Ex vivo characterization of age-associated impedance changes of single vascular endothelial cells using micro electrical impedance spectroscopy with a cell trap

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We aimed to characterize aging of single vascular endothelial cells, which are indicators of senescence, using micro electrical impedance spectroscopy (μ EIS) for the first time. The proposed μ EIS was equipped with two barriers under the membrane actuator near the sensing electrodes, increasing its cell-trapping capability and minimizing the interference between the target cell and subsequent cells. The cell-trapping capability in μ EIS with barriers was considerably improved (90%) with a capture time of 5 s or less, compared to μ EIS without barriers (30%). Cells were extracted from transgenic zebrafish to minimize an initial discrepancy originating from genetic differences. In order to estimate useful parameters, cytoplasm resistance and membrane capacitance were estimated by fitting an electrical equivalent circuit to the data of *ex vivo* sensor output. The estimated cytoplasm resistance and membrane capacitance in the younger vascular endothelial cells were 20.16 ± 0.79 k Ω and 17.46 ± 0.76 pF, respectively, whereas those in the older cells were 17.81 ± 0.98 k Ω and 20.08 ± 1.38 pF, respectively. Discrimination of each group with different aging showed statistical significance in terms of cytoplasm resistance ($p < 0.001$) and membrane capacitance ($p < 0.001$). Considering both of the sensor and cellular level, the optimal frequency was determined as 1 MHz at which the electrical impedance of each group was clearly discriminated ($p < 0.001$). © 2016 AIP Publishing LLC.

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I. INTRODUCTION

The proportion of the world's population aged 60 years or older is continuously increasing and will be ~20% in 2050.¹ This trend has motivated various studies characterizing aging-related changes in cellular molecules and physico-mental capabilities in humans,^{1,2} animals,³ and non-mammals.⁴ There are two major approaches to understanding the aging process.

First, biomarkers are commonly used to determine aging. For instance, reactive oxygen species (ROS) accumulation,⁵ increased senescence-associated β -galactosidase activity,⁶ and telomere length⁷ are used as aging biomarkers. Combinations of several biomarkers in blood such as high sensitivity C reactive protein (hsCRP), high density lipoprotein (HDL) cholesterol, and ApoB100/ApoA1 were also suggested to indicate aging.^{8,9}

Second, decreased physico-mental functionality is another parameter to explain the aging process. Cardiorespiratory fitness, lung function (FEV1/FVC, FEV1), obesity (waist-hip ratio), creatinine clearance, gum health, and cognitive function have all been analyzed in conjunction with aging.^{10,11}

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These two indicators have several limitations for predicting senescence. Although biomarkers are relatively direct indicators, estimation of biomarker levels is a complicated and time-consuming process. In comparison, estimation of functionality is an indirect indicator of senescence and might be biased by subjective decisions of investigators and daily variations in conditions of patients. In this study, we proposed electrical impedance as a physical marker, not biomarker, to effectively characterize aging with simple, fast, and quantitative evaluation.

Micro electrical impedance spectroscopy (μ EIS) is an analytic tool used to characterize the electrical properties of single cells¹² using quantitative information such as conductance, capacitance, and dielectric constant.¹³ μ EIS has been demonstrated as an effective diagnostic tool as it is fast, sensitive, and does not require complicated procedures such as tagging or modifying.^{13,14} Indeed, several studies have shown that μ EIS can detect cancers in breast,^{14,15} prostate,¹³ and bladder¹⁶ by measuring the electrical impedance in a frequency domain.

μ EIS can measure electrical impedance by applying a frequency-dependent excitation signal through single cells. Each cell needs to be trapped during testing to ensure that its position remains stable between the sensing electrodes during the several seconds required for frequency sweeping. Several methods for effectively capturing cells have been reported using vacuum aspiration,^{14,17,18} dielectrophoretic force,^{16,19} surface modification,²⁰ and hydrodynamic fluid trapping.²¹ Recently, we introduced μ EIS integrated with a membrane actuator to capture target cells and measure the electrical impedance.¹³

In this study, we improved this method by employing barriers to increase the trapping capability and minimize the interference between a single cell being measured and subsequent cells. We applied the μ EIS to 4-month-old (younger) and 18-month-old (older) zebrafish cells for *ex vivo* characterization of age-associated changes in electrical impedance of single vascular endothelial cells. Unlike previous studies using different cell lines,^{13–18} our use of transgenic zebrafish can reduce a discrepancy originating from genetic differences. To the best of our knowledge, this is the first study to discriminate cells at different ages by measuring electrical impedance.

II. MATERIALS AND METHODS

A. Cell preparation

The 4-month-old (younger) and 18-month-old (older) adult transgenic zebrafish were used to sort GFP+ vascular endothelial cells for *ex vivo* measurement (gene: *flila*-GFP+). 10 and 15 zebrafish in the younger and older groups, respectively, were selected for heart dissection after anesthesia using tricaine. The hearts were transferred into Dulbecco's phosphate-buffered saline (DPBS) containing 2.5 mM ethylenediaminetetraacetic acid (EDTA) in a 15 ml tube. Subsequently, 100 μ l DPBS and 60 μ l Liberase DH were added to the tube to enhance cell viability. Samples were incubated at 29 °C until the tissue was fully dissociated. Finally, GFP+ vascular endothelial cells were sorted by fluorescence-activated cell sorting (FACS). The numbers of sorted younger and older cells were 3.12×10^4 in 0.1 ml and 5.64×10^4 in 0.2 ml DPBS solutions, respectively.

B. Device operation

Fig. 1(a) shows our μ EIS comprising a fluidic channel for cell flow, sensing electrodes for impedance measurement, and a pneumatic channel for cell trap. When pneumatic pressure is applied, the membrane actuator deflates the fluidic channel to capture single cells on the sensing electrodes, as depicted in Figs. 1(b) and 1(c). The design of our device has several technological merits: (1) the narrower width of sensing electrode than the elongated cell diameter and the tight electrical contact between the cell and electrodes allow the μ EIS to measure the intrinsic electrical properties of the cells with high sensitivity; and (2) two barriers were employed under the membrane actuator near the electrodes, increasing the cell-trapping capability and minimizing the interference between the target cell and subsequent cells.

FIG. 1. Schematic view of the μ EIS. (a) Overall view, (b) working principle of sensing, and (c) cell trap.

C. Fabrication

The three components (fluidic channel, sensing electrodes, and pneumatic channel) of the μ EIS were fabricated by micromachining. Initially, a silicon wafer was thermally oxidized in a furnace. The oxide layer was patterned by reactive ion etching through the photoresist (GXR601) pattern formed by photolithography. The patterns of the oxide mask were transferred to the silicon substrate by wet etching. The resulting fluidic channel contained a $15\ \mu\text{m}$ deep trench with slanted (54.74°) channel sidewalls because the etch rate of silicon is the lowest in the direction of the $\langle 111 \rangle$ crystallographic surface.²²

After the silicon substrate was re-oxidized to prepare the electrical passivation layer, Cr and Au films were conformally sputtered on the slanted sidewalls. The metal films were patterned by a lift-off process using a negative photoresist (PR) (DNR-L300) mask to form $10\ \mu\text{m}$ wide sensing electrodes.

For effective cell trap, two barriers (height: $5\ \mu\text{m}$) were fabricated at the bottom of the fluidic channel by patterning the negative PR, which was reflowed to prevent the barrier edges from damaging the trapped cells. For the top layer of the pneumatic channel, a mold of negative PR (SU-8 2025) was fabricated to produce a polydimethylsiloxane (PDMS) replica. For the membrane actuator, Cytop was spin-coated on a silicon wafer to prepare the hydrophobic layer, and the PDMS was spin-coated on the Cytop. Thereafter, the PDMS replica was bonded onto the spin-coated PDMS after plasma treatment. The bonded PDMS was detached from the Cytop to produce the pneumatic channel for cell capturing.

Finally, the PDMS pneumatic channel after plasma treatment was bonded onto the silicon substrate with the fluidic channel and sensing electrodes. Fig. 2 shows the fabricated μ EIS.

D. Impedance analysis

Electrical impedance, comprised of resistance (R) and reactance (X), can be obtained by applying alternating voltage between two sensing electrodes and measuring the induced current.²³ Conductance and capacitance of corresponding admittance can be expressed by the resistance and reactance according to Eqs. (1) and (2), respectively, where G is conductance, C is capacitance, and ω is radian frequency²⁴

FIG. 2. The fabricated μ EIS. (a) Overall view, (b) microscopic view with pneumatic pump off, and (c) microscopic view with pneumatic pump on.

$$G = R/(R^2 + X^2), \quad (1)$$

$$C = -X/\omega(R^2 + X^2). \quad (2)$$

In this study, we obtained 31 datasets each of magnitude and phase for frequencies from 1 kHz to 1 MHz. The measured electrical responses of the each cell group were analyzed in terms of resistance, reactance, conductance, and capacitance. Statistical analysis was performed to compare the average values between the younger and older cells (Student's *t*-test, PASW Statistics 18).

E. Experimental setup

The electrical impedances for the two groups of zebrafish cells were measured by connecting the μ EIS to an impedance analyzer (Reference-600), a syringe pump (Pump 11 Elite), a pneumatic pump (EFD 1500-XL), and a laptop. A voltage of 100 mV_{pp} was applied to the sensing electrodes of the μ EIS in the sweep frequency range from 1 kHz to 1 MHz. The flow rates of the syringe pumps were 0.5 μ l/min, and pneumatic pressure (350 kPa) was applied to the membrane actuator to capture the cells. All the experiments were conducted within 1 h after FACS to reduce cellular damage.

III. RESULTS AND DISCUSSION

A. Electrical impedance analysis of cells with aging

Two types of vascular endothelial cells in DPBS solutions were injected into the fluidic channel for cell flow. The membrane actuator was inflated by the pneumatic pressure to stop the cell flow and capture a single cell by deflating the fluidic channel. The cells freely passed through the microfluidic channel with a flow rate of 2 μ m/ms, until the flexible membrane made contact with the barriers. At the pneumatic pressure of 350 kPa, a single cell was blocked immediately before entering the trapping zone. Then, the pressure was reduced to allow a single

cell to squeeze between the electrodes. When a single cell was positioned at the center of the sensing electrodes, the pneumatic pressure was increased again, and the membrane actuator captured the cell on the surface of the electrodes. Cell capturing took 5 s or less, and we examined 30 captured cells from each group.

In our previous μ EIS study,¹³ the maximum displacement of the membrane actuator was $10\text{ }\mu\text{m}$, so that the gap between the membrane and the bottom side remained at $5\text{ }\mu\text{m}$. Thus, certain cells located underneath the membrane were free to leak out of the trapping zone. Moreover, subsequent cells to be measured showed a tendency to adhere to the target cell being measured, thereby hindering the impedance measurement for a single cell. In the present study, two barriers (height: $5\text{ }\mu\text{m}$) were newly employed at the front and back sides of the trapping zone in order to more efficiently trap a cell, which could then be easily released after measurement. This enables the μ EIS to increase the cell-trapping capability and minimize the interference between the target cell and subsequent cells. To verify the efficacy of cell capture, two types of μ EIS devices were fabricated with and without barriers, and experimentally compared. The μ EIS without barriers allowed cells to flow freely through the trapping zone even when the membrane actuator was inflated at 350 kPa. Further, the captured cells easily escaped the trapping zone when the pressure was slightly reduced to position the cell at the center of the electrodes. However, almost cells were easily captured in the trapping zone in the μ EIS with barriers, allowed efficient positioning of cells on the surface of the electrodes. Consequently, the acquired cell-trapping capability in the μ EIS with barriers was considerably improved (90%) over that of the μ EIS without barriers (30%). Furthermore, no measurement interference between the cells was observed because the barrier blocked the subsequent cells from adhering to the cell being measured.

The measured electrical responses of the cells were analyzed with regards to resistance, reactance, conductance, and capacitance. The average conductance and capacitance were more

FIG. 3. Age-associated conductance and capacitance changes at the sensor level between the younger (4-month-old, $n = 30$) and older (18-month-old, $n = 30$) cells versus frequency. Scatter plots of (a) average conductance, (b) capacitance, and bar graphs of (c) average conductance, (d) capacitance. The scatter plots show all values in the frequency range of 1 kHz to 1 MHz, while the bar graphs show the values at 1 kHz, 10 kHz, 100 kHz, and 1 MHz. Error bars indicate standard deviation.

TABLE I. The conductance and the capacitance at the sensor level for the younger (4-month-old) and older (18-month-old) cells at different frequencies.

Impedance	Frequency (Hz)	4-month-old (n = 30)	18-month-old (n = 30)	p-value
Conductance (μS)	1 k	0.11 ± 0.03	0.12 ± 0.03	0.141
	10 k	0.91 ± 0.17	1.12 ± 0.18	<0.001
	100 k	9.70 ± 0.32	9.91 ± 0.63	0.098
	1 M	105.32 ± 3.23	117.48 ± 3.01	< 0.001
Capacitance (pF)	1 k	148.48 ± 15.33	158.65 ± 16.64	0.017
	10 k	133.38 ± 5.81	137.18 ± 6.04	0.016
	100 k	101.63 ± 1.02	102.50 ± 1.38	0.008
	1 M	90.56 ± 1.09	91.27 ± 1.27	0.024

clearly differentiated than resistance and reactance in our experiments. Fig. 3 shows the average conductance and capacitance values of the younger and older cells in the sweep frequency range under the 350 kPa pneumatic pressures. Both the conductance and capacitance of the older cells are higher than those of the younger cells throughout the all frequency range. The conductance and capacitance of the younger and older cells are summarized in Table I.

In order to extract accurate values of cellular electrical properties, the diameters of the cells were measured from microscopic images before the cells were injected into the channel. The average diameters, and their standard deviations, of younger and older cells were $14.42 \pm 4.76 \mu m$ and $14.80 \pm 5.51 \mu m$, respectively. Meanwhile, the membrane actuator was designed to sufficiently squeeze the trapped cell, resulting in tight contact with the electrodes. This implies that even the smaller cells used in this study can fully occupy the volume between the sensing electrodes, and their excess portions will protrude out of the electrodes. Consequently, the deviation of the volume fraction occupied by the cell can be reduced, despite the cell-size error. The volume fraction will not be considered in the following procedure for extraction of cellular electrical properties.

Quoting data such as average conductance and capacitance at the sensor level can depend on the actual device being used as well as the measurement set up. Thus, cytoplasm resistance (R_{cyto}) and membrane capacitance (C_{mem}) values were extracted by fitting an electrical equivalent circuit to the data of the sensor output. A model of a cell that has a contact with the electrodes in medium²⁵ was employed in the equivalent circuit to estimate R_{cyto} and C_{mem} , as shown in Fig. 4. The equivalent circuit of the single cell can be represented by R_{cyto} in series with C_{mem} , because it consists of cytoplasm with a highly conductive ionic solution surrounded by a capacitive phospholipid bilayer with low conductivity.²⁶ The solution in the cell-flowing channel is expressed in terms of resistance (R_{sol}), which is connected in series with a single constant phase element (CPE) corresponding to the double layer impedance.^{25,27} R_{seal} represents seal resistance bypassing the trapped cell.^{25,28} Parasitic capacitance (C_p) is also included because a parasitic effect exists in a microdevice and measurement system.^{25,29}

FIG. 4. Electrical circuit models used for fitting the experimental data of the sensor output.

FIG. 5. Electrical circuit fitting to acquire electrical cellular properties. Fitted curves to average impedance of (a) younger (4-month-old) and (b) older (18-month-old) cells.

The best-fitting parameters from 1 kHz to 1 MHz were extracted using non-linear least squares fitting in the Gamry Analyst software. Initially, the impedance data without cells were used to calculate C_p . Then, the other parameters were extracted from the impedance data with cells. The goodness of fit, which is equivalent to the square of the overall percentage error, was calculated to evaluate the agreement between the simulated and experimental impedances.³⁰

Fig. 5 shows the curve fit to the average impedance data of each cell group, where the worst case of the goodness of fit was as small as 5.3×10^{-3} . The estimated R_{cyto} and C_{mem} values in the younger cells were 20.16 ± 0.79 k Ω and 17.46 ± 0.76 pF, respectively, whereas those in the older cells were 17.81 ± 0.98 k Ω and 20.08 ± 1.38 pF, respectively. Discrimination of two cell groups showed statistical significance in terms of R_{cyto} ($p < 0.001$) and C_{mem} ($p < 0.001$), as shown in Fig. 6. The specific membrane capacitances can be estimated by dividing the cell capacitance (C_{mem}) by twice the cross-sectional area projected from the sensing electrodes, considering that the electric field between the electrodes penetrates the two membranes in the front side and the backside. The estimated specific membrane capacitance of younger and older cells was 218.26 ± 9.54 mF/m² and 251.02 ± 17.27 mF/m², respectively.

The standard deviation of the cellular properties in the older cells was larger than that in the younger cells, which might be due to the wider distribution of cell size in the older group. Compensation for cell-size deviation needs to be considered in future researches to enhance the discrimination capability between different cell groups. Experiments using biological cells with relatively well-known electrical properties (for instance, normal and cancerous breast cells) will also be helpful to validate the system and the fit.

Zebrafish are considered mature after 3 months, and their total lifespan is 2–4 years in outbred zebrafish.³¹ The lifespan of inbred zebrafish is reduced by 10%–15% compared to that of outbred zebrafish.³¹ Furthermore, zebrafish lose their reproductive function after 2 years;³² therefore, the electrical impedance of zebrafish cells older than 24 months could be affected by hormonal changes or by epizootic disease. Thus, the older group consisted of healthy 18-month-old zebrafish for appropriate comparison with the younger group. The estimated electrical cellular properties imply that the cytoplasm conductance and membrane capacitance increased with aging. The higher cytoplasm conductance in older cells could be caused by an increase of lactic acid in the cytoplasm of endothelial cells with aging,³³ because the release of hydrogen ions increases with the concentration of acid. In addition, the increase in membrane capacitance might be due to the increase of oxidation by ROS production in the cell membrane with aging.^{34,35}

In flow cytometry, where cells are floating in a medium, several researchers reported that the electrical cellular properties of the cytoplasm and membrane influence the electrical impedance response at high frequency (>1 MHz).³⁶ On the other hand, when a single cell is being squeezed into a constriction channel, the cytoplasm resistance and membrane capacitance of cells were evaluated within 1 MHz.^{17,28,37,38} Other measurements were also carried out within 1 MHz with sensitivity enhanced by the tight contact between the cell and electrodes; the cellular properties were obtained.^{19,25,39,40}

FIG. 6. Electrical cellular properties of the younger and older cell groups. (a) Cytoplasm resistance and (b) membrane capacitance. Cytoplasm resistance is higher in the younger cells ($p < 0.001$), whereas membrane capacitance is higher in the older cells ($p < 0.001$).

B. Determination of optimal frequency

Although the averages of two samples may differ significantly, their error bars (standard deviations) can overlap, which hinder discrimination between them. A t -test is widely used to determine statistically whether two samples are significantly different by using p -values. However, when the p -value is less than 0.001, p -values are given as zero (PASW Statistics 18); therefore, it is difficult to determine optimal frequency at which the impedance of two cell groups is most distinguishable in the case of low p -values. Under these conditions, the discrimination index (D)¹³ can be used to quantify the effectiveness of discrimination and determine optimal frequency more strictly. Both D and p -values for our data are included in Fig. 7 to show their relevance.

D value is defined in Eq. (3), where G_R , A_R , U_m , L_M , U_A , and L_A are the gap ratio, average ratio, minimum value of the upper level, maximum value of the lower level, average of the upper level, and average of the lower level, respectively,

$$D = G_R \times A_R = (U_m/L_M) \times (U_A/L_A). \quad (3)$$

The largest average difference and smallest portion of overlapped error bars are needed for the highest D value. In other words, the D value increases as the data points are clearly differentiated with a low standard deviation. Fig. 7 shows both the D values and p -values in the sweep frequency range. Each value is highly correlated in that higher D values tend to

FIG. 7. The discrimination index (D) and p -value for identifying an optimal frequency. The D value and p -value of the conductance at the (a) sensor level and (b) cellular level. The highest D values at the sensor and cellular levels were found at the same frequency of 1 MHz.

FIG. 8. The electrical conductance at the optimal frequency (1 MHz). (a) Sensor level and (b) cellular level. The graphs show a distinct difference between each cell group ($p < 0.001$).

correspond to lower p -values. More specifically, D values are higher than 1.0 when corresponding p -values are below 0.001, which support the validity of the D value.

In order to speed up the electrical measurement (for so-called real-time operation), the optimal frequency at which discrimination capability is maximized needs to be determined, in terms of the D value and/or p -value.¹³ This is possible by directly utilizing the sensor output, because electrical cellular data require a time-consuming fitting process that hinders the ability for real-time operation. As shown in Fig. 7(a), D values and p -values were calculated from 30 spectral data of the sensor output for each cell group. The highest D value was found at 1 MHz in the sensor conductance within the measurement range, which is supported by the fact that the corresponding p -values were sufficiently low at 1 MHz.

To justify the optimal frequency (1 MHz) of the sensor level, D values and p -values of the cellular level were calculated from 30 spectra for each cell group dealing with the electrical cellular properties (R_{cyto} and C_{mem}) extracted in Sec. III A. As depicted in Fig. 7(b), the highest D value of the cellular level was achieved at the same optimal frequency of the sensor level (1 MHz). This also supports the fact that the optimal frequency can be selected from the sensor output. Figs. 8(a) and 8(b) represent the electrical conductance of each cell group at the sensor and cellular level, respectively, which indicates that the younger and older cells could be clearly discriminated at the optimal frequency ($p < 0.001$).

To examine the repeatability of μEIS measurements, the sensor conductance and capacitance of deionized (DI) water were measured before and after performing the measurements in each cell group. Thus, the repeatability tests were conducted in the following sequence: DI water, younger cells, DI water, older cells, and DI water. At the optimal frequency (1 MHz), the standard deviations of the conductance and capacitance in DI water were as small as $1.82 \mu\text{S}$ and 0.18 pF , respectively, which are sufficiently smaller than the average difference of each cell group at the sensor level (conductance difference: $12.16 \mu\text{S}$ and capacitance difference: 0.71 pF). This indicates that the proposed μEIS method could discriminate the age-associated impedance changes with high repeatability. Meanwhile, the average difference ratio of the conductance (10.35%) was higher than that of the capacitance (0.78%). Furthermore, the overlapped range between the two cell groups was smaller in the conductance than in the capacitance, which was also supported by the p -values shown in Table I. Thus, the conductance is more favorable than capacitance for discriminating the differently aged cells at the sensor level. To the best of our knowledge, no studies have yet measured the electrical impedance of endothelial cells under pressure, making it difficult to compare their measured cell impedance values with those in the literature.

IV. CONCLUSIONS

We have introduced μEIS as a method for effectively characterizing aging of single vascular endothelial cells by measuring electrical impedance. The *ex vivo* experimental results indicate that younger and older cells can be discriminated based on electrical impedance. The

estimated electrical cellular properties show that the cytoplasm conductance and membrane capacitance of 18-month-old cells are higher than that of 4-month-old cells. Discrimination of each cell group with different aging showed statistical significance in terms of cytoplasm resistance ($p < 0.001$) and membrane capacitance ($p < 0.001$). At the optimal frequency (1 MHz), both the sensor and cell conductance were clearly discriminated ($p < 0.001$). Further, the fabricated μ EIS with barriers showed high capability of cell trap (90%) with a capturing time of 5 s or less. To the best of our knowledge, the present study is the first attempt to differentiate cells at different ages using electrical impedance. Our method, which uses electrical impedance as a physical marker rather than a biomarker, can provide a useful tool for estimating senescence.

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