Electronic Biopsy of PC12 Cell Culture

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Received 1 Oct 2012; Accepted 16 May 2013; doi: 10.5405/jmbe.1.330

Abstract

Optical biopsy is the most commonly used method in cell growth studies. This study designs a Petri dish with a 32-electrode array for electronic biopsy. A simplified electrical model of a cell culture is proposed. Electrical impedance spectroscopy has been widely applied to characterize the electrical properties of cells during culture. To determine the electrical properties of PC12 cells for electronic biopsy, the Cole-Cole plot method is used for feature extraction. The analysis uses an equivalent three-element circuit model with a constant-phase element. A distribution curve is also used to determine how parameters affect the impedance loci in the three-element model. A PC12 cell line continuously cultured for 30 hrs without change of medium is used to illustrate the feasibility of the proposed electronic biopsy. The results show that the maximum phase decreases and the other parameter curves increase when cell necrosis occurs. The slope of Cole-parameters and diameter of Cole-Cole plots are useful for cell growth and cell property characterization.

Keywords: Electronic biopsy, Electrode array, Bioimpedance spectroscopy

1. Introduction

The culturing of cells is an essential aspect of research on cellular physiology, biochemical reactions, molecular biology, and stem cells [1-3]. Such research generally deals with cell activity and the effects of external parameters on certain activities, such as cell proliferation, cell viability, toxicity, and the secretion of antibodies [4]. Cell culturing is also important in the production of virus vaccines, anti-virus and anti-cancer medications, and other biotechnological developments [5,6]. Cell behavior during culturing may be usually described as adhesion, motility, proliferation, and detachment in a nutrient medium under controlled environmental conditions [7-9]. Traditionally, cells have been cultured in Petri dishes within a strictly controlled incubation system and the monitoring of cell growth has relied on irreversible methods such as fluorescence staining and dyeing under microscopic observation or chemical analysis [10]. Unfortunately, this approach is susceptible to fluctuations resulting from periodic movement. Mammalian cells are particularly sensitive to extracellular micro-environments; therefore, an incubation system capable of providing a more stable environment is required [11]. Recently, fluorescent bioprobes have been used to distinguish the characteristics of living cells; however, these bioprocesses make cell culture assays irreversible and time-consuming.

Electronic biopsy is a promising method for determining the electrical characteristics of cells and thereby monitoring cell growth.

Culture conditions (particularly growth media) vary widely according to cell type. Giaever and Keese were the first to use evaporated gold electrodes to measure impedance as an indicator of motion in mammalian cells [12]. Their approach is now accepted as an effective means of measuring cell motion, growth, and motility [13-15]. Such noninvasive, reusable techniques for monitoring biological events are crucial to further development in this field, particularly in light of the fact that multi-electrode scans can identify the specific region in which such events occur.

The electrical properties of tissues have been studied using a variety of methods and models [16-18]. The Cole-Cole plot can be useful for the characterization of various tissues and cells. Some studies such as [19,20] developed various application methods that use bioimpedance spectroscopy (BIS) to characterize cell properties during culturing. The electronic properties of pathways are obtained using a switching system within an array of electrodes in the culture. BIS methods are commonly used to study a number of physiological cell events, such as cell growth rate [21]. Many of these BIS applications use Cole parameters as an analytical base, even if events change between frequencies, which prevent the comparison of measurements. BIS applications have been used for analysis of

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body composition, detection of skin and breast cancer, health assessments, perinatal asphyxia diagnosis, and cardiopulmonary testing [22-27]. Nearly all of these studies apply measured spectroscopic data to the Cole equation, in which several Cole parameters are extracted from the impedance locus to explain physiological phenomena.

BIS generally uses a single set of electrodes at a target point; however, the resulting measurements of electrical properties pertain only to the location in which they were placed. To overcome this limitation, the number of sample sites can be increased, but this increases process costs and complexity. Therefore, a method that can provide heterogeneity in the testing process or help to differentiate between sample sites is desirable.

Previous researchers have used characteristic frequency impedance (Zc) to predict total body water and the low-frequency intercept (R0) to predict extracellular water in the analysis of electrical bioimpedance [28]. According to Skourou et al., the size and frequency of MatLyLu tumors are correlated, with large tumors having a high characteristic frequency [29]. Several factors are usually associated with observed changes in impedance, with the center frequency (fc) being a particularly useful measure [30]. Bioimpedance can be used to observe changes in electrical properties as an indication of cell growth and to monitor cell necrosis and apoptosis during culturing. In this study, BIS and Cole-Cole plot are used to characterize the electrical properties of cells during culture. A PC12 cell line continuously cultured without change of medium is used to illustrate the feasibility of the proposed system.

2. Materials and methods

2.1 PC12 cell line preparation

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla, commonly used as a cell model for neuronal differentiation. In this study, the PC12 cell line was obtained from the Bioresource Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan. For PC12 cell line preparation, cells were grown in a 25-T flask containing a medium of RPMI-1640 (Gibco BRL, New York, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were immediately added after being removed from liquid nitrogen and warmed to 37 °C. The cells were cultured at 37 °C in an atmosphere of 5% CO2 and 95% air. Trypsin was then used to separate the cells from the medium, facilitate the use of a centrifuge, and maintain a good cell growth environment. When the cells covered 80% of the visual field of a microscope image, a density of approximately 7 × 10^5 cells/ml was subcultured. Subsequently, 0.5 cc of culture medium containing the PC12 cells was removed and diluted to a density of 8.3 × 10^5 cells/ml using RPMI-1640 medium. 17 µl of penicillin was then added to the culture medium. In order to accelerate cell degeneration, the nutrition medium is not refreshed and replenished for cells during culturing.

2.2 Cell culture with electrode array

Photolithography was used to create a 32-electrode array. The process for fabricating the electrode-array includes four steps: optical lithography, development, etching, and removal of the photoresist. The back polyimide layer of the electrode array was attached to the inside of a culture dish (15 mm in diameter and 10 mm high). A cone-shaped piston was used to press and fix the electrode array to the inner dish surface. To ensure biological compatibility, all electrode surfaces were plated with a 0.07-mm-thick layer of gold. The design of the electrode array was modified slightly from the model provided in [31]. The 32 electrodes were then connected to a bioimpedance measurement system using a pair of connectors for ease of operation during measurement.

2.3 Integrated incubation system

An integrated incubation system was designed to obtain a faithful indication of cell activities within a culture dish. Electrical impedance spectroscopy (EIS) was used to measure associated electrical properties. The incubation system comprised three subsystems: image acquisition, temperature control, and carbon dioxide control [32]. Cell imaging and cultivation were conducted in separate environments. The incubation system integrates an optical microscope within a traditional incubation system to allow real-time recording of cell culturing. This design also reduces the need for manual operations, which could disturb the experiments. An incubation temperature of 37 ± 0.5 °C was maintained throughout the experiment. The gas supply from a steel cylinder (5% CO2 and 95% N2) was controlled directly using a solenoid valve and control circuit. This system captured and recorded data in real time and automatically controlled gas concentrations during cell culturing.

Real-time images (640 × 480 pixels) of the cells were captured in BMP file format using an inverted phase contrast microscope (CKX-41, Olympus, Tokyo, Japan), CCD camera, and acquisition software (MP3.3RTV, Nikon, Qimaging, Canada). The temperature was controlled using a DTA-4848 control circuit (Delta, Taipei, Taiwan) and a quartz heater (C-Blue, Kaohsiung, Taiwan). A negative feedback system was used to maintain these conditions. A PT-100 temperature detector was used to monitor and control the temperature inside the culture chamber.

2.4 Electrical impedance spectroscopy system

This study used the multi-frequency bioimpedance measurement system (MFBii) developed in [33]. The amplitude of the output signal ranged from 0.2 to 2 V, providing measurements of resistance and reactance. Bioimpedance measurements were based on a two-electrode measurement approach. The frequency of MFBii for BIS ranged from 10 to 100 kHz and the scanning frequency was 1 kHz. Every 30 min, a dataset was measured and stored. A switching circuit was designed for the selection of electrodes, resistance, and reactance measurements. MFBii was connected to a computer via UART-RS232 for the transfer of data and signal recording.
2.5 Cole parameters

Cole and Cole proposed an electrical model in 1941, called the Cole-Cole plot [34]. This approach remains a useful method for illustrating the behavior of tissue impedance as a function of frequency. In mathematics, a complex plane is a geometric expression of complex numbers indexed using the real axis and the orthogonal imaginary axis. A Cole-Cole plot shows total impedance $Z_i$ as a function of frequency. Schwan expressed $Z_i$ as Eq. (1) [35], where $j^p$, resistance $R_\alpha$, and reactance $X_\alpha$ are derived using Eqs. (2) to (4), respectively. The plot of the real versus imaginary parts, less than a semicircular arc, is depicted in a complex plane using some discrete frequency inputs. The center of the semicircle is located beneath the real axis. The depression angle is $\varphi = (1 - \alpha) \pi/2$.

$$Z_i = R + jX = R_\alpha + \frac{R_\alpha - R_\infty}{1 + (j\omega \tau)^p}$$  \hfill (1)

$$j^p = \cos(\alpha \frac{\pi}{2}) + j\sin(\alpha \frac{\pi}{2})$$  \hfill (2)

$$R(\omega) = R_\alpha + \frac{(R_\alpha - R_\infty)(1 + (\omega \tau)^p \cos(\alpha \frac{\pi}{2}))}{1 + 2(\omega \tau)^p \cos(\alpha \frac{\pi}{2}) + (\omega \tau)^{2p}}$$  \hfill (3)

$$X(\omega) = -j \frac{(R_\alpha - R_\infty)(\omega \tau)^p \sin(\alpha \frac{\pi}{2})}{1 + 2(\omega \tau)^p \cos(\alpha \frac{\pi}{2}) + (\omega \tau)^{2p}}$$  \hfill (4)

A hypotheses test using a chi-square distribution was employed here, as it makes few assumptions regarding the underlying population. As a result, it is sometimes referred to as a non-parametric test. Equation (5) shows that a chi-square test can be used to determine the square of the standard deviation between the theoretical distribution (known function) and measured data. This is regarded as a fixed error, as it can be traced back to the experiment or measurement environment, including factors such as noise from an A/D converter or amplifier. A weight ratio of $\sigma_0 = \varepsilon^* \frac{1}{\sigma_0}$ can be rewritten as Eq. (6), where $r$ is the radius. The minimal value of the error function was used, which is the same as using the minimal value of $X^2$. The square root of error function $E^2$ in Eq. (6) is extracted; this value is approximately equal to error $\varepsilon$. This describes the curve-fitting method used for the experiments.

$$X^2 = \sum_{i=1}^{n} \frac{\left( (x_i - x_0)^2 + (y_i - y_0)^2 - r^2 \right)}{\sigma_i^2}$$  \hfill (5)

$$E^2 = \frac{1}{n} \sum_{i=1}^{n} \sqrt{\left( (x_i - x_0)^2 + (y_i - y_0)^2 - r^2 \right)} = \varepsilon^* X^2$$  \hfill (6)

Four parameters for $R_\alpha$, $R_\infty$, $\alpha$, and $\tau$ can be obtained using a Cole-Cole plot as given in Eqs. (7) to (10), respectively. Unfortunately, these are not necessarily accurate for describing real BIS measurements. The impedance locus reflects a depressed angle. In a complex plane, an angle of $(1 - \alpha) \times 90^\circ$ exists when the reactance axis and the arc center depression below the real axis is $\alpha \times 90^\circ$, where the value of $\alpha$ ranges from 0 to 1. The maximal phase can also be calculated using a Bode plot. Occasionally, these parameters are related to physiological states. Other parameters include characteristic frequency ($f_0$), center frequency ($f_0$), maximal phase ($\theta$), real center ($x_0$), imaginary center ($y_0$), and diameter. Figure 2 shows the depressed Cole-Cole plot with constant time parameters.

![Depressed Cole-Cole Plot](image)

$$R_0 = x_0 + \sqrt{r^2 - y_0^2}$$  \hfill (7)

$$R_\infty = x_0 - \sqrt{r^2 - y_0^2}$$  \hfill (8)

$$\alpha = \frac{2}{\pi} \sin^{-1} \left( \frac{y_0}{r} \right)$$  \hfill (9)
2.6. Cell circuit model

The basic structure of cells includes the cell membrane, nucleus, intracellular fluid, and extracellular fluid. The equivalent circuit model of culturing cells in this proposed device with electrode-array is presented in Fig. 3(a); the total impedance for the cell culture can be derived as Eq. (11). The intracellular fluid has a resistance \( R_m \), the electrode-electrolyte interface has an impedance \( Z_e \), and the membrane has a parallel combination of a resistance \( R_n \) and a capacitance \( C_n \). The electrical model was simplified into a three-element model, including a parallel combination of a resistance \( R_2 \) and a capacitance \( C \), both in series with \( R_1 \), as shown in Fig. 3(b). Lapicque used a three-element model to study nerve membranes [36] and developed a neuron model that is still widely used [18,37]. Model parameters \( R_1 \) and \( R_2 \) can be calculated analytically using \( R_{\text{solution}}(\omega) \), \( R_n(\omega) \), and \( R_m(\omega) \), as shown in Eq. (12) and (13), respectively.

\[
Z_{\text{sol}}(\omega) = 2Z(\omega) + \left[ R_{\text{solution}}(\omega)/([R_m(\omega) + (C_m(\omega) / R_m(\omega))]) \right] \\
R_1(\omega) = R_m(\omega) / R_{\text{solution}}(\omega) \\
R_2(\omega) = [R_{\text{solution}}(\omega) / (R_m(\omega) + R_n(\omega))] - (R_m(\omega) / R_{\text{solution}}(\omega)) 
\]  

(11) (12) (13)

A simple electrical equivalent circuit and a fitting procedure were used to find the parameters associated with the culturing system for cell degeneration. Equation (14) shows the total impedance. The three-element model has a resistor-capacitor parallel connection in series with a resistor; however, a constant-phase element (CPE) was used instead of a pseudo capacitor. To allow for experiments using electrodes in various positions, a switching circuit was employed. Fitting results obtained using a CPE were better than those obtained using a pure capacitor. The CPE is an empirical impedance function, expressed in Eq. (15). For each current pathway, the Cole-Cole plot was drawn and the pathway of interest was selected for Cole parameter analysis.

\[
Z_{\text{cre}} = \frac{1}{T(\omega)^\alpha} \\
Z_{\text{cap}} = R_1 + \frac{R_2}{1 + R_1T(\omega)^\alpha} 
\]  

(14) (15)

If \( \alpha = 1 \), the CPE acts as pure capacitor. In this case, the center of the circle lies on the real axis and the depression angle is 0°. If \( \alpha < 1 \), the center of the circle falls below the real axis. In this case, the depression angle is greater than 0°.

3. Results and Discussion

3.1 Simulation of electrical impedance spectra

To evaluate the influence of parameter variations on the total impedance of the electrical model, a group of distribution curves was plotted. An empirical three-element circuit model, including CPE simulation, was used to illustrate the effects of variations in \( R_1 \) and \( R_2 \) on the impedance loci. In a study by Pethig, cell membrane capacitance increased from 0.5 to 1.3 \( \mu \text{F/cm}^2 \) when a double phospholipid layer was introduced [38]. Reported values were modified for the Cole-Cole plot to study the electrical properties of biomaterials. Membrane resistance and capacitance were 100 MΩ and 1 \( \times \) 10\(^{-12} \) F, respectively. Intracellular and extracellular fluid resistances were 60 kΩ and 2.5 kΩ, respectively. The values of the three-element model were then obtained using basic circuit theorems. The resistances of \( R_1 \) and \( R_2 \) were 2.4 kΩ and 99 Ω, respectively. The results of electrical impedance spectra simulation, based on these values, ranged from 100 Hz to 1 THz. The Cole-Cole plot R2 loci varied between 100 Ω and 200 Ω in increments of 20 Ω, as shown in Fig. 4(a). For \( R_1 \), the loci varied between 2.4 kΩ and 2.5 kΩ in increments of 20 Ω, as shown in Fig. 4(b). However, the curves are not significantly different from the \( C_m \) value, which was between 5 \( \times \) 10\(^{-13} \) and 5 \( \times \) 10\(^{-9} \) F.

Based on our circuit model analysis, element \( R_1 \) equals the value of \( R_{\text{solution}} \) in parallel with \( R_m \). \( R_1 \) represents the resistance of the medium and intracellular fluid. The Cole-Cole plot moves to the right with a slight change in \( R_1 \). When the stimulation signal frequency trends toward infinity, \( C_m \) is small. \( R_n \) of the equivalent resistance is equal to \( R_{\text{solution}} \) in parallel with \( R_m \) in the three-element model. Similarly, the equivalent resistance is \( R_1 \) in the three-element model. However, as the stimulation signal frequency tends toward zero, \( C_m \) increases. In this situation, the equivalent resistance \( R_{\text{cap}} \) of the four-element model is equal to \( R_{\text{solution}} \) in parallel with \( R_n \) plus \( R_m \). The equivalent resistance in the three-element model is \( R_1 \) plus \( R_2 \).

The physiological significance of \( R_2 \) remains unclear. From the impedance locus, \( R_2 \) is the difference between the low- and high-frequency intercepts. Using a simple parallel RC circuit model, Cho determined that impedance at low frequencies increases with decreasing cell-cell gap and
increasing cell radius [21]. The curve of increasing of R2 impedance in our simulation results as shown in Fig. 4(a) are closely in agreement with his results of the trend of the Cole-Cole plot. Parameter R2 is definitely a function of cellular fluid, as shown in Eq. (12). R2 could be affected by cellular fluid, cell size, and cell-cell gap.

3.2 Cell degeneration

The environmental conditions of the PC12 culture were controlled to be 35.7 °C ± 0.7%, 5.2 ± 6.7% carbon dioxide, and 90% relative humidity. During the experiment, an image of the cells was acquired every 30 min. Figure 5 shows four typical images of cells in different periods of growth. Figure 5(a) shows PC12 cells immediately after the dish was placed in the culturing chamber. The PC12 cells appear as floating clusters with a few scattered cells.

Cells precipitated and adhered to the bottom of the culture dish after 11 hrs (Fig. 5(b)). After 26 hrs, the cell membrane began to lose its integrity (Fig. 5(c)). The culture medium is usually changed daily to provide sufficient nutrition; however, here, the goal was to increase the speed at which cells changed. Thus, in order to encourage the organic development of cell necrosis, the medium was left unchanged. The dark cytoplasm indicates cell expansion and obvious cell atrophy. The content of the cells seeped out after 30 hrs following cell necrosis (Fig. 5(d)).

3.3. Electrical impedance spectroscopy measurements

For each opposite pair of current electrodes, R and C component measurements were obtained; 16 opposite pathways were selected to give 1456 data points. The impedance was first measured during the initial state when the cell culture began. Plots of resistance and reactance as a function of frequency in the range of 10 to 100 kHz in the initial state are shown in Figs. 6(a) and 6(b) for all 16 opposite electrode pathways. The impedance of the medium without cells was also measured for comparison. In 30 hrs of measurement, the volume of culture medium is reduced by 12% due to evaporation, as well as the impedance reduced by 37% at 1 kHz. However, the cell impedance increased by 270% at the end of cell culturing.

Information related to electrical properties may indicate cell functions. This study found a dynamic change in impedance following cell degeneration using MF/BI measurement. The dynamic change values for the resistance and reactance may be obtained as the approximate value of cell impedance. The interface impedance of electrolyte may be neglected.

Each Cole-Cole plot was fitted from 91 data points as a function of frequency in the range of 10 and 100 kHz. Figures 6(c) and (d) show measurement results of resistance and reactance as functions of frequency for one electrode pathway. In Figs. 6(c) and (d), the the slope of resistance curve becomes larger when cells are culturing. The slope of resistance curve is 60 times changes from 4 hrs to 30 hrs, but only 2.5 times changes in reactance.

3.4. Cole-Cole plot and parameters

This study analyzed the electrical pathways of PC12 cells in the three-element model. Impedance consists of resistance $R$ in parallel with complex impedance $Z_{CE}$ and a phase angle, which is independent of frequency. The phase angle of cell
membranes was first mentioned by Cole [39]. Lozano-Nieto and Rezywowski concluded that it is possible to obtain approximate values of impedance from the electrical modeling with predicted results using a constant-phase element (CPE for short) [40]. CPE is used to model the behavior of a double layer as an imperfect capacitor. The Cole-Cole dispersion can be expressed using circuit components as the parallel combination of a resistor and a CPE [17]. The CPE may take the current density disturbance along the surface roughness or porosity of the electrode into account. It is not an intuitive sense that the observed arc of a circle whose center is below the x axis. Nonetheless, a CPE is a useful modeling element, even if the true nature of the system remains unknown [41].

Because the culture dishes were circularly symmetric, opposite current pairs were used for 16 rotational scans. From the experimental results, the measurement with the highest impedance was selected for further analysis. This method provided a highly sensitive current density distribution. During the culture experiment, cell images were acquired to study cell growth and electrical BIS measurements were taken simultaneously. A total of 16 plots were obtained for further analysis of Cole parameters. After 30 hrs of continuous monitoring, six groups of data were selected for Cole-Cole plots from four of the 16 electrode pathways, as shown in Fig. 7. The raw data correspond to the experimental measurements and the semi-circles represent the simulated loci with parameters calculated using the three-element R-C parallel circuit model (Z-View software). The plots express the electrical properties of the cell culture along the vertical plane; the difference between them could be a convenient indicator of the electrical properties in cells along a specific pathway.

After 11 h, the real center (x₀), imaginary center (y₀), diameter, high-frequency intercept (R₀), low-frequency intercept (Rₒ), and depression angle showed similar curve trends. Among these curves, diameter exhibits the largest slope between 26 and 30 hrs. The diameter slope is 20 times larger than that of the low-frequency intercept parameter (Fig. 8(a)). The characteristic frequency (f₀) and center frequency (f_c) decreased for 20 hrs and then increased, which may be due to the cells adhering to the bottom of the culture dish and beginning hyperplasia (Fig. 8(b)). The microscope images show that the cell membranes gradually lost integrity after 26 hrs. The maximal phase decreased 5% and depression angle increased 25% between 26 and 30 hrs. The curve slopes of other parameters increased when cell degeneration occurred (Fig. 8(c)). R₁ is a R(solution)R(intercellular) parallel connection, the value of which increased only 12% from 4 to 26 hrs. Nevertheless, R₂ clearly presents the larger contrast than R₁ in the same periods of time (Fig. 8(d)). The value of R₂ in the cell culture increased to 142% from the initial state. These parameters, particularly the diameter in the Cole-Cole plot and R₂ in the cell circuit model, may provide useful information related to cell functions. Analyzing variations between the electrical parameters and traditional biopsy could be helpful for biological applications.

Figure 6. Plots of the (a) resistance and (b) reactance as a function of frequency for all the 16-electrode pathways in the initial state of culturing. Plots of dynamic changes of the (c) resistance and (d) reactance from the initial state for one pathway.
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Figure 7. Cole-Cole plots for various time periods of cell degeneration for 4 directions of electrode pathways. The electrode pathways 1-17, 5-21, 9-25, and 13-29 denote opposite current paths of (a) 0°, (b) 45°, (c) 90°, and (d) 135°, respectively.

4. Conclusion

This study proposed an electronic biopsy process for monitoring the electrical characteristics of cells during culturing. A culturing dish with an integrated electrode array, an analysis system, a gas and temperature control system, and a microscopic imaging system was developed. Semiconductor fabrication technology was used to design, develop, and attach a 32-electrode array to the inner surface of a culture dish to determine the electrical characteristics of cells during culturing. Finally, an impedance spectroscopy measurement system (MFBii) was employed to obtain EIS signals.

This study cultured a PC12 cell line to demonstrate the efficacy of the proposed electronic biopsy system. The degeneration of PC12 cells for 30 h without changing the medium was investigated. Impedance spectroscopic signals were recorded using Cole-Cole parameters. The slope of Cole-Cole parameter was shown to be a good indicator of cell growth and changes in cell properties. The diameter of the Cole-Cole plot was the most sensitive parameter to the obtained signals. These results demonstrate the feasibility of EIS and multi-electrode arrays in the real-time monitoring of changes in cell properties under electronic biopsy.

Figure 8. Cole parameters varying with time extracted from Cole-Cole plot. (a) Real center, imaginary center, diameter, high-frequency intercept, and low-frequency intercept. (b) Characteristic frequency and center frequency. (c) Depression angle and maximum phase. (d) R1 and R2.
Acknowledgments

This work is partly supported by the National Science Council, Taiwan, under grants NSC 94-2213-E-006-085, NSC 95-2221-E-006-219, and NSC 96-2221-E-006-251.

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