Modeling error and stability of endothelial cytoskeletal membrane parameters based on modeling transendothelial impedance as resistor and capacitor in series

James E. Bodmer,1,3 Anthony English,4 Megan Brady,3 Ken Blackwell,3 Kari Haxhinasto,3 Sunaina Fotedar,2,3 Kurt Borgman,3 Er-Wei Bai,1* and Alan B. Moy2,3*

1Department of Electrical and Computer Engineering and 2Department of Biomedical Engineering, University of Iowa College of Engineering; 3Department of Internal Medicine, University of Iowa College of Medicine and Veterans Administration Hospital, Iowa City, Iowa; and 4Department of Aeronautical, Mechanical and Biomedical Engineering, University of Tennessee, Knoxville, Tennessee

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Bodmer, James E., Anthony English, Megan Brady, Ken Blackwell, Kari Haxhinasto, Sunaina Fotedar, Kurt Borgman, Er-Wei Bai, and Alan B. Moy. Modeling error and stability of endothelial cytoskeletal membrane parameters based on modeling transendothelial impedance as resistor and capacitor in series. Am J Physiol Cell Physiol 289: C735–C747, 2005. First published May 4, 2005; doi:10.1152/ajpcell.00103.2005.—Transendothelial impedance across an endothelial monolayer grown on a microelectrode has previously been modeled as a repeating pattern of disks in which the electrical circuit consists of a resistor and capacitor in series. Although this numerical model breaks down barrier function into measurements of cell-cell adhesion, cell-matrix adhesion, and membrane capacitance, such solution parameters can be inaccurate without understanding model stability and error. In this study, we have evaluated modeling stability and error by using a χ² evaluation and Levenberg-Marquardt nonlinear least-squares (LM-NLS) method of the real and/or imaginary data in which the experimental measurement is compared with the calculated measurement derived by the model. Modeling stability and error were dependent on current frequency and the type of experimental data modeled. Solution parameters of cell-matrix adhesion were most susceptible to modeling instability. Furthermore, the LM-NLS method displayed frequency-dependent instability of the solution parameters, regardless of whether the real or imaginary data were analyzed. However, the LM-NLS method identified stable and reproducible solution parameters between all types of experimental data when a defined frequency spectrum of the entire data set was selected on the basis of a criterion of minimizing error. The frequency bandwidth that produced stable solution parameters based on modeling transendothelial impedance as resistor and capacitor in series and as a repeating pattern of disks is not sufficient to characterize the entire frequency spectrum of experimental transendothelial impedance.

cell-cell adhesion; cell-matrix adhesion; cell membrane capacitance; mathematical computation

ACTIVATION OF SIGNAL TRANSDUCTION pathways and remodeling of endothelial cell-cell and cell-matrix adhesion are critical steps that regulate inflammatory edema, wound injury and repair, and angiogenesis. Inflammatory edema formation, for example, is characterized by dynamic changes in endothelial cell-cell and cell-matrix attachment, which regulate the proper balance of fluid and protein between intravascular and interstitial compartments (3, 4, 11–13, 16, 20–24, 26–30). Endothelial barrier function relies on the mechanical properties of the cytoskeleton and its mechanical connection to the cell’s membrane (1, 4, 10, 15, 17–19, 25, 27). Thus quantification of endothelial cytoskeletal membrane properties is critical to evaluate precisely the mechanisms that regulate endothelial barrier function. By measuring transcellular impedance across a confluent monolayer inoculated on a microelectrode, we previously reported that inflammatory stimuli such as histamine and thrombin regulate human endothelial barrier function in a rapid, nonlinear, and time-dependent fashion (15, 18, 19). Because the measured transendothelial impedance is dependent on endothelial cell-cell and cell-matrix adhesion, transendothelial impedance can be used to quantify cell-membrane properties.

Identifying the specific cell adhesion sites and cytoskeletal membrane properties that regulate membrane integrity and function under physiological and pathological conditions represents a complex task, because an intervening cytoskeletal network mechanically couples cell-cell and cell-matrix adhesion sites. For example, if the cytoskeleton is viewed as an integrative structure, external stimuli can disrupt cell-cell adhesion through two basic mechanisms. Activation of signal transduction events could decrease adhesion at cell-matrix sites and cause cell rounding, which in turn could result in a secondary or reactive loss in cell-cell adhesion. Alternatively, activation of signal transduction pathways may directly target cell-cell adhesion sites and cause a direct loss in cell-cell adhesion with a reactive loss in cell-matrix adhesion. Because there are distinct adhesion proteins at cell-cell and cell-matrix sites that could be affected differentially by signal transduction pathways, it is important to identify the spatiotemporal characteristics by which molecular signals differentially affect cytoskeletal membrane properties. Thus numerical models and simulations are a critical part of evaluating the complexities of these signal transduction pathways.

Giaever and Keese (6) were the first to introduce a closed-form, mathematical model that characterizes cell-cell and cell-matrix adhesion in cultured fibroblasts by measuring transcel-
that thrombin transiently disrupted barrier function through a similar paradigm as histamine, with the exception that thrombin mediated a greater and more sustained loss in endothelial barrier function in part through contractile-dependent disruption of cell-cell adhesion (15). Along this same line of evidence, we more recently (17) reported a comprehensive study in which biophysical and numerical approaches were integrated with microscopic and biochemical approaches to evaluate how phorbol esters and thrombin regulate porcine pulmonary artery endothelial barrier function through actin-dependent mechanical forces. Like the histamine responses reported in cultured human endothelial cells, thrombin-mediated changes in transendothelial resistance occurred without microscopic changes in gap formation in living cells when viewed using time-lapse microscopy. Taken together, these data reinforce the need for new technical approaches that break down endothelial barrier function into separate indices of cell-cell and cell-matrix adhesion. Although there have been reports of modeling cell membrane properties in cultured cells on the basis of the experimental transcellular impedance, the accuracy, predictability, and reliability of this numerical model greatly depend on a thorough understanding of the numerical algorithms, the limitation of model assumptions, and the experimental factors that create model bias and instability. Without understanding these systematic factors that affect the model, the solution parameters of cell membrane properties could be erroneous and could lead to misinterpretation. In this report, we provide a complete description of the boundary conditions and intermediate algorithms that result in the closed-form mathematical model of transendothelial impedance on the basis of the assumptions that 1) a cultured monolayer is treated as an organized disk shape arranged in an ordered pattern (Fig. 1), and 2) the impedance across a cell-covered electrode is treated as a resistor and capacitor in series. By calculating the $x^2$ and using a multiresponse Levenberg-Marquardt nonlinear least-squares (LM-NLS) optimization model of the real-only, imaginary-only, real and imaginary (in complex form), and real and imaginary (in magnitude form) data, we have derived further insight into the modeling error and stability of the model’s solution parameters. On the basis of these analyses, we address the following questions in this report. 1) How much modeling error exists between optimization procedures and visual curve fitting? 2) How much modeling error and instability exist between experimental and calculated measurements? 3) Are modeling stability and error unique and a function of frequency for real and imaginary data? 4) Which numerical approach is required to derive stable and reproducible solution parameters in the real and imaginary data? 5) Is a model that is based on a repeating disk pattern and a resistor and capacitor in series sufficient to characterize the entire impedance data spectrum?

MATERIALS AND METHODS

Materials. Cultured human umbilical vein endothelial cells (HUVECs) were prepared using collagenase treatment of freshly obtained human umbilical veins as described previously (5). Harvested primary cultures designated for cell-adhesion assays were plated on 60-mm tissue culture plates coated with 100 μg/ml fibronectin (Collaborative Research, Bedford, MA). Experiments were conducted after cultures reached 2 days postconfluency. All cells were cultured in medium-199 and supplemented with 20% heat-inactivated

![Fig. 1. General model of current flow around an endothelial cell idealized as a disk shape. Voltage spreads both horizontally in a radial fashion from the cell’s center as well as vertically through the cell’s membrane. $V_I$ is the voltage present at the surface of the electrode, $V$ is the voltage present in the space between the cell’s ventral surface and the electrode surface, and $V_I$ is the voltage along the apical surface of the cell. A change in voltage, $dV$, occurs as the current $I$ spreads horizontally in the space between cells ventral surface and the electrode surface. Current $dl$, flows vertically from the electrode surface, where a change in current, $dl$, crosses vertically across two cell membranes. Finally, a change in current, $dl$, occurs as current spreads horizontally in the space between the cell’s ventral surface and the electrode surface.](http://ajpcell.physiology.org/)

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fetal calf serum, basal medium Eagle vitamins and amino acids, glucose (5 mM), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cultures were identified as endothelial cells by their characteristic uniform morphology, uptake of acetylated low-density lipoproteins, and indirect immunofluorescent staining for factor VIII.

Measurement of transendothelial impedance on a microelectrode biosensor. Endothelial barrier function was measured using a previously reported electrical substrate impedance sensing (ESIS) technique (6–8, 18). In this system, cells were cultured on a small, gold electrode (5 × 10−4 cm²) using culture medium as the electrolyte and barrier function was measured dynamically by determining the electrical impedance of a cell-covered electrode. A variable voltage-alternating signal was supplied through a 1-MΩ resistor between frequencies of 25 and 60,000 Hz. Voltage and phase data were measured using a model SRS830 lock-in amplifier (Stanford Research Systems) and then stored and processed using a personal computer. The same computer also controlled the output of the amplifier and mechanical relay switches to different electrodes using custom software written by Applied Biophysics. Cultured HUVECs were incubated on electrodes at a confluent density of 10⁶ cells/cm². Proprietary algorithms (Applied Biophysics) mathematically converted the in-phase and out-of-phase voltage into the resistance and capacitance, respectively, on the basis of the assumption that both the naked electrode and the cell monolayer were being treated as resistor and capacitor in series.

Software architecture of the numerical modeling. A LabView version 6.0 graphics software application development environment for data acquisition, analysis, signal processing, and instrument control was obtained from National Instruments (Austin, TX). The Microsoft Visual Studio integrated development environment (IDE) was obtained from Microsoft (Redmond, WA). LabView algorithms called Electrical Impedance Modeling Analysis and Simulation (EMAS) were developed to model cell membrane parameters from the measured transendothelial impedance as discussed in the next subsection.

Modeling approach. Transendothelial impedance across a cell-covered electrode was measured at 23 different frequencies. Figure 1 represents a diagram of the primary current flow paths across a confluent monolayer. Each current flow path is affected by small spatial changes in the cellular shape, which dynamically modify transendothelial impedance at each of the measured frequencies.

Three separate cardinal current flow paths govern the total modeled impedance across a confluent monolayer of endothelial cells. The first current flow path lies between the ventral surface of the monolayer and the surface of the naked electrode and is described by the parameter α, which is expressed in units of V/Ω·cm. The α term is defined by the expression α = r,√ρdh, which is dependent on the average separation distance (h) between the ventral membrane surface and the substratum, the solution resistivity, ρ (p), of the culture medium, and the cell radius (rc). The current flow path between the adjacent edges of the cells within the monolayer is labeled with the parameter (Rn), and is expressed in units of Ω·cm². The final current flow path is capacitive in nature and relates to transcellular current flow through a ventral and dorsal plasma membrane. The transcellular current is dominated by the membrane capacitance parameter (Cm) along with a transmembrane resistance and a transcytoplastmic component, which is fixed within the model. The parameter Cm is reported in μF/cm².

Fundamental equations. The following model characterizes endothelial cells as having a disk shape and being arranged in a repeating pattern. If r_c is defined as the cell radius, then the cell area is π·r_c² and the cell perimeter is 2·π·r_c. The general model predicts the experimental impedance spectrum by applying the classical Ohm’s and Kirchhoff’s laws for electrical currents. Eqs. 1–3 are Ohm’s law formulations, and Eq. 4 applies Kirchhoff’s current law, which couples the first three equations:

\[ -dV = \frac{1}{h} \cdot 2\pi r \cdot \rho dI_r \]

\[ V_c - V_i = \frac{Z_m}{2\pi r} \cdot \frac{dI_r}{dr} \]

\[ V - V_i = \frac{Z_m}{2\pi r} \cdot \frac{dI_r}{dr} \]

\[ dI = dI_r - dI_i \]

In Eqs. 1–4 above, V is the voltage present at the surface of the electrode; Vᵣ is the voltage present in the space between the cells’ ventral surface and the electrode surface, and Vᵢ is the voltage along the apical surface of the cell. A change in voltage, dV, occurs as current, I, spreads horizontally in the space between the cells’ ventral surface and the electrode surface. Current dIᵣ flows vertically from the electrode surface. A change in current occurs when part of the current dIᵣ crosses vertically across two cell membrane surfaces and part of the current dIᵢ spreads horizontally in the space between the cells’ ventral surface and the electrode surface. We assume that the electrode voltage potential is the same at any arbitrarily chosen radius from the center of the disk-shaped cell. Equation 1 describes the horizontal drop in voltage in the ventral space below the cell as it spreads radially from the center of the cell toward its outer edges. This horizontal voltage drop is governed by ρ (Rho) and h (height), which have been defined previously. Equation 2 describes the vertical change in voltage from the electrode surface into the medium below the ventral surface of the cell. These voltages are labeled Vᵣ (the voltage of the electrode) and Vᵢ (the voltage associated with the cells’ ventral surface), respectively, in Fig. 1. Equation 3 describes the change in voltage from the ventral to the apical surfaces of the cell membrane and adds the voltages labeled Vᵣ above the cells’ apical surface in Fig. 1. Equation 4 couples the first three equations using Kirchhoff’s current law by the current paths traversed from one electrode to the other. The current forks in two directions. One path is coupled capacitively across the cell membrane, and the other path travels in the paracellular compartments below and between the cells involved. Equations 1–4 lead to the following result, where V and Vᵢ are the first and second derivatives, respectively, of the voltage at the cells’ ventral surface with respect to the radius. Also Zᵣ and Zᵢ are the impedances associated with the naked electrode and cell membrane, respectively. The following equation:

\[ V = \frac{1}{r} V - \frac{\beta}{h} \left( \frac{1}{Z_i} + \frac{1}{Z_r} \right) V + \frac{\beta}{h} \left( \frac{V_i}{Z_i} + \frac{V_i}{Z_r} \right) \]

which is in the following form

\[ \frac{V}{r} + \beta V - \beta V_i = 0 \]

has the following solution

\[ V(r) = A I_0(\gamma r) + B K_0(\gamma r) + \frac{\beta}{\gamma} \]

Because \( K_0(\gamma r) \to \infty \) as \( r \to 0 \), \( B = 0 \) and thus

\[ V(r) = A I_0(\gamma r) + \frac{\beta}{\gamma} = A I_0(\gamma r) + \frac{Z_m}{Z_r} V_r + \frac{Z_i}{Z_r} V_i \]

The constant A depends on the boundary conditions. The radius is \( r_e \) and \( V_0 = 0 \) implies
The total current \( I(r) \) at \( r = r_c \) is given by

\[
I(r_c) = I_{in} - I_{out} = \int_0^{r_c} dI - \int_0^{r_c} dI
\]

(10)

where

\[
I_{in} = \int_0^{r_c} \frac{2\pi r}{Z_m} Vdr = \frac{2\pi}{Z_m} \int_0^{r_c} \left[ A_{ir} I_0(\gamma r) + \frac{Z_m}{Z_n + Z_m} Vr \right] dr
\]

implies

\[
\Rightarrow \frac{2\pi r_c^2 A}{Z_n \gamma r_c} I_1(\gamma r_c) + \frac{\pi r_c^2 V_c}{Z_n + Z_m}
\]

(11)

and

\[
I_{out} = \int_0^{r_c} \frac{2\pi r}{Z_n} (V_c - V)dr = \frac{2\pi r_c^2 A}{Z_n \gamma r_c} I_1(\gamma r_c)
\]

\[
- \frac{2\pi}{Z_n} \int_0^{r_c} \left[ A_{ir} I_0(\gamma r) + \frac{Z_m}{Z_n + Z_m} Vr \right] dr
\]

also implies

\[
\Rightarrow \frac{\pi^2}{Z_n} V_c - \frac{2\pi r_c^2 A}{Z_n \gamma r_c} I_1(\gamma r_c) - \frac{\pi r_c^2}{Z_n} \frac{Z_m}{Z_n + Z_m} V
\]

\[
= \frac{2\pi r_c^2 A}{Z_n \gamma r_c} I_1(\gamma r_c) + \frac{\pi r_c^2}{Z_n} \frac{Z_m}{Z_n + Z_m} V
\]

(12)

Therefore, the total current \( I(r) \) at \( r = r_c \) is obtained as follows:

\[
I(r_c) = I_{in} - I_{out} = - \frac{2\pi r_c^2 A}{Z_n \gamma r_c} I_1(\gamma r_c) \left( \frac{1}{Z_n} + \frac{1}{Z_m} \right)
\]

(13)

Now, we have to determine the coefficient \( A \). The boundary condition that was not described in the original publication (6) is described herein. On the one hand,

\[
V(r_c) = A I_0(\gamma r_c) + \frac{Z_m}{Z_n + Z_m} V_c
\]

(14)

On the other hand, from the boundary condition,

\[
V(r_c) = I(r_c) R_b = \frac{2\pi r_c^2 A}{\gamma r_c} I_1(\gamma r_c) \left( \frac{1}{Z_n} + \frac{1}{Z_m} \right) \frac{R_b}{\pi r_c^2}
\]

\[
= \frac{2}{\gamma r_c} A I_1(\gamma r_c) \left( \frac{1}{Z_n} + \frac{1}{Z_m} \right) R_b
\]

\[
= A \left[ I_0(\gamma r_c) + \frac{2}{\gamma r_c} I_1(\gamma r_c) \left( \frac{1}{Z_n} + \frac{1}{Z_m} \right) R_b \right] = \frac{1}{Z_n + Z_m} V_c
\]

which implies that

\[
\Rightarrow A = \frac{-Z_m}{Z_n + Z_m} V_c \left[ I_0(\gamma r_c) + \frac{2}{\gamma r_c} I_1(\gamma r_c) \left( \frac{1}{Z_n} + \frac{1}{Z_m} \right) R_b \right]
\]

(15)

Now the equivalent impedance is given by the following

\[
\frac{Z}{\pi r_c^2} = \frac{V_c - V_i}{I_{in}} \Rightarrow \frac{1}{Z} = \frac{1}{\pi r_c^2} V_c = \frac{1}{\pi r_c^2} V_i
\]

\[
\Rightarrow - \frac{Z_m}{Z_n + Z_m} V_c \left[ I_0(\gamma r_c) + \frac{2}{\gamma r_c} I_1(\gamma r_c) \left( \frac{1}{Z_n} + \frac{1}{Z_m} \right) R_b \right]
\]

Finally, by letting

\[
\gamma r_c = \alpha \sqrt{\frac{1}{Z_n} + \frac{1}{Z_m}} = \sqrt{\frac{\rho}{h} \left( \frac{1}{Z_n} + \frac{1}{Z_m} \right)}
\]

(17)

the closed form solution is

\[
\frac{1}{Z} = \left( \frac{Z_m}{Z_n + Z_m} + \frac{Z_m}{Z_n + Z_m} \right) \left( \frac{1}{Z_n} + \frac{1}{Z_m} \right)\left( \frac{1}{Z_n} + \frac{1}{Z_m} \right)
\]

(18)

The symbols \( I_0(\gamma r_c) \) and \( I_1(\gamma r_c) \) represent Bessel functions of the first kind, orders 0 and 1, respectively, with arguments of \( \gamma r_c \). In the formulation for \( \gamma r_c \), the parameter for \( \alpha \), which may represent vascular attachment, is shown with alternate relationships exposing \( h \) and \( \rho \). It is important to note that the original report by Giaever and Keese (6) characterized the impedance due to transcellular membrane conductance as two capacitors in series, one capacitor for the basal membrane and the other for the apical membrane (Eq. 19), in which \( j \) is used to represent the imaginary number that results from taking \( V - 1 \) and \( f \) represents the current frequency.

\[
Z_m = \frac{-j}{2\pi f \left( \frac{C_m}{2} \right)}
\]

(19)

In practice, a membrane-resistive component \( R_m \) due to ion transport should be present. We offer a modification of the original formulation as it was originally presented. The membrane impedance \( Z_m \) is formulated herein as a capacitor and resistor in parallel for each membrane encountered vertically through the cell as follows:

\[
Z_m = \frac{1}{\left( R_m + j2\pi f C_m \right)}
\]

(20)
nents simultaneously. The magnitude formulation of the parameter estimation process uses a one-dimensional real value result that combines the real and imaginary components simultaneously while balancing the minimized error in both the real and imaginary components between the simulated and experimental responses. The real or imaginary optimization modes are one-dimensional as well, but optimize only against the real or imaginary component alone, respectively, when selected.

Error evaluation. The real and imaginary experimental data of the cell-covered electrode (Zc) and the naked electrode (Zn) were measured at 23 frequencies between 25 and 60,000 Hz. The values of Zc used in the model were measured 24 h after cell attachment at time points at which the endothelium achieved a steady-state transendothelial resistance (TER). Values of Zn were measured after trypsinization of the cultured monolayers and replacement with fresh medium. Final calculated real and imaginary value solutions (Zc, Zn) using Eq. 21, were generated. The solutions generated were based on a set of (parms), specifically α, Rb, and Cm, over the desired frequency range (f). The function to be fit with an optimum parameter set is, of course, Eq. 18, which describes the simulated or calculated impedance, Zc.

\[ Zc = Zc(f, \text{parms}) \]  

The difference between the calculated and experimental cell impedance (Zerror) is defined by Eq. 22 below:

\[ Zerror = \frac{Zc - Zn}{Zc} - 1 \]  

The error, Zerror, was used to plot a graphical representation of the remaining error after a parametric fit was performed. A \( \chi^2 \) value was also calculated as a result of the analysis to define the modeling error between the simulated and experimental data. A raw \( \chi^2 \) value was reported, along with a red\( \chi^2 \) value divided by the number of data points involved \( n \) minus the degrees of freedom \( df \) or the number of free parameters being fit. In the case of complex data, the analysis in addition takes the square root of the final red\( \chi^2 \) value. The red\( \chi^2 \) value reported after an analysis takes the form provided in Eq. 23 below:

\[ \chi^2(\text{parm}) = \sum_{j=1}^{n} \left( \frac{Zc_j - Zc_j(\text{parm})}{\sigma_j} \right)^2 \cdot \frac{1}{N - df} \]  

In Eq. 23, the Zc values represent the actual collected data and the \( \sigma_j \) values represent the expected values for the observed values. The value assigned to \( \sigma_j \) is assumed to be 1, a unit variance, because the measurement error in this situation is not known.

Simulation procedure. The LabView-based graphical user interface provides user control over the simulation and parameter estimation routines embedded in the C++ dynamic link library. The user interface also provides numerical and graphical feedback of simulation and analytic results. The user supplies both cell-covered and naked electrode data for graphical inspection, along with an overlay of the simulated cell-covered electrode. The user observes the results as the parameters are adjusted until a reasonably close match between the simulated and actual cell-covered responses occurs. This constitutes an initial guess for the parametric estimation process and can be used to update the simulation for greater accuracy.

RESULTS

A comparison of model solution parameters derived using visual inspection and Levenberg-Marquardt nonlinear optimization. Model precision is dependent on the approach with which the numerical model is optimized to fit the experimental data. One approach that has been used previously to find the solution parameters of the numerical model involves finding the best curve fit or match to the experimental measurement using visual inspection. Figure 2 depicts curve fitting between the calculated and experimental data between frequencies of 25 and 60 kHz on the basis of different optimized approaches. Figure 2A depicts the fit between the calculated and experimental data when optimized by a typical visual inspection expressed as the ratio of the cell-covered electrode to the naked electrode resistance, in which the ratio was greatest at ~7 kHz. The fit between calculated and experimental real data was best achieved at frequencies around the peak rather than at lower frequencies.

However, a more accurate visually guided approach to derive cell membrane properties in the endothelium by systematic visual inspection requires consideration of a deterministic set of heuristics by which each solution parameter contributes to the calculated measurement of the real and imaginary data in a defined manner. As shown in Fig. 2B, α, Rb, and Cm uniquely contribute to the real data in a defined fashion. If α is increased in value, characteristic leftward and upward shifts in the peak in real data occur. Conversely, if Rb decreases in value, the calculated peak real data shift rightward and downward. If the value of Rb increases, the net effect is to increase the peak of the real data in a vertical direction. Conversely, if Rb decreases, the net effect is to decrease the peak of the real data in a vertical direction.

Curve fitting between the calculated and the experimental real data appears similar using visual inspection between the different optimization approaches. However, the derived values of α, Rb, and Cm for the different visually based optimization approaches compared with the LM-NLS procedure are distinctly different (Table 1). The values of α, Rb, and Cm were similar between those derived by a LM-NLS procedure and a systematic visual inspection approach that considered the heuristic features of the real data, which would be considered the visual approach of an expert user. The percentage accuracy, defined as the percentage of the solution parameters derived by a heuristic approach of the values derived by the LM-NLS procedure, was small (~5%). However, the values derived using a typical nonheuristic approach, which would be considered the visual approach of a layperson or an inexperienced user, resulted in much greater differences, which ranged from 8% for Cm to 32% for Rb, and to 47% for α compared with the LM-NLS procedure solutions. Taken together, these results demonstrate that deriving parametric solutions solely on the basis of visual inspection lacks accuracy and supports the requirement for numerical nonlinear optimization approaches to derive parametric solutions regarding cell membrane properties.

Effect of frequency on model error and solution parameter stability when modeling the experimental real data. Because the model showed differences in accuracy in curve fitting the calculated real data to the experimental data as a function of frequency, the next step was to quantify this difference or error as a function of frequency. Figure 3 depicts the error (defined as Zerror in MATERIALS AND METHODS) between the experimental
real and imaginary data and the simulated real and imaginary data, whose parameters were derived using the LM-NLS procedure. As shown, the $Z_{\text{error}}$ is very small (<5%) for the real data, except at frequencies <2 kHz. In contrast, the range for relatively small error is a narrower bandwidth between 20 and 60 kHz for the imaginary data. Taken together, these data suggest that deriving numerical solution parameters regarding cell membrane properties are affected by the frequency spectrum selected for the model. Also, the data suggest that the optimal frequency spectrum that is optimized to solve the model solution parameters is dependent on the type of data modeled.

Because the different levels of modeling error were observed as a function of frequency, model solution parameters were correlated with a measured normalized $\chi^2$ assessment at different frequency spectra on the basis of the real data. For this analysis, an upper-frequency limit of 60 kHz was fixed, while the lower-frequency marker was adjusted in predefined steps from 25 Hz to 30 kHz. Increasing the lower limit created a progressively smaller data set over a narrow-frequency bandwidth that was subjected to the LM-NLS procedure to solve the membrane solution parameters. As shown in Fig. 4, the reduced $\chi^2$ when modeling real data was greatest when using the entire frequency spectrum as anticipated. Yet, at a lower frequency marker between 2 and 10 kHz, the reduced $\chi^2$ achieved a relatively low plateau level.

The solution parameters for cell membrane properties on the basis of real data were unstable when using a lower-frequency marker <2 kHz, which corresponded to the highest levels of reduced $\chi^2$ error. Membrane property solutions were particularly unstable for $\alpha$. However, model solutions for cell membrane properties were quite stable over a frequency bandwidth in which the lower frequency ranged between 2 and 10 kHz, which corresponds to the frequency impedance spectrum that includes the left side, the peak, and the right side of the real data curve as shown in Fig. 2B. By increasing the lower-frequency limit >10 kHz, the reduced $\chi^2$ value decreased but was associated with less stable solutions of $\alpha$. This observation was anticipated because real data at frequencies on the left side of the real data peak are required to resolve measurements of $\alpha$ as depicted in Fig. 2B. Taken together, these data indicate that there is a unique frequency spectrum that yields stable solution parameters when modeling the real data, and the solution parameters are unstable or unreliable when the model attempts to solve for solution parameters using a data set selected from a frequency spectrum using a lower-frequency marker outside 2–10 kHz.

**Frequency-dependent error and solution parameter stability when modeling the experimental imaginary, magnitude, and complex data.** If the numerical model is sufficient to derive model solution parameters, then the LM-NLS model should produce the same solution parameters, regardless of whether it is modeling the real data, imaginary data, magnitude data, or complex data.

![Fig. 2](http://ajpcell.physiology.org/)

**Fig. 2.** Several images comparing the curve fitting of the calculated resistance (blue dashed lines) with the measured resistance (solid green lines) expressed as a ratio of a cell-covered to a cell-free electrode. **A:** parametric estimation based on a nonexpert’s visual inspection. **B:** red, yellow, and blue arrows representing the heuristic directions in which the modeled response graph will change its shape when the model parameters for $\alpha$, $R_b$, or $C_m$, respectively, are increased in value during an expert’s visual inspection. When $\alpha$ is increased, the left side of the curve moves leftward and upward. When $R_b$ is increased, the peak of the curve moves upward. Finally, when $C_m$ is increased, the right side of the curve moves leftward and downward. **C:** fit performed by an expert using the rules previously described in **B.** **D:** optimal fit performed using the Levenberg-Marquardt nonlinear least-squares (LM-NLS) numerical curve-fitting method.
the complex data of the impedance. Before we can assess whether the LM-NLS can achieve stable solutions across these different data, it is critical to explore how modeling stability is affected by the frequency spectrum for each type of data. Figure 5 shows the correlation between numerical solution parameters and reduced $\chi^2$ for imaginary data. Modeling only the imaginary data demonstrates similarities and unique behaviors compared with modeling only the real data. Like modeling the real data, model stability and error were worse at low frequencies. However, the impedance spectrum that achieved the most stable model solutions at the least $\chi^2$ error was narrower than that observed when modeling the real data. In addition, the frequency spectrum for identifying the most stable solution parameter at a low plateau $\chi^2$ error for the imaginary data (10–15 kHz) did not overlap with the impedance spectrum for real data (2–10 kHz). Again, like the real data, modeling the imaginary data at >15 kHz resulted in a lower $\chi^2$ error as well as a less stable solution for $\alpha$.

In contrast, the best stability of the numerical model at the lowest plateau of reduced $\chi^2$ error on the basis of the magnitude data overlapped the frequency spectrum between the real and the imaginary data (Fig. 6). The most stable numerical solution parameters on the basis of the magnitude data were observed between 7 and 15 kHz. The best stability of the numerical model at the smallest reduced $\chi^2$ error on the basis of the two-dimensional complex formulation, including both real and imaginary data simultaneously, was observed at a narrow frequency bandwidth between 20 and 30 kHz (Fig. 7). Taken together, these data demonstrate that modeling stability is dependent on the frequency spectrum and that the targeted frequency spectrum data that the LM-NLS optimize are dependent on the type of data modeled.

Table 1. Comparison of the parametric estimates derived by a nonexpert’s visual inspection

<table>
<thead>
<tr>
<th>Method Type</th>
<th>Parametric Estimate</th>
<th>Ratio of Parameter to LM-NLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$</td>
<td>$R_b$</td>
</tr>
<tr>
<td>Layperson</td>
<td>1.96</td>
<td>3.63</td>
</tr>
<tr>
<td>LM-NLS method</td>
<td>3.66</td>
<td>2.75</td>
</tr>
</tbody>
</table>

“Nonexpert” visual inspections were conducted by laypeople. The parametric estimates derived by nonexpert visual inspection are shown in Fig. 2A, and those derived by an expert’s visual inspection are shown in Fig. 2C. Those derived using the Levenberg-Marquardt nonlinear least-squares (LM-NLS) optimization method are shown in Fig. 2D. The right side of the table shows the ratio of the parametric values derived by the two visual inspection approaches compared with the values derived using the LM-NLS approach. See text for discussion.

In contrast, the model achieved the most stable solution parameters when the LM-NLS procedure identified the parameters on the basis of the criteria of choosing the frequency bandwidth that provided a low, reduced $\chi^2$ error (Fig. 9). Using this approach, we found that the model identified very reproducible solution parameters between modeling the real, imaginary, magnitude, and complex data. Taken together, these data demonstrate that the model can solve for stable solution parameters, regardless of the type of impedance data formulation that is used on the basis of an approach that selects the frequency bandwidth that minimizes error. Yet, by the same token, the resistor and capacitor in series model is not sufficient to characterize the entire impedance spectrum, because the most stable solution parameters were achieved within a subset of the impedance spectrum.

Comparison of constant bandwidth vs. minimum $\chi^2$ analysis with consideration of model formulations of real, imaginary, magnitude, and complex data. To illustrate how modeling stability is a function of the type of data modeled and the frequency spectrum used, we compared the mean solution parameters derived using the LM-NLS method for the real, imaginary, magnitude, and complex data on the basis of selecting the same frequency bandwidth that was optimal for the real data (2–60 kHz) (Fig. 8). As anticipated, selecting the same bandwidth to model all types of impedance data resulted in very dissimilar solution parameters. The LM-NLS method had the most difficulty in identifying a reproducible solution for the $\alpha$ parameter, which is most sensitive to frequency. The model solutions for $R_b$ and $C_m$ identified using the LM-NLS procedure were slightly higher for the imaginary, magnitude, and complex data than for the real data.

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Fig. 3. Graphs displaying the Electrical Impedance Modeling Analysis and Simulation (EMAS) front panel depicting the remaining $Z_{error}$ after a parametric fit between the calculated and the experimental data. The left plot displays the $Z_{error}$ of the real data, and the right plot displays the $Z_{error}$ plot of the imaginary data.
Effect of including fixed values of transmembrane resistance \( R_m \) on parameter solution stability. In the original report by Giaever and Keese (6), transcellular membrane conductance was characterized as two capacitors in series: one capacitor for the basal membrane and the other for the apical membrane (Eq. 19). A practical modification includes a membrane-resistive component due to ion transport (Eq. 20). Including this parameter in the list of parameters to be fit generally resulted in severe instability in the resulting parameter estimates. Fixing this parameter to a certain value restored robustness to the solution estimation process. If one fixes values of transcellular membrane conductance \( R_m \) at various values (Fig. 10), one can then observe the effect on perturbing the results of parameter estimation on the remaining parameters. For fixed values of \( R_m > 200 \, \Omega/cm^2 \), the resulting parametric estimates for the remaining parameters were <1% of the difference (0.03% for \( C_m \), 0.83% for \( R_b \), and 0.57% for \( \alpha \)) compared with the asymptotic values to the right side of the graph. These data show that if the likely value for transcellular membrane conductance \( R_m \) is fixed to a value >200 \( \Omega/cm^2 \), then the resulting analysis parameter solutions and \( \chi^2 \) error will not be affected.

DISCUSSION

Endothelial cell-cell and cell-matrix adhesion are critical determinants in regulating endothelial barrier function, angiogenesis, atherosclerosis, and metastatic cancer. Measurement of transendothelial impedance across cultured endothelial monolayers inoculated on a microscopic electrode has increasingly been used as a popular technique to measure endothelial integrity (7, 8, 18, 26). Yet, because the electrode is larger than the diameter of a single cell, the electrical current conducts through three pathways that are impeded by cell-cell adhesion, cell-matrix adhesion, and membrane capacitance (6). Breakdown of transendothelial impedance into the cell-membrane parameters cannot be measured directly but instead must be derived by mathematical modeling. We previously documented and validated a numerical model, originally developed by Moy et al. (19), that characterized transendothelial impedance as a resistor and capacitor in series in which there are two major assumptions. First, the cell’s geometry is considered disk shaped. Second, current travels radially from the electrode and travels under cells and then traverses through and between cells. Accurate and precise measurements of cell-membrane properties are critically dependent on numerical solution parameters derived using optimization procedures that exhibit minimal numerical error and stability. Yet, to date, there have been no systematic studies reported that have quantified numerical stability and error. Without understanding these factors, measurements of endothelial cell-cell and cell-matrix adhesion derived from transendothelial impedance can lead to inaccurate interpretations. In the present report, we have quantified...
tified and used several strategies to assess modeling stability and error on the basis of a LM-NLS optimization algorithm of the real and imaginary components of transendothelial impedance. LM-NLS optimization algorithms are considered a robust and well-recognized optimization approach to execute numerical analyses of nonlinear computational problems.

The data show that the estimations of model solution parameters of cell membrane properties are dependent on the frequency spectrum and the type of impedance data subjected to the LM-NLS procedure. Modeling stability was assessed by examining how the LM-NLS estimated cell-membrane parameters and \( \chi^2 \) error for the real, imaginary, complex, and magnitude transendothelial impedance data as a function of frequency bandwidth. For each type of experimental data, model solution parameters were dependent on unique frequency spectra. The frequency spectra that achieved the most optimal solutions at low plateau level \( \chi^2 \) error were nonoverlapping when the LM-NLS method was used to estimate solution parameters from the real and imaginary experimental data. Optimization of solution parameters on the basis of the magnitude and complex modes took on the partial character of the real and imaginary formulations. The frequency bandwidths to identify stable solution parameters on the basis of magnitude data did not represent the sum of the bandwidths of the real and imaginary data alone. Because the optimal frequency bandwidths for the real and imaginary data were nonoverlapping, the real and the imaginary data have different impacts on the magnitude and the complex data. The magnitude data represent a one-dimensional quantity formed from the real and imaginary data components. If the bandwidths for identifying stable solution parameters for the real and imaginary data components did not overlap, then it would be anticipated that the bandwidth for identifying stable solutions on the basis of magnitude data would not increase, which was the case. In contrast, the complex mode formulation represents a two-dimensional quantity of the real and imaginary input components, which requires satisfying the model for both data types simultaneously. Because the frequency bandwidths for the real and imaginary data did not overlap, it was expected that optimization algorithms to derive stable solutions at minimal error would occur over a narrow-frequency bandwidth, which was the case.

By choosing the appropriate bandwidths for the analysis and by minimizing the \( \chi^2 \) result in each case, the LM-NLS procedure achieved very consistent results. Upon analyzing the model, we found that our data show that the extrapolation error also needs to be minimized. This notion is supported by the notion that the cell membrane parameters were consistent between the real, imaginary, complex, and magnitude data sets when a strategy was used to identify the frequency spectrum.
that minimized error in terms of the measured $Z_{\text{error}}$ or $\chi^2$. Graphed plots with the measured $Z_{\text{error}}$ were used to extract the actual extrapolation error. In contrast, when a fixed frequency spectrum that was suited to the real data was applied to the imaginary, magnitude, and complex data sets, there was added variability in the estimates in the cell membrane parameters, which indicates that such an approach leads to greater modeling error and parametric estimate instability. In particular, the greatest variability in the solution estimates occurred in cell-matrix adhesion when modeling the different types of impedance data. Furthermore, the model solution parameter instability was observed most frequently for the cell-matrix adhesion parameter in a frequency-dependent fashion. Extremely low values for $\chi^2$ can be achieved if not enough data are used for fitting. For example, if only three data points were used to derive solution parameters, a $\chi^2$ of 0 would likely result. However, there is a tradeoff in choosing a too small data subset to minimize $\chi^2$, which would unduly affect the model predictions for the larger original data set.

Under ideal conditions, the instrumental noise would also be known at each frequency and the model would provide a true representation of the experimental system. In these cases, the successful optimization of the model parameters would produce a reduced $\chi^2$ on the order of unity. Frequency data points with large deviations would be weighted less than those with smaller deviations. In cases in which the instrumental noise is not known, one begins by assuming that noise remains constant. If the underlying noise distribution is frequency dependent, large numerical instabilities can arise during the optimization process and determining a stable range of sampling frequencies would be necessary. Frequency-dependent systematic errors can introduce an additional complication.

Although introducing noise measurements into the $\chi^2$ analysis can improve the stability, it can introduce additional numerical artifacts. In cases in which the noise fluctuations are insignificant, singularities could arise during the computation. This can occur, for example, when filtering successfully reduces the electrical fluctuations to the level of the analog-to-digital discretization level. If the noise is non-Gaussian, the estimation would not be a maximum likelihood. Filtering, 60-Hz noise, and other artifacts, for example, could introduce non-Gaussian noise into the data.
By their nature, nonlinear optimization algorithms can produce optimized parameters that are dependent on the starting parameters. By preceding the nonlinear optimization with a visual fit, a more appropriate starting parameter can be chosen.

Identifying the optimal frequency bandwidth was first accomplished by identifying the upper- and lower-frequency bands at which \( \chi^2 \) was typically low (<10%) by first applying the LM-NLS procedure to the entire experimental data set between 25 and 60,000 Hz. Next, the optimization algorithms were repeated with the restricted data subset at the targeted frequency bandwidth. Our data demonstrate that using criteria that derived cell-membrane parameters on the basis of minimizing the \( \chi^2 \) error in the LM-NLS optimization resulted in the most stable and reproducible cell membrane parameters.

The present data also demonstrate the principle that deriving solution parameters on the basis of visual inspection criteria alone is prone to potential error. Choosing a visual fit between the calculated model and the experimental data without regard to the heuristically guided approach potentially leads to significant error. Yet, even with a heuristically guided approach, some remaining error cannot be eliminated. The current data demonstrate the importance of a numerically guided approach that automates and finds model solution parameters on the basis of recognized numerical optimization approaches.

In our study, we also evaluated the impact of the membrane-resistive component of the cell monolayer impedance. Previous reports by Giaever and Keese (6) did not document the impact of the membrane resistance on modeling error and parametric solution estimates. As defined in Eq. 20, transcellular membrane impedance is inversely related to the value of \( R_m \). We observed that the impact of \( R_m \) on model solution parameters of \( \alpha, R_b, \) and \( C_m \) as well as \( \chi^2 \) becomes inconsequential as values of \( R_m \) exceed 2000 \( \Omega \cdot \text{cm}^2 \). The limited role of \( R_m \) is consistent with the empirical evidence showing why it is possible to measure the very low ion conductance using patch-clamping techniques (2). To measure the typical picoampere levels of ion channel conductance on the basis of Ohm’s law, \( R_m \) must be large so that it can be treated as a constant.

The model originally proposed by Giaever and Keese (6) has a systematic \( \chi^2 \) between the experimental and the calculated data on the basis of the model. While the model captures the experimental data for the real data at frequencies >2,000 Hz (<5% error), the model does not fit the data at low frequencies. Furthermore, the model captures an even narrower subset of the data for the imaginary, magnitude, and complex data. To compensate for this systematic error and achieve modeling stability, we developed computational algorithms that can select a subset of the original database at variable-frequency bandwidths. In this fashion, we are able to exclude data in the frequency range <2 kHz, where the error is most prevalent.

There are several potential explanations for the systematic error between the calculated and the experimental data, which require an understanding of the assumptions of the experimental measurement and the numerical model originally proposed by Giaever and Keese (6). First, there may be systematic error introduced by the instrumentation circuit, which affects how biological activity is measured. The original model proposed by Giaever and Keese assumes that both the naked electrode and the cell-covered electrode behave as resistor and capacitor in series. However, this cannot be validated directly, because the ESIS system provides only the resistance and capacitance measurements, which are mathematical conversions of the raw data (in-phase and out-of-phase voltage). The mathematical conversion algorithms are proprietary and protected by trade secret.

Second, the model assumes that there is no drift in the instrumentation system. \( Z_n \) is not modeled but is simply mathematically divided into \( Z_n \), which holds true only if both \( Z_n \) and the transendothelial impedance are both resistors and capacitors in series. If either the monolayer or the electrode does not behave as resistor and capacitor in series, then different numerical expressions for \( Z_n \) and \( Z_c \) are required. Furthermore, the model must assume that \( Z_n \) behaves as a constant and exhibits no measurable drift over time. If there is significant electrical drift, then \( Z_n \) needs to be modeled numerically. Thus, for this reason, we chose experimental data for \( Z_n \) after removing cells from the electrode with trypsin to reduce \( Z_{\text{error}} \).

Third, the model assumes that the cell geometry is disk shaped and contains gaps between cells. Because endothelial cells are not disk shaped, it remains to be validated whether the
model solution parameters and the model stability are affected by selecting a different cell geometry.

Fourth, the LM-NLS optimization assumes a Gaussian distribution of noise across all measured frequencies. For these analyses, the σ value for the $x^2$ was assumed to be unity, because σ was unknown and was not experimentally measured. If there is variable distribution of noise as a function of frequency, then the optimization algorithms require a weighted function to compensate for frequency-dependent noise levels.

The results of these data document that modeling transendothelial impedance as a circuit that consists of a repeating pattern of disks and a resistor and capacitor in series is not sufficient to model the entire impedance frequency spectrum. The present data indicate that a more complicated numerical model is required to characterize the entire impedance spectrum between 25 and 60,000 Hz. More complicated models should provide a more complete fit between the simulated and experimental measurements at all measured frequencies and for both real and imaginary data.

It is important to emphasize that the experimental measurement derived using ESIS does not provide indices of cell-cell adhesion, cell-matrix adhesion, and membrane capacitance directly. Rather, these parameters must be derived by numerical modeling because the electrode area is greater than the diameter of a single cell. Changes in transendothelial resistance in response to physiological stimuli are frequently assumed such that changes are targeted at cell-cell adhesion sites. However, we recently reported that PKC activation initiates a disruption in barrier function that targeted primarily cell-matrix adhesion sites (17). In addition, we recently reported heterologous expression of low-molecular-weight, caldesmon-attenuated, adenovirus-mediated reduction in transcellular resistance in cultured fibroblasts predominately through effects on cell membrane capacitance (9). Only by numerical modeling the experimental transcellular impedance at multiple frequencies can one elucidate and localize the membrane sites at which changes are targeted at cell-cell adhesion, cell-matrix adhesion, and membrane capacitance.

In summary, we provide the first comprehensive assessment of modeling error and stability on the basis of a numerical model that characterizes transcellular impedance across a cell-covered electrode as disk shaped and as a resistor and capacitor in series as originally described by Giaever and Keese (6). We demonstrate that there are potential data-type and frequency-dependent modeling instabilities and systematic errors in the solution parameters. Understanding these experimental factors can allow investigators to produce reproducible and reliable numerical solutions of cell-cell and cell-matrix adhesion and membrane capacitance from measured transendothelial impedance. Use of a numerically stable parameter estimation process and inclusion of the appropriate range of frequencies in the parametric estimation process can allow one to obtain more accurate and reproducible parametric estimates of cell membrane properties. Because the diameter of the electrode exceeds the diameter of a single cell, the experimental measurement of ESIS cannot derive spatial measurements of cell membrane properties without reliable numerical models and computational algorithms. By quantifying the sources of error and parameter estimate instabilities, a method of impedance spectroscopy for determining in vitro cell monolayer properties can elucidate more precisely the cytoskeletal membrane properties that regulate endothelial barrier function.

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Present address of A. B. Moy: Cell Engineering Technologies (CET), Inc., 2660 Crosspark Road, Coralville, IA 52241-3212 (e-mail: moya@celleng-tech.com).

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REFERENCES


