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Impedance-based drug-resistance characterization of colon cancer cells through real-time cell culture monitoring

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ABSTRACT

Interest in impedance-based cellular assays is rising due to their remarkable advantages, including label-free, low cost, non-invasive, non-destructive, quantitative and real-time monitoring. In order to test their potential in cancer treatment decision and early detection of chemoresistance, we devised a new custom-made impedance measuring system based on electric cell-substrate impedance sensing (ECIS), optimized for long term impedance measurements. This device was employed in a proof of concept cell culture impedance analysis for the characterization of chemo-resistant colon cancer cells. Doxorubicin-resistant HT-29 cells were used for this purpose and monitored for 140 hours. Analysis of impedance-based curves reveal different trends from chemo-sensitive and chemo-resistant cells. An impedance-based cytoxicity assay with different concentrations of doxorubicin was also performed using ECIS. The obtained results confirm the feasibility of ECIS in the study of drug resistance and show promises for studies of time-dependent factors related to physiological and behavioral changes in cells during resistance acquisition. The methodology presented herein, allows the continuous monitoring of cells under normal culture conditions as well as upon drug exposure. The ECIS device used, sets the basis for high-throughput early detection of resistance to drugs, administered in the clinical practice to cancer patients, and for the screening of new drugs in vitro, on patient-derived cells.

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2 These two authors share senior authorship.

1. Introduction

Colorectal cancer (CRC) rank third in terms of incidence and second in terms of mortality worldwide, with an estimate of 881.000 deaths per year [1]. Treatment for this kind of cancer includes surgical approaches, classic cytotoxic chemotherapy, molecular targeted therapy and immunotherapy [2]. Among these, chemotherapy is one of the most common options, specifically after surgical removal of tumor and at advanced CRC stages [3]. Despite the various therapeutic options, drug resistance remains a challenge due to intrinsic and acquired resistance [2].

The mechanisms of chemoresistance include genetic and epigenetic mechanisms, e.g. mutations in p53 protein and overexpression of antiapoptotic proteins, and overexpression of ATP-binding cassette (ABC) transporters. Regarding the transport-based mechanism, the ABC transporters clinically involved in multidrug resistance (MDR) are: P-glycoprotein (Pgp/ABCB1), MDR-related protein 1 (MRP1/ABCC1), and Breast Cancer Resistance Protein (BCRP/ABCG2). Overexpression of these transporters in cancer causes an enhanced efflux of drugs out of the cells, decreasing intracellular accumulation, and hence leading to chemotherapy failure [2, 4]. Chemoresistance is diagnosed after a long period of drug administration. The possibility to diagnose it, in a personalized way and before starting the treatment, will save time and prevent the secondary effects of an inefficient drug administration [5]. Understanding the mechanisms of chemoresistance and being able to implement an early detection, could be the key for the development of new therapeutic approaches for cancer treatment, moving towards personalized pharmaceutical regimes [6]. Traditional methods for the assessment of drug resistance include: fresh tumor cell culture tests, cancer biomarker tests and positron emission tomography. Advanced technological tools for the characterization of multi-drug resistance comprise next-generation sequencing, atomic force microscopy and live-cell imaging [7].

Impedance measurement is currently employed in monitoring several cellular processes such as cell growth, cell adhesion, cell migration, cytotoxicity effects of drugs and stem cell differentiation. Impedance measurement of living cells is described as a label free, non-invasive, quantitative electrochemical method [8]. The main advantage of cell impedance measurement is the possibility to perform long term monitoring, allowing the assessment of time-dependent factors and the analysis of the temporal evolution of cellular changes [9, 10]. Continuously monitoring cell cultures represents an improvement compared to conventional endpoint assays that allow measurements only at defined time-points, and very often involve the destruction of cells for detecting specific cellular components, forbidding subsequent assays [9]. Methodologies for measuring cellular impedance include impedance flow cytometry (IFC) [11], electrochemical impedance spectroscopy (EIS) [12] and electric cell- substrate impedance sensing (ECIS) [8]. In IFC, the scope is cell counting of suspended cells in microchannels [13, 14]. EIS is a multifrequency measurement of cells in suspension, while ECIS is a technique based on impedance measurement of adherent cells to substrate electrodes. The principle of ECIS relies on the single-shell model of the cell, in which the cytoplasm is simplified as a sphere of homogeneous phase and the plasma membrane is modelized as a thin shell [8]. With electrodes at the bottom of the culture dish, the insulating membranes of adhering cells interfere with the current flow, thus causing an increase in impedance. The impedance magnitude depends on cell number, morphology and size, as well as the degree of cell attachment [15].

Impendance-based cytotoxicity assays have been performed in different cell types and settings [16]. Using an impedance- based analyzer (xCELLigence, ACEA Biosciences), cardiotoxicity of Etoposide was studied over time in stem cell-derived cardiomyocytes [17]. With the same system, real-time toxicity assessment of aerosolized liposomal amphotericin B formulations in human alveolar epithelial cells was also performed [18]. With another commercial tool (ZTheta, Ap- plied BioPhysics) researchers have recently explored the impact of impedance-based assays in hepatology research [19]. A recently launched commercial system, (Maestro Z, Axion Biosystems), differs from the previously mentioned ones, as it includes the incubating environment within the measuring instrument. Further studies included chitosan cytotoxicity in breast cancer cells [20], and impedance culture monitoring for the dynamics of cell colonies formation in human hepatoma cells [21].

Current commercial systems are very robust and flexible, however the high cost of the systems, and mainly of the culture plates, remains a challenge. The study of chemoresistance through impedance has not yet been explored in detail. In contrast to the traditional approaches of chemoresistance assessment, impedance-based studies take advantage of being real-time, continuous, non invasive, non destructive, and label-free. Chemoresistance impedance-based analysis could help understanding some time-dependent mechanisms of

chemoresistance that might contribute to its early detection. Thus, the importance to devise a simple, flexible, less expensive and multiplexed system for this purpose.

We present herein a custom-made cell impedance measurement system based on ECIS capable of overcoming some of the drawbacks of currently used devices. The proposed label-free methodology, tested on HT-29 cells, allows to bring all the samples to an endpoint and monitor them in a continuous way through real-time data acquisition. This approach has potential applications on point-of-care diagnosis, prognosis and personalized medicine. The system's scalability potential and the use of standard dimensions of culture plates facilitates its direct application in the clinical environment. It could be used for studying patient's response to specific therapeutic agents, and to give novel insights on drug-resistance, and therefore, lead to the optimization of drug doses.

2. Materials and methods

2.1. Cell lines and cell culture

HT-29 cells, both wild type (WT) and doxorubicin-resistant (DX), were grown at 37 °C, 5% CO_2 and 95% air in RPMI 1640 media with L-Glutamine (EuroClone), supplemented with 10% heat inactivated fetal bovine serum (Gibco by Life Technologies) and 1% Penicillin-Streptomycin (Gibco by Life Technologies). The DX subline was produced by a stepwise selection in culture medium with increasing amount of doxorubicin [22]. To maintain the resistant phenotype, cells were cultured in a medium containing 150 nM doxorubicin.

2.2. Cells seeding into 48-well plates with electrodes

Sterile 48-well plates, with interdigitated gold electrodes at the bottom of the culture chamber, were designed and produced by Eltek S.p.A. A preliminary adhesion test was performed by comparing cells adhesion directly to the plastic and to collagen pre-coated wells. Since a better adhesion, and therefore, a better performance of the system was observed with the pre-coated wells, a preconditioning coating step was executed before cells seeding for drug screening tests. Briefly, wells were coated with 10 µg/mL type I Collagen (C7661 from rat tail, Sigma). Impedance measurement was started immediately following cell seeding. Cells plated in conventional 48-well plates served as controls (plating test in Figure S1 in supplementary material).

2.3. Impedance measurement, data acquisition and processing

For impedance measurement, a customized closed system, containing electronic circuits for impedance measurements, was developed by Eltek S.p.A, for use inside incubators (Figure 1). The system is composed of a lid with pins that interface directly with the contact pads of the 48-well plate, integrated electronics for impedance measuring and multiplexing, and a USB cable.
Interdigitated gold electrodes were fabricated through serigraphy on a glass substrate and cover around the 50% of the well surface. Configuration and dimensions of the electrodes are shown in Figure 1. For all tests, a 7-minute interval measurement was set and data was directly transmitted through a serial port to a computer. Data post-processing consisted of an impedance normalization to a culture medium calibration curve. Normalization was performed as in [23, 24]. Normalized impedance (NI) was calculated using the formula in Eq. 1, where Z_m corresponds to the impedance magnitude of culture medium in the absence of cells. After NI computing further normalization of curves to the starting point $Z_{(t0)}$ was performed to allow comparison and statistical analysis as in [25].

$$NI = \frac{|Z_{cell}| - |Z_m|}{|Z_m|}$$

2.4. Quantitative Real-Time PCR (qRT-PCR)

RNA was extracted using the PureLink RNA kit (Ambion, Thermo Fisher Scientific, Italy) and cDNA prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Italy). Target gene expression was analyzed by qRT-PCR and normalized to endogenous 18s expression as previously described [26]. Primers used for PCR are listed in supplementary Table 1.

2.5. Statistical analysis

Results of the qRT-PCR correspond to biological triplicates (n=3). Comparison between WT and DX was done using an unpaired *t* test with GraphPad Prism 8® software. Statistically significant p-values are presented as: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

The impedance curves shown correspond to the mean measurement of four wells (n=4). The tests were performed independently at least twice. Data processing of each test and zero-lag correlation, cosine similarity and Pearson correlation coefficient calculation, for comparison between them was perform in MATLAB R2018b®.

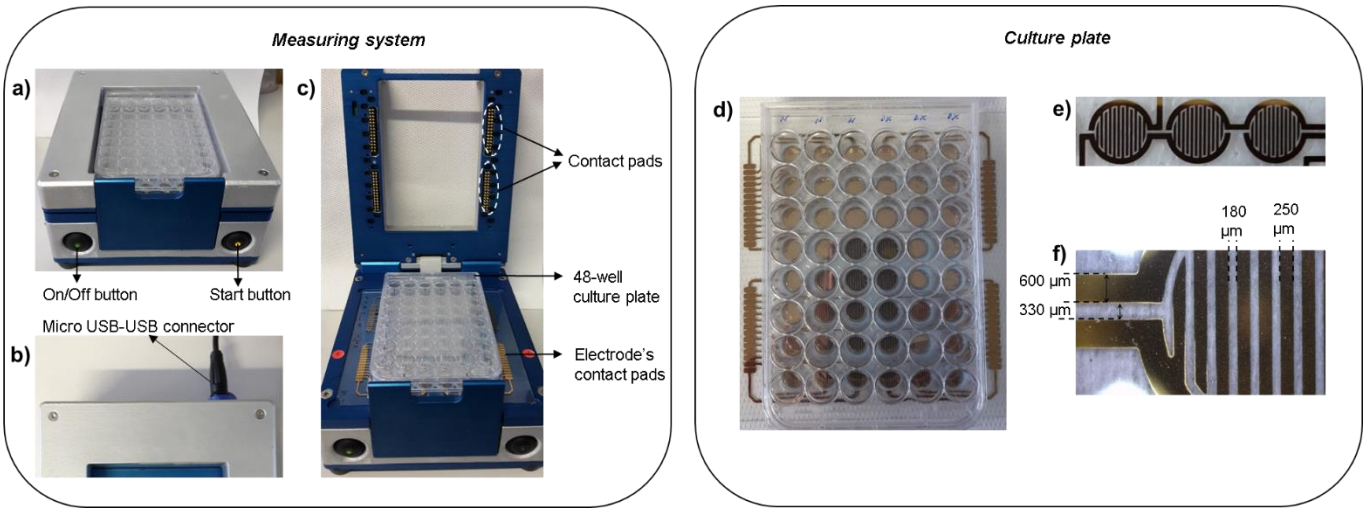

Figure 1: Cell impedance measurement system and culture plate overview. a) Closed device with frontal panel buttons. b) Micro USB-USB connector for data acquisition. c) Open device with 48-well culture plate. d) 48-well plate with interdigitated gold electrodes at the bottom of each well for impedance sensing. e) Interdigitated gold electrodes geometry. f) Electrode's dimensions.

3. Results

3.1. Long-term impedance monitoring curves of WT and DX cells

Two different HT-29 cell concentrations were tested: 4.21×10^4 cells/cm^2 and 2.10×10^4 cells/cm^2. Impedance measurement was performed for 140 hours with a culture medium change at 72 hours. The impedance curves displayed an expected sigmoid pattern. In Figure 2, the stages of adherent cells in vitro were observed: lag phase corresponded to adaptation time and cell adhesion, log phase or exponential population increase, plateau due to contact inhibition, and cell detachment and death due to confluence. [27, 28].

The initial steep decrease in impedance, as observed in raw data of no-cell controls, is attributed to temperature change upon incubation at 37 °C (Figure S2 in supplementary material). As reported also by [29], the spike is characteristic of a culture media change that modifies temperature, pH and dissolved CO_2 values.

The result of a preliminary impedance adhesion test for both phenotypes at the two above-mentioned concentrations are presented in Figure S3. Long-term impedance monitoring curves are shown in Figure 3 along with microscopy images. As expected, for both cell concentrations, after around 24h from seeding, a clear increasing trend is observed due to population doubling. Impedance variation shows a direct correlation to cell density, both in amplitude and slope. There was a significant difference in the impedance curves between the drug resistant and wild type cells, with the latter showing a shorter plateau phase.

Reproducibility tests for both cell lines exhibit a strong correlation with Pearson correlation coefficients higher than 0.96, and values of zero-lag correlation and cosine similarity that back-up the similarity among independent measurements (Table S2 in supplementary material).

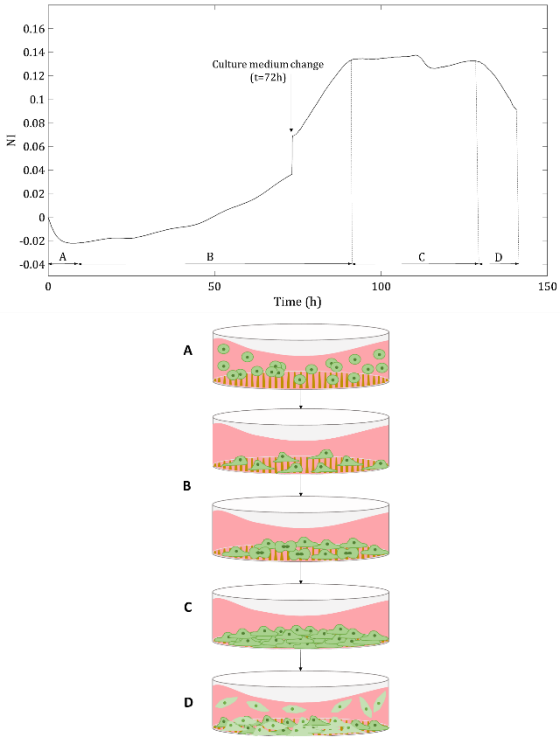

Figure 2: Mean cell impedance monitoring curve for 140h (n=3). Letters correspond to the different phases of the cell culture and are illustrated beneath: A) Lag phase. B) Exponential cell population increase. C) Stationary phase. D) Detachment due to confluence.

3.2. Short-term impedance monitoring curves of WT and DX cells

In order to analyze the trend in impedance after cell seeding, a short-term measurement was performed seeding a high initial cell number (16.84×10 cells/cm^2). Measurement was carried for 40 hours. Mean impedance module and phase values are presented in Figure 4. The main purpose of this measurement was to study the cell impedance response during exponential growth. Initial cell number was calculated in order to analyze the cell layer with an initial estimated surface coverage of the 25%. Clear differences in both module and phase are observed between WT and DX cells.

3.3 Impedance-based cytotoxicity test shows quantitative differences among chemo-sensitive and chemo-resistant cells

For these tests, cytotoxic effects of doxorubicin at different concentrations were analyzed. Cells at a concentration of 3.16×10^4cells/cm^2 were seeded and measurement was performed for 120 hours. Doxorubicin at 7 different concentrations (0 µM, 2.5 µM, 5 µM, 7.5 µM, 10 µM, 12.5 µM, 15 µM) was administered after 48h of seeding. Exposure time to the drug was 72 hours (Figure 5). Impedance curves quantitatively show the cytotoxic effect of doxorubicin. For all concentrations, WT curves exhibit a very small or nonexistent increase, while DX curves at low doxorubicin concentrations increase. Further quantitative analysis was done through drug-response curves at 72 hours of drug exposure (Figure 6).

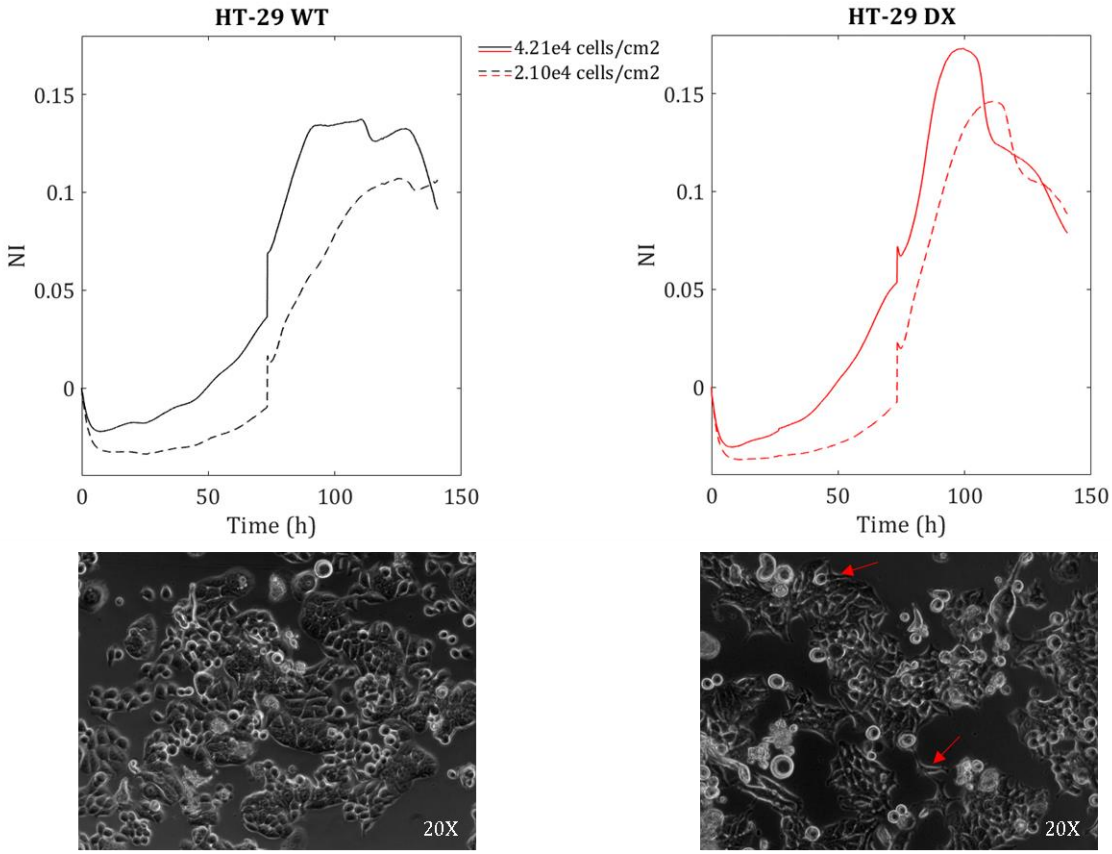

Figure 3: Long-term cell culture monitoring through impedance. Impedance magnitude of HT-29 WT and DX for two different cell seeding densities and microscopy images 20X of the lowest cell concentration at 72h from seeding. Red arrows outline a higher degree of cell spreading in DX cells.

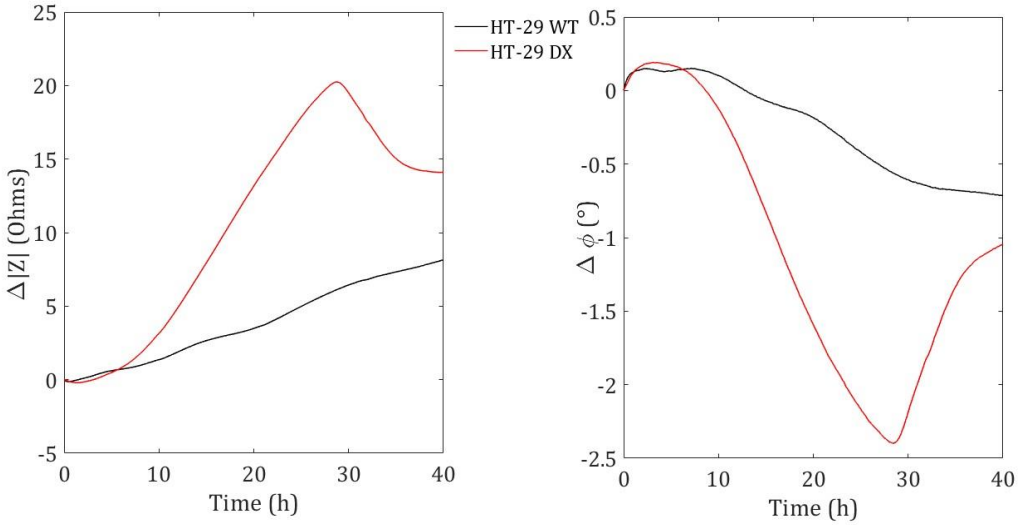

Figure 4: Impedance module and phase variation of high density chemo-sensitive and chemo-resistant cells during exponential growth.

3.4. DX cells show higher levels of drug transporters and epithelial to mesenchymal transition (EMT) markers versus WT HT-29 cells

Significant differences among chemo-sensitive and chemo- resistant cells were found in the quantitative analysis of gene expression. Thirteen different genes were analyzed (Figure S4 in supplementary material). In Figure 7, the results of selected genes, MRP1, MRP2, ABCG2, i.e. three ABC trans- porters expressed in HT-29/DX cells [30], and Vimentin, LGR5 and Snail, three modulators of EMT.

As expected, the drug resistant phenotype showed a considerably higher expression of drug transporters MRP1, MRP2 and ABCG2. DX cells also showed an enhanced mesenchymal gene expression (Vimentin, LGR, Snail) that could be related to an EMT process, that could partially explain the impedance-based results described above.

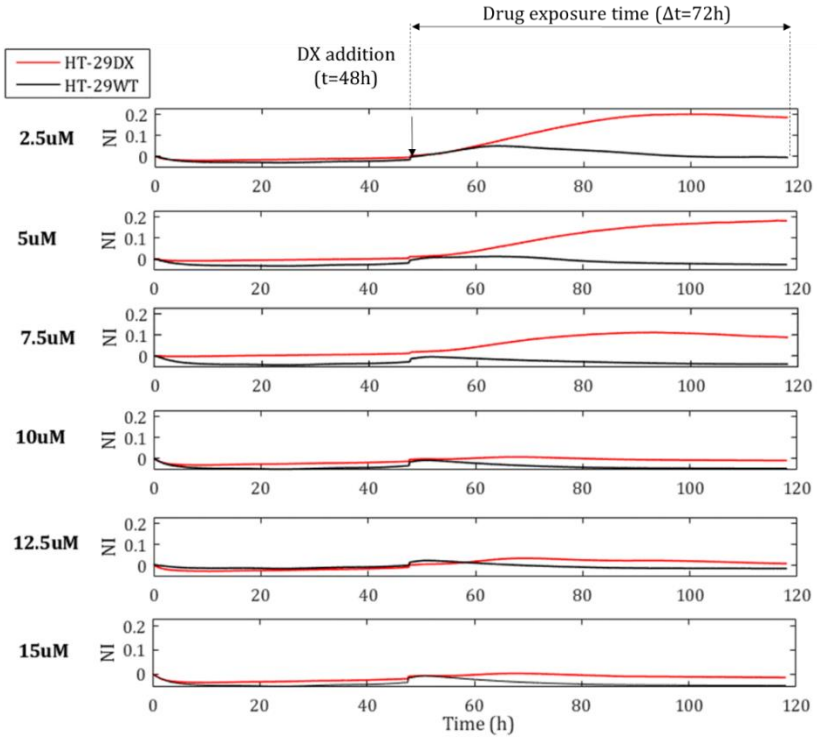

Figure 5: Impedance monitoring curves for different Doxorubicin concentrations. Doxorubicin was administrated after 48h for an exposure time of 72h.

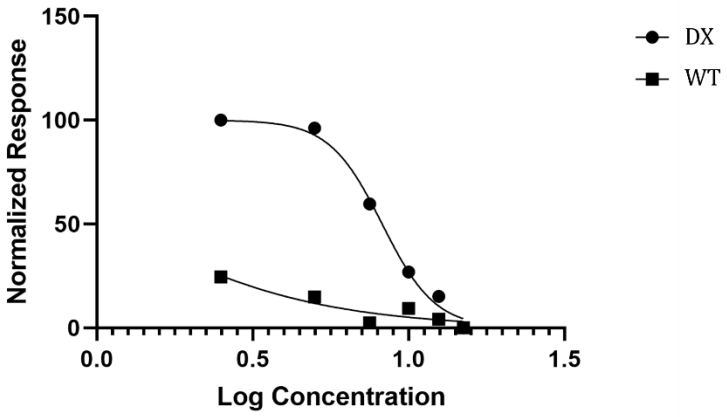

Figure 6: Drug-response curves of DX and WT cells at 72 hours of doxorubicin treatment.

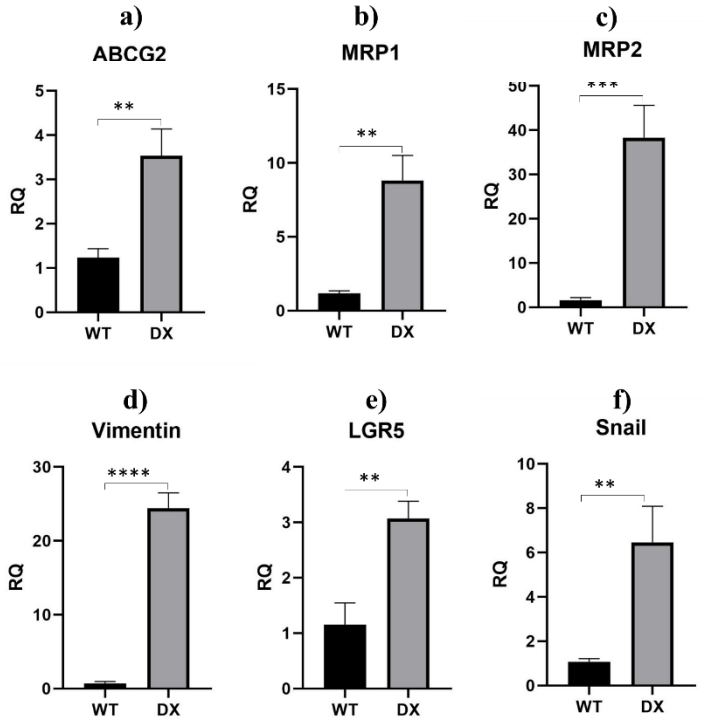

Figure 7: Quantitative PCR results for HT-29 WT and HT-29 DX. a) ABCG2 b) MRP1 c) MRP2 d) Vimentin e) LGR5 f) Snail.

4. Discussion

Multidrug resistance is the main cause of chemotherapy failure and one of the biggest challenges in cancer therapy. Drug resistance is usually diagnosed after a long period of drug treatment, and early assays for diagnosing it, that could optimize the therapy, opening the door to personalized treatment, are scarce [5]. On the other hand, impedance- based cell monitoring presents the great advantage of being a versatile, label free and non-invasive technique, with lower costs compared to traditional in-vitro culture tests. Impedance measurements have been broadly used for real-time monitoring and drug cytotoxicity screening.

Bioimpedance sensing has been studied for cell characterization and as a method to identify cell phenotypes. Srinivasaraghavan et al. were able to distinguish basal and claudin-low sub-types of triple negative breast cancer cells through impedance spectroscopy, using peak frequency and peak phase angle, and obtaining electrical parameters (R and C) of a cell layer under high density conditions [23]. Park et al. also reported impedance spectroscopy for discrimination between normal and cancerous urothelial cell lines. Their measurement was based on single cell trapping and flow cytometry [31].

As a first approach for testing the importance of impedance sensing in detecting drug resistance in cancer cells, we used HT-29 WT and DX cells, and as an initial validation of the system, a traditional impedance-based cytotoxicity test was performed with doxorubicin. A coherent response to the drug was obtained for WT and DX cells. For low doxorubicin concentrations, DX cells curves exhibit a clear increase despite the presence of the drug (Figure 5). Through this tests it is also possible to determine which minimal dose has a cytotoxic effect on the cells. 5 µM doxorubicin is usually a concentration that is cytotoxic in sensitive cells as HT-29 WT, but not in resistant ones as HT-29 DX cells [22]. Higher concentrations - from 10 µM to 25 µM - are progressively more toxic also in resistant cells, according to the common cytotoxicity assays performed in vitro [32]. Our system well reproduces these observations. A better comparison can be established with the half maximal inhibitory concentration (IC_{50}) calculated from the drug-response curve (Figure 6). At 72 hours of exposure to doxorubicin, IC_{50} value for DX cells was 8.23, while the same parameter for WT cells

was 1.09. Through this type of analysis, it is possible to perform a quantitative comparison between chemo-sensitive and chemo-resistant cells solely by means of culture monitoring.

Furthermore, impedance-based cytotoxicity assays offer the possibility to perform the continuous follow up of the cells in the presence of fluorescent drugs or molecules, which is the case of doxorubicin. Traditional colorimetric in vitro cytotoxicity and viability assays are affected by the fluorescent interference of test compounds. Background interference due to inclusion of particles is not always easy to subtract from tests. In fact, in our setting, luminescent viability assay to determine doxorubicin cytotoxicity in DX cells did not give satisfactory results.

We obtained impedance curves from the drug resistant phenotype that differ in morphology compared to the drug-sensitive ones. As shown in Figure 3, the plateau phase in DX cells was significantly shorter, suggesting a higher migratory tendency. This phenomenon could be related to their enhanced mesenchymal gene expression that correlates to EMT processes as shown by our data, and that affects adhesive and migratory characteristics of cells [33]. EMT is the mechanism by which epithelial-derived cells lose their epithelial characteristics and acquire migratory mesenchymal properties. It is a phenomenon that directly affects adhesive and migratory processes. EMT has been identified to occur during three processes: organ development, wound healing and cancer metastasis [33, 34]. Migratory tendency due to an EMT process in DX cells is supported by PCR results: the mesenchymal marker, Vimentin, as well as the EMT signaling molecule, Snail, are highly expressed with respect to WT cells. In agreement with our data, several studies have found Snail-induced EMT in colorectal carcinoma [33].

Other features that could contribute to the difference in the curve's morphology are: the higher level of cell spreading observed in DX cells (outlined by the arrows in the microscopy image of Figure 3) and the difference in lipid membrane composition [35]. Both characteristics are better observed in the short-term impedance measurement in which a greater variation both in module and phase is observed in DX cells (Figure 4).

The presented impedance measurement system was designed with a translational approach for a simplified and easy scalability. It exploits ECIS for a continuous follow-up of cells and it is meant for cell layer measurement of adherent cells in contrast to single cell approaches. Single-cell assays could give more precise information about the cell but are laborious, time consuming and have a low cost-effectiveness ratio, limiting its use in clinical applications. The fact that the ECIS system employed in the current work is based on a standard 48-well plate allows high throughput monitoring of different drug concentrations and the easy application of standard protocols.

5. Conclusions

We successfully validated the custom-made ECIS system for the real-time monitoring of colon cancer cells and screening for doxorubicin resistance, optimizing culture conditions for high throughput measurements. We proved that impedance response of WT and DX phenotypes of HT-29 cells are different due to distinct adhesive and migratory characteristics.

Since morphological and behavioral differences are observed early during chemoresistance acquisition, the use of impedance for cell culture monitoring represents a promising technique for patients-derived cell analysis. Further work should address the measurement of other resistant cell lines and patient-derived cells. Thereby, the presented system and methodology has the potential to become a novel non-invasive technique for drug-resistance identification that allows also the continuous follow up of the cell culture under normal in- vitro conditions. The system could be further used with other adherent cell types and including impedance-phase data might give additional information. The presented work is a fundamental step towards a final goal of a point-of-care device that will enable the practice of personalized medicine approaches.

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Competing interests

Gallo Valentina and Pizzi Marco are employees of ELTEK S.p.A and developed the impedance measurement system and the multi-well plates with electrodes, in their plant, with the support of ELTEK funding. Both authors declare that their affiliation did not influence the presented work.
The other authors have no competing personal or financial interests that could have influenced the presented work.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at Link

CRediT authorship contribution statement

Fuentes-Vélez Susana: Investigation, Formal analysis, Validation, Writing-Original draft. **Fagoonee Sharmila:** Conceptualization, Methodology, Investigation, Writing-review and editing. **Sanginario Alessandro:** Visualization, Writing- review and editing. **Gallo Valentina:** Resources, Investigation, Software. **Riganti Chiara:** Resources. **Pizzi Marco:** Conceptualization, Resources, Project administration, Supervision. **Altruda Fiorella:** Resources, Supervision, Project administration, Funding acquisition. **Demarchi Danilo:** Re- sources, Supervision, Project administration, Funding acquisition.

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