Article

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Single embryo-coupled gate field effect transistor for elective single embryo transfer

Toshiya Sakata[*,1], *Akiko Saito*[1], *Jinji Mizuno*[2], *Haruyo Sugimoto*[1], *Kaori Noguchi*[3], *Eiko Kikuchi*[3] *and Hiroaki Inui*[3]

[1]Department of Materials Engineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

[2] Kato Ladies Clinic, 7-20-3 Nishi-Shinzyuku, Shinzyuk-ku, Tokyo 160-0023, Japan

[3] Inui Maternity Clinic, 3-5-18 Namiki, Koriyama, Fukushima 963-8026, Japan

Corresponding author * E-mail: sakata@biofet.t.u-tokyo.ac.jp

CORRESPONDING AUTHOR FOOTNOTE : Affiliation; Department of Materials Engineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan,113-8656 (Japan), TEL; +81-3-5841-1842, FAX; +81-3-5841-1842

ABSTRACT: In this study, we have proposed and demonstrated experimentally a novel monitoring device of single mouse embryo activity after in vitro fertilization (IVF) using a semiconductor-based field effect transistor (FET). The FET biosensor realized to detect it noninvasively, quantitatively and continuously by change of hydrogen ions with positive charges, which were induced by dissolved carbon dioxide due to cellular respiration activity during cleavage. The electrical signal of FET biosensor should become an effective indication to evaluate objectively single embryo activity as its morphology is observed subjectively after IVF. The platform based on the FET biosensor will contribute to promote elective single embryo transfer (eSET) in human assisted reproductive technology (ART).

KEYWORDS: embryo quality; cellular respiration; semiconductor; quantitative analysis; in vitro fertilization

BRIEFS: We found the possibility of noninvasive, quantitative and real-time monitoring of single mouse embryo activity using semiconductor technology for elective single embryo transfer (eSET), which was based on cellular respiration during cleavage after in vitro fertilization (IVF).

MANUSCRIPT TEXT:

Recently, assisted reproductive technology (ART) has been expected to be one of therapeutic methods of sterility. Engineers other than obstetricians have been required for assured success of ART programs. For in vitro fertilization (IVF) of one of ART programs, how to evaluate embryo quality and select embryo in condition are significant. Morphological evaluation has been widely used to rank embryo quality because microscopic analysis is noninvasive and useful in predicting pregnancy rates.[1,2] However, standard of classification for embryo quality seems to be ambiguous among operators because it is subjective method. Moreover, elective single embryo transfer (eSET) will be recommended in the future in order to prevent multiple pregnancy.[3] Therefore, novel principle to evaluate quality of single embryo quantitatively and noninvasively in a real-time manner is being required for practical use in ART.

In our previous work, the respiration activity of fertilized ova of sea urchin was monitored noninvasively, quantitatively and continuously as the change of pH by use of the principle of semiconductor-based ion sensitive field effect transistor (IS-FET).[4] The detection principle of IS-FET is based on the potentiometric detection of charge density changes at the gate insulator[5] and is applied for various biosensing.[6-9] Since the gate insulator usually consists of Si_3N_4 or Ta_2O_5 with hydroxyl group at the surface in solutions, furthermore, the IS-FET is sensitive to concentration of hydrogen ion with positive charge and should be utilized as the pH sensor. Therefore, pH variation based on respiration activity of embryo will be monitored quantitatively and noninvasively in a real-time manner using a single embryo-coupled gate FET for eSET (eSET-FET), because pH at the interface between embryo and gate membrane of FET will change sensitively according to dissolving of carbon dioxide into medium generated by metabolism and respiration activity in an embryo. Thus, the platform based on eSET-FET sensor will be valuable for the development of an evaluation system to select single embryo with good quality for eSET in the future.

On the other hand, oxygen consumption has been considered to be the parameter that provides the best indication of overall metabolic activity of single embryo,[10-14] although embryo metabolism has previously been assessed by measurement of nutrient consumption, such as glucose, pyruvate and amino acids.[15-18] As one of the detection methods for the evaluation of embryo quality, the electrochemical system is being developed. Shiku et al. reported previously the detection concept of oxygen consumption based on the respiration activity of embryo.[19] In this method, the oxygen reduction current was detected near the surface of single embryo using the cyclic voltammetry technique. However, this method is unsuitable for real-time measurement for a long term such as cleavage of mammalian embryo.

In this paper, we propose a semiconductor-based embryo sensing device for eSET (eSET-FET) to monitor single mouse embryo activity based on cellular respiration in a real-time, quantitative and non-invasive manner. Additionally, we report to have developed the simultaneous analysis system composed of microscopic observation and electrical measurement of eSET-FET under the adequate embryo culture

condition in incubator.

EXPERIMENTAL SECTION

Design of eSET-FET sensor. Insulated gate field effect transistors (FETs) as semiconductor devices were fabricated using the standard integrated circuit technology except for deposition of the gate electrode. N-channel depletion mode FET was designed in a 2 mm x 5 mm chip (ISFETCOM Co. Ltd.). The gate insulator was composed of Ta_2O_5, Si_3N_4 and SiO_2 layers, of which each thickness was 50, 100, and 100 nm, respectively. The gate surface contacting directly with embryo and culture medium was Ta_2O_5 layer, of which the safety was confirmed by estimating the rate of transplantation of cultured embryos on the surface to recipient mice. The fabricated FET chip was mounted on a flexible polyimide film with patterned copper electrodes and bonded. The FET chip was encapsulated with a glass ring except for the sensing area and was utilized in a culture medium as the eSET-FET sensor. The eSET-FET sensor was immersed in a culture medium (KSOM; arc resources JAPAN) with a platinum Pt reference electrode. Polydimethylsiloxane (PDMS) was utilized for mounting of glass ring and Pt electrode on the device because of non-toxicity for embryo culture. Single mouse embryo was put on one gate sensing area of the eSET-FET (**Figure 1(a)**). The embryologist put single mouse embryo on a gate area by use of micro pipette.

Mammalian embryo is very sensitive to external environments such as ion strength of culture medium, temperature and so on. This is why the total system for evaluation of embryo based on IVF should be prepared appropriately. **Figure 1(b)** shows the simultaneous analysis system of embryo activity by microscopic observation and electrical measurement using the eSET-FET sensor in the incubator. Using the simultaneous analysis system, the subjective and objective evaluation of embryo under the adequate condition of embryo culture can be performed in a real-time and non-invasive manner. Moreover, the sensing area was covered by 20 μl droplet culture medium, where single mouse embryo was kept on the gate of semiconductor, as shown in **Figure 1(c)**. The mineral oil of 1 ml was

introduced onto a dropped culture medium of 20 μl to prevent drying of medium. The droplet culture is useful to control position of single embryo on the gate and detect ion concentration change due to low volume.

Electrical measurement using eSET-FET sensor. The electrical characteristics of the eSET-FET sensor such as the gate voltage (V_G)-drain current (I_D) characteristics and the surface potential at the gate surface were measured in a culture medium using a semiconductor parameter analyzer (B1500A, Agilent) and a custom-made potentiometric analyzer (Radiance Ware Inc.), respectively. As the basic electrical characteristic, the threshold voltage shift ΔV_T was determined after embryo was placed on the gate. The ΔV_T was defined as a difference of the V_G-I_D characteristics at a constant drain current of 700 μA. The time course of the surface potential at the gate surface was monitored using a circuit[6] with which the potential change at the interface between an aqueous solution and the gate insulator can be read out directly at a constant drain current. In the present study, the gate voltage and the drain current were set to be 1 V and 700 μA, respectively.

Preparation of mouse embryo based on IVF. All the nutrient media to use for IVF were prepared on the day before experiments, and balanced gas in droplet was controlled through overnight under the adequate condition (5% CO_2 and 37 °C). Male ICR mice (9-10 weeks old; Charles River JAPAN Inc.) were sacrificed by cervical dislocation. Sperms collected from caudal epididymides of ICR male mouse were incubated for 1.5 h at 5% CO_2 and 37 °C. In this case, sperms of 2×10^5 cells/ml were seeded in HTF (human tubal fluid; Arc resource JAPAN) droplet of 200μl and subsequently cultured again in fresh HTF covered by mineral oil (Mineral Oil Light; REPROLINE) at 5% CO_2 and 37 °C. Female B6D2F1 mice (9-10 weeks old; Charles River JAPAN Inc.) were kept for 48 h from intraperitoneal injection of 5 IU PMSG (pregnant mare's serum gonadotropin; ASKA pharmaceutical JAPAN), and subsequently sacrificed by cervical dislocation after 15–16 h from intraperitoneal injection of 10 IU hCG (human chorionic gonadotropin; ASKA pharmaceutical JAPAN) for ovarian hyperstimulation. Oviducts were excised and drew to take out ova with a needle. The ova were introduced into HTF

droplet with sperm for IVF. After about 5 h from insemination, treated ova were washed and moved from the fertilization medium to KSOM (potassium simplex optimized medium; Arc resources JAPAN) droplet for culture. After the release of second polar body from an ovum was confirmed, the embryo was set up on a gate sensing surface of eSET-FET sensor and measured using it in the incubator system with microscopy. Rate of blastocyst-hatching and average of total cells in single embryo at blastocyst-hatching stage were investigated as shown in **Table 1**. Each counting was conducted at 90 h after measurements (IVF). Total cells were counted by use of fluorescent dye (DAPI) and confocal laser scanning microscopy. Moreover, transplantation of embryo to recipient mice cultured on the gate of semiconductor and the culture dish was conducted in order to confirm safety of embryo culture on the eSET-FET sensor. Actually, the blastocysts developed normally on gate during measurement were used for implantation to recipient mice. We got Institutional Review Board approval for this study. This study was overseen by an Animal Care and Use Committee in The University of Tokyo.

RESULTS AND DISCUSSION

In this study, it is very important to confirm firstly the safety of embryo culture on the semiconductor devices like on conventional culture dish. Actually, mouse embryo after IVF was normally cultured on the gate until blastocyst-hatching stage. **Table 1** shows the rate of blastocyst-hatching and the average of total cells in an embryo at the blastocyst-hatching stage for embryos cultured on the gate or the culture dish as control respectively. The rates of blastocyst-hatching were around 90 % for both conditions. The average of total cells for embryos cultured on the gate was also similar with that for embryos cultured on the culture dish. The culture of embryo on the gate was found to be no difference with the one on the culture dish. Furthermore, the safety of embryo culture on the gate was investigated by transplantation to recipient mouse (**Table 2**). The rates of offspring were compared for IVF-embryo transfer (ET) of embryos cultured on the gate or culture dish, respectively. Even embryos cultured on the gate of eSET-FET sensor obtained about the same offspring rate for IVF-ET with embryos cultured on the

conventional dish. These data mean that the semiconductor device itself never show toxicity for mouse embryo under the culture condition. Thus, the embryo sensing by use of eSET-FET sensor was confirmed to be safe for evaluation of embryo quality after IVF.

The detection principle of eSET-FET sensor is based on the potentiometric detection of charge density changes at the gate insulator, on which specific binding between target and probe molecules is made for molecular recognition. **Figure 2** shows the conceptual structure of the eSET-FET sensor for detection of embryo activity after IVF. Single mouse embryo was set on the gate sensing surface. The gate surface of eSET-FET sensor is immersed in a measurement solution together with a platinum (Pt) reference electrode **(Figure 1(c))**. Basically, ionic or molecular charges at the gate interact electrostatically with electrons in silicon crystal through the thin gate insulator and induce electrical signals by the field effect, resulting in the source and drain current (I_D) change at the channel (**Figure 2(a)**). Eventually, the output voltage is monitored as the shift of surface potential using a circuit (**Figure 2(c)**)[6] with which the potential change at the interface between an aqueous solution and the gate insulator can be read out directly at a constant I_D. The charge density changes based on hydrogen ions can be detected as the shift of surface potential of eSET-FET sensor, because a hydroxyl group reacts equivalently with a hydrogen ion with positive charge according to pH (**Figure 2(b)**). Here, this measurement principle is found to be noninvasive for embryo culture. The details of the eSET-FET sensor and the fabrication process have been reported previously by our group[6]. Furthermore, two types of eSET-FET sensors were prepared for differential measurements in this study; one was the eSET-FET sensor on which single mouse embryo was kept, and the other was the control FET sensor without embryo (**Figure 1(a)**). Using them, differential measurements were performed in order to eliminate the common unexpected signals such as temperature change, non-specific adsorption and change in ion concentration.

The change of surface potential at the gate surface of eSET-FET sensor was monitored after IVF (**Figure 3**). The surface potential of eSET-FET sensor with single embryo to blastocyst increased gradually as shown in **Figure 3(a)**. The increase of surface potential at the beginning of cleavage

indicates the increase of hydrogen ions with positive charges based on dissolving of carbon dioxide generated by cellular respiration. Since little surface potential change was measured before fertilization, when virginal ova were kept on the gate surface, the shift of surface potential based on the increase of hydrogen ion would reveal the change of metabolism due to fertilization. Mitochondria play an important role in respiration inside cells. Pyruvate molecules produced by glycolysis are transported into mitochondrion matrix through the inner membrane, where they are oxidized and combined with coenzyme A to form carbon dioxide, acetyl-CoA, and NADH. The mouse embryo after fertilization would become activated accompanied by cleavage resulting in the change of shape and amount of mitochondria,[20] which produce ATP and are closely related to metabolism of embryo. On the other hand, the surface potential change of eSET-FET sensor with single embryo accompanied by 2-cell block decreased gradually, which was similar with that of control sensor without embryo, although it increased drastically only for several hours before 2-cell stage. This electrical signal indicates an abnormal behavior of embryo before 2-cell block and that hydrogen ions with positive charges based on respiration activity increased drastically at the interface between embryo and sensor surface. This may be because metabolism in single embryo was activated accompanied by autophagy before cell death,[21] because proteins would be degraded to amino acids in autolysosome, which would be utilized for cellular respiration. Lastly, the control sensor without embryo showed actually the pH variation of culture medium for 90 h under the embryo culture condition. The surface potential change of control sensor reached to about -20 mV at around 40 h, which could be calculated as pH 7.4 to 7.7 because the semiconductor sensor had the detection ability of about 60 mV/pH. Moreover, the differential electrical signals were calculated as shown in **Figure 3(b)**. Basically, the electrical signal of sample sensor against control sensor should be evaluated considering unexpected signals such as temperature change, pH variation of culture medium and so on. The electrical signal of eSET-FET sensor with normal embryo against control FET (normal eSET-FET) was clearly different from that of eSET-FET sensor with abnormal embryo against control FET (abnormal eSET-FET) after around 2-cell stage. Interestingly, the

surface potential for normal eSET-FET increased gradually after IVF but its inclination changed significantly after around 2-cell stage of 10-15 h. This might show the change of metabolism during cleavage at around 2-cell stage. **Figure 4** shows the differential signal at 40 h after IVF in **Figure 3(b)**, which corresponds to around 8-cell stage for normal eSET-FET particularly. All the signals were calculated based on the differential measurements between sample sensor with embryo and control sensor without embryo. 21 embryos were cultured at 37 °C on the gate in monitoring surface potential. 18 embryos of them were normally cultured to blastocyst-hatching stage, but 3 embryos of them stopped cleavage around 2-cell stage. The difference of surface potential changes at 40 h between normal eSET-FET and abnormal eSET-FET were distinguished as a significant difference ($p<0.01$). Thus, which embryo reaches to blastocyst normally or not can be predicted at the early stage of cell division by evaluating surface potential change of eSET-FET sensor.

The average of surface potential change for normal eSET-FETs, which occurred cleavage to blastocyst-hatching stage, was 36.7 mV with standard error ±4.9 mV (n=18) at 8-cell stage of 40 h after IVF in **Figure 4**, considering the differential measurements. Basically, the eSET-FET sensor utilized in this measurement showed the average change of gate voltage 55.6 mV/pH for pH variation. Therefore, the respiration activity triggered by fertilization caused the change of about $\Delta pH=0.7$ at the interface between embryo and gate surface. Strictly, the shift of pH from 7.4 to 6.7 was detected at the interface by the eSET-FET sensor. The eliminated carbon dioxide was calculated as about 1.6×10^{-7} M based on the concentration change of hydrogen ion corresponds to pH variation, according to the equilibrium of carbon dioxide in solutions ($CO_2 + H_2O \rightleftarrows H^+ + HCO_3^-$). In the calculation, the oxygen consumption, the ATP synthesis and so on in the citric acid cycle inside the mitochondrion could be estimated from the amount of eliminated carbon dioxide, which is worked out from the electrical signal of the eSET-FET sensor. However, the diffusion of carbon dioxide at the interface between embryo and gate surface or from embryo surface apart from gate has to be considered in order to estimate accurately its generation. The volume of culture medium might affect actually on signals of pH variation based on embryos. In

this case, however, the volume of 20 μL used in this study was enough large for the size of embryo (about 100 μm), and actually, we could not find electrical signals when embryo didn't contact with gate area and placed far from the gate in the same volume. Therefore, we need to put directly embryo on the gate surface for measurement. Therefore, the respiration activity of single mouse embryo obtained by the electrical signals of eSET-FET sensor should be significant for convenient and noninvasive evaluation of embryo quality in the future.

CONCLUSIONS

In this study, the unique functionality of semiconductor-based FET sensor was applied for the development of quantitative estimation method of embryo quality for eSET in the interdisciplinary field between engineering and ART. The respiration activity during cleavage of single mouse embryo was monitored as pH variation at the interface between embryo and gate sensing surface using the eSET-FET sensor. The normal embryos proceeded to blastocyst showed significantly larger pH change than the abnormal embryos stopped cleavage at 2-cell stage considering the electrical signals of eSET-FET sensor. The electrical signal of eSET-FET sensor for single mouse embryo activity should become an effective indication to evaluate objectively embryo activity as its morphology is observed subjectively after IVF. The platform based on the eSET-FET sensor will contribute to promote eSET in human ART.

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FIGURE CAPTIONS:

Figure 1 Measurement device for embryo monitoring. A) Photograph of single mouse embryo on the

gate of eSET-FET sensor. The size of gate sensing area was 10 μm x 340 μm. The control sensor was placed at the distance of about 1 mm from the embryo sensor under the same condition. The photograph was taken at the 2-cell stage by upright microscope system in the incubator. B) Simultaneous embryo analysis system. Electrical measurement using the eSET-FET sensor can be continuously performed in various intervals of second or minute to ~ a few weeks or more in the incubator controlled at 37 °C and 5% CO_2. The upright microscopy was set up in the incubator. Therefore, the simultaneous analysis of embryo quality can be realized by microscopic observation and noninvasive electrical measurement under the culture condition in the incubator system. C) Sensing structure. Droplet of 20 μl was covered by mineral oil so that a long term embryo culture can be conducted. Mineral oil is non-toxicity and has permeability of gases such as CO_2 and O_2. Since embryo culture needs to be performed in a safe environment, polydimethylsiloxane (PDMS) was utilized for molding of glass ring and sensor electrodes.

Figure 2 A) Schematic illustration for measurement of surface potential of eSET-FET sensor. A shift of the threshold voltage V_T can be determined from the gate voltage (V_G)-drain current (I_D) characteristics in a culture medium (pH 7.4). The V_G was controlled through the Pt reference electrode. Saturated KCl solution used usually in the field of electrochemistry could never be utilized for measurement of mammalian embryo because of toxicity. B) The Si_3N_4 or Ta_2O_5 gate surface with hydroxyl group in solutions is sensitive to pH based on the concentration of hydrogen ions. The charge density changes at the gate surface induce the electrostatic interaction with electrons at the channel in silicon crystal resulting in the change of drain current. C) Circuit for measuring surface potential of eSET-FET sensor. The output voltage was continuously monitored as the surface potential change of eSET-FET sensor using this circuit, according to the conceptual structure of eSET-FET sensor shown in A)

Figure 3 Real-time and noninvasive monitoring of surface potential of eSET-FET sensor. A) The eSET-FET sensor with embryo and the control sensor without embryo were measured to analyze embryo activity based on respiration after IVF. The normal eSET-FET sensor with blastocyst (V_A) and the

abnormal eSET-FET accompanied by 2-cell block (V_B) were selected as different examples of eSET-FET with embryo, respectively. The control sensor without embryo measured basically pH change in the culture medium (V_C). B) Differential measurements of eSET-FET sensor with embryo against control sensor. The normal eSET-FET sensor with blastocyst against the control sensor indicates V_A-V_C, while the abnormal eSET-FET sensor accompanied by 2-cell block against the control sensor indicates V_B-V_C.

Figure 4 Surface potential change of eSET-FET sensor with embryo against control sensor without embryo at 40 h. 8-cell stage was often observed at around 40 h after IVF for normal eSET-FET. Different letters indicate statistical significance ($P<0.01$).

TABLES:

Table 1 Rate of blastocyst-hatching and average number of cells in single mouse embryo at blastocyst-hatching stage for embryos cultured on the gate of semiconductor and the culture dish.

Table 2 Result of transplantation of embryo to recipient mouse cultured on the gate of semiconductor and the culture dish.

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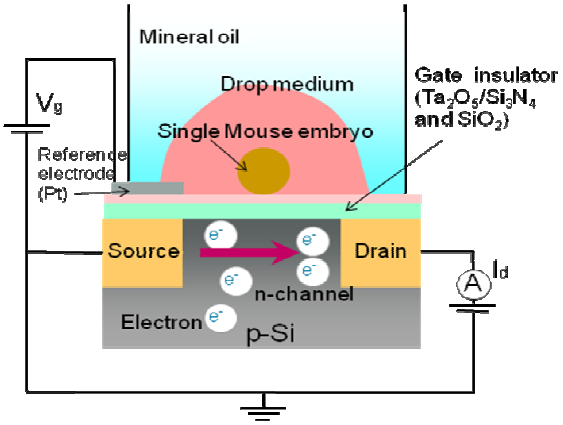
Mineral oil
Drop medium
Single Mouse embryo
Gate insulator
(Ta_2O_5/Si_3N_4 and SiO_2)
V_g
Reference electrode (Pt)
Source
Drain
I_d
A
n-channel
Electron
p-Si

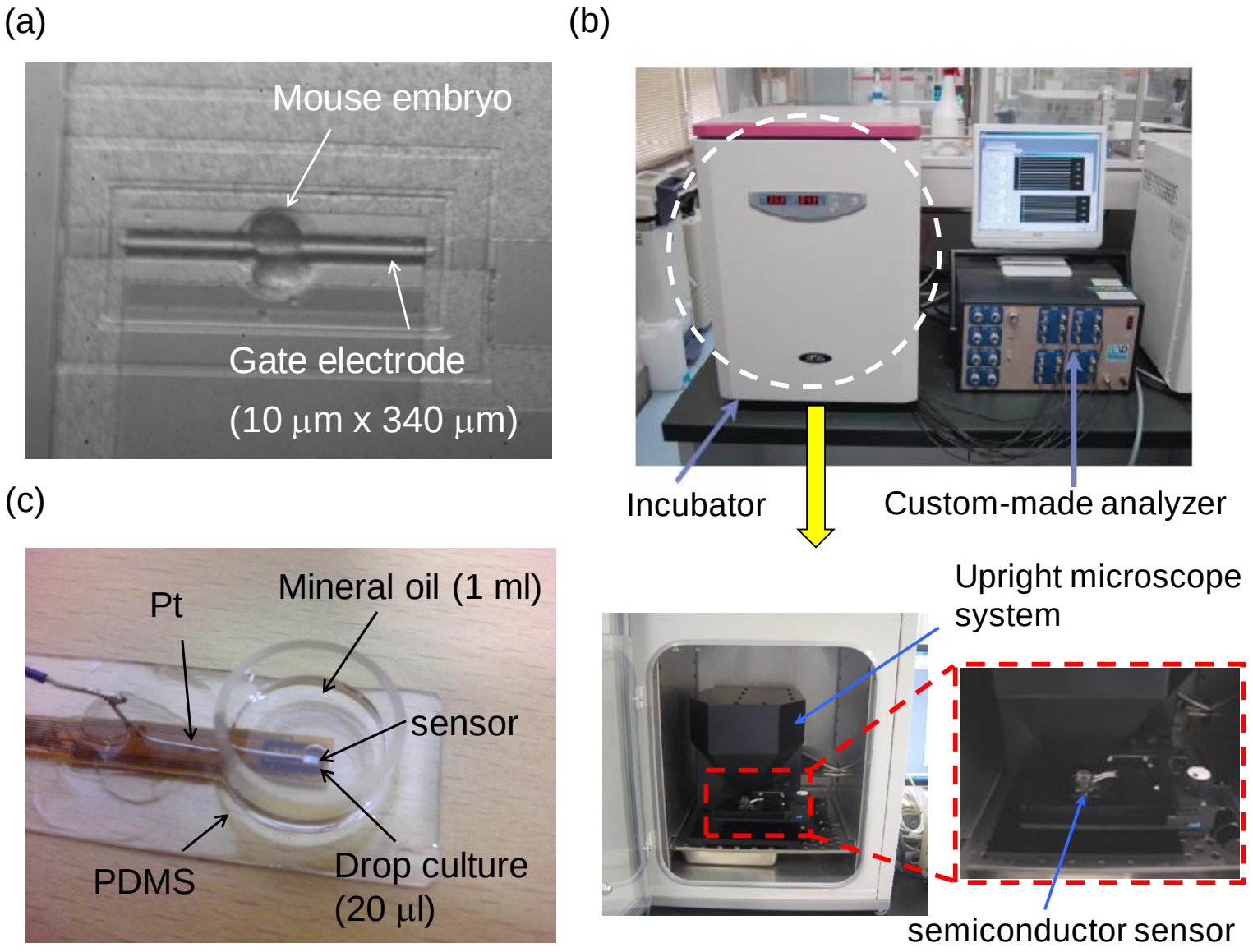
(a)
Mouse embryo
Gate electrode
(10 μm x 340 μm)
(b)
Incubator
Custom-made analyzer
(c)
Pt
Mineral oil (1 ml)
sensor
PDMS
Drop culture
(20 μl)
Upright microscope system
semiconductor sensor

Figure 1

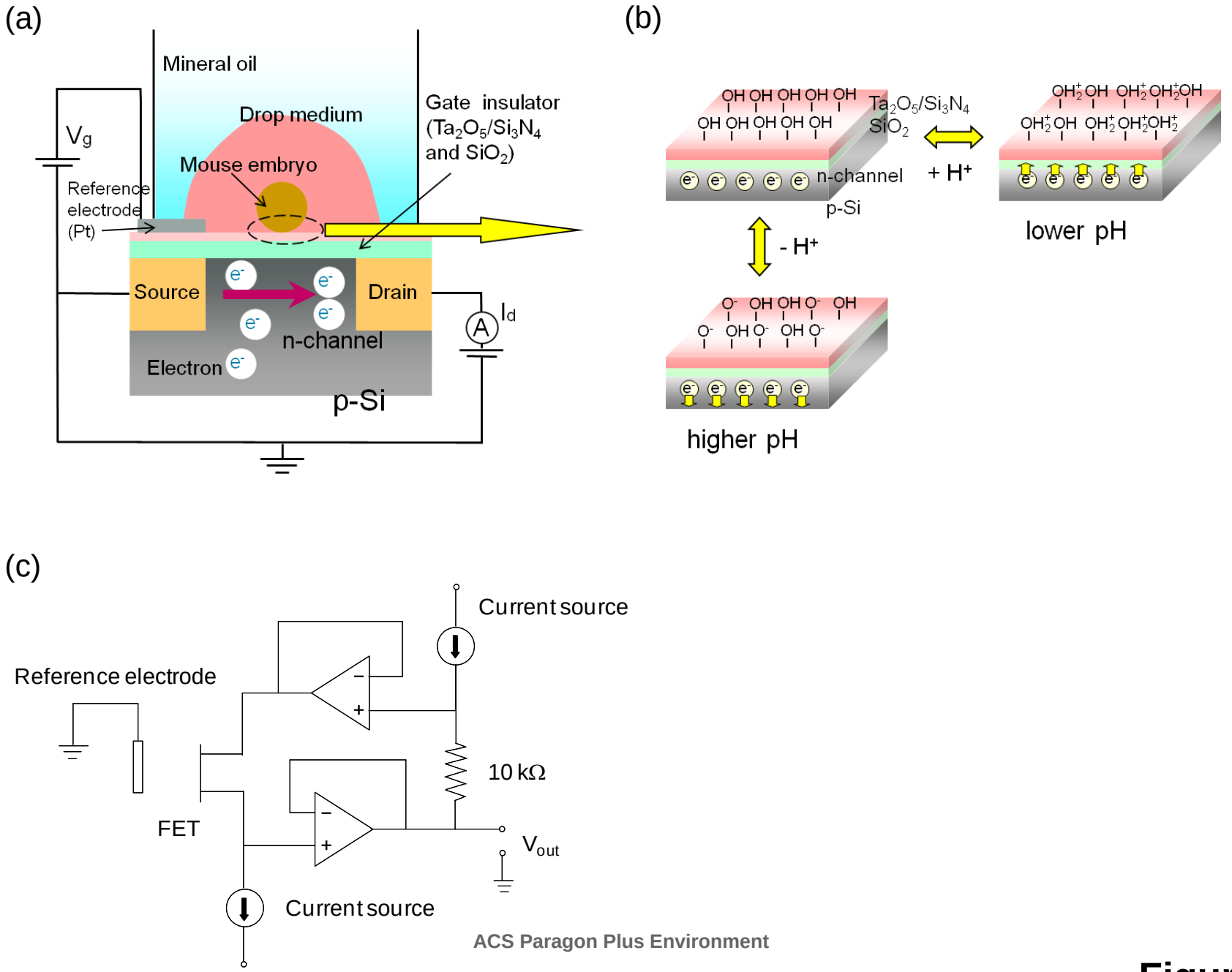
(a)
Mineral oil
Drop medium
Mouse embryo
Gate insulator
(Ta_2O_5/Si_3N_4 and SiO_2)
V_g
Reference electrode (Pt)
Source
Drain
I_d
A
n-channel
Electron
p-Si
(b)
Ta_2O_5/Si_3N_4
SiO_2
n-channel
p-Si
+ H^+
- H^+
lower pH
higher pH
(c)
Current source
Reference electrode
10 kΩ
FET
V_{out}
Current source

Figure 2

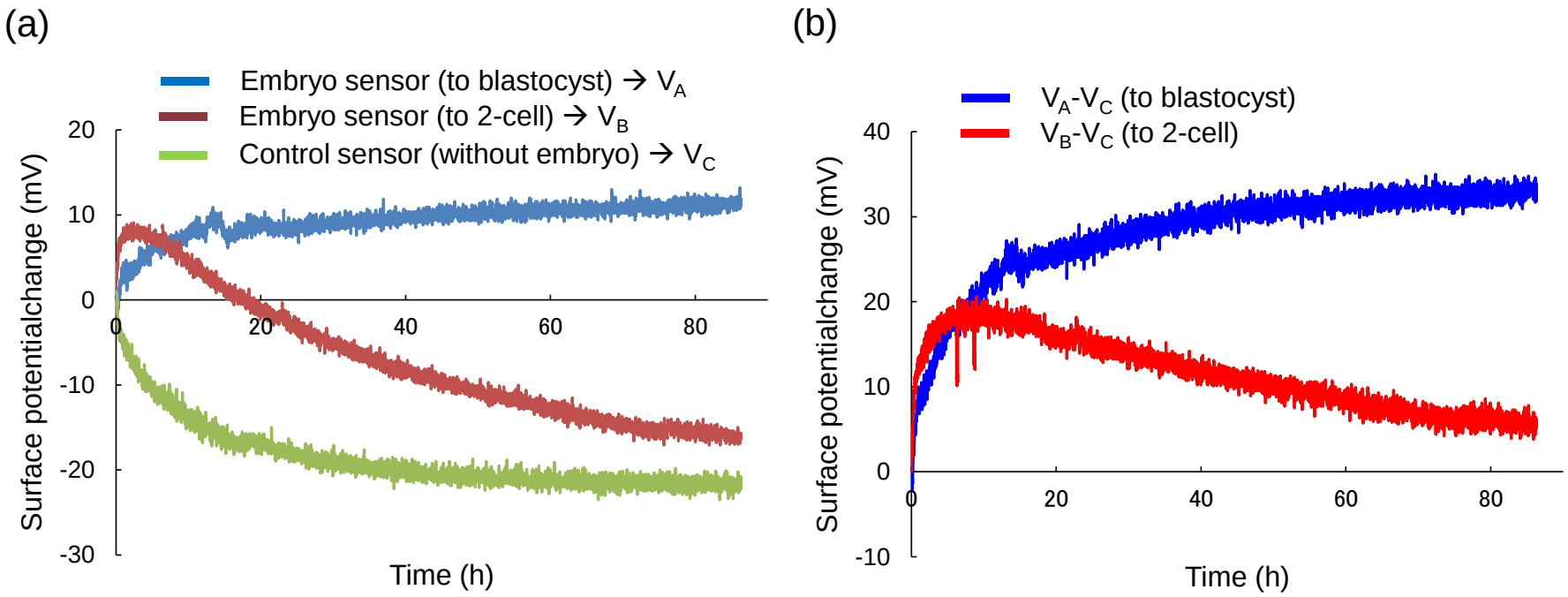
(a)
Embryo sensor (to blastocyst) → V_A
Embryo sensor (to 2-cell) → V_B
Control sensor (without embryo) → V_C
Surface potentialchange (mV)
20
10
0
-10
-20
-30
0
20
40
60
80
Time (h)
(b)
V_A-V_C (to blastocyst)
V_B-V_C (to 2-cell)
Surface potentialchange (mV)
40
30
20
10
0
-10
0
20
40
60
80
Time (h)

Figure 3

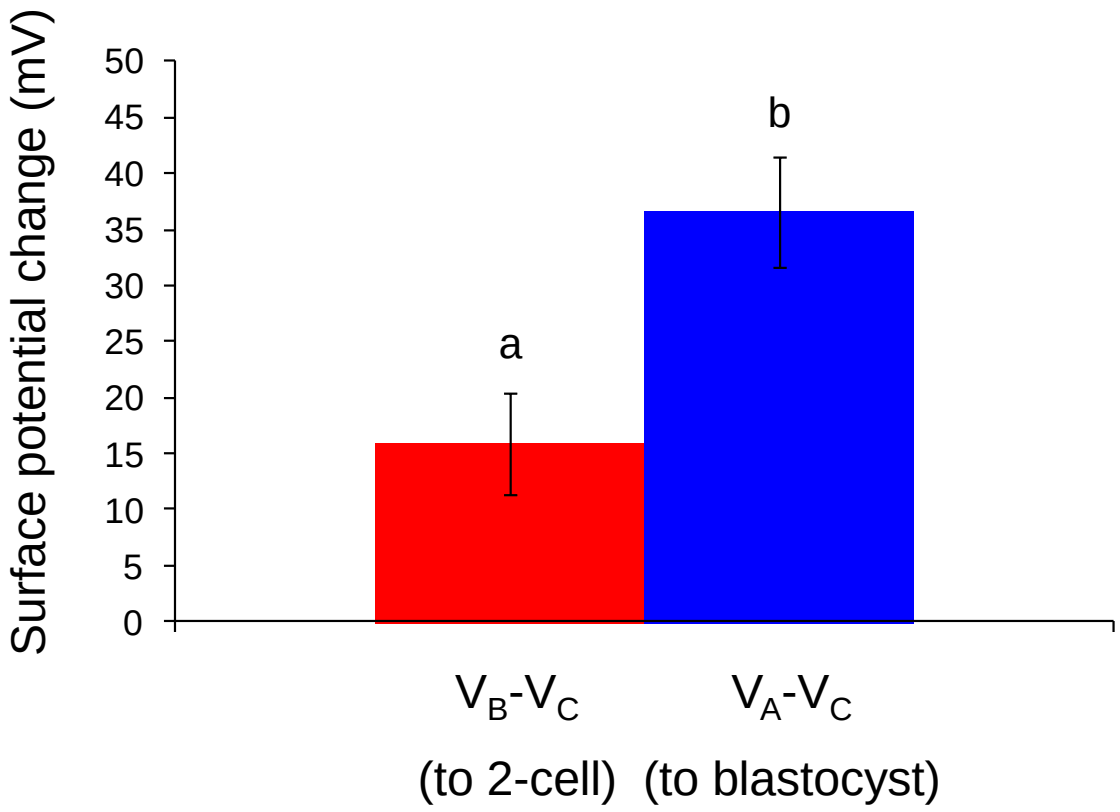
Surface potential change (mV)
50
45
40
35
30
25
20
15
10
5
0
a
b
V_B-V_C
V_A-V_C
(to 2-cell)
(to blastocyst)

Figure 4

	Gate of semiconductor	Culture dish
Rate of blastocyst-hatching (%)	88.1	96.8
Average number of cells in an embryo (%)	71.5	72.4

Number of embryo: 59 on gate, 424 on culture dish

Table 1

	Number of transferred embryo	Number of offspring	Rate of offspring(%)
Culture on gate of semiconductor	20	10 (♂6, ♀4)	50
Culture on dish	20	8 (♂5, ♀3)	40

All offspring grew normally. Deformed babies and abnormal behaviors were not found in this study.

Table 2