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Site-Specific Cytosol Sampling from a Single Cell in an Intact Tumor Spheroid Using an Electrochemical Syringe

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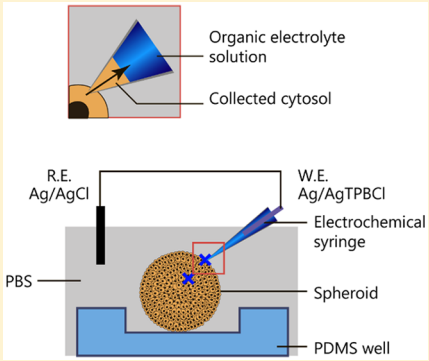
Supporting Information

ABSTRACT: A multicellular tumor aggregate, known as a spheroid, is an indispensable tool to study cancer biology. Owing to its three-dimensional organization, a spheroid exhibits an inherent gradient of nutrients, oxygen, and metabolites within itself. The spheroid provides culture conditions that resemble the microenvironment of certain cancer cells and causes these cells to acquire characteristics relevant to tumors in our body. However, site-specific gene expression analysis in an intact spheroid with single-cell resolution has not been explored. Recently, some types of electrochemical syringes were developed to extract cellular materials from living single cells for transcriptomic analysis. Here, we investigated whether an electrochemical syringe could be used to evaluate site-specific gene expression in a spheroid. A small amount of cytosol (roughly 540−1480 fL, less than the volume of a single cell) was successfully collected from the first, second, and third layers of the spheroid using an electrochemical syringe without causing damage to the spheroid architecture. We found that the *CCNB1* and *CCNA2* expression levels were different between the surface and the average of the entire spheroid, indicating that there are heterogeneous cellular functions across different regions of the spheroid. This method provides opportunities to improve our understanding of spatial gene expression of single cells in a three-dimensional environment.



A tumor microenvironment (TME) is a complex milieu of cells and extracellular matrix (ECM) proteins that plays a critical role in cancer development and progression.[1,2] Spheroids form a multicellular aggregate and have been successfully used as a culture-based method that resembles the TME in vitro.[3−5] The aggregated structure of the spheroids allows cells on the inside to be exposed to heterogeneous nutrients, oxygen, and other physical and chemical stresses, which recapitulates TMEs in our body.[6] The TMEs of different tumor cells vary depending on the spatial position in the spheroid. To understand the effect of TMEs on a tumor cell, it is crucial to integrate each cellular function with the spatial position in the spheroid.

Single-molecule FISH (smFISH), which uses multiple fluorescently labeled oligonucleotide probes that hybridize to target RNA molecules,[7,8] has been widely used to acquire site-specific transcriptome information on target cells.[9,10] Recently, smFISH with combinatorial labeling (seqFISH,[11] MERFISH[12]) increased the number of genes detected.[10] In addition, in situ RNA sequencing methods,[13−16] in principle, allow a complete analysis of all cellular transcripts at subcellular resolution. However, a current drawback of these fluorescence labeling methods is the need to section samples into thin slices (a few micrometers) due to the limited light penetration and the short working distance of the objective. Sample sectioning can be problematic as sample fractures may occur during the sectioning process.[17,18] Therefore, a method for site-specific gene expression analysis in an intact spheroid, without the need for sample sectioning, is highly demanded.

Cytosol sample collection using atomic force microscopy (AFM) probes, nanopipettes, or dielectrophoresis using integrated electrodes in the probes has been previously used for site-specific sampling and transcriptomic analysis.[19−24] More specifically, combining two-photon microscopy[25−27] or optogenetics[28] with patch-clamp pipettes has been a successful approach to target single neurons in deep regions of intact mice brains. Similarly, other studies were able to collect a cytosol sample for PCR[29] and RNA-seq analysis (Patch-seq)[30,31] from a target cell using a patch pipet after electrophysiological recordings. These previous reports indicate that cytosol samples collected by nanopipettes are promising for transcriptomic analysis; however, to our knowledge, there is no report on gene expression analysis of cytosol samples collected from an intact tissue using a nanopipette.

Our group,[32,33] as well as others,[34,35] recently reported the use of an electrochemical syringe[36] to enable site-specific sampling with subcellular resolution.[37,38] Although our previous study showed that cytosol could be collected from a spheroid,[33] it unveiled that the collected cytosol reflects site-

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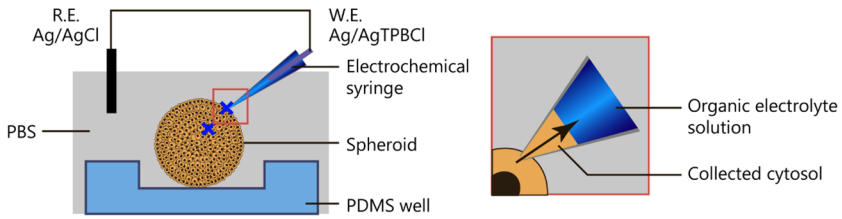

Figure 1. Schematic of the electrochemical syringe setup for the collection of cytosol samples from a tumor spheroid. Blue X marks represent cytosol collection points. The position of the cytosol−organic phase interface was controlled by the voltage between the reference and the working electrodes (R.E., reference electrode; W.E., working electrode). The enlarged view of the red square is shown on the right.

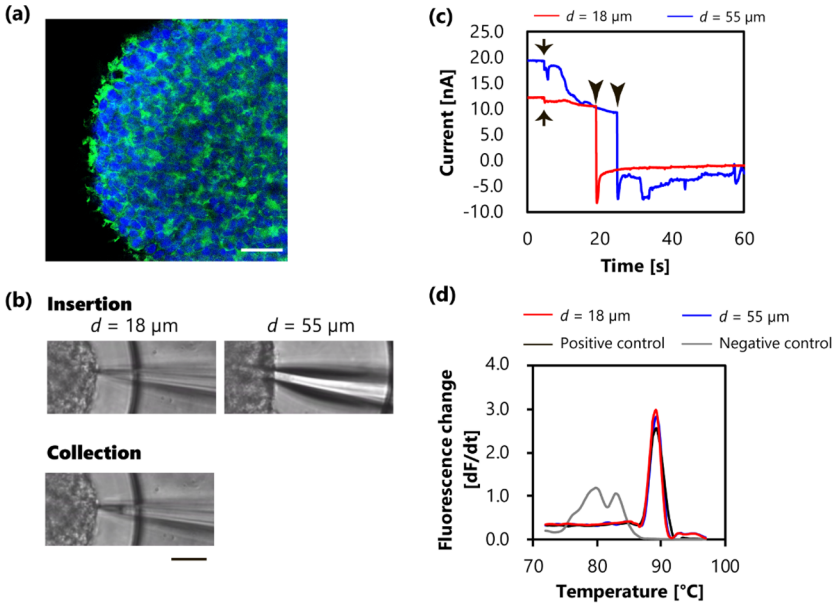

Figure 2. Cytosol collection using an electrochemical syringe. (a) Optical section of the MCF-7 spheroid. Green: F-actin; blue: nuclei. Scale bar is 50 μm. (b) Bright field images during the collection of cytosol from a target spheroid. "*d*" is the distance from the surface of the spheroid. Scale bar is 50 μm. (c) Chronoamperometry measurements during cytosol collection at different depths in the spheroid. Arrows: the point the glass pipet entered the cytoplasm; arrow heads: the point the voltage was switched from +1.0 to −0.5 V vs Ag/AgCl. (d) Melting peak analysis for the *GAPDH* amplicon after qPCR.

specific cell functions. In this study, we used the electrochemical syringe system to investigate site-specific gene expression of an intact spheroid at multiple locations. We collected cytosol samples from multiple positions of the spheroid, and the gene expression levels were analyzed by quantitative PCR (qPCR) (Figure 1). We found that the electrochemical syringe successfully reached the first, second, and third layers from the surface of the spheroid and collected the cytosol from each position. The gene expression analysis showed that cells in the outer layer (first to third layer of the spheroid) have a different gene expression profile from those of the average of the entire spheroid, indicating that site-specific gene expression occurs in the tumor spheroid. This method provides opportunities to improve our understanding of spatial gene expression of single cells in a three-dimensional environment.

■ METHODS

A schematic of the cytosol collecting process is shown in Figure 1. A spheroid in a polydimethylsiloxane (PDMS) well was immersed in a PBS solution (Wako). When the glass pipet was immersed in PBS, an oil−water interface was formed at the tip of the glass pipet. The voltage in the glass pipet was kept at +1.0 V vs Ag/AgCl to prevent PBS from flowing into the glass pipet as it approached the target spheroid.[33,34] After the glass pipet penetrated the cell, the voltage in the glass pipet was changed to −0.5 V so that the oil−water interface could move up and the cytosol could flow into the glass pipet. After collecting a cytosol sample, the glass pipet was retracted and broken in 9 μL of the reaction mixture (Tables S-1, S-2, and S-5). The reaction mixture and the tip of the pipet were mixed well using a Vortex mixer (IKA) for 10 s. The depth of the cytosol collection point was measured on the basis of the images captured during collection. Using the MCF-7 suspension, we prepared a calibration curve to calculate the amount of *GAPDH* from Ct values in the qPCR experiments, and we use this curve to estimate the quantity of mRNA collected in the electrochemical syringe. The other methods (a fabrication of the glass pipet and gene expression analysis) are described in the Supporting Information.

■ RESULTS

Collecting Cytosol Samples from Single Cells Located at Different Depths from the Surface of the Spheroid. First, looking at the optical section, we confirmed that single cells aggregated in a spheroid (Figure 2a). In the spheroid, each MCF-7 cell showed a similar diameter (18−20 μm). To collect a cytosol sample, the glass pipet was inserted at 6−55

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μm in depth from the surface of the spheroid (Figure 2b, insertion). We found that the maximum insertion depth was 55 μm; due to penetration resistance, the MCF-7 spheroid deformed and the glass pipet could not go deeper than 55 μm. The 55 μm depth corresponded to the third layer from the surface of the spheroid (average diameter of the cells: 20 ± 2, 20 ± 3, and 18 ± 2 μm in the first, second, and third layers from the surface, respectively, $n = 9$ for each layer), indicating MCF-7 in the first to third layers can be accessed by the glass pipet in our system. After the applied voltage was switched from +1.0 to −0.5 V vs Ag/AgCl, the oil−water interface moved up and the cytosol was successfully collected in the glass pipet (Figure 2b, collection).

The chronoamperometry measurements taken during sample collection are shown in Figure 2c. When a glass pipet entered the cytoplasm, the ion current decreased (Figure 2c, arrows), implying that there was a high resistance from the cellular membrane, which became more prominent when the glass pipet went deeper into the spheroid (Figure 2c; $d = 55$ μm, 4−26 s and Figure S-1). The current became negative after the applied voltage was switched from +1.0 to −0.5 V (Figure 2c, arrow heads), which allowed the cytoplasm to move into the pipet (Figure 2b, collection). We found that a number of peaks appeared during the cytosol collection, probably due to the collection of cell components with various charges and sizes. The melting peak of the PCR amplicon (*GAPDH*) from the collected cytosol showed a single peak (Figure 2d), corresponding to the positive control. The success rate was 29.0% (9/31). These results indicate that the PCR from the cytosol sample successfully generated target amplicons and that our collection system allows gene expression analysis of the cytosol. The amount of the house keeping gene (*GAPDH*) collected into the electrochemical syringe was equivalent to 0.015 ± 0.006 cells (ave. ± S.E., $n = 5$), although the volume of cytosol collected into the syringe was more than 500 fL, which corresponds to more than 25% of the cytosol from a single cell (the calibration curve is shown in Figure S-2). This result implies that most of the mRNA in the electrochemical syringe might be lost during the collection process. Although the collection ratio needs to improve in future studies, we believe that gene expression analysis is still possible for genes with high expression levels in the target cells, e.g., *GAPDH*. Nonetheless, we investigated whether relative gene expression analysis was possible using the cytosol collected in the electrochemical syringe.

Site-Specific Gene Expression Related to the Cell Cycle. We measured the expression levels of cell cycle related genes (*CCNB1* and *CCNA2*, Figure 3a). Due to the low expression levels and a minute amount of collected cytosol, the qPCR signal of *CDKN1C*, *PCNA*, and *IGF1* could not be detected in this study. We detected significantly higher expression levels in the cytosol sample collected by the electrochemical syringe than that in the cytosol from the entire of the spheroid (Figure 3a). This implies that the expression levels at the surface of the spheroid are greater than in the deeper regions of the spheroid. We did not observe a depth-dependent expression level from the first to the third layers of the spheroid (Figure S-3). We found that some of the cytosol stuck to the outer layer of the glass pipet, which might cause the contamination between the cytosol from first and third layers.

To test this hypothesis, we prepared spheroids with a smaller diameter (diameter = 215 ± 30 μm after 6 days in suspension culture, $n = 3$) and compared the gene expression levels with the lager spheroids (diameter = 458 ± 8 μm after 6 days in suspension culture, $n = 3$, Figures 3b and S-4). As expected, the smaller spheroid showed higher expression levels than the larger one, which is likely due to the higher surface to volume ratio of the smaller spheroid (Figure 3b). These results strongly suggest that the cytosol samples were properly collected from the surface of the spheroid leveraging the electrochemical syringe. At the surface of the spheroid, sufficient nutrients and oxygen are supplied to the cells to maintain proliferation, while in the deep regions of the spheroid, the cells are in a hypoxic microenvironment and show limited growth. The environmental difference between the surface and core might cause the different expression of *CCNB1* and *CCNA2*. Our expression analysis is in accordance with the high proliferation activities of the tumor cells at the surface of the spheroid compared to the average of the entire spheroid. Furthermore, our results demonstrate that relative gene expression can be evaluated from small cytosol samples collected with an electrochemical syringe.

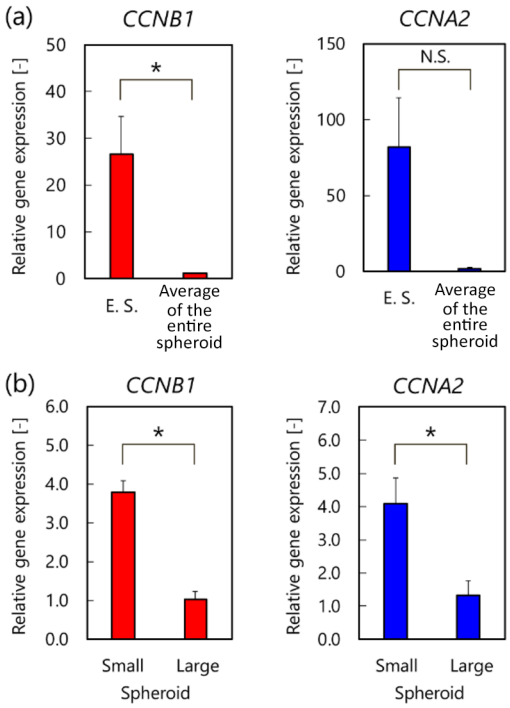

Figure 3. Gene expression analysis of cell cycle genes. (a) *CCNB1* and *CCNA2* expression levels at the surface (collected by the electrochemical syringe (E.S.)) and the average of the entire spheroid. *: $P < 0.05$ (*t*-test); $n = 5$ for E.S. and 6 for the average of the entire spheroid. (b) Comparison of *CCNB1* and *CCNA2* expression between the small (diameter = 215 ± 30 μm) and large (diameter = 458 ± 8 μm) spheroids after 6 days in suspension culture. Cytosol samples were collected from the entire of the spheroids. *: $P < 0.05$ (*t*-test); $n = 6$ for each. Error bars indicate the standard errors.

■ CONCLUSION

In this study, we used an electrochemical syringe as a tool to evaluate gene expression at different locations of a tumor spheroid. The electrochemical syringe successfully collected small amounts of cytosol (540−1480 fL) from the first, second, and third layers of the spheroid without causing severe damage to the spheroid structure. We found that the gene expression levels of genes involved in the cell cycle were different in the

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cells found at the surface of the spheroid compared to those found in the average of the entire spheroid, indicating that there are heterogeneous cellular functions in a tumor spheroid. Although the insertion depth was limited to less than 55 μm, our main goal was to show that site-specific cytosol collection from an intact tissue model using an electrochemical syringe was possible. Our method provides opportunities to increase our understanding of spatial gene expression of single cells in a three-dimensional environment.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b02062.

Additional methods; chronoamperometry measurements; calibration curve for the estimation of the amount of mRNA; relationship between the collection depth and the relative gene expression; bright-field images of the large and small spheroids; tip of the glass pipette obtained by scanning electron microscopy; volume calculation of the collected cytosol; stock solution for primer pair mix; RT and pre-amplification solution; exonuclease solution; nested qPCR solution; primer list for pre-amplification and nested qPCR; thermal conditions for RT and pre-amplification; thermal conditions for the exonuclease reaction (PDF)

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Author Contributions

[‖]Y.N. and M.E. contributed equally to the Article. Y.N. and H.S. conceived the project. M.E. performed the experiments and analyzed the data. K.I. wrote the software program to regulate the electrochemical equipment. Y.N., K.I, and H.S. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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