**One Hour In Vivo-like Phenotypic Screening System for Anti-cancer Drugs Using a High Precision Surface Plasmon Resonance Device**

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In anti-cancer drug (candidate) screening, there is the need for evaluation at physiological concentrations similar to in vivo. This is often performed by three-dimensionally (3D) cultured cells; however, it requires a long culture period of 2 - 4 weeks with tedious experimental procedures. Here, we report on a high precision surface plasmon resonance (HP-SPR)-3D system. We developed the system with average fluctuation of 50 ndeg s\(^{-1}\) using two-dimensionally cultured cells attached onto a sensor chip by applying collagen on the top to change their activity into in vivo-like conditions without cell division. It allowed in vivo-like phenotypic screening for anti-cancer drugs within 1 h of drug addition. The data were collected as the stable linear signal change parts for at least 5 min after 25 min following drug addition. The results provided compatibility to clinically related chemosensitivity test for anti-cancer (\(P < 0.001\)) using two cell lines of pancreatic cancer and three anti-cancer drugs to represent differences in individual gene expression and drug mode of action.

**Keywords** Surface plasmon resonance sensor, label-free biosensing, in vivo-like phenotypic screening, anti-cancer drugs

(Received January 11, 2018; Accepted June 11, 2018; Published October 10, 2018)

**Introduction**

A cell-based assay using human cells is frequently employed as a phenotypic assay for chemosensitivity and toxicity tests for anti-cancer drugs.\(^{1,2}\) For this, two-dimensional (2D) cell culture is used often because of its low cost and easy handling. However, 2D cell culture is performed under an altered environment, resulting in the loss of extracellular matrices and three-dimensional (3D) architecture compared to in vivo condition. The 2D cell culture has a different status from in vivo-like.\(^{6,14}\) As such, the 3D cell culture is used as a restructured in vivo-like status for drug screening.\(^{1,2}\) In the 3D cell culture, reports are categorized into spheroid culture including self-aggregated multicells, scaffold culture and matrix embedded culture, using devices such as 3D printer and hanging drop.\(^{8,13}\) By 3D cell culture, many studies report that gene expression becomes in vivo-like.\(^{6,14}\) The 3D techniques require a cell culture period and live or dead cells in spheroids have to be counted at the endpoint. For this, fluorescence labeling is frequently used and the use of electric measurements such as impedance is also reported.\(^{1,5,8,12,15,16}\) However, the use of a fluorescence agent may not give reliable results due to competitive reaction between the agent and the applied drug, and costs are high.\(^{17,19}\) On the other hand, impedance and electrical current will bring toxicity to live cells and may lead to measurement error.\(^{2}\) Furthermore, in vivo-like 3D techniques well related to clinical tests requires a long cell culture duration of at least 1 week to endpoint, which necessitates periodical culture medium exchange and may cause low reproducibility brought by human errors.\(^{2}\)

Consequently, many systems including micro fluidics are developed for high throughput phenotypic chemosensitivity testing.\(^{5,12,19}\) but these are not widely used with high reliability.\(^{19,20}\) To solve these issues, a rapid and reliable in vivo-like phenotypic screening method and device are demanded to give quantitative evaluation at physiological concentrations of drugs. For this purpose, we have developed a high precision surface plasmon resonance (HP-SPR) device and a method for its application.\(^{2,21-23}\) Our considerations were as follows.

1. An SPR device can detect interactions between materials for label-free and real-time affinity and kinetics research.\(^{24}\) It collects induced refractive index (RI) changes of the dielectric material attached on the metal surface.\(^{25}\) Since cell activity is often expressed as the dielectric polarization magnitude,\(^{26}\) we hypothesized that SPR could detect this.
2. The targeted 1000 cells were considered to have only a couple of mV s\(^{-1}\) of the dielectric polarization demanding a 10\(^{-4}\) deg level of precision.\(^{25,27}\)

We have achieved the device with average fluctuation of 50 ndeg s\(^{-1}\). Subsequently, self-attached live solid cancer cells on a sensor surface were analyzed after application of target anti-cancer drug. The HP-SPR signal change rate was monitored.
in real time for 1 h after the drug addition and the constant change period was picked up for 5 – 10 min. The data were correlated to cell viability of conventional test results of 2D cell culture for 48 h. The signal of the dielectric change originated from mitochondria membrane potential (MMP) caused by a proton gradient across the inner membrane by proton pumping in respiratory chains. This is a label-free method and does not require cell culture (cell division). Additionally, the method was independent of individual differences and to drug mode of action; therefore, this is a rapid phenotypic cell-based assay with possible high-throughput manner.

However, the HP-SPR system uses 2D cells attached onto a sensor chip, which does not allow anti-cancer drug evaluation at physiological concentrations. Theoretically, SPR can detect only materials attached or very close at nearly half of incident light wavelength. Consequently, we cannot use 3D cultured cells for the HP-SPR system.

Here, we report on the newly proposed HP-SPR-3D system, which evaluates the efficacy of an anti-pancreatic cancer drug at physiological concentrations. Because pancreatic cancer does not respond to many anti-cancer drugs efficiently contrary to other types of cancer, the number of deaths is increasing in pancreatic cancer patients. Thus, our first target focused on pancreatic cancer.

First, HP-SPR-3D was examined by the activation of 2D cells into in vivo-like status on a sensor chip without cell division by applying collagen on the top of the cells. Second, the HP-SPR-3D results obtained were compared to the results of a clinically related chemosensitivity test for pancreatic cancer, collagen droplet embedded culture drug sensitivity test (CD-DST), and the HP-SPR-3D was validated as an in vivo-like system.

**Experimental**

**Cell culture**

Human pancreatic cancer cells of MIA PaCa-2 (purchased from RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan) and PANC-1 (donated by Prof. Mitsuo Katano, Kyushu University, Medical Department) were cultured in an incubator (37°C and CO₂ 5% at 91% humidity) using DMEM (Dulbecco’s Modified Eagle’s medium, Thermo Fisher Scientific, Waltham, MA, USA) containing penicillin-streptomycin (5000 U mL⁻¹) (Thermo Fisher Scientific, Waltham, MA, USA), fetal bovine serum (FBS) 10% (v/v) (GE Healthcare Life Sciences, Marlborough, MA, USA) and defined as Medium A. Experiments were performed with cells in the logarithmic growth phase.

**Cell viability assay by CD-DST**

Cell suspension of 3.5 × 10⁶ cells mL⁻¹ was prepared for each cancer cell line with Medium A. Using a collagen drop kit (Primaster® KIT, Kurabo Industries, Osaka, Japan), a total 1/10 concentration of the cell suspension was prepared with Medium B consisting of solution A of (Collagen Cellmatrix Type CD), solution B (10-fold concentrated F-12 medium) and solution C (reconstitution buffer) 8:1:1 (v:v:v). A total of 3 drops containing 30 µL cell suspension each were plated into a 6-well microtiter plate and kept in the incubator for 1 h. Then, 3 mL each of Medium A was poured into each well and the plate was incubated for 24 h in the incubator. After this, anti-cancer drug solutions were added to obtain target concentrations and the plate was incubated for an additional 24 h. Then, the medium was replaced with 4 mL of Medium A for each well and the plate was shaken for 10 min using a plate mixer in the incubator, and this process was repeated again. The medium of each well was replaced with 4 mL of PCM-2 medium (Primaster® KIT, Kurabo Industries, Osaka, Japan) and the medium exchange was repeated every 2 – 3 d for 7 d. Each well medium was replaced with 3 mL DMEM containing 0.1% collagenase (Collagenase L, Nitta gelatin, Osaka, Japan) and was shaken for 30 min in the incubator. Cells were stained by trypan blue (Wako Pure Chemical Industries, Osaka, Japan) after removal of collagen. The number of cells was counted by a hemocytometer and cell viability was calculated. As anti-cancer drugs, doxorubicin, paclitaxel and gemcitabine (Sigma-Aldrich, Saint Louis, MI, USA or Tokyo Chemical Industry, Tokyo, Japan) were used at the concentrations of 0, 25 and 50 nM, 1, 2.5, 5 (MIA PaCa-2 only) and 10 nM (PANC-1 only), and 25, 50 and 100 nM, respectively (Fig. 1). Doxorubicin was diluted with dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) and others were with ultrapure water. The experiments were performed in triplicate except for incubation duration and reproducibility tests.

**Real-time monitoring of surface plasmon resonance angle by HP-SPR**

In an HP-SPR-3D experiment, a polypropylene ring with about 1.5 mm height, 9 mm outer diameter and 8 mm inner diameter was adhered on a sensor chip with a deposit of 45 nm-
thick gold on a BK7 glass slide with collagen (Type A-I, Nitta gelatin, Osaka, Japan), as shown in Fig. 2. Cell suspension of $1 \times 10^6$ cells mL$^{-1}$ was prepared with DMEM and 100 $\mu$L of it was added into the ring and kept overnight in the incubator. After this, 60 $\mu$L of the culture medium was removed and 30 $\mu$L of Medium B was added onto the cells and kept in the incubator for a while. Then, the sensor chip was set on the HP-SPR instrument and filled with 5 mL EMEM (Minimum Essential Medium Eagle) (Sigma-Aldrich, Saint Louis, MI, USA or Thermo Fisher Scientific, Waltham, MA, USA). The measurement was achieved using a p-polarized 3 mW diode laser and a silicon photodiode under 37°C and CO$_2$ 5%. After the HP-SPR signal became stable, the medium was replaced with 5 mL EMEM containing a drug at the same concentration as CD-DST. In the conventional HP-SPR experiment, DMEM was used instead of Medium B.

Stable linear signal change portions were picked up for at least 5 min after 25 min following drug addition and the HP-SPR angle change rate was calculated subtracting the change rate of control. The experiments were triplicated except for collagen overlay time.

**Statistical analysis**

Curve fitting and two-way ANOVA were performed using SigmaPlot 13.0 (Systat Software, San Jose, CA, USA).

**Results and Discussion**

**Requirement of collagen for HP-SPR-3D**

The cancer cells maintain a status characterized by less than normal chemosensitivity and activity during metastases, and the activity is recovered after a few hours of implantation. By this phenomena, we hypothesized that similar cell activation would occur after a few hours when the cancer cells were brought into contact with collagen existing outside of the epithelial cells, imitating implantation.

We tried this first as shown in Fig. 2. Here, different from previous works, we placed a plastic ring to fill the cells with collagen, and measured two samples simultaneously because of high reproducibility to be discussed later. We showed an example of the comparison measurement with or without using collagen in Fig. 3. This is a result of MIA PaCa-2 at 50 nM doxorubicin obtained by HP-SPR. Since the drug requires approximately 30 min to penetrate into the cells, the figure depicts the trend after 25 min following drug addition with the y-axis scale off-set at 25 min. In the case of the cells not covered with collagen (2D cell culture status), almost no trend of HP-SPR signal change was observed, similar to the control, although the cells should respond to the drug at the concentration determined by the conventional 3D cell culture method of CD-DST, which is applied in clinical therapy. In contrast, when the cells were overlaid with collagen for 4 h (3D conditioned cell culture status), chemosensitivity was observed as a trend of MMP decrease. The used drug was classified as across the cell membrane type acting intracellularly. However, the HP-SPR signal was little affected by the drug, unlike the case of the cells not covered with collagen. And it showed a similar trend to the result of the signaling echo method, in which the applied drug was removed after its penetration into the cells. Therefore, when the cells were covered with collagen, it is assumed that an anti-cancer agent would easily pass the cell membrane without disturbing cell membrane potential. On the other hand, in this experiment, it was necessary to supply 5% CO$_2$, which is different from previous works. Consequently, collagen and CO$_2$ are considered to be important for activation of the cells for in vivo-like status because 3D cultured cells accommodate a high penetration degree of the applied agent.

From the above, we considered the cell activity became in vivo-like due to the collagen covering, and then we secondary examined the overlay time. The results are shown in Fig. 4. As a result, from 4 to at least 5.5 h, constant MMP decrease was observed, indicating the feasibility of our proposed HP-SPR-3D method.
Effectiveness of HP-SPR-3D

In order to verify the effectiveness of this new HP-SPR-3D system, the results were compared with a standard 3D cell culture method, the CD-DST method used in clinical therapy, for testing cell viability.

The two cell lines used have mutations in the K-ras and p53, although the mutation point is different for each cell line, expressing individual differences. The anti-cancer drug doxorubicin used here works by inhibiting topoisomerase-II upon insertion into the DNA, leading to cleave the DNA strand. Different from these, gemcitabine is metabolized into di- and tri-phosphorus oxides in cells to work as active nucleotides, which kill cells by inhibiting DNA synthesis both directly and indirectly.

The relationship between cell viability by a standard method of CD-DST and signal change rate by the new method of HP-SPR-3D is shown in Fig. 5. As observed in the figure, a significantly high correlation between signal change rate and cell viability was obtained (P <0.001) regardless of both the type of cell line and anti-cancer drug. Furthermore, the results obtained confirmed the ability to evaluate the drug efficacy regardless of the differences in both individual gene expression and action mechanism of anti-cancer agents. Here, the relative standard deviation (RSD) for the determination of SPR angle change rate by HP-SPR-3D was 7.5% for the four repeated analyses with MIA PaCa-2 at 5 nM paclitaxel. The value is smaller than 12.2% RSD of CD-DST under the same conditions, suggesting adequate reproducibility to elicit cell viability.

In the chemosensitivity test for anti-cancer drugs using 3D cultured cells and devices, imaging methods are frequently utilized. This includes phase contrast microscopy, fluorescence microscopy, confocal laser microscopy, two-photon (multiphoton) microscopy, nuclear magnetic resonance (NMR) microscopy, optical coherence tomography (OCT), and positron emission tomography (PET).

Since test cells require 1 to 14 days of pre-culture before being subjected to the test as far as using 3D cell culture, this problem is commonly pointed out as a factor that limits 3D cell culture tests from being high throughput in manner. In addition to this, chemosensitivity tests for anti-cancer drugs by standard 3D cell culture method require 7 days after drug addition to reach endpoint in the case of CD-DST and often up to about 14 days with medium exchange every 2 - 3 days. In the case of using the above-described imaging techniques, the endpoint measurement is generally employed. In PET, the metabolism is measured using PET radioisotope and it requires about 3 days. Cell viability is predictable at about half the duration to the endpoint, although it is not short enough. On the other hand, there is a similar long-term cell culture problem in NMR microscopy, and additionally no satisfactory resolution can be obtained with over 3 h, leading to drawback this measurement.

Different from the existing methods, our proposed method requires no long-term cell culture and is novel in terms of rapidly predicting endpoint result with the cells simply self-adhering to a sensor chip. This allows for obtaining cell viability results equivalent to the CD-DST method within just 1 h after drug addition, avoiding time-consuming 3D cell culture and tedious medium exchange. In HP-SPR-3D, we have to only adhere the cells to a sensor chip two-dimensionally, and the cells are transformed into in vivo-like activity. The adhered cells do not need to be 3D cultured nor constitute 3D structure and in vivo-like activity can be obtained by simply utilizing 2D cell culture. For these reasons, our proposed method is very quick and suitable for high throughput use.

As described before, in the use of 3D cultured cells, most of them are far from an HP-SPR sensor surface and it is impossible to achieve their HP-SPR measurement in principle. Therefore, the cell activity has to be changed into in vivo-like status while the cells are adhered to the sensor surface two-dimensionally. This method is excellent in achieving this by covering cells with collagen after a certain short time. Further, when the measurement is carried out at a physiological concentration of a drug using the cells with in vivo-like status, the appropriate drug concentration may be reduced to about one hundredth compared to the case of 2D cell culture test, and the corresponding HP-SPR response could be reduced accordingly.

However, we could detect strong enough HP-SPR signal level comparable to the results obtained using 2D cells. Therefore, we thought that the cell activity was enhanced into in vivo-like status. In addition, without the cell division necessary in the existing methods, our HP-SPR-3D method gives an opportunity to perform an accurate and sufficient chemosensitivity test. This is excellent for future clinical applications using biopsy samples.
In phase-contrast microscopy, labeling is not necessary, but it has a problem in that it can handle only transparent or very thin samples. In fluorescence microscopy, including confocal microscopy, fluorescence labeling is required, but the fluorescent dye is chemically unstable and quantitative analysis becomes difficult. Furthermore, the competitive reaction with a drug may lead to erroneous determination results. Our proposed method allows for a label-free experiment avoiding these problems economically without using expensive fluorescent dyes and alleviates us from the complicated labeling operation.

In two-photon microscopy, the measurement can be performed by autofluorescence without labeling. However, this method has been reported to cause photobleaching with an increase in the light power to observe the entire cell culture. On the other hand, OCT has low reaching depth of light and is also not suitable for whole cell observation. The HP-SPR-3D system has an advantage avoiding the photobleaching problem because the measurement is rapidly achieved using the electromagnetic wave generated from weak light.

Conclusions

We reported on an HP-SPR-3D system for anti-cancer drug (candidate) screening to evaluate them at physiological concentrations similar to in vivo. This system utilized an HP-SPR system with average fluctuation of 50 ndeg s⁻¹ using two-dimensional (2D) cells attached onto a sensor chip to monitor MMP for 1 h after the drug addition to predict 2D cell culture chemosensitivity results. We examined the modification of the system by applying collagen on the top of 2D cells on the sensor chip to change their activity into in vivo-like conditions without cell division to create an HP-SPR-3D system. We established the HP-SPR-3D as a rapid in vivo-like phenotypic cell-based assay system using cancer cell lines and anti-cancer drugs to represent individual differences and differences in drug mode of action. We obtained results equivalent to the clinically relevant conventional 3D cell culture chemosensitivity test of the CD-DST method within just 1 h, avoiding time-consuming 3D cell culture and tedious medium exchange.

As shown in Fig. 6, the HP-SPR-3D system offers the prospect of contributing to various fields. It enables the initial screening of newly developed anti-cancer drugs or lead compounds from natural resources. In the future, studies using clinical samples as well as the development of automated devices is expected to be utilized for more efficient tailor-made anti-cancer pharmaceuticals and therapies with the expansion of applications. On the other hand, since our proposed method is based on the measurement of MMP status in principle, it is expected to perform efficacy evaluation of a drug (candidate) for the diseases related to mitochondria. It can be used especially in drug screening at physiological concentrations for the prevention of metabolic syndrome-related hypertension, diabetes and obesity, and also for cardiomyopathy, renal failure, infertility, hearing loss, the development of arteriosclerosis, stroke, Alzheimer’s disease, chronic fatigue syndrome, epilepsy, myocardial infarction, cerebral infarction, migration of the sperm, aging and so on. Furthermore, the HP-SPR-3D system can be applied to diagnosis, screening of active and/or toxic compounds, and stem cell (regenerative) therapy. Consequently, we believe that from the present study, our new method and principle of HP-SPR-3D have the potential for proposing a new methodology both in pharmaceutical and medical industries.

Acknowledgements

This research was partly supported by a grant from the Regional Research and Development Resources Utilization Program of the Japan Science and Technology Agency (JST).
