

Temporal changes in electrical properties of cell culture media in response to different types of electrical stimulation

Elyse Rier

McMaster University

Taylor deVet

McGill University

Gregory Wohl

wohlg@mcmaster.ca

McMaster University

Research Article

Keywords: Electrical stimulation (ES), impedance, cell culture media, stimulation parameters, voltage stimulation, current stimulation

Posted Date: October 19th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11271845/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: Competing interest reported. GW is a principal in Additive Manufacturing International Inc, which provided funding to ER.

Abstract

The use of electrical stimulation (ES) has been found to affect cell movement, proliferation, and growth throughout in-vitro experiments. Many studies performing in-vitro ES do not report changes in the electrical signal through the cell culture media. This study quantified the change in impedance throughout the cell culture media under the application of voltage and current stimulation at three stimulation levels and three waveform types (constant, monophasic and biphasic). It was found that the measured impedance increased throughout the duration of the stimulation, with the change in impedance and the final impedance level varying based on the stimulation level and the waveform, where the biphasic and monophasic waveforms demonstrated lower impedance levels than constant stimulation. This study informed the changes in the media throughout the duration of the applied in-vitro stimulation, suggesting it is important to monitor stimulation parameters as they may vary throughout the experiment. Changes in the expected stimulation may affect the cellular responses and affect the interpretation of stimulation parameters required for cell lines.

Background

Endogenous electric signals are responsible for the movement, proliferation, and growth of cells. (1, 2) This has been extensively recreated *in vitro* to further the understanding of cellular electrical sensitivity, focused mainly on neuronal and connective tissue cells. (3–11) Low voltage exogenous electric fields have been used to trigger cell proliferation, adhesion, and migration. (1, 2, 9)

While performing electrical stimulation (ES) of cells *in vitro*, it is very difficult to get consistent results. (12) The exact pathways that are activated within the cells are unknown and vary across cell types. It is hypothesized that an influx/efflux of ions is triggered through the cell membrane, (12) but the exact trigger varies. Movement of ions causing local concentration gradient changes in the medium, or charge differences opening voltage-gated channels, are hypothesized to be the main triggers of cellular activation. (13)

Electrical current is the movement of electrical charge over time, whereas the strength of an electrical field is the voltage gradient over a distance. In electrical stimulation of tissues, the important factor appears to be the amount of charge delivered as opposed to the field strength. (6, 14) Studies suggest that cells respond best to current in the range of 250 μ Amps. (15, 16) However, this varies depending on the method of stimulation. (8)

A consistent factor across all *in vitro* experiments is the need for a cell culture medium to maintain cells. While culture media come in many forms, they generally share similar compositions and are designed for cellular viability, not with ES in mind. The effects of excessive stimulation can directly affect cell viability, specifically through changes in pH (17), but also through secondary electrochemical reactions that can cause cellular apoptosis. (17, 18) Hydrogen peroxide is one of these products, and the amount generated is directly proportional to the pulse width, frequency, and voltage of ES in the medium. (19) Changing the shape of the electrical signal can mitigate the level of byproduct but does not fully

eliminate its production(20, 21) As a double layer of charged ions forms at the electrode-medium interface, it behaves as a resistor and capacitor (RC circuit) in series,(21) which is likely to cause the medium's electrical properties to behave as an RC circuit over time. The effects of ES in media have not been well quantified, and understanding the behaviour of the media under these conditions would give better insight into the consistency of the stimulation delivered as well as the side effects that are produced.

The purpose of this work was to assess electrical changes to cell culture media during ES to determine its behaviour. Specifically, we looked at spatial and temporal pH changes through changes in phenol red colour, and temporal changes in electrical impedance of the medium through the application of constant, monophasic, and biphasic stimulation at varying EFs and currents.

Methods

Electrode set up

Platinum wires (90% platinum, 10% iridium, Thermo Fisher Scientific Inc.) were bent into an “L”-shape with a horizontal leg 10 mm long and a vertical leg of 20 mm. A 3 mm acrylic plate was laser-cut to hold electrodes parallel with 10 mm separation between the horizontal legs in a standard 12-well plate (Corning Falcon). The electrodes were inserted into holes in the acrylic plate and held in place with polycyanoacrylate (Krazy Glue, Elmer's Products, Inc., Columbus, OH, USA) 10 mm apart and 5 mm from the bottom of a 12-well plate. The long legs of the electrodes (perpendicular to the bottom of the well) were positioned on opposite sides of the well to generate an electric field between the small legs of the electrodes parallel to the bottom of the well (Fig. 1A, B). The tops of the electrodes exposed above the acrylic were connected to the stimulating circuit through alligator clips (Fig. 1C).

Stimulating circuits

Two circuits were designed to apply ES, one delivering a target voltage and the other a target current; referred to henceforth as the voltage circuit and current circuit, respectively. The voltage circuit consisted of a pulse generator (DG535, Stanford Research Systems INC, Sunnyvale, California) as the input to three parallel branches, each consisting of a 1 k Ω resistor in series with a set of PtIr electrodes (Fig. 2A). The tests performed using the voltage circuit included: 1) constant target voltage at three levels (DC) (0.5, 1, and 2.5 V), 2) biphasic target voltage pulses ($\pm$ 0.5, 1, and 2.5 V), and 3), and monophasic target voltage pulses (+ 0.5, + 1, +2.5, -0.5, 1, and - 2.5 V).

The current circuit used a Howland current pump (Fig. 2B). This circuit modifies the voltage across the load to maintain the desired current (22). In this case, the load consisted of a 1 k Ω resistor in series with three sets of electrodes. The input to the Howland current pump was from the function generator. The three sets of PtIr electrodes were placed in series with the resistor, and a single Howland current pump was used for all wells to reduce variations among the wells. The tests performed using the current circuit

included: 1) constant target current (DC) (50, 100, 500 μA), 2) biphasic target current pulses (± 50 , ± 100 , ± 500 μA), and 3) monophasic target current pulses (+ 50, + 100, +500, -50, -100, and - 500 μA).

For both the current and the voltage monophasic and biphasic stimulation, the input was set as a square wave, with a frequency of 1Hz and a 50% duty cycle. The monophasic stimulation was created by adding either a positive or a negative offset to the biphasic waveform. In both cases, a 1 k Ω resistor was added upstream of the electrodes to monitor the current passing through the electrodes. The impedance of the cell culture media was not known, the voltage across the known resistor was used to estimate the current through the media and PtIr electrodes. The voltage across the electrodes and the calculated current were used to estimate the total impedance, which included the electrodes and the cell culture media.

Experimental Design

Cell culture media (McCoy's 5A, Sigma-Aldrich) was removed from 4°C storage 1 hour before testing to allow it to reach room temperature. This was to ensure that any measured changes in impedance or observations of colour change were not due to changes in the temperature of the media. The media contained phenol red, a pH indicator that is yellow in colour at a pH of 6.4 or less and is red at a pH of 8.2 or greater.⁽²³⁾ During the experiments, the voltage across the electrodes and the voltage across the measurement resistors were measured. Observations were collected on physical changes in the media, such as bubbles or accumulation of precipitates on the electrodes, and colour changes in the media were noted. The stimulation was applied for a 1-hour period. Images and visual observations of the stimulated wells (containing electrodes) and the control wells (no electrodes) were taken at 0 minutes, 15 minutes, 30 minutes, and 1 hour.

Voltage measurements were taken with an NI DAQ system (NI USB-6218, National Instruments, Austin, Texas). This system was used to measure the voltage difference between two points in the stimulation system. For the voltage tests, recorded measurements included the voltage across each measurement resistor before each well (R_{m1} , R_{m2} , and R_{m3}), the input voltage (V_{in+} - V_{in-}), and the voltage across the electrodes (V_{E1} , V_{E2} , V_{E3}). For the target current circuit, the recorded measurements included the voltage across the measurement resistor (R_m), the input voltage, and the voltage across the electrodes (V_{E1} , V_{E2} , V_{E3}). Data were collected using LabView (2017, National Instruments, Austin, Texas), saved as an Excel file (Microsoft Excel v. 16.107.4), and data analysis was performed in MATLAB (V 2026a, The Mathworks, Inc., Natick, Massachusetts). Stimulation circuits are shown in Fig. 2, where the inset in Fig. 2B shows the Howland Current Pump with VCC and VEE of + 15 and - 15 V, respectively. In the circuit, R_f and R_g were 1 k Ω , R_s was 4.7 k Ω , and the input signal was a constant, square monophasic, or square biphasic wave depending on the stimulation type.

Data Analysis

For the voltage stimulation, the voltage measured across the measurement resistors (R_{m1} , R_{m2} , and R_{m3}) was minimal and was susceptible to noise. To combat this, the resistor voltages were calculated by subtracting the value of each electrode voltage (V_{E1} , V_{E2} , and V_{E3}) from the input voltage, V_{in} . For the current stimulation, this subtraction was not required, as the measured voltage across the resistor was higher, less noisy, and a single resistor was used to calculate the current passing through all the wells. Before calculating the impedance, a low-pass filter was applied to the data; a cutoff frequency of 0.1 Hz was used for the constant stimulation, and a cutoff frequency of 2Hz was used for the monophasic and biphasic waveforms.

For the constant waveforms, the current through the resistor was calculated by dividing the resistor voltage by its corresponding resistance (1k Ω) following Ohm's law rule of $V = IZ$, where Z is the impedance. The impedance between the PtIr electrodes was calculated by dividing the measured electrode voltages at every point by the corresponding calculated current.

For the monophasic and biphasic waveforms, the first pulse was determined from the input voltage, and groups of 5 data points were used to analyze each pulse. For the monophasic waveforms, the impedance was calculated for the 'ON' positions, and for the biphasic waveforms, all calculations were done on the positive half of the waveform. For each pulse, the max voltage across the PtIr electrodes was divided by the current calculated from the resistor voltage for the same time point.

Calculated impedance values were considered outliers and removed if they were more than three standard deviations away from the mean. The average impedance was calculated for each minute in the test. The average change in impedance between the first and last minute and the average final impedance in the three wells was calculated for every stimulation level and waveform.

The average energy of the voltage signals measured for the three wells was calculated for all combinations of stimulation level and stimulation type. The energy of the signal was calculated for each well using the formula: $E = \frac{1}{f_s} \sum_{n=-\infty}^{\infty} |x[n]|^2$. Where $x[n]$ is the voltage signal at sample n , and f_s is the signal sampling frequency.

The average impedance for each minute was fit with a two-term exponential equation in the form of $y = ae^{bx} + ce^{dx}$ consistent with the second-order RC equivalent circuit models used in other studies. (21, 24–26) The fit was performed with the use of the curve fitting toolbox in MATLAB, with the data centred and scaled based on the time. To determine the time constants, the values of b and d in the equations were multiplied by the standard deviation, followed by the subtraction of the mean that was used to scale the data. The average of the time constants $1/b$ (τ_1) and $1/d$ (τ_2) for all three wells was determined.

Statistical Analysis

All statistical analyses were performed in RStudio (R v4.5.2, R Foundation for Statistical Computing, Vienna, Austria). Statistics were performed on the constant, positive monophasic and biphasic signals.

For simplicity, the negative monophasic signals were not included due to their similarity to the positive monophasic signals; for the remainder of this paper, monophasic will refer to the positive monophasic stimulation. A linear model was fit to include the waveform type and stimulation level as the main effects and included the interaction between the waveform and stimulation level. A two-way ANOVA was performed on the linear model for the change in impedance, the final impedance, and the two time constants (τ_1 and τ_2), for the voltage and current stimulation separately. The factors used were the stimulation level (Low, Mid, and High), and the waveform type (Constant, Positive Monophasic, and Biphasic). The stimulation levels for the current stimulation were 50, 100, and 500 μA , and the levels for the voltage stimulation were 0.5, 1, and 2.5V. Following statistical significance in the ANOVA, a Tukey post-hoc analysis was performed on the estimated marginal means using the emmeans (Estimated Marginal Means, v. 2.0.4) package.⁽²⁷⁾ The voltage and current stimulation were not directly compared as the stimulation method and levels were not equivalent.

Results

Stimulation Levels

For the current circuit, the actual current produced varied slightly from the targeted current values of 50, 100, and 500 μA ; the values that were achieved in the tests were approximately 52, 103, and 470 μA , respectively. The deviation from the target current was due to variations in the circuit and the use of standard-value resistors that varied from the value required to achieve the target current values.

Waveform Shapes – Biphasic and Monophasic Stimulation

When biphasic voltage stimulation was applied, the voltage across the resistor demonstrated a sharp increase in voltage and then a relaxation or discharge with each cycle (Fig. 3A). As expected for elements in series, changes in voltage across the resistor through the stimulation cycle exhibited a reciprocal relationship to changes in the voltage across the electrodes; when there was an increase in voltage across the electrodes, there was a drop in voltage across the resistor, and the balance of the resistor and electrode voltages maintained the targeted voltage value throughout the circuit. The voltage pulses across the electrodes were close to a square wave in shape and exhibited characteristic charging and discharging seen in capacitors (Fig. 3B). When voltage was applied with a monophasic waveform, the response across the resistors (Fig. 3C) was similar to that of the biphasic voltage (Fig. 3A) but with a lower magnitude. The voltage across the electrodes for the monophasic voltage was more square (Fig. 3D) compared to the biphasic voltage (Fig. 3B). Compared to a biphasic voltage, the biphasic current stimulation demonstrated a square wave across the resistor reflecting the consistent current, while the voltage across the PtIr electrodes followed a triangular wave shape (Fig. 3E,F).

Overall, during the voltage stimulation for the biphasic and monophasic stimulation, the voltage across the resistor decreased over the hour of stimulation while the voltage across the PtIr electrodes remained

at the same magnitude.

When the current was applied using a monophasic waveform, the voltage across the resistor followed a consistent square wave (Fig. 3G) while the voltage across the PtIr electrodes was smaller in magnitude than the biphasic current (Fig. 3H). In addition to a smaller magnitude, the PtIr electrode voltage was shifted in the direction of the monophasic pulse (positive or negative) and was similar to the shape of a charging capacitor. The side of the curve that had the highest magnitude (i.e., most positive in positive monophasic and most negative in negative monophasic) had a flattened peak indicative of clipping in electrical signals.

Impedance Measurements

The average impedance for each minute was determined for the three voltage and current levels for constant, monophasic and biphasic stimulation. The middle stimulation levels (100 μ A and 1V) are shown in Fig. 4 as representative changes in the average impedance over the stimulation period. For all stimulation and waveform types, except for the highest biphasic stimulation levels, the average impedance increased throughout the duration of the stimulation period and, in many cases, increased and reached a plateau. The average change in impedance across the three wells for each type of stimulation is shown in Fig. 5. The calculated change in impedance and the time constants for the biexponential fit are in Table 1 for the voltage stimulation and in Table 2 for the current stimulation.

The reported time constants are in minutes, where a positive value represents an increasing exponential while a negative value represents a decreasing exponential (Table 1, Table 2). In general, the time constants were larger for the current stimulation, indicating that it took longer for the impedance to increase or decrease than with voltage stimulation. The change in impedance and the final impedance decreased with a larger stimulation amplitude for both the current and voltage stimulation for all waveform types. The only stimulation where this trend was not seen was the change in impedance for the biphasic current. Overall, the change in impedance and the final measured impedance were larger with voltage stimulation compared to current stimulation.

Table 1

Voltage stimulation numerical results for the average across the three wells of the fitted time constants, change in impedance, final impedance, and energy through the well throughout the duration of the test. Values are displayed with the average value followed by the standard deviation in ().

		Change in Impedance [k Ω]	Final Impedance [k Ω]	τ_1 [min]	τ_2 [min]	Energy [V ² s]
Constant	0.5V	6598	7272	-16	3	897
		(2734)	(2813)	(11)	(24)	(0.1)
	1V	1400	1522	3	-27	3580
		(107)	(107)	(2)	(23)	(0.9)
	2.5V	66	84	33	-28	21681
		(11)	(11)	(38)	(17)	(150)
Monophasic	0.5V	800	1030	39	-55	437
		(135)	(136)	(111)	(36)	(0.1)
	1V	184	244	59	-18	1803
		(31)	(31)	(115)	(18)	(7)
	2.5V	0.3	10	-298	104	9531
		(0.3)	(0.3)	(370)	(134)	(76)
Biphasic	0.5V	37	72	102	-45	890
		(0.9)	(2)	(0.8)	(0.8)	(2)
	1V	11	24	1577	-62	3293
		(1)	(2)	(1445)	(25)	(36)
	2.5V	-3	4	-39	-746	7795
		(0.7)	(0.2)	(1)	(298)	(183)

Table 2

Current stimulation numerical results for the average across the three wells of the fitted time constants, change in impedance, final impedance, and energy through the well throughout the duration of the test. Values are displayed with the average value followed by the standard deviation in ().

		Change in Impedance [k Ω]	Final Impedance [k Ω]	τ_1 [min]	τ_2 [min]	Energy [V ² s]
Constant	50 μ A	9 (0.7)	52 (0.2)	3385 (1626)	-36 (2)	25112 (154)
		100 μ A	5 (0.03)	27 (0.03)	10 (31)	134 (88)
	500 μ A	0.56 (0.01)	7 (0.004)	1615 (93)	-34 (0.2)	25839 (98)
		500 μ A	0.56 (0.01)	7 (0.004)	1615 (93)	-34 (0.2)
	500 μ A	0.56 (0.01)	7 (0.004)	1615 (93)	-34 (0.2)	33859 (36)
		500 μ A	0.56 (0.01)	7 (0.004)	1615 (93)	-34 (0.2)
Monophasic	50 μ A	8 (0.2)	50 (0.06)	1065 (49)	-33 (0.2)	16739 (13)
		100 μ A	4 (0.02)	27 (0.1)	1111 (33)	-34 (0.2)
	500 μ A	0.9 (0.07)	7 (0.02)	3750 (1290)	-34 (0.9)	18756 (106)
		500 μ A	0.9 (0.07)	7 (0.02)	3750 (1290)	-34 (0.9)
	500 μ A	0.9 (0.07)	7 (0.02)	3750 (1290)	-34 (0.9)	24911 (31)
		500 μ A	0.9 (0.07)	7 (0.02)	3750 (1290)	-34 (0.9)
Biphasic	50 μ A	0.3 (0.2)	18 (0.2)	-1089 (974)	366 (714)	1301 (20)
		100 μ A	5 (0.1)	17 (0.1)	2189 (1799)	-36 (2)
	500 μ A	-0.3 (0.02)	4 (0.02)	-56 (9)	352 (140)	3272 (79)
		500 μ A	-0.3 (0.02)	4 (0.02)	-56 (9)	352 (140)
	500 μ A	-0.3 (0.02)	4 (0.02)	-56 (9)	352 (140)	48712 (43)
		500 μ A	-0.3 (0.02)	4 (0.02)	-56 (9)	352 (140)

For the voltage stimulation, the change in average energy between the stimulation levels was approximately a 300% increase from 0.5V to 1V for all types of stimulation. The change in energy between 0.5V and 2.5V was highest for the constant stimulation (2300% increase), followed by monophasic (2100% increase) and the biphasic stimulation (776%). The change in energy delivered relative to 0.5V was lowest for the biphasic stimulation. The constant and biphasic stimulation had similar average energy levels when the same level was compared for 0.5 and 1V, while the monophasic stimulation had a lower energy at the same level. For the 2.5V stimulation, the energy was highest for the constant stimulation, followed by the positive monophasic and biphasic waveforms.

For the current stimulation, the percent increase in the average energy was lower than that of the voltage for all comparisons except for 500 μA compared to 50 μA (3600% increase). The percentage change in average energy was lowest for the constant waveforms (3% and 35% increase for 100 and 500 μA), followed by the positive monophasic (12% and 39% increase for 100 and 500 μA) and the biphasic stimulation (151% and 3600% increase for 100 and 500 μA).

For the voltage stimulation, the initial ANOVA demonstrated significant effects of waveform on the difference in impedance ($F(2, 18) = 23.04, p < 0.001$), the final impedance ($F(2, 18) = 25.72, p < 0.001$), τ_1 ($F(2, 18) = 4.04, p = 0.035$), and τ_2 ($F(2, 18) = 19.47, p < 0.001$). The stimulation level had a significant effect on the difference in impedance ($F(2, 18) = 18.14, p < 0.001$), the final impedance ($F(2, 18) = 21.67, p < 0.001$), τ_1 ($F(2, 18) = 4.16, p = 0.033$), and τ_2 ($F(2, 18) = 8.77, p = 0.002$). Finally, the ANOVA revealed a significant effect of the interaction between the waveform and stimulation level for the difference in impedance ($F(4, 18) = 12.66, p < 0.001$), the final impedance ($F(4, 18) = 14.25, p < 0.001$), τ_1 ($F(4, 18) = 2.97, p = 0.048$), and τ_2 ($F(4, 18) = 16.03, p < 0.001$).

The results from the post-hoc tests following a significant ANOVA for the voltage stimulation can be seen in Table 3. Following the ANOVA, the change in impedance was significantly greater for the constant waveform than the biphasic ($p < 0.001$) and monophasic ($p < 0.001$) waveforms, but monophasic and biphasic waveforms were not significantly different. The change in impedance was significantly greater for 0.5V compared to 1V ($p < 0.001$) and 2.5V ($p < 0.01$), but there was no significant difference between 1 and 2.5V. The interaction between the waveform and stimulation level was significant for the change in impedance only for the constant stimulation 0.5 vs 2.5V ($p < 0.001$) and 0.5 vs 1V ($p < 0.001$). The final impedance demonstrated the same trends and significant differences as the change in impedance. The time constant τ_1 was significantly longer for the biphasic waveform than the monophasic waveform ($p < 0.05$), but not between any other waveform combinations. The time constant was significantly shorter at 2.5V compared to 1V ($p < 0.05$), but not between any other stimulation levels. The interaction effects were significant between the 2.5 vs 1V ($p < 0.01$) and 0.5 vs 1V ($p < 0.01$) for the biphasic waveform and not for any other interactions. The time constant τ_2 was significantly shorter for biphasic waveforms compared to constant ($p < 0.001$) and monophasic ($p < 0.001$) stimulation, but not between the constant and monophasic waveforms. The time constant was significantly shorter at 2.5V compared to 0.5V ($p < 0.01$) and 1V ($p < 0.01$), but not between 0.5 and 1 V. The interaction effects were significant for the biphasic waveform between 2.5 and 0.5V, and 2.5 and 1V ($p < 0.0001$), but not for any other interaction effects.

Table 3

Voltage Stimulation Tukey Post-Hoc test results. Tukey Post-Hoc analysis was performed on the linear model based on a significant ANOVA result. The main effects and the interactions are shown in the table as the t-ratio and the corresponding p-value. Significance is indicated as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

		Comparison	Difference in Impedance	Final Impedance	τ_1	τ_2
Main Effects	Waveform	Biphasic – Constant	t-ratio: -6.2 p: <0.0001***	t-ratio: -6.6 p: <0.0001***	t-ratio: 2.3 p: 0.08	t-ratio: -5.1 p: 0.0002***
		Biphasic – Monophasic	t-ratio: -0.7 p: 0.7	t-ratio: -0.9 p: 0.6	t-ratio: 2.6 p: 0.045*	t-ratio: -5.6 p: <0.0001***
		Constant - Monophasic	t-ratio: 5.5 p: <0.0001***	t-ratio: 5.7 p: <0.0001***	t-ratio: 0.3 p: 0.95	t-ratio: -0.5 p: 0.9
	Stimulation Level	2.5V – 0.5V	t-ratio: -5.7 p: <0.0001***	t-ratio: -6.2 p: <0.0001***	t-ratio: -0.6 p: 0.8	t-ratio: -3.7 p: 0.005**
		2.5V – 1V	t-ratio: -1.2 p: 0.5	t-ratio: -1.3 p: 0.4	t-ratio: -2.7 p: 0.03*	t-ratio: -3.6 p: 0.006**
		0.5V – 1V	t-ratio: 4.5 p: 0.0007***	t-ratio: 4.9 p: 0.0003***	t-ratio: -2.1 p: 0.1	t-ratio = 0.07 p: 1.0
	Interaction Effects	Biphasic	2.5V – 0.5V	t-ratio: -0.05 p: 1.0	t-ratio: -0.09 p: 1.0	t-ratio: -7.8 p: <0.0001***
			2.5V – 1V	t-ratio: -0.02 p: 1.0	t-ratio: -0.03 p: 1.0	t-ratio: -7.5 p: <0.0001***
			0.5V – 1V	t-ratio: 0.03 p: 1.0	t-ratio: 0.06 p: 1.0	t-ratio: 0.2 p: 1.0
		Constant	2.5V – 0.5V	t-ratio: -8.7 p: 1.0	t-ratio: -3.6 p: 0.005**	t-ratio: -0.3 p: 1.0
					t-ratio: 0.1	t-ratio: -0.3

		p: <0.0001***	p: <0.0001***	p: 1.0	p: 0.9
	2.5V – 1V	t-ratio: -1.8 p: 0.2	t-ratio: -1.9 p: 0.2	t-ratio: 0.07 p: 1.0	t-ratio: -0.01 p: 1.0
	0.5V – 1V	t-ratio: 6.9 p: <0.0001***	t-ratio: 7.5 p: <0.0001***	t-ratio: -0.05 p: 1.0	t-ratio: 0.3 p: 0.9
Monophasic	2.5V – 0.5V	t-ratio: -1.1 p: 0.5	t-ratio: -1.3 p: 0.4	t-ratio: -0.8 p: 0.7	t-ratio: 1.7 p: 0.2
	2.5V – 1V	t-ratio: -0.2 p: 0.97	t-ratio: -0.3 p: 0.95	t-ratio: -0.9 p: 0.7	t-ratio: 1.3 p: 0.4
	0.5V – 1V	t-ratio: 0.8 p: 0.69	t-ratio: 1.0 p: 0.6	t-ratio: -0.05 p: 1.0	t-ratio: -0.4 p: 0.9

For the current stimulation, the initial ANOVA demonstrated significant effects of waveform on the difference in impedance ($F(2, 18) = 468.02$, $p < 0.001$), the final impedance ($F(2, 18) = 67685$, $p < 0.001$), τ_1 ($F(2, 18) = 7.13$, $p = 0.005$), but not τ_2 ($F(2, 18) = 2.86$, $p < 0.083$). The stimulation level had a significant effect on the difference in impedance ($F(2, 18) = 1295.1$, $p < 0.001$), the final impedance ($F(2, 18) = 260694$, $p < 0.001$), but not on τ_1 ($F(2, 18) = 1.37$, $p = 0.28$), or τ_2 ($F(2, 18) = 0.29$, $p = 0.75$). Finally, the ANOVA revealed a significant effect of the interaction between the waveform and stimulation level for the difference in impedance ($F(4, 18) = 385.47$, $p < 0.001$), the final impedance ($F(4, 18) = 24524$, $p < 0.001$), τ_1 ($F(4, 18) = 12.05$, $p < 0.0001$), but not τ_2 ($F(4, 18) = 1.41$, $p < 0.27$).

The results from the post-hoc tests following a significant ANOVA for the current stimulation can be seen in Table 4. Following the ANOVA, the change in impedance demonstrated a significant difference between all combinations of waveform types (biphasic < monophasic < constant) ($p < 0.0001$). The change in impedance was significantly different for all combinations of stimulation levels ($50 > 100$, $50 > 500$, and $100 > 500 \mu A$) ($p < 0.0001$). The interaction between the waveform and stimulation level was significant for the change in impedance for all interactions ($p < 0.05$); the individual comparisons can be found in Table 4. All comparisons for the main effects and the interaction effects were significant for the final impedance ($p < 0.0001$); this followed the same trend as the change in impedance. For the time constant τ_1 , the biphasic waveform was significantly shorter than for constant ($p < 0.05$) and monophasic ($p < 0.01$) waveforms, but not monophasic vs constant waveforms. There were no significant effects of the stimulation level on τ_1 . The interaction effects were significant between the 500 vs 100 μA ($p < 0.05$) and 50 vs 100 μA ($p < 0.01$) for the biphasic waveform, between 50 vs 100 μA ($p <$

0.01) for the constant stimulation and 500 vs 100 μA ($p < 0.05$) and 50 vs 500 μA ($p < 0.01$) for the monophasic waveform and not for any other interactions. (Table 4) The current stimulation demonstrated clear trends where the change in impedance and final impedance were greater for constant stimulation followed by monophasic and then biphasic waveforms. There was also a clear trend that the change in impedance and final impedance decreased as the stimulation magnitude increased.

Table 4

Current Stimulation Tukey Post-Hoc test results. Significance is indicated as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. Post-Hoc analysis was not performed for τ_2 because there was no significance found through the two-way ANOVA.

		Comparison	Difference in Impedance	Final Impedance	τ_1
Main Effects	Waveform	Biphasic – Constant	t-ratio: -28.5 p: <0.0001***	t-ratio: -328 p: <0.0001***	t-ratio: -2.9 p: 0.02*
		Biphasic – Monophasic	t-ratio: -23.8 p: <0.0001***	t-ratio: -308 p: <0.0001***	t-ratio: -3.5 p: 0.006**
		Constant - Monophasic	t-ratio: 4.7 p: 0.0005***	t-ratio: 20.8 p: <0.0001***	t-ratio: -0.7 p: 0.8
	Stimulation Level	500 μ A – 50 μ A	t-ratio: -48.6 p: <0.0001***	t-ratio: -722 p: <0.0001***	t-ratio: 1.4 p: 0.3
		500 μ A – 100 μ A	t-ratio: -37.3 p: <0.0001***	t-ratio: -379 p: <0.0001***	t-ratio: 1.4 p: 0.3
		50 μ A – 100 μ A	t-ratio: 11.3 p: <0.0001***	t-ratio: 342 p: <0.0001***	t-ratio: 0.04 p: 1.0
	Interaction Effects	Biphasic	500 μ A – 50 μ A	t-ratio: -173 p: <0.0001***	t-ratio: -1.3 p: 0.4
			500 μ A – 100 μ A	t-ratio: -164 p: <0.0001***	t-ratio: -2.8 p: 0.03*
			50 μ A – 100 μ A	t-ratio: 9.8 p: <0.0001***	t-ratio: -4.1 p: 0.002**
		Constant	500 μ A – 50 μ A	t-ratio: -554 p: <0.0001***	t-ratio: -2.2 p: 0.09

		500 μ A – 100 μ A	t-ratio: -22.4 p: <0.0001***	t-ratio: -250 p: <0.0001***	t-ratio: 2.0 p: 0.1
		50 μ A – 100 μ A	t-ratio: 21.1 p: <0.0001***	t-ratio: 304 p: <0.0001***	t-ratio: 4.2 p: 0.001**
	Monophasic	500 μ A – 50 μ A	t-ratio: -37.5 p: <0.0001***	t-ratio: -523 p: <0.0001***	t-ratio: 3.4 p: 0.009**
		500 μ A – 100 μ A	t-ratio: -16.0 p: <0.0001***	t-ratio: -244 p: <0.0001***	t-ratio: 3.3 p: 0.01*
		50 μ A – 100 μ A	t-ratio: -21.5 p: <0.0001***	t-ratio: 279 p: <0.0001***	t-ratio: -0.06 p: 1.0

Physical Observations

No precipitates were seen on the electrodes when they were removed from the media after the tests; there was, however, a thin yellow film/discolouration on the electrodes. The yellow appeared on both electrodes but was more prominent on the positive electrode. However, this was cleaned easily with wipes and 70% ethanol. Colour changes within the media were isolated to the area around the electrodes and did not demonstrate a full media split throughout the entire well. Overall, there were no colour changes throughout the entire well and a small production of bubbles isolated to the surface of the electrodes. Within the first 15 minutes of the tests, the high stimulation levels for voltage and current stimulation that were held at a constant magnitude produced bubbles from the electrodes, with more on the negative electrode than the positive electrode. Slight yellowing of the media (more acidic) surrounding the positive electrodes occurred and was more prominent at higher stimulation levels.

Discussion

Many studies performing electrical stimulation do not discuss the changes in the signal throughout the stimulation period or the effects on the media (17, 28–33). This study determined that changes in the stimulation type and level change the signal measured across the media. Previous tests by our group saw bleaching of the media at high voltages and currents; the media around one electrode would become more pink (more basic) and the other more yellow (more acidic) as indicated by the phenol red in the media. Changes in media colour were consistent with Dubey et al (34) who reported yellow-coloured media around the positive electrode with stimulation at higher voltages, and this shift toward

acidity resulted in cell death. The change in media colour in our experiments was more prominent when the constant waveforms were applied; media changes were not clearly visible during the monophasic and biphasic waveform stimulations. This is likely due to the cyclic charge and dissipation of any chemical changes during the off-cycle or the switching of charge.

The waveform shapes of the measured voltage between the PtIr electrodes were consistent with waveforms generated through tissue in studies in which current(21, 35, 36) or voltage(21, 35, 37) stimulation was applied. During voltage stimulation, the voltage remained consistent while the current varied due to changes in impedance. During current stimulation, the current remained consistent, and the voltage adjusted based on the impedance between the electrodes to maintain the current. The biphasic responses in this study were similar to those in a similar study by Zimmermann et al.(26) and to those seen in vivo with deep brain stimulation (DBS) (38). It is also interesting to note that the current through the well in the voltage-controlled stimulation was more rectangular in shape at higher frequencies than lower frequencies (26); the current in our stimulation, performed at low frequency, was not rectangular.

In this study, we calculated the impedance using a method similar to other studies (39–41), where the impedance was estimated by dividing the measured voltage within the well by the current, which was often determined from a shunt resistor. This impedance was used as an estimate of the total impedance, which included the impedance of the cell culture media, lead wires, and the impedance at the electrode-electrolyte interface (EEI) (38, 40–42). Other studies have determined the impedance of the media and have reported values in the range of 10–200 Ohms (25, 26, 43). Our estimated impedance was much higher but also reflected the entire circuit, not just the media. The calculated total impedance in this study was lower than values reported for different solutions and those containing cells (42). This could be due to the addition of cells or the differences in measurement techniques. The charge transfer resistance measured by Blandon et al. (43) with electrical impedance spectroscopy (EIS) was approximately 16 k Ω , which is in the same order of magnitude as the calculated final impedance of the biphasic voltage stimulation and all current stimulation waveforms applied in this study. Low frequencies are dominated by the EEI (26, 38, 39, 43), with the capacitive double layer contributing more to the impedance. So, the impedance is higher, and the response is nonlinear (26, 39, 43). At high frequencies (lower limit between 100 and 1000 Hz), the impedance is dominated by the resistance of the electrolyte; (26, 38, 43) this can be modelled as a fixed resistance (26) and is dependent on the composition and volume of the solution (43). This study operated in the low frequency range (DC and 1Hz), and the measured impedance was higher than previously estimated media impedance since the impedance included the electrodes and was dominated by their capacitive nature.

During voltage stimulation, the voltage across the measurement resistor decreased. This indicates a drop in the current within the well. Zimmermann et al., (26) using a similar electrode setup as this study, also reported a decrease in current over time during a 1V DC stimulation. Their voltage magnitude was equivalent to the middle voltage level used in this study; however, a 6-well plate and 25 mm electrode spacing were used, a larger well size and spacing than our study. When a 20Hz AC voltage signal was applied, the current decreased throughout the stimulation. (39) From these other studies, the current did

not scale linearly with the applied voltage. (26, 39) This drop in current was not seen in the current stimulation in our tests because the current was at a fixed value.

Measured impedance has been found to increase with the application of cyclic voltage stimulation. (39, 42) In these studies, cells were seeded on electrodes and stimulated with similar current magnitudes. They found that the lower currents of 50 μ A and 200 μ A had a slight increase in total impedance, while the highest current of 500 μ A saw a decrease in impedance consistent with the trends seen in our study. (40) In our study, the impedance decreased as the stimulation level increased. This is consistent with other studies where the impedance was dependent on the applied stimulation. (26, 39) Lembcke et al. (39) found a decrease in impedance magnitude with an increase in applied voltage.

For many of the stimulation waveforms, the change in impedance and final impedance were greater for the voltage stimulation than the current stimulation. This may be the case as the voltage across the electrodes in the current stimulation was higher in the 50 μ A stimulation than any of the voltage levels for constant and biphasic stimulation. When a biphasic waveform was applied, 500 μ A and 2.5V stimulation had a similar voltage across the electrodes. The difference in electrode voltage may explain why the impedance of the current tests was less than the voltage tests; the impedance decreased as the voltage level of stimulation increased. The current throughout the media was greater for the current stimulation; as shown in Fig. 3, the voltage across the resistors reflects the current. This was especially evident in the monophasic stimulation, whereas the voltage across the resistor for the biphasic stimulation had a higher voltage spike than the corresponding current but decreased rapidly to a value lower than the current stimulation.

The monophasic current stimulation demonstrated a DC offset in the voltage measurement across the electrodes. A similar DC offset was observed by Zimmerman et. al.(26) with the application of a monophasic current waveform (6.5mA, 60 μ s, 130Hz) within 3.5 mL KCl solution. Although the stimulation parameters were different, it is interesting to note that the DC offset in both was in a similar range around 1.5-2V. These DC offsets are potentially damaging to cells as they may cause electrochemical reactions. (26)

From the statistical analyses, it was found that the effects of the stimulation level and the stimulation type on the change in impedance of the media are dependent on one another. It has been reported that the optimal voltage to promote proliferation of mouse fibroblast cells was dependent on the frequency of the applied ES, where the optimal voltage decreased when the frequency increased. (34)

The statistical analyses revealed that some interactions between the stimulation level and the waveform type were significant. More interactions were significant for the current stimulation than the voltage stimulation. The energy calculated for the measured voltage between the two PtIr electrodes changed based on the stimulation level and the waveform for both voltage and current stimulation. This may be an indication as to why these interactions were significant. There may have been fewer interaction terms that were significant for the voltage stimulation because the energy for the constant and biphasic waveforms was similar for the low and mid voltage levels.

Although consistent with the above studies, the calculated impedances in this study vary from the published results due to variations in electrode material, experimental setup, electrical parameters, and media composition. It is difficult to compare directly across studies with these differences. It has also been found that the conductivity of the culture media varies based on frequency. (43) So, stimulation parameters can affect impedance measurements.

Despite variations in this study and others, this work was a comprehensive study on changes within the well during an electrical stimulation period of one hour. The benefit of this study is that it explored two types of stimulation, voltage and current, three applied waveforms, constant, monophasic and biphasic, and three levels of stimulation, providing an understanding of the ES delivered throughout the well. Differences in outcomes from other studies can be affected by the stimulation parameters, materials, cell culture media, and the overall experimental setup. This highlights the importance of measuring changes in individual systems before using them for the application of ES. The results from this study have been used to inform the design of an ES system with the goal of performing in vitro testing with cell cultures.

Limitations

This study made general assumptions about the change in pH based on the colour of the cell culture media. The colour change of the media was difficult to quantify and compare across stimulation trials. The electrodes were held with an acrylic plate that had become increasingly cloudy throughout the tests, making it difficult to see the colour change. It would be beneficial to obtain pH readings of the culture media. This task was difficult due to the design of the system, as removing the electrodes would disturb the media and could remove localized pH changes. Standard pH probes are unable to obtain precise measurements within the small, localized area where the pH change was visually observed. To provide pH monitoring, it would have to be incorporated into the system for real-time measurements. However, this may also alter the electrical properties of the system.

Basal media was used in this experiment due to the cost of other reagents that are commonly added to basal media such as fetal bovine serum (FBS). However, the addition of these reagents may affect the changes in the signal or the changes in pH. FBS contains components including growth/attachment factors, lipids, hormones, electrolytes and factors that protect the media from pH shifts (44). Studies have found that the addition of serum caused a 28% increase in impedance when compared to a saline control. (42) These factors may affect the conductivity, the response of the media to stimulation, and changes in colour/pH of the media during stimulation. Although the complete media was not used, this study provides insight into changes during stimulation and the importance of monitoring the signal applied in vitro.

It would also be advantageous to perform EIS, as numerous studies use these techniques (25, 26, 38, 42, 43), as the properties of the EEI can be inferred from EIS measurements. (26)

Dubey et al. (45) did not find any changes in temperature due to the application of short-pulsed ES. However, their study applied stimulation in bursts of 5 or 10 minutes. The tests performed during this study applied electrical stimulation for 1 hour. With the longer stimulation time and the different stimulation parameters, temperature may play a part in the change in impedance. The measurements throughout this study were performed at 21°C while cell cultures are maintained at 37°C; this limits the study as the behaviour of the media may vary at the higher temperature at which cells are maintained.

Conclusions

Under current and voltage stimulation, the impedance of our cell media circuits increased throughout the length of the hour-long stimulation. In general, the voltage stimulation produced larger impedance values than the current stimulation. The change in impedance for the voltage stimulation was greater for a constant waveform when compared to the pulsed stimulation (biphasic and monophasic), but the two pulsed stimulation waveforms were not significantly different from one another. For current stimulation, the change in impedance and final impedance were greater for constant stimulation, followed by monophasic and biphasic stimulation waveforms. For the voltage stimulation, there was less change in impedance for the 1 and 2.5V stimulation compared to 0.5V, but the change in impedance was not significantly different between 1 and 2.5V. When current stimulation was applied, the change in impedance and the final impedance decreased as the applied stimulation magnitude increased. For both the voltage and current stimulation, the monophasic and biphasic waveforms were an improvement over the constant stimulation, as the change in impedance was less than the constantly held stimulation. The biphasic waveform caused a smaller change in impedance than the monophasic waveform for the current stimulation. These results indicate that the most consistent waveform for the application of current stimulation over an extended period (1 hour) is the biphasic waveform. The monophasic and biphasic stimulation for voltage provides similar changes in impedance and would be most consistent for voltage stimulation compared to a constantly held voltage. The stimulation level and waveforms affected the change in impedance throughout the duration of the hour-long stimulation. This suggests that it is important to monitor changes in electrical stimulation parameters during electrical stimulation protocols as the initial conditions are likely to change over the duration of the protocol. This work has outlined for the first time, a comprehensive look at the temporal changes to cell culture media in a stimulation environment. This is vital to our understanding of cellular activity in stimulation experiments as this directly impacts the media they need to survive and carry out cellular processes and communication.

Abbreviations

DAQ

Data Acquisition

DBS

Deep Brain Stimulation

DC

Direct Current
EEI
Electrode-Electrolyte Interface
EF
Electric Field
EIS
Electrical Impedance Spectroscopy
ES
Electrical Stimulation
FBS
Fetal Bovine Serum
Ptlr
Platinum-Iridium

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing Interests

GW is a principal in Additive Manufacturing International Inc, which provided funding to ER.

Funding

Funding for ER was provided by a Mitacs award in collaboration with Additive Manufacturing International Inc.

Author Contribution

Experimentation and data collection were performed by ER and TD. GW provided supervision and design input. All authors contributed to the writing of the draft and approved the final manuscript.

Acknowledgments

Not applicable.

Data Availability

Data was generated at McMaster University, the descriptive statistics of the data are provided within the manuscript. Raw data files are available upon request.

References

1. Hammerick KE, Longaker MT, Prinz FB. In vitro effects of direct current electric fields on adipose-derived stromal cells. *Biochem Biophys Res Commun*. 2010. 10.1016/j.bbrc.2010.05.003.
2. Nuccitelli R. Endogenous ionic currents and DC electric fields in multicellular animal tissues. *Bioelectromagnetics*. 1992. 10.1002/bem.2250130714.
3. Ariza CA, Fleury AT, Tormos CJ, Petruk V, Chawla S, Oh J, et al. The Influence of Electric Fields on Hippocampal Neural Progenitor Cells. *Stem Cell Rev Rep*. 2010. 10.1007/s12015-010-9171-0.
4. Cao L, Pu J, Scott RH, Ching J, McCaig CD. Physiological Electrical Signals Promote Chain Migration of Neuroblasts by Up-Regulating P2Y1 Purinergic Receptors and Enhancing Cell Adhesion. *Stem Cell Rev Rep*. 2015. 10.1007/s12015-014-9524-1.
5. Kumar A, Nune KC, Misra RDK. Electric field-mediated growth of osteoblasts – the significant impact of dynamic flow of medium. *Biomater Sci*. 2016. 10.1039/C5BM00350D.
6. Brighton CT, Friedenberg ZB, Black J, Esterhai JL, Mitchell JE, Montique F. Electrically induced osteogenesis: relationship between charge, current density, and the amount of bone formed: introduction of a new cathode concept. *Clin Orthop Relat Res*. 1981. 10.1097/00003086-198111000-00015.
7. Ferrier J, Ross SM, Kanehisa J, Aubin JE. Osteoclasts and osteoblasts migrate in opposite directions in response to a constant electrical field. *J Cell Physiol*. 1986. 10.1002/jcp.1041290303.
8. deVet T, Jhirad A, Pravato L, Wohl GR. Bone Bioelectricity and Bone-Cell Response to Electrical Stimulation: A Review. *Crit Rev Biomed Eng*. 2021. 10.1615/CritRevBiomedEng.2021035327.
9. Chao PHG, Roy R, Mauck RL, Liu W, Valhmu WB, Hung CT. Chondrocyte Translocation Response to Direct Current Electric Fields. *J Biomech Eng*. 2000. 10.1115/1.429661.
10. Brighton CT, Wang W, Seldes R, Zhang G, Pollack SR. Signal Transduction in Electrically Stimulated Bone Cells. *J Bone Joint Surg*. 2001. 10.2106/00004623-200110000-00009.
11. Yao L, McCaig CD, Zhao M. Electrical signals polarize neuronal organelles, direct neuron migration, and orient cell division. *Hippocampus*. 2009. 10.1002/hipo.20569.
12. Isaacson BM, Bloebaum RD. Bone bioelectricity: What have we learned in the past 160 years? *J Biomed Mater Res A*. 2010. 10.1002/jbm.a.32905.
13. Mycielska ME, Djamgoz MBA. Cellular mechanisms of direct-current electric field effects: galvanotaxis and metastatic disease. *J Cell Sci*. 2004. 10.1242/jcs.01125.

14. Brummer SB, Turner MJ. Electrical stimulation of the nervous system: The principle of safe charge injection with noble metal electrodes. *Bioelectrochem Bioenerg*. 1975. 10.1016/0302-4598(75)80002-X.
15. Zhang J, Li M, Kang ET, Neoh KG. Electrical stimulation of adipose-derived mesenchymal stem cells in conductive scaffolds and the roles of voltage-gated ion channels. *Acta Biomater*. 2016. 10.1016/j.actbio.2015.12.024.
16. Wang Q, Zhong S, Ouyang J, Jiang L, Zhang Z, Xie Y, et al. Osteogenesis of electrically stimulated bone cells mediated in part by calcium ions. *Clin Orthop Relat Res*. 1998. 10.1097/00003086-199803000-00037.
17. Dauben TJ, Ziebart J, Bender T, Zaatreh S, Kreikemeyer B, Bader R. A Novel In Vitro System for Comparative Analyses of Bone Cells and Bacteria under Electrical Stimulation. *Biomed Res Int*. 2016. 10.1155/2016/5178640.
18. Ehrensberger MT, Tobias ME, Nodzo SR, Hansen LA, Luke-Marshall NR, Cole RF, et al. Cathodic voltage-controlled electrical stimulation of titanium implants as treatment for methicillin-resistant *Staphylococcus aureus* periprosthetic infections. *Biomaterials*. 2015. 10.1016/j.biomaterials.2014.11.013.
19. Srirussamee K. Direct Electrical Stimulation Approaches for Bone Repair and Regeneration [Doctoral Thesis]. The University of Manchester; 2019. <https://research.manchester.ac.uk/en/studentTheses/direct-electrical-stimulation-approaches-for-bone-repair-and-rege/>. Accessed 17 Nov 2025.
20. Brummer SB, Robblee LS, Hambrecht FT. Criteria for Selecting electrodes for Electrical Stimulation: Theoretical and Practical Considerations. *Ann N Y Acad Sci*. 1983. 10.1111/j.1749-6632.1983.tb31628.x.
21. Merrill DR, Bikson M, Jefferys JGR. Electrical stimulation of excitable tissue: design of efficacious and safe protocols. *J Neurosci Methods*. 2005. 10.1016/j.jneumeth.2004.10.020.
22. Vazquez Lam I, Analysis of Improved Howland Current Pump Configurations. 2023. Available from: <https://www.ti.com/lit/pdf/sboa437>. Accessed 5 Sept 2024.
23. Morgan A, Babu D, Reiz B, Whittal R, Suh LYK, Siraki AG. Caution for the routine use of phenol red – It is more than just a pH indicator. *Chem Biol Interact*. 2019. 10.1016/j.cbi.2019.108739.
24. Kulkarni G, Garcia JM, Isasi-Campillo M, González MU, Mobini S. In vitro electrical stimulation devices: practical framework for design, fabrication, and operation. *J Biol Eng*. 2026. 10.1186/s13036-026-00664-7.
25. Abasi S, Aggas JR, Venkatesh N, Vallavanatt IG, Guiseppi-Elie A. Design, fabrication and testing of an electrical cell stimulation and recording apparatus (ECSARA) for cells in electroculture. *Biosens Bioelectron*. 2020. 10.1016/j.bios.2019.111793.
26. Zimmermann J, Budde K, Arbeiter N, Molina F, Storch A, Uhrmacher AM, et al. Using a Digital Twin of an Electrical Stimulation Device to Monitor and Control the Electrical Stimulation of Cells in vitro. *Front Bioeng Biotechnol*. 2021. 10.3389/fbioe.2021.765516.

27. Lenth RV, Piaskowski Jemmeans. Estimated Marginal Means, aka Least-Squares Means. R package version 2.0.4; 2026. <https://CRAN.R-project.org/package=emmeans>
28. Mobini S, Leppik L, Barker JH. Direct Current Electrical Stimulation Chamber for Treating Cells in Vitro. *Biotechniques*. 2016. 10.2144/000114382.
29. Mobini S, Leppik L, Thottakkattumana Parameswaran V, Barker JH. In vitro effect of direct current electrical stimulation on rat mesenchymal stem cells. *PeerJ*. 2017. 10.7717/peerj.2821.
30. Kim JH, Lee TH, Song YM, Kim IS, Cho TH, Hwang SJ et al. An implantable electrical bioreactor for enhancement of cell viability. In: 2011 Annual International Conference of the IEEE Engineering in Medicine and Biology Society. 2011; 10.1109/IEMBS.2011.6090603
31. Kim IS, Song JK, Zhang YL, Lee TH, Cho TH, Song YM et al. Biphasic electric current stimulates proliferation and induces VEGF production in osteoblasts. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2006; 10.1016/j.bbamcr.2006.06.007
32. Hwang SJ, Song YM, Cho TH, Kim RY, Lee TH, Kim SJ, et al. The Implications of the Response of Human Mesenchymal Stromal Cells in Three-Dimensional Culture to Electrical Stimulation for Tissue Regeneration. *Tissue Eng Part A*. 2011. 10.1089/ten.tea.2010.0752.
33. Rabbani M, Rahman E, Al Aishan A, Powner MB, Triantis IF. A Low-Cost, Scalable, and Configurable Multi-Electrode System for Electrical Bio-Interfacing with In-Vitro Cell Cultures. *Appl Sci*. 2024. 10.3390/app14010162.
34. Dubey AK, Gupta SD, Basu B. Optimization of electrical stimulation parameters for enhanced cell proliferation on biomaterial surfaces. *J Biomed Mater Res B Appl Biomater*. 2011. 10.1002/jbm.b.31827.
35. Washburn S, Catlin R, Bethel K, Canlas B. Patient-Perceived Differences Between Constant Current and Constant Voltage Spinal Cord Stimulation Systems. *Neuromodulation: Technol Neural Interface*. 2014. 10.1111/ner.12085.
36. Zhou Z, Wang X, Yicheng Y, Zeng J, Liu H. Mathematical Model of Fingertip Skin Under Constant-Current Electrotactile Stimulation. *IEEE Trans Haptics*. 2023. 10.1109/TOH.2022.3227084.
37. Cao F, Zhang C, Vo Doan TT, Li Y, Sangi DH, Koh JS, et al. A Biological Micro Actuator: Graded and Closed-Loop Control of Insect Leg Motion by Electrical Stimulation of Muscles. *PLoS ONE*. 2014. 10.1371/journal.pone.0105389.
38. Wei XF, Grill WM. Impedance characteristics of deep brain stimulation electrodes in vitro and in vivo. *J Neural Eng*. 2009. 10.1088/1741-2560/6/4/046008.
39. Lembcke L, Bathel H, Zimmermann J, Engel V, Bader R, Frerich B, et al. A validated in vitro approach for low-frequency alternating-current stimulation of stem cells: From electrode characterization to biological responses. *Protoplasma*. 2026. 10.1007/s00709-026-02196-9.
40. Newbold C, Richardson R, Millard R, Seligman P, Cowan R, Shepherd R. Electrical stimulation causes rapid changes in electrode impedance of cell-covered electrodes. *J Neural Eng*. 2011. 10.1088/1741-2560/8/3/036029.

41. Newbold C, Richardson R, Huang CQ, Milojevic D, Cowan R, Shepherd R. An in vitro model for investigating impedance changes with cell growth and electrical stimulation: implications for cochlear implants. *J Neural Eng.* 2004. 10.1088/1741-2560/1/4/005.
42. Merrill DR, Tresco PA. Impedance characterization of microarray recording electrodes in vitro. *IEEE Trans Biomed Eng.* 2005. 10.1109/TBME.2005.856245.
43. Blandón LAQ, Ramón-Azcón J, Gutiérrez MR, Garzón-Alvarado DA, Vaca-González JJ. Dielectric Characterization of Skeletal Muscle Cell Culture Medium Using Electrochemical Impedance Spectroscopy. *Electroanalysis.* 2025. 10.1002/elan.70069.
44. ThermoFisher Scientific. The Basics of Fetal Bovine Serum Use in Cell Culture. 2025. <https://www.thermofisher.com/ca/en/home/references/gibco-cell-culture-basics/cell-culture-environment/fbs-basics.html>. Accessed 17 Nov 2025.
45. Dubey AK, Basu B. Pulsed Electrical Stimulation and Surface Charge Induced Cell Growth on Multistage Spark Plasma Sintered Hydroxyapatite-Barium Titanate Piezobiocomposite. *J Am Ceram Soc.* 2014. 10.1111/jace.12647.

Figures

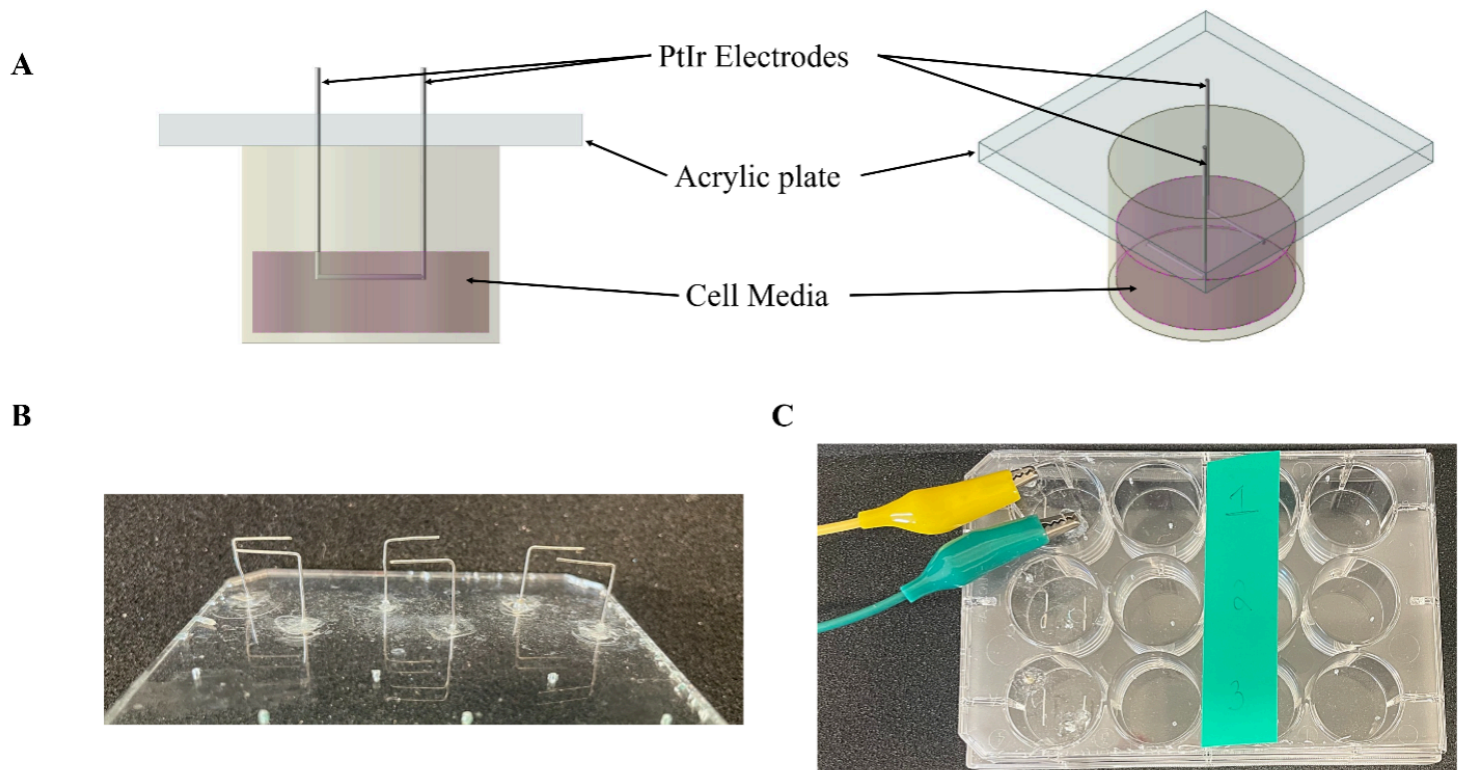


Figure 1

Experimental Setup of PtIr Electrodes. A) 3D model of positioning of electrodes in a single well. B) Bottom view of acrylic plate with three sets of PtIr electrodes glued into it, and C) acrylic plate with

electrodes placed in the cell plate without any culture media, demonstrating connection of electrodes to PtIr electrodes above the acrylic plate.

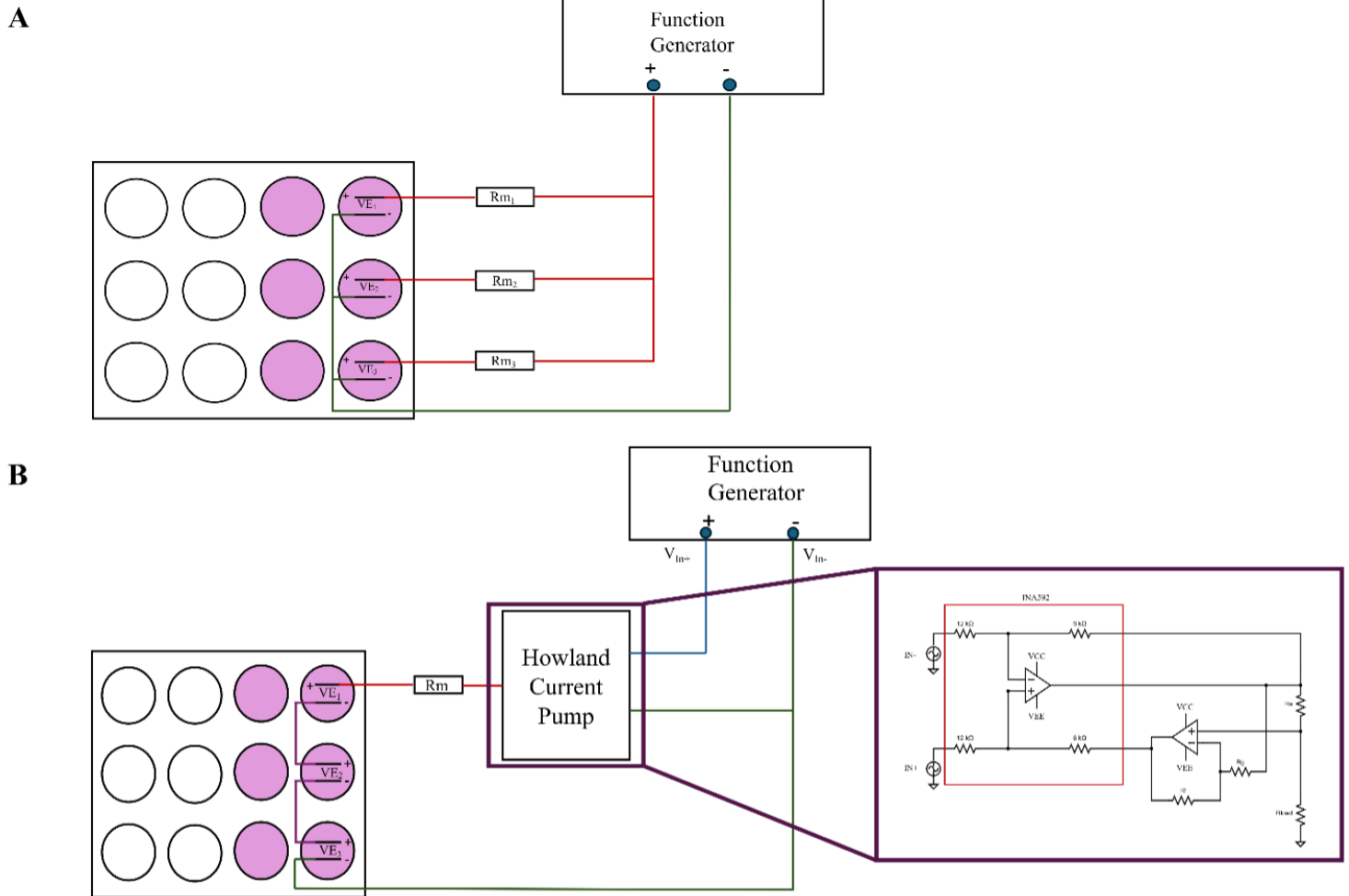
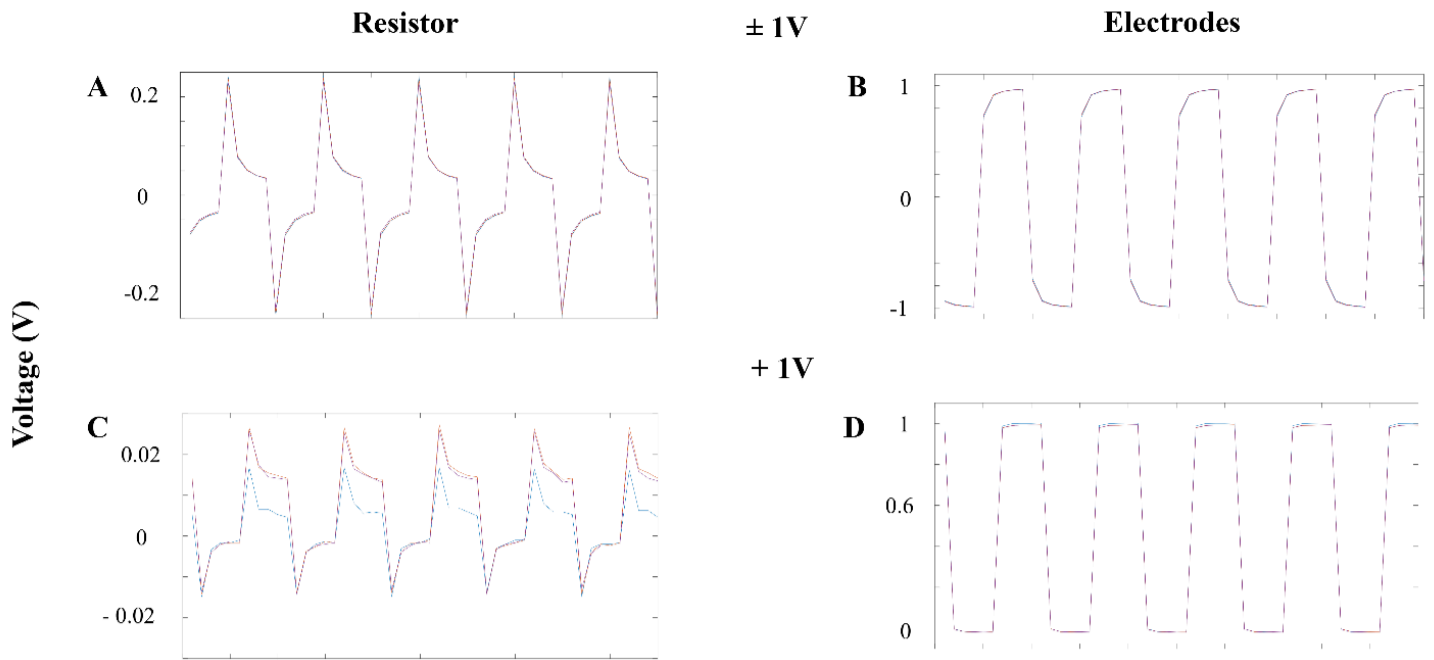


Figure 2

Voltage and Current Stimulation Circuit Experimental Setup. Stimulation circuits where R_m are measurement resistors with a value of $1k\Omega$, and the pink circles represent three control and three stimulated wells filled with culture media for A) Voltage and B) Current stimulation setup.

Voltage Stimulation



Current Stimulation

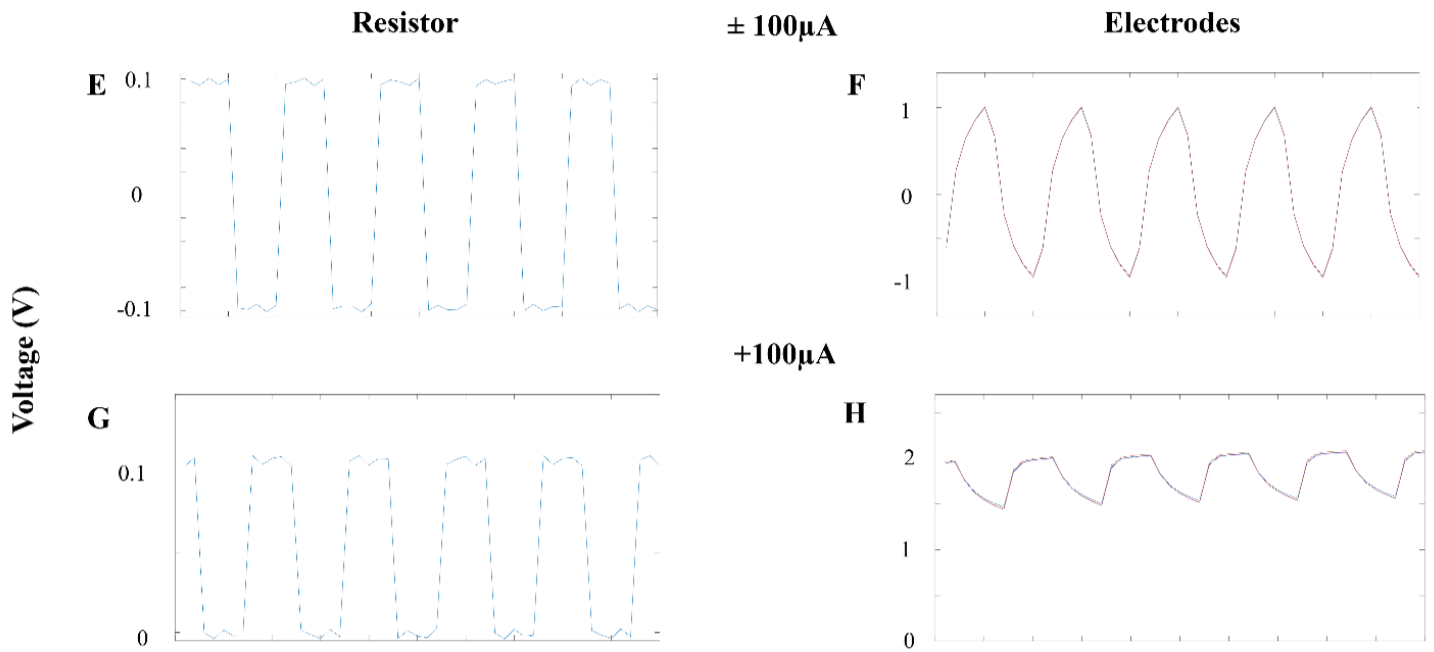


Figure 3

Sample waveform shapes for monophasic and biphasic stimulation voltage and current stimulation. Examples of voltage waveform shapes across the resistors and the electrodes for biphasic ($\pm 1V$ and $\pm 100\mu A$) and monophasic ($+1V$ and $+100\mu A$) stimulation for voltage and current stimulation. A) Resistor voltages for 1V biphasic, B) electrode voltages for 1V biphasic, C) resistor voltages for 1V monophasic, D) electrode voltages for 1V monophasic, E) resistor voltage for 100 μA biphasic, F) electrode voltages

for 100 μ A biphasic, G) resistor voltage for 100 μ A monophasic, and G) electrode voltages for 100 μ A monophasic. In the resistor voltage graphs, R_{m1} is shown in blue, R_{m2} in orange, and R_{m3} in purple. In the electrode voltage V_{E1} is shown in blue, V_{E2} is shown in orange, and V_{E3} is shown in purple. Note that the y-axes have different scales so the waveform shapes could be visualized.

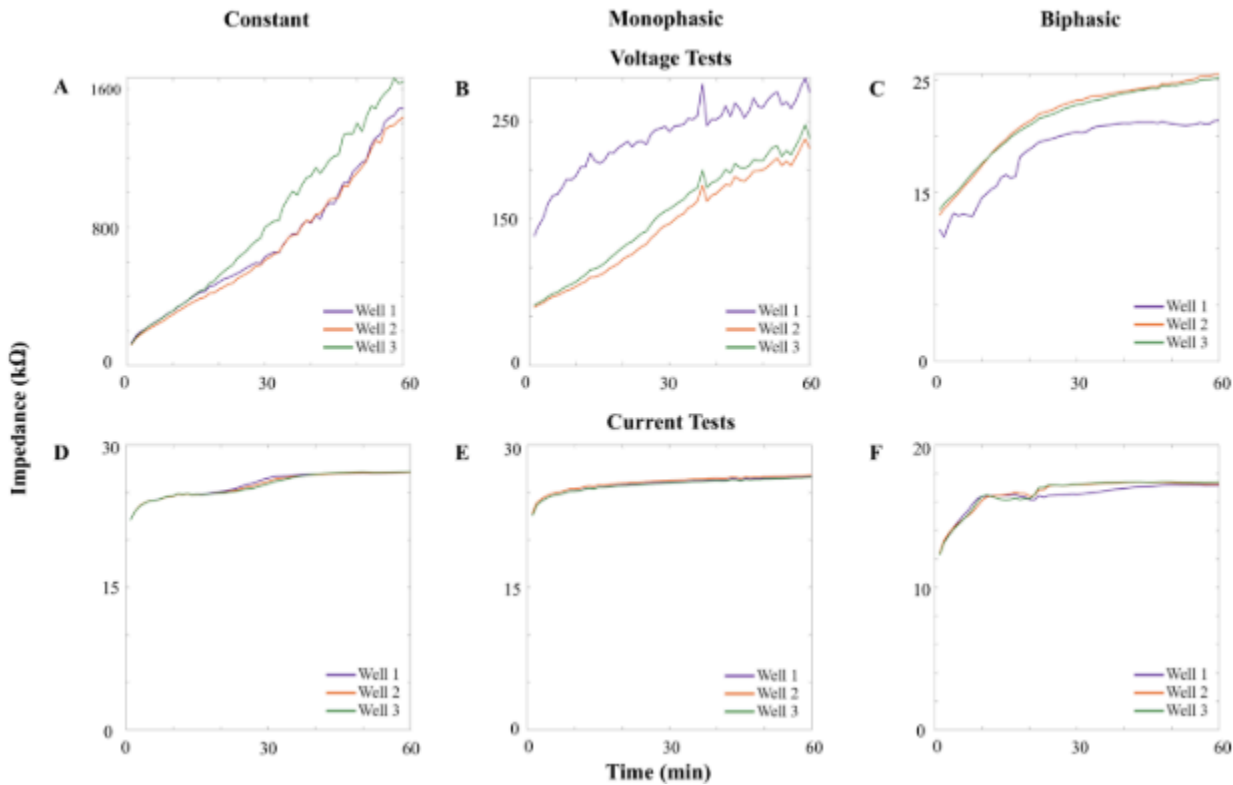


Figure 4

Average impedance per minute over one hour stimulation for 1V voltage and 100 μ A current stimulation. Average impedance over one hour stimulation period for the three tested wells under A) constant, B) positive monophasic, and C) biphasic 1V stimulation and D) constant, E) positive monophasic, and F) biphasic 100 μ A stimulation. Note: y-axis scales are different to demonstrate the respective increase in impedance for each test.

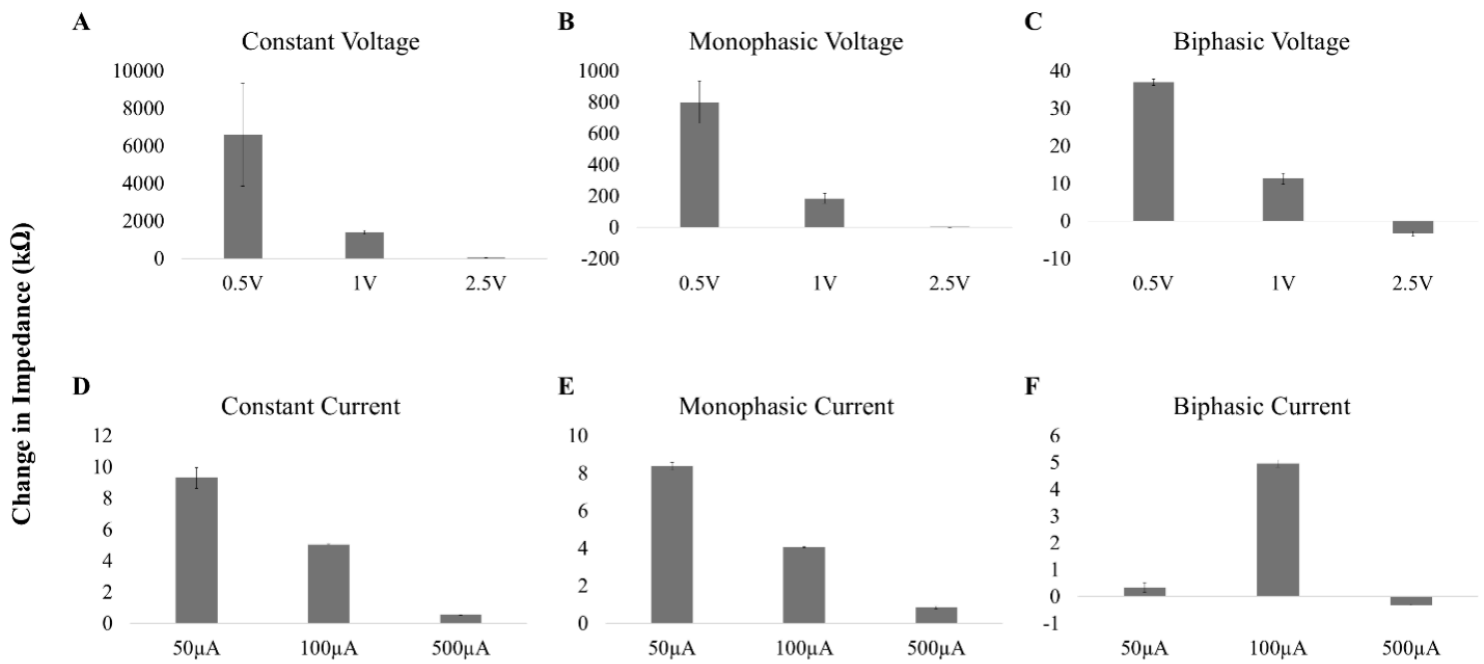


Figure 5

Average change in impedance across three wells over one hour stimulation period. Average change in impedance across three wells over one hour stimulation period for A) constant voltage, B) monophasic voltage, C) biphasic voltage, D) constant current, E) monophasic current, and F) biphasic current stimulation. Note: y-axis scales are different to demonstrate differences in impedance for each test.