Video Article

Regeneration of Arrayed Gold Microelectrodes Equipped for a Real-Time Cell Analyzer

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Abstract

The label-free cell-based assay is advantageous for biochemical study because of it does not require the use of experimental animals. Due to its ability to provide more dynamic information about cells under physiological conditions than classical biochemical assays, this label-free real-time cell assay based on the electric impedance principle is attracting more attention during the past decade. However, its practical utilization may be limited due to the relatively expensive cost of measurement, in which costly consumable disposable gold microchips are used for the cell analyzer. In this protocol, we have developed a general strategy to regenerate arrayed gold microelectrodes equipped for a commercial label-free cell analyzer. The regeneration process includes trypsin digestion, rinsing with ethanol and water, and a spinning step. The proposed method has been tested and shown to be effective for the regeneration and repeated usage of commercial electronic plates at least three times, which will help researchers save on the high running cost of real-time cell assays.

Video Link

The video component of this article can be found at https://www.jove.com/video/56250/

Introduction

Owing to its efficient and less labor-intensive experimental process, label-free cell-based technology has witnessed rapid growth over the past decade for analytical as well as screening purposes such as in the aspect of proteomics[1,2], drug delivery[3], *etc.*[4,5] Compared with traditional biochemical methods aimed at cell analysis, label-free real-time cell assay with the prototype developed by Giaever and coworkers previously[6] is based on the principle of recording electric signal changes on the surface of cell-attached microchips, which permit a continuous measurement of cell growth or migration in a quantitative manner. Following this strategy, a real-time cell electronic sensing (RT-CES) system using electric impedance-based detection principle was introduced[7,8] and more recently a commercial real-time cell analyzer (RTCA) was launched for laboratory research[9].

The commercial real-time cell analyzer mainly reads out the cells' evoked signals of electric impedance, which result from the physiological changes of incubated cells including cell proliferation, migration, viability, morphology, and adherence on the surface of microchips[10,11]. Such electric signals are further converted by the analyzer into a dimensionless parameter named the Cell Index (CI) to assess the cell status. The change of the impedance of microchips mainly reflects the local ionic environment of covered cells at the electrode/solution interface. Therefore, the analytical performance of the cell analyzer relies heavily on the core sensing unit, the disposable microchips (*i.e.*, so-called electronic plates, *e.g.*, 96/16/8-well), which are made of arrayed gold microelectrodes lithographically printed at the bottom of incubation wells. The gold microelectrodes assemble in a circle-on-line format (**Figure 1**) and cover most of the surface area of the incubation wells, which allow for dynamic and sensitive detection of attached cells[3,12,13,14]. The CI will increase in the case of more surface coverage of cells on the chip, and decrease when cells are exposed to a toxicant resulting in apoptosis. Although the real-time cell analyzer has been frequently used to determine cytotoxicity[11] and neurotoxicity[15] and provide more kinetic information than classical endpoints method, the disposable electronic plates are the costliest consumable.

Until now, there have been no available methods for the regeneration of the electronic plate, which is probably due to the fact that harsh regeneration conditions, such as piranha solution or acetic acid are involved[16,17,18], which may alter the electric status of gold microchips. Therefore, a mild and efficient method to remove the adherent cells and other substances from the surface of gold chips will be desirable for the electronic plate regeneration process. We have recently developed a protocol aimed at the regeneration of disposable electronic plates using non-corrosive reagents and the regenerated chips were characterized by electrochemical as well as optical methods[19]. By using readily available

and moderate laboratory reagents including trypsin and ethanol, we have established a general method to regenerate the commercial electronic plate without adverse effects, which is successfully applied to regenerate the two main types of electronic plate (both 16 and L8) used for RTCA.

Protocol

NOTE: In general, the regeneration process includes trypsin digestion and rinsing step with ethanol and water. The digestion time changes according to the number of cells used, and the type and number of cells used may differ depending on the experimental purposes. It is advised to check the regenerated microchips using optical and electrochemical methods to optimize the regeneration conditions. During the experiment, soluble and insoluble chemicals may be involved, and here these two typical cases of the regeneration procedures are detailed.

1. Preparation of Regeneration Solutions

NOTE: Prepare and handle all the solutions under sterile conditions.

1. Prepare fresh 0.25% (g/v) trypsin solution. Trypsin can be purchased or freshly prepared as follows.
    1. Dissolve 0.25 g of trypsin in 100 mL of pH 7.4 phosphate-buffered saline (PBS).
    2. Stir the solution until the trypsin is fully dissolved at 4 ^{o}C. Stir the solution at low speed taking care to avoid any bubble formation.
    3. Filter the solution with a 0.22 µm filter in the biosafety hood.

2. Prepare 75% ethanol using absolute ethanol. Pour 75 mL absolute ethanol into a volumetric flask and add deionized water until the volume reaches 100 mL. Perform further sterilization if necessary.

2. Incubation and Proliferation of A549 Cells in Electronic Plates

NOTE: Electronic plate types 16 and L8 are both made of arrayed gold microelectrodes lithographically printed at the bottom of incubation wells. Electronic plate 16 has 16 wells while electronic plate L8 has 8 wells. The wells of electronic plate 16 are round while the wells of electronic plate L8 are rounded rectangles. The volume of each electronic plate 8 well is about 830 µL and each electronic plate 16 well is about 270 µL.

1. A549 cell culture
    1. Culture A549 cells in Roswell Park Memorial Institute-1640 medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-streptomycin in the cell culture dish.
    2. Passage the cells when the cell density reaches ~75% then wash the adherent cell layer with 1 × PBS and trypsinize with 0.25% trypsin solution for 1 min at 37 ^{o}C. Centrifuge the cells at 179 × g for 5 min to remove trypsin. Resuspend the cells in 10 mL medium for further culture or other experiments.
    NOTE: The solution of cells resuspended in the medium is here designated as the cell suspension buffer.
    3. Take out 10 µL and count the cell number using a hemocytometer. Use the RPMI-1640 medium to further dilute the cell suspension buffer to prepare 30,000-80,000 cells/mL cell suspension buffer for the experiments.

2. **Proliferation/cytotoxicity experiment and data acquisition**
    1. Add 50 µL (150 µL for electronic plate L8) cell medium to each incubation well of the electronic plate 16 and leave it for 30 min in the biosafety hood for equilibration. Manually insert the electronic plate 16 in the real-time cell analyzer in the CO_2 incubator at 37 ^{o}C.
    2. Launch the real-time cell analyzer operation program on the computer. On its default experiment pattern setup page, select "Experiment model" and name the running assay.
    3. On its Layout page, select the corresponding wells that are included in the experiment and input the information like cell type, number and drug names in the edit boxes. On its Schedule page, add the experimental steps, then select the running time (e.g., 12, 24, or 48 h) of each step and interval of real-time data acquisition.
    Note: Here, the interval of step 1 and 2 are 1 min and 15 min afterwards.
    4. Save the file and click "Start" button to measure the background impedance of the media on its Cell Index page; the resulting data will be automatically subtracted. On its Well Graph page, all well curves can be visited. On its Plot page, add wells and the graph of time-cell index will be showed.
    5. For the cell proliferation experiment, firstly click the "Pause" button to pause the program . Then take out the electronic plate and add 100 µL A549 cell suspension buffer per well at room temperature. Leave the plate for 30 min in the biosafety hood to let the cells settle to the bottom of incubation wells.
    6. Manually insert the electronic plate in the real-time cell analyzer station. Click the "Start" button to continue the experiment.
    Note: On the Plot page, the analyzer continuously records Cell Index values or curves throughout the experiment.
    7. When the experiment is complete, enter the Plot page and open the experiment file. Then select all the tested incubation wells and the resulting Cell Index curves that can be averaged or normalized for the last time before adding pharmaceutical drugs or changing medium to reduce variations between assays. On the Data Analysis page, EC50 or IC50 can be calculated

3. Regeneration of Electronic Plates

NOTE: For each rinsing step, the solution in the electronic plate was mixed thoroughly (5-10 times) using a pipette.

1. **Case 1: For cytotoxicity evaluation of *anti* -cancer drug**
    1. Seed A549 cells on the electronic plate as described in section 2.
    2. Dissolve the anti-cancer drug doxorubicin hydrochloride (DOX) in the cell medium first. Slowly add DOX to the cells (final concentration of DOX is 30 µg/mL) using a pipette. Record the CI as described in section 2.

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NOTE: The electronic plate used was regenerated immediately after the experiment was complete. In this case, as DOX is a soluble chemical, the regeneration of the electronic plate following the protocol is relatively easy to handle and no additional steps are needed.

3. **Regeneration**
    1. Take out the electronic plate containing cells from the real-time cell analyzer station. Place the used electronic plate in a sterile biosafety hood at room temperature and mix culture medium thoroughly using a pipette. Pipette out all the medium.
    2. Rinse the electronic plates with 200 µL deionized water for 3 times at room temperature.
    3. Digest the remaining cells on the electronic plates with 0.25% freshly prepared trypsin for 1-2 h (200 µL/well) in an incubator at 37 ^{o}C. When the digestion is completed, mix the incubation solution 5-10 times using a pipette and pipette out all the solution in the biosafety hood at room temperature.
    4. Rinse the electronic plates with 200 µL ethanol and 200 µL water, respectively, as follows: deionized water 2 times, 100% ethanol 2 times; 75% ethanol 2 times; deionized water 2 times. Invert the electronic plate on a sterile gauze or tissue paper to decant the remaining water at room temperature.
    5. Ultraviolet sterilize the regenerated electronic plate for 1-2 h in the biosafety hood at room temperature.
    NOTE: Double the volume of all solutions for the regeneration of electronic plate L8.

2. **Case 2: drug delivery using mesoporous material.**
    1. Use rod-like mesoporous silica materials with an average pore size of 5.6 nm as drug delivery matrices, as previously reported[20].
    NOTE: Here, the electronic plate was employed to monitor the drug-release effect on cells.
        1. Add mesoporous materials into the incubation solution of each well of the electronic plates. As silica materials are not soluble and precipitate in the electronic plate, rinse the plate as follows:
    2. Perform steps 3.1.3.1-3.1.3.3 as described for Case 1.
    3. Rinse the microchips with 200 µL deionized water once in the biosafety hood at room temperature.
    4. Rinse the microchips with 200 µL absolute ethanol 2 times at room temperature.
    5. Rinse the microchips with 200 µL 75% ethanol once at room temperature.
    6. Add 250 µL of 75% ethanol in each well and seal the electronic plate with the sealing film at room temperature.
    7. Spin at 114 x g for 2 min and empty the electronic plate. Invert the electronic plate on a sterile gauze or tissue paper to decant the remaining water at room temperature.
    8. Repeat step 3.2.5-3.2.7 once.
    9. Ultraviolet sterilize the regenerated electronic plate for 1-2 h in the biosafety hood at room temperature.
    NOTE: Double the volume of all solution when rinsing L8 electronic plates.

4. Assessment of Regeneration Effect

1. **Optical characteristics of regenerated electronic plate surface**
    1. Use a steel spoon to carefully disassemble the bottom part of the incubation well of fresh and regenerated electronic plate that contains arrayed gold microelectrodes. Wearing a plastic glove, carefully wipe out the microchip-containing part from the electronic plate using the steel spoon.
    2. Place both parts at the center of the glass microscope slides and collect the resulting Raman spectra by Raman Microscope equipped with a CCD detector[21].
    NOTE: The spectrometer was equipped with a 633 nm laser and the spectra was recorded at an interval of 30 s laser exposure at a wavelength in the range of 500-3500 cm^{-1}. The optical images were obtained by a Raman Microscope using a 10x eye lens and 50x objective. **Figure 1A** and **Figure 1C** were also obtained using the installed Raman Microscope apparatus.
    3. To evaluate the viability of attached cells after anti-cancer drug uptake on the fresh and regenerated electronic plates, use a confocal laser scanning microscope to monitor the spontaneous fluorescence of DOX molecules absorbed by A549.
        1. To record the spontaneous fluorescence of absorbed DOX by the cells (fresh and regenerated electronic plates with A549 cells), use a 63X oil objective and 485 nm as the excitation wavelength.

2. **Electrochemical behaviors of regenerated electrodes**
NOTE: As manual dissembling of a commercial electronic plate is difficult and not ideal for the assessment of surface electric status as checked, the regeneration efficiency of the protocol was evaluated by using normal gold/glassy carbon electrode (GCE) as a test platform, on which electrochemical impedance spectroscopy (EIS) was performed. The diameters of the Au electrode and GCE both are 3 mm. Electrochemical data were obtained by an electrochemical analyzer with a three-electrode system and the impedance measurements were carried out using an alternating current (AC) signal of 10 mV amplitude at a wide frequency range of 100 kHz to 0.01 Hz.
    1. Before the measurement, polish the Au electrode and GCE to a mirror-like surface with an alumina slurry on a polishing cloth, followed by sonication in water to remove any particles.
    2. Activate the Au electrode in 0.5 M H_2SO_4. Obtain EIS data of the bare Au electrode and GCE in 10 mM KCl electrolyte solution containing 1mM $K_3[Fe(CN)_6]$ as described[22].
    3. Prepare stock A549 cell solution according to 2.1.2 and 2.1.3. Drop 10 µL cell suspension on the surface of Au electrode and GCE. Incubate Au electrode and GCE modified with cells in a 37 ^{o}C incubator for 3 h. Obtain EIS data of the Au electrode and GCE modified with cells in 10 mM KCl electrolyte solution containing 1mM $K_3[Fe(CN)_6]$.
    4. **Regenerate the electrodes using the protocol and measure EIS.**
        1. Immerse the Au electrode and GCE modified with cells in 0.25% trypsin for 0.5-2 h. Rinse the Au electrode and GCE softly with water 4 times. Rinse the electrodes softly with absolute ethanol 4 times. Immerge Au electrode and GCE in 75% ethanol for ~5 min. Rinse the chips softly with 75% ethanol 4 times.

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2. Obtain EIS data of regenerated Au electrode and GCE in 10 mM KCl electrolyte solution containing 1mM $K_3[Fe(CN)_6]$.

Representative Results

Surface properties of gold microchips: The regeneration procedures used in this protocol were outlined in **Figure 1**. **Figure 2** shows the microscopic surface pictures of fresh and regenerated electronic plates by the optical microscope. As shown in **Figure 2A** and **Figure 2C**, microscopic observations indicated that there was virtually no difference in the optical properties of the microchip surface between fresh and treated electronic plates. Using the spontaneous fluorescence of doxorubicin molecules included into the cells convenient for assessment of cell status, we observed a similar homogeneity of cultured cells on the gold microchips after the regeneration (**Figure 2B** and **Figure 2D**). Additionally, the incubated cancer cells displayed a well-defined cellular morphology in the electronic plate demonstrating the reusability of chips. **Figure 3** shows the Raman spectra of fresh and regenerated electronic plates. The virtually identical Raman peak distributions between the fresh and treated electronic plates indicated a successful regeneration process of the gold microchip surface. The Raman signals of electronic plates covered with A549 cells were distorted to some extent, probably due to the interference from the spontaneous fluorescence of A549 cells.

Regenerated microchips for the cytotoxicity assay using RTCA: We also applied the regeneration protocol for a test of cell proliferation and material cytotoxicity evaluation (**Figure 4**). A549 cells of optimal density were seeded onto the regenerated electronic plate 16 and growth kinetic curves were recorded by real-time cell analyzer. As shown in **Figure 4A**, all the resulting growth curves of parallel experimental wells showed adequate consistency, which indicated that the surface electric status of electronic plate 16 is greatly retained after the regeneration. The regenerated electronic plate L8 was also tested for cytotoxicity using mesoporous silica materials, as shown in **Figure 4B**. Compared with the control experiment, the CI of mesoporous materials-treated samples decreased to some extent, indicating a slight cytotoxicity of the materials. Further encapsulation and release of the anti-cancer drug of Dox by mesoporous materials caused a dramatic decrease of the growth curve of A549. These experimental results showed consistency with using fresh electronic plates and with previously reported work[19], which verified the efficiency of this proposed regeneration protocol.

Regeneration efficiency assessed by electrochemical methods: Commercial Au electrodes and glassy carbon electrodes were used as test platforms for the evaluation of regeneration efficiency. **Figure 5** shows the EIS of Au electrodes and GCE that were modified with cancer cells. It was shown that after incubation of A549 cells on the electrode, an evident increase of electron transfer resistance (Ret) was observed compared with that of bare electrodes. The Ret was decreased significantly after the Au electrode and GCE were treated according to the protocol in **Figure 5**. The similar changes of Ret of both Au electrode and GCE indicated an effective and universal regeneration process of microchips regarding their surface electric properties.

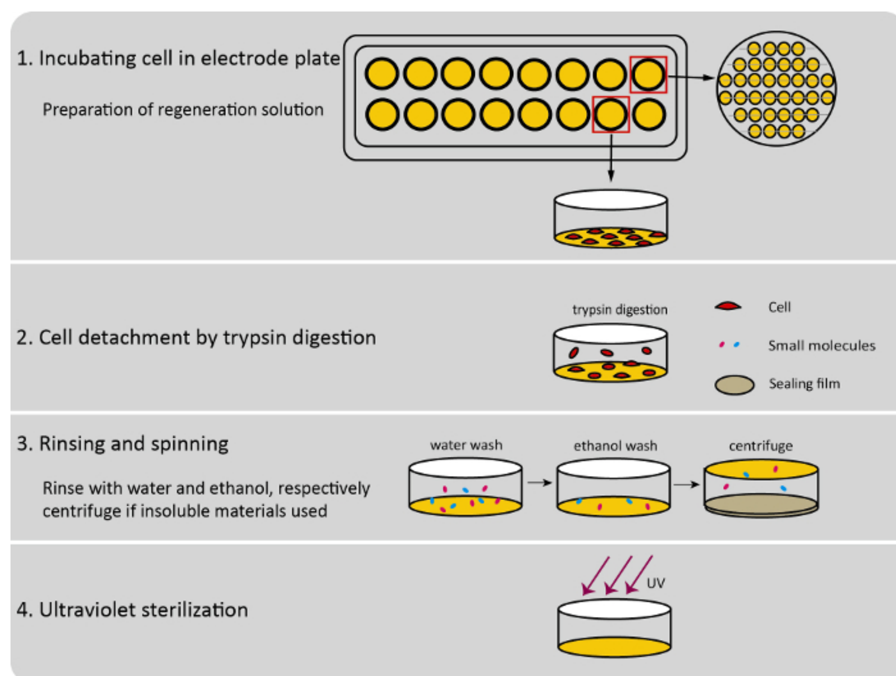

Figure 1: The regeneration process of electronic plates. In a typical regeneration run, four main steps were involved including incubating cells as well as their detachment on the surface of electronic plates followed by the rinsing, spinning, and ultraviolet sterilization steps. Please click here to view a larger version of this figure.

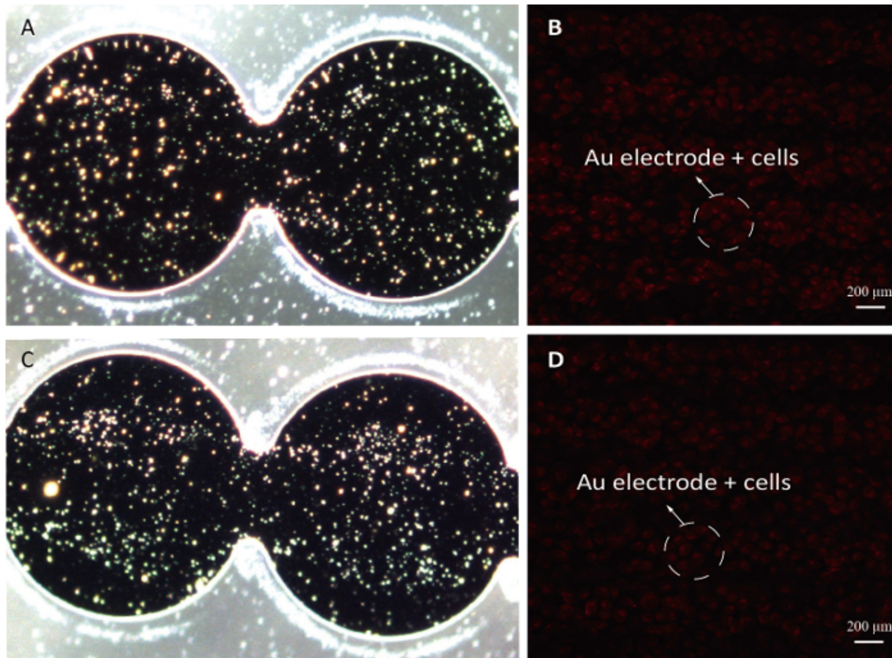

Figure 2: The surface properties of electronic plates. (A) New electronic plates **(B)** A549 cells were plated on A. **(C)** Regenerated electronic plates **(D)** A549 cells were plated on C. In the case of cells attached on gold microchips, DOX was used. **Figure 2A** and **Figure 2C** have been modified from Xu, Z. *et al.*[19] Please click here to view a larger version of this figure.

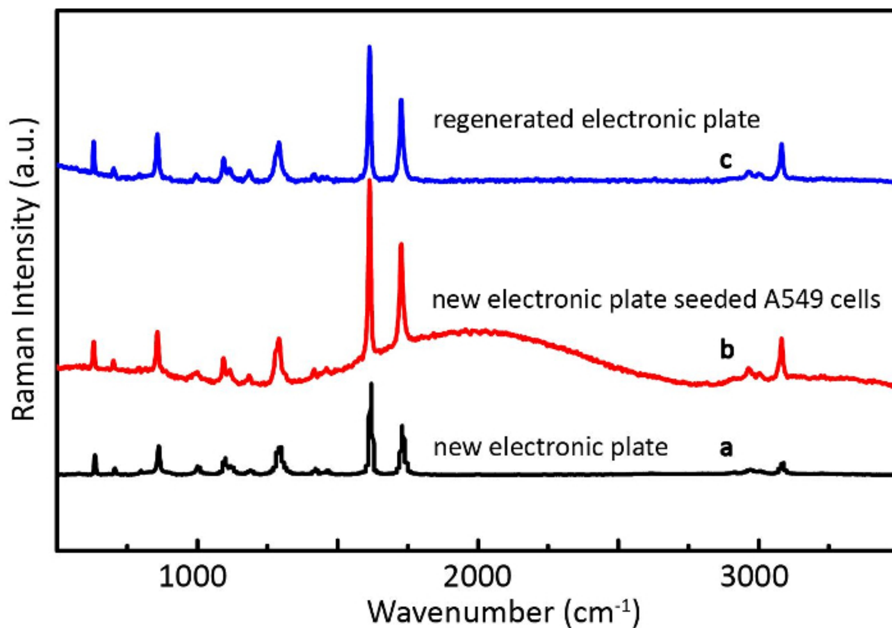

Figure 3: Raman spectra of different treated electronic plates. (A) New electronic plate. **(B)** New electronic plate seeded with A549 cells. **(C)** Regenerated electronic plate. Please click here to view a larger version of this figure.

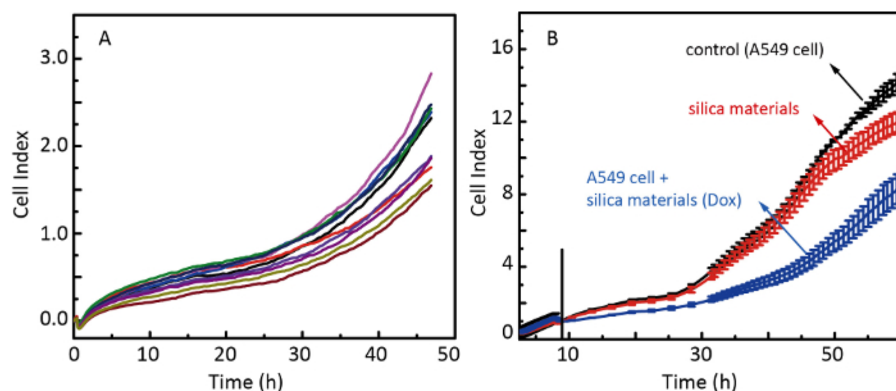

Figure 4: A549 cell growth curves on three-time regenerated electronic plates. (A) Parallel growth curves of cells seeded on electronic plate 16. **(B)** Cytotoxicity test of mesoporous silica materials on electronic plate L8 with error bars (standard deviation, STD) of recorded curves of each incubation well. **Figure 4A** has been modified from Xu, Z. *et al.*[19] Please click here to view a larger version of this figure.

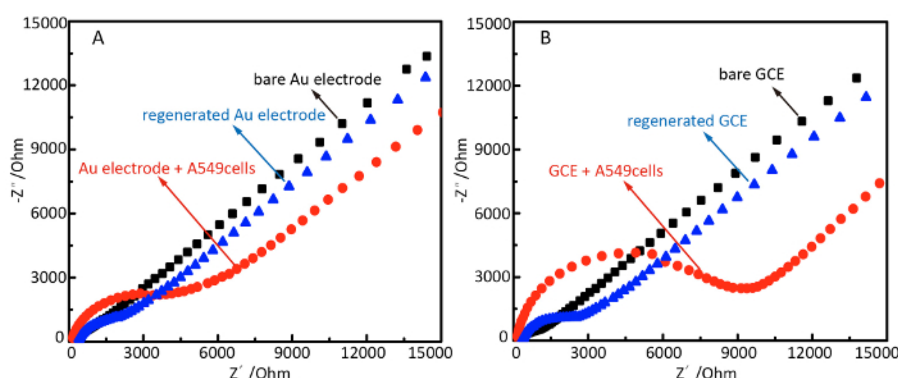

Figure 5: Electrochemical impedance spectra of different modified gold electrodes and glassy carbon electrodes (GCE) in 10 mM KCl electrolyte solution containing 1 mM $K_3[Fe(CN)_6]$. (A) Different treated Au electrodes. **(B)** Different treated GCE. **Figure 5A** has been modified fromXu, Z. *et al.*[19] Please click here to view a larger version of this figure.

Chip application	Regeneration buffer	Regeneration strategy
Surface plasmon resonance (SPR)	Piranha solution (concentrated H_2SO_4/30% H_2O_2 = 3:1, v/v)[17]	strip both the analyte and probe based on strong oxidization
Piezoelectric Microcantilever Sensors (PEMS)	Glycine-HCl mixture[23,24]	dissociate the target-probe complex and remove the analyte
	High salt concentrations solution (2M $MgCl_2$ + 1.5 M Tris)[25]	
Gold film	50mM KOH+25% H_2O_2[26]	Oxidized organics

Table 1: A brief summary of reported regeneration strategies.

Discussion

We summarized several available methods[17,23,24,25,26] for regenerating microchips in **Table 1**. Basically, these methods involved relatively harsh experimental conditions to achieve the complete regeneration of chips because of the presence of strong molecule-molecule interactions such as that of the immune complex used for SPR chips. However, these harsh experimental conditions may be extremely harmful for the surface properties of arrayed gold microchips used in RTCA. Therefore, a mild and efficient method were the major considerations in the development of the presented regeneration strategy. Other factors that are equally important are environmental concerns, since these disposable microchips are frequently used together with targeted cancer cells.

In this protocol, we introduce a general method for regenerating commercial arrayed gold microchips used for RTCA. Our protocol was developed based on trypsin-digestion and rinsing with ethanol and water. Extra spinning steps are considered to be effective in removing the insoluble particles used for the assay for the regeneration process. Compared with the reagents used in previously reported methods, the regenerating reagents trypsin and ethanol are readily available for daily laboratory use and these biological products/chemicals are of low toxicity, and are thus considered to be environmentally friendly, which is desirable for purposes of regeneration.

In this protocol, the critical step is the trypsin digestion to ensure the regeneration performance of gold microchips. The optimization of trypsinization, including concentration and time, should be the first priority to achieve regeneration. Besides the digestion time that is rationalized in our previous report[19], a good assessment for the optimization process is simply to check the surface properties using an optical microscope.

Another concern is the handling of microchips during the regenerating process. As a pipette is frequently used in the experiments, attention should be paid by personnel to avoid scratching the tip on the surface of microchips. Furthermore, it is beneficial to pre-sterilize the tips, gauze, and spoon to ensure regeneration efficiency. Last but not least, the regeneration process should be conducted in a biosafety hood to avoid any contamination.

It should be noted that the rinsing processes involved in the regeneration protocol are manually conducted, which will inevitably cause bias due to differing personnel. To overcome this limitation, automatic or semi-automatic equipment for rinsing purposes could be custom designed, considering the modular electronic plates used. In addition, the regeneration protocol has been tested to be effective for cell-based assays. However, in the presence of strong molecular interactions, such as antibody-antigen formation at the interface of chips, the proposed regeneration conditions may be limited to a great extent and relatively strong buffer conditions should be considered.

Overall, the current regeneration protocol has been tested to allow the repeated usage of commercial gold microchips, as well as other conducting electrodes such as Au and glassy carbon electrodes. We expect that the application of this protocol will save on the high running cost of real-time chip-based cell assays. In the long run, microchip-based assays are becoming more frequently used for proteomic and cellular analysis and the proposed protocol in this study can offer an option for the regeneration, in which automatic or semi-automatic equipment for rinsing could be designed and applied in cases of accumulated disposable chips.

Disclosures

The authors have nothing to disclose.

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