Chapter 16

A Light-Addressable Potentiometric Sensor for Odorant Detection Using Single Bioengineered Olfactory Sensory Neurons as Sensing Element

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Abstract

A light-addressable potentiometric sensor (LAPS), a silicon-based surface potential detector, is combined with bioengineered olfactory sensory neurons (OSN) for odorant detection. A LAPS chip is used as a transducer to monitor cell membrane potential changes. In addition, a focused movable laser with a diameter comparable to cell sizes is employed to select the desirable single cell for measurement under a microscope. Bioengineered OSNs are coupled to the LAPS surface and employed as sensing elements, which are prepared by the expression of an olfactory receptor of *C. elegans*, ODR-10, on the plasma membrane of rat primary OSNs via transient transfection. The responses of bioengineered OSNs to diacetyl, isoamyl acetate, and acetic acid are monitored by extracellular recording using the LAPS chip. Features of the recorded extracellular potential firings are analyzed in frequency and time domains. We have shown that bioengineered OSNs can generate specific response signals upon the stimulation of diacetyl, which is the natural ligand of ODR-10. Moreover, different concentrations of diacetyl can elicit different temporal firing patterns in bioengineered OSNs, which permits the concentration detection of specific odorant molecules in solution.

Key words Light-addressable potentiometric sensor, Bioengineered cell, Olfactory sensory neuron, Biosensor, Odorant

1 Introduction

Since its introduction in 1998 by Hafeman et al., the light-addressable potentiometric sensor (LAPS*See* Light-addressable potentiometric sensor (LAPS)) has been widely applied in bioanalytical and chemical analysis. LAPS is a type of silicon-based field-effect device (FED) for surface potential detection, which consists of an a electrolyte–insulator [SiO_2]–semiconductor [Si] (EIS) structure and a bias voltage applied across the structure [1, 2]. The basic principle of LAPS is based on the scanned light pulse technique towards the measurement in aqueous solutions. A

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focused and modulated light is utilized to illuminate the local area of the semiconductor to select the desirable spot for measurement. The light illumination leads to the generation of electron–hole pairs inside the semiconductor, while the bias voltage results in the separation of electron–hole pairs in the space-charge region. As a result, an alternating photocurrent is generated and can be externally measured. The photocurrent/voltage (*I/V*) curves can be obtained by plotting the photocurrent amplitude versus the applied bias voltage. Therefore, the flat-band voltage shift due to different analyte concentrations can be measured by monitoring the shift of LAPS *I/V* curves along the bias-voltage axis. In addition, as a (bio-) chemical sensor, LAPS is able to determine the analyte concentration of an aqueous solution in a spatially resolved manner. A focused movable light source is employed to define the local area as measurement spot, where a photocurrent is generated depending on the local analyte concentration. This makes it possible to achieve a spatially resolved map of the distribution of analyte concentration on sensor surface. The resolution of the map is determined by the size of focused light and the lateral diffusion of carriers inside the semiconductor. LAPS is thus able to generate chemical images based on the analyte-concentration distribution.

LAPS has also become popular in many cell-based applications due to advantages such as selection of the measurement point, simple structure, and spatial resolution sufficient for measurement on individual cells [3, 4]. This greatly facilitates the coupling of cells with the LAPS chip because there no need to culture cells on discrete active sites such as the gate-electrode of individual field-effect transistors (FETs)*See* Field-effect transistors (FETs) or the tip of individual microelectrodes. Figure 1 shows the basic mechanism

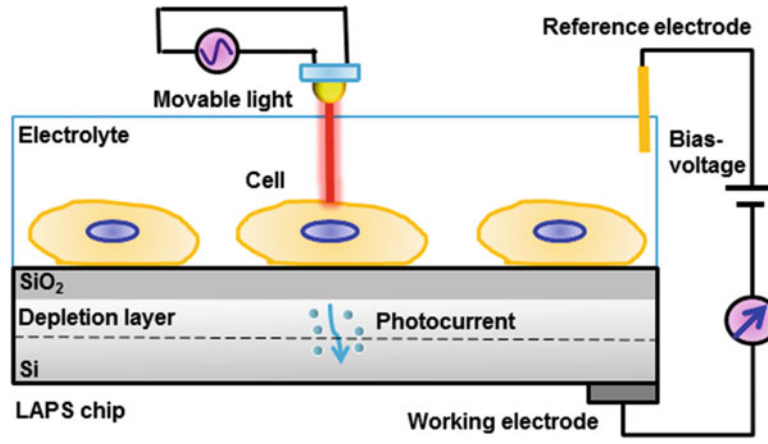

Fig. 1 Schematic diagram showing the structure of LAPS and its mechanism of extracellular recording of cell membrane potential changes from a single cell via a movable light for selection of the desired single cell

of a LAPS chip comprising an EISstructure for the detection of cell membrane potential changes from single individual cells. The LAPS is thus a surface-potential measuring device capable of detecting changes of extracellular potential of cells coupled to the chip surface. The LAPS measurement system uses a movable focused light with a diameter comparable to the sizes of cells to choose the desired single cell for measurement. The focused light also illuminates the local LAPS chip under the single cell being measured. As a result, the local semiconductor inside the LAPS chip can absorb light energy and leads to the generation of electron–hole pairs. If a DC bias voltage is applied to the LAPS chip via a reference electrode so that a depletion layer appears at the insulator/semiconductor interface, rapid recombination of electron and hole and is avoided. This allows generation of a detectable photocurrent through the peripheral circuit. Therefore the AC photocurrent readout by the peripheral circuit is related to the local value of the surface potential, which is in turn influenced by the cell membrane potentials coupled to the local LAPS surface through light illumination. The AC photocurrent will also generate a corresponding fluctuation if the cell membrane potential changes. Based on this mechanism, LAPS has been utilized for the monitoring of membrane potential changes of single cells such as primary olfactory sensory neurons (OSNs)*see* Olfactory sensory neurons (OSNs), where the effects of the specific enhancer and inhibitor on olfactory signals have been successfully recorded in a noninvasive manner [5].

Primary OSNs*see* Primary OSNs have great potential to improve the performance of biosensors for odorant detection in complex environments due to their distinct chemical sensing capabilities. However, the use of primary OSNs as sensing elements for odorant detection has innate limitations that hamper their further development and practical applications. One of the main limitations originates from the unknown type of olfactory receptors in desired OSNs and the corresponding (unknown) olfactory encoding mechanisms; this makes it too complicated and difficult to process and interpret the recorded response signals and use them to discriminate different odorants [6]. Therefore it is essential and desirable to obtain functional OSNs having well-defined olfactory receptors. To accomplish this we introduce a simplified and novel approach for the development of cell-based biosensors for odorant detection using bioengineered primary OSNs as sensing elements and LAPS as transducers [5]. This approach involves four major steps: (1) culture of primary OSNs isolated from the rat olfactory epithelium on the LAPS surface, (2) expression of a well-defined olfactory receptor protein (an olfactory receptor of *C. elegances*, ODR-10) on the plasma membrane of primary OSNs via transient transfection, (3) stimulation of bioengineered OSNs and monitoring the cell membrane potential changes by LAPS extracellular recording, and (4) processing and analysis of recorded responsive

signals in frequency and time domains. Bioengineered OSNs contain not only molecular components necessary for olfactory signal transduction, but also well-defined olfactory receptors. This makes it possible to transduce specific olfactory signals into membrane potential changes. Based on this approach we are able to specifically detect target odorant molecules with high specificity in a concentration-dependent manner.

2 Materials

2.1 LAPS Measurement Setup

1. Silicon wafer (<100>, n-type, resistivity 10–15 Ωcm, 500 μm thick, Sigma-Aldrich Inc., St. Louis, MO).
2. He–Ne semiconductor laser (Coherent Inc., Santa Clara, CA).
3. Chopper (C-995, Terahertz Technologies Inc., Oriskany, NY).
4. Nikon stereoscopic zoom microscope (SMZ1500, Nikon Inc., Kanagawa, Japan).
5. Microscope objective lens (numerical aperture: 0.4, Jiangnan Optic Instrument Co., Nanjing China).
6. Potentiostat (EG&G M273A, Princeton Applied Research, Oak Ridge, TN).
7. Peristaltic pump (BT00-50 M, Longer Inc., Baoding, China).
8. Data-acquisition card and LabVIEW software (DAQmx PCI-6259, National Instruments, Austin, TX).
9. MATLAB software (MathWorks, Inc., Natick, MA)
10. Lock-in amplifier (MODEL SR830 DSP, Stanford Research Systems, Sunnyvale, CA).
11. Reference electrode (Ag/AgCl, 3 M KCl, Metrohm, Herisau, Switzerland).
12. Counter electrode (platinum wire with diameter of 0.1 mm, Changhong Precious Metal Inc. Changshu, China).Rapid Thermal Processing (HG19-RTP-500, Bexin Inc., Beijing, China).
13. Gaertner ellipsometer (L116C, Gaertner Scientific Corporation, Illinois, USA).
14. MULTINANO3–300 (G&N GmBH, Erlangen, Germany).
15. Denton Vacuum Explorer 14 (Denton Vacuum, LLC, Moorestown, USA).
16. Wafer dicing saw machine (ZSH506, Shenyang Academy of Instrumentation Science, Shenyang, China).
17. Epoxy (2 ton Clear Epoxy 14,260, Devcon Inc., Danvers, USA).

18. Conductive glue (HX5849, Shenyang Air Rubber & Plastics Products Industry, Shenyang, China).
19. High-pass filter (home-made, working frequency: 1 M).

2.2 Cell Culture and Transfection

1. Sprague-Dawley (SD) rats (3-day old, Laboratory Animal Center of Zhejiang University, Hangzhou, China).
2. Trypsin solution (0.25% (w/v), pH 7.4~7.8, Sigma-Aldrich Inc., St. Louis, MO).
3. Ringer's solution (6.5 g/L. NaCl, 0.42 g/L. KCl, 0.25 g/L. $CaCl_2$, 0.2 g/L. sodium bicarbonate, pH 7, Sigma-Aldrich Inc., St. Louis, MO).
4. Cell culture medium (Dulbecco's modified Eager's medium (DMEM) with 10% fetal bovine serum (FBS), Sigma-Aldrich Inc., St. Louis, MO).
5. Plasmid containing full-length cDNA of *odr-10* (*pBluescript SK⁻/odr-10*, provided by Professor Cornelia I. Bargmann at the Laboratory of Neural Circuits and Behavior, Howard Hughes Medical Institute, The Rockefeller University, NY).
6. Primers (forward: 5′-GAGTT GGAAT TCATG TCGGG AGAAT TGTGG-3′; reverse: 5′-CAGTA AGGAT CCCGC GTCGG AACTT GAGAC AAATT-3′, Takara Biotechnology (Dalian) Co., Dalian, China).
7. The *rho-tag* import sequence (5′-GTCGC ACTCG AGATG AACGG GACCG AGGGC CCAAA CTTCT ACGTG CCTTT CTCCA ACAAG ACGGG CGTGG TGGAA TTCGC TGGA-3′, Takara Biotechnology (Dalian) Co., Dalian, China).
8. Mammalian expression vector (*pFGFP-N1*, Clontech Inc., Palo Alto, CA).
9. Polymerase (Pyrobest DNA polymerase), dNTPs, PCR buffer solution, and restriction enzymes (*XhoI*, *EcoRI*, and *BamHI*) (Takara Biotechnology (Dalian) Co., Dalian, China).
10. Lipofectamine 2000 (Gibco-BRL/Invitrogen, Carlsbad, CA).

2.3 Odorant Stimulation

1. Phosphate buffer solution (PBS) (135 mM NaCl, 4.7 mM KCl, 10 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4, Beyotime Inc., Nantong, China).
2. Diacetyl (Sigma-Aldrich Inc., St. Louis, MO) is dissolved in PBS (pH 7.4) at a series of concentrations (0.1 μM, 1 μM, 10 μM, 50 μM, and 100 μM) and stored in single-use aliquots at −20 °C. Isoamyl acetate and acetic acid (Sigma-Aldrich Inc., St. Louis, MO) are dissolved in PBS (pH 7.4) at 50 μM and stored in single-use aliquots at −20 °C. All the stimulation solutions are warmed to 37 °C before use.

3 Methods

3.1 Primary OSNs Isolation and Culture

1. Noses of SD rats are dissected after decollation. The intact olfactory epithelium inside the nose is gently peeled away from the underlying bone.
2. The entire intact olfactory epithelium is incubated with trypsin solution for 20 min. After incubation, the olfactory epithelium is subjected to gentle rinsing in the Ringer's solution for 5 min using a pipette in order to dissociate cells from olfactory epithelium. (*see* **Note 1**).
3. Dissociated cells are suspended in freshly prepared cell culture medium after filtration with a sieve of 200 holes per inch.
4. Solutions containing suspended cells are introduced to the surface of LAPS chip by placing them in the detection chamber for 12 h culture at 37 °C with humidified air containing 5% CO_2, which allows for the attachment of cells onto the surface of the LAPS chip.

3.2 Expression Vector Construction and Transfection

1. Full-length cDNA of *odr-10* is amplified from *pBluescript SK⁻/odr-10* by PCR in a 50 μL reaction volume containing 100 ng of *pBluescript SK⁻/odr-10*, 0.2 μg of each primer, 25 mM dNTP, 2.5 U polymerase under the 30 thermal cycles of 94 °C denaturation for 30 s, 55 °C annealing for 1 min, 72 °C extension for 1 min, and finally with a 72 °C extension for 10 min [7, 8].
2. The *rho-tag* import sequence is inserted into multiple cloning sites (MCS) of *pFGFP-N1* between sites *XhoI* and *EcoRI* to construct *pFGFP-N1/rho-tag*.
3. Full-length cDNA of *odr-10* is subclone into the MCS of *pFGFP-N1/rho-tag* between *EcoRI* and *BamHI* of MCS to construct *pFGFP-N1/rho-tag/odr-10*.
4. The constructed vector *pEGFP-N1/rho-tag/odr-10* is sequenced to validate the correction of final sequences.
5. After confirmation by sequencing, the constructed vector *pEGFP-N1/rho-tag/odr-10* is use to transfect cells isolated from rat olfactory epithelium and cultured on the surface of LAPS chip by 6 h incubation at 37 °C with 1 mL mixture solution containing 40 μL Lipofectamine 2000 and 16 μg of constructed vector *pEGFP-N1/rho-tag/odr-10*.

3.3 LAPS Chip Fabrication

1. LAPS chip is fabricated on the basis of n-type silicon wafer (<100>, 10–15 Ωcm) using conventional microfabrication process.
2. A layer of 30 nm SiO_2 is formed on the surface of silicon wafer by thermal oxidization at 1000 °C for 5 min using Rapid

Thermal Processing. The thickness of SiO_2 layer is measured by a Gaertner ellipsometer.

3. Then the silicon wafer is ground to 100 μm in thickness from the rear side of the wafer automatically by wafer-rotating grinding (MULTINANO3–300).
4. Next a 300 nm Al layer is deposited by magnetron sputtering (the thickness is controlled by quartz crystal deposition controller inside Denton Vacuum Explorer 14) onto the rear side of the wafer to create an Ohmic contact and used as the working electrode.
5. Finally, the wafer is cut into separate chips with sizes of 1.0 × 1.0 cm using a Wafer dicing saw machine.
6. For cell culture and measurement, separate LAPS chip is fixed on the bottom of a micro-chamber using Epoxy and integrated with a copper base using conductive glue. Figure 2a is an imaging of LAPS chip integrated with a cell culture micro-chamber. Figure 2b shows the typical bias potential–photocurrent curve for a LAPS chip fabricated from n-type silicon wafer.

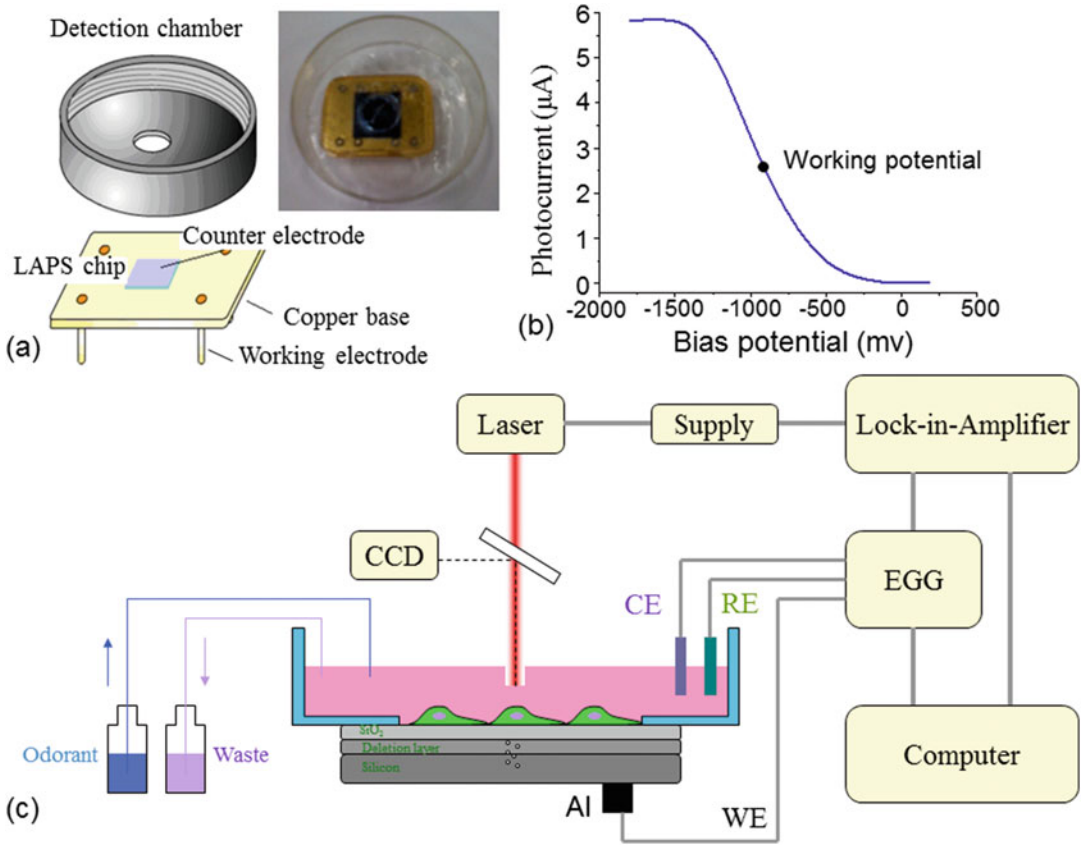

Fig. 2 (**a**) Imaging of LAPS chip integrated with a cell culture micro-chamber. (**b**) The typical n-type LAPS bias potential–photocurrent curve. (**c**) Schematic diagram of LAPS measurement setup for single cell monitoring. (Reproduced with permission from ref. 5. Copyright 2013 Elsevier)

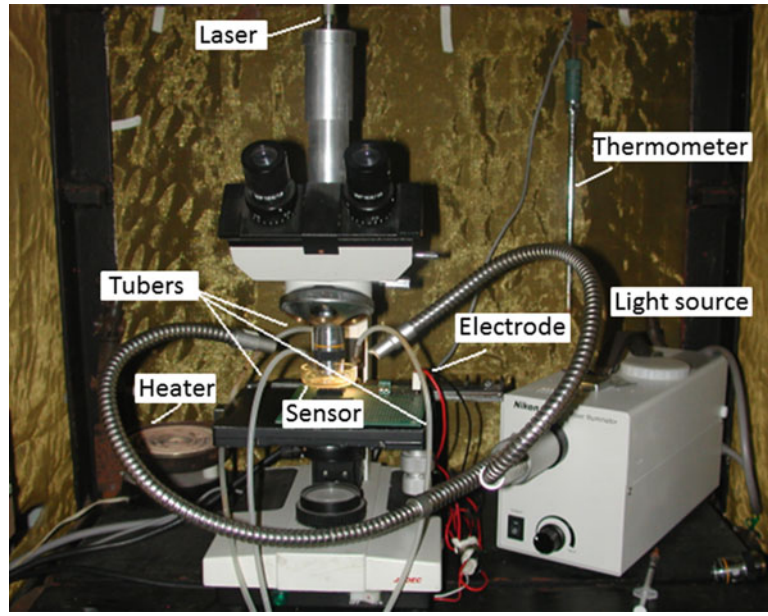

Fig. 3 Photo of measurement setup shield inside a Faraday box including the microscope, the movable laser, the sensor with detection chamber, the thermometer and heater. (Modified from ref. 9)

3.4 LAPS Measurement Setup

1. Figure 2c shows the LAPS measurement setup for extracellular recording from single individual OSNs. During measurements, LAPS chips with cultured OSNs are mounted under a microscope object lens. Figure 3 shows the integration of the microscope, the movable laser, and the sensor with detection chamber, all of which are shielded with a Faraday box to minimize the influence of ambient light and environmental factors on the measurements [9].
2. A He–Ne semiconductor laser with 543.5 nm wavelength and 5 mW power is modulated at a frequency of 4 kHz and focused to less than 10 μm in diameter (Illumination area: ~78.5 μm^2) by a microscope object lens (numerical aperture: 0.4), which is used as the light source to illuminate the desired single cell on the LAPS surface for measurement.
3. A platinum wire and Ag/AgCl electrode are used as the counter electrode and reference electrode, respectively. Counter electrode and reference electrode together with working electrode (rear side of LAPS chip deposited with an Al layer) are connected to the potentiostat.
4. A bias voltage of 2.0 V is applied to the LAPS chip via the reference electrode and working electrode, which is provided by the Potentiostat.

5. The LAPS photocurrent is detected by the potentiostat and recorded by the lock-in amplifier by setting the working potential at the point that LAPS is most sensitive to the surface potential changes. The cutoff frequency of low pass filter (LPF) in lock-in amplifier is set at ~200 Hz.
6. A 16 bit data-acquisition card (DAQmx PCI-6259, National Instruments, Austin, TX) is employed to collect the data at the sampling rate of 10 kHz.
7. A home-made LabVIEW procedures is employed to control the data collection process.
8. The detection chamber is sealed with a plastic cover and connected with a fluidics system with two inlet tubes and one outlet tube. Two inlet tubes are driven by two peristaltic pumps and used for the introduction of cell culture medium and odorant stimulations to the detection chamber, respectively. The pumps are controlled by their built-in software installed in a PC.

3.5 LAPS Extracellular Recording

1. For cell measurement, odorant stimulation such as diacetyl, isoamyl acetate, and acetic acid is introduced to the detection chamber using a peristaltic pump that is controlled by a personal computer.
2. Culture medium or stimulator is pumped alternatively by a peristaltic pump and passes through a degasser, a selective valve, and flows into the detection chamber. PC controls the on–off of pump and the temperature of chip. (*see* **Note 2**).
3. For the first step, PBS solution is introduced to the detection chamber for the extracellular recording of spontaneous potential firings. For the next step, odorant stimulation at a certain concentration is applied to the detection chamber for the recording of responses in extracellular membrane potential changes from single cell. When the recording is finished, the detection chamber is washed with PBS to remove odorant stimulation. (*see* **Note 3**).

3.6 Data Processing and Analysis

1. A custom program is developed by MATLAB software for the processing of LAPS extracellular recording signals.
2. Raw data obtained from LAPS extracellular recordings are smoothed by a high-pass filter in order to get rid of baseline drifts. Figure 4a shows the typical extracellular recording signals after filtered by a high-pass filter, which are recorded from single cell under status of control (without any stimulation) and stimulated by 10 μM diacetyl. LAPS extracellular recording signals show that more firing spikes can be recorded from OSNs under stimulation of 10 μM diacetyl than under control status (without any stimulation).

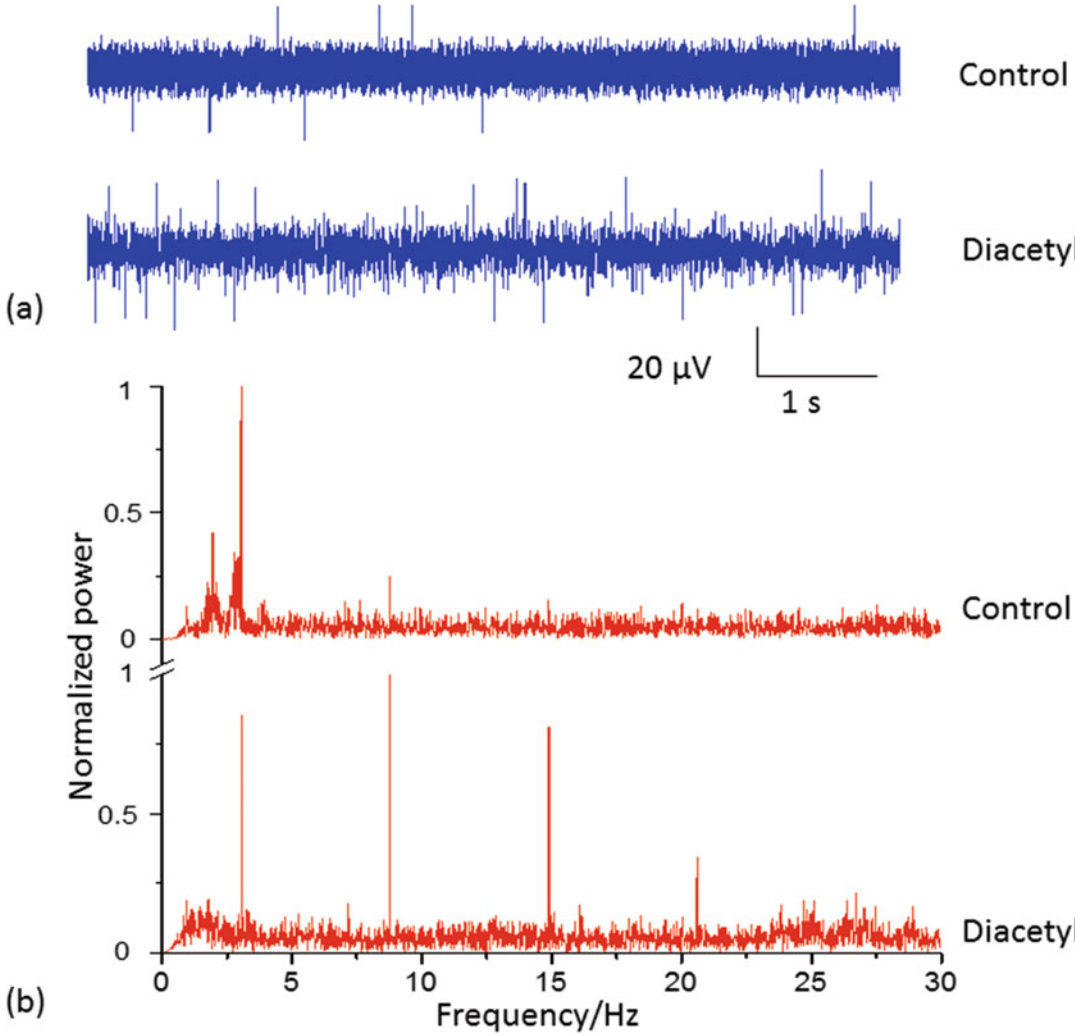

Fig. 4 (**a**) Typical LAPS extracellular recordings from single cell without (*upper*) and with 10 m M diacetyl stimulation (*lower*) after filter. (**b**) Normalized power spectra corresponding to control (*upper*) and stimulation (*lower*) signals. (Reproduced with permission from ref. 5. Copyright 2013 Elsevier)

3. Fast Fourier transform (FFT*See* Fast Fourier transform (FFT)) (Matlab function) is performed on the filtered LAPS extracellular recording signals in order to obtain the power spectrum and study the characteristics in frequency domain. FFT is an efficient algorithm for computing the discrete Fourier transform. In this case, FFT is employed to converse LAPS extracellular recording signals in the time domain to the frequency domain. Figure 4b is the corresponding power spectra of signals shown in Fig. 4a in the low frequency band of 0–30 Hz. Under control status (without any stimulation), the power spectrum of firing spikes is dominated by one major frequency component of 3 Hz and two minor frequency peaks located at

2.4 Hz and 8.4 Hz. The power spectrum of diacetyl-induced firing spikes showed different frequency components dominated by three major frequency peaks (at 3, 8.4, and 15 Hz) and one minor peak (at 21 Hz). In addition, two frequency components (at 15 and 21 Hz) are the distinct frequency characteristics of diacetyl-induced signals, which can be observed in different concentrations of diacetyl-induced signals ranging from 0.1 μM to 100 μM.

4. In the time domain, peak value extraction method is utilized to achieve the properties of the firing spikes. For this, LAPS extracellular recordings from cells under stimulus-free status are employed as the reference signal to quantify the noise of baseline using the mean value "D" of the reference signal. For the extraction of firing spikes, a threshold method is used by setting a threshold of 2.5*D to optimize the balance between error spike detection rate and undetected rate. As a result, all the spikes with amplitude higher than 2.5*D are detected.
5. After the firing spike extraction, the mean firing rate, which is calculated by the total number of firing spikes in 60 s, is used to indicate the activity of cells in cell membrane potential changes. It is indicated that OSNs show much higher mean firing rate under stimulation status compared with stimulation-free status.
6. Peri-stimulus time histograms (PSTHs*See* Peri-stimulus time histograms (PSTHs)) and interspike interval histograms (ISIHs)*See* Interspike interval histograms (ISIHs) are also calculated to further analyze the LAPS extracellular recording signals in time domain. PSTHs is calculated in two steps: the first step is the division of a period of observation time T into N bins with 1 s bin size; and the second step is to count the number of firing spikes in each successive 1 s bin presented as the height of PSTHs bar histogram. The main purposes of PSTHs is to visualize the firing rates at different times post stimulus.
7. ISIHs are calculated based on time intervals between firing spikes, which shows the special firing spike patterns. The time interval is used as the abscissa. The spike amount in each intervals bin is extracted and normalized by means of dividing using the overall amount of spikes in the observation time. Figure 5 shows the characteristics of LAPS extracellular recording indicated by PSTHs (purple) and ISIs (blue) under control (without stimulation) and stimulated by different concentration of diacetyl. (*see* **Note 4**). OSNs show distinct characters in the pattern of firing spikes under different status, which includes status of control (without stimulation) and stimulation by 10 and 50 μM diacetyl.

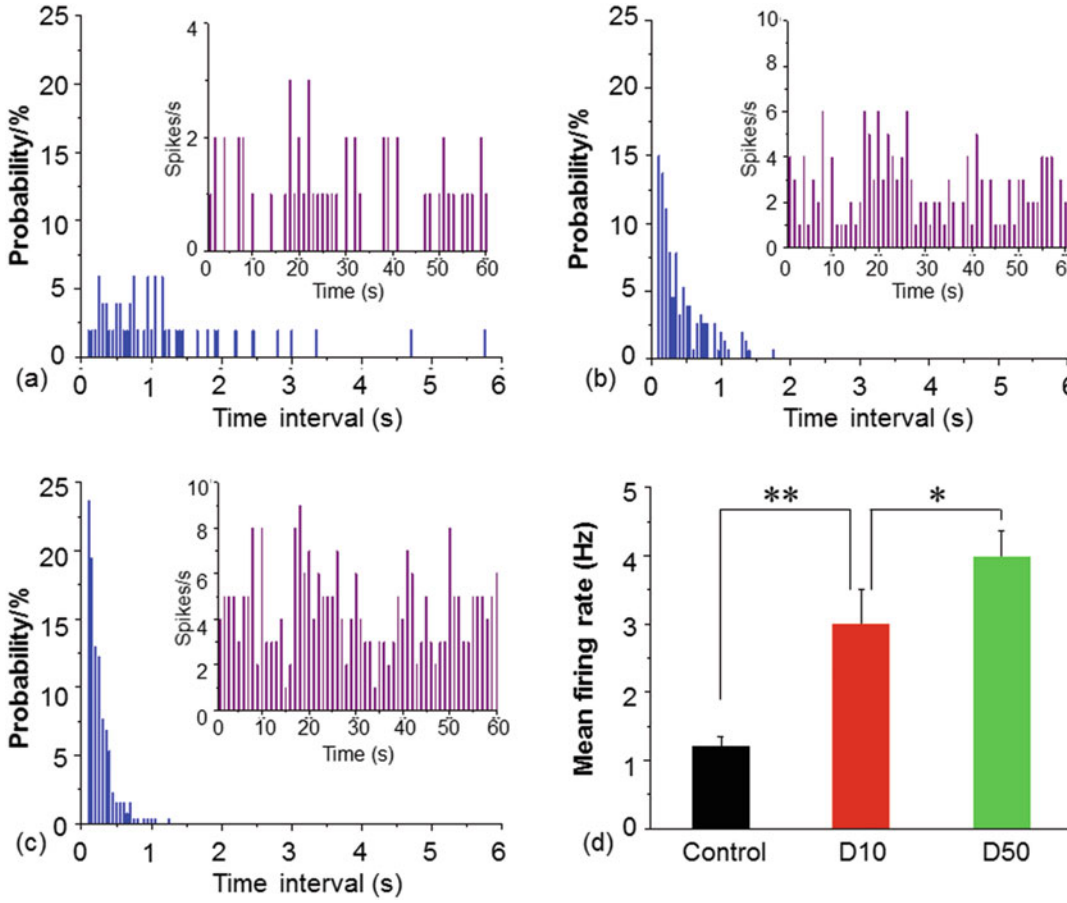

Fig. 5 LAPS extracellular recordings after processing and indicated by PSTHs (*purple*) and ISIs (blue) under (**a**) control (without stimulation) and stimulated by (**b**) 10 μM and (**c**) 50 μM of diacetyl. (**d**) shows the mean firing rate of corresponding status, namely control, 10 μM diacetyl (D10), and 50 μM diacetyl (D50). (*see* **Note 4**) (Reproduced with permission from ref. 5. Copyright 2013 Elsevier)

4 Notes

1. All solutions are warmed to 37 °C before use.
2. All measurements are performed at room temperature (18~25 °C). Phosphate buffer solution (PBS) is used as negative control solution. As shown in Fig. 6, the concentration-dependent responses of OSNs to diacetyl are obtained in the concentration range from 0.1 μM to 100 μM. In addition, the specificity of this biosensor is also demonstrated to be high as shown in the inset of Fig. 6, which shows that this biosensor responds only to stimulation by diacetyl—the natural ligand of ODR-10.
3. Single cells can be stimulated several times only if the interval between two odorant stimulation is at least 5 min, but not over 10 min.

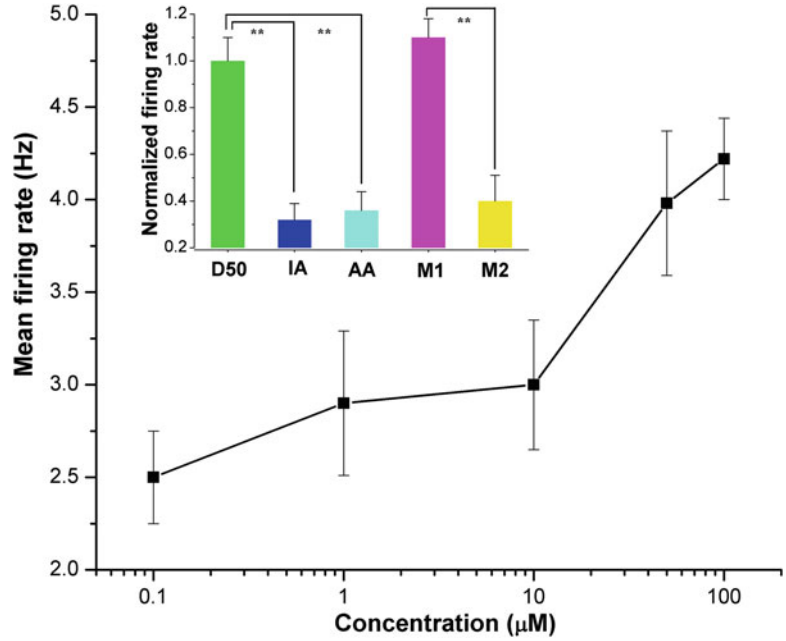

Fig. 6 Responses of OSNs to different concentrations of diacetyl and to various odorant stimulations (*inset*). In the inset, D50 means 50 μM diacetyl; IA, isoamyl acetate; AA, acetic acid; M1, the mixture of 50 μM diacetyl, 50 μM isoamyl acetate, and 50 μM acetic acid; M2, the mixture of 50 μM isoamyl acetate and 50 μM acetic acid. (*see* **Note 4**) (Reproduced with permission from ref. 5. Copyright 2013 Elsevier)

4. All the data are presented as mean ±SD (standard deviation). Each data point represents an average of five replications. Significant differences are indicated by ** ($P < 0.01$) and * ($P < 0.05$) using Student's *t*-test.

Acknowledgments

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