

SYSTEMS AND METHODS FOR ELECTRONICALLY AND OPTICALLY MONITORING BIOLOGICAL SAMPLES

[001] This application claims benefit of priority to US patent application no. 16/833,651 filed March 29, 2020, the entire content of which is herein incorporated by reference in its entirety.

TECHNICAL FIELD

[002] The invention relates generally to systems and methods for electronically and optically monitoring biological samples and more specifically to systems and methods that electronically and optically monitor a same biological sample continuously in real time in a single well.

BACKGROUND OF THE INVENTION

[003] Cell based assays provide a preliminary evaluation of the effects of therapeutics on human biology. While many cell-based assays are endpoint assays, which are limited to a single point in time, a technology known as cell-substrate impedance monitoring permits continuous monitoring of cells. Cell-substrate impedance monitoring assesses the interaction between cells and electrodes, where changes in cell attachment, growth, morphology and motility over electrodes results in a detectable change. To this end, cell-substrate impedance monitoring is a powerful tool to assess cell proliferation and cytolysis.

[004] While cell-substrate impedance monitoring technology can reveal the kinetics of cell responses to potential therapeutics, it has its limitations. In particular, it is limited to detecting changes that occur as cells are cultured on the electrode surface. Therefore, there is a need to develop further advances that expand the ability of cell-substrate impedance monitoring to evaluate the effects of therapeutics on cell biology.

BRIEF SUMMARY OF THE INVENTION

[005] In one aspect of the invention, a system for electronically and optically monitoring biological samples is provided, the system including: a multi-well plate having a

plurality of wells configured to receive a plurality of biological samples, each of the wells having a set of electrodes and a transparent window on a bottom surface of the well that is free of electrodes; an illumination module configured to illuminate the wells; a cradle configured to receive the multi-well plate, the cradle having an opening on the bottom that is configured to expose the transparent windows of the wells; and an optical imaging module movable across different wells of a same multi-well plate to capture images through the windows via the open bottom. In some embodiments the set of electrodes are configured for monitoring cell-substrate impedance.

[006] Although the illumination module can be a single light, preferably the illumination module includes a plurality of lights configured to independently illuminate one or more of the wells. More preferably, the illumination module includes a light emitting diode (LED) array. Most preferably, each LED is arranged to illuminate a single well. In preferred embodiments, the illumination module is a bright field illumination module.

[007] The cradle is configured to receive and preferably cover the multi-well plate and thus can provide a hinged cover. Preferably the illumination module is joined to the inner surface of the cover. The cradle can include a contact sensor that senses receipt of the plate. In some embodiments, the cradle electronically engages both the multi-well plate for electronic communication with the sets of electrodes and the illumination module for communicating illumination instructions.

[008] Since the open bottom of the cradle exposes the transparent windows of the wells, the optical imaging module can be positioned underneath the cradle and configured to move from well to well to capture images through corresponding transparent windows. In some embodiments, the optical imaging module is configured to capture one or more images from a single well at a time. In some embodiments, images are captured under bright field illumination of well(s), such as for determining cell counts or a cell confluence parameter (e.g. percent confluence). In other embodiments, images, such as fluorescence images, are captured after molecule excitation. The optical imaging system can itself include an excitation light source configured to excite one or more molecules, such as fluorescent molecules conjugated to antibodies or antibody fragments for binding to biological molecules such as proteins, polypeptides or nucleic acids, inside cells or on cell surfaces. Examples of excitation light sources can include one or more lights selected from the group consisting of an ultraviolet light, a violet light, a blue light, a green light, a yellow light, an orange light, and a red light.

[009] In some embodiments, the optical imaging module includes a camera capable of

capturing a series of cell images consecutively at sufficiently short time intervals for showing cell motion. In some embodiments the camera captures at least 30 images per second. In other embodiments the camera captures at least 60 images per second. In either or both embodiments, the optical imaging module can include the camera, a bandpass filter, a tube lens, and an objective lens. High-speed imaging not only permits cell movement to be shown visually but also permits superimposing images from different light sources (e.g. bright field illumination and multiple light sources of different wavelengths each for exciting fluorescent molecules to produce a single-color fluorescence image) revealing cell changes occurring during movement. In some embodiments, the captured images from bright field illumination and three or more color fluorescence are superimposed to form a single image for each time point. From this imaging, a cell count or a cell parameter can be determined as well as absence or presence of fluorescence, total fluorescence intensity, and average fluorescence intensity, which further reveals detailed cell kinetics.

[0010] The system can also include or be coupled with a computer processor communicatively coupled to: the cradle for selectively operating each of the sets of electrodes, optionally for electronically monitoring cell-substrate impedance within one or more wells; the illumination module for selectively illuminating the one or more wells; and the optical imaging module for its selective movement and capturing and receiving images from the one or more wells. In some embodiments, the computer processor is programmed to capture images from the one or more wells via the optical imaging module in response to the one or more wells reaching or following a set impedance-based value or parameter from the electronic monitoring. In some embodiments, the computer processor is configured to electronically monitor cell-substrate impedance and optically monitor a same well and is configured to pair impedance and optical data for display or analysis. In some embodiments, the computer processor is configured to electronically monitor cell-substrate impedance over a time period including a specific time interval between two consecutive electronic impedance measurements/monitoring; and optically monitor a same well over a period that is within the time period for electronic monitoring or over a different time period from electronic monitoring. Exemplary impedance monitoring time periods can be programmed to be as short as minutes to as long as hours, days or even weeks. Impedance monitoring time interval(s) between two consecutive impedance measurements can be specified or programmed as short as seconds or less than a second, to as long as one-minute, multiple minutes, one hour even multiple hours. Optical monitoring periods can be programmed inside, outside or over the same time period as the one or more impedance monitoring time

period.

[0011] While the system can be used with a single plate, the system can also include two additional multi-well plates, each having a plurality of wells configured to receive a plurality of samples, each of the wells having a set of electrodes and a transparent window on a bottom surface of the well that is free of electrodes; two additional illumination modules configured to illuminate the wells of the two additional multi-well plates; and two additional cradles configured to receive the two additional multi-well plates, the two additional cradles each having an open bottom that exposes the transparent windows of the wells of two additional multi-well plates; and where the optical imaging module is movable across all wells to capture images through all windows via the exposed bottoms.

[0012] The system can also include a cell or tissue culture vessel that is not configured for electronic monitoring, where the optical imaging module is configured to capture images within the cell or tissue culture vessel. As an exemplary embodiment, the system can include a multi-well plate having a plurality of wells configured to receive a plurality of biological samples, each of the wells having a transparent bottom surface; an illumination module configured to illuminate the wells; a cradle configured to receive the multi-well plate, the cradle having an opening on the bottom that is configured to expose all of the transparent bottom surfaces of the wells; and an optical imaging module movable across different wells of a same multi-well plate to capture images through the windows via the exposed bottoms.

[0013] In a related aspect of the invention, a method of monitoring cells is provided, which includes electronically monitoring cells within wells of a multi-well plate, each of the wells having a set of cell-substrate impedance monitoring electrodes, and a transparent window on a bottom surface of the well that is free of electrodes; and capturing images through the transparent window from at least one well that is being electronically monitored. In some embodiments, the images are captured regularly at a fixed time interval between two consecutive image capturing or irregularly over a time period within the electronic monitoring time period, the method optionally including capturing the images at a same time interval between two consecutive image capturing as the time interval between two consecutive electronic measurements of the cells.

[0014] In some embodiments, prior to the step of capturing images from the at least one well, the electronic monitoring outputs a result from the at least one well that meets a set value, which instructs the optical imaging module to capture the images from the at least one well. Examples of set values can include predetermined impedance-based values.

[0015] In some embodiments, the images being captured are bright field images of the cells. In such embodiments the method can also include counting cells from the bright field images and optionally deriving cell confluence numbers or parameters (e.g. percent confluence) from the bright field images.

[0016] In other embodiments, the captured images include fluorescence images of the cells. In such embodiments, the method can include determining a fluorescence parameter from the images, optionally selected from one or more of the group consisting of total fluorescence counts, total fluorescence intensity, and average fluorescence intensity. The fluorescence parameters are calculated or determined separately for fluorescent images of each color, for example, blue, green and red colors.

[0017] In still other embodiments, the images being captured include bright field images of the cells and fluorescence images of the cells. In such embodiments, the method also includes deriving cell confluence numbers or parameters from the bright field images and optionally counting cells from the bright field images; determining a fluorescence parameter from the fluorescence images, optionally selected from one or more of the group consisting of total fluorescence counts, total fluorescence intensity, and average fluorescence intensity; and optionally, superimposing the bright field images and fluorescence images of one or more colors for one or more of the wells.

[0018] In a related aspect, a method of monitoring cells is provided, which includes electronically monitoring cells within wells of a multi-well plate over a time period, each of the wells having a set of cell-substrate impedance monitoring electrodes, and a transparent window on a bottom surface of the well that is free of electrodes; and capturing images through the transparent window over a time period that is within or outside of the time period for electronic monitoring. The time period used for electronic monitoring may be the same as the time period used for image capturing, or different from that used for image capturing. These time periods for electronic monitoring and optical monitoring can be specified or programmed as short as less than one minute, to as long as hours, days or even weeks. Over the time period of electronic monitoring, electronic monitoring can be continuous at a specified, fixed time interval between two consecutive electronic measurements. Optic monitoring can be continuous over the time period at a specified, fixed time interval between two consecutive image capturing. These time intervals can be specified or programmed as short as seconds or less than a second, to as long as one minute, multiple minutes, one hour even multiple hours.

[0019] In some embodiments, the images being captured are bright field images of the

cells. In such embodiments the method can also include counting cells from the bright field images and optionally deriving cell confluence numbers or parameters (e.g. percent confluence) from the bright field images.

[0020] In other embodiments, the captured images include fluorescence images of the cells. In such embodiments, the method can include determining a fluorescence parameter from the images, optionally selected from one or more of the group consisting of total fluorescence counts, total fluorescence intensity, and average fluorescence intensity. The fluorescence parameters are calculated or determined separately for fluorescent images of each color, for example, blue, green and red colors.

[0021] In still other embodiments, the images being captured include bright field images of the cells and fluorescence images of the cells. In such embodiments, the method also includes deriving cell confluence numbers or parameters from the bright field images and optionally counting cells from the bright field images; determining a fluorescence parameter from the fluorescence images, optionally selected from one or more of the group consisting of total fluorescence counts, total fluorescence intensity, and average fluorescence intensity; and optionally, superimposing the bright field images and fluorescence images of one or more colors for one or more of the wells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] FIG. 1A is a photograph of an exemplary system 10 for electronically and optically monitoring biological samples, where an optional user interface 200 is displaying cell imaging data. FIG. 1B depicts the primary housing 12 with five accessible cradles 14.

[0023] FIG. 2A shows the cradle 14 in a closed configuration. FIG. 2B shows a cradle 14 in an open configuration. FIG. 2C shows a dual cradle 14 in open and closed positions.

[0024] FIG. 3 shows a bright field illumination module 26 joined to a cradle cover 14.

[0025] FIG. 4 depicts an exemplary multi-well plate 20.

[0026] FIG. 5 is a photograph showing: a schematic of a set of electrodes 42 adapted for use with a centrally positioned transparent window 44 (Panel A); a photograph of a well 32 from a multi-well plate 20 (see FIG. 4) having a set of electrodes 42 on a bottom surface and a transparent window 44 that is free of electrodes 42 on the bottom surface (Panel B); and a captured image of cells through a transparent window 44 (Panel C).

[0027] FIG. 6 shows an exemplary optical imaging module 26.

[0028] FIG. 7 is a cross-section of an exemplary optical imaging module 26.

[0029] FIG. 8 shows an exemplary optical imaging support 60 for the imaging module 26 of FIG. 6, that allows for two-dimensional linear motions (including motion along an axis parallel to the linear track 66 and along another axis parallel to the linear track 70).

[0030] FIG. 9A is an impedance-based graph over a time interval after treating MCF7 breast cancer cells transfected lentivirus expressing a red fluorescent protein, with NK92 cells at different Effector:Target (E:T) ratios over time. FIG. 9B provides red fluorescent cell count (indicating live target cells) from the same assay over time. FIGS. 9C-E are images captured under bright field illumination within the same well(s) having an E:T ratio of 2.5:1 before NK92 cell addition (left), 12 hrs. after NK92 cell addition (middle), and 30 hrs after NK92 cell addition.

[0031] FIG. 10 shows exemplary captured and superimposed images, where the left panel superimposes three captured fluorescent images (red, green and blue) with a captured bright field illumination image, and the right panel superimposes three captured fluorescent images (red, green and blue) without bright field illumination. Shown are cells exposed to fluorescence markers for Annexin V (red), caspase 3 (green), cell nuclei (blue).

[0032] FIGS. 11A-B are graphs depicting cell-substrate impedance monitoring over a time period to demonstrate drug-induced apoptosis in real-time. A549-Blue cells were titrated with either MG132 (FIG. 11A) or staurosporine (FIG. 11B). Negative control was DMSO. Error bars represent the standard deviation from samples run in triplicate. Although impedance was continuously measured at a time interval once every 15 minutes, to prevent the error bars from adjacent time points from overlapping, here data points are only shown once per hour.

[0033] FIGS. 12A-C provide a graph plotting impedance monitoring over a time period in combination with live cell imaging at different time points within the impedance monitoring time period. The impedance signal and the number of blue nuclei were tracked after treating A549-Blue cells with either DMSO (FIG. 12A), 50 μ M MG132 (FIG. 12B), or 1 μ M staurosporine (FIG. 12C). Representative images are shown for pretreatment and 1 or 20 hours after treatment. Error bars represent the standard deviation from samples run in triplicate.

[0034] FIG. 13 provides an impedance-based plot before and after treatment with 5.5 μ M MG1322. Also shown are images taken 0 hour after treatment, 20 hours after treatment, and 40 hours after treatment. Top images are each taken under bright field illumination and the bottom images are taken under fluorescence, with Annexin V staining (red), activated caspase 3 (green), and nuclear-localized BFP (blue fluorescent protein). Arrows denote large membrane

blebs that contain phosphatidylserine in their outer leaflet.

[0035] FIGS. 14A-D provide graphs showing image-based continuous tracking MG132- induced apoptosis in A549-Blue cells using number of blue nuclei (FIG. 14A), % confluence (FIG. 14B), number of green (caspase 3 activated) cells (FIG. 14C), and number of red (annexinV-bound) cells (FIG. 14D). Error bars represent the standard deviation from samples run in triplicate.

[0036] FIG. 15 is a graph showing comparing the relative rate and relative abundance of different apoptotic phenomena for cells treated with 50 μ M MG132. Error bars represent the standard deviation from samples run in triplicate.

[0037] FIG. 16 provides a table and graphs showing the use of dose response curves to calculate EC50. Using real-time impedance (as represented by normalized cell index) data (FIG. 11A) and live cell imaging (green cell number, red cell number and blue nuclei number) data (FIGS. 14A-C), area under the curve was calculated, and is plotted here as a function of MG132 concentration. Data were fit to a four-parameter logistic equation to determine the EC50.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0038] The systems and methods described herein enable cell health and behavior to be monitored continuously, and in the same well, from the very different perspectives, namely, from real-time electronic monitoring and live cell imaging. The streamlined workflow, high reproducibility, and quantitative kinetics of the system makes it ideal for a wide range of cell-based assays, including but not limited to, cell health monitoring, proliferation, cytotoxicity, apoptosis, immune cell killing, cell receptor activation, cell differentiation including stem cell differentiation.

[0039] The continuous nature of the systems and methods have two major advantages. In contrast to endpoint assays that provide mere snapshots of a process, real-time tracking ensures that important phenomena do not get missed. Secondly, the continuous nature of the technical approach dramatically reduces that amount of hands-on time required to run an assay. Once cells have been seeded and any treatments added, no further involvement is necessary.

[0040] The systems and methods are preferably used to assess biological samples or effects on cells. The cells can be primary cells isolated from any species or can be cells of cell lines. The cells can be genetically engineered cells. For example, these include cells from a genetically modified organism, such as for example from a "gene knockout" organism, or cells

that have been engineered to over-express an endogenous gene or a transgene, or cells whose normal gene expression has been manipulated (e.g. by use of antisense molecules or silencing RNA.), cells that have been modified by CRISPR and/or other gene editing technologies, or cells that have been engineered to express therapeutic proteins such as CHO cells.

[0041] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications referred to herein are incorporated by reference in their entirety. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

[0042] As used herein, “a” or “an” means “at least one” or “one or more.”

[0043] An “electrode” is a structure having a high electrical conductivity, that is, an electrical conductivity much higher than the electrical conductivity of the surrounding materials.

[0044] As used herein, an “electrode structure” refers to a single electrode, particularly one with a complex structure (as, for example, a spiral electrode structure), or a collection of at least two electrode elements that are electrically connected together. All the electrode elements within an “electrode structure” are electrically connected.

[0045] As used herein, “electrode element” refers to a single structural feature of an electrode structure, such as, for example, a fingerlike projection of an interdigitated electrode structure.

[0046] As used herein, an “electrode array” or “electrode structure unit” is two or more electrode structures that are constructed to have dimensions and spacing such that they can, when connected to a signal source, operate as a unit to generate an electrical field in the region of spaces around the electrode structures. Preferred electrode structure units of the present invention can measure impedance changes due to cell attachment to an electrode surface. Non-limiting examples of electrode structure units are interdigitated electrode structure units and concentric electrode structure units. Additional examples of electrode structures may also include a pair of a small measurement/recording electrode (e.g. microelectrode of circular shape having a diameter between a size as small as less than 10 microns and a size larger as large as 100, or a few hundred microns), and a much larger reference electrode. Multiple small recording electrodes – forming a microelectrode array, may share a common reference

electrode. Such recording electrodes can be used for conducting extracellular recording, by amplifying and recording electrical voltage signals between small recording electrodes and much larger reference electrodes. In extracellular recording embodiments, an extracellular recording system (including voltage signal amplifier and other electronic hardware circuitry for measuring electrical voltage plus signal processing algorithm implemented in software and/or firmware) is used rather than an impedance measurement system (e.g., impedance analyzer system, including electronic hardware circuitry measuring electrical current and voltage, and signal and data processing algorithm in software and/or firmware).

[0047] As used herein, an "electrode bus" is a portion of an electrode that connects individual electrode elements or substructures. An electrode bus provides a common conduction path from individual electrode elements or individual electrode substructures to another electric connection. In the devices of the present invention, an electrode bus can contact each electrode element of an electrode structure and provide an electrical connection path to electrical traces that lead to a connection pad.

[0048] As used herein, "electrode traces" or "electrically conductive traces" or "electrical traces", are electrically conductive paths that extend from electrodes or electrode elements or electrode structures toward one end or boundary of a device or apparatus for connecting the electrodes or electrode elements or electrode structures to an impedance analyzer. The end or boundary of a device may correspond to the connection pads on the device or apparatus.

[0049] As used herein, a "connection pad" is an area on an apparatus or a device (e.g., multi-well plate), which is electrically connected to at least one electrode or all electrode elements within at least one electrode structure on an apparatus or a device and which can be operatively connected to external electrical circuits (e.g., an impedance measurement circuit or a signal source). The electrical connection between a connection pad and an impedance measurement circuit or a signal source can be direct or indirect, through any appropriate electrical conduction means such as leads or wires. Such electrical conduction means may also go through electrode or electrical conduction paths located on other regions of the apparatus or device.

[0050] As used herein, "interdigitated" means having projections coming one direction that interlace with projections coming from a different direction in the manner of the fingers of folded hands (with the caveat that interdigitated electrode elements preferably do not contact one another).

[0051] As used herein, “electrodes (or electrode structures) have substantially the same surface area” means that the surface areas of the electrodes referred to are not substantially different from each other, so that the impedance change due to cell attachment or growth on any one of the electrodes (or electrode structures) referred to will contribute to the overall detectable change in impedance to a same or similar degree as the impedance change due to cell attachment or growth on any other of the electrodes (or electrode structures) referred to. In other words, where electrodes (or electrode structures) have substantially the same surface area, any one of the electrodes can contribute to overall change in impedance upon cell attachment or growth on the electrode. In most cases, the ratio of surface area between the largest electrode and the smallest electrode that have “substantially the same surface area” is less than 10.

[0052] As used herein, “detectable change in impedance between or among electrodes” (or “detectable change in impedance between or among electrode structures”) means that the impedance between or among said electrodes (or electrode structures) would have a significant change that can be detected by an impedance analyzer or impedance measurement circuit when molecule binding reaction occurs on the electrode surfaces. The impedance change refers to the difference in impedance values when cells are attached to the electrode surface and when cells are not attached to the electrode surface, or when the number, type, activity, adhesiveness, or morphology of cells attached to the electrode-comprising surface of the apparatus changes. In most cases, the change in impedance is larger than 0.1% to be detectable. Preferably, the detectable change in impedance is larger than 1%, 2%, 5%, or 8%. More preferably, the detectable change in impedance is larger than 10%. Impedance between or among electrodes is typically a function of the frequency of the applied electric field for measurement. “Detectable change in impedance between or among electrodes” does not require the impedance change at all frequencies being detectable. “Detectable change in impedance between or among electrodes” only requires a detectable change in impedance at any single frequency (or multiple frequencies). In addition, impedance has two components, resistance and reactance (reactance can be divided into two categories, capacitive reactance and inductive reactance). “Detectable change in impedance between or among electrodes” requires only that either one of resistance and reactance has a detectable change at any single frequency or multiple frequencies. In the present disclosure, impedance is the electrical or electronic impedance. The method for the measurement of such impedance is achieved by, (1) applying a voltage between or among electrodes at a given frequency (or multiple frequencies, or having specific voltage waveform) and monitoring the electrical current through said electrodes at the frequency (or multiple

frequencies, or having specific waveform), dividing the voltage amplitude value by the current amplitude value to derive the impedance value; (2) applying an electric current of a single frequency component (or multiple frequencies or having specific current wave form) through said electrodes and monitoring the voltage resulted between or among said electrodes at the frequency (or multiple frequencies, or having specific waveform), dividing the voltage amplitude value by the current amplitude value to derive the impedance value; (3) other methods that can measure or determine electric impedance. Note that in the description above of “dividing the voltage amplitude value by the current amplitude value to derive the impedance value”, the “division” is done for the values of current amplitude and voltage amplitude at same frequencies. Measurement of such electric impedance is an electronic or electrical process that does not involve the use of any reagents.

[0053] As used herein, “at least two electrodes have substantially different surface area” means that the surface areas of any electrodes are not similar to each other so that the impedance change due to cell attachment or growth on the larger electrode will not contribute to the overall detectable impedance to a same or similar degree as the impedance change due to cell attachment or growth on the smaller electrodes. Preferably, any impedance change due to cell attachment or growth on the larger electrode (typically referred to as a “counter electrode”) is significantly smaller than the impedance change due to cell attachment or growth on the smaller electrode (typically referred to as a “working electrode” or a “measuring electrode”). Ordinarily, the ratio of surface area between the largest electrode and the smallest electrode is more than 10. Preferably, the ratio of surface area between the largest electrode and the smallest electrode is more than 20, 30, 40, 50 or 100. Examples where “at least two electrodes have substantially different surface area” include the “Electronic Cell-Substrate Impedance Sensing (ECIS)” approach developed by Giaver and Keese.

[0054] As used herein, “arranged in a row-column configuration” means that, in terms of electric connection, the position of an electrode, an electrode array or a switching circuit is identified by both a row position number and a column position number.

[0055] A “Cell Index” or “CI” is a parameter that can be derived from measured impedance values and that can be used to reflect the change in impedance values. There are a number of methods to derive or calculate Cell Index. CI has been explained previously at length, such as in US 8,344,742; US 7,470,533; US 7,192,752; PCT/US03/22557 and elsewhere. Each is herein incorporated by reference in its entirety. A “Normalized Cell Index” at a given time point is calculated by dividing the Cell Index at the time point by the Cell Index

at a reference time point. Thus, the Normalized Cell Index is 1 at the reference time point. "Normalized Cell Index" has been explained previously at length, such as in US 8,344,742; US 7,470,533; US 7,192,752; PCT/US03/22557 and elsewhere. Each is herein incorporated by reference in its entirety. A "delta cell index" at a given time point is calculated by subtracting the cell index at a standard time point from the cell index at the given time point. Thus, the delta cell index is the absolute change in the cell index from an initial time (the standard time point) to the measurement time. "Delta cell index" been explained previously at length, such as in US 8,344,742; US 7,470,533; US 7,192,752; PCT/US03/22557 and elsewhere. Each is herein incorporated by reference in its entirety. A "Cell Change Index" or "CCI" is a parameter derived from Cell Index and "CCI" at a time point is equal to the 1st order derive of the Cell Index with respect to time, divided by the Cell Index at the time point. "CCI" been explained previously at length, such as in US 8,344,742; US 7,470,533; US 7,192,752; PCT/US03/22557 and elsewhere. Each is herein incorporated by reference in its entirety.

[0056] As used herein, "dose-response curve" means the dependent relationship of response of cells on the dose concentration of a test compound. The response of cells can be measured by many different parameters. For example, a test compound is suspected to have cytotoxicity and cause cell death. Then the response of cells can be measured by percentage of non-viable (or viable) cells after the cells are treated by the test compound. Plotting this percentage of non-viable (or viable) cells as a function of the dose concentration of the test compound constructs a dose response curve. In the present application, the percentage of non-viable (or viable) cells can be expressed in terms of measured impedance values, or in terms of cell index derived from impedance measurement, or in terms of cell change indexes. For example, for a give cell type and under specific cellular physiological condition (e.g., a particular cell culture medium), cell index can be shown to have a linear correlation or positive correlation with the number of viable cells in a well from which cell index was derived from the impedance measurement. Thus, in the present application, one can plot cell index as a function of the dose concentration of the test compound to construct a "dose-response curve". Note that, generally, cell index not only correlate with the number of viable cells in the wells but also relate to the cell morphology and cell attachment. Thus plotting cell index versus dose concentration provides information not only about number of cells but also about their physiological status (e.g. cell morphology and cell adhesion). Furthermore, an important advantage offered by the system and devices of the present application is that in a single experiment, one can obtain "dose-response curves" at multiple time points since the system

allows for the continuous monitoring of cells and provides impedance measurement at many time points over a time range as short as a few minutes to as long as days or weeks.

[0057] As used herein, “each well contains substantially same number . . . of cells” means that the lowest number of cells in a well is at least 50% of the highest number of cells in a well. Preferably, the lowest number of cells in a well is at least 60%, 70%, 80%, 90%, 95% or 99% of the highest number of cells in a well. More preferably, each well contains an identical number of cells.

[0058] As used herein, “each well contains . . . same type of cells” means that, for the intended purpose, each well contains same type of cells; it is not necessary that each well contains exactly identical type of cells. For example, if the intended purpose is that each well contains mammalian cells, it is permissible if each well contains same type of mammalian cells, *e.g.*, human cells, or different mammalian cells, *e.g.*, human cells as well as other non-human mammalian cells such as mice, goat or monkey cells, etc.

[0059] A “known compound” is a compound for which at least one activity is known. In the present invention, a known compound preferably is a compound for which one or more direct or indirect effects on cells is known. Preferably, the structure of a known compound is known, but this need not be the case. Preferably, the mechanism of action of a known compound on cells is known, for example, the effect or effects of a known compound on cells can be, as nonlimiting examples, effects on cell viability, cell adhesion, apoptosis, cell differentiation, cell proliferation, cell morphology, cell cycle, IgE-mediated cell activation or stimulation, receptor-ligand binding, cell number, cell quality, cell cycling, etc.

[0060] An “impedance value” is the impedance measured for electrodes in a well with or without cells present. Impedance is generally a function of the frequency, *i.e.*, impedance values depend on frequencies at which the measurement was conducted. For the present application, impedance value refers to impedance measured at either single frequency or multiple frequencies. Furthermore, impedance has two components, one resistance component and one reactance component. Impedance value in the present application refers to resistance component, or reactance component, or both resistance and reactance component. Thus, when “impedance value” was measured or monitored, we are referring to that, resistance, or reactance, or both resistance and reactance were measured or monitored. In many embodiments of the methods of the present application, impedance values also refer to parameter values that are derived from raw, measured impedance data. For example, cell index, or normalized cell index, or delta cell index could be used to represent impedance values.

[0061] As used herein, a “liquid (fluid) sample” refers to a sample that naturally exists as a liquid or fluid, *e.g.*, a biological fluid. A “liquid sample” also refers to a sample that naturally exists in a non-liquid status, *e.g.*, solid or gas, but is prepared as a liquid, fluid, solution or suspension containing the solid or gas sample material. For example, a liquid sample can encompass a liquid, fluid, solution or suspension containing a biological tissue.

[0062] As used herein, “sample” refers to anything which may contain a moiety to be isolated, manipulated, measured, quantified, detected or analyzed using apparatuses, microplates or methods in the present application. The sample is preferably a biological sample, such as a biological fluid or a biological tissue. Examples of biological fluids include suspension of cells in a medium such as cell culture medium, urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, amniotic fluid or the like. Biological tissues are aggregates of cells, usually of a particular kind together with their intercellular substance that form one of the structural materials of a human, animal, plant, bacterial, fungal or viral structure, including connective, epithelium, muscle and nerve tissues. Examples of biological tissues also include organs, tumors, lymph nodes, arteries and individual cell(s). The biological samples may further include cell suspensions, solutions containing biological molecules (*e.g.* proteins, enzymes, nucleic acids, carbohydrates, chemical molecules binding to biological molecules).

[0063] A “test compound” is any compound whose activity or direct or indirect effect or effects on cells is investigated in any assay. A test compound can be any compound, including, but not limited to, a small molecule, a large molecule, a molecular complex, an organic molecule, an inorganic molecule, a biomolecule such as but not limited to a lipid, a steroid, a carbohydrate, a fatty acid, an amino acid, a peptide, a protein, a nucleic acid, or any combination of these. A test compound can be a synthetic compound, a naturally occurring compound, a derivative of a naturally-occurring compound, etc. The structure of a test compound can be known or unknown.

[0064] As an introduction, beginning at FIG. 1A, an exemplary system 10 configured for electronically and optically monitoring samples continuously in real time is shown in communication with a computer processor 100 loaded with software and user interface 200. By collecting and analyzing different and distinct streams of information from a single assay, the system 10 provides multiple vantage points for studying cellular activity. The information richness of this multiplex assay lies not merely in the number of parameters it reports, but also in the distinctness/uniqueness of the perspectives that it affords. The system 10 described herein

enables cell health and behavior to be monitored simultaneously, and in the same well, from the very different perspectives of real-time cellular impedance and live cell imaging.

[0065] Moving onto **FIG. 1B**, the system **10** is preferably sized for placement in commercially available cell culture incubators for regulating temperature, CO₂ and moisture conditions for cells. That is, the system **10** is sized for placement in a cell culture incubator so that the incubator rather than the system **10** regulates cell culturing conditions, such as temperature, CO₂ and humidity. Though sizing can vary depending on the needs of the user, the system **10** shown in **FIG. 1A** and **FIG. 1B** has a footprint of about 430 mm x 445 mm x 410 mm.

[0066] The top of the primary housing **12** is embodied as a platform having five cradles **14**, where three cradles **14A** are configured for electronically (e.g. cell-substrate impedance) and optically monitoring biological samples and two cradles **14B** are for optically monitoring biological samples without electronic monitoring. While a total of five cradles **14** are shown, one of ordinary skill in the art would recognize that increasing the footprint of the system **10** may allow for additional cradles **14**. In addition, one of ordinary skill in the art would recognize that it is possible to have fewer cradles **14** (e.g. 4, 3, 2 cradles **14** or a single cradle **14**) than those shown in **FIG. 1**, including the same or fewer “electronic” cradles **14A** (e.g. 3, 2 or 1 cradle **14A**) configured for both electronic and optical monitoring of biological samples and/or the same or less number of the “optical only” cradles **14B** (e.g. 2 or 1) configured for only optical monitoring of biological samples.

[0067] As shown more clearly in **FIGS. 2A-2C**, each cradle **14A** configured for electronic monitoring preferably has a hinged cover **16**, which can be locked closed using a movable engagement handle **18**. Locking the cover **16** closed ensures a snug fit for electric engagement between electronic plates **20** (see **FIG. 4**) and pogo pins **22** (see **FIG. 2B**), which connects to an impedance analyzer or impedance measurement circuitry for monitoring impedance inside the system **10**, and ultimately communicate with the external computer processor **100** (**FIG. 1A**) for electronic monitoring. However, as shown in **FIG. 2C**, each cradle **14B** configured solely for optical imaging does not require an electronic engagement between the plate and cradle components and thus does not require a mechanism for locking the cover **16** closed. In both instances, the outside of the hinged cover **16** can be marked with indicia **24** consistent with the intended format of the plate or vessel added to the cradle **14**.

[0068] Inside the cradle, preferably along the cover **16**, is an illumination module **26** configured to illuminate the interior of the cradle **14**. Better shown in **FIG. 3**, in preferred

embodiments illumination module **26** is made up of a light emitting diode (LED) array, which itself is formed from a plurality of discrete LEDs **28** which receive instructions from the computer processor **100** (**FIG. 1A**) via electrical contacts **30**. In some embodiments, LED arrays can be exchanged, such as for different multi-well formatted plates (e.g. 6 well, 12 well, 20 well, 48 well, 96 well, 384 well) or other culture vessels by way of screws. Preferably, each diode **28** is assigned to a single well **32** (See **FIGS. 4-5**) of a 96 well plate and thus configured to independently illuminate the corresponding well **32** for imaging. By providing diodes **28** immediately above the wells **32**, the system **10** clearly illuminates the entire well **32** to ensure quality imaging. In preferred embodiments, the illumination module **26** is configured for bright field illumination, which means that the illumination module **26** emits white light. Consistent with conventional light microscopy, differences in the amount of white light passing through the biological sample provides sufficient contrast to identify cell components such as cell membrane, nucleus and other fine details depending on camera magnification and therefore may be used for cell counting and/or cell confluence analysis or to ensure cells are properly settled against the bottom of the well **32** prior to electronic monitoring. Imaging may be further enhanced by staining biological samples with image enhancing dyes that improve contrast or identification of live cells under bright field illumination (e.g. dead cells absorb Trypan Blue) to assess cytotoxicity, cell viability and/or cell proliferation.

[0069] Control over illumination is performed by way of the computer processor **100**. To this end, the computer processor **100** may selectively illuminate one or more diodes **28** in response to results from electronic monitoring of the biological sample. For example, biological cells may be monitored using cell-substrate impedance monitoring to assess cell growth and upon reaching a set value or parameter indicative of an established cell monolayer or established cell population, the computer processor **100** may instruct illumination of one or more electronically monitored wells **32** using one or more LEDs **28** to capture cell images. Moreover, during an experimental treatment, such as administration of a test compound to assess its cytotoxicity, the computer processor **100** may selectively instruct illumination of one or more LEDs **28** at predefined time points or in response to changes in cell-substrate impedance monitoring for cell imaging to confirm a reduced population of cells. Still further, the computer processor **100** may instruct bright field illumination using one or more diodes **28** from the illumination module **26** to capture images simultaneously while conducting electronic monitoring (e.g. cell-substrate impedance monitoring).

[0070] Returning to **FIGS. 2B** and **FIG. 2C**, white light passing through the biological

sample ultimately exits the cradle **14** through its open bottom **34**. By “open” it is meant that the bottom **34** is not completely opaque. As will be discussed in more detail in paragraphs that follow, this open bottom **34** exposes transparent windows (see **FIG. 5**) providing a path for capturing images. In embodiments configured for electronic monitoring of cells a slotted protective shield **36** may span the open bottom **34** for protection of pogo pins **22** especially during loading and unloading the multi-well plates **22** into or from the cradle **14**, **14A**.

[0071] For completeness, the development of cradle **14** adapted for electronic monitoring had to overcome challenges inherent to electronic measurement of cells using multi-well plates. For example, in conventional electronic monitoring of biological samples, vessels, such as multi-well plates adapted with electrodes require docketing stations that themselves have complex electrode-selection circuitry for communication with and switching the measurements to different sets of electrodes in different wells of the plates. Much of such circuitry for switching and selecting measurement electrodes had to be moved to provide the required opening and thus imaging through the bottom **34** of the cradle **14**. For example, **FIG. 2B**, demonstrates the use pogo-pins **22** (120 shown for communication with a 96 well electronic plate **20**) lining portions of the outer perimeter of the cradle **14**. However, moving or rerouting this circuitry to form the needed opening affects the electrical characteristics of the system **10**. In particular, elongating electric wires to move required electrode switching circuitry farther away from pogo-pins adds to the resistance of the system and may result in interference of electronic noises from other electrical parts (such as the circuitry for driving linear motion stage **66** and **70** in the system **10** of **FIG. 8**), which will ultimately affect the resolution and precision of electronic monitoring. This is especially important in the preferred electrode configurations, where all the wire resistances for switching and selecting each set of the electrodes across all the wells must be equal or about equal. In addition, another challenge with electronic monitoring of cells is that electrode switching circuitry increases local heat generation in the system **10**, which was previously dissipated within each cradle under the docking station. Thus, moving or reconfiguring electrodeswitching/selection circuitry and wiring away from its conventional placement also affects heating of the system **10**, which presented additional challenges in the development of such a system **10**. Thus, achieving a system that is suitable for both electronic monitoring and optical imaging of samples in a same well required special engineering development of the system **10** so that the electrode switching and selection circuitry could be relocated within the system **10** without resulting excessive local heat generation and without affecting the performance of electronic monitoring.

[0072] Through a number of innovative engineering design steps, electrode switching and selection circuitry was arranged in the vicinity of the pogo-pins 22. The locations and orientations of the electrode switching chips were designed to maximize distances among the chips despite the small space available for minimizing local heat generation. In addition, a printed circuit board (PCB board) was designed with appropriate electric trace layouts on different layers of the electrical conduction planes to minimize the variation of the electrode resistance between the pogo pins 22 and circuit switching chips for each set of electrodes across all of the wells on the multi-well plates. In addition, electrical signal wires/lines were designed in appropriate relationships relative to the electrical ground lines and electrical ground planes on the PCB board to minimize the electrical interface from other circuits in the system.

[0073] Now, with reference to FIGS. 2B and 2C, each cradle 14 can include a mechanical sensor 38 that when pressed, signals both receipt of a multi-well plate 20, and a closed cover 16. Upon signaling, the cradle 14 is ready for electronic and/or optical monitoring.

[0074] Moving on to FIGS. 4-5, preferably each well 32 of electronic plates 20 has a set of electrodes 42 and a transparent window 44 that is free of electrodes 42 on a bottom surface of the well 32. By providing zones with electrodes 42 and a transparent zone free of electrodes 42, at least two distinctly different assays can be conducted in a single well 32. In particular, electronic monitoring (e.g. cell-substrate impedance monitoring) can be conducted using the set of electrodes 42 and optical imaging can be performed by focusing an optical imaging module 46 (FIG. 6) through the cradle open bottom 34 (see FIGS. 2B, 2C) and through the transparent window 44. As shown in FIG. 4, the electronic plates 20 are preferably multi-well plates 20, which can be described as having a “plurality of wells” configured for electric connection or electronic monitoring of a sample. The skilled artisan will appreciate that there may be one or more wells 32 in addition to these “plurality of wells” that do not have an electrical connection or are not for electronic monitoring. In such cases, wells 32 that lack electronic monitoring capabilities can serve as control wells or as “optical imaging only” wells 32. To this end, electronically monitoring wells 32 can be described as having a nonconducting substrate (e.g. bottom of the well); an electrode array defined by a set of electrodes 42 fabricated on the substrate; and a transparent viewing window 44 on the substrate that is free of electrodes 42.

[0075] The electrode 42 configuration can vary depending on the needs or desires of the user so long as the transparent window 44 for sample imaging can be retained. In some embodiments, the electrode array is a microelectrode array (MEA) consistent with the

Electronic Cell-Substrate Impedance Sensing (ECIS) systems as described by Gaiver and Keese, where a single large reference electrode is paired with a plurality of small working or measuring electrodes. In ECIS, a small alternating current is applied across the electrode array. This results in a potential across the electrodes which is measured by the ECIS instrument. When cells are added to the ECIS Array and attach to the measuring electrodes, they act as insulators increasing the impedance. As cells grow and cover the measuring electrodes, the current is impeded in a manner related to the number of cells covering the electrode, the morphology of the cells, and the nature of the cell attachment. When cells proliferate or die, the impedance is altered.

[0076] An alternative and more preferred approach has been described previously (e.g. US 8,344,742; US 7,470,533; US 7,192,752; and elsewhere, each of the listed patents is incorporated herein by reference in its entirety), where each electrode array includes two electrode structures **42A**, **42B** and each electrode structure **42A**, **42B** includes electrode elements **42C**, but where innermost electrode elements **42C** are removed to form the transparent window **44** (See schematic shown in **FIG. 5**, panel A). Electrode structures **42A**, **42B** are electrically coupled to connection pads or interfaces, which are located on an edge of the substrate and thus configured to electrically connect to pogo pins **22** on the cradle **14**. Each electrode array has approximately uniform electrode resistance across the entire array. In contrast to the ECIS approach where significant changes in cell-substrate impedance are monitored at working or measuring electrodes only (not at large area reference electrodes), cell attachment or growth on any of the interdigitated electrodes impedes current in a detectable manner relating to the number of cells covering the electrode, the morphology of the cells and the nature of the cell attachment. That is, all electrodes **42** act as measuring or working electrodes in this configuration. Thus, the electrode array can be two or more electrode structures **42A**, **42B** that are constructed to have dimensions and spacing such that they can, when connected to a signal source, such as through the computer processor **100** and cradle **14**, operate as a unit to generate an electrical field in the region of spaces around the electrode structures **42A**, **42B**.

[0077] When electronic monitoring is cell-substrate impedance monitoring electronic circuitry connects the sets of electrodes **42** to the computer processor **100** via the cradle **14**. Preferably, in such embodiments the computer processor **100** communicates with impedance measurement circuitry or an impedance analyzer which may be fully incorporated within the system **10**. The impedance analyzer may include electronic hardware circuitry measuring

electrical current and voltage, and signal and data processing algorithm in firmware and/or software. The system **100**, when connected to an impedance analyzer, can measure differences in impedance values that relate to cell behavior. For example, the system **10** can measure differences in impedance values when cells are attached to the electrode array and when cells are not attached to the electrode array, or can detect differences in impedance values when the number, type, activity, adhesiveness, or morphology of cells attached to the electrodes **42** change. In particular, cell-substrate impedance monitoring can reveal information about cell attachment or adhesion status (e.g. the degree of cell spreading, the attachment area of a cell, the degree of tightness of cell attachment, cell morphology) on the substrate including on the electrodes **42**, cell growth or proliferation status; number of viable cells and/or dead cells in the well; cytoskeleton change and re-organization and number of cells going through apoptosis and/or necrosis.

[0078] In some embodiments, impedance analyzer is capable of measuring impedance between 0.1 ohm and 10^5 ohm in a frequency range of 1 Hz to 1 MHz. More preferably, the impedance analyzer is capable of measuring impedance between 0.1 ohm and 10^3 ohm in frequency range of 100 Hz to 100 kHz. The impedance analyzer is also preferably capable of measuring both resistance and reactance (capacitive reactance and inductive reactance) components of the impedance.

[0079] In addition, the system **10** includes electronic switches that can switch on and off connections to each of the sets of electrodes **42** for selective monitoring. These switches are controlled by a software program preferably loaded into the computer processor **100**. The software program directs connection of electrode arrays to the impedance analyzer and monitor cell-substrate impedance from the electrodes **42**. During impedance monitoring, the impedance analyzer can monitor impedance at one frequency or at more than one frequency. Most often, impedance monitoring is performed at more than one time point for a given assay. Thus, the system can connect individual arrays to an impedance analyzer to monitor one, some, or all of the arrays at one or more time point. Further, the switches allow the selected individual arrays to be monitored in rapid succession for each desired monitoring time point. Each monitoring time point is in fact a narrow time frame (for example from a millisecond to minutes) of measurement in the assay during which impedance monitoring is performed. In some embodiments the software is programmable to direct impedance monitoring of any of the wells **32** of the plate **20** that include arrays at chosen time intervals.

[0080] In furtherance of the above, the system **10** can be used to efficiently and

simultaneously perform multiple assays using circuitry to digitally switch from cell-substrate impedance monitoring over an array in one well **32** to cell-substrate impedance monitoring over an array in another well **32**, whether from a same electronic plate **20** or another electronic plate **20**. In some embodiments, the system under software control is capable of completing an impedance measurement for an individual well **32** at a single frequency within about one second or less. In further embodiments, cell-substrate impedance is monitored at millisecond resolution. Approaches for cell-substrate impedance monitoring at millisecond resolution can be found in US 10,533,985, US 10,012,636; US 9,709,548; and elsewhere. Each of the listed patents is incorporated herein by reference in its entirety. Thus, in some embodiments, two sequential impedance measurements are monitored within 40 ms apart from one another. In some embodiments, two sequential impedance measurements are monitored within 20 ms apart from one another. In some embodiments, two sequential impedance measurements are monitored within 10 ms apart from one another. In some embodiments, two sequential impedance measurements are monitored within 1 ms apart from one another. In some embodiments, two sequential impedance measurements are monitored within less than 1 ms apart from one another.

[0081] While the system is described primarily with respect to cell-substrate impedance monitoring of cells, one of ordinary skill in the art would recognize that the system can also be adapted for conducting extracellular recording. Extracellular recording can be conducted by amplifying and recording electrical voltage signals between small recording electrodes and much larger reference electrodes (note that the use of such small recording electrodes and large reference electrode is similar to those used in ECIS). In extracellular recording embodiments, an extracellular recording system (including voltage signal amplifier and other electronic hardware circuitry for measuring electrical voltage plus signal processing algorithm implemented in software and/or firmware) is used rather than an impedance measurement system (e.g., impedance analyzer system, including electronic hardware circuitry measuring electrical current and voltage, and signal and data processing algorithm in software and/or firmware).

[0082] Proceeding to **FIGS. 6-7**, an exemplary optical imaging module **26** is able to capture images from each well **32**. In preferred embodiments, the optical imaging module **26** is positioned within the primary housing **12** (see **FIG. 1B**) and underneath the cradles **14**. An exemplary optical imaging module **26** includes a long working distance objective **48** at one end, and a sealed CMOS camera **50** at the opposing end. In some embodiments, the camera captures

30 images per second. In some embodiments, the camera captures 40 images per second. In some embodiments, the camera captures 50 images per second. In some embodiments, the camera captures 60 images per second. In some embodiments, the camera captures 70 images per second. In some embodiments, the camera captures more than 70 images per second. This high-speed capturing permits images taken under different conditions or filters to be superimposed. Also shown are tube lenses **52A**, **52B** and a bandpass filter **54** to enhance imaging. The optical imaging system **26** receives instructions from and sends images to the computer processor **100**. Thus, bright field illumination of the well **32** permits the optical imaging system **26** to capture images through the open bottom **34** of the cradle **14** even when the cradle **14** is closed (see **FIGS. 2A-2B**).

[0083] Preferably, the optical imaging system **26** also includes an excitation light source **56**, which is shown as a set of LEDs that direct light through a focusing lens **58** or excitation of molecules within the well **32**, such as to induce fluorescence for fluorescence imaging. While LEDs correspond to yellow **56A**, ultraviolet **56B**, and blue **56C** are shown, the excitation can include any number between one and seven lights. For example, the excitation light source **56** can include one or more lights including an ultraviolet light, a violet light, a blue light, a green light, a yellow light, an orange light, and a red light. Accordingly, the system **10** is configured to not only capture bright field contrast images of cells but also fluorescent tagged markers, such as fluorescent tagged antibodies, antibody fragments or other molecules that bind to the cell. To this end, the system can provide imaging of different stages of cell proliferation, cell death, cell apoptosis, effector cell killing, cell to cell interaction, cell binding, DNA/RNA/protein upregulation, DNA/RNA/protein down regulation and the like by adding a suitable fluorescent dye or fluorescently tagged molecule to the cell sample and capturing fluorescence by way of the imaging module.

[0084] Being part of the optical imaging system **56**, the excitation light source **56** is also controlled by the computer processor **100** loaded with software. Thus, the computer processor **100** can instruct the on/off switching of each LED **56A**, **56B**, **56C** and instruct the high-speed capture of images via the camera **50**. In operation for optical monitoring of the cells, one LED is turned on at a time and the fluorescent image of the corresponding color would be captured by monochrome (black-white) CMOS camera **50**. For example, yellow LED **56A**, ultraviolet LED **56B**, and blue LED **56C** would correspond to red, blue and red fluorescent images, respectively. The captured monochrome images would be displayed with pseudo coloring for representation of the corresponding fluorescent color. Moreover, the computer processor

100 can determine the number of live cells from the imaging, as well as determining parameters such as presence or absence of fluorescence (+/-), total fluorescence intensity of the cells in each well or over many wells, average fluorescence intensity for the cells in one well or over more wells, and others. Further, the computer processor **100** can overlay fluorescence image (or single or multicolors) and bright field illumination image for comprehensive analysis.

[0085] Turning to **FIGS. 5, 6 and 8** collectively, the optical module **56** is mounted to a movable support **60** for movement across the different windows **44**. Generally only movement along the X and Y axis is needed because lens adjustment or computer controlled focusing can account for minor difference focal lengths between wells **32**. As such, the moveable support **60** moves in two directions using an X-axis motor and timing belt **62**, an X-axis cable rack **64**, an X-axis linear motion guide **66**, a Y-axis cable rack **68**, and a Y-axis linear motion guide **70**. A magnetic linear encoder **72** helps identify the X, Y coordinates of the optical imaging module **56**.

[0086] As already eluded to, the monitoring system **10** can also store and display data. Data can be displayed on a screen **200**, as printed data, or both. Preferably the software can allow entry and display of experimental parameters, such as descriptive information including cell types, compound concentrations, time intervals monitored, etc. Moreover, data can be displayed superimposed, such as combinations showing optical imaging from different fluorescence channels (i.e. different fluorescent colors), bright field illumination and electronic monitoring data.

[0087] Preferably, the software can also analyze impedance data. In preferred embodiments, the software can calculate a cell index (CI) for one or more time points for one or more wells. In some preferred embodiments, the software can calculate a cell change index (CCI) from impedance measurements of one or more wells. The software can preferably generate plots of impedance data and impedance values, such as but not limited to CI or CCI, with respect to time. The software may perform other analysis as well, such as calculate cell number from CI, generate dose-response curves based on impedance data, calculate IC values based on impedance values, and calculate kinetic parameters of cell growth or behavior based on impedance values and impedance value curves. The software of the impedance monitoring system can also store and display analyses of the data, such as calculated impedance values and kinetic parameters derived therefrom. Data can be displayed on a screen, as printed data, or both.

[0088] Likewise, the software can also be used to analyze captured images. Preferably,

the software can perform cell counting functions from images and can plot data over time for statistical analysis. Preferably the software can also store and display analyses of the data, such as counts from fluorescence imaging of different wavelength channels, bright field illumination, and pair superimposed cell imaging results with corresponding electronic monitoring time points.

[0089] For example, if the images being captured are bright field images of the cells, the methods of use can include determining cell confluence numbers or parameters from the bright field images or counting cells; or if the images being captured are fluorescence images of the cells, the methods of use can include determining a fluorescence parameter from the images of each color, optionally selected from one or more of the group consisting of total fluorescence counts, total fluorescence intensity, and average fluorescence intensity; or if the images being captured include bright field images of the cells and fluorescence images of the cells, the methods of use can include deriving cell confluence numbers or parameters from the bright field images and optionally counting cells from the bright field images; determining a fluorescence parameter from the fluorescence images, optionally selected from one or more of the group consisting of total fluorescence counts, total fluorescence intensity, and average fluorescence intensity; and optionally, superimposing the bright field images and fluorescence images of one or multiple colors for one or more of the wells.

[0090] The benefits of coupling electronic monitoring of cells with live cell imaging are especially evident viewing **FIGS. 11A-12C** (described in more detail in the below Examples). In particular, whereas cell-substrate impedance was able to detect a rapid staurosporine-mediated effect on A549-Blue cells, the cellular phenomena causing this change could not be deciphered using impedance alone. However, live cell imaging revealed massive cytoplasmic shrinkage, immediately suggesting a mechanistic explanation for the large and rapid impedance response. Likewise, impedance has provided insights that would not have been obtainable by imaging alone. In one such example, MG132 treatment caused cells to detach from electrodes but remain in present in the well (cellular confluence remained >50%, (**FIG. 14B**), where confluence number is derived from bright field images through image analysis algorithms), while cell imaging showed cells present within the wells, only impedance (the impedance signal dropped to zero (**FIG. 11A**)) revealed cell-substrate attachment strength. Beyond the benefit of having two independent measurement techniques, it is important to note the objectivity of the impedance readout, which is reported directly, without any processing or input from the user.

[0091] Returning to FIGS. 1-8 collectively, non-limited uses of coupling cell-substrate impedance monitoring and live cell imaging of a same cell population are now described in more detail. That is, methods of monitoring cells are disclosed, which include, electronically monitoring cells within wells 32 of a multi-well plate 20, each of the wells 32 having a set of electrodes 42, preferably a set of cell-substrate impedance monitoring electrodes 42, and a transparent window 44 on a bottom surface of the well 32 that is free of electrodes 42; and capturing images through the windows 44 from at least one well 32 that is being or has been electronically monitored. From this monitoring, the methods can include generating an impedance-based curve from the monitored impedance over time (e.g. CI curve); and displaying the impedance-based curve and corresponding optical image(s). Images can be captured from bright field illumination or from fluorescence excitation (e.g. excitation of fluorescent molecule labels attached to antibodies, antibody fragments or other binding molecules). As such, the data can provide quantitative kinetics revealing detailed information regarding cell health, behavior, and cell to cell interactions.

[0092] Also disclosed are methods for performing cell proliferation assays. In these assays, an increase in monitored impedance is indicative of an increased cell number, which can be confirmed by corresponding real time imaging. The impedance measurements or impedance values derived from impedance measurements can be plotted versus time to obtain growth curves for cells growing in wells 32 of a multi-well plate 20 and presented with captured cell imaging from the same wells 32, in particular either from bright field illumination and/or from fluorescence imaging of pathways or markers associated with cell proliferation.

[0093] Relatedly, methods of generating at least one cell growth curve are provided, which include: incubating cells over time in a multi-well plate 20, where each well 32 includes a set of electrodes 42 and a transparent window 44 on a bottom surface of the well 32 that is free of electrodes 42; monitoring cell-substrate impedance and capturing optical images from a same well 32 through the window 44; generating an impedance based curve from the monitored impedance; and displaying the impedance based curve and corresponding optical image(s).

[0094] Growth curves of one or more cell types coupled with real time cell imaging can be used to determine kinetic parameters. For example, proliferation rates of different primary cancer cells can be compared, or proliferation rates of primary cancer cells of a same type but different grades. In another example, primary cells of individuals of different genotypes can be compared. In another example, proliferation rates of primary or cell line stem cells can be compared. In yet another example, growth curves or parameters of control and

genetically modified cells of a cell line can be compared. In yet another example, growth curves or parameters of cells infected with virus and control cells can be compared. Moreover, growth can be confirmed using the imaging features of the system, such as by performing cell counts or cell confluence calculation via images captured through bright field illumination or by counting cells stained with a fluorescent molecule or a fluorescent tagged binding molecule and captured under fluorescence imaging.

[0095] The system 10 can also be used to investigate the effect of one or more test compounds on cells. An exemplary embodiment includes incubating cells over time in a multi-well plate 20 where, each well 32 includes a set of electrodes 42 and a transparent window 44 on a bottom surface of the well 32 that is free of electrodes 42; monitoring cell-substrate impedance and capturing optical images from a same well 32 through the window 44; adding a test compound to at least one of the wells 32; continuing to monitor cell-substrate impedance and capturing optical images from the same well 32 through the window 44; generating an impedance based curve from the monitored impedance over time; and displaying the impedance based curve and optical image(s) from corresponding wells. Changes in effect can be determined by comparing results post compound addition to those prior to compound addition/and or by providing a vehicle control to another well 32 having cells and comparing impedance-based curves and/or images between wells 32.

[0096] Also disclosed are methods of comparing the effects of a compound on two or more cell types. An exemplary method includes incubating cells over time in a multi-well plate 20 where, each well 32 includes a set of electrodes 42 and a transparent window 44 on a bottom surface of the well 32 that is free of electrodes 44, where at least one of the wells 32 receives one cell type and at least another well 32 receives a different cell type; monitoring cell-substrate impedance and capturing optical images from each of the wells 32 having cells; adding a same test compound to wells 32 having each of the cell types; continuing to monitor cell-substrate impedance and capturing optical images from each of the wells 32 having cells and test compound through the window 44; generating an impedance based curve from the monitored impedance over time; and displaying impedance based curves and corresponding optical image for each of the wells 32 for comparison with one another. Wells 32 using vehicle controls can also be included as known in the art.

[0097] Also disclosed are methods of comparing the effects of two or more different compounds on cells. An exemplary method includes incubating cells over time in a multi-well plate 20 where, each well 32 includes a set of electrodes 42 and a transparent window 44 on a

bottom surface of the well 32 that is free of electrodes 42, where at least two of the wells 32 receives cells; monitoring cell-substrate impedance and capturing optical images from each of the wells 32 having cells; adding different test compounds to different wells 32 having cells; continuing to monitor cell-substrate impedance and capturing optical images from each of the wells 32 having cells and test compounds through the windows 44; generating an impedance based curve from the monitored impedances over time; and displaying impedance based curves and corresponding optical image for each of the wells 32 for comparison with one another.

Wells 32 using vehicle controls can also be included as known in the art.

[0098] Relatedly, also disclosed are methods of performing assays to test the effect of different concentrations of one or more test compound on cells. Such dose response relationships can be used to derive a time-dependent IC₅, IC₁₀, IC₂₀, IC₃₀, IC₄₀, IC₅₀, IC₆₀, IC₇₀, IC₈₀, IC₉₀, or IC₉₅, all of which could be derived from a dose response curve. Typically the IC₅₀ is of most interest. Determining a range of time-dependent IC₅₀s for a compound provides information on when the effect of the compound on cells is maximal. Accordingly, an exemplary method includes incubating cells over time in a multi-well plate 20 where, each well 32 includes a set of electrodes 42 and a transparent window 44 on a bottom surface of the well 32 that is free of electrodes 42, where at least two of the wells 32 receives cells; monitoring cell-substrate impedance and capturing optical images from each of the wells 32 having cells; adding different concentrations of a test compound to different wells 32 having cells; continuing to monitor cell-substrate impedance and capturing optical images from each of the wells 32 having cells and test compound through the windows 44; generating impedance based curves from the monitored impedances over time; and displaying impedance based curves and corresponding optical image for each of the wells 32 for comparison with one another, such as to compare their dose response curves or dose relationship or compare IC₅₀ values derived from each of the dose response curves. Wells 32 using vehicle controls can also be included as known in the art.

[0099] Also disclosed are methods for performing real-time cytotoxicity assays of a compound. An exemplary embodiment includes incubating cells over time in a multi-well plate 20 where, each well 32 includes a set of electrodes 42 and a transparent window 44 on a bottom surface of the well 32 that is free of electrodes 42; monitoring cell-substrate impedance and capturing optical images from each of the wells 32 having cells; adding a cytotoxic compound or a compound that is suspected of being cytotoxic to one or more wells 32 having cells; continuing to monitor cell-substrate impedance and capturing optical images from each of the

wells **32** having cells and the added compound through the windows **44**; generating impedance based curve(s) from the monitored impedance(s) over time; and displaying impedance based curve(s) and corresponding optical image(s) for each of the wells **32** for comparison with one another. Wells **32** using vehicle controls can also be included as known in the art.

[00100] Also disclosed are methods for analyzing and comparing time-dependent cytotoxic effects of a first compound and a second compound on a cell type. An exemplary embodiment includes incubating cells over time in a multi-well plate **20** where, each well **32** includes a set of electrodes **42** and a transparent window **44** on a bottom surface of the well **32** that is free of electrodes **42**; monitoring cell-substrate impedance and capturing optical image(s) from each of the wells **32** having cells; adding a first cytotoxic compound or a first compound that is suspected of being cytotoxic to one or more wells **32** having cells and adding a second cytotoxic compound or a second compound that is suspected of being cytotoxic to another one or more wells **32** having cells; continuing to monitor cell-substrate impedance and capturing optical images from each of the wells **32** having cells and the added compound through the windows **44**; generating impedance based curves from the monitored impedances over time; and displaying impedance based curves and corresponding optical images for wells **32** associated with the first and second compound for comparison with one another. Wells **32** using vehicle controls can also be included as known in the art. In some embodiments, time-dependent cytotoxic responses are determined for the first compound at multiple dose concentrations. In some embodiments, time-dependent cytotoxic responses are determined for the second compound at multiple dose concentrations. In some embodiments, time-dependent cytotoxic responses are determined for both first compound and second compound at multiple dose concentrations.

[00101] In some embodiment, the first compound is a compound with a known mechanism for its cytotoxic effect and the second compound is a compound with an unknown mechanism for its cytotoxic effect. If the time dependent cytotoxic responses from the second compound are similar to that of the first one, the second compound may follow a similar mechanism for its cytotoxic effect to the first compound.

[00102] Various approaches may be used in comparing the cytotoxic responses of the compounds. A cell index (or cell number index) can optionally be calculated using the impedance values obtained. In some embodiments, time dependent IC₅₀ may be derived for the compounds and comparison between their cytotoxic responses is done by comparing their time dependent IC₅₀ curves based on cell index values. If the IC₅₀ curves follow a similar time-

dependent trend, the two compounds may follow a similar mechanism for inducing cytotoxicity effects.

[00103] In some embodiments direct comparison of time-dependent cytotoxic responses of two compounds are done where the concentrations for the two compounds may be the same or may be different. Direct comparison between time-dependent cytotoxic responses may be done by analyzing the slope of change in the measured responses (that is equivalent to the first order derivative of the response with respect to time) and comparing the time-dependent slopes for the two compounds. In another approach, the time-dependent cytotoxic responses may be analyzed for their higher order derivatives with respect to time. Comparing such high order derivatives may provide additional information as for the mechanisms of compound- induced cytotoxicity.

[00104] In some embodiments analyzing real-time cytotoxicity response may include the derivation of time-dependent IC50 values for the compound on the multiple cell types. In some embodiments, analyzing real-time cytotoxicity response may include derivation of the slope of change in the time dependent cytotoxicity response at a given compound concentration. In some embodiments, analyzing real-time cytotoxicity response may include derivation of high-order derivatives of the time dependent cytotoxicity response with respect to time at a given compound concentration.

[00105] Also disclosed are methods for assessing the effect of proposed anticancer therapeutics on cancer cells. An exemplary embodiment includes incubating cancer cells over time in a multi-well plate 20 where, each well 32 includes a set of electrodes 42 and a transparent window 44 on a bottom surface of the well 32 that is free of electrodes; adding one or more proposed therapeutics to the cells; monitoring cell-substrate impedance and capturing optical image(s) from each of the wells 32 having cells; adding effector cells, preferably from a same subject from which the cancer cells were obtained, to the wells 32; continuing to monitor cell- substrate impedance and capturing optical images from each of the wells 32 through the windows 44; generating impedance based curves from the monitored impedances over time; and displaying impedance based curves and corresponding optical images from the wells 32. In some embodiments, the methods include the addition of CAR-T cells as effector cells. Wells 32 using vehicle controls can also be included as known in the art.

[00106] Also disclosed are methods for assessing cytolysis of cancer cells by engineered effector cells. An exemplary embodiment includes incubating cancer cells over time in a multi-well plate 20 where, each well 32 includes a set of electrodes 42 and a transparent window 44

on a bottom surface of the well **32** that is free of electrodes **42**; monitoring cell- substrate impedance and capturing optical image(s) from each of the wells **32** having cells; adding effector cells engineered to display a binding moiety suspected of binding the cancer cells, preferably from a same subject from which the cancer cells were obtained, to the wells **32**; continuing to monitor cell-substrate impedance and capturing optical images from each of the wells **32** through the windows **44**; generating impedance based curves from the monitored impedances over time; and displaying impedance based curves and corresponding optical images from the wells **32**. Wells **32** using vehicle controls can also be included as known in the art.

[00107] Also disclosed are method for assessing cytolysis of cancer cells by way of a bispecific engager. An exemplary embodiment includes incubating cancer cells over time in a multi-well plate **20** where, each well **32** includes a set of electrodes **42** and a transparent window **44** on a bottom surface of the well **32** that is free of electrodes; monitoring cell-substrate impedance and capturing optical image(s) from each of the wells **32** having cells; adding effector cells to the well **32**, preferably from a same patient as the cancer cells; adding a bispecific engager configured to bridge the effector cells to the cancer cells; continuing to monitor cell- substrate impedance and capturing optical images from each of the wells **32** through the windows **44**; generating impedance based curves from the monitored impedances over time; and displaying impedance based curves and corresponding optical images from the wells **32**. Wells **32** using vehicle controls can also be included as known in the art.

[00108] As will be shown in more detail in the Examples that follow, the systems and methods herein couple the simplicity, analytical sensitivity, and objectivity of real-time impedance monitoring with the highly specific readout of live cell imaging to continuously track cellular processes with unparalleled information richness.

EXAMPLE I

MONITORING OF IMMUNE CELL-MEDIATED KILLING OF CANCER TARGET CELLS IN REAL TIME

[00109] MCF7 breast cancer cells were transfected with a lentivirus expressing a red fluorescent protein (eLenti Red, Cat# 8711011), seeded on an E-Plate (ACEA BIOSCIENCES, San Diego, CA) for 25 hours, and then treated with NK92 cells at different effector:target (E:T) ratios.

[00110] Optical imaging was performed simultaneous with impedance monitoring. As shown in **FIGS. 9A-B**, effector addition causes cancer cell death in an E:T ratio-dependent manner as demonstrated by impedance monitoring (**FIG. 9A**) and optical imaging (**FIG. 9B**). Fluorescent object count (**FIG. 9B**) indicates number of living target cells.

[00111] **FIGS. 9C-9E** are images taken before (**FIG. 9C**), and 30 hr. (**FIGS. 9D, 9E**) at an E:T ratio of 2.5:1 demonstrating target cell death over time.

EXAMPLE II

MULIPLEXING REAL-TIME IMPEDANCE WITH LIVE CELL IMAGING TO TRACK CELL KILLING

[00112] Cell maintenance and assays were conducted at 37 °C/5% CO₂ in F-12K media (ATCC; catalog number 30-2004) containing 10% heat-inactivated FBS (Corning, catalog number 35016CV). While impedance was measured every 15 minutes, images were acquired once per hour. In each well, four fields of view were captured for each channel (brightfield, red, green, and blue). Exposure times were as follows: red (300 ms), green (300 ms), and blue (80 ms). The A549-Blue cell line, which stably expresses nuclear-localized blue fluorescent protein (BFP), was produced by transducing A549 cells (ATCC; catalog number CCL-185) with AgilenteLenti Blue (p/n 8711012) at a multiplicity of infection of 1. From day 2 to day 11 postinfection, 1 µg/mL puromycin was included in the growth medium to select for transductants. For real-time visualization of activated caspase 3, Agilent eCaspase 3 NucView 488 (p/n 8711005) was included in growth medium at a concentration of 5 µM. For real-time visualization of translocated phosphatidylserine, Agilent eAnnexin V Red (p/n 8711007) was included in growth medium at a concentration of 0.25 µg/mL. Agilent E-Plate VIEW microplates (p/n 00300601030) were also used. MG132 (Tocris; catalog number 1748/5) and staurosporine (Calbiochem; catalog number 569396) stocks were dissolved in DMSO.

[00113] A549-Blue cells (described above) were seeded into an E-PATE VIEW at a density of 10,000 cells/well. As the cells proliferate over the first day, they occupy an expanding surface area on the biosensor array, causing the impedance signal to rise steadily (**FIG. 11A** and **FIG. 11B**). If left untreated, the cells grow to confluence, saturating the biosensor array and giving a plateaued impedance signal. Addition of the proteasome inhibitor MG132 or the pan-kinase inhibitor staurosporine at the 25-hour time point induces a marked decrease in the impedance signal in a time- and dose-dependent manner. The kinetics of these

drug-induced responses, and the overall shape of the impedance traces, are distinct for each compound. This is consistent with a broad body of literature spanning over 10 years, which has demonstrated that impedance responses are typically unique for each type of mechanism of action. Although MG132 and staurosporine both induce apoptosis, they elicit distinct cellular behaviors enroute to cell death, which becomes strikingly clear when real-time impedance is multiplexed with live cell imaging.

[00114] Although A549-Blue cells already cover most of the well bottom by the 26-hour time point, in the absence of drug, they continue proliferating for another 50 hours, packing cells together at higher density (**FIG. 12A**). Consistent with this, the impedance signal plateaus at roughly 30 hours while the total number of blue nuclei continues to increase until ~80hours (**FIG. 12A**). Blue nuclei count is obtained by an image processing algorithm to count the number of blue objects in the blue-fluorescent image. Each blue nuclei corresponds to a A549 cell as it was transduced to express nuclear-localized blue fluorescent protein. As such the number of blue nuclei in a well correspond to the number of viable A549-blue cells present in the well. When A549-Blue cells are treated with the four highest concentrations of MG132 (1.9, 5.5, 16.7, and 50 μ m), the total number of blue nuclei decreases over time in a manner that correlates well with the drop in impedance (**FIG. 12B**; only data for the 50 μ m treatment are shown). In contrast, treating the cells with the four highest concentrations of staurosporine (0.125, 0.250, 0.500, and 1 μ m) causes impedance to plummet within the first few minutes, but has a modest impact on the number of blue nuclei over the subsequent 60 hours (**FIG. 12C**; only data for the 1 μ m treatment are shown). Consistent with its ability to cause cells to expel water, one hour after staurosporine addition, the A549-Blue cells have shrunk so severely that their cytoplasm is barely visible and only blue nuclei remain. Although, over time, these nuclei shrink in size and begin clustering together, they largely remain intact, explaining why the number of blue nuclei stays fairly constant in **FIG. 12C**. The above coupling of impedance with imaging clearly provides a more complete and nuanced understanding of drug-mediated A549 cell killing than would be possible using either technique alone. Moving beyond simple cell counts, we next probed the kinetics of biochemical phenomena that are specific to the apoptotic killing pathway.

EXAMPLE III

SIMULTANEOUS TRACKING MG132-MEDIATED APOPTOSIS FROM FIVE DIFFERENT PERSPECTIVES

[00115] Along with impedance and blue nuclei counts, the induction of apoptosis by MG132 was also tracked by percent cellular confluence, caspase 3 activation (causing cells to fluoresce green), and phosphatidylserine translocation (causing cells to fluoresce red). As seen in **FIG. 13**, the drug-induced drop in impedance correlates well with the temporal accumulation of these apoptosis-specific markers. The bold white arrows in the panels for the 20- and 40-hour time points highlight large membrane blebs that contain phosphatidylserine in their outer leaflet.

[00116] Next, the continuous response of A549-Blue cells to MG132 was plotted using each of the image-based readouts. Blue nuclei counts (**FIG. 14A**) display a dependency on drug concentration that closely reflects the impedance responses seen in **FIG. 11A**. Despite the extensive apoptotic response, throughout the course of this assay the % cellular confluence never drops below 50% (**FIG. 14B**), where the cell confluence number is derived from bright field images through image processing algorithms. This is consistent with the fact that, unlike *in vivo* contexts, where apoptotic cells and their debris are removed through phagocytosis, *in vitro*, a large percentage of apoptotic cells continue to occupy the well bottom (**FIG. 12B**). The fact that high concentrations of MG132 cause the impedance signal to drop to zero (**FIG. 11A**) while the % confluence never drops below 50% indicates that the residual cells are no longer attached to the plate bottom. The number of caspase 3 positive (fluorescence green) objects (**FIG. 14C**) and the number of the annexin V positive (fluorescent red) objects (**FIG. 14D**) increase over time, and display clear dependencies on MG132 concentration. Considering the number of cells that were seeded, their rate of growth, and the percentage of cells that display apoptotic markers (**FIG. 13**), the output numbers in **FIGS. 14A-C** are consistent with expectations. To compare the relative rates and relative abundance of different apoptotic phenomena, the impedance response was plotted alongside three different image-based readouts (**FIG. 15**). As expected, the time at which the number of blue nuclei begins to decrease (~10 hours after MG132 addition) is the same time that caspase 3 activation and phosphatidylserine translocation become detectable. For the first 20 hours of drug treatment, the number of cells displaying caspase 3 and phosphatidylserine signals is similar, but over the subsequent 40 hours, the number of caspase 3-activated cells exceeds the number of phosphatidylserine translocated cells by approximately 20%.

EXAMPLE IV

QUANTIFYING DRUG EFFICACY BY SIMULTANEOUS MONITORING OF CELL-

SUBSTRATE IMPEDANCE AND OPTICAL IMAGING

[00117] Using the impedance and image-based readouts presented in Examples II-IV, the EC₅₀ for MG132 was calculated. The area under the curve, spanning from the time of drug addition to 60 hours after drug addition, was plotted as a function of MG132 concentration to yield the dose response curves seen in **FIG. 16**. The quality of the fitting for the four different readouts is quite good, with R² values ranging from 0.96 to 0.98. The calculated EC₅₀ values range from 0.86 to 3.0 μ m, which is consistent with values reported in the literature. See Han, Y. H. *et al.* The Effect of MG132, a Proteasome Inhibitor on HeLa Cells in Relation to Cell Growth, Reactive Oxygen Species and GSH. *Oncol. Rep.* 2009, 22(1), 215–21.