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Fast Serial Analysis of Active Cholesterol at the Plasma Membrane in Single Cells

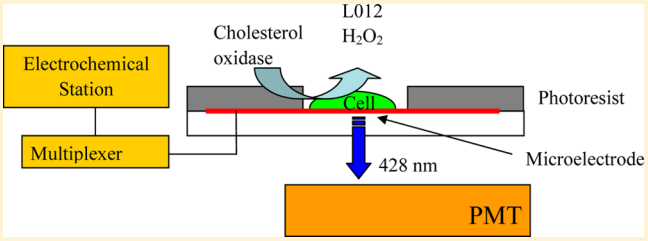
Chunxiu Tian,[†] Junyu Zhou,[‡] Zeng-Qiang Wu,[†] Danjun Fang,*[,‡] and Dechen Jiang*[,†]

[†]State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, China

[‡]School of Pharmacy, Nanjing Medical University, Jiangsu 211166, China

Supporting Information

ABSTRACT: Previously, our group has utilized the luminol electrochemiluminescence to analyze the active cholesterol at the plasma membrane in single cells by the exposure of one cell to a photomultiplier tube (PMT) through a pinhole. In this paper, fast analysis of active cholesterol at the plasma membrane in single cells was achieved by a multimicroelectrode array without the pinhole. Single cells were directly located on the microelectrodes using cell-sized microwell traps. A cycle of voltage was applied on the microelectrodes sequentially to induce a peak of luminescence from each microelectrode for the serial measurement of active membrane cholesterol. A minimal time of 1.60 s was determined for the analysis of one cell. The simulation and the experimental data exhibited a semisteady-state distribution of hydrogen peroxide on the microelectrode after the reaction of cholesterol oxidase with the membrane cholesterol, which supported the relative accuracy of the serial analysis. An eight-microelectrode array was demonstrated to analyze eight single cells in 22 s serially, including the channel switching time. The results from 64 single cells either activated by low ion strength buffer or the inhibition of intracellular acyl-coA/cholesterol acyltransferase (ACAT) revealed that most of the cells analyzed had the similar active membrane cholesterol, while few cells had more active cholesterol resulting in the cellular heterogeneity. The fast single-cell analysis platform developed will be potentially useful for the analysis of more molecules in single cells using proper oxidases.



Cholesterol is a main component at the cellular plasma membrane to establish the proper permeability and fluidity, which also functions in intracellular transport and cell signaling.[1−3] In the membrane, the hydroxyl group on cholesterol interacts with the polar head groups of the membrane phospholipids and sphingolipids, while the bulky steroid and the hydrocarbon chain are associated with the nonpolar fatty-acid chain of the lipids.[4,5] The recent evidence suggested that cholesterol formed complexes with polar bilayer lipids to create the cholesterol-poor and cholesterol-rich domains.[6,7] In the cholesterol-rich domain, excess cholesterol led to an elevated chemical activity (escape tendency), which was termed as active cholesterol.[8,9] The active membrane cholesterol has been observed to prompt several feedback responses, including endoplasmic reticulum (ER) responses, mitochondrial oxysterol responses, and others.[10−12] Thus, the qualification of active membrane cholesterol, especially at the single-cell level, is important to understand the functions of the active membrane cholesterol in the feedback responses.

Previously, our group has developed a strategy based on the luminol electrochemiluminescence to analyze the active membrane cholesterol in single cells.[13] A pinhole was placed below the electrode cultured with the cells so that only one cell was exposed to the detector (PMT). The cholesterol oxidase reacted with the active membrane cholesterol in the cells to generate hydrogen peroxide, which initialized the luminol electroluminescence with a voltage. The luminescence recorded on PMT was only attributed to the target cell above the pinhole to achieve single-cell analysis. A large deviation of active membrane cholesterol in single cells was observed, which exhibited the significance of single-cell analysis. The "pinhole" setup was low-cost, and the measurement was simple; however, it was impossible to multiply analyze single cells on one electrode, which limited the analysis throughput.

To overcome this drawback, a multiple microelectrode array embedded with the cell-sized microwell traps was developed to retain single cells in the microwells. Each cell was in register with one microelectrode, as shown in Figure 1. Cholesterol oxidase reacted with the active membrane cholesterol in single cells to generate hydrogen peroxide in the microwells simultaneously. The structure of the microwells slowed the diffusion of hydrogen peroxide into the bulk solution. The equilibrium between the generation and the diffusion of hydrogen peroxide created a semisteady-state distribution of hydrogen peroxide on the microelectrodes in a time length, in which the serial analysis was achieved by applying voltage on

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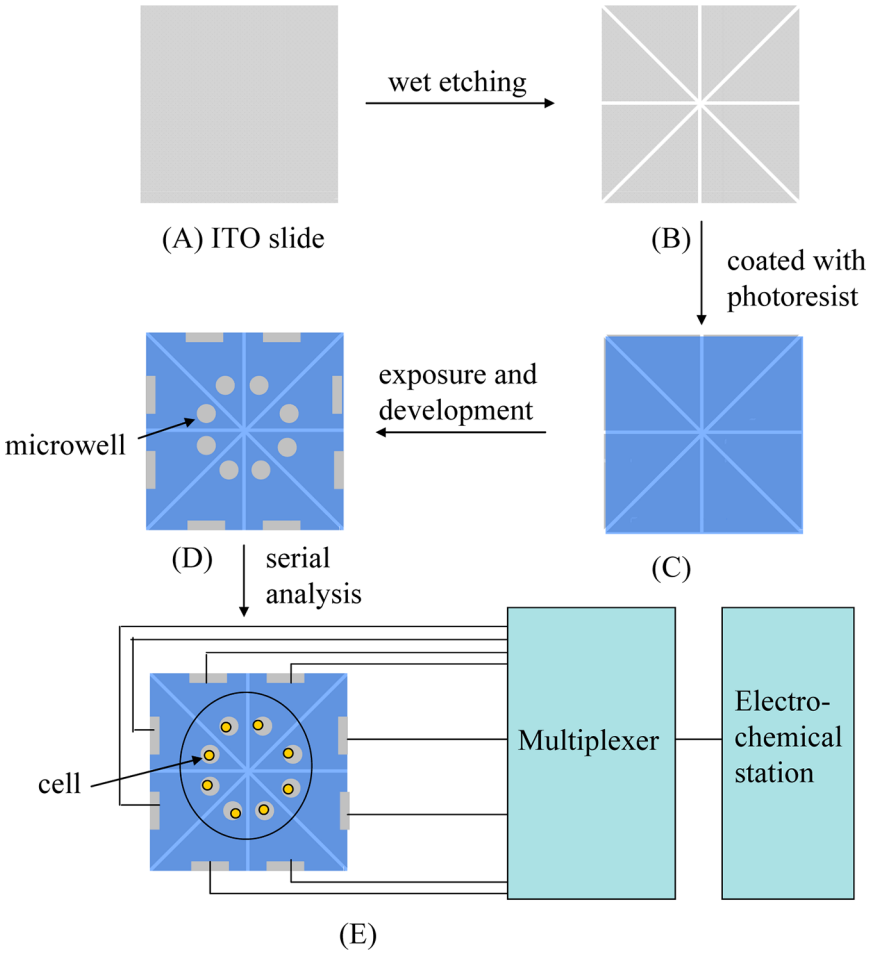

Figure 1. Process flow for the fabrication of the microelectrode array. (A) ITO slide; (B) ITO slide was wet etched into eight blocks; (C) slide was coated with a thick film (~ 30 μm) of SU 8 film; (D) eight microwells and rectangular openings were developed on an ITO slide to obtain the microelectrodes and the connection pads; (E) microelectrodes were connected with the multiplexer and the electrochemical station for the serial analysis of single cells. Single cells were located in the microelectrodes.

the microelectrodes sequentially. The cell/microelectrode gave the luminescence only when the voltage was applied on the microelectrode. The continuous recording of luminescence on PMT from all the microelectrodes gave the information of active membrane cholesterol in multiple single cells. In this setup, the cell analyzed was chosen by the voltage, and thus, no pinhole was needed. The absence of a pinhole facilitated the measurement automation and increased the analysis throughput. The accuracy and throughput of serial analysis were discussed. Using this platform, a relative large number of single cells were analyzed for the further investigation of cellular heterogeneity on active membrane cholesterol.

EXPERIMENTAL SECTION

Chemical. Raw264.7 cells were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences of Chinese Academy of Science (Shanghai, China). The SU-8 photoresist and the developer were obtained from Microchem Corp (Newton, MA). The compound 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4(2*H*,3*H*)-dione (L012) was obtained from Wako Chemical,Inc. (Richmond, VA). All other chemicals were from Sigma-Aldrich, unless indicated otherwise. Buffer solutions were sterilized.

Cell Culture. Raw264.7 cells were seeded in DMEM/high glucose medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin). Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

Fabrication of Eight-Microelectrode Array. The procedure to fabricate an eight-microelectrode array is exhibited in Figure 1. Initially, an indium tin oxide (ITO) slide (2 cm in square) was wet etched into eight blocks, as shown in Figure 1A,B. Then, a SU-8 photoresist layer with a thickness of ~30 μm was spin coated onto the ITO slide and soft baked at 65 °C for 3 min and 95 °C for 7 min, as shown in Figure 1C. The photoresist was illuminated with the lithography system (JB-VIII, Pmled Optoelectronics Co., Ltd., China) through an iron oxide mask and baked at 65 °C for 1 min and 95 °C for 3 min. Unpolymerized photoresist was removed using SU-8 developer, yielding a microwell with 30 μm in diameter and an opening as the connection pad on each block, as shown in Figure 1D. The coverslips with the microwells were hard baked at 120 °C for 20 min. Finally, an O-ring was attached on the ITO slide forming the cell chamber. Eight microwells were located in the cell chamber to create eight microelectrodes for single-cell analysis, as shown in Figure 1E.

Active Membrane Cholesterol. Raw 264.7 cells were cultured with the medium in the flask overnight and then trypsinized. A drop of medium with the cells was placed on the microelectrode array, and eight cells were loaded into eight

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microwells. The cells on the surface of the SU 8 layer were washed away. The cells in the microwells were cultured for 3 h for the adhesion and then activated by treatment in 0.5 mM phosphate-buffered saline (PBS, pH 7.4) with 310 mM sucrose at 37 °C for 1 h. For the ACAT-inhibited cells, the cells in the flask were cultured with 2 μg/mL sandoz 58035 at 37 °C for 24 h and then loaded into the microwells. No pretreatment with low ion strength buffer was needed before the analysis of ACAT-inhibited cells.

Luminescence Detection. The ITO microelectrode array cultured with the cells was used as the working electrodes. Voltage was applied serially on the microelectrodes using an electrochemical station (CHI 630E, CH Instruments) and a multichannel multiplexer (CHI 684, CH Instruments). The minimal time for the channel switching in this electrochemical system was 2 s. An Ag/AgCl electrode and a Pt wire were connected as the reference and counter electrode, respectively. The solution on the microelectrode array for luminescence detection was 50 μL PBS (100 mM pH 7.4) with 200 μM L012. Initially, a voltage cycle from 0.5 to 1.0 V with a scan rate of 1 V/s was applied on the ITO microelectrodes serially to collect the background luminescence. Then, 1 U/mL cholesterol oxidase was added into the buffer, and the luminescence was measured after a certain time. The ratio of luminescence read at 1.0 V before and after the introduction of cholesterol oxidase was determined as the signal, which was termed as "luminance ratio" in this paper.

Finite Element Simulation. The distribution of hydrogen peroxide in the microwell after the introduction of cholesterol oxidase was simulated in 2D axial symmetry model using COMSOL 3.5a software (Comsol, Inc.).

RESULTS AND DISCUSSION

Single-Cell Analysis on Microelectrode. In our previous work, a voltage cycle from −1.0 to 1.0 V with a scan rate of 1 V/s was applied to induce luminol electrochemiluminscence in the presence of hydrogen peroxide. The assay was sensitive for the measurement of active membrane cholesterol in single cells; however, the luminescence collected from single cells was weak, and a PMT voltage as high as 1000 V was required. The high PMT voltage created a large noise in the luminescence to worsen the signal-to-noise ratio.[13] To improve the data quality, L012 as a luminol analog was chosen here in place of luminol. Importantly, L012 was reported to have highly sensitive chemiluminescence and be more active than luminol.[14,15] Our experimental data showed that the electrochemiluminescences of L012 in the absence and presence of hydrogen peroxide were stronger than those of luminol, respectively (data not shown). The enhancement in luminescence lowered PMT voltage to 700 V in single-cell analysis for a clean luminescence trace. Also, the trace showed that the luminescence of L012 and hydrogen peroxide was induced when the voltage was larger than 0.5 V. To decrease the analysis time for higher analysis throughput, a voltage cycle from 0.5 to 1.0 V with a scan rate of 1 V/s was chosen for the following single-cell analysis. Ten continuous voltage cycles were performed on the electrode, and the relative standard deviations of the current read at 1.0 V were 2.92 and 2.70% in the absence and presence of hydrogen peroxide, respectively. The small deviation exhibited the reproducibility in electrochemical property of the electrode.

Before the single-cell analysis, one cell was loaded on one microelectrode and pretreated with low ion strength buffer for 1 h to activate the membrane cholesterol. The voltage cycle was applied on the microelectrode before and after the introduction of cholesterol oxidase. The typical trace of the luminescence collected from the microelectrode is shown in Figure 2. An

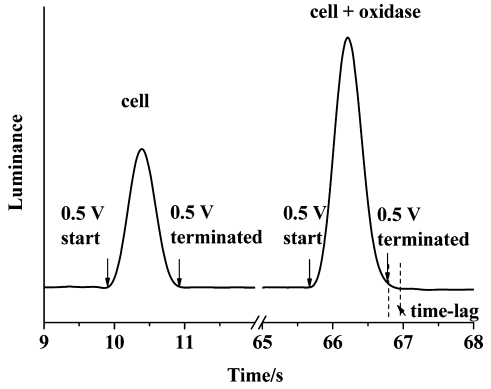

Figure 2. Typical luminescence of one cell loaded on the microelectrode before and after the introduction of cholesterol oxidase. Voltage for PMT was 700 V.

increase in luminescence after the introduction of cholesterol oxidase was observed, which indicated that the active membrane cholesterol in the single cell was measurable on the microelectrode. The luminescence took an additional 0.10−0.60 s to be zero after the voltage cycle, which might be caused by the luminol or oxygen radicals remaining after the ending voltage. This time-lag suggested that an additional 0.60 s was needed to "turn off" the luminescence before the analysis of the next cell, which was the minimal time for channel switching in multiplexer. In our work, a commercial available multiplexer was used to exhibit the feasibility of serial analysis. The time for channel switching was 2 s, which met the requirement of the serial analysis. Thus, the throughput of single-cell analysis in our platform was 3 s per cell including the channel switching time.

Time Length for Serial Analysis. For the serial analysis, the single cells on the microelectrodes were analyzed at different time points. The maintenance of the same situation for all the cells during the serial analysis was important to obtain an accurate result. In our system, active membrane cholesterol from all the cells was continuously converted into hydrogen peroxide in the first minute,[16] which diffused from the microelectrode surface to the bulk solution. The structure of microwells slowed down the diffusion of hydrogen peroxide away from the microwell. Thus, the generation and the diffusion of hydrogen peroxide might create a semisteady-state distribution of hydrogen peroxide on the microelectrode in a limited time length for the serial analysis. To validate the existence of the semisteady state, the distribution of hydrogen peroxide in the microwell was studied using finite element simulation and the experimental measurement. The domain for the finite element simulation is shown in Figure S1(Supporting Information). The microwell was 15 μm in radius and 30 μm in depth. The bottom line in the microwell represented the microelectrode. The cell in the microwell was 5 μm in radius and 5 μm in height. Since the active membrane cholesterol was measured to be 0.33 fmol previously and reacted with cholesterol oxidase in the first minute,[16,13] 0.33 fmol hydrogen peroxide was assumed to be released from the cell surface in 1 min with a constant rate for the simulation. The diffusion coefficient of hydrogen peroxide was 1.5×10^{-9} m^2/s.[17] Figure

dx.doi.org/10.1021/ac403031c | Anal. Chem. 2014, 86, 678−684

3A shows the simulated concentration of hydrogen peroxide on the microelectrode every second. The relative standard deviation of the concentrations in the time length from 20 to 51 s was calculated to be 2.6%, exhibiting the semisteady-state distribution of hydrogen peroxide on the microelectrode. The alteration of the active membrane cholesterol amount to mimic the cells at different disease states changed the simulated concentration of hydrogen peroxide on the microelectrode, but the time length for the semisteady-state distribution of hydrogen peroxide was the same (data not shown). Considering that the heterogeneity of active membrane cholesterol in single cells was as large as 40−50%,[13] we conclude that the error of 2.6% from the serial analysis was not significant and can be considered as the systematic error.

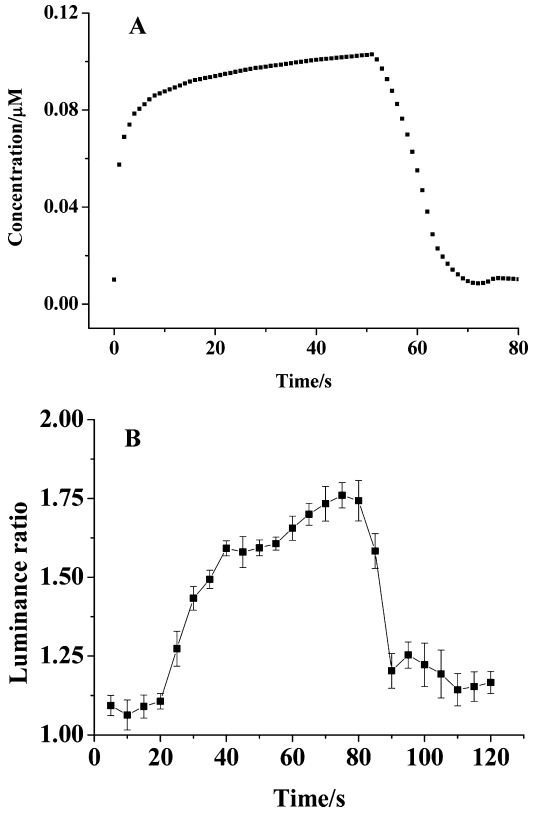

Figure 3. (A) Simulated concentration of hydrogen peroxide at the microelectrode surface over time. (B) Average luminance ratio of the ITO slides with 400 microwells after the introduction of cholesterol oxidase. Voltage for PMT was 700 V. Error bar represents the standard deviation (n = 3).

The experiment was performed to investigate the concentration change of hydrogen peroxide on the microelectrode as well. Due to the high cell heterogeneity, it was difficult to conclude the concentration change of hydrogen peroxide from the result of a few single cells. A relatively large number of cells were needed for the analysis to minimize the cell differences. To achieve this measurement, the ITO slide was coated with SU 8 photoresist without wet etching and 400-cell-sized microwells were fabricated on the ITO slides. When the single cells were loaded into each microwell, the distribution of hydrogen peroxide in these microwells was the same as that in our microelectrode array. By recording the luminescence from the ITO slides, the sum of 400 cell signals was collected. The result was not interfered by the cellular heterogeneity, which provided accurate information on the concentration change of hydrogen peroxide on microelectrodes.

In the experiment, the loading efficiency of single cells in the microwells was typically 75%. The efficiency meant that around 300 cells were loaded on ITO slides and around 100 microwells were empty. After cholesterol oxidase was added on the array, the diffusion time of cholesterol oxidase from bulk solution to the cells was determined. To simplify the measurement, hydrogen peroxide was added into the bulk solution, and a sharp increase in luminescence was observed after 20 s. This 20 s was the diffusion time of hydrogen peroxide from bulk solution to the microelectrode, and it was estimated as the diffusion time of cholesterol oxidase as well. After cholesterol oxidase reached the cells, the luminescence from ITO slide was collected at different time points. As shown in Figure 3B, the luminance ratio increased after the introduction of cholesterol oxidase for 20 s, supporting the diffusion of oxidase to the microelectrode. A semisteady state of luminescence was observed in the time length from 40 to 80 s. The relative standard deviation of the luminescence in this time length was calculated to be 4.4%. After exclusion of the diffusion time of cholesterol oxidase, the time length for the semisteady state distribution was 20−60 s, which was close to the simulation result. The difference in the ending time point from the simulation (51 s) and the experimental result (60 s) might be attributed to the simulation error. In the simulation, the generation rate of hydrogen peroxide was assumed to be constant in the first minute. However, the reaction rate of cholesterol oxidase and cholesterol might involve more kinetic processes and was not constant. Nevertheless, both the simulation and the experimental results exhibited the existence of a semisteady-state distribution of hydrogen peroxide on the microelectrode with a minor measurement error, which supported the relative accuracy of serial analysis. In the following single-cell serial analysis, the time length of 40−80 s after the introduction of cholesterol oxidase in the bulk solution was used.

Difference of the Microelectrodes in the Microarray. An eight-microelectrode array was demonstrated for the serial analysis of single cells. The difference of eight microelectrodes in the response of hydrogen peroxide during the serial analysis was characterized by the luminescence measurement in the presence of 200 μM L012 and 2 μM hydrogen peroxide. The typical trace of luminescence is shown in Figure 4. The increase in the luminescence was observed on all eight microelectrodes

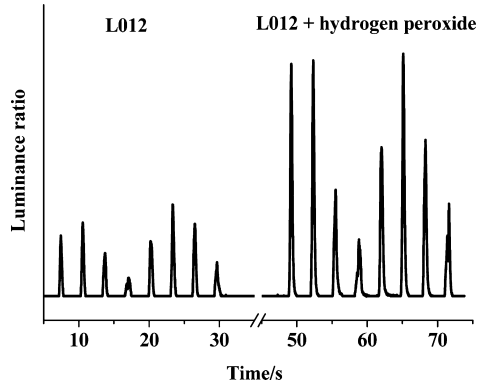

Figure 4. Typical luminescence trace of eight microelectrodes in an array before and after the addition of 2 μM hydrogen peroxide. The concentration of L012 was 200 μM. The voltage for PMT was 700 V.

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after the introduction of hydrogen peroxide, which exhibited that all the microelectrodes in the array were useful for the detection. The luminescence ratios before and after the addition of hydrogen peroxide from 24 microelectrodes in three microarrays were calculated, and the relative standard deviation was 15.6%. The consistence in the luminescence ratio from these microelectrodes supported the serial analysis of single cells on the microarray.

It was noted that the deviation of the microelectrodes was larger than that of 5.0% obtained on five ITO slides previously,[13] which was attributed to the nonuniformity of the ITO layer on the microscale order. Also, ITO slides used in our experiment were not entirely flat, which gave the variation of the thickness of SU 8 layer in microzones. This imperfection in the layer affected the development and created a different area of the exposure surface as the microelectrode. Since the luminescence was strongly dependent on the electrode area, the difference in the exposure area gave the variation of the luminescence from the microelectrodes. Although the luminescence ratio used in our assay excluded the electrode area in the result, the difference in the electrode area might introduce the additional deviation in the analysis.

Serial Analysis of Single Cells. The single-cell serial analysis was performed on the cells activated by the pretreatment in the low ion strength buffer. After the luminescence of eight cell/microelectrodes in the presence of 200 μM L012 was serially recorded, cholesterol oxidase was added in the bulk solution, and the luminescence was measured after 40 s. Figure 5A shows the typical luminescence trace of eight microelectrodes before and after the introduction of cholesterol oxidase. The luminescence increase on all eight microelectrodes after the introduction of cholesterol oxidase exhibited the serial analysis of active membrane cholesterol in eight single cells. The luminescence dropped to zero during the channel switching time, suggesting no cross-talk signal between adjacent cells. The whole time for the analysis of eight cells was 22 s, including 14 s for the channel switching.

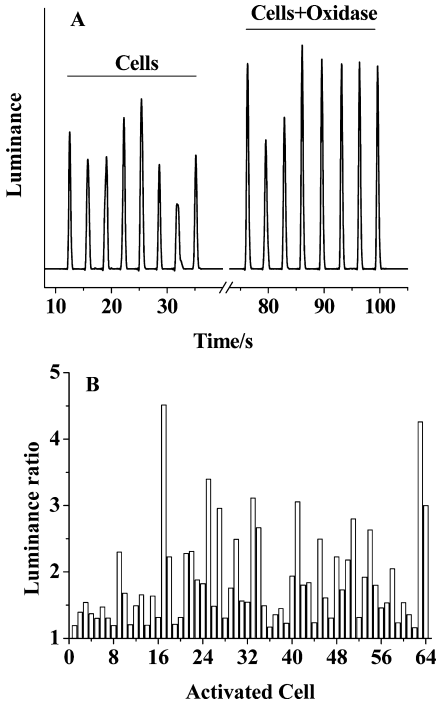

Figure 5. (A) Typical luminescence trace of eight microelectrodes before and after the introduction of cholesterol oxidase. Cells were activated by the pretreatment in low ion strength buffer. (B) The distribution of luminance ratio of 64 single cells. Each bar represents one cell.

Sixty-four single cells were analyzed using the serial analysis platform, and the distribution of the luminescence ratio is shown in Figure 5B. The average luminescence ratio was calculated to be 1.86 ± 0.73, and the relative standard deviation was 39.2%, which was similar to the previous results obtained from the "pinhole" setup.[13] The distribution of the cells revealed that 45 out of 64 cells had the luminescence ratio between 1.0 and 2.0, and the relative standard deviation for these 45 cells was 15.0%. With respect to the error of serial analysis and microelectrode difference, the deviation suggested the similar content of active membrane cholesterol in these 45 cells. For the other 19 cells, 14 cells were between 2.0 and 3.0, and 5 cells were between 3.0 and 5.0. The 19 cells owning a high activation of membrane cholesterol increased the cellular heterogeneity.

Compared with the activation of membrane cholesterol using low ion strength buffer, the activation using intercalating amphipaths gave a better control for the active membrane cholesterol.[18,19] Octanol (75 nM) was applied on the cells at 37 °C for 5 min to activate membrane cholesterol. Sixty-four single cells were analyzed serially, and the result is shown in Figure S2 (Supporting Information). Similar to the activation in low ion strength buffer, 52 out of 64 cells activated by octanol gave the luminescence ratio between 1.0 and 2.0. The relative standard deviation was 17.3%, suggesting a similar effect on active membrane cholesterol. Although the mechanism of high cell heterogeneity in few cells was not clear, the observation of two groups of the cells after the activation using this relatively high throughput platform was significant for the understanding of cellular heterogeneity in active membrane cholesterol.

To further validate our serial analysis platform, the ACAT-inhibited single cells with active membrane cholesterol was analyzed. The inhibition of ACAT decreased the intracellular esterification of cholesterol and generated active membrane cholesterol.[20,21] All the luminescences on eight microelectrodes cultured with ACAT-inhibited single cells increased after the introduction of cholesterol oxidase, supporting the serial analysis of cholesterol-up-regulated cells. Sixty-four single ACAT inhibited cells were analyzed, and the distribution of active membrane cholesterol in single cells is shown in Figure 6A. The average luminescence ratio was 2.56 ± 1.19 with the relative standard deviation of 46.5%. Similar to the result of single cells activated by low ion strength solution, 45 out of 64 cells had the ratio between 1.30 and 2.85 with the relative standard deviation of 24.0%. The other 19 cells had a wide distribution of the ratio between 2.85 and 6.0. The result showed that two groups of cells also existed after the up-regulation of the intracellular cholesterol pool, in which a high percentage of cells had similar active membrane cholesterol, exhibiting cell homogeneity, and a low percent of cells had more active membrane cholesterol, leading to cell heterogeneity. The exploration of these cells with more active membrane cholesterol will enable us to determine whether the heterogeneity was caused by the difference of protein activity or the feedback process after the regulation of protein.

The ACAT-inhibited cells were further treated with 100 μM mevastatin for 24 h to inhibit hydroxy methylglutaryl coenzyme A reductase (HMGCoA), which decreased the intracellular

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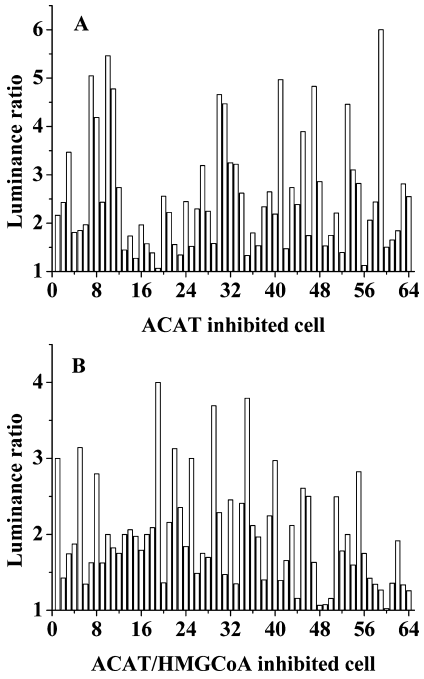

Figure 6. Distribution of luminance ratio of 64 single (A) ACAT-inhibited cells and (B) ACAT/HMGCoA-inhibited cells. Each bar represents one cell.

cholesterol biosynthesis and the active membrane cholesterol.[22,23] No pretreatment with low ion strength medium prior to the inhibition was needed for mevastatin-inhibited cells. Figure 6B shows the distribution of luminescence ratio from 64 single ACAT/HMGCoA inhibited cells using the serial analysis. The average luminance ratio was 1.99 ± 0.68 with the relative standard deviation of 34.2%. Compared with the luminance ratio obtained from the ACAT-inhibited cell, the decrease in the luminance ratio on ACAT/HMGCoA cells was consistent with the less active membrane cholesterol after the statin treatment. Forty-eight out of 64 cells had the luminance ratio between 1.02 and 2.28 with the relative standard deviation of 20.5%, which exhibited the relative homogeneity of active membrane cholesterol in most of cells after the drug treatment. Sixteen cells had the luminance ratio between 2.28 and 4.0. The observation of two cell groups after the drug treatment suggested that the decrease in active membrane cholesterol by the drug did not change the distribution of cells.

CONCLUSION

In this paper, a multimicroelectrode array was developed for the serial analysis of active membrane cholesterol in single cells. The simulation and the experiment results revealed the semisteady-state distribution of hydrogen peroxide on the microelectrode surface after the introduction of cholesterol oxidase, which supported the measurement accuracy of serial analysis. Compared with our previous "pinhole" setup, a relatively high throughput was achieved by analyzing eight cells on the microelectrode array in 22 s. The results from 64 single cells exhibited that most of the cells had similar active membrane cholesterol, whereas few cells had more activation on membrane cholesterol resulting in the cell heterogeneity. Further work will focus on the scale up of the microelectrode array for the serial analysis of more cells. A new multiplexer will be designed by choosing the proper analog chip to shorten the channel switching time into 0.6 s so that the time length of 40 s could be used for the analysis of 25 cells on one ITO slide. The achievement of a higher analysis throughput will analyze a larger pool of single cells for more accurate information on cellular heterogeneity. Also, the platform will be extended for the analysis of membrane lipids in single cells using proper oxidases. The qualification of cholesterol and the associated lipids at plasma membrane in single cell will be important to understand the interaction of cholesterol and lipids.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: djf@njmu.edu.cn. Fax/Tel.: 086-25-86868477 (D.F.).
*E-mail:dechenjiang@nju.edu.cn. Fax/Tel.: 086-25-83594846 (D.J.).

Notes

The authors declare no competing financial interest.

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