olfactory epithelium were recorded by the signal acquisition system with a gain of 1200 and noise of 20 μV. A MC-RACK software (MCS, Reutlingen, Germany) was used to display signals in real time with a sampling rate of 20 kHz.

The signals were analyzed by relevant signal processing methods performed by MATLAB (Mathworks, Inc.). Data were shown as mean ± standard deviation of n samples. Mean values were statistically compared by applying the Student's *t*-test. Differences were considered statistically significant when $P < 0.05$. In time-frequency domain, the visualized analyses of sonogram spectrum and 3-D recognition pattern, were applied to reveal the temporal characteristics of the signals for odor discrimination.

3 Results and discussion

3.1 Olfactory epithelium odor discrimination ability

In our experiments, the odor response signals of olfactory epithelium were recorded in the presence of odorants, and signals of olfactory epithelium in the absence of odorants were used as controls. As shown in Fig. 3 the odorants of ethyl ether, acetic acid, butanedione, and acetone, were added respectively. The preprocessed signals of native state and in-stimulation state are listed in the Figure. Compared to those characteristic of the control group, signals after odor-induced stimulation show evident evoked potentials, with difference in firing rate and amplitude. Meanwhile, the single waveform extracted from those different signals, respectively reflects special properties of responses to different stimuli.

After the stimulations of individual odors, we tried to stimulate the tissue with mixed odor stimuli. When the mixture included two types of odors, different odor-specific response signals were recognized. Figure 4 shows the results obtained with a mixture of acetone and butanedione. The recorded potentials after the stimulation presented a regular signal response pattern. Although there is a similar functional group of $-C=O$ in acetone and butanedione, two patterns of signal responses can be recognized, with the big spikes similar to the typical waveform of acetone and the smaller ones similar to those of butanedione. Therefore, the olfactory epithelium-MEA hybrid system was able to discriminate odors even with similar structure and functional groups. However, mixtures of more odors could not be recognized effectively. The single waveform could not be successfully extracted from the chaotic signals obtained by mixing three or four odor types. It still

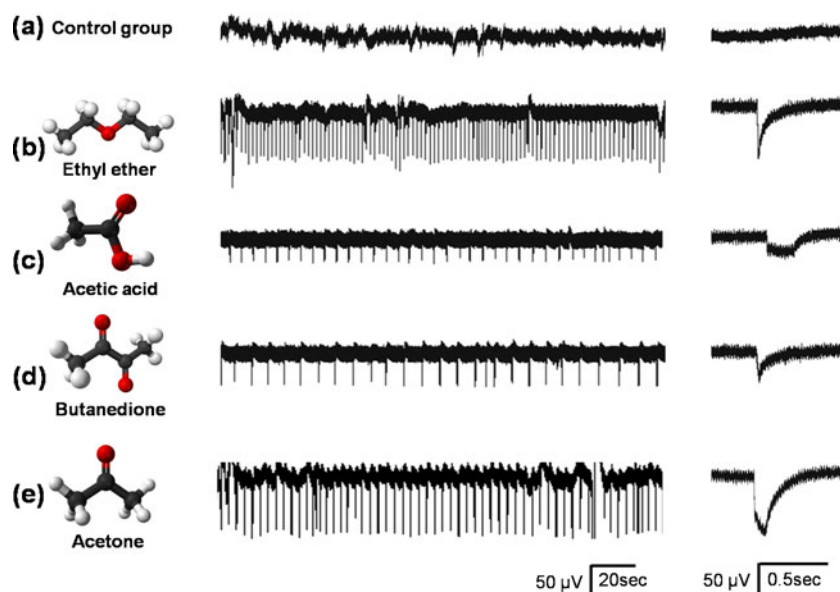
needs to be further investigated whether the system enables to discriminate more than two mixed odors.

Olfaction is initiated by molecular interaction of odorants with the olfactory receptors in the epithelium. The molecular structure and the spatial molecular arrangement of interacting groups are very important for the olfactory sensing (Buck and Axel 1991; Meierhenrich et al. 2004). Olfactory biosensors using living cells expressing special olfactory receptors could even simultaneously distinguish different types of chemicals that differ in double bond isomerisms or functional groups (Misawa et al. 2010). Microelectrode recordings show that many receptor neurons respond to odorants: these cells are distributed across a wide area of the olfactory epithelium itself. The recording of odor-induced signals opens new possibilities for the investigation of the mechanisms underlying the interaction between odorant molecules and olfactory receptors in intact epithelium.

3.2 Olfactory epithelium-deriving signal analysis

The basic characteristics of signal waveforms, such as amplitude, duration and firing rate, represent the response intensity of different stimuli. To further discuss the signals responses to four stimuli, we calculated and plotted the comparison diagram. Figure 5 shows the normalized result of basic characteristics of potentials in our experiment. It was evident that the amplitude and duration of signal waveforms vary depending on the odorant category. The response signals of acetic acid and butanedione had a low amplitude ($36.18 \pm 0.383 \mu V$ and $49.68 \pm 0.3899 \mu V$, respectively), while the amplitude for ethyl ether and acetone was $78.94 \pm 0.6165 \mu V$ and $82.93 \pm 1.233 \mu V$, respectively. In contrast, the duration of acetic acid (687.5 ± 12.5 ms) was higher than that of ethyl ether, acetone and butanedione (582.4 ± 3.784 ms,

Fig. 3 Recording of electrophysiological signal in control conditions (a) and after odor stimulation: ethyl ether (b), acetic acid (c), butanedione (d), and acetone (e). Molecular structures of odorants and corresponding typical signal waveforms are listed respectively



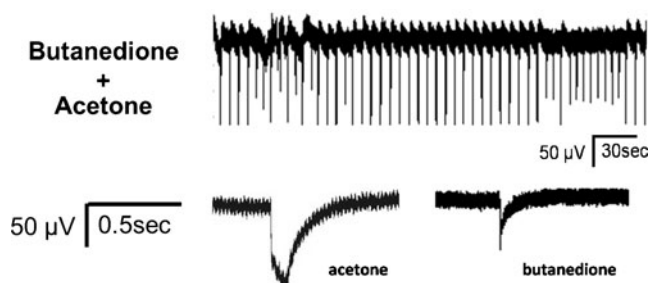


Fig. 4 Response signals to the mixture odor stimuli of butanedione and acetone

674.2 ± 9.967 ms, and 661.2 ± 5.044 ms, respectively). The different stimuli can be classified by calculating the extracting parameters of signal waveform.

The firing rates of olfactory epithelium response to odors were significantly different. Frequency spectrum analysis of olfactory neurons response to odors has been often used to calculate the distribution of frequency band during odor presentation (Lowry and Kay 2007; Ito et al. 2006). The probability density distribution of response signals firing rate is reported in Fig. 6. The epithelium response signals show clear characteristic peaks to different odors. Corresponding to ethyl ether, a characteristic distribution peak was found at a high frequency (0.5 Hz), while in presence of butanedione the peak was located at a lower frequency (0.15 Hz). The frequency peaks of acetic acid and acetone were found at 0.2 Hz and 0.3 Hz, respectively. The specific peaks in the frequency distribution reflected the periodic activities in olfactory system, which may indicate certain odor-dependent firing modes. Probability density

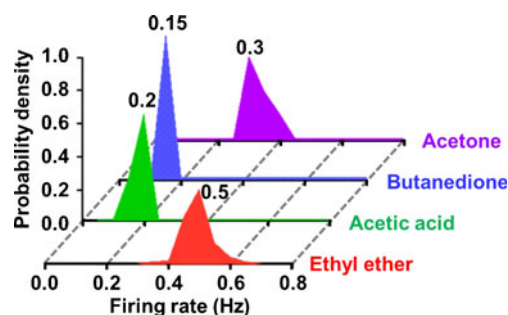


Fig. 6 The probability density distribution of response signals firing rate of olfactory epithelium biosensor exposed to the different odors

distribution of response signals can reflect the firing rate distribution under the odor stimulation. The peak of the distribution was the feature firing rate of each odor response signal.

The different firing rates corresponding to the different odorants might indicate certain coding of olfactory cell population. The statistical results indicated that the individual olfactory receptor neuron response to odors presents specific firing rates, which were summed by the electrical current flowing from nearby olfactory receptor neurons within a region of olfactory epithelium. Thus, combined with the statistics analysis, the odor discrimination can be carried out by the pattern recognition.

3.3 Odor discrimination of olfactory epithelium biosensor

In the study of traditional electronic nose, many typical pattern recognition methods have been used to achieve odors classification (Gopel et al. 1998; Rock et al. 2008). In order to investigate the features of the olfactory epithelium biosensor, we applied signal waveform features to discriminate odor responses by means of analyses both in the frequency and in the time domains. More specifically, sonogram spectrum visualization methods and 3-D recognition patterns were used to analyze the spatio-temporal signal characteristics.

To derive more information from different odors, we calculated the time-frequency distribution by a color-coded method of sonogram. The visualized time-frequency distributions of our recorded typical waveforms in the presence of ethyl ether, acetic acid, acetone, and butanedione, are shown in Fig. 7. The response waveforms of olfactory have evident time-frequency distribution features. Under the stimulation of ethyl ether, the frequency peak emerged with the spike peak, showing a broad frequency band ranging from 0 to about 30 Hz, while in presence of the other odors, the main frequency peak was centralized to the lower frequency band ranging from 0 to 10 Hz. In the whole frequency distribution, there were different response patterns to different odors. Furthermore, the time distributions of the spikes were

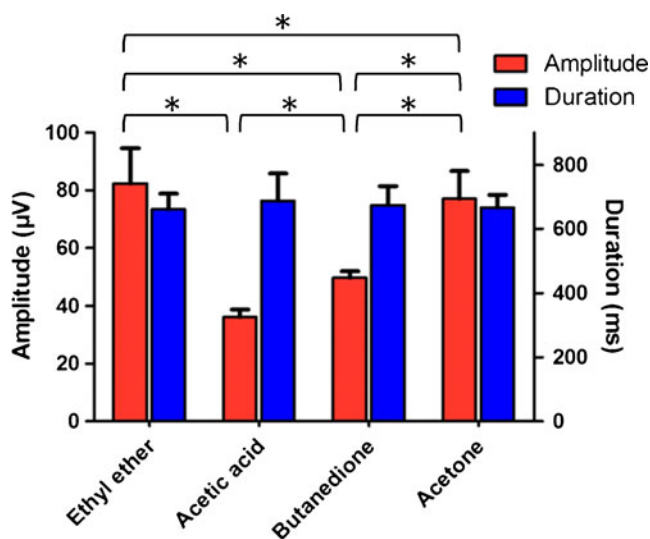


Fig. 5 Response of olfactory epithelium to different odor stimuli. The amplitude and duration were extracted from each signal waveform. Each column represents the mean value $\pm$ S.D. of the response signals ($n \geq 20$). * indicates a statistically significant difference ($p < 0.05$)

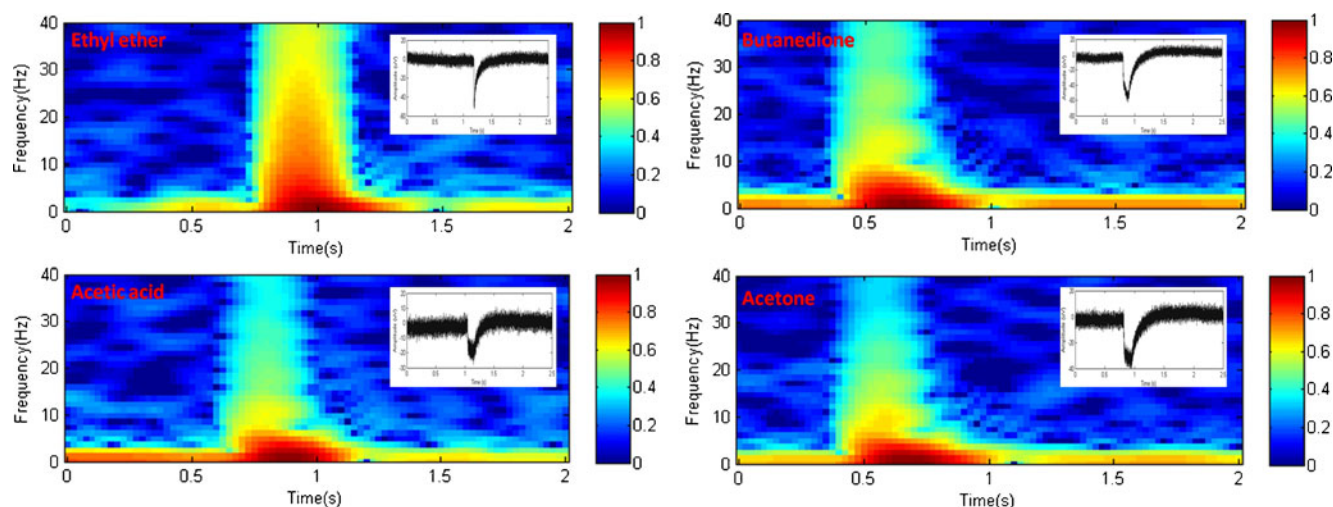


Fig. 7 Sonogram spectrum analysis for different odors. In the upper-right corner of each picture, single waveform with duration of 2 s are represented. The color bar from blue to red represents the normalized magnitude of frequency, ranging from 0 to 1

also displayed in the color maps, with different time-frequency responses to different odors.

At the same time, temporal characteristics of the single waveforms were extracted to analyze odor discrimination in 3-D space. Figure 8 was 3-D pattern sensed by the olfactory epithelium biosensor to different odors. According to characteristics in terms of amplitude, duration and firing rate, the response signals were clustered into several regions. Moreover, the signals were located in four main regions under stimulation of odors, which contained the similar signal features. The 3-D pattern reflects the distribution of the odor response signals, including characteristics of the odor information. Maps of the regions activated by one of chemical stimuli can be visualized with special pattern recognition. Experiments may reveal that many discrete neurons are activated, and the olfactory system can reflect the odorant characteristics. Thus, the smell to a particular chemical can be converted into a specific pattern by deriving the characteristics of the odorants.

Olfactory pattern classification can visualize discrete neuronal network states, which can be a utility method in the future investigation (Niessing and Friedrich 2010). The analysis in time and frequency domains indicated that the olfactory sensor can response specifically to odor stimuli. Compared to the biosensor in our previous investigations (Liu et al. 2010), the research pays more attention on basic characteristics of waveforms, with the odors discriminated with classification. The basic temporal characteristics and time-frequency information of signals in their typical waveforms can reveal the basic olfactory perception patterns and realize visual discriminations of response signal to different olfactory stimuli. These spatio-temporal analyses can provide useful support with pattern recognition for a practical bio-hybrid olfactory system in the future.

Furthermore, the olfactory epithelium can be easily exposed, dissected, and cultured, to keep its native state *in vitro*, with suitable temperature, humidity, and nutrient medium. Considering the long-term survival of olfactory epithelium (Josephson et al. 2004; Nickell et al. 2007), the olfactory epithelium is suitable as sensitive elements to develop a bio-hybrid system which will have high performance in odor detection, comparing to other cell or tissue. It must be pointed out that the odorant-induced cytotoxicity intrinsically reduces the odor sensing ability, as well as sensor stability and repeatability. To achieve practical applications, further investigations are thus needed. Then, with good characteristics of MEA in stability and repetition, the

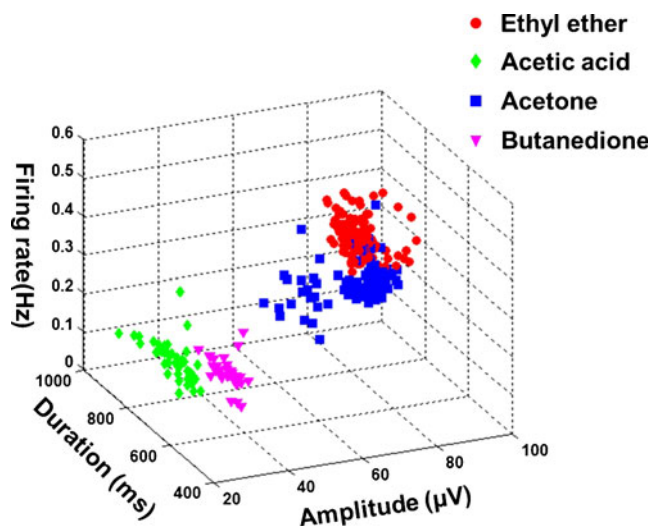


Fig. 8 3-D recognition pattern sensed by olfactory epithelium biosensor under the stimulation of ethyl ether, acetic acid, acetone, butanediol. The amplitude, duration and firing rate were derived from original signals, respectively

bio-hybrid system provides a reliable and fast platform to odor detection, and the intact epithelium studies would potentially bridge the gap between conventional *in vitro* methods and complex *in vivo* experiments for olfactory mechanisms.

4 Conclusion

In this study, we achieved a bio-hybrid olfactory system by employing the olfactory receptor neuron in intact olfactory epithelium as the sensitive element to sense the odors. This bionic olfactory system was suitable for detecting and discriminating the odorants specifically binding to olfactory neurons. The characteristics of odor response signals were analyzed by signal sorting, sonogram, and 3-D odor recognition pattern. This living bio-hybrid system represents a promising tool for detecting and discriminating various odors in the field of food safety, environmental protection and health care.

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