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Real-time Bioimpedance Sensing of Antifibrotic Drug Action in Primary Human Cells

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ABSTRACT: Fibrotic diseases are among the most serious health issues with severe burdens due to their chronic nature and a large number of patients suffering from the debilitating effects and long-term sequelae. Collagenase treatment is a non-surgical option but has limited results. To date, there is no potent non-invasive solution for fibrosis. Part of the reason for this is the lack of appropriate *in vitro* live cell screening tools to assess the efficacy of new therapeutical agents. Here, we demonstrate the utility of a cell-based electrochemical impedance biosensor platform to screen the efficacy of potential antifibrotic compounds. The platform employs a label-free and non-invasive strategy to detect the progression of fibrosis and the potency of the antifibrotic molecules in real-time. The fundamental principle that governs this novel system is that dynamic changes in cell shape and adhesion during fibrosis can be measured accurately by monitoring the changes in the impedance. This is achieved by growing the cells on a transparent interdigitated indium tin oxide (ITO) electrodes. It was demonstrated by monitoring the efficacy of a model antifibrotic compound, PXS64, on cells collected from patients with Dupuytren`s contracture. We confirmed the validity of the developed biochemical impedance biosensor as an tool for *in vitro* screening of antifibrotic compounds and provided quantitative information on subcellular influences of the examined chemical molecules using correlative microscopy analyses that monitor the average cell area, cell morphology, the amount and directionality of the deposited extracellular matrix protein collagen and measurement of cytosolic Ca^{2+} changes,.

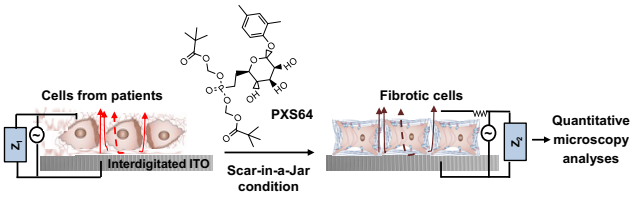
Fibrosis is a major global health burden[1] accompanying with deregulated cell morphology, migration, differentiation, and function that lead to the loss of tissue function and even death.[2] Fibrotic condition is histologically characterized by excessive production of extracellular matrix (ECM) components such as collagen.[3] Different parts of the body such as lung, liver, skin, joints, and tendons can be affected by fibrosis. Dupuytren`s contracture is associated with permanent flexion contracture of digits, which is often painless but severe and debilitating to hand function.[4] Apart from pathological effects, millions of patients develop multiple long-term fibrotic sequelae such as depression and sleep disorders that severely hinder their quality of life.[5] At present, the excision of the palmar aponeurosis, in the form of fasciectomy is the most common therapy for Dupuytren`s contracture. In fact, there is no potent chemical compound available as the curative treatment for various types of fibrosis.[6] This is despite surgery solutions that are associated with complications[7, 8] and a high recurrence rate.[9] There is, therefore, an unmet and critical need to develop antifibrotic drug therapies.

The development of efficient antifibrotic agents has been hampered partly due to the lack of appropriate high-throughput methodologies for accurate *in vitro* validation of drug potency.[10] Currently, most of the analysis methods require fixing cells and high resolution imaging to indicate the amount of deposited collagen or use indirect methods of validation. Due to the time-dependent nature of fibrosis, a desirable extension of this technique would be to develop methods that are amenable to assess fibrosis in the same cells as the condition progresses. Evaluating fibrosis in live cells long-term, without interrupting cells normal function, is important to provide more robust information on the dynamics of cell responses as well as their end result.[11-13]

Electrochemical impedance bioassays can detect minute changes in cell morphology and cells connectivity in real-time, non-invasively.[14-17] These techniques have the capability to assess the disease or therapies that interrupt cell shape and adhesion in a label-free manner over long time frames. During fibrosis, quiescent fibroblasts differentiate to fibrotic myofibroblasts associated with alteration of cell morphology and adhesion.[2] Thus, we hypothesize that bioimpedance measurements may display sensitivity to fibrosis progress. In this regard, the feasibility of screening potential antifibrotic compounds using electrochemical bioimpedance measurements is worthy of assessment to provide a real-time "Scar-in-a-Jar" inspired biochemical sensor. The "Scar-in-a-Jar" is an enhanced collagen production process to speed up the fibrosis progress from several weeks to a few days *in vitro*.[6, 18] However, correlating the acquired raw impedance data to subcellular events is challenging. Therefore, a desirable cell-based electrochemical impedance biosensor that provides the possibility of also performing light microscopy would greatly aid in acquiring information on intracellular changes.[19]

Herein, the feasibility of using cell-based electrochemical impedance measurements in determining the efficacy of potential antifibrotic chemical agents was assessed using primary human cells from Dupuytren`s contracture patients on interdigitated ITO electrodes. We previously have demonstrated the application of ITO for high quality fluorescence microscopy including superresolution imaging.[20] The assay was used to examine the effect of an analogue of mannose-6-phosphate (M6P), namely PXS64, on fibrosis regression. During the entire experiment, fibrosis was modeled *in vitro* by culturing cells under the experimental conditions developed as part of "Scar-in-a-Jar" methodology. Owing to the non-invasive nature of the employed biochemical method and the transparency of the electrodes, fluorescence microscopy was used after finding the useful time window of the drug. The microscopy results not only aid to confirm the validity of the bioelectrochemical impedance assay for evaluating the efficacy of potential antifibrotic compounds, but also provide quantitative information on intracellular events. These techniques were employed to determine the average cell area, the amount of collagen per cell and the alignment of collagen fibrils. In addition, the level of

cytosolic Ca^{2+} for cells cultured under various conditions at different time points was measured to acquire more in-depth insight into the mechanism of PXS64 activation. The principle of the developed biochemical assay by combining impedance measurement with "Scar-in-a-Jar" strategy is shown in Scheme 1.



Scheme 1. Fibrosis is involved with the transition of fibrogenic cells from quiescent fibroblasts collected from patients to fibrotic cells. The electrochemical impedance values of the cell layer before (Z_1) and after (Z_2) applying conditions that accelerate fibrosis by "Scar-in-a-Jar" or inhibit fibrosis by antifibrotic compounds may display different values due to the change in morphology and adhesion state of cells. Impedance measurement aims at determining the efficient time window of the examined chemical compound (PXS64) in a real-time and non-destructive manner prior to performing optical analyses on the same cells which provide quantitative information on intracellular events.

EXPERIMENTAL SECTION

Chemicals. All chemicals were purchased from Sigma-Aldrich (Sydney, Australia) unless otherwise stated. Dulbecco's modified eagle's medium (DMEM), nutrient mixture F-12, PBS, fetal bovine serum (FBS) and fura-2 AM were purchased from Invitrogen (Sydney, Australia). Mouse anti-human collagen I monoclonal primary antibody and mouse monoclonal IgG1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488 conjugated secondary goat anti-mouse antibody, Fluorobrite, and Hoechst® staining solution (Cat. No. H3570) were from Life Technologies (Sydney, Australia). Ficoll PM 400 and 70 were from GE Healthcare (Buckinghamshire, UK). TGF-β_1 was purchased from R&D Systems Inc (Minneapolis, MN, USA).

Preparing Media. Non-crowded condition (control media) represents the DMEM media containing l-ascorbic acid 2-phosphate, penicillin-streptomycin and only 0.5% FBS, similar to a starved media. Fibrotic media (no drug) was prepared according to "Scar-in-a-Jar" method [18], and refers to DMEM-Glutamax™ with 0.5 % FBS, a mixture of 37.5 mg/mL Ficoll PM70 (Fc 70) with 25 mg/mL Ficoll PM 400 (Fc 400), 100 µM of l-ascorbic acid 2-phosphate and 5 ng/mL-1 TGF-β_1 (5 ng/ml) (crowding media). 2-Phosphate hexahydrate is a stable form of ascorbate with a longer lifetime than ascorbate in culture [21]. The neutral macromolecules in a Ficoll cocktail (Fc) increase collagen deposition 10-fold in 6 days and in a reticular deposition pattern [18]. Fibrotic condition containing drug refers to the fibrotic media with 10 µM dissolved PXS64.

Cleaning of Interdigitated ITO Electrodes. The interdigitated ITO electrodes used in this study were purchased from ALS Co., Ltd (Tokyo, Japan). The counter, reference and interdigitated ITO working electrodes were fabricated on rectangular AT-cut quartz crystals. Each working electrode consists of 65 pairs of finger ITO electrodes with 10 µm width and 5 µm spacing between each electrode. An insulating Novolac resin-based membrane on the surfaces confines the sensing area to the interdigitated working electrode. The electrodes were rinsed twice with ethanol and then with Milli-Q water. The electrodes then were plasma cleaned using an oxygen-plasma cleaner (Harrick Plasma Cleaner/Sterilizer PDC-32G, Ossining, NY) for 5 minutes.

Cell Culture. Human primary Dupuytren`s cells were collected from palms of the patients with Dupuytren`s contracture under University of Western Australia ethics approval and with informed consent from all patients in accordance with the NHMRC national statement on ethical conduct in human research. Cells were subsequently grown in T75 flasks in a humidified incubator (Heraeus, Germany) containing 5 % CO_2 at 37 °C, and maintained in DMEM/glutamax media containing 10 % FBS and penicillin/streptomycin (1% v/v). The cell culture medium was changed every 2 days, and all experiments were conducted on cells between passages 3 to 6. Human primary Dupuytren`s cells (50,000 cells/cm^2) were cultured for 24 h on cleaned ITO surfaces in normal DMEM-glutamax media with 10% FBS and 1% penicillin/streptomycin and then the media was changed to one of the control, fibrotic (no drug) or fibrotic plus 10 µM PXS64 media. All the experiments were carried out on bare ITO. Cells attached and grew on ITO surfaces in a consistent manner as shown previously. [19, 22]

Electrochemical Impedance Spectroscopy. Impedance sensing was performed in a three-electrode electrochemical chamber. The cell-covered electrodes were mounted in a custome made electrical cell fabricated by Micrux Technologies (Oviedo, Spain). A Constant AC voltage (10 mV) vs. printed reference electrode with Ag/AgCl ink was applied to the electrode surfaces using a potentiostat (SP 200, BioLogic, Science Instruments). The electrodes were washed two times with the electrolyte (cell media) before cell seeding. The resulting electric potential was measured 24 h after cell seeding across the cell-covered electrode to acquire the baseline impedance spectra. The experimental impedance spectra of the cell layers on the surfaces under different conditions were recorded daily. During long-term experiments the attachment and morphology of cultured cells on ITO surfaces was regularly monitored using

transmitted light microscopy. The measurements continued until the fibrosis occurred and then the effect of the drug became evident. The cell-covered interdigitated ITO surfaces were probed with a weak non-invasive sinusoidal AC signal within the range of frequency between 40000-400 Hz (10 mV) (12 points/decade). This current applies only a negligible electrical stimulation to cells during the impedance measurement and, therefore, do not electrically stimulate them. The impedance value on each surface was normalized by dividing the impedance of cell-covered electrode at each time point by the impedance value of the cell layers obtained 24 h after seeding cells, before applying different experimental conditions. EC-Lab ® V10.33 (BioLogic, Science Instruments) software was used to analyze the impedance results. The cell-covered surfaces were then used for further optical analyses after finding the effective time window of PXS64 for Dupuytren`s cells.

Cell Area Measurement. A measure of Dupuytren`s morphology and cells area was performed after impedance measurements. To this end, cells were fixed and stained with Alexa Fluor® 647-phalloidin. Alexa Fluor® 647-phalloidin stain the cell cytoskeleton through the binding of phalloidin to F-actin. The cell layer was fixed with 4 % paraformaldehyde for 10 min at room temperature. Cells were then incubated in Alexa Fluor® 647-phalloidin solution in PBS (1:100 v/v) for 10 min followed by washing with PBS for three times. The fluorescence images were recorded using a total internal reflection fluorescence (TIRF) microscope (ELYRA, Zeiss) equipped with a cooled, electron-multiplying charge-coupled device camera (iXon DU-897; Andor) with an exposure time of 30 ms. A 20x air objective lens and 15 mW of 633 nm laser illumination were used for imaging. Raw fluorescence intensity images were analyzed using image-analysis software platform ImageJ (US National Institutes of Health).

Immunocytochemistry for Collagen Visualization According to "Scar-in-a-Jar" Method. Cells were exposed to one of the control, fibrotic (no drug), or fibrotic containing PXS64 conditions for the time window that was indicated using impedance measurements. Then, cells were washed with PBS and were blocked with 3% bovine serum albumin (BSA) in Fluorobrite for 10 min. After that, the primary antibody solution was added. This solution was prepared by diluting the primary collagen 1 mouse monoclonal IgG1 antibodies in blocking solution (1:1000 v/v). The cell-covered surfaces were then placed in a cell culture incubator for 90 min, and then cells were washed two times in Fluorobrite (5 min per wash). After that, the cells were fixed in 4% paraformaldehyde solution for 10 min at room temperature. The secondary antibody solution was prepared by diluting the secondary goat anti-mouse antibody conjugated to Alexa-fluor 488 in Fluorobrite (1:500 v/v). The solution was incubated with cells at 37°C for 30 min following washing cells in Fluorobrite twice (5 min per wash). To stain the nuclei, Hoechst® staining solution (1:1000 v/v in Fluorobrite) was incubated for 10 min at room temperature with cells. The coverslips were mounted on the slides using Prolong® Gold anti-fade mounting solution (Life technologies, USA). Nail polish was used to fix the coverslips, and the slides were stored in a light-proof box at 4°C until imaging for quantifying the amount of collagen per cell or for coherency analysis.

Coherence Analysis. The prepared slides with stained collagen fibers and nucleus of cells were imaged using Leica TCS SP2 multiphoton confocal microscope with a Leica confocal software (LCS). Slides were imaged using a 40x oil objective and a 488 nm laser wavelength. Each slide was imaged in at least three separate areas, randomly chosen by the assessor. A z-stack of each region was taken, as determined by the top and the bottom of the collagen layer. Once the top and bottom of the z-plane were set, images were taken at 0.05 µM intervals and then condensed into a single frame using the maximum projection function. The coherency of collagen fibers was analyzed on confocal images using the orientation J plugin of Fiji software [23] and the orientation J package. Briefly, six regions of interest (ROIs) were chosen at random, excluding those areas with deformations or hole [24]. The ROIs were kept as large as possible to examine the orientations of the collagen bundles, as opposed to the local variations within the bundles. The 'measure' feature was then used to determine the coherence of each ROI. The coherency function draws an ellipse that indicates the coherency of the ROI. The coherence formula takes into account the largest eigenvalue (major axis) and the smallest eigenvalue (minor axis). The coherency equal to 0 means that the ellipse has become a circle, with no elongation and no aligned structures present in the position of the image. The coherency value of 1 represents that the ellipse has become a line segment, with very high elongation and oriented structures present in the position of the image.

Ca^{2+} Flux Measurement. The amount of intracellular Ca^{2+} flux in cells was measured after exposing cells to one of the control, fibrotic or fibrotic plus PXS64 culture media for the time window that was determined by impedance results. The measurement was performed after loading cells with fura-2 AM as previously described. Fluorescence at 340 nm/380 nm excitation and 510 nm emission were recorded on an inverted Nikon TE2000-U microscope at 1 min intervals with an exposure time of 50 ms using a Hamamatsu Orca ER digital camera at 37 °C. The ratiometric 340 nm/380 nm signals of individual cells were quantified using Metamorph 6.3 to measure signal

intensity of manually traced cell regions. A cell-free equivalent region was used to measure the background intensity. The background value was subtracted from the value that was obtained from cell areas. Intracellular calcium $[Ca^{2+}]_i$ (nM) was determined as described previously.[25] Ionomycin (5μM) and 5mM Ca^{2+} were added to obtain R_{max}. Media was then replaced with calcium-free imaging solution supplemented with EGTA (3mM) to obtain R_{min}. Intracellular calcium was calculated from ratiometric fluorescent values (R) according to Equation 1.

Equation 1. $[Ca^{2+}]_i = K_d.b.(R-R_{min})/(R_{max}-R)$

where b = ratio of R during illumination at 380nm with 0mM versus 5mM calcium, and K_d (dissociation constant) = 224nM as determined previously.[26] Based on the observed results for cells exposed to different media for the duration that was indicated based on impedance measurement, we decided to check the intracellular calcium ion concentration of Dupuytren`s cells at different time points. Cells were seeded on the surfaces for 24 h first; then the intracellular Ca^{2+} was measured immediately after changing media called 0 h, after 24 h, after 48 h or after 72 h of treating cells in one of the control, fibrotic (no drug), or fibrotic media containing PXS64 culture media. For acute measurement, 0 h, the intracellular calcium ion level of the cells loaded with fura-2 AM was measured 5 min just before and 20 min after changing the experimental condition (when responses had reached plateau). For long term measurements (≥24 h), the media was changed to one of the relevant ones, and at specific time points the fura-2 was loaded, and the intracellular Ca^{2+} flux was measured for cells under various experimental conditions. To study whether the calcium flux is mobilized from endoplasmic reticulum stores, thapsigargin was used [27]. For this purpose, 10 nM thapsigargin was added to cells that were exposed to the fibrotic media for 48 h and the cytosolic calcium ion level was then measured after another 24 h.

Statistical Analysis. Data are displayed as means ± SD unless otherwise stated. Data were analyzed using the GraphPad Prism version 6.0 data management software to conduct one-way ANOVA on groups of data on the number of experiments (n) indicated in the Figure captions.

RESULTS AND DISCUSSION

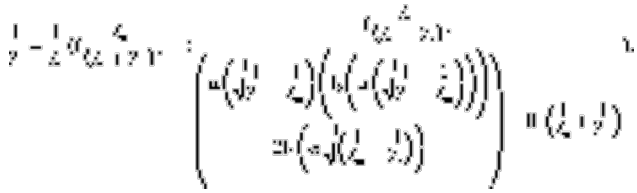
The Principle of The Developed Biochemical Assay. In the "Scar-in-a-Jar" model, specific macromolecules are added to the culture media to provide a crowded environment which results in improved protein folding and protein-protein interactions.[18] Here, we call to this condition as the "fibrotic" condition which leads to enhanced collagen deposition in an accelerated manner in the laboratories. TGF-β is one of more critical components that contributes to the improvement of collagen deposition rate.[9, 28] In contrast, the absence of such crowding molecules slows the fibrosis progress.[29] This condition is referred to as the "control", and fibrosis progression is expected to be slowed compared with the cells in the crowded state. In the current study, the impact of applying the crowded condition on the electrochemical impedance response of cells cultured on interdigitated ITO surfaces was examined. Furthermore, the effects of the PXS64 were also explored by culturing cells in the crowded environment in the presence and absence of this chemical agent. The amount of PXS64 employed was 10 μM in cell media which is a non-toxic and effective concentration according to our previous studies.[30, 31] Cells were treated in one of non-crowded (control), crowded (fibrotic), or drug-containing fibrotic media and their effects on fibrosis progress were assessed using impedance spectroscopy followed by using immunocytochemistry and fluorescence microscopy techniques.

Impedance Measurement. The feasibility of using impedance spectroscopy to assess the efficacy of antifibrotic chemicals on primary human cells was examined using a custom-made chamber (see the Methods section for more detailed information). For this purpose, cells were grown on interdigitated ITO surfaces for 24 h and then the media was transferred to the three media for the three sets of the conditions. These conditions were control media where fibrosis is not expected to advance, fibrotic where fibrosis progression is induced, and fibrotic media plus 10 μM PXS64, where fibrosis is promoted but expected to be inhibited by the drug. The evidence of drug efficacy is that the impedance data for cells under the fibrotic condition containing drug is expected to exhibit very similar values to those under the control environment of the control sample. The impedance data were recorded at different time points until the effect of the chemical drug became evident. To ensure that the drug does not change the impedance by itself, the impedance values of the surface with media containing the drug was recorded for around 75 h (data not shown here). The results confirmed that drug does not have any effect on impedance data variations. The frequency-resolved impedance readings were measured at 12 frequencies spaced evenly on the logarithmic scale ranging from 40000 Hz to 400 Hz. An example of the frequency-resolved impedance data is shown in Figure 1A. According to these results, the most sensitive frequency in displaying the effect of the drug among the examined frequencies was around 40000 Hz. At such a high frequency, the electrical current mainly passes between the ventral surface of the cell membrane and the electrode and through cells.[32] Therefore, the alteration of impedance signals at this range of frequency are mainly dominated by the changes of cell coverage and the cell-electrode gap.[17, 33]

The normalized impedance values at the frequency of 40000 Hz measured immediately 0 h, 24 h, 48 h, and 72 h after changing the normal media to one of the control, fibrotic (no drug) or fibrotic media containing drug are shown in Figure 1B. These normalized impedance data were obtained by dividing the real-time impedance data to the value of the impedance of the cell-covered electrodes 24 h after seeding cells. Subsequently, the changes in normalized impedance values are attributed to the alteration in cellular properties induced by exposing cells to control media, fibrotic media or fibrotic media containing the drug.[34] Herein, impedance values of cell-covered surfaces before adding different media showed no significant difference compared to each other (data not shown here). The magnitude of the impedance of cells under fibrotic condition (no drug) was around 16% higher than that for cells in the fibrotic media containing 10 μM PXS64 after 48 h, and this effect persisted until at least 72 h. The impedance of the cell-covered electrodes cultured in fibrotic media containing PXS64 displayed similar data to those treated under control condition with less than 1.5 % differences in their average values. These results indicated the efficacy of PXS64 in controlling the effects of the fibrotic condition and hence hindering fibrosis progress. The impedance spectroscopy provided real-time nondestructive information on the useful time window of the drug.

The alteration of impedance signals is mainly attributed to three things. These are the current passing 1) beneath the cells (the space between the ventral surface of cell membrane and the surface), 2) in the space between neighboring cells and 3) directly through the cells. Cells are assumed to act as capacitors themselves. Accordingly, the frequency sweep data for the unit area of the confluent cell-covered electrodes can be fitted to a mathematical model developed by Giaever and Keese[35] to discriminate the contribution of three parameters in changing the impedance including the cell-cell connectivity (R_b), cell-surface adhesion (α) and average cell membrane capacitance (C_m) (Figure 1C). This model output is shown in Equation 2, where the values of Z_c ($\Omega.cm^2$) and Z_n ($\Omega.cm^2$), the impedance of the cell covered electrode and cell-free electrodes, respectively, are obtained by running impedance measurement over the frequency (f) range. I_0 and I_1 are attributed to the modified Bessel functions of the first kind of order 0 and 1, respectively. The cell membrane capacitance (C_m) can be calculated based on obtained Z_m, the impedance of cells, as Equation 3 displays. The value of α is dependent on the cell radius (r_c) and average cell-surface distance (h) as is shown in Equation 4, where ρ is the specific resistivity of the cell culture medium.

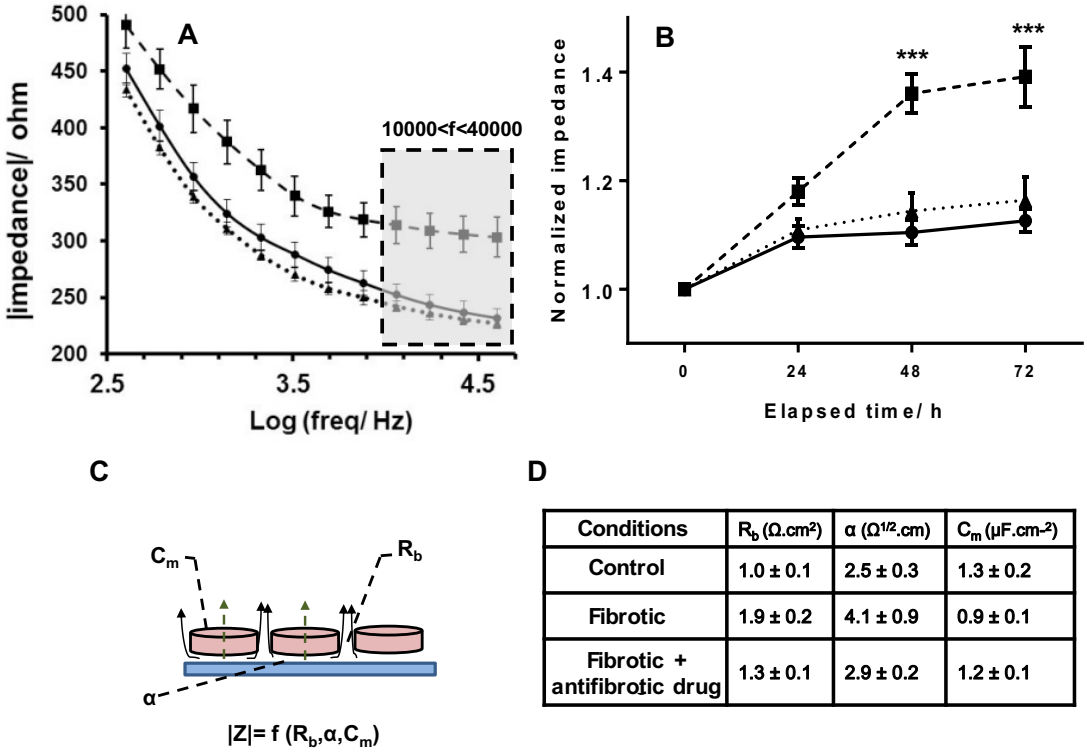
Equation 2.



Equation 3. $Z_m=1/2\pi fC_m$

Equation 4. $\alpha \quad r_c\sqrt{\rho/h}$

The summarized values of R_b, α, and C_m of the cell layer exposed to control, fibrotic (no drug) and fibrotic media containing PXS64 are shown in Figure 1D. All static parameters in this table were obtained by fitting the frequency-resolved impedance data recorded 72 h after changing the media (Figure 1A) to Equation 2. The results indicated that the average value of R_b and α increased from 1.0 ± 0.1 ($\Omega.cm^2$) and 2.5 ± 0.3 ($\Omega^{1/2}.cm$) in control media to 1.9 ± 0.2 ($\Omega.cm^2$) and 4.1 ± 0.9 ($\Omega^{1/2}.cm$) under fibrotic condition, respectively. These indicate significant increases in cell-cell and cell-surface adhesion while cells were stimulated with fibrotic media compared with cells treated under control condition. The modeling results showed that the C_m decreased from 1.3 ± 0.2 ($\mu F.cm^{-2}$) to 0.9 ± 0.1 ($\mu F.cm^{-2}$) for cells cultured in control state compared with cells in fibrotic media. The cell membrane capacitance is proportional to the membrane surface area.[36] Therefore, the decrease in this value can be explained by formation of smoother surface membrane with less degree of folding that leads to less total membrane area of cells in the fibrotic media compared with cells under control condition that does not evoke fibrosis. The values of R_b, α, and C_m of cells grown in fibrotic media in the presence of 10 μM PXS64 showed similar values to those for cells in control state. This confirms the efficiency of the drug in impeding the influence of fibrotic condition on cells morphology and adhesion in accelerating fibrosis progress.



Conditions	R_b (Ω.cm²)	α (Ω$^{1/2}$.cm)	C_m (μF.cm^{-2})
Control	1.0 ± 0.1	2.5 ± 0.3	1.3 ± 0.2
Fibrotic	1.9 ± 0.2	4.1 ± 0.9	0.9 ± 0.1
Fibrotic + antifibrotic drug	1.3 ± 0.1	2.9 ± 0.2	1.2 ± 0.1

Figure 1. (A) The impedance measurement at 12 frequencies in the range of 40000 Hz-400 Hz after 72 h of exposing Dupuytren`s cells to control media (solid curve), fibrotic media (no drug) (dashed curve), or fibrotic plus 10 μM PXS64 media (dotted curve). The frequency of around 40000 Hz (highlighted region) showed the largest relative change for cells. The impedance values at this frequency were reported for further comparisons. (B) Real-time change in impedance of the cell covered electrodes at 40000 Hz treated under control (solid curve), fibrotic (no drug) (dashed curve), or fibrotic plus drug (dotted curve) conditions. The impedance of the cells under the fibrotic state showed a significant increase compared with the cells cultured in control media. The impedance of cells under fibrotic condition plus drug displayed similar values to those in control media. This indicates the efficacy of PXS64 in inhibiting the fibrosis progress in the fibrotic state. The elapsed time shows the time after changing the normal cell media into one of the experimental conditions. (C) The impedance value is a mathematical function of cell-cell (R_b) and cell-surface (α) connectivity and cell membrane impedance that mainly results from alterations in cell membrane capacitance (C_m). (D) The values of R_b, α, and C_m of cell layer after 72 h growing the cells in control media, fibrotic media or fibrotic media containing PXS64 . *** is $p < 0.001$ versus control condition (n=3).

After finding the useful time window of PXS64 to be 72 h using bioimpedance measurement, the cells were studied using immunocytochemistry and fluorescence microscopy assays to examine the validity of electrochemical impedance measurements for antifibrotic drug screening applications. In addition, the microscopy analyses were used to add the important capability of acquiring quantitative and visual subcellular information on cell morphology, spreading and the collagen production.

Cell Area Measurement. Fluorescence microscopy was used to correlate the conclusions made with the electrochemical impedance measurement on cell morphology changes. It has been demonstrated that cell morphology and spreading are downstream events that undergo regulation during fibrosis development.[37] Evaluation of the cell area and shape was performed by staining the F-actin of the cells to obtain the quantitative information on the effect of PXS64 on cell behavior during fibrosis development. For this purpose, cells on the surfaces treated under control, fibrotic (no drug) and fibrotic media containing drug conditions were stained with Alexa Fluor® 647 phalloidin and imaged under fluorescence microscopy (Figure 2). The results showed that under fibrotic condition, cells displayed dendritic morphology, whereas, cells in control media mainly exhibited the stellate shape. Furthermore, the results illustrated that in fibrotic media in the presence of 10 μM PXS64 cells did not develop the dendritic shape but rather had the stellate morphology of the cells under control state. The average area occupied per cell was calculated based on the obtained images (Figure 2D). The results indicate that the fibrotic state (no drug) stimulated cells to display more than 66 % increase in their amount of spreading than cells under control condition. Moreover, it was demonstrated that cells under the fibrotic condition in the presence of 10 μM PXS64 exhibited a similar value of average area per cell to those cultured in control environment. This morphological study shows that the fibrotic condition accelerates

fibrosis in Dupuytren`s cells *in vitro* compared with control samples. Whereas, the cell area and shape in fibrotic media containing the drug remained similar to those for cells treated under the control state. These results provide further evidence, in agreement with impedance data, on the efficacy of the PXS64 in inhibiting the effect of the crowded media in developing fibrotic condition. During fibrosis, cell morphology alters by inducing the polymerization of the actin cytoskeleton from globular to filamentous.[37, 38] Actin polymerization is necessary for impedance alteration.[39] The increased cell coverage limits the bare area of electrode surface exposed to the electrolyte, and thus the current leakage, which consequently causes the increase in the impedance value.[17] In addition, the cell area data evidence that the observed increase in impedance value over time for cells in fibrotic media was partly related to changes in cell coverage and shape occurring during fibrosis progress. The reported drop in C_m of cells in fibrotic media compared with cells in control or fibrotic media containing the drug can be explained by stress fiber formation and the development of smoother cell membrane associated with reduced cell membrane area.[40, 41]

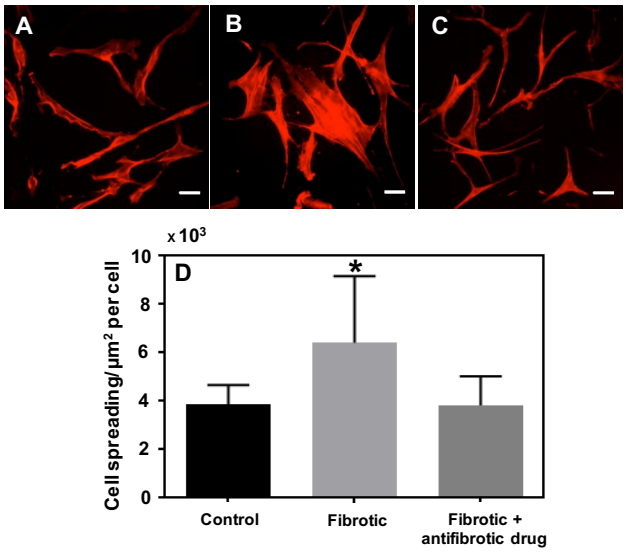


Figure 2. A measure of Dupuytren`s cells morphology and area post Alexa Fluor® 647 phalloidin staining. Epifluorescent images of cells in (A) control, (B) fibrotic media (no drug) or (C) fibrotic media plus PXS65 for 72 h. Considerable alterations in cell morphology under fibrotic condition were observed compared with cells grown in fibrotic media containing PXS64. (D) The values of average area per cell were calculated based on analyzing the obtained epifluorescent images. These results validate the results from the developed impedance sensor in this work. * is $p < 0.05$ versus control condition ($n>35$). The scale bars are 50 µm.

Collagen I Deposition Analysis. An excessive amount of collagen is a salient feature of fibrosis.[42] Several studies have reported the increase of type I collagen fibers in ECM of Dupuytren's cells.[43] Here, fluorescence microscopy was utilized to quantify the amount of collagen per cell after 72 h of exposure to different conditions including control, fibrotic (no drug) and fibrotic containing PXS64 media. Moreover, such data provide one more piece of evidence regarding the applicability of impedance measurement in antifibrotic screenings. The average amount of collagen that was deposited in ECM by each cell was calculated by following the immunocytochemistry strategy used in "Scar-in-a-Jar" model.[10] Figure 3A-C show fluorescence microscopy images of the deposited collagen I (green) and nuclei (blue) from cells deposited on the surfaces in one of the control, fibrotic or fibrotic media containing PXS64. The area of deposited collagen I per cell was calculated by analyzing the recorded fluorescence microscopy images (Figure 3D). The average collagen produced by cells under fibrotic condition (no drug) was around 3-fold higher than cells in control state. The statistical analysis showed no significant difference between the amounts of deposited collagen from cells in fibrotic media containing PXS64 compared with cells under control condition. It is shown that the excessive deposition of collagen increases the matrix stiffness and induces focal adhesions formation and stability through an increase in tension at these sites.[44] Thus the extra deposited collagen by cells cultured in fibrotic media has contributed to raising the impedance value for cells under this state, predominantly, by improving the cell-surface adhesion as the increase in α value in Figure 1D shows.

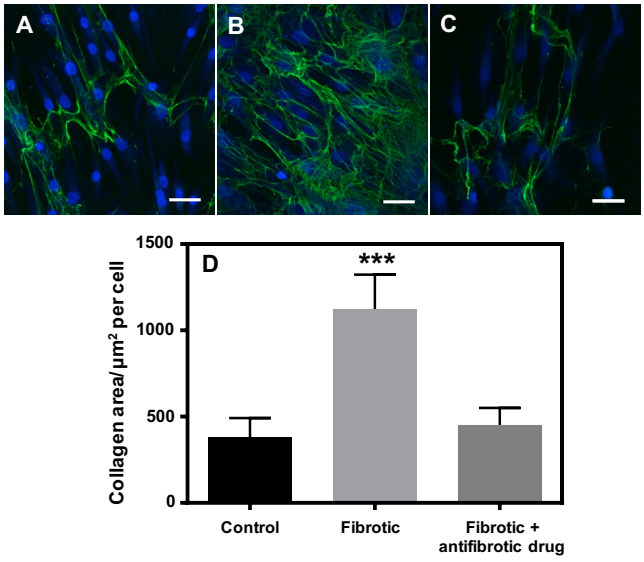


Figure 3. Collagen deposition analyses from the *in vitro* "Scar-in-a-Jar" study. Fluorescence microscopy images of deposited collagen I (green) and nuclei (blue) of cells exposed to (A) control, (B) fibrotic (no drug) or (C) fibrotic media containing PXS64 for 72 h. (D) Quantification of the area of stained deposited collagen I fibers per cell was determined from the recorded

microscopy images as are shown above. Cells under fibrotic condition deposited significantly greater amount of collagen compared with cells in control state. The presence of 10 μM PXS64 significantly reduced the quantity of collagen fibers laid down in ECM under crowded condition of the fibrotic media as was predicted using the developed impedance sensor. *** is $p < 0.001$ versus control condition (n>6). The scale bar is 100 μm.

Study The Alignment of Collagen Fibrils. It has been demonstrated that the functionality of connective tissues directly depends on the alignment of collagen fibrils molecules.[45] During scar and fibrosis development, collagen fibrils undergo many syntheses, breakdown and cross-linking processes that strengthen and stabilize the formed tissue.[46] It has been shown[44, 46] that abnormal alignment of the collagen is one of the characteristics of the formation of scar and fibrosis tissues. Therefore, an effective antifibrotic chemical drug not only reduce the amount of collagen produced by cells but also keep the collagen alignment similar to their organization in control environment. The coherency analysis results of cells after 72 h culturing under one of the experimental conditions are shown in Figure 4. A coherence of 0 means the most random orientation, and a coherence of 1 means the least random alignment. A highly oriented structure was observed for the collagen fibers produced by cells in the fibrotic environment (no drug) compared with cells treated under control state. In addition, there was no major difference between the architecture of collagen fibers deposited in control media and fibrotic media that contains PXS64. These results indicate that PXS64 is effective in keeping the alignment of the collagen fibers in fibrotic environment similar to those produced by cells under control condition. The alignment of collagen fibers can profoundly influence fibroblast cells which orient themselves on rigid collagen fibers to generate maximal traction forces that stabilize integrin adhesions and promote focal adhesion maturation in the direction of collagen fiber orientation.[44, 47]

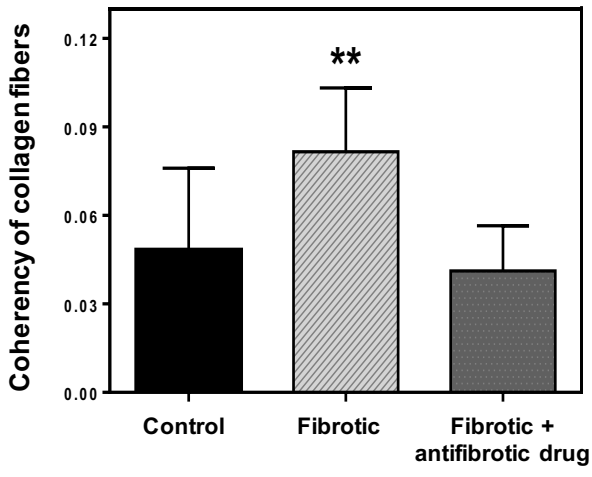


Figure 4. The coherency of collagen fibers deposited by cells exposed to control, fibrotic (no drug) or fibrotic media containing PXS64. The results suggest a significant coherence in the orientation of collagen fibers produced by cells under fibrotic condition versus the random alignment of collagen fibers deposited from cells treated in control media. The collagen fibers exhibited similarity in their alignment when they were stimulated with control or fibrotic media containing 10 μM PXS64 indicating the efficacy of the drug. ** is $p < 0.01$ versus control (n>6).

Ca^{2+} Flux Measurement. There is some evidence demonstrating that several aspects of Ca^{2+} signaling behave abnormally in fibrotic cells.[48, 49] Ca^{2+} is engaged in extensive cellular responses and relaying intracellular messages.[50] To examine whether PXS64 control fibrosis progress through calcium signaling pathways, the cytosolic Ca^{2+} of Dupuytren`s cells was determined before and immediately 0 h, 24 h, 48 h, and 72 h after changing the media to control, fibrotic (no drug) or fibrotic media containing 10 μM PXS64 (Figure 5). The cytosolic calcium level for the cells under fibrotic condition was considerably higher than that for cells in fibrotic media containing the antifibrotic drug after 48 h and 72 h. These are consistent with time points that were recognized using impedance measurement as the time that cell shape and adhesion in fibrotic media become significantly different from those treated under fibrotic condition in the presence of PXS64.

Two possible sources of the increase in cytosolic Ca^{2+} are the release of calcium ions from internal stores and/or influx of Ca^{2+} from the extracellular space through the channels on the plasma membrane.[51] Thapsigargin was used to assess whether the origin of increased Ca^{2+} concentration is endoplasmic reticulum. Thapsigargin elevates cytosolic Ca^{2+} concentration by blocking the ability of the cell to pump calcium into the sarcoplasmic and endoplasmic reticula that, consequently, causes the depletion of these stores.[27] The attenuation of cytosolic Ca^{2+} in the presence of thapsigargin demonstrates that the release of calcium ions from endoplasmic reticulum stores play a major role in increasing the cytosolic Ca^{2+} under fibrotic condition (Figure 5). Previously, the high activity of myosin light chain kinases (MLCK), and thus increase in intracellular Ca^{2+} in Dupuytren`s cells has been shown.[51] However, in the mentioned study a huge inconsistency in the expression of MLCK through Dupuytren`s tissue has been found. Future studies involve assessment of intracellular Ca^{2+} in cells from different parts of Dupuytren`s tissue[51] will provide further clarification regarding the intracellular calcium state in Dupuytren`s cells. This will assist in the design of biochemical therapeutic strategies that target intracellular Ca^{2+} pathways.

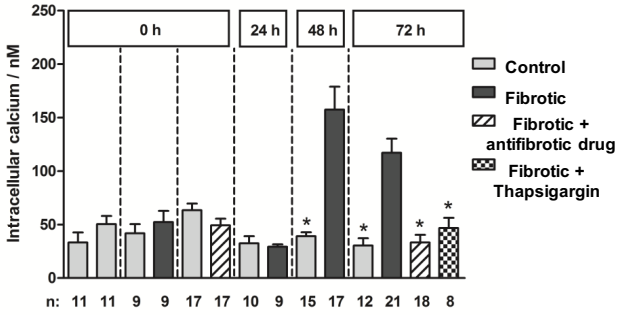


Figure 5. The intracellular Ca^{2+} of cells immediately 0 h and after 24 h, 48 h, and 72 h of changing the media to control, fibrotic (no drug), or fibrotic media containing PXS64. The increase in cytosolic Ca^{2+} after 72 h was significantly attenuated in the presence of either 10 μM PXS64 or 10 nM thapsigargin. The results indicate the capability of PXS64 in controlling the intracellular Ca^{2+} level and show that the source of mobilized calcium was mainly from endoplasmic reticulum stores. * is $p < 0.05$ versus fibrotic condition. The number of cells (n) has been indicated under the bar chart for each condition.

CONCLUSIONS

In conclusion, it was shown that the developed electrochemical bioimpedance sensor has the capability of screening the long-term effects of antifibrotic chemicals non-invasively and instantaneously of live cells on transparent interdigitated ITO electrodes. The real-time impedance measurements were combined with the crowded strategy suggested in "Scar-in-a-Jar" method that models the fibrotic disease *in vitro* in an accelerated manner. It was demonstrated that this device facilitates the determination of the useful screening time window of drugs before performing laborious quantitative measurement processes. The change in impedance magnitude was used as the measure to determine the effect of PXS64 on primary human cells collected from the palms of patients with Dupuytren`s contracture. Here, the fluorescence and immunochemical assays confirmed the validity of the impedance method for monitoring potential antifibrotic agents. The impedance results simply were fitted to a mathematical model and the contribution of change in cell-cell and cell-surface adhesion in defining the impedance value was discriminated.

The introduced bioimpedance sensor can be widely used to screen the potential antifibrotic therapies. However, a challenge is that the presented impedance sensor would not provide subcellular information at single cell level. Therefore, after finding the efficient time window of the drug using the device, as cells were not manipulated, the conventional fluorescence assays were employed to add the important capability of acquiring precise and quantitative subcellular information. This device dramatically reduces the amount of required end-point immune fluorescence assays. The emergence of "Scar-in-a Jar" inspired cell-based electrochemical impedance approach combined with optical analyses opens new avenues to probe the dynamics of fibrosis in live cells and to translate the antifibrotic drug screening *in vitro* results to clinical data.

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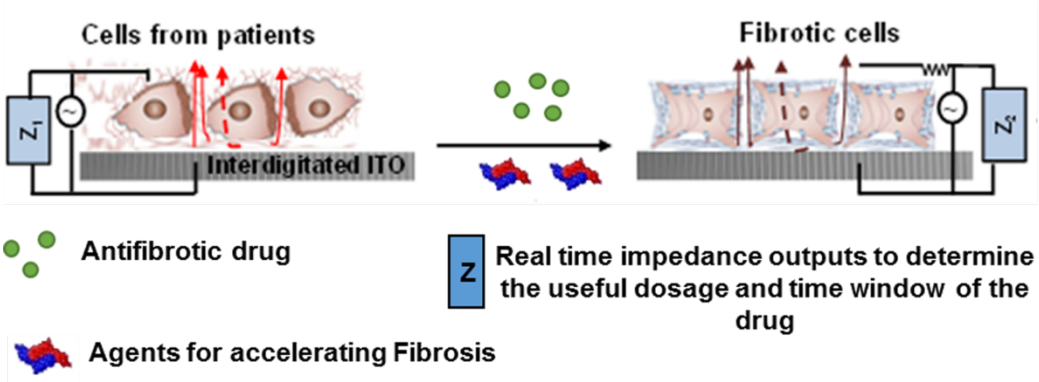
Cells from patients
Fibrotic cells
Interdigitated ITO
Antifibrotic drug
Z
Real time impedance outputs to determine the useful dosage and time window of the drug
Agents for accelerating Fibrosis