1 **Molecular electrochemical switching element based on diffusive**
2 **molecular competition for multipoint electrochemical detection**
3 **of respiration activity of cell aggregates**

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5 Kosuke Ino^{1*}, Yuta Yamada¹, Yusuke Kanno¹, Shunsuke Imai¹, Hitoshi Shiku², and
6 Tomokazu Matsue^{1,3*}

7
8 ¹ Graduate School of Environmental Studies, Tohoku University, Japan

9 ² School of Engineering, Tohoku University, Japan

10 ³ WPI-Advanced Institute for Materials Research, Tohoku University, Japan

11
12 *Corresponding authors:

13 Kosuke Ino (ino.kosuke@bioinfo.che.tohoku.ac.jp)

14 Tomokazu Matsue (matsue@bioinfo.che.tohoku.ac.jp)

15
16 Keywords:

17 Electrochemical analysis

18 Switching element

19 Electrode-array device

20 Detection of respiration activity

21 Cell analysis

22 **Abstract**

23 Electrochemical techniques have been widely utilized for evaluation of oxygen
24 consumption of cells. For high throughput cell analysis and imaging, several
25 electrode-array devices have been developed. However, it is difficult to incorporate
26 many sensors into a small area using a simple arrangement. In the present study, we
27 developed a novel molecular electrochemical switching element for the incorporation of
28 many sensors for detection of oxygen consumption of cells. The switching element is
29 based on the competition of molecular consumption at an electrode pair. The switching
30 elements are incorporated into an electrochemical imaging device, so that n^2
31 electrochemical sensors are prepared with only $2n$ connector pads. The device was then
32 applied to evaluate the respiratory activity of cell aggregates. The detection system is a
33 useful tool for the electrochemical imaging of cell respiratory activity without any
34 damage to samples.

35 1. Introduction

36 The respiratory activity of cells plays an important role in cellular viability.
37 Therefore, measuring the oxygen consumption of cells is important for cell analysis. The
38 oxygen consumption of cells has been studied with spectrophotometric [1], fluorescence
39 [2], and electrochemical techniques such as scanning electrochemical microscopy
40 (SECM) [3, 4]. In an SECM measurement, a probe electrode is scanned to acquire a two-
41 or three-dimensional oxygen concentration profile of a sample. Analysis of the oxygen
42 flux enables evaluation of the oxygen consumption of single embryos, cell aggregates,
43 and single cells without any damage to the samples [3-5]. This method can be performed
44 under conditions that closely resemble those of cell cultures without the use of any
45 markers. SECM has also been applied to viability evaluations of spheroid arrays [6].
46 Although SECM is very useful for the characterization of cell aggregates, it does have the
47 following intrinsic disadvantages: (i) the probe is fragile and difficult to handle, (ii)
48 control of the probe-substrate distance needs technical skills, and (iii) the acquisition of
49 electrochemical images is time consuming due to the scanning of the probe. Therefore,
50 SECM is unsuitable for the rapid analysis of array-based cell aggregates.

51 To overcome these problems and enable rapid measurements, several kinds of
52 electrode-array devices have been developed [7]. However, it is difficult to incorporate
53 many electrochemical sensors (which consist of the sensors, lead connectors, and
54 connector pads) into a small area using a simple arrangement. For the incorporation of
55 many sensors, semiconductor switching elements are incorporated into the
56 electrode-array device. The switching elements are utilized to address detection points
57 in an electrode-array device, which leads to a reduction in the number of connector pads
58 and allows for the incorporation of many sensors. However, the development of such
59 devices requires complicated processes and huge costs.

60 Previously, we proposed a novel type of switching element based on molecular
61 diffusion and an electrochemical reaction, and applied it to an electrochemical imaging
62 system [9-13]. The switching element consists of two electrodes and a solution containing
63 target molecules. One of the electrodes acts as a sensor for detection of the target
64 molecules. Diffusion of the target molecules to the sensor electrode is controlled by
65 adjusting the potential of the other. In this way, detection at the sensor electrode can be
66 switched on and off. We termed this new switching element a "molecular electrochemical
67 switching element." In the electrochemical imaging system where the switching
68 element is used, n row and n column electrodes are perpendicularly arranged to form n^2
69 crossing points, which act as individual sensors. One of the row electrodes induces an
70 electrochemical reaction locally to address the detection points, electrochemical signals

induced by the electrochemical reaction at the crossing points are acquired at the column electrodes. Thus, n^2 sensors can be easily incorporated into the device using only $2n$ connector pads. Furthermore, all of the signals at the n^2 crossing points can be obtained by sequential acquisition.

As an example of a typical molecular electrochemical switching elements, we previously reported a redox cycling-based switching element, and demonstrated its applicability to electrochemical imaging [9-13]. We termed this method "local redox cycling-based electrochemical (LRC-EC) mode". In redox cycling [14], proper potentials are applied to one pair of electrodes. At one electrode (the generator), oxidants (reductants) are reduced (oxidized) to reductants (oxidants), which then diffuse to the other electrode (the collector). The reductants (oxidants) are converted back into oxidants (reductants) at the collector electrodes, and the process repeats. This redox cycling between the electrodes leads to signal amplification. In an LRC-EC system, redox cycling of the target molecules, which is used as the basis of the switching, is locally established between the row and column electrodes. LRC-EC devices have been successfully used for high-throughput assays and electrochemical bioimaging of cell activity, such as cell differentiation of embryonic stem (ES) cells via redox cycling-based detection of their enzymatic product [9, 12]. However, the LRC-EC system can only be used with molecules that are detectable by redox cycling, such as *p*-aminophenol [12] and ferrocenemethanol [11], and the system cannot be used to evaluate the respiratory activity of cells based on oxygen detection because oxygen is an irreversible redox species.

In this study, we developed a novel molecular electrochemical switching element for the detection of irreversible redox species. The switching element is based on the competition of molecular consumption at an electrode pair. We have termed this measurement mode as "molecular competition mode", and applied it to measure oxygen dissolved in solution. In addition, molecular competition mode was applied to evaluate the respiratory activity of MCF-7 aggregates.

2. Results and discussion

The detection system we built based on molecular competition mode is illustrated in Fig. 1. The device consists of 6 row and 6 column electrodes, which form 36 crossing points using only 12 connector pads (Fig. 1A). The row and column electrodes are separated with an insulation layer. Top ring and bottom disk electrodes are placed at the crossing points (Fig. 1A) and connected to the row and column electrodes, respectively. The row electrodes are used for addressing the measurement points,

107 and the column electrodes are used for data acquisition. These electrodes are set to 0.00
108 or -0.60 V vs. Ag/AgCl. Dissolved oxygen is reduced to H₂O at -0.60 V, and no
109 electrochemical reaction takes place at 0.00 V. Figure 1B shows an illustration of the
110 switching mechanism in molecular competition mode. During switch-off, the top ring
111 electrodes are set to -0.60 V to consume oxygen at the electrodes before it has the chance
112 to be reduced at the bottom disk electrode. Therefore, the current at the bottom electrode
113 is low. During switch-on, the top ring electrodes are set to 0.00 V so that no oxygen
114 reduction takes place there. Consequently, oxygen diffusion to the bottom disk
115 electrodes recovers, resulting in an increase in the oxygen reduction currents at the
116 bottom disk electrodes. Thus, on/off switching of the molecular competition at desired
117 crossing points can be used to address the detection points (Movie S1). The detection
118 points are sequentially changed to complete the data acquisition from all of the sensors
119 (Movie S1). If cell aggregates are trapped at the detection points, the respiration activity
120 of the aggregates can be evaluated by measuring their oxygen consumption (Fig. 1C).

121 The fabrication process for the device is illustrated in Fig. 2 and described in
122 more detail in the Experimental section. Briefly, the device consists of five layers on a
123 glass substrate: Pt column electrodes, a polyimide layer, Pt row electrodes, 1st SU-8
124 microwells, and 2nd SU-8 microwells. Figure 3A shows a photograph of an actual
125 fabricated device containing 6 row and 6 column electrodes. The inner and outer
126 diameters of the top ring electrodes were 20 and 40 μ m, respectively, and the diameter of
127 the bottom disk electrodes was 20 μ m. The thickness of the polyimide and 1st SU-8
128 layers were 3.4 and 3.6 μ m, respectively. The center-to-center distance of the sensors
129 was 1.35 mm. The diameter and depth of the microwells of the 2nd SU layer were
130 approximately 350 and 50 μ m, respectively. To avoid cell aggregates covering the ring
131 and disk electrodes completely, the center of the 2nd SU-8 microwells for trapping cells
132 was shifted from that of the sensors (Fig. 3A). An acrylic frame was glued to the
133 device and the device was connected to a multichannel potentiostat using a clip connector
134 (Fig. 3B). A sample solution was introduced into the device, and Ag/AgCl reference and
135 Pt counter electrodes were inserted into the solution (Fig. 3B).

136 Next, we evaluated the performance of molecular competition mode. PBS
137 solutions with various concentrations of oxygen were introduced into the device to
138 generate a calibration curve. The switching condition at one of the row electrodes was
139 changed, as shown in Fig. 4A, and electrochemical signals were acquired from the
140 column electrodes. Figure 4B_i shows chronoamperograms acquired from one of the
141 column electrodes. When the row electrode was switched off, the electrochemical signal
142 increased with the oxygen concentration (Fig. 4B_i), indicating that the top ring

143 electrodes did not consume oxygen completely. When the row electrode was switched on,
144 oxygen diffusion to the bottom disk electrodes from the bulk was recovered at the
145 detection points, resulting in increased oxygen reduction currents at the column
146 electrodes (Fig. 4Bi). Since background current was observed even when the row
147 electrode was switched off, we calculated background corrected signals (Δ current) by
148 subtracting the current measured right before switch-on from that measured after
149 switch-on; background-subtracted chronoamperograms are shown in Figure
150 4Bii. The oxygen reduction current increased after switch-on, and the current 60 s after
151 switch-on was proportional to the oxygen concentration (Fig. 4C). These results indicate
152 that the switching system in molecular competition mode worked successfully for
153 quantitative assays. The device was also evaluated using a computer simulation.
154 (Supporting Information).

155 Next, we evaluated the performance of molecular competition mode for the
156 addressable detection of the respiratory activity of MCF-7 spheroids. Figures 5A and
157 5B respectively show an optical image of living cell aggregates (approximately 400 μ m in
158 diameter) on the device arranged at several sensor points and the corresponding
159 electrochemical image. In this case, we used both live and fixed aggregates. An apparent
160 change in Δ current was observed for live aggregates, while the signals from the fixed cell
161 aggregates were similar to those at sensors without cells because they showed no
162 respiratory activity (Figs. 5B and 5C). These results demonstrate the successful
163 application of molecular competition mode for the addressable detection of the
164 respiratory activity of cells. Figure 5D shows the electrochemical signals for
165 cell aggregates with various diameters (260–500 μ m). The results show that the
166 respiratory activity of the large cell aggregates was higher than that of the small cell
167 aggregates, because the former consisted of a large number of cells. Thus, molecular
168 competition mode can be applied for quantitative assays of the respiratory activity of
169 cells. The respiratory activity depends closely on both Δ current and the diameter of cell
170 aggregates. Therefore, these quantities should be measured during studies of cell
171 respiratory activity using this method.

172 In addition, we simulated the electrochemical reduction of oxygen using the
173 present experimental conditions for cell aggregates of various sizes (Supporting
174 Information). Comparison of the experimental signals with the simulated oxygen
175 reduction currents affords the oxygen consumption of the cell aggregates. Figure 5E
176 shows that the oxygen consumption (i.e., respiratory activity) of the cell aggregates
177 260–500 μ m in diameter was estimated to be 74–515 fmol/s, which is similar to that
178 observed in an SECM measurement.⁴

The cell aggregates with 275 μm diameter showed 72 and 130 fmol/s, respectively (Fig. 5D), indicating that the estimated respiration activity differed although the cell aggregates were almost the same size. The difference might be derived from the inhomogeneity of the cell aggregates and the measurement error. The detailed reason for the difference is under investigation.

Although single cell analysis is important, the present device cannot be applied for single cell analysis because the amount of oxygen consumption from single cell is very lower than that at the electrodes during the consumption mode. To achieve the single cell analysis, it is necessary to reduce the size of the electrodes.

Redox competition was previously utilized for electrochemical imaging in an SECM measurement, which has been reported by Schuhmann and co-workers [15]. In the study, redox competition between a tip in the SECM and a substrate was utilized to locally analyze catalytic activity. In contrast, we utilized redox competition to produce a novel switching element that allowed for an electrochemical imaging system with many sensors, which is unique, compared to other researches.

3. Conclusions

In conclusion, we developed a novel electrochemical imaging device that utilizes a detection mode we have termed “molecular competition mode. The electrochemical imaging device consisted of molecular electrochemical switching elements based on redox consumption at an electrode pair, which are utilized to prepare n^2 electrochemical sensors from only $2n$ connector pads. This configuration allows for the incorporation of many sensors into a small chip device. Molecules that undergo irreversible electrochemical reactions, such as dissolved oxygen, were successfully imaged. This is superior to our previous system using the switching system that only allowed for the detection of molecules via redox cycling. The detection system can be used for the electrochemical imaging of cell respiratory activity, and is a useful tool for cell analysis because it can be used to perform high throughput assays of cells via their oxygen consumption.

4. Experimental Section

4. 1. Detection system

The row and column electrodes of the device were connected to a multichannel potentiostat for 8 working electrodes (HA-1010 mM8, Hokuto Denko, Japan) through a clip connector (CCNL-050-37-FRC, Yokowo, Japan) and a matrix (NI PXI-2529, National Instruments, USA). The connection procedure is described in detail in Movie

215 S1. A sample solution was then introduced into the device. Finally, Ag/AgCl (saturated
216 KCl) and Pt wire electrodes were inserted into the sample solution as reference and
217 counter electrodes, respectively. All of the electrochemical detections were performed in
218 a Faraday cage. The voltage control and data acquisition were performed with a
219 computer program written in LabVIEW (National Instruments).

220

221 **4. 2. Device fabrication**

222 The fabrication process of the device is illustrated in Fig. 2. Briefly, Pt
223 electrodes (column electrodes), utilized for the bottom disk electrodes, were fabricated
224 using a combination of a lithographic technique, Ti/Pt sputtering, and a lift-off
225 process. The wide and thickness of the Ti/Pt electrodes were designed to 100 μm and 210
226 nm, respectively. Next, an insulation layer of polyimide (UR-3100E, Toray, Japan) was
227 fabricated on the device. The thickness of the polyimide was designed to be 3.4 μm . Pt
228 electrodes (row electrodes), utilized for the top ring electrodes, were then fabricated
229 using a combination of a lithographic technique, Cr/Pt sputtering, and a lift-off process.
230 Next, reactive ion etching (RIE) was performed to remove the polyimide layer and
231 reveal the bottom disk electrodes. The polyimide layer was dry-etched with oxygen
232 plasma at 100 W for 3 min and 0.2 Torr ($\text{O}_2:\text{SF}_6 = 4:1$). During the etching process, the
233 row electrodes acted as a mask to protect the polyimide layer under the row electrodes.
234 Then, 1st SU-8 microwells (SU-8 3005, Microchem Co., USA) were fabricated on each
235 sensor to reveal the top ring electrodes. Finally, 2nd SU-8 microwells (SU-8 3050,
236 Microchem Co.) with 50 μm depth were fabricated on each sensor as trapping cell
237 aggregates. The thickness of the polyimide and the 1st SU-8 layers was measured using
238 a surface profiler (Tencor-P10, Tencor. Inc., USA).

239

240 **4. 3. Characterization of the device**

241 The device was placed in a plastic box, and a PBS solution (catalog no.
242 041-20211, Wako) was introduced into the device. Nitrogen gas was supplied to the box
243 to control the oxygen concentration in the solution. A commercial oxygen sensor was
244 inserted into the solution to check the concentration of dissolved oxygen. To prepare a 0
245 mM oxygen solution, Na_2SO_3 was added to the solution as an oxygen scavenger. All of
246 the column electrodes were biased at -0.60 V to monitor the oxygen reduction currents.
247 During switch-off, all of the row electrodes were biased at -0.60 V to consume oxygen
248 and inhibit its diffusion to the bottom disk electrodes (Fig. 4A). During switch-on, one of
249 the row electrodes was stepped from -0.60 to 0.00 V to restore the oxygen diffusion at
250 the desired crossing points. The oxygen reduction currents at the column electrodes

were normalized, as mentioned in the main text, to prepare a calibration curve for oxygen.

4. 4. Cell culture

A human breast cancer cell line (MCF-7) was donated by the Institute of Development, Aging, and Cancer at Tohoku University (Japan). RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) were used for the cell cultures. MCF-7 spheroids were formed by the hanging drop method. Briefly, MCF-7 were suspended in culture medium, 20- μ L droplets of the suspension containing 250, 500, or 1000 cells were introduced onto the cover of a culture dish. The droplets were then hung from the dish cover and cultured for 5 days to form MCF-7 spheroids. As a control, the cell aggregates were fixed with paraformaldehyde. Briefly, cells were immersed in 4% paraformaldehyde (Wako Pure Chemicals Ltd., Japan) for 20 min. Then, the cells were washed with a PBS solution.

4. 5. Electrochemical detection for investigating the respiration activity of MCF-7 spheroids

To investigate the respiration of MCF-7 spheroids, first, the device was filled with a PBS solution, and then cell aggregates were introduced into the microwells with a micropipette.

The imaging process for the detection of dissolved oxygen is shown in Movie S1. The switch was sequentially changed from off, on and off. The duration of the switch-on state was set as 60 s. The switching process was repeated 6 times to complete the electrochemical detection at all of the sensors (Movie S1), and the difference in current between the on and off states, Δ currents was calculated for each sensor.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (A) (No. 25248032), a Grant-in-Aid for Young Scientists (A) (No. 15H05415) and a Grant-in-Aid for challenging Exploratory Research (No. 26600057) from the Japan Society for the Promotion of Science (JSPS). This work was also supported by Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program from the Japan Science and Technology Agency. This work was also supported by the Asahi Glass Foundation.

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398 **Biographies**

399 **Kosuke Ino** received his PhD degree in engineering from Nagoya University in 2008. He
400 received Research Fellow of the Japan Society for the Promotion of Science (2006–2008).
401 He is currently working as an assistant professor in Graduate School of Environmental
402 Studies, Tohoku University.

403 **Yuta Yamada** is a master's student in Graduate School of Environmental Studies,
404 Tohoku University.

405 **Yusuke Kanno** is a PhD candidate in Graduate School of Environmental Studies,
406 Tohoku University.

407 **Shunsuke Imai** is a master's student in Graduate School of Environmental Studies,
408 Tohoku University.

409 **Hitoshi Shiku** received his PhD in engineering from the Tohoku University in 1997. He
410 is currently working as a professor in School of Engineering, Tohoku University.

411 **Tomokazu Matsue** received his PhD in pharmacy from Tohoku University in 1981. He is
412 currently working as a professor in Advanced Institute for Materials Research
413 (WPI-AIMR) and Graduate School of Environmental Studies, Tohoku University.

Figure 1

General outline of the electrochemical imaging device using molecular competition mode (not to scale). (A) The device consists of row (blue) and column (red) electrodes. The row and column electrodes, reference (RE), and counter electrodes (CE) are connected to a multichannel potentiostat. (B) Schematic illustration of the switching system in molecular competition mode. During switch-off, dissolved oxygen (the target molecule) is consumed at the top ring electrode at -0.60 V to inhibit oxygen diffusion to the bottom disk electrodes from the bulk. During switch-on, no oxygen consumption occurs at the top ring electrode at 0.00 V. Under both conditions, the bottom disk electrodes are biased

424 at -0.60 V for the acquisition of oxygen reduction currents. By applying the proper
425 potentials on the electrodes, electrochemical signals from all of the crossing points are
426 acquired. (C) Schematic illustration of the detection of respiration activity of cell
427 aggregates. Cell aggregates are introduced into the sensor wells, and electrochemical
428 images consisting of oxygen reduction currents at the sensors are constructed.
429

Figure 2

Schematic diagram of the device fabrication (not to scale). (A) Glass substrate; (B) Pt bottom electrodes (column electrodes); (C) Polyimide layer; (D) Pt top electrodes (row electrodes); (E) Removal of the polyimide layer by an RIE process to produce bottom disk electrodes; (F) Fabrication of the 1st SU-8 microwells at individual crossing points to produce top ring electrodes; (G) Fabrication of the 2nd SU-8 microwells at individual crossing points to trap cell aggregates.

Figure 3

(A) Photographic image of the device consisting of 36 sensor points with only 12 connector pads. (B) Operation of the device during measurements. First, the device enclosed in the acrylic frame is connected to the multichannel potentiostat and a matrix through the clip connector. Then, a sample solution is introduced into the sensor area. Finally, reference and counter electrodes are inserted into the solution.

Figure 4

Evaluation of molecular competition mode. (A) Schematic illustration of the potentials applied to the electrodes. The green dashed box indicates the sensing area. (B) Chronoamperograms from PBS solutions with various concentrations of oxygen. Electrochemical signals from one of the column electrodes before (B_I) and after (B_{II}) subtracting the background. (C) Calibration curve for oxygen ($N \geq 6$).

Figure 5

Electrochemical imaging using molecular competition mode for evaluation of the respiration activity of MCF-7 spheroids. For preparation of the cell aggregates, 20- μL droplets containing 500 cells were cultured for 5 days. (A) Optical and (B) electrochemical images of the living and fixed cell aggregates. (C) Bar graph showing $\Delta\text{currents}$ from no cell ($N = 58$), living cell aggregates ($N = 6$), and fixed cell aggregates ($N = 6$). (D) $\Delta\text{Currents}$ from the cell aggregates with various diameters. For preparation of the cell aggregates with various diameters, 20- μL droplets containing 250, 500, and 1000 cells were cultured for 5 days. (E) Relationship between estimated respiration activity per cell aggregates and diameter of cell aggregates. The orange line indicates the respiration activity as measured by SECM.⁴

Graphical abstract